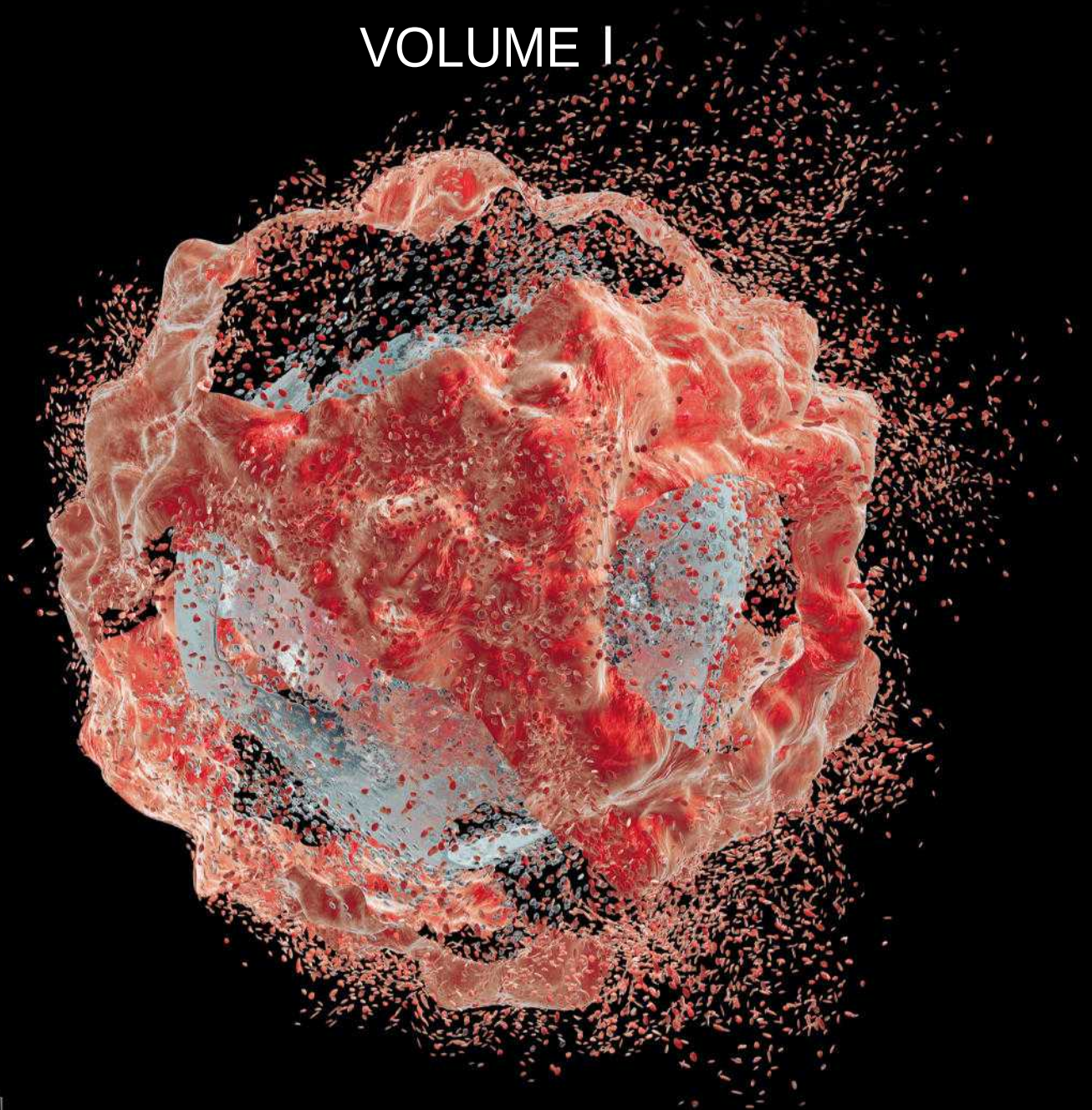


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VOLUME 1

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FOREWORD

It is a great honor to be asked to introduce, with this foreword, the *Encyclopedia of Infection and Immunity*. This work will be read and enjoyed by many students, both graduate and postgraduate, including clinicians, laboratory practitioners, and scientists. Many colleagues have contributed to this important work, with over 230 original chapters assembled in sections and listed alphabetically. I offer my warm congratulations to the editors of this book, for soliciting a wide body of expertise and assembling it in a form that gives so much pleasure and information to the reader.

For those of us who work in the field of infection and immunity, the last two years have been exceptionally challenging. At the current time we are 22 months into a major pandemic caused by a novel coronavirus and its variants, and worldwide, sadly, there have been many hospitalizations and deaths. Clinicians and scientists in our field have been working flat out, caring for patients, generating or utilizing diagnostic tests, generating new knowledge in immunity to coronaviruses, or discovering, testing, and deploying new vaccines. Public Health practitioners have organized our medico-social responses to limit transmission and deal with the consequences of infection. Behavioral scientists have transformed our approach to informing the public, getting the best possible outcomes from effective communication. Governments have learned the danger of ignoring the expertise of scientists, and in many countries, the public look upon clinicians and scientists with gratitude and respect. It is a testament to the advancement of infection and immunity science that the genome of the virus was sequenced within a few weeks of COVID-19 emerging, and that within 12 months, the first trials of novel vaccines had been completed. These vaccines included some with exceptionally novel technologies, including the RNA vaccines and the viral-vectored vaccines.

Underpinning the academic agility of students and practitioners in Infection and Immunity is the universal strength of sound education. Readers of this book will be seeking the knowledge they need for a comprehensive understanding of infectious disease and immunology. We work in a fascinating discipline. Our field is unique in that pathogenesis involves at least two sets of genomes—on the one hand the microbe, and on the other, the host. The microbial genome programs its microenvironment to enable acquisition of and residence in a niche for sufficient time to replicate and further disseminate to other hosts. The human genome has evolved to tolerate and contain a wide range of symbionts and pathobionts, with highly sophisticated immune decision-making at the cellular level, but to respond to invading species with an elaborate coordinated repertoire of innate and acquired immunity. The complex conversation between many different microbes and the human host generates a wide range of disease phenotypes. This makes the job of an infectious disease physician so challenging but at the same time so fulfilling.

Our field is enriched by the many scientists and clinicians who are willing to share their experience with their peers and students. This book reflects the great variety of knowledge that has emerged over centuries of inquiry and endeavor, and I believe you will be rewarded by using it as one of your reference sources.

Robert C. Read

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PREFACE

Although infections have been one of the most important health threats since centuries ago, the mechanisms of human defense against infections have been unclear until last century. The scientific world has remarkable progresses in the field of immunology during recent decades. Indeed, the novel discovery of genes related to different pathogens has enhanced our knowledge about the immune system. Understanding different pathogens and the immune mechanisms promote diagnostic strategies and design more efficient therapeutic agents.

Encyclopedia of Infection and Immunity is a comprehensive text, which covers topics not only on basic immunology and microbiology but also on clinical immunology and infectious diseases. Section 1 focuses on the immune system, while Section 2 describes the interaction between pathogens and immunity. Bacteria, viruses, fungi, protozoan parasites and helminths, and insect and mites are explained in Sections 3–6, respectively. Section 7 discusses the clinical infectious disease challenges, whereas clinical immunology is covered in Section 8. Laboratory tests and treatment protocols are the main focuses of Sections 9 and 10, respectively.

Encyclopedia of Infection and Immunity is the result of valuable contribution of more than 400 scientists from many well-known universities/institutes worldwide. I would like to hereby acknowledge the expertise of all contributors, for generously devoting their time and considerable effort in preparing their respective chapters. I would also like to express my gratitude to Elsevier for providing us the opportunity to publish this Encyclopedia.

Finally, I hope that this comprehensive book will be of special value for researchers and clinicians who wish to extend their knowledge on infection and immunity.

Nima Rezaei, MD, PhD

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General Concepts of Immunity

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Introduction to immune system

Maintaining homeostasis in response to environmental alterations is a significant challenge for living organisms. Elimination of invading pathogens and damaged or harmful cellular contents is an inevitable challenge that all organisms have to contend with it. The body's defense against the potential dangers and re-establish homeostasis rely on "immune system" which consists of the cells and molecules responsible for protecting the host body from harmful agents. In this way, the comprehensive and organized response of immune system components leads to the formation of an "immune response." Although an ideal immune response would eradicate the imminent danger and restore homeostasis to affected tissues, its improper activity can also be detrimental and cause tissue damage. Hence, the regulation of immune responses is crucial, allowing the organism to modulate the intensity and duration of the responses (Chaplin, 2010).

To provoke an efficient response, the immune system must recognize a wide range of infectious agents named pathogens such as viruses, bacteria, parasites, and fungi and discriminate them from the host's own tissues. This process is performed by a variety of receptors expressed on the immune cells (Medzhitov and Janeway, 1998). Any substances recognized by immune system components named "Antigen" and the specific part of an antigen recognized by immune receptors is called "Epitope." One of the unique features of the immune system is the ability to distinguish between "self" and "non-self" antigens. To prevent inappropriate reactions, the immune system must have tolerance against host's own molecules. This is an important event which states the concept of "immune tolerance" theory. Disruption of self-tolerance or inappropriate regulation of immune responses could lead to immune reaction to host cellular components resulting in a state known as "autoimmunity" (Chaplin, 2010).

In overall, immune system can be categorized into two types include “innate” and “adaptive” immunity. The innate immune system is the first line of defense which quickly activated against pathogens. This type of immune response reacts in the same way to all foreign antigens, which is why it is named as the “non-specific” immunity. However, an efficient immune response requires the involvement of adaptive immunity. The adaptive immune system specifically recognizes the antigens and responds slower than innate immune system. The advantage of adaptive immunity is the production of “memory” cells which can react immediately against pathogens. Therefore, when a known antigen is encountered for second time, the adaptive immune system can react faster (McCullough and Summerfield, 2005). In this article, we aimed to describe the basic concepts of immune system including the important components of innate immunity and adaptive immunity as well as the basic effective mechanisms in order to pathogen elimination.

Innate immunity: The first line of defense against pathogens

As an old evolutionary defense mechanism, the innate immune system or native immunity, provide the first line of defense against pathogens. Innate immunity is responsible for respond immediately (within minutes) to microbial components and harmful agents caused by cellular stress or tissue damage. Innate immune system consists of the cellular and biochemical components that are present active even before infection. Although some of them including complement system and cellular compartments become active upon danger exposure. An important feature of the innate immune system is its ability to activate the adaptive immune system (Tosi, 2005; Beutler, 2004).

Innate immune response provides the immunity against pathogens through several ways including:

1. Pathogens blocking from entering the body.
2. Pathogen uptake by phagocytosis or pinocytosis.
3. Pathogen killing via cytotoxic mediators.
4. Increased recruitment of the other immune cells by production of inflammatory mediators.
5. Pathogen processing and antigen presentation to adaptive immune cells.

Effector mechanisms of innate immune response

Recognition of conserved molecular patterns of pathogens

Innate immune response relies on the recognition of molecular patterns existed on the pathogens or damaged associated molecules which produced during injury, infection, or disease condition. These molecular patterns are including “pathogen-associated molecular patterns” (PAMP) such as lipopolysaccharide (LPS) of Gram-negative bacteria, peptidoglycan of bacterial cell wall, and viral RNA; and “damaged-associated molecular patterns” (DAMP) such as heat shock proteins (HSPs) and high mobility group box 1 (HMGB1). PAMPs or DAMPs could be recognized by non-specific receptors named “pattern recognition receptors” or PRRs expressed on immune system components. Several types of PRRs including Toll-like receptors (TLR), nucleotide-binding oligomerization domain (NOD)-Leucine Rich Repeats-containing receptors (NLR), retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLR), and the C-type lectin receptors (CLRs) have been identified which are differently diffused in sub cellular compartments such as cytosol, cell membrane, and endosomal membrane. Ligand-induced activation of PRRs initiates inflammatory responses resulting in the promotion of pathogen elimination as well as tissue homeostasis (Mogensen, 2009).

Phagocytosis: A killing mechanism by phagocytes

Phagocytes are the major innate immune cells act as the first line of defense against microorganisms. The important phagocytes are monocytes and macrophages, neutrophils, eosinophils, basophils, mast cells, and dendritic cells. Anatomically they are abundantly found in the locations where they encounter most pathogens, such as submucosal tissue of the respiratory system, gut, skin, and urogenital tract (Rabinovitch, 1995).

The initiation of phagocytosis is dependent on the interaction of microbial surface with certain receptors on the phagocytes. Subsequently, the bounded microorganism is surrounded by the cell membrane of the phagocytes and internalized in a vesicle named “phagosome.” Then, lysosomes which are enriched in antimicrobial enzymes are fused into phagosome leading to the formation of “phagolysosome.” In this context, pathogen killing would occur through the enzymatic process or by the influence of antimicrobial peptides present in phagolysosomes (Fig. 1) (Flannagan et al., 2012).

Several receptors have been identified that stimulate the phagocytosis and intracellular killing of pathogens. “Dectin-1” is expressed by macrophages, neutrophils, and dendritic cells which recognizes the polymers of glucose especially on the fungal cell wall (Herre et al., 2004; Dambuza and Brown, 2015). Furthermore, “Mannose receptor” (MR) is expressed by macrophages and dendritic cells which recognizes mannose residues in bacteria, virus, and fungi (Ezekowitz et al., 1990). Also, “Scavenger receptor” (SR) on macrophages identifies different anionic polymers and low-density lipoproteins (LDL) (Peiser et al., 2000).

Phagocytosis could be enhanced by binding of some components named “opsonins” to the pathogen. Immunoglobulins and complement fragments (e.g., iC3b) are identified as the key opsonin molecules which induce efficient phagocytosis (Rosales and

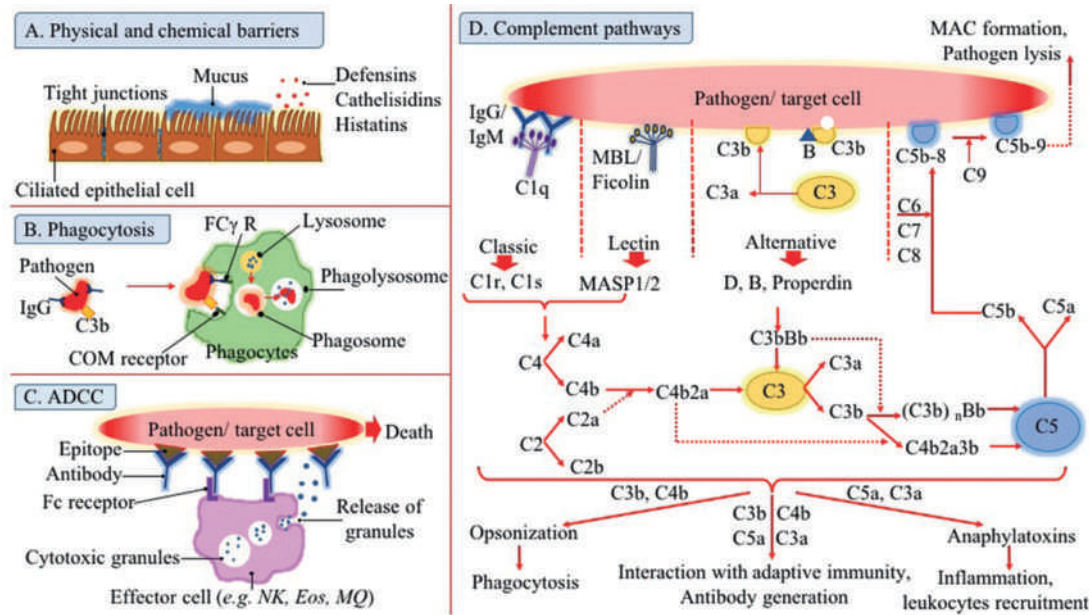


Fig. 1 The effector mechanisms of innate immune system. *Ig*, Immunoglobulin; *ADCC*, antibody-dependent cell cytotoxicity; *NK*, natural killer cell; *Eos*, eosinophil; *MQ*, macrophage; *COM*, complement.

Uribe-Querol, 2017). Therefore, complement receptors (CRs) and Fc receptors (FCRs) are the other receptors that facilitate phagocytosis and enhance pathogen killing function (Fig. 1) (Rosales, 2007).

During the pathogen killing by phagocytosis, some toxic compounds are produced that including superoxide (O_2^-), hydroxyl radical, nitric oxide (NO), hypochlorite (HOCl), and hydrogen peroxide. This process is accompanied by a mechanism known as the “respiratory burst” which included a transient increase in oxygen consumption by the phagocytes. There is another mechanism to pathogen take up known as “macro-pinocytosis.” In this way, large amounts of extracellular fluid along with the microorganism are ingested (Uribe-Querol and Rosales, 2017).

Inflammatory response: A process induced by pathogen recognition and tissue damage

Recognition of pathogens or damage-related molecules elicits an inflammatory response within initial hours of infection that results in increased infiltration of leukocytes to the affected tissue as well as elevated production of acute phase proteins (APPs). This response is accompanied by heat, redness, pain, and swelling at the site of infection (Kotas and Medzhitov, 2015; Nathan, 2002). Interestingly, inflammatory signaling induces blood clotting in small vessels at the site of infection which plays an important role in preventing the dissemination of infection throughout the body (Levi et al., 2003).

During inflammatory response, immune cells produce a variety of inflammatory mediators including the proteins or glycoproteins named “cytokines” and “chemokines.” Interleukin-1 (IL-1), IL-6, and tumor necrosis factor- α (TNF- α) are the most important pro-inflammatory cytokines produced by innate immune cells. These mediators increase the vascular permeability and the recruitment of the other innate or adaptive immune cells leading to the promotion of immune response against pathogen or injury (Gilroy et al., 2004; Headland and Norling, 2015). In this context, inflammatory response induces the expression of cell-adhesion molecules on the endothelial cells leading to the promotion of leukocytes binding and their migration from blood stream to affected tissue, a mechanism known as “extravasation.” Meanwhile, Neutrophils are the first leukocytes attracted to the infection site (Mitroulis et al., 2015).

Surface molecules of microorganisms also activate complement system leading to production of complement-associated molecules such as C5a or C3a which act as “anaphylatoxins” and increase the recruitment of immune cells to the infection site (Merle et al., 2015).

Complement system

Complement system as a pivotal effector mechanism of innate immune system is capable of destruction of a variety of microorganisms including bacteria, viruses, and parasites. This system consists of more than 30 soluble and cell surface proteins (Fig. 1) (Merle et al., 2015). Upon stimulation, these proteins being activated and initiate a cascade which results in:

1. Pathogen destruction by forming pores on the cell membrane of microorganism
2. Increased recruitment of inflammatory immune cells by production of “anaphylatoxins”
3. Enhanced phagocytosis by production of opsonins

The complement system could be activated by three pathways including:

1. Classical pathway: activated by the immune complexes of antigen-antibody
2. Alternating pathway: activated by the spontaneous hydrolysis of C3 component
3. Lectin pathway: activated by the recognition of certain carbohydrates on the microbial surface

An immune complex which consists of antibodies (IgG1 or IgM) attached to the cell surface of pathogens is capable of activating the classical pathway. In this manner, C1 complex (C1q, C1r, C1s) binds to the Fc region of antibodies. Upon activation, C1s acts as a serine protease and cleaves C4 and C2 into C4a, C4b and C2a, C2b, respectively. Subsequently, C4b along with C2a form C4bC2a complex which interacts with pathogen surface. This complex (C4bC2a) acts as an enzyme named "C3 convertase" which cleaves C3 into C3a and C3b fragments. This step is the point in which all complement pathways converge. C3b fragment covalently binds to the microbial surface and its association with C4bC2a makes a complex (C4bC2AC3b) named C5 convertase which in turn converts C5 into C5a and C5b. Binding of C5b to the microbial surface leading to activation of other components including C6, C7, C8, and C9. Ultimately, polymerization of C9 on the pathogen surface results in the formation of several pores named "membrane attack complex" or MAC which can directly lyse targeted pathogen. The lectin pathway is similar to classical pathway, but its activation is antibody-independent. In this pathway, pattern recognition receptors (PRRs), such as mannose-binding lectin (MBL) and ficolins recognize the carbohydrates on the microbial surface. MBL is complexed with MBL-associated serine proteases (MASPs)-1, -2, and -3 which their activation results in cleavage of C2 and C4, and subsequently the generation of the C3 convertase (C4bC2) of both classical and lectin pathways. The alternative pathway is activated with the spontaneous hydrolysis of C3 into C3a and C3b. In the presence of microbial infection, C3b binds to the microbial surface. Subsequently, the association of B and D factors with C3b leads to additional C3 cleavage and formation of alternative pathway C3 convertase (C3bBb) and C5 convertase (C3bBbC3b). A protein named "properdin" stabilizes alternative pathway convertases and facilitates alternative activation. Anaphylatoxins including C4a/C3a/C5a are potent inflammatory molecules which possess several functions such as increases in vascular permeability, leukocyte infiltration, smooth muscle contraction, phagocytosis and production of inflammatory mediators such as pro-inflammatory cytokines (Merle et al., 2015).

Antibody-dependent cell cytotoxicity (ADCC): An extracellular killing mechanism

ADCC could be considered as an extracellular killing mechanism resulting in pathogen elimination. This process requires: (1) immune cells expressing Fc receptor on their surface, and (2) target antigen coated by antibody (Fig. 1). Immunoglobulin G (IgG) and its receptor named FcγRIII (CD16) play an important role in ADCC activation. NK cells which express high levels of CD16 are regarded as the key players in this process. However, in addition to NK cells, macrophages, neutrophils, and eosinophils are able to mediating ADCC (Hubert et al., 2011; Siders et al., 2010; Valerius et al., 1990).

IgG possess a bifunctional structure which is related to the fragment antigen-binding (Fab) and Fc portions of antibody. ADCC is initiated by the engagement of Fab and Fc regions of antibodies with the pathogen and FcγR on effector cells, respectively. Subsequently, degranulation of effector cells (mainly NK cell) leading to pathogen lysis (Nigro et al., 2019).

The components of innate immune system

Physical and chemical barriers

The innate immune system includes anatomical and chemical barriers that impede microbes from entering the body. Epithelial cells which comprise the skin and the lining of the respiratory, gastrointestinal, and genitourinary tracts act as the first line of defense against pathogens. In addition to forming the tight junctions, epithelial cells produce a large amount of "mucus" enriched glycoproteins known as "mucins" which traps microorganisms and prevent their dissemination (Fig. 1). Epithelial cells also provide a chemical barrier by producing various chemical substances with antimicrobial properties. Digestive enzymes and acidic pH of the stomach, lysozyme, phospholipase A2, defensins, cathelicidins, and histatins are identified as the important chemical barriers to infection (Schleimer et al., 2007).

"Lysozyme" is a glycosidase enzyme in tears and saliva which has the ability to disruption of peptidoglycan present in the bacterial cell wall especially in Gram-positive bacteria. Paneth cells, a type of epithelial cells in the small intestine produce several microbiocidal proteins such as lysozyme and phospholipase A2 responsible to killing the pathogens in the gut lumen (Sukhithasri et al., 2013).

"Defensins" as the cationic peptides form a pore in the cell membrane of pathogens like bacteria and fungi as well as the viruses' envelope. Two distinct types of defensins with different activities and structure are implicated in humans including α - and β -defensins. Neutrophils and paneth cells produce α -defensins. The production of β -defensins is induced by infection considerably in skin, airway and urogenital tracts (Ganz and Lehrer, 1997; Ouellette and Selsted, 1996).

"Cathelicidins" are antimicrobial peptides produced by macrophages, neutrophils, mast cells, NK, T, B, sebocytes, and epithelial cells in response to infection (Roby and Di Nardo, 2013).

"Histatins" as the important anti-fungal peptides have a key role in host defense in the oral cavity. Histatins are primarily produced in response to pathogenic fungi such as *Cryptococcus neoformans* and *Candida albicans* (Edgerton and Koshlukova, 2000).

Innate immune cells

The most cells of the innate immune system are derived from myeloid lineage including neutrophils, eosinophils, basophils, mast cells, monocytes, macrophages, and dendritic cells. While others, including NK cells, natural killer T (NKT) cells, and innate lymphoid cells (ILCs) are originated from lymphoid lineage. These cells with distinct phenotypes contribute to pathogen killing through especial functions. Table 1 lists the innate immune cells and their effector mechanisms in host defense.

Monocyte and macrophage

Monocytes and macrophages are the important components of innate immune system which possess various inflammatory and regulatory capabilities. These cells are known as professional phagocytes and play the key roles including cytokine production, antibody-dependent cell cytotoxicity (ADCC), antigen presentation, T cell proliferation and activation, tissue repair and regeneration, resulting in pathogen elimination and tissue homeostasis.

Monocytes are developed in bone marrow and then circulate in the blood stream. These cells emigrate to different tissues throughout the body where they differentiate to macrophages and myeloid-derived dendritic cells. Three subpopulations of monocytes with distinct phenotypes have been found in humans which exhibit different functions. These are including classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺⁺CD16⁺), and non-classical (CD14⁺CD16⁺⁺) monocytes (Ziegler-Heitbrock et al., 2010). Classical type comprises the largest population of monocytes that are highly skilled in phagocytosis and antibody-dependent cell cytotoxicity (ADCC). These cells are important in initiating the inflammatory responses. Non-classical types are known as anti-inflammatory monocytes that are involved in maintaining vascular homeostasis. Intermediate monocytes have several

Table 1 The major components and important functions of innate and adaptive immune systems.

Immune system	Components		Functions
Innate immunity	Physical and chemical barriers		Such as skin, mucus membrane, chemical secretions Prevent pathogens from entering the body, pathogen killing via cytotoxic mediators
	Monocytes and macrophages		Phagocytosis, act as professional APC, ADCC, T cell proliferation and activation, tissue repair and regeneration
	Dendritic cells		Initiating and polarization of the immune responses phagocytosis or macropinocytosis act as professional APC, ADCC, T cell proliferation and activation
	Neutrophils		Most numerous innate immune cells, phagocytosis, ADCC, promotion of both innate and adaptive immune responses formation of NETs
	Eosinophils, Basophils, Mast cells		defense against parasitic infections and allergic reactions, ADCC
	Innate lymphoid cells		promotion the adaptive immune responses, responding to pathogenic tissue damage, tissue homeostasis, repair and regeneration
	Natural killer cells		strong cytotoxic function importantly against virus-infected cells and cancerous cells, ADCC
Adaptive immunity	Natural killer T cells		recognize the lipid derivatives of pathogens presented by CD1 molecule, activation of other cells in innate and adaptive immune system
	B cells	B1 cells	Production of natural antibodies without the presence of any infections
		Follicular B cells	Most frequent B cells in the circulation, production of the majority of antibodies during an infection
		Marginal zone B cells	Defense against blood-born pathogens
	T cells	CD4 ⁺ T helper	Th1: Defense against intracellular pathogens, cytokine production, enhance phagocytosis, inducing B cells to make IgG1 and IgG3 antibodies
			Th2: Defense against extracellular pathogens, cytokine production, inducing B cells to make IgG4 and IgE antibodies
	Antibodies	CD8 ⁺ T cytotoxic	Defense against intracellular pathogens and cancerous cells, enhance phagocytosis
		IgG	The major antibody involved in opsonization and neutralization IgG have four subclasses: IgG1, IgG2, IgG3, IgG4
		IgA	High levels in mucosal tissues, saliva, tear, breast milk Have neutralization activity and moderate opsonization capacity, complement activation
		IgM	The first produced antibody upon infection Acts as membrane bound receptor for B cell Excellent complement activation
		IgD	No opsonization, moderate neutralization Membrane bound receptor for B cell
		IgE	No opsonization and neutralization activities The major antibody involved in parasitic infections and inflammatory conditions including allergy and asthma No opsonization, moderate neutralization

APC, Antigen presenting cell; ADCC, antibody-dependent cell cytotoxicity; TH, T helper; Ig, immunoglobulin; NETs, neutrophil extracellular traps.

functions including the production of reactive oxygen species (ROS), T cell activation by cytokine production and antigen presentation (Sampath et al., 2018).

Macrophages are found approximately in all tissues in the body. These cells are capable to recognition of the pathogens or damaged cells by several surface receptors such as SR, TLR, and NLR (Taylor et al., 2005).

Macrophages could be classified in to two important subtypes, including “M1 or classically activated macrophages” and “M2 or alternatively activated macrophages” that are different in their surface markers, produced cytokines, and functions (Yao et al., 2019).

M1 macrophages exert pro-inflammatory, anti-microbial and anti-tumoral activities, while M2 macrophages exhibit anti-inflammatory and immunomodulatory properties, tissue-repairing, pro-tumoral and pro-angiogenic functions, and possess effective phagocytic activity.

Macrophage polarization is highly dependent on the signals in the microenvironment. For example, LPS and cytokines of Th1 such as interferon gamma (IFN- γ) and TNF- α are identified as the main stimulator of M1 polarization. On the other hand, M2 macrophages are thought to be associated with Th2 cytokines such as IL-4, IL-13, IL-10, IL-33, and TGF- β (Yao et al., 2019).

M1 macrophages are produced in response to intracellular bacteria like *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Salmonella typhi*, *Salmonella typhimurium* (Benoit et al., 2008). These cells are characterized by the expression of CD80 (B7.1), CD86 (B7.2), MHC II, TLR2, and TLR4. During inflammatory responses, M1 macrophages produce pro-inflammatory cytokines and chemokines such as IL-1, IL-6, TNF- α , IL-12, CCL15, CCL20, CXCL9, and CXCL10 upon ligand recognition and promote the Th1-related responses (Yao et al., 2019).

M2 macrophages are mostly related to chronic infectious diseases like chronic brucellosis, chronic mycobacterial infections (Benoit et al., 2008). M2 macrophages secrete anti-inflammatory cytokines such as IL-10, TGF- β and they can stimulate Th2 responses. These cells express the markers including mannose receptor (CD206), CD163, CD209 (DC-SIGN) (Yao et al., 2019).

Dendritic cell

Dendritic cells are tissue-resident or circulating leukocytes which known as the important cells in making a linkage between innate and adaptive immune responses. These cells are important in initiating and polarization of the immune responses. Several functions of DCs are including:

1. Pathogen uptake through phagocytosis or pinocytosis
2. Antigen processing and its presentation to T cells resulting in triggering the adaptive immune responses; these cells are known as the professional antigen presenting cells (APCs)
3. Cytokine production

Functionally, DCs are divided into two types including immature and mature DCs (Hawiger et al., 2001; Steinman et al., 2003). Immature DCs are skilled in antigen (Worbs et al., 2017) phagocytosis or pinocytosis, while mature DCs have the ability to activate T cell responses. DC maturation is initiated by the recognition of pathogens or damage-associated molecules by mediating several receptors such as TLRs (Hemmi and Akira, 2005; Cerboni et al., 2013). Subsequently, they migrate from peripheral tissues to secondary lymphoid organs (e.g., lymph node) where they can activate T cell responses by acting as an APC. Mature DC express high levels of MHCII, CCR7, and co-stimulatory molecules which are essential for T cell activation. Besides, increased levels of pro-inflammatory cytokines have shown to be produced by mature DCs compared with immature DCs. The interaction between DCs and T lymphocytes leading to differentiation of T cells to different subtypes including Th1, Th2, Th17, or regulatory T cells (Treg) which can be influenced by cytokine profile in the tissue microenvironment or the type of recognized pathogen. For example, high levels of IL-12 and IL-4 in microenvironment have shown to be associated with Th1 and Th2 differentiation, respectively (Patente et al., 2019).

Heterogeneous population and different subtypes of DCs have been identified with different origin, phenotypes, and biological functions. Two main subtypes of DCs are including conventional (cDCs) and plasmacytoid dendritic cells (pDCs). cDCs (CD11c⁺ CD123⁻) are regarded as having high ability for antigen uptaking and presentation to naïve CD4⁺ T cells and shown to produce pro-inflammatory cytokines (Haniffa et al., 2013). pDCs (CD11c⁻ CD123⁺) are well-known for the production of type I interferon (IFN-I) during viral infection, although they also have other activities such as stimulation of T cells and NK cells and the production of pro-inflammatory cytokines (Swiecki and Colonna, 2015). In contrast to conventional DCs, these cells are abundantly found in blood stream.

In addition, monocytes could be differentiated to dendritic cells known as monocytes-derived DCs, during infection and inflammation (Schlitzer et al., 2015).

Neutrophil

Neutrophils are regarded as the most numerous innate immune cells which act as the first line of defense against infection. In response to pathogens, neutrophils rapidly migrate from the blood stream to the damaged tissue. CXCL8 as a chemotactic factor plays the important role in their recruitment. These cells play the important role in initiation of the inflammatory response importantly by phagocytosis and release the cytotoxic granules (Rosales et al., 2016). Some pathogens such as *Staphylococcus aureus* is shown to evade phagocytosis and therefore have the potential to suppress defense by neutrophils (Kumar and Sharma, 2010).

Neutrophils display the important functions resulting in the promotion of both innate and adaptive immune responses. Elimination the extracellular pathogens by phagocytosis is the most important function of neutrophils (Rosales et al., 2017). Upon stimulation with various stimuli such as microbial components or increased level of intracellular Ca²⁺, neutrophils release

their granules by a process known as “exocytosis” resulting in pathogen killing. Their granules enriched with various antimicrobial molecules such as cationic peptides, proteases, lactoferrin, myeloperoxidase, gelatinase, cathepsin G, elastase, proteinase 3, and azurocidin (Kumar and Sharma, 2010).

Deficiency in neutrophil-associated granules leads to the increased susceptibility to Gram-positive or Gram-negative bacterial infections. For example, cathepsin G-deficient and elastase-deficient mice are more susceptible to the bacterial infections by *Staphylococcus aureus* and *Escherichia coli*, respectively (Kumar and Sharma, 2010).

In addition to phagocytosis, neutrophils can fight against pathogens by forming neutrophil extracellular traps (NETs). Formation of NETs is important to localizing the early infection. NETs are consisting of neutrophil nuclear material (e.g., chromatin), cytoplasmic and granular proteins. Extracellular release of these components leads to bacterial degradation. NETs play an important role in defense against infections including *Shigella flexneri*, *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, *S. aureus*, *Candida albicans*, fungus, and parasites such as *Leishmania amazonensis* (Kumar and Sharma, 2010).

Eosinophil

Eosinophils are the important cells in defense against parasitic infections and allergic reactions such as asthma, as well as in tissue homeostasis including tissue repair and remodeling. Upon stimulation by several stimuli such as cytokines, complement system proteins, and immunoglobulins, eosinophils are infiltrated from the blood stream to the inflamed tissue (Kita, 1996). Th2 immune response and their cytokines such as IL-4, IL-5, and IL-13 have the significant role in eosinophil recruitment and activation (Sher et al., 1990; Horie et al., 1997).

The important effector functions of eosinophils are including cytokine production, releasing cytotoxic granules, antibody- and complement-mediated cytotoxicity, phagocytosis, and eosinophil extracellular DNA traps. Eosinophils produce several cytokines and lipid mediators in response to pathogens resulting in the vascular permeability, constriction of smooth muscle, and increased secretion of mucus. These are including IL-6, IL-2, IL-8, TNF- α , INF- γ , TGF- α/β , GM-CSF, platelet-activating factor (PAF), leukotriene C4 (LTC4), and matrix metalloproteinases (MMPs). Upon activation, eosinophils release their granules containing different tissue-destructive mediators which contribute to promote the inflammatory response and killing parasitic pathogens. These include eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), major basic protein (MBP), and neurotoxin derived from eosinophils (EDN) (Shamri et al., 2011).

IgG1 and IgG3 immunoglobulins were found to be effective in promoting eosinophil-mediated killing. These antibodies by interacting with immunoglobulin receptor named Fc γ RII expressed on eosinophils, induce degranulation of eosinophils. This process is well-known as ADCC which regarded as a main extracellular killing mechanism (Kaneko et al., 1995).

In addition, eosinophils are shown to exhibit the antibacterial role by releasing the materials consist of mitochondrial DNA as well as cytotoxic granule-derived components which acts as traps in order to pathogen killing. LPS of Gram-negative bacteria considerably induce eosinophil-derived extracellular traps (Yousefi et al., 2008).

Basophil

Basophils are the major immune populations involved in allergy response and hypersensitivity reactions including asthma, anaphylaxis, and allergic rhinitis. Besides, they contribute to immunity against helminthes and bacterial respiratory infection (Chirumbolo et al., 2018). Basophil-mediated responses could also be evoked by several bacterial infections such as *S. aureus*, *non-hemolytic streptococci*, *Haemophilus influenzae*, *S. enteritidis* and *E. coli* (Abraham and Arock, 1998).

These cells are capable to activate adaptive immune responses importantly the stimulation of Th2 immune responses. Basophils produce IL-4 and IL-13 cytokines which promote Th2 responses resulting in hypersensitivity reactions. Upon activation by several stimuli such as immunoglobulin E (IgE) binding, basophils release their granules containing histamine, heparin, and serine proteases. In addition, they can synthesize and release other mediators including leukotrienes and prostaglandins (Chirumbolo et al., 2018).

Mast cell

Mast cells are primarily involved in pathophysiology of allergic reactions and their functional mechanisms are similar to basophils. In contrast to basophils which are found in circulatory system, mast cells are mostly located in the skin, mucosal membranes and around blood vessels. Mast cells possess central functions in response against bacterial infections and certain parasitic infections. They can directly (opsonin-dependent) or indirectly (opsonin-independent) bind to the bacteria or parasites. In the indirect recognition (or opsonin-dependent), mast cells recognize the opsonized pathogens with complement fragments (e.g., iC3b) or immunoglobulins. *Salmonella typhimurium* and the helminth, *Schistosoma mansoni* have been reported to recognize by mast cells via this mechanism. In direct interaction, mast cells possess a striking capacity to recognize and bind to pathogen through their receptors without mediating opsonins. For example, *E. coli* and *K. pneumonia* and *Leishmania* parasites have been recognized to interact directly with mast cells (Abraham and Arock, 1998).

Similar to basophils, mast cells are highly skilled for the synthesis and production of several active mediators including histamine, heparin, serine proteases, leukotrienes, prostaglandins, cytokines, and chemokines in response to certain microorganisms (Galli et al., 1991; Galli, 1993). They produce various cytokines including IL-1, IL-6, TNF, IL-4, IL-5, IL-13, and chemokines MCP-1, MIP-1, RANTES. These mediators play central roles in the promotion of inflammatory responses, pathogen killing, enhanced phagocytosis, and development of adaptive immune responses. Besides, granule exocytosis contribute in pathogenesis of several allergic disorders causing hypersensitivity symptoms (Abraham and Arock, 1998).

Innate lymphoid cells (ILCs)

ILCs are a family of innate immune cells which primarily considered as the innate counterparts of T cells. They are tissue resident cells and abundantly found in mucosal surfaces especially in intestine and lung, and contribute in mucosal immunity and tissue homeostasis. ILCs are involved in several functions including promotion the adaptive immune responses, responding to pathogenic tissue damage, tissue homeostasis, repair and regeneration (Mjösberg and Spits, 2016; Ebbo et al., 2017).

ILCs are divided into three main groups based on their phenotype, cytokine pattern, and their developmental pathways, including ILC1, ILC2, and ILC3 (Vivier et al., 2018). ILC1s are significantly contributed to the promotion of macrophages and DCs activities and Th1 responses. In this context, they produce IFN- γ in response to up-regulation of IL-15 and IL-12 during infection or tissue injury. IFN- γ enhances the elimination of intracellular bacteria as well as antigen presentation by macrophages and DCs (Fuchs et al., 2013).

ILC2s are involved in the immune response against parasites infection and repair the damage caused by parasitosis or viral infections. They secrete cytokines such as IL-4, IL-5, IL-9, IL-13, and amphiregulin in response to IL-25, IL-33, and TSLP which promote Th2 responses and innate defense against parasites (Panda and Colonna, 2019).

ILC3s play an important role in response to extracellular bacteria and fungi. They produce IL-22 and IL-17 in response to IL-23 and IL-1 β and induce Th17 responses and neutrophil recruitment. These cells by the production of IL-22 contribute in the up regulation of antimicrobial peptides and enhancement of barrier integrity especially in the intestine (Aujla et al., 2008; Sugimoto et al., 2008; Zheng et al., 2008).

Natural killer cell (NK)

NK cells are a subset of innate lymphocyte cells that characterize by the strong cytotoxic function importantly against virus-infected cells and cancerous cells. The dominant population of NK cells express CD16 and CD56 molecules (Lanier et al., 1986, 1989). In respect to the NK cell phenotypes, two major subsets of NK cells are identified in humans in according to the expression of CD16 and CD56 markers as follows (Stabile et al., 2017):

1. CD16^{High}CD56^{Low} NK cells possess high levels of perforin mediating cellular cytotoxicity leading to the enhancement of pathogen killing
2. CD16⁺/CD56^{High} NK cells are characterized by the low levels of perforin and they are highly skilled in cytokine production

NK cells have multiple activating (e.g., KIR2DL, KIR3DL, NKG2A, CD85, and CD244) and inhibitory receptors (e.g., NKG2C, NKG2D, KIR2DS, KIR3DS, NKP30, NKP44, and NKP46) allow them to recognize the target cells. Upon activation, NK cells produce several cytokines and chemokines including IFN- γ , TNF- α , granulocyte macrophage colony-stimulating factor (GM-CSF), CCL1-CCL5, and CXCL8 leading to regulation of the function of other innate and adaptive immune cells (Paul and Lal, 2017).

MHC class I molecule which express normally on healthy cells is considered as a ligand for NK-inhibitory receptor and their interaction results in NK cell tolerance and inactivation. On the other hand, decreased level of MHCI is expressed on the virus-infected cells or tumor cells leading to engagement of the activating receptors and propagates cytotoxic activity of NK cells (Lanier, 2008).

NK cell cytotoxic response is dependent on forming an immunological synapse between NK cell and target cell. Upon activation, NK cells release their cytotoxic granules containing "perforin" and "granzyme." Released perforin by polymerization on the cell membrane of target cells forms the pores which facilitates the entry of granzymes into the target cells. Granzyme as a serine protease activates the enzymes known as "caspase" responsible to induction of cell death or "apoptosis" of target cells (Topham and Hewitt, 2009). NK cell-mediated apoptosis of target cells could also perform by engagement the receptors named "death receptor." These receptors such as TNF receptor and Fas receptor bind to cognate ligands on target cells and induce apoptosis of target cells (Sonar and Lal, 2015; Thorburn, 2004).

One of the important NK cell receptors, CD16 or Fc γ RIII, mediates an extracellular death named as antibody-dependent cell-mediated cytotoxicity (ADCC). This receptor by binding to Fc region of antibodies especially IgG, induces the degranulation of NK cells and promote killing the target cells (Mandelboim et al., 1999).

Natural killer T cell (NKT)

NKT cells are a subgroup of innate immune cells with cytotoxic functions which express NK cell markers and a type of T lymphocyte receptor named $\alpha\beta$ T cell receptor or $\alpha\beta$ TCR. These cells can early activate in response to IL-12 and IL-18 produced during microbial infections and is responsible to recognize the lipid derivatives of pathogens presented by CD1 molecule, a non-polymorphic MHC class I molecule on APCs. Upon activation, NKT cells secrete IFN γ and IL-12 resulting in the activation of other cells in innate and adaptive immune system including T cells, NK cells, and DCs (Terabe and Berzofsky, 2008). Gram negative-derived LPS is implied as the important stimulus for activating NKT cells. However, LPS-negative bacteria also have the ability to induce NKT responses (Van Kaer and Joyce, 2005). NKT cells are also contributed to response against parasitic infections like *Leishmania* and viral infections (Terabe and Berzofsky, 2008).

Adaptive immunity

Adaptive immunity, also known as acquired immunity or specific immunity is the second line of defensive response against invading pathogens. This type of immune response occurs to attack non-self pathogens however sometimes may mistakenly attack

self-tissues which results in autoimmune diseases such as lupus, rheumatoid arthritis, and etc. The difference between adaptive immunity and innate immunity is that the adaptive immune response is not immediately started as innate immune response occurs. However, the effect of the adaptive immune response is long-lasting and highly specific.

The adaptive immune system can learn and remembers specific pathogens, thus it can provide long-lasting defense and protection against recurrent infections. While the adaptive immune system is encountered to a new threat, some antigens are memorized to prevent from getting the disease again. One example of immunological memory is vaccinations. Both attenuated whole virus/bacteria and their particles cannot actually cause an active infection but cause an immune response. In other words, fail in the immunological memory system can result in autoimmune diseases. Molecular mimicry of a self antigen by an infectious foreign pathogen, including viruses and bacteria, can trigger autoimmune disease because of a cross-reactive immune response against the infection. *Streptococcus* infection is a good example in which a foreign organism uses molecular mimicry to hide from immunological responses (Chaplin, 2010). Furthermore, the activity of the adaptive immune system relies on the clonal expansion of T and B lymphocytes and also immune response mediators.

In overall, there are two types of the adaptive immune system (Fig. 2):

1. Cell-mediated immunity (or cellular immunity) that occurs against cells infected with viruses, intracellular bacteria, and cancerous cells, and mediated by helper and cytotoxic T lymphocytes.
2. The humoral immunity (or antibody-mediated immunity) is an efficient immunity against extracellular or circulating antigens. This type of immunity involves B cells differentiation into plasma B cells with aid of Type 2 helper T cells ($T_H 2$) that subsequently can produce immunoglobulins called antibodies.

In this section, we discuss in more detail about the components of adaptive immune system and their mechanisms of action in the specific immune response against pathogens.

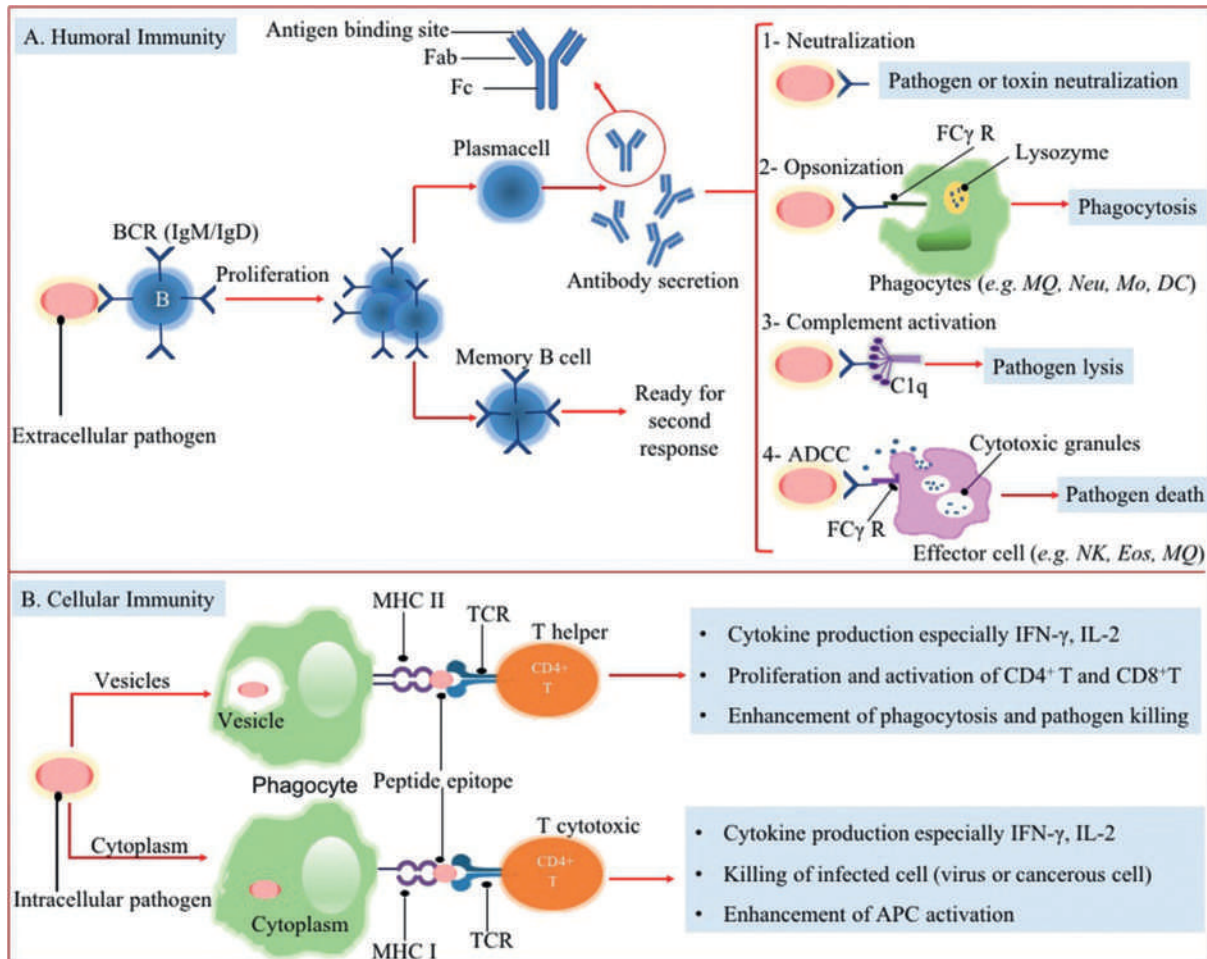


Fig. 2 The effector mechanisms of adaptive immune system and the relation between innate immunity and adaptive immunity. BCR, B cell receptor; TCR, T cell receptor; Fab, fragment antigen-binding region; Fc, fragment crystallizable region; Fcγ R, Fc gamma receptor (receptor for IgG); MQ, macrophage; Neu, neutrophil; MO, monocyte; ADCC, antibody-dependent cell cytotoxicity; MHC, major histocompatibility complex; IFN-γ, interferon gamma; IL-2, interleukin-2; APC, antigen presenting cell.

Adaptive immune cells

The adaptive immune system depends on B and T cells responses. These cells are lymphocytes derived from specific types of bone marrow stem cells, known as multipotent hematopoietic stem cells. Upon formation in the bone marrow, these cells need to mature and activate. In this regard, each type of cell follows different developmental stages to become mature. T cells migrate to the thymus in where they complete their maturation into functional T cells. While, the development of an important part of B cells takes place in the fetal liver and bone marrow and then the immature B cells migrate to the spleen for their final stages of maturation (Huston, 1997).

B and T cells possess antigen-specific receptors which enable them to recognize specific foreign antigens. In this respect, both B cell receptor (BCR) and T cell receptor (TCR) use the process known as “gene rearrangement” to provide the necessary diversity of receptor specificities during developmental process of T and B cells in thymus and bone marrow, respectively (Gellert, 2002).

B lymphocytes

The mature B cells or naïve B cells have distinctive surface antigen-specific receptors (BCR) called membrane-bound antibodies. These surface antibodies are primarily IgM and IgD which can recognize epitopes associated with different types of antigenic structures including proteins, polysaccharides, and lipopolysaccharides. B cell receptors play important roles in antigen binding, internalization and processing of the antigen, and inducing signaling pathways such as the cytokine production pathway in order to communicate with other cells of the immune system.

B cell activation occurs upon antigen recognition in the secondary lymphoid organs such as lymph nodes or spleen. After binding an antigen to BCR, B cells quickly divide to either a memory B cell or an effector B cell (or plasma cell). Memory B cells express the membrane-bound antibody, while plasma B cells can secrete antibodies. Secreted antibodies identify and neutralize the free pathogens circulating throughout the body (Fig. 2) (Pieper et al., 2013).

Two sub-classes of B cells have been identified with distinct phenotype and function including B1 and B2 cells. B1 cells are found in the peripheral organs and they less commonly present in the blood. They are highly skilled in the production of natural antibodies without the presence of any infections. B2 cells are divided into two types including B follicular (BFO) and B marginal zone (BMZ). BFOs are the most frequent B cells in the circulation. They produce the majority of antibodies during an infection. BMZs are found primarily in the marginal zone of the spleen and they are involved in defense against blood-borne pathogens (Pieper et al., 2013).

T lymphocytes

Unlike antibodies, T cell receptors can't bind to antigens directly. They recognize the antigens bound to the membrane surface molecules on APCs, known as Major Histocompatibility Complex class 1 (MHC I) and class 2 (MHC II). During a recombination process in their genes, T cell receptors undergo a gene rearrangement that allows them for diverse binding. However, in order to an appropriate immune response and avoid mistakenly attack against self antigens, mature T cells should recognize only pathogens' antigens bound to the MHC molecules. Therefore, T cells undergo two “selection processes” during their developmental stages in the thymus; Positive selection in which T cells should bind self-MHC: peptide complexes and thus distinguish between self and nonself peptides and proteins. T cells that bind moderately to MHC complexes on the thymic cells receive survival signals. If these cells bind too strongly to these MHC complexes, they fail the positive selection and should be eliminated through apoptosis mechanism in the process of “negative selection.” Negative selection guarantees self-tolerance by testing the specifically binding ability of T cells to self-MHC: peptide complexes. The critical importance of these two selection processes is the protection of own cells and tissues against immune response, and otherwise autoimmune diseases would occur much more common (Chaplin, 2010).

Effector mechanisms of adaptive immune response

In an adaptive immune response, the effector mechanisms by which pathogens are eliminated are essentially similar to those of innate immunity. On the other hand, specific recognition by clonally distributed receptors during an adaptive immune response seems to be evolved as a late additional mechanism to existing innate mechanisms of action.

Antibodies: The effector mechanism of humoral immunity

Antibodies, as the products of plasma B cells in adaptive immune system, are found in the body fluids such as whole blood, serum or plasma, and also extracellular fluids. Thus, immune response mediated by antibodies is known as humoral immune response.

As shown in Fig. 2, an antibody molecule has Y-shaped structure whose identical arms function as two antigen-binding sites known as “antigen binding fragment” or Fab. However, these sites are highly variable from an antibody molecule to another which results in diverse specific antigen recognition. The stem of the Y structure which referred as “fragment crystallizable region” or Fc is a constant region which determines the class of the antibody and its functional properties. Five classes of antibodies including IgG, IgM, IgD, IgE, and IgA have been identified that each class uses diverse distinct set of effector mechanisms for recognition and elimination of antigens (Table 1). Through a mechanism known as “neutralization,” antibodies neutralize pathogens or their toxic products by binding to them and thus preventing them to enter cells and subsequent their infection. This mechanism is critical for protection against bacterial toxins and also pathogens such as viruses. Another mechanism by which antibodies can response to pathogens is known as “opsonization.” By opsonization, antibodies enable phagocytes for ingesting and destroying the extracellular

bacterium. The phagocytes recognize the **Fc region** of the antibodies coating the pathogen and foreign particles (Fig. 2). The third effector mechanism of antibodies is activation of several plasma proteins known as complement system. However, the complement system can also be activated without the function of antibodies in case of many microbial surfaces. Thus, this mechanism has important roles in both innate and adaptive immunity. In fact, the complement components by pore formation in surface of **bacteria** can directly destroy them, a critical function for some bacterial infections. Nonetheless, the main role of complement system is coating the surface of pathogens and enabling phagocytes to ingest and digest bacteria (Forthal, 2015).

Antigen presentation: A mechanism for T cells activation which is mediated by MHC molecules

T lymphocytes receptors are specialized to recognize a foreign antigenic peptide fragment bound to an MHC molecule. MHC molecules are membrane glycoproteins which composed of two types including MHC class I and MHC class II. These molecules are expressed considerably in APCs including dendritic cells, macrophages, and B cells. They trap and carry their cargo of peptide and transport to the cell surface, where the peptide is displayed to T cells (Fig. 2) (Blum et al., 2013).

In overall, antigen presentation process by APCs can be divided into four steps:

1. Antigen recognition and its uptake by APCs
2. Digestion of antigens
3. Loading the peptide epitopes on the MHC molecules
4. Antigen presentation in the form of MHC: peptide complex

The most important differences between the two classes of MHC molecule are the source of the peptides. MHC class I molecules trap peptides derived from proteins synthesized in the cytoplasm, and are thus able to display fragments of viral proteins on the cell surface that are recognized by cytotoxic T cells. MHC class II molecules trap peptides derived from proteins in intracellular vesicles, and thereby display peptides derived from pathogens living in macrophage vesicles or internalized by phagocytes and B cells which eventually are recognized by Th1 or Th2 cells (Blum et al., 2013).

In order to antigen-specific activation of these T cells and distinguish between the two classes of MHC molecule, two types of co-receptors are involved. CD8 co-receptor is expressed in cytotoxic cells and binds MHC class I molecules. Meanwhile, Th1 and Th2 cells express the CD4 co-receptor which binds MHC class II molecules.

Upon recognizing their targets, the effector T cells are stimulated to produce and release various mediators to affect their target cells or help to recruit other effector cells. Such effector mediators include many cytokines have a crucial role in the clonal expansion of lymphocytes and also in innate immune system and in the effector actions of most immune cells (Blum et al., 2013).

T cell response: The effector mechanism of cellular immunity

Compared to the B cells, T cells have a variety of effector actions. Some pathogens including viruses, parasites, and some bacteria for replication needs to enter cells where they don't be detected by antibodies. T cells, as responsible for the cell-mediated immune responses in adaptive immunity, eliminate these intracellular pathogens.

There are two major types of T cells including helper T cell (T_H) and cytotoxic T cell (CTL). As the name indicates, helper T cells help other immune cells to respond against pathogen. T_H cells express CD4 molecule on their cell surfaces and have role in defending the body against pathogens by activate the other immune cells especially through producing cytokine including $IFN\gamma$, IL-2, and TNF. They recognize peptide antigens presented with the form of MHC II: peptide complexes by APCs (Chaplin, 2010).

$CD4^+$ T cells are divided into two major subsets. The first subset of $CD4^+$ T cells are Th1 cells that is important in the inhibition of intracellular bacterial infections. The important examples of such **bacteria** which grow only in the macrophages are *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the pathogens that related to tuberculosis and leprosy, respectively. Upon phagocytosis by macrophages, bacteria grow in intracellular membrane-bounded vesicles, and then destroyed in the lysosomes containing various enzymes and antimicrobial substances. These infections can be controlled by $CD4^+$ T cells that activate macrophages, and induce fusion of their lysosomes with the vesicles containing the bacteria and even also stimulate other mechanisms of the phagocyte in antibacterial action. In overall in order to pathogen clearance, Th1 cells play the important roles including:

1. Enhance macrophage activation and phagocytosis
2. Increase macrophage infiltration to the sites of infection
3. Promote the cytotoxic T cells and NK cells responses
4. Induce B cell responses to make opsonizing antibodies and complement-activating antibodies importantly IgG1 and IgG3

Compared to $CD4^+$ Th1 cells that destroy intracellular pathogens by activating macrophages, another subset of $CD4^+$ T cells called $CD4^+$ Th2 cells have a specialized role in destruction of extracellular bacteria, parasitic helminth infections through activating B cells. Besides, they are involved in inflammatory conditions such as asthma and allergy. They produce several cytokines including IL-4, IL-5, IL-9, and IL-13 which have an important role in controlling the extracellular helminth infections such as *Schistosoma mansoni* (Oliphant et al., 2011).

The important functions of Th2 cells in adaptive immunity are as follows:

1. Induce B cell responses to make antibodies IgG4 and IgE
2. Recruitment and activation of the inflammatory cells including basophils, mast cells, and eosinophils

Cytotoxic T cells are the essential subset of T cells involving in response against intracellular pathogens like viruses as well as cancerous cells. These cells express CD8 molecules on their cell surface and they can control the infection by directly killing the infected cells. They recognize peptide antigens presented with the form of MHC I: peptide complexes by APCs. Their killing mechanisms are depending on two types of cytotoxic proteins in their granules named as perforin and granzymes which allow them to attack and destroy infected cells. CD8⁺ CTLs produce IFN- γ which acts several functions including the inhibition of viral replication, increase MHC I expression, and macrophage activation (Chaplin, 2010).

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Organs and Tissues of the Immune System

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Introduction

The immune system is composed of a number of organs and tissues that work together to promote immune cell development and maturation or to concentrate immune responses against an antigen. The anatomical organization of immune cells in lymphoid tissues and their ability to circulate and migrate between blood, lymph, and tissues play an important role in triggering immune responses. In fact, the immune system must be able to respond quickly to the small number of pathogens that enter the body from anywhere. Therefore, the presence of organs involved in the concentration of immune cells and antigens is essential for an effective immune response.

The physical barriers act as the first line of defense against infection through restricting the ingress of pathogens into the body. These include the skin, mucosal epithelium, and the respiratory cilia. Besides, several chemical barriers are also effective in neutralizing offensive microorganisms and infections, including:

1. The fatty acids present in the sebaceous glands
2. Antimicrobial defensins
3. Hydrolytic enzymes present in body secretions
4. The acidic pH of gastric

In addition, there are special organs that compensate for the probable failure of the first line of defense through generating humoral and cellular responses. In general, the lymphoid organs can be divided into “primary” (e.g., bone marrow and thymus) and “secondary” organs (e.g., spleen, lymph nodes, and mucosa-associated lymphoid tissue) based on anatomical location and immunological role (Fig. 1) (Picker and Siegelman, 1999).

Development of the mammalian immune system organs begins in an early embryonic stage and reaches its peak in early adulthood. However, certain components of the immune system begin to deteriorate around puberty.

Development of the primary organs of the immune system is a genetically determined process, usually completed early after birth, that leads to the organs with typical or normal microstructures. In contrast, the development of secondary lymphoid organs is

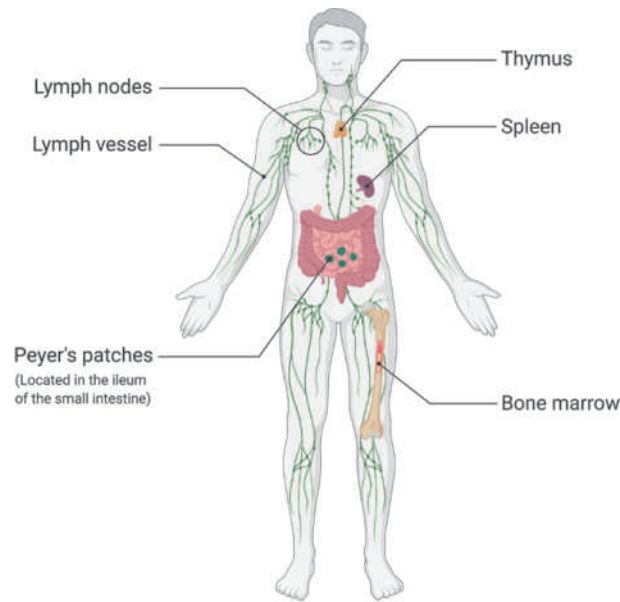


Fig. 1 The primary and secondary lymphoid organs and their locations in the human body. Created by [BioRender.com](https://www.biorender.com) (2021).

affected by environmental factors, in addition to the basic role of genetics. Furthermore, the primary immune organs also affect the development of the secondary lymphoid organs during the latter stages of life ([Parker, 2016](#)).

In this chapter, we will describe the primary and secondary lymphoid organs, their structures, and their role in the production and maturation of the various immune cells, as well as in immunity. Finally, the significance of the lymphoid organs in immune cell trafficking as well as in the fate of tissue transplantation is also discussed.

Primary lymphoid organs

The thymus and bone marrow are key primary lymphoid organs that produce, develop, and select naïve lymphocytes in a process known as lymphopoiesis. While the secondary organs are a place to respond to foreign antigens, the primary lymphoid organs are a place for lymphocyte training, the emergence of specific lymphocyte receptors, and the deletion of autoreactive lymphocytes.

Bone marrow (BM)

The BM is a primary organ where the blood cells are produced through the hematopoiesis process. During this process, all blood cell types originate from a hematopoietic stem cell (HSC). These cells can differentiate into all blood cells, including RBC, platelets (megakaryocytic), myeloid, and lymphoid progenitors. Furthermore, myeloid cells (such as monocytes, dendritic cells (DCs), neutrophils, basophils, and eosinophils), B lymphocytes, as well as natural killer (NK) cells, all develop in the BM. Meanwhile, T cell precursors migrate to the thymus for further maturation. Besides, nonhematopoietic tissues, including chondrocytes, adipocytes, myoblasts, and osteocytes, originate from mesenchymal stem cells (MSCs) in the BM. The BM-derived MSCs have shown immunosuppressive features and prolonged survival properties, especially in transplantation studies.

Interestingly, in order to tolerance induction in organ transplantation, scientists try to simultaneously infuse BM-derived HSCs along with solid organ transplantation from the same donor. However, successful findings achieved from this strategy have been restricted to only studies on small experimental animals ([Andreola et al., 2011](#)).

Well-established evidence suggests that HSCs originated from the yolk sac, migrated to the liver during pregnancy, and eventually implanted in the bone marrow (BM). It seems that the aorta-gonad-mesonephros (AGM), an area in the fetus, acts as an intermediate site of the primitive hematopoietic cells. Indeed, the initial hematopoietic precursor cells in the fetus are developed from hematopoietic stem cells (HSCs) in AGM through a commitment process. These primary progenitor hematopoietic cells then migrate to the liver and spleen through blood vessels, where they transform into the main hematopoietic lineage, to seed the primary (bone marrow and thymus) and secondary (lymph nodes, spleen and MALT) lymphoid organs ([Kyriazis and Esterly, 1971](#)).

In general, there are two types of BM:

1. The red marrow with hematopoiesis activity.
2. The yellow marrow, which contains mostly fat cells, where hematopoietic activity does not take place.

Initially, the marrow of a newborn is thoroughly red, but then gradually replaced by yellow marrow through time. Upon puberty, only flat bones have red marrow, including the sternum, cranium, vertebrae, scapulae, and pelvis, as well as the epiphyseal heads of long bones such as the humerus and femur (Moore and Metcalf, 1970; Parker, 2016).

The red tissue of the BM includes hematopoietic islands and fat cells, as well as vascular sinusoids distributed all over the trabecular bone. These hematopoietic islands provide a microenvironment comprising blood cell progenitors with different levels of maturation, endothelial cells, stromal reticular cells, osteoblasts, osteoclasts, macrophages, and the extracellular matrix. In addition to organizing blood cell maturation, this microenvironment also produces growth factors, chemokines, and cytokines. Upon maturation, blood cells enter the blood stream by migration through spaces between the basement membrane and endothelial cells of the vascular sinusoids (Romaniuk et al., 2016; Travlos, 2006).

Bone marrow as a place for the development of B lymphocytes

There are a variety of cells in the BM microenvironment that promote the development and growth of B cell precursors by providing important growth factors, adhesion molecules, chemokines, and cytokines (e.g., IL-7, thrombopoietin, Flt3 ligand, and CXCL12). B cell precursors are closely related to stromal reticular cells, the inner wall of the bone (endosteum), and the central sinus. The reticular cells next to the endosteum are the first cells involved in the evolution and growth of B cell precursors. There are other cells in the BM called adventitial reticular cells, which facilitate the entry of mature B cells into the bone marrow sinusoids and eventually the central venous sinus. Finally, mature B lymphocytes enter the bloodstream through the central venous sinus. Immature B cells released from the BM migrate to the spleen to become mature B cells.

During B cell lymphopoiesis, the production of specific B cell receptors (BCR) occurs in a process called “receptor rearrangement.” In this process, B cells undergo rearrangement in their immunoglobulin genes to express cell-surface immunoglobulins. In the process of lymphopoiesis in the BM, the developmental stages of B lymphocytes include ProB, PreB, immature B, and mature B, respectively. The ProB cell is the first cell that initiates gene rearrangement, and the heavy chain of IgM is the first chain to be rearranged. This cell contains the enzymes needed for rearrangement, including Recombination-Activating Gene-1 (RAG1), RAG2, and Terminal deoxynucleotidyl Transferase (TdT). Eventually, the ProB turns into a PreB. The PreB begins to rearrange the light chain, and this cell is the first cell to have a receptor called pre B cell receptor (preBCR). The preBCR consists of three chains, including CD79, μ chain, and the surrogate light chain (SLC). PreBCR signal transmission is an important factor in the differentiation of PreB into immature B cells. Immature B cells, after the selection process and, if necessary, undergoing the phenomenon named “receptor editing”, eventually become mature B cells.

As mentioned, immature B cells in the BM are selected in order to delete cells that are potentially autoreactive. At this point, if the B cell receptor expressed on immature B cells binds to insoluble surface antigens of BM stromal cells, the B cell must be deleted or its receptor modified during the receptor editing process to no longer bind to self-antigens. However, if receptor editing also fails, immature B cells that express high-affinity receptors for self-antigens are deleted through apoptosis. On the other hand, a number of B cells that have no interaction with self-antigens will go through the evolutionary process of maturity (Fig. 2) (Kincade, 1981; van de Pavert and Mebius, 2010).

Cell transportation across the BM

Cell transportation/migration to and from the BM is principally modulated by several adhesion molecules, chemokines, and receptors. For instance, the integrin VLA4 (integrin $\alpha 4 \beta 1$) helps maturation of the B cells through its binding to the stromal cells via the ligand VCAM1. Also, the chemokine CXCL12, produced by stromal reticular cells and osteoblasts, maintains HSCs and myeloid and lymphoid cells in the BM through binding to its receptor CXCR4. In this way, some people with WHIM syndrome, who carry the “gain of function” mutations in CXCR4, show increased retention of leukocytes in the BM (pan-leukopenia).

CXCR4 antagonists, such as plerixafor, on the other hand, prevent the transport of HSCs, myeloid and lymphoid cells from the BM into the bloodstream. Animal studies also showed that the receptor CCR2 and its ligand CCL2 play a critical role in monocyte trafficking in the BM of the mouse (Hooper and Macpherson, 2010).

Thymus

The thymus is another primary lymphoid organ placed above the heart in the upper chest of mammals. In most species, the thymus originates from the pharyngeal pouches III and IV and is then located in the anterior mediastinal area. The matrix of most lymphoid organs is developed from fetal mesenchymal cells, but the thymus stroma develops mainly from epithelial cells (endoderm) and from mesenchyme (Anderson and Takahama, 2012).

This lymphoid organ has a central role in the development of the bone marrow-derived thymocytes into T lymphocytes. The thymus is one of the major organs of the mammalian adaptive immune system where CD4⁺ and CD8⁺ T cells are produced. Besides, in the thymus, T lymphocytes are trained in order to distinguish between self- and non-self-antigens. Maturation of the thymus is completed until prior to adolescence, and then its function gradually decreases.

Due to the important role of the thymus in the maturation of T cells, surgical removal of the thymus at birth results in severe immunodeficiency, while its removal after puberty has no significant outcome on the immune responses. One of the well-known examples of human diseases that establishes the vital role of the thymus in T cell development is DiGeorge syndrome. In this disease, the affected individuals are characterized by congenital loss or severe hypoplasia of the thymus, as well as few T cells but a normal population of B cells (Anderson and Jenkinson, 2001).

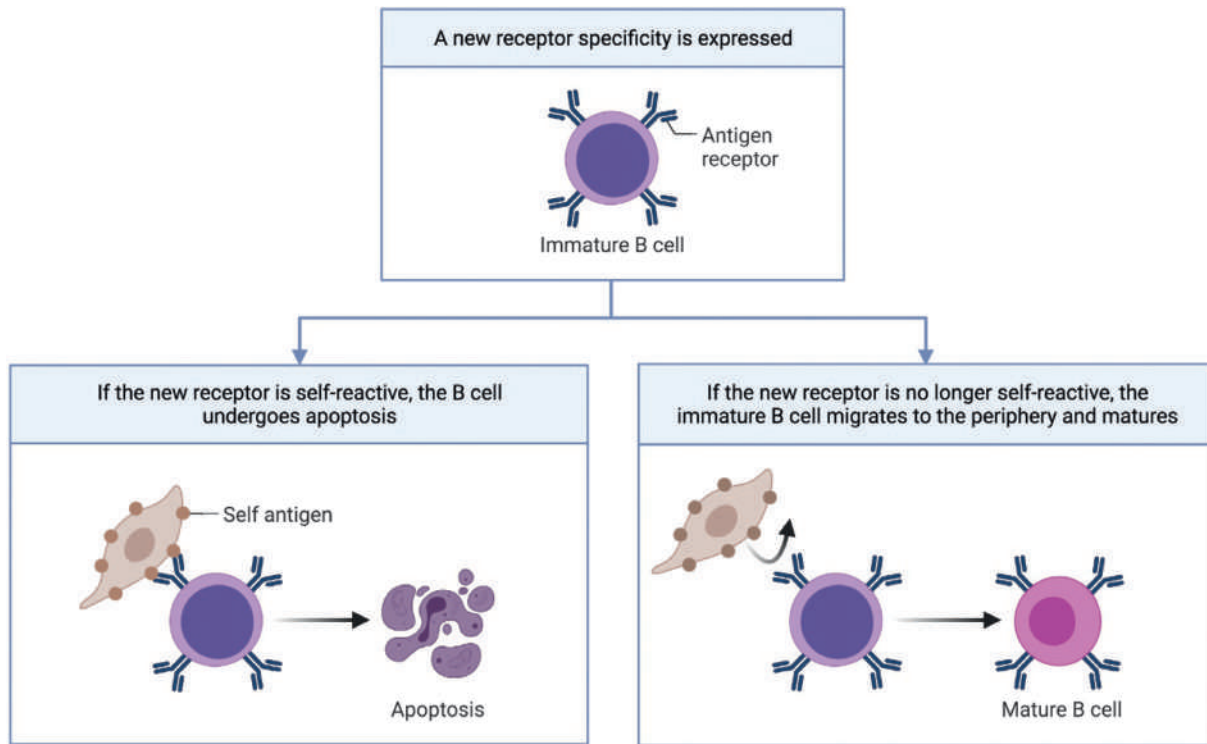


Fig. 2 Immature B cells are tested for autoreactivity before they leave the bone marrow. Created by [BioRender.com](https://www.biorender.com) (2021).

The human thymus matrix derives from three origins, including the endodermal pharyngeal pouch III, the ectodermal branchial cleft, and the stromal pharyngeal arch (i.e., the neural crest). It seems that the interaction between the epithelial cells of the endoderm and ectoderm is necessary for thymus development. Besides, the participation of the neural crest is required for the development of the thymus medulla. The formation of the fetal thymus depends on both the development of epithelial and lymphatic components. The sequence order of the primary lymphocyte origin comes from the hematopoietic sites of the yolk sac and/or AGM, fetal liver, and finally in the postnatal period from BM, respectively. Entrance of a population of naïve T cells into the thymus depends on the successful maturation of the offspring of progenitor cells. Indirect evidence shows that mature thymocyte populations accumulate in the thymic cortex (Anderson and Takahama, 2012).

The thymus plays a central role in tolerance, through the production of nTreg and the negative selection of newly developing T cells. Today, scientists try to use this potential of the thymus to induce tolerance in solid organ transplantation through the mixed hematopoietic chimerism approach (e.g., by co-transplantation of thymus and kidney grafted from the same donor to MHC-mismatched recipients). The basic principle behind this plan is based on the evidence that donor HSC-derived DCs prompt apoptosis in newly developing donor-reactive T cells in the thymus, which results in central tolerance. Furthermore, the epithelial cells of the thymic graft cause central tolerance through their ability to express donor antigens such as donor MHC antigens, and also eliminate donor-reactive T cells from the recipients (Yamada et al., 2020).

Thymus structure

Thymus has a lobular structure consisting of two distinct zones: cortex and medulla (Fig. 3). The cortex is the site of a concentrated population of immature T cells during their development, while the medulla is occupied by mature T cells. It seems that the T precursor cells ingress the thymus and proliferate in the cortex area.

Besides, a highly vascularized corticomedullary boundary separates these areas. Several types of cells are present in the cortex and medulla of the thymus, including epithelial cells, stromal cells, dendritic cells, and macrophages. These cells form a cellular network that facilitates the maturation of thymocytes. Various cell types present in this thymic network contribute to thymocyte development in different ways, including direct interaction (stromal cells); and acting as nurse cells with surrounding thymocytes (epithelial cells).

The constituent stroma of both the cortex and the medulla is a three-dimensional matrix of thymic epithelial cells (TECs) enclosed by T lymphocytes. Depending on whether TECs are placed in the cortex or medulla, they are called cortical TECs (cTECs) and medullary TECs (mTECs), respectively.

The cTECs facilitate positive selection by expression of MHC class I and II molecules that help in processing and presenting self-peptides. At this point, thymocytes that recognize self-peptides:MHC complex with moderate affinity are allowed to survive. Otherwise, they do not receive a survival signal and become apoptotic.

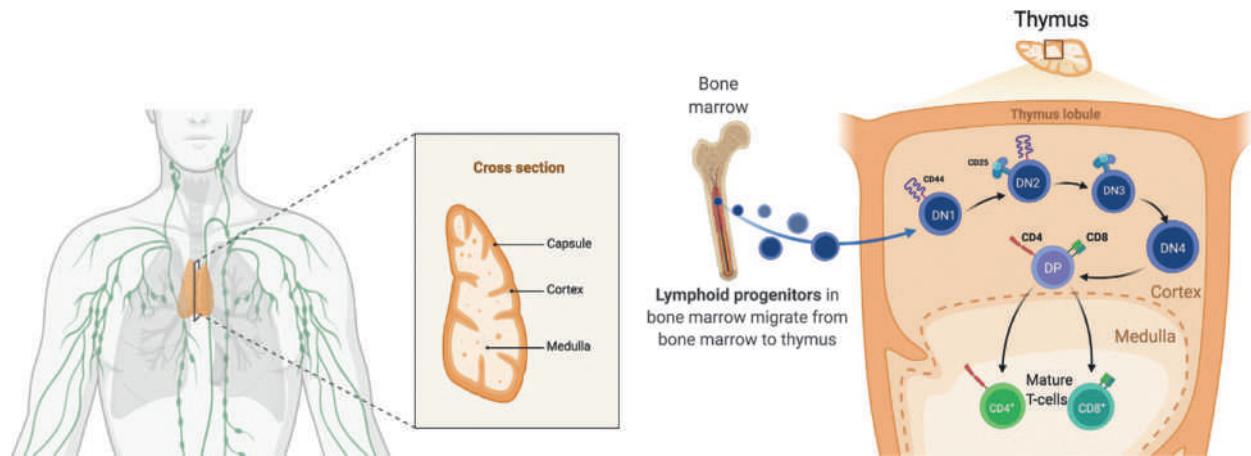


Fig. 3 Thymus as a primary lymphoid organ and its structure, consisting of the cortical and medullary regions. Created by [BioRender.com](https://www.biorender.com) (2021).

Positive selection is a phenomenon involving the restriction of T cells to MHC, which ensures that T cells recognize antigens only if they are presented to them in the form of an MHC:peptide complex.

The mTECs present many types of organ-specific peptides. These cells are the main cause of the negative selection of thymocytes. During negative selection, cells whose TCR detects thymic peptides:MHC complexes presented by mTECs, macrophages, or dendritic cells at the cortico-medullary junction or in the medulla with high-affinity undergo negative selection and apoptosis. But those with low or moderate affinity for thymic peptides:MHC complexes survive and exit the thymus (Itoh et al., 1999).

The medulla in the human thymus also has distinguishable parts called Hassall's corpuscles, containing the dense layers of keratinizing epithelium, where T cell apoptosis and production of thymic stromal lymphopoeitin (TSLP) occur. It seems that TSLP participates in the activation of thymic DCs (Bleul et al., 2006).

Thymus functions in the immune system

As mentioned previously, maturation and selection of T cells are necessary for their ability to identify foreign peptides based on the MHC-restricted antigen recognition, and the thymus has a principal role in this process. For beginning maturation in the thymus cortex, bone marrow-derived T cell progenitors can enter the thymus through the venules next to the corticomedullary barrier. In order to further maturation, T cells acquire classical markers such as CD4 and CD8, then undergo random rearrangement in their T cell receptors (TCR), which are requisite for positive and negative selection. Upon completion of T cell selection in the thymus medulla, they enter the blood stream (Miller, 1961).

Studies have shown that the DCs derived from bone marrow also significantly participate in T cell maturation and selection. Furthermore, a group of TECs located in the corticomedullary region contribute to the production of IL-7, which is vital for T cell survival. The mTECs in the thymus, through expression of a gene known as autoimmune regulator (AIRE), present peptides derived from proteins (such as insulin) to developing thymocytes in order to delete the autoreactive T cells specific to self-antigens. Mutations in the AIRE gene lead to an autoimmune disorder known as autoimmune polyglandular syndrome type I, also called autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) in humans (Yarilin and Belyakov, 2004).

In addition, the thymus plays several important functions, including generating NKT cells, $\gamma\delta$ T cells, and natural regulatory T cells (nTreg). NKT cells are positively selected (having both CD4 and CD8) in the thymus via the non-classical MHC molecule CD1d. These cells help to recognize the glycolipids generated by some pathogens, including mycobacteria. Besides, upon selection of the $\gamma\delta$ T cells in the thymus, they migrate to the gut and skin epithelium. The nTreg, as the CD4⁺ T cells, are also positively selected in the thymus and express the typical $\alpha\beta$ TCR and have a mediocre reaction with self-MHC and self-peptides. These cells regulate immune responses through suppressing the activity of effector T cells. Animal studies have shown that thymectomy upon birth or at the third post-natal day results in immunodeficiency and severe autoimmunity. The absence of nTreg is thought to be the root cause of such autoimmunity. In this way, mutation in Foxp3 (a transcription factor required for the development of Tregs) results in lethal autoimmune diseases, including immunodysregulation polyendocrinopathy, enteropathy X-linked syndrome (IPEX) in humans, as well as scurfy in mice (Takahama, 2006).

Cell transportation across the thymus

The trafficking of the cells in the thymus is critically dependent on the chemokines. Among the chemokines, CCL21 and CCL25 are examples that contribute to the trafficking of precursor cells from BM into the thymus through binding to the receptors CCR7 and CCR9, respectively. Furthermore, the chemokines CCL21 and CXCL12 have an important role in thymocyte maturation via binding to CXCR4 during the corticomedullary migration in the thymus. In addition, CXCL12 is essential for the migration of mature thymocytes from the thymus into the blood. Sphingosine-1-phosphate (S1P) is another mediator in the blood and lymph that aids in the emigration of mature thymocytes by binding to the S1PR1 receptor found on these thymocytes (Yan et al., 2017).

Secondary lymphoid organs

Secondary lymphoid organs are the sites of lymphocyte response to antigens and the production of effector and memory lymphocytes, in a process known as "immunopoiesis." The main secondary lymphoid organs are the spleen, lymph nodes, and mucosa-associated lymphoid tissue (MALT) consisting of cellular aggregates present in various submucosal membrane areas such as the intestinal lamina propria as well as the inner surfaces of the respiratory system, breast, thyroid, salivary glands, lungs, eyes, and skin.

Spleen

The spleen is a secondary lymphoid organ located under the pancreas and has an oval structure. The major function of the spleen is response to systemic infections (especially encapsulated bacteria) through filtering blood and capturing blood-borne antigens (Chadburn, 2000).

Development of the spleen during embryogenesis begins early in pregnancy. Like other secondary lymphoid organs, the complete development of the immune system compartments of the spleen depends on environmental factors. In the human fetus, the primordium of the spleen appears in the 5th week of pregnancy through proliferation of the mesoderm between the two lobes of the dorsal mesogastrium, and joins the stomach to the dorsal wall of the body. The dorsal mesogastrium fuses with the peritoneal wall, causing attachment of the spleen to the dorsal wall of the body through the lienorenal ligament, and to the stomach through the gastrosplenic (or gastrosplenic) ligament (Chadburn, 2000).

Spleen structure

The spleen is a highly vascularized organ. From the histological point of view, it is much more complicated than the thymus. The spleen is composed of two regions: the "red pulp" and the "white pulp" (Fig. 4). The red pulp contains large blood venous sinusoids with RBCs as well as platelets. On the other hand, the immunologic functions of the spleen are more related to the white pulp (Cesta, 2006).

The internal part of the spleen is separated by capsular connective tissue patterns known as trabeculae that further constitute regions called red pulp and white pulp that are spread throughout the spleen. The red pulp consists of a network of sinusoids rich in RBCs as well as macrophages, in which the old and deficient RBCs are eradicated and picked up. The white pulp encloses the arteries, and is enriched mostly with T lymphocytes, which form the periarterial lymphatic sheath (PALS). Furthermore, the marginal area near PALS is composed of the B cells produced in lymphoid follicles. The preliminary activation of B and T cells occurs in PALS, which is rich in T cells. This area is composed of interdigitating dendritic cells that entrap and present antigens via MHC class II to Th cells. This action results in T cell activation, which in turn induces B cell activation. The activated B and T cells subsequently move to the primary follicles in the marginal zone. Upon antigen induction, these primary follicles turn to secondary follicles. The secondary follicles are similar to the germinal parts of lymph nodes, where B cells proliferate (Cesta, 2006).

The role of the spleen in the immune system

The spleen has several functions, including hematopoiesis, storage, clearing of aged and damaged blood cells and platelets, as well as defense actions such as removing opsonized bacteria. Besides, this lymphoid organ has an important role in triggering adaptive immune responses. Thus, the absence of a spleen, as in splenectomy, makes humans and experimental animals extremely vulnerable to sepsis caused by encapsulated bacteria (e.g., *Streptococcus pneumonia*) and certain immunologic defects (Cesta, 2006).

The immune response is mainly concentrated in the "white pulp" composed of PALS and lymphoid follicles. Although PALS is usually considered as a site enriched in T cells, PALS has a major inner area filled with T cells and an outer area filled mainly with B cells, as well as fewer T cells and macrophages. Follicles usually develop at the junction of the T-cell and B-cell areas of PALS, similar to the follicle development in a lymph node. The marginal zone (MZ) surrounding PALS and follicles is occupied by populations of macrophages, and a specific population of B cells called marginal zone B cells (MZB). MZB is mainly found in the spleen and has

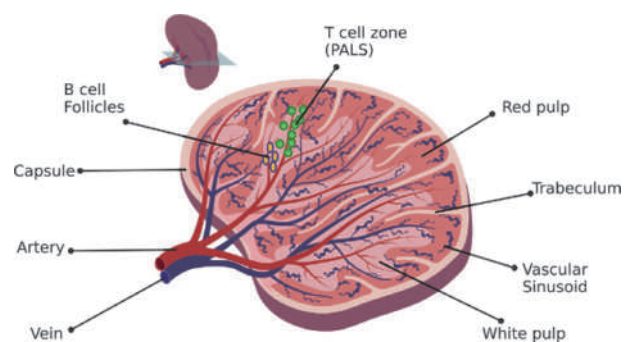


Fig. 4 The spleen and a tissue section of its structure. Created by BioRender.com (2021).

special functions, including high-level response to polysaccharide antigens (e.g., the antigens presented on the capsule of certain bacteria). Deficiency in this specific defense role (e.g., in a traumatic injury situation requiring surgical removal of the spleen) causes the host extremely vulnerable to bacterial infections with the pathogen having polysaccharide-rich capsules such as *Streptococcus pneumoniae*, which causes pneumonia in humans (Cesta, 2006).

Cell transportation across the spleen

The macrophages and immature DCs located in the MZ of splenic white pulp are responsible for removing pathogens such as microbes, antigens, as well as antigen–antibody complexes in the blood. The activation of T cells, as well as differentiation of B cells into memory B cells and antibody-producing plasma cells, are also facilitated in the T cell zones and border of the follicles of the spleen, respectively. Meanwhile, the maturation of the naïve B cells derived from the BM is completed in the spleen.

The existence of marginal zone macrophage and B cell populations are other unique properties of the spleen among the other secondary lymphoid organs. Due to the presence of macrophages, and the B cells expressing IgM and toll-like receptor 9 (TLR9), the spleen is considered as a defense system that mediates the presentation of the captured pathogens to the cells of the adaptive immune system. Furthermore, transmigration of the naïve T and B cells occurs in the white pulp of the spleen through the marginal sinus and then confined to the PALS and follicles stimulated by the chemokines CCL19/CCL21 and CXCL13, respectively. However, the related mechanisms are not well recognized (Lewis et al., 2019).

Lymph nodes

A lymph node has an encapsulated and bean-shaped structure distributed across the lymphatic system throughout the body. The lymphatic system transfers the extracellular fluid between the tissues and the blood via the lymphatic vessels. Due to the existence of antigens and APCs in the lymphatic fluid, lymph nodes act as the sites for antigen presentation to T and B cells. The specialized blood vessels called high endothelial venules (HEVs) localized to specific regions in the lymph nodes provide a place for the entrance of lymphocytes. Based on their function and the difference in the adhesion molecules expressed, lymph nodes are categorized into peripheral or mucosal types that are functionally distributed in the body (Arno, 1980).

The structure of lymph nodes

Lymph nodes are structurally encircled by a capsule along with medullary and subcapsular sinuses (Fig. 5). Lymph fluid infiltrates in lymph nodes via the afferent and efferent lymphatic vessels. A duct network composed of fibroblastic reticular cells, collagen, laminin, and fibrillin layers provides deep penetration of lymph containing antigens into the lymph node parenchyma in which the antigens are picked up by APCs or DCs that reside within lymph nodes. Then, the mature APCs carrying antigens migrate through lymphatic vessels and localize in T cell areas directed by chemokines (Arno, 1980).

HEVs are specialized post-capillary vessels that provide entry of T and B cells to lymph nodes. The cuboidal endothelial cells that cover these vessels express adhesion molecules and chemokines to facilitate T and B cell migration. B cells and follicular dendritic cells (FDCs) are orderly localized in follicles in the outer cortex, and T cells. On the other hand, they are diffused in the paracortical regions under the B cell follicles. In addition, these areas also include macrophages, stromal cells, and DCs. Furthermore, there are germinal centers consisting of the central parts of lymphoid follicles where antibody-producing plasma cells and memory B cells are produced. Finally, an internal area comprising medullary cords is also in the lymph node, which contains macrophages and plasma cells (Arno, 1980).

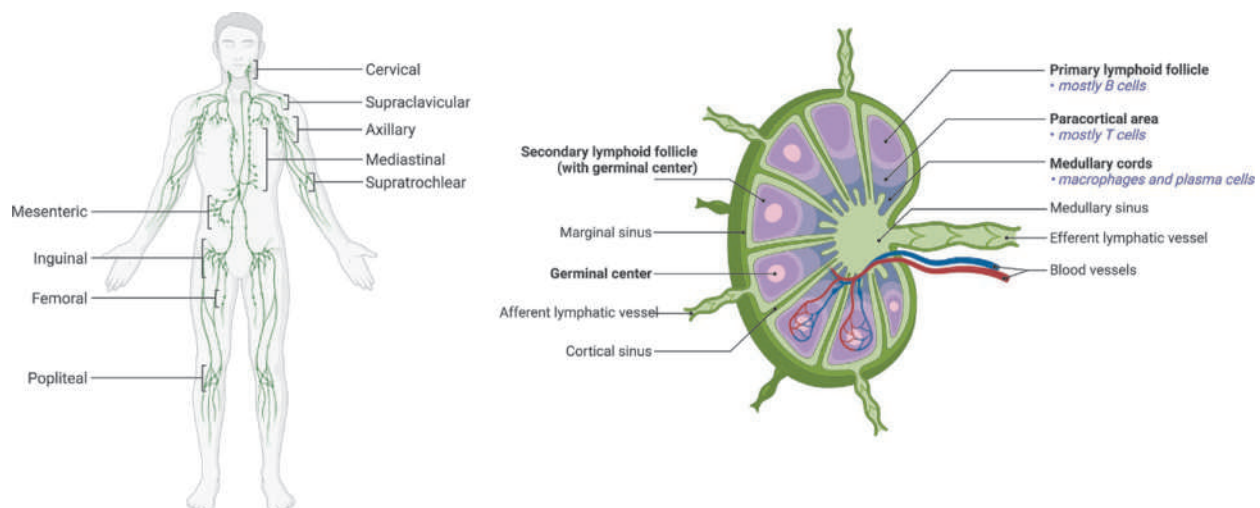


Fig. 5 The lymph nodes and their locations in the human body, along with a tissue section of their structure. Created by BioRender.com (2021).

Lymph node development begins during embryogenesis. For a long time, it has been generally believed that primitive lymph sacs grow from veins, and that lymph sacs act as the origin for the development of lymphatic as well as lymph nodes. Recent studies on mice have shown that primitive lymph nodes develop in the deficient mouse embryos, lacking lymphatic vasculature and lymphatic sacs, and also in the mutant embryos with defective lymph sacs. More recent studies showed that the structure of the lymph sac is not completely essential for the formation of lymph nodes, but they do not challenge the basic hypothesis that lymph nodes come from veins. Lymph node development needs the interaction of hematopoietic-derived lymphoid tissue inducer (LTi) cells with hematopoietic source and lymphoid tissue organizer (LTo) cells with mesenchymal source (Benezech et al., 2014).

Embryonic LTi cells can originate from fetal liver cells, which are the counterparts of common lymphoid progenitor cells in adult BM, while the LTo cells derive from adipocyte progenitors. LTo cells provide specific growth factors and signals that promote LTi cell migration and maturation, and also lymphocyte survival. Both embryonic and adult adipocyte precursor cells can differentiate into lymph node stromal cells, thus adipose tissue remains the source of adult lymph node stroma production. These interactions between adipose tissue and lymphatic structures may explain the appearance of lymphatic tissue in the omentum fat-associated lymphatic clusters, and finally, the association between adipose tissue and lymph nodes (Benezech et al., 2014).

The role of lymph nodes in the immune system

For their activation and maturation, naïve T and B cells initially need to be exposed to antigens in lymph nodes. Priming of naïve T and B cells takes place in the cortical zones (T cell zone), and in the follicles in the outer cortex of lymph nodes, respectively.

Lack of lymph nodes in mice leads to abnormalities in adaptive cellular and humoral immunity. Maintenance of naïve and memory T cells is another important role of lymph nodes that is facilitated via the presentation of self-antigens bound to MHC by the DCs that reside in the lymph nodes, thus providing survival signals to T cells. On the other hand, activated T cells via the membrane lectin CD69 on their surface are held in the lymph node to complete their proliferation and differentiation. However, many T follicular helper cells (Tfh) migrate into the B cell follicles and interact with B cells, which results in the generation of plasma blasts. Certain plasma blasts then migrate to the center of the follicle to divide and differentiate into memory B cells or antibody-producing plasma cells (Arno, 1980).

Cell transportation across lymph nodes

Like in other lymphoid organs, cell transition in lymph nodes is regulated by chemokines. For example, to migrate from non-lymphoid tissues into lymph nodes, DCs express the chemokine receptor CCR7 in response to CCL19 and CCL21 through a process called haptokinesis. Besides, naïve T cells and a group of memory T cells co-localize with DCs in the paracortical regions by expressing CCR7. Furthermore, naïve B cells migrate to follicles in the cortex, via interaction between their chemokine receptor CXCR5 and the chemokine CXCL13. In response to CCL21 expressed by mature DC, B cells express CXCR5 and receive help from activated CD4⁺ T cells. Moreover, T follicular helper cells also express CXCR5, and participate in B cell activation and germinal center formation. More recently, it has been reported that a group of regulatory T (Treg) cells called follicular Tregs inhibit B cell activation and humoral immunity (Cupedo and Mebius, 2005).

In general, T cells enter the lymph nodes initially through the binding of L-selectin (CD62L) located on the T cells to HEV. Depending on whether the lymph node is peripheral or mucosal, this binding is mediated by the peripheral node addressins (PNAd) (i.e., CD34 and GlyCAM-1), and MAdCAM-1 expressed on the endothelial cells, respectively. On the other hand, T cell adhesion and migration, are dependent on the binding of high affinity LFA1 (a 2 integrin) expressed on T cells to ICAM1 expressed on endothelial cells. Furthermore, the egress of effector T cells from the lymph node is mediated by sphingosine-1-phosphate (S1P) (Cupedo and Mebius, 2005).

Mucosa-associated lymphoid tissue (MALT)

Mucosa-associated lymphoid tissues (MALTs) comprise several nodules of partly encapsulated lymphoid tissue as well as feeble-associated lymphoid complexes located in epithelia and lamina propria of mucosal areas such as the gastrointestinal and respiratory tracts. These complexes are further categorized into gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), and nasopharynx-associated lymphoid tissues (NALTs). Lymphoid tissues, including the appendix, tonsils, and Peyer patches, are characterized by their mucosal epithelium structure. By transporting foreign molecules through transcytosis to underlying lymphoid tissue in the gut and the respiratory system, the specialized microfold (M) cells of the follicle-associated epithelium of the MALT have a vital role in the production of immune responses. Furthermore, specific intraepithelial lymphocytes through strong cytolytic and immunoregulatory activities help mucosal tissue in defense against pathogens. Generally, MALT found in different tissues shows certain differences in function, structure, and development. However, all MALTs help to capture antigens through delivery of those antigens from the overlying epithelial surface and their presentation to the underneath cells of the immune system (Randall et al., 2008).

Gut-associated lymphoid tissue (GALT)

GALT is composed of separate or aggregated lymphoid follicles, which form Peyer's patch (PP). Peyer's patches (PP) are visible lymphatic structures that were first discovered in the human small intestine (Cornes, 1965). Due to the capability of PP to manage antigens and bacteria in the luminal areas, they are considered as the immunosensors in the intestine. The PPs comprise both follicle-associated epithelium and immune cells resident in the lymphoid follicles. Their interaction leads to immune defense

against pathogens as well as immunologic tolerance. These immune responses appear to be managed by pathogen recognition receptors, such as Nod2. In addition to the function of Toll-like receptors (TLRs) in PP homeostasis, Nod2 can also modulate the characteristics and population of T cells in PP, and in reply, these T cells can regulate cell permeability. Clinically, it has been reported that Crohn's disease and graft-versus-host disease (GVHD) in humans may be caused by NOD2 mutations, PP dysfunction, and subsequent immune malfunction during response to the normal flora (Randall et al., 2008).

Both the small and large intestines have GALT aggregates, but their GALT components are somewhat different. In other words, GALT is made up of PP, individual lymphoid follicles (ILF), cryptopatches (CP), and lymphocyte-filled villi in the small intestine, while diffuse lymphocytic populations are found in the mucosa of both the small and large intestines. Besides, lymphoglandular complexes are only in the large intestine. Postnatal development of some GALT components, including PP and diffuse lymphocytic populations, is different, and they may even show various immunobiological properties. However, both of them produce immunoglobulin A (IgA), vital for the function and maturity of the human gastrointestinal immune system, which is complete 2 years after birth (Randall et al., 2008).

Bronchus-associated lymphoid tissue (BALT)

BALT is a secondary lymphoid tissue that is only induced in humans during lung inflammation (known as iBALT); however, it is always present in rats and rabbits (Randall et al., 2008).

Nasopharynx-associated lymphoid tissues (NALTs)

The nasopharynx-associated lymphoid tissues (NALTs) are lymphoid aggregates in the posterior nasal cavity in rodents. These are thought to be equivalent to human tonsils and adenoids. In spite of the similarity in their function and structure, NALT and PP present considerable differences in their organogenesis and development. Furthermore, the HEV of NALT expresses the peripheral node address protein (PNAd), while the PP-associated HEV expresses MAdCAM1, and thus various initial B cell populations are found in NALT and PP (Csencsits et al., 1999).

Conclusion

As the primary lymphoid organs, the bone marrow and thymus are sites where the lymphocytes are produced, developed, and learn to distinguish self from non-self. The lymph nodes, spleen, and MALTs are the secondary lymphoid organs. The spleen is a blood filter organ supplying a context for interaction between innate and adaptive components, as well as their response to presented antigens. In other words, lymph nodes are a network connected by lymph vessels, that provide the drainage of antigens and interact with antigen-presenting cell populations to develop the engaged adaptive lymphocytes. MALTs are the diffuse cell aggregates connected to the epithelium and lamina propria of the mucus parenchyma, supplying foreign substances to the underlying lymphocytes.

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Structure and Function of the Immune System

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Introduction

The “immune system” is made up of cells and molecules that protect the host body from harmful stimuli and keep the body in a state of homeostasis. The immune system’s components respond in a coordinated and comprehensive manner, resulting in the emergence of an “immune response.” While an optimal immune response would eliminate the harmful agent and restore homeostasis to damaged tissues, inappropriate responses can be harmful and result in tissue injury. As a result, immune system modulation is critical, allowing the organism to control the strength and duration of responses (Chaplin, 2010).

Any molecule that can be detected by the immune system is called an “antigen.” Parts of an antigen molecule that are recognized by immune system receptors are called “epitopes.” It is important to note that antigens that stimulate the immune responses are also called “immunogens.”

In summary, there are two types of immunity: “innate” and “adaptive” immune systems. The initial line of defense against infections is the innate immune system, which responds rapidly to stimuli. The innate immune response is known as “non-specific” immunity since it reacts to all foreign antigens in the same way. Adaptive immunity, on the other hand, is required for a successful immune response. The adaptive immune system identifies antigens more specifically than the innate immune system and can produce “memory” cells which have the ability to react quickly to pathogens in the second encounter (McCullough and Summerfield, 2005).

The immune system must detect and distinguish a wide range of antigens associated with infectious organisms or pathogens, including parasites, viruses, fungus, and bacteria, from the host’s own antigens in order to elicit an effective response. To this end, a number of receptors are present on immune cells which recognize pathogen-associated antigens (Medzhitov and Janeway Jr, 1998). The immune system must be tolerant to the host’s own molecules in order to avoid unwanted responses. This is a significant occurrence that establishes the theory of “immune tolerance.” Autoimmunity is a condition that occurs when self-tolerance is disrupted or when immune responses are improperly regulated (Chaplin, 2010).

In addition to responding to pathogens, the immune system is also responsible for protecting against other disorders, including cancer. On the other hand, defects or mutations in one or more parts of the immune system lead to the development of immunodeficiency diseases that can predispose a person to a variety of microbial diseases (Gonzalez et al., 2018).

In this chapter, we sought to present the fundamental principles of the immune system, covering the key components of lymphoid organs, immune system structure, innate and adaptive immunity, as well as the basic effective functions of the immune system to pathogen eradication.

Lymphoid organs

The immune system is composed of a variety of organs and tissues aimed at immune cell development and maturation, or concentrating immune responses against an antigen. The organs of the immune system include physical barriers such as skin and mucous membranes, as well as lymphoid organs. Based on the anatomical location and immunological role, the lymphoid organs can be divided into “primary” and “secondary” organs. The primary lymphoid organs include the thymus and bone marrow, which are the sites of lymphocyte production, development, and selection of naive lymphocytes, in a process known as “lymphopoiesis.” Secondary lymphoid organs, including lymph nodes, spleen, and mucosal associated lymphoid organs (MALTs), are the sites of lymphocyte response to antigens and the production of effector and memory lymphocytes, in a process known as “immunopoiesis” (Drayton et al., 2006).

Primary lymphoid organs

Thymus: a lymphoid organ for the development and maturation of T lymphocytes

The thymus is an organ located in the middle-anterior part of the thorax and in front of the aortic artery. This organ is made up of two lobes, each consisting of several lobules. The thymus is surrounded by a capsule of connective tissue, the branches of which extend into the thymus and divide it into different sections. The thymus is the most important center for regulating the function of the immune system and is created in the sixth week of the fetus. This organ is involved in the maturation and training of different types of T lymphocytes. The microenvironment in the thymus, such as the production of interleukin-7 (IL-7) and various hormones including thymosin and thymopoietin, plays an important role in the development of thymocytes. Epithelial cells in the central part of the thymus play a key role in the presentation of the host antigens to developing T cells, leading to the deletion of self-reactive T cells (a process known as self-tolerance or negative selection). Therefore, as mentioned before, the thymus is the most important center for the training and maturation of T lymphocytes. T-lymphocyte progenitor cells migrate to this organ from the fetal liver (during the embryonic period) and the bone marrow, and after development and proliferation, they become mature and functional T lymphocytes (Zdrojewicz et al., 2016).

Bone marrow: where the blood cells are produced through the hematopoiesis process

The bone marrow is the site of production of most blood cells, including red blood cells (RBCs), granulocytes, monocytes, and is the site of development and maturation of B-lymphocytes. The production of all blood cells is first done during the embryonic period in the hematopoietic islands of the yolk sac and mesenchyme around the aorta, then in the third and fourth months of pregnancy is transferred to the liver, and finally the bone marrow is the main site of hematopoiesis. Hematopoiesis occurs at birth in the bone marrow of the entire skeleton. In children, the marrow of the long bones such as the femur and tibia are the major sites of hematopoiesis. However, in adults, more hematopoiesis occurs in the marrow of the pelvis, cranium, vertebrae, and sternum (Jagannathan-Bogdan and Zon, 2013).

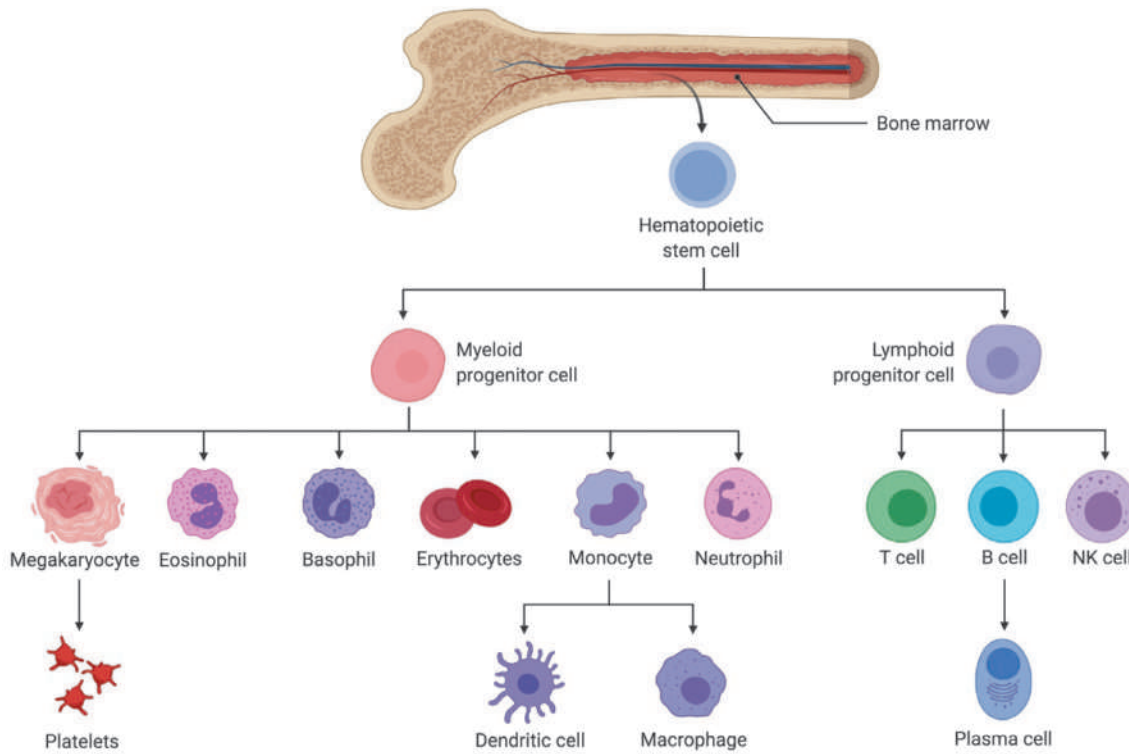


Fig. 1 Stem cell differentiation from bone marrow. Adapted from “Stem cell differentiation from bone marrow”, by BioRender.com (2021). Retrieved from <https://app.biorender.com/biorender-templates>.

Blood cell types, including RBCs, lymphocytes, and other cells, all originate from a hematopoietic stem cell (HSC). HSC have a cell surface marker known as CD34 and do not express lineage-specific markers. HSC generates two types of progenitor cells: one that generates lymphoid progenitors and the other that generates myeloid, erythroid, and megakaryocyte cells. Lymphoid progenitors become multiple precursors of T, B, or innate lymphoid cells such as natural killer cells (NK cells). On the other hand, myeloid precursors can be turned into erythroid, megakaryocytes, granulocytes, and monocytes (Fig. 1) (Jagannathan-Bogdan and Zon, 2013).

In addition to HSCs and developing cells, bone marrow also has a large number of long-lived plasma cells. These antibody-producing cells are produced in peripheral lymphoid tissues by antigen stimulation from B-lymphocytes and then migrate to the bone marrow (Pioli, 2019).

Secondary lymphoid organs

Lymph node

The lymph nodes, as one of the most important secondary lymphoid organs, are located in a regular and extensive network throughout the body. Lymph nodes are round or bean-shaped organs located at the junction of large lymphatic vessels. A capsule of connective tissue surrounds this organ and several lymphatic ducts enter or exit it. Each lymph node is composed of three distinct sections, including the cortex, the paracortex, and the medulla. B and T lymphocytes are present in different areas of the lymph nodes. The cortex and paracortex are where the B and T lymphocytes are located, respectively (Fig. 2). The paracortex is also rich in MHCII⁺ dendritic cells or interdigitating dendritic cells (IDC), which have the ability to pick up antigens and present them to T cells, therefore triggering T cell-mediated immune responses. The cortex possesses lymphoid follicles, and in fact, it is the site of humoral immune response in order to produce antibodies against antigens. The medulla is rich in plasma cells.

Antigens enter the lymph node through the afferent lymphatic vessel. Of course, large antigens are received by subcapsular macrophages and are presented to cortical B lymphocytes. This is the first step in antibody-mediated responses to these antigens. In response to antigens, an area called the “germinal center” is formed in the follicles. The germinal center contains active B lymphocytes and follicular dendritic cells (FDCs), and these are sites of significant proliferation of B cells, selection of B lymphocytes producing high-affinity antibodies, and production of memory cells and long-lived plasma cells (Liao and Padera, 2013).

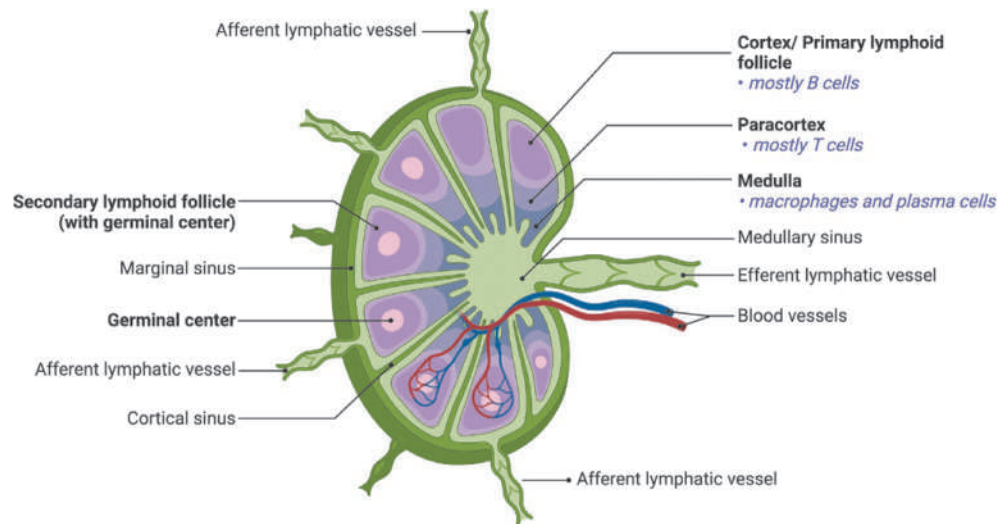


Fig. 2 Lymph node structure and location of immune cells. Adapted from “Lymph Node Structure & Location of Immune Cells”, by BioRender.com (2021). Retrieved from <https://app.biorender.com/biorender-templates>.

Spleen

The spleen is the largest secondary lymphoid organ whose main function is to clear the blood of damaged cells, encapsulated bacteria, and old red blood cells. The spleen is one of the most important organs in the synthesis of antibodies, especially IgM against polysaccharide antigens. In addition, it is a place to store RBC, WBC and platelets. A capsule of connective tissue surrounds the spleen. The splenic parenchyma is anatomically and functionally divided into two important parts: the “red pulp,” which contains sinusoids, and the macrophages present in this part play an important role in clearing damaged cells and opsonized pathogens from the bloodstream; the other is “white pulp,” which is rich in lymphocytes and plays a role in triggering adaptive immune responses to specific antigens in the blood. The white pulp is made up of lymphatic tissue that includes the pale periaarteriolar lymphoid sheath (PALS) and lymphoid follicles. The PALS is rich in T lymphocytes and is similar to the paracortex of the lymph node.

The boundary between the white and red pulp is called the “marginal zone.” This region is made up of B cells, dendritic cells, and macrophages. B cells in this area are called marginal zone B cells (MZB) and function differently than B cells in the follicles (Lewis et al., 2019).

Mucosal associated lymphoid tissue (MALT)

These tissues are the lymphoid organs of the mucosal surfaces of the respiratory, gastrointestinal, and urogenital systems, which are divided into different types depending on their location, including Gut-Associated Lymphoid Tissue (GALT), Bronchus-Associated Lymphoid Tissue (BALT), and Nose-Associated Lymphoid Tissue (NALT).

MALT makes up more than 50% of the body’s lymph tissues. Most body antibodies, secretory IgA (sIgA), and most plasma cells are found in MALT. Gastrointestinal-Associated Lymphoid Tissue includes Peyer’s patches, the appendix, and tonsils.

The main areas of the GALT include small bowel Peyer’s patches, lymphoid follicles throughout the intestine, and mesenteric lymph nodes. Peyer’s patches are lymphoid communities that are surrounded by specialized cells known as “M cells.” These cells actively pinocytosis the macromolecules and transport them from the intestinal lumen to the subepithelial tissues. The intestinal lamina propria contains a variety of cells, including T and B lymphocytes, plasma cells, macrophages, eosinophils, and mast cells. Most lamina propria B cells are plasma cells that produce IgA. Macrophages, especially dendritic cells, are important antigen presenting cells of the lamina propria that play a role in the presentation of lipids and glycolipids to intraepithelial T cells (Cesta, 2006).

An overview of the innate and adaptive immune system

Innate immune system: Components and effector functions

Innate immunity is the first line of defense against pathogens that responds quickly to harmful agents or pathogens. The cellular and molecular components of the innate immune system are present and functioning even before infection. However, some of them, such as the complement system, become active when confronted with stimuli. Although innate immune responses are very effective in eliminating the stimuli in the early minutes after exposure, an efficient immune response requires the cooperation of the adaptive immune system (Tosi, 2005; Beutler, 2004). In this regard, the innate immune system has the ability to activate adaptive immunity through several mechanisms. In the following section we will discuss the components of the innate immune system.

Physical and chemical barriers

The barriers prevent pathogens from entering the body. The epithelial cells lining the respiratory, gastrointestinal, and genitourinary tracts, in addition to providing the tight junctions, can generate a considerable amount of “mucins.” Mucins are mucus-enriched glycoproteins that trap bacteria and impede their spread. Epithelial cells also provide a chemical barrier by generating antimicrobial agents (Schleimer et al., 2007). Chemical barriers against infection include:

1. *Lysozyme*: an enzyme that is found in abundance in secretions such as sweat and tears. Lysozyme is toxic to many pathogens and causes them to lyse. This enzyme is involved in the breakdown of the gram-positive bacteria's cell wall (Sukhithasri et al., 2013).
2. *The acidic pH of the stomach*: prevents the growth and colonization of many pathogens (Smith, 2003).
3. *Defensins*: small cationic peptides that contain two important types of alpha- and beta-defensins in humans. These peptides are produced by mucosal epithelial cells as well as leukocytes such as neutrophils, natural killer cells (NK), and natural killer T cells (NKT). Pant cell defensins are called “Crypticidins,” which are involved in eliminating pathogens in the intestinal lumen (Ganz and Lehrer, 1997; Ouellette and Selsted, 1996).
4. *Cathelicidins*: initially produced as a precursor protein that can be converted to two peptides with protective functions by the effect of microbial products and inflammatory cytokines. They have a toxic effect on a wide range of microorganisms and can also activate various leukocytes to eliminate the pathogen. The C-terminal piece of the cathelicidins is called LL-37 and has the ability to bind to the LPS and neutralize it (Roby et al., 2013).
5. *Lectins*: These are a group of antimicrobial proteins that act particularly against Gram-positive bacteria. They have a carbohydrate recognition domain (CRD) and bind to surface-exposed carbohydrate moieties of peptidoglycan to mediate bacterial death. Reg III is a mouse lectin produced by intestinal Paneth cells which binds to bacterial peptidoglycans. HIP/PAP is an example of a human lectin that causes bacterial death by creating a hole in the bacterial membrane (Breitenbach Barroso Coelho et al., 2018).
6. *Histatins*: these proteins are rich in histidine and found in saliva. Histatins are antibacterial and antifungal proteins that are also involved in wound healing (Edgerton and Koshlukova, 2000).

Innate immune cells: Quickly activated and respond as the first line of defense against infections

The innate immune system contains a variety of cells that can detect and respond quickly to pathogens. These cells include monocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, dendritic cells, NK cells, natural killer T (NKT) cells, and innate lymphoid cells (ILCs). Cellular components of the innate immune system serve a variety of functions aiming to eliminate the pathogen, including inhibition of pathogen entry into the body, antigen capturing by phagocytosis or pinocytosis, killing the pathogens through cytotoxic mediators, and activating the adaptive immune system.

Monocytes and macrophages

These cells are considered professional phagocytes and respond to stimuli via several functions, including antigen presentation and T cell activation, cytokine production, antibody-dependent cell cytotoxicity (ADCC), tissue repair, and regeneration. Monocytes are present in the bloodstream and can move to various body tissues, where they develop into macrophages and myeloid-derived dendritic cells. Interestingly, monocytes are involved in both inflammatory and anti-inflammatory responses. Classical monocytes play an important role in inducing inflammatory responses through phagocytosis and ADCC. On the other hand, non-classical monocytes are more involved in homeostasis maintenance (Sampath et al., 2018). Similarly, two types of macrophages known as “M1 or classically activated macrophages” and “M2 or alternatively activated macrophages” exert pro-inflammatory and anti-inflammatory functions, respectively. M1 macrophages express a number of markers, including Major Histocompatibility Complex II (MHC II), CD80 (B7.1), CD86 (B7.2), and a number of toll-like receptors involved in pathogen recognition and immune response activation. These cells secrete important pro-inflammatory cytokines and chemokines, including IL-1, IL-6, TNF- α , IL-12, CXCL9, and CXCL10. On the other hand, M2 macrophages produce anti-inflammatory cytokines such as IL-10 and TGF- β (Yao et al., 2019).

Dendritic cells (DCs)

These cells are widely found in lymphoid, epithelial, and parenchymal tissues. DCs play a crucial role in the initiation and polarization of immune responses through several functions. DCs in an immature state are present in the blood and after activation, they migrate to the lymph nodes where they interact with T and B lymphocytes to trigger adaptive immune responses. These cells are considered as the professional antigen presenting cells (APCs) so that they have the potential to uptake the antigens by phagocytosis or pinocytosis, processing the antigen and presenting them to T cells, leading to adaptive immune response activation. Therefore, DCs are known as the most important cells, playing an effective role in the relationship between innate and adaptive immunity. DCs express a variety of receptors, including TLRs, to recognize pathogens and microbial components (Hemmi and Akira, 2005; Cerboni et al., 2013).

Immature DCs have a high antigen uptake and processing capacity. Antigen processing by DCs induces high levels of MHC II molecules and co-stimulatory molecules on DCs which are essential for T cell activation. Moreover, active DCs produce a high level of pro-inflammatory cytokines that play an important role in recruiting other leukocytes to the inflammatory site as well as polarization of T lymphocytes. IL-12 is one of the most important cytokines involved in the formation of T lymphocyte responses.

Interestingly, antigen-activated DCs express high levels of chemokine CCR7, which binds to the CCL19 expressed on the high endothelium venule (HEV), leading to the entry of DCs into the lymph node, where they can present antigens to the T cells (Patente et al., 2019).

Different populations of DCs with distinct properties and activities have been found. The two most common DC subtypes are as follows:

1. *Conventional (cDCs)*: They have been found to release pro-inflammatory cytokines and have a high ability for antigen uptake and presentation to naive CD4⁺ T cells (Haniffa et al., 2013).
2. *Plasmacytoid dendritic cells (pDCs)*: They are well recognized for producing type I interferon (IFN-I) during viral infection, but they also have other functions like stimulating T and NK cells and producing pro-inflammatory cytokines (Swiecki and Colonna, 2015).

Neutrophils

These cells develop in the bone marrow under the influence of IL-3, G-CSF, and GM-CSF and then enter the bloodstream. Neutrophils make up 60–70% of peripheral blood leukocytes and, as a professional phagocyte in the peripheral blood, they play a role in defense against extracellular pathogens. These cells are one of the most important innate immune cells involved in response to encapsulated bacteria and fungi (Uribe-Querol and Rosales, 2017). In general, neutrophils can be divided into two groups based on phenotype and function (Wang et al., 2018):

1. *N1 neutrophils*: These cells perform pro-inflammatory functions by producing important inflammatory mediators and they have a high phagocytic potential.
2. *N2 neutrophils*: By producing mediators such as VEGF, IL-1ra N2 neutrophils are more involved in inducing an immunosuppressive microenvironment and therefore resolution of inflammation.

Neutrophils are recruited to the inflammatory site by cytokines and chemokines. Neutrophils are the first cell to be called to the inflammatory site and are an indicator of acute inflammation. An increase in neutrophils in the peripheral blood, known as “neutrophilia,” is caused by various factors such as bacterial infection, stress, hypoxia and even strenuous exercise (Tahir and Zahra, 2021). On the other hand, a decrease in neutrophils or neutropenia is seen in conditions such as chronic infections, leukemia, cytotoxic drugs, aplastic anemia (Justiz Vaillant and Zito, 2021).

Neutrophils have important receptors, including complement receptor-1 (CR1), CR3, and receptors for IgG and IgA that help these cells in the process of phagocytosis (Futosi et al., 2013). Active neutrophils are able to produce many inflammatory cytokines, including IL-12, IL-1, IL-6, IL-18, IL-8, TNF- α , and GM-CSF (Tecchio et al., 2014). These cells are short-lived phagocytes that die shortly after phagocytosis. Dead or dying neutrophils are the majority of pus. Neutrophils possess granules that contain antimicrobial proteins (Cowland and Borregaard, 2016). These granules include the following:

1. *Primary granules*: account for 10–20% of neutrophil granules. Primary granules contain antimicrobial proteins, including myeloperoxidase, lysozyme, and defensin. Besides, it contains proteases such as elastase, proteinase, cathepsin G.
2. *Secondary granules*: account for 80–90% of neutrophil granules. Lysozyme, gelatinase, lactoferrin, and B12-binding proteins are some of the proteins in these granules.
3. *Tertiary granules (gelatinase)*: These granules contain gelatinase (MMP9), arginase 1, and MMP25.

Exocytosis occurs when neutrophils release their granules in response to numerous stimuli such as microbial components or elevated intracellular Ca²⁺ levels, resulting in pathogen death. A lack of neutrophil-associated granules makes individuals more susceptible to Gram-positive or Gram-negative bacterial infections (Kumar and Sharma, 2010).

Another important function of neutrophils is a process known as NETosis. This process is a type of cell death distinguished by the release of decondensed chromatin and granular components into the extracellular environment, resulting in bacterial degradation. By generating neutrophil extracellular traps (NETs), neutrophils can defend against infections (Kumar and Sharma, 2010).

Basophils, mast cells, eosinophils

These cells have certain characteristics in common. All three cells have granules rich in antimicrobial mediators. These cells are involved in the defense against worms and parasites and play a role in the pathogenesis of allergic diseases.

Basophils develop in the bone marrow and then enter the bloodstream. Basophils account for less than 1% of peripheral blood leukocytes. These cells are involved in defending against parasites and causing immediate allergic reactions. Basophils have granules containing histamine, heparin, and serine proteases which play an important role in inducing inflammatory mechanisms in hypersensitivity reactions including asthma, anaphylaxis, and allergic rhinitis. Basophils have the potential to promote Th2 responses through producing IL-4 and IL-13 cytokines. Besides, they can produce other mediators, including leukotrienes and prostaglandins (Chirumbolo et al., 2018).

Mast cells develop in bone marrow under the influence of a cytokine called Stem Cell Factor (or c-Kit ligand). Mast cells are not normally present in the bloodstream, they are mostly found on the skin, in mucosal membranes, and in the vicinity of blood vessels. Similar to basophils, these cells are predominantly involved in allergic reaction pathophysiology. Besides, mast cells have critical roles in the immune response to bacterial and parasite diseases. Like basophils, these cells have high-affinity membrane receptors for IgE antibodies (Fc ϵ RI) (Abraham and Arock, 1998).

In summary, when B cells identify and uptake an allergen, the presentation of these allergens to T cells leads to the differentiation of Th2 cells, which help the B cell to make IgE. These antibodies bind to their receptors, FcεRI, on the basophil and mast cell surfaces. These cells are called sensitive cells at this point. Subsequently, re-entry of the allergen into the body leads to cross-linking of basophil and mast cell surface IgEs by the allergen. As a result, signaling pathways are induced that lead to the activation and degranulation of these cells. Granules contain storage substances such as histamine, heparin, kinase, tryptase, or mediators that are made after cell stimulation, such as IL-3, IL-4, IL-5, IL-13, IL-9, or lipid mediators such as leukotrienes, prostaglandins, thromboxane, and platelet activating factor (PAF). Consequently, the release of these granules leads to the appearance of allergy symptoms (He et al., 2013).

Eosinophils develop in the bone marrow under the influence of GM-CSF, IL-5, and IL-3. In addition to the blood, eosinophils are present in peripheral tissues, especially in the lining of the gastrointestinal tract, respiratory tract, and urogenital tract. These cells have very weak phagocytic potential. Instead, these cells are the most important cells in killing worms, including schistosomiasis, through ADCC. These cells bind to IgG-coated and IgE-coated parasites via FcγR and FcεR receptors, respectively. Subsequently, this stimulates the release of mediators from eosinophil granules. In general, these cells kill the worms, particularly by FcεRI and IL, especially through the ADCC. Besides, due to their role in tissue repair, eosinophils can be involved in establishing homeostasis (Mukai et al., 2016; Weller and Spencer, 2017).

Eosinophils are able to make metabolites such as leukotrienes and prostaglandins after stimulation. Leukotrienes are involved in mucus secretion, vascular permeability, and prolonged bronchospasm. Cytokines (e.g., IL-5, IL-3, IL-4, IL-8, IL-1, IL-13, IL-25, TNF-α), growth factors (e.g., GM-CSF, TGF, VEGF, and NGF), as well as chemokines (e.g., CXCL1, CXCL5, CXCL8, CXCL9) are also important mediators that Eosinophils secrete after stimulation. In addition, these cells contain granules that release their contents in defense against worms and parasites (Shamri et al., 2011). This content includes the following:

1. Major Basic Protein (MBP).
2. Eosinophil Cationic Protein (ECP).
3. Eosinophil Peroxidase (EPO).
4. Eosinophil-Derived Neurotoxin (EDN).

Natural killer (NK) cells

These cells originate from the lymphoid lineage in the bone marrow. IL-7 and IL-15 cytokines play an important role in the development and survival of NK cells. They are known as large granular lymphocytes (LGL) and account for 5–15% of blood and spleen lymphocytes. Although they originate from the lymphoid lineage, they often do not express B and T cell markers, so they are known as non-B, non-T lymphocytes. These cells are the first lymphocytes involved in innate defense against intracellular infections, especially virus-infected cells and cancerous cells (Vivier et al., 2008).

The formation of an immunological synapse between the NK cell and the target cell is required for the cytotoxic response of the NK cell. These cells bind to the surface antigens of the virus or tumor cells through their receptors and exert their cytotoxic activity. One of the most important receptors involved in the NK cytotoxic process is CD16 (FcγRIII). This receptor binds to cells coated with IgG antibodies, resulting in the release of the cytotoxic granules of the NK cell, killing the target cell through ADCC. However, in addition to NK cells, macrophages, neutrophils, and eosinophils are also able to mediate ADCC. NK granules contain lethal mediators such as perforin and granzyme. Perforin polymerization on the cell membrane of target cells creates pores that allow granzymes to enter the cells. Granzyme is a serine protease which activates the enzymes known as “caspases,” resulting in the induction of cell death or “apoptosis” in target cells. Apoptosis of the target cells can also occur through other receptors such as the TNF receptor (TNFR) and the Fas receptor (Topham and Hewitt, 2009; Sonar and Lal, 2015; Thorburn, 2004).

In general, NK cells can be divided into two categories based on markers, each of which has different functions. CD16^{High}CD56^{Low} NK cells have high levels of perforin, therefore they have more cytotoxic effects. On the other hand, CD16^{+/−}CD56^{High} NK cells are distinguished by low amounts of perforin and are extremely proficient in cytokine generation. Once activated, NK cells release a variety of cytokines and chemokines, including IFN-γ, TNF-α, IL-2, granulocyte macrophage colony-stimulating factor (GM-CSF), CCL1-CCL5, and CXCL8 (Paul and Lal, 2017).

Helper innate lymphoid cells (ILCs)

These cells originate from the lymphoid precursor in the bone marrow. They are morphologically similar to lymphocytes. In addition, the function of ILCs is very similar to that of helper T cells. However, they lack the T cell receptor, costimulatory molecules, as well as CD3, and their stimulation is not dependent on the MHC molecule.

These cells include heterogeneous populations and are present in many tissues, including lymphoid tissues, peripheral organs such as the lungs, dermis, liver, and small intestine and play a vital role in mucosal immunity and tissue homeostasis. Three categories of helper ILCs are recognized based on their similarity to helper T cell subtypes in terms of transcription factor and cytokine production (Mathä et al., 2021a,b; Vivier et al., 2018).

1. ILC1s:

These cells produce IFN-γ and TNF-α against intracellular pathogens in the presence of IL-12, IL-15, and IL-18. ILC1s express the transcription factor T-bet and need IL-15 and IL-7 for their development and function. This group is thought to be responsible for defending against intracellular pathogens such as tumors and viruses. Unlike NK cells, these cells lack granzyme B and perforin. These cells also participate in the pathogenesis of inflammatory bowel and lung diseases (Fuchs et al., 2013).

2. ILC2s:

These cells develop under the influence of cytokines such as IL-25, IL-33, and TSLP and produce IL-5 and IL-13. This group expresses the transcription factor GATA3. ILC2 cells induce Th2 responses through IL-4 production. In addition, IL-13 produced by ILC2s plays an important role in the removal of intestinal worms by inducing goblet cell division, eosinophilia, and contraction of colon smooth muscle cells. Pathologic and protective functions of ILC2s have been reported in a variety of human disorders, including fibrosis, asthma, atopic dermatitis, as well as infections (Mathä et al., 2021a,b).

3. ILC3s:

ILC3s play a critical role in the immune response to extracellular bacteria and fungus. This group expresses the transcription factor ROR γ t. In response to IL-23 and IL-1, they generate IL-22 and IL-17, which stimulate Th17 responses and neutrophil recruitment. In addition, these cells contribute to the upregulation of antimicrobial peptides and the improvement of barrier integrity, particularly in the gut, by producing IL-22 (Aujla et al., 2008; Sugimoto et al., 2008; Zheng et al., 2008).

Effector mechanisms of the innate immune system

Identify the stimulus

To generate appropriate responses, the innate immune system must recognize the stimulus properly. A variety of innate immunity receptors have been described, each identifying “pathogen-associated molecular patterns” (PAMP) or “damaged-associated molecular patterns” (DAMP). PAMPs are specific to pathogenic microorganisms and are not present in mammalian cells, such as viral RNA, lipopolysaccharide (LPS) of Gram-negative bacteria, and peptidoglycan of bacterial cell wall. DAMPs may be generated by cellular damage caused by infection or by non-infectious agents or other causes such as chemical toxins, burns, and trauma. Heat shock proteins (HSPs), high mobility group box 1 (HMGB1), urate crystals, and cholesterol are examples of DAMPs. PAMPs and DAMPs can be detected by non-specific receptors known as “pattern recognition receptors”, or PRRs, which are found in immune cells’ sub cellular compartments (e.g., within phagocytic vesicles, in the cytoplasm or cell surface) (Mogensen, 2009).

Several types of PRRs have been identified, including:

1. Toll-Like Receptors (TLR).
2. Nucleotide-binding Oligomerization Domain (NOD).
3. Leucine Rich Repeats-containing receptors (NLR).
4. Retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLR).

By binding to the PAMPs or DAMPs, PRRs induce the signaling pathway, which in turn enhances antimicrobial and proinflammatory functions in the cells expressing these receptors. Other innate immune receptors involved in identifying microbial components and inducing inflammatory responses against pathogens include:

1. Scavenger receptor (SR): Most often expressed on monocytes, macrophages, and dendritic cells, it includes various types such as CD163, CD36, and Macro. They bind to lipids, carbohydrates, oxidized or acetylated LDL. These receptors are effective in phagocytosis of microorganisms and are involved in clearing tissue of apoptotic cells, altered lipoproteins, LPS and lipoteichoic acid (Zani et al., 2015).
2. CD14: It is known as the LPS receptor and can bind to lipid A. It is expressed by most cells except endothelial cells. This receptor plays an important role in inducing an effective response to LPS by TLR4 (Viriyakosol et al., 2000).
3. C-type lectin receptors (CLRs): These receptors detect carbohydrates on the surface of microbes and facilitate the phagocytosis of them. There are a number of receptors in this family, including mannose receptor (MR), DC-SIGN, and Dectins (Yan et al., 2013).
4. Complement receptors (CRs): In addition to binding to complement components and inducing inflammation, these receptors act as opsonin receptors and stimulate phagocytosis or intercellular adhesion. For example, CR3 can be bound to LPS or to the complement components C3b and iC3b (Vorup-Jensen and Jensen, 2018).

Effector mechanisms

Several effector functions are defined by components of the innate immune system, including pathogen killing by phagocytosis or cytotoxic mediators, recruitment of the other immune cells by production of inflammatory mediators, and antigen processing and its presentation to adaptive immune cells. In the following, we will review the most important executive mechanisms of the innate immune system.

Phagocytosis

It is a process in which the pathogen is first surrounded by a phagocytic membrane and is killed in the phagosome vesicle under acidic conditions (Fig. 3). The most important phagocytic cells are neutrophils, monocytes, and macrophages. However, dendritic cells also have high phagocytic ability and act as antigen carriers to secondary lymphatic organs. Eosinophils, basophils, and mast cells are also able to perform phagocytosis with less power (Rabinovitch, 1995).

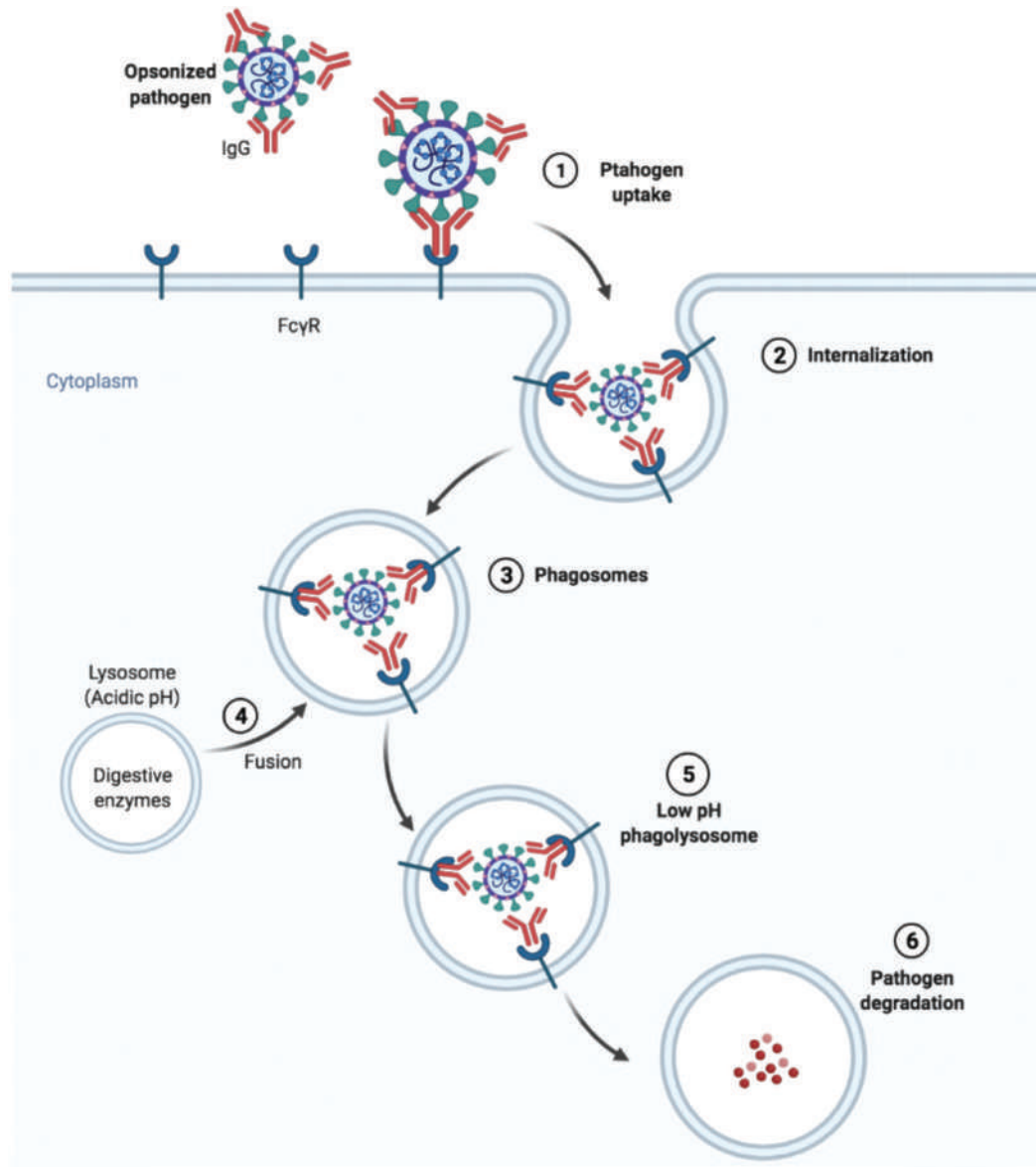


Fig. 3 Schematic diagram of phagocytosis. CR: complement receptor; FcγR: Fc gamma receptor; IgG: Immunoglobulin G. Created by BioRender.com (2021).

Although phagocytosis can occur in the absence of opsonins, antigens coated with opsonins are more easily phagocytosed. In this regard, the opsonized particular antigens with complement components (e.g., C3b) and antibodies bind to the complement receptor (CR) and the Fc receptor (FCR), respectively, and eventually, by contracting actin and myosin and forming a membrane around the antigen, phagosomes are formed. Subsequently, by phagosome and lysosome fusion, phagolysosome is produced and antigen is digested inside the phagolysosome. Receptors that are able to directly detect a wide range of microorganisms without the involvement of opsonins include scavenger C-type lectin receptors, scavenger receptors, and TLRs (Freeman and Grinstein, 2014).

Complement system

The activation of the “complement system” is another important effector mechanism of the innate immune system. Over 30 soluble and cell surface proteins make up this system. The complement system can be activated via three major pathways, including classical, alternative, and lectin pathways (Ghebrehiwet, 2016). Activation of these pathways eventually leads to events including pathogen destruction, increased recruitment of inflammatory immune cells, and enhanced phagocytosis. The “classical pathway” is activated by the immune complexes of antigen-antibody, which consists of antibodies (IgG1 or IgM) attached to the cell surface of pathogens. In this pathway, the C1 complex (C1q, C1r, C1s) binds to the Fc region of antibodies. C1s is a serine protease that cleaves C4 and C2 into C4a, C4b and C2a, C2b, respectively, when activated. C4b and C2a then form the C4bC2a complex, which interacts with the pathogen surface. The C4bC2a complex functions as a “C3 convertase” enzyme, cleaving C3 into C3a and C3b fragments.

The C3b fragment attaches to the microbial surface covalently, and when linked with C4bC2a, it forms the C5 convertase complex (C4bC2AC3b), which converts C5 to C5a and C5b. C5b binds to the microbial surface, causing other components such as C6, C7, C8, and C9 to become activated. Finally, polymerization of C9 on the pathogen surface leads to the creation of numerous pores known as the “membrane attack complex,” or MAC, which can lyse the pathogen directly.

The “lectin pathway” is similar to the classical pathway; however, it does not require antibodies to activate. PRRs such as mannose-binding lectin (MBL) and ficolins identify the carbohydrates on the microbial surface. MBL forms a complex with the MBL-associated serine proteases (MASPs)-1, -2, and -3, whose activation results in the cleavage of C2 and C4, and the subsequent production of the C3 convertase (C4bC2) of both classical and lectin routes.

The spontaneous hydrolysis of C3 into C3a and C3b activates the “alternate pathway.” C3b binds to the microbial surface in the presence of microbial infection. As a result of the interaction of B and D factors with C3b, further C3 cleavage occurs, resulting in the formation of alternative route C3 convertase (C3bBb) and C5 convertase (C3bBbC3b). Properdin is a protein that helps to stabilize alternative pathway convertases and promotes alternative activation.

ADCC

Another important function resulting in pathogen elimination is ADCC, which was previously described in NK cell functions. This process requires immune cells expressing the Fc receptor as well as a target antigen coated by the antibody. As previously mentioned, immunoglobulin G (IgG) and its receptor named FcγRIII (CD16) play an important role in ADCC activation (Mielke et al., 2019).

Antigen presentation

This process can be considered as a key mechanism linking the innate and adaptive immune systems. As mentioned earlier, adaptive immunity is required to establish a proper response against the pathogen. The presentation of antigen by “Antigen presenting cells” or APCs to T cells is one of the vital events in T cell activation. In general, APCs are cells that are able to perform antigen recognition and uptake, antigen processing, loading the peptide epitopes on the MHC molecules, and finally antigen presentation in the form of the MHC:peptide complex to T lymphocytes. Dendritic cells, macrophages, and B cells are considered as the professional APCs (DiLillo et al., 2011; Katz, 1988).

MHC molecules are membrane glycoproteins which are composed of two types, including MHC class I and MHC class II which in turn involve in presentation pathway I and II, respectively. The most important difference between pathway I and II is the source of peptides that are presented. On the presentation pathway by MHC I, peptides derived from proteins synthesized in the cytoplasm (such as viral proteins) are presented to cytotoxic T cells (CTLs). MHC class II molecules are involved in the presentation of peptides derived from proteins in intracellular vesicles (such as peptides derived from pathogens living in macrophage vesicles or internalized by phagocytes) to Th1 or Th2 cells. Upon recognizing their targets, the effector T cells are stimulated to produce and release various mediators to kill the target cells (CTLs) or help to recruit other effector cells (Th cells) (Blum et al., 2013).

Adaptive immune system: Components and effector functions

B lymphocytes and antibodies: The main players in humoral immunity

B lymphocytes are one of the specific immune cells that make up the humoral immunity arm. As described in the organs section, these cells develop in the bone marrow and fetal liver, and their final differentiation occurs in the secondary organs (e.g., lymph nodes or spleen) after stimulation with antigen. These lymphocytes normally circulate between the blood, lymph and organs. Upon exposure to antigens, B lymphocytes proliferate and differentiate into memory cells and plasma cells. Memory cells are involved in the secondary response to antigen, and plasma cells are antibody-producing cells. B lymphocytes possess membrane immunoglobulins (mIg) for specific antigen recognition and uptake (DiLillo et al., 2011).

Antigen recognition and B cell activation

B lymphocytes recognize the antigens through the B cell receptor (BCR) complex, which consists of two important parts (Pleiman et al., 1994):

1. The portion of the protein that binds to the antigen known as BCR
2. Signal transmission units include CD79a (Igα) and CD79b (Igβ), molecules which are known as first signals for B cell activation.

Once the B cell is activated, the second signal, which includes the interaction between CD40 (expressed on B cells) and CD40L (expressed on T cells), is activated. In addition, a complex called the co-receptor on the B cells plays a vital role in B cell activation. This complex consists of three important molecules, including CD21, CR2, and CD81 (TAPA-1).

Types of B cells

B1 and B2 cells are two subclasses of B cells that have diverse phenotypes and functions. B1 cells are mostly located in the peripheral organs and are rarely found in the blood. They are extremely capable of producing natural antibodies in the absence of any infections. These cells are abundant in the fetus and early infancy. B2 cells are classified into two types: B follicular (BFO) and B marginal zone (BMZ). BFOs are the most common type of B cell in circulation and they produce the majority of antibodies during an infection. BMZs are found largely in the spleen’s marginal zone and play a role in defense against blood-borne infections (Pieper et al., 2013).

Antibodies: The mediators of humoral immunity

Antibodies are glycoproteins found in body fluids such as whole blood, serum, or plasma, as well as extracellular fluids. These molecules are the products of plasma B cells and bind specifically to antigens. An antibody molecule has a Y-shaped structure and consists of two similar light chains (L) and two similar heavy chains (H) (Forthal, 2015). In general, immunoglobulins are divided into five classes based on differences in heavy chain structure, including:

IgG: It is structurally a monomer antibody containing two heavy and two light chains. This antibody makes up 60–70% of all serum immunoglobulins and has the longest half-life. This antibody is the strongest antitoxin. IgGs are often produced in the second response to an antigen. IgG1 and IgG3 antibodies are the most important opsonins and are involved in ADCC, especially by the NK cell.

IgA: It is found in various forms of monomer, dimer, trimer, etc. It is the most important antibody in mucous secretions and its key function is mucosal defense. sIgA, a secretory dimer form, is found in gastrointestinal, genital, respiratory, saliva, and tear secretions. Serum IgA can activate complement through an alternative pathway.

IgM: It makes up 10% of serum antibodies and is often found in the form of pentamer in the serum. The monomer IgM is present on the cell membrane of the B lymphocyte. It plays an important role in the identification of carbohydrate antigens. This antibody is involved in stimulating the classical pathway of the complement system.

IgE: This antibody has a monomeric structure and has a short half-life. Of course, it is found on the surface of basophils and mast cells with a long half-life. This antibody plays a major role in type I hypersensitivities.

IgD: This antibody is found in monomer form on the membrane of the B lymphocytes. It accounts for less than 1% of total serum immunoglobulins.

IgG and IgA can be divided into subclasses based on differences in biological activity or other factors such as structure, electrophoretic motion, and hinge length. There are four IgG subclasses, including IgG1, IgG2, IgG3, and IgG4. IgA1 and IgA2 are two subclasses of IgA.

Antibodies are bi-functional molecules; they bind to antigens via an antigen binding site located in the amino terminal (N-terminal) of the antibody. This process is also known as “neutralization”, through which antibodies neutralize pathogens or their toxic products by binding to them and thus preventing them from entering cells. On the other hand, antibodies exert their biological effects via the stem of the Y structure, which is referred as “fragment crystallizable region” or Fc. The most important biological functions of antibodies are the induction of ADCC and activation of the complement system (Fig. 4).

Importantly, different cytokines have distinct effects on B cell activation and antibody production. In this regard, IL-5 is involved in the differentiation of IgA-producing plasma cells and mucosal immunity. IL-2 enhances antibody production by proliferating and stimulating B lymphocytes. In humans, IFN- γ cause antibody class switching to IgG1 and IgG3. These antibodies stimulate the complement system and ADCC. IL-4 is involved in the production of IgE, which plays an important role in hypersensitivity type I and in defense against parasites. IL-6 is involved in the differentiation of plasma cells and IgA production.

T lymphocytes: The main players of cellular immunity

T lymphocytes develop in the thymus and make up approximately 50–70% of peripheral blood lymphocytes. These cells, like B lymphocytes, have specific antigen receptors known as T cell receptors (TCRs) and therefore interact specifically with the target cell. Importantly, the function of these cells depends on the MHC molecules and they respond only when the antigen is presented to them by the MHC. In other words, unlike B cells, T cells are not able to directly detect antigens unless the antigen is associated with the MHC molecule. In this section, we will review how the T cell is activated, its effector functions, and the types of T cells.

Antigen recognition and T cell activation

Similar to B cells, T cells have an antigen-recognizing complex and costimulatory molecules (Griesser and Mak, 1994). TCR acts as an antigen recognition molecule, and CD3, along with TCR, is involved as the first signal in signal transduction and stimulating T cells (Fig. 5). CD28 is expressed as the second signal on T cells. It has a homodimeric structure that binds to B7 molecules (B7–1 and B7–2) on APCs. Involvement of CD28 leads to the activation of several signaling pathways, which eventually leads to the activation of the T cell. Interestingly, when the frequency of B7 is low, such as when APCs are resting or after pathogen elimination, a molecule called CTLA4, which has a higher affinity for B7, interacts with B7 molecules, resulting in diminished T cell responses and establishing homeostasis (Kosmaczewska et al., 2001).

The formation of synapse between the T cell and the APC is required for proper T cell activation. In this region, interactions occur between molecules on the surface of T and APC cells, and cytokines and mediators are transported more rapidly. IL-1, IL-12, and IL-15 are the first cytokines secreted by APC resulting in T cell stimulation. Once activated, T cells produce cytokines including IFN- γ , IL-2, IL-4, and IL-5 which in turn enhance the APC or T cell responses (Malissen et al., 2014).

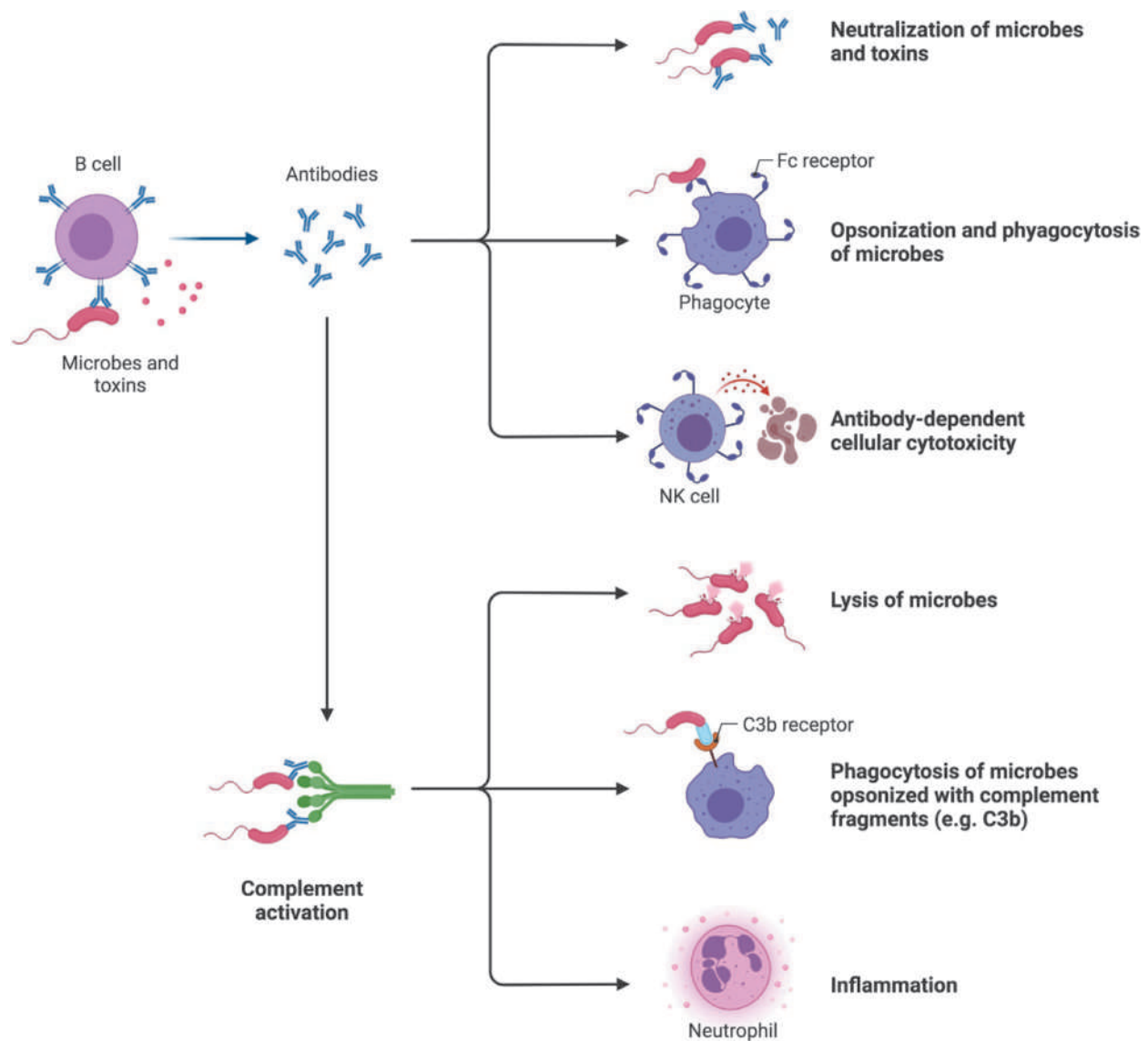


Fig. 4 The effector mechanisms of antibodies. Adapted from “The Effector Functions of Antibodies”, by BioRender.com (2021). Retrieved from <https://app.biorender.com/biorender-templates>.

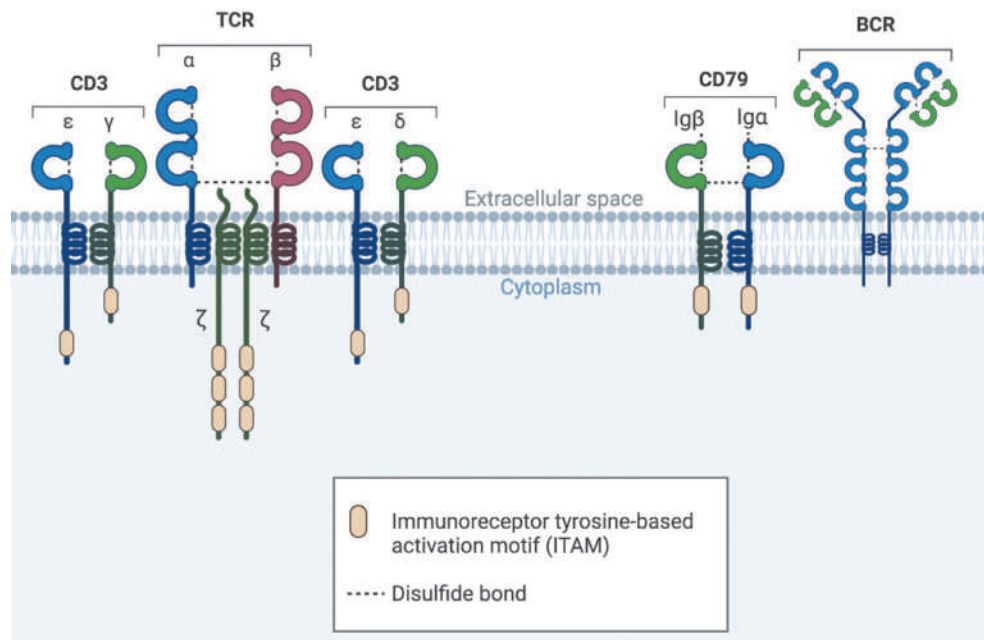


Fig. 5 TCR and BCR complexes. Adapted from “BCR structure” and “TCR/CD3 Subunit Structure”, by BioRender.com (2021). Retrieved from <https://app.biorender.com/biorender-templates>.

The effector mechanism of T lymphocytes

In general, T cells have important functions, including antigen recognition, responding to intracellular antigens such as tumor or virus antigens, helping to produce antibodies from B cells, and regulating immune responses.

Some pathogens, such as viruses, parasites, and bacteria, require entry into cells where they are not identified by antibodies. These intracellular infections are eliminated by T cells. T cells are classified into two types: helper T cells (TH) and cytotoxic T cells (CTL) (Chaplin, 2010).

Helper T cells

Th cells express CD4 marker and they are involved in helping other immune cells in responding to pathogens especially through producing cytokine including $\text{IFN}\gamma$, IL-2, and $\text{TNF-}\alpha$. They identify peptide antigens provided in the form of “MHC II: peptide complexes” by APCs. Th1 and Th2 are the major subsets of the helper T cells. However, other subsets such as Th17, Th22, Th9, Th25, etc. have also been identified which play important roles in immune responses (Romagnani, 1991; De Sousa and Quaresma, 2018; Maddur et al., 2012; Jia and Wu, 2014).

Th1 cells

These cells play a vital role in preventing intracellular bacterial infections such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*. These cells are the most important source of IL-2 and $\text{IFN}\gamma$ cytokines. IL-12 and $\text{IFN-}\gamma$, which can be produced by APCs and NK cells, respectively, play an important role in the differentiation of Th1 cells. These cytokines activate transcription factors T-bet, STAT1, and STAT4 and are considered to be the main regulators of Th1 cells (Constant and Bottomly, 1997).

Dendritic cells and macrophages are the most important cells that present antigen to Th1 cells. Th1 helps kill phagosomal antigens by stimulating macrophages. In this regard, Th1 stimulates the activation of reactive oxygen intermediates (ROI) and nitric oxide (NO) in phagocytes by producing $\text{IFN-}\gamma$, and thus plays an effective role in the death of phagocytosed antigens (Fig. 6). Other Th1 activities include increase macrophage infiltration to the sites of infection, promote the cytotoxic T cells and NK cells responses, induce B cell responses to make opsonizing antibodies and complement-activating antibodies importantly IgG1 and IgG3.

Th2 cells

Th2 cells play a key role in the eradication of extracellular bacteria and parasitic helminth infections through activating B cells (Constant and Bottomly, 1997). They produce a variety of cytokines including IL-4, IL-5, IL-9, and IL-13 which have an important role in controlling the extracellular helminth infections such as *Schistosoma mansoni* (Oliphant et al., 2011). Besides, they are involved in pathogenesis of hyper sensitivity type I by inducing the production of IgE antibodies.

B cell is considered as the APC that present antigen to Th2 cells. Th2 cytokines, including IL-4 and IL-5, are important mediators in the proliferation and differentiation of B cells and the production of IgA and IgE. Besides, IL-4 activates the transcription factor STAT6 which triggers the development of Th2 cells. STAT6 is also leads to the induction of GATA3 expression. GATA3 is the main regulator of Th2 differentiation. Th2 cytokines also induce recruitment and activation of the inflammatory cells including basophils, mast cells, and eosinophils.

Cytotoxic T cells (CTLs)

Cytotoxic T cells are an important subset of T cells that respond to intracellular infections such as viruses and malignant cells. These cells have CD8 molecules on their cell surfaces and can regulate the infection by killing infected cells directly. They recognize peptide antigens provided in the form of “MHC I: peptide complexes” by APCs (Fig. 7) (Henkart, 1997).

By recognizing the target antigen by the TCRs, T cells initiate target cell apoptosis. The main mechanism of killing the target cell by CTL is the release of cytotoxic mediators including perforin and granzyme. CTL cytotoxic granules are similar to NK cells and kill the target cell by creating a pore in it. There are other pathways for target cell apoptosis by CTLs, including FasL (or CD178, expressed

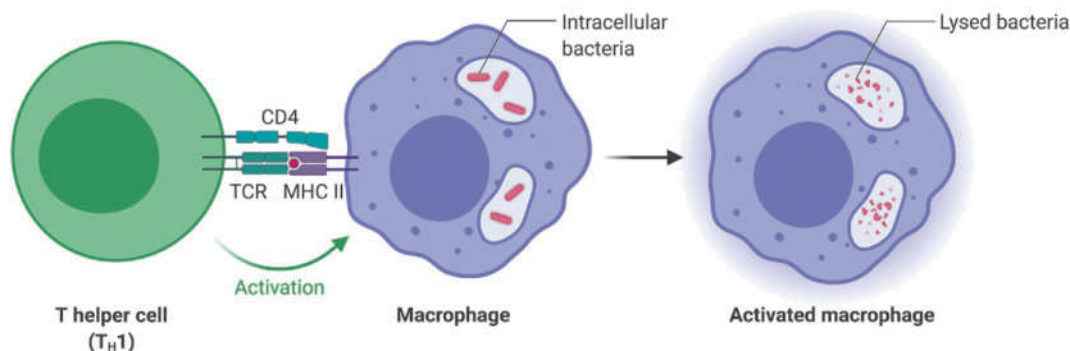


Fig. 6 Th1 activation. Adapted from “Th1 Cells Help Macrophages Kill Intracellular Bacteria” by BioRender.com (2021). Retrieved from <https://app.biorender.com/biorender-templates>.

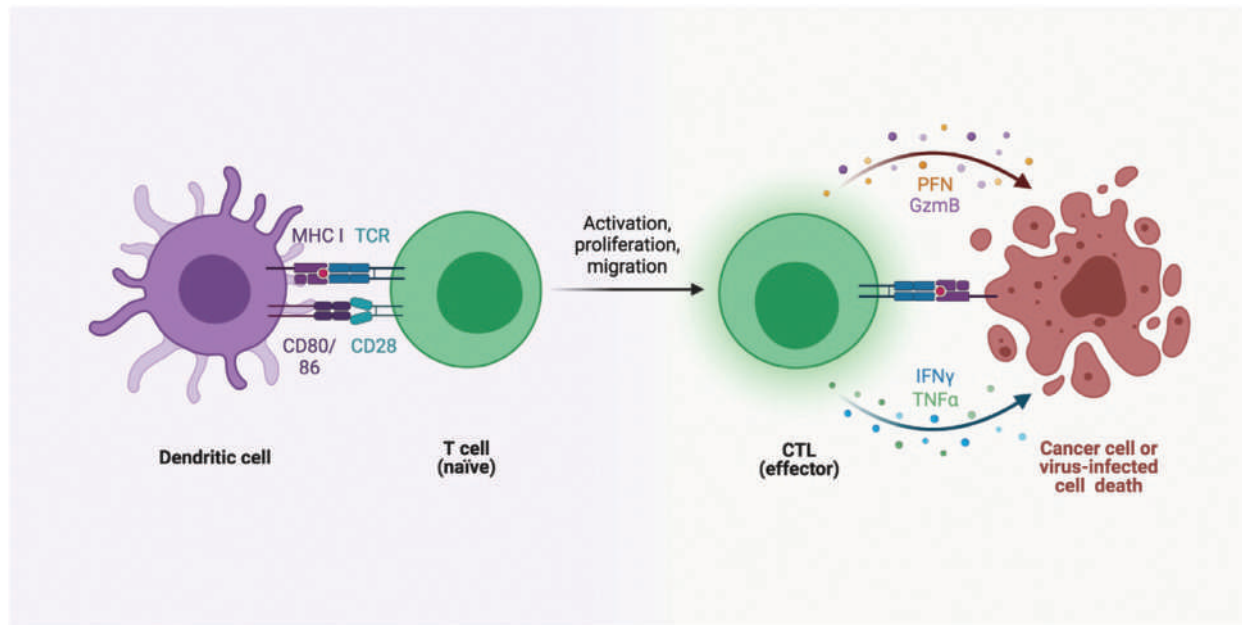


Fig. 7 CTL activation. *PFN*, perforin; *Gzm*, Granzyme B; *IFN*, interferon; *TNF*, tumor necrosis factor; *MHC*, major histocompatibility complex. Adapted from "T Cell Activation in Cancer" by BioRender.com (2021). Retrieved from <https://app.biorender.com/biorender-templates>.

on active T cells) interaction with Fas (or CD95, expressed on cancerous cells or virus-infected cells), which leads to "Caspase 8" stimulation and target cell apoptosis. Another pathway is interaction between TNF and its receptor, TNFRI or CD120a, causing Caspase 8 stimulation and apoptosis of target cell. TNF is produced from various cells, including CTL, NK, and Th1.

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Adaptive Immunity

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Abbreviations

APRIL	A proliferation-inducing ligand
BAFF	B cell-activating factor
BLNK	B cell linker protein
CLIP	Class II-associated invariant chain peptide
ICOS	Inducible T-cell costimulatory
LAT	Linker of activated T cells
PLCγ1	Phospholipase Cγ1
RAG	Recombinase activating gene
SCID	Severe combined immunodeficiency
SLP-65	SH2-binding leukocyte phosphoprotein of 65 Kd
SLP-76	SH2-containing leukocyte protein 76 Kd

TACI	Transmembrane activator and CamL interactor
TAP	Transporter associated with antigen processing
TREC	T-cell receptor excision circle
ZAP-70	Zeta-Associated protein, 70 Kd

Introduction

It has long been believed that innate immune system is non-specific, non-selective and the range of common pathogenic molecular patterns (PAMPS) it can recognize is limited. The extremely high variability of antigenic epitopes as well as the ability of pathogens to rapidly switch their antigenic phenotype has led to the counter-evolution of the adaptive immune response (Flajnik and Kasahara, 2010). The cells of innate immune system continually survey the environment and recognize the pathogens via PAMP-recognition receptors (PRRs) which are all encoded in the germline genome. This is in contrast to the lymphocytes of adaptive immune system that can recognize antigens through diverse, selected antigen-specific receptors that are generated by somatic DNA re-arrangement (Market and Papavasiliou, 2003). After initial exposure to pathogen, memory lymphocytes expressing pathogen-specific receptors are established, providing immunological memory and the capability for rapid and efficient counteract against subsequent exposure. After developing in the primary lymphoid organs (i.e., thymus and bone marrow), mature B and T lymphocytes migrate to secondary lymphoid organs where they can populate in distinct zones to capture and recognize their cognate antigens. Upon antigen-specific recognition, the lymphocytes are activated and differentiated into antigen-experienced effector cells. These cells exert their effector functions through either antibody production (for B cells) or supporting immune responses via production of cytokines or by directly killing virus-infected cells (for T cells). Interaction of antigen presenting cells (APCs) with T cells bridges innate and adaptive immune responses (Palm and Medzhitov, 2009; Iwasaki and Medzhitov, 2015). For example, APCs in concert with the molecules produced during innate immune reactions (e.g., cytokine and complement fragments) provide activation signals for T and B cells. Activated antigen-experienced lymphocytes can circulate in the body and home to the initial site of infection to exert their effector functions. This trafficking is tightly regulated by differential expression of adhesion molecules and chemokine receptors on lymphocytes (Jalkanen and Salmi, 2019). This article describes the principal mechanisms of lymphocyte development, activation and differentiation into various subsets, and ends with a discussion of how effector lymphocytes give rise to memory cells. An emphasis is also placed on the cell- and antibody-mediated immunity.

Types of adaptive immunity

General features

Innate immunity constitutes the first line of defense against invading pathogens. Innate immune responses are restricted to the recognition of specific structures that are conserved within a class of microbes, termed pathogen-associated molecular patterns (PAMPs). The nonclonotypic receptors of the innate immune system that evolved to recognize PAMPs are called pattern-recognition receptors (PRRs). However, many pathogens have evolved to resist innate immunity. Defense against such pathogenic microbes needs the more robust and specialized mechanisms of adaptive immunity (Flajnik and Du Pasquier, 2004). Although adaptive immunity is much slower to respond to threats, antigen-specific lymphocyte clones detect and respond to both microbial and nonmicrobial antigens. The ability to distinguish fine antigenic differences, also known as specificity, and the capacity to respond more powerfully to repeated encounters to the same antigens, also known as memory, are the another distinctive features of adaptive immunity (Table 1). Another cornerstone of the both adaptive and innate immunity is the recognition of “self” versus “non-self” (Hedrick, 2004). Although innate and adaptive immune responses are distinct arms of the immune system, both of them constitute an integrated host defense system that involves many cells and molecules which are function co-operatively (Iwasaki and Medzhitov, 2015).

Table 1 Comparative features of innate and adaptive immunity.

Feature	Innate immunity	Adaptive immunity
Specificity	Limited; only PAMPs	High; PAMPs+ nonmicrobial antigens
Specialization	No	Yes
Clonality	No	Yes
Diversity	Limited; germline encoded	Extensive; receptors are generated by somatic recombination of gene segments
Reactivity against self	No	No
Contraction and homeostasis	No	No
Memory	No	Yes

Phases of adaptive immune responses

The unique components of adaptive immunity are lymphocytes and their secreted products, such as cytokines, cytolytic molecules and antibodies. These lymphocyte-derived molecules in harmony with different components of innate immune system (e.g., phagocytosis) eliminate most of invading pathogens. There are a series of sequential events which play central role in specific reactions of lymphocytes to antigens. They are including antigen recognition, lymphocyte activation and expansion, antigen elimination, cellular contraction and memory formation. Once specific clones of naïve lymphocytes recognize a foreign antigen, they start to activate and proliferate to a large number of the cells that express identical receptors for the given antigen and thus belong to a clone (Burnet, 1959; Huppa and Davis, 2013). Expansion of specific lymphocyte clones with the same antigen specificity is also known as clonal expansion. Some of the progeny of these activated and expanded lymphocytes differentiate into effector cells. As effector lymphocytes have a limited lifetime and, once antigen is eliminated, most of the antigen-specific lymphocyte clones undergo apoptosis probably due to elimination of antigen as a survival signal, downregulation of anti-apoptotic proteins Bcl-2 and Bcl-XL and regulation of proapoptotic proteins Bax and Bim (McKinstry et al., 2010). However, a small number of antigen-specific lymphocyte clones continue to survive after antigen elimination (memory lymphocytes).

T lymphocytes and cell-mediated immunity

T lymphocytes, also called T cells, are essential components of the adaptive immune system. T cells derived from common lymphoid progenitors (CLPs) coming from the early sites of hematopoiesis (i.e., the bone marrow or fetal liver) are matured in the thymus and through expressing highly diverse antigen receptors on their surface they are capable to detect several foreign antigens. Large number of T cells expressing distinct antigen receptors- and therefore specificities- are generated during the development of thymocytes in the thymus (Kondo et al., 2019). In the secondary lymphoid tissues, conventional mature naïve T cells that express antigen-specific TCRs can be activated by their cognate peptide antigens displayed by major histocompatibility complex (MHC). Upon activation, T cells become effector cells and, in coordination with some components of innate immune system (phagocytic cells), they are able to produce anti-microbial cytokines and eliminate foreign antigens from sites of infections. The process of the activation of phagocytes and antigen-specific T cells, as well as the release of various cytokines in response to antigen is called cell-mediated immunity or cellular immunity.

T cell development and diversity

In the thymus, recently arrived progenitor T cells rapidly expand, differentiate and instructed under the effect of IL-7 and several other cell surface receptors (Hosokawa and Rothenberg, 2018). These events not only induce the expression of Notch-1 and other specific transcriptional regulators (e.g., GATA3) which drive progenitors to a T-cell lineage fate but also promote the expression of genes important in the TCR gene rearrangement. Subsequent differentiation of the T-cell lineage committed progenitors in the thymus includes an antigen-independent process in which a harmonized succession of DNA rearrangements results in the establishment of functional TCR genes encoding the γ and δ or α and β chains (Kumar et al., 2018).

The germline structure of the T cell receptor loci comprises of a sets of V (variable), D (diversity) and J (joining) gene segments. Rearrangements of V and J segments in the α and γ chains and V, D, and J segments in the β and δ chains leads to generation of functional and in-frame TCR genes. During a highly ordered procedure, a D segment is first randomly appended to a J segment, and subsequently a V segment is spliced to the preformed DJ complex (Fig. 1). This spatially and serially well-arranged process which is mediated by the V(D)J recombinase leads to combinatorial diversity in TCRs. Through binding to a recombinase signal sequence (RSS) flanking the borders of coding gene segments, V(D)J recombinase cleaves the DNA at these positions and, in consequence, generates hairpin structures on the coding segments. Next, Artemis endonuclease in complex with the DNA-dependent protein kinase (DNA-PK) creates single cuts adjacent to the tips of the hairpins leading to the formation of DNA termini. Further processing including random deletion and addition of nucleotides by a wide range of exonucleases and DNA polymerases is applied to the exposed 3' termini before ligation of the V and D segments and repairing of the DNA occur. The random cleavage of the hairpins by Artemis combined with incorporation of palindromic (P) nucleotides, addition of several non-templated (N)-nucleotides by terminal deoxyribonucleotidyl transferase (TdT) (which is recruited by the X-ray repair cross-complementing protein 4 (XRCC4)-like enzyme Cernunnos and DNA-PK) and exonucleolytic deletions altogether confer extreme variability in the antigen-binding pocket of the TCR which is also known as junctional diversity. Lastly, repair and ligation of the DNA breaks and the processed coding ends are done by DNA ligase IV and XRCC4 in a process called nonhomologous end-joining (NHEJ). Altogether, it is implicated that the defects in the genes involved in the V(D)J recombination (e.g., RAG, Artemis and DNA ligase IV) can lead to severe combined immunodeficiency disease (SCID).

During T cell development, sequential productive rearrangements of the two TCR chain genes (α and β or γ and δ) occurs in the developing thymocytes. These productive rearrangements give rise to generation of a full length TCR protein on the surface of thymocytes and drive the transition from the double-negative to the double-positive stage of thymocyte development. During this process, the TCR chains of the double positive T cells (expressing both CD4 and CD8 co-receptors) are complexed with various CD3 molecules to make a fully functional TCR complex. Double-positive T cells which are reside in the thymic cortex should undertake two selection processes (positive and negative selections) to mature into single-positive thymocytes (CD4⁺ or CD8⁺ T cells) which are found in the thymic medulla (Takaba and Takayanagi, 2017; Kondo et al., 2019). In the positive selection, if thymic double-positive T cells moderately bind to self-MHC molecules they will receive survival signals; otherwise they will die. Conversely, in the

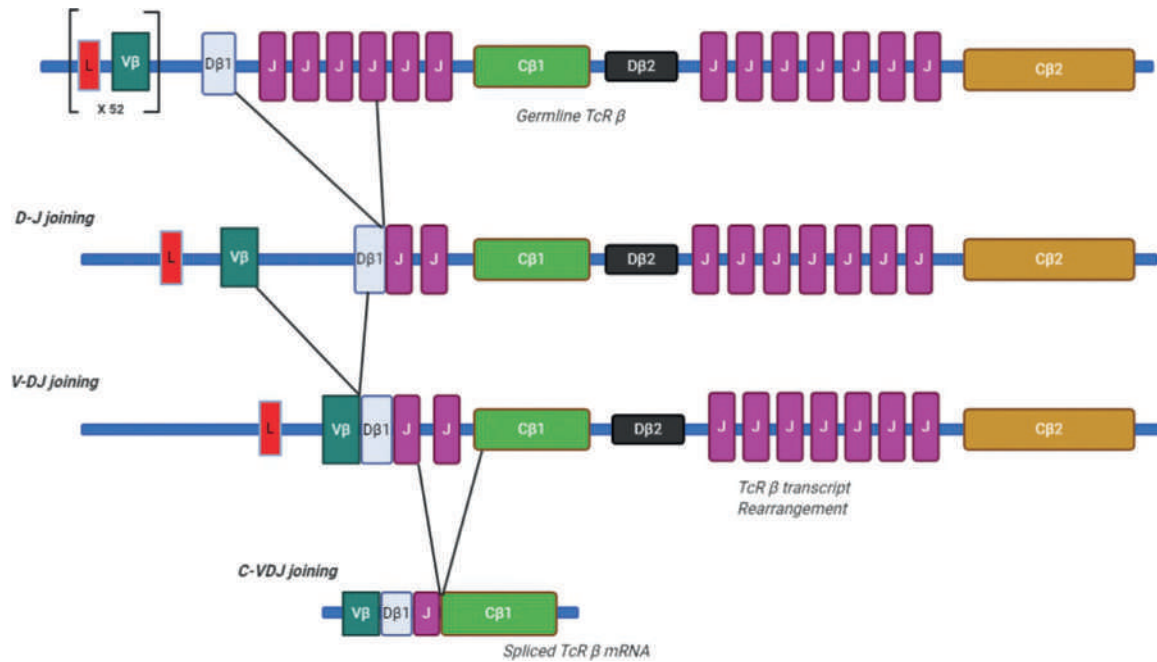


Fig. 1 T cell receptor β (TCRβ) chain gene recombination. The successive recombination of a stochastic assortment of gene fragments determines TCR structure and specificity. During this highly ordered procedure and because of the endonuclease activity of the V (D)J recombinase, a D-J joining is first randomly occurs, and subsequently, a V segment is spliced to the preformed DJ complex leading to the formation of VDJ complex. Final spliced TCRβ mRNA can be translated into β chain protein.

negative selection, if double-positive T cells bind strongly to self-peptide/MHC antigens complexes they either will be deleted or differentiated into the natural regulatory T cells (Kitagawa and Sakaguchi, 2017). Moreover, further interaction of double-positive T cells with thymic epithelial MHC class I and class II molecules promotes their phenotype to CD8 single-positive and CD4 single-positive T cells respectively (Kurd and Robey, 2016; Gascoigne et al., 2016). Both CD4 and CD8 single-positive cells leave thymus and enter to the circulation as fully matured naive T cells (Fig. 2, lower panel). Expression and presentation of self-antigens (self-peptides), which are normally expressed in the distant organs, by thymic epithelial cells (TECs) is mediated by a transcription factor known as autoimmune regulator (AIRE) (Metzger and Anderson, 2011). AIRE plays a significant role in T cell selection and prevention of autoimmunity. Mutations in the AIRE gene can results in a rare autoimmune syndrome called autoimmune polyendocrinopathy syndrome type 1 (APS-1).

T cell activation

Recognition of peptide/MHC complex on an APC by TCR complex (i.e., TCR molecules plus their associated proteins such as CD3 and ζ) is the first step for the T cell activation. Due to rapid clustering of TCR complex, a stable physical interface also known as immunologic synapse is formed between the T cell and the APC (Colin-York et al., 2019). TCR complex specifically binds to the peptide/MHC complex through interaction between CD4/CD8 co-receptors (of T cells) and nonpolymorphic domains of MHC class I or MHC class II, respectively. Moreover, costimulatory signals delivered by receptors (such as CD28) along with TCR signals endorse activation of T cells representing two-signal hypothesis for T cell activation (Kaga et al., 1998). These events altogether facilitate the activation process by bringing the cytosolic domains of CD4/CD8 molecules which is associated with Src family protein tyrosine kinase Lck into proximity of the CD3 and ζ ITAMs (Salmond et al., 2009). Lck-mediated phosphorylation of ITAMs triggers the recruitment of a key signal transduction molecule Zeta-chain-associated protein kinase 70 Kd (ZAP-70) to CD3-associated ITAMs. Recruited ZAP-70 then phosphorylates a number of cytosolic proteins such as transmembrane protein linker of activated T cells (LAT) and SH2-containing leukocyte protein 76 Kd (SLP-76) molecules (Samelson, 2002). Phosphorylated LAT, in turn, serves as a docking site for some signaling molecules including SLP-76. These proteins also serve as docking sites for some signaling molecules and cellular enzymes such as PLCγ1 and exchange factors (e.g., SOS, so named because it is the mammalian homologue of a Drosophila protein called son of sevenless) that activate Ras and other small G proteins upstream of MAP kinases. These enzymes activate several cellular responses. For example, PLCγ1 catalyzes the hydrolysis of membrane inositol phospholipid phosphatidylinositol bisphosphate (PIP2), into inositol trisphosphate (IP3) and diacylglycerol (DAG). Through interaction with IP3 receptors on the endoplasmic reticulum, IP3 stimulates a rapid increase in cytosolic free calcium within minutes after T cell activation. This calcium flux, in turn, stimulates the influx of extracellular calcium (Vaeth et al., 2020; Chakraborty and Weiss, 2014; Hwang et al., 2020; Courtney et al., 2018).

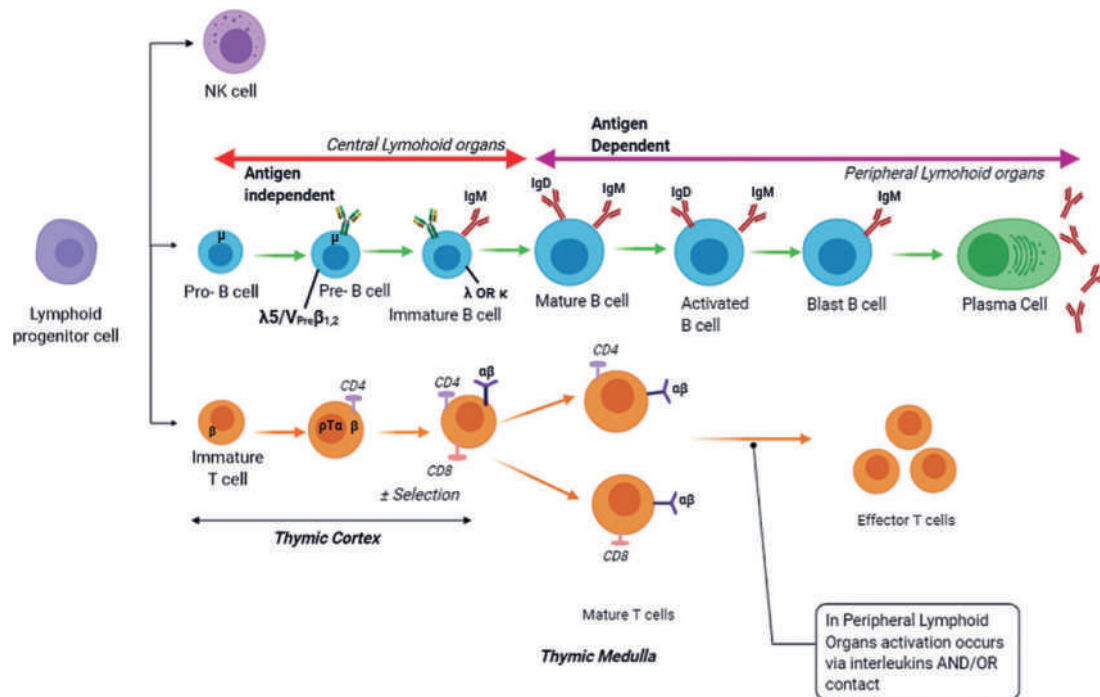


Fig. 2 Stages of B cell and T cell development. Upper panel describes B cell development in the bone marrow in an antigen-independent manner. Later on, immature B cells leave the bone marrow and home to secondary lymph nodes to become mature. Upon exposure to invading pathogens, antigen-specific mature B cells can be activated and differentiated into antibody producing plasma cells. Lower panel represents T cell development in the thymus in an antigen-independent manner. During their development in the thymus, thymocytes undergo positive and negative selections in the thymic cortex and finally give rise to mature single positive $CD4^+$ or $CD8^+$ T cells. In the periphery, following exposure to antigen, mature naïve T cells can be activated and differentiated into different effector T cell subsets.

Cytosolic free calcium binds to a calcium-dependent regulatory protein calmodulin, which in turn activates several enzymes including the phosphatase calcineurin. The calcium-calmodulin pathway activates nuclear factor of activated T cells (NFAT) in the cytosol and facilitates translocation of dephosphorylated NFAT to the nucleus. At the same time, DAG, as the second breakdown product of PIP₂, activates the enzyme protein kinase C (PKC). The PKC- θ isoform which localizes to the immunologic synapse through intermediates activates transcription factor NF- κ B. Activation of the MAP kinase triggers another signaling cascade in the activated T cells. This signaling pathway is mediated by small guanine nucleotide-binding proteins (G proteins). Two most important members of this family activated downstream of the TCR are Ras and Rac. Each activates a different component or set of transcription factors (e.g., AP-1), and together they mediate many cellular responses of T cells (Fig. 3). The harmonized action of transcription factors regulate expression of various genes involved in differentiation, proliferation and effector function of T cells (Oh-Hora and Rao, 2008; Iborra et al., 2013; Monks et al., 1997; Weil and Israël, 2004; Heissmeyer et al., 2004).

Endogenous antigens (MHC class I-dependent pathway)

Mature $CD8^+$ T cells are activated on interaction of their TCRs with antigenic peptides (9–11 amino acids in length) complexed with MHC class I molecules. MHC class I molecules display peptides that are generally either derived from cytosolic protein antigens or the protein antigens phagocytosed and transported from vesicles into the cytosol—collectively called endogenous antigens. As MHC class I molecules present cytosolic peptides, the pathway of MHC class I presentation is generally called cytosolic or endogenous pathway. Upon their presence or expression in the cytosol, peptide antigens are subjected to the proteolytic degradation in the proteasomes. The peptides generated in proteasomes are then translocated by a dimeric protein called transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER), where empty class I MHC molecules are ready to capture the peptides. Empty class I MHC molecules, TAP and tapasin are a portion of a larger peptide-loading complex in the ER that also consists of calreticulin, calnexin and other parts that participate in the MHC class I assembly and loading. In the ER, ER-resident aminopeptidase (ERAP) also further trim peptides to the optimal size for MHC class I binding. The resultant stable peptide/MHC class I complexes are then transported to the cell surface. Once expressed on the cell surface, the peptide/class I complexes can be recognized by $CD8^+$ T cells (Blum et al., 2013; Mantegazza et al., 2013; Neefjes et al., 2011; Blees et al., 2017; Kotsias et al., 2019).

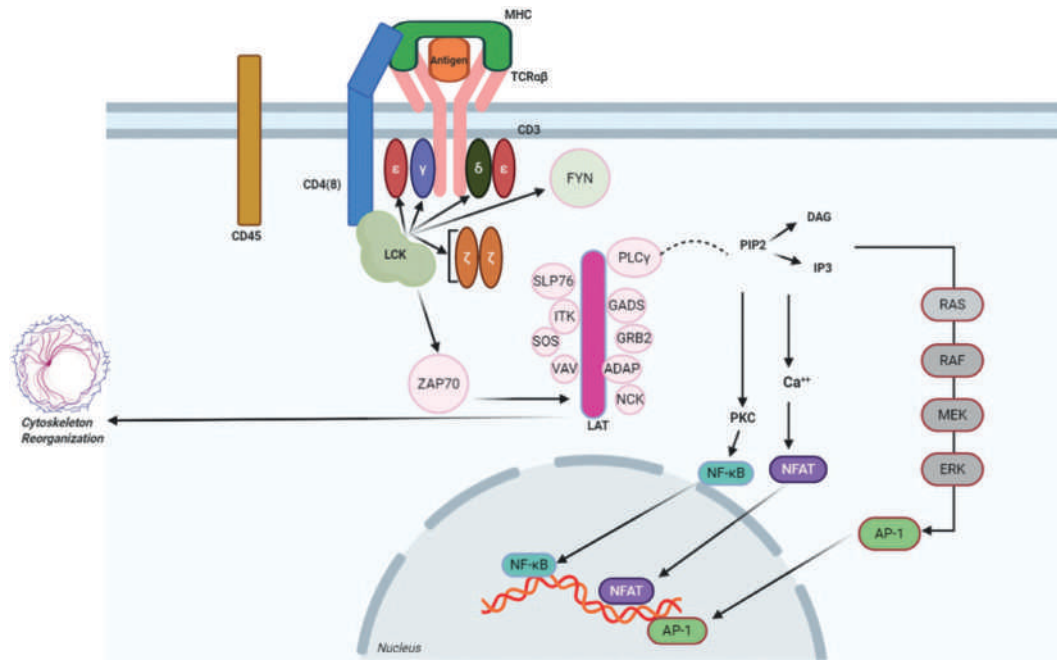


Fig. 3 Abbreviated schematic of the signaling pathways activated by the engagement of the T cell receptor (TCR). Following antigen recognition, TCR complex and co-receptors are clustered within membrane lipid rafts. This conformational alteration in TCR complex leads to activation of a series of signaling enzymes and adaptor proteins followed by activation of various intracellular signaling cascades such as MAP kinase signaling pathways and calcium- and PKC-mediated signaling pathways. These signaling pathways consequently activate transcription factors that bind to regulatory regions of many genes in T cells and thereby increase the transcription of genes involved in the proliferation, differentiation, and survival of T cells.

Exogenous antigens (MHC class II-dependent pathway)

Full functional activation of mature CD4⁺ T cells indeed depends on the interaction of TCR with antigenic peptides presented with MHC class II molecules. MHC class II molecules present peptides that are normally derived from extracellular protein antigens that are captured and internalized into endosomes by APCs. Internalized proteins are then subjected to degradation by proteases (such as cathepsins) that are active in acidic pH in the late endosomes and lysosomes to produce optimal size peptides (approximately 30 amino acids and more) that can bind to the peptide-binding clefts of MHC class II molecules. In simultaneous, newly synthesized MHC class II molecules in the ER are folded and transported to late endosomes and lysosomes with the aid of the invariant chain (Ii). Within the endosomal vesicles, the invariant chain is degraded by proteolytic enzymes (such as cathepsins) and converted to a 24-amino acid remnant called class II-associated invariant chain peptide (CLIP). Next, CLIP peptide is removed from peptide-binding cleft of MHC class II molecules by the action of HLA-DM molecule. The cleft is now accessible to antigenic peptides generated from exogenous antigenic peptides. Loading of exogenous antigenic peptides onto the MHC class II molecules leads to formation of stable peptide/MHC class II complexes. These complexes are next transported to the surface of the APC, where they are presented for recognition by CD4⁺ T cells (Blum et al., 2013; Mantegazza et al., 2013; Roche and Furuta, 2015; Neeffes et al., 2011; Kotsias et al., 2019; Unanue et al., 2016; Watts, 2012).

T cell subsets and their effector functions

The immune system responds in distinct and special ways to different antigens aiming to maximize its effectiveness. This distinct ways of response is obviously reflected in the generation of different subsets of T cells against various classes of the antigens. It is now clear T cell activation and differentiation are influenced by the type of APC and the cytokine milieu at the site of antigen encounter. The generated effector T cell subsets preserve the TCR specificity of their parent antigen-specific naïve T cells however express distinct transcription factors, cytokines and molecules that mediate their unique effector functions (Fig. 4; Kumar et al., 2018). In the following sections different subsets of T cells are described.

CD4⁺ T cell subsets

TCRαβ-bearing CD4⁺ T cells are the biggest class of T cells in the body. By production of cytokines and providing the cognate, these cells help the other immune cells such as B cells and mononuclear phagocytes to enhance their responses to the antigen and this is why these cells are also known as the T helper cells (TH cells).

T helper 1 (TH1) cells

Naïve CD4⁺ T cells differentiate into TH1 cells in a milieu containing IL-12 and IFN-γ (Macatonia et al., 1995). The primary source of IFN-γ may be nonspecific memory T cells or NK cells and the source of IL-12, during priming of naïve CD4⁺ T cells, is antigen-

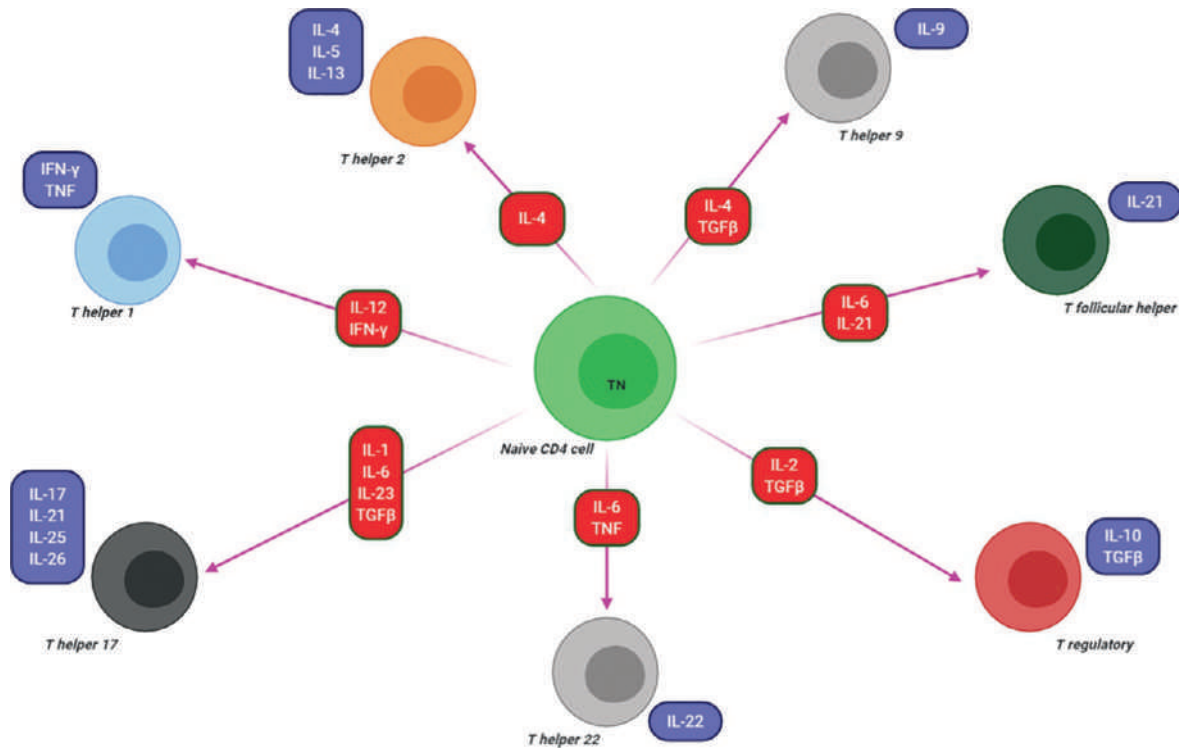


Fig. 4 Differentiation of CD4⁺ T cells into different T cell subsets. Following antigen recognition and activation, naïve CD4⁺ T cells can differentiate into different T cells subsets depending on the type of APC and the cytokine milieu at the site of antigen encounter. The different subsets are characterized by expression of distinct transcription factors, cytokines and molecules that mediate their unique effector functions.

bearing dendritic cells. IFN- γ signals through STAT1 pathway and triggers the expression of T-bet, a master regulator of TH1 differentiation. IL-12 signals via activated Jak2 and Tyk2 and the STAT4 pathway, leading to extension of TH1 responses and augmentation of IFN- γ production by TH1 responder cells. Human TH1 cells can be distinguished by surface expression of CCR5 and CXCR3 (Zhu et al., 2009). TH1 cells play an important role in defense against intracellular bacteria, protozoa and fungi through production of a wide range of cytokines such as IFN- γ , TNF- α and IL-2. Supportive evidence has shown that deficiency in IFN- γ , T-bet, or IL-12 signaling results in a much higher susceptibility to intracellular infections (García et al., 2001). For example, patients with genetic defects in IL-12 and IFN- γ receptors or STAT1 signaling are more susceptible to intracellular infections such as Mycobacterium species. TH1 cells also augment cell-mediated immunity against the intracellular pathogenic protozoans such as Leishmania species (Moll and Röllighoff, 1990). In both above situations, TH1 cells produce IFN- γ that in turn augments the killing ability of these cells against intracellular bacteria and parasites. TH1 cell cytokines, in particular IFN- γ , also activate NK cells and cytolytic T cells for killing of intracellular microbes and virally infected targets.

T helper 2 (TH2) cells

Naïve CD4⁺ T cells differentiate into TH2 cells in a milieu containing IL-4. The primary source of IL-4 may be basophils and mast cells which are in the proximity of T cell priming. IL-4 signals through the STAT6 pathway and promotes expression of GATA3, a master regulator of Th2 differentiation that drives IL-4 expression and downregulates the expression of IFN- γ . Human TH2 cells are differentiated by surface expression of CRTH2, CCR3 and CCR4 (Zhu et al., 2009; Walker and McKenzie, 2018). TH2 cell-driven inflammatory response involves eosinophils and basophils, mast cells and IgE-class switched B cells as effector cells. This response is also characterized by mast cell activation and degranulation due to cross-linking surface-bound IgE. Through production of IL-4, IL-5, and IL-13, Th2 cells coordinate B cell class switching to IgE, thereby priming basophils and mast cells for granule release, augmenting chemotaxis of eosinophils and basophils, and enhancing mucus production, respectively. These events play critical roles in the promotion of type 2 immune responses which are important for hypersensitivity immune responses and expulsion of extracellular parasites (e.g., helminth infections) and bacterial infection (Gurram and Zhu, 2019). Lastly, although a balanced TH2 cell-mediated immune response is critical for elimination of extracellular parasites; imbalanced Th2 response can cause some pathologic circumstances such as allergy and airway hypersensitivity (Nakayama et al., 2017).

T helper 17 (TH17) cells

TH17 cells are the third subset of CD4⁺ T helper (TH) cells. In cytokine milieu of IL-6 and TGF- β , naïve CD4⁺ T cells are developed into TH17 cells. TH17 cells are characterized by production of IL-17, TNF- α , and IL-22 as well as expression of the transcription

factor ROR γ t (Zhu et al., 2009; Ciofani et al., 2012). Unlike TH1 and TH2 cells, TH17 cells have distinctive properties which make these cells well suited for the host protection against some extracellular bacteria and fungi. The pattern of cytokines produced by TH17 cells seem to be critical for the immunity against extracellular bacteria and fungi owing to their role in the recruitment, activation and migration of neutrophils, promoting the expression of tight-junction proteins (e.g., claudins) as well as stimulating the production of antimicrobial peptides (e.g., defensins) (Ouyang et al., 2008). While TH17 cells play a critical role in host defense against extracellular bacteria and fungi, they have been implicated in the immunopathogenesis of a number of T autoimmune diseases such as rheumatoid arthritis (RA) and psoriasis (Stockinger and Omenetti, 2017). TH17 cells also have a key role in chronic allergic inflammatory responses of some inflammatory diseases such as asthma. In human, TH17 deficiency, for example due to mutations in the transcription factor STAT3, can cause the hyper-IgE syndrome (or Job's syndrome) which is associated with recurrent infections and decreased T cell-mediated inflammatory response (Holland et al., 2007).

T helper 9 (TH9) cells

TH9 cells have been most recently described as a subset of CD4⁺ T helper cells. These cells are characterized by the expression of CD4 and CCR6 and the lack of CCR4 as well as production of the IL-9, IL-10 and IL-21. IL-4 and TGF- β signaling drive differentiation of naïve CD4⁺ T cells into TH9 differentiation (Zhu et al., 2009). TGF- β signaling in TH9 cells activates transcription factor Smad and induces the expression of transcription factor PU.1. In TH9 cells, PU.1 directly binds to the *Il9* promoter and recruits chromatin-modifying enzymes which amplify *Il9*-gene transcription (Kaplan et al., 2015). Similar to TH2 cells, TH9 cells are thought to mediate helminthic immunity through production of IL-9. This cytokine induces expansion and recruitment of mast cells. TH9-derived IL-21 can also promote TH17 differentiation, B-cell survival, antibody class switching, NK cell proliferation and activation. Experimental animal models also propose that TH9 cells may play a role in the pathogenesis of atopic and autoimmune diseases (Clark and Schlapbach, 2017).

T helper 22 (TH22)

TH22 cells have been identified as a distinct CD4⁺ T cell subset within the past decade. IL-6 and IL-1 signaling but not TGF- β is required for differentiation of naïve CD4⁺ T cells into TH22 cells. These cells are characterized by the expression of CD4, CCR6, and skin-homing receptors CCR4 and CCR10, as well as production of IL-22, IL-13 and TNF- α . The production of IL-22 in these cells is thought to depend on the expression and activation of aryl hydrocarbon receptor (AhR). The role of TH22 cells in immune responses is still being elucidated, but they seem to not only stimulate wound healing and tissue remodeling but also regulate innate defense mechanisms (e.g., antibacterial protein production) and promote barrier function at mucosal sites. The impact of TH22 cells and IL-22 expression has been assessed in various chronic inflammatory diseases. IL-22 signaling has been shown to have protective roles in experimental models of inflammatory diseases such inflammatory bowel disease (IBD) and asthma through stimulation of various homeostatic mechanisms in the epithelial barriers (Eyerich et al., 2009; Pennino et al., 2013). In contrast, excessive production of IL-22 by skin-resident TH22 cells has been appeared to be an important driver of inflammation in cutaneous inflammatory diseases, such as psoriasis (Ho and Kupper, 2019).

T follicular helper (Tfh) cells

Tfh cells are a distinct activated CD4⁺ T cell subset which resides in B cell zones of lymph nodes and the spleen due to the expression of the CXCR5 (Moser, 2015). IL-6, IL-2, ICOS and TCR signaling altogether induce the differentiation of newly activated CD4⁺ T cells into Tfh cells during priming of dendritic cells through regulation of Bcl6 and CXCR5 (Zhu et al., 2009). Expression of Bcl6 (often referred to as the Tfh master regulator transcription factor) is induced through IL6/IL-6R signaling. This transcription factor not only regulates Tfh cell differentiation but also induces expression of CXCR5 and formation of germinal center. Tfh cells are recognized an indispensable T CD4⁺ cell subset in triggering the formation and maintenance of germinal center immune responses and generation of immunological memory through expression of CD40L, IL-21 and IL-4. Dysregulation of Tfh-mediated immune responses have the potential to trigger the formation of overactivated germinal centers characterized by the presence of aberrantly mutated B cell clones. These aberrantly mutated B cells can become autoreactive and thereby promote antibody-mediated autoimmune diseases. Higher level of Tfh-like cells has been found in the blood of some patients with Sjögren syndrome. However, it remains to be fully elucidated in humans (Crotty, 2014; Vinuesa et al., 2016).

Regulatory T cells (Tregs)

Regulatory T cells (Tregs) are a subset of CD4⁺ T cells characterized by the expression of the transcription factor Foxp3, CD4, and CD25. There are two main types of Tregs: naturally occurring thymus-derived Foxp3⁺CD127^{lo}CD4⁺ Tregs (nTregs) and in vivo-induced Foxp3⁺CD4⁺ T (iTregs) (Zhu et al., 2009). iTregs arise from naïve Foxp3⁻CD4⁺ T cells in the periphery. In the thymus, double positive (CD4⁺ CD8⁺) thymocytes through recognizing self-antigens with high avidity may differentiate into nTregs. Upon stimulation and in the presence of cytokines such as TGF- β and IL-2, naïve CD4⁺ Foxp3⁻ T cells can differentiate into CD4⁺ Foxp3⁺ iTregs (Kitagawa and Sakaguchi, 2017). Tregs balance the immune system by preventing or dampening of immunological responsiveness and maintaining of immunological tolerance in the steady state and under pathophysiological conditions (Sakaguchi et al., 2020). They perform their immunosuppressive functions through different mechanisms such as inhibitory cytokine production, metabolic disruption, cytotoxicity, cell to cell contact, modulation of dendritic cells and competitive uptake of IL-2 (Vignali et al., 2008). Considering the regulatory function of Tregs, defects either in the abundance and/or function of Tregs can disturb immune system responses and immunological tolerance. For example, mutations in Foxp3 gene leads to immune

dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX) syndrome in humans resulting in lethal autoimmunity. IL-10 producing Tregs (Tr1 cells) are another subset of CD4⁺Tregs that do not express Foxp3. In presence of IL-27 and TGF- β , Tr1-like cells are generated. These cells are characterized by CD4⁺ CD49b⁺ LAG-3⁺ CD226⁺ cells. Similar to non-Tr1 cells, activated Tr1 cells exert their immunosuppressive function through various above-mentioned mechanisms (Roncarolo et al., 2018).

CD8⁺ T cell subsets

TCR $\alpha\beta$ -bearing CD8⁺ T cells represent one of the main classes of T cells. CD8⁺ T cells primarily recognize peptides presented by MHC Class I molecules. CD8⁺ effector T cells [also called cytolytic T lymphocytes (CTLs)] can kill the cells harboring intracellular pathogens and transformed cells by three main mechanisms. (i) Secretion of cytokines with anti-tumoral and anti-viral effects; (ii) Production of cytotoxic granules; (iii) Destruction of infected and malignant cells via Fas/FasL interactions (Zhang and Bevan, 2011).

CD8⁺ CTLs are not as diverse as of CD4⁺ TH cells; however, they can differentiate into two different subsets named TC1 and TC2. TC1 and TC2 cells are loosely similar to TH1 and TH2 cells in terms of the cytokines that stimulate their development as well as cytokines they produce. TC1 cells produce IFN- γ , but no IL-4. In the IL-4-enriched milieu, CTLs differentiate into TC2 cells, which produce much more IL-4 and IL-5 than IFN- γ . Although both CTL subsets are potent in the killing of host target cells, TC1 cells perform both perforin and Fas-mediated mechanisms, while TC2 cells only use perforin (Vukmanovic-Stejic et al., 2000; Paul and Ohashi, 2020).

Other T cell subsets

Besides CD8⁺ and CD4⁺ T cells, there are smaller subsets of T cells that represent distinct properties and likely perform particular functions in host immune defense. The best characterized of these subsets are NKT cells and $\gamma\delta$ T cells. Both NKT cells and $\gamma\delta$ T cells are often proposed to be at the intersection of adaptive and innate immunity.

$\gamma\delta$ T cells

$\gamma\delta$ T cells are a small subset of T cells which are mostly populated in the epithelium of tissues. $\gamma\delta$ T cell abundance is largely varied in different tissues and species, however less than 5% of all T cells generally express TCR $\gamma\delta$. Although the TCR is created by DNA rearrangement, unlike conventional TCR $\alpha\beta$, the actual diversity of TCR $\gamma\delta$ repertoire is limited because only a small number of the V, D, and J segments are used in mature $\gamma\delta$ T cells (Pellicci et al., 2020). This limited diversity indicate that $\gamma\delta$ T cells may have evolved to recognize a small group of microbial antigens and serve as an early defense against a small number of frequently encountered pathogens at epithelial linings (Chien et al., 2014). In developing double negative (CD4⁻CD8⁻) thymocytes, rearrangement of TCR loci of β , γ , or δ is plausible. If a cell can make productive rearrangements in γ and δ loci before it makes a productive TCR β rearrangement, the cells will committed to $\gamma\delta$ T cell lineage. Although antigens that activate $\gamma\delta$ T cells are not well-identified, it has been shown that lipid antigens and heat-shock proteins can activate these cells. It has been found that $\gamma\delta$ T cells are not restricted to antigen presentation by MHC molecules and do not require antigen processing (Scanlon, 2019). Some $\gamma\delta$ T cells recognize antigens via nonclassical MHC molecules, whereas others seem to recognize whole antigens without antigen processing. $\gamma\delta$ T cells can recognize target molecules on stressed, infected and/or transformed cells and secrete cytotoxic molecules, inflammatory cytokines and stimulate adaptive immunity. They can also lyse target cells by antibody-dependent cellular cytotoxicity (ADCC). Through secretion of IFN- γ and IL-17, $\gamma\delta$ T cells augment the expression of MHC class I, and thereby positively induce CTL-mediated immune response. $\gamma\delta$ T cells also can interact with DCs and develop Th1 response. Recent investigations have revealed that human V γ 9/V δ 2 T cells are also able to do phagocytosis, a function that was thought to be exclusive to innate immune cells (Lawand et al., 2017).

Natural killer T (NKT) cells

Natural killer T (NKT) cells are a specialized subset of T cells that share morphological and functional characteristics of both NK cells and T cells. Many of NKT cells recognize lipid and glycolipid antigens through interaction with a nonpolymorphic MHC class I molecule-also known as CD1d. NKT cells make up about 0.1% of peripheral blood T cells. NKT cells are recognized by a restricted TCR repertoire and this is why these cells are also called invariant NKT (iNKT) cells (Pellicci et al., 2020). Based on the different features such as reactivity to α -GalCer and CD1-dependent antigen recognition, NK T cells are classified into three subsets (Table 2) (Dhodapkar and Kumar, 2017; Godfrey et al., 2004).

NKT cells are capable to rapidly respond to antigen (without prior activation) and produce cytokines such as IL-4 and IFN- γ . NKT cell-derived cytokines and chemokines can modulate recruitment, activation and immune response of many innate and adaptive immune cells (Krovi and Gapin, 2018). The role of NKT cells in protective immunity or disease in humans is not fully defined. However, recent studies have revealed that deficiency or dysfunction of NKT cells can result in the development of some autoimmune diseases such as diabetes (Griseri et al., 2005). Intriguingly, NKT cells have been reported to exacerbate certain other diseases such as human asthma (Iwamura and Nakayama, 2018).

B cells and antibody-mediated immunity

B cells, also known as B lymphocytes, make up the humoral immunity arm of the adaptive immune system. This type of immunity is largely mediated by antibodies produced by antigen-specific B cells (and plasma cells), so it is often called antibody-mediated

Table 2 Comparative features of NKT cell subsets.

Feature	Type I cells (Classical NKT cells)	Type I cells (non-classical NKT cells)	NKT like cells
Developmental requirements	IL-15	Not established	Not established
CD1d dependency	Yes	Yes	No (MHC I & II)
Reactivity to α -GalCer	Yes	No	No (various lipids and peptides)
TCR α -chain	V α 14-J α 18 (mice) V α 24-J α 18 (humans)	Diverse	Diverse
NK1.1 expression	+ (Resting mature) -/Low (immature or post-activation)	$\pm$	+
IL-4 production	Yes	Yes	Yes
IFN- γ production	Yes	Yes	Yes
Tissue location	Liver and spleen, intestine and pulmonary mucosa	Liver and spleen	Liver, spleen and bone marrow

immunity. Humoral immunity is the major defense barrier against extracellular pathogens and microbial toxins as antibodies can neutralize or flag (opsonize) microbes and their toxins and contributes to their elimination (Cohn and Langman, 1990). Antibody-mediated elimination of microbes is facilitated by various effector mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), antibody-mediated complement activation and antibody (i.e., IgA)-mediated immune exclusion (Lu et al., 2018).

B cell development and diversity

Before birth, B cells arise from committed precursors in the fetal liver and after birth they are developed in the bone marrow. In the bone marrow, under influence of signals received from several cell surface receptors, common lymphoid progenitors (CLPs) are not only induced to express B-cell specific transcriptional regulators (e.g., PU.1, IKAROS, EBF, E2A, IRF8 and Pax-5) that drive cell fate decision to a B-cell lineage but also promote the expression of genes important in the B-cell receptor (BCR) gene rearrangement (Busslinger, 2004; Hardy and Hayakawa, 2001; Zehentmeier and Pereira, 2019; Fistonich et al., 2018; Nagasawa, 2006). During the development of B cells and in a spatially and sequentially ordered events, developing B cells in the absence of exogenous antigen undertake DNA rearrangement, express Immunoglobulin (Ig) genes, undertake selection and proliferation at the pre-antigen receptor checkpoint and finally immature B cells acquire antigen specificity and give rise to mature naïve B cells in the spleen to shape conventional naïve B cell repertoire (Fig. 2, upper panel) (Melchers, 2015). The genes encoding antibodies are rearranged and assembled from gene segments in a way completely resemble to the process for TCR genes. During their maturation in bone marrow, B cell-committed progenitors undertake distinct steps, each featured by diverse cell surface markers and a unique pattern of Ig gene expression. As the earliest bone marrow cell committed to the B cell lineage, pro-B cells express surface markers such as CD19 and CD10. V(D)J recombinase is expressed at this stage and the first gene recombination event arises at the Ig heavy chain locus (IgH). During recombination of IgH locus, D to J joining is randomly occurs and subsequently a V segment is spliced to the DJ complex, giving rise to a rearranged VDJ exon. The rearranged Ig heavy chain gene and C μ region exons are altogether transcribed to produce a primary transcript. The primary transcript is then subjected to RNA processing in which the introns are removed and exons (VDJ and C μ exon) appended together, giving rise to a spliced mRNA for the μ heavy chain (Fig. 5). If the mRNA was in-frame and productive, rearranged μ heavy chain mRNA is translated, leading to synthesis of the μ protein. Only pro-B cells that undergo in-frame and productive DNA rearrangements continue to survive and differentiate into pre-B cells. Pre-B cells are developing B cells that express the μ heavy chain, surrogate light chains (i.e., the λ 5 and V pre-B proteins), and signal-transducing proteins (i.e., Ig α and Ig β) on the cell surface, that altogether make a complex known as the pre-B cell receptor. Following antigen-independent assembly of pre-BCR, a kinase called Bruton's tyrosine kinase (Btk) is activated. This enzyme is required for signal transduction of Pre-BCR and has a prominent role in the proliferation, maturation and survival of pre-B cells. In humans, mutations in the BTK gene leads to a rare genetic disorder called X-linked agammaglobulinemia (XLA), which is characterized by the absence of mature B cells (Satterthwaite and Witte, 2000). The pre-BCR signaling also regulates the allelic exclusion phenomenon. Based on this phenomenon, only an individual B cell clone with monospecificity is generated (Winkler and Mårtensson, 2018; Sonoda et al., 1997). As pre-B cell mature, the pre-BCR signaling also stimulates κ light chain gene rearrangement and inactivates the expression of surrogate light chain gene. DNA recombination in the κ light chain locus happens in a similar way as in the Ig heavy chain locus. However, unlike Ig heavy chain loci, there are no D segments in the light chain loci, and thus recombination involves only the V-J joining. If the developing B cell can productively rearrange, it will produce a κ light chain protein which assembles with the previously produced μ chain to generate a complete IgM molecule. Otherwise, the developing B cell undergoes a λ light chain gene rearrangement and again generates a complete IgM molecule. The λ light chain gene rearrangement arises only if the κ rearrangement was not productive. This phenomenon is called light chain isotype exclusion. Developing B cells that express complete IgM are called immature B cells. Cell membrane IgM in association with signaling molecules Ig α and Ig β makes a BCR complex. Upon encounter with multivalent antigens in the bone marrow, immature B cells that recognize antigen with high avidity do not proliferate and differentiate rather they may undergo receptor editing or clonal deletion. Non-self-reactive immature B cells can finally leave the bone marrow and enter to the spleen to complete their maturation. After maturation, mature B cells (IgM⁺ IgD⁺)

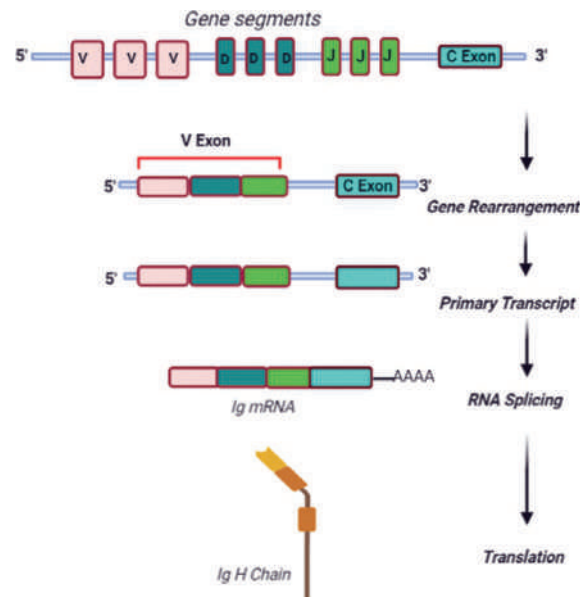


Fig. 5 Ig heavy chain gene recombination and expression. Serial recombination of a random assortment of gene fragments defines heavy chain structure and specificity. During recombination of IgH locus, D to J joining is casual and consequently a V segment is spliced to the DJ complex to create a rearranged VDJ exon. The rearranged Ig heavy chain gene and C μ region exons are altogether transcribed to produce a primary transcript. In the next step, through RNA processing, the introns of the primary transcript are removed and VDJ exon is joined to C μ exon, giving rise to a spliced mRNA for the μ heavy chain.

can migrate to other secondary lymphoid organs. Similar to TCR, combinatorial and junctional diversities hugely contribute to the variability and binding specificity of BCR (Imkeller and Wardemann, 2018).

B cell activation

Membrane IgM and IgD are the antigen recognition receptors expressed by mature naive B cells. In association with two signaling molecules Ig α (CD79a) and Ig β (CD79b), IgM and IgD make B cell receptor (BCR) complex. The class of antibodies IgM and IgD can be switched in different differentiation stages of B cells. For example, in class-switched B cells and memory B cells antigen-recognition domain of BCR complex can be made of the IgE, IgG or IgA classes. In this complex, Ig α and Ig β transduce antigen-recognition-derived signals through their ITAM motifs located in their cytoplasmic tails. BCR cross-linking with multivalent antigen initiates BCR signaling through recruitment and activation of Src family kinases such as Lyn and Fyn which are loosely associated with cell membrane (Shen et al., 2019). Activation of these kinases results in the phosphorylation of the tyrosine residues on the ITAMs of Ig α and Ig β . These phosphorylated ITAMs serve as a docking site for the Syk tyrosine kinase. ITAM-associated Syk is then autophosphorylated and activated and leading to phosphorylation of critical adaptor proteins such as SH2-binding leukocyte phosphoprotein of 65 kD (SLP-65), also called BLNK, or B cell linker protein (Cheng et al., 1995; Pappu et al., 1999). Phosphorylated SLP-65 facilitates the recruitment of guanine nucleotide exchange proteins that can distinctly activate Ras and Rac, PLC γ 2, and PKC- β , each commonly involving in the activation of different signaling pathways and transcription factors such as Fos (downstream of Ras and ERK activation), JunB (downstream of Rac and JNK activation), and NF- κ B (downstream of Btk, PLC γ 2, and PKC- β activation). All of these signaling pathways and their associated molecules are involved in stimulating proliferation, differentiation and survival of B cells (Fig. 6) (Yam-Puc et al., 2018; Schroeder et al., 2019).

In addition to activation of BCR signaling, attenuation of BCR signaling is also required to limit uncontrolled humoral immunity and lymphoproliferation. This is mainly mediated by the action of Fc γ RIIB and CD22/Siglec-2 that serve as a terminator/attenuator of B cell activation. The cytoplasmic tail of CD22 and Fc γ RIIB contain an ITIM. Phosphorylation of tyrosine residues of ITIM serves as a docking site for the SHIP (for Fc γ RIIB) and SHP-1 (for CD22). The recruited SHIP and SHP-1 remove phosphates from the several adaptor proteins and enzymes involved in B cell receptor signaling and hence terminate B cell activation (Nitschke, 2005; Clark and Giltiay, 2018).

T-independent antigens

Some antigens stimulate antibody production without T cell involvement. These antigens are called thymus (or T)-independent (TI) antigens. TI antigens generally do not induce isotype switching and immunological memory which are hallmarks of T cell-dependent antigens. TI antigens are subdivided into two groups based on their ability to activate B cells: T-independent type I antigens and T-independent type II antigens. Most TI type I antigens are molecules (e.g., LPS and endotoxin) that can directly, without considering antigen specificity, induce B cell mitosis and polyclonal antibody production (mainly IgM) (Ulevitch and

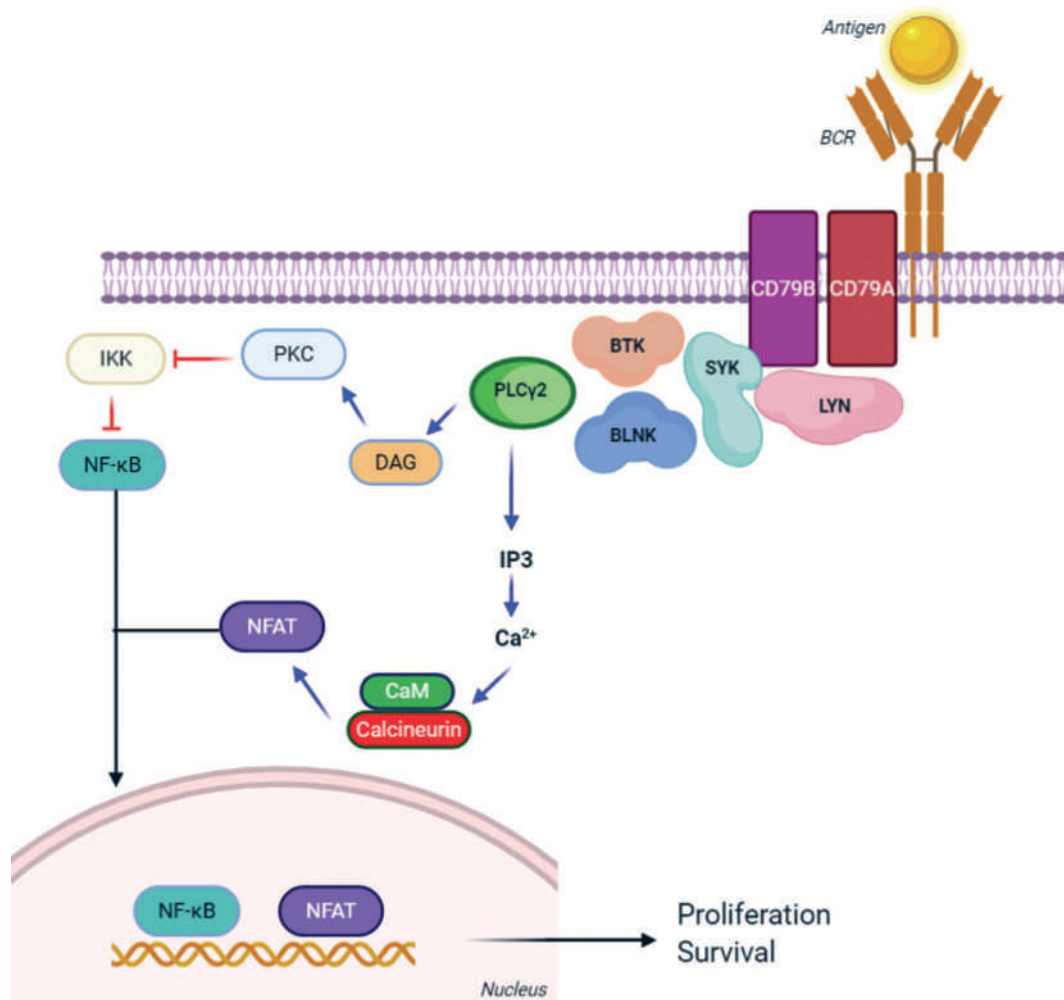


Fig. 6 Abbreviated schematic of the signaling pathways activated by engagement of the B cell receptor (BCR). Following antigen recognition, BCR signaling through recruitment and activation of a wide spectrum of enzymes and adaptor proteins is initiated. Activation of these molecules leads to activation of different signaling pathways and transcription factors such as Fos (downstream of Ras and ERK activation), JunB (downstream of Rac and JNK activation), and NF-κB (downstream of Btk, PLCγ2, and PKC-β activation). All of these signaling pathways and their downstream transcription factors are involved in the induction of proliferation, differentiation and survival of B cells.

Tobias, 1995). Such antigens have the ability to activate B cell in the absence of T cell help probably through the crosslinking of putative LPS receptors (e.g., TLR-4). T-independent antigens types II are typically repeating polymers such as polysaccharides (e.g., dextran, ficoll,), glycolipids, and nucleic acids. They can interact with multiple BCRs on the cell surface and extensively cross-link them, and as a result, trigger signal transduction in B cells (Mond et al., 1995). This activating signal transduction with the additional cues provided by cytokines (e.g., IL-2, IL-3 and IFN-γ) or other cell [e.g., signals induced by transmembrane activator and CamL interactor (TACI) ligands such as a proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF)] provided by dendritic cells can not only altogether induce differentiation of activated B cells into memory B cells or plasma cells but also can promote Ig isotype switching in the absence of T cell help (Bortnick et al., 2010; Defrance et al., 2011). It seems that T-independent humoral immunity in harmony with the innate immunity ensure a rapid initial response to invading pathogens before the recruitment of effective cognate T cell help aiming to restrict the spreading and pathogenicity of microbes at early phases of infection.

T-dependent antigens

Thymus(T)-dependent antigens are typically soluble protein antigens that required to be internalized by specific B cells, processed, and presented to MHC class II-restricted helper T cells, which then activate the B cells for antibody production (Adler et al., 2017). As these proteins antigens only can induce an antibody response with the help of T helper cells, they are classified as T-dependent antigens. To initiate an antibody-mediated immune response, a T-dependent antigen should be primarily delivered to a secondary lymphoid tissue either via dendritic cells and phagocytic cells or the draining lymphatics. In both scenarios, the antigen is processed by the APCs and is presented via MHC class II molecules to antigen-specific CD4⁺ T cells, resulting in the differentiation and

activation of antigen-specific T cells. At the same time, B cells which are populated in the cortex of primary follicles in the lymph nodes can recognize their specific antigen. After initial interaction of B cells and cognate T cells in the B-T border, activated B cells can experience two scenarios. In the first scenario, activated B cells can instantly become short-lived plasma cells characterized by secretion of low-affinity antibody without somatic mutation. In the second scenario, activated B cells accompanied with CXCR5-expressing TH cells move deeper into the B cell zone and form germinal centers (GCs) (Kerfoot et al., 2011). The specialized type of TH cell that facilitate the formation of germinal centers is called Tfh cell. Through GC interactions and in harmony with follicular dendritic cells (FDCs), Tfh cells by production of cytokines (e.g., IL-21) and expression of interacting surface molecules (e.g., CD40L) provide essential signals for Ig class switching somatic hypermutation and affinity maturation, B cell proliferation and differentiation into antibody-producing plasma cells and long-lived memory B cells (Wan et al., 2019). Defects in these events may lead to some immunodeficiencies such as hyper-IgM syndrome.

B cell subsets and their effector functions

B cells are generally described as the cells that can produce antibodies, function as secondary APCs and generate many immunoregulatory cytokines. There are different subsets of B cells that are distinct in terms of phenotype, anatomical locations and functional features. The main B cell subsets are follicular B cells, marginal zone B cells, B-1 cells and regulatory B cells (Bregs). In the following sections, the effector of function of these cells is discussed.

Follicular (FO) B cells

FO B cells are essential for T-dependent humoral immunity. This type of immunity is initiated by crosslinking of highly diverse, clonally distributed sets of antigen-specific BCRs on FO B cells and completed by help of cognate T cells specific for the same antigen. This cognate interaction results in the secretion of a high-affinity and class switched antibodies. During a T-dependent B cell response, if the activated FO B cells get through GC reactions and experience CD40 signaling, they upregulate Bcl6 and hence undergo Ig isotype switching, somatic hypermutation and affinity maturation and finally differentiate into long-lived plasma cells. If the activated FO B cells do not get through the GC reactions and do not receive sustained CD40 signaling, they do not upregulate Bcl6 and hence do not undergo isotype switching or somatic hypermutation and instantly differentiate into short-lived plasma cells (de Maio et al., 2020; Weinstein et al., 2016). Unlike long-lived plasma cells, these cells are populated in the secondary lymphoid tissues and peripheral sites. Short-lived plasma secrete low-affinity IgM antibody for a short period of time (2–3 days) and then the cells are subjected to cell death.

Marginal zone (MZ) B cells

MZ B cells are a distinct subset of B cells characterized by expression of high levels of IgM, CD21, CD1 and CD9 with dim to slight expression levels of IgD, CD23, CD5 and CD11b. MZ B cells respond readily to TI antigens. They play a key role in the very early phases of the splenic humoral immunity against blood-borne antigens (Martin and Kearney, 2002). MZ B cell-mediated antibody responses are initiated in the marginal zone of spleen. MZ macrophages concentrated around the lymphoid follicles in the spleen are mostly effective at catching the blood-borne microbial antigens. These antigens may last for long period of time on the surface of MZ macrophages, where they can be detected by antigen-specific MZ B cells. After activation with antigen (usually a TI antigen), MZ B cells cannot upregulate Bcl6 and hence these cells instantly proliferate and differentiate into short-lived IgM-secreting plasma cells without undertaking somatic hypermutation and isotype switching. It is postulated that MZ-derived IgM-secreting plasma cells contribute to the generation of natural antibodies (Maddur et al., 2020). These antibodies are found in the circulation of normal human subjects and are seemed to be produced in the absence of exposure to the pathogens. Natural antibodies are essentially low affinity antibodies mainly with IgM isotype. By recognition of carbohydrates and oxidized lipids which are found on the bacterial membrane and on apoptotic cells, natural antibodies not only provide a rapid adaptive humoral immunity against blood-borne bacterial infections but also improve the phagocytosis of apoptotic cells (Zouali and Richard, 2011; Cerutti et al., 2013). Interestingly, MZ B cells can act as regulatory cells in autoimmune context through the induction of tolerance by apoptotic cells (Marinkovic and Marinkovic, 2020).

B-1 B cells

B-1 B cells represent another lineage of B cells primarily arisen from the fetal liver-derived stem cells and most of them undergo self-renewal in the periphery (Montecino-Rodriguez and Dorshkind, 2012). During B cell ontogeny, B-1 B cells develop earlier than FO B cells; express an oligoclonal repertoire of variable genes with minimal BCR diversity. Many B-1 B cells express the CD5 (Ly-1) molecule. Through secretion of broadly cross-reactive natural antibodies such as IgM (mainly), IgA and IgG3, these cells respond to TI antigens primarily in the peritoneal and pleural cavities as well as in mucosal tissues (Smith and Baumgarth, 2019). Peritoneal B-1 B cells are seemed to be the primary source of natural antibodies. These antibodies are thought to be stimulated by bacteria that settled down in the gastrointestinal tract. In addition to their capability to produce natural antibodies, B-1 cells are able to produce cytokines (e.g., IL-3 and GM-CSF) as well as uptake and present antigens to T cells during pathogen invasion. B-1 cells can be additionally subdivided into B-1a (CD19, B220, IgM, CD43 and CD5⁺ B-1 cells) and B-1b (CD19, B220, IgM, CD43 and CD5⁻ B-1 cells) subsets. In contrast to B-1a cells, the B-1b subset can be arisen from precursors in the adult bone marrow. Recent functional studies also point out that B-1a B cells are the main source of natural IgM antibody while B-1b cells seem to be the major source of TI antibody production and long-lasting protective immunity against bacterial infections (Baumgarth, 2017; Haas et al., 2005).

Regulatory B cells (Bregs)

Similar to Tregs, the regulatory B cells (Bregs) are now generally recognized as a significant regulatory element of the immune system that suppresses immunological inflammation. However, unlike Treg cells, currently there are no distinctive phenotypic or lineage markers as well as specific cytokine profiles that truly characterize Bregs. Hence, the term Bregs refers to B cells that possess immunoregulatory functions. In this regard, virtually all B cell subset can differentiate into Bregs in the presence of appropriate stimuli such as TLR ligands and anti-CD40 (Rosser and Mauri, 2015). Nevertheless, various subsets of Breg with different phenotypes have been defined. For instance, human blood Bregs are CD19⁺CD24hiCD27⁺, MZ Bregs are characterized by CD19⁺CD21⁺CD1d⁺CD5⁺CD23^{low} and B-1 cell-derived Bregs are defined as CD19⁺CD5⁺IgMhiCD23⁻CD21⁻CD11b⁺ (Iwata et al., 2011; Bankoti et al., 2012; Shimomura et al., 2008). All Bregs can also differentiate into plasmablasts and conventional plasma cells. It seems that Immunoregulatory function of Bregs is mediated through different mechanisms such as secretion of immunosuppressive cytokines, cell-cell contact immunosuppression, secretion of anti-inflammatory antibodies (e.g., IgA and IgG4) and enhancing the production of Tregs (Rosser and Mauri, 2015).

Immunological memory

Immunological memory is a quite unique feature of the adaptive immune system as it can store information of antigen upon first exposure and can elicit a quicker and stronger secondary immune response to the same antigen upon re-exposure probably due to presence of long-lived memory T cells and B cells generated during primary immune response. Immunological memory is not only a key protection mechanism against invading of a wide range of microbes, but also it plays significant role in vaccine development and the success of vaccination (Farber et al., 2016).

Memory T cells

Until recently, it was hard to phenotypically distinguish memory T cells from naïve and effector T cells. Most of memory T cells are in the quiescent state, however unlike naïve T cells, they do not need stringent requirements for their activation. For instance, naïve T cells are almost exclusively activated by DCs, while memory T cells can be activated by various types of APC such as DCs, B cells and macrophages probably due to the expression of different surface adhesion molecules and costimulatory receptors that make memory T cells more receptive for interaction with various types of APC. Due to difference in epigenetic configuration, memory T cells also respond more quickly to antigen re-exposure compared to naïve T cells (Chen et al., 2018). Lastly, recirculation pattern of memory T cells is different with those of naïve or effector T cells (Mueller et al., 2013; Lefrançois, 2006). Memory cells may arise from effector cells based on a linear pathway model, or from naïve T cells that upon antigen stimulation divergently differentiate into effector and memory cells (divergent pathway model) (Moulton and Farber, 2006). It is now unclear how an antigen-activated T cell can differentiate into long-lived memory cells or short-lived effector T cells. Although the signals that drive these two fates are not fully elucidated, it seems that types of transcription factors that are induced during T cell priming determine the T cell fate. For example, T-bet promote naïve T cell differentiation into TH1 cells, whereas Blimp-1 induces the generation of memory T cells. Furthermore, it is not clear whether the expression of these transcription factors is stochastic event or is a response to some specific external cues. Following antigen elimination, activated T cells undergo cellular contraction. During this phase 90–95% of T cells are subjected to apoptosis, however remaining 5–10% of T cells are differentiated into memory T cells. Memory T cells are generally subdivided into four different memory cells including stem memory T cells (Tscm), effector memory T cells (Tem), central memory T cells (Tcm) and tissue-resident memory T cells (Trm). Table 4 summarizes features of various subsets of memory T cell (Rahimi and Luster, 2020; Mueller et al., 2013; Sathaliyawala et al., 2013). Table 3 summarizes features of various subsets of memory T cell.

Memory B cells

Humoral memory participates in the host defense at least at two levels: the primary level is contributed to the presence of pre-existing protective antibodies produced by long-lived plasma cells. This level of humoral memory is considered as constitutive humoral memory. If the primary level was overwhelmed, the second level of humoral memory that is rapid reactivation of microbe-

Table 3 Memory T cell subsets.

Subset	Phenotype	Effector function	Proliferation potential	Trafficking	Function
Tscm	CD44 [±] , CD62L ⁺ , CCR7 ⁺ , CD127 ⁺ , CD69 ⁻ , CD103 ⁻	+	++	Lymph node-homing and with lesser extend in the spleen and bone marrow	Reconstitutes the entire spectrum of memory and effector T cells subsets
Tcm	CD44 ^{high} , CD62L ⁺ , CCR7 ⁺ , CD127 ⁺ , CD69 ⁻ , CD103 ⁻	+	±	Lymph node-homing and circulatory	Confers more powerful immunity against pathogens
Tem	CD44 ^{high} , CD62L ⁻ , CCR7 ⁻ , CD127 ⁺ , CD69 ⁻ , CD103 ⁻	+	-	Tissue homing and circulatory	Mainly responsible for cytotoxic action against pathogens
Trm	CD44 ^{high} , CD62L ⁻ , CCR7 ⁻ , CD11a ^{high} , CD69 ⁺ , CD103 ⁺	+	+	Tissue homing and non-circulatory	Rapid response to pathogens that breach epithelium linings and any relevant pathogen present

Table 4 Human memory B cell subsets.

Subset	Origin	Location/trafficking	Function
D19 ⁺ CD27 ⁺ IgM ⁺ IgD ⁺ (similar to circulating marginal zone B cells)	Early GC, GC independent	Second lymphoid tissues (MZ of spleen), blood	Ti response TD response?
CD19 ⁺ CD27 ⁺ IgM ⁺ IgD ⁺ (IgM-ONLY)	Early GC	Second lymphoid tissues, blood	TD response?
Class-switched CD19 ⁺ CD27 ⁺ IgM ⁺ (IgG ⁺ or IgA ⁺)	Late GC	Mucosal tissues, blood	TD response

experienced memory B cells becomes functional. This level of humoral memory is called reactive humoral memory (Inoue et al., 2018). Although humoral memory is mediated in part by pre-existing protective antibodies produced by long-lived plasma cells, these cells are usually not considered as memory B cells. Instead, long-lived and quiescent B cells that quickly respond to antigen rechallenge are considered as memory B cells. Following T cell-dependent B cell responses in the border of B-T cell zone, activated B cells proliferate and may follow one of the three fates: differentiation into extrafollicular short-lived plasma cells, germinal center-independent memory B cells or move into the follicle to form a GC (Palm and Henry, 2019). Various studies have pointed out that there should be a master regulator of memory B cell differentiation similar to IRF4/Blimp-1/XBP-1 for plasma cells. Although expression of Bach2 in the light-zone germinal center B cells has been supposed to act as a differentiation regulator of germinal center B cells to memory B cells, a memory B cell-specific transcription factor is yet to be discovered (Kometani et al., 2013). Table 4 summarizes features of various human memory B cells.

Concluding remarks

Although innate immunity as the first line of defense is still critical for the protection against many invading microbes, the evolution of microbes that learnt to evade innate immunity through employing a wide spectrum of strategies leads to counter-evolution of more effective adaptive immunity. The adaptive immunity possesses some unique characteristics such as clonal diversity and specificity as well as immunological memory that make it distinct from innate immunity. These characteristics altogether confer adaptive immunity the abilities to vastly increase the opportunities to more specifically recognize and further memorize its experiences of exposures with different pathogens. Since lymphocyte repertoire is shaped in the absence of antigen, it can hugely maximize the risk of unsuitable attack against self-antigens. Hence it is required that adaptive immune responses are finely regulated by several regulatory mechanisms (e.g., regulatory cells) to blunt such self-poisoning attack.

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Cell-Mediated Immunity

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Cell-mediated immune responses, known as cellular immunity, involves recognition of foreign antigens by naïve CD4⁺ or CD8⁺ T cells, their activation, and finally differentiation to effector or memory T cells. As one of the two main arms of adaptive immune responses, cellular immunity contributes to a wide range of defense mechanisms against a variety of pathogens.

T cells not only play a basic role in cell-mediated immunity, but they also have a fundamental role in promoting humoral immune responses.

T lymphocyte development in the thymus

T cells develop from progenitor cells that migrate from bone marrow to the thymus, where they undergo maturation and selection processes and acquire different specificities. Each clone of naïve T lymphocyte expresses a unique TCR when leaves the thymus. Additionally, depending on the type of major histocompatibility complex (MHC, e.g., class I or II) that the developing T lymphocyte encounters as it passes through the thymus, either CD8⁺ or CD4⁺ T cell develops and egresses from the thymus (Kurd and Robey, 2016; Spits, 2002).

Peripheral T cell pool comprises of naïve T cells, memory T cells and also, regulatory T cells. Naïve T cells are the ones that leave the thymus while express TCR and CD4 (or CD8) on their surface and have not exposed to any kind of antigen, yet (Van Den Broek et al., 2018). Naïve T cells circulate throughout secondary lymphoid tissues via high endothelial venules. After specific recognition of an antigen by naïve T cells, they become activated and converted to the effector T cells with diverse functional capacities. These effector T cells migrate preferentially to the peripheral sites of infections. Also, some activated T cells can convert to memory T cells. Memory T cells equipped for early and robust immune responses against subsequent encounters to the same antigen (Masopust and Schenkel, 2013; Sallusto et al., 1999). The third population of T cell pool is regulatory T cells. The majority of regulatory T cells develop in the thymus; however, some of them are differentiated in peripheral tissues (Sakaguchi et al., 2010). Naïve, effector, and memory T cells can be distinguished based on their functions, the receptors they express on their surface, and their preferred homing sites.

T cell aging

Since the thymus undergoes involution following puberty, T cells seem to be age-sensitive. In young individuals, the majority of T cell pool is composed of newly generated naïve T cells which have left the thymus recently. The contribution of the thymus for the generation of naïve T cells declines progressively after puberty. Accordingly, maintenance of naïve T cells after puberty relies on homeostatic proliferation of naïve T cells (Goronzy and Weyand, 2017; Sauce et al., 2012; Surh and Sprent, 2008).

Production of thymus-derived regulatory T cells decrease with age advancement. The peripheral blood regulatory T cells involve both naïve- like and memory-like regulatory T cells even in CD4⁺ or CD8⁺ T cells. However, following the aging decline of naïve- like

regulatory T cells is more prominent compared to memory-like regulatory T cells. By contrast, the memory-like population of T cells is increasing with the aging (Goronzy and Weyand, 2017).

Memory T cell homeostasis is more dynamic compared to naïve T cell; however, response patterns of memory T cells differ according to the type of pathogen (e.g., viruses) that each individual has encounters with. For example, cytomegalovirus (CMV) specific memory cells are dominant in elderly individuals while this is not the case with varicella-zoster virus (VZV); as many elderly have experienced shingles. Although the frequency of memory T cells are upraised with age advancement, inconsistent findings of memory T cells against distinct pathogens, such as the memory T cells against VZV and CMV in the elderly, may be related to the viral load or latency state in these viral infections (Goronzy and Weyand, 2017).

T cell receptor (TCR) and recognition of antigens

Unlike B lymphocytes, T lymphocytes generally recognize peptides in association with MHC molecules, emphasizing the recognition of proteins by T lymphocytes. Also, CD4⁺ T cells could help B lymphocyte to produce antibodies against proteins. Each clone of T lymphocyte expresses a specific T cell receptor (TCR) which identifies a peptide in combination with an MHC molecule (Davis et al., 1998).

TCR is composed of two different transmembrane chains. The majority of T lymphocytes express α and β chains as the TCR; however, a small number of T cells express $\gamma\delta$ TCRs. It is noteworthy that TCR is presented as a complex on the surface of T cells and other molecules such as CD3 and ζ homodimers accompany the TCR. In addition to the TCR complex, each T cell express one of these two co-receptors, CD4 or CD8 (Li and Mariuzza, 2013; Pennock et al., 2013; Smith-Garvin et al., 2009). CD4⁺ T cells are known as helper T cells (Th), while CD8⁺ T cells are called cytotoxic T cells (CTL or Tc) (Geginat et al., 2014; Wan, 2010).

Steps of T cell activation (general features)

Activation of T cells needs a kind of antigen-presenting cell (APC). Naïve T cells could be activated by the most potent APCs, known as dendritic cells (DCs); while effector or memory T cells could be activated by any type of APC, including DCs, Macrophages or B lymphocytes (Croft, 1994). Naïve T cells are generally activated in secondary lymphoid tissues; while effector or memory T cells could be activated at peripheral sites of inflammation in addition to lymphoid tissues (Gasteiger et al., 2016).

T cell activation is maintained through a proper interaction between an APC and a T cell, known as an immunologic synapse. Three signals are needed for the activation of T lymphocytes. The first signal is provided by a peptide, embedded in the cleft of an MHC molecule. This signal is obligatory for the initiation of T cell activation; however, in the case of most T cells, it is not sufficient. The second signal is supplied by costimulatory molecules. These molecules are expressed on the surface of APCs and as the name implies, they stimulate T cells in combination with signals provided by peptide antigens. The third signal is achieved by cytokines which are secreted from the APCs following their interaction with T lymphocytes. These cytokines lead to the differentiation of T cells that are more appropriate for responding to a pathogen that has entered the body (Corthay, 2006).

T cell costimulation

A wide range of costimulatory molecules was identified; most of them belong to B7-family. The most known B7 costimulatory molecules are B7-1 (CD80) and B7-2 (CD86), which are expressed on the surface of professional APCs. These molecules could bind to CD28 which is expressed constitutively on the surface of T lymphocytes. This pathway provides the second signal for initiation of the activation of T cells, particularly naïve T lymphocytes. Expression of B7-1 and B7-2 on the APCs follows a sequential pattern. In this way, B7-2 is detected earlier and augmented upon T cell activation, while B7-1 appears at a later time on the surface of APCs. This sequential pattern could indicate that B7-2 is more important in the initiation of T cell activation; however, B7-1 seems to play a significant role in the maintenance of T cell activation. This means that B7-1 and B7-2 could not replace each other in terms of their functions (Esensten et al., 2016).

CD28 is the most prominent costimulatory receptor on the surface of T lymphocytes, which could ligate with both B7-1 and B7-2 on the surface of APCs. It belongs to the immunoglobulin (Ig) superfamily. The majority of CD4⁺ (80%) and approximately 50% of CD8⁺ T cells express CD28 on their surface (Attanasio and Wherry, 2016; Esensten et al., 2016).

Another member of costimulatory receptors that have demonstrated stimulatory function is inducible-costimulator (ICOS, CD278). ICOS is not expressed constitutively on resting T cells; however, its expression on activated T cells suggests that both TCR and CD28 signals could have a role in ICOS expression on the surface of activated T cells. Nevertheless, it has been demonstrated that ICOS expression is not entirely dependent on CD28, especially in CD8⁺ T lymphocytes. Also, CD28 and ICOS have not shown redundant roles. ICOS binds to ICOS ligand (ICOSL, CD275) instead of B7-1 or B7-2. ICOSL is expressed on the surface of APCs, including dendritic cells, macrophages, and B lymphocytes. Apart from the essential role of the ICOS/ICOSL in humoral immunity, this interaction plays a principal role in T cell proliferation and activation (Dong et al., 2001; Wikenheiser and Stumhofer, 2016). Although IL-2 production increases following the interaction of ICOS and ICOSL, this production seems to be less prominent compared to the engagement of CD28 and B7. Contrary, the production of IL-10 is more eminent in the reaction of ICOS with its ligand. The involvement of ICOS could induce both Th1 and Th2 responses; albeit, Th2 responses are more dependent on ICOS

engagement. Similarly, in CD8⁺ T cells, ICOS interaction with ICOSL could upraise the amount of IL-2 and IFN- γ production by cytotoxic T cells and enhance their activation. It has shown that this interaction could not affect the cytolytic activity of these cells (Carreno and Collins, 2002; Wikenheiser and Stumhofer, 2016).

OX40 (CD134) is a member of the tumor necrosis factor receptor (TNFR) superfamily and represents a marker of T cell activation. This molecule can act as a costimulatory receptor and is appeared on the surface of activated CD4⁺ or CD8⁺ T lymphocytes. OX40 ligand (OX40L, CD252) is not constitutively expressed; however, it could be induced on the surface of APCs in addition to many other immune and non-immune cells. OX40 signaling could enhance T cell survival and proliferation upon their activation and also, augment clonal expansion of effector or memory T cells. OX40 could inhibit the suppressive function of natural regulatory T cells (nTreg) and also, antagonize the generation of inducible regulatory T cells (iTreg) (Croft et al., 2009).

CD40 ligand (CD154) is a member of the TNF superfamily. It is expressed on T lymphocytes upon their activation. It attaches to CD40 which belongs to the TNFR superfamily and is expressed on the surface of different APCs. This interaction can enhance the expression of costimulatory molecules on the surface of APCs as well as the production of cytokines by these cells. Also, CD40-CD40L interactivity could promote the potential of APCs to present antigens. Accordingly, the ability of APCs to activate T cells will improve. This process is named; licensing, which indicates licensing of APCs for the more effective triggering of T lymphocytes (Elgueta et al., 2009). Moreover, this interaction could promote the responses that are related to the production of immunoglobulins in germinal centers such as isotype switching, production of antibodies with higher affinity and also, production of long-lived plasma cells (Chand Dakal et al., 2020; Elgueta et al., 2009).

T cell co-inhibition

Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) or CD152 belongs to the immunoglobulin superfamily. Although approximately 30% identity has been shown in sequences of CTLA4 and CD28, and also similar ligands on the surface of APCs, CTLA4 delivers inhibitory signals to T cells compared to stimulatory signals provided by CD28. CTLA-4 is not expressed on the surface of naïve T cells; however, it is expressed on the surface of regulatory T cells, and its expression increases upon activation on the surface of conventional T cells. CTLA4 is fundamentally an intracellular antigen which transferred to the surface of T lymphocyte upon activation. Also, its expression on the cell membrane is transient due to rapid internalization (Carreno and Collins, 2002; Chambers et al., 2001). CTLA4 remains for a longer duration on the surface of memory and regulatory T lymphocytes (Jago et al., 2004).

CTLA4 represents a co-inhibitory receptor for B7 molecules. CTLA4 inhibits T cell activation through different mechanisms. Some of these mechanisms are related to the effect of CTLA4 on the function of the same T cell that has primarily expressed CTLA4; these mechanisms are known as cell-intrinsic mechanisms. Four mechanisms were categorized as cell-intrinsic mechanisms; First of all, CTLA4 can interfere with signaling pathways of CD3 and CD28. Secondly, compared to CD28, CTLA4 displays a higher affinity for B7-1 and B7-2, resulted in the competition of CTLA4 with CD28 and therefore, suppression of efficient T cell responses. Thirdly, CTLA4 strengthens the adhesion of T cells to the APCs and shortens the duration of their interaction. Finally, splice variants of CTLA4 may attach to B7-1 or B7-2 and prevents T cell activation. On the other side, CTLA4 could affect the expression of costimulatory molecules on the encountered APCs. These mechanisms are categorized as cell-extrinsic mechanisms. Three different extrinsic mechanisms have been explained up to now. First of all, CTLA4 can induce the production of Indolamine 2, 3-dioxygenase (IDO) within APCs, which in turn deprives T lymphocytes of tryptophan and inhibits their activation. The second cell-extrinsic mechanism includes downregulation of costimulatory molecules (B7-1/B7-2) on the surface of APCs and, the third cell-extrinsic mechanism involves reducing the accessibility of B7-1 and B7-2 through triggering the endocytosis of these molecules (Schildberg et al., 2016).

Programmed cell death protein 1 (PD1, CD279) has the potential to appear on the surface of T lymphocytes following their activation. PD1 belongs to the Ig superfamily, and it could attach to two ligands, including programmed death-ligand 1 (PD-L1, CD274) and PD-L2 (CD273). PD-L1 and PD-L2, members of the B7 family, are expressed on the surface of a wide range of immune and non-immune cells. After T cell activation, the interaction of PD1 (on the T cell membrane) with either PD-L1 or PD-L2 (on the surface of APC) can lead to the cell cycle arrest of T cell, reducing the production of IL-2 and finally, attenuation of immune responses. This downregulation plays a fundamental role in returning the immune responses to the baseline state and establishment of a homeostasis condition in the final stages of T cell responses (Carreno and Collins, 2002).

CD4⁺ T cell activation results

Clonal expansion

Following the recognition of peptide antigens in combination with costimulatory signals, T lymphocytes undergo proliferation. T cell proliferation is dependent on the production of interleukin 2 (IL-2) by activated T cells and also the appearance of a complete form of the IL-2 receptor, which is composed of α chain (CD25) in addition to β (CD122) and γ (CD132) chains, on their surface. This stage is called clonal expansion and provides sufficient numbers of a specific clone of T lymphocytes. IL-2 receptor is not expressed in complete form on the surface of naïve T cells; however, it promptly appears on the surface of T cells upon their activation. This time-dependent expression pattern guarantees the proliferation of specific clones of T lymphocytes during adaptive T cell responses (Pekalski et al., 2013).

Th1, Th2, Th17 differentiation

The second stage in T cell responses involves the differentiation of expanded T cells to effector T lymphocytes. Both CD4⁺ and CD8⁺ T cells will undergo differentiation to produce more efficient T cells that could combat more effectually against the pathogen that the human body has encountered. Differentiation of T cells to effector T cells takes place in secondary lymphoid tissues. According to cytokine milieu during the activation process, CD4⁺ T cells could differentiate to a variety of helper T (Th) cells (Zhu et al., 2010). Each of these Th subsets displays a distinctive transcription factor and produces a unique set of cytokines which represents its properties for the eradication of a particular type of invader microorganism. However, plasticity has been shown in CD4⁺ T cells. In a certain microenvironment, upregulated expression of other transcription factors could happen which leads to the differentiation of a distinct subset of T cells. Furthermore, micro RNAs and long non-coding RNAs demonstrate a peculiar role in promoting differentiation of different T cell populations (Sallusto, 2016).

Structurally, T cells categorize to CD4⁺ T cells, known as helper T cells or Th cells; and CD8⁺ T cells as cytotoxic T cells (CTL or Tc). In addition to the distinct expression of these coreceptors on the surface of these subsets, their function is also completely different. Helper T cells could assist other cell populations, such as macrophages or B lymphocytes, for more activation and hence, properly eradication of the pathogens; however, the CTLs are responsible for killing the viral infected or stressed tumor cells. Either of these effector functions can be achieved after the activation of naïve T cells and their further differentiation (Wan, 2010; Zhang and Bevan, 2011).

T helper 1 (Th1) cells

Th1 cells have an essential role in defense against intracellular microorganisms including, viruses, intracellular bacteria, and some intracellular parasites. However, the expression of chemokine receptors is different on the surface of various Th1 cells that defend against these diverse pathogens (Sallusto, 2016).

Th1 cells differentiate in microenvironments that are rich in interleukin-12 (IL-12) (Scott, 1993). Although other cytokines such as IL-18 and interferons type 1 (IFN- α , - β) could promote the differentiation of Th1 cells, IL-12 plays a dominant role in this process (Nakanishi, 2018; Nakanishi et al., 2001; Ramos et al., 2007). Another cytokine with the ability to promote the differentiation of Th1 cells is IFN- γ . IFN- γ , in combination with TCR signaling, can induce the first wave of T-bet expression, which is IL-12 independent. Afterward, IL-12 signaling will initiate that can trigger the second wave of T-bet expression and supports further Th1 differentiation (Lazarevic et al., 2013). The primary sources of IL-12 are DCs and macrophages which phagocytose the microorganism and become activated upon it (Trinchieri et al., 2003). Similarly, IL-18 produced by the same APCs provides synergistic effects with IL-12 (Nakanishi, 2018; Stoll et al., 1998). On the other hand, innate immune responses usually produce IFNs. IFN- α , and IFN- β are produced by virally infected cells, while natural killer cells release IFN- γ (Billiau and Matthys, 2009; Schoenborn and Wilson, 2007).

The chief cytokine that is produced by Th1 cells is interferon γ (IFN- γ). This cytokine can affect dendritic cells and macrophages that had previously driven the differentiation of Th1 cells. Reciprocally, IL-12 signaling in Th cells results in inducing the expression of T-bet; in its turn, T-bet promotes the transcription of IFN- γ (Usui et al., 2006).

One of the consequences related to the activation of CD4⁺ T cells and their subsequent differentiation to Th1 cells is the activation of macrophages. This response is typically known as cell-mediated immunity (CMI) and represents the principal cell-mediated immune response against the pathogens which have been up-taken, processed, and presented as peptides on the surface of macrophages. Activated Th1 cells produce IFN- γ which impress on cognate macrophages and enhance their ability for digestion and presentation of pathogen's antigens. Reciprocally, macrophages will produce IL-12 which could augment the potency of Th1 cells (Trinchieri, 2003). Proper instances of CMI are the infection with intracellular *Mycobacterium tuberculosis* and tuberculoid leprosy (Dannenberg, 1989; Turk, 1969).

T helper 2 (Th2) cells

T helper 2 cells are known to have a significant role in humoral immunity and defense against helminth infections. Besides, Th2 cells play a principal role in a group of prevalent diseases that are allergies. Th2 cells differentiate in the presence of IL-4 that promotes the phosphorylation of STAT6 and also the induction of GATA3. In addition to IL-4, other cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 play fundamental roles in inducing the Th2 cells. GATA3 is the main transcription factor of Th2 cells that promotes the differentiation of Th2 cells while inhibits Th1 differentiation. Also, GATA3 demonstrates a regulatory role in Th2 proliferation. GATA3 seems to be essential for continuous chromatin remodeling of Th2 cytokine gene loci. Although epigenetic modifications induce and maintain GATA3, the expression of all Th2 related cytokines is not under the control of GATA3 (Nakayama et al., 2017; Sasaki et al., 2013).

IL-4, IL-5, IL-13, and IL-9 are the chief cytokines that are produced by Th2 cells, and their expression is under the control of GATA3. Th2 cytokines have a profound effect in defense against intestinal worms. After lodgment of helminths in the intestine, innate immune and non-immune cells recognize derived pathogen-associated molecular patterns (PAMPs). Epithelial cells release cytokines such as IL-33, TSLP, and IL-25 upon activation. These cytokines can influence the other innate immune cells such as DCs, group 2 innate lymphoid cells (ILC2) and, basophils. ILC2 cells will produce IL-13, and this cytokine could modulate dendritic cells in such a way that promotes Th2 differentiation. The cytokines produced by Th2 cells have the potency to reinforce the Th2 responses. Th2 cells could promote the mechanisms that lead to the extrusion of worms from the intestine. IL-13 increases the turnover of intestinal epithelial cells and induces the helminths excretion. Indeed both IL-13 and IL-4 can induce goblet cell

hyperplasia as well as mucus secretion and contractility of intestine muscles, all of them aid in the excretion of the parasite. IL-5 could recruit eosinophils to the intestine, while IL-9 could attract mast cells. Both eosinophils and mast cells demonstrate crucial roles in immune responses against helminths (Cortés et al., 2017; Wynn, 2015).

Another mechanism that is responsible for defense against helminths mediates by the interaction of Th2 cells, B lymphocytes, and eosinophils. Th2 or T follicular helper (Tfh) derived cytokines could promote isotype switching to Immunoglobulin E (IgE) in activated B lymphocytes. Produced IgE covers the worm and facilitates the recognition of opsonized worm by Fc receptors on the surface of eosinophils. This recognition activates the eosinophils which finally, leads to the release of eosinophil granule contents on the surface of worms and the induction of helminth degradation (Huang and Appleton, 2016).

Contrary to Th1 cells that activate M1 macrophages to produce IL-12 and establishment of cell-mediated immune responses, Th2 cells activate M2 macrophages. These macrophages produce anti-inflammatory cytokines and also demonstrate a prominent role in tissue repair by inducing the production of growth factors and angiogenic cytokines. Also, Th2 cells play a fundamental role in the pathogenesis of type 1 hypersensitivity reactions, including allergic reactions (Van Dyken and Locksley, 2013).

T helper 17 (Th17) cells

Th17 cells comprise a distinct subset of CD4⁺ T cells that produce interleukin 17 (IL-17) A, IL-17F, IL-21, and IL-22. Transforming growth factor β (TGF- β) in combination with IL-6 could promote the differentiation of naïve CD4⁺ T cells to Th17 cells. Also, IL-21 and IL-23 could mediate the amplification of Th17 cells. These Th17 cells express ROR- γ t as their main transcription factor. Nevertheless, in human, the combination of IL-1, IL-6, and IL-23 has been reported to be able to induce a category of Th17 cells which are different from the aforementioned Th17 cells, as they express both ROR- γ t and T-bet simultaneously and exhibit more potent pathogenic roles (Patel and Kuchroo, 2015).

Th17 cells are involved in various extracellular bacterial and fungal infections. They recruit neutrophils to the site of infection and induce inflammation. Also, they provide barrier immunity by inducing the production of antimicrobial peptides and maintaining the integrity of epithelial cells. Pathogenic Th17 cells have a role in several autoimmune diseases such as psoriasis, inflammatory bowel disease, and multiple sclerosis (Hou and Bishu, 2020; Li et al., 2020; Moser et al., 2020).

T Follicular helper cells (Tfh)

T follicular helper cells are a distinct population of T cells that are predominantly located in the germinal centers of secondary lymphoid tissues, including lymph nodes and spleen. Their function is to help B lymphocytes for antibody production. Contrary to other effector CD4⁺ (e.g., Th1, Th2, Th17) or CD8⁺ cytotoxic T lymphocytes, they could not act as effector T cells in peripheral sites of infections. It was shown that two kinds of APCs, including DCs and B lymphocytes, are necessary for the differentiation of Tfh cells. Bcl6 represents the essential transcription factor for the differentiation of Tfh cells. Also, it has been shown that the repression of Blimp1 is required for Tfh differentiation. Although Tfh cells have the potential to express transcription factors that are related to other Th subsets, Bcl6 suppresses this expression in Tfh cells which are located in germinal centers. Tfh cells migrate to germinal centers and provide CD40L, IL-21, and IL-4 there. Tfh cells indicate a profound effect in inducing the production of plasma cells and long-lived memory B cells that can produce high-affinity antibodies (Crotty, 2019).

Regulatory T cells (Treg)

Regulatory T cells are a distinct population of T cells with a prominent role in the suppression of aberrant immune responses and induction of self-tolerance. CD4⁺ CD25⁺ T cells express FoxP3 as their main transcription factor and they could be generated in the thymus or induced at the sites of inflammation in peripheral tissues. The former are known as natural regulatory T cells (nTreg) and the latter is recognized as induced regulatory T cells (iTreg). Although FoxP3 is known as the main transcription factor which is related to Treg cells, it has been demonstrated that the expression of FoxP3 is not singly sufficient for acquiring the properties of Treg cells. Epigenetic changes can be responsible for the occurrence of some modifications in Treg cells. Although epigenetic changes can be reversed; however in some loci of Treg cells, these modifications are preserved (Ohkura et al., 2013).

Treg cells can suppress the immune responses in a specific or non-specific manner. Specific suppression is the consequence of direct contact between TCR and MHC, on the surface of a Treg and a dendritic cell, respectively. This interaction can induce a tolerogenic DC that is not able to stimulate T cell responses via several mechanisms. Engagement of B7 with CTLA4 prevents the reaction of B7-1 or 2 with CD28 on the surface of naïve or effector T cells. This event leads to the unresponsiveness of T lymphocytes to autoantigens. Moreover, this interaction could remove peptide-MHC complexes from the cell membrane of DC that impedes the presentation of that particular peptide. Also, the involvement of CTLA4 with B7 can lead to the production of indolamine 2, 3-dioxygenase (IDO) that deprives T cells of tryptophan and inhibits their proliferation.

Treg cells can establish non-specific suppression via a variety of mechanisms. One probable mechanism is the degradation of ATP to adenosine by CD39/CD73 that are existed on the surface of Treg cells. Increased adenosine could suppress the ability of DCs to present antigens. The second mechanism includes the production of IL-10 and TGF- β by Treg cells that repress the activation of T cells (Shevryev and Tereshchenko, 2020; Zhao et al., 2017).

Accordingly, Treg cells could provide tolerance to self-antigens in the thymus and self or non-self-antigens in peripheral tissues. Impairment in the function of Treg cells could lead to a wide range of auto-immune disorders (Zhao et al., 2017).

CD8⁺ T cell activation results

CD8⁺ T cells are developed in the thymus and leave it while they express T cell receptors (TCRs) on their surface. They recognize peptide antigens that are in association with MHC class I molecules on the surface of infected cells. After this recognition, CD8⁺ T cells undergo proliferation, activation, and finally, differentiation to effector or memory T cells. Effector CD8⁺ T cells are known as cytotoxic T lymphocytes (CTLs), acquiring the capacity of killing the infected target cells with viruses or intracytosolic bacteria. Besides, CTLs can lyse tumor cells.

CD8⁺ T cell requires three signals for being activated. These signals include specific antigen recognition, costimulation, and the presence of cytokines in the microenvironment. Activation of naïve CD8⁺ T cells initiates in secondary lymphoid tissues. Infected cells do not express costimulatory molecules. Therefore, the presence of dendritic cells is necessary to provide costimulation for the activation of naïve CD8⁺ T cells. Dendritic cells, as the most potent APCs, can uptake the infected cells or their associated antigens. After the antigen is processed, it is presented by MHCI molecules on the surface of DCs. Concurrently, DCs provide costimulatory signals for CD8⁺ T cells. These two signals induce the activation of CD8⁺ T cells. This process is called cross-presentation and seems to be necessary for the initiation of the activation of naïve CD8⁺ T cells. However, the potential of cross-presentation is diverse amongst different subsets of DCs (Joffre et al., 2012).

Following activation and differentiation of CD8⁺ T cells to CTLs, they acquire the characteristics which are necessary for killing the infected target cells. Transcription factors, such as T-bet and eomesodermin, are specified in CTLs. These transcription factors can induce the expression of granzyme and perforin, as the contents of CTL granules, and also, IFN- γ as the main cytokine of CTLs. Effector CD8⁺ T cells release the content of their granules on the surface of infected target cells upon activation. Perforin creates pores on the surface of target cells, and granzyme penetrates through these pores into infected target cells. Granzyme can activate caspases and induce apoptosis of infected cells (Voskoboinik et al., 2015). Besides this mechanism, CTLs can induce apoptosis of target infected or tumor cells by the interaction of Fas and Fas-ligand (Malyshkina et al., 2017; Rode et al., 2004).

In some cases, the activation of CD8⁺ T cells requires receiving the help of CD4⁺ T cells. In general, the presence of CD4⁺ T cells appears to be necessary for CTL activation when weak innate immune responses evoked. One of the mechanisms of this help is provided by inducing more potent APCs throughout CD40- CD40L interaction (Schoenberger et al., 1998). The other mechanism involves the secretion of cytokines through helper T cells which in turn can affect the CD8⁺ T cells and activate them (Wang and Livingstone, 2003; Wilson and Livingstone, 2008).

CD8⁺ T cell exhaustion

During chronic viral infections and cancers, persistent T cell stimulation causes CD8⁺ T cell exhaustion. The exhausted CD8⁺ T cells exhibit some characteristics, including; loss of effector functions, persistently expression of inhibitory receptors, altered metabolic state, and some emerging epigenetic alterations. Persistent exposure to antigen induces the upregulation of co-inhibitory receptors in T cells. Also, it can increase the expression of their cognate ligands in virally infected or tumor cells. Indeed, in this context, the production of type 1 interferons by infected cells or TGF- β by tumor cells is increased that causes more exhaustion in CD8⁺ T cells (McLane et al., 2019).

Memory T cells

After T cell activation, a population of memory T cells is developed. Activation of CD4⁺ or CD8⁺ T cells can induce memory cells. Compared to naïve T cells, memory T cells have a longer lifespan and respond more quickly after antigen exposure. These are equipped cells that can make more intensely immune responses after activation.

The number of memory T cells gradually increases after each exposure to the antigen which provides more memory T cells with age. On the other hand, memory T cells undergo self-proliferation in the absence of antigen which is maintained by IL7 or IL-15 for CD4⁺ or CD8⁺ T cells, respectively (Surh and Sprent, 2008).

From the functional viewpoint, CD4⁺ or CD8⁺ memory cells are classified as central or effector memory cells. Central memory T cells (TCM) migrate predominantly to the lymph nodes; however, effector memory T cells (TEM) settle more in peripheral sites of infection. These homing characteristics are related to the expression of distinct receptors on the surface of these two subsets of memory T cells. Central memory T cells express CCR7 and CD62L, which cause this population to migrate to the lymph nodes. TCMs can proliferate and supply a higher number of effector cells after exposure to antigen, while TEMs provide more potent effector functions, such as the production of cytokine or cytotoxic activities, after stimulation with antigen. Effector memory T cells do not express CCR7 and CD62L (Sallusto et al., 2004).

A population of memory T cells resides in non-lymphoid tissues such as skin and mucosal surfaces. This population is named tissue-resident memory cells that can defend rapidly against further challenges with the antigen in different tissues. The expression of transcription factors by this population seems to be different and depends on their location (Topham and Reilly, 2018).

For rapid response to pathogens entering a peripheral tissue, tissue-resident memory T cells, as well as effector memory T cells that migrate immediately to the site of infection, play a key role. If these responses fail to control the pathogen, an in-situ response will occur. This in situ response may involve in-situ proliferation and retention of resident or migratory effector memory cells. However, central memory T cell proliferation initiates in draining lymph nodes to provide a sufficient number of memory T cells for later stages. In the next step, the progeny of these central memory T cells migrate to the site of infection to fight the pathogen and control the pathogen completely (Masopust and Schenkel, 2013).

$\gamma\delta$ T cells

A minority of human T cells are $\gamma\delta$ T cells. These T cells express a TCR that is composed of γ and δ chains. Contrary to conventional T cells, $\gamma\delta$ T cells demonstrate lower diversity in their TCRs. Accordingly, the range of their ligands is more limited. There is a limited repertoire of V gene segments in $\gamma\delta$ T cells for rearrangement compared to those that are available in the T cells that express $\alpha\beta$ TCRs. A group of $\gamma\delta$ T cells involves $\delta 1^+$ $\gamma\delta$ T cells that where locate preferentially in the intestines. This population of $\gamma\delta$ T cells can recognize lipids in combination with CD1d and also some stress-induced ligands. They are responsible for defense against pathogens that enter the body through mucosal surfaces. V $\gamma 9$ V $\delta 2$ T cells represent another group of $\gamma\delta$ T cells. This population includes approximately 10% of blood T cells; however, they can locate in other tissues such as the intestine. Ligands of this subset of $\gamma\delta$ T cells are phospho-antigens. $\gamma\delta$ T cells can recognize stress-induced molecules by some other receptors such as NKG2D. Also, transformed cells could express high levels of these stress-induced molecules that can engage NKG2D. Engagement of NKG2D in addition to TCR could cause effector functions of $\gamma\delta$ T cells, including proliferation, T cell activation, and cytokine production (Adams et al., 2015; Poggi and Zocchi, 2014).

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T Cell Development

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Introduction

T lymphocytes are a major component of the adaptive immune response and the immune system as a whole. T cells express a T cell receptor (TCR) assembled through V(D)J recombination, similar to the B cell receptor. The TCR is a heterodimer composed of an α chain and a β chain. Unlike B cells, conventional T cells recognize antigen in the form of short peptide sequences associated with major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs). T cells can be broadly characterized based on the expression of either CD4 or CD8 coreceptors. In addition to $\alpha\beta$ T cells, there is a number of unconventional T cells that differ in the expression of key markers and/or function. Unlike the majority of leukocytes that develop in the bone marrow, T cell development occurs in the thymus. The thymus provides a unique environment that facilitates the development of T cells, while inhibiting other lymphoid lineages. Understanding the factors involved in T cell development is critical for both basic immunology research and biomedical applications. Errors in T cell development have been shown to give rise to cancer, autoimmune disorders and immunodeficiencies, and understanding T lymphopoiesis can provide potential insights for new treatments. This chapter serves to present fundamental concepts pertaining to the development of T cells, with a specific focus on the recent advancements within the field.

Early T cell development

The thymus is seeded by progenitors sourced from the bone marrow

In the postnatal thymus, T cell precursors are derived from bone marrow (BM) resident hematopoietic stem cells (HSCs), which give rise to thymus seeding progenitors (TSP) that migrate into the thymus through the circulatory system and enter at the corticomedullary junction (Fig. 1) (Radojic and Maillard, 2016). This entry is mediated by the chemokines CCL19 and CCL21, which binds CCR7 on the surface of TSPs, as well as CCL25 which binds to CCR9 (Love and Bhandoolla, 2011). Following thymic entry, T cell progenitors undergo a series of developmental stages, which are demarcated by the expression of CD4 and CD8. In mice, the initial stage, characterized by the lack of expression of both CD4 and CD8, can be further broken down into four stages based on their expression of CD44 and CD25 (Fig. 1).

Recent work has further characterized the biology of TSPs as well as the factors involved in their differentiation from hematopoietic progenitor cells. Work conducted by Yu et al. demonstrated that deletion of the Osteocalcin-producing subset of BM stromal cells impairs T cell development despite having a normal thymus. Further investigation revealed that these BM stromal cells express Delta-like ligand 4 (Dll4), which serves to promote T lineage specification ahead of thymus entry, wherein Dll4-expressing thymus epithelial cells support T lineage commitment and further differentiation (Yu et al., 2015). The Dll4⁺ BM stromal cells likely serve to promote TSP generation in the BM prior to thymic seeding. Previously, it was believed that early thymocyte progenitors (ETPs) were derived from common lymphoid progenitors (CLPs) due to their similar phenotype (Radojic and Maillard, 2016). However, it has been shown that lymphoid-primed multipotent progenitors (LMPPs) appear to seed the

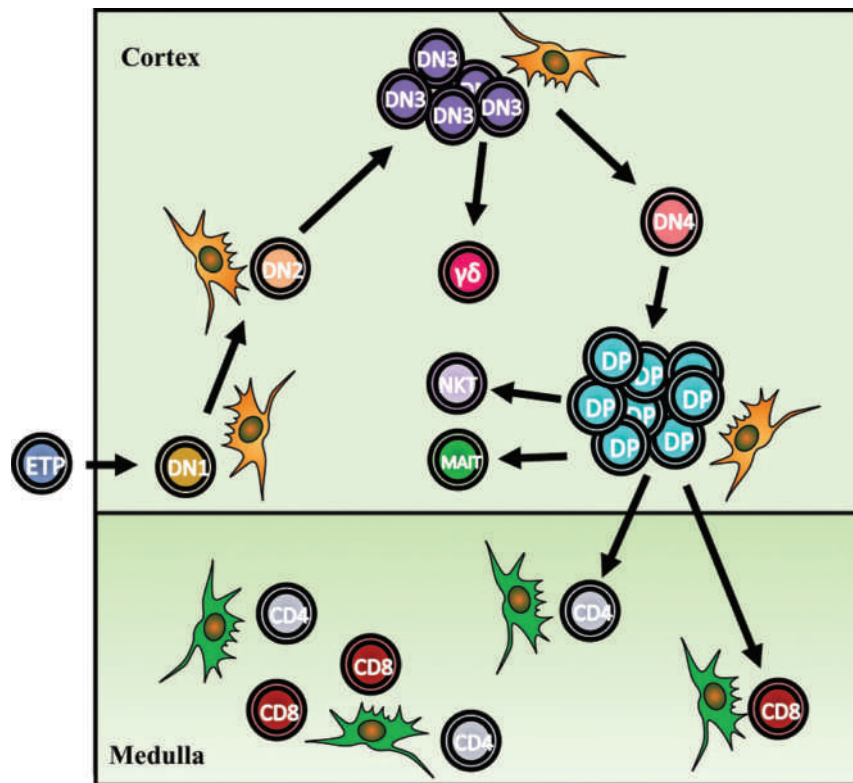


Fig. 1 T cell development occurs in various sections of the thymus. Early thymic precursors (ETPs) enter the thymus at the corticomedullary junction. Following entry, they migrate to the cortex as double negative (DN) 1 cells. DN1 cells interact with cortical thymic epithelial cells (cTECs), which provide a variety of signals to the developing thymocyte. Of note, cTECs express Delta-like ligand 4 (Dll4) and bind Notch1 on the surface of DN1 cells. Notch signaling promotes development and commitment of thymocytes in the early DN stages. As thymocytes develop they move to the outer edge of the cortex, while they rearrange their TCR β gene. At the DN3 stage, if there is a successful TCR rearrangement, the cells would receive signal through the pre-TCR which would allow for progression to the DN4 and double positive (DP) stage. However, if the TCR γ and TCR δ genes are successfully rearranged instead, the cell will develop into a mature $\gamma\delta$ T cell. By the DP stage, the cells have migrated towards the medulla and have rearranged their Tcr α gene. With a fully assembled TCR, DP cells interact with MHC on the surface of cTECs. DP cells that have a TCR specific to NKT or MAIT cells recognize non-classical MHC molecules on the surface of other thymocytes and develop into their respective cell. Cells that are able to successfully bind MHC at an appropriate affinity receive survival signals and continue development. Based on the class of MHC that the TCR is capable of recognizing, the cell develops into either a CD4 or CD8 T cell and migrates into the medulla. Once in the medulla, cells migrate between medullary TECs (mTECs) and dendritic cells and sample tissue restricted peptide expressed on the MHC of these cells. Thymocytes that react too strongly are eliminated through negative selection. Following selection processes, thymocytes leave the thymus as naïve T cells.

embryonic thymus (Luis et al., 2016). Lymphoid development has also been shown to arise from two distinct sets of progenitor cells, both derived from lympho-mono-dendritic progenitors (LMDP). Of the two, the subset that is CD127⁻ (IL-7R α) is thought to give rise to T cells (Alhaj Hussien et al., 2017). Additional research is needed to further define these progenitors and identify the exact subset of hematopoietic progenitors that give rise to TSPs (Chen et al., 2019).

PU.1 (*Spi1*) is expressed in these early progenitors and is critical for both the survival and development of T cells (Champhekar et al., 2015). Several factors have been recently identified as necessary in the promotion of thymic colonization (Alfaro et al., 2015; Lucas et al., 2016; Buono et al., 2016). Lymphotoxin- β receptor has been shown to regulate the expression of VCAM-1 and ICAM-1 in thymic epithelial cells (Lucas et al., 2016). These adhesion molecules were previously shown to be important for the colonization of thymocytes and their interaction with thymic epithelial cells (TECs) (Ehrlich, 2016). EphB2/B3 are a family of tyrosine kinase receptors that bind Ephrins, both of which are expressed by ETPs and TECs (Alfaro et al., 2015). Expression of EphB2 and EphB3 is necessary for the colonization of the thymus by ETPs and defective signaling of EphB2/B3 is associated with reduced expression of adhesion molecules and chemokines (Alfaro et al., 2015). This further illustrates their role in thymocyte seeding. Finally, early thymocytes in the CD4⁻ CD8⁻ double negative (DN) 1 and DN2 stage of development are characterized by expression of c-kit/CD117, however the source of its ligand kit-L/SCF was previously unknown (Buono et al., 2016). It was recently shown that membrane bound kit-L/SCF (mSCF) expressed on vascular endothelial cells (VECs) interacts with c-kit/CD117 on DN1 thymocytes, however DN2 cells instead interact with mSCF on the surface of TECs (Buono et al., 2016). This finding demonstrates a varying niche for early thymocyte entry and development.

Early thymocyte development is dependent on Notch signaling

Once TSPs have entered the thymus, they receive signaling from TECs that promote T cell development (Fig. 1). One of the major signaling molecules expressed on TECs is Dll4, which binds to the Notch-1 receptor on the surface of developing thymocytes (Radojic and Maillard, 2016). This ligand-receptor interaction culminates in the release of a Notch intracellular domain (NICD), which translocates to the nucleus and acts alongside various co-activators to promote the expression of genes associated with T cell development (Shah and Zúñiga-Pflücker, 2014). Using plate-bound Dll4 and VCAM-1, an in vitro niche was developed that was able to promote the development of the T-lineage cells from HSCs (Shukla et al., 2017). The role of VCAM-1 was thought to promote cell adhesion and amplify Notch-1 signaling. This demonstrated that activation of Notch-1 was sufficient to promote T cell development. Notch-1 is expressed prior to seeding of the thymus and its protein level is controlled post-transcriptionally through a suppressive domain in its 3'UTR (Mizuno et al., 2017). This regulatory region promotes expression of Notch-1 within the thymus.

Fringe proteins consisting of Lunatic, Manic and Radical Fringe are also necessary for the development of T cells. These proteins have been shown to modify the glycosylation of Notch-1, which affect its ability to interact with various Notch ligands, and deficiencies of all three Fringes resulted in impaired T cell development (Song et al., 2016). Studies have illustrated that Notch-1 signaling affects the expression pattern of several genes (Dos Santos Schiavinato et al., 2016; Hirano et al., 2015; Kobayashi et al., 2017). One of which is *Gata3*, which was shown to be important for T cell commitment (Mookerjee-Basu and Kappes, 2016; Radojic and Maillard, 2016). Notch-1 signaling directly promotes the expression of GATA3 (Dos Santos Schiavinato et al., 2016). In mice, the protein tyrosine phosphatase PRL2 is downstream of Notch signaling and has been shown to promote T cell development (Kobayashi et al., 2017). In addition, a recent study has demonstrated that the expression of c-Myc is able to promote T cell development from DN3 to DN4 (Hirano et al., 2015). This suggests that c-Myc is potentially one of the critical effector proteins downstream of Notch-1 signaling. Finally, the co-factor Zmiz1 has been shown to interact with Notch-1 directly to promote the DN to DP progression and proliferation (Wang et al., 2018).

DN3 onwards marks commitment to the T cell lineage

From the DN3 stage onward, thymocytes are committed to the T cell lineage and removal from the thymus will not enable differentiation to other lineages. One of the driving stimuli at this point in development arises from the successful V(D)J recombination of the β chain of the TCR. A fully rearranged β chain associates with pT α and CD3 to form the pre-TCR complex, which signals to promote survival and further differentiation (Mookerjee-Basu and Kappes, 2016). Although it is known that *Tcrd* and *Tcrg* rearrange at the same time as *Tcrb*, the significance of *Tcrd* rearrangements at this point in thymocyte development was attributed to an increased capacity to generate a broader repertoire of *Tcr* rearrangements at the DP stage (Carico et al., 2017). This early *Tcrd* rearrangement brings V α and J α regions in a more proximal orientation, which facilitates rearrangement. Thus, demonstrating distinct chromatin conformations at the DN and DP stage.

Recent studies have focused on the factors involved in driving differentiation downstream of the pre-TCR signal as well as factors that promote commitment to the T cell lineage. NFATc1 has been shown to be important for the promotion of both thymocyte survival and T cell commitment. It is regulated by two promoters, P1 and P2. P1 expression requires pre-TCR activity and it promotes differentiation of T cells (Klein-Hessling et al., 2016). In addition, phospholipase C γ (PLC γ) 1 has been shown to be activated by pre-TCR stimulation and functions similar to its role in mature TCR signaling (Fu et al., 2017). Deficiency in PLC γ 1 results in the inability, as thymocytes, to proceed from DN3 to DN4. Further study into the signaling of the pre-TCR has demonstrated that a specific sequence within the CD3 ITAM domains is necessary for DN4 to double positive (DP) transition. In addition, the 10 ITAM domains of CD3 must consist of a $\gamma\epsilon$, a $\delta\epsilon$ and a $\zeta\zeta$ dimer; as other combinations showed reduced cellularity within the thymus (Bettini et al., 2017). Importantly, a recent study demonstrated that the expression of Lck-Cre as a homozygous transgene in mice resulted in defects in thymocyte development. Alterations included reduced DN to DP transition and increased frequencies of $\gamma\delta$ and regulatory T cells (Carow et al., 2016). These alterations can be addressed by using Lck-Cre⁺ T cells as controls, and it should be considered when interpreting data.

Generally, DN thymocytes are dependent on a number of survival signals for proliferation and progression through the T cell lineage developmental program, such as IL-7 (Wang et al., 2016). A recent study demonstrated that conditional deletion in TECs of the gene encoding the protein Rictor, a component of the mTORC2 signaling pathway, induced a large decrease in T cell populations and thymic atrophy (Wang et al., 2016). This suggests that the signals induced by mTORC2 are necessary for the survival of TECs and therefore indirectly, developing thymocytes. Recent studies have demonstrated an important role for epigenetic modifications in the early development of thymocytes. Kat7 is responsible for the maintenance of histone 3 lysine 14 (H3K14) acetylation and was found to be necessary for the development of both conventional and innate-like T cells (Newman et al., 2017). In addition, deficiency of histone deacetylase (HDAC) 3 resulted in an impaired development of DP thymocytes. This phenotype was remedied by the addition of a fully rearranged TCR $\alpha\beta$ transgene (Stengel et al., 2015; Philips et al., 2016). Therefore, it is likely that HDAC3 plays a role in TCR β rearrangement for the transition into the DP stage. In addition, HDAC3 inhibits P2X7, a receptor associated with cell death, and restrains the genes associated with CD8 commitment during the DP stage (Philips et al., 2019a,b). This ensures proper development and cellularity during the DP stage. In addition, ESET is a histone methyltransferase associated with the addition of methyl groups to lysine 9 on histone 3 (H3K9Me) and its deletion resulted in the impairment of thymocyte development through the inhibition of the ERK signaling pathway. H3K9Me often serves to inhibit gene expression, and one of the genes inhibited in T cells is Fc γ RIIb, which has been shown to inhibit the ERK pathway (Takikita et al., 2016), demonstrating the

downstream effect that ESET can have on T cell development. Finally, TCF1 and HEB have been shown to cooperate to establish transcriptional and epigenetic regulation at the DP stage (Emmanuel et al., 2018). Taken together, epigenetic regulation has a clear role in T cell development and further study of epigenetic regulation is imperative for understanding the process.

Selection of thymocytes

Positive selection selects for TCR-MHC interaction

In the DP stage, the cells rearrange their alpha TCR chain and following successful rearrangement, the cells express a mature TCR on their surface and undergo positive selection. Positive Selection is a process that occurs at the DP stage soon after the formation and expression of a fully functional $\alpha\beta$ -TCR (Koehli et al., 2016). Thymocytes sample peptide antigens presented on self-MHC on the surface of cortical TECs and low-affinity TCR binding to peptide-MHC promotes survival of thymocytes (Fig. 1) (Koehli et al., 2016; Neier et al., 2019). At this time, extreme TCR signals outside of this necessary low affinity signal, will result in the death of the cell. Self-peptide presented during positive selection is processed through the thymoproteasome and components such as $\beta 5t$ (*PSMB11*) orchestrate efficient development of DP cells (Apavaloaei et al., 2019; Tomaru et al., 2019). Expression of $\beta 5t$ outside of the cortex of the thymus leads to aberrations in development and increased autoimmunity (Tomaru et al., 2019). These findings support the hypothesis in which the thymoproteasome produces unique self-peptides for the sole purpose of positive selection. This ensures that mature T cells can recognize antigens in the context of self-MHC while preventing the development of a bias towards self recognition within the TCR repertoire. Special AT-rich binding protein 1 (SATB1) is a chromatin regulator that is upregulated in response to TCR signaling and its activity has been associated with positive selection in thymocytes (Gottmukkala et al., 2016). This evidence further supports the fact that TCR activation promotes positive selection.

Recent studies have identified Themis, a protein which is upregulated during positive selection (Garreau et al., 2017). Themis associates with the proteins SHP-1 and Grb2 during positive selection in thymocytes (Choi et al., 2017; Garreau et al., 2017; Paster et al., 2015; Radtke et al., 2016). Themis stabilizes Grb2 allowing it to bind to LAT and recruit the complex to the plasma membrane in proximity to the TCR (Garreau et al., 2017; Paster et al., 2015; Zvezdova et al., 2016). SHP-1 is a tyrosine phosphatase that removes phosphates from ITAM motifs (Martinez et al., 2016). Themis acts as a regulator of SHP-1 to inhibit its phosphatase activity during positive selection (Choi et al., 2017; Mehta et al., 2018). Therefore, Themis acts to reduce the binding threshold needed for TCR activation during positive selection, allowing for TCR activation in response to weak affinity peptide-MHC (pMHC) interactions. Protein kinase D (PKD) is activated in DP cells, and phosphorylates SHP-1, promoting positive selection (Ishikawa et al., 2016). Whether phosphorylation directly inhibits SHP-1 or plays a role in Themis regulation remains to be determined. Grb2 is also found to recruit USP9x to LAT, where it acts to stabilize Themis (Garreau et al., 2017). Finally, CD6 is an adhesion molecule that binds ALCAM/CD166 on the surface of antigen presenting cells (APCs). CD6 deficiency impairs T cell development and lowers regulatory T cell populations (Orta-Mascaró et al., 2016). Generally, these factors function to improve the efficacy of positive selection. However, miRNA-146a has been shown to inhibit NF κ B and other genes associated with positive selection (Li et al., 2018).

Positive selection promotes the production of single positive CD4 and CD8 cells based on affinity for MHC class II or class I, respectively (Koehli et al., 2016). In addition, recent contributions have led to a greater understanding of the molecular factors that influence the development of either subset. Runx3 promotes the development of CD8 single positive T cells, whereas ThPOK promotes the development and commitment of CD4 single positive T cells (Mookerjee-Basu and Kappes, 2016). In CD8⁺ cells, duration of positive selection was similar, independent of TCR-pMHC half-life, however, longer TCR signaling was accompanied by quicker Runx3 induction (Kimura et al., 2016). In addition to γ chain (γ_c) cytokines, primarily IL-7, influencing the development of T cells, non- γ_c cytokines including IL-6 and IFN- γ are associated with the expression of Runx3 (Etzensperger et al., 2017). Therefore, these cytokines support and promote the development of CD8⁺ T cells. Finally, the TLE co-repressors were demonstrated to be imperative for the commitment of CD8⁺ T cells. TLE3, in particular, was shown to bind ThPOK silencer to inhibit the expression of CD4 and related genes (Xing et al., 2018). In contrast, the Ikaros family of proteins, expressed during the transient single positive (TSP) stage, are important for the establishment of single positive cells. CD4 expression is associated with a decrease in Helios and an increase in Ailos (Mitchell et al., 2016). UBC9 modifies NFAT and IL-7 signaling through SUMOylation and is required for efficient development of T cells following positive selection (Wang et al., 2017a).

Negative selection removes auto-reactive T cells

Following positive selection, single positive thymocytes migrate from the cortex into the medulla of the thymus by CCL19/21 chemokine gradients and undergo negative selection, which is a critical component of central tolerance (Fig. 1) (Koehli et al., 2016). Thymocytes sample the autoantigens presented by medullary (m)TECs and medullary DCs, and a strong response to autoantigen triggers the death of the potentially offending T cell (Fig. 1) (Ehrlich, 2016). Nuclear receptor corepressor 1 (NCoR1) has been shown to repress apoptosis induced in negative selection by inhibiting the pro-apoptotic factor BIM (Müller et al., 2017; Wang et al., 2017b). Therefore, this raises the threshold of activation required for the negative selection of thymocytes. This ensures that primarily auto-reactive T cells are eliminated before final maturation. Finally, mechanotransduction loops through the TCR have been indicated in playing a role in negative selection (Hong et al., 2018). Deficiencies in negative selection and central tolerance are heavily associated with auto-immune disease (Koehli et al., 2016). By analyzing the selection pattern associated with known TCRs, it has been shown that the majority of negatively selected TCRs are promiscuous receptors that broadly recognize auto-antigen (McDonald et al., 2015).

During negative selection, thymocytes must migrate between stromal cell niches (Hu et al., 2015; Ki et al., 2017). The chemokine receptor, CCR4, is expressed on thymocytes following positive selection and its ligand is secreted by medullary dendritic cells (mDCs), an APC involved in negative selection. This receptor, alongside CCR7, promotes the entry of thymocytes into the medulla and is necessary for efficient negative selection (Hu et al., 2015). Recently, CCR8 was also identified on the surface of positively selected CD4 thymocytes and its ligand is produced by medullary cells. However, its function was not required for medullary entry (Thyagarajan et al., 2018). This could be explained by redundancy or potentially an alternative role for CCR8. In mouse models, EBI2 is expressed by CD4⁺ T cells and deletions impair migration during negative selection, while also altering the final TCR repertoire (Ki et al., 2017). This indicates that EBI2 is important in mediating the migration of thymocytes during negative selection.

In addition to migration patterns, the thymic medulla is unique due to its expression of autoantigens normally restricted to specific cell types (Kawano et al., 2015; Takaba et al., 2015). The expression of tissue restricted antigens (TRA) in the thymus facilitates T cell exposure to a larger variety of antigens that may be encountered in the periphery. Aire is a transcription factor that is expressed in mTECs and regulates the expression of TRAs as well as adhesion molecules and conditional deletion of Aire gives rise to auto-immune disease (Haljasorg et al., 2015; Pezzi et al., 2016). Recent work has identified a region upstream of the *Aire* gene called CNS1, which contains two NFκB binding sites, deletion of this region results in a similar phenotype to Aire deletion (Haljasorg et al., 2015). Therefore, NFκB likely serves as a regulator of Aire. In addition, Aire was found to be expressed in over 95% of mTECs, however only a small subset of mTECs expressed Aire at one time (Kawano et al., 2015). This would suggest that Aire is temporally regulated. In addition, the expression of TRAs requires specific epigenetic chromatin remodeling that occurs early in mTEC development (Handel et al., 2018). This development is Aire-independent.

Recently, Fezf2 has also been found to promote the expression of TRAs in mTECs, and deletion induced severe autoimmunity (Takaba et al., 2015). Further demonstrating the importance of TRAs in negative selection. Similar to their role in positive selection, Themis, SHP-1 and Grb2 have been found to promote the negative selection of thymocytes (Martinez et al., 2016; Paster et al., 2015). During negative selection, SHP-1 activity attenuates TCR signaling and promotes survival (Martinez et al., 2016). Therefore, the Themis complex functions to modify the TCR signaling threshold necessary for both positive selection and negative selection. Additional research will aid to elucidate what factors are involved in the complex function that Themis has on TCR signaling.

Occasionally, when a TCR exhibits a high affinity for self MHC/peptide, rather than being eliminated, the T cell can be selected to act as a regulatory T cell through the process of agonist selection (Fig. 1) (Koehli et al., 2016; Kitagawa and Sakaguchi, 2016). Regulatory T cells are a component of peripheral tolerance and function to generate an immunotolerant environment to self antigens (Kitagawa and Sakaguchi, 2016). Recent studies have shown that deletion of pro-apoptotic factor BIM results in an increase in the development of agonist-selected T cells (Hu et al., 2017; Li et al., 2017a,b). BIM likely serves to eliminate auto-reactive T cells with an affinity that surpasses the threshold necessary for agonist selection.

Unconventional T cell development

Regulatory T cell development

Regulatory T (T_{reg}) cells are a subset of CD4⁺ T cells which act as the central component of peripheral tolerance (Kitagawa and Sakaguchi, 2016). Generally, these cells regulate homeostasis by impairing effector activation. One method involves the production of anti-inflammatory cytokines such as IL-10 in response to autoantigens, impairing autoreactivity of effector T cells (Kitagawa and Sakaguchi, 2016). Natural T_{reg} (nT_{reg}) make up the majority of T_{reg} cells and this subset develops within the thymus as a result of agonistic selection. Recent evidence suggests that nT_{regs} can be divided into two groups characterized as either CD25⁺ or FoxP3^{low} and these unique types develop from two distinct pathways (Owen et al., 2019). In addition to nT_{regs}, there is a subset that develops in the periphery from naïve T cells referred to as induced T_{reg} (iT_{reg}) cells (Kitagawa and Sakaguchi, 2016). High salt conditions have been found to inhibit the development of nT_{regs}, while having no effect on iT_{reg} (Luo et al., 2019). Further illustrating two distinct mechanisms of development. Early development of nT_{reg} cells occurs in a fashion similar to CD4⁺ effector T cells with differentiation between the subsets occurring in the DP stage of thymocyte development. Evidence has shown that competition among nT_{reg} cells can inhibit their differentiation, resulting in the production of effector T cells (Kitagawa and Sakaguchi, 2016), demonstrating the overlap in their development. During the DP stage, thymocytes undergo selection in which T cells with a high affinity to self-antigen are removed from the population, however some auto-reactive T cells are selected for and differentiate into nT_{reg} cells (Kitagawa and Sakaguchi, 2016). Recently, the cytokines IL-7 and IL-2 has been found to promote the development of nT_{regs} during the DP stage (Tuulasvaara et al., 2016; Chorro et al., 2018). IL-2 was found to regulate SATB1, which controls chromatin accessibility in developing cells (Chorro et al., 2018). Agonistic selection induces the expression of FoxP3 which acts as the master transcription factor for the T_{reg} phenotype. Past studies have demonstrated that FoxP3 is important for both the development and function of T_{reg} cells. FoxP3 is capable of promoting the survival of regulatory T cells during negative selection by upregulating the expression of CTLA-4 on the cell surface that binds to CD80/86 and competes with CD28, a co-stimulatory molecule for TCR activation (Kitagawa and Sakaguchi, 2016). It has also been shown to downregulate the expression of ZAP-70, an important component in the transduction of TCR signals (Kitagawa and Sakaguchi, 2016). This evidence would suggest that FoxP3 can induce T_{reg} survival during negative selection by reducing the intensity of TCR activation. In addition, a region in FoxP3 was shown to directly regulate genes associated with the T_H2 phenotype and mutations in this region led to the de-repression of these genes (Van Gool et al., 2019).

Studies on the development of regulatory T cells have identified molecular factors, in addition to FoxP3, required for their development. Adhesion molecules have been shown to be important for promoting TCR activation and ICAM-1 specifically has been shown to play a role in nT_{reg} development (Gotttrand et al., 2015). In addition, thymic PLZF⁺ innate T cells have been shown to promote the differentiation of CD103⁺ regulatory T cells, which display an activated phenotype, through the production of IL-4 (Kang et al., 2016). Finally, a study conducted by Feng et al. demonstrates a FoxP3 intronic enhancer, CN3, which is necessary for the induction of FoxP3 in the development of nT_{reg} cells but is not necessary in maintaining its expression in mature cells (Feng et al., 2015). Further analysis has shown that this region is epigenetically modified with H3K4Me. This modification occurs early in T cell development and can be added as early as the DN1 stage (Feng et al., 2015). These modifications enhance the cell's sensitivity to FoxP3 induction and are required for a diverse T_{reg} repertoire. Deletion of this domain has been shown to reduce the proportion of regulatory T cells and drastically affect their diversity (Feng et al., 2015). These results provide a potential explanation for how a large variety of TCRs can be found within the population of T_{reg} cells despite the fact that a limited range of TCR affinities are selected to differentiate into regulatory cells.

γδ T cell development

The TCR found on conventional T cells is a heterodimer composed of an α chain and a β chain. However, there exists an alternative class of T cells which contain a TCR composed of a γ chain and a δ chain (In and Anderson, 2016). γδ T cells possess an innate-like phenotype and their development is unique in comparison to conventional T cells (In and Anderson, 2016). γδ T cells proceed through a similar development program as conventional T cells until the DN3 stage (Fig. 1). At this stage, γδ T cells express a fully formed TCRγδ, which provides alternative signaling compared to the pre-TCR found on conventional T cells (In and Anderson, 2016). However, recently a unique class of γδ T cells has been shown to develop from DN2a thymocytes. These cells develop independent of Bcl11b, which is a transcription factor necessary to promote progression from DN2a to DN2b (Hatano et al., 2017). In addition, recent work has demonstrated the role of selecting ligands in shaping the γδ repertoire (Fahl et al., 2018). Finally, PTPN2 is a tyrosine phosphatase that attenuates STAT5 signaling and has been shown to influence T cell commitment, as well as the differentiation between conventional and γδ T cells. Deficiency in PTPN2 has been shown to repress the γδ developmental pathway (Wiede et al., 2017). Additional research should be conducted to understand what factors are involved in the differentiation of these two T cell lineage subsets.

γδ T cells are classified based on the variable region within their TCR as well as their effector function (In and Anderson, 2016), and these effector functions share similarities with the subsets of conventional T cells. The IL-17 producing class of γδ T cells has been shown to rely on different survival factors than the IFN-γ producing cells (Corpuz et al., 2016). Therefore, it is likely that these groups do not share a common niche and likely do not compete for survival. Recent advancements have improved upon the current understanding of what factors influence the development of each γδ T cell subset. Work conducted by Buus et al. has mapped out seven different developmental stages of γδ T cells using CD117, CD200 and CD371 as markers. These stages are labeled A through G and the development of three distinct subsets (IFNγ, IL-4 and IL-17 producing cells) can be mapped using these stages (Buus et al., 2017). Factors involved in the development of IL-17 producing γδ T cells have also been elucidated. HEB has been shown to be required for the expression of RORγT in fetal γδ T cells and promotes the production of related cytokines such as IL-17 (In et al., 2017). In addition, it has been shown that a strong TCR signal inhibits the development of IL-17 producing γδ T cells (Sumaria et al., 2017; Zarin et al., 2018). This supports the previous hypothesis that strength of signaling may play a role in determining γδ T cell subsets. In addition, evidence has demonstrated that IL-17 producing cells are capable of developing extra-thymically. During this process, IL-1β and IL-23 convert CD122⁻ γδ T cells into IL-17 producing cells (Muschawek et al., 2017; Zarin et al., 2018). This evidence suggests that γδ T cell subsets may possess plasticity in the periphery. Finally, it has been shown that subsets expressing Vγ1.1 and Vγ2 diverge at a later stage than previously thought. This divergence occurs after γδ commitment and can be tracked by the expression of CD73 (Coffey et al., 2014). Prior to diversification, these two subsets contain very similar transcriptomes (Buus et al., 2016). In addition, TCR, Notch and cytokine signaling have all been demonstrated to play a role in specifying the various functions of γδ T cells (Zarin et al., 2018). As a whole, it is evident that γδ differentiation is extremely complex and multiple factors are required for the development of each effector function.

Mature double negative TCR-αβ T cells

Double negative T cells are mature cells that lack the expression of the co-receptors CD4 and CD8 (Lamboleze and Cheroutre, 2016). These cells are associated with the intestinal epithelia. During development these cells are selected through agonist selection and can provide immunosuppressive effects. The addition of IL-7 has been shown to inhibit the immunosuppressive function of double negative (DN) T cells through the Akt/mTOR signaling pathway (Lamboleze and Cheroutre, 2016). Overall, the development of these T cells is not well understood other than what has previously been outlined, however recent work conducted by Chowdhary et al. provides a model that could potentially be used to further the understanding of their development. In their experiment, they induced a double knock out of CD4 and CD8 in T cells, then analyzed the phenotype of the affected mice. They found that this simulated the DN T cell phenotype in the periphery and demonstrated DN T cell's role in pathologies such as lupus (Chowdhary et al., 2017). Finally, an additional study has demonstrated that a population of DN T cells develop from autoreactive CD8⁺ T cells. These cells are often short lived but may play a significant role in DN T cell function (Rodríguez-Rodríguez et al., 2016).

NKT cell development

NKT cells are an unconventional class of T cells that have an innate-like phenotype and share similar features with NK cells. These cells have been shown to recognize lipids presented by the non-classical MHC, CD1d. Past studies have demonstrated two types of NKT cells, type 1 or invariant (iNKT) and type 2 which are variant in their TCR. Invariant NKT cells are characterized by the expression of a semi-invariant TCR consisting of V α 14 and J α 18 in mice. These TCRs contain a hydrophobic region that is necessary for CD1d interaction and NKT development (Vieth et al., 2018). Similar to conventional T cells, iNKTs have been shown to possess different functional subsets that produce cytokines such as IFN- γ , IL-4 or IL-17. Recent advancements have focused on understanding the developmental pathway of iNKTs and how their development differs from conventional T cells. Previously, NKT cells have been shown to diverge from conventional T cells at the DP stage, through selection on thymocytes (Fig. 1). However, it has recently been shown that some NKT cells may diverge from the conventional program at the DN rather than the DP stage (Dashtsoodol et al., 2017). In addition, previous work on iNKT development has demonstrated a reliance on miRNA for their development, however the identity and function of these miRNAs were unknown (Blume et al., 2016; de Candia et al., 2016; Frias et al., 2018). Recently, it has been shown that miR-181a/b-1, which is induced by SOX4, functions to tune TCR signaling and is necessary for the development of iNKT cells. In mice deficient for miR-181a/b-1, NKT development was severely impaired, and this impairment could be rescued by the overexpression of V α 14J α 18 TCR transgene (Blume et al., 2016; de Candia et al., 2016; Malhotra et al., 2018). This supports the hypothesis that miR-181a/b-1 acts to promote agonist selection of NKT cells by lowering the TCR activation threshold and therefore promoting their development. TCR activation has also been associated with development of certain subsets of NKT cells (Clancy-Thompson et al., 2017; Cruz Tleugabulova et al., 2016; Tuttle et al., 2018). Strong stimulation of the TCR has been linked to the downregulation of Id2 (Stradner et al., 2016), and Id proteins have been shown to regulate the expression of E2A, which is critical for NKT development (Roy et al., 2018). In addition, Id2 deficiency is associated with decreased expression of T-bet and increased expression of PD-1 (Stradner et al., 2016). However, a double KO of Id2 and Id3 induces rapid growth in NKT cells associated with NKT tumors (Kumar et al., 2017). This suggests that Id proteins may serve multiple and distinct purposes in NKT cells. A higher TCR affinity has also been shown to correlate with increased expression of PLZF and reduced expression of T-bet (Clancy-Thompson et al., 2017). The negative regulatory protein of the NF κ B pathway, TNFAIP3/A20, is downstream of the TCR signaling pathway and a deficiency in this protein results in hyper-reactive NKT cells. In addition, its expression has been shown to influence the development of both IFN- γ producing (NKT1) and IL-4 producing (NKT2) cells (Drennan et al., 2016). This phenotype can be reversed through a deficiency in a member of the NF κ B activation pathway, MALT1. This further illustrates the dependence of TCR signaling on NKT subset development. Overall, the level of TCR signaling is critical for the development of NKT cells. However, the TCR is not the sole contributor to NKT cell development and it has previously been shown that γ chain cytokines are critical for the development of T cells, including NKTs. Recently, arginine methylation by the enzyme PRMT5 has been shown to modulate the strength of cytokine signaling. This enzyme is particularly critical for the development of NKT cells, as deletion of PRMT5 leads to the loss of NKT cells (Inoue et al., 2018). In addition, SHP-1 was shown to regulate iNKT cell differentiation in a TCR-independent manner (Cruz Tleugabulova et al., 2019). In this scenario, it is likely that SHP-1 adjusts NKT cell response to cytokines within their environment. The environment that the NKT cell is exposed to has been shown to influence its maturation. In somatic nuclear transfer studies, the cytokine profile of monoclonal NKT cells was heavily influenced by their tissue localization, despite having identical TCRs (Clancy-Thompson et al., 2017).

Recent work has also demonstrated a strong epigenetic influence on the development of NKT cells (Beyaz et al., 2017; Cui et al., 2016; Northrup et al., 2017; Thapa et al., 2017; Tsagaratou et al., 2017). Histone Demethylase UTX is necessary for the expression of NKT associated genes, specifically PLZF (Beyaz et al., 2017; Northrup et al., 2017). In addition to demethylases, HDAC3, a histone deacetylase, has been shown to be critical, both for the development of NKT cells as well as the production of IL-4 and IL-17 (Thapa et al., 2017). Finally, Uhrf1 is a regulator of DNA methylation and is upregulated during stage 1 of iNKT cell development (Cui et al., 2016). Uhrf1 promotes survival through the Akt-mTOR pathway, which has previously been shown to be necessary for NKT cell survival (Cui et al., 2016). This is further illustrated in the fact that a conditional deletion of Rictor, a protein associated with the mTORC signaling pathway, results in the loss of NKT17 development (Sklarz et al., 2017). The development of NKT cells is also associated with the phenotype of the cells around them. ABCA7 is an ABC transporter and loss of function mutations are associated with reduction of CD1d expression and alterations in lipid rafts (Nowyhed et al., 2017). This extrinsically affects NKT cells by reducing the amount of CD1d available.

CD1d is critical for the selection of NKT cells, and different variants of CD1d possess unique roles in the development of effector functions (Sundararaj et al., 2018). Production of sphingolipids (SL) in cells is also necessary for proper NKT development. In cells deficient for ceramide synthases, SL production is impaired, and this results in a reduced number of NKT cells (Popovic et al., 2017; Saroha et al., 2017). SLs likely function as an endogenous ligand to be presented during selection. Previous entries have defined the importance of SLAM-SAP interaction between thymocytes and NKT cells during selection (Chen et al., 2017). Recently, SLAM receptor alterations have been shown to lead to changes in the expression of Ly108, CD150 and Ly9. Ly108 is especially important since it is necessary for the activation of NKT cells (Baglaenko et al., 2017). SAP deletions in NKT cells decrease the expression of GATA-3 and PLZF while promoting the expression of ROR γ T (Michel et al., 2016). This results in reduction of NKT2 numbers and IL-4 production, while increasing NKT17 numbers. It is evident that NKT development relies on the phenotype of the cells around it, both during selection and maturation.

PLZF is a transcription factor necessary for the innate phenotype in many unconventional T cells and it is important for NKT cell maturation, and recent work has led to a greater understanding of PLZF's role in development. An analysis of the targets of PLZF have

established a temporal hierarchy of PLZF activation. Initially, PLZF acts to regulate the expression of cytokine receptors and adhesion molecules, it then regulates transcription factors associated with effector functions and finally it suppresses Bach2, a repressor of effector functions (Mao et al., 2016). This further demonstrates PLZF's role in promoting innate effector function. Evaluation of the region upstream of PLZF has identified regions homologous to RUNX1 motifs and deletion of RUNX1 resulted in reduced PLZF expression in these cells (Mao et al., 2017). The protein YY1 has also been shown to be critical for NKT development, and directly binds regions of the PLZF gene (Ou et al., 2019). In addition to our increased understanding of PLZF, recent work has revealed other proteins associated with the development of NKT cells. β -Catenin has been shown to be temporally regulated in NKT cells. The protein is initially expressed during development but downregulated in stage 2 and failure to reduce the expression of β -Catenin results in impaired transition to stage 3 (Pyaram et al., 2017). Roquin, a class of mRNA binding protein, has been shown to be a regulator of NKT cell development in mice. Impairment of Roquin leads to impaired cytokine production and increased NKT17 development (Drees et al., 2017). In addition to Roquin, c-MAF and NKAP have been shown to be necessary for proper NKT17 development and function (Thapa et al., 2016; Yu et al., 2017). Finally, CD69 has been shown to be critical for the retention of NKT cells in the thymus. Cells deficient for CD69 prematurely express S1P1R and abort NKT2 development (Kimura et al., 2018). Therefore, the various subsets of NKT cells require different factors for development and further research must be conducted to understand these pathways.

MAIT cell development

Mucosa associated innate T (MAIT) cells are a recently described T cell that, similar to NKT cells, recognizes antigen in the context of a non-classical MHC class 1b (MR1). As their name implies, MAIT cells are found primarily in the mucosa and possess an innate phenotype (Leeansyah et al., 2014). MAIT cells are capable of recognizing vitamin B2 metabolites from bacteria which are presented on MR1 molecules (Rahimpour et al., 2015). This allows for the recognition of a broad spectrum of bacteria. They have been shown to produce IFN- γ and IL-22 in response to antigen (Rahimpour et al., 2015). Recent advancements in the understanding of MAIT cells can be attributed to the development of MR1 tetramers, which can be used to identify MAIT cells using flow cytometry (Rahimpour et al., 2015; Gherardin et al., 2018). MAIT cells develop in the thymus and are positively selected by thymocytes expressing MR1 (Fig. 1) (Seach et al., 2013).

After selection, MAIT cells proceed through three developmental stages similar to NKT cells. Each stage is characterized by their expression of CD24 and/or CD44. Stage one and two of development occurs entirely in the thymus, while stage three cells are initially found in the thymus, but complete maturation extrathymically. Deletion of MR1 in T lineage cells prevents the progression of MAIT cells through the various stages (Koay et al., 2016). This would suggest that MR1 is necessary for checkpoint transition of each stage. Secretion of IFN- γ and IL-22 is only found once the cells have progressed to stage three. This is also the stage where PLZF is expressed. PLZF is a transcription factor associated with innate programming in lymphocytes and is necessary for MAIT cell maturation. Through co-culture with OP9 cells, Koay et al. demonstrated that stage 3 of MAIT cell development requires stromal factors. It was also shown that Dll is initially required for DN development but not required later in MAIT cell-specific development. In addition, the knock outs of DROSHA or DICER, machinery associated with the production of miRNA, inhibited the development of MAIT cells. This would suggest that MAIT cell development is dependent on the production of miRNAs that likely play a direct role. Maturation of MAIT cells is dependent on the presence of microbiota in the gut. This relationship occurs through IL-18 produced as a result of the microbiota. MAIT cells upregulate IL-18R during development and signaling through this receptor promotes development (Koay et al., 2016).

Characterization of the development of MAIT cells demonstrates many similarities with NKT cells. For one, both cells recognize antigen in the context of MHC class 1b and display an innate phenotype. In addition, both cell types are positively selected by thymocytes rather than TECs (Seach et al., 2013). This similarity is further exemplified by the observation that deletion of CD1d leads to an increase in the proportion of MAIT cells (Koay et al., 2016). Since CD1d is necessary for the development of NKT cells but not MAIT cells, this would suggest that these two cell types share a resource niche. However, NKT cells leave the thymus as mature innate cells whereas MAIT cells leave the thymus as naïve cells and complete maturation in the periphery. Evidence suggests that one of the more common MAIT cell phenotypes which express CD8 $\alpha^+\beta^-$ exits the thymus and develops extrathymically (Leeansyah et al., 2014). Recently, a subset of MAIT cells has been characterized that is negative for both CD4 and CD8. These cells are functionally distinct from the conventional CD8 $^+$ MAIT cells, but evidence suggests that chronic TCR stimulation induces development of DN MAIT cells from CD8 $^+$ cells (Dias et al., 2018).

Conclusion and future directions

Based on recent findings, presented above, the complexity of T cell development is even more apparent. However, the past few years have improved our understanding of features in both conventional and unconventional T cell development. Work conducted in the aspects of selection have demonstrated Themis' ability to modulate the threshold of TCR activation required during selection. It has also shown the importance of factors such as Aire and Fezf2 in maintaining effective negative selection. Recent work into unconventional T cells has led to a greater understanding of their development as well as the characterization of MAIT cells. Further research should focus on the mechanisms involved in determining one developmental pathway over another. Special interest should be placed on the development of unconventional T cells, since many of these cells have only been described recently and their development is still poorly understood.

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Antigens

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Introduction

Any substance (molecule) capable of inducing a specific immune response (humoral or cellular) against that substance, or can be recognized by products of an immune response, such as antibodies or lymphocytes, is commonly called an antigen. Antigens exist in many forms: typically they can be simple molecules, toxins, chemicals, proteins, carbohydrates, lipids or nucleic acids derived from invading microorganisms, such as viruses, bacteria, protozoans, and fungi, or other foreign substances that are usually not found in the body (non-self or heteroantigens). In addition, the body's tissue and cells, including cancer cells, can also have antigens (self or autoantigens, or mutated neoantigens) in them and can cause an immune response (Zamvil et al., 1986; Khodadoust et al., 2017). These autoantigens can be used as biomarkers to identify those specific tissues or cells (Schumacher and Schreiber, 2015; Balachandran et al., 2017). Although antigens exist in numerous forms, they all share a common mechanism of triggering an immune response. The immune system in humans and other mammals can be divided into two general categories: the *innate*, which is a more general immune system that can recognize foreignness, but not specific antigens, and the *adaptive*, which is more a specialized immune system with receptors for specific antigens. These two systems work closely together and take on different tasks—while the innate immune response is immediate, the adaptive immune response is not. However, the effect of the adaptive immune response is long-lasting, highly specific, and manifests sustained long-term memory (Janssen et al., 2003; Polonsky et al., 2018).

When an antigen binds to a receptor molecule, it may or may not initiate an immune response. Antigens that have an ability to induce an effective humoral and/or cellular immune response following immunization are called immunogens. This ability to elicit an immune response is referred to as *immunogenicity*. Immunogenicity and antigenicity are related, but are distinct, immunologic properties that sometimes are confused. Antigenicity is the property of being able to be recognized by the immune system, perhaps a pre-existing immune response or its product like an antibody or T cell. It does not require that they be able to elicit that response. All immunogens are antigenic, but not all antigens are immunogenic. In other words, all molecules that possess the property of immunogenicity also possess the property of antigenicity, but the reverse is not true—not all antigens are immunogens. For example, some small molecules called haptens possess the property of antigenicity (can bind to lymphocyte receptors) but do not induce a specific immune response (by themselves). In other words, haptens lack immunogenicity. Although haptens cannot induce an immune response by themselves, they can become immunogenic when joined to a larger and more immunogenic molecule, called a carrier (Martin and Weltzien, 1994). Immunization with hapten-carrier combination elicits an immune response to both hapten and carrier (Boak et al., 1971). Details of haptens will be discussed later in this chapter.

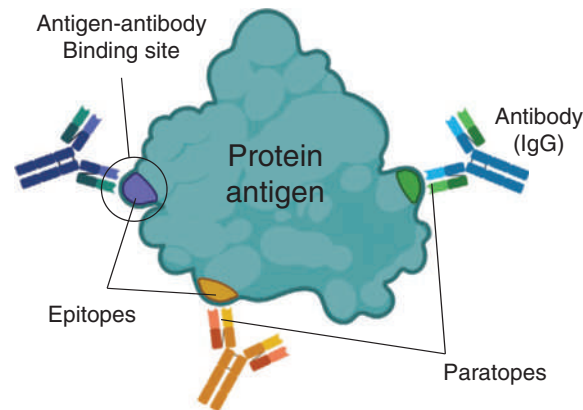


Fig. 1 Diagram of antigen-antibody recognition. Antigens can have multiple epitopes that can be recognized by specific antibody. The antigen-antibody interaction take place between the epitope of the antigen and paratope of the antibody.

Antigens can have several distinct 3D structural patterns on their different surface areas. Each pattern is called an antigenic determinant, or epitope, defined as the portion of the antigen recognized by an antibody or T cell. Each epitope, which is usually made of 6 to 20 amino acids or 1–6 sugars in a polysaccharide, determines the specificity of the antigen-antibody reaction, as usually the antibody binding site cannot accommodate the whole antigen. Thus, the immune system recognizes specific epitopes of the antigen (Fig. 1). There may be multiple epitopes per molecule (Baumhüter et al., 1987; Sahin et al., 2017). Some epitopes are more immunogenic than others. Complex antigens can evoke responses from a variety of specific lymphocytes. Also, it is possible for two or more different antigens to have an epitope in common. In these cases, the immune response induced by one antigen is capable of reacting with the other antigens (cross-reacting antigens) carrying the same or a closely homologous epitope (Wilson et al., 2001; Yewdell et al., 1985). However, in some cases, certain antibodies can distinguish native vs denatured conformational forms of the same protein because they recognize a particular 3D conformation, not just a linear amino acid sequence (e.g., myoglobin, gp160 protein of HIV) (Wrapp et al., 2020; Gorny et al., 2002; Montefiori et al., 1993).

The initiation of durable antigen-specific immune responses requires the activation of T and B lymphocytes, given that they are the only cells able to recognize and respond specifically to each antigenic epitope. T cells recognize antigens through the T-cell receptor (TCR) on their surface, after the antigen has been processed into small fragments and presented on major histocompatibility complex (MHC) molecules (class I, class II, or MHC-like molecules, CD1 molecules, in case of lipids, and MR1 molecule, in case of Vitamin B metabolites or precursors) by antigen presenting cells (APCs), such as dendritic cells (DCs), macrophages, and B cells (Fig. 2; Germain and Margulies, 1993; Chandra and Kronenberg, 2015; Cohen et al., 2009). These T cells either mediate effector functions directly as killer T cells or go on to activate effector cells, as helper T cells activate B-cells or cytotoxic T cells. B cells are activated when their B cell receptor (BCR) on their surface binds to either soluble or membrane-bound antigens. B cells have the ability not only to transform into plasmocytes to produce antibodies but also to act as APCs, although DCs are the primary professional APC (Steinman, 1991; Banchereau and Steinman, 1998). The humoral immune response depends on the B cells while the cellular immune response depends on the T cells (Planz et al., 1997). The capacity of antibodies to recognize specific antigen epitopes resides in their functional binding sites or *paratopes* (Fig. 1). Thus, paratopes on the B cells are the same as those of the antibodies produced from that cell, as these antibodies are displayed in a transmembrane form on the B cell surface. In contrast, paratopes on the TCRs are distinct from those of antibodies, and generally require recognition of a complex of peptide antigen bound to an MHC molecule. T cell and B cell epitopes and their properties will be discussed in greater detail later in this chapter.

Antigen presenting cells

As previously mentioned, T lymphocytes, unlike B lymphocytes, require antigen to first be processed into short peptides and displayed on the host cell's surface as a peptide-MHC complex, which is recognized by alpha-beta TCRs. Complete activation of naive T lymphocytes requires recognition of the appropriate MHC complex with antigen, but additional signals from costimulatory molecules and cytokines produced by the presenting cell are also required (Mueller et al., 1989; Hemmer et al., 1998). MHC class I molecules are expressed on the surface of all somatic cells, except for red blood cells, and present antigen to CD8⁺ T cells. Therefore, any host cell can present antigen to cytotoxic CD8⁺ T lymphocytes. In contrast, MHC class II molecules are expressed primarily on immune cells, in particular DCs, macrophages, and B cells, and present antigen to CD4⁺ T lymphocytes. These immune cells are considered professional APCs and are important for bridging the innate and adaptive immune responses. Thymic epithelial cells and activated T cells (in humans, but not in mice) also constitutively express MHC class II molecules, while other cell types can express MHC class II in response to interferon gamma (Piskurich et al., 1999). Because of the restricted expression of MHC class II molecules, CD4⁺ T lymphocytes mainly recognize their cognate antigen presented by a limited number of professional APCs, the most prominent being DCs (Banchereau and Steinman, 1998).

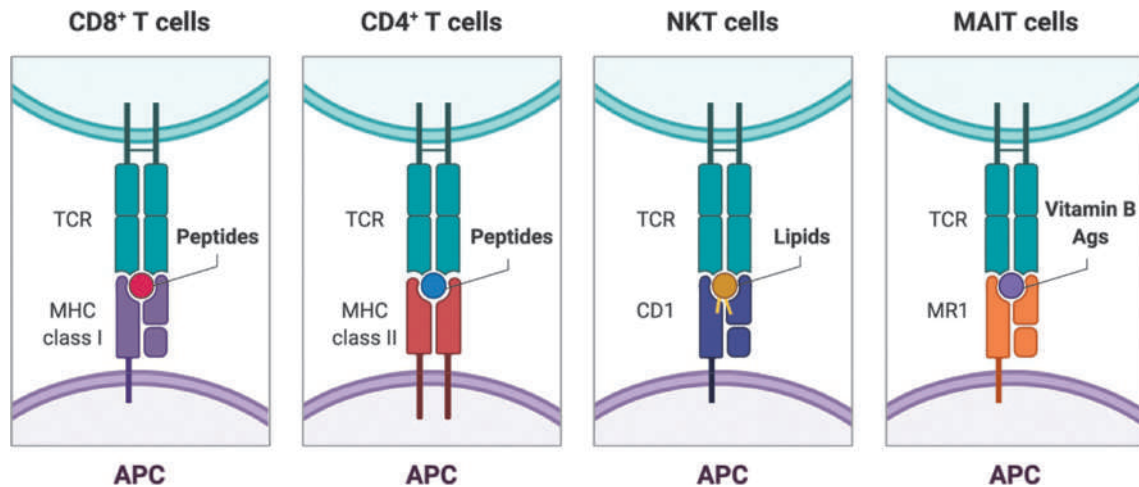


Fig. 2 Antigen presentation by APCs. Antigen presenting cells, including DCs, macrophages, and B cells, can process and present different antigens on their surface that specifically activate the distinct subsets of T cells that express antigen-specific TCRs. The specificity of this activation depends on the type of antigen, the ability of APCs to process the antigen and the MHC molecules used to display the antigen on their surface. For example, intracellular proteins are degraded in proteasome and translocated via the transport associated with antigen processing (TAP) protein into the endoplasmic reticulum (ER) lumen and loaded onto MHC class I molecules. Then they are transported to the plasma membrane to present the antigenic peptide to CD8⁺ T cells. In contrast, endocytosed extracellular proteins are processed in endosomal-lysosomal compartments and loaded onto MHC class II molecules with help of the dedicated chaperone molecule (HLA-DM in human and H2-M in mice) before the complex is transported to cell surface to activate antigen-specific CD4⁺ T cells via TCRs. However, there is also cross-presentation between these two pathways, where extracellular proteins are presented by MHC class I molecules and intracellular proteins are presented by MHC class II molecules. In addition, lipid antigens can be processed and/or presented by MHC class I-like CD1 molecules (CD1a, CD1b, CD1c, CD1d, and CD1e in human and only CD1d in mice) with the help of microsomal triglyceride-transfer protein (MTP) and different lipid-transfer proteins to activate NKT cells. Moreover, mucosal-associated invariant T cells (MAIT cells) can recognize metabolites or precursors of vitamin B (6-formylpterin, or 6-FP, a metabolite of vitamin B9, and several intermediates of the microbial biosynthesis of riboflavin, or vitamin B2) through MR1 molecules, a non-polymorphic MHC class I-like protein, presented on the surface of APCs.

In addition to the differences in constitutive expression of MHC molecules, how antigens are processed and loaded onto MHC molecules also differs. Antigens that are presented via MHC class I molecules are derived from the host cell's cytosol. Here, they are first ubiquitinated and degraded by the proteasome before being transported to the endoplasmic reticulum and directly loaded onto the MHC class I molecule (Basler et al., 2013). This pathway is also known as direct presentation and functions in all nucleated cells, serving as the mechanism for infected host cells to display antigens from intracellular viruses and bacteria to cytotoxic T lymphocytes. In addition, DCs also have the unique capacity to present exogenous antigens not derived from the cytosol via MHC class I molecules in a process called cross-presentation (Ackerman and Cresswell, 2004). For loading peptides onto MHC class II molecules, professional APCs capture exogenous proteins through endocytic vesicles and specific cell-surface receptors (Li et al., 2005). For B lymphocytes, antigen binds the surface receptor (which matches the immunoglobulin secreted by that B cell) and is internalized by receptor-mediated endocytosis. Macrophages and DCs, on the other hand, mainly internalize antigen by phagocytosis and non-specific macropinocytosis. Peptides generated from internalized antigens are loaded onto MHC class II molecules in modified endocytic compartments within APCs (Adorini et al., 1991). MHC class II molecules can also be loaded with peptides from cytosolic proteins by a process known as autophagy. This ubiquitous pathway serves to present self-cytosolic proteins to induce tolerance of self-antigen as well as to present peptides from pathogens found in the host cell cytosol (Dengjel et al., 2005). In DCs, the MHC class II processing pathway is largely regulated during the maturation process. Immature DCs continuously recycle and degrade MHC class II molecules, but upon maturation the molecules become more stable on the cell surface to allow for prolonged antigen presentation, along with increased expression of costimulatory molecules (Villadangos and Schnorrer, 2007).

Pattern recognition receptors

The human body has several barriers in place to prevent microbial pathogens from entering the body, including physical barriers like the skin and chemical barriers such as antimicrobial enzymes in saliva. If bacteria or viruses breach these barriers, host cells expressing receptors known as pattern recognition receptors (PRRs) are responsible for early detection and elimination of the pathogen. In addition to being constitutively expressed by phagocytic immune cells, like DCs, macrophages, and granulocytes, PRRs are also expressed on non-immune cells like intestinal epithelial cells and fibroblasts (Akira et al., 2006). PRRs were first hypothesized by Janeway as a connection between the innate immune response and the initiation of the adaptive immune response (Janeway, 1989). Unlike the variable antigen receptors of T and B lymphocytes, PRRs are encoded in the germline and are not modified via somatic recombination. Instead, PRRs recognize highly conserved molecular structures that are common to pathogens

called pathogen associated molecular patterns (PAMPs). Examples of PAMPs include lipopolysaccharides of gram-negative bacteria, lipoteichoic acid of gram-positive bacteria, peptidoglycan, double-stranded RNA, and flagellar proteins like flagellin, among many others. These antigens are considered essential for pathogen survival and in most cases represent unique molecular characteristics that are not found in host cells, allowing host cells to distinguish generically non-self-antigens (Janeway and Medzhitov, 2002). In addition to PAMPs, PRRs can also recognize proteins and metabolites released by apoptotic and damaged senescent host cells called damage-associated molecular patterns (DAMPs) (Bianchi, 2007).

There are several distinct classes of PRRs based on their protein domain homology, including the Toll-like receptor (TLR) family (O'Neill, 2008), the nucleotide-binding oligomerization domain-like receptors (NLR), the absent in melanoma-2 (AIM2)-like receptors (ALRs), the retinoic acid-inducible gene 1-like receptors (RLR), and the C-type lectin receptors (CLR). Each PRR family differs in the antigens that they recognize, their signal transduction pathways, and their localization within the host cell. PRRs are not only expressed on the cell membrane but are also widely distributed throughout intracellular compartments like the endoplasmic reticulum, endosome, lysosome, or within the cytoplasm. The TLRs were the first family of PRRs to be described and are classical transmembrane proteins that detect PAMPs derived from extracellular bacteria or bacteria taken into vesicular pathways by phagocytosis, or viruses (Akira and Takeda, 2004). The NLR and RLR families are soluble proteins found in the host cell cytoplasm that are important for sensing intracellular pathogens (Creagh and O'Neill, 2006). Downstream signaling pathways are induced when a PRR binds its ligand. This results in a myriad of cellular responses that act to eliminate or control the detected pathogen, some of which include production of inflammatory cytokines and other immune mediators, inducing inflammation, facilitating the elimination of dead cells, and coordinating downstream antigen-specific adaptive immune responses (Pulendran et al., 2001). However, one of the most important functions of PRRs is on DCs, where they serve to mature and activate the cells to be more effective APCs as well as to secrete cytokines and chemokines. Thus it is primarily through DCs that PRRs, especially TLRs, serve to alert the immune system to the presence of pathogens.

Factors influencing immunogenicity

There are a wide number of factors that can impact immunogenicity of antigens. However, it is ultimately determined by the interaction between the properties of the antigen and physiological state of the host. Properties of the immunogen include its foreignness, molecular size, chemical composition and heterogeneity, and the ability of the antigen to be processed and presented by an MHC molecule on the surface of an APC or altered self-cell. The host factors such as self-tolerance, the production of cytokines, and various cellular and regulatory mechanisms can impact the immunogenicity of the antigen. In addition, the quality of the immune response depends on both the amount of the antigen and the route of entry into the body as well (Morgan et al., 1999; de Visser et al., 2000; Kreuwel et al., 2002; Cook et al., 1998). Moreover, the immune system needs an adequate time of exposure to the antigen, so, for example, antigens that degrade quickly may not be able to induce strong immune responses. Also, temperature, pH, and other factors contribute to antigen-antibody interaction and strength of the immune response. Below we discuss the nature of the antigen and major *intrinsic* factors that are known to impact antigen immunogenicity.

Foreignness of the antigens

As mentioned earlier, to induce an immune response, an antigen must be recognized as non-self or foreign by the host. In general, our immune system is tolerant to molecules that are recognized as self-molecules. The degree of foreignness (or non-self) is directly proportional to the degree of immunogenicity. That is, generally the greater the phylogenetic distance between two species, the greater the structural differences, and more immunogenic the substance is (Berzofsky, 1990). In accord with this concept, some antigens are highly conserved evolutionarily across diverse species and therefore display very little immunogenicity. Based on their foreignness, antigens can be classified as: (i) *autologous antigens* (self-antigens, for which there will be no immune response because the immune system develops a tolerance to self-antigens with the exception of autoimmune disorders); (ii) *allogeneic antigens* (antigens from other members of the same species, for which there may be an immune response, for example blood transfusion antigens, organ transplants); and (iii) *heterologous or xenogeneic antigens* (antigens from different species, against which there will likely be a strong immune response).

Molecular size of the antigens

Correlation exists between the molecular size or weight of the antigen and its immunogenicity, such that the higher the molecular weight, the more likely the molecule will be immunogenic, all else being equal. The strong immunogens tend to have a molecular weight of ~100,000 daltons, whereas molecules less than 5000–10,000 daltons are usually less immunogenic with some exceptions. The number of epitopes is generally directly proportional to the size of the antigen. Larger molecules are easily phagocytosed, processed, and presented with multiple epitopes. Also, the more epitopes, the more likely that some of them will be presented by the MHC molecules of the host. Conversely, genetic non-response to certain antigens (so-called *Ir* genes) have been generally seen for small antigens with limited epitopes that may not be presented by a given MHC haplotype (Matis et al., 1982; Heber-Katz et al., 1983; Berzofsky et al., 1977; Berzofsky, 1978; Katz et al., 1982).

Chemical composition and heterogeneity of the antigens

Immunogenicity of the antigen depends upon the chemical structural complexity. Among the biological macromolecules, proteins and carbohydrates (polysaccharides) are the most investigated macromolecules. Other macromolecules, such as nucleic acid and lipids are also antigenic, but poor immunogens. Glycoproteins, nucleoproteins, lipoproteins, peptidoglycans, glycolipids, and other conjugates are also antigens. The more complex chemical structures are generally more potent immunogens than simple chemical structures. For example, homopolymers (polymers composed of a single amino acid or sugar) tend to have poorer immunogenicity regardless of their size. In contrast, copolymers of sufficient size, composed of two or more different amino acids or sugars, are usually more immunogenic than their homopolymer constituents (Cease et al., 1986). The structural stability of the antigens is essential and always needed. If the structure is unstable and degraded easily, then it will be a very poor antigen.

Protein antigens

Most proteins are probably powerful immunogens because of their high molecular weight and structural complexity; however, different proteins may differ in their immunogenicities. All four levels of protein structure (primary, secondary, tertiary and quaternary) contribute to the structural complexity of a protein and hence affect its immunogenicity (see below). Denatured primary structures of proteins are often less immunogenic than the corresponding tertiary or quaternary structure of proteins (Berzofsky, 1985).

Carbohydrate antigens

Carbohydrate antigens are polysaccharides and glycoconjugates of multiple structural configurations. Based on their origins, carbohydrate antigens are divided into microbial antigens and alloantigens (red blood cell surface antigens are alloantigens). Carbohydrates can be strong immunogens by themselves as complete-antigens, or they can be components or antigenic determinants of glycoconjugates (Ishioka et al., 1992; Jensen and Brask, 2005). Antigenic determinants of polysaccharides are usually sequential, consisting of 4–6 sugars. Microbial polysaccharides are located on the cell surface (for example, capsular polysaccharides of pneumococci) and such location is important in immune recognition. Complex lipopolysaccharide antigens (endotoxins) are found in outer membranes of a large variety of microorganisms, especially in gram-negative Enterobacteriaceae, such as *E. coli*, *Salmonella*, and *Shigella* (Mata-Haro et al., 2007; Vatanen et al., 2016; Shi et al., 2014).

Nucleic acid antigens

Nucleic acid molecules, both DNA and RNA, are poor immunogens by themselves. However, when combined with suitable protein carrier molecules they can induce humoral immune responses and produce antibody with high specificity. Microbial pathogens can be recognized by multiple PRRs, including TLRs. Nucleic acids can be recognized by TLRs and induce strong immune responses (TLR3 for dsRNA, TLR7 for ssRNA, and TLR9 for dsDNA) (Kim et al., 1998).

Lipid antigens

Lipid molecules are not soluble in water and poorly immunogenic by themselves. However, lipids are usually associated with cell membranes or lipid-binding proteins in the body. Lipids can be immunogenic when they are presented by an MHC-like molecule, CD1, to which lipid binds, on the surface of APCs and stimulate NKT cells (Prigozy et al., 2001). To date, several lipid antigens of microbial and self origin have been identified (Shamshiev et al., 1999; An et al., 2014; Brennan et al., 2011; Kawano et al., 1997; Kinjo et al., 2005; Mattner et al., 2005; Sriram et al., 2005).

Haptens

Haptens are an additional structural category of antigens that can have any of the above molecular structural compositions, peptide, carbohydrate, nucleic acid or lipid, or even small organic molecules. What makes them haptens is that they are low molecular weight compounds that are capable of specifically binding to an antibody but cannot themselves initiate antibody production; in other words, they are antigenic but not immunogenic. Haptens were first described in the 1930s by Karl Landsteiner, who conducted numerous animal studies to characterize their nature (Landsteiner and Van der Scheer, 1934; Landsteiner and Jacobs, 1935). Much of what was first learned about how antibodies bind to antigen and the variability of antibodies is based upon initial studies of antibodies binding to haptens (Pauling et al., 1941; Eisen and Siskind, 1964). Haptens are unique from antigens in that to be immunogenic they must be covalently linked to a carrier molecule, like a protein. It is only when haptens are conjugated to a carrier molecule that they can directly activate B lymphocytes by cross-linking of multiple surface B cell receptors (Boak et al., 1971; Abbas et al., 1985). Furthermore, it is unlikely that haptens alone are capable of binding to MHC molecules or the T cell receptor directly. The carrier protein provides a source of epitopes to induce CD4⁺ T cell help. Instead, the hapten-carrier conjugate must be linked so the components get into the same DC, which can process and present helper epitopes from the carrier to helper T cells, and the hapten can be presented to B cells that are proximal to the same DC to receive the help. There are also T cells that can recognize a complex epitope consisting of a combination of MHC molecule loaded with a carrier peptide to which the hapten is attached. For example, T cells that see glycopeptides with a sugar moiety (Huang et al., 1996). The peptide conjugate can act to anchor the hapten within the MHC binding groove during loading onto the MHC molecule (Ortmann et al., 1992; Martin and Weltzien, 1994). A similar phenomenon has been described for CD8 T cells instead of B cells, in that the class II MHC-restricted helper epitope needs to be physically associated with or covalently linked to the class I MHC-restricted CTL epitope for immunogenicity (Shirai et al., 1994).

Unintended coupling of a hapten to peptide is responsible for numerous pathologies, including the allergic reaction many people experience to penicillin and other beta-lactam antibiotics. This occurs when the drug is metabolized into prohaptens that can bind to endogenous proteins and elicit deleterious immune responses directed toward conjugated self-proteins (Garratty, 2009; Chung et al., 2016). Hapten-specific T cells are also responsible for inducing skin pathology in contact hypersensitivity reactions to the toxin urushiol in poison ivy, which reacts with host proteins to form a coupled hapten. Many other molecules including metal ions like nickel, industrial chemicals, and even cosmetic additives like fragrance or lanolin, have been shown to illicit adverse immune responses due to hapten-coupling (Dumycz et al., 2019). The mechanisms by which haptens interact with endogenous proteins to induce pathology are still currently being characterized. Most haptens are electrophilic molecules that covalently bind to nucleophilic residues on endogenous proteins to create a new antigenic epitope (Chipinda et al., 2011). It is still unknown, however, if certain self-proteins are more prone to interact with haptens or prohaptens than other proteins. It has also been suggested more recently that haptens could potentially bypass the classic MHC-TCR pathway to directly stimulate T lymphocytes (Pichler, 2005). This new pathway, called the pharmacological interaction with immune receptors concept (or p-i model), suggests that certain metabolites from drugs could bind specifically to antigen receptors directly, acting much like a super antigen. This model proposes that drug metabolites may not be able to activate naïve T lymphocytes directly, but if a hapten were to bind to primed T lymphocytes, which have a lower antigen threshold, stimulation could occur (Pichler et al., 2006).

Ability of the antigen to be processed and presented by APCs

The development of both humoral and cellular immune responses requires interaction of T cells with antigen that has been processed into small fragments within cells, loaded onto an MHC molecule and presented on the surface by APCs (Germain and Margulies, 1993; Crotty, 2011). Recognition of antigen involves close contact binding of the TCR on the surface of T cells and the antigen-fragment-MHC expressed on the surface of APCs, as described earlier in this chapter. Antigens that cannot be phagocytosed, processed, and presented with MHC molecules are poor immunogens. Large, insoluble macromolecules are usually more immunogenic compared to small, soluble molecules because they not only potentially contain multiple epitopes but also are phagocytosed more readily and processed easily to induce immune responses (Berzofsky, 1985; Cease et al., 1986).

Based on their origin, antigens can also be classified as *exogenous* or *endogenous* antigens, where the exogenous antigens enter the cell from the outside (through endocytosis) and endogenous antigens are generated inside the cell as part of cell metabolism or pathogenic infection within the cell. Exogenous antigens phagocytosed and endosomally processed by APCs and presented on MHC class II molecules activate CD4⁺ helper T cells to activate APCs and secrete cytokines to activate B cells, cytotoxic T cells, and macrophages (Berzofsky et al., 1986). In contrast, endogenous antigens are processed in the cytosol and transported to the endoplasmic reticulum where they are loaded onto MHC class I molecules to activate CD8⁺ cytotoxic T cells, which release toxins like granzymes and perforin that induce apoptosis or lysis of the infected cells (Seder and Ahmed, 2003).

To improve immunogenicity, Immunologists have employed what Charlie Janeway (Janeway, 1989) called the immunologists' "dirty little secret," namely adjuvants, as described in the next section.

Adjuvants

Adjuvants are substances that act to enhance the immune response against an antigen and are used to augment and prolong the immunogenicity of vaccine formulations. This phenomenon was first described by a French veterinarian working on the diphtheria and tetanus vaccine in the 1920s. Gaston Ramon observed that vaccinated horses developed a stronger immune response if they also experienced inflammation at the site of injection. He went on to inject substances like starch and breadcrumbs to induce sterile abscesses where he also injected inactivated toxin, which increased the antigen-specific antibodies in serum (Di Pasquale et al., 2015). The molecular mechanisms by which adjuvants work are still not fully understood and most adjuvants use more than one mechanism of action to enhance the immunogenicity of the substance they are administered with. The formation of an injection site depot was the first mechanism of action described for adjuvants. The depot effect involves antigen trapping at the site of injection and ensures slow release and prolonged stimulation of the immune system. The depot effect is thought to be one of the mechanisms of action of many alum-based adjuvants, which are the most widely used adjuvants today, as antigen has been detected for up to 3 weeks in alumina gel granulomas (Awate et al., 2013). After PRRs were first described in the 1990s (Janeway, 1989), the connection between innate cell signaling and the downstream activation of adaptive immune cells became apparent. Today, most adjuvants use bacteria, bacterial or viral components, or mimics such as TLR ligands to enhance immunogenicity through PRR signaling, though the exact molecular mechanisms are still being elucidated. Potential mechanisms of action include regulating cytokines and chemokines, increasing cellular recruitment at the site of injection, enhancing antigen uptake by APCs and their migration to draining lymph nodes, or by activating inflammasomes (Coffman et al., 2010; Pulendran et al., 2021).

Despite nearly a century of research on adjuvants, very few have been approved for use in humans. The FDA says it does not approve adjuvants on their own, but only as part of a specific vaccine formulation. In 1932, aluminum became the first adjuvant to be used in a human vaccine and for 70 years was the only adjuvant product in vaccines licensed for human use. To date there have been only five other adjuvant systems approved for use in the United States. In the 1990s, an oil-in-water emulsion adjuvant called MF59 was introduced as part of a trivalent inactivated vaccine against seasonal influenza. Complete Freund's adjuvant, a water-in-oil emulsion that includes killed mycobacteria is widely used in animal studies but has not been approved for humans because of

injection-site granulomas. It has both the depot effect of trapping the aqueous phase in oil, and the TLR ligands of the mycobacteria. More recently, the adjuvants AS01 and AS04 have been included in vaccine formulations for shingles, malaria, hepatitis B, and human papilloma virus. The adjuvant cytosine phosphoguanosine (CpG) 1018 was approved as part of a hepatitis B vaccine, as CpG oligonucleotides mimic bacterial DNA which is richer in these sequences than mammalian DNA, and the CpG oligonucleotides have been shown to bind to TLR9. Most of the approved adjuvants work as part of a system, with more than one adjuvant substance included in the formulation. For example, AS01 is a liposome-based adjuvant that contains two different immunostimulants: monophosphoryl lipid A (a ligand of TLR4, representing a component of bacterial lipopolysaccharide (LPS) that itself may be too toxic), and QS-21, a natural compound from the Chilean soapbark tree that provides the depot effect and helps transport molecules across cell membranes (Didierlaurent et al., 2014). Also, QS21 in nanoparticles called ISCOMs was found to permit large soluble proteins to enter the cross-presentation pathway and stimulate CD8+ T cells (Takahashi et al., 1990). There have been numerous other formulations used experimentally or tested in clinical trials, but most have been deemed unsafe due to severe side effects or tolerability concerns. For instance, in the early 2000s an intranasal HIV gp120 vaccine using the adjuvant LTK63, a mutant of *Escherichia coli* labile toxin that is an effective mucosal adjuvant (Belyakov et al., 2000, 2001), was pulled from use when several study participants in the trial developed transient facial nerve paralysis (Bell's palsy) (Lewis et al., 2009). Another widely used adjuvant is poly I:C, a double stranded RNA that mimics double-stranded RNA viruses and binds TLR3. This is the only TLR ligand that signals through TRIF, rather than MyD88, so it has been found to be synergistic with some others like TLR 9 and TLR2/6 ligands that signal through MyD88 (Zhu et al., 2008; Sui et al., 2011). Further, targeting TLRs and NLRs simultaneously has also been found to be synergistic in improving an anti-tumor immune response (Garaude et al., 2012; Berzofsky, 2012).

Adaptive immune cell epitopes

For adaptive immune responses, the antigenic epitopes must be recognized by the receptors of T cells or B cells. Thus, it is important to be able to characterize those epitopes and map them within the larger structure of an antigen molecule, as discussed below.

Properties of B-cell epitopes

Antibodies made by B lymphocytes can recognize all classes of antigens, such as proteins, lipids, nucleic acids, and carbohydrates, as well as haptens, as discussed earlier in this chapter. Much early work was done on the structure of carbohydrate epitopes (Kabat, 1978, 1960), including work that helped map the size and shape of the antibody combining site by defining the limits of the carbohydrate size that could fit (Kabat, 1960). However, most structural work in recent years has focused on protein antigens.

Globular proteins have at least 4 levels of structure: primary, secondary, tertiary and quaternary, and many are also glycosylated or otherwise post-translationally modified, creating additional epitopes. Primary structure is just the amino acid sequence. Although primary structure is critical for antibody recognition and can determine specificity, it rarely acts alone as such sequences usually take on some secondary or higher structure. Secondary structure is the local folding of the amino acid sequence, in the form of alpha helices, beta sheets, and many less defined structures. An antibody will certainly distinguish different secondary structures of a given amino acid sequence. As proteins breathe, that is expand, contract and flex in solution, some conformations may bind to an antibody with higher affinity than another conformations (Sachs et al., 1972; Furie et al., 1975). Conversely, the antibody thus can shift this conformational equilibrium toward one of those conformations to which it has higher affinity (Crumpton, 1974; Lando et al., 1982; Dean and Schechter, 1979). These epitopes can therefore be called "conformational epitopes" even though they exist as a continuous amino acid sequence because the antibody recognition depends on the protein conformation.

Tertiary structure, the three-dimensional folding of the protein, can create another more complex type of conformational epitope, by bringing in close proximity regions of amino acid sequence that are far apart in the linear sequence. When such regions fall within the radius of recognition of an antibody binding site, the antibody recognition can depend on a combination of sequence segments brought together by the folding that are juxtaposed only in the tertiary structure, and thus completely dependent on such tertiary structure. We have called these "assembled topographic epitopes" because they are assembled from disparate portions of the amino acid sequence and brought together by its folding (Benjamin et al., 1984). They are also sometimes just called "discontinuous" or conformational epitopes, but these should be distinguished from conformational epitopes that depend only on secondary structure, as the latter can occur even in short peptides that oscillate among different secondary structures (Benjamin et al., 1984; Getzoff et al., 1987, 1988; Van Regenmortel, 1987).

When we look at the surface of most globular proteins and examine the radius of an antibody combining site that could sit on the surface, we usually find that the antibody would very likely span segments of the amino acid sequence that come from different regions of the linear sequence (Barlow et al., 1986), in other words, assembled topographic epitopes (Berzofsky, 1985). Thus, in fact, most epitopes of intact globular proteins may be of this assembled topographic type.

Quaternary structure is the assembly of multiple subunits of a multi-subunit protein. These can be heterodimers/trimers, like the alpha-beta-gamma subunits of the IL-2 receptor, or they can be homodimers, trimers or tetramers. One example is hemoglobin, which forms a tetramer of four chains that can each bind oxygen. Antibodies that favor the oxygen-binding conformation of one subunit can shift the equilibrium of the others to promote oxygen binding by all 4 subunits (Dean and Schechter, 1979). However, these antibodies generally bind one subunit at a time. It is relatively rare to find antibodies that require quaternary structure because the epitope bridges between or spans across more than one subunit. That lack of examples, however, may relate to the relative lack of experiments to detect such quaternary structural epitopes.

Properties of T cell epitopes

In contrast to antibody epitopes, T cell epitopes, at least for conventional CD4⁺ and CD8⁺ alpha-beta TCR T cells, are short peptides presented by class II or class I MHC molecules, respectively (Rötzschke et al., 1990; Germain and Margulies, 1993; Davenport et al., 1997). Therefore, there is no issue with conformational epitopes or assembled topographic epitopes. Only the primary structure needs to be considered for the most part.

In the case of CD8⁺ T cell recognition of peptides presented by class I MHC molecules, like HLA-A, B and C in humans and H-2K, H-2L, and H-2D in mice, the peptides must fit in the groove of the MHC molecule, usually with the N and C termini fitting into pockets at either end of the groove, and anchor residues fitting in pockets in between. The outside pockets binding the terminal amino acids constrict the length of the peptide, generally between 8 and 11 residues, with the majority being 9 or 10 residues long. The other pockets define what type of anchor amino acid side chains can fit in a particular MHC molecule, and thus determine which peptides can be presented by that MHC molecule. These primary anchor residues, as well as secondary anchors and other positions at which side chains can interfere, define a sequence “motif” that is predictive of peptides that can bind to a particular class I MHC molecule. Such motifs were first discovered by Rammensee and colleagues for HLA-A2-binding peptides (Rötzschke et al., 1990; Falk et al., 1990) and by Maryanski, Romero and Corradin for H-2Kd binding peptides (Maryanski et al., 1990; Romero et al., 1991). Rammensee’s group sequenced peptides eluted from MHC molecules, and since in class I molecules they were all aligned starting with the N-terminal residue fitting in a pocket, they could see a pattern that certain positions were highly variable, whereas other positions were conserved among peptides binding to a particular HLA molecule. For example, the first one extensively studied was HLA-A2, the most common class I HLA molecule in humans at least of European descent. They found that the second position and C-terminal position were conserved and virtually always had a hydrophobic aliphatic side chain such as Leucine, Valine, or Isoleucine, or sometimes Methionine. Such motifs were later refined by Sette and coworkers (Ruppert et al., 1993), who examined the effects of different residues at other positions. Such motifs have become the basis of many predictive algorithms for locating CD8⁺ T cell epitopes (see below).

For class II MHC molecules, presenting peptides to CD4⁺ T cells, the situation was more complex because the ends of the peptide could stick out from the ends of the groove, so the peptides were not all aligned from the same starting point. That made it more difficult to identify motifs. Nevertheless, motifs for different class II MHC molecules, such as HLA-DR, DQ and DP in humans and I-A and I-E in mice, have been characterized (Rammensee et al., 1995a). These allow predictions, but perhaps not yet as reliably as for class I MHC molecules.

We also found that non-anchor residues, if too bulky, could interfere with binding by steric hindrance and thus reduce affinity even when appropriate anchors were present (Boehncke et al., 1993). In that case, replacing such a bulky residue with a small alanine (with just a methyl group as side chain) removed the steric hindrance and increased the binding affinity. We conclude that all the residues in the amino acid sequence need to be considered, not just the anchors. Further, based on these observations, we formulated the concept of “epitope enhancement” in which we can modify the amino acid sequence of the epitope to increase affinity for the MHC molecule and thus increase immunogenicity (Berzofsky, 1995; Berzofsky et al., 2001). Sette et al. had shown a strong relationship between peptide affinity for MHC molecules and immunogenicity of the epitope (Sette et al., 1994). The idea is that if certain amino acid residues in the peptide interact exclusively with the MHC molecule and others exclusively with the TCR, then one could modify the former ones to improve affinity for the MHC molecule through either anchor improvements or replacement of interfering residues, without affecting what the TCR could see when it looks down on the peptide-MHC complex and sees the MHC molecules with certain peptide residues exposed out of the groove. Of course, this is overly simplistic, as some residues might interact with both MHC and TCR, and even the residues that interact exclusively with the MHC molecule could still affect the conformation of the part of the peptide exposed from the groove, and thus affect the fit into the TCR. Since the goal is to make a more immunogenic peptide as a vaccine that still elicits T cells that “see” the natural pathogen or cancer antigen, which is not altered, the epitope enhancement process must not alter the specificity of the T cells elicited. In practice, many epitope-enhanced peptides can elicit T cells that react with the natural unmodified sequence, but some do not and have to be screened out empirically by immunization studies. Also, some T cells elicited may crossreact with the wild type sequence and some may not, and the latter would be wasted. Despite all these caveats, the method has been used successfully to improve epitopes from hepatitis C virus, HIV, and other viruses, and cancer antigens (Sarobe et al., 1998; Ahlers et al., 1997; Oh et al., 2004; Huang et al., 2013). At least one of these, from the prostate cancer antigen TARP, has been used successfully in phase I clinical trials and shown evidence of clinical benefit (Wood et al., 2016).

This concept of epitope enhancement has been extended by Kosmatopoulos et al (Tourdot et al., 2000; Gross et al., 2004) in a very interesting idea: T cells specific for the most dominant high affinity cancer antigens (see immunodominance below), may have been tolerized by thymic deletional tolerance or other tolerance mechanisms as self, and thus not be induced by the corresponding cancer antigen in vaccines or in the tumor. However, lower affinity cancer peptides, defining subdominant epitopes, may not have induced tolerance but may be of too weak affinity to elicit a protective response either. The idea is that these weaker epitopes could be strengthened by epitope enhancement to gain higher affinity and thus greater immunogenicity, to be used in vaccines to expand non-tolerized T cells that could then target the cancer. They demonstrated the ability to do so in mouse cancer models.

There are also a set of alpha-beta TCR T cells that recognize non-peptide antigens presented by non-classical MHC like molecules, often using semi-invariant TCRs, as well as gamma-delta TCRs (Godfrey et al., 2015). For example, type I or invariant NKT cells (distinct from NK cells that lack a TCR) recognize lipid antigens presented by the class IB MHC molecule CD1d (Kronenberg and Gapin, 2002; Brutkiewicz et al., 2003; Godfrey et al., 2004; Bendelac et al., 2007; Terabe and Berzofsky, 2008,

2016; Brennan et al., 2013). These provide the T cell immune system a way to detect lipid and glycolipid antigens, which would be invisible to classical peptide-specific T cells. CD1d is the only CD1 molecule in mice, but in humans there are others, CD1a, b and c, which each present different lipids and glycolipids to T cells, for example from mycobacteria (Vincent et al., 2003; Cohen et al., 2009). Another set of semi-invariant TCR T cells that see non-peptide antigens are the Mucosal-Associated Invariant T (MAIT) cells, that see primarily riboflavin precursors, largely from bacteria, presented by the MR1 molecule (Kjer-Nielsen et al., 2012; Koay et al., 2019). Thus, although conventional T cells focus on protein antigens, the adaptive cellular immune system has evolved other T cells, perhaps even more primitive, that allow detection of other types of structures. Another case in point is the ability of even conventional T cells to detect carbohydrate moieties as haptens on peptide epitopes, which thus alter the specificity (Ishioka et al., 1992; Huang et al., 1996). The T cells that see the glycosylated peptide do not crossreact with the non-glycosylated peptide of the same amino acid sequence presented by the same MHC molecule.

Epitope mapping

B cell epitope mapping

The conformational nature of most protein B cell (antibody) epitopes makes epitope mapping difficult. One widespread method is to measure antibody binding to a series of overlapping synthetic peptides covering the whole protein sequence. The problem is that this is limited to detecting epitopes that exist in relatively short peptides, and in the conformation that the peptides can easily adapt when separated from the native protein, and when bound to various media like a microtiter plate. Assembled topographic epitopes would be completely missed by this method.

A second method is to make point mutations in different locations within the intact folded protein to see which ones interfere with antibody binding (this assumes one is dealing with a monoclonal, monospecific antibody). Originally this approach was carried out using natural variants such as myoglobins or lysozymes from different species with known differences in amino acid sequence (Berzofsky et al., 1982; Benjamin et al., 1984). When recombinant methods such as site-directed mutagenesis became available to make point mutations, that became a more widely applicable approach, and this has now become greatly facilitated by use of CRISPR/Cas9 technology (Jinek et al., 2012; Hsu et al., 2014; Ran et al., 2013).

A third method is to carry out competition studies with a monoclonal antibody whose epitope has already been mapped (Streicher et al., 1986). The presumption is that an antibody that competes must be binding in the same region of the protein as the known antibody to mediate steric hindrance. That may define epitopes that are overlapping, but not necessarily identical. Similarly, one can examine antibodies that compete for antibodies known to neutralize a virus. Of course, there are exceptions when allosteric effects on conformation can allow one antibody to change the conformation of the protein antigen in a way that affects binding of a second antibody at a distance. This same caveat also applies to the point mutation method, so one has to have corroborative data to confirm the interpretation.

T cell epitope mapping

T cell epitopes are much easier to map with overlapping peptides as they depend almost completely on primary structure, that is, amino acid sequence. For CD8⁺ T cells, since class I MHC molecules bind peptides in the 8-11-mer range, if the peptides overlap by 10 residues, then any epitope up to 11 residues long would be present in at least one of the overlapping peptides. For peptides presented by class II MHC molecules to CD4⁺ T cells, one has to use longer peptides, but still an overlap of about 15 residues should capture most epitopes. This method can be tedious and labor-intensive, but newer methods to synthesize large numbers of peptides and use array techniques has facilitated this brute force approach.

When it is not feasible to do such empirical mapping, one can use methods developed to predict the location of T cell epitopes based on structural features. An early approach was based on the observation that many T cell epitopes had hydrophobic sides (Stille et al., 1987) or hydrophobic periodicity consistent with that of an alpha helix, before the structure of peptides binding to MHC molecules was determined by X-ray crystallography (DeLisi and Berzofsky, 1985; Margalit et al., 1987; Cornette et al., 1995). When it became obvious that the peptides were not bound in a helical conformation, it was then found that hydrophobic periodicity like an alpha helix was characteristic of the spacing of the anchor residues binding to pockets in the MHC groove, which were often hydrophobic, and thus could be explained by this pocket spacing (Cornette et al., 1995). Anchors were often at positions 2 and 9 (like 2 turns of an alpha helix with 3.6 residues per turn) or at 2 and 5 or 5 and 9, about one turn of a helix apart. Such general methods were supplanted by MHC-motif specific methods when those motifs were identified, so one could look for peptides with the right spacing between residues that served as anchors in a particular MHC molecule, such as the hydrophobic anchors at position 2 and 9 for HLA-A2 (Rammensee et al., 1995b). More refined matrix approaches looked at the probability of a particular amino acid at any position in a peptide binding to HLA-A2, for example (Ruppert et al., 1993; Rammensee et al., 1999). A number of such algorithms are available on the internet. Although motifs for many class II molecules are not as well defined yet, algorithms for these are also available. The reliability of such algorithms is largely dependent on the amount of data (number of known epitopes examined) used to create the algorithm, and so should be more reliable for HLA-A*0201 than for some rare HLA alleles. Other machine learning or artificial intelligence methods have been used to make predictions of T cell epitopes, such as threading approaches and neural networks (Altuvia et al., 1995; Brusica et al., 1994). Further improvements have been made by mass spectrometry analysis of peptides bound to MHC molecules (Hunt et al., 1992; Abelin et al., 2017, 2019). Predictive algorithms

have been greatly refined over the years so that they are extremely reliable (Meister et al., 1995; DeGroot et al., 1997; Gulukota et al., 1997; Zhang et al., 1998, 2007; Lin et al., 2008; De Groot et al., 2020; Peters et al., 2020). Thus, at least for common MHC molecules for which much data are available, predictive algorithms may be able to replace empirical assays for epitope mapping, although the only method to capture all epitopes is by experimental testing of T cell responses to all possible peptides.

Immunodominance

It has been observed that T cell epitopes are not all created equal. Some elicit much stronger responses than others (Vitiello et al., 1996). When one or two dominate the whole response, these are called immunodominant epitopes, and the weaker ones are called subdominant. In fact, these are not just quantitative differences, because removing a dominant epitope can sometimes allow a stronger response to what had been subdominant epitopes, that now move up the hierarchy (Gross et al., 2004; Grossmann et al., 2001; Slifka et al., 2003). This finding suggests some kind of competition, possibly for growth factors, or just for antigen-presenting cells.

Several mechanisms have been proposed for immunodominance. One involves affinity of the peptide for the MHC molecules of the host. It has been shown that higher affinity peptides are more immunogenic and can induce stronger responses (Sette et al., 1994; Alexander et al., 1993). There is also the affinity of the TCR for the peptide-MHC complex, which can affect the response. More generally, the available TCR repertoire may play a big role in immunodominance. If there are more T cells present with a particular TCR for a particular epitope even before exposure to the pathogen, cancer or vaccine, then that epitope will have a greater chance of dominating the response. That greater prevalence could be due to intrinsic genetic factors, like the availability of V, D and J regions or their accessibility for VDJ recombination, or to extrinsic factors, like the history of the host's prior exposure to crossreactive epitopes that could have expanded that repertoire. We found, for example, that when we compared different congenic strains of mice sharing the same MHC but different genetic backgrounds, the response to the same peptide epitope showed different degrees of immunodominance depending on non-MHC genes in that strain (Dzutsev et al., 2007). One key difference in those strains that share the same MHC is the gene cluster of TCR genes, which is the most likely suspect. Whichever set of T cells gets a head start in the response, perhaps by having higher avidity for the peptide-MHC complex, a larger repertoire or a previously expanded one, may out-compete the other epitopes and become dominant.

Immunodominance has translational relevance, because dominant epitopes are often the ones to which T cells are tolerized, or which may select for escape mutations because they exert the most selective pressure. Therefore, targeting non-dominant epitopes may be an important strategy in vaccine development (Slifka et al., 2003), perhaps combined with epitope enhancement as discussed above, to avoid this problem.

Conclusions

In summary, antigens are any type of molecule that can be recognized by specific receptors of the adaptive immune system on T cells and B cells. They can consist of proteins or peptide fragments of proteins, carbohydrates, nucleic acids (DNA or RNA), lipids, or even haptens, which are molecules too small to induce an immune response by themselves but can be recognized by antibodies once those are made. To induce antibodies against haptens, one needs to couple them to a larger immunogenic molecule, called a carrier, to provide a source of T cell help. Unlike B cells or antibodies, T cells don't recognize free antigens in solution, but only peptide fragments of protein antigens presented by an MHC molecule on an APC, such as a DCs. Thus, individuals with different MHC haplotypes (HLA haplotypes in humans) will have different repertoires of antigens they can recognize. In general, CD4⁺ T cells recognize antigens presented by class II MHC molecules such as HLA-DR, DQ or DP in humans or I-A or I-E in the mouse. These generally result from endosomal uptake of antigens from outside the APC. On the other hand, CD8⁺ T cells generally recognize peptide antigens presented by class I MHC molecules such as HLA-A, B or C in humans or H-2K, D, or L in mice. These are most often derived from proteasomal processing of antigens made in the cytosol, such as viral antigens in an infected cells, and are then loaded onto the class I MHC molecules to be carried to the cell surface. Some peptides can be cross-presented to CD8⁺ T cells even from exogenous antigens, as described above.

To be immunogenic, an antigen must not only be recognizable by the immune system (antigenic), generally as foreign (so not subject to self tolerance), but also be able to activate the immune system., which defines them as immunogenic. That often requires triggering PRRs such as TLRs on APCs that activate these APCs and thus alert the immune system. Most pathogens do this naturally, by expressing their own PAMPs such as TLR ligands. However, for individual soluble protein antigens, so called subunit vaccines, one needs to add such signals to the vaccine, in the form of adjuvants. This adjuvant trick is what Charlie Janeway called "the immunologist's dirty little secret" and adjuvants have made possible vaccines composed of single recombinant proteins. Thus, understanding the nature of antigens and how to make them immunogenic has greatly improved our ability to make a wide range of vaccines that have saved millions of lives from infectious diseases, and may soon be able to treat or even prevent cancers as well.

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Antigen Presentation and Major Histocompatibility Complex

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Introduction

Antigen presentation is a process of displaying parts of antigenic fragments—epitopes—to the immune cells bearing corresponding antigen receptors. Antigen can be presented by any nucleated cell of the organism, but there is a specific subset of cells called professional antigen presenting cells (pAPCs), e.g., B cells, macrophages, Langerhans cells, microglial cells or thymic cortical epithelial cells. Antigen presentation is essential for the development of adaptive immune response. For example, germinal center B cells recruit help from follicular helper T cells (T_{fh} cells) to produce high affinity antigen-specific antibodies. At the same time, macrophages which display antigen are activated by helper T cell cytokines thus making antigen presentation important for the innate immunity. The latter is particularly important for the clearance of pathogens residing and replicating inside macrophages, e.g., *Leishmania* and mycobacteria. Major histocompatibility complex (MHC) is a molecular machinery that makes antigen presentation possible. Human MHC molecules were named the human leukocyte antigens (HLA) system. Basic principle of its functioning can be defined as ‘capturing’ of self and foreign antigenic components inside the cell with the consequent transport of these components to the cell surface. Here, the antigen-MHC complex can be recognized by T cells which inspect cells of the organism for the presence of foreign matter or abnormal self proteins. T cells do not recognize native antigens like immunoglobulins do, and they need antigen to be displayed by MHC complexes. Every nucleated cell expresses about 10⁵ major histocompatibility complex class I molecules (MHC class I) in complex with endogenous (cytosolic) antigen peptides which can be recognized by specific CD8⁺ (cytotoxic) T cells. However, the level of expression may differ among different cell types. Lymphocytes exert the highest class I expression while muscle and liver cells are characterized by low expression level (a fact explaining relatively low frequency of liver graft rejection). pAPCs constitutively express MHC class II molecules in complex with exogenous (extracellular) antigen peptides which are recognized by specific CD4⁺ T cells. MHC class I and II display peptide antigens. Native antigenic peptides are prepared before they fit MHC molecules in a pathway referred to as antigen processing. There are also non-classical MHC molecules, such as CD1d, that can bind lipid antigens. MHC expression can be upregulated by interferons (α and β interferons for class I and γ interferon for class I and II) and TNF facilitating immune response against pathogens but also predisposing for the autoreactive T cells activation. IFN- γ action is associated with MHC class II transactivator (CIITA) which promotes MHC class II genes. Loss of CIITA leads to severe immunodeficiency development. Thus, abnormal MHC regulation has strong consequences for the organism health (Abbas et al., 2019).

Basic structure of MHC molecules and MHC-peptide complexes

Both HLA class I and class II molecules are composed of two polypeptide chains that form a structure suitable to hold short antigenic peptides. Class I molecular complex is a heterodimer composed of immunoglobulin superfamily α -chain (45 kDa) noncovalently associated with β 2-microglobulin (12 kDa). α -Chain is presented by one of the three types: A, B, C and is organized into three extracellular domains (α 1, α 2 and α 3) and a cytoplasmic anchor that spans the membrane. MHC class II is constituted by two noncovalently associated nonidentical transmembrane glycoprotein chains α (33 kDa) and β (28 kDa): DP, DQ, DR (Fig. 1). Each chain possesses extracellular α 1 and α 2 domains or β 1 and β 2 domains structurally similar to those of MHC class I domains. Polypeptide chains of HLA complexes form peptide-binding grooves, or peptide-binding clefts, on the top surface of the molecule that fit peptides 8–11 amino acids in length in class I and 13–25 amino acids in length in class II molecules. The structure of grooves determines which peptides will bind here. These peptides are generated during antigen processing. Peptides are contained in grooves via their conserved motifs which can be different for different HLA molecules. Peptide-binding groove is closed at both ends

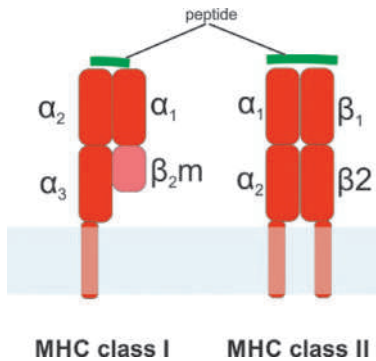


Fig. 1 Structure of MHC molecules.

in class I molecules and is open at both ends in class II molecules and that is a major differences in structure between two MHC classes (Abbas et al., 2019; Male et al., 2020).

Most of the peptides bound to MHC class I molecules are monomers nine amino acids in length that form a 'sandwich' together with α -domains of the MHC molecule. Peptide anchor residues are contained at both ends of peptide; similar anchor residues may be present in different peptides in the same positions in the amino and carboxy termini of the sequence. Given the number of expressed MHC molecules on the surface, this means that any individual cell can simultaneously present a wide range of different peptides to CD8 + T cells. Different peptides are anchored by the same amino acids through hydrogen bonds and ionic interactions. Peptides longer than nine amino acids are accommodated in the peptide-binding groove by making a twist in the peptide backbone, or, in some cases, by letting the residues of the carboxy terminus to hang out of the groove (Abbas et al., 2019; Male et al., 2020).

Peptides bound to MHC class II molecules are longer than the peptides that bind to class I molecules and contain internal binding motifs than distributed along the peptide backbone rather than at the ends of the peptides, which extend beyond the groove edges. It is interesting that number and distance between peptides that interact with MHC binding pockets is conserved: four anchor residues (R) are separated in this manner: Y-R1-X-X-R4-X-R6-X-X-R9-Y, where Y designates up to six residues at the amino and carboxy termini and X designates amino acids between anchors. Properties of anchor residues, such as hydrophobicity or charge, also remain conserved in the corresponding positions. Additional promiscuity of these peptides is often provided by proline residues contributing to structural flexibility. A notable feature of peptide binding both in MHC class I and II is that the binding is sufficiently strong and stable to prevent dynamic peptide exchange at the cell surface. Moreover, peptide binding stabilizes MHC molecules preventing them from dissociation; this also allows purification and analysis of peptide-MHC complexes for research purposes (Abbas et al., 2019; Male et al., 2020).

MHC genetics

MHC molecules are encoded by genes located at the HLA region on chromosome 6 in humans and on murine chromosome 17. However, human β 2-microglobulin is encoded by a non-HLA gene of chromosome 15. Historically these regions were discovered during the search for the graft rejection mechanisms, hence the name 'histocompatibility'. Genes located at the MHC class I and class II regions also code for different molecules that perform different functions. There is also MHC class III gene region which products include complement components and immunoregulatory molecules that are not always directly associated with antigen processing and presentation during the immune responses (Abbas et al., 2019; Male et al., 2020).

Class I genetic loci include genes encoding antigen-binding *HLA-A*, *HLA-B*, and *HLA-C* molecules. Class II genes *DPA*, *DPB*, *DQA*, *DQB*, *DRA*, *DRB* encode corresponding subunits of antigen-binding molecules (Fig. 2). This class II molecules basically have the same function thus MHC gene region is said to be polygenic: multiple genes encode products with only slightly different structures. Impressive number of alleles in these loci constitutes the basis of HLA polymorphism, or allelic variation within the species. Exception is the *DRA* which is monomorphic (Matern et al., 2020). Alleles are designated by the number in humans, e.g., *HLA-A1*, *HLA-A2*, *HLA-A3*, ..., or *HLA-DR1*, *HLA-DR2*, etc. As of December 2020, number of HLA class I and class II identified alleles has

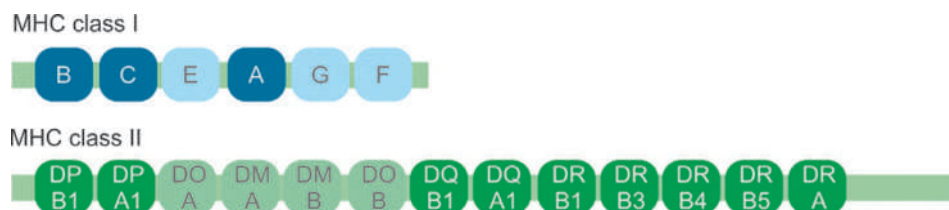


Fig. 2 Simplified map of HLA region on chromosome 6. Classical class I and class II genes are shown in dark blue and green. Nonclassical class I genes and class II genes involved in antigen loading are shown in light blue and green correspondingly.

reached 20,597 and 7723, correspondingly, making it one of the most polymorphic genetic complexes in humans. Most important polymorphisms affect DNA regions that encode protein sequences of the antigen-binding groove. Moreover, multiple genes for α and β chains are present in some individuals encoding different protein isoforms. Also, any DR α -chain can be paired with any DR β -chain. Altogether, MHC diversity is exhibited at both the individual and species level. Genetic polymorphism and versatile α - β chain associations allow a high diversity of antigens that can be presented on individual level and also ensures population resistance to a range of infective agents rather than equal susceptibility to a number of pathogens. At the same time, this HLA diversity makes graft donor-recipient matching quite challenging and some alleles predispose individuals to autoimmune diseases (Murphy and Weaver, 2016; Punt et al., 2018; Rich et al., 2018).

Any individual expresses a small part of MHC repertoire: up to 6 class I molecules and up to 12 class II molecules which are able to bind a bind an enormous array of different peptides. Vice versa, any exact peptide can be presented by several different MHC molecules in contrast to highly specific antigen-antibody or antigen-TCR interactions. However, T cells distinguish between similar peptides in complex with different MHC molecules, that is the essence of MHC restriction.

Allele set in each individual is called haplotype. Some haplotypes are more frequent in population than others which somewhat contradicts expected gene distribution; this phenomenon is called linkage disequilibrium. It reflects human migration history and natural selection process which outsorts allele sets that are less useful in terms of infection resistance. An example is HLA-A11 allele which is frequent in East Asia where many circulating Epstein-Barr virus isolates are characterized by having an epitope presented by HLA-A11. It is important to note that in different organisms same antigens still can be presented, but the specific set of presented peptide fragments (of the same antigen) is determined by the specific haplotype in each individual. The collection of peptides associated with HLA is referred to as the human immunopeptidome (Murphy and Weaver, 2016; Specht et al., 2020).

A set of genes in the MHC I locus encodes nonclassical class I molecules which are expressed on specific cell types. For example HLA-G molecules are expressed on the fetal cells during pregnancy and are known to inhibit CD8 + T cells. Nonclassical MHC class II molecules include HLA-DM and HLA-DO molecules involved in antigen processing and presentation (Murphy and Weaver, 2016; Punt et al., 2018; Rich et al., 2018).

HLA inheritance is codominant (equal expression of haplotypes inherited from the mother and from the father) and follows Mendelian pattern in general (Fig. 3). That means that there is one-in-four chance of having the same paternal and maternal haplotypes for any two siblings, unless they are not identical twins. This makes them histocompatible with one another. However there is also chance of meiotic crossover responsible for about 1% probability of a new haplotype occurrence. Human haplotype initially was identified by mixing lymphocytes with different antisera, but each of serologically identified alleles can be typed by DNA-based methods (PCR or sequencing) which gives rise to a complicated nomenclature reflecting the diversity of HLA alleles in human population. A typical class I HLA haplotype designation thus can be exemplified as HLA-A*23:01:101:01:02N where numbers (which are unique for each haplotype) are used to specify allele group, specific HLA protein, synonymous nucleotide substitution within the coding region and non-coding region differences followed by a letter (in the example above, letter "N" which means "Null") denoting expression pattern. The number of HLA alleles is enormous, as stated above, but probably underestimated due to the low availability of genomic data from African, Indian and other regions (Murphy and Weaver, 2016; Punt et al., 2018; Rich et al., 2018).

Inbred mouse strains, in contrast to human population, are syngeneic and are homozygous at the MHC locus. For example, widely used C57BL/6 strain expresses H2^b haplotype which means that they always have the same set of K, A, E and D H2 alleles. If this strain is crossed with a CBA strain which haplotype is H2^k, the F1 generation will be designated as expresses H2^{b/k} (Murphy and Weaver, 2016).

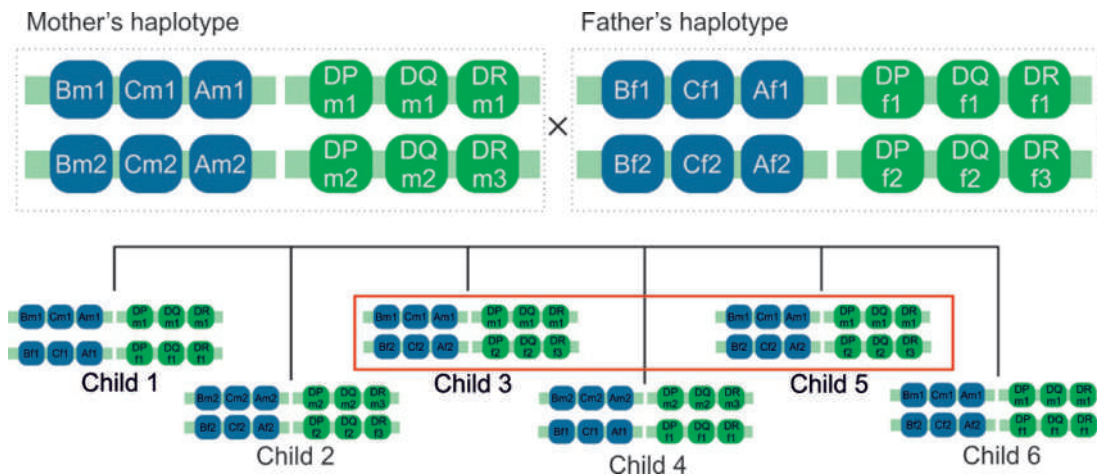


Fig. 3 Inheritance of MHC (HLA) haplotypes. Maternal alleles are designated with letter 'm' and chromosome homolog number (1 or 2). Paternal alleles are designated with letter 'f' and chromosome homolog number. Note identical haplotypes in offsprings 3 and 5. A novel haplotype is recorded in child 6 as a consequence of meiotic recombination in father's chromosomes.

Fully heterozygous individual can express six unique MHC class I molecules on each cell. Remember that MHC class II molecules can form different α - β combinations and there are two DRB molecule types (DRB1 and one of extra DRB3 to DRB5 alleles encoded by DRB paralogue genes). However, Class II DRA allele is invariant and its structure is independent of maternal or paternal origin. Given all above, up to 12 unique class II molecules can be expressed on any single pAPC (Table 1) (Donaldson et al., 2015; Matern et al., 2020).

MHC functions

The diversity of antigens bound by T cell receptors somewhat reflects the diversity of peptides presented by MHC molecules. However, T cells provide recognition of antigen only in context of self MHC molecules. This phenomenon is explained by the term ‘MHC restriction’ which takes place during T cell development in the thymus. If MHC restriction did not exist, multiple ‘useless’ T cells would be differentiated. This T cells would have receptors able to recognize foreign MHC-peptide complexes which seems to be evolutionary impractical resource handling (energy and cell building materials). Despite the enormous diversity of T cell repertoire generated in thymocytes, only 1% survives after thymic selection and only T cells capable of interacting with self MHC are selected for further development. It is natural to conclude MHC restriction is a by-product of positive selection which primary function is to avoid supernumerary wandering lymphocytes generation. However, the inherent ability of T cells to recognize MHC molecules still allows the presence of some alloreactive T cells that recognize nonself MHC molecules as foreign and pose a problem in case of organ transplantation. Negative T cell selection process also makes sure that T cells are able to distinguish between self and non-self since thymocytes with excess affinity to self peptide-MHC complexes are discarded from entering the circulation (Murphy and Weaver, 2016; Punt et al., 2018; Rich et al., 2018).

Summarizing the above, presentation of foreign antigens to CD4+ or CD8+ T cells is a key function of MHC molecules that plays an indispensable role in fighting infection. However, MHC also maintains healthy functioning of an organism even in the absence of an infectious agent. Timing and location of MHC-TCR interactions thus plays crucial role for the consequences of such interaction. Constitutive expression of MHC class I displaying self peptide serves as a marker of healthy cell. It is also necessary to maintain tolerance to self proteins (Murphy and Weaver, 2016; Punt et al., 2018; Rich et al., 2018). It is no wonder then that some immunologists refer to MHC molecules as a “garbage bin” that allow T cells to develop in familiar conditions and to stay aware about the situation in the house.

Loss of MHC class I molecules is called ‘missing self’. Since NK cells have evolved inhibitory receptors activated upon MHC class I binding, the absence of MHC class I on nucleated cells signals that a cell should be destroyed by NK cells since some viruses interfere with MHC class I expression. At the same time, absence of MHC expression in red blood cells makes these cells a privileged site for such parasites as *Plasmodium*, *Babesia* and *Bartonella* (Murphy and Weaver, 2016; Punt et al., 2018; Rich et al., 2018).

It is clear now that different MHC alleles in humans are associated with a range of autoimmune and autoinflammatory diseases, e.g., rheumatoid arthritis, type 1 diabetes, systemic lupus erythematosus, ankylosing spondylitis, etc. However, the pathogenic mechanisms of these alleles are still unresolved. A number of hypotheses have been proposed to explain the contribution of MHC to the autoimmunity. Presentation of autoantigens or molecular mimicry of microorganisms and self antigens may play a role in some cases, while altered T cell repertoire during thymic selection may be another reason.

Table 1 Possible associations of human MHC genes.

MHC class	Origin of first chain	Origin of second chain	Number of possible molecules
Class I	Maternal HLA-A	N/A	3
	Maternal HLA-B		
	Maternal HLA-C		
	Paternal HLA-A	N/A	3
	Paternal HLA-B		
	Paternal HLA-C		
Class II	Invariant DRA	Maternal DRB1	4
		Maternal DRB3/DRB4/DRB5	
		Paternal DRB1	
		Paternal DRB3/DRB4/DRB5	
	Maternal DPA	Maternal DPB	2
	Paternal DPA	Paternal DPB	2
		Maternal DPB	
	Maternal DQA	Maternal DQB	2
		Paternal DQB	
	Paternal DQA	Maternal DQB	2
		Paternal DQB	
Total			18

Endogenous antigen processing and presentation

Antigen presentation is required to initiate immunological responses mediated by antigen-specific T cells. Endogenous antigens, or antigens from intracellular locations are displayed on the surface of nucleated cells in complex with MHC class I molecules. MHC class I makes these otherwise internal antigens accessible for the CD8⁺ T cells. If these antigens recognized as foreign the cell becomes a target for the destruction.

Before antigen is displayed it should be processed. This event is coupled with MHC class I assembly and takes place in the cytosol and in the endoplasmic reticulum (ER). Self proteins are subject to constant turnover inside the cell and a large proportion of newly synthesized proteins are immediately degraded. This is particularly relevant to defective ribosomal products, or DRiPs, which originate from erroneous transcription, translation or protein folding and assembly. Some of the proteins are of viral origin if the cell becomes infected while some proteins recognized as foreign result from malignant cell transformation or aging. This immediate degradation of proteins is crucial for the rapid antiviral response and requires sufficient amount of readily available MHC class I molecules in the ER. Protein breakdown machinery that generates peptides suitable for MHC class I binding involves specific proteasome set: 26S proteasome, immunoproteasome or thymic proteasome depending on the cell type. A complex catalytic structure of proteasomes is represented by different subunits determining the type of the proteasome. Immunoproteasome is a specific type of proteasome which subunits are expressed in pAPCs or infected cells under the exposure to IFN- γ or TNF. The immunoproteasome is more efficient in production of peptides that bind MHC class I molecules since its enzymatic specificity differs from the 'ordinary' constitutive proteasome. Since excessive MHC class I presentation possibly can result in autoimmunity, the immunoproteasome is subject to a quick degradation itself (Specht et al., 2020).

Proteins are tagged for degradation by the ubiquitination process. In the context of cell life cycle, the MHC system can be viewed as an effective system of waste management (Nagy, 2008). In this system, peptides that are not fully degraded or reused and are not destroyed in the autophagy process are transported by MHC molecules out of the cell interior, at the same time providing signal to the T cells.

Peptides produced by proteasome usually occur through proteolytic reaction that requires ATP. However a fraction of the peptides released by proteasomes is formed through a transpeptidation reaction, thus adding a sizeable portion of spliced peptides to the ligand repertoire presented by MHC class I. A number of other modifications may occur, e.g., citrullination, phosphorylation, acetylation or deamidation contributing to neoepitope formation (Sun et al., 2014; Mei et al., 2020; Ostankovitch et al., 2009).

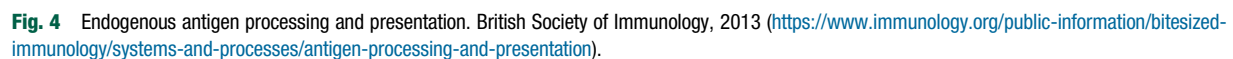
Some of the peptides processed by proteasomes are then picked up by ATP-dependent transporter associated with antigen processing (TAP). TAP is a heterodimer comprising TAP1 and TAP2 proteins. TAP1 and TAP 2 basically form channels across the ER membrane, through which peptides are ferried into the lumen. While TAP has the highest affinity for peptides composed of nine amino acids, it still can transport longer peptides into the lumen of the ER. Here, an additional trimming of the peptide may occur by ER-resident amino peptidases (ERAP1, or ERAAP), before a peptide is loaded onto the MHC. Peptides failing to associate with MHC may re-enter the cytosol by the ERAD (ER-associated protein degradation system) or Sec61 transport complex to undergo further modifications or degradation. ERAD system functions to clear misfolded MHC class I molecules out of the ER as well (Fig. 4) (Murphy and Weaver, 2016; Punt et al., 2018; Rich et al., 2018).

MHC class I molecules manufactured via ribosome synthesis are stabilized by chaperones—calnexin, calreticulin, endoplasmic reticulum p57 (ERp57), TAP-binding protein (also referred to as tapasin) and protein disulfide isomerase (PDI). Calnexin and ERp57 are required for the correct folding of the class I α chain. Upon β 2-microglobulin binding, calnexin dissociates to allow connection between ERp57- α chain complex and calreticulin, as well as tapasin to form the peptide loading complex (PLC). Tapasin is one protein that provides association of MHC class I with TAP transporter forming a bridge between these molecules. In a process referred to as peptide editing, PLC allows dynamic exchange of ER peptides inside the peptide-binding groove until a peptide with high affinity is selected. This affinity is partly determined by the presence of hydrophobic or basic residues at the carboxy terminus. Binding of a peptide to MHC class I molecules releases the chaperones allowing peptide-MHC complexes to leave the ER in a transporting vesicle for presentation at the cell surface. Since peptide still can suddenly dissociate from the MHC class I, this dissociation results in β 2-microglobulin release and MHC class I internalization. This internalization allows MHC class I molecules to enter the endosomal compartments where they can snatch away some peptides used in the MHC class II presentation pathway (Murphy and Weaver, 2016; Punt et al., 2018; Rich et al., 2018). This appears to be one of the mechanisms of cross-presentation described below.

Exogenous antigen processing and presentation

Exogenous antigens derive from outside the cell and thus should be internalized and digested before they are displayed on the cell surface. This processes are realized by APCs that can engulf foreign particles through phagocytosis or clathrin-dependent internalization. Professional APCs express a variety of accessory molecules (costimulatory receptors and cytokines) enabling them to arm T cells for the deployment of immune responses. Three types of pAPCs are known: dendritic cells, macrophages and B cells which differ in origin and manner of antigen handling. However, they all are similar in terms of MHC class II molecules expression, which are synthesized in the ER and pass on to Golgi complex and then to endosomes containing antigens.

Internalized peptides are subject to intracellular digestion by a set of cysteine proteases, or cathepsins, that constitutes peptide processing pathway for MHC class II binding. Processing is facilitated through disulfide bonds reforming by endosomal IFN- γ -induced lysosomal thiol reductase (GILT). Before fusion of MHC II-containing vesicles with an endosome, the newly synthesized



MHC class II molecules are protected from peptide binding by invariant chain (Ii) which forms a trimer stabilized by calnexin during assembly. Another molecule associated with MHC class II is HLA-DM coded in the MHC class II region. Fusion of an endosome and MHC class II-containing vesicle ultimately ends up in a late endosome formation where rapid pH decrease leads to the degradation of Ii-chain by proteases, leaving only a small CLIP fragment (class II-associated invariant chain peptide). CLIP remains bound to the peptide-binding groove, where HLA-DM supports its 'ajar' conformation. This allows CLIP release when a peptide with higher affinity for the peptide-binding groove approaches. Thus, HLA-DM serves to aid the exchange of CLIP with

antigenic peptides inside the groove and resembles peptide-editing role of PLC in MHC class I, ensuring tight binding of the peptide presented on the cell surface. Another nonclassical MHC molecule, HLA-DO, acts as a regulator of HLA-DM function and alters the repertoire of self-peptides presented by thymic epithelial cells (Fig. 5) (Murphy and Weaver, 2016; Punt et al., 2018; Rich et al., 2018).

Exogenous antigens can be found associated with MHC class I molecules, and vice versa, peptide fragments formed in cytosol can bind to MHC class II molecules suggesting that processing pathways are not limited by above described pathway. Notably, cytosolic peptides, including self peptides can be loaded onto MHC class II complex as a result of autophagy. Moreover, successful antiviral response does not solely depend on the MHC class II presentation to engage helper T cells that provide more powerful antiviral response but requires arming of CD8+ T cells via class I molecules. This phenomenon is termed as cross-presentation which is carried out by a subset of CD8 α -expressing dendritic cells or macrophages (Muntjewerff et al., 2020). However, cross-presentation not only used in naïve CD8+ T cell activation (i.e. cross-priming) but is also indispensable to support proper dendritic cell functioning. This is explained as a dendritic cell licensing model where dendritic cell, to be fully activated, requires corresponding signaling from the T helper cell. Taking into account that autoreactive T helper cells are discarded in thymus or become inactive due to the peripheral tolerance mechanisms, dendritic cells licensing provides a mechanism to avoid tolerance breakdown in case

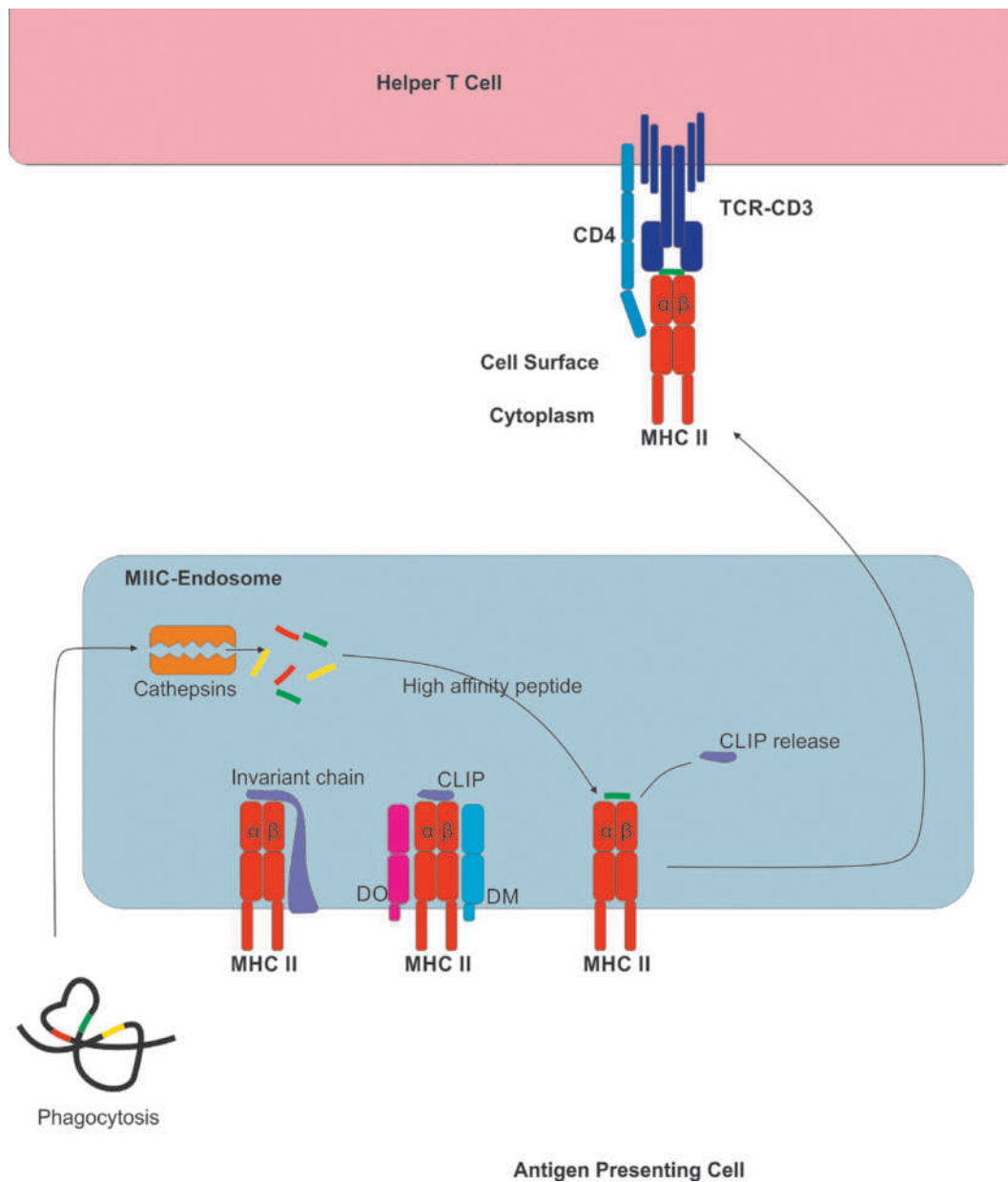


Fig. 5 Exogenous antigen processing and presentation. British Society of Immunology, 2013 (<https://www.immunology.org/public-information/bitesized-immunology/systems-and-processes/antigen-processing-and-presentation>).

self antigen is presented to CD8+ T cell. Once a dendritic cell acquires a stimulatory signal from T helper cell (via cytokine milieu or costimulatory molecules), it becomes capable to activate naïve CD8+ T cells. Otherwise, dendritic cell may provide inhibitory signal to CD8+ T cell to maintain tolerance to self antigens. This is crucial given the fact that dendritic cells constantly present self-peptides originating from dying cells and other sources. Another mechanism proposed to explain self tolerance in case autoantigens are presented is that accidental priming, or activation of CD8+ autoreactive T cell by a dendritic cell will result in a rapid cytotoxic lysis of the dendritic cell, preventing it from further contact with CD8+ T cells (Abbas et al., 2019; Male et al., 2020).

MHC class II molecules can be internalized from the cell surface in case they lose antigen or due to the ordinary recycling process. However, the MHC class II recycling halts in mature dendritic cells upon pathogen encounter. This is mediated by inhibiting the membrane associated ring finger (C₃HC₄)₁ (MARCH-1) protein. MARCH-1 protein is a ligase that targets cytoplasmic tails of MHC II class providing their recycling. Thus, MARCH-1 inhibition ensures lock-on of MHC class II recycling to keep peptide-MHC II complexes on the cell surface upon infection, which is necessary for T cell activation (Abbas et al., 2019; Male et al., 2020).

Nonclassical MHC molecules and presentation of nonpeptide antigens

A scavenger theory of MHC molecules would suggest that a set of molecules discarded from cell should not be limited by peptide molecules. Indeed, there is a class of molecules structurally similar to classical MHC class I molecules capable to display lipid or lipid-linked molecules. These protein families are designated as CD1 and MR1 (MHC class I-related protein) which are expressed by a variety of cells, including APCs and non-immune cells. In contrast to MHC class I, these molecules are coded outside of MHC locus and are not characterized by pronounced polymorphism. A set of CD1-restricted cells is known to recognize the lipid antigens bound to these nonclassical MHC molecules. Among antigens presented via these pathway is mycolic acid from *Mycobacterium tuberculosis* and other Mycobacteriaceae. A fact that a very conserved subtype of T cells which is abundant in skin and mucosa is required to recognize a specific molecule reflects a long history of co-evolution of human immune system and mycobacteria. MR1 displays metabolites of folic acid that are recognized by mucosal associated invariant T cells (MAIT cells) bearing homodimeric CD8 $\alpha\alpha$ co-receptor (Abbas et al., 2019; Male et al., 2020).

Another nonclassical MHC molecule is Qa-1 in mice and its functional homolog in humans, HLA-E. It presents peptides when the function of ERAP1 is inhibited, which can be a consequence of viral infection or tumor transformation. Peptides presented by Qa-1 are conserved and provide signal for CD8+ T cells, preventing pathogens or tumor cells from immunosurveillance evasion (Nagarajan et al., 2012).

TCR-MHC interactions during presentation

Recognition of peptide-MHC complex by the T cell receptor (TCR) provides essential first signal for the T cell activation and the development of immune response. Conventional $\alpha\beta$ TCRs are antigen-recognition molecules that are highly variable and somewhat resemble the structure of an immunoglobulin Fab fragment. Each T cell expresses about 30,000 identical TCRs on the cell surface. TCRs bind short peptide sequences that are usually inaccessible in the native protein structure. Thus, antigen processing and presentation makes these sequences available for TCR recognition. However, TCR reacting with its ligand is also making contacts with the MHC molecule α domains. TCR α chain contacts with $\alpha 2$ domain of class I molecules while TCR β chain contacts with $\alpha 1$ domain. TCR orientation along MHC molecules allows binding of CDR1–CDR3 loops to come in close contact with the presented peptide via slight conformational changes within the CDR loops. While initial stages of the contact are determined by TCR-MHC molecules, subsequent peptide recognition depends on adoptive conformational changes that will affect T cell response. Depending on the type of cells, CD4 or CD8 molecules become associated with TCR when the process of antigen-recognition initiates. CD8 binds to the invariant fragment of MHC class I $\alpha 3$ chain while CD4 binds to such fragment of MHC class II located between $\alpha 2$ and $\beta 2$ domains. Acting as co-receptors, CD4 and CD8 profoundly increase T cell sensitivity to antigen. Once antigen is recognized, other co-stimulatory molecules and cytokines are expressed for the full activation of the T cell (Abbas et al., 2019; Male et al., 2020).

Antigen presentation in thymus

Generation of T cell repertoire requires thymic development where the processes of positive and negative selection ensure self MHC restriction and rejection of autoreactive cells. Starting at the stage of double-positive thymocytes, T cell development substantially depends on TCR-MHC interactions. Double-positive (CD8+/CD4+) thymocytes rely on recognition and binding of self-peptide MHC in order to survive; a process referred to as positive selection. Timing of this interactions coincides with gradual migration of thymocytes from thymic cortex to cortico-medullar region. Here cortical epithelial cells expressing MHC class I and class II reside. Cortical epithelial cells express a set of cathepsins and thymic proteasome distinguishing them from other antigen-presenting cells. Also, they express a significant number of MHC class II that keep binding CLIP. Hence, successful binding to MHC class I or class II is determined by inherent specificity of TCR to MHC molecules rather than their peptide specificity. In contrast, negative selection is provided by screening T cells that bind self antigens with high affinity; these T cells die by apoptosis. Processing of tissue-specific peptide that are expressed in the thymus is driven by autoimmune regulator (*AIRE*) gene which allows expression and presentation of peptides otherwise expressed only outside of the thymus. Cells responsible for negative selection are the thymic epithelial cells, bone marrow-derived dendritic cells and macrophages (Abbas et al., 2019; Male et al., 2020).

Successful positive and negative selection leads to cessation of CD4 or CD8 expression and shutting off TCR α -chain rearrangement. However, the 'second' TCR with a productively rearranged α -chain still can be expressed due to the absence of allelic exclusion in genome during TCR α -chain rearrangement. In mature T cells this TCR is mostly internalized due to the weak or excessively strong peptide-MHC binding, thus maintaining so-called phenotypic allelic exclusion and providing functional monospecificity (Schuldt and Binstadt, 2019). Thymocytes that survive positive and negative selection in the cortex relocate to the medulla as single positive thymocytes where they encounter a new assemblage of self-peptide-MHC. High-affinity interactions here lead to clonal deletion. However, a number of T cells with high affinity to self peptide-MHC survive through the process called agonist selection and give rise to Treg cells (Stritesky et al., 2012).

The role of MHC-mediated T cell selection and tolerance is not limited within the thymus. At the periphery, self peptide-MHC complexes provide tolerogenic signals to eliminate or inactivate autoreactive mature naïve T cells.

MHC-associated immunodeficiencies

Autoimmune diseases comprise only a part of diseases associated with improper MHC functioning. Another large pool of diseases is immunodeficiencies which originate from absence or improper function of certain components involved in antigen processing and/or presentation. Immunodeficiencies manifest as different infections depending on the affected genes. These diseases are classified into different types, e.g., MHC class I deficiency and MHC class II deficiency group A, B, C, D belong to a group of 'Immunodeficiencies affecting cellular and humoral immunity'. They are characterized by genetic defects in *TAP1*, *TAP2*, *B2M*, *CIITA* or other genes directly involved in MHC synthesis and assembly. APECED (APS-1), autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy is a consequence of AIRE defect and is classified into 'Diseases of immune dysregulation' (Tangye et al., 2020). Many immune defects, e.g., ubiquitin system mutations, are not included into the immunodeficiency classification, however they still manifest by impaired immune responses (Etzioni et al., 2017).

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- <http://hla.alleles.org/nomenclature/naming.html>—Nomenclature for Factors of the HLA System.

Phagocytosis

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Glossary

Antibody An antibody is a protein that binds specifically to a particular substance, termed antigen. All antibodies have the same overall structure and are known collectively as immunoglobulins or Igs. They belong to the immunoglobulin superfamily. Antibodies are produced by plasma cells in response to infection or immunization. Antibodies bind to pathogens and prepare them for ingestion and destruction by phagocytes.

Antigen An antigen is any molecule that can bind specifically to an antibody. The term antigen arises from the ability these substances have to generate antibodies. However, some antigens do not, by themselves, evoke antibody production and they are called haptens. Antigens that can induce antibody production by themselves are called immunogens.

Apoptosis Apoptosis is a form of cell death, also known as programmed cell death. It is a 'suicide' program that activates within the cell. This program starts with DNA fragmentation, followed by shrinkage of the cytoplasm, membrane modifications, and finally, cell death without lysis or harm to neighboring cells. It is a normal phenomenon, frequently occurring in a multicellular organism. Proliferating cells usually undergo apoptosis, as part of the natural development process. Also, dividing lymphocytes undergo apoptosis at high rates during development, and during immune responses. Apoptosis contrasts with necrosis cell death, which occurs in circumstances such as anoxia or poisoning. Phagocytes remove apoptotic cells by phagocytosis.

Chemotaxis Process by which phagocytes migrate towards places of infection.

Complement A complement system is a group of plasma proteins that together attack extracellular forms of pathogens. Complement activation can occur spontaneously on some pathogens or by antibody binding to the pathogen. Complement components are deposited on the membrane of the pathogen forming a molecular complex (the membrane attack complex) that forms pores on the membrane. With this, the cell is destroyed by osmotic shock. Complement proteins deposited on pathogens are recognized by phagocytes to remove pathogens via phagocytosis.

Dendritic cells Dendritic cells are bone marrow-derived cells with branched or dendritic morphology, located in lymphoid tissues. They are antigen-presenting cells and the most potent stimulators of T-cell responses.

Fc receptors Fc receptors are receptors for the Fc portion of antibodies. They are named according to the class of antibody they recognize. So Fc γ receptors bind IgG, Fc α receptors bind IgA, and Fc ϵ receptors bind IgE.

Immunoglobulin All antibody molecules belong to a family of plasma proteins called immunoglobulins (Ig). Membrane-bound immunoglobulin serves as the specific antigen receptor on B lymphocytes.

Integrins Integrins are heterodimeric cell-surface proteins involved in cell–cell and cell–matrix interactions. They are essential in adhesive interactions between lymphocytes and antigen-presenting cells and in lymphocyte and leukocyte migration into tissues.

Macrophages Macrophages are large mononuclear phagocytic white blood cells. They are migratory cells deriving from bone marrow precursors found in most tissues of the body. They have a crucial role in host defense.

Monocytes Monocytes are white blood cells with a bean-shaped nucleus. They are macrophages precursors.

Neutrophils Neutrophils are the most abundant type of white blood cell in circulation. They have a multilobed nucleus and multiple granules. They are also known as neutrophilic polymorphonuclear leukocytes. Neutrophils are phagocytes that ingest and kill extracellular pathogens.

Opsonization Process where some plasma components, such as antibodies or complement proteins, cover a pathogen. In doing so, they intensify the process of phagocytosis by neutrophils and macrophages.

Phagocyte A phagocyte is a cell that performs phagocytosis. There are two types of phagocytes: professional and nonprofessional. Professional phagocytes include dendritic cells, eosinophils, macrophages, monocytes, neutrophils, and osteoclasts. Nonprofessional phagocytes include endothelial cells, epithelial cells, and fibroblasts.

Phagocytosis Phagocytosis is a cellular process for ingesting and eliminating particles larger than 0.5 μm in diameter, including microorganisms, foreign substances, and apoptotic cells.

Phagolysosome Phagolysosome is a large vacuole formed from the fusion of the phagosome with one or more lysosomes. The lysosome contains lytic enzymes that are essential in pathogen destruction and degradation to small molecules.

Phagosome The phagosome is a vacuole that forms when extensions of the cell membrane cover and external particle and ingest it into the cell.

Phagocytosis history

Science is supported on fundamental discoveries. One of the main problems to solve during the nineteenth century was, how do we fight infections? There was a major controversy about how the body fights infections. Some people believed it was the humoral immunity (i.e., antibodies) the answer to fight pathogens. Other thought that it was the cellular immunity (i.e., white blood cells) the way to eliminate pathogens. Paul Ehrlich (1854–1915) was the humoral immunity theory leader, and Elie Metchnikoff (Ilya Ilyich Metchnikov; 1845–1916) was the cellular immunity leader. Metchnikov was a Russian pathologist that defined phagocytosis as a defense mechanism (Cavaillon and Legout, 2016). As it turns out both researchers were right. We now know that both arms, the cellular and humoral responses cooperate to fight infections. Elie Metchnikoff shared the Nobel prize in Physiology or Medicine in 1908 with Paul Ehrlich, recognizing this way that together they provided the bases for modern immunology.

First observations for phagocytosis

Elie Metchnikoff was not the first person to observe and report the process of phagocytosis (Table 1), but his deep understanding of the phenomenon let him to outstanding contributions in the field (Cavaillon and Legout, 2016; Rosales, 2018). In the nineteenth century Alexander Ecker, Nathanael Lieberkühn, Giulio Bizzozero saw erythrocytes inside cells (Table 1). Sir William Osler saw carbon particles inside cells while Robert Koch, Paul Albert Grawitz and Alexander Ogston saw bacteria and fungi inside cells (Table 1) (Cavaillon and Legout, 2016; Rosales, 2018).

Phagocytosis theory

During a vacation with his family, Metchnikoff made his original observations in the 1880s while studying marine invertebrate organisms. He stuck rose thorns into starfish larvae and was astonished to see that many special cells were attacking the small thorns (Metchnikoff, 2015; Vikhanski, 2016). Based on his observations, he started realizing that phagocytosis was a defense mechanism. In 1883, he published a paper describing the phagocytosis process (Tauber, 2003). In this publication, he used the word phagocyte, from “phago” (eating) and “cyte” (cell). This term was suggested to Metchnikoff by his friend Carl Friedrich Wilhelm Claus (Cavaillon and Legout, 2016) (Table 2). Succeeding, he moved into immunology and championed the concept of cellular immunity (Metchnikoff, 1890; Cavaillon, 2011). The dispute about humoral and cellular immunity was challenging. There were

Table 1 Initial observations of phagocytosis (Cavaillon and Legout, 2016; Rosales, 2018).

Year	Scientist	Observations
1847	Alexander Ecker	Identified red blood cells (erythrocytes) inside rabbit spleen cells.
1870	Nathanael Lieberkühn	Stated that white blood cells (leukocytes) could ingest red blood cells (erythrocytes).
1871	Giulio Bizzozero	Presented the first pictures of macrophages that had ingested erythrocytes.
1874	Peter Ludwig Panum	Proposed that cell ingestion might represent a method of defense.
1875	Sir William Osler	Reported the intake of carbon particles within alveolar macrophages of miners.
1876	Robert Koch	Identified anthrax bacilli in leukocytes. He believed that the bacteria had invaded the host cells and not that the cells had ingested the bacteria.
1877	Paul Albert Grawitz	Observed that leukocytes ingest fungi.
1881	Alexander Ogston	Described that groups of cocci were inside cells. Introduced the word “ <i>Staphylococcus</i> ” (from the Greek word staphyle) to describe these bacteria resembling a bunch of grapes.

Table 2 Metchnikoff contributions to phagocytosis (Cavaillon and Legout, 2016; Rosales, 2018).

Year	Observations
1882	"If a delicate glass tube, a rose thorn, or a spine of a sea urchin be introduced into one of these larvae, the amoeboid cells of the mesoderm collect around the foreign body in large masses easily visible with the naked eye".
1883	Introduced the term phagocyte in his publication. Described the role of phagocytes during the metamorphosis of tadpole to frog and the disappearance of the tail. Described the process of phagocytosis against foreign microorganisms.
1884	Described the role of phagocytes in daphnia fighting fungal pathogens and in frogs exposed to anthrax bacilli.
1890	Demonstrated that phagocytosis was mainly a function of the microphages (neutrophils).

Table 3 Other observations that supported the phagocytic theory (Bordet, 1886; Gabritschevsky, 1894; Mesnil, 1896; Tchistovitch and Yourevitch, 1908; Tauber and Chernyak, 1989; Cavaillon and Legout, 2016; Rosales, 2018).

Year	Scientist	Observations
1894	George Gabritschevsky	Demonstrated that in rabbits immunized with diphtheria bacilli were within phagocytes 8 h after inoculation of the bacilli. In contrast, in non-immunized rabbits there were still free bacteria. Introduced the word pinocytosis to describe the engulfment of soluble substances.
1896	Félix Mesnil	Demonstrated that in guinea pigs injected subcutaneously with <i>Vibrio cholera</i> the contribution of phagocytosis was much larger than any effect due to humors.
1886	Jules Bordet	Established that phagocytosis was easily observable <i>in vitro</i> .
1901	Ivan Himmel	Reported that by 24 h after injection of <i>Haemophilus ducreyi</i> into guinea pigs, most bacilli had been phagocytized. Intraperitoneal injection of pus from patients infected with <i>Neisseria gonorrhea</i> , guinea pig leukocytes could absorb both free gonococci in pus, and also human leukocytes packed with gonococci. Confirmed that phagocytes could ingest not only microorganisms but also complete cells.
1908	Nicolai Tchistovitch	Demonstrated that the cells responsible for trapping via phagocytosis dust particles in the lung alveoli were alveolar macrophages and not epithelial cells. Found that rabbit alveolar macrophages could phagocytize <i>Erysipelothrix rhusiopathiae</i> and <i>Bacillus anthracis</i> , but not <i>Pasteurella multocida</i> . Coined the word "antiphagin", and showed that pneumococci display a similar capacity to resist phagocytosis.

many opponents to the phagocytic theory, but followers of this theory showed more evidence that supported it (Table 3). Immunization experiments performed by George Gabritschevsky in rabbits; Félix Mesnil and Ivan Himmel in guinea pigs showed that microorganisms were phagocytized by white blood cells (Gabritschevsky, 1894; Mesnil, 1896). Jules Bordet showed *in vitro* phagocytosis (Bordet, 1886) and Nicolai Tchistovitch explore phagocytosis in the respiratory system, showing that macrophages were capable of ingesting dust particles (Tchistovitch and Yourevitch, 1908; Tauber and Chernyak, 1989; Cavaillon and Legout, 2016; Rosales, 2018). However, even though phagocytosis was widely accepted after Metchnikoff won the Nobel prize, its importance was not appreciated until the 20th century.

Phagocytes

There are two types of phagocytes, professional and nonprofessional. Some phagocytes perform phagocytosis very efficiently and were therefore named professional phagocytes by Rabinovitch. These phagocytes include dendritic cells, eosinophils, macrophages, monocytes, neutrophils, and osteoclasts (Rabinovitch, 1995). Professional phagocytes are in charge of eliminating microorganisms and presenting them to cells of the adaptive immune system. Nonprofessional phagocytes include endothelial cells, epithelial cells, and fibroblasts. These cells cannot ingest microorganisms but are essential in eliminating apoptotic cells (Flannagan et al., 2012; Gordon, 2016).

The process of phagocytosis

Phagocytosis is an essential process for nutrition in unicellular organisms. In contrast, in multicellular organisms, just some cell types phagocyte. These cells are called phagocytes. Phagocytes can ingest microbial pathogens, but notably also apoptotic cells. In this way, they contribute to tissue homeostasis by eliminating billions of cells that die every day. Cells use phagocytosis to capture, ingest and destroy all particles larger than 0.5 μm , including cellular fragments and microorganisms.

To understand phagocytosis, we will divide it into steps.

Step 1. Particle recognition: Phagocytes have to recognize the particle to be ingested by using specific receptors (Freeman and Grinstein, 2014). These receptors cooperate for an efficient recognition of the target particle. First, receptors must engage multiple

ligands on the target particle and for achieving this, the receptors must be capable of moving fast in the membrane so that they can aggregate around the target (Rosales and Uribe-Querol, 2017).

Step 2. Particle ingestion: Phagocytic receptors initiate a cascade of signals, change lipids in the cell membrane, and organize the actin cytoskeleton to grow the cell membrane around the particle to be ingested (Freeman and Grinstein, 2014).

Step 3. Early phagosome formation: A phagosome is the vacuole formed to bring the particle inside the cell. Receptors work together in sequential order and cooperate to form the phagosome (Ostrowski et al., 2016). The phagosome rapidly fuses with early endosomes to change its membrane composition.

Step 4. Late phagosome formation: This process happens when the early phagosome fuses with vesicles coming from the endoplasmic reticulum, and the Golgi complex (Wähe et al., 2010; Campbell-Valois et al., 2012; Nunes et al., 2012; Guido et al., 2015).

Step 5. Phagolysosome formation: This process is the fusion of an intermediary phagosome with lysosomes and changing the membrane and interior components. The phagosome turns into a microbicidal vacuole, the phagolysosome (Fairn and Grinstein, 2012; Niedergang and Grinstein, 2018).

Step 1. Particle recognition: Phagocytosis receptors

Phagocytes must recognize many different particles that could potentially be ingested, including all classes of pathogens and dying cells. Phagocytes investigate their surroundings for phagocytic particle targets by extending finger-like membrane projections called filopodia. Various receptors on the plasma membrane of phagocytic cells recognize these particles and then initiate the process of phagocytosis. A single phagocyte can express several receptor types, and they cooperate for the recognition and ingestion of the particle. Phagocytic receptors are classified as non-opsonic receptors or as opsonic receptors. Non-opsonic receptors can directly recognize pathogen associated molecular patterns (PAMPs) and also activate the phagocytosis process (Table 4) (Uribe-Querol and Rosales, 2020). These receptors include C-type lectins, such as Dectin-1 (Dambuza and Brown, 2015), Dectin-2, DC-SIGN and Mincle; and also scavenger receptors (Canton et al., 2013). Toll-like receptors (TLRs) can also detect molecular patterns on pathogens, but they are not phagocytic receptors (Kawai and Akira, 2011). However, TLRs can cooperate with phagocytic receptors to make the phagocytic process more effective (Iwasaki and Medzhitov, 2015).

Opsonic receptors recognize host-derived soluble molecules that bind to foreign particles and target them for ingestion (Table 5). These soluble molecules are called opsonins and include antibodies, complement, fibronectin, mannose-binding lectin, and milk fat globulin (lactadherin) (Flannagan et al., 2012) (Table 5; Fig. 1A and B).

Table 4 Non-opsonic receptors.

	Examples	Ligand	References
Pattern-recognition receptors	CD36 class B scavenger receptor	Plasmodium falciparum-infected erythrocytes. Recognize both apoptotic and microbial polyanionic ligands	Patel et al. (2004) and Canton et al. (2013)
	Dectin-1	Receptor for fungal beta-glucan Polysaccharides of some yeast cells	Herre et al. (2004a), Herre et al. (2004b), and Dambuza and Brown (2015)
	DC-SIGN	Fucosylated glycans	van Liempt et al. (2006)
	Mannose receptor	Mannan	Ezekowitz et al. (1990)
	MARCO	Bacteria	van der Laan et al. (1999)
	Mincle	Trehalose dimycolate of mycobacteria	Ishikawa et al. (2009)
	MCL	Trehalose dimycolate α -Mannan	Lobato-Pascual et al. (2013)
Apoptotic body receptors	Scavenger receptor A	Lipopolysaccharide (LPS) on some gram-negative bacteria and on <i>Neisseria meningitidis</i> , lipoteichoic acid. Recognize both apoptotic and microbial polyanionic ligands	Peiser et al. (2000) and Peiser et al. (2006)
	α V β 3 and lactadherin	MFG-E8 ^a	Hanayama et al. (2002)
	α V β 5	Apoptotic cells	Albert et al. (2000)
	BAI-1 ^a	Phosphatidylserine	Park et al. (2007)
	CD14	Phosphatidylserine (putative ligand)	Devitt et al. (1998)
	CD36	Oxidized lipids	Greenberg et al. (2006)
	Stabilin-2	Phosphatidylserine	Park et al. (2008)
	TIM-1 ^a	Phosphatidylserine	Kobayashi et al. (2007)
	TIM-4 ^a	Phosphatidylserine	Kobayashi et al. (2007)

^aBAI-1, brain-specific angiogenesis inhibitor 1; MFG, milk fat globule; TIM, T cell immunoglobulin mucin.

Table 5 Opsonic receptors.

	Examples	Ligand	References
Complement receptors (CR)	CR1 (CD35)	C1q, C4b, C3b, Mannan-binding lectin	van Lookeren Campagne et al. (2007)
	CR3 (α M β 2, CD11b/CD18, Mac-1)	Bind to iC3b deposited on the particle after complement activation	
	α 5 β 1 (CD49e/CD29)	Fibronectin, vitronectin	Blystone et al. (1994)
	CR4 (α V β 2, CD11c/CD18, gp190/95)	Bind to iC3b deposited on the particle after complement activation	Ross et al. (1992)
Fc receptors (FcR)	Fc α RI (CD89)	IgA1, IgA2	Bakema and van Egmond (2011)
	Fc ϵ RI	IgE	
	Fc γ RI (CD64)	IgG1 = IgG3 > IgG4	Rosales and Uribe-Querol (2013a)
	Fc γ RIIa (CD32a)	IgG3 $\geq$ IgG1 = IgG2	Rosales and Uribe-Querol (2013b)
	Fc γ RIIIa (CD16a)	IgG	Nimmerjahn and Ravetch (2011)

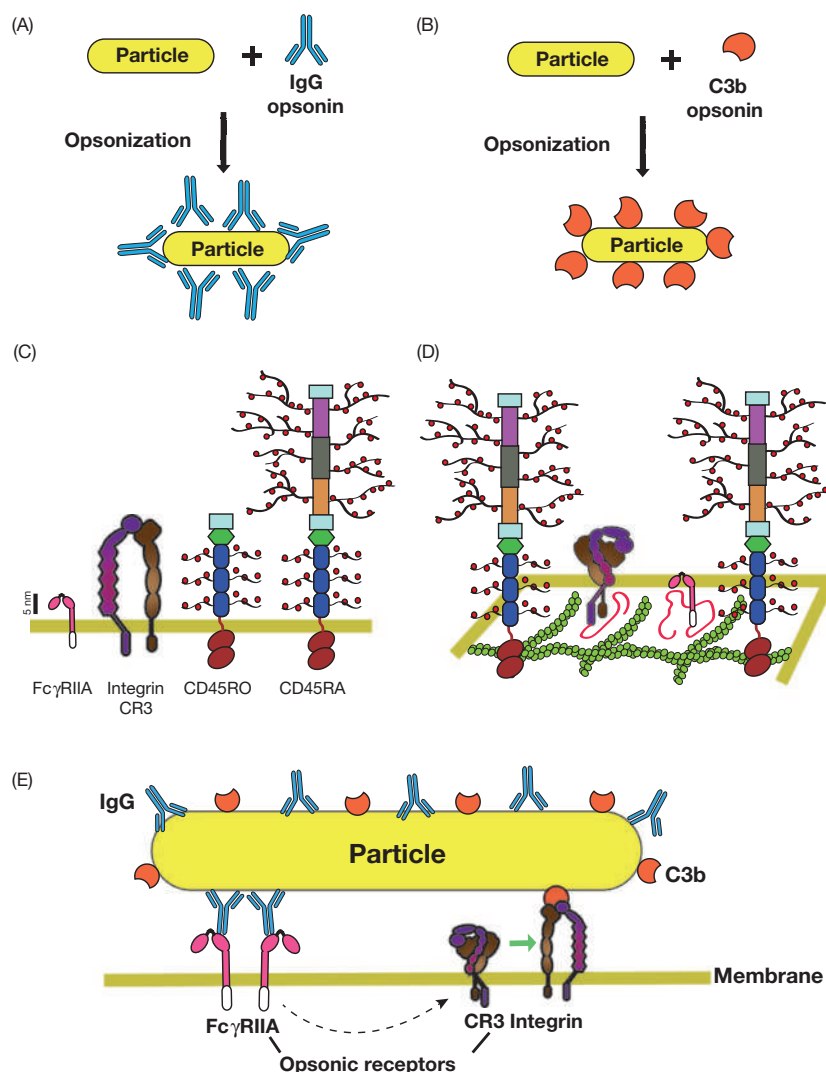


Fig. 1 Particle recognition and cooperation among phagocytic receptors. (A) Opsonization process with antibodies. (B) Opsonization process with complement molecules. (C) Phagocytic receptors, like opsonic receptors for antibody (Fc γ RIIA) and for complement (Integrin CR3) are tiny molecules of only few nanometers. In contrast, transmembrane glycoproteins, such as phosphatases CD45RO and CD45RA, are much longer molecules, of about 40–90 nm, and are usually rigid molecules. (D) At resting state, receptors cannot diffuse freely throughout the membrane. Their movement is restricted by fences of transmembrane glycoprotein attached to an actin mesh. (E) Cooperation among phagocytic receptors. Most phagocytic receptors, such as Fc γ RIIA and receptors for complement (Integrin CR3) cooperate to bind the particle to be ingested. Fc γ R aggregation triggers an inside-out signal that activates integrins (dashed arrow).

By a process of opsonization, antibodies or complement proteins, cover a pathogen and can be recognized by specific receptor on phagocytes (Fig. 1A and B). Antibody receptors and complement receptors (CR) are some of the most studied phagocytic receptors. Antibody receptors are also called Fc receptors (FcR) and are a family of proteins expressed on white blood cell (leukocytes) membranes (Rosales, 2007). FcR bind the Fc portion of antibodies and can induce phagocytosis and other cellular responses (Nimmerjahn and Ravetch, 2011; Rosales and Uribe-Querol, 2013a, b, 2017). Complement receptors recognize the complement components, such as iC3b, deposited on phagocytic targets surfaces (van Lookeren Campagne et al., 2007). There are three types of complement receptors: (1) The immunoglobulin Ig-superfamily member CR1g (Helmy et al., 2006). (2) The short consensus repeat (SCR) modules that code for CR1 and CR2 (Tohyama and Yamamura, 2006; Rosales, 2007). And (3) the $\beta 2$ integrin family members CR3 and CR4 (Tohyama and Yamamura, 2006; Rosales, 2007) (Table 5).

Many cells from our body start a cell dead program called apoptosis. Cells enter apoptosis when they are faulty or when they are no longer needed for a certain function. Apoptosis is a way to get rid of defective cells, but the dead cells must be eliminated in order to maintain homeostasis. Phagocytes are also responsible for removing apoptotic cells. Apoptotic cells release molecules (ATP, lysophosphatidylcholine, and sphingosine 1-phosphate) that function as attractants (chemoattractant) for phagocytes. Apoptotic cells also start expressing molecules on their membrane surface, such as phosphatidylserine and oxidized lipids, that are not normally on the external part of the membrane. These molecules function as an “eat me” signal for phagocytes. There are specific receptors that recognize apoptotic cells such as TIM and stabilin-2 (Table 4) (Rosales and Uribe-Querol, 2017; Uribe-Querol and Rosales, 2020).

Step 2. Particle ingestion: Intracellular activation and actin cytoskeleton

Phagocytes investigate their surroundings for particles to ingest by extending filopodia. These membrane projections covered with receptors contain principally linear actin. When a particle interacts with phagocyte receptors, a signaling cascade inside the cell is triggered to activate particle ingestion. Phagocytic receptors aggregate and cooperate for an efficient recognition of the target particle. The cooperation depends on receptors binding affinity and their relative mobility within the membrane (Patel and Harrison, 2008; Flannagan et al., 2010). Most phagocytic receptors, like receptors for complement (Integrin CR3) and receptors for antibody (Fc γ RIIA), are small molecules that lengthen only a few nanometers from the plasma membrane (Fig. 1C). Glycoproteins like CD45 are much longer and stiff molecules (Fig. 1C). In the resting state, phagocytic receptors cannot diffuse freely throughout the membrane (Fig. 1D). Glycoproteins are attached to an actin mesh that restricts receptor movement (Fig. 1D). Receptors also cooperate to ingest particles. Fc γ R aggregation triggers an inside-out signal that activates integrins (Fig. 1E). Thus, particle recognition by receptor binding and activation are very active processes.

After phagocyte receptors bind to the target, their activation leads to changes in the membrane and the actin polymerization (Fig. 2). At the contact point, the membrane curves and forms a structure called the phagocytic cup. As more phagocytic receptors get engaged around the particle, the cell extends pseudopodia with branched actin fibers around the particle. Next, within a few minutes, the membrane surrounds the particle, leaving a new phagosome formation (Fig. 2).

Actin remodeling is crucial in particle ingestion. The steps of actin remodeling during phagocytosis are as follows. After a target particle is detected, the membrane-associated actin needs to be depolymerized (Fig. 2A). Then, nucleation and actin filament polymerization take place in order to extend pseudopodia around the particle. The Arp2/3 protein complex mediates actin nucleation and actin polymerization (Prehoda et al., 2000) (Fig. 2B). Finally, actin depolymerizes from the phagocytic cup base and polymerizes at the pseudopodia distal end (Fig. 2C). At this distal point is where the membrane will close forming the new phagosome (Fig. 2D), which will detach from the plasma membrane (Fig. 2E) (García-García and Rosales, 2002; Rosales, 2007; Rosales and Uribe-Querol, 2017). After phagosome detachment, the plasma membrane returns to its initial state (Fig. 2F).

Soon after phagosome formation, the process of phagosome maturation starts. This process comprises the events for a phagosome to become a phagolysosome. The process involves successive fusion and fission interactions between the new phagosome and other vesicles called endosomes and lysosomes. At the end of the process, the phagolysosome is a vesicle capable of destroying the particle ingested. It contains a very acidic and degradative contents (Fig. 3) (Fairm and Grinstein, 2012; Canton, 2014).

Step 3. Early phagosome formation

Early Phagosome. The new phagosome quickly gets the characteristics of early endosomes. It fuses with sorting and recycling endosomes (Levin et al., 2016). Membrane fusion between the phagosome and endosomes requires Rab5 (Fig. 3B). This protein is a membrane small GTPase. With the fusion, the interior of the early phagosome becomes a little acidic (pH 6.1–6.5). Nevertheless, this it is not very harmful to microbial pathogens inside the phagosome. The transition from an early to a late phagosome requires Rab5. EEA1 is another protein of the early phagosome that recruits proteins like Rab7 (Christoforidis et al., 1999). Even though many early endosomes fuse to the new phagosome, its size does not change. The size is maintained due to retrieving recycling vesicles to endosomes and to the trans-Golgi network (Fig. 3). The phagosome interior becomes more acidic as a result of the gradual accumulation of active V-ATPase proteins on its membrane. The V-ATPase is an enzyme that moves protons (H⁺) into the interior of the phagosome, using ATP from the cytoplasm as an energy source (Marshansky and Futai, 2008) (Fig. 3). As the phagosome matures, Rab5 is lost, and Rab7 appears on the membrane (Vieira et al., 2003). Rab7 is also a small membrane GTPase. Membrane fusion between the phagosome and endosomes requires Rab7 (Fig. 3C).

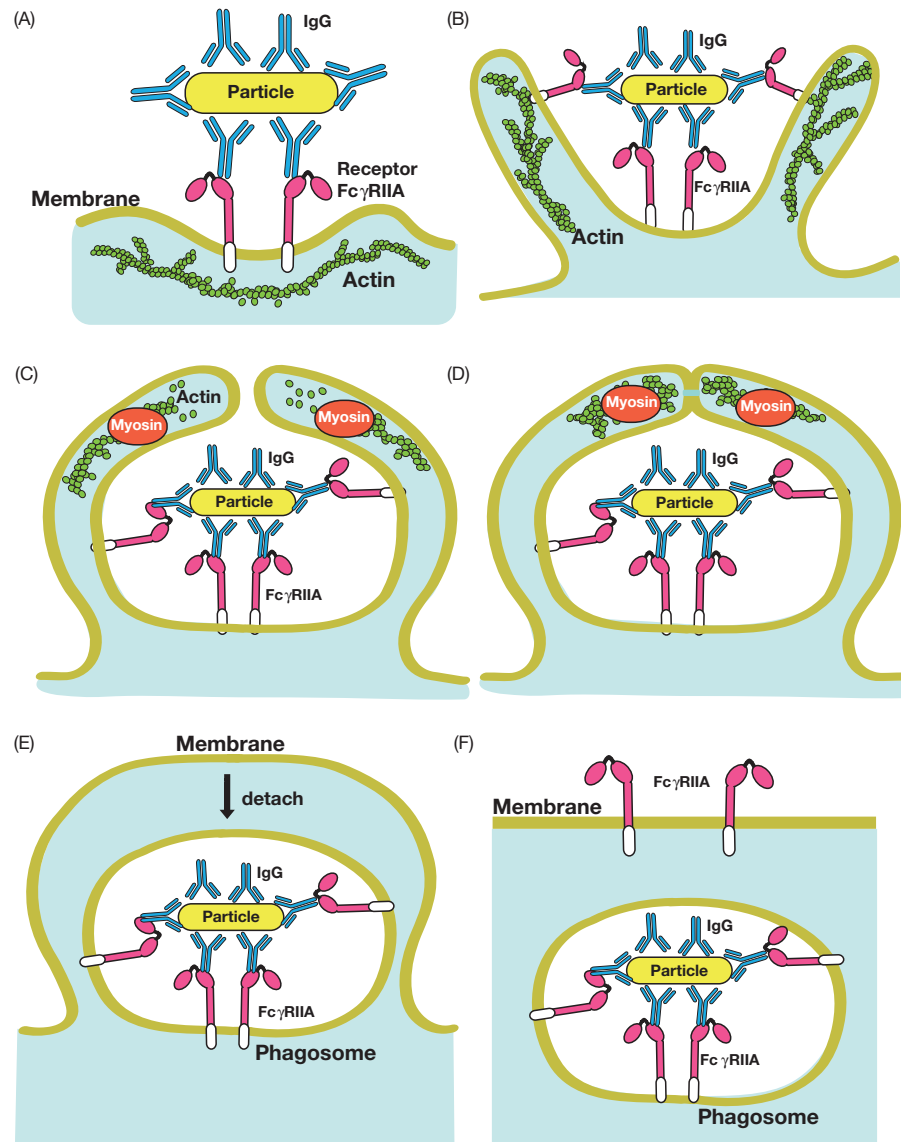


Fig. 2 Particle ingestion. (A) FcγRIIA target particle recognition and the actin cytoskeleton is disrupted at the site where the phagocytic cup starts to develop. (B) The cell extends pseudopodia, which contain new branched actin fibers. (C) Pseudopodia grows and forms the phagocytic cup. (D) The membrane surrounds the particle and fuses at the distal end. (E) New phagosome detach from the plasma membrane. (F) At the last step, depolymerization of actin filaments from the base of the nascent phagosome may facilitate curving of the membrane around the particle and provide room for fusion of internal vesicles, a source of endomembranes.

Step 4. Late phagosome formation

Early to late phagosome formation requires Rab5, but this molecule is lost from the membrane at the late stage (Kitano et al., 2008; Gutierrez, 2013). Instead, Rab7 accumulates and becomes a marker for the late phagosome stage formation. Rab7 recruits new proteins like Rab-interacting lysosomal protein (RILP) (Fig. 3B and C). RILP mediates the fusion between the late phagosomes and lysosomes (Jordens et al., 2001; Harrison et al., 2003). Also, the late phagosome recruits more V-ATPase. The increasing number of this ATPase contributes to a more acidic (pH 5.5–6.0) interior of this later stage of phagosome maturation (Kinchen and Ravichandran, 2008) (Fig. 3). Finally, the late phagosome also recruits lysosomal-associated membrane proteins (LAMPs) and luminal proteases (cathepsins and hydrolases) (Fig. 3C) (Fairn and Grinstein, 2012; Canton, 2014).

Step 5. Phagolysosome formation

The phagolysosome formation in the last stage in the maturation process. This step involves the fusion of late phagosomes with lysosomes. The phagolysosome is the final microbicidal organelle (Levin et al., 2016). It possesses various complicated mechanisms

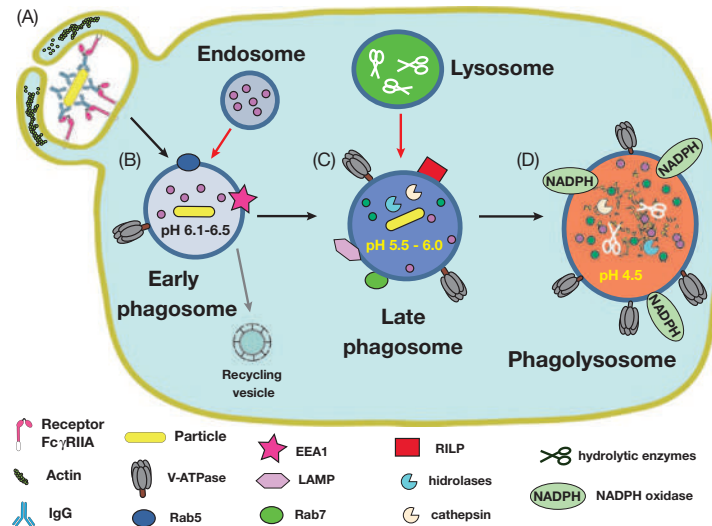


Fig. 3 Phagolysosome maturation. After particle ingestion (A), the new phagosome gets transformed into a phagolysosome by sequential interactions with endosomes and lysosomes. Phagolysosome maturation can be described in three stages: early phagosome (B), late phagosome (C), and phagolysosome (D). In this maturation process, membrane changes to include molecules that control membrane fusion, such as the GTPases Rab5 and Rab7. During maturation stages, these vesicles become more acidic by the action of a proton-pumping V-ATPase. Also different degradative enzymes, such as cathepsins, proteases, lysozymes, and lipases are in charge of degrading microbes. *EEA1*, early endosome antigen 1; *LAMP*, lysosomal-associated membrane protein; *RILP*, Rab-interacting lysosomal protein; *NADPH*, nicotinamide adenine dinucleotide phosphate oxidase.

directed to eliminate and degrade microorganisms. V-ATPase molecules on its membrane continues to accumulate, so the phagolysosome interior is highly acidic (pH as low as 4.5) as more protons (H⁺) are translocated into the lumen of the phagosome (Marshansky and Futai, 2008). The acidic content of the phagosome creates an adverse environment for most microorganisms. The low pH obstructs the normal metabolism of several bacteria and fungi and prevents the use of many essential microbial nutrients (Jabado et al., 2000). Phagolysosome also contains many hydrolytic enzymes. Some of these enzymes are various cathepsins, proteases, lysozymes, and lipases (Kinchen and Ravichandran, 2008). Other recruited molecules at this point are scavenger proteins (Fig. 3). Scavenger proteins can kill bacteria because they sequester important nutrients for the bacteria. For example, the scavenger protein lactoferrin traps the iron required by some bacteria to live (Masson et al., 1969) (Fig. 4). The phagolysosome also produces

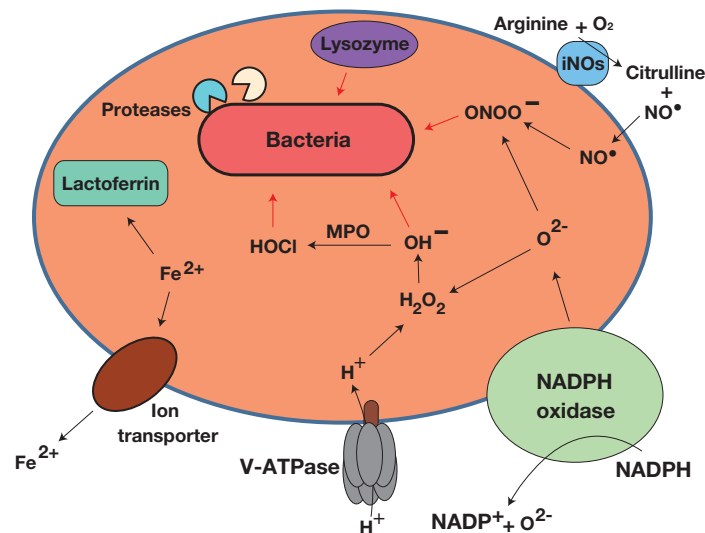


Fig. 4 Inside a phagolysosome. Very low pH is detected inside phagolysosomes. The low pH depends on the V-ATPase, that translocates protons (H⁺) into the lumen of the phagosome. Reactive oxygen and nitrogen species are produced. The NADPH oxidase is an enzymatic complex that produces superoxide (O²⁻). H₂O₂ can react with O₂ to generate other radicals, such as hydroxyl radicals (OH[•]). Myeloperoxidase (MPO) can transform H₂O₂ into HOCl. Also, nitric oxide radicals (NO[•]) are produced by the activation of the nitric oxide synthase 2 (iNOS). NO[•] reacts with O²⁻ and forms ONOO⁻. Lactoferrin captures Fe²⁺ that is essential for bacterial growth. Proteases and lysozyme degrades cell wall peptidoglycan of the bacteria. Red arrows are harmful molecules for the bacteria. Black arrows are chemical reactions or proton movements.

reactive oxygen species (ROS) and reactive nitrogen species. It recruits NADPH oxidase (Panday et al., 2015), a protein complex that produces superoxide ($O_2^{\cdot-}$) (Babior, 2004). Superoxide can dismutate to hydrogen peroxide (H_2O_2). In turn, H_2O_2 can react with $O_2^{\cdot-}$ to generate other radicals, such as hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen (Winterbourn, 1995; Minakami and Sumimoto, 2006). The myeloperoxidase enzyme combines H_2O_2 with Cl^- ions into hypochlorous acid (HOCl) (Nauseef, 2014) (Fig. 4). The various forms of ROS can damage DNA, proteins and lipids of microorganisms (Lam et al., 2010). Also, peroxynitrite ($ONOO^-$) an extremely reactive specie, is formed by combining $O_2^{\cdot-}$ with nitric oxide radicals ($NO^{\cdot}$). Nitric oxide radicals are produced by the nitric oxide synthase 2 (Bogdan et al., 2000). This enzyme converts arginine and oxygen into citrulline and $NO^{\cdot}$ (Fig. 4). $NO^{\cdot}$ is produced on the cytoplasmic side of phagosomes, but it can diffuse across membranes and accumulate inside the phagosome (Webb et al., 2001). As mentioned earlier, once inside the phagosome, $NO^{\cdot}$ can react with $O_2^{\cdot-}$ to generate the highly toxic peroxynitrite ($ONOO^-$) that can alter proteins and DNA of ingested microorganisms (Richardson et al., 2009, 2011). Also, $NO^{\cdot}$ can directly impair bacterial enzymes and affect microbial growth (Grosser et al., 2016).

Conclusion

Phagocytosis is a complex and elegant process performed by specific cells called professional phagocytes. These cells include monocytes, macrophages, neutrophils, dendritic cells, osteoclasts, and eosinophils.

Phagocytosis is crucial for pathogen elimination, dying cell elimination, tissue homeostasis, and control of essential inflammation aspects.

We have presented the main steps of phagocytosis to provide a global picture of the whole process. There are still many questions unanswered about the details of how the final phagolysosome completes its killing functions.

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Dendritic Cells

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Introduction

A concept of dendritic cells (DCs) as a distinct subpopulation of phagocytes was introduced by Ralph Steinman in his seminal work (Steinman, 1991), however many tissue-specific DCs have been discovered decades before. DCs are a class of antigen presenting cells (APCs) that function at the interface of innate and adaptive immune responses, constantly surveying the microenvironment and providing information to T cells. In contrast to macrophages which degrade antigens, DCs activity is biased towards antigen processing for presentation. DCs are widely distributed across different tissues, but their density is relatively low (Shortman, 2020). Different DC subtypes are responsible for the initiation, regulation of the immune response or for maintaining immune tolerance. Major DC subsets are categorized by their ontogeny into conventional DCs (cDCs) or plasmacytoid DCs (pDCs). Functionally, these subsets are characterized by their different ability to recognize molecular patterns, circulate through the body (pDCs) or to reside in thymus and lymphoid tissues (cDCs) and can be distinguished by marker expression and transcription factor set. cDCs are subcategorized into cDC1 or cDC2 types depending on their XCR1 and CD172 expression. A distinct subtype of DC—MoDCs (monocyte-derived DCs)—was shown to differentiate from monocytes during inflammation. A typical characteristic of all DC subtypes is their maturation stage. Immature DCs reside in barrier tissues and express low levels of co-stimulatory molecules. These cells demonstrate poor ability to activate T cells until they uptake an antigen. In a classical model, antigen uptake leads to DC maturation, migration to lymphoid organs, upregulation of MHC class I and class II and other membrane-associated and secreted molecules by means of which DCs drive activation and functional polarization of T cells. However, a concept of “steady-state condition” and “inflammatory condition” dichotomy (Boltjes and van Wijk, 2014) in case of DC maturation is complicated by the fact that DC still can mature in steady state to induce tolerance in peripheral Tregs (Iberg and Hawiger, 2020). Moreover, cDC1s provide constitutive expression of IL-12, promoting Th1 differentiation signaling even in the absence of antigens (Hilligan and Ronchese, 2020) and making Th1 response the ‘default-type’ response in contrast to Th2-response which requires potent antigenic signaling. Many tools have been applied to study DCs in vitro and in vivo, including FACS, single-cell RNAseq, culture with danger-associated and pathogen-associated molecular patterns, co-culture with T cells, genetic knockout (including Cre-based conditional knockout), DC subset depletion and various reporter visualization techniques. A substantial pool of studies has been focused on DC-like cells derived in vitro from blood monocytes in conditioned media, however, sophisticated methods are used to isolate rare DC populations from tissues, such as walkout method (induced migration of DC out of biopsied samples in culture) (Sun et al., 2020).

Phenotype

Each DC subset is characterized by its own set of markers but is lacking lineage markers (such as CD3, CD14, CD15, CD56, CD19 and CD20). DC phenotype differs between species, human DCs lack CD8 molecule while a significant subset of murine DCs expresses high levels of CD8 $\alpha\alpha$. However, murine CD8⁺ DCs express the chemokine receptor XCR1 which is also found on a population of human DC1s with similar characteristics (Guilliams et al., 2014). DC1 subset, in addition to XCR1, is also distinguished by the surface expression of CD11c, BTLA, CD141, CLEC9A, NECL2 and DEC-25. In nonlymphoid tissues cDC1s also express CD103 (Hilligan and Ronchese, 2020). DC2 subset is characterized by surface molecules CD1c, CD172a, DCIR2, CLEC10A, SIRP α , CD301b (MGL2), CD206, PDL2 and CD11b. SIRP α was shown to be a characteristic cDC2 marker, distinguishing this population from cDC1 in human bone marrow and blood (Hilligan and Ronchese, 2020). Two subsets of human blood cDC2s were identified based on the expression of CD32B (also CD5^{high}) or CD163 and CD36 (monocyte-like CD14⁺/CD5^{low} cells termed as DC3) adding to the complexity of DC nomenclature. Ironically, absence of strong subpopulation markers within cDC2 subset

highlights the high heterogeneity of these cells, since no marker can be used to distinguish one population from another while it is known that many subpopulations exist. Single-cell transcriptional studies have demonstrated high variability of cDC2s (Brown et al., 2019).

pDCs express B220, CD123, CD303, CD304, BST2 and Siglec-H (Iberg and Hawiger, 2020; Amon et al., 2020). The exact phenotype and transcriptional profile of each DC subset is governed by the state of development and activation and relies on microenvironmental conditions. E.g., lymphoid tissue-resident DCs express higher levels of H2-DMA and H2-DMab compared to lymph nodes migratory DCs reflecting resident cells ability to process antigens in contrast to mature cells (Hilligan and Ronchese, 2020).

MoDCs in mice express CD64, a monocyte/macrophage marker, upon inflammatory conditions. These cells also bear Ly6C, CD11c and CCR2 (Hilligan and Ronchese, 2020).

Langerhans cells (LCs) represent about one tenth of the epidermis cells in the skin are not DCs, however they resemble some DC properties and phenotypic features. Ontogeny studies and ability of LCs to proliferate in a differentiated state clearly indicate their macrophage origin. LCs can be identified by the expression of CD11c, CD32, CD45, FcεR1, CD1a, CD207 (langerin), CD324, CD326, HLA-DR and lack of XCR1 and CD103 expression (Deckers et al., 2018).

Tissue distribution

Differentiated DCs can be found in hematopoietic tissues and lymphoid organs, as well as in barrier tissues with a varying frequency. Spleen is highly populated with CD141+ cDC1s, bone marrow and blood and cord blood contain CD1c+ cDCs, while tonsils show the highest frequency of pDCs (Amon et al., 2020).

Lymphoid tissue-resident DCs are non-migratory cells that are initially seeded in spleen, lymph nodes or Peyer's patches and exist here on par with tissue-derived DCs. These DCs express low levels of CCR7 and MHC II and high levels of CD11c in steady state but upregulate CCR7 during maturation. cDC1 and cDC2 are positioned at separate regions inside lymphoid tissues due to differential expression of CXCR3 and CXCR1. Resident cDC2s are mostly localized in the proximity to subcapsular sinuses of lymph nodes where they sample soluble antigens arriving with the lymph flow. cDC1s reside in the paracortex where they uptake cell-associated antigens for subsequent cross-presentation in association with MHC class I molecules (Leon and Lund, 2019; Eisenbarth, 2019). Resident cDC1 and cDC2 also have distinct transcriptional profile in contrast to migrating cDC subpopulations in which transcriptome to a greater extent depends on the activation signals than on the ontogeny (Hilligan and Ronchese, 2020). Migratory DCs have a short lifespan and die by apoptosis in 2 days after migration as demonstrated for cDC2s (Tomura et al., 2014).

Human skin contains all three DC subsets and DC-like cells of monocyte origin in the dermis where DC density is highest in the superficial layers and decreases in deeper layers (Ronchese et al., 2020). Superficial DCs show high expression of fascin, an actin-binding protein responsible for cells motility. cDC1 population is underrepresented in the murine and human skin and DC2s are the most abundant. A population of CD301+ DCs inhabits a specific niche in the skin in perivascular areas where these DCs are able to transfer allergens from blood vessels to dermal mast cells, contributing to anaphylactic reactions (Ronchese et al., 2020).

Upon labeled antigen injection, cDC2s make up the majority of cells that reach draining lymph nodes. In mice, skin DCs also include additional cDC population lacking CD11b expression. Many of cDCs in the skin express chemokine receptor XCR1 (lymphotactin receptor/G-protein-coupled receptor 5) and produce chemokine CXCL10 thereby actively interacting with CD8+ T cells and NK cells, including cross-presentation to CD8+ T cells. A minor subpopulation of DCs, CD326+ cells also recruits neutrophils into infected skin via synthesis of VEGFα (Ronchese et al., 2020). CCR7 expression by the skin DCs of the skin allows them to migrate to lymphoid organs (Amon et al., 2020; Hilligan and Ronchese, 2020). While skin cornified layer is sampled by LCs, topically applied antigens can reach hair follicles where they are readily accessed by dermal cDC2s that provide tolerance to commensal microbiota in steady state but initiate IFNγ-mediated inflammatory response if the skin integrity is breached. Importantly, SKH1 hairless mice are unable to respond to epicutaneous OVA application (Tordesillas et al., 2018). Dermal DCs provide a pathway to airborne allergen sensitization, as skin application of house dust mite allergens resulted in antigen uptake by DC2s with consequent initiation of airway inflammation (Ronchese et al., 2020). Filaggrin deficiency which impairs skin barrier function may result in food allergy. This suggests an important role of skin DCs in the atopic march—a progression of allergic diseases from atopic eczema to food allergy, allergic rhinitis and asthma. A crosstalk between skin LCs and underlying cDCs allows the transfer of antigen-MHC class II complexes from LCs to more mobile cDCs. Notably, LCs, due to their proximity to commensal skin microbiota, have been proposed to be intrinsically tolerogenic which is supported by a series of experiments with LC depletion leading to Treg failure (Hilligan and Ronchese, 2020; Ronchese et al., 2020). However, upon proper environmental signals LCs excel in naïve T cell activation via cross-presentation pathway. Thus, skin commensals along with LCs and DCs constitute an immunoregulatory axis which is necessary to immune tolerance and immune protection in the skin.

DCs located in the lung tissues are presented by cDC1 and cDC2 populations with a variable expression of CD207. Human lungs are characterized by a relatively low proportion of cDC1. Lung cDC2s actively migrate due to the CCR7 expression and are distinguished by dynamically changing metabolic profile which reflects their ability to react rapidly to changing environmental cues in lung epithelial barriers (Amon et al., 2020; Hilligan and Ronchese, 2020).

Within the gut, distinct DC populations can be found in Peyer's patches (PP), mesenteric lymph nodes or lamina propria (LP) and lymphoid aggregates (Sun et al., 2020). DCs of intestinal LP are characterized by CCR9 expression. Intestinal cDC2s show

relatively high migratory capability which allows them to relocate into draining lymph nodes where they induce T cell responses, including cross-presentation. cDC2s also comprise a majority of DCs in small intestine LP, while colonic LP DCs are presented mostly by cDC1s. Correspondent ratios of migrating DC subsets is identified in lymph nodes that drain different sites along the gut. Diversity of gut DCs is extended by the lymphoid tissues aggregated along the intestine where cDC1s and pDCs dominate the DC population. In PPs, distribution of DC subtypes resembles that of lymph nodes with cDC2s localized in subepithelial dome (providing direct access to antigens) and cDC1s localized in T-cell regions in the deeper compartment, interfollicular zone (Sun et al., 2020). In contrast to lungs, intestines are densely populated with commensal microbiota and gut DCs are less motile and reactive in steady-state, thus maintaining microbiota-tolerant environment. Intestinal DCs project their dendrites into the intestinal lumen and also are able to obtain antigens via gap junctions indirectly, from macrophages (Hilligan and Ronchese, 2020). Alertness of mucosal DCs along alimentary tract may depend on quantity of commensals and pathobionts. DCs as central regulators of immune responses provide a balance between tolerance and inflammation and accompanying dynamic changes in microbiota composition. This can be described as an axis comprising microbiota, DCs, ILCs, T cells and IgA-producing B cells in a constant cross-talk with each other. Lymphotoxins α and β play crucial role in orchestrating of this axis because LT α or LT β ablation in ILCs results in reduction of IgA levels while LT β controls inducible nitric oxide synthase (iNOS) expression in DCs and impacts intestinal microbiota composition (Kruglov et al., 2013). Induction of oral tolerance among other factors relies on ILC3s that secrete GM-CSF, promoting intestinal cDC population to secrete retinoic acid (Sun et al., 2020).

In the oral cavity, sophisticated interactions between microbiota and immune cells are exemplified by bacteria *Porphyromonas gingivalis* which induce subinflammatory conditions readily converting to inflammation upon excessive growth of this bacteria (Meghil and Cutler, 2020).

Development

Steady-state DC half-lives is thought to be a few weeks, and senescent cells need to be replaced by the newcomers or by proliferating tissue-resident cells (Merad and Manz, 2009). Either way, a progenitor cell is needed to migrate into the tissue. Many insights into DC biology were obtained when the culture method for DC-like cells generation from blood monocytes or HSCs was proposed. The essence of this method was the addition of granulocyte/macrophage colony stimulating factor (GM-CSF) and IL-4 or TNF into culture media containing CD14+ monocytes or CD34+ HSCs (Romani et al., 1994; Sallusto and Lanzavecchia, 1994) to produce cells now referred to as MoDCs or LC-like cells (Luo and Dalod, 2020). Later, DC studies were expanded through identification of several mutations that affected DC development in human patients. Further discovery of many DC subsets complicated the knowledge about DC development. First experiments demonstrated that both CD8+ and CD8- DCs can be produced from either oligopotent common lymphoid (CLP) or myeloid (CMP) progenitors, having multipotent CD34+ hematopoietic stem cells (HSCs) as initial founders (Shortman, 2020). Moreover, there is some experimental evidence that cDC1 and other DC subtypes commitment occurs in early hematopoiesis independently of the lymphoid and myeloid lineages segregation (Luo and Dalod, 2020; Naik, 2020). However, it is largely accepted now that the majority of DCs derive from myeloid precursors. The last progenitor cell from which all DC subsets can be differentiated was described as granulocyte/macrophage progenitor (GMP) expressing CD34 + CD38 + Lin - CD10 + CD45RA+ phenotype in the bone marrow (Lee et al., 2015). Further development of DCs is associated with CD34 downregulation, since a circulating lineage - HLA-DR + CD100 + CD34^{int} cDC progenitor was identified which was able to proliferate and give rise to cDC1 and cDC2 (Amon et al., 2020). Thus, in contrast to cultured DC-like cells, late cDCs progenitors in vivo do not belong to monocytes. Instead, bone marrow precursors seed peripheral lymphoid organs via bloodstream to give rise for cDC1 and cDC2 populations. A commitment to cDC1 or cDC2 seems to take place in bone marrow before precursors enter the circulation (Schlitzer et al., 2015), but some data support an opinion that uncommitted pre-cDCs can be found in bone marrow and blood (Amon et al., 2020).

A series of experiments dedicated to thymic DCs produced ambiguous results indicating that while major source of thymic DCs are bloodstream precursors, there may exist a subset of DCs that has an intrathymic origin. Genetic and pharmacological manipulation of the transcription factors shed light onto identity of distinct DC types. As mentioned above, studies of mutations in human cells also provided significant data on the requirements of DC differentiation. E.g., *TCF4* loss in patients with Pitt-Hopkins syndrome results in decreased pDC differentiation, as well as *IKZF1* mutations. IRF8 deficiency affects development of most DC subtypes (Luo and Dalod, 2020).

In the bone marrow, the fate of early DC precursors (or common DC precursors, CDPs) is largely dependent on the cytokine Fms-like tyrosine kinase 3 ligand (FLT3L). FLT3L is structurally homologous to stem cell factor (SCF) and colony stimulating factor 1 (CSF-1) and regulates proliferation of early hematopoietic cells. CD135, the FLT3L receptor, is downregulated in circulating progenitors, as well as CD116 (GM-CSFR) (Amon et al., 2020). However, FLT3L has been shown to stimulate the DC proliferation in lymphoid tissues in response to inflammatory cues. Experimental administration of FLT3L resulted in the increase of circulating cDC1s and cDC2s that may represent a part so-called "emergency myelopoiesis" in response to systemically disseminated microbial pathogens. This leaves uncertainty about cells that originate the emergency response: experimental evidence suggests that, e.g., bone marrow epithelial cells or tissue macrophages can be the source of myelopoietic factors needed for the proliferation of DC progenitors (Bieber and Autenrieth, 2020). Common transcriptional factor that differentiates cDCs from pDCs is zinc finger transcription factor zBTB46 (Hilligan and Ronchese, 2020). The development of DC1s is directed by the transcription factors Interferon regulatory factor (IRF8), Id2 and Basic Leucine Zipper ATF-Like Transcription Factor 3 (BATF3), while DC2s development

is directed by Zeb2, IRF4, Klf4 and Notch2 (Amon et al., 2020; Hilligan and Ronchese, 2020). IRF4 is also crucial for the development of MoDCs, which was demonstrated in IRF4 knockout cells (Briseno et al., 2016).

TLR ligands may affect DC development. In mice, lymphoid progenitors develop into DCs upon binding with TLR2/TLR4 ligand, while bone marrow CD34+ hematopoietic stem cells give rise to DCs in presence of TLR1/TLR2, TLR7/8 and Nod2 ligands. More differentiated DC progenitors develop into distinct DC populations upon stimulation with LPS, CpG, *Candida albicans* and a range of viruses and parasites (Bieber and Autenrieth, 2020). This stimulation also increases CCR7 expression to promote DC migration into inflammatory sites. However, DC development can as well be impaired by infectious pathogens that stimulate monoopoiesis at the expense of DC generation.

Some functions of cDCs that may attribute them into one or another functional subset, develop in the periphery where they are locally shaped by tissue microenvironment (Eisenbarth, 2019). A representative example is strong cross-presentation capacity classically attributed to cDC1s which is induced by GM-CSF and TLR ligands in lymphoid tissues (Shortman, 2020). However, cross-presentation was also demonstrated in cDC2s and pDCs, especially in the presence of immune complexes (Amon et al., 2020). Mesenteric lymph node (MLN) stromal cells provide essential signals that modulate DC functions in a manner that ensures Treg induction (Pezoldt et al., 2018).

Mouse studies on the origin of pDCs mostly point to their development solely from a lymphoid progenitor common with B cells, in contrast to cDCs. It is no surprise then that many pDCs resemble some features of B cells, including RAG-1 expression and D-J rearrangements in IgH genes (Luo and Dalod, 2020; Rodrigues and Tussiwand, 2020). Murine bone marrow cells were shown to differentiate into pDCs in the presence of GM-CSF and TLR4/TLR9 ligands, and transcription program for DC development involves IRF8 and TCF4 (Bieber and Autenrieth, 2020; Ah Kioon et al., 2018). For human pDCs, their origins still remain to be elucidated. pDC development largely relies on transcription factor E2-2 (TCF4) and interferon regulatory factor 7 (IRF7) (Amon et al., 2020; Hilligan and Ronchese, 2020). However, the same E2-2 in chronic viral infections impairs pDC generation leading to exhaustion of pDC responses (Macal et al., 2018).

In addition to classical method of MoDC generation, many other protocols for generation of DCs have been introduced later on. They include cDC1, cDC2 and pDC differentiation from HSCs or iPSCs, trans-differentiation of fibroblasts into cDC1s and immortalization of primary human DCs. Alignment of these cells to their counterparts in human tissues provided valuable knowledge on DC ontogeny, differentiation and function in vivo. Novel technical advances, e.g., genetic barcoding of HSCs offered additional insights into DC developmental pathways. Barcoding involves labeling of HSCs with lentivirus stock containing a library of DNA barcodes that integrate into single cell genomes. During cell division, barcodes are inherited in the offspring cells. PCR analysis of progeny points to the ancestor cell (Naik, 2020) (Fig. 1).

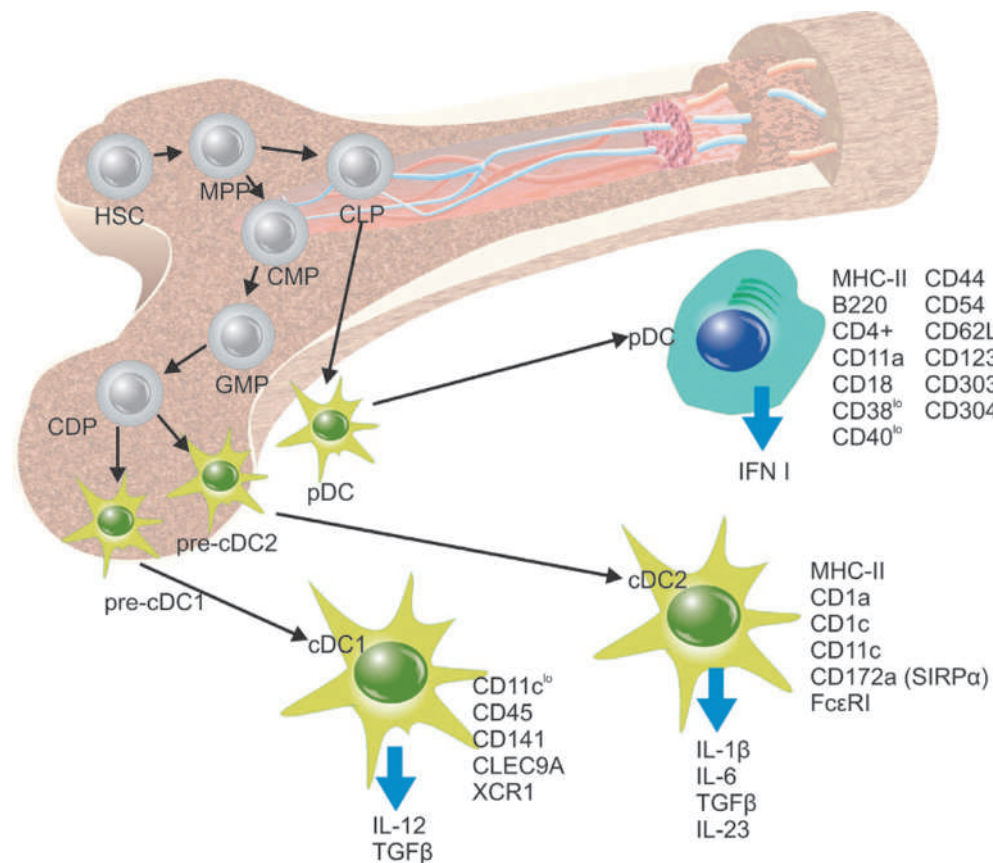


Fig. 1 A simplified scheme of dendritic cells ontogeny, phenotype and secretory profile.

Activation and function of DCs in infection

DCs are crucial for the activation of T cells and maintaining central and peripheral tolerance as well as for providing essential regulatory signals during the immune response. Exact function and activity spectrum are not hardwired, but depend on the subset, location and time, reflecting high adaptability of DCs in changing microenvironment. A functional plasticity in DC subsets may also take place, since intermediate, or transitional types of DCs, such as AXL, SIGLEC1 and SIGLEC6-expressing DCs of myeloid origin were recently characterized. These cells, nonproliferating and failing to produce type I IFNs were designed as AS DCs, exhibiting both pDC and cDC2 properties (Amon et al., 2020; Ah Kioon et al., 2018).

DCs sample their environment at barrier tissues by extending their dendrites through tight junctions of adjacent epithelial cells, gaining access to a variety of antigens. These antigens are further internalized via phagocytosis, receptor-mediated endocytosis or micropinocytosis (Hilligan and Ronchese, 2020). The priming of immune responses by DCs initiates with signaling from pattern recognition receptors (PRRs) indicating the presence of infection, inflammation, or the environmental cues at their patrolling sites. These PRRs include TLRs 1–10 (in humans), C-type lectin receptors (DC-SIGN, SIGNR1, LSECtin, MCL, Langerin, MR and DEC205), Nod-like receptors, RIG-I-like receptors and formyl peptide receptors (FPRs). The signals provide changes in DC motility, metabolism and secretory activity that accompany DC transition from immature to mature state. Depending on the type of the environmental signals, this maturation may lead to the development of inflammatory or tolerogenic DC phenotype, both showing CCR7 expression that allows migration into the draining lymph nodes.

Inflammatory DC maturation occurs upon contact with pathogen-associated antigens or damage-associated molecular patterns derived from necrotic cells. The latter bind to CLEC9A (DNCR-1) receptor on DCs that activates maturation pathway which involves up-regulation of costimulatory molecules (CD80, CD86), maturation markers (CD83), and antigen presenting molecules (MHC class I and II). Migratory capability is another hallmark of maturation which is accompanied by upregulation of CCR7, as mentioned above (Iberg and Hawiger, 2020).

Peptide-MHC complexes on the DC surface interact with antigen-specific TCRs on T cells. Type of presentation (MHC I or MHC II), strength and duration of co-stimulatory signaling and cytokine signaling milieu determine the type of T cell response and the fate of naïve CD4⁺ T cells. Intracellular pathogens are efficiently eliminated largely due to the cross-presentation capability of cDC1 cells, while cDC2s promote Th2 and Th17 responses directed against extracellular pathogens (Amon et al., 2020). Migrating pre-DC1s were shown to express CXCR3 which respond to IFN-inducible CXCL9/10/11 that support their relocation in response to intracellular pathogens (Hilligan and Ronchese, 2020). Mechanisms of antigen processing and presentation are described in the correspondent section (“see ‘Antigen processing and presentation’”). In addition to classic antigen presentation and cross-presentation, DCs are able to produce exosomes (Dex—DC-derived exosomes) containing peptide-MHC complexes that present antigens to CD4⁺ and CD8⁺ T cells. Moreover, Dex provide cross-dressing process where APCs internalize Dex with the subsequent antigen reprocessing and presentation. Upon Dex internalization by tumor cells, the latter may become more immunogenic (Wang et al., 2020).

Upon maturation, DCs of different subtypes may produce distinct effects in T cells, mostly via differential cytokine induction. DC secretome is dependent on the engaged spectrum of PRRs. E.g., IL-12 expression is enhanced by TLR3, TLR8 and TLR9 signaling in cDC1s that secrete high levels of IL-12 and type III interferons to drive Th1 response. CD5^{high} cDC2 produce large quantities of TNF α , IL-6, IL-10, and IL-23, which makes them able to efficiently prime Th2, Th17. Th17 differentiation is guided by IL-6 and TGF β secreted by cDC2s upon ligation of TLR2 and Dectin-1 by fungal glucans. CD5^{low} cDC2 stimulates Th1 CD4⁺ T cell differentiation and are also able to prime Th2 and Th17 in some experiments (Amon et al., 2020). Of note, Th2 differentiation requires lack of IL-12 and presence of IL-4, but there is little evidence that DCs provide initial source of IL-4. Both cDC subsets are able to induce Tregs via TGF β secretion if an antigen is encountered in non-inflammatory microenvironment. DCs bear α v β 8 integrin which serves to convert inactive TGF β into an active molecule, and loss of α v β 8 integrin in DCs results in autoimmunity as well as loss of TSLP, which is exemplified by MLN DCs (Travis et al., 2007; Spadoni et al., 2012). MoDCs stimulate Th1 cells by producing IL-12 (Hilligan and Ronchese, 2020). DCs have a critical role in the Tfh subset differentiation which is essential for germinal center formation and affinity maturation. Besides B cell signaling, Tfh differentiation involves cDC2-derived IL-6 and ICOS co-stimulation as well as type I IFNs, IL-27, IL-1 β and TNFR superfamily member OX40 (Hilligan and Ronchese, 2020). Moreover, pDCs have been shown to induce plasma cell differentiation even in the absence of helper T cells (Ah Kioon et al., 2018).

Neural regulation may play a role in DC functions as it has been shown that cutaneous neurons induce IL-23 synthesis in DCs by the neuropeptide CGRP (calcitonin gene related peptide) and neuron stimulation can drive IL-23-mediated skin inflammation in the absence of pathogen (Cohen et al., 2019).

DCs and their progenitors provide an important to the idea of “trained immunity” which advocated imprinting of DC progenitors towards innate inflammatory responses somewhat representing immunological memory but, of course, without the specificity attributed to adaptive immunity. For example, BCG administration results in the enhanced proliferation of myeloid lineage during second antigen encounter and provides heterologous protection against a broad range of pathogens, including viruses (Cirovic et al., 2020). Epigenetic landscape profiling using mass-cytometry (EpiTOF) have shown changes in histone modifications of DCs which are possibly essential to DC reprogramming by vaccines (Kar and Joosten, 2020). Intriguingly, several observation studies have proposed that countries with high BCG vaccination coverage had fewer COVID-19 cases and associated deaths cause by SARS-CoV-2 that emerged in 2020 (Kleen et al., 2020). “Trained immunity” concept can provide some cues about the role of DCs in this phenomenon.

pDCs are involved in the antiviral immune response associated with rapid production of type I interferons and tolerance. However, the presence of CD4, CCR5 and CXCR4, as well as DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) turns them into targets for HIV infection which are able to pass on the virus to the T cells (Evans et al.,

2011). While pDCs are easily infected by viruses this may have beneficial effects in some cases where viruses, such as VSV, need to be replicate-active to induce type I IFN response. Cross-talk of infected and uninfected pDCs produces effective antiviral response (Ah Kioon et al., 2018).

DCs and cancer

cDC functions are not limited by anti-infectious or tolerogenic responses. Key role of DCs an anti-tumor response has been demonstrated in multiple studies in mouse models, including melanoma and sarcoma. BATF3 knockout in mice results in the loss of ability to reject even highly immunogenic tumors or to respond to checkpoint inhibitors while restoring of cDC pool increases response to immunotherapy against tumor (Gardner et al., 2020). Other experimental data support the indispensable role of cDC1s in the eliciting of anti-tumor CD8+ T cell response. Lack of CCR7 in DCs results in insufficient recruitment of T cells into the sites of neoplasia, and CXCL9/CXCL10 production by cDCs is essential for the tumor infiltration by CTLs, while the loss of cross-presentation function causes a breakdown in tumor rejection. It's interesting that DC recruitment into the tumor relies on other innate player, NK cells which produce Flt3 ligand, CCL5 and XCL1, facilitating DC migration. Synthesis of IL-12 in return promotes IFN γ production by NK cells. Clinical findings proved that the presence of cDC1s in human tumors is also associated with better prognosis. Additionally, there is some experimental evidence for the requirement of cDC2s in tumor control provided by CD4+ T cell response. The role of pDCs and MoDCs in tumors is less defined, while pDC presence in tumors correlated with poor or better prognosis in different settings (Gardner et al., 2020; Del Prete et al., 2020).

DC failure and malfunction

Pathogens have evolved many strategies of immune evasion including induction of DC death. This is particularly the case with regard to systemic infection and sepsis. Many models of systemic infection provide experimental evidence of DC failure both in terms of quantity and function. Infection with *Salmonella typhimurium*, polymicrobial sepsis and parasite invasion were shown to result in attrition of tissue DCs via MyD88-mediated apoptosis and altered cytokine production by DCs (Bieber and Autenrieth, 2020). Consequential immunosuppression leads to secondary infections due to DC and T cell anergy. Similarly, influenza infection in mice have impaired lung DC population thereby increasing susceptibility to secondary infection (Beshara et al., 2018). SARS-CoV-2 was also shown to affect DC maturation and to induce TRAIL-mediated apoptosis. It was proposed that delayed innate immune response in early COVID-19 infection sequenced by excessive inflammatory response is accountable for the severe clinical course, pointing to the possible role of DCs in the course of disease (Kleen et al., 2020). Studies of tumor microenvironment show that tumor-associated cDC2s can be reprogrammed to obtain immunosuppressive functions, adding to detrimental effects of immunosuppressive tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and Tregs (Luo and Dalod, 2020; Maier et al., 2020). DCs themselves are also a subject of tumor transformation, and dendritic cell leukemia is recognized as a distinct hematopoietic neoplasm showing characteristic immunophenotype of pDCs with CD123 and CD303 (CLEC4E) expression (Tsagarakis and Paterakis, 2020).

pDCs have been shown to contribute to autoimmune diseases pathogenesis, including systemic lupus erythematosus (SLE), systemic sclerosis (SSc), type I diabetes and psoriasis. TLR8-induced secretion of CXCL4 and IFN α by pDCs infiltrating the skin in SSc promotes inflammation and fibrosis. In SLE, pDCs react to immune complexes and contribute to disease activity via IFN α secretion. Skin cDCs in psoriasis cross-talk with PGE2-producing fibroblasts and secrete IL-23, TNF α and IL-1 β , that contribute to Th17-mediated inflammation. In graft-versus-host disease (GVHD) pDCs prime alloreactive T cells, however a number of studies have shown protective functions, including Treg induction (Ah Kioon et al., 2018; Hilligan and Ronchese, 2020).

Tolerogenic DCs

Experiments with a range of physiological and chemical signals provided evidence for the tolerogenic DC functions and revealed that cDC1s and cDC2s are mutually redundant in ensuring tolerance via Treg induction (Sun et al., 2020). These functions, in turn, ensure homeostasis at the skin and mucosa, maintaining proper responses to commensal microorganisms and environmental non-pathogenic antigens (Iberg and Hawiger, 2020). E.g., *Mycobacterium vaccae*, a non-pathogenic soil bacterium, abundant in rural areas with livestock farming, provides tolerogenic DC response and exerts anti-allergic properties. Intriguingly, mode of *M. vaccae* lysate administration seems to have different consequences. Epicutaneous lysate application was shown to be feasibly more efficient than heat-killed *M. vaccae* subcutaneous injections, probably owing to LC stimulation (Strygin et al., 2020). This phenomenon is in line with the hygiene hypothesis, or "old friends hypothesis" of allergy development partly confirmed by lower allergy prevalence in livestock farming areas. The signaling pathways that drive tolerogenic response in DCs still remain to be elucidated.

Mechanisms of peripheral tolerance induction involve anergy, T cell deletion and pTreg development (Iberg and Hawiger, 2020). Tolerogenic DCs rely on the synthesis of anti-inflammatory cytokines, including IL-10 and TGF- β , but other soluble factors, such as chemokine CCL3, vitamin D3 and retinoic acid, also play a role in the induction of pTreg. Tolerogenic cDC1s residing in lymphoid organs express immunomodulatory molecules B7h, BTLA, PD-L1, CD80/86 which induce formation of pTregs upon

antigen presentation in steady state and negatively regulate other CD4⁺ T cell subsets (Hilligan and Ronchese, 2020). Antigens used in this presentation derive from apoptotic cells which are ingested via scavenger receptor CD36 which is highly expressed on cDC1. These antigens can as well be displayed via the cross-presentation pathway. Tolerogenic DCs in skin, intestines and airways are in constant contact with a range of pathogens and commensal microbiota and exert their tolerogenic functions in a subinflammatory manner being activated via pattern recognition receptors (PRRs). Since same PRRs may promote an immunogenic response, the exact pathway highly depends on different microenvironmental signals, including IL-10. Moreover, specific associations of activated TLRs may result in different responses. E.g., *Yersinia pestis* antigen LcrV binding to TLR6/TLR2 heterodimer produces tolerogenic response, while binding to TLR1/TLR2 heterodimer leads to an immunogenic response (Depaolo et al., 2008).

Food-derived antigens mostly comprise harmless antigens that require tolerogenic response referred to as oral tolerance. Intestinal DCs provide a non-redundant link ensuring oral tolerance induction which normally prevents hypersensitivity and inflammatory responses. Intestinal DCs express G-protein-coupled receptors (e.g., GPR109a) which serve as ligands for short-chain fatty acids (SCFAs) and other substances inducing tolerogenic pathways (Iberg and Hawiger, 2020). Other factors that promote induction of tolerogenic DCs include ligands of aryl hydrocarbon receptor (AHR) and vitamin D receptor (VDR) as well as vasoactive intestinal peptide (VIP). Of note, cDC2/cDC1 ratio matches vitamin A local concentrations and *Aldh1a2* is highly expressed in proximal intestines where cytokine milieu is also biased towards anti-inflammatory cytokines (Esterhazy et al., 2019). Vitamin A-derived retinoic acid is probably the most distinguished dietary factor that governs gut DC functions. Gut DCs, like many other intestinal cells, express retinaldehyde dehydrogenase (RALDH) encoded by *Aldh1a2* gene (encoding retinaldehyde dehydrogenase 2 or RALDH2), an enzyme involved in retinoic acid production. This enzyme converts vitamin A into retinoic acid that exerts multiple immunomodulatory functions, including cDC2 development with ability to induce Tregs and provide their migration into the LP via CCR9 and FoxP3 induction (Sun et al., 2020). Notably, *Aldh1a2* is upregulated in small intestine DCs, where strong Treg induction was shown (Hilligan and Ronchese, 2020). A variety of other food components also influences DC functions, including SCFAs (particularly, butyrate) produced by anaerobic fermentation of dietary fibers. Polysaccharide A and zymosan that derive from gut microbiota promote tolerogenic DC responses as well as mucin-2 (host mucus component) (Sun et al., 2020).

Clinical utility of DC targeting

Obviously, harnessing of tolerogenic DCs represents promising therapeutic modalities for the treatment of allergy, autoimmune diseases, cancer, and in transplantology. However, clinical studies performed over the last two decades mostly focused on treating cancer and viral infections.

The ability of pDCs to produce high levels of type I IFNs made them most studied human DC subset since these cells offered promising therapeutic applications, namely in HIV infection. Recently, a human monoclonal antibody to type I IFN receptor subunit 1, anifrolumab, have demonstrated significant effectiveness in Phase III trial in SLE patients (Morand et al., 2020). Many studies have backed targeting cDC1s or cells harboring cDC1-like properties for treating cancer or infections by intracellular pathogens. Many preclinical and clinical trials involved DCs derived from monocytes for adoptive cell therapy of viral infections or cancer. Viral antigen-pulsed MoDCs were used recently for enhancing antiviral response in HIV-infected patients (Luo and Dalod, 2020). However, MoDCs fail to efficiently migrate into lymphoid organs. Moreover, restriction of good manufacturing practice (GMP) to MoDCs as the only subset that can be produced in large quantities did not contribute to successful treatments. This partly explains why utilizing DCs for antiviral and anticancer treatment have not reached success as expected. The only approved vaccine that utilizes exogenous expansion of DCs loaded with tumor lysate is sipuleucel-T that shows limited clinical effectiveness. Besides adoptive DC therapies, other approaches seek to improve DC functions in vivo (particularly by TLR agonists) or blocking of inhibitory signals (Gardner et al., 2020). Tumor antigen presentation may be facilitated by fusing tumor antigens to antibodies that bind DC (e.g., anti-CLEC9A, anti-DC-SIGN). One of the targets for these fusion proteins is DEC205 which seems controversial because targeting of DEC205 with OVA-coupled antibody was shown to induce tolerogenic response. This demonstrates complexity of DC-based approaches and the need for a deeper understanding of proper DC engagement, including subtype, timing and location (Lamendour et al., 2020; Wculek et al., 2020; Del Prete et al., 2020). Recently, efforts have been made to engage DC cross-presentation process to facilitate antiviral CD8⁺ T cell generation by vaccines. However, long-term and efficient T cell induction remains challenging; moreover, cross-presentation is a slow process and requires active pathogen replication.

Adoptive DC generation is also under development in solid organ transplantation studies. Potent immunosuppressive capabilities of DCs have been shown in animal experiments where regulatory DCs were able to increase transplant survival time (Luo and Dalod, 2020).

Beyond their multiple specializations, DC types differ in susceptibilities to infection by intracellular pathogens or to hijacking of their immunoregulatory activities by microbes or tumor cells. Targeting of DCs for vaccination or immunotherapy purposes should take into account that the right DC type should be used to avoid unwanted sequela in patients while providing beneficial effects.

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Natural Kills Cells

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Introduction

Natural killer (NK) cells are innate cytotoxic lymphocytes that were first described in the 1970s as a unique population of non-T and non-B cells that spontaneously lysed tumor cells (Kiessling et al., 1975). Today we know that NK cells are complex, professional killer cells that are indispensable for the defense against microbial pathogens (particularly viruses), are involved in tumor surveillance, and have a role in various non-infectious human diseases (Orange and Ballas, 2006). NK cells are functionally unique because their immunological properties are not dependent on prior antigen sensitization—they lack antigen-specific T cell and B cell receptors. They recognize infected or damaged cells using a diverse set of germline-encoded receptors. Integration of the various contact-mediated and cytokine-induced signals activate kill pathways to eliminate the infected or damaged cells. Aberrations in these pathways lead to increased susceptibility to viral infection, malignancy, or autoimmune/inflammatory disorders. In the following chapter, we will review the basic biology of NK cells, their role in infection control and other human diseases, and the genetic defects that contribute to primary NK cell deficiencies (NKD).

NK cell development

NK cells arise in the bone marrow (BM) and differentiate in secondary lymphoid tissues (SLT) (Abel et al., 2018). Studies on human NK cell maturation have demonstrated six distinct stages of development. Briefly, self-renewing CD34⁺ hematopoietic stem cells (HSC) in the bone marrow produce oligopotent common lymphoid progenitor cells (CLPs). CLPs differentiate into pre-T/pre-NK cell progenitor (pre-NKP) cells, Pro-B cells, or innate lymphoid cells (ILCs). Pre-NKP cells (Stage 1 NK cells) express CD244 which is an immunoregulatory transmembrane receptor in the signaling lymphocyte activation molecule (SLAM) family. CD244 mediates both activating and inhibitory signaling in NK cells and remains present throughout all subsequent stages. Pre-NKP cells differentiate into Stage 2a/2b NKP cells which express c-Kit (CD117) and IL-1R1. Expression of IL-15R β -chain (CD122) and IL-15R α during Stage 2b irreversibly commits the cells to the NK lineage and is important for NK cell survival. Stage 3 NK cells are defined by high levels of IL-1R1 and start to express activating receptors (NKP45, NKP30, NKG2D, and NK1.1, discussed later in this chapter). When the expression of these activation receptors reaches maximal levels, the Stage 3 cells mature to Stage 4a and 4b found

in peripheral blood. Another defining feature of Stage 4 NK cells is the very high expression of CD56 (CD56^{bright}) and the absence of the activating receptors FcγRIIIA (CD16) and KIR (CD158). CD56, also known as neural cell adhesion molecule-1, is not believed to have any known function. The function of CD16 and KIR will be discussed later. CD56^{bright} NK cells are potent cytokine-induced, cytokine-producing pro-inflammatory cells with robust anti-tumor activity. CD56^{bright} NK cells produce TNFα, IL-5, and IL-13. Stage 4 NK cells differentiate into Stage 5 NK cells by downregulating CD56 expression (CD56^{dim}) with concomitant increased expression of the activating receptors CD16 and KIR. These CD56^{dim} cells have a mostly cytotoxic effector function (granule and antibody-mediated cytotoxicity) but can also produce cytokines. Together, Stage 4 and Stage 5 NK cells make up the majority of NK cells in the peripheral blood. CD56^{bright} cells are predominantly found in secondary lymphoid tissues with only 5–10% present in the blood. In contrast, CD56^{dim} cells make up >90% of NK cells in peripheral blood circulation. Finally, terminally differentiated (Stage 6) NK have high levels of NKG2C (CD159c) and CD57 and have “memory-like” features which allow them to recognize antigens (Pahl et al., 2018; Newhook et al., 2017; Cerwenka and Lanier, 2016).

NK cells are found in several immune compartments (primary and secondary lymphoid tissue) and non-lymphoid tissue (e.g., skin, liver, and uterus) (Freud et al., 2014). In the peripheral blood of healthy individuals, NK cells make up 3–20% of the mononuclear cell population (Mace and Orange, 2016; Angelo et al., 2015; Shearer et al., 2003) and total levels are affected by age, infection, and conditions that alter the cellular milieu (Mahapatra et al., 2017; Brady et al., 2010; Strauss-Albee et al., 2015). Additionally, NK cells have a half-life of about 2 weeks and can achieve 16 doublings in vitro before becoming senescent (Stringaris, 2017; Zhang et al., 2007; Shimasaki, 2020).

NK cell signaling

The effector function of NK cells depends on (1) distinguishing abnormal cells from normal cells, (2) mediating activating and inhibitory signaling pathways, (3) inducing an effector response via cytolytic proteins and soluble cytokines, and (4) terminating effector function (for comprehensive review, see Ref. Mace et al., 2014).

NK cells express multiple classes of receptors that are critical for chemotaxis, cellular adhesion, and initiating activating or inhibitory signals. Under homeostatic conditions, inhibitory pathways dominate, and the NK cells remain in a “ready and waiting state”. When the NK cells encounter “aberrant” cells with high concentration of activating ligands and reduced expression of inhibitory ligands, this overrides the inhibitory signals and promotes killing of the aberrant cells. Additionally, activated NK cells secrete cytokines and enhance recruitment and activation of accessory immune cells. As such, NK cells serve as a bridge between innate and adaptive immune responses.

Activating receptors

NK cell-mediated cytotoxicity requires signaling via activation receptors which include natural cytotoxicity receptors (NCRs), killer immunoglobulin-like receptors (KIR), NKG2D, and CD16 which will be discussed here (Chan et al., 2014). Other activating receptors including LFA-1, CD27, DNAM-1, CD2, IFNα/βR, IFNγR, IL-2Rα, IL-12Rβ, and IL-15Rβ are reviewed elsewhere (Orange and Ballas, 2006; Watzl and Long, 2010).

Natural cytotoxicity receptors (NCRs) are type I transmembrane (TM) proteins with Ig-like domains on the extracellular surface of NK cells. NCRs interact with multiple pathogen- or host-encoded ligands (e.g., influenza hemagglutinin, *Plasmodium* EMP-1; for comprehensive review, see Ref. Barrow et al., 2019). Nkp30, Nkp44, and Nkp46 are the best characterized human NCRs (Barrow et al., 2019). Nkp30 and Nkp46 are constitutively present on the surface of NK cells, while Nkp44 is expressed upon IL-2 mediated activation of the NK cells. Engagement of ligands with the NCR induces conformational changes that expose positively charged amino acid residues (arginine or lysine) in the transmembrane (TM) domain of the NCRs. These residues form salt bridges with corresponding residues on intracellular adaptor proteins (DAP10, CD3ζ, FcγR) that contain ITAMs, or immunoreceptor tyrosine-based activating motifs (Vivier, 2004). In general, intracellular cytoplasmic domain of the NCRs lack catalytic capacity and, hence, all signal transduction through NCRs must occur through adaptor proteins. The ITAM motif in this covalently-linked receptor-adaptor protein complex is then phosphorylated by Src family of tyrosine kinases (Src, Lyn, Lck) which leads to the recruitment and phosphorylation of Syk and Zap70 tyrosine kinases. This activates multiple signaling cascades including the phosphatidylinositol-3-OH kinase (PI3K), mitogen-activated protein kinase (MAPK), and extracellular signal-regulated kinase (ERK) pathways. The effector function of NK cells is mediated by activation of these different pathways. The ultimate effect is cytoskeletal rearrangements that release cytotoxic granules and activation of various cytokine/chemokine genes.

Killer immunoglobulin-like receptors (KIR; CD158) are an important class of receptors that are either activating or inhibitory. There are >13 KIR genes and although there is no KIR gene rearrangement, there is a very high level of diversity in KIR proteins derived from differences in KIR gene copy number variation and allelic polymorphism (Pende et al., 2019). The nomenclature of KIR genes is based on number of extracellular Ig domains (e.g., KIR3D has 3 Ig domains). Their functionality is determined by whether they have cytoplasmic ITIM domain, or immunoreceptor tyrosine-based inhibitor motifs. If an ITIM domain is present, the KIR protein nomenclature includes the label “L” (“long” domain); in the absence of an ITIM domain, it is labeled with “S” (“short” domain). KIR proteins recognize both classical and non-classical MHC Class I receptors (HLA-A/B/C or HLA-E, respectively). This interaction is crucial for preventing NK cell-mediated lysis of normal host cells (Pende et al., 2019). Importantly, loss of MHC class I is not sufficient enough to activate NK cells which require ligand interaction with activating receptors. There are few known

activating KIRs (aKIR) of which KIR2DS1 is the best characterized (Pende et al., 2019). KIR2DS1 has been demonstrated to play a role in viral and tumor recognition (Naiyer et al., 2017; Thiruchelvam-Kyle et al., 2017). Like NCRs, aKIR do not have ITAM domains and require that signal transduction occurs through adaptor proteins (e.g., KARAP/DAP12).

CD16a, or FcγRIIIa, is the low-affinity transmembrane protein receptor for IgG and is important for NK cell antibody-mediated cellular cytotoxicity (ADCC) discussed below. It co-localizes with CD3ζ and FcεRI on the surface of NK cells. Signaling is mediated via the classical ITAM pathway that leads to PI3K and PLCγ activation. Unlike other ITAM-dependent activating receptors, activation via CD16 alone is sufficient to activate NK cell cytotoxicity and secretion of cytokines including TNFα, IL-2, IL-3, and IL-4 (Bryceson et al., 2009). Mutations in CD16 cause functional NK cell deficiency (NKD), which is discussed later.

Natural-killer group 2, member D, or NKG2D, receptors are C-type lectin-like type II transmembrane proteins expressed on NK, NKT, and CD8 T cells. Endogenous ligands for NKG2D include major histocompatibility complex class I-related chain A and B proteins (MIC-A/B) and ULBP, which are highly expressed in malignant cells including melanoma, leukemia, and neuroblastoma (Paul and Lal, 2017; Pende et al., 2002). Signaling through NKG2D promotes the polarized release of lytic granules to kill the diseased cells. Expression of NKG2D is upregulated by TNFα, IL-1, IL-7, IL-12, IL-15, and IL-18 and downregulated by IFNγ and TGFβ (Paul and Lal, 2017).

Inhibitory receptors

Under normal, physiologic conditions, NK cells activity is restrained. This is crucial for establishing NK cell tolerance to self and is the basis for the “missing self” hypothesis (Ljunggren and Karre, 1990). Briefly, cells expressing “self-molecules” such as MHC class I glycoproteins prevent NK cell activation and subsequent target destruction. However, cytotoxic stress (induced by viral infection or malignancy) can downregulate the expression of MHC-I. Along with other activating signals, this shifts NK cell signaling toward an activating phenotype that kills infected cells while generally sparing autologous, healthy cells.

NK cell inhibitory receptors can be categorized as immunoglobulin-like or lectin-like. Immunoglobulin-like receptors include the KIR family, leukocyte Ig-like (LIR), and Ig-like transcripts (ILT). Separately, lectin-like receptors include NKG2A/B and KLRG1. KIR heterodimers form receptor-ligand interactions with Class-I MHC molecules (HLA-A, -B, and -C) expressed on healthy host cells. Heterodimeric CD94/NKG2A receptor binds to non-classical MHC-molecules (HLA-E). Other inhibitory signaling receptors include TIGIT, CD96, NKR1A. In spite of their structural differences, all NK cell inhibitory receptors have conserved intracytoplasmic ITIM motifs. Negative regulation via ITIM-containing receptors starts with ligand-receptor engagement which induces conformational changes that allows the inhibitory receptor to co-aggregate with activating receptors (FcγRIIIa or FcεR) (Vivier, 2004; Daëron et al., 2008). Src-family tyrosine kinases on the activating receptor phosphorylate the ITIM region (consensus sequence S/I/V/LxYxxL/V) which then recruits phosphotyrosine phosphatases (SHP-1 and SHP-2) and inositol phosphatases (SHIP). These phosphatases dephosphorylate both upstream and downstream signaling molecules in the competing activation pathways that are involved in the clustering and phosphorylation of activation receptors—hence, inhibition dominates (Vivier, 2004). NKG2A has emerged as a reasonable drug target for cancer immunotherapy since inhibition of the receptor can restore NK cell and NK T cells and promotes potent anti-tumor activity in preclinical trials (Creelan and Antonia, 2019).

NK cell effector pathways

NK cells have several non-mutually exclusive mechanisms of function: granule-dependent cytotoxicity, cytokine/chemokine-induced cell signaling, and antibody-dependent cellular cytotoxicity. The specific molecular steps involved in NK cell cytotoxicity are reviewed by Mace et al. (Mace et al., 2014).

Granule-dependent cytotoxicity

NK cells produce numerous specialized membrane-bound granules that contain lysosomal enzymes (cathepsins and hydrolases), lytic proteins (perforin, granzysin, and anti-microbial peptide) and pro-apoptotic molecules (granzymes, TRAIL, and FasL) (Krzewski and Coligan, 2012). Hence, these lytic granules are often called “secretory lysosomes”. Upon NK cell activation, there is polarized degranulation of the lytic granule into the immune synapse. Although NK cell granules contain important lysosomal enzymes required for the activation of cytotoxicity proteins, we will only review the membrane-disrupting and pro-apoptotic proteins contained within them.

One of the best characterized cytolytic molecules stored in NK cell granules is perforin. It is a glycoprotein that has ~20% protein sequence homology with terminal complement proteins. Perforin is constitutively expressed in all developmental stages of NK cells. Cytokine-producing CD56^{bright} NK cells have the lowest perforin expression while cytotoxic CD56^{dim} NK cells have the highest levels of expression. Perforin is produced as an inactive 60–70 kDa globular protein that becomes activated in the presence of calcium ions in the lytic granules (Andrin et al., 1998). Upon release of the granules into the immune synaptic cleft, activated perforin monomers incorporate and polymerize into the plasma membrane of the target cell to form a porous channel (5–20 nm in diameter; 14 nm in length) (Osińska et al., 2014). Although at least 3–4 perforin molecules are sufficient to produce the channel, most channels are comprised of ~20 perforin monomers. Perforin channels are formed not only on the plasma membrane but also

on other membranous structures including the ER/Golgi, endosomes, and the nuclear envelope. The channels allow for the passive transport of water, ions, and macromolecules across all membranes and contribute to osmotic lysis of the cells.

Lytic granules also contain granzysin, a multi-functional protein with cytotoxic, chemoattractant, and pro-inflammatory properties. Like perforin, granzysin is expressed by CTLs and NK cells. Granzysins are 9 kDa proteins that are functionally very similar to defensins in that the positively-charged protein is electrostatically attracted to the negatively charged membrane and permeate through the plasma membrane by a scissoring action (Anderson et al., 2003). Granzysins can also enter via perforin pores. Additionally, granzysin can induce the expression of chemokines (RANTES, CCL8) and cytokines (IL-1, IL-6, type 1 interferons) on the target cell (Krzewski and Coligan, 2012).

Polarized release of the lytic molecules can be organized as a complex in membraneless protein structures called SMAPS, or supramolecular attack particles (Ambrose et al., 2020). Overall, rapid target cell cytotoxicity is induced with perforin and granzysin pathways.

NK cells also produce pro-apoptotic proteins including granzyme B (GzmB), Fas ligand (FasL, CD178), and TNF-related apoptosis-inducing ligand TRAIL (CD253). Similar to perforin, granzyme B is stored in the lytic granules. It is a serine protease that was previously postulated to enter the target cell via perforin pores. More recent models demonstrate that the perforin pores on the target cells induce calcium influx and which activates dynamin-dependent endocytosis of the granzyme that is electrostatically bound to plasma membrane (Chowdhury and Lieberman, 2008; Veugeliers et al., 2004). The protease escapes the endosomal vesicles via perforin pores and enters the cytoplasm. Granzyme B activates multiple cytoplasmic caspases including caspase-3 which triggers DNA and mitochondrial damage pathways. Granzyme B also initiates apoptosis via cleavage of Bid. The net effect is the generation of reactive oxygen species (ROS) which disrupt the mitochondrial membrane potential and release cytochrome C to induce cellular death. GzmB also activates caspase-activated DNase (CAD) which leads to rapid DNA fragmentation and cell death. Separately, FasL and TRAIL are pro-apoptotic molecules that localize to the NK lytic granule and are enriched on the intraluminal membranes of the vesicles (Bossi and Griffiths, 1999; Lee et al., 2018). They initiate apoptosis by engaging death receptors expressed on target cells, Fas (CD95) and TRAIL-R, respectively. This activates pro-apoptotic machinery (namely caspase-8 and -10) which is slow compared to the lytic protein pathways discussed above.

Cytokine dependent function

Unlike perforin and granzyme, NK secreted chemokines/cytokines are packaged into endosomes and are exocytosed in a non-polarized fashion (Reefman et al., 2010). Different subpopulations of NK cells produce proinflammatory and immunosuppressive cytokines that are functionally analogous to T-cell subsets. For example, NK1 cells aid in the induction of a Th₁ response by producing IFN γ and TNF α which is the primary response to intracellular pathogen (Reid-Yu et al., 2013). NK2 cells are analogous to Th₂ cells—they synthesize and secrete IL-4, IL-5, and IL-13 (Peritt et al., 1998; Katsumoto et al., 2004). Finally, regulatory NK cells (NK_{reg}) produce TGF β and IL-10 (Jiang et al., 2018). NK cell cytokine gene transcription is finely regulated by cytokine-induced and activating receptor-mediated activation of transcription factors (STAT-4 and -5, NF κ B, AP-1, and c-Fos/c-Jun). Furthermore, NK cells produce and secrete granulocyte/monocyte colony-stimulating factor (GM-CSF) to activate other leukocytes and chemokines (RANTES, IL-8, MIP-1 α/β) to attract other immune cells (Abel et al., 2018).

Antibody-dependent cellular cytotoxicity (ADCC)

ADCC is a form of cell-mediated immunity whereby IgG-coated target cells are recognized by effector lymphocytes and lead to release of cytotoxic factors that kill the target cells. Importantly, ADCC is not a complement-dependent process. Briefly, the Fab portion of IgG antibodies (IgG1, IgG2, IgG3, and IgG4) recognize antigens on the target cells while the Fc backbone engages the Fc γ Rs on the NK lymphocytes. Natural killer cells have two Fc γ Rs: Fc γ RIIc (CD32c) and Fc γ RIIIa (CD16a), both of which are activating receptors. Fc γ RIIc has a cytoplasmic ITAM that is phosphorylated upon the Fc γ R engaging the antigen-antibody complex. This activates pathways involved in NK cell degranulation, pro-inflammatory cytokine release, and TNF-dependent signaling. In contrast, Fc γ RIIIa associates with Fc ϵ RI- γ or CD3- ζ chains, both of which have ITAMs and activate similar transduction pathways. Polymorphic variants in the Fc γ RIIc and Fc γ RIIIa affect the binding strength to the antibody Fc backbone and subsequent ADCC activity.

NK cells and infectious pathogens

Viral infection

NK cells have important antiviral function and this review will focus primarily on the herpesviridae family.

Herpesviridae: This family of double-stranded DNA viruses includes nine human herpes viruses (HHV) that cause human disease. The viruses are divided into three subfamilies: α -, β -, or γ -herpesviridae. Severe, invasive, and persistent infections by *Herpesviridae* viruses can be seen with various combined immune deficiencies (NEMO deficiency, DOCK8 deficiency, APDS, Wiskott-Aldrich Syndrome, STAT1 GOF, TLR3 deficiency, UNC93b deficiency) and in individuals who have congenital NK cell deficiencies.

α-herpesviridae viruses: this family includes herpes simplex virus-1 (HSV-1, or HHV-1), herpes simplex virus-2 (HSV-2, or HHV-2), and varicella zoster virus (VZV, or HHV-3). The viruses primarily target mucoc epithelial cells and establish latency in neural ganglia. HSV-1 and HSV-2 are major causes of oral/genital herpes infections, gingivostomatitis, eczema herpeticum, herpetic encephalitis, and erythema multiforme. VZV infection causes chickenpox and shingles. Our current understanding of which NK cell receptor-pathways are important for clearance of HSV and VZV come from our knowledge of how the viruses evade these pathways (De Pelsmaeker et al., 2018). HSV-1/2 and VZV have co-evolved with our immune system and developed unique immune evasion mechanisms, different even among the *α-herpesviridae* viruses. In general, virally infected cells express stress-induced ligands which include the polymorphic major histocompatibility complex class I-related chain A and B proteins (MICA and MICB) and UL16 binding proteins (ULPBs). These molecules are activating ligands for NKG2D receptors and natural cytotoxicity receptors (NCRs) on NK cells (Dai and Caligiuri, 2018; Costa et al., 2009). HSV-1 blunts the expression of MICA and ULPBs (Schepis et al., 2009; Campbell et al., 2015). During re-activation, HSV-1 also produces miRH8 to blunt various antiviral pathways. miRH8 decreases expression of the antiviral protein tetherin and decreases expression of GPI-anchored NK cell ligand proteins (ULBP -2 and -3, and the 2B4 ligand CD48) (Enk et al., 2016). In contrast, VZV upregulates MICA expression but reduces ULBP2 and ULBP3 expression (Campbell et al., 2015). VZV can also infect mature NK cell subsets (CD56^{bright}, CD56^{dim}, CD57^{bright}) and alter the expression of NK cell surface receptors (namely CCR4) to enhance NK cell homing to the skin (Campbell et al., 2018). Other immune-evading mechanisms by HSV-1 and -2 include the production of ICP47 (infected cell protein 47, or UL12) which inhibits TAP (transporter associated with antigen presentation), prevent antigen loading on MHC-I, and results in the transport of empty MHC-I molecules (Odom et al., 2012; Cheng et al., 2020). There are known genetic polymorphisms in NKG2D ligands which alter susceptibility to various cancers and rheumatologic conditions (Choy and Phipps, 2010; Zuo et al., 2018), but their role in modulating viral infectivity is less clear.

β-herpesviridae viruses: this family of Herpesviridae viruses includes cytomegalovirus (CMV, or HHV-5) and roseolovirus (HHV-6). Human CMV (HCMV) has the largest genome of the human herpesviruses. It is also the best characterized of the *β-herpesviridae* family with regard to the role of NK cells combating viral infection. CMV is highly prevalent worldwide, particularly in developing countries where >90% of individuals are seropositive (Zuhair et al., 2019). The virus can establish lifelong, latent infections and most individuals with CMV infection are asymptomatic. However, in immunocompromised individuals and neonates, infection can cause hepatitis, gastroenteritis, hearing impairment, retinitis, and pneumonitis. NK cells play an important role in eliminating or containing CMV infection. Like other Herpesviridae viruses, CMV can induce the expression MICA/MICB and ULPBs in infected cell and it has also developed mechanisms to downregulate the expression of these NKG2D ligands. This includes miRNA (miR-UL112)-mediated processes and upregulating UL16 which inhibit MICA/MICB. CMV causes expansion of NKG2C NK cells in both acute infection and with viral reactivation, and alters the NK cell receptor repertoire (Gumá et al., 2004; Barnes et al., 2020). NKG2C recognizes HLA-E, the non-classical class I allele. Furthermore, these NKG2C cells lack expression of other inhibitory receptors (NKG2A) (Jost and Altfeld, 2013). These phenotypic receptor changes are CMV-specific are not present with other viral infections including HSV and EBV (Gumá et al., 2004). There are currently no known NK cell receptors to CMV proteins.

In general, viruses can evade CD8+ T-cell mediated responses by downregulating HLA-I expression or blocking TAP-dependent transport of processed antigen peptides. CMV express US2 and US11 proteins which transfer HLA class I molecules from the endoplasmic reticulum to the cytoplasm where it undergoes degradation. Similarly, the CMV protein US6 blocks TAP-dependent antigen transport. Therefore, clearing CMV infection requires more than cytotoxic T cells.

γ-herpesviridae viruses: this family of Herpesviridae viruses includes Epstein-Bar Virus (EBV, or HHV-4). EBV is transmitted by saliva, infects multiple cell populations (e.g., B cells and NK cells), and establishes latent infection (Howe et al., 2020). While most people with EBV are asymptomatic carriers, EBV can cause infectious mononucleosis (IM) and B-cell and epithelial cell malignancies, namely Burkett's lymphoma and nasopharyngeal carcinoma (Shannon-Lowe and Rickinson, 2019). Additionally, there is preferential expansion of CD56^{dim} NKG2A-expressing cells with acute EBV infections (Png et al., 2021). Additionally, with chronic viral infections, there is an expansion of a subset of NK cells that are CD56-CD16⁺, which most likely represent anergic/exhausted NK cells (Björkström et al., 2010; Lugli et al., 2014). EBV-infected cells expressing CD48 are recognized by the NK cell activating receptor 2B4; non-functional 2B4 contributes to disseminated EBV infection (Tangye et al., 2000). Like other *herpesviridae* viruses EBV evades NK cell recognition by encoding miRNA that downregulate MICA/B and blunting the NKG2D and DNAM-1 activating receptor/co-receptor pathways (Williams et al., 2015). Severe or recurrent EBV infections are seen with most classical NK deficiencies, as discussed later in this chapter.

Other viruses: Recent evidence has demonstrated a robust NK cell response to SARS-CoV2, the causative virus of COVID-19 disease (Maucourant et al., 2020). This includes increased expression of perforin and NKG2C in NK cells (Soleimanian and Yaghoobi, 2020). The antiviral role of NK cells against adenoviruses, respiratory syncytial virus, human papilloma virus, HIV, and influenza virus are reviewed elsewhere (Waggoner et al., 2016; Björkström et al., 2021).

Bacterial infection

Although T-cell and antibody responses are critical for controlling bacterial infections, NK cells are increasingly recognized as important for linking innate immune responses to adaptive immunity (Gabrielli et al., 2016). Activation of NK cells via bacteria-stimulated leukocytes (T/B lymphocytes, PMNs, eosinophils) is reviewed elsewhere (Souza-Fonseca-Guimaraes et al., 2011). NK cells can directly recognize bacterial PAMPs using PRRs and mediate their antibacterial activity predominantly by TNF death receptor signaling, cytotoxic granules, and interferons (Schmidt et al., 2016). For example, *E. coli* fimbrial protein (FimH)

directly activate TLR4/MyD88 signaling in NK cells in an LPS-independent manner and lead to subsequent IFN γ and TNF α secretion (Mian et al., 2010). Clearance of *Klebsiella pneumoniae* lung infection is partly dependent on pathogen proteins (e.g., flagellin, outer membrane protein A) inducing IFN and α -defensin secretion via IRF3, TLR2, TLR4, and TLR5 signaling in NK cells (Ivin et al., 2017; Chalifour et al., 2004). Furthermore, NK cells directly bind to *Mycobacterium* spp. cell wall components (mycolyl-arabinogalactan-peptidoglycan and mycolic acids) and engage in contact-dependent killing of infected cells (Schmidt et al., 2016). For example, vimentin expressed on *M. tuberculosis* (Mtb)-infected monocytes activates Nkp46 to induce non-IFN γ -dependent cell lysis of the infected cells (Garg et al., 2006; Vankayalapati et al., 2002). NK cells also have a role in regulating gut mucosal immunity against *Salmonella* infection (Harrington et al., 2007). Separately, secreted bacterial factors can impair NK cell cytotoxicity pathways or induce NK cell depletion. For example, *B. anthracis* lethal toxin, cholera toxin, and pertussis toxin impair NK cell cytotoxicity and/or motility (Watanabe et al., 1993; Lagadari et al., 2009). Additionally, *Y. pestis* virulence protein YopM inducing global NK cell depletion (Kerschen et al., 2004). Finally, suppression of NK cells by bacteria can increase tumor metastasis. For example, NK cells infected with *Pseudomonas* undergo apoptosis and augment metastasis (Chung et al., 2009). Onco-bacteria such as *Fusobacterium nucleatum* have inhibitor ligands that suppress NK cells and contribute to carcinogenesis (Gur et al., 2015).

Fungal infection

The primary mechanism of antifungal immunity involves a combination of innate (macrophages, polymorphonuclear cells, defensins, cytokines) and adaptive (T and B lymphocytes) immune responses (for a comprehensive review, see Ref. (Romani, 2011)). Recent clinical and scientific evidence have identified novel roles for natural killers cells against fungal pathogens (Schmidt et al., 2017). Clinical studies on patients who have undergone transplants (solid organ; hematopoietic stem cell transplant, HSCT) demonstrated that those with invasive fungal disease (Aspergillosis) often had low number of NK cells (Romani, 2011; Stuehler et al., 2015). It is unclear whether the fungal infection induced the NK cell lymphopenia or if the paucity of NK cells contributed to the development of the infection, but lower absolute NK cells were associated with poor outcome. In vitro studies using human and mouse NK cell lines have demonstrated an important antifungal role for activating NK receptors (NCRs, CD56, and CD16) against *Candida*, *Aspergillus*, and *Cryptococcus* spp. (Li et al., 2013; Ziegler et al., 2017; Nabavi and Murphy, 1986). Furthermore, fungal activation of NK cells induces the secretion of various soluble molecules, namely IFN γ , RANTES, and GM-CSF and can augment classical antifungal pathways in T-cells and PMNs (Bouzani et al., 2011; Marischen et al., 2018). NK cell cytotoxicity against fungi is also mediated per perforin (Ma et al., 2004). Fungal infections are seen in ~10% of patients with classical NK cell deficiency, many of whom have GATA2 deficiency (Spinner et al., 2014; Orange, 2013). Overall, the precise role of NK cells in eliminating fungal pathogens needs to be better characterized both in healthy individuals and immunocompromised patients.

Protozoan infection

Parasites can induce strong host inflammatory responses and activate NK cells. For example, *Plasmodium* spp., the organism responsible for malaria, can directly activate spontaneous and ADCC pathways in NK cells. Patients with uncomplicated malaria infection have high levels of CD56^{bright}, low levels of CD56^{dim} NK cells, and expansion of “adaptive” NK cells (Orago and Facer, 1991; Hart et al., 2019; Tesi et al., 2016). NK cells can directly bind parasite protein during the blood-stage of the parasite life cycle: PfEMP1 with Nkp30 and Nkp46. Interestingly, NK cells lacking CD16 have enhanced protection from malaria. Similar pathways are identified in the cross-talk between NK cells, *Leishmania*, and *Toxoplasma* spp. pathogens (Horowitz et al., 2012).

NK cells in health and human disease

Dysregulation of NK cells has been demonstrated in non-infectious diseases states including malignancy, autoimmunity, and atopic diseases (Mandal and Viswanathan, 2015).

NK cells are the main innate defense cells against cancer cells and have an important role in immunosurveillance (Waldhauer and Steinle, 2008). NK cells are found in tumor cells, albeit in much lower numbers compared to other lymphoid and myeloid cells (Carrega et al., 2014). In fact, upregulation of NKG2D ligands on the surface of many tumor cells recruits NK cells to the tumor (Wang et al., 2019). Furthermore, high numbers of NK cells in the various solid tumors (e.g., GI, lung, breast, brain, and renal cancers) are directly proportional to better survival prognosis (Takeuchi et al., 2001; Best et al., 2020; Nersesian et al., 2020; Sedgwick et al., 2020). Inversely, epidemiologic data has demonstrated that patients with diminished NK cell activity (in the peripheral blood) are at increased risk for developing cancer (Imai et al., 2000). Due to their antitumor potential, NK cells are currently harvested (isolated from umbilical cord, placenta, or peripheral blood) and engineered for cell-based cancer immunotherapy for leukemias and solid-organ cancers (Shimasaki, 2020; Liu et al., 2021a).

NK cells have a significant role in atopic diseases such as atopic dermatitis (AD) and asthma (von Bubnoff et al., 2010; Kim and Jang, 2018). Atopic dermatitis is a complex, type 2 chronic inflammatory skin disease. Patients with severe AD may develop eczema herpeticum and eczema vaccinatum, signs of diminished antiviral immunity. Clinical studies have identified quantitative (diminished CD56^{dim} effector cells) and qualitative dysregulation of NK cells in AD patients in part due to decreased IFN γ production, a potent inhibitor of type 2 inflammation (Mack et al., 2020; Luci et al., 2012; Molofsky et al., 2015). Similarly, NK cells have been

described as “asthma antagonists” and contribute to the resolution of allergic inflammation by suppressing Th₂ cells and IL-17-dependent signaling (Gorska, 2017; Karimi, 2013).

NK cells have been implicated in the development of autoimmune diseases (Type 1 diabetes, systemic lupus erythematosus, rheumatoid arthritis autoimmune encephalitis, multiple sclerosis) and hyperinflammatory disorders. NK cells have protective cytotoxic activity against autoreactive T and B cells, or by cytokine signaling (TGFβ, IL-4, IL-5, and IL-10). However, NK cells can also have a pathogenic role in the development of autoimmune disease by secreting cytokines and chemokines that recruit and activate other immune cells and enhance tissue inflammation and destruction (for a comprehensive review, see Ref. (Liu et al., 2021b)).

NK cell deficiencies

There are numerous immunodeficiencies where abnormalities in NK cells (quantity and/or quality) are present in the setting of other immune defects (Vargas-Hernández and Forbes, 2019). Of the ~430 genes causing primary immune deficiencies (PID) (Tangye et al., 2020), >50 affect NK cells of which only 7 are known to cause true NK cell deficiency (NKD) (Orange and How, 2020). The diagnostic criteria for NKD are as follows:

1. NK cells levels are ≤1% of PBMCs.
2. Depressed NK cell numbers and function on three separate occasions separated by at least 1 month (Bonilla et al., 2015).
3. Exclusion of other PID that affect NK cells (Wood et al., 2011).
4. Exclusion of other causes including malignancy, infection, or medications.

Impaired NK development and/or function is the major immunological defect in these NK cell deficiencies. NKDs which can be categorized based on (1) defects in NK cell receptors, (2) defects in cell transcription factors, or (3) abnormalities in NK cell cycle proteins. Six genes are responsible for classical NKD (cNKD) which include deficiencies in GATA2, MCM4, MCM10, GINS1, IRF8, and RTEL1 (Hsu et al., 2011; Gineau et al., 2012; Cottineau et al., 2017; Mace et al., 2020, 2016; de Vries et al., 1996; Ballew et al., 2013). A hallmark feature of cNKD is the paucity (or complete absence) of NK cell subsets. Additionally, to date there is only one functional NKD (fNKD), due to defects in FCGR3A/CD16 gene (Grier et al., 2012). fNKD present with normal number of NK cell subsets but with abnormal cytotoxicity profile. The following section reviews the biology, clinical and laboratory phenotype, and management of the six known types of NKDs.

GATA2 deficiency (AD)

GATA2 (GATA-binding factor 2; OMIM 614172, Chr. 3) is a zinc-finger containing transcription factor. GATA promotes development and function of hematopoietic stem and progenitor cells (HSPC) and the formation of blood/lymphatic vessels (Ostergaard et al., 2011). GATA2 deficiency was the first identified case of cNKD in a patient that presented with disseminated VZV, CMV, and HSV (Spinner et al., 2014; Mace et al., 2013). GATA2 deficiency has a highly variable penetrance pattern and heterogenous phenotypic expression may be affected by somatic mutations in other genes (RUNX1, ETV6, STAG2, among others) (Bresnick et al., 2020). GATA2 deficient patients are mostly susceptible to viral infections including HPV, HSV, EBC, and molluscum contagiosum. However, they can also develop severe non-tuberculous mycobacterial disease (typically *Mycobacterium avium* complex [MAC], and *M. kansasii*) and fungal infections. GATA2 deficient patients have nearly (or absolutely) absent CD56^{bright} NK cells but can have some CD56^{dim} NK cells with impaired function (Mace and Orange, 2016; Mace et al., 2013). Another prominent feature is multi-lineage cytopenias including B lymphocytopenia, NK lymphopenia, and monocytopenia (Hsu et al., 2011). Lastly, GATA2 deficient patients have increased risk for myeloid malignancy (AML and CMML), pulmonary complications (pulmonary alveolar proteinosis due to pulmonary warts), primary lymphedema, and miscarriage (Griese et al., 2015).

MCM4 deficiency (AR)

MCM4 (Mini-chromosome maintenance complex component 4; OMIM 609981, Chr. 8) is a crucial nuclear subunit of the CMG replicative helicase and is important for maintaining mitotic stability during DNA replication, recombination, and repair. Briefly, the CMG helicase is a 11-subunit complex made up of CDC45 protein, the MCM2-7 complex, and the GINS complex. The MCM2-7 complex is hexameric ring-shaped “motor” that binds to chromatin and gets activated by CDC45 and GINS complex. Defects in the CMG replisome impair cell cycle progression and induces DNA damage (and subsequent activation of DNA damage repair pathways). Partial MCM deficiency was initially reported in six related Irish patients (consanguineous kindred) with variable presentation including intrauterine and postnatal growth restriction, glucocorticoid deficiency, EBV-driven lymphoma, osteosarcoma, and predisposition to viral infections (HSV, VZV) (Gineau et al., 2012; Hughes et al., 2012). Immunophenotyping of the NK cells in these MCMF deficient patients showed reduced total number of NK cells mostly due to the paucity of mature, cytolytic CD56^{dim} NK cells. The selective loss of CD56^{dim} cells resulted in skewing toward higher levels of CD56^{bright} NK cells, similar to other NKDs such as IRF8 and GINS1, as discussed below. The MCM4 deficient CD56^{dim} cells did, however, proliferate with IL-2 and IL-15 stimulation. This suggests that the lack of NK cells was in part due to a large chromosomal genotoxic burden during proliferation of CD56^{bright} cells and inability to generate many CD56^{dim} cells.

MCM10 deficiency (AR)

MCM10 (Mini-chromosome maintenance complex component 10; OMIM 619313, Chr. 10) is a key scaffold protein within the CMG replisome (Di Perna et al., 2013). It is active during cellular proliferation and is crucial for the formation of replication forks, maintenance of telomere integrity, and prevents genomic instability (Miotto et al., 2014; Chattopadhyay and Bielinsky, 2007). MCM10 is upregulated in various tumors including medulloblastoma and breast cancer (Senfter et al., 2017; Murayama et al., 2021). MCM10 deficiency (due to biallelic mutations in the MCM10 gene) was first identified in an infant presenting with febrile illness, organomegaly, diarrhea, and disseminated CMV disease (Mace et al., 2020). He had severely low total NK cells, with an overrepresented CD56^{bright} population, similar to MCM4 deficiency. HSCT was unsuccessful in this patient due to fatal CMV infection. Separately, frameshift variants in the MCM10 genes in three other unrelated patients produced a different phenotype with restrictive cardiomyopathy and splenic/thymic hypoplasia (Baxley et al., 2021). Overall, clinical and basic science data provides convincing evidence for the role of MCM10 in NK cell development/function and its role in other somatic cells.

GINS1 deficiency (AR)

GINS (Go-Ichi-Nii-Sans, or 5, 1, 2, and 3 in Japanese; OMIM 617827, Chr. 20) is an evolutionarily conserved chromosomal DNA replication complex initially identified in budding yeast (Takayama, 2003). GINS subunit 1 (GIN1) is a component of the hetero-tetrameric GINS complex (GIN1, -2, -3, and 4). During the G1/S transition phase of mitosis, the GINS complex interacts with CDC45 and MCM2-7 to form a macromolecular replisome. GINS preferentially binds to ssDNA and may have a direct role in unwinding the DNA during replication (Boskovic et al., 2007). In murine models, deletion of *Gins1* is embryonically lethal. In humans, compound heterozygous mutations of GINS1 were initially identified in two French sisters who presented with intrauterine growth restriction, postnatal growth restriction, facial dysmorphism, lymphadenopathy, and eczema. They had immunological defects including neutropenia, CD8 $\alpha\beta$ T cells lymphopenia, and absent or extremely low number of NK-cells (both CD56^{bright} and CD56^{dim} NK cells) (Bernard et al., 2004). Additional individuals with GINS1 deficiency from other unrelated and non-consanguineous families have been identified and have similar immune phenotypes (Cottineau et al., 2017). GINS1 deficient patients mostly have severe viral infections caused by VZV, CMV, HSV, RSV, and adenovirus. At the cellular level, GINS1 deficient patients have approximately 25–50% of the GINS1 protein expression compared to healthy individuals and their cells have lower number of replication forks, abnormal cell cycle control (slower proliferation), genomic instability, and signs of cellular senescence (Cottineau et al., 2017). While the neutropenia in GINS1 patients can be reversed with G-CSF, there are currently no treatments available for the NK cell deficiency. Additionally, given the important role of GINS in maintaining chromosomal stability, the potential risk for malignancy associated with GINS1 deficiency is unknown.

IRF8 deficiency (AR)

IRF8 (interferon regulatory factor 8; OMIM 601565, Chr. 16) is a transcription factor that is exclusively found in cells with hematopoietic lineage and is highly expressed in plasmacytoid dendritic cells (Sichien et al., 2016). IRF8 binds to the interferon consensus sequence (ICS) upstream of type I IFN and IFN-inducible MHC class I genes which are upregulated during viral infection. IRF8 also has other pleiotropic effects including regulation of apoptotic pathways (Yang et al., 2011). Homozygous and heterozygous IRF8 mutations in mice and humans lead to abnormal differentiation of mononuclear phagocytes and play a critical role in anti-mycobacterial immunity (Hambleton et al., 2011). Additionally, biallelic mutations in IRF8 can cause life-threatening EBV infection (Mace et al., 2016; Fleisher et al., 1982). At the cellular level, viral-infected cells secrete IL-12 or common- γ -chain dependent cytokines (IL-12, IL-15) which upregulate IRF8 expression in NK cells (Adams et al., 2018). IRF8 directly controls Zbtb32, a master regulator of the NK cell proliferative burst, and regulates expression of other cell cycle genes that are critical for cellular maturation and survival (Adams et al., 2018; Beaulieu et al., 2014). In mice, *Irf8* ablation adversely blunts NK-cell proliferation during acute CMV infection (Adams et al., 2018). In humans, IRF8 deficient patients have impaired NK cell function and a paucity of cytotoxic CD56^{dim} NK cells, reflecting impaired NK cell terminal maturation (Mace et al., 2016). There are currently no good treatment strategies available for IRF8 deficiency but cord-blood stem cell transplantation has been previously successful in an infant with IRF8 deficiency and disseminated BCG disease (Hambleton et al., 2011).

RTEL1 deficiency (AR/AD)

RTEL1 (regulatory of telomerase elongation 1; OMIM 608833, Chr. 20) is a DNA helicase with antirecombinase activity. RTEL1 is important for maintaining telomere length and limiting excessive meiotic crossovers. In general, mutations in helicases can cause developmental delay, dyskeratosis congenita (DC), pulmonary fibrosis, hepatic disease, bone-marrow failure, and myelodysplastic syndrome. Mutations in the RTEL1 helicase can cause a very severe variant of DC called Hoyerall Hreidarsson (HH) syndrome. HH patients present with immunodeficiency, enteropathy, IUGR, and CNS hypoplasia. RTEL1 deficient patients are at risk for viral infections (e.g., VZV) and immune phenotyping reveals either isolated NK cell lymphopenia and impaired cytotoxicity (Etzioni et al., 2005), or B/NK cell combination quantitative and qualitative defects (Speckmann et al., 2017). Importantly, although this helicase is important for DNA repair, V(D)J recombination and T-circle accumulation is not affected in RTEL1 deficient patients (Speckmann et al., 2017). This is consistent with the fact that NK cells do not undergo V(D)J recombination to generate their large repertoire of antigen receptors, which are germline encoded (Karo and Sun, 2015). HSCT has been successful in two cases of RTEL1 deficiency (Speckmann et al., 2017).

FCGR3A (CD16) deficiency (AR)

FCGR3A (Fc fragment of IgG receptor 3a, or CD16; OMIM [146740](#), Chr. 1) encodes for a low affinity, IgG-binding (IgG1 or IgG3), transmembrane glycoprotein receptor. CD16 is expressed on NK cells, activated monocytes/macrophages, and natural killer T cells. Activation of CD16 stimulates Ca^{2+} -dependent pathways that increase expression of various cytokines and exocytosis of secretory lysosomes in both spontaneous and antibody-dependent cytotoxicity assays, as discussed above. Patients with homozygous mutations in FCGR3A can present with otitis media, sinusitis, URIs, and susceptibility to various viral infections (HSV, HPV, CMV, EBV; stomatitis, whitlow, Castleman's disease) (de Vries et al., 1996; Jawahar et al., 1996). Most of the known human mutations in FCGR3A (e.g., L66H) are located distal to the IgG-binding domain of the receptor (recognized by B73.1 anti-CD16 antibody) (Orange, 2013). In healthy patients, this region of CD16 engages with as co-stimulating receptor for CD2 and is important for spontaneous cytotoxic function. The variant does not affect receptor expression or binding of IgG to CD16. Hence, L48H mutations in FCGR3A are associated with abnormal spontaneous cytotoxicity and normal ADCC. To date, there are no known mutations in the IgG-binding domain of CD16 (recognized by 3G8 anti-CD16 antibody). Overall, patients with FCGR3A deficiency have quantitatively normal number of NK cell subtypes but have abnormal cytotoxicity profiles. Hence, unlike the other NK deficiencies discussed above, FCGR3A is a functional NK cell deficiency.

Summary

NK cells are innate lymphocytes involved in defenses against pathogen-infected, senescent, and malignant cells. Advances in medical and basic sciences in the last 50 years have allowed us to better characterize the complex biology of NK cells. We are now in an exciting, yet challenging, position to translate the wealth of knowledge into therapeutic application in various disease conditions.

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Toll-Like Receptors

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Pattern recognition receptors: As the sensors for pathogen-associated molecular patterns

The innate immune system is defined as the first line of host defense against invasive pathogens by discrimination of “non-self” from “self” antigens. Recognition by innate immune cells depends on germline-encoded pattern-recognition receptors (PRRs) which have specialized to recognize the conserved structures of foreign pathogens called pathogen-associated molecular patterns (PAMPs). In this regard, the engagement of PRRs with their ligands triggers the pro-inflammatory responses resulting in pathogen elimination (Takeuchi and Akira, 2010).

PRRs are also responsible for detecting the endogenous molecules released by dying or damaged cells referred to as Damage-Associated Molecular Patterns (DAMPs). Recognition of DAMPs as danger signals initiate or propagate sterile inflammation leading to tissue repair and regeneration (Takeuchi and Akira, 2010).

In addition to their roles in host defense, PRRs signaling plays an important role in immune system homeostasis. In this context, inappropriate receptor activation could result in developing a variety of diseases such as neurodegenerative disorders (Hanke and Kielian, 2011), autoimmune diseases (Farrugia and Baron, 2017), cardiovascular disorders (Moghimpour Bijani et al., 2012), and cancer (Khajeh Alizadeh Attar et al., 2018).

Four primary families of PRRs including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-Leucine Rich Repeats-containing receptors (NLR), retinoic acid-inducible gene 1 (RIG-1) -like receptors (RLR), and the C-type lectin receptors (CLRs) have been identified which are differently diffused in subcellular compartments such as cytosol, cell membrane, and endosomal membrane (Takeuchi and Akira, 2010).

TLRs are the well-known PRRs which play an important role in priming the inflammatory responses and mounting efficient immune responses. This chapter attempts to survey the biology and significance of TLRs activation in host defense.

Toll-like receptors (TLRs)

TLRs were the first discovered PRRs which acknowledged as important receptors involved in homeostasis of the immune system and establishing host immunity to infections. TLRs are mostly expressed on antigen-presenting cells (APCs), in particular macrophages and dendritic cells promoting the phagocytic activity such as pathogen uptake and killing by APCs (Medzhitov, 2001). The TLR functions depend closely on where they are expressed; in the epithelial cells they promote the production of anti-microbial peptides as the first line of defense at mucosal sites. For example, activation of TLR4 in the Paneth cells of the intestine results in high production of defensins; however, in the absence of infection, they are involved in the enhancement of tight junctions and tissue homeostasis (Menendez et al., 2013; Hug et al., 2018).

TLRs also act as a bridge between innate and acquired immune responses. Ligand-induced activation of these receptors leads to recruitment of leukocytes to the infected tissues. This process is enhanced by the expression of adhesion molecules such as E-selectin and ICAM1 on endothelial cells induced by TLRs. Besides, TLR engagement on APCs induces the expression of co-stimulatory molecules such as B7.1 (CD80) and B7.2 (CD86), which are essential for CD4⁺ T cells activation and triggering adaptive immunity (Iwasaki and Medzhitov, 2004).

TLRs tend to be involved in tissue homeostasis, in addition to their functions in host immunity against infection. In this manner, the production of prostaglandins, cyclooxygenase 2 (COX2), and the expression of anti-apoptotic proteins induced by TLR signaling plays a crucial role in tissue repair and regeneration (Fukata et al., 2006; Brown et al., 2007). This function is of utmost importance; so that the improper or inappropriate activation of such receptors might contribute to the development of various disorders such as cancer, autoimmunity, brain, and cardiovascular diseases, whether in the context of infection or not.

Structure of TLRs

TLRs are classified as type I integral membrane proteins that consist of three domains as follows (Fig. 1) (Jin and Lee, 2008):

- 1- Extra-cellular or N-terminal domain is a glycosylated protein composed of tandem repeats of a motif called leucine-rich repeat (LRR) responsible for ligand recognition.
- 2- Intra-cellular or C-terminal region of TLRs includes a domain known as Toll IL-1 receptor (TIR) contributing to signaling cascade upon TLR activation.
- 3- The transmembrane region is a single-pass helix containing typical hydrophobic amino-acids. This part is involved in TLR dimerization and stabilization.

Of note, all TLRs form a hetero- or homo-dimer structure upon ligand binding that facilitates their signal transmission process.

Cellular expression of TLRs

TLRs are widely expressed in both immune- and non-immune cells enabling antimicrobial responses to be triggered in several tissues. Dendritic cells (DC), macrophages, and B cells are the prominent immune cells expressing TLRs. Fibroblast cells, epithelial cells, osteoblasts, endothelial cells, and hematopoietic stem cells (HSC) are the other TLRs-expressing cells. Up to now, 10 TLRs in humans (TLR1–10) and 12 TLRs in mouse (TLR1–9, TLR11, TLR13) have been identified. They are mostly classified based on their cellular localization; TLR1, 2, 4, 5, 6, 11 are located on the cell surface, while others like TLR3, 7, 8, 9, 10, 12, 13 are found in the intracellular compartments such as the membranes of endosomes, endoplasmic reticulum (ER), and lysosomes (Table 1) (El-Zayat et al., 2019).

TLRs recognize both intra- and extra-cellular molecular patterns

Any particular TLR is devoted to identifying a distinct molecular pattern. For clarify, Table 1 displays TLRs along with their associated ligands. In contrast to the cell surface TLRs which are contributing to the recognition of the external antigenic structures of pathogens such as proteins, lipids, and lipoproteins, intracellular TLRs are responsible for responding to pathogens- or host-derived nucleic acids.

Cell surface TLRs: The sensors for external antigenic patterns

TLR2 by forming heterodimers with TLR1 or TLR6 identifies various molecular patterns of Gram-negative bacteria including lipoteichoic acid and lipoproteins. TLR2/1 heterodimers mostly recognize triacyl lipoproteins, while TLR2/6 heterodimers tend to interact with diacyl lipoproteins (Hedayat et al., 2012). They are expressed on DCs, macrophages, basophils, eosinophils, mast cells, endothelial, and fibroblast cells. Recent studies also illustrate the formation of TLR2/TLR10 heterodimers; however, their function and ligands have not been clearly described (Guan et al., 2010).

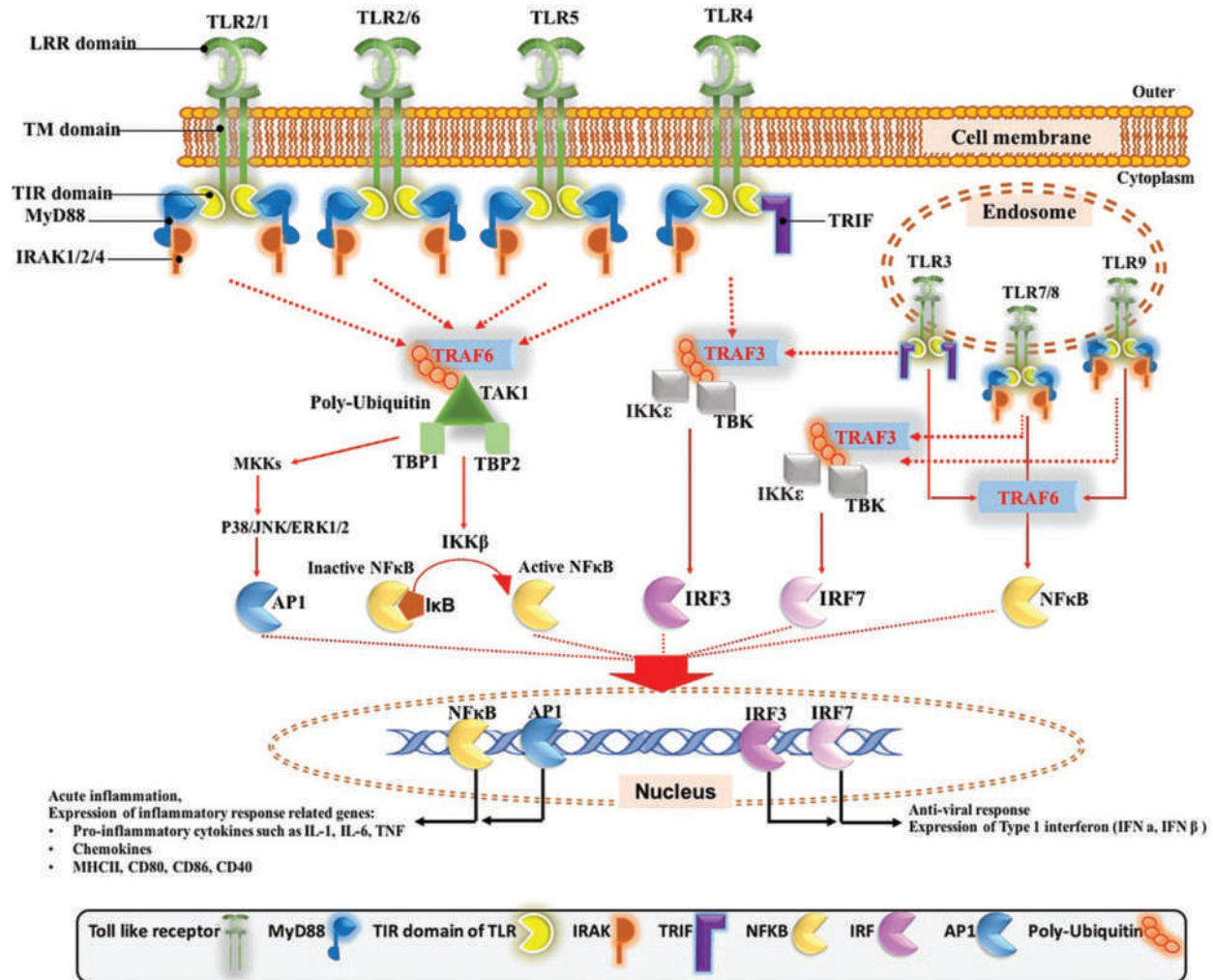


Fig. 1 TLR structure and signaling pathway. TLRs consist of three domains: 1) Extra-cellular or N-terminal domain responsible for ligand recognition; 2) Intra-cellular or C-terminal region contributing to signaling cascade upon TLR activation; 3) The transmembrane region involving in TLR dimerization and stabilization. All TLRs form a hetero- or homo-dimer structure upon ligand binding. TLR signaling induces a wide spectrum of intracellular responses resulting in the production of pro-inflammatory or anti-viral cytokines. Most TLRs use MyD88 for initiating downstream signaling pathway, except for TLR3 which interacts only with TRIF. TLR4 employs both of MyD88-dependent or TRIF signaling pathways. MyD88 consists of two main domains including TIR domain at C-terminal which interacts with TIR domain at cytoplasmic tails of TLRs, and death domain at N-terminal which recruits IRAKs including IRAK4 and IRAK1.

Recently, a number of accessory molecules and co-receptors have been identified for accumulation of microbial products on the cell surface or within phagosomes to facilitate TLR2 responses. For example, CD36 binds to diacylglycerol and acts as a transporter to deliver it to accessory molecule CD14 which in turn, facilitates ligand loading onto TLR2/TLR6 or TLR2/1 heterodimers (Hedayat et al., 2012). Although CD14 and CD36 are not the essential prerequisites for TLR2 signaling, it is notable that the function of these molecules is to improve responses and decrease the ligand concentrations required for recognition and receptor signaling.

TLR4 is expressed on the numerous cell types including antigen-presenting cells, endothelial cells, myocytes, thyroid cells, endometrial cells, mesangial cells, and adipocytes. TLR4 has been identified as the most important receptor for inflammatory response against lipopolysaccharide (LPS) derived from gram-negative bacteria. For its recognition, TLR4 requires accessory molecules including myeloid differentiation 2 (MD-2) and CD14, which are essential for enhancing the downstream signaling cascade. In the serum, LPS is directed to CD14 by forming a complex with LPS binding protein (LBP). Association of CD14 with TLR4 forms an effective receptor for LPS recognition. TLR4 is also responsible for responding to other components of gram-positive and gram-negative bacteria such as teichuronic acid and mannuronic acid polymers, respectively (Vaure and Liu, 2014).

In addition to PAMPs, some endogenous molecular patterns were also identified as the ligands for TLR4 and TLR2 which could trigger inflammatory responses during tissue damage or necrosis; including heat shock proteins (HSPs), uric acid crystals, fibronectin type III, high mobility group box 1 (HMGB1), surfactant protein A, saturated fatty acids, and heparan sulfate (Roh and Sohn, 2018).

Table 1 Cellular distribution, and known ligands of human Toll-like receptors.

TLR type	Cellular distribution ^a	Expressing cell type	Exogenous ligands ^b	Endogenous ligands ^b	Transcription factor Activation		Cytokine production
					NF- κ B	IRF	
TLR1	Cell membrane	Monocyte/macrophage, Neutrophils, B cell, PDCs MDSCs	Bacteria: • Triacyl Lipopeptides (e.g. <i>N. meningitidis</i>)	N/A	+	–	Pro-inflammatory cytokines
TLR2	Cell membrane	Monocyte/macrophage, Neutrophils, MDCs, Mast cells	Bacteria: • Peptidoglycan, Lipoprotein/lipopeptides, Lipoteichoic acid (Gram positive bacteria) • Modulin (<i>Staphylococcus</i>) • Lipoprotein/lipopeptides (<i>Mycoplasma</i> , <i>Mycobacteria</i> , <i>Spirochetes</i>) • Glycolipids (<i>Spirochetes</i>) • Heat-killed bacteria (<i>Listeria</i>) • lipoarabinomannan (<i>mycobacteria</i>) • Porins (<i>Neisseria</i>), Soluble factors (<i>N. meningitidis</i>) • OmpA (<i>K. pneumoniae</i>) • Outer membrane protein A (<i>Klebsiella</i>) • Soluble factors (<i>Neisseria meningitidis</i>) Virus: • Hemagglutinin (<i>measles virus</i>), structural viral proteins (<i>herpes simplex virus</i> , <i>cytomegalovirus</i>) • Fungi: Zymosan (<i>Saccharomyces</i>) phospholipomannan (<i>C. albicans</i>) Protozoa: • Glycoinositolphospholipids (<i>Trypanosoma cruzi</i>) Yeast: • Zymosan	HSP60, HSP70; hyaluronic acid, HMGB1, versican, GP96	+	–	Pro-inflammatory cytokines
TLR3	Endosomal membrane	MDCs, T cell, B cell	Virus: • dsRNA	mRNA	+	+	Proinflammatory cytokines, type I IFNs
TLR4	Cell membrane	Monocyte/macrophage, Neutrophils, MDCs, Mast cells, B cell, intestinal epithelium	Bacteria: • Lipoteichoic acid (Gram positive bacteria) • LPS (Gram negative bacteria) Virus: • Fusion proteins (<i>respiratory syncytial virus</i> and mouse mammary tumor virus) Fungi • Mannan Protozoa: • Glycoinositolphospholipids (<i>Trypanosoma cruzi</i>)	Hsp60, HSP22, HSP70, HSP72, Gp96, HMGB1, Oxidized phospholipids, Heparin sulfate, Fibronectin, Tenascin-C, b-Defensin 2, Versican, Hyaluronic acid	+	+	Proinflammatory cytokines, type I IFNs
TLR5	Cell membrane	Monocyte/macrophage, Neutrophils, MDCs, intestinal epithelium	Bacteria: • Flagellin (flagellated bacteria)	N/A	+	–	Proinflammatory cytokines

(Continued)

Table 1 (Continued)

TLR type	Cellular distribution	Expressing cell type	Exogenous ligands	Endogenous ligands	Transcription factor Activation		Cytokine production
					NF- κ B	IRF	
TLR6	Cell membrane	Monocyte/macrophage, Neutrophils, Mast cells, MDCs, PDCs, B cell	Bacteria: • Diacyl lipopeptides (e.g. <i>mycoplasma</i>), Lipoteichoic acid Fungi: Zymosan (<i>Saccharomyces</i>)	N/A	+	–	Proinflammatory cytokines
TLR7	Endosomal membrane	Monocyte/macrophage, Neutrophils, DCs, B cell	Virus: • ssRNA	ssRNA (immune complex)	+	+(IRF5, IRF7)	Proinflammatory cytokines, type I IFNs
TLR8	Endosomal membrane	Monocyte/macrophage, Neutrophils, MDCs, Mast cells	Virus: • ssRNA	ssRNA (immune complex)	+	+(IRF5, IRF7)	Proinflammatory cytokines, type I IFNs
TLR9	Endosomal membrane	Monocyte/macrophage, Neutrophils, PDCs, B cell, T cell	Bacteria and virus: • Unmethylated CpG DNA Protozoa: • Unmethylated CpG DNA • Hemozoin (<i>Plasmodium</i>)	N/A	+	+(IRF5, IRF7)	Proinflammatory cytokines, type I IFNs
TLR10	Cell membrane	Monocyte/macrophage, B cell	Bacteria: • Triacylated lipopeptides	N/A	+	–	Proinflammatory cytokines

NF- κ B: Nuclear factor- κ B; IRF: Interferon regulatory factor; MDSCs: Myeloid dendritic cells; PDCs: Plasmacytoid dendritic cells; HSP: Heat shock protein; GP96: Glycoprotein96; HMGB: high mobility group box; OmpA: outer membrane protein A; ssRNA: Single-strand RNA; dsRNA: double-strand RNA; IFN: interferon; CpG: cytosine-guanine dinucleotide; NA: not available.

^aFrom El-Zayat SR, Sibaii H, and Mannaa FA (2019) Toll-like receptors activation, signaling, and targeting: An overview. *Bulletin of the National Research Centre* 43: 187.

^bFrom Hedayat M, Takeda K, and Rezaei, N (2012) Prophylactic and therapeutic implications of toll-like receptor ligands. *Medicinal Research Reviews* 32: 294.

TLR5 is expressed on macrophages, DCs (especially intestinal lamina propria DCs), and basolateral pole of intestinal epithelial cells. This receptor acts as an immune sensor for capture flagellin, a protein that is found on motile bacteria (Uematsu et al., 2006). TLR11 was also initially determined to detection of flagellin, but is not expressed in humans. TLR11-deficient mice have shown to be susceptible to pathogens carrying flagellin (Hatai et al., 2016).

Intracellular TLRs: The sensors for nucleic acids

Intracellular TLRs are involved in immune response to pathogens- or host-derived nucleic acids. They are mostly located on endosomal compartments, and the acidification of endosomes is required for their activation. UNC93B1, an ER protein, facilitates intracellular trafficking of TLRs from the ER to the endolysosomes (Blasius and Beutler, 2010).

TLR3 is expressed by macrophages, myeloid DCs (importantly CD141⁺ DCs), epithelial cells, fibroblasts, neuronal cells, astrocytes, and skeletal muscle cells. This receptor intrinsically plays a fundamental role in detecting virus-derived double-strand RNA (dsRNA) and activating inflammatory responses by inducing the production of pro-inflammatory cytokines. In particular, the expression of TLR3 is up-regulated by type I interferons (IFN α , IFN β) which promote the antiviral responses. Intriguingly, although several studies suggest that TLR3 improves the eradication of particular viruses, some reports indicate that certain viruses may benefit from TLR3 activation (Vercammen et al., 2008). For example, up-regulation of TLR3 induced by Influenza A virus in the respiratory tract causes acute pneumonia; While, TLR3 was suggested to have a protective role during encephalopathy caused by the influenza A virus (Hidaka et al., 2006).

TLR7 and TLR8 are endosomal sensors for the detection of single-strand RNA (ssRNA) derived from multiple viruses such as HIV-1 or the influenza virus. TLR9 identifies unmethylated CpG (cytosine-guanine dinucleotide) motifs that are present in the genome of viruses and bacteria especially in HSV-1 (Herpes simplex virus 1), MCMV (Murine cytomegalovirus), adenovirus, and poxviruses (Carty and Bowie, 2010).

TLR7 and TLR9 are primarily expressed by plasmacytoid dendritic cells (pDC), B cells, and eosinophils, while TLR8 has shown to be expressed on monocytes and macrophages. All these receptors induce the expression of interferon type I and pro-inflammatory cytokines upon RNA virus detection (Cervantes et al., 2012).

TLR10 expression can be induced in dendritic cells, eosinophils, B cells, neutrophils, and trophoblast cells. It is proposed that TLR10 can form heterodimers with TLR2. In contrast to the other TLRs, little is known about the ligands and biological function of TLR10. Interestingly, its inhibitory functions were shown in one study using transgenic mice. In this context, TLR10 suppresses inflammation by inducing the anti-inflammatory cytokine IL-1Ra through PI3K/Akt signaling pathway (Oosting et al., 2014).

TLR signaling pathway

TLR signaling induces a wide spectrum of intracellular responses resulting in the production of pro-inflammatory or anti-viral cytokines. Upon ligand binding, TLRs actively form dimers which cause their TIR domains to be placed next to each other. Further, the interaction between TIR domains and cytoplasmic adaptor molecules initiates intracellular signaling (Fig. 1) (Kawasaki and Kawai, 2014).

Four adaptor molecules have been identified to interact with distinct TLRs:

- 1- Myeloid differentiation primary response 88 (MyD88)
- 2- Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP) which also known as MAL
- 3- TIR-domain-containing adapter-inducing interferon- β (TRIF)
- 4- TRIF-related adaptor molecule (TRAM)

Most TLRs employ MyD88 for initiating downstream signaling cascade, except for TLR3 which interacts only with TRIF. TLR4 uses both of MyD88-dependent (MyD88/TIRAP) or MyD88-independent (TRIF/TRAM) signaling pathways.

MyD88-dependent pathway

The MyD88 adaptor protein is used by both cell surface TLRs (TLR1/2, TLR2/6, TLR4, TLR5) and endosomal TLRs (TLR7, TLR8, TLR9) to activate signaling pathway (Kawasaki and Kawai, 2014).

MyD88 consists of two main domains including: 1) TIR domain at C-terminal of MyD88 protein which interacts with TIR domain at cytoplasmic tails of TLRs; 2) Death domain at N-terminal of MyD88 which recruits IL-1R-associated kinases (IRAKs) including IRAK4 and IRAK1 (Fig. 1). These kinases being activated via self- and cross-phosphorylation and form an appropriate signaling scaffold that mediates phosphorylation of the other recruited molecules. TRAF6 (tumor necrosis factor receptor-associated factor 6) is one of the important downstream molecules recruited and activated through phosphorylation by IRAKs (Deguine and Barton, 2014).

TRAF6 as an E3 ubiquitin ligase by forming a poly-ubiquitin chains makes a complex with other molecules including transforming growth factor β -activated kinase (TAK1), TAK1-binding protein 1 (TBP1), and TAK1-binding protein 2 (TBP2). Consequently, the activation of TAK1 leads to the initiation of NF κ B signaling pathway.

In the inactivated state, NF κ B is regulated and inhibited by I κ B protein in the cytoplasm. Activating NF κ B as a transcription factor requires phosphorylation and degradation of I κ B that is mediated by a kinase known as IKK β .

Besides, activation of TAK1 may stimulate MAP kinase and JNK signaling pathways resulting in activation of another transcription factor known as activator protein-1 (AP-1) (Chen, 2012; Ajibade et al., 2013).

Notably, with regards to the endosomal TLRs which use MyD88 as an adaptor molecule, IRAK1/4 recruitment may also trigger another signaling pathway. In this way, IRAK1/4 recruits TRAF3 which in turn engages other signaling molecules including IKK and TBK1. These two kinases phosphorylate and activate IFN regulatory factor 7 (IRF7) which translocates to the nucleus where it can derive transcription of interferon type I gene as the anti-viral response (Kawasaki and Kawai, 2014).

Both NF κ B and AP-1 induce multiple inflammatory immune responses leading to the promotion of T cells activation; including the expression of many pro-inflammatory cytokines such as IL-1, IL-6, TNF- α , expression of co-stimulatory molecules B7.1 (CD80), B7.2 (CD86), and up-regulation of CD40 and MHC II (Khajeh Alizadeh Attar et al., 2018).

The activation of NF κ B and AP-1 transcription factors by TLRs is critical to enhancing the immune responses against bacterial pathogens. For instance, mutations in IRAK4 or MyD88 lead to a type of immunodeficiency characterized by recurrent bacterial infections. We will describe the important immunodeficiencies affected by the impaired TLR signaling in the next part.

MyD88-independent pathway

As described earlier, some TLRs such as TLR3 and TLR4 use MyD88-independent signaling pathway upon ligand binding. In contrast to TLR4 which uses either MyD88 or TRIF, TLR3 initiates its signaling cascade only by mediating TRIF adaptor molecule. Signaling using TRIF leads to activation of NF κ B and AP-1 transcription factors, same as MyD88 (Kawasaki and Kawai, 2014).

One of the important downstream mediators of TRIF signaling, known as TRAF3, by making poly-ubiquitin scaffold (like TRAF6) recruits IKK and TBK1 kinases which phosphorylate and activate IRF3. Finally, IRF3 translocates to the nucleus and mediates transcription of interferon type I cytokine (Ajibade et al., 2013). Therefore, activation of both pathways, whether by MyD88 or not, contributes to the enhancement of inflammatory and antiviral responses resulting in the enhancement of pathogen clearance.

Primary immunodeficiencies associated with TLR signaling: Increased susceptibility to infectious diseases

A few numbers of rare immunodeficiencies have been reported associated with impaired TLR signaling and NF κ B activation. Besides, different single-nucleotide polymorphisms (SNPs) were identified in TLRs associated with the increased susceptibility to infectious diseases (Mukherjee et al., 2019) (Table 2).

The important genetic disorders related to TLR signaling are as follows:

Table 2 Association between TLR and susceptibility to infectious diseases.

TLR	Association with diseases					TLR's SNPs associated with infectious diseases ^b
	Cancer ^a			Autoimmunity	Other disorders	
	Cancer type	Role in tumor progression	Role in tumor regression			
TLR2	Gastric, hepatocellular carcinoma	• Immunosuppression • Angiogenesis and Metastasis of tumor • Increase inflammation and Vascularization • Upregulation of Cox-2 and IL8 up-regulation	• Increase of chemosensitivity	• RA: Increased TLR2 on both peripheral blood monocytes and synovial tissues • MS: Increased TLR2 on CNS endothelial cells, microglia, astrocytes and oligodendrocytes, peripheral blood, and in demyelinating lesions of the patients • SSC: Interaction of SSA with TLR2 results in high production of IL-6 which plays an important role in fibrotic effects in dermal fibroblasts. • Psoriasis: Increased expression of TLR2 in keratinocytes • Sjogren's syndrome: Increased expression of TLR2/1 and TLR2/6 heterodimers in salivary gland epithelial cells and PBMCs	• Brain disease: - Astrocytes: express TLR2 which contribute to inflammatory responses - Oligodendrocytes: express TLR2 associated with CNS repair process, immune response regulation, and gliosis. - Bacterial infections and meningitis: anti-bacterial immunity - Parasite infections: protective role or disease severity - Neuronal damage • Diabetes: TLR2 expression associated with higher inflammatory responses, progression of atherosclerosis • Cardiovascular disease: Induction of apoptosis	• Susceptibility to leprosy and tuberculosis • Increased severity of genital herpes • Susceptibility to staphylococcal disease and tuberculosis • Protection from Lyme disease
TLR3	Breast, colon, cervical, head and neck, lung, and prostate cancers; hepatocellular carcinoma, melanoma, myeloma	• Tumor cell proliferation • Tumor cell survival	• Apoptosis • Production of type-1 interferon	• RA: Increased TLR3 on synovial tissues • MS: TLR3 engagement on the normal astrocytes stimulate the production of anti-inflammatory cytokines such as IL-10, which have an important role in down-	• Brain disease: - Astrocytes express TLR3 which contribute to inflammatory responses - Oligodendrocytes express TLR3 associated with CNS repair process, immune response regulation, and gliosis.	• Susceptibility to HSV encephalitis

Table 2 (Continued)

TLR	Association with diseases					TLR's SNPs associated with infectious diseases
	Cancer			Autoimmunity	Other disorders	
	Cancer type	Role in tumor progression	Role in tumor regression			
				regulating inflammation and homeostasis regulation in the brain • SSc: Increased expression of TLR2 associated with disease progression • Psoriasis: Increased expression of TLR2 in keratinocytes, interaction of dsRNA-LL37 complex with TLR3 • Sjogren's syndrome: Increased expression of TLR3 in salivary glands associated with apoptosis and the production of inflammatory cytokines	- Neurons: express TLR3 associated with both physiological and pathological conditions. - Viral infections: TLR3 expression associated with anti-viral immunity and neuronal repair	
TLR4	Breast, colon, gastric, and lung cancers; hepatocellular carcinoma, melanoma	• Tumor cell Proliferation • Immunosuppression • Up-regulation of VEGF, NO, MMPs • Tumor cell growth • Reduced apoptosis • Treg stimulation	• Increase of chemosensitivity • Apoptosis induction • Treg inhibition • Increased antigen presentation	• RA: Increased TLR2 on both peripheral blood monocytes and synovial tissues • MS: TLR4 is increased in the CSF mononuclear cells of MS patients • SSc: Involvement of TLR4 with its ligands leading to enhance fibrotic process and tissue damage. • Sjogren's syndrome: Increased expression of TLR4 in salivary glands associated with glandular inflammation	• Brain disease: - Alzheimer's disease: expression of TLR4 associated with response to amyloid beta - Bacterial infections and meningitis: anti-bacterial immunity - TLR4 may associate with neuronal damage • Diabetes: TLR4 expression associated with higher inflammatory responses, progression of atherosclerosis • Cardiovascular disease: decrease of apoptosis, Increased expression of TLR4 in atherosclerotic lesion	• Susceptibility to infection with gram-negative bacteria, <i>malaria</i>, and <i>RSV</i> • Susceptibility to meningococcal sepsis • Protection from Legionnaires' disease

(Continued)

Table 2 (Continued)

TLR	Association with diseases				Autoimmunity	Other disorders	TLR's SNPs associated with infectious diseases
	Cancer						
	Cancer type	Role in tumor progression	Role in tumor regression				
TLR5	Breast, head and neck, colon, and gastric cancers	• Treg stimulation • Up-regulation of IL-8 and TNF	• Treg inhibition • enhanced antigen presentation • cell death	• Psoriasis: Increased expression of TLR2 in keratinocytes	• Brain disease: - Alzheimer's disease: expression of TLR5 associated with response to amyloid beta	• Susceptibility to Legionnaires' disease	
TLR7	Lung cancer, basal cell and squamous cell carcinoma	• Tumor cell Proliferation and survival • Drug resistance • Increase metastasis • up-regulation of various cytokines	• Increased chemosensitivity • Inhibition of angiogenesis • Apoptosis induction • Treg inhibition • Increase antigen presentation	• SLE: Recognition of the complexes of autoantibodies and self-nuclear antigens • RA: Increased TLR3 on synovial tissues • SSc: disease progression	• Brain disease: - Neurons: express TLR7 associated with both physiological and pathological conditions. - Alzheimer's disease: expression of TLR7 associated with response to amyloid beta	• Protection from hepatic fibrosis in chronic HCV infection	
TLR9	Breast, gastric, lung, ovarian, and prostate cancers, glioma, hepatocellular carcinoma, neuroblastoma	• Tumor metastasis, MMP and Cox-2up-regulation • Drug resistance • Apoptosis inhibition	• Angiogenesis inhibition • Apoptosis • Treg inhibition • enhanced antigen presentation • cytotoxicity • cell cycle arrest	• SLE: Recognition of the complexes of autoantibodies and self-nuclear antigens • SSc: disease progression • Psoriasis: Increased expression of TLR2 in keratinocytes	• Brain disease: - Astrocytes: express TLR9 which contribute to inflammatory responses - Neurons: express TLR9 associated with both physiological and pathological conditions. - Alzheimer's disease: expression of TLR9 associated with response to amyloid beta - Bacterial infections and meningitis: anti-bacterial immunity - Viral infections: TLR9 expression associated with anti-viral immunity and neuronal repair - Parasite infections: protective role or disease severity	• Susceptibility to SLE • Rapid progression of HIV infection • Increased risk of low birth weight in malaria during pregnancy	

TLR: Toll-like receptor; Cox: cyclooxygenase; HSV: herpes simplex virus; RA: rheumatoid arthritis; MS: multiple sclerosis; SSc: Systemic sclerosis; SLE: systemic lupus erythematosus; HIV: human immunodeficiency viruses; HCV: hepatitis C virus; VEGF: vascular endothelial growth factor; NO: nitric oxide; MMP: matrix metalloproteinase; Treg: T regulatory; RSV: respiratory syncytial virus; TNF: tumor necrosis factor; IL: interleukin; PBMC: peripheral blood mononuclear cell; CNS: central nervous system.

^aFrom Khajeh Alizadeh Attar M, Anwar MA, Eskian M, Keshavarz-Fathi M, Choi S, and Rezaei N (2018) Basic understanding and therapeutic approaches to target toll-like receptors in cancerous microenvironment and metastasis. *Medicinal Research Reviews* 38: 1469–1484.

^bFrom Mukherjee S, Huda S, Sinha Babu SP (2019) Toll-like receptor polymorphism in host immune response to infectious diseases: A review. *Scandinavian Journal of Immunology* 90(1): e12771. <https://doi.org/10.1111/sji.12771>.

Anhydrotic ectodermal dysplasia with immunodeficiency (EDA-ID)

EDA-ID is an X-linked recessive immunodeficiency characterized by abnormalities in ectodermal development, including absent or decreased dental and hair growth. This disorder caused by mutation in NEMO, a central member of the IKK complex leading to improper NF κ B activation. The patients are susceptible to infection with encapsulated pyogenic bacteria, such as *S. aureus*, *S. pyogenes*, and *Haemophilus influenza*, as well as mycobacteria, herpesviruses, fungal infections, importantly *P. jirovecii* pneumonitis. NK abnormalities, hyper immunoglobulin M syndrome, decreased response to LPS, TNF, and IL-1 are the important features of EDA-ID patients (Keller et al., 2011).

IRAK4 deficiency

This is an autosomal recessive genetic disorder. The affected patients revealed a decreased inflammatory responses and persistent pyogenic bacterial infections, considerably with *S. pneumonia*. Given the central role of IRAK4 in TLR signaling, its mutation results in the impaired NF κ B and MAPKs activation. Interestingly, the isolated blood cells from these patients fail to produce the inflammatory cytokines in response to different TLR agonists. A similar phenotype was reported in MyD88 deficiency characterized by recurrent pyogenic infections particularly early in life. Thus, it is concluded that both IRAK4 and MyD88 are essential in protective immunity against bacterial infections (Picard et al., 2010).

TLR3 deficiency

TLR3 deficiency is a rare genetic disorder in which the patients are more susceptible to HSV encephalitis. Therefore, TLR3 plays an important role in immunity against central nervous system infections. In this regard, the lack of UNC-93B is also reported to be associated with HSV encephalitis (Lim et al., 2014).

TLR: Roles in human diseases

In recent years, TLRs have attracted attention not only for responding to pathogen components, but also for their function in several disorders including cancer, autoimmunity, brain disease, and cardiovascular diseases (Table 2).

Cancer

Anti-tumoral roles of TLRs

TLR agonists have shown to be effective agents in the treatment of several cancers. OK-432 as a ligand for TLR4 was used to treat gastric, cervical, and oral squamous cell carcinomas (Kikkawa et al., 1993; Maehara et al., 1994; Sato et al., 1997; Okamoto et al., 2006; Hironaka et al., 2006). Bacillus Calmette–Guérin (BCG) which has long been used in treatment of bladder cancer, is an effective stimulator of TLR2 and TLR4 signaling pathways (Tsuji et al., 2000; Uehori et al., 2005; Razack, 2007). LPS as a potent activator of TLR4, has been administered in phase II clinical trial to treat patients with colorectal and lung cancers (Otto et al., 1996). The agonists of TLR7 and TLR8 such as ssRNA and imiquimod were shown favorable results in the treatment of chronic lymphocytic leukemia and skin tumors (Scheel et al., 2006; Stockfleth et al., 2003; Spaner and Masellis, 2007). Administration of CPG as a ligand for TLR9 has been used to treat lymphoma, skin, brain, and renal cancers (Krieg, 2007; Carpentier et al., 2006).

TLR agonists are thought to promote anti-cancer immune responses through several ways. Their signaling cascade increases the recruitment of leukocytes particularly cytotoxic T, helper T, and NK cells which destruct tumors by the production of IFN- γ and cytotoxic mediators such as perforin. Besides, TLR signaling by up-regulation of the co-stimulatory molecules on APCs and stimulating the adaptive immune system plays an important role in raising anti-tumor responses (Rakoff-Nahoum and Medzhitov, 2009).

Pro-tumoral roles of TLRs

Chronic infection has been identified as the important factor involving in development of malignant neoplasms (Michelson et al., 2016). For instance, gastric cancer and colon cancer have been shown to be related with chronic *Helicobacter pylori* infection and chronic inflammatory bowel diseases, respectively (Kapetanakis et al., 2012).

The association between improper activation of TLRs and tumor progression is currently being investigated in several studies (Khajeh Alizadeh Attar et al., 2018). Up-regulation of NF κ B induced by inflammation has a central role in tumor development which explained in various malignancies including prostate cancer, hepatocarcinoma, myeloid leukemia, and multiple myeloma (Palayoor et al., 1999; Griffin, 2001).

TLR signaling leads to high production of pro-inflammatory cytokines such as TNF, IL-1, IL-6, and MCP-1, MIF, GRO chemokines by mediating NF κ B activation resulting in more infiltration of leukocytes to the infectious tissue. In this context, the production of anti-apoptotic factors provides a suitable milieu for promoting tumor growth (Philip et al., 2004).

In preclinical studies, the increased invasion, angiogenesis, and metastasis have been shown after LPS administration in mammary and colon adenocarcinomas models. Interestingly, TLR4 has shown to be required for growth of tumor cells in an

experimental murine model of colon adenocarcinoma in which tumor growth was stimulated by LPS. The proposed mechanism was the increased level of TNF and NF κ B activation in tumor cells which results in the up-regulation of anti-apoptotic proteins (Harmey et al., 2002; Luo et al., 2004).

Listeria monocytogenes has indicated to have a pro-tumoral role by activating TLR2 in ovary cancer cells via NF κ B stimulation. Infection with *Mycoplasma* is also associated with decreasing apoptosis and the promotion of tumor development by activation of TLR2 (Huang et al., 2007).

In cervical cancer, increased expression of TLR5 and TLR9 on the cervical epithelial cells was shown to be associated with cancer progression (Kim et al., 2008). The elevated levels of TLR9 were also found in lung and prostate cancer cells. In vitro stimulation of these cells with TLR9 ligands such as CpG-oligodeoxynucleotide or bacterial DNA was associated with tumor cell invasion (Droemann et al., 2005; Ilvesaro et al., 2007).

Besides, TLR interaction with DAMPs released from necrotic or damaged cells has shown to be associated with tumor development. Heat shock proteins (HSP60, HSP70), HMGB1 protein, ATP, Ca²⁺-modulating protein family (S100), and uric acid are the most important DAMPs stimulating tumor growth (Lotze et al., 2007; Ellerman et al., 2007). During oxidative stress, cytoplasmic organelles such as mitochondrial DNA could be released which interact with TLR9 and thereby activates its downstream signaling pathway leading to inflammation and tissue damage in tumor microenvironment (Oka et al., 2012).

Autoimmunity

Autoimmune disorders are characterized by various coincident mechanisms leading to decreasing immunological tolerance and uncontrolled activation of immune cells (Anders et al., 2005).

Growing body of evidence from human and animal models describes that dysregulation of TLR signaling contributes to the development of autoimmune disorders and tissue damage. Inappropriate TLR activation has shown to be associated with the pathogenesis of various autoimmune disorders including Systemic lupus erythematosus, Rheumatoid arthritis, Multiple Sclerosis, Scleroderma, and Sjogren's syndrome.

Systemic lupus erythematosus (SLE)

SLE is a systemic autoimmune disease identified by the production of autoantibodies against self-nuclear antigens resulting in the formation of immune complexes in serum of the patients. These complexes could stimulate TLR7 and TLR9 as the nucleic acid sensors especially on plasmacytoid dendritic cells leading to activation of both innate and adaptive immune responses.

Impaired clearance of apoptotic cells has been reported in patients with SLE. Accumulation of apoptotic debris would stimulate TLRs which subsequently lead to B cell activation and anti-nuclear antibody production.

The elevated level of serum IFN- α is found in SLE patients that plays an important role in disease progression and severity. IFN- α contributes to inducing the production of autoantibodies in these patients. As described earlier, the production of IFN- α could be a consequence of TLR7 and TLR9 signaling pathways (Horton and Farris, 2012).

Rheumatoid arthritis (RA)

RA is an inflammatory autoimmune disease characterized by the production of autoantibodies destructing joints. The increased expression of TLR2 and TLR4 has been found on peripheral blood monocytes in patients with RA. Besides, the elevated levels of both cell membrane- and endosomal-TLRs including TLR2, TLR3, TLR4, TLR7 have been shown in RA synovial tissues. The endogenous TLR ligands such as released RNA from necrotic synovial fluid cells cause the activation of TLR3 in synovial fibroblasts. An acute phase protein, serum amyloid A, has been identified in the peripheral blood of RA patients which acts as a TLR2 ligand.

Damage associated molecular patterns including HSPs (HSP60, HSP70, HSP22), HMGB1, fibrinogen, fibronectin, and hyaluronic acid fragments can activate TLR4 signaling pathway in rheumatoid synovium. Increased level of hyaluronic acid fragments in RA synovial fluid by interaction with TLR4 activates myeloid dendritic cells (Huang and Pope, 2009).

Multiple sclerosis (MS)

MS is an autoimmune disorder that affects the central nervous system (CNS) leading to immune-related demyelination and damage to oligodendrocytes and neurons.

TLR2 is up-regulated on CNS endothelial cells, microglia, astrocytes and oligodendrocytes, peripheral blood, and in demyelinated lesions of the patients with MS. TLR4 is increased in the CSF mononuclear cells of MS patients. Endogenous ligands such as HMGB1 by activating TLR2 and TLR4 stimulate the production of inflammatory cytokines like IL-1, IL-6, IL-23, and IL-12. This process enhances the polarization of naive T cells into Th1 and Th17 cells; these cells subsequently promote leukocyte recruitment to CNS by the production of INF γ and IL17, respectively, which leads to neurons damage.

TLR3 is expressed on neurons, cerebral endothelial cells, microglia, astrocytes, and oligodendrocytes. Interestingly, TLR3 engagement on the normal astrocytes stimulates the production of anti-inflammatory cytokines such as IL-10, which have an important role in down-regulation of inflammation and regulation of homeostasis in the brain. In the MS patients, a TLR3 ligand known as stathmin was increased in astrocytes, microglia, and neurons, which has shown to have the neuroprotective role (Miranda-Hernandez and Baxter, 2013).

Systemic sclerosis (SSc)

SSc or scleroderma is a heterogeneous autoimmune disease affecting connective tissue characterized by vascular injury and ischemia of the skin. Tissue damage and inflammation leading to release the molecules which can initiate inflammatory signaling pathways by interaction with TLRs.

SSA and S100A7 have been identified as two known ligands for TLR2 in SSc patients. Interaction of SSA with TLR2 results in high production of IL-6 which plays an important role in fibrotic effects in dermal fibroblasts.

Involvement of TLR4 with ligands such as Tenascin-C, HMGB-1, hyaluronan, and fibronectin are also indicated in the patients with SSc leading to enhance the fibrotic process and tissue damage.

Besides, the role of intracellular TLRs including TLR3, TLR7, and TLR9 has been shown in disease progression of SSc patients (O'Reilly, 2018).

Psoriasis

Psoriasis is an autoimmune disorder affecting skin characterized by hyper-proliferation of the epidermis. Damaged skin cells release DAMPs such as ssRNA and dsRNA which lead to triggering inflammatory responses.

keratinocytes have shown to express TLRs 1, 2, 3, 5, 9 in psoriasis patients. Upon DAMP or PAMP ligation, keratinocytes can produce several pro-inflammatory cytokines such as TNF, IL-6, IL-1, IL8, IFN β , IL36, IL25, and CXCL10 which stimulate T cell activation.

Intriguingly, antimicrobial peptide LL37 produced during skin injury can make a complex with dsRNA. This complex interacts with TLR3 resulting in the production of IFN- β from keratinocytes and plasmacytoid DCs. LL37 is also interacts with ssRNA or DNA which subsequently recognized by TLR7 or TLR9 leading to the production of IFN- α (Sun et al., 2019; McInturff et al., 2005).

Sjogren's syndrome

Sjogren's syndrome (SS) is a systemic autoimmune disease affecting exocrine, lacrimal, and salivary glands. High expression and inappropriate signaling of TLRs are described in both salivary cells and peripheral blood mononuclear cells (PBMCs) of the patients with SS.

Although the precise ligands for activating TLRs in the context of SS are not described in detail, TLR2/TLR1 and TLR2/TLR6 heterodimers have shown to highly expressed in salivary gland epithelial cells and PBMCs derived from SS patients. In vitro stimulation of salivary epithelial cells derived from SS patients with TLR2 ligands resulted in NF κ B and IL-15 up-regulation. This process leads to NK generation, increased expression of immune-regulatory molecules including ICAM-1, CD40, and MHC-1 on epithelial cells resulting in increased inflammatory immune responses.

High expression of TLR4 is also reported in salivary glands which was associated with glandular inflammation. Besides, TLR3 expression mediates apoptosis and increased production of inflammatory cytokines in salivary gland cells in SS. Activation of TLR7, TLR8, and TLR9 causes immune dysregulation and increased production of IFN α cytokine (Kiripolsky and Kramer, 2018).

Brain disease

TLR signaling has shown in host defense against CNS microbial infection. Recent studies have also demonstrated the involvement of TLRs during neurogenesis in the absence of any infectious causes. These receptors contribute to CNS homeostasis, cellular differentiation, immune regulation, and tissue repair.

Various cells in CNS including microglia, neuron, astrocyte, and oligodendrocyte have shown to express distinct TLRs. Microglia in the CNS parenchyma, are the important innate immune cells which express all TLRs (TLR1–9) and respond to several infectious agents. Astrocytes express TLR2, 3, 9 and they are involved in inflammatory responses through the production of different chemokines. Oligodendrocytes have shown to express TLR2 and TLR3 which their expression appears to be associated with CNS repair process, immune response regulation, and gliosis. Neurons by expressing endogenous TLRs including TLR3, 7, 8, 9 play important roles in both physiological and pathological conditions.

Besides, in Alzheimer's disease, TLR2, 4, 5, 7, 9 have shown to be important in response to amyloid beta accumulation in this disease (Momtazmanesh et al., 2020). In bacterial infections and meningitis, TLR2, 4, 9 are necessary for triggering anti-bacterial immune responses. In viral infections, TLR3 and 9 are involved in anti-viral immunity and neuronal repair. TLR1, 2, and 9 are indicated to participate in immunity against parasite infections of the brain, although they also can worsen disease severity. In contrast to the protective role of TLRs, TLR2 and 4 are also implicated in stimulating neuronal damage (Hanke and Kielian, 2011).

Diabetes

Increasing evidence suggests the elevated levels of inflammatory biomarkers in diabetes which promote susceptibility to vascular complications. Several studies have shown that the higher expression of TLR2 and TLR4 in patients with diabetes is associated with increased inflammatory responses and production of inflammatory cytokines including IL-1 β , IL-6, TNF, MCP-1, and IP-10. These TLRs and their ligands such as HSP60 and HMGB1 are related to the progression of atherosclerosis in diabetes patients (Jialal and Kaur, 2012).

Cardiovascular disease

Paradoxical functions of TLRs have been shown in cardiovascular disorders. TLR2, 3, 4, 6 are expressed on cardiac myocytes. In contrast to the role of TLR2 in the apoptosis induction of cardiac myocytes, TLR4 contributes to attenuating the apoptosis of these cells. However, TLR2 and TLR4 targeting is shown to be effective in septic cardiomyopathy.

Atherosclerotic lesion expressed an increased level of TLR4 leading to high inflammatory cytokine production. Lipid oxidation which plays an important role in the formation of atherosclerotic plaques results in the production of HSPs which act as TLR4 ligands. Interestingly, Chlamydia pneumonia as a ligand for TLR4 is closely related to atherosclerosis (Moghimpour Bijani et al., 2012).

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The Complement System

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Nomenclature

aHUS	Atypical hemolytic uremic syndrome
AP	Alternative pathway
ARMG	Age-related macular degeneration
C1-INH	C1 esterase inhibitor
C1q, 2C1r, 2C1s	C1 complex
C2a	Small C2 cleavage fragment
C2b	Large C2 cleavage fragment-enzyme
C3(H ₂ O)	Thioester-hydrolyzed form of C3
C3bBb	Alternative pathway C3 convertase
C3bBbC3b	Alternative pathway C5 convertase
C3bBbP	Alternative pathway C3 convertase with properdin
C3G	C3 glomerulopathies
C4bC2b	LP/CP C3 convertase
C4bC2bC3b	LP/CP C5 convertase
C4BP	C4b binding protein
CCP	Complement control protein
CL	Collectins
CLU	Clusterin
CP	Classical pathway
CR1/CD35	Complement receptor type 1
CR2/CD21	Complement receptor type 2
CR1g	Complement receptor of the immunoglobulin family
CRP	C-reactive protein
CSMD1	CUB and sushi domains protein 1
DAF/CD55	Decay-accelerating factor
DAMP	Damage-associated molecular pattern
FB	Factor B
FD	Factor D
FH	Factor H
FHL-1	Factor H-like protein-1
FHR	Factor H-related protein
FI	Factor I
GAGs	Glycosaminoglycans
HMGB1	High mobility group box 1
IC	Immune complex
IgAN	IgA nephropathy
LP	Lectin pathway
MAC (C5b-9)	Membrane attack complex

MASP	MBL-Associated serine protease
MBL	Mannose-binding lectin
MCP/CD46	Membrane cofactor protein
MDSC	Myeloid-derived suppressor cells
MPGN	Membranoproliferative glomerulonephritis
P	Properdin
PAMP	Pathogen-associated molecular pattern
PNH	Paroxysmal nocturnal hemoglobinuria
PTX-3	Pentraxin 3
RCA	Regulators of complement activation
SCR	Short consensus repeat
SLE	Systemic lupus erythematosus
sMAC (sC5b-9)	Soluble MAC
TP	Terminal pathway
Vn	Vitronectin

Introduction

Within this article we will be learning about the overall functions of the complement system, including how the complement system is activated through three pathways (classical, lectin and alternative), how the enzymatic complexes that lead to complement activation and amplification are formed, how the membrane attack complex is formed, what the main consequences of normal complement function are, as well as what are the pathological consequences of defects in complement proteins, defects in complement regulatory proteins, and exacerbated activation of the complement system as a consequences of disease, even when complement proteins are normal.

The complement system is an ancient system for detecting danger and is a major component of the innate immune system. It comprises a group of more than 50 proteins and protein sub-fragments, which participate in a cascade-like activation process. It efficiently protects the host from pathogenic microorganisms, contributes to several housekeeping functions, including immune complex regulation, and represents an essential link between the innate and specific/adaptive immune system. For example, it is a major effector system for antibody-mediated killing and it affects cell-mediated immunity. In order for complement to carry out its normal functions, it needs to be tightly regulated.

The complement system can be activated through three pathways, the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP). Each pathway is activated through different mechanisms and they all lead to the activation of a central component known as C3. C3 is present in the blood at about 1.2 mg/mL and is one of the most abundant proteins in blood. After C3 is activated, there are a series of events that occur in sequence, leading to several outcomes. The three best characterized consequences are: (a) direct killing of pathogens by the formation of a macromolecular complex that will insert itself into the membrane, leading to osmotic lysis of the cell; (b) the opsonization of pathogens by complement fragments that are formed during activation and bind to the surface on which complement is activating. These fragments make the pathogen surface more attractive to phagocytic cells for it to be taken up and destroyed; and (c) generation of small cleavage fragments of complement proteins that go into circulation and serve as chemoattractant molecules and increase vascular permeability, for effectively recruiting inflammatory and immunocompetent cells. Although these are the functions that are most commonly known about complement, it is actually very pleiotropic, resulting in a series of additional events including effects on adaptive cellular immune responses, participation in critical housekeeping functions that result in the efficient removal of dead and dying cells and clearance of immune complexes from circulation, among many other functions. Some examples of each of these functions are depicted in [Fig. 1](#), and throughout this article, several of these will be examined.

The liver is the major source of many complement proteins and their production increases significantly during an acute-phase response (3–50 fold). However, many complement proteins are either exclusively or mainly produced by a variety of immune cells, indicating the relevance of complement beyond the circulation, in local tissue microenvironments where these cells are found [reviewed in [Cortes et al. \(2013\)](#), [Hosszu et al. \(2012\)](#), and [Graier et al. \(2013\)](#)]. Complement components are largely secreted as inactive stable zymogens (enzyme precursors) and circulate widely through body fluids and tissues. Activation causes a precursor zymogen to be cleaved and become an enzymatically active serine protease. This enzyme will cleave and activate a different zymogen. Thus, it is a triggered enzyme cascade where each successive enzymatic cleavage causes amplification.

The complement components were named in the order of their discovery, not in the order of the reactions. In general, complement cleavage fragments are designated with letters according to their relative size, with “a” fragments smaller than “b” fragments. The standard nomenclature used in this article has been reviewed elsewhere ([Kemper et al., 2014](#); [Bohlson et al., 2019](#)) and will follow that recommended by the Boards of International Complement Society and the European Complement Network.

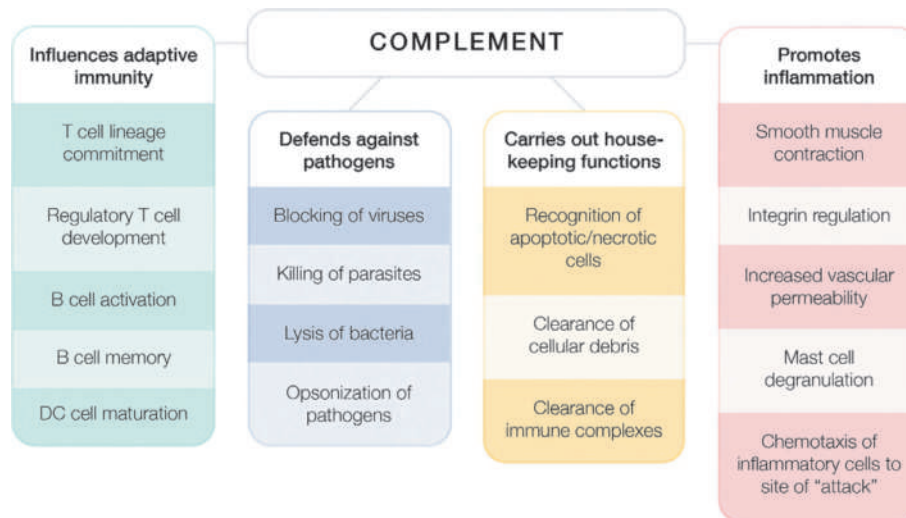


Fig. 1 Pleiotropic functions of the complement system. The complement system participates in several processes including adaptive immunity, defense against pathogens, host homeostasis or “housekeeping functions”, and inflammatory processes.

Biomedical relevance of the complement system

Deficiency in complement cascade proteins leads to increased susceptibility to severe bacterial infections, development of systemic lupus erythematosus (SLE), immune complex disease, and other diseases. One of the most compelling clinical examples of how relevant the complement system is to the proper functioning of the whole immune system is a published case of a child with complete C3 deficiency (Channam et al., 2008). A 2-year-old boy had suffered from recurrent pyogenic infections (severe meningitis, pneumonia, otitis) since early infancy. He had undetectable total complement hemolytic activity (CH_{50} ; assay discussed in a later section) and C3 values in blood. Vaccination of the child did not lead to any long-term antibody response. *In vitro* development of immature dendritic cells and their maturation capacity was greatly impaired. Regulatory T cell development was also significantly impaired. Thus, complement is an essential component of the immune system.

Given all the essential functions, the complement system needs to be tightly regulated. The complement system is regulated at various levels and deficiencies in one or more complement regulatory proteins leads to pro-thrombotic hematological diseases, renal and ocular diseases, and others. Finally, under limited/normal activation conditions, complement activation leads to a transient increase in local inflammation or removal of damaged cells by phagocytosis. However, inflammatory diseases (e.g. ischemia/reperfusion, sepsis, asthma, arthritis, cardiovascular diseases, chronic transplant rejection, neurodegenerative disorders, etc.) usually overwhelm complement regulatory mechanisms and lead to pathologic, excessive inflammation and complement-mediated tissue damage. Functions of complement and diseases related to malfunctions in the complement system or to excessive complement activation will be discussed later in this article.

Historical background

Complement was first described more than 124 years ago, and since then, components of each pathway (alternative, classical, and lectin) and the pleiotropic functions of complement in innate and adaptive immunity have been elucidated. Detailed information about the origin of complement can be found in (Pillemer, 1943; Lachmann, 2006). In summary, in the late 1800s it was determined that blood serum was able to destroy bacteria and that this effect is thermosensitive. At the end of the 1800s and early 1900s it became clear that efficient destruction of pathogens by serum required the presence of a heat-stable substance, later called hemolysin and now known as antibodies, and a heat-sensitive component that “complemented” the role of antibodies, now called the complement CP. The biochemical characterization of the complement system began in the early 1900s. During the 1950s, the first evidences of an “alternative” pathway (AP) capable of activating the complement system on surfaces, without the presence of antibodies, emerged. During the 1950s–70s the characterization and order of the components improved substantially due to the improvement of chromatographic and electrophoretic procedures. Further purification of AP and CP proteins occurred and some complement deficiencies and disease phenotypes were described. The 1980s and 1990s were marked by the discovery of several other regulatory components and their ligands, genome sequences of complement components, and characterization of the thioester bond in C3 and C4. In the 1990s, discoveries included characterization of complement genes and protein domain-based identification of families of complement proteins and their structural characterization via NMR and X-ray

crystallography. From 1990 to now, the LP was discovered, as well as the interrelationship between complement and the coagulation system, and therapeutic targeting of complement function has taken place. The pleiotropic functions of complement effects on adaptive immunity and diseases, including more detailed description of mechanisms of action of complement regulatory proteins in diseases and non-canonical functions of certain complement proteins, has dominated the field thus far in this century.

Pathways of the complement system

Overview

Each of the three pathways of the complement system will be described in detail, first up to the point of the generation of the C5 convertase for each pathway, followed by the non-enzymatic assembly of the membrane attack complex (MAC), which is common for all three pathways. While the CP and LP rely on an initiating recognition molecule for activation, the AP does not require this and is constitutively active, relying mainly on the characteristics of the surface to allow activation to proceed.

Classical pathway (CP)

The CP (Fig. 2) is an antibody-dependent and -independent way of recognizing “danger” (e.g. pathogens, apoptotic self-cells).

Antibody-dependent activation of the CP and formation of the C3 and C5 convertases

When antigens on a surface, such as a pathogen surface, are recognized by antibodies, these antibodies can be recognized by the C1 protein of the CP. C1 is a complex of three different proteins, C1q, C1r and C1s. C1q is produced mainly by immature dendritic

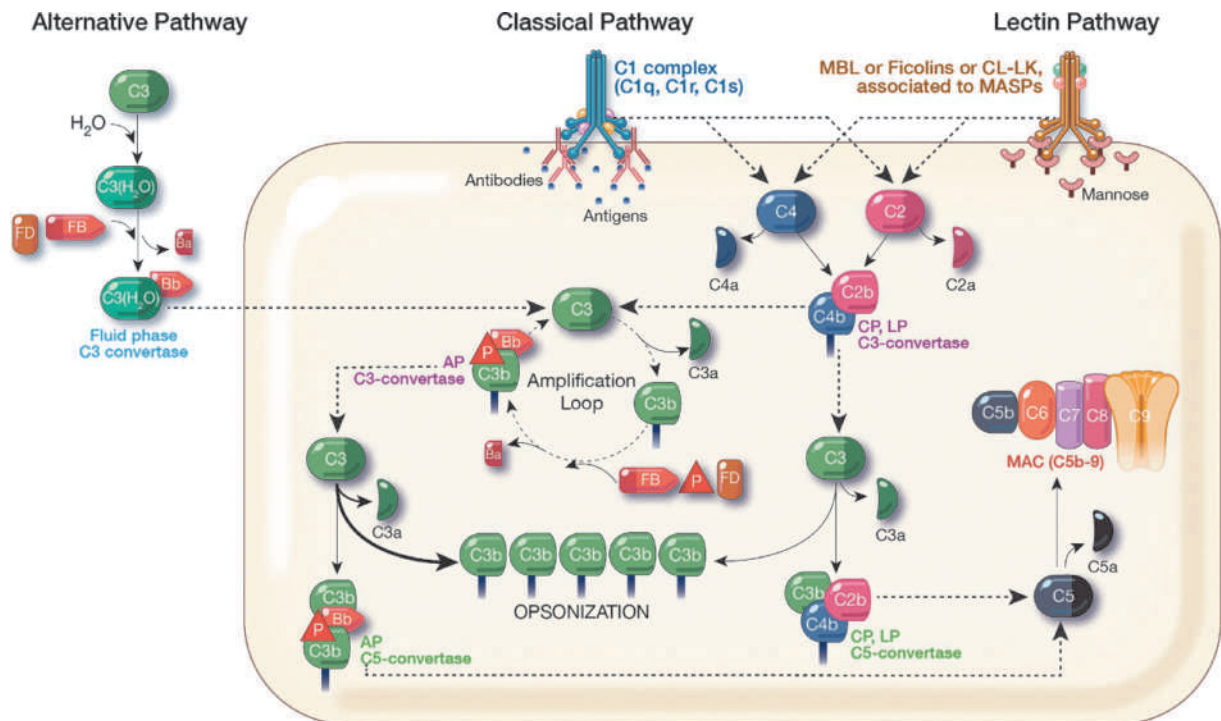


Fig. 2 Overview of the complement system. Complement is activated by three pathways: the alternative, classical, and lectin pathways. The alternative pathway (AP) initiates when soluble C3 hydrolyzes to C3(H₂O). This conformational change allows Factor B (FB) to bind, which subsequently recruits Factor D (FD). FD cleaves FB to form Bb and Ba, resulting in the fluid phase AP C3 convertase, C3(H₂O)Bb. C3(H₂O)Bb cleaves C3 to C3a and C3b, and C3b covalently attaches to nearby surfaces to form the AP membrane-bound C3 convertase, C3bBb. The classical pathway (CP) initiates when the C1 complex (C1q, C1r, C1s) recognizes surface-bound immunoglobulins, circulating immune complexes, or pentraxins (not shown here). The activation of the C1 complex results in cleavage of C4 to form C4b and C4a, whereby C4b covalently attaches to cell surfaces, followed by cleavage of C2 to form C2b and C2a, forming the surface bound C3 convertase, C4bC2b. The lectin pathway (LP) initiates when mannose-binding lectin (MBL), Ficolins, or collectins recognize molecular patterns (i.e. carbohydrates) on foreign surfaces. Activated MBL-associated serine proteases are able to cleave C4 and C2, to form the C3 convertase, C4bC2b, similar to the CP. C3 convertases derived from all pathways cleave C3 to C3a and C3b. C3b generated by C3 convertases binds to or near the CP/LP C3 convertase to form a C5 convertase (C4bC2bC3b) or binds to or near the AP C3 convertase to form an AP C5 convertase (C3bBbC3b_n). C5 convertases cleave C5 to C5b and C5a to initiate the common terminal pathway, which culminates in the formation of the membrane attack complex (MAC or C5b-9). C3b produced from the cleavage of C3 by C3 convertases from all pathways forms an amplification loop that contributes to the generation of additional AP C3 convertases. Properdin (P) binds to AP convertases, significantly increasing the convertase half-life, leading to more efficient C3b deposition on cells surfaces.

cells, monocytes, and macrophages (Chai et al., 2007) and consists of six triple helical strands, each composed of three polypeptide chains (A chain, B chain and C chain) arranged in a hexameric structure that resembles an up-side-down bunch of tulips. The stalks are formed by N-terminal collagen-like sequences, while the globular heads are formed by the C-terminal portion of the chains. These globular heads at the end of each arm are the contact regions for the CH₂ region in the Fc region (constant region) of immunoglobulins. C1r and C1s are serine proteases that form a Ca⁺⁺-dependent tetramer composed of two C1r and two C1s molecules (C1s-C1r-C1r-C1s), that bind to C1q, also in a Ca⁺⁺-dependent manner, to form the C1 complex (C1q, 2C1r, 2C1s) (Fig. 3).

C1q must bind two or more immunoglobulin Fc portions to initiate the CP (Fig. 4). These Fc regions need to be at a critical distance from each other (<40 nm) in order to properly engage the C1q globular heads, explaining why soluble individual IgG do not activate C1 in circulation. In order of preference, C1 binds to IgM, IgG3, IgG1, and IgG2. IgE, IgG4, and IgD do not activate C1. The Fc portions of soluble pentameric IgM are normally not accessible to C1; however, when IgM binds to antigens on a surface, it changes its conformation (from planar to staple-like) in a way that exposes the CH₂ regions for binding to C1.

Once C1q binds to the Ig on the surface (recognition step), its structure is slightly modified, leading to sequential activation of C1r and C1s serine proteases. Activated C1s cleaves C4 into C4a and C4b. C4b exposes a reactive thioester site that, if close enough to the surface, allows it to bind covalently to the membrane via hydroxyl and amino groups present on carbohydrates and proteins, respectively. C4b that is not close enough to a surface is quickly inactivated by hydrolysis of the thioester site. C1s also cleaves C2 into C2a (small fragment) and C2b. C2b will bind in a Mg⁺⁺-dependent manner to the C4b that is on the cell surface, forming C4bC2b, which is the C3 convertase of the CP. It is important to note that some resources reverse the nomenclature for the C2 fragments, where the smaller fragment is referred to as C2b and the larger fragment as C2a. The standard nomenclature for C2 has not been agreed upon by the international community (Bohlson et al., 2019). Herein, we will use the more simple nomenclature, whereby C2b is the larger fragment. Within the C4bC2b convertase, the C2b fragment contains the proteolytically active site. C4bC2b cleaves C3, generating C3a (anaphylatoxin; chemotactic; to be discussed in a later section) and C3b. Proteolytic cleavage of the C3 α chain exposes a highly reactive thioester site in C3b, which is exposed and susceptible to nucleophilic attack by nitrogen or oxygen atoms (Fig. 5). These interactions result in the formation of covalent bonds with proteins (amino, -NH₂) or carbohydrates (-OH) if the C3b is close enough to the cell surface. If C3 is not close enough to bind, the activated thioester is quickly hydrolyzed and the C3b is inactivated. Approximately 90% of the C3b or C4b binds a water molecule, and becomes inactive and unable to bind within ~60 microseconds, avoiding unnecessary damage to nearby bystander surfaces. C4 is structurally homologous to C3 and has an identical thioester group.

Each C4bC2b convertase can cleave several C3 molecules, leading to opsonization of the surface. When a generated C3b binds to the C4bC2b convertase, it now forms part of a C4bC2bC3b complex, known as the C5 convertase of the CP. The C5 convertase will cleave circulating C5 into C5a (a small, soluble, pro-inflammatory fragment; to be explained in a later section) and C5b that will be the starting point for the assembly of the MAC (to be discussed in a later section).

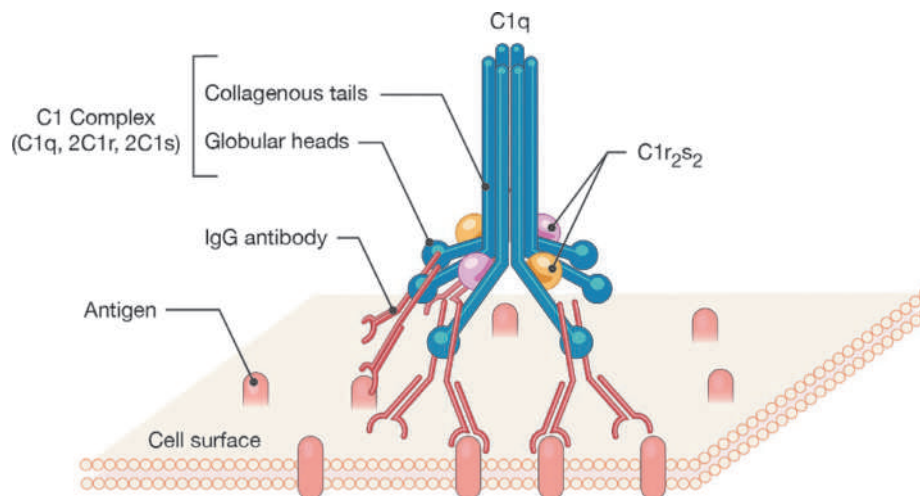


Fig. 3 C1 complex and its role in classical pathway activation. The C1 complex (C1q, 2C1r, 2C1s) is the first component of the classical complement pathway and is composed of six C1q subunits, two C1r units, and two C1s units. C1q binds through its globular heads to antigen-antibody complexes. The globular heads of C1q are mainly responsible for target recognition, and specifically recognize the Fc region (constant region) of IgG or IgM bound to cells surfaces, but also bind bacterial and viral surface proteins, apoptotic cells, pentraxins, amyloid, and prion proteins. The collagenous tail mediates immune effector mechanisms, including complement action through interaction with the C1r and C1s proteases. Binding of C1q to immunoglobulins leads to conformational changes in the collagenous tail of C1q, which activates the associated serine proteases, C1r, which then cleaves the C1s molecules. Activated C1s then cleaves C4 into C4a and C4b and C2 into C2a and C2b, forming the CP C3 convertase (C4bC2b complex) (Fig. 2).

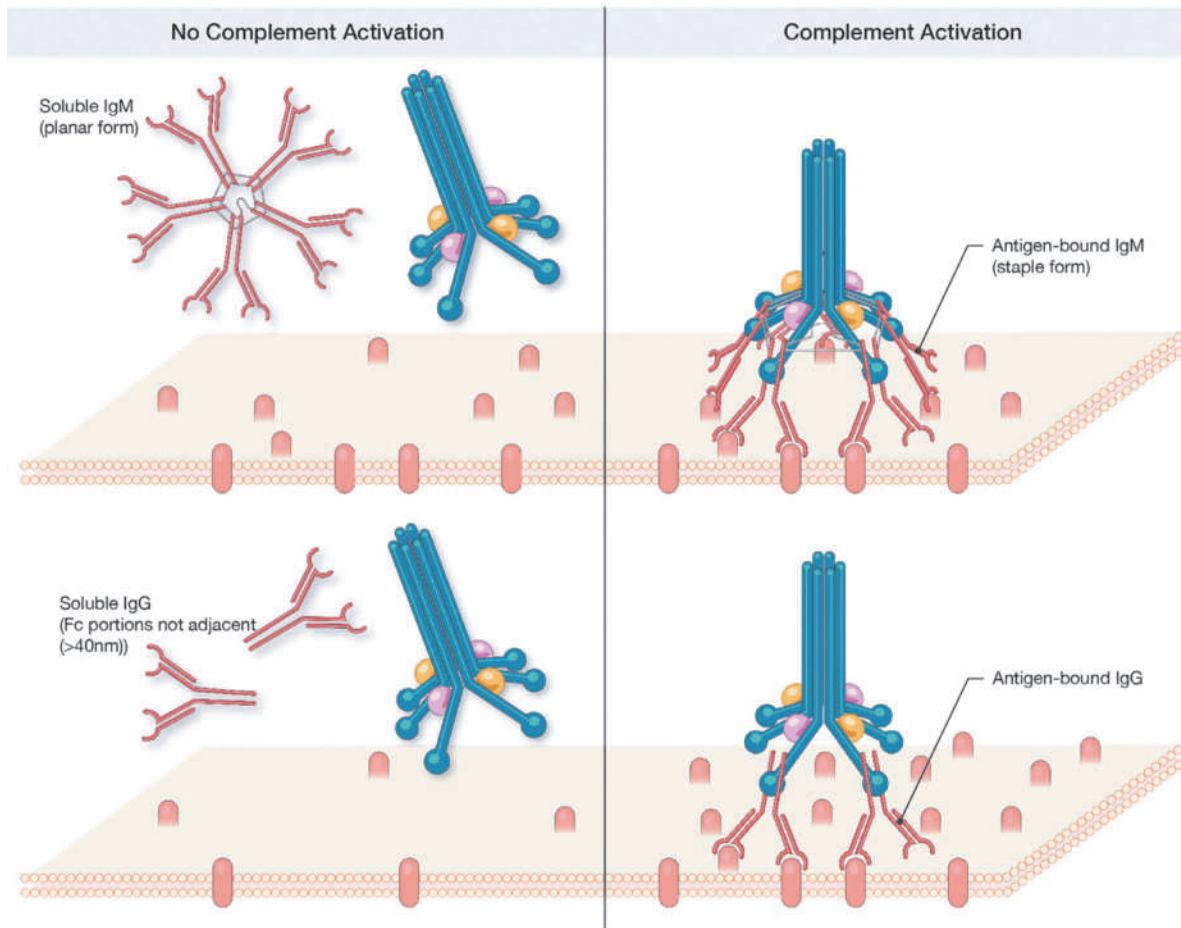


Fig. 4 Classical pathway activation vs no activation. A single pentameric IgM molecule has the potential to provide multiple binding sites for C1q and activate C1 to initiate the CP. However, soluble IgM has a planar conformation (top left panel), which does not allow C1 complex to bind. Once a single IgM binds its targets (top right panel), it undergoes a conformational change (staple form) in the constant region of immunoglobulins, exposing the C1 binding sites on IgM. In contrast, a single IgG cannot bind a C1 complex in the fluid phase (bottom left panel) nor when bound to cell surfaces unless several bound IgGs bound are close enough to be recognized by C1 (bottom right panel).

Antibody-independent activation of the CP

As mentioned above, the CP can also be initiated by antibody-independent mechanisms. C1 also recognizes many different target molecules on the cell walls and membranes of microorganisms and fragments of cellular and sub cellular membranes, including surface-bound pentraxins such as C-reactive protein (CRP) and pentraxin 3 (PTX-3), the damage-associated molecular pattern (DAMP) HMGB1 (Kishore et al., 2004; Merle et al., 2015a), phosphatidyl serine, DNA, certain annexins, among other ligands on dead and dying cells [reviewed in Merle et al. (2015a) and Galvan et al. (2012)], and LPS (Roumenina et al., 2008) and bacterial porins (Alberici et al., 1996). C1 binding to these ligands leads to C1 activation and CP activity.

Lectin pathway (LP)

The LP is very similar to the CP, but differs in the recognition molecules that initiate the pathway (Fig. 2). The LP relies on antibody-independent recognition of “danger” (sugar residue patterns), by mannan binding lectin (MBL), Ficolins (M, L, H, also known as 1, 2, and 3, respectively) and collectin CL-LK (heterodimer of CL-L1 and CL-K1) present on bacteria, viruses, and dead/dying cells (Kjaer et al., 2013; Matsushita et al., 2013). The LP can also be triggered by the DAMP HMGB1 (Ratajczak et al., 2019).

Although structurally similar to C1q, MBL, Ficolins, and CL-LK are instead collectins (collagen-like domain and a carbohydrate-binding domain). MBL binds specifically to mannose and certain sugars that form distinctive patterns on microbial surfaces and it must bind multiple sugars to activate. Ficolins and MBL are very similar structurally; although their carbohydrate-binding moieties are different. MBL recognizes glucose, mannose, and *N*-acetyl-glucosamine, Ficolins have a lectin activity toward *N*-acetylglucosamine through their fibrinogen-like domains, and CL-LK binds mannose-containing carbohydrates. MBL is formed by 3–6 oligomers of trimeric subunits, Ficolins form homooligomers of 4–6 trimeric subunits, and CL-LK is a

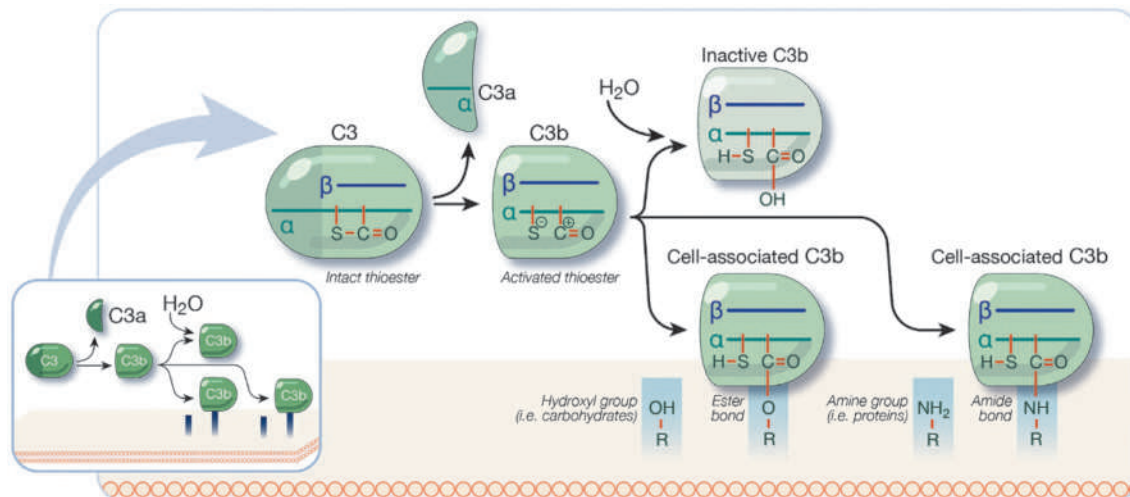


Fig. 5 Complement system targets cells surfaces. C3 has an intact internal thioester bond which is formed between cysteine and glutamic acid residues found in the α -chain of C3. When C3 or C4 (not shown in this figure) are cleaved during the process of complement activation, the thioester bond becomes reactive (activated thioester), which is quickly hydrolyzed in the fluid phase forming inactive C3b or attaches to surfaces by forming ester or amide bonds (or transacylated thioester bond) with carbohydrates or proteins found on surfaces.

heteromeric complex between CL-L1 and CL-K1 [reviewed in Cedzyński et al. (2018), Matsushita (2018), and Ohtani and Wakamiya (2018)]. MBL, the Ficolins, and CL-LK form a Ca^{++} -dependent complex with homodimers of two serine proteases, MASP-1 and MASP-2 (related to C1r and C1s) and can also associate with MASP-3. The MASP acronym stands for mannan binding lectin-associated serine protease. Once MBL, Ficolins or CL-LK are bound to a surface, MASP-1 activates MASP-2 or MASP-2 autoactivates (Héja et al., 2012; Endo et al., 2015; Hansen et al., 2016). MASP-1 and MASP-2 cleave C2, while MASP-2 also cleaves C4 (Takahashi et al., 2008). The role of MASP-3 within the LP remains undefined; however, it does have a function within the AP, which will be discussed in the next section. All steps of the LP after cleavage of C4 are identical between the CP and LP, including having identical C3 and C5 convertases (C4bC2b and C4bC2bC3b, respectively).

Alternative pathway (AP)

Activation of the AP and formation of the C3 convertase

The AP is the first to appear in evolution (most ancient of all the pathways) and is distinct from the CP and LP with regard to how it activates. Unlike the CP and LP, the AP does not require a recognition molecule to activate. It is active by default and will only effectively activate and amplify on surfaces that “allow it to” due to the lack of negative regulation of the AP on that surface. In simple terms, if C3b lands on a surface that has the ability to regulate complement, activation will not proceed. Conversely, if C3b lands on a surface without regulators, activation will proceed uncontrolled. So where does the C3b that lands on the surface come from and how is the AP regulated? These are the key questions to answer in order to understand the AP (Figs. 2 and 6). Spontaneous cleavage of C3 is postulated to occur at a low but constant rate in blood (Pangburn et al., 1981; Lachmann et al., 2018), and is known as tickover phenomena. In order for this to occur, C3, undergoes spontaneous hydrolysis at a low rate, where water is introduced in the thioester site of uncleaved C3. The hydrolyzed C3 in blood is known as C3(H₂O). The hydrolysis of soluble C3, changes the structure of C3 in such a way that C3(H₂O) can now bind Factor B (FB) in a Mg^{++} -dependent manner. Once FB binds to C3(H₂O) it can be cleaved by mature Factor D (FD) into a smaller fragment known as Ba (with no known function) and a larger fragment Bb that remains bound to the C3(H₂O), generating C3(H₂O)Bb. In order for FD to be able to cleave FB, MASP-3 (introduced in the previous section) must cleave zymogenic pro-FD to generate mature FD (Fig. 6). Thus, MASP-3 serves as a bridge between the LP and the AP (Takahashi et al., 2010; Dobó et al., 2016). C3(H₂O)Bb is the soluble C3 convertase of the AP that, via its enzymatic site (Bb), cleaves C3 into C3a (pro-inflammatory, chemoattractant molecule) and C3b. If the C3b is close enough to the membrane, it will bind covalently to the membrane or be quickly inactivated by hydrolysis in the same way as described for the CP and LP. The C3b that does bind to the surface is structurally similar to the intermediate form of C3 [C3(H₂O)] and thus C3b on the surface (Fig. 6) can also bind FB, which will be cleaved by mature FD into a smaller fragment known as Ba that is released into the fluid phase and a larger fragment Bb that remains bound to the C3b. This C3bBb complex is known as the membrane-bound C3 convertase of the AP. This convertase cleaves C3 into C3a and C3b, and if the C3b is close enough to the membrane it will bind covalently to it, as described in Fig. 5.

It is important to note that there are other ways in which the AP can activate (in addition to tickover), including (a) generation of C3(H₂O) via contact activation [reviewed in Nilsson and Nilsson Ekdahl (2012)]; (b) C3b and C3(H₂O) may bind directly to certain cell surfaces via P-selectin, CR2 and/or other ligands, some unknown (Saggu et al., 2013; Hamad et al., 2010; Merle et al.,

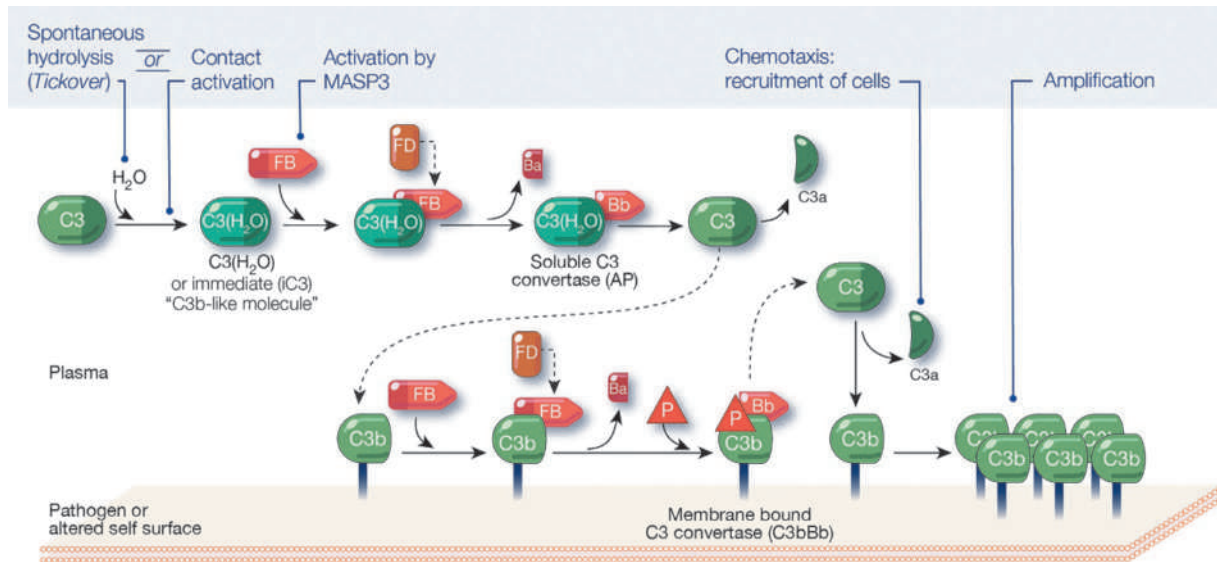


Fig. 6 Alternative pathway activation on pathogen or altered self surfaces. Spontaneous hydrolysis of C3 induces a conformational change of C3, forming C3(H₂O). Factor B (FB) binds to C3(H₂O) and subsequently Factor D (FD) cleaves FB into Bb and Ba, forming the soluble AP C3 convertase, C3(H₂O)Bb. This convertase can cleave soluble C3 leading to deposition of C3b on targeted surfaces and production of C3a, a small pro-inflammatory molecule that is released into solution. Since pathogen surfaces inefficiently recruit FH (see Fig. 7C), AP complement activation proceeds unchecked leading to formation of membrane bound C3 convertases (C3bBb), C5 convertases, and membrane attack complex (MAC or C5b-9) formation. Properdin (P), a positive regulator, assures efficient amplification of C3b deposition by increasing the half-life of AP C3 convertases.

2019; Mold et al., 1988); (c) LPS on cell walls of Gram negative bacteria, although *in vivo* relevance is uncertain [reviewed in Harboe and Mollnes (2008)]; and (d) the physiological forms properdin, a positive regulator of the AP (to be further discussed in a later section), can bind certain surfaces such as dying host cells and certain bacteria, and activate the AP by recruiting C3b and FB, forming a C3 convertase de novo, *in vitro* (Hourcade, 2006; Ferreira et al., 2010a). The *in vivo* relevance of this phenomena has been controversial due to methodological limitations that are discussed elsewhere (Ferreira et al., 2010a; Chen et al., 2018). One recent study supports the role of properdin as a possible DAMP recognition molecule *in vivo* indicating physiological properdin binds to proximal tubular cells in the kidney in a syndecan-1/heparan sulfate-dependent, but not C3-dependent manner (Lammerts et al., 2020).

Stabilization of the C3bBb convertase by properdin and amplification of the AP, CP and LP

In order for AP activation to occur fast due to efficient function of the C3 convertase (C3bBb), which is key for effective activation and amplification, C3bBb needs to interact with a blood protein known as properdin, forming C3bBbP (Fig. 6). The main function of properdin is to stabilize the AP convertases. When properdin binds to C3 convertases, it increases their half-life by 5–10-fold (Fearon and Austen, 1975; Schreiber et al., 1975), allowing effective cleavage of C3, surface opsonization, and amplification given the long half-life of the convertase. It is important to note that the C3b that lands on a surface due to CP and LP activation (Fig. 2) can serve as a starting point for the AP, thus amplifying each of those pathways. Amplification by the AP may account for up to 80% of the C5a and MAC generated by CP activation (Harboe et al., 2004). Additional information related to properdin will be discussed in later sections.

Generation of the C5 convertase of the AP

When there is one or more C3b fragments, generated by the C3 convertases, that land on or near a C3 convertase, this convertase shifts its affinity from C3 to C5, becoming a C5 convertase of the AP (C3bBbC3b; Fig. 2). In some texts the C5 convertase can be found as C3b_{2-n}Bb, where the "2-n" refers to 2 or more C3b molecules. The C3bBbC3b convertase is also stabilized by properdin, increasing its half-life ~10-fold (Fischer and Kazatchkine, 1983; Medicus et al., 1976). The C3bBbC3b convertase will cleave circulating C5 into C5a (a small, soluble, pro-inflammatory fragment; to be discussed in a later section) and C5b that will be the starting point for the assembly of the MAC (to be discussed in a later section).

How does the AP know when to activate?

As mentioned, the AP is always active by default, but activation only proceeds on surfaces that allow it to. Fig. 7A explains what this means in simple terms. C3b can land on a non-activating surface (e.g. host) or on an activating surface (i.e. pathogen or altered-self cell, such as a dead or dying cell). If C3b lands on a "non-activating" surface, it will be able to effectively regulate complement, and

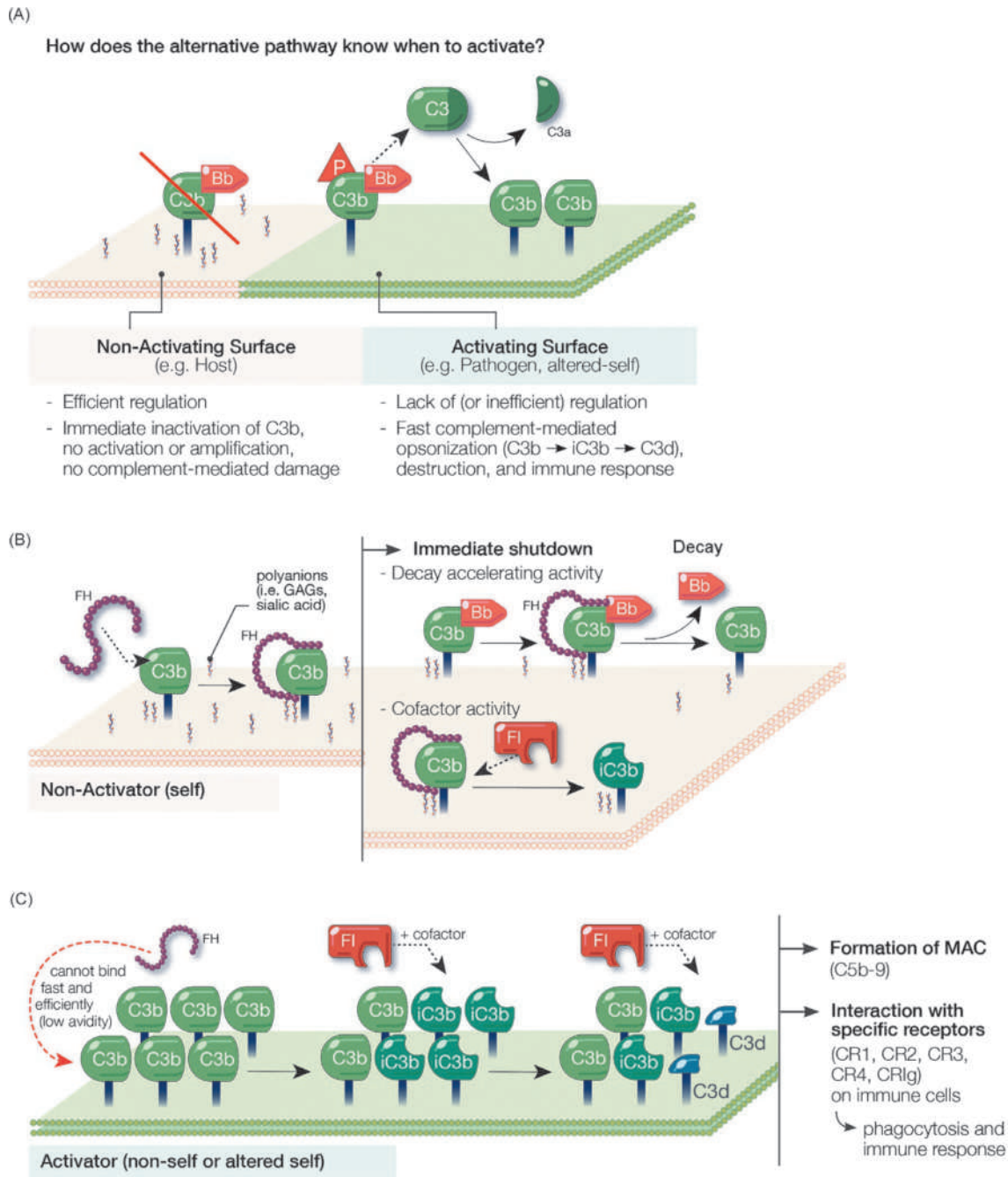


Fig. 7 Alternative pathway activation on self vs non-self surfaces. (A) Panel representing the main differences between non-activating surfaces and activating surfaces. (B) Non-activating surfaces (host cells). Activation of AP is efficiently regulated by Factor H (FH). FH efficiently protects host surfaces when it binds to C3b and poly-anions (i.e. GAGs, sialic acid) found on non-activating surfaces. The consequences of recruiting FH to non-activating surfaces is an immediate shutdown of AP activation by both accelerating the decay of the AP C3 convertase, C3bBb, and recruiting Factor I (FI) to cleave C3b and inhibit the formation of the AP C3 convertases. (C) Activating surfaces. Pathogens (non-self) or altered cells surfaces do not allow efficient binding of FH, thus leading to formation of AP C3 convertases that promote abundant C3b deposition and formation of the membrane attack complex (MAC or C5b-9) on targeted surfaces. Eventually, FI cleaves C3b into iC3b and C3d that interact with specific receptors found on immune cells to promote phagocytosis and enhance immune responses.

thus the C3b will be immediately inactivated, no activation or amplification will occur, and there will be no complement-mediated damage. However, if C3b lands on an “activating” surface, there will be a lack of or inefficient regulation, which will result in complement-mediated opsonization with C3b and C3b sub-fragments that will serve as important ligands for specific receptors on immune cells, leading to phagocytosis and immune responses. In addition, the cascade will proceed to form membrane attack

complexes (MACs). One key example of a regulator for understanding what defines a surface as an activator or non-activator, is the ability of the negative complement regulator Factor H (FH) to discriminate between host/self (non-activating) and non-self/ altered-self (activating) surfaces (Fig. 7B). FH circulates at ~200–500 mg/mL in blood and has multiple binding sites for C3 fragments and polyanions [e.g. sialic acid and glycosaminoglycans (GAGs)] (Pangburn and Muller-Eberhard, 1978; Fearon, 1978; Meri and Pangburn, 1990; Ram et al., 1998; Kazatchkine et al., 1979; Blackmore et al., 1998; Pangburn et al., 2009; Haque et al., 2020). The non-activating surface needs to have both host markers (i.e. polyanions, which are present on most of our cells and tissues) as well as initial deposits of C3b in order to facilitate the binding of FH to the interface of both ligands simultaneously (Fig. 7B). The presence of both C3b and polyanions allows FH to bind with sufficient affinity and avidity to the surface in order to remain bound long enough to carry out its complement regulatory functions, which will be discussed in a later section. Briefly, these include (Fig. 7B): (a) Decay acceleration that consists in accelerating the decay of the C3 convertases by displacing the enzymatic portion of the C3bBb complex (Bb), and (b) Cofactor activity, in which FH acts as a cofactor for Factor I (FI)-mediated cleavage of C3b to iC3b, which can no longer bind FB to form new convertases. Thus, activation and amplification do not proceed and insignificant amounts of iC3b are left on the surface. In addition to the fluid phase regulator FH, host cells are also protected by membrane-bound complement regulatory proteins (e.g. CD55, CD59, MCP, CR1, etc.) that carry out decay acceleration, cofactor activity, and other functions. The LP and CP are also controlled by regulatory proteins; most are common to all pathways, while others are pathway specific. All regulatory proteins and their functions will be discussed in the next major section.

In the case of an “activating” surface, fast and efficient activation and amplification of the AP will occur because the initial C3b lands on a surface (such as a pathogen) where there are (a) no or low host polyanionic markers, leading to FH not being able to bind effectively fast enough due to lack of adequate affinity and avidity to the surface and (b) no or low amounts of membrane-bound regulatory proteins (Fig. 7C). This allows activation and amplification to proceed very quickly, unimpaired, leading to a surface that is covered with thousands of C3b fragments. These fragments, will lead to the formation of MACs or, over time, will be further cleaved into iC3b and then C3d/C3dg by a regulator known as FI with the help of cofactors, such as FH, and others. Thus, the surface will be decorated with a variety of thousands of opsonic C3 fragments that will play central roles in destruction by phagocytosis and in generating effective immune responses by interacting with specific receptors on immune cells (e.g. CR1, CR2, CR3, etc.). These receptors and their associated functions will be discussed in a later section. Most pathogens have “activating” surfaces and are cleared by complement-mediated lysis and/or phagocytosis. However, many pathogens have evolved the ability to make their own complement regulatory proteins or sequester host regulatory proteins, making their surfaces “non-activating” as a complement evasion strategy (further discussed in a later section).

Cleavage of C3 and C5 in a complement-independent manner

C3 and C5 can be cleaved in the absence of complement activation due to a cross-talk between the complement and coagulation pathways. An example of this is that the extrinsic pathway of the coagulation system involves proteases that have isolated convertase activity, including factor IXa, Xa, XIa, thrombin, and plasmin, which cleave C3 and C5 (Amara et al., 2010), and renin and kallikrein, which cleave C3 (Békássy et al., 2018; Irmischer et al., 2018).

Generation of the membrane attack complex (MAC)

Once the C5 convertase is formed in each pathway, the rest of the cascade is identical for all the pathways. Cleavage of C5 by the C5 convertases releases soluble C5b (and pro-inflammatory fragment C5a; to be discussed in later section). C5b binds C6 and C7 in the blood, which exposes a hydrophobic site on C7, leading to the binding of soluble C8. Binding of C8 to this complex exposes a hydrophobic site, allowing C8 to penetrate the membrane partially. The C5b-C8 complex promotes polymerization of C9, which completely penetrates the membrane, forming a membrane pore that leads to osmotic lysis of the cell. All reactions after C5b production are nonenzymatic (assembly). A MAC complex can have a wide range of C9 molecules (as many as 22). A fully formed MAC is an asymmetrical pore, where the polymerized C9 does not completely close and overlaps at the origin point (Serna et al., 2016). The MAC can also be sub-lytic, with a variety of functions other than cell lysis that include inducing proliferation, protein synthesis, and apoptosis, amongst others (Morgan, 2016). Regulation of MAC will be discussed in the next section and consequences of deficiencies in components that make up MAC will be discussed in a later section.

Regulation of the complement system

Overview

Regulation of all complement pathways is essential to protect the host from unwarranted complement-mediated attack. Deficiencies in any of these regulators will lead to a variety of disease pathologies, as will be discussed in a later section. There are several proteins that are essential in modulating and controlling complement activation on self surfaces. These regulators are considered negative or positive, depending on whether these factors reduce or promote complement activation on cell surfaces, respectively.

Most of negative regulators are members of a network of proteins known as regulators of complement activation (RCA), which includes CR1, DAF, MCP, C4BP, FH, FHL-1, CSMD1 and FH-related family proteins [reviewed in Hourcade et al. (1989) and Skerka et al. (2013)]. These RCAs are mainly composed of complement control protein (CCP) domains, also known as a short consensus

repeat (SCR) or sushi domains. Each domain consists of ~60 amino acids in size with four conserved cysteine residues in which the first and third and second and fourth residues are cross-linked. The number of CCPs varies from 2 to 37 depending on the regulatory protein (Barnum and Schein, 2018a). CRiG is the only complement regulatory protein that is not part of the RCA, but rather part of the immunoglobulin family (Langnaese et al., 2000; Helmy et al., 2006). All the RCA proteins are able to control complement activation by either binding to proteolytic fragments of C3 and C4, or serve as cofactors in their degradation, or by inhibiting their biological activities (Alcorlo et al., 2015). Another complement regulator, C1-esterase inhibitor (C1-INH), which belongs to the serpin family, regulates the initial steps of the AP and LP pathways. Another subset of regulators includes those that inhibit the terminal pathway or the formation of the MAC, either on cell surfaces or in the fluid phase, and share no structural homology (i.e. CD59, clusterin, vitronectin) (Zipfel and Skerka, 2009). Finally, there is a small group that are considered positive regulators that promote C3b deposition on cell surfaces (i.e. properdin and possibly FH-related proteins) (Fearon and Austen, 1975; Schreiber et al., 1975; Jozsi et al., 2019). The regulators of complement are explained below and summarized in Table 1.

Negative regulation of the complement system

Inhibition of C1, MBL, and Ficolins

C1-INH is a multiple-function serine protease inhibitor, regulator of several plasma cascade systems, such as the CP of the complement system and the plasma bradykinin-forming cascade [reviewed in Davis (1988) and Beinrohr et al. (2008)]. C1-INH has an anti-inflammatory function via the irreversible displacement of complement serum proteases C1r and C1s from C1q in the CP pathway, and displacement of MASP2 from MBL or Ficolins from the LP (Fig. 8A and Table 1). C1-INH has non-complement-related functions such as controlling vascular permeability by inhibiting factor XIIa and plasma kallikrein, which are involved in the formation of bradykinin, a potent vasodilator molecule. Other possible functions of C1-INH include the role of inhibiting factor XIa, representing the initial protease of the intrinsic pathway of coagulation, the fibrinolytic proteases plasmin, and interacting with immune cells (neutrophils and macrophages) and enhancing phagocytosis [reviewed in Drouet et al. (2018), Zeerleder and Levi (2016), Davis et al. (2010), Liu et al. (2007), and Liu et al. (2003)].

Decay acceleration

The regulatory proteins that have decay acceleration function will “dismantle” the C3 and C5 convertases, thus inhibiting the cleavage of C3 and C5 (Gigli et al., 1979; Pangburn and Müller-Eberhard, 1986; Nicholson-Weller et al., 1982; Medof et al., 1984; Pangburn, 1986; Fujita et al., 1987). Specifically, decay acceleration protects host cells from complement-mediated attack by preventing the formation of, as well as accelerating the decay of, the CP/LP and AP C3 and C5 convertases (Fig. 8B and Table 1). During decay acceleration, the regulator either (a) competes for FB or C2b binding to C3b or C4b, respectively, impairing the formation of the convertase, or (b) displaces the enzymatic portion of the convertase (Bb for the AP and C2b for the CP), effectively shortening the half-life of the convertase. Circulating soluble negative regulators, FH and C4 binding protein (C4BP), have decay accelerating activity on the cell surface mainly for the AP and CP, respectively. FH also is an essential regulator of the fluid-phase AP convertase C3(H₂O)Bb. Membrane-bound complement negative regulators with decay accelerating activities in both the CP/LP and AP pathways include complement receptor 1 (CR1/CD35), decay accelerating factor (DAF/CD55), membrane cofactor protein (MCP/CD46) and soluble FH-like protein 1 (FHL-1) (Kühn et al., 1995).

Cofactor activity

FI plays a fundamental role in protecting host cells from complement-mediated attack by quickly cleaving the α' -chains of newly formed membrane-bound C4b and C3b, generating so-called “inactive” forms of C3b (iC3b) and C4b (iC4b). The iC3b and iC4b molecules remain bound to the cell and can no longer bind FB or C2b, respectively. Thus, C3 convertases can no longer be formed, inhibiting all complement pathways. This FI regulatory function can only be accomplished in the presence of a cofactor, including FH, CD46, CR1, C4BP, or CSMD1 (CUB and sushi domains protein 1) (Roversi et al., 2011; Nilsson et al., 2011; Whaley and Ruddy, 1976; Pangburn et al., 1977; Harrison and Lachmann, 1980) (Fig. 8C and Table 1). FI also plays a central role, using FH as a cofactor, in cleaving fluid phase C3(H₂O) to iC3(H₂O), thus also regulating fluid phase complement activity.

As initially mentioned in a previous section, a surface on which complement has successfully activated (such as on pathogens) will be covered in several thousand C3b molecules, which will be converted to iC3b, and C3dg molecules by FI with the help of cofactors (Fig. 7C), as mentioned above. These iC3b and C3dg fragments will be recognized by specific receptors on immune cells promoting phagocytosis and adaptive immunity (van Lookeren Campagne et al., 2007; Carroll and Isenman, 2012). These receptors and their associated functions will be described in the next major section.

Inhibition of the terminal pathway (MAC)

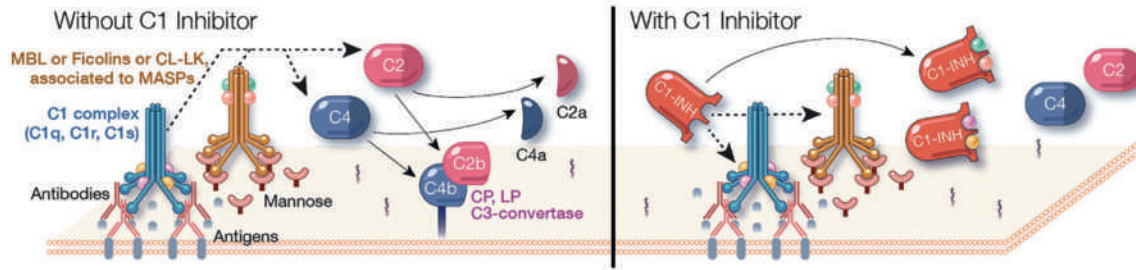
Inhibition of MAC (C5b-9) formation is an essential step that assures host cells are protected from complement-mediated lysis. Fig. 8E illustrates a subset of regulators that inhibit the formation of the MAC either on cell surfaces (CD59) or in the fluid phase (clusterin, vitronectin). CD59 inhibits the polymerization of C9, while Clusterin and Vitronectin inhibit soluble C5b-7 and/or soluble C5b-9 (sMAC), from binding to the cell surface (Zipfel and Skerka, 2009).

Table 1 Summary of complement regulatory proteins and associated functions.

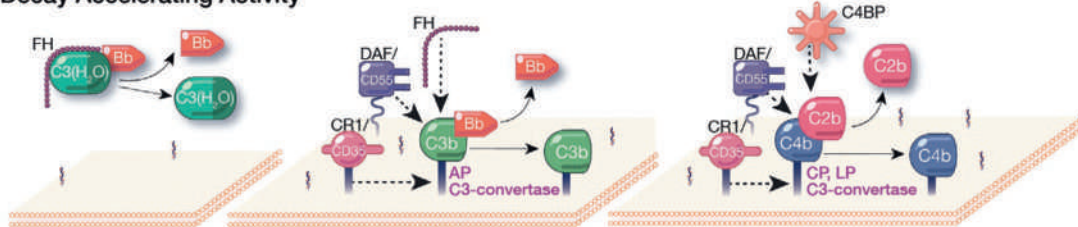
		<i>Name (symbol)</i>	<i>Concentration in serum and MW</i>	<i>Role in the regulation of complement system^a</i>
Negative regulators	Fluid phase complement regulatory proteins	C1 inhibitor (C1-INH)	0.15–0.30 mg/mL Predicted: 52.9 kDa, 71.1 kDa (glycosylated)	Dismantles C1, MBL and Ficolins complexes by removing C1r and C1s from C1q and MASP-2 from MBL and Ficolins, respectively. Inhibits Factor XIIa and plasma kallikrein of the contact system. Essential for control of bradykinin generation.
		C4-binding protein (C4BP)	200 µg/mL 500 kDa (oligomeric glycoprotein)	Binds C4b, displacing C2b; cofactor for C4b cleavage by Factor I.
		Factor H (FH)	0.3–0.5 mg/mL 155 kDa	Main regulator of the fluid phase AP convertase C3b(H ₂ O)Bb, by displacing Bb from C3b(H ₂ O). Regulates the cell-bound AP convertases by (a) binding to a combination of C3b and polyanions on surfaces and competing with binding of FB to C3b and/or displacing Bb from C3b and (b) acting as a cofactor for C3b cleavage by Factor I.
		Factor I (FI)	~35 µg/mL Predicted: 65–75 kDa	Serine protease that cleaves C3b and C4b to generate iC3b, C3d/dg, or iC4b, C4d; aided by cofactors Factor H, MCP/CD46, C4BP, or CR1/CD35.
		Factor H like protein-1 (FHL-1)	30–50 µg/mL Predicted: 49 kDa	Regulates the fluid phase AP convertase C3b(H ₂ O)Bb by displacing Bb from C3(H ₂ O). FHL-1 also has Factor I-cofactor activity.
	Membrane bound complement regulatory proteins	Complement receptor 1 (CR1/CD35)	Predicted depending of allotypes: 190–280 kDa	Displaces Bb from C3b and C2b from C4b. Cofactor for C4b and C3b cleavage by Factor I.
		Complement receptor of the immunoglobulin family (CRiG)	Predicted: 42 kDa	Cofactor for C3b cleavage by Factor I.
		Decay-accelerating factor (DAF/CD55)	Observed: 70 kDa	Displaces Bb from C3b and C2b from C4b.
		Membrane Cofactor proteins (MCP/CD46)	Predicted: 39 kDa	Cofactor for C4b and C3b cleavage by Factor I.
		CUB and sushi domains protein 1 (CSMD1)	389 kDa	Cofactor for C3b cleavage by Factor I.
Positive regulators	Fluid phase complement regulatory proteins	Properdin (P)	5–15 µg/mL Predicted 53 kDa	Binds C3b and Factor B or Bb and stabilizes the alternative pathway C3 and C5 convertases.
		Factor H related -1, -4, -5 (FHR-1, -4, -5)	CFHR1: 35.7 kDa, CFHR2 25.9 kDa, CFHR3 35.5 kDa, CFHR4A: 63.4 kDa, CFHR4B: 35.3 kDa, CFHR5: 62.5 kDa Predicted 8.96 kDa	CFH-1, -4, -5 compete with Factor H, acting as potential positive regulators of the AP. All FHR interact with C3b, C3d/dg and iC3b.
Terminal pathway inhibition	Membrane bound complement regulatory protein	CD59	Predicted 8.96 kDa	CD59 inhibits the formation of MAC by locking onto C8 in the forming MAC to block the recruitment of C9 into the complex.
	Fluid phase complement regulatory proteins	Mature form of clusterin (sCLU)	22.6–87.2 µg/mL 70–85 kDa	Binds to a structural motif common to C7, C8 and C9β inhibiting the correct complex assembly and prevents their insertion into cell membranes.
		Vitronectin (Vn)	200–400 µg/mL Predicted: 52.5 kDa	Binds to pre-membrane attack complex (C5b-7); hinders the insertion of C5b-7 into the cell membrane, inhibiting C9 to form part of the MAC. Interacts with soluble C5b-7, C5b-8 and C5b-9, in which sC5b-7 further attracts free C8 and C9 to form the sC5b-8 and sC5b-9, respectively.

^aThe table summarizes information in the text; thus references are cited within the text.

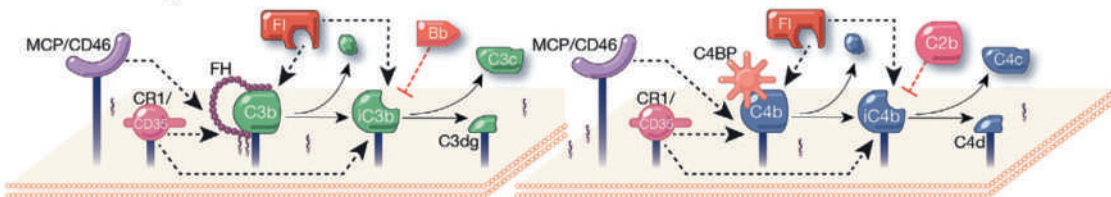
A. C1 Inhibitor



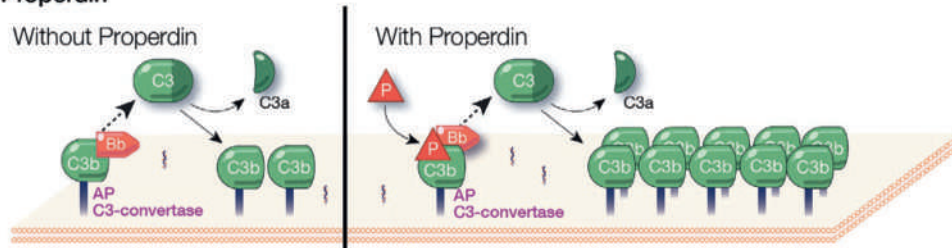
B. Decay Accelerating Activity



C. Cofactor Activity



D. Properdin



E. MAC Inhibition

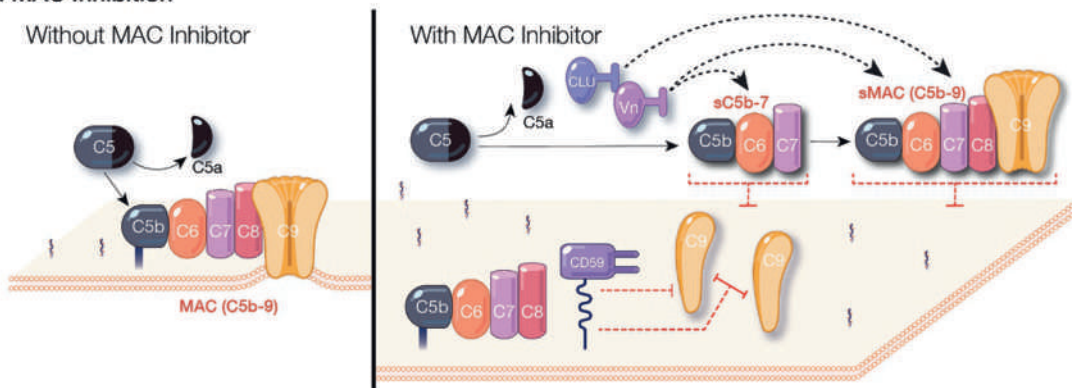


Fig. 8 Function of complement regulatory proteins. (A) C1 inhibitor function. C1 and MBL complexes are able to cleave C4 and C2 to generate the CP/LP C3 convertase, C4bC2b (left side of panel). C1-inhibitor (C1-INH) prevents complement activation by removing the serine proteases C1r, C1s, or MASPs from C1 and MBL complexes, respectively. (B) Decay accelerating activity. Several factors including DAF/CD55, CR1/CD35, and Factor H (FH) are able to displace Bb from the AP C3 convertase, C3bBb, while DAF/CD55, CR1/CD35, and C4BP displace C2b from the CP/LP C3 convertase, C4bC2b. (C) Cofactor activity. MCP/CD46 and CR1 act as cofactors for Factor I (FI)-mediated cleavage of C3b into iC3b, and C4b into iC4b. FH acts as a cofactor for FI-mediated cleavage of C3b into iC3b, while C4BP acts as a cofactor for FI-mediated cleavage of C4b into iC4b. CR1 can also act as a cofactor for FI to further cleave iC3b and iC4b into C3dg and C4d, respectively. Inactivated fragments, iC3b and iC4b, are no longer able to form C3 convertases. (D) Properdin function. AP C3 convertase, C3bBb, cleaves C3 into C3a and C3b. Properdin (P) is a positive complement regulator that stabilizes the AP C3 convertases increasing 10 times its half-life to promote efficient C3b deposition. (E) MAC inhibitor. C5 is cleaved by all C5 convertases to generate C5a and C5b. C5b is the focal point for the formation of the pore-forming membrane attack complex (MAC or C5b-9). CD59 inhibits the formation of MAC by binding to C8 and inhibiting the polymerization of C9 in the bilayer membrane of cell surfaces. Clusterin (CLU) and Vitronectin (Vn) bind soluble MAC (sMAC) to inhibit its deposition on cells surfaces. CLU also can bind to soluble C5b-7 (sC5b-7) and inhibit its binding to cells membranes.

Inactivation of C5a, C3a

During complement activation, C3 and C5 are cleaved, releasing small soluble fragments known as C3a and C5a, respectively. These molecules are pro-inflammatory and carry out several key functions (to be discussed in the next major section). In order to restrict the action of these potent molecules close to where they are synthesized, plasma carboxypeptidases N and R remove the C-terminal arginine from the C5a and C3a peptide forming the C5a desArg and C3a desArg derivatives (Matthews et al., 2004; Mueller-Ortiz et al., 2009).

Positive regulation of the complement system

Properdin

AP C3 convertases have a short half-life of ~90 s, under physiological conditions, in the fluid phase and on cell surfaces (Pangburn and Müller-Eberhard, 1986). Properdin is a 53 kDa plasma glycoprotein that has the main function of binding to and stabilizing surface-bound AP C3 convertases (C3bBb) and C5 convertases (C3bBbC3b) by extending the half-life of the nascent convertases 5–10-fold, leading to accelerated and efficient amplification of C3b deposition on surfaces (Fig. 8D) (Berends et al., 2015; Fearon and Austen, 1975; Schreiber et al., 1975). Properdin is found at 4–25 µg/mL in plasma and each monomer is composed of seven thrombospondin repeat (TSR) type 1 domains that, under physiological conditions, form mainly cyclic dimers (P2; parallel strand shaped), trimers (P3; triangle shaped) and tetramers (P4; square shaped) in an approximate 1:2:1 ratio via head-to-tail association of its identical monomers (Pangburn, 1989; Smith et al., 1984). The convertase-stabilizing activity of the P4 is greater than the P3, and both are greater than the P2 (Pangburn, 1989; Blatt et al., 2016b). The vertices of the oligomers interact with both C3b and FB in the convertases (Alcorlo et al., 2013; Pedersen et al., 2017), helping explain why the oligomers vary in functional potency. Properdin is synthesized by various pro-inflammatory cell types, including activated neutrophils, monocytes, and T cells and is thus important for regulating complement in local cellular microenvironments. Finally, deficiency in properdin leads to increased susceptibility to fulminant systemic Neisserial infections (to be discussed in a later section).

FHR proteins

In humans, there are five FH-related (FHR1-5) proteins, whose functions are not completely characterized. However, studies support a role for some FHRs that is the opposite of the role of FH and FHL-1. Specifically, they enhance complement activation by competing with the FH and FHL-1 [reviewed in Cserhalmi et al. (2019) and Harrison (2018)]. The magnitude of their physiological effects as positive regulators *in vivo* remains to be determined.

Functions of complement and associated receptors

Overview

The complement system is highly pleiotropic and aside from generating MACs on surfaces to induce direct osmotic lysis, several opsonic fragments (i.e. C3b, C4b, iC3b, C3d/dg) and pro-inflammatory mediators (e.g. C3a, C5a) are generated, which need to interact with specific receptors on a variety of cells in order to carry out their functions. In addition, certain intact complement proteins, such as C1q and others, also bind specific receptors, leading to essential functions. In this section, some critical roles of complement will be discussed, and, when applicable, explained in the context of the receptors that are required for a particular function.

Functions associated with killing of pathogens

Direct killing via MAC

The main role of MAC is to kill certain gram-negative bacteria that cannot be efficiently removed by opsonophagocytosis. Examples of bacteria that require MAC formation on their surface for elimination are *Neisseria*, *Haemophilus*, *Moraxella*, and others [reviewed in Morgan et al. (2017)]. Deficiencies in MAC components leave individuals highly susceptible to disseminated Neisserial infections, as will be discussed in a later section. Susceptibility to MAC varies widely among Gram-negative bacteria (i.e. smooth strains, but not rough strains, are resistant to killing because they are protected from the MAC by long-chain LPS), while Gram-positive bacteria are not susceptible to MAC, due to complement evasion strategies. Enveloped viruses and parasites may also be susceptible to MAC, and those that are not have developed complement evasion strategies.

Opsonization for efficient pathogen phagocytosis and killing

There are many routes by which C3b sub-fragments and C4b deposited on target cells can lead to efficient phagocytosis and elimination. Complement activation on a pathogen or altered self surfaces leads to abundant C4b and/or C3b molecules that are converted to iC3b and C3d/dg by FI with the help of cofactors (Fig. 7C). For phagocytosis to occur, most of these fragments will be recognized by complement receptors CR1, CR3, and CR4 on phagocytic cells. These receptors each have a multitude of functions and ligands; this section summarizes how they participate specifically in phagocytosis for elimination of pathogens. CR1 (CD35) that is present on several immune cells, including mononuclear phagocytes and neutrophils, recognizes mainly C3b on pathogens,

although it can also recognize C4b and iC3b (with less affinity). CR3 (Mac-1, CD11b/CD18) and CR4 (p150,95, CD11c/CD18) are present on several types of immune cells, including mononuclear phagocytes and neutrophils. Both receptors recognize opsonizing iC3b on the pathogen surface and lead to destruction by phagocytosis.

It is important to note that CR3 and CR4 have a multitude of additional functions that will also contribute to pathogen killing. Aside from phagocytosis, CR3 participates in neutrophil activation, respiratory burst, degranulation, netosis, and regulating cytokine production (Bullard, 2018), while CR4 promotes cytokine production from monocytes, triggers neutrophil respiratory burst, and plays a role in monocyte and neutrophil adhesion (Georgakopoulos et al., 2008; Ren et al., 2004; Keizer et al., 1987; Stacker and Springer, 1991).

Initiating molecules of the CP and LP (C1q, MBL, and Ficolins) can bind directly to pathogen-associated molecular patterns (PAMPs) on certain pathogens and directly promote their phagocytosis. For example, (a) C1q can bind *Trypanosoma cruzi*, *Schistosoma mansoni*, *Listeria monocytogenes* (Alvarez-Dominguez et al., 1993; Rimoldi et al., 1989), and others; (b) MBL enhances phagocytosis of *Neisseria meningitidis* (Jack et al., 2005) and others; and (c) Ficolins promote phagocytosis of *E. coli*, *Salmonella typhimurium*, and others (Hajishengallis and Russell, 2015).

Pathogen complement evasion strategies that prevent killing

Although the complement system is a highly sophisticated innate defense mechanism that eliminates pathogens via direct killing and/or opsonophagocytosis, healthy individuals still succumb in infections, because many pathogens have evolved effective complement evasion strategies. Some of these include the secretion of convertase inhibitors, the expression of complement regulators that mimic the function of human regulatory proteins, capturing host regulators to protect themselves, preventing insertion of the MAC due to cell wall structure, capturing and inactivating initiating molecules of complement (e.g. immunoglobulins, C1q, etc.), and others. Some intracellular pathogens even use opsonization to their advantage for gaining entry into cells via complement receptor CR3, or bind directly to complement receptors CR3 and CR1. These concepts have been extensively reviewed elsewhere (Lambris et al., 2008; Hovingh et al., 2016; Ogembo et al., 2013).

Functions related to the generation of proinflammatory mediators

Small soluble cleavage fragments of some complement proteins can initiate inflammatory responses

When complement C3, C4 and C5 are cleaved during the course of complement activation, small fragments (C3a, C5a, C4a) are released into the blood or surrounding cellular microenvironment. Fragments C3a and C5a have specific receptors (C3aR, C5aR1, C5aR2) on a wide variety of cell types including neutrophils, eosinophils, mast cells, monocyte/macrophages, as well as endothelial cells, mast cells, smooth muscle cells, and others (Martin et al., 1997; Ohno et al., 2000). They can even be found intracellularly on T cells, with effects on T cell biology. C3a and C5a are also called anaphylatoxins due to their ability to induce smooth muscle contraction, increased vascular permeability, and basophil and mast cell degranulation. C4a, due to the lack of an identified receptor and very low anaphylatoxin activity (100–1000-fold less than C3a), may not be an actual biological anaphylatoxin (Hugli et al., 1983; Barnum, 2015; Klos et al., 2013). C3a and C5a are also chemotactic molecules that directly attract neutrophils, eosinophils, basophils, and monocytes to the sites where complement is activating.

C3a, once bound to C3aR (a G-coupled receptor) on myeloid receptors, participates in cellular activation, chemoattraction, degranulation and cytokine production [reviewed in Coulthard and Woodruff (2015)]. C3a-C3aR interaction has several proinflammatory properties overlapping with C5aR (below); however, C3a is much less potent than C5a in its functions and also can mediate anti-inflammatory functions in a cell-specific manner, likely dependent on the concentration of C3a (e.g. enhancement or inhibition of cytokine production) [Ember and Hugli, 1997; reviewed in Barnum and Schein (2018b) and Coulthard et al. (2018a)].

C5a carries out anaphylatoxin functions, as mentioned above and also is a very potent chemoattractant molecule (more potent than C3a), that, once bound to its primary specific receptor, C5aR1 (CD88; a G-coupled receptor), attracts all cells mentioned above, and tissue resident macrophages, including microglia. C5a-C5aR1 interaction controls effector functions of these cells, inducing, for example, degranulation and proinflammatory mediator release, and enhancing phagocytosis. C5a can also have opposing modulatory functions (pro-inflammatory or anti-inflammatory) on chemotaxis, degranulation, apoptosis, and cytokine production, depending on the extracellular concentrations of C5a and the cell type [reviewed in Coulthard et al. (2018b)]. This variability in C5a consequences makes it capable of either contributing to low grade inflammation needed for normal control of infection, healing, and homeostasis, or to severe inflammation, inducing even a cytokine storm. C5a can also bind to C5aR2 (C5L2; not coupled to G-proteins). Less is known about the consequences of the C5a-C5aR2 interaction. It may have both pro-inflammatory and anti-inflammatory consequences, and one possible function may be to serve as a modulator of C5aR1 signaling (Strainic et al., 2013; Arbore et al., 2016).

Functions related to housekeeping and homeostasis

The complement system plays essential roles in housekeeping functions including clearance or solubilization of immune complexes and clearance of dead and dying cells in a way that does not induce pathological inflammation. These two phenomena will be described below. Research in the complement system is also in the process of defining mechanisms by which both extracellular and intracellular complement pathways participate in cellular metabolism and homeostasis (i.e. regulation of autophagy, adipose tissue

metabolism, etc.), and this novel field has been reviewed elsewhere (Kolev and Kemper, 2017; King et al., 2019; Rahman et al., 2020).

Clearance of immune complexes

Antigen/antibody complexes in the blood activate the CP, because the antibody Fc regions are close enough together to bind C1q. Once the immune complexes are opsonized with C3b fragments, they are recognized by complement receptor CR1 on erythrocytes. These erythrocytes transport the opsonized immune complexes to the spleen or liver, where the immune complexes are taken up by resident tissue macrophages via CR3, CR4, or CR1. The erythrocytes then return to the circulation. Removal of immune complexes avoids immune complex-mediated inflammatory responses, including vasculitis due to deposition of excess immune complexes in the vasculature [reviewed in Walport and Lachmann (1988)].

Clearance of apoptotic cells

Recognition and removal of apoptotic and necrotic cells needs to occur efficiently and in a tightly controlled manner in order to avoid excessive inflammation and autoimmune responses. Apoptotic and necrotic cells are recognized by complement C1q, MBL, and Ficolins, via recognition of various ligands (e.g. C-reactive protein that binds to these cells, phosphatidylserine, and others), which triggers complement activation and deposition of opsonizing C3b. These opsonized cells are phagocytosed without pathological inflammation (Mevorach et al., 1998; Gershov et al., 2000). As discussed in previous sections, normal healthy cells are protected from complement activation by complement regulators. The reason that complement can activate on apoptotic cells is because membrane-bound regulators are partially downregulated (Elward et al., 2005) allowing limited complement activation, while CD59 (regulator of MAC) is still present; thus MAC is not formed. FH and C4BP also participate in regulating the process. This allows the opsonized apoptotic cells to be removed by phagocytosis, without inflammatory consequences. If dead and dying cells are not cleared (i.e. to deficiency in early CP components), there is an increase of dangerous autoantigens released from the cells and increased susceptibility to immune complex disease (e.g. SLE).

Another mechanism that contributes to elimination of apoptotic and necrotic cells involves binding of C1q and MBL to the dying cell, followed by the recognition of their collagenous tails by cC1qR (calreticulin) receptor on macrophages. cC1qR does not have a transmembrane domain and uses CD91 ($\alpha 2$ -macroglobulin) as an adaptor molecule. The apoptotic cell/C1q/cC1q/CD91 complex is taken up by micropinocytosis (Ogden et al., 2001).

Functions related to the adaptive humoral and cellular immune responses

B cell function

The best characterized complement-mediated function related to B cells is to opsonize antigen or immune complexes for uptake by complement receptor type 2 (CR2, CD21) expressed on B cells. B cells have on their surface a receptor (immunoglobulin) for recognizing a specific pathogen antigen, as well as a B cell co-receptor (CR2/CD19/CD81 complex). CR2 is a complement receptor present on B cells, follicular dendritic cells, and others, that recognizes sub-fragments of C3b [i.e. C3d/dg and iC3b (to a much lesser extent)]. Microbial antigens that are opsonized with C3d/dg can simultaneously engage both CR2 in the context of the B cell co-receptor and the microbe-specific immunoglobulin on the surface of a B cell, effectively activating the B cell. The presence of C3d/dg on the antigen and its interaction with CR2 lowers the amount of antigen needed to induce B cell responses by up to 10,000-fold (Carter et al., 1988; Dempsey et al., 1996). CR2 is also involved in B cell antigen presentation to T cells (Boackle et al., 1998) and has a role in the determination of B-cell tolerance toward self-antigens [reviewed in Boackle (2018)].

Interplay with T cells

Complement influences T cell immunity in many essential ways and unveiling the mechanisms involved is a hot topic that is ever-evolving in the field. There are several excellent reviews that address this topic in detail (Lo and Woodruff, 2020; West et al., 2018; Merle et al., 2015b; Hess and Kemper, 2016); herein, a few examples will be given to illustrate the variety and relevance of complement in T cell immunity.

The anaphylatoxins C3a and C5a, and their respective receptors (C3aR and C5aR), have roles in T cell function. C3a provides homeostatic signals to T cells and modulates metabolism, C5a is involved in polarization of T cell responses, modulation of T cell effector functions, and regulation of apoptotic cellular pathways. These effects are primarily thought to occur via C5a modulation of several different cytokines and interleukins. C3a, C5a, C3aR and C5aR have also been found intracellularly (Arbore et al., 2016). Intracellular C3a is generated by Cathepsin L-mediated cleavage of intracellular C3, and the source of intracellular C3 may come from the uptake of the hydrolyzed form of C3, C3(H₂O), from the blood by immune cells (Elvington et al., 2017). C3a in resting T cells is required for homeostatic CD4⁺ T cell survival. Intracellular C3a and C3b can transfer to the cell surface and engage C3aR and CD46, respectively, which is needed for interferon IFN γ secretion and induction of protective human T_H1 responses [reviewed in West et al. (2018)]. In the case of intracellular C5, the protease that cleaves it has not been identified. In activated T cells, intracellular C5aR1 signals have various functions in T cells, including inducing generation of reactive oxygen species and promoting NLRP3 inflammasome assembly, increasing T_H1 responses to infection (Arbore et al., 2016).

C1q also has several functions that influence T cells. One way is mediated by C1q interaction with dendritic cells via cC1qR receptor (calreticulin; receptor for the collagenous tails of C1q). Depending on what C1q is coupled to (e.g. antigen or apoptotic

self-cells), it will induce differential cytokine production by dendritic cells which will either promote T_H1 polarization or inhibit it, respectively [reviewed in Lo and Woodruff (2020)].

Functions related to complement system and coagulation system cross-talk

The complement system is not only a significant contributor to inflammation, but also to thrombosis. There are several points of intersection between the complement and coagulation systems that, when excessive, can lead to complement-mediated pro-thrombotic disorders, such as paroxysmal nocturnal hemoglobinuria, atypical hemolytic uremic syndrome, and thrombosis in infectious states, such as COVID-19. Some examples include: (a) C5a-mediated tissue factor (TF) expression on neutrophils (Johnstone, 2006) and endothelial cells, von Willebrand factor secretion from endothelial cells (Foreman et al., 1994), and upregulation of CR3 on neutrophils with resulting increase in platelet/neutrophil aggregates in blood (Blatt et al., 2016b; Hamad et al., 2015); (b) C5b-7-induced TF expression on monocytes (Langer et al., 2013) and C5b-8/C5b-9-mediated platelet activation (Wiedmer et al., 1986); (c) gC1qR (p33), a receptor for the globular heads of C1q, can also bind to high-molecular-weight kininogen (HK) and factor XII (FXII) on endothelial cells, leading to activation of the contact coagulation system (Ghebrehiwet et al., 2006); (d) C1-INH negatively regulates both factor XIa of the coagulation system and complement C1 (Meijers et al., 1988).

The crosstalk can also occur in the opposite direction. Certain coagulation enzymes, such as thrombin and factor Xa can directly activate components of the complement cascade. For example, C5 can be directly cleaved, leading to C5 convertase formation in the absence of C3 (Huber-Lang et al., 2006).

The complement system in disease

Overview

The essential and pleiotropic nature of complement functions and the relevance of the regulatory mechanisms involved, are clearly illustrated when the underlying pathophysiology of a wide variety of diseases is examined. Complement deficiencies account for ~5% of primary immunodeficiencies. This section will cover examples of: (a) diseases due to deficiencies in complement proteins; (b) diseases due to deficiencies in complement regulatory proteins; and (c) complement activation-mediated pathology, without defects in the complement system itself.

Deficiency in complement proteins

Deficiency in early CP complement components

Deficiency of early components of CP (C1q, C1r/s, C4, C2) predisposes to the autoimmune disease systemic lupus erythematosus (SLE) and immune complex disease, due to an increase in dangerous autoantigens and immune complexes that cannot be properly removed via the normal housekeeping functions of the CP (described in a previous section). Over 50% of individuals with deficiencies in C1q, C1r, C1s, and C4 develop SLE (Kaul et al., 2016). In addition, autoantibodies against C1q are observed in ~ a third of patients with SLE, which may block C1q function, mimicking C1q-deficiency (Leffler et al., 2014). SLE is one of most prevalent immune complex diseases that is 10× more common in females. Glomerulonephritis and arthritis are two of the most frequent symptoms and vasculitis is also found. Immune complexes trapped or formed in tissues (e.g. kidney and synovial tissue) lead to complement-mediated tissue injury to kidneys and joints (Ballanti et al., 2013).

Deficiencies in CP components also predispose to recurrent respiratory infections from encapsulated bacteria such as *S. pneumoniae*, *H. influenzae*, and *N. meningitidis* (Macedo and Isaac, 2016).

Deficiencies in LP proteins

Deficiencies in MBL (~5% of Caucasians) or Ficolins increase susceptibility to secondary infections and autoimmunity in infants and immunocompromised patients, while healthy individuals are usually asymptomatic due to intact CP and AP activity (Degen and Thiel, 2013). Deficiency in MASP-1 causes 3MC syndrome (Malpuech-Michels-Mingarelli-Carnevale syndrome), which is a rare disease characterized by developmental abnormalities and mental and growth retardation (Sirmaci et al., 2010). Some MASP-2-deficient individuals (~4% of Caucasians and up to 18% of some African populations) have increased risk of infection or autoimmune disease, but most are asymptomatic (Thiel et al., 2007 reviewed in Brodzski et al., 2020).

C3 deficiency

C3 is the most abundant protein in the complement system, with serum levels of approximately 1 g/L in both adults and newborns. Given all the roles that C3 cleavage fragments are needed for, including cytolysis, increasing vasopermeability, opsonization, clearance of immune complexes, facilitation of B cell proliferation and differentiation, and dendritic cell maturation, its deficiency leaves the individual severely immunocompromised. Individuals lacking C3 are highly susceptible to encapsulated bacteria (e.g. *S. pneumoniae*) and all pyogenic infections (recurrent, systemic), such as from *S. aureus*, *S. pyogenes*, and *N. gonorrhoeae*, and frequently suffer from immune complex-related disorders, such as glomerulonephritis and SLE. The rare deficiency is caused by mutations in the C3 gene, leading to absence or markedly reduced levels, or to dysfunction of the protein (Ghannam et al., 2008;

Figueroa and Densen, 1991; Macedo and Isaac, 2016; Okura et al., 2016; Grumach and Kirschfink, 2014; Pekkarinen et al., 2015; Reis et al., 2006).

Deficiencies in terminal complement components (C5, C6, C7, C8, C9) or FD or FB

C8 binds C5b67 and initiates C9 polymerization, leading to MAC formation. Genetic deficiency in C5, C6, C7, C8, C9 leads to increased susceptibility to invasive meningococcal infections, in particular with *Neisseria sp. (meningitidis and gonorrhoeae)*, resulting in dissemination of gonococcal infection, meningitis, and septic arthritis. Individuals with these deficiencies can lead a mostly normal life by receiving a tetravalent meningococcal vaccine (e.g. Menactra) as prophylaxis (Fijen et al., 1998). Similarly, FD deficiency leads to meningitis and gonococcal infections (Sprong et al., 2006). FB deficiency was thought to be non-existent and lethal until recently identified in an individual that presented recurrent bacterial infections including meningitis by *Neisseria*, pneumococcal peritonitis, and pneumococcal pneumonia (Slade et al., 2013).

Deficiency in complement regulatory proteins

A fine balance of positive and negative regulation of complement is essential in order to avoid disease.

Deficiency in C1-esterase inhibitor (C1-INH)

As explained in a previous section, C1-INH regulates C1, MBL, and Ficolins of the complement system, but also controls the contact activation system by inhibiting factor XIIa and plasma kallikrein, which are involved in the formation of bradykinin, a potent vasodilator molecule. C1-INH deficiency is rare and causes a disease known as hereditary angioedema or hereditary angioneurotic edema (HAE or HANE, respectively) that manifests as spontaneous episodes of skin, laryngeal and intestinal subcutaneous and submucosal edema. Although C1-INH leads to dysregulation of the CP and LP of complement, the edema is due to bradykinin production by the uncontrolled contact system [Nussberger et al., 1998; reviewed in López-Lera et al. (2019)]. In some textbooks the C2a fragment of C2 is still referred to as “C2 kinin” and implicated in the pathogenesis of HAE as well, but this is not the case, as the C2a fragment has been shown to not have kinin activity, and thus C2 kinin does not actually exist (Kaplan and Ghebrehiwet, 2005). HAE is not an allergic disease (attacks not mediated by histamine) and there is no itching associated to the swelling. The edema in the gastrointestinal tract may cause severe abdominal pain and vomiting and sometimes diarrhea. HAE episodes are infrequent in early childhood, but exacerbations occur during adolescence and continue throughout life. Because HAE patients still have an intact AP, patients are not unduly susceptible to infection. HANE is corrected by replacing C1-INH (FDA-approved, purified C1-INH: Berinert®, CINRYZE®, and HAEGARDA®, or recombinant C1-INH: RUCONEST®). This is an ideal alternative to using anabolic androgens (e.g. testosterone ethanate, danazol) for treatment, especially in women.

Properdin deficiency

Properdin is required for efficient function of the AP, by promoting AP activity through AP convertase stabilization and potentially initiating convertase formation. Deficiency of properdin is inherited as a recessive X-linked trait and leads to reduction in C3 cleavage and all down-stream effects. Patients are thus highly susceptible to *Neisseria* meningitis, otitis media, and pneumonia [reviewed in Chen et al. (2018) and Figueroa and Densen (1991)].

Factor I deficiency

One of the essential functions of FI is to control complement in the fluid phase and on cell surfaces by cleaving C3(H₂O) and C3b fragments into iC3(H₂O), iC3b and C3d/dg, with the help of cofactors. New convertases cannot be formed on these C3 fragments. In the absence of FI, the C3(H₂O) and C3b will not be cleaved by FI and new convertases will be readily formed, leading to complete consumption of C3 and FB (very low or undetectable levels in plasma). The essential function of FI becomes evident when considering that it is needed for cleaving the thousands of C3b molecules that are on surfaces where complement activates and amplifies successfully (such as pathogens). The abundant iC3b and C3d/dg molecules that are formed will serve as opsonic and immunostimulatory molecules to achieve adequate immune responses (Fig. 8C and 7C). Thus, the absence of FI leads to severe immunodeficiency, increasing susceptibility to pyogenic infections (e.g. otitis media, bronchitis, sinusitis, tonsillitis, etc.) [reviewed in Reis et al. (2006)]. Heterozygous individuals produce about half of the normal amounts and this is sufficient to prevent clinical symptoms (Barrett and Boyle, 1984). Clinical findings are similar to C3 deficiency and to agammaglobulinemia (i.e. no formation of C3d leads to no co-stimulation of B cell activation).

Defects in the function of Factor H and other regulators that lead to renal and ocular pathologies

FH is a critical negative regulator of both fluid phase and cell-bound complement activity, in order to protect our cells and tissues from complement-mediated attack. FH carries out its function via decay acceleration and by acting as a cofactor for FI (Fig. 7B and C). FH consists of 20 SCR domains, arranged like beads on a string, where the N-terminal domains 1–5 contain the functional site of the protein, while polyanion- and C3b/d-binding sites (for recognizing cell surfaces) are distributed throughout the protein. SCR domains 7 and 19–20 are considered particularly relevant for recognition of host cells for anchoring FH to the surface to carry out its protective functions [reviewed in Ferreira et al. (2010b) and Haque et al. (2020)]. Understanding the distribution of these sites allows for a better understanding of how diseases are associated to FH dysfunction, depending on whether FH is completely deficient, or if a particular domain of FH is defective. Thus, FH deficiency leads to the inability to accelerate the decay of AP

convertases and to act as a cofactor for FI in the fluid phase and on cell surfaces. Because FH is the main regulator of complement activity in the fluid phase of blood, FH deficiency leads to complete consumption of C3. This massive, chronic consumption of C3 (C3 synthesis is normal) leads to uncontrolled deposition of C3b, iC3b, and C3d/dg on cells, with damage particularly to the basement membrane of the kidneys that rely mainly of FH for protection, resulting in type II membranoproliferative glomerulonephritis (MPGN). Some individuals also have increased risk to infections.

On the other hand, when congenital mutations occur in the C-terminus of FH, FH can still regulate complement in the fluid phase of blood (the levels of C3 remain normal), but it cannot bind properly to host cell surfaces in order to regulate complement, resulting in cell damage. This results in the potential development of atypical hemolytic uremic syndrome (aHUS), a hematological disease that causes hemolysis, thrombi formation, renal failure and death. For aHUS to manifest, it usually requires a triggering event, including renal endothelial cell damage secondary to pregnancy/postpartum, infections, autoimmune disorders, or certain pharmacological treatments [reviewed in [Nester et al. \(2015\)](#)]. The current FDA-approved treatment for aHUS is C5-inhibition with anti-C5 monoclonal antibody Eculizumab or Ravulizumab.

aHUS can also be caused by mutations in other regions of FH, or in complement regulators FI, MCP/CD46, or by anti-FH autoantibodies, as well as gain-of-function mutations in C3 or FB [reviewed in [Nester et al. \(2015\)](#)]. Other renal pathologies caused by AP dysregulation include rare C3 glomerulopathies (C3G) and IgA Nephropathy (IgAN), the most frequent adult glomerulonephritis [reviewed in [López-Lera et al. \(2019\)](#)].

In the case of ocular pathology, when SCR domain 7 of FH or FHL-1 have a particular polymorphism, Y402H, individuals are significantly more likely to develop age-related macular degeneration (ARMD), the main cause of blindness in the elderly, due to deficient control of complement activation within the retina [reviewed in [López-Lera et al. \(2019\)](#)].

Deficiency in CD55 and CD59

CD55 and CD59 are glycosylphosphatidylinositol (GPI)-anchored membrane proteins. Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic stem cell disorder in which mutations in the PIG-A (phosphatidylinositol glycan class A) gene result in a deficiency of GPI anchor, and thus absence CD55 and CD59. The lack of complement control by these regulators on cells derived from the defective clone (red blood cells, platelets, etc.) results in intravascular and extra-vascular hemolysis and thrombi formation. PNH patients have dark early morning urine due to hemolysis and are diagnosed by a positive HAM test that consists of a serum lysis assay where PNH red blood cells readily lyse in the presence of acidified normal human serum, while normal cells do not. Flow cytometry using red blood cells from patients for detection of CD59/55 is now more common for diagnosis. Although bone marrow transplant is the only cure for PNH, patients can be treated for life with the FDA-approved anti-C5 monoclonal antibody Eculizumab or Ravulizumab ([Risitano et al., 2019](#); [Rondeau et al., 2019](#); [McKeage, 2019](#)).

Complement-mediated pathology where defects in complement proteins or regulators are not required

Although complement is known as an essential defense mechanism, complement activation on our own cells is possible and sometimes necessary, such as occurs during housekeeping functions and when local promotion of inflammation is needed (e.g. under limited/normal activation conditions this could lead to transient increase in reparative local inflammation or removal of damaged cells by phagocytosis) [[Camous et al., 2011](#); reviewed in [Blatt et al. \(2016a\)](#)]. However, excessive complement activation, as a consequence of certain diseases, will overwhelm complement regulatory mechanisms and lead to pathologic, excessive inflammation and complement-mediated tissue damage. Some examples are explained below.

Immune complex diseases

Immune complexes (ICs) are produced wherever there is an antibody response to a soluble antigen. IC disease is a state in which vascular injury occurs due to circulating antigen-antibody complexes, formed by exacerbated and/or chronic immune reactions or when normal levels of ICs cannot be removed efficiently. The normal process for IC elimination involves complement and Fc receptors. The excess ICs end up depositing in the basement membrane of small blood vessels, serving as a focal point for complement-mediated damage, and complement-mediated and Fc-mediated recruitment and activation of inflammatory cells. Certain pathological situations generate levels of ICs that overwhelm the normal elimination processes, even when complement proteins and regulators are “normal”. Common manifestations include glomerulonephritis, synovitis and dermal vasculitis. Examples of immune complex diseases are antibody-mediated glomerulonephritis, bacterial endocarditis, SLE (can also have complement-independent etiology), mixed essential cryoglobulinemia (various infectious, autoimmune or cancer-driven etiologies leading to hypergammaglobulinemia), rheumatic disease, and (HIV)-associated immune complex renal disease [reviewed in [Poppelaars and Thurman \(2020\)](#) and [Leffler et al. \(2014\)](#)].

Complement in inflammatory diseases

Complement plays a role in the pathology of most inflammatory diseases including, but not limited to, ischemia/reperfusion, asthma, arthritis, cardiovascular diseases, acute and chronic transplant rejection, skin diseases, infectious diseases that lead to severe systemic and/or local inflammation (e.g. sepsis, viral infections such as that by COVID-19, etc.), and chronic inflammation in cancer, etc., and have been reviewed extensively by others ([López-Lera et al., 2019](#); [Lappegård et al., 2014](#); [Lo and Woodruff, 2020](#); [Roumenina et al., 2019](#); [Lo et al., 2020](#)). For example, in ischemia-reperfusion injury, such as that which can occur during transplantation reperfusion or traumatic injury, systemic inflammation and can result in multi organ failure from complement

activation. This is due to hypoxia-induced alteration of the cell surfaces with exposure of neoantigens that now can be activators of the AP or be recognized by natural antibodies to trigger the CP, triggering sterile inflammation. In the case of skin diseases, complement plays a role in the pathogenesis of pemphigus and bullous pemphigoid (BP) due to the presence of autoantibodies against skin antigens.

Complement in neuroinflammation/neurodegenerative diseases

Local synthesis of complement components by astrocytes, microglia and neurons, and oligodendrites in the brain (e.g. C1, C2, C3, C4, C5, C6, C7, C8, C9, FB) allows complement to play important roles in normal function and homeostasis in the brain, such as removal of apoptotic cells and cellular debris, developmental functions, neurogenesis, and synaptic pruning [reviewed in [Tenner et al. \(2018\)](#), [Hammad et al. \(2018\)](#), and [Lo and Woodruff \(2020\)](#)]. In the context of disease, when complement-dependent synapse elimination is aberrant, it may contribute to neuropsychiatric and neurodevelopmental disorders. Expression of complement components increases in the brain during aging and especially in Alzheimer's disease. Also, excessive complement-mediated synapse pruning occurs in aging, Alzheimer's disease and other disorders such as Parkinson's disease, multiple sclerosis, and Huntington's disease that correlate with cognitive or behavior impairments [reviewed in [Morgan \(2015\)](#) and [Tenner et al. \(2018\)](#)]. In addition, complement is involved in traumatic brain injury pathology, where due to loss of blood/brain barrier integrity, excessive amounts of complement from leukocytes and blood contribute to massive inflammation [reviewed in [Hammad et al. \(2018\)](#)].

Complement in cancer

Complement has a several functions in the context of cancer including directly promoting proliferation and metastasis, stimulating angiogenesis, and regulating or generating an immunosuppressive microenvironment. The tumor microenvironment highly influences the growth and spread of tumors, thus impacting the clinical outcome. In the tumor microenvironment, complement may either act to kill antibody-coated tumor cells, or on the contrary, support local chronic inflammation and/or hamper anti-tumor T cell responses, favoring tumor progression. For example, C5a stimulates myeloid-derived suppressor cells (MDSC), which dampen the immune response, suppressing cytotoxic T cells, and stimulate T regulatory cells. C3a, C5a, and C5b-9 promote angiogenesis, helping in tumor nutrient support and dissemination [reviewed in [Roumenina et al. \(2019\)](#)]. Normal mature neutrophils also acquire a suppressor phenotype within the tumor microenvironment that requires a number of neutrophil effector functions, including complement activation ([Singel et al., 2019](#)). The role of complement is highly variable depending on the type of cancer; thus, modulation of the complement system as potential cancer therapy will likely need to be used within the context of personalized medicine.

Autoimmune hemolytic anemia

Autoimmune hemolytic anemia is an uncommon type II autoimmune disease that results in complement- and Fc receptor-mediated hemolysis after recognition of the autoantibodies on the surface of red blood cells. In approximately half of cases, the cause of autoimmune hemolytic anemia is idiopathic or primary; however, it can also be associated with other disorders (secondary) such as autoimmune disease, chronic lymphocytic leukemia, and other blood cancers, hepatitis, HIV, etc. It can also occur following the use of certain drugs (such as penicillin) or after a bone marrow stem cell transplant [reviewed in [Braunstein \(2020\)](#)].

General overview of therapeutic targets

As explained in the previous section, (a) the complement system must be tightly controlled otherwise it leads to unwanted complement-mediated damage of host cells, causing several possible diseases, and (b) complement activity plays a role in disease pathogenesis, even when regulation is intact. Anti-complement therapeutics targeting a wide variety of stages of the complement system is proving effective in many of these disease scenarios and are prevalent in clinical trials, which are quickly evolving and extensively reviewed elsewhere [reviewed in [Zipfel et al. \(2019\)](#), [Ricklin et al. \(2018\)](#), [Tomlinson and Thurman \(2018\)](#), and [Zelev et al. \(2019\)](#)]. So far, the only FDA approved drugs with complement connectivity, used in controlling complement-mediated pathology, include: (a) the anti-C5 antibodies, Eculizumab and Ravulizumab, for the treatment of PNH and aHUS, and (b) purified or recombinant replacement therapy of C1-INH (i.e. Berinert®, CINRYZE®, and HAEGARDA®: Human derived C1-INH, and RUCONEST®: recombinant C1-INH) in hereditary angioedema (HAE) where C1-INH deficiency causes dysregulation of the complement and kinin systems. Individuals receiving anti-C5 therapy need to be concomitantly vaccinated with the meningococcal vaccine as well as vaccines to prevent *Streptococcus pneumoniae* and *Haemophilus influenza* type b.

Basic laboratory assays for assessing overall complement function and disease diagnostics

Common clinical tests for assessing complement consist of measuring overall CP and AP activity, and C3 and C4 levels by hemolytic assays [Morgan \(2010\)](#) or ELISA-based assays ([Seelen et al., 2005](#)). Additional immunometric tests exist to measure levels and functions of all complement components, as well as to identify autoantibodies against certain components (e.g. C1q or FH), but not

all tests are available in all clinical laboratories. Depending on the suspected disease and following identification of quantitative functional defects, an underlying genetic cause can be confirmed by genetic screening. Complement tests and the criteria used for selecting them, as well as their limitations, have been thoroughly reviewed by the European Society for Immunodeficiencies and European Reference Network on Rare Primary Immunodeficiency, Autoinflammatory and Autoimmune Diseases (Brodzski et al., 2020). Often, complement analysis in clinical practice is restricted to cases in which complement deficiency is suspected in patients due to increased susceptibility to severe infections. However, complement defects may also present as autoimmune diseases (e.g. SLE), ARMD, renal disorders (e.g. aHUS and other C3 glomerulonephropathies) and angioedema. Early diagnosis of complement deficiency decreases the risk of severe infections during childhood and may avoid irreversible tissue damage when prophylactic treatment (i.e. vaccination and anti-complement therapeutics) is administered.

Basis of hemolytic assays that measure general CP and AP activity

Although ELISA kit assays are available commercially for obtaining CH_{50} and AP_{50} values, herein, the traditional hemolytic assays will be described as a way to review how the CP and AP pathways function.

CH_{50} hemolytic assay

The CH_{50} assay consists of CP-mediated hemolysis of IgG or IgM-coated sheep erythrocytes. The anti-sheep antibodies used are also referred to as hemolysin. Sheep erythrocytes are used because of the high levels of the polyanion, sialic acid, on the cell surface, which allows them to be very effectively protected by FH. Thus, these erythrocytes are resistant to the AP. This assay also requires Ca^{++} for stability of C1 and Mg^{++} for stability of the convertases. Patient serum is incubated with the antibody-coated sheep erythrocytes and hemolysis is measured. The assay is performed in parallel with normal human reference serum. The result is expressed as the reciprocal of the amount of patient serum necessary to lyse 50% of the erythrocytes (due to MAC formation). Thus, the lower the CH_{50} value, the less active complement is in the serum (compared to values from the normal human serum standards). The reduced activity may be due to primary deficiencies in C1(q, r, s), C2, C3, C4, or C5b-9, or to complement consumption due to defective or absent C1-INH, C4BP, FI, and FH, or certain chronic infectious or inflammatory diseases.

AP_{50} hemolytic assay

The AP_{50} assay relies on AP-mediated hemolysis of erythrocytes lacking surface expression of polyanions (e.g. rabbit, guinea pig, chicken). Without polyanions, these erythrocytes are effectively lysed by the AP because they will not be protected by FH. This assay requires Mg^{++} for stability of the convertases. Because these erythrocytes are not coated with antibodies and Ca^{++} is absent, the CP does not readily activate, thus AP function is adequately assessed. Patient serum is incubated with erythrocytes and hemolysis is measured. The assay is performed in parallel with normal human reference serum. The result is expressed as the reciprocal of the amount of patient serum necessary to lyse 50% of the erythrocytes (due to MAC formation). Thus, the lower the AP_{50} value, the less active complement is in the serum (compared to values from normal human serum standards). The reduced activity may be due to primary deficiencies in C3, FB, FD, C5b-9, properdin, or complement consumption due to defective or absent FI and FH.

Conclusion

The complement system is multifaceted and comprises a versatile set of molecules that participate in innate and adaptive immunity. The main points discussed in this article emphasize the extraordinary characteristics of complement (no longer considered just a set of circulating molecules restricted to innate immunity), including participating in circulating and local tissue defense and homeostatic processes. These processes include fighting off infections by direct killing and removal by phagocytosis, modulating adaptive cellular immune responses, removing dead and dying cells and immune complexes from circulation, among others. Given the essential functions of complement, it needs to be tightly regulated in order to not cause unwanted damage to self. When integrity of either a complement cascade protein or of a complement regulator is compromised (via primary or secondary deficiencies or loss or gain-of function mutations), a wide range of disease scenarios ensue, including severe infections, autoimmune disorders, eye and/or renal disorders, or angioedema, etc. Moreover, even when all complement cascade proteins and regulators are functionally intact, complement can contribute to disease pathogenesis, for example, via fluctuating concentrations of complement cascade proteins and/or complement regulatory proteins, locally and/or systemically, and/or modulation in expression, due to consumption during infections or acute and chronic inflammatory diseases. Moreover, in many disease scenarios, such as immune complex disease, ischemia reperfusion injury, and others, the normal housekeeping functions of complement as well as the ability to regulate itself are overwhelmed, contributing to pathology. Currently, there are few FDA-approved therapeutics to target complement, but there are many promising ongoing clinical trials aimed at assessing the efficacy of inhibiting complement at multiple stages of the cascade. The continued study of molecular mechanisms involved in complement-mediated disease pathology will continue to be essential. Finally, a large number of investigators groups have made essential contributions over the past century, only a portion of which have been cited here due to space constraints. Excellent reviews have been cited in an attempt to compensate for omissions.

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Cytokines in Innate Immunity

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Glossary

Cytokine storm and COVID-19 Cytokine storm is a hyperinflammatory state due to overactivity of major pro-inflammatory cytokines like IL-1, IL-6, and TNF. It is a major obstacle for the treatment of severe COVID-19. The latest analyzes of the SARS-CoV-2 infection show IL-6 as a potential biomarker for the severity of the disease. Anti-IL-6R (tocilizumab) and remdesivir antiviral therapies show promise in the treatment of severe disease. Prevention measures are in place with many auspicious novel vaccines.

IL-6 IL-6 family of cytokines is structurally distinct from IL-1/IL-18 or TNF families. They are characterized by four α -helical bundle structure that signals via the JAK-STAT pathway. IL-6 has a major role in innate and adaptive immunity. IL-6 classic signaling is implicated in acute-phase responses, whereas IL-6 *trans*-signaling (and *trans*-presentation/cluster-signaling) characteristically initiates pro-inflammatory pathways with a major impact on adaptive immunity, including plasma cell development and antibody production, and T-cell helper 17 response.

Interferons Antiviral cytokines or interferons are classified into three types. Type I interferons like IFN- α , β are early molecular effectors capable of inducing an antiviral state in cells. Almost all nucleated cells can produce IFN- α . Its predominant source is plasmacytoid dendritic cells (pDCs). IFN-type I and III (IFN- λ) stimulate both innate and adaptive immune responses. Type I IFNs can positively influence both cellular and humoral adaptive immunity. Type II interferon (IFN- γ) has a weak antiviral ability, but its major role is in regulating adaptive immunity, particularly in Th1 responses.

The IL-1 family Members of the IL-1 family of cytokines are similar in structure and function. Most receptors of this family have one chain (IL-1RAcP) in common in their heterodimeric receptors (IL-1R, IL-33R, and IL-36R). The functions include agonistic (pro-inflammatory) and antagonistic (anti-inflammatory) activities. There are seven agonists: IL-1 α , β , IL-18, IL-33, and IL-36 α , β , γ , and four antagonists: IL-1Ra, IL-36Ra, IL-37, and IL-38. Additional antagonists are IL-18BP and several soluble receptors (ST2, IL-1RAcP, IL-1R2-decoy). With these activities, the IL-1 family of cytokines can signal damage of tissue integrity, distress, and danger to the organism, ultimately alarming innate and adaptive immunity to fight pathogens.

TNF and LT TNF and LT are trimeric proteins belonging to a structurally related TNF superfamily of cytokines. They are pleiotropic cytokines capable of signaling an array of pro- and anti-inflammatory activities. TNF- α has soluble (sTNF) and membrane (mTNF) forms. Both forms can bind to two TNF receptors (TNF-R1 and TNF-R2), but mTNF can alter the structure of the TNF-R2 such that it binds to sTNF with a lower affinity. Thus, sTNF primarily binds to TNF-R1 and plays an important role in the inflammatory immune response, whereas mTNF- α interacts preferentially with TNF-R2 and promotes cellular proliferation and survival and some other biological effects.

Abbreviations

ADAM	A disintegrin and metalloproteinase
APC	Antigen-presenting cell
CTL	Cytotoxic T lymphocyte
DCs	Dendritic cells
FDA	Food and Drug Administration of USA
FDC	Follicular dendritic cells
GALT	Gut-associated lymphoid tissue
IBD	Inflammatory bowel disease
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
IL-1R1	IL-1 receptor chain 1
IL-1RAcP	IL-1 receptor accessory protein
ILCs	Innate lymphoid cells
iNOS	Inducible nitric oxide synthase
IRAK4	Interleukin-1 receptor-associated kinase 4
LT	Lymphotoxin
LT β R	Transmembrane lymphotoxin receptor
MHC I	MHC class I molecule
MHC II	MHC class II molecule
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response protein 88
RA	Rheumatoid arthritis
SOCS1	Suppressors of cytokine signaling
STAT	Signal transducers and activators of transcription
TACE	TNF- α converting enzyme
Th	T helper cell
Th1	Th type 1 response
TIR	<i>Toll</i> /IL-1 resistance domain
TIRAP	TIR domain-containing adaptor protein
TLR	<i>Toll</i> -like receptor
TNF	Tumor necrosis factor
TNF-R	Tumor necrosis factor receptor
TOLLIP	<i>Toll</i> interacting protein
TRAF	TNF receptor-associated factor

Introduction

Cytokines are small proteins with a major role in inflammation and cell survival. Cytokines can be classified into several structural classes. We recognize the (1) helical cytokines (Nicola and Hilton, 1998), (2) the trimeric tumor necrosis factor (TNF) family (Idriss and Naismith, 2000), (3) the cysteine knot growth factors (Sun and Davies, 1995), and (4) the β -trefoil growth factors (Murzin et al., 1992). Cytokines can also be classified according to the type of receptor that they engage (Wang et al., 2009), and that has

been mostly used in chemokine descriptions. The cytokine receptors can be grouped into six major families based on shared structural features: (1) class I cytokine receptors, (2) class II cytokine receptors, (3) TNF receptors, (4) IL-1 receptors, (5) tyrosine kinase receptors, and (6) chemokine receptors (Fitzgerald et al., 2001; Thomson and Lotze, 2003).

Innate immunity cytokines have agonistic, antagonistic, and receptor antagonistic actions that also include soluble receptors and reverse signaling. They are essential regulators of tissue homeostasis (including wound healing). By provoking inflammation and distress in the body, some of them can alarm innate and adaptive immune systems to make a proper type of response to pathogenic infections. They are helped in these matters with many factors, including other cytokines, because most have multiple, sometimes overlapping functions.

IL-1, TNF, and interferons (IFNs) are important mediators in immunity. They were among the first ones identified. They differ in basic structure, but they are similar in functioning as a homeostatic mechanism to maintain integrity of an organism. Many shared functions include pro-inflammatory, anti-inflammatory and immunoregulatory activities.

The IL-1 family

IL-1 is a pleiotropic cytokine with pro-inflammatory, immunoregulatory, and hematopoietic effects. It can cause an increase in body temperature (Evans et al., 2015), bone resorption, muscle proteolysis, cachexia (indirectly via TNF), an increase in circulating acute-phase proteins such as C-reactive protein (CRP), and a decrease of iron and zinc plasma concentrations (Dinarello, 2009; Garlanda et al., 2013).

The IL-1 can be secreted by various stimuli. Pattern recognition receptors, including TLRs and their counterparts in inflammasomes, are the critical mediators in sending the alarm to activate defensive actions of innate and adaptive immune responses. Thus, pattern recognition, senescence, dysbiosis, necroptosis, tissue damage, and coagulation of blood are provoking stimuli that, with increasing strength, influence and amplify local innate and, in turn, adaptive immune responses, all of which could lead to a systemic inflammation with grave and sometimes fatal outcomes. The latter is called a *cytokine storm*, implicated in patients with severe COVID-19 disease. It is characterized by an acute increase in circulating levels of various pro-inflammatory cytokines, including IL-1 β , IFN- γ , TNF, IL-6, IL-10, and IL-18 (Ragab et al., 2020; Shimizu, 2019).

Target tissues for IL-1 are cells of the hematopoietic (lymphoid and myeloid) origin that subsequently regulate innate and adaptive immune responses by various cytokines, including IFN- γ and IL-17. The endothelial lining of blood vessels secretes chemokines and expresses adhesion molecules that lead to leukocyte recruitment to the pathological event site. The brain is affected and sets in motion effects like fever and hormone production (via hypophysis-pituitary axis, steroids) as a reaction to stress. These processes tend to help heal an organism when wounded, infected, or otherwise compromised by necrotic cell death in tissues. The central function of IL-1 in the connective tissue in wound healing, probably due to the angiogenic effect (Dinarello, 2011).

IL-1 α is a dual function cytokine similar to IL-33 and IL-37 (Li et al., 2019). It signals via surface membrane receptors, but it also translocates to the nucleus. If it is released from chromatin during necrosis or necroptosis (Werman et al., 2004), it could promote inflammation (and is the leading cause for sterile inflammation) (Cohen et al., 2010). However, if it stays bound to the chromatin (for unknown reason), as in the apoptotic cell death, then it would not cause inflammation (Cohen et al., 2010). Secretion of IL-1 α requires cleavage of the precursor pro-IL-1 α by the caspase-1 and calpain enzymes.

Thus, necrotic death, exemplified by the IL-1 precursor release, is a signal of distress for the innate and adaptive immune systems, as most necrosis is either caused by or present during infection by pathogens or parasites. On the other hand, apoptotic death is physiologic during embryonal development, tissue regeneration, and maintenance, and, if sterile, will not alarm the immune system, thereby avoiding the generation of autoreactivity and possibly autoimmune disease (Chen et al., 2007).

During T-cell activation, IL-1 promotes the development of the Th17 phenotype of helper T cells, accompanied by IL-21, IL-23, and polarizing (required) cytokines IL-6 and TGF- β , whereas IFN- γ , IL-2, IL-4, IL-12, and IL-27 block Th17 development.

The gene coding for human IL-1 α is IL1A, and the one encoding IL-1 β is the IL1B gene. They are on the chromosome 2q12–21. Other group members are also encoded in this segment of the chromosome and form the IL1 gene cluster. The IL-1 receptor antagonist (IL-1Ra) is encoded by a gene in the same *locus*, called IL1RN (Fig. 1).

The IL1 gene cluster includes nine genes encoding IL-1-alike cytokines on human chromosome 2q14.1 (at 112–113 Mb). Six of these genes are located in-between IL1A/IL1B on the one side and IL1RN gene on the other in the following order (in the direction from centromere to telomere): IL37 (alias: IL1F7), IL36G (alias: IL1F9), IL36A (alias: IL1F6), IL36B (alias: IL1F8), IL36RN (alias: IL1F5) and IL38 (alias: IL1F10).

Interleukin-1 receptor 1 chain (IL-1R1) is also called “antigen CD121a”. Its gene is named IL1R1. It is located on chromosome 2q12 (at 102 Mb), 10 Mb centromeric to the IL-1 gene cluster, close to the receptor of the IL18R gene. The gene for the accessory chain (IL-1RAcP; IL-1R3) of the IL-1R1 (and IL-1R2) is located on chromosome 3q28 (at 190 Mb) and is named IL1RAP (alias: IL1R3). Interleukin-1 decoy receptor chain (IL-1R2) is also called “antigen CD121b.”

In Fig. 2, IL-1 functions and its family members are illustrated. The IL-1, IL-12, IL-23, IL-25, IL-33, and TSLP affect innate cells *innate-like cell type 1* (ILC1), ILC2, ILC3, and natural killer (NK) cells each in its specific way (Garlanda et al., 2013).

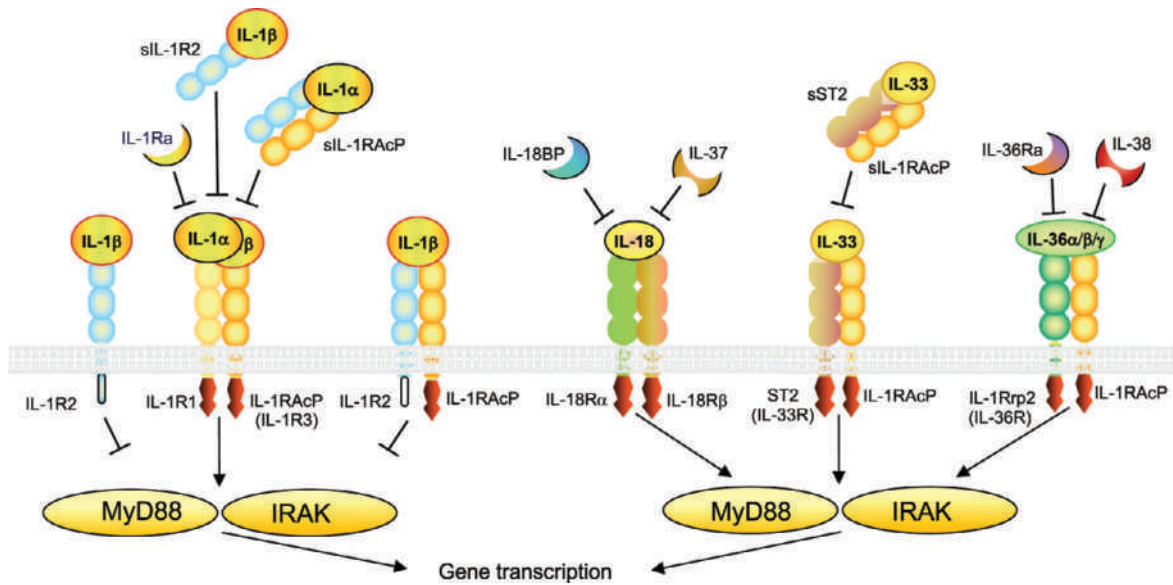


Fig. 1 Agonists and antagonists in the IL-1 family. IL-1α, IL-1β, IL-18, IL-33 and IL-36 cytokines are agonists. Receptor antagonists (IL-1Ra, IL-36Ra, IL-37, and IL-38), soluble receptors' extracellular domains (sIL-1R2, sST2, and sIL-1RAcP), and IL-18 binding protein (BP) can downregulate their signal transduction.

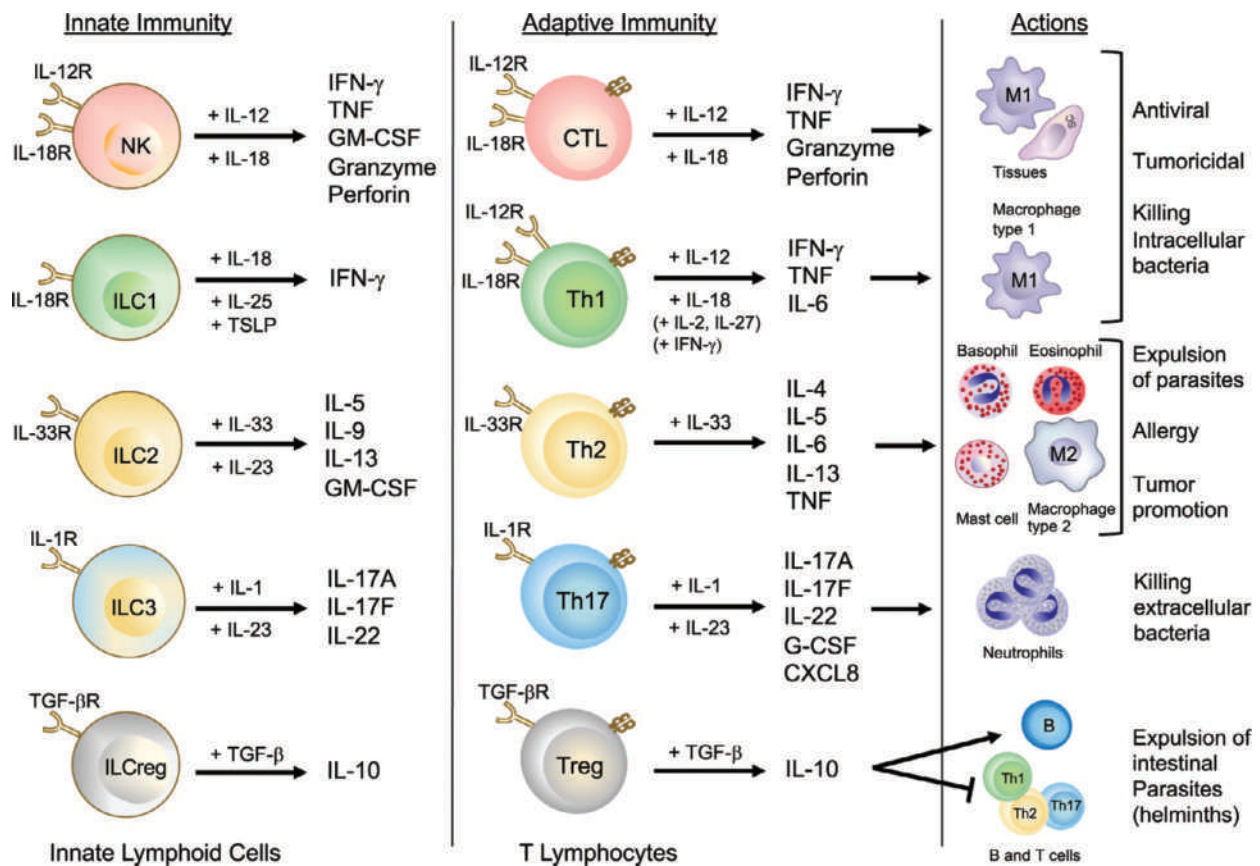


Fig. 2 The IL-1 family of cytokines (IL-1, IL-18 and IL-33) actions: effects on innate and adaptive immune responses.

Innate lymphoid cells

Innate lymphoid cells are non-lymphoid-tissue-resident immune cells that lack antigen receptors or NK receptors, as has been reviewed (Fan and Rudensky, 2016; Vivier et al., 2018). ILCs are divided into five groups based on cytokines produced and transcription regulators involved in their generation and function: natural killer (NK) cells, ILC1, ILC2, ILC3 (which include

lymphoid tissue inducer, LT α cells), and ILCreg (Mortha and Burrows, 2018; Schenkel and Masopust, 2014; Godfrey et al., 2015; Spits et al., 2013; McKenzie et al., 2014; Klose and Artis, 2016). ILCs are derived from early innate lymphoid precursors (EILP), which can differentiate into common helper innate lymphoid precursors (CHILP) (Mortha and Burrows, 2018).

The ILCs resemble CD4 helper or CD8 cytotoxic T cell responses of adaptive immunity. The ILC1 type produces IFN- γ and has T-bet transcription factor like Th1 cells, whereas ILC2 produces IL-5, IL-13 and shares GATA-3 transcription factor expression with the Th2 type. The ILC3 group corresponds to the Th17 type as they express the ROR γ t transcription factor, and they can be further subdivided into CCR6 positive and CCR6 negative cells. Regulatory ILCreg cells express Id3 transcription factor, produce IL-10 and can suppress activation of ILC1 and ILC3 (Wang et al., 2017). This is similar to Tr1 or Tregs that can inhibit adaptive immune responses of Th1, Th2, and Th17 types. Then, innate immunity's conventional NK cells derive from EILPs and express transcription factors T-bet and Eomes that lead to a phenotype similar to CD8 cytotoxic T cells, as they both have cytotoxicity, produce and secrete IFN- γ , TNF, GM-CSF, and other similar proteins (reviewed in Mortha and Burrows, 2018; Klose and Artis, 2016; Vivier et al., 2018).

The plasticity of the development of ILCs is uncovered by finding that ILC2 and ILC3 can trans-differentiate into other ILC types. So, we have ex-ILC2 cells that produce IFN- γ and function as ILC1. Similarly, we have ex-ILC2 cells that produce IL-17A resembling the ILC3 group. Lastly, there is an ex-ROR γ t/ILC3/ILC1 group expressing T-bet nuclear factor, secreting IFN- γ and TNF cytokines, and producing granzyme, perforin, and natural cytotoxicity receptors similar to ILC1 and NK cells (Mortha and Burrows, 2018).

ILC2 and ILC3 integrate multiple signals consisting of predominantly soluble factors in exerting their actions. In detail, ILC2 cells receive activation from signals via IL-33 and TSLP from epithelial cells and myeloid cells, and IL-25 from tuft cells. Furthermore, IL-1 α and IL-1 β , derived from myeloid and epithelial cells together with IL-23 can activate ILC3.

While circulating adaptive lymphocytes (B and T cells) and conventional NK replenish their numbers from hematogenous sources, the resident ILCs populate their niches early in ontogeny and remain in their environment throughout life. Their renewal depends on the support of local tissue-resident progenitor cells (Bando et al., 2015). The markers on ILC include integrin α 4 β 7 (that binds to MadCAM-1, which is expressed on high endothelial venules of MALT like Peyer's patches) and the chemokine receptor CXCR6 that guides them to the intestine (Klose et al., 2014; Satoh-Takayama et al., 2014). Additional chemokines involved in intestinal residence are CCR9 receptors and CCR6 that can guide ILCs to mesenteric lymph nodes.

In concert with other factors, ILC1 can be stimulated by IL-18 to secrete IFN- γ and TNF after activation by myeloid-cell-derived IL-12. IL-1 can stimulate ILC3s to produce IL-17, IL-22, and GM-CSF. IL-33 can similarly induce ILC2 to secrete IL-5, IL-9, and IL-13. Furthermore, IL-1, IL-18 and IL-33 affect adaptive immune cells Th1, Th2, Th17, T γ δ 17 and CD8 T cells to secrete IL-4, IL-5, IL-15, IFN- γ , G-CSF and chemokine CXCL8 (IL-8). These cytokines influence macrophages (M1 and M2), neutrophils, mast cells, and other granulocytes (eosinophils and basophils) to fight against viruses, tumors, extracellular parasites, or extracellular bacteria. In addition, these actions may provoke allergic reactions or promote tumor growth, too (Fig. 2).

IL-1 with its cytokine family members like IL-18, IL-33, IL-36, IL-37, and IL-38 affect almost all cells and organs in the body.

Medical aspects

IL-1 is the major pathogenic mediator of autoinflammatory, autoimmune, infectious, and degenerative diseases. IL-1 cytokine and its receptor are involved in the etiology of various acute and chronic inflammatory diseases, including *Alzheimer's disease*, *atherosclerosis*, *endometriosis*, *kidney diseases*, *migraine*, *rheumatoid arthritis*, *septic shock*, and in the tumors like *bladder*, *gastric*, and *liver cancer* (Dinarello, 2009; Gabay et al., 2010; Sims and Smith, 2010). For example, IL-1 β , TNF, and IFN- γ stimulate gamma-secretase-associated cleavage of *amyloid precursor protein* (APP) via JNK-dependent MAPK, which may be the reason for increased production and accumulation of amyloid in *Alzheimer's disease* (Alasmari et al., 2018).

Because IL-1 is a key mediator of inflammation, for clinical purposes, there are attempts to inhibit the action of IL-1, particularly in *autoimmunity*, *osteoarthritis* (Jotanovic et al., 2012), and other inflammation-related diseases, some using the addition of anti-TNF treatment (Dinarello, 2011).

Tumor necrosis factor (TNF) and lymphotoxin (LT)

Historical aspect

Tumor necrosis factor (TNF) is a pleiotropic cytokine involved in immunologic processes of infection resistance, autoimmunity, allergic disease, and anti-tumor activity (Ruddle, 2014; Old, 1985), originally discovered in 1968, under the name lymphotoxin (LT), as a factor that promotes the killing of mouse fibrosarcoma (L-929) tumor cells (Kolb and Granger, 1968; Ruddle and Waksman, 1968). Then, in 1975, a factor named "tumor-necrotizing factor" TNF was discovered in experiments when serum from bacillus *Calmette-Guérin* (BCG)-infected mice treated with lipopolysaccharide (LPS) could cause hemorrhagic necrosis in tumors (Carswell et al., 1975). It was subsequently shown that TNF increase could lead to endotoxic shock accompanying malaria (Clark et al., 1981). When the cDNAs were cloned for TNF (Pennica et al., 1984) and LT (Gray et al., 1984), their sequence predictions reveal to be similar as well as their gene organization (Nedwin et al., 1985). Furthermore, functionally, they were homologous, too, as LT could displace TNF from binding to its receptor on target cells' cell surface. These discoveries led to the renaming of TNF as TNF- α and LT (also called LT- α) as TNF- β .

The story continues when another factor called cachectin (which induces cachexia, exhaustive loss of body weight) was cloned and discovered to be actually TNF (Beutler et al., 1985). TNF was discovered to be the key mediator in lethal septic shock, which could be successfully prevented by mAbs against it (Tracey et al., 1986; Tracey et al., 1987).

In humans, both TNF- α and TNF- β bind to two types of receptors (Hohmann et al., 1990) of apparent molecular masses of 55/60 kDa and 75/80 kDa that were identified by monoclonal antibodies (Brockhaus et al., 1990). The receptors were subsequently molecularly cloned and denoted TNF-R1 (Loetscher et al., 1990; Schall et al., 1990) and TNF-R2 (Smith et al., 1990; Dembic et al., 1990), respectively.

Finally, in 1993, a novel LT/TNF-like cytokine was discovered and was named LT- β . It could make heterotrimers with LT- α resulting in functionally important complex LT- $\alpha_1\beta_2$ for gut physiology (Browning et al., 1993). This event encouraged the reversal of the name TNF- β back into LT- α .

TNF- α and LT- α (TNF- β)

TNF- α and TNF- β (LT- α) share 30% similarity at the amino acid level. They belong to the superfamily of tumor necrosis factor (TNFSE, *tumor necrosis factor superfamily*), which has over 20 members and includes molecules such as FasL and CD40L (Smith et al., 1994; Locksley et al., 2001). The superfamily members are structurally glycoproteins with the transmembrane regions.

Human TNF- α can be expressed as a 26-kDa transmembrane-bound homotrimer (mTNF- α) (Kriegler et al., 1988) or as a soluble 17-kDa form (sTNF- α), which is produced by cleavage of the mTNF- α by ADAM17 metalloproteinase (Black et al., 1997).

The TNF- α and LT- α (TNF- β) genes, named TNFA and TNFB, respectively, are located within the MHC (HLA) locus on human chromosome 6.

Although TNF- α expression is ubiquitous, it is typically produced by activated macrophages, lymphocytes, and other cells. TNF- β (LT- α) is mainly produced by B-, T-, NK cells, and ILCs.

TNF is involved, together with IL-1, in the development of the Th17 response.

There are two receptors that TNF can bind: type 1 TNF receptor or TNF-R1 (CD120a), and TNF receptor type 2 or TNF-R2 (CD120b). TNF-R1 has MW of 55/60-kDa, and TNF-R2 has 75/80-kDa, and both are expressed as homotrimers in the cell membrane. The molecular cloning of receptors revealed that they belong to a large TNF/TNF-R-related superfamily (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990, 1994; Dembic et al., 1990; Wallach et al., 1999; Locksley et al., 2001). TNF-R1 structure includes the death domain, which is not present in the TNF-R2. The TNF-R2 is mainly found on immunocytes (monocytes/macrophages, dendritic cells, B and T cells), endothelial cells, fibroblasts, oligodendrocytes, cardiomyocytes, keratinocytes, and some particular subsets of neurons (Medler and Wajant, 2019). TNF-R1 can be fully activated by both the membrane-bound and soluble trimeric forms of TNF. However, it seems that TNF binding to TNF-R1 is irreversible, whereas binding to TNF-R2 is reversible (Szondy and Pallai, 2017). The interaction between mTNF- α and TNF-R2 induces bi-directional (forward and reverse) signaling in both mTNF- α - and TNF-R2-expressing cells. Increasing evidence shows that the forward and reverse signaling mediated by mTNF- α and TNF-R2 might play a significant role in the tumor microenvironment (Szondy and Pallai, 2017).

A simplified version of TNF signaling is depicted in Fig. 3. It gives the basic principles of signaling through TNF-R1 and TNF-R2. The pathways are deposited and curated on the web at the WikiPathways database (ReactomeTeam et al., 2021).

Two TNF receptors (TNF-R1 and TNF-R2) have different intracellular domains and have variable outcomes (Dembic et al., 1990). Also, sTNF and mTNF differ in signaling, with mTNF being able to signal in a reverse direction. In short, TNF-R1, having the death domain, can promote cell death and pro-inflammatory actions, whereas TNF-R2 can collaborate with TNF-R1 signaling promoting cell survival (protection) and anti-inflammatory activities (Fig. 3). It seems that cell survival, proliferation, and death are a matter of quantitative balance between TNF-R1 and TNF-R2 and the key components of their respective signaling complexes (Gough and Myles, 2020).

Concerning the membrane-bound form of TNF (mTNF) - receptor interaction, mTNF was found to bind TNF-R2 (Grell et al., 1995) preferentially, and by doing so, alters structurally (via proline-rich stalk region) the organization of monomers, thus inhibiting the ability of this receptor to bind to sTNF. It appears that mTNF can control the quantitative balance of TNF-R1 and TNF-R2 in cells that express them, and that can have serious consequences for the outcome of the TNF response. Namely, when sTNF levels rise in circulation due to infection, sTNF actions in tissues would depend on the "imprint" left from previous interaction with mTNF.

The function of reverse signaling via mTNF- α is thought to be anti-inflammatory, probably in connection with secretion of IL-10 and TGF- β by the cell that signals in reverse (Kirchner et al., 2004; Pallai et al., 2016).

Membrane-bound mTNF and soluble sTNF have distinctive functions as indicated by knockout and knockin transgenic animal studies. It seems that mTNF can mediate a subset of beneficial TNF activities while lacking the systemic inflammatory effects of sTNF. One of the first functional consequences observed in mice deficient in both sTNF and mTNF is the lack of normal secondary lymphoid organ structure. These mice fail to form germinal centers upon immunization, are unable to mount host defense responses to infections (Pasparakis et al., 1996; Marino et al., 1997), and have none of the inflammatory responses known to be involved in models of brain-related autoimmune disease (*multiple sclerosis*) like *experimental autoimmune encephalomyelitis* (Körner et al., 1997). Targeted deficiencies in transgenic knockout mice of two ligand-two receptor systems (sTNF, mTNF, and TNF-R1 and TNF-R2 receptors) revealed an array of CNS effects and characteristics, which have been reviewed (Probert, 2015).

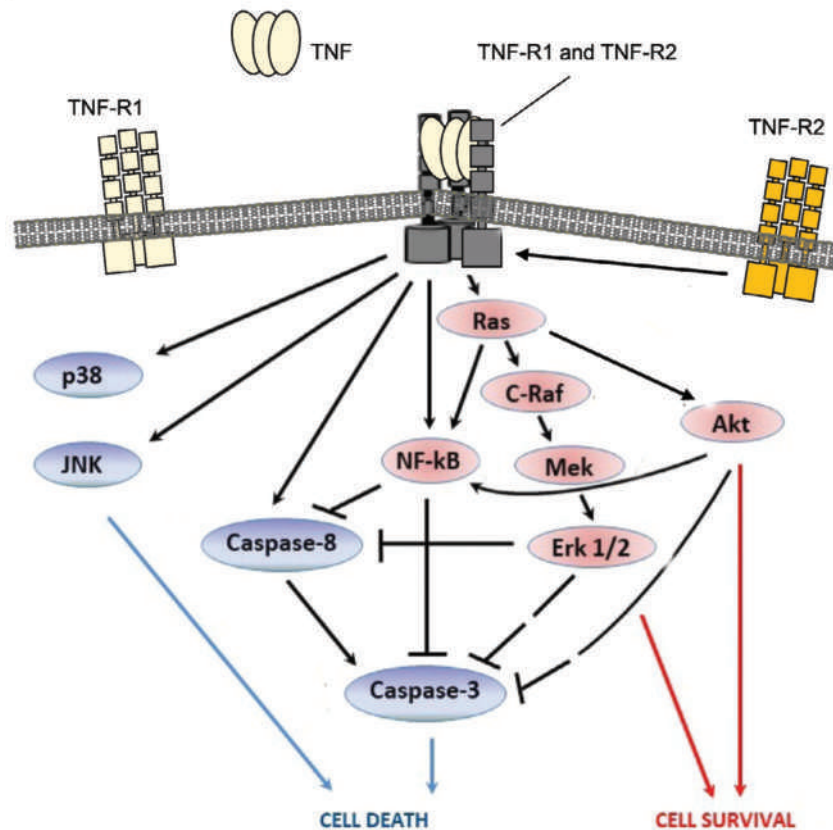


Fig. 3 Simplified TNF signal transduction and function. The proteins colored in blue are related to cellular distress or cell death, whereas the proteins in red are factors promoting cell survival. The resulting biological activities of TNF are indicated by blue (cell death) and red (survival) lines.

LT- β

In 1993, a novel LT- and TNF-like cytokine was discovered and was named LT- β . It could make heterotrimers with LT- α resulting with LT- $\alpha_1\beta_2$ complex (Browning et al., 1993) or LT- $\alpha_2\beta_1$ (Weinstein and Storkus, 2015) (Fig. 4). It prompted the reversal of the name TNF- β back into LT- α .

Several years later, the knockout mice revealed some cytokine functions (Koni et al., 1997).

LT- α (TNF- β) and LT- β are necessary for the development of secondary lymphoid tissue, and this requires the binding and activation of LT- β receptor (LT- β R) with the predominant form of LT- β , namely, LT- $\alpha_1\beta_2$ complex (Gubernatorova and Tumanov, 2016) (Fig. 4). LT- β R is required for the development of gut-associated lymphatic tissue (GALT), including Peyer's patches, mesenteric lymph nodes, and isolated lymphoid follicles. In the latter, T-independent IgA production occurs (as well as in the gut's mucous plate—lamina propria).

In LT- β R- and LT- β -knockout mice, there is a defect in IgA production in the intestine. It was demonstrated that T-cell independent IgA induction (via DC activation) depends on the LT- $\alpha_1\beta_2$ complex (TNF- β /LT- β_2) on ILC3 cells. However, soluble LT- α homotrimer (sTNF- β_3) secreted by ILC3 cells is necessary for T-cell dependent IgA induction in the intestinal lamina propria by promoting T cell migration to the gut (Kruglov et al., 2013; Kang et al., 2002).

The presence of LT- α (TNF- β) is necessary on the cell surface of lymphoid tissue inducer cells to interact with stromal cells (carrying LT- β R) during embryogenesis (Randall et al., 2008; van de Pavert and Mebius, 2010; Bar-Ephraim and Mebius, 2016).

Medical aspects

Production dysregulation of TNF has been implicated in a variety of human diseases. These include *Alzheimer's disease* (Swardfager et al., 2010), autoimmune diseases, major *depression* (Dowlati et al., 2010), and *cancer* (Locksley et al., 2001). Of autoimmune disorders, *rheumatoid arthritis* (RA), *juvenile RA*, *psoriasis*, *psoriatic arthritis*, *insulinitis*, and *inflammatory bowel disease* (IBD) have been linked to high levels of TNF (Keffer et al., 1991; Picarella et al., 1993; Victor and Gottlieb, 2002; Brynskov et al., 2002; Kontoyiannis et al., 1999; Neurath, 2014). Although *septic shock* involves high TNF levels, so far, it has not been seen as its dysregulation.

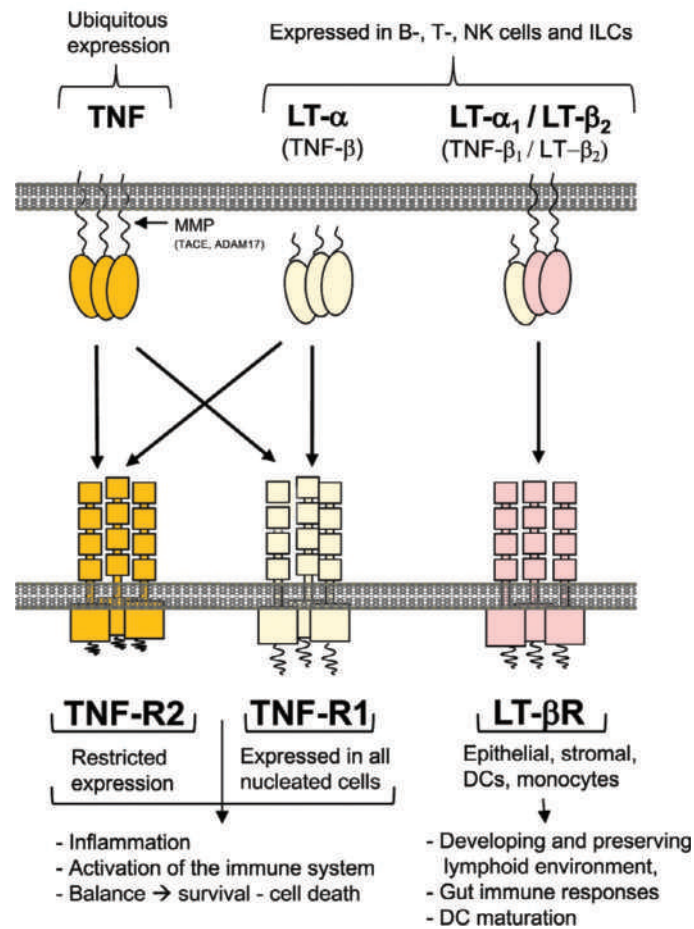


Fig. 4 Ligands and receptors of the TNF/LT family, their sources, and suggested function.

Monoclonal antibody (mAb) Infliximab (Remicade; Janssen Biotech, Horsham, PA, United States) was developed as a remedy to block TNF activity (Scallion et al., 2002) after animals' studies have shown benefit in the *rheumatoid arthritis* autoimmunity model. The introduction of infliximab in the clinics 20 years ago resulted in initial excitement because of successful treatment with lasting remission of many *rheumatoid arthritis* patients (reviewed in Melsheimer et al., 2019). Later on, several problems arose, including insensitive cases to TNF-treatment, but the most significant problem was immunization with the therapy. Namely, as the mAb needed to be repeatedly applied, rejection of the injected remedy occurred quite often, diminishing the clinical outcome in the end.

Also, constructs like Etanercept (Enbrel, Amgen) were made and approved for use in RA. Etanercept is a biologic comprising human TNF-R2 extracellular domain (for binding to TNF) that is attached to the human IgG1 constant region (to prolong serum half-life) (Scallion et al., 2002).

In the following years, novel TNF inhibitors, including humanized mAbs (Adalimumab, Humira; Golimumab, Simponi; and Certolizumab pegol, Cimzia) that efficiently bind and neutralize TNF were also developed (Kaymakcalan et al., 2009; Shealy et al., 2010).

Etanercept has been successfully used in RA patients. It is approved by the Food and Drug Administration of USA (FDA) to treat other autoimmune diseases such as *plaque psoriasis*, *psoriatic arthritis*, *polyarticular juvenile arthritis*, and *ankylosing spondylitis*.

Regarding *inflammatory bowel disease* (IBD), comprising *Crohn's disease* and *ulcerative colitis*, despite higher levels of TNF found in gut mucosa (Murch et al., 1991; Breese et al., 1994) and serum (Murch et al., 1991) of patients compared to a healthy population, its particular etiology and pathogenesis are still unclear as has been reviewed (Neurath, 2014). Recent advances point to ILC3 cells in gut mucosal homeostasis, which can be a double-edged sword because when ILC3 dysregulate, they can contribute to the progression and aggravation of IBD by overexpression of inflammatory cytokines IL-17, IL-22, and IFN- γ (Zeng et al., 2019). In animal models of IBD (reviewed in Gubernatorova and Tumanov, 2016), both TNF (sTNF and mTNF) were implicated in the disease (resembling mostly *Crohn's disease*), as blocking both forms of TNF led to stable continuous remission. Neutralization of mTNF seems crucial for treating experimental IBD *colitis* (Perrier et al., 2013). The therapy of *inflammatory bowel disease* using anti-TNF antibodies infliximab, adalimumab, golimumab, and certolizumab pegol has been a revolutionary improvement

(Tracey et al., 2008; Olesen et al., 2016; Ueda et al., 2013; Slevin and Egan, 2015). Unfortunately, one-third of patients have not responded to initial therapy, and up to 50% of patients ultimately have stopped responding (Nielsen and Ainsworth, 2013; Steenholdt et al., 2014).

Anti-TNF therapy has many systemic side effects, including those linked to infections and inflammation in the skin and joints (Tracey et al., 2008; Mocci et al., 2013). The most serious complication is the (re-)activation of latent *tuberculosis* (Cleynen and Vermeire, 2012). Other interesting side-effects of anti-TNF therapy are the Lupus-like syndrome and psoriasiform lesions, despite being anti *psoriatic* remedy (Mocci et al., 2013).

In the future, the receptor-specific antagonistic mAbs might be developed as treatment is specifically targeting TNF-resistant RA, MS or heart failure, or neurodegeneration.

The members of the large TNF-/TNFR-related superfamily are being targeted for treatments against human diseases or conditions such as *atherosclerosis*, *autoimmune* disorders, *cancer*, *osteoporosis*, and *transplant rejection*.

The IL-6 family

IL-6 is a product of activated T and B lymphocytes, monocytes and fibroblasts, and activated macrophages. IL-6 is produced after bacterial or viral challenge.

In innate immunity, the role of IL-6 is pro-inflammatory, stimulating the production of acute-phase proteins in liver cells, and in a concerted fashion helping in wound healing. The major function of IL-6 in the adaptive immune system is plasma cell differentiation as the final step of peripheral B lymphocyte development with antibody secretion.

Human IL-6 is a glycoprotein of 21–26 kDa, which contains 185 amino acids. The IL6 gene is located on human chromosome 7p21. IL-6 family of cytokines share four-helix bundle structures.

The receptors for the IL-6 family of cytokines have two polypeptides and are characterized by the presence of the shared transmembrane receptor subunit called gp130 (Fig. 5) or interleukin-6 signal transducer (IL-6ST, CD130) (Bravo et al., 1998; Bravo and Heath, 2000). Alternatively, some cytokine receptors in this group have a second chain very similar to IL-6ST. IL-6ST is a common signal-transducing chain for other cytokine receptors such as IL-11, IL-27, ciliary neurotrophic factor—CNTF, cardiotrophin-1—CT-1, cardiotrophin-like cytokine—CLC, leukemia inhibiting factor—LIF, neuropoetin (NPN), oncostatin M—OSM, and viral vIL-6 of Kaposi sarcoma associated herpesvirus HHV8 (Taga et al., 1992; Wang et al., 2009; Spangler et al., 2015). Receptors having chains similar to gp130 include IL-27R, IL-31R, G-CSF-R, LIF-R, OSM-R IL-12 β 1, and IL-12 β 2. These receptors have three structurally unique cytokine-binding sites on their cytokine ligands to assemble multimeric signaling complexes.

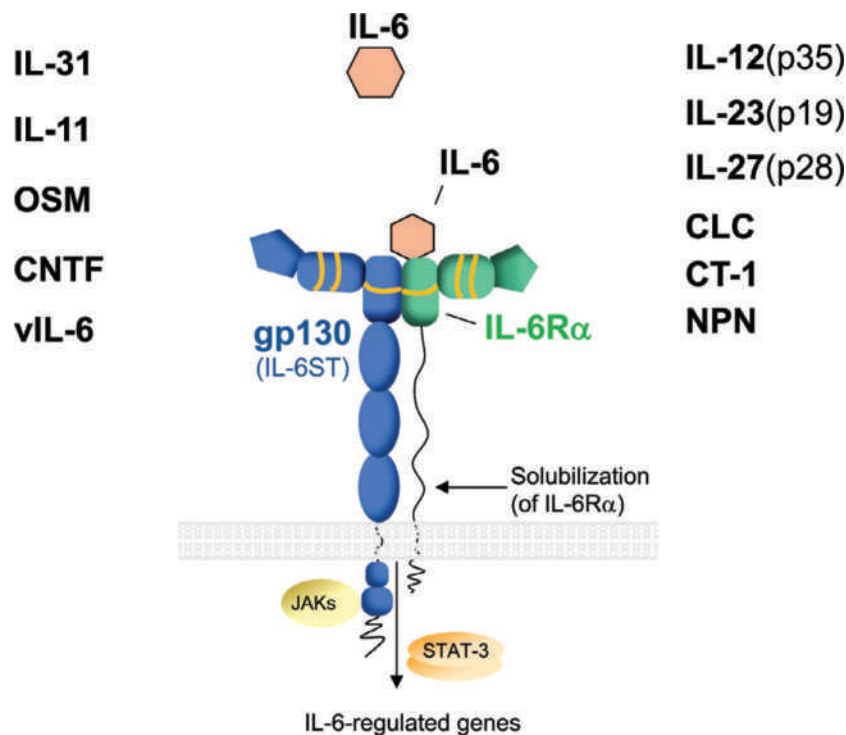


Fig. 5 Extended IL-6 family of cytokines characterized by shared four-helix bundle structure. In the middle, schematic representation of the ligand (IL-6) and its receptor comprising the gp130/IL-6 signal transducer (IL-6ST) chain and the IL-6R α chain (which can be solubilized, or trans-presented by a neighboring cell). These three molecules form a heterohexamer, which can signal into the cell. Each IL-6 family member can bind gp130 and additional specific receptor chain, or associate with biosimilars to gp130 (not shown).

Regarding the T-cell part of adaptive immunity, IL-6 is essential in forming the Th17 phenotype of helper T cells together with IL-1, IL-21, IL-23, and TGF- β (whereas IFN- γ , IL-2, IL-4, IL-12, and IL-27 can block their development). The hallmark of Th17 cells in the production of IL-17A, IL-17F, and IL-22 cytokines with neutrophil (granulocyte) mobilization.

IL-6 is also required for the development of Th22 helper cells, together with TNF (whereas TGF- β blocks their development). IL-6 can be secreted from some DCs, an action with which DCs can inhibit regulatory Treg cells' activity (and thereby, by suppression of suppression, they might activate helper T cells and, hypothetically, break tolerance).

Normal cells typically do not secrete IL-6. Secretion of IL-6 can be activated with the NF- κ B, a factor that is activated in many signaling pathways, including those by TNF and TLRs. Stimulation by bacterial LPS (that binds to TLR4) causes the secretion of IL-6 from monocytes and fibroblasts. Activated macrophages (with IFN- γ) also secrete IL-6.

Neutrophils that have migrated first to inflammatory sites do not release the receptor chain's membrane form (sIL-6R α) as previously thought. Instead, soluble sIL-6R α comes from the circulation (Schumacher et al., 2016). And this, in turn, activates the migration of pro-inflammatory cells and secretion of other mediators. Thus, soluble IL-6R α controls the IL-6 action (Fig. 5).

The trans-presentation or cluster signaling can happen across the thin divide between cells in close proximity when the IL-6R α anchored in the membrane of one cell binds to IL-6, and then together they attach to the gp130 fastened in the membrane of another cell.

Medical aspects

Increased serum concentrations of IL-6 and sIL-6R α were found in RA and juvenile forms of RA, which correlate with faster disease progression as well as the degree of joint destruction in RA. In *systemic juvenile RA*, sIL-6R was also significantly increased. Furthermore, osteoclasts produce large amounts of IL-6 in *Paget's disease*.

IL-6 is thought to play a role, along with other cytokines and factors, in the pathogenesis of *postmenopausal osteoporosis*, *Castleman's disease*, *multiple myeloma*, and *pituitary adenoma*.

Recently, in COVID-19 patients, high levels of serum IL-6 in the severe stage of the disease were detected, which was suggested to be an informative prognostic marker for severity (Santa Cruz et al., 2021).

Inhibition of IL-6 signaling pathway has potential to be used as treatment for many diseases. Anti-IL-6R α mAb tocilizumab (Actemra, Roche) has shown success in the clinical use of severe COVID-19 patients and some trials with autoimmune diseases. Sarilumab is also an IL-6R α binding mAb with inhibitory properties (Kevzara, Sanofi and Regeneron) that might be also used for the treatment of COVID-19 patients in severe cases. Furthermore, Sarilumab was approved as monotherapy (and in combination with methotrexate or other disease modifying drugs) for moderately to severely active *rheumatoid arthritis* in people who have not responded to, or did not tolerate, more conventional treatments (Burmester et al., 2017).

Cytokines with antiviral properties—Interferons

Interferons are proteins produced by many cell types in response to viral infections that can affect the uninfected neighboring cells thus, being an alarming signal for adaptive immunity and a mechanism for clearing the infection by preventing replication of particular viruses. Upon discovery over 60 years ago (Isaacs and Lindenmann, 1957), they were divided according to their properties like pH stability and antiviral potency (Wheelock, 1965; Stewart, 1980). Type I interferons are pH insensitive like IFN- α and IFN- β . However, pH-sensitive and 1000-fold less potent was type II interferon, so far with a single member—IFN- γ . In 2003, type III interferons were identified by genome analysis and named IFN- λ and they resemble type I in the potency of antiviral activity (Kotenko et al., 2003; Sheppard et al., 2003).

Type I interferons

Type I interferons are early molecular effectors capable of inducing an antiviral state in cells (Pestka et al., 1987). Originally classified as leukocyte (α) and fibroblast (β) IFN, novel advances led to their renaming. Although it was realized that almost all nucleated cells could produce IFN- α (Levy, 2002), its predominant source are plasmacytoid dendritic cells (pDCs).

Type I interferons comprise a large group of very similar proteins. In humans, the group includes 12 IFN- α , IFN- β , IFN- ϵ , IFN- κ , and IFN- ω . All members can bind to the single heterodimeric receptor IFN- α / β -R (encoded by IFNAR1/IFNAR2 genes) to exert their action, which is also ubiquitously expressed (Walter, 2020).

Genes encoding IFN- α / β members are located on human chromosome 9p22. There are over 20 known IFN alpha and the like genes, but only a single IFN- β gene—IFNB1.

The transcription of type I IFNs is stimulated by nuclear factor IRF7, a consequence of TLR3 signaling, which detects double-stranded RNA.

IFN- α R1/IFN- α R2 receptor signals via *Janus kinases* Jak1 and Tyk2 and these can, in turn, activate other factors like STAT-1, STAT-2, and IRF9 family of nuclear factors (Fig. 6). STAT-1 and -2, together with IRF9, constitute ISGF3 factor that can act on ISRE (interferon-stimulated response elements) in the genome and upregulate the expression of several hundred interferon-stimulated genes (Walter, 2020). The products of interferon-stimulated genes (ISGs) provide the biologic role of type I IFN.

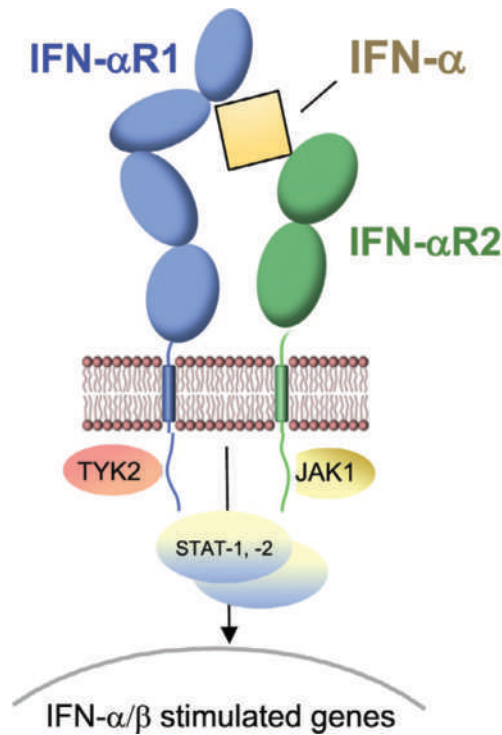


Fig. 6 Signal transduction of type I interferons. IFN- α signals via IFN- α R1 (blue) and IFN- α R2 (green) receptor inducing *Janus kinases* Jak1 and Tyk2, and in turn, STAT family of nuclear factors.

The role of type I interferons is antiviral and immunomodulatory. The type I interferons have potent antiviral activities. IFN-type I stimulate both innate and adaptive immune responses, a characteristic which is shared with type III IFN, despite having a different receptor (Hoffmann et al., 2015). Type I IFNs can positively influence both cellular and humoral adaptive immunity.

Type I IFNs are produced rapidly following the infection with a virus. The anti-viral effects necessitate interaction with the receptor with consecutive induction anti-viral gene products such as ISG15, Mx proteins, OAS-ribonuclease and PKR enzyme. The ISG15 (IFN-stimulated protein of 15 kDa) has a role in the ubiquitin-like pathway. The Mx (myxovirus resistance) proteins have a role in surveying vesicle trafficking and preventing viral exocytosis. The 2',5'-oligoadenylate synthetase (OAS)-regulated ribonuclease L (RNase L) has a role in degrading single-stranded RNA in virus-infected cells. And, protein kinase R (PKR) has a role in inhibiting the translation of viral proteins (Sadler and Williams, 2008).

Type I IFNs enhance adaptive immune responses to viruses in numerous ways. In antigen-presenting cells (like DCs), they induce MHC class II molecules that would carry viral peptides and present them to cytotoxic CD8 T cells during priming. They also upregulate the co-stimulatory molecules (CD80/86) and initiate IL-15 synthesis in DCs (Fig. 9). Subsequently, the activation of DCs induces NK cells and T-cell proliferation and differentiation, with enhanced IFN- γ secretion (Tough et al., 1996; Sun et al., 1998; Kolumam et al., 2005; Surh and Sprent, 2008). Furthermore, type I IFN can directly promote expansion and differentiation of CD8⁺ T cells (Sun et al., 1998; Kolumam et al., 2005) and CD4⁺ T cells, like follicular helper (T_{fh}) cells (Loetsch et al., 2017). The T_{fh} cells help B cells in germinal centers within secondary lymphoid organs to differentiate into plasma cells and secrete specific antibodies that can neutralize the virus. The timing of type I IFN production seems to be critical for its successful influence on adaptive immunity.

Medical aspects

IFNs are used therapeutically, with the most significant example being the treatment of *hepatitis C virus* (HCV) infection, and they are also used against various other disorders, including numerous malignancies and *multiple sclerosis* (Borden et al., 2007). For example, IFN- α is used in therapy against *hairy cell leukemia*, *malignant melanoma*, and AIDS-related *Kaposi's sarcoma* under IFN Alfacon-1 (Advaferon, Infergen) as listed in DrugBank (Wishart et al., 2018, Drugbank, 2021).

IFN- α/β are essential for human anti-HSV-1 immunity in the CNS, as immunodeficiency of type I receptor (hereditary IFNAR1-null mutation) can lead to *encephalitis* caused by *herpes simplex virus-1* (Zhang et al., 2008; Bastard et al., 2021a).

Additionally, autoantibodies (auto-Abs) against IFN- α/β have been detected in some other pathological circumstances. High titers of circulating auto-Abs against at least 14 of the 17 individual type I IFNs were found in some recipients of the *yellow fever*

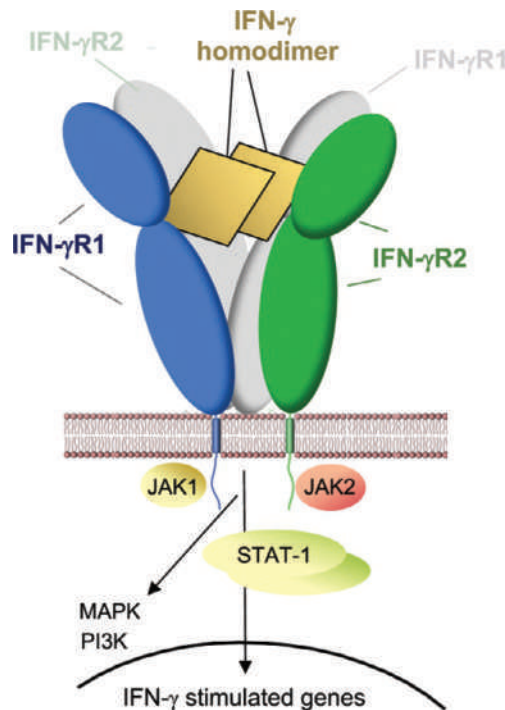


Fig. 7 The signal transduction of IFN- γ involves Jak/STAT pathways and MAPK/PI3K/Akt.

vaccine. These antibodies were recently shown to underlie at least 10% of cases of life-threatening COVID-19 pneumonia. The auto-Abs were neutralizing in vitro, blocking the protective effect of IFN- α 2 against Yellow fever vaccine strains (Bastard et al., 2021b).

Moreover, auto-Abs to type I IFN resemble a functionally hereditary lack of genes involved in type I IFN signal transduction. Such auto-Abs have been found against other cytokines, including anti-IL-6 in patients with the *staphylococcal* disease, against IFN- γ in *mycobacterial* disease, and against IL-17A/F in *mucocutaneous candidiasis* (Bastard et al., 2020). Such individuals are likely at higher risk of acquiring life-threatening not only COVID-19 but also other viral or bacterial diseases, including adverse effects of vaccinations in general.

Type II interferons

IFN- γ is the sole member of type II interferon kind. Its activity includes antiviral, but it is a 1000-fold lower than that of other types of interferon. The major role IFN- γ has is in regulating adaptive immunity.

IFN- γ is a homodimer (Yphantis and Arakawa, 1987). It binds to two different transmembrane receptor molecules in forming a heterohexamer (Fig. 7). The α -receptor chain, IFN- γ R1, is constitutively expressed on all nucleated cells and, historically, was the first cytokine receptor molecularly cloned and analyzed (Aguet et al., 1988). On the other hand, the β -receptor chain, IFN- γ R2, is expressed in a more restricted fashion and is tightly regulated. This feature of the receptor confers receptiveness to the ligand (Bach et al., 1995; Pernis et al., 1995; Tau et al., 2001) and has major consequences, especially in peripheral T helper cell development.

IFN- γ has a weak antiviral activity (Walter, 2020). One of the most important actions of the IFN- γ is activating macrophages and dendritic cells and inducing or enhancing the MHC molecules' expression to enhance the presentation of viral, bacterial, parasitic, or tumor antigens to T cells. In Fig. 8, some of the essential activities of the IFN- γ are listed. The full list of induced genes is accessible in databases like WikiPathways or NetPath.

The naïve T cells are usually localized in the immune system's periphery (secondary lymphoid tissue like lymph nodes and spleen). Upon encountering a pathogen in infected and usually damaged tissue, local dendritic cells pick up this destructed mixture of proteins, mature, and re-locate to the paracortex of draining lymph nodes via lymphatics. There they meet naïve and quiescent memory T cells of the helper kind. Then they present antigens to the T cells. In detail, when a particular T cell bind (using its T-cell receptor, TCR) with sufficient affinity to the peptide-loaded MHC class II molecules on DCs, such naïve CD4 T cell can differentiate further into one of the classes depending on the cytokine environment (and perhaps some putative additional signals) including Th1 type characterized by T-bet signature, and by producing IFN- γ (Dembic, 2015a).

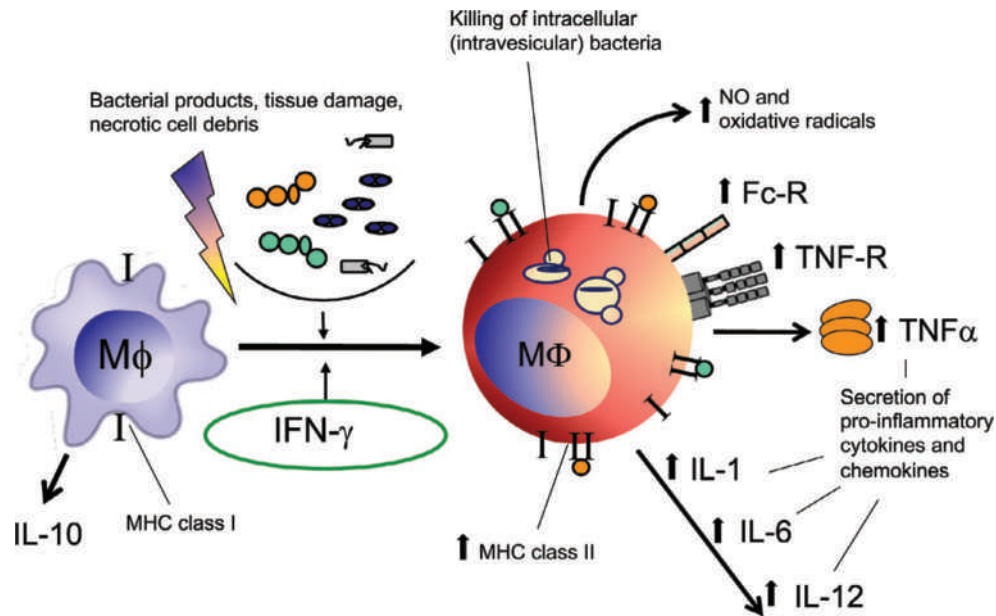


Fig. 8 IFN- γ is the macrophage activating factor and polarizing cytokine for Th1 response. Only actions on macrophages are shown. M ϕ : quiescent (not activated) macrophage; aM ϕ : activated macrophage. I & II: MHC class I and class II molecules. Adapted from Dembic Z (2015a) (Chapter 5) Cytokines of the immune system: Interferons. In: Dembic, Z. (ed.) *The Cytokines of the Immune System*. Amsterdam: Academic Press. Chapter 5 with permission.

IFN- γ plays an important role in guiding the development of Th1 response and inhibits the formation (induces apoptosis) of other types like Th2, Th9, Th17, and Tregs. The Th1 cells downregulate the IFN- γ R2 and achieve an IFN- γ resistant state (Pernis et al., 1995), which is likely the reason for their survival.

IFN- γ is also a master checkpoint regulator for many cytokines. It operates via an autocrine mechanism to elevate STAT1 and induce internalization of gp130, a common component of many cytokine receptors. This targeting of a receptor subunit common to many members of an otherwise diverse family of cytokines indicates how a master regulator can control so many diverse receptors (Zha et al., 2017).

Type III interferons

Type III IFNs play a similar role as type I IFN and are especially important at airway epithelial surfaces (Crotta et al., 2013). In addition to their antiviral activity against hepatitis C virus, they have an immunomodulatory role. Type III IFNs comprise four members: IFN- λ 1 (IL-29) and IFN- λ 2, IFN- λ 3 (IL-28A, IL-28B), and IFN- λ 4 (Prokunina-Olsson et al., 2013).

IFN- λ s are small proteins conformationally similar to type I IFNs (Mendoza et al., 2017). The heterodimeric IFN- λ R1/IL-10R β receptor, upon binding the ligand, activates Jak1 and Tyk2 kinases, leading to the initiation of IFN-mediated signaling cascades. Thus, type III IFNs signal via the same STAT-1/STAT-2/IRF9 complex (ISGF3) as type I IFNs to exert antiviral activity (Zhou et al., 2007). A review has been recently reported about the structural organization of ligand-receptor complexes and their signaling, comparing all three types IFNs (Walter, 2020).

Cytokine storm (and COVID-19)

The *cytokine storm* is a hyperinflammatory condition triggered by infection (bacterial septic shock or viral load in COVID-19) and characterized by a systemic and persistent increase in many cytokines. Here, it will be discussed mainly in relation to COVID-19.

Biology

The septic shock can be caused by superantigens, endotoxins, exotoxins, or possibly other molecules produced by an infectious agent(s), which penetrate tissues and ultimately enter a victim's circulation. The infection site (usually respiratory, urogenital, or gastrointestinal tract) gets an influx of immune cells such as monocytes, dendritic cells, macrophages, neutrophils, and T cells from the circulation (*hemophagocytic lymphohistiocytosis*).

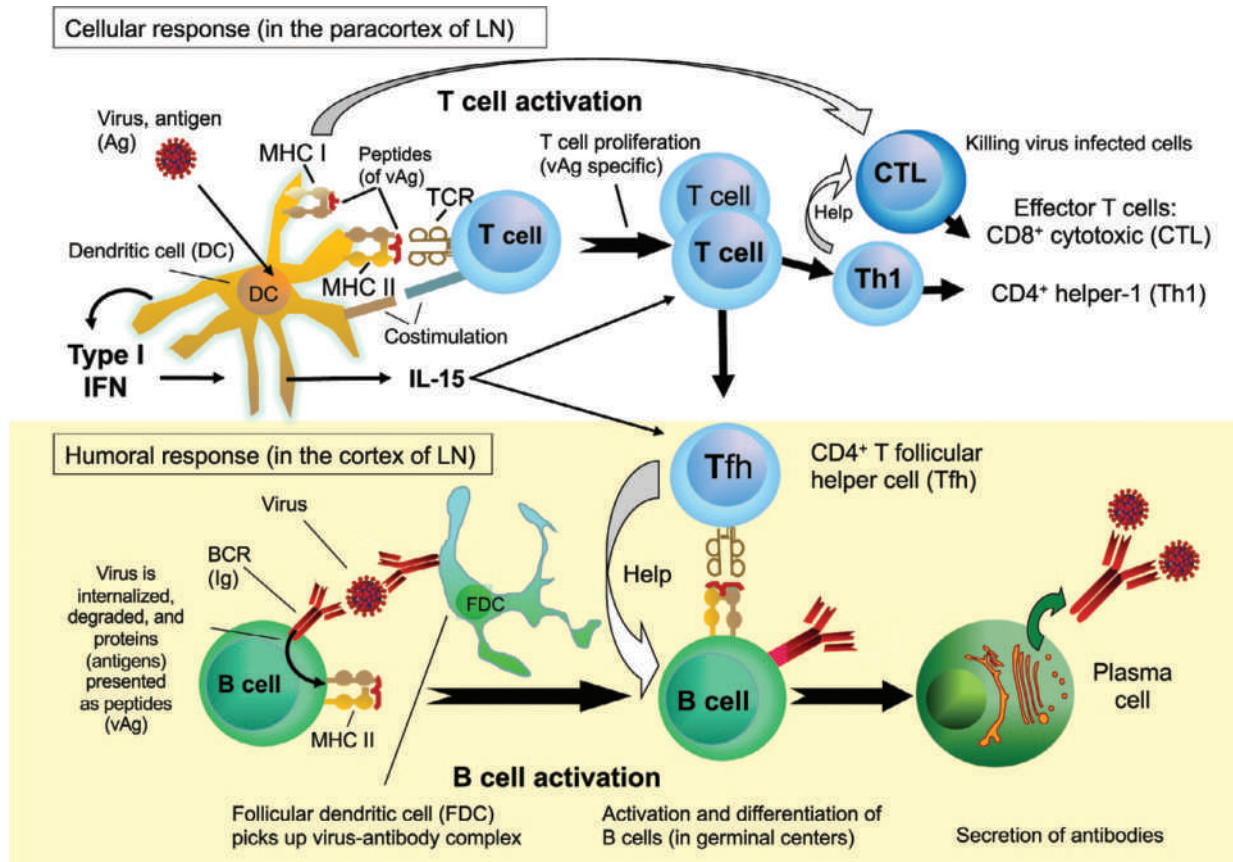


Fig. 9 Adaptive immunity responses to viral infection like SARS-CoV-2. The schematic representation of cellular and molecular events in the secondary lymphoid tissue like the cortex and paracortex of lymph nodes (LN). BCR, B cell receptor; TCR, T cell receptor; MHC, Major histocompatibility complex.

The immune dysregulation seen in septic shock is likely triggered by a particular programmed cell death called pyroptosis. Pyroptotic cell death induces an abundance of cytokines produced locally, including IL-1, IL-2, IL-6, IL-7, IL-10, interferon- γ -induced-protein-10 (IP-10), monocyte chemoattractant protein 1 (MCP-1) alias C-C motif chemokine ligand (CCL2), macrophage inflammatory protein (MIP)-1 α also known as CCL3, and TNF- α .

These events can further lead to tissue destruction by breaking endothelial cell-to-cell interactions, damage of vascular barrier, an increase of capillary permeability, neutrophil and cytotoxic T cell recruitment, production of oxygen free radicals, and leukotrienes (Vardhana and Wolchok, 2020) with consequential multiorgan failure and death (Huang et al., 2020; Bhaskar et al., 2020; Tleyjeh et al., 2021).

This scenario is typical for the *septic shock*, and it resembles the cytokine storm encountered in COVID-19. The critical part is still unclear, namely, why the immune system dysregulation happens, or was it there from the start and only uncovered by the “terrible” virus?

Clinical history of COVID-19

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a single-stranded positive-strand RNA virus that infects humans, birds, and some other vertebrates. It belongs to the Coronaviridae (CoV) family, Nidovirales order. CoVs are further divided into four categories: α , β , γ , and δ (Peck et al., 2015). COVID-19 disease is a consequence of infection with SARS-CoV-2 beta-coronavirus. The virus caused a world pandemic infecting within a year and 4 months over 150 million people with over 3 million deaths (Worldometer, 2021).

The virus probably emerged in late 2019 in Wuhan (China). The phylogenetic trees are shown on the Nextstrain site—a database maintained by the dedicated team (Nextstrain, 2019), depict evolutionary relationships of SARS-CoV-2 virus variants from the ongoing COVID-19 pandemic. There are hundreds of thousands of genome sequences of SARS-CoV-2 available. However, only around 3000 are shown on the site, pointing to the mutated virus’s major substrains (starting with the Wuhan strain as number 1). Its mutation rate seems to be around 8×10^{-4} substitutions/site/year (Nextstrain, Hadfield et al., 2018). In other words, in the genome of SARS-CoV-2 (about 30 kB) 24 mutations are expected per year.

COVID-19 is divided into three clinical stages concerning severity and prognosis (Siddiqi and Mehra, 2020; Romagnoli et al., 2020). First of all, 43% of those infected have no symptoms at all (Gudbjartsson et al., 2020a; Lavezzo et al., 2020), but can infect others because they shed the virus 15–25 days (median 19) after they got infected (Long et al., 2020). People with symptoms go through three stages of the disease. In the first stage (I), some mild symptoms usually prevail (headache, dry cough, myalgia, or sub febrility). These can occur between 2 and 14 days after infection (with a median of 6 days), probably depending on the viral load. The second stage (II) presents with dyspnea, cough, and high fever within a week or two. Clinical examination shows abnormal thoracic radiographic imaging, and blood tests show lymphopenia and raised levels of inflammatory markers, including cytokines (more details follow below). The second stage is further divided into two substages, relating to the absence (IIa) or presence (IIb) of hypoxemia. Lastly, the third stage (III) is characterized by clinical manifestations of the cytokine storm (systemic inflammatory syndrome), culminating in severe lung failure with an ominous prognosis.

About 14% of COVID-19 patients develop a severe form of the disease, while most develop only mild symptoms. Some 5% become critically ill with respiratory failure and multiorgan damage (Wiersinga et al., 2020). Those with co-morbidities have a higher risk for death in severe stages. So far, indices report 2–3% deaths in over 100 million closed cases (Worldometer, 2021). It seems that widespread damage caused by the cytokine storm is proportional to the severity of COVID-19 (Del Valle et al., 2020). The risk of death from infection is 0.3% (infection—fatality ratio) in Icelanders, and it is similar to those in the US (Gudbjartsson et al., 2020b). This is about three times higher than influenza. However, higher infection rate and more deadly outcome are feared in infection with the UK (B.1.1.7; alpha), South African (B.1.351; beta), Brazilian (P.1; gamma), or Indian (B.1.617.2; delta) strains of the SARS-CoV-2 virus.

Those that were diagnosed with COVID-19 developed neutralizing antibodies against the virus. The humoral immune response initially peaked but subsided within several weeks after clearing the virus. Then another protective wave of neutralizing antibodies takes over and probably lasts much longer, as we have observed for similar viral diseases. In fact, 91% of people who tested positive for the infection with SARS-CoV-2 had such protective antibodies 4 months after initial diagnosis (Gudbjartsson et al., 2020b).

Post-acute sequelae of COVID-19 (PASC)

In about 10% of patients with COVID-19, symptoms like fatigue, shortness of breath, “brain fog,” and chest pain do not resolve in subsequent months (Carfi et al., 2020). These after-effects are referred to as post-acute sequelae of COVID-19, or PASC. The long-lasting health effects of SARS-CoV-2 infection can lead to some severe clinical complications.

The culprit for PASC might be several: (1) First, viral persistence because 4 months after acute disease, the virus was found alive in biopsies of gastrointestinal mucosa in third of patients (Gaebler et al., 2020). (2) Then, PASC could be the long-lasting effect of the virus (similar to late consequences of Ebola, CMV or EBV). (3) The next possibility is the upsurge of autoreactive antibodies and autoimmunity that could develop alongside the protective immune response to SARS-CoV-2 and cause widespread damage (Zuo et al., 2020; Ehrenfeld et al., 2020). An example is that neutralizing autoantibodies against type I and type III interferons were found in about 10% of patients with the life-threatening disease (Bastard et al., 2021b).

The latter explanation was also suggested to be the main cause for severe COVID-19 disease. It is endorsed by the fact that persons with hereditary lack of key proteins in the type I/III interferon signaling system have been shown to have a higher risk of viral diseases and can show adverse effects after vaccinations (Bastard et al., 2020, 2021a,b), indicating that hereditary dysregulation in the innate immunity can lead to severe COVID-19 and PASC in survivors.

And, lastly, PASC is not necessarily correlated with disease severity.

Immune responses in COVID-19

Infection with SARS-CoV-2 usually starts by airborne virus invading cells of the upper respiratory tract. The virus achieves this by first binding to angiotensin-converting enzyme (ACE)2 and transmembrane serine protease (TMPRSS)2 on the respiratory epithelium (Hoffmann et al., 2020). Upon entry, SARS-CoV-2 binds to Toll-like receptors (TLR)3 and TLR7 in endosomes and in the cytosol to retinoic-acid-inducible gene I (RIG)-like receptors (RLRs), which engage with the mitochondrial antiviral signaling (MAVS) protein (Seth et al., 2005). These actions recruit nuclear factors like interferon regulatory factor (IRF)3, IRF7, AP-1 and NF- κ B to stimulate the production of type I and type III IFNs, and pro-inflammatory cytokines IL-1, IL-6 and TNF- α (Takeuchi and Akira, 2010).

IFN- α/β binds to heterodimeric IFN- α/β R (Fig. 6), which signals via Jak1/Tyk2 and STAT1/STAT2/IRF9 pathway leading to induction of interferon-stimulated genes. The latter encode enzymes like 2'-5'-oligoadenylate synthetase 1 (OAS-1), OAS-2, OAS-3 and protein kinase R (PKR), critical for innate immunity (Pico et al., 2020). The OAS enzymes activate latent RNase L, which can degrade any intracellular RNA resulting in the inhibition of viral replication.

Further, type I IFNs stimulate adaptive immune responses to SARS-CoV-2 (and others) in many ways. In antigen-presenting cells like DCs (Fig. 9), IFN- α/β enhances antigen presentation to T cells via enhancement and induction of the expression of MHC class I and class II molecules, respectively. DCs, in turn, can activate cytotoxic CD8 T cells specific for viral peptides presented on them. Prompted by the type I/III IFN, DCs upregulate the co-stimulatory molecules (CD80/86) and initiate IL-15 synthesis. In the next step, the activated DCs induce NK cell activation with enhanced IFN- γ secretion (Tough et al., 1996; Sun et al., 1998; Kolumam et al., 2005; Surh and Sprent, 2008). Furthermore, type I IFNs promote expansion and differentiation of CD8⁺ T cells (Sun et al., 1998; Kolumam et al., 2005) and CD4⁺ T cells, like follicular helper (T_{fh}) cells (Loetsch et al., 2017). The T_{fh} cells help B cells in

germinal centers within secondary lymphoid organs to differentiate into plasma cells and secrete specific antibodies that can neutralize the SARS-CoV-2 virus. The timing of type I IFN production seems to be critical for their beneficial influence on adaptive immunity (Fig. 9).

Through these initial steps in activating innate and adaptive immunity, the SARS-CoV-2 virus can be easily eliminated from the body. However, since it does not happen readily in all infected persons, and some might die without clearing the virus, we wish to understand why. The methods to realize this are observations and analyzes of clinical data and laboratory findings of affected patients, including cytokines.

It seems that the timing of the interferon response is crucial for a successful outcome. SARS-CoV-2 can induce IFN- β and type III IFNs; however, its effect *in vitro* is weaker or delayed than other coronaviruses with similar tropism (Blanco-Melo et al., 2020; Chu et al., 2020). Coronaviruses can evade innate immunity during the infection in humans, probably due to increased viral load within the first 10 days. Higher viral load is associated with widespread inflammation and increased severity of the disease (Sariol and Perlman, 2020). Furthermore, a reduced percentage and counts of circulating ILCs were found in COVID-19 patients, with a particularly interesting drop in frequencies of ILC2 cells in severe but not moderate disease, suggesting that they indicate disease severity (García et al., 2020).

In patients with moderate and severe COVID-19, very low production of IFN- β (and insufficient ISG induction) was observed (Blanco-Melo et al., 2020). However, this was contradicted by others that found the opposite (Del Valle et al., 2020; Wilk et al., 2020). Yet, the 30-kb genome of SARS-CoV-2 encodes several accessory proteins (N, ORF6, ORF8 and ORF3b) that can strongly inhibit the induction of type I IFNs, and ORF3a protein can reduce the quantity of IFN- α R on the cell surface (Li et al., 2020; Sa Ribero et al., 2020; Konno et al., 2020). Another fact is that SARS-CoV-2 is sensitive to exogenous type I IFN *in vitro* (Lokugamage et al., 2020). Given these arguments, and assuming the high mutation rate of the SARS-CoV-2 virus (10^{-8} changes/site/year Nextstrain, 2019), it is possible that differences in the above-mentioned controversial studies are related to distinctive substrains of the virus or unknown difference in the genetic background of the patients, as there were too few analyzed samples (Blanco-Melo et al., 2020; Wilk et al., 2020; Del Valle et al., 2020).

The SARS-CoV-2 elicits in most people neutralizing antibody response. As these circulate and clear the virus, the cells that get infected can only be eliminated by CD8 cytotoxic T cells (CTLs) specific for viral antigenic peptides (within the MHC class I molecules on target cells). These CTLs have a major role in the antiviral response (Fig. 9). In severe COVID-19 patients, there is a pronounced diminished frequency of CTL in the blood. Among these inadequate numbers of T cells, CTLs often have an exhausted phenotype, expressing PD-1, which is a marker of ineffective immunity. The number of T cells rises after recovery (Ruan et al., 2020; Sun et al., 2020).

However, in the fatal cases, a hypothesis has recently been upheld that the inhibition of type I (and III) IFNs by the SARS-CoV-2 virus in some individuals might lead to severe COVID-19 because of the power balance shift. Namely, the power in inducing adaptive immunity has changed for the worse. In such a scenario, DCs would produce less IL-15, and in turn, fewer T-follicular helper (Tfh) cells would be generated (to help B cells and hence fewer neutralizing antibodies), as well as reduced numbers of ineffective CTLs (with PD-1 phenotype) would not eliminate virus-infected cells (Fig. 9). The result would be the stage with continued, persistent, and ominous production of pro-inflammatory cytokines such as IL-1, IL-6, and TNF- α from infected monocytes and macrophages—or the *cytokine storm* (King and Sprent, 2021).

Cytokines in COVID-19 patients

Detailed analyzes of numerous cytokines in COVID-19 patients revealed higher plasma levels than healthy adults of IL-1 β , IL-1Ra, IL-7, IL-8 (CXCL8), IL-9, IL-10, Fibroblast growth factor (FGF), Granulocyte-colony stimulating factor (G-CSF), Granulocyte-Macrophage-CSF (GM-CSF), Interferon- γ (IFN- γ), CXCL10, CCL2, CCL3, MIP-1 β (CCL4), Platelet-derived growth factor (PDGF), TNF- α , and Vascular endothelial growth factor (VEGF). Some cytokines like IL-5, IL-12p70, IL-15, Eotaxin (CCL11), and RANTES (CCL5) were not elevated (Huang et al., 2020). Interestingly, severe COVID-19 patients (in the intensive care unit) showed even higher plasma concentrations for IL-2, IL-7, IL-10, G-CSF, CXCL10, CCL2, CCL3 TNF- α than the non-intensive care unit patients (Huang et al., 2020; Bhaskar et al., 2020).

Others identified, besides the IL-1 β , IFN- γ , MIG (CXCL9), CXCL8, CXCL10, CCL2, and TNF- α , also IL-6, IL-12, and TGF- β in the early phase of the disease. In the late stage of COVID-19 disease, IL-2, IL-10 and TGF- β were elevated, whereas in the disease with fatal outcome, only CXCL9, CXCL8, CXCL10, and CCL2 were detected with higher levels. In the lung tissue, massive infiltration of pro-inflammatory cells, including T helper-17 (Th17) lymphocytes, have been found by *postmortem* examination (Tang et al., 2020).

The most recent report points to the correlation between increased serum IL-6 levels and respiratory failure in COVID-19 pneumonia. According to these authors, quantification of IL-6 levels during various stages of the disease could discriminate between survivors and non-survivors, which may be useful for prognosis and treatment (Santa Cruz et al., 2021). However, not all fatalities can be ascribed to the cytokine storm. High titers of circulating auto-Abs against type I IFNs were shown to be associated with at least 10% of cases of life-threatening COVID-19 pneumonia (Bastard et al., 2020).

In conclusion, IL-6, IL-8, and TNF- α are independent biomarkers regarded as essential for the prognosis of COVID-19 severity (Del Valle et al., 2020), thus shaping potential treatment methods (Fig. 10).

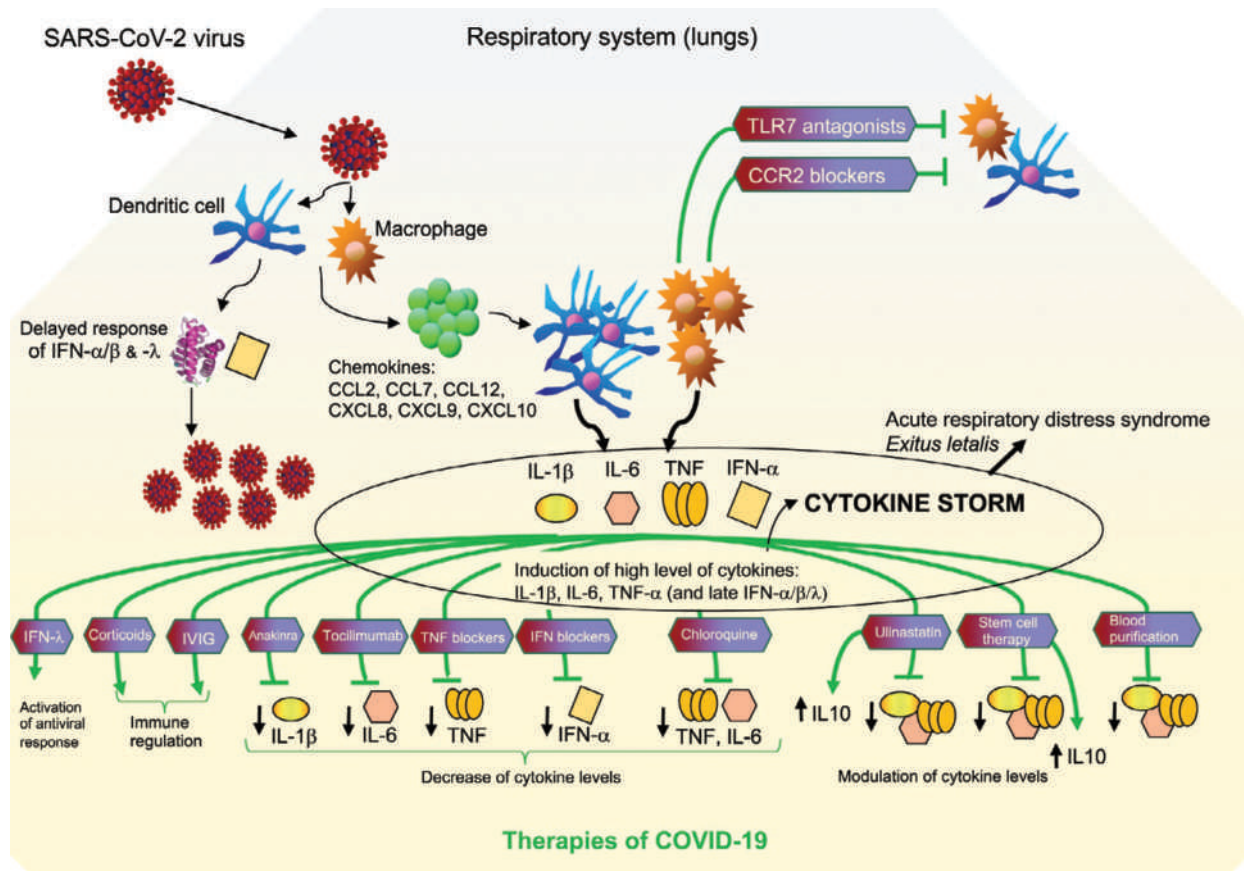


Fig. 10 Cytokine storm in COVID-19 and potential treatments. Adapted from Ye Q, Wang B and Mao J (2020) The pathogenesis and treatment of the 'Cytokine Storm' in COVID-19. *Journal of Infection* 80: 607–613, with permission.

Genetic risk

Risk factors for life-threatening COVID-19 pneumonia are gender (male), age (elderly), and having other medical conditions (comorbidity) (Bastard et al., 2020).

Regarding prognosis, it is noteworthy that genetic (hereditary) polymorphisms in some cytokine and cytokine receptor genes can modulate variations in infection- or vaccine-induced cytokine responses (Morel and Turner, 2010). Some could lead to a hyperreactive state like cytokine storm, but the exact causative relationship remains unclear.

Recent research showed several genetic risk factors in causing severe COVID-19 disease. The first is the type I interferon receptor gene *IFNAR2* coding for the IFN- α/β receptor second chain (Fig. 6). Other predisposing genetic factors for COVID-19 include genes that code for antiviral restriction enzyme activators (*OAS1*, *OAS2*, and *OAS3*), *TYK2*, encoding a tyrosine kinase (implicated in signal transduction of several cytokines), and the dipeptidyl peptidase gene *DPP9*. Further analysis revealed a causal link between severe COVID-19 disease, low expression of *IFNAR2*, and high expression of *TYK2*. Lastly, a transcriptome-wide association study showed the monocyte/macrophage chemotactic receptor *CCR2* associated with severe COVID-19, too (Pairo-Castineira et al., 2020). Perhaps, people with such defects should be singled out and taken care of in preventing infection, not only with the SARS-CoV-2, as they might not be eligible for vaccinations.

Therapies

Treatment for COVID-19 disease (illustrated in Fig. 10) involves specific therapies like antagonizing biologics, including receptor antagonists, monoclonal antibodies (mAbs) directed to cytokine-receptor interaction (anti-IL-6, anti-IL-1, anti-GM-CSF, and anti-TNF- α) to prevent its action, and small molecules that could inhibit signal transduction. Non-specific treatments include hormones like corticosteroids that counteract cytokines' actions and re-purposed drugs previously used in treating other diseases (reviewed in (Badary, 2021; Ye et al., 2020).

mAbs used, so far, in the treatment of COVID-19 are anti-IL-6-receptor, tocilizumab (Actemra, Roche) and Sarilumab (Kevzara, Sanofi & Regeneron), and anti-IL-6 mAbs like olokizumab (R-Pharm) and siltuximab (Sylvant, EUSA Pharma and BeiGene)

(Badary, 2021). Further, mavrilimumab (anti-GM-CSF) and (PD)-1/PD-L1 checkpoint inhibition ones (pembrolizumab, nivolumab) are also considered. So far, anti-IL-6 therapies yielded mixed results, but treatment with anti-IL-6R α *tocilizumab* may reduce the risk of invasive mechanical ventilation or death in patients with severe COVID-19 pneumonia in randomized controlled trials (Kewan et al., 2020; Tleyjeh et al., 2021). Moreover, bevacizumab (a VEGF-inhibiting mAb) also showed promise recently in treating severe COVID-19 patients (Pang et al., 2021).

Regarding anti-IL-6R α therapy with *tocilizumab*, some argue that Vitamin D has advantages. Namely, *tocilizumab* non-selectively blocks both anti-inflammatory and pro-inflammatory actions of IL-6 and Vitamin D reduces IL-6 production (by immune cells), thus reducing only pro-inflammatory effects of IL-6 (Silberstein, 2021).

Other therapies used antiviral agents (remdesivir), cytokine adsorption devices, antimalarials (chloroquine, hydroxychloroquine), antibiotics (azithromycin), intravenous immunoglobulin (IVIG) from immune donors, and other small molecules (Tang et al., 2020; Badary, 2021).

Antiviral agents like remdesivir have shown success in treating COVID-19 (Buckland et al., 2020).

It seems that antimalarials were effective against SARS-CoV-2 because they inhibit the production and release of IL-6 and TNF, potentially preventing the cytokine storm (Yao et al., 2020). They can also work in synergy with azithromycin (Gautret et al., 2020). Still, the US Food and Drug Administration (FDA) revoked its approval due to adverse effects, showing that the drug's potential benefits may not outweigh its known and potential risks (FDA, 2020), and azithromycin could have an additive effect on the adverse effects of hydroxychloroquine.

Dexamethasone was also shown to reduce COVID-19-associated mortality (Recovery, 2020), as corticosteroids can reduce cytokines' uncontrolled expression during the COVID-19's cytokine storm.

Anakinra is a biologic medicine (IL-1 receptor antagonist, IL-1Ra) that inhibits the IL-1 α and IL-1 β . It has been used to treat *rheumatoid arthritis* in combination with methotrexate. Anakinra has been used with some success in several small studies in patients with COVID-19 (Cavalli et al., 2020). An overall strategy in COVID-19 therapies regarding the cytokine storm is depicted in Fig. 10.

It is not known what are the effects of these remedies on PASC patients.

Prevention

Over 200 vaccine candidates are underway to prevent the development of COVID-19 disease (World Health Organization, Coronavirus disease web site, Nature briefings). Some countries have already approved vaccines that use SARS-CoV-2 mRNA encoding spike protein (that binds the angiotensin receptor helping the virus penetrate and infect human cells) with 90–95% protection (Pfizer/BioNTech, International, and Moderna, United States). Similar technology (mRNA encapsulated in lipid nanovesicles) was also used to produce CureVac (Belgium and Germany) vaccine, underway in phase 3 trials.

Other vaccines use non-replicating types of adenovirus as vectors carrying SARS-CoV-2 spike antigens encoded in its DNA. For example, the *Covishield*, produced by Oxford University and Astra-Zeneca (UK, US, Brazil, and South Africa), is a chimpanzee adenovirus (ChAdOx5) based vaccine. It has lower protection (around 70% in a single dose) against severe disease, but if applied in two dosages separated more than 3 weeks apart, the protection could increase to comparable levels. It can protect against B.1.1.7 type of SARS-CoV-2 (the UK variant), but not against South African B.1.351 type (Nature briefings, update 25, March 2021). Novel formulations are under development that would be able to do so in the future. Oxford-AstraZeneca vaccine might be preferable for use because it is cheap and can be stored at 2–8 °C (while the other, mentioned so far, need deep-freezer conditions).

Some vaccines use human adenovirus types Ad5 and Ad26; for example, Johnson & Johnson (International) vaccine uses Ad26. It is approved for a single-dose use. The vaccine was 66% effective at preventing moderate and severe COVID-19 at 28 days after vaccination including all countries and variants, but 85% effective at preventing severe or critical disease. When split by country, the vaccine was 72% effective in the U.S., 66% effective in Latin American countries and 57% effective in South Africa at preventing moderate to severe disease at 28 days (Nature briefings).

On the other hand, CanSino Biologics and the Academy of Military Medical Sciences (China, Canada, Russia and Saudi Arabia) uses Ad5 in a single-dose regimen with 90% efficacy in preventing severe disease. Interestingly, Gamaleya Institute, Health Ministry, and "Acellena Contract Drug Research and Development" (Russia) uses Ad5 in the first injection, followed by Ad26 type in the second dose of the vaccine known as *Gam-COVID-Vac*, or *Sputnik V* and reported over 90% prevention of severe disease.

Vaccines like *Valneva* (France and Austria) and several Chinese-based producers, including *SinoVac Biotech* (China, Brazil, Bangladesh, and Indonesia), Wuhan Institute of Biologic Products with Sinopharm (China and UAE), and Beijing Institute of Biologic Products with Sinopharm (China) use previously established protocols for vaccinations with inactivated SARS-CoV-2 virus. Valneva's vaccine *VLA2001*, currently in phase 3 in Europe, consists of inactivated whole virus particles combined with two adjuvants, alum and CpG1018. Preclinical experiments with this adjuvant combination showed higher antibody levels than single adjuvant formulations, including the Th1 type immune response development. The novel CpG1018 adjuvant has previously been used to prepare the recently FDA-approved hepatitis B vaccine (HEPLISAV-B).

Novavax, a biotechnology company supported by Glaxo-Smith-Kline (GSK) and the UK government, produced a COVID-19 vaccine (Australia and South Africa), that contain nanoparticles carrying antigens derived from the SARS-CoV-2 spike protein (with Matrix-M adjuvant). It demonstrated around 90% efficacy against most types of the virus (but only 50% against B.1.351 South-African type) according to Phase 3 trial in the UK in the beginning of 2021 (DDW, 2021).

In addition, Shenzhen Geno-Immune Medical Institute (China) is testing a novel approach using immune cells (human dendritic cells and T cells, or artificial antigen-presenting cells) that are engineered to express a synthetic minigene based on

SARS-CoV-2 proteins and injected or infused into the patient. In detail, the dendritic cells previously taken out from the patient's blood are incubated in cell culture in order to introduce SARS-CoV-2 antigens into them (using a lentiviral vector) and then returned to the patient. The aim is to stimulate the correct T cell helper response (likely Th1) that would be beneficial in preventing COVID-19.

The immune response and inflammation caused by SARS-CoV-2 virus infection, as well as the variety of COVID-19 clinical consequences, were recently extensively reviewed (García, 2020).

The side effects are usually mild, start during the first days after vaccination, and stop within the following few days. Majority reports low pain sensation at the site of injection. Other usual side effects include weakness, headache, muscle pain, freezing feelings, joint pain and fever. They are more frequent after the second dose. Rare side effects that are serious include thrombosis - blood-clotting ($1:10^5$ – $1:10^6$) which can lead to stroke or heart attack. The possible reason for these events is a rare occasion when DNA from the vaccine forms with proteins like tissue factor (a coagulation factor) an immunogenic complex (in some persons for unknown reasons). The latter can provoke the generation of auto-antibodies against this factor. When tissue factor binds to platelets, autoantibodies can start coagulation by activating them. Thus, increased blood clotting tendency can occur in some individuals, but the reason for making such auto-antibodies is still unclear. Furthermore, if *disseminated intravascular coagulation* develops (like in the septic shock), then the complete consumption of coagulation factors and depletion of fibrinogen in the blood would lead to overall bleeding. Recent surveys show that vector-based vaccines have reportedly 20–30 times higher bleeding incidents than mRNA-based ones. The reasons for bleeding incidents might be due to aptamer formation between vector DNA and some of the coagulation factor cascade proteins or their regulatory ones in some individuals thereby preventing (or prolonging) hemostasis.

Concluding remarks

We cannot predict how long vaccine protection would last; we can only infer it from the observation. Similarly, we cannot predict how would the immune system combat cancer. We observed that it could win in some 20% (to at best 40%) of treated cancer patients, and the success rate is correlated with the type of cancer (Dembic, 2020). Furthermore, we cannot predict with certainty how successful our treatments will be in stopping autoimmune diseases or curing allergies. We do not have a treatment to tolerate transplanted organs without immune-depressants, yet, the nature has provided a (perhaps complex, but finite) solution each time a child is being carried by its mother. The reason why we cannot predict and find correct treatments is simple—we still lack knowledge to form a unifying theory on immunity.

We have quite a few models starting with the Self-nonsel self discrimination (Bretscher and Cohn, 1970; Cohn, 2006; Cohn et al., 2019), the Pathogenicity (Janeway, 1989; Medzhitov and Janeway, 2000), the Danger (Matzinger, 1994, 2007), the Co-stimulation and co-inhibition (Sinclair and Anderson, 1994, 1996), the Ignorance (Zinkernagel, 1996), and the Integrity (Dembic, 1996, 2000, 2015b, 2019) model, but we could not use them so far for predictive and preventive purposes, or at least not reproducibly in most cases. Perhaps, inventing more models about the inner workings of the immune system would help like the most recent example in which the immune system employs quorum sensing to act and regulate its responses (Butler et al., 2013; Bosch et al., 2017; Al-Yassin and Bretscher, 2018; Postat and Bousso, 2019; Schrom et al., 2020). A quote from the famous British statistician, George E.P. Box, says “Essentially, all models are wrong, but some are useful.” Then, perhaps, we only need more time for the useful concept to emerge.

Maybe this pandemic helped make the awareness about what we can do and what we cannot: however, if we invest in science for our collective health, the goal would be reachable perhaps in our lifetimes. The success would save humanity from another, perhaps even more deadly, pandemic in the future (we know from the history of plague that such scenario is possible). Should we invest in deciphering the immunity with more vigor? I strongly advocate this pathway. Conceivably, some funds could be used to make computer-aided predictions about the immune system's working in healthy and diseased.

These past achievements would not have been possible without the groundbreaking research of the heroes in science working and giving their lives for the cause in previous decades and centuries. And although, superficially, biomedical science looks like a never-ending story—I believe in the happy ending—we only have about 20 thousand genes, and that is not an *infinite* but *useful* number (to start with computer-aided predictions).

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Chemokines and Chemokine Receptors

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Chemokines

Chemokines are a group of small, secreted molecules known for stimulating directed communication and migration of various cells via signaling through G-protein coupled receptors (Bachelier et al., 2013). In humans, the genes encoding chemokines are found on chromosomes 4 and 17 (Zlotnik et al., 2006; Nomiya et al., 2010). In their primary amino acid sequence, the arrangement of important cysteine residues connected with disulfide bonds forms the conserved chemokine fold and constitutes the basis for the nomenclature and classification of chemokines (Miller and Mayo, 2017; Hughes and Nibbs, 2018). The systematic nomenclature introduced in 2000 is based on the structure of the two cysteines closest to the N terminus which can be directly juxtaposed (CC), separated by a single amino acid (CXC) or by three amino acids (CX₃C) (Murphy et al., 2000). Certain chemokines lack the first cysteine residue and are known as XC chemokines (Kennedy et al., 1995). Structurally, a chemokine monomer consists of a central three stranded β -sheet, an overlying C-terminal α -helix, and a short unstructured N terminus which plays a critical role in receptor activation (Miller and Mayo, 2017). The rigid loop, the N-terminus and the glycosaminoglycan (GAG) binding sites within the β -strands or in the C-terminus are considered important domains for chemokine functions (Rossi and Zlotnik, 2000). Chemokines are active as monomers, yet physiologically they also form homodimers and heterodimers during an immune reaction, hence eliciting a more pronounced immune response compared to a single chemokine (Paoletti et al., 2005; Kischer et al., 2009; Proudfoot and Uguccioni, 2016).

Functionally, chemokines can be classified into two main groups: the first is involved in the inflammation process while the second is involved in homeostatic cell migration. However, the biological functions of chemokines extend to many others such as controlling cell proliferation and differentiation, regulating angiogenesis, inflammatory responses, tumor growth and metastasis (Hughes and Nibbs, 2018). The various functions are dependent on the signaling pathways induced upon the binding of chemokines to their cognate receptors and are affected by several factors such as GAGs and the post-translational modifications of chemokines. GAGs play an important role in chemokine-chemokine and chemokine-receptor interactions (Salanga and Handel, 2011). Moreover, GAGs influence the immobilization of chemokines from the cell surface and extracellular matrix (ECM) which in turn affects the activity and distribution of chemokines post secretion (Salanga and Handel, 2011; Monneau et al., 2016). On the other hand, chemokines can undergo several post-translational modifications that impact their functions including citrullination (Proost et al., 2008), nitration/nitrosylation (Barker et al., 2017), cleavage by matrix metalloproteinases (MMPs), cathepsins, thrombin, plasmin, elastase, and the dipeptidyl peptidase CD26 (Van Lint and Libert, 2007; Metzemaekers et al., 2016). Moreover, the binding of chemokines to the ECM is affected by the proteolytic cleavage which aids several chemokines such as CCL21 (Schumann et al., 2010), CX₃CL1 (Hundhausen et al., 2003) and CXCL16 to detach from the ECM (Gough et al., 2004) to be released more readily allowing efficient GAGs binding. As a result, proteases help in modifying interstitial chemokine gradients regulating half-life and distribution (Barreira da Silva et al., 2015).

Chemokine receptors

Chemokine receptors can be classified as conventional (cCKRs) or atypical. To date, there are 22 chemokine receptors named based on the predominant type of chemokine to which they bind followed by R for “receptor” and a number annotating the receptor discovery (Murphy et al., 2000; Zlotnik and Yoshie, 2000; Stone et al., 2017). Accordingly, there are currently about 10 CCRs, 6 CXCRs, a single CX₃CR and XCR as well as 5 atypical receptors (Acr1, Acr2, Acr3, Acr4 and Acr5) (Hughes and Nibbs, 2018). A specific immune cell does not express fixed cCKRs and can undergo receptor switching that is crucial for altering the function based on the immunological response induced by a particular pathogen. For instance, antigen experienced B-lymphocytes upregulate CCR7 to direct activated B cells to T cell area (Reif et al., 2002), while CXCR4 and CXCR5 direct activated B cells to germinal centers (Allen et al., 2004), and CCR6 expression defines memory B cells (Suan et al., 2017).

Conventional receptors exist as homodimers and oligomers, yet they can also heterodimerize with atypical chemokine receptors, other cCKRs, or membrane proteins (Mellado et al., 2001; Chen et al., 2004; Wilson et al., 2005; Kumar et al., 2006; Sohy et al., 2009). Moreover, cCKRs are not only activated by chemokines. β -defensins, alarmin, high mobility group box 1 protein (HMGB1) and migration inhibitory factor (MIF) are among the non-chemokine ligands that can also activate cCKRs (Schiraldi et al., 2012; Tirone et al., 2018). Conversely, chemokines like CCL18 can bind to non-GPCR such as phosphatidylinositol transfer protein (PITPMN3) in the context of tumor cell invasion (Chen et al., 2011). Importantly, chemokines do not always activate a signal upon binding to their cognate receptors, but instead may act as natural antagonists (Nibbs et al., 2000; Loetscher et al., 2001; Petkovic et al., 2004).

On the other hand, atypical chemokine receptors are structurally related to cCKRs upon binding to chemokines with high affinity. However, they do not activate the same signaling pathways induced by cCKRs due to the absence or alteration of the intracellular signaling motifs (Bachelier et al., 2013; Nibbs and Graham, 2013). Functionally, atypical receptors control the localization, distribution and abundance of chemokines and hence, are considered efficient scavengers compared to cCKRs (Cardona et al., 2008). Accordingly, atypical receptors play an important role in controlling chemokines/cCKRs interactions by preventing unnecessary desensitization upon exposure to excess chemokines (Fukuma et al., 2003; Lee et al., 2003; Pruenster et al., 2009).

Chemokine families

The main function of the chemokine system is cell migration, especially leukocytes. Previously, chemokines were classified based on their function, whether inflammatory or homeostatic. However, the used nomenclature is based on their structural differences in their cysteine residues. For the past 3 decades, chemokine ligands and receptors were intensely reviewed in health (Hughes and Nibbs, 2018; Murphy, 2019; Schall and Proudfoot, 2011; Schall and Bacon, 1994; Zlotnik and Yoshie, 2012; Zimmerman et al., 2020; Capucetti et al., 2020; Sokol and Luster, 2015; Gustavsson, 2020) and disease (Rolin and Maghazachi, 2014a, 2014b; Aldinucci et al., 2020; Dhaiban et al., 2020; Elemam et al., 2020; Miyabe et al., 2019; Miao et al., 2020).

The CC chemokine family is composed of 28 ligands and 10 receptors in both human and mouse species. In this family, some receptors bind to multiple ligands while others are specific to one or two ligands only. For example, CCR1 binds CCL3, CCL4, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16 and CCL23. Similarly, CCR2 binds CCL2, CCL7, CCL8, CCL11, CCL13, and CCL16, while CCR3 binds various ligands including CCL5, CCL7, CCL11, CCL13, CCL15, CCL24, CCL26, and CCL28. Another inflammatory associated chemokine receptor CCR5, is known to bind CCL3, CCL4, CCL5, CCL7, CCL8, CCL14 and CCL16. CCR8 possesses the ability to bind CCL1, CCL8 and CCL18. On the other hand, CCR4 is known to bind CCL17 and CCL22, while CCR7 binds CCL19 or CCL21, and CCR10 binds CCL27 or CCL28. However, some CC receptors bind to only one ligand, as known with CCR6 binding CCL20, and CCR9 binding CCL25.

The second group of chemokines, CXC family, is composed of 6 conventional receptors and 16 ligands in human and mouse. Similar to the CC group, this family is composed of shared receptors that bind multiple ligands including CXCR1, CXCR2, and CXCR3. CXCR1 binds CXCL6 and CXCL8, while CXCR2 binds CXCL1-3, and CXCL5-8. Interferon regulated chemokines CXCL9, CXCL10, CXCL11 as well as CXCL4 bind CXCR3. The other receptors are more specific and bind to one ligand including CXCR4 which binds CXCL12, CXCR5 which binds CXCL13, and CXCR6 which binds to CXCL16. XC chemokine family is composed of one receptor (XCR1) and 2 ligands (XCR1 and XCR2) while the CX₃C family is composed of one receptor, that is CX₃CR1 which binds CX₃CL1.

The family of atypical receptors constitutes of 5 receptors: Acr1/DARC, Acr2/D6, Acr3/CXCR7, Acr4/CCRL1 and Acr5/CCRL2. These receptors bind to CC and CXC chemokine ligands, thus scavenging them and halting their effect on conventional chemokine receptors. The conventional and atypical chemokine receptors and ligands are illustrated and summarized in Fig. 1.

Chemokine signaling

Cell migration and adhesion are among the major biological responses occurring upon the binding of chemokines to their cognate receptors. Initially, chemokines tether to the extracellular loops leading to the entry of the N terminus to the heptahelical bundle,

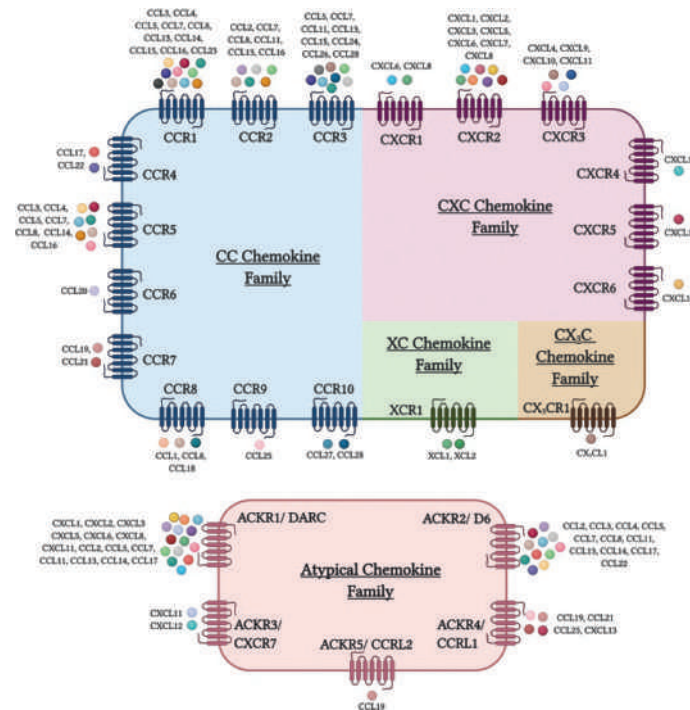


Fig. 1 Conventional and atypical chemokines and chemokine receptors families. The upper panel shows the conventional chemokine receptors and their corresponding ligands including the CC chemokine family, CXC chemokine family, XC and CX₃C chemokine families. The lower panel shows the five different atypical receptors that play a role in scavenging certain ligands leading to the inhibition of conventional chemokine signaling. Chemokines are shown in colored circles.

thus inducing a conformational change (Kufareva et al., 2015; Kleist et al., 2016) that leads to activation of intracellular signaling pathways such as G α i, β -arrestins (Kehrl, 2006), or JAK-STAT (Bachelierie et al., 2013). The complexity of chemokine signaling is attributed to several reasons. First, the alternative splicing of transcripts encoding cCKRs not only alters the signaling pathways induced by a chemokine receptor, but also affects the ligand-binding specificity (Lasagni et al., 2003). Second, chemokines can bind to multiple cCKRs with different affinities. Accordingly, a chemokine receptor may have different ligands that induce different functions (Yu et al., 2000). For instance, CXCR3 binds CXCL11 with higher affinity compared to CXCL10 which results in skewing T cells toward Th1 by the former and toward Th2 by the latter (Zohar et al., 2014). This feature of promiscuity applies more to chemokines involved in inflammation as a robust mechanism against microbial infections. On the contrary, chemokines and cCKRs responsible for homeostatic cell migration tend to be more specific having a maximum of one or two ligands. For instance, CXCL12 binds CXCR4 which is responsible for myelopoiesis, B cell development, lymphopoiesis, differentiation and homing of hematopoietic stem cells and plasma cells in the bone marrow (Bachelierie et al., 2013). Another homeostatic chemokine is CXCL13, the ligand for CXCR5, which is a key regulator of leukocyte trafficking in secondary lymphoid organs (Bachelierie et al., 2013). In addition, the cell context where chemokines bind to their cCKRs is considered an important determinant of the induced signaling pathway (Steen et al., 2014). The signaling pathways induced upon binding of chemokines to their receptors contribute to many pathological diseases.

Chemokines in innate and adaptive immunity

The role of the chemokine system in immunity is a very wide topic given the vast roles it has on various innate and adaptive immune cells at different levels. The involvement of chemokines begins as soon as the immune cells reside in primary lymphoid organs and continue to control their localization in secondary and tertiary lymphoid organs as well as their homeostatic presence in the circulation. Further, chemokines and their respective receptors control the effector functions of distinct immune cells by influencing their migration and modulating their function within peripheral tissues. Moreover, chemokines play an important role in controlling memory cell function in lymph nodes (LNs) and in the periphery. In this section, we will try to summarize the most important roles of chemokines in innate and adaptive immunity aiming to illustrate how the redundant and non-redundant functions of the chemokine/chemokine receptor system contribute to fighting infections and malignancies.

Role in the innate immunity

Effects on monocytes and macrophages

Early during acute inflammation and in response to pattern recognition receptors (PRR) activation and cytokine stimulation, activated endothelium and immune cells release different chemokines to attract monocytes. Among these chemoattractant molecules, CCL2 and CCL7 are rapidly produced. CCL2 establishes a stable gradient for the migration of CCR2⁺ inflammatory monocytes into peripheral tissues by dimerizing and binding to extracellular matrix glycosaminoglycans (Kuziel et al., 1997; Proudfoot et al., 2003). Soluble CCL2 enters the draining LNs, providing access to monocytes. It can as well be detected in the serum during infection where it induces the migration of inflammatory monocytes from the bone marrow (Palframan et al., 2001; Shi and Pamer, 2011). Other chemokine receptors expressed on monocytes have distinct roles in adhesion and migration. These include CXCR2, CCR1, CCR5, CCR6 and CX3CR1 (Weber et al., 2001). The promiscuity of chemokine receptors allows CXCR2 to bind CXCL8 which induces a firm adhesion of monocytes to the vascular endothelium, while the binding of CXCR2 to the atypical ligand macrophage migration inhibitory factor (MIF) induces integrin-dependent chemotaxis of monocytes (Gerszten et al., 1999; Bernhagen et al., 2007). Although the role of CCL20/CCR6 axis in inducing monocyte migration in vitro is not well defined, experiments showed that CCR6 is necessary for inflammatory monocytes migration inside cutaneous tissues post immunization (Le Borgne et al., 2006; Shi and Pamer, 2011). Regarding the fractalkine receptor CX₃CR1, it is mainly expressed on anti-inflammatory or patrolling monocytes although it is also present but to a lesser degree on proinflammatory monocytes (Griffith et al., 2014). CX₃CR1 expressed on anti-inflammatory monocytes, promotes integrin-mediated adhesion within the vessel and provides pro-survival signals (Auffray et al., 2007; Landsman et al., 2009).

Effect on Dendritic Cells (DCs)

Dendritic cells are important initiators of the adaptive immune response. The release of CCL3, CCL4 and CCL20 during early pathogen encounter brings immature DCs into immediate contact with pathogen-associated molecular patterns (PAMPs) which bind to the various toll-like receptors (TLRs) expressed on DCs and allow their maturation (Banchereau and Steinman, 1998; Michelsen et al., 2001; Esche et al., 2005). CCR1, CCR2, CCR5, CCR6 and CXCR1 allow the recruitment of immature DCs to inflamed tissues while the expression of these receptors is reduced post activation (Caux et al., 2000; Sozzani et al., 2000). Mature DCs express CCR7 and follow a stable gradient of CCL21 and CCL19 (Dieu et al., 1998; Sozzani et al., 1998). CCL21 is produced by the lymphatic endothelium and released into the periphery where it binds GAGs (Sallusto et al., 1998; Weber et al., 2013). This mediates the entry of peripheral mature DCs to the draining LN (Tal et al., 2011). Furthermore, upon the entry of chemokine-rich interstitial fluid to the high endothelial venules (HEV) lumen, inflammatory monocytes are attracted via CCR2 and differentiate to monocytes-derived DCs which in turn play a crucial role in Th1 differentiation (Palframan et al., 2001; Shi and Pamer, 2011). The switch in chemokine receptor is essential for DCs migration into the lymphatics specifically to the T cell-rich regions of the LN where they act as professional antigen presenting cells and induce adaptive immune response.

Effects on neutrophils

Together with macrophages, neutrophils are among the earliest cells recruited to the sites of inflammation by chemokines such as CXCL8, CXCL5 and CXCL1, given these cells express CXCR1 and CXCR2 (Rot and von Andrian, 2004). Other chemokines attract neutrophils in an organ specific manner such as CCL3 and CCL4 which bind CCR1 (Lee et al., 2000). In addition to promoting directional motility and effector function, chemokines are vital during an infection for the persistent release of neutrophils from the bone marrow given they have a short life span of 6 h in the periphery. During infections, systemic increase of granulocyte-colony stimulating factor (G-CSF) induces the production of neutrophils and their release from the bone marrow by decreasing the production of CXCL12, increasing the release of CXCL2 and reducing CXCR4 expression on neutrophils (Kawakami et al., 1990; Semerad et al., 2002; Kim et al., 2006; Eash et al., 2010). The role of chemokines does not end at this stage and is further needed for neutrophils to migrate and gain access to areas of acute inflammation via rolling along the luminal surface of the endothelium where they bind CXCL1, CXCL2 or CXCL8. It is worth mentioning that the Duffy antigen receptor for chemokines (DARC/ACKR1) sequesters bound chemokine on the luminal surface of the endothelium and this immobilization of chemokines is important for binding immune cells, promoting their transmigration and regulating their entry in order to avoid undesired excessive inflammation (Proudfoot et al., 2003; Hillyer and Male, 2005; Pruenster et al., 2009). Other neutrophil chemo-attractants produced by macrophages and endothelial cells such as CCL3, CCL4, CCL5, CXCL1 and CXCL2 bind their respective receptors on neutrophils to enhance their entry into infected tissues, amplifying the immune response (Chou et al., 2010). Importantly, despite the redundancy in the action of chemokines in promoting and amplifying immune cell migration, different forms of the same chemokine can still exhibit distinct roles probably due to the differential binding capacity to a specific receptor (Griffith et al., 2014). For instance, monomeric CXCL8 dimers bind CXCR2 and induce initial neutrophil recruitment whereas the binding of the monomeric form to the same receptor is rather responsible for sustaining the recruitment (Das et al., 2010). Following their entry into the infection sites, neutrophils execute their phagocytic function, trapping pathogens in extruded DNA, and forming neutrophil extracellular traps (Griffith et al., 2014).

However, in order to ensure additional inflammatory cell influx, neutrophils continue to produce various inflammatory mediators based on the type of stimulus or immunological context. For instance, G-CSF induces the release of CXCL5 (Suzuki et al., 2002), and CXCL2 (Nguyen-Jackson et al., 2012). The Wntless-related integration (Wnt) signaling pathways trigger the release of CXCL8 and CCL2 (Jung et al., 2013) and organic dust triggers the release of CXCL8 and CCL3 (Blidberg et al., 2012).

Other chemokines released by neutrophils include CCL4, CCL5, CCL19, CCL20, CXCL1, CXCL9, CXCL10 and CXCL11 (Gasperini et al., 1999; Bennouna et al., 2003). These chemokines recruit additional neutrophils (CXCL1 and CXCL8), or other innate immune cells such as monocytes (CCL3 and CCL4), macrophages, DCs (CCL19, CCL20) and natural killer (NK) cells. Moreover, different subsets of T cells are also influenced by neutrophil chemokines. For instance, CXCL9 and CXCL10 harvested from IFN- γ plus lipopolysaccharide (LPS)- or TNF- α -treated neutrophils promoted the migration and the integrin-dependent adhesion of CXCR3-expressing Th1 cells (Gasperini et al., 1999). On the other hand, the release of CCL2 and CCL20 chemoattract Th17 cells, which plays important roles in adaptive immunity, as well as amplifies innate immunity by releasing CXCL8, CXCL1 and G-CSF to recruit additional neutrophils to the site of infection (Pelletier et al., 2010; Annunziato et al., 2015). This illustrates a clear and an important role of the chemokine system in linking the adaptive and innate arms of the immune system (Tecchio and Cassatella, 2016).

Natural killer cells

Two subsets of NK cells are found in the blood circulation characterized by the density of CD56 and CD16 markers on their surfaces. These are designated as CD56^{dim}CD16^{bright} and CD56^{bright}CD16^{dim} (Maghazachi, 2005). Earlier studies demonstrated that CXCL8 induced the in vitro chemokinesis of IL-2-activated NK cells (Sebok et al., 1993), whereas CCL2, CCL3, CCL4 and CCL5 induced the chemotaxis of these cells (Maghazachi et al., 1994). Other chemokines such as CXCL10 (Maghazachi et al., 1997; Taub et al., 1995), CXCL12 (Maghazachi, 1997), CCL1 (Inngjerdingen et al., 2000, 2001), CCL7, CCL8 (Allavena et al., 1994; Loetscher et al., 1996; Taub et al., 1995), CCL17 (Inngjerdingen et al., 2000), CCL19 (Al-Aoukaty et al., 1998; Kim et al., 1999), CCL20 (Al-Aoukaty et al., 1998), CCL21 (Kim et al., 1999), CCL22 (Godiska et al., 1997; Inngjerdingen et al., 2000), XCL1 (Bianchi et al., 1996; Hedrick et al., 1997; Maghazachi et al., 1997), and CX₃CL1 (Al-Aoukaty et al., 1998; Fraticelli et al., 2001; Imai et al., 1997), also promoted the in vitro chemotaxis of human NK cells. Inngjerdingen et al. (2001) reported the expression of messenger RNA for XCL1, CCR3, CCR4, and CCR5 in three different NK cell subsets. Those authors described in details the expression of chemokine receptors in these NK cell subsets. Berahovich et al. (2006) also demonstrated the expression of chemokine receptors and reported that human NK cells depending whether they were CD56^{bright} or CD56^{dim} variably express CXCR1, CXCR3, and CXCR4, and contain subsets expressing CCR1, CCR4, CCR5, CCR6, CCR7, CCR9, CXCR5, and CXCR6.

In addition, chemokines play positive roles in NK cell tissue localization (reviewed in Maghazachi, 2010). The main stimuli that regulate the expression of chemokine receptors which are involved in NK cell homing to normal or inflamed tissues, including tumors or organs such as the lungs and liver have been previously reviewed (Castriconi et al., 2018). CCL5 recruited NK cells corroborated with tumor regression, and abolished the anti-tumoral effects of immunotherapy (Bhat et al., 2020). In addition to chemotaxis, chemokines induced NK cell lysis of tumor target cells (Maghazachi et al., 1996). These observations clearly demonstrate that chemokines play vital roles in NK cell biology.

Effects on mast cells and eosinophils

Mast cells express a wide variety of PRRs and contain large granules of inflammatory molecules and hence, can immediately respond to acute inflammation. Moreover, degranulation of mast cells is considered an important source of chemokines required for neutrophil recruitment (Griffith et al., 2014). While responding to various stimuli, mast cells produce different chemokines including CCL2, CCL3, CCL4, CCL5, CCL11, CCL20, CXCL1, CXCL2, CXCL8, CXCL9, CXCL10, and CXCL11 (Marshall, 2004).

On the other hand, eosinophils respond to a wide variety of chemokines including CCL11, CCL24 and CCL26 upon binding to CCR1 and CCR3. These chemokine/chemokine receptors signaling pathways possess overlapping roles by promoting eosinophil migration to the peripheral tissues (Humbles et al., 2002; Mattes et al., 2002; Kalomenidis et al., 2005; Pope et al., 2005). In turn, when eosinophils are stimulated by thymic stromal lymphopoietin (TSLP), not only they exhibit increased adhesion by increasing their expression of ICAM-1 and CD18, but can also produce CCL2, CXCL1 and CXCL8 to further attract additional inflammatory cells (Wong et al., 2010). In this context, it is worth noting that tight regulation of chemokine effects in general is of high importance to prevent excessive and harmful inflammation. In vivo, CCL11 is cleaved and rendered inactive by a surface-associated protease, CD26 (Struyf et al., 1999).

Role in adaptive immunity

Effects on T lymphocytes

Different T cells express distinct chemokine receptors that define their functions. For instance, CCL1, CCL11, CCL17, CCL22, CCL24 and CCL26 selectively recruit Th2 lymphocytes which express CCR4, CCR8 and CCR3 (Mathew et al., 2001; Ono et al., 2003). During helminth infection and in response to B-cell derived CXCL13, Th2 and Tfh are induced upon CXCR5-mediated signaling in DCs and CD4⁺ T cells (León et al., 2012). Moreover, activated T follicular helper (Tfh) cells downregulate CCR7 and upregulate CXCR5, which allows their migration toward the follicle to instruct B cells in class switching (Schaerli et al., 2000; Kim et al., 2001b).

On the other hand, Th1 cells preferentially express CCR5, CXCR3 and CXCR6 and migrate in response to CCL3, CCL4, CCL5 or the ELR negative CXC chemokines (Luther and Cyster, 2001). Among the different chemokine receptors, CXCR3 plays a specific role in Th1 priming (Bromley et al., 2008; Groom et al., 2012). CXCL9 and CXCL10 produced in the draining LNs by mature DCs promote the migration of CD4⁺ T cells, leading to stable contacts with DCs which are necessary for optimal Th1 differentiation (Groom et al., 2012). In addition, this localization places Th1 in close proximity with IFN- γ producing cells such as CXCR3⁺ cells including NK, NKT, $\gamma\delta$ T, and innate-like CD8⁺ T lymphocytes (Bajénoff et al., 2006; Groom and Luster, 2011; Kastenmüller et al., 2012; Oghumu et al., 2013).

In response to viral infection and post antigen-specific activation, CD8⁺ T lymphocytes release large amounts of CCL3, CCL4 and CCL5 from their cytotoxic granules which recruit other inflammatory cells including phagocytes to amplify the immune response (Wagner et al., 1998). However, the optimal priming of CD8⁺ T cells requires their coordinated migration and interaction with DCs and CD4⁺ T cells which release CCR5 ligands that bind to CCR4 and CCR5 on CD8⁺ lymphocytes within the LN (Castellino et al., 2006). As developing immune memory is at the heart of adaptive immunity, it is important to highlight the chemokines involved in the function of central memory CD8⁺ T cells. Unlike naïve CD8⁺ T cells, the expression of CXCR3 on central memory T cells allows their preferential migration and localization within CXCL10 rich regions of the LNs where they interact with macrophages and DCs and rapidly produce IFN- γ . In response to IFN- γ , macrophages and stromal cells release CXCL9, directing other immune cells toward the sites of infection (Sung et al., 2012). In this context, CXCL9 and CXCL10 exhibit non-redundant roles in positioning central memory T cells to induce a rapid response during secondary infections.

When discussing the role of chemokines on lymphocytes, we ought to mention the role of T regulatory cells (Tregs) in regulating chemokine production in the LNs. The absence of Tregs causes an increase in the levels of chemokines and cytokines in the draining LNs which eventually retain the immune cells necessary to control viral replication instead of migrating toward infected tissues (Lund et al., 2008). Accordingly, Tregs have a critical function in controlling the levels of CCL3, CCL4 and CCL5 in order to restrict the priming of antigen-specific cells to high-avidity CD8⁺ T cells which establish protective memory (Pace et al., 2012). Moreover, B cell responses are also controlled by FoxP3⁺ Tregs which express CXCR5. This subset of T cells is controlled by the receptor programmed cell death protein-1 (PD-1) molecule which limits the numbers of Tfh and germinal center (GC) B cell numbers post immunization to reach optimal antigen-specificity (Linterman et al., 2011).

Effect on B lymphocytes

CXCR4 and CCR7 are required in attracting newly arriving B cell entry into the LNs (Okada et al., 2002). On the other hand, the expression of CXCR5 on mature B cells mediate their entry into the Peyer's patches, whereas the CXCR4/CXCL12 axis is rather important for the egress of B cells from the draining lymphatics (Schmidt et al., 2013). Post encountering antigens, B cells upregulate CCR7 while maintaining the expression of CXCR5 and this alteration of chemokine receptors guide B cells to get in contact with CD4⁺ Tfh cells (Reif et al., 2002; Okada et al., 2005; León et al., 2012). This interaction leads to a decrease in CCR7 receptor and the migration of B cells to germinal center where they undergo proliferation and hypermutation in multiple cycles via bidirectional migration between the dark and light zones of the germinal center mediated by switching the expression of CXCR4 and CXCR5 (Allen et al., 2004). Another important axis exhibiting potential role in the coordination and regulation of the humoral immune response is the CCL20/CCR6 where its disruption results in ineffective T-B cell conjugates and poor antibody quality (Lee and Körner, 2019). At present, CCR6 is the only known receptor for CCL20 (Baba et al., 1997). CCR6 is upregulated on B-cells after their activation in secondary lymphoid organs and it is also upregulated on Tfh cells and their precursors (Lee and Körner, 2019).

Role of chemokines in microbial infections

The role of the chemokines in health and disease and especially viral infections was previously reviewed in humans and mice (Melchjorsen et al., 2003; Raman et al., 2011). Additionally, serum levels of chemokines may be potential disease biomarkers as well as indicators of response to treatment. Chemokines are considered crucial in the antiviral response, as they enhance the innate and adaptive immune system to be cytolytic or to release antiviral mediators (Mahalingam et al., 2003). In order to escape this, viruses try to evade the immune system and bypass their effect by mimicking the host chemokine ligands and receptors (Fleming et al., 1999; Murphy, 2001). For example, respiratory syncytial virus (RSV) viral protein mimics the chemokine fractalkine CX₃CL1 and binds to its respective receptor CX₃CR1 (Tripp et al., 2001). Among the CC chemokines, CCL3 and CCL5 were highly associated with viral infections such as RSV and hepatitis C virus (HCV) (Harrison et al., 1999; Haeberle et al., 2001; Sung et al., 2001; Apolinario et al., 2002). This was further supported by CCL3^{-/-} mice that displayed a delay in viral clearance upon influenza virus, paramyxovirus, or cytomegalovirus (Cook et al., 1995; Domachowske et al., 2000; Salazar-Mather et al., 2000). CCL3 plays a detrimental role in infections, as CCL3-deficient mice displayed a lower inflammatory state in herpes simplex virus (HSV) and RSV infections (Tumpey et al., 1998; Haeberle et al., 2001). Likewise, CCR2^{-/-} mice had a decreased inflammatory reaction and increased viral titers in influenza virus infection (Dawson et al., 2000). On the other hand, RSV viral proteins were found to inhibit the expression of multiple chemokines such as CCL2, CCL3, CCL4 and CXCL9 in pulmonary leukocytes (Tripp et al., 2000). Other chemokines include CCL2 and CXCL10 were highly detected and associated with influenza infection (Dawson et al., 2000). CXCL10 along with CCL2 present in the cerebrospinal fluid (CSF) were identified to play a role in viral meningitis, promoting chemotaxis of immune cells (Lahrtz et al., 1997). Likewise, CCL2 was found to be one of the differentially expressed chemokine in HSV-1 encephalitis and is inversely correlated with the clinical status (Rösler et al., 1998). Also, it was found to be elevated in human immunodeficiency virus (HIV) infection and plays a role in promoting viral replication in T lymphocytes and macrophages (Fantuzzi et al., 2003; Campbell and Spector, 2008; Sabbatucci et al., 2015). CCL5 was associated with herpesvirus infections, as high levels were detected in murine and human herpesvirus infections, even in latent phases (Halford et al., 1996; Weinberg et al., 2002). CCL19 was found to be elevated at early and late time points of HIV infection, and is positively correlated with plasma HIV RNA levels (Damás et al., 2009; Fontaine et al., 2010). In contrast, its receptor CCR7 was downregulated in CD4⁺ T cells during HIV infection, thus promoting viral replication (Ramirez et al., 2014).

In viral infections, a cascade of events leads to the release of cytokines and chemokines that interact in order to establish viral clearance. For example, (IFN)- α/β were recognized as main inducers of CCL3 expression, which in turn, triggered recruitment of the

potent anti-viral NK cells (Salazar-Mather et al., 2000, 2002). Subsequently, NK cells released IFN- γ , which induced the expression of the interferon associated chemokines that promote the migration of T cells. These chemokines include CXCL9, CXCL10 and CXCL11, which bind CXCR3, that is highly affected in viral infections (Luster, 1998; Dawson et al., 2000; Haeblerle et al., 2001; Liu et al., 2001; Willemse et al., 2016). Reports have indicated the correlation between expression of CXCL9 in the liver with the viral activity of chronic hepatitis C (Apolinario et al., 2002). However, others indicated that CXCL9 and CXCL10 play a role in immune system overactivity and pathology such as that observed in hepatitis B virus (HBV) infection, where their neutralization led to a reduction in the severity of disease (Kakimi et al., 2001). CXCL10 was found to be elevated in the early stages of viral infections, as well as being positively associated with disease progression (Jiao et al., 2012). Moreover, CXCL10 was considered to be a prognostic factor upon antiretroviral therapy (Stylianou et al., 2000). CXCR3⁺ T cells are recruited through CXCL10 to HIV-infected lymph nodes, leading to the persistence of infection acquired immunodeficiency syndrome (Foley et al., 2005).

In HIV, the envelope glycoprotein (gp120) binds to CD4 on T cells along with a coreceptor binding via CXCR4 or CCR5, that are responsible for leukocyte signaling/chemotaxis and apoptosis (Deng et al., 1996; Dragic et al., 1996; Berger et al., 1999; Iyengar et al., 1999). On the other hand, the respective ligands CXCL12 and CCL5 were found to inhibit HIV infection (Cocchi et al., 1995; Bleul et al., 1996). Lack of CCR5 receptor is the natural resistance to HIV infection. This was first identified with the CCR5 delta32 (Δ 32) homozygous donor, where there is a 32-bp deletion in the CCR5 gene, thus producing a non-functional protein on T cells, which halts HIV entry. Upon transplantation of the bone marrow into the HIV patient, remission with undetectable viral load was achieved (Samson et al., 1996; Doms, 2000; Hütter et al., 2015). Trials were initiated where the CCR5 antagonists such as maraviroc and cenicriviroc showed great efficacy to block viral entry (Mehellou and De Clercq, 2010; Kim et al., 2016). On the other hand, viruses that utilizes CXCR were associated with worse clinical outcome (Connor et al., 1997). Hence, multiple non-peptide small-molecules and peptides that antagonize CXCR4 were investigated including AMD3100, AMD3465, AMD070 as well as isothioureas, indole, KRH, piperidine (Tamamura et al., 2006; Thoma et al., 2008; Mosley et al., 2009; Das et al., 2015; Wu et al., 2015). The role of chemokines in HIV infection was previously reviewed (Maghazachi and Al-Aoukaty, 1998).

Similar to HIV infection, CCR1, CCR2, CCR5 and CXCR3 are known to contribute to the pathogenesis of HCV infection (Wald et al., 2007; Larrubia et al., 2008). CCR1 and CCR5 were differentially expressed in the peripheral blood and intrahepatic T cells from HCV patients, especially CD8⁺ T cells showing a cytotoxic phenotype (Lichterfeld et al., 2002; Ahlenstiel et al., 2004). CCL5 binds CCR1 or CCR5 receptor and play a critical role in HCV infection (Fahey et al., 2014). The virus possesses the ability to affect CCL5 expression and secretion by T cells via interferon regulatory factors and the NF- κ B signaling pathway (Nattermann et al., 2006; Katsounas et al., 2012). CCR2 and its ligand CCL2 were increased in the HCV patients, and showed a correlation with the liver inflammation state as well as progression to cirrhosis (Asselah et al., 2005; Neuman et al., 2007; Farci et al., 2012). CXCR3 ligands such as CXCL10 and CXCL11 are enhanced in HCV infection by the presence of Th1-type cytokines IFN- γ and TNF- α in the liver (Helbig et al., 2009). Another vital chemokine is CXCL16, which was reported to induce the recruitment of CXCR6⁺ lymphocytes such as inflammatory Th1 and Th17 cells, leading to excessive tissue damage (Kim et al., 2001a; Oo et al., 2010; Guidotti and Iannacone, 2013).

Chemokines connect several members of the innate and adaptive immune system in viral infections. For example, in HCV infection, resident Kupffer cells in the liver secrete CCL2 stimulating the recruitment of CCR2⁺ DCs and CD8⁺ T cells (Boisvert et al., 2003; Decalf et al., 2007). This leads to the secretion of proinflammatory cytokines and chemokines such as TNF- α , CXCL10 and CCL4 that attract NK cells and promote IFN- γ production. Subsequently, macrophage activation occurs followed by production of other chemokines such as CCL3 and CXCL9 that would recruit CD4⁺ T cells (Simpson et al., 2002; Salazar-Mather and Hokeness, 2006; Heydtmann, 2009). Other players of inflammation in viral infection are neutrophils that secrete CXCL8 (Wisniewska-Ligier et al., 2006). CXCL8 causes the migration of neutrophils through CXCR1 and CXCR2 to the liver leading to further exacerbation of the inflammation (Polyak et al., 2001; Neuman et al., 2007). In HCV infection, CXCL8 production is a downstream effect of the binding of viral proteins to TLR2, TLR3, and TLR7 and activation of the NF- κ B pathway (Szabo et al., 2007; Wagoner et al., 2007). On the other hand, CXCL8 and CCL5 levels were elevated in the nasal lavages of RSV patients (Noah et al., 1995; Sheeran et al., 1999). Similar to lymphocyte choriomeningitis virus, West Nile virus, RSV, and HCV infections (Asensio and Campbell, 1997; John et al., 2003; Nischalke et al., 2004; Shirato et al., 2004), high expression levels of CCL2, CCL3, CCL5, and CXCL10 were found in avian influenza virus infections, and even much higher than in human influenza infection (Zhou et al., 2006). Upon CCL5/CCR5 axis engagement and activation, it was found that prevention of apoptosis of influenza virus-infected macrophages takes place, permitting viral persistence (Tyner et al., 2005).

In bacterial infections, CCL4 was elevated while CXCL8 expression was unchanged. Also, CCL2 was high in bacterial infections and even higher than in viral infections (Holub et al., 2013). Sepsis, community-acquired pneumonia, and skin abscess bacterial infections displayed high levels of CCL5, while CXCL10 was elevated in septic cases. This indicates that this chemokine contributes to the pathogenesis of bacterial infections (Cavalcanti et al., 2016). Levels of CCL2, CXCL1, CXCL8, CXCL9, and CXCL10, were found to be higher in infants with suspected late-onset infection, with CXCL10 as a sensitive early marker of infection (Ng et al., 2007). Interestingly, a study by Liu et al. reported CXCL8 to be higher in patients with bacterial infection more than those with viral infections, as well as showing a differential expression between gram negative and gram-positive bacteria. On the other hand, CXCL10 showed an opposite pattern with higher expression in the virally infected patients (Liu et al., 2016).

Regarding the parasitic infections, chemokines are involved in *Plasmodium* infection, where CCL2 induces the recruitment of CCR2⁺ cells to the site of infection (Belyaev et al., 2013). Also, parasite antigens and tissue damage caused by *Schistosoma* cercariae were found to be the cause of production of CXCL1, CXCL2 and CXCL8, thus inducing the migration of neutrophils and antigen presenting cells to the skin (Mountford and Trottein, 2004; McGovern and Wilson, 2013). CCL5 was reported to play a critical role

in B cell proliferation in *Trypanosoma cruzi* infection (Sullivan et al., 2011). Furthermore, CCL5 along with CXCL9 and CXCL10 were highly elevated in parasitic infections as they aid in controlling these infections (Talvani et al., 2000). Similar to viruses, parasites try to mimic the chemokine system and bind to chemokine receptors, thus enhancing cell migration of specific immune cells (Stein et al., 2009).

Conclusions

Chemokines is a large group of soluble ligands that bind to respective receptors that belong to GPCRs family. They have fundamental roles in the homeostasis, survival and migration of the immune system, including the innate (macrophages, neutrophils, DCs, NK cells) and adaptive (B and T cells) arms. Furthermore, they are involved in several physiological processes such as cell proliferation, survival, angiogenesis and differentiation, as well as pathological diseases like inflammation, autoimmunity, cancer growth, and microbial infections (viral, bacterial and parasitic). In infections, chemokines promote the migration of immune cells to the infection site in order to stimulate pathogen elimination. However, excessive stimulation and secretion of certain chemokines could cause more damage, excessive inflammation and disease progression.

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Immunological Tolerance

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Introduction

Immune tolerance is an essential process in the immune system that prevents T cells and B cells from auto-reactive generation, and it is an important topic in auto-immunity, cancer, organ transplantation, and infectious diseases.

B cells have two types of immune tolerance, including central tolerance in the bone marrow and peripheral tolerance in peripheral tissues. If immature B cells respond to bone marrow cells' auto-antigens with high avidity in central tolerance, they will undergo receptor editing by somatic recombination or clonal deletion via apoptosis. If immature B cells react to self-antigens with low avidity in central tolerance, they will undergo anergy. Anergy is a state in which immune cells would be unresponsiveness in terms of function. Apropos B cells peripheral tolerance, If mature B cells interact with self-antigens, they will experience anergy or deletion. Sometimes, self-antigens can provoke inhibitory receptors such as CD22, CD32b on mature B cells, contributing to inhibiting B cells (Singh and Schwartz, 2006). It is noteworthy that Cbl-b plays an important role in anergy (Jeon et al., 2004).

During the T cells' development in the thymus and central tolerance, auto-reactive immature T cells are purged by negative selection via apoptosis. This event happens when T cells recognize self-peptides in the context of thymic epithelial cells' MHC with high affinity. Recognition of self-peptides in the context of thymic epithelial cells' MHC with low affinity—also known as positive selection—leads to generating the single positive T cells, including CD4⁺/CD8⁻ or CD4⁻/8⁺ T cell. Death by neglect is a phenomenon in the thymus cortex in which double-positive (CD4⁺/CD8⁺) immature T cells do not recognize the peptide-MHC complex, and they are undergone apoptosis. Autoimmune regulator (AIRE) has a vital role in T cell central tolerance. AIRE mutations cause autoimmune polyendocrine syndrome type 1 (APS1), also called autoimmune polyendocrinopathy-candidiasis-ectodermal

dystrophy (APECED). Increased susceptibility to candidiasis is an APECED-related symptom. AIRE acts as a tissue-restricted antigens (TRAs) expression regulator in the medullary thymic epithelial cell, where it orchestrates self-antigens presentation to autoreactive T cells to negative selection (Singh and Schwartz, 2006).

T cell-related peripheral tolerance includes anergy by lacking co-stimulator such as B7 molecule, suppression by Treg cells, and deletion via apoptosis (Singh and Schwartz, 2006).

Immune tolerance in cancer

The failure of immune surveillance has been understood in tumor progression. One of the causes of this progression is the induced immune tolerance in the tumor microenvironment (TME). Generally, the involving factors in cancer immune tolerance include host T cell direct deletion, direct tolerization by tumor cells, Treg cell-mediated immunity suppression, tumor ignorance, and tumor antigens cross-presentation-induced T cell tolerization (Mapara and Sykes, 2004). Here, we will expatiate involving elements in inducing immune tolerance in the TME.

Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells from the immature myeloid lineage. These cells' expansion in the TME leads to suppressing T-cell responses. MDSCs are comprising myeloid progenitor cells and immature macrophages, immature granulocytes, and immature dendritic cells. MDSCs two main subsets are including monocytic MDSCs, which have a CD11b⁺ LY6G[−] LY6C^{hi} phenotype, and granulocytic MDSCs, which have a CD11b⁺ LY6G⁺ LY6C^{low} phenotype. MDSC suppressive activity mechanisms include Arginase 1 and inducible nitric oxide synthase (iNOS), L-cysteine degradation, reactive oxygen species (ROS), peroxynitrite (ONOO[−]), production of prostaglandin E2 (PGE₂), and T_{reg} cell induction (Gabrilovich and Nagaraj, 2009; Veglia et al., 2018). We are going to expatiate these mechanisms below.

iNOS, arginase, and L-cysteine degradation

iNOS, which generates nitric oxide (NO), has been upregulated by STAT1 in monocytic MDSCs (Movahedi et al., 2008). NO suppresses T cell activation via Jak3 and STAT5 inhibition (Bingisser et al., 1998), inhibition of MHC-II transcription (Harari and Liao, 2004), and induction of T cell apoptosis (Vig et al., 2004; Rivoltini et al., 2002). Also, NO contribute to promoting metastasis via vascular endothelial growth factor-C (VEGF-C) production in vitro (Nakamura et al., 2006). NO inhibition with radiotherapy leads to promote anti-tumor responses (Xu et al., 2020). iNOS and arginase 1 (which catalyze L-arginine to urea and L-ornithine) use L-arginine as a substrate. Arginase 1 overexpression has been indicated on MDSCs infiltrating tumors, which destroys L-arginine non-essential amino acid from the TME (Rodriguez et al., 2005; Ochoa et al., 2007). Consequently, L-arginine deficiency accounts for the downregulated CD3 ζ -chain expression (Rodriguez et al., 2002), impaired the expression of cyclin D3 and cyclin-dependent kinase 4 (cdk4) (Rodriguez et al., 2007), arrested T cell proliferation (Rodriguez et al., 2010). MDSCs can degrade L-cysteine amino acid and L-cystine, consequently inhibiting T cell activation and function (Srivastava et al., 2010).

Reactive oxygen species

ROS serve as an essential factor for MDSC suppressive activity in cancer (Kusmartsev et al., 2004; Schmielau and Finn, 2001; Kusmartsev et al., 2005; Mantovani et al., 2003). H₂O₂, which formed from MDSC-derived superoxide anion (O₂[−]), affects T cells, including reduced CD3 ζ -chain expression (Schmielau and Finn, 2001), decreased cytokine production (Schmielau and Finn, 2001), and downregulated IFN- γ expression (Corzo et al., 2009). NADPH oxidase (NOX2) mediates increased ROS production in MDSCs in patients with head and neck cancer (Corzo et al., 2009). It has revealed that integrins on MDSCs such as CD11b, CD18, and CD29 interact with T cells, contributing to increased ROS formation and suppression of CD8⁺ T cell responses (Kusmartsev et al., 2004).

Peroxynitrite. The reaction between NO and O₂[−] products ONOO[−]. Tumor progression has been associated with high peroxynitrite levels in many cancer types (Nakamura et al., 2006; Cobbs et al., 2003; Bentz et al., 2000; Dairou et al., 2005; Ekmekcioglu et al., 2000; Kinnula et al., 2004). A study has found that MDSCs induce immune tolerance in antigen-specific CD8⁺ T cells in cancer via nitrosylation of tyrosines within the TCR/CD8 complex (Nagaraj et al., 2007). In this situation, T cells are unresponsiveness. ROS and peroxynitrite overexpression during direct cell-cell contact incites nitration of TCR/CD8 (Nagaraj et al., 2007). Furthermore, peroxynitrite plays a role in the nitrosylation of CCL2 chemokine. CCL2-CCR2 axis has a pivotal role in MDSCs infiltration into the TME (Huang et al., 2007). Besides, CCL2 mediates MDSCs recruitment in cancer, contributing to colorectal carcinogenesis (Chun et al., 2015). Nitrosylation of CCL2 in the cancer environment prevents cytotoxic T lymphocyte trafficking to the tumor, promoting cancer development and immune tolerance (Molon et al., 2011).

Production of PGE₂

PGE₂ is one of the prominent factors to promote an immune tolerance state in favor of tumor progression. PGE₂ incites suppressive function of MDSCs, contributing to tumor progression (Sinha et al., 2007a; Tomić et al., 2019). In prostate cancer, obesity leads to recruit macrophages into the TME and induces tumor-associated macrophages (TAMs) polarization via PGE₂/COX-2 pathway. Besides, obesity increases CCL2, PGE₂, and COX-2 expression in prostate cancer cells (Galván et al., 2017). PGE₂ reduced MDSCs'

capacity to produce proinflammatory cytokines and raised their ability to produce IL-27, IL-33, and TGF- β (Tomić et al., 2019). Recently, a paper has reported that the P50 NF- κ B-dependant differentiation of MDSCs has been mediated by tumor-derived PGE₂ (Porta et al., 2020). It has been indicated that sulforaphane drug inhibits PGE₂ synthesis in breast cancer cells via NF-E2-related factor 2 (Nrf2) and prevents to accumulation MDSCs (Rong et al., 2020). Epigenetics processes have regulated MDSCs tolerogenic functions. In this context, a study revealed PGE2-mediated upregulated DNA methyltransferase 3A (DNMT3A) in MDSCs that DNMT3A amplifies MDSC-suppressive properties (Rodríguez-Ubreva et al., 2017).

Treg cell induction and cell anergy

MDSCs can induce the development of forkhead box protein P3⁺ (Foxp3⁺) T_{reg} in vivo (Huang et al., 2006; Yang et al., 2006a). In A20 B-cell lymphoma model, It has been identified that T_{reg} induction by MDSCs is dependant on arginase but is TGF- β independent (Serafini et al., 2008). On the contrary, another study reported that MDSCs suppress iT_{reg} differentiation from naive CD4⁺ cells and require ROS and indoleamine 2,3-dioxygenase (IDO) but do not require arginase 1, iNOS, NO, cystine/cysteine depletion, PD-1, and PD-L1 signaling, or COX-2 independent (Centuori et al., 2012). A cancer immune tolerance in breast cancer can be induced by MDSCs-expressing IDO, increasing T_{reg} cell expansion (Li et al., 2018). Inducing anergy in NK cells can be incited by MDSCs via membrane-bound TGF- β , STAT5 activity, ARG-1, or as well as the NKp30 receptor (Li et al., 2009; Liu et al., 2007; Hoechst et al., 2009; Oberlies et al., 2009). In glioma, NKG2D and IFN- γ production can also be inhibited by MDSCs (Alizadeh et al., 2010). MDSCs can expand and differentiate T_{reg} in the tumor-bearing host by IL-10 and TGF- β upregulation (Huang et al., 2006). Using tumor mouse models, It has demonstrated that MDSCs can differentiate macrophages into M₂ phenotype by producing IL-10 and decreasing macrophage production of IL-12 (Kerkar et al., 2011; Sinha et al., 2007b).

Regulatory T cells

First, T_{reg} participation in anti-tumor immunity has been introduced by two studies (Onizuka et al., 1999; Shimizu et al., 1999). A growing body of evidence has indicated that T_{reg} cells are increased and infiltrated into many types of tumor, including head and neck (Schaefer et al., 2005), breast (Liyanage et al., 2002), lung (Wolf et al., 2003), liver (Ormandy et al., 2005), gastrointestinal tract (Ichihara et al., 2003; Sasada et al., 2003), pancreas (Hiraoka et al., 2006), prostate (Watanabe et al., 2019), ovary (Curiel et al., 2004; Sato et al., 2005), brain (El and Lesniak, 2006), kidney (Giraldo et al., 2017), and B cell non-Hodgkin-lymphoma (Yang et al., 2006b). Significantly, there is a negative correlation between the high frequency of tumor-infiltrating Foxp3⁺ cells and patients' survival in solid tumors, including cervix, kidney, breast, lung, and melanomas (Shang et al., 2015; Shou et al., 2016; Tao et al., 2012). There are discrepancies in other studies. Two studies have reported the high frequency of tumor-infiltrating Foxp3⁺ cells associated with improved colorectal cancer survival (Salama et al., 2009; Frey et al., 2010). High ratios of Foxp3⁺ T_{reg} cells to tumor-infiltrating CD8⁺ T cells have been associated with patients' poor prognosis. It has been seen in patients with breast (Bates et al., 2006), gastric (Sasada et al., 2003), and ovarian cancer (Curiel et al., 2004; Sato et al., 2005). T_{reg} can inhibit high endothelial venules neogenesis via inhibition of T-cell activation's self-amplifying loop, which causes decreased lymphocyte infiltration and development of the tumor tissue (Colbeck et al., 2017; Hindley et al., 2012).

Here, we are going to highlight the suppressive mechanisms of Treg cells. Treg cells constitutively express IL-2 receptor subunit- α or CD25, which causes to deplete of IL-2 from surroundings; thus, effector T cell functions are reduced (Chinen et al., 2016). T_{reg} cell releases IL-10, TGF- β , and IL-35 to inhibit effector T cells (Collison et al., 2007; Taylor et al., 2006). Treg cells derived from the TME can also induce apoptosis in NK and CD8⁺ T cells via granzyme B, perforin, and Fas/Fas-L interaction (Cao et al., 2007; Strauss et al., 2009; Ohue and Nishikawa, 2019). IDO is an enzyme in tryptophan (Trp) metabolism which degrades Trp to kynurenine in TME, and Trp starvation results in T cell dysfunction (Uyttenhove et al., 2003). IDO secretion is mediated by the interaction between CTLA-4 on T_{reg} cells and B7 on APCs (Mellor and Munn, 2004). Adenosine metabolism has been seen in T_{reg} cells. Oxidative stress causes T_{reg} cells apoptosis, contributing to the release of ATP from them. CD39 and CD73 molecules are expressed considerably by T_{reg} cells, and these molecules orchestrate metabolism ATP to adenosine. Binding adenosine to the A2A receptor (A2AR) inhibits effector T cells (Maj et al., 2017). Another Treg cell inhibitory mechanism in TME is immune checkpoint involvement. These immune checkpoints are including lymphocyte activation gene-3 (LAG-3) (Camisaschi et al., 2010), PD-1 (Kumar et al., 2017), CTLA-4 (Matoba et al., 2019), T-cell immunoglobulin mucin (TIM-3) (Liu et al., 2018), TIGIT (Kurtulus et al., 2015), and inducible T-cell costimulatory (ICOS) (Li and Xiong, 2020). T_{reg} cells inhibit DC maturation and antigen presentation via CTLA4-B7 interaction (Ohue and Nishikawa, 2019).

Tumor-associated macrophages

TAMs have different roles in tumor progression and cancer immune evasion. TAMs also play an essential role in angiogenesis, migration, invasion, metastasis, and epithelial-mesenchymal transition (EMT) (Aras and Zaidi, 2017). TAMs suppress tumor immunity via mediators production, including IL-10, TGF- β , Arginase-1, IDO 1/2, and prostaglandins (Aras and Zaidi, 2017; Mantovani et al., 2017). Hypoxia and HIF-1 α upregulate PD-L1 expression on TAMs, inhibiting effector T cell via interaction with PD1 (Doedens et al., 2010). In squamous cell carcinoma, TAMs phenotype polarization has been mediated by cancer-associated fibroblasts (CAFs) via IL-10, TGF- β , and arginase-1 release (Takahashi et al., 2017). In bladder cancer, DC-SIGN⁺ TAMs infiltration into the TME is associated with promoting tumor immunity tolerance (Hu et al., 2020). In breast cancer, progranulin (multi-functional growth factor) mediates PD-L1 expression on TAMs via the STAT-3 signaling pathway, promoting breast tumor immune

escape (Fang et al., 2021). In breast cancer and ovarian cancer, the lncRNA-Xist/miR-101-3p/KLF6/C/EBP α axis causes TAMs polarization (Zhao et al., 2021). TAMs express immune checkpoints such as PD-L1, PD-L2, B7-H4, and VISTA, which trigger inhibitory signals to effector T cells (Mantovani et al., 2017).

T helper 2 (Th2)-mediated immunity

Th₂-mediated immunity plays a role in the inhibition of anti-tumor immunity. Type 2 cytokines such as IL-4 and IL-13 induce a tolerogenic state in TME, contributing to tumor development. However, Th₂-mediated immunity acts as a double-edged sword. Indeed, studies have revealed that Th₂ and its cytokines also harm tumor growth. For example, IL-13 has reduced tumorigenicity in fibrosarcoma tumor models and B16F1 melanoma (Ma et al., 2004).

Several studies have shown positive effects of Th₂-mediated immunity on tumor progression. IL-13, IL-4R, STAT6 caused to reduce tumor immunosurveillance (Terabe et al., 2000). It has been reported that lacking B cells have been associated with enhanced anti-tumor responses (Shah et al., 2005; Tadmor et al., 2011). In a study, IL-4 exerted a negative effect on tumor clearance, and it interrupted tumor-induced CD8⁺ T-cell responses (Olver et al., 2006).

T helper 17

Th17 cell has contrasting dual roles in cancer immunity in terms of anti-tumoral and pro-tumoral (Dahal, 2017). Here, we emphasize its pro-tumoral role.

IL17A-producing Th17 can promote lung cancer metastasis in murine models (Kulig et al., 2016). Tosolini et al. (2011) have confirmed a relationship between poor prognosis and high expression of the Th17 in patients with colorectal cancer samples. In the tumor's animal model, IL-17 facilitated MDSCs activation, MDSCs accumulation, and decreased CD8⁺ T cells infiltration (He et al., 2010), suggesting Th17 cells may suppress anti-tumor immunity.

Exhausted T cells and NK cells

In chronic infections and cancer, T cells are affected by persistent antigens and inflammatory signals. This condition is usually related to T cell dysfunction that has been termed exhaustion. Exhausted T cells lose their powerful effector functions, express multiple inhibitory receptors such as PD-1, TIM-3, LAG-3, and become exhausted, causing induce immune tolerance. T cell exhaustion is usually related to ineffective control of tumors, but repairing exhausted T cells can restore immunity (Wherry and Kurachi, 2015). Immune-suppressive factors in the tumor microenvironment such as IL-10, TGF- β , NOS, IDO, and hypoxia can induce exhaustion phenotype in tumor-specific T cells (Schietinger and Greenberg, 2014). In turn, These cells suppress anti-tumor-specific responses.

Although NK cells have possible cytolytic activity against tumors or infected cells, NK cells show impaired effector functions in hosts suffering from tumors or chronic infections (Bi and Tian, 2017).

Taken together, it has been concluded that exhausted cells have been invigorated by TME elements and these cells incite an excess tolerogenic state in TME.

Immune checkpoints

Inhibitory immune checkpoints are molecules that transduce negative signals to the immune cell. Blocked of these molecules is a novel way in cancer immunotherapy. The ligands of immune checkpoints have been expressed on tumor cells, causing anti-tumor immunity tolerance via interaction with immune checkpoints on the immune cell in TME. Inhibitory immune checkpoints are including CTLA-4, PD-1, T cell membrane protein 3 (TIM-3), lymphocyte activation gene 3 (LAG-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), B and T lymphocyte attenuator (BTLA), adenosine A2a receptor (A2aR), and killer cell immunoglobulin-like receptor (KIR) (Pardoll, 2012; Anderson et al., 2016). Besides, several novel immune checkpoints have introduced in recent years, including V-domain Ig suppressor of T cell activation (VISTA) (Gao et al., 2017), ST2 (Van der Jeught et al., 2020), CD47 (Logtenberg et al., 2019), Siglec (Fudaba et al., 2021), annexin A5 (Kang et al., 2020), CD200 (Xiong et al., 2020), SLAMF6 (Yigit et al., 2019), and CD39/CD73 (Moesta et al., 2020).

Immune checkpoints expression in TME can be affected by epigenetic mechanisms, contributing to inducing immune tolerance. Several studies have shown that DNA hypomethylation on H3K9me3 and H3K27me3 in colon and breast TME leads to expressing the immune checkpoints, including CTLA-4, PD-1, TIM-3 LAG-3 in transcription level (Sasidharan Nair et al., 2018a,b; Elashi et al., 2019).

MicroRNAs (miRNAs) and long non-coding RNAs (lncRNAs) can also regulate immune checkpoints expression in cancer. In lung cancer, p53/miR-34/PD-L1 axis mediated tumor immune evasion (Cortez et al., 2016). In contrast, miRNA-424(322) downregulated PD-L1 expression in human cancer cell lines (Xu et al., 2016). In nasopharyngeal carcinoma, co-expression PD1 and AFAP1-AS1 as a lncRNA have associated with poor prognosis (Tang et al., 2017).

Tolerogenic dendritic cells

Derivative factors of tumor which induce tolerogenic DCs include PGE2, TGF- β , Wnt signaling, HMGB1, CXCL12, GCSF, CCL2, VEGF, tumor-derived Exosomes delivering arginase-1 and mir-212-3p (DeVito et al., 2019). It has been reported that the CD31 molecule incites DCs polarization toward tolerogenic DCs (Clement et al., 2014). Tolerogenic DCs' inhibitory mechanisms include IDO expression, TGF- β , IL-10 secretion, Fas-L expression, PD-L1/PD-L2 expression, ILT3/4 up-regulation, and TRAIL interaction with TRAIL receptor (Fucikova et al., 2019; Brenk et al., 2009; Izawa et al., 2012). By contrast, activational receptors and functions have been downregulated in these cells, including CD40, CD80, CD86, TNF- α , IL-12, IL-6, and NF- κ B (Fucikova et al., 2019). In the animal models, DCs promote Treg cells' induction via TGF- β signaling, orchestrated by tumor cells (Ghiringhelli et al., 2005). In breast cancer, DCs incite CD4⁺ T cells to IL-13 secretion, contributing to tumor development (Aspord et al., 2007). It has been identified that M2 macrophages and tolerogenic DCs express MerTK molecule, which is a family of tyrosine kinase receptors (TAM-R). Besides, mononuclear cells-infiltrating human breast cancer tumors such as activated B lymphocytes and regulatory T cells and CD4⁺ and CD8⁺ T cells express MerTK. Gas6 and PROS1 are the ligands of TAM-R. Notably, IL-10 production is regulated by MerTK/PROS1 axis in tolerogenic DCs, promoting immunosuppressive TME (Giroud et al., 2020).

Regulatory B cells

Several B_{regs} phenotypes have been known in human cancers, including B10 B_{reg}, granzyme B (GrB⁺) B_{reg}, TIM-1⁺ B_{reg}, PD-1^{hi} B_{reg}, and PD-L1⁺ B_{reg} (Guan et al., 2016; Hu et al., 2019; Ye et al., 2018; Xiao et al., 2016; Wu et al., 2020a). Cross-talk between regulatory B cells (B_{regs}) and TME components has revealed that B_{regs} account for tumor progression. The mechanisms that B_{regs} impede anti-tumor immunity in TME are including immunosuppressive cytokine production such as IL-10, TGF- β , and IL-35, the expression of inhibitory molecules such as PD-1, PD-L1, and Fas-L, and provoking MDSCs (Shang et al., 2020). Glioma-derived ADAM10 and placental growth factor can provoke B_{reg} cells differentiation (Ye et al., 2014; Han et al., 2014). Consequently, tumor-specific CD8⁺ T cells are suppressed. In patients with lung cancer, peripheral Breg cells' frequency has increased compared to healthy individuals (Zhou et al., 2014). In gastric cancer, CD19⁺CD24^{hi}CD38^{hi} B_{reg} cells play a crucial role in suppressing tumor immunity via the inhibition of IFN- γ and TNF- α secretion and the up-regulation of the T_{reg} cells mediated by TGF- β 1 (Wang et al., 2015). IL-35 production from CD1d^{hi}CD5⁺B cells stimulates a protumorigenic state in pancreatic cancer (Pylayeva-Gupta et al., 2016).

Immunosuppressive cytokines

Immunosuppressive cytokines are including TGF- β , IL-10, and IL-35. In melanoma, MDSCs can inhibit T cells by producing TGF- β (Filipazzi et al., 2007). We have discussed apropos to the immunosuppressive cytokines in previous sections.

Immunosuppressive metabolites

Several critical immunosuppressive metabolites in TME and cancer metabolism induce immunotherapy resistance and tumor immunity tolerance. These metabolites include adenosine, R-2-hydroxyglutarate, glutamine, and methylglyoxal (Jiang et al., 2020).

We earlier discussed adenosine. In NK cells, adenosine can incite an immune tolerance state via decreasing the production of IFN- γ and TNF- α . Besides, adenosine reduced the proliferation and differentiation ability of NK cells (Beavis et al., 2013; Raskovalova et al., 2006). Adenosine/A2AR axis in macrophages leads to M2 macrophages phenotype polarization via down-regulating IL-2, TNF α , and NO expression and upregulating arginase-1, IL-10, and VEGF (Jiang et al., 2020; Ramanathan et al., 2007). Adenosine facilitated CD11b⁺ Gr1⁺ MDSCs expansion and their immunosuppressive activity (Ryzhov et al., 2011).

One of the important oncometabolites is R-2-hydroxyglutarate that release into the TME by glioma tumor cells, catalyzed by isocitrate dehydrogenase (IDH) mutations. R-2-hydroxyglutarate is taken up by T cells; consequently, NFAT activation, polyamines biosynthesis, T cell proliferation, and function are inhibited (Bunse et al., 2018).

One of the essential factors for cancer growth is glutamine. Glutamine induced a tolerogenic state in effector T cells tumor-bearing mice. Using glutamine antagonism named JHU083 reduced hypoxia and acidosis, increasing effector T cells activation and anti-cancer immunity (Leone et al., 2019). JHU083 inhibited tumor growth, MDSCs recruitment, expansion, and IDO/kynurenine expression; in turn, it increased MDSC to M1 macrophage conversion, tumor sensitivity, and immunotherapy and MDSC apoptosis (Oh et al., 2020). It is noteworthy that glutamine metabolism is essential for CD8⁺ T cells differentiation and function (Sinclair et al., 2013).

The role of methylglyoxal in tumor malignancy has been proven (Antognelli et al., 2019; Nokin et al., 2019). Methylglyoxal is a side biochemical product arising from aerobic glycolysis (Nokin et al., 2019). MDSCs attenuated CD8⁺ T cells via dicarbonyl radical methylglyoxal accumulation. In murine cancer models, methylglyoxal blockade hindered T cell suppression mediated by MDSCs, resulting in tumor immunotherapy improvement (Baumann et al., 2020).

Immune tolerance in autoimmunity

Robust immune response to self-antigens leads to the incidence of the broad spectrum of autoimmune maladies. Here, we will expatiate the immunoregulatory molecules involving immune tolerance, which their deficiencies cause autoimmunity.

B cell and T cell tolerance

B cells and T cells are central elements of adaptive immunity that can distinguish self-antigens from non-self-antigens. These cells are trained to respond to foreign antigens, not self-antigens, in the bone marrow and thymus during their development. Earlier, we have explained immune tolerance processes in B cells and T cells.

Genetic susceptibility

Autoimmunity's genetic basis is classified into human leukocyte antigen (HLA) alleles, non-HLA alleles, and monogenic defects. Monogenic defects are the type of Mendelian disorders associated with rare autoimmune diseases.

Celiac disease (CD) and type 1 diabetes (T1D) are significantly associated with HLA DQA1*05 and DQB1*02 alleles encoding the DQ2.5 molecule and HLA DQA1*03 and DQB1*03 alleles encoding DQ8 molecules (Farina et al., 2019). Ankylosing spondylitis (AS) susceptibility and intensity are associated with HLA-B27 haplotypes (Pimentel-Santos et al., 2013). The most susceptible HLA class II alleles to multiple sclerosis (MS) are DRB1*15:01 for Caucasians and DRB1*04:05 and DRB1*15:01 for Japanese (Watanabe et al., 2020). HLA-DRB1*0405, HLA-DRB1*1502, and HLA-DRB1*1602 alleles are related to an elevated risk of developing systemic lupus erythematosus (SLE), while HLA-DRB1*1201 and HLA-DRB1*1202 alleles are associated with a lower risk of developing SLE (Selvaraja et al., 2021). Besides, HLA-DRB1*04 significantly was associated with risk of SLE (Selvaraja et al., 2021).

Studies have shown that several genes orchestrate the maintenance of tolerance to self-antigens. These genes are including AIRE, complement system C4 component, CTLA-4, Fas/Fas-L, FoxP3, IL10/IL10R, IL2/IL2R $\alpha\beta$, and SHP-1 (Cheng and Anderson, 2012; Wu et al., 2009; Facciotti et al., 2020; Lee et al., 2014; Croker et al., 2008). Monogenic defects in AIRE, Fas/Fas-L, and FoxP3 account for APS, autoimmune lymphoproliferative syndrome (ALPS), and immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), respectively (Cheng and Anderson, 2012). C4 deficiency is associated with inappropriate immune complex clearance and causes SLE (Wu et al., 2009). IBD and colitis have arisen due to IL10/IL10R and IL2/IL2R $\alpha\beta$ mutations (Lee et al., 2014; Yamanouchi et al., 2007).

The PTPN22, a lymphoid tyrosine phosphatase, negatively regulates the T-cell response. The single nucleotide polymorphism of PTPN22 has been linked to autoimmune conditions such as RA, SLE, T1D, and autoimmune thyroid diseases (Majorczyk et al., 2007; Namjou et al., 2013; Valta et al., 2020; Wu et al., 2020b). NOD-2, IL-23R, and ATG16L1 gene polymorphisms are hereditary risk factors correlated with Crohn's disease (Wildenberg et al., 2017; Rochereau et al., 2021; Xu et al., 2015). AS has been linked to genetic polymorphisms in ARTS1 (Choi et al., 2010). The peptidyl arginine deiminase 4 (PADI4) haplotype is linked to a higher risk of developing RA since it raises the synthesis of citrullinated peptides that function as autoantigens (Suzuki et al., 2003). IL-7 receptor alpha chain-related gene polymorphism is a major risk factor to MS (Gregory et al., 2007). Polymorphisms in the IL2/IL21 locus have been associated with CD (Sherrill et al., 2010). While in patients with SLE, BLK polymorphisms are associated with renal dysfunction (Di et al., 2020), in Mexican patients, the genes BLK and BANK1 are associated with RA susceptibility (Ramírez-Bello et al., 2020). STAT4, IRF5, FC γ RIIB, mannose-binding lectin type 2 (MBL-2), and IL-10 gene polymorphisms are associated with a higher risk of SLE (Wang et al., 2020, 2021; Song et al., 2020; Zhu et al., 2016; Mahto et al., 2020).

Environmental factors

Several environmental factors have been suggested to promote autoimmune diseases, including infection, weather, stress, occupation, smoking, drugs, and diet (Marrie, 2004; Jörg et al., 2016). Also, the consumption of "westernized foods," including intakes high in salt, fat, protein, and sugar, is already associated with an increasing prevalence of various diseases (Jörg et al., 2016; Thorburn et al., 2014). Changes in eating habits have been intensively investigated, showing a direct effect on the gut microbiome leading to immune homeostasis and the gastrointestinal tract (GIT) (Jörg et al., 2016).

One of the most potent environmental compounds related to autoimmunity is silica. The spatial interaction of ANCA-associated vasculitis (AAV) with quarries in an area with a high density of quarries supports the importance of silica as a particular environmental element in autoimmunity (Giorgiutti et al., 2021).

Evidence on the effects of antibiotic usage in childhood on the likelihood of certain autoimmune disorders is missing. The most often used antibiotics had no link to T1D or CD autoimmunity progression within the first four childhood years (Kemppainen et al., 2017).

CTLA4/B7 axis

CTLA-4, also known as CD152, is an inhibitory receptor that aids in maintaining self-antigen immunity. CTLA-4 is structurally and functionally relevant to CD28 since it shares 31% homology and has a higher affinity for the B7 family molecules CD80 and CD86. CTLA-4, on the other hand, has antagonistic effects on T cell activation, and evidence suggests that its inhibitory activity extends beyond the ligand-binding interaction. CTLA-4 competes with CD28 for B7 binding and interacts with clathrin adaptor proteins and tyrosine phosphatases via its cytoplasmic domain to control cell trafficking and set the activation threshold in T cells. CTLA-4 is essential for regulatory T cell function. CTLA-4 has been linked to many autoimmune maladies, including SLE, due to its role in maintaining peripheral tolerance (Romo-Tena et al., 2013). CTLA-4 gene polymorphisms associated with graves syndrome (Esteghamati et al., 2009; Ting et al., 2016), hashimoto disease (Ting et al., 2016), rheumatoid arthritis (RA) (Lei et al., 2005), and T1D (Kavvoura and Ioannidis, 2005). A study reported that the CTLA-4 rs231775 gene polymorphism was linked to a lower risk of RA, while the CTLA-4 rs16840252 and CD86 rs17281995 gene polymorphisms were not (Liu et al., 2021).

In a study to better understand CTLA-4's function in adulthood, Paterson and colleagues (Paterson et al., 2015) developed a model in which tamoxifen-mediated conditional ablation of CTLA-4 can be induced pharmacologically. This study has demonstrated that adult mice with CTLA-4 deletion develop resistance to experimental autoimmune encephalomyelitis (EAE). T_{reg} cells deficient in CTLA-4 produce excessive IL-10 and have greater levels of co-inhibitory receptor expression such as PD-1 and LAG-3. T_{reg} cells without CTLA-4 remain capable of reducing in vitro and in vivo the proliferation of T cells (Paterson et al., 2015).

Abatacept is a soluble fusion protein of CTLA-4 and the Fc tail of IgG that is fully human. Co-stimulation is inhibited by this biological disease-modifying anti-rheumatic drug (DMARD), inhibiting T-cell activation (van Nimwegen et al., 2020). Abatacept-related clinical trials have been done in several autoimmune maladies such as systemic sclerosis (Chung et al., 2020), juvenile idiopathic arthritis (Ruperto et al., 2008), sjögren's syndrome (SS) (van Nimwegen et al., 2020), T1D (Orban et al., 2011), RA (Genovese et al., 2005), and SLE (Group TAT, 2014).

FOXP3

Foxp3 is a critical regulator of T_{reg} cell gene expression responsible for most of these cells' suppressive ability. The coordinated activity of transcription factors and comprehensive epigenetic control mechanisms regulates Foxp3 expression in T_{reg} cells; additionally, post-translational modifications modulate Foxp3 activity. Besides T_{reg} cells, Foxp3 expression has been shown on activated CD4⁺ T cells, CD8⁺ T_{reg} cells, natural killer T cells (NKT), macrophages, B cells, and cancer cells (Lu et al., 2017). Cytokines such as TGF- β and IL-2 and other elements in the tissue microenvironment like retinoic acid, vitamin C, and short-chain fatty acids (SCFAs) can positively affect the induction and maintenance of Foxp3 transcription (Lu et al., 2017; Yue et al., 2016; Hill et al., 2008; Arpaia et al., 2013; Chen et al., 2011). In comparison, pro-inflammatory cytokines such as TNF and IL-6 and other stimuli such as Toll-like receptor (TLR) activation or robust T cell receptor (TCR) and co-stimulatory molecule signaling have a negative effect on Foxp3 (Lu et al., 2017; Gerriets et al., 2016; Nie et al., 2013; Gao et al., 2012). Foxp3 gene expression is initiated and stabilized by hierarchical and synchronized Foxp3 conserved non-coding sequences (CNSs) such as CNS0 and CNS3 activation, which is essential for T_{reg} cell growth, maintenance, and immunological self-tolerance. CNS0 and CNS3 deficiency results in fatal autoimmunity. Foxp3 expression is induced and maintained by CNS0 in an IL-2-dependent manner (Kawakami et al., 2021).

AIRE

In thymic epithelial cells (TEC), AIRE mediates the expression of tissue-specific antigens (TSAs) to facilitate tolerance against self-reactive T lymphocytes. Brg1 allows AIRE to act by promoting accessibility at loci encoding tissue-specific antigens. Brg1 promotes peripheral-tissue-specific gene accessibility while AIRE suppresses it. In medullary TEC, the effects of AIRE and Brg1 on chromatin state are opposed (Koh et al., 2018).

Biomolecular assemblies that resemble aggregates are evolving as modern practical conformational states. AIRE assembles into broad aggregates that can be used as nuclear foci. AIRE forms filamentous homo-multimers in vitro using its caspase activation recruitment domain (CARD), and that this assembly mediates foci forming and transcriptional function. AIRE mutants shape foci with enhanced promyelocytic leukemia protein (PML) body association. AIRE's transcriptional function is impaired as it is directed to PML bodies, but it is restored when PML bodies are dispersed with a viral antagonist (Huoh et al., 2020).

Multiple endocrine defects, chronic mucocutaneous candidiasis, and high serum autoantibody titers characterize APS1, a monogenic recessive condition. An APS1 mouse model was used to test the effectiveness of adeno-associated virus serotype 9 (AAV9)-AIRE (AAV9 bearing AIRE cDNA) gene therapy. AAV9-AIRE was injected intrathymically and showed strong transduction ability, restoring AIRE expression in the thymus. In treated versus control mice, AIRE gene transmission resulted in a substantial improvement in TSA expression and, more significantly, a significant decrease in serum autoantibodies (Almaghrabi et al., 2020).

FAS/FAS-L pathway

Fas (also known as CD95 or APO-1 or TNFRSF6) and Fas-L belong to TNF-R and TNF superfamily, respectively. The initiation of a caspase cascade that initiates apoptosis occurs when Fas is ligated with Fas-L. Fas-FasL-mediated apoptosis or activation-induced cell death (AICD) is an effective pathway for immune homeostasis maintenance. Programmed cell death (apoptosis) plays an

important function in a physiological immune response by removing potentially pathogenic autoreactive lymphocytes from the bloodstream and tissues, reducing the tissue damage that immune responses eventually do. When Fas binds, the protein adaptor Fas-associated death domain (FADD) of procaspase-8 is recruited and activated, resulting in the forming of the death-inducing signaling complex (DISC). Active caspase-8 cleaves caspase-3 immediately, triggering the caspase cascade, which results in cell death (Volpe et al., 2016).

MRL-lpr/lpr mice have mutations in the Fas gene and are used to study autoimmune maladies, including SLE, RA, SS, and ALPS (Yamada et al., 2017). In MRL-lpr/lpr mice, Fas-mediated apoptosis of peripheral T cells, which is a critical mechanism for maintaining immunological tolerance, is inhibited. In MRL-lpr/lpr mice, AICD fails to delete peripheral T cells, resulting in an uptick in autoreactive T cells, which causes autoimmune lesions to develop in several organs (Yamada et al., 2017; Singer et al., 1994). ALPS patients also have mutations in the Fas gene (Hsu et al., 2012).

Mice with the gld/gld genotype have mutations in the gene that codes for Fas-L, and they are commonly used as an autoimmune disease model (Nagata and Suda, 1995). Since some enzymes allow Fas-L to be shed from the cell surface, it is unstable (Garcia et al., 2013). When soluble Fas-L (sFas-L) binds to Fas, it causes cell proliferation rather than apoptosis (Herrero et al., 2011).

Autoreactive CD8+ T cells

Autoreactive CD8⁺ T cells tend to be essential players in various animal models of autoimmunity such as T1D and MS in the non-obese diabetic (NOD) mouse and EAE mouse, respectively; their roles in human autoimmune disorders are still being investigated. Autoreactive CD8⁺ T cells can serve as pathogenic effector cells, causing tissue damage, while other autoreactive CD8⁺ T cells can act as regulators, protecting the body from autoimmune attacks (Tsai et al., 2008). Activated CD8⁺ T cells as a juggernaut can destroy target cells via the Fas/Fas-L pathway or release cytolytic granules at the effector/target cell junction. TNF and IFN- γ are two pro-inflammatory cytokines secreted by activated CTLs that can trigger tissue harm (Tsai et al., 2008).

Type 1 diabetes mellitus (T1DM), or autoimmune diabetes, is a progressive condition that causes hyperglycemia due to insulin deficiency caused by pancreatic-cell failure (Katsarou et al., 2017). This malady is characterized by a CD4⁺ and CD8⁺ T cell-dependent autoimmune pathway that directly targets the pancreatic β -cell (Katsarou et al., 2017). Epidemiological experiments have demonstrated that inheriting specific human MHC class I alleles, such as HLA-A*0201, increases the hereditary susceptibility to T1D when expressed with particular MHC class II alleles (Tsai et al., 2008). In order to identify the effect of type of autoreactive CD8⁺ T cells phenotype on T1D progression, a study has revealed that autoreactive T cells were phenotypically diverse, and their phenotype varied with disease progression rate (Wiedeman et al., 2020). Also, in subjects with T1D who underwent rapid loss of C-peptide, activated islet-specific CD8⁺ memory T cells were prevalent (Wiedeman et al., 2020). In the islets of non-obese diabetic mice, IL-21 controls SOCS1 expression in autoreactive CD8⁺ T cells, but it is not needed for CTL activity acquisition (Sutherland et al., 2019).

Molecular mimicry and infectious agents

Molecular cross-reactivity is the existence of common bacterial and pathogenic or toxic agent epitopes in molecular response to Microorganisms that mimic the host molecules, which is known as pathogenesis. The molecular mimicry was seen to exist in various ways, including complete protein identification, protein level homology, amino acid sequence similarity, and structural similarity. The mimicry between *Campylobacter jejuni* lipooligosaccharide (LOS) and human GM1 ganglioside, as well as the mimicry between the dietary antigen butyrophiline and myelin oligodendrocyte glycoprotein, are two examples of molecular mimicry (Peterson and Fujinami, 2007).

Group A Streptococcus (GAS) antigens stimulate antigen-specific B and T cells after GAS adhesion to and penetration of the pharyngeal epithelium. An autoimmune reaction may result from a molecular mimicry between GAS group A carbohydrate or serotype-specific M protein and the host heart, brain, or joint tissues, which triggers the clinical manifestations of acute rheumatic fever (ARF) (Carapetis et al., 2016).

It has been shown that viruses such as Epstein-Barr virus (EBV), Coxsackie virus, and human T-lymphocyte virus (HTLV) are linked to the development of SLE (Singh et al., 2013). In lupus patients, EBV infection causes the synthesis of the viral protein Epstein-Barr virus nuclear antigen-1 (EBNA-1) antibodies, which cross-react with lupus-associated autoantigens such as Ro, Sm B/B', and Sm D1 (Poole et al., 2006). In SLE patients, anti-C1q antibodies against the main antigenic residues of C1q known as A08 directly cross-reacted with the EBV-related EBNA-1-derived peptide EBNA348 (Csorba et al., 2019). Anoctamin 2 participates in an EBNA-1-associated molecular mimic, which is a decisive etiopathogenesis factor in MS (Tengvall et al., 2019). It was shown that the Coxsackievirus B1 virus was correlated with an enhanced risk of β -cell autoimmunity. CVB3 and CVB6 were not previously thought to be associated with β -cell autoimmunity; however, the new research found that CVB3 and CVB6 positively affect decreasing it (Laitinen et al., 2014).

HTLV-1 infection results in antibody response to HTLV-1-tax protein. Tax-specific antibodies, which are immunodominant for its C terminus, cross-react with the M9 sequence of heterogeneous nuclear ribonuclear protein A1 (hnRNP A1), expressed by CNS, especially corticospinal neurons. This immune response inhibits neuronal firing, potentially leading to neurodegeneration and death (Lee et al., 2005).

A particular immunologic cross-reactivity was seen in the human acetylcholine receptor (HuAChR) alpha-subunit 160–167 residues, with the homologous domain of herpes simplex virus (HSV) glycoprotein D, residues 286–293. This cross-reaction supports the hypothesis that HSV being associated with the onset of the myasthenia gravis (Schwimmbeck et al., 1989).

Epitope spreading

Epitope spreading (ES) phenomenon is the emergence of an immune reaction to epitopes other than the disease-causing epitope that are non-cross reactive (Powell and Black, 2001). ES is not a trait exclusive to systemic autoimmune maladies. Additionally, it has been identified in T cell-dependent organ-specific autoimmune disorders such as T1D, MS, and EAE (Monneaux and Muller, 2002). In autoimmune diseases, there are two main pathways as regards ES, including independent and dependent. Tissue injury, inflammation, and cytokines provoke T cells to detect cryptic epitopes and trigger B cells in the “independent” pathway. A “dependent” pathway, on the other hand, is dependent on the processing and presentation of physical antigens to activate T and B cells (Didona and Di Zenzo, 2018). ES can also be induced in response to some pathogen; antigen release and intermolecular ES may occur due to viral and other cytolytic infections (Ruff et al., 2020). In EAE mouse models, an investigation was conducted to indicate that naive T cells are recruited to the inflamed CNS and are activated by local APCs such as DCs, beginning ES (McMahon et al., 2005).

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Immunologic Memory

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Glossary

Bacteriophages A virus that infects and replicates within bacteria and archaea.

Variable lymphocyte receptors (VLR) A group of receptors with the Leucine-rich repeat (LRR) property which mediate adaptive immune responses in the jawless vertebrates, lampreys and hagfish.

Natural killer T cell (NKT cells) A heterogeneous group of T cells that share properties of both T cells and natural killer cells which recognize an antigen-presenting molecule that binds self and foreign lipids and glycolipids.

Gamma delta T cells ($\gamma\delta$ T cells) A less common subset of T cells that have a distinctive antigen receptor (made up of one γ and one δ chain) and they are mainly located in the gut mucosa and have prominent role in recognition of lipid antigens.

Mucosal associated invariant T cells (MAIT cells) A subset of T cells with special MHC class I-like protein presenting foreign antigens like bacterially produced vitamin B metabolites.

B1 cells A subset of B cells with marker profile of CD20 + CD27 + CD43 + CD70- recognize and produce antibody against more types of antigens including intracellular antigens.

Marginal zone B cells This subset of B cells with profile CD19 + CD27 + IgD + IgM^{high} is non-circulating mature B cells that locate in marginal zone of the secondary lymphoid organs.

Piwi-interacting RNA (piRNA) This is the largest class of small non-coding RNA molecules expressed in animal cells and are mostly involved in the epigenetic and post-transcriptional silencing of transposons and other repeat-derived transcripts.

Follicular helper T cells (TFH) This subset of CD4 + T cells are antigen-experienced T cells in B cell follicles of secondary lymphoid organs and upon cross-signaling with their cognate B cells, TFH cells trigger the formation and maintenance of germinal centers.

Rh blood group, D antigen (RHD) This membrane protein is cluster of differentiation 240D (CD240D) that expressed in erythrocyte and define blood group system.

Toll-like receptors (TLRs) These single, membrane-spanning receptors are a class of proteins that play a key role in the innate immune system that recognize structurally conserved molecules derived from microbes.

Cytosine triphosphate deoxynucleotide oligodeoxynucleotides followed by a guanine triphosphate deoxynucleotide (CpG) This is a unmethylated short single-stranded synthetic DNA molecule that contain a cytosine followed by a guanine which a phosphodiester link between consecutive nucleotides.

Fragment, crystallizable region (Fc) receptor This is a protein found on the surface of certain immune cells bind to antibodies that are attached to infected cells or invading pathogens.

Historically the ability of memorization in the immune system was the first characteristic that hints early scientists nearly 2500 years ago to propose the presence of immunity. Greek historian "Thucydides" mentioned the powerful secondary response survivor of the plague of Athens in the returns of the disease around 430 BC (Doherty and Robertson, 2004). Theory of recognition and remembering previously encountered diseases, however, described as adaptive immunity by "Rhazes" 930 CE when he refers to when a disease affects only children in a family but not affecting the parents caring for the patient (Doherty and Robertson, 2004).

During the last 50 years, we now define the immunologic memory as the second goal of immune components after successful primary immune response and termination of infection using short-lived effector cells. To achieve this goal, immunity creates a quantitatively and qualitatively advanced response (with higher speed or more robust and stronger) than the first encounter. Until less than a decade ago, our knowledge regarding immunologic memory was restricted to T and B cells since they were capable to rearrange their cell receptors for unique antigen specificity, and generate long-lived memory cells, however, the new evidence suggests that prokaryotes and other eukaryotes without these adaptive immune cells can also possess memory traits (Table 1) (Sun et al., 2014).

Table 1 Immunological memory from prokaryote to higher vertebrate.

Immunity	Prokaryotes	Invertebrates eukaryotes	Lower vertebrate eukaryotes	Higher vertebrate eukaryotes
Receptors	CRISPR–Cas (genetic elements)	Toll, DSCAM, (antigen receptor)	VLRA, VLRB, VLRC (antigen receptor)	TCR, BCR (antigen receptor)
Mechanism of diversity	CrRNA	?	Leucine-rich repeats	VDJ recombination
Specificity	Yes (sequence specific elements)	Yes	Yes	Yes
Immune memory	Yes	Yes	Yes	Yes

CRISPR: clustered regularly interspaced short palindromic repeats, CAS: CRISPR Associated protein, CrRNA: CRISPR transcript, DSCAM: Down syndrome cell adhesion molecule, VLR: variable lymphocyte receptor, TCR: T cell receptor, BCR: B cell receptor, VDJ: variable, diverse, and joining regions.

Immunologic memory in prokaryotes

Prokaryotes including ~50% of bacteria and almost all archaea present an adaptive immunity against infectious genetic elements (e.g. viruses, bacteriophages and plasmids) using CRISPR–Cas (clustered regularly interspaced short palindromic repeats–CRISPR associated) system (Labrie et al., 2010). CRISPR–Cas is a complicated mechanism that prokaryotes use to silence foreign nucleic acids upon the re-infection of the specific invader. CRISPR genomic loci are like a library in the genome of prokaryotes recording, in a very well managed format, a segment of infectious nucleic acids originated from viruses or plasmids in the first encounter (Westra et al., 2012). The process of adaptation and incorporating of the foreign nucleic acids is known as spacer acquisition. Subsequently, the CRISPR locus is transcribed to produce a transcript of captured foreign nucleic acids that becomes processed into short RNAs (Charpentier et al., 2015). This transcribed short RNAs at the time of the second infection can target the foreign nucleic acids and interfere with their complementarity sequence with the help of Cas proteins (Fig. 1) (Brouns et al., 2008). Although 20% of CRISPR library contains spacers originating from the host's genome suggesting other functions of the CRISPR–Cas in endogenous gene regulation, spacer acquisition was evident to be significantly dependent on DNA replication and invader-derived nucleotide acids have 100–1000-fold higher affinity for acquisition versus self-derived spacers (Levy et al., 2015).

Immunologic memory in invertebrates and low vertebrates

In eukaryotes also recent evidence changed our previous thoughts that only early jawed vertebrates pose adaptive immunity due to attaining the antigen receptor diversification machinery. Although in 1960s some experiments on “sea lampreys” suggested antigen-specific agglutinating antibody and delayed-type hypersensitivity against bacteria and second skin allografts (Finstad and Good, 1964), after almost 20 years variable lymphocyte receptors (VLRs) in primordial lymphocyte-like cell subsets and receptors (mainly in the surviving jawless fish or agnathans) were described (Boehm et al., 2012). Despite the common consensus for evaluation of innate immunity in invertebrates (>97% of known species in the animal kingdom), in the early 21st century an adaptive immunity and resistance to subsequent infection were discovered in crustacean species against a parasitic tapeworm (Kurtz and Franz, 2003). During the recent 15 years, more evidence has been provided regarding adaptivity immunity in invertebrates including somatic re-combinatorial diversity (in immunoglobulin superfamily domains of hemolymph proteins of freshwater snail) (Zhang et al., 2004), pathogen-specific splice variant repertoires (of pattern recognition receptor in Anopheles mosquitoes against malaria (Dong et al., 2012), stronger established Toll pathway (in primed fruit fly against secondary bacterial challenge with *Streptococcus pneumoniae*) (Pham et al., 2007) and improved circulating granulocyte numbers (in the Gambiae mosquitoes following the primary infection against *Plasmodium falciparum*) (Rodrigues et al., 2010). Another sophisticated adaptive immune response has been labeled as “olfactory imprinting” where immune networks of invertebrates (mainly worms) activated during first encounter with pathogen influence nervous system memory (e.g. dopaminergic neurons/receptors or epigenetic memory mediated by piRNAs) to avoid subsequent toxic exposure (Anyanful et al., 2009; Inoue et al., 2013; Shirayama et al., 2012). Although many aspects of the nervous system–immune system cross-talk remains to be clarified, evidence such as these indicate that efferent neural circuits that recognize injury or infection modulating immune reaction and impact immune memory (Tracey, 2009).

Non-B non-T immunologic memory in higher vertebrate

A strong plethora of studies supports the importance of innate immune cells crosstalk for the development and programming of the adaptive immune system. New additional insights, however, identified intrinsic immunologic memory of the RAG-dependent “innate” lymphocytes (NKT cells, $\gamma\delta$ T cells, mucosa-associated invariant T cells or MAIT, other “non-classical” T cells, B1-B cells and

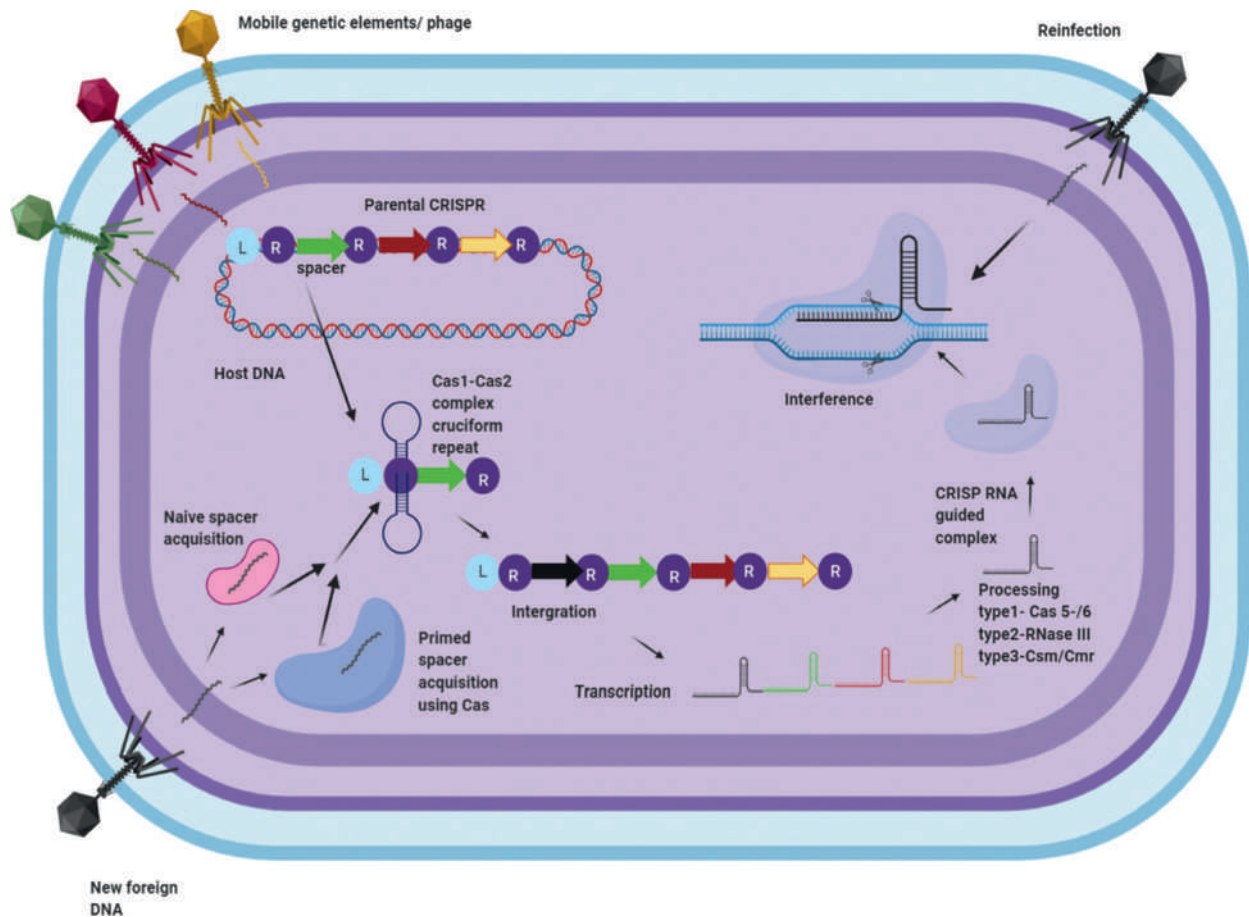


Fig. 1 CRISPR defense mechanism in prokaryotes including bacteria and archaea including spacer acquisition (naive or primed Cas dependent), integration, transcription, processing (three different types) and interference providing general adaptive immunity with memory against invader genetic elements. R = repeated sequences, L = leader sequences, arrows = incorporated spacer sequences.

marginal zone B cells) and RAG-independent “innate lymphoid cells” (ILC1-NK cells, ILC2, and ILC3) (Spits et al., 2013). These data showed that these innate cells may not restrictively respond rapidly and non-specifically with the production of short-lived cells, a phenomenon that further blurring the previous misconception that separated innate from adaptive immunity. Still, the physiological mechanism for imprinting at a genetic level by initial stimulation and epigenetic modifications of these cells is not very well illustrated. However, the first line of evidence has been rooted in the role of NK cells in hybrid resistance and intense/rapid rejection in a pre-treated receiver of parental bone marrow graft compared to graft from the other parent (Cudkowicz and Stimpfling, 1964). In the following years after this experiment, immunologists discovered NK cell specificity and memory against a wide range of antigens (particularly in anti-viral immune responses) including species-specific limited-repertoire against cytomegalovirus which can lead to the generation of long-lived memory NK cells (Sun et al., 2009; Sun and Lanier, 2009). Interestingly, memory NK cells could perform self-renewal (persist for years by maintaining IL-15 signals), and susceptibility to locate within the spleen and create better secondary responses (by production of activating CD94/NKG2C receptor, IL-12 and STAT4 early pro-inflammatory cytokines and producing IFN- γ more robustly than resting NK cells, Fig. 2) (Firth et al., 2013; Foley et al., 2012; Romee et al., 2012; Sun et al., 2010). Further, this notion was backed up with studies that evaluated NK-cell clonal proliferation and memory in a wide range of viral models in mice, including vaccinia virus (Gillard et al., 2011), influenza virus (van Helden et al., 2012), and herpes simplex virus infection (Theyerl-Abele et al., 1990). Moreover, CXCR6-expressing NK cells residing in the liver in primed RAG-deficient mice with specific antigens of viral infections (e.g. influenza and HIV) can result in secondary challenge protection (Paust et al., 2010). Memory function of $\gamma\delta$ T cells against homologous *Listeria* bacteria challenge, but not against a heterologous bacterium (Sheridan et al., 2013), modulated longevity of macrophage or dendritic cell subsets like Langerhans cells with longer half-life tissue residency due to their adopted Toll-like or Nod-like receptors or intracellular sensors (Sun et al., 2014) suggesting that other innate immune cells and even cells derived from the myeloid lineage have characteristic resembling adaptive immunity.

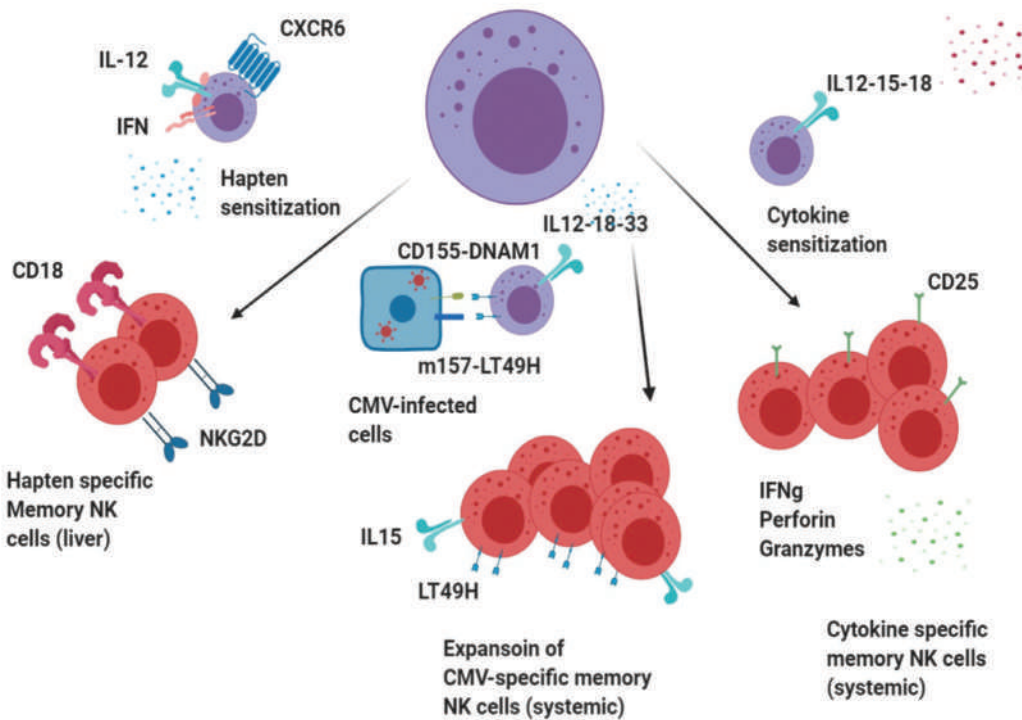


Fig. 2 Pathways of priming of adaptive immune and memory cell generation in NK cells during Hapten-sensitization, cytokine stimulation (increased inflammatory response after cytokine priming of IL12, IL15 and IL18) and viral infections (long-lived cells after contraction phase dependent of IL-15 and microRNA-155).

Humoral immunologic memory in higher vertebrate

Given the cornerstone role of B and T cells in the adaptive immunity of higher vertebrates up to mammals, immunologic memory is tightly connected with the function of these cells. The first important component that provides non-lifelong antigen-specific protection for a long period is short-lived plasma cells (PCs) since that can generate high-affinity pathogen-specific antibodies in the reticuloendothelial system and mucosal surfaces preventing reinvasion for several months. Their produced antibodies harmonize innate and adaptive immunity and improve immediate neutralization and rapid opsonization against seasonal viral infection which minimizes the pathogen load in wintertime. However true memorization can be assigned to long-live PCs located in bone marrow (which produce less compared to short live PC). These long-live PCs make the steady-state level of immunoglobulin in the healthy individual and they receive their long-term survival signal by interaction with IL-6 secreting stromal cells. However, if the secondary pathogenic encounter happens, the new antibody-antigen complex enhances the apoptosis in old PC in bone-marrow and replace them by competition toward stromal cells by newly generated more-specific PCs (Nutt et al., 2015).

A higher level of complexity in adaptive immunity poses by long-lived memory cells which can both be identified in humoral and cell-mediated memory. B and T memory cells develop inside of secondary lymphoid tissues and originate during the first activation of naïve T and B cells in the primary response. If these memory cells survive till the secondary infection their stochastic frequency is 10–100 folds higher than naïve compartment with the same T cell or B cell receptors. Memory cells signaling machinery retain the ability of response with accelerated kinetic compared to naïve cells (higher co-stimulatory molecules and therefore receive more cognate interaction for proliferation), improved antigen presentation (in case of memory B cells [MBC] higher level of MHC-class II complex) and have a better antigen recognition due to prior somatic hypermutations and improved affinity. These advantageous mechanisms in MBCs together with their anatomical localization facilitate their rapid differentiation toward new PCs generation within 4 days in secondary immune response (still with capacity for class switching recombination and further somatic hypermutation for better refinement) compared to 8 days in the primary response. Another important inhibitory mechanism by Fc receptor $\text{Fc}\gamma\text{RIIB1}$ on naïve B cells suppressing activation of naïve B cells which guarantees the proliferation of MBCs in the secondary antigen exposure and continues the repetition of antibody development for successive infections with the specific pathogen by age. Altogether the memory generation leads to decreasing the rate of infection in children (normally with 5–10 episodes per year) compared to adults (normally with 0–2 episodes per year) (Kurosaki et al., 2015).

Regulation of MBCs and effector PCs differentiation in the first infection takes place in two anatomical places of secondary lymphoid organs including pre- and post-germinal center (pre-GC and post-GC). In the pre-GC responses, at the T-B border region activated B cells choose between initiating the GC, short-lived PCs or MBCs generation, but in post-GC, the fates of activated B cells

are migration to the dark-zone, long-live PCs or MBCs generation (MacLennan et al., 2003). Recent studies showed that most of the clones against different epitopes of specific pathogens have only one fate (only 10% of clones can generate fate subsets) and this is tightly connected with the strength of signals that B cells receive from the receptor (Fig. 3). The stronger affinity of antigen receptor determines the PC generation (by enhanced expression of BAFF receptor, RelA, Blimp1, PRDM1, IL-4 and anti-apoptotic factor MCL-1) and intermediates signal favor GC- or dark-zone migration, in pre or post GC responses (by increased expression of BCL-6), respectively. Of note, if the signal of B cell receptor is slightly over than apoptotic threshold, then it presents the memory fate (with lower metabolic activity and less specific antigenicity, low IRF4 expression, upregulated BCL-6 in response to IL-21 and expression of MITF, PU.1/IRF8, and BACH2) (Shinnakasu and Kurosaki, 2017).

MBCs constitute multiple subsets but all express CD27 on their surface and they are reactive in secondary lymphoid tissues with cognate interaction with pathogen-specific effector CD4 TFH cells to produce new MBCs with higher average affinity and effector PCs. IgM only (IgM + IgD-CD27+) and class-switched (IgD-IgM-CD27+) MBCs are GC-dependent and thus they classify as classical MBCs with somatically mutated Ig V genes. However, IgM + IgD + CD27+ MBCs can be both the result of T-dependent GC response (the majority of this subset with BCL6 expression) and non-MBCs independent of GC (a minority of this subset with TLR signaling dependency and location in the marginal zone of spleen and bone marrow) (Tangye and Good, 2007). Among classical MBCs, IgM-only MBCs are longer-lived, propagate more and re-enter secondary GC for further class switching and diverse their BCR (higher level of FOXP1) but class-switched MBCs disappear faster and preferentially direct to PCs generation (higher level of IRF4, CD80 and PDL2) (Pape et al., 2011). Typical MBCs recirculate between secondary lymphoid organs (expressing RUNX1) and they localized later to inflamed tissue (expressing TBX21 and CXCR3) or re-enter GC in a secondary infection (expressing CCR6). IgA + MBCs also prefer localization in the mucosal epithelium and mucosa draining lymph node (expressing ROR α and CCR1/CCR5). Of note, IgE+ MBCs rarely arise from subsequential switching since autonomous GC formation toward IgE signaling actively prevent IgE+ MBCs and long-term PCs by apoptosis. Therefore, the IgE level of normal individuals is the lowest level of serum immunoglobulin (Talay et al., 2012).

Of note, the memory process of humoral immunity gain insight toward several pathologic events and therapeutic strategy. A physician can treat hemolytic anemia of the newborn due to discordant Rhesus D between mother and fetus by injection of IgG anti-RhD (RhD) antibodies or RhoGAM which mimic secondary immune response and suppress activation of naïve B cells and

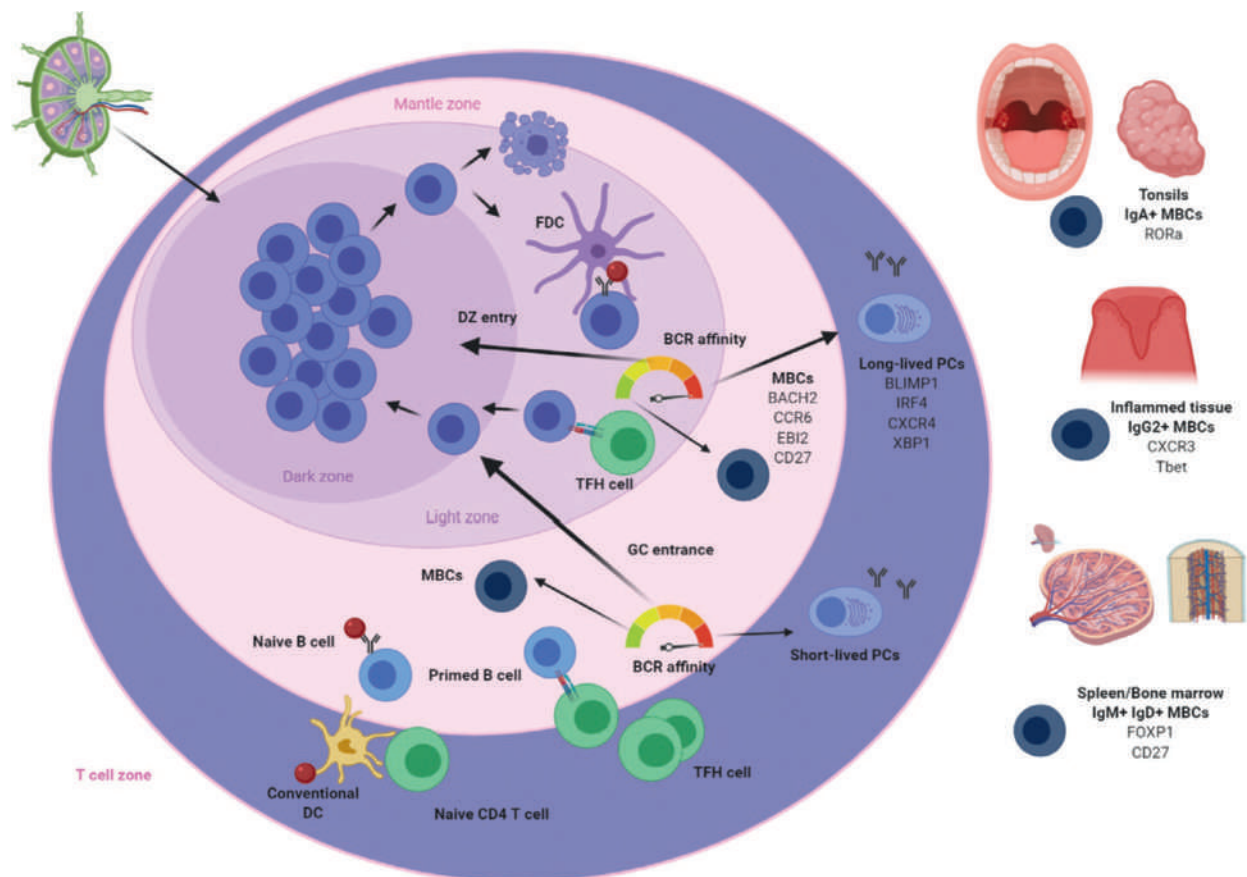


Fig. 3 Pathways of priming of naïve B cells and relocation of the generated memory B cell (MBCs) based on the threshold of B cell receptor (BCR) signaling in pre and post germinal center (GC) reactions. BCR = B cell receptor, DC = dendritic cells, DZ = dark zone, FDC = Follicular dendritic cells, PC = plasma cells.

MBCs generation in mothers with RhD-. This should be performed when they might encounter red blood cells of their RhD+ fetus at 28th week of pregnancy and 3 days after delivery to prevent MBCs generation (Brinc and Lazarus, 2009). The suppression of naïve B cells activation in the secondary immune response is also challenging for pathogens that change their epitope to escape immune response, mainly influenza virus, and it may lead that after several years all epitopes will be not-recognizable for MBCs and cause full-blown influenza and simulate primary immune response since they need activation of naïve B cells (Chen et al., 2018). Although self-reactive post-GC class-switched MBCs are more abundant in peripheral blood of normal individuals, proliferation of an activated group of CD27-IgD-CD95+ MBCs with a predisposition to differentiate into IgE+ PCs correlates with biomarkers in serum and disease severity of autoimmune disorders (Butt et al., 2015).

Cellular immunologic memory in higher vertebrate

During a primary response of cellular immunity, 95% of each activated naïve T cells (1 to 50,000 cells proliferation) will be effector and only 5% has potential for long-term survival and escape apoptosis during contraction phase as memory T cells (MTCs expressing IL7R and BCL2 which is essential for the cell renewal). These cells persist in either secondary lymphoid organs (central MTCs) or in the recently infected peripheral tissue (effector MTCs and tissue resident MTCs, Fig. 4) (Wakim and Bevan, 2010). Central MTCs express L-selectin or CD62L and CCR7 chemokine receptors which allow them to enter secondary lymphoid organs and being activated slowly by antigens presented and cytokine produced by antigen-presenting cells. Central MTCs also presents a higher tendency for activation combined with increased capacity for IL-2 secretion, cellular expansion and differentiation to T effector cells. In contrast to central cells, effector and tissue specific MTCs are already differentiated and do not circulate through lymphoid organs but due to expression of CCR6, CCR4, CXCR3, CCR5 can enter mucosal tissues and inflamed regions patrolling peripheral tissues response immediately to in site infection (Pepper and Jenkins, 2011). MTCs have common features including evidence of previous activation, independency to cognate antigen and co-stimulation with CD28 (compared to naïve compartment T cell) and enhanced function upon re-exposure to the specific antigen. MTCs is well-studied in CD8+ compartment (surface

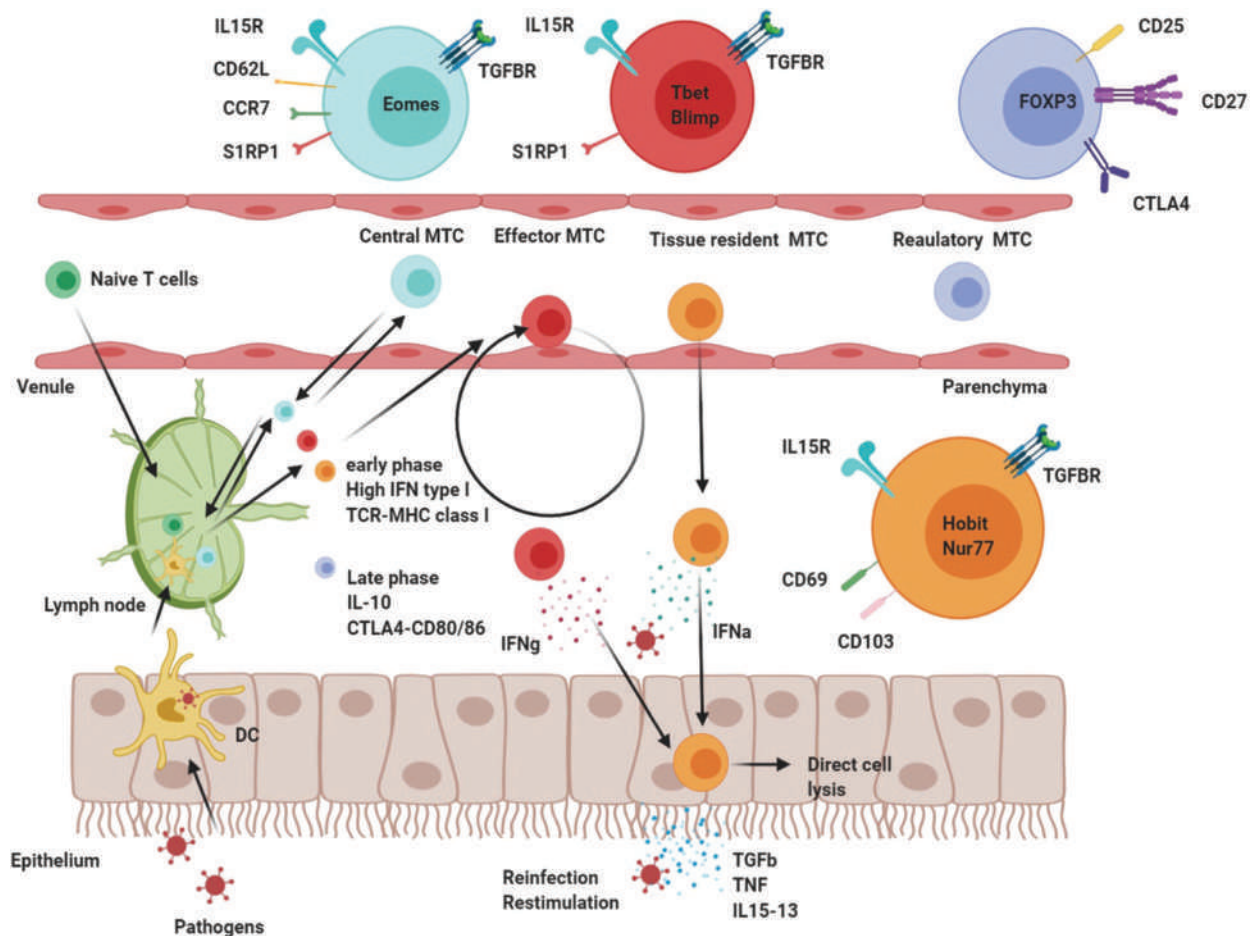


Fig. 4 Pathways of priming of naïve T cells and relocation of the generated memory T cell (MTCs) based on the phase of infection and the function of generated MTCs.

markers, epigenetic profile and metabolic states), but less characterized in CD4+ T cells due to high inherent plasticity and diverse polarity (TH1, TH2, TH17, TFH) as well as rapid decline in number over time compared to CD8+ MTCs (Chang et al., 2014). Contrary to MBCs, MTCs and naïve T cells both can be stimulated in the secondary immune response but MTCs contribution is more pronounced. Among MTCs two other subsets have been discovered recently including stem cell MTCs (low level of IFN- γ and IL2 with potential to self-renew and the multipotent programming machinery to diverse polarization of effector and memory subsets) and regulatory MTCs (previously activated FOXP3 T cells required for mitigation of tissue damage during the function of pro-inflammatory MTCs and reinforcing of fetal tolerance during pregnancy) (Rosenblum et al., 2016).

Intriguingly, the memory process of cellular immunity gain insight toward several pathologic events and therapeutic strategy. How MTCs are produced and kept long-term is an essential question were can help immunologist to treat autoimmune disorders using regulatory MTCs and cancer and metastasis using tissue resident MTCs. By studying MTCs we understood that systemic vaccine can be improved by the concomitant injection of adjuvant molecules into the targeted peripheral tissues or used for tumor treatment (Mueller and Mackay, 2016; Rosenblum et al., 2016). Few studies also suggested that treatments promoting the generation of stem cell MTCs could induce more durable and effective T cell-based immunotherapies (Kugelberg, 2016).

Using the state of art of humoral and cellular immunological memory, the theory of protection before infection (vaccination) becomes reality before knowing the complete mechanism. Induction of primary immune response and generation of immunological memory with a harmless form of the pathogens stimulate protective adaptive immunity which has been practiced in China 1000 CE using crusts of smallpox lesion (Doherty and Robertson, 2004). In 1796 these practices were reconsidered by Edward Jenner using variolation (variola: Latin name for pustule, intranasal or intradermal confrontation with pustule of the patient with risk of death $\sim$ 1:100) or inoculation with cowpox (vacca: Latin name for cow, due to antigen mimicry of natural safe relative) to prevent smallpox infection. Hence the term vaccination arose and lead to the global eradication of smallpox in the 20th century due to long life immunity. However long-life immunity is not achievable for all pathogens since this aim needs vaccination via the same pathologic route of infection, conserved antigenicity of the pathogen and no animal reservoir for the pathogen. Moreover, immunologic components including memory PCs and MTCs against those pathogens should remain in the circulation (e.g. measles and smallpox stay for 75 years) but for the majority of pathogens due to non-optimal vaccination conditions (antigen-independent survival due to IL7 and IL15 signaling are dropped down e.g. diphtheria halved after 19 years), the immunization should be repeated for maintenance or re-generation of memory cells.

Application of immunologic memory and vaccination

Louis Pasteur and Emile Roux in 1880 showed immunity could be induced against cholera using attenuated cultures (Doherty and Robertson, 2004) which provide inactivation of the pathogen virulence while preserving its immunogenicity. Live attenuated vaccines were later produced by an infection in non-human cell lines to adopt them with new cell lines. A mutant form of live viruses that grow poorly in humans were also other versions of attenuated vaccines that pose a better immunity compared to totally inactivated viruses since they infect to some extent target cells mimicking real infections. Among those anti-viral vaccines, oral polio vaccine (OPV, sabin), measles, mumps and yellow fever are well known worldwide. Some anti-bacterial vaccines (e.g. bovine strain against *Mycobacterium tuberculosis* and salmonella typhi with a mutation in an enzyme necessary for LPS synthesis) are nowadays in common medical practices. Killed or inactivated pathogens can be produced as vaccines using chemical components (with formalin) or physical methods (with heat or irradiation) against viruses (influenza, rabies, polio in IPV from) bacteria (formalin embedded bacterial toxins diphtheria and tetanus called toxoids and *Bordetella pertussis* cause of whooping cough) (Greenwood, 2014). Combination of inactivated and live attenuated virus were also gain some achievement to solve side-effect of OVP Sabin strain 3 and back-mutation making wild type polio during vaccine manufacturing or after vaccination in prolonged replication in an individual with immunodeficiency. Recently the new strategy of combining IPV and OPV has been suggested to make the first dose with an inactivated vaccine which may generate memory and antibodies again strain 3 and then boosting vaccination can be continued with trivalent OPV (Tang et al., 2018). The other generation of vaccines is recombinant DNA technology to produce a subunit vaccine. This new method usually targets surface molecules of the pathogens to generate neutralizing antibodies with the highest protective repertoire to discover superior antigens. The most significant examples are HBV vaccine using surface antigen from baker's yeast culture (85% of vaccinated people are protected), rotavirus (against 5 major cattle or most common VP4 and VP7 assortment) and meningococcus vaccine.

To improve the quality and quantity of memory generation vaccination conjugate and the adjuvant vaccine can be helpful. To stimulate high-affinity antibodies against polysaccharides antigen and improve feeble T-cell independent B cell response which gave rise to low-affinity IgM antibodies and no MBCs, som carbohydrate from the other capsids of encapsulated bacteria (including pneumococcus, *Salmonellae* spp., meningococcus, *Hemophilus influenzae*, *Escherichia coli* and *Klebsiella* spp.) can be conjugated by tetanus or diphtheria toxoid. This method improves the interaction of naïve B cell-specific for an epitope of bacterial polysaccharide by endocytose of the intact conjugate and accelerates processing of the toxoid. Therefore, they present peptide antigens to the activated toxoid specific TFH which drive GC reactions and produce high-affinity antibodies and long-term MBCs. The adjuvant component can be added to stimulate innate immunity via TLR and pattern recognition receptors to mimic inflammation required for the initiation of the adaptive immune response. Mainly alum aluminum hydroxide is the adjuvant used in tetanus or diphtheria vaccines (DT vaccine), although this adjuvant is not effective as conjugate form with pertussis carbohydrate (DTP). Other newly

invented adjuvants include oil-in-water MF59 and virosomes, TLR agonists (CpG nucleotides, PolyIC RNA analogs, flagellin, Pam3Cys lipopeptide) which may improve vaccination quality in the future (Zimmermann and Lepenies, 2015).

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Inflammation[☆]

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Introduction to inflammation

Understanding the role of inflammation as a function of clinical disease states has become invaluable in design of treatment modalities and the development of therapeutics. A greater knowledge of inflammatory responses at all levels of disease is critical for health care workers, physicians, and students of all biological systems. Inflammation is critical for everyday health and homeostasis, without which the body cannot discern regular biological turnover and events from outside interference that causes harm. I.R. Cohen stated it most directly when he wrote “The function of the immune system is to produce inflammation commensurate with the body’s need to keep itself fit for living” (Segel and Cohen, 2001).

There is a basic assumption that the objective of the immune response is to protect an individual from pathogenic assault. However, when closely examined, inflammation as part of the immune system involves factors for both protection and health maintenance. Protection is the outcome of an immune-regulated response to non-host molecules, with the end result triggering production of factors involved in effector processes. Inflammation is a by-product of those early induced events, where it functions primarily to elicit reactive mechanisms that clear pathogens. Longer term, well-regulated inflammation is also critical for homeostasis. These pathways are critical for effector maintenance, such as normal cellular turnover during aging, healing of tissue due to trauma, and keeping the immune response in a “ready” state of surveillance to guard against unwanted pathogenic components that may enter the body.

Inflammation: A working definition

It is critical to define “inflammation” to allow discussion of responses to be placed within a clinical or pathological setting. A simplistic working definition includes the response of living tissue to damage or assault of any kind. At its root, inflammation

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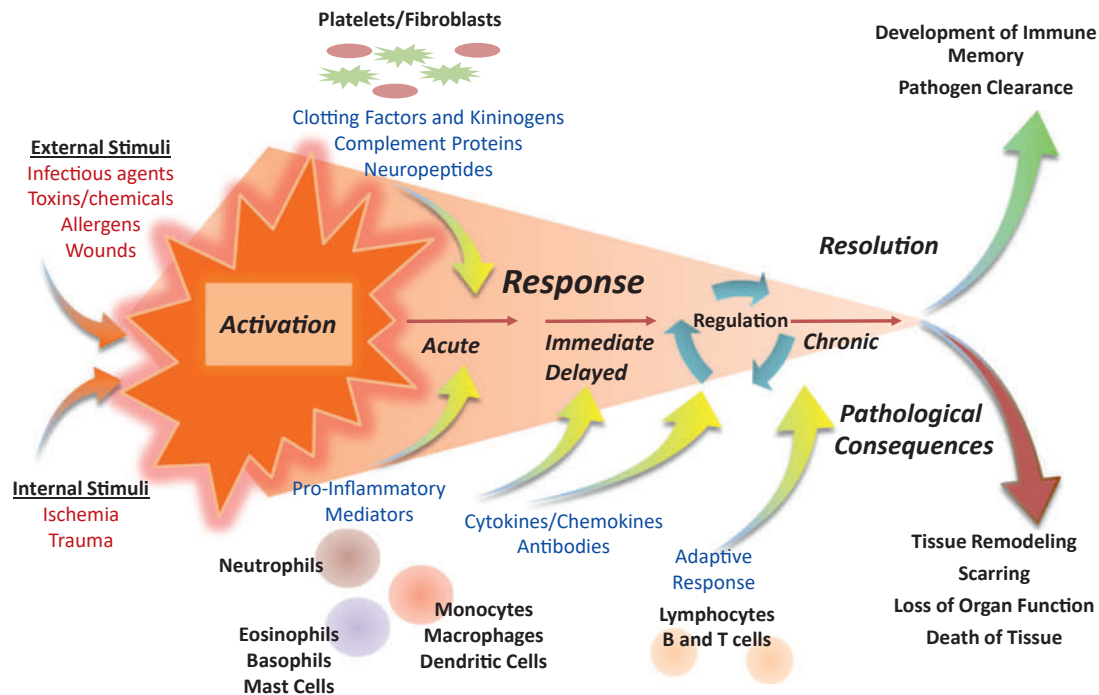


Fig. 1 Biological elements of inflammation. Multiple parameters drive the inflammatory response, leading to development of pathological consequences and clinical disease states.

represents a response to insult of or to vascularized tissues, with a purpose to deliver defensive materials (blood cells and fluid) to a site of injury (Kumar et al., 2017; Actor, 2014; Murphy and Weaver, 2017; Owen et al., 2013). Main components of inflammation revolve around vascular reactions and cellular responses that activate and release chemical or cell-derived mediators. A strong feedback mechanism controls active participation of leukocytes and factors involved in maintaining healthy tissue status (Fig. 1). Upon injury, released factors in the form of chemical mediators diffuse from the region to function on vascular beds, triggering endothelial cells to alter surface conformation to manipulate delivery of circulating factors and cell populations into subvascular space. A process ensues whereby chemotactic factors allow activation in localized blood vessels (capillaries, venules, or arterioles) to permit diapedesis of specific leukocytes into the underlying parenchyma. Cells migrate toward inflammatory sites by following a chemotactic gradient. In a similar manner, blood-borne components carried in exudate accumulate outside of vascular beds, result in the delivery of serum factors essential for a functional response. The optional endpoint of this feedback reaction is resolution of tissue damage, and protection of the host from outside elements (pathogenic or physical). However, the inflammatory response may be caustic in and of itself; if it persists or triggers damaging adaptive immune function it may result in extended deleterious pathology.

Historically defined acute inflammation

The clinical consequences of inflammation are varied and unique to each organ or tissue. Why then ascribe a single condition of “inflammation” to the processes that lead to this damage? The combination of signs and symptoms observed following injury or illness are visible and consistent. The word “inflammation” is derived from the Latin “inflammare”, which literally means “to set on fire.” Greek literature suggests that ancient healers had an understanding of inflammatory response. Indeed, the first written definition of the condition was provided in the 1st century by Cornelius Celsus who noted four cardinal signs: *RUBOR ET TUMOR CUM CALORE ET DOLORE* (redness and swelling with heat and pain) (Rivas, 2010). It is now understood that redness occurs due to changes in localized blood flow (vasodilation); swelling occurs from influx of fluid and cells leaving blood vessels to enter tissue; heat is the result of increased blood arriving to areas of damage; and, pain results from released molecular mediators and edema (fluid accumulation) which increases pressure on, and activates, local nerves surrounding the damaged site. The concept that this response was more than tissue damage in response to injury was introduced in the 3rd century by Galen, whose description of “laudable pus” suggested that inflammation was beneficial and necessary for wound healing. The advent of microscopy in the late 19th century allowed for the identification of the cellular components of inflammation, further underscoring the role of infiltrating immune cells in the response. At this time, Virchow added a fifth “classical sign” of an inflammatory response—*FUNCIO LAESA* (loss of function) (Rather, 1971). It is this loss of tissue function that clinicians grapple with today.

A bird's eye view of the inflammatory process

Reaction to cellular injury can take multiple forms, due to initiating stimuli that either directly or indirectly manipulate responding cells in the area affected. The innate immune system is exquisitely sensitive to inflammatory triggering signals, and has the ability to quickly ramp up recognition upon real or perceived alterations in homeostasis (Kumar et al., 2020). It is now understood that infectious agents contain molecular motifs (e.g. danger signals) which trigger resting innate immune cells to initiate molecular activation of internal processes. However, it should be appreciated that physical agents, such as toxins and chemicals, can also activate similar responses. In fact, simple tearing of cellular membranes and subsequent release of phospholipids can result in vasoactive change (dilation or constriction) with the result being altered permeability for blood derived components to enter damaged areas (Galvão et al., 2018). Often, these signals arise from localized cell death due to ischemia, yet they can also be elicited by way of blunt physical trauma.

The initiation through stimuli induces further cellular responses, generally characterized as those mediated by locally residing innate cells (macrophages, dendritic cells or fibroblasts), and those recruited from vascular regions (neutrophils and specialized first responding lymphocytes such as natural killer cells). The cellular response is immediate, with explicit release of both preformed and induced substances from innate cell phenotypes. These effector factors (cytokines and chemokines) subsequently diffuse from the area of damage or danger to nearby blood vessels where they induce changes in endothelial cell membranes. A directed process of recruitment then occurs, based primarily on adhesion and migration properties of multiple circulating cell populations (Walzog and Gaetgens, 2000). Concurrently, fibrous matrix ladders assemble, providing a cellular thoroughfare for trafficking through subcapillary beds.

Overt changes in vascular permeability result in the delivery of a fluid exudate, which allows nutrients and oxygen to flow into areas of need. With respect to microorganisms, the sheer influx of fluid (edema) creates a safety buffer by diluting toxins. This vasodilation also permits fluids (serum) containing circulating antibodies, complement and acute phase proteins to accumulate within damaged tissue and submucosal spaces. The antibodies that are present combine with toxins, neutralizing their inherent destructive activities. Complement components, either by themselves or with the help of antibodies, directly lyse microorganisms, and coat them (opsonization) with breakdown products of the complement enzymatic cascade. This greatly assists in directed uptake and phagocytosis of encountered pathogens.

Fluid and cellular flow into damaged tissue is typically thought of as a one-way process, it should be noted that it is in fact dynamic. Signals from reactive components leave damaged sites and enter the blood stream with the purpose of systemic activation. Distant organs are affected, including those responsible for thermoregulation (e.g. the hypothalamic, pituitary and adrenal axis), and those responsible for production of additional enzymes and acute reactants (such as those produced by the liver). Over time, activated innate immune cells make their way to draining lymphatics and to lymph nodes where they condition lymphocytes for secondary hypersensitivity reactions. This step is critical for the adaptive second phase of response when lymphocytes are actively recruited to the area of inflammation; these lymphocytes have the capability to respond with exquisite specificity to non-host antigenic stimuli. It is this second wave of immune cells that are ultimately responsible for prolonged pathological consequences resulting from the initial stimuli. If the situation is limited in duration and scope, the acute reaction has minimal lasting effect. Resolution occurs with limited tissue reorganization. However, chronic persistence with repeated stimulation due to a contribution of innate and antigen-specific adaptive components can lead to progressive damage to tissue with lasting consequence and clinical scope.

Inflammation is thus a combination of desirable and non-desirable events, with these terms defined according to the underlying stimuli initiating reactivity. The clinical outcome is truly a balance between responses which benefit the health of the host, versus responses that lead to destructive pathology.

Inflammatory danger signals

PAMPs and DAMPs

Encounter with external stimuli can initiate the inflammatory response (Table 1), with the end result often dictating downstream (adaptive) immunity (Schenen and Medzhitov, 2011). Response to invading bacteria or viruses has evolved to combat their attempts at colonization. Basic phagocytic innate cells, macrophages and dendritic cells in particular, are hardwired to respond to motifs, or danger signals, present on and within microorganisms (Janeway, 1989; Akira et al., 2006). These signals are common pathogenic patterns (pathogen-associated molecular patterns; PAMPs) specific for non-eukaryotic cells, usually linked to pathogen survival. Examples of PAMPs include peptidoglycan and lipomannans, lipopolysaccharide and lipoteichoic acid, bacterial DNA, double-stranded RNA, and flagellin. Recognition occurs via unique surface or intracellular molecules expressed by phagocytes (e.g. mannose receptors, scavenger receptors, Toll-like receptors (TLRs), or C-type lectin receptors (CLRs)) which trigger subsequent endocytosis and/or internal activation (Brubaker et al., 2015).

Binding of the specific motifs to intracellular NOD-like receptors (NLRs), allows translation of encounter of the “danger” signal to initiate a protective proinflammatory response (Takeuchi and Akira, 2010), often via the assembly of an “inflammasome” (Dolasia et al., 2017; Platnich and Muruve, 2019; Triantafyllou, 2021). These are multicomponent molecular structures that link the bound NLR to the downstream activation of caspase-1; ultimately culminating in the production of small chemical mediators

Table 1 General inflammatory stimuli.

Type	Examples
Microbial infectious organisms	Pathogen associated molecular patterns Bacterial endo- and enterotoxins
Physical agents	Trauma Radiation Carcinogens
Irritants	Excessive temperature Foreign bodies (e.g. splinters, dirt) Corrosive chemicals Inhaled inorganic metals/dust Allergens
Tissue necrosis	Infarction and hypoxia Oxidative stressors

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(cytokines, chemokines) that work in concert to allow cells to communicate with each other and facilitate cross-talk. These molecules also lay the groundwork for destruction of the unwanted pathogen. Because of their varied nature, these mediators are quite pleiotropic and exert actions on multiple different cell types, often with overlapping and redundant functionality.

Realize that in addition to pathogens, danger signals presented by physical agents may also induce inflammation. Damage-associated molecular patterns (DAMPs) represent a second class of inflammatory inducing signals, usually host-derived biomolecules that initiate and perpetuate a noninfectious inflammatory response (Matzinger, 1998). In many cases, these “alarmins” are released by stressed tissue undergoing necrosis (Yang and Oppenheim, 2017; Rider et al., 2017). A class of molecules that should not be overlooked are those represented by foreign objects that trigger this type of stress inflammation. Irritants and corrosive chemicals (acids, alkalis, oxidizing agents) can cause cellular damage. This can also be seen in response to trauma, ultraviolet light, radiation, and even frostbite. In all of these examples, inflammation is elicited; the underlying mechanisms are initiated via damage to cellular membranes and/or apoptotic induced events. Released membrane phospholipids, combine with natural lipases to produce arachidonic acid, which then triggers prostaglandin and leukotriene production which in turn mediates vasoactive changes, endothelial cell permeability, and chemotaxis of polymorphonuclear cells. In fact, any cause of gross tissue necrosis, be it lack of oxygen or nutrients, inadequate blood flow due to infarction, or physical dysplasia, will initiate similar inflammatory episodes.

By definition, a toxin initiates a destructive process which often triggers inflammation. Bacterial toxins are biological virulence factors that prepare the host for colonization. A classic example is the lipopolysaccharide (LPS), a powerful endotoxin representing a portion of the outer membrane of Gram-negative bacteria. In contrast to endotoxins, bacterial exotoxins are soluble mediators released during bacterial cell lysis or destruction. A specific class of exotoxins, called enterotoxins, are especially toxic to the intestinal tract causing vomiting and diarrhea. Well defined toxins (neurotoxins, leukocidins or hemolysins) are classified in terms of the specific target cell or site affected.

The cellular players in the inflammatory response

To understand the development and control of the inflammatory response, it is imperative to understand the roles that each phenotypic cell plays in the process. A global chart for these cells is given in Table 2. Each individual cell type is further described to allow a context for activity in the overall process. These cells include the myeloid and lymphoid phenotypes, as well as fibroblasts, endothelial cells, and platelets. Many of these cell phenotypes exhibit great levels of functional plasticity, helping to shape the overall inflammatory environment (Galli et al., 2011).

Myeloid cells (monocyte/macrophages and polymorphonuclear cells)

Monocytes/macrophages

Monocytes and macrophages are directly involved in phagocytosis and intracellular killing of microorganisms (Bieber and Autenrieth, 2015). Macrophages are differentiated monocytes, which are one of the principal cells found to reside for long periods in the reticuloendothelial system. These monocytes/macrophages are highly adherent, motile and phagocytic; they marshal and regulate other cells of the immune system, such as T lymphocytes; they also serve as antigen processing-presenting cells. Upon

Table 2 Cells and general roles in inflammation.

<i>Phenotype</i>	<i>Morphology</i>	<i>Main effector function</i>	<i>Mediators of inflammation</i>
<i>Myeloid leukocytes</i>			
Neutrophil	PMN granulocyte	Phagocytosis and digestion of microbes; vaso-responsiveness	Leukotrienes, oxygen metabolites, complement activators, chemoattractants, defensins, hydrolytic enzymes
Eosinophil	PMN granulocyte	Immediate hypersensitivity (allergic) reactions; defense against helminths; vaso-responsiveness	Leukotrienes, cytokines, chemoattractants, peroxidases, major basic proteins
Basophil	PMN granulocyte	Immediate hypersensitivity (allergic) reactions; vaso-responsiveness	Chemoattractants, histamines, heparine, proteolytic enzymes, arachidonic acid, leukotrienes, prostaglandins
Mast cell			
Monocyte macrophage	Monocytic	Phagocytosis and digestion of microbes; antigen presentation to T cells; vascular permeability	Proinflammatory cytokines, anti-inflammatory cytokines, chemokines, vasoactive mediators, reactive oxygen metabolites
Dendritic cell	Monocytic	Antigen presentation to naïve T cells; initiation of adaptive responses	Proinflammatory cytokines; regulatory cytokines for T cell development
<i>Lymphoid leukocytes</i>			
B cell	Monocytic	Humoral immunity	Cytokines, immunoglobulins
Plasma cell	Monocytic	Terminally differentiated, antibody secreting B cell	Secreted immunoglobulins
T cell	Monocytic	Cell-mediated immunity (helper and cytotoxic); modulation of APC function (regulatory)	Regulatory cytokines, chemoattractants, cytotoxic factors (granzymes, perforins)
Natural killer T cell (NKT)	Monocytic (rare)	Cell-mediated immunity (lipids)	Cell-mediated immunity (lipids), cytokines, chemoattractants, cytotoxic factors
Natural killer cell (NK)	Monocytic	Innate response to microbial or infection; antibody dependent cell cytotoxicity	Cytotoxic factors (granzymes, perforins), cytokines
<i>Non-leukocytes</i>			
Platelet	A nuclear	Clotting and tissue repair	Prostaglandins, chemoattractants, coagulation factors
Fibroblast/keratinocyte	Elongated	Connective tissue, structural support	Chemoattractants, regulatory factors for PMN function
Endothelial cell	Monocytic	Vascular composition and blood fluidity; leukocyte trafficking; coagulation control	Vascular permeability, cellular adhesion factors, chemoattractants, platelet activating factor, prostaglandins, fibrinolytic constituents

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encountering different stimuli, monocytes differentiate into highly microbicidal macrophage (M1), or into immunosuppressive macrophage (M2) phenotypes (Arango Duque and Descoteaux, 2014; Arora et al., 2018). Macrophages are immediate responders during inflammation, changing phenotype in concert with immune evolution in response to threats and needs for homeostatic and surveillance tasks, as well as for tissue regeneration and repair (Lichtnekert et al., 2013). They bear functional pattern recognition receptors on their surface, as well as complement and antibody receptors, which make them key regulators for recognition of inflammatory stimuli. Upon activation, they secrete chemokines and cytokines that both regulate and attract other immune cells, as well as having strong impact on local vascular endothelium (Arango Duque and Descoteaux, 2014) as well as tissue repair and wound healing (Arora et al., 2018). Indeed, release of these inflammatory mediators strongly impacts the direction of subsequent immune function, both in a localized manner as well as systemically to affect tissues throughout the body. It should be stressed that while macrophages are critical in initial inflammatory responses, they also contribute to chronic responses. Finally, macrophages also regulate healing properties such as renewed vascularization of tissue, and wound closure (Hesketh et al., 2017).

Monocytic macrophages are called “macrophages” when they are in the connective tissue, spleen and lymph nodes. However, many submucosal macrophages have specific nomenclature, dependent upon tissue localization. For example, they are called Kupffer cells in the liver, Dust cells in the lung, Microglia in the central nervous system, osteoclasts in the bone, and Langerhans cells when found in the skin.

Dendritic cells

Dendritic cells (DCs) provide a link between innate and adaptive immunity by interacting with T cells in a manner to deliver strong signals for development of functional and memory responses (Yin et al., 2021). They represent an intriguing and diverse population (Steinman, 2012; Malissen et al., 2014) whose phenotypic control is under precise regulation (Hammer and Ma, 2013). They direct local responses during infection (Bieber and Autenrieth, 2015) and inflammation, as well as participate in the generation of matured responses post trafficking to lymph node tissues (Aguzzi et al., 2014; Chistiakov et al., 2014a). Immature DCs are called to

the site of inflammation via chemokines, in a complex signaling path which is dependent upon both physical location and necessity for specific triggering by other immune cells responding to the insult or assault (Chistiakov et al., 2014b). Dendritic cells recognize foreign agents and pathogens through a series of pattern recognition receptors (non-specific), and, are similar to macrophages in that they are able to present antigen to lymphocytes to direct maturation and immune functionality. They play critical roles within the skin (where they are referred to as Langerhan's cells) and have special properties to assist in wound healing and repair (Deckers et al., 2018; Gregorio et al., 2010).

Neutrophils

Neutrophils are the most highly abundant, motile, phagocytic leukocytes which are one of the first cells recruited to acute inflammatory sites. They are extremely important in development of acute and chronic inflammation (Pinegin et al., 2015; Jorch and Kubes, 2017). They ingest, kill, and digest pathogens, with their functions dependent upon special proteins, such as adherence molecules, or via biochemical pathways (respiratory burst). Neutrophils contain primary lysosomes which contain myeloperoxidase and acid hydrolases; they also contain secondary granules containing inflammatory mediators, such as lactoferrin, which can be released upon activation. Lactoferrin is especially important as a homeostatic modulator; lactoferrin can work both ends of the immunological spectrum to increase low response or dampen aggressive ones (Kruzel et al., 2017). Neutrophils express high levels of antibody receptors and complement receptors which allow increased phagocytosis of invading organisms. As a mechanism to help control the spread of intracellular pathogens, neutrophils are capable of deploying extra cellular traps (NETs) which consist of decondensed nucleosomes, neutrophil elastase, and myeloperoxidase (Brinkmann et al., 2004). Critical to the inflammatory process, neutrophils are recruited via specific chemokines (interleukin-8; CXCL8), through a dynamic process where they extravasate from endothelial lined vessels to enter tissue (Pick et al., 2013). Activation leads to respiratory burst producing reactive oxygen and nitrogen intermediates, and subsequently release primary and secondary granules containing proteases, phospholipases, elastases and collagenases (Kolaczowska and Kubes, 2013).

Eosinophils

Eosinophils defend against larger parasites and participate in hypersensitive reactions via cytotoxic events (Strandmark et al., 2016). Their cytotoxicity is mediated by large cytoplasmic granules, which contain eosinophilic basic and cationic proteins. These granules are somewhat unique and contain a dense filamentous core of major basic protein, forming structures known as crystalloids. The matrix of the granules contains lysosomal enzymes, and in particular has a high content of peroxidase, arylsulfatase, acid phosphatase, RNase and cathepsin. While the functionality of eosinophils are classic ascribed to parasitic infections, they also play a major role as responders in allergic reactivity to a variety of stimuli including pollen, and in some drug hypersensitivity reactions. These cells are known to phagocytose antigen-antibody complexes, which can also trigger responsiveness. They also have effects on leukotrienes, one major mediator of the inflammatory response.

Basophils/mast cells

Basophils, and their counterparts mast cells which reside in tissue spaces, produce cytokines that help defend against parasites (Gieseck 3rd et al., 2018; Symowski and Voehringer, 2017), and also cause allergic inflammation (Miyake and Karasuyama, 2017; Ryan et al., 2007). These cells display high affinity surface membrane receptors for IgE antibodies, and have many cytoplasmic granules containing heparin and histamine. The cells degranulate when cell-bound IgE antibodies are cross-linked by antigens, and produce low-molecular weight vasoactive mediators (e.g. histamine). Their granules contain high concentrations of heparin anticoagulant, and multiple vasodilating agents which cause increased vascular permeability. They also contain modulating agents such as slow reacting substance of anaphylaxis (a specific leukotriene), and serotonin. Basophils (and mast cells) typically have their IgE receptors completely saturated, so that when subsequently exposed to the corresponding antigen, they quickly release vasoactive substances leading to hypersensitive allergic reactions (defined as a "Type I" hypersensitivity). Of note, the mast cells respond aggressively during tissue trauma, upon which they release granules containing histamine, heparin, platelet-activating factor, and other substances.

Natural killer cells

NK cells are large granular "innate" lymphocytes that nonspecifically kill certain types of tumor cells and virus-infected cells (Ignacio et al., 2017; Mujal et al., 2021), with recent emerging data to indicate properties that assist during bacterial infections as well (Guo et al., 2018). They are a critical component in multiple facets of inflammation, including being participants in chronic inflammatory disorders (Parisi et al., 2017). NK cells share many surface molecules with T lymphocytes. These circulating large granular lymphocytes are able to kill "self" in the absence of antigen-specific receptors. NK cells are especially effective against virus-infected cells, and keep the expansion of virus in check until the adaptive immune response develops. In this regard, they also secrete interferon-gamma, which is an effective immunoregulator. NK cells can also kill via antibody-dependent cellular cytotoxic mechanisms (ADCC) via their receptors for the constant portion of antibodies (Fc receptors). NK cells express a large number of receptors that deliver either activating or inhibitory signals, and the relative balance of these signals controls NK cell activity.

Endothelial cells

Specialized epithelial cells play a key role in both acute and chronic inflammation by facilitating the transfer of fluid and cells from the blood into the local tissue (Sturtzel, 2017; Greven et al., 2018). In response to chemical mediators such as histamine, endothelial cells contract and the intercellular junctions separate, allowing for rapid and transient vascular leakage near the area of inflammation. Signals from pro-inflammatory cytokines including tumor necrosis factor (TNF) and interleukin-1 (IL-1) trigger endothelial cells to express adhesion molecules on their cell surface, which facilitate binding and subsequent extravasation of leukocytes from the blood into surrounding tissue (Pick et al., 2013). Vascular endothelial cells also play an important role in tissue repair by participating in angiogenesis. In response to vascular endothelial growth factor (VEGF), endothelial cells can migrate, proliferate, and mature in newly formed blood vessels. Endothelial cells secrete multiple factors which affect tissue repair (Godo and Shimokawa, 2017), and play a key role in the clotting cascade. Specifically, they respond to the presence of activated thrombin by releasing tissue-type plasminogen activator (t-PA) as well as plasminogen activator inhibitors. Recent advances have begun to describe additional activities for endothelial cells in overall homeostasis and vascular remodeling events during inflammatory disease (Sachdev and Lotze, 2017) and chronic fibrosis (Specia et al., 2012; Duffield et al., 2013).

Fibroblasts

Fibroblasts are critical within the inflammatory process because of their ability to modify connective tissue and parenchymal structure (Jordana et al., 1994). They are readily found as resident cells in submucosal loose connective tissue, and contribute to both innate and adaptive inflammatory functions (Mack, 2018; Richards, 2017). They produce collagen that makes up collagen fibers which are important components of tissues requiring rigidity, flexibility, and strength. A critical product made by fibroblasts is fibronectin, which mediates normal cell adhesion and migration. Indeed, the extracellular matrix proteins made by fibroblasts certainly influence the shape, movement and state of activation of inflammatory cells in localized tissue. It should be noted that many of the components involved in the production of extracellular matrix are active in regulation of the inflammatory process, and play substantial roles in homing of inflammatory cells to certain sites. Finally, differentiated subsets are known to produce neuropeptides, lipid mediators, cytokines and growth factors, which all actively contribute to regulation of inflammatory events that culminate in fibrosis (Duffield et al., 2013; Wynn, 2008).

Platelets

The process of coagulation and repair of injured tissue results in multiple pathways involved in development and control of inflammation. Platelets are integrally involved in thrombosis (bleeding cessation), and contain potent immune effector molecules (Carestia et al., 2016; Jenne and Kubes, 2015). Platelets aggregate on damaged endothelium and exposed collagen, releasing contents of alpha and dense granules which actively promote the coagulation cascade. Multiple plasma factors correlate and connect to form a blood clot, with ultimate formation of a platelet plug referred to as "primary hemostasis." The entire coagulation pathway, both involving the kinin and clotting cascades, results in fibrin degradation, which heavily influences the activation and alteration of complement factors. Released complement factors, specifically complement C3a and C5a, are powerful contributors as chemotactic and anaphylactic agents to the inflammatory response. Platelets are also integral in inducing production of Neutrophil Extracellular Traps (NETs).

Lymphoid leukocytes

The lymphoid leukocytes are considered secondary responders to inflammation, contributing to both acute and chronic reactivity (Hwang and Actor, 2014). As such, they are included within this discussion, albeit in a limited overview. In many cases they function only after pre-exposure to external antigen or developed reactivity to self-antigen. They must work in concert with antigen presenting phagocytic cells, and do so in a specific antigen receptor based manner. Because of their unique ability to recognize antigen, these types of cells fall into the acquired immune category, which includes the B lymphocytes and the T lymphocytes. The B lymphocytes secrete antibodies. The T lymphocytes operate in a supervising role to mediate cellular and humoral immunity. A third group, the NK cells (mentioned above), work in concert with antibodies in defense against viral agents. Finally, innate lymphoid cells, a relatively new-comer to the appreciation of inflammatory processes, should also be recognized as influencers in the acute inflammatory process (Kumar, 2021).

B lymphocytes

The majority of the effects of B lymphocytes in inflammation are caused by their differentiation into antibody-secreting plasma cells (Lebien and Tedder, 2008). The role of antibodies is outlined in detail later in this chapter. Importantly, memory B cells are more reactive to antigen stimulation, and can provide antigen-independent help for T cells that drive adaptive inflammatory responses (Good et al., 2009). Recent evidence points to a significant role for specialized subsets of B lymphocytes in the modulation of inflammatory response. The regulatory B cell subset produces IL-10 that results in down-regulation of MHC on APC cell surface.

On the other side of the inflammatory coin are pro-inflammatory B cells that produce IL-6. CD5 + B1 cells may represent an inherent regulatory population of cells that produce “natural” IgM and IgG autoantibodies that are anti-idiotypic in nature that may keep autoimmune response under control.

T lymphocytes

The T lymphocytes are the true ringleaders of the adaptive response. Different subsets control different functions (Hwang and Actor, 2014; O’shea and Paul, 2010; Nakayamada et al., 2012). Some help B cells produce antibodies. Others help the myeloid cells more efficiently destroy pathogens, and thus elevate the inflammatory response in a localized and directed manner which is dictated by unique stimulating antigens at the root of the response. Some preferentially function to kill viral infected cells or tumor cell targets. Finally, some function as regulators, conferring immune tolerance or establishing limits on responsiveness.

The T lymphocytes are critical in inflammatory responses that involve specific recognition of antigens, small shapes which are presented via major histocompatibility markers on antigen presenting cells. As such, they do not directly initiate the inflammatory response, but rather direct the level of responsiveness after the antigen has been processed. With that said, they are critically important in both acute and chronic inflammation. The ability to recognize specific foreign antigenic stimuli places these cell phenotypes in a directive role to regulate pathological damage during infection; when the stimuli is “self,” they function to control autoimmune reactivity. The involvement of T lymphocytes in development of pathology is the definition of a classical adaptive inflammatory hypersensitive response (Cosmi et al., 2014). While a complete and exhaustive role of specific T cell subsets is beyond the scope of this discussion, their functional presence in adaptive inflammation leads to multiple distinct disease states. These cells are indeed critical in the maturation of responses forming the basis of the type IV hypersensitivities.

Chemical mediators of inflammation

The chemical mediators of inflammation have been extensively studied (Griffith et al., 2014). These factors were historically identified as molecules which are capable of inducing edema upon injection, stimulating smooth muscle contraction, or altering blood pressure following administration. These substances can act via direct stimulation of cell surface receptors (e.g. histamine) or function as plasma derived factors that cause direct tissue damage (e.g. complement). Alternatively, they may function as chemotactic agents to attract specific cell phenotypes to areas of inflammatory response (e.g. chemokines; arachidonic acid metabolites). Some of the major chemical mediators of inflammation are listed in Table 3.

Many of these molecules represent systems that are interrelated (Kumar et al., 2017). For example, the complement components (lytic, vasoactive, chemotactic) are linked to the kinin pathway (vasoactive mediation, pain) which is linked to the fibrinolytic system (clotting, vasoactive) (Kaplan et al., 1981; Kaplan and Joseph, 2014). Molecules effective at coagulation and repair work to direct the clotting cascade and the production of fibrin. Plasma serine proteases function to produce kinins, which mediate vascular permeability and pain. Proteins and enzymes released by platelets help to further drive and direct the response. Keep in mind that these factors initiate molecular changes that are extremely beneficial from an immunological standpoint. Clotting and formation of a fibrin matrix impedes movement of microorganisms. In addition, breakdown products generated in the cascading events also attract and activate incoming leukocytes.

Neurogenic inflammation should be regarded as protective mechanism to buffer trauma (Corrigan et al., 2016). Molecules associated with neurogenic inflammation include substance P (SP) and calcitonin gene-related peptide (CGRP), as well as neurokinin A, neuropeptide Y and vasoactive intestinal polypeptide (VIP) (Brain, 1997; Luger, 2002; Duan et al., 2017). These agents are under investigation as strong modulators of the inflammatory response.

Role of antibodies in inflammation

A major benefit of the influx of fluid is the direct delivery of antibodies. The circulating plasma is filled with relatively high levels of antibodies that, as a group, have the ability to recognize just about any shape or form of antigen. Depending on prior exposure or positive vaccination status, there may also be an abundance of specific antibodies reactive to antigenic features present on destructive pathogens.

Antibody entry to the area confers multiple functions, the most critical of which resides within the variable region of the antibody molecule that can recognize unique shapes and forms, with a specific and unique binding site to allow direct interaction with a pathogen or to pathogen-derived proteins (Cohen and Milstein, 1967). Antibodies recognizing toxins and deleterious pathogenic factors can inhibit and/or neutralize their toxic properties. Direct binding to the pathogen also results in inhibition of organism movement. In the case of viruses, antibodies can inhibit infection of host cells by blocking attachment and adhesion. Antibodies can also limit direct expansion of bacterial organisms; for example, recognition of multiple regions on the microorganism can result in a latticework precipitate which inhibits movement and promotes organism clearance.

The constant region of the antibody molecule has a constant structure which confers biological function that is critical for initiation of the inflammatory response (Franklin, 1975; Schroeder and Cavacini, 2010). Innate phagocytic cells contain receptors on their surface for this constant portion of the antibody. This assists the phagocyte in targeted pathogen engulfment and subsequent intracellular destruction. The term for coating the organism with antibodies is opsonization.

Table 3 General mediators of inflammation.

<i>Molecules</i>	<i>Effects</i>	<i>Source</i>
<i>Vasoactive amines</i>		
Histamine	Vasodilation	Preformed, released by local cells
Serotonin		
<i>Neuropeptides</i>		
CGRP	Transmit pain	Secreted by nerve fibers
Substance P	Regulate vessel tone	
VIP	Modulate vascular permeability	
<i>Plasma proteases</i>		
Bradykinin	Vasodilation Increased vascular permeability Pain	Synthesized in liver, circulate in plasma, cleaved to active forms downstream of Factor XII activation
Thrombin	Vasodilation Increased vascular permeability Chemotaxis	
C3a	Vasodilation Increased vascular permeability Increased phagocytosis, chemotaxis	
C5a	Vasodilation Increased vascular permeability Leukocyte activation and chemotaxis	
<i>Arachadonic acid metabolites</i>		
Prostaglandins	Vasodilation Pain	Short range hormones produced by local cells; derived from membrane phospholipids; produced via the cyclooxygenase or lipoxygenase pathways
Leukotrienes	Increased vascular permeability Leukocyte activation and chemotaxis	
Lipoxins	Leukocyte activation and chemotaxis	
<i>Cytokines/chemokines</i>		
IL-1, TNF α , IL-6	Vasodilation	Short range molecules produced by local cells (macrophages, dendritic cells, lymphocytes)
IL-8 (CXCL8)	Leukocyte activation and chemotaxis	
Eotaxins, IL-5	Eosinophil attractant	
Interferons	Immune cellular activation	
TGF- β , VEGF	Tissue repair	
<i>Other</i>		
Platelet activating factor	Platelet aggregation, vasoconstriction and vasodilation, vascular permeability, increases leukocyte adhesion, increases oxidative burst	Produced by platelets, endothelial cells and leukocytes
Reactive oxygen species	Oxygen derived free radicals, increased vascular permeability, smooth muscle control	
C-reactive protein	Binds phosphorylcholine on microorganisms' enhances macrophage phagocytosis	Produced in the liver; general marker for inflammation
Lactoferrin	Immune modulation to maintain inflammatory homeostasis	Produced by neutrophils; exocrine secretions; present/secreted in high concentrations on mucosal surfaces

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Complement

Overall, complement and its related components exert multiple biological functions that are critical components of inflammation, including activation and regulation of both innate and adaptive immune functions (Ricklin and Lambris, 2013; Hajishengallis et al., 2017). It has been well accepted for decades that tissue damage caused by both immunological mechanisms as well as non-immunological trauma can initiate surface dependent pathways of coagulation, fibrinolysis, and kinin formation (Kaplan et al., 1981). Complement is important in direct cytolysis of invading organisms, in coating organisms to enhance phagocytosis for targeted destruction, and as a specific director of leukocyte chemotaxis (Noris and Remuzzi, 2013). Complement is a term that originally referred to the heat labile factor in serum that causes immune cytolysis. It comprises at least 30 distinct serum proteins that affect multiple biological functions in a tightly regulated manner. These components are inert when normally present in serum, however, a “cascade” of events occur when these molecules interact with signaling agents. Activation of complement enzymes results in subsequent cleavage of proteins to yield specific polypeptide fragments with short-lived enzymatic functions related to inflammation. In essence, proteolytic degradation of the complement components drives many aspects of immunity.

Different pathways exist to initiate complement activation. One pathway, referred to as the Classical Pathway, utilizes antibodies as the initiating signal. Antibody opsonization (coating) of organisms targets them for attack. The “first” complement component (called C1) interacts with the constant portion of specific classes of antibody bound to the surface of the bacteria. This initiates a cascading series of enzymatic reactions whereby a complex structure is built upon the bacterial cell surface. Synthesis of this structure culminates in a pore channel, called a membrane attack complex, which causes osmotic lysis of the pathogen or infected cell. Two additional pathways of complement activation, the Alternate Pathway and the Lectin Pathway, also function to allow direct lysis of microorganisms in the absence of antibodies. In these enzymatic cascades, deposition of complement components directly to pathogens occurs via recognition of bacterial sugars and lipids, with eventual pore channel assembly on the organism surface. Furthermore, breakdown of components during the cascading enzymatic process creates smaller molecules, opsonins, which remain deposited on the pathogens. These opsonins act as molecular beacons, allowing interaction with receptors on macrophages, monocytes, and neutrophils to enhance phagocytosis and elevate mechanisms of targeted organism destruction.

One of the most prevalent byproducts of the complement enzymatic cascade is the production of molecules with the ability to directly affect migration of leukocytes (Sarma and Ward, 2011). Proteolytic degradation of complement components releases leukocyte chemotactic factors referred to as anaphylatoxins. For example, breakdown products of complement component C3 and C5 produces responses triggering smooth muscle contraction and increased vascular permeability, as well as chemotactic functions. C3 is chemotactic for eosinophils. Proteolytic digestion of component C5 produces a much more potent chemokine, attracting neutrophils, monocytes and macrophages, and eosinophils. Another major property of the enzymatic cascade is the production of factors that activate those incoming cells. For example, interaction of components C3a, C4a or C5a with mast cells and basophils leads to release of histamine, serotonin, and other vasoactive amines which further drive vascular permeability. One can readily see that byproducts of the complement cascade can directly influence local inflammatory responses. Indeed, directed targeting of these products may well dictate future therapeutic modalities to control both acute and chronic inflammatory responses (Morgan and Harris, 2015). Finally, it is important to consider that recent investigations now identify multiple roles for complement outside of the cytolytic cascade; there are identified roles for phagocytic activities and subsequent cytokine production by antigen presenting cells, and functionality in the T-lymphocyte maturation process (Thielens et al., 2017; Lu and Kishore, 2017; West et al., 2018).

Consequences of hypersensitive inflammation

So far, the concept of inflammation has been presented as an action in response to disease. Yet it should be stressed that inflammation may be the cause of disease as well (Hwang and Actor, 2015). Inflammation is the basis for all hypersensitive reactions, defined as an immune response that leads to elements of host pathology. The hypersensitivity reactions fall readily into four established subgroups characterized on their mechanisms and the ability to passively transfer response through antibodies or through T lymphocytes. These responses include “inappropriate” antigenic response, excessive magnitude of response, prolonged duration of response, and innocent bystander effect reactions leading to tissue damage. The hypersensitivities, whether they be acute or chronic in nature, can lead to clinical inflammation (Table 4).

Hypersensitivities

Molecular pathways for each hypersensitivity class have been detailed, according to classifications defined by Gell and Coombs (1963), although there is a certain amount of mechanistic overlap between groups (Abbas et al., 2020; Actor, 2012), and plenty of room for interpretation over the past 50+ years that “hypersensitivities” have been described (Descotes and Choquet-Kastylevsky, 2001). Type I hypersensitivity (also called immediate or allergic hypersensitivity) is due to aberrant production and activity of IgE against normally nonpathogenic antigens (commonly called allergens). The response is relatively quick (15–30 min post exposure). The IgE binds to mast cells via high affinity IgE receptors; antigen exposure results in crosslinking of mast cell bound IgE with release of preformed mediators (e.g. histamine, leukotrienes, etc.). Type II hypersensitivity is dependent on antibody directed against cell membrane-associated antigen that results in cytolysis. This reaction occurs minutes to hours after contact with the antigen. The mechanism may involve complement (cytotoxic antibody) or effector lymphocytes that bind to target cell-associated antibody and effect cytolysis via a complement independent pathway (Antibody dependent cellular cytotoxicity, ADCC). Type III hypersensitivity results from soluble antigen-antibody immune complexes that activate complement. Accordingly, reactions take time to develop (up to 8 h). The antigens may be self or foreign (i.e. microbial); the byproducts of complement activation (C3a, C5a) are chemotaxins for acute inflammatory cells. Type IV hypersensitivity (also called Delayed Type Hypersensitivity, DTH) involves macrophage-T cell-antigen interactions that regulate activation and cytokine secretion. These reactions develop over the course of 2–3 days, thus they are described as “delayed-type” in nature. Diseases such as tuberculosis, leprosy and sarcoidosis as well as contact dermatitis are all clinical examples where the tissue injury is primarily due to the vigorous inflammatory immune response rather than the inciting pathogen itself (Hwang and Actor, 2015).

Fortunately, most acute inflammation is self-limiting with resolution of immediate effects that result in normal homeostasis of tissue regions involved. There may be residual scarring or fibrosis related to the healing process post inflammation. When considering chronic inflammation, the level of tissue damage is related in part to length of response, clearance of reactive components, and overall proliferative and regenerative capacity of the parenchyma affected.

Table 4 Clinical consequences of acute and chronic inflammation due to hypersensitivities.

Type	Classification	Clinical examples
I	Allergic (immediate)	• Anaphylaxis • Allergic rhinoconjunctivitis • Allergy to penicillin, bee sting, food or pollen
II	Cytotoxic	• Autoimmune hemolytic anemia • Myasthenia gravis (antibodies to acetylcholine receptors) • Granulocytopenia or thrombocytopenia • Goodpasture's nephritis or pemphigus
III	Immune complex	• Arthus reaction • Systemic lupus erythematosus; lupus nephritis • Rheumatoid arthritis • Polyarteritis
IV	Delayed type (DTH)	• Tuberculin (Mantoux) reaction • Poison ivy • Tuberculosis, leprosy, toxoplasmosis, leishmaniasis • Blastomycosis, histoplasmosis

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Conclusions

Multiple events are involved in normal physiology of tissue maintenance. In reality there is a balance between factors necessary for response to damage and subsequent tissue repair. Indeed, it is critical to view inflammation as a homeostatic fulcrum, integral in both sides of the equation to allow flexibility in function in normal homeostasis. Most often, inflammation is beneficial to the host, but dysregulation or unbalanced response may also cause tissue damage. Understanding how pathways control this balance will be critical for therapeutic intervention and development of translational aspects to treat disease and disorders.

The focus of this overview defines a perspective of the inflammatory response in a way that permits discussion of its control as a therapeutic mechanism to combat disease. Understanding the role of inflammation in various disease states allows those in medical and related theoretical fields to gain insights into the inflammatory response. Thus, knowledge of the inflammatory pathways, and defining elements involved in those pathways as they relate to disease development, furthers understanding on how inflammation impacts manifestation of clinical disorders.

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Hypersensitivity

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Definition

In a normal condition, the immune system is designed to defend against hazardous organisms without causing severe damage to the human body. However, in hypersensitivity disorders, the immune response is either exaggerated or inappropriate, which leads to tissue damage.

Etiology

Three major mechanisms by which hypersensitivity responses are mediated are as follow.

Excessive response to micro-organisms

Mounting an exaggerated response to a microbe or upon exposure to a persistent antigen can result in various types of tissue damage. For instance, antibodies produced against a specific antigen may bind with their ligands and form immune complexes (ICs), which can then be trapped in tissues, causing inflammation and tissue injury. Additionally, in tuberculosis, an example of a persistent antigen, an excessive T-cell mediated immune response gives rise to inflammation, which is a driving force in formation of inflammatory structures called “granuloma” (Abul et al., 2017a).

Autoimmunity

Self-tolerance is a process leading to the unresponsiveness of the immune system to self-antigens, molecules, and cells (Wang et al., 2014). The failure of self-tolerance can lead to autoimmune diseases. Autoimmune diseases are chronic illnesses with a growing prevalence of 3–5% (Lerner et al., 2015). These diseases are most common among women, and the age of onset is usually between the 20s–40s (Wang et al., 2015).

Inappropriate response to innocuous antigens

In a healthy status, the immune system usually does not respond to harmless environmental antigens such as pollen, animal dander, dust mites, drugs, milk, egg, fish, soybeans, peanuts, latex, tree nuts, and wheat. However, in 20–30% of the world population, these antigens initiate a damaging immune response (Pawankar et al., 2012). They can trigger production of immunoglobulin (Ig)E and mast cell degranulation, resulting in allergic reactions (Valenta et al., 2018). Skin contact of these antigens can also activate T-cells, leading to increased secretion of pro-inflammatory cytokines (Ho et al., 2015).

Classification

Hypersensitivity disorders can be divided into four major groups; namely, immediate (type I) hypersensitivity, antibody-mediated (type II) hypersensitivity, IC-mediated (type III) hypersensitivity, and delayed-type hypersensitivity (type IV). More than 40 years ago, Philip Gell and Robin Coombs coined this classification, which is still widely used (Cell and Coombs, 1963). Table 1 illustrates the different types of hypersensitivity and corresponding diseases.

Type I (immediate) hypersensitivity

Introduction

Type I hypersensitivity, the most prevalent hypersensitivity disorder, is also the most common immune disease, generally known as allergy or atopy. Type I hypersensitivity can manifest in less than 24 h; therefore, it is also called immediate hypersensitivity (Justiz Vaillant AA, 2019). In patients suffering from this disorder, excessive production of IgE in response to harmless antigens can cause a wide variety of symptoms ranging from moderate reactions, such as itching, to severe responses, such as death (Sheldon et al., 2014). T-helper 2 cells, IgE, mast cells, and eosinophils are the key role players in type I hypersensitivity (Delves et al., 2017b).

Mechanism

We can divide the underlying mechanism of type I hypersensitivity into two stages: sensitization stage and effector stage.

Sensitization stage

Subsequent to exposure to an allergen, a cascade of events leads to sensitization of the immune cells to the particular antigen. At first, dendritic cells residing in epithelia process the antigen, transfer it to lymph nodes and present it to CD4+ naive T-helper cells (Zhu, 2015). Then these cells differentiate into mature T-helper 2 cells and release cytokines; namely, IL-4, IL-5, and IL-13. These cytokines play several roles in the pathophysiology of allergy. For instance, IL-5 results in eosinophil activation, and IL-13 induces mucus production by epithelial cells (Wynn, 2015). Class switching of the B cells to IgE production is the most prominent effect of these cytokines. CD40 ligand, which is expressed by the activated T cells, is the other crucial factor in the class switching of B cells.

A large proportion of the IgE produced as a result of B cell class switching enters the circulation and binds with the high-affinity Immunoglobulin E receptor (FcεRI) of basophils and mast cells while the rest of IgE may act locally. Normally, the concentration of circulatory IgE is less than 1 µg/mL. However, this concentration soars in allergic reactions (Abul et al., 2017b). The remaining IgE in the region where the allergen penetrated binds to the allergen or cross-reacts with the FcεRI of mast cells in the tissue (Li et al., 2019). Subsequent to the linkage of IgE and FcεRI, mast cells are sensitized to the allergen and will react to the next exposure (Ishizaka and Ishizaka, 2016).

Table 1 Different types of hypersensitivity and their correspondent diseases.

<i>Hypersensitivity type</i>	<i>Characteristic feature</i>	<i>Diseases</i>
Type I hypersensitivity	Immediate	● Anaphylaxis ● Asthma ● Allergic rhinitis ● Atopic eczema ● Urticaria ● Food allergy
Type II hypersensitivity	Antibody-mediated	Cellular destruction ● Autoimmune hemolytic anemia ● Immune thrombocytopenia ● Hemolytic disease of the newborn Inflammation ● Rheumatoid fever ● Good pasture syndrome ● Rheumatic fever ● Pernicious anemia ● Pemphigus(ref:primer) Cellular dysfunction ● Myasthenia gravis ● Graves' disease
Type III hypersensitivity	Immune complex-mediated	● Systemic lupus erythematosus ● Post-streptococcal glomerulonephritis ● Polyarthritis nodosa ● Serum sickness
Type IV hypersensitivity	Cell-mediated and delayed	● Contact dermatitis ● Hashimoto thyroiditis ● Multiple sclerosis (MS) ● Rheumatoid arthritis (RA) ● Type1 diabetes ● Guillain-Barre syndrome ● Celiac disease ● Crohn's disease ● Graft-versus-host disease (GVHD) ● Autoimmune myocarditis

Effector stage

When the immune system becomes sensitized to the allergen, the subsequent exposures trigger the effector stage. The primary immune cells involved in this stage are mast cells, basophils, eosinophils, and T-helper 2 cells. This stage can be divided into immediate and late-phase responses, which are explained in the following lines.

● Immediate phase response

Mast cells are the principal elements of the immediate response. Upon the subsequent exposures, allergens cross-link with several IgE molecules attached to FCεR1s on the mast cells which leads to their activation (Renke et al., 2019). After activation, mast cells release their intracellular granules, which were previously formed and stored (Amin, 2012). The degranulation is triggered by an increased Ca^{2+} influx (Borriello et al., 2017). The granules contain histamine, serotonin, chemotactic factors, proteoglycans, amines, cytokines, and enzymes such as lysosomal hydrolases and proteases (Wernersson and Pejler, 2014). Histamine and serotonin can cause vasodilation and bronchial smooth muscle cell contraction. Histamine can also increase vascular permeability, increase mucus production (in asthma), and affect sensory nerves, which may lead to itching and eczema or may cause sneezing (in hay fever) (Thangam et al., 2018; Mygind, 2014; Yang and Kim, 2019; Tak Mak and Jett, 2014).

After degranulation, the reaction of mast cells is continued by rapid de novo synthesis of lipid mediators and cytokines (Gonzalez-De-Olano and Alvarez-Twose, 2018). Lipid mediators, including prostaglandin D_2 ; leukotrienes C_4 , D_4 , and E_4 ; and platelet-activating factor (PAF), are derived from arachidonic acid (Fanning and Boyce, 2013). Prostaglandins and PAF promote aggregation of platelets and contraction of respiratory smooth muscles (Tak Mak and Jett, 2014). Moreover, PAF is known to play a key role in development of nasal congestion in hay fever (Munoz-Cano et al., 2019). Leukotrienes can cause vasodilation, eosinophil recruitment, increased vascular permeability, and mucus production in addition to bronchoconstriction (Jo-Watanabe et al., 2019). Additionally, the cytokines are also responsible for initiation of the inflammatory response (Ngoc et al., 2005).

● Late-phase response

The late-phase response takes place approximately 3–5 hours after the exposure to the allergen and lasts for roughly 24 hours. T-helper 2 cells, eosinophils, neutrophils, basophils, and macrophages are the involved cells in this stage (Tak Mak and Jett, 2014).

Due to the chemotactic factors produced during the immediate response, the immune cells transfer from the circulation to the site of allergy (Tak Mak and Jett, 2014). These cells can produce inflammatory mediators causing tissue injury. For instance, eosinophils, one of the major effector cells in this step, secrete (1) enzymes, which can be toxic to the tissue; (2) leukotrienes, which can lead to bronchoconstriction, increased mucus production and vascular permeability; and (3) cytokines, which are chemotactic factors and can trigger eosinophil production (Fulkerson and Rothenberg, 2013; Abul et al., 2017b).

Examples of diseases caused by type I hypersensitivity

Anaphylaxis

Anaphylaxis is defined as “a serious, generalized or systemic, allergic or hypersensitivity reaction that can be life-threatening or fatal” by the International consensus on (ICON) anaphylaxis (Simons et al., 2014). The prevalence of this rapid-onset hypersensitivity reaction is estimated to be 0.3–5.1% (Tejedor Alonso et al., 2015). Except for idiopathic cases, it can be caused by drugs, insect venoms, foods, biological agents, and human seminal fluid (Lieberman et al., 2015). The rapid and extensive releases of vasodilators and inflammatory mediators such as histamine, PAF, and cysteinyl leukotrienes (CysLTs) by mast cells and basophils can have detrimental effects on the respiratory, cardiovascular, digestive systems and the skin. The most common signs and symptoms of the disease include dyspnea, wheeze, hypotension, tachycardia, nausea, abdominal pain, urticaria, cutaneous angioedema, and flushing (Reber et al., 2017). The immediate management of anaphylaxis includes removal of the triggering factor, injection of epinephrine, using supplemental oxygen, and administering intravenous fluids to resuscitate the volume (Brown, 2005; Simons, 2004). Interestingly, omalizumab, an anti-IgE antibody, is found to reduce the risks of anaphylaxis (Brosby-Olsen et al., 2018).

Food allergy

Food allergy is an inappropriate and potentially dangerous response of the immune system to certain innocuous protein antigens in food. With an estimated prevalence of 6–8% and less than 3%, respectively, food allergy is more common among children compared to adults (Loh and Tang, 2018). It occurs both dependent and independent of IgE. However, IgE-mediated reactions are more common (Sampson, 2016). In IgE-mediated food allergy, exposure to minimum amounts of specific foods can lead to a soar in IgE production, leading to local and/or systemic symptoms (Renz et al., 2018). While the foods triggering food allergy may vary in different regions, egg, milk, and wheat are the most common triggers (Yu et al., 2016). The symptoms of food allergy include involvement of the skin and the mucosal membrane (causing urticaria, erythema, angioedema, and pruritus), the respiratory system (causing hoarseness, dysphagia, coughing, wheezing, and dyspnea), the digestive system (causing nausea, diarrhea, vomiting, and abdominal pain), the cardiovascular system (causing decreased blood pressure and arrhythmia), and the nervous system (causing weakness and headache) (Ebisawa et al., 2017). In severe cases, it can also lead to anaphylaxis (Devdas et al., 2018). Currently, there is no optimum treatment available for food allergy. Avoidance is the recommended management. Additionally, to alleviate the systemic and localized allergic reactions, adrenalin, and antihistamines can be used respectively (Boyce et al., 2011; Iweala et al., 2018).

Asthma

Asthma is a chronic heterogeneous condition causing inflammation of airways which leads to tightening of bronchial tubes and difficulty in breathing (Mims, 2015). Asthma, with an estimated global prevalence of 4.3%, is more common in the developed countries (Loftus and Wise, 2016; To et al., 2012). Environmental, epigenetic, and genetic factors are involved in its pathogenesis. Notably, the underlying mechanism of asthma can be widely varied (Russell and Brightling, 2017; Portelli et al., 2015). For instance, paucigranulocytic asthma is associated with increased number and activity of eosinophils, neutrophils, and inflammatory cells. Asthma can also either result from allergy or not (Papi et al., 2018). Fig. 1 summarizes different immunopathologies of asthma. Immune responses can cause remodeling of mucosa (epithelial hyperplasia and metaplasia of goblet cells) and submucosa (hypertrophy of smooth muscle and enlargement of mucous gland). These changes give rise to bronchial obstruction and excess production of mucous (Holgate et al., 2015). Shortness of breath, wheeze, cough, and chest pain are the most common manifestations of asthma. The diagnosis can be established when these clinical features are accompanied by evidence of variable airflow restriction, which is usually provided by spirometry (Brigham and West, 2015). Management of asthma, a key factor of which is avoiding triggers of exacerbation, aims to reduce both current impairment and future risks. While treatment options vary in different patients, inhaled and oral corticosteroids, short and long-acting β_2 -receptor agonists, and leukotriene receptor antagonists are used in a stepwise approach in asthma management (Papi et al., 2018).

Type II (antibody-mediated) hypersensitivity

Introduction

Type II hypersensitivity is mediated by antibodies, which are typically IgG or sometimes IgM, directed to endogenous antigens or exogenous antigens, such as penicillin. Type II hypersensitivity, which is the underlying mechanism of several autoimmune disorders, is usually limited to one type of tissues or organ. Binding of these antibodies with their antigens can result in cytotoxicity (mainly mediated by components of the complement system) or abnormal cell function without cytotoxicity.

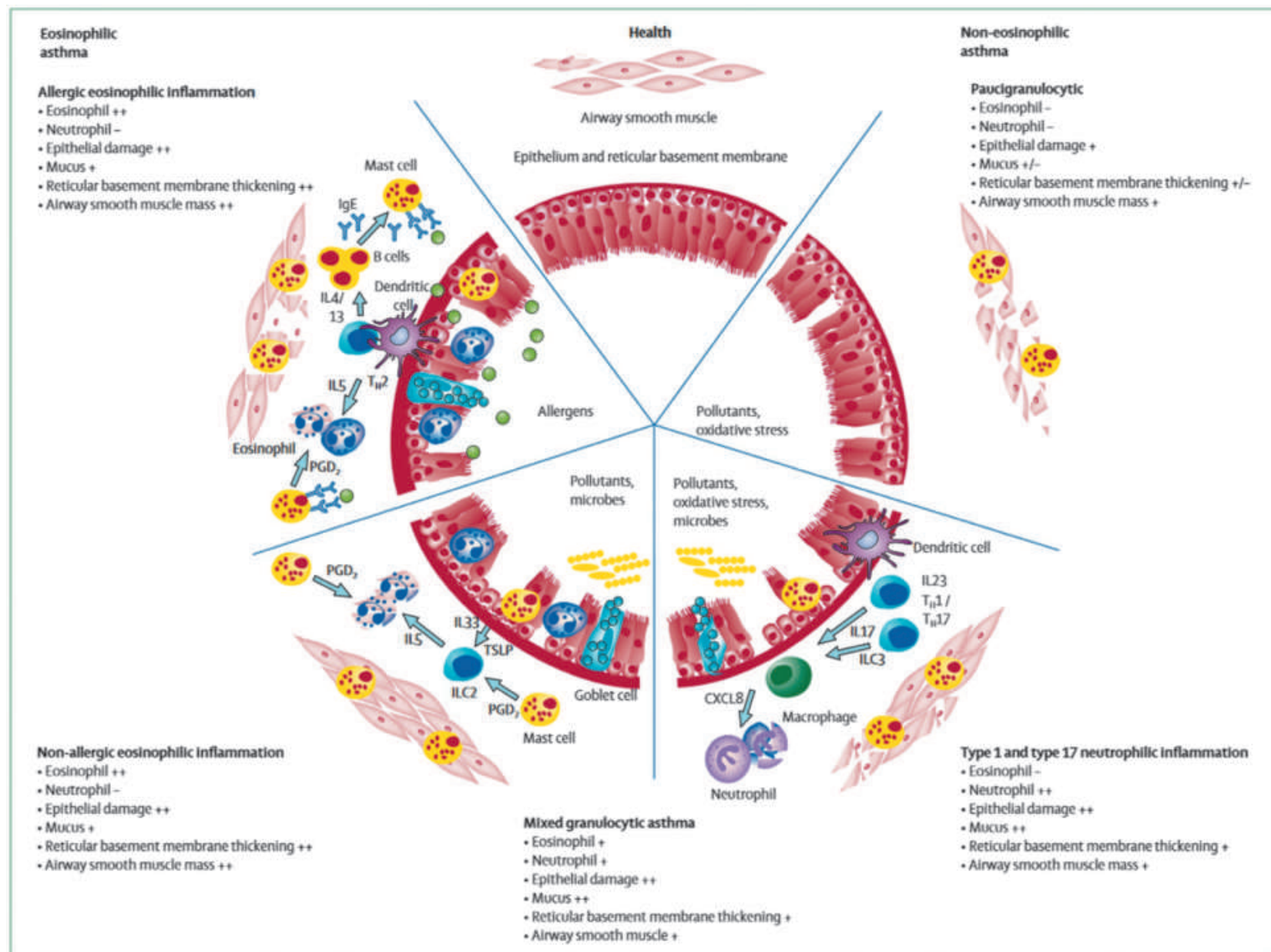


Fig. 1 Immune Mechanisms involved in the pathophysiology of asthma: Five distinctive patterns may be seen in asthma: 1- *Allergic eosinophilic inflammation*, in which after encountering an allergen, activation of dendritic cells results in production of IL4, IL-5, and IL-13 from T-helper2 lymphocytes. The produced IL-5 promotes maturation of eosinophils, and the produced IL-4 leads to antibody class-switching of B cells to IgE, which stimulates mast cells. 2- *Non-allergic eosinophilic inflammation*, in which subsequent to epithelial injury, the epithelium promotes release of TSLP, IL-25, and IL-33. In response to these mediators and PGD₂, ILC2 releases IL-5 and IL-13. 3- *Non eosinophilic asthma*, which can be seen in some patients; however, its mechanism is not fully elucidated. 4- *Neutrophilic inflammation*, in which the dominance of neutrophils can stem from bacterial colonization or usage of corticosteroids (suppressing type 2 immune responses and increasing neutrophil survival). In this pattern, cytokines released from T-helper1 lymphocytes, T-helper17 lymphocytes, or ILC3 result in activation of macrophages. The activated macrophages produce neutrophil chemokines, like CXCL8. 5- *Mixed granulocytic asthma*, which can be caused from co-occurrence of all of the four aforementioned mechanisms. **Abbreviations:** IL = interleukin. TH = T helper. PGD₂ = prostaglandin D₂. TSLP = thymic stromal lymphopoietin. ILC2 = type 2 innate lymphoid cells. CXCL8 = C-X-C motif chemokine ligand 8. ILC3 = type 3 innate lymphoid cells. From Papi A, Brightling C, Pedersen SE and Reddel HK (2018) Asthma. *Lancet*, 391: 783–800.

Mechanism

These autoantibodies can cause various diseases via three major mechanisms; namely, induction of cellular destruction, induction of inflammation, and damaging physiological responses without tissue injury. In the following lines, each one of these is discussed in more depth.

Cellular destruction

Phagocytosis (by neutrophils or macrophages), antibody-dependent cell-mediated cytotoxicity (ADCC), and formation of membrane attack complex (MAC) are the primary mechanisms of cell death. Phagocytosis is caused by opsonization of the cells by antibodies and C3b, which binds to the antibody-antigen complex (Tak Mak and Jett, 2014). ADCC is another cytotoxic mechanism in the type II hypersensitivity. In ADCC, natural killer (NK) cells detect the antigen-antibody complexes by the Fc tail of the antibody and become activated. The activated NK cells release cytotoxic compounds such as perforins which lead cell death and apoptosis (Gómez Román et al., 2014). Lastly, the antibody-antigen complex activates the complement system, leading to formation of the MAC. The MAC disrupts the integrity of the cell membrane and causes cell lysis (Beenhouwer, 2018).

Inflammation

The inflammation caused by the antibody-antigen complex initiates with binding of C1 with the Fc tail of the antibody and complement activation. This attachment triggers a cascade during which chemotactic factors, such as C3a, C4a, and C5a, are produced. These mediators recruit immune cells, particularly neutrophils. When neutrophils are in the site, they release cytotoxic enzymes, such as peroxidase, proteinase B, and myeloperoxidase, leading to production of reactive oxygen species (ROS), which results in tissue damage (Abul et al., 2017a).

Cellular dysfunction

In contrast to the two previous mechanisms, this process is not cytotoxic. In this mechanism, attachment of the antibody to self-antigens, particularly receptors, leads to changes in the normal functions of the cell, which causes cellular dysfunction (Tak Mak and Jett, 2014).

Examples of diseases caused by type II hypersensitivity

Conditions mediated by cellular destruction

- Autoimmune hemolytic anemia (AIHA)

AIHA is an acquired hemolysis caused by production of antibodies against antigens of red blood cells (RBC) (Hill and Hill, 2018). AIHA is a rare disease with an estimated prevalence of 0.001 to 0.009% (Michel, 2010). Two different major types of autoantibodies are involved in AIHA: (1) warm and (2) cold anti-RBC antibodies (Quist and Koepsell, 2015). Warm autoantibodies (IgG), bind to the RBC at 37 °C and account for about 70% of AIHA cases (Kalfa, 2016). They typically induce phagocytosis of the RBCs (extravascular hemolysis) (Naik, 2015). However, they can also activate the complement system, which leads to intravascular hemolysis (Brodsky, 2019). Cold autoantibodies (IgM) bind to RBC at lower temperatures, resulting in complement activation and intravascular hemolysis (Berentsen et al., 2015). In almost 8% of patients, both cold and warm autoantibodies exist (Liebman and Weitz, 2017). Direct Coombs test is used for identification of the autoantibodies and diagnosis of AIHA (Shulman et al., 1985). However, in approximately 5% of cases the Coombs test can be negative. The most common paraclinical and clinical manifestations of AIHA include anemia, spherocytosis, fatigue, dizziness, pale skin, activity-induced breathlessness, and jaundice (Brodsky, 2019; Hill, 2015). The first line of AIHA treatment are glucocorticoids. Additionally, rituximab, cyclophosphamide, and azathioprine are also used in AIHA management (Liebman and Weitz, 2017). The last line of treatment is splenectomy, which is performed in resistant cases (Zanella and Barcellini, 2014).

- Immune thrombocytopenia (ITP)

ITP, with an estimated prevalence of 5.6 to 20 per 100,000 population (Moulis et al., 2014; Fogarty, 2009), is an autoimmune disease leading to destruction of platelets and causing a platelet count of $<100 \times 10^9/L$ (Lambert and Gernsheimer, 2017). ITP can affect children and adults. In children, the condition is typically self-limited, while in adults, it is usually chronic and more severe. Moreover, primary ITP is more common among children, but in adults, secondary ITP, which may result from viral infections, systemic lupus erythematosus, etc. is more common (Despotovic and Grimes, 2018). Anti-platelet antibodies which promote the destruction of platelets by the macrophages and circulating CD₈₊ T cells, are a key component in ITP pathogenesis (Zufferey et al., 2017). The spleen is the major site for the elimination of the platelets, with macrophages phagocytosing the opsonized platelets. Moreover, the immune system impairs the function of the bone marrow and reduces platelet production (Marini and Bakchoul, 2019). ITP can present with mild to severe bleeding (happening commonly in the skin or mucosal membranes), fatigue, and thrombocytopenia (Kohli and Chaturvedi, 2019). As ITP does not have an ideal diagnostic test, it is diagnosed by ruling out other differential diagnoses (Kelton et al., 2018). ITP treatment aims to provide an adequate platelet count to prevent severe hemorrhage. The first line of treatment are glucocorticoids and intravenous immunoglobulin. Splenectomy, immunosuppression, and

thrombopoietin receptor agonists are in the second line of treatment in ITP management (Cervinek, 2018; Neunert et al., 2019). Notably, in patients with severe bleeding, immediate platelet transfusion is required (Neunert and Cooper, 2018).

Conditions mediated by inflammation

- Goodpasture's syndrome or anti-glomerular basement membrane (GBM) disease

Goodpasture's syndrome is a rare rapidly progressive and potentially fatal autoimmune condition mediated by anti-GBM antibodies, which are targeted against the $\alpha 3$ chain of type IV collagen (McAdoo and Pusey, 2017). The $\alpha 3$ chain is inclusively expressed in the basement membrane of the kidneys and lungs. Thus, Goodpasture's syndrome can cause glomerulonephritis (by involving the kidneys) and alveolar hemorrhage (by involving the lungs) (Fig. 2) (DeVrieze BW, 2020). With an annual incidence rate of fewer than 10 cases per million, Goodpasture's syndrome accounts for 10–20% of rapidly progressive glomerulonephritis cases (Shiferaw et al., 2016). An interplay of genetic and environmental factors is involved in the etiology of this disease (Podzolkov et al., 2019; Henderson and Salama, 2018). In Goodpasture's syndrome, binding of autoantibodies with their ligands initiate inflammation through either activation of the complement system or the Fc receptor-dependent mechanism. In addition to the aberrant humoral immunity, at least in a subgroup of patients, glomerular autoreactive T-cells also play a vital role in the pathogenesis of Goodpasture's syndrome (Gulati and McAdoo, 2018). The clinical and paraclinical manifestations of this condition include acute kidney injury, hematuria, proteinuria, shortness of breath, hemoptysis, cough, pulmonary infiltrates on chest X-Ray, and iron deficiency anemia (Greco et al., 2015). Diagnosis is established by detecting anti-GBM antibodies in the serum or by kidney biopsy (Hellmark and Segelmark, 2014). Plasmapheresis, together with immunosuppression, is used in the treatment of Goodpasture's syndrome (Predecki and Pusey, 2019). In case of severe renal failure, renal transplantation may be required as well (Greco et al., 2015).

- Acute rheumatic fever (ARF)

ARF is an autoimmune response presenting two to three weeks after pharyngeal infection with *Streptococcus pyogenes* in 0.3–3% of cases (Siegel et al., 1961). Developing countries show the highest prevalence of ARF (Carapetis et al., 2016). Worldwide, the annual incidence of ARF is estimated at 5–51/100000 population, whereas in endemic regions, the annual incidence is more than 20/100000 population (Karthikeyan and Guilherme, 2018; Tibazarwa et al., 2008). The pathophysiology of ARF is speculated to be based on molecular mimicry and associated with polymorphisms of several genes involved in the innate and adaptive immunity (Azevedo et al., 2012; Baker et al., 2019). The streptococcal infection triggers production of antibodies and activation of T cells, both of which can target host tissues, including heart, joints, brain, and skin (Webb et al., 2015). The similarity of some antigens of *Streptococcus pyogenes* and several cardiac proteins is the main underlying cause of heart involvement, which can be life-threatening and is called rheumatic heart disease (RHD) (Marijon et al., 2012). RHD results from excessive inflammation, which can involve all three layers of the heart, but mostly affects endocardium. RHD is diagnosed by echocardiography (Liu et al., 2015). Cross-linking of antibodies in basal ganglia and joints can cause Sydenham's chorea and transient migratory polyarthrititis, respectively (Carapetis et al., 2016). Diagnosis of ARF is based on the modified Jones criteria (Table 2) (Pereira et al., 2017). Eradicating the infection with penicillin or erythromycin (in patients with penicillin allergy) is the primary treatment of ARF (Karthikeyan and Guilherme, 2018).

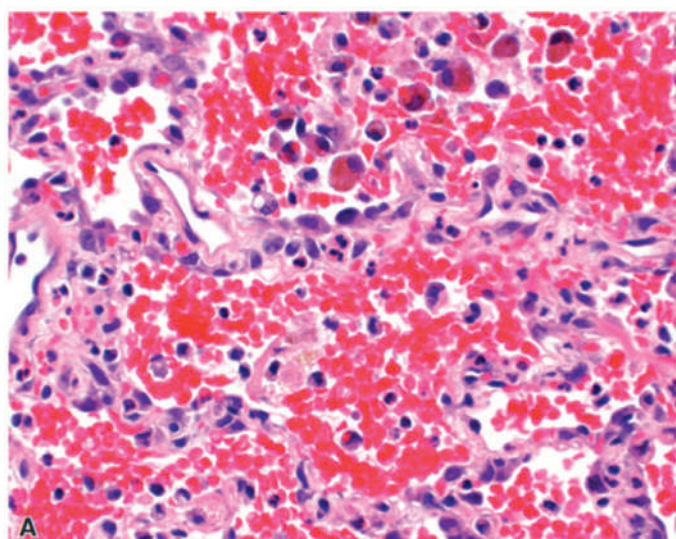


Fig. 2 Diffuse alveolar hemorrhage in addition to capillaritis in Goodpasture's syndrome. From Travis WD, Leslie KO, and Beasley MB (2018) Pulmonary vasculitis and pulmonary hemorrhage. In: Leslie KO and Wick MR (eds) *Practical Pulmonary Pathology: A Diagnostic Approach*, 3rd edn., Elsevier, ch. 11, 365–400.e5, ISBN: 9780323442848.

Table 2 Revised Jones criteria.

Major criteria	Minor criteria
Myocarditis	Fever
Joint involvement	Arthralgia
Erythema marginatum	Prolonged PR interval
Chorea	Elevated CRP/ESR
Subcutaneous nodules	

Diagnosis is established when there is laboratory confirmation of a recent group A streptococcal pharyngeal infection in addition to two major or one major and two minor criteria (Pereira et al., 2017).

Conditions mediated by cellular dysfunction

- Myasthenia gravis (MG)

MG is a chronic autoimmune disease causing weakness by affecting skeletal muscles and neuromuscular signal transmission (Gwathmey and Burns, 2015). Its prevalence is estimated to be 20 in 100,000 population (Phillips 2nd, 2003). Of note, in 10–15% of cases, MG is associated with an underlying thymoma (Romí et al., 2017). In MG, there is a disruption of stimulation of skeletal muscles. This stimulation is mediated by the attachment of presynaptic acetylcholine and acetylcholine receptors (AChR) in the membrane of these cells in the neuromuscular junctions (Hughes et al., 2004). In about 80% of cases, the disease is mediated by IgG1-3 autoantibodies against AChR (Gilhus et al., 2016). However, autoantibodies targeting either muscle-specific tyrosine kinase (MuSK) or low-density lipoprotein receptor-related protein 4 (LRP4) can also be responsible for the development of MG (Binks et al., 2016). AChR autoantibodies lead to its conformational changes, blockade of acetylcholine binding, and destruction of AChR by complement activation (Gilhus, 2016; Mantegazza et al., 2018). Apart from generalized disease of the muscles, MG may manifest as isolated eye involvement. In both conditions, ptosis, diplopia, and weak eye movements commonly occur due to involvement of extraocular muscles. In the generalized type, skeletal, bulbar, and respiratory muscles are also affected. As a consequence, the patient experiences fatigable chewing, dysarthria, dysphagia, expressionless face, dropped-head syndrome, and proximal weakness in limbs (Drachman, 2016). Diagnosis is established by detection of autoantibodies and electrophysiological studies. Acetylcholinesterase, immunosuppressive drugs, and corticosteroids are used in the treatment of this condition (Drachman, 2016; Mantegazza et al., 2018).

- Graves' disease (GD)

GD, the most common cause of hyperthyroidism, is an autoimmune disorder distinguished by over-stimulation and hypertrophy of the thyroid gland. With an estimated prevalence of 0.5%, GD commonly develops in the third-fifth decades of life. Notably, the lifetime risk of GD is six times higher in women compared to men (Brent, 2008). Genetic and epigenetic susceptibility are the key risk factors in GD (Cortes and Zeron, 2019; Bartalena, 2013). Environmental factors, like consumption of iodine and smoking, account for almost 20% of the risk (Bartalena, 2013). GD pathophysiology includes production of anti-thyrotropin receptor IgG1 subclass antibodies by B cells activated by autoreactive T cells. These antibodies act as an agonist and stimulate the production of thyroid hormones (Wemeau et al., 2018). Other autoantibodies can be found in GD as well. Some of these antibodies are involved in development of one of the characteristic features of GD; namely, ophthalmopathy (Fig. 3) (Smith and Hegedus, 2016). Manifestations of GD are similar to other disorders causing hyperthyroidism, including weight loss, fatigue, tremor, irritability, change of menstrual cycle, and erectile dysfunction. However, ophthalmopathy, dermatopathy, and acropachy (rare) are unique to GD (Antonelli et al., 2020). Presence of these symptoms, in addition to hyperthyroidism and diffused goiter (Fig. 4), suggests the GD diagnosis. In complicated cases, the diagnosis can be confirmed by radionuclide scanning or serological detecting of anti-thyrotropin receptor autoantibodies (Tozzoli et al., 2012). Treatments of GD include anti-thyroid drugs and ablative therapy, including ¹³¹I-radiotherapy and thyroidectomy (Bobanga and McHenry, 2019).

Type III (immune complex-mediated) hypersensitivity

Introduction

In this type of hypersensitivity, cross-linking of antigen-antibody pairs, which are produced during the normal immune response, leads to formation of unsolvable ICs. These complexes are usually positively charged; therefore, they have a high avidity to the basement membrane of kidney glomeruli and vessels, which have negative charges.

Mechanism

In case of excess production of immune complexes or impaired clearance of them, these complexes may get stuck in narrow channels of the body like capillaries and glomeruli. The deposition of these ICs activates the classical pathway of the complement which causes MAC mediated endothelial damage. After the endothelial injury, ICs infiltrate into the underlying tissue aggravating

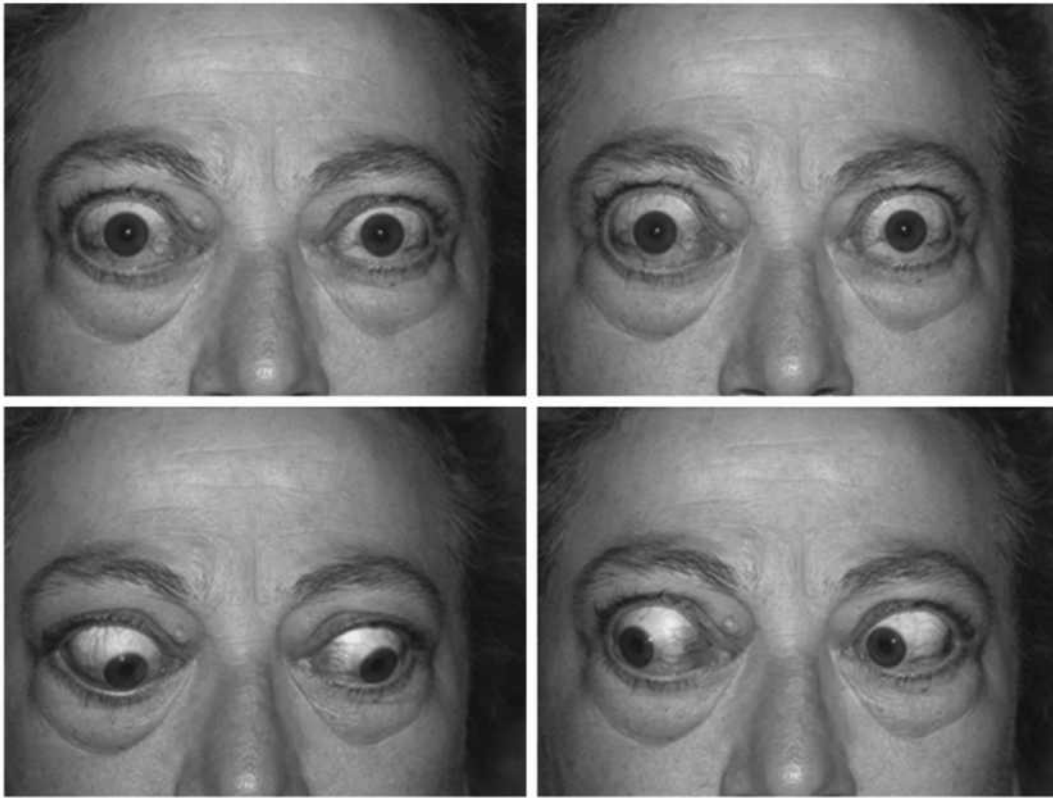


Fig. 3 Typical Graves' ophthalmopathy. From Marino M, Vitti P, and Chiovato L (2016) Graves' Disease. In: Jameson JL, De Groot LJ, de Kretser DM, Giudice LC, Grossman AB, Melmed S, Potts JT, Weir GC (eds.) *Endocrinology: Adult and Pediatric*, 7th edn., ch. 82, 1437–1464.e8, W.B. Saunders, ISBN: 9780323189071, doi: <https://doi.org/10.1016/B978-0-323-18907-1.00082-2>.

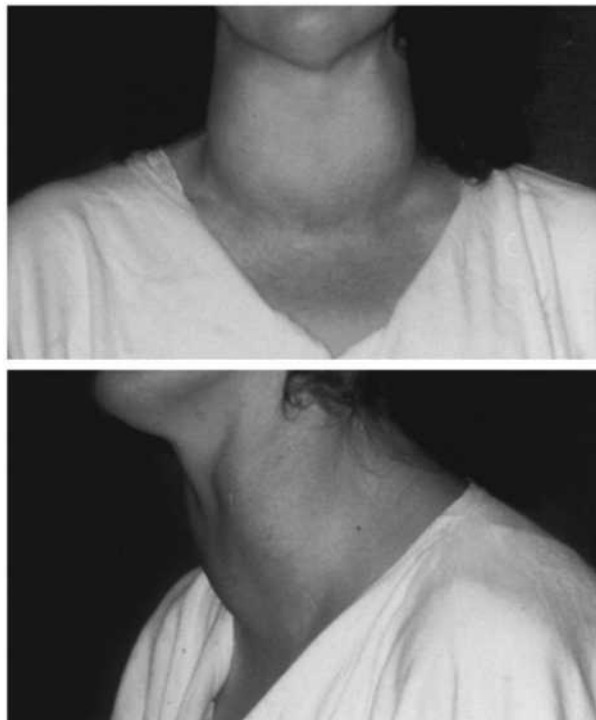


Fig. 4 Symmetrical and diffused enlargement of the thyroid in graves' disease. From Marino M, Vitti P, and Chiovato L (2016) Graves' Disease. In: Jameson JL, De Groot LJ, de Kretser DM, Giudice LC, Grossman AB, Melmed S, Potts JT, Weir GC (eds.) *Endocrinology: Adult and Pediatric*, 7th edn., ch. 82, 1437–1464.e8, W.B. Saunders, ISBN: 9780323189071, doi: <https://doi.org/10.1016/B978-0-323-18907-1.00082-2>.

complement activation, accumulation of the ICs, and leading to production of anaphylatoxins. Anaphylatoxins recruit mast cells, the secretion of which results in an increased vascular permeability. As a result of the increased vascular permeability and inflammation, leukocytes enter the tissue. In addition to the complement activation, neutrophils, macrophages, and NK cells can cause cellular damage by promoting phagocytosis or releasing lytic mediators. Although the deposition of the ICs can occur throughout the body, the most common sites of accumulation are small arteries, the synovia of joints, and glomeruli (Abul et al., 2017a; Delves et al., 2017a).

Examples of diseases caused by type III hypersensitivity

Serum sickness

Serum sickness is the first known type III hypersensitivity, recognition of which led scientists to identify pathogenicity of ICs for the first time in the early 1900s. Those days, serum of horses that had encountered diphtheria and had produced antibodies against it was used in the treatment of diphtheria in humans. Clemens von Pirquet, an Austrian scientist, noticed that approximately 1 week after the injection, patients develop arthritis, fever, and rash in response to the antiserum (Huber, 2006). This response was justified by production of antibodies directed to the antigens of the injected serum, which formed ICs by pairing with the antigens (Shulman, 2017). Nowadays, we know that serum sickness results from impaired clearance of ICs and their deposition in joints and parenchymal tissue (Lawley et al., 1984). These ICs can activate the classical complement pathway causing histamine release and inflammation (Rixe and Tavaréz, 2020). Medications such as rituximab, penicillin, cephalosporins (especially cefaclor), ciprofloxacin, and fluoxetine can cause this disorder (Cheong and Ooi, 2018; Maker et al., 2019). Notably, serum sickness is a self-limiting condition with a favorable prognosis (Joint Task Force on Practice et al., 2010). The symptoms can be managed by non-steroidal anti-inflammatory drugs (NSAIDs), antihistamines, and glucocorticoids (Clark et al., 2006).

Systemic lupus erythematosus (SLE)

SLE is a systemic chronic autoimmune disorder, the clinical manifestations of which are widely varied (Tsokos, 2011). With an estimated and increasing prevalence of 3–53/10000 people, SLE is most common among 15 to 45 year-old women (Stojan and Petri, 2018). While its pathophysiology is not fully understood, genetic susceptibility, like complement deficiencies, together with environmental factors, such as ultraviolet (UV) radiations, play a crucial role in its development (Lisnevskaja et al., 2014). The mechanism of SLE is mediated by defective clearance of apoptotic bodies, which is a result of impaired innate and adaptive immune responses. As a result, autoreactive antigen-presenting cells (APCs) capture the excessive nuclear antigens, which leads to activation of B and T cells. Activation of these cells gives rise to production of anti-nuclear antibodies, proliferation of CD8+ cytotoxic T cells, release of inflammatory cytokines, and tissue injury (Lisnevskaja et al., 2014; Fortuna and Brennan, 2013). Manifestations of SLE can be widely heterogeneous, including fatigue, fever, myalgia, arthritis, arthralgia, vasculitis, malar rash (Fig. 5), discoid, and photosensitivity. SLE can involve kidneys, gastrointestinal system, lungs, and eyes as well (Durcan et al., 2019). SLE patients are also more susceptible to coronary heart disease, and about 10% of them develop end-stage renal disease (Liu and Kaplan, 2018; Hanly et al., 2016). Diagnosis of SLE is based on the clinical manifestations, presence of anti-nuclear (ANA) or anti-double-stranded DNA (anti-dsDNA) or anti-Smith (Sm) antibodies, and evidence of SLE compatible nephritis in the kidney biopsy (Yu et al., 2014). Hydroxychloroquine, glucocorticoids, B-cell modulators, and immunosuppressive agents are some of the pharmacological treatments used in SLE management (Fanouriakis et al., 2019; Lisnevskaja et al., 2014; Tsokos, 2011).

Post-streptococcal glomerulonephritis (PSGN)

PSGN, the most common cause of childhood nephritis worldwide, occurs approximately 2 weeks after a group A streptococcus (GAS) skin or throat infection (Savige et al., 2019). Compared to the developed countries, where PSGN is more prevalent among



Fig. 5 Malar rash in a SLE patient, with a butterfly shape, crossing the nasal bridge, and sparing the nasolabial folds. From Nouh A, Speiser J, and Biller J (2015) Acquired neurocutaneous disorders. In: Islam MP and Steve Roach E (eds.) *Handbook of Clinical Neurology*, Elsevier, vol. 132, 29–73, <https://doi.org/10.1016/B978-0-444-62702-5.00003-2>.

elderly patients with debilitating conditions, in the developing countries, pediatric PSGN, with an annual incidence of about 9/100000, is more common (Rodríguez-Iturbe and Haas, 2016). Pathophysiology of PSGN is mediated by immune complexes. Antigens of GAS entering the circulation following the infection reside in glomeruli. Meanwhile, antibodies directed at these antigens are produced and form ICs with them either in the glomeruli or in the circulation, some of which aggregate in the glomeruli as well. Notably, before the activation of the adaptive immune response, streptococcal cell wall antigens activate innate immunity via the lectin pathway of the complement system. To date, two nephrogenic antigens; namely, nephritis-associated plasmin receptor (NAPlr) and streptococcal pyrogenic exotoxin B (SPE-B) have been found in GAS, which activate the alternative complement pathway, increase the production of circulatory ICs, and have high affinity to glomerular proteins (Eison et al., 2011; Rodríguez-Iturbe and Haas, 2016). Autoantibodies can also play a role in the PSGN pathophysiology (Balasubramanian and Marks, 2017). While PSGN is usually asymptomatic, some cases present with nephritic syndrome, exhibiting as hematuria, elevated creatinine levels, hypertension, and oliguria (Walker et al., 2014). The prognosis of PSGN is generally favorable. However, it can rarely lead to acute renal failure (VanDeVoorde, 2015). Detection of antistreptolysin O (in pharyngitis) and anti-DNase B (in impetigo) antibodies, together with low C3 levels, can help in the PSGN diagnosis (Maness et al., 2018). PSGN management is supportive treatment, unless an active infection is present, in which case antibiotics are used (Hunt and Somers, 2019).

Type IV (cell-mediated or delayed-type) hypersensitivity

Introduction

Type IV hypersensitivity is mediated by T cells and macrophages while other types are mediated by antibodies. Therefore, this reaction, which occurs at least 1–3 days after the initial exposure, is called delayed-type hypersensitivity (DTH) (Luo and Dorf, 2001). Type IV hypersensitivity is the underlying mechanism of many autoimmune disorders.

Mechanism

Activation of CD4+ T cells by tissue APCs can lead to tissue damage via two mechanisms: (1) cytokine-mediated inflammation and (2) direct cell killing by CD8+ cytotoxic T cells (CTLs). These mechanisms are described in the following lines.

Cytokine-mediated inflammation

Cytokines and other mediators produced by the immune cells play a key role in type IV hypersensitivity. For instance, T-helper 1 can promote activation of macrophages by interferon (IFN)- γ , recruit monocytes by tumor necrosis factor (TNF)- α and IFN- γ , aggravate proliferation of T-helper 1 cells by IL-2, and activate fibroblasts and angiogenesis by cytokines such as growth factors. Moreover, IL-17, which is released from T-helper 17, helps in the recruitment of neutrophils. The recruited neutrophils and macrophages produce toxic factors such as reactive oxygen species (ROS) and inflammatory mediators, which can lead to tissue injury (Tak Mak and Jett, 2014).

CTL mediated direct cell killing

Cross-linking of APCs with CD4+ T-helper cells trigger generation of CTLs. These T cells can be directed to foreign or autoantigens. Activation of CD8+ CTLs directed at self-tissue can lead to direct cell injury, an example of which is type 1 diabetes caused by CTL-mediated destruction of β cells of the pancreatic islets. Additionally, excessive reaction of CTLs directed at foreign antigens, whose main function is to defend against intercellular pathogens such as viruses, can lead to an excess damage in viral infections. The underlying cause of this extra damage is that CTLs are generally not able to differentiate between viral infections causing cell death and the less toxic ones. For instance, in some cases, like some types of hepatitis, the viral infection itself might not cause cell injury. Therefore, an excessive CTL response in these cases can be the primary cause of tissue damage (Welz et al., 2018).

Granulomas, which are accumulations of immune cells, are the hallmark of type IV hypersensitivity. Activated T-helper 1 cells produce pro-inflammatory cytokines, such as TNF- α and IFN- γ , leading to recruitment of monocytes to the tissue and massive activation of the macrophages. Some of these macrophages fuse and form giant cells, which are a key component of granuloma. In the granuloma, which is surrounded by fibroblasts, T-helper 1 cells can be detected as well. Generally, granulomas form in response to infections, such as tuberculosis, and play a protective role. However, they can also form in autoimmune disorders like Crohn's disease (Timmermans et al., 2016).

Examples of diseases caused by type IV hypersensitivity

Multiple sclerosis (MS)

MS is a demyelinating disease of the central nervous system (CNS) mediated by immune dysfunction. The prevalence of MS varies from 2 to 164 per 100,000 population in different regions (Collaborators, 2019; Howard et al., 2016). A complex interplay of mostly environmental factors, e.g., vitamin D deficiency, smoking, Epstein–Barr virus infection and to a lesser extent genetic susceptibility, e.g., HLA DRB1*15:01, is involved in the development of MS. MS pathology is characterized by multiple demyelinating plaques in the brain, particularly near bilateral ventricles, and the spinal cord. The exact pathogenesis and initial mechanisms of MS are widely unknown. Disruption in the integrity of the blood-brain barrier and aberrant adaptive immune responses seem to

play a pivotal role. Production of inflammatory mediators by T cells can damage oligodendrocytes; activate B cells, which produce antibodies against myelin molecules; and activate microglia and macrophages, which may phagocytose oligodendrocytes or aggravate the inflammation. Moreover, perforins secreted by CTLs may lead to axonal injury secondary to demyelination, which seems to be the leading cause of disability (Lemus et al., 2018). MS can present in four different forms, which are (1) relapsing-remitting multiple sclerosis (which is the most common form), (2) primary progressive MS, (3) secondary progressive MS, and (4) progressive-relapsing MS (which is the rarest form). Fig. 6 depicts the disease course in each form. The symptoms of MS are widely varied depending on the location of the plaques and the severity of the inflammation. Optic neuritis, fatigue, spasticity, bowel, bladder, sensory, and sexual dysfunction are among the most common manifestations of MS. Presence of at least two neurological attacks separated in time and space, detection of demyelinating lesions in magnetic resonance imaging (MRI), in addition to laboratory tests (such as blood and CSF analysis) are the clues to MS diagnosis. Treatment of MS is comprised of three sections: management of exacerbations, slowing the course of the disease with disease-modifying therapies, and symptomatic treatments (Michel et al., 2015; Dobson and Giovannoni, 2019).

Rheumatoid arthritis (RA)

RA is a chronic autoimmune condition characterized by inflammation, which primarily affects joints and can lead to disability. With an estimated prevalence of 0.5–1% worldwide, RA has a great burden for the patient and society (Silman and Pearson, 2002). Genetic susceptibility, epigenetic modifications, female gender, and smoking are the most prominent risk factors in RA (Deane et al., 2017). APCs Present foreign or autoantigens to naïve T cells, leading to their activation and differentiation. This results in activation of B-cell and macrophage (Aletaha and Smolen, 2018). The activated B cells may give rise to production of autoantibodies, like against citrullinated peptides antibody (ACPA), which activate macrophages, osteoclasts, and formation of ICs. These ICs aggregate in the synovium, trigger production of anti-IgG antibody (rheumatoid factor), and may cause complement activation. The activation of macrophages results in production of pro-inflammatory cytokines, which results in recruitment of immune cells and activation of fibroblasts (McInnes and Schett, 2011). The fibroblasts stimulate generation of osteoclasts via interaction of receptor activator of nuclear factor κ B ligand (RANKL) and RANK (Smolen et al., 2016). All of these processes lead to symmetric

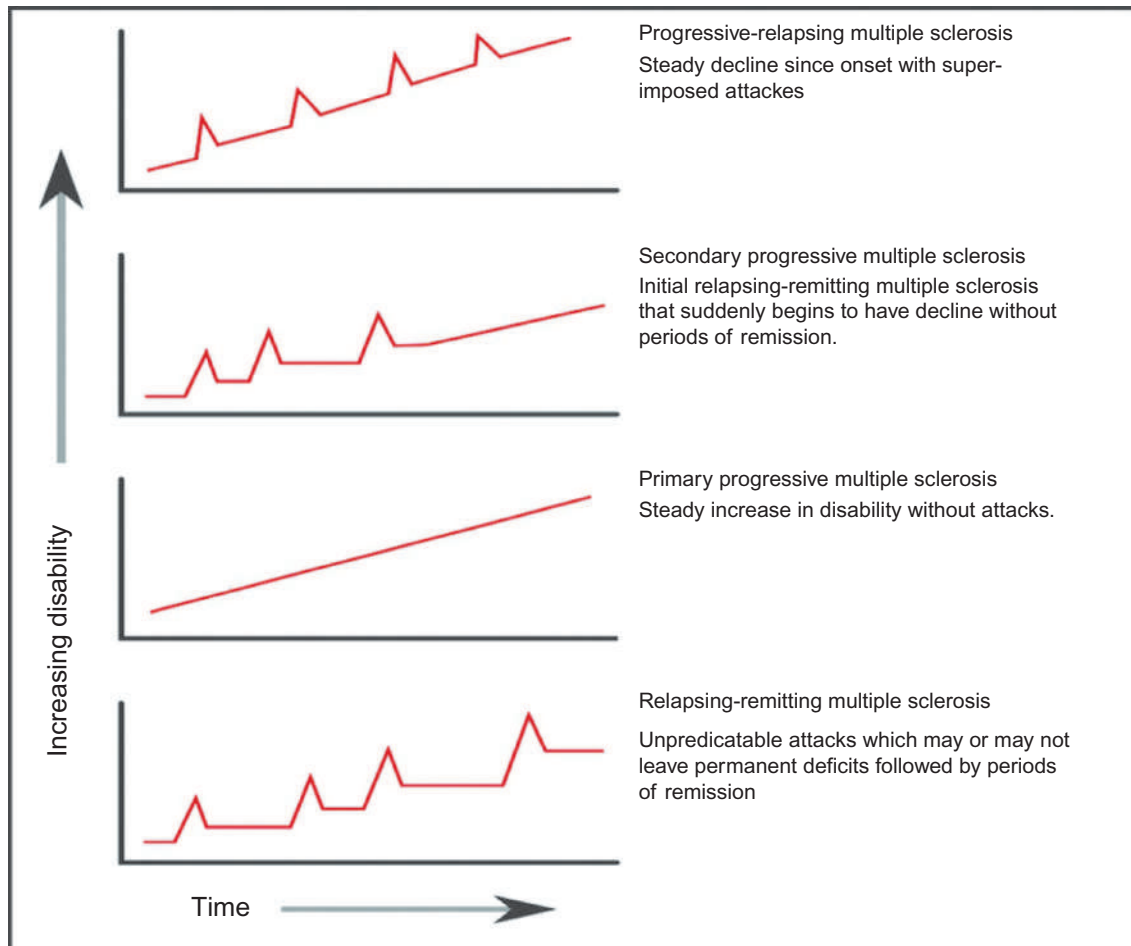


Fig. 6 Four different disease courses in multiple sclerosis: The X axis shows time (age of the patients) and the Y axis shows disability.



Fig. 7 Rheumatoid nodules. From Erickson AR, Cannella AC, Mikuls TR (2017) Clinical features of rheumatoid arthritis. In: Firestein GS, Budd RC, Gabriel SE, McInnes IB, and O'Dell JR (eds.) *Kelley and Firestein's Textbook of Rheumatology*, 10th edn., 1167–1186, ch. 70, Elsevier, <https://doi.org/10.1016/B978-0-323-31696-5.00070-X>.

pain and swelling in small or large joints and morning stiffness. RA commonly involves joints of hand and feet. Additionally, 18–41% of patients present with extra-articular manifestations, the most common one of which is rheumatoid nodules (Fig. 7). Other extra-articular manifestations include interstitial lung disease, rheumatoid vasculitis, neurological involvements, and increased risk of cardiovascular disease (Marcucci et al., 2018). Early diagnosis of RA, which is mainly based on clinical examination, plays a vital role in RA management and prevention of disability (Allen et al., 2018). The number of involved joints, duration of symptoms, and presence of acute-phase reactants and autoantibodies are components of the 2010 RA classification criteria (Aletaha et al., 2010). RA management encompasses strategies targeting remission or reducing disease activity. Pharmacological agents used in the treatment include disease-modifying anti-rheumatic drugs (DMARD) and anti-inflammatory medications (Aletaha and Smolen, 2018; Littlejohn and Monrad, 2018).

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Immunosenescence

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Introduction

The alterations observed regarding the immune system during aging are summarized as immunosenescence (Singhal et al., 1978). Since the populations in the western countries are aging, due to an increased life expectancy and low birth rates, immunosenescence becomes more and more important for the western societies (Luy et al., 2020). Aging impacts all organ systems and mostly results in their decreased functionality. Regarding the immune system, most functions decrease as well. However, some activities are increased as part of immunosenescence, resulting in an overall complex alteration of the immune response and higher incidence of infections, tumors and autoimmunity (Fulop et al., 2019). The immune system is modified by dynamic changes throughout life. Two major factors drive these changes: sex and age. Both are obviously hard to influence in contrast to other factors impacting on the immune system like nutrition, physical activity, environmental pollution, etc. (Rink et al., 2015). The functionality of the immune system is therefore directly related to wellbeing and life expectancy. Investigations on elderly over 100 years, so-called centenarians, showed that these people have a better functioning immune system than the average of normal aged people, which includes a high number of frail elderly (Borras et al., 2020).

Whereas all healthy humans are born with a powerful innate immunity, the adaptive immunity improves during childhood through the contact to harmless and harmful foreign organisms and particles. The immune system undergoes major changes during puberty, resulting in an initially stronger immune response in women than in men. During menopause, the efficiency of the immune response in women starts to decline. After the age of 65 years the immunity of men and women is nearly equal and starts to deteriorate, which results in increased numbers of infections and cancers. However, the number of autoimmune diseases increases with age as well. Interestingly, the immune response of centenarians shows a less pronounced decline and is comparable to adults under the age of 60 years.

In a long-term observational study, starting in 1989 with elderly over 80 years, which were monitored until the age of 90 years (i.e., nonagenarians), an immune risk phenotype (IRP) has been defined by Swedish scientists. Changes leading to the IRP are not observed in young people, so that life expectancy could not be estimated. However, in elderly the IRP shows characteristic changes of the immune system with a decreased functionality which resulted in death within the next years (Wikby et al., 2008). In the normal population the decreased function of the immune system becomes obvious long before, since the rate of infections and tumors increases strongly with age (Katzir et al., 2021). The infection-dependent death rate increases steadily from the age of 50 years and is ten times higher at the age of 80 years compared to the rate at an age below 50 years. Interestingly, the increase in the death rate is delayed in women, which is related to the onset of menopause. After reaching the age of 75 years the infection-related death rate in men and women is equal (Rink and Dalhoff, 2004).

Since we cannot stop aging, the only way to improve the immune response is to decrease the biological aging by a positive impact via a healthy lifestyle, including balanced nutrition and exercise. It is important to mention that the aim of immunosenescence research or immune gerontology is not immortality, since human life is restricted to around 120 years (the oldest person on earth became 122 years) (Niedermüller and Hofecker, 2004). The aim of this field of research is to improve the immune capacity, consequently increasing the number of healthy years so that an individual's optimal life expectancy will be reached. Therefore, the most important question is how to reach the immune capacity of centenarians and to live a healthy life until the personal genetics

determine life's end. Most centenarians die between the age of 104–106 years, but without a long period of illness or frailty (Borras et al., 2020). To explain this miracle, the age-related differences between the immune system of normal elderly and centenarians must be understood to uncover and address the immunological issues responsible for frailty (Nielsen et al., 2021). Based on a number of studies with SENIEUR-elderly, a group specified by a complex protocol, those differences were clearly defined (Ligthart et al., 1984, 1990a). This definition is important since in contrast to the field of pediatrics, still no age-specific standard values for laboratory parameters in geriatrics are defined, although it is well known that a number of parameters show age-related alterations without any related diseases (Harm, 1997). However, these various alterations of standard clinical laboratory parameters seems to appear randomly up- or down-regulated. Often one parameter is always altered in the same direction, but biochemically related parameters are changed into a different direction. Therefore, it helps to categorize these observations for “normal elderly,” SENIEUR-elderly and centenarians. “Normal elderly” describes the average population over 65 years including frail elderly and elderly with diseases, since a couple of diseases have an age-independent effect on the immune system. In comparison to the SENIEUR-elderly, the normal elderly are often non-healthy, but the regular medical case, since the SENIEUR protocol excludes 90% of the elderly population. Therefore, normal elderly could be split into healthy (but not selected with SENIEUR criteria) and frail elderly. Table 1 shows some major differences of the immune system of these four major groups of elderly. In the following paragraphs, the age-related alterations of the different parts of the immune system will be discussed in detail in the light of healthy elderly (Compte et al., 2019). Fig. 1 shows a summary of all functions disturbed in the aged immune system.

Innate immune system

The innate immune system includes physical barriers of the body like skin and mucosa as firewalls. These barriers are less effective in elderly, since their skin is thinner and drier (Wlaschek et al., 2021). On dry skin, less fat soluble, antimicrobial defensins are present. Mucosal surfaces show a loss of moisture as well, which facilitates the settlement of bacteria. Elderly have a decrease in cilia function, which leads to a reduced export of bacteria from the body and facilitates bacterial settlement (Bailey et al., 2018). In addition, the cough reflex to eject bacteria is decreased (Lalley, 2013). Those disturbances specifically explain the age-related increase in pneumonia susceptibility. Gastrointestinal and bladder infections increase with age as well. This is related to a reduced acidity of the gastric acid since the low pH normally kills bacteria. In the bladder, the bacteria stick to the surface more easily, due to a decreased diuresis since normally the urine flow flushes out the bacteria. The reduced diuresis is boosted by less drinking as a consequence of the absence of thirst. The weakness of the physical barriers in the elderly reduces the threshold for bacterial numbers, sufficient to establish an infection (Jump and Canaday, 2017).

Table 1 Immunological differences between frail, healthy and SENIEUR elderly as well as centenarians.

Parameter	Frail elderly	Healthy elderly	SENIEUR elderly	Centenarians	References
Immune risk phenotype	YES	YES	YES	NO	Nilsson et al. (2003), Strindhall et al. (2013) and Strindhall et al. (2007)
Inflammaging	YES	YES	YES	YES	Mysliwska et al. (1998), Franceschi et al. (2000), Ferrucci et al. (2005) and Compté et al. (2013a)
Cytokine production induced by Toll-like receptor activation	↓	↓	↑	↑	Gabriel et al. (2002), Della Bella et al. (2007), Compté et al. (2013b), Verschoor et al. (2014)
Inflammatory monocytes	↑	↑	↑	?	Sadeghi et al. (1999), Seidler et al. (2010)
Number of NK cells	↑	↑	↑	↑	Cakman (1996) and Miyaji et al. (2000)
NK cell activity	↓	↓	↓	↑	Lesur et al. (2019)
Interleukin-2 secretion	↓	↓	↔	↔	Cakman (1996), Mysliwska et al. (1998), Ahluwalia et al. (2001) and Palmeri et al. (2012)
Naïve B cells	↓	↓	↓	↔	Colonna-Romano et al. (2009) and Colonna-Romano et al. (2010)
Memory B cells	↑	↑	↑	↔	Colonna-Romano et al. (2009) and Colonna-Romano et al. (2010)
B cell repertoire	↓	↓	↔	↔	Gibson et al. (2009)
Naïve T cells	↓	↓	↓	↔	Derhovanessian et al. (2010)
Memory T cells	↑	↑	↑	↔	Derhovanessian et al. (2010)
T cell repertoire	↓	↓	↓	↔	Naylor et al. (2005) and Britanova et al. (2014)
CD4/CD8 T cell ratio	↓	↑	↑	↑	Cakman et al. (1996), Strindhall et al. (2007) and van der Geest et al. (2014)

Increased ↑, decreased ↓, normal ↔, unknown ?

Table adapted and updated from Compté N, Pepersack T and Goriely S (2019) Frailty in old age is associated with altered cytokine production in response to TLR ligation. In T Fulop, C Franceschi, K Hirokawa and G Pawelec (eds.), *Handbook of Immunosenescence*. 2nd edn, pp. 2417–2434, Springer International Publishing: Cham. doi:10.1007/978-3-319-99375-1_152.

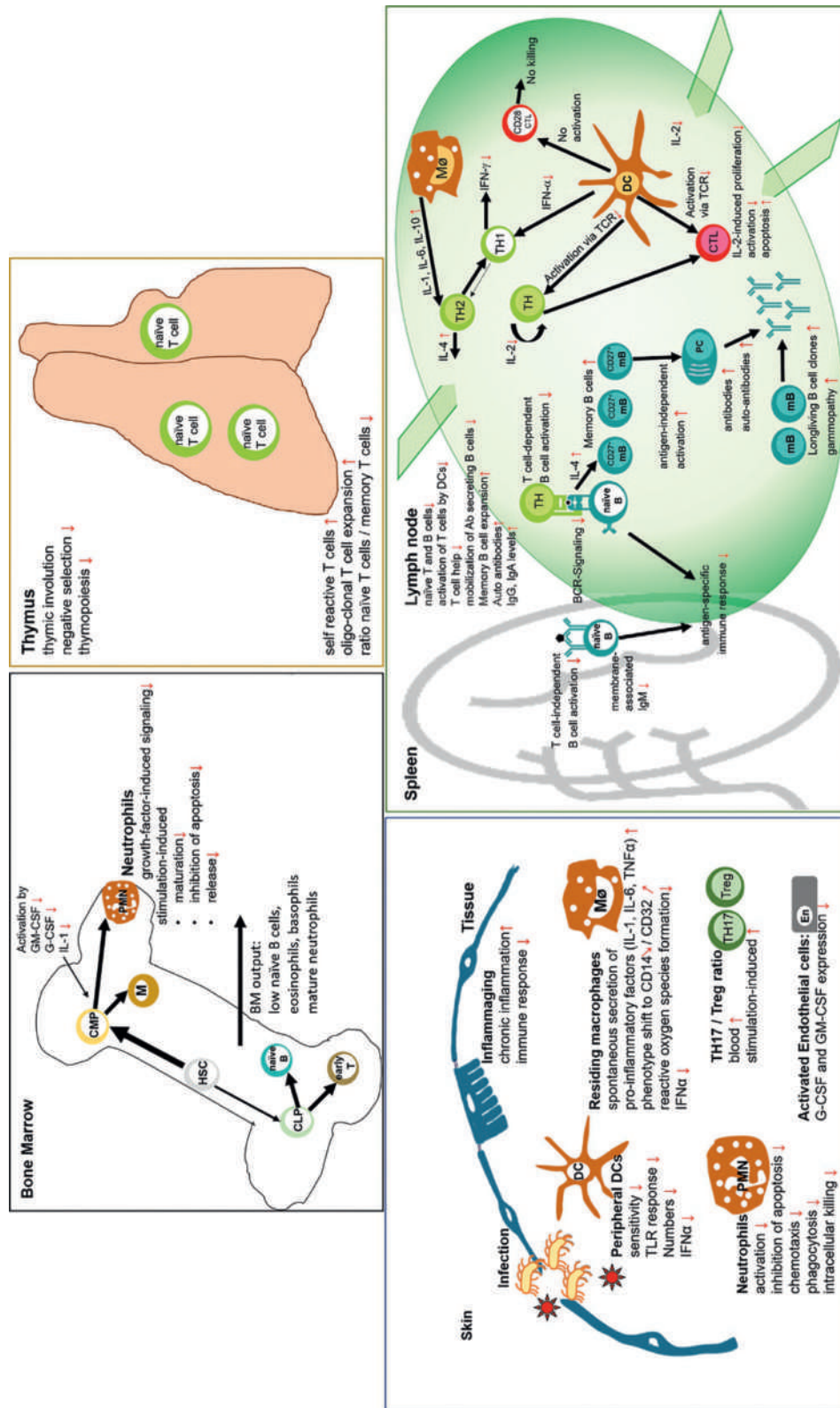


Fig. 1 Summary of immunosenescence. All stages of the immune response are altered in aging, starting with the disruption of the maturation of leukocytes in the bone marrow (BM) (upper left). Hematopoietic stem cells (HSC) differentiate more towards common myeloid precursors (CMP) than to common lymphoid precursors (CLP), leading to a reduced production of lymphocytes in contrast to a normal production of myeloid cells (mainly monocytes (M) and polymorph nuclear neutrophils (PMN)). However, the up-regulation of PMN release from the bone marrow is disturbed in the elderly, due to a reduced signal transduction of the cytokines (e.g., interleukin (IL)-1, granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage CSF (GM-CSF)) responsible for their differentiation. Since the thymus

(upper right) is shrinking with age (thymic involution), the production of new T cells without antigen contact (naïve T cells) is reduced. Furthermore, in both T and B cells, the number of long living clones increases. The interaction between different leukocytes as part of the immune response in the spleen and lymph nodes (lower right) is disturbed as well. Both T cell-dependent and T cell-independent B cell activation are reduced in aging, mainly due to a decreased signal transduction of the B cell receptor (BCR) and a reduced BCR expression in naïve B cells (membrane-associated IgM). However, the antigen-independent B cell activation, e.g., by cytokines like IL-4, is increased, leading to a proliferation of memory B cells (mB) and an increased production of IgG and IgA by mB and plasma cells (PC). The interaction between T cells and dendritic cells (DC) or macrophages (Mφ) is disturbed as well. The activation through the T cell receptor (TCR) is reduced and major cytokines for inducing a T helper (TH) 1 response (e.g., interferon (IFN)- α and IL-12) are less produced. This leads to a reduced production of TH1 cytokines (e.g., IL-2, IFN- γ) as well. On the other hand, as part of inflammaging, Mφ produce increased amounts of the pro-inflammatory cytokines IL-1 and IL-6, as well as IL-10, which stimulates TH2 cells and the production of IL-4. This results in a dysbalance of TH1 and TH2 cells. In the skin and peripheral tissues (lower left) this leads to a chronic inflammatory process even in the absence of a pathogen, i.e., without a danger signal. On the other hand, this chronic inflammation results in a reduced activation-induced immune response. Furthermore, the sentinel cells of the immune system (i.e., DC and Mφ) as well as the PMN show reduced reactivity and killing activity, but a spontaneous release of pro-inflammatory cytokines. Although the ratio between TH17 cells and regulatory T cells (Treg) increases in the blood, upon stimulation this turns around preventing an activation of the adaptive immune system. Finally, the activated endothelial cells produced fewer recruiting factors for leukocytes (e.g., G-CSF, GM-CSF) and thereby closing the vicious cycle of the disturbed immune response in aging. This figure is adapted and updated from Rink L, Kruse A and Haase H (2015) *Immunologie für Einsteiger*. 2nd edn, Springer Spektrum: Berlin. doi: 10.1007/978-3-662-44843-4.

The functions of the complement system itself are almost unaffected by aging, as long as the liver metabolism is intact. Some hepatic factors like acute phase proteins are even elevated in serum. However, it is a matter of debate if this increase is due to a chronic inflammatory process in the elderly, called inflammaging, or due to the frequent dehydration and thus concentrating serum factors. However, the alterations on the cellular level are more prominent.

Granulocytes and mast cells

The overall number of granulocytes in the blood remains largely stable during aging but the numbers for the three subtypes change differently. In contrast, the function of all granulocyte subtypes is altered markedly in the elderly (Uciechowski and Rink, 2019). Changes in granulocytes and mast cells are summarized in Table 2.

Basophils

The total number and percentage of basophils in the blood is significantly decreased in aging. Functions of basophils are altered as well. Basophils show a delayed activation, but an increased release of histamine in the elderly. However, the knowledge on basophils in aging is still limited (Uciechowski and Rink, 2019).

Eosinophils

Research data on eosinophils in aging is scarce as well. The number of eosinophils is decreased in elderly. Eosinophils seem to have a lower chemotactic activity in aging, but data are controversial (Uciechowski and Rink, 2019). The production of reactive oxygen species (ROS) is decreased and stimulation-induced degranulation is delayed in the elderly (Mathur et al., 2008).

As a result from dysfunctions in eosinophils and basophils, the defense against parasites and fungi is markedly decreased in elderly.

Neutrophils

The changes of neutrophils (or polymorphonuclear neutrophils (PMN)) during aging are well studied. The number of PMN in the elderly is normal or slightly increased. Although the basal number of PMN in the blood might be increased, the production rate in the bone marrow is unchanged. The infection-induced increase in granulopoiesis is markedly decreased (Uciechowski and Rink, 2019), mostly due to a disturbed signal transduction of G-CSF (granulocyte colony-stimulating-factor), whereas GM-CSF-induced signaling (granulocyte/macrophage CSF) is unaffected (Chatta et al., 1993; Fulop et al., 2019). Normally, G-CSF and GM-CSF inhibit apoptosis in PMN during an infection, but not in aging (Fulop Jr et al., 1997; Tortorella et al., 1998). In addition to the higher production, the inhibition of apoptosis is a second reason why the number of PMN in young people quickly increases as an early response of the immune system. Together these two age-related changes lead to a relative deficiency or even an exhaustion of PMN during infection in the elderly. This is also the reason why a decreased lifespan of PMN is observed in the elderly. The main functions of PMN, i.e., chemotaxis, phagocytosis, oxidative burst (ROS production) and intracellular killing, are all decreased in aging (Uciechowski and Rink, 2019). The reduced chemotaxis is once more based on the disturbed signal transduction of chemotactic factors. In contrast to the chemotaxis, the rolling and attachment of PMN to the endothelium before diapedesis is even elevated due to an increased expression of adhesion molecules (e.g., CD15). However, this increased contact between PMN and the endothelium is a disadvantage, since it causes damage of the endothelium. PMN exert enhancing effects in the late phase of the immune response, which is disturbed in the elderly due to a reduced expression of the Fc γ -Receptor-III (CD16) on the surface of the PMN (Uciechowski and Rink, 2019). However, although the total number of PMN does not change, hematopoietic stem cells (HSC) of elderly preferentially differentiate into myeloid cells (Garrrick et al., 2019).

In conclusion, the defense against bacteria and fungi is markedly decreased in elderly due to the reduced recruitment and killing capacity of neutrophils.

Table 2 Altered granulocyte and mast cell functions in the elderly.

Parameter/Function	Alteration	References
Basophils		
Number and percentage	↓	Song et al. (1999)
Activation	*	Schwarzenbach et al. (1982)
Histamine release	↗	Marone et al. (1986)
Eosinophils		
Number	↘	Starr and Deary (2011)
Chemotactic activity	↘	Mathur et al. (2008)
ROS production	↘	Mathur et al. (2008)
Degranulation	*	Mathur et al. (2008)
Neutrophils		
Number	↗	Chatta et al. (1993)↔, Born et al. (1995)↔, Cakman (1996)↑ and Cakman et al. (1997)↑
Number of precursor in bone marrow	↔	Chatta et al. (1993)↔
Precursor activation with G-CSF	↓	Chatta et al. (1993)↓
with GM-CSF	↔	Chatta et al. (1993)↔
with interleukin-3	↔	Chatta et al. (1993)↔
Chemotaxis	↘	Biasi et al. (1996)↔, Esparza et al. (1996)↔, Niwa et al. (1989)↓, and Wenisch et al. (2000)↓
Expression of adhesion molecule	↔	Esparza et al. (1996)↔ and Butcher et al. (2001)↔
CD11a		
CD11b	↔	Rao (1986)↔, Esparza et al. (1996)↗, and Butcher et al. (2001)↔
CD15	↗	Esparza et al. (1996)↗
Expression of Fcγ-receptor RIII (CD16)	↓	Butcher et al. (2001)↓
Oxidative burst (ROS production)	↓	Biasi et al. (1996)↓, Braga et al. (1998a)↓, Braga et al. (1998b)↓, Tortorella et al. (1998)↓, Lord et al. (2001)↔↗, and Butcher et al. (2001)↔↗
Intracellular killing	↓	Lesur et al. (2019)
Ca ²⁺ release upon stimulation with fMLP	↓	Varga et al. (1988)↓, Fülöp et al. (1989)↓, and Lipschitz et al. (1991)↓
Intracellular Ca ²⁺ level in resting cells	↑	Varga et al. (1988)↑, Mohacsi et al. (1992)↑, and Wenisch et al. (2000)↑
Inhibition of apoptosis after stimulation with G-CSF	↓	Tortorella et al. (1998)↓
GM-CSF	↓	Fulop Jr et al. (1997)↓ and Tortorella et al. (1998)↓
IL-2	↓	Fulop Jr et al. (1997)↓
LPS	↓	Fulop Jr et al. (1997)↓, Tortorella et al. (1998) and Tortorella et al. (1998)↓
Toll-like receptor signaling	↓	Lesur et al. (2019)↓
Mast cells		
Number	↑	Kutukova et al. (2016)↑
Preactivation	↗	Kutukova et al. (2016)↗
Activation upon stimulation	↘	Kutukova et al. (2016)↘

Significantly increased ↑, increased ↗, significantly decreased ↓, decreased ↘, normal/unchanged ↔, delayed *, unknown ?

Table updated from Rink L and Dalhoff K (2004) Altersspezifische Veränderungen des Immunsystems und deren assoziierte Krankheitsbilder. In D Ganten, K Ruckpaul and A Ruiz-Torres (eds.), *Molekularmedizinische Grundlagen von altersspezifischen Erkrankungen*, 1st edn, pp. 429–464, Springer: Berlin. doi:10.1007/978-3-642-18741-4_16.

Mast cells

The number of mast cells in skin and other tissues increases significantly with age and is related to tissue fibrosis. Mast cells show an unspecific activation in aging but their function and activation upon stimulation are decreased (Kutukova et al., 2016). Moreover, their main function as early sensors of pathogens is disturbed. Altogether, the knowledge on mast cells in aging is very limited in contrast to that on granulocytes. In conclusion, the elevated numbers of mast cells in aging have rather a negative effect.

NK cells

The number of natural killer (NK) cells and more prominently NKT cells increases with age. The increase in NK cells is mostly based on long-lived NK cells, whereas the NK cell production in the bone marrow decreases (Campos et al., 2019). However, the functionality of NK cells decreases even in SENIEUR elderly. The reduced activity becomes apparent in a decreased killing rate, proliferation, activation, interferon (IFN)-γ and chemokine production of the NK cells (Borrego et al., 1999a,b). The decreased expression of CD25 (high affinity interleukin (IL)-2 receptor) (Bartoloni et al., 1990) and CD69 (general activation marker of lymphocytes) (Borrego et al., 1999a,b) indicates a reduced activation rate. Since the perforin concentration in the NK cells of elderly remains stable, the decreased activation seems to be the primary explanation for the disturbed killing (Mariani et al., 1994). The lower production of the mentioned cytokines leads to a reduced activation of other leukocytes in the early phase of the immune response. Interestingly, the NK and NKT cell activity in centenarians is normal or even slightly elevated (Levy et al., 1991; Steinmann and Hartwig, 1995). Age-dependent alterations in NK cells are summarized in Table 3.

Table 3 Age-related changes in NK cells.

Parameter/function	Alteration	References
Number of CD56 ⁺ NK cells	↗	Sanson et al. (1993) ↔ and Solana et al. (1999) ↑
Percentage of CD56 ⁺ NK cells	↑	Sanson et al. (1993) ↑, Cakman et al. (1996) ↗, Solana et al. (1999) ↑
Number of CD16 ⁺ NK cells	↑	Sanson et al. (1993) ↑, Xu et al. (1993) ↑, Born et al. (1995) ↔
CD57 ⁺ NK cells	↑	Ligthart et al. (1986) ↑, Sansoni et al. (1993) ↑, Kudlacek et al. (1995) ↑, and Solana et al. (1999) ↑
CD2 ⁺ /CD16 ⁺ NK cells	↑	Xu et al. (1993) ↑
NKT cells	↑	Tarazona et al. (2001) ↑
CD3 ⁺ and (CD16 ⁺ or CD56 ⁺ or CD94 ⁺)		
NKT cells	↓	DelaRosa et al. (2002) ↓
Vα24 ⁺		
NK cell activity in SENIEUR elderly	↘	Mariani et al. (1994) ↘ and Borrego et al. (1999a) ↘
NK cell activity in centenarians	↗	Levy et al. (1991) ↗, Steinmann and Hartwig (1995)
Killing rate of NK cells	↘	Solana and Mariani (2000) ↘
Expression of CD25	↘	Bartoloni et al. (1990) ↘
CD69	↘	Borrego et al. (1999a) ↘, Borrego et al. (1999b) ↘
Cytokine-induced proliferation	↘	Borrego et al. (1999a) ↘ and Borrego et al. (1999b) ↘
Ca ²⁺ -release	↘	Borrego et al. (1999b) ↘ and Solana and Mariani (2000) ↘
IFN-γ-production	↘	Borrego et al. (1999b) ↘ and Solana and Mariani (2000) ↘
Chemokine-production	↘	Borrego et al. (1999b) ↘ and Solana and Mariani (2000) ↘
TNF-production	↔	Solana and Mariani (2000) ↔
Perforin release	↔	Solana and Mariani (2000) ↔

Significantly increased ↑, increased ↗, significantly decreased ↓, decreased ↘, normal/unchanged ↔.

Table updated from Rink L, Kruse A and Haase H (2015) *Immunologie für Einsteiger*. 2nd edn, Springer Spektrum: Berlin. doi: 10.1007/978-3-662-44843-4.

In conclusion, the defense against viruses and cancer is markedly decreased in the elderly due to the reduced killing capacity of NK cells and the diminished recruitment and activation of other cells by the NK cells in the early phase of the immune response.

Monocytes, macrophages, and dendritic cells (DC)

Antigen presentation is a prerequisite for activation of the adaptive immune system. The main antigen presenting cells in the early immune response are monocytes/macrophages and dendritic cells (DC), including their tissue resident subtypes. The number of monocytes and DC in the blood of the elderly is equal to that of young adults (Fulop et al., 2019). In contrast, numbers of skin DC (Langerhans cells) decrease with age (Chambers and Vukmanovic-Stejic, 2020). Data on other tissue resident macrophages and DC are rare and not consistent but decreased numbers can be expected as well. In contrast to most other cell types, the monocytes/macrophages of elderly show an augmented inflammatory response, e.g., an overproduction of IL-1, IL-6 and other cytokines (see section “Immune regulation by cytokines”). In contrast to granulocytes, the phagocytic activity of monocytes/macrophages is close to normal. However, the antigen presentation and their capability to activate T cells are reduced in monocytes/macrophages and DC (Rich et al., 1993; Agrawal et al., 2007). Pinocytosis and endocytosis decline with age in DC as well. Collectively, the activity of antigen presenting cells is disturbed. However, recent data indicate that the number of MDSC (myeloid-derived suppressor cells) is increased in aging (Verschoor et al., 2013; Alves et al., 2018), which augments the deregulated balance within the myeloid system. Age-related changes in monocytes/macrophages and DC are summarized in Table 4 (Della Bella, 2019).

In conclusion, the defense against all types of pathogens is markedly disturbed in elderly due to the impaired antigen presentation by monocytes/macrophages and DC and the imbalance triggered by an overproduction of pro-inflammatory cytokines and the increased number of MDSC.

Adaptive immune system

The adaptive immunity, especially the T cell system, is stronger impaired by aging than the innate immune system. The differentiation of hematopoietic stem cells to the lymphoid lineage is impaired, whereas the differentiation to the myeloid lineage is favored due to epigenetic changes (Garrick et al., 2019). In general, the number of naïve lymphocytes decreases, and the number of memory cells increases. Thus, the response to novel antigens becomes weak, whereas elderly are protected from most common infections as long as the memory system is stable. However, elderly are prone to infections by pathogens with a high mutation rate, like the influenza virus, where the constant change in antigens becomes a new challenge for the aging immune system.

Table 4 Changes in the function of antigen presenting cells.

Parameter/function	Alteration	References
Monocytes/macrophages		
Number of microglia cells	↑	Wong (2013) ↑
Number of monocytes	↔	Sansoni et al. (1993) ↔, Fagiolo et al. (1993) ↔, Franceschi et al. (1995) ↔, Cakman et al. (1996) ↔, Cakman et al. (1997) ↔ and Verschoor et al. (2013) ↓
Number of CD14 ⁺ monocytes	↘	Nijhuis et al. (1994) ↘
CD68 ⁺ cells in bone marrow	↘	Ogawa et al. (2000) ↘
Chemotaxis	↔	Gardner et al. (1981) ↔ and Schwab et al. (1985) ↔
Phagocytosis	↔	Gardner et al. (1981) ↔ and Schwab et al. (1985) ↔
Intracellular killing	↔	Gardner et al. (1981) ↔ and Schwab et al. (1985) ↔
Accessory function	↘	Rich et al. (1993) ↘
Pro-inflammatory cytokine production	↑	Fagiolo et al. (1993) ↑, Paganelli et al. (1994) ↑, Cakman et al. (1997) ↑ and Gabriel et al. (2002) ↑
Interferon- α production	↓	Kita et al. (1991) ↓, Sindermann et al. (1993) ↓, Uno et al. (1996) ↘ and Cakman et al. (1997) ↓
Expression of adhesion molecule CD11a	↘	Chiricolo et al. (1995) ↘
CD11b	↔	Stohlawetz et al. (1998) ↔ and de Martinis et al. (2000) ↔
CD15	↔	Stohlawetz et al. (1998) ↔ and de Martinis et al. (2000) ↔
CD29	↗	Stohlawetz et al. (1998) ↔ and de Martinis et al. (2000) ↔
CD54	↗	Stohlawetz et al. (1998) ↔ and de Martinis et al. (2000) ↔
CD58	↔	Rich et al. (1993) ↔ and Fagiolo et al. (1993) ↔
Expression of (Fc γ -receptor II) CD32	↗	Stohlawetz et al. (1998) ↗ and de Martinis et al. (2000) ↗
Expression of HLA-DR	↔	Rich et al. (1993) ↔ and Fagiolo et al. (1993) ↔
Myeloid-derived suppressor cells (MDSC)		
Number of MDSC	↑	Verschoor et al. (2013) ↑ and Alves et al. (2018) ↗
Monocytic MDSC (CD33 ⁺ /HLA-DR ⁺)	↘	Verschoor et al. (2013) ↔ and Alves et al. (2018) ↓
Granulocytic MDSC (CD11b ⁺ /CD15 ⁺)	↑	Verschoor et al. (2013) ↑, Alves et al. (2018) ↑
Dendritic cells (DC)		
Number of Langerhans cells	↓	Chambers and Vukmanovic-Stejic (2020) ↓
Number of myeloid (m)DC	↘	Pietschmann et al. (2000) ↔, Perez-Cabezas et al. (2007) ↔, Agrawal et al. (2007) ↔, Della Bella et al. (2007) ↓, Jing et al. (2009) ↔, Panda et al. (2010) ↔, Garbe et al. (2012) ↔, Orsini et al. (2012) ↓ and Schulz et al. (2015) ↓
Expression of TLR-1,-3,-8 by mDC	↓	Panda et al. (2010) ↓
Number of plasmacytoid (p) DC	↘	Shodell and Siegal (2002) ↓, Perez-Cabezas et al. (2007) ↓, Agrawal et al. (2007) ↔, Della Bella et al. (2007) ↔, Jing et al. (2009) ↓, Panda et al. (2010) ↓, Canaday et al. (2010) ↓, Garbe et al. (2012) ↓, Orsini et al. (2012) ↓ and Schulz et al. (2015) ↓
Expression of TLR-9 by pDC	↘	Panda et al. (2010) ↓, Sridharan et al. (2011) ↔ and Garbe et al. (2012) ↓
Expression of TLR-7 pDC	↔	Sridharan et al. (2011) ↔
Interferon- α production by pDC	↓	Jing et al. (2009) ↓, Panda et al. (2010) ↓, Canaday et al. (2010) ↓, Sridharan et al. (2011) ↓ and Qian et al. (2011) ↓
T cell priming by pDC	↓	Agrawal et al. (2007) ↓

Significantly increased ↑, increased ↗, significantly decreased ↓, decreased ↘, normal/unchanged ↔.

Table updated and adapted from Rink L and Dalhoff K (2004) Altersspezifische Veränderungen des Immunsystems und deren assoziierte Krankheitsbilder. In D Ganten, K Ruckpaul and A Ruiz-Torres (eds.), *Molekularmedizinische Grundlagen von altersspezifischen Erkrankungen*, 1st edn, pp. 429–464, Springer: Berlin. doi:10.1007/978-3-642-18741-4_16 and Della Bella S (2019) Dendritic cells and aging. In T Fulop, C Franceschi, K Hirokawa and G Pawelec (eds.), *Handbook of Immunosenescence*. 2nd edn, pp. 651–671, Springer International Publishing: Cham. doi:10.1007/978-3-319-99375-1_92.

T cells

The impaired function of the T cell system was one of the first observations of immunosenescence. The function of no other cell type is as strongly disturbed during aging as found for T cells. The resulting problems are often compared to HIV (human immunodeficiency virus) infections as a model of advanced aging of the immune system (Quiros-Roldan et al., 2020). One common problem of aging and HIV infection is the chronic stimulation of T cells by latent herpes viruses, which continuously stimulate the reactive T cells clones and thereby decreases the remaining T cell repertoire, due to enhanced numbers of the herpes virus reactive T cell clones (Nikolich-Zugich, 2008). The most prominent change during aging is the involution of the thymus (Henry, 1967). Since all T cell populations mature in the thymus, the loss of thymic function results in a decrease in the number of T cells (Sansoni et al., 1993). However, in contrast to the original discovery, it is now clear that the thymus is only partially replaced by fat tissue and that

centenarians have a nearly functional thymus. Therefore, the thymic output, i.e., the number of newly produced naïve T cells (indicated by a TREC (T cell receptor rearrangement excision cycle)), is a marker of thymic activity and the immunological age (Pinti et al., 2010). However, normally the thymus starts to degenerate from the third decade of life and is replaced by fat tissue (Elyahu and Monsonego, 2021). The thymic involution is mainly induced by the male and female sex hormones, i.e., testosterone and estrogen, and by ROS (Bodey et al., 1997). Some cytokines like IL-7 can slow down or even reverse the thymic involution, which is currently evaluated in clinical studies in a clinical experimental phase. The endogenous IL-7 production declines with age (Pangrazzi et al., 2017; Bradburn et al., 2018). The decreasing thymic output is directly correlated to the low number of T cells in the blood. However, the functional disturbances are much more significant, since nonfunctional CD28⁻ T cell clones accumulate with age, suggesting only a slight decrease in T cells (Grabstein et al., 1993; Posnett et al., 1994). Furthermore, some original papers did not observe decreased T cell numbers, probably due to using CD2 as T cell marker instead of CD3. The latter is currently state of the art and CD2 is expressed by NK cells, which are increased with age (Xu et al., 1993).

Numbers of CD8⁺ cytotoxic T cells (CTL) decrease stronger in the elderly than the numbers of CD4⁺ T helper cells, which results in an increased CD4/CD8 ratio (Sansoni et al., 1993). Since CTL are mostly needed to defend viral infections, viral infections increase with age. As pointed out before, the numbers of memory T cells (CD45R0⁺) increase with age, whereas the numbers of naïve T cells (CD45RA⁺) decrease (Xu et al., 1993). This explains the weak response to new (neo) antigens, whereas the response to well-known antigens (recall antigens) remains quite normal.

In addition to the decline in T cell numbers, their function is disturbed. T cells of elderly show an impaired response to stimulation since they are already chronically pre-activated. This pre-activation becomes apparent by the higher numbers of CD25⁺ (high affinity IL-2 receptor) and HLA-DR⁺ T cells (Sansoni et al., 1993). However, the pre-activation is the main reason for the overall reduced T cell response upon activation and the high clonal exhaustion, observed in the elderly. The poor activation of the T cells of elderly results in less proliferation of the T cells. Proliferation is a prerequisite for the adaptive immune response (Pawelec et al., 1999), since this depends on the clonal selection of the antigen-specific lymphocytes. Without proliferation of the T cells, the number of effector T cells will be too low for pathogen clearance. Furthermore, the activated T cells may be clonally exhausted, and a new memory cannot be established. The clonal exhaustion due to AICD (activation-induced cell death) is increased by the overall higher apoptosis rate of leukocytes in the elderly (McLeod, 2001). The reduced activation is based on an impaired T cell receptor (TCR) signal transduction and on a lack of survival signals. The ζ -chain of the CD3 signal transduction complex of the TCR shows a reduced phosphorylation and the transcription factor c-fos is lowly expressed in the elderly (Song et al., 1992; McLeod, 2001). Additionally, the balance between pro-apoptotic (e.g. Bax) and anti-apoptotic proteins (e.g., Bcl-2 and p53) in T cells is shifted towards the pro-apoptotic ones. Furthermore, the number of T cells expressing the death receptor CD95 and its ligand is increased. Elevated apoptosis is more prominent in the naïve T cells of elderly, which further contributes to the lower quotient of naïve to memory T cells (CD45RA⁺/CD45R0⁺).

As mentioned above, the number of CD28⁻ T cell clones increases with age. These cells are anergic, which means that they cannot respond to their specific antigen. Naïve and resting T cells need at least two signals for their activation: First an antigen-specific signal through the TCR and second via the costimulatory molecule CD28. Consequently, these CD28⁻ long-living T cell clones cannot be activated. So far it is unclear, why these unresponsive CD28⁻ T cell clones have a much longer half-life than other T cells. In addition, these T cell clones lack CD40L (CD154), which is the ligand for CD40 expressed by B cells and other antigen presenting cells and necessary for T cell help (Weyand et al., 1998). Most of the CD28⁻ T cell clones are CD8⁺ and represent the T cell counterpart to the long-living B cell clones in MGUS (monoclonal gammopathy of undetermined significance). The survival of the anergic T cell clones is maintained by signal transduction through the IL-2-receptor and CD2, which is not impaired in contrast to TCR-induced signaling via CD3 (Song et al., 1992). However, the anergic T cell clones displace normal T cells in blood and lymphatic tissues, resulting in a higher functional loss than estimated due to reduced T cell counts.

The number of regulatory T cells (Treg) in the blood is decreased in elderly, while the number of TH17 cells is increased, leading to an increased TH17/Treg ratio. However, during stimulation this ratio turns around in the elderly, resulting in a reduced TH17/Treg ratio, which explains the disturbed antimicrobial response (Schmitt et al., 2013). Furthermore, the number of memory Treg cells is increased in the elderly (van der Geest et al., 2014). The age-related changes in the T cell system are summarized in Table 5. In conclusion, the capacity of CTL to kill virus infected cells is reduced in the elderly and the function of T helper cells is disturbed, leading to a dysbalanced immune response against other pathogens as well. Finally, the impaired proliferative capacity of T cells limit the strength and duration of the immune response

B cells and antibodies

The humoral immunity is disturbed in aging as well. Clinically, this is revealed by a disturbed vaccination response (Grubeck-Loebenstein et al., 2009). The number of B cells decreases with age (Sansoni et al., 1993). The production of B cells in the bone marrow is reduced, due to a differentiation stop at an early stage (Quaglino et al., 1996; Zheng et al., 1997). However, the number of B cells in the spleen remains stable, since these B cells have a prolonged lifespan (Klinman and Kline, 1997). Therefore, it is still surprising that the T cell-independent B cell activation is reduced in the elderly, since this is normally due to a reduced number of marginal zone B cells in the spleen. A possible explanation is the reduced expression of IgM on the B cell surface in aging (Klinman and Kline, 1997).

Comparable to the increased number of memory T cells, numbers of CD27⁺ memory B cells increase as well, leading to a reduced antibody repertoire with increasing age. This problem is augmented by single highly proliferating B cell clones. Mostly, these B cell

Table 5 Alterations in the T cell system in aging.

Parameter/function	Alteration	References
Number and percentage of T cells (CD3 ⁺ lymphocytes)	↓/↓	Sansoni et al. (1993)↓/↓, Xu et al. (1993)↓/↓, Nijhuis et al. (1994)↓/↓, Born et al. (1995)↓/↓, Cakman et al. (1996)↘/↘ and van der Geest et al. (2014)↘/?
T cells (CD2 ⁺ lymphocytes including T & NK cells)	↔/↔	Xu et al. (1993)↔/↔
T helper cells (CD3 ⁺ /CD4 ⁺ cells or CD4 ⁺ lymphocytes)	↘/↔	Sansoni et al. (1993)↓/↓, Xu et al. (1993)↔/↔, Nijhuis et al. (1994)↔/↔, Born et al. (1995)↓/↔, Cakman et al. (1996)?/↗, van der Geest et al. (2014)↑/?
CTL (CD3 ⁺ /CD8 ⁺ cells or CD8 ⁺ lymphocytes)	↓/↓	Sindermann et al. (1993)↘/↘, Sansoni et al. (1993)↓/↓, Xu et al. (1993)↓/↓, Nijhuis et al. (1994)↘/↘, Born et al. (1995)↓/↓, Cakman et al. (1996)?/↗ and van der Geest et al. (2014)↓/?
Naïve T cells (CD45RA ⁺ T cells)	↓/↓	Xu et al. (1993)↓/↓, Franceschi et al. (1995)↓/↓ and Cakman et al. (1996)?/↓
Memory T cells (CD45RO ⁺ T cells)	↑/↗	Xu et al. (1993)↑/↑, Franceschi et al. (1995)?/↑ and Cakman et al. (1996)?/↓
γ/δ-T cells	↓/↔	Nijhuis et al. (1994)↓/? , Cakman et al. (1996)?/↔ and Argentati et al. (2002)↓/↔
Anergic T cells (CD28 ⁻ /CD40 ⁻ T cells)	↗/↗	Grabstein et al. (1993)↗/↗, Vallejo et al. (1998)↗/↗ and Weyand et al. (1998)↗/↗
T cells clones	↗/↗	Posnett et al. (1994)↗/↗ and Schwab et al. (1997)↗/↗
CD4/CD8 ratio healthy elderly	↗	Sindermann et al. (1993)↑, Kudlacek et al. (1995)↔, Cakman et al. (1996)↑ and van der Geest et al. (2014)↑
CD4/CD8 ratio frail elderly	↓	Muller et al. (2015)↓
Percentage of naïve T helper cells (CD45RA ⁺ /CD3 ⁺ /CD4 ⁺ cells or CD45RA ⁺ /CD4 ⁺ lymphocytes)	↓	Xu et al. (1993)↓, Fagiolo et al. (1993)↓, Nijhuis et al. (1994)↓, Kudlacek et al. (1995)↓, Stulnig et al. (1995)↓ and Cakman et al. (1996)↓
Memory T helper cells (CD45RO ⁺ /CD3 ⁺ /CD4 ⁺ cells or CD45RO ⁺ /CD4 ⁺ lymphocytes)	↑	Xu et al. (1993)↑, Fagiolo et al. (1993)↑, Nijhuis et al. (1994)↑, Kudlacek et al. (1995)↑, Stulnig et al. (1995)↑ and Cakman et al. (1996)↔
Naïve CTL (CD45RA ⁺ /CD3 ⁺ /CD8 ⁺ cells or CD45RA ⁺ /CD8 ⁺ lymphocytes)	↓	Xu et al. (1993)↓, Stulnig et al. (1995)↓, Ruiz et al. (1995)↓ and Cakman et al. (1996)↓
Memory CTL (CD45RO ⁺ /CD3 ⁺ /CD8 ⁺ cells or CD45RO ⁺ /CD8 ⁺ lymphocytes)	↗	Xu et al. (1993)↑, Fagiolo et al. (1993)↑, Stulnig et al. (1995)↑, Ruiz et al. (1995)↔, Kudlacek et al. (1995)↔ and Cakman et al. (1996)↓
Activated T cells (CD3 ⁺ /HLA-DR ⁺ or CD3 ⁺ /CD25 ⁺)	↑	Sansoni et al. (1993)↑, Born et al. (1995)↑ and Stulnig et al. (1995)↑
Naïve regulatory T cells (Treg) (CD45RA ⁺ /CD3 ⁺ /CD4 ⁺ /Foxp3 ⁺ cells)	↓	van der Geest et al. (2014)↓
Memory Treg (CD45RA ⁻ /CD3 ⁺ /CD4 ⁺ /Foxp3 ⁺ cells)	↑	van der Geest et al. (2014)↑
Blood Treg (CD4 ⁺ /Foxp3 ⁺ lymphocytes)	↘	Schmitt et al. (2013)↘
After stimulation Treg (CD4 ⁺ /Foxp3 ⁺ lymphocytes)	↔	Schmitt et al. (2013)↔
Naïve T helper 1 cells (CD45RA ⁺ /CD3 ⁺ /CD4 ⁺ /IFN-γ ⁺ cells)	↑	van der Geest et al. (2014)↑
Memory T helper 1 cells (CD45RA ⁻ /CD3 ⁺ /CD4 ⁺ /IFN-γ ⁺ cells)	↓	van der Geest et al. (2014)↓
Naïve T helper 2 cells (CD45RA ⁺ /CD3 ⁺ /CD4 ⁺ /IL-4 ⁺ cells)	↗	van der Geest et al. (2014)↗
Memory T helper 2 cells (CD45RA ⁻ /CD3 ⁺ /CD4 ⁺ /IL-4 ⁺ cells)	↗	van der Geest et al. (2014)↗
Naïve T helper 17 cells (CD45RA ⁺ /CD3 ⁺ /CD4 ⁺ /IL-17 ⁺ cells)	↗	van der Geest et al. (2014)↗
Memory T helper 17 cells (CD45RA ⁻ /CD3 ⁺ /CD4 ⁺ /IL-17 ⁺ cells)	↓	van der Geest et al. (2014)↓
Blood TH17 (CD4 ⁺ /IL-23R ⁺ lymphocytes)	↑	Schmitt et al. (2013)↑
After stimulation TH17 (CD4 ⁺ /IL-23R ⁺ lymphocytes)	↓	Schmitt et al. (2013)↓
Blood TH17/Treg ratio	↑	Schmitt et al. (2013)↑
After stimulation TH17/Treg ratio	↓	Schmitt et al. (2013)↓
Expression of CD7	↘	Kukel et al. (1994)↘
CD57	↗	Kukel et al. (1994)↗
CD62L	↘	Kukel et al. (1994)↘
Proapoptotic molecules (CD95, CD95L, Bax)	↗	McLeod (2001)↗
Antiapoptotic molecules (Bcl-2, p53)	↘	McLeod (2001)↘
Proliferation rate	↓	Pawelec et al. (1999)↓
Activation with anti-CD3	↓	Song et al. (1992)↓

(Continued)

Table 5 (Continued)

Parameter/function	Alteration	References
Anti-CD2	↔	Song et al. (1992)↔
Ca ²⁺ -influx after TCR stimulation	↘	Gupta (1989)↘, Song et al. (1992)↘, Whisler et al. (1993)↘, Liu et al. (1997a), Liu et al. (1997b)↘ and Whisler et al. (1998)↘
Production of IFN-γ	↓	Sindermann et al. (1993)↓ and Cakman et al. (1996)↓
IL-10	↗	Cakman et al. (1996)↗
IL-2	↗	Cakman et al. (1996)↗
sIL-2R	↓	Sindermann et al. (1993)↓ and Cakman et al. (1996)↓
Rate of apoptosis	↗	Pawelec et al. (1999)↗ and McLeod (2001)↗

Significantly increased ↑, increased ↗, significantly decreased ↓, decreased ↘, normal/unchanged ↔, unknown ?

Table updated from Rink L and Dalhoff K (2004) Altersspezifische Veränderungen des Immunsystems und deren assoziierte Krankheitsbilder. In D Ganten, K Ruckpaul and A Ruiz-Torres (eds.), *Molekularmedizinische Grundlagen von altersspezifischen Erkrankungen*, 1st edn, pp. 429–464, Springer: Berlin. doi:10.1007/978-3-642-18741-4_16.

clones are MGUS and only a minority are malignant B cell neoplasia. 38% of elderly have a monoclonal gammopathy, whereas this is observed in only 1–3% of middle-aged adults. Even SENIEUR elderly have a MGUS rate of 11% (Ligthart et al., 1990b)

Functional B cells show a reduced expression of IgM on the cell surface and a disturbed signal transduction of the B cell receptor (BCR) (Whisler et al., 1991a,b), leading to a reduced T cell-dependent and—independent B cell activation. In combination with the reduced T cell response and since most B cell responses are T cell-dependent, the resulting impairment is even worse since the functional disturbance in either the T cell or the B cell is sufficient to disrupt their interaction. This results in a reduced antibody production in response to pathogens as well as to vaccination. Furthermore, the quality of the antibodies produced is lower in aged people, since the affinity maturation by hypermutation is disturbed as well (Miller and Kelsoe, 1995; Zheng et al., 1997). However, the antigen-independent B cell activation by IL-4 and other mediators is normal (Fernandez-Gutierrez et al., 1999). Since the induction of memory B cells is disturbed in the elderly, booster vaccinations are required in shorter intervals to provide protection. Alternatively, the use of higher antigen doses or adjuvants are recommended for vaccination of the elderly.

Although the B cell system is impaired, the immunoglobulin concentration in the serum of elderly is increased (Cakman, 1996). This increase is based on significantly higher amounts of IgG1, 2, 3 and IgA, whereas the levels of the other immunoglobulins are stable (de Greef et al., 1992). Interestingly, specific IgE levels, responsible for type-I allergies, are reduced in the elderly (Nakazawa et al., 1994). However, the number of autoantibodies and thereby the incidence of autoimmune diseases increases with age. Despite the detection of autoantibodies in asymptomatic elderly, the onset of autoimmune diseases after the age of 65 years is rare (Bryl and Witkowski, 2019). While both organ-specific and non-organ-specific autoantibody levels increase with age, in SENIEUR elderly and centenarians mostly the non-organ-specific autoantibodies are observed. Table 6 summarizes the changes in the B cell system.

In conclusion, the decreased capacity to produce antigen-specific antibodies upon stimulation leads to a higher frequency of infections. The low vaccination response and shortened memory function further add to the decreased protection.

Immune regulation by cytokines

The regulation of the immune response is largely governed by cytokines and adhesion molecules. Since differences in the expression of adhesion molecules were discussed already for the respective cell types, the following paragraphs will focus only on disturbances in the cytokine system. In contrast to the alterations in the leukocyte populations, with increasing or decreasing numbers, the changes in the cytokine system are quite heterogeneous. Production of most pro-inflammatory cytokines is elevated upon stimulation and cytokines are frequently even produced constitutively without a danger signal. This phenomenon is called inflammaging (Franceschi et al., 2000), describing the chronic inflammation found regularly in the elderly. Some cytokines like IL-6 are even used as aging markers, since increased IL-6 levels correlate with the grade of frailty and with lower response to vaccination. Interestingly, disturbances are cytokine but not cell type specific. For example, monocytes/macrophages and DC produce more pro-inflammatory IL-1, IL-6 and tumor necrosis factor (TNF)-α, whereas the same cells have a defect in the production of the antiviral IFN-α (Cakman et al., 1997). The increased production of the pro-inflammatory cytokines leads to a chronic inflammation accompanied by an acute phase response, resulting in increased serum levels of C-reactive protein (CRP) and α2-macroglobulin. Furthermore, this pro-inflammatory cytokine cocktail activates lymphocytes antigen-independent, which is one reason for the observed unspecific T cell activation and increased antibody production. Unfortunately, this chronic activation has rather negative side effects and does not enhance the memory function, since pre-activated cells cannot efficiently be further activated, which is a prerequisite for an effective immune response.

Beside this pro-inflammatory response, the balance between immune cells is affected by aging. The ratios of TH1/TH2 and TH17/Treg are disturbed. Lymphocytes of elderly produce less of the TH1 cytokine IFN-γ, whereas the production of the TH2 cytokine IL-4 is unchanged, resulting in a reduced TH1/TH2 ratio. The production of the TH17 cytokine IL-17 is diminished as well. Together this leads to a reduced activation of effector T cells (Cakman et al., 1996, 1997; Schmitt et al., 2013). In conclusion, the alterations in the cytokine system, which are summarized in Table 7, lead to a weak immune response against viruses and bacteria.

Table 6 Alterations in the B cell system in aging.

Parameter/function	Alteration	References
Number and percentage of B cells (CD19 ⁺ lymphocytes)	↘↘	Sansoni et al. (1993)↓↓, Nijhuis et al. (1994)↔↔, Born et al. (1995)↓↓, Cakman et al. (1996)↘↘, Globerson and Effros (2000)↘↘ and Colonna-Romano et al. (2002)↘↘
B cell clones	↗↗	Ligthart et al. (1990a)↗↗, Yang et al. (1994)↗↗ and Yang et al. (1996)↗↗
Percentage of CD5 ⁺ B cells	↘	Geiger et al. (2000)↘ and Colonna-Romano et al. (2003)↘
Membrane bound IgM	↘	Klinman and Kline (1997)↘, Ginaldi et al. (1999a)↘ and Ginaldi et al. (1999b)↘
Plasma cells (CD27 ^{high} /CD38 ^{high} /CD138 ⁺)	↓	Pritz et al. (2015)↓
T cell-dependent B cell activation by anti-CD3	↘	Fernandez-Gutierrez et al. (1999)↘
T cell-dependent B cell activation by IL-4 and phorbol ester	↘	Whisler et al. (1991a)↘, Whisler et al. (1991b)↘ and Klinman and Kline (1997)↘
T cell-dependent B cell activation by anti-IgM	↔	Fernandez-Gutierrez et al. (1999)↔
Vaccination response	↓	Huang et al. (1992), Arreaza et al. (1993)↘, Govaert et al. (1994), Brydak et al. (2003)↘, Hainz et al. (2005), Hainz et al. (2005)↓ and Grubeck-Loebenstein et al. (2009)↓
Period of immunization protection	↘	Brydak et al. (2003)↘ and Hainz et al. (2005)↘
Total immunoglobulin	↗	Consens in results for the tested immunoglobulins: de Greef et al. (1992), Paganelli et al. (1992), Paganelli et al. (1994), Cakman et al. (1996), Listi et al. (2006), Schwarzenbach et al. (1982) and Al-Rayes et al. (1992)
Total IgG	↑	
IgG1, IgG2, IgG3	↑	
IgG4	↔	
IgA	↑	
IgM	↔	
IgE	↔	
Specific IgE	↘	Nakazawa et al. (1994)↘
Somatic hypermutations in V-region	↘	Miller and Kelsoe (1995)↘
Benign gammopathy	↗	Ligthart et al. (1990a)↗
Autoantibodies	↗	Mariotti et al. (1992) and Mariotti et al. (1995)↗
Autoreactive antibodies (ab) observed in asymptomatic elderly: anti-thyroglobulin ab, anti-parietal cell ab, anti-mitochondrial ab (AMA), antinuclear ab (ANA), anti-smooth muscle ab (SMA), anti-basement membrane zone ab, rheumatoid factor IgM, dsDNA ab, anti-phospholipid ab, anti-cardiolipin ab	↗	Bryl and Witkowski (2019)

Significantly increased ↑, increased ↗, significantly decreased ↓, decreased ↘, normal/unchanged ↔.

Table updated from Rink L and Dalhoff K (2004) Altersspezifische Veränderungen des Immunsystems und deren assoziierte Krankheitsbilder. In D Ganten, K Ruckpaul and A Ruiz-Torres (eds.), *Molekularmedizinische Grundlagen von altersspezifischen Erkrankungen*, 1st edn, pp. 429–464, Springer: Berlin. doi:10.1007/978-3-642-18741-4_16.

Infectious diseases in the elderly

Infectious diseases increase dramatically with age, with the exception of the sexual transmitted diseases. The risk for pneumonia in those older than 65 years is 3–5 times higher. 50% of people dying from pneumonia are over 65 years. The mortality from pneumonia increases from 5–8% to 80% in elderly over 85 years. In the US, pneumonia is the most common cause of infection-associated deaths in the elderly and the fourth most frequent overall cause of death (Castle, 2000; Rink et al., 2015). 90% of the estimated excess deaths by influenza occur in the elderly over 65 years. Therefore, vaccinations against pneumococci, influenza and now corona virus are of high importance, especially for the elderly. However, even if the seasonal influenza vaccine matches well and protects 70–90% of the adults under 65 years, only 30–40% of the elderly are protected. For pneumococcal vaccination, the protection is also age-dependent. Five years after vaccination, 70% of elderly under 75 years are protected, whereas it declines to 53% in the 75–85 year-olds and finally reaches only a protection rate of 22% in the very old (>85 years) (Castle, 2000). During the COVID-19 (corona virus disease 2019) pandemic in Germany, 97% and 89% of the 73.445 deaths between March 2020 and March 2021 were 60 years and older and 70 years and older, respectively. Therefore, old age is the highest risk factor for a critical illness from the corona virus (Robert Koch-Institute, 2021).

Herpes zoster, i.e., the reactivation of chicken pox, is a typical age-related disease. The lifetime incidence is 10–20% counting all infected people, which increases to 50% for those older than 80 years. This increased reactivation is directly related to the decline of the T cell response, since all latent herpes viruses are under control of T cells (Rink et al., 2015). In addition to chicken pox, reactivation is often observed for cytomegalovirus (CMV) and tuberculosis. However, since the tuberculosis rate dramatically decreased in Western countries after the Second World War, this is mostly a problem in the generation born before the 1940s. In contrast, CMV is still the most frequently transferred virus during pregnancy and therefore the infection rate in the population is very high. Studies on the IRP imply, that the chronic stimulation of the immune system by CMV accelerates the decline of the T cell system. This seems comparable to HIV infection, where forced aging of the immune system is observed as well (Quiros-Roldan et al., 2020).

Table 7 Dysbalanced immune regulation in aging.

Parameter/function	Alteration	References
APRIL (a proliferation-inducing ligand)	↓	Pangrazzi et al. (2017)↓
CCL3 (macrophage inflammatory protein (MIP)-1α)	↘	Wang et al. (2017)↘
CCL5 (regulated and normal T cell expressed and secreted (RANTES))	↗	Wang et al. (2017)↗
CCL2 (monocyte chemoattractant protein (MCP)-1)	↗	Wang et al. (2017)↗
CCL11 (Eotaxin-1)	↑	Hoefler et al. (2017)↑, Wang et al. (2017)↑, Bradburn et al. (2018)↑
CXCL10 (10 kDa interferon gamma-induced protein (IP-10))	↑	Bradburn et al. (2018) and Wang et al. (2017)↑
EPO (erythropoietin)	↓	Pasqualetti and Casale (1997)↓
FGF-2 (fibroblast growth factor)	↓	Bradburn et al. (2018)↓
GM-CSF (granulocyte-macrophage colony stimulating factor)	↓	Bradburn et al. (2018)↓
IFN-α	↓	Kita et al. (1991), Sindermann et al. (1993), Katschinski et al. (1994), Cakman et al. (1997)↓ and Uno et al. (1996)↘
IFN-γ	↓	Kita et al. (1991), Paganelli et al. (1994), Cakman et al. (1996), Bradburn et al. (2018), Born et al. (1995), Sindermann et al. (1993)↘, and Wang et al. (2017)↑
IL-1	↑	Fagiolo et al. (1993), Paganelli et al. (1994), Gabriel et al. (2002)↑, Wang et al. (2017)↗, and Bradburn et al. (2018)↓
IL-1RA (IL-1 receptor antagonist)	↑	Ferrucci et al. (2005), Bradburn et al. (2018) and Jylha et al. (2007)↑
IL-2	↓	Gillis et al. (1981), Born et al. (1995), Huang et al. (1992), Bradburn et al. (2018), Paganelli et al. (1994)↓ and Nijhuis et al. (1994)↘
sIL-2R (soluble IL-2 receptor)	↓	Sindermann et al. (1993), Cakman et al. (1996)↓, and Wang et al. (2017)↘
IL-3	↑	Paganelli et al. (1994)↑
IL-4	↗	Paganelli et al. (1994), Paganelli et al. (1996), Nijhuis et al. (1994)↑, Wang et al. (2017)↘, and Bradburn et al. (2018)↓
IL-5	↓	Bradburn et al. (2018)↓
IL-6	↑	Sindermann et al. (1993), Cakman et al. (1997)↗, Fagiolo et al. (1993), Paganelli et al. (1994), Bruunsgaard et al. (1999), Arai et al. (2001), Giuliani et al. (2001), Forsey et al. (2003), Pangrazzi et al. (2017), and Wang et al. (2017)↑
IL-7	↓	Pangrazzi et al. (2017) and Bradburn et al. (2018)↓
IL-8	↑	Cakman et al. (1996), Gabriel et al. (2002) and Wang et al. (2017)↑
IL-10	↗	Wang et al. (2017), Cakman et al. (1996)↑ and Forsey et al. (2003)↓
IL-12	↗	Salazar et al. (2013), Wang et al. (2017)↑ and Bradburn et al. (2018)↓
IL-13	↔	Wang et al. (2017)↑ and Bradburn et al. (2018)↓
IL-15	↗	Pangrazzi et al. (2017), Gangemi et al. (2005)↑ and Bradburn et al. (2018)↓
IL-17	↓	Bradburn et al. (2018) and Alvarez-Rodriguez et al. (2012)↓
IL-18	↑	Gangemi et al. (2003)↑
TGF-β1 (transforming growth factor)	↑	Carrieri et al. (2004)↑
TNF-α	↑	Fagiolo et al. (1993), Born et al. (1995), Bradburn et al. (2018)↑ and Paganelli et al. (1994)↗
sTNFR-I (soluble TNF receptor)	↑	Gerli et al. (2000)↑
sTNFR-II	↑	Gerli et al. (2000) and Bruunsgaard et al. (1999)↑

Significantly increased ↑, increased ↗, significantly decreased ↓, decreased ↘, normal/unchanged ↔.

Table updated from Rink L and Dalhoff K (2004) Altersspezifische Veränderungen des Immunsystems und deren assoziierte Krankheitsbilder. In D Ganten, K Ruckpaul and A Ruiz-Torres (eds.), *Molekularmedizinische Grundlagen von altersspezifischen Erkrankungen*, 1st edn, pp. 429–464, Springer: Berlin. doi:10.1007/978-3-642-18741-4_16.

Urinary tract infections (UTI) increase with age as well. For example, the asymptomatic bacteriuria is enhanced, from a prevalence of 1–5% in healthy premenopausal women to 4–19% in healthy elderly and finally 15–50% in institutionalized elderly. Symptomatic UTI in women aged 65–74 years showed an increased incidence of 9–11 cases per 100 person-years, which further increases to 11.4–14.3 cases in women aged 75–84 years and ended up in 14.7–19.8 cases per 100 person years in those over 85 years. UTI in men increased as well by age, but on a lower level (Rodriguez-Manas, 2020).

Although the efficient treatment of bacterial, viral, and other infectious diseases with antibiotics, virostatics, antimycotics and other drugs has much improved, an intact immune system is needed to prevent infections and to slow down the spreading of the pathogens before starting the treatment. Also, opportunistic pathogens may take their chance in elderly. Thus, improvement or stabilization of the immune functions during aging is important to prevent death from infectious diseases.

Conclusion

The increasing knowledge on the differences between frail and healthy elderly, as well as on the distinctive immune characteristics of SENIEUR-elderly and centenarians gives us the chance to understand why people may reach their potential maximal age or not. Standard health check-ups should be improved by compiling immunological parameters to generate an individual immune risk profile. In the future, we may be able to push the immune system towards a healthy decline like in centenarians, which will increase the healthy life span, but not the overall life expectancy. If we are successful, this will decrease the burden of infections and cancers in the very old.

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Cancer Immunology

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Introduction

Cancer is one of the prevalent diseases affecting a lot of people around the world. It has been predicted that new cases of cancer will be increasing to 22.2 million in 2030 while it was 18.1 million in 2018 (Bray et al., 2018). It is the second cause of death worldwide and accounted for 9.6 million deaths in 2018 (Bray et al., 2018). Researchers and physicians have tried their best to cure cancer; however, limited improvement in overall survival has been achieved and the mortality rate is still high (Pishvaian et al., 2020). Therefore, a deep understanding of cancer pathophysiology is necessary to discover bench-to-bedside diagnostic, predictive, and prognostic biomarkers and develop novel preventive and therapeutic modalities. Immune evasion is one of the hallmarks of cancer highlighting the remarkable role of the immune system in eradication of cancer. It has also been revealed that the immune system can promote cancer development (Vinay et al., 2015). Hence, improving the understanding of cancer immunology can assist researchers to develop improved modalities and potent biomarkers.

Immunotherapy is the fourth pillar of cancer treatment recently applied to improve the survival of patients with cancer (Hunter, 2017). The first evidence of successful cancer immunotherapy was reported in 1891 by Coley. He examined a bacterial toxin consisted of killed *Streptococcus pyogenes* and *Serratia marcescens* for treatment of a young patient with sarcoma, and the toxin led to tumor shrinkage. Although the fact that an infection can induce immunity and eventually tumor regression had been discussed prior to his work, he was the first person who released this result as a report. Therefore, he is known as the father of cancer immunotherapy (Coley, 1891; McCarthy, 2006). By contribution of other scientists, the field has been promoted; many targeted immunotherapies were developed, and lots of them received approval for treatment of different types of cancer.

In concert with the advancement of cancer immunotherapy, it has been figured out that conventional approaches such as the TNM tumor staging system are not sufficient to predict the response to immunotherapy and survival of patients. Therefore, some immune-based strategies such as immunescore and Tumor Immunity in MicroEnvironment (TIME) Classification have been developed (Bruni et al., 2020; Zhang and Chen, 2016; Mlecnik et al., 2011).

To summarize the significant role of the immune system in eradication or progression of tumors, we will review the antitumor and protumor functions of various immune subsets, immune regulation of metastasis, immune based interventions for cancer treatment and prevention, and main immune-based predictive and prognostic biomarkers in this chapter.

Cancer immunosurveillance and immunoediting

The notion of endogenous cancer suppression was proposed by Paul Ehrlich in 1909. He believed that host defense mechanisms are capable of preventing cancer development in most people (Ehrlich, 1908). This hypothesis was supported by Lewis Thomas and Macfarlane Burnet in the 1950s. Lewis Thomas suggested that immunological surveillance mechanisms occur by the recognition of tumor neo-antigens and subsequent elimination mechanisms, similar to the mechanism of homograft rejection (Thomas and Lawrence, 1959). Macfarlane Burnet by consideration of the natural evolution supported the presence of protective mechanisms against tumor cells which can be developed due to genetic alterations in long-lived animals and human. He proposed that immunological responses can eliminate tumor cells because they are activated by antigenic properties of tumor cells attained during malignant transformation (Burnet, 1970). Cancer immunosurveillance theory formed based on these hypotheses and subsequent experimental studies on knockout mice which displayed the roles of various immune components in cancer immunosurveillance (Dunn et al., 2002; Kim et al., 2007).

Aside from the ability of the immune system in eradication of transformed cells, it was revealed that the immune system also plays an essential role in development of a clinically detectable tumor. The cancer immunoediting hypothesis explains the role of the immune system in progression of tumor cells in three phases e.g., elimination, equilibrium, and escape (Dunn et al., 2004). These phases are illustrated in Fig. 1

Immunoediting describes the steps preceding the development of a clinically detectable tumor. Initially, immune effectors eradicate highly immunogenic tumor cells in the elimination phase similar to what happens in cancer immunosurveillance. Although cytotoxic T lymphocytes (CTL) are the key players to recognize and attack malignant cells (Mukherji et al., 1990), both innate and adaptive arms of immunity are essential for eradication of malignant cells in elimination phase; natural killer (NK), CD4⁺ and $\gamma\delta$ T cells are other necessary components in tumor rejection (Mukherji et al., 1990; Krasnova et al., 2017; Terabe and Berzofsky, 2018; Zhao et al., 2018). In equilibrium phase, whose occurrence was questioned for a long time, tumor cells are kept quiescent by both innate and adaptive immunity. In other words, tumor cells with low immunogenicity are selected to survive (Quezada et al., 2011; Wu et al., 2013). In escape phase, survived tumor cells elude the immune system and present a clinically detectable tumor (Dunn et al., 2004). The transformed cells exploit various mechanisms such as downregulation of tumor antigen, impairment of antigen processing and presentation, decreased frequency and activity of immune effectors, increased immunosuppressive components, and alteration of tumor metabolism to escape from the immune system (Corrias et al., 2001; Johnsen et al., 1999; Idos et al., 2020; Schaefer et al., 2005; Huber et al., 2017).

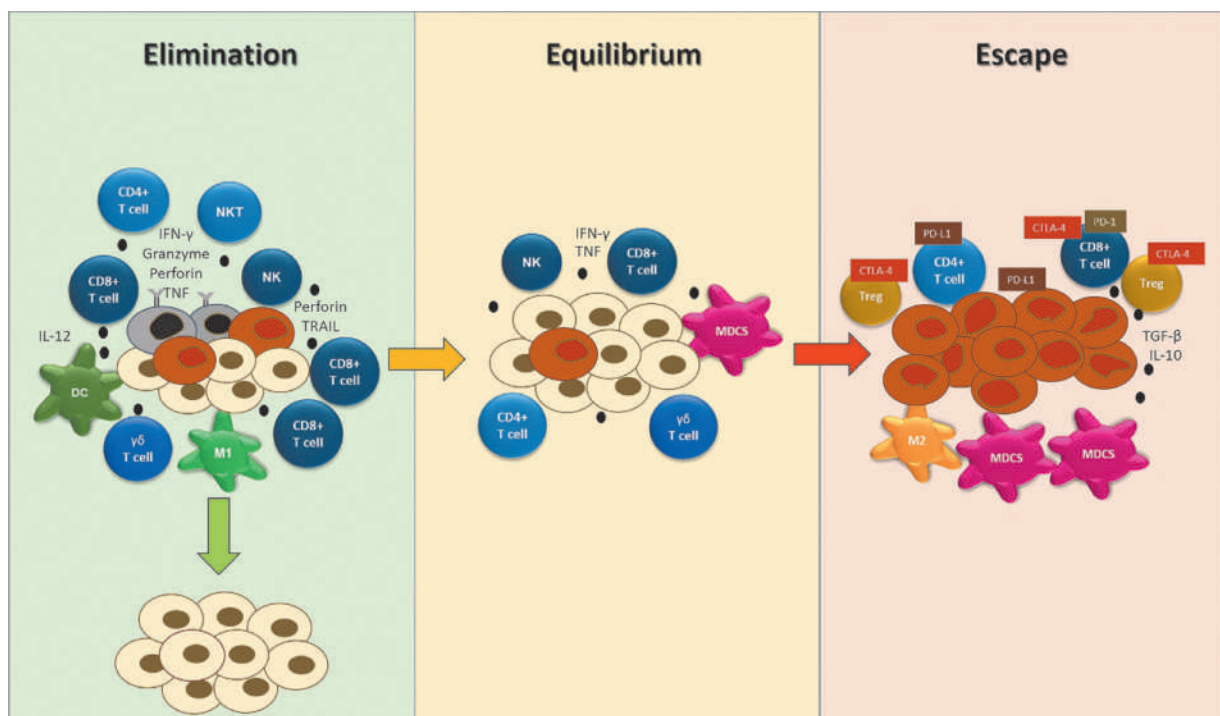


Fig. 1 Cancer immunoediting. The figure displays three phases of cancer immunoediting including elimination, equilibrium, and escape. The Innate and adaptive immune cells contribute to tumor rejection in the elimination phase. Tumor dormancy occurs in the elimination phase under influence of both innate and adaptive immunity. As a result of immunosuppressive microenvironment and dysfunctional T cells, immune evasion occurs in the escape phase.

Tumor antigens

Tumor antigens are the most important markers for the recognition and elimination of tumor cells by CTLs. Tumor antigens mainly are peptides or glycoproteins. They are produced by tumor cells and stay in one of the cellular compartments including the membrane, cytosol, or nucleus. They might also be secreted to the blood stream (Zarour et al., 2003). There are various categories for tumor antigens and they are generally classified into tumor associated antigens (TAA) and tumor specific antigens (TSA). TAAs are normally expressed in healthy tissues but overexpressed or posttranslationally altered in malignant tissues. In contrast, TSAs are exclusively expressed by tumor cells and caused either by mutation or viral oncogenes (Zarour et al., 2003; Feng et al., 2008; Dalet et al., 2011). Based on the origin, there are also other categories of tumor antigens summarized in Table 1.

Initially, tumor antigens were identified by using a mouse tumor-specific CTL which recognized a peptide derived from tumor cells (Lurquin et al., 1989). Indeed, MHC class I molecules on tumor cells present short peptides, which contain 8-10 amino acids originated from mutated, or overexpressed intracellular proteins, to T cells (Vigneron and Van den Eynde, 2011). Nowadays, direct or forward immunology and indirect or reverse immunology are used for identification of tumor antigens. Forward immunology approaches use CTLs isolated from patients and cDNA expression libraries to identify cDNAs encoding CTL epitopes while reverse immunology approaches use an algorithm-based method to predict the binding of a peptide to MHC class I. The later might also predict peptides that are not naturally processed and presented by MHC I molecules to induce T cell response. This disadvantage necessitates validation methods for the candidate peptides (Traversari et al., 1992; Popović et al., 2011).

The identified tumor antigens might be used for screening, diagnosis, monitoring and prediction of prognosis (Even-Desrumeaux et al., 2011). They are also great targets for immune-based interventions such as monoclonal antibodies (mAbs), vaccines, and chimeric antigen receptor (CAR) T cell therapy (Kantoff et al., 2010a; Braendstrup et al., 2020; Cobleigh et al., 1999; Kimura et al., 2013). An ideal tumor antigen for clinical targeting is the one with (1) high immunogenicity, (2) oncogenic function, (3) extracellular expression which makes it accessible for recognition by T cells and immunoglobulins, (4) specificity and (5) high expression level compared to the normal tissue (Zarour et al., 2003; Engelhard et al., 2002; Khong et al., 2004; Kessler and Melief, 2007; Cheever et al., 2009). Overall, ideal tumor antigens can make an appreciable difference in the field of cancer treatment and prevention.

Immune subsets in cancer immunopathology

Various immune cell lineages are present in the tumor microenvironment which contribute to either progression or rejection of tumors. Generally, immune cells with cytotoxic capabilities are involved in tumor rejection while immune cells with suppressive properties foster tumor progression and metastasis. In this section, we will review the functions of various immune subsets in cancer immunopathology.

Dendritic cells (DCs)

Nucleated cells contain the major histocompatibility complex (MHC) class I and can present antigens to CD8+ T cells; however, tumor cells are not potent T cell stimulators. Instead, professional antigen presenting cells (APCs) stimulate CD8+ T cells through cross presentation of tumor antigens i.e., uptake and processing of tumor antigens obtained from dead cells and their presentation to CD8+ T-cells. In addition, APCs contain not only MHC I but also MHC II enabling them to stimulate both CD8+ and CD4+ T cells (Petersen et al., 2010; Joffre et al., 2012; Jongbloed et al., 2010).

Dendritic cells (DCs) originate from myeloid and lymphoid progenitors. Conventional DCs (cDCs) usually originate from myeloid progenitors while CD123+ plasmacytoid DCs (pDCs) originate from both myeloid and lymphoid progenitors (Manz et al., 2001; Rodrigues et al., 2018). DCs might also be differentiate from monocytes and develop monocyte-derived DCs (MoDCs) in inflammatory states (Schlitzer et al., 2015). Generally, pDCs do not perform a strong antigen presentation. They stimulate

Table 1 Categories of tumor antigens.

Category	Characteristics	Example
Oncoviral antigens	Originate from oncogenic viruses, not present in non-infected tissues	HPV E6 and E7 proteins (Kessiss et al., 1993)
Neo-antigens	Mutated or transcriptionally modified peptides, not presented in normal tissues	Mutant PTEN (Keskin et al., 2019)
Cancer testis antigens	Normally expressed by testis and placenta	MAGE-A (van der Bruggen et al., 1991)
Oncofetal antigens	Prominently expressed by fetal tissues	AFP (Wepsic, 1983)
Overexpressed antigens	Expressed by normal tissues overexpressed by tumor cells	HER2 (Slamon et al., 1987)
Differentiation antigens	Lineage specific, expressed by tumor cells and the corresponding normal tissue	Melan-A/MART-1 (Berset et al., 2001)
Retired antigens	Expressed prior to maturity	alpha-lactalbumin (Jaini et al., 2010)
Cancer stem cell antigens	Expressed on cancer stem cells and responsible for recurrence	TLR2 (Conti et al., 2013)

Abbreviations: HPV Human papillomavirus; PTEN Phosphatase and tensin homolog; MAGE-A Melanoma antigen-A; AFP alpha-fetoprotein; HER2 human epidermal growth factor receptor 2; Melan-A Melanoma antigen recognized by T cells; TLR2 Toll-like receptor 2.

expansion of regulatory T cells (Tregs) through the inducible T cell costimulator ligand (ICOSL), and associate with poor outcome in cancer (Aspord et al., 2013; Liu et al., 2019; Villadangos and Young, 2008). Although pDCs produce type I interferons (IFN) exerting antitumor functions, these cytokines can be inhibited in an immunosuppressive tumor microenvironment (TME) (Demoulin et al., 2013). cDCs are classified into cDC1 (CD141+), and cDC2 (CD1c+) subsets. cDC1s contribute to the activation of both cytotoxic CD8+ T cells (through cross-presentation of endogenous antigens) and T helper 1 (Th1) CD4+ cells, while cDC2s mostly activate CD4+ T cells which display an anti-tumor function (Macri et al., 2018; Merad et al., 2013; O'Keefe et al., 2015; Binnewies et al., 2019).

Maturation of DCs is important for their antitumor activities. Immature DCs contain pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and cytoplasmic NOD-like receptors (NLRs) which are involved in maturation of DCs by recognition of danger signals or damage-associated molecular patterns (DAMPs) (Khajeh Alizadeh Attar et al., 2018; Saxena and Yeretssian, 2014). Mature DCs express MHCs, chemokine receptors and co-stimulatory molecules (Table 2) (Villadangos et al., 2005; Caux et al., 1994). The chemokine receptor CCR7 on mature DCs plays the main role in the migration of DCs to tumor-draining lymph nodes (TDLN) (Roberts et al., 2016). DCs are also involved in T cell recruitment to the TME through expression of CXC-chemokine ligand 9 (CXCL9)/10 (Spranger et al., 2017).

Cytotoxic activity of CD8+ T cells is the central determinant of tumor rejection. Therefore, CD8+ T cell priming upon cross-presentation of tumor antigens is the key antitumor function of DCs, especially cDC1s the main DC subset accountable for antigen cross-presentation. Three signals are required for this purpose: (i) presentation of tumor antigen on MHC to T cell receptor (TCR), (ii) co-stimulation through specific molecules on DCs and T cells and (iii) secretion of proper cytokines (Wculek et al., 2020). MHC I presents antigens to the TCR on CD8+ T cells while MHC II matches with the TCR on CD4+ T cells. The second signal is performed through co-stimulatory molecules involved in T cell priming, including CD80/CD86-CD28, CD137L-CD137, and ICOSL-ICOS (Table 2) (Chen and Flies, 2013), and The third signal depends on the secreted cytokines. IL-12 is the cytokine required for Th1 differentiation and cytotoxic activity of CD8+ T cells. It is secreted by basic leucine zipper transcription factor ATF-like 3 (BATF3)-dependent cDCs through stimulation of TLRs (Mittal et al., 2017; Nizzoli et al., 2013). IL-12 causes IFN- γ secretion by NK cells and the subsequent reinforcement of IL-12 secretion by cDCs (Hochrein et al., 2000). Signaling through co-inhibitory

Table 2 Co-stimulatory and co-inhibitory molecules on DCs and their counterparts on T cell.

	<i>Expressed by DCs</i>	<i>Counterpart on T cells</i>
Co-stimulatory	CD80	CD28
	CD86	CD28
	ICOSL	ICOS
	CD137L	CD137
	4-1BBL	4-1BB
	OX40L	OX40
	CD70	CD27
	CD40	CD40L
	TL1A	DR3
	GITRL	GITR
	CD30L	CD30
	SLAM	SLAM
	CD48	CD2
	CD58	CD2
	CD155	CD226
	CD112	CD226
Co-inhibitory	CD80	CTLA-4
	CD86	CTLA-4
	PD-L1	PD-1
	PD-L2	PD-1
	VISTA	unknown
	MHC II	LAG-3
	CD31	CD31
	Galectin9	TIM3
	CD112	TIGIT
	CD113	TIGIT
	CD155	TIGIT

Abbreviations: ICOS Inducible T Cell Costimulator; ICOSL Inducible T Cell Costimulator Ligand; TL1A Tumor necrosis factor-like cytokine 1A; SLAM Signaling lymphocytic activation molecule; GITRL Glucocorticoid-induced tumor necrosis factor receptor family-related protein ligand; GITR glucocorticoid-induced TNFR family related gene; VISTA V-domain Ig suppressor of T cell activation; TIGIT T cell immunoreceptor with Ig and ITIM domains; CTLA-4 cytotoxic T-lymphocyte-associated protein 4; PD-1 Programmed cell death protein 1; PD-L1 Programmed death-ligand 1; PD-L2 Programmed death-ligand 2.

molecules on T cells such as CTLA-4 and metabolic alterations namely activation of indoleamine 2,3-dioxygenase 1 (IDO1) which uses tryptophan, a necessary factor for T cell function, are among the barricades of T cell priming (Buchbinder and Desai, 2016; Hwu et al., 2000).

DCs might also show protumor properties and become tolerogenic due to the effect of interleukin-10 (IL-10) or 1 α ,25(O)2D3, a vitamin D3 metabolite (Gur-Wahnon et al., 2009; Ferreira et al., 2015). Tolerogenic DCs with immature or semi-mature characteristics upregulate receptors and ligands such as immunoglobulin like transcript (ILT3) and ILT4 which contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and stimulate expansion of the immunosuppressive T cells, regulatory T cells (Treg) (Li et al., 2020; Manavalan et al., 2003).

Tumor cells also contribute to the impairment of antitumor functions of DCs. They express T cell immunoglobulin mucin receptor 3 (TIM3) to prevent the danger signal high mobility group protein B1 (HMGB1) which is involved in the activation of DCs and antigen presentation (Chiba et al., 2012). They produce agonists of liver X receptor- α (LXR α) to inhibit CCR7 necessary for DC migration to TDLN and versican to increase IL-10 and IL-6 and to impair DC functions (Villablanca et al., 2010; Tang et al., 2015).

T-cells

Both conventional T cells containing $\alpha\beta$ TCR and unconventional T cells containing $\gamma\delta$ TCR contribute to tumor rejection. T cells originate from lymphoid progenitors in bone marrow and mainly develop in the thymus. Initially, they are immature cells which express CD3 and either the $\alpha\beta$ or $\gamma\delta$ TCR but not CD4 or CD8. The conventional or $\alpha\beta$ T cells undergo negative and positive selection and most of them with high affinity to self-peptides are eliminated. After selection, TCR rearrangement, and expression of either CD4 or CD8, naïve T cells enter the peripheral blood (Katz and Rabinovich, 2020). It is noteworthy to mention that during selection some subclasses with high affinity to self-peptides including natural killer T cells (NKTs) and Tregs remain (Stritesky et al., 2012). $\gamma\delta$ T cells also undergo negative and positive selection but these processes are not necessary checkpoints for $\gamma\delta$ T cell development. Despite conventional T cells which recognize peptides through a classic MHC-mediated mechanism, $\gamma\delta$ T cells are responsible for classic MHC-independent antigen recognition (Prinz et al., 2006; Jensen et al., 2008). Further differences and functions of these subtypes will be discussed in next sections.

Initially, conventional T cells are naïve and in resting state. They are featured with low cell volume, metabolism, and proliferation rate. IL-7 and a weak baseline function of the TCRs recognizing healthy self-peptides support the proliferation of naïve T cell (Geltink et al., 2018). Following antigen presentation by MHC to the corresponding TCR, clonal expansion and differentiation of naïve T cells occurs, mTORC1 is activated, and the metabolic rate and cell volume increase (Katz and Rabinovich, 2020; Jones and Pearce, 2017). The clonal expansion is performed by sequential asymmetric cell division which produce memory T cells and terminally differentiated effector T cells which might be CD8+ CTLs or CD4+ helper T cells (Arsenio et al., 2015).

In addition to IL-7, other cytokines are also important for activation and proliferation of T cells. IL-2 and IL-4 are also involved in proliferation and survival of T cells. As mentioned, IL-12 is also required for T cell activation upon antigen recognition. A deprivation in these cytokines, for instance, by Tregs, can cause T cell apoptosis (Pandiyan et al., 2007). The development of memory T cells which survive to provide a long-term protection and rapid anamnestic reaction to future stimulations is also dependent on cytokines namely IL-7 and IL-15. IL-7 accounts for the upregulation of Bcl-2, the antiapoptotic protein, to develop memory T cells and IL-15 supports their proliferation. (Kim et al., 2008). Memory T cells are divided into central and effector memory T cells. Effector memory T cells circulate in the blood and non-lymphoid tissues to exert a rapid response to the target antigen while central memory T cells mostly available in lymph nodes can give rise to effector T cells and effector memory T cells (Sallusto et al., 1999).

As mentioned before, the second signal for T cell activation is provided by CD28 which can be expressed by both CD4+ and CD8+ T cells and binds to CD80/CD86 on DCs (Riha and Rudd, 2010). However, CTLA-4 competes with CD28 and exert inhibitory effects through binding to CD80/CD86.

In addition to competition, CTLA-4 exploits further mechanisms to inhibit T cell priming. It mediates endocytosis of CD80/CD86 to suppress their ligation with CD28 and decrease IL-2 production (Qureshi et al., 2011a; Greenwald et al., 2001; Krummel and Allison, 1995). It also disrupts cytoskeleton and through this interferes with antigen presentation by DCs (Schneider et al., 2006). The most important function of CTLA-4 is inhibition of phosphorylation of the CD3 ζ -subunit of the TCR and the adaptor protein linker of activated T cells (LAT) (Lee et al., 1998). CTLA-4 performs this function through SH2 domain-containing tyrosine phosphatase 2 (SHP2) (Marengère et al., 1996). It also uses protein phosphatase 2A (PP2A) to inhibit the downstream molecule of the TCR, extracellular signal-regulated kinase (ERK) (Chuang et al., 2000). CTLA-4 also contributes to development of anergic T cells, by decreasing some transcription factors including activator protein 1 (AP-1), nuclear factor- κ B (NF- κ B) and nuclear factor of activated T cells (NFAT) (Greenwald et al., 2001; Fraser et al., 1999).

CTLA-4 is restored within intracellular vesicles in naïve T cells and can be secreted to the immunological synaptic space to inhibit T cell priming (Linsley et al., 1996). It is also expressed by T cells early after activation to develop T cell anergy (Greenwald et al., 2001). Interestingly, CD4+CD25+ Tregs constitutively express CTLA-4 and whenever present contribute to trans-endocytosis of CD80/CD86 and prevention of co-stimulation (Linsley et al., 1996; Qureshi et al., 2011b). PD-1 is another inhibitory molecule expressed on the surface of T cells in the later steps of immune response. It binds to PD-L1 and PD-L2 its ligands on tumor cells and T cells and advances T cell exhaustion (Prall and Hühns, 2017). PD-1/PD-L1 are expressed during inflammation to make a restricted T cell function and prevent the expansion of inflammatory response (Chen and Han, 2015). However, it is not of interest in the immune rejection of tumors.

Overall, T cells might be dysfunctional through different fates: (i) anergy is the state of T cell inactivity due to the lack of signal 2 or 3, low production of IL-2 and subsequent impaired T cell differentiation. (ii) Exhaustion is the state of T cell dysfunction due to excessive and chronic stimulation with signal 1 or 3, and decreased level of the required cytokines. These effects weaken the effector activity of T cells. (iii) Senescence is the state of stopping the cell cycle which might be caused by aging and short telomere, Tregs, or tumor associated stress. (Wherry and Kurachi, 2015; Crespo et al., 2013; Huff et al., 2019). Stemness is also another fate of T cells which is favorable for tumor destruction. In this state, stem-cell like memory T cells re-establish themselves to produce central and effector memory T cells (Vodnala et al., 2019).

CD8+ CTLs

CD8+ CTLs are the key immune subsets for tumor elimination. Cytotoxic functions of CD8+ T cells against cancer has been revealed by isolation of tumor antigen-specific CTLs in restricted and regressing tumors (Boon et al., 1997; Echchakir et al., 2000). To play their role, CD8+ T cells need to be differentiated and infiltrated into the TME (Nolz, 2015). Naïve CD8+ T cells progress to effector and then to cytotoxic and memory CD8+ T cells upon antigen presentation and secretion of appropriate cytokines (Nolz, 2015). Their recruitment to the TME is dependent on chemokines and chemokine receptors i.e., CXCL9, CXCL10, CXCL11 - CXCR3 and CXCL16 - CXCR6 (Slaney et al., 2014).

To destroy tumor cells, CD8+ CTLs secrete perforin and granzyme as the main direct mechanism of cytotoxicity (Yasukawa et al., 2000). They also can use other cytotoxic pathways including Fas(CD95)/Fas ligand (FasL), TNF- α /TNF receptor 1, and TNF-related apoptosis-inducing ligands (TRAILs)/DR4 or DR5 (Rossin et al., 2019; Martínez-Reza et al., 2017). They induce indirect tumor destruction through secretion of interferon γ (IFN- γ) and tumor necrosis factor- α (TNF- α) (Bossi et al., 2002). After clearance of the diseased cells, T cells undergo apoptosis in the contraction phase (Rossin et al., 2019; Martínez-Reza et al., 2017).

As mentioned before, T cells might become dysfunctional. Chronic weak antigen presentation to TCRs leads to an impaired response known as T cell exhaustion characterized by diminished stimulatory cytokines including IL-2, restricted T cell proliferation, differentiation, and trafficking, increased co-inhibitory molecules such as PD-1, CTLA-4, lymphocyte activation gene 3 protein (LAG-3), and T-cell immunoglobulin domain and mucin domain protein 3 (TIM-3), and decreased cytotoxic activity due to impaired production of TNF- α , IFN- γ , and granzyme b (Wherry and Kurachi, 2015; Maimela et al., 2019).

Tumor cells and immunosuppressive components in TME restrain the anti-tumor function of CTLs as well. Tumor cells prevent T cell infiltration into the TME through various mechanisms including impaired chemokine secretion, tumor angiogenesis and co-inhibitory signaling (Maimela et al., 2019). Immunosuppressive cells also contribute to this state through production of vascular endothelial growth factor (VEGF) transforming growth factor- β (TGF- β), IL-10, CTLA-4, indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), and reactive oxygen species (ROS) (Gajewski et al., 2006). Regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSC) are the main immunosuppressive cells in the TME and will be discussed in next sections. In addition to immune subsets and tumor cells, other cell lineages such as cancer-associated fibroblasts (CAF) have an effect on the immune response. Interestingly, stromal components including CAFs shape a structure for tumor growth and impede the immunological destruction of tumor cells (Singh et al., 1992). They express fibroblast-activating protein (FAP) to suppress antitumor functions of the immune system (Kraman et al., 2010). They are immunosuppressive, produce IL-6, CXC-chemokine ligand 9 (CXCL9) and TGF β to hinder the antitumor activity of T cells and indicate a poor prognosis in cancer (Qiao et al., 2018; Fearon, 2014).

The infiltration of CTLs to the TME correlates with tumor regression and better clinical outcome. The higher CTLs in the TME the better prognosis in many cancer types including breast and colorectal cancer (Stanton and Disis, 2016; Pagès et al., 2009). Due to the importance of the infiltration and function of CTLs in antitumor immunity, the composition and spatial distribution of CTLs are used for classification of the tumor immune microenvironment (TIME). There are 2 main types of TIME: (i) An infiltrated-excluded (I-E) TIME is defined as a milieu infiltrated with multiple immune cells but a lack of CTLs in the tumor core (Binnewies et al., 2018). In this phenotype, CTLs instead are arranged along the border of the tumor mass and prevented to infiltrate to the tumor core by Ly6C^{lo} F4/80^{hi} tumor-associated macrophages (TAMs) (Beatty et al., 2015). It seems that the I-E TIMEs are not potently immunogenic to destroy tumor cells (Spranger, 2016). Compared to inflamed TIME, it is composed of CTLs with low expression of GZMB (GRZB) and IFNG. The features of low infiltration to the tumor core and low expression of activation markers demonstrate immunologic tolerance (Binnewies et al., 2018). I-E TIMEs are observed in epithelial-originated malignancies including melanoma, colorectal carcinoma (CRC) and pancreatic ductal adenocarcinoma (PDAC) (Herbst et al., 2014; Mlecnik et al., 2016; Ademmer et al., 1998). (ii) An infiltrated-inflamed (I-I) phenotype is the state of a TIMEs with infiltrating CTLs expressing PD-1 as well as tumor cells and immune cells expressing PD-L1. A good example of this TIME is CRC with microsatellite instability high (MSI-H) which associates with higher frequency of neoantigens, PD-1+ CTLs, and a better response to immune checkpoint inhibitors (Binnewies et al., 2018). An infiltrated-TLS TIME is a subtype of I-I TIME which indicates the features of a tertiary lymphoid structure (TLS) at the invasive tumor border. A TLS is a structure resembling lymph nodes composition (Binnewies et al., 2018). It contains DCs, naïve and activated T cells, and Tregs and is a location for activation of immune cells upon inflammation for example after receiving autologous cancer vaccine (Neyt et al., 2012; Lutz et al., 2014). Therefore, infiltration of CTLs to the tumor core is of great importance to predict immunological rejection of tumors.

CD4+ T-helper cells

CD4+ T cells recognize tumor antigen presented on MHC II (König et al., 1992). Helper functions of CD4+ T cells foster antitumor CD8+ CTLs (Borst et al., 2018). It has been demonstrated that the lack of CD4+ T cells leads to tumor progression since CD4+ T

cells improve both the frequency and activity of CD8⁺ CTLs (Marzo et al., 2000; Ossendorp et al., 1998). Through secretion of IL-2, CD4⁺ T cells directly increase differentiation and proliferation of CD8⁺ CTLs (Hor et al., 2015). Tumor-antigen-specific CD4⁺ T cells also foster the infiltration of CD8⁺ T cells to the TME (Wong et al., 2008). By expression of CD40L, CD4⁺ T cells indirectly foster CD8⁺ CTLs. The CD40L on CD4⁺ T cells binds to the CD40 on DCs to propel DCs toward type I subtype capable to prime naïve CD8⁺ T cells (Schoenberger et al., 1998). CD4⁺ T cells also have a role in development and maintenance of CD8⁺ T cell memory. Their presence during CD8⁺ T cell priming is crucial for development of memory T cells (Shedlock and Shen, 2003; Janssen et al., 2003). Tumor antigen-specific CD4⁺ memory T cells can increase the expansion of the cognate CD8⁺ memory T cells as well (Hwang et al., 2007). They also participate in induction of antitumor humoral response. The CD40L on CD4⁺ T cells through binding to CD40 on B cells promote differentiation and maturation of plasma cells, indeed development of antibodies against tumor antigens which correlates with clinical response (Reed et al., 2015).

CD4⁺ T cells can exert direct cytotoxicity as well. Upon induction by various cytokines and transcription factors, CD4⁺ T cells develop different subclasses and exert various effector functions (Fig. 2). The polarization of naïve T cell into Th1, Th9, and Th17 leads to the antitumor functions and Th1 cells are the main antitumor CD4⁺ T cells (Li et al., 1874). Upon polarization toward Th1 cells and under the influence of IL-12, they can secrete IFN γ and TNF α (Kennedy and Celis, 2008). IFN- γ increases antigen presentation through the upregulation of MHC molecules (Dighe et al., 1994). The concurrent presence of these two cytokines foster tumor dormancy and inhibition of escape phase (Müller-Hermelink et al., 2008). CD4⁺ T cells might also perform direct cytotoxic activity similar to what is done CD8⁺ CTLs under special circumstances (Quezada et al., 2010).

CD4⁺ T cells have been identified as a prognostic biomarker in non-small cell lung cancer (NSCLC). It has been revealed that the presence of CD62L^{lo} CD4⁺ T cells which are T-bet⁺ CXCR3⁺ CD27⁺ FoxP3⁺ correlates with the presence of CD8⁺ effector T cells and improved response to PD-1 blockade (Kagamu et al., 2020). It has also been shown that the presence of TERT-specific Th1 CD4⁺ T cells is a prognostic biomarker for NSCLC (Laheurte et al., 2019). Altogether, CD4⁺ T cells are significant agents for tumor rejection.

Tregs

Despite effector CD4⁺ T cells which exert antitumor functions, Tregs are CD4⁺ CD25⁺ T cells which express the FoxP3 transcription factor (Fontenot et al., 2003). They are necessary for maintenance of immune homeostasis and preventing autoimmune diseases. However, this attribute promotes immunosuppression and protumor functions in TME (Sakaguchi et al., 2010a). FoxP3⁺ CD4⁺ T cells are classified to 3 subtypes: CD45RA⁺ FoxP3^{low} CD4⁺ T cells or naïve Tregs, CD45RA⁺ FoxP3^{high} CD4⁺ T cells or effector Tregs which are the main immunosuppressive immune cells, and CD45RA⁺ FoxP3^{low} CD4⁺ T cells or non-Tregs which perform immunostimulatory functions and secrete IFN- γ and IL-17 (Miyara et al., 2009).

The most important immunosuppressive activities of Tregs are mediated through CTLA-4 and IL-2 consumption (Wing et al., 2008). To suppress the immune response, they consume IL-2 via CD25 (the IL-2 receptor α -chain) and degrade ATP (Sakaguchi et al., 2010b). The FoxP3 decreases expression of IL-2 and increases the expression of CTLA-4 and CD25 (Hori et al., 2003). Without CTLA-4, Tregs are not able to perform immunosuppressive functions (Wing et al., 2008). CTLA-4 binds to the CD80/86 molecules

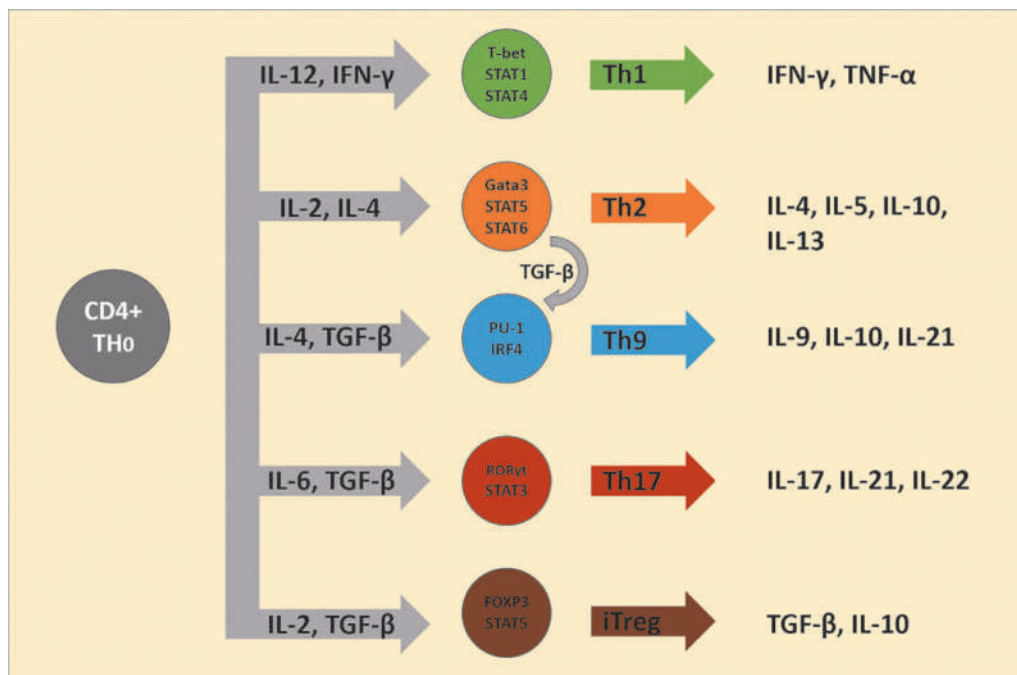


Fig. 2 Polarization of naïve CD4⁺ T cells. Various subtypes of CD4⁺ T cells with distinct functions are developed under the influence of a variety of cytokines and transcription factors.

on APCs with more affinity than CD28. CTLA-4 alongside with CD80/86 moves to Tregs which impairs APC maturation (Walker and Sansom, 2011). Tregs also produce inhibitory cytokines such as IL-35, IL-10, and TGF- β targeting tumor antigen presentation by DCs (Jørgensen et al., 2019). Production of TGF- β by Tregs inhibit antitumor functions of NK cells as well (Ghiringhelli et al., 2005).

Tregs can be derived from both effector and naïve CD4+ T cells and they might also be reversely converted to effector T cells (Tay et al., 2021). In colorectal cancer, it has been shown that there is a population of Tregs with low expression of FoxP3 which are induced by IL-12 and exert antitumor functions. The higher infiltration of this population associated with better prognosis (Saito et al., 2016). Therefore, non-Tregs show antitumor functions despite the effector Tregs which are potent immunosuppressive cells.

Gamma Delta T-cells

$\gamma\delta$ T cells compose around 1-10% of CD3+ T cells in the peripheral blood (Parker et al., 1990). They provide a protective effect on tumors and deficiency of TCR δ chain leads to a higher incidence of malignancy (Girardi et al., 2001). $\gamma\delta$ T cells by expression of the NK cell receptors show the feature of innate immunity and by expression of TCR share the characteristics of adaptive immunity (Melandri et al., 2018). They can recognize TCR-specific tumor antigens. However, despite $\alpha\beta$ T cells, they are not dependent on classic MHC molecules (Katz and Rabinovich, 2020). They use natural killer cell receptors (NCRs) including NKG2D, NKP30, and DNAM-1 to recognize various tumor antigens in an MHC-independent manner (Bonneville et al., 2010; Kabelitz et al., 2020). The cytotoxic function of $\gamma\delta$ T cells is dependent on the activation by ligation of NKG2D and its ligands MHC I-related chain A (MICA), MICB, and UL16-binding proteins 1, 2, and 3 (ULBPs) expressed on cells under stress such as activation of Ras, ultraviolet radiation, and epidermal growth factor receptor (EGFR) signaling (Nausch and Cerwenka, 2008; Shafi et al., 2011). $\gamma\delta$ T cells are in a pre-activated condition and are not dependent on clonal expansion and differentiation (Vantourout and Hayday, 2013). Upon activation, $\gamma\delta$ T cells can directly perform cytotoxicity, secrete perforin, granzyme, chemokines, and Th1 cytokines to exert antitumor functions. They can activate a variety of immune cells including APCs, $\alpha\beta$ T cells and B cells (O'Neill et al., 2020; Nussbaumer and Koslowski, 2019). They also play an APC role to present antigens to $\alpha\beta$ T cells (Meuter et al., 2010).

The most frequent subtype of $\gamma\delta$ T cells consisting more than half of the $\gamma\delta$ T cells in the peripheral blood is V γ 9V δ 2T cells which contain a TCR made by the variable (V) gene V γ 9 and V δ 2 (Hinz et al., 1997; Wesch et al., 1998). V γ 9V δ 2T cells or in short V δ 2 cells perform various functions via recognition of pyrophosphate antigens through butyrophilin-3 subfamily molecules such as BTN2A1 and BTN3A1 (Yazdanifar et al., 2020). They use specific TLRs for costimulation (Pietschmann et al., 2009) and can differentiate into different T helper cells to produce the related cytokines such as IL-4, IL-10, IL-9, or IL-17 (Wesch et al., 2001; Ness-Schwickerath et al., 2010; Peters et al., 2016). They displayed antitumor functions in both solid and hematologic malignancies (Vantourout and Hayday, 2013; Airolidi et al., 2015) and contribute to tumor rejection.

Natural killer T (NKT) cells

NKT cells are another subtype of T cells which are not dependent on the classic MHC molecules. They are among the first immune responders to tumors and rapidly produce cytokines (Terabe and Berzofsky, 2008). They mainly have a protective role against tumor; however, they might also play a protumoral role (Terabe and Berzofsky, 2008). They express T cell receptor (TCR)-CD3 complexes as well as the NK cell receptor CD161 (Godfrey et al., 2010). The invariant NKT (iNKT) or type I NKT cells in human express a semi-invariant TCR, V α 24-J α 18 paired with V β 11 (Lantz and Bendelac, 1994). iNKT cells recognize glycolipid antigens such as α -Galactocylceramide (α -GalCer) the most important glycolipid presented on CD1d. Upon recognition, iNKT cells become activated and produce IFN- γ , perform clonal expansion, and stimulate other immune cells (Lam et al., 2017). iNKT cells are able to perform direct tumor destruction; however, they mainly activate other cytotoxic cells (Metelitsa et al., 2001; Crowe et al., 2002). The antitumor activity of NKT cells is dependent on endogenous IL-12 (Smyth et al., 2000). iNKT cells by expression of CD40 binding to CD40L on immature DCs as well as IFN- γ production have a role in the maturation of DCs which produce IL-12. IL-12 gives rise to the production of IFN- γ and IL-2 by iNKT cells. iNKT cells can activate other tumor killer cells including NK cells and CD8+ T cells through IL-2 and IFN- γ production (Terabe and Berzofsky, 2008; Metelitsa et al., 2001). IFN- γ has an antiangiogenic role in tumor immunity as well (Hayakawa et al., 2002). Production of Th1 cytokines is another antitumor activity of iNKT cells (Terabe and Berzofsky, 2008).

There is another type of NKT cells, Type II NKT cells which do not express the CD1d-restricted TCR, express more variable TCRs, and foster tumor outgrowth. Type II NKT cells produce IL-13, which provoke production of TGF- β by myeloid cells which in turn suppress CTL functions (Terabe et al., 2000, 2003). Moreover, thymic NKT cells show less antitumor immunity than liver NKT cells due to the production of IL-4 (Crowe et al., 2002). Therefore, NKT cells consisting of type I and II exerts antitumor and protumor functions, respectively.

Natural killer (NK) cells

In addition to T cells, there is a key agent from the innate immunity responsible for tumor rejection (Deng et al., 2015). Natural killer (NK) cells originate from the common lymphoid progenitor and undergo the "education" process in which their immunoreceptor tyrosine-based inhibitory motifs (ITIMs) recognize MHC I to become licensed ie, recognize healthy cells expressing MHC I

and do not destroy them (Bauer et al., 1999). NK cells recognize malignant and virus-infected cells in the pattern of missing self and induced self (Wu et al., 2020). Therefore, NK cells are necessary for tumor killing since downregulation of MHC I by tumor cells is one the escape mechanisms to flee from the CD8+ cytotoxicity (Bauer et al., 1999). NK cells do not contain TCR or CD3 and utilize the NKG2D receptor to recognize tumor cells expressing the ligands including MICA, MICB, and ULBPs (Eagle and Trowsdale, 2007; Smyth et al., 2005). Transcription factors namely Nfil3, Id2, Tox, EOMES and T-bet as well as cytokines including IL-2 and IL-15 are necessary for the development, maturation and function of NK cells (Wu et al., 2020). NK cells are CD3-CD56+ cells and, based on the CD56 expression, are classified into CD56bright and CD56dim subtypes. CD56 is an important molecule for the differentiation and cytotoxicity of NK cells and is necessary for the development of CD56dim which is finally matured NK cell and the main subset of antitumor NK cells in the peripheral blood (Ferlazzo et al., 2004). However, CD56bright NK cells can also perform antitumor activities upon stimulation with IL-2 or IL-15 (Ferlazzo et al., 2004; Wagner et al., 2017).

NK cells exert direct cytotoxic functions through perforin and TRAIL to destroy tumor cells (Takeda et al., 2001). Upon expression of IL-15 and its receptor IL-15 receptor α in the TME, the infiltration and induction of cytotoxic granulated NK cells occurs (Liu et al., 2012). They are capable to produce cytokines including IFN- γ , TNF, and GM-CSF (Fehniger et al., 1999). The function of NK cells depends on the activating and inhibitory receptors. The activating receptors include NCRs, integrins, and cytokine-binding receptors (Wu et al., 2020). Inhibitory receptors include MHC I-specific receptors including killer cell immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LILRs), killer lectin-like receptors (KLRs), and MHC-I non-related receptors namely NKG2A (Wu et al., 2020).

Myeloid-derived suppressor cells (MDSCs)

Myeloid-derived suppressor cells (MDSC) are immature myeloid-lineage cells which play an immunosuppressive role in the TME. They foster tumor progression and immune escape, as well as metastasis through angiogenesis, formation of pre-metastatic niche, and epithelial-mesenchymal transition (EMT) (Li et al., 2017; Sceneay et al., 2013; Toh et al., 2011). They suppress the antitumor activities of T cells by induction of T cell dysfunction and inhibition of T cell activation, expansion, and infiltration. They decrease the production of IL-12 and prevent CD8+ T cell infiltration which is reversible by IL-12 treatment (Steding et al., 2011). MDSCs disturb T cell trafficking through increased production of reactive nitrogen species (RNS) and expression of a disintegrin and metalloproteinase 17 (ADAM17) to destroy the CD62L on T cells (Hanson et al., 2009a, b; Molon et al., 2011).

There are two subtypes of MDSCs: (i) granulocytic or polymorphonuclear MDSCs (PMN-MDSCs) which are CD11b⁺CD14⁻CD15⁺ or CD11b⁺CD14⁻CD66b⁺ and mimic neutrophils. (ii) monocytic MDSCs (M-MDSCs) which are CD11b⁺CD14⁺HLA-DR⁻/lowCD15⁻ cells and similar to monocytes. Both subtypes can be involved in the proliferation and invasiveness of tumor cells (Bronte et al., 2016).

MDSCs can inhibit antigen-specific T cell response through production of short-lived substances i.e., reactive oxygen species (ROS) and peroxynitrite (PNT). PNT is a potent oxidant which impairs T cell function by nitrating the TCR complex and Tyr394 of lymphocyte-specific protein tyrosine kinase (LCK), a tyrosine kinase involved in TCR signaling (Nagaraj et al., 2007; Feng et al., 2018). Through this alteration the ability of MHC to bind and present tumor antigens to T cells decreases (Lu et al., 2011). Production of ROS and subsequent decreased expression of T-cell receptor (TCR) ζ -chain is another mechanism by which MDSCs impair the function of antigen-specific T cell (Kusmartsev et al., 2004). This mechanism is dependent on the close contact of MDSCs and T cells. MDSCs provide this close contact with T cells by expression of MHC I and presentation of tumor antigen (Nagaraj et al., 2007). There is some evidence showing that MDSCs are able to hamper the function of CD8+ T cells which are only specific for the antigen presented by themselves but no other antigens (Nagaraj et al., 2010).

MDSCs are also able to block non-specific T-cell response. They produce long-lived cytokines, iNOS and arginase 1 (ARG1) for this purpose (Kumar et al., 2016). MDSCs produce inducible nitric oxide synthase (iNOS) to provide nitric oxide (NO). NO inhibits T cell proliferation through the blockade of Jak/STAT5 pathway as well as antigen presentation by DCs (Markowitz et al., 2017; Bingisser et al., 1998). NO is a factor provoking T cell apoptosis as well (Wang et al., 2010). NO also interferes with T cell trafficking by decreasing E-selectin (Gehad et al., 2012). MDSCs decrease the essential amino acids for T cell activity. By expression of ARG1, they hamper L-arginine and ultimately CD3 ζ -chain of the TCR complex (Rodríguez and Ochoa, 2008). By importing cystine but not releasing cysteine, MDSCs deplete cysteine, another amino acid vital for T cell activity (Srivastava et al., 2010). MDSCs produce adenosine which impairs T cell functions (Ryzhov et al., 2011). They produce PD-L1 which binds to the PD-1 on T cells and induce T cell anergy (Weber et al., 2018). They can also impair the CD4+ T cell function if they present a sufficient level of MHC II (Nagaraj et al., 2012). It has also been shown that they also inhibit IFN- γ secretion from CD4+ tumor specific T cells (Chalmin et al., 2010).

MDSCs produce various cytokines namely IL-6, IL-10, and TGF- β (Chandra and Gravekamp, 2013). MDSCs produce the majority of IL-10 in the TME, and there is a correlation between the number of MDSCs, the level of IL-10, and cancer progression (Arihara et al., 2013; Zhang et al., 2015). IL-10 inhibits the activity of DCs and T cells and provokes Treg response. MDSCs produce TGF- β which hampers the function of T cells, NK cells, and NKT cells (Yang et al., 2020). They provoke induction and trafficking of Tregs; the cytokines TGF- β and IL-10 are involved in the induction and the CCR5 ligands CCL3, CCL4, and CCL5 are the main factors for Tregs infiltration (Huang et al., 2006; Schlecker et al., 2012). By and large, MDSCs exploit a variety of mechanisms to hinder the T cells functions.

Tumor-associated macrophages (TAMs)

Macrophages are classified into M1 and M2 subtypes which exert antitumor and protumor functions, respectively. IFN- γ , tumor necrosis factor (TNF), lipopolysaccharide, and GM-CSF participate in development of classically activated or M1 macrophages while inhibitory cytokines including IL-4, IL-10, IL-13, and M-CSF are involved in development of alternatively activated or M2 macrophages (Quatromoni and Eruslanov, 2012; Orecchioni et al., 2019). M1 macrophages exert antitumor functions. They produce proinflammatory cytokines namely IL-1, IL-6, IL-12 and TNF, toxic substances including RNS and ROS, present tumor antigens, and foster a Th1 response (Petty and Yang, 2017). M1 macrophages foster the activation and infiltration of effector T cells through expression of CXCL9, CXCL10 and CCL5 (Mantovani et al., 2013). They also induce ADCC and can perform a direct cytotoxicity by production of cathelicidin (Bruns et al., 2015). M2 macrophages produce IL-10 and TGF- β , COX-2 derived prostaglandin E2 (PGE2), CD206, and CD163 for suppression of T cell responses and CCL2, CCL17 and CCL22 for infiltration of Tregs to TME (Mantovani et al., 2013; Curiel et al., 2004; Ge and Ding, 2020). TAMs are a group of macrophages which exert protumor functions similar to M2 macrophages. However, a distinct subtype might not be attributed to TAMs only by markers; other determinants such as function, milieu, and progression of malignancy should be taken into consideration (Biswas et al., 2006; Nasrollahzadeh et al., 2020).

The high frequency of TAMs in TME is an indicator of poor prognosis. They are involved in angiogenesis and prevention of T cell recruitment (Tsutsui et al., 2005; Dannenmann et al., 2013). They produce different angiogenic stimuli including VEGF, PDGF and bFGF, and other matrix metalloproteinase (MMP) and other molecules which support EMT. They foster establishment of cancer stem cells by production of various cytokines such as TNF- α , IL-6, and EGF (Petty and Yang, 2017). Overall, TAMs mainly support progression of tumors.

Immune regulation and metastasis

Metastasis is a state of tumor cell dissemination from the primary site to the secondary one(s). After growth, tumor cells migrate and invade the surrounding tissue through epithelial-to-mesenchymal transitions (EMTs) and pass into the blood or lymphatic vessels (intravasation), move and survive there, leave the vessels (extravasation), adhere to the secondary site and proliferate there (Guan, 2015; Pearson, 2019). Some immune subsets can prevent metastasis and some contribute to metastasis in different phases (Blomberg et al., 2018). In this section, we will review the dual role of the immune system in cancer metastasis.

Remodeling of ECM is performed by various immune cells (Lu et al., 2012). The ECM remodeling leads to tumor cell invasion through structural alteration of the surrounding tissue, and spread of the growth factors and signaling molecules necessary for tumor progression (Ghajar and Bissell, 2008; Cox and Erler, 2011). In addition to CAFs, immune cells including TAMs, tumor associated neutrophils (TANs) and mast cells are involved in ECM remodeling by secretion of proteases (Gocheva et al., 2010; Nozawa et al., 2006; Ribatti et al., 2003). Bregs also stimulate the production of MMPs by tumor cells and contribute to ECM remodeling (Ou et al., 2015). ECM remodeling is among the early required steps for metastasis.

Angiogenesis and lymphangiogenesis are vital for metastasis both as a channel for migration and as a provider of factors required for tumor survival (Zetter, 1998; Achen et al., 2005; Mazzone et al., 2009). The formation of new blood and lymphatic vessels is also modulated by immune cells (Christiansen and Detmar, 2011; Bruno et al., 2014). Angiogenesis correlates with the frequency of TAMs, Tregs, and mast cells, which produce VEGFA (Leek et al., 1996; Zidlik et al., 2015; Yano et al., 1999; Esposito et al., 2004). Hypoxia which is induced by the growing tumor is one of the factors stimulating angiogenesis (Mazzone et al., 2009). It also attracts TAMs, which produce VEGFA (Ramanathan et al., 2003). IL-17 produced by Th17 is the other inducer of the production of VEGF by tumor cells (Pan et al., 2015). Also, the ECM remodeling modulating enzymes such as MMPs make a loose ECM releasing the matrix-bound VEGF (Lee et al., 2005). Through the abovementioned mechanisms, tumor cells and immune cells contribute to the formation of vessels necessary for metastasis.

Intravasation, survival in the vessels, and extravasation are necessary steps undertaken by tumor cells to migrate into a secondary site. TAMs are involved in intravasation of EGFR+ tumor cells. It has been shown that IL-4/IL-13-activated TAMs produce EGF under the influence of colony-stimulating factor-1 (CSF-1) which is secreted by tumor cells (DeNardo et al., 2009; Wyckoff et al., 2004). The production of IL-4 by CD4+ T cells is also prometastatic since IL-4 regulates macrophage phenotype and inhibit antitumor activities (Qian et al., 2011). Circulating tumor cells (CTCs) must survive various threats including immune destruction, high blood flow shear stress, and anoikis in the challenging environment of vessels (Fu et al., 2016; Liotta and Kohn, 2004; Labelle and Hynes, 2012). NK cells, T cells, and liver-resident macrophages are involved in immune destruction of CTCs (Santos et al., 2014; Ye et al., 2017; Denève et al., 2013). Other cells however support CTC survival. For example, Tregs produce the receptor activator of nuclear factor- κ B ligand (RANKL) and platelets establish a fibrin clot to protect CTC from destruction by immune cells (Tan et al., 2011; Palumbo et al., 2005). CTCs also exploit other approaches to survive. They upregulate CD47 the anti-phagocytic molecule (Steinert et al., 2014), produce HLA-G a driver of immunosuppression (König et al., 2016), and their presence associate with the expression of FAS on T cells which promote T cell apoptosis (Gruber et al., 2013). Myeloid cells also perform prometastatic functions by ingestion of the dying tumor cells and hiding their antigen from DCs (Borghaei, 2015).

For extravasation, tumor cells require to adhere to the endothelium, a process which is supported by some immune cells including neutrophils, and metastasis-associated macrophages or the very inflammatory monocytes (Cools-Lartigue et al., 2013; Qian et al., 2009a, 2011). Neutrophils by production and release of neutrophil extracellular traps (NETs) insulate tumor cells and

induce their invasion and migration into the secondary site (Cools-Lartigue et al., 2013; Park et al., 2016). The adhesion of tumor cells is mediated through the ligation of intercellular adhesion molecule 1 (ICAM-1) on tumor cells with the integrin CD11b on neutrophils (Spicer et al., 2012) as well as the formation of fibrin clot, activation of TGF β -dependent pathways, and releasing ATP-containing granules by platelets (Stegner et al., 2014; Labelle et al., 2011; Schumacher et al., 2013). Inflammatory monocytes also contribute to extravasation. They increase vascular permeability by production of VEGF and provide mechanical help for extravasation (Qian et al., 2011, 2009b).

Based on the “seed and soil” hypothesis, tumor cells do not incidentally metastasize to an organ. There are some intrinsic factors such as expression of specific chemokines on tumor cells which direct them to secondary sites expressing the cognate ligands, and extrinsic factors such as immune and stromal cells which prepare the secondary site or (pre)metastatic niche (Paget, 1989; Müller et al., 2001; Doglioni et al., 2019). Immunosuppression is important for making a (pre)metastatic niche. Among immune cells, immature myeloid cells are recruited from the bone marrow (bone marrow-derived cells [BMDCs]), to establish the (pre-)metastatic niche (Jablonska et al., 2017). Tumor-derived factors induce recruitment of BMDCs and tumor cells as well as preparing the secondary site for their arrival (Erler et al., 2009a; Hiratsuka et al., 2008). The infiltration of myeloid cells alters the local environment of the secondary site to a one with enhanced angiogenesis, vascular permeability, inflammation, immunosuppression, and ECM remodeling (Liu and Cao, 2016). Neutrophils, macrophages and fibroblasts are the major supporters of tumor cell infiltration and extravasation in the (pre)metastatic niche by angiogenesis and ECM remodeling. They produce proangiogenic factors such as Bv8 (Kowanetz et al., 2010) and ECM remodeling factors, such as fibronectin and lysyl oxidase (LOX) (Erler et al., 2009b; Yamamura et al., 2015). Neutrophils might show either prometastatic or antimetastatic functions (Wculek and Malanchi, 2015; Granot et al., 2011). Prometastatic neutrophils can be developed by IL-17 produced by $\gamma\delta$ T cells. These immunosuppressive neutrophils suppress the activity of CD8+ T cells to foster metastasis in early steps (Coffelt et al., 2015). They also support metastasis by production of leukotrienes helping colonization of tumor cells in the secondary site (Wculek and Malanchi, 2015). On the other hand, to prevent metastasis to the secondary site, CD103+ DCs in the secondary site activate CD8+ T cells and inhibit metastasis (Roberts et al., 2016; Headley et al., 2016).

Immune-based interventions for cancer treatment and prevention

Upon understanding of the antitumor and protumor functions of the immune system, various therapeutic approaches have been developed to hinder the barricades of an effective tumor rejection. The majority of these approaches have been assessed in clinical trials and due to the improved clinical outcomes received approval for treatment or prevention of cancer from the US Food and Drug Administration (FDA). However, some of them have yet to be optimized (Keshavarz-Fathi and Rezaei, 2019a, 2021). Herein, we will provide a quick review on the advancement of immune-based interventions for cancer treatment and prevention.

Nonspecific immunomodulators are among the first modalities evaluated for cancer treatment. IL-2, a cytokine predominantly produced by CD4+ T cells has a significant function in the proliferation and activity of T cells (Valle-Mendiola et al., 2016). High-dose IL-2 was approved for the treatment of metastatic renal cell carcinoma and melanoma; however, the monotherapy is not desired since IL-2 participates in expansion of Tregs too (Choudhry et al., 2018). Nowadays, it is mostly used for combination therapy (Razavi et al., 2021). TLR agonists another drug of this class are used for different purposes. They are effective vaccine adjuvants (Keshavarz-Fathi and Rezaei, 2019b) and are potent modalities for treatment and prevention of cancer. For instance, Bacillus Calmette-Guérin (BCG) a TLR2 agonist has been approved for treatment of superficial bladder cancer (Morales and Nickel, 1986), and imiquimod a TLR7 agonist has been approved for immunoprevention of skin squamous cell carcinoma (SCC) (Swanson et al., 2014).

To block the activity of molecules contributing to tumor progression and invasion, monoclonal antibodies (mAb) have been developed. The molecules CD20, HER2, VEGF, EGFR, SLAMF7, CD38, and RANKL are among the targets toward which mAbs have been showed improved clinical outcomes and approved for cancer treatment (Grillo-Lopez et al., 2000; Goldenberg, 1999; Hurwitz et al., 2004; Cunningham et al., 2004; Lonial et al., 2015; Usmani et al., 2015). By addition of a linker and a cytotoxic agent to mAbs, antibody–drug conjugates (ADCs) are constructed which destroy tumor cells expressing the target antigen. ADCs targeting CD22, CD30, and CD33 for hematologic malignancies and an ADC targeting HER2 for breast cancer have been approved (Kantarjian et al., 2016; Connors et al., 2018; Amadori et al., 2016; Verma et al., 2012). Recently, bispecific Abs (BsAbs) which have scFv against both tumor markers and CD3 have been used to engage tumor cells and T cells. They have been approved for treatment of acute lymphoblastic leukemia (ALL) (Kantarjian et al., 2017). mAbs, ADCs, and BsAb are interesting modalities for cancer treatment and are rapidly growing.

Vaccines have been primarily used for prevention of infectious diseases; however, they are interesting modalities with desirable safety profile and long-term protection for treatment and prevention of cancer. Various constructs of vaccines including whole tumor cell, peptide, APCs, mRNA, and DNA have been evaluated and demonstrated improved antigen presentation and CTL response (Keshavarz-Fathi and Rezaei, 2019a, 2021). However, only one APC vaccine which showed improved overall survival in an advanced clinical trial has been approved for prostate cancer (Kantoff et al., 2010b). Due to the advantages of cancer vaccines, their effectiveness, and the recent approval of an mRNA vaccine for COVID-19, there is a hope to rapidly promote cancer vaccines specially mRNA vaccines (Miao et al., 2021).

Immune checkpoint Inhibitors (ICIs) another type of antibodies are directed against inhibitory molecules such as PD-1, PD-L1, CTLA-4, LAG-3, and TIM-3. The PD-1 inhibitors received approval for treatment of many solid tumors including melanoma, lung,

colorectal, renal, bladder cancer, hepatocellular carcinoma, and SCC (Keshavarz-Fathi and Rezaei, 2019a; Weber, 2015; Borghaei, 2015; Overman et al., 2017; Motzer, 2015; Sharma et al., 2017; El-Khoueiry et al., 2017; Kudo et al., 2019; Ahmed et al., 2019), as well as a hematologic malignancy Hodgkin lymphoma (Ansell et al., 2015; Younes et al., 2016). An anti-PD1 Ab is also being evaluated in an ongoing clinical trial for treatment of oral proliferative verrucous leukoplakia (OPVL), indeed prevention of oral SCC (NCT03692325). The PD-L1 inhibitors received approval for treatment of several solid tumors including lung, breast, urothelial, and renal cancer (West et al., 2019; Schmid et al., 2018, 2019; Balar et al., 2019; Motzer et al., 2019). The antibody against CTLA-4 also received approval for treatment of colorectal cancer and melanoma (Eggermont et al., 2015; Overman et al., 2018). Other ICIs directed against LAG-3 and TIM-3 are under evaluation in early clinical trials (NCT02061761, NCT03066648). ICIs are promising interventions which showed significant improvement of survival in patients with cancer; however, resistance to ICIs and their immune-related adverse effects can restrict their usage.

Adoptive cell therapy (ACT) is an immunotherapeutic method which is based on transferring autologous or allogeneic immune cells such as T cells, NK cells, and macrophages to empower antitumor cytotoxicity. Chimeric antigen receptor (CAR) T cell therapy a subtype of ACT equips T cells to 3 main components including a scFv to recognize tumor antigens, the CD3 ζ for signal transduction and a costimulatory molecule such as CD28 and/or 4-1BB (Hosen, 2020; Boyiadzis et al., 2018). It has been approved for treatment of hematologic malignancies including ALL, large B-cell lymphoma, multiple myeloma, and follicular lymphoma in recent years (Maude et al., 2018; Schuster et al., 2019; Munshi et al., 2021) (NCT03105336). CAR macrophages and CAR NK cells are also being evaluated in early clinical trials (NCT04660929, NCT00995137, NCT02944162). Tumor-infiltrating lymphocyte (TIL) therapy is another subtype of ACT in which the T cells infiltrated to the TME are isolated from the patient, activated, expanded and re-infused to the patient. If the isolated T cells are equipped with a new TCR, the approach is known as engineered TCR therapy (Linnemann et al., 2011). CAR T cell therapy and other ACTs are a major breakthrough in immunotherapy for cancer especially hematologic malignancies. They have faced some challenges for solid tumors and yet to be optimized.

Immune-based biomarkers for cancer

Various immune-based biomarkers are used for prediction of prognosis as well as response to immunotherapies. The importance of the TIME in cancer prognosis has recently gained attention. It has been demonstrated that the immune contexture of tumors is indicative of response to treatment and patient survival. Interestingly, a preexisting adaptive immune response in TME associates with a better response to immunotherapy. Therefore, to consider the effect of T cell infiltration to the TME, the immunescore developed (Bruni et al., 2020; Galon et al., 2014). It has been reported that the immunescore surpasses the classic tumor-node-metastasis (TNM) staging system as a prognostic biomarker in CRC (Mlecnik et al., 2011). The immunescore was defined as the frequency of two lymphocyte populations including CD3+ T cells, CD8+ CTLs, and CD45RO+ memory T cells (CD3/CD8, or CD3/CD45RO, or CD8/CD45RO) in the core of the tumor (CT) and the invasive margin (IM) of the tumor (Pagès et al., 2009). It is assessed by immunohistochemical methods and ranges from 0 to 4 which respectively indicate low densities and high densities of both cell types in the CT and IM (Lanzi et al., 2020). Immunescore is a powerful prognostic biomarker for disease-free survival and overall survival in CRC (Pagès et al., 2018).

The immune contexture of tumor can be classified based on other markers. Since the blockade of PD-1/PD-L1 has been a major breakthrough in cancer treatment but some patients do not respond to this intervention, determining the responders is of great importance. It has been revealed that tumor-infiltrating lymphocytes (TILs) and the expression of PD-L1 molecule on T cells are significant biomarkers (Zhang and Chen, 2016; Brahmer et al., 2012; Topalian et al., 2012; Tumei et al., 2014). Accordingly, tumors might display 4 possible TIME classifications including T1 (PD-L1-, TIL-), T2 (PD-L1+, TIL+), T3 (PD-L1-, TIL+), and T4 (PD-L1+, TIL-) (Zhang and Chen, 2016; Taube et al., 2012). Interestingly, there is a correlation between TIL and PD-L1 expression because the production of IFN- γ by T cells, which occurs upon their tumor antigen recognition, provoke the expression of PD-L1 on nucleated cells including T cells (Taube et al., 2012). It is predicted that T2 tumors with high TIL and PD-L1 expression are great responders to the therapy by PD-1/PD-L1 blockade. These tumors contain high level of TIL but are unarmed due to the expression of PD-L1. The majority of tumors are nonresponders to anti-PD therapy. They are either T1 or T4 ie, tumors without TIL. T4 tumors express PD-L1 because of the activation of oncogenic pathways. Several reasons including impaired tumor antigen presentation and immunosuppressive components underlie the lack of TIL (Taube et al., 2012; Parsa et al., 2007). However, some modalities such as local radiation, cryotherapy, and local oncolytic viruses can compromise this state and enhance the tumor antigen shedding and presentation. Likewise, immunotherapies such as vaccines, adoptive T cell therapy, and CTLA-4 blockade can also improve the filtration of TIL (Gupta et al., 2012; Waitz et al., 2012; Luo et al., 2020; van der Burg, 2018; Chruściel et al., 2020). T3 tumors contain TIL but do not express PD-L1 probably due to the lack of IFN- γ secretion by T cells (Sanmamed and Chen, 2014). Upon administration of OX-40 or 4-1BB agonists, T cell tolerance could be reversed. In this case, T3 tumors can turn into T2 tumors which are good responders to PD-1/PD-L1 blockade (Zhang and Chen, 2016). Altogether, the TIME classification can assist the prediction of response to PD-1/PD-L1 blockade. However, since only small slides of the tumor are assessed to detect PD-L1 expression and TIL, novel technologies are warranted to obtain more comprehensive data.

As explained, TIL and PD-L1 expression are the main predictive biomarkers of response to PD-1/PD-L1 blockade. There are also other immune-based biomarkers for prediction of response to ICIs; heterogeneity in the HLA genes, expression level of CTLA-4 and PD-1 on TILs, expression of immune-related genes such as *IFNG* and *GZMB*, and neutrophil to lymphocyte ratio (NLR) are some

examples (reviewed in Mahdavi Sharif et al., 2021). Altogether, exploiting immune-based and other biomarkers is recommended to specify patients benefiting from immune-based interventions.

Conclusion

The immune system has a significant role in determining suppression or progression of tumors, and immune escape is one of the hallmarks of cancer. A comprehensive understanding of the antitumor and protumor functions of various immune subsets led to development of immune-based interventions for treatment and prevention of cancer. Based on the improvement of overall survival and disease-free survival of patients, the majority of interventions have been approved for cancer treatment. However, some of them such as vaccines have failed to show a dramatic clinical response and resistance was developed to ICIs. To overcome the challenges, immunosuppressive phenotype of advanced cancers needs to be inhibited and predictive biomarkers especially immune-based biomarkers should be exploited to include the subgroups of patients benefiting from the treatment.

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Normal Microbial Flora

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Glossary

Anaerobic A condition in which there is no oxygen present.

Cariogenesis The generation of tooth decay.

Colonization The growth of a microorganisms after it has accessed host cell tissues.

Commensal Opportunistic microorganisms that reside on outer surfaces and inside the human body that do not cause infections unless dysbiosis occurs.

Dental biofilm A community of microorganisms adhere to a solid surface in an intercellular matrix composing of saliva, gingival crevicular fluid and bacterial products. Exopolysaccharides are produced by the present bacteria maintaining integrity and protect the cells from antimicrobials and harmful agents.

Dental caries Tooth decay.

Dimorphic Microorganisms able to occur as two separate forms. *Candida albicans* can grow as both yeast and filamentous cells.

Disease A disorder of structure or function in organism, especially one that produces specific symptoms or that affects a specific location and is a direct result of physical injury.

Dysbiosis An imbalance between microbial communities within the host.

Enteric virus A virus that resides within the GI tract.

Gastric fluid The acidic fluid secreted by glands in the stomach to aid digestion.

Genera A taxonomic classification that falls below family.

GI Tract Gastrointestinal tract.

Goblet cells Cells found within the respiratory and intestinal tracts that secrete mucins.

Host An organism that harbors a parasite.

Infection The growth of microorganisms in a host that results in eliciting of a immune response.

Monosaccharides Simple sugars forming the basis of carbohydrates.

Mucins A glycoprotein component of mucus.

Mucus Soluble glycoproteins secreted by epithelial cells that coat the mucous membranes.

Mycobiome The fungal component of the microbiome.

Next Generation Sequencing (NGS) Next-generation sequencing.

Normal microbial flora The microorganisms found in association with healthy host tissues.

Pathogen A microorganism that causes disease in a host.

Phyla A taxonomic classification that falls below kingdom.

Pyrosequencing Method of DNA sequencing that detects the order of nucleotides using sequencing by synthesis, revealing the genetic code of a section of DNA.

Stomach Ulcer A form of peptic ulcer disease that present as open sores within the stomach lining.

Symbiosis A mutually beneficial interaction between two organisms.

The commensal microbiome

The commensal microbiome contributes to nutrition, energy and a healthy immune response in humans (Littman and Pamer, 2011) (Fig. 2). Microbes will occupy a niche in which they persist and survive in a mutually beneficial state; however, dysbiosis may result in proliferation of pathogenic microorganisms. An example of this is growth of the Gram positive spore former *Clostridioides difficile* which proliferates when the commensal gut microbiome is altered by the use of broad spectrum antibiotics (Leffler and Lamont, 2015).

Commensal microbial communities are often complex in nature and have thus presented as a challenge to study (Malla et al., 2019). The human gut microbiome for example has been most widely studied through fecal analysis via in vitro culture of single microbial species within the laboratory (Scanlan and Marchesi, 2008). However, standard culture techniques are limited when analyzing the microbiome as many microorganisms have yet to be grown in culture, and their optimal growth conditions remain unknown. The advent of culture-independent approaches, such as metagenomic sequencing, has significantly enhanced our understanding of host microbiome interactions.

Initial studies examined the presence of the 16S ribosomal RNA genes which are suitable conserved sequence for species identification (Madigan et al., 2008). However, advanced sequencing technologies such as next generation sequencing (NGS) have enabled comprehensive study of the environmental and human microbiome. The most notable is the NIH Human Microbiome Project (Peterson et al., 2009; NIH, 2016 <https://www.hmpdacc.org/hmp/>) These include, but are not limited to, metagenomic sequencing of the skin microbiome and the gut microbiome, where whole genome shotgun sequencing could be undertaken (Weinstock, 2012). We are now in an era where sequencing is becoming commonplace to analyze microorganisms, even in real time via the Oxford Nanopore using short read sequencing data (Moss et al., 2020).

The normal microbiota of the skin

The human skin is a physical barrier that prevents invasion by pathogenic microorganisms or any foreign elements. The normal microbiota of the skin comprises beneficial bacteria, viruses and eukaryotes such as fungi and arthropods which exist as commensals in either transient or residential population (Grice and Segre, 2011). The colonization sites of the skin are either dry, moist or sebaceous areas; all of which have varied microbial populations that play key roles in preventing pathogens from infecting the skin. This can be related to the pH and/or moisture content of areas of the skin, where the pH is between 5.3 and 6.5 (Neugent et al., 2020) (Fig. 3).

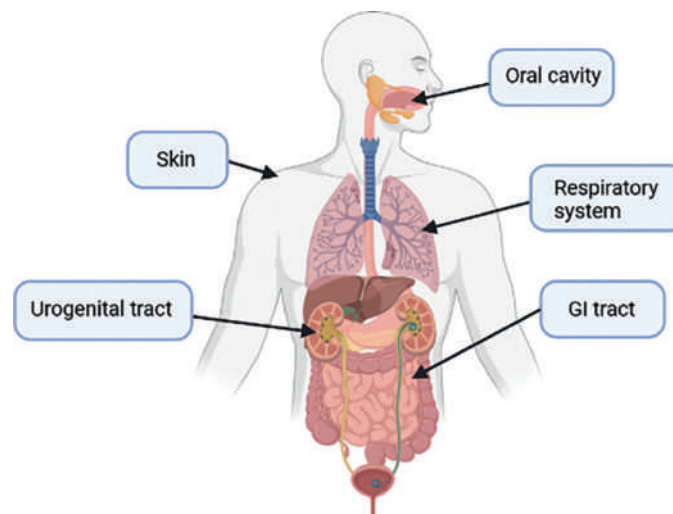


Fig. 1 The human body. The skin, oral cavity, respiratory system, urogenital tract and gastrointestinal tract (GI Tract) are covered within this chapter. Created using Biorender (2021). <https://app.biorender.com/user/signin>.

However, when the barrier is broken, the balance between commensal microbiota and the invading pathogens is disturbed which can lead to skin infections (Madigan et al., 2008). The skin microbiome is known to harbor a great variety of microorganisms.

Bacteria

Analysis of skin samples via culture-based methods has found that Gram positive bacteria are the most common commensals. These include those of the genus *Staphylococcus* (e.g., *Staphylococcus aureus*, *Staphylococcus epidermidis*), *Streptococcus* (*Streptococcus pyogenes*), *Propionibacterium* (*Cutibacterium acnes*) and *Corynebacterium*. However, through 16S rRNA gene phylotyping a greater diversity of microbiota has been found including Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes (Byrd et al., 2018). *Propionibacterium* has been found to colonize sebaceous sites; *Corynebacterium* in moist sites and a mixture of Proteobacteria and Actinobacteria are mostly found on dry skin sites. Gram negative bacteria such as *Escherichia coli* do not usually grow on the skin; however, they are often found in skin flora samples due to occasional fecal contamination of the skin via feces. Gram positive microorganisms are better able to colonize these dry skin environments compared to their counterparts and thus out compete Gram negatives (Percival et al., 2012). The only exception to this is bacteria from the genus *Acinetobacter* which are able to colonize the skin.

Fungi and Eukarya

The most common fungal species able to colonize the skin are *Malassezia*. These lipophilic yeasts include *M. globosa* (back), *M. restricta* (scalp) and *M. sympodialis* which all reside in the sebaceous areas of the skin (Schommer and Gallo, 2013). *Pityrosporum ovalis* is another lipophilic yeast that is found on the scalp. Extremities such as the foot are often colonized by varying fungi from the genus *Aspergillus*, *Epicoccum* and *Cryptococcus* (Findley et al., 2013). Other eukaryotes in the form of arthropods also comprise the normal skin microbiota. These include mites that are found on hair follicles (*Demodex folliculorum*) and sebaceous glands (*Demodex brevis*) (Lacey et al., 2009; Clavaud et al., 2013).

Virome

There is currently limited knowledge of viruses that comprise the skin's normal microbiota. Metagenomic and genetic analyses are revealing more about the viruses that could potentially be a part of the normal microbiota, mainly human papillomaviruses; however, this is still an area of ongoing research (Foulongne et al., 2012).

Summary of skin microbiota

The skin has a residential microflora that comprises varying microorganisms. Their ability to remain in constant balance relies on the environment of the skin and host factors which can influence their survival. The microbiota composition can vary according to the host's age, hygiene and even the weather. Usually, however, microorganisms which are transient will not survive the low pH of the skin or its low moisture content.

The normal microflora of the oral cavity

The oral cavity

The oral cavity is made up of habitats whereby microbial colonization takes place. The mouth is mostly anaerobic, and bad breath—known as halitosis—is caused by the anaerobic flora of the mouth due to the excretion of sulfur compounds after protein digestion (Winkel, 2020). Oral structures such as the teeth, saliva and hard and soft palates are home to specific species within the oral microbiome (Kilian et al., 2016; Gao et al., 2018). Other structures that contribute to this heterogeneous environment include: keratinized gingiva (gums), gingival sulcus, attached gingiva, supra gingival and subgingival dental plaque (tooth biofilm above and below the gum), throat and tongue soft tissues, buccal mucosa (cheek) and lips (Kilian et al., 2016; Gao et al., 2018). Depending on which surface is colonized determines whether the microflora is more aerobic or anaerobic.

The flora of the oral microbiome was identified in the 1670s by the 'Father of Microbiology' Antonie van Leeuwenhoek. This pioneering scientist constructed his own microscopes and observed his own dental plaque samples, becoming a founder into the discovery and studies of the oral microbiome (Gao et al., 2018; Deo and Deshmukh, 2019). The oral cavity is made up of the second most diverse microbiome, comprising of over 700 species of microorganisms (Duran-Pinedo and Frias-Lopez, 2015; Gao et al., 2018; Deo and Deshmukh, 2019). Additionally, the oral cavity is the best studied microbiome as sample collection is non-invasive and easy to perform. Despite this, culturing and studying the oral microbiome has been difficult in the past due to the anaerobic environment required to imitate the anaerobic oral habitat. Currently, the ability to sequence genes and set up databases such as the Human Oral Microbiome Database (HOMD) (www.homd.org) has made studying the microbiome and oral diseases much more accessible and reliable. Dysbiosis of the oral commensal species leads to a disruption in homeostasis, which then leads to development of oral disease.

Microflora of the oral cavity

Bacteria

The predominant bacterial species in the oral microbiota may be anaerobic, facultative or obligate anaerobic. Table 1 provides insight to the many bacterial species that live symbiotically in the oral cavity (How et al., 2016). It is estimated around 500 species colonize the oral cavity, with Streptococci spp. making up around 60–90% of the bacteria that colonize teeth first after cleaning. An estimated 415 of these species are found in the subgingival plaque and supragingival plaque (Kolenbrander et al., 2002).

The Gram-negative obligate anaerobe *Porphyromonas gingivalis* (*Porphyromonas gingivalis*) is a well-known cause of gingivitis leading to chronic periodontitis—also known as gum disease and chronic inflammation of the gingiva (gums), respectively. *P. gingivalis* is a stable member of the oral microflora and able to co-habit the oral cavity, however dysbiosis leading to an increase of the bacterium causes gum disease. Good oral hygiene can prevent dysbiosis and is required to prevent biofilm formation and plaque build-up of commensal bacteria. Brushing and flossing teeth daily using dental products that contain fluoride; this controls tooth demineralization and helps to replenish calcium and phosphate ions, known as remineralization. Other bacteria present in the oral microbiome may belong to the Streptococcus or Actinomyces spp. which reside as a dental biofilm on the surface of teeth (Lemos et al., 2019). *Streptococcus salivarius* produces alkali by expressing a gene for urease when under acidic conditions and in the presence of carbohydrates (Duran-Pinedo and Frias-Lopez, 2015). The commensal bacteria may act as a barrier against pathogens, however pathogenic *Streptococcus mutans* manipulates dysbiosis due to the presence of sucrose to compete with normal microflora in the oral cavity. The first bacteria to naturally colonize teeth around 4 h after cleaning are *Streptococcus gordonii*, *Streptococcus mitis*, and *Actinomyces* spp. attach to the tooth enamel and form biofilm normally, at an almost neutral pH. At this stage, overproduction of H₂O₂ by peroxigenic bacteria may lead to the growth of pathogenic bacteria such as *S. mutans*. The take-over of biofilm by *S. mutans* modifies the biofilm microenvironment and the secretion of mutacins kills nearby competitors such as commensal streptococci (Lemos et al., 2019). The highly acidic environment leads to demineralization of the enamel, ending with dental caries. This type of cariogenesis can be prevented by practicing good oral hygiene and a limited-carbohydrate diet. However, as antimicrobial resistance is a growing concern with almost all bacterial species, *S. mutans* has been found to exhibit fluoride resistance (Liao et al., 2017).

Viruses

Viruses such as human Herpes viruses (HHV) can be found in over 70% of healthy individuals. The most common HHV commensal detected in the oral cavity is the Epstein-Barr virus (EBV) at around 60% of immunocompetent individuals (Yap et al., 2020). Invading viruses have a complex relationship with oral microflora as they can either compete with or promote one another's pathogenicity (Ma et al., 2019).

Fungal and mucosal flora

There is a high fungal oral diversity, also known as the oral mycobiome, which has only recently been discovered and studied due to the advancements of Next generation Sequencing (NGS). Recent studies have shown 75% of healthy subjects were colonized by *Candida* spp., 65% had *Cladosporium* and 50% carried *Aureobasidium* and *Saccharomycetales*. It has also been found more recently that *Malassezia* species have been found as part of the oral mycobiome, evading classical techniques, and now able to be detected due to pyrosequencing (Bandara et al., 2019). *C. albicans* is a commensal dimorphic yeast found in the oral cavity and in

Table 1 Examples of the bacterial species that make up the oral microflora.

Microbial group	Genus	Species
Gram-positive Aerobic or facultative	Streptococcus	<i>Streptococcus gordonii</i> , <i>S. mitis</i> , <i>S. auralis</i> , <i>Streptococcus salivarius</i>
	Staphylococcus	<i>S. aureus</i> , <i>Staphylococcus epidermidis</i>
	Enterococcus	<i>Enterococcus faecalis</i>
	Lactobacillus	<i>Lactobacillus casei</i> , <i>Lactobacillus fermentum</i>
	Corynebacterium	<i>Corynebacterium matruchotii</i>
Obligate anaerobic	Actinomyces	<i>Actinomyces naeslundii</i> , <i>A. israelii</i> , <i>A. viscosus</i>
	Bacillus	<i>B. cereus</i>
	Propionibacterium	<i>Propionibacterium acnes</i>
	Peptostreptococcus	<i>P. micros</i> , <i>Peptostreptococcus anaerobius</i>
	Campylobacter	<i>C. rectus</i> , <i>Campylobacter concisus</i> , <i>C. gracilis</i>
Gram-negative Aerobic or facultative	Actinobacillus	<i>A. actinomycetemcomitans</i>
Obligate anaerobic	Fusobacterium	<i>Fusobacterium nucleatum</i>
	Porphyromonas	<i>Porphyromonas gingivalis</i>
	Prevotella	<i>Prevotella melaninogenica</i> , <i>P. oralis</i> , <i>P. intermedia</i>

Adapted from How KY, Song KP and Chan KG (2016) *Porphyromonas gingivalis*: An overview of periodontopathic pathogen below the gum line. *Frontiers in Microbiology* 7: 53.

the female reproductive tract (Rossoni et al., 2018). The *Candida* spp. form biofilms on the surface of the oral cavity using yeast and hyphae of which, during dysbiosis leading to disease, cause oral pseudomembranous candidiasis—known as oral thrush. Treatment with antifungals such as clotrimazole are given for 7–14 days. Commensal *Lactobacillus* strains such as *Lactobacillus fermentum* have been shown to decrease *C. albicans* infections in healthy individuals by disrupting biofilm and hyphal formation, which further highlights the benefits of commensal flora (Rossoni et al., 2018). Other genera found in the oral mycobiota include *Penicillium*, *Aspergillus*, *Geotrichum*, *Hemispora*, *Fusarium* and more, however there is little wider literature currently to explain the roles of each within the oral mycobiome and their relation to disease.

The normal microbiota of the gastrointestinal tract

Stomach

Conditions within the stomach are highly acidic, therefore only microbes evolved to withstand these acidic conditions may survive. Consequently, the stomach typically hosts fewer commensals than other locations along the gastrointestinal tract (Ohno and Satoh-Takayama, 2020). The most influential bacterium within stomach microflora is *Helicobacter pylori* (*H. pylori*) of the Proteobacteria phyla. Proteobacteria, along with Actinobacteria, Bacteroidetes, Firmicutes, and Fusobacteria are common commensals found within gastric fluid. The colonization of commensal *H. pylori* promotes the growth of other commensal bacteria such as *Streptococcus* species (including *Streptococcus pneumoniae*, *S. thermophilus*, *S. pyogenes*, *Streptococcus parasanguinis*, *Streptococcus dysgalactiae*, *S. salivarius*, *Streptococcus suis*, *S. mutans*, and *Streptococcus agalactiae*) which maintain gut biosis (Boer et al., 2019). However, upon dysbiosis *H. pylori* may become pathogenic, reducing microflora diversity and leading to the development of stomach ulcers (Narayanan et al., 2018; von Rosenvinge et al., 2013; Jandhyala et al., 2015). Fungal *Candida* and *Phialemonium* colonies may also be found in gastric fluid. Interestingly, increased colonization of *C. albicans* within the gut has been found to exacerbate autistic behaviors. This demonstrates the influence of the microbiome on the brain (Clapp et al., 2017; Sam et al., 2017).

Duodenum

The luminal flora of the duodenum consists mainly of *Lactobacillus*, Enterococci and Bifidobacteria, with a total microbe count of 10^3 mL/luminal contents (Angelakis et al., 2015). Lactic acid bacteria produce a range of metabolites that aid the host, the most useful being Vitamin B, serotonin and (LeBlanc et al., 2013; Harper et al., 2018; Visconti et al., 2019). *Lactobacillus* populations make up 6% of total bacteria within the duodenum, however, their densities fluctuate with certain disease states. For example, colony numbers increase in cases of Rheumatoid arthritis, but decrease in cases of HIV (Heeney et al., 2018). Commensal viruses, such as the double-stranded DNA species Caudovirales, may also be found in the duodenum. Enteric viruses influence the host mucosal immune response and play an important role in the modulation of intestinal intraepithelial lymphocytes. Not only this, but they also act as indicators of disease. For example, a reduction in viral diversity is linked to irritable bowel disease (Liu et al., 2019). Moving through the intestine to the jejunum, there are high levels of *Streptococcus* and *Bacteroides* colonies. *Bacteroides thetaiotaomicron* commensals are beneficial to food digestion within the gut, for example by helping with carbohydrate metabolism. By-products from this process are then counteracted by *Lactobacillus* species; this symbiosis between commensal microbes is essential for the maintenance of healthy gut flora (Jandhyala et al., 2015). Table 2 presents the variety of bacterial flora found along the GI tract (Sekirov et al., 2010).

Table 2 Common commensal bacteria found along the GI tract.

GI Tract Location	pH	Bacteria per gram contents	Common Commensal Bacteria
Stomach	2	10^1	<i>Streptococcus</i> , <i>Lactobacillus</i> , <i>Prevotella</i> , <i>Enterococcus</i> , <i>Helicobacter pylori</i>
Small intestine	5–7	10^3 – 10^7	<i>Bacteroides</i> , <i>Clostridium</i> , <i>Streptococcus</i> , <i>Lactobacillus</i> , <i>Proteobacteria</i> , <i>Enterococcus</i>
Caecum	5.7	10^7 – 10^{11}	<i>Lachnospira</i> , <i>Roseburia</i> , <i>Butyrivibrio</i> , <i>Ruminococcus</i> , <i>Faecalibacterium</i> , <i>Fusobacteria</i>
Colon	5–5.7	10^{12}	<i>Bacteroides</i> , <i>Clostridium</i> , <i>Prevotella</i> , <i>Porphyromonas</i> , <i>Eubacterium</i> , <i>Ruminococcus</i> , <i>Streptococcus</i> , <i>Enterobacterium</i> , <i>Enterococcus</i> , <i>Lactobacillus</i> , <i>Peptostreptococcus</i> , <i>Fusobacteria</i>

Adapted from Jandhyala SM, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M and Nageshwar Reddy D (2015) Role of the normal gut microbiota. *World Journal of Gastroenterology* **21**(29): 8787–8803; Sekirov I, Russell SL, Antunes LC and Finlay BB (2010) Gut microbiota in health and disease. *Physiological Reviews* **90**(3): 859–904.

Caecum

The caecum is the location between the small and large intestines; therefore, the caecal microbiome is affected by the fluids and microbes that pass through from the upper gastrointestinal tract (Marteau et al., 2001). The bacterial density of the caecum averages at of 10^8 CFU, consisting of a variety of bacterial genera (Marteau et al., 2001; Jandhyala et al., 2015). Approximately 50% of commensal bacteria within the caecum are facultative anaerobes such as Lactobacilli and Enterococci. However, as the caecum develops into the colon, the decrease in oxygen concentrations leads to an increase in the density of anaerobic bacteria, such as *Clostridioides difficile* (*C. difficile*) (Marteau et al., 2001; Jandhyala et al., 2015). Although harmless under normal physiology, *C. difficile* can proliferate during a course of antibiotics and lead to *C. difficile* colitis (Quigley, 2013). Other potentially pathogenic commensals include *Campylobacter jejuni* and *Salmonella enterica*, of which don't become pathogenic until dysbiosis occurs. Fortunately, many beneficial commensals colonize too, including Lactobacillus and Bifidobacterium. For example, *Lactobacillus acidophilus* and *Bifidobacterium animalis* contribute to the alleviation of irritable bowel symptoms through fermentation of non-digestible prebiotics such as inulin, beta-glucans and fructo-oligosaccharides which produce short chain fatty acids (SCFAs) that help to boost the numbers of these beneficial lactic acid bacteria and bifidobacteria (Quigley, 2013; Jandhyala et al., 2015; Harper et al., 2018). Thus the presence of Bifidobacteria colonies also has been shown to improve mood in patients with chronic stress, another example of how gut microflora can affect many bodily functions (Clapp et al., 2017). Furthermore, many commensals take advantage of host tissue functions. Within the colon, commensal bacteria reside within the outer mucus layer. Every hour, crypt goblet cells secrete mucin monosaccharides to renew the inner mucus layer, of which is then converted to the outer mucus layer. The bacteria then metabolize these mucin secretions into SCFAs, harboring a continuous supply of energy (Hansson, 2012; Johansson et al., 2013).

The normal microflora of the respiratory tract

The human respiratory tract is composed of mucous membranes with two parts- the upper respiratory tract and the lower respiratory tract. The upper respiratory tract comprises the nasopharynx, oral cavity, larynx and pharynx. Microorganisms usually reside in areas where there are moist mucosal membranes and there are differences in the microbiome of each part of the respiratory tract (Unger and Bogaert, 2017). Microbes enter the upper respiratory tract during breathing and are usually trapped in the nasal passages and expelled via nasal secretions. There are some microbes however that do colonize respiratory mucosa and nasopharynx. These species include bacilli, staphylococci, streptococci, and other Gram negative species such as *Pseudomonas aeruginosa* or *Haemophilus influenzae* (Fothergill et al., 2014; Mitchell and Glanville, 2018; Watson et al., 2019). The staphylococci and streptococci residing in nasopharynx of healthy individuals remain a part of the microbiota without causing disease; likely due to the competition of nutrient resources and space from other resident microorganisms previously described. These individuals, however, are carriers of these opportunistic pathogens (Madigan et al., 2008).

The lower respiratory tract comprises the trachea, bronchi and lungs have been traditionally considered as naturally sterile sites in adults. However, recent advances in culture-independent metagenomic analyses suggest there may be commensal microbiota in healthy lung environments (Moffatt and Cookson, 2017; Carney et al., 2020; Sulaiman et al., 2020). Microorganisms and dust particles can be inhaled into the upper respiratory tract, landing on the ciliated lung epithelial cells; however, these are usually pushed up toward the upper respiratory tract where they are expelled in nasal secretions, saliva and mucus (Lanaspa et al., 2017). Few pathogens manage to reach the lungs and can cause diseases such as pneumonia. Thus, due to the inhalation process, the lungs are in contact with a range of microorganisms which have colonized the lung mucosa, some of which include the opportunistic pathogens *Pseudomonas* and *Staphylococcus* species (Blainey et al., 2012; Sommariva et al., 2020). The most common phyla include Proteobacteria, Firmicutes and Bacteroidetes of which dominant species include Prevotella, Pseudomonas, Streptococcus, Staphylococcus and Veillonella (Hilty et al., 2010; Erb-Downward et al., 2011; Beck et al., 2012; Fig. 2).

The normal microflora of the urogenital tract

The Urogenital tract in male and females comprises the urethra and bladder, and in females, the vagina. The bladder itself is another sterile site where there are no commensals. However, the urethra, downstream from the bladder, often has its own microbiota which reside on the uroepithelial cells. The gastrointestinal microflora is thought to play a significant role in urological microbiota due to the ability of bacterial population to colonize the urethra. Bacterial pathogens such as *E. coli*, *Staphylococcus saprophyticus*, *Enterococcus faecalis* and *Proteus mirabilis* are common pathogens of the urethra, often causing urinary tract infections (Ravel et al., 2011).

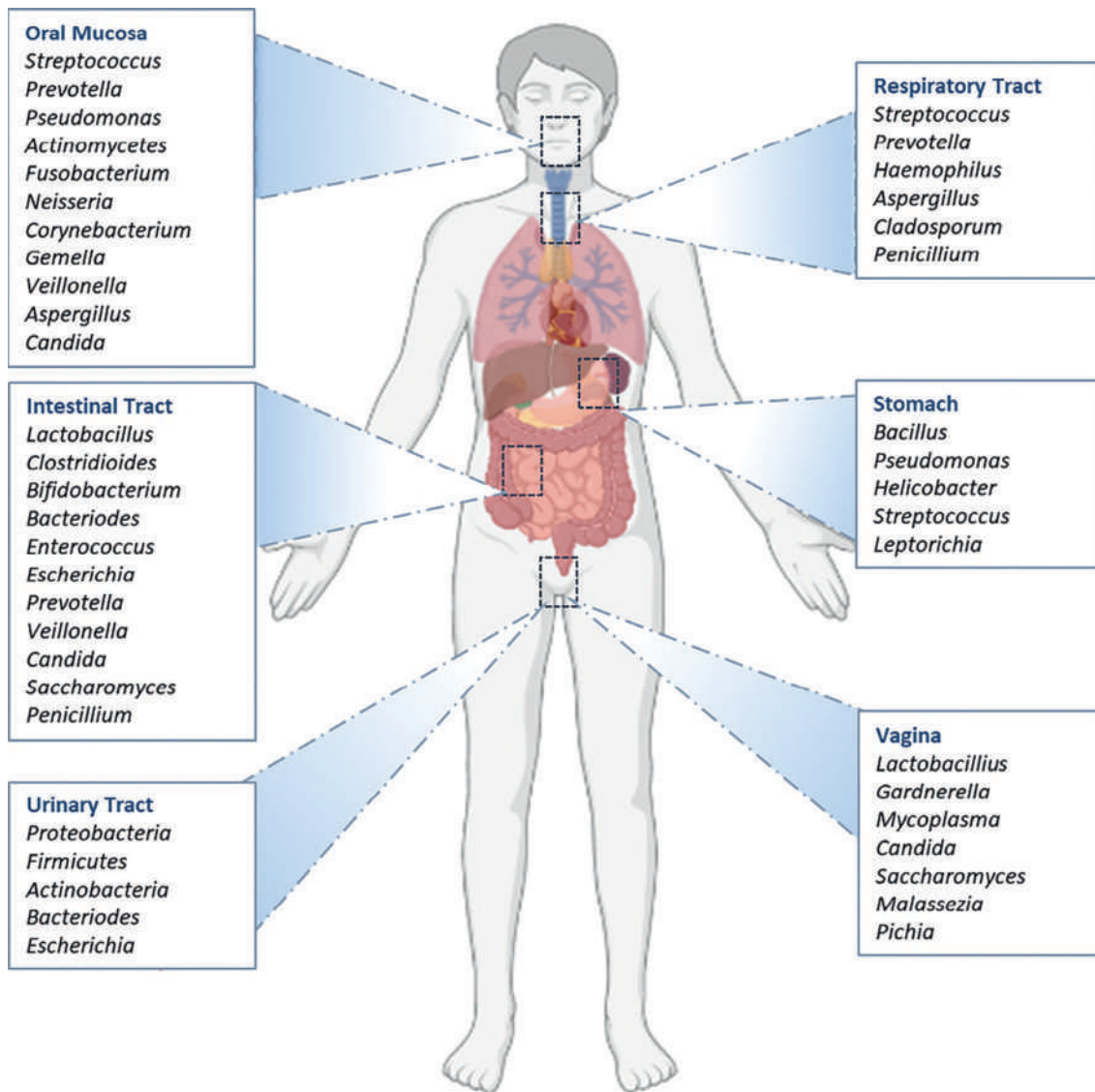


Fig. 2 Commensal Microbiome of the Human Body. The human body harbors microorganisms of different Kingdoms. The main phyla and species are shown above. Created using Biorender (2021).

The vagina microflora in adult females is complex. The vagina has a pH of between 3.5 and 4.5 and contains glycogen which is fermented upon by commensals of the *Lactobacillus* genus (Boskey et al., 1999). The lactic acid produced from this fermentation maintains the acidic environment, resulting in a homogenous environment for lactobacilli (such as *L. acidophilus*) to survive. Changes in this balance often result in increased risk of pathogenic infections (Fettweis et al., 2019). Yeasts such as *C. albicans* are also part of the normal vaginal microflora. Interestingly, other bacteria such as streptococci and *E. coli* can also be found in the vaginal microbiome.

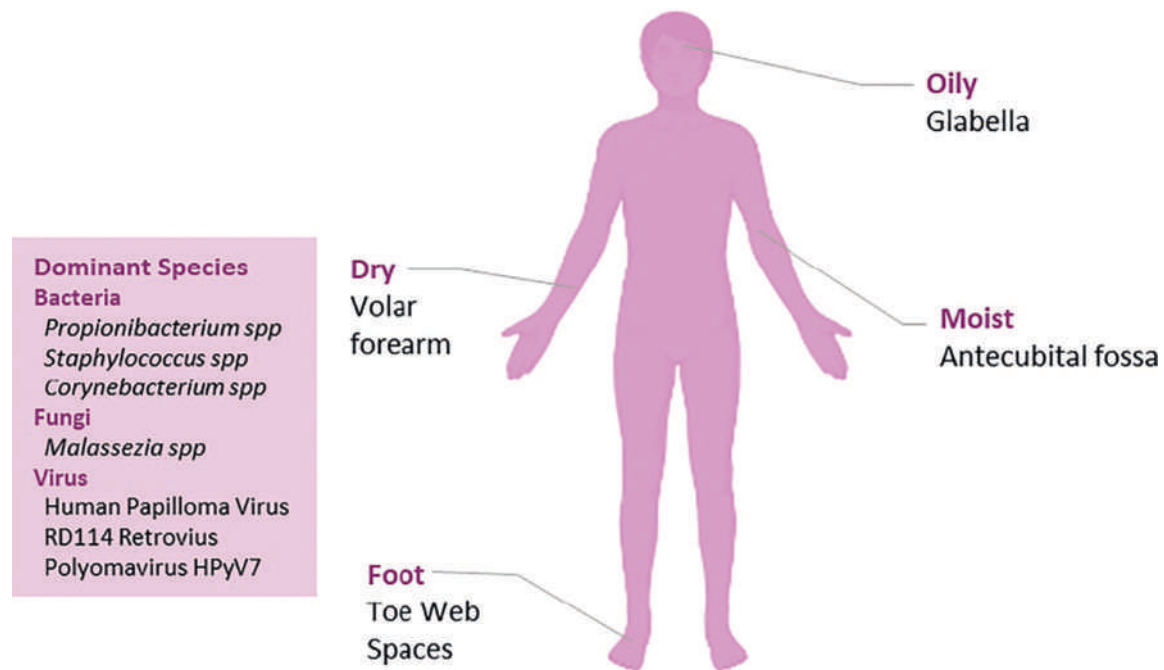


Fig. 3 Physical characteristics of the Human Body. Microbial communities on the skin are based on the individuals physiological characteristics. These are dependent on whether the area is oily, moist or dry. Adapted from Byrd AL, Belkaid Y and Segre JA (2018) The human skin microbiome. *Nature Reviews Microbiology* 16(3): 143; Created using Biorender (2021).

Conclusions

The normal microflora of humans is complex and has relied on using culture based methods to determine its composition. With the wider use of metagenomic analysis, scientists are now able to examine non-culturable microorganisms in the microbiome, building up a more comprehensive picture of microorganisms from sterile and non-sterile sites in/on the human body.

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Microbial Diversity and Classification

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Glossary

Amplicon The source or product of an amplification event, typically the product from a polymerase chain reaction.

Atopy The genetic tendency to develop allergic diseases.

Carcinogenic Having the potential to cause cancer.

Dysbiosis The imbalance between organisms found within the human microbiome.

Endodontic Relating to the soft tissues within the tooth, such as dental pulp.

Gastric Referring to the stomach.

Germ-free mice These are a subsection of gnotobiotic mice which are free of all micro-organisms.

Gnotobiotic mice Mice with only defined micro-organisms present.

Lamina propria A type of connective tissue found under the thin layer of tissues covering a mucous membrane.

Metabolome Large scale analysis of small molecules, metabolites, directly isolated from environmental or clinical samples.

Metagenomics The sequencing and analysis of genetic/genomic material extracted directly from environmental or clinical samples.

Microbiome The combined genetic material of the micro-organisms within a particular environment.

Microbiota The micro-organisms present at a particular site or habitat.

Neonates Referring to a new-born child, typically less than four weeks of age.

Operational Taxonomic Units Clustering of sequences based on similarity, typically with a similarity threshold of 97%.

Papillae Small rounded projections on part of the body.

Perinatal Covers the time a woman becomes pregnant up to one year after birth.

Periodontitis A microbial related inflammatory disease of the supporting tissues of teeth, resulting in the progressive destruction of the periodontal ligament and alveolar bone with periodontal pocket formation or gingival recession.

Virome The metagenomic sequencing of viral nucleic acids isolated directly from an environmental or clinical sample.

Introduction

Traditional classification of micro-organisms, predominantly bacteria, was borne out from the first observations of micro-organisms by Van Leeuwenhoek and focused on their corresponding morphology and physiology (Porter, 1976). From the 1960's onwards classification has alternatively centered on DNA-DNA hybridization (DDH), with both phenotypic characteristics and a high DDH value (>70%) required to class two bacteria as the same species (Stackebrandt and Goebel, 1994). The advent of high-throughput genome sequencing has allowed another method of classification based on average genome nucleotide identity (ANI). Both DNA methods of classification are used to determine taxonomy on a phylogenetic format, with organisms grouped on their evolutionary relationship. Subjectivity can arise when classifying microorganisms regarding the scientific standpoint, for example level of pathogenicity (De La Cal et al., 2012). In addition to the subjectivity used to classify micro-organisms, a further complication arises from the horizontal gene transfer between micro-organisms which cannot be easily mapped in a phylogenetic format. In 2015 an alternative method for the comparison of micro-organisms based on molecular functionality was proposed (Zhu et al., 2015). The alteration to the classification of micro-organisms over time has resulted in the re-classification of many microbial species (Burroughs and Brown, 1974; Thynne et al., 2015; Tran et al., 2017; Li et al., 2018).

Within recent years there has been increasing interest and publicity on the human microbiome both within literature and in the mainstream media, with many commercial products now marketed as supporting the health of the 'microbiome.' The human microbiome is composed of the genomes of symbiotic micro-organisms living inside and on humans including bacteria, archaea, eukaryotes (i.e. fungi and protozoans), skin mites and viruses (Balato et al., 2019), with bacteria often deemed the most predominant. These micro-organisms are located on a variety of locations within the human body with external access such as skin, gastrointestinal tract, urogenital tract, and respiratory tract. Furthermore, these micro-organisms are known to provide traits which humans did not evolve on their own (Gill et al., 2006).

Cell for cell, bacteria have long been described as significantly outnumbering human cells with a ratio of 10: 1 often quoted from the work by Thomas Luckey in 1972 (Luckey, 1972). This statement has however been brought into question and in 2016 Sender et al. reviewed a wide range of experimental data and estimated that within a 70 kg reference man there would be 3.8×10^{13} bacterial cells, corresponding to a mere 0.2 kg, with a comparable order of human cells at 3.0×10^{13} (Sender et al., 2016). This suggests that the actual ratio of bacterial to human cells is far closer to a 1:1 ratio. With bacteria only one component within the human microbiome, it is possible that when taken in its entirety (including fungi, archaea, protozoa and viruses) in terms of absolute numbers the total microbiome would indeed be far greater than cells of human origin.

Determination of diversity within the human microbiome

Traditionally, studying the microbiome has been reliant on the presence of cultivable microbes using techniques such as culture, PCR, or gel-based methods. Advances in culture-independent techniques and metagenomic sequencing analysis have aided the breadth of micro-organisms identified. Despite these advances there continues to be a dearth of information regarding non-bacterial species (Andersen et al., 2013; Amato et al., 2019) as well as a bias for known bacterial species (Jenkins et al., 2018; Watson et al., 2019). The following section discusses the methodologies used to determine microbial diversity and the corresponding advantages and disadvantages of each (Table 1), as well as the importance in study design and models for human microbiome research.

Methodologies

Historically, the identification of bacterial taxa related to the human microbiome would have involved culture-based methodologies (Rodríguez-Valera, 2002; Schleifer, 2004). This could involve differential growth on selective media (carbon source utilization) or growth under selective conditions (presence of antibiotics). Despite widespread use across literature there are many drawbacks to this approach. The key problem is that the majority of bacteria are deemed 'unculturable' (Stewart, 2012) and these methods for example can only identify around 50% of the estimated 700 bacterial species within the oral microbiome (Zhao et al., 2017). This problem is exacerbated by the use of selective medias which can bias towards the growth of particular strains (Gorski, 2012). This can result in the exclusion of slow-growing species and preference species which would ordinarily be at low abundance within the sample.

Over the years, researchers have moved towards more DNA-based methods for determining microbial diversity within the microbiome. This has included the use of gel-based techniques, PCR methods, DNA micro-arrays and nucleotide sequencing (Deo and Deshmukh, 2019). Gel-based methods include Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE), which are inexpensive methods, sensitive to one base pair difference and can be performed rapidly (Miller et al., 1999; Liu et al., 2006). Polymerase Chain Reaction (PCR) methods include Restriction Fragment Length Polymorphisms (RFLP), Real-Time PCR, and Random Amplified Polymorphic DNA (RAPD) (Nocker et al., 2007). These methods typically show high sensitivity and are fast but can introduce PCR bias potentially missing the species at lower abundance (Gonzalez et al., 2012). Phylogenetic DNA micro-arrays can prove valuable for high-throughput, systematic, quantitative analysis of bacterial communities within different microbial ecosystems (Parolin et al., 2017).

Table 1 The advantages and disadvantages for different methods for studying the microbiome (Everett et al., 2010; Green et al., 2015; Escobar-Zepeda et al., 2015; Environmental Chemicals, the Human Microbiome, and Health Risk: A Research Strategy, 2018).

<i>Method</i>	<i>Advantages</i>	<i>Disadvantages</i>
<i>Culture- based methods</i>	<i>Selective for viable micro-organisms</i>	<i>Bias for easily cultured micro-organisms</i>
<i>DNA-based methods</i>	<i>High sensitivity and reproducible</i>	<i>Bias towards most abundant species</i>
TGGE/DGGE analysis		
DNA microarrays	Reproducible, high sensitivity and rapid	PCR bias Co-migration of DNA molecules
Nucleotide sequencing:	High capacity Offers phylogenetic analysis of microbial populations	Complexity in design and time required for analysis Offers no differentiation between viable and non-viable micro-organisms
Amplicon sequencing	High sensitivity Identification from mixed samples (containing human and other non-microbial contamination) Readily used and reasonably inexpensive	Only generates genus level phylogenetic profiles High reliance on method for nucleotide extraction Only really used for bacterial analysis and to a much lesser extent Eukaryotic microbes
Metagenomics	Resolves species and strain level phylogenetic profiles Provides genome content and functional potential based on metabolic gene analysis	Expensive technique Lower tolerance of non-microbial contaminants within samples Requires complex and computationally expensive analytic approach
Culture-independent molecular profiling	Allows identification of molecules/proteins key to micro-organism to micro-organism or micro-organism to host signalling	Samples specifically prepared for relevant target molecules, preventing analysis of multiple molecules simultaneously Potentially poor differentiation of human or microbial derived molecules
Metabolomic		Sensitivity varies dependent on precise method used
Metaproteomic	Can identify key secreted and intracellular microbial products Can improve understanding of relevant proteins and post-translational modifications accordingly	
Direct Observations	Provides detailed spatially relevant information regarding host-microbe interaction Can provide in situ information	Technically challenging to conduct and verify Less depth in taxonomic results
Electron microscopy	TEM/SEM allow direct visualization of microbial community within fixed sample	No identification of microbial taxa or traits
FISH	Evaluates taxonomy, location, and organization of microbial communities within fixed sample Offers insight into spatial distribution of bacteria Allows quantification and sorting of microbes from microbiome sample (following dissociation of microbial community	Lower resolution, particularly in host tissues with high levels of opacity
FACS		

More recent studies have utilized nucleotide sequencing to characterize microbial populations within the human microbiome (Willing et al., 2010; Brubaker and Wolfe, 2015; DiGiulio et al., 2015). This can be broadly split into amplicon sequencing and metagenomics, with both methods utilizing either Sanger sequencing (Franc et al., 2002) or next generation sequencing (Goodwin et al., 2016). Amplicon sequencing is a method which utilizes the amplification of a specific gene which is deemed unique to the micro-organism, typically at the genus level (Escobar-Zepeda et al., 2015). Typically ribosomal ribonucleic acids (rRNA) genes are utilized for amplicon sequencing, 16S rRNA for bacteria and 18S rRNA for eukaryotes, although the internal transcribed spacer (ITS) is also used for eukaryotic microbes (Hamady and Knight, 2009; Findley et al., 2013). Amplicon sequencing is one of the most prevalent methods used in microbiome research, however some caution is required with this approach as *Escherichia* spp. and *Shigella* spp. were found to have identical sequence along all of the 16S ribosomal RNA (rRNA) genes (Zuo et al., 2013). Furthermore, nucleotide extraction is of key importance for full utilization of this method, with some techniques destroying subsets of micro-organisms (Gohl et al., 2016; Environmental Chemicals, the Human Microbiome, and Health Risk: A Research Strategy, 2018). Advances in next generation sequencing have led to a rise in the use of whole-genome shotgun sequencing/metagenomics in studying the human microbiome (Venter et al., 1998). This method involves the random shearing of DNA by a shotgun method and can result in the complete profiling of gene content within the sample (Venter et al., 1998). Providing information on metabolic pathways and functional profiles of the microbial communities, as well as removing bias that can be observed with amplicon sequencing. This methodology has yet to fully replace amplicon sequencing due to the associated costs and the increased complexity in detailed analysis of results (Environmental Chemicals, the Human Microbiome, and Health Risk: A Research Strategy, 2018).

Both amplicon sequencing and metagenomics produce valuable information regarding the microbial composition and diversity within a sample, however they do not differentiate between viable and non-viable cells.

Methods to study viable microbial communities within the human microbiome include direct observation methods such as electron microscopy (Croucher et al., 1983), fluorescent *in situ* hybridization (FISH) (Franks et al., 1998), and cytometric microbiome profiling (utilizing flow cytometry and sequencing) (Vandeputte et al., 2017). These analyze the microbiome *in situ* and offer insights into spatial distribution, however this can be limited by the behavior of microbes within their environment and the opacity of the host tissue (Wiles et al., 2006). Viability tests including growth studies (based on optical density), colony structure or direct enzymatic screens, can be used to support *in situ* analysis however these can bias towards easily cultured micro-organisms (Environmental Chemicals, the Human Microbiome, and Health Risk: A Research Strategy, 2018).

Culture-independent molecular profiling using metabolomics or meta-proteomics, via nuclear magnetic resonance (NMR) or mass spectrometry techniques, provides information on host: micro-organism or micro-organism: micro-organism interactions (Zhang et al., 2019). Varying sensitivity levels and the specific requirements for monitoring target molecules, however, prevents simultaneous analysis from single sample collection (Environmental Chemicals, the Human Microbiome, and Health Risk: A Research Strategy, 2018).

Sampling

The type and methods of sample collection for studying the human microbiome can have a substantial impact on the quantity and quality of data obtained. Sampling methods differ depending on the body site of interest and range from swabbing the area of interest to collection of stool samples (Aagaard et al., 2013). Different sampling methods for the same area can result in alterations in observed microbial composition. The plethora of micro-environments within the oral cavity has resulted in a variation in findings between individual studies dependent on sampling methods, for example site-specific sampling (sub-lingual plaque) (Aas et al., 2007) versus global sampling (oral rinses) (Ghannoum et al., 2010). Another consideration with regards to sampling is the methods of storage prior to analysis. Temperature, length of storage, and hypoxic conditions all contribute to the observed microbial composition (Jenkins et al., 2018), with repeated freeze-thaw cycles also shown to alter the microbial composition (Sergeant et al., 2012).

Study design

A large proportion of microbiome studies utilize animal models, particularly rodents, as they are effective models and their environment can be tightly controlled. The way the animals are born and raised has a large impact on the microbiome studies, with the maternal effect (inoculation of rodents at birth) as well as individual diet and co-habitants factoring into the diversity and composition of the microbiome (Ley et al., 2005; Rawls et al., 2006). The use of germ-free mice can circumvent the maternal effect by allowing the introduction of identical pre-determined inoculum to mice of different genetic make-up. Environmental factors can also alter the results of microbiome studies, with mice obtained from different vendors found to contain varying levels of segmented filamentous bacteria within their gut microbiome (Ivanov et al., 2009).

Alternatively, human samples can be used in microbiome studies with the main advantage that these studies directly relate to the human microbiome. Controlling the environment is far harder in these studies as there are many factors which can influence the microbiome including age, pregnancy, diet, and the use of antibiotics (Goodrich et al., 2014). Correspondingly, many will involve the use of exclusion criteria when recruiting individuals. Further problems can involve the identification of effective controls, although temporal studies can establish a control by utilizing an initial baseline for the individual. Comparison of human microbiome studies would be greatly enhanced by the introduction of standardization of protocols and study design, with recent literature suggesting ways of achieving this (Goodrich et al., 2014; Poussin et al., 2018; Greathouse et al., 2019).

The Human Microbiome Project

In 2008 the NIH Common Fund Human Microbiome Project (HMP) was established, with the aim of creating resources to allow the comprehensive understanding of the human microbiome and its role in health and disease. This project has resulted in the sequencing of >2200 reference strains isolated from five body sites (Table 2) of 300 healthy adults between 18–40 years old and a plethora of publications (Gevers et al., 2012; Huttenhower et al., 2012; Ward et al., 2012; Proctor et al., 2019). It is a consortium which studies many clinically relevant body-sites with paired 16S profiling and deep metagenomic sequencing coverage using a large cohort of participants to accommodate for the high intra- and inter-personal variation observed within the human microbiome (Gevers et al., 2012). As multiple sequencing centers were used over a prolonged duration for the HMP, effort was taken to standardize and benchmark protocols for sample collection (Huttenhower et al., 2012), handling and 16S profiling (Ward et al., 2012).

Table 2 Specimens taken from the five major body sites for the Human Microbiome Project, with 18 samples taken for women and 15 for men (minus three vaginal samples) (Huttenhower et al., 2012).

Body site	Specimens taken
Oral Cavity and Oropharynx	Saliva
	Buccal mucosa (cheek)
	Keratinized gingiva (gums)
	Palate
	Tonsils
	Throat and tongue soft tissue
	Supra and Subgingival dental plaque (tooth biofilm above and below the gum)
Nasal cavity	Anterior nares (nostrils)
Skin	Retro auricular creases (behind each ear)
	Antecubital fossae (inner elbows)
Gastrointestinal tract	Self-collected stool sample
Urogenital tract	Vaginal introitus
	Vaginal midpoint
	Posterior fornix

Diversity across the microbiome

Microbial diversity across body sites

The majority of microbiome studies, including the Human Microbiome Project, have focused on five major body sites (Table 2). The level of diversity observed across different body sites is variable, with variation observed at an inter-personal level (between subjects), intra-personal level (between body sites of the same subject) as well as temporally (within a single body site) (Costello et al., 2009; Grice et al., 2009; Turnbaugh et al., 2009a). In this section what is known about the diversity in specific body sites and how these are known to be affected between individuals is discussed.

Oral cavity

In 2019 the oral cavity was described as second only to stool samples regarding microbial richness determined by operational taxonomic units (Deo and Deshmukh, 2019). The oral cavity and associated nasopharyngeal areas are well suited to microbial growth due to consistent temperature, neutral pH (6.5–7) and high levels of hydration (Lim et al., 2017). In addition, it is a complex body site because of the number of individual microenvironments located within it including the subgingival dental plaque, supragingival dental plaque, keratinized gingiva (gums), palate, buccal mucosa (cheek), tonsils and throat (Gao et al., 2018). Research into the oral microbiome is supported by both the human oral microbiome database (Chen et al., 2010), which contains approximately 700 culturable prokaryotic species analyzed by 16S rRNA sequencing, and the Human Microbiome Project (Huttenhower et al., 2012).

The micro-environments found within the oral cavity provide niche environments for different types of bacteria, with microbial diversity linked to the micro-environments. High microbial diversity was observed on the tongue linked to numerous papillae with few anaerobic sites, whereas conversely the buccal and palatal mucosae were found to be low in microbial diversity (Sultan et al., 2018). Despite the association between microbial diversity and specific oral micro-environments, *Streptococcus* spp. were still shown to dominate at most micro-environments (Chen et al., 2010; Huttenhower et al., 2012). Composition of bacterial communities does however vary between the oral micro-environments showing higher prevalence of genera at individual sites, with a higher abundance of *Hemophilus* spp. at the Buccal mucosa, *Actinomyces* spp. at supragingival plaque and *Prevotella* spp. at the subgingival plaque (Huttenhower et al., 2012; Gao et al., 2018).

High inter-personal variation within the oral microbiome relating to carriage of taxa, species and strain level has been noted, despite observed conservation across metabolic pathways within a healthy population (Ahn et al., 2011). One study also found the conservation of fifteen bacterial genera within the healthy oral microbiome across ten different individuals including conservation of the following species: *Streptococcus oralis*, *Hemophilus parainfluenzae*, *Actinomyces odontolyticus*, *Prevotella melaninogenica* and *Campylobacter gingivalis* (Bik et al., 2010).

Studies into the difference between the oral microbiome and pathogens of modern populations versus ancient population, suggest that microbial diversity is comparatively decreased in modern populations with the suggestion that this could relate to the chronic oral disease observed in post-industrial lifestyles (Ursell et al., 2012; Adler et al., 2013; Metcalf et al., 2014). This observation is in part put down to alterations to diet and a further study into the mobile genetic elements from oral samples of North Americans compared to Fijians showed high variation (Brito et al., 2016) suggesting diet and environmental factors are key.

One study into the oral fungal microbiome found 74 culturable and 11 unculturable fungal genera (Ghannoum et al., 2010). *Candida* spp. were the most dominant, present in 75% of samples, with other prevalent genera including *Cladosporium*,

Aureobasidium, *Saccharomycetales*, *Aspergillus*, *Fusarium* and *Cryptococcus* (Ghannoum et al., 2010). These findings share some overlap with previous studies on the oral fungal microbiome (Salonen et al., 2000; Jabra-Rizk et al., 2001), with differences likely relating to alternative sequencing techniques or sample size. The identification of environmentally sourced fungal species within the oral microbiome is hypothesized to link to inhalation/consumption of spores from the environment (Ghannoum et al., 2010). Just as bacterial commensals within the oral cavity can offer protection against pathogenic bacteria, it is thought that a similar protective effect could be observed with fungal species.

Skin

The greatest level of bacterial diversity is observed within the skin microbiome, in particular at the palms, fingers and forearms with these sites showing higher levels of diversity than those observed within the gut or oral cavity (Costello et al., 2009), although more recent studies have brought this into question (Deo and Deshmukh, 2019). One of the reasons for the high bacterial diversity is that the skin microbiome is consistently altered by both lifestyle and environmental factors (Ursell et al., 2012; Prescott et al., 2017). There are still, however, predominant bacterial species observed, with >90% of the microbiota on the forearm composed of Actinobacteria, Proteobacteria and Firmicutes (Gao et al., 2007). Regardless of this there is very high inter-personal variation, with only 2% of species level operational taxonomic units (OTUs) for the volar forearms shared between individuals (Gao et al., 2007). Furthermore, within the same individual there is only 28% (13.5 out of 48) of species-level OTUs shared between the left and right forearms (Gao et al., 2007), and only 17% between hands of the same individual (Fierer et al., 2008).

One study into the fungal microbiota within the skin, showed that majority of sequences obtained from the forearm resembled *Malassezia* (Paulino et al., 2006). This study was not extensive however due to the low level of 18S rDNA sequences present within GenBank at time of publication. Future advances in sequencing techniques could allow for the better determination of the true breadth of fungal species observed within the natural microbiome.

Gastrointestinal tract

The gastrointestinal tract (GI tract) encompasses microbiota for the mouth (although oral cavity will be treated as a separate body site here), stomach, duodenum, colon, and fecal samples. Due to ease and non-invasive nature of sampling, the majority of studies look at fecal samples when establishing microbiome data (Arumugam et al., 2011; Le Chatelier et al., 2013; Ren et al., 2019).

Studies looking into the observed diversity across the gastrointestinal tract, showed highest diversity within the oral cavity and lowest diversity within the stomach but importantly demonstrated an observable gradient of increasing diversity moving from the stomach, to the duodenum, to the colon through to the fecal samples (Stearns et al., 2011). With greatest clustering of bacterial species between variant sites rather than between individuals or across sexes (Stearns et al., 2011). Further research has supported the idea of a gradient across the GI tract with Enterobacteriaceae observed with greater frequency towards the distal end of the GI tract and *Streptococcus* spp., *Enterococcus* spp., and *Corynebacterium* spp. showing greater abundance at the proximal end of the GI tract (De Cárcer et al., 2011). Gut microbiota have been classed as one of three enterotypes based on the dominance of either *Bacteroidetes*, *Prevotella* or *Ruminococcus* however these enterotypes are better described as microbial gradients rather than discrete communities (Arumugam et al., 2011).

The prevalence of these bacterial groups within the gut microbiome has been shown to be heavily dependent on diet, with *Bacteroidetes* commonly associated with long-term diets high in protein and animal fat and *Prevotella* conversely associated with long-term diets high in carbohydrates (Wu et al., 2011). It has been suggested that the gut microbiota has co-evolved with mammals to suit their respective diets resulting in more specialized communities for herbivores, carnivores or omnivores (Ley et al., 2008; Muegge et al., 2011). Both studies using mono/dizygotic twins and gnotobiotic mice have been used to study the impact of diet on a genetically similar background. The GI tract microbiome has been linked to the weight of individuals, with obese individuals shown to have lower diversity compared to those of lean weight (Turnbaugh et al., 2009a). This has been further typified by the ratio of *Bacteroidetes*: *Firmicutes* within the gut microbiota, with *Bacteroidetes* levels increasing within the ratio in people consuming fat-restricted or carbohydrate-restricted diets for one year (Ley et al., 2006). A study looking at this within gnotobiotic mice found that mice colonized by obese microbiota could have their microbiome rescued by the invasion of a particular *Bacteroidetes* species from lean microbiota when co-housed (Ridaura et al., 2013). It is important to note, however, that the observed effect resulting from fecal transplant was completely overridden by diet of the mice.

With diet such a key determinant in the diversity in the GI Tract it is of little surprise that the diversity of the gut microbiota varies greatly during the first two years of a human's life. Several studies across the USA and Europe have explored the impact of breastfeeding versus bottle feeding on the baby gut microbiome (Penders et al., 2006; Fallani et al., 2010; Levin et al., 2016; Ho et al., 2018). Human breast milk is known to contain immunoglobulins, fatty acids, hormones and cytokines (Ballard and Morrow, 2013) and in more recent years has been shown to be both prebiotic and probiotic in nature (Moossavi et al., 2018). Consequently, it is now believed that breastmilk can both directly and indirectly impact the gut microbiome of the nursed infant. The microbiota of breastmilk has been shown to have high levels of inter-personal variation with common bacteria including *Streptococcus* spp., *Staphylococcus* spp., *Propionibacteria* spp., lactic acid bacteria and *Bifidobacterium* spp. (Collado et al., 2009; Hunt et al., 2011; Fernández et al., 2013). Studies comparing breastfeeding against formula feeding have consistently shown lower bacterial diversity within breastfed infant's gut microbiota (Bäckhed et al., 2015; Thompson et al., 2015; Rautava, 2016). It is also apparent that breastfeeding is a large predictor of infant gut microbiota (Kim et al., 2019).

Recent data has also suggested a possible link between diversity within the gut microbiome and personality traits (Christian et al., 2015; Kim et al., 2018; Johnson, 2020). Greater microbial diversity within the gut microbiome has been observed in extraverted

individuals both as children (Christian et al., 2015) and adults (Johnson, 2020) compared to introverted individuals. Increased sociability has been linked to an abundance of *Akkermansia*, *Lactococcus* and *Oscillospira* species, and neurotic tendencies (anxiety and stress) have been described as a negative predictor for the abundance of *Corynebacterium* and *Streptococcus* species (Johnson, 2020).

Urogenital tract

The majority of research into the urogenital tract microbiome has been focused on women of reproductive age, predominantly due to the increased burden of urogenital diseases within women (~81% of urinary tract infections occur in women) (Salvatore et al., 2011; MacIntyre et al., 2017). Urogenital tract microbiome research covers three key areas, the lower reproductive tract (vagina and cervix), the upper reproductive tract (uterus) (Moreno and Simon, 2019), and the urinary tract (Burton et al., 2015).

The lower reproductive tract has low microbial diversity and is typically dominated by *Lactobacillus* spp. (Huttenhower et al., 2012). The microbiome of the lower reproductive tract have been classified as one of five identified groups (named community state types (CST) I-V) (Ravel et al., 2011). CST groups I, II, III and V contain high abundances of particular *Lactobacillus* species, with CST I dominated by *L. crispatus*, CST II dominated by *L. gasseri*, CST III dominated by *L. iners* and CST V dominated by *L. jensenii*. The CST group IV however contains low levels of *Lactobacillus* spp. and instead demonstrates a high diversity of facultative/strict anaerobes including, *Prevotella*, *Gardnerella*, *Megasphaera*, *Sneathia*, *Atopobium* species.

The upper reproductive tract was originally thought to be sterile (Ansbacher et al., 1967) but more recent research has implied the presence of a uterine microbiome (MacIntyre et al., 2017). Overall, the uterine microbiome is thought to share certain similarities to that of the lower reproductive tract, however around one fifth of individuals sampled (6 out of 26) showed a marked difference between the two sites (Moreno et al., 2016). The majority of uterine microbiomes can be divided into three classifications: low diversity, *Lactobacillus* spp. dominated profiles or high diversity (MacIntyre et al., 2017).

The urinary tract was also originally thought to be sterile due to the hostile environment created by urine, which is a hypertonic solution of low pH containing high concentrations of urea and other antibacterial compounds, coupled with the regular mechanical voiding and filling (Ipe et al., 2016; Tang, 2017). Microbes that can therefore thrive within the urinary tract require mechanisms to protect them from or negate the harsh conditions. Bacteria associated with the urinary tract microbiome include *Lactobacillus*, *Staphylococcus* and *Gardnerella* species (Wolfe et al., 2012; Pearce et al., 2014; Brubaker and Wolfe, 2015). There are, however, limitations to detecting microbial composition within urine dependent on the sampling method. More recent studies looking at suprapubic aspiration (Wolfe et al., 2012) or transurethral catheterization samples (Hilt et al., 2014; Karstens et al., 2016; Thomas-White et al., 2016) are detecting more complex microbial communities which differ from those seen in either the vagina or gut. As with many other body site microbiomes there is a high degree of inter-personal variation (MacIntyre et al., 2017). The urinary tract microbiome is still believed to be of a lower abundance and less diverse than other body sites (Pearce et al., 2014).

Sex-dependent microbial variation

The diversity and composition of several human microbiota (skin, gut and urogenital) vary between males and females, these differences have been linked to the anatomical and hormonal differences between the two sexes.

Differences between the sexes has been noted within the skin microbiomes (Sanmiguel and Grice, 2015; Ying et al., 2015). One study looking into the similarities of skin microbiomes between sexually active cohabiting couples also identified various micro-organisms which were disproportionately abundant on each biological sex (Ross et al., 2017). Diversity in female skin microbial communities was significantly higher than the male counterparts. Interestingly, the differences between the sexes was so significant for the skin microbial communities on the thighs that they were able to correctly classify the sex from which the sample originated with 100% accuracy (Ross et al., 2017). Microbial communities on the thighs in females contained significantly more Proteobacteria and 11-fold more *Lactobacillus* spp. (commonly found within the vaginal microbiome), whilst males showed a greater abundance of Actinobacteria.

The composition of bacterial species observed within the gut microbiota are significantly different between the sexes. In one study males were seen to cluster together and were enriched for *Faecalibacterium prausnitzii*, *Bifidobacterium* spp., *Bacteroidetes* spp., *Clostridium* spp., *Enterococcus* spp., and *Prevotella* spp. (De Cárcer et al., 2011). Conversely, females showed enrichment of *Streptococcus* spp., *Veillonella* spp., *Mannheimia* spp., and *Ruminococcus* species. With diet deemed the major determinant of the gut microbiome (Conlon and Bird, 2014) these observed differences could have multiple interpretations, from the implication of a basal difference between sexes to highlighting the potential variation between the diets of males compared to females.

With arguably one of the greatest differences in anatomy between the two sexes, it is unsurprising that there are differences in microbial composition of the urinary tract. Regardless, both sexes contain *Lactobacillus* spp., *Streptococcus* spp. and *Corynebacterium* species (Fouts et al., 2012; Nelson et al., 2012; Lewis et al., 2013). Within women of child-bearing age *Lactobacillus* spp. are predominant within the urinary tract microbiome, conversely within men a higher abundance of *Corynebacterium* are observed (Fouts et al., 2012). Differences between the microbial compositions of men and women is also related to the changes in urine composition between the sexes, with women having more citrate and less calcium and oxalate and men containing an increased amount of creatinine (Ipe et al., 2016).

Environmental factors and microbial diversity

The role of the environment in altering the human microbiome has been reviewed (Laursen et al., 2015; Ross et al., 2017), particularly the effect of co-habiting with other humans or pets. The microbial taxa observed within skin, oral and gut microbiomes of co-habiting families have been shown to resemble each other (Song et al., 2013). In 2017 Ross et al. showed that partners of sexually active cohabiting couples could be predicted 86% of the time based on their skin microbiome profiles, with greatest similarity in microbial skin population observed across their feet (Ross et al., 2017). In addition to these observations between co-habiting humans, similar observations have been noted for pet owners. Indoor pets, such as dogs, are known to alter the microbial content within the vacuumed house dust and on surfaces within the household (Sitarik et al., 2018). Research into how pets alter the composition of the gut microbiome in infants, demonstrated an altered gut microbial composition in neonates at one month with an increased abundance of an animal derived *Bifidobacterium pseudolongum* strain (Nermes et al., 2015). There have been other studies investigating the effects on infants at different ages of three, four, nine, and eighteen months which have found enrichment and depletion of various bacterial genera (Azad et al., 2013; Laursen et al., 2015; Tun et al., 2017). Overall, the research suggests that although pets were not associated with overall gut bacterial diversity/richness, they did determine variations in the observed abundance of specific genera. Various studies have looked into the reduction in the development of atopy in children exposed to pets at a young age, the majority of research has generated mixed results with only protection against the development of allergy to the type of pet exposed consistently noted (Wegienka et al., 2010; Kim et al., 2019).

Geography and ethnicity observed microbial diversity

With lifestyle and environmental factors shown to impact the composition and diversity observed within the microbiome (Conlon and Bird, 2014; David et al., 2014; Laursen et al., 2015; Ross et al., 2017), it is perhaps logical that geography also impacts variation within microbiomes. One study using twins from South Korea and the USA showed that there was great variation within the composition of the gut microbiome despite similarities at the OTU level (Lee et al., 2011). Most gut microbiome studies have focused on what is commonly described as western populations, including individuals from Europe, the USA and Canada. With diet such a key aspect to determining the composition of the gut microbiome (David et al., 2014; Hullar and Fu, 2014), this has been seen as a limitation within gut microbiome studies, given the expected similarity between many westernized diets. One study comparing the gut microbiome of children from Europe to those from Burkina Faso, showed the latter had an increased level of Bacteroidetes and reduced levels of Firmicutes when compared to those from Europe (De Filippo et al., 2010). Another study looking at the Japanese gut microbiome found increased prevalence of *Bacteroides plebius*, a species which encodes the enzyme porphyranase which is known to naturally degrade seaweed (Hehemann et al., 2010). This study is interesting as it highlights how cultural diets can have an impact on microbiome communalities within a geographical area. This is equally seen for the Hazda hunter-gatherers which have a seemingly unique gut microbiome composition, presumed to be linked to their foraging lifestyle. These studies highlight the importance of geography, and potentially key dietary differences, when establishing a baseline for a healthy gut microbiome. Bacterial diversity within the oral cavity, unlike that within the gut, demonstrates no clear geographical patterns with greater variation seen between individuals of the same location than those between different locations (Nasidze et al., 2009). Variations in composition of microbial communities have however been observed between different geographical areas. An example of this is the identification of Enterobacteriaceae in 28% samples taken from the Congo, whereas members of this taxa was not observed in any samples from China, Germany, Poland, Turkey or California (Nasidze et al., 2009).

The impact of ethnicity on microbiota is complex, with a multifactorial approach required to establish true associations (Fortenberry, 2013). One study looked at the variation within the vaginal microbiome of women of different ethnicities and found a greater dominance of lactic acid producing *Lactobacilli* in women of Caucasian and Asian ethnicities compared to those of African American or Hispanic ethnicities (Zhou et al., 2007; Ravel et al., 2011). The observed variation was proposed to link to the lower pH levels observed in Caucasians and Asian women compared to the other ethnicities studied.

Temporal variation in microbial diversity

Alterations to the bacterial diversity within the human microbiome over time have been noted for various body sites (Dekaboruah et al., 2020). The top three body sites showing temporal variation include the skin, the gut and the oral cavity (Caporaso et al., 2011).

The gut microbiome is perhaps one of the more interesting and classical examples of temporal variation within microbial diversity. The guts of new-born babies are rapidly colonized by their mother's microflora (either from the vagina or skin depending on delivery method) which is then followed by a linear increase in diversity to 2 years old at which point it resembles a healthy adult (Koenig et al., 2011). One reason for the observed temporal change in gut microbiome at this stage is the prominent alteration in diet within the first two years of life. Interestingly, microbial diversity in the oral cavity will continue to evolve from childhood all the way to the age of eighteen when it will represent an adult oral microbiome (Crielaard et al., 2011).

There are four key components to understanding temporal change: constitutive differences observed between subjects, time-varying dynamics over several months, biological noise (background variations between time points), and technical noise (variation between replicates at a single time point) (Lloyd-Price et al., 2017). Assessing temporal changes using these four components allows the determination of genuine variations in diversity from background noise. Using this methodology, a paper in 2017 on the expanded Human Microbiome Project found that the gut microbiome composition altered in a temporal

fashion (Lloyd-Price et al., 2017). Within adults Firmicutes demonstrated greatest variation by temporal dynamics within an individual whereas conversely Bacteroidetes primarily showed inter-individual variation.

Temporal changes can also be observed within the urogenital tract with the composition and diversity of the lower reproductive tract in women shown to change throughout their reproductive lifecycle. Following microbial seeding of the site during birth (Dominguez-Bello et al., 2010), a surge in maternal estrogen results in the thickening of epithelium and production of glycogen (a key carbon source) which supports growth of lactic acid producing bacteria (Cruickshank and Sharman, 1934; Spear et al., 2014). As the levels of maternal estrogen drop there is then an increase in bacterial diversity and consequently pH, as the abundance of lactic acid producing bacteria drops (Hammerschlag et al., 1978a, b; Farage and Maibach, 2006). During pregnancy, levels of the hormone estrogen increase substantially and the microbial diversity within the vagina is known to reduce with an increase in dominance of *Lactobacillus* spp. (MacIntyre et al., 2015). Post-birth the estrogen levels reduce by around 1000-fold (Nott et al., 1976) and this results in a reduction of *Lactobacillus* spp. and increase in bacterial diversity persisting for one year (DiGiulio et al., 2015). Post-menopause the composition is shown to alter again with estrogen levels reducing alongside a thinning of the epithelium and glycogen levels, resulting in vaginal atrophy and dryness and correspondingly a depletion of *Lactobacillus* spp. and increase in bacterial diversity (Brotman et al., 2018). There is a strong connection between hormone levels and microbial diversity observed within the lower reproductive tract and women who receive hormone replacement therapy continue to have *Lactobacillus* dominated microbiomes (Muhleisen and Herbst-Kralovetz, 2016). Within the urinary tract the microbiome has also been noted to change between children and adults (Tang, 2017). In part this is related to the alterations observed in diet, personal hygiene, and voiding habits. This alteration continues throughout life with abundance of *Lactobacillus* spp. notably increased during puberty and decreased in post-menopausal women (Burton et al., 2015; Karstens et al., 2016; Thomas-White et al., 2016).

Dysbiosis within microbiota and disease

The dysregulation of parts of the human microbiome have long been hypothesized to link to various disease states either directly by reduction in competition for pathogenic species (Blaser and Falkow, 2009) or indirectly by activating the immune response (Kau et al., 2011). The last decade has seen an abundance of research on the potential links between alterations in the diversity of the human microbiome and related diseases (Cho and Blaser, 2012; Karlsson et al., 2013a; Wade, 2013; Scher et al., 2015).

Obesity

According to the World Health Organization (WHO) the prevalence of obesity, individuals with a body mass index (BMI) ≥ 30 , has drastically risen over the last five decades with >650 million adults worldwide classed as obese in 2016. Obesity is listed as a major risk factor in the development of cardiovascular disease (Van Gaal et al., 2006), diabetes (Lazar, 2005), musculoskeletal disorders (Wearing et al., 2006), certain cancers (Wolin et al., 2010) and poor outcomes from infectious diseases such as SARS-CoV-2 (Simonnet et al., 2020). Multiple studies have investigated the role of the gut microbiota in altering and determining obesity (Turnbaugh et al., 2009a; Schwiertz et al., 2010; Ravussin et al., 2012; Ridaura et al., 2013). Firmicutes and Bacteroidetes are commonly found within the healthy gut microbiome (Ley et al., 2006; Lagier et al., 2012). The ratio of Bacteroidetes to Firmicutes has been linked to the bodyweight of mice, with a lower ratio observed in obese mice compared to their lean counterparts (Ley et al., 2005; Turnbaugh et al., 2006). The alterations within this ratio has however been contradicted by other studies (Schwiertz et al., 2010), most probably due to alterations in methodologies, sample size and study design. A study within twins showed obesity was associated with a decreased abundance of Bacteroidetes species coupled with overall reduced bacterial diversity (Turnbaugh et al., 2009a). This study also highlighted an enrichment of genes related to lipid and carbohydrate metabolism in obese individuals. Gastric by-pass surgery within obese individuals was shown to alter specific metabolic markers such as the production of urinary amines and cresols (Li et al., 2011). Interestingly, transplantation of obese microbiota to germ-free mice resulted in an obese phenotype (Turnbaugh et al., 2006). This observation has been supported by a study using human donors, whereby the introduction of fecal transplants resulted in the host phenotype (Turnbaugh et al., 2009b), so suggesting a direct correlation between the microbiome and the obese phenotype. The observed correlation is however overridden by diet (Turnbaugh et al., 2009b; Ravussin et al., 2012). A further study showed perinatal administration of *Lactobacillus rhamnosus* decreased excessive weight gain during childhood (Luoto et al., 2010). Collectively these studies suggest that there is a modifiable factor regarding the gut microbiome which can alter the level of obesity within individuals.

Diabetes

Diabetes is a chronic, metabolic disease which is characterized by the elevated levels of blood glucose of individuals with the disease. Over time this can result in serious damage to the heart, blood vessels, eyes, kidneys, and nerves. There are two key types of diabetes classed as Type I (pancreas produces very little to no insulin) or Type II (body becomes insulin resistant or fails to produce enough insulin). A link between diabetes and bacteria has been established with both diabetic mice and humans containing increased plasma levels of lipopolysaccharide (LPS), a component of Gram-negative cell walls (Cani et al., 2007; Creely et al., 2007). LPS has also been shown to impair glucose metabolism in mice (Creely et al., 2007). Furthermore, both Type I and Type II diabetes have been linked with the GI Tract microbiome (Larsen et al., 2010; Brown et al., 2011).

In Type II diabetes, metagenomics has shown an alteration to the gut microbiome compared to healthy controls (Larsen et al., 2010). Metagenomic studies within Chinese and Swedish cohorts showed markedly different bacterial composition of the gut microbiota at the species level but much like within obesity studies showed similar functionalities between healthy and diabetic microbiomes (Karlsson et al., 2013b). A lower abundance of butyrate-producing bacteria, which are known to be anti-inflammatory (Rivière et al., 2016), were observed in patients with Type II diabetes compared to healthy controls (Wang et al., 2012a; Karlsson et al., 2013b).

Type I diabetes has strong genetic links (Steck and Rewers, 2011) but more recent research has increased the evidence of environmental factors relating to the disease (Harrison et al., 2008). One metagenomic study into children with Type I diabetes found reduced bacterial diversity compared to controls (Brown et al., 2011). Furthermore, an altered serum metabolome was seen in children who go on to develop Type I diabetes compared to those that do not with many of the metabolites microbially regulated (Orešič et al., 2008).

Dental caries and gingivitis

Dysbiosis of the oral microbiome has been associated with multiple oral health issues including tooth infection (dental caries (Gross et al., 2010) and endodontic infections (Munson et al., 2002)) as well as gum related infections (periodontal disease (Huang et al., 2011) and periodontitis (Costalonga and Herzberg, 2014)).

Dental caries result from the dissolution of tooth structure resultant from acid produced from the fermentation of carbohydrates by oral bacteria (Walsh and Walsh, 2006). Correspondingly, the level of carbohydrates within the diet and the composition of the oral microbiome have been shown to impact development of dental caries (Jensen, 1999; Caufield et al., 2015). A number of commensal bacteria within the oral cavity are capable of producing acid from carbohydrates (Takahashi and Nyvad, 2011; Wade, 2013), however *Streptococcus mutans* and *Lactobacillus* spp. are known to continue producing acid from carbohydrates under acidic conditions (Caufield et al., 2015) so exacerbating the rate of tooth decay. Other bacterial genera implicated in the development of dental caries include *Bifidobacterium*, *Propionibacterium* and *Scardovia* (Munson et al., 2004; Downes et al., 2011; Tanner et al., 2011; Kaur et al., 2013). Conversely, some members of the oral microbiome have been noted to raise the pH within the mouth via production of urea and arginine, offering a neutralizing effect so allowing homeostasis (Burne and Marquis, 2000). Endodontic infections result from the progression of dental caries to the tooth pulp (Persoon and Özok, 2017). Bacteria associated with these infections are typically anaerobic proteolytic bacteria (Munson et al., 2002). *Enterococci* are associated with endodontic infections (Portenier et al., 2003; Wang et al., 2012b); these grow well within the tooth pulp and outside of this habitat are usually outcompeted by the normal microflora.

Gingivitis is the swelling of gums which bleed either spontaneously or upon probing (Page, 1986). Gingivitis is known to arise from the increase in number of Gram negative and anaerobic species within dental plaque which commonly occurs due to poor dental hygiene (Wade, 2013). The increase in abundance of dental plaque and these species within it produce endotoxins and enzymes which result in the observed inflammation and irritation of gums (Ebersole et al., 2010), however there are no specific species currently associated with this disease. Periodontitis derives from the loss of attachment between gingivae and teeth resulting in a periodontal pocket which goes on to be heavily colonized by anaerobic bacteria and ultimately can lead to tooth loss (Wade, 2013). Unlike gingivitis there are specific bacterial species associated with the development of periodontitis. Those identified in by culture-dependent methods include *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* (Socransky et al., 1998). Those identified by culture-independent methods include *Eubacterium saphenum*, *Anaeroglobus geminates*, *Filifactor alocis*, *Porphyromonas endodontalis*, and *Prevotella denticola* (Kumar et al., 2003).

Skin diseases

In 2015 a study by Scher et al. evaluated the link between the gut microbiota and patients with psoriasis and the associated condition psoriatic arthritis (Scher et al., 2015). Psoriasis is a disease which results in red, itchy scaly patches of skin, whereas psoriatic arthritis is a form of arthritis (swollen joints) affecting individuals with psoriasis (Moll and Wright, 1973). Comparative to healthy individuals a lower relative abundance of several intestinal bacteria were identified using 16S rRNA sequencing analysis. Patients with psoriasis had gut microbiota with a lower abundance of *Parabacteroides* and *Coprobacillus* compared to healthy individuals (Scher et al., 2015).

Acne and skin ulcers have been linked to components of the skin microbiomes. Acne is a common skin condition causing spots and oily or inflamed skin. The bacterial species *Cutibacterium acnes* (formerly *Propionibacterium acnes*) has long been associated with this condition (Bojar and Holland, 2004), with the secretion of enzymes from this species known to cause local injury and inflammation (Perry and Lambert, 2011). Individuals with chronic skin ulcers (open wound on the skin), treated with antibiotics, were shown to contain a higher abundance of *Pseudomonadaceae* within the skin microbiome (Price et al., 2009). The same study found those with type II diabetic ulcers instead had greater levels of *Streptococcaceae*. Alterations within the skin microbiome are thought to potentially cause aberrant expression of cutaneous defense response genes (Grice et al., 2010).

Cancer

The connection between the human microbiome and cancer is well reviewed in literature (Schwabe and Jobin, 2013; Bultman, 2014; Rajagopala et al., 2017; Goodman and Gardner, 2018). The microbiome has been linked directly and indirectly to the development of certain cancers as well as being altered in the presence of cancers.

Specific bacterial species shown to be carcinogenic include *Helicobacter pylori* with gastric cancer (Lee et al., 2008; Lofgren et al., 2011), *Salmonella enterica* with gallbladder cancer and mucosa-associated lymphoid tissue (MALT) lymphoma (Welton et al., 1979; Caygill et al., 1994), as well as *Campylobacter jejuni* (Lecuit et al., 2004), *Borrelia burgdorferi* (Cerroni et al., 1997) and *Chlamydia psittaci* (Ferrer et al., 2012) which are associated with certain lymphomas. Various viral pathogens have also been associated with cancers such as human papilloma virus with cervical cancer (Crosbie et al., 2013), Epstein Barr virus and nasopharyngeal cancer (Young and Dawson, 2014) or Burkitt lymphoma (Brady et al., 2008), hepatitis B and C viruses and liver cancer (Ringelhan et al., 2017) as well as HIV with a variety of cancers such as Hodgkin's lymphoma, lung, mouth and throat, skin and liver cancer (Engels et al., 2008; Patel et al., 2008; Silverberg et al., 2009).

Although many of listed microbes are not common commensals, known commensals such as the colonic commensal (in mice) *Helicobacter hepaticus* have been shown to promote the development of hepatocellular carcinoma (Ward et al., 1994; Yang et al., 2013). *Helicobacter pylori*, a common commensal found within the gastric microbiome, is linked to several diseases when at high levels including peptic ulcer disease, MALT lymphoma, and gastric adenocarcinomas (Testerman and Morris, 2014). Individuals with *H. pylori* within the gastric microbiome (three of those analyzed) are found to have it as a dominant species accounting for $\geq 90\%$ (Andersson et al., 2008). *Enterococcus faecalis*, a commensal within the gut, is genotoxic to cells and can induce aneuploidy and tetraploidy in colonic epithelial cells (Wang et al., 2008). This in turn could promote the development of cancer by stimulating an exaggerated immune response, with previous literature demonstrating a link between the immune system and cancer (Karin and Greten, 2005; Karin et al., 2006).

The composition of the microbiome is equally altered by the presence of cancer. The bacterial species *Fusobacterium nucleatum* has been shown to be significantly enhanced in colon cancer tumor samples both when compared to healthy individuals or tissue adjacent to the tumors (Castellarin et al., 2012; Kostic et al., 2012). Comparison of gut microbiota in individuals with tumors compared to those without showed a lower abundance of both Bacteroidetes and Firmicutes compared to other *Fusobacterium*-rich malignancies (Kostic et al., 2012).

Autoimmune diseases

Autoimmune diseases are conditions which arise from an abnormal immune response to a normal body part. A number of autoimmune conditions have been associated with the human microbiome including arthritis (Scher and Abramson, 2011) and inflammatory bowel disease (Halfvarson et al., 2017).

Inflammatory bowel diseases (IBD), namely ulcerative colitis and Crohn's disease, are chronic conditions involving the inflammation of the gut (Ishihara et al., 2009). Patients with IBD contain polymorphisms within the host bacterial sensor genes such as NOD2 (Hugot et al., 2001; Ogura et al., 2001) and TLR4 (Franchimont et al., 2004). There are also strong links between the microbiome and host immune responses with Th17 cell differentiation in mouse lamina propria reliant on segmented filamentous bacteria (SFB) (Cytophaga, Flavobacter, Bacteroidetes) (Ivanov et al., 2008) and polysaccharide A produced by *B. fragilis* mediating the conversion of CD4⁺ T cells to regulatory T cells (Round and Mazmanian, 2010). These studies suggest the emergence of IBD could therefore result from dysbiosis within the gut microbiome. Analysis of gut microbiota from individuals with ulcerative colitis compared to that of healthy individuals has shown significant difference in microbial composition, with reduced diversity and increased abundance of *Enterococcus faecium* and several Proteobacteria (Mondot et al., 2011). Specific members of Enterobacteriaceae have been shown to act in conjunction to increase the risk of developing ulcerative colitis (Garrett et al., 2010). Interestingly, treatment with antibiotics in early life has been linked to the development of Crohn's disease, suggesting that microbial perturbations could have an important role in the risk of developing Crohn's disease (Hviid et al., 2011).

Rheumatoid arthritis, a chronic idiopathic inflammatory condition, has been linked to alterations within the gut microbiota (Scher and Abramson, 2011). Studies in mice have suggested that the SFB results in local expansion of Th17 cells (Ivanov et al., 2009) which migrate to peripheral immune compartments and activate B-cells to antibody producing plasma cells (Cho and Blaser, 2012). Antibody production has been linked to immune-mediated destruction of joints, such as that observed in rheumatoid arthritis (Scher and Abramson, 2011). Psoriatic arthritis (PsA), a type of arthritis linked to patients with psoriasis, has also been associated with perturbations within the gut microbiota (Scher et al., 2015). Individuals with PsA were found to differ in bacterial compositions compared with healthy counterparts with the following showing greatest variation *Akkermansia*, *Ruminococcus*, and *Pseudobutyryvibrio* (Scher et al., 2015). The altered microbiome composition shares strong similarities with those observed in individuals with IBD, with both Ruminococcaceae and *Akkermansia* underrepresented in both PsA and IBD patients compared to healthy microbiomes (Willing et al., 2010; Vignani et al., 2012; Rajilić-Stojanović et al., 2013).

Conclusion

The human microbiome is the symbiotic association of microbes with the human body and is comprised of a plethora of microbial organisms including bacteria, viruses, eukaryotic microbes, and archaea. The importance of the composition and diversity within the microbiome has been explored in terms of both determining health and in the development of disease. Most research has focused to date on the bacterial populations, with recent years starting to see an increase in the characterization of eukaryotic microbiomes (Parfrey et al., 2011; Miller, 2016; Laforest-Lapointe and Arrieta, 2018) and viromes (Manrique et al., 2016; Carding et al., 2017; De Sordi et al., 2017). The NIH Common Fund Human Microbiome Project, launched in 2008, has allowed for the analysis of five body sites from large number of healthy adults. It has provided a wealth of information regarding the 'healthy' microbiome and >2200 reference strains. One key aspect to studying the microbiome has included establishing the diversity of microbial populations within different body sites and how varying confounding factors alter these including gender, diet, geography, time, and even personality traits. The importance of each of these factors has been shown to vary and furthermore it is virtually impossible for one to be truly studied in isolation. There have been some factors which have outweighed the importance of others such as the effect of diet on the gut microbiota.

The technologies for determining microbial diversity has changed over the years from culture, PCR, and gel-based methods to amplicon sequencing and more recently metagenomics, metabolomics, and meta-proteomics. Bias can occur across sampling and analysis methods used with distinct advantages and disadvantages to each. Standardization of protocols and study designs have been widely suggested to allow for better cross-sectional analysis of microbiome research (Goodrich et al., 2014; Poussin et al., 2018; Greathouse et al., 2019). The inclusion of multiple methods within a single study would also serve to produce a more complete picture of the microbiome and its role in human health.

Dysbiosis of the human microbiome has been affiliated with the development of various diseases including obesity, diabetes, as well as infectious and autoimmune diseases. The impact of the dysregulation of the microbiome can result in disease directly by reducing competition with pathogenic microbes or indirectly by the activation of the immune system. A key question to ask when analyzing the impact of these studies is whether the observed results are the cause or an effect of the disease. The case for it being causative can be supported by consistency in results, biological plausibility, and strength of association. Furthermore, as discussed earlier within this chapter there are many confounding factors in determining the diversity and composition of the microbiome, research into disease association with the microbiome requires ample controls to account for all confounding factors.

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Microbial Growth

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Introduction to chapter

At its most basic level microbial (both bacterial and fungal) growth is the division of a single (parent) cell into two (daughter) cells (Walther and Wendland, 2003). The process is named cytokinesis and is tightly regulated, with multiple redundant systems controlling it. Cell division is also tightly linked to other essential cell process such as DNA replication, spatial organization of cells, maintenance of their shape and size, and cell membrane and wall synthesis. This interconnectedness and regulation allow microbial populations to be maintained over multiple generations. Although bacterial and fungal species belong to two different kingdoms of life, much of their basic cell division principles remain similar, at least at a functional level. As such the term “microbial” is used within this chapter when discussing principles which are conserved in both bacterial and fungal organisms, with the two terms “bacterial” and “fungal” being used when discussing aspects of cell division which are specific to each kingdom.

Microbial growth and its control is an especially important driver of pathogenicity. Barreto et al. (2006) defined the term infection as “the entrance and development of an *infectious agent* in a human or animal body, whether or not it develops into a disease”. Such persistence would not be possible without the infectious agent being able to not only persist within the host, but also expand and/or maintain its population. Experimentally, microbial growth is a particularly useful output which can be easily and rapidly measured. Within the lab populations are typically cultured planktonically (i.e., suspended in broth) and with gentle shaking to ensure good aeration. Microbial growth medium is typically rich, with an excess of nutrients to support rapid population expansion. Under such conditions bacterial populations can expand rapidly, for example it is often quoted that under such conditions *Escherichia coli* populations can double every 20 min (Gibson et al., 2018). It must be recognized that this growth type, although very useful for microbiological investigations, is not typical outside the laboratory (Jaishankar and Srivastava, 2017).

In natural habitats nutrients are typically limited and there is intense competition for both nutrients and habitat. As such doubling times are significantly longer than described for lab grown planktonic populations. One recent study showed that within the gut environment *E. coli* had a doubling time of 15 h, compared to just 30 min under laboratory conditions (Gibson et al., 2018). Single species populations are also uncommon outside the laboratory. Instead, microbes commonly form “biofilms,” complex interspecies or even inter-kingdom populations which are anchored and surrounded in a self-produced extracellular matrix (Costerton, 1999; Hall-Stoodley et al., 2004).

Despite the uncommonness of rapidly growing planktonic populations outside the laboratory, measurement of growth underpins much of our understanding of microbial persistence, metabolism, pathogenicity and antimicrobial compound efficacy. Knowledge of microbial growth and division systems can lead to novel treatment strategies for combating infectious agents and increasing the lifespan of perishable goods. Accurate measurement of growth is important in testing antimicrobial treatments. There are multiple methods of measuring both microbial growth and population viability, the most common of which will be briefly described here along with their advantages and disadvantages. Typically, microbial growth is measured at the population levels, with little knowledge of the destination of single cells. However recent advances in technology have begun to allow investigation of gene expression, protein production and metabolism of single cells (Rosenthal et al., 2017).

This section will focus mainly on division of unicellular organisms, in particular bacterial species. The growth and division of fungal species is closely related to other eukaryotic species and detailed overviews of their cell cycles and division can be found in most molecular cell biology text books, for example Alberts et al. (2015). The chapter will initially discuss the phases of growth, before moving to a description of the major methods of population measurement which are used within the laboratory. We will then provide an overview of the molecular mechanisms involved in bacterial and fungal cell division along with some examples of atypical growth. Finally, we will describe three mechanisms of controlling population expansion, utilized by either the microbes themselves or via human intervention.

Unicellular growth (i.e., growth of bacteria and yeast—Binary fission)

Binary fission and the stages of population growth

Unicellular organisms (like bacteria, yeast and archaea) typically grow using a method called binary fission. Here each single cell (mother cell) expands, replicates its genetic material, and divides into two cells (daughter cells). In this way, each time a new generation is produced the population doubles (Fig. 1A).

Growth by binary fission is controlled by the availability of nutrients, space and environmental conditions. Nutritional and environmental factors are rarely optimum, slowing growth from the outset. In turn, binary fission also rapidly depletes nutrients and oxygen, regulating the pace of growth and causing it to further slow. Microbial growth within a closed system typically follows four phases: lag, log/exponential, stationary and death. These can be visualized as shown in Fig. 1B. and are described in detail in sections “Lag phase”, “Log/exponential phase”, “Stationary phase” and “Death phase”.

As well as understanding growth on a population scale, each cell undergoes a series of tightly regulated phases during its growth cycle. These cycles are named G (growth) 1 phase, S (synthesis) phase, G2 phase and finally M (mitosis) phase. G1 is a period of time in which the cell is actively growing in size, S phase encompasses the stage in which DNA and other cellular components are synthesized. Finally, G2 is a second period of growth, allowing the mother cell to become large enough to successfully divide into two daughter cells via mitosis. In addition to the four phases cells are able to move from an actively replicating cell cycle into “G0.” Cells in G0 are not actively replicating but still metabolically active. The ability to move from the cell cycle into G0 is particularly important for unicellular organisms, or static populations.

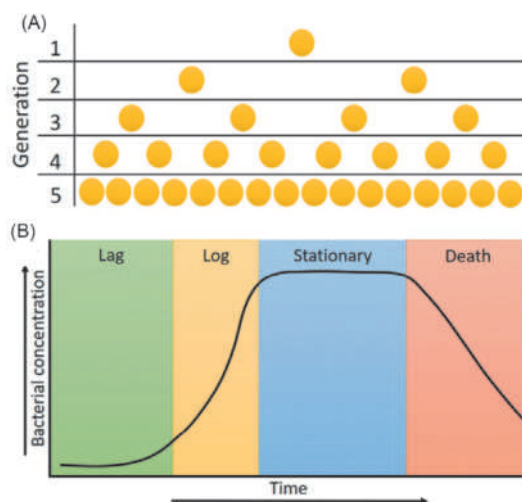


Fig. 1 In optimum growth conditions microbial populations can rapidly expand. An example of binary fission is provided (A) showing the rapid population expansion over five generations. (B) describes a typical microbial growth curve, highlighting the four stages of microbial growth.

Lag phase

When microbes enter a new environment, they require time to sense the new environmental and nutritional conditions, upregulate relevant genes and produce proteins. During this time little growth is observed. The period of time that lag phase lasts is dependent on not only on species but also the conditions and stresses encountered (Hamill et al., 2020). In rapidly growing organisms such as *E. coli* the lag phase is relatively short in duration whereas for slower growing bacterium such as *Mycobacterium tuberculosis* lag phase can last for a period of several days (Von Groll et al., 2010).

Log/exponential phase

During this phase populations are growing rapidly, typically via binary fission. During log phase the cells are highly metabolically active and dividing rapidly, increasing population density quickly and, in the case of some pathogenic species, causing overwhelming infection. In an open system, where nutrients are continually replaced and toxic products removed, exponential growth continues until no more physical space is available. However, nutrients within a closed system (for example an aliquot of broth or agar plate) are rapidly used up by the expanding population and toxic waste products (such as ethanol) rise. Many experimental systems use cells which are in log phase. Rapid population expansion is particularly useful when assessing the effect of antimicrobials on microbial populations, since it allows easy measurement of any inhibition compared to untreated, positive controls.

Stationary phase

During stationary phase the population is static, with the number of cell deaths and cell divisions balancing each other. Most of the population are still metabolically active, but population expansion is slowed and the majority of the microbes within the population are not undergoing division. In an open system once stationary state is reached this becomes the steady state of the microbial population and can be maintained for prolonged time periods. In closed systems a period of stationary phase is followed by death phase once nutrients are depleted to a critical level.

Death phase

In death phase the viability of the population decreases as nutrients become scarcer and toxic products accumulate. Microbes are no longer able to replicate due to both the lack of nutrients and cellular damage incurred. Death phase may be reversed by replenishment or replacement of the growth medium although, this is not possible if the damage to the cells is too extensive. Typically, medium replenishment will move microbes back through the growth cycle, with a short lag phase while the cells carry out repair to damaged systems and up regulate metabolic and replicative genes followed by a log phase until nutrient depletion again reaches a critical stage, and the cells can no longer replicate exponentially.

Measurement of microbial growth

Accurate measurement of populations is important as many experimental microbiological techniques require initial inoculums at specific cell concentrations and/or at specific points in their growth cycle. Commonly, growth is considered at the population level although it is important to remember that individual cells will be at different stages of the cell cycle. Not all the techniques discussed here are suitable for all microbial species and researchers must carefully consider the pros and cons of techniques before making a choice. It is not uncommon for more than one method to be used in parallel. Here we have provided an overview of four commonly used techniques, along with some information of both positive and negative aspects of the techniques to assist researchers in their choice (an overview of the techniques is presented in Table 1).

Optical density

Optical density (OD) is a method of quantifying microbial growth in relation to the turbidity of an inoculum, i.e., its optical density. An assumption is made that the number of microbes within any suspension is directly proportional to the turbidity of the culture and as such, dilution to a set optical density represents a specific microbial concentration. Density measurement is widely used in research and clinical labs as it provides a rapid and inexpensive method of adjusting microbial suspensions to set concentrations. The technique is particularly powerful when used in conjunction with cell counting methods, allowing researchers to rapidly dilute suspensions to an estimated concentration using optical density and then confirm the estimate was correct using cell concentration counting.

McFarland developed a method of preparing suspensions of set turbidity, to which microbial inoculum of unknown concentration could be compared (McFarland, 1907). These “McFarland Standards” have equivalent density to bacterial cultures meaning a researcher can prepare a standard concentration of bacteria without the need for counting (Lahuerta Zamora and Pérez-Gracia, 2012). Typically, measurement is done either by comparing the density of inoculum to pre-prepared standards or by using a spectrophotometer measuring light scatter.

Table 1 Overview of microbial quantification techniques.

Name	Description	Positive aspects	Considerations
Cell count	Initial inoculum is serially diluted, plated and allowed to grow. Counts of the number of colonies grown directly relate to the viable microbial concentration in the initial inoculum	The method allows direct enumeration of inoculum without confounding factors such as debris or slowed metabolism. The method is commonly used, allowing easy comparison of data to previously published examples.	Time must be allowed for colony growth meaning instant assessment of inoculum concentrations is not possible. The method also requires a greater number of materials and reagents than other common methods.
Optical density	The concentration of an inoculum is directly related to its turbidity. As such, dilution of an inoculum to a specific turbidity should provide a specific microbial concentration	Instant method of determining concentrations. It requires minimal materials and equipment.	The method is based on turbidity so care must be used to ensure the microbe concentration-turbidity relationship is known in advance and confounding factors (i.e., debris) are limited.
Viability staining	Dyes either convert to a colored solution upon metabolism or bind selectively to viable cells	There is a wide range of viability stains available, so the methods are highly adaptable to budget, application and specific research need.	Viability dyes can be influenced by the growth phase of the inoculum, leading to over/under estimation of populations. Some are also expensive, require specialist equipment to measure or long incubation periods before reading
Quantification by qPCR/sequencing	Conserved/variable DNA regions are amplified and quantified from whole communities to enumerate the gross community or specific members.	The technique requires no culture and can be used on highly complex communities. In combination with sequencing, it can give significant information about the community members, their genes and biology	Many steps in the process have a bias and expertise is needed to interpret data correctly. Sequencing is relatively expensive, time consuming and requires specialist equipment (although qPCR only techniques much less so).

It should be noted that although turbidity measurement can be linked to bacterial density it is not necessarily a direct correlation. Turbidity can be impacted by the presence of debris, microbial cell size, extracellular products and/or microbial clumping within the sample (Zapata and Ramirez-Arcos, 2015). Some bacterial species, such as *Bdellovibrio* sp. are so small that they are unable to register a density measurement. In contrast, yeast species such as *Candida* sp. are much larger than *E. coli* and so will have much lower cell number than *E. coli* when diluted to the same McFarland standard. Density measurements also give no indication of growth stage or viability of the sample.

Cell counting

Microbial growth counts provide a highly accurate estimate of the microbial population, which is not confounded by the presence of debris or dead cells. One of the most accurate ways of determining microbial numbers in clonal population is to count a subset of the microbial population and scale that number up to give a concentration for the whole population. Typically, this is done by producing a series of dilutions (serial dilutions are usually logarithmic, i.e., 10-fold). The dilutions are then added to agar and incubated in optimum growth conditions to allow microbial growth and colony formation (Fig. 2).

It is assumed that each single microbe within a dilution will grow by binary fission to produce a clonal colony. Colony counting methods express the concentrations as the number of colony forming units in each mL of the initial microbial culture or “CFU/mL.” The term “colony forming unit” is used in preference to a cell/mL value since although the assumption is that each colony on the plate is derived from a single cell, this is not directly known. To extrapolate the total number of cells within the starting inoculum the following formula is used:

$$\text{CFU/mL} = \frac{\text{Number of colonies} \times \text{dilution factor}}{\text{Volume spread on agar plate}}$$

Many videos are available online demonstrating colony counting methods and how to calculate CFU/mL.

Although colony counting methods are commonly used and considered to be one of the best methods of estimating inoculum concentrations, they do have some negative aspects which should be fully considered before use. It is important to have some understanding of the growth dynamics and phenotype of the species to be worked with before counting. For example, if the species to be worked with produces aggregates which cannot be separated then it is very likely that any CFU/mL values will underestimate the actual number of viable cells. Some bacterial species which have a mucoid or swarming phenotype, for example *Pseudomonas aeruginosa* and *Proteus mirabilis* respectively, will also be difficult to count accurately since they do not produce well defined and separated colonies when grown on some agars. Similarly fungal species which form complex multicellular bodies or hyphae are difficult to count, although this technique can work well with species, such as yeast, which have a unicellular, non-hyphenated growth stage. Finally, methods for determining CFU/mL are time consuming, requiring a period of incubation to allow visible microbial growth and require a large number of agar plates. This can be mitigated slightly using direct methods such as counting cells within a hemocytometer, but again this is not suitable for all species—in particular those which are very small or motile.

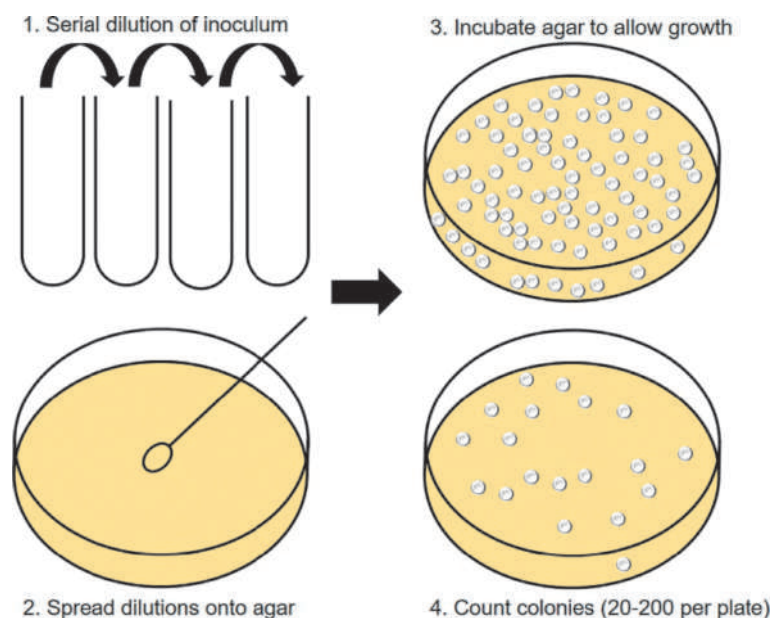


Fig. 2 Overview of a basic method of counting the number of colony forming units within a population.

Viability staining

Various dyes can be used to determine the viability of a microbial population, with viability being both a measure of concentration and population growth phase. In their most basic form viability dyes are altered by metabolic activity or able to preferentially enter only live or dead cells, leading to the conversion of the dye or selective labelling of specific populations. Quantification can be colorimetric, fluorescent or luminescent, with the measurement mechanism linked to the specific dye used. The choice of dyes is restricted only by the experimental system, output method and budget. Here an overview of commonly used viability stains/techniques is given.

High throughput-based dye methods

Tetrazolium salt-based dyes are commonly used as they are cheap, readily available from suppliers and easily measured colorimetrically. Examples include: 2,3,5-Triphenyltetrazolium chloride (TTC) which changes color from clear to a red solution (Brown et al., 2013), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) changing color from yellow to purple solution (Benov, 2019) and sodium 3,3'-[1-[(phenylamino)carbonyl]-3,4-tetrazolium]Bis[(4-methoxy)-6-nitro]benzene sulfonic acid hydrate (XTT) which changes from yellow to red solution (Xu et al., 2016; Pérez et al., 2010). Another commonly used dye is resazurin, which develops from a deep blue to bright pink color when exposed to metabolically active cells. Resazurin relies on redox reactions to stimulate the color change, making the dye also useful as an indicator of anaerobic conditions. It can be quantified fluorescently, although the obvious color change makes viability easy to see by eye. Due to the dramatic color change resazurin is often used in antimicrobial susceptibility assays, allowing researcher to clearly see the exact concentration at which a compound becomes inhibitory to the population. Finally, luciferase dyes luminesce upon interaction with ATP, a reaction which can be quantified by spectrophotometers able to measure luminescence.

As with all the techniques discussed here the use of viability dyes is particularly powerful when used in combination with other microbial growth techniques. For example, when populations move from lag to log or log to stationary phase there may be little difference in CFU/mL or turbidity, but metabolic activity will alter significantly at these transition points and can be tracked by using both metabolic dyes and cell counts. Although tetrazolium salts and resazurin are relatively cheap and stable at room temperature some other fluorescent and luminescent dyes are expensive and require storage in chilled or frozen conditions. Fluorescent and luminescent measurement also requires specialist consumables, such as black or white microtiter plates, and spectrophotometers able to measure the specific light spectrums required. This can make them too costly for routine measurement of cell growth, although for more specialist or focused techniques, such as microscopy and flow cytometry, they are a powerful tool.

Microscopy/cytometry-based viability dyes

Some dyes are also able to distinguish between live and dead cells using a combination of two dyes, one of which typically binds DNA and will penetrate all cells, examples include propidium iodide and 4',6-diamidino-2-phenylindole (DAPI), and a second dye which binds only to viable cells. Live/dead staining can be useful to determine total and viable numbers in a population and is suitable for use with both microscopes and flow cytometers. Live/dead dyes are frequently used when assessing the efficacy of

antimicrobial treatments and can give rapid information on both total microbial numbers and population viability. They can also provide information about the fate of individual community members something which is impossible when using the other techniques mentioned sections “Binary fission and the stages of population growth” and “Lag phase”.

Quantification of genetic material

All the techniques mentioned above require laboratory culture of micro-organisms for a period, however the vast majority of known microorganisms are not able to be cultured, so could not be quantified by standard techniques. Examples include soil, marine and gut microbiome communities. To investigate such communities, molecular techniques can be used. Commonly these techniques involve amplifying “universal” DNA fragments, such as the 16S rRNA gene in bacteria or internal transcribed spacers (ITS) in fungal species. These sequences contain both conserved and variable sequences. The conserved sequences are “universal,” meaning that primers designed to bind in these areas will be able to bind to DNA from a wide range of species. In contrast amplification of variable DNA regions allows greater specificity, allowing investigators to identify what organisms are present and their proportion within a community. At the most basic level primers can be used in combination with quantitative PCR (qPCR) to determine either the total size of a community or subsections (Jian et al., 2020). The technique is commonly combined with next generation sequencing, allowing a single set of primers to be used to amplify DNA from the whole community. This mix is then sequenced and *in silico* analysis used to match the sequences back to specific genus or species (Tringe and Hugenholtz, 2008). As the cost of sequencing has decreased, 16S rRNA sequencing can be replaced by Shotgun Sequencing, which sequences all the DNA within a community, allowing functional information such as antimicrobial resistance to also be discovered. Shotgun sequencing also allows all members of the community, whether bacterial, viral or eukaryotic to be identified in a single step.

Although these methods are very powerful and have significantly improved our knowledge of the diversity of microbial communities, there are some considerations researchers should think about before beginning a study. Firstly, it is not possible to determine if all the DNA present in a sample originated only from viable organisms. DNA is a very stable structure and able to persist in the environment. As such using DNA for enumeration can be difficult unless the amount of non-viable organism DNA present in the sample can be accurately estimated. All DNA extraction methods and primers have a bias towards specific organisms. For example, Gram-positive organisms are more difficult to lyse than Gram-negative ones and as such less Gram-positive DNA may be present, even if they were present in equal numbers in the originating community. As such, researchers must recognize that their data will be skewed, with some community members being under- or over-represented as a result of the choice of DNA extraction method, primer design and amplification (Poretsky et al., 2014). It is very important to understand the bias of the methods chosen in order to give a correct interpretation of data. The number of copies of the 16S rRNA gene in an organism is also variable, again potentially leading to overrepresentation of particular members of the community (Kembel et al., 2012). Finally, techniques which including sequencing, although powerful, are costly and take several weeks to complete, so would be unlikely to be chosen for routine enumeration. They also require a significant element of *in silico* processing, which again can introduce bias or be open to false interpretation. Identification of organisms is carried out by software which matches sequences provided to online, publicly available, databases. If sequences from an organism are not present in the database, or the sequence quality obtained is poor, matches will not be found, and no identity assigned. Experimental design is particularly important in these types of studies and there are many online resources and training courses to help researchers.

Bacterial division

Our knowledge of microbial division largely comes from the study of division in two model organisms: the Gram-negative *E. coli* and *Bacillus subtilis*, which is Gram-positive. As with the study of many other molecular mechanisms, these two organisms were utilized because they are genetically tractable and multiple molecular tools have been developed to work effectively with them. It should be noted that neither of the organisms present a particular “representative” model of either Gram-negative or -positive organisms. Both organisms are rod shaped and as such do not provide an effective model of the growth of other bacterial shapes, such as coccus or spiral shaped species such as *Staphylococcus* sp. and *Campylobacter* sp. Despite their limitations as model organisms, the action of binary fission is largely conserved across bacterial and archaeal species and as such information drawn from these two species can be applied to other bacterial species.

In its simplest form, binary fission must involve the formation of a ring (called the Z ring) in a mid-point of the parent cell after replication of the cellular components is completed. This ring then constricts, and finally separates, forming two daughter cells (Fig. 3). It therefore follows that, if the basic mechanism of division is the same then several the major microbial division proteins may also be conserved. This was found to be the case; the major protein forming the Z ring, a protein called FtsZ is highly conserved across all bacteria and archaea. FtsZ is also very similar in structure to tubulin, which is a major eukaryotic cytoskeleton component, suggesting that the evolution of this protein occurred at a very early stage in the evolution of life (Erickson, 1997).

Differences do however occur in the mechanisms supporting ring formation and its positioning. Although many of these mechanisms are again conserved across species, there is a greater degree of redundancy, so species may not contain copies of all the genes. In many species inactivating the genes involved in, for example, positioning of the Z ring does not lead to complete defect in the cell division process and, as in the case of nucleoid occlusion systems (described in detail in section “Nucleoid occlusion”), several genes must be inactivated before clear phenotypes are discovered (Wu and Errington, 2004; Bernhardt and De Boer, 2005).

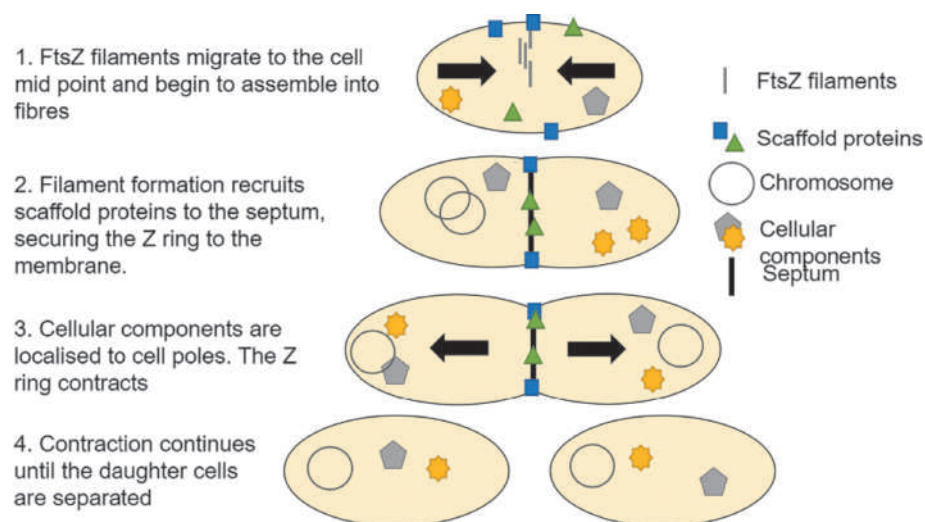


Fig. 3 Overview of the process of cell division and the major steps.

In this section some of the major mechanisms controlling the cell division machinery will be described, with a focus on proteins identified as important to *E. coli* and *B. subtilis* since these are among the best studied (Errington et al., 2003). Although the Z rings formation and regulation is carried out by proteins of similar function in different microbial species, except for FtsZ these proteins may not have homologous amino acid sequences, being functionally rather than structurally similar.

FtsZ and the Z ring

FtsZ is a homologue of tubulin, the protein which in eukaryotes is involved in cytoskeleton structure. FtsZ is highly conserved and present in all bacteria and archaea. When FtsZ proteins are allowed to assemble *in vitro* (i.e., outside of the cellular environment) they assemble into long fibers (Mukherjee and Lutkenhaus, 1994). The fibers assemble at a specific point of the cell, typically the mid-point within elongated, rod shaped, cells, and form a ring structure closely associated with the inner surface of the cytoplasmic membrane. This structure is called the Z Ring and is the fundamental structure required for cell division. Assembly of the Z ring recruits other proteins involved in cell division (the divisome) to the area. The Z ring is also the point at which the cell wall begins to invaginate, due to constriction of the Z ring. The wall invagination eventually leads to the division of one cell into two daughter cells. Key members of the divisome are described in Table 2.

The positioning and formation of the Z ring is highly regulated, since its incorrect positioning or poor timing of division could lead to serious viability problems within the daughter cells. The Z ring must be placed away from nuclear material and division must be timed so that the cells are able to maintain not only their correct volume and shape but also genetic material and (in the case of eukaryotic organisms) organelles. The Z ring and divisome are typically assembled in two stages. Initially the FtsZ proteins assemble and begin to recruit “core” divisome proteins to the structure. There is then a lapse in activity as the cell cycle continues and the cell expands to the volume necessary to produce two daughter cells (G2 phase). Finally, when the cell reaches the desired size and volume, the second stage of recruitment occurs, and the parent cell divides. The major proteins involved in the divisome, and key cell division processes are listed below in Table 2. Although multiple proteins have been identified as having a critical role in cell division, there is still little definitive information about the exact role many play.

Regulation of Z ring formation and positioning

The regulation of cell division is as important as cell division itself since division at the wrong time in the cell cycle or in the wrong part of the cell could lead to irreparable damage and cell death. As such, just as is the case with the cell division apparatus, the cell division regulatory systems are highly conserved. Here we will discuss two of the most common regulatory systems: nucleoid occlusion and Z ring positioning. As with the cell division apparatus, these systems have been most closely studied in model organisms such as *B. subtilis* and *E. coli*, but homologues of the major proteins involved are present in a wide range of bacterial species.

Nucleoid occlusion

Nucleoid occlusion is a system which inhibits placement of the Z ring in the vicinity of nuclear material (Wu et al., 2009). This means that the nuclear material is remote from the area where the Z ring forms and so cannot be damaged during the separation of

Table 2 Table of proteins typically involved in formation and regulation of the divisome.

Name	Role	References
<i>Core divisome</i>		
FtsZ	FtsZ units assemble into protein filaments which form the Z ring. This provides a point of anchor for constriction of the membrane, separating the cell into two daughter cells. FtsZ is closely related to the eukaryotic protein tubulin.	Silber et al. (2020) and Margolin (2005)
FtsA	Closely related to the eukaryotic protein actin, FtsA binds to the FtsZ polymers and secures them to the cytoplasmic membrane.	Ozyamak et al. (2013) and Chen et al. (2017)
ZipA	Inner membrane protein which also plays a role in securing the Z ring to the membrane. There is some redundancy between the role of FtsA and ZipA, with both providing a similar end function (via a different mechanism).	Chen et al. (2017) and Hale and De Boer (1997)
FtsK	FtsK (and its <i>B. subtilis</i> homologue: SpoIIIE) are involved in positioning chromosomes so they can be separated and translocated to the daughter cells. FtsK localizes at the septum site and assists in recruiting other divisome components.	Stouf et al. (2013) and Besprozvannaya and Burton (2014)
FtsQ, FtsL and FtsB	Transmembrane proteins which form the FtsQBL complex. Role of the complex is still poorly understood but hypothesized to be part of the Z ring scaffold.	Choi et al. (2018)
FtsW	Transporter protein which exports Lipid II, a cell wall component, allowing expansion of the cell wall during elongation.	Mohammadi et al. (2011)
FtsI	Also named Penicillin binding protein 3. FtsI forms a complex with FtsW and has a role in peptidoglycan biogenesis. This allows expansion of the cell wall and subsequent elongation of the cell.	Marmont and Bernhardt (2020)
MatB, ZapA, ZapB	Responsible for chromosome separation and anchoring the chromosome to the Z ring via the replication terminus region.	Männik et al. (2016)
XerC, XerD	Tyrosine recombinases involved in separation of replicated chromosomes. Their activity is coordinated with that of FtsK.	Castillo et al. (2017) and Spiers and Sherratt (1999)
<i>Z ring position regulation</i>		
Noc/SlmA	DNA binding protein. Noc is a major factor in effecting nucleoid occlusion and inhibiting division (SlmA is the <i>E. coli</i> homologue). Noc is able to bind to both DNA and FtsZ, disrupting normal Z ring formation when bound to both.	Tonthat et al. (2011)
MinC	Interacts directly with FtsZ to destabilize the Z ring and inhibit its formation.	Park et al. (2018)
MinD	ATPase which recruits MinC to the site of the bacterial membrane. MinC cannot begin to oscillate without MinD.	Margolin (2001)
MinE	MinE oscillates between the cell poles, moving the MinC/D complex with it. Due to the oscillation more MinE accumulates at the poles, along with the MinC/D complex it accompanies. This leaves the center of the cell free for binding of FtsZ and limits the potential of FtsZ to bind away from the center of the cell.	Hu and Lutkenhaus (1999)
MinJ and DivIVA	Within the <i>B. subtilis</i> min system MinE is replaced by these two proteins. The proteins form a complex which allows MinC/D to oscillate, accumulating at the cell poles and limiting the potential of Z ring formation away from the center of the cell.	Errington and Wu (2017)

This list is not exhaustive but gives those major proteins identified to be involved in *E. coli* division.

the daughter cells. Within the division cycle the chromosome, and associated genetic material such as plasmids, are replicated and then moved to opposite poles of the cell. This movement allows the center of the cell, where the Z ring typically forms, to remain relatively clear. The physical distancing of the genetic material from the area of division helps to ensure that genetic material is not damaged or lost during separation of the two daughter cells. In *B. subtilis* the protein controlling nucleoid occlusion is named Noc, the *E. coli* homologue is SlmA. Noc/SlmA can bind directly to FtsZ and the bacterial chromosome via specific DNA sequences (Wu and Errington, 2004; Wu et al., 2009). When bound to both DNA and FtsZ Noc is able to interfere with normal Z ring formation (Tonthat et al., 2011). This double binding means that if DNA is within the vicinity of the Z ring formation site the Z ring cannot form, limiting the potential for chromosomes damage during Z ring contraction and daughter cell separation.

As with many of the systems involved in cell division, nucleoid occlusion does not appear to be essential for position of the Z ring, at least in *E. coli*. In cells where no nuclear material is present (anucleate) the Z ring still formed at or near the midpoint of the cell, suggesting other mechanisms also support Z ring localization (Sun et al., 1998). One such system involved in the positioning of the Z ring is called the Min system.

The min system

The Min proteins form a negative regulation system which allows correct positioning of the Z ring in the center of rod-shaped cells. In *E. coli* three proteins (MinC, MinD and MinE) form the Min system whereas in *B. subtilis* there are four major proteins (MinC, MinD, MinJ and DivIVA) (Singhi and Srivastava, 2020). MinC and MinD form a complex, named MinCD, which is preferentially positioned at the poles of the cell. This positioning leads to a concentration gradient of MinCD, with the poles having the highest concentration of the complex. This presence of this complex inhibits binding of FtsZ, thereby localizing FtsZ binding (and therefore Z ring formation) near the center of the cell, away from the nucleoid containing poles. When minC and minD are

inactivated in *B. subtilis* multiple Z ring structures form, although this effect can be mitigated by providing minimal nutrients, indicating the Min system is also in part redundant and works alongside other, currently unidentified, systems (Levin et al., 1998).

Min systems can either be oscillating, moving continuously from pole to pole of the cell, or non-oscillating, where the protein complexes are near the poles of the cells (Taviti and Beuria, 2019). MinD and MinE are needed for oscillation. The *E. coli* min system contains all three proteins and is an oscillatory system, *B. subtilis* lacks the MinE protein and as such its system is non-oscillatory. As with many of the other proteins involved in cell division the Min system can assemble and function *in vitro*, with no additional cellular components present (Zieske and Schwillie, 2014).

Division in atypical organisms and circumstances

The majority of bacterial cells position their Z ring in the center of the mother cell and following cytokinesis two daughter cells are produced which are identical to the mother cell. The growth of cells is also typically initiated at the site of Z ring formation, with expansion occurring equally at both sides of the Z ring site. This however is not always the case, and some species are able to divide asymmetrically or expand in size from a single pole.

The genus *Mycobacterium* expands not from the central septum site, but from one of the cell poles. Regulation of the expansion sites position is still not understood, but position always appears to occur at the distal end of each cell (Baranowski et al., 2019). For species where heterogeneity is required in daughter cells then polar growth and division is particularly common. Since growth only occurs from one pole, rather than the site of septum formation, cell membrane and wall components are temporally separated, with the oldest parts of the cell wall inherited by the daughter cell. In cells elongating from the central septum both mother and daughter cells will contain a mix of new and old components. Although growth is polar in *Mycobacterium* sp. the Z ring is still positioned in the cells mid-point. The mechanism of this positioning is still not well understood since *Mycobacterium* sp. contain no nucleoid occlusion homologous genes and only a single, distantly related, Min protein (Baranowski et al., 2019).

Yet other bacteria produce two distinctly different daughter cells during division. Perhaps the best studied model of this is the aquatic Gram-negative species *Caulobacter crescentus*. When undergoing division this species produces one flagellated daughter cell, while the aflagellated mother cell remains tethered to a surface via a stalk. The *C. crescentus* lifecycle is particularly complex with cells changing function and morphology multiple times before successful division occurs. Cells initially have a swimming lifestyle, before attaching to surfaces via tether or stalk, losing its flagella in this process. Swimming cells are unable to begin DNA replication (Skerker and Laub, 2004). Once tethered the mother cell initiates cell division and enters a continuous cycle of producing flagellated daughter cells. Although the daughter cells cannot begin replication until they themselves are tethered their ability to swim allows them to explore the environment, only settling and becoming stalked mother cells when a suitable environment is encountered (Xu et al., 2020).

Finally, many bacterial species can form spores. Spores are produced by parent cells when conditions are unsuitable for growth. Spores are a highly resistant, dormant cell type which can persist in a vegetative state until local conditions are able to once again support growth. At this point the spore germinates, become metabolically active and initiating cytokinesis. *B. subtilis* is an example of a spore forming bacterium and much work has been carried out in this species to understand the basic sporulation apparatus. For a *B. subtilis* spore to be formed the mother cells first initiates asymmetrical cell division. Rather than forming in the center of the mother cell, during sporulation the septum is much closer to one pole, producing a much smaller daughter cell (termed a “forespore”). Septum formation and closure is not completed however, so the daughter cell is not released. Instead the mother cell engulfs the forespore, allowing it to mature within the cytosol. Finally, the mother cell undergoes a programmed lysis, releasing the spore (Tan and Ramamurthi, 2014). Sporulation is an important virulence factor utilized by several opportunistic pathogens. The ability to form spores allows bacteria to remain in an environment indefinitely without incurring a metabolic cost. *Clostridioides difficile* is a slow growing spore forming anaerobic member of the gut microbiome. In the healthy gut *C. difficile* is unable to outcompete other members of the microbiome, maintaining a small population. Its ability to form spores allows it to persist during antibiotic treatment, which dramatically reduces the abundance of other microbiome species. Following antibiotic treatment *C. difficile* spores quickly germinate and can become the dominant species within the gut, causing a range of gastroenteritis symptoms ranging from mild diarrhea to potentially fatal colitis (Shen, 2020). Infection is difficult to eradicate as further antibiotic treatment results in the production of spores, which again can germinate once antibiotic treatment is stopped. To date the most effective treatment for *C. difficile* infection is replacement of the gut microbiome via fecal transplant (Baunwall et al., 2020).

Fungal division

Fungi and yeast are eukaryotic rather than prokaryotic and as such have a similar cell division machinery and processes to other animal and plant cells. In depth information about the eukaryotic cell cycle and division can be found in standard cell biology text books such as (Alberts et al., 2015), but much of our understanding of the process comes from investigation in model yeast organisms such as *Saccharomyces cerevisiae*. In its most basic form fungal cell division follows the same process as that of bacterial cells, although different terminology is used for key components and processes. The mother cell must replicate its DNA (and in the case of eukaryotes organelles must also be multiplied), expand the size of the cell, form a septum (analogous to the bacterial Z ring) and then constrict and separate at this point until two daughter cells are formed. Yeasts and fungi are able to exist as both unicellular and multicellular forms, both requiring different cell division processes, with only the unicellular form discussed here. Cell division

is a highly choreographed process, with specific processes only occurring at set points in the cell cycle (described in Section “Unicellular growth (i.e., growth of bacteria and yeast—Binary fission)”).

Before entering G1 cells must commit to the division process, passing a checkpoint called “Start.” Once Start is passed the cell is committed to division and although the process can later be delayed at further checkpoints if key processes are not completed the cell cannot exit cell division once committed to the process. Following Start the cell moves through the cell cycle before finally separating into two cells at M phase. Progression through the cell cycle is controlled by the interaction of cyclins and Cyclin Dependent Kinases. Cyclins are so named because they are only expressed at specific points within the cell cycle. There are two major types of cyclin in *S. cerevisiae* G1 cyclins, regulating events in G1 and the initial commitment to cell division and B-type cyclins, which are usually expressed between Start and the end of M phase (Dörter et al., 2016). Size also plays a role in progression through the cell cycle, with cells not able to undertake S and M phase activities until they have reached specific mass (Berman, 2006).

As with bacterial cells, not all fungal species divide symmetrically. Again, *S. cerevisiae* is one such example. In this species most new cellular components created during each division cycle are inherited by the daughter cell, with the mother cell retaining older (and potentially damaged) molecules. This system of inheritance allows the population overall to remain healthy, with only the oldest cells containing any potentially damaged components (Manzano-López and Monje-Casas, 2020). Daughter cells are also smaller than the mother cell, requiring a prolonged G1 period to reach the size required for transition into S phase (Berman, 2006).

Limiting microbial growth

In vitro culture of organisms typically allows greater growth of microbial populations than they would experience in a natural setting. The conditions encountered by microbes when infecting a host are typically very nutrient deficient. Hosts deliberately limit the availability of nutrient such as iron to limit the potential for a microbial population to exponentially increase (Hershko, 1993). The immune system also constantly screens the host for the presence of microbes. If microbes, and particularly rapidly growing population of potentially pathogenic microbes, are encountered within tissues the immune system is rapidly activated, limiting the possibility of microbes to become established or cause symptomatic infections. Exponential growth is the exception rather than normality in nature. To maximize survival in these sub-optimal conditions most microbial species have sophisticated signaling, sensing and migration mechanisms. These systems allow them to sense not only the local environmental conditions, but gradients of nutrients and toxins and the density of the surrounding microbial population. Unlike the systems controlling cell division these systems are numerous, less conserved and often species specific.

Quorum sensing (QS)

QS is particularly important during infection as it is the mechanism by which population density is detected. QS systems are two-component systems comprising of both a small soluble marker which is secreted by the microbe and a cell wall bound receptor (Portnoy et al., 2000). The soluble marker is secreted by the cell and detected via the receptor. In areas of low diffusion or high population density there will be high levels of soluble marker present, conversely when few cells are present, or diffusion is rapid little marker will be available to bind to the receptors. In this way microbes are able to co-ordinate communal behaviors such as virulence factor production, sporulation, log phase growth and biofilm formation (Abisado et al., 2018). Alongside nutrient detection systems the QS system is also able to drive population expansion or a move to a stationary growth phenotype.

Biofilm formation

Biofilms are defined as attached microbial populations which are surrounded in a self-produced extracellular matrix (ECM) (Costerton, 1999). Biofilms typically contain more than one species of bacteria or fungus, with many containing a mix of fungal, bacterial and viral species. Since their discovery approximately 40 years ago they have been shown to be the primary lifestyle of microbes and are now known to be an important virulence factor. It is estimated that 65% of all infections and 80% of chronic infections involve biofilms (Jamal et al., 2018). Biofilms are such an important pathogenicity factor for several reasons. Firstly, the biofilms size limits the ability of phagocytic cells to engulf them. Since the biofilm cannot be engulfed host cells instead initiate a pro-inflammatory response, secreting cytokines and chemokines and causing further local tissue damage. Damage to the tissue surrounding the biofilm in turn releases more nutrients which can be utilized by the microbes within the biofilm. Secondly, the ECM has a protective function, limiting diffusion in and out of the biofilm. This property not only inhibits penetration of antimicrobials but stops diffusion of nutrients or communal goods such as β lactamase, an inhibitor of β lactam antibiotics. Finally, biofilms predominantly contain stationary phase organisms which means that large numbers of organisms can be sustained in an environment with limited nutrients.

Targeting microbial growth mechanism with antimicrobials

The term antimicrobial is a collective term for all substances which can limit the growth, replication and/or metabolism of microbes, be they bacteria, fungi or virus. They can be synthetically made or derived from natural products or cellular secretions. Although it may therefore seem obvious to state that all antimicrobials inhibit microbial growth, many of them specifically target

aspects of the cell division machinery. The cell division machinery is a particularly attractive target as these proteins are highly conserved within bacteria, but often are different to those found in Eukaryotic cells. This means that any drugs targeting cell division apparatus are likely to have broad spectrum activity, while also not proving toxic to the host (for a review see (Den Blaauwen et al., 2014)). Curcumin, a phenolic compound produced within the Turmeric root, was demonstrated to reduce expansion of a *B. subtilis* population with treated cells showing a filamentous (elongated) cell morphology (Rai et al., 2008). Further investigation highlighted the curcumin inhibited accumulation of FtsZ, stopping Z ring formation and initiation of cell division (Rai et al., 2008).

Several antibiotics also target the cell division machinery. For example, β lactam antibiotics, including Penicillin, limit the ability to expand bacterial cell walls, stopping the enlargement of cells prior to Z ring formation and division (Yocum et al., 1980). Quinolones target DNA replication mechanisms, specifically inhibiting the activity of DNA gyrase (Aldred et al., 2014) so that DNA cannot be efficiently translated. A note of caution must be sounded however as the majority of antimicrobials targeting microbial growth systems are most effective against rapidly growing bacterial populations. As discussed earlier, rapid (exponential) growth is not the normal mode of growth outside of the laboratory where environmental factors typically limit population expansion. As such antimicrobials inhibiting cell division may not be effective against slow growing or static populations, such as those found in biofilms. In these instances, antimicrobials which target essential cellular functions such as protein synthesis may be a more appropriate treatment choice.

Summary and conclusion

Within this chapter we have discussed binary fission and its key stages, the major systems involved in bacterial and fungal cell division and techniques commonly used for the measurement of microbial growth. Much of the cell division machinery is conserved across all prokaryotes. Many microbes also appear to utilize multiple checkpoints and regulatory mechanisms to ensure that cell division occurs smoothly and at the correct pace. Many of the proteins involved in cell division appear to be in part redundant, increasing the chance that division will occur without damage to the daughter cells. To date we have a detailed knowledge of how division occurs in model organisms however, there is still much to be discovered. Recent advances in genomics, transcriptomics and proteomics as well as microscopy have allowed us to begin to investigate fundamental biological systems, such as cell division, in individual organisms. This means that rather than measuring populations as a whole, the fate of single cells and their offspring can now be followed and modelled. This will provide a further insight into microbial growth, its control and the factors, both intrinsic and extrinsic, which control it.

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Microbial Infections: The Good, the Bad and the Ugly

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Skin infections

Infections of the skin can be caused by bacteria, viruses, parasites and fungi and across the population they are extremely common, with an estimated 24% of adults in England and Wales suffering from a skin infection during their lifetime (Schofield et al., 2011). Worldwide, it is estimated that 1.9 billion people, which equates to one third of the world's population will suffer with a skin condition (Hay et al., 2015). The most common bacterial skin pathogens are *Staphylococcus aureus* and group A β -hemolytic streptococci; Herpes simplex is the most common viral skin disease and of the dermatophytic fungi, *Trichophyton rubrum* is the most prevalent cause of skin and nail infections (Aly, 1996). Normally, it is a single pathogen that causes issues with the skin, depending on the location of the infection with variation between the upper and lower body (Ki and Rotstein, 2008). Most primary skin infections usually occur in normal skin. However, secondary infections occur in skin that is already diseased, for example with intertrigo, due to the underlying disease the clinical picture and course of these infections vary (Aly, 1996). With primary skin infections, impetigo, folliculitis and boils are common and these are usually down to infections with common skin pathogens including *S. aureus*, β -hemolytic streptococci, and coryneform bacteria (Sukumaran and Senanayake, 2016). In most skin infections these organisms enter the body through a break in the skin or hair follicle (Ki and Rotstein, 2008), commonly due to a cut or insect bite for example as outlined in Fig. 1. Other entry points into the body have also been identified (Fig. 1).

There are some systemic infections that involve skin reactions and symptoms caused by a pathogen or toxin, for example measles, varicella, gonococemia, and staphylococcal scalded skin syndrome (Aly, 1996). Dermatophytic fungi have a strong affinity for keratin and therefore invade keratinized tissue of the nails, hair, and skin (Aly, 1996). Clinically most skin infections follow a similar pattern of disease and cause erythema, edema, and more localized redness and inflammation. Focal accumulations of pus or fluid (vesicles, bullae) may form. Alternatively, lesions may be observed with no obvious inflammation. Nail infections cause discoloration of the nail and thickening of the nail plate (Aly, 1996).

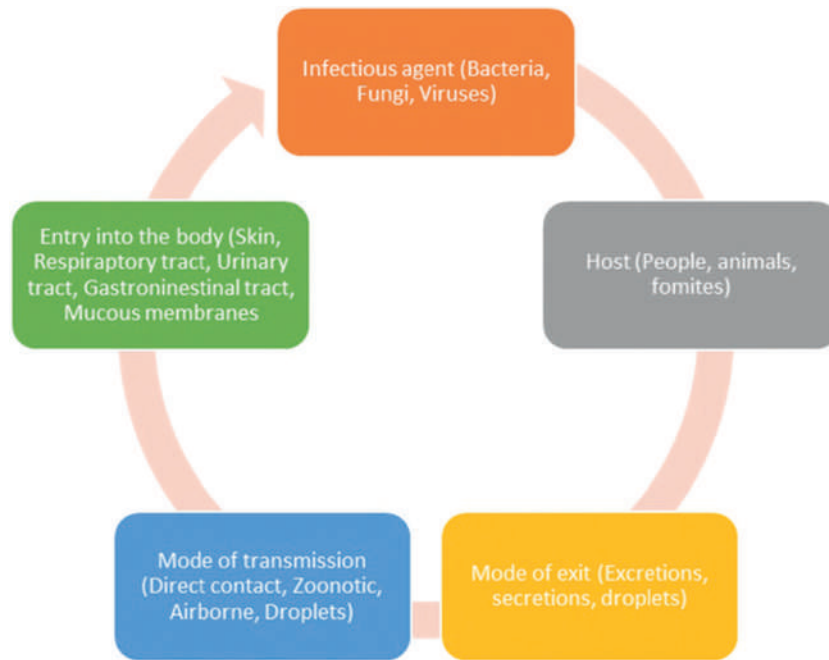


Fig. 1 Infection routes into the human body, including infectious agents, hosts and mode of transmission.

Bacterial skin infections

Primary infections

Impetigo

Impetigo is the most common skin infection and there are three forms of impetigo that are recognized on the basis of clinical, bacteriologic, and histologic results (Aly, 1996). Impetigo causes lesions that may contain group A β -hemolytic streptococci, *S. aureus*, or both, and controversy exists about which of these organisms is the primary pathogen (Pereira, 2014). The lesions have a thick, adherent, recurrent, dirty yellow crust with an erythematous margin (Pereira, 2014). This form of impetigo is the most common skin infection in children and is highly contagious and requires prompt treatment which tends to be antibiotics that can be taken either topically or orally.

In staphylococcal impetigo, the lesions are always caused by *S. aureus*, and are superficial, thin-walled, and bullous (Hartman-Adams et al., 2014). When a lesion ruptures, a thin, transparent crust appears which can be distinguished from the stuck-on crust of common impetigo. This distinctive appearance of bullous impetigo results from the local action of the epidermolytic toxin (exfoliation), the lesions are often found in clusters (Hartman-Adams et al., 2014).

Ecthyma is a deeper form of impetigo. Lesions usually occur on the legs and other areas of the body that are generally covered, and they often occur as a complication of debility and infestation. The ulcers have a punched-out appearance when the crust or purulent materials are removed, the lesions heal slowly and leave scars (Hartman-Adams et al., 2014). Treatment is dependent on the severity of the infection, but can include antibiotics.

Cellulitis

Streptococcus pyogenes is the most common agent of cellulitis (Stevens and Bryant, 2016). Which is inflammation of loose connective tissue, particularly subcutaneous tissue and most common to occur in legs (Stevens and Bryant, 2016). The pathogen generally invades through a breach in the skin surface, and infection and a tissue oedema occur (Stevens and Bryant, 2016). Cellulitis can happen in normal skin, but the lesion in cellulitis is erythematous, oedematous, brawny, and tender, with borders that are poorly defined (Stevens and Bryant, 2016).

No absolute distinction can be made between streptococcal cellulitis and erysipelas (Morris, 2008). Clinically, erysipelas is more superficial, with a sharp margin as opposed to the undefined border of cellulitis, lesions usually occur on the cheeks (Morris, 2008).

Staphylococcal scaled skin syndrome

Staphylococcal scalded skin syndrome, also known Lyell's disease or toxic epidermal necrolysis, starts as a localized lesion, followed by widespread erythema and exfoliation of the skin (Mishra et al., 2016). This disorder is caused by phage group II staphylococci which elaborate an epidermolytic toxin (Mishra et al., 2016). The disease is more common in infants than in adults (Mishra et al., 2016).

Folliculitis

Folliculitis can be divided into two major categories: superficial and deep. The most superficial form of skin infection is staphylococcal folliculitis, manifested by minute erythematous follicular pustules without involvement of the surrounding skin (Aly, 1996), with the scalp and extremities being favorite sites. Gram-negative folliculitis occurs mainly as a superinfection in acne vulgaris patients receiving long-term, systemic antibiotic therapy (Aly, 1996) with pustules are often clustered around the nose (Aly, 1996). *Cutibacterium acnes* (formerly *Propionibacterium acnes*) folliculitis is often misdiagnosed as staphylococcal folliculitis (Sun and Chang, 2017). The primary lesion is a white to yellow follicular pustule, Gram stain of pus reveals numerous intracellular and extracellular Gram-positive pleomorphic rods. The lesions are more common in men than in women (Sun and Chang, 2017). The process may start at the age when acne usually appears, yet most cases occur years later (Aly, 1996).

In deep folliculitis, infection extends deeply into the follicle, and the resulting perifolliculitis causes a more marked inflammatory response than that seen in superficial folliculitis (Aly, 1996). The primary lesion is a follicular pustule pierced by a hair. Bearded men tend to be more prone to this infection than shaven men (Aly, 1996).

Many people suffer from boils, these are due to a staphylococcal infection of a follicle with involvement of subcutaneous tissue (Lin et al., 2018). The preferred sites of boils are the hairy parts or areas that are exposed to friction (Aly, 1996).

Secondary infections

Secondary skin infections of pre-existing wounds, skin conditions, burns or retention cysts are common. Broken or damaged skin is prone to infection with bacteria, including *Staphylococcus* spp., *Streptococcus* spp. and *Corynebacteria*. These bacteria infect pre-existing skin conditions and can cause severe infections that need to be treated with antibiotics.

Intertrigo

Intertrigo is most commonly seen in overweight adults and children (Janniger et al., 2005). In the skin fold which is associated with obesity, heat, moisture, and rubbing produce erythema, maceration, or even erosions (Janniger et al., 2005). Overgrowth of resident or transient flora may produce this problem, and the bacteria involved can vary on a case by case basis (Janniger et al., 2005).

Viral skin diseases

Viral skin diseases covers a wide range of conditions, with the most common symptom being a rash (Ramdass et al., 2015). Most viral skin conditions are self-limiting, treatment would be given in severe cases, especially where sequela could occur.

Herpes simplex virus

Herpes simplex virus is a neurotropic pathogen, with clinical disorders ranging from harmless skin manifestations, such as oral or facial lesions to severe infections of the central nervous system (Duarte et al., 2019). The virus is acquired in childhood and produces infections throughout a person's life, due to the ability of the virus to infection and remain latent in neurons (Kramer et al., 2003). Worldwide approximately 60% of the population have antibodies against this virus, with 20–40% of those that are infected developing symptoms (Looker et al., 2015). Common symptoms of this virus including, itching and burning sensations before any blisters appear. One or more painful fluid filled blister can appear, these are most common on the lips, but can occur anywhere on the body and commonly referred to as cold sores. Blisters break open and form a crust before healing. Sores appear between 2 and 20 days after contact with an infected person and can last up to 10 days. Antiviral medications, such as acyclovir, famciclovir, and valacyclovir, are often given and are effective, they can help to reduce the severity and frequency of symptoms, but cannot cure the infection.

Chickenpox virus

Chickenpox, also called variella, is an extremely common virus which is common in children but can also infect adults, particularly if they did not have this virus in childhood (Gershon et al., 2015). It is a highly contagious infection that causes an itchy, spotty rash with blisters being able to occur anywhere on the body. Chickenpox is easily transmissible with infection happening by being in close contact with an infected individual for example, being in the same room as well as touching fomites that have been handled by an infected person (Fig. 1). Chickenpox, is self-limiting and symptoms usually last for 1-week, topical treatments can help to ease the symptoms (Gershon et al., 2015). Shingles, which tend to occur in older people, is caused by the same virus that causes chickenpox, when a person has chickenpox, the virus remains in the body, it can be reactivated later in life and cause shingles particularly if the immune system is lowered. You cannot pass shingles from person to person, but a person could get chickenpox if they have not had it previously. There is a vaccine for shingles, it commonly given to those aged 70 or over.

Fungal skin diseases

Several genera of fungi are responsible for skin diseases and are known as dermatophytes, but some yeasts which are not classed in this group can also cause skin infections.

Athletes foot

Athlete's foot (tinea pedis) is a fungal infection that usually begins between the toes. It commonly occurs in people whose feet have become very sweaty while confined within tightfitting shoes. A variety of fungi all belonging to a group called dermatophytes is responsible (Crawford, 2009). Signs and symptoms of athlete's foot include a scaly rash that usually causes itching, stinging and burning. Athlete's foot is contagious and can be spread via contaminated floors, towels or clothing (Crawford, 2009).

Athlete's foot is closely related to other fungal infections such as ringworm. It can be treated with over-the-counter antifungal medications, but the infection often recurs (Crawford, 2009). Keeping the area clean and dry limits re-infection.

Ring-worm

Ringworm of the body (tinea corporis) is a rash caused by a fungal infection. It's usually a red, itchy, circular rash with clearer skin in the middle. Ringworm gets its name because of its appearance. Ringworm is related to athlete's foot (tinea pedis) and ringworm of the scalp (tinea capitis; Leung et al., 2020). Ringworm often spreads by direct skin-to-skin contact with an infected person or animal. Mild ringworm often responds to antifungal medications that are applied to the skin. For more-severe infections, antifungal pills can be taken, usually for several weeks. Signs and symptoms of ringworm include a scaly ring-shaped area, particularly on the trunks arms or legs that can be itchy (Leung et al., 2020). Ringworm, is a zoonoses and so is transmitted between animals and humans and from human to human, it is highly infectious and easily transmissible (Leung et al., 2020).

Nail infections

Many infections of the nail and nail bed are caused by fungus, these are common problems and any nail of the hand or foot can become infected (Christenson et al., 2018). However, they are more common on toe nails (Christenson et al., 2018). Fungal nail infections are characterized by discolouration, thickening or distortion of the nail. The nails may also become brittle or crumbly and there is usually some pain and discomfort associated with infection. In addition, the area around the nail and nail bed can become itchy, swollen and red. Nail and nail bed infections are often associated with Athletes foot. Treatment is not always needed for mild infections, these are normally self-limiting and unlikely to cause further problems. For more severe infections, anti-fungal medication may be used.

Bacterial infections

Bacterial infections can be mild infections of the skin or of an internal organ, they can multiply rapidly within the body and can produce toxins (Aly, 1996), in more severe cases the result could be septicaemia. This occurs when a bacterial infection elsewhere in the body enters the bloodstream, it can quickly become life-threatening. There are many bacterial species that can result in infections.

Bacterial meningitis

Meningitis can be both viral and bacterial, viral is the most common and least serious type (Hussein and Shafran, 2000). Bacterial meningitis is rarer, but is more serious. Several different bacteria can cause meningitis including, meningococcal bacteria which is caused by *Neisseria meningitidis* and pneumococcal bacteria. For *Neisseria meningitidis*, disease-causing strains are classified according to the antigenic structure of their polysaccharide capsule. Serotype distribution varies markedly around the world. Among the 13 identified capsular types of *N. meningitidis*, six (A, B, C, W135, X, and Y) account for most disease cases worldwide (Rouphael and Stephens, 2012). Meningitis is an infection of the membranes surrounding the brain and spinal cord (Rouphael and Stephens, 2012), this can interfere with the blood flow which in turn could lead to paralysis or a stroke. It can be acute with a rapid onset of symptoms or it can be chronic, both forms require rapid treatment with antibiotics (Hussein and Shafran, 2000). Anyone can potentially get meningitis, but it's more common in babies and young children, teenagers and young adults, elderly people and people with a weakened immune system—for example, those with HIV and those having chemotherapy. There are numerous vaccines that can provide protection against the bacteria responsible.

Urinary tract infections

Urinary tract infections are very common infections that can affect the bladder, the kidneys or the tubes that connect them. The urinary tract becomes infected usually by bacteria, that come from the gut, they enter the urinary tract through the urethra (Flores-Mireles et al., 2015). Urinary tract infections (UTIs) are a severe public health problem and are caused by a range of pathogens, but most commonly by *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus saprophyticus* (Flores-Mireles et al., 2015). They are more common in women than men, with some women experiencing them regularly. They are painful and uncomfortable with common symptoms including the need to urinate more often, pain or discomfort during urination, strong odor from the urine and blood in the urine. In elderly person, urinary tract infections are often associated with confusion (Flores-Mireles et al., 2015). They are easily treatable with antibiotics, with symptoms lasting for a 3–5 days following the commencement of treatment (Flores-Mireles et al., 2015). Reoccurring infections are common, some patients are more susceptible to these than others, they normally occur due to failure to eradicate the infection in the first place, a re-lapse or re-infection from the original source.

Respiratory tract infections

Respiratory tract infections can affect the sinuses, throat, airways and lungs, they can be caused by both viruses such as the common cold and bacteria. This section is going to focus on bacteria. The upper respiratory tract is considered to be the airway above the glottis and includes the nose, sinuses, pharynx and larynx (Mete and Akbudak, 2018). Typical infections in this section include tonsillitis and laryngitis. Tonsillitis is commonly caused by viral infection but up to 40% of cases can be attributed to a bacterial infection with group A *Streptococcus* spp. being responsible for the majority of cases (Georgalas et al., 2014). However, other bacteria including *Neisseria gonorrhoeae*, *Corynebacterium diphtheriae*, or *Haemophilus influenzae* may be the cause (Georgalas et al., 2014). Typically, the infection is spread between people through the air. Lower respiratory tract infections are more severe than upper respiratory infections, as these are infections of the trachea, bronchial tubes, bronchioles and lungs (Dasaraju and Liu, 1996). The two most common infections are bronchitis and pneumonia. Bronchitis is inflammation of the bronchi in the lungs. Symptoms include coughing up sputum, wheezing, shortness of breath, and chest pain. Bronchitis can be acute or chronic, with *Mycoplasma pneumoniae* or *Bordetella pertussis* being the bacteria responsible. Pneumonia is an inflammatory condition of the lung primarily affecting the small air sacs known as alveoli. Symptoms typically include dry cough, chest pain, fever and difficulty breathing. Pneumonia is usually caused by infection with bacteria, with *Streptococcus pneumoniae* isolated in nearly 50% of cases (Dion and Ashurst, 2020), with drug-resistant versions becoming more common, including drug-resistant *Streptococcus pneumoniae* (DRSP) and methicillin-resistant *Staphylococcus aureus* (MRSA). All respiratory tract infections that are attributed to a bacterial source can be treated with antibiotics.

Legionella

Legionella species are Gram-negative bacilli (Winn, 1996). Legionellosis is a collective term for diseases caused by legionella bacteria including the most serious Legionnaires' disease. This disease is a potentially fatal form of pneumonia and everyone is susceptible to infection (Winn, 1996). The risk increases with age but some people are at higher risk including those aged 45 or over, those that smoke or drink heavily and those that are suffering from pre-existing medical conditions including diabetes and heart disease (Winn, 1996). *Legionella pneumophila* and related bacteria are common in natural water sources such as rivers, lakes and reservoirs, but usually in low numbers. They may also be found in purpose-built water systems such as cooling towers, evaporative condensers, hot and cold-water systems and spa pools (Winn, 1996). Most infections can be treated with antibiotics.

Aspergillus

Aspergillus is a fungus. All members of the genus *Aspergillus* are members of the Ascomycota phyla. *Aspergillus* species are highly aerobic and are found in almost all oxygen-rich environments, where they commonly grow as molds on surfaces (Latgé and Chamilos, 2019). *Aspergillus* species are common contaminants of starchy foods (such as bread and potatoes), and grow in or on many plants and trees. *Aspergillus niger* can be found growing on damp walls, as a major component of mildew. Several species of *Aspergillus*, including *A. niger* and *A. fumigatus*, will readily colonize buildings, favoring warm and damp or humid areas such as bathrooms (Latgé and Chamilos, 2019). Aspergillosis is caused by breathing in small particles, called spores, of aspergillus in the air. Symptoms include, shortness of breath, cough or wheezing along with a high temperature. There are several different types of aspergillosis including allergic bronchopulmonary aspergillosis, chronic pulmonary aspergillosis and invasive pulmonary aspergillus (Latgé and Chamilos, 2019). Infections are treated with anti-fungal medication and possibly steroids.

Systemic infection

Systemic bacterial infection, commonly referred to as Sepsis is defined as an overwhelming innate inflammatory response to an infection, caused by bacteria entering the bloodstream (Singer et al., 2016). Sepsis is a very severe condition that can result in fatality if not treated promptly and can be caused by several bacterial species. The most common Gram-positive bacteria isolated in such sepsis cases are *S. aureus* and *Strep. pneumoniae*, and the most common Gram-negative bacteria are *E. coli*, *Klebsiella* spp. and *P. aeruginosa* (Ramachandran, 2014; Opota et al., 2015). Among those who are most vulnerable are the immunocompromised, including the elderly, new-born babies, and patients with previous or current trauma and conditions, such as diabetes and cancer (Lever and Mackenzie, 2007). Treatment is with antibiotics which are normally given intravenously due to the severity of this condition.

Foodborne illness

Foodborne illness, more commonly known as food poisoning, is the illness resulting from the consumption of contaminated food. This contamination can be viral, parasitic or due to toxins, but it is most likely to be bacterial. Two thirds of human foodborne illness worldwide can be attributed to bacteria as the causative agent, with a high burden in developing countries (Abebe et al., 2020). Most cases of foodborne illness can be attributed to the consumption of animal products. Meat, dairy products, and eggs are the main vehicles that people are exposed to zoonotic bacteria. *S. aureus*, *Salmonella* species, *Campylobacter* species, *L. monocytogenes*, and *E. coli* are the major zoonotic bacterial pathogens which are the causative agents of food-borne illness and death worldwide.

Table 1 Overview of infectious dose for common bacterial pathogens associated with foodborne illness.

Microorganism	Infectious dose, Colony forming units (cfu)	References
<i>Salmonella</i> spp.	10,000 cfu	Catford et al. (2017)
<i>Campylobacter jejuni</i>	<1000 cfu	Janssen et al. (2008)
<i>L. monocytogenes</i>	>100 cfu per g	Swaminathan and Garner-Smith (2007)
<i>E. coli</i> O157	<50 cfu	Griffin et al. (1994)

(Abebe et al., 2020). According to the World Health Organisation (WHO), an estimated 600 million—almost 1 in 10 people in the world—fall ill after eating contaminated food and 420,000 die every year, with the biggest burden (40% of infections) being in the under 5 age group (WHO, 2020). In the UK, the number of reported foodborne illness is recorded by Public Health England (PHE), to date in 2020 there have been 20,188 reports of *Campylobacter* spp., followed by 2002 reports of *Salmonella* spp. and 1735 of *Giardia* spp. (Food Standards Agency (FSA), 2020). The infectious dose in humans differ depending on the bacterial species responsible for the foodborne illness, these are summarized in Table 1.

Campylobacter species

Campylobacter spp. is the leading cause of bacterial foodborne illness worldwide, with the majority of cases being attributed to the consumption of poultry and poultry products (Wilson et al., 2010; Hermans et al., 2011; Levin, 2007; Williams et al., 2014). Despite poultry being the number one agent responsible for the majority of human cases of *Campylobacter* there have been reported cases associated with other animals including pigs, cows, sheep, companion animals and wild birds, although human infection from these sources remains low (Denis et al., 2008; Horrocks et al., 2009; Zweifel et al., 2004; Little et al., 2008; Oporto et al., 2007; Gilpin et al., 2008; Kwan et al., 2008; Inglis et al., 2004; Busato et al., 1999; Kemp et al., 2005; Brown et al., 2004; Andrzejewska et al., 2013; Meerburg et al., 2006). In addition to the above sources there have also been reported cases, particularly in the developing world from un-pasteurized milk and water (WHO, 2020). In the UK, in 2017 there were 56, 729 reported cases of *Campylobacter* spp. (Food Standards Agency (FSA), 2020) and it is thought that 82% of hospital admissions with a diagnosis of food poisoning can be attributed to a *Campylobacter* infection (Adak et al., 2002). In humans, the infectious dose is believed to be low, at around 500 cells in an adult male (Robinson, 1981) with an incubation period of up to 10 days, with most people exhibiting symptoms by day four. Symptoms include diarrhea, which may be bloody, particularly in children, acute abdominal pain and fever. Most infections are self-limiting and those who suffer an infection recover quickly after resting and maintenance of fluid levels; a fatal outcome is rare and would usually occur in the elderly or those already suffering from another serious illness (Skirrow and Blaser, 2002). Despite treatment with antibiotics being rare, resistance to clinically important antimicrobials including macrolides and fluoroquinolones has been reported (Humphrey et al., 2007). Approximately 1% of cases develop long-term sequelae, such as inflammatory bowel syndrome or reactive arthritis.

Salmonella

When the general public think of food poisoning they tend to think of *Salmonella* spp. this is due to the publicity received in the press, particularly in the 1980s in the UK, when an outbreak of *Salmonella* linked to eggs made headlines, this led to most UK laying hens, reared under the Lion Code, to be vaccinated against *Salmonella*. This control measure led to a reduction in the number of cases in the human population seen in the UK. Despite this intervention there are still reported cases of foodborne illness attributed to *Salmonella* spp. each year, although not surprisingly in the UK, hens eggs are rarely the cause providing they are from vaccinated flocks. Chicken, pork and dairy produce can contain *Salmonella*, in addition fruit and vegetables have been implicated in some outbreaks if they have been washed with contaminated water. In the UK, in 2016 there were 8630 reported cases of *Salmonella* (Food Standards Agency (FSA), 2020), significantly less than *Campylobacter*. The symptoms of *Salmonella* include diarrhea, stomach cramps, nausea, vomiting and fever (Eng et al., 2015), with these symptoms developing between 12 and 72 h post infection, with symptoms lasting up to 7 days (Eng et al., 2015). Medical intervention is not normally required unless symptoms are severe or prolonged.

Listeria

L. monocytogenes is the bacterium responsible for Listeriosis in humans. The bacteria are widely present in the environment and infection occurs after the consumption of foods, particularly raw, chilled or ready to eat foods, unpasteurised milk and dairy products such as soft cheeses and meat and meat products are the most common sources of infections (Buchanan et al., 2017). Infections are usually sporadic, but outbreaks can occur. People with listeriosis have been reported to develop symptoms between 1 and 70 days after consuming food contaminated with *Listeria*, and this prolonged time between exposure and onset of symptoms makes it difficult to get accurate dose information (Buchanan et al., 2017). Symptoms include fever and diarrhea, similar to other foodborne illnesses. However, *Listeria monocytogenes* infection can cause severe disease, for example septicemia in vulnerable groups,

including the elderly, pregnant women, unborn and new-born babies, and people with impaired immunity. In these groups, listeriosis can present as infection of the bloodstream or brain. Due to the severity of infection and high case fatality rate, listeriosis is an important public health concern. Compared to other foodborne pathogens, infections in humans are relatively rare. In 2018, 156 cases of listeriosis were reported in England and Wales (Food Standards Agency (FSA), 2020), with the greatest number of cases being reported in those over the age of 80. The most serious cases of listeriosis are treated with antibiotics, with ampicillin used on its own or conjunction with another antibiotic (most likely to be gentamicin) is the treatment of choice (Temple and Nahata, 2000). In vulnerable groups where the most serious infections can occur which can lead to septicemia, then intravenous antibiotics will be given, treatment can last up to 6 weeks.

Escherichia coli and *Clostridia*

E. coli has also been associated with foodborne outbreaks of infection. This bacterium lives in the intestine of most animals and humans and is considered to be part of the normal healthy gut flora. There are numerous types of *E. coli* strains, whilst most are harmless there are some for example *E. coli* 0157 which can be more serious. The more virulent strains can cause stomach cramps, diarrhea and fever. Most infections are self-limiting but the more serious cases require treatment with antibiotics. The number of infections in the human population attributed to *E. coli* is lower compared to the other food-borne pathogens, with 608 cases of *E. coli* 0157 being reported in England and Wales in 2018 (Food Standards Agency (FSA), 2020).

Whilst lower in numbers compared to other bacteria, clostridia is also associated with foodborne illness. There are 2 species commonly implicated *C. perfringens* and *C. botulinum*. These are spore-forming bacteria, found in many environmental sources as well as in the intestines of animals and humans. *C. perfringens* is commonly found on raw meat and poultry. It prefers to grow in conditions with very little or no oxygen, and under ideal conditions can multiply very rapidly. Some strains of *C. perfringens* produce a toxin in the intestine that causes illness (Uzal et al., 2014). The symptoms include diarrhea and abdominal cramps, with the onset of symptoms being rapid (within 24 h) of ingesting the bacteria (Kiu and Hall, 2018), there can be a rapid onset of symptoms with most infections clearing within 24 h.

Norovirus

More commonly known as the winter vomiting bug, this is a highly contagious infectious bacterium and can easily spread from person to person (Robilotti et al., 2015) and so outbreaks are common in hospitals, schools and care homes. The symptoms include fever, diarrhea, nausea, vomiting and fever (Robilotti et al., 2015). Most cases are self-limiting and do not require treatment. The UK Food standards agency, estimated that 380,000 cases of norovirus linked to food occur annually in the UK (Food Standards Agency (FSA), 2020).

Conclusion

Microbial infection is a broad topic that covers infections caused by bacteria, viruses and fungi. There are several species within each microbe that are responsible for infections all over the body. Some infections can be self-limiting and will clear up without any medical intervention. However, other require medical treatment and in the case of bacterial infections this is antibiotics. Some infections can lead to serious post infection complications that can persist throughout a persons' life and so with this in mind any infection cannot be regarded as trivial. Whilst, a lot of research has been carried out in infection diseases there are still many unknowns but with the advancements in technology and whole genome sequencing these unknowns are reducing.

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Microbial Cell Structure and Organization: Bacteria

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Glossary

Acid-fast staining A staining method used on acid-fast bacteria such as Mycobacteria, involves using a stain such as basic fuchsin and then decoloring with acid alcohol, which acid-fast bacteria resist.

Biofilm A structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface.

Capsule A dense polysaccharide or protein matrix surrounding the bacterial cell surface.

Cell wall associated proteins (CWAs) Surface proteins, that are covalently bound to the cell wall peptidoglycan, responsible for adhesion and colonization of host cells, and evasion of host immune responses.

Chemotaxis Movement towards or away from a chemical or stimuli.

Commensal bacteria Bacteria that are part of the normal human flora/microbiota.

Conjugation Transfer of genes from one bacterium to another by direct cell-to-cell contact or by a bridge-like connection between two cells called a pilus.

Copy number The number of copies of a plasmid found within a single bacteria cell.

Cytoplasm Fluid filled inner most area of the bacteria cell contained within the cytoplasmic membrane and contains the bacterial chromosome, mRNAs, ribosomes, proteins and metabolites required for growth and survival.

Cytoplasmic membrane Semi-permeable lipid bilayer that separates the inside of the cell (cytoplasm) from the surrounding environment.

Eukaryotic Cell has a membrane-bound nucleus and various other organelles such as mitochondria and Golgi body.

Fimbriae (singular fimbria) Small hair-like filamentous protein structures on the bacteria cell wall, numerous in number and have a role in adhesion to surfaces including host cells.

Flagella A long, thin appendage composed of flagellin, that is responsible for bacterial motility.

Gram staining A differential staining technique where bacterial cells either stain pink-red (Gram-negative) or purple (Gram-positive) depending on their cell wall structure.

Gram-negative bacteria Cell wall composed of a thin peptidoglycan layer and an outer lipid membrane. In a Gram stain, they do not retain crystal violet so stain red.

Gram-positive bacteria Cell wall composed of a thick peptidoglycan layer and no outer lipid membrane. In a Gram stain, they retain the crystal violet color and stain purple.

Human flora/Microbiota All the microorganisms (bacteria, fungi and protozoans) that live on or in humans.

Inclusion body Aggregates of stable substances, usually proteins found in the cytoplasm, and function as energy stores or carbon reservoirs.

Lipopolysaccharide (LPS) An endotoxin and powerful inflammatory stimulant that forms the major part of the outer

membrane in Gram-negative bacteria, composed of lipid A, core polysaccharide chain, and O-specific polysaccharide/O antigen.

Lipoteichoic acid (LTA) A macroamphiphile with a glycolipid anchored covalently to the cytoplasmic membrane.

M protein Protein that alters surface of cell wall and inhibits phagocytosis.

Messenger RNA (mRNA) A single-stranded RNA molecule that is complementary to one of the DNA strands of a gene, contains the genetic information to encode proteins.

Nucleoid Area within the cytoplasm that contains the bacterial DNA/chromosome.

Opportunistic bacteria Bacteria that are part of the normal human flora/microbiota but under certain circumstances such as weakened immunity or altered microbiota can cause infections.

Opsonin A receptor such as immunoglobulin G (IgG) or complement molecule used by neutrophils, macrophages, dendritic cells, and B lymphocytes to bind bacteria and initiate phagocytosis.

Opsonophagocytosis Phagocytosis initiated by an opsonin.

Outer membrane proteins (OMP)/Porins Transmembrane proteins that function as channels for the movement of solutes in and out of the cell.

Outer membrane Found external to the peptidoglycan layer in Gram-negative bacteria, consists of an inner layer composed of mainly phospholipids and an outer layer composed of lipopolysaccharide.

Pathogen-associated molecular pattern (PAMP) A macromolecule on the inside or outside of the bacteria cell wall that serve as a marker by which phagocytes can identify the pathogenic bacteria/microbes.

Pathogenic bacteria Bacteria that cause infections in healthy persons and are not part of the normal human flora/microbiota.

Peptidoglycan (PG) Main structural component of cell wall composed of alternating repeats of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) in adjacent layers that are cross-linked by short peptides.

Periplasmic space/periplasm A gel-like space between the inner peptidoglycan layer and outer membrane of Gram-negative bacteria.

Phagocyte pattern recognizing receptors (PRR) Soluble or membrane-bound proteins on the surface of phagocytes that recognize and bind to PAMPs. The interaction of a PAMP and PRR activates the phagocyte to ingest and destroy the bacteria.

Phagocytosis A process by which an immune cell ingests/engulfs a particle such as a bacterial cell and digests it.

Pili (singular pilus) Small hair-like filamentous protein structure that extends from cell wall, and depending on type involved in adhesion, genetic exchange, or twitching motility.

Plasmid An extrachromosomal self-replicating genetic element that are expendable as they are not part of the bacteria's permanent chromosome but carry genetic information that can provide a selective advantage such as antibiotic resistance.

Prokaryotic Cell lacking membrane-bound nucleus and other organelles.

Ribosomal RNA (rRNA) An RNA molecule that makes up part of the ribosome, some actively participate in protein synthesis.

Ribosome Site of protein synthesis.

Slime layer Similar to a capsule but less structured.

Teichoic acid (TA) Major component of Gram-positive bacteria cell wall, composed of wall teichoic acids and lipoteichoic acids.

Transcription Synthesis of an RNA molecule complementary to one of the two strands of the DNA.

Transduction Transfer of genes from one bacterium to another by a virus/phage.

Transformation Transfer of genetic information by free DNA from one bacterium to another.

Translation Synthesis of a protein by a ribosome using information provided by mRNA.

Twitching motility A flagella-independent form of bacterial motility by Type IV pili. The bacteria move due to the extension and retraction of the Type IV pili so cell is dragged across a surface in a gliding-like motion.

Type III secretion systems Commonly used by pathogenic Gram-negative bacteria to inject toxic proteins directly into eukaryotic host cells.

Type IV pili/secretion system Pilus extends through the outer membrane of the bacteria and can aid the bacteria in twitching motility or transfer of DNA to other cells via conjugation.

Virulence factor Molecules produced by the bacteria that help them cause infections, invade the host and evade host defences.

Wall teichoic acid (WTA) Composed of glycerol or ribitol residues containing phosphodiester links and attached to *N*-acetylmuramic acid of the peptidoglycan.

Introduction

Microorganisms are the smallest organisms on earth, and comprise of bacteria, archaea, fungi, protozoa, and viruses. Except for viruses, microorganisms are living single cell or a cluster of cells and are found in both aquatic and terrestrial environment (Beveridge, 1980; Madigan et al., 2019). The microbial cell has many shared features (Fig. 1), but the differences seen between

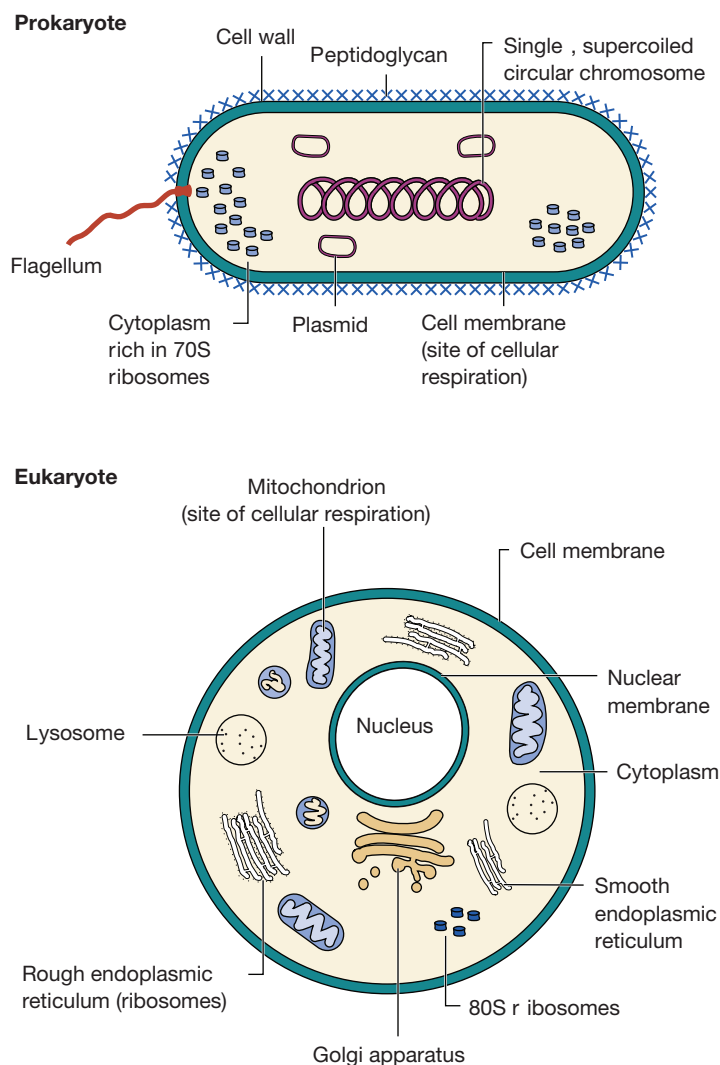


Fig. 1 Diagram of the major features of Prokaryote and Eukaryote cells. From Murray PR, Rosenthal KS and Pfaller MA (2015) *Medical Microbiology*. Philadelphia: Elsevier, pp. 107.

their internal cell structure is used to differentiate bacteria from fungi and protozoa (Beveridge, 1980; Madigan et al., 2019). Bacteria and archaea are defined as prokaryotic cells as they lack a nucleus and other membrane-bound organelles and their DNA is a single circular molecule, whereas fungi and protozoa are eukaryotes as they have a nucleus and an assortment of membrane bound organelles (Fig. 1; (Murray et al., 2015)). This chapter will focus solely on bacteria and aims to provide a basic understanding of the bacterial cell structure and organization and how it influences their ability to cause human infection and diseases.

Bacteria are a very large diverse group of microorganisms that are classified according to their morphology (size and shape), cell wall structure, and their metabolic and genetic features (which are discussed in other chapters). Most bacteria are defined as rod (bacillus), spherical (coccus) or spiral (spirillum) in shape (Fig. 2; Murray et al., 2015). Coccus-shaped bacteria are seen as single cells, pairs, long chains or clusters, whereas rod-shaped bacteria are simply cells that are longer in one dimension than the other, resulting in fat, thin, short or long rods. Spirillum-shaped bacteria are curved rods. However other bacteria groups are recognized by the unusual shape of their individual cells (Fig. 2), such as spirochetes, which are tightly coiled bacteria; filamentous bacteria, that form long thin cells or chains of cells; and budding or appendaged bacteria, that have long tubes or stalks as extensions of their cells. Bacteria vary in size from about 0.2 μm to more than 700 μm in diameter. Most rod-shaped bacteria are between 0.5 μm and 4 μm wide and less than 15 μm long, while coccus-shaped bacteria have an average diameter of about 0.5–1.0 μm (Murray et al., 2015). The size of bacteria is limited by their volume and ability to transport compounds in and out of the cell. A bacterial cell less than 0.1 μm in diameter has too small a volume to house the cells' essential components i.e., nucleic acids, proteins and ribosomes. Whereas if the bacteria cell is too large it cannot transport enough nutrients into the cell, as surface to relative to cell volume is too small. Hence, a significant advantage to being small is that the cell has a larger surface-to volume ratio so able to transport more nutrients into the cell, resulting in faster growth rates and larger populations. The surface-to volume ratio therefore also influences the ability of bacteria to adapt and evolve to changing environmental conditions (Madigan et al., 2019; Murray et al., 2015).

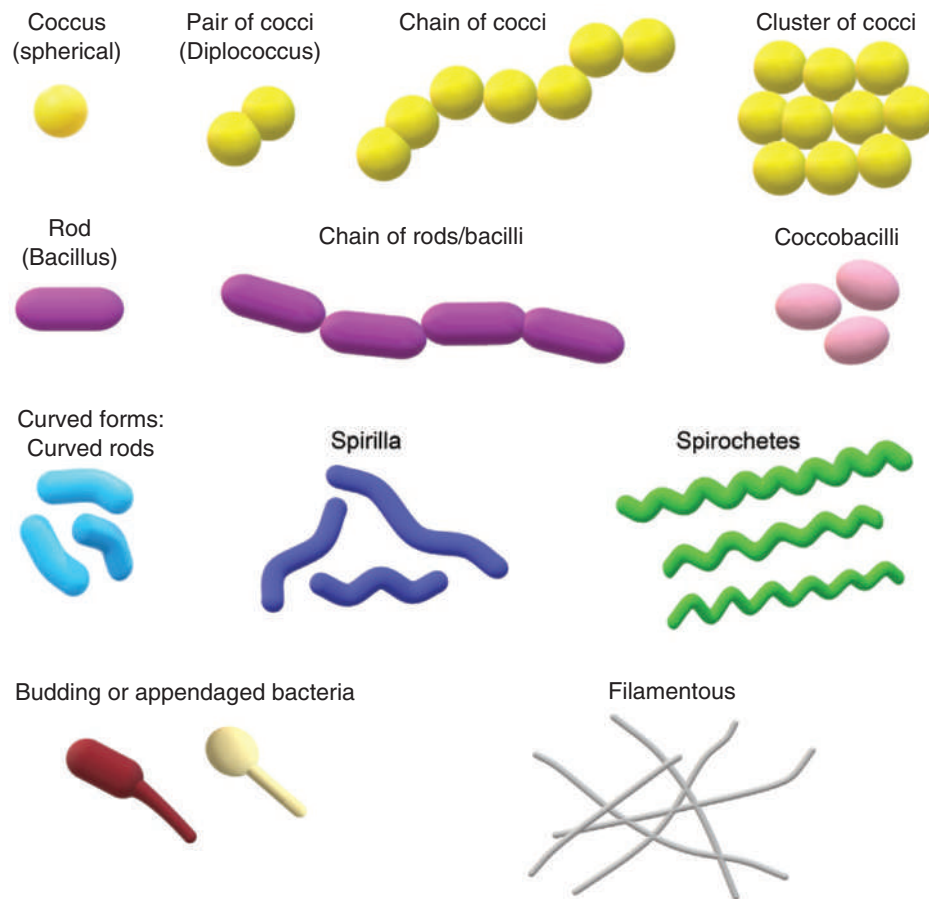


Fig. 2 Bacterial cell morphologies.

Most bacteria are harmless to humans and some are even useful and used in the food, drug, agriculture and energy industries. Some of these harmless bacteria have developed a symbiotic relationship with humans and are deemed part of the normal human flora or microbiota, also referred to as commensal bacteria. These bacteria are found on the human skin, and in the oral cavity, gastrointestinal (GI), respiratory and urogenital tracts, and contribute to human health and well-being by producing beneficial products and inhibiting the growth of harmful bacteria (Beveridge, 1980; Wilson et al., 2002). In return, the bacteria have access to nutrients. Harmful bacteria can cause infections and are defined as opportunistic or pathogenic bacteria. The severity of the infection depends on the molecules the infecting bacteria have on the cell surface and secrete into the surrounding environment, also termed virulence factors (Wilson et al., 2002). However, regardless of bacteria's morphology or whether they are harmless or not, the one thing they all have in common is a similar basic cell structure.

Bacterial cell structure

The bacterial cell can be split into three parts, the cytoplasm, cell envelope and external cell surface structures as shown in Fig. 3. Certain features are found in all bacterial cells, while others are specific to the bacterial species. This chapter will look at some of these features and how they may influence the ability of the bacteria to cause infections.

Cytoplasm

The inner most part of the bacterial cell is the cytoplasm where functions for cell growth, metabolism and replication are carried out. It is a predominately aqueous environment containing a nucleoid, ribosomes, inclusion bodies and plasmids. However, with the advancement in imaging and fluorescent proteins, different macromolecules within the cytoplasm including proteins, RNAs, DNA and lipids have been found to be organized and display complex subcellular localization patterns and are homologous to the eukaryotic cytoskeleton components actin, tubulin and intermediate filament protein (Ingerson-Mahar and Gitai, 2012). These cytoskeletal structures undergo dynamic reorganization and self-assembly, and are essential for processes ranging from cell division to differentiation, and localization of biochemical reactions for distributing metabolites (Govindarajan and Amster-Choder, 2016).

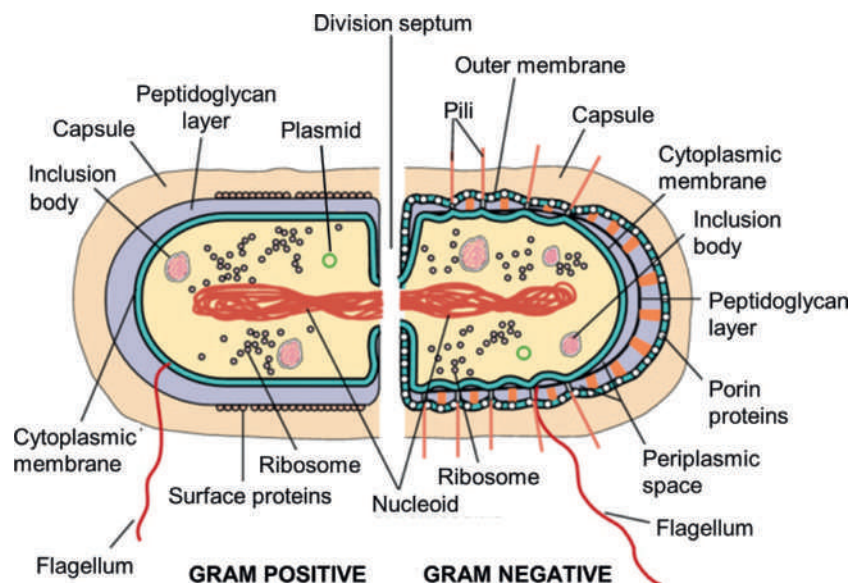


Fig. 3 Bacterial cell structures. From Murray PR, Rosenthal KS and Pfaller MA (2015) *Medical Microbiology*. Philadelphia: Elsevier, pp. 107.

Detailed information on the bacterial cytoskeleton which is beyond the scope of this chapter can be found in various open access reviews (Shih and Rothfield, 2006; Ingerson-Mahar and Gitai, 2012; Cho, 2015; Govindarajan and Amster-Choder, 2016).

Nucleoid

The nucleoid is a chromatin-dense area within the cytoplasm and contains the bacterial DNA, associated proteins and RNA that are responsible for controlling the bacteria's activity and reproduction (Fig. 3). As bacteria are haploid organisms, they have one chromosome that carries all the genes and genetic information it requires to survive as a living organism. The bacterial chromosome is a long circular molecule of double-stranded DNA, that is tightly looped and coiled into a supercoiled state and is not membrane bound (Thanbichler et al., 2005; Barer, 2018). The absence of a nuclear membrane means that when bacterial genes are transcribed the messenger RNA can be immediately transcribed and translated into protein, and that signals that influence bacterial transcription factors can do so directly on entering the cytoplasm (Dorman, 2013). The nucleoid is associated with several nucleoid-associated proteins (NAPs) that were formerly known as 'histone-like' proteins. NAPs are abundant within the chromatin as they bind to the chromosome DNA. Specifically, they have an important role in chromosome condensation and organization during DNA replication, and they also influence gene expression at the transcription level replication (Thanbichler et al., 2005; Dorman, 2013).

Plasmids

Many bacteria contain other naturally occurring genetic elements within the cytoplasm called plasmids (Fig. 3). Plasmids are small circular double stranded DNA that are distinct from the bacteria's chromosomal DNA; linear plasmids have also been found in a variety of bacteria such as *Borrelia* (cause of Lyme disease) and *Streptomyces* (largest antibiotic-producing genus) (Clark et al., 2019). They are supercoiled DNA molecules that vary in size from approximately few hundred to over a million base pairs (Barer, 2018). Plasmids are autonomous self-replicating molecules that are expendable as they rarely contain genes required for growth, but they often carry genes that provide the bacteria cell with a selective advantage such as antibiotic resistance. As mobile elements, some plasmids encode all the genetic information required to transfer from one bacterial cell to another via conjugation, while others can be transferred into the cell by transformation or transduction. There are two mechanisms that plasmids can use to spread, first is by direct transfer from one bacterium to another in a specific microenvironment, and secondly by being carried in a host to another such as in a hospital (Barer, 2018).

Bacteria may possess different plasmids in different numbers, this is called copy number, so may possess as few as one in the case of large plasmids or several hundred for smaller ones (Novick, 2002). The copy number influences the effect of plasmid related phenotypes such resistance to antibiotics, because more copies of the plasmid will mean more antibiotic genes resulting in increased antibiotic resistance (Murray et al., 2015). With the advent of genomic sequencing, plasmid DNA is also being sequenced, thus plasmids can now be typed according to their genetic features (Barer, 2018). Pathogenic bacteria are known to possess plasmids that carry genes for various virulence factors enabling them to successfully colonize hosts and cause infections (Thomas and Summers, 2008). In some cases plasmids can integrate into the bacterial chromosome, which results in the transfer of plasmid DNA/genomes into the bacteria's genome (Clark et al., 2019). Certain plasmids can also mediate the transfer of host chromosomal genes from one bacterial cell to another (Novick, 2002), and this is the basis of genetic engineering (which will not be discussed in this chapter).

Ribosomes

Ribosomes serve as the site of messenger RNA (mRNA), translation and protein synthesis in bacteria (Fig. 3). Bacterial ribosomes are composed of 30S and 50S subunits resulting in a sedimentation coefficient of 70S (Barer, 2018). Each ribosomal subunit comprises of specific ribosomal RNA (rRNA) and proteins. The 30S subunit comprises of 16S rRNA and 21 proteins, while 50S comprises of 5S and 23S rRNA, and 31 proteins (Madigan et al., 2019). The 16S, 23S and 5S rRNA are transcribed together with some tRNAs to give a single pre-rRNA (Clark et al., 2019). The rRNA then form part of the ribosome mechanism that translates the mRNA. Thus transcription and translation are coupled during protein synthesis, meaning the mRNA is read before the mRNA has been completed and released from the transcription complex, allowing the bacteria to grow and reproduce quickly (Grogan, 2011). Bacterial ribosomes and proteins are also very different to eukaryotic ribosomes making them a major target for antimicrobial compounds (Murray et al., 2015).

Inclusion bodies

Under certain growth conditions, inclusion bodies are produced in the cytoplasm of some bacteria and function as energy stores or carbon reservoirs (Fig. 3). They can often be seen without staining under a light microscope and are usually enclosed by a non-unit membrane protein layer, while others are defined as non-membrane (Hobot, 2015). Inclusion bodies are normally seen as dense, large spherical or cylindrical particles or aggregations, ranging from 0.2 μm to 1.2 μm , and composed of 80–95% of the heterologous expressed protein (Ramon et al., 2014). They may also contain other proteins such as heat shock proteins (e.g., IbpA), chaperons (e.g., DnaK system), phospholipids from membranes and nucleic acids (Ramon et al., 2014). Storing carbons such as glycogen, lipids such as poly- β -hydroxybutyric acid, inorganic phosphate in the form of polyphosphate and other substances in an insoluble form can be advantageous to the bacteria as it reduces osmotic stress and is a major energy storage mechanism for future use (Madigan et al., 2019). In recent years, inclusion bodies have been deemed as undesirable by-products in recombinant protein production, while their ability to form aggregates has been utilized as a model to understand protein aggregation in human diseases such as Huntington disease and Alzheimers (Ramon et al., 2014).

Cell envelope

Cytoplasmic membrane

The cytoplasmic membrane is the innermost layer of the cell envelope and surrounds the cytoplasm, vitally separating it from the environment (Fig. 3). It is structurally weak giving little protection from osmotic lysis hence, if the cytoplasmic membrane is damaged or compromised the cell's integrity is destroyed and the cytoplasm leaks into the environment leading to cell death. However, the cytoplasmic membrane structure is a semipermeable barrier perfect for the movement of solutes in and out of the cell.

The cytoplasmic membrane comprises of a phospholipid bilayer similar to eukaryotic cells but without the sterols/cholesterol (Murray et al., 2015). The bacterial phospholipid bilayer is made up of two hydrophobic fatty acid tails that point inward towards each other with hydrophilic glycerophosphate heads pointing out into the environment or cytoplasm (Fig. 4). The phospholipid bilayer's oily nature gives flexibility and fluidity to the membrane making it a semifluid environment (Madigan et al., 2019). The cytoplasmic membrane also has a high protein content and contribute to the mechanical structure of the membrane (Barer, 2018). Integral membrane proteins are firmly embedded in the membrane bridging the whole membrane, while peripheral membrane proteins have one end anchored in the membrane with the other end pointing in or out of the cell (Fig. 4). Some peripheral membrane proteins are lipoproteins as they have a lipid tail that anchors them to the cytoplasmic membrane. Peripheral and

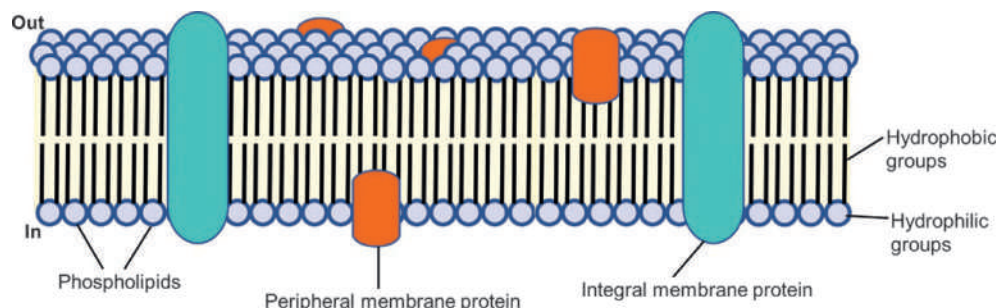


Fig. 4 Structure of cytoplasmic membrane. In—faces the cytoplasm; Out—faces the environment.

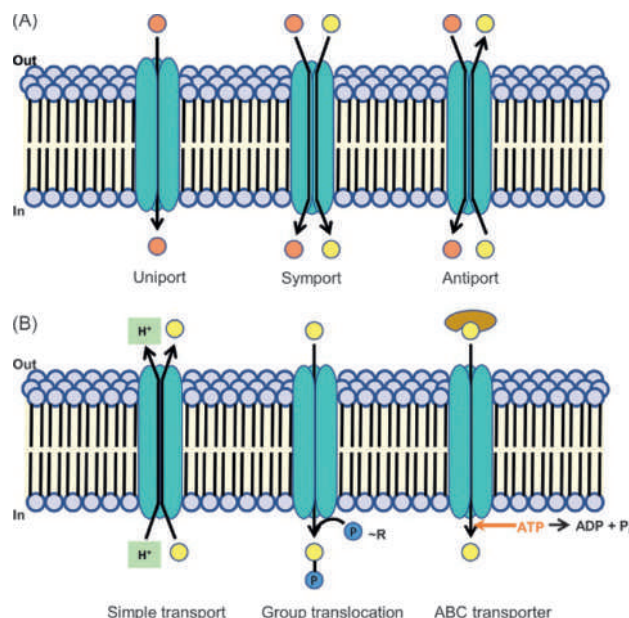


Fig. 5 Membrane-spanning transport systems. (A) Carrier protein systems; (B) Classes of transport systems.

integral membrane proteins interact for important cellular processes such as energy metabolism and transport (Grogan, 2011; Madigan et al., 2019). Such proteins usually cluster together so they remain close to each other in the semifluid environment of the cytoplasmic membrane.

One of the cytoplasmic membrane's functions is as a selective permeable barrier that regulates the movement of substances in and out of the cell and maintaining and regulating the bacterial cell's internal ionic concentration and osmotic pressures (Hobot, 2015). The hydrophobic component of the membrane acts as a tight barrier allowing the passive diffusion of water and some small hydrophobic molecules but not the passage of larger or charged molecules, which have to be transported through the membrane via transport proteins, also known as transport systems or permeases (Hobot, 2015; Madigan et al., 2019). Transport proteins generally span the membrane to form a channel, and it is through this channel that they mediate the passage of substances by one of three types of transport processes catalyzed by a protein called porter (Fig. 5).

Uniporters carry the substance across the membrane unidirectionally, Symporters or cotransporters transport two substances across the membrane at the same time, typically the substance and a proton, while Antiporters transport one substance into the cell and simultaneously transport a second substance out of the cell (Madigan et al., 2019). Transport proteins operate by one of three transport mechanisms (Fig. 5B), all of which require energy from ATP, proton motive force or some other energy-rich organic compound (Grogan, 2011). The simple transport mechanism (Fig. 5B) consists only of a membrane spanning transport protein that is driven by the proton motive force. Essentially the mechanism exploits the principle that a proton gradient can be used as an energy source by a symporter transport protein to move the substance across the membrane (Grogan, 2011). A well understood example is the transportation of lactose into *Escherichia coli* by the simple transport protein, lactose permease (Deutscher et al., 2006). Group translocation involves modifying the chemical structure of the substance as it is transported across the membrane and is driven by an energy-rich organic compound rather than proton motive force or ATP (Fig. 5B). One of the best studied examples is the phosphotransferase system, used to transport sugars such as glucose across the membrane of *E. coli* (Saier Jr, 2015). The system is driven by phosphoenolpyruvate, an energy-rich intermediate of glycolysis and involves the phosphorylation of the sugar as it passes through the membrane. The third mechanism is the ABC transporter that consists of three proteins, one of which hydrolyzes ATP for energy (Fig. 5B). This transport mechanism utilizes proteins bound to the external surface of the cytoplasmic membrane or periplasmic binding proteins along with the transporter proteins and ATP to move sugars, amino acids, inorganic nutrients such as sulfate and phosphate and some metals across the cytoplasmic membrane (Madigan et al., 2019).

The bacterial cytoplasmic membrane not only acts as a permeability barrier, but also carries out many of the functions carried out by the organelles in eukaryotic cells, therefore it is a major site for electron transport and energy production (Hobot, 2015). One important energy source associated with the membrane is the proton motive force. Since the membrane can have positively charged protons (H^+) on the outside of the membrane with hydroxyl ions (OH^-) on the inside, a charged energized state is created which can drive many energy-requiring functions such as transport proteins, motility and biosynthesis of ATP (Madigan et al., 2019). The cytoplasmic membrane also contains proteins and enzymes involved in cell wall synthesis including membrane synthesis and septum formation during replication, detecting the concentration of molecules in the surrounding environment, and translocating signals to genetic and metabolic processes in the cytoplasm (Salton et al., 1996; Wilson et al., 2002).

Cell wall

On the outside of the cytoplasmic membrane and covering the whole cell is the cell wall (Fig. 3). The cell wall's main functions are to maintain the mechanical strength and shape of the bacterial cell while protecting it from the internal osmotic pressure found in the cytoplasm, as its integrity is critical to the cell's viability (Scheffers and Pinho, 2005). Thus, if the cell wall is damaged, the cytoplasmic content will be released into the surrounding environment resulting in cell death (cell lysis (Hobot, 2015)). The cell wall is composed of a rigid layer of peptidoglycan (PG), also known as murein (Weidel and Pelzer, 1964). PG is a heteropolymer that consists of long glycan chains of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) sugars that are cross-linked by short peptides of D- and L-amino acids (Fig. 6A) (Coleman and Smith, 2014). The chemistry of the glycan chain only differs slightly between bacteria, while the peptide cross-links vary considerably and are responsible for giving the peptidoglycan structure the flexibility needed to allow the bacteria to alter its shape and cell wall mechanical properties during growth and differentiation (Knox and Wicken, 1973; Scheffers and Pinho, 2005). Differences in the structure of the PG are used to differentiate between Gram-positive and Gram-negative bacteria and will be discussed in detail below. The PG can be destroyed by enzymes such as lysozyme found in tears, saliva and other bodily fluids, and is a defence mechanism used by other bacteria, leading to the lysis of the cell wall (Vollmer, 2015). It is also a target for various antibiotics such as β -lactams (penicillin, cephalosporin) and glycopeptides (vancomycin), as they inhibit peptidoglycan formation (Coleman and Smith, 2014).

Gram-positive bacteria

Gram-positive bacteria have a thick PG layer approximately 10–20 layers thick that retains the crystal violet stain in a Gram stain giving the bacteria a purple color (Fig. 6C; Scheffers and Pinho, 2005). Most Gram-positive bacteria cell walls are also composed of teichoic acids (TAs) or related glycopolymers, capsular polysaccharides and cell wall associated proteins, and collectively they make up a polyanionic mesh-like matrix that surrounds the cytoplasmic membrane giving the Gram-positive bacteria cell wall elasticity, porosity and tensile strength (Xia et al., 2010; Neuhaus and Baddiley, 2003). TAs are composed of wall teichoic acids (WTA) and lipoteichoic acids (LTA). WTAs are water-soluble anionic polymers of glycerol phosphate or ribitol phosphate linked by phosphodiester bonds covalently bound to MurNAc residues and known WTA structures have a great diversity between bacterial species and even between clonal groups. While LTAs are a macroamphiphile with a glycolipid anchored covalently to the cytoplasmic membrane (Neuhaus and Baddiley, 2003). Together WTAs and LTAs may contribute to the net negative charge of Gram-positive bacteria cell walls (Xia et al., 2010), but their overall function remains unclear, although it has been suggested that they have a role in adhesion, cell wall maintenance, and host-mediated immune responses (Neuhaus and Baddiley, 2003; Xia et al., 2010).

Gram-negative bacteria

Gram-negative bacteria have a more complex multi-layered cell wall that stains pink-red in a Gram stain. The Gram-negative cell wall consists of a thin PG layer next to the cytoplasmic membrane and an outer membrane external to the PG (Fig. 6D) (Bower and Rosenthal, 2006). In-between the PG and outer membrane is the periplasm or periplasmic space. Within this 10–25 nm gel-like space are proteins that facilitate the transport of molecules in and out of the cell, and hydrolytic enzymes such as proteases, phosphatases and lipases involved in metabolism (Graham et al., 1991; Bower and Rosenthal, 2006; Murray et al., 2015). The outer membrane consists of an inner layer composed of mainly phospholipids and an outer layer composed of lipopolysaccharide (LPS, Fig. 6C) (Bower and Rosenthal, 2006). LPS is composed of three parts: lipid A, core polysaccharide chain, and O-specific polysaccharide or O antigen (Bower and Rosenthal, 2006). Lipid A is a hydrophilic region composed of fatty acids attached to a disaccharide backbone and is a strong stimulator of the human innate and immune responses. Lipid A as an endotoxin will be discussed in more detail later in the chapter. The core region is a branched polysaccharide of 9–12 sugars, including negatively charged 2-keto-3-deoxy-ocanate, and is phosphorylated (Hancock, 1997). Together with lipid A the core region is an essential part of the LPS structure and bacteria viability, as divalent cations bridge the phosphates on lipid A and the core region to give the outer membrane strength (Murray et al., 2015). The O antigen region is attached to the core region and consists of 50 to 100 repeating units of 3–7 sugars that extend away from the bacteria. Differences in this region are used clinically to distinguish different bacterial strains by their O antigen serotype, for example, *E. coli* O157:H7 (O antigen: flagellin) serotype for the enterohemorrhagic *E. coli* (Bower and Rosenthal, 2006).

Importantly the outer membrane maintains the bacteria structure but also functions as a selective permeability barrier (Hancock, 1997). To aid with the transport of small molecules through the membrane are proteins called outer membrane proteins (OMP) or porins (Fig. 6C). Porins are made of three subunits that traverse the outer membrane to form a 1 nm in diameter channel that allows the passage of small and hydrophilic molecules but restricts access to larger and hydrophobic molecules such as bile salts, toxins, lysozyme, and many antibiotics (Bower and Rosenthal, 2006). The outer membrane also contains secretion systems that traverse the membrane to allow for the secretion of molecules such as toxins hydrolytic enzymes and other proteins out of the bacterial cell. To date there are 6 known secretion systems called type I to VI, which either secrete the molecule directly out of the cell in a single process (Type I, III, IV and VI) or move it into the periplasm to await transport across the outer membrane (Type II and V; Dalbey and Kuhn, 2012; Madigan et al., 2019). Type III and IV secretion systems are discussed in detail later in this chapter.

Acid-fast bacteria—Mycobacterium

Mycobacteria are Gram-positive bacteria but their cell wall is composed of a thick PG layer covered by covalently bound arabinogalactan (AG) polysaccharides and mycolic acids (2-alkyl-3-hydroxy fatty acids), and a variety of glycolipids, sulfolipids, and glycerophospholipids (Coleman and Smith, 2014). The high lipid content gives the cell wall a wax-like coat that is very hydrophobic in nature resulting in such bacteria being resistant to Gram staining, therefore they are stained using an acid-fast test such as Ziehl-Neelsen, and hence being called acid-fast bacteria (Murray et al., 2015). The complex nature of the acid-fast cell wall also makes it more resistant to antibiotics as they are unable to penetrate the cell and has anti-phagocytic properties. To facilitate the uptake of nutrients the cell wall layer also contains porin proteins (Coleman and Smith, 2014).

Cell surface structures and components

Bacteria also have structures or components on the outside of their cell wall that are in contact with the environment, many of which are classed as virulence factors (Fig. 3). The structures described in detail below are important in the survival of the bacteria, and in clinically relevant bacteria help initiate infections.

Capsules and slime layers

Many bacteria produce a capsule or slime layer on their cell surface. These terms are often used interchangeable, but they are actual two different structures, neither of which confer any structural strength to the bacteria cell or are required for growth (Roberts, 1996). A capsule also known as a capsular polysaccharide is a dense, hydrated, tightly organized layer of complex polysaccharide that covalently adheres to the bacteria cell wall phospholipid or lipid-A molecules (Fig. 7A; Roberts, 1996). Not all capsules are polysaccharide based, some Bacilli have simple polypeptide capsules. In comparison, a slime layer is a diffuse layer, composed of extracellular polysaccharide (EPS) molecules that are of loosely bound to the cell wall so can easily be lost from the cell wall (Fig. 7B; Roberts, 1996).

Capsular polysaccharides are composed of over 95% water and repeating monosaccharides coupled by glycosidic linkages. The composition of capsules is very diverse, and is species and strain dependent (Corbett and Roberts, 2008). In human pathogens such as *E. coli*, *Streptococcus pneumoniae*, Group A *Streptococci*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, a large number of different capsule serotypes have been identified (O’Riordan and Lee, 2004; Roberts, 1996) and this knowledge is often used to type these bacteria. For example, *E. coli* has over 80 different capsular polysaccharides designated K antigens or a polymer derived from 170 different O antigens (Schembri et al., 2004). Certain serotype K1 and certain types of O antigens are associated with specific infections, such as K1 capsule with neonatal meningitis and urinary tract infection (UTI), and K5 capsule with UTI and sepsis (Schembri et al., 2004). Thus, capsular polysaccharides are often used as a typing tool in the diagnosis and identification of clinical bacteria (Table 1).

In pathogenic bacteria capsular polysaccharides and EPS are classed a key virulence factors, as they have a role in colonization and are involved in microbe-host interactions due to their antigenic properties (Table 2) (Roberts, 1996; O’Riordan and Lee, 2004; Schembri et al., 2004). Bacteria with a capsular polysaccharide are known to have an enhanced ability to adhere and colonize epithelial and endothelial cells, and mucosal surfaces compared to unencapsulated bacteria (Wen and Zhang, 2015). The primary role of the bacterial capsule is to protect the bacteria from the host innate and adaptive immune systems. Capsular polysaccharide expression has been shown to confer resistance to complement-mediated killing in the absence of a specific antibody as the capsule

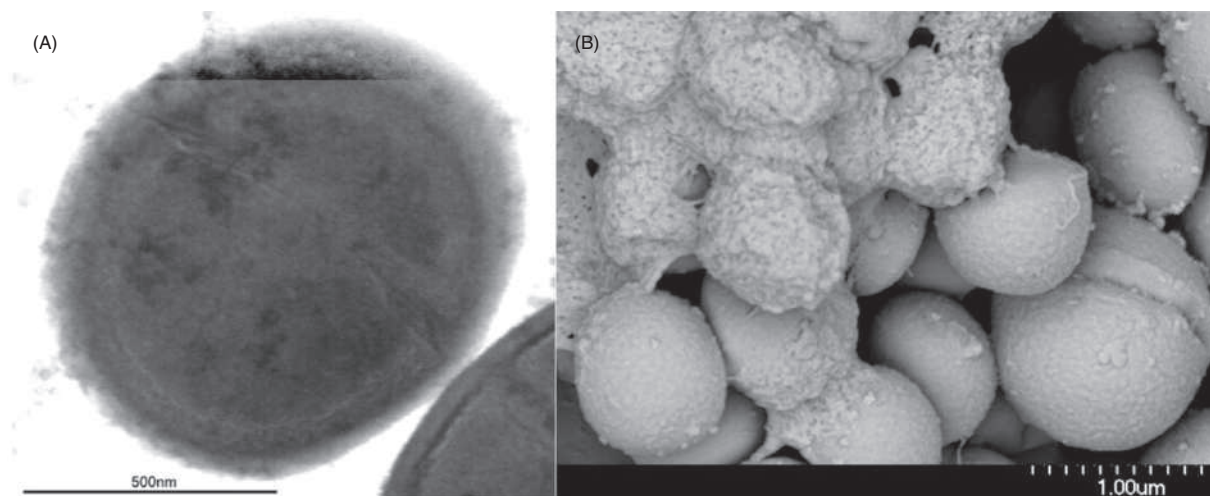


Fig. 7 Examples of bacteria with (A) a capsular polysaccharide surrounding the bacteria outer surface, and (B) EPS in association with a bacterial biofilm.

Table 1 Pathogenic bacteria with capsules.

Bacteria	No. serotypes
<i>Acinetobacter baumannii</i>	n/d
<i>Campylobacter jejuni</i>	47
<i>Escherichia coli</i>	80
<i>Haemophilus influenzae</i>	6
<i>Klebsiella pneumoniae</i>	82
<i>Neisseria meningitidis</i>	13
<i>Pseudomonas aeruginosa</i>	1
<i>Salmonella enterica</i> serovar Typhi	1
<i>Streptococcus pneumoniae</i>	94
<i>Streptococcus agalactiae</i>	9
<i>Streptococcus pyogenes</i>	1
<i>Streptococcus suis</i>	35
<i>Staphylococcus aureus</i>	11

n/d, not determined.

Modified from Wen Z and Zhang J (2015) Bacterial capsules. In: Tang YW, Poxton I and Schwartzman J (eds.), *Molecular Medical Microbiology*. London: Elsevier.

Table 2 Capsular polysaccharide virulence associated functions.

Function	Virulence role
Prevention of desiccation	Transmission and survival
Adherence	Colonization of oral surfaces Colonization of indwelling catheters
Resistance to nonspecific host immunity	Complement-mediated phagocytosis Complement-mediated killing
Resistance to specific host immunity	Poor antibody response to the capsule

acts as a permeable barrier to complement factors, so the underlying bacteria cell surface structures that would have been potent activators of the alternative pathway are masked (Roberts, 1996). The capsule may also confer resistance to complement-mediated opsonophagocytosis by polymorphonuclear leukocytes (PMNL) (Corbett and Roberts, 2008; Wen and Zhang, 2015). Evidence also suggests that certain capsular polysaccharides may affect cytokine release by host's cells, thus disrupting the immune response cascade (Roberts, 1996). While most capsular polysaccharides can trigger an immune response, a small set of capsular polysaccharides such as *E. coli* serotype K1 are poorly immunogenic, thus the infected person mounts a poor antibody response to the bacteria while the capsule gives the bacteria some form of resistance against host's specific immune responses (Roberts, 1996).

The EPS in bacteria such as *Staphylococcus epidermidis*, *Streptococci* and *Pseudomonas aeruginosa*, is associated with biofilm formation. Biofilms are a thick layer of bacteria bound together by the EPS (Fig. 7B) and defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum, interface or to each other, and are embedded in a matrix of EPS that they have produced (Donlan and Costerton, 2002). The ability of such bacteria to adhere to surfaces is a critical first step in biofilm formation and how they adhere is discussed in detail below. Once bacteria have adhered to a surface they accumulate and form biofilms with the production of EPS by the sessile bacteria (Fig. 7). Bacterial EPS is composed of polysaccharides, proteins and/or extracellular DNA (eDNA), and similar to capsule composition, EPS components and the amount produced varies between and within species (Donlan and Costerton, 2002). Biofilm formation has been implicated as the cause of chronic and persistent infection in a variety of diseases as highlighted in Table 3. Once a biofilm has formed, the biofilm facilitates resistance against the host's immune system response (Sabate Bresco et al., 2017) and confers antibiotic resistance as the antibiotics cannot penetrate the biofilm effectively (Donlan and Costerton, 2002; Mack et al., 2006). Biofilm formation also complicates medical and surgical treatment protocols as implant removal is often required to eradicate the biofilm. Further information on biofilm formation by staphylococci and streptococci and its implication in the pathogenesis of these bacteria can be found in (Geoghegan and Speziale, 2015).

The antigenic properties of the capsular polysaccharide have been used as targets in the development of vaccines against clinical bacteria such as *Strep. pneumoniae* (Geno et al., 2015) and *Haemophilus influenzae* type B (Giesker and Hensel, 2014). However due to the lack of immunogenicity in young children and the elderly, polysaccharide vaccines have been conjugated to a carrier protein (glycoconjugated vaccine) to stimulate an immune response against bacteria that cause meningitis and pneumonia (De Gregorio et al., 2013; Murray et al., 2015). Vaccines against bacteria that form biofilms such as *S. epidermidis*, *S. aureus* and *P. aeruginosa* have been researched for years (McKenney et al., 2000; Tümmler, 2019), but there are no licensed vaccines at present against such bacteria, mainly because of the variation in the expression of the targeted EPS molecules and failure to show efficacy in clinical trials (Moriarty et al., 2016; Tümmler, 2019).

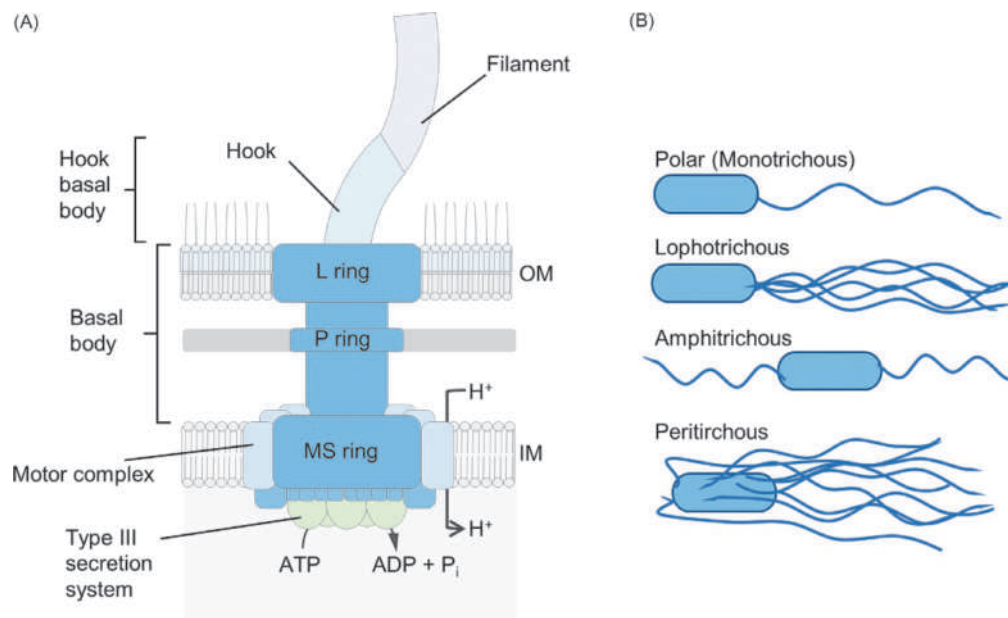
Table 3 Bacteria commonly associated with biofilm-related infections.

Biofilm-related infection	Bacteria species
Cystic fibrosis lung infection	<i>S. aureus</i> , <i>Haemophilus influenzae</i> , <i>P. aeruginosa</i> , <i>Klebsiella pneumoniae</i>
Central venous catheter	<i>S. aureus</i> , <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>Enterococcus faecalis</i>
In-dwelling urinary catheter	<i>Staphylococcus epidermidis</i> , <i>Enter. faecalis</i> , <i>E. coli</i> , <i>Proteus mirabilis</i> , <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>Enterobacter aerogenes</i>
Contact lens	<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>S. epidermidis</i> , <i>E. coli</i> , <i>Serratia</i> spp., <i>Proteus</i> spp.
Chronic wound	<i>Staphylococcus</i> spp., <i>Pseudomonas</i> spp., <i>Enterobacter</i> spp., <i>Stenotrophomonas</i> spp., <i>Proteus</i> spp., <i>Morganella</i> spp., <i>Serratia</i> spp.
Orthopaedic device related infections	<i>S. epidermidis</i> , <i>S. aureus</i> , coagulase negative staphylococcus (CoNS), <i>Propionibacterium acnes</i> , <i>E. coli</i> , <i>P. aeruginosa</i> , Non- <i>E. coli</i> Enterobacteriaceae, <i>Enterococcus</i> spp., <i>Streptococcus</i> spp.
Endocarditis	<i>Streptococci</i> (including viridans streptococci, enterococci, pneumococci, and <i>Streptococcus bovis</i>), <i>S. aureus</i> , CoNS including <i>S. epidermidis</i>
Otitis media (middle ear infection)	<i>Strep. pneumoniae</i> , <i>H. influenzae</i> , <i>Moraxella catarrhalis</i> , group A beta-hemolytic streptococci, enteric bacteria, <i>S. aureus</i> , <i>S. epidermidis</i> , <i>P. aeruginosa</i>

Flagella

Many bacteria are motile by swimming due to structures called flagella (flagellum singular) and found on Gram-negative and -positive bacteria. Flagella are long, thin helical shaped appendages that are anchored to the bacterium in the cytoplasmic membrane and cell wall and a rope-like filament protruding out of the cell wall (Fig. 8; Murray et al., 2015). Depending on the species, a bacterium can have one or several flagella on their cell surface. In rod shaped bacteria, flagella can be attached at one or both ends of the cell (polar flagella), or several can arise like a tuft at one end of the cell (lophotrichous flagella), or they can be inserted at many locations along or around the cell (peritrichous flagella) (Fig. 8; Madigan et al., 2019). The latter is commonly seen in motile coccus shaped bacteria. Flagella type is also used in the classification of bacteria.

The flagellum is composed of several components (Fig. 8) and like a tiny motor, propels the bacteria towards nutrients and away from harmful compounds (chemotaxis). The basal body comprised of multiple ring-like subunits anchors and powers the flagella, the hook connects the filament to the basal body, and the filament extends out of the cell (Fig. 8). The filament is composed of multiple subunits of the protein flagellin, which to some extent determines the structure and shape of the filament, and the rotation direction (Aizawa, 2015). Flagella rotate in an anticlockwise or clockwise direction, but not at a constant speed, rather they increase and decrease their rotational speed as needed. Depending on the number of flagella and their location on the cell surface the bacteria can move in a linear direction or tumble (Aizawa, 2015). Bacteria with peritrichous flagella tend to move slowly in a straight line, while polar flagella allow the bacteria to move rapidly, tumbling or spinning around in a zig zag motion (Madigan et al., 2019). Swarming motility is also seen in bacteria that are hyperflagellated such as *Proteus* spp. where thousands of flagella

**Fig. 8** Flagellum in Gram-negative bacteria (A) structure of flagellum, and (B) different flagella arrangement.

propel the bacteria forwards in a smooth motion (Aizawa, 2015). Motility is an important virulence factor for many bacteria as the flagella not only allows the bacteria to move but also expresses antigenic properties that activate innate immune responses (Aizawa, 2015; Murray et al., 2015).

Fimbriae and pili/fibrils

Fimbriae and pili are small hair-like filamentous protein appendages that extend from the bacterial cell surface of Gram-negative and -positive bacteria (Fig. 3). Fimbriae are morphologically distinct from flagella as they are much shorter, with smaller diameters, straighter without the curved spiral structure of flagella, and are much more numerous on the cell surface (Murray et al., 2015). Multiple types of fimbriae exist, and their functions are varied. One of the first roles associated with pili was in facilitating genetic exchange between bacteria as sex pili confer the ability to bind to other bacteria and initiate the process of conjugation, and they also function as receptors for bacteriophages (Barer, 2018). In pathogenic Gram-negative bacteria such as *E. coli* (UTIs, gastrointestinal infections), *Salmonella* spp. (salmonellosis, food poisoning), *Neisseria gonorrhoeae* (gonorrhoea) and *Bordetella pertussis* (whooping cough) fimbriae promote adherence and/or invasion to host tissues (Lukaszczuk et al., 2019), while in *E. coli* and Gram-positive bacteria such as *Staphylococcus* spp. and *Streptococcus* spp., curli amyloid fibrils are associated with adhesion and biofilm formation (Volkan et al., 2015). Pili are longer in length, and Type IV pili are found in various Gram-negative bacteria such as *P. aeruginosa*, *N. gonorrhoeae*, *Neisseria meningitidis*, and *Vibrio cholerae* (cholera), as they assist in adhesion but are also involved in twitching motility (Lukaszczuk et al., 2019). In twitching motility, the pili are found on the poles of rod shaped bacteria and by extending and retracting, drag the cell across a surface in a gliding-like motion, thus Type IV pili have been implicated as important in the colonization of bacteria such as *N. gonorrhoeae* and *V. cholerae* as they assist the bacteria in finding suitable adhesion sites for the bacteria to initiate an infection (Madigan et al., 2019). As mentioned above, Type IV pili also have a role in the transfer of DNA into other cells via conjugation.

Other virulence factors

Cell wall associated proteins in Gram-positive pathogens

Pathogenic Gram-positive cocci such as *Staphylococci* and *Streptococci* express a variety of virulence factors on their cell wall, including surface proteins, that are covalently bound to the cell wall peptidoglycan (Fig. 6; Nobbs et al., 2009; Foster, 2019). These cell wall associated (CWA) proteins have an important role in adhesion, colonization of host tissues and evasion of host defences.

The most prevalent CWA proteins are the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). They have been described in great detail in *S. aureus* but are also present in *S. epidermidis*, other *Staphylococcus* spp., *Streptococcus* spp. and *Enterococcus* spp. (Foster, 2019). However, the precise number of CWA proteins expressed varies from strain to strain and within a species, and is dependent on growth conditions and growth phase (exponential vs. stationary phase; Foster, 2019). Due to the limited number of proteins expressed on the surface of the bacteria, a single MSCRAMM can carry out several functions. However, all promote bacterial adhesion to abiotic surfaces and synthetic surfaces coated with host extracellular matrix components (such as fibrinogen, fibronectin collagen and laminin), and various host cells (Table 4; Pietrocola et al., 2018; Foster, 2019). A typical MSCRAMM contains a secretory Sec-dependent signal sequence at the N-terminus, and a sorting signal comprising of an LPXTG (Leu-Pro-X-Thr-Gly; X denoted any amino acid) sortase cleavage motif, a hydrophobic domain and positively charged residues at the C-terminus (Fig. 9; Nobbs et al., 2009; Foster, 2019). The proteins are bound to the cell wall peptidoglycan via the C-terminus through the activity of Sortase A, while the N-terminal A domains are projected away from the cell wall surface ready to bind to a suitable host ligand.

CWA proteins and MSCRAMMs have been well studied in *S. aureus*. Depending on strain, *S. aureus* can express up to 24 different CWA proteins, that have multiple roles in promoting adhesion to host cells and tissue, survival in blood during an invasive infection, and in the formation of abscesses in the skin and internally (Table 4; Foster et al., 2014; Foster, 2019). Most *S. aureus* strains have two fibronectin binding proteins (FnBPA and FnBPB), up to five proteins that bind fibrinogen, and several that promote adhesion to squamous epithelial cells and complement factors of the host immune system (Table 4; Foster, 2019). While Group A streptococci such as *Strep. pyogenes* have a MSCRAMM called M protein on their cell surface (Fig. 9; Table 4). They function like a capsule but are a CWA as they have an LPXTG motif and bind both fibrinogen and fibrin, thus inhibiting the alternative complement pathway. M protein is also responsible for inhibiting neutrophil phagocytosis by altering the bacteria's cell surface. The importance of CWA proteins and MSCRAMMs in the pathogenesis of Gram-positive cocci are described in detail in the following reviews (Nobbs et al., 2009; Foster et al., 2014; Pietrocola et al., 2018; Foster, 2019).

Type III secretion system

Type III secretion systems also known as injectosomes are an important virulence factor as they have a molecular syringe-like structure that injects proteins, known as effector proteins directly into eukaryotic cells (Bower and Rosenthal, 2006). Therefore, they are not a true secretion system as they do not export bacterial products into the surrounding environment, but are an effective system that delivers specific virulence factors such as toxins directly into the cytoplasm of the host cell (Fig. 10; Wilson et al., 2002). A type III secretion system consists of a multi-protein ring that attaches it to the cytoplasmic membrane, an inner

Table 4 CWA proteins identified in various Gram-positive bacteria, collated from (Nobbs et al., 2009; Foster et al., 2014; Pietrocola et al., 2018; Foster, 2019; Shivshankar et al., 2009; and Suits and Boraston, 2013).

CWA protein/ MSCRAMM	Bacteria	Ligand	Role in pathogenesis
ClfA	<i>S. aureus</i>	Fibrinogen	Adhesion to fibrinogen, thrombus—endocarditis, medical device infections
ClfB	<i>S. aureus</i>	Complement factor I Fibrinogen	Inhibition of opsonophagocytosis—bacteremia, sepsis Adhesion to fibrinogen, thrombus—endocarditis, medical device infections Adhesion to desquamated epithelial cells—nasal colonization Enhanced survival from neutrophils—bacteremia
FnBPA	<i>S. aureus</i>	Fibronectin	Adhesion to fibronectin, thrombus—endocarditis, medical device infections Biofilm formation—medical device infections Invasion adjacent endothelium and epithelial cells in mammary glands—mastitis Abscess development
FnBPB	<i>S. aureus</i>	Fibronectin	Adhesion to fibronectin—medical device infections Biofilm formation—medical device infections Invasion epithelial cells in mammary glands—mastitis Abscess development
Cna	<i>S. aureus</i>	Collagen Complement protein C1q	Adhesion to collagen-rich tissue e.g., cartilage within joint and blood—septic arthritis Prevention of complement activation, and enhanced survival in
SdrE	<i>S. aureus</i>	Complement factor H	Immune evasions, inhibition of complement in degradation of C3b
SdrD	<i>S. aureus</i>	Desquamated epithelial cells	Nasal colonization?
Protein A	<i>S. aureus</i>	IgG von Willebrand factor	Inhibition of opsonophagocytosis Increased inflammation Endovascular infection, endocarditis
SasG	<i>S. aureus</i>	Fibronectin	Biofilm formation—medical device infections Adhesion to desquamated epithelial cells
Embp	<i>Staphylococcus epidermidis</i>	Fibronectin	Adhesion to fibronectin, promotes accumulation Adhesion to conditioned biomaterial?
SdrG/Fbe	<i>S. epidermidis</i>	Fibrinogen	Adhesion to fibronectin Promotes adhesion to conditioned biomaterial—medical device infections
SdrF/Geh	<i>S. epidermidis</i>	Collagen	Adhesion to collagen deposited on ex vivo biomaterial—medical device infections
SdrI	<i>Staphylococcus saprophyticus</i>	Fibronectin Collagen	Adhesion to fibronectin and collagen—persistence in urinary tract infection
Fbl	<i>Staphylococcus lugdunensis</i>	Fibrinogen	Adhesion to fibrinogen—medical device infections?
SpsD	<i>Staphylococcus pseudintermedius</i>	Fibrinogen Fibronectin Cytokeratin 10	Adhesion to canine fibrinogen, fibronectin and cytokeratin 10—colonization of the host and skin infection
SpsL	<i>S. pseudintermedius</i>	Fibrinogen Fibronectin	Adhesion to canine fibrinogen and fibronectin—colonization of the host and skin infection
M protein	<i>Strep. pyogenes</i>	Fibrinogen Fibronectin Plasminogen Complement factor H	Adhesion to fibrinogen—pro-inflammation, anti-phagocytic activities, Adhesion to epithelial cells Adhesion to plasminogen—increase bacterial extracellular proteolytic activity. Inhibits complement activation
FbaA	<i>Strep. pyogenes</i>	Fibronectin Complement factor H	Adhesion to fibronectin
SfbI	<i>Strep. pyogenes</i>	Fibronectin	Adhesion to fibronectin
ScIA, ScIB	<i>Strep. pyogenes</i>	Collagen	Adhesion to collagen

SrpA	<i>Strep. pyogenes</i>	Multiple substrates	Adhesion to platelets
Mrp	<i>Strep. pyogenes</i>	Fibrinogen	Adhesion to fibrinogen
Lbp,	<i>Strep. pyogenes</i>	Laminin	Adhesion to laminin and epithelial cells
Protein H	<i>Strep. pyogenes</i>	IgG	Escape detection by immune system
SfbI/PrtF1	<i>Strep. pyogenes</i>	Fibronectin	Adhesion to fibronectin—adhesion epithelial/endothelial cells
		IgG	Escape detection by immune system
SibA	<i>Strep. pyogenes</i>	IgG, IgA, IgM	Escape detection by immune system
Srr1/Srr2	<i>Strep. agalactiae</i>	Fibrinogen	Adhesion to fibronectin, epithelial cells, platelets, and salivary glycopeptides
		Cytokeratin	Role in thrombus formation?
FbsA, FbsC	<i>Strep. agalactiae</i>	Fibrinogen	Adhesion to fibronectin—adhesion epithelial/endothelial cells
PbsP	<i>Strep. agalactiae</i>	Plasminogen	Adhesion to plasminogen
			Role in transmigration across brain endothelial cells
PfbA	<i>Strep. pneumoniae</i>	Fibronectin	Adhesion to and evasion of epithelial cells
		Plasminogen	
PsrP	<i>Strep. pneumoniae</i>	Keratin 10	Adhesion to lung epithelial cells

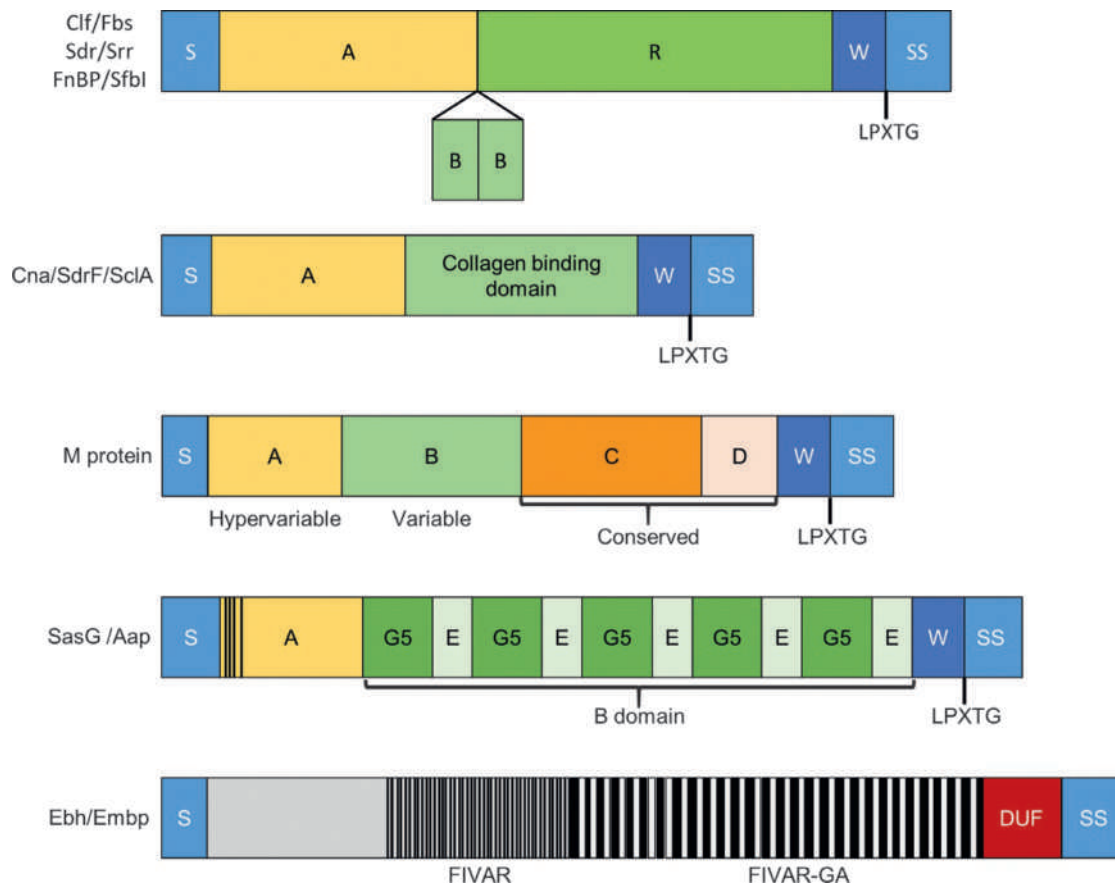


Fig. 9 Structural motifs of some CWA proteins in *S. aureus* (Clf, Sdr, FnBPs, Cna, SasG and Ebh), *S. epidermidis* (Sdr, Aap and Embp), and *Strep. pyogenes* (Srr, Sfbl, SclA, and M protein). Each CWA protein contains a secretory signal sequence (S) at the N-terminus, and a proline-rich wall-spanning region (W), LPXTG motif, and sorting signal (SS) at the C-terminus that promote covalent anchorage to the cell wall PG. A, ligand binding domains; R, serine-aspartate dipeptide repeats (Clf, Sdr, Srr) or fibronectin-binding repeats (FnBP/Sfbl); B, variable repeat domain with ligand-binding properties; C, albumin-binding domain; G5E repeat region promote adhesion and biofilm formation when the A domain is removed; FIVAR, found in various architecture repeats and FIVAR-GA, FIVAR-G-related albumin binding are two long repeat regions with fibronectin binding properties; DUF, domains of unknown function. Adapted from Nobbs AH, Lamont RJ and Jenkinson HF (2009) Streptococcus adherence and colonization. *Microbiology and Molecular Biology Reviews* **73**: 407–50; Foster TJ (2019) The MSCRAMM family of cell-wall-anchored surface proteins of Gram-positive cocci. *Trends in Microbiology* **27**: 927–941; Foster TJ (2020). Surface proteins of *Staphylococcus epidermidis*. *Frontiers in Microbiology* **11**: 1829.

tube that traverses the bacterial peptidoglycan and outer membrane layers, and a needle-like tip that pierces through the eukaryotic cell membrane by forming a pore, delivering the effector proteins directly into the host cell cytosol (Fig. 10; Bower and Rosenthal, 2006). Type III secretion systems have been found in Gram-negative bacteria such as enteropathogenic *E. coli* (EPEC, diarrhoea), *P. aeruginosa*, *Shigella flexneri* (shigellosis, diarrhoea), *Salmonella typhimurium* (typhoid), *Yersinia* spp., and *Chlamydia trachomatis* (chlamydia). Of those identified, they have been found to mediate the bacteria adhesion and invasion to host cells, interfere with host cell signaling, induce cytoskeletal rearrangements, promote host cell apoptosis, and up- or down-regulate cytokine production (Wilson et al., 2002; Bower and Rosenthal, 2006). The actual effector proteins and their virulence properties vary among the different bacteria but without a type III secretion system the bacteria are less virulent (Wilson et al., 2002).

Lipopolysaccharide (LPS)

Lipopolysaccharides (LPS) as mentioned earlier are specific to Gram-negative bacteria and are part of their outer membrane. The lipid A portion is the endotoxin and is a potent pathogen-associated molecular pattern (PAMP) toxin, and as it is cell bound is only released or shed from the outer membrane when the bacterial cell lyses. In pathogenic bacteria that cause food poisoning like *E. coli* and *Salmonella*, the shed LPS binds to the phagocyte pattern recognizing receptors (PRR) on phagocytes, activate B cells and stimulate expression of pro-inflammatory cytokines that induce fever, diarrhoea and the life threatening condition, sepsis/septic shock (Murray et al., 2015; Wooldridge, 2018). *N. meningitidis* (meningitis) also shed a related

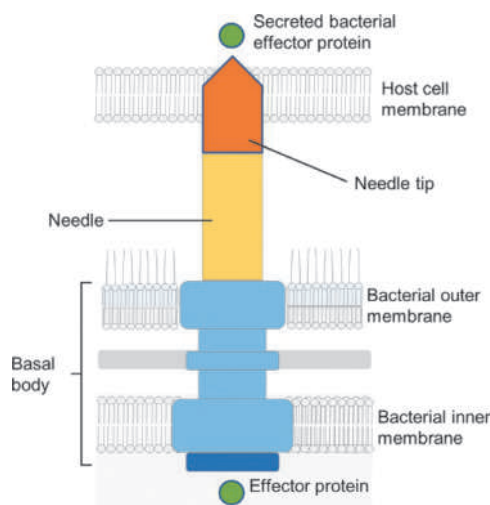


Fig. 10 Schematic of a type III secretion system. Adapted from Hotinger JA and May AE (2020) Antibodies inhibiting the type III secretion system of Gram-negative pathogenic bacteria. *Antibodies* 9: 35.

molecule, lipooligosaccharide that also stimulates an innate immune response due to its lipid A portion (Bower and Rosenthal, 2006). Further details on the host immune response to LPS exposure can be found in Chapter 49 (Immunity to bacterial infections).

Summary

Bacteria are small unicellular prokaryotic cells with one common feature is their cell wall structure and organization, that can be split into three parts, the cytoplasm, cell envelope and external cell surface structures. The cytoplasm is the inner most part of the bacterial cell, within which is the nucleoid that contains a single chromosome/DNA stand, ribosomes, inclusion bodies, and in some bacteria, plasmids. The cell envelope is composed of the cytoplasmic membrane, a semipermeable phospholipid bilayer that regulates movement of substances in and out of the cell; and the cell wall which covers the whole cell giving it its strength and shape while protecting it from the external environment. Differences in the cell wall PG composition are used to differentiate between Gram-positive and Gram-negative bacteria. The external cell surface structures are associated with virulence. These include capsule/capsular polysaccharide and slimes that help with surface adhesion and protection from phagocytosis; flagella for motility; fimbriae for adherence; and pili for adhesion, twitching motility, and DNA transfer. Finally, many clinically relevant bacteria have additional virulence factors on their cell wall surface, such as CWA proteins in Gram-positive bacteria, which are important for adhesion, and eukaryotic host cell colonization and evasion; and in Gram-negative bacteria, Type III secretion systems used to inject toxic proteins directly into eukaryotic host cells, and LPS an endotoxin and powerful inflammatory stimulant. Thus, these small microorganisms are well adapted and continually evolve to survive in various environments.

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Microbial Metabolism

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Glossary

Anabolism The process by which living organisms synthesize complex molecules from simpler ones, coupled with energy storage.

Autotrophs Organisms which use carbon dioxide as their sole source of carbon for organic matter synthesis, also known as "primary producers".

Catabolism The process by which living organisms break down complex molecules into simpler ones, coupled with energy release.

Chemolithotrophs Organisms which utilize inorganic compounds found in the environment as their main energy source.

Chemoorganotrophs Heterotrophic organisms which utilize organic compounds found in the environment as their major carbon and energy-yielding substrates.

Endergonic A reaction that absorbs free energy. The net change in free energy at the end of the reaction is positive.

Exergonic A reaction that releases free energy. The net change in free energy at the end of the reaction is negative.

Fermentation Enzyme-catalyzed breakdown of energy-rich compounds by microbes under anoxic conditions.

Heterotrophs Organisms which source their carbon from organic compounds which they oxidize to yield energy and generate the building blocks for biosynthetic processes.

Oxidative phosphorylation The use of substrate oxidation to drive an [electron transfer chain](#), coupled to energy conservation via an electrochemical transmembrane gradient under aerobic conditions.

Phototrophs Organisms which contain colored pigments which allow the conversion of solar energy to chemical energy through photosynthesis.

Redox Reactions involving simultaneous reduction and oxidation of chemical species.

Nomenclature

G Gibbs free energy

ΔG° The change in free energy during a chemical reaction under standard conditions (pH 7, 25 °C, 1 atm and 1 M concentration), expressed in kilojoules (kJ)

E_0' Reduction (or redox) potential expressed in Volts (V) under standard conditions

Introduction

Metabolism encompasses all chemical reactions which occur within a living organism. Its main purposes are, (1) to convert nutrients into a usable form of energy to sustain key cellular processes, (2) acquire the building blocks for cellular growth and (3) eliminate waste products. In the context of infection and immunity, host-microbe interactions are governed by the dynamic relationship which exists between the metabolic pathways of these players. The outcome of these metabolic interactions may be beneficial or destructive to the host. Commensal microbes, for example, can break down indigestible carbohydrates which are consumed by the host, releasing nutrients which are essential for human health (Gibson and Roberfroid, 1995; Gibson et al., 2004). They also suppress the growth of pathogens through nutrient depletion (Abt and Pamer, 2014). When the metabolic balance is tipped in favor of pathogens, this may lead to serious consequences to the host (Carding et al., 2015). Understanding the metabolic pathways employed by our resident microbial population is therefore key in developing new strategies to boost the immune system and suppress pathogen growth, thereby promoting human health.

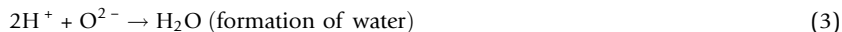
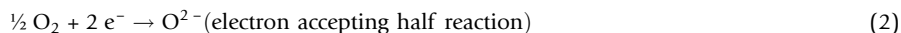
In order to understand how and why metabolic reactions arise, it is important to consider the Laws of Thermodynamics. The First and Second Laws of Thermodynamics were defined by Rudolf Clausius and William Thomson (Kelvin) ca. 1850. These laws are unifying principles in biology and govern all chemical reactions in living organisms. The First Law of Thermodynamics states that all the energy within a closed system, e.g., the Universe, is constant. Energy may transform from one form to another, but it cannot be created or destroyed. The Second Law of Thermodynamics states that each of these energy transformations increase the disorder, or entropy, within a system. This means that no energy transfer is completely efficient due to some energy being lost in an unusable form (e.g., via heat or waste products). The more energy that is lost from a system the more disordered that system becomes. Microbes have evolved ingenious ways to mitigate this tendency towards entropy, via for example, DNA repair, antioxidant defense and sporulation or encystation in response to hostile environments. Many are also able to switch their mechanisms of energy conservation in response to nutrient availability in the environment (Passalacqua et al., 2016). All such processes are energetically demanding, therefore living organisms are engaged in a constant battle to maintain their highly ordered, low entropy state, otherwise known as homeostasis.

This chapter covers the various biochemical mechanisms utilized by microorganisms to conserve and consume energy. It begins by reviewing the basic building blocks and mechanics of energy conservation ("Bioenergetics and redox reactions" and "Energy sources" sections) followed by an overview of the main pathways for energy generation utilized by microorganisms under a range of environmental constraints ("Catabolic pathways" section). The chapter concludes with a short description of key energy-consuming biosynthetic pathways which facilitate cellular growth and survival ("Anabolic pathways: Biosynthesis" section).

Bioenergetics and redox reactions

The definition of energy is the ability to do work. Energy can be quantified in units of kilojoules (kJ), which is a measure of heat energy. The remaining energy following a chemical reaction which is not released as heat and still available to do work is known as Gibbs free energy (G). The change in free energy during a chemical reaction under standard conditions (pH 7, 25 °C, 1 atm and 1 M concentration) is abbreviated as ΔG^0 . If ΔG^0 is negative the reaction is exergonic or energy-yielding. If ΔG^0 is positive the reaction is endergonic, or energy-requiring.

Oxidation-reduction or redox reactions play a key role in cellular energy conservation. Oxidation and reduction involve the removal or addition of electrons (e^-) to a substance, respectively. In biochemistry, the transfer of an electron is often accompanied by the transfer of a proton (H^+). Redox reactions require an electron donor, from which electrons are removed and transferred to an electron acceptor. During this process, the electron donor becomes oxidized and the electron acceptor becomes reduced. For example, water (H_2O) is formed from a redox reaction between hydrogen (H_2 , the electron donor) and $\frac{1}{2}$ oxygen (O_2 , the electron acceptor), as per Eqs. (1)–(4).



Reduction potentials

The tendency of a compound to become oxidized or reduced depends on the reduction (or redox) potential, expressed as E_0' under standard conditions (pH 7), of their half reaction. Most molecules can either behave as electron donors or acceptors, depending on the substances with which they react. For example, when water is formed (see Eqs. 1–4), the constituents on each side of the arrow represent a redox couple, e.g., $2H^+/H_2$ or $\frac{1}{2} O_2/H_2O$. E_0' refers to the reduced constituent of a redox couple (by convention is always written on the right). A negative E_0' value indicates that the reduced constituent of a redox couple has a greater tendency to donate electrons than does the oxidized constituent to accept them. This is the case for the $2H^+/H_2$ couple where E_0' is -0.42 V. A positive

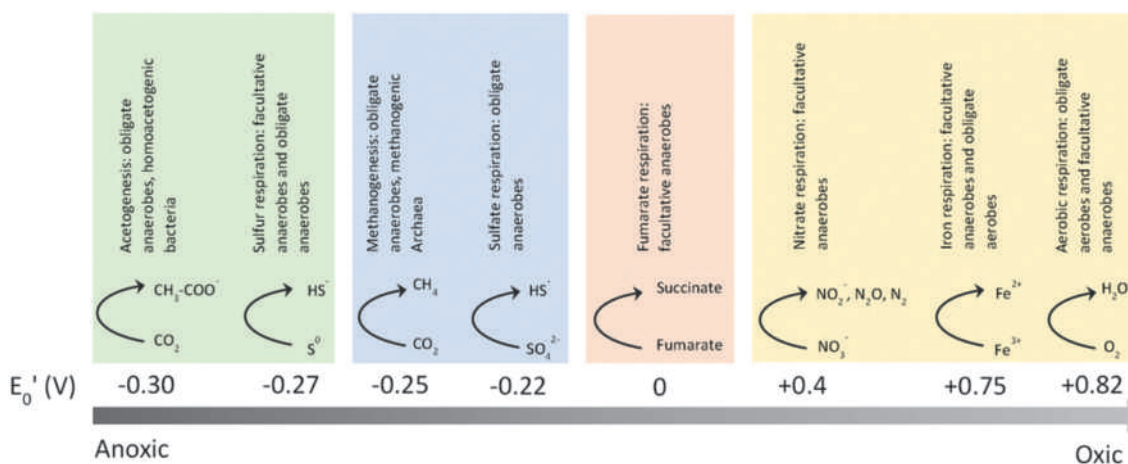


Fig. 1 A summary of the key redox couples involved in anaerobic and aerobic respirations within different microorganisms. The reduction potentials (E'_0) are ordered from the most electronegative to the most electropositive under anoxic to oxic habitats. Madigan MT and Martinko JM (2006). *Brock Biology of Microorganisms*, 11th edn., pp. 558. Upper Saddle River, NJ: Pearson Education, Inc., Fig. 17.35.

E'_0 value indicates that the reduced constituent has a lower tendency to donate electrons, whilst the oxidized constituent has a greater tendency to accept them. This is the case for the $\frac{1}{2} \text{O}_2/\text{H}_2\text{O}$ couple where E'_0 is +0.82 V. The greater the difference in E'_0 between the electron donor and acceptor in a reaction, the greater the energy that is released. $\Delta E'_0$ is therefore proportional to ΔG^0 . A summary of the key redox couples utilized in the metabolic pathways of different microorganisms is presented in Fig. 1, along with their reduction potential values (Madigan and Martinko, 2006).

Electron carriers

Electron transfer in redox reactions usually rely on the presence of electron carriers. There are two main classes of electron carriers: (1) coenzymes, which are freely diffusible, and (2) prosthetic groups, which are tightly bound to the enzymes involved in redox reactions. Nicotinamide-adenine dinucleotide (phosphate), in its oxidized, NAD(P)^+ , or reduced, NAD(P)H , form is an example of a diffusible coenzyme which acts as an intermediary for reactions between chemically dissimilar molecules. $\text{NADP}^+/\text{NADPH}$ is typically used in anabolic reactions whereas NAD^+/NADH is used in catabolic reactions. The $\text{NAD(P)}^+/\text{NAD(P)H}$ redox couple has a $\Delta E'_0$ value of -0.42 V, making NAD(P)H an efficient electron donor. NADH dehydrogenases contain a prosthetic group which can reduce or oxidize NAD^+ (by accepting 2H or $2\text{e}^- + 2\text{H}^+$) and $\text{NADH} + \text{H}^+$ (by donating $2\text{e}^- + 2\text{H}^+$), respectively. Flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) are examples of flavin prosthetic groups, bound to flavoproteins. Flavins can accept 2e^- and 2H^+ but can only donate electrons. Cytochromes have prosthetic groups containing a porphyrin ring (heme), which undergo redox reactions involving a single electron at the iron-containing active site. Non-heme iron-sulfur proteins, e.g., ferredoxin, are another important prosthetic group, which like cytochromes can only carry electrons. Finally, hydrophobic molecules called quinones are also involved in electron transport and can accept 2e^- and 2H^+ but can only donate electrons.

Energy conservation

The energy released during redox reactions needs to be conserved by the cell in order to fuel energy-demanding processes. As energy conservation in itself is an energy-requiring process, cells tend to rely on compounds with a ΔG^0 of $>30 \text{ kJ mol}^{-1}$ as "energy currencies." The main energy currency produced through catabolic reactions is the molecule adenosine triphosphate (ATP). This is an energy-rich molecule which is universal across the domains of life. ATP stores potential energy in the form of two high energy phosphoanhydride bonds. When hydrolyzed in endergonic reactions, each of these bonds release 32 kJ mol^{-1} of energy under standard conditions. Derivatives of coenzyme A are also used to conserve the energy released during exergonic reactions. These derivatives, e.g., acetyl-CoA ($\Delta G^0 = -31 \text{ kJ mol}^{-1}$), contain high-energy thioester bonds. The energy released when these bonds are hydrolyzed is conserved in the production of ATP. As demand for ATP is high within an actively growing cell, adenosine monophosphate (AMP) - adenosine diphosphate (ADP)—ATP cycling is a dynamic process. For long-term storage, cells must conserve energy in the form of insoluble polymers. In the absence of an external substrate, these can be oxidized to release ATP as maintenance energy in order to maintain cellular growth. Polyglucose such as glycogen and starch, and lipid-based polymers such as poly- β -hydroxybutyrate, are examples of polymers used by microorganisms as long-term energy reserves.

Energy sources

All life on Earth is carbon-based. This makes carbon a basic requirement as a metabolic substrate. The ubiquity of carbon as a nutrient is due to its ability to form four simultaneous bonds with other atoms. This important feature facilitates the assimilation of long molecular chains, forming the basis of DNA, proteins and sugars. Heterotrophic organisms source their carbon from organic compounds which they oxidize to yield energy and generate the building blocks for biosynthetic processes. Autotrophs on the other hand use carbon dioxide (CO_2) as their sole source of carbon for organic matter synthesis. These are also known as “primary producers” as all organic material on Earth is synthesized by such organisms.

Over 4 billion years of evolution, microbes have developed innumerable metabolic strategies for energy conservation, allowing them to flourish in almost every environment on Earth. All microbes fall into one of three broad metabolic classifications, according to their choice of energy-yielding substrate. (1) Chemoorganotrophs are heterotrophic organisms which utilize organic compounds found in the environment as their major carbon and energy-yielding substrates. (2) Chemolithotrophs utilize inorganic compounds found in the environment as their main energy source. For example, the sulfur-oxidizing lithotrophs convert hydrogen sulfide (H_2S) to elemental sulfur (S_0) and S_0 to sulfate (SO_4). So far, chemolithotrophy has only been identified in prokaryotes. This lifestyle means that the organism does not need to compete with chemoorganotrophs for organic substrates, as they can exploit the substrates that are inaccessible to most, and furthermore can live alongside such organisms by feeding on their waste products (e.g., H_2 and H_2S). Many chemolithotrophic organisms are autotrophs (also known as lithoautotrophs or chemoautotrophs), sourcing their carbon from CO_2 . (3) Phototrophs contain colored pigments which allow the conversion of solar energy to chemical energy through photosynthesis. Most phototrophs are autotrophs. Although of great ecological significance, phototrophic microorganisms do not play a significant role in human infection and immunity therefore will not be discussed further in the context of this chapter.

Catabolic pathways

Fermentation

Fermentation involves the extraction of energy from carbohydrates under anoxic conditions. Glycolysis, or the Embden-Meyerhof pathway, is one of the most common fermentative pathways (see Fig. 2). In this pathway, the exogenous electron donor, most commonly glucose, is oxidized to pyruvate (and H^+) via three main enzymatic stages. Stage I involves a series of preliminary reactions which requires the expenditure of two molecules of ATP. The first is used by the enzyme hexokinase to phosphorylate glucose to glucose-6-phosphate. Isomerase then converts glucose-6-phosphate into an isomeric form, fructose-6-phosphate. This is then further phosphorylated by phosphofructokinase to yield fructose-1,6-bisphosphate at the expense of another molecule of ATP. The final step of Stage I involves aldolase, which splits fructose-1,6-phosphate into two units of the 3-carbon molecule, glyceraldehyde-3-phosphate.

Stage II marks the first of a series of redox reactions involved in glycolysis with the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglyceric acid via glyceraldehyde-3-phosphate dehydrogenase. This enzyme contains the coenzyme NAD^+ , which forms NADH (used later in Stage III) by accepting 2e^- and 2H^+ from the phosphorylation of glyceraldehyde-3-phosphate. Note that this reaction, and all subsequent reactions occur twice simultaneously due to the presence of two molecules of glyceraldehyde-3-phosphate from Stage I. In total, four molecules of ATP are synthesized during Stage II, due to the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate, via the action of phosphoglycerokinase and later, the conversion of phosphoenolpyruvate to pyruvate, via pyruvate kinase. A number of steps precedes the latter, notably the conversion of 3-phosphoglycerate to 2-phosphoglycerate and subsequently to phosphoenolpyruvate, via enolase. Thus, for every glucose molecule that has fermented, the net gain is two molecules of ATP.

Stage III serves to recycle the two molecules of NADH, produced in Stage II, back into its oxidized form (NAD^+), so that it is once again available to accept electrons from the phosphorylation of glyceraldehyde-3-phosphate. Different organisms complete this fermentation in a variety of different ways. For example, lactic acid bacteria reduce pyruvate to lactate via lactate dehydrogenase, whereas yeasts reduce pyruvate to ethanol and CO_2 via acetaldehyde, via the sequential action of pyruvate decarboxylase and alcohol dehydrogenase. As well as restoring redox balance through NAD^+/NADH cycling, Stage III also enables atomic balance to be achieved, where the end products of the fermentation, e.g., 2 ethanol ($\text{C}_2\text{H}_5\text{OH}$) + 2CO_2 , equal that of the starting substrate, i.e., glucose ($\text{C}_6\text{H}_{12}\text{O}_6$). The end products of fermentation are disposed of from the cell as waste. Organisms which produce a single end product, e.g., lactic acid, from glycolysis are known as homofermenters. Heterofermenters on the other hand produce multiple end products from glycolysis, e.g., acetate, ethanol, formate and CO_2 .

Other fermentative pathways also exist which utilize glucose as the exogenous electron donor. These are the heterolactic, or phosphoketolase, pathway and the Entner-Doudoroff pathway. The key enzymes involved in these pathways are phosphoketolase and 2-keto-3-deoxy-6-phosphogluconic acid, respectively. The main difference between these pathways and glycolysis is the timing of NAD-mediated oxidations. These occur before the substrate is cleaved during the phosphoketolase and Entner-Doudoroff pathways and generate 1 ATP, whereas in glycolysis, these occur after substrate cleavage, generating 2 ATP. The phosphoketolase pathway is prominent in probiotic lactic acid bacteria such as *Lactobacillus* and *Leuconostoc*, whereas the Entner-Doudoroff pathway dominates in some pathogenic bacteria such as *Pseudomonas aeruginosa* and *Vibrio cholerae*.

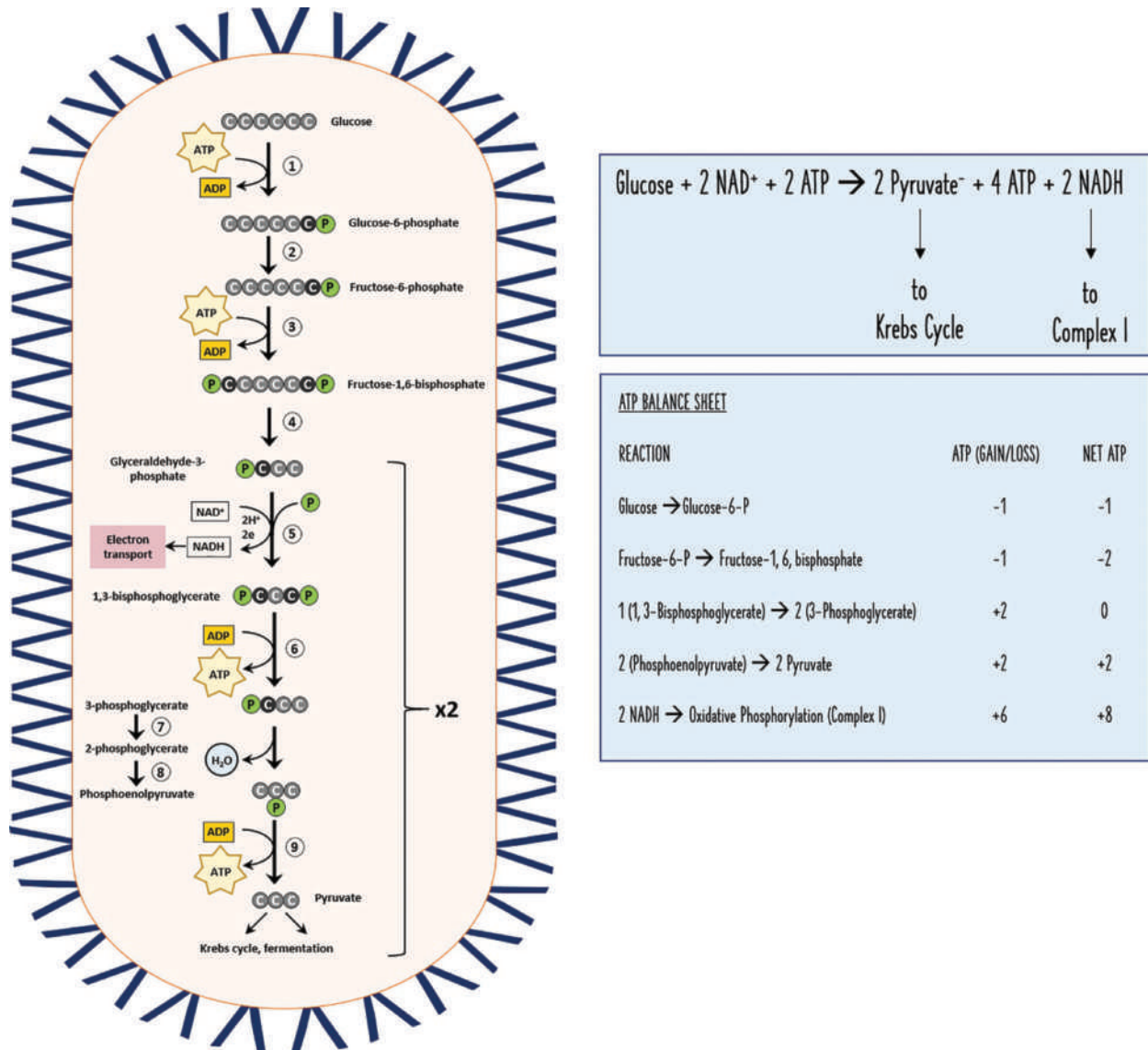


Fig. 2 Glycolysis, or the Embden-Meyerhof pathway. The sequence of enzymatic reactions involved in the generation of pyruvate from glucose. The key enzymes involved are: (1) hexokinase, (2) isomerase, (3) phosphofructokinase, (4) aldolase, (5) glyceraldehyde-3-phosphate dehydrogenase, (6) phosphoglycerokinase, (7) phosphoglyceromutase, (8) enolase, and (9) pyruvate kinase. Note that two molecules of glyceraldehyde-3-phosphate are produced after step 4 therefore the subsequent sequence runs twice, ultimately forming two molecules of pyruvate. The overall reaction as well as a summary of the number of ATP molecules consumed and generated is also provided.

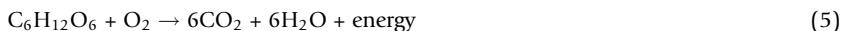
Bacteria are also able to metabolize longer chained derivatizations of glucose or other carbohydrates. Examples of these are nondigestible oligosaccharides including fructooligosaccharide (a chain of fructose residues bound terminally to glucose) and galactooligosaccharide (a chain of galactosyl residues bound terminally to glucose), which are hydrolyzed to their constituent monosaccharides, thus releasing glucose. Oligosaccharides, found in bananas, garlic and onions, are known for the prebiotic potential, meaning that they are selectively metabolized by probiotic bacteria found in the human colon. Dominance of probiotic bacteria in the gut is linked with several host advantages including the breakdown of indigestible fibers to usable nutrients, modulation of the immune system and inhibition of pathogenic microbes.

Aerobic respiration

Respiration is a process whereby glucose can undergo complete oxidation to CO_2 . This is facilitated by the presence of a final electron acceptor. The formation of ATP coupled to an electron transport chain is known as oxidative phosphorylation. In aerobic

respiration, the final electron acceptor is O₂. Other anaerobic forms of respiration also exist and will be discussed in “*Anaerobic respiration*” section. The theory of ATP synthesis driven by the proton motive force or “chemiosmosis” was one of the most monumental scientific discoveries of the 20th century. Its founder, Peter Mitchell, was awarded the Nobel Prize for Medicine (Mitchell, 1961). It involves the sequential transfer of electrons from an organic substrate to the final electron acceptor across a semi-permeable membrane. Here, each electron transport step results in the synthesis of ATP, establishing a highly energized state. Electron transport relies on the presence of electron carriers which span the cell membrane, allowing separation of electrons and protons on the inside and outside, respectively. In Gram positive bacteria, these protons are released into the environment, whereas in Gram negative bacteria they accumulate in the periplasm. This differential separation of protons and electrons results in a pH gradient, whereby the outside of the membrane is slightly acidic, or electro-positive, when compared to the inside, which is electro-negative. This generates an electrochemical potential across the membrane, and the resulting electrical energy is conserved by the cell in the form of ATP.

Aerobic respiration in heterotrophic organisms can be summarized as per Eq. (5).



Here, glucose is completely oxidized to the given reaction products. The net energy yield from the oxidation of a single glucose molecule is 38 molecules of ATP. This equates to the generation of approximately 380,000 calories (cal), as each ATP molecule corresponds to 10,000 cal mol⁻¹. Thermodynamically, this net calorific value is much higher (around 688,000 cal), however the vast majority of this energy (around 308,000 cal) is lost as heat, making the process of cellular respiration approximately 55% efficient (Jurtshuk, 1996). The above equation is however a highly simplified representation of the total dissimilatory reaction, which typically involves three fundamental biochemical steps. The first of these is glycolysis, described previously, yielding 4 ATP molecules. The second and third steps, the Krebs cycle and the Electron Transport Chain (ETC), respectively are described in the subsequent sections.

The Krebs cycle

The second step in respiration is the Krebs cycle (also known as the tricarboxylic acid or citric acid cycle, see Fig. 3). It involves a series of oxidative decarboxylation steps, beginning in bacteria with acetyl -CoA and results in complete oxidation of the starting substrate (i.e., glucose) coupled with release of CO₂. In addition, this metabolic cycle results in the generation of 10 pairs of electrons, which feed into the final step of respiration, the ETC. Each electron pair produced results in the generation of 2–3 molecules of ATP through the ETC. As two molecules of the intermediary substrate pyruvate are formed during glycolysis, this cycle runs twice for every molecule of glucose which is oxidized.

The first step of this pathway begins with the decarboxylation of pyruvate to the Krebs cycle starting molecule, acetyl-CoA. This itself is a three-step reaction, whereby (1) a carboxyl group is removed from pyruvate to produce CO₂ and a hydroxyethyl group bound to the enzyme pyruvate dehydrogenase, (2) an acetyl group is formed from the oxidation of the hydroxyethyl group, and the resulting electrons are transferred to NAD⁺, forming NADH + H⁺, (3) this acetyl group is then transferred from the enzyme to coenzyme A (CoA) resulting in the formation of acetyl-CoA. There are two things to note from this initial step of the Krebs cycle: (1) the decarboxylation of pyruvate represents the first of six decarboxylations within this reaction cycle, one for each of the six carbon atoms present in the initial substrate, glucose, facilitating its complete oxidation; (2) the high energy electrons, stored within the eight NADH molecules generated will be used later to generate ATP via the ETC.

Next, CoA is released from the acetyl group following its condensation with oxaloacetate to form the tricarboxylic acid, citrate. This is the first stable intermediary of the Krebs cycle, which is initially isomerized to isocitrate, before being oxidized and decarboxylated to α-ketoglutarate. The oxidation of α-ketoglutarate to succinyl CoA via α-ketoglutarate dehydrogenase is analogous to the action of pyruvate dehydrogenase described previously, involving CoA and NAD⁺. The conversion of succinyl CoA to succinate completes the decarboxylation of pyruvate, and also yields a molecule of guanosine triphosphate (GTP, equivalent to ATP), via succinyl-CoA synthetase. The remaining steps of the Krebs cycle result in the regeneration of oxaloacetate (via fumarate and malate) and complete the oxidation of succinate.

Both the Krebs cycle and glycolysis are amphibolic, meaning that a number of the intermediary molecules produced may be used for assimilative (biosynthetic) pathways (see “*Anabolic pathways: Biosynthesis*” section), as well as the dissimilative pathway outlined here.

Electron transport chain

The ETC is the final step of respiration where electrons are transferred through a series of oxidation-reduction reactions (see Fig. 4). Here a series of membrane-associated proteins and mobile accessory carriers transport electrons from the substrate and along the plasma (in prokaryotes) or mitochondrial membrane (in eukaryotes) to the final electron acceptor. In eukaryotic micro-organisms the substrate for this process is usually NADH + H⁺, succinate or fatty acids. In addition to these substrates, bacteria can also oxidize a larger selection of low redox potential substrates such as H₂, glutamate, α-glycerophosphate, lactate, malate and formate (Jurtshuk, 1996) via non-pyridine nucleotide-dependent pathways. The electron carriers themselves are separated into four major classes: cytochromes, flavoproteins, iron-sulfur proteins and quinones, and are more varied in prokaryotes than in eukaryotes. These electron carriers are responsible for the sequential removal of electrons from the substrate at the various steps, due to their redox potential. In order to accept electrons, the protein or carrier must be more electronegative (or have a lower E₀') than the electron

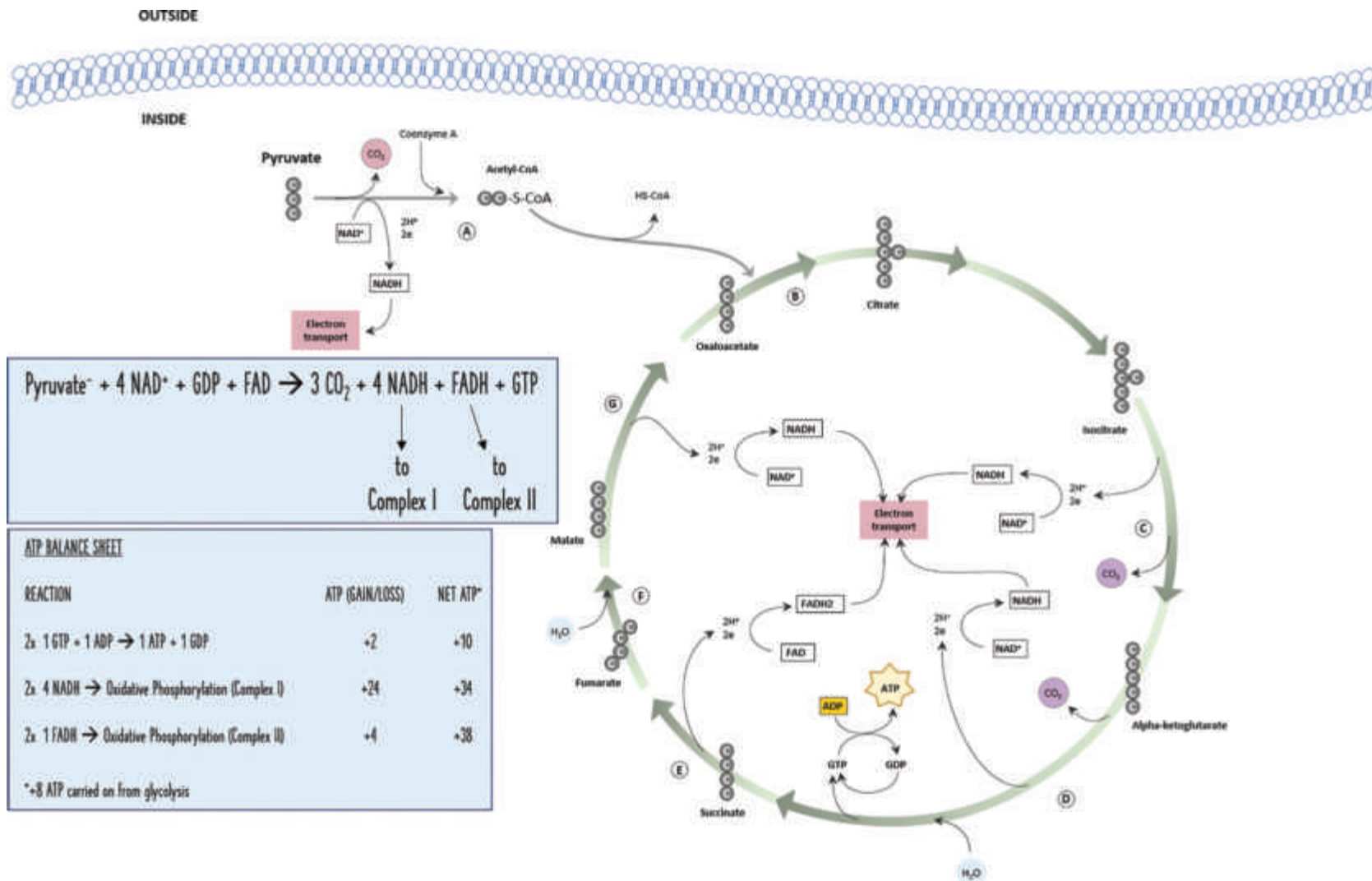


Fig. 3 The Krebs, or citric acid, cycle. Pyruvate, derived from glycolysis, is firstly converted into acetyl-CoA (2-C) which is subsequently condensed with oxaloacetate (4-C) to form the 6-C compound citrate. This is cycled back to oxaloacetate via a series of reactions which form CO₂ (as a waste product) as well as NADH and FADH. Note that this cycle runs twice for every molecule of glucose which undergoes oxidation during glycolysis, as two molecules of pyruvate are formed. Although some ATP is generated via the GTP derived from the Krebs cycle, most of the ATP generated during aerobic respiration comes from oxidative phosphorylation. The NADH and FADH generated during the Krebs cycle fuels the electron transport chain which drives this process, ultimately producing 38 molecules of ATP. The key enzymes involved are: (A) pyruvate dehydrogenase; (B) citrate synthase; (C) isocitrate dehydrogenase; (D) α-ketoglutarate dehydrogenase and succinyl-CoA synthetase; (E) succinic dehydrogenase; (F) fumarase; (G) malate dehydrogenase.

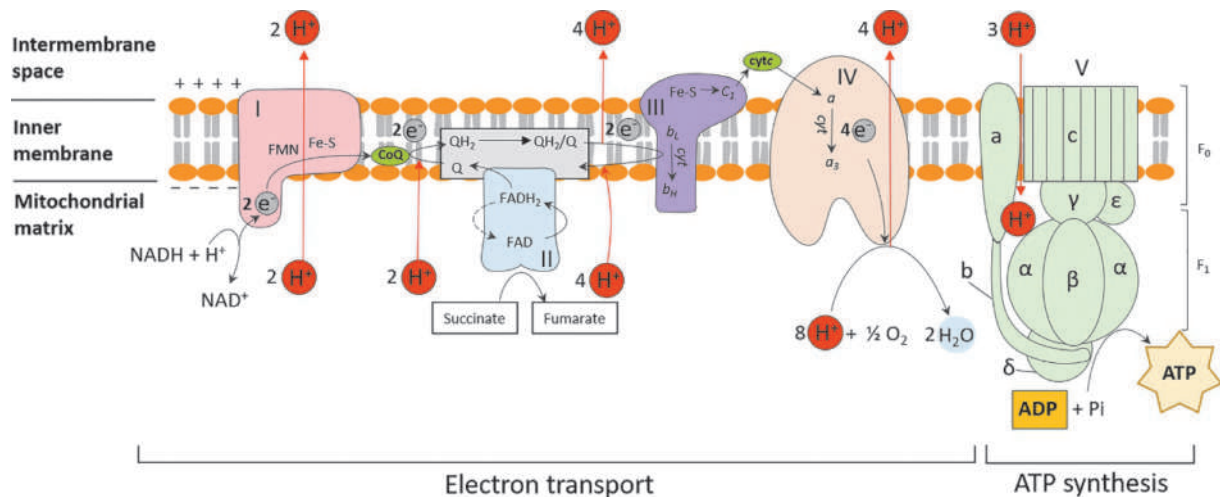


Fig. 4 Oxidative phosphorylation in eukaryotes. ATP synthesis is coupled to the sequential transfer of electrons via an electron transport chain from NADH or FADH₂ to oxygen. This process is facilitated by a series of electron carriers, which also serve to establish the proton motive force by translocating protons outside the membrane. This generates an electrochemical gradient which fuels the final ATP synthesis step. Abbreviations are as follows: complex I, NADH coenzyme Q reductase; complex II, succinate dehydrogenase; complex III, cytochrome *bc*₁; complex IV, cytochrome *c* oxidase; complex V, F₀F₁ ATP synthase; CoQ, coenzyme Q; cytC, cytochrome *c*. The subunits (a, b, c, α, β, δ, ε and γ) which make up the F₀ and F₁ units of complex V are also indicated.

donor, e.g., NADH and FADH₂. Electrons therefore enter this chain at low E_0' (electronegative) and exit at a higher E_0' (electro-positive). This drop in E_0' supplies energy to drive oxidative phosphorylation which results in the generation of ATP. Essential to this process is a final electron acceptor which is derived from outside the cell (e.g., O₂ in aerobic respiration and S, SO₄, NO₂ or NO₃ in anaerobic respiration) and an ATP synthetase (ATPase) which spans the membrane. Two protons are consumed by this enzyme, which are shuttled from the F₀ to the F₁ units where the phosphorylation of ADP to ATP takes place as per Eq. (6).



The ATPase serves as a channel for H⁺ to flow across the membrane in a process called chemiosmosis, where these ions flow along their electrochemical gradient from a region of higher concentration (outside membrane) to a region of lower concentration (inside membrane). The potential energy stored within this electrochemical gradient serves as a second mechanism for ATP synthesis.

Inhibitors and uncouplers are chemicals that can be used to study the ETC and oxidative phosphorylation, respectively. Inhibitors prevent the establishment of the proton motive force by blocking electron flow. Cyanide (CN⁻) and carbon monoxide (CO) are examples of inhibitors which bind to a-type cytochromes such as those forming Complex IV. These inhibit terminal electron transfer to O₂ or compete with O₂ for binding to Complex IV. Uncouplers prevent ATP synthesis without affecting the ETC. Dinitrophenol (DNP) is an example of an uncoupler which as a protonophore, allowing protons to leak across the membrane, thereby bypassing ATP synthase. This greatly reduces the efficiency of oxidative phosphorylation and the excess energy accumulated via the ETC is lost as heat.

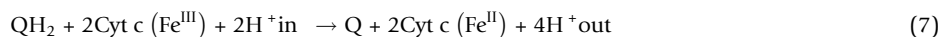
Electron transport chain in eukaryotes

In aerobic unicellular eukaryotes which possess traditional mitochondria (e.g., *Acanthamoeba* and *Naegleria*), the ETC is comprised of four complexes (I-IV), each of which are composed of multiple proteins (see Fig. 4). Each complex either receives electrons from a coenzyme carrier or directly from one of the other complexes. These proteins sit on the inner mitochondrial membrane and can pump protons extracted from the oxidized substrate into the intermembrane space. This establishes the electrochemical gradient (ΔpH) and largely contributes towards the mitochondrial membrane potential ($\Delta\Psi_{\text{M}}$) across the inner mitochondrial membrane, which is important for the final ATP synthesis step. The ETC begins with **complex I** (NADH coenzyme Q reductase, also known as NADH:quinone oxidoreductase). This enzyme removes 2e^- from NADH, the main electron carrier derived from the Krebs cycle, and subsequently transfers them to coenzyme Q (ubiquinone), producing the reduced product ubiquinol (QH₂). This reaction is facilitated by FMN, which also accepts two protons from the dissociation of water in the cytoplasm, becoming its reduced form FMNH₂. Each electron from the FMNH₂ is transferred to an iron-sulfur (Fe-S) cluster before being passed on to ubiquinone (Q). This releases two protons which are translocated into the intermembrane space, and recycles FMNH₂ back to its oxidized form, FMN. The transfer of electrons from the Fe-S cluster to Q is a two-step reaction, whereby transfer of the first electron results in the formation of a semiquinone intermediate (the free radical form of Q), and transfer of the second electron results in reduction of this semiquinone to QH₂. This process, known as the Q cycle, results in four H⁺ being translocated across the membrane. The energy

needed to actively transport these protons into the intermembrane space is derived from the electron current which is established through the continuous oxidation and reduction of electrons across the complex at a ratio of 4H^+ per 2e^- derived from NADH.

The resulting QH_2 is mobile within the membrane and is also capable of bypassing complex I by receiving electrons from succinate via **complex II** (succinate dehydrogenase or succinate-CoQ reductase) or other electron donors (e.g., fatty acids and glycerol-3-phosphate). All such electrons are transferred to Q via FADH_2 . The pathways of electron transport through complex II is similar to that of complex I, but without the translocation of protons across the membrane.

The electrons are next transferred from QH_2 in **complex III** (cytochrome bc_1 or CoQH_2 -cytochrome c reductase) to two molecules of cytochrome c , which is water soluble and located within the intermembrane space. Complex III is composed of several proteins containing heme or metal centers, including b-type hemes (b_L and b_H), a c-type heme (c_1) and Fe-S protein ("Rieske"). Complex III is conserved across most respiring organisms. The transfer of electrons to cytochrome c by complex III is coupled with the translocation of four protons, of which two are used to reduce QH_2 to quinol and a further two are released from two molecules of ubiquinol (see Eq. 7).



Complex IV (cytochrome c oxidase) transfers four electrons (derived from four molecules of cytochrome c) to O_2 , reducing it to two molecules of H_2O . This complex is composed of cytochromes a and a_3 , where the latter acts as the terminal oxidase. During this process, eight protons are taken from the mitochondrial matrix, four of which are used to produce H_2O and the remaining four are pumped into the intermembrane space. This complex is comprised of several copper and heme groups, including Fe^{3+} . The latter makes complex IV a target for CN^- poisoning, which blocks electron transport thereby depleting ATP synthesis, resulting in cell death. The oxidase test is a standard biochemical test for the identification of microbes which possess cytochrome c oxidase. Here, the reagent, tetra-methyl- p -phenylenediamine dihydrochloride (e.g., Kovac's oxidase reagent) acts as an artificial electron acceptor for the oxidase enzyme inducing a color change from clear to purple, indicating an oxidase positive result. *Pseudomonas*, *Campylobacter*, *Vibrio* and *Neisseria* species are examples of oxidase positive organisms.

The final step in respiration involves coupling the ETC, and the resulting electrochemical gradient, to oxidative phosphorylation via the F_0F_1 ATP synthase (or **complex V**). This culminates in the synthesis of ATP, which is a reversible reaction depending on the application. The F_0 part of the enzyme spans the membrane providing a channel through which H^+ ions can flow back into the mitochondrial matrix. The protons move sequentially through the α , β and δ subunits of the F_0 unit. Once this revolution is complete, the protons are released back into the mitochondrial matrix. This process releases the free energy derived from the ETC, which is used by the F_1 component of complex V, located at the matrix end of the complex, to drive ATP synthesis.

The overall structure of ATP synthases is conserved across the domains of life, which suggests an early evolutionary origin in biological energy conservation. This structure allows the complex to behave as a motor (see complex V structure in Fig. 4). The flow of protons through subunit a of F_0 causes the c proteins to rotate. This torque is transmitted to F_1 via the coupled rotation of the $\gamma\epsilon$ subunits. These subunits also transfer energy to F_1 which is converted to potential energy via conformational changes of the β subunits. This allows the binding of $\text{ADP} + \text{P}_i$, the substrates for ATP synthesis. When this is complete, the β subunits return to their original conformation. The ATPase usually consumes 3H^+ for every ATP produced. As mentioned, the reversibility of the F_1/F_0 motor is facilitated by the hydrolysis of ATP, which induces the torque to allow the γ subunit to rotate in the opposite direction. Here, protons are also translocated in the opposite direction, i.e., inside the cytoplasm or mitochondrial matrix, thus generating the proton motive force, rather than dissipating it. This explains why some anaerobic microbes which cannot undergo oxidative phosphorylation also synthesize ATPase and use this for important cellular processes such as motility and transport.

Electron transport chain in prokaryotes

As discussed above, prokaryotes can use a wide variety of electron donors, which enter the ETC at various levels, depending on the energy source of that organism. Organotrophs typically use NADH or succinate, whereby electrons enter the ETC through complexes analogous to complex I and II of mitochondria, respectively. Alternative dehydrogenases are required to process electron donors such as H_2 , formate, lactate and glyceraldehyde-3-phosphate. Many of these dehydrogenases are inducible depending on the metabolic requirements of the organism and their external environment. In terms of quinone carriers, many bacteria use ubiquinone as used by eukaryotic mitochondria. Others employ menaquinone (vitamin K_2). Some archaea, e.g., *Sulfolobus* spp., use caldariellaquinone. The choice of quinone depends on the redox potential of the electron donor. In bacteria, the ETC components sit on the plasma membrane, therefore a proton gradient must be established between the cytoplasm and periplasmic space to provide the energy for oxidative phosphorylation to occur. Bacteria must therefore also possess proton pumps, which maintain this gradient, in much the same way as their eukaryotic counterparts. Membrane-bound dehydrogenases, oxidases and reductases serve as bacterial proton pumps. The bacterial cytochrome bc_1 for example is very similar to complex III found in mitochondria, however it is not ubiquitous among all bacteria (absent from *E. coli*). In lithotrophic bacteria, electrons derived from inorganic electron donors (e.g., nitrite and ferrous iron) may enter the ETC at the level of the mobile electron carriers, i.e., cytochromes or quinones. This means that the electron donor has a reduced E_0' as compared to NADH, therefore the ETC must firstly run in reverse in order to generate this higher-energy molecule. Bacteria have two classes of oxidases. Class I oxidases are comprised of cytochrome oxidases, using O_2 as the terminal electron acceptor, whereas Class II oxidases are quinol oxidases, which use a wide range of final electron acceptors.

Anaerobic respiration

Anaerobic respiration occurs when an energy-yielding electron transport chain terminates with any final electron acceptor other than O_2 . It is important to note, however, that facultative anaerobes growing in an aerobic environment invariably use O_2 as the terminal electron acceptor, as this produces the most energy via the Gibbs free energy change. Many of the key enzymes involved in anaerobic respiration are oxygen-sensitive, rendering such pathways inactive in the presence of O_2 . Obligate anaerobes are not able to use O_2 , therefore rely solely on the availability of alternative electron acceptors. The terminal reductases of anaerobic bacteria are tailored to their terminal electron acceptor (see Fig. 1). This section outlines four key pathways of anaerobic respiration which have evolved in different microbes, these are methanogenesis, acetogenesis, denitrification/nitrate reduction and sulfate reduction.

Methanogenesis

Methane (CH_4) production through anaerobic respiration is reserved to a specialist group of strictly anaerobic archaea, known as methanogens. The initial substrate for this reaction is CO_2 and this is ultimately reduced to CH_4 via a series of reactions involving two types of cytochromes, classified according to their role in methanogenesis (Madigan and Martinko, 2006; see Fig. 5), as per Eq. (8).



The first class of cytochromes carries the C_1 unit from CO_2 to the end-product. The second class provide the electrons required to perform the necessary redox reactions. The electrons required to reduce CO_2 to CH_4 are typically sourced from H_2 , however CO , formate and some organic compounds (e.g., methanol), are also known to perform this function. The initial step of methanogenesis involves the coenzyme methanofuran. This binds CO_2 to the amino nitrogen atom attached to its furan ring, and using H_2 , reduces CO_2 to formyl. The methanogenic coenzyme, methanopterin, then carries the C_1 unit from methanofuran, at which point it is dehydrated to methylene and subsequently reduced to a methyl group (CH_3). The F_{420} coenzyme (a flavin derivative, structurally similar to FMN) acts as an electron donor during these steps. From methanopterin, the methyl group is then transferred to coenzyme M. Whilst bound to CoM, the CH_3 is reduced to CH_4 via methyl reductase enzyme complex. Coenzyme B and F_{430} play a key role during this terminal step of methanogenesis. The CH_3 group is removed from CoM and binds to the nickel ion (Ni^{2+}) group of coenzyme F_{430} . Electrons from CoB are then used to reduce the Ni^{2+} - CH_3 complex, resulting in the generation of CH_4 , as

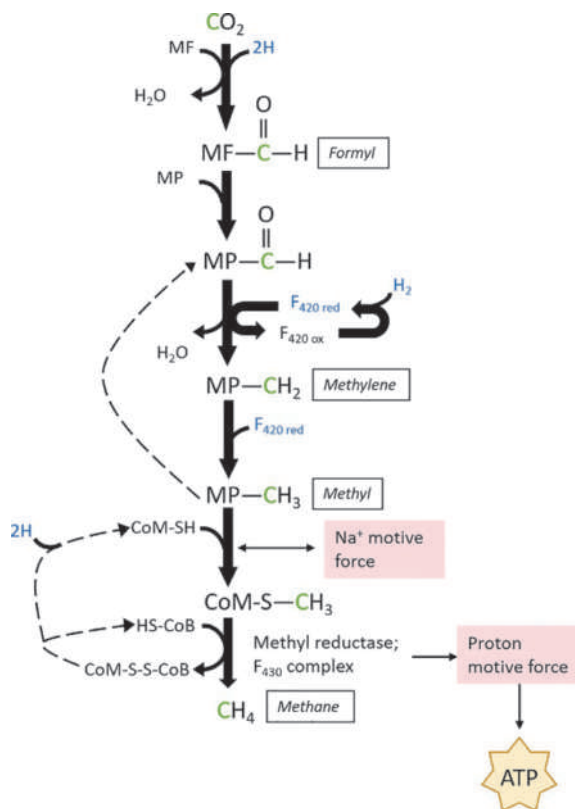


Fig. 5 Methanogenesis. The sequential series of enzymatic reactions which leads to the formation of methane (CH_4) from CO_2 using H_2 as an electron donor. The reduced C-atom is indicated in green and electron sources are indicated in blue. Abbreviations are as follows: MF, methanofuran; MP, methanopterin; CoM, coenzyme M; F_{420}^{red} , reduced coenzyme F_{420} ; F_{430} , coenzyme F_{430} ; CoB, coenzyme B. Adapted from Madigan MT and Martinko JM (2006). *Brock Biology of Microorganisms*, 11th edn., pp. 566. Upper Saddle River, NJ: Pearson Education, Inc., Fig. 17.44.

well as a CoM and CoB disulfide complex (CoM-S—S-CoB). Coenzymes F_{420} , M and B are recycled through reduction by H_2 . Energy conservation in methanogenesis is facilitated during the reduction of the CoM-S—S-CoB complex cytochromes to CoM-SH and CoB-SH. This is carried out by cytochrome F_{420} and heterodisulfide reductase. The latter is an enzyme which spans the membrane and generates a proton motive force by extruding protons across the membrane. This is linked to an ATPase. ATP is produced at a rate of approximately 1 ATP molecule for every reduction of CO_2 to CH_4 via H_2 . When methanol is the substrate for methanogenesis, methyl groups are instead donated to a corrinoid protein (containing a porphyrin-like ring and Co), which transfers the CH_3 group to CoM. A corrinoid protein also plays a similar role when acetate is the substrate. In this reaction, acetate firstly undergoes activation to acetyl-CoA and carbon monoxide dehydrogenase from the acetyl-CoA pathway. When methanol is the substrate, some of this compound must firstly be oxidized to CO_2 in order to supply the electrons required for reduction of CH_3 to CH_4 . The energy required for methyl oxidation is provided by a second type of ATPase powered by a sodium (Na^+) motive force, rather than H^+ .

Acetogenesis

Like the methanogens, homoacetogens also use CO_2 and H_2 as an electron acceptor and donor, respectively. These organisms use the acetyl-CoA pathway to reduce CO_2 to acetate ($C_2H_3O_2^-$) as per Eq. (9) and detailed further in Fig. 6 (Madigan and Martinko, 2006).



During this process, the two molecules of pyruvate synthesized during glycolysis are converted to 2 molecules of $C_2H_3O_2^-$, in addition to two molecules of CO_2 and 4 hydrogen atoms. The $2CO_2$ molecules are reduced to the methyl and carbonyl groups of acetate using the electrons derived from the $4H$ atoms, as well as four additional electrons derived from the $2NADH + H^+$ also generated during glycolysis. Coenzyme tetrahydrofolate (THF) is involved in the generation of the CH_3 group of acetate from one CO_2 molecule, which is firstly converted to CHO-THF (at the expense of one molecule of ATP) and then CH_3 -THF before being transferred to an enzyme containing a vitamin B_{12} cofactor. The carbonyl group (CO) of acetate, derived from the second CO_2 molecule, is synthesized via the action of carbon monoxide dehydrogenase. This contains a number of metal cofactors, Ni, Fe and Zinc (Zn), which facilitate its catalytic function. In the final step of the pathway the CH_3 group is combined with the CO group, facilitated by coenzyme A to form acetyl-CoA. This step helps to establish a Na^+ motive force across the cytoplasmic membrane, which is coupled to an ATPase, forming one molecule of ATP. An additional ATP molecule is produced during the conversion of acetyl-CoA to acetate.

A wide variety of alternative electron donors are also available for acetogenesis, e.g., C_1 compounds, organic/amino acids, sugars and some N-bases. In addition to CO_2 , many homoacetogens are also able to reduce NO_3^- and $S_2O_3^{2-}$. Most acetate-excreting homoacetogens are Gram positive bacteria, many of which belong to the genus *Clostridium*. *Clostridium difficile*, for example, is an opportunistic pathogen associated with hospital-acquired antibiotic-associated diarrhea. In addition to its role in energy conservation, many homoacetogens also use this pathway for autotrophic growth, e.g., sulfate-reducing bacteria, in addition to the methanogens mentioned previously. In some acetotrophic methanogens and sulfate-reducing bacteria this pathway runs in the reverse direction, whereby acetate is oxidized to CO_2 .

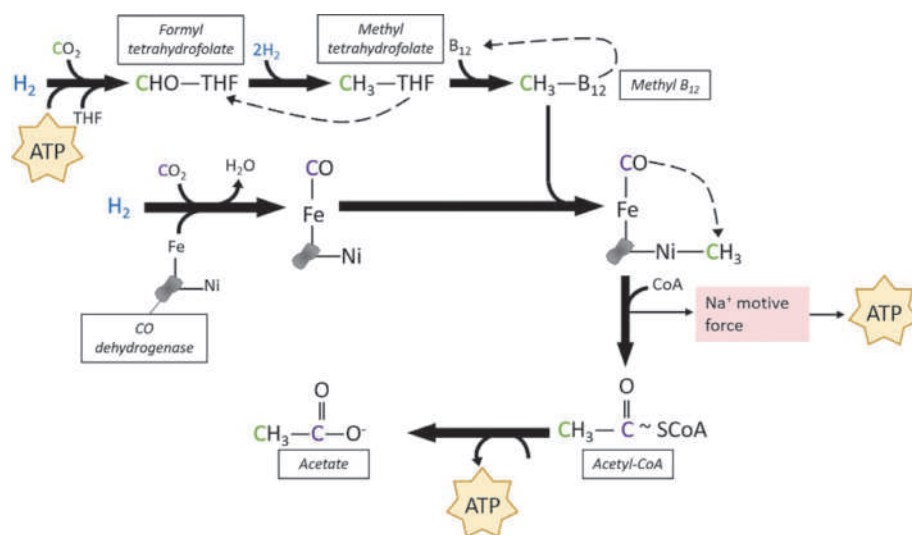


Fig. 6 Acetogenesis. The sequential series of enzymatic reactions which leads to the formation of acetate ($C_2H_3O_2^-$) from CO_2 via acetyl-CoA, using H_2 as an electron donor. The reduced C-atom is indicated in green and electron sources are indicated in blue. Abbreviations are as follows: THF, tetrahydrofolate; B_{12} , vitamin B_{12} . Adapted from Madigan MT and Martinko JM (2006). *Brock Biology of Microorganisms*, 11th edn., pp. 564. Upper Saddle River, NJ: Pearson Education, Inc., Fig. 17.41.

Nitrate reduction and denitrification

Nitrogen gas (N_2), ammonia (NH_3) and nitrate (NO_3^-) are among the most commonly used alternative electron acceptors among anaerobes. Reduction of NO_3^- to NH_3 or N_2 involves a series of sequential redox reactions involving an ETC (Madigan and Martinko, 2006). The enzymes which facilitate this process are usually found in different microorganisms which live in the same niche and thus live synergistically. The opportunistic pathogen *Pseudomonas stutzeri*, however, possesses the full repertoire of enzymes necessary to perform the entire process of NO_3^- reduction to N_2 under aerobic conditions. Denitrification is the loss of the end products derived from NO_3^- reduction, e.g., nitric oxide (NO), nitrous oxide (N_2O) and N_2 (which are all gaseous) to the environment. This represents the major route of biological N_2 formation and is of great environmental significance. This is because it involves the removal of fixed nitrogen sources, nutrients which are vital to plants and therefore agriculture, and can result in accumulation of greenhouse gasses. The entire process of nitrate reduction in microbes, as well as the enzymes involved at each step in various organisms are summarized in Fig. 7.

Due to the lower redox potential of the NO_3^-/NO_2^- couple (+0.43 V, see Fig. 1) a smaller amount of ATP is generated via ATPase. The complete dissimilation of NO_3^- to N_2 yields a greater amount of ATP due to the ability of NO reductase to translocate additional protons within the ETC. Most denitrifying bacteria are facultative anaerobes belonging to the Proteobacteria phylum (e.g., *Escherichia coli*). Other forms of anaerobic respiration found within members of this phylum involve the use of ferric iron (Fe^{3+}) and organic molecules as final electron acceptors, in addition to fermentation.

Sulfate reduction

Dissimilative sulfate (SO_4^{2-}) reduction is limited to the sulfate-reducing bacteria (SRB, e.g., *Desulfovibrio* spp. found in the intestine) in addition to *Archaeoglobus*, an archaeon. The members of this group are ubiquitous in nature and excrete H_2S as a product of SO_4^{2-} reduction. The reduction potential of SO_4^{2-} ($E_0' = +6$) as an electron acceptor is lower than that of O_2 and NO_3^- , thereby yielding less ATP see Fig. 1). A range of electron donors are employed by SRB to complete SO_4^{2-} reduction, these include H_2 , lactate, pyruvate, alcohols (e.g., ethanol), fumarate, malate and long-chain fatty acids. The reaction is summarized in Fig. 8 (Madigan and Martinko, 2006). Firstly, the sulfate ion requires ATP activation due to its stability. ATP sulfurylase catalyzes this reaction which results in the formation of adenosine phosphosulfate (APS). APS reductase then reduces the SO_4^{2-} group in APS to sulfite (SO_3^{2-}), the first product of SO_4^{2-} reduction, releasing AMP. Next, SO_3^{2-} reductase reduces SO_3^{2-} to sulfide (HS^-). A proton motive force is established via electron transport during SO_4^{2-} reduction, which drives ATP synthesis via an ATPase. Cytochrome C_3 plays a key role as an electron carrier, accepting 8 electrons from hydrogenase and transferring them to a cytochrome complex (Hmc). All three proteins are located on the periplasmic side of the membrane. Electrons are shuttled across the membrane from Hmc to an Fe-S protein located

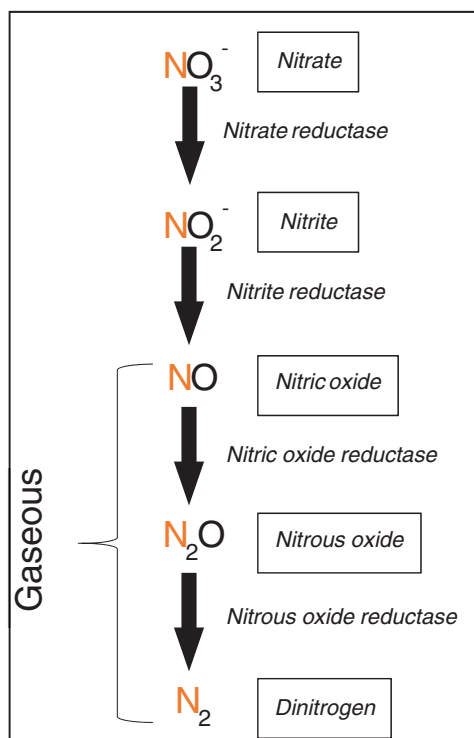


Fig. 7 A summary of the steps involved in dissimilative nitrate reduction to N_2 . The reduced N-atom is indicated in orange. The final three steps result in the release of gaseous products in a process called denitrification. Adapted from Madigan MT and Martinko JM (2006). *Brock Biology of Microorganisms*, 11th edn., pp. 559. Upper Saddle River, NJ: Pearson Education, Inc., Fig. 17.36.

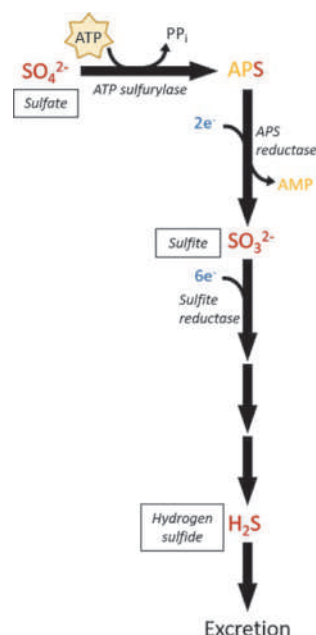


Fig. 8 A summary of the steps involved in dissimilative sulfate reduction to hydrogen sulfide (H_2S) via adenosine 5'-phosphosulfate, a derivative of ADP. The reduced S-atom is indicated in red. Adapted from Madigan MT and Martinko JM (2006). *Brock Biology of Microorganisms*, 11th edn., pp. 561. Upper Saddle River, NJ: Pearson Education, Inc., Fig. 17.38.

on the cytoplasmic side, which in turn supplies these electrons to APS reductase and sulfate reductase within the cytoplasm. Externally sourced H_2 or that generated through the oxidation of organic acids, e.g., lactate to acetate (via pyruvate) through lactate dehydrogenase, can be utilized as electron donors. When H_2 is the electron donor, around one molecule of ATP is generated for every SO_4^{2-} reduction to HS^- . When lactate or pyruvate are electron donors, an additional ATP is produced during the conversion of pyruvate to acetate and CO_2 via acetyl CoA or acetyl-phosphate (see Acetogenesis section).

Some SRB, such as *Desulfovibrio sulfodismutans*, are capable of sulfur disproportionation. This involves splitting a compound, e.g., thiosulfate ($\text{S}_2\text{O}_3^{2-}$), into one more oxidized product, e.g., SO_4^{2-} , and another more reduced product, e.g., H_2S , than the original substrate. The initial substrate is usually a sulfur compound of intermediate oxidation state, additional examples of which include SO_3^{2-} and elemental sulfur (S^0). This allows SRB to coexist with chemolithotrophic bacteria which produce these sulfur intermediates through the oxidation of H_2S . The autotrophic SRB *Desulfotignum phosphitoxidans* is a strict anaerobe which uses phosphite (HPO_3) and CO_2 as its sole carbon and energy source, respectively. This bacterium couples SO_4^{2-} reduction to HPO_3 oxidation, producing phosphate (HPO_4^{2-}) and HS^- chemolithotrophically.

Anabolic pathways: Biosynthesis

Monomers, hexoses such as glucose and its six-carbon sugar derivatives, are the building blocks of the polymers required for cellular growth. The majority of the ATP molecules synthesized during the catabolic processes described above are directed towards protein and nucleic acid synthesis. A comparatively smaller amount of energy is required for lipid and polysaccharide biosynthesis. In the absence of monomers in their surrounding environment, microbes must synthesize these building blocks from simpler constituents through anabolic processes.

Gluconeogenesis is an example of an anabolic process, whereby compounds containing 2–5 carbon atoms are used as substrates to synthesize glucose. In general, the reverse steps of glycolysis are performed from phosphoenolpyruvate, which can be synthesized from oxaloacetate derived from the Krebs cycle. For polysaccharide biosynthesis activated forms of glucose, uridine diphosphoglucose (UDPG) or adenosine diphosphoglucose (ADPG), are used as starting material. UDPG is a precursor for *N*-acetylglucosamine or *N*-acetylmuramic acid, which are present in peptidoglycan or lipopolysaccharide in the Gram-negative cell wall. ADPG is a precursor for glycogen, used in long-term energy storage. Pentose (5-carbon) sugars, e.g., ribose and deoxyribose, are required for nucleic acid synthesis. For this a carbon atom is removed from a hexose sugar and released as CO_2 . The NADPH-dependent ribonucleotide reductase enzyme reduces ribose to deoxyribose, which are precursors of RNA and DNA, respectively.

Synthesis of amino acids, the building blocks of proteins and nucleic acids, consist of complex, multi-step processes. Most of the carbon skeleton required to synthesize all amino acids are derived from either glycolysis or the Krebs cycle. The amino group is derived from a source of inorganic nitrogen in the environment. Glutamate dehydrogenase and glutamate synthase, for example,

incorporate NH_3 into the amino acids glutamate or glutamine, respectively. These amino acids can then shuttle NH_3 through transaminase (via oxaloacetate) or aminotransferase (via α -ketoglutarate) reactions into carbon skeletons which undergo further biosynthetic reactions culminating in the synthesis of all 21 amino acids which form the building blocks of proteins in the cell. Purines and pyrimidines, the monomeric precursors of nucleotides, are constructed from several different carbon and nitrogen sources. Inosinic acid and uridyate are the precursors for purine (i.e., adenine and guanine) and pyrimidine (i.e., cytosine, thymine and uracil) nucleotide synthesis, respectively.

Fatty acids, a major component of lipids in bacteria and eukaryotes, are synthesized via an acyl carrier protein (ACP). This holds the fatty acid chain in position and binds with a 3-carbon compound, malonate, which donates 2-carbon atoms at a time to build the chain. The remaining carbon atom is released as CO_2 . ACP releases the saturated (even carbon number chain length) fatty acid when the desired chain length has been achieved (around C_{12} – C_{20} in bacteria). Unsaturated (odd carbon number chain length) fatty acids are synthesized through the desaturation of a saturated fatty acid and contain at least one double bond. Different microbial species vary in their fatty acid composition and this is temperature-dependent: the higher the temperature, the longer the chain. Lipid assembly in bacteria and eukaryotes involves the addition of glycerol to fatty acids through esterification. Complex lipids may also have phosphate, ethanolamine or a sugar attached to one of the glycerol carbon atoms. Instead of fatty acids, the lipids of archaea contain phytanyl sidechains. In all cases however, the glycerol head is hydrophilic and the tail is hydrophobic. These characteristics allow phospholipids, where the glycerol backbone is bound to a phosphate group and two fatty acid tails, to play a key structural role in cellular membranes.

Conclusion

This chapter summarizes key metabolic pathways present in medically relevant microorganisms. It is important to note, however, that, rather than being passive players, many microbes can remodel their metabolism in response to the host microenvironment. This can facilitate evasion of the immune system (Traven and Naderer, 2019), antibiotic resistance (Davies, 1994) and out-competing rivals (Bauer et al., 2018), ultimately facilitating growth and survival in the host. Microbes are very seldom found as singular entities. Their existence relies on complex metabolic interactions with other microbial species, their host and environment. Understanding this complex web of interactions is facilitated by metabolomics, the study of substrates and products of metabolism (or metabolites). Techniques for characterizing the metabolome include nuclear magnetic resonance (NMR spectroscopy) and chromatography coupled to mass spectrometric detection, e.g., gas chromatography-mass spectrometry (GC-MS, David and Rostkowski, 2020). The metabolic fingerprint generated through metabolomic techniques is thought to be the closest representation of phenotype as it reflects the biochemical activity of a cell or particular biological niche. This approach therefore has great potential in enhancing our understanding of cellular processes in normal and diseases states, biomarker discovery and personalized medicine (Singh, 2020).

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Primary Metabolism of Human Pathogenic Fungi, Importance for Virulence and Potential for Drug Development

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Introduction

Global burden of human pathogenic fungi

Over one billion people worldwide suffer from mycoses. These range from superficial, non-life-threatening infections to potentially mortal chronic and invasive diseases that result in approximately 1.6 million deaths each year (Bongomin et al., 2017). Among the millions of fungal species only around 300 are capable of causing human disease and four genera are responsible for 90% of deaths: *Candida*, *Aspergillus*, *Cryptococcus* and *Pneumocystis* (Taylor et al., 2001; Wu et al., 2019; Brown et al., 2012). The variety of niches within the human body in which fungi are able to grow is astounding, and indeed mycotic infections have been described in every organ system (Denning, 1998; Spampinato and Leonardi, 2013; Urs et al., 2016; Corr, 2011; Gavito-Higuera et al., 2016; Kuttin, 2009; de Moraes et al., 2011; Lionakis et al., 2008). *Aspergillus* and *Pneumocystis* predominantly infect the respiratory system (Brown et al., 2012; Kosmidis and Denning, 2015) but both may also have extrapulmonary manifestations; for example *Aspergillus* is one of the dominant causes of fungal keratitis (Xie et al., 2008; Karam and Mosadegh, 2014). Similarly, *Cryptococcus* infection commences in the lungs, but this fungal pathogen can reach almost every organ by hematogenous dissemination, with infection of the central nervous system being most common and the leading cause of mortality (Kwon-Chung et al., 2014). *Pneumocystis jirovecii* has a highly compacted genome with substantial reduction of metabolic pathways, which explains its nature as an obligate fungal parasite that can only thrive in human lungs (alveoli), where it causes pneumonia in immunodeficient patients (Brown et al., 1980;

Skalski et al., 2015; Nevez et al., 2020). In contrast, *Candida* is a generalist that can thrive as a commensal in niches throughout the body, such as the gastrointestinal tract, vaginal mucosa or the buccal cavity. Under certain circumstances it can cause mucosal infections, which affect millions, and invasive candidiasis, the most common type being candidemia or *Candida* bloodstream infection (Köhler et al., 2015; Bongomin et al., 2017). Worryingly, the incidences of fungal invasive infections are increasing due to the rise of the at-risk populations, and, despite of the availability of antifungal drugs, the mortality rates are intolerably high (Brown et al., 2012; Bongomin et al., 2017; Bitar et al., 2014). This is usually attributed to delayed diagnosis, inappropriate antifungal therapy and emergence of resistance, but also other factors such as high fungal burden, underlying disease, the antifungal pharmacokinetics and pharmacodynamics and toxicity (Fisher et al., 2018; Pagano and Mayor, 2018). This chapter will provide a broad overview of fungal primary metabolism, a prominent source of potential molecular targets for the development of novel antifungal drugs and diagnostic biomarkers.

Importance of primary metabolism and its potential for antifungal exploitation

Primary metabolism can be defined as the group of biochemical pathways that are necessary for energy production, growth and maintenance of life in an organism. Fungi have an extremely versatile metabolism, as they can synthesize virtually all primary metabolites *de novo*, and also use a huge variety of extracellular nutritional sources. Indeed, central to the ability of fungal pathogens to colonise human tissues is their capacity to assimilate essential nutrients and to adapt to changes in nutritional availability between disparate microenvironments within the dynamic mammalian host (Mayer et al., 2013; Kronstad et al., 2012). Therefore, primary metabolism can be considered one of the most important virulence traits for fungal pathogens and its targeting is widely considered a promising strategy for the development of novel antifungals (Martin et al., 2011; Kaltdorf et al., 2016; Amich and Krappmann, 2012).

In this chapter we present a broad overview of primary metabolism in pathogenic fungi. We first explain the mechanisms of regulation and assimilation of essential nutrients. Subsequently, we give a brief overview of the metabolism of the four molecules of life (amino acids, lipids, nucleotides and sugars). We discuss the relevance of all processes for fungal pathogenesis and, furthermore, identify opportunities for the development of new therapeutics.

Macronutrients

Macronutrients or macroelements are those elements that fungi need in large amounts and are integral components of the molecules of life. Here we review the metabolism of the main macronutrients: carbon, nitrogen and sulfur.

Carbon metabolism

Carbon is a fundamental element of organic matter. In living organisms, carbon is essential for energy and macromolecule production and thus for growth. Therefore, fungi, as any other organism, must be able to assimilate it from the environment in large amounts. In order to do so, fungi have evolved signalling networks to identify and prioritize available carbon sources to ensure that the appropriate metabolic response is mounted. In particular, the system of carbon catabolite repression (CCR) inhibits metabolic pathways involved in exploiting alternative sources to guarantee the most energetically favorable source, glucose and other sugars (Ene et al., 2014), is utilized preferentially (Ruijter and Visser, 1997). This is important for pathogenic fungi, which need to acquire large amounts within mammalian hosts, where nutrient availability between and even within a niche is dynamic (Ramachandra et al., 2014). For instance, glucose concentrations in respiratory fluids, a primary site of host-pathogen interaction in many fungal infections, is estimated to be around 0.4 mM of healthy individuals, which can be increased by certain diseases and inflammation (Baker et al., 2007, 2006) and rises to about 5.5 mM in plasma (Danaei et al., 2011). However, alternative organic sources are present in host microenvironments and decision to exploit them or not is important for any pathogen's fitness. For instance, amino acids concentration in pulmonary microenvironments increase during inflammation (Lorenz and Fink, 2001; Grahl et al., 2011; Hu et al., 2010). Lactate is found at concentrations of between 0.5 and 2.2 mM in the plasma of healthy individuals and its concentration increases during inflammation as a result of its production by neutrophils (Bensel et al., 2011). Acetate is present at relatively high concentrations (10–30 mM) in the colon as it is produced by the intestinal microflora, and this correlates to concentrations of approximately 50–200 μ M in plasma (Schug et al., 2016). Importantly, proteins, such as collagen, elastin and mucins, are found across all host tissues and may be used as alternative carbon sources to support fungal growth (Ries et al., 2019; Farnell et al., 2012; Kronstad et al., 2012).

Regulation of carbon metabolism

The main mechanism that fungi use to regulate the acquisition of carbon from the environment is CCR. This system is partially regulated by a zinc finger transcription factor called CreA in *A. fumigatus* and Mig1p in *C. albicans* and *C. neoformans*, which represses the transcription of genes involved in the uptake and catabolism of alternative carbon sources when a preferable one, such as glucose, is present.

CCR has been extensively studied in the model organism *Saccharomyces cerevisiae*. In this yeast, the extracellular glucose levels are sensed through the Snf3p/Rgt2p signalling pathway (Kaniak et al., 2004). In low glucose, this pathway leads to the activation of the

AMP-activated kinase (AMPK) Snf1p, which phosphorylates Mig1p allowing release of glucose repression and expression of glucose-repressed genes. In high glucose, Mig1p remains dephosphorylated and present in the nucleus where it exerts repression, together with the Snf6p/Tup1p complex (Hedbacker and Carlson, 2008; Gancedo, 1998). Snf1p also controls other transcription factors, such as Rgt1p, crucial for fine control of expression of the hexose transporters, Cat8p, required for gluconeogenesis, or Adr1p, important for ethanol utilization and fatty acid metabolism (Palomino et al., 2006; Hedges et al., 1995; Rande-Gil et al., 1997; Young et al., 2002). In addition, extracellular glucose concentrations are also sensed by the G-protein coupled receptor (GPCR) Gpr1p, which regulates Rgt1p and thus expression of hexose transporters via the cAMP/PKA pathway (Xue et al., 1998; Brown et al., 2014b).

In *C. albicans* the Snf3p/Rgt2p homologue Hgt4p is implicated in glucose sensing and regulation of the expression of at least six hexose transporter encoding genes and was found to be relevant for virulence (Brown et al., 2006). Snf1 has not been well characterized yet; it was initially described to be an essential enzyme, although more recently it has been shown that null mutants are viable, and it seems to be regulated by ATP levels rather than glucose availability (Thewes et al., 2007; Zhang et al., 2018; Mottola et al., 2020). One way in which Snf1p maintains ATP homeostasis is through regulation of carbon metabolism during normoxia, a function carried out by Snf5p during hypoxia (Burgain et al., 2019). Snf5p is critical for intestinal colonization in mice and virulence in *Galleria mellonella*. In *C. albicans* Mig1p, and its paralogue Mig2p, primarily act as inhibitors and play an important role in the regulation of genes involved in the import of carbohydrates and catabolic factors (Murad et al., 2001a; Lagree et al., 2020). Surprisingly Mig1p was dispensable for growth in a murine model of systemic candidiasis (Zaragoza et al., 2000). Tup1p co-represses a subset of Mig1p targets, in addition to regulating some genes independently of Mig1p, and contributes to pathogenicity (Ruben et al., 2020; Murad et al., 2001b).

In *C. neoformans*, the proteins responsible for glucose sensing have not been identified yet (Liu et al., 2013). In contrast, Snf1p has been characterized and linked to CCR and its relevance in virulence was demonstrated (Hu et al., 2008a; Yang et al., 2010). Mig1p regulates enzymes involved in synthesis of and energy release from alternative carbon sources (Caza et al., 2016). It also acts on genes involved in heme biosynthesis, and together with Tup1p it has been indicated to play a role in adaptation to iron limiting conditions (Lee et al., 2009). However, Mig1p was found to be expendable for virulence in a murine model of cryptococcosis (Caza et al., 2016).

In filamentous fungi, GPCR and the cAMP/PKA pathway have also been shown to mediate glucose sensing (Lafon et al., 2006). However, the presence of Snf3/Rgt2- type of sensors has not been shown to date, although in *A. nidulans* it was suggested that the transporter HxtB may be involved in glucose signalling (dos Reis et al., 2017). As in yeasts, SnfA controls nuclear/cytoplasm localization of CreA, but the role of this kinase is not well characterised in filamentous fungi yet (Brown et al., 2013; Ries et al., 2018). The role of the transcriptional factor CreA has mostly been studied in *A. nidulans*. In the presence of glucose CreA is translocated to the nucleus and represses genes encoding enzymes for the utilisation of alternative carbon sources (Kulmburg et al., 1993; Tamayo et al., 2008; Ries et al., 2016). In contrast to yeasts, the Tup1p homologue, RcoA, appears not play a direct role in CCR of filamentous fungi, although it affects chromatin structure and indirectly CreA binding to some promoters (García et al., 2008). Deletion of CreA is lethal in *A. nidulans* and other filamentous fungi such as *Fusarium oxysporum*, but not in *A. fumigatus* (Dowzer and Kelly, 1991; Jonkers and Rep, 2009; Beattie et al., 2017). In this pathogen, transcriptome data indicated that CreA is a critical regulator of carbon metabolism and cell energetics and that, unlike yeast Mig1, it both inhibits and activates gene expression. Inhibited pathways include gluconeogenesis and the transport and synthesis of alternative carbon sources such as carbohydrates and amino acids. Interestingly, it has been shown that CreA plays a role in regulating these genes during infection to ensure appropriate responses to changing nutrient availability during infection and is thus crucial for diseases progression in a murine model of invasive pulmonary aspergillosis (Beattie et al., 2017). Additionally, its deletion increases sensitivity to mitochondrial inhibitors and azoles (Furukawa et al., 2020; Beattie et al., 2017). Three additional proteins regulate CCR in filamentous fungi, a deubiquitination enzyme CreB, a WD-40 repeat protein CreC and an arrestin protein CreD. CreB and CreC act in complex to accomplish correct glucose repression, likely through deubiquitination of CreA, although there is still some controversy on the implication of ubiquitination in CreA regulation (Lockington and Kelly, 2002, 2001; Ries et al., 2016; Alam et al., 2016). CreD seems to oppose CreB-CreC action, by ubiquitinating CreA in conjunction with the ubiquitination ligase HulA (Boase and Kelly, 2004).

Uptake of carbon sources

Transport across the membrane is the first step in the metabolism of extracellular carbon sources. As a result, fungi possess numerous transporters and permeases which have evolved to facilitate this critical function. In this section we will focus on transporters of the preferred carbon source, sugars, while the mechanisms involved in transportation of secondary C-sources will be explored in the relevant sections of this chapter.

S. cerevisiae has approximately 20 hexose transporters which impact the pattern of sugar utilization as they vary in their affinity and specificity to particular monosaccharides including glucose, fructose and mannose (Wieczorke et al., 1999; Reifemberger et al., 1997; Guillaume et al., 2007). The induction of these genes is controlled by Snf3p and Rgt2p in response to source availability (Ozcan et al., 1996). In the absence of glucose, Rgt1p forms a complex with Mth1p, Std1p and Snf6p-Tup1p, repressing the expression of hexose transporter-encoding genes. In the presence of glucose, Rgt2p and Snf3p mediate phosphorylation, via casein kinase I (Yck1p/Yck2p), of Mth1p and Std1p, which leads to their degradation by the proteasome, and consequently Rgt1p dissociation from the promoter regions (Kim et al., 2013). Many of the yeast transporters have different affinities for multiple sugars and as such are able to sense sugar ratios which has positive implications for fitness (Escalante-Chong et al., 2015).

S. cerevisiae also has transport systems for the active uptake of disaccharides such as sucrose and maltose, with different affinities to ensure uptake of complex sugars in different conditions (Badotti et al., 2008; Henderson and Poolman, 2017). *C. albicans* has a similar system for the uptake of sugars, with at least 20 known glucose transporters (Fan et al., 2002). Similarly to in *S. cerevisiae*, the Snf3p/Rgt2p orthologue, Hgt4p, regulates the transcription of hexose transporters in response to carbon availability (Brown et al., 2006). This allows the fungus to exploit varying carbon sources encountered in disparate niches and to simultaneously assimilate preferred and alternative carbon sources (Sandai et al., 2012). The expression of numerous hexose transporters is upregulated in murine models of candidiasis showing that glucose sensing and/or transport is important for infection (Fanning et al., 2012; Cheng et al., 2013a; Xu et al., 2015). *C. neoformans* encodes over 50 hexose transporter homologues, however few have been characterized (Liu et al., 2013). Hxt1p is one high affinity glucose transporter that is highly expressed during the initial stages of pulmonary infection, suggesting glucose limitation is experienced by the fungus during the establishment of infection in this niche (Hu et al., 2008a). Hxs1p is an additional high affinity glucose transporter which is essential for full virulence in a murine inhalation model (Liu et al., 2013). In *C. neoformans* mannose is a relevant sugar, which is required in large quantities to maintain its polysaccharide capsule (Bose et al., 2003). Accordingly, it has been found to encode two GDP-mannose, a mannose donor, transporters which are differentially expressed in various environmental conditions and have different contributions to normal capsule formation (Cottrell et al., 2007).

A. nidulans has multiple energy dependent glucose transporters, which similarly to the situation in yeast, can transport multiple sugars with varying affinity (dos Reis et al., 2013; Forment et al., 2014, 2006; Wei et al., 2004; Colabardini et al., 2014). However, none were found to possess the cytosolic tail present in Snf3 and Rgt2 that are required for intracellular signalling (dos Reis et al., 2013). The signalling mechanisms remain uncharacterized, however transcription of the transporters is increased under glucose limiting conditions (dos Reis et al., 2013, 2017). In *Aspergillus niger* numerous putative sugar transporters, with varying substrate specificity, have been identified and shown to display differing expression profiles on various carbon sources (Mäkelä et al., 2018). In this species five transcription factors were found to regulate subsets of sugar transporters, some transporters were regulated by multiple transcription factors (Peng et al., 2018). This allows the fungus to finely tune the response to dynamic nutritional environments. In *A. fumigatus* large numbers of potential sugar transporters have been identified which are differentially induced on various carbon sources (de Gouvêa et al., 2018); however, glucose transporters and their regulation have not been investigated in detail in this species.

After acquisition of sugars, their phosphorylation is often the next metabolic step. *S. cerevisiae* encodes three glucose phosphorylation enzymes, Hxk1p, Hxk2p and Glk1p, which accept different sugars as substrates and phosphorylate these at different rates (Lobo and Maitra, 1977). The hexokinase Hxk2p is a critical glucose kinase that has both glycolytic action and a role in the nucleus as a transcription regulator (Randez-Gil et al., 1998). Transportation into the nucleus is partially determined by its phosphorylation status which is governed by Snf1p and the phosphatase Glc7p (Fernández-García et al., 2012). Research indicates that Hxk2p's conformation is determined directly by cytosolic glucose levels, so that at high glucose levels binding to Mig1p to form a stabilized repressor complex is promoted (Vega et al., 2016). *C. albicans* has four sugar kinases that play a role in glucose metabolism, Hxk1p, Hxk2p, Glk1p and Glk4p (Laurian et al., 2019). Hxk2p and both Glk glucokinases directly contribute to glucose phosphorylation and their expression is positively regulated by Hxk1p (Wijnants et al., 2020). The deletion of *HXK1* singly decreases biofilm formation, a virulence factor in *Candida*, whereas deletion of all four sugar kinases, or the combination of *HXK2*, *GLK1* and *GLK2*, attenuates virulence in a murine model of systemic candidiasis (Wijnants et al., 2020). In *C. neoformans*, a *HXK1* and *HXK2* double deletion strain is unable to utilize glucose and displays severely reduced virulence in a murine inhalation model (Price et al., 2011). In *A. fumigatus* sugar phosphorylation is dependent on a glucokinase, GlkA, and hexokinase, HxkA, which have different efficacies for activating various sugars (Fleck and Brock, 2010). Both enzymes are required for proper CCR and growth, deletion of *glkA* results in delayed germination, whereas *hxkA* has a stronger impact on mycelial growth (Fleck and Brock, 2010).

Nitrogen metabolism

Nitrogen is an essential nutrient that is required for most biosynthetic processes. To facilitate growth within the human body pathogenic fungi are able to utilize a wide variety of nitrogen sources. However, particular sources are favored as their efficient assimilation makes them energetically favorable (Tudzynski, 2014). Ammonia and asparagine are among these preferred nitrogen sources and are present in all major tissue locations (Marzluf, 1997; Lee et al., 2013b). Ammonia concentrations in the plasma of healthy individuals is limiting at approximately 20 μM , although it can be elevated by a range of health conditions including cystic fibrosis (Ucar et al., 2016). Some species, such as *A. fumigatus*, can produce ammonia from nitrate in the absence of a preferred nitrogen source (Zhou et al., 2002). Glutamine is another preferred source found in a more limited number of tissues, including the gut, kidney and blood where it is the most abundant free amino acid, at concentrations of $\sim 650 \mu\text{M}$ (Lee et al., 2013b; Blomstrand and Essén-Gustavsson, 2008; Mandal et al., 2012). An additional favored source, glutamate, is an important signalling molecule in various tissues throughout the human body (Bai and Zhou, 2017). Concentrations of the amino acid vary among tissue types, reaching around 100 μM in plasma and 6 μM in the cerebral spinal fluid (Madeira et al., 2018a, b). For many fungi the primary site of infection are the mucosal membranes of the gastrointestinal, ocular, respiratory, reproductive or urinary tracts. These epithelia secrete large quantities of mucin glycoproteins which likely act as alternative nitrogen and/or carbon sources (Linden et al., 2008). Similarly, upon invasion of underlying tissue their predominate source of nitrogen will likely be proteins, including collagen and elastin, peptides and amino acids (Farnell et al., 2012; Brandão et al., 2018).

In addition to the difference in nitrogen availability encountered between tissues the health status, antimicrobial exposure, activity level and diet of the host can cause rapid shifts (Blomstrand and Essén-Gustavsson, 2008; Schmidt et al., 2016; Gaston et al., 2002). To maximize growth, fungi are able to inhibit the use of alternative nitrogen sources under dynamic conditions when preferred sources are available, by a process termed nitrogen catabolite repression (NCR) (Wong et al., 2008). Indeed, the nitrogen sources utilized influences morphological transitions and the production of virulence factors by various pathogenic fungi (Lee et al., 2013b).

Regulation of nitrogen metabolism

When preferred nitrogen sources are limited GATA transcription factors regulate expression of many nitrogen catabolic enzymes (Marzluf, 1997). These regulators play a key role in the exploitation of a range of nitrogen sources in three major fungal pathogens: *A. fumigatus*, AreA, *C. albicans*, Gat1p and Gln3p, and *C. neoformans*, Gat1p (Liao et al., 2008; Lee et al., 2011; Hensel et al., 1998). They allow the utilization of alternative sources by activating the expression of proteins that facilitate their breakdown and uptake. NCR forms part of a very complex regulatory network that includes many enzymes, some of which play roles in processes beyond nitrogen utilization. Therefore, when specific enzymes are shown to be relevant for virulence, it is not always possible to establish a direct correlation with their role in nitrogen metabolism (Lee et al., 2011).

The critical role of NCR and AreA in determining nitrogen utilization has been most extensively studied in *A. nidulans*; in fact the latter was the first GATA transcription factor identified as coordinating nitrogen utilization and is highly conserved in *Aspergillus* species (Arst and Cove, 1973; Han et al., 2016). The level of AreA within the cell must be tightly controlled so the fungus maintains a hierarchy of utilization of preferred nitrogen sources (Morozov et al., 2001). One mechanism by which the levels of AreA are modulated involves the sensing of intracellular nitrogen availability: AreA mRNA is degraded when preferred nitrogen sources are abundant, this is primarily but not exclusively determined by glutamine levels (Morozov et al., 2001). In addition, a co-repressor, NmrA, competes with DNA to bind AreA in the presence of preferred nitrogen sources, therefore reducing expression of nitrogen catabolic enzymes that are upregulated by AreA (Kotaka et al., 2008; Morozov et al., 2001). Expression of NmrA is positively regulated by the transcription factor MeaB during nitrogen sufficiency (Wong et al., 2007). When preferred nitrogen sources are limiting three *A. nidulans* proteases cleave NmrA, thereby removing its inhibition of the utilization of alternative nitrogen sources (Zhao et al., 2010). The transcriptional function of AreA requires nuclear localization, which is facilitated by its unusually high number of nuclear localization signals (Hunter et al., 2014). Once in the nucleus, AreA can interact with its co-activator TamA to initiate the expression of nitrogen catabolic enzymes (Small et al., 2001). An additional GATA transcription factor, AreB, is involved in nitrogen and also carbon catabolite repression in *A. nidulans*, seemingly via regulation of key factors as AreA, TamA or CreA (Chudzicka-Ormaniec et al., 2019). AreA is not necessary for *A. fumigatus* growth on ammonium or glutamine, although growth is reduced compared to wildtype at low concentrations (Hensel et al., 1998). It is essential for growth on many amino acids, including glutamate and asparagine, and when using collagen as the primary source of nitrogen when a preferred carbon source is available (Hensel et al., 1998). In a murine model of invasive pulmonary aspergillosis loss of AreA in *A. fumigatus* resulted in a minor delay and slight reduction of mortality (Hensel et al., 1998). This delay in virulence might be explained by an interruption in germination, as AreA is upregulated ~9 fold during the initiation of germination in wildtype conidia (Lamarre et al., 2008).

C. albicans processes two important GATA transcription factors, Gln3 and Gat1, with partially redundant functions. Their deletion allows growth on a wider range of nitrogen sources than upon deletion of AreA in *A. fumigatus* (Liao et al., 2008; Limjindaporn et al., 2003). Single and double mutants are able to utilize ammonium as the sole nitrogen source, however Gln3p is necessary for normal filamentous growth in nitrogen limiting conditions (Dabas and Morschhäuser, 2007). Both Gat1p and Gln3p contribute significantly to the virulence of *C. albicans* in murine models of disseminated candidiasis (Limjindaporn et al., 2003; Liao et al., 2008). Similarly to the theory proposed for *A. fumigatus*, this might be due to the misregulation of nitrogen utilization but also to impaired filamentation during infection. In *C. neoformans* Gat1p is responsible for the utilization of many nitrogen sources, including ammonium, and has a complex regulatory role during infection as it contributes to a wide variety of pathways (Cui et al., 2019; Lee et al., 2013b; Kmetzsch et al., 2011); its deletion, however, did not compromise virulence in a mouse model of pulmonary cryptococcosis (Kmetzsch et al., 2011). GATA repressors are not well characterized however, the genomes of *C. albicans* and *C. neoformans* do not appear to encode homologues of NmrA (Ries et al., 2018). One putative GATA repressor, Gzf3p, has been identified in *C. albicans*, but its function has not to date been thoroughly investigated (Holland et al., 2014). However, the homologue of Gzf3p in *Candida parapsilosis* has been shown to mediate NCR (Turner et al., 2018). These three species highlight that within NCR systems utilized to exploit appropriate nitrogen sources, the mechanisms involved can vary significantly and that there are many opportunities to advance the field's knowledge of the role of nitrogen metabolism during fungal infection. In line with this, the importance of investigating NCR in *Paracoccidioides* pathogens has recently been highlighted (Milhomem Cruz-Leite et al., 2020).

Uptake of nitrogen sources

The enzymes that sense and transport extracellular nitrogen containing compounds are central to the ability of fungi to correctly utilize available nitrogen sources, and are therefore a potential source of promising antifungal targets. Fungi express a broad spectrum of transporters and G-protein-coupled receptors to allow the uptake of preferred and alternative nutrient sources (Diallinas, 2017; Rutherford et al., 2019), several of which have been implicated in virulence (Brown et al., 2018).

Fungal ammonium permeases are critical as they allow the detection and uptake of preferred sources, even when present at limited concentrations (Rutherford et al., 2008). *C. albicans* has two ammonium permeases, Mep1p and Mep2p, that allow growth

even at low levels of ammonium (Biswas and Morschhauser, 2005). At high concentrations the transporters are not required for growth, presumably as ammonium uptake is possible through nonspecific uptake or simple diffusion (Biswas and Morschhauser, 2005). Mep2p has an additional role in pseudohyphal growth induction when it is not utilized for ammonia uptake, thereby using low ammonium conditions as an inducer of morphogenesis (Biswas and Morschhauser, 2005). Expression of *MEP2* is induced under limiting conditions by Gat1p and Gln3p (Dabas and Morschhäuser, 2007). *C. neoformans* has two ammonium transporters, Amt1p and Amt2p, which are also essential for growth in ammonium limiting conditions and the formation of pseudohyphae, but are dispensable for virulence (Lee et al., 2012b; Rutherford et al., 2008). A homologous putative ammonium transporter, MeaA, has been identified but not characterized in *A. fumigatus* (Escobar et al., 2018).

The ability of fungal pathogens to breakdown complex polypeptides and to assimilate the breakdown products from the extracellular space is key to survival within many host niches, particularly in environments such as the lung where proteins dominate as a nitrogen source (Farnell et al., 2012). *A. fumigatus* secretes an impressive variety of secreted proteases, with the particular combination depending on nutrient availability (Farnell et al., 2012). However, elimination of the transcription factors PrtT and XprG, positive regulators of the expression of secreted proteases, alone or in combination did not reduce *A. fumigatus* virulence in models of systemic and pulmonary invasive infection (Sharon et al., 2009; Bergmann et al., 2009; Shemesh et al., 2017). A high degree of redundancy within secreted proteolytic activities may explain why none have been found to be essential for growth within host tissues (Ries et al., 2018). Therefore, the relevance of proteases for *A. fumigatus* virulence still needs to be clarified. Nevertheless, fungal secreted proteases are important allergens, which has been proposed to be caused by their activity to cleave fibrinogen and activate TLR4 signalling (Millien et al., 2013). For instance, the serine protease Alp1/Aspf13 has been shown to be relevant in asthma where it causes degradation of the extracellular matrix within the bronchioles of patients (Druey et al., 2020).

C. albicans produces 10 secreted aspartic proteases (SAP) which are differentially secreted as infection progresses and are implicated in epithelial damage (Naglik et al., 2008). SAPs can cleave a variety of human proteins that could be used to provide nitrogen and six have been shown to contribute to virulence of *C. albicans* in rodent infection models (Cassone et al., 2016; Naglik et al., 2003; Hube et al., 1997; Sanglard et al., 1997).

In *C. neoformans* the urease Ure1p is an important virulence factor in murine cryptococcosis models of intravenous and inhalation infections (Cox et al., 2000). The enzyme is not required for growth in the brain but plays a role in dissemination and central nervous system invasion (Olszewski et al., 2004). Similarly, deletion of the urease and ureidoglycolate hydrolase genes in *Coccidioides posadasii* significantly reduced virulence in a murine model of infection (Wise et al., 2013). Serine proteases were also implicated as playing a role in the permeabilization of the blood brain barrier (Xu et al., 2014a). The *C. neoformans* genome encodes 8 putative global amino acid permeases, two of which are essential for virulence in an inhalation model of murine cryptococcosis (Martho et al., 2016).

Sulfur metabolism

Sulfur is an essential nutrient involved in a multitude of crucial metabolic processes in fungi. For instance, it is constituent of the proteogenic amino acids cysteine (Cys) and methionine (Met). Sulfur is very relevant for oxidative stress defense, as the thiol group in the redox-active molecules glutathione, thioredoxin, peroxiredoxin and ergothioneine is fundamental for their activity (Ulrich and Jakob, 2019). Sulfur is also fundamental for methylation reactions, as it is an integral constituent of the major cellular methyl donor S-adenosylmethionine (SAM), the synthesis of which is essential for fungal viability (Chiang et al., 1996; Gerke et al., 2012). Sulfur is also crucial for enzymatic functions, as it is integral component of several major co-factors as Fe-S clusters (which are also fundamental for iron sensing), vitamins (biotin, thiamine, pantothenic acid) and co-enzyme-A (Misslinger et al., 2018; Sprenger et al., 2020; Dietl et al., 2018; Nakamura et al., 2012).

Regulation of sulfur metabolism

The regulation of sulfur metabolism in pathogenic fungi has been mostly investigated in filamentous fungi, where it is tightly regulated by catabolite repression (Marzluf, 1993). The Cys3/MetR bZIP transcriptional factor activates its own expression and that of genes required to acquire and utilize sulfur sources upon S-starvation (Paietta et al., 1987; Petra et al., 1992; Natorff et al., 2003). This activator is crucial for *A. fumigatus* and important for *C. neoformans* virulence and is important for the regulation of sulfur assimilation in the dimorphic switch of *Paracoccidioides brasiliensis* (Amich et al., 2013; de Melo et al., 2019; Ferreira et al., 2006). In *A. nidulans*, an additional transcriptional factor, MetZ, has been described, seemingly involved in the regulation of sulfur metabolism (Pilsyk et al., 2015). In conditions of sufficient sulfur the so-called sulfur controller (*scon*) genes negatively impact Cys3, most likely through its proteolytic degradation (Natorff et al., 1993; Kumar and Paietta, 1998; Paietta, 1990; Peterson et al., 2014). Regulation of sulfur metabolism has also been extensively studied in *Saccharomyces cerevisiae*, where a heteromeric complex of three proteins (Met4p-Met28p-Cbf1p) is responsible for transcriptional regulation (Kuras and Thomas, 1995; Thomas et al., 1992; Kuras et al., 1996). Sulfur regulation has not been investigated in detail in *Candida* species, but it seems to resemble the situation in *S. cerevisiae* (Hébert et al., 2011).

Uptake of sulfur sources

Fungi can assimilate sulfur from inorganic and organic sources. Assimilation of sulfate has been well characterized in yeasts and filamentous fungi, where it is first transported into the cell by a sulfate permease and subsequently reduced to adenosine

5'-phosphosulfate (APS) by an ATP sulfurylase. APS is then phosphorylated by an APS kinase to form 3'-phosphoadenosine 5'-phosphosulfate (PAPS) followed by sulfite release from PAPS by action of a PAPS reductase. Sulfite is reduced to sulfide by a sulfite reductase and can then be incorporated into cysteine via O-acetylserine through a process catalysed by a cysteine synthase or into homocysteine via O-acetylhomoserine through a process catalyzed by a homocysteine synthase (Marzluf, 1997). Homocysteine can be methylated to form methionine, and cysteine and methionine can be interconverted within the trans-sulfuration pathway. Fungi can assimilate sulfur from chemically diverse organic sources, as reviewed in (Linder, 2018). Many of these routes converge in the sulfate assimilation pathway, as the rhodanese dependent processing of thiosulfate, the arylsulfatase dependent assimilation of sulfate esters or the assimilation of volatile sulfur compounds (Chen et al., 2018; Linder, 2018; Scott et al., 2019). However, an *A. fumigatus* sulfite reductase mutant was fully virulent, suggesting that the assimilation of inorganic and complex organic S-sources is not relevant in the mammalian tissues (Amich et al., 2016). Nevertheless, high levels of sulfate esters can be found on the airway and intestinal epithelial surfaces (e.g. sulfated mucins) and of sulfamates in the extracellular matrix (e.g. heparan sulfate), so more studies are needed to definitely exclude these compounds as S-sources. Most likely fungal pathogens obtain sulfur from the S-containing amino acids cysteine and methionine and their derivatives (SAM, glutathione, etc...), based on uptake by amino acid permeases and oligopeptide transporters (Amich et al., 2016). This idea is also supported by the impossibility of several dimorphic fungi to assimilate inorganic sulfur source in the pathogenic yeast form (Medoff et al., 1987; Paris et al., 1985; Boguslawski and Stetler, 1979). However, even if there are specific transporters described, as Mup permeases for methionine uptake, the presence of general amino acid permeases makes it difficult to specifically prevent uptake of the S-containing amino acids to validate this hypothesis (Isnard et al., 1996; Garbe and Vylkova, 2019; Martho et al., 2016).

Micronutrients

Micronutrients or microelements are those that fungi require in trace amounts and are usually cofactors and coordinators of the molecules of life.

Iron

The biological functions of iron are based on its ability to cycle between two oxidation states: ferrous (Fe^{2+}) and ferric (Fe^{3+}). The corresponding redox potential makes it an ideal catalyst for a multitude of cellular and metabolic processes, including respiration and DNA replication. In humans most of the iron in circulation is complexed with heme, bound by hemoglobin within red blood cells. In plasma, iron is bound to transferrin, which is used to safely distribute this element to cells. In cells, excess of iron (not incorporated in iron-sulfur clusters or redox co-factors) is stored in ferritin. From a host side of view, the availability of iron and other trace elements is actively reduced during infection as a strategy to limit pathogen growth. This process has been termed nutritional immunity and is paramount for antimicrobial defense (Hood and Skaar, 2012). In order to scavenge extracellular iron, neutrophils secrete lactoferrin, an iron-binding glycoprotein (Lönnerdal and Iyer, 1995; Zarembek et al., 2007). Furthermore, in response to inflammatory stimuli hepatocytes secrete hepcidin, a hormone that triggers the internalization and degradation of the cellular iron exporter ferroportin, therefore reducing iron export from macrophages and enterocytes and decreasing the levels of extracellular iron (Nemeth and Ganz, 2009).

Fungi have evolved four different strategies for iron uptake. Fungal pathogens have the genes required to deploy several but not all of the below strategies. The relative relevance of each strategy (set of genes) for fungal virulence mostly depends on the source of iron at the site of infection.

One strategy is low affinity iron uptake, which is based on permeases (Fet4p, Smf1p) that are not specific for ferrous iron, but also can transport other metals (Kaplan and Kaplan, 2009). Although this system is present in many pathogenic fungal species, it does not seem to be important for virulence (Jung et al., 2008). Nevertheless, it appears to be relevant for iron uptake under hypoxic conditions, a condition that is known to impact iron homeostasis, and therefore a role of low-affinity iron uptake for fungal virulence cannot be completely excluded yet (Jensen and Culotta, 2002; Chung et al., 2012; Blatzer et al., 2011).

Another strategy for iron uptake is reductive iron assimilation, which involves two sequential steps: first a reduction of ferric iron to soluble ferrous iron by membrane-located ferric reductases, encoded by the *FRE* genes, to be followed by subsequent re-oxidation to the ferric form by a high-affinity heterodimeric transport complex consisting of the multicopper ferroxidase Fet3 and the permease Ftr1. *FRE* reductases are integral membrane proteins that require heme, FAD and or NAD(P)H (Philpott, 2006). Most pathogenic fungi encode several ferric reductases, such as 18 in *C. albicans*, 15 in *A. fumigatus* or 8 in *C. neoformans*, but likely not all of them exert a ferric reductase activity (Jeeves et al., 2011; Saikia et al., 2014; Fourie et al., 2018). Ferrous iron needs to be re-oxidized in a reaction that is copper dependent, and copper ions are inserted into Fet3 action of the chaperone Atx1 and the pump Ccc2 (Lin et al., 1997; Yuan et al., 1995). The transport of ferric iron involves a classic metabolite channelling mechanism where amino acid residues in both Fet3 and Ftr1 interact with each other to drive the iron into the cell (Kwok et al., 2006). Reductive iron assimilation has been shown to be important for virulence of various fungal pathogens, as *C. albicans* (where is also required for iron acquisition from transferrin and ferritin), *C. neoformans*, *C. glabrata* (although the latter does not contain ferric reductases), *Rhizopus oryzae*, *Mucor circinelloides* (Navarro-Mendoza et al., 2018; Ibrahim et al., 2010; Gerwien et al., 2017; Srivastava et al., 2014; Xu et al., 2014b; Cheng et al., 2013b; Knight et al., 2005; Ramanan and Wang, 2000; Almeida et al., 2008; Saikia et al., 2014; Jung et al., 2008, 2009). *Paracoccidioides* spp., have a non-traditional reductive iron assimilation, involving iron reduction and

zinc-regulated transporter homologs, which has been suggested to be important for persistence in macrophages (Camacho and Niño-Vega, 2017; Bailão et al., 2015).

An alternative mechanism is the acquisition of iron from heme, most of which is bound to hemoglobin, which contains about 70% of the total body iron in humans. Some fungi have evolved a distinct pathway to scavenge iron from heme and hemoglobin. This pathway can be divided in three steps. Firstly proteins containing a domain exclusively found in fungi, named Common in Fungal Extracellular Membrane (CFEM), extract heme from the host hemoglobin and mediate its delivery to the fungal cell (Weissman and Kornitzer, 2004). At least three of the six CFEM proteins present in *C. albicans* genome seem to form a transfer cascade for heme, from extracellular secreted Csa2p to the cell wall anchored Rbt5p and then to Pga7p on the plasma membrane (Kuznets et al., 2014; Nasser et al., 2016). Subsequently, heme is delivered through the endocytic system to the vacuole compartment via action of several ESCRT (endosomal sorting complex required for transport) proteins and a type I myosin (Myo5p) (Weissman et al., 2008). Finally, the oxygenase Hmx1p metabolizes heme and has been described to be required for its utilization, although other studies suggested that alternative heme-degrading enzymes may exist in the vacuole (Pendrak et al., 2004; Santos et al., 2003; Roy and Kornitzer, 2019). Heme uptake seems to contribute to *C. albicans* virulence (Kuznets et al., 2014). Other *Candida* species can also utilize heme (Pinsky et al., 2020). In *Cryptococcus neoformans* it is still unclear whether CFEM proteins are involved in heme binding and internalization; a secreted mannoprotein (Cig1p) is likely able to perform this function and was reported to contribute to *C. neoformans* virulence (Bairwa et al., 2019). For heme endocytic delivery to the vacuole in *C. neoformans* has been shown that in addition to ESCRT proteins, several components of the clathrin mediated endocytosis are required for hemoglobin utilization, elaboration of virulence factor and pathogenesis (Hu et al., 2015; Bairwa et al., 2019). Hemoglobin uptake also seems to be required for *Paracoccidioides* ssp. virulence (Bailão et al., 2014).

A relevant strategy that fungi use to acquire iron is the utilization of siderophores, heterogeneous small molecules secreted by bacteria and fungi, which bind extracellular ferric iron with extremely high affinity. Many fungi produce hydroxamate-type siderophores to solubilize and take up iron (Haas et al., 2008). Siderophores are important for the virulence of fungal pathogens that produce them, for instance *Histoplasma* and *A. fumigatus* and its synthesis has been extensively studied in the latter (Haas, 2014; Schrettl et al., 2004; Hwang et al., 2008). *A. fumigatus* produces four siderophores: two extracellular fusarinine-type siderophores, fusarinine-C and triacetylfusarinine-C for iron uptake, and two intracellular ferrichrome-type siderophores, ferricrocin for iron distribution and storage in hyphae and hydroxyferricrocin for storage of iron in spores (Schrettl et al., 2007). The biosynthetic pathway starts with the N^5 -hydroxylation of L-ornithine catalysed by the L-ornithine- N^5 -monooxygenase SidA (Schrettl et al., 2004). Subsequently, the pathway splits in two branches to form extracellular (by action of SidF, SidD and SidG) and intracellular (by action of SidL, SidC) siderophores (Haas, 2014). Siderophore-iron complex uptake is mediated by the siderophore-iron transporters (SITs), which belong to the major facilitator protein superfamily (Yun et al., 2000a, b). SIT transporters are present in all fungal species, even in those not producing siderophores. Species such as *Candida* spp. and *C. neoformans* can exploit xenosiderophores (i.e. siderophores produced by other bacteria or fungi), which probably confers an advantage in competing microbial interactions, but is not required for virulence (Hu et al., 2002; Tangen et al., 2007). Finally, iron is released from the internalized siderophores by action of specific esterases to incorporate it in the fungal metabolism or transfer it to intracellular siderophores for storage (Kragl et al., 2007; Ecker et al., 2018).

Transcriptional regulation of iron homeostasis is conserved in virtually all pathogenic fungi, with the prominent exception of *C. glabrata* (Gerwien et al., 2016). This homeostatic system comprises two transcriptional factors, a GATA transcription factor that downregulates iron acquisition (called Sfu1, Cir1, SreA) and a CCAAT-binding complex that upregulates iron acquisition and downregulates iron consumption pathways (Hap43, HapX) (Chen et al., 2011; Lan et al., 2004; Hwang et al., 2012; Hsu et al., 2011; Jung et al., 2010; Schrettl et al., 2010). These factors are interconnected in a negative feed-back loop: Sfu1/SreA represses expression of *hap43/hapX* during iron sufficiency, while Hap43/HapX represses *sfu1/sreA* during iron starvation (Singh et al., 2011; Chen et al., 2011; Haas, 2014). *A. fumigatus* HapX has been shown to be important not only under iron starvation, but also to activate iron detoxification mechanisms at high iron concentrations, a double function that appears to be conserved in other fungal pathogens (Gsaller et al., 2014). Both Sfu1/SreA and Hap43/HapX function seems to be regulated by direct iron binding, which apparently block HapX and activates SreA function (Hortschansky et al., 2007; Haas et al., 1999). In addition, the monothiol glutaredoxin (Grx4/GrxD) is essential for sensing the availability of iron and to control the activity of the transcriptional factors (Attarian et al., 2018; Misslinger et al., 2019). Given the lower availability of iron in human tissues, it is not surprising that Hap43/HapX are essential for virulence in all fungal pathogens studied so far (Schrettl et al., 2010; Hsu et al., 2011). In *C. albicans* Sfu1 is required for persistence in the iron-replete gut, illustrating the importance of adaptation to different iron availability conditions (Chen et al., 2011). Indeed, possibly due to this two lifestyles of *C. albicans* (as a commensal and as a pathogen), this species has incorporated a third regulator into the system (Sef1), to serve as an activator of Hap43 expression and that is required for full virulence (Chen and Noble, 2012).

Given the importance of iron homeostasis, it is not surprising that its regulation is tightly linked to the homeostasis of other elements (as copper, zinc or sulfur) and to the adaptation to particular conditions that can affect its availability or its cellular need (Hsu et al., 2011; Vicentefranqueira et al., 2019; Amich et al., 2013; Baek et al., 2008; Eisendle et al., 2004; Chung et al., 2012).

Zinc

Zinc is an essential metal crucial for many cellular functions and an indispensable micronutrient for all living organisms. Approximately 9% of all eukaryotic, including fungal, proteins specifically bind zinc (Andreini et al., 2009). This metal is co-factor of more than 300 enzymes and is the only metal present in all six types of enzymes (according to the functional

classification of the International Union of Biochemists) (Vallee and Galdes, 1984; Auld, 2001). Among its many functions, zinc is particularly important in DNA replication and transcription, as it is required for the activity of all DNA and RNA polymerases and a significant number of transcription factors contain zinc-finger domains (Wu and Wu, 1987; Andreini et al., 2006).

Acquisition of zinc is therefore essential for fungal viability and virulence. The concentration of free zinc in tissues and cells is however very low, as it is predominantly bound to proteins (Tapiero and Tew, 2003; Maret, 2015). Furthermore, similarly to iron, upon infection the host nutritional immunity can further reduce the availability of zinc through the activity of transporters or the expression of zinc-binding proteins, such as calprotectin (a neutrophil-produced member of the S100 family of metal-binding proteins) (Corbin et al., 2008; Urban et al., 2009). In addition, macrophages can intoxicate fungi inside lysosomes with zinc (Sacks et al., 2018). Accordingly, fungi contain specialized and tightly regulated systems for zinc uptake and homeostasis, which have been proven to be required for virulence in all the species studied to date (Wilson, 2015).

As for other metals, zinc homeostasis must be finely regulated to ensure uptake in restrictive conditions and prevent toxic effects. In fungi, the zinc responsive activator protein Zap1 is the major transcriptional factor regulating zinc homeostasis (Zhao and Eide, 1997). Under zinc limiting conditions, Zap1 (also known as Csr1 in *C. albicans* and ZafA in *A. fumigatus*) upregulates its own transcription, as well as of zinc importers (ZIP, Zrt-, Irt like Proteins) and cytosolic exporters (CDF cation diffusion facilitator or ZnT) (Kim et al., 2008b; Moreno et al., 2007; Gaither and Eide, 2001). The relevance of zinc importers for fungal virulence has been shown in all species studied (Amich et al., 2014; Crawford et al., 2018; Do et al., 2016; Schneider et al., 2015; Dade et al., 2016). In addition, some species as *C. albicans* and *A. fumigatus* secrete a zinc-binding protein ("zincophore") that can sequester the metal from host cells and facilitate its uptake (Cititolo et al., 2012; Amich et al., 2009; Wilson, 2015). Upregulation of ZnT transporters ensures prevention of toxic effects, and has been also shown to be important for fungal virulence (Crawford et al., 2018). Zap1 has also been implicated in the regulation of other virulence factors in some species, such as capsule and melanin formation in *C. neoformans*, *C. albicans* morphogenesis, biofilm formation and adhesion, or amino acid and gliotoxin biosynthesis in *A. fumigatus* (Liu et al., 2008; Jung et al., 2015; Kim et al., 2008b; Nobile et al., 2009; Finkel et al., 2012; Vicente-franqueira et al., 2018). Besides, in most fungal pathogens zinc transporters are co-regulated by the pH master regulator PacC/Rim101 and the activity of transporters is pH optimised, which is likely due to the lower solubility of Zn in alkaline pH (Wilson, 2015; Bensen et al., 2004; Amich et al., 2009, 2014; Crawford et al., 2018).

Copper

Copper is essential for eukaryotic life. The ability of copper to cycle between oxidized (Cu^{2+}) and reduced (Cu^+) states underlies its function as cofactor for many enzymes to catalyze biochemical processes as aerobic respiration (cytochrome-c oxidase), superoxide detoxification (superoxide dismutase), iron acquisition or pigmentation (tyrosinase) (Cox et al., 2003; Askwith et al., 1994; Gruhn and Miller, 1991). However, copper also rapidly becomes toxic at increased levels, as it can inactivate other metalloenzymes, inappropriately engage with intracellular metallophilic ligands and generate reactive oxygen species through Fenton-like chemistry. Therefore, copper homeostasis must be finely tuned to maintain its levels within homeostatic ranges. In fact, host nutritional immunity appears to utilize both copper sequestration and poisoning to fight fungal (and other) infections (Besold et al., 2016).

Upon copper limitation, a transcriptional factor called Mac1 in *A. fumigatus* and *C. albicans* and Cuf1 in *C. neoformans* activates uptake, which is based on membrane metalloredutases (FRE), that reduce Cu^{2+} to Cu^+ , and subsequent import via high-affinity Cu^+ importers, Ctr1 in *C. albicans*, Ctr1 and Ctr4 in *C. neoformans* and CtrC and CtrA2 in *A. fumigatus* (Park et al., 2017, 2014; Kusuya et al., 2017; Marvin et al., 2004, 2003; Waterman et al., 2007; Woodacre et al., 2008; Ding et al., 2011). Copper is then bound by chaperones that direct copper to the desired location: Atx1 to Ccc2 Cu-transporting ATPases of the Golgi membrane, Ccs1 to the Cu/Zn-superoxide dismutases (SODs) and Cox17 for transporting copper into mitochondria for the assembly of cytochrome c oxidase (Lin and Culotta, 1995; Lin et al., 1997; Culotta et al., 1997; Gleason et al., 2014; Boyd et al., 2018; Horng et al., 2004; Glerum et al., 1996; Punter and Glerum, 2003).

High intracellular copper levels are sensed by the same factor in *C. neoformans* (Cuf1), but a different factor in other species, named Cup2 in *C. albicans* and AceA in *Aspergillus* (Ding et al., 2011, 2013; Schwartz et al., 2013; Antsotegi-Uskola et al., 2017; Wiemann et al., 2017). These factors activate different mechanisms to prevent and counteract copper toxicity, as P1-type ATPase copper exporter, copper metallothioneins, accumulation in vacuoles and Fe-S biogenesis (Riggle and Kumamoto, 2000; Weissman et al., 2000; Wiemann et al., 2017; Oh et al., 1999; Ding et al., 2011; Rees and Thiele, 2007; Garcia-Santamarina et al., 2017).

As mentioned above, fungi may encounter copper limiting and toxic niches during infection, which implies that both Cu^+ acquisition and detoxification systems are likely relevant for fungal virulence. For instance, *C. neoformans* seems to face copper limitation in the brain and poisoning in the lung (Waterman et al., 2007; Ding et al., 2013). Copper detoxification is also important for *A. fumigatus* and *A. flavus* virulence, while the role of copper uptake is somewhat controversial as some studies reported its relevance for virulence, other reported no effect (Wiemann et al., 2017; Yang et al., 2018; Park et al., 2018; Cai et al., 2017). Similarly, studies in *C. albicans* have shown that a dynamic response is required to counteract both host-imposed limitation and poisoning (Besold et al., 2018).

Other metals

Many elements are required in trace amounts for the correct functioning of fungal cells. Here we discuss some of the most important elements which have been studied and are related with fungal virulence.

Calcium is a paramount signalling molecule in eukaryotes. In fungi it is essential for many processes such as growth and development, reproduction, stress adaptation and virulence. Calcium signalling is initiated by a sharp rise in cytosolic Ca^{2+} , which binds and changes conformation of Ca^{2+} -binding proteins, followed by a fast decrease in its concentration. These changes in intracellular calcium concentration are exerted by six major types of transporters that uptake Ca^{2+} from the extracellular space and mobilize or store it in organelles: high-affinity calcium system (HACS), low-affinity calcium system (LACS), Ca^{2+} pumps, $\text{Ca}^{2+}/\text{H}^{+}$ exchangers, TRP-like Ca^{2+} channels, and mitochondrial Ca^{2+} uniporters (MCU) (Lange and Peiter, 2020; Tisi et al., 2016). Changes in intracellular Ca^{2+} concentration are detected by sensors containing EF-hand motifs, as calmodulin or Neuronal calcium sensor-1 (Ncs-1, also known as Frq1 in *S. cerevisiae*), which activate specific downstream signalling cascades (Davis et al., 1986; Hendricks et al., 1999). One of the better characterized downstream pathways involve calcineurin and the transcription factor calcineurin-responsive zinc finger 1 (Crz-1), which are important in stress responses, morphogenesis and pathogenesis (Agranoff et al., 2005; Juvvadi et al., 2014; Tisi et al., 2016).

Manganese is required for polymerases, Golgi sugar transferases and Mn-SODs (Reddi et al., 2009a). Mn is taken up from the environment by transporters of the Nramp family, Smf1 and Smf2 (Portnoy et al., 2000; Agranoff et al., 2005). These transporters are constitutively transcribed and are mainly regulated post-translationally, targeted for vacuolar degradation when the intracellular content is sufficient (Reddi et al., 2009b). It is currently unknown if Mn^{2+} can be mobilized from the vacuole to respond to starvation. The host defense protein calprotectin can chelate Mn^{2+} to limit its access to pathogens, which has been shown to reduce *A. fumigatus* growth (Amich et al., 2014; Clark et al., 2016).

Magnesium is a critical cofactor of many enzymes and participates in essential processes as oxidative phosphorylation, nucleic acid synthesis and glycolysis. In *S. cerevisiae* several Mg^{2+} transporters have been described: two membrane importers (Alr1 and Alr2), a vacuolar transporter (Mnr2), and three mitochondrial transporters, two importers (Lpe10 and Mrs2) and one exporter (Mme1) (da Costa et al., 2007; Pisat et al., 2009; Cui et al., 2015). Mg^{2+} has been shown to modulate *C. neoformans* virulence factors, particularly capsule formation, and *C. albicans* virulence traits (Suo et al., 2018; Hans et al., 2019).

Nickel is a cofactor of urease in some fungal species, which is an important virulence factor for some pathogens as *C. neoformans* and *C. posadasii* (Singh et al., 2013; Fu et al., 2018; Olszewski et al., 2004; Wise et al., 2013). Although not much is known about nickel homeostasis, a Ni-permease has been described in *S. pombe* and a vacuolar storage detoxification response in *S. cerevisiae* (Hinnebusch, 1984; Eitinger et al., 2000), suggesting that a complete acquisition and homeostatic system must exist in fungi.

Metabolism of the four molecules of life

The four molecules of life (amino acids, lipids, nucleotides and sugars) are all vital for all organisms. They are the building blocks of biological macromolecules and their metabolism is at the core of primary metabolism. A deep understanding of fungal primary metabolism and its exploitation with modelling studies (bioinformatics and structural) has the potential to revolutionize the identification of valuable molecular targets (against which novel antifungal compounds can be developed) (Bencurova et al., 2018).

Amino acids

Amino acids are the crucial building blocks of all proteins. Unlike humans, pathogenic fungi are capable of biosynthesizing all 20 proteinogenic amino acids, however their uptake is energetically more favorable (Jastrzębowska and Gabriel, 2015). In host niches amino acids may serve as carbon and nitrogen sources such as in plasma, where free amino acid concentrations reach around 3.4 mM depending on diet and other factors, or in the lungs where concentrations can rise in response to inflammation (Blomstrand and Essén-Gustavsson, 2008; Hu et al., 2008b). Indeed, they are found in relatively large quantities in all tissues, contained within host proteins and peptides (Brosnan, 2003). To exploit these nutritional sources, pathogenic fungi possess mechanisms which allow the breakdown of exogenous proteins and the efficient assimilation of amino acids (Pérez et al., 2013; Farnell et al., 2012). Crucially, many of the enzymes involved in sensing, uptake and metabolism of amino acids are essential for fungal viability and virulence but are absent in humans, making them attractive targets for antifungal development (Amich and Bignell, 2016; Schobel et al., 2010; Pascon et al., 2004).

To ensure that preferred nitrogen sources, such as glutamine and glutamate, are utilized when available, fungi use a system of nitrogen catabolite repression. These responses are mediated by the upregulation of GATA transcription factors in nitrogen limiting conditions, allowing the uptake of alternative sources such as proline and arginine (Lee et al., 2013a; Coffman et al., 1995). Central to the ability of pathogenic fungi to respond to nutrient availability appropriately is the capacity to sense and differentiate between different nutrient concentrations (Crespo et al., 2002). The target of rapamycin (TOR) pathway is conserved as the main sensor of nutrient state in eukaryotes and plays an important role in mediating the response to different nutritional conditions, including alteration of amino acid transport and metabolism (Hardwick et al., 1999; Beck and Hall, 1999; Cardenas et al., 1999; Peng et al., 2002; Kim et al., 2008a; De Virgilio and Loewith, 2006). It is activated by amino-acid derived signals, including glutamine, and able to differentiate between nutrient availability conditions so the appropriate response is induced (Crespo et al., 2002; Baldin et al., 2015; Bastidas et al., 2009). In *A. fumigatus*, *C. albicans* and *C. neoformans*, Tor protein kinases are essential for viability and control a range of important metabolic and morphogenic features which contribute to pathogenicity (Bastidas et al., 2009; Martins et al., 2008; Cruz et al., 1999; Martho et al., 2016; So et al., 2019; Baldin et al., 2015; Böttcher et al., 2016). Unsurprisingly, interruption of downstream Tor signalling pathways results in attenuated virulence in murine models of invasive pulmonary aspergillosis, disseminated candidiasis and cryptococcosis (Han et al., 2019; Lee et al., 2004, 2012a; Rohde et al., 2004; Bastidas et al., 2009; de Castro et al., 2016; Bom et al., 2015).

An additional global regulator of metabolic and morphogenic responses to amino acid availability in fungi is the cross-pathway control (CPC) response or general amino acid control (GAAC), as it is called in filamentous fungi or yeasts, respectively (Carsiotis et al., 1974; Hinnebusch, 1984). Utilizing these systems fungi respond to limiting concentrations of a single amino acid by activating transcription of numerous amino acid biosynthetic genes (Tripathi et al., 2002; Lavoie et al., 2009; Lee et al., 2018). In *A. fumigatus*, intracellular amino acid starvation is sensed by a highly conserved eIF2 α sensor kinase, CpcC, to result in increased translation of the CPC transcriptional effector, CpcA (Sasse et al., 2008). CpcA activates signal transduction pathways to promote amino acid biosynthesis, and this process contributes to pathogenicity as deletion of CpcA resulted in attenuated virulence in a murine model of pulmonary aspergillosis (Krappmann et al., 2004). In *C. albicans*, the GAAC response is mediated by the CpcA orthologue Gcn4. Its expression is tightly regulated by transcriptional and translational control mechanisms, including inhibition by TOR, as it regulates critical virulence determinants that are induced by amino acid starvation (Sundaram and Grant, 2014; Bastidas et al., 2009; Tripathi et al., 2002). Ingestion of *C. albicans* by neutrophils results in a Gcn4- dependent induction of arginine biosynthesis, revealing that the fungus experiences amino acid starvation within these immune effector cells (Rubin-Bejerano et al., 2003). As in *A. fumigatus*, the transcriptional activator is necessary for full virulence in a model of candidiasis (Brand et al., 2004). To date an orthologue of CpcA/Gcn4 has not been identified in *C. neoformans* (Garbe and Vylkova, 2019).

C. albicans possesses a sensor system capable of sensing extracellular amino acid availability and subsequently enhancing the breakdown and import of external proteins (Zhang et al., 2020). The so called SPS sensor complex comprises membrane bound Ssy1, which senses extracellular amino acids and relays the signal to the other components, scaffold protein Ptr3, and the signalling endopeptidase Ssy5 (Brega et al., 2004; Martínez and Ljungdahl, 2005). Ssy5 removes cytoplasmic retention domains from the transcription factors Stp1, that stimulates extracellular protein degradation (via enzymes such as the major secreted aspartyl proteinase (SAP) Sap2) and oligopeptide transport (as Opt1), and Stp2, which increases expression of amino acid permeases including transceptors such as Gap1 and Gap2 (Martínez and Ljungdahl, 2005; Silao et al., 2019). The functionality of Ssy1 and amino acid permeases (AAPs) are dependent on a chaperone, Csh3, the deletion of which prevents amino acid uptake and virulence in a murine model (Martínez and Ljungdahl, 2004). Levels of Stp1, but not Stp2, are controlled by Gln3 and Gat1- mediated NCR to allow amino acids to be utilized as a preferred nitrogen source over extracellular proteins when abundant (Dabas and Morschhäuser, 2008; Martínez and Ljungdahl, 2005). In vivo evidence supports the critical role of this pathway in nutrient acquisition within the host as SAPs, including Sap2, contribute to the virulence of *C. albicans* in models of candidiasis (Hube et al., 1997; Sanglard et al., 1997; De Bernardis et al., 1999; Staib et al., 2002). A subset of Stp2- regulated genes, including amino acid biosynthetic pathways and transporters, are controlled independently of Ssy1-mediated activation (Miramón et al., 2020). This might explain Stp2's significant contribution to virulence in models of candidiasis (Miramón et al., 2020; Miramón and Lorenz, 2016; Vylkova and Lorenz, 2014). Deletion of Stp2 results in increased activation of Gcn4 and sensitivity to rapamycin, highlighting the interconnected nature of the TOR, GCN and SPS nutrient response pathways (Böttcher et al., 2020). The SPS system also facilitates the amino acid driven neutralization of the acidic environment encountered upon phagocytosis, and is therefore critical to normal macrophage resistance in *C. albicans* (Miramón and Lorenz, 2016; Vylkova and Lorenz, 2014). The SPS system has not been characterized in *A. fumigatus* or *C. neoformans*, although putative orthologs of a very limited number of the proteins involved have been identified (Garbe and Vylkova, 2019; Fernandes et al., 2015).

The genomes of pathogenic fungi encode numerous enzymes which allow the utilization of external proteins as nutrient sources. *C. albicans* possesses 10 SAPs which produce oligopeptides and individual amino acids from the degradation of extracellular proteins and eight oligopeptide transporters and 22 AAPs that facilitate their uptake (Monod et al., 2002; Naglik et al., 2003; Ramachandra et al., 2014; Reuß and Morschhäuser, 2006; Sychrová and Souciet, 1994; Kraidlova et al., 2011). Proteases are critical to amino acid acquisition and the penetration of the blood brain barrier by *C. neoformans*, and therefore for its pathogenicity (Vu et al., 2014; Xu et al., 2014a; Clarke et al., 2016). One of *C. neoformans*' six predicted oligopeptide transporters (OPTs), Opt1 has also been implicated in the regulation of virulence factors and four of its 10 AAPs play a role in virulence (Homes et al., 2016; Martho et al., 2016). *A. fumigatus* encodes a particularly high number (>100) of putative proteases that, as mentioned before, have redundant functions, making it difficult to establish their importance for virulence but are known to be implicated in allergic responses (Garbe and Vylkova, 2019; Gifford et al., 2002; Druey et al., 2020; Tomee et al., 1997). *A. fumigatus* proteases are differentially expressed on various nutrient sources, with at least two transcription factors identified, PrtT and XprG, that control their expression, indicating that this fungus can distinguish between complex protein sources and regulate secretion of proteases to exploit them (Farnell et al., 2012; Sharon et al., 2009; Bergmann et al., 2009; Shemesh et al., 2017). It has been proposed that during infection *A. fumigatus* exploits amino acids as C-source, as a methylcitrate synthase deletant (which accumulates propionyl-CoA from the catabolism of valine, isoleucine, and methionine) showed a strong decrease in virulence (Ibrahim-Granet et al., 2008). Interestingly, deletion of all eight *A. fumigatus* OPTs did not inhibit in vitro growth or virulence in a murine model of pulmonary aspergillosis, indicating the existence of unidentified methods of oligopeptide uptake or that the fungus effectively degrades extracellular proteins to individual amino acids (Garbe and Vylkova, 2019; Hartmann et al., 2011).

Lipids

Lipids are a functionally and structurally heterogeneous group of macromolecules that are soluble in nonpolar solvents, insoluble in water and have hydrophobic or amphiphilic properties. Common lipid molecules in cells are fatty acids, phospholipids, sphingolipids, glycerolipids, and sterols (Huang and Freter, 2015).

Fatty acids

Fatty acids are building blocks for other lipids and also act as energy reservoirs and signalling molecules. They have a common basic structure of carboxylic acids with hydrocarbon tails that vary in chain length and the degree of unsaturation. De novo synthesis of fatty acids can take place in the cytoplasm and in the mitochondria, followed by elongation and desaturation in the endoplasmic reticulum (ER). It starts with the carboxylation of acetyl-CoA by the action of ACC1 (cytosol) or HFA1 (mitochondria) to form malonyl-CoA, which serves as the two-carbon building block for the subsequent reactions (Hasslacher et al., 1993; Hoja et al., 2004). Synthesis in the cytosol is carried out by the hexameric fatty acid synthase (FAS) complex, which consists of 6 Fas1p and 6 Fas2p subunits, each exhibiting more than one enzymatic activity (Stoops and Wakil, 1978; Leibundgut et al., 2008). The mitochondrial FAS complex consists of six proteins, each producing only one enzymatic activity (Hiltunen et al., 2010). Both complexes add two carbons from malonyl-CoA to a saturated acyl chain, to produce mostly lipoic acid (mitochondrial), or a chain length up to C18 (cytosolic). Further elongations by the addition of two carbons from malonyl-CoA takes place in the ER by different elongases (Toke and Martin, 1996). Desaturation and hydroxylation also occur in the ER, performed by the acyl-CoA Δ^9 -desaturase Ole1p (Stukey et al., 1989).

Fungal cells can also acquire fatty acids from the environment in a process termed vectorial acylation that links transport with metabolic activation and involves fatty acid transport proteins (FATP) and long-chain acyl CoA synthetases (ACSL) (Black and DiRusso, 2007).

Excess of fatty acids are stored in the form of triacylglycerols, which serve as energy reservoirs and are stored in distinct organelles called lipid droplets (Daum et al., 2007). Mobilization of triacylglycerols is based on the action of three lipases Tgl3p, Tgl4p and Tgl5p (Athenstaedt and Daum, 2003, 2005). Fatty acids can then enter β -oxidation, a catabolic process which in fungi exclusively takes place in peroxisomes and that produces a high number of ATP molecules (Hiltunen et al., 2003).

Phospholipids

Phospholipids have an amphipathic character due to their structure. They consist of a diacylglycerol as hydrophobic moiety and different polar head groups at the hydrophilic side. Diacylglycerol is esterified in the positions 1 and 3 with combinations of fatty acids and the head group is linked to the 2 position, which constitutes the diversity of phospholipids. Due to their amphipathic properties, phospholipids form bilayers, which are the basis of biological membranes (Daum et al., 1998). The major phospholipids in *S. cerevisiae* are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) (Klug and Daum, 2014).

In this model fungus, biosynthesis of phospholipids starts with the synthesis of phosphatidic acid (PA). PA is generated by acylation of glycerol 3-phosphate by Sct1p (GAT2) and Gpt2p (GAT1) to form lyso-PA and subsequent acylation by acyltransferases Slc1p, Slc4p (Athenstaedt and Daum, 1997; Jain et al., 2007; Benghezal et al., 2007). Sct1p and Gpt2p can also use dihydroxyacetone phosphate (DHAP) as a substrate to form acyl-DHAP, which is then converted to Lyso-PA by the reductase Ayr1p (Zheng and Zou, 2001; Athenstaedt and Daum, 2000). PA serves as a central metabolite in the de novo synthesis of all phospholipids by a complex metabolic network that has substantial differences with humans (Klug and Daum, 2014; Pan et al., 2018). At least three enzymes of the pathway, PS synthase, PS decarboxylase, and phospholipid methylase, have been described as potential antifungal targets (Cassilly and Reynolds, 2018).

Sphingolipids

Sphingolipids are also important components of cell membranes and act as signalling molecules. In *S. cerevisiae*, de novo sphingolipid synthesis begins with the condensation of a serine and a fatty acyl-CoA, normally palmitoyl-CoA, by the action of the serine palmitoyl-transferase complex, which consists of three enzymes encoded by LCB1, LCB2, and TSC3 (Nagiec et al., 1994; Gable et al., 2000). The product of this reaction, 3-ketodihydrosphingosine, is converted to dihydrosphingosine by the 3-ketosphinganine reductase Tsc10p (Beeler et al., 1998). Dihydrosphingosine can be hydroxylated by Sur2p to form phytosphingosine. Then, the ceramide synthase complex, comprising Lag1p, Lac1p and Lip1p, catalyzes acyl-CoA-dependent acetylation of dihydrosphingosine or phytosphingosine with long fatty acids to yield dihydroceramide and phytoceramide, respectively (Schorling et al., 2001; Vallée and Riezman, 2005). Complex sphingolipids are formed by addition of an inositol phosphate group to ceramide, catalyzed by the inositolphosphoryl ceramide synthase Aur1p (Nagiec et al., 1997). Inositol-P-ceramide can then be mannosylated by Csg1p, Csg2p and Csh1p complex to form mannose-inositolphosphoryl ceramide and then a second inositol phosphate group can be attached by Ipt1p to synthesize mannose-diinositolphosphoryl ceramide (Uemura et al., 2003; Dickson et al., 1997).

Sterols

Sterols are fundamental components of cell membranes, regulating their fluidity and dynamically aggregating in conjunction with sphingolipids to form lipid rafts. The major sterol in fungi is ergosterol, the equivalent of mammalian cholesterol.

Ergosterol is synthesized through a complex pathway that begins with acetyl-CoA and involves more than 20 enzymes (Klug and Daum, 2014). In addition, some species as *A. fumigatus* encode multiple genes for the same key enzymatic activities (Mellado et al., 2001; Alcazar-Fuoli et al., 2006). Balance and regulation of sterol synthesis is important to avoid accumulation of free sterols which may become toxic for the cell. Such regulation of sterol levels is mainly achieved by feedback mechanisms at transcriptional, translational and post-translational levels (Espenshade and Hughes, 2007). For instance, hydroxy-3-methylglutaryl-CoA (HMG-

CoA) is reduced to mevalonate by HMG-CoA reductases and this step is subjected to feedback inhibition by sterol intermediates through enzyme proteasome dependent degradation (Burg and Espenshade, 2011). Interestingly, there seems to be a strong link between sterols and oxygen, where sterols act as oxygen sensors, serve as defense against oxidative damage and their synthesis being an intensive oxygen-consuming process (Galea and Brown, 2009). The Sterol Regulatory Element Binding Protein (SREBP) class of transcription factors maintain cellular sterol levels through transcriptional activation of target genes upon steroid depletion. In addition, SREBP factors monitor oxygen dependent sterol synthesis as an indirect measure of oxygen availability and are required to adapt to low oxygen (Hughes et al., 2005; Todd et al., 2006). These factors are essential for *A. fumigatus* and *C. neoformans* virulence (Willger et al., 2008; Chung et al., 2014; Chang et al., 2007; Chun et al., 2007). Apparently, *C. albicans* does not encode SREBPs orthologs, while its *UPC2* product seems to carry out similar functions (Silver et al., 2004; MacPherson et al., 2005).

Excess sterols are acetylated and stored as sterol-esters in lipid droplets (together with triacylglycerols) (Yu et al., 1996; Kim et al., 2004; Daum et al., 2007). Sterol-esters can be mobilized by various hydrolytic enzymes, which can help to balance shortage of sterols (Daum et al., 2007; Brown et al., 1980).

Polyketides

Polyketides are a rare type of lipids, normally referred as secondary metabolites, that have a biosynthetic chemistry highly similar to fatty acids (Smith and Tsai, 2007; Chiang et al., 2010). This diverse group of compounds has great significance for the virulence of fungal pathogens. Particularly, *Aspergillus* species can produce many polyketides, by action of polyketide synthase enzymes type I, with high relevance to human health (Bhetariya et al., 2011). For example, *A. flavus* and *A. parasiticus* produce aflatoxin, by a polyketide pathway consisting of at least 27 reactions, which is a toxin that contaminates food and causes hepatotoxicity, teratogenicity, and immunotoxicity (Caceres et al., 2020; Kumar et al., 2017). Furthermore, *A. fumigatus* produces (among approximately 8 polyketides) DHN-melanin, which covers spores and that is known to be a very important fungal virulence factor (Bignell et al., 2016; Heinekamp et al., 2013; Akoumianaki et al., 2016).

Sugar metabolism

As detailed in the carbon metabolism section, glucose is the preferred carbon source for fungi. Glucose is catabolized through glycolysis pathway to form precursors of other macromolecules (such as lipids and amino acids) and to obtain energy through fermentation and/or the TCA cycle and respiration. Glucose can also be metabolized to enter the pentose phosphate pathway to create redox equivalents and to form nucleotides.

When glucose is not available, fungi can obtain carbon and energy from a broad range of fermentable and non-fermentable sources (Ene et al., 2014). These alternative carbon sources can fuel anabolism by generating glucose and its derivatives through gluconeogenesis. In order to assimilate non-fermentable two-carbon sources (such as ethanol and acetate), fungi use the glyoxylate cycle. This is basically a shunt in the TCA cycle in which two enzymes (isocitrate dehydrogenase and α -ketoglutarate dehydrogenase) are excluded and the alternative glyoxylate cycle comprises two key enzymes: isocitrate lyase and malate synthase. In essence, isocitrate lyase breaks down isocitrate (C6) to glyoxylate (C2) and succinate (C4), and malate synthase condensates the released glyoxylate with acetyl-CoA (C2) to generate malate (C4). In this way, malate produced from C2 compounds can replenish the TCA cycle or serve as the precursor of oxaloacetate, an essential substrate for gluconeogenesis (Dunn et al., 2009).

In fungi, two glucoses can join via a α,α -1,1-glycosidic linkage to form the disaccharide trehalose. Human-pathogenic fungi use one or two biosynthetic pathways. The canonical pathway involves two enzymes: trehalose-6-phosphate synthase (Tps1p) and trehalose-6-phosphate phosphatase (Tps2p). Tps1p transforms UDP-glucose and glucose-6-phosphate into UDP and trehalose-6-phosphate, which is then converted by Tps2p into trehalose and free inorganic phosphate (Pi). The non-canonical pathway, present in some filamentous fungi, consists in one enzyme, trehalose phosphorylase (TreP) that converts glucose-1-phosphate (G1P) and glucose into trehalose (Shinohara et al., 2002; Thammahong et al., 2017b). Trehalose is an important molecule in fungal biology and required for virulence of *C. albicans*, *C. neoformans*, *C. gattii* and *A. fumigatus* (Zaragoza et al., 1998; Petzold et al., 2006; Ngamskulrungron et al., 2009; Al-Bader et al., 2010; Puttikamonkul et al., 2010). It is proposed to serve as energy reserve, although there is still some controversy on this topic (Wiemken, 1990; Thammahong et al., 2017b). Trehalose seems to play an important role as a general stress protectant in fungi, providing protection against dehydration, thermal stress and free radicals (Thammahong et al., 2017b).

The requirement of specific pathways for infection depends on the nutritional niche that the fungal pathogens encounter during infection (Barelle et al., 2006; Price et al., 2011). For instance, glycolysis is important for the virulence of most fungal pathogens, but not for the intracellular pathogen *Histoplasma capsulatum* (Shen et al., 2020). The glyoxylate cycle is required for *C. albicans* and *C. glabrata* virulence, but not in *C. neoformans* or *A. fumigatus* (Lorenz and Fink, 2001; Barelle et al., 2006; Chew et al., 2019; Rude et al., 2002; Idnurm et al., 2007; Schöbel et al., 2007; Olivas et al., 2008). In addition, metabolic flexibility and temporal adaptation are essential traits for pathogenesis. For instance, *C. albicans* adapts its metabolism to different host niches and can assimilate glucose and alternative carbon sources simultaneously, which provides an advantage in the interaction with the host (Brown et al., 2014a; Sandai et al., 2012; Tucey et al., 2018). *A. fumigatus* seems to perform both fermentation and respiration during infection, and a temporal metabolic plasticity of the assimilation and metabolization of carbon sources is fundamental for its virulence (Grahl et al., 2011, 2012; Beattie et al., 2017). Such an adaptation to different carbon sources is also important for *C. neoformans* virulence (Hu et al., 2008a).

Cell wall

The metabolism of sugars is particularly relevant in fungi as polysaccharides are core components of their cell wall. These are dynamic structures, pivotal for cell viability, morphogenesis, and pathogenesis. Indeed, the structure of cell wall adapts to the environment, which is crucial for fungal survival under conditions of infection (Shepardson and Cramer, 2013). In particular, there is a directly link of the carbon source with the cell wall architecture, which modulates fungal virulence (Ene et al., 2012; Ballou et al., 2016). Major cell wall polysaccharides include glucans, chitin and mannans. Glucans are polysaccharides of D-glucose linked by glycosidic bonds. Common glucans in cell walls are β -1,3-glucan, α -1,3-glucan, β -1,6-glucan and mixed β -1,3-/ β -1,4-glucans. Chitin is a long-chain polymer of N-acetylglucosamine, Mannans are linear polymers of mannose, usually with α (1,6)-mannoside linear links and α (1,2) or α (1,3) linked branches (Gow et al., 2017). Being in contact with the exterior, these molecules are important pathogen associated molecular patterns (PAMPs) that are recognized by host pathogen recognition receptors (PRRs) to induce and orchestrate innate and adaptive immune responses. For instance, the C-type lectin receptor Dectin-1 recognizes β -glucans, a variety of receptors can recognize different mannans and mannosylated moieties (C-type receptors as Dectin-2, Dectin-3, Mincle, Mannose receptor (MR) or DC-SIGN and Toll-like receptors as TLR-2 or TLR-4), and chitin has been suggested to be a ligand for NOD2 and TLR-9 (Brown and Gordon, 2001; Lionakis et al., 2017; Wagener et al., 2014).

Nucleotides

Nucleotides are critical players in a myriad of cellular processes. Purine and pyrimidine nucleotides are basic components of nucleic acids, major energy carriers, signalling molecules (as cyclic AMP) and precursors for the synthesis of cofactors such as Nicotinamide adenine dinucleotide (NAD) and SAM. Purine and pyrimidine biosynthesis pathways are highly conserved in prokaryotes and eukaryotes.

Pyrimidine de novo biosynthesis requires 6 enzymatic activities and consumes 2 ATPs (Garrett and Grisham, 2010). In *S. cerevisiae*, the pathway is regulated at the enzymatic level through feedback inhibition of the first committed enzyme of the route (Ura2) by the final product UTP and at the transcriptional level by the zinc-finger transcription factor Ppr1, which can sense levels of intermediates of the pathway (Jones and Fink, 1982; Kammerer et al., 1984; Flynn and Reece, 1999). Transcription of URA2 seems to be Ppr1-independent and be instead controlled by alternative transcription start-sites that lead to production of unstable mRNAs ("cryptic unstable transcripts") when there is sufficient uracil (Thiebaut et al., 2008). Interestingly, Ppr1 has a different function in *C. albicans* and *A. nidulans* (UaY), where it controls purine catabolism (Suárez et al., 1995; Tebung et al., 2016). Therefore, even if transcription of the genes responds to the concentration of pyrimidines, the exact mechanism has not described (Aleksenko et al., 2002). The pyrimidine biosynthetic pathway has been shown to be important for *C. neoformans*, *A. fumigatus*, *H. capsulatum*, and *C. albicans* virulence (de Gontijo et al., 2014; D'Enfert et al., 1996; Retallack et al., 1999; Cole et al., 1995; Brand et al., 2004).

Purine de novo biosynthesis requires 11 enzymatic activities and consumes 6 ATPs (Garrett and Grisham, 2010). The pathway is regulated at the transcriptional level by the regulators Bas1 and Bas2/Pho2 (Daigan-Fornier and Fink, 1992). These two factors need to interact to activate transcription, which is promoted in the presence of the metabolite intermediate SAICAR. In low adenine concentrations, levels of ADP and ATP decrease, liberating feedback inhibition of the first enzyme of the pathway (Ade4), which in turn increases the levels of SAICAR (Rébora et al., 2001). Transcription of genes for GTP synthesis is independently regulated by guanine (Escobar-Henriques and Daignan-Fornier, 2001). In addition, the gene encoding the first enzyme of the branch (IMD2) is regulated by cryptic unstable transcripts, via GTP-dependent start site selection (Davis and Ares, 2006; Steinmetz et al., 2006; Kuehner and Brow, 2008). Integrity of the biosynthetic pathway has been shown to be important for *C. albicans*, *C. neoformans* and *A. fumigatus* virulence (Donovan et al., 2001; Jiang et al., 2010; Rodriguez-Suarez et al., 2007; Morrow et al., 2012; Blundell et al., 2016; Chitty et al., 2017).

As building blocks of DNA, nucleotide biosynthesis is regulated by the growth phase. When cells enter stationary phase, several genes of the pathway are downregulated and some enzymes become inactivated (Escobar-Henriques et al., 2003; Escusa et al., 2006; Narayanaswamy et al., 2009). In contrast, nucleotide biosynthetic genes are upregulated under growth proliferation conditions (Walvekar et al., 2018).

Most fungi can use purines and some can use pyrimidines as nitrogen source (Rasmussen et al., 2014). The purine catabolic pathway is present in most filamentous fungi, while it is degenerated in most yeasts (Pantazopoulou and Diallinas, 2007). The pathway, which consists in 6 enzymatic reactions that sequentially degrade xanthine to ammonia, is present in all human fungal pathogens except *Pneumocystis jirovecii*, but its relevance for virulence has only been tested in *C. neoformans* (Chitty and Fraser, 2017; Cissé et al., 2014; Cox et al., 2000). Purines and pyrimidines are taken up by transporters belonging to three different protein families: the nucleobase cation symporter family 1 (NCS1 or PRT), the nucleobase-ascorbate transporter family (NAT or NCS2) and the AzgA-like family (Pantazopoulou and Diallinas, 2007). Transcription of most genes encoding the enzymes involved in purine catabolism and uptake are regulated by nitrogen catabolite repression and the positive-acting transcription factor UaY (Scazzocchio et al., 1982; Scazzocchio, 1994).

Prospectives for new antifungal treatments

As highlighted in this chapter, many points within primary metabolism have been described to be crucial for fungal growth and/or virulence. In this section, we provide an overview of some of the most promising targets identified from primary metabolism and the existing novel agents developed so far against them.

Targeting the central routes of carbon, nitrogen and sulfur metabolism have been repeatedly suggested as a promising strategy to fight fungal infections (Bachhawat and Yadav, 2010; Traynor et al., 2019). For example, sulfite reductase, cystathionine γ -synthase and methionine synthase, in the sulfur assimilation and trans-sulfuration pathway, are considered good targets for the development of novel antifungals (Scott et al., 2020; Jastrzębowska and Gabriel, 2015); active agents have already been described, as azoxybacilin to inhibit gene expression of sulfite reductase (Aoki et al., 1996), or anilino-pyrimidines, which apparently inhibit cystathionine- γ -synthase and/or cystathionine- β -synthase (Jastrzębowska and Gabriel, 2015).

Targeting acquisition of microelements is also considered a promising strategy to fight fungal infections. Blockage of iron acquisition, either by direct inhibition of uptake systems/biosynthetic enzymes or by iron chelation appears to be a good strategy to treat fungal infections and augment antifungal therapy (Bairwa et al., 2017). In addition, siderophores have the potential to be exploited for diagnosis, both by its direct detection as a biomarker in urine or by the use of radiolabelled siderophores as trojan horse for the targeted imaging of infection by positron emission tomography (Skriba et al., 2018; Hoenigl et al., 2019; Petrik et al., 2012, 2014). Actually, specific internalization of antifungal drugs by siderophore transporters opens new possibilities for specific delivery, as described for the novel drug in development VL-2397 (formerly ASP2397) (Dietl et al., 2019). Additionally, compounds with zinc-chelation activity have been found to have potent antifungal activity in murine models of infection (Clark et al., 2018; Cohrt et al., 2018; Laskaris et al., 2016; Li et al., 2018; Vicentefranqueira et al., 2015; Simm and May, 2019).

Interference with calcium homeostasis is one of the best studied new strategies to fight fungal infections. In particular, inhibiting calcineurin with compounds such as cyclosporine-A or tacrolimus (FK506) has been demonstrated to be an effective antifungal therapy (Juvvadi et al., 2017, 2019) and to synergize with azoles (Uppuluri et al., 2008; Li et al., 2019b). The main holdback is that calcineurin is not a fungal specific target and consequently these compounds are immunosuppressive. Encouraging, a novel promising compound, APX879, with higher specificity and lower toxicity was recently developed (Juvvadi et al., 2019). Other approaches to interfere with calcium homeostasis have also shown value as antifungal therapies, as the use of the calcium-channel blockers verapamil or disruption of activity of the Golgi transporter Pmr1 (Yu et al., 2013; Gupta et al., 2003).

Amino acid metabolism is directly and indirectly crucial for fungal virulence. Many of its biosynthetic pathways have been shown to be required for pathogenicity and thus blocking them is proposed as a promising antifungal strategy, with many inhibitors already described (Jastrzębowska and Gabriel, 2015; Amich and Bignell, 2016). Proline catabolism has been implicated in *C. neoformans* ROS homeostasis and production of virulence factors and in *C. albicans* filamentation (Lee et al., 2013a; Silao et al., 2019). Disruption of the tryptophan, threonine, isoleucine/valine and leucine biosynthetic pathways impacts *C. neoformans* viability and/or virulence (Do et al., 2015; Kingsbury and McCusker, 2008; Fernandes et al., 2015; Kingsbury et al., 2004). In *C. albicans*, disturbance of the threonine and branched-chain amino acid biosynthetic pathways reduces virulence in mice (Kingsbury and McCusker, 2010b, a) and methionine biosynthesis intertwines metabolism, signalling and virulence (Maidan et al., 2005; Schrevens et al., 2018) hyphal morphogenesis (Vylkova et al., 2011; Schrevens et al., 2018; Silao et al., 2019) and biofilm formation (Rajendran et al., 2016). In *A. fumigatus*, interference with methionine synthase triggers a complex metabolic imbalance that causes growth halt and decreased virulence (Scott et al., 2020), the biosynthesis of aromatic amino acids and histidine are crucial for pathogenicity, and arginine biosynthesis impacts on siderophore biosynthesis (Dietl et al., 2020, 2016; Sasse et al., 2016). This all supports the premise that amino acid metabolism presents a promising target for antifungal drug discovery (Amich and Bignell, 2016; Kaldorf et al., 2016). In addition, amino acid transport mechanisms have been recently been proposed as potential antifungal targets (McCarthy and Walsh, 2018).

Lipid metabolism is arguably the major source of antifungal targets. Indeed, several existing antifungals target ergosterol, including the three major classes: azoles, polyenes and allylamines. Azoles, the first line of treatment of *Aspergillus*-related infections, inhibit 14- α sterol demethylase (Cyp51/Erg11), blocking the biosynthesis pathway and causing accumulation of precursor sterols, accumulation of reactive oxygen and nitrosative stress as well as cell wall stress (Ullmann et al., 2018; Patterson et al., 2016; Ghannoum and Rice, 1999; Vandenbosch et al., 2010; Ferreira et al., 2013; Geißel et al., 2018). The polyene Amphotericin-B, which continues to be an important antifungal for the treatment of invasive infections, directly binds ergosterol in the membrane (Gray et al., 2012). The allylamine group of antimycotics inhibit the enzyme squalene epoxidase (Erg1) causing high intracellular squalene concentrations, which seemingly interfere with membrane function and cell wall synthesis (Geißel et al., 2018). Fatty acid synthesis has been proposed as an antifungal target in *C. neoformans* and *C. parapsilosis* (Chayakulkeeree et al., 2007; Nguyen et al., 2009). Fatty acids seem to serve as carbon source for *C. albicans* during infection but not to *A. fumigatus* (Lorenz and Fink, 2001; Ramírez and Lorenz, 2007, 2009; Schöbel et al., 2007). In addition, *C. albicans* morphogenesis (an important virulence factor) is influenced by extracellular fatty acids (Noverr and Huffnagle, 2004). Hydrolysis of phospholipids by phospholipases releases various types of molecules that are involved in signalling and that are important for fungal virulence (Köhler et al., 2006; Barman et al., 2018; Ghannoum, 2000; Park et al., 2013). A few compounds to target fungal phospholipase B have been described in vitro (Ng et al., 2006; Ganendren et al., 2004). Sphingolipids play essential roles in fungal virulence (Rittershaus et al., 2006; Bertini et al., 2007; Oura and Kajiwar, 2008); however, targeting sphingolipids is not easy as many biosynthetic steps between mammals and fungi are conserved (Rollin-Pinheiro et al., 2016). One of the most promising drug target candidates is inositolphosphoryl ceramide synthase, a fungal specific enzyme against which various inhibitors with strong antifungal effect have already been described (Sugimoto et al., 2004; Mandala et al., 1998, 1997; Aeed et al., 2009; Wuts et al., 2015; Harris et al., 1998). In addition, a novel family of drugs that inhibit glucosylceramide synthesis has been found to have broad antifungal activities (Mor et al., 2015).

Targeting sugar metabolism, including energy producing routes is also a promising antifungal strategy. As a prominent example, the novel antifungal drug Arylamidine T-2307, which has broad spectrum antifungal activity in vitro and in vivo, acts by selective disruption of yeast mitochondrial function (Mitsuyama et al., 2008; Yamashita et al., 2019). Several other enzymes of glucose

metabolism have been proposed as antifungal targets, with various specific inhibitory compounds shown to exert antifungal activity (Chen et al., 2020). Moreover, the cell wall is an attractive target for antifungal therapeutics, as it is fungal specific and thus treatment might be associated with low host toxicity. Echinocandins, which are still the newest class of antifungal agents approved for clinical treatment, act through inhibition of the catalytic subunit of the β -(1,3)-D-glucan synthase, leading to osmotic instability and cell death (Denning, 2003; Odds et al., 2003). The novel drug Fosmanogepix (APX001) targets Gwt1p, which catalyzes an early step in glycosylphosphatidylinositol (GPI)-anchor biosynthesis, resulting in a defect in cell wall assembly (Hata et al., 2011; Umemura et al., 2003). Targeting chitin synthesis is considered a promising strategy for the development of novel drugs (Li et al., 2019a, b; Nix et al., 2009; Kovács et al., 2019). Enzymes related to trehalose biosynthesis have been shown to modulate chitin biosynthesis, and targeting them has also been proposed as an attractive strategy to develop novel antifungals (Thammahong et al., 2017a, b, 2019; Vahedi-Shahandashti and Lass-Flörl, 2020). Galactosaminogalactan, an *A. fumigatus* and *A. flavus* specific heteropolysaccharide with multiple immunomodulatory activities, has been proposed as a good target for novel therapeutics (Speth et al., 2019).

Finally, targeting nucleotide metabolism is as well a promising strategy to fight fungal infections. Indeed, the novel drug Olorofim (formerly F901318) targets dihydroorotate dehydrogenase, the enzyme catalyzing the fourth step of the pyrimidine biosynthesis pathway, and is currently undergoing clinical trials (Oliver et al., 2016). Furthermore, this pathway connects with cell integrity and potentiates action of the antifungal amphotericin-B (Banerjee et al., 2014, 2016). Besides, fungal purine biosynthesis can be targeted by anticancer and immunosuppressive agents such as mycophenolic acid (which inhibits Inosine 5'-monophosphate dehydrogenase, IMPDH, in the de novo synthesis of guanosine) (Rodriguez-Suarez et al., 2007; Köhler et al., 2005; Morrow et al., 2012).

Conclusions

In this chapter we have provided an in-depth overview of fungal primary metabolism, focusing on human pathogens (Fig. 1). We have highlighted the many processes that are known to be relevant for virulence and specified those that are considered promising for the development of novel therapies. The high number of possible metabolic targets supports the idea that primary metabolism is a paramount virulence trait in pathogenic fungi. Many of these targetable metabolic processes are fungal specific, suggesting the possibility to develop new drugs with low host toxicity. Interestingly, the multitude of points in the metabolism that can be attacked, even in the same pathway or convergent pathways, multiplies the possibilities to develop combinatorial strategies to maximize efficacy of treatment and to reduce the emergence of resistance. In addition, as many of the processes are conserved among the fungal kingdom, pan-fungal approaches can likely be developed, including therapies for opportunistic fungal species without obvious virulence factors to target. Nevertheless, it is evident that there are many gaps in our understanding of fungal metabolism that need to be filled to fully exploit the potential of metabolism to fight fungal infections. Particularly, it is clear that there are significant differences among the various pathogenic species, and we have very little knowledge about the specifics in fungal pathogens other than “the big four”. Hence, fungal primary metabolism remains an active and vibrant research field that holds the promise to provide novel options for the development of antifungal therapies.

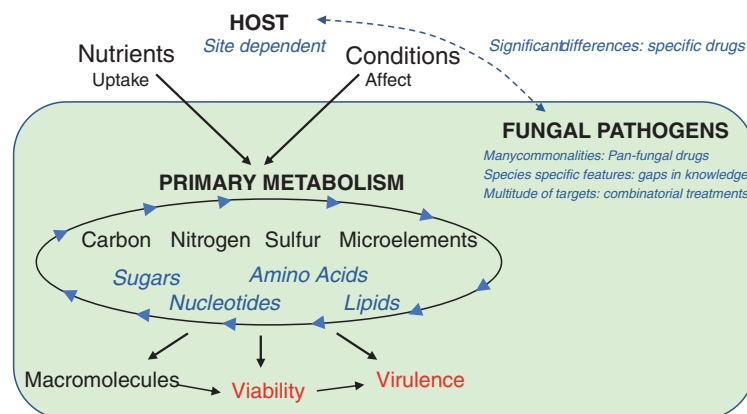


Fig. 1 Primary metabolism of human fungal pathogens directly links with virulence. The specific host niche in which the pathogen locates determines the nutritional milieu and the environmental conditions to which the fungus is exposed. Both directly impact primary metabolism, the regulation of the acquisition of macro- and micronutrients and thus the metabolism of the molecules of life. A balanced primary metabolism supports the biosynthesis and metabolism of macromolecules, underlie viability and confers the capacity to thrive and grow in the host tissues, therefore sustaining virulence of fungal pathogens. Primary metabolisms of fungi and mammals have significant differences, which highlights the opportunities to develop specific (non-toxic) drugs. Within the primary metabolism of fungal pathogens, there are many commonalities, which suggests that pan-fungal drugs can be developed. Nevertheless, there are as well species specific differences, which in fact are not well understood yet. Finally, the multitude of potential targets in primary metabolism multiplies the possibilities to develop combinatorial strategies to maximize efficacy of treatment and to reduce the emergence of resistance.

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Immunity to Bacterial Infections

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Introduction

The continuous exposure of the human body to bacteria requires constant monitoring. The most obvious reason for this is to prevent infection, but the most relevant reason is to provide up to the minute environmental samples to educate the immune system on “dangerous” or threatening materials and to differentiate those from innocuous substances or microorganisms that actually provide an advantage to the host. Nowhere is this more obvious than in the gut where current estimates suggest there are at least an equal number of bacteria as human cells (Sender et al., 2016). In comparison, certain bacteria such as *Salmonella enteritidis*, cause disease following a dose as small as 100 cfu. Understanding the differences in this dramatic contrast in immune responses is important for host development and survival and is the subject of this review.

It is also clear that a number of variables exist that affect the immune response that will be impossible to cover in detail, but we have drawn attention to many (Table 1). Therefore, we will not go through immunity to every bacteria, rather we will give a temporal view of the immune response describing each mechanisms within a generalized immune response to bacteria and provide examples of organisms that are targeted by that mechanism. In this case, immune responses against bacteria, will follow three lines including (i) Barriers-physical, chemical and microbiological mediators and cells that block bacterial colonization (ii) innate immunity- receptors and cells that respond immediately over a broad range of specificities and (iii) adaptive immunity-mediators and cells that have a delayed response but have a very narrow range of specificity tailored to the infectious bacteria. These lines of defence work in sequence against each individual organisms, but may overlap depending on the number of infecting organisms.

Table 1 Summary of factors affecting protective immunity to bacteria.

Host	Bacteria
• Body site/route of inoculation/infection • Systemic or local infection • First or subsequent infection • Vaccination • Age • Immunocompromise or immunosuppression • Mucosal immunity	• Dose/number inoculated • Commensal or pathogen • Extracellular or intracellular phase • Virulence factors • Evasion mechanisms • Biofilm production • Antimicrobial resistance • Growth/nutrient requirements

Each bacteria will produce an individualized response, but generalizations can be made due to the property of the immune system in recognizing conserved structures.

Barriers: Repelling and restriction of bacterial infections

The important sites forming the first line of defence as barriers to bacterial infection include the skin, respiratory tract and gastrointestinal tract. Here barriers may be defined as chemical “physical or anatomical,” and microbiological. Nowhere is chemical anti-bacterial immunity more easily demonstrated, than in the secretions from the fluids bathing these sites (Fig. 1). One hundred years ago, Alexander Fleming made the observation that agar plates supplemented with “imbedded tears” had antibacterial activity, identifying “lysozyme” in the process (Fleming, 1922). More recently, Glaser and colleagues identified that the skin protein “psoriacin” has anti-bacterial activity against *E. coli* (Glaser et al., 2005). These visual observations summarize a collection of large and small antimicrobial proteins and peptides present in bodily secretions that lyse and kill bacterial cells. These agents may be present in many areas or anatomically restricted to a few. Thus, lysozyme, lactoferrin, and the cathelicidin, LL-37 are found in the mucosal epithelium like the mouth, intestine, respiratory and urogenital tract. In addition, to these sites, secretory leukocyte protease inhibitor (SLPI), Trappin-2, S100 proteins (such as psoriacin and calprotectin), the α and β defensins can be found in the skin. The surfactant molecules SP-A and SP-D are largely confined to the respiratory tract and the regenerating-islet derived (Reg)-3 β / γ proteins are predominantly found in the intestinal tract. Similarly, histatins and dermcidin are largely confined to the saliva and skin respectively. Many of these proteins are produced constitutively, but can also be induced in response to bacterial stimulation.

Despite being overlooked when discussing immunity to bacterial infection, anatomical factors provide some of the most efficient non-specific host mechanisms to remove pathogenic bacteria. Throughout the respiratory tract, physical defences at the barrier interface include cough and sneeze reflexes which have dramatic power in expelling foreign respiratory pathogens (Bonvini and Belvisi, 2017; Bourouiba et al., 2014; Bourouiba, 2020; Duguid, 1945).

Please see video link in Relevant Websites section.

These reflexes are induced by direct (irritants/bacteria) or indirect (by host derived products) stimulation of sensory vagal and trigeminal nerves respectively. Studies to date have implicated the transient receptor potential (TRP) family of ion channels in this process (Bonvini and Belvisi, 2017) which has recently been shown to be activated by bacterial products (Meseguer et al., 2014).

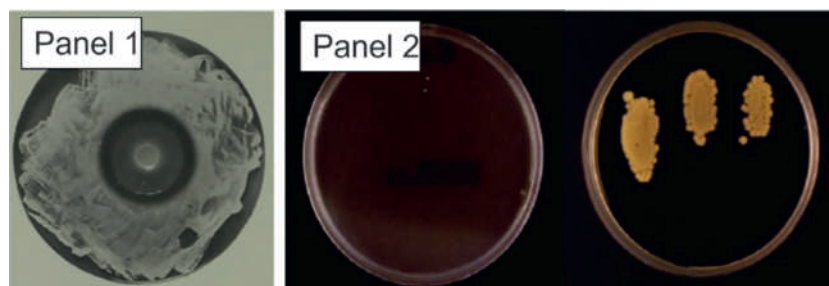


Fig. 1 Barrier secretions protect against bacterial infection. Panel 1: Agar embedded “tears” in the central circle clearing a lawn of *M. lysodeikticus* (*Micrococcus luteus*) in Fleming’s seminal studies. Panel 2: Psoriacin protects the skin against pathogens. Washed (left) and gloved (right) fingertips were inoculated with 10^4 cfu/mL *E. coli* and incubated for 30 min. Then fingertips were pressed into agar and the plate incubated for 24 h. Note the growth inhibition in the washed hands and the uninterrupted growth in the gloved hand. Antibacterial activity of barrier fluids: Fleming A (1922) On a remarkable bacteriolytic element found in tissues and secretions. *Proceedings of the Royal Society B (Biological Sciences)* 93: 306–317; Glaser R, Harder J, Lange H, Bartels J, Christophers E, and Schroder JM (2005) Antimicrobial psoriacin (S100A7) protects human skin from *Escherichia coli* infection. *Nature Immunology* 6: 57–64.

In contrast, a more subtle but equally effective mechanisms are employed by the mucociliary apparatus to defend the lung against bacterial pathogens. Composed of tiny hair-like projections called cilia, bathed in low viscosity periciliary fluid and an upper mucus layer, the mucociliary system is synchronized to direct mucus associated bacteria and particles to the oro-pharynx for swallowing. The system is present in both the nasal and bronchial tracts, moving in opposite directions, and toward the oro-pharynx. The physiological importance of the mucociliary escalator is highlighted in the rare autosomal recessive disorder primary ciliary dyskinesia (PCD) where cilia are immotile or unable to synchronize resulting in recurrent chronic infections and inflammation of the respiratory tract (Lucas et al., 2020). In the gastrointestinal tract, the emphasis on continued movement remains important. Smooth muscle peristalsis not only pushes food along the gut but discourages bacterial adhesion to the digestive tract wall and encourages the appropriate growth of microbiota (Cremer et al., 2016). Mechanisms of emesis (vomiting) allow the rapid expulsion of stomach contents through the oral cavity to protect the host against food poisoning organisms and their toxins (Horn, 2014; Popa et al., 2019; Hu and Nakane, 2014). Likewise, very recent work on the expulsion from the rectum at the other end of the gastrointestinal tract suggest that mechanisms of diarrhea and defecation are essential for successful clearance during the early phase of bacterial infections (Tsai et al., 2017). Changes in the pH and concentrations of digestive enzymes also contribute to restricting bacterial invasion across the gut wall.

Barriers cannot be discussed without reference to the mucus that they secrete, which is largely composed of mucin molecules. These form a heterogeneous group of high molecular weight, polydisperse, richly glycosylated proteins, transcribed from the MUC genes. There are at least 21 genes for mucin proteins in human and mouse and they are either secreted or membrane bound and show anatomical site specificity (Dharmani et al., 2009; Ridley and Thornton, 2018). For instance, the mucociliary escalator of the lung has been shown to express five main mucins including the membrane associated MUC-1, 4, and 16 and the secreted mucins MUC-5B and MUC-5AC. In contrast the major mucin of the gastrointestinal tract is MUC2. The main functions of mucins are in hydration, lubrication and protection (from host proteases) and in host defence. Here they act to restrict bacterial commensals and pathogens from invading the underlying epithelium. These protective functions are observed in MUC2 deficient mice, that have spontaneous colitis (Van der Sluis et al., 2006), dramatic changes in microbiota (Wu et al., 2018) and have a markedly decreased immunity to *Listeria monocytogenes* (Zhang et al., 2021). Consistent with this, MUC5b deficient mice show abnormal lung architecture (Valque et al., 2019) and confirm its requirement for lung defence against multiple bacterial types, including *S. aureus* (Roy et al., 2014).

Microbiological barriers, in the form of commensal flora, often termed “good bacteria” have been known and studied for many decades. However, it is only in the last 10 years that tools to determine the true diversity have been available. While “microbiota” has accepted effects on innate and adaptive immunity, its major activity contributing to the microbiological barrier is colonization resistance (McKenney and Kendall, 2016; van der Waaij et al., 1971). This parameter measures how long an inoculated bacterial pathogen can be detected in the feces of a host for a given dose giving a proxy measure of colonizing ability (van der Waaij et al., 1971). It is also clear that obligate anaerobic bacteria remain vitally important for health, including species such as *Bifidobacterium*, *Akkermansia*, *Bacteroides*, or *Faecalibacterium* (Andrade et al., 2020). However, colonization resistance is ‘expressed in a variety of ways’ (Sorbara and Pamer, 2019; Fehervari, 2019). These may be summarized as (i) passive-through, out-competing other bacteria for space and nutrients; (ii) active-through the production of anti-bacterial factors or processes. At least 6 mechanisms exist for direct colonization resistance (Fig. 2; Sassone-Corsi and Raffatellu, 2015). While the detailed mechanisms remain unknown, recent work suggests that the microbiome can prime two different host response pathways in the intestine and lungs that protect against bacterial pathogens (Solis and Levy, 2020).

Targeting and responding to bacteria

Immune responses to bacteria (and other organisms) can be organized into those that protect and sample the “extracellular” space and those that target and sample the “intracellular” space (Table 2). Coincidentally, bacteria have also been categorized as “extracellular pathogens,” obligate intracellular pathogens and facultative intracellular pathogens. It is now becoming clear over the last 10 years that strict definitions such as this do not fit the bacterial life cycle and survival within the host (Silva, 2012). Thus, it is perfectly reasonable to find traditionally defined extracellular bacteria, such as *S. aureus* and *E. coli* inside the cell. Likewise, studies have also reported on the presence of intracellular bacteria such as *Chlamydia* and *Campylobacter* outside the cell. Indeed, for successful survival the ability to survive and multiply in different cellular niches is vital for a successful organism. It is clear that “the facultative intracellular bacteria” may well account for a great proportion of organisms that interact with a human host (Silva, 2012). Therefore, while immune mechanisms may be characterized in terms of targeting extracellular and intracellular bacteria, the total immune response should be viewed as that targeting different parts of its life cycle in the host. Thus, every bacteria, will spend a different proportion of time inside or outside the cell and will therefore stimulate specific parts of immunity to different extents.

Sensing bacteria inside and outside the cell

The ability to detect bacteria, quickly and reproducibly, is done through host expressed pattern recognition receptors (PRR) (Paul-Clark et al., 2012). There are germ-line encoded receptors that recognize conserved molecular structures, termed pathogen

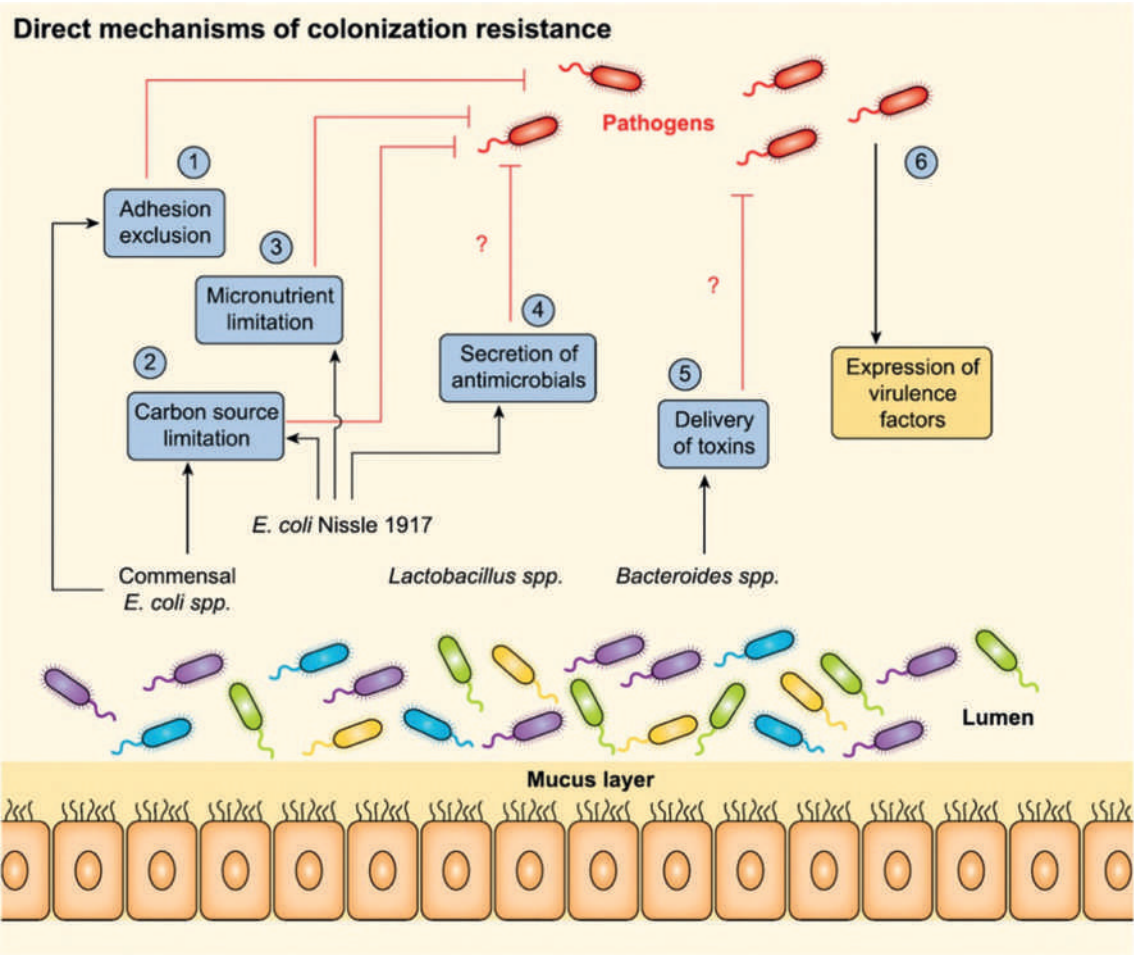


Fig. 2 Direct mechanisms of colonization resistance against enteropathogens. The microbiota provide a barrier against incoming enteric pathogens via multiple mechanisms. (1) Adhesion exclusion: certain commensals reduce pathogen adherence to the intestinal mucosa. (2) Carbon source limitation: human commensal *E. coli* strain HS and probiotic strain *E. coli* Nissle both metabolize multiple sugar molecules, which limits the availability of nutrients in the gut to certain pathogens. (3) Micronutrient limitation: the probiotic strain *E. coli* Nissle can uptake iron via several mechanisms, limiting its availability to pathogens, such as *Salmonella typhimurium*. (4) Secretion of antimicrobials: commensals act against pathogens by limiting nutrients, as well as by the production of antimicrobial compounds, such as bacteriocins and microcins. (5) Direct delivery of toxins: although not yet demonstrated *in vivo*, commensals can express T6SSs and contact-dependent inhibition systems, means by which to deliver growth-inhibiting toxins to close competitors. (6) Circumventing colonization resistance: pathogens use a variety of virulence factors to colonize the host and cause disease. Direct colonisation resistance. Sassone-Corsi M and Raffatellu M (2015) No vacancy: How beneficial microbes cooperate with immunity to provide colonization resistance to pathogens. *Journal of Immunology* 194: 4081–4087.

Table 2 Summary of major immune mechanisms against extracellular and intracellular bacteria.

Intracellular immunity	Extracellular immunity
• Barriers • ILC/NK cells • CD8, cytotoxic T cells • Th1 immunity • TLRs and NLRs • AMP • Cytokines IFN	• Barriers • Complement • Soluble PRR • IgG, IgM and IgA • Phagocytes/phagocytosis • CD4 T helper lymphocytes • Th17 immunity • TLRs • AMP • Cytokines IL-1, CXCL8, CCL2

Bacteria will be present in both the intracellular and extracellular space. The table lists the important immune components that will target intracellular and extracellular spaces.

Table 3 Toll like receptors and their agonists.

TLR	PAMP	Pathogen	Species
2/1 (heterodimer)	Pam3/Csk4	Synthetic diacylated lipoprotein	Mouse
2/6	FSL-1	Synthetic diacylated lipoprotein	Guinea pig
		Synthetic triacylated lipoprotein	
2	Pam3/Csk4	Gram Pos bacterial cell wall	Mouse
2	LTA, MDP	Mycobacterial	Human/mouse
2	PGN	Extra cellular matrix protein	Human/mouse
2	BCG	Fungal	Human/mouse
2/6	Hyaluronan, biglycan	Bacterial	Human/mouse
2/6	Zymosan		
	MALP-2		
3	Poly I:C	dsRNA viruses	Human
4	LPS	Gram negative bacteria	Human
	Lipid A moiety	LPS moiety	Human
	Ozone	Oxidant	Mouse and human
	BCG	Mycobacterial	Mouse
	Hyaluronan	Extra cellular matrix protein	Human
5	Flagellin	Bacterial flagella	Human
2/6	Zymosan	Fungal	Human/mouse
2/6 (heterodimer)	MALP-2	Bacterial	
7	Imidazoquinolines [imiquimod, resiquimod (R-848) and gardiquimod] isatoribine, loxoribine	ssRNA viruses and nonviral	Human
		Origin (poly dT; thymidine homopolymer phosphorothioate)	Human
			Human
7/8	Resiquimoid (R848), CL097		Human
7/8			Human
8	3M-002/CL-075	ssRNA viruses	Human
		Pure thiazoloquinolone TLR8 agonist	
9	CpG DNA	TLR9 recognizes unmethylated CpG motifs of bacterial and viral DNA	Human
	Imidazoquinoline (852 A), ODN2006		
		Agonists of the TLR9 are major activators of type 1 IFNs (IFN α /b). They can produce other cytokines, for example, IFN-g, IL-6, IL-10 and IL-1ra.	
10/1	Pam3CSK4	Synthetic triacylated lipoprotein	Human
10/2	Zymosan		
11	Profilin	Uropathogenic bacteria	Mouse

Known PRR, organised as a table on TLR and a table on NLR. Paul-Clark MJ, George PM, Gatheral T, Parzych K, Wright WR, Crawford D, Bailey LK, Reed DM, and Mitchell JA (2012) Pharmacology and therapeutic potential of pattern recognition receptors. *Pharmacology & Therapeutics* 135: 200–215.

associated molecular patterns (PAMPs). Therefore, these initial sensing molecules scan for “similarities” between all bacteria. The major PRR for bacteria include the toll like receptors (TLR, [Table 3](#)) and the nucleotide binding and oligomerization (NOD)-like receptors (NLR) ([Table 4](#)).

These two families of PRRs recognize their conserved targets in different parts of the host cell, including the surface, the cytoplasm and within endocytic vesicles and therefore allow detection of bacteria in the extracellular environment and interstitial space together with the intracellular environment and cytoplasmic organelles. Important conserved structures include peptidoglycan (PGN), lipoteichoic acid (LTA), lipopolysaccharide (LPS) and flagellin that bind to TLR-2, TLR-4 and -5 respectively. Numerous cells constitutively express or can upregulate PRR and this together with their anatomical locations provide opportunity for bacterial sensing.

Macrophages

Originating from blood derived monocytes and recruited to reside in tissue spaces, macrophages are regarded as local sentinels and scavengers of the local tissue spaces ([Orecchioni et al., 2019](#); [Mills et al., 2000](#); [Martinez and Gordon, 2014](#); [Murray et al., 2014](#); [Shapouri-Moghaddam et al., 2018](#)). They are the resident “phagocyte” (eating cell) responsible for removing bacteria and dead cells.

Table 4 NOD like receptors and their agonists.

Family	Members		Location	Agonists
	Human	Mouse		
NLRA	CIITA	Clita	Cytosol (Mφ, Tφ, Bφ)	IFN γ
NLRB	NAIP	Naip1 Naip2 Naip3 Naip4 Naip5 Naip6 Naip7	Cytosol	<i>Legionella</i> , flagellin
NLRC, NLRs/CATERPILLARS	NOD1 NOD2 NLRC3 NLRC4 NLRC5	Nod1 Nlrc3 Nlrc4 Nlrc5	Cytosol	Meso-DAP (mesodiaminopimelic acid from Gm $^-$, some Gm $^+$, MTb), <i>H. pylori</i> , <i>B. anthracis</i> MDP (PGN from Gm $^+$, Gm $^-$, <i>Mycobacterium</i>), <i>T. gondii</i> , RSV <i>Pseudomonas</i> , <i>Salmonella</i> , <i>Legionella</i> , <i>Shigella</i> , flagellin
NLRP	NLRP1 NLRP2 NLRP3	Nlrp1a-c Nlrp2 Nlrp3	Cytosol	Anthrax lethal toxina, MDP, <i>B. anthracis</i> Pore-forming toxins, extracellular ATP, crystals, chemical sensitizers, silica, asbestos, amyloid β , nanoparticles, Bleomycin, * <i>L. monocytogenes</i> (LLO), <i>S. aureus</i> , aluminum adjuvants, uric acid
	NLRP4 NLRP5 NLRP6 NLRP7 NLRP8 NLRP9 NLRP10 NLRP11 NLRP12 NLRP13 NLRP14 NLRX1	Nlrp4a-g Nlrp5 Nlrp6 Nlrp9a-c Nlrp10 Nlrp12 Nlrp14 Mitochondrial		Sterile inflammation-hyaluronan, biglycan
NLRX				
RIG-I/MDA5			Cytosol	Cytoplasmic ds-RNA
AIM2			Cytosol	DNA

Known PRR, organised as a table on NLR. Paul-Clark MJ, George PM, Gatheral T, Parzych K, Wright WR, Crawford D, Bailey LK, Reed DM, and Mitchell JA (2012) Pharmacology and therapeutic potential of pattern recognition receptors. *Pharmacology & Therapeutics* 135: 200–215.

The last 10–20 years has confirmed a significant phenotypic diversity in tissue macrophages, which directly effects their responses to individual bacteria (Orechioni et al., 2019). While there is still debate over exact definitions the classical or M1 activated macrophage releases huge quantities of proinflammatory cytokines, including TNF α , IL-6, IL-12 and IFN- γ and antibacterial agents such as nitric oxide (NO) and reactive nitrogen species (RNS). They kill bacteria through nitrogen mediated radicals (RNS) produced by inducible nitric oxide synthase (iNOS) following efficient phagocytosis and are potently antibacterial. In contrast, alternative or M2 activated macrophages have enhanced production of anti-inflammatory cytokines IL-4, IL-10 and arginase and have roles in reducing bacteriocidal activity. This is particularly important with respect to bacterial biofilms where *S. aureus* and *P. aeruginosa* induce M2 macrophage activation whereas *E. coli* induces the M1 activation phenotype (Thurlow et al., 2011; Yu et al., 2020a). Macrophages have a life span of weeks and their importance to protect against bacterial infections has been confirmed using macrophage depletion strategies. Here removal of macrophages increases bacterial loads in *P. aeruginosa* (Horino et al., 2009), *Bacillus anthracis* (Cote et al., 2004) and in natural neonatal *Pasteurella pneumotropica* (Saini et al., 2016) infection.

An important note must be addressed with respect to infectious dose. While, unsurprisingly an increase in the number of infectious bacterial cells often leads to increased pathogenesis and tissue damage, it is very important to note that dose is also intricately linked to immune system activation. Low inocula of bacteria, as that associated with nocturnal aspiration into the lungs from the oropharynx, will likely be cleared by macrophages alone. In contrast, higher doses, will require recruitment of blood derived phagocytes and may also stimulate adaptive immunity and the generation of lymphocyte memory to protect against future infections. This point is particularly important when considering the impact of high virulence bacteria (e.g. *S. aureus*, *P. aeruginosa* and *E. coli*) versus low virulence bacteria (*S. epidermidis* and *P. acnes*) that are responsible for chronic low grade infections such as in medical device failure (Lovati et al., 2017).

Epithelial cells

Situated at the interface between the host and the outside world, epithelial cells act as a barrier to protect underlying tissues. They form an integral monolayer held together by tight junctions whose integrity prevents bacterial invasion while at the same time allowing solute absorption. Epithelial cells are not just neutral barriers (see above), but synthetic cellular machines capable of producing molecules essential for host defence against bacteria. Epithelial cells express PRR constitutively, but these can be up-regulated upon stimulation to produce an anti-bacterial shield. Epithelial derived mediators include mucins, antimicrobial peptides and cytokines and chemokines. Epithelial cells have different antibacterial phenotypes based on their anatomy. In the gut epithelial cells are situated adjacent to commensal and pathogenic bacteria continuously. This requires a greater tolerance to activation and damage, while sensing for danger (Peterson and Artis, 2014; Soderholm and Pedicord, 2019; O'Callaghan and Corr, 2019). In the lung, a delicate balance of air-exchange must be maintained against a background of inspired bacterial pathogens (Bissonnette et al., 2020; Whitsett and Alenghat, 2015; Kirkpatrick et al., 2018; Leiva-Juarez et al., 2018). In the skin, multiple layers of epithelial cells form a water permeable seal to retain moisture and respond to bacterial pathogens (Bitschar et al., 2017; Chieosilapatham et al., 2021).

Innate lymphoid cells and NK cells

Recent advances in the understanding of early immune responses to bacteria have also detected PRR on a heterogeneous group of lymphocytes, termed innate lymphoid cells (ILCs) (Montalban-Arques et al., 2018; Beck et al., 2020). While they are derived from lymphoid progenitors, they do not express the T-cell receptor (TcR/CD3) suggesting that they do not participate in major histocompatibility (MHC) restricted detection. They reside in the lamina propria below the epithelial cell monolayer and respond rapidly to infection. To date, three groups have been identified, termed ILC-1, ILC-2 and ILC-3, each having slightly different roles in immunity. Responses to bacteria are mainly associated with ILC-1 and ILC-3 cells in gut and lung. Specifically, IL-22 producing ILC cells appear in the intestine upon *Citrobacter rodentium* infection (Cella et al., 2009) and in response to microbial flora (Satoh-Takayama et al., 2008). Furthermore, this response is essential for protection against *C. rodentium* (Basu et al., 2012; Zheng et al., 2008). ILC 1 and 3 are also essential for protection against human pathogens of the gastrointestinal tract, such as *C. difficile* (Abt et al., 2015) and *S. typhimurium* (Kupz et al., 2013) as well as respiratory pathogens such as *Klebsiella pneumoniae* (Xu et al., 2014) and *Streptococcus pneumoniae* (Van Maele et al., 2014). In contrast, ILC-2 cells generate inflammatory injury induced by *S. aureus* (Hardman et al., 2017).

Innate T cells

While T lymphocytes are commonly associated with adaptive immunity and restricted activation through binding of their $\alpha\beta$ /gamma delta ($\gamma\delta$) T cell receptors (TcR) to MHC class I and II receptors (see T-cells) the "innate T cells" form a heterogeneous collection of three groups expressing high levels of the marker CD161 (Gao and Williams, 2015; Trottein and Paget, 2018; Krovi and Gapin, 2018; Fichtner et al., 2020). These groups include; (i) mucosal associated invariant $\alpha\beta$ -T cells (MAIT), (ii) invariant natural killer $\alpha\beta$ -T cells (iNK) and (iii) $\gamma\delta$ T-cells. Current research suggests that these groups may be separated further but this is beyond the scope of this review. The iNKT, MAIT, and gamma delta ($\gamma\delta$) T cells play an important role in anti-bacterial defence through cytokine production, perforin release, and cross-talk to other innate and adaptive cells. Specifically, iNKT knockout mice succumb to enhanced infection to *Streptococcus pneumoniae* infection, showing increased mortality, higher bacteria load and decreased early neutrophil specific chemokines compared to wild-type mice (Kawakami et al., 2003). Furthermore, recruitment of iNKT to the lung is observed in response to *Chlamydia pneumoniae* infection with the cells recruited being strain specific (Joyee et al., 2007). Consistent with this are studies suggesting that iNKT cells play a protective role against *Salmonella*-induced reactive arthritis (Noto Llana et al., 2017). Likewise, MAIT cells have been shown to increase in response to live *Francisella tularensis* intracellular infections and produce key protective cytokines IFN γ , TNF α and IL-17A (Meierovics et al., 2013). The cytolytic potential of MAIT granular contents is highly regulated with resting states showing low granzyme B and perforin, which are activated upon bacterial stimulation (Kurioka et al., 2015). $\gamma\delta$ T-cells produce important protective cytokines IFN- γ , TNF α and IL-17A, but are also thought to establish long lived "memory" populations essential for the defence against *M. tuberculosis*, *S. aureus*, *B. pertussis*, *L. monocytogenes*, and *Salmonella enterica* (Shepherd and McLaren, 2020; Khairallah et al., 2018).

Targeting and marking bacteria

Soluble PRR (Smole et al., 2020) are structurally quite diverse but share binding properties that allow them to eliminate bacteria in the extracellular space through agglutination and opsonization, complement activation and ultimately phagocytosis (Fig. 3). They are constitutively produced by hepatocytes and immune cells, but are rapidly upregulated by bacterial stimulation and are known to provide specific anti-bacterial defence.

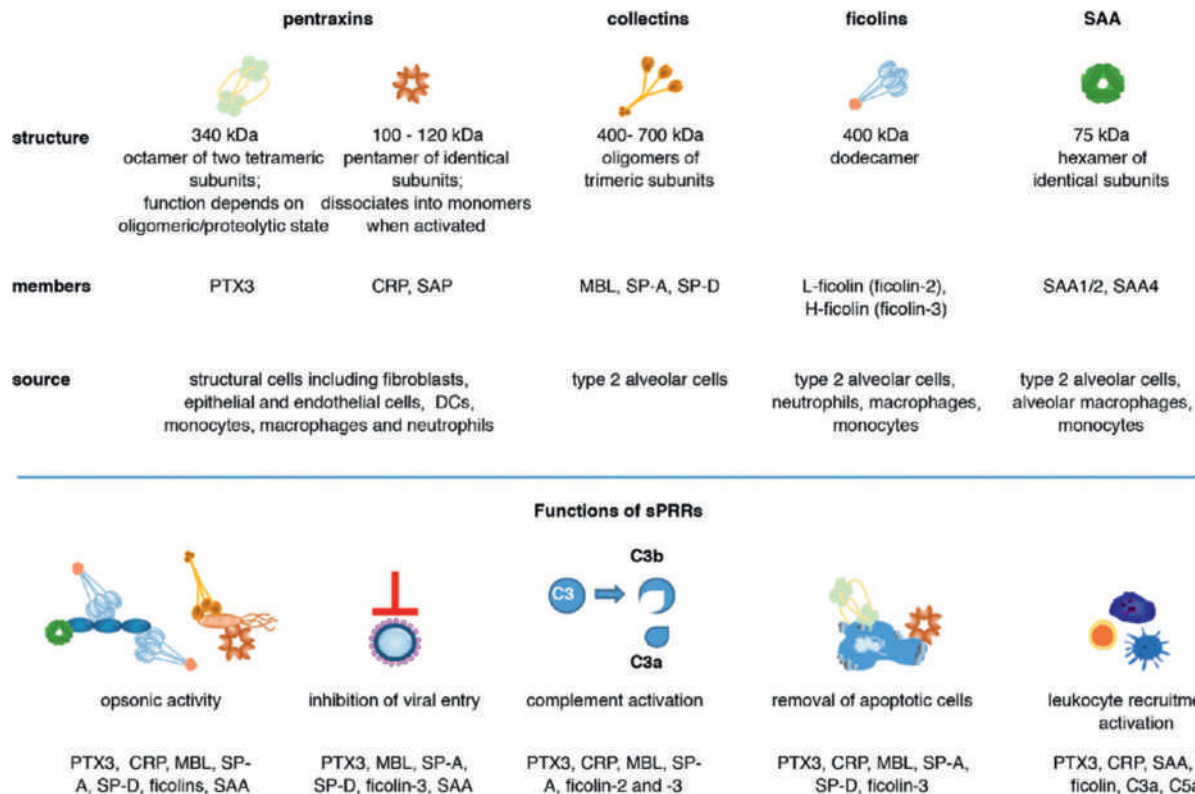


Fig. 3 Protein structures, source and roles of sPRRs in the airways. Shown are the functional structures of pentraxins, collectins, ficolins and SAA proteins and their prominent family members. Local production of pentraxins in the lung could be detected in structural and myeloid cells as well as neutrophils. While the lung (type 2 alveolar cells) is the main synthesis site of the collectins SP-A and SP-D, MBL is primarily produced by the liver. Ficolins are produced in the liver by hepatocytes and in the lung by type 2 alveolar cells, as well as by neutrophils, macrophages and monocytes. SAA proteins, similar to pentraxins, were long thought to be liver-derived serum factors but are also produced locally in the lung by type 2 alveolar cells, macrophages and monocytes. sPRRs share overlapping but also nonredundant roles in the first line of defence against microbial intruders through opsonic activity, inhibition of viral entry and the activation of the complement cascade. Some sPRRs play essential roles in the removal of apoptotic bodies, which is crucial for pulmonary immune tolerance and tissue homeostasis. In addition, several sPRRs are involved in leukocyte recruitment to the lung and local proinflammatory cytokine production. Smole U (2020) Soluble pattern recognition molecules: Guardians and regulators of homeostasis at airway mucosal surfaces. *European Journal of Immunology* 50: 624–642.

The pentraxins (PTX) (Haapasalo and Meri, 2019)

Pentraxins are a superfamily of cyclic multimeric sPRR and include the long pentraxin (PTX-3) (Porte et al., 2019) and the short pentraxins, C-reactive protein (CRP) and Serum amyloid P (SAP). All the pentraxins are intricately involved in complement activation through their opsonizing capacity. However, their anti-bacterial activities remain complex due to binding at multiple levels of the pathway and furthermore an ability to bind activators (e.g. C1q) as well as inhibitors (Factor H). It is clear that PTX-3 has a protective role against bacterial infections, especially to *S. flexneri* (Ciancarella et al., 2018) and uropathogenic *E. coli* (Jaillon et al., 2014). In contrast, CRP was originally named by its ability to bind to the phosphocholine segment of the C-type lectin of pneumococcus in a calcium dependent manner. There is also good evidence to suggest that high CRP levels are associated with bacterial infections and low levels are associated with viral infections (Thomas et al., 2020). Less data exists on SAP, however Yuste and colleagues confirmed that SAP binds to *S. pneumoniae* and activates complement by the classical pathway, improving the phagocytic efficiency (Yuste et al., 2007).

Serum amyloid A (SAA)

Serum amyloid A (SAA) binds to outer membrane protein A (Hari-Dass et al., 2005) and acts as an opsonin for Gram negative bacteria, like *E. coli* and *P. aeruginosa* (Shah et al., 2006). Indeed, recent work suggests that SAA may bind bacterial lipoproteins for delivery and stimulation of TLR-2 (Burgess et al., 2018). Very recent work using a double knockout mouse for SAA1 and 2 confirmed that SAA is essential for protection against *S. aureus* by a mechanisms involving IL-6 (Zheng et al., 2020). Furthermore, it was clear

that its killing action was pH dependent. It is clear that SAA is a retinol binding protein needed for defence against *S. typhimurium* *in vivo* (Derebe et al., 2014) and *K. pneumoniae* *in vitro* (Zheng et al., 2016). However, while physiologically relevant concentrations of SAA seem to have little effect on urinary pathogenic *E. coli* (UPEC), they do seem to inhibit biofilm formation (Erman et al., 2012).

Collectins: Mannan binding lectin (MBL) (Beltrame et al., 2015) and surfactant proteins A and D (Kuroki et al., 2007)

Collectins are collagen containing C-type lectins and play important specific roles in anti-bacterial defence and include mannose-binding lectin (MBL) and surfactant protein A (SP-A) and SP-B. Specifically, SP-A has been shown to opsonise *S. aureus* for uptake by macrophages. In contrast, SP-D opsonises Gram negative bacteria including *E. coli* and activates respiratory burst in macrophages and uptake by dendritic cells. Mice deficient in SP-A and SP-D support unique roles for each molecule. SP-A is required for appropriate protective defence against group B streptococcus infection (LeVine et al., 1997, 1999) and *P. aeruginosa* infection (LeVine et al., 1998). The same author compared group B streptococcus infection and *H. influenzae* infection in both SP-A and SP-D deficient mice and found that SP-A, but not SP-D, was associated with increased bacterial killing (LeVine et al., 2000) despite both molecules playing a role in inflammation. MBL monomers self-associate to form trimers which provide the correct conformation for MBL-associated serine protease (MASP) to activate the complement cascade through the lectin pathway. MASP-2 deficient mice have poor defence against *S. pneumoniae* due to a failure in opsonization (Ali et al., 2012) but do not play a role in *P. aeruginosa* infection (Kenawy et al., 2012).

Ficolins (Bidula et al., 2019)

Ficolins represent a class of opsonins that are characterized by the presence of a C-terminal fibrinogen-like and an N-terminal collagen-like domain (fi-col-ins) that have distinct ligand specificities (GlcNAc GalNAc, sialic acid, D-fucose). All 3 types, M-, L-, and H-ficolin (also termed Ficolin-1, -2 and -3 respectively) interact with bacteria by primarily acting as opsonins. However, they can also activate the lectin pathway. L-ficolin (ficolin 2) is by far the best studied and it is clear that it has a diverse binding capability that provides protection against numerous bacteria, including *M. tuberculosis* (Kobayashi et al., 2019), enteroaggregative *E. coli* (Adler Sorensen et al., 2018), *M. leprae* (de Messias-Reason et al., 2009) and *S. pneumoniae* (Endo et al., 2012). Indeed one important general mechanism that might be employed here is through natural antibody (not antigen induced) binding to ficolin-opsonised bacteria (Panda et al., 2013). Furthermore, bacterial infections associated with immunocompromised patients, including diabetes mellitus, primary immunodeficiencies, liver cirrhosis and B cell leukemia may be due to polymorphisms in ficolin genes (Barkai et al., 2019; Babaha et al., 2020; Foldi et al., 2017; Pana et al., 2014).

Complement

Paul Ehrlich described the heat-labile activity of the soluble fraction of blood as complement as it supported the activity of the cellular leukocyte fraction (Kaufmann, 2008). Today we understand complement as a protein zymogen cascade where members are cleaved for system activation. It has been termed the guardian of the extracellular space (Heesterbeek et al., 2018). Complement has three major pathways and three major downstream functions to clearing bacteria. Critically complement is essential for immunity to extracellular phases of bacterial infection and moreover it is required for the killing of Gram-negative bacteria. In particular, *Neisseria meningitidis* are sensitive to these effects. In contrast, the much thicker layer of peptidoglycan and specific evasion mechanisms are thought to provide complement resistance in Gram positive organisms such as *S. aureus* despite an ability of complement to deposit on their surfaces (Berends et al., 2013). Interestingly however, it is clear that the distinction may be slightly more complicated as *E. coli* pathotypes have unique sensitivities to complement, including extraintestinal strains showing strong serum resistance (Miajlovic and Smith, 2014).

Immunoglobulin (antibody) (Hey, 2015)

Immunoglobulins provide protection against extracellular bacterial infection through a variety of routes. Their specific and avid binding to target bacteria provide, an early signpost for six different mechanisms of killing once antibody has bound (Fig. 4); (i) complement activation (by classical pathway) and complement dependent cellular cytotoxicity (CDCC) through MAC formation; (ii) activation of ILC-1 cells (NK cells) through Fc receptors to deliver cellular lysis through antibody dependent cellular cytotoxicity (ADCC); (iii) binding and neutralization of bacterial toxins; (iv) opsonization of bacteria for antibody dependent phagocytosis; (v) degranulation of granulocytes (e.g. neutrophils and mast cells) through receptor aggregation following antibody crosslinking (Menon et al., 1986); (vi) agglutination of bacteria though antibody crosslinking and complex formation restricts bacterial adhesion and invasion.

The importance of antibody in the immune response to bacterial infections is confirmed in the X-recessive primary immunodeficiency agammaglobinaemia, where patients have severely reduced numbers of peripheral B-cells (less than 2% of normal), severely reduced serum immunoglobulin levels of all classes, and a lack of a humoral response to antigens (Bousfiha et al., 2018; Bruton, 1952). A recent long-term follow-up in 168 patients confirmed bacterial infections in the skin, respiratory and

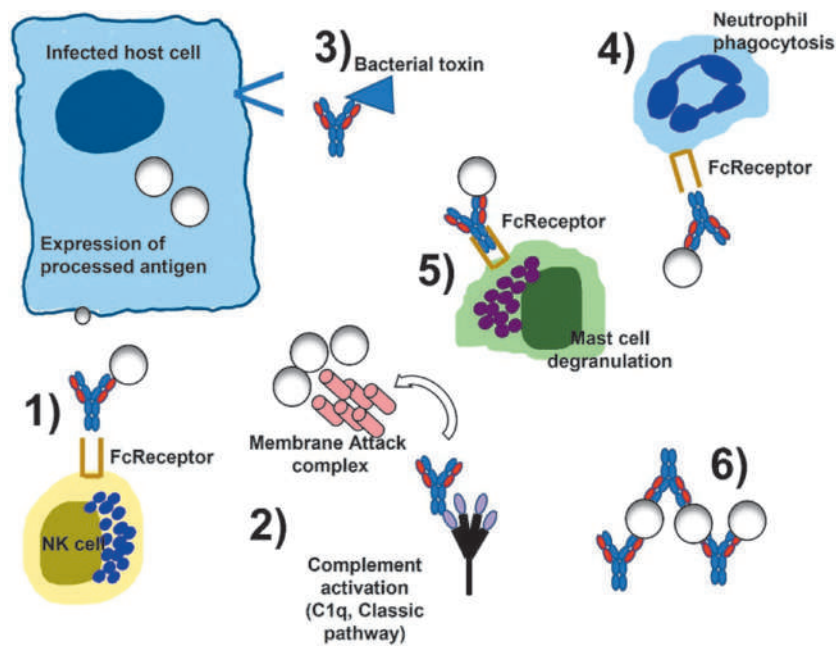


Fig. 4 A summary of the protective functions of antibody. (1) Antibodies bind to pathogen-derived or endogenous antigens expressed on the surface of an infected cell, which triggers binding to Fc receptors on natural killer cells and lysis of the infected cell by antibody-dependent cellular cytotoxicity. (2) Antibodies bind to pathogen-derived or endogenous antigens expressed on the surface of infected cells, which triggers activation of complement through binding of complement factor C1q. (3) Neutralization, here a bacterial toxin neutralized by bound antigen. (4) Antibody bound to bacterial surface proteins binds to Fc receptors on phagocytic cells, e.g., macrophages, and triggers endocytosis and destruction of bacteria in endolysosome; (5) Antibody binding to bacterial antigen can trigger the degranulation of neutrophils and mast cells by Fc receptors; (6) Agglutination of bacteria through antibody crosslinking. Antibacterial functions of antibodies. Hey A (2015) History and practice: Antibodies in infectious diseases. *Microbiology Spectrum* 3: AID-0026-2014.

gastrointestinal tracts (Lougaris et al., 2020). In most cases bacteria were encapsulated species with a significant proportion of their pathogenesis spent in the extracellular space confirming the importance of antibody in this tissue space. The most frequently isolated pathogen in the skin was *S. aureus* (~75%), in the lung *Haemophilus influenzae* and *Streptococcus pneumoniae* (53 and 18% respectively) and in the gut *Salmonella* (24%), *Campylobacter* (19%), and *E. coli* (5%).

Responding to bacteria and recruiting appropriate cells

Neutrophils are short lived blood phagocytes that are recruited to the sites of infection to phagocytose and kill extracellular bacteria. They are almost essential for protection against extracellular phases of infection as demonstrated by chronic granulomatous disease (CGD) (Yu et al., 2020b). This is a very rare primary immunodeficiency (~1 in 250,000) where patient suffer from recurrent bacterial (and fungal) infections in areas patrolled by neutrophils including the lungs, lymph nodes, skin, liver and bones. Specifically, infections are associated with catalase positive bacteria including *S. aureus*, *Burkholderia cepacia* complex, *Serratia marcescens* and *Nocardia species*, but extend to others including *P. aeruginosa*, *K. pneumoniae*, *E. coli* and *Salmonella species*.

Neutrophils and the CXCL2-CXCR2 axis

Both CXCL1 and CXCL2 act through CXCR2 expressed on neutrophils. Antibody neutralization of CXCR2 has confirmed the need for neutrophil recruitment in models of *P. aeruginosa* (Tsai et al., 2000). Consistent with this, CXCR2 deficient mice have severely perturbed recruitment of neutrophils (and exudate macrophages) resulting in bacterial outgrowth in pneumococcal pneumonia (Herbold et al., 2010).

Monocyte recruitment and the CCL2-CCR2 axis (Serbina et al., 2008; Shi and Pamer, 2011) and mononuclear phagocyte system

Monocytes are important for providing a major source of macrophages and dendritic cells in peripheral tissues. At least two distinct subsets can be distinguished in humans and mice that are responsible for constitutive macrophage production and recruitment during infection respectively (Shi and Pamer, 2011). Inflammatory monocytes are extremely important for immune defence against certain bacterial infections. Indeed *L. monocytogenes* is even named for the observation that part of its pathology is the accumulation

of monocytes in peripheral tissues. Transgenic mice overexpressing CCL2 in epithelial cells have improved bacterial clearance and mortality following pneumococcal (Winter et al., 2007) and *M. bovis* (Schreiber et al., 2008) challenge compared to their wild-type counterparts. In the same pneumococcal infection model CCL2 knockout mice had greater lung burden and sepsis than their wild-type counterparts and this was due to numbers of recruited monocytes and dendritic cells (Winter et al., 2009). Consistent with this were results in a neonatal *E. coli* pneumonia model where CCR2 deficient mice had increased mortality compared to their wild-type controls (McGrath-Morrow et al., 2017). Similarly, *L. monocytogenes* infection cannot be cleared in CCR2 deficient mice because of defective monocyte recruitment (Kurihara et al., 1997).

Cytokines responsible for host resistance to bacterial infections have been identified in knockout mice and in human disease (Netea et al., 2004)

Cytokines provide essential intercellular signaling between immune cells during a bacterial infection. Their efficacy to activate the correct immune program, to provide appropriate immunity for a given bacteria is central to their importance. They may be grouped by structure, by function, by their importance to innate or adaptive immunity or by their cellular source, however in this review, focus will be made to their requirement for defence against specific bacterial infections. Specifically, the major pro-inflammatory cytokines IL-1 β , IFN- γ , TNF α , IL-12 and IL-18 have been shown to be critical to defence against a variety of bacterial infections. IL-1 β is needed for optimal neutrophil dependent control of infection (Mayer-Barber and Yan, 2017). Similarly, early work in TNF receptor deficient mice confirmed that they were protected from lethal doses of toxins such as LPS and staphylococcal enterotoxin, but died following *L. monocytogenes* infection (Pfeffer et al., 1993). Indeed the importance of IL-1 β and TNF α is underlined in treatment of Crohn's disease and arthritis with anti-TNF monoclonal antibodies (infliximab) and IL-1 receptor antagonists (anakinra), where therapy can induce a state of secondary bacterial infection (Salliot et al., 2009; Keane et al., 2001; Fernandez-Ruiz and Aguado, 2018). IFN- γ has specific activities to "acidify" the phagolysosome within infected macrophages. This activity combined with enhancing reactive nitrogen species and the induction of autophagy allows IFN- γ to increase macrophage bactericidal capabilities. IFN- γ is important for defence against *M. tuberculosis*, *S. typhimurium* and *L. monocytogenes* (Kak et al., 2018).

IL-12 (and its family member IL-23) and IL-18 are major IFN- γ inducing cytokines and many of their effects can be abolished in the absence of IFN- γ (Flynn et al., 1995). Indeed, mutations in genes of the IL-12/IFN- γ axis are responsible for increased susceptibility to *M. tuberculosis* and *Salmonella* infections (Ramirez-Alejo and Santos-Argumedo, 2014). As such, they have clearly overlapping activities with IFN- γ and protect against bacterial infections such as *L. monocytogenes*, *Y. pestis* and *S. typhimurium*. IL-18 seems to have a broader spectrum showing protection against *S. aureus* (Wei et al., 1999), *S. pneumoniae* (Lauw et al., 2002), and *P. aeruginosa* (Huang et al., 2002).

IL-17 (and its family members IL-21 and IL-22 (Koenders and van den Berg, 2010)) is the guardian of the mucosal barriers (Cypowyj et al., 2012). It is required for protection in a variety of mucosal infections including lung infections caused by *Klebsiella pneumoniae*, *Bacillus anthracis*, and *Mycobacterium bovis* BCG, oral infections to *Porphyromonas gingivalis*; gastric immunity to *Helicobacter pylori*; urogenital immunity to *Neisseria gonorrhoeae*; intestinal immunity to polymicrobial sepsis; cutaneous skin immunity to *S. aureus*.

IL-10 is the prototype anti-inflammatory cytokine, responsible for inhibiting the early proinflammatory responses to stimulate tissue homeostasis (Penaloza et al., 2016). This regulatory activity has huge implications in bacterial infections. Recent hypotheses on the functions of IL-10 suggest that it is required to limit inflammation in response to extracellular bacteria like *S. pneumoniae* and *P. aeruginosa* and highly pro-inflammatory intracellular bacteria like *M. tuberculosis*, *E. coli* and *F. tularensis*. This would allow bacterial clearance without tissue damage and bacterial dissemination. In contrast, excessive IL-10 suppression of the immune response can allow intracellular bacteria like *L. monocytogenes*, *B. abortus* and *S. enterica*, to survive and disseminate but critically could also allow commensal bacteria to survive in the host.

Finally, it is very clear that the cytokine system is based on a significant amount of biological redundancy. The ability to substitute and overlap biological activities is a strength in generating appropriate immunity to bacteria. While the number of interactions here is almost limitless, much can be learned from studies in mice deficient in cytokine signaling molecules (Matsukawa, 2007), but also in polymorphisms associated with cytokines and their receptors that produce susceptibility to infectious disease (van Deventer, 2000; Smith and Humphries, 2009).

Generating a bespoke specific immune response (Boes et al., 2009; Gatfield et al., 2000; Ramachandra et al., 1999a, b; Watts, 2004)

Production of specific immunity and memory toward a given bacterial pathogen requires the "processing" and "presentation" of parts of its structure in the form of peptides or lipids on the surface of host cells through the major histocompatibility complex (MHC) and CD1 receptors. Distinctions can be made on how bacteria are managed. Extracellular bacteria are phagocytosed by macrophages and dendritic cells, with bacterial antigens entering the endosomal-lysosomal pathway for surface presentation on MHC-class 2 molecules. Binding of the correct T-helper cell (CD4 positive) to this MHC with co-stimulatory molecules activates immune mechanisms targeting the extracellular environment through the production of appropriate cytokines. Intracellular or

cytosolic bacteria identified in any other nucleated cells are degraded by the cytosolic proteolytic machinery, and proteasome for transport to the endoplasmic reticulum and cell surface presentation on MHC-class 1 molecules. Binding of the correct cytotoxic T-lymphocyte (CD8 positive) and co-stimulatory molecules alerts that lymphocyte toward the infected cells resulting in induction of apoptosis or cell lysis. These pathways have resulted in a clear understanding of adaptive immunity to a variety of pathogens and the diversity in their responses.

With respect to bacteria that have largely extracellular lifestyles. *S. aureus* induces a protective Th2 response in the skin associated with increased phagocytic neutrophils (Nippe et al., 2011). This is related to induction of epithelial derived Th2 cytokines such as IL-5 and IL-13 (Lan et al., 2018). In contrast, *S. epidermidis* induced no Th2 cytokines (Lan et al., 2018) but has shown strong Th17 protective immunity in the skin (Naik et al., 2015). *Campylobacter* has demonstrated robust Th1 immunity (Rathinam et al., 2008) which changes with time from Th1 to Th2 response while assessing for the related neurological syndrome Guillain Barre (Nyati et al., 2012). Furthermore, *Campylobacter* infection in chickens has also been associated with a Th17 response (Reid et al., 2016). *S. pneumoniae* induces Th1 and Th17 responses to experimental infection (Kim et al., 2013a; Olliver et al., 2011) and a similar response to attenuated vaccination (Gao et al., 2016; Zhang et al., 2017; Qiu et al., 2017). *S. pyogenes* induces a Th1 response (Agren et al., 1998). Furthermore, parenteral administration of an anti-GAS vaccine was able to protect against intranasal inoculation through Th17 immunity involving specific IgA induction (Mortensen et al., 2017). *Haemophilus influenzae* generates a Th1 response (Agren et al., 1998). However, comparative vaccination by a variety of routes, including nasal, oral, tracheal or peritoneal confirmed that the nasal route provided the best protection against an intranasal challenge through a Th1 and Th2 response with local IgA and systemic IgG protection (Kurono et al., 1999). Consistent with this, conjugate vaccines, such as Hib-CRM(197) also generated a mixed Th1 and Th2 response and vigorous T-cell memory (Kamboj et al., 2001). *E. coli* heat-labile enterotoxin induces strong Th17 responses in experimental models through IL-1 β and IL-23 (Brereton et al., 2011). This response has been applied to vaccines which generate IgG2a and IgA responses (Norton et al., 2012) to enterotoxins. Similar responses have been observed to *E. coli* outer membrane proteins (OMP) which also share Th1/Th17 immunity, although protection is offered by T cells (Kim et al., 2013b). Indeed, consistent Th1/Th17 responses or also observed to uropathogenic *E. coli* pathotypes (Quick et al., 2013).

With respect to bacteria that have a high proportion of intracellular lifestyle. For *S. typhimurium* MHC-class 1 knockouts show increased susceptibility to infection and increased CD8 positive T-cells can be detected (Lo et al., 1999). Vaccination studies using non-lethal Aro mutants of *Salmonella* show increased survival in IFN- γ knockout mice. Wild type mice have increased CD4 and CD8 specific cells (Bao et al., 2000). Furthermore, vaccines to *S. typhimurium* in humans generate Th1 cytokines, including IFN- γ and TNF α . This results in classical MHC class Ia-restricted responses and novel, nonclassical MHC class Ib-restricted CD8(+) cytotoxic T cell responses (Sztein, 2007). Other species of *Salmonella* differ slightly. Vaccination of pigs against *S. choleraesuis* using C500, induces mucosal IgA and CD8 immunity (Zhu et al., 2017). In contrast *S. enteritidis* infection depends on strong early IL-12 production to reduce infected neutrophils and generate CD4 and CD8 specific responses (Lehmann et al., 2001). Adaptive immunity to *L. monocytogenes* as a facultative intracellular pathogen requires IL-12 induced CD4+ Th1 immunity (Hsieh et al., 1993). Indeed this IL-12 dependent response is so strong that heat-killed *Listeria* can convert established Th2 immunity (characterized by high specific IgE) into Th1 immunity (characterized by high IgG, IFN- γ and low IL-4) (Yeung et al., 1998). However, these effects are subject to local tissue differences in the liver, spleen and mucosal tissues (Marzo et al., 2002; Kuranaga et al., 2005) and the Th1 response is also dependent on the atypical NF κ B inhibitor I κ BNS (Frentzel et al., 2019). There is very good evidence that *M. tuberculosis* responses are dominated by CD4+ Th1 cytokines like IFN- γ (Robinson et al., 1994). Consistent with this is that successful antimycobacterial therapy also induces Th1 immunity (Marchant et al., 2001). Pathogenesis requires lymphocytes as severe combined immunodeficient (SCID) mice succumb to infection and virulent strains of *M. tuberculosis* are successful because they subtly suppress Th1 immunity (Manca et al., 2001). Indeed, in African countries poor outcomes are observed due to a reduced Th1/Th2 ratio (Lienhardt et al., 2002) potentially due to endemic helminth infections (Amelio et al., 2017). Finally, *Chlamydia trachomatis* generates Th1 immunity and IFN- γ is critical in combination with CD4+ immunity (Marks et al., 2007; Gondek et al., 2009). However, the situation is complicated by anatomical segregation in the genital tract where Th1 immunity dominates in the upper genital tract but Th2 immunity in the lower genital tract (Marks et al., 2010). This is consistent with recent findings of both phenotypes in women with *C. trachomatis* infection (Ogendi et al., 2018).

Body compartments-local mucosal and systemic responses

Mucosal associated lymphoid tissue (MALT)

On a macroscopic scale it is clear that immune responses to bacteria are anatomically specific, despite a great degree of overlap in the cellular mechanisms and soluble mediators. Body sites that are at an interface with the external environment have their own specialized local combined innate and adaptive immune mechanisms to sample and respond to bacteria. These collections of cells are termed mucosal-associated lymphoid tissue (MALT). Recent reviews provide important information on the differences at each site including iSALT (Chen et al., 2018; Belkaid and Tamoutounour, 2016; Honda and Kabashima, 2016), NALT (Pabst, 2015; Takaki et al., 2018), BALT (Randall, 2010; Foo and Phipps, 2010), GALT (Morbe et al., 2021), and vaginal mucosal host defence (Smith and Ravel, 2017; Cole, 2006) are provided elsewhere in this book.

Blood

In normal health, the blood compartment is sterile, with limited numbers of bacteria. However, surgery, intravenous catheters or highly virulent invasive bacteria can reach the blood compartment. Numerous mechanisms exist for protecting this vital compartment from bacterial infection. The systemic effects of bacterial PRR activation are often termed the acute phase response, which is a systemic immune response program of events. These include (i) fever and hyperalgesia; (ii) release of acute phase proteins by the liver; (iii) vasodilation of blood vessels; and (iv) recruitment of leukocytes from the blood (Slaats et al., 2016). The resident liver macrophage, termed a Kupffer cell, clear bacteria from the circulation through the complement receptor of the immunoglobulin family CRIg, through binding the opsonin molecules C3b and iC3b (Helmy et al., 2006). Recently, it has been suggested that clearance of bacteria from the blood compartment is a dual process involving parallel “slow” and “fast” mechanisms of clearance (Broadley et al., 2016). “Slow clearance” involves bacterial opsonization, platelet binding, and capture of bacteria-platelet-complexes via the complement receptor of the immunoglobulin superfamily, CRIg. “Fast clearance” involves uptake and destruction in the liver via Kupffer cell scavenger receptors. This dual mechanism is important as the “slow clearance” spares some bacteria for adaptive immunity induction by splenic CD8a+ dendritic cells. Indeed, it is clear that this small portion of bacteria associated with platelets are dependent on glycoprotein, GPIb and complement C3, diverting them to splenic dendritic cells to generation of longer-term immunity (Verschoor et al., 2011).

Differentiating bacterial pathogens and commensals

The state of homeostasis active in health is in a state of continuous antigenic challenge and flux. The best example of this is in the gut where continuous monitoring is essential to maintain barrier integrity (Fig. 5). However, what mechanisms are in place to allow the immune system to differentiate between a bacterial commensal and pathogen? Recent research has identified hundreds of potential mechanisms, which have been organized here with a few relevant examples and broader reference to appropriate manuscripts (Sassone-Corsi and Raffatellu, 2015; Sun et al., 2015) :

Commensals enhance epithelial barrier function

There is a clear link between the production of functional two layer intestinal mucus by specific PAMPs such as LPS or commensal bacteria (Petersson et al., 2011). It is also well known that *Bifidobacterium infantis* (90% of the microbiota in healthy infants) secretes bioactive factors that improves epithelial barrier integrity (Ewaschuk et al., 2008). In addition, *Bifidobacterium* has been shown to protect against enteropathogenic infection through the production of short-chain fatty acids (SCFA), such as acetate (Fukuda et al., 2011) through carbohydrate transporter molecules (Fukuda et al., 2012).

Commensals enhance innate immunity

Commensal bacteria are required for Paneth cell secretion of the antimicrobial C-type lectins REG3 γ and REG3 β through a TLR mediated MyD88 pathway. Consistent with this is the ability of enteric bacteria to activate a Paneth cell transcriptional panel of antimicrobial factors, including REG3 β , REG3 γ , CRP-ductin, which agglutinates Gram-positive and Gram-negative bacteria; the inflammatory modulator resistin-like molecule- β (RELM- β); and several members of the α -defensin family of antimicrobial peptides (Vaishnava et al., 2008). Under normal circumstances commensal bacteria generate poor pro-inflammatory responses, however in the presence of pathogens a regulated pro-inflammatory response is generated through targeting the processing of IL-1 β and IL-18 (Hasegawa et al., 2012). Consistent with this is work implicating the NLRC4-inflammasome in processing IL-1 β signals to differentiate commensal and pathogens (Franchi et al., 2012). Thus commensal organisms produce unprocessed proinflammatory signals, whereas pathogens appear to provide the necessary extra signals from production of mature pro-inflammatory cytokines.

Commensals enhance of adaptive immunity

It is clear that some commensal bacteria have the ability to drive the differentiation of specific T-cell clones. Early, work confirmed that dendritic cells could use “snorkeling” mechanisms to sample luminal bacteria prior to migration to lymph nodes to activate a specialized transcriptional program for T-regulatory cells (Rescigno et al., 2001) following induction by TLR ligands (Chieppa et al., 2006). Indeed, T cells primed by DCs activated by commensals express integrin α 4 β 7 and CCR9 and home to the gut where they generate tolerogenic responses to commensals (Iwata et al., 2004). This response requires retinoic acid for full T-cell homing. In contrast, the Clostridiales family of segmented filamentous bacteria (SFB), specifically induce the differentiation of mucosal Th17 cells in the lamina propria of the intestine, which, in turn, secrete the proinflammatory cytokines IL-17 and IL-22 which enhance antimicrobial peptide generation (Ivanov et al., 2009). In impressive state of the art experimentation, members of the family Clostridia were isolated from healthy human feces based on their ability to induce CD4+FOXP3+ regulatory T-cells promoting TGF β and IL-10 production. Critically these strains were shown to reverse pathological colitis (Atarashi et al., 2013). In the skin, commensal *S. epidermidis* are critical in generating protective Th17+CD8+ T cells that home to the skin generating immunity to protect against pathogen invasion (Naik et al., 2015). Equally important mechanisms exist for B-cells. Indeed, dendritic cells

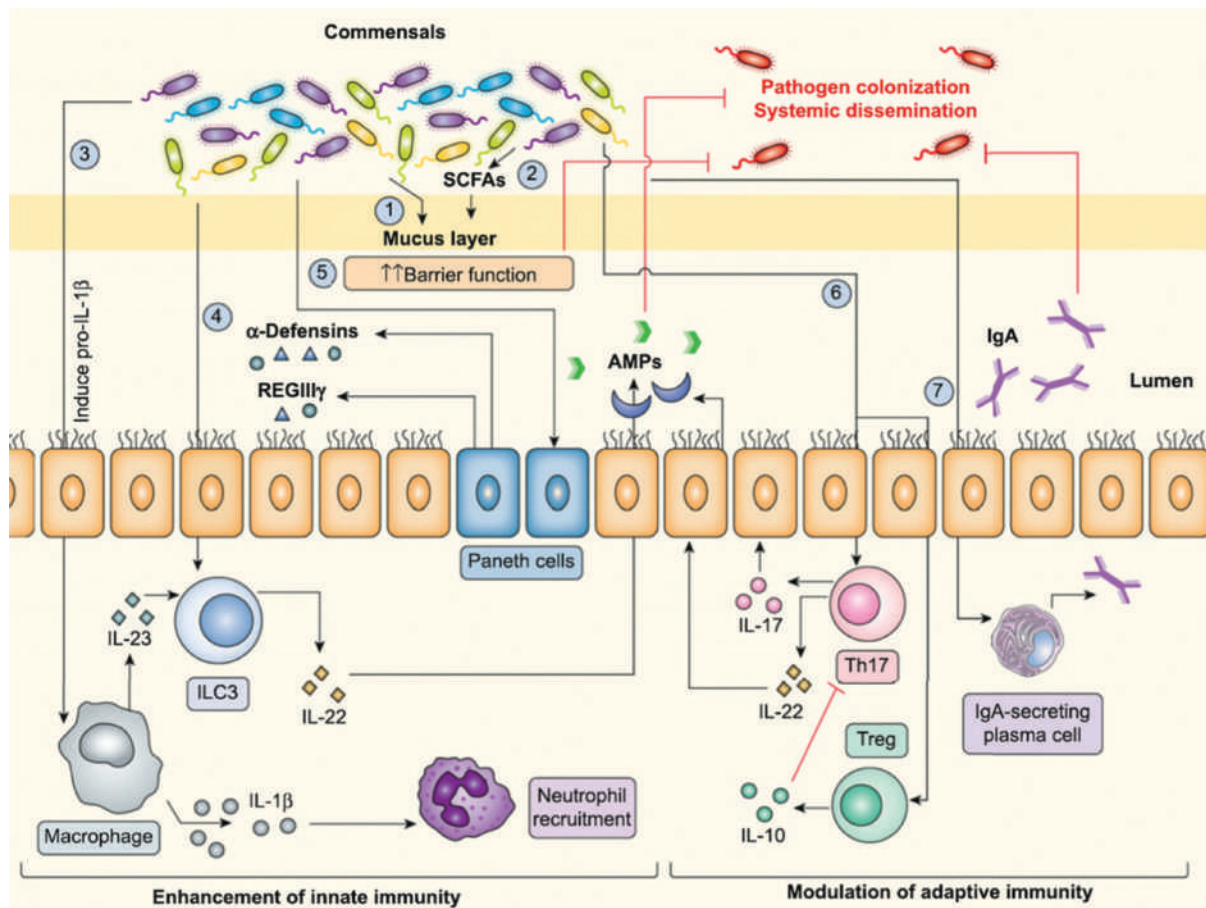


Fig. 5 Microbiota-stimulated host immunity provides colonization resistance. Commensal bacteria can indirectly control pathogen colonization by a variety of means. (1) Barrier function: the commensal microbiota upregulate host barrier function by contributing to the development of the mucus layer. (2) SCFA production: members of the microbiota, such as *Bifidobacterium* spp., can enhance epithelial barrier function by producing SCFAs, such as acetate. (3) IL-1 β -mediated neutrophil recruitment: commensals can promote protection against certain pathogens by stimulating IL-1 β processing and secretion, resulting in the recruitment of neutrophils to the site of the infection. (4) IL-22-dependent release of antimicrobials: certain commensals (such as *L. reuteri*) induce the secretion of IL-22 by ILCs, which, in turn, can protect against some pathogens via the induction of antimicrobial release by epithelial cells. (5) Direct stimulation of antimicrobial production by the host: secretion of antimicrobial proteins (AMPs), including α -defensins and REG3 γ , is a key component in controlling pathogen growth and is mediated, in part, by commensal-dependent mechanisms. (6) Induction of T cell differentiation: commensals can promote adaptive immunity by inducing the differentiation of T cells, such as by stimulating Th17 cell and Treg differentiation and activation. (7) sIgA: commensals can facilitate host-barrier function by inducing B cells and by regulating the secretion of IgA. Differentiating pathogens and commensals. Sassone-Corsi M and Raffatellu M (2015) No vacancy: How beneficial microbes cooperate with immunity to provide colonization resistance to pathogens. *Journal of Immunology* 194: 4081–4087.

containing low levels of commensal microbes (e.g. *Enterobacter cloacae*) can selectively generate IgA to protect the host from its own microbiota (Macpherson and Uhr, 2004). Indeed, SFB, but not commensal *E. coli*, were shown to contribute to the development of intestinal lymphoid tissue, including Peyer's patches, and to the development of intestinal sIgA boosting intestinal defence. The authors suggest that SFB have a remarkable capacity to induce and stimulate multiple types of intestinal lymphoid tissues that cooperate to generate potent IgA and Th17 cell responses displaying only limited target specificity (Lecuyer et al., 2014).

Commensals induce subtle changes in cytokine networks

The ability of *Lactobacillus reuteri* to metabolize tryptophan to indole-3-aldehyde, which activates the aryl hydrocarbon receptor (Ahr) on ILC to induce the barrier protective cytokine IL-22. Indeed, it is now clear that the Ahr is important in providing a docking site for microbiota derived ligands for activation of the immune system (Bock, 2020). Studies with probiotic strains of bacteria like *E. coli* Nissle have shown its ability to change the direction of immune responses from Th2 to Th1 protective immunity (Bickert et al., 2009). In the last decade thymic stromal lymphopoietin (TSLP) has been shown to be induced by intestinal bacteria and is important for controlling inappropriate Th17 responses through the maturation of FOXP3 T-regulatory cells (Mosconi et al., 2013).

Conclusions

It is clear that the immune system has evolved a variety of mechanisms to detect, mark, kill and remove bacteria. Our knowledge of the balance and individualized immune responses to different bacteria has advanced by a greater understanding of the host pathogen interactions within the gut (Fig. 6; Sun et al., 2015). Here the three lines of defence, including barriers, innate and adaptive immunity combine to provide subtle variations that allow discrimination between bacteria. Variations on these mechanisms are present at different body compartments (e.g. lung, skin), in different cellular compartments (intra or extracellular), and with dramatic differences in virulence potential. Understanding this network of events is important to understanding bacterial infection processes, but is essential for the design of effective strategies to protect the host from pathogenic bacteria (Blander et al., 2017; Kamada et al., 2013; Chu and Mazmanian, 2013; Brown et al., 2013; Goldszmid and Trinchieri, 2012).

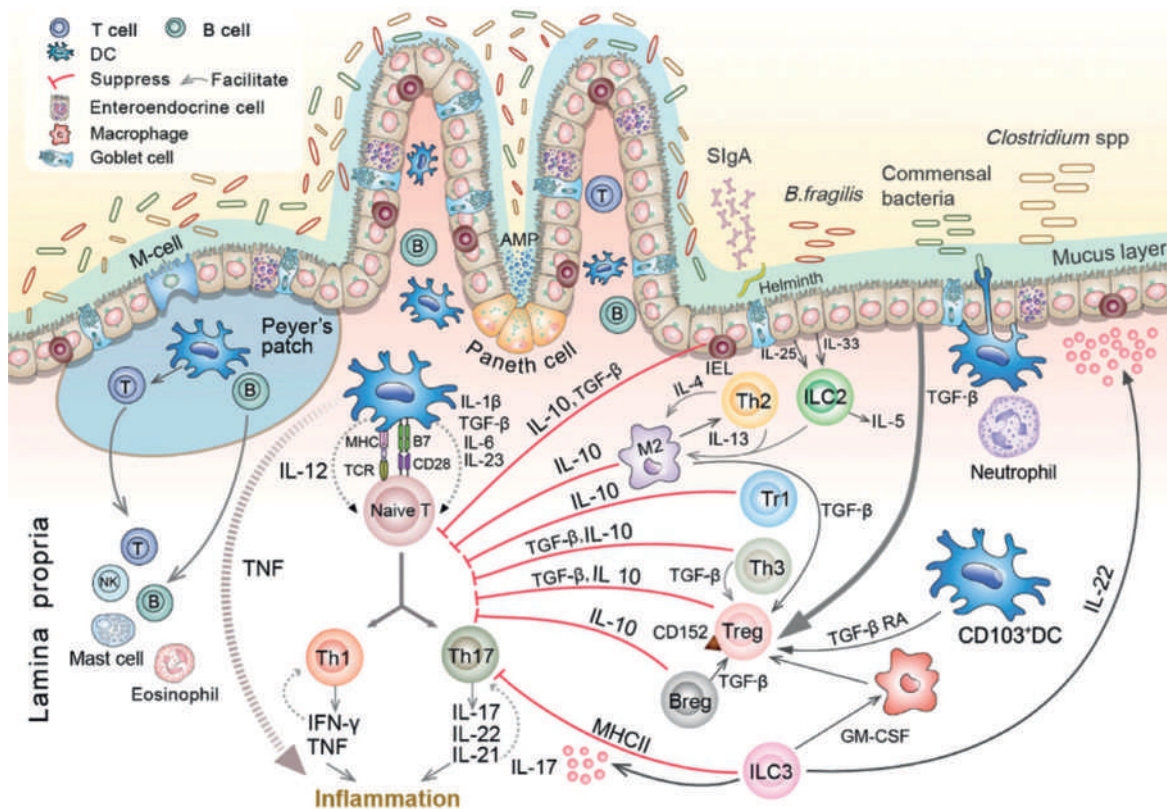


Fig. 6 Microbiota and immune regulatory cells in gut. Schematic shows the location of immune regulatory cells and other immune cells and their signaling cascades in the lamina propria of gut. The luminal microbiome lies adjacent to the intestinal epithelium. The microbiota plays an important role in the maintenance of mucosa homeostasis. Breach of the tolerance leads to the development of inflammatory bowel diseases (IBD), inflammation, and tumor. Intestinal epithelial barrier is the first line of defense to prevent the pathogens invasion, including the epithelial tight junction complexes, mucus layer secretion by goblet cell, secretory immunoglobulin A (sIgA), antimicrobial protein (AMP) secretion by plasma cells, and Paneth cells. With regard to the innate immune response in intestine, neutrophils could be earlier recruited to inflammatory sites to clear the pathogens. In addition, macrophages, dendritic cells (DCs), and innate lymphoid cells (ILCs) could sense antigens and then secrete cytokines or chemokines to regulate the inflammatory responses. Some innate immune regulatory cells such as M2 induced by interleukin (IL)-4 and IL-13 could directly inhibit effector T cells through IL-10 secretion and promote regulatory T cell (Treg) suppressive function by producing transforming growth factor- β (TGF- β). CD103⁺ DCs could regulate intestinal inflammation via facilitating Treg differentiation in a TGF- β - and retinoic acid (RA)-dependent manner. ILC3 serve as an antigen-presenting cell (APC) that uniquely restrain the CD4⁺ T-cell response in a major histocompatibility complex (MHC) II-dependent manner, and could secrete IL-22 that participates in the repair of impaired epithelium. In response to epithelium-derived IL-25 and IL-33, ILC2 could produce IL-5 and IL-13 to play the role of anti-helminth. The primarily regulatory cells are Tregs that suppress effector T-cell differentiation and proliferation by producing IL-10 and TGF- β or expressing CD152. The IL-10-producing type 1 regulatory T (Tr1) cells perform the anti-inflammatory function. Moreover, T helper type 3 (Th3) cells, regulatory B cells (Bregs), and intraepithelial lymphocytes (IELs) have the capacity of suppressing the excessive Th1 and Th17 responses through IL-10 and TGF- β production. Therefore, these immune regulatory cells collectively maintain the intestinal homeostasis by interacting with each other. Cellular and cytokine networks responsible for the immune response to bacteria. Sun M, He C, Cong Y, and Liu Z (2015) Regulatory immune cells in regulation of intestinal inflammatory response to microbiota. *Mucosal Immunology* 8: 969–978.

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Immune Response to Viruses

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Immune system

The immune system is a tightly regulated, complex system of tissues and cellular components (immune cells, lymph nodes and mucosal tissues), and humoral components (antibody, antimicrobial proteins, and complement proteins), that have evolved to work in unison to protect us from infectious diseases. The immune system can also be broken down into innate immunity and adaptive immunity. The innate immune system is a rapid non specific response that helps the body prevent infection from pathogens never encountered before, prior to an antibody response being developed. The adaptive immune system is a slower more pathogen specific response (B cells/T cells). In this chapter we will discuss the above components of the immune system in relation to viral infections. Specifically, how we recognise and respond to viral infections, and ways in which viruses have evolved to try to avoid the host immune system.

Innate immunity - First line of host defense

Before we progress to the key components of the immune system we will first look at the initial barriers that viruses must cross in order to initiate infection, and ways in which the body tries to counteract this. The main routes of pathogen entry into the body are via the mucosal surfaces and the skin.

The skin

The skin, the largest organ of the body, is a very strong physical barrier, that many pathogens find impenetrable. Alongside the physical barrier, the skin is also inhospitable to many pathogens, containing antimicrobial peptides that can kill pathogens (Handfield et al., 2018). It is also covered in commensal skin flora that promote wound healing and restrict the growth of other microbes (Robinson and Pfeiffer, 2014).

Despite this many viruses utilise this as a route of entry into the host, discussed in detail in Bomsel and Alfsen (2003) and Lei et al. (2020). This includes as examples, Human papilloma viruses (HPV) and Herpes Simplex viruses (HSV). Although these infect via the skin an abrasion of some sort is required for viral entry into the body and for infection to occur. Other viruses bypass the skin barrier by transmitting via vectors, that again penetrate the skin barrier. Some examples of common vectors include Mosquitos, Ticks, Fleas, Sandflies. With mosquitos being a common vector aiding transmission of many human viruses, such as Zika virus, Dengue fever virus (DENV), Yellow Fever virus (YFV) and Chikungunya virus (Onyango et al., 2020).

Mucosal surfaces

Other key entry points for viruses and other pathogens into the body include the mucosal surfaces (Bomsel and Alfsen, 2003). This includes the mouth, nose, eyes, respiratory tract, gastrointestinal tract, urinary tract and reproductive tract. With the respiratory and gastrointestinal tract being the main sites of viral entry. All these sites have an arsenal of antimicrobial agents, specialist cells types, mucosal antibody (mainly secretory IgA) and some have inhospitable conditions (low pH). Further they are resident to an array of immune cells that patrol these entry points to prevent pathogen invasion and infection. Some of which we will discuss briefly here in relation to viral infections and are reviewed in Sato and Kiyono (2012) and Holmgren and Czerkinsky (2005).

One clear example of how viruses are able to manipulate these mucosal sites to aid infection and transmission, is seen during infection of the respiratory epithelium by respiratory viruses. The respiratory tract is lined by specialised cell types, mainly the ciliated epithelial and goblet cells, both key for the proper functioning of the mucociliary escalator (Kilburn, 1968; Tam et al., 2011). Ciliated airway epithelial cells (AECs) line the vast majority of the respiratory epithelium and beat in a coordinated directional manner to remove mucus from the airways. This mucus, produced by goblet cells, lines the epithelium and traps pathogens and other inhaled particulates, which is then removed from the airways by ciliated AECs (Jeffery, 1983). Malfunctioning of these cells and the mucociliary escalator, like that seen in conditions such as cystic fibrosis and primary ciliary dyskinesia, results in chronic respiratory infections and lung damage (Wijers et al., 2017; Afzelius, 1976; Boucher, 2007).

The functioning of the mucociliary escalator can also be temporarily damaged due to infection (Pittet et al., 2010). Either through generalised damage to the respiratory epithelium as seen during Influenza virus infection (Plotkowski et al., 1986; Nugent and Pesanti, 1983; Short et al., 2014), or by targeted infection of the ciliated epithelial cells. The latter is a feature seen for many of the common respiratory viruses, such as Respiratory Syncytial Virus (RSV), Rhinovirus (RV) C and some of the Severe Acute Respiratory Syndrome associated Coronaviruses (SARS-CoV). RSV has been shown to specifically targets the ciliated AECs, causing ciliary loss (shedding) and ciliary dyskinesia (uncoordinated movement of the ciliated cells) (Zhang et al., 2002; Smith et al., 2014). The most recently discovered RV species, RVC, has also been shown to target the ciliated epithelium for viral entry and infection leads to shedding of the infected cells, reducing the number of ciliated cells present within the epithelium (Griggs et al., 2017). The receptor used for SARS-CoV infection, angiotensin-converting enzyme 2 (ACE2), has also been shown to be preferentially expressed on ciliated AECs, and targeted infection of these cells causes cell death and shedding into the airway (Li et al., 2003; Sims et al., 2005). Due to the fact SARS-CoV-2 also used ACE2 as its receptor, the viral cause of the 2019 global pandemic, this virus has also been shown to target ciliated cells for infection (Ahn et al., 2021; Zhu et al., 2020). Studies have similarly shown this leads to damage to the ciliated cells and cell shedding into the lower airways, and has been shown to perturb mucociliary clearance (Robinot et al., 2021). Some strains of Influenza A virus (IAV) also show a tropism for ciliated cells (Ibricevic et al., 2006).

Other routes of viral entry into the host and examples of viruses that utilise these routes are summarised in Table 1.

Table 1 Examples of viral pathogens that utilize different entry routes into the host.

Entry route	Viruses
Mouth, Nose, Eyes	RV, Ebola virus (EBOV), Cytomegalovirus (CMV), Epstein-Barr Virus (EBV)
Respiratory tract	IAV, SARS-CoV, SARS-CoV-2, RSV, RV
Gastrointestinal tract	Norovirus, Rotavirus
Urinary tract	Uncommon in immunocompetent hosts
Reproductive tract	Human immunodeficiency virus (HIV), Hepatitis C virus (HCV), EBOV, CMV
Skin	Abrasion – Human papilloma virus (HPV), Herpes Simplex virus (HSV), EBOV, HIV Vector – Zika virus, YFV, DENV

Innate immunity - viral recognition

Once these barriers are breached pathogens are detected by epithelial cells, patrolling immune cells, or antibody, which initiate an immune response to aid pathogen clearance.

Pattern recognition receptors (PRRs)

Key component of the innate immune system are pattern recognition receptors (PRRs). These are present on the cell surface of a number of different cell types (immune cells, epithelial cells, endothelial cells etc), and recognise components shared by many pathogens, which are not present in the host, distinguishing self from non self (Janeway, 1989, 2013; Takeuchi and Akira, 2010). These are known as Pathogen Associated Molecular Patterns (PAMPs). PAMPs include things such as lipopolysaccharide (LPS), flagellin, peptidoglycan, which are present on the surface of different bacterial pathogens. They also recognise the genetic material of some viruses (dsRNA/ssRNA), and other ubiquitous components shared by other microorganisms (fungi, parasites) (Takeuchi and Akira, 2010). The ability to recognise different pathogens depend on which PRRs cells have, and therefore dictate the ensuing immune response. Pathogens will often activate multiple PRRs allowing cross talk between receptors and a more robust immune response.

The location of PRRs also varies within the cell, some are present on the cell surface (plasma membrane) and recognise extracellular pathogens. These are mainly present on immune and epithelial cells. Other PRRs are present within the cells and recognise intracellular pathogens, such as viruses (endosome membrane/cytoplasm). Intracellular PRRs can also be membrane bound and present within the endosome membrane or free within the cytoplasm. These are often found in all cell types (Chan and Gack, 2016). PRR cellular localisation is summarised in Fig. 1.

There are four main groups of PRR. Toll Like receptors (TLRs) are the most widely studied, C-type lectin receptors (CLRs), Retinoic acid inducible gene-I (RIG-I) like receptors (RLRs) and Nucleotide-binding oligomerisation domain-like receptors (NLRs). Cytosolic DNA sensors are another group of recently discovered PRRs (Chan and Gack, 2016). We will discuss the above in more detail below in relation to their role in recognition of viral pathogens.

Toll like receptors (TLRs)

In humans there are 10 functional TLRs (Medzhitov, 2001; Takeda and Akira, 2005). These are found as heterodimers or homodimers in the plasma membrane or endosome membrane (Lester and Li, 2014). TLR1, 2, 4, 5, 6, 10 are found in the plasma membrane, and TLR3, 7, 8, 9 in the endosome membrane (recognise intracellular pathogens). These signalling molecules consist of an extracellular domain that recognises PAMPs and an intracellular domain that has cell signalling capabilities, known as the Toll Interleukin Receptor (TIR) domain. TLRs present within the endosome have the signalling domain present within the cell cytoplasm and PAMP recognition domain present within the endosome. TLR3, 7 and 8 are the main TLRs that detect the presence of viral genetic material. TLR3 recognises dsRNA, and TLR7/8 ssRNA, which can either be the composition of the viral genome or it can be an intermediate produced during viral replication (Alexopoulou et al., 2001; Heil et al., 2004; Lund et al., 2004).

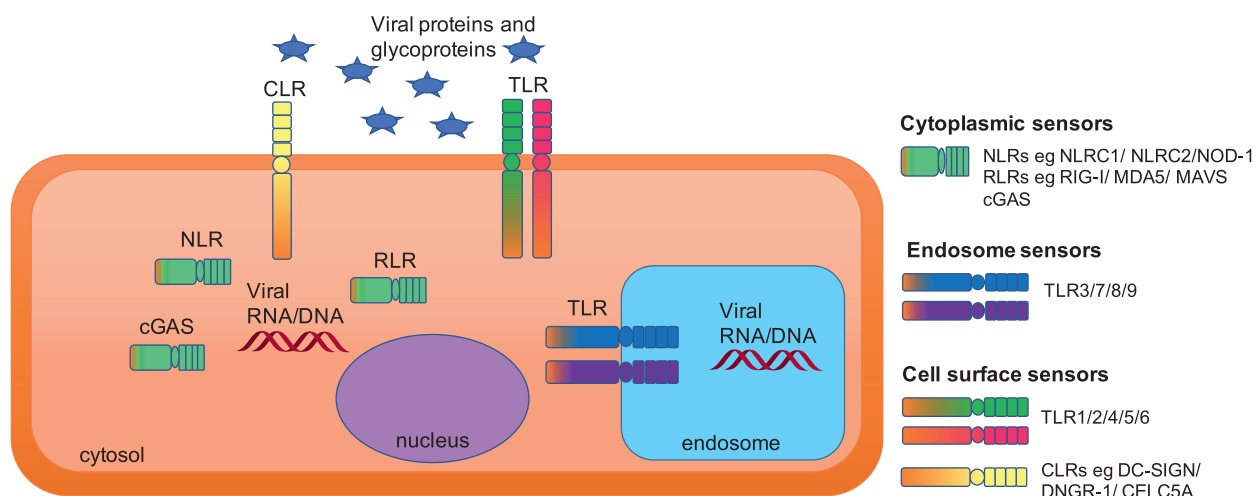


Fig. 1 Cellular localisation of Pattern recognition receptors (PRRs). Schematic shows the cellular localisation of PRRs. TLRs are located in the plasma membrane and endosome membrane. Those present in the plasma membrane detect extracellular pathogens and those within the endosome intracellular pathogens. The pathogen recognition domain is present outside of the cell (plasma membrane TLRs) or within the endosome, with the c-terminal signalling domain being present within the cytosol for both. CLR PRRs are also present within the plasma membrane and recognise extracellular pathogens. NLRs, RLRs and cGAS PRRs are all present within the cell cytosol and recognise intracellular pathogens.

As well as recognising viral genetic material, viral proteins have also been shown to activate a number of the cell surface exposed TLRs. Some examples include the cell surface exposed F protein of RSV, which can directly activate TLR4 (Funchal et al., 2015). This is also the case for the non structural protein (NSP) 1 of DENV, and surface glycoprotein of EBOV, which both also directly activate TLR4 (Modhiran et al., 2015; Okumura et al., 2010). Examples of TLR2 being directly activated by viral proteins include that of the measles hemagglutinin protein and the NSP4 of rotavirus (Bieback et al., 2002; Ge et al., 2013).

Once activated the TLRs undergo a conformational change which leads to recruitment of adaptor proteins to the signalling domain, this includes proteins such as Myeloid differentiation factor 88 (MYD88) (TRL7/8) or TIR-domain-containing adapter-inducing interferon- β (TRIF). This can also include Toll-interleukin-1 Receptor domain-containing adaptor protein (TIRAP) and TRIF-related adaptor molecules (TRAM)) (Takeuchi et al., 2000; Hayashi et al., 2001; Kawai et al., 1999). These activate a downstream signalling cascade which eventually leads to the translocation of transcription factors into the cell nucleus, which alters gene expression. This includes transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), Activator Protein 1s (AP1s) and Interferon Regulatory Factor (IRF) 3/IRF7, which are the end products of the NF- κ B, Mitogen activated protein (MAP) kinase and Interferon (IFN) signalling pathways respectively, resulting in expression of key genes of the innate immune system (discussed below). Signalling through TLRs is discussed in more detail in (Kawasaki and Kawai (2014); Takeda and Akira (2005))

C-type lectin receptors (CLRs)

The function of C-type lectin receptors in antiviral immunity and downstream signalling is nicely summarised in Bermejo-Jambrina et al. (2018) and Geijtenbeek and Gringhuis (2009). C-type lectin receptors are present within the plasma membrane of the host cell and recognise extracellular pathogens. They are predominantly on immune cells, such as dendritic cells (DCs) and macrophages. These PRRs recognise carbohydrate structures (recognised through their carbohydrate recognition domain), present on the pathogen surface glycoproteins such as mannose, glucan and fucose. Mannose being the one most predominantly recognised on viral pathogens. Some examples of these receptors which recognise viral pathogens includes DC-SIGN, which recognises mannose on Human immunodeficiency virus (HIV) and IAV (Gringhuis et al., 2007; Yu et al., 2017), DNCR-1 is able to recognise the vaccinia virus (Iborra et al., 2012) and CELC5A has been shown to recognise Japanese encephalitis virus (JEV) glycoproteins (Chen et al., 2012).

Downstream signalling after activation of the CLR is complex and the pathways activated varies depending on the CLR activated. Signalling through CLRs is summarised nicely in Geijtenbeek and Gringhuis (2009). Some CLR signalling pathways also cross talk with TLR signalling pathways further complicating the downstream signalling (Meyer-Wentrup et al., 2009). Activation of the IFN and NF- κ B signalling pathways, is similarly to TLRs, a key feature of activation of CLRs. However, as these receptors are predominantly on professional antigen presenting cells (APCs) the viral antigens are internalized and processed for presentation to T cells on major histocompatibility complex (MHC) molecules. Due to the antigen being from an extracellular source these are processed and expressed on MHC class II molecules and presented to CD4+ T helper cells (Engering et al., 2002; Gurer et al., 2008). DCs also possess a special ability to cross present exogenous antigen (extracellular) on MHC class I molecules (normally present intracellular antigens to T cells), allowing presentation of antigen to CD8+ cytotoxic T cells, which are key for the antiviral immune response (Schreibelt et al., 2012; Iborra et al., 2012; Zelenay et al., 2012).

Although activation of these PRRs is important in the induction of the antiviral immune response (Long et al., 2013; Chen et al., 2012). There are also multiple cases where viruses have adapted to avoid these receptors and can also use these receptors to aid infection and propagation, some examples include HIV, EBOV and Hepatitis C virus (HCV) (Hodges et al., 2007; Simmons et al., 2003; Ludwig et al., 2004).

Retinoic acid inducible gene-1 (RIG-1) like receptors (RLRs)

RIG-like-receptors are a trio of RNA helicases proteins with the RIG-I being the most recognisable member of the RLR family. Followed by melanoma differentiation antigen 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). All three have a key role in the cellular recognition of RNA viruses (Kell and Gale, 2015; Loo and Gale, 2011). During non-infectious periods, RLR proteins act as sentinels for viral PAMPs and are ubiquitously expressed at low levels in the cell. Both RIG-1 and MDA5 have a c-terminal caspase activation and recruitment domains (CARDs) which directly interacts with other downstream signalling molecules activating an immune response (Loo and Gale, 2011). Upon viral RNA recognition, these proteins change their structural conformation activating a downstream signalling cascade, eventually forming a complex with the mitochondrial antiviral signalling (MAVS) adaptor protein. Resulting in preferential expression of the type III IFNs and other interferon stimulated genes (ISGs) (Dixit et al., 2010; Chan and Gack, 2016).

These receptors have been shown to be key in recognising infection by a number of important viruses such as IAV, HCV, Hepatitis B virus (HBV), West Nile virus (WNV), picornaviruses etc. However, these viruses have evolved mechanisms to avoid detection via RLRs and some of these are summarised in Chan and Gack (2016).

Nucleotide-binding oligomerization domain-like receptors (NLRs)

Nucleotide-binding and oligomerisation domain NOD-like receptors (NLRs) are PRRs expressed in the cytosol. NLRs constantly assess the cytosolic environment for signs of infection, molecules that can affect the cellular equilibrium (such as toxins), or even imbalances in metabolic processes. There are 22 NLRs with nucleotide-binding oligomerization domain-containing protein (NOD) 1/NOD-2 and NOD-, LRR- and pyrin domain-containing 3 (NLRP3) being the most well characterised. These receptors detect

different stimulus including viral RNA and DNA. NLRs contain multiple domains, core NLR domains such as NLRC1 and NLRC2, have a pivotal role in the activation of the mitogen-activated protein kinase (MAPK) producing Type I IFNs (Anand et al., 2011). Whereas some receptors activate the inflammasome and caspase pathways. Activation of inflammasome is achieved through NLRP1, NLRP3, and NLRC4 (Malireddi et al., 2010; Masters et al., 2012; Thomas et al., 2009), resulting in the breakdown of pro-interleukin-1 β (pro-IL-1 β) and pro-IL-18. This caspase-1-modulated breakdown initiates the mechanisms for pyroptosis, which is cellular death due to pro-IL-1 β and pro-IL-18 (Malireddi et al., 2010).

RNA viruses can activate the NLRP3 inflammasome, as demonstrated in a study where murine macrophages exhibited caspase-1 activation due to influenza virus and rotavirus (Kanneganti et al., 2006). IL-1 β secretion was NLRP3-dependent in an *in vitro* model where encephalomyocarditis virus (ECMV) and vesicular stomatitis virus (VSV), challenged DCs and macrophages (Rajan et al., 2011). The NLRP3 inflammasome in THP-1 cells can be activated by measles virus, leading to the expression of IL-1 β (Komune et al., 2011) where in other instances the NLRP3 inflammasome can be activated by human RV and viroporin 2B protein (a pore-forming protein of HRV) (Triantafilou et al., 2013). This is achieved through the increase of Ca²⁺ concentration in the cytosol (Shrivastava et al., 2013).

Similarly to the above PRRs these viruses have evolved mechanisms to avoid detection via NLRs and some of these mechanisms are summarised in Chan and Gack (2016).

Cytosolic DNA sensors

Recognition of viral DNA is also important to identify infection by viruses with DNA genomes or RNA viruses that use DNA intermediates during viral replication (such as HIV). This is recognised by cytosolic DNA sensors such as cyclic GMP-AMP synthase (cGAS) which works in unison with the adaptor protein STING (Ishii et al., 2006; Ishikawa and Barber, 2008), and interferon- γ (IFN γ)-inducible protein 16 (IFI16) (Goubau et al., 2013). These are ubiquitously expressed in almost all cells. These sensors do not distinguish between host and pathogen derived DNA (White et al., 2014; Barber, 2015). However host DNA is localised in the cell nucleus so responses to self-DNA are avoided. Upon activation a downstream signalling cascade ensues resulting in activation of the IFN and NF- κ B signalling pathways, similar to other PRRs (Ishikawa et al., 2009). A detailed understanding the role of these PRRs in viral infection is reviewed in Abe et al. (2019) and Chan and Gack (2016).

Downstream signalling after PRR activation

Three main pathways are activated by PRRs, is summarised in Fig. 2. Focus in this chapter will be on the Interferon (IFN) pathway due to its critical importance in the immune response to, and clearance of viral infections.

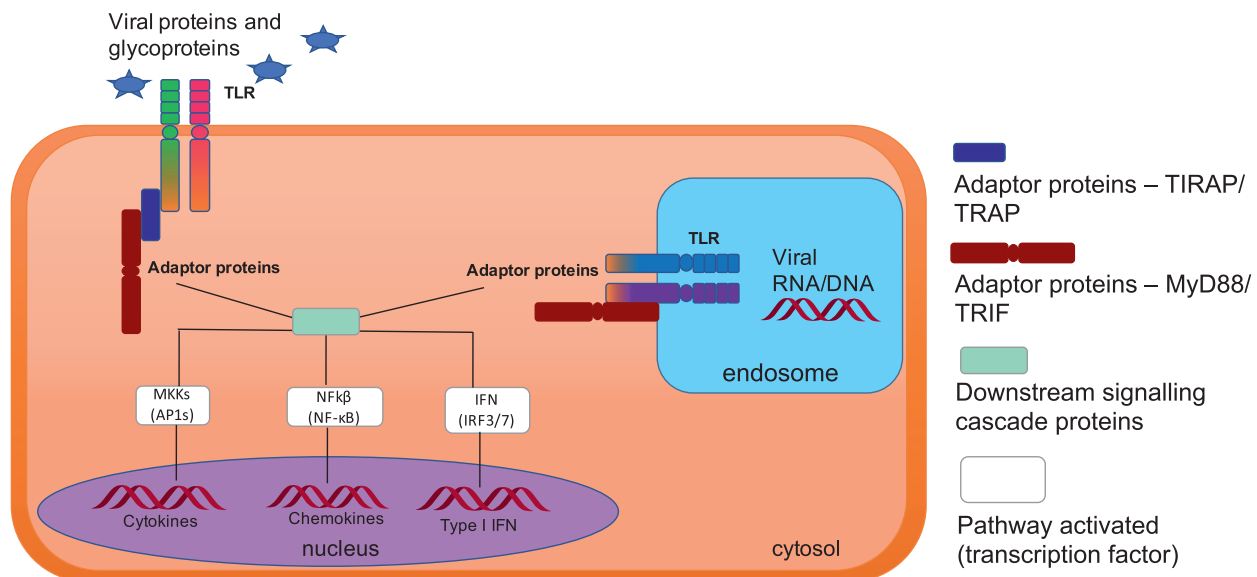


Fig. 2 Signalling through pattern recognition receptors (PRRs). Schematic shows signalling through PRRs, using TLRs as an example. Upon activation adaptor proteins bind to the c-terminal (TIR) signalling domain of the TLR. For TLRs this is TIRAP/TRIF/MyD88/TRIF. This activates a signalling cascade which activates further proteins in the cascade, eventually resulting in activation of transcription factors NF- κ B, AP1s and IRF3/7. These transcription factors are subsequently translocated into the cell nucleus where they bind to the promoter regions of target genes, turning on transcription of these genes. This includes cytokines, chemokines and Interferons. All PRRs function similarly however the adaptor proteins and signalling cascade proteins differ. The pathways activated may also differ with some PRRs preferentially activating certain pathways.

Interferon (IFN) pathway

Interferons (IFNs) are key members of the cytokine family that are vital components of the anti-viral innate immune response (Levy et al., 2011; Schulz and Mossman, 2016; Hoffmann et al., 2015). There are three types of IFN, type I, II and III. Type I IFNs, are made up of 17 cytokines, type II a single cytokine, and type III IFNs 4 cytokines. Type I (especially IFN- α and IFN- β) and Type III IFNs (IFN- λ) are the key families of cytokines that play essential roles in the anti-viral immune response. These families of cytokines are mainly produced in response to activation of PRRs that detect viral nucleic acids (such as TLR3, RIG-1, MDA5, TLR7). Activation and production of IFNs are able to restrict viral replication and spread through modulating cell survival, replication and protein translation, they also promote tissue repair, and induction of adaptive immunity. Eventually facilitating viral clearance.

Due to their wide ranging effects on the host their expression is tightly controlled. Partly due to the fact these small proteins can effect multiple host cells. Type I IFNs signal through IFNAR1 (interferon alpha and beta receptor subunit 1) and IFNAR2, which is present on most nucleated cells (Gibbert et al., 2013). Whereas Type III IFN signal through a IL-10R2 (most cells)/IFNLR1 (epithelial cells) heterodimeric receptor, whose effects are more restricted to epithelial cells (Sommereyns et al., 2008).

Once expressed and secreted IFNs bind to IFN receptors on the host cell surface, these activate a downstream signalling cascade, through phosphorylation of Janus tyrosine kinase (JAK) proteins, the JAK1 and tyrosine kinase 2 (TYK2) (Schneider et al., 2014). This promotes recruitment of other signalling proteins, signal transducer and activator of transcription proteins (STAT1 and STAT2), which again are activated through phosphorylation. This is referred to as the JAK/STAT pathway. These STAT proteins then combine with Interferon regulatory protein (IRF) 9 to form a heterotrimeric transcription factor complex called IFN-stimulated gene factor (ISGF) 3. This complex translocates into the host cell nucleus, where it binds to the interferon stimulated response elements (ISREs) promoter, inducing the expression of numerous interferon stimulated genes (ISGs) (Schneider et al., 2014).

These genes have far reaching effects on the host and target all aspects of the viral life cycle (reviewed in Schoggins, 2019; and Yang and Li, 2020). Some examples include the family of Tripartite Motif Family (TRIM) proteins which have been shown to inhibit translation of numerous viral pathogens, this includes TRIM22 which has been shown to prevent transcription factor binding to the HIV-1 promoter region (Hattmann et al., 2012). This protein is also upregulated during viral infection with HBV/HCV, Epstein Barr virus (EBV) and Rubella virus infection, suggesting it may also play a role here in viral infection control (Su et al., 2002; Wieland et al., 2004; Mo et al., 2007). TRIM32 ubiquitinates the IAV PB1 polymerase marking it for degradation (Fu et al., 2015). These are just a few examples and more are discussed in detail in reviews by Schoggins (2019) and Yang and Li (2020). However, because of their importance in the antiviral immune response, viruses have adapted ways to avoid this response, which is discussed later in this chapter.

NK- κ B pathway

NK- κ B pathways is one of the three main pathways activated in response to pathogen recognition. The family of five structurally related transcription factors (NF- κ B1, NF- κ B2, RelA, RelB and c-Rel) are involved in the activation/expression of cytokine and chemokine genes and genes involved in the inflammasome (Oeckinghaus and Ghosh, 2009). The activity of these transcription factors are regulated by being coupled to inhibitory proteins (Ik- β family) in the cell cytoplasm, and are therefore inactive. Activation of the NF- κ B pathway eventually results in the degradation of Ik- β and translocation of NF- κ B transcription factors into the nucleus, and ultimately induction of NF- κ B controlled genes. Signalling through the NF- κ B pathway and its role in the ensuing immune response is covered in detail in Liu et al. (2017).

Numerous PRRs activate this signalling pathway, which results in production of pro-inflammatory cytokines, chemokines and inflammatory mediators that have far reaching effects on different innate immune cells. For instance these cytokines impact T cell differentiation and activation (Oh and Ghosh, 2013) and recruit neutrophil to the site of infection (Liu et al., 2017).

Mitogen-activated protein kinase (MAPK) pathway

The final pathway activated by PRRs is the MAPK pathway. The MAPK pathway is comprised of highly conserved serine/threonine kinases that are activated in response to many stimuli, including viral components. Signalling through this pathway is propagated via phosphorylation of upstream MAP kinases. This pathway is responsible for regulating many cellular functions including cell differentiation, proliferation, and survival. The role of the MAPK pathway in viral recognition and removal is discussed in detail in DuShane and Maginnis (2019) and Kumar et al. (2018), but will not be discussed further here.

Innate immune cells in the antiviral response

Although the immune system is all interlinked and many cell types contribute to the antiviral immune response, we will specifically focus on a few key cells/mechanisms of viral removal. Other cells of the innate immune response, such as the professional phagocytes, macrophages and DCs, are also important in the antiviral immune response. Especially dendritic cells, which bridge the innate and adaptive arm of the immune response. However, these will not be discussed further, and their functions in regards to viral infections are reviewed in Gracia-Hernandez et al. (2020); Sang et al. (2015); Soto et al. (2020), and Marongiu et al. (2021).

Natural killer (NK) cells

Natural Killer (NK) cells have cytotoxic functions and can secrete cytokines when activated (Vivier et al., 2008). NK cells target host cells which are either infected, have genetic mutations, or have increased stress markers, removing cells that are no longer functional and restore homeostasis (Cichocki et al., 2014).

Once virally infected, host cells are a potential targets for NK cells, which recognise compromised cells by the activation of NK cell surface receptors. NK cells have two different types of cell surface receptor, activating and inhibitory receptors (Bartel et al., 2013; Biassoni and Malnati, 2018; Bryceson et al., 2006). Normal healthy host cells express MHC class I molecules on their surface, which are recognised by NK cell inhibitory receptors and promote self-tolerance (Paul and Lal, 2017; Abel et al., 2018). At the same time NK cell activating receptors bind to host cell surface motifs. Virus infected cells often (also tumor cells) lose surface MHC class I expression, preventing the NK cell inhibitory signal (renders them undetectable by CD8⁺ cytotoxic T cells), activating the NK cell.

NK cells once activated kill infected cells using similar mechanisms to cytotoxic CD8⁺ T cells, discussed in more detail below. Cell death occurs through release of cytotoxic granules (perforin/granzyme) onto infected cells, or via death receptor mediated apoptosis (Fas/FasL interaction). NK cells can also recognise and kill cells via antibody-dependent cellular cytotoxicity (ADCC) (Ochoa et al., 2017). Recognising antibody bound to the surface of infected cells. NK cells also produce a variety of cytokines, mainly IFN- γ and TNF. These potent inflammatory cytokines activate other immune cells at the site of infection (Nutt and Huntington, 2019).

Studies indicate the importance of these cells during viral infection as they actively remove viral infected cells. This has been shown for a number of viruses such as IAV, where NK numbers correlated to reduced disease severity (Björkström et al., 2021; Jegaskanda et al., 2013). However, these cells can also exacerbate chronic viral infections, through elimination and manipulation of viral infected immune cells, rendering the immune system less functional, allowing the virus to propagate further (Waggoner et al., 2011; Lang et al., 2012). Interestingly some viruses can also directly infect these cells rendering them non functional (van Erp et al., 2019).

Neutrophils and neutrophil extracellular traps (NETs)

Neutrophils are the most abundant circulating white blood cell in the body and are the first immune cells to move to the site of infection. Although predominantly associated with the anti-bacterial and anti-fungal immune response they have also been shown to play a role in the removal of viruses. The three main mechanism of pathogen removal are degranulation, phagocytosis and the most recently discovered mechanisms of Neutrophil Extracellular Trap (NET) formation. Their importance in viral infection is clear as in animal models where neutrophils numbers are depleted increased viral load and clinical symptoms are observed (Tate et al., 2011; Stacey et al., 2014; Haick et al., 2014).

Although phagocytosis is a key mechanisms for removal of bacterial pathogens by neutrophils, the role of this anti-microbial removal is controversial for viruses, as the presence of some viruses within neutrophils is not necessarily due to phagocytic uptake but could be due to viral infection of the neutrophil itself (Orenstein, 2000; Halfhide et al., 2011). In some instances active uptake of viruses has been confirmed and this has also been shown to be modulated through uptake of apoptotic viral infected cells, leading to virus removal (Grundy et al., 1998; Hashimoto et al., 2007).

NETs are the newest mechanism of neutrophil killing discovered in 2004 by Brinkmann and colleagues (Brinkmann et al., 2004). These are web like structures composed of the nuclear DNA of the neutrophil studded with the granular proteins, such as neutrophil elastase, myeloperoxidase and citrullinated histones. These have been shown to trap and kill pathogens including viruses, and also enable the neutrophil to increase its surface area to help prevent further spread of the pathogen. Again although mainly studied in the context of bacterial and fungal pathogens there are many examples where they have been shown to trap and neutralise viral pathogens, such as HIV, RSV and IAV (Saitoh et al., 2012; Cortjens et al., 2016; Hoeksema et al., 2015).

Viral induced NETs however have also been shown to cause damage to the host. This is the case for RSV Bronchiolitis in young infants. The virus itself seems to cause little damage to the host (Deng et al., 2018; Villenave et al., 2011; Herbert et al., 2020), but the level of neutrophils and neutrophil extracellular traps (NETs) found in the airways of young children during severe RSV bronchiolitis positively correlates to disease severity (Cortjens et al., 2016). This phenomenon has also been observed during IAV infection, RV infection in asthmatics and during SARS-CoV-2 infection (Cortjens et al., 2016; Zhu et al., 2018; Toussaint et al., 2017; Veras et al., 2020). These are just a few examples.

Adaptive immune cells in the antiviral response

B cells and antibody

The function of B cells, specifically those that produce antibodies (plasma B cells), have a well characterised role in the immune response to viruses (Radbruch et al., 2006). Production of antibody is the premise for all vaccines and these are effective against many viral pathogens (measles, rubella, smallpox, yellow fever, SARS-CoV-2, influenza etc) producing neutralising antibodies. In many cases these also produce long lived memory B cells, which recognise the same pathogen on future encounters, rapidly produce antibody (von Behring and Kitasato, (1890); Ahmed and Gray, 1996). Production of these two cell types in response to viral infection is summarised in Palm and Henry (2019) and Dörner and Radbruch (2007)). However, in some instances

production of antibody to viral infections are short lived such as that observed for SARS-CoV-2 and RSV, and this allows reinfection to occur (Antia et al., 2018; Lyski et al., 2021; Gaebler et al., 2021; Habibi et al., 2015; Siggins et al., 2021).

B cells also have antibody independent functions during viral infection. This includes primarily production of pro and anti-inflammatory (Bregs) cytokines which interact and modulate the immune response of other cells (Iwata et al., 2011; Shen and Fillatreau, 2015). They also present antigen to T cells on MHC class II molecules, which helps dictate the T cell response to viral antigens (Constant et al., 1995). Further information on these are summarised in Upasani et al. (2021) and Shen and Fillatreau (2015).

Cytotoxic T lymphocytes (CD8+)

Cytotoxic T cells (CTLs) are a key component of the adaptive immune system and are key for destruction of viral infected cells (Nutt and Huntington, 2019). These T cells recognise antigen, via their T cell receptor, presented in MHC class I molecules. MHC class I molecules are present on the surface of all nucleated cells within the body. Peptides generated within these cells, that are loaded onto MHC class I molecules, are from endogenous (intracellular) peptides. This includes peptides generated from host cell proteins, but also from intracellular pathogens such as viruses. There are multiple MHC class I molecules on a single cell, which can have give a readout of up to 10,000 proteins on the cell surface, this array is interpreted by CTLs and NK cells. Processing of peptides for the MHC class I pathway is nicely reviewed in Neeffes et al. (2011).

There are two main host cell killing mechanisms employed by CTLs to remove infected or tumour cells. Loss of function of these killing mechanisms by CTLs through genetic deficiencies such as, familial hemophagocytic lymphohistiocytosis (perforin dysfunction), shows increased susceptibility of these individuals to severe uncontrolled viral infections (Katano and Cohen, 2005).

One mechanism includes production and release of cytotoxic granules (similar to those produced by NK cells), which are mainly composed of perforin and granzyme (Voskoboinik et al., 2015). These are released from the CTLs in a directional manner towards the target cell to reduce bystander damage. This occurs as the T cell forms an immunological synapse between itself and the target cell, which is a highly organised structure made through the interaction with receptors and ligands on target cell and T cell surfaces. Perforin once released forms a pore in the host cell membrane, damaging it, but this also allows entry of the granzyme protein into the host cell. This protease once within the cell degrades cellular components such as protein or DNA, shutting down the production of viral proteins, and ultimately killing the infected host cell.

The second killing mechanism is through death receptor mediated apoptosis (Fas/FasL interaction) (Keckler, 2007). FasL expression is restricted to CTLs and NK cells, whereas Fas is expressed on the surface of many different cell types. The interaction of Fas, which has a death domain, with FasL on CTLs causes Fas to trimerise on the target cell surface, activating a downstream signalling cascade which ultimately leads to the activation of the apoptosis initiating caspase cascade, including caspase 8 and 10, which eventually results in the target cell undergoing apoptosis. This process is discussed in more detail in Yamada et al. (2017).

Finally CTL produce a number of pro-inflammatory cytokines such as TNF- α , IFN- γ and IL-2 which interact with other immune cells, promoting a T helper cell response and further CTL differentiation (Nutt and Huntington, 2019; Cox et al., 2013). One mechanism by which viruses are able to avoid these is through downregulating the expression of MHC class I molecules on the infected cells surface.

Cytotoxic T lymphocytes (CD4+)

CD4⁺ T cells are most commonly associated with T helper (Th) cell subsets, and recognise antigen presented in MHC class II molecules (on immune cells) with their TCR (Sant and McMichael, 2012). Naïve CD4⁺ T cells, when stimulated, can differentiate into a number of different effector T cell populations. The type of T cell produced depends on the specific cytokines within the microenvironment, which ultimately coordinates the type of immune response. The cytokines IL-12 and IL-4 respectively, induce differentiation into Th1 and Th2 subsets, and these were the first to be described (Mosmann et al., 1986). Further and more recent studies elucidated other Th subsets, such as Th17 cells, regulatory T cells (Treg), Th22, Th9 and follicular helper T cells (Tfh) (Raphael et al., 2015). These cells functions mainly through production of cytokines which interact with other cells of the immune systems and dictate the ensuing immune response.

More recently an unexpected CD4⁺ T cell subset was recognised, which have similar cytotoxic function as CD8⁺ CTLs. Their function and role in antiviral immunity is nicely summarised in Muraro et al. (2017). CD4⁺ CTLs, similar to CD8⁺ T cells, can release cytotoxic granules containing granzyme B and perforin (Fleischer, 1984), which when in direct contact with the target cell can kill them (Brown et al., 2016). They also kill target cells using the Fas/FasL interaction, as noted above. These mostly rare cells increase significantly in their numbers during transient and chronic viral infection, and have been shown to have cytolytic activity against viral infected cells (Nemes et al., 2010; Brown et al., 2016). This has been shown during Cytomegalovirus (CMV), Epstein-Barr virus (EBV), HCV, HBV and HIV infections (Facchinetti et al., 2005; Aslan et al., 2006; Appay et al., 2002; Rosenberg et al., 1997; Musey et al., 1997; van Leeuwen et al., 2004). Such is the importance of CD4 CTLs, they can replace CD8⁺ CTLs during chronic infections, as CD8⁺ CTLs lose their functionality. Further studies are being undertaken on this cell population to elucidate further their role in antiviral immunity.

Natural killer T cells (NKT)

Another cell type of potential importance during viral infections is the Natural Killer T cell (Gumperz et al., 2002; Gapin et al., 2001). These are innate like cells and can respond rapidly to antigen that has not been encountered previously. These cells contain an invariant TCR which recognise lipid antigens presented in a MHC class I like molecule (CD1d). They also have a number of cell surface molecules in common with NK cells, hence the name. Once activated these cells can produce a wide range of cytokines and chemokines which influence other cells of the immune system (similar to T helper cells) (Coquet et al., 2008; Paget et al., 2012), such as DC maturation (Fujii et al., 2003), B cell antibody production (King et al., 2011) and T cell polarisation (Godfrey and Kronenberg, 2004). These cells can also directly lyse infected cells using cytolytic proteins (granzyme/perforin), similarly to CTLs, however this is a CD1d-dependent mechanism (Kok et al., 2012).

Some evidence suggests these cells help protect against viral infection, but more research is still needed in this area. During influenza (H1N1) viral infection NKT cell numbers are reduced in patients with severe disease (Chen et al., 2010). However, when present these cells have been shown to selectively lyse influenza infected cells and reduce lung pathology (Kok et al., 2012; Paget et al., 2012). During chronic HBV infection, the frequency of circulating CD4⁺ NKT cells is lower in asymptomatic carriers (Jiang et al., 2011), but numbers increase after treatment (Tripathy et al., 2011). NKT cell numbers are also reduced during HIV-1 infection, due to viral infection of the CD4⁺ NKT cell subset, which occurs preferentially to conventional CD4⁺ T cells, due to the expression of multiple co-receptors (CD4/CCR5, CXCR4, and CXCR6) involved in viral fusion and cell entry (Motsinger et al., 2002; Fernandez et al., 2014; van der Vliet et al., 2002; Sandberg et al., 2002; Fleuridor et al., 2003). NKT cell numbers recover following effective antiretroviral therapy (van der Vliet et al., 2006). More information on NKT cells and their role during viral infection is summarised in Slauenwhite and Johnston (2015) and Tupin et al. (2007).

Viral avoidance of the immune response

Despite the arsenal of antiviral mechanisms in place to prevent viral infection and recognise and destroy viral infected cells, viruses have evolved ways to avoid detection by the immune system in a number of different ways. Some of these will be discussed in more detail below.

Interference of viral antigen presentation on MHC class I molecules

As discussed above CTLs can recognise and kill viral infected cells through recognition of viral peptides in MHC class I molecules (on all nucleated cells). A commonly used viral strategy to evade CTL killing mechanisms is to interfere with the presentation of antigen in MHC class I molecules. This can occur through multiple mechanisms, but mainly via interfering with antigen processing in the infected cell, or by reducing the number of MHC molecules on the target cell surface. Examples of this are discussed in the following reviews Schuren et al. (2016),; Loch and Tampé (2005), ; Horst et al. (2011).

Ways in which viruses have been able to interfere with antigen processing within the target cell, which ultimately leads to reduced presentation of viral peptides to CTLs, includes: producing proteins that are resistant to antigen digestion by the proteasome. Proteins must first be digested within the target cell by proteases to allow short peptides to be loaded onto MHC class I molecules, preventing this degradation prevents these viral peptides being presented to CTLs. This mechanism is used by the EBNA-1 (Epstein Barr Nuclear Antigen 1) protein of EBV (Levitskaya et al., 1995). Some viruses also interfere with the Transporter associated with Antigen Processing (TAP) protein, which transports peptide antigens into the endoplasmic reticulum for loading into MHC class I molecules. This is the case for Herpes Simplex virus (HSV) where ICP47 (infected cell protein 47) interferes with the binding of the peptides to the TAP protein (Ackerman et al., 2006). This is also a mechanism used by CMV through protein US6 (Unique short 6 glycoprotein) (Lehner et al., 1997).

Other viruses reduce viral antigen presentation through interfering with MHC I presentation on the cell surface. This can target multiple steps in the production and trafficking of MHC in the cell (van de Weijer et al., 2015). Adenovirus is able to do this using its E3 protein, which forms a complex with MHC class I molecules to prevent antigens from being processed (Fu et al., 2011). CMV achieves this through multiple mechanisms, proteins US2 and US11 promote the rapid degradation of newly synthesised MHC class I complexes (Gabor et al., 2020). CMV gpm152 protein causes retention of the MHC class I molecules in the Golgi compartment through which MHC class I loaded with peptide are trafficked. Varicella zoster virus open reading frame 66 protein also performs this immune evasion tactic (Purohit et al., 2021). Further some viral protein of the Kaposi's Sarcoma virus (Kaposi's Sarcoma Virus ubiquitin ligase) promote rapid endocytosis of surface expressed MHC Class I and their subsequent degradation (Coscoy and Ganem, 2000).

Antigenic variation (antibody escape)

Antigenic variation is a key mechanism by which many different viral pathogens are able to avoid the adaptive arm of the immune system (antibody recognition). This is most notably utilised by the respiratory RNA genome viruses, such as Influenza viruses, coronaviruses and RVs (Duffy, 2018). One key feature that allows this to occur more readily in RNA genome viruses is the fact the RNA polymerase enzyme required for replication of the viral genome has no proof reading functionality (not the case for

coronaviruses) (Smith and Denison, 2012). Meaning during genome replication mutations are constantly being introduced into the genome. If these are beneficial genetic changes they will be selected for and that strain will thrive. These mutations can provide many benefits, such as increased ability to bind to the host receptor, increased replication capabilities, increased transmission capabilities, but also an ability to evade antibody binding if the mutation is present within a key cell surface epitope (Harvey et al., 2021).

Key examples of this have frequently been seen during the SARS-CoV-2 pandemic (Harvey et al., 2021). Many novel strains have emerged, which have been shown to have genome changes that give that strain a selective advantage. Changes specifically in the viral cell surface spike protein, which binds to the host receptor ACE2, are common (Forni et al., 2020). The B.1.1.529 variant (given the name Omicron), is a highly mutated variant with a significant number of gene alterations, including 26–32 mutations in the spike protein, some of which are associated with immune escape and higher transmissibility (Kannan et al., 2021; Zhang et al., 2021). This is also the protein target for all vaccines to date.

RVs also use this mechanism to avoid immune system detection. These are some of the most genetically diverse group of viruses (Stobart et al., 2017). They are categorised into three distinct serotypes, RVA, RVB and RVC. Within these serotypes there are over 150 different types, which are antigenically distinct from one another (Lau et al., 2007). It has been shown that antibodies produced against RV infection are robust and protective, however, antibodies produced against one type are not cross protective against another, allowing different strains to circulate annually (Lee et al., 2016). This has hindered vaccine design.

Influenza viruses also use this mechanism to avoid the immune system. This is known as antigenic drift, where genetic changes accumulate in the regions targeted by antibodies (Shao et al., 2017). For influenza viruses this is normally within the genes that encode the cell surface proteins haemagglutinin and neuraminidase, which bind to the host cell receptor (sialic acid) and cleave sialic acid to release the virus, respectively (Byrd-Leotis et al., 2017). Antigenic drift where we see minor genetic changes results in influenza strains that circulate annually causing epidemics. These strains can reinfect the host with antibodies being less effective, but in general a level of protection still exists, limiting the severity of infection (Shao et al., 2017; Wang et al., 2017).

Another phenomenon occurs in influenza viruses known as antigenic shift (Shao et al., 2017). This occurs in IAV only, which can infect humans and other animals, most notably pigs and poultry. This process which leads to the genetic rearrangement of two viral strains, is facilitated through the fact influenza viruses have a segmented RNA genome (Krammer et al., 2018). This allows easier reassortment of the genes during infection (two distinct viruses infect the same cells), which produces novel strains from reassortment of the zoonotic strains that can jump species barrier into humans, or from reassortment of a human and zoonotic strain (Sun et al., 2020). These strains have never before been encountered by humans, therefore there is no immunity (antibody) to prevent infection and disease can often be very severe. This phenomenon generates pandemic influenza strains, and since 1918 (Spanish flu) has generated 4 pandemics, with varying severity (Saunders-Hastings and Krawski, 2016).

Although not technically avoidance of the immune system, RSV for reasons unknown does not produce long lasting immunity. This means that antibodies produced against one strain one year are no longer present the following year, meaning that strain can reinfect the same person. This allows this virus to cause seasonal epidemics. This also seems to be the case in relation to antibodies produced against SARS-CoV-2, with evidence showing antibodies only last for roughly 6 months (Antia et al., 2018; Lyski et al., 2021; Gaebler et al., 2021; Habibi et al., 2015; Siggins et al., 2021).

Other mechanisms used by respiratory RNA viruses to avoid the immune system are nicely summarised in Kikkert (2020).

Modulation of PRR signalling

To avoid activation of the key signalling pathways many viruses have adapted to subvert the PRR signalling pathways, this is nicely reviewed in Bowie and Unterholzner (2008). This occurs either through preventing their activation or blocking the downstream signalling cascade. Vaccinia virus is able to subvert signalling via its protein A46R, which contains a TIR domain (Stack et al., 2005). This is able to sequester TIR domain binding adaptor proteins, preventing activation of the downstream signalling pathways (Stack et al., 2005). TRIF, a TLR signalling adaptor protein is cleaved by the NS3–4A viral protease of HCV. Cleavage of TRIF results in its inactivation rendering it unable to activate the downstream signalling pathways during HCV infection (Li et al., 2005). Viruses such as EBOV and IAV can also sequester their genetic material from the environment to prevent activation of the TLRs (Cárdenas et al., 2006; Hatada and Fukuda, 1992).

Avoidance of the RLR PRR is also a common adaptation of viruses. They modify the structure of their genetic material to avoid detection by these important PRRs. This can involve adding a cap structure to viral RNA making it mimic host mRNA. The ability to do this can be using viral encode capping enzymes or can involve stealing host encoded cap proteins, known as cap snatching (e.g. IAV) (Plotch et al., 1981). Alternatively viruses can protect their genetic material via adding a protein to protect it (e.g. VPg protein of picornaviruses) (Flanagan et al., 1977). Genetic material can also be sequestered to prevent detection by PRRs. Many viruses encode RNA binding proteins, that shield the dsRNA species from recognition by PRRs. Examples include Vaccinia virus E3L protein, EBOV viral protein (VP) 35 (Haasnoot et al., 2007) and HIV Tat protein (Weeks et al., 1990). Other viruses produce proteins that directly interact with signalling molecules involved in the RLR pathways, such as the V proteins of many paramyxoviruses (Andrejeva et al., 2004) and NS1 protein of IAV (Mibayashi et al., 2007).

Viral avoidance of cytoplasmic DNA sensors using similar strategies is summarised in review Abe et al. (2019).

Modulation of the IFN response

IFN, especially type I and type III IFN are key players in the antiviral immune response, as discussed above. Therefore many viruses have evolved ways in which to avoid this key immune response. Some key examples of this will be discussed below.

EBOV has multiple mechanisms that allow it to avoid the host IFN response. Two key viral proteins involved in this include the VP24 and VP35, which inhibits the activation of the IFN-stimulated genes through preventing STAT1 translocation into the host nucleus, and inhibits IFN production through preventing phosphorylation of IRF-3, respectively (Messaoudi et al., 2015; Fanunza et al., 2019). *In vitro* and *in vivo* (murine infection model) studies, where mutations have been introduced into these proteins, show reduced viral replication, increased host IFN response and severe attenuation compared to infection with strains containing the wild type proteins (Hartman et al., 2008a,b, 2006; Lubaki et al., 2016).

Deficiencies in the IFN response to SARS-CoV-2 infection have also been identified as a hallmark of disease, aiding viral replication and disease pathology. It is suggested to use multiple mechanisms to prevent the IFN response, including: inhibiting IFN induction by evading host PRR sensing or disrupting IFN signalling cascades, interfering with host protein production which included IFNs, and suppressing IFN action (reviewed in (Min et al., 2021)). Some examples of how this is achieved is via methylating the 5'-end of viral mRNA by NSP16/NSP10, which then mimics host mRNA (Viswanathan et al., 2020). This makes the viral genome undetectable by host PRRs. Multiple other viral proteins have the ability to interfere with the IFN signalling cascade, including SARS-CoV-2 PLpro (papain like protease) which can directly cleave IRF3, NSP6 inhibits IRF3 activation, NSP1 can suppress STAT1 transcription factor phosphorylation and its nuclear translocation, all which suppress IFN production (Xia et al., 2020; Moustaqil et al., 2020).

Some viruses also inhibit the initial binding of IFNs to their cognate receptors (IFNAR) on host cells. This includes IAV haemagglutinin protein, which tags the IFNAR1 protein for degradation (Xia et al., 2015). The NS5 protein of WNV can inhibit IFNAR1 intracellular trafficking and glycosylation and therefore surface expression of this receptor (Lubick et al., 2015).

Hiding within immune cells

Some viruses are also able to modulate the immune system through the infection of, destruction of and modulation of immune cells function. The most notable example of this is HIV, which infects CD4+ T cells, NKT cells, macrophages and DCs (Kruize and Kootstra, 2019; Harman et al., 2011). This phenomenon may be due to the virus having a broad cell tropism, as the host receptor is expressed on many different cell types, or this may be a more immune cell targeted infection. In some instances it can also be hard to prove whether the virus is able to actively infect and replicate within immune cells, or whether this is an artefact of viral phagocytosis by antigen presenting cells.

EBOV has a broad cell tropism and can infect many different cell types, this however includes some antigen presenting cells such as macrophages and dendritic cells, for which EBOV seems to have a preference for. This is also the case for other filoviruses and is reviewed in Olejnik et al. (2011). Evidence suggests that active replication of EBOV occurs within these cells (Geisbert et al., 2003; Ryabchikova et al., 1999; Johnson et al., 1995). Both of which play a key role in the ensuing immune response, especially dendritic cells, which bridge the adaptive and innate immune response. Although work is still ongoing as to the effect of infection of immune cells in EBOV infection, current studies suggest this induces a pro-inflammatory state (Bray and Geisbert, 2005). This over exuberant immune response and apoptosis of lymphocytes is associated with poor disease outcomes (Baize et al., 1999).

RSV has been found inside neutrophils (Halfhide et al., 2011). These are important cells in RSV disease as their number in the lungs, as mentioned above, directly correlate with disease severity (McNamara et al., 2003). The presence of RSV within neutrophils is controversial and it is still unclear if this is due to infection of these immune cells or if this is due to phagocytosis of the virus (Halfhide et al., 2011). Whether this virus can infect and manipulate these cells for their own benefit is yet to be elucidated.

Viral latency

Another way viruses can avoid the immune system is to reside within the host in a latent form. This is a common feature of herpesviruses where after initial infection they become transcriptionally inactive. For herpes simplex virus the viral genetic material is retained within infected cells as circular episomes (not integrated into host DNA), however some herpesviruses can integrate their DNA into the host chromosome, such as EBV. Establishment of latency allows these viruses to infect their host for life. Viral latency is discussed further in Sorel and Dewals (2018); Brar et al. (2020) and Bennett et al. (2005), in regards to how these viruses avoid and manipulate the host immune response.

This chapter summarizes how viruses are detected during infection, the key cells involved in the antiviral immune response and ways in which viruses have evolved to avoid the host immune system. More in depth discussion on each topic can be found in the cited reviews.

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Immunity to Fungal Infections

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Glossary

CR3 Complement receptor 3. Also known as Mac-1 (macrophage-1 antigen) or CD11b/CD18.

LC3 Microtubule-associated protein 1A/1B-light chain 3mAb—monoclonal antibody.

TRIF TIR-domain-containing adapter-inducing interferon β .

Abbreviations

APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
BCL10	B-cell lymphoma/leukemia 10 protein
BTk	Bruton's tyrosine kinase
CARD9	Caspase recruitment domain-containing protein 9
Fas/FasL	Fas/Fas ligand
ITAM	Immuno-receptor tyrosine activation motif
KIR	Killer Ig-like receptor
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
PAD4	Protein arginine deiminase 4
PAR-2	Protease-activated receptor 2
PKC	Protein kinase C
PLC	Phospholipase C
SNP	Small nucleotide polymorphism
STAT1	Signal transducer and activator of transcription 1
STAT6	Signal transducer and activator of transcription 6
Syk	Spleen tyrosine kinase
α-GalCer	alpha-Galactosylceramide

Introduction

Human fungal pathogens are often ubiquitous in the environment or are members of the human commensal microbiota. Despite this high exposure rate, fungal infections are generally opportunistic and only cause disease in immunocompromised hosts. Fungal infections represent a wide range of human diseases from common superficial mucosal infections to life-threatening invasive infections (Brown et al., 2012). Mucosal/skin fungal infections are the most common human mycoses and include vulvovaginal candidiasis, oropharyngeal candidiasis and dermatophytosis (Fig. 1) (Brown et al., 2012; White et al., 2014). These fungal infections often associate with risk factors (such as antibiotic pre-treatment and poor hygiene) but can also affect apparently immunocompetent individuals. While they are not life-threatening, they are a significant healthcare burden and can severely affect quality of life and mental health, especially for those with recurrent infections. In contrast, invasive fungal infections mainly affect people who are immunocompromised as a result of iatrogenic factors, inherited immunological defects or acquired immunodeficiency (Table 1) (Brown et al., 2012).

The incidence of invasive fungal infections has been rising over the last few decades due to a growing at-risk population of immunosuppressed individuals. This is partly due to significant increases in the use of antibiotics, steroids and immune-modulating drugs in modern medicine (Clark and Drummond, 2019). Furthermore, there are now several documented congenital risk factors for severe fungal infections, including primary immunodeficiency disorders such as chronic granulomatous disease (CGD) and CARD9 deficiency. Global spread of viral infections which predispose to fungal infections (e.g. HIV) and the recent emergence of SARS-Cov-2 (COVID19) have also contributed to increased numbers of fungal infections and/or a worse

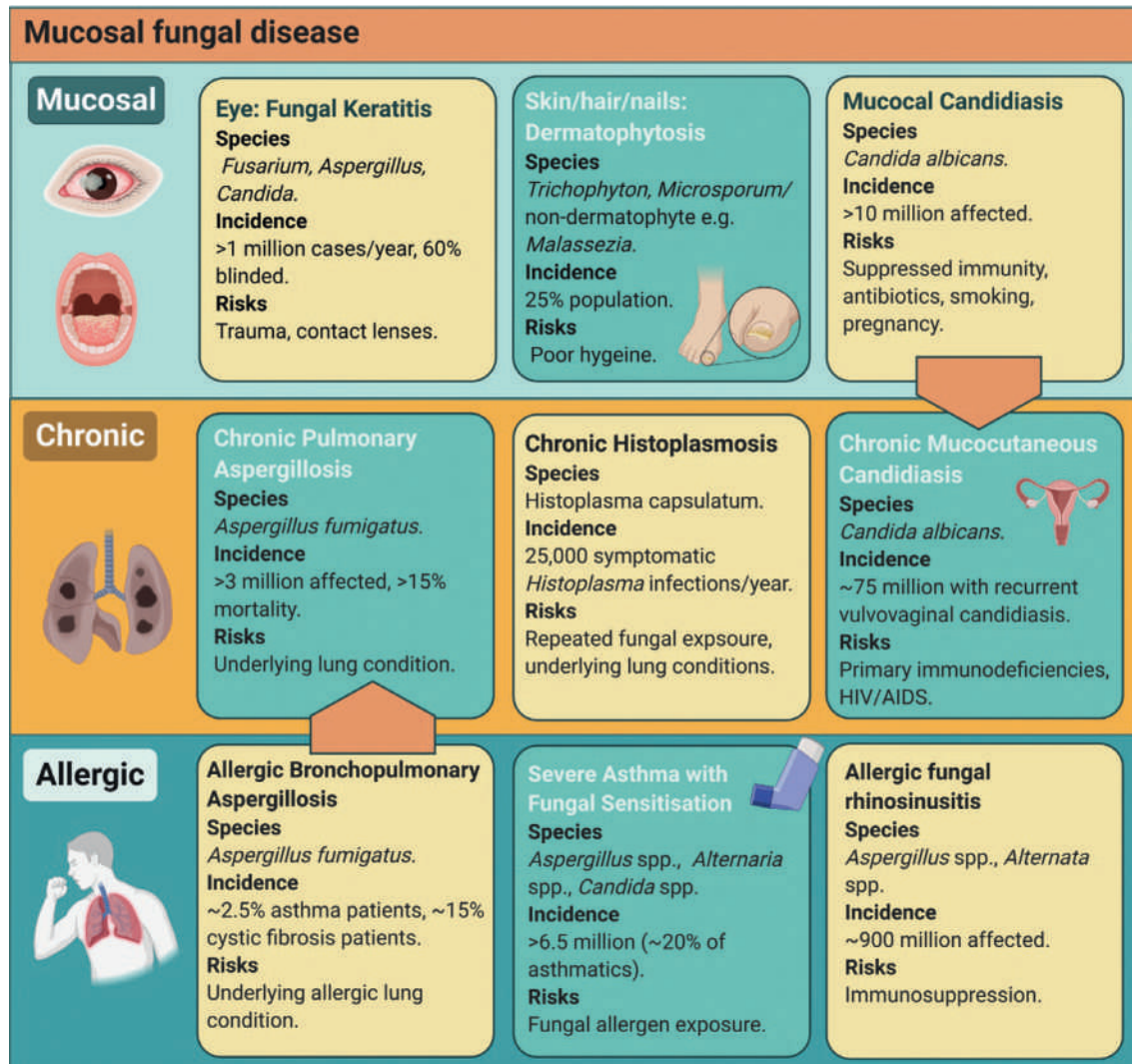


Fig. 1 Mucosal fungal diseases. Non-disseminated mucosal fungal infections are mostly non-life threatening, albeit they have a significant healthcare burden as they are often recurring or chronic in nature. Many are associated with a variety of risk factors and caused by a wide range of fungal species. Created with BioRender.com.

Table 1 Incidence and risk factors predisposing to invasive fungal infections in humans.

Fungal disease	Infectious agent	Disease burden	Fatality rate	Number of deaths	Tissues affected	Risk factors
Cryptococcosis	<i>Cryptococcus neoformans</i> , <i>Cryptococcus gattii</i>	~1.5 million	15–50%	~600,000	CNS, lung, kidney	Particularly prevalent in HIV/AIDS
Invasive aspergillosis	<i>Aspergillus</i> spp., mostly <i>A. fumigatus</i>	>1 million	30–70%	~500,000	Lung, CNS, kidney, heart, skin, eye, bone	Cancer, organ transplant, COPD
Invasive candidiasis	<i>Candida</i> spp.	>750,000	40%	~350,000	Lung, CNS, kidney, heart, skin, eye, bone, abdominal, liver, spleen	Cancer, organ transplant, surgery, hospitalization
Disseminated histoplasmosis	<i>Histoplasma capsulatum</i>	~100,000	15–30%	~80,000	Any, mostly liver, spleen or gastrointestinal	Particularly prevalent in HIV/AIDS
Pneumocystis pneumonia	<i>Pneumocystis jirovecii</i>	~500,000	15–50%	>250,000	Lung	HIV/AIDS, immunosuppressant drugs e.g. corticosteroids
Mucormycosis	Mucormycetes	~10,000	~50%	~5000	Multiple, mostly lung and skin	Diabetes, organ transplant, trauma e.g. burn

Data derived from (Bongomin et al., 2017).

clinical outcome (Lanternier et al., 2013; van Arkel et al., 2020). Mortality rates for invasive fungal infections have remained high in part due to inadequate diagnostics and treatments, and limited access to gold-standard antifungal drugs in low-middle income countries which carry the highest burden of infections (Benedict et al., 2017; Brown et al., 2012; Kozel and Wickes, 2014; Rodrigues and Albuquerque, 2018). Additionally, the efficacy of currently available anti-fungal drugs can be limited by the development of anti-fungal drug resistance. For example, *Candida auris* is a recently discovered multi-drug resistant species that causes significant mortality and morbidity, and has spread to at least 30 countries following its discovery in 2009 (Sabino et al., 2020).

Despite the impact of fungal diseases on human health, there remains much to be learned about the induction and regulation of anti-fungal immune responses. Yet, a greater understanding of antifungal immunity will be critical for the future development of diagnostics, vaccines and adjunctive immune-based treatments for fungal infection (Brown et al., 2012; Lionakis et al., 2017a). In this chapter, we summarize mechanisms of cellular antifungal immunity within the innate and adaptive immune systems, highlighting recent advances in our understanding and the areas requiring further research.

Innate anti-fungal immunity

Macrophages

Macrophages are myeloid cells of the innate immune system with a range of functions including tissue repair, removing dead cells, maintaining homeostatic organ function and uptake and clearance of invading microbes. During embryonic development, macrophages first arise from progenitors in the yolk sac, some of which seed developing organs to generate specialized tissue-resident macrophage populations (Fig. 2). Thereafter, hematopoietic stem cells (HSCs) in the foetal liver and the bone marrow give rise to monocytes which circulate in the blood and differentiate into macrophages upon entering tissue. The phenotype and function of monocyte-derived macrophages is shaped by environmental signals and the nature of the inflammatory insult (Epelman et al., 2014; Gordon and Plüddemann, 2017; Wynn et al., 2013). For example, high concentrations of IFN γ (as a result of bacterial or fungal infection) drives upregulation of the transcription factor STAT1 which supports polarization towards a pro-inflammatory and phagocytic phenotype, termed M1 or classical activation. M1 macrophages are particularly important for protective immunity against intracellular bacteria and fungi. On the other hand, exposure to IL-4 or IL-10 stimulated by extracellular pathogens such as dimorphic fungi, upregulates STAT6 resulting in an anti-inflammatory/tissue repair macrophage phenotype termed M2 or alternative activation (Italiani and Boraschi, 2014; Martinez and Gordon, 2014).

Macrophages are critical anti-fungal immune cells since individuals with genetic mutations that disrupt macrophage responses are highly susceptible to fungal infections. For example, patients with the dysfunctional *CX3CR1-M280* allele have a greater risk of developing disseminated candidiasis, which is related to the role of CX3CR1 in the recruitment and survival of monocyte-derived macrophages within the fungal-infected kidney (Lionakis et al., 2013; Salazar and Brown, 2018). Moreover, macrophage-depletion in mice results in higher fungal burden and decreased survival following infection with *C. albicans* (Lionakis et al., 2013; Salazar and Brown, 2018) and *C. neoformans* (Osterholzer et al., 2009; Sun et al., 2019). However, there are some contexts in which macrophages may promote fungal infection. For example, depletion of macrophages at a late stage of *C. neoformans* infection

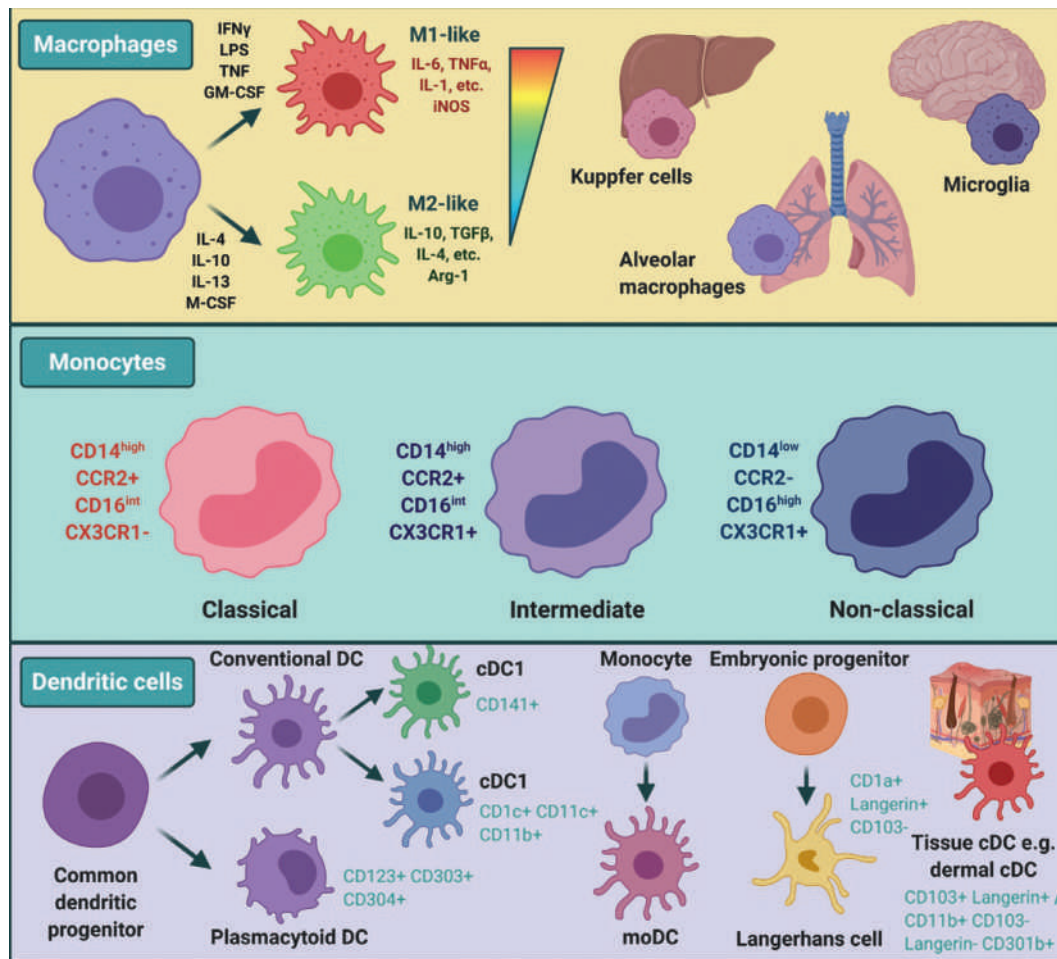


Fig. 2 Heterogeneity of myeloid cells. Macrophages, monocytes and DCs exist as functional subsets driven by environmental cues and/or developmental pathways. The myeloid cell subsets discussed in this chapter are presented along with their defining markers and/or anatomical location. Created with BioRender.com.

reduced dissemination of the fungus to the spleen and brain in mice, indicating that some fungi may utilize macrophages for immune evasion and trafficking to distant organs (Charlier et al., 2009).

Macrophages detect fungal pathogens via pattern recognition receptors (PRRs) which detect PAMPs (pathogen-associated molecular patterns) within the fungus (see Box 1). Once activated by PRRs, macrophages initiate fungal uptake (phagocytosis) and killing and produce pro-inflammatory cytokines and chemokines. Macrophages phagocytose fungi using either opsonic receptors (e.g. CR3, Fc γ R) or non-opsonic receptors (e.g. Dectin-1) (Becker et al., 2015; Brown, 2011). Immune-modulating drugs that disrupt fungal phagocytosis by macrophages have demonstrated the importance of macrophage-mediated phagocytosis for fungal clearance. For example, ibrutinib is a BTK inhibitor prescribed to patients with chronic lymphocytic leukemia and has been associated with the development of invasive fungal infections, particularly aspergillosis, which is thought to be related to the role of BTK in regulating Dectin-1-dependent phagocytosis of *A. fumigatus* (Bercusson et al., 2018; Fiorcari et al., 2020; Ghez et al., 2018; Lionakis et al., 2017b). Following uptake, fungi-containing phagosomes fuse with lysosomes to form an acidic phagolysosome compartment, which contains a milieu of anti-microbial enzymes and drives nutrient starvation within the fungus, facilitating fungal death. This is a highly dynamic process which helps accommodate the shape-shifting nature of some fungal species. For example, *C. albicans* can form long filaments called hyphae, and lysosome fusion with the phagosome enables extensive growth of the phagolysosomal compartment and is required for macrophage killing of this fungus (Westman et al., 2020). Fungal morphology and cell wall structure play a significant role in determining the outcome of phagocytosis. For example, *C. neoformans* fungi form giant Titan cells that are resistant to phagocytic uptake (Dambuza et al., 2018) and form an anti-phagocytic capsule that prevents immune recognition and phagosome acidification (De Leon-Rodriguez et al., 2018; Gilbert et al., 2015). Several fungal species produce melanin which has been shown to interrupt LC3-dependent phagocytosis, driven by Dectin-1 signaling (Akoumianaki et al., 2016; Andrianaki et al., 2018; Sprenkeler et al., 2016). These evasion mechanisms lead to persistence of fungal pathogens within macrophages, which has been implicated in enabling dissemination of some fungi, such as *C. neoformans* whose persistence within lung interstitial macrophages has been indicated to drive dissemination to the CNS (Santangelo et al., 2004).

Box 1 Innate recognition of fungi

Innate recognition of fungi by myeloid cells is mediated by several classes of PRRs including the C-type lectin receptors (CLRs), Toll-like receptors (TLRs) and NOD-like receptors (NLRs) which bind to components of the fungal cell wall and nucleic acids leading to the activation of intracellular signaling cascades and antifungal immune responses (Fig. 3) (Salazar and Brown, 2018).

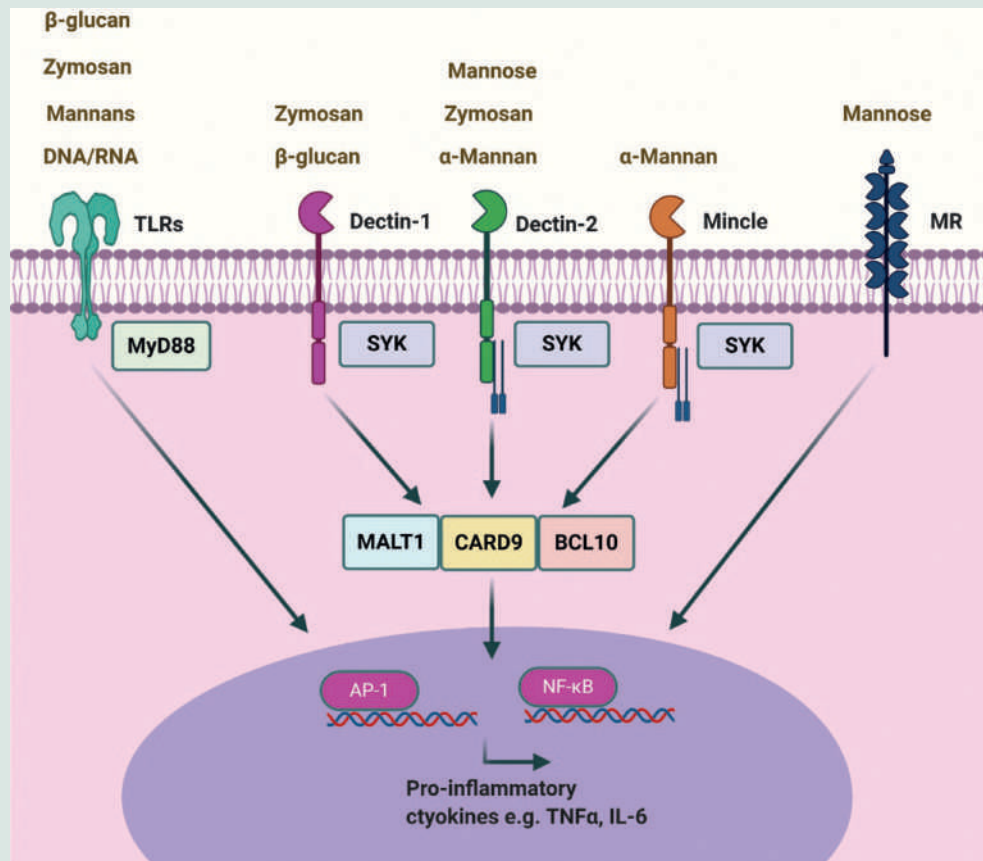


Fig. 3 Innate recognition of fungi. Fungal PAMPs deriving from the fungal cell wall are bound by TLRs or CLRs expressed by myeloid cells. Recognition by these receptors activates intracellular signaling cascades mediated by MyD88 (TLRs) or SYK/CARD9 (CLRs), culminating in a cellular immune response (e.g. cytokine production). Created with BioRender.com.

CLRs: The CLRs are a large family of transmembrane receptors which share a carbohydrate recognition domain (CRD) that is either calcium-dependent (C-type lectin) or calcium-independent (C-type lectin-like) (Zelensky and Gready, 2005). Several CLRs have been shown to activate antifungal immunity in mice and humans, particularly the CARD9-coupled CLRs Dectin-1, Dectin-2, Dectin-3, and Mincle (Patin et al., 2019). Dectin-1 is the best-studied CLR in the context of antifungal immunity and recognizes β -glucan in the fungal cell wall. Upon β -glucan binding, the ITAM signaling motif within the cytoplasmic tail of Dectin-1 (or Fc γ R, the signaling partner of Dectin-2/3) becomes phosphorylated, resulting in the recruitment and activation of Syk kinase. This initiates a signaling cascade via the kinases PLC γ and PKC δ leading to the formation of the CBM signaling complex, composed of CARD9, BCL10 and MALT1. This activates phagocytosis and fungal killing pathways, and also drives production of pro-inflammatory cytokines through activation of transcription factors (e.g. NF κ B and AP-1) and the NLRP3 inflammasome (see below). Dectin-1 can also alternatively activate the Syk-independent Raf1 signaling pathway, driving IL-23 production to promote Th17 differentiation (Drummond et al., 2018; Lionakis et al., 2017a; Patin et al., 2019; Speakman et al., 2020).

Mice deficient in CARD9 or CARD9-coupled receptors have increased susceptibility to *A. fumigatus*, *C. albicans* and *C. neoformans* infection (Drummond et al., 2018). This is replicated in humans where deleterious mutations in *CARD9* result in a primary immunodeficiency disease that specifically predisposes to fungal pathogens, but not bacterial or viral infections. CARD9 deficiency presents with an organ-specific pattern of susceptibility with patients predominantly presenting with either subcutaneous skin infections, CNS candidiasis or extra-pulmonary aspergillosis (Lanternier et al., 2013, 2015; Rieber et al., 2016). Human deficiencies in single CLRs have also underscored the importance of this receptor family in antifungal immunity. Dectin-2 deficiency has been shown to promote susceptibility to pulmonary cryptococcosis (Hu et al., 2015), while Dectin-1 deficiency, caused by the Y238X polymorphism, was found to increase the risk of vaginal and oropharyngeal candidiasis, as well as invasive aspergillosis in stem cell transplant patients (Chai et al., 2011; Ferwerda et al., 2009).

TLRs: TLRs are a family of PRRs consisting of 12 members in mice and 10 in humans, which are expressed at the cell surface or on intracellular organelles. All TLRs have a PAMP-recognizing ectodomain, a transmembrane domain and a Toll/IL-1 receptor domain for signaling (Kawasaki and Kawai, 2014). TLRs bind a diverse array of fungal ligands including cell wall components (TLR2), mannans (TLR4) and fungal DNA (TLR9) (Patin et al., 2019). TLR signaling is reviewed extensively elsewhere (Kawasaki and Kawai, 2014); briefly, extracellular TLRs recruit the signaling adaptor MyD88 to activate AP-1 and NF κ B resulting in production of inflammatory cytokines, while intracellular TLRs signal through the adaptor TRIF to activate interferon responsive transcription factors, such as IRF3. MyD88-deficient mice have an increased susceptibility to multiple fungal infections, including *A. fumigatus*, *C. albicans*, *C. neoformans*, and *Histoplasma capsulatum*, while deficiencies in single TLRs

(Continued)

Box 1 (Continued)

promote susceptibility in a context-dependent manner (Salazar and Brown, 2018). However, MyD88 deficiency in humans does not appear to be associated with an increased susceptibility to fungal infections. Instead, MyD88-deficient patients die in early childhood from profound bacterial infections, presumably because CLR-CARD9 signaling is sufficient to protect against fungal infection in the absence of the TLRs (Von Bernuth et al., 2008).

NLRs: NLRs are an intracellular family of PRRs which can be divided into inflammasome forming (e.g. NLRP3 and NLRC4) and non-inflammasome forming (e.g. NOD1 and NOD2). Inflammasomes are large protein complexes that drive proteolytic cascades mediated by caspase enzymes culminating in the production of bioactive cytokines IL-1 β and IL-18. In mice, deficiencies in NLRP3 and NLRC4 promotes susceptibility to several fungal pathogens including *C. albicans*, *C. neoformans* and *A. fumigatus* (Patin et al., 2019). Until recently, the fungal ligands responsible for inflammasome activation were unknown, but exciting new studies have shed light on how these pathways become activated by *Candida* and *Aspergillus* fungi. *Candida* hyphae secrete a peptide toxin called Candidalysin which helps the fungus invade epithelial barriers and establish mucosal infections (Moyes et al., 2016). Candidalysin was recently found to activate the NLRP3 inflammasome in macrophages, promoting both fungal escape (by driving macrophage death) (Kasper et al., 2018) and the induction of IL-1 β -dependent protective immunity by microglia in the brain (Drummond et al., 2019). Fungal activation of inflammasomes can therefore have diverse consequences that are organ- and cell type-specific. Galactosaminogalactan, a component of the *Aspergillus* cell wall, was recently found to bind mammalian ribosomes and block translation, which acted as a trigger for NLRP3 inflammasome activation and induction of protective immunity (Briard et al., 2020). Thus, by studying fungal-mediated activation of these pathways, we have discovered novel mechanisms controlling inflammasome function.

To kill fungi, macrophages produce toxic compounds such as reactive oxygen species (ROS) which is generated by the enzyme NADPH oxidase. CGD patients have mutations in components of the NADPH oxidase complex (e.g. p47^{phox}) and are particularly susceptible to aspergillosis, although cases of candidiasis and mucormycosis have also been reported (Falcone and Holland, 2012). In addition to ROS, macrophages produce reactive nitrogen species (RNS) and nitric oxide (NO) following activation of the iNOS enzyme (Warris and Ballou, 2019). M1 macrophages express high levels of iNOS which enables the conversion of arginine to NO and citrulline. In contrast, M2 macrophages alternatively upregulate arginase to convert arginine to ornithine and urea, and so produce little NO (Rath et al., 2014). Nitrogen-dependent fungal killing by M1 macrophages has been shown to be protective in many fungal infections including cryptococcal meningitis. STAT1 knockout mice, which lack an M1 response, are highly susceptible to pulmonary *C. neoformans* infection resulting in increased lung/brain fungal burden and accelerated mortality (Hardison et al., 2012). STAT1 regulates expression of CXCL9 and CXCL10, which drive recruitment of CXCR3⁺ Th1 CD4⁺ T cells that promote fungal clearance by enhancing phagocytic and killing functions of macrophages (Hardison et al., 2012; Xu et al., 2020). The NO produced via iNOS can react with superoxide to generate RNS, which are important in host defence by inhibiting growth and killing of fungi, particularly *Candida* species (Ahmadi et al., 2016; Warris and Ballou, 2019). Mice deficient in iNOS show increased susceptibility to *C. albicans* infection, while application of NO has been shown to be beneficial in treating cutaneous fungal infections (Balish et al., 2005; Stasko et al., 2018; Warris and Ballou, 2019). As with phagocytosis, fungal pathogens may employ evasion strategies to circumvent macrophage RNS-dependent killing. For example, *C. albicans* inhibits NO secretion by down-regulating iNOS expression and activity while upregulating arginase, a process mediated by exposure to fungal cell wall chitin, which drives an M2-like phenotype in macrophages thereby aiding fungal survival (Wagener et al., 2017). Similar perturbations of iNOS/NO pathways have been observed with *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis* (Rocco et al., 2011) and *C. neoformans* (Kawakami et al., 1997; Leopold Wager et al., 2016).

Tissue-resident macrophages often act as the front-line defence against invading fungi and promote rapid responses via chemokine production, which recruit and activate leukocytes. For example, microglia are CNS-resident macrophages that protect against *C. albicans* brain infection via the CARD9-dependent production of IL-1 β and CXCL1, which acts to recruit neutrophils that are required for fungal clearance (Drummond et al., 2015b, 2019). In the fungal-infected lung, alveolar macrophages are a major source of pro-inflammatory chemokine CXCL2 (Xu-Vanpala et al., 2020; Xu and Shinohara, 2017), which recruits neutrophils and monocytes. Interestingly, alveolar macrophages develop into heterogeneous populations during fungal infection, in which CXCL2⁺ populations drive inflammation and uptake more fungi, whereas CXCL2⁻ populations express C1q which limits excess neutrophil recruitment and pathological inflammation (Xu-Vanpala et al., 2020). Macrophage responses to fungi, particularly of tissue-resident macrophages, are therefore heterogeneous and dependent on fungal species/organ. As we learn more about the environmental cues shaping tissue-resident macrophage identity, it will be interesting to determine how fungal infection impacts on these pathways and how initial responses by tissue-resident macrophages contribute towards antifungal defence.

Monocytes

Monocytes are circulating myeloid cells that differentiate into macrophages or dendritic cells (DCs) following recruitment into tissues (Italiani and Boraschi, 2014). However, monocytes can also act as immune effector cells in their own right, mediating fungal uptake and producing inflammatory cytokines. Monocytes are classed into three subtypes: classic (inflammatory), intermediate, and non-classical (patrolling). These subsets are identified by expression of surface receptors, in which “classic” are identified as CD14^{high}CCR2⁺CD16^{int}CX3CR1⁺, “intermediate” as CD14^{high}CCR2⁺CD16^{int}CX3CR1⁺ and “non-classical” as CD14^{low}CCR2⁻CD16^{high}CX3CR1⁺ (Fig. 2). Equivalent subsets exist in mice which are defined by expression of Ly6C in addition to CCR2 and/or CX3CR1 (Fu and Drummond, 2020; Italiani and Boraschi, 2014). Classical monocytes account for the majority of circulating monocytes and are the main precursor for inflammatory macrophages/DCs. Patrolling and intermediate monocytes are

less well understood, however patrolling monocytes are believed to be involved in homeostatic functions and tissue repair, while intermediate monocytes have been observed to perform both classical and non-classical functions, dependent on context (Fu and Drummond, 2020; Italiani and Boraschi, 2014).

Monocytopenia, a condition defined by reduced numbers of circulating monocytes, promotes susceptibility to systemic fungal infections in humans including histoplasmosis, aspergillosis and cryptococcosis (Salazar and Brown, 2018; Vinh et al., 2010). Murine pulmonary *A. fumigatus* infection induces an influx of inflammatory monocytes into the lung, with similar observations seen in the kidneys of mice infected systemically with *C. albicans*. Monocyte depletion in these studies has shown that these cells are essential for clearance of fungal pathogens, either by acting as a source of macrophages and DCs which then uptake/kill fungi and activate adaptive immune responses (see “Dendritic cells” below), and/or by recruiting leukocytes via cytokine and chemokine production (Espinosa et al., 2014; Fu and Drummond, 2020; Hohl et al., 2009; Ngo et al., 2014). For example, monocyte-derived DCs produce CXCL9/10 in the fungal-infected lung to recruit plasmacytoid DCs (pDCs) which activate ROS production in neutrophils and promote fungal killing (Guo et al., 2020). Although monocytes exert many protective functions in the context of fungal infection, there is evidence to suggest that their role can also be detrimental to fungal clearance. For example, monocyte depletion during early *C. neoformans* infection was actually found to be protective by preventing an accumulation of M2 macrophages which support *C. neoformans* proliferation and survival (Fu and Drummond, 2020; Heung and Hohl, 2019). Furthermore, *C. neoformans* has also been shown to hijack monocytes and use them as “Trojan Horses” to invade the blood-brain-barrier (Santiago-Tirado et al., 2017).

In recent years, monocytes have been implicated in mediating innate immune memory, termed “trained immunity”, to fungal pathogens (Heung, 2020; Netea et al., 2016). Monocytes stimulated with either *C. albicans* or β -glucan undergo epigenetic and metabolic reprogramming, characterized by histone H3 acetylation and a switch from oxidative phosphorylation to glycolysis. These changes prime monocytes to respond faster and in a greater magnitude to a secondary fungal exposure (Heung, 2020; Netea et al., 2016). For example, mice infected with a non-lethal dose of *C. albicans* had a higher survival rate following challenge with a lethal infection dose, an effect that did not require lymphocytes (Quintin et al., 2012). This monocyte reprogramming requires Dectin-1 and Raf-1-signalling (Quintin et al., 2012) and appears to provide a broad spectrum of protection, since mice trained with the BCG vaccine are also more resistant to *C. albicans* infection (Netea et al., 2016). These studies have inspired interest in trained immunity as a therapeutic strategy, particularly for patients with defects in adaptive immunity and therefore unable to mount classical memory responses.

Dendritic cells

DCs are professional antigen-presenting cells that interact with T-cells and activate the adaptive immune response. DCs possess long dendrites decorated with PRRs which enable them to sample their surroundings and rapidly detect an inflammatory insult or infection, often resulting in the release of cytokines to activate other effector cells (Banchereau et al., 2000). DCs can be found circulating in the blood, but are also resident in lymphoid tissues and in various mucosal tissues such as the skin and gut (Eisenbarth, 2019). Once activated via PRRs, DCs migrate to lymph nodes where they present antigenic peptides to CD8 and CD4 T cells on MHC-I or MHC-II molecules, respectively (Banchereau et al., 2000). This process of antigen presentation leads to the development of an adaptive immune response (see “adaptive anti-fungal immunity”) which is critical for the clearance of some fungal pathogens, such as *C. neoformans* and endemic dimorphic fungi (e.g. *Blastomyces*, *Histoplasma*) (Verma et al., 2015). DCs are divided into different subsets based on developmental origin, location and immune function (Fig. 2) (Eisenbarth, 2019). HSCs in the bone marrow give rise to the common DC precursor, which can then go on to become conventional DCs (cDC, further subdivided into cDC1 and cDC2) or plasmacytoid DCs (pDC). Alternatively, monocytes can give rise to monocyte-derived DCs (moDCs), or embryonic precursors give rise to tissue-resident DC populations (e.g. Langerhans cells in the skin) (Eisenbarth, 2019; Satpathy et al., 2012).

During fungal infection, DC subsets exhibit context-specific functions that are necessary for fungal clearance. For example, pDCs play crucial protective roles in the *A. fumigatus*-infected lung by producing IFN α and TNF α in a TLR9-dependent manner, but have also been shown to dampen excessive inflammation in the fungal-infected vaginal mucosa (LeBlanc et al., 2006; Ramirez-Ortiz et al., 2011; Ramirez-Ortiz and Means, 2012). In the skin, DC subsets are classified by location and surface expression of Langerin, CD103 and CD11b into epidermal Langerhans cells and various dermal DC subtypes (Igyártó et al., 2011; Kaplan, 2010). These DC subsets exhibit distinct functions in the context of *C. albicans* skin infection, which depends on IL-17-dependent immunity and neutrophil recruitment for clearance. For example, Langerhans cells specifically promote the differentiation of IL-17-producing CD4 T-cells via IL-6 production, which requires recognition of *C. albicans* by Dectin-1 (Igyártó et al., 2011; Kashem et al., 2015a). In contrast, CD301b⁺ dermal DCs indirectly promote IL-17 mediated immunity following activation by the neuropeptide CGRP, released by nociceptive neurons in response to *C. albicans* skin invasion. Binding of CGRP by these dermal DCs drives their production of IL-23, resulting in the rapid activation of $\gamma\delta$ T cells to produce IL-17 (Cohen et al., 2019; Kashem et al., 2015b).

Neutrophils

Neutropenia and inherited defects in neutrophil function are strongly associated with an increased risk for candidiasis, aspergillosis and mucormycosis in humans (Desai and Lionakis, 2018; Hassan and Voigt, 2019). Neutrophils are short-lived phagocytes specialized for pathogen killing, particularly extracellular pathogens, that are rapidly recruited in large numbers to the site of

infection (Rosales, 2018). Neutrophils release granules that contain microbicidal enzymes such as elastase and myeloperoxidase, and also produce large amounts of ROS (Rosales, 2018). Furthermore, neutrophils can form neutrophil extracellular traps (NETs), a mesh of chromatin containing citrullinated histones and antimicrobial proteins (e.g. calprotectin) to trap and kill pathogens (Urban and Nett, 2019).

Mouse studies have shown robust recruitment of neutrophils into *Candida* and *Aspergillus*-infected tissues, driven by the upregulation of neutrophil-attracting chemokines and cytokines (e.g. IL-8, CXCL2) (Lionakis et al., 2011). This recruitment is a tightly controlled process involving a range of signaling pathways that are activated in a fungal species- and organ-specific manner. For example, we recently showed that *C. albicans* brain infection drives production of the neutrophil chemoattractants IL-1 β and CXCL1 from microglia in a CARD9-dependent manner, whereas production of other neutrophil chemoattractants (e.g. CCL3) were not required for neutrophil influx into the CNS (Drummond et al., 2019). In contrast, neutrophil recruitment to the *C. albicans*-infected kidney does not require CARD9 and is dependent on CCR1 signaling, which is upregulated by neutrophils at a late stage of infection (Drummond et al., 2015b; Lionakis et al., 2012). Although neutrophil recruitment is required to control fungal proliferation within the kidney, CCR1 knockout mice were found to have significantly reduced inflammation resulting in improved survival following *C. albicans* infection (Lionakis et al., 2012), highlighting the need to regulate neutrophil recruitment in order to strike a balance between fighting the infection and preventing immunopathology. In pulmonary *A. fumigatus* infection, neutrophil recruitment into the lung occurs in two distinct phases. First, epithelial cells in the lung produce CXCL2 via a IL-1 β -IL-1R/MyD88 signaling pathway, followed by CARD9-dependent signaling within monocytes/macrophages that controls the second wave of neutrophil recruitment and is required to activate fungal killing by neutrophils (Caffrey et al., 2015; Desai and Lionakis, 2018; Jhingran et al., 2015).

Neutrophil fungal killing is regulated by several signaling pathways. For example, neutrophil killing of unopsonized *C. albicans* requires CR3-mediated recognition and activation of a Syk/PI3K/CARD9 signaling pathway, while killing of opsonized *C. albicans* requires recognition by Fc receptors resulting in signaling through Syk/PKC and activation of NADPH oxidase (Gazendam et al., 2014). Another fungal killing mechanism employed by neutrophils is the activation of an apoptosis-like cell death pathway within fungal spores, which depends on neutrophil NADPH oxidase activation (Shlezinger et al., 2017). Lastly, the formation of NETs is thought to be particularly important for entrapment and killing of large fungal structures, such as hyphae formed by *Candida* and *Aspergillus* species (Urban and Nett, 2019), while NET components such as calprotectin have been shown to restrict nutrient availability to fungal cells (Urban et al., 2009). The generation of NETs requires NADPH oxidase (for ROS production) and the enzyme PAD4, which generates the citrullinated histones (Urban and Nett, 2019). However, the relative dependence on these pathways in different disease contexts, including fungal infection, is not yet well understood. Opsonized *C. albicans* yeasts and hyphae have been shown to drive ROS-dependent PAD4-independent NETosis, whereas un-opsonized *C. albicans* drives Dectin-2-dependent PAD4 signaling to generate NETs independently of NADPH oxidase (Guiducci et al., 2018; Wu et al., 2019). Formation of NETs against *A. fumigatus* also appears to be strictly dependent on NADPH oxidase/ROS, while the role of PAD4 is still unclear with some studies indicating PAD4-dependence for optimal NET-based fungal killing, and other studies finding no requirement for PAD4 (Clark et al., 2018; Röhm et al., 2014; Silva et al., 2020).

Platelets

Platelets are short-lived anucleate blood cells with established roles in hemostasis by preventing bleeding through thrombus formation (van der Meijden and Heemskerk, 2019). In recent years, platelets have been appreciated to also help regulate immune responses. Following vasculature damage and inflammation, platelets become activated and release an array of cytokines and chemokines through their α -granules, including platelet factor 4 (PF4), RANTES and IL-1 β , which recruit and activate leukocytes. Activated platelets also express specific surface receptors including P-selectin, CD40L and GPIb which enable their interaction with leukocytes (Assinger et al., 2019; Mezger et al., 2019).

Thrombocytopenia is a common complication of organ transplantation and hematological malignancies (both of which enhance the risk of invasive fungal infections), and has been independently associated with an increased risk of aspergillosis and candidiasis (Eberl et al., 2019; Tischler et al., 2020). In vitro studies have shown platelet release of activation factors (including CD40L and RANTES) in response to *A. fumigatus*, resulting in recruitment of innate immune cells and disruption of hyphal growth (Rambach et al., 2015; Rødland et al., 2010; Speth et al., 2014). In a mouse model of pulmonary *A. fumigatus* infection, platelet depletion significantly enhanced mortality, which was the result of significant bleeding caused by fungal invasion and reduced neutrophil-mediated fungal killing (Tischler et al., 2020). Thrombocidin-1, a component of human platelet α -granules, has a potent antifungal effect against *C. neoformans* which further supports the theory that platelets can have direct fungicidal functions (Krijgsveld et al., 2000). Indeed, high resolution microscopy has shown that platelets aggregate and directly bind to *A. fumigatus* and *C. albicans* (Perkhofer et al., 2008; Robert et al., 2000). Moreover, an in vitro study showed that *Mucor circinelloides* potently induced platelet aggregation and activation, mediated by recognition through GPIIb/IIIa and the platelet Fc receptor Fc γ RIIA (Ghuman et al., 2019). The receptors mediating direct interactions between fungi and platelets are still poorly understood, and it is not always clear whether the consequences of platelet activation are beneficial for host protection. For example, platelets can activate complement via P-selectin which enhances fungal clearance by driving opsonization and phagocytosis (Speth et al., 2004, 2014). However, excessive complement activation has been associated with pathologic thrombosis and infarction, serious complications that have been observed in patients with angioinvasive fungal infections such as mucormycosis and aspergillosis, particularly those with CNS involvement (Gundlach et al., 2019; Sherif and Segal, 2010).

In addition to direct fungicidal functions, platelets may mediate fungal killing indirectly via the recruitment of phagocytes. For example, neutrophils are strongly attracted to thrombi formed *in vitro* with *A. fumigatus* conidia and human whole blood (Fréalles et al., 2018). Platelet/macrophage interactions can also boost antimicrobial immunity. For example, CLEC-2/podoplanin interactions, which mediate platelet-macrophage binding, has been shown to be protective since CLEC-2^{-/-} mice have excessive inflammation and increased mortality following the induction of bacterial sepsis (Rayes et al., 2017). Indeed, GP1b/Mac-1 (CR3) interactions between platelets and macrophages can help drive an M1 phenotype which helps to promote bacterial clearance (Carestia et al., 2019). Further research is needed to establish whether similar interactions occur during fungal infection and what effect they exert on disease outcome.

NK cells, NK T cells and innate lymphoid cells

NK (natural killer) cells and innate lymphoid cells (ILCs) are lymphoid cells that do not have rearranged antigen receptors and respond rapidly to inflammatory stimuli in an innate-like fashion (Becker et al., 2015). NK T cells are a subtype of T cell which express the invariant T cell receptor (TCR) through which they recognize lipid antigens presented by CD1d (an MHC-like molecule) on antigen presenting cells (Tupin et al., 2007). ILCs are grouped by their functional/cytokine profiles into ILC1 (IFN γ /TNF α producing, includes NK cells), ILC2 (IL-4, IL-5, IL-13 producing) and ILC3 (IL-17, IL-22 producing) subpopulations. Most ILCs are found in high numbers at mucosal sites, allowing them to rapidly respond to invading microbes and barrier breaches (Eberl et al., 2015).

NK cells (ILC1) are cytotoxic cells involved in the direct killing of fungal pathogens, however they also secrete inflammatory mediators to activate and recruit other immune cells including macrophages. NK cells recognize fungal pathogens via their expression of TLRs, CLRs and NK activation/inhibitory receptors (Schmidt et al., 2017). The latter includes Ly49 and KIR receptors, which mediate either activation or inhibition signals in NK cells (Pegram et al., 2011). For example, NKp30 directly recognizes β -glucan on the surface of *C. albicans* and *C. neoformans* (Li et al., 2013), while NKp46 mediates recognition of adhesins in the cell wall of *Candida glabrata* (Vitenshtein et al., 2016). These recognition events lead to NK cell activation, resulting in the release of perforin-containing granules and fungal killing (Schmidt et al., 2017).

NK T cells produce significant amounts of IFN γ and are particularly important in *C. neoformans* infection where Th1 responses are protective (Kawakami et al., 2001). Indeed, a specific subset of NK T cells expressing TCR chain V α 14 was found to accumulate in the lungs of *C. neoformans*-infected mice in a CCL2-dependent manner, and depletion of these cells (e.g. using CCL2 KO mice) resulted in reduced Th1 immunity and increased fungal burden (Kawakami et al., 2001). Furthermore, non-specific activation of NK T cells using the lipid α -GalCer was shown to increase IFN γ production by NK T cells resulting in enhanced fungal clearance (Kawakami et al., 2001). However, NK T-cells can also promote pathologic inflammation and prevent induction of protective antifungal immunity. In mice infected with *C. albicans*, non-specific activation of NK T-cells led to reduced survival and increased tissue fungal burdens, which correlated with enhanced inflammation and neutropenia (Tarumoto et al., 2014). Indeed, animals deficient in NK T cells (*Ja18*^{-/-}) are more resistant to *C. albicans* infection (Haraguchi et al., 2016). Thus, the role of NK T-cells is clearly dependent on fungal species but whether organ-dependence also exists has yet to be determined.

The role of other ILC subtypes in fungal infection is still poorly understood and sometimes controversial. ILC2 are associated with a type 2 (Th2) response characterized by the production of IL-33 and IL-13, which worsen pulmonary *C. neoformans* disease severity and may be involved in driving allergic fungal disease (see “Fc ϵ R-expressing granulocytes”) (Castanhinha et al., 2015; Flaczyk et al., 2013; Müller et al., 2007). ILC3s produce IL-17, IL-22 and GM-CSF and are required for maintaining barrier integrity in the gut and oral mucosa (Mortha et al., 2014; Sonnenberg et al., 2012). In murine models of oral candidiasis, there is significant production of IL-17 which is critical for protection, since animals deficient in the production of this cytokine or its receptor are highly susceptible to uncontrolled infection. Both CD4 T-cells and ILC3s have been shown to produce IL-17 during oral *C. albicans* infection, however the relative contribution of these cell types and their importance remains controversial (Conti et al., 2014; Gaffen et al., 2011; Gladiator et al., 2013).

$\gamma\delta$ T cells

$\gamma\delta$ T cells are found in mucosal tissues such as the gut, and are characterized by their TCR which has γ and δ chain as opposed to the α and β chain found in conventional CD4 and CD8 T cell subsets, (Van De Veerdonk and Netea, 2010). $\gamma\delta$ T-cells rapidly produce large amounts of IL-17, similar to ILC3, and thus these cells have also been implicated in protective antifungal immunity within mucosal tissues (Drummond et al., 2015a). However, mucosal fungal infections that depend on Th1 immunity for protection may be limited by the action of IL-17A⁺ $\gamma\delta$ T cells, evidenced during *Cryptococcus neoformans* infection where these lymphocytes downregulate the Th1 response and are associated with poor outcome (Sato et al., 2020). Furthermore, depletion of $\gamma\delta$ T cells improved resistance to vaginal *C. albicans* infection, albeit a mechanism for this has not yet been established (Wormley et al., 2001). $\gamma\delta$ T cells may also enhance humoral immunity (mediated by B cells). For example, *in vitro* *P. brasiliensis* infection was shown to activate $\gamma\delta$ T cells which stimulated the production of antibodies, particularly IgM, by B cells (Munk et al., 1995). $\gamma\delta$ T cells therefore have diverse roles in antifungal immunity, although specific mechanisms underlying $\gamma\delta$ T-cell activation and influence on the infection outcome remain poorly defined.

FcεR-expressing granulocytes

FcεR-expressing granulocytes are particularly important in the development of hypersensitivity and allergic inflammation due to their function and location. These cells include basophils, eosinophils and mast cells, which are myeloid cells activated by IgE and Th2-associated cytokines, and share high expression of the IgE receptor (FcεR) (Stone et al., 2010). Due to the prevalence of fungal spores in the environment, fungi are a common cause of exacerbation of allergic inflammation. Fungi can therefore cause a severe form of asthma, termed severe asthma with fungal sensitization (SAFS), which is believed to affect at least 10 million people worldwide. Fungal allergy has been associated with several fungal species including *A. fumigatus*, *C. albicans* and *Alternaria alternata* (Agarwal, 2011; Denning et al., 2014; Simon-Nobbe et al., 2008).

Mast cells are found in mucosal tissues, releasing inflammatory mediators including cytokines, histamines and prostaglandins following activation and degranulation in response to various inflammatory stimuli including bacteria and fungi (Krystel-Whittemore et al., 2016). Mast cells express Dectin-1 and TLR2 which bind zymosan in the cell wall, leading to Syk signaling and the release of leukotrienes which promote vasoconstriction and mucus production (Olynych et al., 2006), as well as production of inflammatory mediators to recruit macrophages (Lopes et al., 2015) and activate IL-9-producing T-cells (Moretti et al., 2017). In vitro studies using mouse and human mast cells have shown *C. albicans* causes rapid degranulation and release of ROS to drive extracellular fungicidal activity, while other studies have shown that mast cells can phagocytose *C. albicans* yeasts resulting in intracellular NO production, but this does not appear to effectively induce fungal killing (Lopes et al., 2015; Nieto-Patlán et al., 2015; Pinke et al., 2016; Trevisan et al., 2014). Indeed, in vivo mast cells at the intestinal mucosa de-granulate and phagocytose *C. albicans* in the gut, but are unable to kill it, resulting in cell death and loss of barrier integrity (Renga et al., 2018).

Patients with fungal allergy and asthma commonly present with eosinophilia (Cockrill and Hales, 1999) indicating that these cells may regulate mechanisms of fungal allergic inflammation. Eosinophils release granules in response to *A. alternata* conidia and hyphae, both predominant fungal sensitizers in asthmatics. Eosinophils recognize *A. alternata* through β-glucan/CD11b binding or through eosinophil PAR-2 activation by fungal proteases, resulting in degranulation and cytokine release and ultimately facilitating fungal killing as eosinophil granules contain several cytotoxic molecules including MBP (major basic protein) and EDN (eosinophil-derived neurotoxin) (Matsuwaki et al., 2009). Basophils are also activated by IgE, as well as cytokines (e.g. IL-33) and TLR signaling, upon which they leave the blood and enter inflamed tissue where they release histamine and cytokines (Stone et al., 2010). Basophil recruitment has been implicated in the development of chronic allergy (Ghosh et al., 2013), but their function in fungal sensitization has not been extensively explored. Early studies indicated that basophil function in the context of *A. fumigatus* infection was regulated by surfactant proteins, which reduced basophil histamine release and inhibited IgE binding to *A. fumigatus*, helping to reduce pathologic inflammation that is typical of allergy and asthma (Madan et al., 1997).

Adaptive anti-fungal immunity

CD4 T cells

CD4 T cells recognize specific peptide antigen via their TCR in the context of MHC-II expressed on the surface of antigen-presenting cells (such as DCs, B-cells and macrophages). CD4 T-cells differentiate into several subtypes (including Th1, Th2, Th9, Th17 and Treg) in response to environmental signals and APC-derived instructions, which are classified based on expression of master transcription factors and cytokine profiles (Fig. 4) (Verma et al., 2015).

Th1 cells are defined by their production of TNFα and IFNγ, and are generally protective against invasive fungal infections. In humans, genetic mutations in *IL12R* and autoantibodies against IFNγ are both associated with increased susceptibility to candidiasis, cryptococcosis, coccidioidomycosis and histoplasmosis (Romani, 2011; Verma et al., 2015). Furthermore, adjunctive immune-based therapy with recombinant IFNγ has been shown to improve clinical outcome in patients with *Candida* and *Aspergillus*-induced sepsis (Delsing et al., 2014) and cryptococcal meningitis (Jarvis et al., 2012). Indeed, mice deficient in IFNγ or its receptor are highly susceptible to infections with *C. albicans* (Balish et al., 1998), *A. fumigatus* (Cenci et al., 1999) and *C. neoformans* (Chen et al., 2005). IFNγ secreted by Th1 cells drives macrophages towards an M1 phenotype, which is required for clearance of *C. neoformans* and *H. capsulatum*, as discussed above (Drummond et al., 2015a).

While Th1 cells appear to provide protection against disseminated fungal infections, Th17 cells are particularly important for activating mucosal antifungal defence. In the oral mucosa, Th17 cells drive the recruitment of neutrophils and activate production of antimicrobial peptides in response to *C. albicans* infection (Hernández-Santos and Gaffen, 2012). Human deficiencies in IL-17-mediated immunity (e.g. Hyper IgE syndrome or *IL17RA* mutations) increase susceptibility to chronic mucosal candidiasis (CMC) (Lionakis et al., 2017a; Romani, 2011; Verma et al., 2015), and this has been replicated in murine models of CMC where deficiencies in IL-17, IL-23 or CD4 T-cells leads to uncontrolled fungal proliferation at the oral mucosa (Gaffen et al., 2011; Hernández-Santos and Gaffen, 2012; Verma et al., 2015). The susceptibility of APECED patients to CMC was also thought to be due to dysregulated IL-17 immunity. APECED is an autoimmune disease in which a single infection, CMC, is a defining symptom (Ferre et al., 2016). Since many APECED patients were found to have autoantibodies against IL-17, this was thought to explain their susceptibility to oral *Candida* infections. However, a recent study has shown that APECED is associated with excessive IFNγ production at the oral mucosa by pathologic CD4 T-cells, damaging the epithelial barrier and presenting an opportunity for *Candida* invasion and infection (Break et al., 2021). Thus, the interplay between Th17 and Th1 at the oral mucosa must be exquisitely balanced to promote control of fungal proliferation (Th17) while suppressing barrier damage and initiation of infection (Th1). Why IFNγ overproduction at the oral mucosa solely promotes susceptibility to CMC in APECED patients, and not to other infections, is as yet unknown.

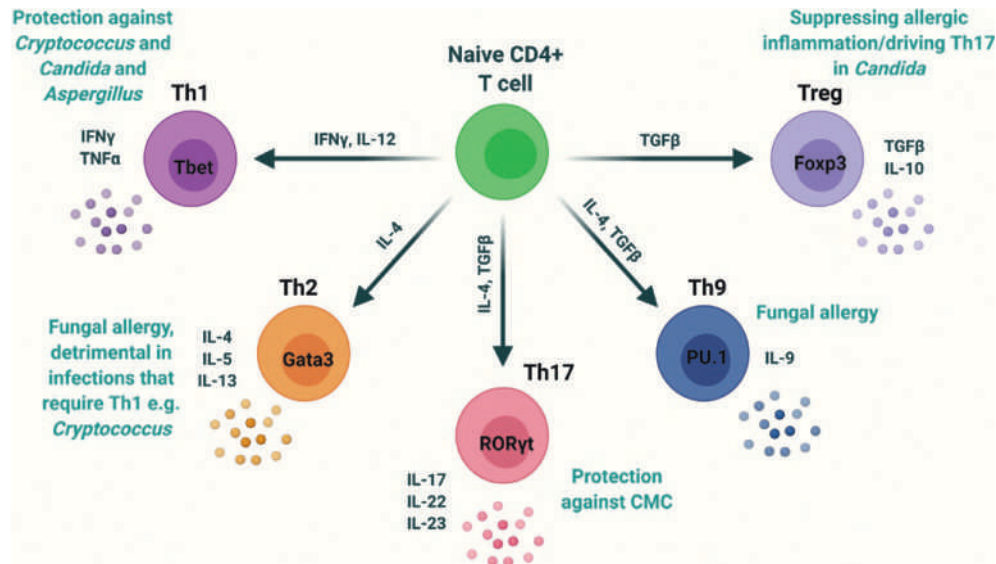


Fig. 4 CD4 T cell differentiation. Naive CD4 T cells differentiate into functional subsets following environmental cues from antigen-presenting cells. Each subset has unique context-specific functions regulated by the production of cytokines which is controlled by the expression of a master transcription factor. Created with BioRender.com.

Tregs are a subset of CD4 T cells that suppress inflammation in order to limit potential tissue damage (Vignali et al., 2008). For example, Tregs have been shown to downregulate Th2 responses in the lungs of *A. fumigatus* infected mice, reducing allergic inflammation within the airways (Montagnoli et al., 2006). Treg-mediated suppression can also promote specific types of immune responses by removing inhibitory signals. Th17 differentiation is antagonized by IL-2 signaling, and by acting as a sink for excess IL-2, Tregs can indirectly promote Th17 responses (Adler, 2007). This mechanism has been shown to be protective in oral *Candida* infection, where Treg-binding to IL-2 was shown to promote Th17 immunity and fungal clearance (Pandiyan et al., 2011).

CD8 T cells

CD8 T cells are cytotoxic T-cells activated by recognition of cognate antigen presented in the context of MHC-I, upon which they secrete pro-inflammatory cytokines and cytotoxic granules containing perforins and granzymes, resulting in killing of target cells and driving inflammation. CD8 T cells can also induce apoptosis of infected cells through Fas/FasL, and they are therefore important for killing viruses and cancers. CD8 T cell cytotoxicity occurs rapidly, but is controlled and limited by their antigen specificity (Zhang and Bevan, 2011).

CD8 T cells secrete several protective antifungal cytokines such as IFN γ and IL-17, and can also directly exert cytotoxic effects on fungal pathogens, including *C. albicans* and *H. capsulatum* (Kumaresan et al., 2018). In several fungal diseases, it has been shown that CD8 T cells may be able to compensate in the absence of CD4 T cells (e.g. in HIV-infected patients). For example, protection against *C. neoformans* pulmonary infection primarily relies upon Th1 CD4 T-cells. However, when CD4 T-cells are depleted in mice, the number IFN γ producing CD8 T cells was found to increase which helped to control fungal growth. Interestingly, the protective capacity of CD8 T-cells appears to be organ-specific as they could not compensate for the lack of CD4 T-cells within the brain, which was profoundly dependent on Th1 immunity for protection (Buchanan and Doyle, 2000; Huffnagle et al., 1994; Lindell et al., 2005). Since CD8 T-cells may be able to provide protection in a CD4 depleted environment, albeit in a context-dependent manner, there has been interest in stimulating CD8 T cell responses as a therapeutic strategy in patients at risk for fungal infections with compromised CD4 T cell function (Kumaresan et al., 2018). Indeed, mouse models have indicated that this strategy works in the context of blastomycosis and histoplasmosis, for which CD8 responses were found to be sufficient to provide vaccine immunity in CD4 T-cell-depleted mice (Wüthrich et al., 2003).

B cells

B cells provide humoral immunity and act as antigen-presenting cells. Once B cells exit the bone marrow, they can either circulate through the blood and secondary lymphoid tissues, searching for antigen, or become resident subsets such as spleen marginal zone (MZ) B cells (Hauser and Höpken, 2015). Antigen recognition occurs via the B cell receptor (BCR) and subsequently results in activation and differentiation into antibody-secreting plasma cells. Naïve B cells express IgM/IgD antibodies, and upon activation undergo T-cell-dependent somatic hyper-mutation and class-switching resulting in the production of high-affinity functional antibodies. Alternatively, activated B cells can develop into memory cells, a self-renewing population that remains in the body for many years and enables a rapid humoral response upon re-exposure to antigen (Cyster and Allen, 2019).

Patients with defects in humoral immunity, such as X-linked agammaglobulinemia or hypogammaglobulinemia (in which patients have a mutation in the BTK gene resulting in low numbers of mature B cells and therefore produce little or no antibodies), have increased susceptibility to some fungal infections including cryptococcosis and pneumocystis (Casadevall and Pirofski, 2012; Plebani and Lougharis, 2014; Verma et al., 2015). Similarly, patients prescribed therapies that deplete B cells (e.g. rituximab) have an increased risk of mucosal and disseminated candidiasis (Lin et al., 2007). Studies using B cell and/or IgM deficient mice have demonstrated a protective role for these cells in controlling *C. neoformans* infection, since animals lacking B-cells exhibited increased fungal burdens in the lung and brain and reduced survival rates (Rohatgi and Pirofski, 2012; Subramaniam et al., 2010; Szymczak et al., 2013). Antifungal antibodies, which most commonly target cell wall components such as β -glucan, promote protection against infection by enhancing macrophage phagocytosis and killing by binding Fc receptors expressed by myeloid cells (Casadevall and Pirofski, 2012; Lionakis et al., 2017a; Verma et al., 2015). This appears to be particularly important for *C. neoformans* infection, where antibodies are needed to overcome the resistance of these encapsulated yeast to phagocytosis, and likely explains the susceptibility of B-cell-deficient mice to this fungal infection (Rivera et al., 2005). Antibodies may also directly bind the fungal surface, disrupting growth and virulence (Brena et al., 2011; McClelland et al., 2010). Antibody binding to the fungal cell surface can further activate complement, and induce antibody-dependent cellular cytotoxicity mediated by NK cells (Nabavi and Murphy, 1986). In addition, antibodies can act intracellularly by driving maturation of fungi-containing phagosomes. For example, it was shown that opsonizing *H. capsulatum* with monoclonal antibodies increased phagosome acidification, thus reducing intracellular survival of the yeast (Shi et al., 2008). Within barrier tissues, antibodies (particularly IgA) are crucial for providing antifungal defence and limiting dissemination and invasive infection. For example, plasma cells residing in the meninges provide critical antifungal defence in the CNS, by secreting antifungal IgA which prevents *C. albicans* invasion of the brain, which is often lethal (Fitzpatrick et al., 2020). The GI tract is also protected by antifungal IgA; CD37-deficient mice, which overproduce IgA, were found to be significantly better protected from *C. albicans* gut infection, indicating that IgA provides critical control of commensal fungal populations and limits their dissemination (Van Sriel et al., 2009).

These protective mechanisms have resulted in optimism for vaccine development for fungal pathogens, as well as providing an opportunity to boost immunity in immunocompromised patients (specifically those lacking T cell responses) using immunoglobulin therapy. mAbs against *C. auris* have been shown to increase phagocytosis of the fungus by macrophages, and thus may be a therapeutic route for this inherently drug-resistant species (Rudkin et al., 2018). Furthermore, there have been a small number of clinical trials testing the efficacy of anti-fungal mAbs against *C. albicans*, *A. fumigatus* and *C. neoformans* that have yielded promising results, however none have yet resulted in a licensed therapy in humans (Di Mambro et al., 2019).

Conclusions

Recent years have seen numerous significant advances of our understanding of antifungal immune defence. Many of these advances have centered on analyzing organ-specific mechanisms of immunity. For example, the discovery of how meningeal B-cells and CARD9-expressing tissue-resident macrophages protect the CNS against *Candida* infection, and identification of heterogeneous alveolar macrophage populations promoting fungal clearance and limiting tissue pathology in the lung. These studies have highlighted how it is critical to focus on context-dependent functions of immune cells, which are shaped by their environment and interplay with local cells and molecules. Indeed, by specifically analyzing the cellular interactions occurring at the oral mucosa, recent work has uncovered the mechanism underlying the susceptibility of APECED patients to CMC, while studies analyzing platelet behavior in the peritoneum has revealed important interactions with macrophages that help control sepsis. These important new insights not only hold significant clinical potential in helping to treat invasive fungal diseases, but have also underscored how the study of antifungal immunity can enable a wider understanding of the functioning immune system.

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Microbial Genetics in Mycology

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Abbreviations

bp	Base pair
CCNV	Chromosomal copy number variation
CNV	Copy number variation
DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
GATK	Genome Analysis Toolkit
Gb	Gigabase
GC	Guanine-cytosine content
GWAS	Genome wide association study
HPD	Highest posterior density
ICU	Intensive care unit
INDEL	Insertion/deletion
ITS	Internal transcribed spacer
MLST	Multi-locus sequence typing
mtDNA	Mitochondrial DNA
N50	50% of the assembly is in contigs of this length or larger
NCBI	National Center for Biotechnology Information
ONT	Oxford Nanopore Technologies™
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PNW	Pacific Northwest
RFLP	Restriction fragment length polymorphism
RIP	Repeat Induced Point
RNA-seq	RNA sequencing
SNP	Single nucleotide polymorphism
SRA	Short Read Archive
TB	Tuberculosis
TE	Transposable element
TMRCa	Time to most recent common ancestor
WGS	Whole genome sequencing

Introduction

Fungi are a globally ubiquitous Kingdom, which diverged from the animal and plant Kingdoms approximately 1.5 million years ago (Wang et al., 1999). There are thought to be at least 140,000 fungal species that are heterogeneously defined based on morphological, physiological, mating and molecular features (Xu, 2020), making this Kingdom taxonomically diverse. Taxonomy has

separated this Kingdom based on morphological features into macroscopic fungi, such as those that produce fruiting bodies (i.e., mushrooms), and microscopic fungi, such as yeasts and molds. Yet the advancement of genomics has allowed other features, such as DNA sequences, to be incorporated to help define species (Taylor et al., 2000), and genotype information is now increasingly used in the identification of new and cryptic fungal species.

Current taxonomic classifications group fungi into eight phyla: zoosporic fungi (Cryptomycota, Chytridiomycota and Blastocladiomycota), Microsporidia, Zoopagomycota, Mucoromycota and Dikarya fungi (Ascomycota and Basidiomycota). There are increasing numbers of emerging fungal pathogens in all phyla, either due to altered pathogenicity or the availability of new biological niches, infecting animals, plants and humans (Fisher et al., 2016), providing public health and conservation threats, as well as threats to food security (Fisher et al., 2018). There have been a number of emerging pathogenic fungal species which have needed the application of high-throughput genomics in order to understand the mechanisms driving evolution of virulence, drug resistance and pathogenicity. For example, in the last decade, the newly described chytrid fungus *Batrachochytrium salamandrivorans* (Bs) has caused rapid declines in salamander populations in Europe (Martel et al., 2013, 2014); *Cryptococcus gattii* has expanded its range from endemic to non-endemic regions (specifically the Pacific Northwest (PNW)), causing fatal disease in humans (Fraser et al., 2005); and the emergence of *Candida auris*, a pathogenic yeast causing nosocomial disease (Sato et al., 2009). Research into these emerging fungal pathogens, among others, have highlighted elaborate species complexes, alongside highly recombinogenic and dynamic genomes (Farrer et al., 2013a; Fisher et al., 2013), which drive micro- and macro-evolutionary changes capable of ultimately overcoming host defenses, and survival in non-endemic regions. The complex life cycles of fungi means fungal genomes are capable of undergoing recombination due to sexual and parasexual reproduction, and clonal propagation due to asexual reproduction, leading to novel genetic diversity and evolving pathogenic lineages. It is clear that there is a need to understand the mechanisms of evolution, which can only be addressed using genomics.

Genomics in mycology came into its own after the completion of the yeast *Saccharomyces cerevisiae* draft genome in 1996 (Goffeau et al., 1996; Mewes et al., 1997). Since then, the number of complete genome sequences for fungi have increased exponentially, with re-sequencing projects producing hundreds of genome sequences for one fungal species. With this large amount of data, our understanding of genome evolution, ecological and geographical distributions (Vanhove et al., 2016), population structure (Rhodes et al., 2017b) and fungal reproduction (Farrer et al., 2011) have advanced. The fall in the cost of WGS (Balloux et al., 2018; Rhodes et al., 2014) has also enabled large projects dedicated to the evolutionary processes acting within populations and species that lead to adaptation and phenotypic differences (Liti et al., 2009; Peter et al., 2018; Rhodes et al., 2017a,b).

In addition to WGS, other “omics” approaches have been utilized, with great success, for various species of fungi to illuminate their biology. Transcriptomics, proteomics and metabolomics have impacted our current understanding of fungal biology, which when used in combination with WGS can comprehensively scrutinize an entire system. Whilst RNA-seq can confirm gene predictions and enhance gene annotations (Bruno et al., 2010; Zhao et al., 2013), it can also be a useful tool for identifying changes in gene expression and alternate gene splicing, which may have roles in drug resistance (for example, the increase in *erg11* expression in *Cryptococcus neoformans* (Sionov et al., 2010)) and virulence respectively (Grützmann et al., 2014). Some studies have used RNA-seq to identify changes in gene expression required for host survival, highlighting the complex nature of crosstalk between fungal pathogens and their hosts, especially when combined with host RNA-seq during infection (Chen et al., 2013; Muñoz et al., 2019).

Other advancements in ‘omics’ approaches can also infer methylation, a hallmark of epigenetic changes. Whole-genome bisulphite sequencing can profile cytosine methylation to 5-methylcytosine (5-meC) within cytosine-rich genomic CpG isolates. Whilst 5-meC seems to be absent in some fungi (e.g., *Saccharomyces* and *Pichia* (Capuano et al., 2014)), in *Neurospora* 5-meC within CpG islands are found within ex-transposons that are targets of RIP mutations (Selker et al., 2003). Hosseini et al. combined whole-genome bisulphite sequencing with RNA-seq for 10 *Neurospora* isolates to investigate genome-wide DNA methylation; they confirmed a conserved link between DNA methylation and RIP mutations, with TE content and DNA methylation patterns showing a phylogenetic signal also (Hosseini et al., 2020). It is likely that *Neurospora* genomes are influenced on an evolutionary level by the interplay between TEs and the host defense. It is perfectly feasible, therefore, that similar studies could be carried out in other fungi, such as *Cryptococcus* species, which are associated with TEs (Wang et al., 2010), to investigate the role of epigenetics in mechanisms of infection.

In recent years, rapid “real-time” sequencing has been used to sequence fungal isolates, particularly within an outbreak. The development of the Oxford Nanopore MinION (discussed further below) device allows sequencing to be completed in under 48 h within one’s own laboratory, instead of relying on a centralized sequencing center. MinION has been used successfully on fungal isolates (Dutreux et al., 2018; Rhodes et al., 2018), and it is feasible to assume that this could be used to provide rapid transcriptomic data also, akin to what has been observed in viral genomics (Kim et al., 2020; Moldován et al., 2018; Tombácz et al., 2018). The generation of long reads (as opposed to short reads up to 500 bp in length) of 1 kb or more using this rapid sequencing technology also has the potential to make the generation of draft reference genomes more common (Rhodes et al., 2018). This in turn would allow alignment-based analyses to be carried out. However, alignment-free approaches also have their place, which will be discussed below.

Whilst these approaches have mostly been applied to fungal pathogens of crops, humans and wildlife, there is also a need to apply to populations of fungi that are important for food production and ecosystem maintenance. The baker’s yeast *S. cerevisiae* has been used in fermentation throughout human history, creating domestication events, but is largely neglected by scientific research. Liti et al. applied genomics to produce WGS of more than 70 *S. cerevisiae* isolates, finding phenotypic variation and populations that were delineated along geographic boundaries (Liti et al., 2009), and found that human influence has led to cross-breeding and production of new variants. This study was followed up in 2018 with a large scale population genomics survey of over one thousand

S. cerevisiae genomes which supported an “out-of-China” origin for this fungal species (Peter et al., 2018). By using additional ‘omics approaches, such as transcriptomics and proteomics, there could be significant advancements on the understanding of significant gene regulation differences during alcohol fermentation, and potentially link these to specific phenotypic differences.

Using genomics to characterize variation in complex fungal genomes

Due to the complex nature of fungal genomes, variation can arise and manifest as single nucleotide polymorphisms (SNPs), INDELs, recombination, hybridisation, CNVs and CCNVs. There may also be larger, structural variation such as chromosomal rearrangements (such as inversions, translocations or deletions), which can be responsible for gains or losses of genes or entire segments of the genome. Finally, transposable elements can replicate through transposition, impacting the plasticity, architecture and evolution of fungal genomes (Lorrain et al., 2021).

These variations are detected post-sequencing during bioinformatic analyzes, where two or more isolates are aligned and compared for changes indicative of SNPs; these SNPs may cause an amino acid substitution (a non-synonymous mutation), or due to redundancy in the genetic code, the amino acid may remain the same. The advancement of next-generation sequencing has allowed the identification of CNVs and CCNVs (and partial CCNVs, such as a chromosome arm) by noting changes in the depth of sequencing relative to normalized coverage.

Hybridisation between organisms can generate progeny with novel genetic variations, and has been reported in most eukaryotes. It is hypothesized that as the genetic distance between parental isolates increases, heterosis (where progeny display traits that are superior to their parents) becomes more prevalent (East, 1936). Whilst heterosis is associated with phenotypic effects, it is possible to identify heterotic quantitative trait loci through genome-wide association studies (GWAS) using whole genome SNPs.

Whilst these sources of variation aid our understanding of the epidemiology of infection, virulence factors and potential drug resistance, they require careful detection due to the complex nature of fungal genomes. Fungal genomes are, on average, smaller than their animal and plant counterparts, yet they are generally larger than bacteria and viruses. The average genome sizes of Ascomycota and Basidiomycota fungi are 36.91 and 46.48 Mb respectively, and range from 8.97 to 177.57 Mb. Therefore, the computational processing power required is greater than that of viral and bacterial genomics, along with a need for more storage space for those data, which may be beyond the financial capacity of some laboratories. Prior to bioinformatic analysis, the quality of the sequence data needs to be high, with sufficient coverage to confidently call these variant. In order to do this, the DNA extraction method and library building protocols need to be carefully considered, before deciding on multiplexing and the sequencing machine used. For example, comparing Illumina TruSeq Nano DNA extraction kit provided better quality data and higher coverage at regions of low GC content when compared to the New England Biolabs NEBNext Ultra DNA extraction kit (Rhodes et al., 2014); it is therefore important to consider the percentage GC content of the genome prior to selecting a DNA library preparation kit.

Before undertaking sequencing, researchers must consider the quality of the reference genome (if there is one) and whether there is a thorough understanding of the organisms’ genetic makeup and relatedness to other known species. If one of these points are unknown, or even debateable, then de novo assembly, using long reads or implementing a hybrid assembly of long and short reads, would be required. Otherwise, alignments using short or read reads against a preselected reference genome can be completed. Alignments are generally quicker than assemblies, and the “best practices” for variant calling are more established from alignments than from assemblies. Generating alignments will be discussed in more detail below.

Assemblies

Giordano et al. presented a comprehensive comparison of two long read sequencers, PacBio and MinION, and the short-read Illumina MiSeq platform (Giordano et al., 2017). Long read sequencers are capable of producing reads of over 10,000 base pairs, with reports of maximum lengths of 100,000 bp, which can overcome complex genetic features such as repetitive regions (repeats) and copy number variations. As such, long reads are deemed as the “gold standard” for reference genome assembly. In comparison, the typical length sequenced for short reads is between 50 and 400 bp (Goodwin et al., 2016); as a result, attempts at assembling creates a fragmented genome, with contigs that are much smaller than the actual chromosome size. However, a drawback of long read sequencing is the higher rate of sequencing errors (5–20%) compared to the short read data (<1%) (Goodwin et al., 2016). These errors could negatively impact de novo assembly (where there is no a priori knowledge of the correct sequence order) for genome construction as the errors are not always randomly distributed. Therefore, it is common practice to combine short- and long-read sequences in a hybrid assembly, where scaffolding of high quality short-read assemblies is carried out by bridging the gaps using long-read data, and therefore resolving the high error rate and repetitive regions.

The cost of sequencing cannot be overlooked, as it is a major consumable, and sometimes financially unachievable, especially for laboratories in developing nations. Currently, the initial cost for the PacBio RS II platform is approximately US\$700,000, and costs US\$300 per Gigabase (Gb) of data generated. The recently released PacBio Sequel (costing US\$350,000 initially for the platform) has, however, decreased the cost per Gb sequenced to US\$13–26 (Fatima et al., 2020).

Oxford Nanopore Technologies™ (ONT) developed a disruptive sequencer, the handheld MinION, in 2014. ONT offer the MinION Mk1B platform for US\$1000, and charging approximately US\$300 per Gb (when purchasing flow cells in bundles). Coupled with the low computing requirements and short laboratory preparatory work, this was an attractive option for many genomics groups. More recently, ONT produced high-throughput platforms, such as the GridION (5 flow cells) and PromethION

		Initial platform cost	Cost per Gb
MinION		£240	US\$9
PromethION 48		£24,000	US\$0.16
MiSeq		US\$125,000	US\$350
Sequel		US\$350,000	US\$13-26
RS II		US\$700,000	US\$300

Fig. 1 Comparison of sequencing platforms, their initial setup cost and the cost per Gb sequenced. Third-generation sequencing platforms, namely those producing long read sequences, include the MinION and PromethION (both from Oxford Nanopore Technologies) and the Sequel and RS II from PacBio. Short read technology has been dominated by sequencing platforms such as MiSeq, developed by Illumina.

(24 or 48 flow cells), bringing the cost of sequencing in parallel with the PacBio Sequel platform (approximately US\$21–24 per Gb). ONT do however charge a software license and annual warranty for the PromethION of between US\$20–30,000, for PromethION 24 and 48 respectively. These sequencing platforms are compared in Fig. 1.

In their report, Giordano et al. found that both PacBio RS II and ONT MinION provided excellent continuity and completeness for nuclear chromosomes of *S. cerevisiae*, but not for the mitochondrial genomes (although the PacBio data did provide a more complete mitochondrial genome compared to MinION data (Giordano et al., 2017)). This presents a significant challenge, as mitochondrial genomes are essential for molecular dating as mtDNA provide uniparental inheritance. Mitochondrial genomes are used for phylogenetic and phylogeographic analyses of taxa as there is a lack of recombination, and is therefore more likely to approximate the history of divergence among species than other loci (Pozzi et al., 2014). In addition to lower depth with respect to the PacBio data, the MinION data also had a higher than average homopolymer content by at 30% (Giordano et al., 2017). In order to rectify this, improving in the polishing step of de novo assembly is required, which is both time consuming and computationally intensive.

These long-read only assemblies did improve when scaffolding the MiSeq data with the long reads (a hybrid assembly), but varied depending on the assembler used; whether scaffolding with ONT MinION reads or PacBio reads, the npScarf assembler (Cao et al., 2017) performed better than HybridSPAdes (Antipov et al., 2015) and SMIS (available from <https://github.com/fg6/smis.git>), assembling a more contiguous sequence, a better N50, and overall, requiring less computational power. Therefore, it is extremely important to consider the sequencing technology and software for handling sequence data from fungi when performing de novo assemblies.

Alignments

Sequence alignment arrange two or more sequences (whether nucleic or protein sequences) in order to identify regions of similarity and dissimilarity, which may indicate regions of evolution and functionality. Aligners that arrange two sequences (pairwise alignment) are usually based on the Smith-Waterman or Needleman-Wunsch algorithms. Both create a substitution matrix based on a scoring scheme, but the former is primarily concerned with local alignments, whilst the latter is concerned with global, complete sequences. The most commonly used aligner, BWA-mem (Li and Durbin, 2009), is based on the Needleman-Wunsch algorithm, and is recommended for high-quality queries, and is faster and more accurate than other versions of BWA. Bowtie2 (Langmead and Salzberg, 2012) is also memory and time efficient, and is also optimized for the alignment of RNA-seq data to a reference genome.

The importance of a high quality reference genome cannot be overstated. Reference genomes differ from draft reference sequences due to their completeness i.e., a low number of gaps in the sequence. It is also expected that a high-quality reference genome will have a low number of errors, and high percentage of sequence assembled into chromosomes (The Vertebrate Genome Project, 2018). Ideally reference genomes will also have gene annotation data also. If the reference genome is mis-assembled or there has been PCR error introduced into the reference genome, this will likely be reflected in the variant calling. If the assembly quality of the reference genome is poor, this could also lead to larger variation, such as structural variation, being mischaracterized or missed completely. Lastly, the alignment of short read sequences will also not be of high quality in the presence of high genetic variability, which may be experienced in genomes that have introgression events or high mutation rates. The Fungal Genome Initiative at Broad Institute, United States, has amassed a large collection of gold-standard, high-quality fungal reference genomes spanning a wide diversity of fungi. These reference genomes can be downloaded from the Broad Institute FTP site or NCBI, which is the primary repository for all genomic data. Links to examples of good quality reference genomes, the majority of which have gene annotation, can be found here: <https://www.broadinstitute.org/science/projects/fungal-genome-initiative/current-fgi-sequence-projects>.

Once sequence data has been aligned to a reference genome, variant calling can be performed in order to identify SNPs and INDELs. The GATK (McKenna et al., 2010) variant caller “HaplotypeCaller” can call SNPs and indels simultaneously via local de novo assembly of haplotypes in an active region. This allows for more accurate calling of variants, particularly in regions containing different types of variants close together. It is worth considering the ploidy of your organism of study, as some variant callers can only consider homozygous or heterozygous (biallelic) sequences, whereas some fungal species display trialleles and/or polyploidy (such as *B. dendrobatidis*, which display polyploidy (Farrer et al., 2013a)). Reads that contain multiallelic loci may not map to the reference genome correctly, resulting in the underrepresentation of diverging haplotypes (Pfeifer, 2017). GATK HaplotypeCaller currently requires ploidy to be defined as a parameter, as either haploid or diploid; therefore, alternatives for aneuploidy and polyploidy are required. The variant caller FreeBayes (Garrison and Marth, 2012) has become more popular as it calls variants based on Bayesian statistical frameworks and is capable of modeling multiallelic loci. The variant caller BISCAP (Farrer et al., 2013b) is computationally inexpensive and is able to call variants from polyploid organisms due to its use of the binomial probability distribution for expected error rates post-alignment. SNP calling has been extensively reviewed elsewhere (Altmann et al., 2012; Mielczarek and Szyda, 2016; Pfeifer, 2017), however there are some drawbacks, especially when applied to fungal genomes which are classed as “non-model organisms” (with the exception to *S. cerevisiae* and *Schizosaccharomyces pombe*). For example, some genes related to pathogenicity are located within repetitive regions. *Phytophthora infestans*, whilst strictly not a fungal pathogen but an oomycete, has been shown to possess a “two-speed genome,” with gene-dense and gene-sparse, yet repeat-rich, regions (Raffaele et al., 2010). In the case of *P. infestans*, it has been shown that gene-sparse regions (GSRs) contain effectors, such as a class of translocated proteins called RXLRs, and other pathogenicity factors (Haas et al., 2009) which have been shown to evolve rapidly and adapt to host defenses (Raffaele et al., 2010). Two-speed genomes, with GSRs rich in effector-like genes involved in pathogenicity, have also been identified in plant fungal pathogens such as *Fusarium* species (Laurent et al., 2016; Fokkens et al., 2018; Wang et al., 2017). In *F. graminearum* for instance, within these “fast subgenomes,” genes known to be involved in pathogen-host interactions (PHI) and pathogen-associated genes are all overrepresented (Wang et al., 2017). In some cases, such as the barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (Frantzeskakis et al., 2018), and the wheat stripe rust fungus *Puccinia striiformis* f.sp. *tritici* (Schwessinger et al., 2018), secreted effector proteins are not predominantly found in GSRs; a one-speed genome has been proposed for these fungal pathogens (Frankzeskakis et al., 2019). Current research projects are focusing on assessing two-speed genomes in other fungal pathogens, including those affecting animals and humans.

Alignment-based approaches may not be able to characterize these repetitive regions, and indeed, most gold-standard variant calling pipelines for various fungal organisms exclude repetitive regions due to false positives (Chen et al., 2017; Lockhart et al., 2017; Rhodes et al., 2018). Therefore, some variants relating to pathogenicity are likely being missed in a multitude of genomics studies.

It is therefore extremely important to consider the tools and data currently available before undertaking a study involving short read sequencing. Whilst fungal genomes are relatively small, and can mostly be sequenced in the haploid phase, some fungi display aneu- or polyploidy, heterozygosity and phasing which can present issues in downstream analyses.

Taxonomy

Although nearly 150,000 species of fungi have been described, it is estimated that between 2.2 and 3.8 million are estimated to exist (Hawksworth and Lücking, 2019). Ascomycota and Basidiomycota, which together form the subkingdom Dikarya, diverged 1.2 billion years ago; this is in stark contrast to some diversifications within the plant kingdom, where mosses and vascular plants only diverged 680 million ago (Berbee and Taylor, 2010).

Previously, fungi were characterized based on macro- and/or micromorphological features (Singer, 1986). However, with advancements in technology and biomedical science, classification of fungal species has grown to include ecological factors, physiology, reproduction, and recently, DNA sequences. Whole genome level approaches are becoming increasingly employed as they overcome resolution issues faced in single- or multi-loci studies. Commonly used species concepts are summarized in Table 1.

The human fungal pathogens *C. neoformans* and *Cryptococcus gattii* have been the subject of much discussion over its species concept in the last decade. In their study, Hagen et al. using phylogenetic species concept and genetic diversity of 11 genetic loci in 115 isolates to recognize seven species: *C. neoformans* var. *grubii* (now referred to as *C. neoformans*) and *C. neoformans* var. *neoformans*

Table 1 Species concepts in fungi.

Species concept	Key criterion	Early proponent	Fungal examples
Biological	Interbreeding to produce viable and fertile offspring	Mayr 1942	Kerrigan et al. (1994)
Ecological	Shared ecological niche	Van Valen 1976	Trudell et al. (2017)
Evolutionary	Shared evolutionary history	Simpson 1951	Kwon-Chung et al. (2017)
Genotype	Distinct genotypes	Mallet 1995	Banke et al. (1997)
Morphological	Distinct features	Regan 1926	Linnaeus (1753) and Zang (1987)
Phenetic	High phenotypic quantitative similarity	Michener 1970	Singer (1986)
Phylogenetic	Monophyly	Rosen 1979	Hagen et al. (2015) and Wu et al. (2016)
Recognition	Shared mate recognition	Paterson 1985	Callac et al. (2003)

Adapted from Xu J (2020) Fungal species concepts in the genomics era. *Genome* 63: 459–468. <https://doi.org/10.1139/gen-2020-0022>.

(now referred to as *C. deneoformans*), and five species within *C. gattii* (Hagen et al., 2015). Kwon-Chung et al. proposed the new naming system was compatible with advances in phylogenetic theory, and recognized the complex biodiversity within the agents of cryptococcosis (Kwon-Chung et al., 2017). However, there were disadvantages to the proposal put forward by Hagen et al., including the low number of isolates tested (115), and the inclusion of only 11 genetic loci, which were used for an MLST-based phylogenetic analysis, representing 43% of the *Cryptococcus* chromosomes. Therefore, the full effect of genetic diversity and recombination on taxonomic classification of *Cryptococcus* species cannot be properly inferred. It has also been shown that *C. neoformans* and *C. gattii* are able to reproduce asexually, producing clonal isolates, therefore the biological species concept model may not be appropriate here. Given the production of lineage hybrid isolates, it was not clear whether reproductive barriers prevented gene flow between the newly proposed species. Indeed, genetic exchange is thought to occur between *C. gattii* lineages (now recognized as five separate species: VGI as *C. gattii*, VGII as *C. deuterogattii*, VGIII as *C. bacillisporus*, VGIV as *C. tetragattii* and VGIV/VGIIIc as *C. decagattii*), and is proposed to be responsible for the high genetic diversity, as well as the existence of clonal populations and populations resulting from sexual reproduction (Muñoz et al., 2018b).

Despite these concerns, Hagen, along with a substantial amount of researchers within the *Cryptococcus* community published their rebuttal in 2017 stating these species complex should be accepted (Hagen et al., 2017). As such, scientific literature generally accepts and uses the proposed nomenclature for *C. neoformans*, yet lineage names are still widely used for *C. gattii*. The proposed species nomenclature seems to have been premature here, especially with the discovery of a new lineage, VGV, where the typing method RFLP could not distinguish between this new lineage and VGIV (Farrer et al., 2019). Indeed, this lineage could only be distinguished using WGS, and subsequently, a novel RFLP method developed specifically for this lineage; it is therefore crucial to attain all the available genetic data via WGS before assigning novel species nomenclature, and document historical reproductive isolation through a genealogical concordance phylogenetic species recognition (GCPSR). This approach would identify shared genealogical partitions between lineages across multiple loci (up to whole genome level) as evidence of isolation, and thus, evidence of a species (Taylor et al., 2000). Given the availability of over 1000 whole genome sequences for *Cryptococcus* species, the resolution is accessible in order to document GCPSR within *C. neoformans* and *C. gattii*.

Xu (2020) proposed a Genome-sequence based classification and identification (GSCI) system, which would contribute to the stability of fungal taxonomy and accelerate the discovery of new fungi (Xu, 2020). Currently, nearly 6000 fungal genome sequences are available in NCBI, covering over 1000 fungal species; this wealth of data could provide enough resolution for investigations to establish species boundaries.

Sequence-based classification via barcoding

Many taxa cannot currently be classified by current species concept (Table 1) and nomenclature rules. It is hoped that with the increased availability of WGS that classification efforts will shift towards genome-based identification. However, DNA barcoding will likely remain important for some time.

Due to challenges in modern fungal taxonomy, there is an emphasis on genealogical approaches, including genealogical concordance (discussed briefly above) and phylogenomics. However, in many cases, DNA barcoding using the internal transcribed spacer (ITS) remains an option in metabarcoding studies for the identification of fungi. These metabarcoding studies trade accuracy and precision for speed, however they rely on the curation of databases in order to assign taxa. Therefore, definitive answers are not always possible as the reference database may be incomplete or improperly labeled (Nilsson et al., 2006). Some metabarcoding pipelines, such as QIIME (Caporaso et al., 2010), utilize reference OTU picking, which are highly sensitive to the level of curation in the reference database, although open reference OTU picking allows recognition of sequences that do not have a close match.

Environmental DNA (eDNA) metabarcoding is a novel method, and can rapidly document unknown fungal diversity, however the fungal community remains divided as to how best to name these species. Sequence-based nomenclature seems to be a viable approach. Environmental metabarcoding studies have provided 16 billion fungal ITS reads (Lücking et al., 2021), encompassing a huge amount of fungal diversity. Yet this diversity is not captured by culture-based sequencing, and a large amount of these data

may be incorrectly or incompletely named. There is also potential error here, hidden in the lowest common ancestor (LCA), which is included in pipelines such as QIIME and is commonly used in environmental metabarcoding. Therefore, metabarcoding data have to be interpreted with great care.

The use of genomics in the detection and management of fungal outbreaks

Genomics has proven to be a valuable tool for the analysis of viral and bacterial pathogen outbreaks (Faria et al., 2016; Quick et al., 2015, 2016); it was therefore a logical step to apply the use of genomics to outbreaks of fungal pathogens.

Recently, the human fungal pathogen *Candida auris* has emerged as a major global public health concern due to high drug resistance and high mortality rates, and high propensity for nosocomial transmission (Lockhart et al., 2017; Schelenz et al., 2016). Although first described in 2009, the first hospital outbreak was reported in 2016 in a central London specialist cardio-thoracic hospital (Schelenz et al., 2016); at the time it was the largest outbreak of *Corynebacterium auris* observed globally. Since then, *C. auris* has caused a “stealthy pandemic” (Rhodes and Fisher, 2019), emerging in all continents with the exception of Antarctica.

WGS has played a crucial role in shaping our understanding of *C. auris* and its life history. Considering prior to 2015 little was known about the genetics of this important fungal pathogen, as of early 2021 there are nearly one thousand deposited whole genomes within NCBI SRA. These genomic studies have allowed thorough investigation into the *C. auris* genome, showing it to possess over 5000 protein coding genes (Lockhart et al., 2017; Rhodes et al., 2018) and expresses several virulence factors (Larkin et al., 2017; Sherry et al., 2017), such as biofilm formation and adherence, similar to *C. albicans* (Borman et al., 2016; Larkin et al., 2017). These discoveries have been made possible by combining WGS and gene annotation pipelines, and later with RNA-seq guided gene predictions (Muñoz et al., 2018a).

Yet the largest role genomics have played in improving our understanding of *C. auris* has been in the investigation of outbreaks and transmission events. Whilst genomics are commonly used in viral and bacterial outbreaks (Ebola (Quick et al., 2016), Zika (Grubaugh et al., 2017), SARS-CoV-2 (Lu et al., 2020) and cholera (Weill et al., 2017, 2018), for example), genomics has not been regularly employed in outbreak investigation due to the complexity of fungal biology and large genomes. And whilst fungal infections are not new, they have only recently increased in prevalence (for both human and plant infections) over the last three decades (Fisher et al., 2013), as illustrated in Fig. 2.

Chow et al. estimated a Bayesian molecular clock phylogeny, dating the origin of each *C. auris* clade to within the last 360 years, and outbreak forming clades originating in the last 40 years (Chow et al., 2020). A recent study has suggested a marine origin for *C. auris* (Arora et al., 2021); it is therefore a logical step to include these marine isolates within the current global collection of WGS *C. auris* isolates to date the emergence of outbreak forming lineages and identify a likely geographic origin.

Genomic data can also be used to reconstruct infectious disease transmission; software such as *TransPhylo* (Didelot et al., 2017) can indicate who infected whom, including the potential existence of unsampled individuals who may act as missing transmission links. *TransPhylo* has been used successfully to create transmission networks for viral outbreaks of mumps (Stapleton et al., 2019) and bacterial outbreaks of TB (Hatherell et al., 2016), yet has not been applied to a fungal pathogen. Despite infection control measures being in place, an outbreak of *C. auris* in a neurology ICU was only reduced upon removal of temperature probes, thus preventing transmission (Eyre et al., 2018). It would be interesting to use WGS combined with transmission network software such as *TransPhylo*, to see whether the incidence of new cases could be reduced in a shorter timeframe.

Identification of mutation rates and molecular clocks

In order to understand molecular change(s) in terms of geological time, we need a molecular clock to infer the relationship between evolution and the genotypes and phenotypes observed. This is of particular interest for outbreaks of pathogenic bacterial and viral infections, and is becoming increasingly questioned in relation to outbreaks of pathogenic fungal infections to determine when the

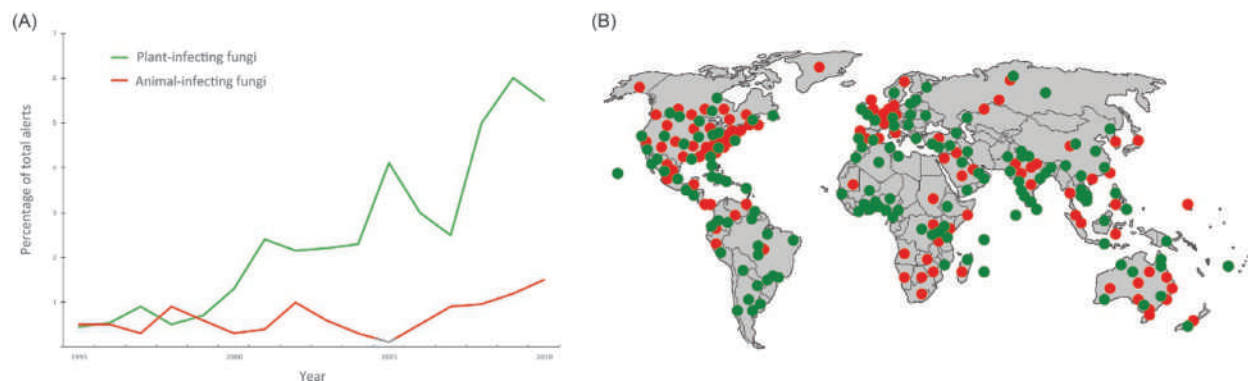


Fig. 2 Recently, there has been an increase in prevalence of both plant- and animal-infecting fungi worldwide.

organism was introduced into the setting (whether this is the country or a specific place, e.g., a hospital) and emergence of drug resistance. In recent years, the dating of a molecular clock has been investigated in outbreaks of many fungal pathogens, including *B. dendrobatidis* (O'Hanlon et al., 2018), *C. gattii* (Roe et al., 2018) and *C. auris* (Chow et al., 2020; Rhodes et al., 2018). Heckman et al. were the first to apply multiloci data to date fungi, and assumed that sampling many genes would average out gene-specific selective pressures (Heckman et al., 2001). As a result, their study produced inconsistencies between fossil records and molecular data, which raised the question: which one is correct? If the molecular dates were incorrect, this would suggest a misunderstanding in molecular evolution (Berbee and Taylor, 2010).

One assumption that underpinned Heckman's study was that the molecular clock had the same rate for all eukaryotes. However, a few years later Peterson et al. showed that it was possible to assume multiple calibration points to show that molecular clocks do not have a uniform rate (Peterson et al., 2004). Within the same study, Peterson showed that by picking a slowly evolving clade for calibration, Heckman et al. estimated older ages than expected. Therefore, rate variation needs to be a feature when estimating molecular evolution in modern approaches to molecular clock rates. This can be achieved by allowing rates to vary across the different branches of a tree, which is implemented in Bayesian coalescent-based algorithms, such as BEAST (Drummond and Rambaut, 2007); this is referred to as a "relaxed molecular clock." As previously discussed with other computational tasks, dating the molecular clock in fungi is intensive.

The data type used for molecular clock analysis needs to be carefully considered also; some studies have used high quality non-recombining segments of the mitochondrial genome (O'Hanlon et al., 2018) (because, as discussed above, mitochondria provide uniparental inheritance, less recombination and can provide better estimates of divergence times), whilst others have used whole genome SNPs without regions of recombination removed (Chow et al., 2020; Roe et al., 2018).

C. gattii had predominantly been regarded as a tropical pathogen, causing a range of symptoms, including cryptococcal meningitis. The origin of *C. gattii* has been identified as being in South America, likely Brazil (Engelthaler et al., 2014; Hagen et al., 2013), whilst some studies place its origin in Australia (Billmyre et al., 2014). However, a lineage of *C. gattii*, VGII, first appeared in British Columbia in the PNW in 1999 (Byrnes et al., 2009; Kidd et al., 2007). Roe et al. used whole genome SNPs before assessing the temporal signal of the dataset, implementing root-to-tip regression analysis, whereby the tips were dated with isolate collection date (Roe et al., 2018). They then inferred evolutionary rates and the TMRCA in BEAST. The TMRCA for VGIIa, VGIIb and VGIIc were estimated at 87.99, 81.43 and 66.29 years prior to 2018. A logical progression of this study would be to estimate the divergence of VGII and other *C. gattii* lineages, and the date of *C. gattii* lineage arriving in the PNW.

Roe et al. tested the statistical fit of 10 different clock and demographic model combinations, which resulted in the relaxed lognormal clock being the best fit for VGIIa, and a strict clock being the best fit for lineages VGIIb and VGIIc. This benchmarking analysis highlights how the same molecular rate is not necessarily the same across eukaryotes; the VGII lineages are closely related, with chromosome structure highly conserved between the lineages (Farrer et al., 2015), yet the molecular rates are different. Despite this, the mutation rates for VGIIa and VGIIc, which used different molecular clocks, had similar rates: 1.59×10^{-8} SNPs per base per year (95% HPD, 5.54×10^{-9} to 2.93×10^{-8} and 5.54×10^{-9} to 2.04×10^{-8} respectively). VGIIb on the other hand, was calculated to have a mutation rate of almost double that of VGIIa and VGIIc, at 2.70×10^{-8} SNPs per base per year (95% HPD 6.75×10^{-9} to 5.23×10^{-8}). These rates were an order of magnitude higher than common neutral fungal mutations rates ($\sim 2.0 \times 10^{-9}$), which the authors suggested was due to microevolutionary rate because of successive clonal generations in the laboratory as opposed to a slower rate when using fossil records. The latter would likely be affected by purifying selection, whereas the PNW lineages are clonal in nature, leading potentially to higher mutation rates. Interestingly, whilst investigations into serially collected isolates of *C. neoformans* have also shown clonal relationships, some isolates display an inflated substitution rate due to nonsense mutations in DNA mismatch repair pathways leading to hypermutator evolution (Rhodes et al., 2017a).

The occurrence of hypermutator states has been confirmed in other studies on *Cryptococcus* species (Billmyre et al., 2017; Boyce et al., 2017), and also in *Candida* (Healey et al., 2016). Therefore there is a need to properly investigate and accurately estimate the substitution rates, which can be achieved by partitioning regions of recombination. This approach was used to date the origin of the *B. dendrobatidis* hypervirulent lineage BdGPL to the 20th century (Farrer et al., 2011). Another approach to molecular dating is to use mitochondrial DNA, which is non-recombining. This approach was used to date the emergence of *B. dendrobatidis* lineage BdASIA-1, which displayed hallmarks of an ancestral lineage, as the early 20th century, coinciding with the global expansion of commercial trade in amphibians (O'Hanlon et al., 2018).

Concluding remarks

Currently, whole genome sequencing techniques have surpassed typing methods to provide a wealth of data on fungal genomes for detection and characterization of genomic epidemiology, virulence traits and life histories. These data are an important weapon in the arsenal in the war against contemporary epidemics in plants, animals and humans (Fisher et al., 2018). Whilst there are still some challenges with these approaches, it is clear there are many advantages to using genomic techniques. It is therefore important to consider the tools being used in fungal species, and adopt "best practices." Whilst some of the gold-standard techniques require substantial financial and computational power, recent advancements (such as ONT sequencers) are making these approaches more available to those in developing countries; since the burden of fungal disease is highest in developing countries (particularly those that affect crops, which are mainly grown in developing countries (Fisher et al., 2013)), we in developed countries need to do more to ensure scientists in these countries are financially able to carry out research and avoid "helicopter science."

Finally, the rapid emergence of outbreaks caused by fungal pathogens requires collaboration between researchers on a global level in order to fully exploit and utilize the expertise available; it is clear that the current systems for the generation, storage and distribution of genomic data cannot be sustained unless collaborative efforts are initialized and maintained between global research groups. Together, these multi-factorial approaches will ultimately improve our understanding of fungal biology, and any threats posed to ecosystems, health, and biodiversity in an ever changing world.

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Immunology of Parasitism[☆]

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Glossary

Antigen presentation Process by which antigens are taken up, processed, and presented to the immune system.

Autoimmunity Pathology arising as the consequence of self-reactive immune cells failing to distinguish self and non-self and attacking host cells and tissues.

Cytokines Signaling molecules that are produced by immune cells to regulate the immune response.

Immune evasion Diverse set of mechanisms that allow the parasite to escape the host immune response.

Immunopathology Pathology arising as a consequence of an over-reactive, unregulated, immune response.

Resistance Host defence strategy aiming at clearing the infection or at least controlling parasite population size.

T-cell subpopulations CD4⁺, CD8⁺, CD25⁺ Classes of lymphocytes expressing different surface molecules. CD4⁺ T cells are helper lymphocytes that contribute to polarize the immune response towards a Th1 or a Th2 response. CD8⁺ T cells are cytolytic cells that participate in cytotoxic reactions. CD25⁺ T cells are regulatory lymphocytes that contribute to the resolution of ongoing immune response by the production of anti-inflammatory cytokines (IL-10, TGF- β).

Th1 and Th2 CD4⁺ cells Division of the CD4⁺ T cells depending on the type of cytokines they produce. Th1 response is characterized by the production of Interferon- γ and Tumor Necrosis Factor- α and is usually triggered by infection with intracellular parasites; Th2 response is characterized by the production of Interleukin-4, IL-5, IL-6 and is usually triggered by infection with extracellular parasites.

Tolerance Host defence strategy aiming at limiting the cost induced by the infection.

Introduction

Parasites, both micro and macroparasites, represent a pervasive threat for their hosts. Microparasites usually refer to viruses and pathogenic bacteria, whereas the term macroparasite indicates parasitic protozoa and helminths. Macroparasites have higher genomic complexity and life cycles that require either vectors either one or several intermediate hosts to be completed (complex life cycles). To give a glance of the diversity of the possible life cycles adopted by parasites inducing human diseases, we might mention malaria parasites that are transmitted from vertebrate host to vertebrate host by competent mosquitoes, filarial nematodes relying on black flies, or schistosomes that have water snails as intermediate hosts (Fig. 1).

Upon entering the vertebrate host, parasites elicit a complex array of immune defenses that may or may not clear the infection. A relatively broad feature of parasitic infections is chronicity. In other words, contrary to many microparasite infections that can induce acute fatality, macroparasites usually tend to establish long-lasting, chronic infections. This pattern suggests that macroparasites can withstand the host immune response. Indeed, protozoa and helminths have evolved a diversity of mechanisms that subvert the host immune system to allow their persistence within the host.

Humans still pay a high burden to infectious diseases and parasitic infections. Although many parasitic diseases are nowadays mostly distributed in the rural areas of tropical countries, during the last decades, we have witnessed the emergence/re-emergence of a number of infectious diseases in the countries of the northern hemisphere. Broadly speaking mechanisms of parasite pathogenicity can be divided into two large classes: the direct effect of parasite growth and/or multiplication within the host; the indirect effect of an over-reacting immune response.

[☆]Change History: January 2021. G Sorci updated all sections except the introduction. The section on resistance/tolerance and Figure 1 have been updated.

Schistosomiasis

Schistosoma haematobium, *S. intercalatum*, *S. japonicum*, *S. mansoni*, *S. mekongi*

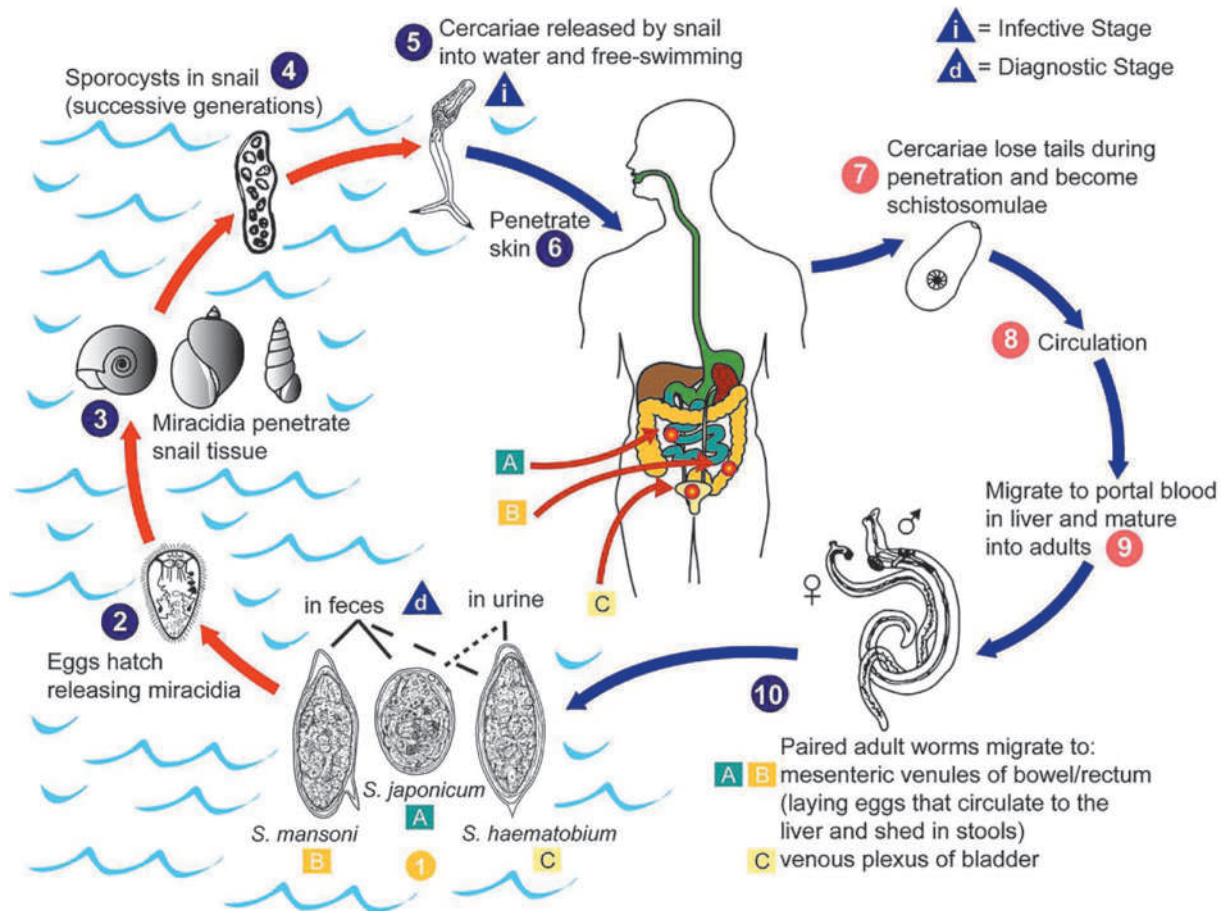


Fig. 1 Schistosome life cycle. Schistosome eggs are shed in the water with feces or urine. After hatching, miracidia infect different species of snails (the intermediate host), where they undergo successive generations of sporocysts. The infective stage (cercariae) is released into the water and infects humans through the skin. Within the human body, schistosomulae migrate through different organs. Adult male and female worms establish and reproduce in the mesenteric venules or the venous plexus of bladder, depending on the species. Source: Centers for Disease Control and Prevention, Public Health Image Library.

Parasite diversity and the diversity of immune effectors

As mentioned above, macroparasites regroup a large diversity of species with complex life cycles and a large spectrum of sites of infection in the final host. This diversity of parasite strategies elicits a variety of immune effectors in the final host; there is no universal response that provides protection to all macroparasites. This diversity of immune responses is illustrated by the following list of immune effectors and the corresponding parasite target: complement-fixing antibodies play a role against larval stages of cestodes, opsonizing antibodies can target extracellular protozoa, eosinophils play a role against schistosome eggs and larvae (Behnke, 1990; Kaufmann et al., 2002).

CD4⁺ T helper lymphocytes can differentiate into two distinct types that polarize the immune response into a cellular-type immune response (with the production of cytotoxic T-cells) and a humoral-type immune response (with production of antibodies). The two phenotypes (Th1 and Th2) are induced by a multitude of "environmental" factors, such as the activation of antigen presenting cells, the cytokine environment, or the nature and the dose of the stimulating antigen. Th1 and Th2 cells are characterized by a different profile of cytokine production. The production of interferon- γ mostly characterizes a Th1 polarized response, whereas interleukin-4 (IL-4), IL-5, or IL-13 are markers of a Th2 response.

Induction of Th1 versus Th2 response is associated with a variable pattern of protection/susceptibility to different parasites. For instance, infection with the protozoan *Leishmania major* in mice induces a Th1 response that confers protection, whereas Th2-prone individuals fail to control the infection. On the contrary, protection to nematodes and other helminth species is conferred by Th2-type response and Th1 contributes to establish a chronic infection. These observations subsequently led to the conclusion that

Th1 response should mostly be involved in the protection against intracellular parasites, whereas Th2 response should confer a protection towards extracellular parasites and pathogens.

The Th1/Th2 paradigm has been very useful to identify immune pathways that can lead to protection or susceptibility to specific parasites. However, the picture appears to be slightly more complex than this simple view, since other Th-cell phenotypes (Treg, Th17) and immune regulatory cytokines (IL-10, TGF- β) also intervene to establish the pattern of susceptibility/resistance to a given parasite.

As mentioned above, another source of complexity during a parasitic infection is that, contrary to viruses and bacteria, macroparasites express a diversity of antigens during their maturation within the final host. For instance, many nematode infections are acquired when the host ingests infective stages (eggs or L3 larvae). Upon ingestion, the larvae molt a few times until the adult stage is reached. Maturation can require the migration through different host tissues. For instance, *Ascaris* and *Strongyloides* parasites migrate into the lungs before establishing in the gut of infected humans. Other species, such as hookworms, that are acquired by a transdermal route (larvae penetrate through the skin) also migrate through different tissues before establishing in the gut. These infections clearly have the potential to elicit different immune responses in different tissues and to express a diversity of antigenic stimuli as long as they mature into the adult stage and they start to produce eggs.

Similarly, protozoa such as *Plasmodium* parasites have different stages that exploit different host tissues (liver, blood) and the immune response that is produced against one stage can be very ineffective against the other stage.

Innate immunity

Innate immunity to protozoa and helminths is the first line of defence that is activated within minutes/hours after the infection. Innate immunity is more and more recognized as playing a pivotal role in shaping the pattern of resistance/susceptibility. Innate effectors allow the immune system to keep parasite multiplication under control (for protozoa) or to kill infective stages of helminths until adaptive immunity takes effect.

As for the adaptive immune response, innate effectors can be divided into cellular and humoral mechanisms. Macrophages and granulocytes have the capacity to engulf and phagocytize invading organisms. The recognition by macrophages and granulocytes of non-self structures is based on receptors that bind to parasite antigens. For instance, the glycosylphosphatidylinositol (GPI) lipid anchor of many protozoa is recognized by protein kinases and induces macrophages to produce cytotoxic compounds and pro-inflammatory cytokines. Pro-inflammatory cytokines, such as TNF- α , IFN- γ and IL-12 stimulate another class of lymphocytes, the natural killer (NK) cells. NK cells further contribute to the production of pro-inflammatory signals such as IFN- γ and this early activation of NK has been shown to be important in the clearance of several intracellular protozoa (*Leishmania*, *Toxoplasma*, and *Plasmodium*).

Whereas macrophages, granulocytes and NK lymphocytes are cellular effectors mostly involved in the control of proliferating intracellular protozoa, mast cells and eosinophils have been suggested to be involved in the resistance to helminths. Eosinophilia is commonly observed in many helminthic infections. However, experimental evidence in support of the idea that eosinophilia is a defence against helminths is mixed and as it is usually the case, mast cells and eosinophils seem to play a role in conjunction with other effectors that facilitate their action, such as opsonic antibodies or NK cells. To date, the best evidence seems to indicate that eosinophils might play a role in the resistance to larval and egg stages of the trematode parasite *Schistosoma mansoni*.

Innate immunity can also involve humoral effectors. Complement activation is probably the most effective innate mechanism involving humoral effectors towards extracellular parasites. The complement system encompasses glycoproteins that are produced by monocytes and macrophages. Complement can be activated through two different pathways (antigen/antibody complex and mannose-binding lectin pathways). The cleavage of complement components (C) gives rise to elements that produce a cascade of events, such as the deposition of C3b on the surface of extracellular parasites, the production of C5a, a chemotaxis factor, or of C5b, initiating the membrane attack pathway.

More recently, group 2 innate lymphoid cells (ILC2) have been recognized to play a key role during the process of Th2-response against helminths in humans and animal models (Pelly et al., 2016). ILC2 produce key Th2-cytokines (IL-4, IL-5, and IL-13) and orchestrate immune (mast cell recruitment, alternative macrophage activation) and non-immune (mucus production, hypercontractility of smooth muscles) responses that help to expel helminths from the gut. Cross-talk between ILC2 and mast cells has also been shown, since Spi-B-deficient mice that have increased numbers of mast cells are more resistant (expel the parasite at a faster rate) to the infection with the gut nematode *Heligmosomoides polygurus*. This better resistance is due to the induction of IL-13 producing ILC2 (Shimokawa et al., 2017). In the same line, enhanced renewal of intestinal epithelial cells can also promote parasite expulsion in mouse models infected with the nematode *Trichuris muris*. Such "epithelial escalator" has been shown to be under control of IL-13 and the chemokine CXCL10 (Cliffe et al., 2005).

Adaptive immunity

As already mentioned, adaptive immunity relies on both cellular and humoral effectors. The universal feature of the triggering mechanism is the recognition of parasite epitopes by receptors that are expressed in different immune cells (T- and B-cells). Other cell types, such as the antigen presenting cells (APC), are devoted to the process and presentation of parasitic peptides to T- and B-cells through the action of transporter associated with antigen processing molecules (TAP) and MHC molecules that respectively transport and present parasite peptides to the cell surface where they can bind to T-cell receptors (TCR). Based on the type of CD4⁺

T cells that are activated during the infection, the acquired immune response will either involve the activation of another subfamily of lymphocytes (CD8⁺), with cytolytic properties, or the production of neutralizing antibodies by B-cells. Even though the two responses are mostly involved in the clearance of parasites with different ecological niches (intracellular vs. extracellular parasites), and although effectors of the Th1 response tend to have inhibitory effects on the Th2 response and vice versa, the two responses can also operate in concert to eliminate different stages of the same parasite exploiting different tissues. The dichotomy between innate and adaptive immunity is also quite artificial since innate and adaptive effectors do strongly interact to determine the issue of the interaction between the host and the parasite.

A Th2-type response induced by the activation of CD4⁺ T cells is the predominant mechanism of clearance of helminths. Studies on mice infected with gastrointestinal nematodes, such as *Heligmosomoides polygyrus* or *Trichuris muris*, have shown that Th2 cytokines (IL-4) are essential for a rapid expulsion of adult parasites from the gut. It should nevertheless be noted that other gastrointestinal nematodes might require the activation and production of other cytokines, such as IL-13, to be cleared and certainly there exists synergistic effects due to the interactive effect of different cytokines. These synergistic effects are nicely illustrated by studies where genes of different cytokines have been simultaneously knocked down.

Another interesting aspect of nematode infection is that they tend to persist over long period of time in humans and animal hosts. Chronicity of infection is generally due to the inhibition of the Th2 response by two different mechanisms. In certain cases, it has been shown that infection with *Trichuris muris* leads to a Th1 response that is beneficial to the parasite because it inhibits the most threatful Th2 response. Under natural conditions, hosts are exposed to a range of micro and macroparasites that elicit different branches and types of the immune response. It is therefore possible that microparasite infections that lead to a Th1 response set a favorable ground for the establishment of helminth infection. An alternative pathway by which gut nematodes might achieve to persist into the host for long periods of time involves the specific down-regulation of Th2 cytokines by regulatory cytokines such as IL-10 and TGF- β . A fascinating observation is that helminths produce mimics of these regulatory cytokines to manipulate the host immune system for their own benefit. This capacity of many parasite species to overrule the host immune system will be briefly treated in the following section.

Immune subversion

By definition, a successful parasite is a parasite that manages to complete its life cycle and to transmit to a novel host. To achieve this goal, parasites have to go through the selective filter that represents the immune system. A parasite that is recognized and killed by the immune effectors will have nil fitness and would not be able to establish a persistent infection. Therefore, there exists quite strong selection pressure to promote mechanisms that allow parasites to subvert the host immune system.

Parasites can subvert the host immune system using strikingly diverse pathways. They can interfere with the very early stage of immune recognition, they can block complement, avoid phagocytosis, escape vacuoles, interfere with antigen presentation, hide and become invisible to the immune system by coating their surface or change identity by expressing different sets of surface proteins (antigenic variation) (Sacks and Sher, 2002). Finally, some parasites can directly down-regulate the host immune response by stimulating the production of anti-inflammatory and regulatory cytokines (IL-10 and TGF- β) or even by producing mimics of these regulatory cytokines.

Antigenic variation has been reported for many protozoa that induce disease in humans. Among the best-known examples are *Trypanosoma* and *Plasmodium*. Trypanosomes escape the humoral immune response by coating the entire cell surface with a very dense layer of glycosylphosphatidylinositol (GPI)-anchored variant surface glycoprotein (VSG). During the infection, VSG express intense antigenic variation since different VSG genes are transcribed during the course of the infection. Although only one VSG gene is expressed at time, the pool of genes is fairly large and switches from one gene to another are relatively frequent. Therefore, during the course of the infection, new antigenic variants emerge and even though B-cell clones can keep track of the most prevalent antigenic variant, the emergence of new variants allows the persistence of the infection in the face of the host immune response.

A similar phenomenon has been described for *Plasmodium* parasites that express very variable surface proteins (*var*). As for trypanosomes, *Plasmodium* expresses different *var* genes during the course of the infection and therefore antibodies produced at a given time are effective to attack the most predominant variant but cannot keep track of the newly emerged variants.

Rodent malaria (*Plasmodium yoelii*) has also been shown to activate a specific family of T lymphocytes that have a regulatory function (Hisaeda et al., 2004). Treg cells are characterized by the CD25⁺ receptor and account for 5–10% of peripheral CD4⁺ T cells in mouse and humans. Treg cells contribute to ensure self-tolerance by suppressing auto-reactive lymphocytes, mostly through contact or via the secretion of anti-inflammatory cytokines, such as IL-10 and TGF- β (Belkaid, 2007). However, parasites might take advantage of these regulatory mechanisms for their own benefit.

Helminths are masters in the down-regulation of the immune response of their host (Maizels, 2005). Helminths usually occur with high prevalence in natural populations. In many instances, the infection persists in face of the host immune response, suggesting that helminths have evolved effective strategies of immune evasion and suppression. Adult helminths often survive within immunocompetent hosts by inducing immunoregulatory mechanisms involving regulatory T cells that result in a shift towards an anti-inflammatory environment characterized by high levels of anti-inflammatory cytokines. The hypo-responsiveness of the immune system prevents the elimination of the worm, but can also protect the host against excessive inflammation. *Heligmosomoides polygyrus*, a rodent nematode phylogenetically close to the human hookworms, has been shown to drive the differentiation of Treg cells from naïve CD4⁺ T cells by secreting a product that binds to host TGF- β receptors. Tregs appear to be

involved in the long-term establishment of infection with *Litomosoides sigmodontis*. Susceptible strains of mice are able to clear the infection when Tregs are experimentally removed.

Tregs also play a role in the dampening of Th2 response in humans infected with helminths. In some African populations, people carrying a schistosome infection have higher numbers of circulating Tregs compared to people who do not carry the infection. Interestingly, there might be a certain degree of heterogeneity in the potential of Tregs to shut down the immune response and this heterogeneity might be partly due to an active role of helminths. In agreement with this hypothesis, it has been shown that Tregs isolated from people carrying helminthic infections were more effective in reducing TNF- α production upon stimulation with *Plasmodium* antigens.

Immunopathology

Although the immune system is an essential component of organismal homeostasis involved in both parasite protection and surveillance of malignant cells, it can also represent a serious threat for the host structures (cells and tissues). To be effective the immune system has first to make a distinction between self and non-self. To achieve this task the immune system has central and peripheral control mechanisms. Negative selection of auto-reactive T-cell clones in the thymus and suppression of auto-reactive T-cells that have escaped the thymic filter are examples of the two types of control. In addition to avoiding self-recognition, the immune system has to keep control of an over-reactive response. Over reactive inflammatory responses can be particularly threatening because they do not rely on a specific recognition of self/non-self. Oxidative burst, production of cytotoxic compounds, cytokine storms, septic shock are a few examples of potentially very harmful side effects of the inflammatory response. Actually, immune disorders are now common diseases in western societies where the incidence of these autoimmune diseases has increased in a very substantial way during the last decades (Bach, 2002). In addition to autoimmune diseases, the immune system has been identified as representing a component of the most severe symptoms induced by some of the major infectious and parasitic diseases. Among the 10 most important tropical infectious diseases recognized by the WHO, all of them have an immunopathological component (Graham et al., 2005). For instance, the most severe symptoms of human malaria (cerebral malaria) are due to uncontrolled inflammatory response (van der Heyde et al., 2006). Similarly, uncontrolled immune response increases skin lesion and liver damage during the *Leishmania* infection and unregulated response damages the central nervous system in trypanosome infection. Therefore, unregulated immune response is an additional component of pathogenesis of most parasitic diseases (Sell, 2001).

Parasites that attenuate the immune response have, therefore, the potential to reduce the severity of infection where the symptoms are due to a dysfunction of the immune response (Sorci et al., 2013). In line with this argument, murine cerebral malaria induced by *Plasmodium berghei* is ameliorated by the inoculation of the filarial parasite *Brugia pahangi* (Yan et al., 1997) and the serum of *Heligmosomoides polygyrus*—*Plasmodium chabaudi* co-infected mice has lower concentration of pro-inflammatory (IFN- γ) and higher concentrations of anti-inflammatory cytokines (IL-10 and TGF- β) than *Plasmodium chabaudi*-infected mice. In humans, increased levels of pro-inflammatory cytokines associated with cerebral malaria are down regulated by a concomitant infection with the nematode *Ascaris lumbricoides* (Nacher et al., 2000). Again, the protective effect is due to the activation of regulatory T-cells and the secretion of high levels of IL-10 and TGF- β induced by *Ascaris lumbricoides* infection.

Autoimmune disorders are not exclusively produced by an ongoing infection. A noticeable proportion of cancers appear as the outcome of excessive inflammation and many other age-related diseases have an inflammatory origin (Coussens and Werb, 2002). Here, again, infection with parasites that rely on the dampening of immune reactivity for their own persistence within the host can provide the host with a benefit in terms of protection towards risk of lethal autoimmunity.

As for the reduction of disease severity, parasites seem to play a key role in the protection from autoimmunity (Table 1). Under certain circumstances, parasites that down-regulate the immune response might contribute to reduce the risk of autoimmune diseases or possibly alleviate the symptoms of the disease (Table 1). There are several epidemiological and experimental reports showing that infection with helminths is negatively correlated with the incidence of several autoimmune disorders (Dunne and Cooke, 2005). For instance, experimental infection with the trematode *Schistosoma mansoni* prevents the development of Type-1 diabetes mellitus in non-obese diabetic mice (Cooke et al., 1999), as well as allergic encephalomyelitis and Grave's thyroiditis. Intestinal nematodes, such as the murine parasite *Heligmosomoides polygyrus*, have also been shown to have immunomodulatory properties. Mice with chronic *Heligmosomoides polygyrus* infections are less likely to develop airway inflammation and allergy (Wilson et al., 2005). Finally, infection with *Litomosoides sigmodontis* reduces the risk of becoming diabetic in NOD mice.

Resistance vs. tolerance

Host immunity is often seen as a mechanism conferring either protection against parasitic infection either allowing the host to kill the intruder as fast as possible. Immune effectors that allow clearing the infection (killing the parasite or limiting its proliferation) make the host resistant to that particular parasite. However, as already mentioned above, immunity also confers costs in terms of overreacting or misdirected responses. An alternative strategy that might produce higher fitness rewards, might be to minimize the cost of the infection, or in other words to tolerate the infection (Soares et al., 2017). Contrary to resistance, tolerance mechanisms do not aim at hitting the intruder, but instead at limiting the damage it induces. Tolerance to infection has now been investigated in several species, and to this purpose, helminths have proved be particularly useful model systems. As already said, helminths are

Table 1 A non-exhaustive list of parasites (protozoa and helminths) that have been reported to protect from autoimmune diseases.

Parasite	Disease	References
<i>Protozoa</i>		
<i>Trypanosoma brucei</i>	Collagen-induced arthritis	Mattsson et al. (2000)
<i>Helminths</i>		
<i>Acanthocheilonema vitae</i>	Asthma, acute colitis	Schnoeller et al. (2008)
<i>Ascaris suum</i>	Arthritis	Rocha et al. (2008)
<i>Heligmosomoides polygyrus</i>	IBD ^a	Elliott et al. (2004)
<i>Heligmosomoides polygyrus</i>	Allergy	Bashir et al. (2002)
<i>Hymenolepis diminuta</i>	Colitis	Reardon et al. (2001)
<i>Nippostrongylus brasiliensis</i>	Allergy	Wohlleben et al. (2004)
<i>Schistosoma mansoni</i>	T1DM ^a	Zaccone et al. (2003)
<i>Schistosoma mansoni</i>	Colitis	Moreels et al. (2004)
<i>Schistosoma mansoni</i>	Encephalomyelitis	La Flamme et al. (2003)
<i>Schistosoma mansoni</i>	Hyperthyroidism	Nagayama et al. (2004)
<i>Strongyloides stercoralis</i>	Allergy	Wang et al. (2001)
<i>Trichinella spiralis</i>	Colitis	Khan et al. (2002)
<i>Trichuris suis</i>	Crohn's disease	Summers et al. (2005)
<i>Trichuris suis</i>	IBD ^a	Summers et al. (2003)

^aT1DM, Type 1 Diabetes Mellitus; IBD, Inflammatory Bowel Disease.

large metazoan and several species migrate through the body of the host, causing tissue damage. Interestingly, the canonical type-2 response that characterizes helminth infection also plays a key role in tissue healing and repair. Therefore, it has been suggested that a polarized type-2 response during a helminth infection might contribute to limit the cost of the infection by restoring tissue integrity (Gause et al., 2013). In addition to this, the activation of regulatory pathways and anti-inflammatory cytokines, that are both under host and parasite control, also contributes to tolerance (King and Li, 2018).

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General Characteristics of Bacteria

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The bacterial world is a universe that we are just beginning to understand. Until a few decades ago, our view of the bacterial world was limited by the technology available to study it, and in many aspects, it had changed relatively little in the last century. Our vision was basically limited to cultivable bacteria, when today we know that this is a probably a very minority group of the total. Now we know that we have managed to cultivate bacteria belonging to just 30 *phyla*, of which 95% belong to four *phyla* (*Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*), when the List of Prokaryotic Names with Standing in Nomenclature (LPSN) recognizes more than 40 *phyla*, and the SILVA database recognizes around 90.

Bacteria are prokaryotic cells. Prokaryotic cells are those that lack a proper nuclear membrane and lack membrane-bound organelles such as endoplasmic reticulum, Golgi complex or mitochondria. There are two kingdoms of the six into which living beings are divided, made up of prokaryotic cells: *Eubacteria* and *Archaea*.

Though *Archaea* are microorganisms that have been associated habitually to extreme conditions of life of temperature, salinity, pH, etc. (extremophiles) they can also be found in environments where it is more likely to interact with other living organisms. However, no microorganisms belonging to the *Archaea* Kingdom have been described by now that cause or are associated to diseases in animals or plants. Thus, in this chapter we will refer only to *Eubacteria*, since it is the Kingdom that integrates all prokaryotes (bacteria) pathogenic for humans and animals.

Although bacteria are mostly microscopic organisms, their size is highly variable. While the bacteria most frequently isolated as members of the normal human microbiota, or as causing infections in humans, measure a few microns (staphylococci about $1 \times 1 \mu\text{m}$, enterobacteria about $1 \times 2 \mu\text{m}$), and therefore are clearly microscopic organisms, some bacteria are even at the limit of visibility without the need for a microscope, such as *Epulopiscium fishelsoni*, a large, symbiotic bacteria found within the intestinal tracts of some species of tropical surgeonfish (Family *Acanthuridae*), or are directly visible with the naked eye, such as *Thiomargarita namibiensis*, a chemolithotrophic bacterium that is capable of using the nitrate anion as a terminal electronic acceptor in the electronic transport chain, and that is capable of storing high concentrations of nitrate in a massive vacuole, which is responsible for 98% of its size. Anyway, as a rule they are smaller than eukaryotic cells. As an example, *Escherichia coli*, one of the most abundant bacteria in the intestine of many mammals, measures approximately $1 \times 2 \mu\text{m}$, while a hepatocyte can have a diameter between 25 and 40 μm .

From a morphological point of view, bacteria are grouped into **cocci** (bacteria with approximately round or oval morphology), **bacilli** (elongated, approximately cylindrical morphology), **vibrio** (curved, comma-shaped microorganisms), **spirillum** (rigid helical morphology), **spirochetes** (flexible helical morphology, which gives them a characteristic undulating movement). In addition, it must be considered that this “basic” morphology can be modified by other factors such as the presence of spores, which can sometimes noticeably deform the bacterial cell (Fig. 1).

Bacteria divide by binary fission, as we will see in detail later. However, the division of the wall in some bacterial genera and species tends to be incomplete, which makes these bacteria, not completely separated, appear forming characteristic groups. Thus, cocci can appear grouped in pairs (**diplococci**), as frequently happens in *Streptococcus pneumoniae* or *Neisseria*, in chains (**streptococci**), in cubic structures (**tetrads**) or in irregular groupings, which have classically been morphologically compared to bunches of grapes (**staphylococci**), hence its name, from the Greek *staphylé*, bunch. **Filamentous bacteria** are bacilli that remain attached end to end when divide, thus leading to long filaments composed by several individual bacilli (Fig. 2).

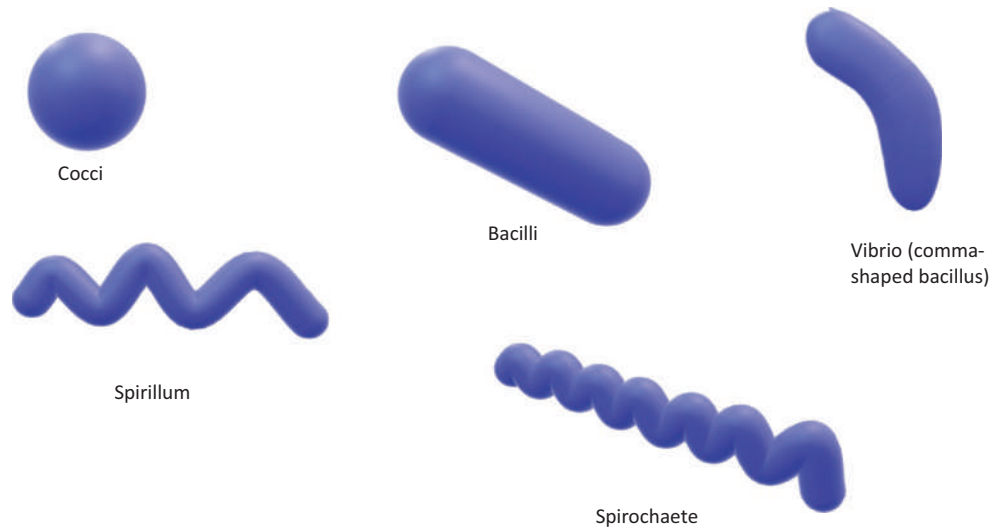


Fig. 1 Bacterial morphologies.

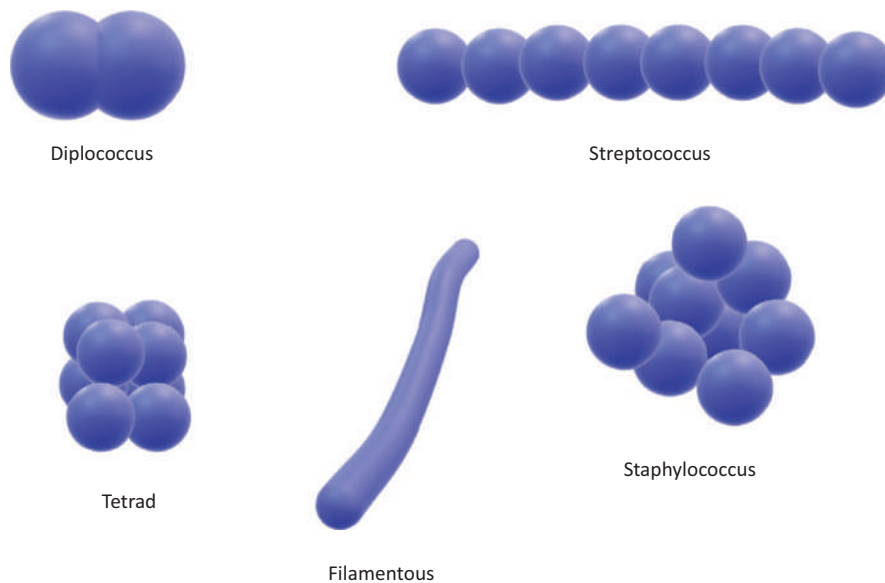


Fig. 2 Types of bacterial aggrupation.

As mentioned in the previous paragraph, bacteria divide by **binary fission** (Fig. 3). The division of eukaryotic cells poses two problems from a biological point of view. It is necessary to duplicate a DNA present in large quantities, and grouped in complex structures, the chromosomes, and it is necessary to duplicate all the organelles, especially membrane-covered organelles. Bacteria, however, do not have membrane-covered organelles to duplicate, and they have a much smaller amount of DNA (between 1000 and 2000-fold smaller) than eukaryotic cells, allowing directly to duplicate DNA and divide the cell, generating two new cells, each of which contains one of the DNA copies. This simplicity of replication conditions another of the characteristics of bacterial division, which is speed. *E. coli* can replicate, in optimal conditions, every 20 min. In more real conditions, its replication time can be around an hour. Still, this means that each individual *E. coli* cell would have generated, within 24 h, between 15 and 20 million of new bacteria.

Although it has important differences with eukaryotic cells, prokaryotic cells also have some characteristics in common with them. They are covered by a cytoplasmic membrane that allows them to maintain a stable environment in terms of its characteristics

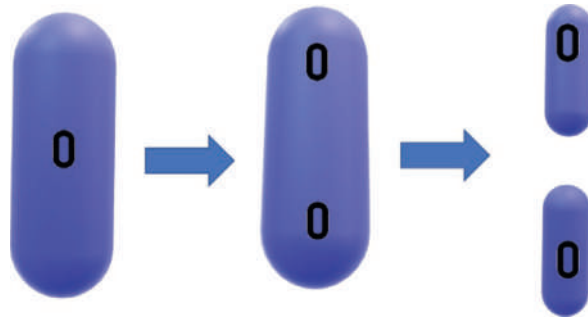


Fig. 3 Binary fission.

(composition, pH, etc.) inside. They also have a genetic endowment based on DNA, and a protein synthesis machinery (mRNA, tRNA, ribosomes) functionally like that of eukaryotic cells, although structurally the ribosomes show differences that, i.e., are responsible for selective toxicity (toxicity for bacteria but not for eukaryotic cells) of various groups of antimicrobials (macrolides, aminoglycosides, tetracyclines, among others).

The lack of an endomembrane system means that the bacteria do not have specialized spaces to carry out specific functions, so that all reactions must be carried out in the cytosol.

Bacterial genome

Biological information in bacteria follows the universally accepted paradigm. The basic unit of genetic information is the **gene**, which codes for the amino acid sequence of a protein. Genes are grouped primarily in **chromosomes**, but also in other **genetic elements**, and the set of all genetic elements constitutes the **bacterial genome**. This way, bacterial genome is the whole genetic information of a microorganism, and is formed by the genes that encode proteins, the RNA and regulatory sequences, as well as all the non-coding DNA that may be present.

We can divide the genome into the following types:

- Bacterial genome is made up of the total set of genes that a bacterium possesses and comprises both its chromosome and all the extrachromosomal genetic elements, if any.
- Genome core is formed by the set of genes present in all individuals of a specific bacterial species.
- Accessory genome which is the set of variable genes between members of the same species.
- Metagenome that is reserved to refer to the set of genes of microbial origin present in a ecosystem.

As in other living organisms, genetic information is stored in **DNA**, which is translated into **messenger RNA (mRNA)** by RNA polymerases. This mRNA will be the one that, in ribosomes, directs protein synthesis and establishes its exact amino acid sequence. For its part, the **transference RNA (tRNA)** will be the one that, following the instructions of the mRNA, incorporates each amino acid in the corresponding position into the ribosome, to give rise to the synthesis of the complete protein.

The main genetic elements in bacteria are the **chromosome** and **plasmids**.

Most bacteria contain a single circular chromosome, although some specific species may contain two and up to three chromosomes.

Plasmids are linear or circular DNA strands, usually much smaller than the chromosome, with the ability to replicate independently of the chromosome. They can be transferred from one bacteria to another directly (conjugation) or through phages (transduction).

Transposable elements are DNA fragments integrated into other DNA molecules (plasmids, chromosomes ...) but that can move within the same DNA molecule, or to another DNA molecule.

Furthermore, some bacteria can capture free bacterial DNA in the medium and integrate it into their own genome (**transformation**). Some human pathogens, such as *S. pneumoniae*, have a particularly marked ability to take up DNA in this way.

Both plasmids, transposable elements and transformation have been shown in recent years as decisive elements in the horizontal transmission of different characteristics of microorganisms, including antimicrobial resistance. Thus, plasmids and transposable elements are decisive elements in the horizontal transmission of resistance to antimicrobials, and transformation has also been shown to be an efficient way to develop resistance in pneumococci, through the uptake by transformation of DNA from streptococcal species more resistant to penicillin.

Plasmids and transposable elements are decisive elements for the genetic variability of bacterial populations. While the semi-conservative reproduction of eukaryotic organisms guarantees recombination and sufficient genetic heterogeneity in populations, bacterial reproduction by binary fission implies that the new bacteria are identical copies of the previous one. This would suppose, without corrective elements, a genetic homogeneity in the bacterial populations that would be very dangerous for the

survival of the species in the event of any alteration in the habitat conditions. These genetic elements, which reproduce independently of the chromosome, are also transmitted independently of it and are capable of transposing with great freedom between some major genetic elements and others, represent an element of genetic heterogeneity that is vital, in the long term, for the survival of the species.

The bacterial chromosome is integrated into a structure called the **nucleoid**. The lack of a proper nucleus is one of the basic characteristics that differentiates prokaryotic cells from eukaryotes. The nucleoid is an area within the bacterial cell that groups most of the bacterial genome, particularly the chromosome, but is not delimited, as in eukaryotic cells, by a cell membrane. The composition of the nucleoid is at least 60–70% DNA. The rest are mainly mRNA and proteins with different functions. This includes both proteins responsible for regulating the transcription of certain genes, and proteins helping to maintain the supercoiled structure of the nucleic acid (**nucleoid proteins** or **nucleoid-associated proteins**). These nucleoid proteins are distinct from histones of eukaryotic cells. While in eukaryote DNA is wrapped around a histone protein core, the nucleoid DNA-binding proteins do not form nucleosomes. Instead, they often use other mechanisms, such as DNA looping, to promote compaction.

Ribosomes

Ribosomes are particles made up of ribosomal RNA (rRNA) and proteins, and are the place where protein synthesis takes place, therefore it is a fundamental element for living beings. The ribosome is responsible for reading the sequence of the messenger RNA and through the genetic code it translates the base sequence of the messenger RNA into a specific amino acid sequence.

We can find ribosomes in mitochondria, cytosol, chloroplasts, and endoplasmic reticulum. They are small and therefore can only be viewed under an electron microscope. They have no membranes in their structure.

Ribosomes are made up of subunits that are named through the sedimentation coefficient in Svedberg units (S). It indicates the relationship between the sedimentation speed of a particle and the applied acceleration that causes the sedimentation.

Ribosomes are dynamic structures, and their subunits associate and dissociate alternately and will also interact with other proteins.

Prokaryotic cells have ribosomes with a 70S sedimentation coefficient and in turn it is divided into two subunits:

- The largest subunit (L subunit) that has a 50S sedimentation coefficient and contains 5S and 23S rRNA and 31 proteins.
- The minor subunit (S subunit) that has a 30S sedimentation coefficient and contains 16S rRNA and 21 proteins.

Eukaryotic cells have a sedimentation coefficient of 80S. They are also made up of subunits:

- The largest subunit that has a sedimentation coefficient of 60S and contains 5S 28S 5.8S rRNA and 49 proteins.
- The minor subunit that has a 40S sedimentation coefficient and has a single 18S rRNA molecule and 33 proteins.

Although the functions of ribosomes are similar in eukaryotic and prokaryotic cells, their structures, although similar, present crucial differences. These structural differences are those that confer selective toxicity to some of the main groups of antibiotics (aminoglycosides, tetracyclines, chloramphenicol, macrolides...) allowing them to inhibit bacterial protein synthesis without significantly interfering with protein synthesis in prokaryotic cells.

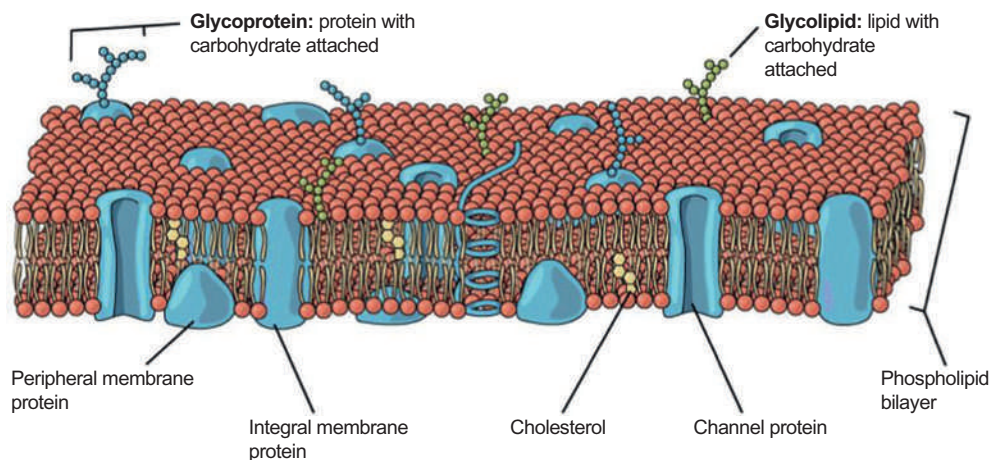
Cell envelope

The cell envelope is formed by a group of structures surrounding the cytoplasm. These structures have several crucial functions: they are involved in attachment to biological and inert surfaces, can protect the microorganisms from the action of the immune system, protect the bacteria from mechanical stress and has have a decisive role in the exchange of substances with the environment.

Cytoplasmic membrane

Unlike other structures, such as the cell wall, the cytoplasmic membrane is highly conserved in all bacteria, and bears great similarities to the cytoplasmic membrane of eukaryotic cells. Its main function is to act as a semi-permeable selective membrane, which allows or not the passage of different molecules. However, its structure incapacitates it, due to its almost semi-liquid consistency, to act as a barrier or physical protection, as occurs with the wall.

The cytoplasmic membrane of both prokaryotic and eukaryotic cells is made up of a phospholipid bilayer, organized in such a way that the hydrophobic lipid parts are oriented inwards, and therefore contiguous, while the polar parts are oriented outwards, therefore in contact with the external environment on one side, and with the cytoplasm on the other. Proteins appear integrated, to a greater or lesser extent, in the cytoplasmic membrane. These proteins can be attached loosely to the outer part of the cytoplasmic membrane (peripheral proteins), embedded more extensively in it (integral proteins) or completely cross the cytoplasmic membrane (transmembrane proteins). These proteins play an important role in different cellular processes crucial for the bacteria, such as energy production and transport. (Fig. 4).



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Fig. 4 Cytoplasmic membrane. Structure.

The characteristics of the cytoplasmic membrane make it an effective barrier that prevents the free diffusion of substances between the inside and outside of the cell. Thus, most of the substances must enter and leave the cell through transporter proteins. This system not only guarantees a selective transport into and out of the bacteria, but also allows substances to accumulate against a concentration gradient, thus ensuring an adequate intracellular concentration of the different compounds necessary for bacterial metabolism, regardless of its concentration in the external environment. These carrier proteins can be highly specific for certain compounds or have a broader range of substrates.

These transport systems require energy. The cytoplasmic membrane is also a point of energy production. The accumulation of protons on the outside of the membrane generates an electrochemical potential difference between the inside and outside of it. This potential difference (proton motive force) is used by the cell for different functions, such as transport against a gradient, the movement of flagella that confers mobility to the cell or the production of high-energy molecules, such as ATP.

Cell wall

The high concentration of solutes inside the bacterial cell leads to a high intracellular osmotic pressure (around 2 atm). This pressure would cause the bacterial cell to burst, if it did not have a rigid cover, which does not exist in eukaryotic cells, which is the cell wall. The cell wall is also responsible for maintaining bacterial morphology.

The bacterial wall can be structured in two clearly differentiated ways: the Gram positive wall and the Gram negative wall, named for their different behavior with the staining method devised in the 19th century by the Danish microbiologist Hans Christian Joachim Gram.

The Gram positive bacterial wall is almost exclusively made up of a thick layer of a polysaccharide compound called peptidoglycan, while the more complex Gram negative wall is made up of several layers with different composition, only one of which contains peptidoglycan. Although most bacterial families have a cell wall, there are some families, such as the *Mycoplasma-taceae* family, that naturally lack this structure.

Structure of the cell wall. Peptidoglycan

Peptidoglycan is a polysaccharide compound that is present in all bacterial walls, both Gram positive and Gram negative, and is what confers rigidity and resistance to the wall. It has a central structure made up of the alternative repetition of two polysaccharide molecules, *N*-acetyl-muramic acid and *N*-acetyl-glucosamine, linked by a β -1,4 bond. Attached to each residue of *N*-acetyl-muramic there is a tetrapeptide that can vary discretely from one microorganism to another (Alanine-Glutamic Acid-Lysine-Alanine) or (Alanine-Glutamic Acid-diaminopimelic Acid (DAP)-Alanine). These peptidoglycan chains, which extend longitudinally throughout the cell, establish covalent bonds with the adjacent ones through these tetrapeptides, between the third amino acid of one of them (lysine or DAP) and the fourth of the adjacent one (alanine), either directly or through a glycine pentapeptide, as occurs for example in staphylococci. In this way, a large macromolecule is formed that covers all the bacteria (Fig. 5).

The peptidoglycan thus formed constitutes a sufficiently strong and stable structure to withstand the high intracellular osmotic pressure and avoid osmotic lysis of the cytoplasmic membrane.

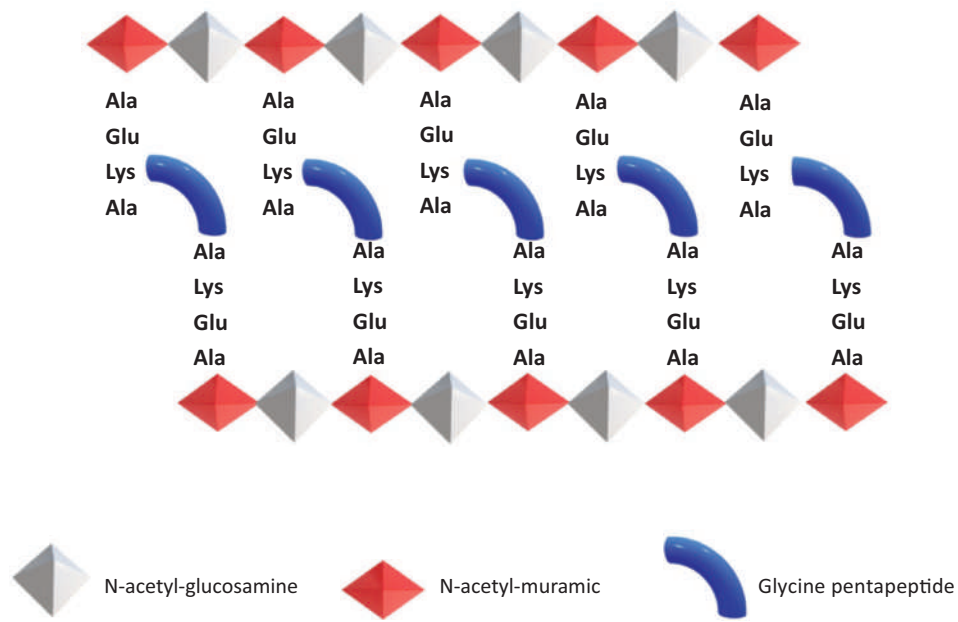


Fig. 5 Peptidoglycan structure.

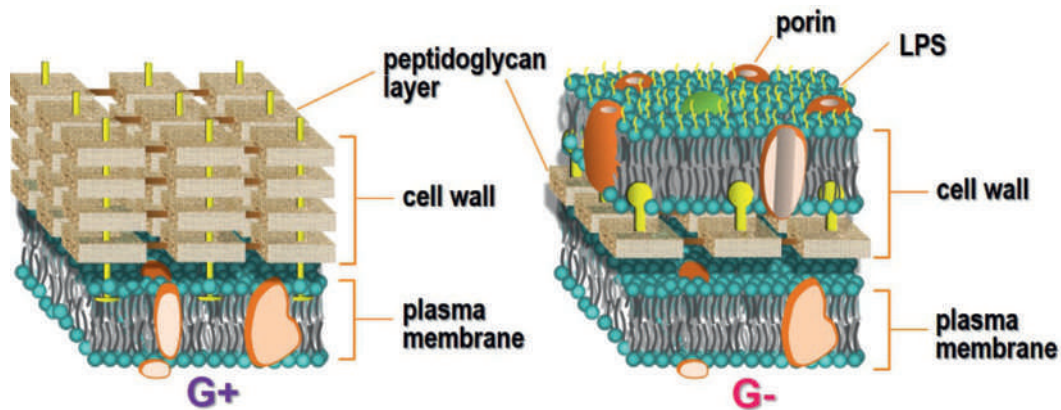
Cell wall. Gram positives

The cell wall of Gram positives is considerably thicker than that of Gram negatives and is more than 90% made up of overlapping layers of peptidoglycan.

The remaining 10% is largely made up of glycerol phosphate or ribitol phosphate compounds, embedded in peptidoglycan and generically called teichoic acids, if they are covalently bound to the peptidoglycan, or lipoteichoic acids, if they are bound to the cytoplasmic membrane. (Fig. 6).

Cell wall. Gram negatives

The Gram-negative wall has a much lower amount of peptidoglycan, generally formed by a single layer of the polysaccharide compound, that is included in the so-called periplasmic space, limited by the cytoplasmic membrane on the inside and the outer membrane on the outside. This outer membrane, which does not appear in Gram positives, is a second phospholipid bilayer external to the peptidoglycan, which contains associated proteins, like the cell membrane, but also contains polysaccharides associated with the lipids of the bilayer, therefore it is also commonly referred to as a lipopolysaccharide (LPS) (Fig. 6).



Laurent, E. (2016, December 31). Bacterial cell wall. OER Commons. Retrieved October 27, 2021, from <https://www.oercommons.org/authoring/19267-bacterial-cell-wall>.

Fig. 6 Structure of Gram-positive and Gram-negative wall.

The outermost layer of LPS is composed of polysaccharides and consists of core polysaccharides and O-specific polysaccharides, frequently branched. The innermost part of the lipopolysaccharide is lipid A, which is associated with the outer layer of the phospholipid bilayer and harbors the endotoxic action of Gram-negative LPS.

This outer membrane is an impermeable structure, so most of the substances must penetrate through specific pathways. These pathways are mainly the so-called **porins**, transmembrane proteins made up of three identical peptides, which form aqueous channels through which polar substances can pass through the LPS. There are **nonspecific porins**, through which numerous compounds can penetrate, especially small hydrophilic compounds, without any structural relationship, and **specific porins**, which show binding sites for specific compounds, or groups of compounds structurally related, which will be the only ones capable of penetrating. Through that channel.

The LPS is separated from the cytoplasmic membrane by a space, called the **periplasmic space**, which houses the thin layer of peptidoglycan present in this type of bacteria. This periplasmic space houses different structures and molecules important in the physiology of the microorganism and shows an important metabolic activity. The LPS is anchored to the peptidoglycan through lipoproteins that join both structures crossing the periplasmic space: they are the so-called Braun lipoproteins.

In this periplasmic space, a metabolically very active area, proteins with various functions are found, from hydrolytic enzymes to proteins with transport functions, or synthesis of extracellular structures, such as penicillin binding proteins (PBP), proteins involved in the final phases of peptidoglycan synthesis, and target of beta-lactam antibiotics.

Pili and fimbriae

Pili are thin, hair-like appendages found on the surface of bacteria. Short pili, especially adapted to mediate adherence functions are also called **fimbriae**. They are usually more numerous than pili. Both are filamentous proteins and can fulfill many functions. They facilitate adherence to surfaces, allow the formation of biofilms, and can be a fundamental factor of virulence by mediating adherence to animal tissues.

Some pili have specific functions beyond adherence. This way, **conjugative pili** facilitate the genetic exchange between bacteria through a process called **conjugation**.

This process of bacterial conjugation serves to transfer genetic information from a donor cell to another recipient, involving plasmids and specialized surface structures with specific functions such as pili.

Pili are characteristic of Gram-negative bacteria. In fact, virtually all Gram-negative bacteria produce pili of one type or another but can also be found in Gram positive microorganisms.

Another type of pili are **electrically conductive pili** or **nanowires**. These pili are capable of establishing a flow of electrons from or to a specific cell, thus having an important role in energizing the affected cells.

Finally, **type IV pili** are capable not only of establishing adhesion functions, but of giving rise to a special type of bacterial movement called *twitching motility*, that allows bacteria to move along a surface by extension, adhesion to the surface and retraction of this type of pili. These filaments are thin and flexible but have great mechanical strength. They can appear at one or both poles of the bacteria individually or in groups. They are also the most frequently found in both Gram positive and Gram negative bacteria.

In addition, these type IV pili play a role in the infectivity of different human pathogens (*Neisseria gonorrhoeae*, *Streptococcus pyogenes* . . .), by allowing them to move on the epithelial surfaces to locate the most suitable place to start the infection. This class of pili are subdivided into two groups, IVa and IVb, based on the major subunit of pilin that makes them up.

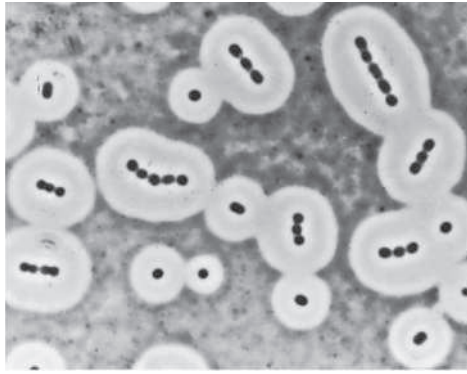
The motility mediated by these pili is independent of that produced by flagella, and occurs on solid surfaces. This movement can be described as jerking or jumping. The correct retraction of these structures allows them to get closer and to adhere to the host cell, achieving greater stability.

Capsule and slime

Many bacteria produce substances external to the cell wall, usually of a polysaccharide and sometimes protein composition, which form a kind of external envelope for the bacteria, with functions of physical and mechanical protection, adherence, and defense against various external factors (phagocytosis, antibodies. . .). It tends to be called a **capsule** when it is a more individualized covering, tightly attached to the bacteria and readily visible with light microscopy, and **slime** when it tends to form a matrix more loosely attached, that usually can't be seen by light microscopy and includes larger groups of bacteria.

One of their main functions is adherence. Many pathogens, to colonize and invade an organism, need to adhere to certain cell surfaces. This union, at least in its initial stages, is usually done non-specifically through these structures. Later this binding can be made more specific through the interaction between certain structures of the bacterial surface and specific receptors.

The capsule also plays an important role in protection against the immune system. The presence of a capsule makes it difficult for phagocytes to engulf the bacteria and the interaction of antibodies with specific epitopes of the bacterial wall. The importance of the capsule is evident in cases such as *S. pneumoniae* or *Haemophilus influenzae*. In the first case, the capsule is a determining factor for the pathogenicity of the microorganism, so that, in the absence of the capsule, *S. pneumoniae* virtually loses its invasive potential. In the case of *H. influenzae*, the presence of a capsule, especially capsular type B, is crucial for the organism to be capable of producing serious invasive infections, such as bacteraemia, meningitis, or epiglottitis. (Fig. 7).



From W.H. Taylor and E. Juni, "Pathways for Biosynthesis of a Bacterial Capsular Polysaccharide," *Journal of Bacteriology* (May 1961)

Fig. 7 Bacterial capsule.

These structures are also decisive in the ability of bacteria to bind to inert surfaces, giving rise to structures called biofilms, which consist of groups of bacteria firmly attached to each other and to inert surfaces by means of this *slime*. This adherence is important in the biology of many pathogenic bacteria and the pathogenesis of the infections caused by them. Thus, the biofilms that *Legionella* forms on the surface of pipes, water pipes, etc., have a decisive role in maintaining the colonization of these structures and, eventually, in that these colonized structures behave as a source of microorganisms that eventually they can pass to man and cause infection. Similarly, the ability of some microorganisms to form biofilms in prosthetic materials used in medicine (catheters, orthopedic prostheses, cardiac prostheses ...) play a fundamental role in infections associated with this type of devices.

Cell movement. Flagella

Some cells can move within liquid media, or even along the surface of solid media, thanks to rotating structures capable of propelling them, called flagella. They are elongated structures, attached to the cell at one end and free at the other end, which can be single or multiple, and present several forms of association:

- *Monotrichous*—Bacteria have one only polar flagellum.
- *Lophotrichous*—All flagella are grouped at one cell end.
- *Amphitrichous*—Bacteria have two tufts of flagella grouped at both poles.
- *Peritrichous*—Bacteria present flagella distributed throughout its perimeter (Fig. 8).

The flagella are too thin to be observed by conventional stains, but there are specific stains that allow them to be observed. Obviously, they can also be observed by electron microscopy.

The flagella have a rotary motion, similar to that of a ship's propeller, and are capable of moving in both directions, and therefore propel the bacterium forward or backward. This movement is obviously carried out with a consumption of energy, which is provided by the proton motive force. The flagella do not have a fixed rotational speed, but this mobility is modulable, reaching a maximum of about 1000 rpm, which implies a mobility for the bacteria of 100–120 $\mu\text{m/s}$. To evaluate this mobility in real terms, we must understand that this speed implies moving a length equivalent to 50–60 times its own length in 1 s. If we applied this same parameter to a 4 m long car, this would mean traveling at about... 800 km/h!

Flagella are protein structures. The free part of the flagellum, called the *filament*, is made up entirely of a protein called *flagellin*. There are also a series of structures related to its anchorage to the bacterial wall and to the energization of the process, which constitute the motor of the flagellum. It is a central rod surrounded by a series of discs, attached to the filament through a proteic area called *hook*. In Gram negatives, there are several discs: a disc called *L disc*, anchored to the LPS, a second, more internal disc, denominated *P disc*, anchored to peptidoglycan, an *MS disc*, anchored to the cytoplasmic membrane, and a *C disc*, included within the cytoplasm. Gram positive bacteria lack the outer (L and P) discs, and only the inner discs (MS and C) are found.

Outside the internal discs, and anchored to the cytoplasmic membrane and peptidoglycan, a structure called *stator* appears, formed by a protein called *Mot*, and whose mission is to provide torsional force. It would be the device that would facilitate the energization of the turbine, which is ultimately the entire base of the flagellum, through the proton motive force. The translocation of protons through channels present in this structure would generate the energy necessary to rotate the internal discs, and consequently the flagellum (Fig. 9).

Motility, even though it is not a vital characteristic for the bacteria (there are immobile bacteria), confers great advantages on being able to move towards optimal areas from the nutritional point of view, temperature, pH, etc. On the other hand, this motility is important in the pathogenicity of some bacteria. Thus, the motility conferred to *Helicobacter pylori* by its polar flagellum, allows it to migrate rapidly towards the gastric wall and penetrate it, minimizing the time of exposure to gastric pH, and thus facilitating its survival.

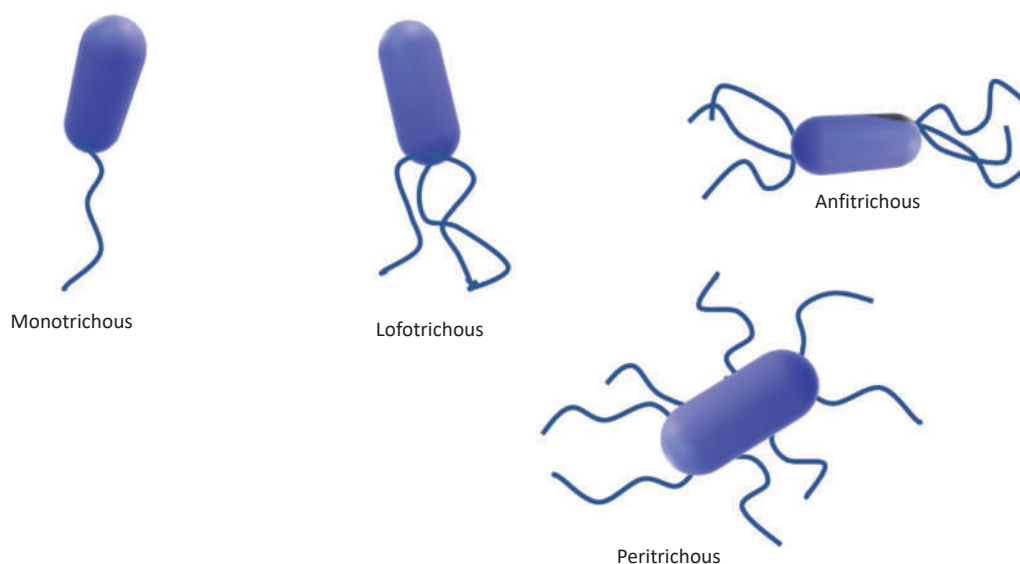
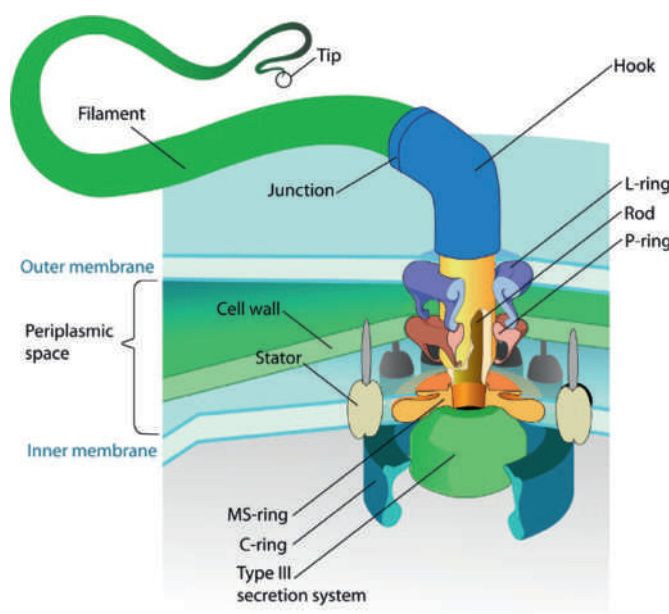


Fig. 8 Classification of bacteria according flagella disposition.



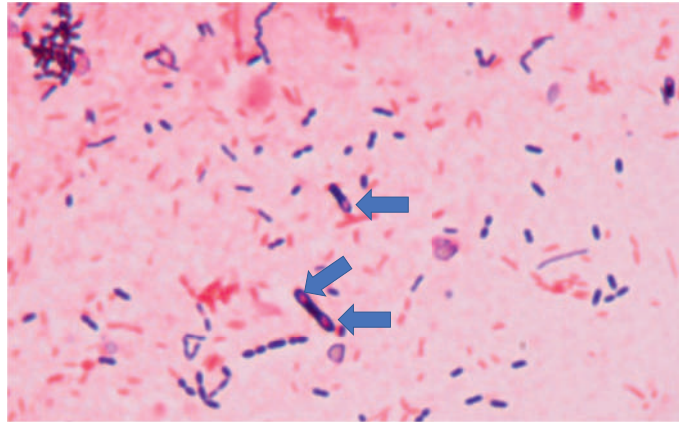
LadyofHats, public domain, via Wikimedia Commons

Fig. 9 Flagellum structure.

Endospores

Endospores are forms of bacterial resistance capable of surviving in extreme conditions. They must be differentiated from **spores**, which are active reproductive structures produced mainly by fungi, some plants, and some bacterial genera, such as *Streptomyces*.

Endospores are not reproductive structures. They are highly differentiated, dormant cells, which can withstand very unfavorable conditions such as extreme temperatures (they can survive for hours in boiling water), aggressive chemicals, radiation, dryness, or lack of nutrients. Endospores are a reversible status in bacterial cycle life. A vegetative form can convert to an endospore when environmental conditions are unfavorable to it, and this endospore can in turn revert to a vegetative form when conditions become favorable again. Not all bacterial species can produce endospores. It is a characteristic that is more common in bacteria whose habitat usually gives unfavorable conditions for the bacteria (high or low temperatures, droughts, high irradiation . . .) in a cyclical way, so the bacteria must be prepared to resist in these conditions, and to be able to return to the vegetative form when the situation



Endospore in *Clostridium* spp. Gram stain, 1.000x.
Juan Luis Muñoz Bellido, personal collection.

Fig. 10 Sporulated clostridia in a mixed infection.

allows it. In fact, only two bacterial orders are known, the order *Bacillales* and the order *Clostridiales*, capable of producing endospores. Among them, the two most frequent genera in human infections are the genera *Bacillus* and *Clostridium*, and in the main human diseases caused by microorganisms of these genera (anthrax, caused by *Bacillus anthracis*; botulism, caused by *Clostridium botulinum*; tetanus, caused by *Clostridium tetani*; gas gangrene, caused mainly by *Clostridium perfringens* among others), the ability to sporulate is a decisive factor in its infective cycle (Fig. 10).

On the other hand, they are extremely long-lived biological forms, since they can survive for hundreds of years, and can be easily spread by wind, water and through animal stools.

Endospores are refractive structures in the light microscope, and they do not stain with conventional methods, so they often appear within cells as unstained areas. However, they can be dyed with specific procedures, such as Schaeffer-Fulton's method, also known as malachite green staining.

The process that leads to the formation of endospores is called **sporulation**.

In the process of sporulation, vegetative cells will become non-growing structures that resist heat and are light-refractive. The sporulation process is usually triggered when growth stops because one of its essential nutrients is depleted.

In turn, this is a reversible process since they can remain dormant for years and easily revert to a vegetative cell through a process called **germination**. In the same way that sporulation is triggered by the absence of certain essential nutrients, germination is precipitated by their availability. This process consists of three phases: **activation**, **germination** and **outgrowth**.

During the **activation phase**, the endospore rehydrates, losing its resistance to heat and chemicals, and becoming less refractile under the microscope. During the **germination phase** it loses the structures characteristic of the endospore and recovers the typical structures of the vegetative cell and finally, during the **outgrowth phase**, it fully recovers the functionalities of a vegetative cell and begins to divide by binary fusion. This can be a very fast process that, if the circumstances are favorable, can be completed in a few minutes.

The endospore has a series of chemical components and structures absent in the vegetative cell. From the inside out, it is made up of an internal area or **core**, which contains DNA and ribosomes from the cytosol of the vegetative cell. Immediately outside there is an **inner membrane**, which develops from the cytoplasmic membrane of the vegetative cell, the **cortex**, which is made up of peptidoglycan also from the vegetative cell and an **outer membrane** that, unlike the previous layers, is formed during the sporulation phase. Outside is the **endospore coat**, made up of endospore-specific proteins. A final, inconsistent protein layer may exist, which is not present in all cases, called **exosporium**.

There are two key compounds for endospore resistance characteristics, which are dipicolinic acid and calcium. Dipicolinic acid is a characteristic component of the endospore and can account for up to 15% of its total weight. Dipicolinic acid, inside the core, combines with calcium, giving rise to **calcium dipicolinate**, which binds to free water in the core, producing intense dehydration (the endospore has approximately 25% of the water present in the cell vegetative). This dehydration inactivates the existing enzymes in this area, which, however, are not denatured, so that they can be reactivated when the endospore rehydrates and germinates. In addition, calcium dipicolinate is capable of intercalating between the bases of DNA, stabilizing its structure.

Another important component of the endospore are the so-called **small acid-soluble spore proteins (SASP)**. These proteins are specifically produced during the sporulation process, and they bind to bacterial DNA, compacting it. Because of this, DNA becomes more resistant to heat, desiccation, radiation. ... On the other hand, they also become a source of nutrients and energy, in the final stages of the germination process.

Inclusion bodies

Inclusion bodies are structures frequent in bacteria. They are granules of organic or inorganic material, which are usually surrounded by a single-layer protein membrane which isolates the inclusion body from the rest of the cytosol, though they can also be found free in the cytoplasm. Inclusions can usually be seen directly under the microscope without special staining.

The function of **inclusion bodies** is mainly to store energy reserve elements such as lipids or polysaccharides to use them as an energy source in conditions of nutrient scarcity or for the biosynthesis of molecules. Nevertheless, there are some inclusion bodies that accumulate other kind of substances, with very specific functions.

Why has this method of storing energetic compounds been evolutionarily successful? Probably because storing these elements as insoluble compounds allows the bacteria to accumulate more chemical energy in less space, and with less osmotic stress than if they had to store it as soluble substances, which would inevitably contribute to increasing the already high intracellular osmotic pressure.

These bodies allow to store a great variety of substances that make up the different inclusion bodies that exist. The most important are:

- **Poly-beta-hydroxybutyrate granules:** They are surrounded by a membrane and their formation occurs under conditions of excess carbon and will be degraded as carbon or energy sources when conditions demand it.
- **Polysaccharide inclusions:** Formed mainly by glucans such as starch or glycogen. They are also produced under conditions of excess carbon, and are degraded as carbon or energy source.
- **Biopolymers:** They can be completely degraded by the bacteria that produce them and by other types of microorganisms.
- **Sulfur globules,** in which sulfur is fundamentally accumulated and in which, for example, chemolithotrophic organisms use for their oxidation.
- **Magnetosomes:** Allows bacteria to orient themselves in a magnetic field. They are intracellular particles of magnetite (Fe_3O_4). The bacteria that present these elements have a property called magnetotaxis that allows them to move along the magnetic lines of the earth. They are more frequent in microorganisms that live under water.
- **Gas vesicles:** They are typical of planktonic microorganisms that live in the water columns of oceans and lakes. Thanks to these structures they can float. They are typical of cyanobacteria.

In Enterobacteria, the main element stored is glycogen and constitutes 40% of their weight. Mycobacteria have polyphosphate granules and some *Pseudomonas* accumulate carbon.

Further reading

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Pathogenesis of Bacterial Infections and Bacterial Persistence

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Introduction

Pathogenesis could be defined as a multi-factorial process which depends on several circumstances such as the nature or virulence of the species of microorganism, the number of these microorganisms in the initial exposure and the immune status of the host. Pathogenesis refers both to the mechanism of infection and to the mechanism by which disease develops. On the other hand, pathogenicity is the capacity to initiate any disease. It requires some characteristics such as the transmissibility to a host, infectivity in this new host and virulence or the capacity of the pathogen to cause disease. The capacity of a bacterium to cause disease reflects its relative pathogenicity; thus, the bacteria can be included in three groups according to its pathogenicity: primary pathogen, when a bacterium is isolated from a patient and is considered to be the probable agent of this infection in previously healthy individuals with intact immunological defenses. Opportunistic microorganisms are those isolated from patients whose host defense mechanisms have been impaired or compromised. Finally, other bacteria are considered to be non-pathogens due to the fact that they rarely or never cause human infection.

Another aspect of the bacterial infections is the bacterial persistence phenomenon because many bacteria can infect and persist inside their hosts for long periods of time. This fact can be due to immunosuppression of the host, immune evasion by the pathogen and/or ineffective killing by antimicrobials.

The purpose of this article is to provide an overview of the main mechanisms of pathogenicity, to describe the main virulence factors and their interactions with host defense mechanisms and their role in the pathogenesis of infections. In addition, description of the phenomenon of persistent bacterial infections and the presence of persister cells has been also developed.

Pathogenesis of bacterial infections

Pathogenesis refers both to the mechanism of infection and to the mechanism by which disease develops (Finlay and Falkow, 1989). It is well known that the pathogenic mechanisms of several infections are poorly understood but, basically, bacteria may cause disease by two mechanisms: direct damage of host cells by virulence factors, and indirectly by stimulating a great host inflammatory/immune response. There are some steps involved in the bacterial pathogenesis: transmission into the body by several routes, adhesion which is necessary to avoid innate host defense mechanisms, invasion or penetration in host cells and tissues, survival in the host, and tissue injury.

Host susceptibility

Susceptibility to bacterial infection is related with the immunologic and physiologic condition of the patient and with the virulence of the bacteria. When the microorganism has invaded the host, the first line defense barrier is a “nonspecific” mechanism which includes polymorphonuclear neutrophils and macrophages (Eisenstein et al., 1983) and the skin which contains a great variety of microorganisms in an enormous quantity. The normal microbiota of the skin and mucosal surfaces also protect the host against colonization by bacterial pathogens. Most bacteria present in this location and in the environment are relatively benign to persons with normal immune system. However, in immunosuppressed individuals like those with VIH infection or receiving chemotherapy, opportunistic microbial pathogens may cause severe infections. In addition, mucous membranes of the body (e.g., urogenital, gastrointestinal and respiratory systems) are locations through which microorganisms can entry to the body. In these locations, the presence of lysozyme, lactoferrin and lactoperoxidase can eliminate the bacteria or inhibit their growth.

After this, a specific immunity such as the antibody response to the bacteria is established in several weeks. People develop local specific antibodies for these bacteria. Antibodies cause lysis of the organisms and/or their opsonization by phagocytes and they are quickly killed. After phagocytosis, the bacterial microorganisms usually are killed. When an inflammatory process occurs, phagocytic cells, along with lymphocytes, play an important role in innate immunity to bacterial infections.

The mucous membranes also contain immunoglobulins, especially IgA, produced by plasmatic cells present in the submucosal tissue. In healthy persons, a bacterium present in the normal microbiota that may penetrate into the body is eliminated in most cases by the host's humoral and cellular mechanisms. On the other hand, persons with an altered immune system could be infected by these less virulent microorganisms causing recurrent infections. Moreover, in several circumstances such as the elderly or the infants, a decrease of the mechanisms of defense could be observed, being especially susceptible to some pathogens. Other patients have several genetic deficiencies of the complement system or cellular defenses or may develop a decrease of some mechanisms of defense as a consequence of various predisposing diseases like cancer, organ transplantation or chemotherapy.

Host defenses may be disrupted by other several circumstances or diseases such as cystic fibrosis, urinary tract obstructions, medical procedures or devices' implantation.

On the other hand, intracellular parasites are primarily killed by cell mediated immunity. Continuously generated antigens released from persisting microbes will elicit humoral antibodies and cell mediated immunity which will kill these bacteria.

Pathogenic mechanisms

Bacterial infectivity

Virulence factors are factors that are produced by some microorganisms and may cause disease in the host (e.g., toxins, surface receptors, ...). Many virulence factors are produced only by specific virulent strains of a bacterium (e.g., enterotoxins for some *E. coli* strains). The clinical course of a disease usually depends on the interaction of virulence factors and the response of the host. When the balance between virulence factors and host response is favorable to the first one, the infection begins.

Host-mediated pathogenesis

In some infections such as Gram-negative bacterial sepsis, tuberculosis and leprosy, the pathogenesis of these infections cannot be taken into account apart from the host immune response; in these cases, the tissue damage may be produced by the host response to the infection rather than by specific virulence factors of the bacteria. It is to highlight that the tissue damage in these circumstance is caused by several toxic factors produced by polymorphonuclear neutrophils, lymphocytes and macrophages which infiltrate the site of infection. When this cellular reaction occurs, the host response can be of great severity and intense and may cause proliferation of the bacteria and destruction of host tissue.

Intracellular growth

The capacity of bacteria to survive and multiply within host cells is an important factor of the pathogenesis to cause infection. The majority of bacteria do not enter within the cells and they prefer to proliferate in the extracellular matrix; some of them are able to attach to the epithelial surfaces secreting toxic substances or toxins. In most cases, bacteria present within cells or in the extracellular environment are eliminated from humoral antibodies or by a cellular immune response. However, other pathogenic bacteria can proliferate inside the cells due to some protection mechanisms. Some bacteria are able to survive intracellularly by releasing phospholipases which eliminate the phagocytic vesicle surrounding them (e.g., *Rickettsia rickettsii*). On the other hand, *Legionella pneumophila* may grow within the macrophages blocking the lysosomal fusion by unknown mechanisms. Other bacteria are able to grow in low-pH environment within the lysosomal granules such as *Coxiella burnetii*. In addition, some bacteria like *Salmonella* and *Mycobacterium* species are very resistant to the action of phagocytic cells.

Molecular and genetic basis for virulence

Virulence factors in bacteria may be encoded on both chromosomal and bacteriophage DNA, plasmids or transposons. Other virulence factors are acquired by bacteria after infection by a bacteriophage which integrates its genome into the bacterial chromosome. Other virulence factors are encoded on the bacterial chromosome (e.g., cholera toxin).

Specific virulence factors

Virulence corresponds to the pathogenicity of a microorganism. This characteristic is closely related to the ability of the pathogen to cause disease despite host resistance mechanisms. Virulence factors can be divided into two categories: those that cause damage to the host (e.g., toxins), and those that produce colonization and survival of infecting bacteria.

Bacterial toxins

There are two types of toxins: exotoxins and endotoxins (Berry, 1977). Exotoxins are protein molecules liberated from viable bacteria. Most of them are heat labile and are produced by some members of both Gram-positive and Gram-negative genera. They are excellent antigens that elicit protective antitoxic antibodies called antitoxins. The exact functions of these exotoxins for the bacteria are usually unknown and the site of action of most exotoxins is often localized in particular cell types or cell receptors. Exotoxins can be produced in several different ways: ingestion of preformed toxin (e.g., botulism), colonization of wound or surface followed by toxin production (e.g., diphtheria, cholera), and production by bacteria in tissues (e.g., *Clostridium perfringens*).

Based on their biologic effect on host cells, exotoxins may be classified into some categories such as neurotoxins, enterotoxins and cytotoxins. Cytotoxins constitute a heterogeneous group with a wide spectrum of toxic manifestations (e.g., diphtheria toxin produced by *Corynebacterium diphtheriae*). An example of neurotoxin may be those produced by *Clostridium* spp. (e.g., botulinum toxin produced by *Clostridium botulinum*). On the other hand, enterotoxins can stimulate hypersecretion of water and electrolytes from the intestinal epithelium that cause watery diarrhea (e.g., shiga-enterotoxin produced by *Shigella* spp., shiga-like enterotoxin produced by *E. coli*, cholera toxin...).

Other classification of exotoxins includes:

- (a) A-B toxins: these toxins act intracellularly, and they are composed of two components (A and B portions). The B portion binds to a specific host cell receptor and the A portion passes into the cells and has biological activity against a specific target.
- (b) Membrane damaging toxins: these toxins cause damage digesting enzymatically the components of membranes which leads to osmotic lysis and cell death. Some examples of these toxins are the enzymes that hydrolyze phospholipids (e.g., phospholipase, sphingomyelinase), toxins that disrupt by membrane solubilization (e.g., delta toxin of *S. aureus*) and pore forming toxins that insert in the membrane and form a hydrophilic pore.
- (c) Superantigens: they are toxins that bind directly to major histocompatibility complex II (MHC II) on macrophages and form a crosslink with T cell receptors. As a consequence of this, increased amounts of IL-2 are produced and further stimulation of other cytokines lead to shock production (e.g., staphylococcal toxic-shock syndrome).
- (d) Extracellular enzymes: they may cause severe tissue damage aiding in dissemination (e.g., coagulase, hyaluronidase and protease, collagenase and DNase).

The second group of toxins is the endotoxins which are toxic components of the bacterial cell envelope. Endotoxins are promoters of inflammation; the most potent endotoxin is the lipopolysaccharide (LPS) produced by Gram-negative bacteria. In addition, in Gram-positive bacteria peptidoglycan and teichoic acid are also endotoxins. The toxic effects of endotoxins include leukopenia followed by leukocytosis, pyrogenicity, complement activation, hypotension, induction of prostaglandin synthesis, hypothermia and mitogenicity. LPS binds CD14 and this complex binds Toll-like receptor 4 (TLR4) on macrophages and monocytes. On the other hand, TLR1 binds peptidoglycan and TLR2 teichoic acids. Macrophages and monocytes release cytokines inducing prostaglandin and leukotriene release. Thus, the complement and coagulation cascades are immediately activated. At the end of these events, sepsis and lethal shock may be produced with multiple organ system failure.

Factors involved in bacterial invasion of tissues

In this case, the host damage is caused by direct disruption of function or an increased immune response that affects tissue function. The invasive bacteria could be classified as obligate intracellular microorganisms (e.g., rickettsia, chlamydia), facultative intracellular bacteria (e.g., *Mycobacterium*) and extracellular pathogens. Overall, there are some steps in bacterial invasion:

1. Motility that uses several elements such as flagella, corkscrew, gliding motility or chemotaxis using a gradient.
2. To cause infection, many bacteria must first adhere to a mucosal surface and multiply. Bacteria have developed some attachment mechanisms by using two strategies: fimbriae (*pili*) and monomeric protein adhesins mediated by cell surface proteins.
3. Invasion of cells (in intracellular microorganisms): the specific bacterial surface factors that mediate invasion are unknown in the majority of cases, but there are two main mechanisms for invasion: bacteria can present ligands on their surface allowing to bind to host cells and initiate the entry process (zippering), and bacteria can inject effectors into host cells to regulate phagocytosis (triggering).
4. Manipulation of host cell functions: pathogens can alter some functions of host cells by injecting effector proteins directly into the host cell cytoplasm.

Survival in the host

Some microorganisms are able to resist the cytotoxic action of plasma and other body fluids by antibodies, complement or lysozyme. A major survival mechanism is the derision of phagocytosis by extracellular pathogens. With regard of this, the presence of capsule or secretion of protein A and/or protein M may have this function.

Protein A is a surface component of *S. aureus* binding to the Fc portion of immunoglobulins. Bacteria can activate the classical complement cascade which results in the attachment of fragments of C3. After this, phagocytosis can occur, but in the presence of protein A, interaction of bacteria with C3 receptors will be inhibited.

On the other hand, peptidoglycan such as LPS, can activate the alternate complement cascade. The M protein of the group A streptococci binds fibrinogen which blocks complement binding; thus, streptococci are non phagocytosed.

Finally, intracellular pathogens can survive within phage lysosomes or within phagosomes.

Bacterial persistence

Several bacterial infections are capable to persist in the host for long periods of time, even if antimicrobial treatment has been applied. Persisters were firstly described after the discovery of antibiotics by Joseph Bigger in 1944, when a culture of *Staphylococcus aureus* was not sterilized with high doses of penicillin (Bigger, 1944; Hobby et al., 1942). Several factors in both the host and the bacteria can contribute for the start-up and maintenance of persistent infections, including antimicrobial resistance and immunosuppression factors. Another important fact is the presence of persister cells that have been well demonstrated in *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus* (Helaine and Kugelberg, 2014; Harms et al., 2016; Michiels et al., 2017). Persisters may cause antimicrobial treatment failure and can lead to the emergence of antibiotic resistance (Cohen et al., 2013; Levin-Reisman et al., 2017). Moreover, a decrease in metabolic activity has been demonstrated in the persister phenotype (Amato et al., 2014). These persisters could contribute to treatment failure of bacterial infections and to the evolution of genetic resistance. The effect of persistence could be applied for causing resistance to the majority of antibiotics.

Persister cell formation

It is well known that several circumstances increase the possibility of formation of persister cells such as nutrient limitation, extreme pH and DNA damage (Helaine et al., 2014; Maissoneuve et al., 2013; Dörr et al., 2010). The presence of biofilms that could be inhabited by bacteria can be also be associated with the formation of persisters by the fact of the presence of some or all of the cellular stresses factors above mentioned. Inside the biofilms, not all bacterial population form persisters due to the fact of the limitation of nutrients. The presence of macrophages can also induce the presence of persisters restricting nutrient availability and decreasing the pH.

On the other hand, phenotypic resistance permits a subpopulation of bacteria to keep growing in the presence of an antimicrobial.

Asymptomatic persistent infections may be caused by latent growth-arrested bacteria (e.g., tuberculosis, typhoid fever, urinary tract infections by *E. coli*, ...). On the other hand, symptomatic persistent infections have clinical manifestations over a long period of time usually despite antimicrobial therapy (Grant and Hung, 2013). Repetitive use of drugs for infections may also increase the selection of antibiotic-resistant strains and decrease of the normal microbiota (Jernberg et al., 2010). They often may be associated to the use of indwelling medical devices or to appear in patients with suppression of immunity (e.g., HIV infection, cancer, chemotherapy, ...).

The bacteria that cause persistent infections usually have never been cleared from the host during antimicrobial treatment.

The survival of persister cells is often explained by their transition to a dormant state characterized by a reduction of growth rate and metabolism. It has been proposed that bacterial communities generate a diverse quantity of dormant cells, but the nonreplicating state is not sufficient for survival because bacteriostatic conditions do not necessarily induce antimicrobial tolerance. In addition, persister formation involves specific qualitative changes of bacterial physiology to permit survival and resuscitation. Some studies have demonstrated that the signaling that controls bacterial persistence and the mechanisms related to persister formation are genetically encoded (Lewis, 2010). Another hypothesis explains the presence of persisters as nongrowing cells arising from errors and glitches (Levin et al., 2014).

Causes of persistent infections

Absence of clearance by the host

If not antimicrobial treatment has been applied, a persistent infection may be due to the failure of the host immune system to eliminate the pathogen as a consequence of absence of detection of the microorganism by the immune system. Some bacteria have developed mechanisms or strategies to avoid detection by the host; for example a variation of the expression of surface antigens in *Borrelia* species (Norris, 2006). Another mechanism is the possibility of the modulation of host immune response resulting in an ineffective clearance of the pathogen. In this case *L. monocytogenes* and *M. tuberculosis* induce the production of interleukin-10 in macrophages to suppress the interferon- γ -response (Redpath et al., 2001). Other mechanisms include the inhibition of maturation of phagosomes (Scott et al., 2003) and the inhibition of MHC class II antigen presentation in dendritic cells (Bayer-Santos et al., 2016).

On the other hand, the formation of biofilms has also been proposed as a mechanism to the persistence of some infections (Costerton et al., 1999). Finally, the formation of granulomas such as in *M. tuberculosis* leads to the absence of macrophages interaction and persistence of infection.

Absence of clearance by antimicrobials

When antibiotic treatment is being applied and no cure of infection can be observed, treatment failure could be due to some factors such as poor pharmacokinetics of drugs, low patient compliance and/or acquired antibiotic resistance (Kardas, 2002; Gonçalves-Pereira and Pôvoa, 2011; Blair et al., 2015). If not antimicrobial resistance is detected, bacteria can survive due to antibiotic tolerance. Both mechanisms, antibiotic resistance and tolerance can either occur in a whole population of bacteria or in a subpopulation.

Persistent infections and persister cells

During treatment with antibiotics, there are some bacteria that cause infections which were never cleared from the host; in this sense, studies with *Salmonella typhimurium* isolates have demonstrated that in cases of recurrent fever in these patients, around 80% were caused by the same *genovar* as the first infection (Okoro et al., 2012).

Genetic mutations in bacteria may increase antibiotic tolerance by slowing growth (Fridman et al., 2014). In addition, mutations can also increase the probability of persister formation in a clonal population. These mutations were firstly demonstrated in *E. coli* (*hipA7* mutation) being called high-persistence (*hip*) mutations (Moyed and Bertrand, 1983). Regarding to the patients affected by cystic fibrosis, they are usually infected by strains of *P. aeruginosa* and these isolates are more likely to be *hip* mutants if obtained later in infection (Mulcahy et al., 2010).

On the other hand, it has been observed that periodic treatment of *Candida* species with antifungals is able to select for *hip* mutants (Lafleur et al., 2010).

An important fact is the link established between the presence of persisters and biofilm formation; thus, several studies have showed that the formation of biofilms is associated with persistent infections (Costerton et al., 1999; Grant and Hung, 2013). Some examples of formation of biofilms are *P. aeruginosa* in patients with cystic fibrosis and uropathogenic strains of *E. coli* on the bladder epithelium.

Clinical relevance of persisters

Many patients may suffer from bacterial infections and despite the antimicrobial treatment these infections can be often chronic and are never fully cleared because these bacteria can persist in different ways (e.g., biofilm formation). Some clinical examples are the infections due to *M. tuberculosis*, urinary tract infections caused by uropathogenic strains of *E. coli*, opportunistic infections of implanted devices typically caused by *Staphylococcus* spp. and *P. aeruginosa*.

In some animal models of bacterial infections with these microorganisms, a heterogeneous population of persisters has been observed. In vitro, bacterial persisters show increased tolerance to antibiotic treatment and then induce microorganism persister formation. The usual treatment of chronic infections is linked to increased persister levels of clinical isolates and rapid selection for mutants. In this sense, different stress signaling pathways commonly involved in persister formation are known to increase mutations.

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Staphylococcus and Other Catalase-Positive Cocci

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Introduction

Staphylococci belong to the Micrococcaceae family, which also includes Micrococcus, Stomatococcus, and Planococcus. These bacteria are catalase-positive gram-positive cocci that divide into irregular clusters and form a “grape bunch”-like clustering when viewed under the microscope. The genus *Staphylococcus* comprises microorganisms that are present in the mucosa and on the skin of humans and other mammals and birds, including a total of 51 species, of which 17 can be isolated from humans and cause a wide spectrum of systemic diseases that can be life-threatening (Tong et al., 2015). From a microbiological point of view, considering the ability to produce coagulase and the clinical picture, they can be divided into two groups:

- Coagulase positive staphylococci: *Staphylococcus aureus* is the most important; *S. argenteus*, *S. schweitzeri*, *S. intermedius*, *Staphylococcus pseudintermedius* and *Solanum coagulans* (previously *Staphylococcus schleiferi* ssp. *coagulans*).
- Coagulase negative staphylococci (CoNS): *Staphylococcus epidermidis* is the most prevalent; *Staphylococcus capitis*, *Staphylococcus cohnii*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus hyicus*, *Staphylococcus lugdunensis*, *Staphylococcus pettenkoferi*, *S. saccharolyticus*, *Staphylococcus saprophyticus*, *S. schleiferi*, *Staphylococcus sciuri*, *S. simulans*, *Staphylococcus warneri* and *Staphylococcus xylosus*.

Species most frequently associated with human disease are *S. aureus* (the most virulent and best-known member of the genus), *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, and *S. saprophyticus*. Staphylococcal infections are one of the most prevalent, both in the community and in the hospital, according to the latest EPINE (Prevalence Study of Nosocomial Infection in Spain). *Staphylococci* are among the first causative agents of skin and soft tissue infections (IPPB), osteoarticular infections (IOA), bacteremia and endocarditis (Chalmers and Wylam, 2020). About 20% of the population is permanently colonized by *S. aureus*. The main reservoir for *S. aureus* is the nostrils of the carriers. Spread occurs from person to person through direct contact or exposure to contaminated fomites. The infection generally begins in areas with previous alterations of the mucocutaneous barrier. Colonization increases the risk of infection (Sakr et al., 2019).

It can act through toxins (alpha-hemolysin, Pantone-Valentine leucocidin [LPVL]) or superantigens (TSST-1, enterotoxins), or by invasion of any organ or tissue and cause suppuration, tissue necrosis, vascular thrombosis and bacteremia (McGuire et al., 2020). It is the microorganism with the greatest capacity to cause hematogenous metastasis. It can also form biofilms and lead to persistent and chronic infections (Oliveira et al., 2018).

Based on the susceptibility to oxacillin/methicillin, *S. aureus* is divided into two groups: methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). MRSA causes resistance to almost all beta-lactam drugs except ceftaroline and ceftolozime (Chalmers and Wylam, 2020). The importance of MRSA has been made evident not only by the implications for a worse prognosis, but also by the increased demand for healthcare resources. Patients infected with MRSA require a greater number of diagnostic and antimicrobial tests and are associated with prolonged hospital stays and higher mortality (Morosini et al., 2012). For this reason, programs have been established to detect colonized individuals trying to reduce the risk of acquiring a MRSA infection (European Centre for Disease Prevention and Control, 2020a,b,c).

Epidemiology

According to the prevalence study recently published by the European Center for Diseases Control and Prevention (ECDC), *Escherichia coli* (44.2%) is the most important pathogen in nosocomial infection, the second being *S. aureus* (20.6%). Thirty countries in the European Union (EU) or the European Economic Area (EEA) reported 2019 data to the European Antimicrobial Resistance Surveillance Network (EARS-Net). (European Centre for Disease Prevention and Control, 2020a,b,c). For *S. aureus*, there is a decrease in the percentage of methicillin-resistant isolates (i.e., MRSA) reported compared to previous years. However, MRSA remains an important pathogen in the EU/EEA, with levels still high in several countries, and combined resistance to another group of antimicrobials was common (European Centre for Disease Prevention and Control, 2020a,b,c). In general, northern European countries reported lower percentages of resistance and southern and eastern European countries reported higher percentages (World Health Organization, 2015).

EU/EEA population-weighted mean percentage of MRSA was 15.5% in 2019. This denotes a significantly downward trend for the period 2015–2019. Among MRSAs, combined resistance to another group of antimicrobials was common. The most common resistance combination was MRSA and fluoroquinolone resistance. Many countries have developed and implemented national recommendations and guidance documents on preventing the spread of MRSA, focusing both on improving infection prevention and control and the prudent use of antimicrobials (European Centre for Disease Prevention and Control, 2018). Despite this positive development, MRSA remains a major pathogen in Europe. *S. aureus* is one of the most common causes of bloodstream infections and has a high burden in terms of morbidity and mortality. Hospital clones (HA-MRSA) were the first to emerge and responsible for the large increase in MRSA in hospitals during the 1980s and 1990s. In some Asian countries, the dispersal of HA-MRSA clones in the community has been observed, behaving like community clones (CA-MRSA) (Cantón and Ruiz-Garbajosa, 2013).

For this reason, universal screening programs have been established for patients admitted to hospitals to detect colonized individuals, trying to reduce the risk of acquiring a MRSA infection. This action has allowed a drastic decrease in MRSA infection (Cantón and Ruiz-Garbajosa, 2013).

Microbiology

These bacteria are catalase-positive gram-positive cocci that are characterized by not forming spores, they are spherical in shape. Most staphylococci are 1.5 µm tall, immobile, and capable of growing under a variety of facultative aerobic and anaerobic conditions in the presence of high salt concentrations (e.g., 10% sodium chloride) and at temperatures 18–40 °C, except for *S. saccharolyticus* and *S. aureus* ssp. *anaerobius*, which are strictly anaerobic. *S. aureus* produces acute infections, associated or not with toxins, of any location, although the most common are skin and soft tissue (IPPB), osteoarticular (IOA), bacteremia, endocarditis and pneumonia. In contrast, CoNS infections are more long-standing and are mainly related to the presence of foreign bodies, with bacteremia associated with vascular catheters being very common (Murray et al., 2021).

Coagulase-negative staphylococci are normal commensals on the skin and mucosa of various mammalian hosts. *S. epidermidis* is the most prevalent species on human skin. Given the variable characteristics of human skin, including its moisture, nutrients, pH range, and temperature, *S. epidermidis* can adapt to different environmental conditions. Some species of coagulase-negative staphylococci are well adapted for survival in specialized niches, such as *S. capitis* (in the human scalp), *Staphylococcus auricularis* (in the human external auditory canal), and *S. saprophyticus* (in the human scalp, digestive and genitourinary tracts of the human population) (Becker et al., 2014).

Mechanisms of pathogenicity

Virulence factors include structural components that facilitate adherence to host tissues and prevent phagocytosis, and a variety of toxins and hydrolytic enzymes. Among the structural components of *Staphylococcus* are the following:

- Capsule: it is the outermost layer of the staphylococcal cell wall, 11 capsular serotypes of *S. aureus* have been identified:
 - Serotypes 1 and 2 are associated with thick capsules and colonies with a mucoid appearance, but they rarely cause disease in people.

- o Serotypes 5 and 8 are responsible for the majority of human infections. The capsule protects bacteria by inhibiting phagocytosis of these microorganisms by polymorphonuclear leukocytes (PMN).
- Silt or biofilm layer: most *staphylococci* have a loose water-soluble layer made up of monosaccharides, proteins, and small peptides. Facilitates adherence to tissues and foreign bodies, such as catheters, grafts, valve and joint prostheses.
- Peptidoglycan: it is formed by layers of glucan chains built with 10 or 12 alternating subunits of *N*-acetylmuramic acid and *N*-acetylglucosamine. Gram-positive microorganisms are composed of numerous interlocking layers, which confer greater rigidity to the cell wall. The enzymes that catalyze the construction of the peptidoglycan coat are called penicillin-binding proteins because they are the targets for penicillins and other β -lactam antibiotics. Bacterial resistance to methicillin and related penicillins is mediated by the acquisition of a gene (*mecA*) that encodes a new penicillin-binding protein, PBP2a. The *mecA* gene is located on the staphylococcal *mec* cassette (SCCmec) chromosome and six gene sequences are described in this cassette (types I-VI). This information is important because MRSA strains, previously limited to hospital infections, now appear in the community and are responsible for the majority of staph infections. These strains usually have type IV SCCmec, these strains have moved from the community to the hospital (Kwiecinski and Horswill, 2020).
- Teichoic acids: they are specific phosphate polymers bound to *N*-acetylmuramic acid residues of the peptidoglycan layer or through a lipophilic bond to the cytoplasmic membrane. They stimulate a specific humoral response.
- Surface adhesion proteins: they are covalently bound to the peptidoglycan of the cell wall in staphylococci and have been designated as MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules) proteins, components of the microbial surface that recognize matrix molecules adhesive. The best characterized MSCRAMM proteins are staphylococcal protein A, fibrin-binding proteins A and B, and aggregation factor proteins A and B. The aggregation factor proteins (also called coagulase) bind to fibrinogen and convert it into fibrin; this constitutes the identification test of *S. aureus* (Balasubramanian et al., 2017).

Staphylococcal infections depend on the ability of bacteria to evade phagocytosis, produce adhesion proteins of the bacteria to host tissues, and produce tissue destruction through the manufacture of specific toxins and hydrolytic enzymes. In the expression of virulence factors, the most important is the AGR (accessory gene regulator). This quorum sensing control system allows the expression of adhesion proteins and promotes tissue colonization when the density of bacteria is low and of hydrolytic enzymes and toxins when the density is high.

Among the components of staphylococcal toxins are the following:

- Five cytolytic toxins (hemolysins) that damage the membrane (alpha, beta, delta, gamma, and Panton-Valentine [P-V] leucocidin).
- Two exfoliative toxins (A and B).
- 18 Enterotoxins (A to R).
- Toxin-1 from toxic shock syndrome (TSST-1).

Cytotoxins can cause lysis of neutrophils, resulting in the release of lysosomal enzymes that subsequently damage surrounding tissues. A cytotoxin, P-V leucocidin, has been linked to severe lung and skin infections. Exfoliative toxin A, enterotoxins, and TSST-1 belong to a class of polypeptides known as superantigens (Murray et al., 2021).

Cytotoxins

- Alpha toxin is a 33,000 D polypeptide produced by most *S. aureus* strains that cause disease in humans. The toxin disrupts the smooth muscle of the blood vessels and is toxic to many cells. It is a mediator of tissue damage in staphylococcal disease.
- Beta toxin is a 35,000 D thermolabile protein produced by most of the *S. aureus* strains that cause disease in humans. Catalyzes the hydrolysis of membrane phospholipids in susceptible cells. It is responsible for the differences in susceptibility of different species to the toxin.
- Delta toxin is a 3000 D polypeptide produced by almost all *S. aureus* strains and most staphylococcal species. It has cytolytic activity. It alters cell membranes through a detergent-like action.
- Gamma toxin (manufactured by most strains of *S. aureus*) and Leucocidin PV are toxins made up of two components that consist of two polypeptide chains: the S component (slow eluting proteins) and the F component (proteins of rapid elution). Proteins S (and two proteins F) have been identified. Bacteria that produce both toxins code to produce six different toxins. These six toxins can lyse neutrophils and the most intense lytic activity. It is encoded by mobile phage. PV leucocidin toxin is leucotoxic, but it lacks hemolytic activity.
- Leucocidin toxin PV has generated great interest because, although it is found in less than 5% of all hospital MRSA strains, it is identified in virtually all MRSA strains associated with community infections (soft tissue infection and pneumonia in young people) (Oliveira et al., 2018).

Exfoliative toxins

Scalded skin syndrome (SPEE) is characterized by exfoliative dermatitis. It is seen in young children and is rarely described in older children or adults.

Two different forms of exfoliative toxin have been identified (ETA and ETB), and both can cause disease. ETA is heat stable and the gene associates with a phage, while ETB is heat labile and is located on a plasmid. The toxins are serine proteases that break down desmoglein-1, so staphylococci are not present in the affected epidermis layer. After exposure of the epidermis to the toxin, protective neutralizing antibodies develop, leading to resolution of the toxic process.

Enterotoxins

18 staphylococcal enterotoxins (A to R) have been identified:

- Enterotoxin A is the one most frequently associated with food poisoning.
- Enterotoxins C and D are found in contaminated dairy products.
- Enterotoxin B produces pseudomembranous colitis.

They are perfectly designed to cause foodborne diseases, as they are stable even when heated to 100 °C for 30 min and resist hydrolysis by gastric and jejunal enzymes. Therefore, when a food product is contaminated with enterotoxin-producing staphylococci and toxins are produced, neither mild reheating of the food nor exposure to gastric acids will be protective. These toxins are produced in 30–50% of *S. aureus* strains (Oliveira et al., 2018).

Toxin-1 toxic shock syndrome toxin-1

It is estimated that 90% of the strains of *S. aureus* that cause menstrual toxic shock syndrome (TSS) and half of the strains that cause other forms of TSS produce TSST-1. Enterotoxin B and enterotoxin C originate half of the TSS cases not associated with menstruation. TSST-1 is a superantigen that stimulates the release of cytokines and causes extravasation of endothelial cells, it has a cytotoxic effect on cells. The ability of TSST-1 to cross mucosal barriers causes the systemic effects of SST. Death in patients with TSS occurs as a consequence of hypovolemic shock that causes multiple organ failure.

Staphylococcal enzymes

- Coagulase: *S. aureus* strains have two forms of coagulase: bound and free.
 - o The bound coagulase/aggregation factor/clumping factor binds to the surface of the staphylococcal cell wall directly converting fibrinogen to insoluble fibrin to form staphylococcal aggregation (*S. aureus*, *S. lugdunensis*, *S. schleiferi* ssp. *schleiferi* and *S. sciuri*).
 - o Free coagulase achieves the same result by reacting with a globulin-like plasma factor (coagulase-reactive factor) to produce a thrombin-like enzyme. This factor catalyzes the conversion of fibrinogen to insoluble fibrin (*S. aureus*, *S. intermedius*, *S. schleiferi* sub *coagulans*, and *S. lutrae*).
 - o The role of coagulase can cause the formation of a fibrin layer around the staphylococcal abscess, the infection is localized, and the microorganisms are protected from phagocytosis (Tam and Torres, 2019).
- Hyaluronidase: hydrolyzes hyaluronic acids, present in the acellular matrix of connective tissue. Staphylococci hydrolyze host tissue components and aid in the spread of bacteria.
- Fibrinolysin: can dissolve fibrin clots.
- Lipases: All *S. aureus* strains and more than 30% of all coagulase negative *Staphylococcus* strains produce several different types of lipases, which hydrolyze lipids and ensure survival of staphylococci.
- Nuclease: *S. aureus* also produces a thermostable nuclease, which can hydrolyze viscous DNA.

S. aureus is one of the microorganisms with the most pathogenic mechanisms for developing infections. It can act through toxins (alpha-hemolysin, Panton-Valentine leucocidin [LPVL]) or superantigens (TSST-1, enterotoxins), or by invasion of any organ or tissue and cause suppuration, tissue necrosis, vascular thrombosis and bacteremia (Sadeghi et al., 2017). It is the microorganism with the greatest capacity to cause hematogenous metastasis. It can also survive in the cell cytoplasm, form biofilms and lead to persistent and chronic infections or remain quiescent and reactivate later. Likewise, in the cell cytoplasm it can undergo phenotypic changes consisting of the formation of so-called Small Colony Variants with intracellular persistence capacity, which is one of the mechanisms by which this microorganism can cause chronic or recurrent infection. They are slow-growing, punctate colonies, not pigmented, not beta-hemolysis. They usually occur in cystic fibrosis, osteoarticular devices and infections. Need for prolonged treatments with rifampicin associations. But the most common cause of chronic staphylococcal infection that is resistant to antibiotic treatment or recurrent is the formation of biofilms in vascular catheters, bone sequestration, pacemakers or any type of endovascular foreign material (Melter and Radojevič, 2010). The permanence of venous catheters with *S. aureus* infection is often a cause of persistent or recurrent bacteremia and distant septic complications.

Biofilm formation is also the main pathogenetic mechanism of CoNS, with the production of toxins and extracellular enzymes occupying a secondary place (Lister and Horswill, 2014).

S. saprophyticus causes urinary tract infections due to certain proteins that allow it to bind to the urothelium and a urease that contributes to cytopathic action and tissue invasion.

S. lugdunensis behaves like *S. aureus*, producing acute and severe infections. Its virulence has been related to multiple mechanisms, among which the following stand out:

- Production of adherent surface proteins that bind to tissues and foreign bodies.
- Formation of biofilms

- Resistance to lysozyme and elaboration of a delta-like hemolysin similar to the delta-hemolysin of *S. aureus*.
- The presence of sequences related to the *agr* (accessory gene regulator), a quorum-sensing system, which acts as a global regulator of virulence factors.

Clinical manifestations

Suspicion of a staphylococcal infection should be raised in certain situations. While *S. aureus* infections are acute and severe, those caused by CoNS are usually subacute and related to foreign bodies, with the exception of *S. saprophyticus*, which causes mild urinary tract infections in young women in late summer and *S. lugdunensis* with a behavior similar to that of *S. aureus*.

MRSA-C produces outbreaks with great dissemination capacity in groups of homosexuals, injecting drug users, residents of geriatric centers, athletes, soccer players, prisoners and the military, among others. These are mainly IPPB of varying severity and pneumonia with a tendency to necrosis (Tong et al., 2015).

Clinical manifestations of *Staphylococcus aureus*

- Scalded skin syndrome (SPE) due to exfoliative toxins A and B. Locally or generalized, blisters of clear fluid with positive Nikolsky's sign appear and self-limit in 4–7 days, probably the time needed to synthesize antitoxin immunoglobulins. It mainly affects infants producing a disseminated desquamation of the epithelium generating blisters devoid of microorganisms or leukocytes. Treatment focuses on hydric replacement. Antibiotic treatment is not indicated because the disease has been caused by a preformed toxin (Leung et al., 2018; Del Giudice, 2020).
- Food poisoning by enterotoxins due to ingesting contaminated food (processed meats, cream puffs, etc.) with the thermostable toxin, presents a rapid onset of intense vomiting, watery diarrhea and colic, resolving within 24 h.
- Toxic shock syndrome (TSS) caused by toxic shock toxin 1 or exotoxin B or C behaving like a superantigen. It can be given during menstruation or not related to it. In the first case, it has been associated with the use of high-absorption buffers, and in the second, the responsible *S. aureus* can colonize any location in the patient. Clinically, it is characterized by high fever, hypotension, generalized edema, hypoalbuminemia, and a morbilliform rash that peels off within a few days. The toxin is produced locally and blood cultures are typically negative. It presents high mortality in the absence of immediate antibiotic treatment and elimination of the focus of the infection (Silversides et al., 2010).
- Impetigo: localized superficial skin infection characterized by the presence of pus-filled vesicles on an erythematous base on the face and extremities, especially in young children.
- Folliculitis: pyogenic infection that affects the hair follicles (Del Giudice, 2020).
- Boils: large painful skin nodules filled with pus and necrotic tissue accumulated underneath.
- Anthrax: union of boils with extension towards the subcutaneous tissues and signs of systemic disease (fever, chills, bacteremia).
- Bacteremia and endocarditis: spread of bacteria into the blood from a source of infection, most are acquired in the hospital after surgery or after a contaminated intravascular catheter; endocarditis is characterized by damage to the endothelial lining of the heart, which can lead to peripheral septic embolization (*S. argenteus*, *S. intermedius*, *S. pseudintermedius* and *S. coagulans*) (Taquin et al., 2019).
- Pneumonia and empyema: consolidation and abscess formation in the lungs; it is observed in very young and elderly subjects, and in patients with underlying lung disease; A severe form of necrotizing pneumonia with septic shock and high mortality has been recognized. Empyema affects 10% of patients with pneumonia, purulent material is drained (*S. intermedius*, *S. pseudintermedius*) (Grau, 2017).
- Osteomyelitis: destruction of bones in the metaphyseal area of long bones after hematogenous bone spread or infection as a consequence of trauma or extension of an adjacent infection. It is characterized by bone pain and high fever.
- Septic arthritis: painful erythematous joint with accumulation of purulent material in the joint space, usually in the form of vertebral osteomyelitis, presenting low back pain with fever. It occurs in young children and adults who receive intra-articular injections or joints with mechanical abnormalities.

Clinical manifestations of coagulase negative staphylococci (CoNS)

Previously considered non-virulent contaminants, CoNS have been increasingly recognized as true pathogens, related to foreign bodies and prosthetic devices.

CoNS are the cause of approximately 30% of bacteremia cases. Immunosuppressed patients are at increased risk of bacteremia.

Since CoNS (especially *S. epidermidis*) occupy a prominent place in the commensal flora of human skin and mucosa, they often appear as contaminants in cultures.

Patients with clinical signs of sepsis and multiple positive cultures revealing bacterial growth in less than 24 h are more likely to experience true bacteremia.

When the blood in the catheter is positive 2 or more hours before the peripheral blood, this can be considered an indication that the catheter is the origin of the bacteremia (Murray et al., 2021).

- Wound infections: characterized by the presence of erythema and pus at the site of a traumatic or surgical wound; *S. aureus* and CoNS can cause foreign body-associated infections.
- Infections of the genitourinary tract: dysuria and pyuria in sexually active young women (*S. saprophyticus*), subjects with urinary catheters (other CoNS) or after inoculation of the genitourinary tract due to bacteremia (*S. aureus*).
- Catheter and shunt infections: chronic inflammatory response to bacteria lining a catheter or shunt (most often by CoNS).
- Prosthetic infections: chronic device infection characterized by localized pain and mechanical failure of the device (most often due to CoNS).
- Endocarditis is characterized by damage to the endothelial lining of the heart, *S. epidermidis* and *S. lugdunensis* can infect prosthetic valves more frequently.

Apart from *S. epidermidis*, there are other species of coagulase negative staphylococci that must be specifically exposed due to their pathogenic potential and other special characteristics.

Staphylococcus haemolyticus

It is the second or third most frequent species of CoNS implicated in infectious conditions. They are usually nosocomial bacteremia related to intravascular catheters, skin and soft tissue infections, urinary tract infections, meningitis, endocarditis, and device-associated infections. *S. haemolyticus* has been implicated in outbreaks in neonatal ICUs. The most notable feature of *S. haemolyticus* is that it is often resistant to multiple antibiotics, including glycopeptides (Becker et al., 2014).

Staphylococcus lugdunensis

It behaves clinically similar to *S. aureus* and has been shown to cause fulminant endocarditis of native and prosthetic valves, suppurative skin infections, bacteremia, eye, urinary tract, central nervous system, bone and joint infections, and peritonitis. *S. lugdunensis* has been reported to be more virulent because it can produce several virulence factors, as well as various enzymes such as DNase and lipase, as well as being resistant to lysozyme and can form biofilm.

Identification can be difficult. *S. lugdunensis* is easily confused with *S. aureus* when identification is based solely on the latex agglutination test. Fortunately, this organism is usually susceptible to most antistaphylococcal antibiotics because only about 25% of strains show beta-lactamase production and also because resistance to methicillin is rare. Infections caused by *S. lugdunensis* are treated similarly to those caused by *S. aureus*.

Staphylococcus schleiferi

S. schleiferi appears to be a significant cause of skin infections and otitis in companion animals. It produces very aggressive infections, presenting a small beta-hemolysis.

Staphylococcus saprophyticus

S. saprophyticus colonizes the rectum or urogenital tract of approximately 5–10% of women and is the second most common cause of uncomplicated urinary tract infections in young, sexually active women, after *E. coli*. They occur in late summer and fall, often appear after intercourse or menstruation, and can occur simultaneously with vaginal yeast infection.

90% of the patients with urinary tract infections due to *S. saprophyticus* present symptoms with dysuria, urgency and polyuria, and 80% present pyuria or hematuria.

It is resistant to novobiocin and is susceptible to other antibiotics, and urinary tract infections caused by *S. saprophyticus* are adequately treated with specific antibiotics (Becker et al., 2014).

Diagnosis

Diagnosis of staphylococcal infections is based on the data that make them suspect comorbidity, existence of entry points and clinical manifestations. Common comorbidity is mainly diabetes mellitus, chronic skin diseases, chronic kidney failure on dialysis, ADVP, previous flu, etc. The principal entry points are both skin lesions and devices such as vascular catheters, endotracheal tubes, implants, etc. Characteristic clinical presentation involves abscesses, septic metastases, bacteremia, endocarditis, joint and soft tissues infections.

According to previous studies, the recommended locations for sample collection are nasal swab and cutaneous exudate from the perineal area. The nasal swab is the most appropriate location, although it can be replaced by pharyngeal exudate. With greater susceptibility is the triple sample of nasal, pharyngeal and perirectal or perineal exudates. In patients with mechanical ventilation or tracheostomy, samples of respiratory secretions are also recommended. Other samples, depending on the clinic, are: exudate from ulcers or wounds in patients with broken skin, urine culture in patients with a urinary catheter, blood cultures, ocular and auricular exudates, sterile fluids (articular, pericardial, CSF, etc.) (Mandell et al., 2020).

These samples can be stored for less than or equal to 24 h, at room temperature or in a refrigerator between 2 °C and 8 °C. They do not require any type of preparation prior to inoculation in the culture media. For its culture, the swab is discharged in approximately one third of each plate, rotating it on itself and finally it is extended with a sterile loop in a qualitative way to obtain isolated colonies (Datta et al., 2011).

From a microbiological point of view, in any moderate or severe staphylococcal infection, blood cultures should be performed, samples of the possible primary or metastatic focus obtained, and a nasal swab. The latter is of particular interest when it is not possible to obtain samples from the source of sepsis, since it allows early identification of the state of colonization by *S. aureus* and to know its susceptibility to methicillin. If the patient has not received antibiotic treatment and the blood culture is extracted by venipuncture, a growth time of less than 14 h suggests the existence of an endovascular focus. If the initial blood cultures confirm the existence of staphylococcal bacteremia, they should be repeated every 3 days, as long as they remain positive. The susceptibility profile will allow a targeted treatment.

Microscopy

Staphylococci are gram-positive cocci that form clusters when grown on agar medium, but are generally seen in clinical specimens as single cells or small clusters of microorganisms. Swab or scrape samples from the base of the abscess show large numbers of organisms on Gram stain. The pus aspirate contains necrotic material with a relatively low number of organisms (Murray et al., 2021).

Culture

Clinical specimens should be inoculated onto enriched agar media supplemented with sheep's blood. The most widely used culture medium is blood agar, where the microorganism is subjected to definitive identification. *Staphylococci* grow rapidly on non-selective media, both aerobically and anaerobically, and large, smooth colonies can be seen within 24 h, gradually turning golden. Almost all strains of *S. aureus* and some strains of coagulase-negative staphylococci produce hemolysis on sheep blood agar (Harbarth et al., 2011).

S. aureus in a variety of special media:

- Champagne Medium or Mannitol-Salt Agar: It is a selective medium for staphylococci due to its high content of 5% sodium chloride (Han et al., 2007). Incorporates mannitol and phenol red, allows differences:
 - o Positive mannitol: forming yellow colonies (*S. aureus*, *S. simulans*, *S. sciuri*, *S. cohnii*, *S. capitis*, *S. xylosus*, *S. saprophyticus*, *S. haemolyticus*, *S. warneri*).
 - o Negative mannitol: forms white colonies (*S. epidermidis* and others).
- CHROMAgar *S. aureus*: selective chromogenic medium for *S. aureus* (Chihara et al., 2009).
- CHROMAgar MRSA: Chromogenic medium for the detection of methicillin resistant *S. aureus* (MRSA) (Malhotra-Kumar et al., 2010).
- Medium for the detection of DNase: It is a standard medium for the determination of deoxyribonuclease. Positive organisms will be surrounded by transparent areas of depolymerized DNA (*S. aureus*, *S. schleiferi*, *S. intermedius*, *S. hyicus*).

In the case of a positive gram-positive coconut, catalase, DNase, mannitol and coagulase with growth on MRSA agar (composed of Mueller-Hinton agar supplemented with 4% NaCl and 6 µg/mL oxacillin), the microorganism is identified as MRSA and it is performed antimicrobial susceptibility tests (Wendt et al., 2010; Glick et al., 2014).

Identification

Relatively simple biochemical tests (e.g., positive reactions for coagulase, protein A, thermostable nuclease, and mannitol fermentation) can be used to differentiate *S. aureus*. The identification of coagulase-negatives is more complex and requires the use of commercial identification systems or the detection of species-specific genes through nucleic acid sequencing (Murray et al., 2021).

Phenotypic or genotypic methods can be used to confirm methicillin resistance.

- Phenotypic
 - o Disc diffusion method: cefoxitin disc (30 µg) which is a potent inducer of PBP2a production, a transpeptidase with low affinity for beta-lactam antibiotics but which retains its function in cell wall synthesis, being responsible for resistance to methicillin.
 - o Automated or commercial systems such as Microscan, Vitek and Phoenix systems that use oxacillin and cefoxitin for the detection of MRSA (Sáinz-Rodríguez et al., 2019).
- Genotypic
 - o Confirmation of methicillin resistance in a culture dish can be performed by detecting the *mecA* gene (encoding PBP2a) by conventional or real-time polymerase chain reaction (PCR). It can also be done by latex agglutination to detect the presence of PBP2a. These confirmation methods require prior identification of the microorganism species (*S. aureus*) because resistance to methicillin in coagulase negative strains of staphylococci is also mediated by the *mecA* gene and its product, PBP2a (Arnold et al., 2016; Dupieux et al., 2016; Tasse et al., 2016).

- o PBP2a Immunochromatography: This is a qualitative chromatographic immunoassay for the rapid detection of penicillin binding protein 2a (PBP2a) in previously identified colonies, to help identify methicillin-resistant *S. aureus* (MRSA). It is based on nitrocellulose membrane technology where highly sensitive recombinant monoclonal antibodies (rFabs) and a control protein bind. Results are visually read after 15 min (Sáinz-Rodríguez et al., 2019).

The test can be performed from a direct sample of positive blood cultures with good results. Others are also obtained by performing the identification by MALDI TOF mass spectrometry (Bruker®) from microcolonies a few hours after subculturing the positive blood cultures and the detection of PBP 2a when it comes to *S. aureus*, producing results in a few hours. of great repercussion in the management of the patient (Delpont et al., 2016).

Molecular methods

Based on the performance of a PCR whose objective is to amplify a DNA fragment so that it is easier to identify the microorganism that causes a disease. In the case of MRSA, they detect DNA sequences related to resistance, specifically SCCmec, a mobile genetic element called the mec chromosomal cassette, which carries the mecA gene responsible for resistance (Rocchetti, 2018).

The need to obtain a rapid result in surveillance studies for MRSA has favored the development of molecular tests for its detection. These rapid methods mainly include real-time PCR amplification and allow results to be obtained within 2–6 h.

Currently, the methods based on the PCR reaction for the detection of the mec gene are considered of reference for the detection of MRSA. Detection of the mec gene allows differentiation from other strains of *S. aureus*, very rare, BORSA. MRSA strains with a negative mecA PCR result should be tested for the possible presence of the mecC gene (Paterson et al., 2014). Recently, commercial methods have been developed that include the detection of the mecC gene (Patel et al., 2015).

In hospital outbreak situations in which a rapid response is necessary to avoid the spread of this pathogen, it is of great relevance to have a technique capable of identifying these strains quickly and easily, generating great benefit in socio-economic terms and sanitary as it facilitates early isolations and effective treatments (Larsen et al., 2014).

Antibody detection

Many patients with long-standing *S. aureus* infections have antibodies to teichoic acids in the cell wall. However, this test is no longer performed in many hospitals because it is less sensitive than culture-based or nucleic acid-based tests.

Treatment

Depending on the susceptibility to oxacillin/methicillin, *S. aureus* is divided into two groups, those susceptible to it and those resistant (MRSA) since resistance to this compound causes resistance to almost all beta-lactam drugs, the main group of antibiotics used in its treatment (Rao et al., 2016).

This microorganism is usually susceptible to macrolides, clindamycin, fluoroquinolones, aminoglycosides, rifampicin, and cotrimoxazole, although strains resistant to each of these compounds may appear.

Clinical isolates of coagulase-negative staphylococci are usually more resistant to antibiotics and more than 50% of strains are resistant to oxacillin.

There are different ways to determine the antibiotic susceptibility of these microorganisms:

- Disc-plate system: it uses discs with an established concentration of each antibiotic and the inhibition zone produced by each antibiotic is determined.
- Epsilon test system: uses strips with a gradient of various concentrations of each antibiotic.
- Automated microdilution systems that are capable of determining the minimum inhibitory concentration (MIC) of each antibiotic. The cut-off points for a strain to be susceptible or resistant to an antibiotic depend on the antibiotic and are established by CLSI (Clinical Laboratory Standard Institute) or EUCAST (European Committee on Antimicrobial Susceptibility Testing) (Larrosa et al., 2020).

Fortunately, there are several new antibiotics that have been successfully marketed that possess adequate antibacterial activity against staphylococci, such as daptomycin, linezolid, and tigecycline. On the other hand, there are also several antibiotics activity against coagulase-negative staphylococci, such as dalbavancin, telavancin, oritavancin, iclaprim, ceftobiprole and ceftaroline. An important aspect of the treatment of most infections caused by CoNS is the ability of these infections to form biofilms on biomaterials (e.g., catheters, prostheses). However, combinations of antibiotics with rifampin appear to show promise in the treatment of biofilm-associated staphylococcal infections (Khan et al., 2018).

Staphylococcus aureus infections

Intravenous (IV) cloxacillin is the treatment of choice for moderate or severe SASM infections. It is recommended to administer it at intervals of no more than 4 h and continuous infusion is even preferable, in order to maintain the serum concentration, between consecutive doses, 4–5 times above the MIC. It is indicated in any type of infection with the following exceptions:

1. If there is biofilm formation on prosthetic material, or phenotypic variants of small colony.
2. Severe forms caused by strains that produce LPVT.
3. Localization in areas not easily accessible to beta-lactam administered by the systemic route, such as the eyeball and suppurative collections that for whatever reason cannot be drained immediately and completely.

Mild SASM infections can be treated orally with amoxicillin-clavulanate or cephalosporins whose minimum inhibitory concentration (MIC) against *S. aureus* is ≤ 4 mg/L (Rao et al., 2016).

Vancomycin is no longer the treatment of choice for methicillin-resistant staphylococcal infections (MRSA and most CoNS) due to:

- a) Its lower efficacy than isoxazole penicillins or cefazolin in the treatment of MSSA infection.
- b) The observation of failure rates above 30% in bacteremia, endocarditis, pneumonia and ATI.
- c) The worst prognosis seen in MRSA infections when the MIC₉₀ is > 1 mg/L. This last aspect has been related to the difficulty of complying with the PK/PD index that best predicts the efficacy of vancomycin (Fu et al., 2020).

Another option is to use vancomycin in continuous infusion that allows reaching the desired serum concentration earlier and maintaining it without fluctuations, although it has not improved the clinical response, it does seem to have a lower risk of toxicity. These facts have led to the consideration of the use of vancomycin for the treatment of MRSA infection (or MSSA infection in patients allergic to beta-lactams) when:

- a) The MIC against the causative strain is ≤ 1 mg/L or is unknown but the infection is mild or moderate.
- b) The infection does not occur with the formation of biofilms, nor is it located in areas of limited diffusion.
- c) Glomerular filtration rate is ≥ 40 mL/min and the patient does not receive other potentially nephrotoxic medication.
- d) The means are available to determine the serum concentration of vancomycin in the first 48 h of treatment and to know the result in the 24 h after taking the sample, in order to adjust the dose to the value of the desired trough concentration (Fu et al., 2020).

Linezolid is considered the antibiotic of choice for the treatment of pneumonia, meningitis and endophthalmitis caused by MRSA (or by SASM in patients with anaphylactic allergy to penicillin). It is also associated, associated or not with rifampicin, in the infection of prosthetic material by MRSA and is an alternative to the association of a fluoroquinolone with rifampicin for the oral treatment of MSSA infection. Linezolid is one of the antibiotics of choice in moderate or severe IPPB caused by MRSA (or SASM in patients with anaphylactic allergy to penicillin); and in monotherapy or association in any staphylococcal infection caused by a strain of *S. aureus* that produces enterotoxins or LPVT, regardless of the location of the infection and the susceptibility of the isolate to beta-lactams (Shariati et al., 2020).

Daptomycin is the treatment of choice in:

- a) Primary or catheter-associated bacteremia due to MRSA (or anaphylactic allergy to beta-lactam SASM).
- b) MRSA endocarditis (or MSSA in patients with anaphylactic allergy to beta-lactams) at a dose of 10 mg/kg/day, alone or in combination with other antimicrobials (fosfomycin and/or gentamicin—depending on the susceptibility of the strain and the risk of deterioration of renal function in the native valve, and the addition of rifampicin in the prosthetic valve).
- c) Severe IPPB caused by MRSA (or MSSA in patients with anaphylactic allergy to beta-lactams), especially if there is bacteremia, in monotherapy or associated with clindamycin or linezolid.
- d) Infection of the joint prosthesis or osteosynthesis material by *S. aureus* (MRSA or SASM), in doses of 8–10 mg/kg/day (Shariati et al., 2020).

Use of oral cotrimoxazole should be considered in the following circumstances:

- a) Mild or moderate IPPB produced by MRSA (or by SASM in allergic to beta-lactams), although in empirical treatment it should be remembered that its efficacy is not optimal when the infection is produced by beta-hemolytic streptococci of groups A, C or G.
- b) Infection of prosthetic material and in chronic osteomyelitis by MRSA (or by SASM in case of allergy to beta-lactams), as an alternative to linezolid, associated with rifampicin.

Clindamycin can be used in:

- a) Mild or moderate IPPB produced by MRSA (or by SASM in patients allergic to beta-lactams).
- b) As an alternative to linezolid in osteomyelitis and infection of prosthetic material caused by MRSA (or by SASM in patients allergic to beta-lactams).
- c) Pneumonia due to a strain of *S. aureus* that produces LPVT and IPPB due to an enterotoxin-producing strain (Khan et al., 2018).

Minocycline and doxycycline can be used as oral treatment alternatives for mild or moderate MRSA IPPB. Minocycline is more active, has a lower risk of resistance development, and is not metabolized through CYP3A4. Tigecycline is another alternative intravenous treatment of IPPB due to MRSA of moderate severity, especially when the infection is polymicrobial.

Use of rifampicin in staphylococcal infection is always in association and is indicated:

- a) Biofilms (infection on prosthetic material or bone sequestration) (Suresh et al., 2019).
- b) Growth of the microorganism in the vascular endothelium (persistent or recurrent bacteremia without focus).

The most commonly used antibiotics are quinolones, cotrimoxazole, and linezolid. The association with levofloxacin is the guideline of choice for the treatment of infection on osteoarticular prosthetic material by SASM (Otto, 2018).

Fosfomycin, like rifampicin, is used in association with other drugs with which it generally has a synergistic or additive behavior. The main indications are the following:

- a) infections in territories where the diffusion of the antibiotic is limited (CNS, eyeball, abscesses and other suppurative collections when drainage is difficult or incomplete).
- b) left endocarditis and MRSA infections with criteria of severe sepsis.
- c) infections of moderate or high severity caused by a MRSA strain with high limit susceptibility (MIC of vancomycin of 2 mg/L, daptomycin of 1 mg/L or linezolid of 4 mg/L).
- d) moderate or severe infections caused by a strain of SASM with a vancomycin MIC >1 mg/L (Davis et al., 2015).

Coagulase-negative staphylococci infections.

The antibiotic treatment of infections caused by most of the CoNS species (bacteremia and associated with foreign bodies) is similar to what was previously mentioned for those caused by *S. aureus*. As 80% of the species are resistant to methicillin, the antibiotics of choice are vancomycin, linezolid, and daptomycin. If the species is methicillin-susceptible, cloxacillin should be used. Vancomycin and daptomycin are preferred for bacteremia, and for infections associated with implant materials, linezolid or cotrimoxazole, associated or not with rifampin, unless the species is methicillin-susceptible, in which case the first option is rifampicin with levofloxacin.

S. lugdunensis and *S. schleiferi* are often susceptible to penicillins, and urinary tract infection due to *S. saprophyticus* can be treated with amoxicillin-clavulanate, a first-generation cephalosporin, cotrimoxazole, or oral nitrofurantoin (Heilmann et al., 2019).

Antimicrobial resistance

90% of *S. aureus* are resistant to penicillin produced by β -lactamases (blaZ gene). MRSA methicillin resistance phenotype is also frequent due to the acquisition of the *mecA* gene that encodes the penicillin-binding protein PBP2a that has low affinity for β -lactams. It implies resistance to all β -lactams, except ceftaroline and ceftobiprole. Currently in Spain it is 10–25%. They present nosocomial transmission, although lately also community transmission. It is associated with the MRSA CC398 clone that has a multidrug resistance phenotype. It is detected using 30 μ g cefoxitin discs: (a) *S. aureus* and *S. lugdunensis*, halo of ≤ 21 mm indicates the presence of the *mecA* gene. (b) CoNS, a halo of ≤ 24 mm indicates the presence of this gene (Bartash and Nori, 2017).

The phenotypic expression of methicillin resistance can be heterogeneous (MIC for oxacillin: 1–16 μ g/mL) or homogeneous (MIC >16 μ g/mL). Strains with heterogeneous expression are characterized by the fact that only a small proportion of the population ($\leq 0.1\%$) survives with oxacillin concentrations greater than 10 μ g/mL; they show great heterogeneity in the size of the colonies when grown on agar. In strains with homogeneous expression, most of the population expresses resistance and the size of the colonies is also homogeneous.

In 2011, a new methicillin resistance gene called the *mecC* gene was described with a 69% homology with the *mecA* gene: it presents strains resistant to ceftaroline (MIC >1 mg/L). It is associated with clonal complexes CC5, CC8, CC22. Screening is performed: cefoxitin <22 mm; MIC oxacillin >2 mg/L. It is confirmed when no *mecA* gene (GeneXpert) or PBP-2 agglutination is detected with PCR.

There are strains of *S. aureus* with low-level resistance or borderline resistance to oxacillin (borderline-oxacillin-resistant *S. aureus* or BORSA) that are characterized by intermediate resistance to oxacillin and MIC values of this antibiotic in the range 1–8 μ g/mL. Presents elevated oxacillin MIC without *mecC* gene-mediated resistance (Hryniewicz and Garbacz, 2017).

Staphylococcus strains have maintained a high susceptibility to glycopeptides, so that most strains are susceptible to vancomycin and teicoplanin. However, since 1997 the presence of strains of *S. aureus* with decreased susceptibility to vancomycin has been described in Japan. They are rare. It has been detected in the United States, Europe, South America and Asia according to:

- EUCAST: resistant if vancomycin/teicoplanin MIC >2 mg/L.
- CLSI:
 - Sensitive if vancomycin/teicoplanin MIC ≤ 2 mg/L.
 - Intermediate if MIC vancomycin/teicoplanin 4–8 mg/L.
 - Resistant if vancomycin/teicoplanin MIC ≥ 16 mg/L.

These strains were named vancomycin-intermediate *S. aureus* (VISA) with MIC 4–8 mg/L. They have poorly defined resistance mechanisms (Fu et al., 2020).

There are strains called vancomycin-resistant *S. aureus* (VRSA) with MIC ≥ 16 mg/L. Mediated by the *vanA* gene, located in transposon Tn1546. It is associated with clones CC5, CC8 and CC30 (Gardete and Tomasz, 2014).

Resistance of *Staphylococcus aureus* to other antibiotics

Macrolides: the presence of *erm* (A) and *erm* (C) genes generally confers a resistance phenotype called MLSB (resistance to group B macrolides, lincosamides and streptogramins) and this phenotype can be constitutive or inducible expression (cMLSB or iMLSB). In strains with the iMLSB phenotype, erythromycin induces the expression of the resistance mechanism (Feßler et al., 2018).

Aminoglycosides: the susceptible phenotype is the most frequent in staphylococci. Among the resistance mechanisms, the most frequent is that mediated by the *aac*-(6')-*aph*-(2'') gene. The strains appear resistant to gentamicin and tobramycin and susceptible to amikacin when a diffusion antibiogram is performed with discs. It is due to the action of modifying enzymes transmitted by plasmids or transposons (Nazarchuk et al., 2020).

Quinolones: the main mechanism of resistance to fluoroquinolones in staphylococci consists of mutations in the targets of action of the antibiotic, specifically in DNA topoisomerase IV (GrlA and GrlB) and in DNA gyrase (GyrA and GyrB). They are common in nosocomial MRSA strains.

Lipoglycopeptides (dalbavancin*, telavancin, oritavancin): Presents excellent activity against MRSA and multi-resistant. *On some occasions, there are strains with increased MIC (0.25–0.5 mg/L).

Oxazolidinones (linezolid): They are very rare. Mutations occur in 23S rRNA and ribosomal proteins. They are generally 100% susceptible. Resistance to this antibiotic has been observed both in vivo and in vitro in staphylococci, although it is rare in patients with prolonged treatment with this antimicrobial, by the dissemination of resistant clones in hospital units, or by the dissemination of transposons or plasmids that contain the gene *cfr* (Shariati et al., 2020).

Lipopeptides (daptomycin): They are very rare. It is produced by changes in the cell membrane and wall. They are active in 100% of the strains. Resistance (MIC >1 mg/L) could appear in infections such as osteomyelitis, endocarditis, devices, foreign bodies and poorly perfused tissues (Shariati et al., 2020).

Mupirocin: The CLSI does not define cut-off points to interpret susceptibility to this antibiotic. Two resistance phenotypes can be distinguished:

- Low level resistance (MIC 8–256 µg/mL) produced by *ileS* native gene mutations.
- High level resistance (MIC ≥ 512 µg/mL) by acquisition of plasmid containing the *mupA* gene.

In the first case, although it was initially considered to be clinically irrelevant resistance, it must be taken into account that these strains do present problems when using mupirocin for nasal decolonization. Regarding high-level resistance, this antibiotic is totally ineffective (Dadashi et al., 2020).

Tetracycline: resistance to tetracycline and doxycycline in staphylococci is quite frequent and may be due to two mechanisms: increased active expulsion and ribosome protection. The first mechanism is encoded by the *tetK* and *tetL* genes and the second by the *tetM* and *tetO* genes (Shariati et al., 2020).

In previous studies of colonization by MRSA, the susceptibility observed depending on the type of sample has been: 31–48% for pharyngeal exudate, 19–38% for perineal exudate, 78–82% for nasal exudate, 85–92% for nasal along with the pharyngeal, 88–93% the nasal along with the perineal, and 98% the triple nasal, pharyngeal and perineal intake. Therefore, a nasal swab is considered the most suitable sample if a single sample is chosen for surveillance cultures (Sakr et al., 2019).

Isolation usually lasts throughout admission or until at least three consecutive surveillance samples are negative. With greater agreement is the use of disposable gloves, masks and gowns when handling patients and always washing the hands of healthcare personnel after handling patients. Nasal decolonization with mupirocin with or without the use of chlorhexidine hygiene, restriction of the use of antimicrobials (cephalosporins and fluoroquinolones) and training of healthcare personnel.

The control of MRSA must combine better use of antimicrobials and epidemiological measures, including the detection of carriers, which reduces the pressure of colonization and, therefore, its transmission (Dadashi et al., 2020).

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Streptococcus pneumoniae

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Abbreviations

BBB	Blood brain barrier
CBP	Choline binding proteins

CPS	Capsular polysaccharide
CRP	C-reactive protein
CSF	Cerebrospinal fluid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H ₂ O ₂	Hydrogen peroxide
IgA1	Immunoglobulin A1
MHC	Major histocompatibility complex
PAF-R	Platelet activating factor-receptor
PAMP	Pathogen associated molecular patterns
PavA	Pneumococcal adhesion and virulence A
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PPSV	Pneumococcal polysaccharide vaccine
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
TLR	Toll-like receptors

History and introduction

The first description of *Streptococcus pneumoniae* in the literature can be attributed to Klebs in 1875 and Eberth and Ma'tray in 1880. They later used the term "Pneumoniekokken" for their findings based on observations from sputum and lung fluid of pneumonia patients (White et al., 1938). The discovery of *S. pneumoniae* is, however, accredited to two scientists, George M. Sternberg in the United States and Louis Pasteur in France, who concurrently identified the organism in 1881. Both these scientists injected human saliva in rabbits; Sternberg used his saliva, whereas Pasteur inoculated saliva from a child who had died from rabies. Both of them identified and isolated diplococci in the blood of rabbits (White et al., 1938). In 1886, the organism was termed as "Pneumococcus" by Fraenkel (1886) due to its tendency to cause pulmonary disease. Based on its appearance on gram-stain, it was re-named *Diplococcus pneumoniae* in 1920 (Winslow et al., 1920) and finally in 1974, due to its morphological characteristics and ability to grow as cocci chains, the current name *Streptococcus pneumoniae* was allotted (Deibel and Seeley Jr., 1974).

S. pneumoniae normally colonizes the upper respiratory tract. It causes a variety of diseases in adults as well as in children, including lobar pneumonia, sinusitis, meningitis, and otitis media. It is a gram-positive diplococcus that grows in chains in liquid culture mediums. It is non-motile, non-spore-forming, and can ferment glucose to lactic acid (Bogaert et al., 2004).

Structure

S. pneumoniae is lancet-shaped, which means that the bacterium is pointed at one end. Single bacterium measures 0.5–1.25 µm in diameter (Todar, 2020). *S. pneumoniae* consists of an outer capsule, an inner cell wall, a periplasmic space, plasma membrane, cytoplasm, and nuclear material (DNA).

Capsule and serotypes

Most pneumococci consist of an external polysaccharide capsule (PSC); however, some non-encapsulated variants also exist (Keller et al., 2016). The capsule is almost 400 nm thick and accounts for more than half of the pneumococcal volume. It is made up of repeating oligosaccharides, which are synthesized in the cytoplasm and are transported to the surface of the bacteria by cell membrane transferases after polymerization. The capsular polysaccharide (CPS) is covalently linked to the outer peptidoglycan of the cell wall (Calix et al., 2012; Geno et al., 2015).

Multiple genes are responsible for CPS synthesis, which are present in a cassette-like arrangement (Griffith, 1928; Dawson, 1930). The capsule locus (*cps locus*) has type-specific genes in between the genes mutual to many or all pneumococci. This arrangement allows the recombinational exchange of genetic material and the formation of different serotypes (Coffey et al., 1998).

To date, 98 different types of CPS (serotypes) have been discovered, and two systems are used to classify these serotypes. The American system (less preferred), which enlists serotypes in the order of their discovery, and the Danish System (more preferred) in which similar, antigenically-related serotypes are grouped together (serogroups). Lower number serotypes were identified earlier due to their clinical relevance, and hence they were the first ones to be numbered. Serotypes are of significant importance due to their implication in current and new vaccines (Geno et al., 2015, 2017).

Cell wall

The Pneumococcal cell wall lies inner to capsule, has six layers, and is mainly composed of peptidoglycan covalently linked to teichoic acid. Peptidoglycan is made up of alternating N-acetyl-D-glucosamine and N-acetylmuramic acids arranged in long chains

(Fig. 1). The pneumococcal cell wall also contains lipoteichoic acids (LTA), which are similar to wall teichoic acids (WTA) except that they have a lipid part attached to them (Vollmer et al., 2019; Yamaguchi et al., 2019). WTA is attached covalently to peptidoglycan; in contrast, LTA is non-covalently linked to the cytoplasmic membrane with the help of a lipid anchor (Fischer, 2000). Both WTA and LTA contain phosphorylcholine as a structural component that binds choline-binding proteins. Phosphorylcholine plays an essential role in the virulence of the organism. C-polysaccharide is a component of the cell wall in all capsular pneumococci, and it is made up of teichoic acids and tightly attached peptidoglycan fragments. Cell wall plays a vital role in maintaining the bacterial shape, division, and growth. Components of the cell wall interact with the host, e.g., C-polysaccharide reacts with C-reactive proteins (CRP) and other acute-phase reactants during inflammatory processes. Elevated CRP levels are observed in pneumococcal infections, but it is a non-specific marker and is raised in many other infections (Vollmer et al., 2019; Yamaguchi et al., 2019).

Cytoplasmic structures and metabolism

Like other gram-positive bacteria, the *S. pneumoniae* cell membrane is a phospholipid bilayer, made up of proteins and lipids, chiefly phospholipids, but lack sterols. The protein-to-lipid ratio is approximately 3:1 and has lipoteichoic acid linked to the outer surface of membrane lipids. The cytoplasm is abundant in 70S ribosomes but lacks endospores (Salton and Kim, 1996).

S. pneumoniae is a fermentative bacteria that rely heavily on carbohydrate metabolism for energy, biosynthesis, and growth. However, the bacteria has numerous transporter systems (e.g., ATP-binding cassette transporters, phosphotransferase systems) and enzymes (e.g., glucokinase, transketolase) that enable it to use a variety of substrates such as amino acids, iron, and other compounds (Leonard and Lalk, 2018). The bacterial metabolism is regulated by Catabolite control protein A and CodY (nutritional regulator). An effective metabolic process is vital for the growth and survival of bacteria (Carvalho et al., 2011; Hendriksen et al. 2008).

Genome

The genome of *S. pneumoniae* consists of a closed, circular DNA containing 2.0–2.1 million base pairs along with small plasmids. The guanine-cytosine content makes up 39.7% of the genome. There are 1553 core genes that are essential for viability and an extra 154 genes in virulome, which play a role in the virulence of the organism. An additional 176 genes are responsible for maintaining a noninvasive phenotype. Up to 10% variation in the genome can exist between different strains of pneumococcus (Tettelin et al. 2001; Lanie et al., 2007; Van der Poll and Opal, 2009).

Pili

Pili are hair-like extensions from the surface of the bacteria and are made up of proteins called pilins. They extend out of PSC and are approximately 6 nm wide and over 1 µm long (Hilleringmann et al., 2009). Pili enhance the attachment of pneumococci to the epithelial lining (Barocchi et al., 2006).

Surface proteins

It has been estimated that pneumococcus has more than 500 surface proteins, either attached physically to cell wall or lipoproteins associated with the cell membrane (Todar, 2020). These surface proteins aid in the pathogenesis of bacteria and protect it against the

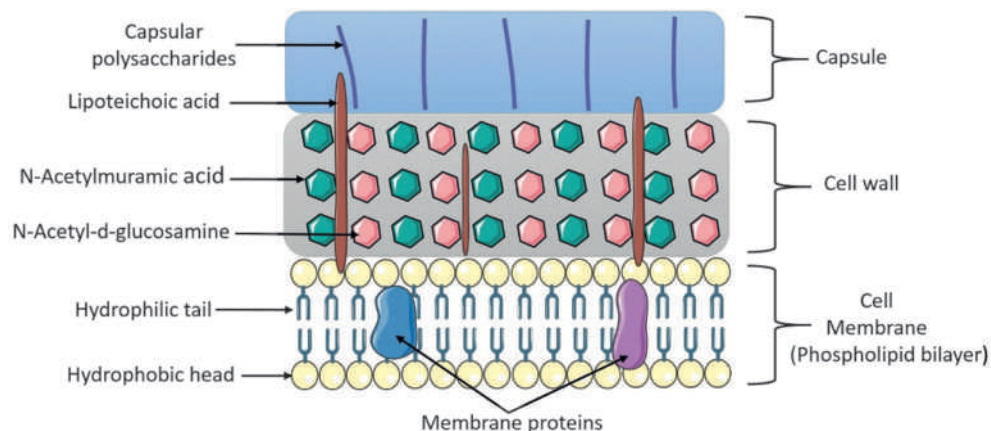


Fig. 1 Simplified view of *Streptococcus pneumoniae* cell wall and cell membrane. Adapted with permission from Brooks LR, and Mias GI (2018) *Streptococcus pneumoniae's* virulence and host immunity: Aging, diagnostics, and prevention. *Frontiers in Immunology* 9: 1366; Made from <https://smart.servier.com/>.

host's defense (Brooks and Mias, 2018). Surface proteins include (1) penicillin-binding proteins; (2) choline binding proteins (e.g., pneumococcal surface protein, autolysins, and CbpF); (3) metal-binding proteins; (4) LPXTG cell wall-bound proteins (e.g., neuraminidase); and (5) non-classical surface proteins (Brooks and Mias, 2018).

Important features

Transformation

S. pneumoniae is capable of natural transformation. Transformation is a process of horizontal gene transfer in which bacterium picks up genetic material (naked DNA) from the neighboring environment, or genetic material is transferred from one bacterium to another through surrounding media. This process was first shown by Griffith (1928). In natural transformation and recombination process, the beneficial mutations in bacteria are fixed and dispersed. Through transformation, mobile genetic elements are disseminated, and new genes are gained (Straume et al., 2015). The normal genetic exchange occurs between different co-existing strains of pneumococcus during nasopharyngeal colonization as pneumococcus has very high transformation efficacy in the nasopharynx (Marks et al., 2012). The process of transformation plays a crucial role in the evolution of genomes and the development of resistance against antibiotics and evading vaccines conferred immunity (Lattar et al., 2018).

During the transformation process, the pneumococcus enters a particular physiologic state of "competence" which is triggered by peptide pheromone called a competence-stimulating peptide (Johnsborg and Håvarstein, 2009). The state of competence can also be induced by factors that damage the DNA of the organism, such as mitomycin C, fluoroquinolone, and topoisomerase inhibitors (Gagne et al., 2013). Competence increases the resistance of *S. pneumoniae* to oxidative stress by increasing the repair of damaged DNA. The virulence and nasal colonization depend on intact competence system as the bacteria are successfully able to repair DNA damages produced by oxidative bursts (Salvadori et al., 2019).

Gram positivity

Streptococcus is a gram-positive bacteria. The peptidoglycan of the bacterial cell wall takes up crystal violet stain and appear purple under the microscope. Fig. 2A shows gram stained Pneumococci.

Culturing of pneumococcus

S. pneumoniae is a fastidious bacteria, i.e., it requires a specific environment and nutrients to grow. It grows best at 35–37 °C with 5% carbon dioxide. Usually, the bacteria are cultured on blood agar, but chocolate agar can also be used. Catalase (also present in the blood) is required for the growth of bacteria as it produces a large amount of H₂O₂. In blood agar, the doubling time of bacteria is 20–30 min (at 37 °C). The bacteria form small (~1 mm), grey, and moist or mucoid colonies with a zone of green (alpha) hemolysis (WHO and CDC, 2011; Todar, 2020). Pneumococci show phase variation where the opaque colonies turn transparent. The opaque phase is more suited for survival in the blood, whereas the transparent phase is a better fit for nasopharyngeal colonization (Li and Zhang, 2019). The young colonies of pneumococci appear raised, but after 24–48 h, flattened colonies with a depressed center are seen (Fig. 2C) (WHO and CDC, 2011). This happens when the central (older) bacteria start undergoing autolysis, thus leading to a depression in the center.

Alpha-hemolysis

S. pneumoniae (in aerobic conditions) and *S. viridans* are alpha-hemolytic, i.e., they produce a green or dark hue when cultured on blood agar (Fig. 2D). *S. pneumoniae* produces pneumolysin (previously called alpha-hemolysin), which produces greenish pigment on the breakdown of hemoglobin in blood or chocolate agar. Alpha-hemolysis is also called incomplete or partial hemolysis, and it is due to H₂O₂ produced by the bacteria which oxidize hemoglobin to methemoglobin (WHO and CDC, 2011). In anaerobic conditions, however, the bacteria can cause beta-hemolysis due to oxygen-labile hemolysin (Todar, 2020).

Optochin sensitivity

Optochin, also called ethylhydrocupreine hydrochloride, is used to differentiate *S. pneumoniae* from *S. viridans* group, which is also an alpha-hemolytic streptococcus. *S. pneumoniae* is optochin sensitive and will show a zone of inhibition (>14 mm) around the optochin disk (6 mm, 5 µg), whereas resistant bacteria (e.g., *S. viridans*) continue to grow without inhibition (WHO and CDC, 2011). Fig. 2E shows an example of optochin sensitivity test. In recent years optochin resistant *S. pneumoniae* has emerged (Nunes et al., 2008), thus reducing the reliability of the test.

Quellung reaction

Quellung reaction, also called Neufeld reaction (Neufeld, 1902), is used to determine the serotype of pneumococcus (Fig. 2F). Typing serum is added to bacteria and then observed under a microscope for capsular swelling, which happens due to the binding of

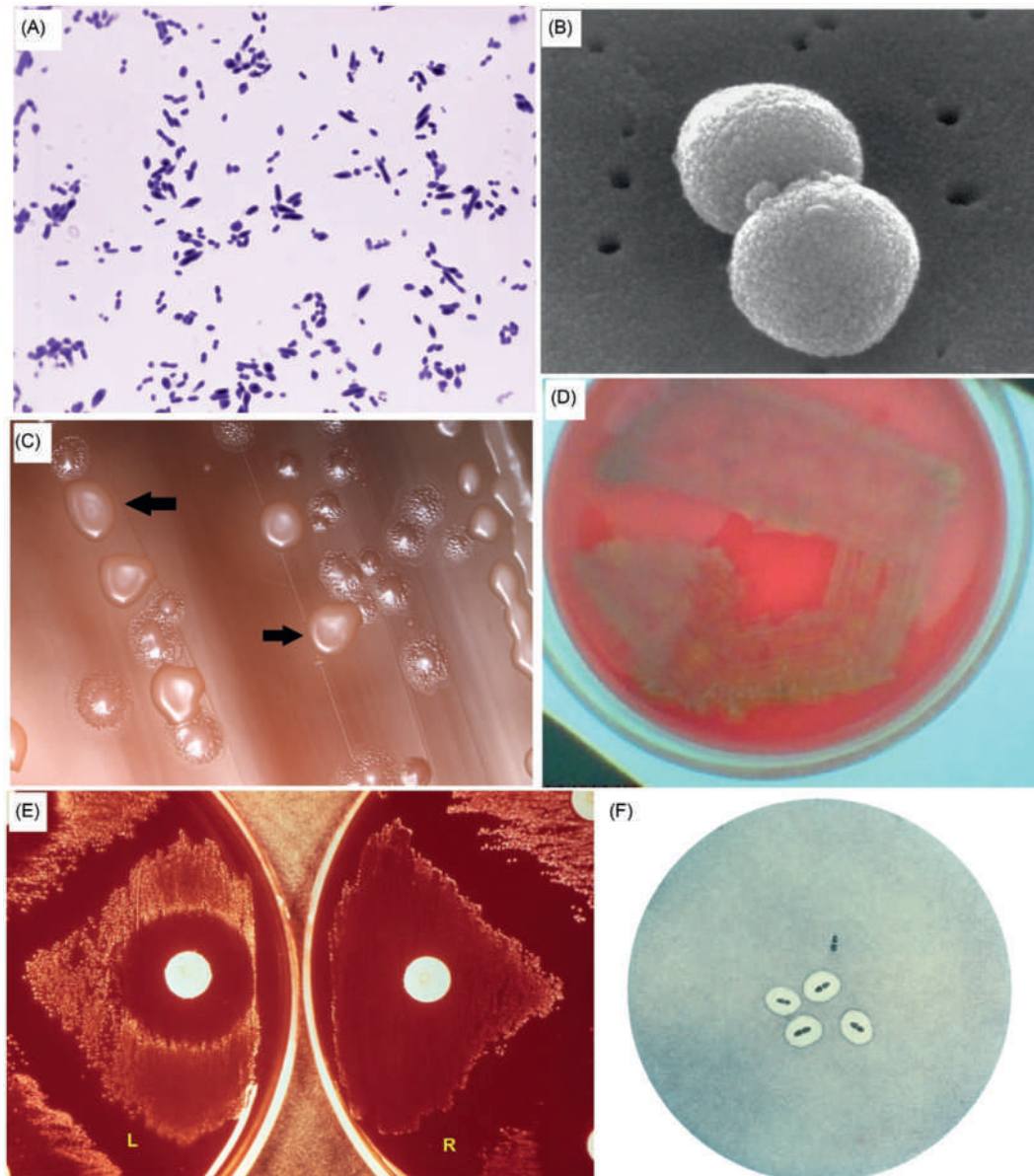


Fig. 2 Images showing different properties of *Streptococcus pneumoniae* (A) Gram staining of bacterial culture shows gram-positive, lancet-shaped diplococci; (B) Scanning electron microscopic image of *S. pneumoniae* depicts two, round-shaped cocci; (C) Doughnut-shaped *S. pneumoniae* colonies with depressed centers (black arrows). The colonies without depression are bacteria other than *S. pneumoniae*; (D) *S. pneumoniae* shows alpha hemolysis (green) on blood agar. Alpha hemolysis can also be produced by *S. viridians* group; (E) Optochin-sensitivity test shows *S. pneumoniae* on the left with a zone of inhibition around the optochin imbibed disc, i.e., optochin-sensitive, whereas, on the right side, optochin-resistant bacteria show uninhibited growth up to the edge of the disc; (F) Capsular swelling observed on Neufeld-Quellung test. Antibodies bind to the capsule of *S. pneumoniae*, which then appears thickened under the microscope. Source: (A) CDC/Arnold Kaufman; (B) CDC/ Dr. Richard Facklam; (C) CDC/ Dr. Richard Facklam; (D) Wikimedia/Netha Hussain; (E) CDC/Dr. Richard Facklam; (F) CDC. All images from CDC are available at PHIL.

the type-specific antibody with the CPS. The reaction can also be seen in other encapsulated organisms such as *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Escherichia coli* (WHO and CDC, 2011).

Catalase negative

Catalase breaks down H_2O_2 into H_2O and O_2 . The O_2 produced is visible in the form of bubbles in the liquid (Fig. 3A). Catalase test is used to differentiate between the members of gram-positive cocci. *S. pneumoniae* and *Enterococci* are catalase-negative, whereas *Staphylococci* are catalase-positive (WHO and CDC, 2011).

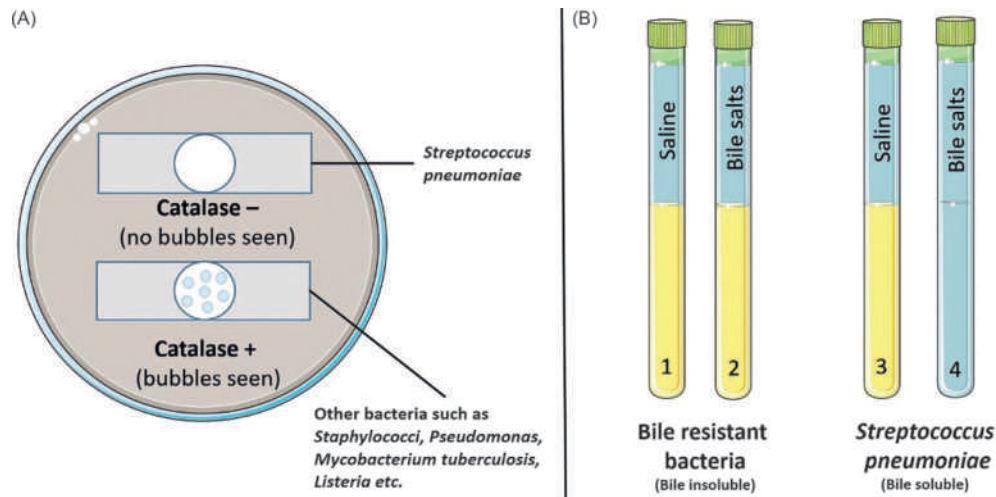


Fig. 3 (A) Illustration A shows the results of a catalase test. *S. pneumoniae* being catalase-negative will not produce bubbles on the slide; (B) Illustration B shows bile solubility test to identify *S. pneumoniae*. Since *S. pneumoniae* is bile soluble, when pneumococcus is added to the test tube containing bile salts, pneumococcal cells rupture. This makes the fluid clear, as seen in test tube 4. If any bile resistant bacteria are added to bile salts, bacterial cells do not rupture, and the fluid in test tube remains turbid as seen in test tube 2. Made using <https://smart.servier.com/>.

Bile solubility

Bile (sodium deoxycholate) solubility test is also used to differentiate *S. pneumoniae* from *S. viridans* group (Fig. 3B). Except for *S. pneumoniae*, all alpha-hemolytic streptococci are bile resistant (WHO and CDC, 2011).

Virulence factors

S. pneumoniae has numerous virulence factors in the form of physical structures, surface proteins, and toxins. All of these virulence factors help the bacteria in the invasion of the host and evading the body's immune response to cause disease (Jedrzejak, 2001). Therefore, it is essential to recognize the role played by these virulence factors to understand the pathogenesis of diseases caused by *S. pneumoniae*. Some of the most important virulence factors are shown in Fig. 4 and discussed below.

Capsule

The PSC of *S. pneumoniae* is regarded as its primary virulence determinant (Geno et al., 2015). CPS plays an integral part in nasopharyngeal colonization by reducing entrapment of *S. pneumoniae* in nasal mucus and subsequently evading the clearance by mucus ciliary flow. CPS aids in the access and attachment of *S. pneumoniae* to the surface of epithelial cells by repelling sialic acid-rich mucopolysaccharides present in the mucus (Nelson et al., 2007). It helps to evade the host's immune system by interfering with both the classic and alternative complement pathways. CPS can also attenuate the interaction between the bound C3b/iC3b or Fc regions of immunoglobulin on the bacterial cell surface and surface receptors of phagocytic cells thus inhibiting opsonization (Hyams et al., 2010; Abeyta et al., 2003).

Cell wall

S. pneumoniae, being a gram-positive bacteria, has a thick cell wall comprising of peptidoglycan along with LTA and WTA. Human lysozyme is an antimicrobial protein that helps the degradation of the bacterial cell wall by enzymatically targeting the bond between N-acetyl-d-glucosamine and N-acetylmuramic acid, thus breaking the integrity of peptidoglycan (Ganz and Lehrer, 1997). *S. pneumoniae*, to resist this bactericidal action, undergoes secondary modification in its glycan chains rendering the target moieties unrecognizable by lysozymes (Bui et al., 2012). WTA, LTA, and peptidoglycan are identified as pathogen-associated molecular patterns (PAMPs) by the host's immune system and elicit an inflammatory response. The inflammatory response caused by LTA is possibly carried out by the activation of the C-type lectin pathway of complement (Gisch et al., 2013).

Choline-binding proteins

Choline binding proteins (CBPs) are pneumococcal surface protein found anchored to phosphorylcholine residue present in the cell wall (Gosink et al., 2000). CBPs hinder both the innate and adaptive immune system by blocking the activation of the complement system and attenuating the ability of immunoglobulin to clear the bacteria. *S. pneumoniae* has approximately 10–16

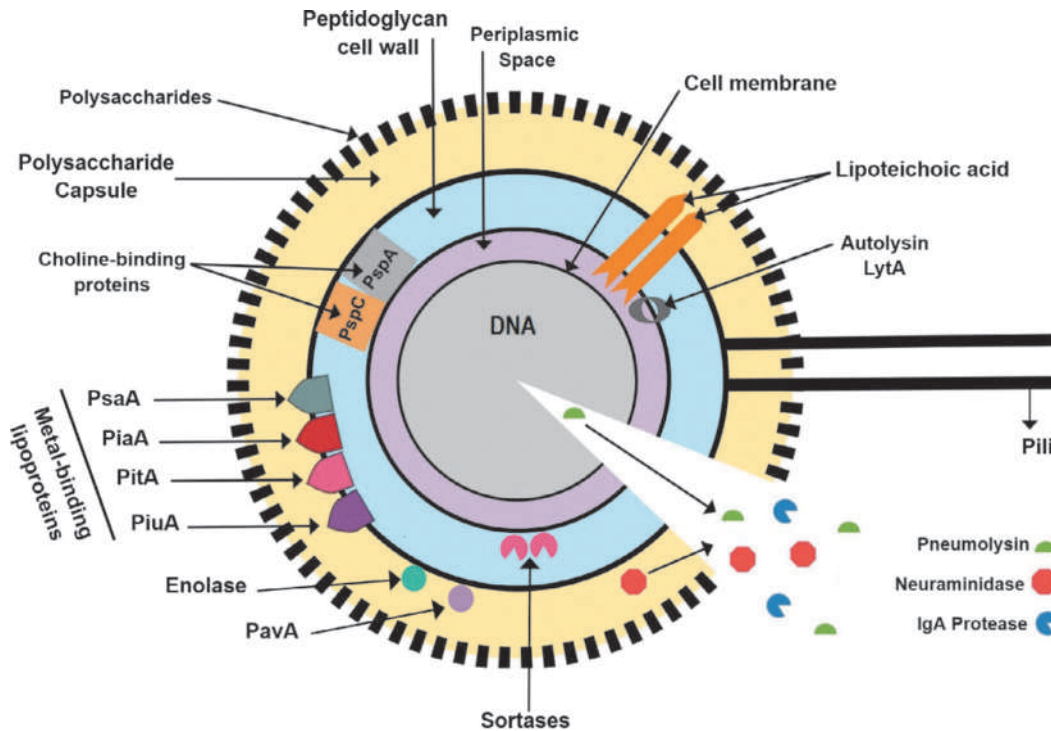


Fig. 4 Diagrammatic representation of the structure and different virulence factors of *Streptococcus pneumoniae*. PavA, Pneumococcal adhesion and virulence A; PiaA, pneumococcal iron acquisition A; PiuA, pneumococcal iron uptake A; PitA, pneumococcal iron transporter; PsaA, pneumococcal surface adhesin A; PspA, pneumococcal surface protein A; PspC, pneumococcal surface protein C. Adapted with permission from Brooks LR and Mias GI (2018) *Streptococcus pneumoniae*'s virulence and host immunity: Aging, diagnostics, and prevention. *Frontiers in Immunology* 9: 1366; Made using <https://mindthegraph.com/>

CBPs present on its cell wall, including Pneumococcal surface protein A (PspA), Pneumococcal surface protein C (PspC), and LytA (Bergmann and Hammerschmidt, 2006).

Pneumococcal surface protein A (PspA)

PspA inhibits opsonization of *S. pneumoniae* by binding to complement (Jedrzejewski et al., 2001). PspA also binds to apolactoferrin (an iron-depleted form of lactoferrin) and protects the pathogen from the bactericidal effect of apolactoferrin (Shaper et al., 2004).

Pneumococcal surface protein C (PspC)

PspC plays a significant role in adhesion and nasopharyngeal colonization of *S. pneumoniae* by binding to the polymeric immunoglobulin receptors present on epithelial cells (Rosenow et al., 1997). Additionally, it also blocks the alternative pathway in the host cell by binding to factor H, thus interfering with opsonization (Dave et al., 2004).

LytA

LytA is an autolysin that causes bacterial cell lysis and release of intracellular products like pneumolysin, inflammatory peptidoglycan, and teichoic acids (Kadioglu and Andrew, 2004).

Immunoglobulin A1

Immunoglobulin A1 (IgA1) protease is a zinc metalloprotease secreted by *S. pneumoniae* to target and cleave human immunoglobulin A1 (IgA1) (Chi et al., 2017). The IgA1 isotype comprises more than 90% of the total IgA in the human airway (Wani et al., 1996). Thus, through the action of IgA1 protease, the humoral protection and inflammatory response offered by IgA1 on mucosal surfaces is hampered.

Pneumolysin

Pneumolysin is a potent toxin secreted by *S. pneumoniae*. It belongs to a family of cholesterol-dependent cytolysins and exerts its main cytolytic effect by forming pores on cholesterol-containing cell membranes (Marshall et al., 2015). Pneumolysin activates the classical complement pathway and causes a subsequent decrease in C3b opsonization (Paton et al., 1984; Mitchell and Dalziel,

2014). Pneumolysin exerts its proinflammatory effect by activating Toll-like receptor (TLR)-4 (TLR-4) and stimulating cytokine and chemokine production (Van der Poll and Opal, 2009; Los et al., 2013; Steel et al., 2013).

Metal-binding proteins

Metal-binding proteins are lipoproteins that bind to ATP-binding cassette transporter and help metal ions such as iron, zinc, and manganese to be transported across membranes by utilizing energy generated from ATP binding and hydrolysis (Brooks and Mias, 2018). These metal ions are vital for the survival and growth of bacteria, especially in conditions when free ions in the host are low. The four most crucial metal-binding proteins are described below.

Pneumococcal surface adhesin A (PsaA)

PsaA is involved in the transport of magnesium and zinc into the cell (Rajam et al., 2008). Mutations that hamper the ability of PsaA to obtain manganese can cause decreased resistance of *S. pneumoniae* to oxidative stress (Mitchell and Mitchell, 2010).

Pneumococcal iron acquisition A (PiaA), pneumococcal iron uptake A (PiuA), and pneumococcal iron transporter (PitA)

PiaA, PiuA, and PitA are involved in iron transport and uptake (Whalan et al., 2005, 2006). Mutations in PiaA and PiuA can inhibit the growth and virulence of *S. pneumoniae* (Brown et al., 2001; Honsa et al., 2013).

LPXTG-anchored proteins

LPXTG-anchored proteins are cell surface proteins that contain an LPXTG motif (X denotes any amino acid). They are linked covalently to the bacterial cell wall by sortase. Sortase transpeptidase recognizes the particular amino acid sequence present in LPXTG protein. After recognition, it cleaves and anchors the protein to the cell wall.

Neuraminidase is an LPXTG-anchored protein that cleaves terminal sialic acid from glycoprotein, glycolipid, and oligosaccharides present on the host's cell surface and exposes new potential receptors for binding (Mitchell and Mitchell, 2010). Furthermore, the removal of sialic acid from lactoferrin can impede its bactericidal effect (Brooks and Mias, 2018). Neuraminidase also plays a pivotal role in nasopharyngeal colonization and pathogenesis of otitis media (Wren et al., 2017).

Pili

Pili promotes colonization of pneumococcus in the host's nasopharynx and lungs by helping it to bind human epithelial cells (Barocchi et al., 2006; Steel et al., 2013). It can also aid bacteria in evading phagocytosis by immune cells (Van der Poll and Opal, 2009) and can stimulate the release of large amounts of proinflammatory cytokines, e.g., large amounts of tumor necrosis factor (TNF) (Barocchi et al., 2006).

Non-classical surface proteins

Non-classical surface proteins (NCSPs) are a miscellaneous group of proteins that lack attachment to the membrane via leader peptide or anchor motif. They perform several functions and hence given the name moonlight proteins (Bergmann and Hammerschmidt, 2006). NCSPs include pneumococcal adhesion and virulence A (PavA) and glycolytic enzymes.

Pneumococcal adhesion and virulence A (PavA)

PavA is an adhesin present on the outside of the *Pneumococcus* cell wall. It binds to fibronectin and mediates adherence to cells of the host. PavA plays a major part in virulence, and it has been observed that the load of bacteria in the host considerably decreases when PavA is not present (Holmes et al., 2001).

Glycolytic enzymes

Glycolytic enzymes such as enolase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are also NCSPs that mediate their virulence by binding to and stimulating host's plasminogen on the pneumococcal surface. Enolase is found on the surface of pneumococcus, while GAPDH is found on the surface as well as in the cytoplasm (Kolberg et al., 2006; Bergmann et al., 2004). Enolase binds to plasminogen and helps *S. pneumoniae* to adhere to host cells, disseminate, and avoid the host's immune cells (Bergmann et al., 2013). GAPDH can bind to hemoglobin and is postulated to play a part in the attainment of iron for the bacteria (Zomer et al., 2015).

Epidemiology

The incidence of pneumococcal infection varies in different populations and age groups. *S. pneumoniae* colonization may be asymptomatic or can cause mucosal and invasive infections (WHO, 2018). *S. pneumoniae* is the leading cause of severe pneumonia and associated mortality worldwide, and it is the most common cause of bacterial pneumonia in children (WHO, 2018; WHO, 2019).

In 2015, over 9 million pneumococcal infections were recorded with >318,000 deaths in children under five years. Most of these deaths occurred in developing countries. Global incidence and mortality rate of pneumococcal pneumonia in children under-five was 1356 per 100,000 and 36 per 100,000, respectively (Wahl et al., 2018). *S. pneumoniae* is the most common cause of community-acquired pneumonia, accounting for almost a quarter of all identified cases (Said et al., 2013). In the United States, it has been estimated that each year almost 502,600 cases of non-bacteremic pneumococcal pneumonia, 29,500 cases of invasive pneumococcal disease (IPD), and 25,400 pneumococcal-related deaths occur (Weycker et al., 2010). The occurrence of IPD is age-related, with more cases occurring in individuals above 50 years (Drijkoningen and Rohde, 2014). In Europe, the incidence rates of IPD range from 11 to 27 per 100,000 population, whereas in North America, the incidence rate is estimated to be 15–49 per 10,000 (Kyaw et al., 2003; Reinert et al., 2005; Lynch and Zhanel, 2009). Patients suffering from comorbidities like diabetes, lung, heart, or liver disease are at higher risk of pneumococcal disease (Zhang et al., 2018).

Pathogenesis

Colonization and transmission

S. pneumoniae is a commensal of the nasopharynx. In humans, colonization begins within the first week of life, increases in the initial few years, and then decreases as age progresses. In children, rates of colonization vary between 27–65% but can be as high as up to 90% (Yahiaoui et al., 2016; Smith et al., 2020). Less than 10% of adults are carriers of pneumococcus (Smith et al., 2020). Usually, one serotype exists at a time in most individuals, but the co-existence of multiple serotypes is also possible (Hare et al., 2008). Along with the rate, the duration of pneumococcal carriage also decreases with age (Högberg et al., 2007). Over-crowding (e.g., in daycares), lower socioeconomic status or poverty, respiratory viral infections, smoke exposure either due to smoking or cooking fires, and recent antibiotic use increases the risk of colonization (Van der Poll and Opal, 2009; Koliou et al., 2018). Changes in the natural biome of nasopharynx can also influence colonization as microbes compete for binding sites and nutrients (Van der Poll and Opal, 2009; Shak et al., 2013).

S. pneumoniae is mainly transmitted via respiratory droplets and secretions produced by coughing or sneezing of a carrier or infected individual. Also, transmission through fomites is possible when inanimate objects are contaminated with biofilms by chronic carriers (Marks et al., 2014). The transmitted bacteria can either be cleared by host defense or produce disease.

Body defense against infection

The elimination of bacteria depends on the host's immune system. The host's body fights off pneumococcal invasion by innate (early) and adaptive (late) immune system. After colonization, pneumococcus comes in contact with the host's epithelial lining (e.g., in the respiratory tract). The body tries to remove the bacteria via mucociliary clearance. The mucous traps the bacteria, and cilia beat to expel it. Epithelial cells produce antimicrobial peptides that can directly kill bacteria and secrete cytokines that recruit inflammatory cells (neutrophils and macrophages).

PAMPs are recognized by TLRs (e.g., TLR-2, TLR-4, and TLR-9), leading to stimulation of adaptive immune system cells. TLR-2 recognizes and binds to components of the pneumococcal cell wall (lipoproteins and peptidoglycan), and TLR-4 recognizes pneumolysin. TLRs are activated by recognition and binding of PAMPs, and this leads to the production of cytokines and co-stimulatory molecules, which eventually activate T cells. Antigen-presenting cells present (bacterial) antigen to T cells via major histocompatibility complex-II (MHC-II). T cells differentiate into Th1 and Th2 cells that produce interferons (IFN- γ) and interleukins, which provide signals for B cell maturation, further macrophage activation, and neutrophil recruitment. All these steps aid in the elimination of pathogens from the body (Brooks and Mias, 2018; Weiser et al., 2018; Henriques-Normark and Tuomanen, 2013; Loughran et al., 2019).

Invasion and disease

Using wide array of virulence factors, *S. pneumoniae* is able to evade host's immune response, decrease clearance, and cause infection. If the bacteria successfully escape host defense, it replicates and disrupts normal, non-pathogenic flora of the respiratory system (Van der Poll and Opal, 2009). *S. pneumoniae*'s negatively charged capsule avoids mucous entrapment. The bacteria secrete neuraminidase that cleaves mucin and provides sites for bacterial attachment as well as decrease mucociliary clearance. *S. pneumoniae* firmly adheres to epithelial cells using pili and CBPs (Löflying et al., 2011; Henriques-Normark and Tuomanen, 2013). *S. pneumoniae* also produces pneumolysin and H₂O₂, which forms pores and disrupts epithelium leading to infection of sterile organs and tissues. Pneumolysin inhibits cilia on respiratory epithelial cells, lyse host cells, and trigger inflammation via complement activation. It is also toxic to cochlear hair cells and contributes to otitis media. The bacteria can infect lungs (pneumonia), ear (otitis media), paranasal sinuses (sinusitis), and seed into the blood leading to sepsis, meningitis, and other complications as shown in Fig. 5 (Henriques-Normark and Tuomanen, 2013; Subramanian et al., 2019). *S. pneumoniae* uses phosphorylcholine (in the cell wall) to gain access to blood using the platelet-activating factor receptor (PAF-R). Binding of phosphorylcholine to PAF-R initiates uptake of bacteria in the vacuole (receptor-mediated endocytosis) to be transported across host cells into the bloodstream (Radin et al., 2005).

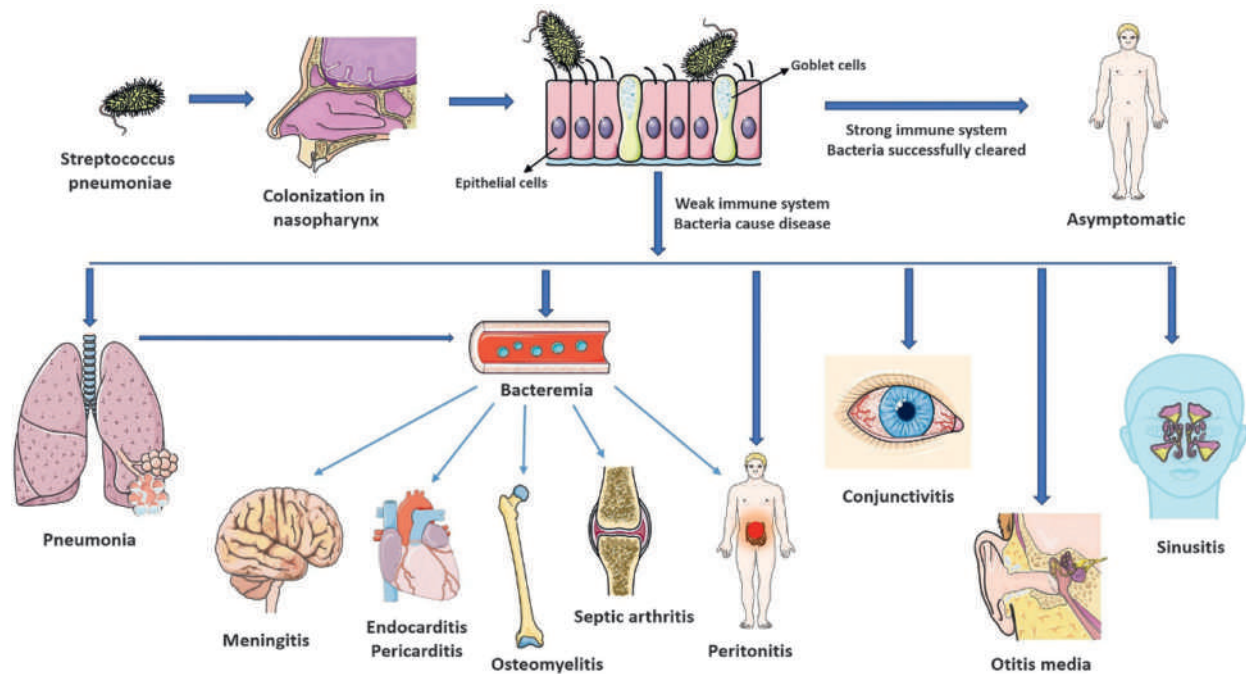


Fig. 5 Illustration showing colonization of nasopharynx by *S. pneumoniae* and progression to diseased states. *S. pneumoniae* colonizes the nasopharynx and is unable to cause disease in the presence of strong host defense; thus the individual remains asymptomatic. If the host immune system is weak, pneumococcus can invade into different sterile tissues and organs to cause diseases such as pneumonia, otitis media, sinusitis, and conjunctivitis. If the bacteria enters the bloodstream, it is known to cause meningitis, endocarditis, pericarditis, septic arthritis, osteomyelitis, and peritonitis. Made using <https://smart.servier.com/>

Meningitis develops when the pneumococcus can enter into CSF via choroid plexus or by crossing the blood-brain barrier (BBB) in cerebral capillaries. It is a two-step process. Initially, bacterial PspC binds to endothelial laminin receptor. Secondly, phosphatidylcholine binds to PAF-R, and bacteria translocate across BBB by receptor-mediated endocytosis. Once inside the CSF, pneumococcus can trigger an inflammatory response and cause neuronal damage and mediate meningitis (Orihuela et al., 2009; Henriques-Normark and Tuomanen, 2013; Loughran et al., 2019).

Diseases

Community-acquired pneumonia

Community-acquired pneumonia (CAP) causes significant clinical and economic burden of disease in both developed and developing nations and is a cause of considerable morbidity and mortality (File Jr and Marrie, 2010; Shibl et al., 2010). The most common pathogen which causes CAP is *Streptococcus pneumoniae*, independent of age (Drijkoningen and Rohde, 2014; Howard et al., 2005).

The clinical presentation of pneumococcal pneumonia is highly variable. Classically, pneumonia is characterized by the acute onset of chills and pleuritic chest pain followed by fever, fatigue, dyspnea, cough, sweats, and the production of purulent rusty color sputum (Brandenburg et al., 2000). However, these symptoms are more apparent and well-defined in younger patients as compared to older patients. In the older population, patients having pneumococcal pneumonia can be afebrile and disoriented with no other specific signs and symptoms. Usually, elevated respiratory rate is the only sign speculative of respiratory disease (Metlay et al., 1997). This puzzling clinical presentation often makes the diagnosis of CAP in the elderly a diagnostic conundrum.

The diagnosis of pneumococcal CAP starts with establishing the presence of pneumonia with the help of symptoms, clinical examination, and chest radiographs. The chest radiograph shows air space consolidation and infiltrates in one or more segments in a lobe (Janoff and Musher, 2015). Fig. 6 shows an image of a chest radiograph of pneumonia patient. Gram staining of sputum is regarded as a traditional and reliable test. The test strongly suggests positive for pneumococcus pneumonia, if it demonstrates high-quality (<10 squamous epithelial cells and >25 polymorphonuclear cells at a magnification of 100×) and predominance of gram-positive diplococci (Murray and Washington, 1975; Musher et al., 2004).

The detection of streptococci from blood and pleural fluid, which are otherwise sterile tissues, provide a definite diagnosis of pneumococcal pneumonia. However, less than 10% of patient who have pneumococcal pneumonia test positive for presence of streptococci in their blood, making the clinical usefulness of these tests very restricted (Luna et al., 2000). The main drawbacks of diagnosing CAP with the conventional microbiological investigation is its extensive time frame to isolate the bacteria and determine its antibiotic susceptibility, inadequate sampling, and high rate of a false negative.



Fig. 6 Chest X-ray (anteroposterior view) showing pneumonia of left lower lobe with some consolidation. Source: CDC/Dr. Thomas Hooten. *Image is available at PHIL.*

One of the diagnostic methods recently gaining momentum is a rapid immunochromatographic assay to detect C-polysaccharide of the pneumococcal cell in urine (Binax NOW *S. pneumoniae* assay). Children showed poor specificity for Binax NOW *S. pneumoniae* assay, mainly due to a higher rate of nasopharyngeal carriage and subsequent urinary excretion of teichoic acid (Charkaluk et al., 2006). In contrast, in the adult population, the sensitivity and specificity for Binax NOW *S. pneumoniae* assay were considerably high, especially in bacteremic patients (Sinclair et al., 2013). Prior antibiotic use has less influence on the diagnosis of this test (Said et al., 2013); however, the test can become false positive because of immunization (Navarro et al., 2004) and stays positive even one month after infection (Marcos et al., 2003).

For molecular testing, using polymerase chain reaction (PCR) assay of blood and respiratory tract specimens to detect specific *S. pneumoniae* targets e.g., *lytA*, *pneumolysin*, and *PsaA*, has shown promising results (Abdeldaim et al., 2008; Albrich et al., 2012).

Pneumonia caused by pneumococci that are susceptible or intermediately resistant to penicillin responds to treatment with penicillin, 1 million units intravenously every 4 h; ampicillin, 1 g every 6 h; or ceftriaxone, 1 g every 24 h (Janoff and Musher, 2015).

Acute otitis media

S. pneumoniae is the most common pathogen to cause otitis media (Ngo et al., 2016). Acute otitis media is characterized by otalgia, otorrhea, fever, vomiting, and the presence of fluid in the middle ear (Schilder et al., 2016). It is one of the most common bacterial infections in children between the ages of 6 months to 3 years (Klein, 2000). Otitis media has also been described as the leading cause of physician visits and antibiotic prescription in children (Zhou et al., 2008; Coco et al., 2009), and cause a significant strain on health care systems worldwide due to its chronicity, recurrence, and repeated antibiotic prescriptions.

The conversion of harmless nasopharyngeal colonization of pneumococcus to middle ear inflammatory disease is usually attributed to a concomitant upper viral respiratory infection (Heikkinen and Chonmaitree, 2003). The viral infection can cause eustachian tube obstruction, nasal mucosa alterations, and fluid retention, facilitating the entry of microorganisms to the middle ear and triggering otitis media.

On physical examination, the tympanic membrane is erythematous, with loss of light reflex and decreased mobility. Otitis media is usually diagnosed clinically by tympanic membrane visualization and testing hearing function (Vergison et al., 2010). If the tympanic membrane ruptures, a specimen of the exudate can be analyzed with gram staining and culture that would show the presence of gram-positive diplococcus streptococci. Otitis media in children is usually treated by a 7–10 days regimen of oral broad-spectrum antibiotics, usually amoxicillin. Guideline of the American Academy of Pediatrics suggests that otitis media in older children can be managed symptomatically. On the other hand, otitis media in infants is best managed with antibiotic therapy (Lieberthal et al., 2013). In the case of bacterial resistance or failure to resolve, amoxicillin-clavulanate and cefdinir can also be used (Coco et al., 2010).

Sinusitis

Acute bacterial rhinosinusitis is inflammation of the paranasal sinuses, usually maxillary sinus, due to bacterial infection (Rosenfeld et al., 2015). The most common pathogens causing the disease are *S. pneumoniae* and *H. influenzae* (Gwaltney Jr et al., 1992; Brook, 2002). Viral infection, allergens, and environmental pollutants can impair mucociliary clearance, which results in stasis of mucus and bacterial proliferation in the sinus (Gwaltney Jr et al., 1992, 1994).

Symptoms include purulent nasal discharge, nasal congestion, facial pain, and slight anosmia. Diagnosis is usually made based on clinical symptoms and findings. Treatment is mostly symptomatic because it is usually self-limiting.

Antibiotics can be prescribed if the infection persists, a 5–10 days regimen of amoxicillin is the standard therapy. Patients having a penicillin allergy are usually prescribed trimethoprim/sulfamethoxazole or a macrolide as an alternative (Rosenfeld et al., 2015).

Meningitis

Meningitis is the inflammation of the meninges and subarachnoid space, which can extend to involve the cortex and parenchyma of the brain as well. Meningitis can be caused by an infectious (bacterial, viral, or fungal) or non-infectious (autoimmune, drugs, or neoplasia) etiology. In nearly 70% of the cases of bacterial meningitis, the causative pathogen is *S. pneumoniae* and is associated with the highest rates of mortality (30%) and morbidity (Schedl et al., 2002; van de Beek et al., 2016). *S. pneumoniae* can reach the meninges and subarachnoid mainly through two routes: (i) Hematogenous spread and (ii) Spread from contiguous infected sites, e.g., paranasal sinuses or middle ear.

Pneumococcal meningitis cannot be differentiated from other causes of bacterial meningitis solely based on clinical manifestation. All patients of bacterial meningitis present with similar symptoms such as headache, fever, neck stiffness, and impairment of consciousness (Glasgow Coma Scale <14). The “classical” triad of meningitis (fever, neck stiffness, and altered mental status) occurs only in almost one-third of the patients. The involvement of brain parenchyma and cortex can cause focal neurological deficits and behavioral changes (Bijlsma et al., 2016). A petechial rash, which has been considered the hall-mark of meningococcal infection, can occur in pneumococcal meningitis as well (van de Beek et al., 2004). The physical examination will reveal neck stiffness, positive Kernig sign, and Brudzinski sign; however, the accuracy of these signs in identifying patients of meningitis is a topic of debate (Thomas et al., 2002).

Microbiological diagnosis of pneumococcal meningitis is made by gram staining and culture of CSF (Dunbar et al., 1998). However, it is clinically pertinent to point out that if a patient presents with a new CNS lesion, focal neurological deficit, or onset of seizures, cranial imaging (by CT scan) might be warranted before lumbar puncture to rule out elevated intracranial pressure. Antibiotics taken before the collection of CSF specimens can decrease the diagnostic sensitivity of the specimen. PCR of CSF can be a valuable tool, especially in those patients in which prior antibiotic intake has rendered gram stain and culture-negative (Châtelet et al., 2005). Latex agglutination testing in CSF has also been used for the rapid identification of *S. pneumoniae*, although its sensitivity is still variable (Brouwer et al., 2010).

Pneumococcal meningitis can be treated with 12–24 million units of penicillin every 24 h or 1–2 g ceftriaxone every 12 h. This regimen can successfully treat antibiotic-susceptible *S. pneumoniae*. For the resistant *S. pneumoniae*, β -lactam antibiotics are less likely to reach therapeutic levels in CSF and eradicate the pathogen. Thus vancomycin is included along with a β -lactam until susceptibility results are reported (Young and Thomas, 2018).

Unusual manifestations of pneumococcal infections

Before the advent of antibiotics or routine immunization with pneumococcal vaccine, unusual pneumococcal diseases were not uncommon. Albeit, their incidence has rapidly declined over the years, few cases can still be seen in clinical practice (Sousa et al., 2018). The unencapsulated type of *S. pneumoniae* has been implicated in causing epidemics of conjunctivitis (Martin et al., 2003). Endocarditis due to *S. pneumoniae* is a rare manifestation and usually occurs in patients who otherwise have healthy heart valves (Daudin et al., 2016). Another rare and potentially fatal cardiac disease caused by pneumococcus is purulent pericarditis (Zmora et al., 2019). *S. pneumoniae* can also cause osteoarthritic complications like septic arthritis and vertebral osteomyelitis (Suzuki et al., 2013; Dernoncourt et al., 2019). Spontaneous bacterial peritonitis (SBP) can also occur due to *S. pneumoniae*, while the bacteria are not part of normal gastrointestinal flora, it is postulated that SBP occurs due to primary respiratory tract infection like pneumonia and consequently bacteremia (Capdevila et al., 2001).

Risk factors for pneumococcal infections

The extremes of age are associated with increased risk of pneumococcal infection, children under the age of two years, and the elderly (>65) have the highest incidence of IPD. The increased susceptibility for *S. pneumoniae* in infants is usually due to their naive

immune system, while in elderly is due immunosenescence; a phenomenon in which the effectiveness of adaptive immune system declines with age leaving individuals disposed to a variety of infections (Infante et al., 2015; Krone et al., 2014).

In healthy children, crowded households and exposure to cigarette smoke are crucial risk factors for developing IPD (Pereiró et al., 2004). In non-elderly immuno-competent adults, cigarette smoking is one of the strongest risk factors for IPD (Nuorti et al., 2000). Alcohol abuse has also been described as one of the most common preventable risk factors for developing IPD (Grau et al., 2014). The incidence of IPD also increases with pre-existing comorbidities such as congestive heart failure (Kyaw et al., 2003), chronic lung disease (Kyaw et al., 2005), asthma (Talbot et al., 2005), and diabetes mellitus (Musher et al., 2000).

Individuals with a weakened immune system have a greater risk of developing IPD (Wong et al., 1992; Picard et al., 2003). Acquired disorders causing immune deficiency and increased risk of IPD are malignancy (Kyaw et al., 2003), HIV (Feldman and Anderson, 2016) and asplenia (Bisharat et al., 2001; Schutze et al., 2002).

Similarly, inherited immune deficiencies like Bruton's X-linked agammaglobulinemia and hemoglobinopathies e.g., sickle cell disease, carry a greater risk of IPD (Battersby et al., 2010).

Antibiotic resistance

In the early 1940s, the treatment of *S. pneumoniae* was straightforward, with the bacteria being highly susceptible to penicillin. In 1967, the first resistant isolate of *S. pneumoniae* was isolated, and to date, numerous strains with varying antibiotic resistance have made the treatment of pneumococcal disease increasingly multifaceted (Appelbaum, 2002).

Penicillin was first discovered in 1928, and for two decades after its discovery, *S. pneumoniae* was highly susceptible to it. In 1967, the first nonsusceptible *S. pneumoniae* strain was isolated, and this resistance was attributed to the alteration of penicillin-binding proteins, which were the prime target of the drug (Hansman, 1967). Similarly, resistance to other class of antibiotics (e.g., cephalosporins and macrolides) have also emerged raising concern about the use of these agents in the empiric treatment of clinical diseases frequently caused by the pneumococcus (Jorgensen et al., 1994). Pneumococcal isolates resistant to erythromycin, azithromycin, and clarithromycin have been isolated and are increasing at a steady rate (Jenkins and Farrell, 2009). Fortunately, *S. pneumoniae* resistance to fluoroquinolone is considerably low as of now.

The resistance of *S. pneumoniae* to a plethora of antibiotics poses a severe and grave health concern. With resistance to a large number of drugs, minor infections can lead to considerable mortality and morbidity. Thus, it is imperative to form strict guidelines for antibiotic use in the health sector. Awareness should be spread among the masses about the dire consequences of the misuse of antibiotics. Implementation and enhancement of pneumococcal conjugate-vaccines (PCV) and the development of other therapeutic regimens for the treatment of pneumococcal infections should be encouraged.

Vaccination for prevention

Vaccines play a chief role in the prevention of pneumococcal diseases. Currently, two types of subunit vaccines (conjugate and polysaccharide) are used in clinical practice. Pneumococcal conjugate vaccine (PCV) contains polysaccharide antigens conjugated with a carrier protein (e.g., diphtheria toxoid). Examples of PCV include PCV7 (Pneumovax®), PCV10 (Synflorix®; Fig. 7), and PCV13 (Pneumovax 13®) (Kim et al., 2017). PCV13 contains polysaccharides of the 13 most common pneumococcal serotypes and is currently recommended for use in the US and other parts of the world (CDC, 2019; Van Werkhoven and Huijts, 2018). These vaccines have reduced the burden of childhood pneumococcal diseases, especially the incidence of pneumococcal pneumonia has markedly decreased after the use of PCV13 (Olate et al., 2017; Takeuchi et al., 2020).

Pneumococcal polysaccharide vaccine (PPSV) uses capsule components as antigen. PPSV23 protects against 23 serotypes and reduces the risk of IPD in the elderly (CDC, 2019; Van Werkhoven and Huijts, 2018). PCV13 causes a T cell-dependent immune response that is stronger and long-lasting whereas, PPSV23 does not contain conjugated protein and therefore causes a T cell-independent response, which is weaker and requires booster (Kim et al., 2017). Table 1 shows the comparison between PCV and PPSV.

Both PCV and PPSV are age and serotype-dependent, and guidelines for vaccine use differ in different countries. In the US, PCV13 is recommended for children <2 years, whereas in elderly, smokers, immunocompromised, and individuals with selected comorbidities, PPSV23 is also given with PCV13 (CDC, 2019). A booster dose is usually recommended for (1) elderly >65 years who were vaccinated >5 years ago, (2) elderly >65 years who received vaccination when they were younger than 65, and (3) individuals between 2–64 years who are at increased risk of infection due to asplenia, HIV infection, or receiving immunosuppressive/cancer chemotherapy (Levinson, 2014).

Newer vaccines such as whole-cell vaccine (containing all bacterial components) and protein-based vaccines (e.g., PspA or pneumolysin) are being developed to reduce the burden of disease. The aim is to provide immunity against serotypes not covered by prior mentioned vaccines (Masomian et al., 2020).



Fig. 7 Pneumococcal conjugate vaccine 10 (PCV10) available under tradename Synflorix™. It contains polysaccharides of serotypes 1, 4, 5, 6B, F, 9V, 14, 18C, 19F, and 23F conjugated to carrier proteins (CP) such as tetanus toxoid CP, protein D, and diphtheria toxoid CP. Synflorix™ vial contains four doses and is administered intramuscularly. Courtesy of Dr. Dilshad Ahmed.

Table 1 Summary of vaccines used in clinical practice.

Pneumococcal vaccine	Example		Mode of action	Recommended populations	Strength	Weakness
	Names	Serotypes covered				
Conjugate	PCV7	4, 6B, 9V, 14, 18C, 19F and 23F	T cell-dependent activation of immune system.	Children <2 years.	Immunity lasts longer.	Expensive and limited serotype coverage.
	PCV10	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F		Children (≥ 2 years) and adults with specific medical conditions.	Protective against pneumonia and IPD.	
	PCV13	PCV10 serotypes + 3, 6A, and 19A			Reduces nasopharyngeal carriage.	
Polysaccharide	PPSV23	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F	T cell-independent activation of immune system.	Elderly >65 years.	Wider serotype coverage.	Immunity is short-lasting compared to PCV.
				Individuals between the age of 2-64 years with specific medical conditions. Smokers between the age of 19-64 years.	Reduces the severity of pneumonia and protective against IPD.	Has limited to no effect on nasopharyngeal carriage.

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Other *Streptococcus* Species and *Enterococcus*

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Introduction

The genus *Streptococcus* is a group consisting of various Gram-positive cocci that are usually arranged in pairs or chains. Most species are facultative anaerobes, and some of them grow only in an atmosphere enriched with carbon dioxide (caphnophilic growth). Their nutritional requirements are complex, and their isolation requires the use of media enriched with blood or serum. They are able to fermenting carbohydrates, a process that produces lactic acid, and are catalase-negative. There are species that are important pathogens for humans, but most of them are commensal, members of the normal human skin and mucosal microbiota (Milagrosa and García Arenzana, 2007).

They can be classified in three ways:

- (a) Serological properties: Lancefield groups (originally, A to W).
- (b) Hemolytic patterns: complete hemolysis (beta), incomplete hemolysis (alpha), and no hemolysis (gamma).
- (c) Biochemical properties.

From a practical point of view, streptococci can be divided into two groups:

- β -hemolytic streptococci, which are classified according to Lancefield groups.
- α -hemolytic and γ -hemolytic streptococci, which are classified by biochemical tests. The latter group is collectively called streptococci viridans.

In this chapter the most important *Streptococcus* species that cause human diseases will be analyzed.

Streptococcus pyogenes

Streptococcus pyogenes is an important human-specific bacterial that causes a wide range of clinical manifestations from mild localized infections to life-threatening invasive infections. It is commonly associated with skin or throat infections (Ibrahim et al., 2016).

Etiology

S. pyogenes is a Gram-positive, catalase-negative, β -hemolytic *Streptococcus*. The type A antigen of *S. pyogenes* is a polysaccharide consisting of *N*-acetylglucosamine attached to a rhamnose polymer main chain (Cunningham, 2000).

This microorganism usually colonizes the pharynx, anus and genital mucosa. Transmission can occur through airborne droplets, hand contact with nasal secretions or with objects or surfaces contaminated with bacteria, skin contact with contaminated lesions or contaminated food sources (Murray et al., 2013).

Epidemiology

Skin lesions are the most important risk factor, but also the presence of a number of preexisting medical conditions increases the risk of invasive group A streptococcal disease, like diabetes, heart disease, neoplasms, injection drug users and poor living conditions.

Overall, *S. pyogenes* disease is due to newly acquired strains that cause infection of the pharynx or skin before specific antibodies are produced or the competing microorganisms are able to proliferate. *S. pyogenes* pharyngitis is a disease that primarily affects children between 5 and 15 years old, although infants and adults are also vulnerable to this entity. The transmission is mainly person to person via respiratory droplets. Overcrowding, such as in classrooms and day care centers, increases the likelihood of spread of the microorganism, especially during the winter months. Soft tissue infections are usually preceded by an initial colonization of the skin by group A streptococci, after which the microorganisms are introduced into superficial or deep tissues through disruption of the skin barrier.

Clinical manifestations

Suppurative streptococcal diseases

Pharyngitis

S. pyogenes is the most common bacterial causing pharyngitis in children and teenagers. Main symptoms consist of sore throat, sudden onset of fever and absence of cough. Physical examination findings include cervical lymphadenopathy, pharyngeal swelling, and tonsillar exudate. Palate petechiae and uvular edema are also suggestive of this disease. But, despite these clinical signs and symptoms, it is difficult to distinguish streptococcal pharyngitis from viral pharyngitis. A diagnosis definitive can only be achieved by specific laboratory tests (Ashurst and Edgerley-Gibb, 2021).

A complication of strep throat is the scarlet fever, which is characterized by a bright red rash that affects to most of the body. It is almost always accompanied by a sore throat and high fever. Some of the symptoms produced by the scarlet fever, in addition to the red rash are red lines (Pastia lines), where the folds of skin around the groin, armpits elbows, knees and neck usually become deeper red than the surrounding rash and strawberry tongue, that is often covered with a white coating early in the disease.

Impetigo

The lesion of streptococcal pyoderma begins as a papule that rapidly evolves into a vesicle, surrounded by an area of erythema. Vesicular lesions are evanescent and rarely recognized clinically; they give rise to pustules that gradually enlarge and then break down over a period of 4–6 days to form characteristic thick crusts. Streptococcal impetigo occurs on exposed areas of the body, most often on the lower extremities or in the face. Although regional lymphadenitis may occur, systemic symptoms are usually not present (Stevens and Bryant, 2016).

Erysipela

Erysipela is a skin infection that affects the dermis layer of the skin, but may also extend to the superficial lymph nodes. It is characterized by a well demarcated, raised area of erythema often affecting the lower extremities, with the face being the second most common site affected (Michael and Shaukat, 2021).

All age groups may be affected, but it is more common at the extremes ages (Klotz et al., 2019).

Cellulitis

It is an acute disseminated inflammation of the skin and subcutaneous tissues. Unlike erysipela, the lesion is not raised, the demarcation between affected and unaffected skin is indistinguishable, and the lesions are pinkish rather than salmon red in color. However, the clinical differentiation between these entities is not clear.

Clinical findings include local pain, tenderness, swelling and erythema. The process may spread rapidly to involve large areas of skin. Systemic manifestations include fever, chills, and malaise, and there may be associated with lymphangitis and/or bacteremia (Stevens and Bryant, 2016).

Necrotizing fasciitis

It is defined as a rapidly progressive infection affecting the skin, subcutaneous cellular tissue, superficial fascia and occasionally deep fascia, resulting in tissue necrosis and systemic toxicity (Bueno Rodríguez et al., 1999).

It is usually polymicrobial, but in the majority of cases in which only a single microorganism is isolated, it is produced by *S. pyogenes* (Headley, 2003). Some signs can help differentiate necrotizing fasciitis from cellulitis such as toxic appearance, generalized erythematous rash, high fever, tachypnea and low platelet count (Clark et al., 2000).

Given the characteristics of this disease, early medical treatment is necessary to save the patient. Unlike cellulitis, which can be treated with antibiotics, fasciitis must also be treated aggressively by surgical debridement of the infected tissue.

Streptococcal toxic shock syndrome (STSS)

STSS is one of the complications of invasive streptococcal disease and is defined as the isolation of group A *Streptococcus* from a normally sterile site in the setting of shock and multiorgan failure. It should be suspected and recognized early in order to offer appropriate treatment and support measures (Zavala et al., 2018).

An increased risk of the disease can be observed in patients with human immunodeficiency virus (HIV) infection, cancer, diabetes, lung or heart disease, varicella zoster virus infection, as well as those addicted to injecting drugs and alcoholics.

Non-suppurative streptococcal diseases

Rheumatic fever

Rheumatic fever is a multisystemic immune-mediated inflammatory disease that occurs predominantly in childhood and teenagers and follows infection with *S. pyogenes*. It is characterized by fever, migratory polyarthritides of predominantly large joints, carditis, skin and soft tissue changes (erythema marginatum and subcutaneous nodules) (Carapetis et al., 2005; Lozano et al., 2012).

There is no single diagnostic test for rheumatic fever. The diagnosis is based on clinical criteria and evidence of preceding infection with *S. pyogenes* like (1) positive results of pharyngeal swab cultures or test based on specific nucleic acids; (2) detection of group A antigen in pharyngeal smear, or (3) an elevation of anti-ASLO, anti-DNAase B or antihyaluronidase antibodies (Steer, 2015).

Acute glomerulonephritis

The glomerulonephritis is characterized by acute inflammation of the renal glomeruli with edema, hypertension, hematuria and proteinuria. It is a sequelal of pyoderma and pharyngeal streptococcal infections. Diagnosis is based on clinical manifestations and finding of recent *S. pyogenes* infection (Satoskar et al., 2020).

Laboratory diagnosis

Microscopy

Gram's staining of tissue samples affected by *S. pyogenes* infection is crucial for rapid and preliminary diagnosis and for starting empirical treatment. However, many streptococcal species are part of the normal oropharyngeal microbiota, and the observation of streptococci in such samples has low predictive value (Abbour et al., 2016).

Antigen detection

There are many immunological tests that use antibodies that react with the specific carbohydrates of the bacterial cell wall to identify group A streptococci in pharyngeal swabs directly. Simple rapid antigen detection tests are widely used to diagnose pharyngitis by Streptococci group A at the point of care. The sensitivity in children is approximately 85%, and specificity is high, at approximately 95%. It is not recommended relying on negative tests results without culture confirmation (Cohen et al., 2016).

Culture

Throat culture is the gold standard for the diagnosis of streptococcal pharyngitis and it has a sensitivity from 90% to 95%. Samples should be taken from the posterior oropharynx despite the difficulty of obtaining pharyngeal exudates in the pediatric population (Anjos et al., 2014).

Isolation of *S. pyogenes* from skin infection is not difficult. The scab is lifted and the purulent material and the base of the lesion is cultured. These microorganisms are easily recovered from both tissue and blood cultures from patients with necrotizing fasciitis.

Identification

The *S. pyogenes* colonies measure >0.5 mm in diameter, are white or gray and some have a mucous appearance. The edges are surrounded by a large zone of beta-hemolysis, which is bigger in areas of low oxygen concentration. Streptococci does not produce catalase. In addition, this microorganism is susceptible to bacitracin, the presence of any inhibition halo with a disk containing 0.04 units of bacitracin is diagnostic. Another definitive test for the identification of *S. pyogenes* is the detection of pyrrolidonyl arylamidase (PYR) and the detection of Lancefield's A antigen using commercially available systems (Batista et al., 2006).

Treatment

Penicillin is the antimicrobial of choice for the treatment of *S. pyogenes* infections. However, in penicillin allergic patients, an oral cephalosporin or a macrolide are alternatives treatments. *S. pyogenes* may exhibit macrolide resistance because active efflux (mef genes) or target modification (erm genes), the latter conferring cross resistance to lincosamines and streptogramin B. The combined use of intravenous penicillin with clindamycin is recommended in severe systemic infections (Silva-Costa et al., 2015). Patients with severe penicillin hypersensitivity can be treated with linezolid, vancomycin or daptomycin (Stevens et al., 2014). In patients with severe soft tissue infection, drainage and surgical debridement should be initiated promptly.

Streptococcus agalactiae

Streptococcus agalactiae, also known as group B *Streptococcus* (GBS) was described as a colonizer of the vaginal tract of asymptomatic women. However, its pathogenesis was not described until 1938 when reports of fatal postpartum infection were published. Invasive GBS disease remains an important pathogen in both infants and adults (Raabe and Shane, 2019). The serotypes of GBS are based on the capsular polysaccharide and it has been divided into nine serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII). The most common serotype causing invasive disease is group V (Edwards and Baker, 2015). GBS can cause septicemia, pneumonia and meningitis in newborns and serious illness in adults.

Epidemiology

Vaginal colonization by *S. agalactiae* represents the most important risk factor for the development of invasive neonatal infections. The rate of GBS colonization in pregnant women in the vagina and/or rectum varies by geographic area, from 6.5% to 36% in Europe, from 10% to 30% in the United States, from 7.1% to 16% in Asia, from 9.1% to 25.3% in the Middle East, and from 11.9% to 31.6% in Africa (Verani et al., 2010).

Because GBS colonization status is intermittent and may be transient during pregnancy, the timing of microorganism detection and specimen collection is important in predicting colonization status at delivery (Hansen et al., 2004). CDC guidelines determine that the best time for detection is late in the third trimester, no more than 5 weeks before delivery (Verani et al., 2010).

Currently, up to 10 serotypes (Ia, Ib and II-IX) based on a capsular polysaccharide rich in sialic acid, which acts as a virulence factor in the persistence and survival of GBS within the host, are recognized (Le Doare and Heath, 2013). Although, colonization rates appear quite similar in different geographical areas, the prevalence and distribution of serotypes is not the same in the different regions of the world (Xia et al., 2015). In Europe and the United States the more prevalent serotypes are Ia, Ib, II, III and V. Serotype III accounts for the majority of cases of late-onset neonatal meningitis (Alhhazmi et al., 2016).

Microbiology

Group B streptococci are Gram-positive cocci that form short chains in clinical specimens and longer chains in culture, that make them indistinguishable from *S. pyogenes* on Gram staining. *S. agalactiae* colonies have a buttery appearance and a narrow zone of beta-hemolysis.

The polysaccharide capsule is the most important virulence factor of GBS, which interferes with phagocytosis until the patient generates specific antibodies. In the absence of maternal antibodies, the newborn is at risk of infection. The genital colonization by group B streptococci is associated with an increased risk of preterm partum, and infants are at increased risk for the disease.

Pathogenesis

Many of the virulence determinants of GBS contribute to neonatal disease, based on adherence to epithelial surfaces, penetration of host cellular barriers, evasion of immune clearance mechanisms, and inflammatory activation (Doran and Nizet, 2004).

The main virulence factor is the polysaccharide capsule. The antibodies developed against the capsule antigen are protective, and in the absence of these, the neonate is at increased risk for acquiring the infection. The fact that GBS colonizes the genital area is associated with an increased risk of preterm delivery, and newborns are at increased risk for the disease (Murray et al., 2013).

Clinical manifestations

Early-onset neonatal disease

Early-onset disease is mainly associated with bacteremia, pneumonia or meningitis. Lung involvement is seen in the majority of neonates, but meningeal involvement may be apparently undetectable, so examination of cerebrospinal fluid would be necessary in all infected neonates.

A few years ago the mortality rate was quite high, but thanks to early diagnosis and improved treatment it has decreased to less than 5%.

Late-onset neonatal disease

It has an exogenous origin and develops between the first week and 3 months of life. It is usually associated with bacteremia and meningitis, and although it has a low mortality rate, 25–50% of children with meningitis develop neurological complications.

Infections in pregnant women

During gestation or even immediately after delivery, infection often leads to postpartum endometritis, wound infection and genitourinary tract infections. The outcome is usually favorable in pregnant women who receive appropriate treatment.

Infections in men and non-pregnant women

The most frequent forms of infection are bacteremia, pneumonia, bone, skin and soft tissue infections. Affecting men and women of advanced age and suffering from other pathologies such as diabetes mellitus, cancer, and/or immunosuppression. Mortality is higher in this population (Murray et al., 2013).

Diagnosis

As mentioned above, the timing of specimen collection is very important for the detection of GBS. A swab should be taken from both the distal vagina and rectum, as it significantly increases the recovery of this microorganism, between the 35th and 37th week of pregnancy (Verani et al., 2010).

Swabs can be seeded on blood agar, colistin-nalidixic acid agar (CNA), Granada agar or chromogenic agar. Plates are incubated at 35–37 °C and should be examined for GBS-like colonies at 24 and 48 h. Orange colony growth should be observed on Granada agar. Colonies growing on other media can be confirmed by additional tests such as latex agglutination or the CAMP test (Nickmans et al., 2012).

To distinguish GBS from other beta-hemolytic streptococcal species, detection of the reddish polyene pigment grenadaene, which is linked to the expression of GBS β -hemolysin, is used (Spellerberg et al., 2000) (Fig. 1). As no other species produces this pigment, it can be used as a simple and specific method for one-step detection of SGB. It is carried out on Granada agar media, already mentioned above (Rosa-Fraile et al., 2014). But not all strains produce pigment, as 2–5% of human colonizing GBS isolates are not hemolytic and do not produce pigment (Tazi et al., 2009).

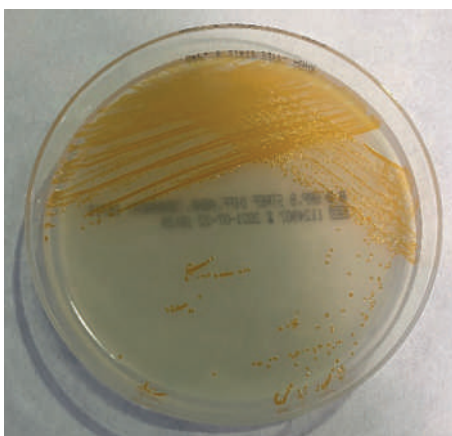


Fig. 1 *S. agalactiae* in Granada agar.

In addition to identification by biochemical tests such as CAMP, or the pyrrolidonyl arylamidase (PYR) test, it can be identified by MALDI-TOF MS mass spectrometry because it is fast and accurate (Lartigue et al., 2009).

Regarding to molecular diagnosis, in recent years several specific PCR assays have been developed for the detection of GBS colonization, employing different genetic targets. Most are currently incorporated in automated molecular platforms such as the BD MAX system (Becton, Dickinson), SmartCycler and Xpert (Cepheid). It can be performed directly with the clinical swab. The main advantage of PCR is the short time to report results regarding GBS colonization status at delivery. Despite being a rapid and sensitive technique, it is recommended to perform culture (Verani et al., 2010).

Treatment

SGB is considered susceptible to penicillin, although reduced sensitivity has been detected in some isolates, which is believed to be secondary to reduced expression of the PBP2 protein (Seki et al., 2015). Thus, penicillin G remains the treatment for invasive disease. GBS is sensitive to beta-lactams such as ampicillin, first, second and third generation cephalosporins and carbapenems. In penicillin-allergic patients, alternative treatment includes clindamycin, erythromycin, fluoroquinolones and vancomycin. However, increased resistance has been detected in these antibiotics and they should only be used if the patient is allergic to penicillins and their susceptibility has been proven (Piccinelli et al., 2015).

The duration of treatment is usually 10 days in infections such as bacteremia, pneumonia, pyelonephritis and skin and soft tissue infections. For meningitis, a longer duration, about 2 weeks, is recommended (Edwards and Baker, 2015).

In newborns with early-onset disease, empirical treatment consists of ampicillin in combination with an aminoglycoside. A 10-day therapy is recommended for uncomplicated bacteremia and 14 days for uncomplicated meningitis, whereas a complicated infection may require a longer duration of treatment (Kimberlin et al., 2015).

Anginosus group

Among the remaining β -hemolytic *Streptococci*, the *Streptococcus anginosus* group is the most important and includes *S. anginosus*, *S. constellatus* and *S. intermedius* among others. It is usually associated with suppurative infections and abscesses. It can see as small colonies, which may be observed with alpha, beta or without hemolysis and could belong to Lancefield groups A, C, G or F, and they usually have a characteristic caramel odor. They colonize human mucous membranes, oral, genital and gastrointestinal (Whiley et al., 1992).

Viridans group

This group includes several species, most of which have similar characteristics, they form part of the mucous membranes of mammals, have low virulence, are in the form of small colonies (<0.5 mm) and most are alphahemolytic (from which comes its name viridans, green).

This group includes *Streptococci* belonging to the mitis group (except *S. pneumoniae*), *anginosus*, *salivarius* and *mutans* (Alam et al., 1999).

Bovis group

This group of *Streptococci* belongs to Lancefield's group D and grows in the presence of bile-esculin, they are related to isolations in patients with endocarditis or any underlying disease affecting the colon. The most important species of this group is *S. gallolyticus* which have two subspecies, *gallolyticus* and *macedonicus* is isolated in bovine mastitis and also in cases of human endocarditis associated with colon neoplasia (Schlegel et al., 2003).

Enterococcus

Introduction

The genus *Enterococcus* comprises a ubiquitous group of Gram-positive bacteria that have become highly relevant to human health because of their role as major causative agents of health care-associated infections. They are able to survive in difficult conditions, and are therefore well adapted to the hospital environment. They show intrinsic resistance to several common antibiotics, including some beta-lactams such as cephalosporins, aminoglycosides, trimethoprim-sulfamethoxazole and clindamycin. This fact supposes a prolonged hospitalization time, increases both the cost of treatment and increases the risk of treatment failure and death (García-Solache and Rice, 2019).

This genus consists of several species but most of them are caused by *Enterococcus faecalis* and *Enterococcus faecium*, which can easily acquire resistance to other antibiotics, such as high-level ampicillin resistance or resistance to glycopeptides such as vancomycin, either by mutation or by horizontal transfer of genetic elements that confer resistance determinants.

These microorganisms are common residents of the gastrointestinal tract of almost all terrestrial animals, including humans, and the main infections that they produce include urinary tract infections, bacteremia, intraabdominal infections and endocarditis (Selleck et al., 2019).

Epidemiology

Enterococci have been isolated from soil, surface water and seawater; in plants; in fermenting food products, and from the intestinal microbiota of vertebrates and invertebrates (Švec et al., 2005).

The most common species isolated in the human gastrointestinal tract are *E. faecium* and *E. faecalis*, being *E. faecium* isolated from production animals and *E. casseliflavus* from plant sources (Klein, 2003). The number of *E. faecalis* in human feces varies from 10^5 to 10^7 per gram, and of *E. faecium* from 10^4 to 10^5 per gram (Franz et al., 1999).

Epidemiological studies have reported that *E. faecalis* and *E. faecium* species are regularly isolated from food such as cheese, fish, sausages and pork meat (Foulquié Moreno et al., 2006).

The distribution of *Enterococcus* species varies throughout Europe. In countries such as Spain and the United Kingdom, the most commonly isolated species are *E. faecalis* and *E. faecium*, both from clinical and environmental sources (Kühn et al., 2003).

Some risk factors that are associated with enterococcal infections include the use of urinary or intravascular catheters, prolonged hospitalization and the use of broad-spectrum antibiotics (Murray et al., 2013).

Regarding to the resistance of these microorganisms, they can acquire some resistance genes. Acquired resistance to beta-lactams is due to hyperproduction or alterations in PBP5, while the production of betalactamase enzymes is rare in these microorganisms. Most *E. faecium* isolates are resistant to ampicillin. However, resistance of *E. faecalis* to this antimicrobial is rare. Acquired resistance to glycopeptides is due to the acquisition of resistance operons called VanA, vanB, vanD, VanE. In Spain, resistance to vancomycin and teicoplanin is uncommon and generally below 5% (Cercenado, 2016).

Microbiology

Enterococcus spp. are Gram-positive, catalase negative, non-spore-forming bacteria, which can appear either as single cocci or as pairs, chains or clusters (Woodford, 1998). They are chemoorganotrophic facultative anaerobes with homofermentative metabolism, with lactic acid as the predominant end product of carbohydrate fermentation (Švec and Franz, 2014). They are identified with a low G + C content of 34–45% (Zhong et al., 2017).

The term *Enterococcus* was first described by Thiercelin in 1899, when he described intestinal commensal bacteria with the ability to become pathogenic (Bouvet and Couvry, 1994). Based on a 1970 study, proposed that they should be placed in the genus *Enterococcus* (Scheleifer and Kilpper-Bälz, 1984). This genus consists of 58 described species. It belongs to the family *Enterococcaceae* which is in the order *Lactobacillales*, class *Bacilli* in the phylum *Firmicutes*.

Most *Enterococci* are oxidase and catalase negative, salt tolerant, resistant to 40% bile, and esculin hydrolytic. *E. faecalis* and *E. faecium* will grow in a wide pH range (4.6–9.9). *E. faecalis* can grow in 6.5% NaCl and has a cation homeostasis that is believed to contribute to its resistance to Ph, salt, metals and desiccation. They can growth between 10 and 45 °C, with optimal growth for most species between 35 and 37 °C. Both microorganisms can survive heating 60 °C for 30 min, which makes *Enterococcus* species different from other closely related genera such as *Streptococcus* (Foulquié Moreno et al., 2006).

When it is cultured on horse blood agar, *Enterococci* can show both alpha and beta-hemolysis and no hemolysis. They form 1 to 2 mm colonies with a wet appearance. Given their metabolic capabilities, different selective culture media have been developed for the isolation of *Enterococci*, containing bile salts, antibiotics, esculin salts or tetrazolium. The most clinically relevant species grow well on these media. Clinical tests for identification of *Enterococci* include the catalase production test, the pyrrolidonyl arylamidase/pyrrolidonyl aminopeptidase (PYP) test and the bile esculin hydrolysis test (England, 2014).

Pathogenesis

The virulence of *Enterococcus* species is mediated by several factors: ability to colonize the gastrointestinal tract, which is the normal habitat; ability to bind to a variety of extracellular matrix proteins, including thrombospondin, lactoferrin and vitronectin; and ability to adhere to urinary tract epithelium, oral cavity epithelium, and human embryonic kidney cells.

Colonization of the gastrointestinal tract is directly related to the risk of infection (Taur et al., 2012), which occurs when the microorganism overwhelms host defenses, replicates at high rates that exceed clearance, and when pathological changes result from direct toxin activity (Garsin et al., 2014).

There are several proteins described as virulence factors of pathogenic *Enterococci*. Microbial surface components that recognize adhesive matrix molecules are surface elements that help *Enterococci* adhere to host tissues.

Pilin gene clusters (PGCs) are present in both *E. faecalis* and *E. faecium* and encode surface proteins, called pili that can function as adhesins. The PGC ebp (endocarditis- and biofilm-associated pilus) in *E. faecalis* is associated with initial adhesion and biofilm formation and has been implicated in the pathogenesis of endocarditis and urinary tract infections (Nallapareddy et al., 2006).

Another protein involved in the virulence of *E. faecalis* is the cytolysin (Cyl), encoded by the cylL L and cylL S genes. It can damage host cells and promotes infection (Haas et al., 2002).

The gelatinase (GelE) of *E. faecalis*, is a matrix metalloproteinase that hydrolyzes gelatin, collagen, and other proteins. It is implicated in the development of endocarditis. The cell wall associated enterococcal surface protein (Esp) contributes to cell adhesion in both microorganisms and it is implicated in urethral colonization and endocarditis and promotes biofilm formation (Thurlow et al., 2010).

Genes encoding several of these virulence factors are often placed on PAIs or mobile elements, which facilitates their spread between isolates. PAIs are large elements that can be acquired by horizontal transfer and confer virulence to bacterial pathogens (Gal-Mor and Finlay, 2006).

In addition to secreted proteins, it has been shown that *E. faecalis* and *E. faecium* can produce toxic oxygen metabolites that can cause cellular damage.

Clinical manifestations

Bacteremia

Enterococcal bloodstream infections are associated with a high mortality rate and are due to the result of translocation of *Enterococci* from the intestine into the bloodstream (Billington et al., 2014).

Risk factors associated with enterococcal bacteremia include severity of illness, patient age, and use of third-generation cephalosporins (Stroud et al., 1996). Common sources for community-acquired bacteremia are the gastrointestinal tract and urinary tract (Arias and Murray, 2015). They are usually acquired from intravascular or urinary catheters, but they have also been associated with intraabdominal, burn wound, pelvic, biliary and bone sources.

Bacteremia caused by glycopeptide-resistant *Enterococci* is associated with a 2.5 fold increase in mortality compared to vancomycin-sensitive *Enterococci* bacteremia (Salgado, 2008).

Urinary tract infection

Enterococci are one of the most commonly isolated gram-positive bacteria from catheter-associated urinary tract infections, being the majority of them *E. faecalis* isolates (Weiner et al., 2016).

Some studies showed a potential role of plasmid-encoded aggregation substance in enterococci adhesion to renal epithelial cells. Several recent studies have identified a pilus in *E. faecalis*, and have shown that Ebp pili are important for adhesion to urinary epithelium and urinary catheters through binding of fibrinogen released into the bladder during catheterization (Tomita and Ike, 2004).

Urinary tract infections tend to be more common in men and are associated with recurrent urinary tract infections, previous antibiotic treatment, indwelling catheters, instrumentation and abnormalities of the gastrointestinal tract (Arias and Murray, 2015).

Endocarditis

Infective endocarditis caused by *Enterococci* represents one of the most therapeutically challenging clinical manifestations. *Enterococci* cause 10–20% of all cases of infective endocarditis and *E. faecalis* cause the majority of cases (Giannitsioti et al., 2007).

One of the most important virulence factors causing this infection is the adhesion to host tissues by cell surface adhesins. In *E. faecalis*, aggregating substance (AS) represents a class of large surface proteins involved in mediating cell-to-cell adhesion (Clewett et al., 2014). These proteins also appear to be key players in both cardiac tissue attachment and biofilm development during infective endocarditis. Three proteins are related, Asa1, Asc10 and Asp, and they help in cell-to-cell adhesion and also contribute to the exopolymeric matrix that accumulates within the biofilm (Chuang-Smith et al., 2010).

As in bacteremia production, the pili encoded by the Ebp group in *E. faecalis* are important for both adherence and biofilm formation. Other important virulence factors in *E. faecalis* IE include the metalloprotease GelE, the collagen-binding adhesin Ace, the fibronectin-binding protein EfbA, cytolysin, and the PTS permease BepA.

In *E. faecium* specific virulence factors and the role they play in infective endocarditis are unknown. However, the enterococcal surface protein Esp and the collagen-binding protein Acn have been shown to be important for the pathogenesis of this species (Heikens et al., 2011).

As for endocarditis caused by vancomycin-resistant strains of *E. faecalis*, these are associated with central venous tract, liver transplantation and mitral valve infections, whereas endocarditis caused by vancomycin-resistant *E. faecium* is usually associated with tricuspid valve infection (Forrest et al., 2011).

Enterococcal endocarditis usually presents as a subacute course, with the most common clinical manifestations being the presence of a murmur, fever, weight loss, malaise, and generalized aches and pains (Arias and Murray, 2015).

Intra-abdominal and pelvic infections

Since *Enterococci* are part of the microbiota of the gastrointestinal tract, they are commonly isolated with pelvic and intra-abdominal infections, usually along with other microorganisms. Treatment of intra-abdominal enterococcal infections is considered acceptable in immunocompromised patients and the critically ill associated with abscesses, wounds or peritonitis in patients with damaged heart valves to prevent bacteremia or endocarditis (Arias and Murray, 2015; Harbarth and Uckay, 2004).

Skin and skin structure infections

These microorganisms colonize the skin and may be associated with infections of the skin and skin structures. They are usually isolated in polymicrobial infections and their pathogenic role is unclear. They can be isolated from decubitus ulcers and diabetic foot ulcers, and sometimes in osteomyelitis, septic arthritis and soft tissue abscesses (Agudelo Higuera and Huycke, 2014).

Diagnosis

Enterococci can grow readily on non-selective media such as blood agar and chocolate agar. On Gram stained these microorganisms can be seen as cocci in pairs or forming chains and can be easily differentiated by simple biochemical reactions. *Enterococci* are optoquine resistant, while *S. pneumoniae* is susceptible, do not dissolve when exposed to bile and produce L-pyrrolidonyl-arylamidase (PYR). Catalase-negative and PYR-positive cocci that are arranged in pairs or short chains are identified as *Enterococci*.

Identification of *Enterococci* to species level has clinical relevance due to antibiotic resistance profiles of the different pathogenic *Enterococci*. New molecular techniques are also useful for epidemiology and surveillance and in the diagnosis of difficult cases. Molecular-based methods have the advantages of increased diagnostic accuracy, provide information of antimicrobial resistance, and reduce time and costs compared to traditional culture and phenotypic testing.

New systems for the classification and identification of these microorganisms include matrix-assisted laser desorption ionization- time-of-flight mass spectrometry (MALDI-TOF MS), nucleic acid amplification testing (NAAT), in situ hybridization, and multilocus sequence typing (MLST).

Clinical use of MALDI-TOF MS based methods allows rapid identification of *Enterococci* directly from blood culture bottles, potentially reducing the time to initiation of antimicrobial treatment. This technique has a high sensitivity, being able to identify about 94% of isolates to species level, including differentiation between closely related species (Stepień-Pyśniak et al., 2017). In addition, it could be quite useful for antibiotic resistance profiling, for detecting the presence of van genes, although it is not yet used in clinical practice (Singhal et al., 2015; Bizzini and Greub, 2010).

NAAT methods are based on PCR amplification and subsequent sequencing or array/hybridization or real-time PCR amplification of genes that are useful for identification of organisms to genus or species level and important for detecting antimicrobial resistance genes (Ljungström et al., 2015).

Sequencing of the 16S rRNA gene is commonly used to identify bacterial species and allows differentiation of enterococci to the species level. Multiplex real-time PCR allows testing for more than one gene, allowing determination of both species and antibiotic resistance genes at the same time (Bosshard et al., 2004).

Hybridization allows rapid detection of the presence of *Enterococci* in blood culture bottles. These tests enable differentiation of *E. faecalis*, *E. faecium* and other less common *Enterococci* species (Deck et al., 2014).

Another technique used in strain identification is multilocus sequence typing (MLST) which is used to study molecular epidemiology and to study outbreaks, which is more reproducible and less laborious than pulsed-field electrophoresis (PFGE) analysis (Ruiz-Garbajosa et al., 2006).

Treatment

Antimicrobial treatment of enterococcal infections is complicated. Treatment of severe infections has traditionally consisted of the synergistic combination of an aminoglycoside and an antibiotic able to inhibit cell wall synthesis.

The genus *Enterococcus* is characterized by reduced susceptibility to many infectious agents, active against other Gram-positive microorganisms. Enterococci are intrinsically resistant to almost all cephalosporins (with possible exceptions such as ceftaroline and ceftobiprole), antistaphylococcal penicillins, and aztreonam (Murray, 1990).

Enterococci are usually susceptible to vancomycin, although in recent years resistance to this antimicrobial is increasing, which we will discuss in this section. The emergence of vancomycin-resistant *Enterococcus* as an important nosocomial pathogen is due to its propensity for colonization of the gastrointestinal tract, persistence in hospital settings, genome plasticity, mobile genetic elements and increased mortality. In addition, they are resistant to clindamycin, trimethoprim-sulfamethoxazole and clinically achievable concentrations of aminoglycosides (Florescu et al., 2008).

New antimicrobials such as linezolid, tedizolid, daptomycin, telavancin, and oritavancin are active against *Enterococci*. In the clinical setting, the treatment of choice remains ampicillin in susceptible strains and patients who can tolerate this drug (de Lastours et al., 2017).

The mechanism of resistance of *Enterococci* against β -lactams, is attributed to the expression of a low-affinity penicillin-binding protein (PBP), PBP4 in *E. faecalis* and PBP5 in *E. faecium* (Duez et al., 2001). Therefore, most *Enterococci* are tolerant to the bactericidal activity of β -lactams, making them bacteriostatic. Another mechanism by which resistance to beta-lactams can occur is the production of β -lactamases, but this is not very common in *Enterococci*, although it can lead to a high level of resistance to ampicillin by hydrolyzing β -lactams before they reach their target in the cell wall. To treat severe infections such as endocarditis or meningitis, a synergistic bactericidal combination of a β -lactam with an aminoglycoside can be used. Treatment of endocarditis is currently based on combinations of ampicillin, which is active against *E. faecalis* and ceftriaxone, although the mechanism of this synergism is not clear, it has been postulated that the combination of the two antibiotics inhibits all PBPs of *E. faecalis* more effectively than either antibiotic alone (Pericas et al., 2014).

Acquired antimicrobial resistance

β -lactams resistance

As described above, *Enterococci* are intrinsically resistant to most β -lactams, being susceptible only to a small number of penicillins such as ampicillin, penicillin and piperacillin (Rice and Murray, 1995). Resistance to these penicillins can be produced through two mechanisms, as summarized above, the first and less important, the production of β -lactamases; this β -lactamase has been

identified as identical to that produce by *S. aureus* in some cases within genetic regions identical to that of the Tn 551 transposon (Rice and Marshall, 1992). The other, more important mechanism, also mentioned above is high-level penicillin resistance in *E. faecium* due to the expression of low-affinity PBP5 (Rice et al., 2004). In addition in these species a mutation occurs in the *pbp5* gene leading to amino acid substitutions near the active site of the enzyme (Rice et al., 2004; Rybkine et al., 1998). Epidemiological sequencing data suggest that strains with a high level of ampicillin resistance belong to relatively few lineages that have been spreading, mainly in hospitals, causing infections and colonization of patients exposed to a variety of antibiotics. In the case of *E. faecalis* higher level penicillin resistance is usually rarer than in *E. faecium*. It is due to a higher expression of low affinity PBP4. The fact that deletion of PBP4 or PBP5 results in susceptibility to β -lactams of *E. faecalis* and *E. faecium*, respectively, indicates that these proteins are necessary for resistance (Rice et al., 2018). But other proteins necessary for resistance expression have been found. In *E. faecalis*, the CroRS regulatory locus is necessary for cephalosporin resistance and the presence of genes for two of the three class A PBPs of this microorganism (*ponA* and *pbpZ*) are also necessary for cephalosporin resistance (Kellogg et al., 2017). Deletion of the equivalent class A PBPs in *E. faecium* also increases susceptibility to cephalosporins (Arbeloa et al., 2004).

Glycopeptide resistance

Vancomycin has been an active antibiotic against *E. faecalis* and *E. faecium* for almost three decades after its clinical introduction. In the early 1980s, resistant strains began to emerge, expressing high-level inducible resistance to vancomycin and the more recently introduce antibiotic teicoplanin (Shales et al., 1989). This resistance was attributed to the acquisition by these microorganisms of operons that altered the nature of the peptidoglycan precursors, producing a substitution of a D-lactate for the terminal D-alanine in the UDP-MurNac pentapeptide (Arthur and Courvalin, 1993). The mechanism of action of vancomycin is due to binding to the D-terminal alanine of the cell wall precursor, preventing access to PBPs. Since this antibiotic binds to D-lactate-terminating pentapeptide stalks with much lower affinity than to D-alanine-terminating ones, it is not an effective inhibitor of cell wall synthesis in these strains.

The first glycopeptide resistance operon to be described was the *vanA* operon, and this remains the most common operon in the clinical setting. In addition, other phenotypic variants of acquired glycopeptide resistance have been described in *Enterococci* (*VanB*, *VanD*, *VanE*, *VanG*, *VanL*, *VanM* and *VanN*), with one type of intrinsic resistance (*VanC*) that is unique to *E. gallinarum* and *E. casseliflavus* (Reid et al., 2001). *VanA* is responsible for the majority of human cases of VRE worldwide, and is mainly carried by *E. faecium*. These operons are divided into two groups, those that replace the D-alanine terminus with a D-lactate (*VanA*, *VanB*, *VanD* and *VanM*) and those that replace the terminus with a D-serine (*VanC*, *VanE*, *VanG*, *VanL* and *VanN*) (Sassi et al., 2018). Operons encoding proteins that result in precursors ending in D-serine confer lower levels of resistance to vancomycin but remain susceptible to teicoplanin. In contrast, operons terminating in D-lactate confer resistance to vancomycin and teicoplanin, although the *vanB* operon is not induced by the presence of teicoplanin, so strains in which the induction mechanism is intact.

Aminoglycoside resistance

Enterococci are intrinsically resistant to clinically achievable concentrations of aminoglycosides. They are used in the treatment of enterococcal endocarditis in combination with cell wall-active antimicrobials to achieve bactericidal synergism.

Some strains of *Enterococcus* spp. which have expressed high levels of resistance to these antibiotics with very high MICs for gentamicin and streptomycin have emerged, which is due to the expression of aminoglycoside-modifying enzymes and negates the synergistic benefit of these combinations. The gene encoding the most common enzyme conferring resistance to gentamicin is *aac-6'-Ie-aph-2*. Another enzyme that has been associated with lower gentamicin MICs is phosphotransferase *APH(2'')-1c*. In *E. faecium* intrinsic resistance to kanamycin and tobramycin is due to chromosomally encoded *AAC(6')-II*.

Fluoroquinolones resistance

Fluoroquinolones such as ciprofloxacin and levofloxacin are only used in the treatment of urinary tract infections due to susceptible strains of *Enterococci*. Highly resistant strains contain mutations in *gyrA* and *parC* (El Amin et al., 1999).

Linezolid resistance

Linezolid is an antimicrobial active against *E. faecalis* and *E. faecium*. Resistance to linezolid is rare, but has been described in the literature and is associated with duration of pretreatment with linezolid (Vega and Dowzicky, 2017). Resistance to linezolid may be due to two mechanisms, a decrease in binding due to mutations in the 23S ribosomal RNA or to the acquisition of a *cfr* gene (resistance to chloramphenicol-florfenicol resistance) through horizontal transmission, which causes methylation of 23S ribosomal RNA. This enzyme confers resistance to a wide variety of antimicrobial classes, including phenicols, lincosamides, oxazolidinones and streptogramin A (Vester, 2018).

Linezolid is effective in the treatment of vancomycin-resistant *E. faecium* bacteremia and is recommended as a first-line treatment option for infective endocarditis due to ampicillin- and vancomycin-resistant *Enterococci* (Scheetz et al., 2008).

Linezolid has good central nervous system (CNS) penetration and has been used successfully as monotherapy for vancomycin-resistant *Enterococci* CNS infections (Knoll et al., 2013).

A new oral and parenteral oxazolidinone called tedizolid with a broad spectrum of bacteriostatic activity against resistant gram positive bacteria has emerged. Tedizolid has a fourfold lower MIC against vancomycin-resistant *Enterococci* compared to linezolid. In addition, it is active against linezolid-resistant strains carrying the *cfr* mutation (Rybak et al., 2014).

Daptomycin resistance

Daptomycin is an antibiotic with dependent bactericidal activity used against some resistant Gram positive microorganisms, including *E. faecalis* and *E. faecium* vancomycin-resistant. Some recent studies have compared daptomycin with linezolid used for the treatment of vancomycin-resistant enterococcus bacteremia and found higher mortality in patients treated with daptomycin. But these studies are limited by several factors, so linezolid and daptomycin should still be used as first-line options for the treatment of VRE bacteremia, although high doses (8–12 mg/kg) should be considered for daptomycin (Chuang et al., 2014).

Due to therapeutic failures and the development of resistance during the use of daptomycin monotherapy for the treatment of enterococcal endocarditis, the possibility of combination therapies and the use of high-dose daptomycin has been explored (Reyes and Zervos, 2013). Resistance to daptomycin is associated with longer durations of therapy and is a function of genetic mutations in genes responsible for biogenesis, permeability, and resistance potential (Arias and Murray, 2015).

Daptomycin monotherapy is not recommended for CNS infections due to its poor penetration with inflamed meninges (Knoll et al., 2013).

Some in vitro studies have shown synergy for combinations of daptomycin with beta-lactams in VRE, including new generation cephalosporins. This combination increases the bactericidal activity of daptomycin and reduces resistance formation (Werth et al., 2015).

Lipoglycopeptides

Telavancin and dalbavancin possess concentration-dependent bactericidal activity against several resistant Gram positive bacteria, including VRE (Zhanel et al., 2010).

Oritavancin has the broadest spectrum of lipoglycopeptides that have bactericidal activity against nearly all resistant gram positive bacteria, including both VRE-expressing VanA and VanB. It represents a promising option for the treatment of VRE infections of the skin and skin structure and possibly bacteremia or even endocarditis, when administered with gentamicin for synergy (Lefort et al., 2000).

Tigecycline

Tigecycline is a glycylcine, which has activity against Gram positive and negative microorganism resistant to tetracycline, including VRE faecalis and faecium. Resistance to tigecycline has not yet been reported in VRE (Meagher et al., 2005).

Tigecycline has been used successfully together with daptomycin for the treatment of VRE infective endocarditis, but should not be used in the treatment of VRE bacteremia due to its high volume of distribution with low serum levels (Jenkins, 2007).

It achieves high penetration into the peritoneal space and has a broad spectrum of activity, which is why it can be used in the treatment of polymicrobial intra-abdominal infection associated with VRE (Scheetz et al., 2006).

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Genus *Neisseria*

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Genus *Neisseria*

The genus *Neisseria* is a group of Gram-negative bacteria, and the different species that make it up appear generally (although with some exceptions) in the form of diplococci, with flattened adjacent walls (Tønjum, 2005).

The genus owes its name to the German microbiologist Albert Neisser, who was the first to describe the species *Neisseria gonorrhoeae*. Although 15 species are listed in the latest edition of the Bergey's manual (Bergey's Manual of Systematic Bacteriology) (Tønjum, 2005), a good number of new species have been proposed whose taxonomic position is not yet resolved (Diallo et al., 2019).

Of all the species described, only two, *Neisseria meningitidis* and *Neisseria gonorrhoeae* are pathogenic for humans, while the rest are usually found as commensals of humans and animals, on mucosal surfaces, such as the epithelium of both the nasopharynx or the genital (Rotman and Seifert, 2014).

Although conventionally the species of the genus have been distinguished based on their phenotypic properties, such as the use of carbohydrates and enzymatic tests, which generally give satisfactory results for the identification of meningococcus (*N. meningitidis*) and gonococcus (*N. gonorrhoeae*), misclassification using these methods are not uncommon (Dossett et al., 1985), so it is increasingly common to use molecular methods for identification purposes (Bennett et al., 2012) and phenotypic differences are less and less used in the determination of species, being replaced by a genetic approach.

Neisseria meningitidis

Neisseria meningitidis, also known as meningococcus, is one of two pathogenic species in the genus. Its history begins in 1887, when Weichselbaum first identified the bacteria in cerebrospinal fluid from a patient with meningitis, although its association with the production of epidemics of cerebrospinal meningitis had already occurred in 1805 in Geneva (Switzerland), in 1806 in Massachusetts and in the early 1900s in the African meningitis belt (Rouphael and Stephens, 2012).

To be more precise, *N. meningitidis* can be considered as an accidental pathogen, with the bacterium only rarely causing life-threatening disease. Its only known natural habitat is the mucosa of the human nasopharynx. This phenomenon of colonization of the upper respiratory tract is known as “carriage,” and represents a successful commensal relationship between the host and the bacteria (Caugant and Maiden, 2009), without the host showing any pathology. However, the development of the disease represents in some way a dysfunctional failure in that commensal balance between both protagonists.

Invasive meningococcal disease

The bacteria *N. meningitidis* is the etiological agent associated with Invasive Meningococcal Disease (IMD), a devastating disease that remains a public health concern because of its rapid onset and significant risk of death and long-term disability (Wang et al., 2018). Even with proper therapy, 5–10% of patients die 24–48 h after the onset of symptoms (Olbrich et al., 2018). Additionally severe permanent sequelae in those surviving to septicemia include severe skin necrosis and scarring requiring skin grafts and amputation of limbs, with long-term sequelae in 10–20% of survivors (Olbrich et al., 2018).

Meningococcus usually causes meningitis and sepsis in susceptible individuals. However, the presentation of unusual clinical forms or symptoms that have not been classically associated with meningococcal infection is becoming more and more frequent (Stinson et al., 2020). The unusual clinical presentations of meningococcal disease include: abdominal presentation (abdominal pain, gastroenteritis, diarrhea and peritonitis) (Vienne et al., 2003), complicated forms of meningitis (Toubiana et al., 2013), cardiac manifestations and arthritis (Vienne et al., 2003), meningococcal pneumonia (Vossen et al., 2016). Strains belonging to the cc11 either from serogroup W or C appear overrepresented in most of these unusual clinical presentations except in pneumonia, where serogroup Y cc23 are the most prevalent (Campbell et al., 2019).

Meningococcal carriage (reservoir and transmission)

The only known ecological niche of *N. meningitidis* is the human nasopharynx. The acquisition of the bacteria requires a previous person-to-person transmission through direct contact or contact with drops from the respiratory tract of an infected individual to another susceptible (Caugant and Maiden, 2009). The phenomenon by which an individual is infected in the nasopharyngeal mucosa without developing any symptoms or signs of disease is known as carriage.

Carriage rates are very variable among human populations, and it is associated with the age. Global carriage rates in Europe and the United States have been estimated to range from 10% to 35% in young adults (Caugant and Maiden, 2009) and it is very likely that throughout his life an individual will be a nasopharyngeal carrier at least once in his life.

Age has been shown to be a risk factor for being a carrier of meningococcus, so that in studies carried out in Europe and the United States, the highest rates of carriage are observed in the group of adolescents around 20 years of age, while that carriage is very low in the first years of life, then gradually increasing until adolescence, later decreasing until being around 10% in older ages (Christensen et al., 2010). One of the factors that explain the greater prevalence of bacteria in the group between 17 and 24 years old is more and closeness social contacts in this group of age (MacLennan et al., 2006).

In contrast to isolates from IMD patients, the greater proportion of isolates from asymptomatic carriers are noncapsulated, with a greater presence of unusual serogroups found in IMD cases (Glitza et al., 2008).

Obtaining in-depth knowledge of the transmission dynamics of *N. meningitidis* strains is essential to be able to understand the dynamics of the disease itself, as well as for the selection of vaccination-based strategies (Cartwright et al., 1991). For this purpose, the molecular characterization of the strains has allowed a great qualitative leap in the understanding of these mechanisms (Caugant and Maiden, 2009).

Detection of the presence of the microorganism in clinical samples: Microbiological diagnosis

Early signs and symptoms are usually unspecific, with a range of signs and symptoms that may or may not appear at different times of the disease (Vázquez et al., 2016). Although meningitis and sepsis are the two most frequent symptoms, other presentations may also be observed (e.g., arthritis and pericarditis), and different guidelines recognize different clinical criteria.

Microbiological methods are used to confirm the case. Meningococci are Gram-negative diplococci and can be found extracellularly or intracellularly in peripheral mononuclear leukocytes. Although the Gram stain can be used as a first approximation in the confirmation of a case using CSF samples or blood films, the probability of visualizing a meningococcus in the staining depends

largely on the bacterial load, and the previous administration of antibiotic prior taking the sample slightly reduces the chances of success (Tunkel et al., 2004). Although it is a widely used method due to its low cost and speed of implementation, its low sensitivity and a specificity that largely depends on the operator, does not allow it to be considered the “gold standard method.”

Several rapid diagnostic tests based in antisera directed against the capsular polysaccharide have been used (Vázquez et al., 2016), including counterimmunoelectrophoresis, coagglutination, and latex agglutination. The sensitivity for *N. meningitidis* range between 50% and 93% (Tunkel et al., 2004), being particularly low in clinical samples, so a negative result cannot rule out the presence of a specific meningial pathogen, and false positive results have been also described. For that reasons those test are not usually included in guide lines for meningococcal diagnostic. However, latex agglutination has swon very effective in identifying capsular groups A, B, C, W and Y from culture (Vázquez et al., 2016).

CSF and blood culture are valuable for detection of the causative organism and establish susceptibility patterns. Blood culture is particularly useful if CSF cultures are negative or unavailable, e.g., when lumbar puncture is contraindicated. Growth is optimal at 35–37 °C in ~5% CO₂, on blood agar or chocolate agar plates (Vázquez et al., 2016; Olcen and Fredlund, 2001). The timing of specimen collection is crucial for successful processing of *N. meningitidis* culture and also false-negative results may appear from early antibiotic treatment and incorrect handling or culturing of samples (Olcen and Fredlund, 2001). Carbohydrate utilization tests are used to validate the presence of *N. meningitidis* further. Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDITOF) has also been described for routine identification but some improvement of meningococcal identification might be required (Cunningham et al., 2014).

Finally, molecular methods such as the polymerase chain reaction (PCR), real-time PCR (rtPCR), qualitative or quantitative PCR (qPCR), and loop-mediated isothermal amplification assays (LAMP), have been developed and used in the diagnosis and characterization of *N. meningitidis*, with the advantage that they are not constrained by the presence of cultivable organisms (Diallo et al., 2021). Recommended genetic target is the capsule gene *ctrA* that detects encapsulated meningococci. In order to detect both, capsulated and also non-capsulated strains, a combined *cnl* and *ctrA* assay may be needed to increase sensitivity.

Bacteria structure

Meningococcal capsule

Meningococcal strains present a polysaccharide capsule with defined functions in the invasion and survival of the bacteria in usually sterile liquids such as blood, spinal fluid, etc., providing resistance to host immune response (Rouphael and Stephens, 2012). The capsule is not present in many strains but it is almost always present in those isolates from invasive disease and for that reason the capsule is considered essential for the survival of the bacteria during the invasive disease (Uria et al., 2008).

The finding that antibodies directed against the capsule played a fundamental role in the protection against invasive meningococcal disease (IMD) (Goldschneider et al., 1969), has led to this polysaccharide envelope forming the basis for the development of vaccines as well as for the classification of the meningococcus in serogroups (Snape and Pollard, 2005).

Although there are 13 serogroups described, a proposal has recently been made to revise the scheme used, which reduces the number of serogroups to 12 and is widely accepted by the scientific community (Harrison et al., 2013); of these, six serogroups, A, B, C, W, X, and Y, cause disease, and are associated with the vast majority of the cases globally (Peterson et al., 2019).

Each serogroup is associated with a chemically distinct capsular polysaccharide; those capsule polysaccharides associated with invasive disease are sialic acid derivatives, with only one exception corresponding to the serogroup A capsule, with a number of repeating units of *N*-acetyl-mannosamine-1-phosphate (Rouphael and Stephens, 2012). The incorporation of sialic acid in the capsule allows the bacteria to be less visible to the immune system, since the sialic acid is also incorporated in the host's cell surfaces. This mimicry reaches its highest degree of expression in the case of serogroup B, where the structure of sialic acid is identical to that of a component of human neuronal cells known as NCAM (neural cell-adhesion molecule). This equality would explain the poor immune response obtained against group B polysaccharide in humans (Hill et al., 2010).

The process of synthesis and translocation to the cell surface is complex, and the genes involved are well identified and located at a single locus termed *cps* (Harrison et al., 2013).

Outer membrane

In meningococci, as in the rest of Gram negative bacteria, the subcapsular cell envelope consists of an outer membrane (OM), a layer of glycan peptide and an inner membrane or cytoplasmic membrane. Most of the interaction of the bacteria with the external environment, including the host's immune response, antimicrobial response, exchange of nutrients and metabolites, etc., takes place at the OM level. For this reason the main components of OM have been extensively analyzed.

The outer membrane would be the subcapsular cell envelope of the bacterium, and it is basically composed of an outer layer of lipooligosaccharide (LOS) and proteins and an inner part composed of phospholipids (Rouphael and Stephens, 2012).

Meningococcal LOS constitute the endotoxin of the bacteria, and three different parts can be distinguished in the LOS of meningococcus: lipid A with hydroxy fatty acid chains and phosphoethanolamine, a core oligosaccharide containing 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) and heptose residues, and a part of short and highly variable oligosaccharides (Jennings et al., 1999). LOS antigenic variability have been the basis for the immunological classification of *N. meningitidis* in different immunotypes (L1–12) (Frasch et al., 1985). Although this classification has been useful for the study of the pathogenesis of the microorganism (Zughaier et al., 2004), it is not a widely used characterization method (Jolley et al., 2007).

At the OM level we can find numerous adhesins. The first step in the infection is the adhesion and attack of the bacteria to the epithelial cells of the mucosa of the upper respiratory tract, colonizing and constituting what is known as the “carrier state” that has already been commented previously. The pili are responsible for a first adhesion to the cells of the mucosa, having proven their role when pilate and non-pilate meningococci are compared (Stephens and McGee, 1981). Additionally, the pili are also involved in the process for the uptake of DNA by meningococci and also facilitating the adherence to erythrocytes.

Also playing a role in adhesion, two types of opacity proteins are found in *N. meningitidis*, known as Opa and Opc (Virji et al., 1994). Opa proteins, also expressed in *Neisseria gonorrhoeae*, are encoded by multiple genes and have four extracellular loops, three of which have variable regions, and sequence diversity within these regions conferring specificity for host receptors (Sadarangani et al., 2011).

In addition, meningococcal strains can express other minor adhesins including NadA (Neisseria adhesin A), NspA (neisserial surface protein A), and the autotransporters MspA (meningococcal serine protease) and Msf (meningococcal surface trimeric autotransporter fibril) (Hollingshead and Tang, 2019). Of these, the most relevant is NadA because it was included in the 4CMenB vaccine formulation, having been studied more in depth (Giuliani et al., 2006).

At the OM level we can find a group of proteins that intervene in the acquisition of iron, which is an essential growth factor for the survival of the bacteria, both in colonization in the nasopharynx and during the disease (Guilhen et al., 2013). They are generically known as “iron-binding proteins” and include HmbR (hemoglobin), TbpA and TbpB (transferrin), HbpA and HbpB (lactoferrin), HpnA and HpnB (hemoglobin-haptoglobin complex), and possible siderophore homologues (Rouphael and Stephens, 2012).

Other well-known proteins found at the OM level are porins, of which *N. meningitidis* expresses two, PorA and PorB. Among the main functions of these proteins is the passage of small nutrients into the bacteria, although they are also involved in host cell interactions since they are target antigens for bactericidal antibodies (Mosaheb and Wetzler, 2018; Peak et al., 2016). Both porins have been used in the characterization of isolates: By using panels of monoclonal antibodies, PorB defined the serotype while PorA allowed classification into serosubtypes. When molecular characterization has been implemented, PorB is no longer used and serosubtypes have been called genosubtypes, based on the peptide sequence of two variable regions (VR1 and VR2) of the protein (Jolley et al., 2007). As we will see later, PorA has been the subject of multiple studies as it is the main component of vaccines against serogroup B, although the great variability of the protein makes it very difficult to achieve a wide coverage of isolates with only this component (Feavers et al., 1992).

With the advent of reverse vaccinology (Rappuoli, 2000) as a vaccine development tool against serogroup B, a good number of new antigens located in OM have been described, which will be later mentioned in the section corresponding to vaccines.

Genetic of *Neisseria meningitidis*

An important characteristic of the meningococcus is the plasticity of its genome that allow a tremendous capability to diversify the phenotype.

Phase variation

Phase variation can be defined as the ON and OFF switching of gene expression mediated by mutations in simple tandem repeats or homopolymeric nucleotide tracts situated upstream region or in the open reading frame of genes (Hollingshead and Tang, 2019; Saunders et al., 2000). A large number of antigens present in the cell envelope, including the polysaccharide capsule, have this genetic regulation mechanisms (Hollingshead and Tang, 2019). This allows the bacteria to regulate the expression of the capsule during the colonization process since it is a structure that, although it facilitates the survival of the bacteria during the invasion process, does not favor the process of adhesion to the cells of the mucosa.

Capsule switching

This phenomenon is possible because meningococcus is naturally competent for genetic transformation, either with DNA from other meningococci or with other species, both of the *Neisseria* genus and others. “Capsule switching” is a horizontal genetic exchange process through transformation and recombination at the locus of serogroup-specific genes involved in capsule biosynthesis, and is favored by a high genetic identity in parts of said genes (Swartley et al., 1997). Therefore, the result is a meningococcus that presents most of the microbiological markers the same as the original strain, but that expresses a capsule of a different serogroup. It constitutes an immune response evasion mechanism, and although it was initially a cause for concern, when starting to use serogroup-specific conjugate vaccines (Castilla et al., 2009), immunization-associated capsule switching has not been observed until now (Snape and Pollard, 2005).

Typing meningococci

Initially the characterization methods were based on an immunological approach, which identified capsule variants (serogroups), outer membrane porins (serotypes and serosubtypes based on PorB and PorA respectively) and lipooligosaccharide (immunotypes). These methods were widely used, and the availability of panels of monoclonal antibodies favored their usefulness and interchangeability (Jolley et al., 2007).

However, because the high variability of most of the proteins that gave an increase number of Non-Typeable strains, but also the need to apply characterization methods in non-culture samples (early treatment with antibiotics results in culture negative samples) led to the development and application of molecular characterization methods that do not produce non-typeability results and that can be used directly on clinical samples, therefore they do not depend on the culture of the meningococcus.

The analysis by multilocus enzyme electrophoresis (MLEE) represent the first step in molecular characterization for meningococci (Caugant et al., 1987), although MLEE was still depending of the culture of the strains. However the methodology was technically complex and presented great difficulties in exchanging information (profiles) between different laboratories, so its use was not wide. In addition, greater accessibility to sequencing methods allowed the development of schemes based on genes or specific fragments thereof, and the emerged genogroups (based in caular genes), PorA types (genosubtypes), FetA types (FetA is a protein that has been a candidate to be included in vaccine preparations), and even PorB types (genotypes) although, as previously commented, their use has disappeared (Jolley et al., 2007). To these typing schemes the Multilocus Locus Sequence Typing (MLST) was added, a methodology that came to replace the MLEE and which had the great advantage of simplicity and portability of results (Maiden et al., 1998). MLST analyzes the variation in housekeeping genes by determining the nucleotide sequence in seven alleles. MLST identify major clones, or clonal complexes, in *N. meningitidis* populations (Maiden et al., 1998) and it has been key for a better understanding of the meningococcal epidemiology, defining hyperinvasive lineages, etc. (Brehony et al., 2007).

More recently, when the Whole Genome Sequence (WGS) has become accessible to many laboratories, MLST is being replaced by core genome MLST (Bratcher et al., 2014) that is based on a core set of genes, that are present in most of the isolates, providing higher resolution than MLST.

Meningococcal epidemiology: Unstable and unpredictable

Even now a days the burden of meningococcal disease is unknown for many parts of the world mainly because of inadequate surveillance, which severely hampers evidence-based immunization policy. This is probably the main reason for the creation of the so-called Global Meningococcal Initiative (GMI), which was established in 2009 (Harrison et al., 2011), and is a forum of independent experts that is organized to develop regional and global recommendations on different aspects of IMD, and that has produced in the last 10 years a wealth of information on relevant aspects of epidemiology in many regions where there was little prior information.

Since IMD epidemiology changes temporally and geographically, a review by region can give a more precise idea about the current situation:

IMD in Africa

In Africa we must distinguish three different sub-regions: North Africa, African Meningitis Belt and the South, each with its own well-defined characteristics.

In the North data are scarce and mainly based in a regional GMI meeting (Borrow et al., 2017b). The data shows that the incidence rate range between 0.09 and 3.6 cases per 10⁵ inhabitants, being the serogroup B predominant followed by serogroup C, and just few group W or Y cases.

The African Meningitis Belt is an area that include 26 countries, from Senegal in the West to Ethiopia in the East, and where large-scale epidemics occur every 5–12 years when IMD attack rates can reach 100–800/100,000 (Mustapha and Harrison, 2018). Historically, the epidemics have been associated with serogroup A strains (LaForce et al., 2007). In 2001 the Meningitis Vaccine Project was created through a collaboration between WHO, the Bill & Melinda Gates Foundation and the Program for Appropriate Technology in Health (PATH) developing a conjugated serogroup A vaccine (MenAfriVac) produced at the Serum Institute of India Pvt., Pune, India (Fall et al., 2018). Between 2010 and November 2015, more than 270 million individuals aged 1–29 years were vaccinated in mass campaigns across 16 countries. This has resulted in a near elimination of serogroup A in the region, although epidemic outbreaks have occurred by serogroups W and X, and more recently by a group C strain not previously described, and which has spread to Nigeria and Niger. producing more than 15,000 cases in Nnadi et al. (2017).

Data coming from SouthAfrica are robust because a well stablish surveillance (Borrow et al., 2017b), with a high proportion of cases associated with serogroup W followed by B (Borrow et al., 2017b).

IMD in Asia

The incidence of IMD in the Asia-Pacific region appears to be low but probably some countries might have some level of underestimation (Borrow et al., 2016).

In China five epidemics associated with serogroup A have been reported between 1938 and 1977 (Borrow et al., 2017b) but serogroup A isolates have been declining following the universal introduction of the MenA polysaccharide vaccine in China in the 1980s (Borrow et al., 2016). Current data indicates a shifts in serogroups distribution, with the emergence of new clones of serogroup B from serogroup C clonal complex (cc) 4821 due to capsular switching (Zhu et al., 2015), and it is also affected by the international spread of serogroup W cc11.

In India, most of the reported cases express serogroup A. The incidence rate has been decreasing during the last years but there is an increasing number of outbreaks and some change in the affected ages has been observed in these outbreaks with a majority of cases between 20 and 30 years old, which could be indicating the appearance of new epidemic clones (Borrow et al., 2016). Nevertheless the lack of a well establish surveillance system in India might be offering underestimation of the actual IMD burden.

In Japan, with a strong surveillance system, the incidence rate is particularly low (0.03 per 10⁵ inhabitants) being the serogroup Y the most prevalent followed by C and B (Borrow et al., 2016).

In the Republic of Korea surveillance for IMD is passive and it appear that there are not many reliable epidemiological data (Borrow et al., 2016).

In the Philippines only the septicemic form, meningococcemia, is reported to the surveillance and response systems probably giving a very skewed view of the actual burden of the IMD. Serogroup A is the most prevalent but serogroup B has also been documented (Borrow et al., 2016).

Australia and New Zealand

In Australia the incidence rate of IMD is around 1.5×10^5 and the serogroups distribution is quite different in different geographical areas: Serogroup B was predominant until 2015 all over the country; however, from that year on, serogroup W had a strong increase, particularly in the North, West and Queensland, which led to the inclusion of MenACWY tetravalent conjugate vaccine in these territories. An exception was the territory of the South, where the increase of W strains was much lower, and serogroup B is still predominant (Parikh et al., 2020).

In New Zealand, serogroup B has historically been the majority, although epidemic outbreaks have been described by serogroup A and C, and more recently an increase in cases associated with serogroup W has been reported, which is part of the global expansion of W cc11 strains (Parikh et al., 2020). In 1991, an epidemic associated with a strain B: 4: P1.7b, 4, cc41/44 began in the country. The incidence rate reached 17.4 per 100,000 population in 2001, and this led to the development of a strain-specific vaccine based on outer membrane vesicles (Taylor-made vaccine concept) that was used between 2004 and 2008, reaching 68% efficacy (Walls et al., 2018).

IMD in America

The incidence rate ranges from 0.01 in Mexico to 0.54 in Brazil, although some Central and South American countries have scarce data and probably an underestimation due to the existence of not very robust surveillance systems, limited laboratory capacity, etc. (Parikh et al., 2020; Sáfadi et al., 2015). There are also significant differences in the serogroup distribution: in Canada serogroup B account for most of the IMD cases, but epidemic outbreaks associated with serogroups C and W has been also reported; in United States an unusual high proportion of serogroup Y cases has been observed since the 1990s of the last century, which has been maintained until today, with serogroup Y representing around a third of cases (Harrison et al., 2009; Parikh et al., 2020) (in a context of a very low rate of incidence). In Central and South America there are great differences between nearby countries, with huge contrasts in incidence rates, which surely reflects fragile surveillance systems, poor accessibility to molecular diagnostic methods, etc. (Sáfadi et al., 2015): while many countries have exceptionally low incidence rates, those with higher incidence rates (Brazil, Argentina and Chile) also have more established surveillance systems, which would give an idea of an underestimation of the actual burden of IMD in the region. In Brazil serogroup C is predominant and a decreasing trend in the incidence rate has been observed recently (Parikh et al., 2020); by contrast, in Argentina and Chile, serogroup W has been predominant during the last years (Abad et al., 2014).

IMD in Europe

In Europe, the incidence rate has been falling during the last 20 years, with an average rate of 0.6 with extremes in the United Kingdom with 1.1 and Bulgaria with 0.1 (Peterson et al., 2019). The serogroup distribution is quite heterogeneous in European countries, with the presence of very diverse vaccination schedules being one of the factors potentially associated with this diversity: serogroup W has increased in a good number of countries, although the most affected have been the United Kingdom and the Netherlands (Krone et al., 2019); around the year 2000, a good number of countries were affected by an epidemic outbreak of serogroup C cc11 strains, while others were not affected or were affected to a lesser extent (Borrow et al., 2013). Nevertheless, serogroup B is still predominant, although the circulating strains associated with clinical cases show some variations in different countries (Abad et al., 2016).

Europe has been the scene of a good number of interventions with vaccines against different serogroups, including the most recent protein vaccines against serogroup B (Davis et al., 2021).

Antibiotics resistance

Except in the case of sulfonamides, with meningococcus rapidly developing resistance, the bacterium has remained susceptible antimicrobials used both clinically and in prophylaxis in close contact of cases.

Attention to a decrease in penicillin susceptibility appeared in the 1980s (Sáez-Nieto et al., 1992) and rapidly this type of isolates was described in a large number of countries, although the level defined as “moderate resistance” remained at similar levels for a long time. However, strains have recently been described, especially isolated in the urogenital tract, in which the penA327 allele is present (Deghmane et al., 2017). This allele has been associated with a significant increase in Minimum Inhibitory Concentration (MIC) against beta-lactams, including 3rd generation cephalosporins. Although the MICs of these strains do not exceed the MIC that defines resistance (250/mL), they are already on the border of it and therefore surveillance should be reinforced to see what its evolution is.

On the other hand, the antibiotics used in prophylaxis of close contacts include both rifampin and ciprofloxacin. Resistance to rifampicin only appears sporadically and no expansion has been detected (Skoczynska et al., 2009). Resistance to ciprofloxacin is still relatively rare except in China, where it is associated with cc4821 strains (Zhao et al., 2020), the majority in the country, and in outbreaks associated with serogroup A in India (John et al., 2013). Although they were already described at the beginning of this century (Block and Vázquez, 2006), only recently an increase in reported cases has been noted, including ciprofloxacin resistant, beta-lactamase producing serogroup Y strains isolated in United States and El Salvador (McNamara et al., 2020; Marín et al., 2021). These strains contain a ROB-1 β -lactamase gene (blaROB-1) most probably acquired from *Haemophilus influenzae*.

Great efforts have been made to organize databases that include alleles associated with resistance genes in order to predict susceptibility in the absence of culture (an increasingly frequent situation in the case of *N. meningitidis*): *penA* gene for penicillin (Taha et al., 2007), *rpoB* gene for rifampicin (Taha et al., 2010) and *gyrA* gene in the case of ciprofloxacin (Hong et al., 2013). All alleles are hosted on the MLST website (Jolley et al., 2018) (<https://pubmlst.org/organisms/neisseria-spp>).

Vaccines

Fig. 1 shows a complete history of the development and authorization of vaccines against IMD that constitute a critical tool for the prevention of the disease.

Already in 1907 a vaccine against IMD was tested with whole cells of *N. meningitidis* killed with heat, although inconclusive results on its effectiveness in the following 30 years, together with the arrival of sulfa drugs, with which it could be prevented but also cured the disease, completely stopped this path of development (Hollingshead and Tang, 2019). In the 1960s Gotschlich et al.

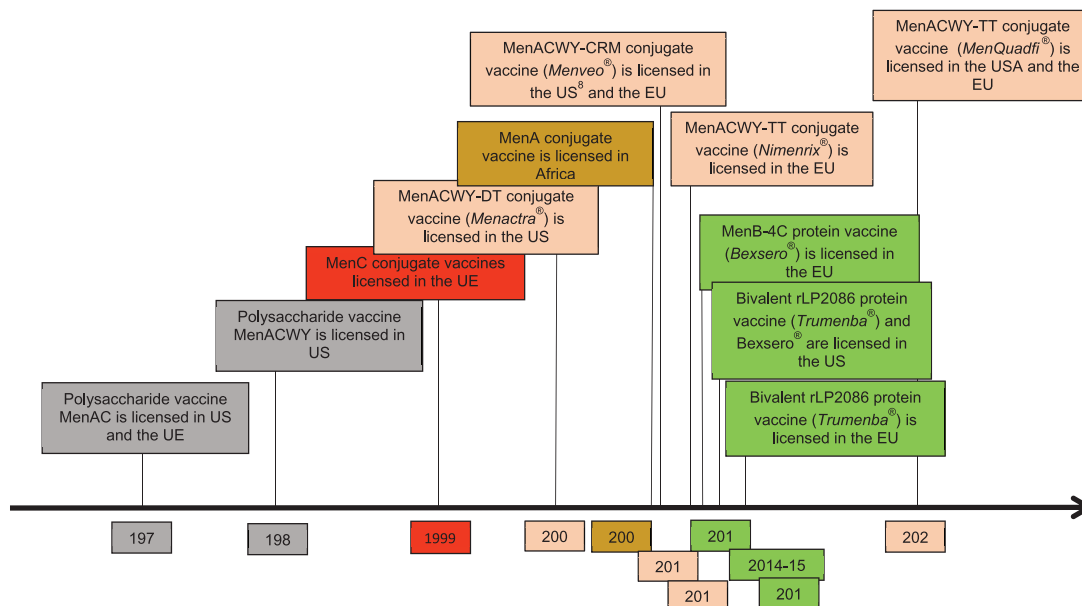


Fig. 1 History of meningococcal vaccines. Vaccines shown on timeline may not be approved/available in all countries and may not all remain available currently (Pizza et al., 2020; Piazza et al., 2021).

(1969) demonstrated the potential of some of the meningococcal capsular polysaccharides to induce a good immune response, which led to the development of monovalent purified polysaccharide vaccines against group C, bivalent against serogroups A and C and finally tetravalent against serogroups A, C, W and Y. However, these vaccines had a number of limitations, such as not being effective in children under 2 years of age, produce short lived, T-cell independent responses and not generate immune memory (Borrow et al., 2013).

The situation improved in the 1980s with the development of glycoconjugate vaccines, in which polysaccharide capsule was coupled to a carrier protein such as tetanus or diphtheria toxoid (Borrow et al., 2013). These conjugated vaccines activate long lived T-cell dependent responses, generating memory B cells, with the possibility to be used in children older than 2 months, generating immune memory effect and with a significance impact in carriers. The first conjugate vaccines were monovalent vaccines against serogroup C, and they were included in a good number of national vaccination schedules worldwide, following the expansion of serogroup C cc11 strains in the late 1990s (Tin Tin Htar et al., 2020).

However, the capsule corresponding to serogroup B has molecular mimicry with the envelope of human neuronal cells and is therefore poorly immunogenic (Freudenburg-de Graaf et al., 2020). Therefore, the development of vaccines against this serogroup has had to be based on an alternative strategy through the search for subcapsular antigens. Gram negative bacteria continuously form the so-called outer membrane vesicles (OMVs) which are small blisters excreted to the outside of the bacteria and which largely reflect the natural composition of the bacteria's own outer membrane (van der Pol et al., 2015). The discovery in the 1970s by Frasci et al. (1976) that the inoculation of meningococcal OMVs produced a protective antibody response was the beginning of what we might call the era of OMV tailor made vaccines. Following this strategy, strain-specific vaccines were developed in Norway, Cuba, Brazil, Chile, to control clonal MenB outbreaks in the 1980s, and also in New Zealand from 2004 to 2008 (Acevedo et al., 2019). However, OMV vaccination only provide protection when the strain associated with an IMD outbreak shares identical or very similar PorA to that included in the vaccine (Acevedo et al., 2019).

With the publication of the first meningococcal genome, but also with and a generalization of the use of genomics and proteomics methods, a more advanced stage is reached in the development of vaccines against serogroup B. Following the strategy of reverse vaccinology (Rappuoli 2000), several new antigens were identified: factor H-binding protein (fHbp), Neisserial adhesin A (NadA), and Neisseria Heparin-Binding Antigen (NHBA). The three antigens have been used to develop a multicomponent vaccine against serogroup B meningococci (Bexsero®) that additionally includes the OMV from the New Zealand vaccine, which expresses a PorA P1.4 (Borrow et al., 2017a). The vaccine has now been implemented in several national immunization programs with very promising data in the England experience with 75% of reduction in IMD cases in age groups eligible for vaccination (Ladhani et al., 2020a).

A second subcapsular vaccine, Trumenba®, containing two fHbp variants (Shirley and Dhillon, 2015) has been also approved for use in adolescents and young adults aged 10–25 years by the FDA in United States and by the EMA in Europe. It has been used in outbreaks control in the United States (Fiorito et al., 2018). There is not available information about effectiveness or impact in “real life.”

Interestingly, Bexsero® vaccine also confers protection against other serogroups (Biolchi et al., 2021), which has also been suggested for Trumenba (Harris et al., 2018).

It has recently been suggested that the use of Bexsero® may produce some level of cross protection against its close relative *Neisseria gonorrhoeae* (Leduc et al., 2020). More studies are necessary to elucidate this finding and to adequately characterize the antigen or antigens involved in this cross-reactivity, but this finding represents an added value in the design of vaccination programs with Bexsero®.

Neisseria gonorrhoeae

Neisseria gonorrhoeae, also known as gonococcus, is the other pathogenic species, together with the meningococcus, appearing in the genus *Neisseria* and it is the etiologic agent of the sexually transmitted disease, gonorrhea. The disease was initially described (or at least symptoms compatible with the clinical characteristics that we know today) 3500 years ago, although the etiological agent of the disease was not described by Albert Neisser until 1789 (Hill et al., 2016). It is a microorganism that has adapted very well to colonize its ecological niche in such a way that, being microaerophilic, it is able under certain conditions of growing under anaerobic conditions (Kellogg et al., 1963).

The disease

N. gonorrhoeae is the etiological agent of a sexually transmitted disease known as gonorrhea, although it can also cause some related pathologies. The gonococcus is able of colonizing a wide range of mucosal surfaces, including the urethra, endocervix, pharynx, conjunctiva, and rectum (Kirkcaldy et al., 2019), being mainly associated with urethritis in men and endocervicitis in women. The disease is asymptomatic in less than 10% of the males infected but most of 50% of women will not develop apparent symptoms (Unemo and Shafer, 2014). Rectal and pharyngeal infections are mostly asymptomatic and usually mainly associated with sexual practices that favor the colonization of these locations. In 2012, the World Health Organization (WHO) estimated that there were 78 million new cases among adults worldwide (Wi et al., 2017).

When the infection is not detected and therefore is not treated, or is not adequately treated, the bacteria can ascend to the upper genital tract and produce severe pathologies such as endometritis, pelvic inflammatory disease, penile edema, and epididymitis, resulting in infertility or ectopic pregnancy these complications affecting mainly women. Conjunctivitis in adults is rarely seen and it is more frequent in the newborn as ophthalmia neonatorum. One form of invasive disease is disseminated gonococcal infection, although it is currently very rare (Bignell and Unemo, 2013). Gonococcal infection has been associated with increased risk for other Sexually Transmitted Infections (STIs) including HIV (Unemo and Ison, 2013).

Detection of the presence of the microorganism in clinical samples: Microbiological diagnosis

Microscopy of clinical samples using either Gram stain or methylene blue stain can be a good diagnostic alternative in laboratories with very limited resources. In symptomatic men, microscopy has a sensitivity of 95% and a specificity of 97%. In endocervical samples, the sensitivity is between 40% and 60%, and in asymptomatic patients or in oropharyngeal and rectal samples, the sensitivity decreases so much that its use is not recommended (Meyer and Buder, 2020).

Culture in a selective medium has been the gold standard and is currently the only diagnostic strategy that allows determining antimicrobial sensitivity, which in the case of *N. gonorrhoeae* acquires great relevance. Its sensitivity in urethral or cervical samples ranges between 85% and 95% and the specificity reaches 100% when the identification of the strains obtained by culture is carried out. However in samples from other locations (conjunctival, rectal, and oropharyngeal samples) these high sensitivity values are not obtained (Meyer and Buder, 2020).

The gonococcus is one of the so-called fastidious microorganisms and because they do not tolerate dehydration, samples have to be inoculated immediately after swab collection onto selective culture media (Unemo and Ison, 2013). To confirm the identification, biochemical (based in enzymatic and metabolic reactions), immunological (coagglutination tests with gonococcal-specific monoclonal antibodies), spectrometric (MALDI-TOF to identify bacteria taken directly from the agar plate), or molecular test (based in Nucleic Acid Amplification Tests (NAATs)) are applied (Meyer and Buder, 2020).

NAATS, based on polymerase chain reaction (PCR) or isothermal transcription-mediated amplification, are the most sensitive and specific method for the detection of *N. gonorrhoeae* in clinical samples, and can be used from swabs collected by the same patient or first-catch urine, often constituting the method of choice for the diagnosis of gonococcus. Its application does not require the presence of viable microorganisms, in most cases they allow automation and consume less time than culture. Additionally, many of those that have been developed allow the simultaneous detection of *N. gonorrhoeae* and other microorganisms associated with Sexually Transmitted Infections (STIs) (Unemo et al., 2019). The main disadvantage of these methods is that they do not allow the analysis of antimicrobial sensitivity, which is critical in the case of *N. gonorrhoeae*, and molecular methods have not been developed for its determination in the absence of culture (Meyer and Buder, 2020).

On the other hand, although NAATs are considered as the diagnostic method of choice globally, a good number of low-income countries have difficult access to this methodology due to its relatively high price, which means that in many of them the diagnosis is based in clinical symptoms and the use of syndromic treatments, which facilitates the risk of development and spread of resistance due to the use of inappropriate treatments (Meyer and Buder, 2020).

Bacteria structure

Capsule

Although gonococcus shares some of the DNA regions involved in the production and transport of capsular polysaccharide in meningococcus, there are other regions that are essential for the synthesis and expression of the polysaccharide that are absent in *N. gonorrhoeae* (Petering et al., 1996). This opens up two possible explanations: one would be the loss of capacity for expression of the capsule by the gonococcus in the course of its evolutionary process of divergence with the meningococcus, and another would be the acquisition by the meningococcus of the ability to express said structure. Although there are not enough data to affirm one thing or the other, it does seem clear that the presence of capsules is not necessary for colonization in mucosa (niche of *N. gonorrhoeae* but also of many non-capsulated strains of *N. meningitidis*) and both its loss and its non-gain, it adapts well to a potential evolutionary process of gonococcus.

Outer membrane

The gonococcal outer membrane contains the most important structures in the colonization and pathogenesis of the microorganism. Bacterial structures located in the OM being involved in attachment and invasion include lipooligosaccharide (LOS), porin, pili (assembled from pilin protein) and opacity associated proteins (Opa proteins) (Unemo et al., 2019).

As with meningococcus, gonococcal LOS has three oligosaccharide (OS) chains, attached to a lipid A core and represent 50% of the mass of the outer membrane of the bacteria, and the number of branches and the length of OSs in each branch vary among gonococcal strains and, indeed, in the same strain during growth in vitro and in vivo (Gulati et al., 2019).

Regarding porins, both gonococcus and meningococcus seem to be the only two species of the genus *Neisseria* that have genes that code for two different types: *porB* and *porA*. However, in the case of gonococcus, the gene *porA* is present but is not expressed due to frameshift and promoter mutations (Derrick et al., 1999). The porin Por is encoded by a single copy of the *porB* gene. There are two major variants of porin protein in *N. gonorrhoeae*, PorB-1A and PorB-1B allowing the classification of the strains in serotypes 1A and 1B, being further classified into serovars through a panel of monoclonal antibodies (Knapp et al., 1984). PorB-1A is often associated with strains causing disseminated gonococcal infection (DGI), being more resistant to complement (C)-dependent killing by normal human serum, while PorB-1B are associated with local gonococcal infection (Shaughnessy et al., 2019). Although gonococcal strains show antigenic differences from PorB, which are the result of mutations or recombination events, the variability is not high. While Gonococcal strains show antigenic differences PorB, which are the result of mutations or recombination events, the variability is not high. Although it was a vaccine antigen candidate a few years ago, the data do not seem to indicate that the antibodies generated are protective in experimental vaccines (Shaughnessy et al., 2019).

Proteins associated with colony opacity (Opa) are a family of proteins that mediate adhesion and invasion of gonococci in human cells (Dehio et al., 1998). Opa proteins play an important role in the pathogenesis of the microorganism, and it has been seen that Opa + gonococci are associated with localized infection (urogenital, cervical and rectal) while Opa-strains are generally isolated from disseminated infection (Draper et al., 1980). Gonococci have a wide repertoire of up to 11 different opa genes coding for distinct opacity proteins which are distinguishable at their variable domains (Bhat et al., 1991). The expression of each gene is governed by independent events that turn on or off the expression of each opa gene. A single strain may express none of the Opa proteins, a single Opa, or a combination of several (Unemo et al., 2019).

Pili are filamentous structures composed of the main subunit (pilin, PilE) with an adhesion associated protein (PilC) located both at the end of the pilus and within the bacterial membrane (Shaughnessy et al., 2019). Gonococcal pili are essential for a correct adhesion to mucous epithelial cells, and they also intervene in self-adhesion and adherence functions with other gonococci, protection from PMNL killing mechanisms, and horizontal genetic transfer (HGT) by DNA transformation (Stohl et al., 2013; Unemo et al., 2019). Their expression seems to be linked to strong selective processes throughout the infection, since all clinical isolates present pili, but they quickly stop expressing them in subcultures in the laboratory (Unemo et al., 2019). The PilE protein is made up of around 160 amino acids and has a highly conserved N-terminal fraction, while the C-terminal is extremely variable (Schoolnik et al., 1984). On the other hand, PilC seems to have a determining role in the entire adhesion process, so its expression would be decisive for it to occur successfully (Shaughnessy et al., 2019).

Other bacterial structures present in OM and of great importance in the pathogenesis of gonococcus are the so-called binding iron-containing host proteins such as transferrin, lactoferrin and hemoglobin. Iron is essential for the growth of the bacteria, and the bacteria obtain it from their host through an acquisition system for transferrin (TbpA–TbpB), one for lactoferrin (LbpA–LbpB) and one for heme (HpuA–HpuB), which can be found, for example, in hemoglobin (Rohde and Dyer, 2003). These systems are known as TonB-dependent Transporters (TdT). The uptake and transport of iron from the outside to the cytoplasm of the bacteria is generally a complex system, which is shared with *N. meningitidis* and other Gram-bacteria (Noinaj et al., 2010).

Finally, other important components of *N. gonorrhoeae* OM are flow pump systems. Gonococcus expresses up to a total of five different systems (MtrC–MtrD–MtrE, MacA–MacB–MtrE, NorM, FarA–FarB–MtrE and MtrF64–66). Some of these flow pump systems are shared with *N. meningitidis*, although the regulatory mechanisms of expression are different in both species, they are different and their role in the pathogenesis and production of antimicrobial resistance could also be different (Shafer et al., 2001; Enríquez et al., 2010).

Genetic of *Neisseria gonorrhoeae*

The gonococcus is an extremely competent bacterium to acquire DNA from other bacteria via horizontal gene transfer (HGT), which translates into a great facility for the acquisition of antimicrobial resistance mechanisms, etc. The transfer of genetic material is mediated by pili, and particularly by type IV-pili. This ability extraordinarily complicates the study of clones due to the instability of the alleles, and suggests that the presence of mixed infections could be more frequent than reported, and that their frequency could be underestimated (Unemo et al., 2019).

Unlike meningococcus, almost all gonococcal strains have a small cryptic plasmid (of unknown function), and the presence of plasmids that code for the production of penicillinase (mostly TEM-1 or TEM-135 β -lactamase), as well as conjugative plasmids that sometimes carry the tetM determinant associated with high resistance to tetracycline. These plasmids have been classified according to a nomenclature based on their place of origin, although at present this classification is rarely little used (Cehovin et al., 2020).

Gonococcus shows high genetic plasticity and even the same isolate can give rise to different antigenic identities after repeated subcultures in the laboratory (Russell et al., 2019). Most of its main surface antigens continually change their sequence and/or use phase variation mechanisms for ON and OFF switching of gene expression. Thus, for example, type IV pili are encoded by pilE genes, and each strain has another set of pilE genes that lack a promoter (pilS) so they are silent genes, which represent a large repository of sequences that can be randomly shuffled to generate new antigenic variants through intra-chromosomal recombination (Haas and Meyer, 1986). This is possible thanks to the addition or loss of a pentanucleotide repeat sequence in the structural gene encoding the amino-terminal leader peptide (Stern and Meyer, 1987).

Typing gonococci

Some of the phenotypic characterization methods that were widely used in the past, such as plasmid profile, auxotyping (profiles of nutritional requirements) and classification in serovars, have fallen into disuse since they did not offer detailed and precise information on the evolution of the clones of isolates with antimicrobial resistance and their level of discrimination could be improved. Molecular typing schemes have very quickly replaced those old traditional phenotypic typing methods due to their greater resolution power, reproducibility and portability and power of analysis of inter-relationships between strains (Unemo and Dillon, 2011).

In the case of *N. gonorrhoeae*, the most commonly used molecular typing schemes have been MLST (Maiden et al., 1998) and *N. gonorrhoeae* Multi-Antigen Sequence Typing (NG-MAST) (Martin et al., 2004). The latter has a significantly higher discriminatory power than the former since, even though it is based on the analysis of the sequence of only two loci (*porB* and *tbpB*), these encode highly variable proteins of the outer membrane exposed to the surface.

However, lower prices both in reagents and equipments and simplifying them have allowed WGS replace these molecular methods in many laboratories, which will improve not only the usual parameters of accuracy and discrimination, allowing to analyze with great precision the appearance and spread of resistance mechanisms (Golparian et al., 2021).

Gonococcal epidemiology

In 2016 the WHO estimated that there would be 86.9 million cases of gonorrhea (global prevalence 0.9%). In the epidemiology of the disease there are a series of determinants such as sexual orientation, socioeconomic, demographic and geographic status, access and quality of sexual education, prevention, tests and diagnoses, as well as the provision of health services (Unemo et al., 2019).

In the United States, the number of gonorrhea cases increased by almost 70% from 2013 ($n = 333,510$) to 2017 ($n = 556,413$) and the proportion of cases in men who have sex with men (MSM) has gone from 4% in 1989 to 38.5% in 2017, which probably reflects, among other factors, a significant improvement in the collection of information on sexual orientation in the United States. In the United States in 2017, the rate of reported cases of gonorrhea was much higher among black populations, American Indians and Alaskan Natives, Native Hawaiians and individuals with Hispanic origin compared with white populations whereas the rate among individuals with Asian origin was half the rate among white individuals (Unemo et al., 2019).

In the EU/EEA, the number of reported cases has increased since 2008, from 29,434 cases to 89,239 cases in 2017, being the United Kingdom, France, the Netherlands and Spain the most affected countries. MSM represented 25–30% of all the cases and it is 57% of the cases reporting sexual orientation, perhaps reflecting that information on sexual orientation in Europe still has a wide room for improvement (Unemo et al., 2019).

Although with many limitations, the WHO has estimated the prevalence of gonorrhea globally and it is the African Region that would have the highest prevalence, followed by the Region of the Americas, the Southeast Asia Region, and the European Region (Rowley et al., 2019), with low-income countries, territories and areas showing the highest prevalence of gonorrhea.

Antibiotics resistance

Gonococcus has been able to develop resistance to all antibiotics used in the first line of treatment over the last 80 years. Gonococcal antimicrobial resistance (AMR), particularly resistance to the only remaining currently recommended antimicrobials, is of grave concern. This is particularly relevant in MSM, where the existence of sexual networks is very frequent, which greatly facilitate the dissemination of these resistances (Kwong et al., 2018).

The evolution of resistance to different antimicrobials used in the treatment of the disease has been extraordinarily rapid, and there is currently a real risk that gonorrhea will become a disease without an antibiotic alternative of choice. Dual antimicrobial therapy (mainly parenteral ceftriaxone plus oral azithromycin) is currently recommended for empirical first-line therapy by the WHO global guidelines but also it is the recommended regimen in most high-income countries (Unemo et al., 2019); however, some other high-income countries like Japan and the United Kingdom are inclined towards the use of monotherapy with ceftriaxone high dose (1 g).

A wide battery of mechanisms responsible for AMR have been described (Table 1), including point mutations, small insertions and deletions in the bacterial chromosome, both in the genes involved and in their regulatory regions. Furthermore, the acquisition of new genetic material on a larger scale, via recombination or through plasmids can also generate AMR.

However, the prediction of resistance from genomic data has not always been possible. It is well known that mosaic structures of the *penA* gene are associated with a lower susceptibility to extended spectrum cephalosporins (ESCs). However, not all mosaic structures explain all cases of resistance to ESCs, and some mosaic alleles of *penA* do not cause a decrease in susceptibility or resistance to these antibiotics. Other factors such as overexpression of the MtrCDE flow pump, mutations in the *poB1b* gene, mutations in the *rpoB* and *rpoD* genes (which code for RNA polymerase subunits) have also been associated with a lower susceptibility to ESCs. Mutations in the 23S rRNA gene (A2059G and C2611T in *Escherichia coli*) are associated with high resistance to azithromycin, as are variants in *mtrR* or its promoter that increase the expression of the MtrCDE efflux pump. Mutations in *rplD*

Table 1 Mechanisms and main determinants of gonococcal antimicrobial resistance.

Sulphonamides	<i>folP</i> gene region (Qvarnstrom and Swedberg, 2006)
Penicillin G	<i>penA</i> mutations producing four -eightfold MIC increases: ✓ Insertion of asparagine: 345 Asp345A (Dowson et al., 1989) ✓ Mosaic <i>penA</i> alleles encoding mosaic PBP2 (A311V, I312M, V316T, etc.) which reduce acylation rate (Unemo et al., 2019). <i>penB</i> (<i>porB1b</i>) mutations (G120K/G120D and A121D) (Olesky et al., 2006). <i>penC</i> (<i>pilQ</i> gene) mutation (E666K) reducing penicillin entry (<i>penB</i> and <i>mtrR</i> mutations are needed for being effective) (Zhao et al., 2005). <i>ponA</i> mutations (L421P in PBP1) (Ropp et al., 2002). Deletion or insertions in the <i>mtrR</i> promoter or in the <i>mtrR</i> gene with an increased expression of the <i>MtrC-MtrD-MtrE</i> efflux pump (Lewis, 2010). Production of plasmid-encoded beta-lactamases (<i>bla</i>_{TEM1} or <i>bla</i>_{TEM135}). Penicillinase-producing plasmids include the Asia, Africa, Toronto, Rio, Nîmes and New Zealand plasmids (Pagotto et al., 2000)
Tetracyclines	<i>rpsJ</i> mutation (V57M) giving ribosomal protection (Hu et al., 2005). <i>penB</i> mutations (G120D and A121D in <i>PorB1b</i>). (Warner et al., 1980). <i>penC</i> (<i>pilQ2</i>) mutation (E666K) reducing tetracycline entry (<i>penB</i> and <i>mtrR</i> mutations are required for the <i>penC</i> mutation effect) (Zhao et al., 2005). Deletion or insertions in the <i>mtrR</i> promoter or in the <i>mtrR</i> gene with an increased expression of the <i>MtrC-MtrD-MtrE</i> efflux pump (Lewis, 2010). Plasmid-mediated tetracycline-resistant N gonorrhoeae (TRNG): the streptococcal tetM determinant responsible for tetracycline resistance is located in a 25.2 MDa plasmid as result of insertion in the gonococcal conjugative plasmid (Morse et al., 1986).
Spectinomycin	16S rRNA (C1192U) reducing the affinity to ribosomal target (Galimand et al., 2000). <i>rpsE</i> mutations (encoding the 30S ribosomal protein S5) (Ilin et al., 2013).
Azithromycin	Mutations in the peptidyltransferase loop in domain V of 23S rRNA alleles (Galarza et al., 2010). Overexpression of the MtrCDE efflux pump associated with mutations in either the <i>mtrR</i> gene or the <i>mtrR</i> promoter (Unemo and Shafer, 2014; Zarantonelli et al., 2001).
Quinolones	<i>gyrA</i> mutations (for example, S91F or D95N or D95G) (Shultz et al., 2001). <i>parC</i> mutations (for example, D86N, S88P and E91K) (Shultz et al., 2001). Overexpression of NorM efflux pump, resulting in decrease susceptibility (Rouquette-Loughlin et al., 2003).
Cephalosporins	Mosaic genes: <i>penA</i> mutations G545S, I312M and V316T mutations are the key for PBP2 with reduced affinity for cephalosporins (Takahata et al., 2006). Non-mosaic: For example A501V or A501T produce increases in the cephalosporins MICs in the order of four times higher (Takahata et al., 2006). Deletion in the <i>mtrR</i> promoter, alterations in amino acid residues 101 and 102 in putative loop 3 of <i>PorB1b</i>, <i>ponA1</i> polymorphism resulting in the alteration L421P have been also associated with reduce susceptibility working with synergy between them (Ohnishi et al., 2011). <i>pena</i> mosaic genes with seven specific alterations associated with high level of resistance (Singh et al., 2020).

have also been associated with decreased susceptibility to this antibiotic, and, conversely, loss-of-function mutations in *mtrC* have been associated with increased susceptibility to several antibiotics, including azithromycin (Sánchez-Busó et al., 2021).

In this sense, WGS is a very effective tool to be able to predict AMR in gonococcus with great precisión (Eyre et al., 2017; Unemo and Shafer, 2014). With WGS, multiple AMR genetic mechanisms, as well as regions of virulence, can be simultaneously analyzed and typed, using the appropriate bioinformatics tools. Compared with traditional epidemiology, it presents a significant improvement in resolution and precision, which allows a comparison of the entire genome of the strains to be analyzed, identifying RAM clones, outbreaks, transmission networks, national and international extension, new resistance mechanisms as well as acquaintances etc. The future in this field involves the organization of platforms that are easy to use by laboratories and in which the comparison of results is accessible and reliable (Sánchez-Busó et al., 2021).

Vaccines

The idea of having an effective vaccine against *N. gonorrhoeae* has acquired great importance in recent years because it would be a very useful tool to stop the advance of antimicrobial resistance and that the infection does not become a disease with little or no treatment option. However, despite the efforts made by different researchers in recent decades, the only slightly optimistic news is that already mentioned for *N. meningitidis* of a certain level of protection against gonorrhea when the 4-component vaccine (Bexsero®) is used in young population and adults (Leduc et al., 2020). The absence of a specific vaccine against gonococcus is explained by a combination of different factors that have made its design and development very complicated up to now. In the first place, gonococcal infection does not confer protection against other re-infections, which does not allow us to define correlates of protection. Second, only recently have animal models of infection been developed (gonococcus is an exclusively human pathogen) in genetically modified female mice (Jerse, 1999).

The absence of a specific vaccine against gonococcus is explained by a combination of different factors that have made its design and development very complicated up to now. First, gonococcal infection does not confer protection against other re-infections, which does not allow the definition of protection correlates. Second, only recently have animal models of infection been developed (gonococcus is an exclusively human pathogen) in genetically modified female mice (Jerse, 1999) and thirdly, and probably as the most relevant factor is the extraordinary antigenic variability that the gonococcus has, with most of those expressed on surfaces being genetically regulated through various mechanisms as has already been commented previously (Russell et al., 2019). And in this regard, *N. gonorrhoeae* has no rival, being able to express different variants of the same antigen in a single strain during passage in the laboratory.

The potential development of an effective vaccine against gonococcus has had a strong push in recent years, the most promising strategies being the use of the mimetic peptide 2C7 (Gulati et al., 2013), use of gonococcal OMV administered vaginally with microencapsulated IL-12 (Liu et al., 2017), and the use of recombinant refolded porin (Zhu et al., 2011).

Both, gonococci and meningococci, constitute pathogenic bacteria of high interest in Public Health and both share a good number of antigens and genetic information (between 80% and 90% homology). However, their adaptive process has followed different paths, probably because they have inhabited different ecological niches with different requirements (Vázquez et al., 1993). However, now it is increasingly common to find gonococcus colonizing extragenital areas such as the nasopharyngeal mucosa and the anal mucosa (Foschi et al., 2020) as well as isolates of *N. meningitidis* in genital and anal mucosa (Retchless et al., 2018; Ladhani et al., 2020b), even in the case of meningococcus, adapting genetically to a more efficient colonization of these new habitats (Seifert (2019); Retchless et al., 2018). This situation produces greater opportunities for genetic exchange between both species, therefore future developments must be closely monitored.

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Corynebacterium diphtheriae

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Corynebacterium diphtheriae

Introduction

The genus *Corynebacterium* is located within the order Actinomycetales and has 88 species, of which *C. diphtheriae* is the most important species from the clinical point of view. It is a Gram-positive or Gram-variable, nonmotile, aerobic rod that does not present a capsule or develop spores. In the Gram stain, it usually presents a characteristic distribution that looks like Chinese letters. If stained with methylene blue, purple, or reddish polyphosphate granules can be seen at the ends of the rod (Murphy, 1996).

The solid medium most used for its culture is Tinsdale agar, a selective and differential medium developed in 1947 based on Loeffler medium (Moore and Parsons, 1958). It is a medium that contains potassium tellurite, which inhibits the growth of most of the microbiota of the upper respiratory tract and, by producing SH₂, forms a dark halo around the colony. This halo is observed in different species of *Corynebacterium* sp., which can be differentiated by mass spectrometry or biochemical techniques such as urease production, as described later.

Within the species *C. diphtheriae*, there are four biotypes (*belfanti*, *gravis*, *intermedius*, and *mitis*) that can be distinguished according to the morphology of their colonies, the presence or absence of hemolysis, different biochemical reactions, and by sequencing the 16S ribosomal RNA (Khamis et al., 2005). These biotypes are clinically indistinguishable, so their identification is due to epidemiological and outbreaks control reasons.

C. diphtheriae may carry the *tox* gene, which encodes the diphtheria-causing toxin (Holmes, 2000). This gene, which is transferable to non-toxigenic strains through a lysogenic cycle and site-specific recombination, is repressed by the DtxR protein, which is inactivated in low iron media. The activity of the toxin will be described in the Pathogenesis section. Other species that can carry the *tox* gene are *C. ulcerans* and *C. pseudotuberculosis*.

Historical background

The French physician Pierre Bretonneau observed in the cities Chenonceaux and Tours during several epidemics in the early 19th century that many patients suffered from a hitherto unknown variety of pharyngeal infections. This disease was associated with the development of an observable pseudomembrane in the throat of the patients. For this reason, he named this disease “diphtheria,” which means “made of leather” (English, 1985).

The first isolation of the bacterium was made by Klebs in 1883 from a pseudomembrane, and Loeffler identified it as the etiological agent of the infection in 1884. The existence of exotoxin was first described in 1888 by Roux and Yersin and von Behring and Kitasato developed antitoxin as a treatment (Behring and Kitasato, 1890).

The first vaccine was developed in 1923 by the French veterinarian Gaston Ramon. The development of vaccines and their massive use led to a 99% decrease in incidence in countries such as the USA (Roush and Murphy, 2007).

Epidemiology

Diphtheria mainly affects the pediatric population and immunocompromised adults and was one of the main causes of infant mortality in the prevaccine era. Cutaneous infection, however, has not been diminished by the action of the vaccine, and data on outbreaks in impoverished and resource-limited communities are regularly recorded.

Due to the introduction of the vaccine, the incidence rate of respiratory infection has decreased globally by more than 90% between 1980 and 2000 (Van Panhuis et al., 2013). Despite this, outbreaks continue to occur in environments with limited resources and low vaccination coverage. Currently, the highest incidence data is registered in India, where 50% of the 16,611 cases registered in 2018 by the WHO occurred, followed by Yemen, Nigeria, and Indonesia (WHO (World Health Organization), 2018a). In Europe, the ECDC recorded 63 infections with *C. diphtheriae* or *C. ulcerans* in 2018 (ECDC, 2021), of which only two were respiratory infections. In the United States, the CDC reported 14 cases of diphtheria between 1996 and 2018 (Acosta et al., 2020).

The mortality of diphtheria is 5–17% in the unvaccinated population, even in immunocompetent individuals (Wagner et al., 2012). Diphtheria is currently a notifiable disease. According to WHO data, vaccination coverage was 79% in 2000 and 88% in 2010. The WHO objective is to reach a minimum of 90%, a number that was reached in 2018 in practically the entire northern hemisphere and Australia and New Zealand. The countries of South America, Central and South Africa, as well as countries such as Pakistan, Yemen, the Philippines, Indonesia, and Ukraine, present vaccination coverage of 50–90% (WHO (World Health Organization), 2018b).

Pathogenesis

C. diphtheriae produces a toxin that is the main responsible for the clinical manifestations, with a lethal dose of 100 ng/kg (Gill, 1982). It is an exotoxin made up of two fragments, A and B, of which the second binds to the receptor. Fragment A is composed of the catalytic domain (C domain) and fragment B is divided into two domains: the host cell-binding domain (R domain) and a transmembrane domain (T domain). Fragment A catalyzes the hydrolysis of nicotinamide adenosine dinucleotide (NAD), transferring ADP-ribose to elongation factor 2, which supposes its inhibition. Elongation factor 2 is a protein responsible for coordinating transfer RNA with messenger RNA and ribosome during protein synthesis (Collier, 1975).

Other virulence factors are neuraminidase and trans-sialidase proteins, involved in binding to the host cell. Other proteins that facilitate adherence to epithelial cells of the respiratory tract are the Spa proteins, divided into SpaA–SpaI, adhesins and hemagglutinins (Ton-That and Schneewind, 2003).

Clinical manifestations

Respiratory infection

The incubation period is 2–5 days (WHO (World Health Organization), 2017a,b). Transmission normally occurs by air, although it can also occur through direct contact or fomites. The first symptoms of the infection at the pharyngeal level are nonspecific: sore throat, malaise and low fever. About 70% of patients develop a pseudomembrane in the tonsils in the following days. It can extend into the nasopharynx, larynx, and bronchi or into the nasopharynx and soft palate. Its appearance is cerulean at the beginning and it turns greenish, gray and black. Sometimes this condition is accompanied by swelling of the lymph node and submandibular edema, evolving to a “bull neck” appearance. The main complications of respiratory infection are caused by airflow obstruction, which represents a 10% mortality rate (Dobie and Tobey, 1979).

Systemic infection

There are complications derived from the circulation of diphtheria toxin through the bloodstream, the most common being myocarditis and peripheral neuropathy. Myocardial dysfunction can be accompanied by both bradyarrhythmias and tachyarrhythmias and is seen in 60–70% of deaths during the acute phase of diphtheria (Jayashree et al., 2006). Neuropathy is an autoimmune sequela due to the similarity between the B subunit of the toxin and the extracellular domain of the epidermal growth factor receptor (EGFR). Neurological involvement usually manifests through muscular paralysis or alteration of the facial nerves, which can evolve into bulbar palsy (Hadfield et al., 2000).

Cutaneous infection

Skin infection is usually caused by non-toxigenic strains. Transmission can be through direct contact with the skin or fomites. The most common symptom is an ulcer lesion with possible pseudomembrane formation. Coinfections with other bacteria have also been described, mainly *Staphylococcus aureus* and *Streptococcus pyogenes* (de Benoist et al., 2004).

Diagnosis

For the diagnosis, it is essential to know the epidemiological data, as well as the vaccination status. Differential diagnosis should be made by ruling out other infections with similar symptoms: streptococcal or viral pharyngitis, mononucleosis, oropharyngeal candidiasis, oral syphilis, Vincent angina. . . If there is a suspicion of acute infection, contact precautions should be taken in case of a cutaneous syndrome and, in addition, droplet precautions in a respiratory profile.

Samples indicated for culture are pharyngeal or tonsillar exudates and skin lesions. The sample must be collected with a sterile cotton swab, which will be placed in a tube with a transport medium (Stuart-Amies, Cary-Blair or similar). The sample should be stored at room temperature and processed as soon as possible. Direct Gram staining will be performed and it will be plated on sheep blood agar, and on culture media with tellurite (Tinsdale or modified Loeffler), which inhibits the flora of the upper respiratory tract and also differentiates the *Corynebacterium* sp. colonies with a brown halo. As discussed in the introduction, in the Gram stain, the bacterium appears as a Gram-positive rod-shaped bacillus, usually forming groups in the form of Chinese characters. The culture media will be incubated for 48 h in a 5% CO₂ atmosphere at 37 °C (SEIMC (Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica) et al., 2006).

C. diphtheriae can be differentiated biochemically from *C. ulcerans* and *C. pseudotuberculosis* by the production of urease, which is negative in *C. diphtheriae*, or by the nitrate test, which is positive only for *C. diphtheriae*. However, the latter is not usually used since it is also negative for *C. diphtheriae* serogroup *belfanti*. These three species can also be differentiated by mass spectrometry (MALDI-TOF), with very high levels of identification (Konrad et al., 2010).

In order to determine if the strain is toxigenic, there are several techniques available. Traditionally, a modification of the immunodiffusion test developed by Elek (1949) has been used. This test is based on using a known toxigenic strain as a positive control, a non-toxigenic strain as a negative control, and the test sample. The three strains are plated on an agar medium with a diphtheria antitoxin disk (10 IU) in the center. At 24 h of incubation, a precipitate line is observed in the junction area between the toxin of the test strain and the antitoxin of the disk. This technique has become obsolete due to the appearance of molecular techniques. These allow the *tox* and *rpoB* genes to be detected by real-time PCR, the latter specific for *C. diphtheriae* (Khamis et al., 2005). Enzyme immunoassays can also be performed to detect exotoxin fragment A, although it is a rarely used technique (Engler and Efstratiou, 2000).

Therapy

The main treatment for respiratory diphtheria is the use of antitoxin. Its usual dose is 20,000–40,000 U (up to 120,000 U in severe or advanced cases) and is administered intravenously or intramuscularly. Additionally, the use of antibiotics can prevent toxin production and reduce transmission. CDC guidelines advise 14 days of oral or parenteral erythromycin at a dose of 40 mg/kg/day or intramuscular penicillin G (600,000 U twice daily; at half dose in children <10 kg) (CDC (Centers for Disease Control and Prevention), 2021).

In the management of respiratory diphtheria, it is also essential to ensure airflow. For this, endotracheal intubation or tracheostomy can be used in severe cases.

Cutaneous diphtheria is treated only with antibiotics; the use of antitoxin is not useful because most cases are caused by non-toxigenic strains. The usual treatment is a beta-lactam alone or in combination with an aminoglycoside. Vancomycin can also be used as a substitute for beta-lactam (Zou et al., 2020). However, there is little information on the treatment of this type of diphtheria; therefore, there are no specific recommendations on dosage or duration of treatment.

Prophylaxis

The main prophylaxis is vaccination, which is 97% effective in preventing the development of an infection by a toxigenic strain (CDC (Centers for Disease Control and Prevention), 2021). Four doses (three in adults) are necessary to reach the level of 0.1 IU/mL of antitoxin considered protective against infection (Galazka and Roberson, 1996). A British study showed that, in the absence of booster doses, the protection of the vaccine in those over 60 was reduced by 50% (Myers et al., 1982).

The vaccine has been administered since 1948 in combination with tetanus toxoid and acellular remains of *B. pertussis* (DTPa). According to the vaccination schedule, three doses should be administered between 4 and 8 weeks from 2 months of age and up to 1 year of life. In Spain, the vaccination calendar establishes the doses at 2, 4 and 11 months. Subsequently, a fourth dose will be administered at 6 years of age and a fifth at 14 years with possible successive doses every 10 years. In patients who have acquired the infection, vaccination is also recommended as natural immunity may not reach protective levels. In pregnant women, a dose of DTaP is recommended at 27 weeks of gestation (SNS (Sistema Nacional de Salud), 2021).

In the case of a patient with a respiratory infection, precautionary measures should be instituted to avoid droplet transmission. These should be maintained until two cultures of samples taken in consecutive days are negative after the end of the 2 weeks of antibiotic therapy. If a person comes into contact with a patient with a respiratory infection, antibiotic prophylaxis will be administered regardless of their vaccination status. Prophylaxis can be performed with erythromycin (7–10 days) or a single intramuscular dose of benzathine penicillin G (CDC (Centers for Disease Control and Prevention), 2021).

Corynebacterium spp.

Coryneform bacteria are part of the skin microbiota of humans and some animals, as well as being distributed in the environment. They can also colonize surfaces and materials forming a biofilm, which is very important when it comes to medical or healthcare equipment. Due to its role in the microbiota, its finding in the microbiology laboratory is often interpreted as contamination without clinical significance. However, since the beginning of the century, there has been increasing evidence on the etiological role

of coryneform bacteria in some infections, especially in the nosocomial context and in immunocompromised patients (Funke and Bernard, 1999). The most frequent corynebacterial infections in the health care setting are infections associated with implantation of devices, catheter-related septicemia, endocarditis, peritonitis in dialysis patients, etc. On the other hand, some corynebacteria that have been described as recognized pathogens in animals have been associated with certain zoonoses in humans. These infections are usually acquired in the community and can present as skin, genitourinary, or oropharyngeal infections.

As general characteristics, corynebacteria are irregularly shaped, pleomorphic, non-spore-forming aerobic Gram-positive rods. As previously described, in the Gram stain they usually present a very characteristic Chinese letter-shaped distribution.

Fermentative corynebacteria: Nonlipophilic group

The classification of corynebacteria is based on their ability to ferment and to grow or not in the presence of lipids such as Tween 80. Fermentative corynebacteria can ferment sugars and produce acids. Non-fermentative corynebacteria make up a group that will not be treated due to their practically harmless role for humans, forming part of the respiratory microbiota.

Corynebacterium ulcerans and Corynebacterium pseudotuberculosis

These are two pathogenic bacteria in animals that can occasionally cause zoonotic infections in humans. Both can produce a toxin similar to that of *C. diphtheriae*.

C. ulcerans is associated with skin ulcers and pharyngitis, as are *C. diphtheriae* cutaneous infections. In fact, in some countries such as the United Kingdom, it has been postulated as a more common diphtheria agent than *C. diphtheriae* (Both et al., 2015). *C. ulcerans* is similar to *C. diphtheriae* therefore not only in clinical presentation but also in diagnostic and treatment methods. The difference is that human-to-human transmission is unclear and seems unlikely (Konrad et al., 2015).

C. pseudotuberculosis is a common pathogen of cattle and its infection in humans is rare and is related to farmers or veterinarians (Dorella et al., 2006). It causes the same condition as in animals, lymphadenitis, and it can also produce an infection similar to *C. diphtheriae*. In addition to the antibiotic treatment already mentioned, it may be necessary to remove the affected nodes.

Corynebacterium striatum

It is a ubiquitous bacterium, which is part of the skin microbiota in humans. For this reason, it has always been considered a contaminant; however, it has been described as the main pathogen in immunosuppressed patients, COPD patients or catheter carriers. This is related to its ability to produce biofilms, which usually means that the infections are mainly nosocomial. Although less frequently, its role as an etiological agent of infections in immunocompetent patients has also been described, producing septic arthritis, osteomyelitis, empyema and endocarditis, among others (Lee et al., 2018).

Resistance to penicillin, erythromycin, clindamycin and ciprofloxacin has been described. It is usually treated with vancomycin in hospitalized patients or oral daptomycin; however, resistance has also begun to be observed (McMullen et al., 2017).

Corynebacterium minutissimum

It is also a bacterium present on human skin, and for years it has been considered the cause of erythrasma; however, their role is controversial and in doubt. Erythrasma is a skin condition, especially in intertrigo folds: fingers, armpits and groin. It causes redness in the area and itching. The lesions acquire a characteristic coral-reddish color under the ultraviolet light of Wood lamp (Groves et al., 2021). Like *C. striatum*, it can cause endocarditis in immunosuppressed or catheter patients, pyelonephritis, and skin abscesses (Ahmad and Ahmad, 2005).

Corynebacterium glucuronolyticum

It is part of the microbiota of the genitourinary tract of animals and humans and can sometimes act as an opportunistic pathogen (Ruiz-Pino et al., 2019). It has traditionally been found in semen samples from men and is associated with non-gonococcal male urethritis and chronic prostatitis (Riegel et al., 1995). It is sensitive to beta-lactams and vancomycin.

Corynebacterium amycolatum

It is part of the skin microbiota of healthy individuals, and it is the non-lipophilic corynebacterium most frequently isolated in clinical samples (Bernard et al., 2002). It has been associated with catheter infections, nosocomial endocarditis, and septic arthritis. It is usually treated with vancomycin and daptomycin; it is also sensitive to beta-lactams, macrolides and quinolones (Dragomirescu et al., 2020).

Corynebacterium xerosis

Also present in the mucocutaneous microbiota of humans, it can be considered an opportunistic pathogen in immunocompromised patients. Although rare, endocarditis, septic arthritis, and osteomyelitis have been reported (Eliakim et al., 1983). Infections are also related to a surgical history.

Fermentative corynebacteria: Lipophilic group

This group of microorganisms is characterized by their slow and fastidious growth, with small non-hemolytic colonies (Funke and Bernard, 1999). Its growth is favored if lipids such as Tween 80 are added to the culture medium, which gives the group its name.

Corynebacterium urealyticum

This is another bacterium that usually colonizes the skin of hospitalized patients. It is related to urinary tract infections, such as encrusting cystitis, especially in patients with bladder catheters. The clinic is characterized by pyuria, leukocyturia, and struvite crystals in the urine (Costales et al., 2019).

Treatment is usually carried out with vancomycin since it has resistance to beta-lactams and variable sensitivity to quinolones and macrolides. In addition, the removal of incrustations in the bladder mucosa can be considered in cases of encrusting cystitis.

Corynebacterium jeikeium

It usually colonizes the skin of hospitalized patients and can cause infection in those with risk factors such as immunosuppression and the use of a catheter or prosthetic valves. The most usual symptoms are peritonitis, external otitis, osteomyelitis and postsurgical infections (Mookadam et al., 2006). It is a corynebacterium with high resistance to beta-lactams, aminoglycosides and macrolides, which is why it is usually treated with vancomycin or daptomycin.

Other corynebacteria

Arcanobacterium haemolyticum

Formerly named *Corynebacterium haemolyticum*, it is described as an etiological agent of pharyngitis and skin infections. It is a nonmotile, Gram-positive or Gram-variable, catalase-negative and non-spore-forming bacillus. On blood agar it produces weak β -hemolysis and does not grow well in tellurite-rich media, which differentiates it from *C. diphtheriae*.

Pharyngitis caused by *A. haemolyticum* is clinically indistinguishable from streptococcal pharyngitis and usually includes a scarlatiniform rash on the neck, trunk, and extremities. Together with skin infections, polymicrobial wound infections and cellulitis have been described. In addition to these infections, others that can occur less frequently are necrotizing fasciitis, osteomyelitis, and endocarditis (Vu and Rajnik, 2021).

Treatment can be with vancomycin, clindamycin, or doxycycline. It is sensitive to beta-lactams, but they show low activity in vivo, probably due to the difficulty of intracellular penetration.

Turicella otitidis

It is part of the microbiota of the ear canal, which is why it has been associated with otitis media. However, its implication is questioned since it is isolated from both patients and healthy individuals. It has been described as a cause of mastoiditis and atrial abscess (von Graevenitz and Funke, 2014). It is sensitive to beta-lactams, vancomycin, linezolid, and quinolones.

Rothia mucilaginosa

- It is a bacterium of the oral microbiota, rarely identified as a causative agent of bacteremia or endocarditis in hematological patients and pneumonia in immunosuppressed patients (Maraki and Papadakis, 2015).

Trueperella bernardiae

It is a rare pathogen in humans. It has been associated with sporadic cases of bacteremia, necrotizing fasciitis and joint, wound and ophthalmic infections (Rattes et al., 2016).

Listeria monocytogenes

Introduction

L. monocytogenes is the main pathogen of the genus *Listeria*, which contains two other species that may be opportunistic pathogens (*L. ivanovii* and *L. grayi*) and 14 more non-pathogenic species (Orsi and Wiedmann, 2016). It is a non-sporulated Gram-positive rod, facultative anaerobic and mobile due to the presence of peritrichous flagella. However, it has often been described as Gram-variable and even Gram-negative because it can discolor relatively easily. On blood agar it forms round white colonies and produces weak beta-hemolysis. It is halophilic and psychrophilic, capable of growing in refrigeration temperatures (4–8 °C), although its optimum growth temperature is 30–37 °C. Biochemically it is characterized by being oxidase negative and catalase-positive. It presents different serotypes, of which the most predominant in gastrointestinal infections are 1/2a and 1/2b, while 4b is an invasive serotype. These serotypes are differentiated according to the type of somatic antigen and flagellar antigen (Datta and Burall, 2018).

Historical background

In 1926, the South African doctor Everitt G. D. Murray and his collaborators Webb and Swann studied in Cambridge an epidemic that affected animals, mainly rabbits. Murray described the presence of a bacillus in the observed samples and, because this disease was accompanied by monocytosis, he named this bacterium *Bacterium monocytogenes* (Murray et al., 1926). In 1940 it was officially named *Listeria monocytogenes*, based on the name *Listerella hepatolytica* given to it by physician James H. H. Pirie in 1927 in honor of Joseph Lister, a surgeon committed to antisepsis (Pirie, 1940).

Epidemiology

L. monocytogenes is normally transmitted through the consumption of contaminated food and can produce small outbreaks. The most related foods are unpasteurized dairy, smoked fish, ice cream, pâté, and raw vegetables. In Europe, it is the third bacterium that causes the most deaths related to food contamination, after non-typhoid *S. enterica* and *Campylobacter* spp. (WHO (World Health Organization), 2017a,b). In 2017, the CDC recorded 887 cases of listeriosis in the US (CDC (Centers for Disease Control and Prevention), 2017) and the ECDC 2502 cases in Europe, with Germany, France and Spain as the countries with the highest incidence (ECDC (European Centre for Disease Prevention and Control), 2020).

Pathogenesis

It is a facultative intracellular pathogen with virulence factors that facilitate its entry into cells, as well as the evasion of the immune response. The main proteins it produces for this are internalin A (InlA) and listeriolysin O (LLO). The first allows it to invade host cells by endocytosis; the second is activated in the acidic environment of the phagosome and prevents the formation of the phagolysosome, slowing down the cellular response (Nguyen et al., 2019).

Normally, the route of entry is the gastrointestinal tract. For *L. monocytogenes* to survive, the stomach pH must be greater than 3. Once in the duodenum, the bacteria can cross the gastrointestinal membrane and spread via bloodstream through the lymph nodes. It presents tropism for the liver and spleen and can cross the blood-brain barrier in certain cases, moving retrogradely through the nerve axons to the brain parenchyma. This happens when *L. monocytogenes* travels within lymphocytes by the method called “Trojan horse” (Drevets and Bronze, 2008). In this same way, it can also cross the transplacental barrier.

Clinical manifestations

Gastrointestinal infection

It is the most common presentation in immunocompetent individuals, both adults and the pediatric population. The incubation period is less than 24 h and usually presents a self-limited condition lasting 1–3 days, although in some cases it can last up to 7 days (Ooi and Lorber, 2005). The most common symptoms are nausea, diarrhea, fever and headache.

Listeriosis in pregnancy

16% of invasive listeriosis cases occur in pregnant women, making pregnancy a risk factor for developing invasive listeriosis (Goulet et al., 2012). The main risk in these cases is a neonatal infection due to vertical transmission, which can lead to preterm delivery, neonatal listeriosis or stillbirth. During pregnancy, cellular immunity may decline in the third trimester, making it the most likely time for a woman to become infected with *L. monocytogenes*. However, the prognosis is worse in the first trimester of pregnancy (Wang et al., 2021).

The incubation period is longer than in gastrointestinal infection and can last 1–2 months. Symptoms in the mother are nonspecific: fever, flu-like syndrome, abdominal pain, diarrhea, contractions... By crossing the placenta, *L. monocytogenes* causes chorioamnionitis and neonatal infection, which can also result in premature delivery, spontaneous abortion or fetal death. The infections most associated with neonatal listeriosis are pneumonia, meningitis, and bacteremia. A rare condition but associated with neonatal listeriosis is infantile septate granulomatosis, which is characterized by the presence of microabscesses and hepatosplenic granulomas. The mortality of neonatal listeriosis is around 20–30% (Lamont et al., 2011).

Bacteremia

It is the most common presentation of invasive listeriosis and is practically restricted to immunocompromised patients or those over 70 years of age. It usually presents with nonspecific symptoms (fever, tachycardia, nausea) 5–10 days after infection. It can lead to neuroinfection, native or prosthetic valve endocarditis, or septic arthritis (Goulet et al., 2012).

Neuroinfection

L. monocytogenes is one of the most common pathogens in CNS infections, especially in patients with risk factors such as immunosuppression or diabetes (Brouwer and van de Beek, 2018). The most common condition is meningoencephalitis, where a parenchymal infection occurs in the cerebral cortex before a suppurative process and the development of abscesses. It presents with altered consciousness and cognitive dysfunction, similar to herpetic encephalitis. Another less common condition is rhombencephalitis, which affects the brainstem and presents with a prodromal onset of 4 days followed by abrupt onset asymmetric cranial

neuropathy, hemiparesis and cerebellar symptoms. It is associated with respiratory failure (40%) and high mortality. It can also affect the brain stem (unlike other bacteria) and cause brain abscesses. The incubation period is 10–14 days and the most common symptoms are fever, headache, chills, myalgia, and diarrhea. In addition, neuropathies and neck stiffness may occur. Mortality is 30%, and about 50% of surviving patients have neurological sequelae (Mylonakis et al., 1998).

Diagnosis

Depending on the clinical presentation, *L. monocytogenes* can be isolated in blood cultures or cultures of CSF, amniotic fluid, placenta. . . In cases of suspected neurolisteriosis, it can be detected by PCR in the CSF. The Gram stain has low sensitivity in both CSF (30%) and blood (45%) (Shoham and Bartlett, 2018). *L. monocytogenes* can be identified by mass spectrometry or by biochemical tests, which can also be used to differentiate it from *L. ivanovii* (xylose positive) and *L. grayi* (mannitol positive) (Pine et al., 1989). In addition, the CAMP hemolysis test is positive. This test consists of a synergy of *S. aureus* β -hemolysis on sheep red blood cells, which is enhanced when sown together with *L. monocytogenes*. The name of this test is an acronym of the authors who described it when observing it in group B streptococci such as *Streptococcus agalactiae*: Christie, Atkins and Munch-Petersen (Christie et al., 1944).

Therapy

Due to its self-limited nature, listeriosis often does not require antibiotic treatment, but simply support. In severe cases, different antibiotics have been used; however, there are no official guidelines for the management of the infection, so this is a glance over the traditional and more frequent use in the literature. The treatment of choice is ampicillin or amoxicillin (2 g IV/4 h) for 2 weeks in pregnant women, 3 in cases of meningoenzephalitis and 6 in rhomboencephalitis and endocarditis. Gentamicin (5 mg/kg/24 h) is usually associated, especially if the cellular immune response is affected (Pagliano et al., 2017).

Second-line treatment; for example, in cases of penicillin intolerance, it is erythromycin, cotrimoxazole or meropenem. Cotrimoxazole is contraindicated in the first trimester of pregnancy since it can alter the cardiac and CNS development of the fetus (Hernández-Díaz et al., 2001). Gastrointestinal infection cases, being normally self-limited, do not require antibiotic treatment.

Prophylaxis

Prophylaxis is mainly based on avoiding the consumption of potentially contaminated food, such as raw, smoked or undercooked fish and meat, pâtés, raw vegetables such as prepared salads, unpasteurized milk or cheeses. . . It is also advisable not to store these foods in the refrigerator for more than 4–7 days (Iwamoto et al., 2014).

Bacillus anthracis

Introduction

B. anthracis is a large Gram-positive rod ($1.5 \times 5 \mu\text{m}$), spore-forming and non-motile. On blood agar the colonies are white and have irregular edges, which has traditionally been called the “Medusa head” shape. On Gram stain, the bacilli tend to form chains and may have a faded center. The spores are non-deforming. Apart from these characteristics, it differs from other species of the genus by being catalase positive.

Historical background

During the 19th century, numerous physicians and biologists in Europe studied anthrax disease. The German physician Alois Pollender is considered the discoverer of the etiological agent in 1849 when he described that elongated cylinders were always observed in the samples of anthrax lesions. The microbiologist Ferdinand Cohn proposed that anthrax was then caused by a bacterium, which he named *Bacillus anthracis* (from Greek “anthrax,” “charcoal”). Robert Koch proved Cohn’s hypothesis in 1877 by isolating the bacterium, inoculating it into a healthy animal, and observing the onset of the disease. From this event, Koch developed the postulates that allow establishing the relationship between a microorganism and a disease (Koch, 1876). In 1881, Pasteur developed a veterinary vaccine from non-virulent strains (Scorpio et al., 2006).

Epidemiology

It is a zoonosis that affects 2000–20,000 humans per year and has a worldwide distribution, with special incidence in Central African, Central American and Asian countries (WHO (World Health Organization), 2008). It was precisely in Africa that the largest anthrax epidemic in humans was recorded, which occurred between 1979 and 1985 in Zimbabwe, with more than 10,000 cases and 182 deaths (Clegg et al., 2007).

Spores are the infectious form of the disease, and humans often acquire it from exposure to infected animals or contaminated animal products. The route of entry of the spores can be by inhalation of spore-loaded dust, by ingestion of contaminated meat or water or skin through injuries (WHO (World Health Organization), 2008).

It is convenient to remember that when an infected animal dies, humans can become contaminated by ingesting vegetative forms that may be in the meat. Furthermore, these vegetative forms can be distributed by flies and transmitted to humans or other animals. Finally, some scavengers such as vultures can shed *B. anthracis* spores in their feces after feeding on these infected carcasses.

When the route of entry is the ingestion of contaminated meat, *B. anthracis* usually produces a gastrointestinal infection.

Medical and social interest in anthrax grew in 2001 when *B. anthracis* spores were first used as a biological weapon. These were sent in mailing envelopes and resulted in 22 cases of anthrax, with 5 deaths (Borio et al., 2001).

Pathogenesis

The main virulence factor of *B. anthracis* is its production of exotoxins. These are encoded in the plasmid pX01 and have been used both in the diagnosis of the disease and in the development of vaccines. The toxins are: edema toxin and lethal toxin (Tournier and Rougeaux, 2020).

Edema toxin is composed of an “edema factor” linked to a protective antigen and it is an adenylate cyclase that alters cellular function, especially those associated with innate immunity. Therefore, phagocytosis, the production of free radicals, and the performance of lymphocytes and macrophages are altered. It also produces an increase in permeability and hypotension, which is why it is called edema toxin.

The lethal toxin is a calmodulin-dependent zinc metalloprotease that will also alter cell function. This toxin also increases vascular permeability; in addition, it produces lesions in the intestinal epithelium that can lead to superinfections by enteropathogenic bacteria.

Clinical manifestations

Cutaneous infection

The incubation period, normally after contact with the infected animal, is 1–5 days. The site of inoculation is usually a small trauma to the skin of the head, neck, or extremities, and will result in an itchy papule. Small vesicles with a fluid rich in bacteria form around the papule. When the vesicles rupture, an ulcer forms that blackens over time and a painless induration of the skin or edema forms around it. This is important for the diagnosis since the rest of cutaneous infections by other microorganisms do not usually form edema or induration of the lesion. The ulcer usually heals spontaneously within 2–3 weeks and the eschar falls off without scarring. Although it is a local infection and there is no bacteremia, it is usually accompanied by headache and low-grade fever. The lethality of untreated infection is 15–20% and is almost nil with treatment (Dogany et al., 2010).

Respiratory infection

Disease caused by accidental inhalation is extremely rare; however, it is of great importance because it is practically always lethal, even with antiserum treatment (Holty et al., 2006). The incubation period is usually less than a week. In order to be inhaled and to gain access to the bronchioles and alveoli, spores must measure less than 5 µm. There the spores are phagocytosed and travel to the mediastinal lymph nodes. This first phase, called the early prodromal phase, is characterized by flu-like symptoms with low-grade fever, myalgia, and malaise (Lucey, 2005). It is followed by the intermediate progressive phase with high fever, intense sweating and respiratory symptoms: dyspnea, chest pain and syncope. The third and final phase is the fulminant late phase, which presents with bacteremia, sepsis, and multiple organ failure. Patients in this phase require intubation and mortality in less than 24 h is high (Sweeney et al., 2011).

Gastrointestinal infection

This presentation, which is the most common in animals, is quite rare in humans and is restricted to rural areas of developing countries. The infection period is 1–5 days and may present an oropharyngeal or intestinal presentation. The first presents symptoms such as dysphagia, odynophagia and pharyngitis. In addition, there is usually fever, lymphadenitis, and edema of the face and neck. Sometimes an ulcer may develop in the mouth or pharynx; for example, in the tonsils. These ulcers are usually covered with a pseudomembrane, which facilitates their diagnosis. The intestinal disease presents with nausea, vomiting and abdominal pain, in addition to fever and diarrhea with bloody stools. Gastrointestinal infection is usually associated with bacteremia and sepsis and has a mortality of 40% (Beatty et al., 2003).

Central nervous system infection

One of the possible complications of *B. anthracis* bacteremia is meningitis. It is rare since it occurs in 30% of cases of inhalational anthrax, which is uncommon. The most frequent symptoms are acute headache, fever, vomiting, altered mental status and neurological signs (Katharios-Lanwermyer et al., 2016). In addition, it is usually accompanied by edema and parenchymal cerebral hemorrhage or subarachnoid hemorrhage, atypical signs in meningitis caused by other microorganisms. It is therefore a presentation with high mortality that requires antibiotic combination therapy to increase survival. Microbiological diagnosis can be made from CSF by staining, PCR, or culture.

Diagnosis

In skin infection, the clinical diagnosis must be made from a differential diagnosis that allows ruling out other diseases that present with similar lesions, such as those caused by *Francisella tularensis*, *Streptobacillus moniliformis*, *Blastomyces* sp. and other skin fungi and rickettsiae. The features that should guide the suspicion of *B. anthracis* are the painless lesion and the large surrounding edema.

Suitable samples for the biological diagnosis of skin infection are aspirate of the gallbladder fluid, smear from the edge of an eschar, or biopsy of the lesion (CDC (Centers for Disease Control and Prevention), 2020). In cases of suspected sepsis or systemic infection, blood cultures are recommended due to their high sensitivity. If there is pulmonary involvement, it is advisable to obtain pleural fluid and not sputum, since a productive cough is not usually observed. Identification is usually carried out in reference laboratories and can be done by PCR or direct immunofluorescence (ASM (American Society for Microbiology), 2017). Other less used but useful techniques are serological techniques such as ELISA, which make it possible to detect antibodies against the protective antigen. The main drawbacks are that these techniques are not useful in the first period of the disease, since they become positive from 1 week after the onset of symptoms.

Therapy

Antibiotic treatment has historically been based on the use of penicillin. However, due to increased resistance, other treatments are currently used depending on whether anthrax is cutaneous, respiratory, or systemic (Hendricks et al., 2014). *B. anthracis* is sensitive to first-generation cephalosporins, carbapenems, clindamycin, macrolides, fluoroquinolones, aminoglycosides, tetracyclines, rifampin, daptomycin, and linezolid. In cases of cutaneous anthrax, the treatment of choice is oral, with fluoroquinolones or doxycycline for 7–10 days. In severe cases or with CNS involvement, the intravenous combination of a fluoroquinolone with a carbapenem (or penicillin if sensitive) and a protein synthesis inhibitor, such as linezolid, clindamycin, or rifampicin is recommended. The duration can be from 2 to 3 weeks to 2 months depending on the clinic.

Prophylaxis

The mainline of prophylaxis is the veterinary vaccine, which has been used for decades and has led to a great decrease in cases in both animals and humans.

There is also a vaccine for humans that was licensed in the US in 1970, developed by Wright and his colleagues. It is a vaccine that contains the protective antigen (PA) adsorbed on an aluminum hydroxide gel. This vaccine is used to immunize mainly exposed professionals (veterinarians, farmers) and soldiers to prevent possible bioterrorist attacks. This vaccine is for intramuscular administration to avoid adverse effects locally from subcutaneous administration. The dosing regimen is 0, 1, 6, 12 and 18 months, in addition to annual boosters (Bower et al., 2019).

Bacillus spp.

Introduction

The rest of the species in the genus *Bacillus* are spore-forming bacilli, Gram-positive or Gram-variable, facultative anaerobes. In blood agar cultures they usually show beta-hemolysis, unlike *B. anthracis*. They are ubiquitously distributed as they tolerate extreme temperatures, desiccation, and heat. For this reason, they have been used for decades in the industry to control sterilization and hygiene processes (e.g., *B. subtilis*, *B. stearothermophilus*) (Turnbull, 1996).

Normally, the isolation of *Bacillus* spp. in clinical samples it is considered contamination since they are not usually identified as etiological agents of infections. The syndromes most related to species of this genus are food poisoning and infections at different levels: cutaneous infection, meningitis and systemic infection. *B. cereus* is the main *Bacillus* species responsible for these syndromes, so it will be the main object study in this chapter.

Clinical manifestations

Food poisoning

Food poisoning is caused by the ingestion of toxins produced by different species, mainly *B. cereus*, and is widely described, although it is rare compared to gastrointestinal infections produced by *Campylobacter*, *Salmonella* or *Clostridioides* genera. There are two types of toxins: emetic toxin and diarrheal toxin. The emetic toxin is a heat-stable peptide toxin found primarily in rice and is similar to the enterotoxin of *S. aureus*. It acts quickly (<6 h) and produces a disease that occurs with vomiting, cramps and diarrhea in some cases. The syndrome is usually self-limited in less than 24 h. The diarrheal toxin is polypeptidic and has a higher molecular weight than the emetic toxin. It is heat-labile, so it can be removed by heating food to a high temperature. It takes 6–18 h to act and produces acute diarrhea that usually remits spontaneously in less than 24 h (Jessberger et al., 2020).

Systemic infection

Although rare, the etiological role of *B. cereus* in systemic infections has been described. Its role as an infectious agent is highly controversial, since, as said before, it is often considered a contaminating agent. However, there are studies where it has been considered the causal agent of bacteremia and skin infections, mainly in patients with central venous catheters (Weber et al., 1989). Likewise, infections by *Bacillus* spp. in neonates by perinatal transmission have been described.

Cutaneous and mucosal infection

B. cereus has been associated with wound infections and cellulitis, usually after severe trauma and skin erosion or with postoperative wounds. These are not normally serious infections, but they make wound healing difficult and can favor the appearance of superinfections (Ehling-Schulz et al., 2019).

Central nervous system infection

CNS infection is rare and is related to the implantation of CSF bypass valves. It can present with encephalitis, meningitis and brain abscesses, especially in neutropenic patients (Gaur et al., 2001).

Diagnosis

In cases of gastrointestinal infection, the search for *Bacillus* spp. in stool samples is not routinely performed in the laboratory, nor are there any commercially available toxin detection techniques. For these reasons, the diagnosis of food poisoning is usually clinical and based on the patient's history, and not microbiological.

Therapy

The main treatment of food poisoning is supportive, ensuring the rehydration of the patient. For catheter-associated skin infections or bacteremia, the infected catheter or prosthetic material must be removed. *B. cereus* is resistant to penicillins and cephalosporins. In the case of using antibiotics, it is recommended to use ciprofloxacin, vancomycin, linezolid or carbapenems. Clindamycin efficacy has been observed in cases of native valve endocarditis (Wright, 2016).

Prophylaxis

Proper cooking of food eliminates viable *Bacillus* spp. and it destroys the diarrheal toxin, but not the emetic. It is advisable to avoid leaving the rice already cooked at room temperature and it is recommended to refrigerate it as soon as possible since the production of toxins is inhibited at low temperatures (Ehling-Schulz et al., 2019).

Erysipelothrix rhusiopathiae

Introduction

E. rhusiopathiae is a facultative anaerobic, non-sporulated Gram-positive or Gram-variable rod. On blood agar it forms small transparent colonies and can produce α -hemolysis. This bacterium was first isolated by Robert Koch in 1878 and was proposed by Löffler in 1886 as the causative agent of erysipelas in pigs (Löffler, 1886). In 1909 Rosenbach isolated it from the skin lesions of a man, establishing its role as a human pathogen (Rosenbach, 1909). The disease it produces was called erysipeloid to distinguish it from the previously described erysipelas associated with *S. pyogenes*.

Epidemiology

Erysipeloid is a worldwide zoonosis, mainly related to pigs and fish. Transmission occurs through direct contact with the lesions, so it is a disease related to farmers, butchers, fishermen, veterinarians and other professionals susceptible to occupational exposure.

Clinical manifestations

Erysipeloid, or "Rosenbach's disease", is a cellulitis that is located mainly on the fingers by direct contact with the infected animal. The incubation period is 3–7 days, after which local inflammation with pain or itching occurs. The lesion is defined and reddish or purplish, it spreads over time and may blister. Systemic symptoms such as fever or arthralgia are rare. The main difference with staphylococcal or streptococcal cellulitis is the subacute onset, the absence of suppuration, and severe pain. The condition remits without treatment 3–4 weeks after exposure (Wang et al., 2010).

Systemic infection is very rare; however, cases of bacteremia and even endocarditis due to *E. rhusiopathiae* have been described (Wang et al., 2020).

Diagnosis

Due to the depth of the lesions, biopsies must include the entire dermis, which makes it difficult to take the sample correctly. In the case of suspected *E. rhusiopathiae* endocarditis or bacteremia, blood culture is the best diagnostic technique. Given their slow growth, the cultures should be incubated for at least 7 days (Micaelo et al., 2016).

Therapy

Although data on antibiotic sensitivity are scarce, it is known to have high sensitivity to beta-lactams, clindamycin, quinolones, linezolid, and daptomycin. It is resistant to vancomycin, which is important since it is an antibiotic widely used empirically in Gram-positive bacteremia (Wang et al., 2010).

Prophylaxis

Prophylaxis is focused on exposure, promoting the use of adequate hand hygiene, the use of gloves when handling fish or coming into contact with animals and the correct disinfection of potentially contaminated surfaces. There are veterinary vaccines that can help control swine erysipelas (Opriessnig et al., 2020).

Tropheryma whipplei

Introduction

T. whipplei is a small bacillary actinomycete ($0.25 \times 1.5\text{--}2.5\ \mu\text{m}$) with the ability to invade cells of the immune system such as macrophages of the small intestine, polymorphonuclear cells and intraepithelial lymphocytes, as well as epithelial or muscle cells. It was first described in 1907 by the American physician George H. Whipple, who pointed to it as a possible etiological agent of the disease (Whipple, 1907). It is a very slow-growing bacterium, genetically close to *Nocardia* sp. and other actinomycetes. It is found in wastewater, so it is suspected that infection in humans is acquired through the consumption of contaminated water (Maiwald et al., 1998).

Clinical manifestations

T. whipplei causes a rare disease known as Whipple's disease, considered a sort of intestinal lipodystrophy. It is a systemic infection that affects the intestine, causing diarrhea, abdominal pain and weight loss. In some cases, it can also affect other organs such as the CNS, lungs or heart. As in many infections, the severity of the disease is determined by risk factors such as immunosuppressive treatment.

The digestive picture is characterized by prolonged weight loss, steatorrhea, watery diarrhea, and abdominal cramps. This condition can lead to a syndrome of malabsorption and cachexia (Raoult et al., 2010).

Other symptoms related to Whipple's disease are arthropathies, fever, lymphadenopathy (especially at the mesenteric level), hyperpigmentation of the skin exposed to the sun, hypotension and occult blood in the stool. Regarding cardiac involvement, *T. whipplei* has been associated with endocarditis, aortic or mitral regurgitation, and heart murmurs (Fenollar et al., 2012). The pulmonary picture is associated with chronic non-productive cough, chest pain, and pulmonary hypertension. It is also related to *T. whipplei* with CNS involvement, which can cause myoclonus, nystagmus, dementia and memory disorders (Compain et al., 2013).

Diagnosis

Diagnosis of intestinal infection is usually made by endoscopy, where a mucosal pattern of erythematous areas mixed with pale yellow areas, with whitish plaques, is observed. The characteristic evidence is that the lesions show positive Schiff inclusions. The Schiff technique uses periodic acid Schiff (PAS) as a stain, which turns red in contact with aldehyde groups (Schiff, 1866). The development of a PCR has allowed the rapid and accurate detection of *T. whipplei* from biopsies, although it is normally performed in specialized or reference centers and not routinely in hospitals (El-Abassi et al., 2017).

Diagnosis of cardiac infection is made by histology and PCR of the removed valve.

Therapy

Standard treatment is ceftriaxone (2 g daily) for 2 weeks (up to 4 weeks in cases of CNS infection or endocarditis). It is usually recommended to continue treatment with cotrimoxazole (two daily doses of 160/800 mg) for 1 year. In case of allergy to ceftriaxone, it can be substituted for meropenem. Likewise, cotrimoxazole can be substituted for doxycycline in combination with hydroxychloroquine (Lagier et al., 2014).

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Listeria and Erysipelothrix

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Listeria

Introduction

Listeria monocytogenes was first described as a pathogen in 1926 by Murray EGD in an outbreak affecting rabbits (Murray et al., 1926). *Listeria* is an intracellular gram-positive rods widely distributed in the environment, transmitted primarily through contaminated food. The infection is mild and self-limiting in healthy adults but causes severe disease as sepsis or meningitis in people over 50 years of age, neonates and immunocompromised individuals, especially patients with solid or hematological malignancies, solid organ transplant recipients, or patients on chronic immunosuppressive therapy. Although listeriosis has low incidence (0.42 cases/100000 population), this disease is of great public health concern due to outbreaks (European Centre for Disease Prevention and Control, 2020).

Microbiology

The genus *Listeria* consists of a group of gram-positive, non-sporulating, facultative anaerobic rods. They are observed under the microscope singly or in short chains.

Listeria spp. is a saprophytic and ubiquitous bacterium that are isolated from a diversity of environmental sources, including water, soil, a wide variety of foods and the feces of humans and animals. *Listeria* can survive at extreme temperatures or pH making this microorganism a serious threat to food safety in food industry (Vázquez-Boland et al., 2001).

This genus includes six species: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi*. *Listeria monocytogenes* is the main human pathogenic species and will be the focus of this article, although *L. grayi* bacteremia has also been reported in immunocompromised patients. Bacteremia, infection of ventriculo-peritoneal shunts by *L. innocua*. Bacteremia in immunocompromised patients, infections of vascular prosthesis or gastroenteritis due to *L. ivanovii*. And meningitis due to *L. seeligeri* has been reported (Radoshevich and Cossart, 2018).

The characterization of the isolates using multilocus sequence typing (MLST) has divided the species into clonal complexes (CC). They are classified into virulence categories: (1) CC1, CC2, CC4 and CC6 are associated with infections, (2) associated with food and hypovirulent (CC9 and CC121) and (3) the intermediate isolates associated with clinical and food environments (CC5, CC8, CC3, CC37, CC101, CC90, CC7, CC155 and CC77). The whole genome MLST has subdivided CC into clonal types and has revealed the great heterogeneity of isolates in terms of virulence (Chenal-Francisque et al., 2011; Moura et al., 2016; Kwong et al., 2016; Kuch et al., 2018; Lepe, 2020).

Physiopathogenesis and immunity

The main pathogenic factor of *Listeria monocytogenes* is that it is an intracellular pathogen that can grow inside macrophages and epithelial cells. Infection begins after ingestion of contaminated food in high concentrations and its subsequent multiplication in the endothelial cells of the intestine (Vázquez-Boland et al., 2001; Pamer, 2004).

At cell level, the transition from saprophyte to pathogen requires the co-ordinated regulation of bacterial factors that determine pathogenesis and intercellular dissemination. An essential determinant of *L. monocytogenes* pathogenesis is the transcriptional activator PrfA, which activates most of the genes required for cell entry and intracellular parasitism. *L. monocytogenes* induces its own internalization by cells that are normally phagocytic. Its entry into cells is mediated by host surface proteins classified as internalins. Internalin-mediated entry is important for crossing intestinal, blood-brain and foetoplacental barriers. *L. monocytogenes* transits from the gut to the brain or fetus (Nikitas et al., 2011; Bierre et al., 2018; Radoshevich and Cossart, 2018).

The most important factor determining the pathogenesis of *L. monocytogenes* is its beta-hemolysin, listeriolysin O (LLO). Listeriolysin O mediates the breakdown of the phagosomal membrane that forms after phagocytosis of the micro-organism. It probably inserts into an acidifying phagosome preventing vesicle maturation. In addition, it acts as a transposition pore for one or both phospholipases of *L. monocytogenes*, which also contributes to vacuolar lysis. Listeriolysin O synthesis and activity are controlled at multiple levels to ensure that its lytic activity is limited to acidic vacuoles and does not affect the cytosol. Mutations in listeriolysin O that influence its synthesis, cytosolic half-life or pH optimum reduce premature toxicity in infected cells (Nikitas et al., 2011; Bierre et al., 2018; Radoshevich and Cossart, 2018).

Once in the cytosol, *L. monocytogenes* proliferates quickly. One of the PrfA-regulated genes encodes the synthesis of a hexose-phosphate transporter that facilitates the proliferation of cytosolic bacteria on phosphorylated glucose derivatives originating from the host. Then in the cytosol it produces ACTA, another PrfA-regulated surface protein that mediates the nucleation of host actin filaments to propel bacteria into and between cells. ActA mimics host proteins by promoting the actin nucleation properties of the Arp2/3 complex. Consequently, *L. monocytogenes* enters the cytosol of almost any cell (Nikitas et al., 2011; Bierre et al., 2018; Radoshevich and Cossart, 2018).

After the process of endocytosis and activation, they can be ingested by macrophages and reach the liver and spleen, leading to the spread of infection. By multiplying in macrophages, they prevent them from being eliminated by antibodies, so individuals with impaired cell-mediated immunity are more susceptible to this infection while humoral immunity is of no importance.

The incubation period varies from 3 to 70 days, with a median of 21 days (Coulet et al., 2013). It is variable depending on the clinical manifestation of the disease: for gastroenteritis cases the median incubation period is 24 h, for bacteremia is 1–12 days, for neurolisteriosis 1–14 days and in cases related to pregnancy 17–67 days (Lepe, 2020).

Iron overload is a virulence factor as the siderophores of *L. monocytogenes* enable it to take up iron from transferrin, hence iron-containing culture media favor its cultivation.

Epidemiology

Listeriosis is a relatively uncommon but severe invasive infection caused by *Listeria monocytogenes*. It is an environmental bacteria, which can inhabit water, soil and plants. It can be present in the human microbiota, being present in 5% of healthy adults in their feces (Swaminathan and Gerner-Smidt, 2007).

Listeria monocytogenes infection is uncommon in immunocompetent individuals and may present with fever or self-limiting gastrointestinal infection. In pregnant women, neonates, elderly and immunocompromised individuals, may cause severe infections such as bacteremia, meningoencephalitis or maternal-fetal listeriosis.

The most frequent cases of human listeriosis occur through ingestion of contaminated food (unsanitized (soft) milk and cheeses, prepared foods such as paté, sliced meat products, contaminated vegetables or smoked fish products), or in the case of newborns through transplacental transmission, which is the only case of inter-human transmission.

Severe clinical cases occur in children younger than 30 days or adults older than 65 years, in pregnant women and in immunocompromised populations such as recipients of solid organ transplants, solid or hematological malignancies, immunosuppressed or patients treated with chronic corticosteroids.

Thirteen serotypes of *Listeria monocytogenes* and four distinct lineages that are related to the serotypes have been identified. The serotypes most frequently identified in food and clinical samples are 1/2a, 1/2b, 1/2c and 4b (96% of isolates). Most outbreaks are caused by 4b. Pulsed field gel electrophoresis (PFGE) or ribotyping techniques can be used to detect outbreaks for epidemiological purposes (Radoshevich and Cossart, 2018).

Despite being sporadic, listeriosis has caused outbreaks worldwide. The largest outbreak to date has occurred in South Africa from ready-to-eat processed meat product. A total of 1060 associated cases were reported, with an estimated case fatality from or with the infection of 30% (Thomas et al., 2020; European Centre for Disease Prevention and Control, 2020).

Listeriosis incidence in Europe

From 2013 to 2017, between 1905 and 2527 cases of listeriosis were reported to the European Surveillance System (TESSy) annually in 30 EU countries. Of the cases reported in this period, Germany, France and Spain accounted for 26%, 17% and 10%, respectively. Severe *L. monocytogenes* infections were more common in men (54%) and in people over 65 years of age (65%). The majority of cases (98%) were of autochthonous origin.

According to surveillance data in Europe coordinated by the ECDC, the incidence rate in the European Union in 2017 was 0.42 cases per 100,000 population and the highest rate was detected among people over 64 years (1.7 cases per 100,000 population) (European Centre for Disease Prevention and Control, 2020).

In Spain since the 2015 Ministerial Order, listeriosis has been included among the diseases under compulsory surveillance in Spain. Previously, it was reported to the National Epidemiological Surveillance Network (RENAVE) through the Microbiological Information System (SIM) on a voluntary basis.

In the period 2015 to 2018, fifteen autonomous communities and cities notified 1369 confirmed cases to the RENAVE, so that around 300–400 cases are being recorded annually. In Andalusia, an average of 65 cases per year have been reported. In 2018, 432 cases were reported. Its usual presentation is in the form of sporadic cases or small intra-familial outbreaks. Specifically, there are eight outbreaks reported since 2015, with two to three cases per outbreak, and they are usually clusters due to vertical, mother-to-newborn transmission (six outbreaks) or small family foodborne outbreaks (two outbreaks). The average age of cases reported to the Surveillance Network during these years is around 65–70 years and there have been 124 deaths, representing a case fatality rate of around 9%. In general, case fatality increases with age (Centro Nacional de Epidemiología, n.d.).

The most significant outbreak of listeriosis recorded in Spain is in Andalusia in August 2019, and the only one that has been associated with such widely distributed commercial products, specifically in larded pork (Lepe, 2020).

Recommendations on Listeriosis prevention

Cooking food at temperatures above 65 °C kills bacteria. However, *Listeria monocytogenes* can contaminate food after processing (e.g., contamination can occur after food is cooked but before packaging). Unlike many other foodborne bacteria, *Listeria* tolerates salty environments and can even multiply at cold temperatures between +2 °C and 4 °C. To prevent listeriosis it is important to follow good manufacturing practices, good hygienic practices and effective temperature control throughout the food production, distribution and storage chain, including in the home. The resistance of this bacterium, together with high mortality rates in humans, makes safe food handling paramount to ensure public health (Ministerio de Sanidad, n.d.).

In pregnant women, they are reminded of the need to take certain additional hygiene measures and to avoid consumption of certain foods in order to prevent risks which, although rare, may have negative consequences for the fetus or the pregnant woman.

Clinical manifestations

Listeria monocytogenes can cause two forms of listeriosis, non-invasive gastrointestinal and invasive listeriosis.

Listeria infections cause a variety of clinical syndromes, the most common are meningitis and septicemia, especially in immunocompromised patients.

Gastroenteritis: Occurs within 48 h of ingestion of a significant inoculum of bacteria in contaminated foods such as milk, deli meats and salads. Clinical manifestations are fever, diarrhea, headache and general symptoms. Isolated gastrointestinal disease does not require antibiotic treatment (Ooi and Lorber, 2005).

Bacteremia: *L. monocytogenes* septicemia is manifested by fever, chills, myalgia and arthralgia, indistinguishable from bacteremia caused by other bacteria. Meningeal symptoms, neurological or focal data or changes in mental status suggest the diagnosis. In the MONALISA study where patients with invasive listeriosis between 2009 and 2013 were included, of which 427 were primary bacteremia. Mortality was 46% at 90 days (Charlier et al., 2017).

Infection during pregnancy and neonatal infection: Listeriosis in pregnancy causes a serious threat to the fetus that may result in miscarriage.

Listeriosis occurs up to 20 times more frequently in pregnant women than in the general population (Mulonakis et al., 2002). Listeriosis occurs mainly during the third trimester and most of the time asymptotically or with minor symptoms, but the effects on the fetus are greater the less advanced the stage of gestation and can cause miscarriage or death in up to 20% of cases (Silk et al., 2012).

The usual clinical presentation is an acute or subacute non-specific febrile illness with myalgias, arthralgias, dorsalgia and headache. Pregnant women with listeriosis often suffer from bacteremia. Central nervous system involvement is rare. Even without treatment, the prognosis for the mother is very good but not for the fetus. Up to 2/3 of live births to women who have had listeriosis during pregnancy may develop neonatal listeriosis, hence the importance of early diagnosis (Craig et al., 2019).

Infection in the fetus can be via the transplacental route or by passage through the birth canal. If infection occurs in utero, infantiseptic granulomatosis, a form of listeriosis characterized by microabscesses and disseminated granulomas mainly in the liver and spleen, can occur, with a mortality rate of 50% (Lamont et al., 2011; Wadhwa Desai and Smith, 2017).

Neonatal listeriosis can be early-onset (less than 7 days of life) or late-onset (more than 7 days). In early-onset listeriosis, the neonate may present with early-onset sepsis often associated with respiratory distress or pneumonia, skin lesions with or without conjunctivitis. In late onset, they develop sepsis and even meningitis. Many of these cases involve full-term infants who become infected while passing through the birth canal and whose mothers remained asymptomatic during pregnancy. Mortality is around 25% in these cases.

The prospective Monalisa study included 107 infections in pregnant women. The most common diagnosis was fever with obstetric symptoms (contractions, labor or fetal loss). In 83% of the infections there were serious consequences for the fetus: fetal death (24%), premature delivery (45%) (Charlier et al., 2017).

Central nervous system infections: Invasive *Listeria monocytogenes* infection causes CNS involvement relatively frequently due to the tropism of the bacterium for the meninges and brain parenchyma, especially the brainstem, and mortality is high and neurological sequela can affect more than 60% of survivors (De Valk et al., 2005). *L. monocytogenes* causes approximately 5–10% of cases of adult out-of-hospital bacterial meningitis in the United States (Harrison). The presentation is subacute and nuchal rigidity and meningeal signs are less common. Photophobia is rare. Cerebrospinal fluid chemistries in listerogenic meningitis most often show leukocyte counts in the range of 100–5000/ μ L, rarely higher; 75% of patients have leukocyte counts below 1000/ μ L, with neutrophil predominance more modest than in other bacterial meningitis. Low glucose concentrations and the observation of typical gram-positive bacilli on Gram stain are found in about 30–40% of cases (Hohmann and Portnoy, 2020).

L. monocytogenes can directly invade the brain parenchyma and produce cerebritis or focal abscess (Hohmann and Portnoy, 2020). In these cases the predominant clinical presentation is altered consciousness or cognitive dysfunction and may mimic Herpes encephalitis. Blood cultures are often positive and concomitant meningitis may develop (Pelegri n et al., 2014).

Other focal infections: Focal infections of visceral organs, eyes, pleural, peritoneal and pericardial spaces, bones and joints have been reported in low percentages (about 3%) (Bartolom   et al., 2005; Benjelloum et al., 2011; Charlier et al., 2012; Morgand et al., 2018; Shoai-Tehrani et al., 2019; Lepe, 2020).

Diagnosis

A microbiological diagnosis is based on conventional staining and culture techniques of samples taken from a sterile location (blood, cerebrospinal fluid or less frequently pleural, articular fluid, pericardial, etc.). The study of other samples as placenta, amniotic fluid may be indicated within the first 48 h after delivery. The samples should be submitted to the laboratory as soon as possible and if not, they can be stored at 4  C for a maximum of 48 h.

Listeria monocytogenes is a tiny, Gram-positive rod exhibiting tumbling motility at room temperature. The sensitivity of gram staining in cerebrospinal fluid is low but Multiplex PCR assay can be performed in patients with suspected central nervous system infections to confirm the diagnosis (Caillaux et al., 2020). It is easily isolated on enriched media such as blood agar or chocolate, is a gram positive catalase positive and oxidase negative rod, which produces hemolysis and grows readily on blood agar. The presence of a small zone of beta-hemolysis on blood agar. *L. monocytogenes* is CAMP test positive. Its growth is optimal at 37  C but it also grows at low temperatures (Anand et al., 2016). It is resistant to low pH and high salt environments making it an important pathogen in the food industry. For identification to species level, Maldi-Tof is currently used. For epidemiological studies, typing techniques such as PFGE or ribotyping are used.

Treatment

In listeriosis, recommended treatment is based on the result of antibiotic susceptibility testing (Mart  nez-Macias et al., 2012). Treatment is ampicillin 200–300 mg/kg/day. Consideration should be given to the association with gentamicin 5–7 g/kg 7/day (during the first week) in case of central nervous system infection, endocarditis or immunosuppression (Lorber 1997).

Associations of ampicillin with ceftriaxone and ampicillin with daptomycin have been shown to be synergistic in vitro and in animal models of *Listeria* endocarditis (Radoshevich and Cossart, 2018; Lepe et al., 2019; Mensa and Soriano, 2021).

The association of ampicillin with co-trimoxazole (trimethoprim 20 mg/kg/day in 3–4 doses) is at least as effective as the association of ampicillin with gentamicin and may be more appropriate if the infection is localized in the central nervous system or the risk of nephrotoxicity is high. Treatment is maintained for 2 weeks for isolated bacteremia, 3 weeks for meningitis, 4–6 weeks for endocarditis and more than 6 weeks for rhombencephalitis, brain abscesses or neurological focality (likely microabscesses). Alternatives are co-trimoxazole/TMP 20 mg/kg/day in 3–4 doses alone or in combination with rifampicin in case of central nervous system infection. Clarithromycin or doxycycline in isolated bacteremia. The following antibiotics: rifampicin, moxifloxacin, levofloxacin, meropenem and linezolid are active against *Listeria* but clinical experience with their use is limited. The use of linezolid and/or rifampicin may be considered in the treatment of brain abscess. Vancomycin is active, but clinical efficacy is irregular. Cephalosporins are not active against *Listeria* (Radoshevich and Cossart; Lepe et al., 2019; Mensa and Soriano, 2021).

In immunosuppressed, pregnant and over 65-year-old people, consider amoxicillin for 7 days in situations of invasive infection as a complication of gastrointestinal listeriosis. In the recent outbreak in 2019 in Andalusia used this recommendation until the blood culture result was known (Lepe, 2020).

Antimicrobial resistance

Listeria species are intrinsically resistant to several antibiotics, including first-generation quinolones, broad-spectrum cephalosporins, monobactams and fosfomycin. The mechanism associated with cephalosporin resistance in *Listeria* spp. is complex and is thought to be secondary to unique penicillin binding protein in the cytoplasmic membrane, which is the primary target for other beta-lactams but does not bind well to cephalosporins (Anand et al., 2016).

Trimethoprim resistance may not be detected if the anti-biogram is performed with co-trimoxazole discs. Recognizing this, is clinically important, as they are commonly employed to empirically treat sepsis and meningitis of unknown etiology (Anand et al., 2016). The mortality rate is high, reflecting the combination of an immunocompromised host and an often delayed diagnosis (Schlech, 2019).

Erysipelothrix

Introduction

Erysipelothrix species are ubiquitous in nature and have been isolated from a variety of animals species.

The genus *Erysipelothrix* is composed of at least five species, *E. rhusiopathiae*, *E. tonsillarum*, *E. inopinata*, *E. larvae* and *E. piscicarius* sp. nov.

E. rhusiopathiae is a facultative anaerobic non-spore-forming Gram-positive rod. It is a zoonotic pathogen causing human disease rarely. It occurs mainly in individuals with exposure to contaminated animal and fish products (Rihana et al., 2018). There are three well-defined manifestations in humans: a localized cellulitic form known as erysipeloid of Rosenbach, a diffuse cutaneous form, and a septicemic form that is associated with endocarditis (Brooke and Riley, 1999; Lorenz et al., 2018). Other rare manifestations in humans are abscess formation, septic arthritis, and osteomyelitis (Reboli and Farrar, 1989; Brooke and Riley, 1999; Principe et al., 2016).

The term erysipeloid was introduced by Rosenbach, who isolated it from a localized lesion in a man and established its human pathogenicity to avoid confusion with typical bacterial erysipelas caused by *Streptococcus pyogenes* or *Staphylococcus aureus*, less so by group B, C or G Streptococci.

Microbiology

Surface swabs are not useful for isolation of *E. rhusiopathiae* from cutaneous lesions since the organisms reside deep in the subdermal layer. Biopsy specimens are optimal for isolation of the organism. In bloodstream infections, can be recovered from standard blood cultures.

Gram staining shows short, thin, straight or slightly curved gram-positive bacilli, sometimes with a tendency to form long filaments. After growth for 24 h at 37 °C on standard culture media such as blood agar, the colonies are small and clear. These bacteria are catalase-negative, oxidase-negative and capable of producing SH2. The microbiological differential diagnosis should be made with other gram-positive bacilli such as *Listeria monocytogenes* and *Corynebacterium* species.

The introduction of mass spectrometry (MALDI-TOF) in clinical microbiology laboratory has improved identification of microorganism at species level.

This approach could allow to reduce the duration of empirical treatment and improve the appropriate therapy for infections caused by fastidious pathogen.

Epidemiology

It is a globally distributed microorganism, commensal or pathogenic to many animals, including mammals, birds and fish. The main reservoir is the domestic pig. It is able to live for prolonged periods in soil. Zoonotic infection is the most important route of transmission of the bacterium to humans. Transmission from animals to humans occurs by direct skin contact (abrasions or wounds), so most human cases are related to occupational exposure, veterinarians, farmers, slaughterhouse workers, etc.

In the absence of occupational exposures, risk factors for invasive infection include immunosuppressing condition such as diabetes mellitus, chronic kidney disease and treatment with high-dose steroids. In patients with endocarditis, excessive alcohol use, in addition to underlying heart conditions has also been identified as a risk factor (Jean et al., 2019).

No inter-human transmission has been reported. *E. rhusiopathiae* cannot resist temperatures above 55 °C, but can resist salting and smoking of food.

Clinical manifestations

Infections can manifest in one of the following three forms:

- Erysipeloid: This is the localized cutaneous lesion that manifests as subacute cellulitis. It usually occurs on the upper limbs after cuts or abrasions. It is the most common presentation in humans. The incubation period is 2–7 days. The clinical presentation is characterized by severe pain with edema. The skin is reddish-violet in color and appears bordered and raised. Vesicles may appear. Adjacent joints may also be inflamed. Is typically limited to the fingers or hand.
- Diffuse cutaneous infection: an extensive area of rash may be seen from the initial lesion at the site of inoculation and/or the appearance in remote areas of erysipeloid skin lesions with a typical rhomboidal appearance. General symptoms are frequent and include fever and severe arthralgias. Blood cultures are usually negative.

These patients usually have the same exposures as those with erysipeloid. However eating contaminated seafood or undercooked has been associated with this infection form.

Bacteremia, whether or not associated with endocarditis: Rare and in most cases associated with endocarditis on native valves (Artz et al., 2001; Nielsen et al., 2018). Complications include heart failure, myocardial abscess, aortic valve perforation, meningitis and glomerulonephritis. Brain abscess, osteomyelitis, chronic arthritis and primary bacteremia are rarely reported in immunocompromised patients. Chronic liver disease is an important predisposing factor, more than one-third of patients with systemic infection are alcoholics (Schuster et al., 1993; Balkhair et al., 2019).

Uncommon forms of infection due to *E. rhusiopathiae* include brain abscess, endophthalmitis, meningitis, psoas abscess, intra-abdominal abscess, prosthetic joint infection, tenosynovitis, pneumonia, epidural and paravertebral abscesses, periosteal dialysis-related peritonitis with bacteremia, necrotizing fasciitis and osteomyelitis (Kim et al., 2007; Elvy et al., 2008; Hocqueloux et al., 2010; Feasi et al., 2010; Meric and Keceli Ozcan, 2012; Groeschel et al., 2019).

Diagnosis

Identification of *Erysipelothrix* species can be difficult. They can have an uneven Gram stain appearance and appear as gram negative. Colonies are alpha-hemolytic and can resemble streptococci or enterococci. Similar to enterococci is also positive for pyrrolidonyl aminopeptidase (PYR) activity, but *Erysipelothrix* produce hydrogen sulfide (H₂S) on TSI agar and not enterococci. Positive catalase reactions differentiate *Corynebacterium* spp. and *Listeria* spp. from *Erysipelothrix* spp. which are negative for catalase, oxidase, indole, methyl red, Voges-proskauer and hydrolysis of esculin or urea. *Lactobacillus* spp. are catalase negative, slender Gram positive bacilli associated with endocarditis and bloodstream infection and can be vancomycin resistant as *Erysipelothrix* but *Lactobacillus* are negative for H₂S production (Jean et al., 2019).

Differential diagnosis

Clinically, the classic erysipeloid lesion is similar to staphylococcal or streptococcal cellulitis. In the differential diagnosis, a history of occupational exposure, lesions on the hands, absence of suppuration, subacute course, red-violet skin discoloration and pain apparently disproportionate to the extent of the lesion are typical of *E. rhusiopathiae* infection.

E. rhusiopathiae may be underdiagnosed and undertreated. It's important a good history including occupational exposure and be vigilant to add coverage for this uncommon fastidious organism.

Treatment

E. rhusiopathiae are generally susceptible to penicillin, cephalosporins, fluoroquinolones and carbapenems (Venditti et al., 1990).

Cellulitis of limited extent and without systemic damage can be treated with oral amoxicillin or ciprofloxacin.

More severe infection is treated with penicillin G sodium 12–20 × 10⁶ IU/day iv. In endocarditis treatment should be prolonged by 4–6 weeks (2 weeks iv and 2–4 weeks oral) (Mensa and Soriano, 2021).

Antimicrobial resistance

It is intrinsically resistant to co-trimoxazole, aminoglycosides and glycopeptides.

Patients with osteomyelitis usually receive empiric treatment with vancomycin and in abscesses is common to choose trimethoprim-sulfamethoxazole so this should be taken into consideration when *E. rhusiopathiae* is suspected (Lorenz et al., 2018).

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Coryneform Gram-Positive Bacilli

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Introduction

Corinebacteriae are immobile gram-positive bacilli, mostly catalase positive, also called “diphteromorphs or corineform” bacilli because they possess many strains of similar morphology. Called “diphtheromorphs” due to the possession of a morphology like *C. diphtheriae*, this being the oldest known pathogen of the genus and causal agent of diphtheria and “corineforms” by its form of mace or cane word whose etymology derives from the ancient Greek word “coryne” (Bernard, 2005; Funke and Bernard, 2007).

They have traditionally been considered as harmless saprophytic bacteria and their microbiological finding has been considered as contamination in sampling or as colonization with no clinical significance to the patient, however, their pathogenicity is believed to be underestimated in previous years and begins to take on importance, strengthening its role as a producer of certain opportunistic infections and in immunocompromised or prosthetic carriers. This has helped advance its more accurate and simple identification through the implementation in laboratories of more advanced molecular diagnostic methods and bacterial identification based on mass spectrometry, specifically in the MALDI-TOF MS.

The main pathogen of this genus and best known remains *C. diphtheriae*, a cause of diphtheria, a serious epidemic infectious disease that in developed countries has virtually disappeared thanks to immunization through the vaccine containing diphtheric toxoid and which is included in universal vaccine schedules (Wagner et al., 2012). In this article we will talk about the species of the genus *Corynebacterium* of medically relevant interest.

Microbiology

The genus *Corynebacterium* has more than 88 validated species that are increasing over the years, characterized by their high heterogeneity. It includes species that are of medical relevance because they produce rare infections in humans, although they do not always act as opportunistic pathogens. They are also important in veterinary medicine, where they are sometimes producers of zoonoses and also have uses in biotechnology (Bernard, 2012; Soares et al., 2013; Eikmanns and Blombach, 2014). They are bacteria that are present in the human microbiome but also in the environment, in water, in food, on the surface of inert materials.

They come from the bacterial family Corynebacteriaceae belonging to the phylum Actinobacteria. In this family the two genera *Corynebacterium* and *Turicella* (Busse, 2012) are closely related, their difference being that in the former the presence of mycolic acids

is detected in their cell wall except for three species (*C. amycolatum*, *C. atypicum* and *C. kroppenstedtii*) and absence of tuberculostearic acid (TBSA) except for some species where it is also present (*C. appendicis*, *C. bovis*, *C. confusum*, *C. kroppenstedtii*, some strains of *C. minutissimum*, *C. mucifaciens*, *C. tuberculoestearicum*, *C. urealyticum*) (Funke and Bernard, 2011) and *C. ureicelerivorans* (Yassin, 2007). In the genus *Turicella*, TBSA is always present but never mycolic acids.

In the last 30 years there have been multiple changes in the number of species assigned to this group of *Corynebacterium*. Many species have been included thanks to the improvement in identification techniques, even in some cases, all those species whose genetic characteristics were incompatible have been eliminated and have subsequently been included in other genus.

Corynebacteria of interest in human pathology are part of the normal microbiota of the skin, upper respiratory tract, digestive and genitourinary tract such as *C. jeikeium* and *C. amycolatum*. They are Gram-positive, aerobic or facultative anaerobic bacilli because they possess an enzyme called catalase which allows them to grow properly in the presence of oxygen. They are non-motile and non-sporulating.

Most corynebacteria grow well in the usual culture media that are available in microbiology laboratories. They multiply adequately by incubation at a temperature of 37 °C, with the exception of some strains that benefit from the addition of 5% CO₂, or a temperature of 30 °C or culture in media enriched with fosfomycin or 0.1–1% Tween 80 (Bernard, 2012).

Identification methods

There are three main methods of identification:

Method based on phenotype: Through the observation of the morphology of colonies after 24 or 48 h of incubation in those of slow growth. Using Gram staining, oxidase and catalase reactions and checking if the isolate has lipophilic or non-lipophilic character. In addition, the strains of present a wide variety of different metabolic processes.

According to this there is a useful way to classify corinebacteria based on their lipophilic or non-lipophilic character. These different lipophilic and non-lipophilic species, in turn, can behave as fermenters and non-fermenters of sugars (Funke et al., 1997).

	Fermentative	Oxidative
No Lipophilia	<i>C. amycolatum</i> , <i>C. argentoratense</i> , <i>C. auricanis</i> , <i>C. aurimucosum</i> , <i>C. canis</i> , <i>C. confusum</i> , <i>C. coyleae</i> , <i>C. diphtheriae</i> (biotipos <i>gravis</i> , <i>mitis intermedius y belfanti</i>), <i>C. durum</i> , <i>C. falsenii</i> , <i>C. flavescens</i> , <i>C. freneyi</i> , <i>C. glucuronolyticum</i> , <i>C. hansenii</i> , <i>C. imitans</i> , <i>C. matruchotii</i> , <i>C. minutissimum</i> , <i>C. pseudotuberculosis</i> , <i>C. riegliei</i> , <i>C. simulans</i> , <i>C. striatum</i> , <i>C. sundsvallense</i> , <i>C. thomsseni</i> , <i>C. timonense</i> , <i>C. tuscaniae</i> , <i>C. ulcerans</i> , <i>C. xerosis</i>	<i>C. afermentans</i> subsp. <i>Afermentans</i> , <i>C. auris</i> , <i>C. massiliense</i> , <i>C. mucifaciens</i> , <i>C. propinquum</i> , <i>C. pseudodiphtheriticum</i>
Lipophilia	<i>C. appendicis</i> , <i>C. accolens</i> , CDC grupo F1, CDC grupo G, <i>C. diphtheriae</i> biotipo <i>intermedius</i> , <i>C. kroppenstedtii</i> , <i>C. macginleyi</i> , <i>C. resistens</i> , <i>C. tuberculoestearicum</i> , <i>C. ureicelerivorans</i>	<i>C. afermentans</i> subsp. <i>Lipophilum</i> , <i>C. bovis</i> , <i>C. jeikeium</i> , <i>C. lipophiloflavum</i> <i>C. urealyticum</i>

Corynebacteria with relevance in human infections. Classification according to your oxidative or fermentative metabolism and lipophilia.

The phenotypic methods have as a limitation that they do not correctly identify 30% of the strains. The number of substrates to test with each strain is limited and are difficult to apply in strains of difficult growth. The results are sometimes obtained in days and the databases for establishing identification after the completion of the tests are updated infrequently.

Method based on the sequence of a gene: if we sequence the 16S rRNA gene we will have a good starting point to be able to discriminate between different species of the genus *Corynebacterium* and will allow in most cases its definitive identification. In exceptional cases where typing of the strain is not possible, we can resort to sequencing a secondary gene, such as the *rpoB* gene (Khamis et al., 2004).

Methods based on the use of MALDI-TOF: This bacterial identification tool is becoming a common resource in microbiology laboratories. It has an affordable cost and does not require to be carried out by highly trained technical personnel. A mass spectrometer detects proteins ionized and released from bacteria thanks to a laser. A pattern is then generated that is compared in a database giving a degree of coincidence (Konrad et al., 2010).

Virulence factors

Species of the genus *Corynebacterium* have different virulence factors:

Adhesion mechanisms: They can adhere to the eukaryotic cells of the host expressing molecules such as lipoglycans that interact with human respiratory cells. In addition, they have another type of adhesins of protein character anchored in the surface of the cell wall like pilis and fimbrias that act by means of mechanisms mediated by sortases.

Toxicity: some strains such as *C. diphtheriae* after being infected by lysogenic bacteriophages that hold the *tox* gene and after a conversion mechanism, can produce diphtheria exotoxin. Another toxin is phospholipase D, which also facilitates the spread in host tissues.

Intracellular survival: It can present lipids on the surface that allow them to alter the intracellular medium of macrophages and be able to live inside, preventing their elimination and causing cell death (Oliveira et al., 2017).

Mechanisms of resistance to antibiotics

In the evolution of antibiotic resistance of the genus *Corynebacterium* most studies conclude that extrachromosomal genetic elements are involved in resistance gene transfer. Horizontal gene transmission plays an important role in their pathogenicity. Antibiotic resistance genes are localized in large plasmids, for example, the pTP10 plasmid is responsible for the resistance to chloramphenicol, tetracycline, streptomycin, and erythromycin described in *Corynebacterium xerosis* (Oliveira et al., 2017).

Many species such as *C. striatum*, *C. pseudodiphtheriticum*, *C. jeikeium* and *C. coyleae* (Fernández-Natal et al., 2008) are resistant to macrolides because they possess ermX genes. For this reason, erythromycin, which had traditionally been the treatment of choice, has ceased to be used unless the sensitivity report of the laboratory confirms its sensitivity. It has also been linked to resistance to clindamycin, and trimethoprim-sulfamethoxazole.

Another example is the existence of multidrug-resistant strains, such as *C. amycolatum*, one of the most common strains of corynebacteria. This property is defined by the existence of resistance to three or more groups of antibiotics commonly used in the treatment of infection such as beta-lactam, aminoglycosides, macrolides, MLSB group and quinolones. The presence of mutations in the gyrA gene is related to resistance to treatment with quinolones (Ortiz-Perez et al., 2010).

Main species of *Corynebacterium*: Pathogenesis, clinical features, diagnosis and treatment

In the table below, we can visualize the most important pathogenic species of *Corynebacterium* along with the clinical manifestations attributed to them (Oliveira et al., 2017).

Taxonomy	Pathogenesis
<i>C. accolens</i> (Ang and Brown, 2007; Wong et al., 2010)	Pelvic Osteomyelitis; Granulomatous Mastitis, endocarditis, keratoconjunctivitis, infection of wounds and soft parts, otitis media, sinusitis, meningitis, bacteremia
<i>C. afermentans</i> subsp <i>afermentans</i>	Bacteremia
<i>C. afermentans</i> subsp <i>lipophilum</i>	Endocarditis. Liver and lung abscesses, lung abscesses and empyema in aids.
<i>C. amycolatum</i>	Endocarditis, sepsis. Bacteremia in immunocompromised patients, osteoarticular infections. Meningitis. Urinary tract infection. Wound infection. Otitis media. Prepuberal vaginitis. Breast abscess.
<i>C. argentoratense</i> (Riegel et al., 1995)	Tonsillitis
<i>C. aurimucosum</i> (Lo et al., 2015)	Urinary tract infection. Bacteremia. Osteoarticular infections.
<i>C. auris</i>	Otitis media in children
<i>C. auriscanis</i> (Collins et al., 1999)	Dog bite infection
<i>C. bovis</i> (Burr et al., 2012)	Hyperkeratosis, mastitis. CNS infection, Endocarditis. Chronic Otitis media infection of coma ulcers of coma joint prosthesis of ventricle-jugular bypass and glomerulonephritis. Bacteremia. Eye infection.
<i>C. camporealensis</i>	Mastitis
<i>C. canis</i>	Dog bite
<i>C. confusum</i>	Abscesses. Bacteremia
<i>C. coyleae</i>	Bacteremia. Urinary tract infection, soft parts infection, pancreatic abscess.
<i>C. diphtheriae</i>	Diphtheria
<i>C. durum</i> (Riegel et al., 1997; James et al., 2011)	Respiratory infection. Abscesses. Bacteremia
<i>C. falsenii</i>	Bacteremia. Isolated cerebrospinal fluid
<i>C. flavesces</i>	Axillary trichomycosis
<i>C. freneyi</i> (Auzias et al., 2003; Funke and Frodl, 2008)	Bacteremia. Granulomatous mastitis. Isolated in urine.
<i>C. genitalium</i>	
<i>C. glucuronolyticum</i>	Bacteremia, peritonitis, prostatitis, urethritis. Embedded cystitis. Granulomatous mastitis.
<i>C. imitates</i>	Pharyngitis. Bacteremia.
<i>C. jeikeium</i>	Nosocomial infections in long-hospitalized patients such as neutropenia or AIDS patients receiving broad-spectrum antibiotic treatment or carrying a vascular catheter. Sepsis. Bacteremia, pulmonary infiltrates, rash, endocarditis, meningitis. Prosthetic or foreign infections, gangrene. Malignant external otitis in diabetics
<i>C. kroppenstedtii</i>	Endocarditis, breast abscess, neutrophilic cystic granulomatous mastitis, pulmonary infections, bacteremia.
<i>C. kutscheri</i>	Lung abscess
<i>C. lowii</i>	Eye infection
<i>C. macginleyi</i>	Eye infections post-surgical endophthalmitis conjunctivitis queratitis. Rarely endocarditis. Aortic abscess. Bacteremia. Surgical infection. Respiratory infection (Trachee bronchitis, pneumonia, pneumonia associated with mechanical ventilation)
<i>C. massiliense</i>	Hip arthritis
<i>C. mastitidis</i>	Subclinical mastitis

(Continued)

Taxonomy	Pathogenesis
<i>C. matruchotii</i>	Oral infections
<i>C. minutissimum</i> (Granok et al., 2002)	Erythrasma. Bacteremia. Endocarditis. Vascular prosthetic infection. Peritonitis in patients on peritoneal peritonitis secondary dialysis. Meningitis. Pseudo meningococcal infection. Mastitis
<i>C. mucifaciens</i>	Bacteremia. Infection of soft parts by cat bite. Arthritis. peritonitis. Corneal ulcer. Implantable cardioversion defibrillator infection
<i>C. oculi</i>	Eye infection
<i>C. pilbarensae</i>	Ankle abscess
<i>C. pilosum</i>	Cystitis
<i>C. propinquum</i> (Abdolrasouli and Roushan, 2013)	Urethritis. Endocarditis. Bacteremia. Osteosynthesis infection. Axillary trichomycosis. Keratitis. Osteitis. Joint prosthetic infection. Pneumonia. Sharpening COPD. Pleural effusion. Sinusitis.
<i>C. pseudodiphtheriticum</i> (Martaresche et al., 1999)	Nosocomial Pneumonia; Bronchitis. Tracheitis in intubated patients. Endocarditis. Skin ulcer. Urinary tract infection. Keratitis. Conjunctivitis. Iatrogenic arthritis.
<i>C. pseudogenitalium</i>	Urinary tract infection
<i>C. pseudotuberculosis</i>	Caseous Lymphadenitis in patients in contact with sheep or have drunk non-sterile milk. Lab-acquired pneumonia.
<i>C. renale</i> (Honda and Yanagawa, 1973)	Cystitis; Pyelonephritis
<i>C. resistens</i> (Schroder et al., 2012)	Bacteremia. Abscesses.
<i>C. riegliei</i> (Funke et al., 1998)	Urinary tract infection. Bacteremia. Isolated in umbilical cord blood.
<i>C. simulans</i> (Wattiau et al., 2000)	Bacteremia. Biliary infection. Breast implant infection. Joint prosthetic infection
<i>C. striatum</i> (Sheldon and Coudron, 1990)	Endocarditis. Foreign material infections. Bacteremia. Endocarditis. Infection of surgical wound and soft parts. Visceral abscesses. Conjunctivitis. Keratitis. Osteomyelitis Epidural abscess. Synovitis. Arthritis. Meningitis. Respiratory infections (Pneumonia, Empyema). Lung abscess. Peritonitis. Endometritis. Urinary tract infection. Mastitis.
<i>C. sundsvallense</i>	Bacteremia. Gynecological infection. Infection soft parts.
<i>C. thomsseni</i>	Isolated in pleural liquid
<i>C. timonense</i> (Henrique et al., 2011; Merhej et al., 2016)	Isolated in blood
<i>C. tuberculoostearicum</i>	Arthritis. Osteomyelitis. Granulomatous mastitis. Pancreatic pancreatitis.
<i>C. tuscaniae</i>	Endocarditis
<i>C. ulcerans</i> (De Zoysa et al., 2005)	Pharyngeal Diphtheria in people exposed to livestock. Lung infection.
<i>C. ulceribovis</i>	Ulceration
<i>C. urealyticum</i>	Urinary tract infection. Chronic cystitis embedded. It rarely causes pneumonia, peritonitis, endocarditis, bacteremia, osteomyelitis, surgical wound infection.
<i>C. ureicelerivorans</i>	Septicemia. Ascites.
<i>C. xerosis</i>	Bacteremia. Neonatal sepsis. Endocarditis. Foreign material infections. Brain abscess. Meningitis. Arthritis. Spinal osteomyelitis. Pneumonia. Mediastinitis. Intra-abdominal and surgical wound infection.

This table of the genus *Corynebacterium* has been modified from references Bernard (2005), Bernard and Funke (2012), Funke and Bernard (2011), Wattam et al. (2014), Mensa et al. (2021).

We detail below some of the most important species of corinebacteria with human clinical relevance, specifying which disease is caused by the infection and what will be the appropriate antibiotic regimen. Treatment for the different species of *Corynebacterium* is obtained by antibiotic sensitivity tests according to the criteria of the reference laboratories EUCAST and CLSI.

***Corynebacterium diphtheriae*, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*: A disease called diphtheria**

Diphtheria is an epidemic acute bacterial disease of worldwide distribution rare in developed countries due to universal vaccination against the disease. The disease mainly affects the upper respiratory tract such as tonsils, nasal mucosa, larynx and pharynx. Less often it can affect the skin, eye conjunctiva and vagina.

The species *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* form the group *C. diphtheriae*. These three species share different characteristics being the most important the possibility of being able to host the bacteriophage that induces the generation of the diphtheria exotoxin and/or the production of phospholipase D (Belchior et al., 2009).

Man is the only reservoir of *C. diphtheriae* and animals are the reservoir of *Corynebacterium ulcerans* like sheep and to a lesser extent domestic animals. In poor countries, cases of diphtheria continue to appear because they do not have adequate immunization in the absence of well-structured vaccination plans for the population. The development of European active immunization programs against diphtheria has been successful and is now an uncommon disease in our midst.

Other infections caused by *C. diphtheriae* produced by strains that do not produce diphtheria exotoxin are arthritis, endocarditis, sepsis and osteomyelitis mainly in parenteral drug users.

Etiology

Diphtheria is produced by bacteria of the genus *Corynebacterium*. The disease occurs when the subject becomes infected with a toxigenic strain of *C. diphtheriae* and is less frequently caused by *C. ulcerans*. The species *Corynebacterium pseudotuberculosis* is usually the etiological agent of granulomatous lymphadenitis located in subjects who are in contact with sheep or have taken unsterilized milk, but there are also strains capable of producing toxin.

There are four biotypes of *C. diphtheriae*: *gravis*, *mitis*, *intermedius* and *belfanti*. Everyone can carry the gene that encodes the diphtheria toxin.

Epidemiology

It is a disease that mainly affects young children but the good vaccination coverage in this age group has led to cases being observed in adult individuals.

Sixty-two cases of diphtheria confirmed by reference laboratories were reported in Europe in 2018. Twenty-nine cases due to *Corynebacterium diphtheriae* and 33 cases caused by *Corynebacterium ulcerans*. The overall reporting rate was 0.01 cases per 100,000 population.

Diphtheria caused by *C. diphtheriae* was reported by nine countries. Most cases of diphtheria due to *C. diphtheriae* were imported. Among the affected countries, Latvia reported the highest number of autochthonous cases followed by France (European Centre for Disease Prevention and Control, 2021; Centers for Diseases Control and Prevention, 2018).

Since 1904 diphtheria is a notifiable disease in Spain. The skin form of diphtheria began to be declared in 2014. Confirmation of a case of diphtheria requires isolation in a clinical sample of a strain of *C. diphtheriae*, *C. ulcerans* or *C. pseudotuberculosis* capable of producing toxin (National Epidemiological Surveillance Network (RENAVE), 2019).

The last two cases reported during the twentieth century in Spain were recorded in 1986. No cases of respiratory diphtheria were reported over a period of 28 years. From 2014 to 2019, eight cases of diphtheria have been reported, three of them of imported origin from an endemic area. In two cases the clinic was respiratory, in two others the disease was located in the cutaneous and the other case the clinical manifestation was respiratory and cutaneous. In the reference laboratory, *C. diphtheriae* and *C. ulcerans* were identified as etiologic diphtheria agents and were not associated with a single clinical presentation (National Epidemiological Surveillance Network (RENAVE), 2019).

Diphtheria is a life-threatening infectious clinical picture. The lethality if the disease is not treated is 5–10%, being 20% in children under 5 years.

Pathogenesis and clinical manifestations

The disease is produced by toxigenic strains. For a strain to produce toxin it must have been infected by a lysogenic phage containing the diphtheria toxin gene which is type A/B.

Transmission of *C. diphtheriae* is from person to person by air or by close physical contact with the patient or asymptomatic carrier. When the microorganism reaches the susceptible subject, it begins its multiplication and after 2–7 days of incubation period begins to excrete the toxin that is its main virulence factor and justifies the appearance of symptoms at a distance from the focus of respiratory infection, such as myocarditis, renal failure, neurological involvement, etc.

The clinic is characterized by the appearance of characteristic lesions located in the mucous membranes have the appearance of pseudomembranes and that are in the respiratory mucosa of the upper respiratory tract. It usually starts in tonsils and thickens, acquiring a grayish color. The lesion, called diphtheria membrane, is drawing a kind of mold of pharynx and trachea that ends up compromising the adequate respiratory function of the patient. When trying to separate it bleeds easily so that the necrotic cells together with inflammation and exudate favors the own elaboration of more toxin. Symptoms such as fever, chills, characteristic enlargement of the nodes of the neck, producing an appearance of bull's neck, sore throat and hoarseness, general malaise, runny nose and difficulty breathing, which can be aggravated and require orotracheal intubation, are accompanied. At 10–14 days due to the spread of the toxin, cardiac complications such as myocarditis, renal complications appear and between 1 and 4 weeks later peripheral neuritis may appear. It is a life-threatening disease (American Academy of Pediatrics, 2018).

The transmission of *Corynebacterium ulcerans* is through contact with animals including domestic animals or the consumption of unpasteurized milk. Vesicular inflammatory lesions appear that evolve into a chronic grayish ulcer with defined edges.

The individual's vulnerability to diphtheria and its severity depends on its ability to generate toxins, the infective dose and the immune status of the person—(National Epidemiology Center, 2013).

Microbiological diagnosis

For the confirmation of the disease, it is necessary to take samples from the patient, ideally before the start of antibiotic treatment. Samples will be taken with swabs of Dacron or calcium alginate from the throat, pseudomembranes and nose. In the case of skin diphtheria, samples of the lesions will be sent. The samples will be sent to the reference laboratory for the realization of a culture.

Crops in which there is growth of *C. diphtheriae* will have to be biotyped and the toxigenicity of the strain will have to be confirmed. This will require phenotypic and genotypic testing.

The phenotypic tests are based on the Elek test which confirms the strain's ability to produce diphtheria toxin. The production of this toxin allows to confirm the disease. The phenotypic test is the reference test.

Genotypic tests detect the tox gene by performing a PCR. They are easy to interpret and quick to perform, but a positive result does not confirm the disease since there are strains of *Corynebacterium* that have the tox gene and yet biologically do not express it, so they are not toxigenic strains.

Treatment

As soon as there is a suspected case, start treatment with diphtheria antitoxin, after taking clinical samples, although there is no diagnostic confirmation. The antitoxin does not neutralize the diphtheria toxin once it has penetrated the cell interior and the delay in its administration is related to the lethality of the disease due to remote complications. Administer 20,000–120,000 U (children 2000–5000 U/Kg) preferably via i.v.

Antibiotic treatment has the utility of destroying microorganisms to stop the production of diphtheria toxin. An intravenous or oral macrolide or penicillin procaine 600,000 IU/12 h i.m may be used until symptoms improve. Complete with amoxicillin 500 mg/8 h until 14 days. Therapeutic alternatives in case of allergy can be Cotrimoxazole, clindamycin, rifampicin and linezolid (Mensa et al., 2021).

If there is respiratory distress, it may be necessary to perform a tracheostomy or intubation in order to maintain the supply of oxygen to the body.

The patient will have to be vaccinated after treatment since the disease does not confer in all cases natural immunity (Vaccine Advisory Committee (CAV-AEP), 2018; Diphtheria Vaccine, 2017).

It is important to confirm pharyngeal eradication by performing a culture from 2 weeks after the end of the antibiotic regimen. In the case of persistence of the bacterium, it is necessary to elongate the treatment 14 more days (European Centre for Disease Prevention and Control, 2021).

Patients with pharyngeal diphtheria require drop-type or contact-type isolation precautions in the case of cutaneous diphtheria. Action by public health agencies is needed to limit the spread of the disease through contact tracing.

Early administration of diphtheric antitoxin because it only neutralizes circulating toxin. Adult dose of 20,000–120,000 U. Treatment for children should be adjusted according to weight, 2000–5000 U/kg. It should be administered intravenously or intramuscularly according to severity and days of disease evolution. Antibiotic treatment with penicillin procaine 600,000 IU/12 h or a macrolides i.v. Administer if there is improvement of the patient amoxicillin 500 mg/8 h orally for 10–14 days in total. In case of intolerance to the previous treatment clindamycin, rifampicin, linezolid and cotrimoxazole may be used (Mensa et al., 2021).

It may be necessary to intubate the patient if the breathing is compromised. The patient will have to be vaccinated once the disease has been overcome, along with the request for a culture in the oropharyngeal zone to confirm the eradication of the microorganism.

Corynebacterium striatum

Pathogenesis and clinical manifestations

Multidrug-resistant strains of *Corynebacterium striatum* have frequently been associated with nosocomial outbreaks with high mortality. Some research has described this microorganism as potentially resistant. The main theory is based on the combination of two main factors:

The first factor is the existence of antibiotic resistance emerging from cross-resistance to antiseptics and disinfectants that are widely used in industrialized countries for cleaning hospitals to control nosocomial infections.

The second factor is the presence of these infections more frequently in immunocompromised patients, in patients with chronic respiratory diseases, with prolonged hospital admissions in surgical or intensive care rooms, in patients in whom invasive medical devices have been used and who had been treated with various antibiotic guidelines (Silva-Santana et al., 2021).

To this should be added its ability to modify the wall in order to reduce its permeability, the high lipid content and the ability to form biofilms (Rasmussen et al., 2020) implies favorable conditions for these bacteria to adhere to venous catheters constituting a risky environment and that can also contribute to increase their tolerance to various antimicrobials (Souza et al., 2019, 2020).

Because of the above, *Corynebacterium striatum* can produce foreign material infections (Kalt et al., 2018), catheter infections, bacteremia and endocarditis, surgical wound and soft tissue infection, abscesses, synovitis, arthritis, meningitis, respiratory infections such as empyema pneumonia, peritonitis endometritis, mastitis, urinary tract infection and eye infections such as conjunctivitis and keratitis.

Treatment

Hospital *C. striatum* strains may have resistance genes, such as the erm(X) gene against erythromycin and clindamycin, the tetA and tetB genes against tetracyclines and oxacillin, the cmx and aphA1 genes encoding resistance to aminoglycosides and chloramphenicol and may also acquire resistance to beta-lactams and quinolones (Mensa et al., 2021; Campanile et al., 2009).

Drugs such as vancomycin, teicoplanin, daptomycin (resistance to this antibiotic can appear quickly), linezolid and imipenem maintain good activity (Reddy et al., 2012).

Corynebacterium amycolatum, Corynebacterium jeikeium, Corynebacterium resistens and Corynebacterium urealyticum

Pathogenesis and clinical manifestations

C. amycolatum: it is part of the normal microbiota of the skin and being one of the Corinebacteria found most frequently in human clinical samples. In the clinical laboratory is isolated in blood cultures as a possible producer of bacteremia in immunocompromised patients, sepsis, endocarditis on native valve, infection of prosthetic material (catheters, heart valves, arthroplasties, pacemakers, etc.), osteoarticular infections, meningitis, surgical wound infection, urinary tract infection, prepubertal vaginitis, breast abscess and as a producer of otitis media.

C. jeikeium: It is part of the axillary and perineal microbiota. It causes diseases in patients with long periods of hospitalization who have been prescribed broad-spectrum antibiotics. This microorganism causes infections such as sepsis (Rasmussen et al., 2020), pleuropulmonary infection, rash, intravascular catheter infections, meningitis or endocarditis in prosthetic valves, especially in immunocompromised persons, neutropenic patients or patients with acquired immunodeficiency syndrome. In diabetics it can produce gangrene and malignant otitis externa.

C. resistens: in bacterial cultures it is visualized as grayish-white, bright, pearlescent colonies up to 1.0 mm in diameter after 48 h of incubation in TSA with sheep blood of 5%. It has lipophilic characteristics. If Tween 80 is added to a concentration of 1% the colony increases its growth to 2–3 mm. It mainly produces rapidly deadly bacteremia in immunocompromised patients and abscesses in different anatomic zones (Otsuka et al., 2005).

C. urealyticum: usually is an opportunistic pathogen of the urinary tract. It takes advantage of favorable circumstances, such as previous antibiotic regimen associated with hospital maneuvers such as probing, urological surgery, cystoscopies. It produces acute or chronic cystitis, fouling cystitis, pyelonephritis and sepsis of urinary origin. It can also produce other pathologies typical of an opportunistic pathogen, such as bacteremia, endocarditis, osteomyelitis, skin infections and soft tissues.

Treatment

Because these strains may have resistance genes to several antibiotics, it is essential to perform sensitivity tests in microbiology laboratories. These tests are intended to confirm the possibility of treatment with erythromycin, quinolones, rifampicin, doxycycline and fosfomycin.

It is best to start treatment with antibiotics that are often sensitive such as linezolid, tigecycline, daptomycin and glucopeptides.

In the case of *C. urealyticum* it may be necessary along with antibiotic treatment the elimination of foreign bodies such as urinary tubes. Endoscopy may also be required to remove mucosal fouling from the urinary bladder along with treatment to acidify urine (Mensa et al., 2021).

Corynebacterium glucuronolyticum

Pathogenesis and clinical manifestations

Corynebacterium glucuronolyticum is usually an opportunistic pathogen can produce infections in humans, such as bacteremia, peritonitis and granulomatous mastitis, but is mainly involved in pathologies of the male genitourinary tract by which it has some specificity.

It highlights its important role as an infectious agent of embedded cystitis in men, in addition to generating male non-gonococcal urethritis and also persistent bacterial prostatitis chronic paucisymptomatic monomicrobial, appearing abundantly in semen samples (Mestrovic, 2018).

Its role has become more relevant recently because its presence in clinical samples was not investigated and its detection was not part of the basic diagnostic protocols of genitourinary tract infections (Ruiz-Pino et al., 2019).

Treatment

Corynebacterium glucuronolyticum is usually sensitive to beta-lactam antibiotics, gentamicin, cotrimoxazole and vancomycin. It is often resistant to fluoroquinolones and macrolides.

Corynebacterium macginleyi

Pathogenesis and clinical manifestations

Bacteria with lipophilic characteristics. Fermentative metabolism. In the bacterial culture there are colonies of corynebacteria of convex morphology and small size, typical of lipophilic *Corynebacterium*, with pink appearance (Riegel et al., 1995). It causes eye infections such as keratitis, postsurgical endophthalmitis and conjunctivitis. Less frequent as etiology of bacteremias, respiratory infections associated with mechanical ventilation, aortic abscess, endocarditis and surgical infection.

Treatment

It may show antimicrobial susceptibility to amikacin, gentamicin, chloramphenicol. Increased resistance to fluoroquinolones is described (Muñoz Lorenzo, 2015).

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Nocardia, *Rhodococcus*, *Streptomyces* and Other Aerobic Actinomycetes

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Introduction

The aerobic actinomycetes is a group of Gram-positive filamentous bacillary organisms that grow better under aerobic than anaerobic conditions. The term “actinomycete” derives from the Greek words “actino” as ray and “mycete” as fungus. Because, they are characterized by their peculiar morphology to undergo complex cellular differentiation like filamentous fungi. They tend to form branches, filaments and spores, being in the past, known as fungi as they true hyphae. The aerobic actinomycetes are known to be an evolutionary heterogeneous assemblage of divergent genera belonging to the phylum *Actinobacteria*, in the class *Actinobacteria* (McNeil and Brown, 1994; Conville and Witebsky, 2011). The heterogeneity of this phylum, one of the largest within the Bacteria domain, in terms of morphology, physiology, metabolism and genome, reflects its biodiversity (Ventura et al., 2007; Barka et al., 2015). Most of them are free-living organism, widely distributed in different terrestrial and aquatic ecosystems, natural or man-made (Goodfellow and Williams, 1983), with interest in human and veterinary medicine, ecology and biotechnology. They have an full-scale secondary metabolism, producing about two-thirds of natural derived antimicrobials, as well as anticancer, immunomodulators agents and others bioactive compounds (Mochon et al., 2016; Barka et al., 2015; Nouioui et al., 2018). The taxonomy of aerobic actinomycetes is not an easy matter, with frequent incongruities, pitfalls and emmendations. Fortunately, the data of the new genomic and proteomic technologies can provide a better understanding of this phylum than those previously available with the polyphasic taxonomy (genotypic and phenotypic data) (Nouioui et al., 2018; Tamura et al., 2018). In fact, the backbone of taxonomic studies, the analysis of the 16S ribosomal RNA (rRNA) gene sequence, is too conserved to distinguish two closely related species in many cases (Conville et al., 2017). The common application of whole-genome sequencing (WGS) for comparative genome hybridization (CGH), average nucleotide sequence identity and average amino acid sequence identity (ANI)/ (AAI), differences of guanine-plus-cytosine (G + C) content in bacteria with their respective cut-offs (>70%, >94%, and <1%, respectively) could be of help to delineated taxonomy of this phylum (Achtman and Wagner, 2008; Sangal et al., 2016; Moore et al., 2010).

At the time of writing, the majority of human pathogens of the aerobic actinomycetes group are placed in the order *Mycobacteriales* with the genera *Corynebacterium*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* (Tindall, 2019; Parte et al., 2020). Until recently, they were included in the order *Corynebacteriales* (Goodfellow and Jones, 2012; Nouioui et al., 2018). With the

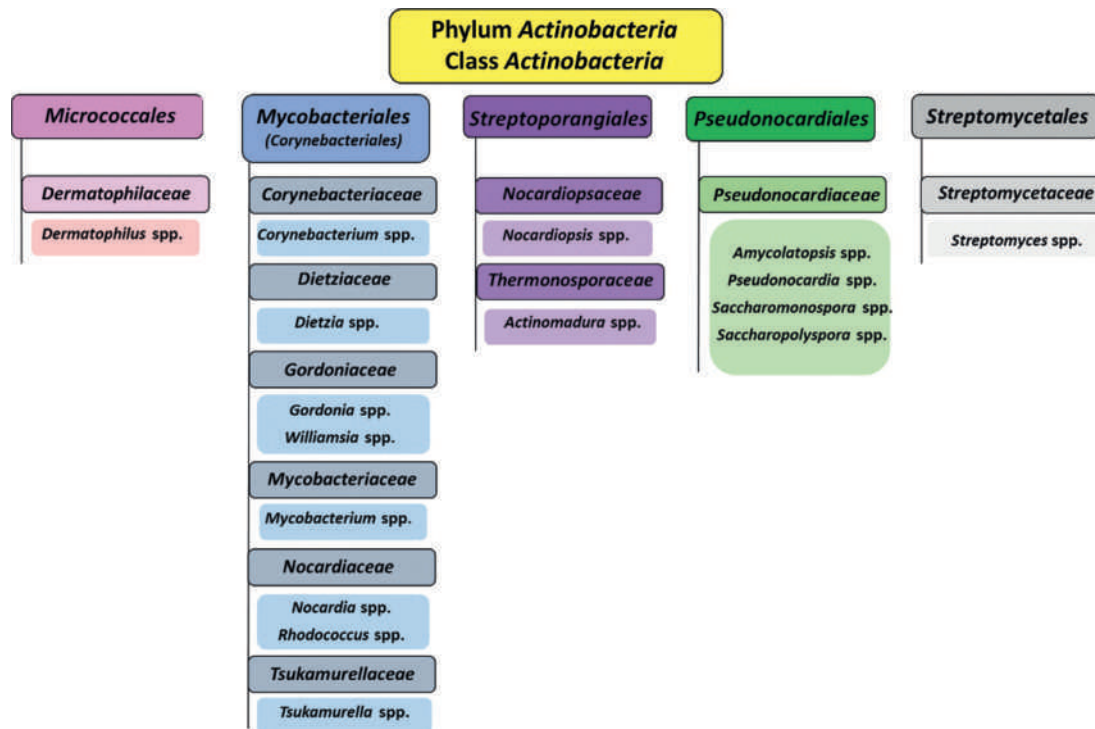


Fig. 1 Current classification of genera of aerobic actinomycetes considered to be human pathogens according to the List of Prokaryotic Names with Standing in the Nomenclature (<https://lpsn.dsmz.de/>) (date of accession 18 May 2021) (Parte et al., 2020). The name *Corynebacteriales* (Goodfellow and Jones, 2015) is not the earliest validly published name, being replaced by *Mycobacteriales* (Cavalier-Smith, 2002), meanwhile a solution may be in sight (Tindall, 2019).

phylogenomic data, a possible reclassification of this order *Mycobacteriales/Corynebacteriales* is possible (Vázquez-Boland and Meijer, 2019; Salam et al., 2020). They have a high content of G + C in their genomes and mycolic acids (chemotype IV) in their cell walls. The current classification according to the List of Prokaryotic Names with Standing in the Nomenclature (<https://lpsn.dsmz.de/>) (Parte et al., 2020) is shown in Fig. 1.

Among the extensive phylum *Actinobacteria*, only few genera possess clinical significance in human or veterinary or plant health. They are rarely considered a part of the normal human flora, and mainly cause opportunistic infections, except *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, and *Dermatophilus* (Saubolle, 2009). In the last decades, these infections have raised due to the increase of the immunosenescent population, and of those patients with immunosuppression conditions and underlying diseases (transplant recipients, neoplasia and leukemia, chronic lung disease, AIDS, corticosteroid treatment). With an acquisition from environmental source by inhalation or traumatic inoculation, the spectrum of disease in human is wide, which may differ greatly between the genera and even between species, but which also may have a great amount of overlap. Many times the infections caused by aerobic actinomycetes are infradiagnosed, because the clinical significance of many species is not clear or the methods for identification fail (McNeil and Brown, 1994; Conville and Witebsky, 2011; Barka et al., 2015; Conville et al., 2017).

This chapter deals only genera with most impact on human health care, excluding *Corynebacterium* and *Mycobacterium* (deal in others chapters). Despite the fact, the prevalence of specific genera and species varies geographically, *Nocardia* spp. is the main etiological agent followed by far by *Rhodococcus* spp., *Gordonia* spp. and *Tsukamurella* spp., with the increase of the mycetoma cases by *Streptomyces* spp. and *Actinomadura* spp. A better understanding of their characteristics as habitat, pathogenesis and immunity, diagnosis, identification and typing, clinical and epidemiological features, antimicrobial susceptibility and therapy is recommendable, to achieve a good outcome of the infections produced by aerobic actinomycetes.

Nocardia

Nocardia genus is the most common aerobic actinomycete isolated as human pathogen. Genus type of the family *Nocardaceae*, *Nocardia asteroides* is the type species, although in the past it was also *Nocardia farcinica* (Goodfellow, 2012). From the description of this genus (Nocard, 1888; Eppinger, 1891), its taxonomic classification has always been a challenge due to the misidentification of the type strain, the reassignment of species to diverse genera (*Actinomadura*, *Amycolatopsis*, *Oerskovia*, *Rhodococcus*, and *Rothia*), the presence of complexes in the same or neighboring species, and the description of new species through identification by genetic, genomic and proteomic techniques (Lechevalier, 1976; Goodfellow and Jones, 2012; Nouiouei et al., 2018). At the time of writing, the genus contains 114 species with validly published names (Parte et al., 2020). More than 90 *Nocardia* species have been recovered

in human samples, being several species unusual causes of a wide spectrum of clinical diseases in both humans and animals. The most common species isolated in clinical specimens are *N. asteroides*, *Nocardia brasiliensis*, *Nocardia cyriacigeorgica*, *N. farcinica*, *Nocardia nova*, *Nocardia otitidiscaviarum*, and *Nocardia transvalensis* (Ambrosioni et al., 2010; Tremblay et al., 2011; Chen et al., 2014; Hardak et al., 2012; Valdezate et al., 2017; Lebeaux et al., 2019; Huang et al., 2019; Tan et al., 2020). *Nocardia* spp., is an opportunistic pathogen, that mainly affects lungs and frequently disseminates to other sites including the central nervous system both immunosuppressed and in apparently immunocompetent people (Saubolle and Sussland, 2003; Coussement et al., 2016; Paige and Spelman, 2019; Wilson, 2012).

Members of the genus are aerobic, Gram-stain positive or gram-variable, weakly acid-fast, non-motile, and mycolic acid-containing actinomycetes. They form an extensively branched mycelia and substrate hyphae (0.5–1.2 µm in diameter) that fragment into rod-shaped or coccoid elements. Macroscopically, visible aerial hyphae may be lacking, sparse, or very abundant, which grow on the surface and penetrate agar media (Goodfellow and Jones, 2012; McNeil and Brown, 1994). The genome sizes of *Nocardia* strains can range from 6.00 Mb (*Nocardia paucivorans*) to 10.52 Mb (*Nocardia miyunensis*), with an average of 7.86 Mb. *Nocardia vinacea* showed the lowest DNA G + C content (65.5 mol%), whereas *Nocardia harenae* has the highest (72.0 mol%) (Tamura et al., 2018).

Habitat

This organism possesses a ubiquitous nature, able to localize in different types of ecological niches. In fact, they are among the most predominant actinobacteria of the soil microbiota, including that of the extreme biosphere (Bull and Asenjo, 2013). *Nocardia* spp. species are found everywhere from sludge and soil to contaminated soil water, foaming coastal marine waters, deep-sea sediments, lake, and desert habitats (Goodfellow and Jones, 2012; Mohammadipanah and Wink, 2016). Some species even infect plants and animals (Yaemsiri and Sykes, 2018; Yasuie et al., 2017). Climate, temperature, humidity, pH and type of soil influence the concentration and diversity of *Nocardia* species founded in the different geographical areas (Andalibi et al., 2015). Like other actinomycetes, *Nocardia* spp. contribute to soil health, playing major roles in the cycling of organic matter, inhibiting the growth of plant pathogens, and decomposing complex mixtures of dead plants and animals (Bhatti et al., 2017).

Pathogenesis and immunity

Nocardia species are an optional intracellular organism, able to survive and multiply inside the macrophage and host tissue. This genus neutralize the activity of the macrophage and the oxidative killer mechanism, with the reduction of the phagosome-lysosome fusion and the intracellular acid phosphatase levels in macrophages, with the production superoxide dismutase, catalase, toxins and cell wall glycolipids (Beaman et al., 1985). Other virulence genes were observed in the analysis of the genome as caseinolytic peptidases, cytolysin, hemolysin, lipases, metalloproteases, phosphatases, phospholipase able to destroy tissues along with hydrolases, mammalian cell invasion genes, esterases, hemagglutinin, the antigen 85 complex family, ESX-1, and sortase A among others (Vera-Cabrera et al., 2012; Gonzalez-Carrillo et al., 2016; Yasuie et al., 2017; Carrasco et al., 2017; Nouiouui et al., 2020). The *Nocardia* cell wall deficient variants (L-forms) are involved in pathogenesis in in vivo animal models and contribute to latency of disease (Beaman, 1980). Lung macrophages and T-cell mediated immunity play a key role in the local control of *Nocardia*. So, invasive nocardiosis mostly affect patients receiving any immunosuppressive agent and particularly with systemic corticosteroid therapy that causes a selective suppression of the Th1-cellular immunity (Margalit et al., 2020a; Lafont et al., 2020).

Diagnosis, identification and typing

In the direct Gram smears, *Nocardia* species appear as very long, branching, thin, and finely beaded rod. But, in the smears of cultures they appear as streptococcus-like chains or as small filaments. They are usually partially acid-fast due to the presence of intermediate-length mycolic acids in their cell wall. Although, Gram stain is more sensitive than the modified acid-fast (Kinyoun) stain (Saubolle and Sussland, 2003; Brown-Elliott et al., 2006). *Nocardia* spp. grow on most routine media, but buffered charcoal-yeast extract agar or modified Thayer-Martin medium may enhance their recovery. The colonial morphology will vary according to species, isolate, medium, incubation temperature and with the time of growth. Growth is aerobic, producing chalky, matt or velvety colonies of different colors (brown, tan, pink, orange, red, purple, grey or white) as it is shown in Fig. 2A–H. Soluble brown or yellow pigments may be produced. They are susceptible to the decontamination methods (*N*-acetyl-L-cysteine, etc.) Prolonged incubation from samples of patients with suspicious nocardiosis are recommended (Conville and Witebsky, 2015; UK Standards for Microbiology Investigations, 2016).

Conventional culture identification based on phenotypic methods (casein, tyrosine, xanthine, hypoxanthine together with other substrates) lacks enough sufficient discriminatory power and it has long turnaround time. The drug pattern type is also of help to the presumptive identification (Brown-Elliott et al., 2006). So, both are surpassed by the great advantage of the molecular techniques, essential to obtain an accurate identification (Tamura et al., 2012). PCR base on the sequencing of housekeeping genes of *rrs* (gene coding for 16S rRNA), *hsp65* (heat-shock protein 65), *secA* (preprotein translocase ATPase), *rpoB* (β-subunit of RNA polymerase), *gyrB* (β-subunit of DNA gyrase), *sodA* (superoxide dismutase) genes, and 16-23rRNA intergenic spacer region (Chun and Goodfellow, 1995; Clinical and Laboratory Standards Institute, 2008; Rodríguez-Nava et al., 2006; Kong et al., 2010; Takeda et al., 2010; Sánchez-Herrera et al., 2017). The 16S rRNA gene sequencing is the gold standard for *Nocardia* identification. But, due to the

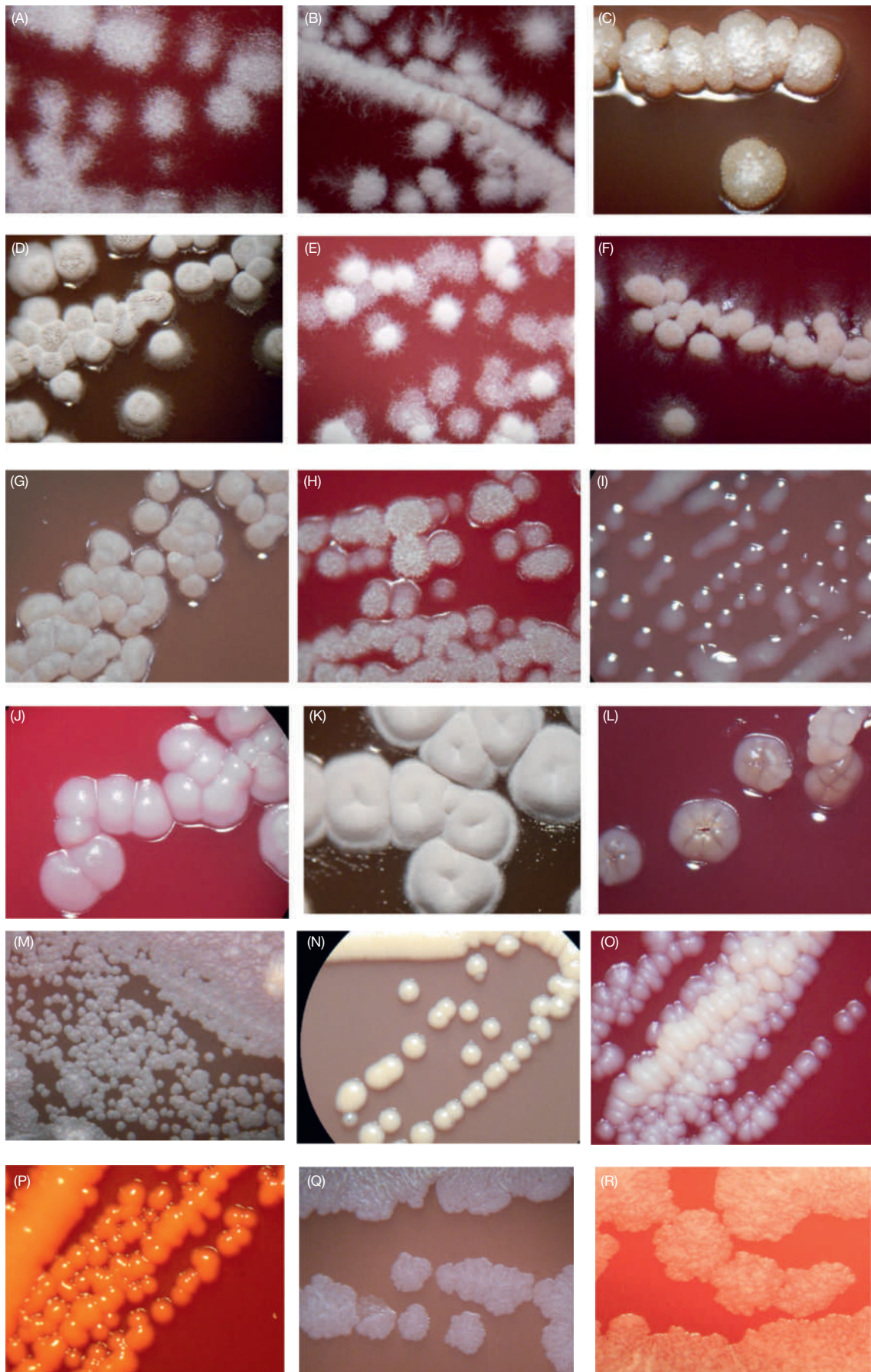


Fig. 2 Morphology of colonies of clinical strains of several aerobic actinomycetes grown on Columbia agar (with 5% of sheep blood) with 3–5 days of incubation: (A) *Nocardia abscessus*; (B) *Nocardia asteroides*; (C) *Nocardia brasiliensis*; (D) *Nocardia cyriacigeorgica*; (E) *Nocardia farcinica*; (F) *Nocardia nova*; (G) *Nocardia otitidiscaviarum*; (H) *Nocardia transvalensis*; (I) *Rhodococcus equi*; (J) *Streptomyces albus*; (K) *Streptomyces cacaoni*; (L) *Actinomadura madurae*; (M) *Gordonia bronchialis*; (N) *Gordonia polyisoprenivorans*; (O) *Gordonia sputi*; (P) *Gordonia terrae*; (Q) *Tsukamurella tyrosinosolvens*; (R) *Tsukamurella pulmonis*. Photos have been taken by N. Garrido (National Centre of Microbiology. Reference and Research Laboratory for Taxonomy).

insufficient interspecies polymorphisms to differentiate some species, multiple copies to cause species misidentification, polymorphisms for a single species, and inability to distinguish species belonged to the same complex, make it necessary to complement the identification with another the mentioned above molecular targets or to combine them in a MLSA (multilocus sequence analyzes). The PCR-RFLP (restriction fragment length polymorphism) 16S rRNA and *hsp65* genes was also applied for identification, but the presence of different patterns and/or extrabands limits its use (Fatahi-Bafghi, 2018).

Nocardia species can be rightly identified by the matrix-assisted laser desorption ionization-time of flight mass spectrometry [(MALDI-TOF MS)] when a wide and varied *Nocardia* library is used. However, there are limitations of commercial databases for providing accurate identifications. While some species are only identified at complex level (*N. abscessus* complex, *N. brevicatena*, *N. paucivorans* complex, *N. nova* complex, and *N. transvalensis* complex), others species showed variation in the spectral profiles (as *N. cyriacigeorgica*) and uncommon species are not enough included in the library (Blosser et al., 2016; Carrasco et al., 2016; Conville et al., 2017). In addition, incubation time, culture media and extraction method influence the specie assignation (Verroken et al., 2010; McTaggart et al., 2018). For typing purposes, *gyrB* gene, MLSA, together with pulsed-field gel electrophoresis and random amplified polymorphic DNA-polymerase PCR and amplified fragment length polymorphisms were used (Kalpoe et al., 2007; McTaggart et al., 2010; Carrasco et al., 2013; Gnanam et al., 2020).

The most current taxonomy for *Nocardia* species (Conville et al., 2017) showed the presence of the following complexes: the *Nocardia abscessus* complex includes *N. abscessus*, *Nocardia arthritis*, *Nocardia asiatica*, *Nocardia beijingensis*, and *Nocardia pneumoniae*; the *Nocardia nova* complex includes *Nocardia africana*, *Nocardia aobensis*, *Nocardia cerradoensis*, *Nocardia elegans*, *Nocardia kruczakiae*, *Nocardia mikamii*, *N. nova*, *Nocardia vermiculata*, and *Nocardia veterana*; the *N. farcinica* complex includes *N. farcinica* and *Nocardia kroppenstedtii*; the *Nocardia transvalensis* complex includes *Nocardia blacklockiae*, *N. transvalensis*, and *Nocardia wallacei*. Their relationships among them and respect to other *Nocardia* species could be observed in the phylogenomic tree of Fig. 3.

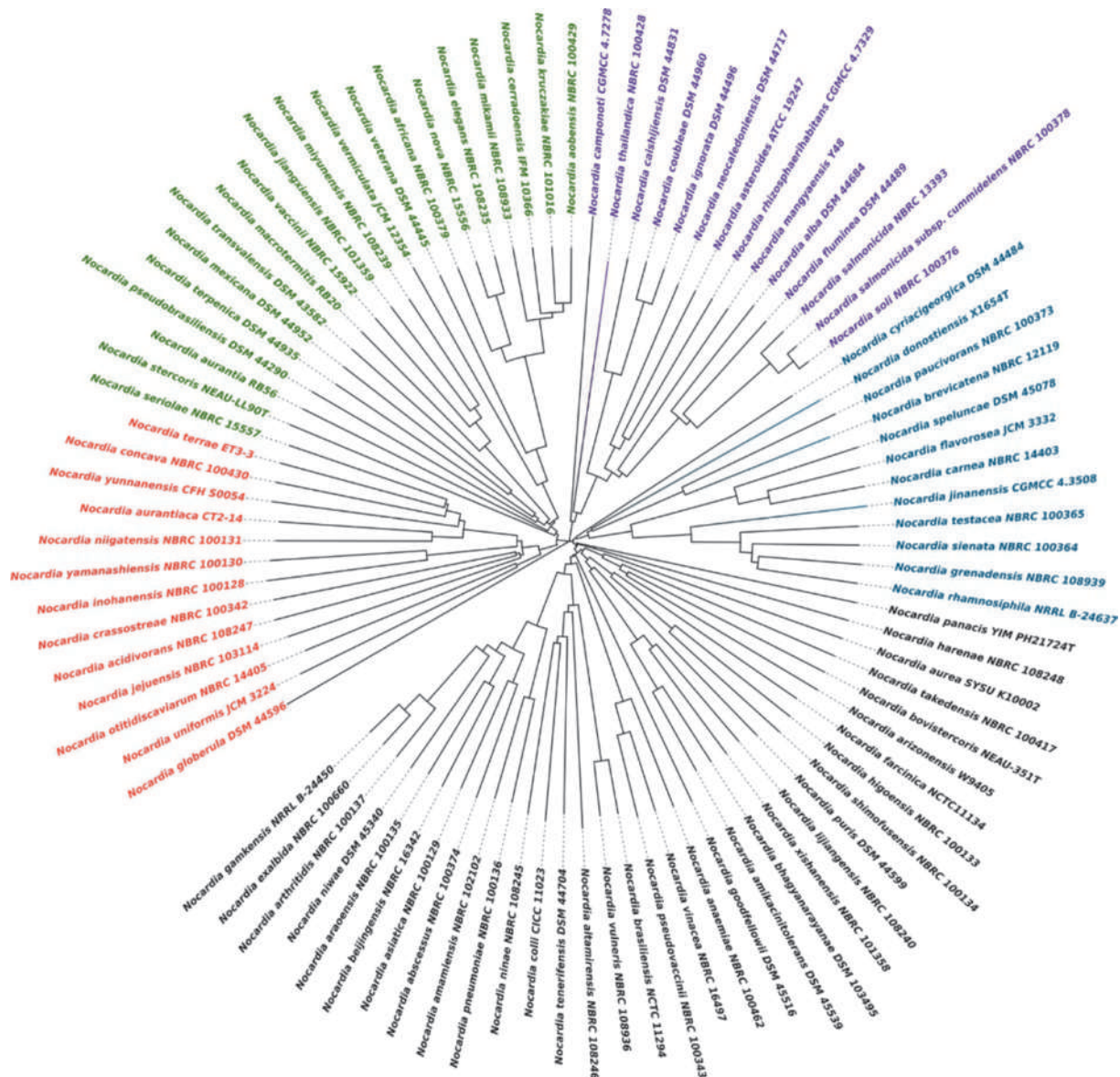


Fig. 3 *Nocardia* species phylogenomic tree inferred with FastME from GBDP distances determined with NCBI's assemblies. The results were provided by the Type Strain Genome Server (TYGS) (<https://tygs.dsmz.de>, accessed on 2nd April 2021) (Meier-Kolthoff and Göker, 2019). The adscription to different nodes is indicated by the different colors of species' names.

Clinical and epidemiological features of *Nocardia* infections

This environmental organism is mainly acquired by inhalation and traumatic inoculation, being pulmonary nocardiosis and mycetoma the most common entities. More than 60% of the nocardiosis cases present pulmonary involvement (Ambrosioni et al., 2010; Tremblay et al., 2011; Hardak et al., 2012; Valdezate et al., 2017; Fatahi-Bafghi, 2018; Huang et al., 2019). Cough and fever were the most common symptoms, but dyspnea, sputum and weight loss also appear. The inflammatory parameters (C-reactive protein and white blood cell count) were moderately elevated. One or more nodules or masses and pulmonary airspace consolidations were the most frequent radiologic abnormalities, and cavitation mostly occurred within 2 weeks (Chen et al., 2014). Some of these manifestations can be seen in Figs. 4–6 Chen et al. (2014). Right upper lobe is mainly involved (53.9%), perhaps because is one of the best ventilated areas (Ott et al., 2019). Owing to the non-specific clinical appearance of pulmonary nocardiosis, which mimic with pulmonary tuberculosis, invasive fungal disease and lung cancer, the median time to final diagnosis was 14–25 days (range 0–54 days) (Chen et al., 2014; Ott et al., 2019). In Germany, the overall average annual age-adjusted hospitalization rate was estimated in 0.04/100.000 inhabitants. With highest rate among men aged 75–84 years (0.24/100000) (Ott et al., 2019). Although, pulmonary nocardiosis associated with an underlying autoimmune disease showed a female predominance and presentation at younger age (Li et al., 2015). The mortality rate has been estimated about 18%, being death most frequent among patients who received immunosuppressive drugs (Chen et al., 2014). In those immunocompromised patients who present new nodules or masses evolving into cavitation in a short amount of time, and suffered from pneumonia with poor response to treatment, a high clinical index of suspicion of pulmonary nocardiosis is necessary for an early diagnosis and timely treatment (Chen et al., 2014).

An important trait of this organism is its ability to disseminate, when infection was present in two non-contiguous organs or in the central nervous system (CNS), and more prevalent in immunocompromised patients. *N. asteroides*, *N. abscessus*, *N. farcinica*, and *N. nova* cause the majority of invasive infections (Saubolle and Sussland, 2003; Lafont et al., 2020). Extrapulmonary sites of infection include the skin and soft tissue, CNS, and bloodstream. Being the CNS the most frequently affected site (Wilson, 2012), due to the special tropism of *Nocardia* for neural tissue from pulmonary or cutaneous infection. Responsible of 2% of all cerebral abscesses, high mortality rates are described in immunocompromised and in immunocompetent patients (55%, and 20%, respectively). Rates that increase to 66% with multiple abscesses (Patel et al., 2019). *Nocardia* brain abscess presents as a slowly progressive mass lesion, with variable signs and symptoms: focal neurologic abnormalities (hemiparesis, and cranial nerve palsies, among others), headache, fever, altered mental status, seizures, visual changes, nausea and vomiting, ataxia and falls (Anagnostou et al., 2014). As consequence that, more than 43% of the CNS nocardiosis case are asymptomatic (Coussement et al., 2016). Routine contrast-enhanced brain imaging is recommended in patients with nocardiosis. Magnetic resonance imaging is preferred

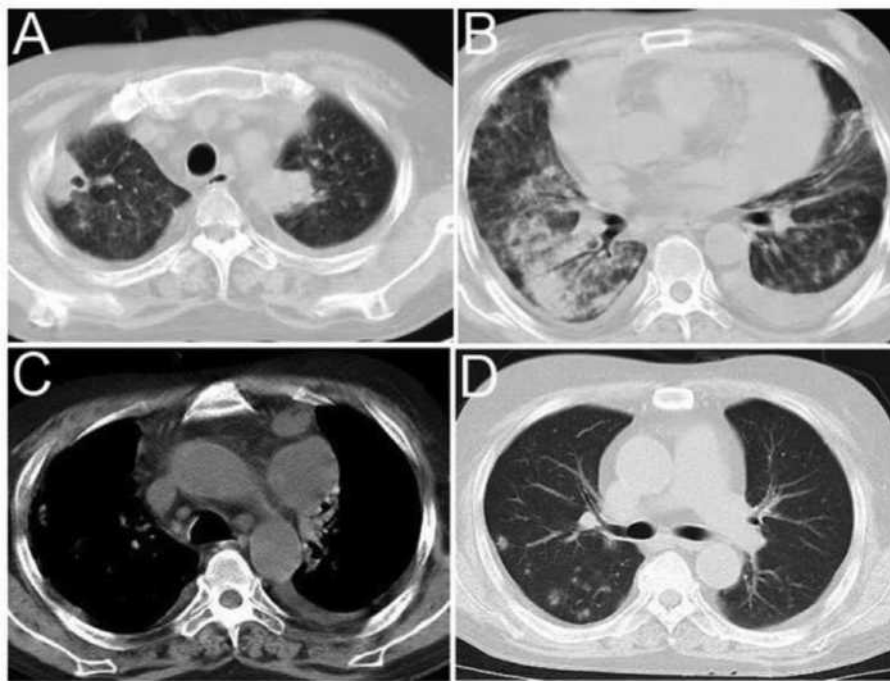


Fig. 4 A 45-year-old man on immunosuppressive therapy after renal transplantation was infected with *Nocardia asteroides*. (A–C) Axial CT images reveal multiple patterns of disease and lymphadenopathy; (D) An axial CT image reveals small pulmonary nodules with relapsed infection two years after the initial diagnosis (Chen et al., 2014). Courtesy of PLoS One. Chen J et al. (2014) Clinical and radiographic characteristics of pulmonary nocardiosis: Clues to earlier diagnosis. *PLoS One* 9(3): e90724. <https://doi.org/10.1371/journal.pone.0090724.g002>.

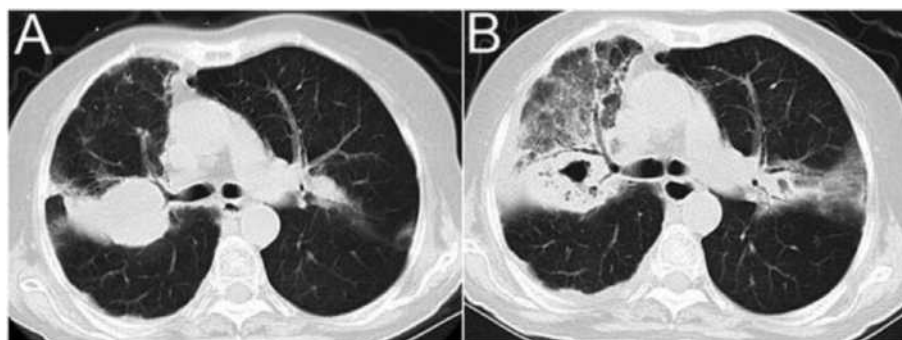


Fig. 5 A 63-year-old woman with COPD and *Nocardia* spp. infection. (A) An axial CT image reveals a lobar consolidation in the right upper lobar; (B) Axial CT imaging was performed 12 days later and the images reveal a cavitary consolidation on the right and GGO changes. Note the prominent air bronchogram (Chen et al., 2014). Courtesy of PLoS One. Chen J et al. (2014) Clinical and radiographic characteristics of pulmonary nocardiosis: Clues to earlier diagnosis. *PLoS One* 9(3): e90724. <https://doi.org/10.1371/journal.pone.0090724.g002>.

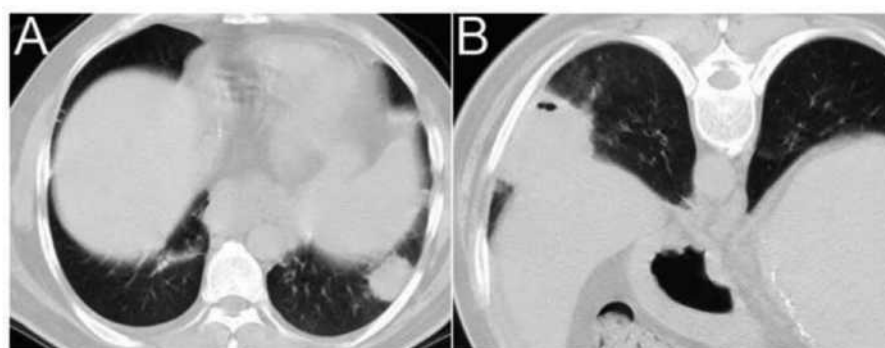


Fig. 6 A 42-year-old man after liver transplantation. He suffered from fever 15 days after the liver transplant. (A) Thoracic CT shows a solitary nodule in the left lower lobe. (B) CT-guided puncture of the left lower lobe was performed 20 days after the liver transplant, and a punctured tissue culture revealed *Nocardia* spp. infection (Chen et al., 2014). Courtesy of PLoS One. Chen J et al. (2014) Clinical and radiographic characteristics of pulmonary nocardiosis: Clues to earlier diagnosis. *PLoS One* 9(3): e90724. <https://doi.org/10.1371/journal.pone.0090724.g002>.

over computerized tomography, appearing as ring-enhancing lesions (Margalit et al., 2021). According to the literature, surgical excision or aspiration of the brain abscess seems to provide good chances of eradication of the disease if the patient demonstrates clinical deterioration or no improvement with the appropriate antibiotic therapy (seen within 1 week) (Patel et al., 2019). *N. farcinica* has been described as a more virulent pathogen with a tendency for disseminated infection, being the main causative agent in CNS nocardiosis (Brown-Elliott et al., 2015; Valdezate et al., 2017; Lebeaux et al., 2019).

Skin and soft tissue infections (SSTIs) could be primary cutaneous nocardiosis due to trauma or other inoculation, including iatrogenic, or secondary cutaneous nocardiosis due to disseminated infection. They include lymphocutaneous, mycetoma, and superficial skin infection, that can be misdiagnosed with fungal, viral or other bacterial infections. Lymphocutaneous infection typically presents as an ulcerated draining lesion at the site of injury accompanied by proximal lymphangitic spread with overlying subcutaneous nodules and often regional lymphadenopathy. Mycetoma is a chronic, slowly progressive infection that often starts as a small nodule at the site of traumatic entry of the pathogen, usually the foot. If left untreated, it can lead to mass-like lesions with destructive granulomata and fistula formation, affecting the underlying tissue and bone with significant disability. Superficial SSTIs can have a quite variable appearance including ulcers, papules, pustules, plaques, nodules, diffuse erythema, and cellulitis as well as abscess and fistula formation. Although many *Nocardia* species produces SSTIs, *N. brasiliensis* is most often associated with skin and soft tissue infections and seen more commonly in tropical and subtropical climates (Brown-Elliott et al., 2006; Hemmersbach-Miller et al., 2019).

Bacteremia, is a rare but serious entity, with high associated overall mortality (40%). It is acquired by disseminated (pulmonary, CNS or SST infections) mainly in immunocompromised patients or by intravascular device-related infection. Frequently, concurrent infections with other pathogens occur (Al Akhrass et al., 2011; Williams et al., 2020). Other nocardiosis infections involve eyes, joints, bones, kidneys (Soleimani et al., 2020; Gnanam et al., 2020). *Nocardia* also affect to cattle, pets, wild animals and fishes, with big economic losses (Eroksuz et al., 2017; Yasuike et al., 2017).

Nocardiosis is rare in humans, with an incidence ranging from 0.33 to 0.87 for 100,000 inhabitants, with high values in some countries (Lafont et al., 2020). It reaches high values in patients with higher degree of immunosuppression. The organ transplant recipients is the most affected population, especially those with lung and heart transplants (Coussement et al., 2016; Paige and Spelman, 2019). But also, nocardiosis can affect until 45% of apparently immunocompetent patients with impaired immunity

(Peleg et al., 2007; Tremblay et al., 2011; Coussement et al., 2017; Majeed et al., 2018; Steinbrink et al., 2018). The population susceptible to infection is increasing due to the increased prevalence of immunosenescent population or/and immunosuppression conditions (Fatahi-Bafghi, 2018; Li et al., 2015) by the presence of some comorbidities as chronic lung disease, neoplasia and leukemia, organ transplant recipients, hematopoietic stem cell transplantation, autoimmune diseases, chronic alcoholism, diabetes mellitus, and AIDS (Ambrosioni et al., 2010; Tremblay et al., 2011; Wang et al., 2014; Li et al., 2015; McGuinness et al., 2016; Margalit et al., 2021).

Immunosuppressive therapy has been associated with nocardiosis (prednisone, calcineurin inhibitor, mycophenolate, cytostatic agent, mTOR inhibitor, and TNF-binding protein), and especially the corticosteroid therapy. Being the systemic corticosteroids strongly associated with pulmonary nocardiosis. In fact, when systemic corticosteroids are adjusted in a study with multivariable model, the positive association between solid organ transplantation and nocardiosis was attenuated. Among individuals diagnosed with nocardiosis, corticosteroid therapy is prevalent, especially in patients with chronic pulmonary disease and pulmonary nocardiosis (Margalit et al., 2020a). Other described risk factor for *Nocardia* acquisition is the traumatic inoculation in the outdoor activities, which may cause skin nocardiosis. Clinical setting in patients with catheterization or surgery could also ease the acquisition (Al Akhrass et al., 2011).

Not always the isolation of *Nocardia* means an evident clinical infection which required therapy. *Nocardia* colonization of the upper respiratory airways can exist in healthy individuals, and in the lower respiratory airways of cystic fibrosis patients or non-CF bronchiectasis, lung transplant recipients or in immunocompromised patients (Vetor et al., 2016; Dagan et al., 2015). A recent study has showed that a significative proportion (20%) of colonization occurs in patients with chronic pulmonary diseases and less frequently in patients with corticosteroid treatment (Margalit et al., 2020b).

Overall *Nocardia* species distribution depends of the geographic location, host and sites of infection, because some species may have a predilection to certain sites (Saubolle and Sussland, 2003; Brown-Elliott et al., 2006; Hemmersbach-Miller et al., 2019). The major pathogenic *Nocardia* species vary depend on the country: *N. nova* predominates in some countries as United States, Canada, and Australia (Uhde et al., 2010; Schlager et al., 2014; Hamdi et al., 2020; Tremblay et al., 2011; Tan et al., 2020); *N. cyriacigeorgica* in Spain (Valdezate et al., 2017); *N. farcinica* in France (Lebeaux et al., 2019); and *N. brasiliensis* in Japan and Taiwan (Kageyama et al., 2004; Wang et al., 2015).

Antimicrobial susceptibility and therapy

A key element for the antimicrobial stewardship is the right *Nocardia* specie assignation. For the most clinically significant *Nocardia* species/taxa, correlation among a determinate drug pattern type is traditionally described as it can be observed in Table 1 (Wallace Jr. et al., 1988; Brown-Elliott et al., 2015). However, the susceptibilities varied for a single species. As consequence, antimicrobial susceptibility testing of the isolates always has been done for an efficient and effective therapeutic management. According to Clinical and Laboratory Standards Institute guidelines (CLSI) (Clinical and Laboratory Standards Institute, 2018a,b), broth microdilution is the recommend method (Uhde et al., 2010; Brown-Elliott et al., 2012; Schlager et al., 2014; Hamdi et al., 2020) with standardized susceptible and resistance breakpoints for *Nocardia* spp. and other aerobic actinomycetes (Clinical and Laboratory Standards Institute, 2018a) as it is seen in Fig. 7. Other alternatives are the E-test or the disk diffusion (Valdezate et al., 2017; Lebeaux et al., 2019). The inoculum preparation due to the bacterial clumps and the time (low/slow) and type growth can difficult the minimum inhibitory concentrations (MICs) reading.

In overall, amikacin, linezolid, and trimethoprim-sulfamethoxazole showed high in vitro activity against most *Nocardia* species. The keystone of nocardiosis treatment is the trimethoprim-sulfamethoxazole, applied empirically before susceptibility results, alone or in combination after testing with other agents (third-generation cephalosporin, imipenem, or amikacin). Linezolid and tedizolid, very active in vitro against almost all *Nocardia* species, enlarged the therapeutic options (Margalit et al., 2021; Soueges et al., 2021).

Resistance against trimethoprim-sulfamethoxazole have been detected in some strains, in special in those belonged to the *N. transvalensis* complex. Regarding other antimicrobials, higher resistance rates are observed for some of main species against: amoxicillin-clavulanic acid in *N. cyriacigeorgica* and in *N. nova* complex; ceftriaxone in *N. brasiliensis*, *N. farcinica*, *N. nova*, and in *N. transvalensis* complex; against imipenem in *N. abscessus* complex, *N. brasiliensis*, and *N. transvalensis* complex. Clarithromycin, minocycline, and ciprofloxacin have showed higher resistance rates except for *N. nova* complex, *N. abscessus* complex, and *N. transvalensis* complex, respectively (Uhde et al., 2010; Brown-Elliott et al., 2012; Schlager et al., 2014; Valdezate et al., 2017; Lebeaux et al., 2019; Hamdi et al., 2020).

Two tentative definitions of multidrug-resistant (MDR) for *Nocardia* have been proposed: (i) an isolate is defined as MDR if it is non-susceptible (resistant or intermediate) to 2 or more of the most commonly used empirical agents (amikacin, ceftriaxone, imipenem, and TMP-SMX) (Schlager et al., 2014); (ii) MDR (non-susceptibility to ≥ 1 agent in ≥ 3 antimicrobial categories of CLSI), XDR (non-susceptibility to ≥ 1 agent in all but ≤ 2 categories), and pandrug resistance (PDR; non-susceptibility to all antibiotics) (Valdezate et al., 2017), excluding intrinsic non-susceptibility to a specific antimicrobial for a given specie (Magiorakos et al., 2012). Higher MDR rates were detected in *N. brasiliensis*, *N. farcinica*, and *N. transvalensis* complex (Valdezate et al., 2017; Hamdi et al., 2020) highlighting the importance of survey in the main species.

At this moment, no consensus exists on the optimal management of nocardiosis, but some proposals have been done, as it can see in Table 2 (Margalit et al., 2021). A combination of targeted antimicrobial treatment based on the antimicrobial susceptibility profile of the infecting nocardial strain, which include trimethoprim/sulfomethoxazole for more than 6 months, and neurosurgical intervention is recommended for CNS nocardiosis (Anagnostou et al., 2014). Always, therapy must be tailored to the individual

Table 1 Major antimicrobial susceptibility patterns of clinically significant *Nocardia* species and complexes.

Species and complexes ^a	Drug pattern type	Susceptibility ^b									
		AMC	CRO	IMI	AMK	TOB	CLA	MIN	CIP	SxT	LZD
<i>N. abscessus</i> complex	I	S	S	R	S	V	R	V	R	S	S
<i>N. brevicatena</i> , <i>N. paucivorans</i> complex	II	S	S	R	S	NA ^c	R	V	R	S	S
<i>N. nova</i> complex	III	R	S	S	S	V	S	V	R	S	S
<i>N. transvalensis</i> complex	IV	V	S	S	V	R	R	V	S	S	S
<i>N. farcinica</i> complex	V	R	R	S/V	S	R	R	V	S/V	S	S
<i>N. cyriacigeorgica</i>	VI	R	S	S	S	V	R	V	R	S	S
<i>N. brasiliensis</i>	NA	S	R	R	S	V	R	S	R	S	S
<i>N. pseudobrasiliensis</i>	NA	R	V	R	S	V	S	R	S	S	S
<i>N. otitidiscaviarum</i>	NA	R	R	R	S	V	V	V	S	S	S
<i>N. asteroides</i> ATCC 19247 ^T	VII	R	S	S	S	V	R	V	R	S	S

^aThe *N. abscessus* complex includes *N. abscessus*, *N. arthritidis*, *N. asiatica*, *N. beijingensis*, and *N. pneumoniae*; the *N. nova* complex includes *N. africana*, *N. aobensis*, *N. cerraodensis*, *N. elegans*, *N. kruczakiae*, *N. mikamii*, *N. nova*, *N. vermiculata*, and *N. veterana*; the *N. farcinica* complex includes *N. farcinica* and *N. kroppenstedtii*; the *N. transvalensis* complex includes *N. blacklockiae*, *N. transvalensis*, and *N. wallacei*.

^bAMC, amoxicillin-clavulanic acid; CRO, ceftriaxone; IMI, imipenem; AMK, amikacin; TOB, tobramycin; MIN, minocycline; CIP, ciprofloxacin; SxT, trimethoprim-sulfamethoxazole; LZD, linezolid.

^cNA, not applicable.

Modified from Brown-Elliott BA, Conville PS, Wallace RJ Jr (2015) Current status of *Nocardia* taxonomy and recommended identification methods. *Clinical Microbiology Newsletter* 37: 25–32. <https://doi.org/10.1016/j.clinmicnews.2015.01.007>.

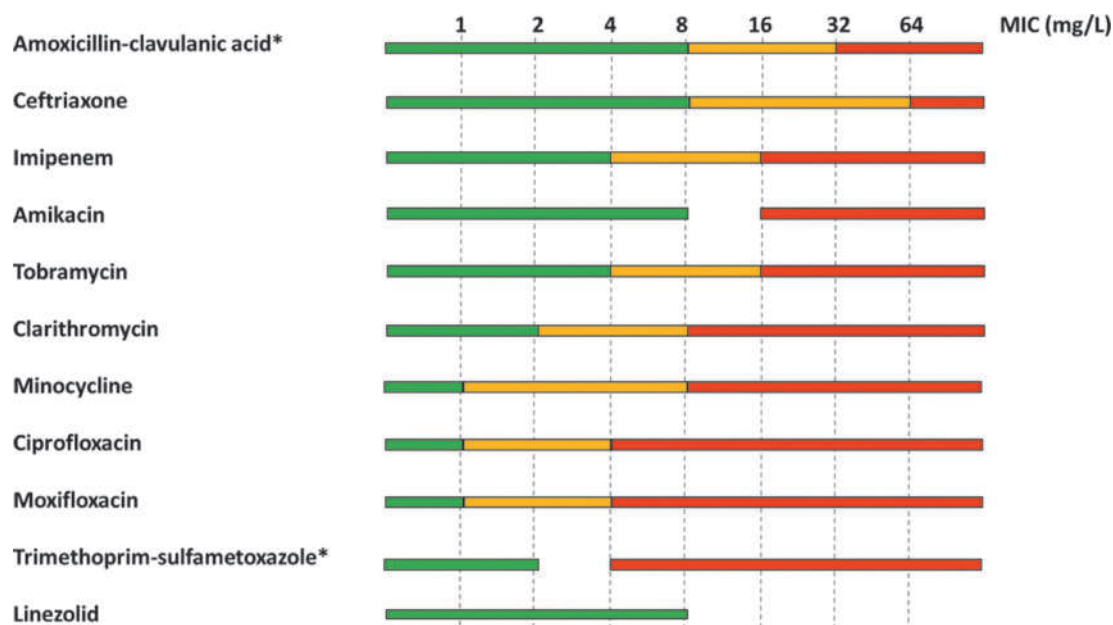


Fig. 7 Antimicrobial categories traffic light. Tentative breakpoints proposed for *Nocardia* species and other aerobic actinomycetes (except *Rhodococcus equi*). Categories are indicated as susceptible with a green bar, intermediate with an orange bar, and resistant with a red bar. *Combinations for amoxicillin-clavulanic acid (8/4, 16/8 and 32/16 mg/L) and trimethoprim-sulfamethoxazole (2/38 and 4/76 mg/L) were considered. Source: CLSI standard M24-A2, Clinical and Laboratory Standards Institute (2018b) *Performance Standards for Susceptibility Testing of Mycobacteria, Nocardia spp., and Other Aerobic Actinomycetes*. CLSI Supplement M62. Clinical and Laboratory Standards Institute: Wayne, PA.

patient based on species-specific susceptibility patterns and formal susceptibility testing, site(s) of infection, and patient tolerability (Hemmersbach-Miller et al., 2019). The prognosis for nocardiosis is variable, depending on the affected organ, the immune status of the patient, and the characteristics of the implicated specie.

Bioactive secondary metabolite production

When the metabolic potential of *Nocardia* is assessed, a plethora of putative biosynthetic gene clusters of various classes, including polyketide, nonribosomal peptide, and terpenoid pathways have revealed. *Nocardia* represent a prolific source for natural products rivaling better-characterized genera such as *Streptomyces* (Barka et al., 2015; Männle et al., 2020).

Table 2 A proposed algorithm for the management of a patient with nocardiosis.

<i>Nocardiosis extent</i>	<i>Primary skin</i>	<i>Isolated pulmonary</i>	<i>Dissemination^a without CNS involvement</i>	<i>CNS involvement</i>
Initial treatment (i.e., before species identification and AST results)	SxT ^b Possible alternative: LZD ^b	SxT ^b ± IMI or AMK or CRO or CTX ^b Possible alternatives: LZD ± IMI or CRO or CTX ^b Monotherapy (e.g., with SxT) is probably appropriate in selected patients with non-severe pulmonary nocardiosis	SxT ^b ± IMI or AMK or CRO or CTX ^b Possible alternatives: LZD ± IMI or CRO or CTX ^b	SxT with IMI ± AMK ^{b,c} Possible alternative: LZD with IMI ^b
Microbiology	Systematic species identification and antimicrobial susceptibility testing (if <i>Nocardia</i> identified to the species level but AST still pending)			
Brain imaging	Systematic brain imaging (MRI or contrast-enhanced CT) for all individuals with nocardiosis (only possible exception: immunocompetent patients with primary skin nocardiosis)			
Chest imaging	Systematic chest CT for all individuals (only possible exception: immunocompetent patients with primary skin nocardiosis)			
Maintenance therapy	If initial treatment was a multi-drug regimen with intravenous agents: continue for 2–6 weeks Therapeutic modifications based on response to therapy, microbiology results, and patient specificities			
Switch to oral therapy (if indicated, when the clinical response is favorable)	SxT ^b (possible alternatives, including MIN or AMC ^b)			SxT ^b
Total duration of antimicrobial therapy	3–6 months (shorter durations may be appropriate for immunocompetent patients)	6 months (3–4 months may be appropriate for selected patients with good response to therapy and treatment complicated by side-effects)	6–12 months (depending on the response to therapy and individual specificities)	9–12 months (possibly longer)
Secondary prophylaxis	Not indicated in immunocompetent patients (very low risk of relapse) SxT prophylaxis ^b may be considered in immunocompromised patients (if well tolerated)			
Intravascular device removal	Not indicated			Required in device-associated bacteremia and endocarditis

CNS, central nervous system; SxT, trimethoprim-sulfamethoxazole; AMC, amoxicillin-clavulanic acid; CTX, cefotaxime; CRO, ceftriaxone; IMI, imipenem; AMK, amikacin; MIN, minocycline; LZD, linezolid; MRI, magnetic resonance imaging; CT, computerized tomography; IV, intravenous.

^aDissemination is defined as the involvement of ≥ 2 non-contiguous organs.

^bTreatment regimens: trimethoprim-sulfamethoxazole: 10–20 mg trimethoprim/kg/day (possibly lower dose in patients with primary skin nocardiosis), secondary prophylaxis: daily oral at least 800/160 mg (unclear effectiveness, and breakthrough nocardiosis described while on 800/160 mg once daily); linezolid: IV, oral: 600 mg twice daily; minocycline: oral: 100–300 mg twice daily; amikacin: IV: 20–30 mg/kg/day; ceftriaxone: IV: 2000 mg once daily or twice daily if CNS involvement; cefotaxime: IV: 2000 mg three times daily or 4000 mg three times daily if CNS involvement; imipenem: IV: 500 mg four times daily: Dose adjustment for renal function is required for the following drugs: trimethoprim-sulfamethoxazole, amikacin, cefotaxime, and imipenem.

^cThe addition of amikacin may be discussed despite limited CNS penetration.

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Rhodococcus

Previously classified as *Corynebacterium*, *Prescottia*, and *Prescottella*, this genus belonged to the family *Nocardiaceae*, being the type specie *Rhodococcus rhodochrous*. At the present time, *Rhodococcus* genus (Zopf, 1891) includes 51 species with validly published and correct names (Parte et al., 2020) and about 800 genome assemblies of 37 species are available. Two species are recognized as pathogenic *Rhodococcus fascians*, which causes leafy gall in plants (Stes et al., 2013), and *Rhodococcus equi* (Vázquez-Boland and Meijer, 2019). The genus *Rhodococcus* is obligate aerobic, Gram-positive, partially acid-fast, catalase-positive, non-motile, and none-endospore bacteria. Without flagellum, sometimes a few numbers of pili may exist in this genus. They showed a pleomorphic shape with coccobacillus appearance in solid media and tissue, and filamentous and branched form in liquid media. Members of this genus producing red pigment. Most strains of this genus grow well in conventional medium, but some species require thiamin (vitamin B1) for their growth (McNeil and Brown, 1994).

Rhodococcus equi is a zoonotic organism that can cause rare, potentially fatal, pulmonary and extrapulmonary pyogranulomatous disease in animals and people. It is well-known as a major horse pathogen, and it predominantly affects immunocompromised people (Verville et al., 1994; Yamshchikov et al., 2010; Majidzadeh and Fatahi-Bafghi, 2018). Its circular chromosome has a G + C content of 68.8% with a size of 5.04 Mbp, more compact than environmental rhodococci. It appears genetically stable, with absence of significant recombination and unfrequent insertion sequences, but with a significant contribution of horizontal gene transfer (Anastasi et al., 2016). A special feature of *R. equi*, also shared with *M. tuberculosis* within Actinobacteria is the absence of phosphoenolpyruvate:carbohydrate transpot system (PTS), it might be associated with adaptation to intracellular niche. Other special feature, but in the *Rhodococcus* genus, is the presence of large circular or linear conjugative plasmids which carry genes encoded virulence determinants and detoxification pathways to survive in xenobiotic-contamination ecosystems (Vázquez-Boland and Meijer, 2019).

Habitat

Rhodococcus species is a soil-borne organism that is widespread in the environment, particularly in soil but also in aquatic reservoirs as rivers, lakes. This organisms is eliminated through the feces of animals. Exposure to soil contaminate with herbivore manure is likely the major route of both animal and human infection. This pathogen has been isolated from feces of a wide variety of animal species, including equines, bovines, small ruminants, dogs, pigs, and some wildlife (McNeil and Brown, 1994; Majidzadeh and Fatahi-Bafghi, 2018). Recently, the main responsible to cause infections, *R. equi* has been isolated from the feces of cats which had a history of contact with horses and their environment (Lechinski de Paula et al., 2019).

Pathogenesis and immunity

R. equi is an intracellular pathogen, which enter in the phagosomes of the macrophages. It is thought that immunity to *R. equi* relies on cell-mediated immunity via T helper type 1 lymphocytes, with a minor role of the humoral immunity. The inhibition of lysosome-phagosome fusion by this bacteria generates a macrophage killing resistance producing in tissue destruction and neutrophil influx, which ultimately leading to necrotizing pneumonia. The lack of macrophage killing in *R. equi* pneumonia can provokes a granulomatous disease with basophilic intracellular accumulations of iron and calcium inside macrophages named Michaelis Gutman bodies (Giguere et al., 2011).

The intra-macrophage proliferation of *R. equi* is facilitated by virulence associated proteins (VAP), encoded by *vap* genes located on circular and linear plasmids. Three *R. equi* *vap* plasmids have been identified: *vapA*, *vapB*, and *vapN*. According to mouse pathogenicity, the *vapA*-positive strains are classified as virulent and they carry large and circular plasmids (80–90 kb). While *vapB*-positive strains are considered as intermediately virulent and less pathogenic in horse, and carrying plasmids of 20 kb. Virulent strains and intermediately virulent strains are associated with severe pulmonary and systemic infections in foals and *vapB* in lymph node of swine. The last described, *vapN* is initially associated with strains collected from lymph nodes of ruminants. The presence of a specific *vap* plasmids is related with animal host adaptation (MacArthur et al., 2017), being an active exchange across *R. equi* population (Anastasi et al., 2016). In strains with human origin, *vapN* and *vapB* plasmids were more prevalent than *vapA* (43%, 30% and 16%, respectively) (Bryan et al., 2018) or absents (Takai et al., 2020). In addition, the transfer of this plasmids is done by the relaxe TraA.

Diagnosis, identification and typing

A common feature of human *R. equi* infection is the delay in diagnosis. The insidious course of disease contributes to delay, as does failure to identify the organism (Verville et al., 1994). This organism replicates slowly and may not even produce visible colonies unless culture plates are incubated for 48–72 h, contributing further to under-recognition (Fig. 21). Furthermore, *R. equi* on a culture plate may suggest as normal respiratory flora. So, it has recommended to pay attention to microscopic examination of gram-stained specimens (with large numbers of white blood cells with pleomorphic gram-positive coccobacilli) and the replace of sputum sample by aspirated or biopsies samples. In pulmonary infection, *R. equi* positive blood cultures would provide a definitive diagnosis. Although, bacteremia is seen more often in immunocompromised than immunocompetent patients (80%, and 30%, respectively). Too, the presence of necrotic tissue with dense histiocytic infiltrates and eosinophilic granular cytoplasm has been useful to identify *R. equi* pulmonary infection (Scott et al., 1992; Lin et al., 2019).

Take in mind, the importance of the diagnosis of rhodococci, several selective media have been used for isolation of *R. equi* as TINSDALE (selective medium for *Corynebacterium diphtheria*), NANAT medium (Nalidixic acid-Novobiocin-Actidione-Potassium tellurite), CAZ-NB (with Mueller-Hinton agar base, novobiocin, ceftazidime, and cycloheximide) among others (Majidzadeh and Fatahi-Bafghi, 2018). In the last two decades, molecular identification techniques have displaced phenotypic tests [Christie-Atkins-Munch-Petersen test (CAMP), serology, etc.] to distinguishing *Rhodococcus* spp. as 16S rRNA gene sequencing. But, sometimes it is unable to differentiate between closely related species. To specific species detection has been use the *choE* gene (cholesterol oxidase) sequencing and PCR-restriction fragment length polymorphism (RFLP) of the 65 kbp-heat shock protein (*hsp65*) gene. The sequencing of other genes (*catA*, *gyrB*, *alkB*) and of those genes included in the multilocus sequence typing (MLST) scheme (<https://pubmlst.org/organisms/rhodococcus-spp>) [glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), RNA polymerase β /*rpoB*], malate dehydrogenase (*mdh*), adenosine kinase (*adk*), triosephosphate isomerase (*tpi*), isocitrate lyase (*icl*), and recombination and repair systems (*recA*)] allowed more accurate *Rhodococcus* species identification and *R. equi* typing (Duquesne et al., 2017; Dhaouadi et al., 2020). For virulent strains of *R. equi* detection or typing, the detection of the presence of the *traA* gene and *vap* plasmids is of usefulness (Morego et al., 2009; Bryan et al., 2018; Takai et al., 2020).

In a whole-genome phylogeny, the genus *Rhodococcus* appears as a distinct, well-defined monophyletic grouping of the *Mycobacteriales* order. All *R. equi* isolates group in the well-supported monophyletic cluster no. 3 or “equi” subclade, which also contains *Rhodococcus defluvi* Ca11Ts as the closest relative, and *Rhodococcus triatoma*. Other subclades, in general congruent with 16S rDNA clustering are: subclade 1 or “rhodochrous” with two sublineages encompassing *Rhodococcus ruber*, *Rhodococcus rhodochrous* and *Rhodococcus pyridinivorans*; subclade 2 or “erythropolis,” with two sublineages, one containing *Rhodococcus opacus*, *Rhodococcus jostii*, *Rhodococcus imtechensis* and *Rhodococcus watislaviensis*, and the other, *Rhodococcus erythropolis* and *Rhodococcus qingshengii*; a subclades 4 and 5 that correspond to *Rhodococcus rhodnii* LMG 5362 and *R. fascians* isolates (Anastasi et al., 2016).

Clinical and epidemiologic features of *Rhodococcus equi* infections

The vast majority of rhodococcal human infections are caused by *R. equi*. The infections due to *R. defluvi*, *R. erythropolis*, *R. fascians*, *Rhodococcus gordoniae*, and *Rhodococcus ruber* have also been reported as cause of pneumonia, encephalitis, bacteremia and eye infections (Canetti et al., 2019; Baba et al., 2009; Bagdure et al., 2012). *R. equi* infection is characterized by diverse pyogranulomatous clinical disorders in humans, domestic animals, and wildlife. Human diseases consist of endophthalmitis, wound infection, pulmonary infection, osteomyelitis, peritonitis, chronic corneal ulcer, sarcoidosis, brain abscesses, skin and lymphadenitis, actinomycetoma, pulmonary malacoplakia, and purulent meningitis. *R. equi* has also been reported as a case with diffuse large B cell lymphoma (Verville et al., 1994; Majidzadeh and Fatahi-Bafghi, 2018; Lin et al., 2019; Takai et al., 2020).

The most common lesions associated with *R. equi* infection in susceptible patients are cavitating pulmonary abscesses, bacteremia, and lymphadenitis. The involvement of pulmonary sites (lungs, pleura or thoracic cavity) and extrapulmonary sites (brain abscess, skin/soft-tissue, intrathoracic lesion) were reported in nearly 90% and 20%, respectively of AIDS patients (Topino et al., 2010). Meanwhile, in immunocompetent patients are more frequent the extrapulmonary infections (Drancourt et al., 1992; Giguere et al., 2011; Lin et al., 2019). In this way, around 95% of the immunocompromised patients develop an infection with pulmonary involvement, meanwhile do it less than 50% of the immunocompetent patients. For both type of patients, the presence of cavitary lesions located in the upper lobe of lung appears as differential feature of *R. equi* infection by radiologic imaging (Gundelley et al., 2016).

The *R. equi* human infection mainly occur in patients with an immunocompromised status, with organ transplants, with hematological malignancies, with immunosuppressive drugs (corticosteroids, tumor necrosis factor- α inhibitors, prednisone), and with HIV infection. Being HIV infection, with CD4 count <200 cell/mm³ (Capdevila et al., 1997), the main risk factor in *R. equi* pneumonia (61.5%) (Scott et al., 1995). Due to the marked feature of opportunistic infection by *R. equi*, it is important to search in immunocompetent subjects with pneumonia a possible status of immunodepression and in the case of no pulmonary infections, the presence of localized infections such as cutaneous wounds or osteomyelitis (Gundelley et al., 2016).

Similar to humans, zoonotic diseases produced by *R. equi* have been reported as: pneumonia in foals (the major cause), calfs, pigs, sheeps, koalas and marmosets; pyaemia (a type of sepsis that leads to widespread abscesses of a metastatic nature) in foals; pyometra (uterine infection) and mastitis in cows and she-buffalos; abortion in lambs; lymphadenitis in cats, cattles, and seals; abscesses, skin infection and cellulitis in dogs and cats; and liver and spleen lesion in foals in goats. As plant pathogens, *R. fascians* and *Rhodococcus* spp. have been reported in in sweet peas, leafy gallin various plants, cauliflower, strawberry and pistachio plants (Majidzadeh and Fatahi-Bafghi, 2018).

Antimicrobial susceptibility and therapy

Different antimicrobial susceptibility testing methods has been used as broth dilution, E-test, disk diffusion, with $>85\%$ of agreement between them (Riesenberg et al., 2013; Berghaus et al., 2015). Although the first one is the recommended method by CLSI, there is not an available interpretative criteria for the specific categorization of *R. equi* (susceptible-intermediate-resistant) strains. Susceptibility up to 90% for vancomycin, macrolides, carbapenems, ciprofloxacin, and rifampin, with lower values for doxycycline and minocycline haven reported. With resistance rates $>40\%$ for trimethoprim-sulfamethoxazole, ampicillin-sulbactam and clindamycin (Topino et al., 2010). The emergence of resistance to macrolides and rifampin have been cause by erythromycin-resistant methylase gene *erm*(46) and mutations in the resistance-determining region of the RNA-polymerase β -subunit encoded by of *rpoB* gene, respectively. Difference in the antibacterial resistance has been seen in different geographic regions due to selective pression (Giguère et al., 2017).

The *R. equi* infections treatment it not easy. Because to the ability of *R. equi* to survive and replicate in macrophages, the presence of mass lesions or pulmonary cavities, the tendency to recurrence and the immunocompromised status of patient. Being a prolonged (it could be at least 6 months) and combined treatment (two antimicrobials) needed. In fact, the main factor of the success of therapy and its length is the immunological status and the nature of the lesion (Giguère et al., 2017; Lin et al., 2019). So, treatment with bactericidal antibiotics with an effective antiretroviral therapy is mandatory in HIV/AIDs patients, because high mortality (Drancourt et al., 1992). Failures in treatment had been produced in immunocompetent patients with short course treatment with single antibiotics (Lin et al., 2019). Combination treatment with two or three drugs is the mainstay of therapy, with the inclusion of antibiotics with intracellular activity, such as rifampicin and azithromycin, because intrahistiocytic survival remains a major virulence determinant for *R. equi* pathogenesis. For veterinary use, choice of drugs is the a combination of macrolide (erythromycin, clarithromycin or azithromycin) with rifampicin (Yamshchikov et al., 2010). In addition, resection or surgical drainage for abscess or lesions could be required.

Streptomyces

Streptomyces is the largest genus in the Actinobacteria, and also within the bacteria, that includes more than 950 species and 50 subspecies (Parte et al., 2020). The importance of this genus is consequence that they are a valuable source for bioactive commercially significant products (Barka et al., 2015). They belonged to the order *Streptomycetales*, family *Streptomycetaceae*, being the type genus *Streptomyces* and the type species *Streptomyces albus* (Waksman and Henrici, 1943). *Streptomyces* genus has been

subjected to extensively isolation and screening, with a large number of isolates and poor species definition which has provoked an overspeciation and taxonomic chaos (Anderson and Wellington, 2001; Labeda et al., 2012). More than 4000 genome assemblies of 537 species are available. Unlike other bacteria, this genus have a linear chromosome, G-C rich (about 70%), with a large size (6 Mbp–12 Mbp). This linear chromosome is a district property of this genus, and it is highly unstable suffering large rearrangements (Zhou et al., 2011). In addition, three linear plasmids have been detected in the *Streptomyces rochei* specie (Nindita et al., 2019).

Streptomyces produce an extensive branching substrate and aerial mycelium. Growth occurs at the hyphal apices and is accompanied by branching, thus producing a complex tightly woven matrix of hyphae during the vegetative growth phase. As the colony ages, aerial mycelia (sporophores) are produced which develop into chains of spores (conidia) by the formation of crosswalks in the multinucleate aerial filaments. This is followed by separation of individual cells directly into spores (Wildermuth and Hopwood, 1970; Anderson and Wellington, 2001).

The pathogenic role of this genus is difficult to assign, being considered as a mainly as a contaminant. Prevalent in tropical and subtropical regions, *Streptomyces* rarely causes severe invasive infections, but it is the most common cause of actinomycetoma (Dunne et al., 1998; Siddig et al., 2019).

Habitat

Streptomyces species are widespread environmental bacteria, distributed in terrestrial and aquatic ecosystems (deserts, ice in South Pole, insects, plants, lakes, seas, oceans) with soil, fodder, and compost as their primary habitat. In soil, they represent around 1–20% of the total viable count and are known to prefer the characteristic earthy smell (geosmin). *Streptomyces* are chemoheteroorganotrophs and are distinguished by their tough, leathery colonies and filamentous growth. They play an important ecological role in the turnover of organic material participating in numerous biodegradation and bioremediation processes. They are able of using complex organic materials such as carbon and energy sources in the breakdown of these products in the soil. The number and type of *Streptomyces* strains present in a particular soil are greatly influenced by environmental conditions such as temperature, pH, amount of organic matter, aeration etc. (McNeil and Brown, 1994; Núñez-Montero et al., 2019; Yisong et al., 2019).

Pathogenesis and immunity

Streptomyces spp. may be isolated from various clinical specimens, but they rarely represent true infections, particularly in a polymicrobial setting. Except of specimens from actinomycotic mycetoma, the isolation of this genus from clinical specimens is frequently considered as contaminants or colonizers (McNeil and Brown, 1994). To diagnose true *Streptomyces* infections is need to relate the clinical manifestations of the infection with the isolation of the organism from sterile sources (better in large quantities), direct microscopic identification in infected tissue with the exclusion of other pathogens or other causes (Kapadia et al., 2007). With at least of two of these criteria, the diagnosis true infection by *Streptomyces* could be done as occurred in the pneumonia case reported by Ariza-Protá et al., where *S. atratus* reached a pure and high growth (4×10^6 colony-forming units per mL) in the quantitative cultures of bronchoalveolar lavage (BAL) specimens and in two blood cultures (Ariza-Protá et al., 2015).

Diagnosis, identification, and typing

Some features of *Streptomyces* spp., as long filamentous gram-positive bacteria that grow aerobically and are negative for partial acid-fast stain, distinguish them from other morphologically similar genera within actinomycetes. In addition, the appearance as a hairball or a mesh of filaments in their culture (Fig. 2J–K) suggested the presence of this genus (McNeil and Brown, 1994). The characterization and systematics of the high number of species belonged to the *Streptomyces* genus has been developed from a large morphology-based classification system (spore color, spore surface ornamentation, morphology of the sporophores) with the evaluation of phenotypic traits. To clarify the confused state of the *Streptomyces* taxonomy, cooperative projects such as the International *Streptomyces* project (ISP) have been done with type strains (Anderson and Wellington, 2001).

The identification at genus level is reached by the use of 16S ribosomal RNA gene sequencing, providing presumptive species identification. For well supported *Streptomyces* clades, there are a good correlation among phylogeny based in 16S rRNA and morphological properties or phenotypic numerical taxonomy studies (Labeda et al., 2012). With a breakpoint score of 99.6% (Clinical and Laboratory Standards Institute, 2008) for the specie assignment, the minor and single membered clusters were considered to be species and the major clusters species-groups. For instance, the largest cluster is considered for the *S. albidoflavus* species-group. However, there is insufficient variation in the sequence of the *rrs* gene within species to perform an accurate species identification or to clarify relationships between close related species or established clades (Labeda et al., 2012). As occur for *Streptomyces griseus* 16S clade, one of the most taxonomically complex group and most prolific in the production of bioactive secondary metabolites (Guo et al., 2008).

In this way, the phylogenetic study of the family *Streptomycetaceae* based on sequences of the 16S rRNA gene showed that several species (*Streptomyces almquistii*, *Streptomyces flocculus*, *Streptomyces gibsonii* and *Streptomyces rangoonensis*), are very closely related phylogenetically to *Streptomyces albus* subsp. *albus*, the type species of the genus. The high similarity of 16S rRNA sequences among

Streptomyces species weakens the statistical support for the backbone structure of their phylogenetic tree. To avoid these issues other ribosomal genes (23S rRNA or 5S rRNA) or intergenic 16S-23S rRNA spacer regions together with other phenotypic and genotypic characters had been used (Anderson and Wellington, 2001). A preliminary study tries to postulate MALDI-TOFF MS as efficient first-line step for rapid identification of *Streptomyces* isolates (Loucif et al., 2014). But, the application of a scheme of multi-locus sequence typing (MLST) with the concatenate of the partial sequences of five house-keeping genes [ATP synthase F1 β -subunit (*atpD*), DNA gyrase B-subunit (*gyrB*), recombinase A (*recA*), RNA polymerase β -subunit (*rpoB*), and tryptophan synthase, β -subunit (*trpB*)] (Guo et al., 2008) is being more efficient to the study of the complex systematic of the taxa of the *Streptomycetaceae* family at inter- intra-species level, showing higher bootstrap support for the different clades than 16S rRNA (Labeda et al., 2012). The coexistence of homologous recombination and ecological divergence has been revealed in the evolution of streptomycetes (Andam et al., 2016; Cheng et al., 2015). By this way, phylogenies of some of the *Streptomyces* species and their ancestry have been studied (Andam et al., 2016). As mentioned above, *Streptomyces* genome is now one of the most highly sequenced. The study of some of these genomes has demonstrate that ecological adaptation promotes genetic differentiation in *S. albidoflavus*, suggesting a model of ecological speciation with gene flow in streptomycete (Li et al., 2019; Yisong et al., 2019).

Some species as been described as responsible of different types of truly human infections such as *Streptomyces albus*, *Streptomyces anulatus*, *Streptomyces atratus*, *Streptomyces bikiniensis*, *Streptomyces coelicolor*, *Streptomyces griseus*, *Streptomyces lanatus*, *Streptomyces lavendulae*, *Streptomyces paraguayensis*, *Streptomyces pelleteri*, *Streptomyces rimosus*, *Streptomyces somaliensis*, *Streptomyces thermovulgaris* and *Streptomyces violaceoruber*. In many cases, the definitive identification could be not possible as occur for *Streptomyces maritimus* vs. *Streptomyces olivaceus*, *Streptomyces nobilis* vs. *Streptomyces parvulus*, *Streptomyces humidus* vs. *Streptomyces pilosus*, *Streptomyces indigoferus* vs. *Streptomyces herbaricolor*. Or only it could be reached at genus level as *Streptomyces* spp. (Kapadia et al., 2007; Riviere et al., 2012; Joseph et al., 2010).

Clinical and epidemiologic features of *Streptomyces* infections

Streptomyces species are a common cause of actinomycetoma or mycetoma, a chronic suppurative infection of the skin and underlying soft-tissue and bone, characterized by tumefaction and draining sinuses. If lower extremity is affected, it is known as Madura foot. *Streptomyces* being frequent in those mycetomas affecting head and neck (McNeil and Brown, 1994; Kapadia et al., 2007). This entity is more prevalent in some areas of Africa and Asia. *S. somaliensis* is one of the major bacterial implicated agents (Develoux et al., 2003), together with *Actinomadura madurae* (van de Sande, 2013; Siddig et al., 2019), with the descriptions of new *Streptomyces* species (Quintana et al., 2008). Mycetoma infection is usually established after a disruption of mucosa through abrasion or traumatism, facilitated by the collagenase activity to access into deeper tissues.

Streptomyces species are rarely known to cause invasive infections (Kapadia et al., 2007), as it is seen in Table 3. But, severe infections as pneumoniae (Kofteridis et al., 2007; Riviere et al., 2012), lung nodules (Kapadia et al., 2007) bacteremia (Moss et al., 2003; Ekkelenkamp et al., 2004), brain abscess (Rose 3rd et al., 2008), endocarditis of the prosthetic valve (Mossad et al., 1995), arthritis, lymphadenitis, pericarditis and peritonitis (Dunne et al., 1998), cervical lymphadenitis, intraspinal mycetoma, and otitis media have been described. In some reported cases of bacteremia the acquisition source was catheter, prosthetic valve, intravenous infusion, primary lesion in internal organs (Ariza-Protá et al., 2015) or a subcutaneous actinomycetoma (Joseph et al., 2010) have been reported. Sometimes, the specific histological description of *Streptomyces* infection shows the presence of sulfur granules (Dunne et al., 1998). In *Streptomyces* pneumonia, the chest X-ray revealed a right lung basal alveolar infiltrate and a reticular interstitial pattern at the lung bases (Fig. 8) (Ariza-Protá et al., 2015).

These infections were mainly considered to be limited patients with immunosuppression linked to malignancies or antineoplastic chemotherapies (Moss et al., 2003), acquired immunodeficiency syndromes, AIDS (Dunne et al., 1998), Cushing Syndrome (Ataiekhorsgani et al., 2014), Crohn's disease, the presence of central venous catheter (Carey et al., 2001) and prosthetic heart valve (Ghanem et al., 2007), treatment with immunosuppressive and oral or inhaled glucocorticoids (Kapadia et al., 2007), and pregnancy (Joseph et al., 2010). But, some studies have report respiratory infections in immunocompetent patient (Kofteridis et al., 2007; Ariza-Protá et al., 2015). Depends on the underlying disease associated with *Streptomyces* infection, exitus could be produced. However, if antimicrobial susceptibility testing is carried out and treatment length recommendations is followed, death linked to *Streptomyces* infection is not produced (Riviere et al., 2012). In any case, if *Streptomyces* infection occurred in patients, immunosuppression status should be suspected.

Similar to humans, susceptible animals can suffer *Streptomyces* infections such as actinomycotic mycetoma (Walton et al., 2015). Plant pathogenicity is rare in this genus, nevertheless dozen or so species, cause important crop diseases in potato, carrot, radish, beet and other tap root crops. The potato common scab, characterized by lesions that form on the potato tuber surface, is produced mainly by *Streptomyces scabies* and *Streptomyces europaieiscabiei* (Wanner, 2009). A family of phytotoxins called the thaxtomins which function is cellulose synthesis inhibitors are the responsible of the plant pathogenicity enhanced with other traits with predicted function is the suppression of plant defense responses. These diseases have had a significant impact on agricultural economies throughout the world. However, beneficial roles have been described as the tripartite mutualism between *Streptomyces* from rhizosphere and flowers, strawberry plants and pollinating bees. Where *Streptomyces* protect both against phytopathogenic fungus and pathogenic bacteria (Kim et al., 2019). Similar to do *Streptomyces* isolates from soils surrounding the nests of termite colonies (Chouvinc et al., 2018).

Table 3 Epidemiologic and clinical characteristics of cases of invasive infection with *Streptomyces* species in the first two decades of the 21st century.

Reference (case no.)	Sex/ Age ^a	Species	Type of infection	Underlying conditions	Immunosuppressive and/or antineoplastic drugs	Outcome	Location
Carey et al. (2001), (16)	F/49	<i>Streptomyces</i> spp.	CVC ^b -related bacteremia	Metastatic breast cancer	Not receiving chemotherapy	Exitus of metastases	New York, EEUU
Moss et al. (2003), (17)	F/14	<i>S. bikiniensis</i>	CVC-related bacteremia	Osteosarcoma with recent cadaveric bone graft	Unspecified chemotherapy, not neutropenic	Recovered	Maryland, EEUU
Ekkelenkamp et al. (2004), (18)	F/81	<i>S. thermovulgaris</i>	Bacteremia	Crohn's disease	Corticosteroid	Exitus of bowel perforation	Utrecht, the Netherlands
Kapadia et al. (2007), (1)	F/21	<i>S. maritimus</i> / <i>S. olivaceus</i>	Lung nodule	Acute myelogenous leukemia (AML)	Cytarabine, fludarabine	Exitus of AML	Houston, EEUU
Kapadia et al. (2007), (2)	F/21	<i>S. albus</i>	Lung nodule	Systemic lupus erythematosus	Corticosteroids	Recovered	Houston, EEUU
Kapadia et al. (2007), (3)	M/71	<i>S. nobilis</i> / <i>S. parvulus</i>	CVC-related bacteremia	Metastatic cholangiocarcinoma	Gemcitabine, cisplatin	Recovered	Houston, EEUU
Kapadia et al. (2007), (4)	F/51	<i>S. humidus</i> / <i>S. pilosus</i>	CVC-related bacteremia	Metastatic breast cancer	None	Recovered	Houston, EEUU
Kapadia et al. (2007), (5)	M/25	<i>S. indigoferus</i> / <i>S. herbaricolor</i>	CVC-related bacteremia	Ewing sarcoma, neutropenia	Vincristine, doxorubicin, ifosfamide	Recovered	Houston, EEUU
Kapadia et al. (2007), (6)	M/18	<i>Streptomyces</i> spp.	Hypersensitivity Pneumonitis and/or drug toxicity	Burkitt lymphoma	Methotrexate and others	Recovered	Houston, EEUU
Ghanem et al. (2007), (1)	M/71	<i>Streptomyces</i> spp.	CVC-related bacteremia, septic thrombosis	Gallbladder carcinoma	Gemcitabine, cisplatin	Recovered	Houston, EEUU
Kofteridis et al. (2007), (1)	M/52	<i>S. lanatus</i>	Pneumonia	Recurrent pneumonia, immunocompetent	Periodic inhalation corticosteroids	Recovered	Crete, Greece
Rose 3rd et al. (2008), (1)	M/19	<i>Streptomyces</i> spp.	Brain abscess	Craniotomy after injury	None	Recovered	Georgia, EEUU
Riviere et al. (2012), (1)	M/57	<i>Streptomyces</i> spp.	Lung nodule	Multiorgan sarcoidosis, splenectomized	None	Recovered	Bordeaux, France
Ariza-Protá et al. (2015), (1)	M/77	<i>S. atratus</i>	Bacteremic pneumonia	Smoked, immunocompetent	None	Recovered	Oviedo, Spain
Ataiekhorsgani et al. (2014), (1)	M2/4	<i>Streptomyces</i> spp.	Pneumonia	Cushing Syndrome	none	Recovered	Isfahan, Iran

^aM, male; F, female, age in years.

^bCVC, central venous catheter.

Antimicrobial susceptibility and therapy

There are no standard guidelines for optimal therapy of invasive *Streptomyces* infection. Because the low number of reported cases of *Streptomyces* infections restricts their clinical management, mainly respect to the best choice and length of the antimicrobial treatment. Due to its slow growing nature is need a prolonged treatment period of 6–24 weeks, or 1 year of length (Ameen and Arenas, 2009; Kofteridis et al., 2007; Riviere et al., 2012). The different in vitro susceptibility studies which included different species showed consistent susceptibility to aminoglycosides (amikacin), frequent susceptibility to imipenem, macrolides, and minocycline, and infrequent susceptibility to ampicillin, ciprofloxacin and trimethoprim-sulfamethoxazole (McNeil et al., 1990; Kapadia et al., 2007). As antibiotic-producing bacteria, *Streptomyces* species have self-resistance mechanisms to protect themselves from the attack of their own antibiotics, avoiding suicide (Ogawara, 2016). In this way, it has been described in *S. clavuligerus* the presence of penicillin-binding protein PBPs in both sides of the clavulanic acid/cephamycin gene production cluster and the β -lactamase expression before the production of β -lactams (Coque et al., 1993). For a successful treatment of *Streptomyces* infection, the antimicrobial treatment choice should be based on the specific *in vitro* susceptibility profile of the isolated strain. But, some factors as patient immune status, location of the infection and response to treatment could be modified and guided the treatment (Kapadia et al., 2007).

Bioactive secondary metabolites production

Actinobacteria constitutes the most outstanding group of microorganisms for the production of bioactive compounds, producing nearly 40% of the bioactive secondary metabolites. From them, the genus *Streptomyces* produces nearly 80%. It had been predicted that less of 10% of their bioactive metabolite have been discovered. This ability relies on their genomic potential, a large number of biosynthetic gene clusters (25–70 BCGs) which encode for the enzymes involved in peptide assembly, regulation, resistance, and synthesis of a secondary metabolites. These BCGs include genes encoding for polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS) and ribosomally synthesized and post-translationally modified peptides (RiPPs) (Belknap et al., 2020; Kloosterman et al., 2020). A large diversity of structurally distinct compounds with variety of biological properties is produced by over 900 species of *Streptomyces*. *Streptomyces* species are considered as antibiotic factories (Barka et al., 2015), being responsible for the production of more the half of all known antibiotics like tetracyclines, chloramphenicol, streptomycin, and clavulanic acid, among others compounds. The genus is also a bountiful source of antiviral, antifungal, antiparasitic, antitumor, immunostimulatory, immunosuppressive, anti-hypertensive agents producers, as well as of bioherbicides (Barka et al., 2015; Watve et al., 2001). The great and diversified biosynthetic potential of this genus is steadily explored to discover new active compounds.

Other aerobic actinomycetes

Actinomadura

The genus *Actinomadura* is one of the member of the family *Thermomonosporaceae* (Lechevalier and Lechevalier, 1970). At the time of writing, 69 species are validly published with a correct name, being the *Actinomadura madurae* the type specie (Parte et al., 2020). Its microscopy and colonial morphologies resembles to those of the *Streptomyces* genus (Conville and Witebsky, 2015). This genus has, a median G–C% content about 70%, with median total length of 9.0 Mb. Nearly to 150 genome assemblies of 41 species are available. *Actinomadura* spp. are common soil-borne organisms that may have a role in the decomposition of organic material. Members of this genus can cause the development of a mycetoma, a term used to describe the clinical nature of the infection and not the causative agent (McNeil and Brown, 1994). Mycetoma refers to localized chronic infections of the skin and underlying tissues indicated by pyogranulomatous inflammation adjacent to the bacteria or fungus. As a result, the infected areas become swollen, often with the development of a draining fistula. Depends of the causative agent aerobic actinomycete or fungus, it is described as actinomycetoma or eumycetoma, respectively. Infection typically mainly results from skin trauma contaminated, via a thorn prick, a wood splinter or a stone cut, with environmental material (e.g., dust, soil, or vegetable matter) enabling enter to the human body (Nenoff et al., 2015; Zijlstra et al., 2016). Different *Actinomadura* species together with *N. brasiliensis* and *N. asteroides*, and *Streptomyces somaliensis* are described as the main responsible agents of actinomycetoma (Cárdenas-de la Garza et al., 2020).

Species reported as human pathogens include *Actinomadura bangladeshensis*, *Actinomadura madurae*, *Actinomadura mexicana*, *Actinomadura pelletieri*, *Actinomadura sputi*, or *Actinomadura vinacea* in cat. Among them, to note *A. madurae* followed by *A. pelletieri* as the most prevalent species in the actinomycetoma (Quintana et al., 2008; Yassin et al., 2010; Mencarini et al., 2016; Bérot et al., 2019; Cárdenas-de la Garza et al., 2020; Bessis et al., 2020; Siddig et al., 2021). There is a limited information regarding in the infections caused by *Actinomadura*, and rarely implicated in other types of infection different to mycetoma. But, intracranial granuloma caused by *A. pelletieri* (Samson et al., 2005), peritonitis by *A. madurae* (Wüst et al., 1990), and pneumonia (Bär et al., 2003) have been reported.

The diagnosis of mycetoma is mainly based on clinical characteristics and microbiological identification of the causative agent. Clinical diagnosis is characterized by a symptomatic triad: a subcutaneous mass (tumefaction), draining sinuses and grain discharge. Spontaneous drainage and manually expressed material from sinus should be carefully examined macro and microscopically to visualize the grains (Izri et al., 2020). The atypical round to irregular cells and the degree of pleomorphism including the large, multinucleated giant cells on cytology were concerning for a neoplastic cellular population. Surgical biopsy, including



Fig. 8 Community-acquired bacteremic *Streptomyces atratus* pneumonia in an immunocompetent adult. (A) Posteroanterior chest X-ray of the right lower lobe with alveolar infiltrate and a reticular interstitial pattern at the lung bases; (B) Chest computed tomographic scan showing right lower lobe alveolar consolidation with minimal pleural effusion, severe bilateral diffuse emphysema, and signs of chronic bronchitis (Ariza-Prata et al., 2015). Courtesy of BioMed Central Ltd. Ariza-Prata et al. (2015) Community-acquired bacteremic *Streptomyces atratus* pneumonia in an immunocompetent adult: a case report. *Journal of Medical Case Reports* 9: 262. <https://doi.org/10.1186/s13256-015-0753-y>.

special stains, confirmed that the origin of the wound was secondary to an infectious and inflammatory nature rather than a neoplastic process. These findings are clinically significant since treatment and prognosis are drastically different between an underlying infectious agent and a neoplastic process, and also between the responsible organism. Actinomycetoma is usually more aggressive than eumycetoma, spreading more quickly and with a greater tendency to be extrapedal than eumycetoma (Zijlstra et al., 2016). The grain appearance, the hematoxylin and eosin stain of tissues, Ziehl-Neelsen stain, cytopathological techniques, the microbiological culture (colonies with different morphology) (Fig. 2L), biochemical test, MALDI-TOFF, and genetic targets (by restriction fragment length polymorphism or by sequencing) allows the definitive diagnosis of the mycetoma entity (Siddig et al., 2021). Among the latter, the sequencing of the 16S rDNA gene of the bacterial DNA in the grains isolated from the patient or in growth cultures is the main choice (Salipante et al., 2013).

Mycetoma is a major health problem in many tropical and subtropical areas, which is noted for social stigmatization. The World Health Organization (WHO) included mycetoma as a neglected tropical disease in 2016. The distribution of the mycetoma causative agents is not equal around the globe. It is commonly observed in the barefoot walking population of tropical and subtropical regions in a zone, known as the "Trans-African or Mycetoma belt" (latitudes 15 degrees south and 30 degrees north) with dry and arid climates. In overall, actinomycetoma is more commonly found in Middle and South-America while eumycetoma is more commonly found in Africa. But within a country, this could also differ per region (van de Sande, 2013). Most cases of actinomycetoma were reported from North African countries (Morocco, Tunisia), but occur, e.g., in Senegal and Sudan, too. They are endemic in Middle and South America (Mexico, Venezuela, Colombia, Argentina) and some cases are reported from Asian countries (India, Laos) (Zijlstra et al., 2016). Rarely, mycetomas were described in the Mediterranean or South East countries of Europe, or in the EUU. The cases of this zone are uncommon and usually imported by migrants from endemic areas (Mencarini et al., 2016). There is an association of mycetomas to distinct species of fungi and actinomycetes in the environment, depending on the concrete amount of rainfall, temperature and humidity (Cárdenas-de la Garza et al., 2020).

The typical clinical presentation of mycetoma is a painless subcutaneous mass of slow progression forming multiple sinuses that drain pus and clusters of bacterial and fungal structures called "grains". The host's inflammatory response consists of a neutrophilic infiltrate and the formation of granulomas that typically contain grains. Single or multi-fistulized pseudotumor appears in the mycetoma. The grains can vary in size, color, and consistency, depending on the etiological species. Untreated infections will eventually destroy the tissues, including muscles and bones (Izri et al., 2020). The most frequent radiological finding in mycetoma is soft tissue swelling followed by bone sclerosis, bone cavities and periosteal reaction (Figs. 9 and 10) (Izri et al., 2020). The mycetoma is usually painless and processes slowly, over months and years. Although mycetoma could be painful in case of secondary bacterial infection.

Mycetoma, a neglected infection of the poor, is considered occupational disease of individuals who work in rural areas, such as farmers or agricultural workers, gardeners, shepherds, construction workers, housewives, or that engage in activities related to the environment, with or without previous trauma (Cárdenas-de la Garza et al., 2020). Mycetomas is more prevalent in people between 20 and 40 years old, with a low socio-economic level, and it is more common in men rather than women (3:1). This infection occurs mostly in the lower extremities of the body, particularly in the feet but other parts of the body can also be involved. In immunocompetent patients dissemination or a generalized invasive mycetoma infection will be prevented. In case of malnutrition or undernourishment and associated illnesses (e.g., malaria, HIV/AIDS, leishmaniasis, diabetes mellitus, etc.) with immunodeficiency, a more intense and faster progression of the disease takes place and exitus may occur (Nenoff et al., 2015).

Similar clinical manifestations appearing in actinomycetoma and eumycetoma, but therapy differs depending of causal microorganisms. Eumycetoma treatment primarily relies on wide surgical excision of the lesion and administration of antifungal agents, while actinomycetoma therapy is based on antimicrobials. The cure rate of patients with *A. madurae* and *A. pelletieri* was up to 80% (Siddig et al., 2020). Sulfonamides, particularly trimethoprim-sulfamethoxazole (TMP/SMX) is the mainstay therapy, often in combination with other drugs such as amikacin or amoxicillin/clavulanic acid. Being the combination of TMP/SMX plus amikacin which provides a higher cure rates (90%) (Cárdenas-de la Garza et al., 2020). Since antibiotics have a difficult time penetrating the dense granulomatous tissue associated with these lesions, the treatment is time extended (weeks, months, sometimes years). Moreover, therapy continues after the resolution of clinical disease. The average length of treatment can vary according to the involved species: *A. madurae* (24 months), *A. pelletieri* (16 months), and *A. mexicana* (6–9 months) (Siddig et al., 2020). The role of surgery varies with the progression of the disease and the inadequate response to chemotherapy (Welsh et al., 2014). As consequence of the significant clinical presentations and complexity in therapeutic implications, the disease diagnosis in the early stages is of high importance. But, mycetoma is mostly a painless infection leading to delayed medical consultation, and aggressive therapies, like amputation of affected body members, and causing consequently lifelong disability.

Gordonia

Proposed *Gordonia* as a new genus by Tsukamura (1971), some representatives were transferred to *Rhodococcus* genus, being returned to *Gordonia* genus through 16S rRNA analysis (Stackebrandt et al., 1988). After, the etymologically correct name of *Gordonia* replaced the previous. At present, the genus *Gordonia* included 44 validly published species, being *Gordonia bronchialis* the type species. It forms the *Gordoniaceae* family together with the *Williamsia* and *Jongsikhunia* genera (Parte et al., 2020). *Gordonia* is arylsulfatase-negative, weakly acid-fast bacilli, non-motile, short rods or circular, with G + C content from 63% to 69% and with a chromosomal size of 4.3–5.6 Mbp. More than 200 genome assemblies of 38 species are available for their study.



Fig. 9 The case of a 58-year-old man with a left foot chronic tumefaction of 5 years caused by *Actinomadura madurae*. (A) Presence of mycetoma grains and sinuses on the top side of the left foot; (B) Left foot mycetoma presenting grains and sinuses comparing to healthy right foot; (C) Close vision of the grains and sinuses (red arrow) on the left foot (Izri et al., 2020). Courtesy of BioMed Central Ltd. Izri et al. (2020) Molecular identification of *Actinomadura madurae* isolated from a patient originally from Algeria; observations from a case report. *BMC Infectious Disease* 20: 829. <https://doi.org/10.1186/s12879-020-05552-z>.

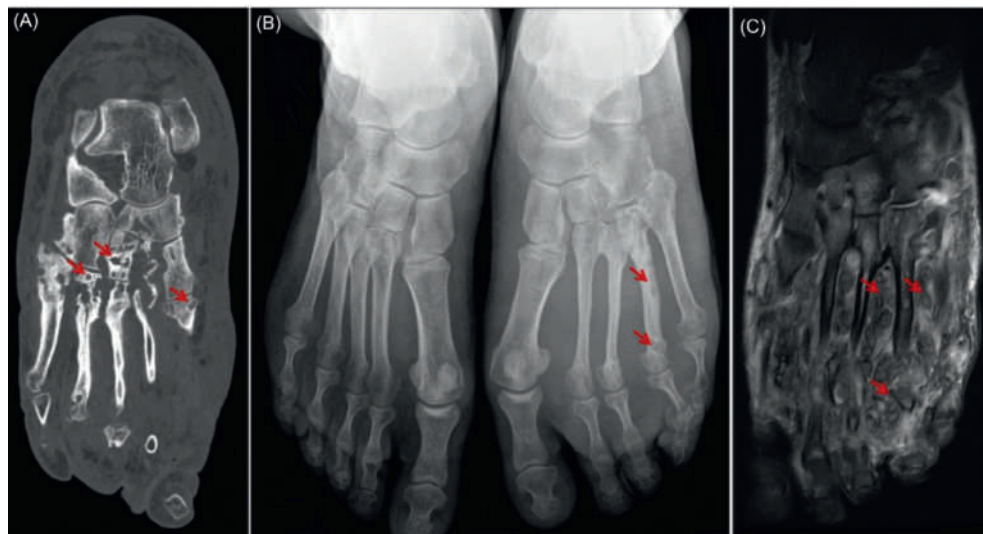


Fig. 10 The case of a 58-year-old man with a left foot chronic tumefaction of 5 years caused by *Actinomadura madurae*. (A) Left foot tomodensitometry demonstrating metatarsals osteolysis and thickened soft parts; (B) Foot X-rays with thickened soft parts of the left foot and the 4th metatarsal osteolysis; (C) Soft tissue infiltration by bacteria and bone involvement of the left foot demonstrated using T2 sequence fat-sat pulses in MRI (Izri et al., 2020). Courtesy of BioMed Central Ltd. Izri et al. (2020) Molecular identification of *Actinomadura madurae* isolated from a patient originally from Algeria; observations from a case report. *BMC Infectious Disease* 20: 829. <https://doi.org/10.1186/s12879-020-05552-z>.

Some *Gordonia* species produce pigmented colonies with colors of orange to orange-red and shiny surfaces (Fig. 2O-P) (McNeil and Brown, 1994). Morphological differences are seen as *Gordonia sputi* colonies are smooth, mucoid, and sticky on the medium, whereas *G. bronchialis* colonies are large and dry. This genus does not produce aerial hyphae, except for *Gordonia amarae* and *Gordonia defluvi* (Arenskötter et al., 2004). Plasmids can be detected in some *Gordonia* species from clinical and environmental source (Lee et al., 2020).

Gordonia species have been isolated from various native biotopes such as soil or mangrove rhizosphere, from extensively industrially influenced habitats such as oil-producing wells or hydrocarbon-contaminated soil, from artificial sources such as wastewater treatment bioreactors or biofilters, and from human diseases (Arenskötter et al., 2004). But also this soil bacteria has been isolated from the intestinal contents of mammals (Gil-Sande et al., 2006). As other actinomycetes, *Gordonia* spp. is transferred through aerosols from environmental sources to the respiratory tract.

The major human pathogens in the *Gordonia* species are *G. sputi*, *G. bronchialis*, *Gordonia polyisoprenivorans*, and *Gordonia terrae*. But, other species as *Gordonia aichiensis*, *Gordonia amicalis* (Lai et al., 2012), *Gordonia aarii*, *Gordonia effuse*, *Gordonia otitidis*, *Gordonia rubropertincta*, *Gordonia westfalica* (Gueneau et al., 2020) are also responsible of human infections (Blaschke et al., 2007; Johnson et al., 2011; Ramanan et al., 2013; Akrami et al., 2017; Andalibi and Fatahi-Bafghi, 2017).

Infections caused by *Gordonia* spp. are difficult to diagnose (Fang et al., 2017). This underdiagnosis is due to its slow growth (72 h), the misidentification with other medically important aerobic actinomycetes (*Nocardia* spp. or *Rhodococcus* spp.) by phenotypic test (Gil-Sande et al., 2006), and the role of this environmental bacteria as cause of true infection. Often, the obtained results by biochemical test for species assignment are inconclusive (Andalibi and Fatahi-Bafghi, 2017; Eribi et al., 2020). And in some occasions, MALDI-TOF does not resolve the species (Teng et al., 2018). So, molecular methods as the 16S rRNA gene, *gyrB*, and *secA1* sequencing provide definitive identification of *Gordonia* species (Kang et al., 2009; Lam et al., 2015; Andalibi and Fatahi-Bafghi, 2017; Ding et al., 2017). For typing purpose, these targets are useful together with *hsp65* PCR-RFLP and pulsed-field gel electrophoresis (Johnson et al., 2011; Wright et al., 2012).

A wide spectra of infections are produced by *Gordonia* species (Lai et al., 2010; Andalibi and Fatahi-Bafghi, 2017) as central venous-catheter related bacteremia (Blaschke et al., 2007; Ramanan et al., 2013), pneumonia (Ding et al., 2017), endocarditis (Verma et al., 2006), meningitis (Martín et al., 2017), brain abscess (Eribi et al., 2020), ventriculitis (Blaschke et al., 2007), cutaneous infection (Lai et al., 2012), actinomycetoma (Gueneau et al., 2020), breast abscess, ocular infections (Fang et al., 2017; Choi et al., 2019), pleural infections (Johnson et al., 2011), otitis, arthritis, osteomyelitis, peritonitis (Lam et al., 2015), and surgical site infections (Akrami et al., 2017). Sternal wound infections are caused mainly by *G. bronchialis* (Johnson et al., 2011), being also responsible of two outbreaks, wherein nurse scrub were identified as the vector for contamination of the surgical wounds (Richet et al., 1991; Wright et al., 2012).

Susceptible patients to suffer *Gordonia* infections are those ones with immunocompromised status due to congenital or acquired immunodeficiencies (AIDS infection, hypogammaglobulinemia, leukemia, solid organs cancer, transplant recipients, Hodgkin's disease, etc.), or other underlying diseases chronic hepatitis B virus infection, diabetes mellitus, chronic obstructive pulmonary disease, vasopressor medication, obesity (Blaschke et al., 2007; Johnson et al., 2011; Guerrero et al., 2014; Kempf et al., 2004; Ding et al., 2017). However, many reported cases occurred in immunocompetent patients, being the main risk factor the presence of foreign bodies including central lines and shunts (Blaschke et al., 2007; Lai et al., 2010; Bartolomé-Álvarez et al., 2016; Akrami et al., 2017). This event is in relation with the ability of some *Gordonia* species to attach and colonize the surface of medical devices by biofilm formation, leading to bacteremia. For instance, *G. polyisoprenivorans* produces biosurfactants that help to form biofilm onto the catheter decreasing antibiotic penetration (Verma et al., 2006).

There is not a standardized treatment for *Gordonia* infections, and little is the information about susceptibility. Fortunately, in contrast to some other actinomycetes, *Gordonia* are generally susceptible to many antimicrobial drugs. Amoxicillin-clavulanic acid, ceftriaxone, carbapenems, aminoglycosides, linezolid and fluoroquinolones showed better activity against *Gordonia* species than macrolides and trimethoprim-sulfamethoxazole (Blaschke et al., 2007; Moser et al., 2012; Bartolomé-Álvarez et al., 2016). Frequently, patients are treated with a variety of antimicrobial regimens, usually for several weeks or months, with good outcome in most cases (Guerrero et al., 2014; Ding et al., 2017). Being the prognosis for *Gordonia* bloodstream infections is variable, depending on the underlying conditions of the host, the clinical factors related to the infection, and the time to diagnosis. For catheter-associated *Gordonia* infections, catheter removal is a common practice. Depend on infection type and its evolution, surgical debridement could be required.

Gordonia is a bacteria potentially useful for environmental and industrial biotechnology, because of their abilities to degrade xenobiotics, environmental pollutants, or biodegradable natural polymers as well as to transform or synthesize possibly useful compounds (Arenskötter et al., 2004; Drzyzga, 2012).

Tsukamurella

According to the current state of the taxonomy, *Tsukamurella* (Collins et al., 1988) is the single genus of the family *Tsukamurellaceae*, which is composed of 16 validly identified species being *Tsukamurella paurometabola* the type species (<https://lpsn.dsmz.de/family/tsukamurellaceae>) (Parte et al., 2020). *Tsukamurella* is a partially acid fast, obligatory aerobic bacterium that does not have an aerial hyphae. It is a non-spore forming and non-motile bacterium with rod-shaped cells that can be straight or curved and can be seen in pairs or groups of coccobacillary forms by Gram stain. Some *Tsukamurella* species produce pigmented colonies (Fig. 2Q–R) (Safaei et al., 2018). This genus possess a circular chromosome with a G + C content close to 70% and a size range 4.6–5.2 Mbp (Teng et al., 2016). A limited number of genomes assemblies are available.

Tsukamurella spp. is environmental saprophyte which is isolated from different environmental sources (soil, water, sludge, foam) as well as in animals (arthropods, sponges, snakes). The *Tsukamurella* species involved in human infections have been: *Tsukamurella incheonensis*, *T. paurometabola*, *Tsukamurella strandjordii*, *Tsukamurella tyrosinosolvens*, *Tsukamurella pulmonis*, *Tsukamurella hongkongensis* and *Tsukamurella sinensis* (Yassin et al., 1996, 1997). Incorrect species identifications via MALDI-TOF were due to the absence of the corresponding reference spectra in the database (Teng et al., 2016). The most commonly used molecular method for bacterial identification, 16S rRNA gene sequencing is probably not discriminative enough to provide a species-level identification of *Tsukamurella* spp. differing with less than 1% among them. In contrast, *secA1* gene appears to be a better and more reliable target to discriminate *Tsukamurella* species (Bernal and Balbin, 2017). The phylogenomic analyses, concordant to the clustering patterns observed in phenotypic and MALDI-TOF MS analyses, reclassified some species (*T. spongiae* as *T. pulmonis*, *T. carboxydivorans* as *T. tyrosinosolvens*, and *T. sunchonensis* as *T. pseudospumae*) (Teng et al., 2016).

They are opportunistic pathogens and can be spread through clinical instruments (e.g., catheters) resulting in sporadic episodes of nosocomial infections. So, the most common *Tsukamurella* infections in human are related to the presence of indwelling devices. They therefore can cause various infections in humans, such as respiratory tract infection, meningitis, brain abscess, bacteremia,

endocarditis, catheter related bloodstream infection, peritonitis, conjunctivitis keratitis, cutaneous infection, and acute otitis media (Teng et al., 2020; Safaei et al., 2018; Ismayilov et al., 2020; Malik et al., 2020). *T. tyrosinosolvens* was the most common species encountered in patients with *Tsukamurella* infections (Leroy et al., 2020).

Susceptible patients for *Tsukamurella* infections are mainly patients with immunosuppressive diseases, renal failure, and foreign bodies (e.g., clinical equipment). In immunocompromised patients, mostly with hematology malignancies, the bacteremia's length was often notably long, up to 4 weeks. The leading causes of persistent bacteremia were noticed: the time to positivity of the bacterial vial; the frequent consideration of Gram-positive bacilli rods as possible contaminant microorganisms; and the time required reaching an accurate bacterial identification that might have led to inappropriate empirical antibiotic choices (Leroy et al., 2020).

The antibiotic susceptibility profile of different *Tsukamurella* species remains poorly reported. The best antibiotic susceptibility testing is microbroth dilution. *Tsukamurella* spp. are resistant to penicillin, oxacillin, piperacillin/tazobactam and cephalosporins, which are prescribed for treatment of nontuberculous mycobacteria infection. Whereas *Tsukamurella* is susceptible to amikacin, ciprofloxacin, imipenem, doxycycline, linezolid and sulfamethoxazole. But, susceptibility testing is necessary for proper treatment of *Tsukamurella* infections. Effective results resulted from a combination of β -lactam or macrolide with aminoglycoside antibiotic agents for a long treatment period. In addition to optimal antibiotic treatment, probably to be defined due to the limited number of isolates reported, catheter removal seems to be the cornerstone of the treatment (Safaei et al., 2018; Leroy et al., 2020).

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- <https://lpsn.dsmz.de/>—List of Prokaryotic names with Standing in Nomenclature (LPSN).
- <https://www.ncbi.nlm.nih.gov/assembly>—Assembly NCBI.
- <https://pubmlst.org/>—Public databases for molecular typing and microbial genome diversity PubMLST.
- <https://www.gov.uk/uk-standards-for-microbiology-investigations-smi-quality-and-consistency-in-clinical-laboratories>—Public Health England.
- <https://tygs.dsmz.de/>—Type Strain Genome Server DSMZ.

Infections Caused by Anaerobic Microorganisms

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Introduction

Anaerobic bacteria are the most numerous components of the normal human microbiota colonizing the mucous membranes. They are dominant commensals in mucosal surfaces such as the oral cavity and the gastrointestinal and female genital tracts. In some circumstances, especially after the breakdown of mucosal barriers, these microorganisms may spread from indigenous microbiota into normally sterile body sites and they can be responsible for severe disease like in blood infections. The presence of anaerobic microorganisms predominates in several clinical syndromes, and this fact could be attributed to the large numbers of these bacteria residing on mucous membranes, the production of a wide variety of virulence factors, the synergy with other aerobic bacteria and the increased resistance of these microorganisms to some antimicrobials. However, most clinically significant anaerobes are involved in mixed infections alongside aerobic bacteria.

Infections due to anaerobic isolates may sometimes be missed because of the special measures required for their transportation. Other critical factors for the successful isolation of these microorganisms in the microbiology laboratory include incubation in anaerobic atmosphere, the use of specialized culture media, and prolonged culture (Finegold, 1995). Moreover, several infections caused by anaerobes are important due to the fact of the observation of higher resistance rates of these microorganisms to some antimicrobial agents over the last years (Brook et al., 2013). The combination of all those factors makes it crucial to know when an anaerobic infection is vital in order to use appropriate microbiologic methods to identify the bacteria and to select the correct treatment.

This chapter focuses on the description of the main genera of anaerobic bacteria that causes human infections and their main syndromes in which they are implicated (Table 1).

Table 1 Main anaerobic bacteria and clinical syndromes in human population.

Type of bacteria	Microorganism	Infectious syndrome
Gram-negative bacilli	<i>Bacteroides</i> spp.	Abdominal infections, peritonitis, bacteremia, genital infections, abscess
	<i>Porphyromonas</i> spp.	Orofacial infections
	<i>Prevotella</i> spp.	Orofacial infections, periodontitis, abdominal infections, genital infections, bacteremia
	<i>Fusobacterium</i> spp.	Cerebral abscess, bacteremia, orofacial infections, pneumonia, Lemière syndrome
Gram-positive bacilli	<i>Clostridium</i> spp.	Bacteremia, skin and soft-tissue infections, diarrhea (colitis pseudomembranous), necrotizing enterocolitis, tetanus, botulism
	<i>Actinomyces</i> spp.	Cerebral abscess, mastoiditis, genital infections, neck and head infections, lung infections
	<i>Cutibacterium acnes</i>	Medical devices infections
	<i>Bifidobacterium</i> spp.	Cervical lymphadenitis, abdominal infections, periodontitis
	<i>Eubacterium</i> spp.	Periodontitis, odontogenic abscess, bite infections
Gram-positive cocci	<i>Finnegoldia magna</i> , <i>Parvimonas micra</i> , <i>Peptoniphilus</i> spp., <i>Anaerococcus</i> spp., <i>Peptostreptococcus</i> spp.	Skin and soft-tissue infections, abdominal infections, abscess, bacteremia
Gram-negative cocci	<i>Veillonella</i> spp.	Polymicrobial infections, bacteremia

Anaerobic bacteria present in the human microbiota

Human microbiota is integrated by different kind of microorganisms of which anaerobic bacteria are the most frequent of them (Huttenhower et al., 2012). Until now, culture of specimens has been the gold standard of microbiology for the characterization of microorganisms that make up the microbiome. However, many of the bacteria isolated from these samples were no cultivable, as it was demonstrated in a study (Gill et al., 2006). Today, new techniques based on DNA analysis are being used in order to characterize new pathogens as well as anaerobic bacteria. Anaerobes are especially present in mucosal surfaces like the gastrointestinal and female genital tract and the oral cavity. The asymptomatic carriage of *Clostridioides difficile* has been confirmed in the human gastrointestinal tract in a small number of healthy people. The number of colonized persons increases during hospitalization and among those who are taking antimicrobial agents. The microbial species and concentrations varies depending on the location; the highest anaerobes concentration is found in the oral mucosa with concentrations ranging $10^2/\text{mL}$ to $10^{12}/\text{mL}$. In the gingival area, the anaerobe:aerobe ratio is 1000:1. The main anaerobic microorganisms present in this location are species of the genera *Prevotella*, *Porphyromonas*, *Fusobacterium* and *Bacteroides*.

Regarding to the microbiota of the stomach and upper intestine, low number of anaerobic bacteria are present in this area; in persons with decreased gastric acidity, the microbiota of the stomach is similar to those present in the oral cavity. However, the microbiota of the upper intestine resembles those present in the colon. The predominant anaerobes present in this last location are species of *Bacteroides* spp., *Clostridium*, *Peptostreptococcus* and *Fusobacterium*.

Regarding to the normal female genital tract, the ratio anaerobic:aerobic range of 1:1 to 10:1. The predominant anaerobic species are *Prevotella*, *Bacteroides*, *Fusobacterium*, *Clostridium* and *Lactobacillus*. *Bacteroides* spp. is found in the genital tract of approximately 50% of women and supposes a 15% of the microbial population.

Anaerobic microorganisms also may be present in areas that are exposed to air; the skin microbiota contains species of anaerobes such as *Cutibacterium* (formerly *Propionibacterium*) *acnes*, other species of *Cutibacterium* and *Peptostreptococcus*.

Fig. 1 shows macroscopic aspect of some anaerobic microorganisms.

Taxonomic and microbiologic classification

Despite the great number of anaerobes present in the normal human microbiota, only relatively few species are involved in human infections. A significant fact is that infections involving anaerobic pathogens are often polymicrobial and usually are produced as a consequence of the disruption of mucosal surfaces by surgery, trauma, tumors or ischemia and the spread of microbiota to sterile sites.

Anaerobic microorganisms are divided in Gram-negative and Gram-positive and in bacilli and cocci. There are also some Gram variable microorganisms such as *Actinomyces* spp., *Clostridium tetani* and *Cutibacterium acnes*, alternating between a Gram-negative and Gram-positive phase depending on the replication-stationary stage (Beveridge, 1990).

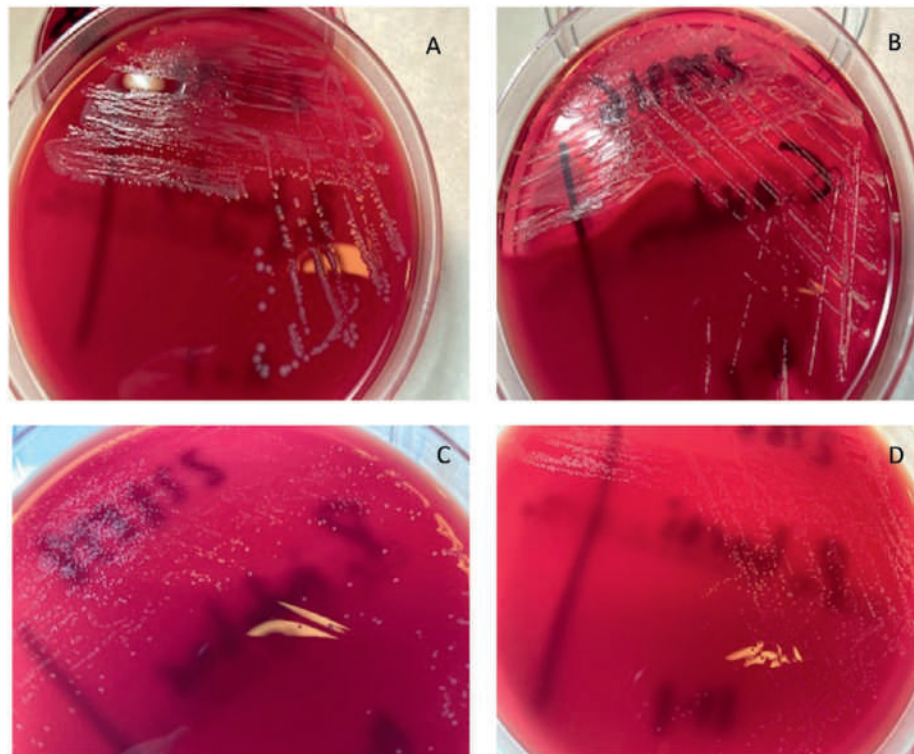


Fig. 1 Microphotographs showing the macroscopic aspect of some anaerobic microorganisms isolated in blood agar: (A) *Bacteroides fragilis*. (B) *Cutibacterium acnes*. (C) *Peptoniphilus hareii*. (D) *Finegoldia magna*.

Gram-negative anaerobic bacilli

Bacteroides, *Prevotella*, *Porphyromonas* and *Fusobacterium* species are the most frequent anaerobes in this group isolated in clinical samples and involved in human infections, especially the *B. fragilis* group (Wexler, 2007). Of this group, *B. fragilis* is the species most often isolated from human infections. Other members of this group include *B. thetaiotaomicron*, *B. ovatus*, *B. vulgatus* and *B. distasonis*. A definitive taxonomic classification of this genus was performed based on the study of 16S rRNA (Paster et al., 1994). These are microorganisms commonly involved in intraabdominal infections and other conditions such as decubitus ulcer and diabetic foot ulcers. Moreover, the disruption of the mucosal barrier becomes in abscess formation and bacteremia. *B. fragilis* virulence is mostly by a toxin production. Enterotoxigenic *B. fragilis* strains secrete a termed enterotoxin and may have a role in inflammatory diarrhea and flare-ups of inflammatory bowel disease (Sears, 2009; Basset et al., 2004).

Prevotella (both pigmented and non-pigmented species) and *Porphyromonas* species are anaerobic Gram-negative bacilli involved in oral cavity infections and also they are the most often anaerobes isolated from respiratory infections and their complications. As a consequence of their presence in infections of the oral cavity, these microorganisms can spread within the intracranial cavity and could produce brain abscesses.

Finally, regarding to *Fusobacterium* species they are mainly part of the microbiota of the oral cavity and the intestinal tract and they are able to produce abscesses and necrotizing infections such as pneumonia and brain infections.

Gram-positive anaerobic bacilli

Among the anaerobic Gram-positive bacilli, *Clostridium* spp. is most commonly isolated from human infections. These microorganisms are usually associated with wound and bloodstream infections and abscesses. There is more than 200 species of *Clostridium*, but the most common species isolated from clinical samples are *C. perfringens*, *C. septicum*, *C. ramnosum*, *C. novyi* and *C. sordellii* (Brook, 2016). *C. difficile* may cause several intestinal diseases that range from mild sickness to severe intestinal infections called pseudomembranous colitis.

Regarding to the anaerobic Gram-positive non-spore-forming rods, these bacteria are part of the microbiota of different body sites like vagina, skin, gingival crevices and gastrointestinal tract. This group include *Cutibacterium* (formerly *Propionibacterium*), *Bifidobacterium*, *Lactobacillus*, *Actinomyces*, *Eubacterium*, *Atopobium* and others. These anaerobes may be isolated from aspiration pneumonia, peritonitis, periodontitis, gynecological infections and intracranial abscesses. *C. acnes* is the most frequent species included in genera *Cutibacterium* and usually has low virulence, but it might cause infections in implanted prostheses and medical devices as well as in central nervous system (CNS) shunts and drains.

Gram-positive anaerobic cocci (GPACs)

Among the GPAC associated with clinical infections, the most prominent isolated bacteria from human samples are *Anaerococcus*, *Finegoldia*, *Parvimonas*, *Peptoniphilus* and *Peptostreptococcus*. GPACs are mostly isolated from polymicrobial infections so their relevance has often been overlooked. Moreover the taxonomy of GPACs has undergone various changes over the years, mainly due to the introduction of new molecular identification methods. These anaerobic bacteria are part of the oral, upper respiratory, genitourinary and intestinal tract and skin microbiota and are isolated from infections such as lung abscesses, aspiration pneumonia, soft tissue infections, female genital tract and chronic sinusitis.

Gram-negative anaerobic cocci

From the three described genera of Gram-negative anaerobic cocci, *Veillonella*, *Acidaminococcus* and *Megasphaera*, the first of them is that it has epidemiological relevance. Its clinical meaning is unknown and they are considered microorganisms of low virulence. The most relevant species are *V. parvula*, *V. atypica* and *V. dispar*, which are part of the microbiota of the oral cavity and the intestinal and genitourinary tract. They are mainly isolated within polymicrobial infections, although in some circumstances such as malignancies may cause bacteremia (Cobo et al., 2020a, b).

Pathogenesis and virulence factors of anaerobic bacteria

Once anaerobic microorganisms are present in tissue with a lowered oxidation-reduction potential, they may proliferate and cause local infection. After this, there are some factors involved in the pathogenesis of these kinds of infections such as bacterial synergy, virulence factors of the bacteria, and mechanism of abscess formation. Some of these virulence factors are showed in Table 2. Virulence factors may contribute to anaerobic infection due to its ability to evade host defenses, produce enzymes and toxins, adhere to cell surfaces and exhibit some surface structures like capsular polysaccharides.

Regarding to the GPACs, in two of them has been possible to identify some virulence factors. The well-described virulence factors of *F. magna* are Protein L (Björck and Protein, 1988), subtilase (SufA) (Karlsson et al., 2007), peptostreptococcal albumin binding protein (PAB) (De Château and Björck, 1994) and *F. magna* adhesion factor (FAF) (Frick et al., 2008).

Protein L induces an effect on human basophils inducing the synthesis of leukotriene C₄ (LTC₄) (Patella et al., 1990). LTC₄ is a proinflammatory mediator that induces a greater histamine release and the synthesis of the prostaglandin D₂. These two mediators have significant importance in inflammation, so expression of protein L is correlated with virulence (Kastern et al., 1990).

Table 2 Main virulence factors of anaerobes.

Microorganism	Virulence factor
<i>Finegoldia magna</i>	Protein L SufA (subtilase) PAB (peptostreptococcal albumin binding protein) FAF (<i>F. magna</i> adhesion factor)
<i>Parvimonas micra</i>	Capsule formation Adhesins Immunoglobulin Fc-binding proteins Gelatinase and protease activity Production of TNF- α and IL-1 β
<i>Bacteroides fragilis</i> group	Hemagglutinin Protease and neuraminidase activity Capsular polysaccharides Enterotoxin production
<i>Prevotella</i> species	Protease production Lipopolysaccharides
<i>Fusobacterium</i> species	Proteases production Lipopolysaccharides Leukotoxin production Hemolysin and phospholipase (<i>F. necrophorum</i>) Adhesins (<i>F. nucleatum</i>)
<i>Porphyromonas gingivalis</i>	Capsule Hemolysin Proteases Lipopolysaccharides

In addition, protein L is a potent inducer of the synthesis and release of IL-4 and IL-13 and classifies this protein as a bacterial super-antigen (Genovese et al., 2003).

Protein PAB is a human serum albumin present in several isolates of *F. magna* whose presence suggests a link between this phenotype and the presence of suppurative infections. Therefore, protein PAB plays an important role in enhancing bacterial virulence during infection.

On the other hand, subtilisin-like proteinase of *F. magna* whose presence may result in enhanced pathogenicity of this anaerobe during the infection. The ability of SufA to cleave and inactivate some antimicrobial peptides might lead to increase survival and proliferation during infection. Moreover, several studies showed that SufA was able to modulate the activity of chemokines to promote bacterial survival during epithelial inflammation decreasing the inflammatory response (Karlsson et al., 2009a). In addition, the role of SufA in the interaction with the host coagulation system was also studied. SufA was found to cleave fibrinogen which affects to fibrin polymerization which prevent the formation of a fibrin network around *F. magna* and to the wound healing that leads to the delay of wound repair (Karlsson et al., 2009b). The final effect is that the bacteria are trapped and this fact facilitates the establishment of infection and the promotion of virulence (Karlsson et al., 2009a).

Finally, the FAF protein is a factor that mediates bacterial aggregation. The presence of this protein in the *F. magna* surface could impair wound healing in chronic wounds. Moreover, this factor was found to have a neutralizing capacity on bacterial proteins such as LL-37, MK and hBD-3 (Frick et al., 2011).

Regarding to *Parvimonas micra*, this is one of the best studied microorganisms belonging to the GPAC group in terms of characterization of its virulence factors. The capacity of capsule formation and the ability to form hydrogen sulfide have been demonstrated to be as important virulence factors (Carlsson et al., 1993). Moreover, *P. micra* have the capacity to adhere to gingival epithelial cells (Dzink et al., 1989) and to express immunoglobulin Fc-binding proteins (Grenier and Michaud, 1994). In addition, some data in several strains of *P. micra* demonstrated to have cell-associated gelatinase activity (Ng et al., 1998) and elastase activity (Mikamo et al., 1999). Finally, some studies also reported the *P. micra* production of TNF- α and IL-1 β which are important determinants of the periodontitis progression (Tanabe et al., 2007).

In Gram-negative anaerobic microorganisms, some virulence factors have been also identified. Regarding to *B. fragilis*, this microorganism has a high ability for abscess formation. *Bacteroides* species have involvement in the prolongation of the intrinsic pathway of clotting in human blood (Murphy et al., 2011). *B. fragilis* and other *Bacteroides* species such as *B. thetaiotaomicron* cause the release of significant levels of bradykinin in human plasma that will provide the bacteria the ability to spread by inhibition of clot formation (Murphy et al., 2011). However, the capsular polysaccharide has been identified as the major virulence factor of *B. fragilis*, playing a central role in the abscess production (Onderdonk et al., 1977). *B. fragilis* may express different surface polysaccharide combinations through a reversible inversion of DNA segments (Krinos et al., 2001). The most important and most studied of these polysaccharides are PSA and PSB; PSA was demonstrated to activate host CD4⁺ T cells and facilitate the release of interleukin-17, interferon- γ , and chemokines (Chung et al., 2003; Wang et al., 2006). Moreover, the capsule promotes the release of the proinflammatory cytokines tumor necrosis factor- α and IL-1 β that are found to potentiate the increase of cell adhesion molecules in order to increase the binding of neutrophils to these cells and initiates abscess formation (Gibson et al., 1998).

On the other hand, *B. fragilis* produces other virulence factors such as fimbriae, pili and hemagglutinin molecules that help in attachment to host cell surfaces. Moreover, they produce several toxins and enzymes like neuraminidase, protease, hydrolase and superoxide dismutase. Enterotoxigenic *B. fragilis* strains secrete an enterotoxin termed *B. fragilis* toxin (BFT) and may have a role in inflammatory diarrhea and flare-ups of inflammatory bowel disease (Basset et al., 2004; Sears, 2009). This toxin has a cytopathic effect for epithelial intestinal cells inducing tissue damage.

On the other hand, *C. difficile* produces two toxins, an enterotoxin (toxin A) and a cytotoxin (toxin B). In some strains a third toxin (binary toxin) could be produced, although the importance in the pathogenesis of this disease has not been clearly established. Apparently, toxins A and B are able to interact synergistically in the pathogenesis of *C. difficile* infection, although toxin-A negative strains can also produce disease. Highly virulent *C. difficile* (BI/NAP1/PCR ribotype 027) strains have caused some outbreaks. These isolates are characterized by high level toxin production due to a deletion in the negative regulator gene (tcdC) of toxin A and B. Other *Clostridium* species produce some toxins such as neurotoxins, enterotoxins, lecithinases, DNAses and others and this fact justify their virulence and involvement in aggressive clinical pictures.

F. necrophorum and *F. nucleatum* produce a numerous types of toxins such as leukotoxin, endotoxin and hemolysin causing several necrotic diseases and oral infections. *F. nucleatum* also co-aggregates with other oral bacteria to increase attachment to plaque helped with some adhesin molecules. Both species of *Fusobacterium* produce a lipopolysaccharide responsible for the production of cytokines and other inflammatory mediators playing a central role in periodontal disease.

Regarding to *Prevotella* species little is known about the virulence factors, although production of proteases and metabolic products has been described, especially IgA proteases. The destruction of Ig A produced by mucosal surfaces permit this anaerobe to evade the first line of defense.

The last Gram-negative anaerobe which is known producing virulence factors is *P. gingivalis*. This bacterium produces proteases that can help to cause attachment, degradation or cleavage of host cell proteins and surface receptors, adhesins and hemagglutinins. The capsular polysaccharide of this microorganism may facilitate the spread of infection because it has a proinflammatory activity implicated in the periodontal disease.

Clinical syndromes associated with anaerobic microorganisms

Anaerobic bacteria are common pathogens in humans and they can produce a great variety of infections in different body locations. Table 3 summarizes the type and location of these infections. Although most clinically significant anaerobes are involved in mixed infections alongside aerobic bacteria, they can be responsible for severe disease in certain circumstances, such as in blood infections or when present in normally sterile body sites. Infections due to anaerobic isolates may sometimes be missed because of the special measures required for their transportation. Other critical factors for the successful isolation of these microorganisms in the microbiology laboratory include incubation in anaerobic atmosphere, the use of specialized culture media, and prolonged culture (Finegold, 1995). The main feature of infection caused by anaerobes is the abscess formation, both in the site of direct bacterial contamination and in distant sites due to contiguous and hematogenous spread.

Central nervous system infections

In this region, the main types of infections in which anaerobic bacteria are involved include subdural empyema and both brain and epidural abscesses. Meningitis caused by anaerobes is quite rare and often suggests a parameningeal collection or shunt infection. However, anaerobic microorganisms are common pathogens producers of brain abscesses (Brook, 2017; Le Moal et al., 2003). This clinical entity is caused by microorganisms belonging to the normal oral cavity microbiota as a consequence of its spread from otitis, sinusitis or oral infections. On the other hand, it is not unusual the hematogenous dissemination from intraabdominal or pelvic infections, leading to the production of infections located at the brain region. In these last circumstances, the microorganisms implicated are those present in the intestinal and/or female genital tract microbiota. The microbiology of these abscesses may include a great variety of pathogens; it may present as an isolated infection by anaerobes or a mixture of aerobic and anaerobic bacteria, being the anaerobic bacteria common constituents of brain abscesses. The main anaerobes implicated in the production of brain abscesses are GPACs, *Prevotella*, *C. acnes*, *Fusobacterium*, *Bacteroides*, and *Actinomyces* (Brook, 2017).

Table 3 Types and location of main infections caused by anaerobic microorganisms.

Location of infection	Infection
Central nervous system	Epidural abscess Subdural empyema Brain abscess
Head, neck and oral cavity	Sinusitis Gingivitis Dental infections Periodontal infections Perimandibular infection
Chest cavity	Pneumonia Pneumonitis (aspiration) Pulmonary abscess
Abdominal cavity	Empyema Intraabdominal abscess Liver abscess Biliary tract infection Peritonitis
Female genital tract	Appendicitis Pelvic inflammatory disease Pelvic abscess Bartholin gland abscess Endometritis Bacterial vaginosis Post-surgery infection
Blood	Bacteremia
Skin and soft tissue	Cutaneous abscess and wound Diabetic foot ulcer Pressure ulcer Gas gangrene
Bone and joints	Arthritis and osteomyelitis

Head, neck and oral cavity infections

Anaerobic microorganisms are involved in some infections of the oral cavity and adjacent tissues. The bacteria isolated from these infections reflect the microorganisms present in the normal oral microbiota, especially *Bacteroides non-fragilis*, pigmented *Prevotella* species, *Porphyromonas*, *Fusobacteria* and *Peptostreptococcus*.

Dental infections

Dental infections include a group of diseases such as periapical or dental abscesses, pulpitis and perimandibular space infections and anaerobes are involved in most clinically important infections in this area. The initial lesion is endodontal and the infection progress to the periapical region and it may spread through the mandible to involve adjacent tissues causing osteomyelitis of the maxillary sinuses and infection of submandibular spaces. Factors involved in this progression are unknown but the presence of gingival microbiome forming a “network of bacteria” may contribute (Siqueira and Rôças, 2013).

Also, in the gingival crevices and gums, anaerobic microorganisms are able to cause gingivitis, periodontitis and periodontal abscesses. Formation of dental plaque influenced by several factors like oral hygiene, leads to overgrowth of pathogenic bacteria and development of infections in this location. The bacteria mainly involved in these kinds of infections are *Prevotella*, *Fusobacterium*, *Porphyromonas* and *Treponema* spp.

The most common type of periodontitis is chronic periodontitis, a major cause of tooth loss. However, gingivitis may become a more serious infection known as Vincent angina or trench mouth. This disease is a necrotizing ulcerative process associated with severe pain, tender bleeding gums, tissue destruction, pseudomembrane formation and putrid discharge. Patients may have fever, cervical lymphadenopathy and leukocytosis. This infection may spread to adjacent tissues causing bone or soft tissue destruction or acute necrosis of the pharynx.

Another necrotizing infection of the oral mucous membranes is noma or cancrum oris. As in the Vincent angina, this infection is characterized by destruction of bone and soft tissue. In the most severe cases, it can evolve quickly to from gingival inflammation to orofacial gangrene (Enwonwu et al., 2006). The majority of cases are encountered in sub-Saharan Africa in young children with malnutrition or systemic disease (Falkler et al., 1999).

Deep neck space infections

The deep neck spaces are formed by fascial planes of the head and neck such as the submandibular space. The infections in these areas often emerge from dental infections rather than from pharynx or tonsils infections.

There is two severe and life-threatening perimandibular infections known as Lemierre syndrome and Ludwig’s angina.

Ludwig’s angina consist of a bilateral infection of the sublingual and submandibular spaces accompanied by marked local tissue swelling (base of the tongue), tongue displacement and potential airway compromise. This disease is typically a polymicrobial infection involving the oral cavity microbiota.

On the other hand, Lemierre’s syndrome is an infection of the posterior compartment of the lateral pharyngeal space with subsequent septic thrombophlebitis of the internal jugular vein, bacteremia and lung metastatic abscesses. This entity is usually caused by *F. necrophorum* but other anaerobic microorganisms such as *Bacteroides*, *Peptostreptococcus*, *Porphyromonas* and *Prevotella* have been involved in this infection (Kuppalli et al., 2012; Klug et al., 2016).

Other infections

Although rare, anaerobic microorganisms are able to produce chronic sinusitis in both adults and children. The predominant anaerobes involved in these infections are *Fusobacterium*, *Peptostreptococcus*, *Prevotella* and *C. acnes*. However, the majority of these infections are caused by mixed infections of aerobic and anaerobic bacteria (Brook, 2008a).

Chest cavity infections

Anaerobic bacteria are relatively common pathogens isolated from infections of the pleuropulmonary area. These infections are predominantly acquired due to aspiration of oral or dental secretions by patients with predisposing conditions such as neurologic disorders, periodontal or gingival disease and/or situations with temporary loss of consciousness (Bartlett, 2012).

The anaerobic microorganisms most commonly associated with pleuropulmonary infections are those that form part of the oral cavity and include *Prevotella*, *Peptostreptococcus*, *Bacteroides* and *Fusobacteria*. However, a great quantity of these infections are mixed anaerobic and aerobic, especially microaerophilic streptococci (Takayanagi et al., 2010; El-Sohl et al., 2003).

Clinical presentation may occur in four ways: aspiration pneumonitis, necrotizing pneumonia, lung abscess or empyema. Aspiration pneumonitis is generally an indolent form of pneumonia in contrast to the abrupt course of acute pneumonia. Patients often present with symptoms of chronic disease, including anemia and weight loss. Initially, patients rarely have putrid sputum although can become malodorous in the later stages of disease. It can see a mixed microbiota in the Gram stain and the infection is produced in a dependent pulmonary segment (Bartlett, 2012). Samples obtained by transtracheal and/or transthoracic aspiration may be adequate for the diagnosis.

On the other hand, necrotizing pneumonia is a complication of the aspiration pneumonitis and is characterized by the presence of many abscesses within the lung parenchyma.

Regarding to the lung abscesses, the majority of them are secondary to the presence of periodontal disease and in these cases the predominant bacteria are those present in the oral microbiota. Finally, empyemas are long-term complications of anaerobic pulmonary infection and are characterized by pleuritic chest pain.

Abdominal cavity infections

The main intraabdominal infections caused by anaerobic microorganisms are the peritonitis, both generalized and localized, and the abscesses. These entities are usually polymicrobial and mixed infections (aerobic and anaerobic bacteria), with a predominance of coliforms, anaerobes and *Streptococcus/Enterococcus*. They accounted due to a break in continuity of the mucosal surface and the leak of the normal intestinal microbiota into the sterile peritoneal cavity. The main causes of the mucosal break are diseases such as neoplasms, diverticulitis, appendicitis, inflammatory bowel disease, surgery or trauma. The majority of bacteria isolated in these kinds of infections are *Escherichia coli* and *Bacteroides* spp., especially *B. fragilis* group. Other anaerobic microorganisms commonly isolated are *Prevotella*, *Peptostreptococcus* and *Fusobacterium* spp. Regarding to the proximal bowel infections, the perforation of this area is followed of infection due to aerobic and anaerobic Gram-positive bacteria and *Candida* spp. Moreover, infections in this region involve other microorganisms like *C. septicum* and other clostridia.

The toxin-producing *C. difficile* is responsible for antibiotic-associated gastrointestinal diseases ranging from a relatively benign, self-limited diarrhea to a severe, life-threatening pseudomembranous colitis. The antibiotics alter the normal microbiota allowing the overgrowth of these relatively resistant microorganisms. The disease is produced due to the proliferation of this bacterium in the colonic mucosa and the production of toxins.

Female genital tract infections

The female genital tract is a body region that constitutes a major reservoir for anaerobic microorganisms. The majority of infections of the female genital tract that are not caused by sexually transmitted agents involve anaerobes. The main infections that can be considered within this section are endometritis, Bartholin gland abscesses, bacterial vaginosis, salpingitis, pelvic cellulitis, septic thrombophlebitis, wound infections, tubo-ovarian abscesses, pyometra, adnexal abscesses and pelvic inflammatory disease, and septic abortion. The main bacteria implicated in these kinds of infections include *Prevotella*, *Porphyromonas*, *Peptostreptococcus*, *Actinomyces*, *Eubacterium* and *Clostridium*. Like in the majority of anaerobic infections, most cases are produced by mixed infections, involving both aerobes and anaerobes (Cereija et al., 2013).

Anaerobic bacteremia

Anaerobic microorganisms remain an important cause of bloodstream infection being still a major cause of morbidity and, despite all the advances in medical practice in recent years, the presence of anaerobes in the bloodstream continues to have a high associated mortality requiring appropriate treatment (Raymond et al., 2006; Blairon et al., 2006). The detection rate of anaerobes in blood cultures is around 0.5–11.8% of all bacteremic episodes, depending on geographic location, and both patient age and condition (Arzese et al., 1995). Mortality from anaerobic bacteremia has remained high over the past few years. Mortality rates reported range from 14% (De Keukeleire et al., 2016) to 27% (Robert et al., 2008). In a very recent report, the mortality rate was 25.7%, similar to other investigations (Cobo et al., 2020a, b). A study obtained a mortality rate of 14% for patients with adequate antimicrobial therapy compared with 63% for patients with inappropriate treatment (Zahar et al., 2005).

Some factors frequently associated with high mortality from anaerobic bacteremia have been published including underlying malignancies, especially hematologic, diabetes and gastrointestinal surgery (Blairon et al., 2006; Lassmann et al., 2007). A below mentioned report (Cobo et al., 2020a, b), moreover, found three variables significantly and independently associated with mortality with anaerobic bacteremia: hospitalization in an ICU, presence of septic shock and presence of any type of cancer.

Most anaerobic bacteriemias are caused by Gram-negative bacilli, especially by members of *B. fragilis* group (60% cases), followed by *Clostridium*, *Peptostreptococcus* and *Fusobacterium* species (Brook, 2010). Some of these bacteriemias could be polymicrobials (Cobo et al., 2018).

Anaerobic bacteremia is often secondary to an infectious process derivate from an intraabdominal, female genital tract, respiratory tract or soft tissue source. Predisposing factors for this infection are mainly debilitating diseases such as malignancies, diabetes, organ transplantation and abdominal and pelvic surgeries (Brook, 2010).

Skin and soft tissue infections

Infections in the skin and soft tissue caused by anaerobic microorganisms are mainly due by contamination with the flora from adjacent mucosal surfaces, although can be also caused by cutaneous anaerobic microbiota, especially *Peptostreptococcus* (Brook, 2007). The main clinical entities in this type of infections are cutaneous abscess and wounds, diabetic foot ulcers, pressure ulcers and gas gangrene. As in other areas, infections most often are polymicrobials, yielded mixed microbiota, including aerobes and anaerobes, mainly the *B. fragilis* group and *Clostridium* spp. along with Enterobacteriaceae and *Enterococcus* spp. However, in infections caused near to oropharynx region, predominant pathogens are those present in the oral microbiota such as *Porphyromonas*, *Prevotella*, *Fusobacterium* and *Peptostreptococcus* spp. Anaerobic microorganisms can also cause deep soft tissue infections like

synergistic cellulitis, necrotizing fasciitis and gas gangrene; these infections are usually mixed infections with aerobes and anaerobes. The most important pathogens involved in deep infections are *Clostridium*, *Bacteroides*, *Peptostreptococcus* and group A β -hemolytic streptococci. A subtype of cellulitis such as Fournier gangrene may require also surgical treatment and it is characterized for affection of perineum, scrotum and the anterior abdominal wall.

Bone and joint infections

Arthritis is rarely caused by anaerobic microorganisms and most cases involve a single isolate and are secondary to hematogenous spread, trauma, or a prosthetic joint. The most frequent anaerobes encountered in this kind of infection are *B. fragilis* group, *Fusobacterium*, and *Peptostreptococcus* spp. (Brook, 2008b). In infections involving prosthetic devices, especially in shoulder joints, *Cutibacterium* could be sometimes isolated these types of samples (Levy et al., 2008).

Regarding to osteomyelitis, the predominant anaerobes in this disease are *Bacteroides* spp., while *Prevotella*, *Porphyromonas*, *Peptostreptococcus* and *Fusobacterium* are the predominant anaerobes in skull and bite infections. On the other hand, in osteomyelitis associated with wound contamination after trauma or exposure to gut microbiota, *Clostridium* spp. may be also isolated.

The main sources encountered in osteomyelitis infections are infected adjacent soft tissue areas, such as foot and decubitus ulcers, in which is frequent the isolation of aerobes and anaerobes.

Laboratory diagnostics of anaerobic infections

Samples collection and transportation

The samples should be properly collected and transported. Samples should be collected minimizing contamination by indigenous microbiota of mucosal surfaces and it is also important to remember that the samples should be taken, if it is possible, before starting antimicrobial therapy. The optimal samples are usually sterile fluids such as blood, pleural and peritoneal fluids, and also biopsy specimens. Table 4 describes the main acceptable collection procedures for the recovery of anaerobes. As it can see, in general, collection of tissues or sterile fluids is better than swab samples. The percutaneous aspiration from an abscess requires decontamination of the skin or mucosal surface. The sample should be immediately inoculated in a commercially available anaerobic-transport medium or, as alternative, to inoculate the sample in a sterile tube and cover up to the surface eliminating or minimizing the quantity of air. After the collection, a rapid transportation into the microbiology laboratory is necessary in order to preserve the anaerobes and to permit prompt microbiologic processing, because a contact with O₂ makes unviable the anaerobic microorganisms in less than 1 h.

Direct examination of the sample and gram staining

Direct macroscopic examination of the specimens for study of anaerobes may reveal several features that cause suspicion of anaerobic infection such as foul odor (due to the presence of volatile fatty acid and amine-end products of metabolism), red fluorescence due to pigmented *Prevotella* or *Porphyromonas* species, and black necrotic tissue, discharge, gas or purulence. However, the absence of these characteristics does not rule out anaerobic infection.

On the other hand, microscopic examination of the sample should include a Gram stain that may reveal the number and morphology of microorganisms and the presence of neutrophils. Because anaerobic infections are usually polymicrobial, Gram stain of exudates showing a polymicrobial microbiota could be indicative of anaerobic infections. Some anaerobes have special characteristics when they are observed by microscopy: *F. nucleatum* is a fusiform bacterium with pointed ends, *F. necrophorum* is a long "ropy" Gram-negative bacillus and *Clostridium* spp. are large "boxcar"-like Gram-positive bacillus. In some circumstances, the direct microscopic examination can lead to final diagnosis like in case of bacterial vaginosis due to the presence of "clue" cells. The presence of branching Gram-positive bacilli can mean the presence of *Actinomyces* spp., *Propionibacterium* spp. or *Bifidobacterium* spp.

Table 4 Acceptable collection procedures for anaerobic microorganism culture.

Blood collection for culture
Aspiration of sterile fluids (pleural, peritoneal, . . .)
Suprapubic puncture for urine collection
Percutaneous tracheal aspiration
Bronchoalveolar lavage
Transthoracic pulmonary puncture
Abscess percutaneous aspiration
Deep aspiration of cutaneous ulcer
Transvaginal aspiration
Biopsy samples

Sample processing: Anaerobic culture techniques

Selective and nonselective media should be used for culture of samples. As a nonselective medium, it can use the anaerobic *Brucella* blood agar (containing horse or sheep blood, additional hemin, and vitamin K₁). For the selective isolation of the *Bacteroides fragilis* group and *Bilophila*, *Bacteroides* bile-esculin agar could be used; for the selective isolation of pigmented and non-pigmented *Prevotella* and other Gram negative anaerobic rods, kanamycin-vancomycin agar with laked sheep blood to select could also be used; other media such as phenylethyl alcohol sheep blood agar may be used to inhibit facultative Gram negative rods. Finally, to inhibit swarming of several clostridia, an anaerobic broth could be inoculated. After inoculation, the media should be placed in an anaerobic atmosphere as soon as possible. The best method for this incubation is the use of an anaerobic chamber. Other methods are being also used such as boxes, plastic envelopes shorter jars and automated gas flushing devices. The only caution using jars and boxes is that they should not be opened until after 48 h of incubation to prevent premature death of anaerobes. The period of incubation in the majority of cases should be of 5–7 days.

Identification of anaerobic bacteria

Initially, a presumptive identification could be performed with antibiotic disks such as vancomycin (5 µg), colistin (10 µg), penicillin (2 U) and kanamycin (1000 µg). Table 5 shows the presumptive identification of the main anaerobic microorganisms with the use of these antibiotic disks. There have been numerous ways in which bacteria could be identified: phenotypic (physiological/biochemical characteristics), chemical analysis (MALDI-TOF, nuclear magnetic resonance (NMR) spectroscopy), and genetic and molecular analysis (including nucleic acid sequencing). Until recently, biochemical methods have been used for genus and/or species identification. However, these methods are time consuming (1–5 days of anaerobic incubation), too cumbersome and costly for many laboratories (Holdeman et al., 1977). Now, there are simple and rapid micromethod identification systems which would permit laboratories with limited facilities to identify clinically important anaerobic bacteria (e.g. API ANA BioMérieux, ANI Card, BioMérieux, MINITEK Becton-Dickinson). Application of gas liquid chromatography (GLC) for the detection of anaerobes has been the confirmation method; however, the introduction of mass spectrometry (MALDI-TOF MS) in the routine of laboratories has supposed a great revolution in the diagnosis. This technique is a useful a simple method for rapid identification of microorganisms for the routine identification of clinical isolates (Nagy et al., 2009, 2012). Recent advances in detection of anaerobic microorganisms from clinical samples include 16S rRNA gene-based methods, DNA hybridization, multiplex polymerase chain reaction, and oligonucleotide array technologies (Coltella et al., 2013). 16S rRNA aids in taxonomic placement of sequences comparing the sequences to others (Song et al., 2003).

Laboratory diagnosis of *Clostridium difficile* infection

Isolation of the bacterium from liquid or semisolid stool specimens is necessary for epidemiological investigation including typing. Diagnosis of *C. difficile* infection has been based on the detection of the microorganism by culture in CCFA (cefoxitin-cycloserine fructose agar), CCEY (cefoxitin-cycloserine egg yolk agar) or CCEYL (cefoxitin-cycloserine egg yolk agar with lysozyme) and further demonstration of the toxins. Pretreatment of the stools with a similar volume of alcohol increases the isolation rate of the bacterium. After this, direct detection of the toxins from the isolated strain by the cytotoxic assay using different cell lines is necessary. However, at this moment, the most widely method used for the diagnosis is the detection of *C. difficile* specific antigens such as glutamine dehydrogenase (GDH), and toxin A and B, by immunocromatography or ELISA techniques. A diagnostic algorithm of *C. difficile* infection is showed in Fig. 2. More recently, real-time PCR methods have been developed to detect the

Table 5 Presumptive identification with antibiotic disks of main anaerobes.

Microorganism or group	Vancomycin (5 µg)	Kanamycin (1000 µg)	Colistin (10 µg)	Penicillin (2 U)
Gram-positive	S	V	R	V
Gram-negative	R	R ^a	S ^b	
<i>B. fragilis</i> group	R	R	R	R
Other <i>Bacteroides</i> species	R	R	V	S
<i>Porphyromonas</i>	S	R	R	
<i>Fusobacterium</i>	R	S	S	
<i>Clostridium perfringens</i>	S	S	R	
<i>Prevotella</i>	R	V	V	
<i>Parabacteroides</i>	R	R	R	

S = sensitive; R = resistant; V = variable.

^aSome strains are sensitive.

^bUnusual Gram-negative anaerobes may be resistant.

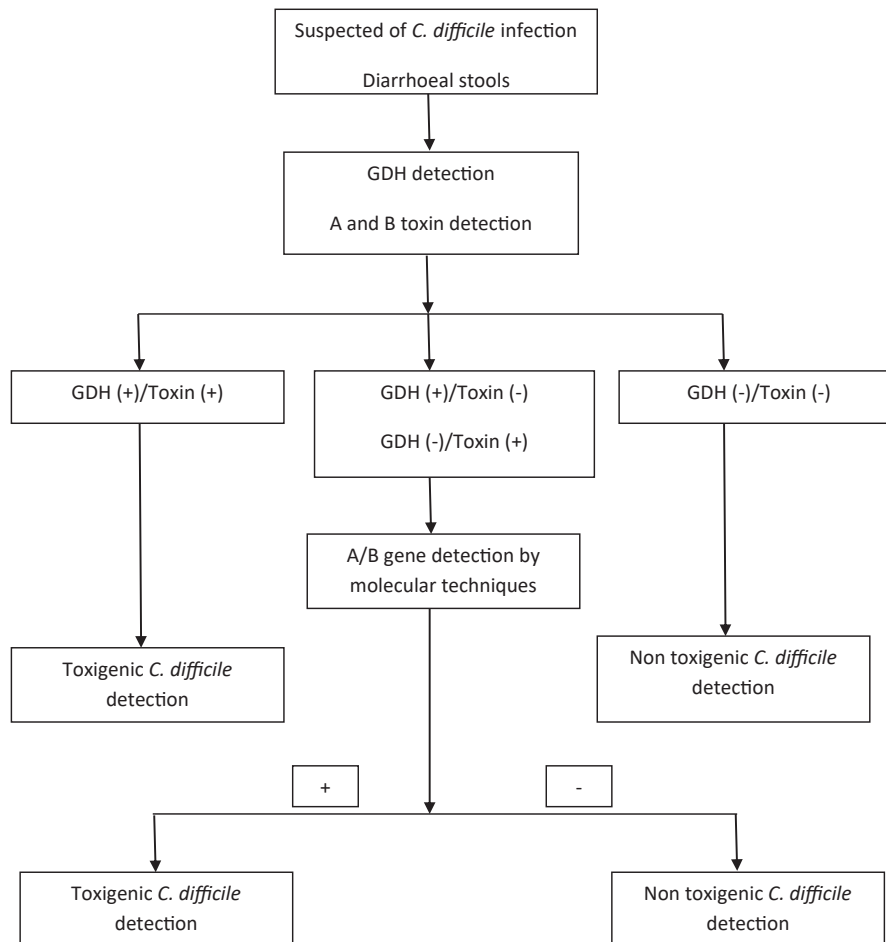


Fig. 2 Diagnostic algorithm of *C. difficile* infection.

toxin B gene (*tcdB*) directly from the stool or from the strain. Molecular methods for typing (PCR-ribotyping REA, PFGE, MLST) could be used for study of outbreaks.

Treatment of anaerobic infections: Antibiotic selection

Because anaerobic infections may cause abscess formation and tissue damage, therapy for these infections usually requires the administration of effective drugs, surgical debridement and/or abscess drainage. Moreover, as many of these infections are of mixed etiology (aerobes and anaerobes), the drugs used should be active against both aerobic and anaerobic microorganisms. Most anaerobic infections are treated empirically on the basis of some features such as the result of the Gram stain, the type of infection, the microorganisms often present in these infections, and the antimicrobial resistance pattern. Antimicrobial susceptibility testing of anaerobic bacteria is performed by a minority of laboratories (Goldstein and Citron, 2011; Smith et al., 2010) due to some circumstances like that cultures usually yield a polymicrobial flora or may be falsely negative. Current recommendations emphasize the fact that antimicrobial susceptibility testing of anaerobic isolates is only needed for severe infections, to test new antimicrobial agents or to monitor local and regional resistance patterns, although a rise in the resistance of anaerobes to some drugs may indicate a greater need for this testing (Brook et al., 2013; Schuetz, 2014).

Currently, the main drugs used against the majority of anaerobic infections include metronidazole, carbapenems, β -lactam/ β -lactamase inhibitor and clindamycin. Table 6 summarizes the spectrum of activity of various antibiotics that can be used against anaerobic pathogens.

Depending on the clinical entity, a specific antimicrobial treatment should be applied due to the predominant microorganisms that may be encountered. For intraabdominal infections, antimicrobial coverage of *Bacteroides* spp. and Gram-negative aerobic microbiota is mandatory. For patients with mild-to-moderate community-acquired infections single agents such as carbapenems and β -lactam/ β -lactamase inhibitor combinations are suitable. Other valid options could be tigecycline, moxifloxacin and

Table 6 Activity spectrum of main drugs used against anaerobic microorganisms.

Antimicrobial agent	Spectrum of activity/characteristics
Penicillin, ampicillin and amoxicillin	Active against anaerobes that do not produce β -lactamase. Almost all GPACs are susceptible to them, but up to 80% of <i>B. fragilis</i> strains are resistant to penicillins. Many strains of <i>Prevotella</i> spp. and some Clostridia are also resistant.
Cephalosporins	Overall, this group has less activity in vitro than penicillin versus most anaerobes. Many strains of <i>B. fragilis</i> group, <i>Prevotella</i> , <i>Porphyromonas</i> and <i>Fusobacterium</i> species produce cephalosporinases. 85% of isolates are susceptible to cefoxitin. Cefoxitin has poor activity against Clostridia. Cefotetan is less effective than cefoxitin.
β -lactam/ β -lactamase inhibitor	Good option against β -lactamase producing anaerobes. Ceftazidime-avibactam has limited action against anaerobic microorganisms.
Metronidazole	This drug is active against Gram-negative anaerobes, including the <i>B. fragilis</i> group. Inactive versus <i>Cutibacterium</i> , <i>Actinomyces</i> and microaerophilic streptococci. Penetrates well in abscesses.
Clindamycin	This antibiotic is active against many anaerobes. Rates of resistance have increased in last years. Some Clostridia other than <i>C. perfringens</i> are resistant.
Carbapenems	All components of this group of antibiotics are equally active against anaerobes. They are resistant to most <i>Bacteroides</i> β -lactamases.
Fluoroquinolones	The most active in this class for anaerobic infections is moxifloxacin. Increased resistance in <i>B. fragilis</i> group, so no recommended for intra-abdominal sepsis
Vancomycin	Active against Gram-positive anaerobes; inactive against Gram-negative anaerobes
Macrolides	They have moderate to good in vitro activity against anaerobic microorganisms; inactive against many <i>Fusobacterium</i> spp. and some <i>B. fragilis</i> spp.
Tigecycline	Active against some anaerobes, including strains of <i>B. fragilis</i> that are resistant to β -lactams, clindamycin and fluoroquinolones

GPACs: Gram-positive anaerobic cocci.

cefoxitin. A two-drug therapy with an antibiotic active against coliforms and the other against anaerobes can be an alternative. Clindamycin is now no longer recommended for intraabdominal infections because increasing rates of resistance in *B. fragilis* group.

For oral infections, drugs active against both anaerobic microbiota of the mouth and the Gram-positive aerobic microbiota should be applied. β -lactams antibiotics (e.g. penicillins, cephalosporins) alone are not suitable because the β -lactamase production of anaerobic microorganisms. Antimicrobial treatment for these infections may include β -lactam/ β -lactamase inhibitor combinations, clindamycin or penicillin along with metronidazole.

Antimicrobial susceptibility testing

Susceptibility testing should be performed for clinically relevant anaerobic microorganisms involved in human infections. The disk diffusion method is not recommended for anaerobic pathogens, so the gradient diffusion method using Etests is the choice method (CLSI, 2012). The anaerobic strains should be sub-cultures in Brucella agar supplemented with 5% laked sheep blood, hemin, and 10 μ g/mL vitamin K1. Plates should be incubated in anaerobic atmosphere at 35–37 °C for 48 h. However, agar dilution is the gold standard in the case of MIC determination for anaerobes, although this method is not suitable for routine laboratories.

Antimicrobial resistance

Antibiotic resistance among anaerobic microorganisms has increased significantly over the past decades (Hecht, 2004; Karlowsky et al., 2012). However, resistance rates vary widely among different geographic regions. Regarding to *B. fragilis* group, resistance to penicillin may be observed in around 80–90% of isolates, while a higher proportion of the isolates (20%) are also resistant to amoxicillin-clavulanate. The overall resistance rate to carbapenems is very low (<1%), although some studies have reported higher rates (Snydman et al., 2011). The most significant changes last years in *Bacteroides* spp. have been the increase of resistance rate to clindamycin, ranging 30–50%. The most active drug against *Bacteroides* spp. is metronidazole and resistance to this antibiotic remains rare, although reports are emerging worldwide (Boyanova et al., 2015; Cobo et al., 2019).

Regarding to *Prevotella*, the resistance rate to penicillin is also increasing (Cobo et al., 2019), and their resistance rate to clindamycin is ranging 11 to 40%. Overall, *Prevotella* isolates found no resistance to metronidazole, although in some studies this resistance has been detected (Cobo et al., 2017a; Shilnikova and Dmitrieva, 2015).

On the other hand, very few isolates of *Fusobacterium* shows resistance to antibiotics, except for penicillin due to β -lactamase production.

Among *Clostridium*, *C. difficile* shows high resistance rate to imipenem (90%) and clindamycin (40%), but no resistance to metronidazole or vancomycin. Among other *Clostridium* species, resistance may be observed for all antimicrobials, except for imipenem. Non-spore-forming Gram-positive bacilli are intrinsically resistant to metronidazole, but they are highly susceptible to penicillin, β -lactams/ β -lactamase inhibitor, and carbapenems. The rate of resistance to clindamycin is highest for *Actinomyces* and lowest for *Cutibacterium*.

Finally, regarding to GPACs no resistance to carbapenems and β -lactams/ β -lactamase inhibitor may be observed in any genera. On the other hand, resistance to moxifloxacin is high in all genera, ranging between 25% and 46%. In this group of microorganisms,

the resistance rate to clindamycin is much higher for *Finegoldia* (around 50%) and *Peptoniphilus* (around 40%). Some GPAC strains have shown resistance to metronidazole (Cobo et al., 2017b).

In conclusion, routine antimicrobial susceptibility testing for anaerobes contributes information on the global situation and permits empirical therapies to be selected in accordance with local data on resistant strains.

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Human Pathogenic *Enterobacterales*

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General characteristics

The *Enterobacterial* family contains numerous and varied species, being the largest and most varied group of gram-negative bacilli of clinical importance. These have characteristics in common and are classified according to their biochemical properties, structural antigens, molecular analysis of the genome through sequencing and by analyzing the composition of their proteins using mass spectrometry (Murray et al., 2014).

They are ubiquitous microorganisms, we can find them in soil, water and vegetation, and the most important thing is that as a common niche they are part of the intestinal microbiota of animals, including humans. They produce a great variety of infections in humans, about one third of all bacteremias, more than 70% of urinary tract infections (UTI), as well as many abdominal infections (Eliason, 1940).

Depending on the clinical infections they produce, members of the *Enterobacterales* family can be divided into two broad categories: opportunistic pathogens and primary pathogens.

Primary pathogens (*Yersinia pestis*, *Salmonella* serotype Typhi, *Shigella*) cause disease whenever they are detected in clinical samples; while those that are part of the commensal microbiota (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus* sp., . . .) can produce opportunistic infections.

Physiology and structure

They are all gram-negative, intermediate-sized ($0.3\text{--}1.0 \times 1.0\text{--}6\text{ }\mu\text{m}$), non-spore-forming, facultative anaerobes. They can grow on general media (e.g., blood agar) or on selective media (e.g., MacConkey agar). As nutritional requirements they are undemanding, glucose fermenting, nitrate reducing, catalase positive and oxidase negative.

Selective and differential media allow us a presumptive identification since these media contain one or more carbohydrates (lactose, sucrose) that can be fermented by some of these bacteria. This change is visible thanks to a color change in the medium, caused by a decrease in pH detected by a pH indicator incorporated in the medium (Eliason, 1940).

Lactose fermentation allows us to differentiate fermenting strains (*Escherichia*, *Klebsiella*, *Enterobacter*, *Citrobacter* and *Serratia*; pink colonies on MacConkey agar) from non-fermenting or slow fermenting strains (*Proteus*, *Salmonella* and *Yersinia* species; colorless colonies on MacConkey agar). There are other media in which resistance to bile salts allows us to differentiate enteric pathogens (*Salmonella*, *Shigella*) from commensals that are inhibited by bile salts.

Virulence and antigenic factors

The ability to adhere, colonize, produce toxins and invade tissues are some of the many factors that affect the virulence of enterobacteria. Some species harbor plasmids through which resistance gene transfer can occur (Eliason, 1940).

Regarding the antigenic structure of the cell wall, lipopolysaccharide (LPS) is the main antigen of the wall, consisting of three components.

- Polysaccharide O or somatic, the most external. Important for epidemiological classification within a species.
- A central polysaccharide, shared by all enterobacteria, is used to classify a microorganism as a member of the *Enterobacterales* family.
- Lipid A, responsible for endotoxin activity (important virulence factor).

The classification into serological groups is based on three groups of antigens. These antigens include the following:

- antigen or somatic antigen: it is a thermostable antigen located in the cell wall, present in every genus and species.
- H antigen or flagellar antigen: it is a thermolabile antigen found on the surface of flagella, structures responsible for motility.
- K antigen or capsular antigen: a thermolabile polysaccharide found only in some encapsulated species (K1 antigen of *Escherichia coli* and Vi antigen of *Salmonella enterica* subsp. *enterica* serotype Typhi).

The detection of these antigens has not only epidemiological but also clinical relevance. Some species are associated with specific O and H serotypes, such as *E. coli* O157:H7 associated with hemorrhagic colitis.

As for virulence factors, there are some common to all genera, while others are specific to virulent strains.

Endotoxin

It is a virulence factor shared by aerobic and some anaerobic gram-negative bacteria. Its activity depends on lipid A, released during cell lysis.

Capsule

It serves as protection during phagocytosis by hydrophilic capsular antigens, which interfere with the binding of antibodies to bacteria and are poorly immunogenic or complement activators.

Antigenic phase variation

Somatic O antigens, capsular K antigens and flagellar H antigens can be alternatively expressed or not expressed (phase variation), thus providing protection against antibody-mediated cell destruction.

Type III secretion systems

This is an effector system for transferring their virulence factors to target eukaryotic cells. In the absence of this system, bacteria are less virulent.

Opportunistic members of the family Enterobacteriaceae and associated infections

Escherichia coli

E. coli is the most frequent and important member of the genus. It is associated with multiple diseases, including gastroenteritis, urinary tract infections (UTI), meningitis and sepsis (Croxen and Finlay, 2010).

Most strains of *E. coli* are motile and generally possess adhesive fimbriae, sexual pili and O, H and K antigens. The O groups of *E. coli* have shown remarkable cross-reactivity with the O antigens of other members of the Enterobacteriaceae, particularly *Shigella* spp. (Eliason, 1940).

Pathogenesis

E. coli, in addition to possessing the general virulence factors of the Enterobacteriaceae family, possesses a wide variety of specialized virulence factors such as adhesins and exotoxins.

Adhesion to the host cells occurs in all *E. coli* cells with the exception of ECEI (enteroinvasive *E. coli*), and is often achieved by means of the fimbriae or pili. Following adhesion, *E. coli* counteract host cell mechanisms using secreted proteins. Hijacking and manipulation of host cell signaling pathways can result in coordinated invasion of host cells, evasion of host immune responses, and efficient colonization, ultimately leading to disease (Croxen and Finlay, 2010).

Epidemiology

E. coli resides in large numbers in the gastrointestinal tract, but can behave as an opportunistic pathogen. Most diseases are therefore endogenous, due to an alteration in the patient's defenses (e.g., through trauma or suppression of immunity) they gain access to the peritoneal cavity and can cause extraintestinal or digestive symptoms, with the exception of meningitis and neonatal gastroenteritis.

Most *E. coli* cause disease by acquiring virulence factors encoded in plasmids or bacteriophage DNA (Kolenda et al., 2015).

Clinical manifestations

Gastrointestinal

E. coli can cause several different gastrointestinal syndromes. According to virulence factors, clinical manifestations, epidemiology and the different O and H serotypes, there are five main categories.

(a) Enterotoxigenic *E. coli* (ECET)

It is associated with diarrhea in adults and especially children in tropical and subtropical climates in developing countries, where it is a major cause of bacterial diarrhea in children. In developed countries it is the most common cause of diarrheal disease, sometimes referred to as "traveler's diarrhea". TCD infection is acquired primarily through consumption of contaminated food or water. Poor hygienic hygiene, poor availability of drinking water sources and inadequate sanitation are the main factors contributing to the transmission of this disease. A high infectious dose (10^6 – 10^{10} microorganisms) is needed to initiate disease in an immunocompetent host (Murray et al., 2014).

Watery diarrhea occurs after an incubation period of 1–2 days and lasts 3–5 days. Symptoms are non-bloody watery diarrhea with abdominal cramping, less frequently nausea and vomiting.

It is known that colonization of ECET in the proximal small intestine is mediated by fimbriae or surface proteins (colonization factors (CF)), which allow ECETs to bind to specific receptors on intestinal microvilli. These CFs are encoded in a transmissible plasmid. Once attached, they can release one and/or two types of toxins:

- Thermostable toxin (STa and STb)
- Thermolabile toxin (LT-I, LT-II)

STa toxin is detected in 75–80% of TSEs, alone or in association with LT, and is responsible for the majority of severe cases. It is a monomeric peptide that binds to the guanylate cyclase receptor, causing an increase in cyclic guanosine monophosphate concentrations and thus hypersecretion of fluids and inhibition of fluid absorption (Isidean et al., 2011).

As for LT, LT-II is not associated with disease in humans, whereas LT-I is functionally and structurally similar to cholera toxin. LT consists of two fragments (A and B), where A is the enzymatically active portion. The B, or binding, portion confers specificity. The B portion binds to the GM1 ganglioside of the intestinal mucosa, allowing entry of the A portion. During infection, the A portion activates cellular adenylate cyclase, leading to increased conversion of adenosine triphosphate to cyclic adenosine monophosphate (cAMP). The consequence of cAMP accumulation is hypersecretion of electrolytes and fluids into the intestinal lumen, resulting in cholera-like watery diarrhea (Croxen and Finlay, 2010).

(b) Enteropathogenic *E. coli* (ECEP)

Although the EPEC strain has been known to cause childhood diarrhea since the 1940s, its pathogenic role has remained controversial in recent decades.

The disease is sporadic and outbreaks are infrequent in developed countries; currently cases occur in impoverished countries and mainly affect infants. Transmission is fecal-oral through contaminated surfaces or food (Croxen and Finlay, 2010).

The disease is characterized by watery diarrhea, with low-grade fever, malaise, and vomiting. The stool usually contains large amounts of mucus, but no blood is apparent. Onset may be rapid, and although it usually resolves within a few days, persistent diarrhea may occur, necessitating hospitalization.

(c) Enteraggregative *E. coli* (ECEA)

It is a heterogeneous group of strains characterized by producing diarrhea by adhering to the epithelium of the intestinal mucosa forming a stacking pattern in "brick piles."

It causes watery diarrhea, vomiting, dehydration and occasionally abdominal pain, especially in children. It is one of the few bacteria associated with chronic diarrhea and growth retardation in children. Symptoms usually persist for at least 2 or more weeks.

(d) Shiga toxin-producing *E. coli* or Enterohemorrhagic *E. coli* (EHEC)

Members of this group are defined by the presence of Shiga toxin 1 (Stx1) or 2 (Stx), with serotype O157:H7 being the most frequent.

Stx-1 is a cytotoxin identical to the Shiga toxin produced by *Shigella dysenteriae*, while Stx-2 has 60% homology, both encoded from lysogenic bacteriophages.

Transmission of the disease most often occurs through consumption of undercooked beef or other meat products, water, unpasteurized milk or raw fruit and vegetable juices such as spinach. A small ingestion of bacteria is needed to cause disease, and person-to-person transmission has also been described.

The classic disease caused by ECEH produces a watery diarrhea progressing to bloody diarrhea with abdominal cramping and low fever or absence of fever. The stool does not contain leukocytes, which distinguishes it from dysentery caused by *Shigella* spp. or ECEI infections. The infection is life-threatening and is usually associated with serotype O157:H7.

Hemolytic uremic syndrome (HUS), a disorder characterized by acute renal failure, thrombocytopenia, and hemolytic-microangiopathic anemia, is a complication affecting 5–10% of children under 10 years of age. It usually resolves within 4–10 days in uncomplicated cases, but 3–5% of patients may die or severe sequelae may appear (Amézquita-López et al., 2018).

(e) Enteroinvasive *E. coli* (ECEI)

ECEI strains are rare in both developed and developing countries.

Enteroinvasive strains produce dysentery with direct penetration, invasion and destruction of the intestinal mucosa.

Direct transmission is from person to person by the fecal-oral route. Clinical infection is characterized by fever, severe abdominal cramps, malaise and watery diarrhea.

Although ECEI and *Shigella* spp. are morphologically similar, ECEI and *Shigella* spp. produce similar clinical disorders, the infectious dose of ECEI necessary to produce the disease is much greater (10^6) than that of shigellae (about 100 bacterial cells) (Croxen and Finlay, 2010).

Extraintestinal infections

(a) Urinary tract infection

Urinary tract infections caused by gram-negative bacilli occur mostly by ascension from the colon through the urethra to the bladder, and may reach the kidneys or prostate. In most of the cases the pictures are produced by more virulent strains, with capacity to produce adhesins and hemolysins (Bennet et al., 2016).

(b) Neonatal meningitis and septicemia

E. coli is a major cause of septicemia and meningitis in neonates, accounting for about 40% of gram-negative meningitis.

The K1 capsular antigen present in certain strains of *E. coli* is the virulence factor that is associated with neonatal meningitis infections, it is immunochemically identical to the capsular antigen of *N. meningitidis* group B. (Eliason, 1940).

Klebsiella

Members of this genus are commonly found as part of the intestinal tract of humans and animals.

These microorganisms are associated with various opportunistic infections both at the nosocomial and community level (especially pneumonia, wound infections and urinary tract infections) (Murray et al., 2014).

The genus *Klebsiella* consists of several species, among which are *Klebsiella pneumoniae* subsp. *pneumoniae*, *K. oxytoca*, *K. pneumoniae* subsp. *ozaenae*, *K. rhinoscleromatis* and *K. ornithinolytica*. All species possess a capsule that confers the mucoid appearance to the isolated colonies, in addition to protection against phagocytosis and absorption of antimicrobials (Eliason, 1940).

The most common species of pneumonia are *K. pneumoniae* and *K. oxytoca*, which can produce lobar pneumonia. This type of pneumonia generally produces necrotic destruction of the alveolar spaces, cavity formation and the production of hemoptotic sputum. In addition to this type of infections we can also find them as producers of skin and soft tissue infections and UTIs.

In the case of *Klebsiella granulomatis* it is the etiological agent of granuloma inguinale or Donovanosis, a granulomatous disease affecting the genitals and groin area. It can be transmitted through sexual intercourse or genital trauma. After an incubation period of weeks to months, subcutaneous nodules appear on the genitals or in the inguinal region. These granules rupture giving rise to painless lesions which may resemble syphilitic lesions.

Klebsiella pneumoniae subsp. *rhinoscleromatis* has been isolated from patients with rhinoscleroma, an infection of the nasal cavity that manifests as intense swelling and malformation of the entire face and neck (Murray et al., 2014).

Enterobacter, Citrobacter and Serratia

Genus *Enterobacter*

The genus *Enterobacter* is composed of at least 12 species, among which *Enterobacter cloacae*, *Enterobacter aerogenes*, *Enterobacter gergoviae* and *Enterobacter hormaechei* are most frequently isolated from clinical specimens. Members of this genus are motile. The macroscopic appearance of colonies in many cases resembles *Klebsiella* when grown on MacConkey agar. *E. cloacae* and *E. aerogenes* are the two most common isolates of this group and have been isolated from wound, urine, blood and CSF samples.

E. gergoviae is found in respiratory samples and is rarely isolated in blood cultures. *Enterobacter sakazakii* has been isolated as a pathogen in bacteremia, meningitis, respiratory and wound infections (Mezzatesta et al., 2012).

Genus *Citrobacter*

The genus *Citrobacter* consists of at least 11 species that have been isolated from clinical specimens. Most *Citrobacter* spp. slowly hydrolyze urea and ferment lactose, producing colonies on MAC agar that resemble those of *E. coli*.

Bacteria of this genus are commensals of the gastrointestinal tract and are associated with hospital-acquired infections, most frequently UTI. The three most frequently isolated species are *C. freundii*, *C. koseri* and *C. braakii*.

C. freundii mainly causes nosocomial infections (urinary tract infections, pneumonias and intra-abdominal abscesses).

C. amalonaticus is frequently found in feces, but has not been described as a causative agent of diarrhea, it has been isolated as a producer of extraintestinal infections (Lipsky et al., 1980).

Genus *Serratia*

The genus *Serratia* includes *S. marcescens*, *S. liquefaciens*, *S. rubidaea*, *S. odorifera*, *S. plymuthica*, *S. ficaria*, *S. entomophila*, and *S. fonticola*.

Serratia spp. is an opportunistic pathogen associated with outbreaks in healthcare settings. *Serratia* spp. are also known to be resistant to a wide range of antimicrobials.

S. marcescens, *S. rubidaea* and *S. plymuthica* usually produce a characteristic pink to red pigment, prodigiosin, especially when cultures are incubated at room temperature. The pigment is a typical characteristic of strains of environmental origin.

S. marcescens is the species considered most clinically important. It has been frequently encountered in hospital urinary or respiratory tract infections and in respiratory outbreaks and bacteremic outbreaks in day care centers and cardiac surgery and burn units.

S. odorifera contains two biogroups. *S. odorifera* biogroup 1 is predominantly isolated from the respiratory tract, *S. odorifera* biogroup 2 has been isolated from blood and CSF (Murray et al., 2014).

Proteus, Morganella and Providencia

The genera *Proteus*, *Morganella* and *Providencia* belong to the tribe *Proteeae*. They are widely distributed in the environment and are part of the normal intestinal microbiota.

Genus *Proteus*

This genus consists of five species, *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. myxofaciens* and *P. hauseri* (O'Hara et al., 2000).

P. mirabilis and *P. vulgaris* are widely recognized human pathogens. Both species have been isolated from urine specimens, wounds and ear infections (Eliason, 1940).

P. mirabilis is the most frequent member of this genus, producing mainly urinary tract infections. This microorganism produces large amounts of urease, cleaving urea into carbon dioxide and ammonium. This causes an increase in urinary pH, and therefore the precipitation of magnesium and calcium in the form of struvite and apatite crystals, which results in the formation of kidney stones. In addition this increase in pH is also toxic to the urothelium (Murray et al., 2014).

Genus *Morganella*

The genus *Morganella* contains a single species, *M. morganii*, with two subspecies: *M. morganii* subsp. *morganii* and *M. morganii* subsp. *sibonii*. Neither *M. morganii* subsp. *M. morganii* is a documented cause of UTI. It has also been identified as a cause of neonatal sepsis. *Morganella* is motile but does not swarm (Eliason, 1940).

M. morganii is widely distributed in nature, commonly found in the environment and in the intestinal tract of humans, mammals and reptiles as part of the normal flora. Drug resistance of *M. morganii* is increasing in recent years, and this resistance is introduced mainly through extra-genetic and mobile elements. Infections caused by multidrug-resistant (MDR) or even extensively drug-resistant (XDR) *M. morganii* often result in clinical treatment failure. In general, *M. morganii* can produce virulence factors, such as urease, hemolysins, and lipopolysaccharide (LPS); these virulence factors make *M. morganii* an opportunistic pathogen that mainly causes wound and urinary tract infections (Liu et al., 2016).

Genus *Providencia*

The genus *Providencia* consists of five species: *P. alcalifaciens*, *P. stuartii*, *P. rettgeri*, *P. rustigianii* and *P. heimbachae* (Shah et al., 2019).

P. rettgeri is a documented pathogen of the urinary tract and has caused occasionally outbreaks in healthcare settings. Similarly, *P. stuartii* has been implicated in outbreaks in burn units and has been isolated in urine cultures. Infections caused by *P. stuartii* and *P. rettgeri*, especially in immunocompromised patients, are particularly difficult to treat because of their antimicrobial resistance.

P. alcalifaciens is most frequently found in the feces of children with diarrhea; however, its role as a cause of diarrhea has not been demonstrated.

P. rustigianii, previously identified as a strain of *P. alcalifaciens*, is rarely isolated, while *P. heimbachae* has not yet been isolated from any clinical specimens (Eliason, 1940).

P. rettgeri and *P. stuartii* are generally resistant to gentamicin and tobramycin, but susceptible to amikacin. Urine isolates are susceptible to oral extended-spectrum cephalosporins, ciprofloxacin, and amoxicillin-clavulanate. *Providencia* spp. are also susceptible to ceftazidime, cefotaxime. Alternative options for antimicrobial therapy include ceftriaxone, imipenem, and trimethoprim-sulfamethoxazole (O'Hara et al., 2000).

Primary intestinal pathogens of the family Enterobacteriaceae

Salmonella, *Shigella* and *Yersinia* are microorganisms that cause gastrointestinal disease in humans, they are not part of the normal microbiota of the human gastrointestinal tract.

Humans acquire the infection by ingesting the microorganisms through food products of animal origin, poultry, milk, and undercooked dairy products. Some *Salmonella* infections can be transmitted through human carriers.

Infections caused by *Shigella* spp. are associated with human carriers responsible for the spread of the disease; an animal reservoir has not yet been identified. *Shigella* dysentery usually indicates inadequate sanitary conditions and poor personal hygiene. *Yersinia* spp. infections are transmitted by a wide variety of wild and domestic animals. *Yersinia* infections include gastrointestinal disease, mediastinal lymphadenitis, fulminant septicemia and pneumonia (Eliason, 1940).

Salmonella

Members of the genus *Salmonella* cause important infections in humans and certain animals. Many *Salmonella* serotypes are typically found in cold-blooded animals, as well as in rodents and birds, which are their natural hosts.

Salmonella spp. are gram-negative, facultative anaerobic bacilli that morphologically resemble other enteric bacteria. On selective and differential media used primarily to isolate enteric pathogens, *Salmonella* spp. (e.g., MAC), grow as clear, colorless colonies without lactose fermentation. On media with indicators for H₂S production (Hektoen or XLD), colonies with centers are observed (Eliason, 1940).

Most of the isolates of clinical significance belong to the *Salmonella enterica* species. Within the species *S. enterica* there are six subspecies (subspecies VI).

Pathogenesis

Once ingestion occurs and they reach the stomach, they bind to the small intestinal mucosa and invade M cells (microfolds), which are located in Peyer's patches and enterocytes. Bacteria can: replicate within endocytic vacuoles and transport through the cytoplasm to be released into the blood or lymphatic circulation.

The processes of regulation of anchoring, engulfment and replication are due to two aggregates of genes, called pathogenicity islets I and II.

Epidemiology

Salmonella is able to colonize multiple animals (poultry, reptiles, cattle, rodents, domestic animals. . .). The spread from one animal to another and the use of feed contaminated with *Salmonella* maintain an animal reservoir. In the case of *Salmonella* Typhi and Paratyphi, the reservoir is only human and therefore only produce disease in humans.

In most cases, infection occurs by ingestion of contaminated products (poultry, eggs, dairy products or products prepared on contaminated surfaces) or by the fecal-oral route. The incidence is higher in children under 5 years of age and adults over 60 years of age. It is estimated that there are about 1.2 million infections and 400 deaths per year (Murray et al., 2014).

In the case of *Salmonella* Typhi infections, the infection is contracted through contaminated food and water by an infected handler. An estimated 27 million infections and 200,000 deaths occur from *Salmonella* Typhi and Paratyphi (ECDC, 2018).

Clinical manifestations

There are different pictures of *Salmonella* infection:

- Gastroenteritis
- Typhoid fever, the most severe form being enteric fever.
- Septicemia
- Asymptomatic colonization

It is the most common form of food poisoning, produced by the ingestion of contaminated food. *Salmonella* strains associated with gastroenteritis are usually strains found in animals, most of these strains are members of *S. enterica* subsp. *enterica* (Eliason, 1940).

Symptoms usually appear between 6 and 48 h after ingestion of contaminated food or water, with nausea, vomiting and non-bloody diarrhea. Fever, abdominal spasms, myalgia and headache are also common. Most cases of *Salmonella* gastroenteritis are self-limiting, with symptoms persisting for 2–7 days (Murray et al., 2014).

Antimicrobial therapy is not usually indicated in uncomplicated cases. Antimicrobial therapy is believed to prolong the carrier state (Eliason, 1940).

- Typhoid fever

Enteric fever caused by *Salmonella* Typhi is known as typhoid fever, a febrile illness resulting from ingestion of food contaminated with the organisms from infected individuals or carriers. Clinical features of enteric fevers include:

- Prolonged fever
- Bacteremia
- Reticuloendothelial system involvement.

A mild form of the disease is produced by *Salmonella* Paratyphi A, *Salmonella* Schottmuelleri and *Salmonella* Hirschfeldii (Murray et al., 2014).

Typhoid fever develops around 9–14 days after ingestion of the bacilli, beginning with a progressively increasing fever and nonspecific symptoms such as headache, myalgia, malaise and myalgias. These symptoms last about 1 week, and are followed by gastrointestinal symptoms. This cycle corresponds to an initial bacteremic phase which is followed by colonization of the gallbladder and subsequent reinfection of the intestine.

- Septicemia

Prolonged fever and intermittent bacteremia, identical to that of other gram-negative bacilli, occurs. The serotypes most commonly associated with bacteremia are *S. Typhi*, *Paratyphi* and *Choleraesuis*.

The highest risk of developing *Salmonella* bacteremia is in pediatric, geriatric and immunocompromised patients.

- Asymptomatic colonization

Chronic colonization for more than 1 year after symptomatic infection occurs in 1–5% of patients, mainly by *Salmonella* species responsible for typhoid fevers, the reservoir in most cases being the gallbladder. In the case of infections by other *Salmonella* species, chronic colonization occurs in less than 1% (Murray et al., 2014).

Shigella

Four species have been described from almost 50 serogroups based on the O antigen: *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*. It is known from DNA molecular studies that *Shigella* and *Escherichia* are closely related, and therefore these 4 species constitute a biogroup within the *E. coli* species. However, they are kept distinct so as not to lead to confusion (Murray et al., 2014).

Pathogenesis

The disease occurs when *Shigella* invades the cells of the colon where they replicate. The genes that regulate the process of adherence, replication, invasion and dissemination are located on a virulence plasmid that is regulated by a chromosomal gene (Murray et al., 2014).

Shigella binds to M cells, once attached and by means of a type III secretion system are phagocytosed. They then lyse the vacuole and replicate in the cytoplasm. These bacteria are known to survive phagocytosis as they induce a process of apoptosis (Kotloff et al., 2018).

In the case of *S. dysenteriae* it produces an exotoxin, Shiga toxin. It contains one A and five B subunits. The B subunits facilitate the passage of the A subunit into the cell, the A subunit separates the 28S rRNA from the 60S unit, alternating protein synthesis. It mainly causes damage to the intestinal epithelium but can also cause damage to glomerular endothelial cells (Niyogi, 2005).

Epidemiology

Humans are the only reservoir for *Shigella*. About 90 million cases occur annually, with *S. sonnei* being the main culprit in developed countries, while in developing countries *S. flexneri* is predominant. In addition, epidemics of *S. dysenteriae* occur with mortalities of 5–15% (Niyogi, 2005).

Shigellosis affects mainly pediatric ages, reaching 60% in children under 10 years of age (ECDC, 2020).

Shigellosis is transmitted from person to person by the fecal-oral route, primarily from people with contaminated hands (Murray et al., 2014).

Clinical manifestations

The clinical manifestations of shigellosis vary from asymptomatic to severe forms of the disease. The main symptoms are high fever, chills, abdominal spasms, diarrhea and bloody stools, appearing between 24 and 72 h after ingestion of the bacteria.

The organisms, which originally multiply in the small intestine, move to the colon, where they can be isolated 1–3 days after infection develops (Eliason, 1940).

The main feature of shigellosis is abdominal spasms and tenesmus with abundant pus and blood in the stool, this is due to the invasion of the colonic mucosa by the bacteria.

Resolution of the disease is usually spontaneous, although antimicrobial treatment is recommended to reduce the course of disease (Niyogi, 2005).

Yersinia

The genus *Yersinia* consists of 14 species, most of which are considered environmental species.

Only three species are considered pathogenic in humans: *Y. pestis* (causative agent of plague), *Y. pseudotuberculosis* and *Y. enterocolitica* (enteric pathogens).

Pathogenesis

Yersinia species are able to resist phagocytosis by dephosphorylating proteins necessary for phagocytosis (*YopH* gene), inducing cytotoxicity by alternating actin filaments (*YopE* gene) and inducing apoptosis in macrophages (*YopJ/P* gene).

As for *Yersinia pestis* it possesses two additional plasmids, the fraction 1 (*f1*) gene encoding an antiphagocytic protein capsule and the plasminogen activator protease (*pla*) gene, which prevents opsonization and phagocytic migration by degrading complement components (Iriarte and Cornelis, 1996).

Epidemiology

Yersinia infections are all zoonotic, with humans acting as accidental hosts.

In the case of *Yersinia pestis* infection, two forms can be distinguished:

- Urban plague (rats as reservoir)
- Wild plague (squirrels, rabbits, wild rats as reservoir)

In *Yersinia enterocolitica* infections the reservoir are pigs, rodents, cattle and rabbits; in *Yersinia pseudotuberculosis* infections the reservoir are rodents, wild animals and game birds (Black and Slome, 1988).

Plague has been one of the most devastating diseases in history, causing up to three epidemics. Currently, around 10 cases per year are described, mostly of the wild type.

In urban plague, the reservoir is rats and transmission to humans occurs through the bite of the rat flea, which has been eliminated in most communities by controlling rat populations and improving hygiene. In the case of feral plague, it is very difficult to control due to the universal distribution of reservoirs (Fàbrega and Vila, 2012).

Y. enterocolitica is a frequent cause of enterocolitis in northern Europe and North America. Ninety percent of infections are due to consumption of contaminated meat, milk or water. The virulence of infection is associated with certain serotypes, such as O3 and O9.

Clinical manifestations

- Bubonic plague (*Y. pestis*)

Following the bite of an infected flea, symptoms begin within 7 days. The picture presents with high fever and a painful bubo (inflammatory lymphadenopathy) in the groin or axilla area. Bacteremia develops rapidly and up to 75% die.

- Pneumonic plague (*Y. pestis*)

In this case the incubation period is shorter (2–3 days), they present fever and general malaise with pulmonary symptoms appearing on the first day. The mortality rate in untreated cases can reach 90%.

- Enterocolitis (*Y. enterocolitica*)

The incubation period is between 1 and 10 days, the symptoms that develop are diarrhea, fever and abdominal pain. The disease affects the terminal ileum and acute appendicitis may develop.

Laboratory diagnosis of enterobacterales

Isolation and identification

Enterobacteriaceae grow easily in culture media, they are facultative anaerobes and grow at an optimum temperature between 35 °C and 37 °C.

Some species (*Serratia*, *Yersinia*) can grow at low temperatures (1–5 °C).

On non-selective and differential media they grow after 18–24 h of incubation.

Cultivation

Most laboratories use a variety of non-selective media, such as blood agar and chocolate agar, as well as selective media, such as MacConkey, especially for samples where polymicrobial flora may be growing.

On chocolate agar or blood agar plates, enteric bacteria produce large, grayish, smooth colonies. On blood agar, colonies may be β -hemolytic or nonhemolytic. Differential media allow separation of lactose-fermenting enterobacteria (pink color) from non-fermenting enterobacteria (transparent color). In the case of the diagnosis of ECTS O157:H7 (non-sorbitol-fermenting), sorbitol MacConkey agar is used, allowing differentiation of these colonies because they are colorless colonies in this medium. Subsequently, it is confirmed by serogroup tests. Unfortunately there are strains that ferment sorbitol, therefore the preferred diagnosis is the detection of Shiga toxin by immunoassay or molecular methods.

To recover microorganisms such as *Salmonella* or *Shigella*, there are media such as Hektoen agar, XLD or SS in which H_2S production can be appreciated (Eliason, 1940).

Biochemical identification

Currently most laboratories use different automated systems that allow the identification of virtually all family members within 24 h. In addition, the sequencing of species-specific genes such as the 16S rRNA gene or the detection of protein profiles using mass spectrophotometry can be performed.

Treatment

Treatment of enterobacterial infections should be based on in vitro sensitivity testing and clinical experience. Certain enterobacteria such as *E. coli* and *P. mirabilis* are usually sensitive to many antibiotics, but there may be production of enzymes that inactivate penicillins and cephalosporins, such as extended spectrum beta-lactamases (EBLECs) or enzymes that inactivate carbapenemics. BLEEs are usually quite widespread in *E. coli* and *K. pneumoniae* (Cercenado, 2015).

Antibiotic resistance is more frequent in nosocomial infections than in community infections, and multidrug-resistant profiles may appear more and more frequently, complicating the treatment of infections.

There are also certain infections where antibiotic treatment is not recommended if not symptomatic treatment, as in the case of gastroenteritis caused by Shiga toxin-producing *E. coli* or *Salmonella*, as they can prolong the carrier state or increase the risk of secondary complications (Gut et al., 2018).

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Microbiology

The genus *Vibrio* belongs to the family *Vibrionaceae*, is composed of Gram-negative curved rods 0.5–0.8 µm wide and 1.4–2.6 µm long, and mobile. They are part of natural aquatic habitats, preferably warm and saline waters. Most species of this genus are halophilic and need NaCl requirements in culture media for growth, although *V. cholerae* and *Vibrio mimicus* can grow in most common culture media without this requirement. They tolerate a wide pH range (6.5–9) well, but are sensitive to acidic pH, which is why people with reduced gastric acid are more susceptible to *Vibrio* infections.

In its structure we can find the presence of a polar flagellum, responsible for motility, and pili on the surface, which are involved in the virulence of the different species. The cell wall has lipopolysaccharides formed by Lipid A (endotoxin), central polysaccharide and polysaccharide O, the latter is used to classify *Vibrio* species by serogroups.

There are about 30 species of *Vibrio*, but only 12 have been recognized as pathogenic to humans, including: *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. mimicus*, *V. fluvialis*, *V. cincinnatiensis*, *V. hollisae*, *V. furnissii*, *V. damsela*, *V. metshnikovii* and *V. carchariae* (Baker-Austin et al., 2018).

All these pathogenic *Vibrios* are responsible for foodborne diseases, but the most important ones are considered to be *V. cholerae* O1 and O139, *V. parahaemolyticus* and *V. vulnificus* because of the serious clinical manifestations they can cause (Fig. 1).

Phenotypically these species are oxidase and catalase positive, able to ferment glucose without gas production.

V. parahaemolyticus and *V. vulnificus* are phenotypically similar, but can be differentiated biochemically because *V. vulnificus* ferments lactose and β-D-galactosidase sugars unlike *V. parahaemolyticus*.

In the case of *V. cholerae* can differentiate more than 200 serogroups, which includes pathogenic and nonpathogenic species, so *V. cholerae* O1 and *V. cholerae* O139 are cable of synthesizing the cholera toxin and be responsible for the clinical manifestation called cholera, these serogroups are differentiated by the presence or absence of methylated O antigen. The remaining species are referred to as *V. cholerae* non-O1, non-O139. *V. cholerae* O1 is further subdivided into serotypes and biotypes. The biotypes are called Classical and El Tor, which differ in phenotypic traits, such as biochemical tests and different susceptibility to bacteriophages. Both can be serotypes Inaba, Ogawa and Hikojima, which express different antigens, the latter being a transitional state that expresses antigens of the previous two (Fig. 2). The Inaba and Ogawa serotypes differ in the absence or presence, respectively, of a methyl group on the terminal sugar of the O antigen (Harris et al., 2012).

V. cholerae O139, arose after the acquisition of a genomic island containing the *rfb* region, which includes the genes required for the synthesis of the O139 antigen.

The prevalence of one serotype or another has changed over time and they have been responsible for the different pandemics, such that classical *V. cholerae* O1 is attributed to the fifth and sixth pandemics (and possibly the previous ones) and the seventh to the El Tor biotype (Lekshmi et al., 2018).

These bacteria have in their genomic structure two circular chromosomes, formed by genetic recombination and horizontal gene transfer, we can also find the presence of plasmids responsible for antimicrobial resistance.

For *V. cholerae*, on circular chromosome 1, we find most genes responsible for DNA replication, translation and transcription, while on chromosome 2, we find genes responsible for pathogenicity, such as those encoding choleric toxin, toxin co-regulated pilus and chemotactic agents (Baker-Austin et al., 2018).

V. parahaemolyticus is also differentiated into different serogroups based on somatic O antigen and capsular K antigen, recognizing a total of 76 serotypes (González-Escalona et al., 2008).

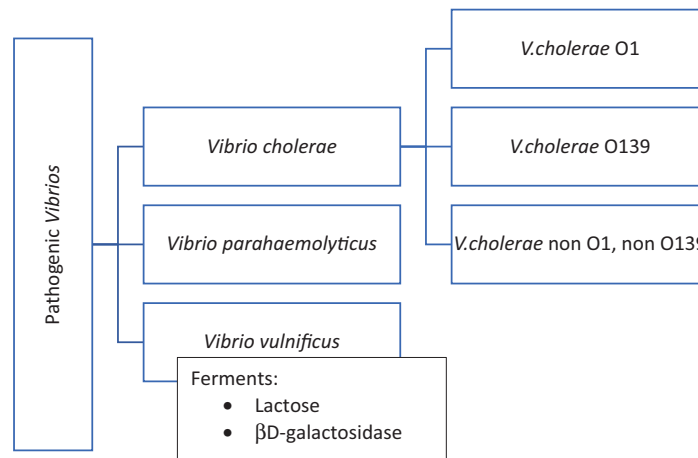


Fig. 1 Classification pathogenic *Vibrios*.

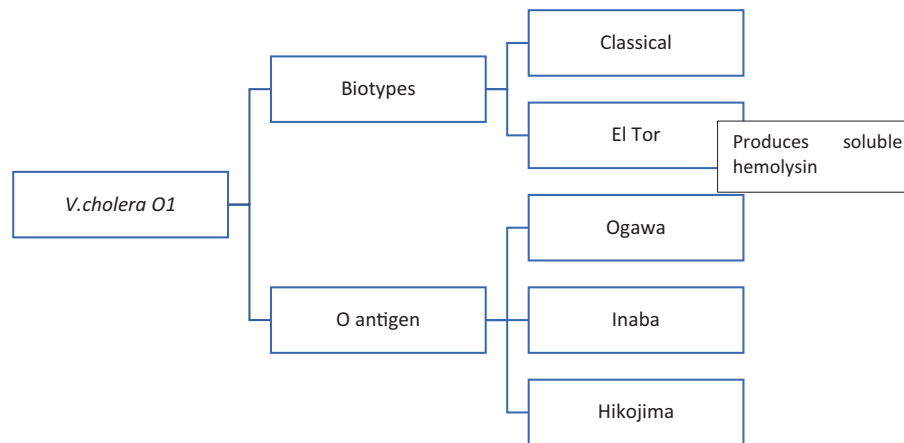


Fig. 2 Classification *V. cholerae* O1.

Pathogenesis and immunity

The mechanism of transmission of cholera, both *V. cholerae* O1 and O139, is the fecal-oral route, and both have the same mechanism of pathogenicity. After ingestion of *V. cholerae*, either by consumption of water or food contaminated with this water, it reaches the intestine where the cholera toxin will perform its action and is the main virulence factor responsible for the characteristic diarrheal symptoms of cholera.

The infectious process begins when the person ingests water or food contaminated with *V. cholerae*, a high infective dose is required, at least an inoculum of $10^6 - 10^8$, this reaches the cells of the digestive mucosa and secretes the cholera toxin, without the destruction of these cells.

The choleric toxin is an exotoxin formed by the A-B complex, the A fraction is bound by non-covalent bonds to the B subunit of a pentameric complex, which binds to the GM1 ganglioside receptors of intestinal epithelial cells, The A fraction is then internalized and interacts with G proteins, which activate the enzyme adenylate cyclase, which catalyzes the catabolic reaction of ATP to cAMP, thereby increasing the intracellular cAMP concentration. This increase in cAMP leads to hypersecretion of water and electrolytes such as chloride ion through apical channels of transmembrane conductance (CFTR) and bicarbonate and decreased sodium reabsorption, all leading to a large loss of fluid with high ion concentrations (Yoon and Waters, 2019; Weil and Ryan, 2018).

The choleric toxin is encoded by a filamentous bacteriophage (CTXφ) and consists of two subunits, ctxA and ctxB. The bacteriophage binds to the toxin co-regulated pilus (TCP) and inserts its genetic material into the genome of the host bacterium. This TCP in turn is encoded within a genomic pathogenicity island called the *Vibrio* pathogenicity island (VPI).

In addition to the action of the toxin itself, there are other factors that contribute to virulence, such as the toxin co-regulated pilus gene complex (TCP) and chemotactic proteins that act as colonization factors allowing *V. cholerae* to adhere to the mucosal cell layers of the digestive tract, preventing the elimination of the pathogen.

Other virulence factors that contribute to pathogenesis is the enzyme sialidase, known as NanH or VcN, which remodels the GM1 toxin receptor, facilitating its binding by increasing the availability of this receptor.

Occlusive zonula toxin (zot) and cholera accessory enterotoxin (ace) are also involved. Occlusive zonula toxin increases intestinal membrane permeability by relaxing the junctional zones of the intestine, and cholera accessory enterotoxin increases fluid secretion, so the two complement each other (Chowdhury et al., 2017).

In general, cholera affects the entire population equally, although it is true that it is more serious in children, due to their smaller body volume, and their immune system has not fully matured, so therapeutic support is of vital importance to prevent death after hypovolemic shock that causes cholera. There are also certain characteristics of the host that can make it more susceptible to infection, so a person who has previously acquired the infection or has been vaccinated has some immunity, but this protection is specific to the serogroup and for *V. cholerae* O1 is dependent on the biotype and serotype.

In people suffering from achlorhydria problems are more likely to infection, since the pathogen is sensitive to gastric acids, and is an important defense barrier prior to colonization of the small intestine (Lippi et al., 2016).

As for the rest of the non-cholera-producing *Vibrio* species, the following virulence factors stand out.

V. parahaemolyticus produces a thermostable direct hemolysin (Vp-TDH) called Kanagawa, this enterotoxin produces the secretion of chloride ion, which is accompanied by fluid loss and causes watery diarrhea in the patient.

The action of enterotoxin can be seen by observing the beta-hemolysis produced by these strains when grown on a modified blood agar medium, called Wagatsuma-agar.

It has been proven that strains with this enterotoxin are responsible for the diarrhoeal clinical manifestations.

In addition to the presence of this toxin, there are others that collaborate in the virulence of the strain, they are called hemolysins related to Vp-TDH (Vp-TRH), and there are also strains carrying genes encoding type III secretion systems this virulence factor has been seen present in the main *Enterobacteria* causing diarrhea such as *Salmonella*, *Shigella* and *Enteropathogenic Escherichia coli*.

The pathogenicity mechanism of *V. vulnificus* involves several elements. It has polysaccharide capsule which gives it the ability to avoid phagocytosis by macrophages and evade the host immune response. Activates *cadC*, *cadB*, *cadA* genes that mediate *V. vulnificus* tolerance to gastric acid. Secretes cytotoxins, extracellular proteases (ECPase) and collagenase enzymes involved in tissue destruction. It has several adhesins that participate in bacterial adhesion, as well as protein U (OmpU) a protein present in the outer membrane and immunogenic lipoprotein A (LpA) both play the same function of adhesion; the latter also has immunogenic capacity and is involved in the activation of various signal transduction cascades (such as MAP kinases pathway) that activate different transcription factors of NF- κ B type and Toll-like receptors 1 and 2 which recognize various molecular patterns that are expressed by pathogens. All these elements are part of the innate immune system and by one mechanism or another causes the host to release a large amount of inflammatory mediators causing an exacerbated immune response (Yokochi et al., 2013).

Epidemiology

Vibrio is a pathogen found in various aquatic environments, able to withstand wide pH ranges and grow better in saline conditions. Asymptomatic patients can be considered reservoirs, since transmission is fecal-oral route, either through water or contaminated food such as crustaceans that inhabit these aquatic habitats. Person-to-person transmission is more complicated due to the high bacterial inoculum necessary to cause infection.

Cholera is a disease that occurs both endemically and epidemically. In South Asia and in some areas of Africa and Latin America, it is an endemic disease due to conditions of poverty and lack of sanitation and where access to safe drinking water is difficult. That is why this disease often emerges also after devastating natural events, in which conditions of poor sanitation, difficult access to safe food and inadequate sanitation are evident, as was recently the case after the earthquake in Haiti in 2010 and whose strain was subsequently linked to outbreaks in the countries of Cuba, Mexico and the Dominican Republic.

In countries where cholera is endemic, a seasonal course coinciding with peaks before and after rainy seasons can be observed; epidemic outbreaks are affected by climate the proliferation of *Vibrio* spp. is favored by warm temperatures and by population immunity.

In countries where they enjoy health resources, the cases that arise are imported cases of travelers from endemic or epidemic areas (Lekshmi et al., 2018; Chowdhury et al., 2017; Mutreja and Dougan, 2020).

According to WHO data, it is estimated that this disease affects approximately 1.3–4 million people, and between 21,000 and 143,000 deaths are due to this cause, although it is true that it is an under-diagnosed disease and morbidity and mortality data are not properly documented, so it is estimated that the actual number of cases is higher.

There is evidence of this disease throughout the 19th century, its original reservoir is located in India and from there, due to intercontinental travel, spread to the rest of the world. In history, a total of seven epidemics have been recorded, six due to *V. cholerae* O1 classical biotype and the seventh was caused by serotype O139, which began in Asia and spread to Africa and America.

Molecular epidemiology has allowed us to know that the current cholera pandemic is due to *V. cholerae* O1 El Tor which has displaced the classical biotype. The second wave of this pandemic differs because *V. cholerae* has acquired the antibiotic resistance gene SXT/R391 which encodes resistance to Trimethoprim/Sulfamethoxazole (Spagnoletti et al., 2014; Kiiru et al., 2009; Bordeleau et al., 2010).

For pathogenic *Vibrio* species other than cholera, such as *V. parahaemolyticus* and *V. vulnificus*, infection is due to consumption of crustaceans and shellfish exposed to contaminated water.

V. parahaemolyticus, like other *Vibrios*, is found in aquatic environments, and the increase in the number is favored by warm temperatures and salinity. Thus, it has been observed how global warming has affected the count of this microorganism in water.

In Japan and Southeast Asia this pathogen is the most frequent cause of bacterial diarrhea (Ndraha et al., 2020; Ndraha and Hsiao, 2021).

V. vulnificus is mainly associated with wound infections that can cause septicemia. It has regional and seasonal characteristics. Most of these infections occur in subtropical areas, and it is spreading to other regions as temperatures rise as a result of climate change that is affecting marine ecology. The peak of *V. vulnificus* infection is reached in summer and most cases occur from May to October coinciding with the fishing of fish and shellfish from fish farms, and with increased exposure to the sea by the population, which may have wounds that are the route of entry for the bacterium (Baker-Austin and Oliver, 2020).

Clinical illnesses

Vibrio cholerae

In *V. cholerae* infection we must distinguish between toxigenic *V. cholerae* (*V. cholerae* O1, *V. cholerae* O139) and *V. cholerae* non-O1, non-O139. In such a way that we can speak from asymptomatic colonization to gastrointestinal symptoms that compromise the patient's life.

V. cholerae O1, O139, is the causative agent of the disease called cholera, whose clinical manifestation is characterized by watery diarrhea. Symptoms begin after an incubation period of 3–5 days, abruptly with diarrhea and vomiting. The loss of liquid can be up to 1 L per hour, so as liquid is lost, the stool becomes colorless, which is why it is called "rice water stool." Due to the mechanism of pathogenicity of the bacteria, in which the loss of liquid is accompanied by loss of chloride and bicarbonate ions, which causes metabolic acidosis, and loss of sodium and potassium ions, this electrolyte imbalance causes muscle cramps, heart rhythm is affected, causing arrhythmias. Renal function and the nervous system are also compromised due to hypoperfusion of the organs.

The clinical features manifested by these patients are associated with the dehydration they suffer, without rapid rehydration. Thus, these patients present: thirst, irritability, lethargy, loss of skin turgor, decreased urine production, low blood pressure and sunken eyes.

Dehydration in its severe form causes hypovolemic shock in the patient, compromising the patient's life. It is of vital importance to establish a treatment of liquid and electrolyte replacement to avoid this outcome. Before rehydration therapy was established, cholera in its severe form could reach a mortality rate of 50% (Clemens et al., 2017).

Complications of cholera are infrequent, but the possibility of sepsis and aspiration pneumonia due to vomiting should be mentioned. Cases called "cholera sicca" have been described in which liquid accumulates in the intestinal lumen without causing diarrhea and circulatory collapse occurs.

Both *V. cholerae* serotypes O1 and O139 cause the same severe form of cholera, but they are clinically indistinguishable. *V. cholerae* non-O1, non-O139, do not produce cholera toxin, and cause mild diarrheal illness (Baker-Austin et al., 2018; Hsueh and Waters, 2019; Aydanian et al., 2015).

Vibrio parahaemolyticus

V. parahaemolyticus is responsible for gastrointestinal clinical manifestations, being diarrhea the most common symptom, but unlike that caused by *V. cholerae*, these are less serious for the patient. They are usually self-limited diarrhea and this infection is associated with the ingestion of contaminated raw seafood, mainly with the consumption of oysters, which involves greater risk, because they are water filters and accumulate higher concentrations of *V. parahaemolyticus*.

The gastrointestinal symptoms may be accompanied by other symptoms such as cramps, nausea and headaches, all of which are a consequence of hydrolytic loss (Martinez-Urtaza and Baker-Austin, 2020).

Vibrio vulnificus

V. vulnificus can present with a variety of clinical manifestations including primary septicemia following ingestion of contaminated raw seafood, secondary septicemia following infection of a wound exposed to contaminated water, traumatic wound infection and gastroenteritis.

Sepsis, whether primary or secondary, begins when the pathogen reaches the bloodstream. The patient will present with manifestations of sepsis including fever, chills and general malaise.

If the infection occurs via the digestive tract, this symptomatology may be accompanied by vomiting, diarrhea and abdominal pain. If the sepsis is due to a complication of the wound infection, this lesion presents tumefaction, blistering erythema, with exudation, cellulitis, tissue necrosis and muscle inflammation and finally the appearance of systemic signs.

In these septic conditions the mortality of these patients can be up to 50%. Within 24–48 h there may be a rapid evolution of symptoms reaching septic shock and multiple organ failure (Li and Wang, 2020; Baker-Austin and Oliver, 2020).

Diagnosis

The diagnosis of *V. cholerae* can be carried out by various techniques, although it is true that in most cases the clinical symptoms of the patient with diarrhea in "rice water" along with the anamnesis, will serve for the presumptive diagnosis, which will be confirmed by various microbiological tests.

Microscopy

Darkfield or fluorescence microscopy is used to observe small curved rods with polar flagellum and moving in the direct specimen. Microscopy is quite specific, but lacks sensitivity, so it is not appropriate to use it as the only diagnostic tool.

Culture

Vibrios are not species that need additional requirements, they grow well in the usual culture media used in microbiology laboratories, although it is true that selective media can be used as TCBS agar medium (Thiosulfate, Citrate, Bile, Sucrose), or tellurite taurocholate gelatin agar medium (TTGA) and enrichment broths as alkaline peptone water that allow a recovery of *Vibrio* in samples in which there are more microorganisms, as is the case of feces. Once the pathogen has been isolated, identification can be carried out by means of biochemical tests, and it will also allow the sensitivity study to be carried out. The serogroup and serotype can be determined using specific antisera by means of an agglutination reaction.

Nucleic acid amplification test (NAAT)

Genomic sequencing techniques, either of the whole genome or of highly conserved regions such as 16S rDNA and *rpoB* gene, have allowed to know the genome of the different species of *Vibrio*.

It is these techniques that have contributed to the knowledge of the different lineages. In such a way that it has allowed an expansion of knowledge in the field of molecular epidemiology.

It allows to establish the different relationships between species and to build the phylogenetic tree, which helps to understand the spread of the different pandemics and to know which strains have caused each one of them.

In addition, these techniques also allow to know the acquired genetic material responsible for antibacterial resistance, virulence genes and greater environmental stability of the different species.

Sequencing techniques have been used primarily for epidemiological research and surveillance (Liu et al., 2021; Yen et al., 2021).

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF) is a powerful tool used in most microbiology laboratories for the identification of bacteria, fungi and yeasts. This technique allows the structural determination based on the mass/charge ratio (m/z) and the relative abundance (intensity) of the molecules that constitute the microorganism, which is represented by characteristic peaks.

MALDI-TOF identifies at genus level *Vibrio* spp. and at species level, from culture growth, without the need for biochemical tests which are more tedious techniques. The use of MALDI-TOF allows a fast and accurate alternative for colony identification. The appearance of different peaks that only appear in this genus, makes possible its typing thus distinguishing it from other closely related bacteria, such as species of the *Aeromonadaceae* family that also inhabit aquatic environments and are present in the same environment (Dieckmann et al., 2010; Cho et al., 2017; Cheng et al., 2015).

There are commercially available rapid techniques that can detect the presence of O1 and O139 antigens, allowing a diagnosis of *V. cholerae* in a direct sample that complements the diagnosis by culture, which is considered the gold standard.

It can also be identified by PCR, by quantitative real-time PCR (qPCR) techniques, which detect the DNA of the bacterial species, but not the viability of the species and thus the causative agent of the infection (Moussa et al., 2021).

Treatment, prevention and control

The main treatment in the case of cholera is water and electrolyte replacement; it is vitally important to hydrate the patient to avoid hypovolemic shock. This rehydration is usually done orally, but in severe cases intravenous administration is justified. Fluid management is carried out according to the degree of dehydration, which according to WHO criteria is classified according to the loss of fluid in relation to body weight:

- None if the loss is <5%.
- Medium if between 5% and 10%.
- Severe if >10%.

WHO recommends the use of oral rehydration salts (ORS), a standard sachet that dissolves in 1 L of drinking water and contains several vital electrolytes (sodium, potassium and chlorine) and a carbon source (glucose).

The administration of antibiotics to treat this disease is only advised in moderate-severe cases of cholera, since the massive administration of antibiotics will not interfere with the spread of cholera and will contribute to the emergence of bacterial resistance.

Antimicrobial treatment will be complementary with the aim of reducing the production of cholera toxin and therefore diarrhea, thus improving the clinical symptoms of these severe patients.

The empirical antibiotic of choice is usually Azithromycin, because resistance to this antibiotic is not common. Quinolones and tetracyclines can also be used, although it is true that the empirical use of these antibiotics is not the most advisable because resistance to these antibiotics is increasing and therapeutic failure may occur (Hsueh and Waters, 2019).

Due to the increase of antimicrobial resistance, it is important to perform antibiotic sensitivity studies, so that the choice of antibiotics will depend on the sensitivity profile of the microorganism and the type of patient to be treated. Likewise, it is recommended to periodically monitor the sensitivity in epidemic outbreaks in order to evaluate the possible emergence of resistance.

The determination of antimicrobial susceptibility can be performed by the disc diffusion method or E-test, and also by broth microdilution.

Antibiotics generally tested for *V. cholerae* are: tetracyclines (whose result can be extrapolated to Doxycycline), Trimethoprim/Sulfamethoxazole, Ampicillin (representative for Amoxicillin), Chloramphenicol, Nitrofurantoin, Ciprofloxacin, and Azithromycin.

For *Vibrio* spp. third generation cephalosporins (Cefotaxime, Ceftazidime) can also be used.

Also, it is revealing to know the serotype and biotype of *V. cholerae*, because most *V. cholerae* O139 and *V. cholerae* O1 El Tor are usually resistant to Trimethoprim/Sulfamethoxazole, which is another therapeutic alternative (Janda et al., 2015).

In the case of gastroenteritis caused by *Vibrio haemolyticus*, it is usually self-limiting, and like cholera, the main treatment is fluid and electrolyte replacement, and in severe cases antibiotics are added to the treatment regimen.

In clinical manifestations, in which *V. vulnificus* is involved, in wound infections and septicemia, should be treated early with appropriate antimicrobial treatment according to sensitivity studies. In general, the use of tetracyclines and/or third generation cephalosporins is recommended, to which quinolones can be associated. Depending on the resistance profile of each country, one or other antibiotics will be used as the first line of treatment.

In cases of traumatic infection, surgical debridement of the wound is very important and on extreme occasions it may be necessary to amputate the affected limb if the tissue necrosis is too severe, all in order to improve the patient's prognosis.

The main prevention is an improvement of sanitary conditions, water potabilization, good control of waste, sewage and the introduction of measures to avoid food contamination. In cases of travelers to endemic areas, the use of bottled water and avoidance of raw or undercooked seafood are recommended (George and Sack, 2017). In cases of *V. vulnificus* infection, which is highly lethal, it is very important to avoid exposure to seawater and consumption of raw seafood. And upon suspicion and appearance of clinical signs, early antibiotic treatment is essential (Park and Lee, 2018).

In addition to these essential measures, there are also oral vaccines that reduce the risk of contracting cholera and attenuate the effects of the infection, and these vaccines are intended not only to immunize the population but also to reduce transmission. As oral vaccines, the aim is to achieve immunity at the mucosal level of the intestine, preventing colonization and multiplication of *V. cholerae*.

There are two types of oral vaccines, inactivated vaccines, which contain whole killed *V. cholerae* cells, and live attenuated vaccines, which contain genetically modified strains of *V. cholerae* that are not pathogenic. The latter are usually single-dose vaccines as opposed to killed-cell vaccines that require two or three doses over an interval of time.

Inactivated vaccines have reduced efficacy in children under 5 years of age, who are usually the most vulnerable population. In contrast, live attenuated vaccines confer greater immunity, as they mimic infection by producing antigens and colonization factors, which are targets of protective immunity. Despite this, these vaccines have not been approved for use in cholera-endemic regions (Shaikh et al., 2020; Sinclair et al., 2011).

The following vaccines are currently available on the market:

- Wc-rBS (Dukoral®): Monovalent inactivated vaccine, contains whole killed cells of *V. cholerae* O1 and the recombinant B fraction of the cholera toxin. It is administered in a buffered solution to prevent degradation of the B subunit by the action of gastric acid. This complicates the logistics of administering the vaccine, as well as its storage and shipment to vaccination sites.
- BivWC (Shanchol®) bivalent inactivated vaccine, containing killed cells of *V. cholerae* O1 and O139. This vaccine lacks the recombinant B subunit in order to facilitate administration by avoiding buffer solution and also to reduce vaccine costs. This vaccine was reformulated from the mORCVAX® vaccine and allowed its purchase by the ONU agency, fulfilling the requirements of the WHO.
- Euvichol-Plus®: It is the same as the Shanchol® vaccine, the manufacturing competences were transferred to the company Eubiotics, in order to be able to supply the demand in the population.
- BivWC (mORCVAX®) is identical to the Shanchol® vaccine, but is only available in Vietnam.
- Vaxchora®: Monovalent live attenuated vaccine, single dose, which is indicated for immunization against *V. cholerae* O1. Exposure to attenuated strains of *V. cholerae*, causes an increase in antibody titer against *V. cholerae*, thus generating some immunity and protection against future infections.

It is only marketed in the United States and is indicated for use in travelers to endemic areas, in order to reduce the risk of infection (Mosley et al., 2017).

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***Pseudomonas* Infections**

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Infections due to *Pseudomonas* spp. are caused by members of the genus *Pseudomonas*, from the family Pseudomonadaceae, a group of gram-negative rods. The genus *Pseudomonas* encompasses more than 140 species, which inhabit a variety of niches in soil and water. Several species of *Pseudomonas* may cause infection in humans (Table 1); but, by far, the most frequently recovered pathogen in the family is *P. aeruginosa* (D'Agata, 2015; Falagas and Rafailidis, 2020).

Related gram-negative bacillary diseases include infections due to *Stenotrophomonas maltophilia* (formerly known as *Pseudomonas maltophilia* and *Xanthomonas maltophilia*), *Ralstonia pickettii* (formerly *Pseudomonas*, then *Burkholderia pickettii*), *R. mannitolilytica* (formerly *Pseudomonas thomasi*, then *R. pickettii* biovar 3/"thomasi") and members of the genus *Burkholderia* (formerly *Pseudomonas*, then *B. pseudomallei*, *B. mallei*, and *B. cepacia* complex) (Currie, 2015; Falagas and Rafailidis, 2020; Steinberg and Burd, 2015). This article refers only to infections caused by *Pseudomonas* spp.

Microbiology

Pseudomonas aeruginosa is a gram-negative, obligate aerobic, lactose nonfermenting, straight or slightly curved rod with a length ranging from 1 to 3 µm and a width of 0.5–1.0 µm (D'Agata, 2015; Falagas and Rafailidis, 2020). It is catalase positive, oxidase positive, and motile with one or more polar flagella. Most species oxidize glucose and reduce nitrate to nitrite or nitrogen gas. The exterior surface of this pathogen consists of a polysaccharide capsule with lipopolysaccharides, pili and polar flagella. Furthermore, it is capable of elaborating a large number of toxins. On culture plates, *P. aeruginosa* grows at 37–42 °C, forming smooth round colonies with a characteristic sweet grape-like odor and a typical green-blue coloration. This coloration is due to the production of pigments, notably a pigment called pyocyanin (blue) and pyoverdine (green or yellow-green). In fact, the name "*aeruginosa*" derives from the green-blue hue seen in colonies of many clinical isolates. However, some strains produce red or black colonies because of the synthesis of the pigments termed pyorubin and pyomelanin, respectively. Isolates from patients with cystic fibrosis and other chronic infections may have a distinct mucoid appearance (Pang et al., 2019; Kanj and Sexton, 2019d). This is the consequence of the overproduction of an extracellular polysaccharide known as alginate. *Pseudomonas* strains with acquired resistance to multiple antibiotics have become common in some hospitals and regions (Del Barrio-Tofiño et al., 2019; Falagas and Rafailidis, 2020).

Table 1 *Pseudomonas* species of clinical significance in alphabetical order.

<i>P. aeruginosa</i>
<i>P. alcaligenes</i>
<i>P. fluorescens</i>
<i>P. fulva</i>
<i>P. luteola</i>
<i>P. mendocina</i>
<i>P. mosselii</i>
<i>P. oryzae</i>
<i>P. pseudoalcaligenes</i>
<i>P. putida</i>
<i>P. stutzeri</i>

Epidemiology

P. aeruginosa is ubiquitous in nature and it can be found in most fresh water environments (Kanj and Sexton, 2019d). Apart from infecting humans, *P. aeruginosa* is also an important plant pathogen, affecting tobacco, tomatoes, and lettuce (Kanj and Sexton, 2019d). This organism can survive in environments with minimal nutrients. Colonization by *P. aeruginosa* in human usually takes place in moist areas, such as perineum, auditory canal, axillae, and the lower alimentary canal. It is also isolated from other moist, inanimate environments within the hospital, including sink traps and drains, toilets, showers, ice machine, kitchen settings, cleaning mops, water in vases of flowers, and even cleaning solutions. It can become a particular problem when it contaminates multidose medications or medical devices with a moist environment including endoscopes, ventilators, pressure monitors, dialysis equipment, and the like (Falagas and Rafailidis, 2020; Kanj and Sexton, 2019d).

P. aeruginosa is often an opportunistic pathogen, causing infections in patients with physical, phagocytic, or immunologic defects in host mechanisms. Thus, this organism is one of the most common pathogens in health care-associated infections. According to data reported to the National Healthcare Network at the Centers for Disease Control and Prevention in the United States from 2011 to 2014, *P. aeruginosa* was the sixth most common cause of hospital-acquired infections in general (7.3%); the second most common cause of ventilator-associated pneumonia (16.5%); and the third most common cause of urinary tract infections associated with urinary indwelling catheter (10.3%) (Weiner et al., 2016). Ventilator-associated pneumonia, primary bacteremia associated with intravenous catheters or secondary bacteremia in relationship to infections at different sites of the body, urinary tract infections, and surgical site infections are the main types of infections caused by *P. aeruginosa* in the hospital setting (Falagas and Rafailidis, 2020).

A range of hosts are particularly prone to infections by this pathogen. Historically, patients with neutropenia, burns, and cystic fibrosis (CF) were considered especially susceptible to infections by this organism (Falagas and Rafailidis, 2020; Kanj and Sexton, 2019d). However, for unknown reasons the incidence of *Pseudomonas* infections in all these patients is decreasing in Western Europe and North America (Kanj and Sexton, 2019d). Nonetheless, *P. aeruginosa* remains the most common pathogen isolated from adults with CF (Kanj and Sexton, 2019d). It is also the most common cause of respiratory failure in CF and is responsible for the deaths of the majority of these patients. In addition, a study from Spain has shown that *P. aeruginosa* was the most common cause of gram-negative bacteremia among neutropenic patients with malignancy and pneumonia (Gudiol et al., 2016).

In CF patients, acquisition of *P. aeruginosa* begins early in childhood (median 1 year); and the initial acquisition is with nonmucoid strains (Kanj and Sexton, 2019d). Over time (median 11 years), there is a transition to a mucoid phenotype of *P. aeruginosa* that is associated with a deterioration in symptoms, chest X-ray and pulmonary function in these patients. Furthermore, Oliver et al. (2000) have demonstrated that patients with CF are colonized with hypermutable strains of *P. aeruginosa* which may persist for years in the respiratory tree of these patients.

Other patients prone to *P. aeruginosa* infection include patients with compromised immunity (cancer patients, transplant recipients, diabetics, and patients with AIDS) and patients with foreign bodies (e.g., vascular grafts, orthopedic implants) (Falagas and Rafailidis, 2020).

Infection due to *P. aeruginosa* is characteristically nosocomial. Notwithstanding, there has been increasing recognition of *P. aeruginosa* as a cause of community-acquired infections (Kanj and Sexton, 2019d). In the nonhospital setting, pseudomonas infection is related to exposure to the water of recreational baths (swimming pools, whirlpools, hot tubs, spas), as well as to the use of contact lenses, particularly the extended-wear variety (Falagas and Rafailidis, 2020). Puncture wounds, including those through contaminated trainers, can give rise to *P. aeruginosa* osteomyelitis of the foot. *P. aeruginosa* endophthalmitis after eye trauma can result in visual compromise, and *P. aeruginosa* endocarditis can be found in intravenous injection drug users (Falagas and Rafailidis, 2020; Kanj and Sexton, 2019d).

Clinical manifestations

P. aeruginosa possesses a vast array of virulence factors. Some studies have pointed out that certain virulence factors may be related to infection severity (Peña et al., 2015). As mentioned earlier, *Pseudomonas* may produce varied clinical manifestations; and no symptoms or signs effectively allow to discriminate *Pseudomonas* infection from infections caused by other pathogens.

Febrile neutropenia

P. aeruginosa is an important pathogen causing infection in patients with neutropenia. The classic clinical syndromes in febrile neutropenic patients are bacteremia, pneumonia, and soft tissue infection, typically manifested as ecthyma gangrenosum (see later). Mortality rate increases if appropriate treatment of infection is delayed. Thus, *P. aeruginosa* is the organism against which empirical coverage must always be included in neutropenic patients with fever (Falagas and Rafailidis, 2020).

Bacteremia

Pseudomonas aeruginosa bacteremia usually occurs in patients with serious underlying diseases and presents a high mortality rate. The great majority of reported crude mortality rates from large surveillance studies range from 39% to 60% (D'Agata, 2015); although recent studies report lower mortality rates around 30% (Peña et al., 2015; Babich et al., 2019). The large range in mortality rates from different studies reflects the multitude of factors that affect outcomes associated with blood stream infections (D'Agata, 2015). Risk factors for mortality in *P. aeruginosa* bacteremia include advanced age, high APACHE II score, sepsis, poor functional status, polymicrobial bacteremia, and inappropriate initial antimicrobial treatment (D'Agata, 2015; Kanj and Sexton, 2019c). It usually derives from primary infection at different sites, such as pneumonia, urinary tract infection, complicated intra-abdominal infection (peritonitis, abscess), and endocarditis. The source of the bacteremia may be unknown in up to 40% of cases (Kanj and Sexton, 2019c). Manifestations can include those of sepsis and septic shock; that is, fever, tachycardia, tachypnea, hypotension, and mental status changes ranging from confusion to coma. Multiorgan failure with adult respiratory distress syndrome and acute renal failure along with coagulation defects may occur (Kanj and Sexton, 2019c).

Infective endocarditis

P. aeruginosa may cause infective endocarditis (IE), mainly of native valves but also of prosthetic ones. Complications are common, and mortality rates are high, ranging from 36% to 60% (D'Agata, 2015). In earlier years, more than 90% of cases of IE due to *P. aeruginosa* had been reported in intravenous drugs users (IDU) (Kanj and Sexton, 2019c). Nonetheless, IDU was recognized in only 4% of cases of IE caused by non-HACECK Gram-negative aerobic bacilli in a multinational survey that included 49 patients seen between 2000 and 2005 (Baddour et al., 2015). The majority of IDU with pseudomonal IE had used tripeleminamine and pentazocine with contaminated water or paraphernalia (D'Agata, 2015; Kanj and Sexton, 2019c). Patients predominantly present with right-sided endocarditis; and the clinical manifestations of *P. aeruginosa* endocarditis resemble those of other forms of acute endocarditis in addicts (Falagas and Rafailidis, 2020). *P. aeruginosa* left-sided endocarditis occurs also in non-intravenous injection drug users, although infrequently. It is predominantly a nosocomial infection occurring after invasive procedures (D'Agata, 2015).

Ventilator-associated pneumonia and hospital acquired pneumonia

The respiratory tract is a frequent site of infection due to *P. aeruginosa*. This microorganism can occasionally cause community-acquired pneumonia; but it is a well-established cause of hospital exposure pneumonia, either associated with or without mechanical ventilation (D'Agata, 2015; Kanj and Sexton, 2019e). It is the second most common pathogen involved in ventilator-associated pneumonia (VAP) after *Staphylococcus aureus*; and it causes 9% of all episodes of nosocomial pneumonia among non-ventilated patients (D'Agata, 2015). According to the definitions by the 2016 Infectious Diseases Society of America and American Thoracic Society, hospital acquired pneumonia is defined as pneumonia not incubating at the time of hospital admission, and occurring 48 h or more after admission, and not associated with mechanical ventilation (Kalil et al., 2016). VAP is a pneumonia occurring >48 h after endotracheal intubation. The clinical manifestations of *P. aeruginosa* pneumonia are similar to infections caused by other microorganisms.

Currently, the evidence suggests that outcomes for pneumonia are similar regardless of whether respiratory specimens are obtained invasively or noninvasively, and whether microbiologic cultures are performed quantitatively or semiquantitatively. Therefore, the etiologic diagnosis in patients with nosocomial pneumonia should be pursued through noninvasively obtained respiratory samples with semiquantitative cultures (Kalil et al., 2016) (see Diagnosis).

Chronic respiratory tract infections

P. aeruginosa is responsible for chronic colonization of the airways associated mainly with CF, advanced chronic obstructive pulmonary disease and bronchiectasis (D'Agata, 2015; Eklof et al., 2020; Falagas and Rafailidis, 2020; Kanj and Sexton, 2019d,

e). In these cases, recurrent pneumonia may ensue. Another chronic infection of the respiratory tract associated with *P. aeruginosa* is diffuse panbronchiolitis, which mainly affects Asian populations (Falagas and Rafailidis, 2020).

Urinary tract infections

Urinary tract infections (UTIs) due to *P. aeruginosa* are typically hospital acquired (Kanj and Sexton, 2019a). *P. aeruginosa* is the third most common pathogen to cause hospital acquired UTIs, after *Escherichia coli* and *Enterococcus* spp. (Kanj and Sexton, 2019a). Pseudomonal UTIs usually occur as a complication of the presence of a foreign body, such as a catheter or stent in the urinary tract, or an obstruction (mainly a stone or malignant neoplasm) of the urinary system, or after instrumentation or surgery in the urinary tract (Fig. 1). For patients with a chronic indwelling bladder catheter, the isolation of *P. aeruginosa* is not necessarily indicative of a UTI, since bacteriuria and even pyuria in the absence of overt infection are common in such settings (Kanj and Sexton, 2019a). Notwithstanding, there have been descriptions of *P. aeruginosa* UTIs in outpatient children and adults without relevant evidence of urinary tract obstruction or foreign bodies (Falagas and Rafailidis, 2020). Urinary tract infection is frequently a source for bacteremia.

Eye infections

P. aeruginosa may produce eye infections, generally in relationship with eye trauma or surgery. Corneal infections caused by *P. aeruginosa* may manifest as focal or diffuse inflammation of the cornea (keratitis) or as corneal ulcers (Kanj and Sexton, 2019a). Pseudomonas causes a virulent form of keratitis which predominantly appears in subjects who wear contact lenses (Kanj and Sexton, 2019a; Puig et al., 2020). Clinical findings of keratitis include eye redness, pain and decreased visual acuity. Perforation of the cornea may occur and the disease can progress quickly and lead to loss of vision.

Endophthalmitis is another *P. aeruginosa* eye infection that may result from penetrating injuries, surgery, perforation of a corneal ulcer, or seeding from bacteremia (Kanj and Sexton, 2019a). The course of pseudomonal endophthalmitis is typically fulminant, developing over hours (Kanj and Sexton, 2019a). The initial symptom is decreased vision followed by dull severe ocular pain. Other findings include chemosis, conjunctivitis, lid edema, anterior uveitis and vitritis (Falagas and Rafailidis, 2020; Kanj and Sexton, 2019a).

Orbital cellulitis and gangrene necrosis of the eyelids have rarely been reported in neutropenic patients as metastatic foci of bacteremia (Falagas and Rafailidis, 2020).

Ear infections

P. aeruginosa causes acute external otitis, chronic otitis media, and malignant external otitis (D'Agata, 2015; Falagas and Rafailidis, 2020; Kanj and Sexton, 2019a). Acute external otitis due to *P. aeruginosa* is common in swimmers, mostly in residents of warm or



Fig. 1 Urinary cast from a nephrostomy tube. A 55-year old woman presented with right ureteral-hydronephrosis after gynecologic surgery. A percutaneous placement of a nephrostomy tube into the right dilated renal pelvis was carried out. The patient had high fever and right costovertebral angle pain. Cultures from urine and blood yielded *P. aeruginosa*. She eliminated a cast into the urine bag which accommodated to the form of the nephrostomy tube. The nephrostomy tube was removed and the patient was treated with antibiotics. Courtesy of Dr. A. Ceballos.

humid climates ("swimmer's ear"). The source of the organism is likely to be insufficiently chlorinated water from hot tubs or swimming pools. Patients with external otitis typically complain of ear itching and pain (otalgia), worsened by traction on the pinna. On examination, the external auditory canal is usually tender, edematous and contains debris. The infection is usually self-limited without sequelae. In chronic otitis media caused by *P. aeruginosa*, the main clinical manifestation is fluid discharge. Cultures are usually polymicrobial; although *P. aeruginosa* is found in isolation in a third of patients.

Malignant external otitis is a life-threatening *Pseudomonas* infection, which particularly affects elderly patients with diabetes (D'Agata, 2015; Falagas and Rafailidis, 2020; Kanj and Sexton, 2019a). Malignant otitis occurs when *P. aeruginosa* penetrates the epithelium of the external auditory canal and invades the surrounding soft tissue, cartilage and cortical bone. Infection can spread to the retromandibular, parotid and mastoid spaces, and even to the bones of the base of skull (skull base osteomyelitis). Infection presents with pain and discharge from the affected ear, sometimes accompanied by fever and hearing loss. Cranial nerve palsies (VI–XII) appear later in the course. Altered mental status may be seen, indicating the spread of infection into the brain, which heralds an ominous clinical course. Prompt diagnosis and treatment of this condition is essential. Cultures specimens should be taken from the ear canal and neuroimaging should be performed (computed tomography or magnetic resonance imaging). Gallium scan may be used to evaluate the bone extension of the disease.

Bone and joint infections

P. aeruginosa is a common cause of bone infections in patients with indwelling devices or prosthesis used in orthopedics and neurosurgery (D'Agata, 2015; Falagas and Rafailidis, 2020). Furthermore, vertebral osteomyelitis, sacroiliac septic arthritis, and sternoclavicular septic arthritis have been well documented in IDU (D'Agata, 2015; Falagas and Rafailidis, 2020). Sternoclavicular and sacroiliac septic arthritis from *P. aeruginosa* is seen almost exclusively in IDU. Before 1981, *P. aeruginosa* sternoclavicular septic arthritis was related with the injection of tripeleminamine and pentazocine (D'Agata, 2015). These infections are the result of bacteremia secondary to either injection of contaminated illicit drugs or IE. The clinical manifestations of vertebral *P. aeruginosa* osteomyelitis are more indolent than those of staphylococcal osteomyelitis. Patients present after 1–3 months of symptoms, which include neck or back pain, with fever present in fewer than half (D'Agata, 2015).

Pseudomonas osteomyelitis of the foot may follow nail puncture wounds to the plantar surface and inoculation of the pathogen through contaminated trainers. Most of these cases are reported in children, but it is also seen in adults. The main manifestation is pain in the foot and there may be superficial cellulitis around the puncture wound. *P. aeruginosa* also has the potential to affect the diabetic foot, especially when there is frequent exposure to water (Falagas and Rafailidis, 2020).

Central nervous system infections

Intracranial infections caused by *Pseudomonas* are rare clinical events. They appear following penetrating head trauma or neurosurgery and most patients have serious underlying diseases (Falagas and Rafailidis, 2020; Kanj and Sexton, 2019a). The entities seen most often are postoperative or post-traumatic meningitis, subdural empyema, and epidural infections resulting from initial contamination of access areas or wounds. Brain abscess secondary to embolic disease from endocarditis has also been described in drug addicts. Clinical features and diagnosis are similar to central nervous system infections caused by other gram-negative pathogens.

Skin and soft tissue infections, including burns

Ecthyma gangrenosum is a characteristic manifestation of *P. aeruginosa* bacteremia among immunocompromised patients, especially those with neutropenia (D'Agata, 2015; Falagas and Rafailidis, 2020; Kanj and Sexton, 2019b). It has been observed in approximately 1.3–3% of *P. aeruginosa* bacteremia cases (Kanj and Sexton, 2019b). Ecthyma gangrenosum, however, can also be caused by numerous other bacteria (including *S. aureus* and *Citrobacter freundii*), fungi, and viruses, and has also been documented to occur among immunocompetent hosts, but with much lower incidence; and even, in the absence of bacteremia (D'Agata, 2015; Falagas and Rafailidis, 2020; Kanj and Sexton, 2019b). However, the presence of this skin lesion should raise high suspicion for *P. aeruginosa* bacteremia (D'Agata, 2015). Ecthyma lesion results from perivascular bacterial invasion of the tunica media and adventitia of arteries and veins with secondary ischemic necrosis (D'Agata, 2015; Falagas and Rafailidis, 2020; Kanj and Sexton, 2019b). Lesions begin as single or multiple red macules progressing to vesicles and later bullous or pustular lesions. Hemorrhage and necrosis in the central part ensues, followed by the development of a grey-black eschar with surrounding erythema, within 12–18 h (Fig. 2). All body parts can be involved, but the anogenital and axillary areas are most commonly affected (D'Agata, 2015; Kanj and Sexton, 2019b). On histology, there is epidermal and upper dermal necrosis, inflammatory cell infiltrate, necrotizing vasculitis and vascular thrombosis. *P. aeruginosa* is isolated from skin biopsy specimens and blood (D'Agata, 2015; Kanj and Sexton, 2019b).

Complication of burn injuries by *P. aeruginosa* skin infection is a severe and significant problem (Falagas and Rafailidis, 2020; Kanj and Sexton, 2019b). Patients present an expanding black necrotic eschar along with a high colony count of *P. aeruginosa* of exceeding 10^5 organisms per gram of skin tissue (Falagas and Rafailidis, 2016; Kanj and Sexton, 2019b). Signs of sepsis may be present.



Fig. 2 Ecthyma gangrenosum in 21-year-old woman with acute myeloid leukemia. The lesion started on her left knee after 5 days of chemotherapy, as a painless erythematous macule (A), which progressed rapidly into a violaceous pustule (B). Blood and wound cultures grew *P. aeruginosa*. From Korte AKM and Vos JM (2017) Ecthyma gangrenosum. *The New England Journal of Medicine* 377: e32, with permission from the Publisher.

Paronychia is the acute or chronic inflammation of the proximal and lateral nail folds. *P. aeruginosa* can cause acute or chronic paronychia. In these cases, the affected digit is painful and swollen with pus discharge localized to one corner of the proximal nail fold. Green nail syndrome is an extension of chronic paronychia with a bluish-green discoloration of the nail plate, due to the diffusion of pyocyanin and pyoverdine produced by *Pseudomonas* spp. through the nail bed (Kanj and Sexton, 2019b).

Hot tub folliculitis is an infection of the infundibulum (upper segment) of the hair follicle, just beneath the skin surface, caused by *P. aeruginosa* (D'Agata, 2015). It is a recreational skin infection associated with immersion in contaminated swimming pools, hot tubs, and whirlpools (D'Agata, 2015; Kanj and Sexton, 2019b). The main presentation is that of a maculopapular superficial folliculitis that is painful and pruritic and that becomes vesiculopustular within 24–48 h after exposure. It remits spontaneously within 7–14 days.

Intra-abdominal and gastrointestinal infections

P. aeruginosa may be involved in complicated intra-abdominal infections. It may be recovered (in isolation or as part of a polymicrobial flora) in secondary and tertiary peritonitis, intra-abdominal abscesses and peritonitis associated with continuous ambulatory peritoneal dialysis (Falagas and Rafailidis, 2020). Necrotizing enterocolitis due to *P. aeruginosa* in children, mainly from Taiwan and China (Shanghai fever), and typhlitis in neutropenic patients have also been described (Falagas and Rafailidis, 2020; Kanj and Sexton, 2019a).

Infection due to *Pseudomonas* spp. other than *Pseudomonas aeruginosa*

Infection due to other *Pseudomonas* spp. may occur, usually among immunocompromised hosts (D'Agata, 2015; Falagas and Rafailidis, 2020) (see Table 1). The great majority of strains are susceptible to third-generation cephalosporins, piperacillin, carbapenems, and fluoroquinolones. However, antimicrobial susceptibility patterns should always guide therapy (D'Agata, 2015).

P. fluorescens belongs to the fluorescens group of *Pseudomonas* species, along with *P. aeruginosa* and *P. putida*. *P. fluorescens* has been implicated in catheter-related bloodstream infections and in some outbreaks related to blood transfusions and other contaminated fluids (D'Agata, 2015; Falagas and Rafailidis, 2020). *P. putida* has been reported as a causative pathogen in bacteremia, pneumonia, cholecystitis, cholangitis, urinary tract infections, and war wounds. Reports of *P. stutzeri* infections include osteomyelitis, arthritis, bacteremia, endocarditis, endophthalmitis, pneumonia, empyema, urinary tract infections, and meningitis. Species of *Pseudomonas* other than the aforementioned have only been documented as a human pathogen in isolated case reports (D'Agata, 2015).

Diagnosis

From a clinical stand point, there are no symptoms or signs to distinguish *Pseudomonas* infection from diseases caused by other pathogens. However, some epidemiological data should be a clue to consider *P. aeruginosa* as the causative organism of infection. Clinicians should consider *Pseudomonas* infections in the differential diagnosis of hospital-acquired infections and in specific population of patients, such as neutropenic patients, patients with burns, CF patients, and external otitis in diabetics.

Obviously, the etiologic diagnosis of infectious diseases implies the culture isolation of the pathogen from pertinent clinical samples. Moreover, newer methods, including multiplex polymerase chain reaction (PCR) tests, which are based on the detection of the bacterial DNA, have received clearance from the Food and Drug Administration (FDA) for use in clinical practice. PCR offers the advantage of speed in diagnosis. Serology is not helpful in the diagnosis of *P. aeruginosa* infections. Pulsed-field gel electrophoresis, restriction fragment length polymorphism, multilocus sequence typing, and random amplified polymorphic DNA PCR are used mainly for epidemiologic purposes (Falagas and Rafailidis, 2020).

For the diagnosis of IE, the American Heart Association recommends three blood culture sets, obtained from different venipuncture sites, with the first and last drawn at least 1 h apart and early echocardiography (Baddour et al., 2015). In patients with VAP, endotracheal aspiration with semiquantitative microbiologic culture is currently preferred over invasive sampling (bronchoscopy for bronchoalveolar lavage or obtaining samples through a protected specimen brush or blind bronchial sampling [mini-BAL]) and quantitative culture to diagnose pneumonia (Kalil et al., 2016). In non-ventilated patients with hospital acquired pneumonia, non-invasively obtained samples (expectorated sputum, induced sputum or nasotracheal suctioning) and semiquantitative culture are also preferred (Kalil et al., 2016). For the etiologic diagnosis of osteomyelitis, the acquisition of bone through fine-needle aspiration guided by CT or open bone biopsy is necessary when blood cultures are sterile (Falagas and Rafailidis, 2020; Osmon et al., 2013).

A challenge clinicians face frequently is to differentiate frank infection due to *P. aeruginosa* from colonization. Local or systemic symptoms and signs of infection along with clinical judgment may aid to resolve this difficult dilemma.

Treatment

General principles

P. aeruginosa is intrinsically resistant to many antibiotics and can acquire resistance to other agents during therapy. This happens either by selection of mutations in chromosomal genes and from the growing prevalence of horizontal gene transfer (Del Barrio-Tofiño et al., 2019; Falagas and Rafailidis, 2020). Antibiotics used to treat *P. aeruginosa* infections belong to eight categories, including aminoglycosides (gentamicin, tobramycin, amikacin, netilmicin), carbapenems (imipenem, meropenem, doripenem), cephalosporins (ceftazidime, cefepime), fluoroquinolones (ciprofloxacin, levofloxacin), antipseudomonal penicillins plus beta-lactamase inhibitors (ticarcillin-clavulanic acid, piperacillin-tazobactam), monobactams (aztreonam), fosfomycin, and polymyxins (colistin, polymyxin B). Attending to these categories and antimicrobials, Magiorakos et al. (2011) standardized definitions for acquired antimicrobial resistance of *P. aeruginosa*, as multi-drug resistant (MDR), extensively-resistant (XDR), and pandrug resistant (PDR). PDR is a strain non-susceptible to all antimicrobial agents mentioned above. XDR is an isolate non-susceptible to ≥ 1 agent in all but ≤ 2 categories. MDR is a *P. aeruginosa* strain non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories. In Spain, a recent nationwide survey on *P. aeruginosa* resistance has shown a high resistance rate for most antipseudomonal antibiotics (Table 2) (Del Barrio-Tofiño et al., 2019). In this study, 26% of the isolates were classified as MDR, 17% as XDR, and 0.1% as PDR. Patterns of antibiotic resistance in *P. aeruginosa* evolve over time and vary geographically and by type of infection (Del Barrio-Tofiño et al., 2019; Fernández-Cuenca et al., 2020; Kanj and Sexton, 2020; Weiner et al., 2016). This geographical variation highlights the importance of local data when choosing empiric therapy. Appropriate antibiotics for *P. aeruginosa* treatment and their dosages, provided the renal clearance is >50 – 60 mL/min, are shown in Table 3.

Antipseudomonal penicillins in combination with a β -lactamase inhibitor include piperacillin-tazobactam and ticarcillin-clavulanate. Tazobactam inhibits AmpC-type β -lactamases but does not have direct antipseudomonal activity (Kanj and Sexton, 2019d). Recently, newer beta-lactamase inhibitor combinations have been introduced. These compounds include ceftazidime-avibactam, ceftolozane-tazobactam, imipenem-cilastatin-relebactam, meropenem-vaborbactam, and aztreonam-avibactam. Neither varbobactam nor avibactam enhance the bacterial activity of meropenem and aztreonam against carbapenem resistant *P. aeruginosa* (Bassetti et al., 2018; Morris and Cerceo, 2020; Kanj and Sexton, 2019d). Imipenem-cilastatin-relebactam is a novel

Table 2 Antimicrobial susceptibility data for 1445 *P. aeruginosa* isolates according to EUCAST 2018 breakpoints.

Antibiotic	Susceptible (%)	Resistant (%)
Ticarcillin	18.8	81.2
Ceftazidime	79.7	20.3
Cefepime	79.4	20.6
Piperacillin-tazobactam	73.5	26.5
Ceftolozane-tazobactam	94.6	5.4
Ceftazidime-avibactam	94.2	5.8
Aztreonam	–	14.8
Imipenem	72.8	15.6
Meropenem	70.1	14.1
Ciprofloxacin	61.6	38.4
Tobramycin	83.7	16.3
Amikacin	91.6	4
Colistin	94.6	5.4

Modified from Del Barrio-Tofiño E, Zamorano L, Cortes-Lara S, et al. (2019) Spanish nationwide survey on *Pseudomonas aeruginosa* antimicrobial resistance mechanisms and epidemiology. *Journal of Antimicrobial Chemotherapy* 74: 1825–1835.

Table 3 Intravenous antibiotics effective against *Pseudomonas aeruginosa* infections in adults (D'Agata, 2015; Falagas and Rafailidis, 2020; Kalil et al., 2016; Kanj and Sexton, 2019c, 2019d; Morris and Cerceo, 2020).

Class	Agent	Dose ^a
Penicillin-beta-lactamase combinations	Ticarcillin-clavulanate	3.1 g q 4 h
	Piperacillin-tazobactam	4.5 g q 6 h ^b
Cephalosporins	Ceftazidime	2 g q 8 h
	Cefepime	2 g q 8 h or q 12 h
	Cefoperazone	2 g q 12 h
	Cefiderocol	2 g q 8 h
Advanced beta-lactamase inhibitor combinations ^c	Ceftazidime-avibactam	2.5 g q 8 h
	Ceftolozane-tazobactam	1.5–3 g q 8 h ^d
	Meropenem-vaborbactam	4 g q 8 h (infused over 3 h)
	Imipenem-relebactam	1.25 g q 6 h (infused over 30 min)
Monobactams	Aztreonam ^e	2 g q 8 h
Fluoroquinolones	Ciprofloxacin	400 mg q8
	Levofloxacin	750 mg q 24 h
Carbapenems ^f	Imipenem	500 mg q 6–8 h ^g
	Meropenem	1 g q 8 h
	Doripenem	500 mg q 8 h
Aminoglycosides ^h	Tobramycin	5–7 mg/kg q 24 h
	Gentamicin	5–7 mg/kg q 24 h
	Amikacin	15–20 mg/kg q 24 h
Polymyxins ⁱ	Colistin	6–9 MU loading dose then 4.5 MU q 12 h (maintenance dose) OR 5 mg/kg loading dose then 2.5 mg × (1.5 × CrCl +30) q 12 h (maintenance dose)
	Polymyxin B	2.5–3 mg/kg q 24 h divided in two daily doses
Phosphonic antibiotic ^j	Fosfomycin	16–24 g q 24 h in 3–4 divided doses

^aDoses are for intravenous administration. Doses listed are for patients with normal renal function; dose adjustments may be warranted for renal impairment.

^bPiperacillin-tazobactam is generally infused over 30 min. Some evidence suggests that an extended infusion (4.5 g over 4 h every 8 h) may be associated with better outcomes among critically ill patients.

^cCombinations agents that include a cephalosporin or carbapenem plus a β -lactamase inhibitor are generally reserved for specific types of infections (such as complicated intra-abdominal or complicated urinary tract infection) resistant to other agents.

^dPulmonary infections are treated with the 3 g every 8 h dose.

^eIn the absence of other options, it is acceptable to use aztreonam as an adjunctive agent with another β -lactam-based agent because it has different targets within the bacterial cell wall.

^fCarbapenems given as a single therapy have the propensity to induce resistance during treatment.

^gImipenem can be given up to a dose of 1 g every 6–8 h for severe infection.

^hAminoglycosides are generally used in combination with a beta-lactam and should NOT be used as a single agent for pseudomonal infections other than those of the lower urinary tract. When using an aminoglycoside as part of therapy for an infection with *P. aeruginosa*, some authorities prefer tobramycin over gentamicin because the former seems to have greater intrinsic antipseudomonal activity; however, other experts favor amikacin.

ⁱPolymyxins are generally reserved for the treatment of serious infections caused by *P. aeruginosa* isolates resistant to other agents. In such cases, they are often administered in combination with other antimicrobial agents and with a loading dose preceding the standing dose.

^jOptimal efficacy against *P. aeruginosa* is obtained with intravenous continuous infusion.

β -lactam- β -lactamase inhibitor combination approved in July of 2019 for the treatment of complicated urinary tract infections and complicated intra-abdominal infections (Karlowsky et al., 2020; Lob et al., 2020; Smith et al., 2020). In November of 2019, cefiderocol gained its approval for the treatment of complicated UTI in adults with little to no other treatment options. It is the first antibiotic in its class of siderophore cephalosporins, with a broad spectrum of activity for Gram-negative bacteria. Cefiderocol binds to ferric iron and gains entrance into bacteria via ferric iron transporter systems, with subsequent destruction of cell wall synthesis (Morris and Cerceo, 2020; Wright et al., 2017).

Experts do not advise using quinolones other than ciprofloxacin or levofloxacin in the treatment of *Pseudomonas* infection (Kanj and Sexton, 2020). In addition, levofloxacin has no advantage over ciprofloxacin for *P. aeruginosa* therapy, since its additional spectrum of coverage is usually unnecessary. Fluoroquinolones are the only class of antibiotics available as an oral formulation that is reliably active against *P. aeruginosa*. For the oral route, ciprofloxacin should be dosed 750 mg every 12 h and levofloxacin 750 mg once daily.

All carbapenems given as single therapy have the propensity to induce resistance during treatment. However, meropenem and doripenem have lower propensity to develop resistance during therapy than imipenem (Kanj and Sexton, 2019d). For these reasons, some experts use carbapenems for the treatment of *P. aeruginosa* infections when the microorganism is resistant to other agents or in

polymicrobial infections (Kanj and Sexton, 2019d). In vitro studies have shown that the carbapenem minimum inhibitory concentration (MIC) is lowest with doripenem, followed by meropenem, then imipenem (Luyt et al., 2014). It is unknown whether this in vitro potency difference translates into any difference in clinical outcome (Kanj and Sexton, 2019d).

Treatment of serious *P. aeruginosa* infections focuses around the controversy of using monotherapy versus combination therapy (Kanj and Sexton, 2020; Paul and Leibovici, 2013). Combination therapy (either empirical or definitive) has been proposed for patients with known or suspected *P. aeruginosa* infection as a means to prevent the emergence of resistance, and to achieve a synergistic effect. Neither of these goals have been convincingly demonstrated to be achieved with combination therapy so far (D'Agata, 2015; Falagas and Rafailidis, 2020; Kanj and Sexton, 2020). However, combination therapy should be strongly considered in the severely ill patient for the empirical treatment of *P. aeruginosa* infections, especially in health care institutions with a high rate of resistant *Pseudomonas* (e.g., >10–15% to the chosen antibiotic class). Using combination therapy will thus ensure that at least one antimicrobial agent is effective against the infecting *P. aeruginosa* strain. De-escalation to a single antimicrobial agent, once antimicrobial susceptibility profiles are available, should then be undertaken (Bassetti et al., 2018; D'Agata, 2015; Falagas and Rafailidis, 2016; Kanj and Sexton, 2020). Aminoglycosides are a suboptimal treatment as monotherapy of *P. aeruginosa* infections; except for lower urinary tract infections or topical usage in other infections. Classically, a β -lactam as first agent and an aminoglycoside as the second have been used for combination therapy, provided there are no contraindications to its use. Addition of ciprofloxacin 400 mg every 8–12 h IV (instead of an aminoglycoside) has been suggested to fare equally well (Falagas and Rafailidis, 2016; Kanj and Sexton, 2020). When using an aminoglycoside as part of therapy, some experts favor tobramycin over gentamicin as it has greater intrinsic antipseudomonal activity (Kanj and Sexton, 2020). Other authorities, however, prefer amikacin when using aminoglycosides (Falagas and Rafailidis, 2020). Additionally, initiation of a second antipseudomonal agent may be reasonable in infections that are slow or fail to respond to a single agent, although there are few data to support this practice (Kanj and Sexton, 2020).

MDR in *P. aeruginosa* infections is a growing clinical problem. These infections should be managed with the assistance of an expert in the field (Kanj and Sexton, 2020). When *P. aeruginosa* is resistant to first line agents (including piperacillin, cephalosporins and carbapenems) the susceptibility to β -lactams-beta-lactamase inhibitors, such as ceftazidime-avibactam and ceftolozane-tazobactam should be checked (Hirsch et al., 2020; Kanj and Sexton, 2020). Antibiotics that have to be considered on a compassionate basis if resistance to other antipseudomonal agents is encountered include colistin (polymyxin E), polymyxin B and the parenteral formulation of fosfomycin sodium. These agents are used when no other options are available and typically associated to a second antibiotic, even if it only has partial activity (Kanj and Sexton, 2020). Care must be taken when dosing colistin, as formulations differ between the United States and European product, each having distinct dosing recommendations (Falagas and Rafailidis, 2020; Kanj and Sexton, 2020). One must also remember that ertapenem, ceftaroline, tigecycline, eravacycline, and plazomicin do not have antipseudomonal activity (Bassetti et al., 2018; Falagas and Rafailidis, 2020; Morris and Cerceo, 2020; Wright et al., 2017).

Finally, the control of the source of infection is of paramount importance when treating *Pseudomonas* infections. Adequate surgical control of the infectious foci (drainage, debridement) and/or removal of the infected foreign body (catheter, implant) is an essential measure in therapy (Falagas and Rafailidis, 2020; Kanj and Sexton, 2020).

Treatment of *Pseudomonas aeruginosa* bacteremia

In the management of *P. aeruginosa* bacteremia prompt active antimicrobial therapy is crucial, since delayed treatment is associated with a higher mortality rate (Falagas and Rafailidis, 2020; Kanj and Sexton, 2020). Therefore, combination therapy to ensure appropriate antibiotic coverage is advisable for empiric treatment of known or suspected *P. aeruginosa* bacteremia, especially in neutropenic patients (Martínez-Nadal et al., 2020). An extended-spectrum β -lactam and either an aminoglycoside or a fluoroquinolone may be used in combination therapy. De-escalation to the most appropriate single-agent therapy should be performed as soon as the susceptibility profile is known (Bassetti et al., 2018; Falagas and Rafailidis, 2020; Kanj and Sexton, 2019c). Neither monotherapy with a quinolone (Falagas and Rafailidis, 2020) nor an aminoglycoside is suggested for the treatment of *P. aeruginosa* bacteremia, because they are associated with poor clinical outcomes (D'Agata, 2015; Falagas and Rafailidis, 2020; Kanj and Sexton, 2019c, 2020; Paul and Leibovici, 2013).

Another important consideration is the prolonged intravenous infusion of the antibiotic to exploit the pharmacokinetic/pharmacodynamic (PK/PD) properties of β -lactams in the treatment of *P. aeruginosa* (Falagas and Rafailidis, 2020; Kanj and Sexton, 2019c; Valero et al., 2020). The therapeutic efficacy of β -lactam antibiotics depends on the time their blood concentration is above the MIC. Thus, with prolonged intravenous administration of β -lactams, their blood concentration is above the MIC for longer periods. There are data, mainly from nonrandomized studies and a relevant meta-analysis, that support the fact that prolonged infusion of piperacillin-tazobactam for 4 h may provide a survival benefit (Falagas and Rafailidis, 2016; Kanj and Sexton, 2019c). Meropenem is infused in a relatively short infusion of 30 min; an extended meropenem infusion is one that extends to 3 h.

Treatment duration is largely dictated by the primary site of the infection and the patient response. In nonneutropenic hosts, if the source of the bacteremia could be removed (e.g., an indwelling urinary catheter) and the patient has responded promptly to therapy, treatment can be stopped after 7–10 days (Kanj and Sexton, 2019c). In cases with undrainable foci of infection or slow clinical resolution, longer antimicrobial courses are necessary. In neutropenic patients, antimicrobial treatment should be continued at least 14 days or longer, until the absolute neutrophil count is equal to or greater than 500 cells/mm³ (Falagas and Rafailidis, 2020; Kanj and Sexton, 2019c).

Treatment of pneumonia

Ventilator-associated tracheobronchitis may be difficult to distinguish from pneumonia and may progress to it. However, the 2016 Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA) and the American Thoracic Society (ATS) do not recommend antibiotic therapy for ventilator-associated tracheobronchitis (Kalil et al., 2016). In these cases, a watchful and vigilant clinician's eye is necessary. In patients with nosocomial pneumonia, the IDSA and ATS guidelines recommend using clinical criteria alone to initiate antibiotic therapy rather than clinical criteria plus biomarkers (i.e., serum procalcitonin determination, PCR, or bronchoalveolar lavage fluid soluble triggering receptor expressed on myeloid cells [sTREM-1]); or clinical criteria plus the Modified Clinical Pulmonary Infection Score (Kalil et al., 2016).

Patients with nosocomial pneumonia may have *P. aeruginosa* isolates resistant to many antibiotics, a problem that appears to be progressively worse (Bassetti et al., 2018; Falagas and Rafailidis, 2020; Fernández-Cuenca et al., 2020; Kalil et al., 2016). In patients with suspected or known VAP, the 2016 Clinical Practice Guidelines by the IDSA and the ATS have recommended empirical treatment with an antipseudomonal β -lactam agent associated with a fluoroquinolone or an aminoglycoside if the patient has risk factors for MDR *Pseudomonas*, or >10% of the patient's isolates are resistant to the antibiotic chosen, or the patient is in an ICU where the local antimicrobial susceptibility is unknown. Patients with risk factors for MDR *Pseudomonas* isolates are those with prior intravenous antibiotic use within 90 days, septic shock at the time of VAP, acute respiratory distress syndrome preceding VAP, five or more days of hospitalization prior to the occurrence of VAP, and acute renal replacement therapy prior to VAP onset (Kalil et al., 2016). β -lactams based agents include piperacillin-tazobactam, ceftazidime, cefepime, imipenem, meropenem and aztreonam. In the setting of penicillin allergy, aztreonam can substitute the other β -lactams (Kanj and Sexton, 2019e). Moreover, in the absence of other options, it is acceptable to use aztreonam as an adjunctive agent with another β -lactam antimicrobial, because it has different targets on the bacterial cell wall (Kalil et al., 2016). Antibiotic dosages are shown in Table 3. These 2016 Guidelines suggest avoiding both aminoglycosides and colistin if alternative agents with adequate activity against *Pseudomonas* are available. Despite some reports indicating the opposite (Luyt et al., 2014), doripenem is not approved by the FDA to treat any type of pneumonia, based on a study comparing imipenem-cilastatin with doripenem in patients with pneumonia while on ventilators (FDA, 2019). The rationale to use combination therapy as empirical treatment of VAP is to ensure that at least one antimicrobial agent is active against *P. aeruginosa*. Once susceptibility profiles are available, monotherapy should also be considered because data supporting a higher efficacy of combination therapy are lacking (D'Agata, 2015; Falagas and Rafailidis, 2020; Kalil et al., 2016; Kanj and Sexton, 2019e). Patients with septic shock should also receive combination therapy (Kalil et al., 2016).

Aminoglycosides are not optimally active in the lungs at concentrations used for intravenous administration and are not recommended as monotherapy (D'Agata, 2015; Falagas and Rafailidis, 2020; Kalil et al., 2016; Kanj and Sexton, 2019e, 2020). However, the administration of aerosolized aminoglycosides may provide adequate drug levels in the tracheobronchial tree. The 2016 IDSA guidelines recommend inhalational therapy as an adjunct to systemic antibiotics in patients with nosocomial pneumonia in two situations: (i) patients with microorganisms that are susceptible only to aminoglycosides or polymyxins (colistin or polymyxin B); and (ii) as a last resort for patients who do not respond to intravenous antibiotics alone, regardless of the sensitivity of the microorganism (Kalil et al., 2016). Tobramycin (300 mg inhaled daily) and aztreonam lysine (75 mg inhaled three times daily for 28 days) have been shown to be safe and effective for patients with cystic fibrosis and have FDA approval only for these patients (Falagas and Rafailidis, 2020). Another antibiotic that has been used on a compassionate basis is the inhaled form of colistin. Inhaled colistin does not have FDA approval for patients without CF (Falagas and Rafailidis, 2020; Kanj and Sexton, 2019e).

The duration of antimicrobial treatment should depend upon the rate of improvement of clinical, radiological, and laboratory parameters. However, a 7-day course of antibiotics is the current recommendation by the IDSA and the ATS for patients with nosocomial *Pseudomonas* pneumonia (Kalil et al., 2016). Nonetheless, some experts advise for a course of 10–15 days or even longer, arguing that a shorter course may be suboptimal and associated with higher recurrence rates (D'Agata, 2015; Kanj and Sexton, 2019e). *P. aeruginosa* may continue to colonize the airway despite a clinical response; therefore, symptomatic improvement is a better indicator for antibiotic discontinuation (Kanj and Sexton, 2019e).

Treatment of other infections

The American Heart Association recommends cardiac surgery in combination with prolonged courses of combined antibiotic therapy in *P. aeruginosa* IE. Consultation with an expert in IE treatment should be sought. Combination antibiotic therapy with a β -lactam (penicillins, cephalosporins, or carbapenems) and either an aminoglycoside or fluoroquinolone for 6 weeks is reasonable (Baddour et al., 2015). If splenic abscess occurs as a complication of pseudomonal endocarditis, splenectomy should be performed prior to valve replacement (Kanj and Sexton, 2019c). In right-sided *Pseudomonas* IE, medical treatment is successful in 50–75% of cases (Baddour et al., 2015; Kanj and Sexton, 2019c). In the remaining patients, partial tricuspid valvectomy or "vegetectomy" without valve replacement is indicated. Placement of prosthetic valves should be avoided in these patients; because, typically, they have been IDU and have a high risk of recidivism (Baddour et al., 2015; D'Agata, 2015).

The majority of *P. aeruginosa* UTIs are complicated. Thus, the management of *P. aeruginosa* UTI involves antibiotic administration as well as correcting any obstruction and removing an indwelling catheter to prevent relapse (Falagas and Rafailidis, 2016; Kanj and Sexton, 2019a). Eradication of the organism is usually impossible in patients with *P. aeruginosa* UTI with a chronic indwelling bladder catheter (Kanj and Sexton, 2019a). Asymptomatic bacteriuria should not be treated, unless the patient is pregnant or is

undergoing an urologic procedure for which mucosal bleeding is expected (D'Agata, 2015; De Cueto et al., 2017; Nicolle et al., 2019). The duration of treatment for *P. aeruginosa* UTI is 3–5 days for cystitis; 7–10 days may suffice for pyelonephritis; although up to 2–3 weeks may be necessary for severe cases (Falagas and Rafailidis, 2016; Kanj and Sexton, 2019a). Fluoroquinolones (ciprofloxacin, levofloxacin) have the pharmacodynamic advantage of excellent concentrations in the urinary tract. Antipseudomonal β -lactam/ β -lactamase inhibitors and carbapenems also achieve high levels of urinary excretion (Falagas and Rafailidis, 2016).

The treatment of corneal infection usually consists of topical antipseudomonal formulations, containing aminoglycosides or fluoroquinolones (Kanj and Sexton, 2019a). In cases in which involvement is extensive, ceftazidime or gentamicin have been used via subconjunctival injection (Falagas and Rafailidis, 2016). Therapy for endophthalmitis includes vitrectomy and intravitreal antibiotics (Kanj and Sexton, 2019a). In addition, adjunctive systemic antibiotics are administered at high doses to achieve an adequate concentration in the eye. Ceftazidime and aminoglycosides are the antibiotics most frequently used in this entity (Falagas and Rafailidis, 2016).

Management of otitis externa involves the use of topical antibiotics, such as aminoglycoside-containing otic solutions (neomycin plus polymyxin B and hydrocortisone) and quinolone-containing otic solutions (ciprofloxacin 0.2% or ofloxacin 0.3%). Protection of the ear against moisture and avoidance of further mechanical injury by scratching are also important. For malignant external otitis, antipseudomonal antibiotics are the mainstay of treatment nowadays. Ciprofloxacin is the antibiotic of choice (D'Agata, 2015; Grandis and Yu, 2019). In cases of fluoroquinolone-resistant *P. aeruginosa*, other antibiotics should be used, such as piperacillin-tazobactam, ceftazidime, cefepime, or meropenem. In any case, a long treatment period of 6–8 weeks is necessary (D'Agata, 2015; Falagas and Rafailidis, 2016; Grandis and Yu, 2019). Therapy of malignant external otitis also involves surgical debridement of the ear canal, including any necrotic tissue cartilage and adjacent bone, rather than extensive bone debridement and facial decompression as was previously practiced (Falagas and Rafailidis, 2016; Grandis and Yu, 2019).

The IDSA 2013 guidelines for prosthetic joint infections caused by *P. aeruginosa* recommend treatment with cefepime, 2 g IV every 12 h, or meropenem, 1 g IV every 8 h, for 4–6 weeks. Alternative antibiotics to consider include ciprofloxacin, 750 mg orally twice a day or 400 mg IV every 12 h, or ceftazidime, 2 g IV every 8 h (Osmon et al., 2013). In these guidelines, the use of an aminoglycoside or two other active antibiotics could be considered. There are no recommendations for treatment of *Pseudomonas* arthritis or osteomyelitis (D'Agata, 2015). Therefore, the mentioned guidelines can be applied to the treatment of these infections. Ciprofloxacin has been used extensively in the treatment of chronic osteomyelitis due to *Pseudomonas* and the oral route may be an alternative to parenteral treatment (Falagas and Rafailidis, 2016). There are relatively few data regarding the appropriate duration of treatment. A duration of 6–12 weeks is most often reported (Falagas and Rafailidis, 2016).

In central nervous system infections, ceftazidime, cefepime or meropenem are preferred because of their good cerebrospinal fluid (CSF) penetration (Kanj and Sexton, 2019a; Tunkel et al., 2017). Alternative therapies are aztreonam and ciprofloxacin. Aminoglycosides may be added to these agents (Falagas and Rafailidis, 2016). The duration of treatment for meningitis extends to 10–21 days and repeat CSF cultures should confirm clearance of the microorganism (Kanj and Sexton, 2019a; Tunkel et al., 2017). Intra-ventricular antimicrobials are not approved by the FDA and specialists do not recommend their general use (Tunkel et al., 2017). In CSF shunt infections, removal of all components of the infected shunt, along with appropriate antimicrobial therapy appears to be the most effective treatment (Rodríguez-Lucas et al., 2020; Tunkel et al., 2017). Placement of a new shunt necessitates 10 days of sterile cerebrospinal culture (Tunkel et al., 2017). Brain abscess and epidural and subdural empyema generally require surgical drainage in addition to antibiotics (Falagas and Rafailidis, 2020).

Concluding remarks

Despite all the advances in medical care in recent years, infection due to *Pseudomonas* spp. continues to have an unacceptably high mortality rate, ranging from 30% to 60%. Furthermore, severe disabilities may be caused by *Pseudomonas* infection, such as loss of vision or impairment of neurological function. On the other hand, these infections are associated with increased length of hospitalizations and medical costs.

Antibiotic resistance in association with *Pseudomonas* infections is a growing clinical problem worldwide and will pose an enormous burden on human lives and financial resources in the near future. Once MDR *Pseudomonas* infection or carriage is detected in hospitalized patients, contact precautions should be implemented to prevent hospital spread. Factors that shorten the length of hospitalization and the judicious use of antibiotics are likely to decrease the incidence of this dreadful infection.

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Stenotrophomonas, Burkholderia and Other Related Microorganisms

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Introduction

Stenotrophomonas maltophilia, *Burkholderia cepacia* complex (Bcc) and other related microorganisms are a group of fastidious nonfermenters gram-negative bacillus. These environmental organisms can complicate treatment in those who are immunocompromised, critically ill in the intensive care unit and cystic fibrosis patients (CF).

Through a range of intrinsic antimicrobial resistance mechanisms, virulence factors, and the ability to survive in biofilms, these opportunistic pathogens are well suited to persist, both in the environment and the host.

Epidemiology and transmission

Stenotrophomonas, *Burkholderia*, and *Achromobacter* species are all ubiquitous environmental organisms found in water, soil, the rhizosphere, and in and on plants. All three organisms are capable of causing a variety of infections, including bacteremia, pneumonia, meningitis, urinary tract infections, and nosocomial infections from contaminated environmental sources (e.g., medications, nebulizers, dialysis fluids, saline solution, disinfectants, and contact lens fluid) (Brooke, 2012; Liu and Tong, 2019). A major virulence factor of these organisms is their ability to produce and survive with in biofilms (Tacconelli et al., 2014; Flores-Treviño et al., 2019).

Person-to-person transmission of these multidrug-resistant pathogens, especially among CF patients, remains a preoccupation. Unlike Bcc, where evidence for cross-transmission is well reported (Barchitta et al., 2009), less is known for *S. maltophilia*. However, case reports have documented incidences of patient cross-transmission (Diniz Rocha et al., 2020). The transmission of *S. maltophilia* to susceptible individuals may occur through direct contact with the source. The hands of health care personnel have been reported to transmit nosocomial infection in an intensive care unit.

S. maltophilia has been co-cultured with *P. aeruginosa* in respiratory samples obtained from CF patients. Cough generated aerosols from CF patients have the potential to provide airborne transmission of *S. maltophilia* (Wainwright et al., 2009).

The incidence of *S. maltophilia* hospital-acquired infections is increasing, particularly in the immunocompromised patient population. *S. maltophilia* infections can occur in both children and adults (Nayyar et al., 2017). The reported incidence of *S. maltophilia* infections seems to be increasing as the population of patients at risk increases. This increase is probably due to advances in the treatment of malignancy, increased use of invasive devices, and widespread use of broad-spectrum antibiotics.

S. maltophilia was among the 10 most common bacterial pathogens causing pneumonia in hospitalized patients from Europe, China, and the United States. The Bcc is a serious problem for cystic fibrosis patients and is increasingly occurring in hospital outbreaks in intensive care settings (Ramsay et al., 2013; Flamm et al., 2019).

Although *P. aeruginosa* and *S. aureus* continue to be the main colonizers in patients with CF, in recent years there has been an increase in the prevalence of *S. maltophilia* (4–15%) and *Achromobacter xylosoxidans* (3–8%) in these patients. Cases of colonization by two or more of these microorganisms have been described.

Microbiological and identification characteristics

S. maltophilia, formerly named *Pseudomonas* and then *Xanthomonas maltophilia*, is the only species in the genus. The name signifies “a unit feeding on few substrates,” based on the Greek roots *stenos* (narrow), *trophos* (one who feeds), and *monas* (a unit). *Maltophilia* means “affinity for malt,” based on the Greek roots *maltum* (malt) and *philia* (affinity). *S. maltophilia* bacteria are motile, free-living, glucose-non-fermentative, gram-negative aerobic bacilli with multitrichous polar flagella. *S. maltophilia* grows readily on most

bacteriologic media, typically appearing pale-yellow, grayish, or lavender-green when grown on blood agar. Preliminary identification may be facilitated by its ammonia-like odor. Most clinical isolates are oxidase negative and use maltose and usually dextrose and xylose. *S. maltophilia* may produce extracellular deoxyribonuclease on selected media, can hydrolyze esculin and orthonitrophenyl- β -D-galactopyranoside, and produces catalase and a strong acid reaction in oxidation-fermentation in maltose medium.

The *Burkholderia* genus, also originally of the genus *Pseudomonas*, contains more than 60 species and is found within the Betaproteobacteria class and Burkholderiales order. *B. cepacia* is referred to as a “complex” as it contains at least 17 genetically related species, formally designated as numbered genomovars. *B. cepacia*, now represents genomovar I of the large and diverse Bcc. Like *Stenotrophomonas* species, Bcc bacteria are motile, free-living, glucose-nonfermentative, gram-negative aerobic bacilli with multitrichous polar flagella. The appearance of Bcc colonies is variable, depending on the strain and the culture medium used. At present, MLST represents the most promising way for identifying species and strains of the Bcc.

Achromobacter and *Alcaligenes* species are gram-negative, motile, oxidase and catalase positive, non-fermenting rods. *Achromobacter xylosoxidans* was described in 1971 after being isolated from otic discharge samples from patients with chronic otitis media. The name stems from the ability of the organism to oxidize xylose readily, which is unique to the species. *A. xylosoxidans* was briefly renamed *Alcaligenes xylosoxidans*; however, it was placed back in the genus *Achromobacter* in 1998.

Modern laboratory identification techniques, such as matrix-assisted laser desorption ionization, time-of-flight mass spectrometry (MALDI-TOF MS) appears to identify and discriminate these organisms well.

On isolation, few biochemical reactions used to differentiate Bcc, *B. gladioli*, *Pandoraea* spp., *R. pickettii*, *A. xylosoxidans*, and *S. maltophilia* are enlisted in Table 1.

Mechanisms of resistance

S. maltophilia, Bcc and *A. xylosoxidans* have numerous intrinsic and acquired mechanisms of antibiotic resistance. Intrinsic β -lactamases, a wide range of efflux pump systems, enzymatic modifications, changes in the outer membrane and target site modification are just several of the mechanisms harbored by these organisms. Importantly, however, is the ability of these organisms to acquire new resistance determinants and to rapidly induce resistance. Intrinsic antibiotic resistance patterns in *S. maltophilia*, Bcc and *A. xylosoxidans* are important for physicians to consider when deciding on empiric therapy (Table 2).

Forms of clinical presentation

Stenotrophomonas, *Burkholderia* and *Achromobacter* species are capable of causing a variety of infections, including bacteremia, pneumonia, meningitis, urinary tract infections, and nosocomial infections from contaminated environmental sources (e.g., medications, nebulizers, dialysis fluids, saline solution, disinfectants, and contact lens fluid) have been reported (Abbott and Peleg, 2015). A major virulence factor of these organisms is their ability to produce and survive within biofilm.

The ability of *S. maltophilia* to form biofilm on various abiotic surfaces and tissues is an important feature of its virulence (Passerini de Rossi et al., n.d.). Biofilm consists of a community of bacterial cells that are attached to a surface and are embedded in an extracellular matrix composed of polysaccharides and proteins. This specific structure provides resistance to various antimicrobial drugs, antiseptic solutions and to the host immune defence. Biofilm formation contributes to the progression of CF lung disease and other chronic respiratory diseases (Pompilio et al., 2010). Biofilms were associated with 65% of the hospital-acquired infections, and *S. maltophilia* is a contaminant of a variety of medical devices and equipment (Brooke, 2012). Additionally, *S. maltophilia* can form biofilms on moist surfaces in direct or indirect contact with patients, such as respiratory tubing, catheters, intravenous lines, dialysis equipment, dental equipment and hospital plumbing systems (sinks and faucets) (Flores-Treviño et al., 2019).

S. maltophilia is not a highly virulent pathogen, but it has emerged as an important nosocomial pathogen associated with crude mortality rates is around 33% in children (Büyükcami et al., 2020; Alsuhaibani et al., 2021) and ranging from 16% to 60% in adults with bacteremia (Sumida et al., 2015; Bao et al., 2020; Hamdi et al., 2020).

Stenotrophomonas is increasingly recognized as an opportunistic pathogen responsible for nosocomial infections in intensive care unit patients (such as ventilator associated pneumonia and sepsis), life-threatening diseases in immunocompromised patients with hematological malignancies and cancers, and chronic pulmonary infections in patients CF (Nseir et al., 2006; Scholte et al., 2016).

The first challenge regarding management is to establish the clinical significance of culturing one of these nonfermenters from a clinical specimen. This question is largely irrelevant if these organisms are identified from sterile sites (e.g., cerebrospinal fluid, blood, and joint aspiration), but when they are identified either alone or with other organisms from nonsterile sites (e.g., sputum, wound swabs, and urine cultures), their role in disease may be difficult to ascertain. However, the repeated isolation of these organisms in the context of clinical disease or in unwell patients, antimicrobial therapy directed against these nonfermenters is often warranted.

The clinical manifestations of Bcc vary largely, ranging from no symptoms to severe respiratory infections and septicemia, especially in patients with cystic fibrosis or chronic granulomatous disease (Mahenthalingam et al., 2002). Uncommon presentations associated with Bcc were described in the literature, including but not limited to spontaneous bacterial peritonitis in

Table 1 Biochemical characteristics to differentiate *B. cepacia* complex, *B. gladioli*, *Pandoraea* spp., *R. pickettii*, *A. xylosoxidans*, and *S. maltophilia*.

	<i>B. cepacia</i>							<i>B. gladioli</i>	<i>Pandoraea</i> species	<i>R. pickettii</i>	<i>A. xylosoxidans</i>	<i>S. maltophilia</i>
	<i>Genomovar</i> <i>I</i>	<i>Genomovar</i> <i>II</i>	<i>Genomovar</i> <i>III</i>	<i>Genomovar</i> <i>IV</i>	<i>Genomovar</i> <i>V</i>	<i>Genomovar</i> <i>VI</i>	<i>Genomovar</i> <i>VIII</i>					
Oxidase	+	+	+	+	+	+	+	—	v	+	+	—
Oxidation of:												
Sucrose	v	—	v	—	+	—	+	—	—	—	—	v
Adonitol	v	+	v	v	—	+	+	+	—	—	—	—
Lactose	v	+	v	+	+	+	+	—	—	—	—	+
Lysine decarboxylase	+	v	+	+	+	—	+	—	—	—	—	+
Ornithine decarboxylase	v	—	v	+	—	—	—	—	—	—	—	—
Gelatin liquefaction	v	—	v	+	—	—	+	v	—	—	—	+
Aesculine hydrolysis	v	—	v	—	—	—	v	v	—	v	—	+
β -Galactosidase activity	+	+	+	—	+	+	+	+	—	—	—	+
Growth at 42 °C	v	+	v	—	+	+	v	—	v	v	NK	v
β -Hemolysis	—	—	—	—	v	—	v	—	—	—	NK	NK

Table 2 Intrinsic antibiotic resistance.

	S. maltophilia		B. cepacia complex		A. xylosoxidans	
	EUCAST	CLSI	EUCAST	CLSI	EUCAST	Other ^a
Amoxicillin-clavulanate	R	R	R	R	—	R
Ticarcillin-clavulanate	—	—	R	n/r	—	—
Piperacillin-tazobactam	R	R	—	R	—	—
Ceftriaxone	R	R	—	R	R	R
Ceftazidime	R	—	—	—	—	—
Cefepime	R	—	n/r	R	n/r	R
Aztreonam	R	R	n/r	R	n/r	R
Ertapenem	R	R	R	R	R	R
Imipenem	R	R	R	R	—	—
Meropenem	R	R	—	—	—	—
Ciprofloxacin	—	n/r	R	n/r	—	R
Aminoglycoside	R	R	R	R	—	R
Trimethoprim	R	R	R	R	—	R
Trimethoprim-sulfamethoxazole	—	—	—	—	—	—
Fosfomycin	R	R	R	R	—	R
Minocycline/Tigecycline	—	—	—	—	—	—
Colistin	—	—	R	R	—	—
Chloramphenicol	—	—	R	—	—	—

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; n/r, not reported.

^aIntrinsic resistance patterns for *A. xylosoxidans* gathered from other reports in the literature.

From Abbott I and Peleg A (2015) *Stenotrophomonas*, *Achromobacter*, and Nonmelioid *Burkholderia* species: Antimicrobial resistance and therapeutic strategies. *Seminars in Respiratory and Critical Care Medicine* 36(1), 99–110. <https://doi.org/10.1055/s-0034-1396929>.

patients with liver cirrhosis, endophthalmitis post-ocular surgery and/or eye trauma, necrotizing fasciitis in an immunocompetent patient, genitourinary infections e.g., prostatic abscess in a child with chronic granulomatous disease, bladder wall abscess post-urethrocytostomy, and vaginitis in a patient with smoldering myeloma, skin and soft tissue infections with and without deep seated abscesses, especially in children with chronic granulomatous disease, etc. (Sfeir, 2018).

Treatment strategies

Reported rates of in vitro antibiotic resistance are very broad. The fact that these organisms are also frequently part of a mixed infection, especially when it comes to pulmonary involvement, adds to the complexity of management. In general, isolates from CF patients demonstrate higher rates of resistance than those found in other patient groups.

Stenotrophomonas maltophilia

There are many difficulties regarding the treatment of *S. maltophilia* infections, high level of intrinsic resistance against a large number of antimicrobial agents being the most significant one. It was reported that trimethoprim/sulfamethoxazole could be the treatment of choice. On the basis of in vitro pharmacodynamics modelling and the bacteriostatic action of trimethoprim-sulfamethoxazole, it is recommended that a higher dose be used (daily dose of 15 mg per kg of the trimethoprim component, split 6–8 h). Other approaches included treatment with a combination of antibiotics (trimethoprim/sulfamethoxazole and tigecycline, tigecycline and amikacin, aerosolized colistin and doxycycline), treatment by inhalation with aerosolized levofloxacin and tobramycin powder, and innovative treatments (nano-emulsions, phage therapy, plant oils) (Chang et al., 2015).

S. maltophilia is inherently resistant to carbapenems, and in fact, use of this class of antibiotic often selects for *S. maltophilia* in patients who are heavily immunosuppressed (e.g., neutropenic patients). The inclusion of a specific biofilm active agent, such as moxifloxacin or levofloxacin has also been shown to be of benefit (Di Bonaventura et al., 2004; Wu et al., 2013) although caution should be applied when used as monotherapy because of the risk of rapid induction of resistance. Minocycline and tigecycline have also shown some promise to assist with the treatment of *S. maltophilia* (Farrell et al., 2010; Church et al., 2013). Recent studies revealed that *S. maltophilia* was highly susceptible to cefiderocol (MIC₉₀ 0.5 mg/L) and cefiderocol was at least as active as comparators, including trimethoprim/sulfamethoxazole (MIC₉₀ 2 mg/L) (Delgado-Valverde et al., 2020). The evidence for combination therapy often comes from in vitro synergy testing data, and highlights the need for further research into optimal therapy for this troublesome organism. The combinations are often reported involving trimethoprim-sulfamethoxazole, ticarcillin-clavulanate, moxifloxacin, levofloxacin, aztreonam, ceftazidime, colistin, rifampicin, tigecycline, and minocycline (Falagas et al., 2008; Milne and Gould, 2012; Church et al., 2013).

Burkholderia cepacia complex

Trimethoprim-sulfamethoxazole remains a recommended first-line therapy. Combination therapy is often used for patients who are more severely unwell, and includes double and triple combinations of first and second line agents. In contrast to *S. maltophilia*, Bcc are often sensitive to meropenem, which is another first-line therapy, but are inherently resistant to polymyxin and colistin. Tigecycline demonstrates poor activity against Bcc owing to drug efflux, although minocycline maintains activity.

The role of penicillins, namely, piperacillin-tazobactam and ticarcillin-clavulanate remains controversial given the different intrinsic resistance reports between EUCAST and CLSI. Inhaled tobramycin has the potential to achieve high pulmonary concentrations to inhibit Bcc isolates, despite widespread resistance reported (Trapnell et al., 2012).

Achromobacter xylosoxidans

Less is known about the optimal therapy for *Achromobacter* spp. In addition to the recognized intrinsic antibiotic resistance patterns, acquired resistance is also widely reported.

The most active agents are piperacillin-tazobactam, meropenem, and trimethoprim sulfamethoxazole, whereas ceftazidime is more active than cefepime (Almuzara et al., 2010; Atalay et al., 2012; Wang et al., 2013). Tetracyclines (e.g., minocycline) have variable activity and may be vulnerable to a multidrug efflux pump (Bador et al., 2013).

Aminoglycosides, fluoroquinolones, fosfomycin, and aztreonam all have poor activity. Multidrug-resistant phenotypes and carbapenemase-producing isolates have been reported, especially for the CF patient population, further complicating therapeutic options (Bador et al., 2013). Combination therapy has been recommended for the treatment of *A. xylosoxidans* pulmonary exacerbations in CF. Although the use of concurrent inhaled antibiotics, such as inhaled colistin, could also be considered.

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Moraxella and Other Non-Fermentative Gram-Negative Bacilli

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Introduction

The non-fermentative Gram-negative bacilli such a *Moraxella* spp. and other genus that will discuss in the chapter, are a group of aerobic, non-spore-forming bacilli, that do not use carbohydrates as a source of energy or degrade them in another way than fermentation. Over the past 3 decades, *Moraxella* (*Branhamella*) *catarrhalis* has emerged as an important and common human respiratory tract pathogen. This bacterium was transferred to a new genus named *Branhamella* on the basis of differences in fatty-acid content and DNA hybridization studies compared with other *Neisseriaceae* (Rossau et al., 1991). At this time, *Moraxella catarrhalis* is the most widely accepted name. The genus *Moraxella* comprises more than 20 species that are part of the normal microbiota of the human respiratory tract. *Moraxella* species are coccoid or coccobacillary microorganisms appearing usually in pairs and sometimes in short chains, tending to resist decolorization in the Gram stain. *M. catarrhalis* and *M. canis* are *Neisseria*-like diplococcic.

In this chapter, we have summarizes the main characteristics of the genus *Moraxella* and we have also included other non-fermentative Gram-negative bacilli such as *Oligella* and *Elizabethkingia* species which recently have demonstrated to be pathogens in the human population.

Moraxella (Branhamella) catarrhalis

Throughout history, *Moraxella catarrhalis*, has several taxonomic changes. It was initially named as *Micrococcus catarrhalis*, later it was renamed as *Neisseria catarrhalis*, owing to they share a similar morphology, phenotype and ecological niche with *Neisseria* species. Seeing the limited DNA homology with these species, it was described a new genus, *Branhamella*. Then, it was suggested that *Branhamella catarrhalis* was close to others species of *Moraxella* spp. There is no evidence to separate *B. catarrhalis* from *Moraxella* genus, so it should be included in this genus. Actually, *Moraxella* genus englobe cocci and short bacilli genetically related. Some authors prefer the name of *B. catarrhalis* because of other *Moraxella* species are short bacilli while *M. catarrhalis* (Rossau et al., 1991; Enright and McKenzie, 1997).

M. catarrhalis it is accepted as the third most important pathogen in the human respiratory tract, although until recently it was considered as a normal flora of the upper respiratory tract. Since it was recognized as a possible pathogen it was further investigated.

Epidemiology

It is believed that the only natural habitat is exclusively human. *M. catarrhalis* and other species of medical importance as *M. lacunata*, *M. osloensis*, *M. atlantae*, *M. nonliquefaciens*, can be found on nasopharynx and throat and other parts of the upper respiratory tract. They can also be found in conjunctiva and some species, like *M. osloensis*, in urogenital tract (Naheed et al., 2003; Forbes et al., 2002). Unlike *M. catarrhalis*, most of them are rarely associated with infections and are considered opportunistic. *M. catarrhalis* is the most frequently causes disease in humans.

M. catarrhalis is capable of colonizing without causing disease. The percentage of colonization depends on several factors. Age is one of them. The percentage of colonization in infants and children is high (up to 75%) while in healthy adults is very low (about 1–5%). There is not a clear reason for the difference in the carriage rates. One possibility could be the age-dependent production of the secretory immunoglobulin A (IgA). However, IgG antibody levels are not correlate with the percentage of colonization (Murphy, 1998; Verduin et al., 2002). According to the season, different levels of colonization have also been found, being higher during autumn and winter than in spring and summer (Van Hare et al., 1987). Some studies performed with respiratory samples from patients with chronic respiratory diseases have analyzed and described that some adults with chronic lung diseases appear to have a higher colonization rate than those who are healthy (Murphy, 1998). Some authors have described a relationship between geographical or socio-economic situation and colonization. A lower purchasing power has been associated with a higher possibility of overcrowding and less hygiene, which contributes to a higher possibility of colonization (Karalus and Campagnari, 2000).

Microbiology

Members of genus *Moraxella* are aerobic non-fermentative Gram-negative bacilli. In Gram stain they are tiny and look like bacilli or elongated diplococci. All of them are non-motile.

Colonies of *M. catarrhalis* grow well on both blood and chocolate agars. Generally, colonies on blood agar are non-hemolytic, round, gray to white, smooth, convex, opaque, and after 24 h of incubation they measure about 1–3 mm. Those colonies can be move and remain intact on the agar surface, like a “hokey puck” (Schreckenberger et al., 2007).

M. catarrhalis is catalase positive and strongly oxidase positive, but other tests are needed to identify it. Tests can be done on the strains to confirm the production of DNase and reduction of nitrate and nitrite. They do not produce acid from glucose, maltose, sucrose, lactose, and fructose but they have ability to hydrolyze glycerol tributyrat (Catlin, 1990).

In addition to *M. catarrhalis*, another species frequently isolated in human specimens is *M. nonliquefaciens*. These isolates are semiopaque and smaller (0.1–0.5 mm) than *M. catarrhalis* and some of them can pit the agar. Colonies of *M. osloensis* are similar but pitting is not common. *M. lacunata* y *M. atlantae* have small colonies and pitting is common. Growth of *M. atlantae*, *M. nonliquefaciens* and *M. lacuna* are similar in many of their features but, for example, growth of *M. atlantae* is stimulated by bile salts and sodium deoxycholate, while *M. nonliquefaciens* and *M. lacunata* are not (Schreckenberger et al., 2007).

Pathogenicity

The increasing importance that *M. catarrhalis* has acquired has increased the study of this bacterium. Research has promoted the study of virulence factors involved in the pathogenesis of infection and its immunogenicity. Several bacterial factors have been identified as contributing of virulence. Depending on the factor, it will be present in some or all isolates. Some of these factors have been considered as potential vaccine candidates.

Lipopolysaccharide

Lipopolysaccharide (LPS) or lipooligosaccharide (LOS) is a glycolipid exposed on the surface of the outer membrane. Unlike enteric Gram-negative bacteria it consists only of lipid A and an oligosaccharide part, that lacks O-antigen side chain. It appears to be more antigenically conserved and it is similar to other gram-negative bacteria like *Haemophilus influenzae* (Holme et al., 1999). The lipid A part is similar to that other bacteria. Although *M. catarrhalis* do not require LPS to be viability, it is an important virulence factor and it is involved in adherence and invasion epithelial cells and serum resistance (de Vries et al., 2009; Blakeway et al., 2017).

Three serotypes have been described: A, B and C. Serotype A is the predominant one (60%) while B (30%) and C (5%) represent a smaller number. Serotypes A, B and C represent 95% of the strains while 5% are unidentified. They have common epitopes that cause cross reactions between serotypes but also with other bacteria (Holme et al., 1999; de Vries et al., 2009).

Peptidoglycan

M. catarrhalis has multiple layers of peptidoglycan. That peptidoglycan triggers an efficient macrophage response, inducing the secretion of NO/NO₂ and TNF- α -independent tumoricidal activity (Keller et al., 1992).

Outer membrane proteins

Outer membrane proteins (OMPs) have been classified based on its electrophoretic mobility using sucrose gradient purification and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Eight types were identified (A-H). In addition to OMPs A to H, a new OMPs has been described, which will be developed below.

- Ubiquitous surface protein A (UspA)

The UspA family consists of UspA1 and UspA2, which are structurally related. Different functions are attributed to this family of proteins. Although they are structurally related, they are involved in different biological functions. UspA1 having been shown that is mainly involved in adherence and attachment to epithelial cells and biofilm formation. However, UspA2 is essential for complement evasion and serum resistance (Aebi et al., 1998; Verduin et al., 2002; de Vries et al., 2009; Blakeway et al., 2017).

- Outer membrane protein CD (OMP CD)

It has been shown that the initially called OMPs C and D are two stable forms of the same protein, the OMP CD. This protein is heat-modifiable and it is highly conserved among strains, which produce an immunogenic response. It has been seen that it is involved in adherence to epithelial cells and mucin from nasopharynx and middle ear, therefore, it is a way of promoting the invasion of the bacteria (Holm et al., 2004; Blakeway et al., 2017).

- Outer membrane protein E (OMP E)

OMP E is a heat-modifiable protein that is abundantly expressed in the bacterial surface and is highly conserved among strains, but it appears not to be very immunogenic (Blakeway et al., 2017). It has been described its implication in the acquisition of nutrients as well as serum resistance (Murphy et al., 2000; Verduin et al., 2002; de Vries et al., 2009; Blakeway et al., 2017).

- Outer membrane protein B2 (OMP B2) or CopB

This protein is present and is fairly well conserved in most of strains. It seems to be involved in iron acquisition and serum resistance (Verduin et al., 2002; de Vries et al., 2009; Blakeway et al., 2017).

- Hemagglutinin/Moraxella IgD binding protein (Hag/MID)

This protein, designated Hag (hemagglutinin) is also known as MID, is expressed by most strains of *M. catarrhalis*. It is involved in hemagglutination, autoagglutination and adherence to respiratory human cells (Murphy et al., 2019) and also biofilm formation (de Vries et al., 2009), which it leads to colonization of the host.

Fimbriae

Fimbriae, also called pilus, are spread along the bacterial surface. Type IV pilus (T4P or TFP) are polymers of the major pilin protein that are present in most of strains. It is associated with bacterial adherence to the host epithelial cells that plays an essential role in colonization (Luke et al., 2007; de Vries et al., 2009).

Biofilm formation

It is known that biofilm formation is important for persistence and colonization. Biofilm formation capacity of *M. catarrhalis* is already demonstrated by in vitro assays, and it is shown that play a role in chronic middle ear infections. Self-produced matrix that adheres to a surface makes relatively resistant to antibiotics and action of the immune system (Pearson et al., 2006; de Vries et al., 2009).

Other virulence factors

- *M. catarrhalis* adherence protein (McaP)

It was classified as a conventional autotransporter. It is highly conserved OMP in most of *M. catarrhalis* strains. McaP may be involved in adhesion, playing an important role in colonization. It also may display the enzymatic activity of an esterase and phospholipase, involved in a diverse range of cellular functions (Lipski et al., 2007; de Vries et al., 2009).

- Filamentous Hemagglutinin-Like Proteins (FHA-like proteins)

FHA-like proteins were shown to function as a two-partner secretion locus (TPS), involved in adherence to epithelial cells. These proteins were designated as MhaC (transporter), MhaB1 (exoprotein), and MhaB2 (exoprotein). TPS are present in other Gram-negative bacteria. They are found on the outer membrane and directly mediate attachment to human epithelial cells (Lipski et al., 2007; Plamondon et al., 2007).

- Toll-like receptor (TLRs) activation

TLRs are important components of the innate immune system that recognize pathogen. TLRs are involved in defense of the airways. *M. catarrhalis* induces an inflammatory response upon contact with TLR2 in respiratory airways, starting a cascade of reactions and having an important role in COPD exacerbation (de Vries et al., 2009; Perez Vidakovics and Riesbeck, 2009).

Complement resistance

Colonization means survival; therefore, it is necessary to interfere with the different components of the immune system. Complement system is the first line of innate defense against pathogenic microorganisms. Activation of the complement system triggers a protein cascade. Deposition of complement proteins on the bacterial surface provokes attack and opsonization of bacteria and its phagocytosis.

LOS structures are involved in serum resistance. Modifications in its structure have an impact on the response to the complement, making it more susceptible to it.

In addition to LOS, OMPs are also involved in complement resistance. UspA proteins play an important role in serum resistance. Both UspA1 and UspA2, are involved, but UspA2 affinity to complement proteins is higher than that of UspA1. It influences both in the classical and the alternative pathways. It is capable of binding to C4b binding protein (C4BP), C3 and vitronectin. C4BP is a regulator of the classical pathway of complement system. The binding of the α -chain of C4BP to the bacterial surface inhibits the classical pathway. Union also occurs with C3. UspA proteins bound to C3d fragment of C3 and inhibit the alternative pathway, increasing its survival. Also binds with vitronectin, which is involved in the regulation of the terminal pathway of complement system. It recruits vitronectin which allows to regulate the membrane attack complex (MAC) formation. Other OMPs like OMP CD, OMP E and CopB are also involved in complement resistance. Mutants without those proteins are more susceptible to the complement action (de Vries et al., 2009; Riesbeck, 2020).

Clinical manifestations

Otitis media

Otitis media (OM) is an inflammation of the middle ear. *M. catarrhalis* was recognized as a presumable OM pathogen in the 1980s and since then the reported cases have been increasing, particularly in children. Approximately 80% of children have had an episode of acute otitis media (OMA) in the first 3 years of their life. It is one of the most common reasons for medical consultation and prescription of antibiotics in children (Murphy and Parameswaran, 2009). Therefore, OMA has an important impact on the health of children.

Culture of the middle ear fluid (MEF) obtained by tympanocentesis is the standard for etiological diagnosis (Verduin et al., 2002; Murphy and Parameswaran, 2009; Sillanpää et al., 2016). The most common bacteria we found causing OMA are *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae* and *M. catarrhalis*. Around 15 to 20% of cases of OMA isolated from MEF are caused by *M. catarrhalis* (Murphy and Parameswaran, 2009). OMA caused by *M. catarrhalis* is clinically milder and less virulent than that caused by *S. pneumoniae* (Palmu et al., 2004; Murphy and Parameswaran, 2009).

There is an association between nasopharyngeal colonization and OMA. Nasopharyngeal carriage of the OMA major pathogens may carry an increased risk of OMA (Faden et al., 1991). It is relatively frequent that after a viral infection of nasopharynx an OMA appears. This is because the middle ear mucociliary clearance is less effective than in a normal situation, owing to the bacteria will stay longer in fluid ear and multiply, causing OMA (Owen, 2002).

Clinical manifestations of OMA include acute onset of otalgia, presence of MEF or inflammation with or without other nonspecific symptoms such as fever, rhinitis or vomiting. In bacterial OMA is characteristic of the bulging tympanic membrane that has purulent fluid. This is the best predictor of OMA, but is not always present (Lieberthal et al., 2013; Ngo et al., 2016). Tympanocentesis and culture is not always possible and it is not done routinely. This is because in most cases diagnosis is established using signs and symptoms. These characteristics do not allow differentiating the etiologic agent so generally OMA is empirically treated (Verduin et al., 2002; Murphy and Parameswaran, 2009).

M. catarrhalis can also produce recurrent otitis media (ROM) and otitis media with effusion (OME). OME is the presence of MEF without clinical signs of OMA. Children who have at least 3 episodes of OMA in 6 months or at least 4 episodes of OMA in 12 months or at least 8 months with MEF in a year are prone to recurrent otitis. Biofilm formation has been correlated with ROM and OME. This makes it more resistant to the antibiotic and immune system action (Murphy and Parameswaran, 2009; Ngo et al., 2016).

OME is more common than OMA. OME can be accompanied of a viral nasopharyngeal infection and be predictive of a future OMA.

Sinusitis

Most sinus infections are of viral origin. Sinusitis generally develops after an episode of viral upper respiratory infection and only a small number of patients may develop acute or subacute purulent sinusitis. Other predisposing event but in a lower percentage, is allergic inflammation. Sinus are connected with the nasal cavity, so the microbiology of sinusitis is related with that resides in the nasopharynx. As in otitis media, sinusitis is a very common disease in childhood and is most common after a viral upper respiratory infection. *M. catarrhalis* is the third most common cause of acute sinusitis in adults and children after *S. pneumoniae* and non-typeable *H. influenzae*. *M. catarrhalis* produce 20% of cases in children, but in adults is a smaller portion (Verduin et al., 2002; Murphy and Parameswaran, 2009). Sinus aspiration and culture determinate the etiological agent but is not performed routinely. In addition, infection is underdiagnosed because its symptoms are nonspecific.

Lower respiratory tract infections

The most common bacterial causes of respiratory infections are *S. pneumoniae*, non-typeable *H. influenzae* and *M. catarrhalis*.

M. catarrhalis is not a common pathogen causing a lower respiratory tract infection in healthy persons. Healthy people respiratory tract is sterile beyond the vocal cords, but in some cases, patients with predisposing factors can develop a pulmonary infection caused by *M. catarrhalis*.

This pathogen can produce respiratory infection in persons with chronic obstructive pulmonary disease, person with various forms of immunosuppression or pneumonia in elderly and can play a role as nosocomial respiratory tract pathogen.

- Lower respiratory tract infections in chronic obstructive pulmonary disease (COPD)

COPD is one of the most common causes of deaths in the world, causing a high mortality, morbidity and health care cost. COPD exacerbation is sustained worsening of a patient's condition. It is known that a lot of exacerbation is caused by bacterial infection, without forgetting those produced by viruses or non-infectious causes. These periods of increased airway inflammation may accelerate the progressive loss of pulmonary function in these patients.

M. catarrhalis and non-typeable *H. influenzae* strains are the most common bacterial pathogens causing acute exacerbations and chronic lower respiratory airway infection in COPD (Murphy et al., 2019).

The possible implication of *M. catarrhalis* in exacerbation of COPD has been ignored during decades. In patients with COPD, lower respiratory tract is colonized by bacteria, including *M. catarrhalis*, as a result of deficient mucociliary clearance. Around 5 to 35% of patients appear to be chronically colonized by *M. catarrhalis* and sometimes with different strains (Murphy et al., 2005). The acquisition of a new strain may play a role in episodes of exacerbation, thus triggering an immune response and its corresponding airway inflammation. During these episodes is seen an elevation of inflammation markers compared with no bacterial exacerbation.

The clinical presentation of *M. catarrhalis* during COPD exacerbation is similar to those due to another pathogen as *S. pneumoniae* or not-typeable *H. influenzae* (Verduin et al., 2002). The typical clinical presentation is an increased sputum production, increased sputum purulence and increased dyspnea. Other variability symptoms are fever and fatigue. Pleuritic pain, empyema and bacteremia are uncommon. Examination of the chest show ronus and generally decreased air entry with localized crackles. If patient has a coinfection with other low respiratory tract pathogens, infection may be more severe.

Gram stain smear of sputum or other lower respiratory tract samples reveals polymorphonuclears and abundant gram-negative elongated bacilli.

- Pneumonia in the elderly

Pneumonia due to *M. catarrhalis* does not occur frequently but it causes an important portion of pneumonia in elderly persons (older than 65 years). Most elderly patients who present community-acquire pneumonia have underlying cardiopulmonary disease including COPD, diabetes or are smoker or ex-smoker. About 10% of pneumonia cases in the elderly are caused by *M. catarrhalis* (Murphy, 2005). Seasonal variation may play a role in the ability to cause disease of *M. catarrhalis*, being more common in autumn and winter months.

- Nosocomial respiratory tract infection

Nosocomial transmission has been observed in some units in hospital environment. There is evidence of spread of *M. catarrhalis* within health care environment (McGregor et al., 1998; Verduin et al., 2002). Analysis of some isolates indicates that some cases were caused by the same strain, confirming person-to-person transmission.

Ocular infections

Infections of the eye can be caused by different microorganisms. Conjunctivitis is a frequent infection and it is more remarkable in pediatric population. *M. catarrhalis* is a frequent cause of acute conjunctivitis in children along with *H. influenzae* and *S. pneumoniae*. These episodes of conjunctivitis are commonly associated with upper respiratory infections but also can be transmitted directly from infected persons or for proliferation of normal flora. Blepharitis, that can be associated with conjunctivitis, can also be caused by *M. catarrhalis* among other microorganisms. Blepharitis is an inflammatory process involving the eyelids. *M. catarrhalis* can be isolated from swab or scraping culture. However, the isolation of these organisms may be carefully interpreted because it is part of the normal flora. Hence, is desirable collect sample in parallel from both eyes (healthy and infected) (Azari and Barney, 2013; Díaz López et al., 2019).

M. catarrhalis can also produce keratitis which affect cornea, and may progress to endophthalmitis (Berrocal et al., 2001).

Bacteremia

Bacteremia is an infrequent manifestation of *M. catarrhalis* infection. It has been described in patients with a wide range of age. Most of cases were associated with a respiratory focus or have some underlying pathology (Verduin et al., 2002; Murphy and Parameswaran, 2009).

Other Moraxella species infections

Other *Moraxella* species are unusual pathogens in humans. It has to be emphasized that other *Moraxella* species are frequently associated as an ocular pathogen. *M. lacunata* has been typically associated with eye infections. Moreover, *M. lacunata* is more common in chronic than acute conjunctivitis. Other species like *M. nonliquefaciens* and *M. osloensis* are also involucrate in these infections. Thus, *Moraxella* species can also cause conjunctivitis, keratitis and rarely endophthalmitis (Murphy, 2005).

Some case reports published in the medical literature have shown that these species can be as unusual cause of invasive infection in human, including endocarditis, septic arthritis, purulent pericarditis, cellulitis and meningitis. Patients experiencing meningitis due to *Moraxella* species are characterized by a high frequency of inherited and acquired complement deficiencies (Murphy, 2005).

Microbiological diagnosis

Colony morphology can change depending on the culture medium used and it is difficult to differentiate between *Moraxella* species.

Identification using biochemical has been traditionally used to identify these bacteria. The most used criteria for its identification are the following:

- Absence of pigmentation in blood agar (non-hemolytic).
- Production of oxidase and catalase.
- Production of DNase.
- Hydrolysis of tributyrin.
- Absence of acid production from glucose, maltose, sucrose and fructose.
- Reduction of nitrates and nitrites.

Biochemical tests have poor resolution to differentiate between some species because most *Moraxella* species have similar biochemical responses. The introduction of the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has allowed to the microbiology laboratory's ability to differentiate *Moraxella* species (Takahashi et al., 2019). This method is relatively rapid and accurate in comparison with biochemical identification.

Serodiagnostic techniques are not generally used for the laboratory diagnosis of infection caused by *Moraxella* species.

New DNA-based technologies have opened new possibilities for detection of *M. catarrhalis* and other *Moraxella* species in clinical samples without needing bacteriological culture. Polymerase chain reaction (PCR) based analyses have been tested for direct detection of *M. catarrhalis* DNA. There are several limitations of conventional culture methods to detect etiological agents involved in middle ear infections (Post et al., 1995). PCR has been used to improve the sensitivity and specificity of bacterial detection in middle ear effusion. 16S rRNA gen was targeted, which is found in multiple copies (Hendolin et al., 2000). It has been described multiplex PCR to detect main pathogens involved in these infections.

Treatment

Before 1970s, almost all isolates of *M. catarrhalis* were described as susceptible to penicillins (McGregor et al., 1998; Murphy, 2005). Therefore, these antibiotics were considered an adequate treatment for *M. catarrhalis*. But at the end of 1970s, first cases of beta-lactamase-producing strains were described. Now, more than 90% of *M. catarrhalis* strains produce beta-lactamase. This dramatic rise could be influenced by the increased prescription of beta-lactam antibiotics. If beta-lactamase production is demonstrated it should be assumed that hydrolyze penicillin, ampicillin and amoxicillin, but not all beta-lactams antibiotics are inactivated by these enzymes. It remains susceptible to cephalosporins (McGregor et al., 1998). Therefore, empiric use of penicillin or amoxicillin cannot be recommended (Wallace Jr et al., 1990). Beta-lactamase enzyme can be inactivated by beta-lactams inhibitors so it can be used amoxicillin in combination with clavulanic acid. The beta-lactamase stable cephalosporins or amoxicillin-clavulanic acid are generally the drugs of choice.

Antimicrobial susceptibility testing is generally recommended, although *M. catarrhalis* remains susceptible to a wide number of antibiotics such as trimethoprim-sulfamethoxazole, tetracyclines, oral cephalosporins, macrolides, fluoroquinolones and aminoglycosides (Murphy, 2005).

All isolates of *M. catarrhalis* are intrinsically resistant to trimethoprim, vancomycin and clindamycin (McGregor et al., 1998).

Treatment of the most common infectious diseases caused by *M. catarrhalis* is generally empirical. Thus, the chosen treatment is usually active against the pathogens that most commonly cause these infections (*S. pneumoniae*, *H. influenzae* and *M. catarrhalis*).

- Beta-Lactamase Production

The first beta-lactamase-producing strain was described in 1976. This beta-lactamase was called BRO (BRanhamella mOraxella). Two related beta-lactamase, BRO-1 (previously Ravasio-type enzyme) and BRO-2 (previously 1908-type enzyme) have been described. At the end of the decade of the 1990s, the percentage of this type of strains was more than 90%, but these percentages remain until today (McGregor et al., 1998; Bootsma et al., 2000). Genes of both BRO-1 (*bro1*) and BRO-2 (*bro2*) are located at the same chromosomal locus. Both alleles differ only in 5 bases and by only a single amino acid. The majority beta-lactamase produced by *M. catarrhalis* strain is BRO-1 and express higher levels of ampicillin resistance than those produced by BRO-2. Differences in the promoter region have been observed, that may explain this different expression (Bootsma et al., 2000).

It has been demonstrated that other *Moraxella* species produce similar beta-lactamase enzymes. *M. nonliquefaciens* and *M. lacunata* produce BRO-1 and BRO-2. *M. osloensis* produce beta-lactamase but is different to BRO-1 and BRO-2. Another beta-lactamase with a different hydrolysis pattern have described too. It seems to be a predominant beta-lactamase enzyme produced by other *Moraxella* and *Oligella* species (Wallace Jr et al., 1989).

Oligella species

The genus *Oligella* is composed of two species: *O. ureolytica* and *O. urethralis*. *O. ureolytica* was assigned by CDC (Center for Disease Control) to group IVe and *O. urethralis* to CDC group M-4 and formerly *Moraxella urethralis*. In 1970 the analysis of the DNA of both species, allowed to create the genus *Oligella* (Lautrop et al., 1970; Rossau et al., 1987).

Epidemiology

Both species are present as a normal flora in the human genitourinary tract and most isolates were described from human urine (Koneman, 2006; Steinberg and del Rio, 2005). *Oligella* species have been described in respiratory samples, so that indicates that it could colonize the human respiratory tract (Fernández-Olmos et al., 2012).

Microbiology

Oligella species are small and non-capsulated Gram-negative bacilli. Moreover, its name, *Oligella*, refers to its small size. Both species are catalase and oxidase positive, do not ferment or oxidize carbohydrates, do not produce hydrogen sulfide, indole and do not hydrolyze esculin and gelatin. Most strains can grow on MacConkey agar, being visible small non-lactose fermenting colonies after 2–4 days (Baqi and Mazzulli, 1996).

O. urethralis is similar to *Moraxella* species. Colonies, when grow on blood agar, are non-hemolytic, convex, and semiopaque to whitish. Colonies of *O. urethralis* are smaller than those of *M. osloensis*. Colonies of *O. ureolytica* have a relatively slow growth. It is found very small punctate colonies after 24 h of incubation on blood agar medium. After 3 days of incubation, it is found non-hemolytic, white, opaque and large colonies (Koneman, 2006; Schreckenberger et al., 2007).

The main differences between both species are that *O. urethralis* non-motile, does not produce urease and cannot reduce nitrate to nitrite. However, *O. ureolytica* is motile because it has peritrichous flagella, produce urease and can reduce nitrate to nitrite (Schreckenberger et al., 2007).

Pathogenicity

Both *Oligella* species are considered opportunistic pathogens and their role in pathogenicity is low or uncertain. Generally, infection chiefly occurs in persons with underlying conditions (Beauruelle et al., 2019).

Clinical manifestations

Infections due to *Oligella* species are rare but some cases have been described, mainly in patients with underlying predisposing factors.

These bacteria have mainly been found causing urinary infection, including urosepsis. Some cases of bacteremia produced by these species have also been described (Pagotto et al., 2016). Even less frequent are infections with extragenitourinary location, but cases of septic arthritis or lymph node infection have been described (Mesnard et al., 1992; Baqi and Mazzulli, 1996). Patients with cystic fibrosis or chronic obstructive pulmonary disease have also been reported (Beauruelle et al., 2019).

Microbiological diagnosis

A presumptive identification can be performed using Gram stain, colony morphology and some biochemical characteristic such as positive catalase and oxidase reactions. Final identification using biochemical tests is unreliable in laboratory practice routine. Misidentification of these two pathogens may be involved in the few cases of infections have been described. The introduction of

techniques such as MALDI-TOF MS or molecular techniques facilitates the identification of these microorganisms (Branda et al., 2014; Almuzara et al., 2015; Fernández-Olmos et al., 2012).

Treatment

O. urethralis is susceptible to most antibiotic, including penicillins, cephalosporins, aminoglycosides, chloramphenicol, erythromycin, tetracycline and trimethoprim sulfamethoxazole (Pagotto et al., 2016; Beauruelle et al., 2019). It has been reported several cases of fluoroquinolones resistance therefore empiric treatment with these antibiotics may not be recommended (Pugliese et al., 1993; Riley et al., 1996).

Most strains of *O. ureolytica* are susceptible to aminoglycosides, cephalosporins and colistin but using usual achievable serum concentrations it has been described resistance to penicillin, ampicillin, erythromycin, tetracycline, chloramphenicol and trimethoprim sulfamethoxazole (Welch et al., 1983; Koneman, 2006).

There are some beta-lactamase-producing strains which are resistant to penicillin and ampicillin (Welch et al., 1983; Pugliese et al., 1993). These bacteria are capable of acquiring resistance mechanisms. It has been described a chromosomal integration of a cephalosporinase gene from *Acinetobacter baumannii* into *O. urethralis* (Mammeri et al., 2003).

Elizabethkingia spp.

Currently, six species belong to the genus *Elizabethkingia*, namely, *E. meningoseptica*, *E. miricola*, *E. anophelis*, *E. ursingii*, *E. bruuniana*, *E. occulta*.

It was first described in 1959 by Elizabeth O. King associated with neonatal meningitis and it was named *Flavobacterium meningosepticum* (King, 1959). In 1994 a new genus was created, *Chryseobacterium*, and it was moved to this new genus: *C. meningosepticum* (Vandamme et al., 1994). Later, in 2005, the genus *Elizabethkingia* was proposed and *C. meningosepticum* was renamed as *E. meningoseptica* (Kim et al., 2005).

E. miricola was first described in 2003 as *Chryseobacterium miricola* which later, in 2005, was renamed and included in the genus *Elizabethkingia* (Li et al., 2003; Kim et al., 2005).

E. anophelis was first isolated from midgut of the mosquito *Anopheles gambiae* in 2011 (Kämpfer et al., 2011).

Finally, in 2017, three new species were proposed: *E. bruuniana*, *E. occulta* and *E. ursingii* (Nicholson et al., 2018).

Epidemiology

They are ubiquitous in nature. We find them in the soil, in both salt and fresh water, as well as in treated water, including in the tap water of hospitals. Generally, it is not found forming part of the human flora. In recent years there has been an increase in the number of cases, some of them associated with the hospital environment. The prevalence varies between geographic areas. The incidence of *E. meningoseptica* is variable and not clear but most of them are associated with hospitals (Vaneechoutte et al., 2015; Lin et al., 2018; Moore et al., 2016).

Microbiology and pathogenicity

Elizabethkingia species are gram-negative, aerobic, non-spore-forming, nonmotile, oxidase positive, catalase positive, nitrate negative and indole positive bacilli and they do not ferment glucose. The colonies on blood agar are smooth, circular, non-hemolytic and some strains are pale yellow-pigmented. The colonies are non-lactose fermenting having a pale and translucent appearance when grown on MacConkey agar. The strains cannot reduce nitrate and can hydrolyze esculin and gelatin.

Virulence factors are not well known for *Elizabethkingia* species but the ability to adapt to different environments and the existence of multi-resistant strains must be considered.

Clinical manifestations

Elizabethkingia species should be considered as a new emerging opportunistic pathogen. There are several cases associated with intensive care units and those associated with patients with underlying comorbidities or immunosuppression (Maraki et al., 2009; Weaver et al., 2010; Hsu et al., 2011).

For all six species, cases of bacteremia (associated or not with catheter), urinary tract infection and pneumonia have been described (Green et al., 2008; Jean et al., 2014; Lin et al., 2019). *E. miricola* has been isolated several times in patients with cystic fibrosis (Kenna et al., 2018). Both *E. meningoseptica* and *E. anophelis* have been isolated as causing meningitis, especially in pediatric patients (King, 1959; Frank et al., 2013; Joshi et al., 2019). They can also cause skin and soft tissue infections and other invasive infections such as bone infection (Calatrava et al., 2020). The infections are usually related to the hospital environment and many of them causing sepsis and having a high mortality rate (Jean et al., 2014; Lin et al., 2019).

Microbiological diagnosis

Overall, the usual identification methods are unreliable to identify *Elizabethkingia* species, so they are and have been misidentified. The identification performing biochemical properties has shown low correlation with the studies carried out by sequencing the 16S ribosomal RNA gene (rRNA). It has been seen that many species are misidentified as *E. meningoseptica*. The application of techniques such as MALDI-TOF mass spectrometry is useful to identify infections caused by *Elizabethkingia* species. However, MALDI-TOF systems will better or worse identify between different species according to the database they have and some errors are detected with some species. In this case, molecular methods are the techniques of choice to differentiate between the species of the genus *Elizabethkingia* (Eriksen et al., 2017; Lin et al., 2019; Burnard et al., 2020).

Treatment

Antibiotic susceptibility varies between species. Usually, isolates of the genus *Elizabethkingia* are resistant to beta-lactams.

Overall, *Elizabethkingia* species have two types of beta-lactamases; class A extended spectrum β -lactamase (ESBLs) and class B metallo-beta-lactamase (MBL), which confer intrinsic resistance to beta-lactams and carbapenems. It is typically resistant to third and fourth generation cephalosporins, carbapenems and aminoglycosides. The strains tend to be sensitive to piperacillin-tazobactam, ciprofloxacin, levofloxacin, rifampicin, trimethoprim sulfamethoxazole and minocycline. There are many genes involved in antibiotic resistance, i.e. *bla_B* and *bla_{COB}* (MBL) or *bla_{CME}* (ESBLs). Management should be based on sensitivity results of the antibiogram (Bellais et al., 2000; Yum et al., 2010; Han et al., 2016; Lin et al., 2012; Lin et al., 2019).

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Francisella, Brucella and Pasteurella

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In this chapter we include three microorganisms that are a common cause of zoonotic related human infection: *Francisella* spp., *Brucella* spp. and *Pasteurella* spp.

These microorganisms are bacilli or coccobacilli Gram negative bacteria, with different requirements for growing on laboratory culture media. *Francisella* spp. and *Brucella* spp. share a concern for their potential use in bioterrorism.

Francisella

Taxonomy

Francisella is one of the three genera of the *Francisellaceae* family, a member of the gamma-proteobacteria. This genus comprises several species of which the most studied, because of their potential to cause human infection, are *Francisella tularensis*, *Francisella novicida*, *Francisella philomiragia* and *Francisella noatunensis*. The genus also comprises several non-classified *Francisella* species. Edward Francis, an American bacteriologist subdivided these four species into 6 subspecies (Versalovic et al., 2011; Rosenberg et al., 2013a; Parte, 2014).

Francisella tularensis is subdivided into three subspecies, two of which are in turn subdivided into several subclades. These subspecies are: *Francisella tularensis* subsp. *tularensis* (type A), which is subdivided in three subclades: A1a, A1b and A2; *Francisella tularensis* subsp. *holarctica* (type B), which is comprised by 10 subclades; and *Francisella tularensis* subsp. *mediasiatica* (Versalovic et al., 2011).

Francisella novicida is mainly isolated from water; it was originally described as *Pasteurella novicida*. The most recent additions to the genus *Francisella* are *F. hispaniensis*, isolated from human blood and *F. haliotica*, from a marine gastropod mollusk in the family Haliotidae (Rosenberg et al., 2013a).

Despite a 75% DNA relatedness and >99.8% identity in 16S rRNA gene sequences between *F. novicida* and *F. tularensis*, whole-genome comparisons provide enough evidence to consider both being two separate species (Versalovic et al., 2011).

Using pulse field gel electrophoresis, type A strains have been separated into two main genetic groups (AI and AII) and subgroups (e.g., AIa and AIb). More recent studies based on whole genome sequencing and canonical SNP (canSNP) genotyping have revealed a higher population complexity, with three main subgroups A.I.3, A.I.8 and A.I.12. Type B strains are classically separated into three biovars (I, II and Japonica). Whole genome sequencing and canSNP genotyping methods have also revealed multiple clades, including the three major subpopulations B.4, B.6 and B.12. The B.16 clade is phylogenetically more deeply rooted and correlates perfectly with biovar Japonica (Maurin, 2014).

Genus description

The *Francisella* genus bacteria are small (0.7–1.7 µm), Gram-negative bacilli or coccobacilli. These microorganisms are nonmotile, non-spore-forming, heterotrophic, strict aerobes, urease negative and weakly catalase positive. Most of the species, but not all, produce H₂S. They react with a limited number of carbohydrates and utilize a few sugars (glucose, maltose, sucrose and glycerol), also produce acid without gas. They have a relatively slow growth that can be enhanced with sulfhydryl supplementation. Less metabolically competent members of the genus require enhanced levels of cysteine or cystine in culture media (Versalovic et al., 2011; Rosenberg et al., 2013a).

Epidemiology

Francisella species are zoonotic. Mainly found in the Northern hemisphere in more than 300 species of mammals, birds, reptiles, amphibians, fish and invertebrates.

Francisellaceae is widely distributed. Some species are obligate pathogens of animals and humans (*F. tularensis*), other species are present in the environment and are opportunistic pathogen of humans (*F. novicida* and *F. philomiragia*). Other species have been associated only with animals (*F. noatunensis*) (Versalovic et al., 2011).

F. tularensis is only found in the North Hemisphere, the Holarctic region. *F. novicida* and *F. noatunensis* are both found in the North and South Hemispheres. *F. tularensis* subsp. *tularensis* is only found in North America, and *F. tularensis* subsp. *holartica* have much wider distribution. *F. tularensis* subsp. *mediasiatica* is restricted to central Asia. *F. novicida* is primarily found in North America. *F. philomiragia* is mostly found in North America, but also in central and Eastern Europe and Australia. *F. noatunensis* is isolated from Scandinavia, Chile and Japan (Versalovic et al., 2011).

In North America we only found infection caused by *F. tularensis* subsp. *tularensis* (type A) or *F. tularensis* subsp. *holartica* (type B). In Eurasia, type A is not observed; type B is the main subspecies causing disease, with fewer cases due to *F. tularensis* subsp. *mediasiatica*. Types A and B are found in USA but differ in virulence and ecological niche. Type A1a and A1b predominate in the eastern half of USA while type A2 appears restricted to the western. Type A1, which is the most virulent and invasive subspecies, is endemic in the central states, and is associated with lagomorphs, cats and ticks. Type A2 is mostly found in the western USA, and it is less virulent. Type B is associated with water bodies, rodents and ticks (Versalovic et al., 2011; Mani et al., 2016).

F. holartica is separated into three biovars. Strains from biovar 1 are erythromycin sensitive and are reported in Western Europe; strains from biovar 2 are erythromycin resistant and are found in Eastern Europe. These strains can overlap in their geographical spread. The third one, biovar japonica, is mainly found in Japan, but also reported in China and Turkey (Maurin and Gyuranecz, 2016).

There is a known focus of endemicity of tularemia in Russia, Kazakhstan, Finland and Sweden. Reports of annual cases are frequent in Eastern Europe. Tularemia cases are reported less frequently from Western Europe, however, several outbreaks have occurred in Spain (Versalovic et al., 2011).

F. tularensis has many animal reservoirs, the most often associated with tularemia in humans are small rodents, like mice, rats and lemmings, but also it can be transmitted by arthropods and mosquitoes. In Europe, the most prevalent Ixodidae ticks infected with *F. tularensis* belong to the species *Dermacentor reticulatus* and *Ixodes ricinus*. *Aedes cinereus* is the mosquito species most often associated with the transmission of tularemia in Sweden and Finland (Maurin and Gyuranecz, 2016).

The ecology of human tularemia in the USA has changed over the past century. During the first half of the twentieth century, the majority of human cases were attributed to exposure to infected lagomorphs, while during the latter part of the century, human tularemia has been predominantly a tick-borne disease (Mani et al., 2016).

The major modes of *F. tularensis* transmission to humans are direct transmission from animal reservoirs through handling or ingestion of undercooked meat from an infected animal, arthropod and mosquito bites and transmission through contaminated water and soil environments (Maurin and Gyuranecz, 2016).

Pathogenicity and clinical significance

Francisella tularensis

Francisella tularensis is one of the most infectious bacterial pathogens known, as few as 10 microorganisms delivered subcutaneously or 25 microorganisms delivered by inhalation can lead to life threatening infection in humans. It is considered a potential bioterrorism agent due to its high infectivity and lethality, and the ease with which it can be dispersed (Maurin, 2014; Clemens et al., 2018).

F. tularensis is an intracellular pathogen that infects several types of cells: endothelial cells, alveolar type II cells, neutrophils and hepatocytes, but it is in macrophages where it replicates, and these cells are considered to be the vehicles for bacterial dissemination within the host. Two subspecies, *F. tularensis* subspecies *tularensis* (type A) and *F. tularensis* subspecies *holarctica* (type B) causes human tularemia (Kingry et al., 2014; Kinkead and Allen, 2016).

Tularemia is a serious and potentially life-threatening disease in humans. It has a short incubation period, usually from 3 to 5 days, but with a maximum of 2 weeks. It has been known by a number of synonyms attesting to the variety of clinical presentations, the ubiquitous presence in nature of the infectious agent and the means by which humans may acquire the infection, some of these names are rabbit fever, deerfly fever, Ohara's disease, glandular type of tick fever, ... (Versalovic et al., 2011; Rosenberg et al., 2013a; Maurin and Gyuranecz, 2016).

First, *F. tularensis* multiplies at the site of inoculation, where it causes an acute inflammatory reaction which involves neutrophils, macrophages and lymphocytes, and can lead to tissue necrosis. Then it can spread to regional lymph nodes and may then spread systemically through a lymphohematogenous route. Macrophage uptake is a unique process called "looping phagocytosis" that involves pseudopod loops. This entry is dependent upon complement and complement receptors. It also may be directly transferred from infected cells to uninfected macrophages during exchange of cytosolic material. After being taken up, phagosome maturation and phagosome-lysosome fusion are impaired and *F. tularensis* escapes into the cytosol inducing macrophage cell death and release of bacteria. Neutrophils are also unable to kill *Francisella* because it inhibits NADPH oxidase, repressing the oxidative burst. *F. tularensis* also can inhibit apoptosis and prolong survival of infected neutrophils (Clemens et al., 2005, 2018; Kinkead and Allen, 2016).

The clinical spectrum of tularemia depends on the mode of acquisition, the virulence of the infecting strain, the immune status of the host and timely diagnosis and treatment. Tularemia can be misdiagnosed since its symptoms are not unique. The differential diagnosis includes cat scratch fever, mycobacterial infections, anthrax, brucellosis, legionellosis and plague. Early clinical presentation corresponds to influenza-like symptoms (fever, chills, headache, myalgia and arthralgia). Infections can initiate through the skin, mucosal membranes, lungs and gastrointestinal tract, to produce different tularemia manifestations depending on the site of infection: ulceroglandular, glandular, oropharyngeal, oculoglandular and pneumonic forms. The ulceroglandular is the most common form (45–80% of the reported cases), the usual portal of entry is via arthropod bite or other inoculation through the skin barrier. This form combines a skin inoculation long-lasting ulcer with chronic suppuration, and a localized lymphadenopathy. An isolated lymphadenopathy without detectable skin ulcer corresponds to the glandular form. The oropharyngeal form comprises chronic pharyngitis occurring after oral contamination due to ingestion of contaminated water or food, sometimes with mucosal ulcers and often with swollen and painful cervical lymph nodes. The oculoglandular form is a painful conjunctivitis with localized lymphadenopathy occurring after conjunctival contamination, usually as a result of the mechanical transfer from an infectious source to the eye by the fingers. Pneumonic tularemia is acquired through inhalation of a contaminated aerosol or through hematogenous spread. It is the most severe form of the disease. There is also a Typhoidal tularemia which is a severe systemic disease with acute onset, high fever, headaches and neurological symptoms, but no localized lesions (nor skin ulcers, nor lymphadenopathy). It is the most difficult form to recognize because there is no identified portal of entry and localized signs are absent. If untreated, bacterial dissemination can lead to secondary clinical presentations, such sepsis and meningitis (Versalovic et al., 2011; Rosenberg et al., 2013a; Maurin and Gyuranecz, 2016).

The severity can range from mild and self-limiting to fatal and is largely dependent on the infecting strain. Little tularemia-related mortality is reported in Europe and Asia, where only *F. tularensis* subsp. *holarctica* causes tularemia, while in the USA, where tularemia is caused by *F. tularensis* subsp. *tularensis*, the mortality ranges from 2.3% to 9%. Among *F. tularensis* subsp. *tularensis* clades, type A1b shows a significantly higher mortality (24%) compared to types A1a (4%) and A2 (0%) (Kugeler et al., 2009; Versalovic et al., 2011; Kingry et al., 2014).

Other Francisella species

- *Francisella novicida*, *F. philomiragia*, *F. hispaniensis* and *F. opportunistica* are rarely associated with human disease and cases that do occur are associated with immunocompromised patients or patients that have other underlying health problems (Huber et al., 2010; Kingry et al., 2014; Siebert et al., 2020).
- *Francisella novicida*: rare opportunistic human pathogen. Usually causes disease in immunocompromised patients. Disease in healthy individuals it is being associated to consumption of contaminated water or following near-drowning events (Kingry et al., 2014; Siebert et al., 2020).
- *Francisella philomiragia*: it is commonly found in soil and water. It is an opportunistic pathogen that infect immunocompromised patients, but also causes disease in healthy individuals after near-drowning accidents (Siebert et al., 2020).

- *Francisella hispaniensis*: it was first isolated from the blood of a patient in Spain. Only two cases have been reported. The second one was a male fisherman with a cutaneous ulcer in China. It is considered an emerging human pathogen, but its epidemiology and pathogenicity are still unknown (Huber et al., 2010; Zhou et al., 2020).
- *Francisella opportunistica*: it was first isolated from cerebrospinal fluid and blood from two immunocompromised patients in the USA. There are no additional reports of this species causing disease in humans (Kugeler et al., 2008; Dietrich et al., 2020).

Diagnosis

Collection and transport of specimens

Francisella spp. has an extremely low infectious dose and cultures have a high concentration of microorganism so it must be handled with great precaution. Tularemia has been commonly reported as a laboratory associated infection. Clinical specimens should be handled under biosafety level 2 (BSL-2) conditions and go into biosafety level 3 as soon as a suspect *Francisella tularensis* is isolated in culture (Versalovic et al., 2011).

The clinical specimen for diagnostic should be chosen accordingly with the clinical form of the disease (sputum, aspirates, local and lesions swabs, biopsies, whole blood, serum. . .). Whole blood could be used for all clinical forms, but it could be negative at the early stages of the disease. The specimens should be delivered to the laboratory within 24 h (preferably within 2 h), swabs in an appropriated transport medium as Amies, and if the transport is delayed, specimens should be stored at 2–8 °C. *F. tularensis* can survive up to 7 days at room temperature when placed in Amies medium, but Stuart medium or saline are inadequate for keeping it viable. Aspirates, respiratory secretions and biopsies should be collected into sterile containers. If biopsies are too small, saline should be added to keep them moist. Serum samples should be separated from blood as soon as possible and stored at 2–8 °C for up to 10 days, or frozen (WHO, 2007; Versalovic et al., 2011; Maurin and Gyuranecz, 2016).

Direct detection in specimens

Fresh clinical specimens can be directly examined by microscopy after a Gram staining, but it has little diagnostic value due to the small size of *Francisella* spp. and their poor staining with safranin. It can be improved by using basic fuchsin but remains a poor diagnostic method. Antigen detection by DFA staining with fluorescein isothiocyanate-labeled hyperimmune rabbit polyclonal antibody directed against whole killed *F. tularensis* cells can presumptively identify *F. tularensis* in clinical specimens. DFA can also confirm the identification of recovered isolates, as well as slide agglutination. Immunohistochemical staining using monoclonal antibody against the lipopolysaccharide (LPS) can be used to visualize *F. tularensis* in formalin-fixed tissues (WHO, 2007; Versalovic et al., 2011).

Molecular detection is a valuable diagnostic tool. PCR methods have been used mainly for diagnostic of the most prevalent form, ulceroglandular tularemia. The majority have been conventional PCR assays targeted at *fopA* and *tul4* genes. The *tul4* PCR has been validated with wound specimens from patients with ulceroglandular tularemia and displays a sensitivity of 75%, greater than culture (sensitivity of 62%). There is also a real-time TaqMan PCR assay, with increased specificity, that includes multiple target genes (*ISFtu2*, *iglC*, *tul4*) and can be applied to different clinical specimens (aspirates, bronchial washes, ulcer swabs, . . .) (WHO, 2007; Versalovic et al., 2011).

Culture isolation and identification

Culture provides a conclusive diagnosis, but *F. tularensis* is a fastidious microorganism that requires cysteine or cystine supplementation to grow on artificial medium. It could grow on chocolate agar, buffered charcoal yeast extract (BCYE), Thayer-Martin, and thioglycolate broth. It also can grow on general media supplemented with 1–2% IsoVitalEx. Subcultures must be performed on cysteine-enriched media as *F. tularensis* will usually lose its viability with continued passage. There is a specialized medium of cysteine heart agar supplemented with 9% of chocolated sheep blood (CHAB) where *F. tularensis* displays a distinctive colony morphology that can aid in its identification (green, opalescent, raised, shiny colonies). The organisms grow slowly (60 min generation time), so cultures must be kept a minimum of 48 h incubated at 36 °C ± 1 °C aerobically and observed daily for up to 14 days. Clinical samples from non-sterile sites should be plated on antibiotic-containing media, such Thayer-Martin or BCYE, in order to prevent the microbiota from overwhelming *Francisella* (WHO, 2007; Versalovic et al., 2011; Rosenberg et al., 2013b).

Suspicion of *F. tularensis* may be raised when small gray-white colonies (1–2 mm) grow on chocolate agar after 48 h and scant to no growth on blood agar is observed. *F. tularensis* is oxidase negative, weakly catalase positive, beta-lactamase positive; X and V factor negative and urease negative. Some of these tests can help differentiate it from other similar gram-negative organisms as *Haemophilus influenzae* or *Brucella* spp. On chocolate agar, BCYE and Thayer-Martin, *F. tularensis* colonies are gray to white, smooth, raised and moist. *F. novicida* and *F. philomiragia* are cysteine independent and grow more robustly, with a >5 mm on chocolate agar after 24–48 h (WHO, 2007; Versalovic et al., 2011).

Identification of isolates can be achieved using antigen or molecular detection methods, as well as matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS).

Serologic test

Clinically significant antibody titers may be detected on the first week after onset of symptoms and peak at 3–4 weeks. Residual titers might persist for over 10 years. Immunoglobulins, IgM, IgA and IgG may appear at the same time and IgM can last months to

years, so its presence does not always indicate early or recent infection. Various immunoassays have been used for serological diagnosis of tularemia, including microagglutination, ELISA and western blot. These tests have a high specificity but can cross-react with some other microorganisms as *Brucella* spp. (WHO, 2007; Versalovic et al., 2011; Rosenberg et al., 2013b; Maurin and Gyuranecz, 2016).

Antibiotic susceptibility testing and treatment

The Clinical Laboratory Standards Institute (CLSI) establish that the antibiotic susceptibility testing of *Francisella tularensis* strains should be performed using Mueller-Hinton broth enriched with 2% defined growth supplement. The pH must be adjusted to 7.1 ± 0.1 after addition of growth supplement. The inoculum should be calibrated at 5×10^5 CFU/mL of final concentration. Incubation in 5% CO₂ enriched atmosphere may lead to acidification of the medium and overestimation of aminoglycoside and macrolide MICs, or underestimation of tetracyclines MICs (Caspar and Maurin, 2017).

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) does not have antibiotic break-points nor recommendations on antibiotic susceptibility testing for *Francisella* spp.

Several agar media were used for MIC determination using the *E*-test strip method, appearing to be a convenient alternative to the broth microdilution method, however it has not been standardized (Caspar and Maurin, 2017).

Antimicrobials with well-established clinical efficacy include aminoglycosides, tetracyclines, fluoroquinolones and chloramphenicol. Resistance to these drugs has not been reported. Among antibiotic recommended for first-line treatment of tularemia, ciprofloxacin displayed the lowest MICs ranges. The other most active fluoroquinolone is levofloxacin. Streptomycin is the preferred aminoglycoside because its high efficacy, however, gentamicin is more available and has less vestibular toxicity. Chloramphenicol use is restricted to meningitis, because of potential bone marrow toxicity. Beta-lactams have been associated with clinical failure. Two β -lactamase genes (*bla1* and *bla2*) have been found in the Live Vaccine Strain (LVS). A class A β -lactamase (FTU-1) is present in, at least, 14 strains of *F. tularensis* subspecies and corresponds to the *bla2_{LVS}* gene. There has been other β -lactam resistance mechanisms described that conferred resistance to all β -lactams, therefore, these antimicrobials are not recommended to treat tularemia (Penn, n.d.; Versalovic et al., 2011; Caspar and Maurin, 2017).

Antimicrobial susceptibility testing of *F. tularensis* is not usually performed in clinical microbiology laboratories because of safety concerns and because resistance to antibiotics used for tularemia treatment has not been reported (Versalovic et al., 2011).

Brucella

Taxonomy

Brucella genera belong to the *Brucellaceae* family along with other free living soil organisms like *Ochrobactrum* and *Mycoplana* genera. The *Brucellaceae* are part of the order *Rhizobiales* (which includes other genera that causes human disease like *Bartonella* and *Roseomonas*) in the class *Alphaproteobacteria* of the phylum *Proteobacteria*. Other organisms that cause human disease in the *Alphaproteobacteria* class are, for example, the genera *Rickettsia* and *Ehrlichia* (Ficht, 2010; Versalovic et al., 2011).

The different species of *Brucella* show more than 90% similarity at the genome level which leads some authors to propose that *Brucella* are actually one species composed by biovars of *B. melitensis*, but as the distinct species display different host preferences and virulence, separating the genus based on phenotypic characteristics is more compelling (Versalovic et al., 2011; Olsen and Palmer, 2014; Suárez-Esquivel et al., 2020).

As of today, the genus *Brucella* comprises 12 species, nine terrestrial and three marine (Table 1).

Table 1 *Brucella* species and preferred hosts.

	Species name	Biovars	Preferred hosts
Terrestrial	<i>Brucella melitensis</i>	Three	Goats, sheep, camels
	<i>Brucella abortus</i>	Seven	Cattle, bison, buffalo
	<i>Brucella suis</i>	Five	Swine and wild animals
	<i>Brucella canis</i>		Dogs
	<i>Brucella ovis</i>		Rams
	<i>Brucella neotomae</i>		Desert and wood rats
	<i>Brucella papionis</i>		Baboons
	<i>Brucella vulpis</i>		Foxes
	<i>Brucella inopinata</i>		Unknown
	<i>Brucella delphini</i>		Marine mammals
Marine	<i>Brucella pinnipediae</i>		
	<i>Brucella cetaceae</i>		

Versalovic et al. (2011) and Whatmore et al. (2016).

Genus description

Brucella are small (0.5–1.5 µm), nonmotile, facultative intracellular Gram-negative coccobacilli that lack capsules and do not form spores. These bacteria are slow-growing and aerobic, but some prefer CO₂ atmosphere for their growth. *Brucella* species are catalase and oxidase positive, do not ferment sugars and can grow on wide range of culture media. Urease activity differs between species and biovars (*B. suis* and *B. canis* give strong reaction within minutes). *Brucella* spp. may occur either as smooth or rough strains depending on the presence of LPS. Rough strains contain little LPS and are less virulent than smooth strains (Versalovic et al., 2011; Percin, 2013; Castaño et al., 2016; Yagupsky et al., 2020).

Epidemiology

Brucellosis is one of the most important zoonotic diseases and is reemerging in some countries. It is endemic in the Mediterranean basin, Middle East, Central Asia, China, Indian subcontinent, sub-Saharan Africa, Mexico and Central and South America. It causes more than 500,000 infections annually worldwide, mostly in agrarian societies and places where handling of animal products such as dairy products is less strict (Olsen and Palmer, 2014; Al-Nassir, 2017; Bosilkovski, 2020).

Worldwide, *Brucella melitensis* is the most prevalent species causing human brucellosis and it is the most virulent. It is normally associated with infection in sheep and goats, but other species can be infected. In the Middle East, *B. melitensis* infection of cattle is an emergent problem (Corbel, 2006).

Brucella abortus is the most widespread, but associated with much less human disease. Cattle is the most common source, but other animals are an important sources in some areas (Corbel, 2006).

Brucella suis is more restricted but can cause severe human disease. Biovars 1 and 3 are zoonotic; the preferred hosts are domestic pigs, are mainly present in the United States, Australia and China and are highly virulent. Biovar 2 is mainly restricted to hares and wild boars in Europe and Asia, it rarely infects humans. Biovar 4 infects reindeer and caribou and is a zoonotic risk in the Arctic region. Biovar 5 usually infect rodents in Eastern Europe and seems confined to their natural hosts, human infection is rarely reported (Corbel, 2006; Suárez-Esquivel et al., 2020).

Brucella canis is widespread in dogs, but rarely produces disease in humans (Corbel, 2006).

Human brucellosis occurs among the general population in endemic areas, with no differences among sexes nor ages and it is mostly due to ingestion of unpasteurized goat, sheep, cow and camel milk or its products. Human infection can also occur by direct contact with tissues or fluids associated with abortion in infected animals, such exposure is more common seen in non endemic areas and it is occupational related (e.g., veterinarians, abattoir workers, clinical and research microbiology staff, ...). *Brucella* spp. can survive for long periods in dust, water, soil, meat and dairy products. Infection is acquired through direct damage skin contact, inhalation of aerosolized infected particles or mucosal accidental inoculation. Human-to-human transmission of brucellosis has been reported through breast milk and venereal transmission, which is very rare, but blood donation and bone marrow transplant are of more potential significance as a source of infection (Corbel, 2006; Versalovic et al., 2011; Olsen and Palmer, 2014).

Pathogenicity and clinical significance

Brucella spp. enters the host via inhalation, ingestion or through damaged skin. Once the bacteria penetrate the mucosal epithelium they encounter polymorphonuclear and mononuclear phagocytes, then *Brucella* spp. inhibit the phagosome-lysosome fusion escaping from phagocytic killing and evading the immune system. This allows the bacteria to be transported to regional lymph nodes within phagocytes, which will result in lymph node hypertrophy and inflammation. If the local infection it is not resolved rapidly, the bacteria will spread via blood or lymph to other tissues and organs, with tropism for the reticuloendothelial system, where it will replicate, resulting in different clinical phases of the disease (Versalovic et al., 2011; Olsen and Palmer, 2014).

In animals, brucellosis is usually sub-acute or chronic. In sexually mature animals, the infection localizes in the reproductive system causing placentitis in the pregnant female, followed by abortion, and epididymitis and orchitis in the male. Although abortion or premature birth are characteristic, in some areas are relatively uncommon, being hygromas and abscesses the major clinical signs (Corbel, 2006).

The clinical categories of human brucellosis are based on arbitrary criteria and there is not uniform definition. Some authors describe them based on the duration of manifestations: acute (≤ 2 months), subacute (2–12 months) and chronic (≥ 12 months); other based on clinical manifestation: subclinical, localized, chronic and active (Versalovic et al., 2011).

Human brucellosis has an incubation period that varies from 1 to 4 weeks, with an insidious onset on almost half the cases. It presents with a wide spectrum of nonspecific clinical manifestations, such as fever, sweats, arthralgia, myalgia, fatigue, weight loss, hepatomegaly and splenomegaly. Usually, patients feel better in the morning, with symptoms worsening as the day progresses. The pattern of the fever presents with peaks and valleys ("undulant fever") over several days if untreated (Corbel, 2006; Versalovic et al., 2011).

Brucella virulence factors include urease (to avoid stomach stress through oral passage) and vacuoles that enable the escape from immune system recognition and provide acidic environment to hamper antibiotic activity. *Brucella* has several antigens characterized. The LPS is the major one, and dominates antibody response. Based on their O side chain, smooth strains were reported to be composed of two antigenic epitopes (A, in *B. abortus*, and M, in *B. melitensis*). *Brucella* LPS O-polysaccharide appears to be the key for interaction with lipid rafts on host cells and prevents complement-mediated bacterial lysis and host cell apoptosis. It also is a poor

inducer of gamma interferon and tumor necrosis factor alpha, both of which are essential for T-helper 1 mediated immunity. The outer membrane protein 25 (OMP 25) has also been reported as a helper in surviving within mononuclear phagocytes (Versalovic et al., 2011; Olsen and Palmer, 2014).

The most common focal complications are osteoarticular, genital (both males and females), neurological, cardiac, pulmonary and renal. Mortality is very low and is mostly associated to cardiac complications (Versalovic et al., 2011).

Brucellosis during pregnancy carries the risk of spontaneous abortion during the early trimesters or vertical transmission to the fetus. Very rare transmission through breastfeeding has been reported (Corbel, 2006).

- Osteoarticular complications: It occurs mostly as arthritis, up to 70% of cases, and rarely as osteomyelitis, less than 1%. Sacroiliitis is especially common and presents with fever and back pain, often radiating down the legs (sciatica). Other joints commonly affected are, in descending order, knee, hip, vertebra and ankle. It can be misdiagnosed as rheumatoid arthritis, tuberculosis and other entities (Corbel, 2006; Versalovic et al., 2011).
- Gastrointestinal complications: As brucellosis is often foodborne, it could present with nausea, vomiting and abdominal discomfort. It resembles typhoid fever because systemic symptoms predominate over gastrointestinal ones (Corbel, 2006).
- Hepatobiliary complications: *B. abortus* may cause granulomas that are indistinguishable from sarcoidosis lesions. *B. melitensis* causes small foci of inflammation, resembling viral hepatitis. *B. suis* could cause chronic suppurative lesions and hepatic abscesses. Brucellosis may present with acute and chronic cholecystitis (Corbel, 2006).
- Respiratory tract complications: As brucellosis can be acquired through aerosol inhalation, pulmonary complications may be present, such as paratracheal lymphadenopathy, interstitial pneumonitis, bronchopneumonia, lung nodules and empyema. *Brucella* is rarely isolated from expectorated sputum (Corbel, 2006).
- Genitourinary complications: In males are mostly orchitis, usually unilateral, and epididymitis. In females, abortion in the first trimester. Other less common complications are cervicitis, salpingitis and abscesses (Corbel, 2006; Versalovic et al., 2011).
- Neurological complications: Neurobrucellosis can affect both adults and children with diverse symptoms (fever, headache, meningeal signs, coma or paresis). Direct invasion of the central nervous system occurs about 5% of cases of *B. melitensis*. *Brucella* meningitis can be acute or chronic. Analysis of cerebrospinal fluid (CSF) reveals an elevated protein content with normal or low glucose concentration and lymphocytic pleocytosis. The yield of *Brucella* culture from CSF is very low, but specific antibodies can be found in CSF and serum (Corbel, 2006; Versalovic et al., 2011).

Relapse is one of the most important features of brucellosis. Factors associated with it include the use of less effective antibiotic therapy, positive blood culture during the initial presentation and less than 10 days duration of symptoms before treatment initiation (Versalovic et al., 2011).

Diagnosis

Collection and transport of specimens

Collection of specimens of adequate volume should be performed prior to initiation of antimicrobial therapy. Specimens can be sent for culture, serology and molecular testing. Culture can be performed on many different specimens, such as blood, serum, bone marrow, CSF, other sterile body fluids, urine, abscesses and tissues. Specimens should be sent to the laboratory within 2 h, if not, they should be refrigerated. Since brucellosis is highly contagious, specimens from a patient suspected to have brucellosis should be labeled appropriately, specifying that brucellosis should be ruled out (Versalovic et al., 2011).

Direct detection in specimens

There are several gene targets in *Brucella* that can be used for detection using real-time PCR (BCS P31, BP26, 16 rRNA and IS711), but the sensitivities have a great variation and there is no standardization, so their use is relegated mostly for investigation and for reference laboratories (Versalovic et al., 2011).

Culture isolation and identification

Culture is the gold standard for brucellosis diagnosis. *Brucella* is a Class 3 pathogen and specimens should be handled with precaution in a biosafety cabinet (Versalovic et al., 2011; Percin, 2013).

Since human brucellosis always involves a bacteriemic stage, peripheral blood cultures are a suitable tool for microbiological confirmation of diagnosis. Although the sensitivity varies widely due to diverse factors like the *Brucella* species causing the disease, the duration of symptoms, volume of blood collected for culture, number of cultures obtained, blood culture system, incubation period and the performance of subculture (Percin, 2013; Yagupsky et al., 2020).

It usually takes long incubation times due to the slow growing nature of *Brucella* spp. The yield of culture is higher among patients with acute disease and bone marrow cultures demonstrate to have a 20% higher sensitivity than peripheral blood cultures (Versalovic et al., 2011).

Blood cultures using a continuously monitored system such as Bactec (BD Diagnostics, Sparks MD) and BacTAlert (bioMérieux, Durham, NC) show high sensitivities and detect bacterial growth within 1 week. There is no need to incubate bottles longer than 14 days. Subculture should be performed on conventional blood enriched media such as blood agar or chocolate agar, incubated at 36 ± 1 °C in aerobic conditions, but better with a 5% CO₂ enriched atmosphere, there is no need to keep subcultured plates longer than 4 days before discarding them as negative. CSF and other sterile body fluids can be inoculated into blood culture bottles (Versalovic et al., 2011; Percin, 2013; Yagupsky et al., 2020).

Table 2 *Brucella* species phenotypic features.

Brucella species	Growth on dyes			Agglutination in serum			H ₂ S production	Urease activity (time to positivity)	CO ₂ requirement
	Thionin	Basic Fuchsin	Safranin	A (abortus)	M (melitensis)	R (rough)			
<i>B. melitensis</i>	+	+	+	–	+	–	–	+ (24 h)	–
<i>B. abortus</i>	–	+	+	+	–	–	+	+ (24 h)	+/v
<i>B. suis</i>	+	–	–	+	–	–	–	+ (15 min)	+/v
<i>B. neotomae</i>	–	–	ND	+	–	–	+	+ (ND)	–
<i>B. canis</i>	+	–/v	–	–	–	+	–	+ (15 min)	–
<i>B. ovis</i>	+	–	ND	–	–	+	–	–	+

+, positive; –, negative; v, variable (mostly among biovars); ND, no data (Percin, 2013; Castaño et al., 2016; Yagupsky et al., 2020).

For the isolation of *Brucella* from samples contaminated with local microbiota, selective media containing antibiotics such as Thayer-Martin or Martin-Lewis can be used. Specimens inoculated directly onto solid media should be incubated at 36 ± 1 °C in a 5% CO₂ enriched atmosphere, for up to 14 days (Versalovic et al., 2011; Percin, 2013).

Brucella species colonies are usually raised and convex, 1–2 mm in diameter, smooth and glistening after 24–48 h of incubation on blood agar and chocolate agar. Rough variants can occur with *B. canis*. *Brucella* fails to grow on MacConkey agar, what can help for discriminating *Brucella* spp. from *Bordetella* spp. *Brucella* spp. are oxidase and catalase positive, majority of species are urease positive, and all are indole negative and Voges Proskauer negative. Catalase test is particularly dangerous because it is strongly positive in *Brucella* and causes aerosolization of microorganism when bubbling, every test should be performed into a biosafety cabinet. Gram staining demonstrates single, Gram-negative coccobacilli of 0.5–0.7 µm × 0.6–1.5 µm size. For confirmation and identification to the species level, slide agglutination can be performed with monospecific antiserum for *B. abortus* and *B. melitensis*, susceptibility to lysis by different phages and incubation in the presence of different dyes, such as basic fuchsin, thionin or safranin, but PCR remains as the best method to differentiate *Brucella* at the species level. Table 2 shows different phenotypic features of some *Brucella* species (Versalovic et al., 2011; Percin, 2013; Castaño et al., 2016; Bosilkovski, 2020; Yagupsky et al., 2020).

Serologic test

Human immune response to *Brucella* is characterized by an initial production of IgM, followed by appearance of IgG within 10–14 days. The general evolution of these immunoglobulins depends on response to treatment. Persistence of IgG and IgM for months (sometimes years) is observed in about 20% of asymptomatic patients who have undergone treatment and cure (Corbel, 2006; Versalovic et al., 2011).

There is no gold standard in serodiagnostic of brucellosis, consequently these tests must be evaluated by comparing results with other serological assays (Yagupsky et al., 2020).

Several antigens are used for serologic diagnostic assays, LPS is used mainly in ELISAs (Versalovic et al., 2011).

The most common test are serum agglutination test (SAT) and enzyme-linked immunosorbent assays (ELISAs). Rose Bengal agglutination test and immunochromatographic lateral flow assay are commonly used as screening tools due to their short turnaround time. Other test used for confirmation are the 2-mercaptoethanol (2-ME) agglutination test, the immunocapture agglutination test (Brucellacapt) and the indirect Coombs test. ELISA and indirect fluorescent antibody (IFA) tests can differentiate the type of antibody involved, while agglutination-based tests cannot (Bosilkovski, 2020).

- Serum agglutination test (SAT) is a tube agglutination commonly used for diagnosing *B. melitensis*, *B. abortus* and *B. suis* infections. SAT detects antibodies to brucellar smooth LPS, so it is not useful for rough *B. canis*. In general, positive SAT titers consists of >1:160 outside endemic regions and >1:320 within endemic areas. A fourfold or greater increase in titer between acute and convalescent phase serum specimens obtained at least 2 weeks apart is indicative of brucellosis, but this approach may delay therapy (Bosilkovski, 2020; Yagupsky et al., 2020).
- Rose Bengal test (RBT) is an agglutination test that detects agglutinating and non-agglutinating antibodies and does not have the drawback of the prozone phenomenon like the SAT does. The RBT has a high sensitivity (>99%) for all stages of the infection, but it has cross-reactivity with some bacteria. To provide quantitative information, after a positive RBT result, serum may be subjected to twofold dilutions and rested, positive reactions at a $\geq 1:8$ dilution correlated with acute disease and lower titers suggested contact with the organism without clinical disease but requires further investigation (Yagupsky et al., 2020).
- 2-mercaptoethanol (2-ME) agglutination test is performed identically to SAT and is run in parallel. Lately, 2-ME has been replaced by dithiothreitol (DTT) because is less toxic and also inactivates IgM antibodies, eliminating this way the cross-reactivity and the confounding caused by IgM long persistence. This assay is mainly used for serological monitoring of the response to antibiotic treatment. A 2-ME titer of $\leq 1:20$ is considered negative, titers between 1:40 and 1:80 are indicative of active brucellosis in low incidence regions, and titers $\geq 1:80$ are interpreted as diagnostic in endemic areas (Yagupsky et al., 2020).

- Coombs antiglobulin agglutination test consist of serial dilutions of patients sera. This test is useful for confirmation of brucellosis because it can detect incomplete antibodies. It is generally ordered in patients with chronic brucellosis and in relapses (usually with titer of 1:80 or greater). There is a modified Coombs test, the Odak test, that is performed in microcolumns with gel matrix and Coombs antibodies, which is more rapid and simple, but more experience is needed (Yagupsky et al., 2020).
- Complement fixation (CF) test consists in a serial dilution of patient sera, previously inactivated by heat (56 °C, 30–60 min). If IgG1 isotype antibodies are present in patients' sera, they will attach to the antigen, the complement will be activated and no residual complement will remain for lysing the erythrocytes. If no antibodies are present, the hemolysis will occur. The resulting hemoglobin concentration represents an inverse measure of antibody activity in the serum. This test it is used to diagnose brucellosis in livestock, but it is not used in clinical laboratories to diagnose human infection due to its technical complexity and lack of standardization (Yagupsky et al., 2020).
- Immunocapture agglutination test is a single-step assay (Brucellacapt test; Vircell, Santa Fé, Granada, Spain) recently introduced for serodiagnostic of human brucellosis. It operates in acidic medium to avoid the low specificity of IgM, and uses *B. abortus* as antigen. This assay detects agglutinating IgG and IgM, and non-agglutinating antibodies to the three *Brucella* species with smooth LPS strains. In general, the titers obtained with this assay are concordant with those in the Coombs test in the early stages of infection. Titers $\geq 1:640$ are observed in long evolution brucellosis with non-diagnostic SAT titers ($< 1:160$). If the antimicrobial therapy is successful, Brucellacapt titers decrease rapidly (Yagupsky et al., 2020).
- ELISAs can detect different immunoglobulins using LPS or cytosolic proteins as antigen, but it is also used to detect the IgG avidity. It is performed in microtiter plates coated with *Brucella* antigen, filled with serially diluted patients' sera and incubated. For the IgG avidity assay, pairs of wells of a microtiter plate coated with *B. abortus* LPS are incubated with the patients' sera. For each serum, one well is washed with phosphate-buffered saline (PBS) and the other well is washed with highly concentrated urea in PBS. Low-affinity antibodies are removed from the binding sites by the urea, leaving only the high-affinity ones attached to solid phase. Then, the ELISA for IgG is performed. The avidity index (AI) is calculated by dividing the absorbance of urea-treated microwells by the absorbance of untreated wells and multiplying by 100. A high AI suggest immunological memory, thus a advance infection, whereas a low AI is consistent with recent infection (Yagupsky et al., 2020).

Usually, brucellosis diagnosis by serology testing it is done by a combination of two of these tests. This approach also give us an idea of the stage of the disease: in acute disease any of the assays may be positive, but in chronic, complicated or focal disease, SAT may be negative while 2-ME, Brucellacapt, Coombs and ELISA IgG may be positive (Bosilkovski, 2020).

ELISAs measure IgM, IgG, and IgA, and typically use whole cells or purified lipopolysaccharide (LPS), protein extracts as well as other antigens. The sensitivity of ELISA IgM or IgG is lower than SAT (45% and 79% versus 95%, respectively), but if ELISA IgM and IgG are used in combination, the sensitivity and specificity were comparable with SAT (94% and 97%, respectively) (Castaño et al., 2016).

The Brucellacapt and indirect Coombs tests have similar sensitivity and specificity; the Brucellacapt is more rapid and less cumbersome to perform (Castaño et al., 2016).

There are many disadvantages associated with serologic tests, like cross-reactivity with other bacteria, which is a problem especially with standard tube agglutination. Cross-reacting organisms include *Francisella tularensis*, *Yersinia enterocolitica*, *Escherichia coli*, *Salmonella urbana*, *Vibrio cholerae*, and others. False-negative results are common in the early stages of infection, in immunosuppressed patients and in the presence of incomplete or blocking antibodies. In addition, a "prozone" phenomenon (the inhibition of agglutination at low dilutions due to an excess of antibodies or to nonspecific serum factors) may also be observed with serum agglutination testing (Castaño et al., 2016).

The interpretation of serologic tests can be difficult in the setting of chronic infection, reinfection, relapse, and in endemic areas with a high seroprevalence. Positive serologic test results can persist long after recovery in treated individuals, so it is not always possible to distinguish serologically between active and past infection (Castaño et al., 2016).

Antibiotic susceptibility and treatment

Routing susceptibility testing of *Brucella* spp. is not indicated due to poor correlation between *in vitro* activity and clinical efficacy, laboratory safety concerns and the rare development of antibiotic resistance against the main antimicrobials used in its treatment (Versalovic et al., 2011).

Tetracyclines, especially minocycline and doxycycline, are the most effective drugs for brucellosis. Defervescence usually occurs within the first week of treatment, but six o more weeks are required. Because the relapse with tetracycline monotherapy is about 10–20%, most authorities recommend combination with aminoglycosides, rifampicin, trimethoprim-sulfamethoxazole or quinolones. The most effective regimen in adults without complications is combined doxycycline for 45 days with streptomycin for 14 days or gentamicin for 7 days. A good alternative is the combination of doxycycline and rifampicin for 45 days. A secondary alternative therapy is the use of fluoroquinolones or trimethoprim-sulfamethoxazole in combination with other drugs (doxycycline or rifampicin). These antibiotics should be used always in combination therapy due the high rate of relapse when used alone. The duration of therapy must be individualized attending to the complications that may be present, thus neurobrucellosis and spondylitis usually require longer courses of antibiotherapy (Corbel, 2006; Castaño et al., 2016).

Pasteurella

Taxonomy

The genus *Pasteurella* belongs to the *Pasteurellaceae* family. The main specie causing human infection is *Pasteurella multocida*, which is separated into three subspecies: *multocida*, *septic* and *gallicida*. Other species isolated from humans are *P. canis*, *P. dagmatis* and *P. stomatis*. *Pasteurella multocida* has a high genotypical homogeneity, but its phenotypic characteristics allow to differentiate diverse lineages (Versalovic et al., 2011).

Pasteurella multocida strains are classified into five serogroups based on capsule antigens: A, B, D, E and F. Each is generally associated with a specific host, although not completely restricted to them (Ewers et al., 2006; Harper et al., 2006).

Genus description

Pasteurella spp. are small, pleomorphic, nonmotile, non-spore-forming, Gram-negative coccobacilli. *P. multocida* is facultatively anaerobic, grows well on blood agar, chocolate agar, Mueller-Hinton agar and brain-heart infusion (BHI), but it shows no growth on MacConkey agar. It can be seen as a single bacillus, in pairs, in short chains and in clusters. Bipolar staining with Giemsa or methylene blue is frequent (Versalovic et al., 2011; Wilson and Ho, 2013; Mogilner et al., 2019).

Epidemiology

Pasteurella spp. are distributed worldwide. It is part of the normal oral, nasopharyngeal and upper respiratory tract microbiota of fowl and mammals. Dogs and cats have the highest carriage rate of *P. multocida*. It is associated with several distinct disease syndromes. In animals it causes avian cholera, which is a systemic disease that all bird species appear to be susceptible to, hemorrhagic septicemia, atrophic rhinitis, snuffles and pneumonia. Zoonotic transmission to humans is mostly from direct contact with oral or nasal secretions of animals. Generally occur after cat or dog bites or scratches, but also from licking of wounds or injured skin (Wilkie et al., 2012; Wilson and Ho, 2013; Mogilner et al., 2019; Weber and Kaplan, 2019).

Pathogenicity and clinical significance

Many virulence factors are described in *P. multocida* such as capsule, LPS, adhesins (fimbria), toxins (PMT), iron-regulated and iron acquisition proteins and several membrane proteins (OMPs) (Peng et al., 2019).

Pasteurella multocida is associated with several distinct disease syndromes in wild and domestic animals.

- Avian cholera: it is caused largely by strains of capsular type A, but other capsular types are occasionally incriminated, like type F in turkeys. *P. multocida* will colonize the mucosa of the upper respiratory tract and spread to the air sacs and lungs. It also can enter the circulation directly from the mucosa. The clinical course ranges from a few hours to several days. Morbidity and mortality vary with the virulence of the strain involved (Wilkie et al., 2012).
- Hemorrhagic septicemia: it affects ungulates mostly. It is a systemic disease caused by serogroup B in Asia and Europe, and by serogroup E in Africa. These strains can be isolated from tonsils of healthy animals that can just carry and shed without getting sick, or may develop fulminating hemorrhagic septicemia. Bacteria multiply in peritonsillar soft tissue and will be carried by macrophages to local lymph nodes from where they will spread. Mortality rate is close to 100% without prompt antibiotic treatment (Wilkie et al., 2012).
- Atrophic rhinitis: it affects domesticated dogs and housed rabbits, mostly. It is associated with a dermonecrotxin (PMT: *Pasteurella multocida* toxin), which is produced by capsular type D mostly, but other serogroups may produce it as well. After bacteria colonize the upper respiratory tract mucosa, without the necessity of invasion, it will produce PMT. This toxin will cause osteopaenia and destruction of the local bones (Wilkie et al., 2012).

In humans, opportunistic infections of soft tissue are relatively common. Severe infections can occur in previously healthy individuals. However, immunocompromised host are at particular risk (Wilkie et al., 2012; Weber and Kaplan, 2019).

Human infections with *P. multocida* can be divided into three categories:

1. Local infection after animal bites or scratches. This includes cellulitis, abscesses, necrotizing soft tissue infection, septic arthritis and osteomyelitis.
 - Soft tissue infections usually occur after cat bites or scratches, or dog bites, but also can occur after cat or dog licks of damaged skin. The incubation period for bite wound infections with *P. multocida* is usually less than 24 h, and symptoms often progress rapidly. The most common symptoms include wound site swelling, erythema, tenderness and discharge, but fever, chills and lymphadenopathy may be present as well. Most common complications include abscesses, osteomyelitis, tenosynovitis and septic arthritis (Mogilner et al., 2019).
 - Bone and joint infections occur after a cat or dog bite distal to the involve joint. Most commonly involves a single joint, usually the knee, with a predilection for prosthetic joints or joints previously damaged by another disease (Weber and Kaplan, 2019).

2. Respiratory infections. They usually occur in patients with underlying lung disease (COPD, bronchitis, ...). Includes glossitis, pharyngitis, sinusitis, otitis media, tracheobronchitis, pneumonia, empyema and lung abscess. Pneumonia is the most common respiratory infection caused by *P. multocida*. The clinical course is nonspecific and common symptoms include fever, malaise, dyspnea and chest pain (Weber and Kaplan, 2019).
3. Systematic infections. Often unrelated to animal bites. Include meningitis, intraabdominal infection (peritonitis and appendicitis), endocarditis or ocular infection. Sepsis and septic shock present with purpura fulminans (Weber and Kaplan, 2019).

Diagnosis

Collection and transport of specimens

Specimens from skin and mucosal infections should be collected with a swab and placed into transport media. Specimens from joint infection should be collected into an empty, sterile container, as well as respiratory tract samples, such sputum (Jorgensen et al., 2015).

Samples must be delivered to the laboratory within 2 h. If the deliver to the laboratory will be delayed, samples must be keep refrigerated (Jorgensen et al., 2015).

Direct detection in specimens

For direct detection, Gram staining can be performed on the specimens. *Pasteurella* spp. are small, Gram-negative rods or cocci that may appear singly, in pairs or in short chains. Bipolar staining is frequent (Jorgensen et al., 2015).

Culture isolation and identification

Pasteurella spp. grows well on sheep blood agar and does not require hemin nor CO₂, so it will grow on media without blood. The majority of the species are oxidase, catalase and indole positive; urease negative (except *P. dagmatis*) and they do not grow on MacConkey agar. For separation of the three subspecies of *P. multocida* sorbitol/dulcitol fermentation must be performed (Jorgensen et al., 2015) (Table 3):

Antibiotic susceptibility and treatment

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has published breakpoints for antimicrobial dilution and disk susceptibility testing. According to their guidelines, broth microdilution testing should be performed on Mueller-Hinton broth +5% lysed horse blood and 20 mg/L β-NAD (MH-F broth); and for disk diffusion method, Mueller-Hinton agar +5% defibrinated horse blood and 20 mg/L β-NAD (MH-F) should be used. In both cases the incubation must be at 35 ± 1 °C for 18 ± 2 h. For disk diffusion method, a 5% CO₂ enriched atmosphere is preferable (The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 11.0, 2021, available at https://www.eucast.org/clinical_breakpoints/).

Pasteurella spp. are usually susceptible to penicillins, third-generation cephalosporins, fluoroquinolones and trimethoprim-sulfamethoxazole. Recommendations on antibiotic therapy are based on experience. For monomicrobial infections, penicillin is the drug of choice, although rare beta-lactamase-producing strains have been isolated. It is usually resistant to clindamycin, erythromycin and aminoglycosides. Trimethoprim-sulfamethoxazole may be used in patients with allergy to penicillin. Local infections are usually treated for 10–14 days. Bone and joint infections require longer duration of treatment, around 4–6 weeks (Mogilner et al., 2019; Weber and Kaplan, 2019).

Table 3 *Pasteurella multocida* subspecies separation based on sugar fermentation.

<i>P. multocida</i> subspecies	Sorbitol fermentation	Dulcitol fermentation
<i>multocida</i>	+	–
<i>septica</i>	–	–
<i>gallicida</i>	–	+

+, positive; –, negative (Jorgensen et al., 2015).

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Relevant Websites

<https://eucast.org/>—European Committee on Antimicrobial Susceptibility Testing.
<https://www.bacterio.net/>—List of Prokaryotic names with standing nomenclature.

Campylobacter* and *Helicobacter

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Campylobacter

Introduction

Campylobacter spp., and particularly *C. jejuni*, is one of the most frequent zoonotic pathogens foodborne and its incidence is increasing in recent years throughout the world (Elhadidy et al., 2018).

It is found commensally in the gastrointestinal tract of wild and domestic animals. Transmission to humans is by the fecal-oral route through the ingestion of undercooked food (especially poultry), unpasteurized milk or contaminated water (Little et al., 2010).

The symptoms that it mainly produces are self-limited diarrhea but can cause severe dysentery and systemic manifestations, especially in immunosuppressed patients, including post infectious complications such as Guillain-Barré syndrome (Elhadidy et al., 2018).

In recent years, an increase in strains resistant to several selected antibiotics of choice has been observed, especially to fluoroquinolones, which highlights the importance of preventing this increasingly frequent frequency (Elhadidy et al., 2020).

Epidemiology

Campylobacter spp. is one of the leading causes of foodborne illness, causing acute bacterial enteritis worldwide. The first infection recognized by *Campylobacter* was at the beginning of the 20th century, where a microorganism with a curved morphology that caused abortions in cattle was observed, first being called *Vibrio fetus* and in the 1970s reclassified as *Campylobacter fetus*. But it was not until the 1980s that its importance as a human pathogen was highlighted thanks to the development of selective media for its isolation (Costa and Iraola, 2019).

It is the most frequent foodborne illness in Europe since 2005 and the second cause in the United States, where the main cause is *Salmonella* spp. (Facciola et al., 2017). The main food source for *Campylobacter* infection is the consumption of undercooked poultry (mainly liver pate) or other cross-contaminated foods, on the farm or during cooking in the kitchen (Little et al., 2010). To a lesser extent, infection can occur through the consumption of unpasteurized milk or contaminated water with excrements of infected animals, since it is capable of surviving on these foods at 4 °C for several weeks. To eliminate it effectively, the liver must be cooked at high temperatures, the water disinfected with chlorine and the milk pasteurized (Hope et al., 2014).

Campylobacteriosis is a worldwide zoonosis found as a commensal in the gastrointestinal tract of domestic or wild cattle. The reservoir of *C. jejuni* is varied, with poultry being the main source of infection. *C. coli* are isolated mainly from pigs, *C. upsaliensis* from dogs and cats, and *C. fetus* from sheep, cattle, poultry, pigs and reptiles (Labarca et al., 2002).

Diarrhea affects all age groups but with a higher incidence in the first 5 years of life according to a study carried out in the United States. In children under 5 years of age *Campylobacter* was the second cause of diarrhea after non-typhoid *Salmonella* (Scallan et al., 2013). According to a cohort study, this incidence is higher in the first 2 years of life, placing *Campylobacter* spp. among the six most frequent microorganisms that cause diarrhea, along with rotavirus, norovirus, astrovirus, *Shigella* spp. and *Cryptosporidium* spp. (Platts-Mills et al., 2015).

Campylobacter infections are usually sporadic and are more frequent in summer and autumn and may be due to increased air temperature and the presence of a greater number of flies, considered possible vectors of transmission (Nichols, 2005).

There are epidemiological differences in different countries, since campylobacteriosis is endemic in developing countries, affecting up to 40% of children with or without symptoms (Buss et al., 2019). In these countries, person-to-person transmission seems to be higher than in developed countries, since there is greater contact with animal vectors of the infection, as occurs in rural areas of developed countries where the incidence is also higher (Shah et al., 2009).

Due to the high incidence of diseases caused by *Campylobacter*, its low infectious dose in humans ($5-8 \times 10^2$ cells/mL) and its potentially serious sequelae. It should be considered as a significant risk to public health (Moore et al., 2005).

Microbiology

Campylobacter spp. belongs to the Campylobacteraceae family and is composed of 32 species and 9 subspecies (Costa and Iraola, 2019). *C. jejuni* and *C. coli* are the species that most frequently cause pathology in humans and represent 90% of infections, and less frequently *C. fetus* (Elhadidy et al., 2020).

They are Gram-negative rods, curved (comma-shaped) and mobile, due to the presence of one or two polar flagella. They are small in size, 0.5–5 µm long and 0.2–0.5 µm wide. They are non-spore forming; they react positively to oxidase and variably to catalase. More than 90 different serotypes have been described based on somatic antigens (O) and thermolabile antigens (flagellar and capsular).

It grows easily in microaerophilic conditions (5–10% oxygen) and its optimal growth temperature is wide, from 25 °C to 42 °C. Although they tend to grow well at 37 °C, incubation at 42 °C allows to specifically recovering those thermophilic species responsible for most cases of enteritis, such as *C. jejuni* and *C. coli*. Another characteristic that differentiates *C. jejuni* from the rest of the species of the genus is the hydrolysis capacity of hippurate, although 5–10% of the strains do not present this characteristic (Snelling et al., 2005).

Pathogenesis

Campylobacter is able to invade the gastrointestinal epithelial barrier due to the presence of multiple adhesins responsible for binding to fibronectin and the production of the cytotoxic strain toxin. This toxin has DNase activity that causes DNA damage and is responsible for invading and altering the density of endocrine cells in the colon and even reaching the rectum, which can lead to post infectious irritable bowel syndrome (El-Salhy et al., 2013). Another important virulence factor that allows it to cross this epithelial barrier is its motility, due to the presence of flagella, and its characteristic corkscrew shape (Fouts et al., 2005). These virulence factors along with a low infective dose are responsible for the clinical symptoms.

In cases where there is a prolonged excretion of *Campylobacter*, as in immunosuppressed patients, there is an alteration of the integrity of the epithelium that leads to permanent damage to the mucosa and subsequent complications (El-Salhy et al., 2013).

The main virulence factor of *C. fetus*, responsible for systemic manifestations, is the presence of a protein in its surface layer that acts as a capsule. This prevents their phagocytosis and facilitates extra intestinal spread, mainly in immunosuppressed patients (Dworkin and Blaser, 1997).

Clinical manifestations

The genus *Campylobacter* most frequently produces intestinal diseases, especially *C. jejuni*, followed by *C. coli*. It can also produce extra intestinal manifestations, especially in immunosuppressed patients, produced in most cases by *C. fetus*.

Campylobacter jejuni infection

The main manifestation produced by *C. jejuni* is acute enteritis. It mainly affects children under 5 years of age and young adults, although it can occur at any age. The incubation period is 2–5 days, in which the patient has watery diarrhea, abdominal pain and/or fever, most of the time being a mild and self-limited condition that resolves in a week. *Campylobacter* is one of the main causes of traveler's diarrhea, especially in Southeast Asia. In people with previous pathologies or in immunosuppressed people it can produce a more serious picture of dysentery, with fever and blood in the stool, and even later complications (Ternhag et al., 2007).

Extraintestinal manifestations occur exceptionally and more frequently in immunosuppressed patients, especially with immunoglobulin or HIV deficiencies, where infections are more severe, prolonged and recurrent. Bacteremia is observed in less than 1% of patients, being normally transient or with good evolution in immunocompetent patients (Nielsen et al., 2010).

It has been related, mainly in developing countries, with malnutrition in young children, even asymptomatic, since in children under 2 years old it usually produces dysentery. This highlights the importance of its early detection and subsequent treatment (Lee et al., 2013a,b).

The complications that can occur, especially in immunosuppressed patients, are:

- Guillain-Barré syndrome (GBS): it is an autoimmune disease of the peripheral nerves that occurs because *C. jejuni* lipooligosaccharides can induce a cross-reactive immune response with nerve gangliosides due to molecular mimicry. 20–50% of GBS cases are preceded by a *C. jejuni* infection (Heikema et al., 2013).
- Post-infectious irritable bowel syndrome: this condition is characterized by intestinal disorders and chronic abdominal pain that can persist up to 10 years after the episode of acute enteritis. It can be caused by *Campylobacter* or other enteropathogens (Dai et al., 2020).
- Reactive arthritis: it is a sterile joint inflammation that can occur after a gastrointestinal infection, being *C. jejuni* the most common bacterial enteropathogen. Its association with the presence of HLA-B27 histocompatibility antigens is not yet clear (Hannu et al., 2004).

Campylobacter fetus infection

Infection with *C. fetus* is rare but can cause serious symptoms due to its ability to spread extraintestinally, especially in immunosuppressed patients.

C. fetus can produce local symptoms of gastrointestinal involvement, although much less frequently than *C. jejuni* and most of the time it is responsible for invasive conditions such as bacteremia, pneumonia and meningoenitis. It seems to have a predilection for endovascular tissue, as it can lead to serious conditions such as endocarditis or fungal aneurysms (Haruyama et al., 2011).

It mainly affects adults with previous diseases or immunosuppressed; and in patients with immunoglobulin deficiencies it can cause recurrent infections difficult to treat, such as persistent bacteremia and other local symptoms (septic arthritis, cellulitis or osteomyelitis) (Yamagami et al., 2014).

Infection with other *Campylobacter* species

The clinical manifestations of *C. coli* are similar to those produced by *C. jejuni*, although it appears to be somewhat milder.

C. upsaliensis, like *C. fetus*, produces diarrhea in immunocompetent patients and bacteremia in immunosuppressed patients, although it is believed to be underdiagnosed since it cannot be detected with the incubation conditions of the stool culture media used for the diagnosis of the rest of *Campylobacter*, being necessary its incubation at 37 °C. It is believed that it is transmitted through contact with feces of domestic pets or food contaminated with them (Nakamura et al., 2015).

Diagnosis

The microbiological diagnosis of diarrhea is not usually necessary in most patients because it is often a mild and self-limited condition. On the opposite, in the case of bloody stools, fever or prolonged diarrhea, a stool sample should be taken for culture and subsequent identification of the most frequent pathogens that cause diarrhea, among which is *Campylobacter*.

For the identification of thermophilic species, which are responsible for acute diarrhea, selective culture media with blood and antibiotics (Skirrow medium, Butzler agar and Campy-BAP medium) are used, which inhibit the growth of other species of *Campylobacter*. In cases in which diarrhea due to *C. fetus* or other atypical species is suspected, the plates should be incubated at 37 °C. It can also be isolated from blood cultures, although it may take up to 14 days to grow, so if it does not exist clinical suspicion

will not be possible a correct identification. The drawback of cultivation is that it is a slow method, it takes 48–72 h to grow and requires special media for its growth. It also has lower sensitivity than molecular methods, approximately 70%, which may be due to the loss of viability when kept refrigerated until culture or to the low bacterial load of some samples (Buss et al., 2019).

The use of mass spectrometry (MALDI-TOF) allows an excellent and rapid identification of *Campylobacter* species and is used as confirmation against a colony that grows under microaerophilic conditions and that presents the characteristic morphology in the Gram stain.

In recent years, rapid diagnostic techniques such as immunochromatography tests are being used that allow a diagnosis in less than 30 min with good sensitivity and specificity. They are very useful to establish an adequate treatment earlier and to detect cases with a low bacterial load that cannot be recovered by culture, but they do not detect all pathogenic *Campylobacter* species and are less sensitive than culture (Franco et al., 2021; Balsalobre-Arenas and Alarcón-Cavero, 2017).

Molecular biology techniques increasingly used in laboratories are more sensitive and faster than culture and are usually part of a panel of enteropathogens that allows the screening of the main gastrointestinal infections in a single step. Their main drawback is that they need specific automation, trained personnel, and they must be interpreted with caution as they can detect non-viable organisms. These techniques have the disadvantage that they do not allow us to know the resistance to antibiotics, this being fundamental due to its current increase, so it is always necessary to also perform the culture considered the gold standard technique for the identification of *Campylobacter* infection (Poppert et al., 2008).

Treatment

Most of the patients present a mild and self-limited condition, being only necessary support treatment for the replacement of fluids and electrolytes and thus avoid dehydration (Snelling et al., 2005).

The use of antibiotics for the treatment of diarrhea due to *Campylobacter* is only beneficial in those patients at risk such as the immunosuppressed, the extreme ages of life, severe diarrhea (more than eight stools a day) or of prolonged evolution. In these situations, complications and systemic manifestations can also occur, so the use of antibiotics in these patients reduces the elimination of bacteria through the feces and the duration of the symptoms, especially if they are administered early (González-Abad and Alonso-Sanz, 2013). The difficulty lies in knowing in which patients should be administered antibiotics and in which not, since the ideal is that these are given early but in most cases it will not be necessary because it is a self-limiting disease, which leads to an increase in resistors (Ternhag et al., 2007).

The antibiotics of choice include macrolides, fluoroquinolones and aminoglycosides, but fluoroquinolone-resistant strains of *C. jejuni* have emerged since the 1990s. This is related to its indiscriminate use and greater administration at the veterinary level, mainly in poultry (Dai et al., 2020). These resistances are largely due to mutations in the topoisomerase gene (*gyrA*), which confers high-level resistance to nalidixic acid and ciprofloxacin (Elhadidy et al., 2018). Several studies report levels of resistance to ciprofloxacin higher than 80% in countries such as Spain and Thailand, and this resistance is spreading to other parts of the world, which is why its use as an empirical treatment is discouraged (Mason et al., 2017).

In countries with a high prevalence of resistance to fluoroquinolones, the first line treatment is macrolides, although resistance to these drugs is being observed in developing countries due to ribosomal mutations or through the efflux pump. To this day, the incidence of macrolide resistance in Europe and the United States remains low (Dai et al., 2020). Thus, azithromycin is the treatment of choice for travelers, especially to areas of Southeast Asia, since it is also effective against other bacteria that cause traveler's diarrhea (Mason et al., 2017).

For the treatment of systemic infection, the antibiotics of choice are intravenous carbapenems and aminoglycosides for at least 2 weeks. It is not advisable to administer fluoroquinolones due to the increase in resistance observed or 3rd generation cephalosporins, since they present resistance of up to 20% (Yamagami et al., 2014).

Furthermore, it has been observed in recent studies that patients infected by these resistant strains present more severe and longer-lasting symptoms, a fact that highlights the importance of continuing to study the molecular mechanisms responsible for resistance in *Campylobacter* (Elhadidy et al., 2020). For these cases of multidrug resistant *Campylobacter*, the association of fosfomicin-tromethamine is proposed as an alternative therapy with good results (Aguilar-Company et al., 2016).

Prevention

There are regulations in the food sector aimed at the prevention of food-borne illnesses, including *Campylobacter* infection. These controls are based on improving slaughter hygiene, especially poultry, and reducing microbial contamination by establishing limit values for microorganisms such as *Campylobacter*.

The population should also be informed of the measures to be taken when handling food for consumption, including thorough cooking of the meat and good cleaning of both the kitchen equipment and the handler's hands.

Currently, the development of an effective vaccine for *Campylobacter* infection is still in the research phase, this difficulty is due to the fact that it presents a great antigenic diversity (Kreling et al., 2020).

Helicobacter

Introduction

Helicobacter pylori (*H. pylori*) is a highly mobile, curved Gram-negative rod that colonizes the human gastroduodenal mucosa of approximately half the world's population (Gravina et al., 2018). Most people have no symptoms, but their presence is associated with an increased risk of peptic ulcer and gastric carcinoma (Chmiela and Kupcinskas, 2019).

In recent years the prevalence of *H. pylori* is decreasing in developed countries while it remains high in developing countries related to low socioeconomic status.

H. pylori eradication therapy is one of the pillars of the strategy to eliminate gastric cancer and peptic ulcer, but in recent years an increase in antibiotic resistance has been observed, which has resulted in therapeutic failures. Hence the importance of detecting resistance before starting treatment thanks to the use of new diagnostic techniques (Pohl et al., 2019).

Epidemiology

H. pylori is normally acquired during childhood and can last for a lifetime (Eed et al., 2019). In developing countries more than 70% of children older than 10 years are carriers. Transmission of the infection occurs by the fecal-oral route, through contaminated food and water; and to a lesser extent it can also be transmitted by oral-oral route since it has been isolated in dental plaque and saliva (Gravina et al., 2018).

The global prevalence of *H. pylori* varies between different regions and countries, being highest in Africa, followed by Latin America and Asia; and it is lowest in North America and Oceania. *H. pylori* infect approximately half of the world's population.

The prevalence of *H. pylori* colonization varies according to the socioeconomic development of the country and according to age. Since the 21st century, the incidence has been decreasing in developed countries due to improvements in sanitation, access to drinking water... and even within the same country there are differences between regions, being greater in the most disadvantaged (Hooi et al., 2017).

Microbiology

Within the genus *Helicobacter* there are several species that cause pathology in humans, such as *H. fennelliae* and *H. cinaedi*, although the most important species is *H. pylori* and it is the one that we will talk about in this topic.

Due to its typical S-shaped morphology, this microorganism was published in the 1980s, known as *Campylobacter pylori* or *pyloridis* and later classified as *H. pylori* (Serena et al., 2018).

H. pylori are a small, microaerophilic, curved (corkscrew-shaped) Gram-negative rod (0.5–1.0 µm wide and 2.5–4 µm long). It is mobile thanks to the presence of 4–8 polar flagella that allow it to reach the protective mucus layer of the stomach and are covered by a lipid structure that is believed to be responsible for its protection from the acidic environment of the stomach. Its reaction to oxidase and catalase is positive, and its biochemical characteristics include the presence of a urease with a more powerful action than that of other bacteria (Frydman et al., 2015).

Pathogenesis

H. pylori are able to survive in the acidic environment of the stomach thanks to its curved shape and the presence of flagella that allow it to move through the mucous layer of the stomach. In addition, the activity of urease allows it to neutralize stomach acid by converting urea from gastric juices into ammonia and carbon dioxide (Serena et al., 2018).

H. pylori colonize the gastric mucosa thanks to the presence of adhesins that interact with the mucin and produce an increase in it, which facilitates bacterial adherence. *H. pylori* activates gastric epithelial cells that produce pro-inflammatory cytokines and activate polymorphonuclear and mononuclear cells, triggering an inflammatory response and a decrease in mucus in the epithelial glands (Tham et al., 2001).

Virulence factors include a cytotoxin-associated protein (CagA) produced by some strains of *H. pylori*, which has demonstrated its oncogenic capacity by producing a massive vacuolation of gastric epithelial cells, playing a key role in the development of gastric cancer (Serena et al., 2018). Another pore-forming protein (VacA) is also produced whose toxicity is increased by the ammonia produced by urease (Chmiela and Kupcinskas, 2019).

H. pylori produces an increase in gastrin-producing cells and acid-producing parietal cells, this being responsible for the long-term chronic active gastritis that can progress to premalignant stages of atrophic gastritis, intestinal metaplasia and dysplasia up to get to gastric cancer (Lu and Li, 2014).

Clinical manifestations

The presence of *H. pylori* is related to various gastric pathologies, although most of the time it is asymptomatic, being the most frequent cause of chronic gastritis and peptic ulcer, and less frequently it can produce systemic manifestations. Its presence has also been observed to protect against other diseases (Vaezi et al., 2000).

Gastritis

Exposure to *H. pylori* can cause acute gastrointestinal symptoms or be asymptomatic. Usually, this colonization will remain for years and in many cases without symptoms and in some people it will cause symptoms of gastritis or non-ulcer dyspepsia, being responsible for 10% of these cases.

Duodenal ulcer

H. pylori is found in more than 90% of patients with idiopathic duodenal ulcer, which is one not related to the use of non-steroidal anti-inflammatory drugs or Zollinger-Elison syndrome. The risk of developing an ulcer is even higher in patients with CagA-producing strains of *H. pylori*.

A causal association between *H. pylori* and duodenal ulcer formation has not been demonstrated, although a reduction in duodenal ulcer recurrence rates has been observed when *H. pylori* eradication therapy is administered in conjunction with antacids. In contrast, recent studies have shown that the incidence of reflux esophagitis increases after *H. pylori* eradication, demonstrating that this eradication therapy has both benefits and harms (Lee et al., 2013a,b).

Gastric ulcer

The proportion of patients with gastric ulcers colonized by *H. pylori* is lower than in the case of duodenal ulcers (50–80%), due to the more frequent association of gastric ulcer with the use of non-steroidal anti-inflammatory drugs. The presence of gastric ulcers, especially in the area of the body of the stomach, is associated with the development of diffuse gastric cancer. This is because *H. pylori* are able to decrease gastric mucosal bicarbonate secretion by reducing two cellular bicarbonate transporters, thereby removing its barrier against stomach acid damage. *H. pylori* eradication therapy in these patients is associated with the non-development of cancer (Chmiela and Kupcinskas, 2019).

Gastric cancer

Gastric cancer is the third leading cause of cancer death and the fourth most common cancer worldwide. About half of gastric cancers are diagnosed in Asia, mainly China, and it is more common in men.

In approximately 89% of cases of gastric cancer, not heart cancer, *H. pylori* is involved, considered a necessary but not sufficient cause for cancer to develop (Hooi et al., 2017). Among the risk factors for developing gastric cancer is host, environmental and bacterial factors, such as the expression of the CagA protein. It is also related to dietary factors, such as a diet low in fruits and vegetables and rich in salt (Ang and Fock, 2014).

Gastric cancer of the intestinal type and diffuse type has been shown to be closely related to the presence of intestinal metaplasia and atrophic gastritis, and these are in turn associated with persistent *H. pylori* infection. Furthermore, worldwide, a correlation is observed between areas with high rates of *H. pylori* infection and high rates of cancer of the antrum and the gastric body (Lee et al., 2016).

H. pylori eradication therapy has been shown to stop the progression of gastric mucosal injury and restore the microbiome, resulting in a decrease in the incidence of gastric cancer. This reduction occurs when there are premalignant lesions, such as atrophic gastritis, but not if there is already irreversible intestinal metaplasia (Lee et al., 2013a,b). This protective effect is greater in those with a higher risk of initial gastric cancer, so *H. pylori* eradication therapy is considered the main gastric cancer prevention strategy (Lee et al., 2016).

MALT type gastric lymphoma

Most gastric lymphomas originate from B lymphocytes and are called mucosa-associated lymphoid tissue (MALT) lymphomas. These lymphomas are associated with chronic *H. pylori* infection and are believed to be due to antigenic stimulation and subsequent polyclonal lymphoid response. Complete remissions of these lymphomas have been described after *H. pylori* eradication therapy in both immunocompetent and immunosuppressed patients (Verduzco-Rodriguez et al., 2017).

Extra-digestive manifestations

H. pylori has also been linked to extradigestive diseases including vitamin deficiencies, such as vitamin B12, childhood asthma and other related diseases, and hematological diseases such as idiopathic thrombocytopenic purpura (ITP). In recent decades, an association has been observed between the presence of *H. pylori* and ITP. The exact cause is not known but it appears to be because the CagA protein stimulates anti-CagA antibodies that are cross-reactive with platelet surface antigens, leading to platelet aggregation. Currently, patients with ITP are evaluated for the presence of *H. pylori* and if it is positive, eradication therapy is recommended in highly endemic areas (Frydman et al., 2015).

In recent years, an increase has been observed in a series of pathologies related to the decrease in colonization by *H. pylori* due to eradication therapy. These include pathologies of the esophagus, such as gastroesophageal reflux disease, Barrett's esophagus and

adenocarcinoma of the esophagus. These diseases have increased since *H. pylori* eradication therapy has been introduced. An inverse correlation has been observed between the presence of CagA positive *H. pylori* strains and esophageal diseases, so that these strains could act as a protective factor against these diseases due to the decrease in stomach acid, among other mechanisms (Vaezi et al., 2000).

Diagnosis

For the diagnosis of *H. pylori* infection, two types of techniques can be performed, invasive or non-invasive, with their respective advantages, disadvantages and limitations. Nowadays, the diagnosis should only be made in symptomatic cases, and it is not routinely indicated (Pohl et al., 2019).

Invasive techniques

Invasive testing is performed using endoscopically obtained gastric biopsy specimens, including histology, culture, rapid urease test and molecular techniques. Endoscopy has the advantage that other lesions such as masses and strictures can also be evaluated. Due to the irregular distribution of *H. pylori* in the stomach, it is recommended to take two samples from the antrum and another two from the greater curvature of the gastric body, since in this way fewer false negative results are obtained.

Histology

Histology was the first method used for the diagnosis of *H. pylori* infection. Different stains are used, such as Giemsa or silver staining, which together with the presence of bacteria with a typical morphology and the inflammatory reaction of the tissue, the diagnosis is made. It presents a series of limitations that reduce its precision, such as the size and number of biopsies, the experience of the pathologist, the staining methods or the use of proton pump inhibitors (it is recommended not to consume these drugs 2 weeks before endoscopy to avoid false negative results). In recent years, specificity has been improved with non-morphology-based techniques, such as fluorescent nucleic peptide in situ hybridization (PNA-FISH). It is also a useful method to detect clarithromycin resistance, although it requires experienced personnel and a fluorescence microscope (Wang et al., 2015).

Culture

The samples are cultured in selective media, such as Skirrow medium or Columbia Agar base broth or Brain Heart Infusion (BHI) supplemented with blood agar, for 5–7 days at 37 °C in a humid microaerobic atmosphere (5% oxygen). The growing colonies are confirmed with a Gram stain to observe their characteristic shape and the positivity of the oxidase, catalase and urease tests. Culture has the advantage that it allows the study of sensitivity to antibiotics, which is very important due to the current increase in resistance, but has the disadvantage that it is a slow method and requires special media and conditions, as it is a bacterium demanding.

Rapid urease test

This test is performed by inoculating the biopsy in urea broth and observing the color changes to pink after a few minutes, due to the activity of its powerful urease (Balsalobre-Arenas and Alarcón-Cavero, 2017). It has a sensitivity of 60% that increases to more than 90% after 24 h of incubation. It has the advantage of being quick and easy to perform, but the disadvantage of low sensitivity if there is a low bacterial concentration, since a minimum of 10^4 microorganisms is needed per biopsy piece (Patel et al., 2014).

Molecular methods

The real-time polymerase chain reaction (PCR) is used both for the identification of the bacteria and for the detection of mutations related to antibiotic resistance in samples such as gastric biopsy, gastric juice, feces or saliva. Resistance to macrolides such as clarithromycin (23S rRNA gene), fluoroquinolones (gyrA gene), tetracyclines (16S rRNA gene), rifabutin (rpoB gene), and amoxicillin (pbp-1a gene) can be detected. These techniques have the advantage of being faster and more sensitive than culture, and they are also capable of detecting heterogeneous resistance.

Non-invasive techniques

Serology

There are several techniques that detect antibodies anti-*H. pylori* IgG in the patient's serum, but its main limitation is that it does not differentiate between active and past infection. It is a useful test in epidemiological studies and in those situations with a low bacterial load in the stomach, where the rest of the tests will show a decrease in sensitivity.

Carbon-13 (13C) urea breath test

It consists of administering a food with ^{13}C or ^{14}C -labeled urea and at the hour the amount of CO_2 marked in the breath is measured. This CO_2 is released when urea is hydrolyzed by the *H. pylori* enzyme urease (Balsalobre-Arenas and Alarcón-Cavero, 2017). It has a sensitivity and specificity greater than 90%. It is used to evaluate the efficacy of *H. pylori* eradication therapy and false positives may occur as there are other urease-producing bacteria in the stomach (Patel et al., 2014).

Stool antigen test

It is a test that is usually used for the initial diagnosis of *H. pylori* infection in symptomatic patients, being very common in Microbiology laboratories. It is also used for monitoring treatment (4 weeks later) and for epidemiological studies. False negatives can occur with the presence of some preservatives in the stool such as formaldehyde (Wang et al., 2015; Balsalobre-Arenas and Alarcón-Cavero, 2017).

Treatment

H. pylori eradication therapy is one of the pillars for the prevention of peptic ulcer and gastric cancer. Today the World Health Organization (WHO) has established that all *H. pylori* infected patients should be treated regardless of whether or not they present symptoms, since it is the best strategy to prevent gastric cancer (Serena et al., 2018). If the treatment is carried out correctly, it has a high efficacy rate with a very low probability of reinfection (2%), but its efficacy is decreasing due to the increase in resistance to 1st-line antibiotics (Pohl et al., 2019). The treatment consists of a combination of antibiotic and antisecretory agents, like as proton pump inhibitors (PPIs), which increase the gastric pH necessary for the bactericidal effect of antibiotics. The antibiotics included in eradication therapy are amoxicillin, clarithromycin, fluoroquinolones, metronidazole and tetracycline. For the therapy to be effective, a combination of two or more antibiotics are required due to increased resistance, known as standard triple therapy that includes two antibiotics (clarithromycin and amoxicillin/metronidazole) and a PPI. This therapy has been considered 1st line but in recent years an eradication rate of less than 80% has been observed in several areas due to the increase in antibiotic resistance, which makes it necessary to use quadruple or bismuth salt therapies for 14 days, with cure rates greater than 90% (Yan et al., 2014).

Genes for resistance to clarithromycin, tetracycline, fluoroquinolones and metronidazole have been described. (Eed et al., 2019). The most frequent resistance is to clarithromycin and is currently increasing, with an overall rate of almost 20% in Europe with variations between different countries, and can reach up to 50% in China. Resistance to metronidazole is less common but it also reduces the effectiveness of triple therapy and is increasing in recent years. Due to this, an alternative therapy must be evaluated in regions with high rates of resistance to 1st-line antibiotics and the ideal is to carry out sensitivity studies before administering the treatment to avoid failure (Pohl et al., 2019). Because culture is a slow and demanding method, faster techniques are needed and several commercial kits are currently available that detect, directly from the biopsy, the presence of *H. pylori* and the main resistance mutations to clarithromycin and fluoroquinolones with very good sensitivity and specificity (Balsalobre-Arenas and Alarcón-Cavero, 2017).

In countries with a low rate of resistance to clarithromycin (<15%), triple therapy is administered based on the association of a PPI, clarithromycin and metronidazole or amoxicillin; or quadruple bismuth therapy. In countries with a high rate of resistance to clarithromycin (>15%) and low resistance to metronidazole, triple therapy with a PPI, amoxicillin and metronidazole can be administered (Pohl et al., 2019). For those patients who present resistance to the 1st line, fluoroquinolones can be used as 2nd line therapy, although resistance to them is also increasing (Eed et al., 2019).

There is currently no effective vaccine for *H. pylori* infection. More studies are needed and research is continuing (Pohl et al., 2019).

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Haemophilus, Bordetella and Bartonella

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Introduction

Haemophilus, Bordetella and Bartonella are pleomorphic gram-negative bacteria with fastidious growth, which can produce a wide variety of clinical symptoms. This chapter briefly summarizes and updates the microbiological characteristics, epidemiology and laboratory diagnosis of these fastidious bacteria and describes the pathophysiology and treatments caused by these microbes (Fig. 1).

Haemophilus

The genus Haemophilus includes gram-negative, facultative anaerobic, pleomorphic bacteria found in the mucous membranes of humans. They are classified within the family Pasteurellaceae, as are the genera Actinobacillus, Aggregatibacter and Pasteurella. The number of Haemophilus species includes a long list of pathogenic and non-pathogenic bacteria, but 6 species affecting humans are particularly relevant: *H. aegyptius*, *H. ducreyi*, *H. haemolyticus*, *H. paraemolyticus*, *H. parainfluenzae* and *H. influenzae*, being *H. influenzae* the most commonly associated with human disease.

Microbiology

The genus Haemophilus consists of small gram-negative, immotile, aerobic or facultative anaerobic bacilli or coccobacilli with fastidious growth requirements. For their development they require the addition of growth factors present in the blood, especially hemin (called factor X) and nicotinamide dinucleotide (NAD), also called factor V. Because of their requirement of factors, they are demanding bacteria and grow only in specific media (chocolate agar or supplemented chocolate agar) or with an exogenous source of essential nutrients (satellitism around a *Staphylococcus aureus* streak or supply of specific nutrients). Optimal growth is obtained by incubating the plates in a 5% CO₂ atmosphere at 35–37 °C (Kilian, 1976, 2009).

The requirements of factors X and V, the porphyrin test and the use of sugars are the most important properties for the classification of the genus Haemophilus (Table 1).

H. influenzae strains may produce one of six distinct capsular polysaccharides or may be nonencapsulated. The capsule consists of repeating units of one of six different disaccharides, which provides the basis for differentiating six distinct antigenic serotypes from

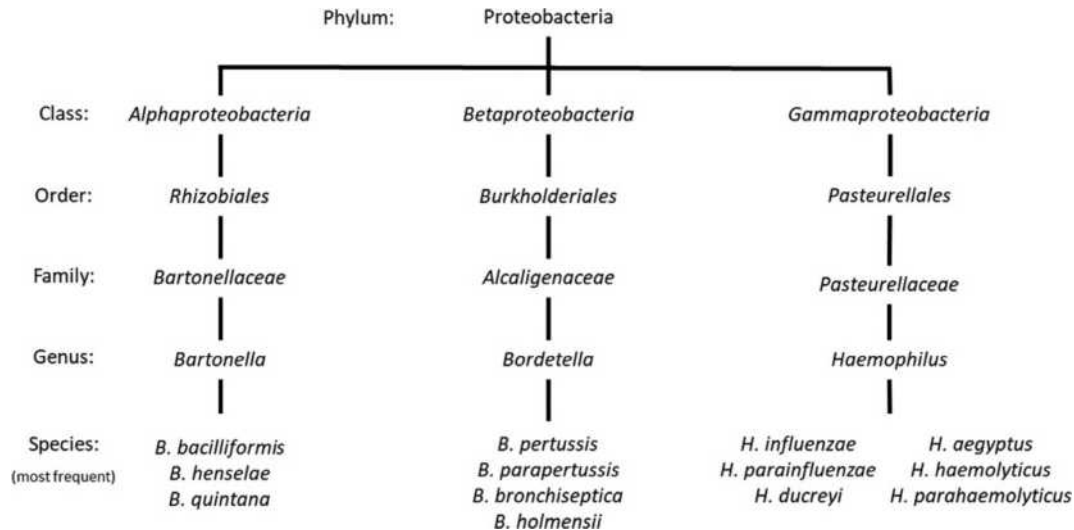


Fig. 1 Phylogenetic distance and most frequent species of different genera of bacteria described in this article.

Table 1 Growth requirements of the most common *Haemophilus* species.

Species	Dependence		
	X Factor	V Factor	Hemolysis
<i>H. influenzae</i>	+	+	–
<i>H. parainfluenzae</i>	–	+	–
<i>H. ducreyi</i>	+	–	–
<i>H. aegyptius</i>	+	+	–
<i>H. haemolyticus</i>	+	+	+
<i>H. parahaemolyticus</i>	–	+	+

a to f (Hia-Hif) (Lâm et al., 2011). *H. influenzae* strains that are not encapsulated are commonly referred to as nontypeable *H. influenzae* (NTHI). Assignment of the different serotypes can be done by slide agglutination or by nucleic acid amplification techniques (NAATs); agglutination on slides leads to a high discordance rate compared to polymerase chain reaction (PCR)-based methods (Nørskov-Lauritsen, 2014). Of all the serotypes, *H. influenzae* type b (Hib) stands out, since it contains a polysaccharide antiphagocytic capsule, formed by ribose, ribitol and phosphate (polyribosyl ribitol phosphate; 5-D-ribitol-(1 → 1)-β-D-ribose-3-phosphate; PRP) which constitutes its main virulence factor.

Epidemiology

Haemophilus species occur in almost all individuals colonizing mainly the nasopharyngeal and genital mucous membranes. *H. parainfluenzae* and unencapsulated *H. influenzae* are predominantly found in the mouth and upper respiratory tract (Kuklinska and Kilian, 1984). Carriage of *H. influenzae* Hib, associated with severe and invasive infections, has decreased from up to 5–0.05% since introduction of the Hib conjugate vaccine in the 1980s. By contrast, non-typeable *H. influenzae* strains together with strains of *H. parainfluenzae* still represent a major portion of the cultured bacterial microbiota of the upper respiratory tract of healthy humans (Morris et al., 2008).

With regard to the incubation period for *H. influenzae* infection, it is still a question poorly understood. Preceding viral infections, Hib capsule, and host-deficient antibody responses may lead to increasing bacterial proliferation, tissue invasion and colonization of inflamed or damaged mucosa. Accession to the bloodstream may further lead to bacterial dissemination to multiple sites and severe disease, including: meninges, subcutaneous sites, joints, pleura and lungs (Agrawal and Murphy, 2011; Nørskov-Lauritsen, 2014).

H. ducreyi infection is mainly prevalent in parts of Africa, being uncommon in Europe and North America. Until 1999, a high rate (>35%) of *H. ducreyi* infections was reported in African countries (published studies in Côte d'Ivoire, Gambia, Kenya, Lesotho, Senegal, South Africa, and Swaziland). However, after 2000, a clear decrease in the proportion of *H. ducreyi* cases was observed as a result of social changes and improved antimicrobial stewardship in these countries. Despite this favorable trend, the CDC (Centers for Disease Control and Prevention) has documented that the disease goes largely unrecognized and underreported, so its true incidence is unknown (González-Beiras et al., 2016).

Clinical manifestations

- Bloodstream infections caused by *H. influenzae* have been nearly eliminated in populations where polysaccharide conjugate vaccine programs have been implemented. However, invasive infections caused by other serotypes and non-typeable strains have increased due to Hib and pneumococcal conjugate vaccines. Therefore, non-Hib *H. influenzae* strains still represent an important cause of community onset bacteremia, particularly for very young infants and patients aged >65 years. Upper respiratory tract appears to be the most important focus and reduced susceptibility to ampicillin, age >90 years and dissemination to central nervous system are factors associated with a poor prognosis (Laupland et al., 2011).
- Meningitis caused by *H. influenzae* type b was the most frequent cause of pediatric meningitis until the vaccine became available. It is caused by blood-borne dissemination of the microorganisms from the nasopharynx. It begins with mild upper respiratory symptoms lasting 3 days, followed by signs and symptoms characteristic of bacterial meningitis. Currently, cases of invasive *Haemophilus* infection have greatly decreased, and are associated with cases of immunosuppression, HIV infection and respiratory disorder. Although rare, there are reported cases of non-type b *H. influenzae* meningitis due to the spread of the vaccine (Fickweiler et al., 2004; Sawardekar, 2017).
- Orbital cellulitis due to *H. influenzae* is an invasive clinical entity that frequently affects children under 36 months. Orbital cellulitis is usually associated with high fever, leukocytosis and blue-purple discoloration of the eye affected areas, in which abscesses may appear. Bacteremia and central nervous system infection with neurological complications may also appear (Sharma et al., 2015).
- Epiglottitis caused by *H. influenzae* type b has considerably decreased its incidence thanks to the conjugate vaccine, as has happened with meningitis. It used to represent a pediatric emergency because affected children presented with pharyngitis, fever and respiratory distress, which rapidly progressed to complete airway obstruction and death. Currently, Hib is no longer the dominant organism causing epiglottitis, being replaced by a variable microbiome of causal pathogens of this entity (Baird et al., 2018).
- Otitis, sinusitis and lower respiratory tract infections. *Haemophilus influenzae* may gain access to different focus of infection by direct contagious spread from its frequent reservoir in the respiratory tract. Conjunctival infection may appear through direct inoculation of the bacteria in the eyes, probably by contact with hands contaminated with respiratory secretions. Previous viral infections and inflammation of the eustachian tubes may favor subsequent infection of the middle ear cavity and maxillary sinuses. Additionally, establishment of infection in the lungs is facilitated by underlying conditions that may affect mucociliary clearance, such as smoking, EPOC, other bacterial infections or other conditions such as cystic fibrosis and bronchiectasis (Murphy, 2003).
- Acute purulent conjunctivitis is associated with *H. aegyptius* (Koch-Weeks bacillus). This pathogen is also the cause of Brazilian purpuric fever (BPF), a fulminant pediatric disease, with a clinical course very similar to meningococcal sepsis (high fever, vomiting, abdominal pain, rapid progression of petechiae and vascular collapse) (Cury et al., 2014).
- Chancroid or soft chancre is a sexually transmitted disease caused by *Haemophilus ducreyi*. A painful ulcer with an erythematous base form on palpation in the genital or perianal region 5–7 days after exposure. It is often accompanied by inguinal lymphadenopathy which may suppurate. Recently, *H. ducreyi* has been identified as a possible cause of chronic skin ulcers on the legs of children and adults living in the Pacific and African regions (Lewis and Mitjà, 2016).

Diagnosis

Diagnosis of the different *Haemophilus* species is made by traditional culture from a wide variety of clinical samples (respiratory samples, ear exudates and urethral exudates, cerebrospinal fluid, synovial fluid, etc.). The culture is highly specific, but may have low sensitivity in those patients who have received antibiotic treatment or in those samples that have not been taken properly. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry allows identification to species level of *Haemophilus* with high sensitivity and specificity, however, MALDI-TOF cannot differentiate between typeable and nontypeable *H. influenzae* and does not permit susceptibility testing (Chen et al., 2017). The diagnosis of *H. influenzae* can also be confirmed by PCR-based assays. The advantages of these tests are based above all on their high sensitivity and specificity, and they can be used in patients who have received antibiotics (Abdeldaim et al., 2009). Currently, there are commercial PCR-based assays that allow the detection of *H. influenzae* directly from clinical specimens (in less than 2 h), saving valuable diagnostic time (e.g., BIOFIRE FILMARRAY® Meningitis/Encephalitis Panel, Curetis Unyvero® P55 /P50, etc.).

Treatment

Treatment for *Haemophilus* infections varies according to severity and location. In severe systemic infections by capsulated *H. influenzae* (meningitis or epiglottitis), early antimicrobial treatment is required, generally with broad-spectrum cephalosporins, ceftriaxone 2 g/12 h or cefotaxime 2 g/4–6 h iv 7–10 days. In cases of meningitis, dexamethasone 0.15 mg/kg/6 h iv 2 days would be added. In less severe infections, such as sinusitis and otitis, they can be treated with oral antibiotics, such as amoxicillin, cefixime, azithromycin or a fluoroquinolone (avoiding its use in children). *H. ducreyi* infections can be treated with single doses of ceftriaxone, azithromycin or ciprofloxacin or with longer regimens with erythromycin.

Both *H. influenzae* and *H. parainfluenzae* can present resistance to ampicillin alone or in association with multiple resistance to other drugs, such as co-trimoxazole, chloramphenicol, tetracyclines and aminoglycosides. The most frequent mechanism of ampicillin resistance is the plasmid production of a β -lactamase type TEM-1 and, more rarely, of the ROB-1 type, both inhibited by clavulanic acid, so that these strains retain their sensitivity to the amoxicillin-clavulanic acid combination and to third-generation cephalosporins (Tristram et al., 2007). In the United States, approximately 25% of *H. influenzae* strains are estimated to produce beta-lactamase (Heilmann et al., 2005). Occasionally there are strains of *H. influenzae* that do not produce beta-lactamase but are nevertheless resistant to amoxicillin and ampicillin due to changes in penicillin binding proteins (PBPs). These strains are designated as "beta-lactamase negative, ampicillin resistant" or BLNRA strains and are prevalent in Asia (Han et al., 2019). Most BLNRA strains, like beta-lactamase positive strains, maintain susceptibility to third generation cephalosporins (Ohno et al., 2007).

Resistance to third generation cephalosporins, such as ceftriaxone or cefotaxime in *H. influenzae* is a growing cause of concern, since these antimicrobial agents are considered mainstays for severe *H. influenzae* infections. Resistance to third generation cephalosporins in *H. influenzae* occurs through the selection of mutations in key conserved residues of the *ftsI* gene, which codes for the essential penicillin-binding protein PBP3, which is the target of cefotaxime and ceftriaxone. The levels of resistance may vary, but high-level resistance to these agents is achieved after the stepwise acquisition of the mutations R517H or N526K in combination with L389F (Sanbongi et al., 2006). High-level cephalosporin resistance is still rare outside Japan and South Korea, although resistant strains are increasingly reported in countries from Asia, Europe and America (Deghmane et al., 2019).

Vaccination

As mentioned above, vaccination against *Haemophilus influenzae* type b helps prevent severe *H. influenzae* infections, reducing disease incidence and colonization by this pathogen. Several Hib conjugate vaccines have been developed and approved. All vaccines use polyribosylribitol phosphate (PRP) isolated from the Hib capsule. The immunogenicity of PRP is limited in children under 2 years of age so it needs to be conjugated with a protein carrier. Four different types of protein carrier have been used diphtheria toxoid (PRP-D), tetanus toxoid (PRP-T), CRM197 (a non-toxic variant of diphtheria toxin HbOC), and *Neisseria meningitidis* outer membrane protein complex serogroup B (PRP-OMP). The vaccine is administered in infancy in three doses at 2, 4, and 6 months of age, or in two doses at 2 and 4 months, depending on the formulation. In either case, a second dose is recommended between 12 and 15 months of age.

Bordetella

The genus *Bordetella* belongs to the *Alcaligenaceae* family and currently consists of 12 species: *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, *B. brachialis*, *B. flabilis*, *B. sputigena*, *B. avium*, *B. hinzii*, *B. holmesii*, *B. trematum*, *B. petrii* and *B. ansorpii*. Of these, *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. holmesii* are associated with respiratory infections in humans.

Microbiology

The genus *Bordetella* consists of gram-negative, non-sporulating coccobacilli of very small size (0.2–0.5–1 μ m). At the genomic level, *B. pertussis* and *B. parapertussis* show smaller genomes (4.0–4.8 Mbp) than many other species of the genus (more than 5 Mbp). Only the 50% of the genome of the species of the genus *Bordetella* is conserved in all species, and the signature of multiple genomic rearrangements, such as acquisition of insertion sequences, horizontal gene transfer, and acquisition of new genes and point mutations has likely driven the diversification and development of the different species of the genus (Park et al., 2012).

Bordetella are catalase positive and oxidize peptides but are not able to ferment carbohydrates. Some species are able to grow in simple synthetic media, however, the most clinically relevant species *B. pertussis* and *B. parapertussis* are very sensitive to toxic substances and metabolites present in multiple culture media. As a consequence, they need special media supplemented with blood and growth factors as niacin, in which they grow slowly at 35–37 °C. Other less pathogenic species can be easily isolated by routine microbiological procedures.

Bordetella also express a wide array of virulence factors that are usually controlled by complex expression systems that may act in response to different environmental conditions (BvgAS) (Gerlach et al., 2004). The most relevant virulence factors include adhesins, the filamentous hemagglutinin or the pertussis toxin (PT). The pertussis toxin is only expressed in *B. pertussis* by the *ptx* gene, since other species such as *B. parapertussis* or *B. bronchiseptica* the *ptx* gene is also present but lacks a functional promoter. The PT suppresses chemotaxis, oxidative responses and the activity of macrophages and neutrophils. On the other hand, the filamentous hemagglutinin and the fimbria type proteins FIM2 and FIM3 play important roles in adhesion and colonization of the ciliated epithelium of the upper respiratory tract (Mattoo and Cherry, 2005).

Epidemiology

B. pertussis and *B. parapertussis* are transmitted by droplets and cause human disease. *B. pertussis* is highly transmissible, since susceptible contacts transmission contact may be close to 90% and the infectious dose is around 100 colony-forming units (CFU). Once the bacteria are inhaled and introduced to the organism, they attach to the ciliated epithelial cells of the nasopharynx, leading to tissue lesion and the respiratory condition known as pertussis or whooping cough (Mattoo and Cherry, 2005).

Although the incidence and morbidity and mortality associated with *B. pertussis* infection has decreased since the introduction of the vaccine, pertussis is distributed throughout the world and causes outbreaks that can result in more than 15 million infections and 160,000 deaths per year, especially in less developed countries (Black et al., 2010; Yeung et al., 2017). The greatest morbidity and mortality associated with pertussis infection occur in young infants (<3 months of age) who often requiring hospitalization in an intensive care unit. In Europe, according to the European Centre for Disease Prevention and Control (ECDC), 33,133 cases of *B. pertussis* were confirmed during 2018. The highest notification rate was observed among infants below the age of 1 year (44.4 cases per 100,000 population), followed by 10–14-year-olds (22.0 cases per 100,000 population) (European Centre for Disease Prevention and Control, 2020). In developed and vaccinating countries, the cases of whooping cough are mostly restricted to neonates and unvaccinated young infants. A stage of permanent carrier is not observed with these pathogens, and the appearance of outbreaks in certain settings has been linked to insufficient vaccine coverage, changes in the circulating strains and decreased longevity of protection after vaccination (Watanabe et al., 2006).

B. bronchiseptica, *B. parapertussis*, and *B. holmesii*, can cause respiratory infections in humans but also in other mammals. *B. bronchiseptica* infects a wide range of hosts and occasionally causes cough illnesses in humans; in particular severe infections have been noted in persons who are immunocompromised such as patients with acquired immunodeficiency syndrome (AIDS).

Clinical manifestations

B. pertussis infection causes whooping cough, which has an incubation period of 7–10 days (maximum 21 days) (Cherry, 2005). The clinical presentation usually has three well-defined phases:

- 1st or catarrhal phase: After an initial incubation period of up to 10 days, the primary infection starts with symptoms that last 1–2 weeks and are indistinguishable from a common cold (including rhinorrhea, sneezing, malaise and fever). During this stage, the infection may be underdiagnosed due to lack of specificity of symptoms. This stage is the most contagious since in this period there is a peak in bacterial inoculum on the respiratory tract, posing a high risk to their close contacts which may lead to outbreaks.
- 2nd or paroxysmal phase: In this stage, the classic symptoms of pertussis appear, raising the suspicion of a diagnosis of *B. pertussis* infection. The patient has bursts of numerous rapid coughs, apparently due to difficulty expelling thick mucus from the tracheobronchial tree. At the end of the paroxysm, a long inspiratory effort is usually accompanied by a characteristic high-pitched whoop. It is common during the attack to become cyanotic. and children and young infants, especially, appear very ill and distressed (Hamborsky et al., 2015). Post-attack fatigue and vomiting episodes are common. The average number of daily episodes of paroxysmal cough is between 5 and 20, predominantly at night, triggered by stimuli such as laughter, crying or physical exercise. These episodes progressively decrease after the 3rd week. In adults, symptoms tend to be less severe than in children, but pertussis causes a long illness, with a mean duration of 6 weeks during which choking and vomiting are frequent.
- 3rd phase or convalescence phase: arrives after 2–4 weeks, where the number and severity of paroxysms decreases, but secondary complications may appear, such as secondary bacterial pneumonia, atelectasis, recurrent epistaxis, urinary incontinence, syncope, etc. (Kilgore et al., 2016).

Pertussis caused by *B. parapertussis* manifests with symptoms similar to those caused by *B. pertussis*, although it is generally less severe. Approximately 40% of cases are asymptomatic and if symptoms appear, they tend to be more moderate than those observed in infections caused by *B. pertussis* (Wirsing Von König et al., 2002). Other pathogens that may cause pertussis-like symptoms that should be considered for diagnosis include adenoviruses, respiratory syncytial virus, human parainfluenza viruses, influenza viruses, *Mycoplasma pneumoniae* and other agents (Paddock et al., 2008).

With regard to other infections caused by *Bordetella* spp., *B. holmesii* may cause respiratory symptoms and has also linked with different clinical conditions in immunocompromised hosts. *B. bronchiseptica*, *B. petrii*, *B. avium* and *B. hinzii* have been also exceptionally associated with respiratory symptoms and severe infections (e.g., bacteremia) in immunocompromised hosts with different underlying clinical disorders, including asplenia, HIV infections, or hematological malignancies (Toubiana et al., 2019).

Diagnosis

Several microbiological techniques are readily available, such as cultures, serology, and nucleic acid amplification tests for the diagnosis of *Bordetella* spp. infection. Although culture remains the gold standard since allows further typing and antimicrobial susceptibility testing, fastidious growth requirements make *Bordetella* spp. difficult to culture (only positive 12–60%) (Wendelboe and Van Rie, 2006). Different media are available for culture, such as RL medium, Bordet-Gengou, and Stainer-Scholte. Incubation should be performed for at least 1 week at 35–37 °C. Some media may include antimicrobials, such as cephalixin, to suppress concomitant growth of other bacteria.

Serology is applicable for the diagnosis of pertussis. Detection of a high level of anti-PT antibodies in the serum of an unvaccinated individual indicates infection. The use of serology in infants is not recommended because their immune system is immature and there is interference with maternal antibodies. The use of serology as a diagnosis is not recommended in those patients vaccinated for a period of 1 year, since it is not possible to differentiate the anti-PT antibodies from the vaccine or natural antibodies from the infection (Guillot et al., 2014).

Sensitive and specific NAATs are being increasingly implemented to overcome the limitations of culture and serology and can provide a quick result. Analysis of nasopharyngeal samples by PCR should be done between the first and third week after the onset of the cough. From the fourth week, false negative results can be obtained due to the decrease of bacterial DNA in the nasopharynx. NAATs targeting against the insertion sequence 481 (IS481) and insertion sequence 1001 (IS1001), are commonly used for the diagnosis of *B. pertussis* and *B. parapertussis*, respectively. However, both targets are present in other *Bordetella* strains, such as *B. holmesii* and *B. bronchiseptica* (Tizolova et al., 2013). For this reason, the CDC Pertussis and Diphtheria Laboratory has developed its own PCR to diagnose pertussis. The multi-target RT-PCR assay combines a pertussis toxin subunit 1 (ptxS1) singleplex assay and a multiplex assay targeting the multicopy gene IS481, *B. parapertussis* IS1001 (pIS1001), and *B. holmesii* IS1001-like (hIS1001) to distinguish between *Bordetella* species and identify co-infections (Lee et al., 2018) (Valero-Rello et al., 2019).

Molecular typing and discrimination of circulating strains may be important for outbreak management and surveillance. Despite *B. pertussis* show low genomic heterogeneity, some genomic targets show enough polymorphisms: the toxin gene (ptx), the toxin promoter (ptxP) and the pertactin gene (prn). A vast array of methods are available, including multilocus variable-number tandem repeat analysis (MLVA), pulsed-field gel electrophoresis (PFGE) and classic and next-generation sequencing (Williamson et al., 2012).

Treatment

B. pertussis and *B. parapertussis* are intrinsically susceptible in vitro to a wide range of antibiotics, including penicillins, macrolides, quinolones, tetracyclines and trimethoprim-sulfamethoxazole. However, they show resistance to most orally available cephalosporins.

The first line treatment for pertussis is macrolides. The accepted guideline for azithromycin is 500 mg 1st day, followed by 250 mg/day for 4 days, while for clarithromycin it is 500 mg/12 h for 7 days and for erythromycin 500 mg/8 h 7–14 days. If there is allergy or intolerance to macrolides, the alternative is trimethoprim-sulfamethoxazole (TMP 160 mg + SMX 80 mg) 12 h for 14 days. Antibiotic treatment does not modify the evolution of the infection if started after the third week of the disease, but it is considered indicated to reduce the risk of transmission. Symptomatic treatments (dexamethasone, salbutamol, etc.) have not shown superiority in the management of whooping cough (Pillay and Swingle, 2003).

Macrolide-resistant strains of *B. pertussis* are rare in most countries, although recently, an increasing number of strains carrying the A2047G mutation in the 23S rRNA, which is associated with macrolide resistance, have been reported in China and Vietnam (Kamachi et al., 2020).

There is no consensus on the best treatment for non-pertussis *Bordetella* infections. Because confirmatory diagnosis usually requires some time, patients with *B. holmesii* and *B. bronchiseptica* infections are usually treated for a *B. pertussis* respiratory infection. *B. holmesii* and *B. bronchiseptica* are generally poorly sensitive to macrolides and some cephalosporins, but low MICs have been reported for carbapenems and fluoroquinolones (Pittet et al., 2014; Kadlec and Schwarz, 2018). Further microbiological research is needed to determine breakpoints and the best antimicrobial treatment for these species.

Vaccination

Two acellular vaccines (dTdap for children and Tdap for adults) indicated for active immunization against diphtheria, tetanus and pertussis are currently approved by the FDA. Its composition includes detoxified pertussis toxin (PT), filamentous hemagglutinin (FHA) and Pertactina (PRN) (Sanofi Pasteur. DAPTACEL (Diphtheria and Tetanus Toxoids and Acellular Pertussis Vaccine Adsorbed) [package insert] U.S. Food and Drug Administration, n.d.).

Bartonella

The genus *Bartonella* encompasses fastidious and intracellular gram-negative bacteria belonging to the family Bartonellaceae. Currently this genus consists of more than 40 species that infect animals, with the majority of human diseases being caused by *B. bacilliformis*, *B. henselae* and *B. quintana*. The association of human diseases with other *Bartonella* species (*B. vinsonii*, *B. grahamii*, *B. washoensis*, etc.) is based on very limited information from case reports.

Microbiology

Bartonella are gram-negative, pleomorphic, intracellular, small (0.2–0.5 by 0.5–2 µm), intracellular bacilli. Because of the very low dividing time (approximately 24 h) of these bacteria on agar plates, conventional identification of this bacteria cannot be achieved through conventional biochemical tests and are therefore not useful for clinical diagnosis of infection. *Bartonella* species are oxidase and catalase and highly hemin-dependent, and can be isolated in agar cultures of enriched blood-containing media. The optimal growth temperature may vary between species, being from 25 to 30 °C for *B. bacilliformis* and from 35 to 37 °C for *B. quintana* and *B. henselae*. They can be cultured on solid media from blood samples, biopsies of eruptive lesions or subcutaneous nodules, requiring a prolonged incubation period (2–6 weeks).

Epidemiology

The main reservoir of *Bartonella* species are mammals (e.g., humans, rodents, cats and sheep, among others) and they are transmitted by vectors, mainly hematophagous arthropods and insects, such as fleas, sand flies, body lice and ticks. In addition, transmission may also occur through animal bites and scratches. At the geographical level, some species are circumscribed to specific regions in which the specific vector is distributed. Such is the case of *B. bacilliformis*, the etiologic agent of Carrion's disease, which only lives in areas between 1.000 and 3000 m altitude in the Andes Mountains of South America, as this is the endemic area of its vector, *Lutzomyia verrucarum* (Gomez and Ruiz, 2018).

Others, such as *B. quintana* and *B. henselae*, appear to have a worldwide distribution. Outbreaks due to *B. quintana* are mainly associated with homeless patients living under very poor conditions of hygiene and sanitation. This favors the infestation of the human body with the main vector of *B. quintana*, *Pediculus humanus*, and subsequent bacterial infection through inoculation of contaminated arthropod excreta through skin fissures. On the other hand, *B. henselae* main reservoir are cats, which are responsible of transmitting infection to humans through direct transmission of infective flea feces at the time of the scratch, causing the disease known as Cat Scratch disease. The disease tends to be more prevalent in regions with high prevalence of stray cats living outdoors and warm and humid climates that favor a major abundance of sand fleas (Boulouis et al., 2005).

Clinical manifestations

Bartonella species may infect humans and cause a plethora of different clinical manifestations. Here we summarize some of the most clinically relevant diseases caused by these species:

- Carrion's disease: caused by *B. bacilliformis*, it is characterized by a hemolytic bacteremia, consisting of a first acute phase (known as Oroya fever) of fever and severe anemia caused by *B. bacilliformis*-infected erythrocytes. The clinical course in this phase is severe and without treatment can be fatal (40–85% mortality) and is frequently observed in nonnative populations. After weeks of months after infection, the acute phase is followed by a chronic phase with the appearance of one or multiple skin lesions of vascular appearance, papular and pruritic, which bleed easily (known as Peruvian Wart, in Spanish "Verruga Peruana" (Kosek et al., 2000)). Myalgias, arthralgias, headaches and superinfections are also frequent, especially by *Salmonella* spp. These lesions may persist for months, but prognosis is favorable at this stage. The link between acute and chronic forms was documented in 1985 by Daniel Carrion, who died of Oroya Fever after inoculating himself with infected material from a verruga.
- Trench fever: 5-day fever or Wolhynia fever. It is caused by *B. quintana* and transmitted from person to person by the body louse, *P. humanus*. It was widely described in World War I, where the overcrowded conditions of the European armies in the trenches favored the spread of the disease. Nowadays we are assisting to the reappearance of this clinical disease, especially in disadvantaged social groups, which is called urban trench fever (Brouqui et al., 1999). The infection can range from asymptomatic to severe headache, high fever, asthenia and bone pain, especially in the pretibial area, and has an incubation period which ranges from 15 to 25 days. The fever is characterized by recurrence at 5-day intervals, hence the name of the entity. The disease may last several weeks without treatment during which bacteremia and the onset of endocarditis are common. *B. quintana* endocarditis is one of the most frequent cause of blood culture-negative endocarditis (Jackson and Spach, 1996).
- Cat scratch disease whose main agent is *B. henselae*. After scratching, an inflammation of the area that often resembles an insect bite occurs after 10–14 days, followed by enlargement of the lymph nodes draining at the site of contact (Lamps and Scott, 2004). The skin lesion usually disappears after some weeks. In 25% of cases, suppurative complications occur, although most infections are self-limited and resolve spontaneously. The large majority of cases the lymphadenopathy is accompanied with fever, chills, malaise and anorexia. Serious complications or dissemination to other organs rarely appear, and may include Parinaud's oculo-glandular syndrome, retinitis, osteolytic lesions, encephalopathy and thrombocytopenic purpura.
- Bacillary angiomatosis produced mainly by *B. quintana* and *B. henselae*, is a vascular proliferative disease that mainly affects immunocompromised patients (patients with immunosuppressive treatment, HIV with less than 100/mm CD4, etc.) and rarely immunocompetent patients. Skin lesions of unspecific size occur, which can be superficial and/or deep and may involve the subcutaneous tissues and bones. They are very vascular lesions, which bleed easily (similar to a Peruvian wart), and are accompanied by adenopathy in the area. When the vascular proliferations have hepatosplenic localization it is known as Peliosis. Hepatic and splenic peliosis may present with fever, abdominal pain, hepatosplenomegaly and increased acute phase reactants (Koehler et al., 1997).
- Subacute endocarditis. *B. quintana* and *B. henselae* account for most cases of this entity. The clinical presentation is characterized by nonspecific symptoms such as weight loss, fever and fatigue, accompanied by a mild and progressive infection of the endocardium. In patients who have not received antibiotic treatment, the most common etiologies of blood culture-negative endocarditis are *Coxiella burnetii* and *Bartonella* species, the former being responsible for 28–37% and the latter for 12–28% of cases (Chomel and Kasten, 2010).

Diagnosis

Most of specimens used for isolation or detection of *Bartonella* are blood or tissues with suspected infection. However, confirmation of the diagnosis of *Bartonella* infection is often a major challenge. Definitive diagnosis is made by isolation of *Bartonella* in

blood or tissue culture, which is usually difficult in most cases due to fastidious Bartonella growth. Often the diagnosis is made by complementary tests such as serology and molecular biology tests. Close communication with the Microbiology service is often key to getting to this point, since for Bartonella species the available clinical specimens and the patient underlying disease are particularly relevant for choosing the most optimal diagnostic approach. Indirect fluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA), are available for the diagnosis of Bartonella infections and are of particular relevance for diagnosis of Bartonella endocarditis. Although they can be helpful for diagnosis, these techniques often result in lack of specificity and cross-reactivity between the different Bartonella species (Bergmans et al., 1997). As for molecular tools, an increasing number of laboratories allow the amplification of Bartonella DNA in various samples (valvular tissue, angiomas lesions, whole blood) by RT-PCR or the detection and sequencing of Bartonella 16S rRNA gene or other housekeeping genes that allow confirmation of the diagnostic suspicion and identify the pathogen at the species level (Fenollar and Raoult, 2004; Liesman et al., 2017).

Treatment

As we have seen, *B. bacilliformis*, *B. henselae* and *B. quintana* lead to different clinical manifestations, leading to a different therapeutic approach depending on the involvement they cause:

Treatment for Carrion's disease is differentiated for either the acute or chronic phase. In Oroya fever, the recommended treatment is ciprofloxacin 500 mg/12 h vo, 10 days or chloramphenicol 14 days. In the phase known as Peruvian wart, rifampicin 300 mg/12 h 14 days or streptomycin 15–20 mg/kg/day, 10 days. For cat scratch disease, no efficacy of antibiotic treatment (azithromycin) versus placebo has been demonstrated, except for a faster decrease in the size of the adenopathy in patients treated with azithromycin. When adenopathy is large, it can be drained by aspiration puncture. Treatment of trench fever and chronic bacteremia is successfully performed with the association of doxycycline 100 mg/12 h vo 6 weeks with gentamicin 3 mg/kg/day iv 2 weeks. In patients with bacillary angiomatosis and hepatic peliosis, the treatment of choice is erythromycin, and doxycycline can be used in those patients who do not tolerate erythromycin. Treatment may be prolonged to avoid the risk of recurrence, especially in immunocompromised patients. In cases of suspected Bartonella endocarditis, the consensus treatment according to The American Heart Association is ceftriaxone and gentamicin with or without doxycycline. When the diagnosis of Bartonella is confirmed, the consensus treatment is doxycycline 100 mg/12 h for 6 weeks and 3 mg/kg/day gentamicin for 2 weeks (Baddour et al., 2005). Valve replacement is often necessary due to embolic events and the formation of large valvular vegetations (Prutsky et al., 2013).

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***Mycobacterium* infections**

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Mycobacterium

The family *Mycobacteriaceae* are a diverse group of bacteria that differ widely in several traits, such as their pathogenic potential in humans and animals, reservoirs, and growth dynamics in culture. The only genus of this family can be divided into four major groups based on fundamental differences in epidemiology, association with disease, and the ability to grow in vitro: those that belong to the *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, and *Mycobacterium ulcerans* and those referred to as nontuberculous mycobacteria (NMT).

Mycobacterium spp. contain mycolic acids in their cell wall and share this characteristic with bacteria of other genera such as *Gordonia*, *Nocardia*, *Rhodococcus* and *Tsukamurella*. This characteristic enables these bacteria to be differentiated from other bacteria based on staining techniques since the high mycolic acid content in the cell wall makes organisms resistant to discoloration with acid alcohol.

Mycobacterium spp. are aerobic, non spore forming, Gram positive, acid –fast bacilli (AFB). They are non motile, and most of them straight or slightly curved rods, with only a small number of species exhibiting some branching. Some species are pigmented, and this pigment may be constitutive (scotochromogenic), or induced only with exposure to light (photochromogenic) and finally others never produce pigment (nonphotochromogenic). Mycobacteria are different from most of other bacteria, these are slowly growing, require at least 5 days of incubation, and many of them one or more weeks for visible growth.

From 41 valid species in 1980, the genus *Mycobacterium* currently encompasses almost 200 recognized species and subspecies, many of which either can cause clinical disease, have been associated with disease, or have been isolated from clinical specimens without any known clinical correlation (Tortoli, 2014).

Mycobacterium tuberculosis

Tuberculosis continues to cause considerable morbidity and mortality globally. According to estimates of the World Health Organization (WHO), in 2018 there were approximately 10 million active cases, of which 3–4 million cases were infectious, with positive sputum smears. 8–7 million (87%) of these individuals reside in 30 high burden countries. Deaths due to tuberculosis occur in 1–3 million people worldwide each year (WHO, 2019). Thus tuberculosis is still a major cause of disease and death, and its elimination will be extremely difficult as long as, overpopulation, and multidrug-resistant (MDR) disease characterize large portions of the earth. Every year, more than half a million people become sick with rifampicin-resistant forms of tuberculosis, but in 2017, only 160,684 people were diagnosed or notified, and only 139,114 had started treatment (WHO, 2018). Currently, prevalence of rifampicin-resistant tuberculosis is increasing in several key countries including Russia, Myanmar, China and South Africa (Law et al., 2017).

Global tuberculosis incidence is estimated to be slowly declining by 1.6% per year, far from the 4–5% estimated to be required to reach WHO End TB Strategy targets (GBD Tuberculosis, 2015).

Although tuberculosis can affect any organ of the body, the lungs are virtually always the portal of entry. The bacilli are most commonly discharged into the atmosphere by aerosolization of pulmonary secretions by a diseased pulmonary patients in coughing, sneezing, speaking, and singing. Aerosol droplets dry rapidly, leaving tiny droplet nuclei, some of which contain a few bacilli (Wells, 1934). Droplets nuclei in the range of 1–10 µm can be inhaled, with the larger ones trapped in the upper nasal passages or expelled into the pharynx by the mucociliary mechanism of the lower respiratory tract and harmlessly swallowed and digested; smaller droplet nuclei may reach the alveoli and establish infection. Although, in theory, one droplet nucleus may be sufficient to establish infection, prolonged exposure and multiple aerosol inocula are usually required. Although homes are the focus of TB contact investigations, evidence is mounting that in high –TB settings, approximately 85% of transmission occurs in other social spaces including medical facilities, public transportation vehicles, workplaces, schools, churches, bars and other areas where people congregate. Other methods of transmission are rare. In the past, transmission of infection with *Mycobacterium bovis* through consumption of milk from infected cows was common, but these means have been brought under control in all developed countries by elimination of disease cattle and pasteurization of milk and milk products (Ayele et al., 2004).

The close contact of a smear-positive patient is at maximum risk of being infected, however, the disease is not as highly infectious as some of the viral infections. The ability of the bacilli to cause infection in newly exposed contacts depends on the adequacy of the innate antibacterial defenses of the person. Studies have shown that the infection rate among close contacts ranges from 25% to 50% even in the worst overcrowded and substandard conditions (Templeton et al., 1995; Ting et al., 1999). On the basis of epidemiological data, it appears that exposure generally must be close and sustained, the environment heavily laden with droplet nuclei, and the prospective host unprotected by inborn defenses, previously activated immune mechanisms, or both if an infection sufficient to produce disease is to be established.

In a recent study, immune responses found within individual granulomas suggest that local immune responses at the site of infection are as important in controlling tuberculosis infection as systemic immunity (Torrelles and Schesinger, 2017). Other studies show that some individuals exposed to tuberculosis do not become infected, whereas others rapidly succumb to infection and disease even, with minimal exposure (Seshadri et al., 2017).

In relation to the time course of progression from infection to disease, until now, the people newly infected with *M. tuberculosis* have the highest risk to progression to disease within the first several years after the infection. Analysis of historical data has

confirmed that incubation of *M. tuberculosis* is probably shorter than previously thought around 24 months (Behr et al., 2018). In general, 3–4% of infected individuals acquire active TB during the first year after tuberculin conversion and an additional 5% do so thereafter.

Various factors may influence the risk of developing tuberculosis in an individual or a population. The age and sex variables are also influenced by the timing of epidemic in the population, showing in developing world tuberculosis rates are higher among young adults, which indicates that primary transmission occurs in this age group. Children are at higher risk of contracting tuberculosis infection and disease. The majority of children of less than 2 years of age get infected from the household source case, whereas, with children older than 2 years of age, the majority of them become infected in the community.

Considerable evidence has documented the linkage of tuberculosis with HIV/AIDS (Glynn, 1998). Tuberculosis infection progresses to active disease in persons infected with HIV at a rate of 10% per year. HIV infected persons are highly susceptible to exposure to tuberculosis (CDC, 1993). HIV coinfection greatly increases the chances of reactivation of latent infection of tuberculosis and increases the rapid tuberculosis progression following primary infection or reinfection with tuberculosis (Miles Braun et al., 1991; Shafer et al., 1995).

Important work has also been done on socioeconomic risk factors that might be just as predictive of who becomes infected with, and sick from tuberculosis. In this manner, people from low socioeconomic status populations are known to be at high risk of becoming sick from tuberculosis (Oxlade and Murray, 2012). Although there are many factors involved, such as racial differences, crowding and availability of health care.

There are special populations from tuberculosis: prisoners have a higher chance of getting infected with tuberculosis mostly because of crowded living conditions and coinfection with HIV and injection drug abuse (Raina MacIntyre et al., 1997), patients in chronic mental hospital (Stead, 1981). Alcoholics have been found to have 10 times higher risk of developing tuberculosis than the general population in urban areas. This risk of active tuberculosis is substantially elevated in persons who consume more than 40 g of alcohol per day (Lonnroth et al., 2008). Cigarette smoking increases a relative risk of 1.5–2 for the development of tuberculosis (Bates et al., 2007). Individuals with immune mediated inflammatory disorders are also known to be at increased risk of developing active tuberculosis, particularly after the use of tumor necrosis factor (TNF). Now, screening for latent infection for *M. tuberculosis* has been recommended before TNF alpha inhibitor therapy is initiated, (Smith et al., 2011). Different studies have shown that malnutrition increases the risk of tuberculosis because of an impaired immune response. Tuberculosis disease can itself lead to malnourishment because of decreasing appetite and changes in metabolic processes (Cegielski and McMurray, 2004).

Other factors associated with an increased risk of developing tuberculosis are diabetes mellitus. A systematic review comparing 13 studies examining the association between diabetes and tuberculosis found that diabetic patients had about a threefold increased risk of developing tuberculosis when compared to those without diabetes (Jeon and Murray, 2008). Another review on treatment outcomes among patients with diabetes mellitus and tuberculosis found that the risk of death was 1.89 times higher compared to those without diabetes, with the risk increasing to five times higher for those with diabetes mellitus after adjustment for potential confounders (Baker et al., 2011). Healthcare workers are at increased risk of exposure to tuberculosis, a review by Sleider et al. showed that among healthcare workers in high income countries, the overall incidence of tuberculosis disease in the general population and native born healthcare workers was less than 10–25 per 100,000 per year (Seidler et al., 2005).

Clinical manifestations of tuberculous disease

Pulmonary and thoracic tuberculosis

Pulmonary TB has nonspecific clinical manifestations, and can even be detected in asymptomatic individuals in the course of contact studies or other examinations. It is usual for the patient to usually presents with symptoms of several weeks' duration, consisting of coughing, cough, expectoration, sometimes hemoptoic, chest pain and general symptoms (fever or fever, sweating, asthenia, anorexia, asthenia, anorexia and weight loss). Thoracic examination may be unremarkable or localized or asymmetrical sounds or rales may be auscultated or asymmetrical breath sounds may be heard. It is paramount to suspect the disease, and in any patient with respiratory symptoms and/or general symptoms of more than 2–3 weeks duration initiate diagnostic procedures, as well as in any patient with hemoptysis, irrespective of duration.

Pleural TB can occur in isolation or concomitantly with pulmonary TB. Chest radiograph shows the typical picture of pleural effusion which is usually unilateral and variable in size. Thoracentesis yields a yellowish pleural fluid, usually with the characteristics of a lymphocytic exudate, which should be used for the various diagnostic procedures.

TB may also involve the hilar and mediastinal nodes, and may or may not be associated with other forms of thoracic and/or cervical lymphadenitis.

Another thoracic structure that may be affected by TB is the pericardium, which manifests as a pericardial effusion or constrictive pericarditis, which may occur alone or in association with pleuritis or another form of thoracic TB. The clinical presentation is usually insidious. Ultimately, the symptomatology of pulmonary and thoracic TB is not specific to the disease, so a high index of suspicion is needed in patients with suggestive symptoms in order to make a diagnosis as early as possible and institute appropriate treatment (Perlman et al., 1997; Berh et al., 1999; Kline et al., 1995).

Extrathoracic tuberculosis

M. tuberculosis is preferentially found in well-oxygenated areas of the lung. However, by hematogenous route, it can implant itself in any other part of the body and multiply there to give rise to different forms of clinical manifestations depending on the site of

infection. Extrathoracic TB may be accompanied by systemic symptomatology, although this is less frequently observed. Patients with HIV or other immunodeficiencies, women and people of non-Caucasian race are more likely to manifest extra-thoracic forms of TB (Cagatay et al., 2004; Yang et al., 2004). Once the diagnosis of extrapulmonary TB has been made, the association with pulmonary TB must always be investigated, as they can coexist and, although the basis of treatment is the same, the diagnosis of pulmonary TB implies contagiousness, which requires contact studies to be carried out (CDC, 2005).

Tuberculosis of the central nervous system

Tuberculous meningitis is the most common form of TB in the central nervous system. With a fatal prognosis without treatment, treatment should be initiated as soon as there is a well-founded diagnostic suspicion. Onset symptoms are nonspecific (asthenia, anorexia, headache) and may last for several weeks. If left untreated, decreased level of consciousness and signs of neurological focalities appear. The British Medical Council (BMC) proposed a three-stage classification of the disease, from the absence of neurological involvement and consciousness to coma, which still retains its prognostic value today.

(NCCCC, 2006)

Osteoarticular tuberculosis

Mainly due to bacillary hematogenous dissemination, vertebral localization is the most frequent. The initial symptomatology is very unspecific, with a slow progression and a long delay in diagnosis. In advanced cases, the infection spreads to adjacent soft tissues, causes cold abscesses or abscesses to the back of the vertebra and can affect the spinal canal and cause spinal cord compression, sometimes requiring urgent surgery.

The most frequent locations are lumbar in older people and thoracic in younger people.

Urinary tuberculosis

Caused by hematogenous dissemination, the symptomatology is unspecific or even asymptomatic in early stages. The most frequent signs and symptoms are pollakiuria, dysuria and hematuria.

Genital tuberculosis

In men, the prostate and epididymis are the most frequent sites, and to a lesser extent the testicles and seminal vesicles. In women, the fallopian tube is the most frequent location and is responsible for 80% of genital TB. It is one of the most common causes of infertility in the world.

Lymph node tuberculosis

The most frequent localization is in the cervical and supraclavicular ganglia. Cervical tumor are the most frequent form of progression. Adenopathies tend to grow gradually and are initially rigid and painless in consistency. Adenopathies may undergo necrosis, show signs of inflammation with fistulization and drainage of caseum to the outside, known as scrofula (Cagatay et al., 2004).

Miliary tuberculosis

Due to hematogenous dissemination to multiple organs. Small nodules may be seen in the affected organs. Chest X-ray may show the typical millet-grain pattern with small pulmonary nodules, although it may be normal. Diagnostic suspicion is important in order to treat as early as possible.

Laryngeal tuberculosis

Currently occurs in less than 1% of patients with tuberculosis, the most frequent sign is dysphonia, which should raise suspicion of this condition, although it can occur with dysphagia, cough, hemoptysis. The differential diagnosis is with laryngeal neoplasia. Laryngeal tuberculosis is accompanied by bacillary pulmonary TB, and is therefore highly contagious.

Cutaneous tuberculosis

It is a manifestation of systemic disease, although it can be acquired by direct inoculation or by extension from a contiguous focus. It has different forms of presentation in immunosuppressed and immunocompetent patients such as verrucous tuberculosis or papulonecrotic tuberculous tuberculosis.

Gastrointestinal tuberculosis

Although the ileocecal location is the most frequent, can affect any part of the digestive tract. On endoscopy, it may present as a carcinoma or as warty or fistulized images.

Clinical manifestations in immunocompromised patients

The clinical presentation, radiology, severity and course of tuberculosis may be altered in the context of immunosuppression caused by some diseases such as solid neoplasms, hematological diseases, solid organ transplantation and HIV infection. The latter being the paradigm of the influence of immunosuppression on tuberculosis disease. In HIV-infected patients, exclusive pulmonary

involvement occurs in less than 50% of cases, with the remainder having exclusive or mixed extrapulmonary involvement. The signs and symptoms of tuberculosis in HIV-infected patients are often nonspecific and do not clearly distinguish the disease from other opportunistic infections.

The radiological presentation of pulmonary tuberculosis is highly variable, ranging from normal chest radiographs to extensive bilateral patterns. There are a large number of intrathoracic adenopathies. In extrarespiratory forms, peripheral lymph node involvement such as intrathoracic or intra-abdominal is very frequent. The development of explosive forms of tuberculosis has been reported at the beginning of antiretroviral treatment and anti-tuberculosis treatment. They do not have a worse prognosis or evolution and do not require specific treatment.

In patients with immunosuppression other than HIV, the typical presentation is extrapulmonary or disseminated. In solid organ transplant recipients, it has been associated with the use of anti-CD3 monoclonal antibodies or anti-TNF administration (Narita et al., 1998; DeSimone et al., 2000).

Mycobacterium leprae

M. leprae is an intracellular pathogen, cause of leprosy a chronic granulomatosis disease that affects the skin, peripheral nervous system, and mucous membranes. Patients with leprosy are classified as having paucibacillary or multibacillary disease. Paucibacillary (low numbers of organisms) disease is the milder form and is characterized by one or more skin macules. Multibacillary (large numbers of organisms) leprosy is associated with symmetric skin lesions, nodules, plaques, thickened dermis, and frequent involvement of the nasal mucosa. Person to person spread via inhalation of infectious droplet is the usual mode of transmission of *M. leprae* (WHO, 2020).

M. leprae incubation period can last up to 11 years. Several types of leprosy have been described, including indeterminate leprosy, tuberculoid leprosy, borderline tuberculoid leprosy, mid-borderline leprosy, borderline lepromatous leprosy and lepromatous leprosy. A few hypopigmented macules that can heal naturally, endure or advance to other types can be observed for intermediate leprosy. Tuberculoid leprosy is characterized by hypopigmented macules, with loss of thermic, tactile and pain sensations, and enlargement of nerves which can heal naturally, persist or advance to other types. In borderline tuberculoid leprosy, smaller but more lesions like tuberculoid leprosy and less nerve enlargement can persist, regress to tuberculoid leprosy or progress to other types. Mid-borderline leprosy is described by asymmetrically dispersed reddish plaques, with moderate loss of thermic, tactile and pain sensations, with swollen lymph nodes which can revert or progress to another type or persist. Numerous skin lesions including flat lesions, raised bumps, nodules and plaques and sometimes loss of thermic, tactile and pain sensations that can persist, revert to lepromatous leprosy or progress to other types are the characteristic features of borderline lepromatous leprosy. For lepromatous leprosy, initial lesions are observed as pale flat diffuse and symmetric regions that contains *M. leprae*, and initially hair loss is noted, and limb weakness, aseptic necrosis, lepromas, and disfigurement is observed as the disease progresses. Unlike other forms of leprosy, lepromatous leprosy does not regress to less serious types (Lockwood and Reid, 2001; Saonere, 2011).

In the past 20 years, 16 million cases of leprosy have been recorded according to the WHO. In 2013 to 2015 to the WHO, more than 200,000 new cases were reported each year, indicating that the disease is still progressing without hindrance. In 2017 and 2018 over 200,000 new cases were reported, whereby in 2018, 71% of cases was described in South East Asia, while the Americas and African region represented 15% and 10% of the new cases. The countries with most new cases were India, Indonesia and Brazil (WHO, 2020).

Mycobacterium ulcerans

Mycobacterium ulcerans is a strict pathogen of humans. Disease ranges from a localized nodule or ulcer to widespread ulcerative or nonulcerative disease, including osteomyelitis. If untreated, severe limb deformities with contractures and scarring are common. In Africa, the disease is referred to as Buruli ulcer, while in Australia, it is called Baringsdale ulcer. Nevertheless, disease mediated by *M. ulcerans* is the third most common mycobacterial disease in the world (Pfyffer, 2015; WHO, 2017a,b). In human, the unique pathogenic capacity of *M. ulcerans* is due to the secretion of a potent macrolide toxin, mycolactone, encoded by a plasmid that causes tissue damage and inhibits the immune response (Adusumilli et al., 2005). It is difficult to determine the true incidence of disease caused by *M. ulcerans* since it is not easily isolated in laboratories. Individuals of all ages and both sexes may be infected, many are children under 15 years of age (Marras et al., 2007).

Although *M. ulcerans* can lead to deep infections, such as osteomyelitis, the predominant clinical picture is cutaneous lesions. This may take the form of different clinical pictures depending on the stage of development of the disease. It initially appears as a painless papular or nodular lesion, which in women occurs on the upper extremities and in men on the lower extremities. Subsequently, due to subcutaneous necrosis, the epidermis becomes necrotic. Deep tissues may be affected. The lesions can evolve toward healing, giving rise to scars that can become disabling.

Nontuberculosis mycobacteria

Nontuberculous *Mycobacterium* spp. have been called a variety of names, from atypical mycobacteria to MOTT (mycobacteria other than TB), and today are most frequently called nontuberculous mycobacteria or NTM. For many years, the Runyon classification of *Mycobacterium* was utilized in clinical laboratories to provide a convenient way to differentiate among NTM based on the rate of growth of the organism from subculture and pigment production. With the increasing use of molecular and other nontraditional biochemical means of identification, this classification system is no longer universally used and has limitations. The increase of newly described species seen in the last few decades is in part the consequence of the availability and increased reliability of new DNA sequencing methods that can differentiate even closely related species and, in part, the consequence of an increased frequency of isolation of mycobacteria (Al Houqani et al., 2011; Adjemian et al., 2012). NTM can form biofilms on a wide range of organic (plastic, silicone, rubber and PVC) and inorganic (glass, metals, and metallic fluids of machines) materials due to their hydrophobic cell wall and their resistance to disinfectants, antibiotics or heavy metals.

According to the American Thoracic Society (AST)/Infectious Diseases Society of America (IDSA) 2007 statement, when NTM are suspected as the etiology of disease, definitive diagnosis should always be supported as the etiology of disease, definitive diagnosis should always be supported by repeated isolation of NTM from several specimens of the patient single specimen if is collected aseptically from a sterile body site (Griffith et al., 2007). Laboratory results should always be correlated with the individuals clinical presentation and radiological and histological findings to determine the clinical significance.

Mycobacterium avium complex

Pulmonary infection caused by The *Mycobacterium avium* complex (MAC) can occur in immunocompetent host; disseminated infections usually occur in people living with HIV. The most common presentations of MAC lung infections in immunocompetent individual are TB like apical fibrocavitary or interstitials nodular infiltrates and bronchiectasis. In children, cervical lymphadenitis is the most common presentation (Cassidy et al., 2009).

The MAC is acquired from the environment and is not thought to be spread from person to person. Recently, *M. chimera*, a species within the MAC, was implicated in causing disease from the use of contaminated heater-cooler devices used in open heart surgery (Haller et al., 2016; Perkins et al., 2016). Historically, the MAC included two species, *M. avium* and *M. intracellulare*. From *M. avium* specimens, four subspecies have been assigned; *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *silvaticu* (Turenne et al., 2007).

M. avium subsp. *avium* is associated with avian TB, while *M. avium* subsp. *hominissuis* is the agent of disseminated disease in humans AIDS with low CD4 counts, chronic lung diseases in patients with cystic fibrosis (CF), or cervical lymphadenitis in children. *M. intracellulare* is primarily a respiratory pathogen in humans, and an association with disseminated diseases is less common.

Mycobacterium kansasii

M. kansasii it is the second most common cause of NTM disease in some regions of the United States and different countries of Europe. The isolation of the bacteria in human specimens is almost always associated with disease. The primary manifestation of *M. kansasii* infection resembles that of pulmonary TB, with cavitary infiltrates in the upper lobes, but noncavitary and nodular bronchiectatic lung disease has also been observed. Risk factors include pneumoconiosis, chronic obstructive lung disease, malignancy, and alcoholism (Griffith et al., 2007; Falkingham III, 2015).

Mycobacterium mageritense. It was first isolated from a patient in Malmo, Sweden and it is usually recovered from sputum or cervical lymph nodes of children or adults with underlying chronic pulmonary disease (Griffith et al., 2007).

Mycobacterium xenopi

The clinical and radiological disease manifestations of this bacteria vary according to the patients immunological status, and can be classified into three groups: a cavitary form in patients with pre-existing pulmonary disease, a solitary nodular form in immunocompetent patients, and an acute infiltrate form in immunosuppressed patients. Extrapulmonary cases with joint and soft tissue infections have also been observed (Okano et al., 2016).

Mycobacterium haemophilum

This mycobacteria have special growth conditions (optimal growth temperature of 28°–30° and medium solid with hemin or hemoglobin) is often underrecognized. Most infections are reported in AIDS patients or with solid organ transplants, bone marrow recipients, or long term steroid user. The classical clinical presentation is multiple skin lesions or ulcerations appearing on the extremities that are occasionally associated with abscesses, fistulas, or osteomyelitis. In children caused cervical lymphadenitis.

Mycobacterium marinum

M. marinum is the cause agent of swimming pool or fish tank granuloma, which is the result of finger, hand, arm, elbow, knee or toe soft tissue injuring freshwater or saltwater due to punctures from fins of fish or shrimp or from cleaning of fish tanks or swimming pools. The typical presentation is a single papule on an extremity that may progress to a shallow ulcer, usually 2–3 weeks after inoculation. Severe complications may also include osteomyelitis of the bones and tenosynovitis and arthritis of the joints (Griffith et al., 2007).

Mycobacterium szulgai

Recovery of *M. szulgai* from the environment is rare and very unusual. Isolation of the bacteria in clinical laboratories almost always has clinical significance. The clinical presentation of pulmonary infections with *M. szulgai* is indistinguishable from that of TB and usually develops in middle aged men with alcohol abuse, smoking, obstructive pulmonary disease. Other extrapulmonary clinical manifestations may include cervical lymphadenitis, cutaneous infections, osteomyelitis, tenosynovitis, bursitis or disseminated infections in both AIDS patients and immunocompetent patients (Griffith et al., 2007).

Mycobacterium scrofulaceum. Its name is derived from the histological term scrofula, used to describe infections of the cervical lymph nodes by mycobacteria. It is a common cause of mycobacterial cervical lymphadenitis in children (Wolinsky, 1995).

***Mycobacterium abscessus* complex**

These organisms often cause infections of skin and soft tissue, severe lung infections can occur in persons with underlying chronic lung disease, including patients with CF, and disseminated disease. Although the taxonomy of organisms belonging to the *Mycobacterium abscessus* complex has been somewhat controversial, whole genome sequencing data strongly support the presence of three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense* (Tortoli et al., 2016).

M. abscessus is a frequently isolated respiratory NMT, after the MAC, and accounts for more than 80% of all rapidly growing mycobacterial respiratory infections. Patients with lung disease due to *M. abscessus* are usually white, female nonsmokers older than 60 years of age without predisposing factors. Predisposing conditions that are commonly associated with *M. abscessus* pulmonary infections are bronchiectasis with reticulonodular lung infiltration, CF and rarely lipoid pneumonia and gastrointestinal disorders.

***Mycobacterium chelonae* complex**

It is a pathogen a key human opportunistic pathogen, rapidly growing mycobacterium that is widely recoverable from human made environments as tap water or from fresh water and seawater. The most common clinical manifestations are skin, soft tissue and bone infections. Disseminated diseases have been described in immunocompromised individuals, especially in those receiving high dose steroids. Infection may also be associated with ophthalmic surgery or contact lens wear (keratitis). Pulmonary infections by *M. chelonae* are less common than are those by *M. abscessus* (Umrao et al., 2016).

***Mycobacterium fortuitum* complex**

Includes the species *M. fortuitum*, *M. peregrinum*, *M. senegalense*, *M. setense*, *M. septicum*, *M. porcinum*, *M. houstonense*, *M. boenickei*, *M. brisbanense* and *M. neworleansense*.

Frequently is associated with skin, soft tissue, and bone infections, while it rarely causes pulmonary diseases, except in cases of lipoid pneumonia, gastrointestinal disorders, or disseminated diseases. Skin and soft tissue infections are common after mastectomy and similar plastic surgery interventions or cardiac surgery.

Microbiological diagnosis

Although multiple advances have been made in the diagnosis of tuberculosis, no reliable, simple, point of care exists to definitively diagnose the disease. Clinicians often seek bacteriological diagnosis, but this evidence is also supplemented by clinical findings, radiological evidence.

The samples should be representative of location and symptoms. The most common location in TB and NTM infections is the lung. In expectorating patients 2–3 series samples of early morning sputum is sufficient. When there is no expectoration, samples may be obtained with a bronchoscopy, through bronchial suction or bronchoalveolar lavage. In children or in situations without access to bronchoscopy, gastric suction may be used as an alternative. With kidney disease series samples of early morning urine is used. In cases of tissue or organ compromise, the samples are obtained by puncture or preferably biopsy. If there is suspicion of disseminated infection, blood, bone marrow may be useful. Biological fluids, such as cerebral spinal fluid, articular pleural fluid or others fluid will be indicated in their clinical presentations, when it is not possible to obtain a relevant biopsy.

Achieving optimum sensitivity should take into consideration: obtainment of sterile recipients, appropriate minimum volume, transport, quality of the samples, repetition of multiples test in the samples reduces.

Microscopy

Direct smear microscopic examination with Ziehl-Neelsen (ZN) staining is commonly used to detect AFB, especially in low-income countries, owing to its rapidity, low cost and predictive value for detecting pulmonary TB. The ZN staining, which colors the mycobacteria red and the remaining structures blue. Furthermore, fluorescence microscopy (FM) using auramine O/auramine rhodamine staining has approximately 10% greater sensitivity than ZN with similar high specificity and rapid diagnosis. The FM method has been further improved by a more advances, cost effective and stronger light source using light-emitting diodes (LEDs). The florescent colored mycobacteria on a dark background. As with all laboratory procedures, strict quality control is needed for acid fast staining or rates of false positives and negative quickly rise.

Microscopy is the primary method of examination for people with TB symptoms, but it has limitations such as laborious and times-consuming procedures for the technician. The specificity of the stain is high, with few distinguishable exceptions from its morphology (*Nocardia*, *Rhodococcus*, *Tsukamurella*, *Cryptosporidium*, *Cyclospora* and *Isospora*) since the majority of the microorganisms do not stain red o fluorescent.

Because acid –fast artifacts may be present in the AFB smear, morphology must be examined for the presence of long and typically acid-fast rods or coccobacilli that may appear slightly curved, beaded, isolated, in pairs or in groups and stand out clearly the background. Using auramine O stain, acid –fast organisms stain yellow-green (if auramine and rhodamine are used, AFB will fluoresce orange-yellow. Depending on the mycobacterial species, different morphologies may be observed on stained AFB smears. When stained from liquid medium *M. tuberculosis* often demonstrates cording. Some NTM species can exhibit cording as well, albeit rarely, such as the *M. avium* complex, *M. kansasii*, *M. goodii*, *M. chelonae* and *M. marinum*.

Currently recommended that all positive AFB smears be confirmed either by a second reader, by retaining the slide (if a fluorescent stain was used) using a Ziehl-Neelsen stain, or by initially preparing two AFB smears, one for the fluorescent stain and other for the Ziehl-Neelsen stain.

The sensitivity is conditions by factors such as the extent of the disease, the prevalence, the sample volume, concentration of mycobacteria and above all, the sample type and number of organisms in the specimen, laboratory processing. The limit of sensitivity would be between 50,000 and 100,000 mycobacteria/ml overall sensitivity may range between 20% and 80% of processed samples, depending on their anatomical type and origin. In respiratory samples it is higher, particularly in carious lesions, and in tissues, such as enlarged lymph nodes (50–80%). Sensitivity is very poor in biological fluids due to the low concentration of mycobacteria.

The stain from the concentrate after decontamination of the sample for the culture is 11–26% more sensitive than that directly made from the sample. Stains may be closely observed, at least 300 fields in optic microscope and 30 for florescent staining, prior to issuing a negativity report.

The optimum number of sputum specimens to establish a diagnosis has been examined in several studies that have served to support recommendations to decrease the minimum number of sputum specimens examined from 3 to 2, assuming that they are examined in a quality–assured laboratory. In a systematic review of 37 studies on the yield of sputum AFB smear microscopy, it was found that, on average, the initial specimen was positive in 85% of the all patients ultimately found to have AFB detected. The second specimen was positive in an additional 11, 9% of patients, and the third specimen was positive in a further 2.3% of patients.

Culture methods

The culture is the most sensitive diagnostic method. For the isolation of mycobacteria, the use of a liquid medium in combination with at least one solid medium is essential for good laboratory practice (Esther et al., 2011) It has been estimated that detection limit between 10 and 100 bacteria/ml. Its main drawback is the long incubation period, from several days up to 6 weeks before a final negativity report may be issued. Samples where flora or other bacteria are present, such as those of respiratory origin, they should be previously eliminated through a chemical decontamination process with the preservation of mycobacteria. The methods most well known is based on the use of NaOH and N-acetyl-L-cysteine (Kubica method). Of significance, some decontamination/concentration procedures are know to be compatible with egg-based media only and may not be used with any other media not containing egg yolk. These procedures include the use of Zephirantrisodium phosphate (Z-TSP), sodium lauryl sulfate, cetylpyridinium chloride or other quaternary ammonium compounds. The proportion of cultures will be contaminated despite decontamination, between 3% and 5% is considered acceptable, although it depends on the type of samples of each laboratory. Samples which contain *Pseudomonas aeruginosa*, capsulated bacteria, fungi and yeast, among others, will be more likely to contaminate the cultures. At present, for primary isolation from clinical samples the combined use of a liquid culture and solid culture is recommended. The most well known of both types are Lowenstein–Jensen and Middlebrook 7H11. The majority of liquid media used in diagnosis are semi-automated, based on the use of incubators which are capable of detecting growth by O₂ consumption or production of CO₂. They usually use Middlebrook 7H9 as the base medium, with enriched supplements and antibiotic solutions to neutralize microorganisms which have resisted decontamination, Three methods are the most common: VersaTREK (Thermo Fischer Scientific, Oakwood Village, OH, US), BactT ALERT 3 D (BioMerieux, Marcy L' Etoile, France) and MGIT960 (Becton Dickinson, Sparks; MD, US).

The cultures for the majority of mycobacteria are incubated a 35–37 °C. The species which cause skin and soft tissue diseases, such as *Mycobacterium ulcerans*, *Mycobacterium marinum*, *Mycobacterium haemophilum* and *Mycobacterium chelonae*, grow better at 25 °C and 30 °C. In contrast, *Mycobacterium xenopi* grows better a 42 °C and *Mycobacterium thermoresistibile* at 52°. Others species need specific supplements to grow in cultures, *M. haemophilum* which requires hemin, hemoglobin of ferric ammonium citrate solution

or *Mycobacterium genavense*. Tubes media should be incubated in slanted position with screw caps loose for at least 1 week to ensure an even distribution of the inoculum. Plated medium, ideally placed into CO₂ permeable plastic bags or sealed with CO₂--permeable tape, should be incubated with the medium side down if all the inoculum has not been absorbed.

The cultures are monitored continuously in liquid culture equipment, detecting potential positivity when they are produced. The solid cultures are inspected 1–2 times/week for the first 3 weeks and once during the following 3 weeks. All solid media should be examined within 3–5 days after inoculation to determine if there is contamination. The performance in culture is observed with the use of automated liquid cultures. With regard to the detection period of positive results, the majority become positive between the first and third week. The mean is 14 days. For samples with positive bacilloscopy, the positivity drops to 13 days for *M. tuberculosis complex* isolation. When the bacilloscopy is negative, this takes place between 17 and 16 days for MTC and NTM respectively. The solid cultures are positivized between the second and fourth week. Overall, almost 100% of solid and liquid cultures are positivized during the first 4 weeks. Recovery in liquid media is considerably superior to that of solids, especially for isolation of NTM. Specimens with positive AFB smears that are culture negative should be held an additional 4 weeks. Culture negative specimens that are positive for mycobacteria by one of NAATs or for cases with a high suspicion of tuberculosis should also be held an additional 4 weeks.

Nucleic acid amplification test

Algorithms for testing specimens from patients suspected of having tuberculosis should include a nucleic acid amplification test (NAAT). Guidelines from the CDC recommend the performance of a TB NAAT on at least one respiratory specimen from patients with signs and symptoms consistent with pulmonary tuberculosis. This is especially important if diagnosis of tuberculosis has not yet been made and the test result would alter patient care and/or TB control activities and can predict drug resistance (CDC, 2009). The NAAT should ideally be performed on all first time patients with suspected TB, and results should be reported within 48 h of specimen receipt.

It is important to consider that the sensitivity of amplification techniques is lower than that of the culture in those samples with negative stain, and therefore they cannot be used to rule out disease. Although the specificity of NAATs for tuberculosis diagnosis is very high, the sensitivities vary widely and depend on both the AFB smear status of the specimen (positive or negative) and the specimen type (Cepheid, 2015). Disadvantages of the molecular detection of the *M. tuberculosis complex* directly from clinical specimen include the inability to differentiate among species within the *M. tuberculosis complex*. In addition, a positive NAAT results does not differentiate between live and dead organisms. For this reason, amplification technologies should not used on specimens collected from patients who have received antituberculosis drugs for more than 7 days or have received such therapy in the last 12 months prior to collection. Furthermore, approximately 4% of pulmonary and 19% of extra pulmonary specimens have substances that are inhibitory to amplification (determined by a failed internal amplification control). Since it is rare that all specimens from a given patient show inhibition, testing of multiple samples can be advantageous (Hologic, 2013; Cheng et al., 2005).

To increase the sensitivity of these test and achieve similar results to the culture, the inhibitors found in the sample must be efficaciously eliminated to detect ≤ 10 –100 mycobacteria/ml, the lower limit of culture detection.

In 2010, the Xpert MTB/RIF (Cepheid, Sunnyvale, CA, US) appeared as a totally automated method, with minimum handling prior to the introduction of the sample in the cartridge where the whole reaction was to take place. It is authorized for use with AFB smear –positive and AFB smear negative expectorated and induced raw sputum specimens as well as processed sputum sediment (Cepheid, 2015). The Xpert MTB/RIF technique consist in a nested PCR at real time of the *rpoB* gene, which codes for sub-unit of the polymerase RNA. It uses 5 genetic probes of molecular beacon type, each one marked with a different fluorophore. It totally covers the area of 81 base pairs, between codons 426 and 452 of the *rpoB* gene determinant of rifampicin resistance. In this way it simultaneously reports the presence of MTC and the most frequent mutations in the *rpoB* gene, predicting resistance to rifampicin which in the majority of cases is connected to multiresistance. The low sensitivity of this system in paucibacillary samples has driven the same company to develop an Xpert MTB/RIF Ultra system which collects a larger sample volume than the previous model and adds 2 new targets which are found in a greater number in the MTC (IS6110 y IS1081) genome. Both changes have successfully increased sensitivity, which has led the WHO to propose its use, particularly in cases of patients infected by HIV and pediatric patients (WHO, 2017a, b). Other methods available on the market in our environment for the direct detection are those produced by Hain Lifescience Fluorotype MTB (Brucker) is a semi automated method which has recently been marketed, based on real time PCR. Initial assessment indicates a specificity of 98.9% with sensitivity of 100% for samples with microscope positivity and 56,3% for microscope negativity (Hofmann-Thiel et al., 2010). In order to increase sensitivity in diagnosis recent literature reports different proposals which may possibly be introduced in the future. It combines several DNA targets with several lavages to reduce the presence of the qPCR inhibitors with posterior amplification of 2 specific MTC targets, IS6110 and *sen X3-regX3* (XtractTB system) (Reed et al., 2017), to raise test sensitivity and specificity and minimize the probability of false negatives.

Lastly although trends lie in the use of commercial automated or semi-automated test, the WHO has recommended the use of loop mediated isothermal amplification or LAMP technique in its rapid diagnostic strategy of TB (WHO, 2016). Amplifies target DNA with high specificity, efficiency and rapidity under isothermal conditions requiring only a heat block and allows to detect visually DNA directly from clinical sputum samples without purification process of DNA, in less than 2 h and minimal equipment.

Detection of mycobacterial antigens in urine

Detection of *M. tuberculosis* antigens in urine may have some usefulness in diagnosing TB in patients with advanced AIDS, particularly in countries where coinfection is highly prevalent. Urine based antigen testing is attractive at the point of care (POC) level because urine is easy to collect and store, and lacks the infection-control risks associated with sputum collection. Especially, urinary detection of mycobacterial lipoarabinomannan (LAM) as a target antigen has emerged as potential POC test for active TB and/or HIV patients. LAM antigen is a lipopolysaccharide present in glycolipids of mycobacterial cell walls, which is released from metabolically active or degenerating bacterial cells and appears to be present only in people with active TB disease and/or with severe immunosuppression in their urine (McNerney and Daley, 2011; Chatterjee et al., 1991). A simple low cost lateral flow version, urine lateral flow LAM (LF-LAM) assay has been developed, and this lateral flow device is easy to perform rapidly within 30 min at the POC test diagnosis of TB and/or VIH using the Determine TB-LAM (Alere, Waltham, MA, USA). LF-LAM has been evaluated and reviewed in several studies for the detection of active TB infected with HIV, who are severely immunocompromised, and WHO is not recommended for diagnosis of TB (pulmonary or extrapulmonary) the use of LF-LAM, with the exception of people living with HIV, who has low CD4 counts or who is seriously ill in 2015 (WHO, 2015).

Genetic methods applied to the direct samples in diagnosis of nontuberculosis mycobacteria (NTM)

Molecular diagnosis of the NTM directly in the clinical sample has been the focus of less attention than the diagnosis of TB, probably due to incidence and epidemiological consideration. Anyplex Plus MTB/NTM combines the identification of MTC and of *Mycobacterium* sp. in a single amplification in real time. Another would be the Genotype NTM DR (Hain Lifescience Bruker) test which is able to detect mutations of resistance to macrolides and aminoglycosides while simultaneously identifying several species of NTM frequently isolated in clinical samples such as *M. chelonae*, *M. avium*, *M. intracellulare*, *M. chimera* and 3 sub-species of *M. abscessus* (sub. abscessus, sub bolletii and sub massiliense). No contrasted experience exists as of yet with regard to its use directly on clinical samples.

Immunological diagnosis

Individuals infected with *M. tuberculosis* with no clinical evidence of disease have latent tuberculosis infection (LTBI). They have been infected with *M. tuberculosis* but do not have TB disease. To test LTBI, a skin or blood test is performed to detect a cellular immune response to tuberculosis antigens, indicating prior infection.

The tuberculin skin test (TST) has been performed by injecting a small amount of TB-purified protein derivative (PPD) into the skin in the lower part of the arm. A person given the TST must return within 48–72 h to have a trained health care worker look for a reaction on the arm. If the skin area reacted to the antigen and is larger than or equal to 10 mm, it is considered a positive reaction (Froeschle et al., 2002). However, the TST gives false-positive results in people who have been vaccinated with *Bacillus Calmette–Guérin* (BCG), a vaccine for TB, non tuberculous mycobacterial infection, and false-negative responses in immunocompromised individuals. False negative reaction occur in at least 20% of all persons with known active TB. Protein malnutrition diminishes all cutaneous delayed hypersensitivity reactions. Sarcoidosis may cause false negative TST results. Vaccination with live virus vaccines (measles, smallpox), corticosteroid therapy may cause false negative.

Although tuberculin cannot sensitize an uninfected person, it can restimulate remote hypersensitivity that has deteriorated. This booster effect (a positive tuberculin test result after a negative one) develops within several days after a first injection and may be persistent. This problem is circumvented by retesting nonreactive 1–3 weeks after the initial test. If the second test result is positive, this indicates boosting rather than recent tuberculin conversion.

Others test, with interferon gamma release assay (IGRA) measures host cellular immune response to *M. tuberculosis* antigens in whole –blood samples.

The presently available blood IGRA is QuantiFERON-TB Gold In-Tube test (QFT-GIT, Cellestis, Australia) and T-SPOT.TB test (T-Spot, Oxford Immunotec, UK) which are the US FDA approved as indirect and adjunct tests for the TB infection (WHO, 2011). For the QFT-GIT test, fresh blood containing viable white blood cells is incubated with controls and single mixture of synthetic peptides representing ESAT-6, CFP-10, and TB7.7. Concentration of IFN- γ released in the plasma separated from blood is determined by enzyme-linked immunosorbent assay (ELISA). The next generation QuantiFERON-TB Gold Plus (QFTPlus) test has been FDA approved and is stranding to be rolled out in the United States and Europe. QFTPlus contains a fourth tube with short peptides from CFP-10, which are designed to elicit increased CD8⁺ T cell production of interferon- γ . There are only a few studies comparing the QFT-GIT and QFTPlus, and these suggest comparable results or perhaps slightly improved sensitivity of the QFTPlus in elderly or immunocompromised populations. For T-Spot test, peripheral blood mononuclear cells (PBMCs) are incubated with controls and two mixtures of peptides representing ESAT-6 and CFP-10, and the number of IFN- γ producing cells (spots) in the response to antigens is measured (Mazurek et al., 2010).

These test overcome several limitations of the TST including false positive results from environmental mycobacterial exposure or BCG vaccination, operator-dependent variability in test placement and regarding, and the need for a follow-up visit to examine skin induration.

Data on the performance of the interferon- γ release assays in children are limited, and guidelines differ on the optimal test. The ATS/IDSA/CDC guidelines recommend use of the same criteria as in adults for children aged 5 years and older. For children younger than 5 years, these guidelines recommend the TST, recognizing the limited data.

Antimicrobial susceptibility testing for the *Mycobacterium tuberculosis* complex

Antimicrobial susceptibility testing (AST) should be performed on at least the initial isolate from each patient with TB. The AST can be repeated after 3 months or earlier, if a patient remains culture positive and/or is not responding to treatment (Lewinsohn et al., 2017). Once a culture has been confirmed as *M. tuberculosis* complex, AST should be performed by a growth based method. This method should include at a minimum, AST for the first-line antituberculous drug rifampin and isoniazid (2 concentrations), ethambutol and pyrazinamide. If Rifampicin resistance or resistance to any two these drugs is detected, testing for susceptibility to additional second line drugs should be performed.

There are others genotypic testing, which correlates specific mutations in the isolates genome with known drug susceptibility patterns.

The agar proportion (AP) method is most commonly used to determine phenotypic drug resistance. The AP compares growth on the of appropriately diluted inocula on drug containing media versus growth on the drug-free media and is reported as proportion resistant. For most drugs, resistance is significant when growth on drug containing media exceeds 1% control; 6–10% resistance or more indicates that the drug will and nothing to multidrug therapy. Liquids broth systems, such as BACTEC, in MGIT tubes containing the critical concentrations of drugs can be inoculates from solid or liquid *M. tuberculosis* isolates. A categorical susceptibility or resistant result is provided. The VersaTREK method is an automated system used for growth and detection of mycobacteria as well as AST. Both methods are FDA cleared for rifampicin, isoniazid, ethambutol, pyrazinamide.

The TREK Sensititre method (ThermoFischer Scientific, Oakwood Hills, OH) is a semiautomated research use only broth microdilution method for susceptibility testing of mycobacteria in a microtiter plate format. A total of 12 drugs are provided, each with a minimum of 7 dilutions on the standard plate. This test determines MIC values, but there are no guidelines for the interpretation of susceptibility or resistance results.

Molecular methods detect mutations in genes associated with resistance to anti-TB drugs. These methods are rapid and specific, but phenotypic AST must still be performed, as genotypic resistance may not always translate to phenotypic resistance.

Most commonly used are test for rimpacin resistance, which predicts poor treatment outcomes and is a surrogate marker for multidrugs TB. Assays detect mutations in the 81 base pair *rpoB* gene, which encodes the β subunit of RNA polymerase and correlates with greater than 96% of RIF resistance. Resistance to isoniazid is more complex and is encoded by multiple genes, including the catalase peroxidase gene *kat G*, the gene *ihnA* gene involved in fatty acid biosynthesis. The X pert assay is an FDA cleared, rapid, real-time PCR test that simultaneously detects the presence of the *M tuberculosis* complex and the mutations in the RRDR of *rpoB*. The Genotype MTBR plus Version 2 assay is a PCR based line probe assay for the detection of *M. tuberculosis* complex, mutations in the *rpoB* gene associated with resistance to rifampicin, and mutations in the *lhnA* and *KatG* genes associated with resistance to isoniazid. These fast test can be performed directly on the specimen or from culture isolates. Genotype MTBDRsl assay, which detects resistance to fluoroquinolones and second line injectable drugs in culture isolates and smear positive sputum samples and thereby can be used to identify XDR TB. Sequencing methods can be used to target a variety of genes to determine mutations associated with resistance. These sequencing methods are laboratory developed test but can still play an important role in AST for the *M. tuberculosis* complex.

Antimicrobial susceptibility testing for nontuberculous mycobacteria

The role and relevance of in vitro AST of nontuberculous micobacteria to guide the treatment and clinical management of patients with NMT disease are under continuous debate. The base of this debate is that the clinical response to antimycobacterial drugs or antibiotics has been shown to correlate with only some compounds and in only some NMT. On the other hand, most clinically significant NTM isolates already show intrinsic resistance or high in vitro breakpoints for several antibiotics upon first isolation (Brown-Elliott et al., 2012).

The gold standard method for AST of rapidly and slowly growing NTM is broth microdilution assay, a commercial research use only product is the TREK Sensititre plate. AST of rapidly growing NTM should have rigorous quality control using CLSI recommended reference strains to ensure not only quality testing but also reproducibility of MICs within the recommended and acceptable ranges from antimicrobials tested. Isolates that are susceptible to clarithromycin should be further incubated for 14 days to rule out inducible macrolide resistance. MIC results for imipenem, meropenem and tetracycline may be invalid after more than 5 days of incubation because of stability related problems with these drugs (Brown-Elliott et al., 2012. Genotype NTM-DR ver 1.0 (Bruker) detect resistance macrolide and aminoglycosides *rrl* gen, *erm* gen and *rrl* gen. (Brown-Elliott et al., 2015).

For slowly growing NTM recommendations of the CLSI include clarithromycin susceptibility testing for new and previously untreated MAC isolates. There is no recognized value for testing of MAC isolates against first line antituberculosis drugs. Previously untreated *M. kansasii* isolates should be tested against rifampin only. If resistance to rifampin is detected, should be tested against a panel of secondary agents, including ethambutol, isoniazid, clarithromycin, fluoroquinolone, amikacin, sulfonamides and linezolid.

M. marinum isolates do not require susceptibility testing unless the patients fails treatment after several months. There are no current recommendations for one specific method of in vitro AST for fastidious NTM species (*M. haemophilum*) and some less commonly isolated NTM.

Treatment of tuberculosis

Treatment regimens that meet all the favorable requirements and have been universally recommended are 6 months with rifampicin, isoniazid, ethambutol and pyrazinamide for the first 2 months, followed by 4 months with rifampicin and isoniazid.

When the isolate is identified as sensitive to these drugs, ethambutol can be withdrawn. Exceptionally in paucibacillary cases, three drugs could be used in the initial phase.

Children generally have a good tolerance to pharmaceuticals. Treatment is the same as for adults, but the dose is adjusted to weight. The regimen should not be changed during pregnancy or breast-feeding.

Treatments in special situations

1. Patients with chronic liver disease

Treatment should be attempted with a standard regimen, with close monitoring of liver function. In cases of advanced liver disease, one of the three most hepatotoxic drugs, preferably pyrazinamide, should be withdrawn.

In the case of acute hepatitis or terminal stages of chronic liver disease, treatment without potentially toxic drugs should be chosen. These include a quinolone, an injectable and ethambutol or cycloserine.

2. Chronic renal failure

No modification of standard treatment is necessary. Only in patients with a creatinine clearance of less than 30 ml/min or in patients on hemodialysis programs it is recommended to administer the treatment 3 times a week, maintaining the same dose as the daily regimen.

3. Extrapulmonary forms of tuberculosis

It is usually recommended to use the same treatment regimens as for pulmonary tuberculosis and to prolong the duration of treatment in some special situations, e.g. tuberculous meningitis and tuberculous spondylitis.

4. Administration of corticosteroids

In the first weeks of meningeal forms and in pericarditis.

5. Administration of pyridoxine

Isoniazid may cause peripheral neuropathy due to deficiency of this substance in certain situations. Prophylactic pyridoxine supplementation is recommended in patients with alcoholism, malnutrition, pregnancy, diabetes, renal insufficiency and HIV co-infection.

6. Treatment in HIV-infected patients

Rifampicin is a potent inducer of cytochrome P450 and accelerates the metabolism of many antiretroviral drugs, and lowers their plasma levels. Of the antiviral drugs, efavirenz is the drug of choice for treating HIV-infected patients who must also receive rifampicin.

Treatment of tuberculosis infection

Screening for possible infection should be done with a view to the possibility of initiating treatment in cases where it has been best demonstrated to be effective. (REF 7 T), such as in recent infection, HIV infection, recurrent lesions on chest X-ray and without previous treatment and in the case of patients who are to start treatment with anti-TNF- α .

The most studied treatment with proven efficacy is isoniazid. This efficacy would increase if given for 6 months or longer (up to 9 or 121 months). In co-infection with HIV, the usual recommendation is to extend it for 6–9 months if isoniazid is used. An alternative to isoniazid is rifampicin, especially when there is resistance to isoniazid, being used for 4 months. Finally, there is the possibility of using rifampicin and isoniazid for 3 months, with similar efficacy to the isoniazid regimen and better compliance (Ena and Valls, 2005; Spyridis et al., 2007).

In MDR-TB contacts, there is no recommended and proven effective scheme, so it is advisable to maintain surveillance with regular visits.

Treatment of infections caused by *Mycobacterium leprae*, *Mycobacterium ulcerans* and non-tuberculous mycobacteria

Mycobacterium leprae

The guidelines recommend a 3-drug regimen of rifampicin, dapsone and clofazimine for all patients with leprosy, with a treatment duration of 6 months for paucibacillary leprosy and 12 months for multibacillary leprosy. This represents a change from the current standard treatment for paucibacillary leprosy, which is rifampicin and dapsone for 6 months, due to some evidence indicating better clinical outcomes with a 3-drug, 6-month regimen over a 6-month regimen. For rifampicin-resistant leprosy, the guidelines recommend treatment with at least two second-line drugs (clarithromycin, minocycline), or quinolone plus two second-line drugs (clarithromycin, minocycline or quinolone) plus clofazimine per day for 6 months, followed by clofazimine per day for 6 months, followed by clofazimine plus one of these drugs for an additional 18 months. When ofloxacin resistance is also present, a fluoroquinolone should not be used. Fluoroquinolone should not be used as part of second-line treatment. The regimen of choice in such cases will consist of 6 months of clarithromycin, minocycline and clofazimine followed by clarithromycin, minocycline and clofazimine for an additional 18 months.

Mycobacterium ulcerans

It has been considered a disease of basically surgical treatment based on wide debridement and grafting. However, several studies suggest better results with early combination of drug treatment with a combination of rifampicin, clarithromycin and streptomycin (Nienhuis et al., 2010).

Mycobacterium avium complex

The introduction of macrolides (clarithromycin and azithromycin) has been a major breakthrough in the treatment of MAC infection. They have improved response rates compared to the classical regimens based on rifampicin, isoniazid and ethambutol in both pulmonary and disseminated HIV infection. Clinical response and in vitro resistance only correlates well with macrolides, where an MIC of 32 is clearly associated with treatment failure.

In MAC disseminated infection, treatment should be maintained for a minimum of 12 months, and may be suspended in patients with effective antiretroviral treatment who achieve virological suppression and CD4 counts above 100 cells for more than 3–6 months. The treatment of choice is the combination of clarithromycin and ethambutol.

In the case of pulmonary MAC infection, a combination of macrolide, ethambutol and rifampicin should be included. Differentiating between fibrocavitary (with the addition of an aminoglycoside) and nodular bronchiectasis forms is necessary for treatment. The duration should be 18–24 months and a minimum of 12 months from culture negative. Cervical lymphadenitis in children due to MAC can resolve spontaneously or produce spontaneous fistulas. The best results are obtained with surgical excision of the affected ganglia, although it is not clear whether it is necessary to add pharmacological treatment.

Mycobacterium Kansasii

The rifampicin, isoniazid and ethambutol regimen can be used for a minimum of 12 months from culture negative. In the initial phase or until sensitivity is known, a fourth drug (streptomycin, clarithromycin or quinolones) may be added. The in vitro activity of isoniazid against *M. kansasii* is lower than against *M. tuberculosis*. Despite this, treatments are effective, with rifampicin resistance accounting for the majority of therapeutic failures.

Mycobacterium xenopi

Sensitivity studies are difficult to interpret because of its slow growth, although it is usually considered sensitive to rifampicin, ethambutol, clarithromycin and high concentrations of isoniazid. It is recommended to treat with combinations of these drugs for 18–24 months, and a minimum of 12 months from negative culture in pulmonary disease (Philly and Griffith, 2015).

Mycobacterium malmoense

It is treated on schemes similar to those used against MAC, and is considered sensitive to rifampicin, ethambutol and clarithromycin, despite difficulties in the interpretation of in vitro sensitivity studies (Hoefsloot et al., 2008).

Mycobacterium szulgai

It is usually sensitive to rifampicin, isoniazid, ethambutol, quinolones and macrolides. Treatment with at least three active drugs for a minimum of 12 months from negative culture in lung disease is recommended (Tortoli et al., 1998).

Mycobacterium marinum

Systematic sensitivity studies are not recommended, as surgical excision of the lesion is often curative. It is usually sensitive to rifampicin or ethambutol, as well as tetracycline, cotrimoxazole or clarithromycin. Empirical treatment with ethambutol associated with clarithromycin and sometimes rifampicin is usually considered for at least 2 months after clinical cure (Aubry et al., 2000).

Rapidly growing mycobacteria

A special feature of fast-growing mycobacteria is their antimicrobial sensitivity, which is very different from that of slow-growing strains. In general, most fast-growing strains are resistant to the usual anti-tuberculosis drugs (isoniazid, rifampicin, ethambutol, pyrazinamide, and streptomycin), but are usually sensitive to other antibiotics used in the treatment of various bacterial infections, such as macrolides, linezolid, quinolones, imipenem or tigecycline.

It is considered necessary to perform sensitivity studies on those isolates that are considered significant as there is a wide variety of sensitivity patterns. In general, most strains are sensitive and species are sensitive to amikacin and have low MICs to tigecycline (although no specific cut-off point has been established for this antibiotic). *M. abscessus* and *M. chelonae* are usually the most resistant species, and macrolides (except for strains possessing inducible methylase), cefoxitin and amino-glucosides are useful in these cases. *M. fortuitum* complex are usually sensitive to quinolones, aminoglycosides, cotrimoxazole and linezolid, while they are usually resistant to macrolides. Other species such as *M. peregrinum*, *M. mucogenicum* or *M. mageritense* are usually sensitive to numerous antibiotics (Esteban et al., 2012; Brown-Elliott and Philley, 2017).

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Leptospira, Borrelia and Treponema

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Leptospira

Introduction

Leptospire belong to the order *Spirochaetales*, family *Leptospiraceae*, genus *Leptospira*. The traditional system divided the genus into two species: the pathogenic *L. interrogans* and the non-pathogenic *L. biflexa*.

Currently, *Leptospira* can be genetically classified based on DNA hybridization technique into at least 20 species: pathogenic species, intermediate, or saprophytic (Fouts et al., 2016; Levett, 2015). *Leptospira* species are further divided into hundreds of serovars, defined by cross-agglutinin absorption testing with rabbit antiserum (Naze et al., 2015). For convenience, serovars are often grouped into serogroups, which have no taxonomic significance but can be identified by microscopic agglutination testing (MAT) of acute and convalescent serum from infected patients (Haake and Levett, 2015). The genetic classification of *Leptospira* does not correlate with the phenotypic classification because serovars of the same serogroup may be distributed between different species.

Pathogenic species causes leptospirosis. The classical description of leptospirosis is that of Weil's disease, an acute, febrile and sometimes epidemic illness characterized by jaundice, splenomegaly and nephritis. Some infected persons, however, may have no symptoms at all.

Transmission

Leptospirosis is a zoonotic disease transmitted directly or indirectly from animals to humans (Faine, 1982). About 160 mammalian species have been identified as natural carriers of pathogenic leptospire. These include feral, semi-domestic and farm and pet animals as important infection sources. The disease occurs worldwide but is more common in tropical and subtropical areas. Infection usually results from direct or indirect exposure to the urine of infected animals, or indirect exposure through contaminated soil and water (Faine, 1982). It is an occupational hazard for many people who work outdoors or with animals, such as farmers and veterinarians. Leptospirosis has also been associated with swimming, or people who work in contaminated water. Human-to-human transmission occurs only very rarely. Transmission may occur via sexual contact and mother's milk. In-uterus transmission can lead to reproductive failures such as abortion.

Clinical significance

Leptospire can enter the body through cuts and abrasions in the skin, through the mucosa (conjunctiva, respiratory tract); They can occasionally enter in the human body via the inhalation of droplets of urine or via drinking-water.

After infection, leptospire appear in the blood and invade practically all tissues and organs. They are subsequently cleared from the body by the host's immune response.

The incubation period for leptospirosis ranges from 2 to 20 days, with an average of 10 days, but in rare cases very long incubation periods have been reported (Faine, 1982; Kelly, 1992). The infection is associated with a very broad spectrum of severity, ranging from subclinical illness to a self-limited biphasic illness, and a severe potentially fatal illness. Biphasic systemic illness has been seen in approximately 90% of infection. The initial, or systemic phase begins abruptly with fever, headache, myalgia, nausea and vomits. Skin rash, pharyngitis and conjunctival injection develops in the first or second days. Often the skin is warm and erythematous, with the patient complaining of feeling warm. Psychological changes are often seen, with patients feeling depressed, confused, aggressive and sometimes psychotic. After 3–7 days, patient improves for 1–3 days, reflecting the beginning of the immune response; the second or immune phase begins. Immune phase is generally shorter and slighter than systemic phase. The initial symptoms and fever return, accompanied with chest and abdominal pain, some renal problems and psychological changes. Meningitis and leptospire in urine are the most important features.

Distinct phases of illness may not be observed, particularly for patients with severe disease (Levett, 2001). About 10% of patients develop icteric severe leptospirosis. Symptoms are the same as for the mild type but more pronounced, and multiple organs are damaged—liver and kidney failure can appear, leading to jaundice and may cause potentially fatal conditions such as Weil's syndrome and the emerging Severe Pulmonary Hemorrhagic Syndrome (SPHS).

In a recent systematic review, mortality was low in patients with anicteric leptospirosis or meningitis (median, 0%), but mortality increased with patient age and was higher in patients with jaundice (19.1%) or renal failure (12.1%) (Taylor et al., 2015).

Immune response

Humans react to an infection by leptospire by producing specific anti-*Leptospira* antibodies. In the initial phase, patients with leptospirosis may produce antibodies that react with several serovars (cross-reaction) but in the course of weeks or months cross-reactive antibodies gradually disappear, while serogroup- and serovar-specific antibodies often persist for years. The genus-specific antibodies usually remain detectable for months, the serovar specific antibodies for years.

Seroconversion may occur as early as 5–7 days after the onset of disease but sometimes only after 10 days or longer. IgM class antibodies usually appear somewhat earlier than IgG class antibodies, and generally remain detectable for months or even years but at low titer.

Diagnostic

Laboratory diagnosis included: culture, detection of specific immune response and detection of specific DNA by polymerase chain reaction (PCR).

Culture and isolation

During the systemic phase, the most suitable sources to culture are blood and CSF. After the first 7 days of illness the most appropriate source for isolation of leptospira is urine.

1 or 2 drops of blood are inoculated into several tubes, each containing 5 mL of a suitable medium semisolid medium such as Fletchers, Ellinghausens or polysorbate 80 (Faine, 1982; Sulzer and Jones, 1978). Inoculate 3–5 tubes. For CSF, a volume of 0.5 mL of sample per 5 mL of medium is recommended. For urine one drop of undiluted urine is inoculated into a tube containing 5 mL of culture medium. Alternatively, urine samples may be centrifuged and the pellet resuspended in medium, after which 10-fold serial dilutions are made immediately in 1 or 2 additional tubes. Cultures should be incubated at 30 °C and examine a drop of culture by dark-field microscopy regularly for a period of 4–6 months, although leptospire are usually detectable in positive cultures within 1–2 weeks.

Isolation of *Leptospira* spp. from clinical samples has low diagnostic sensitivity, requires specialized expertise, and most importantly takes too long to be of use to the treating team (Picardeau, 2013).

Serological test

Anti-*Leptospira* antibodies become detectable around day of illness 5–7.

Microscopic Agglutination Test (MAT) and Enzyme-linked Immunosorbent Assay (ELISA) are the methods generally used in laboratory diagnosis.

The MAT is a micro-agglutination test carried out using live antigen pools, and detects both immunoglobulin M (IgM) and immunoglobulin G (IgG) class agglutinating antibodies. Serially diluted from the dilution of 1:100, serum specimens were added to the antigen suspension in 96 well round bottomed microtiter plates. The plates are gently shaken, covered and incubated for 2 h at room temperature. Agglutination was examined using dark field microscopy. The reciprocal of the highest dilution agglutinating at least 50% of the *Leptospira* organisms, was considered the end point.

A serologically confirmed case to leptospirosis is defined by a fourfold rise in MAT titer between acute-phase and convalescent serum samples run in parallel. A titer of at least 1:800 with compatible symptoms is strong evidence of recent or current infection (Faine, 1982).

Limitation to MAT is the requirement for paired acute and convalescent serum samples for optimal performance, low sensitivity when acute serum samples are tested (Cumberland et al., 2001), and cross-reactivity with Lyme disease, legionellosis, autoimmune disease, and viral hepatitis (Bajani et al., 2003) and in regions where leptospirosis is common, there may be a substantial proportion of the population with elevated titers.

The IgM ELISA test detects early production IgM and therefore can become positive earlier than the MAT, but suffers from both false positive and false negative problems. IgM-ELISA's sensitivity ranges from 48% to 100% and specificity ranges from 88.6% to 98% (Niloofa et al., 2015).

An immunochromatographic test is available that identifies specific IgM and provides results in 15 min with sensitivity and specificity similar to the ELISA tests (Niloofa et al., 2015).

Polymerase chain reaction

A variety of molecular techniques, including conventional and real-time polymerase chain reaction (PCR), have been developed for the specific detection of pathogenic *Leptospira* species (Jesse et al., 2016). PCR allows the diagnosis in early stages, before the appearance of specific antibodies. Specific DNA can be detected from blood, CSF, urine and tissue although serum is the optimal sample. A limitation of PCR-based diagnosis of leptospirosis is the inability of most PCR assays to identify the infecting serovar (Bal et al., 1994).

A study on 103 patients of meningitis of unknown cause showed that 39.08% were positive by PCR, 3.88% by ELISA & 8.74% by MAT (Romero et al., 1998). The use of real-time RT-PCR for testing serum/plasma and cerebrospinal fluid, when available, may improve the detection of *Leptospira*.

Treatment

Antimicrobial therapy is indicated for the severe form of leptospirosis, but its use is controversial for the mild form of leptospirosis.

Antibiotic therapy is most effective when begun early in the infection.

In severe illness, one of the following is recommended (Brett-Major and Coldren, 2012):

- Penicillin G 5–6 million units IV every 6 h.
- Ampicillin 500–1000 mg IV every 6 h.

In less severe cases, one of the following may be given for 5–7 days:

- Doxycycline 100 mg orally every 12 h.
- Ampicillin 500–750 mg orally every 6 h.
- Amoxicillin 500 mg orally every 6 h.

In severe cases, supportive care, including fluid and electrolyte therapy, is also important.

Prevention

Rodents and fresh water are the primary risk factor for leptospirosis and once dispersed through the urine of contaminated animals, such as rats, the bacteria can survive for up to 6 months in fresh water or a humid location. There are several steps to help prevent getting leptospirosis. These include: Avoid contact with animal urine or body fluids, do not swim in, walk in, swallow or submersing head in potentially contaminated freshwater (rivers, streams), wear protective clothing or footwear near soil or water that may be contaminated with animal urine and keep rodent populations (rats and mice) or other animal pests under control.

Some studies have shown that chemoprophylaxis with doxycycline might be effective in preventing clinical disease and could be considered for people at high risk and with short-term exposures. But a systematic review concluded that regular use at 200 mg weekly was associated with nausea and vomiting, and there was no clear benefit for reducing the risk of leptospirosis (Brett-Major and Lipnick, 2009).

Borrelia

Introduction

Borrelia species are members of the family *Spirochaetaceae*, so present the characteristic spirochete shape. *Borrelia* species have an outer membrane and an inner membrane, with axial filaments called endoflagella in their periplasmic space. The axial filaments rotate in this space, propelling the bacterium. *Borrelia* includes several species transmitted by lice and ticks and causing relapsing fever (*B. recurrentis* and others) and Lyme disease (*B. burgdorferi*) in humans.

Lyme borreliosis

B. burgdorferi sensu lato (s.l.) species complex causes Lyme borreliosis (LB), which are transmitted by several species of *Ixodes* ticks (Stanek et al., 2012).

The *B. burgdorferi* complex comprises at least 15 genospecies worldwide; human infections are caused by three *B. burgdorferi* sensu lato genospecies: *B. garinii*, *B. afzelii*, and *B. burgdorferi* sensu stricto. All three species cause LB in Europe, whereas only *B. burgdorferi* sensu stricto causes LB in the United States (European Centre for Disease Prevention and Control, 2016).

Two other pathogenic genospecies, less frequent, have been identified in Europe: *B. bavariensis*, associated with neurological complications and *B. spielmanii*. *B. valaisiana* and *B. lusitaniae* rarely cause disease in humans.

LB is the most prevalent tick-transmitted infection in temperate areas of Europe, North America and Asia. The mean annual number of LB notified cases in Europe is more than 65,400 with a strong heterogeneity in spatial distribution: the level of antibodies to *B. burgdorferi* s.l. is highest in residents of northern and central countries and lowest in those in the southern countries. Countries with high incidence rates include Slovenia, Germany and Austria, the Baltic coastline of southern Sweden, and some Estonian and Finnish islands (Rizzoli et al., 2011).

Transmission

The vectors of *B. burgdorferi* s.l. are hard ticks of the genus *Ixodes*. The main vector of Lyme borrelia in Europe is *Ixodes ricinus*, whereas *Ixodes persulcatus* is the main vector in Asia. *Ixodes scapularis* is the main vector in northeastern and upper Midwestern United States and *Ixodes pacificus* is the vector in Western United States (Stanek et al., 2012).

Ticks normally feed on animals, ground-feeding birds, or reptiles. Humans are a secondary blood source. The Lyme disease ticks can attach to any part of the human body but are often found in hard-to-see areas such as the groin, armpits, and scalp. Most humans are infected through the bites of immature ticks called nymphs. Nymphs are tiny (less than 2 mm) and difficult to see. The prime seasons for human tick bites are late spring and summer. Adult ticks can also transmit Lyme disease bacteria, but they are much larger and are more likely to be discovered and removed before they had time to transmit the bacteria. Adult *Ixodes* ticks are more active during the cooler months of the year.

Clinical presentation

LB can involve several organs and may have different clinical presentations. The clinical expression of borreliosis is divided into three stages (Stanek et al., 2012; European Centre for Disease Prevention and Control, 2016).

Stage I: Early manifestations or erythema migrans

Erythema migrans (EM) occurs in about 80–90% of cases, few days to several weeks after the infection. It is an erythematous rash that gradually expands from the site of a tick bite and is not significantly raised or painful. EM is often accompanied by influenza-like symptoms such as fever and sweats, chills, fatigue, neck pain or stiffness, headaches, joint or muscle pains. Less common are backache, nausea and vomiting, sore throat, lymphadenopathy, and splenomegaly.

Without therapy, erythema migrans typically fades within 3–4 weeks.

Stage II: Early disseminated stage

If the infection remains untreated, bacteria can spread to other tissues and organs. The second stage of the disease causes more severe manifestations that can involve the skin, nervous system, joints, or heart.

Neuroborreliosis (NB) is the main complication of LB (it appears in 10% of patients with EM). Most common are lymphocytic meningitis or meningoencephalitis, or neuritis. Other uncommon features of disseminated infection include multiple erythema migrans, intermittent arthritis and carditis. These also present within a few weeks to several months of infection.

Myocardial abnormalities appear about 8% of patients in weeks of EM. They include fluctuating degrees of atrioventricular block and, rarely, myopericarditis.

Stage III: Late stage

The late stage begins months to years after initial infection. The late or disseminated LB includes acrodermatitis chronica atrophicans (ACA), arthritis, or more severe stages of NB.

Arthritis develops in about 60% of patients within several months, occasionally above to 2 years, of disease onset. Intermittent swelling and pain in a few large joints, especially the knees, typically recur for several years. Affected knees commonly are much more swollen than painful; they are often hot but rarely red. Baker cysts may form and rupture. Malaise, fatigue, and low-grade fever may precede or accompany arthritis attacks. In about 10% of patients, knee involvement is chronic (unremittent for ≥ 6 months).

Other late findings (occurring years after onset) include an antibiotic-sensitive skin lesion (acrodermatitis chronica atrophicans) and chronic central nervous system abnormalities, either polyneuropathy or a subtle encephalopathy with mood, memory, and sleep disorders.

Some patients have symptoms such as fatigue, headache, joint and muscle aches, and cognitive problems after successful antibiotic treatment (post-treatment Lyme disease syndrome).

Stage 2 and stage 3 symptoms may be the first signs of Lyme disease in people who didn't have a rash or other symptoms of early infection.

Laboratory diagnosis**Culture**

B. burgdorferi s.l. strains have been isolated from the blood, cerebrospinal fluid (CSF) and synovial fluid in complex media. Culture sensitivity depends on sample and clinical manifestation. The relative complexity of the culture makes it available in specialized laboratories.

Nucleic acid amplification techniques

Nucleic acid amplification techniques are available for the detection of genospecies of Lyme borrelia in specialized laboratories. This method cannot establish whether or not an infection is active because polymerase chain reaction (PCR) detects borrelial DNA of both viable and non-viable organisms.

PCR testing of CSF or synovial fluid is often positive when those sites are involved. PCR for detection of borrelial DNA in synovial fluid specimens is positive in up to about 80% of untreated patients with Lyme arthritis (Aguero-Rosenfeld et al., 2005; Cerar et al., 2010). The sensitivity of PCR in CSF tends to be much lower than it is in synovial fluid (Wilske et al., 2007). It should be emphasized that a negative result for PCR does not exclude active infection.

PCR on blood or urine samples are not recommended for clinical use (Stanek et al., 2012).

Serology

Serological testing is the principal means of laboratory diagnosis of Lyme disease. Assays available for serology are immunofluorescence, enzyme-linked immunosorbent assay (ELISA), chemiluminescence and immunoblot. Third generation immunoassays use recombinant or synthetic antigens like C6, OspC, p100, p18, p41 and VlsE.

Serological assays for antibodies to *B. burgdorferi* are negative frequently at EM stage, and typical erythema is usually diagnosed clinically. In atypical EM cases serologic evidence of infection is best obtained by testing of paired acute- and convalescent-phase serum samples.

Serum samples from persons with disseminated or late-stage LB almost always have a strong IgG response to *Borrelia* antigens.

Serodiagnosis of LB is currently based on a sensitive screening assay and subsequent confirmation by an specific immunoblot system. Most screening assays use VlsE or C6 as a single antigen for detection of specific IgG antibodies. OspC (as a single antigen or in a mixture with other antigens) is used for detection of specific IgM antibodies.

If the screening test result is positive, it should be followed up by a confirmatory test. The immunoblot with recombinant antigens is the method of choice because it allows a reliable differentiation between individual antigens.

In the first 4 weeks of disease onset (early LB), both IgM and IgG should be tested. If only IgM bands are detected on immunoblot, especially in persons with illness greater than 1 month's duration, the results are often false positive.

However, more recently, the results of several studies suggest that the screening test that use Vlse and OspC as show sufficiently sensitivity and specificity to replace the two-step test principle (Steere et al., 2008; Branda et al., 2011; Moore et al., 2016).

Serological testing for neurological Lyme disease is based on demonstrating intrathecal synthesis of *Borrelia*-specific antibodies in CSF. IgG serology assay is performed on CSF and paired serum and the results compared (Stanek and Strle, 2018).

Immunological tests for cellular response: These are tests that measure cellular immunological response. Examples are T-cell activity and decrease or increase of certain lymphocyte subpopulations. These tests are labor intensive, require laboratory expertise and are still under development (Stanek and Strle, 2018).

Treatment

People treated with appropriate antibiotics in the early stages of Lyme disease usually recover rapidly and completely. Antibiotics commonly used for oral treatment include doxycycline, amoxicillin, or cefuroxime axetil. Macrolides such as azithromycin are used as second-line treatment.

Neurological or cardiac forms of illness may require intravenous treatment with antibiotics such as ceftriaxone or penicillin. More recent studies suggest that oral doxycycline treatment of NB is as effective as intravenous ceftriaxone (Ogrinc et al., 2006). Antibiotics should be administered for 2 weeks in cases of early localized and early disseminated disease. For chronic manifestations, a 4-week course is recommended. Pregnant women with Lyme borreliosis receive the same antibiotic treatment as women who are not pregnant, except that doxycycline should be avoided.

Antibiotic treatment and doses are as follows (Wormser et al., 2006):

In erythema migrans, doxycycline (100 mg twice per day) or amoxicillin (500 mg³ times per day) or cefuroxime axetil (500 mg twice per day). Alternative: Clarithromycin 500 mg orally twice per day or azithromycin 500 mg orally per day. Duration of treatment must be 14–21 days, except for azithromycin (7–10 days).

In meningitis or cardiac manifestation, doxycycline 100–200 mg orally twice a day or ceftriaxone 2 g IV once a day or cefotaxime 2 g IV q 8 h or penicillin G 3–4 million units IV q 4 h. Duration of treatment will be 14–28 days.

In arthritis, amoxicillin 500 mg orally three times a day, or doxycycline 100 mg orally twice a day or cefuroxime axetil 500 mg orally twice a day. Duration of treatment will be 28 days.

Prophylaxis and prevention

Transmission of *B. burgdorferi* does not usually occur until the infected tick has been in place for >36 h. Thus, searching for ticks after potential exposure and removing them promptly can help prevent infection.

Antibiotic prophylaxis (doxycycline) to prevent Lyme disease after a recognized tick bite is recommended if, the tick is estimated to have been attached ≥ 36 h and patients have visited an area where $\geq 20\%$ of these ticks are infected with *B. burgdorferi* (Wormser et al., 2006).

Treponema

Introduction

The genus *Treponema* consists of pathogenic and non-pathogenic spirochaetes. Human diseases caused by the treponemes are syphilis (*Treponema pallidum* subsp. *pallidum*), endemic syphilis (*T. pallidum* subsp. *endemicum*), yaws (*T. pallidum* subsp. *pertenue*) and pinta (*T. carateum*). While syphilis is a venereal disease, the remaining are not sexually transmitted.

Syphilis

Syphilis is a systemic infection usually contracted through sexual activities, and is characterized by an extraordinary variety of clinical manifestation. Vertical transmission can occur in the first 4 years after infection.

According to the WHO, in 2014 there were a rate of 25.1 cases per 100,000 adults. Regions with the highest incidence were Africa and western Pacific (European Centre Disease Prevention and Control, 2018). In Europe, 29,365 cases were reported in 2016 with an incidence of 6.1 cases per 100,000 inhabitants (European Centre Disease Prevention and Control, 2018).

Depending on the time elapsed from infection to diagnosis, it is classified as early syphilis or late syphilis. Early syphilis is syphilis that has been acquired in the last year and late syphilis is syphilis acquired more than a year previous. Early syphilis includes primary syphilis, secondary syphilis and early latent syphilis. Late syphilis includes late latent syphilis and tertiary syphilis (European Centre Disease Prevention and Control, 2018). For WHO early syphilis is that acquired in the last 2 year.

Clinical manifestations

Early syphilis

After an incubation period of 9–90 days a primary lesion or chancre appears, often at the site of infection. It is usually solitary, indurated, painful and a serous exudate is formed in the center. The exudate have many spirochetes and is highly infectious. The chancre usually lasts 3–6 weeks and disappears regardless of treatment. During this stage the treponemes spread throughout the body via the lymphatic system and the blood. Atypical manifestations include multiple painful, destructive, usually extragenital (most frequently oral) chancres, and sometimes without chancre.

Secondary syphilis occurs in 25% of untreated infected individuals and begins between 2 and 8 weeks after the onset of the initial chancre. There can be fever, anorexia and arthralgias. The central nervous system can be affected and in fact, any organ can be affected. It often presents as a disseminated mucocutaneous rash and generalized lymphadenopathy. The lesions can be macular, maculopapular, papular or pustular, with no vesicular lesions. The different morphologies can be present at the same time and affect any cutaneous location, especially palms of the hands and soles of the feet. Lesions may appear on the tongue, mucous membranes and scalp (alopecia). In relapses, secondary syphilis lesions are usually less florid.

In latent syphilis, which can persist over time, there are no clinical manifestations, only serological reactivity.

Tertiary syphilis

Late or tertiary syphilis would appear 10–30 years after the infection began and it is divided into gummatous syphilis (15% of patients), cardiovascular syphilis (10%) and late neurological complications (7%). Most people with untreated syphilis do not develop tertiary syphilis.

Neurosyphilis is the infection of the central nervous system by *T. pallidum* and this can happen during any of the stages of syphilis. There are different forms of neurosyphilis: symptomatic, asymptomatic meningitis and meningovascular neurosyphilis, the most serious form of meningeal neurosyphilis. In late disease there is usually overlap of meningovascular and parenchymal involvement. Symptoms of neurosyphilis include headache, difficulty coordinating muscle movements, paralysis, and mental disorder.

Congenital syphilis

Congenital syphilis (CS) appear when bacteria *T. pallidum* is transmitted from a pregnant woman to her fetus. Overall risk of transplacental infection of the fetus is about 60–80%, and likelihood is increased during the 2nd half of the pregnancy. Untreated primary or secondary syphilis in the mother usually is transmitted, but latent or tertiary syphilis is transmitted in only about 20% of cases. Infection can result in stillbirth, prematurity, or a wide spectrum of clinical manifestations.

In infected neonates, manifestations of syphilis are classified as early congenital (i.e., birth through age 2 year) and late congenital (i.e., after age 2 year).

Clinical manifestations in early congenital syphilis: in untreated infants usually appear by 3 months of age, most often by 5 weeks. An infant or child (aged less than 2 years) may have signs such as: hepatosplenomegaly, rash, condyloma lata, snuffles, jaundice, pseudoparalysis, anemia or edema.

Clinical manifestations in late congenital syphilis includes: Hutchinson's triad (Hutchinsons teeth, interstitial keratitis, and eighth nerve deafness), facial features (frontal bossing, saddle nose, short maxilla, protuberant mandible), interstitial keratitis secondary glaucoma, corneal scarring, optic atrophy, sensorineural hearing loss associated with late congenital syphilis typically develops suddenly at 8–10 years old and often accompanies interstitial keratitis, Hutchinson teeth, gummas in the skin or mucous membranes, intellectual disability skeletal and hematologic manifestation.

Laboratory diagnosis

Direct detection methods include the detection of *T. pallidum* by microscopic examination of samples from lesions or nucleic acid amplification methods such as PCR. Indirect diagnosis is based on serological tests for the detection of antibodies and is indicated for routine diagnosis of suspected syphilis cases, monitoring of treatment of diagnosed syphilis, and as part of prenatal screening, and donor screening.

Direct detection of *Treponema pallidum*

Darkfield microscopy

Exudates and fluids from a chancre or lymph node aspirate are examined as a wet mount using dark-field microscopy to directly visualize spirochetes. This technique requires a trained, experienced microscopic and is subject to both false positive and (many) false negative result (Wheeler et al., 2004).

Nucleic acid amplification methods

It is widely accepted that routine PCR could be an effective supplement for early diagnosis, especially suitable for the clinical sample cases, such as chancre secretions and other lesions where contamination with comensal treponemes is likely (Janier et al., 2016; Zhou et al., 2019). Additionally, routine PCR has been reported to detect atypical cases in tonsillar, vertebral, and ocular syphilis

(Zhou et al., 2019). CSF PCR is considered to have little value for the diagnosis of neurosyphilis because it has low sensitivity and specificity (Arando and Otero, 2019).

Serological test

There are two categories of tests for syphilis: non-treponemal tests and treponemal tests. Antibody to non-treponemal antigens is found in active disease and the levels subside after successful treatment, while *Treponema*-specific antibodies persist for a long time after the infection has been successfully treated. Both tests are needed for aiding the diagnosis of syphilis.

Non-treponemal tests (NTT): these detect antibodies to antigens such as cardiolipin and lecithin released from the damaged host cells, as well as lipoprotein-like material released from the *Treponema* and are not specific for syphilis. The most common NTT are the Rapid Plasma Reagin (RPR) and Venereal Disease Research Laboratory (VDRL). The RPR tests are macroscopic agglutination tests and require no microscope and the VDRL tests are microflocculation tests and are read under a microscope. NTT are often used as a screening test against syphilis, because are cheap, simple and allow semiquantification; these techniques help to establish the phase of the infection and are generally considered to be sensitive in early syphilis and poor sensitivity in the late stage of infection (Table 1). NTT are used to monitor patients response to treatment. RPR titers are usually higher than those of the VDRL. The sensitivity of serological tests for syphilis is detailed in Table 1 (data from Ratnam, 2005; Park et al., 2019).

These tests can have a high false-positive rate, especially in low prevalence populations. False-positive results are usually, but not always, of low titer (<1:8) and occur due to autoimmune disease, drug addiction, acute viral infections, recent immunizations, or age (10% of people over 80 years of age have false-positive tests results). False-negative reactions can occur due to the prozone phenomenon, especially in secondary syphilis.

Treponemal test (TT): include the FTA-Abs (fluorescent treponemal antibody absorption) test, TPHA (*Treponema pallidum* Hemagglutination), TPPA (*Treponema pallidum* particle agglutination), EIA (enzyme immunoassays), Immunoblot, CLIA (chemiluminescence immunoassay) and rapid treponemal assays. TT detect antitreponemal antibodies and indicate exposure to syphilis during the patient's lifetime. A positive result, however, does not mean the patient currently has untreated syphilis. Treponemal tests have equivalent sensitivity but greater specificity for *T. pallidum* infection compared to non-treponemal tests. Positive results from both tests support a diagnosis of syphilis with a very high positive predictive value. A treponemal test remains positive even after successful treatment of the disease and cannot be used to monitor therapy.

The sensitivity of serological tests for syphilis is detailed in Table 1 (data from Ratnam, 2005 and Park et al., 2019).

Syphilis screening

The traditional syphilis testing algorithm starts with a NTT test followed by a TT if the first test is reactive, but in many laboratories the new reverse algorithm is used. The initial test performed is a treponemal antibody test (EIA or CLIA). If the specimen is reactive, a NTT semi-quantitative RPR or VDRL (titer) will be performed. If the NTT test is reactive, the results are interpreted as consistent with current or past syphilis infection. If the NTT is non-reactive, a second treponemal test (TPHA or TPPA) is performed and charged. If this test is reactive, the results indicate past or potential early syphilis infection. If it is non-reactive, the presence of treponemal antibodies is not confirmed and the testing algorithm is inconclusive for syphilis infection. This could indicate that the initial treponemal test was a false positive. The reverse algorithm is illustrated in Fig. 1.

Special circumstances

Neurosyphilis: the VDRL is the test of choice for cerebrospinal fluid (CSF) to assist in the diagnosis of neurosyphilis (Kent and Romanelli, 2008; Marra, 2004). It is of particular importance that CSF is not contaminated with blood. VDRL is highly specific but insensitive, therefore, a positive can be used for diagnosis but a negative does not rule out neurosyphilis. The FTA-Abs treponemal

Table 1 Sensitivity (%) of serological test for syphilis.

Stage of syphilis	Test			
	Non-treponemal		Treponemal	
	VDRL/RPR	FTA-ABS	TPHA	CLIA
Primary	80–86	79	95	96
Secondary	95	100	100	100
Latent	97	100	100	98

VDRL, Venereal Disease Research laboratory; RPR, Rapid Plasma Reagin; FTA-ABS, Fluorescent treponemal antibody absorption; TPPA, *Treponema pallidum* particle agglutination; CLIA, Chemiluminescence immunoassay.

Data from references Ratnam S (2005) The laboratory diagnosis of syphilis. *Canadian Journal of Infectious Diseases and Medical Microbiology* **16**(1): 45–51. and Park IU, Fakile YF, Chow JM, Gustafson KJ, Jost H, Schapiro JM, Novak-Weekley S, Tran A, Nomura JH, Chen V, Beheshti M, Tsai T, Hoover K, Bolan G (2019) Performance of Treponemal Tests for the Diagnosis of Syphilis. *Clinical Infectious Diseases* **68**: 913–918.

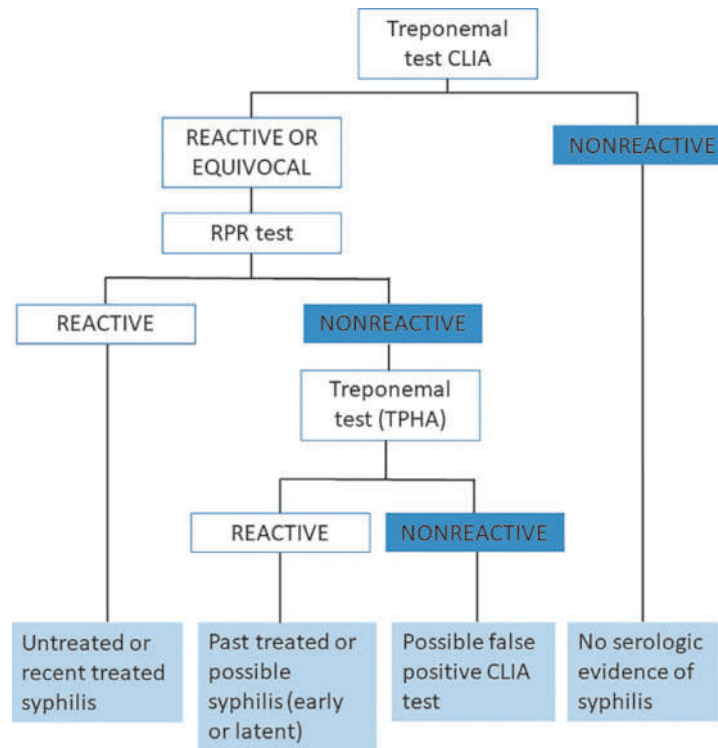


Fig. 1 Syphilis serology algorithm in Virgen de las Nieves University Hospital.

test is another test that has been used in CSF fluid. This test has high sensitivity and low specificity; a negative test is consistent with the absence of neurosyphilis. False-positive CSF results are common with the FTA-Abs test as a consequence of passive diffusion of treponemal antibodies through blood-brain barrier. PCR methods are not very helpful due to their low sensitivity and specificity in CSF.

Neonatal syphilis: Newborn infants should not be discharged from the hospital without determination of the mother's serologic status for syphilis. Testing of the infant's blood is inadequate for screening because false-negative and false-positive results can occur. Maternal IgG antibodies are passively transferred across the placenta. Therefore, a positive syphilis IgG or FTA-Abs test (both treponemal tests) is not conclusive of active syphilis infection; the NTT on the infant must be positive as well. The tests performed on the infant should be the same as those performed on the mother to enable comparison of titer results: if the infant's titer was higher than that of the mothers, then the infant had congenital syphilis. A lower titer in the infant's serum than in the mother does not rule out congenital syphilis (Norris and Larsen, 1995).

Treatment

The recommended treatment at all stages of this disease is penicillin benzathine administered parenterally. In primary, secondary or early-stage latent syphilis (by definition, less than a year), the recommended treatment is a single injection IM of Benzathine penicillin G 2.4 million units. Additional doses of 2.4 million units should be given 7 and 14 days later for late (>1 year) latent syphilis or latent syphilis of unknown duration because treponemes occasionally persist in the CSF after single-dose regimens. Treatment is the same regardless of HIV status.

For non-pregnant patients with a significant penicillin allergy (anaphylactic, bronchospastic, or urticarial), the first alternative is doxycycline 100 mg orally twice a day for 14 days (28 days for late latent syphilis or latent syphilis of unknown duration). Azithromycin 2 g orally in a single dose is effective for primary, secondary, or early latent syphilis caused by susceptible strains.

For pregnant women with syphilis penicillin is the only recommended treatment. Women who are allergic to penicillin can undergo a desensitization process that may allow them to take penicillin.

The treatment for neurosyphilis and ocular syphilis is penicillin G sodium 3,000,000–4,000,000 IU intravenously every 4 h (18–24 million IU/day) for 10–14 days.

For congenital syphilis Centers for Disease Control and Prevention (CDC) recommend aqueous crystalline penicillin G 50,000 units/kg IV q 12 h for the first 7 days of life and q 8 h thereafter for a total of 10 days or procaine penicillin G 50,000 units/kg IM once/day for 10 days. If ≥ 1 day of therapy is missed, the entire course must be repeated.

Most patients with primary or secondary syphilis, especially those with secondary syphilis, have a Jarisch-Herxheimer reaction within 6–12 h of initial treatment. Signs and symptoms include a fever, chills, nausea, achy pain and a headache. This reaction usually doesn't last more than 1 day.

Follow-up

Patients with early syphilis who have been treated with appropriate doses and preparations of benzathine benzylpenicillin should be evaluated clinically and serologically, using a non-treponemal test, after 3 months to assess the results of therapy. A second evaluation should be performed after 6 months and, if indicated by the results at this point, again after 12 months. Following adequate therapy for primary and secondary syphilis, there should be at least a fourfold decline in titer by the third or fourth month and an eightfold decline in titer by the sixth to eighth month. Patients treated in the latent or late stages may show a more gradual decline in titer, and low titers persist in approximately 50% of these patients. This persistent titer does not signify treatment failure (Norris and Larsen, 1995).

Repeat treatment should be considered when clinical signs or symptoms of active syphilis persist or recur or there is confirmed increase in the titer of a non-treponemal test.

Patients should be re-treated with the schedules recommended for syphilis of more than 2 years duration. In general, only one re-treatment course is indicated because adequately treated patients often maintain stable, low titers of non-treponemal tests.

Bejel, pinta and yaws

Bejel, pinta, and yaws (endemic treponematoses) are chronic, tropical, non-venereal spirochetal infections spread by body contact.

Yaws (also known as frambesia or pian) is caused by *T. pallidum* subspecies *pertenue*. Transmission occurs mainly in poor communities in warm, humid tropical regions of Africa, Asia, Latin America and the Pacific.

Bejel is caused by *T. pallidum* subspecies *endemicum*. Transmission occurs principally in the Sahel region of Africa and the Arabian Peninsula.

Pinta is caused by *T. carateum*. Transmission occurs only in the American region.

They may be differentiated by clinical manifestations, geographic distribution, and molecular diagnostic testing.

Bejel, yaws, and pinta are spread by any close contact with the skin of an infected person. Bejel also may be spread by mouth-to-mouth contact or when eating utensils are shared. Also unlike syphilis, bejel, yaws, and pinta are not spread through contact with contaminated blood or from mother to fetus during pregnancy.

Clinical manifestations

Bejel

Bejel usually begins in childhood as a small mucous patch, often on the interior of the mouth, followed by the appearance of raised, eroding lesions on the limbs and trunk.

Periostitis (inflammation) of the leg bones is commonly seen, and gummas of the nose and soft palate develop in later stage.

Yaws

After an incubation period of several weeks, begins at the site of inoculation as a red papule that enlarges, and ulcerates (primary yaws). Within next 3–6 weeks generalized eruption develops that resembles condylomata lata of secondary syphilis (secondary yaws). During the next 5–10 years, destructive lesions (tertiary yaws) may develop in skin, mucous membrane, and bone.

Pinta

The skin is the only organ involved in pinta. Lesions begin at the inoculation site as a small papule that enlarges and becomes hyperkeratotic; pinta usually progresses and some lesions become slate blue or depigmented.

Diagnosis

Diagnosis of endemic treponematoses is based on the typical appearance of lesions in people from endemic areas. Serologic test for syphilis, NTT and TT are positive; differentiation from venereal syphilis is clinical.

Treatment

Yaws is treated with 1 dose of penicillin benzathine 1.2 million units IM. Children <45 kg should receive 600,000 units IM. A single dose of azithromycin 30 mg/kg orally (maximum 2 g) is the preferred treatment because of the ease of administration.

Alternative agents for treatment of endemic treponematoses in non-pregnant adults include doxycycline (100 mg orally twice daily for 15 days) or tetracycline (500 mg orally four times a day for 15 days) (Perine et al., 1984).

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Mycoplasma and Ureaplasma

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Introduction

Ureaplasma spp. and *Mycoplasma* spp. belong family *Mycoplasmataceae*, order *Mycoplasmatales* and to the class *Mollicutes*, which means “soft skin” since it is composed of microorganisms whose main characteristic is the absence of cell wall and presences of sterols in the cell membrane. This characteristic differentiates them from the rest of the prokaryotes, phylogenetically, *Mycoplasmas* are more closely (Kashyap and Sarkar, 2010). *Mycoplasmas* are the smallest free-living microorganisms, both in cell size (0.2–0.8 nm), and in the size of their genome, the smaller being that of *M. genitalium* with only 381 genes and 580 kb. An intimate microorganism-host relationship is established that manifests as surface parasitism with epithelial cells and immune system cells and as an intracellular location, in some species (Acosta et al., 2011b).

Mycoplasmas take pleomorphic forms. Some of them can pass 45 µm filters, so they were originally thought to be viruses. However, these microorganisms are divided by binary fission, some of them can grow in acellular artificial media and contain RNA and DNA. They behave like optional aerobics (except *M. pneumoniae* which is strict aerobic) and need exogenous sterols for growth. Their generation time is high, so they need prolonged incubation to be detected. The extremely small size (150–200 nm) and its limited metabolic and biosynthetic capabilities are responsible for many of the biological characteristics of the organisms. It explains the parasitic and saprophytic existence of the organisms and its fastidious growth requirements which may complicate its detection by culture (Kashyap and Sarkar, 2010; Zhou et al., 2018).

Ureaplasma urealyticum was first isolated in 1954, 17 years later than *Mycoplasma hominis*. Subsequently, with the development of nucleic acid amplification (PCR) techniques, other species such as *Mycoplasma genitalium* and *Ureaplasma parvum*, formerly considered a serovariety of *U. urealyticum*, could be differentiated. To date, *Ureaplasma* spp. consists of 14 serovars which could be divided into two species, *Ureaplasma parvum* (UPA) and *Ureaplasma urealyticum* (UUR), based on differential growth responses to manganese, 16S rRNA gene sequences, the 16Se23S rRNA intergenic region, urease gene, and differences in the multiple-banded antigen (MBA) genes. UPA comprises four serovars (1, 3, 6, and 14), while UUR includes the remaining 10 serovars (Feng et al., 2016; Zhou et al., 2018).

Epidemiology

They are widely distributed in nature and, today, the growing number of established species approaches 200, many of which behave like diners, mainly in the oropharynx and genitourinary tract, while others are pathogenic to man, animals and plants.

In humans, 14 species have been isolated, 12 belong to the genus *Mycoplasma* and 2 species (*Ureaplasma biovar parvum* and *Ureaplasma urealyticum*) belong to the genus *Ureaplasma*. Six of the 14 species have the genitourinary tract as their main place of colonization, so they are known as genital mycoplasmas (Latino et al., 2018).

Ureaplasma spp. has been isolated in 30–80% of vaginal/cervical samples from sexually active women (Castellano-González et al., 2007; Leli et al., 2018; Wang, 1992). In pregnant women, prevalences of 10% for *M. hominis* and 26.25% for *Ureaplasma* spp. were found. Among the pregnant, *Ureaplasma* spp. was the most frequently isolated, specially primigravidas and during the second gestational trimester (Castellano-González et al., 2007) while the recovery rate of *U. urealyticum* in girls with no history of sexual contact was significantly lower (Wang, 1992).

Table 1 Species of *Mycoplasma* spp. and *Ureaplasma* spp. with clinical interest.

Species	Location	Metabolism	Classification
<i>Mycoplasma pneumoniae</i>	Respiratory tract Rarely genital or other locations	Glucose	Pathogen
<i>M. salivarium</i>	Nasopharynx and oropharynx	Arginine	Commensal
<i>Mr. Oral</i>	Nasopharynx and oropharynx	Arginine	Commensal
<i>Mr. Oral</i>	Nasopharynx and oropharynx	Arginine	Commensal
<i>M. faucium</i>	Nasopharynx and oropharynx	Arginine	Commensal
<i>M. lipophilum</i>	Nasopharynx and oropharynx	Arginine	Commensal
<i>M. primatum</i>	Nasopharynx and oropharynx	Arginine	Commensal
<i>M. hominis</i>	Genitourinary tract, tissues, blood	Arginine	Pathogen
<i>M. genitalium</i>	Genitourinary tract, Respiratory tract rarely other locations	Glucose	Pathogen
<i>M. fermentans</i>	Genitourinary tract, blood, tissues, urine	Glucose, Arginine	Opportunistic
<i>M. spermatophilum</i>	Genitourinary tract	Arginine	
<i>M. pirum</i>	Rarely in peripheral blood (HIV)	Glucose, Arginine	Unknown
<i>M. penetrans</i>	Genitourinary tract, urine	Glucose, Arginine	Opportunistic
<i>Ureaplasma urealyticum</i>	Orofaringe and genitourinary tract	Urea	Pathogen
<i>U. parvum</i>	Orofaringe and genitourinary tract	Urea	Pathogen

Adapted from Acosta B, Codina M, Mata L, and Meseguer M (2011) Diagnóstico microbiológico de las infecciones por *Mycoplasma* spp. y *Ureaplasma* spp. <https://www.seimc.org/contenidos/documentoscience/procedimientosmicrobiologia/seimc-procedimientosmicrobiologia40.pdf>; Winn WC, Allen SD, Janda WM, Koneman EW, Procop G, Scheckenberger PC and Woods GL (2008) *Koneman Diagnóstico Microbiológico: Texto y Atlas en Color*. p. 1475.

Genital ureaplasmas (*U. urealyticum* and *U. parvum* *M. genitalium* and *M. hominis*) are natural inhabitants of male urethra (Gdoura et al., 2007). In males, prevalence of *U. urealyticum* are 15–25% while for *M. hominis* are prevalence are 4–13.3% (Andrade-Rocha, 2003; Choi et al., 2018; Veiga et al., 2019).

Among the species that can be found in oropharynx, *M. pneumoniae* is the only clearly related to disease. *M. pneumoniae* occurs endemically worldwide. Infections can be observed throughout the year but tend to be more common in summer and early fall. Epidemic peaks can be observed every 3–7 years. Outbreaks of *M. pneumoniae* infections have been reported within families, schools, institutions and military bases. *M. pneumoniae* infections can occur in all ages. However, *M. pneumoniae* is reported to be most frequent among school age children 5–15 years of age and adolescents and young adults (Ding et al., 2020; Sauteur et al., 2018; Waites et al., 2017). Epidemic peaks can be observed every 3–7 years. Outbreaks of *M. pneumoniae* infections have been reported within families, schools, institutions, and military bases (Sauteur et al., 2018).

Other oropharynx colonizing species have rarely been described as respiratory pathogens or in other locations (Ding et al., 1988; Edouard et al., 2016; Ketchersid et al., 2020) (Table 1).

Pathophysiology

Mycoplasma pneumoniae

Mycoplasma pneumoniae respiratory tract infections can range in severity from mild to life-threatening (Waites et al., 2017).

M. pneumoniae can induce both upper and lower respiratory infections and occurs both endemically and epidemically worldwide. Although tracheobronchitis is a more common clinical manifestation, pneumonia is the most clinically important illness associated with *M. pneumoniae* infections. *M. pneumoniae* may be responsible for about 4 to 8% of community-acquired bacterial pneumonias (CABP) during periods of endemicity (Waites et al., 2017).

Other investigations have assessed the role of *M. pneumoniae* in upper respiratory tract infections such acute pharyngitis (Esposito et al., 2004).

M. pneumoniae reinfections are quite common because natural immunity to microorganism is short-lived, which can lead to poor pathogen elimination and in cases resulting in a carrier state (Waites et al., 2017). Some studies from different localization reveal colonization percentages of more than 50% in asymptomatic children, although percentages vary greatly depending on the study.

M. pneumoniae is an obligate parasite. Adherence of *M. pneumoniae* to host cells is critical for virulence, as evidenced by the lack of virulence in strains deficient (88). Binding of *M. pneumoniae* to the host cell surfaces relies on proteins modified with sialic acid and sulfated glycolipids (Krivan et al., 1989). Cytoadherence and motility require an attachment organelle, a polar cellular projection about 290 nm in length that is continuous with the cell membrane. Transmembrane proteins P1, B (P90), C (P40), and P30 are most directly implicated in cytoadherence. The major adhesion is due to P1 adhesin. Antibodies against P1 block cytoadherence and a mutant lacking P1 is nonadherent and avirulent (Waites et al., 2017). Cytoadhesion protects mycoplasmas from mucociliary clearance transmembrane proteins P1, B (P90), C (P40), and P30 are most directly implicated in cytoadherence. Following cytoadherence, *M. pneumoniae* stimulates mast cells to produce IL-4 and activates and induces cytokine production by peripheral blood leukocytes, respiratory epithelial cells, and macrophages. The more vigorous the cell-mediated immune response and cytokine stimulation, more severe is the clinical illness and pulmonary injury (Furr and Taylor-Robinson, 1993).

Hydrogen peroxide is produced locally, which has a cytopathic effect on airway epithelium and cilia and is responsible for persistent cough (Kashyap and Sarkar, 2010; Waites et al., 2017).

Community-acquired respiratory distress syndrome (CARDS) is mediated by an exotoxin initially identified as a surfactant protein A (SP-A)-binding protein, CARDS toxin, with sequence homology with the S1 subunit of pertussis toxin that have ADP-ribosyltransferase activity. Antibody responses to CARDS toxin can be demonstrated in humans with *M. pneumoniae* infection (Kannan and Baseman, 2006).

Clinical presentation

The clinical presentation of *M. pneumoniae* respiratory disease is often similar to what is seen with other atypical pathogens, particularly *Chlamydia pneumoniae*, various respiratory viruses and bacteria (Deeks et al., 2005).

The most common manifestation is tracheobronchitis, with a cough that may either be dry or productive of mucoid or mucopurulent sputum. Many patients may have nonspecific symptoms resembling those of an upper respiratory infection, which may include headache, sore throat, coryza, and otitis media.

Chest auscultation may reveal coarse rales and rhonchi if disease is limited to tracheobronchitis and fine inspiratory rales and dullness at lung bases if pneumonia has developed.

Radiographically, *M. pneumoniae* infection may be indistinguishable from a viral lower respiratory infection, with patchy airspace consolidation and round glass opacities.

This disease often spreads slowly through families, with an incubation time of approximately 23 days. *Mycoplasma pneumoniae* is a primary pathogen of mucous membranes, while other non-pathogenic *Mycoplasma* species colonize the surface of the respiratory mucosa. It is transmitted from person to person by air but, due to its high sensitivity to changes in temperature and humidity. The index case is likely to be an older child, 5–14 years of age, and children under five are more likely to have milder disease. In one study, *M. pneumoniae* infection typically resulted in a prolonged cough that lasted 3–4 weeks. In older children and adolescents and an average of 54 days in adults (Foy, 1993).

M. pneumoniae pneumonia is typically mild and characterized by a persistent dry cough or self-limiting pneumonia, that resolves with no medication. However, respiratory failure and severe acute respiratory distress syndrome (ARDS) occur in 0.5–2% of all *M. pneumoniae* pneumonia cases and primarily affect young adults (Ding et al., 2020).

Uncommon fulminant and/or fatal cases of *M. pneumoniae* have been reported in healthy younger persons, as well as older persons with underlying diseases. Persons with severe illness may experience a hyperimmune response in the lung, they may also be unable to eradicate the organism from the lung, resulting in a prolonged infection which further stimulates the immune system (Waites et al., 2017).

Some authors have investigated the role that colonization by *M. pneumoniae* can play in chronic respiratory diseases such as asthma in both neonates and the pediatric population (Patel et al., 2011).

Extrapulmonary manifestations

Even though the humoral response to infection does not provide complete immunity to future infections, the importance of an intact humoral immune system takes on great importance. Patients with antibody deficiency can develop severe and prolonged respiratory illness and the risk of extrapulmonary also increases (Waites et al., 2017).

Central nervous system manifestations are the most frequent extra-pulmonary complications of *M. pneumoniae* infection and can at times be life threatening (Kashyap et al., 2008). Frequently, a manifest respiratory infection precedes the central nervous system symptoms. Involvement can occur in the brain, spinal cord, meninges, and peripheral nerves. Encephalitis, which occurs more commonly in children than in adults and may not have a respiratory component to the illness (Waites et al., 2017). Early-onset encephalitis is more likely to be PCR positive, indicating a direct effect of the organism, while late-onset encephalitis is usually PCR negative and associated with antiganglioside antibodies which may develop because of cross-reactivity with mycoplasmal glycolipids (Waites et al., 2017).

Nonspecific gastrointestinal symptoms commonly accompany mycoplasmal dermatological disorders, development of the phenomenon of “cold agglutinins” that can reach levels sufficient to trigger a brisk hemolytic anemia pericarditis, cardiac tamponade, myocarditis, myopericarditis (Kashyap and Sarkar, 2010; Waites et al., 2017).

Septic arthritis due to invasion of the joints mainly in persons with antibody deficiency or Hepatitis with severe liver dysfunction and pancreatitis has also been reported.

Other nonspecific manifestations can include conjunctivitis, iritis, and uveitis, which are presumed to have an immunological basis since the organism has not been detected in ocular tissues (Waites et al., 2017).

Other species of *Mycoplasmas* such as *M. orale* and *M. faucium*, commonly considered oropharyngeal commensal, have also been described as causing non-respiratory disease (Edouard et al., 2016; Ketchersid et al., 2020).

Genital mycoplasmas and ureaplasma

Urogenital diseases

Genital mycoplasma *M. genitalium* and *M. hominis* causes between 10% and 30% of genitourinary diseases (Beeton et al., 2019; Gdoura et al., 2007) mainly in non-gonococcal urethritis. The role of *Ureaplasma* spp. (*Ureaplasma urealyticum* and *Ureaplasma*

parvum) in nongonococcal urethritis and infertility among males still remains controversial, due to historically high rates of isolation of these bacteria from the urethra in healthy men, however, there is growing evidence to support that they can be considered true genitourinary pathogens especially in the male (Marovt et al., 2015).

Symptomatic patients and, if observed, those with a visible discharge should be assessed for the presence of urethritis. The discharge is mucopurulent and often sparse but may be cloudy or clear (Moi et al., 2015).

M. genitalium, has a strong association with acute, persistent UNG after treatment and recurrent, in both males and women. *M. genitalium* also has a significant association with balanitis and/or posthitis. *U. urealyticum*, has been related to UNG, *Ureaplasma* spp. it is also related to a small proportion of chronic prostatitis cases and as a rare cause of epididymitis. Symptomatic patients and, if observed, those with a visible discharge should be assessed for the presence of urethritis. The discharge is mucopurulent and often sparse but may be cloudy or clear (Moi et al., 2015).

Genital mycoplasmas and *Ureaplasma* have been linked to urinary tract infection in patients with chronic unexplained urinary tract symptoms. *Ureaplasma* urinary tract infection also has been evaluated primarily in pregnant women and their isolation in the first trimester of pregnancy has been linked to the development of preeclampsia in the third trimester. Some authors have obtained up to 48% of positive crops for mycoplasmas (predominantly *Ureaplasma* spp.) in women with chronic symptoms of urinary tract infection, once the most common etiological agents have been excluded. *Ureaplasma urease* induces the crystallization of struvite phosphate and calcium in urine, in vitro and animal models. In addition, these microorganisms have been isolated in renal stones of patients (Combaz and Kuhn, 2017).

Ureaplasma spp. and *M. hominis* participate in chronic reflux pyelonephritis with 7% and 9%, respectively, of isolations from urine obtained by suprapubic puncture (Combaz and Kuhn, 2017).

They have also been reported from renal stone and suppurative arthritis (Kokkayil and Dhawan, 2015).

In women, *M. genitalium* is significantly associated with cervicitis, although co-infections with other sexually transmitted infection agents are common. Also *M. hominis* and especially *M. genitalium* have been linked to the development of Inflammatory Pelvic Disease (PID) while *U. urealyticum* has also been found in PID and cases of miscarriage (Meseguer-Peinado et al., 2012).

Infertility

The fact that genital mycoplasmas are part of the urogenital microbiota calls into question its role in infertility (Gnanadurai and Fifer, 2020; Zhou et al., 2018). Some of these infections may be paucisymptomatic or asymptomatic, but acute infections such as chronic infections can compromise spermatogenesis (Solomon and Henkel, 2017). The prevalence of these microorganisms has been seen to be higher in samples from patients undergoing fertility treatments and that their detection in high count is related to lower sperm quality (Beeton et al., 2019; Díaz-García and Flores-Medina, 2013; Gdoura et al., 2007; Solomon and Henkel, 2017; Zhou et al., 2018). On the other hand, the presence of genital mycoplasmas with genital disease in women has also been linked so some authors recommend the systematic search of these microorganisms in infertile partner samples (Veiga et al., 2019).

Disease in pregnancy, childbirth and postpartum

Bacterial Vaginosis is a microbiological entity in which the normal vaginal microbiota is altered in its composition, with loss of the main components and predominance of other species such as *Mycoplasma* or *Ureaplasma* (Meseguer-Peinado et al., 2012). Genital mycoplasmas were isolated from gravid women at approximately the same recovery rate as in non-pregnant women; being *M. hominis* the most frequently isolated in non-pregnant women and *Ureaplasma* spp. in the pregnant group (Patel et al., 2011). The prevalence of bacterial colonization in women has been shown to correlate with number of sexual partners in previous months. Sex hormones have a profound influence on colonization, multiplication, and persistence of mycoplasmas/ureaplasmas in the genital tract (Furr and Taylor-Robinson, 1993; Taylor-Robinson, 2017).

The prevalence of bacterial colonization in women has been shown to correlate with number of Genital mycoplasmas are capable of hampering reproductive outcome by attaching to and penetrating the human sperm plasma membrane, which in turn affects male fertility, cause cervicitis, acute chorioamnionitis, preterm labor or preterm premature rupture of membranes, postpartum fever, and infection transmission to the newborn infant or (Feng et al., 2016; Goldenberg et al., 2008; Kwak et al., 2014; Latino et al., 2018; Solomon and Henkel, 2017; Suzuki et al., 2018). Colonization with *U. parvum* serovar three in early pregnancy is associated with preterm delivery at very and extremely low gestational age (Rittenschöber-Böhm et al., 2019).

The transmission of ureaplasmic infections from the mother to the fetus occurs in the uterus or during labor. They can cause ascending infection after rupture of membranes (Bartkeviciene et al., 2020). Membrane phospholipases A1, A2, and C of *Ureaplasma* spp. hydrolyze the phospholipids of the placenta, with release of arachydonic acid, and the consequent production of prostaglandins triggering childbirth. In addition to both *M. hominis* and *U. urealyticum*, they can produce active proteases against IgA which facilitates mucosal invasion. On the other hand, the presence of phospholipases alters the function of the pulmonary surfactant of the fetus, with the consequent risk of bronchopulmonary dysplasia in preterm newborns, because they increase lung inflammation and alter their normal development (Acosta et al., 2011a).

Respiratory distress syndrome (RDS), a major cause of morbidity and mortality in preterm infants, is caused by cardiopulmonary immaturity with a deficiency of surfactant in the alveolar space. RDS is often associated with patent ductus arteriosus (PDA), intraventricular hemorrhage and chronic lung diseases. *Ureaplasma urealyticum* and *Mycoplasma hominis* have been linked to the development of chronic lung disease or bronchopulmonary dysplasia and RDS (Cultrera et al., 2006; Goldenberg et al., 2008).

Diagnosis

Microbiological diagnosis of infections caused by mycoplasma and ureaplasma has long been limited by the fastidious growth of these microorganism in conventional culture media which could lead to an underdiagnosis. This situation has changed markedly in recent years thanks to advances in serological diagnostic methods and the application of nucleic acid amplification methods what has down drawn an increase in their detection and, consequently, in the appreciation of the clinical importance of these microorganisms (Acosta et al., 2011a).

Culture of human mycoplasmas is useful only for the isolation of pathogenic species with a reasonably satisfactory growth in specific media, such as *Ureaplasma* spp., *M. hominis* and *M. pneumoniae* but not for extremely annoying growth species, such as *M. genitalium*, *M. fermentans* and *M. penetrans*, etc.

Growth in artificial media these need a supply of sterols, fatty acids, cholesterol amino acids and other compounds. These media are generally based on a brain-heart infusion broth (BHI) with nutritional supplements and antibiotics to inhibit the accompanying microbiota, in addition to a metabolite (glucose or urea, depending on the species) and a pH indicator such as phenol red.

Currently there are commercial galleries with microwells containing lyophilized antimicrobials, usually in two or more concentrations corresponding to the cut-off points proposed for classification of the isolate as sensitive, intermediate, and resistant. The reading of the test is also based on the presence/absence of color change of the culture medium. Some of these teams also incorporate the growth of the organism providing an approximate colony count and differentiation between mycoplasma and ureaplasma (Acosta et al., 2011a).

Respiratory, genital samples or other locations such as CSF, pericardial fluid, synovial fluid, skin or tympanic vesicle fluid and blood or serum may be cultured, taking into account that they are collected in swabs, these should be calcium alginate, dacron or polyester with plastic or aluminum stem, since cotton and wood exert an inhibitory effect on the microorganism.

M. pneumoniae grows very slowly and culture are not widely available in clinical microbiology laboratories. Sensitivity is less than 50% compared to serology so microbiological diagnosis, in clinical practice, has generally been based on the demonstration of specific antibodies.

Immunoglobulin M (IgM) assays that are more sensitive than the complement fixation (CF) test ha, but the IgM response may be nonspecific 7 or absent, particularly in adults 8. Hybridization with DNA probes proposed as a rapid and specific procedure but lacks sensitivity (Wang et al., 2017).

Passive agglutination techniques use a support, such as latex or gelatin, for a mixture of *M. pneumoniae*-specific antigens can detect IgG and IgM, also allowing their quantification. They are techniques of very easy realization and considerable specificity, although, to achieve maximum sensitivity (66%) it is recommended to make the determination in two serial samples (Templeton et al., 2003) enzymeimmunoassay techniques (EIA) using purified proteins, synthetic peptides, mixture of raw antigens or purified glycolipids to the detection of IgG or IgM with different presentations like capture-μ techniques, microplates, in membrane support can be used. EIA tests appear very sensitive for the detection of specific antibodies. Presentations in membrane support allow qualitative determinations of IgM, very quickly with good sensitivities and specificities.

Detection of IgA is considered to be the best indicator of acute infection, without.

However, using reagents marketed, found 68.5% positivity for this immunoglobulin in blood donors (Csángó et al., 2004).

Serological tests have been developed for the diagnosis of genital *Mycoplasmas* or *Ureaplasma* spp. using highly immunogenic membrane proteins with high specificity. These diagnostic tests may be useful to help clarify the participation of these microorganisms in certain clinical entities but not for daily clinical practice and are only used in the field of research (Winn et al., 2008).

Molecular-based tests such as the PCR assay are becoming more widely used for diagnostics because they are more time-efficient and sensitive than culture because of their high sensibility, specificity and suitability for various types of samples, including vulvovaginal swabs, urethral exudate y and first-catch urine, both individually and in Multiplex PCR for the diagnosis of the most common sexually transmitted disease-producing pathogens (Carneiro et al., 2020; Hilmarisdóttir et al., 2020; Le Roy et al., 2012).

The genes used to detect these microorganisms are detailed in Table 2.

Table 2 Target for detection of Mycoplasma and Ureaplasma.

Species	Target gen
<i>Mycoplasma</i> spp.	16S RNAr
<i>M. genitalium</i>	Adhesina MgPa
<i>M. hominis</i>	Gap (glycolytic enzyme glirelandahide-3 phosphate dehydrogenase)
<i>Ureaplasma</i> spp.	16S ARNr
<i>Ureaplasma urealyticum</i>	ureB (Urease complex component)

Treatment

Since they do not have a cell wall, these microorganisms are intrinsically resistant to antimicrobials whose target is this, such as β -lactams. Mycoplasmas are generally sensitive to tetracyclines, macrolides, lincosamides, streptogramins, fluoroquinolones, aminoglycosides, chloramphenicol and linezolid. *M. pneumoniae* and *Ureaplasma* spp. They are intrinsically resistant to lincosamides and *M. hominis* to macrolides. The most used antimicrobials in the treatment of infections caused by these microorganisms are fluoroquinolones, macrolides and tetracyclines. Sensitivity levels against ciprofloxacin range from 20% to 50%, reaching 80% in some series, while against ofloxacin, moxifloxacin and levofloxacin sensitivity levels, they are generally higher than 50% for both ureaplasma and mycoplasma species. Sensitivity to tetracycline and doxycycline is generally high (above 80% in most series), being somewhat lower for macrolides, among which Azithromycin has lower resistance values.

PCR and sequencing techniques have detected mutations that confer resistance against different antimicrobial agents such as tetM present in the tetracycline-resistant isolate and a mutation within the parC or gyrA in the quinolone-resistant isolate, as well as mutations in the gene encoding for 23S rRNA gene, which confers resistance against macrolides (Orellana and Gómez-Lus, 2011; Choi et al., 2018; Lee and Yang, 2020; Leli et al., 2012; Piñeiro et al., 2019; Redelinguys et al., 2014; Valentine-king and Brown, 2017).

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Legionella, Chlamydia and Chlamydophila

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Glossary

BGMK Cell line from African green monkey kidney cells, commonly used for the isolation of *Chlamydia trachomatis* and Enterovirus.

HeLa Cell line from epithelial cell from human cervix adenocarcinoma.

Hep2 Cell line from epithelial human carcinoma.

HL Cell line from human promyeloblasts.

McCoy Cell line from fibroblast cell from mouse, commonly used for the isolation of *Chlamydia trachomatis*.

Nomenclature

CAP	Community acquired pneumonia
CDCs	Centers for Disease Control and prevention
ECOFFs	Epidemiological cut off values
EIAs	Enzyme immunoassay
HAP	Hospital acquired pneumonia or Nosocomial pneumonia
IC	Immunochromatographic
IFA	Indirect fluorescent antibody

IV	Intravenous
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
mg	Milligram
MIC	Minimum inhibitory concentration
NAATs	Nucleic acid amplification techniques
PCR	Polymerase chain reaction
PO	Orally
spp.	Species
STDs	Sexually transmitted diseases
μm	Micrometer

Legionella

Taxonomy

The order Legionellales belongs to the phylum proteobacteria and class gammaproteobacteria. This order includes two families Coxiellaceae and Legionellaceae. The most representative species of those families, *Coxiella burnetii* and *Legionella pneumophila*, respectively; show similar growth characteristics and close homologies in virulence genes (Edelstein, 2019; Sauer et al., 2005; Segal et al., 2005).

Legionellaceae has a single genus, *Legionella*, with at least 59 different species, 3 subspecies and more than 70 serogroups (Boyd, 1995a; Brenner et al., 1980).

Some of the most commonly isolated in clinical specimens are (Mensa et al., 2016a):

Legionella anisa, *L. bozemanii*, *L. cardiaca*, *L. cincinnatiensis*, *L. dumoffii*, *L. feeleii*, *L. gormanii*, *L. hackeliae*, *L. jordanis*, *L. lansingensis*, *L. londiniensis*, *L. longbeachae*, *L. macaechernii*, *L. micdadei*, *L. nagasakiensis*, *L. oakridgensis*, *L. parisiensis*, *L. pneumophila*, *L. quinlivanii*, *L. sainthelensi*, *L. steelei*, *L. tucsonensis*, *L. wadsworthii*, *L. waltersii*.

The majority of species are considered pathogenic, even so *L. pneumophila*, *L. micdadei*, *L. longbeachae* and *L. dumoffii* are the most significant from a clinical point of view. *Legionella pneumophila* has 17 known serogroups, being serogroup 1 the most prevalent in human disease.

There are a number of *Legionella*-like bacteria, which only grow within free amoeba, known as “LLAPs” (*Legionella*-like amoeba pathogen) that have been reported to be involved in human disease usually as co-pathogen (Marrie et al., 2001; Newsome et al., 1998; Adeleke et al., 2001).

General characteristics

The family Legionellaceae includes a diverse group of mesophilic, obligate aerobic small gram-negative bacilli. Most of species are motile due to a single polar flagellum and possess fimbriae. The surface of its cell wall contains cellular branched-chain fatty acids and ubiquinones which is not common on gram negative bacteria.

Their size and shape varies from small rods to long filamentous bacilli depending on developing conditions. The intracellular bacteria from clinical specimens or environmental samples range between 0.3 and 0.4 μm in diameter and 2.0–3.0 μm in length. However those bacteria isolated in agar plates has shown up to 25 μm of length.

Legionella species (*Legionella* spp.) are asaccharolytic and nutritionally fastidious; their growth depends on L-cysteine and is enhanced by iron (Winn Jr, 1996). Temperatures from 35°C to 45 °C are optimum for Legionellaceae, although they can tolerate up to 55 °C; regarding pH they can only grow on a narrow range of pH 6.7–6.9.

The reservoir of *Legionella* organisms is usually fresh water. The species of this family have been isolated from rivers, lakes, wet soil, fountains, ponds, water supply systems, cooling towers, aerosol therapy equipments, mist humidifier as well as air-conditioned facilities, among others. *Legionella* attach to elements such as pipes, rubber, plastics and sediments. They multiply and survive inside amoebas and ciliated protozoa often found within microbial biofilms, this allows the bacteria to remain protected from external aggressions (e.g., chlorination procedures or extreme temperatures). *Legionella longbeachae* is found in wet soil, specially potting soil and compost, where it may parasite insects or worms (Edelstein, 2019; Boyd, 1995a; Currie and Beattie, 2015; Steele et al., 1990).

Inside the human body it is a facultative intracellular parasite of macrophages (lysosomes and endoplasmic reticulum) usually within the lungs of infected individuals (alveolar macrophages).

Epidemiology and transmission

The Legionnaire's disease and its causal agent were first distinguished from other atypical pneumonia in 1977, after an outbreak of unknown pneumonia developed in Philadelphia at the Pennsylvania State Legionnaires Convention, in July 1976. More than 200 individuals were infected and the mortality rate ascended to a 15%. (Edelstein, 2019). Although that outbreak in 1976 allowed identifying and classifying the agent; Legionellaceae have been isolated since 1940s and a previous outbreak of atypical pneumonia that took place in the summer of 1957 in Austin, Minnesota at a local meat packing plant has been ascribed to Legionnaire's disease bacteria in retrospective (Osterholm et al., 1983).

Mechanism of transmission is inhalation of contaminated aerosols. It is less common by aspiration of contaminated water or ice in susceptible hospital patients. A possible person-to-person transmission has been documented (Borges et al., 2016).

Legionnaire's disease distributes worldwide. The incidence is underestimated and varies depending on the level of diagnosis and reporting, as many countries lack of neither appropriate methods to identify the infection nor surveillance systems. In Europe, United States and Australia there are about 10–15 cases per million population per year. Of the reported cases 75–80% are over 50 years old and 60–70% are male. Other risk factors for community-acquired and travel-associated legionellosis include:

- Cigarette smoking (current or historical).
- Chronic heart and lung disease.
- Underlying illness such as diabetes, renal or hepatic failure.
- Immune system disorders due to disease or medication.
- Systemic malignancy.
- Exposure to a contaminate source (overnight travel as well as overnight stay in a healthcare facility, use of well water, recreational water, etc.).

Patients at particularly high risk of Legionnaires disease are those receiving high dose of glucocorticosteroids, solid organ transplant and those receiving antitumor necrosis factor therapy.

Death rate depends on the severity of the disease, the administration of the appropriate antibiotic as soon as possible, host factors as mentioned before, as well as the identification of the infection source. Patients with no underlying illness receiving the correct antimicrobial treatment can be cured in 95–99% of the cases; untreated disease has a mortality rate of 15%, for this group of patients, increasing to a 75% on several immunocompromised ones (Edelstein, 2019; Joly et al., 1986).

Community-acquired Legionnaire's disease is caused in 95–98% by *Legionella pneumophila* serogroup 1 where Pontiac subtype constitutes about 80–90% of clinical isolates. Other serogroups described less frequently are 6, 5, and 10 (Byrne et al., 2018; Visca et al., 1999; Chen et al., 2006; Zhang et al., 2014; Graham et al., 2020).

Nosocomial infection may be caused up to a 60% by other serotypes of *Legionella pneumophila* serogroup 1, as well as other serogroups of *Legionella pneumophila* and *Legionella* spp.

Pneumonia caused by *Legionella* occurs along the year with seasonal peaks of the disease during the warmer seasons due to the use of air-conditioning, water spray cooling systems, water pools and amusement water parks. About 60% of the reported cases from Europe and United States take place between June and October.

Pathogenesis

Legionella invades and grows within alveolar macrophages, mistaking them for their natural host and causing disease. Most of the known species of this family have been isolated in cases of pneumonia, sometimes being more than one type of legionella organisms involved in the same infection.

- Legionnaire's disease (community-acquired pneumonia and hospital-acquired pneumonia) shows an acute onset of lower respiratory illness with fever and/or cough. Additional symptoms are myalgia, shortness of breath, headache, malaise, chest discomfort, confusion, nausea, diarrhoea or abdominal pain. Pathogenesis is due to the replication of bacteria inside the alveolar macrophages. The incubation period ranges from 2 to 10 days, with a median of 4 days.
- Pontiac fever is an influenzae-like illness caused by *Legionella* spp. in humans. Symptoms include fever, chills, myalgia, malaise, headache, fatigue, nausea and/or vomiting. Pathogenesis is due to the inflammatory response to endotoxin. The incubation period vary from 24 to 72 h after exposure.
- Extra-pulmonary infections have been reported although it is extremely rare. They are due to hematogenous dissemination of the pathogen from a primary focus (Table 1). Those include endocarditis, myocarditis, pericarditis, pancreatitis, peritonitis, skin and soft tissue infections as well as arthritis among others. In neonates can cause bacteremia and/or pneumonia.

Table 1 Shows a summary of infections caused by some *Legionella* spp.

	Respiratory infection	Extra-pulmonary infection
<i>L. anisa</i>	Pontiac fever Pneumonia Empyema	Osteomyelitis
<i>L. bozemanii</i>	Pneumonia (immunocompromised patients) Lung abscess	Arthritis Soft tissue infection
<i>L. cardiaca</i>		Endocarditis
<i>L. cincinnatiensis</i>	Pneumonia	Recurrent soft tissue abscess
<i>L. dumoffii</i>	Pneumonia Lung abscess	Arthritis Prosthetic valve endocarditis
<i>L. feeleyi</i>	Pontiac fever Pneumonia	Cellulitis
<i>L. hackeliae</i>	Pneumonia	Multi-organ failure on splenectomy patients
<i>L. jordanis</i>	Chronic respiratory disease	
<i>L. londiniensis</i>	Pneumonia on immunocompromised patients	
<i>L. longbeachae</i>	Pneumonia (30% of Legionnaires disease in Australia and New Zealand)	Endocarditis Eye infections Skin infections on immunocompromised patients Soft tissue infection
<i>L. macaecheirii</i>	Pneumonia Lung abscess	
<i>L. micdadei</i>	Pneumonia (immunocompromised patients) Lung abscess	Brain abscess Prosthetic joint infection Prosthetic valve endocarditis Severe cellulitis

Methods of diagnostic

Radiologic features

Chest X-ray features on Legionnaires disease are non-specific but may aid in diagnosis and management of the patient with *Legionella* infection. Findings include a mid-to-lower zone predominance of patchy consolidation; infiltrates frequently progress despite initiation of appropriate antibiotic therapy. Pleural effusion is common and occasionally appears in absence of lung infiltrates. Peripheral densities and cavitations are common in immunocompromised patients (Muder et al., 1987; Kim et al., 2007).

Culture

Clinical specimen culture is the gold standard method for *Legionella* identification; it allows detecting every specie and serogroup of *Legionella*. Clinical and environmental isolates may be compared by culture on epidemiologic studies.

Sputum, lower respiratory tract secretions, sterile fluids and tissues from any suspected location of infection are acceptable for culture. A better yield of bacteria from the specimen can be achieved by dilution (1:10) in tryptic soy broth or distilled water for sputum and respiratory secretions. Tissues (1 g, ideally) should be grounded with 1 mL of broth, except from spleen which requires a 1:10 dilution. Sterile fluids as well as blood sub-cultured from blood culture bottles don't need to be diluted before plating, although centrifugation may be useful to concentrate bacteria in pleural fluid for example (Edelstein, 2019).

The buffered charcoal yeast extract medium with α -ketoglutaric acid (BCYE α) yields optimal recovery of Legionellaceae from clinical specimens and environmental samples after a short incubation period. Selective media are supplemented with antimicrobial agents to inhibit the growth of yeast, fungal and other bacteria. To optimize the isolation of Legionellaceae it is necessary to use at least a non-selective and a selective media (Table 2).

Plates should be incubated at 35 °C–37 °C in humidified air. A CO₂ supplementation (2–5%) enhance the growth of some *Legionella* spp. without affecting *L. pneumophila*, however this is not used by clinical laboratories due to the low prevalence of those capnophilic species in human infection. Incubation should last for at least 14 days; cultures should be inspected on days 1, 5 and 14 of incubation. *Legionella* spp. never grows from clinical specimens after 24 h of incubation.

Size and morphology of the colonies vary with time. Diameter may range from 0.5 mm after 3 days of incubation to 7 mm on day 5. Colonies are gray, glistening, convex and circular with an entire edge, although the aspect would change along the course of incubation. Several species exhibit a blue-white auto-fluorescence, and most species produce a diffusible brown pigment on tyrosine-containing agar.

To identify presumptive *Legionella* spp. colonies from other gram negative bacteria, a sub-culture on BCYE α and BCYE α -L media can be done; Legionellaceae won't grow on BCYE α -L medium as require L-cysteine to do so.

Biosafety level 2 precautions should be used for *Legionella* spp. cultures

Table 2 Description of some of the most commonly used media.

Medium	Selective agent	Selectivity	Main use
BCYE α	None	None	Isolation and culture maintenance
BCYE α -L	None	<i>Legionella</i> spp. doesn't grow on this medium	Organism identification
BMPA	Cefamandole, Polymyxin B and Anisomycin	Respiratory and intestinal microbiota, yeasts and some molds	Clinical and environmental
GVPC	Glycine, Vancomycin, Polymyxin B and Cycloheximide	Respiratory and intestinal microbiota yeasts and molds	Clinical specimens and environmental water samples
GVPN	Glycine, Vancomycin, Polymyxin B and Natamycin	Respiratory and intestinal microbiota yeasts and molds	Environmental water and related materials
MWY	Glycine, Polymyxin B, Anisomycin and Vancomycin	Respiratory and intestinal microbiota, some environmental bacteria, yeasts and some molds	Environmental samples

Legionellaceae are faintly staining Gram-negative filamentous bacilli, this morphology is completely different from the one found in clinical specimens (coccobacilli). Legionellaceae are relatively inert biochemically, some key reactions for identification may include: catalase positive (some may show a weak reaction), oxidase-variable, urease-negative and nitrates negative.

Alternative identification methods include MALDI-TOF MS (matrix-assisted laser desorption ionization time of flight mass spectrometry) that is a rapid and cost effective method, available in most routine laboratories now a days, or DNA sequence-based methods used in public health or reference laboratories to identify species and serogroup level for outbreaks investigation or scientific projects.

Antigen detection

Antigen detection test is performed in urine samples. The assay detects *Legionella pneumophila* serogroup 1 and in particular the Pontiac/mab2/Mab3-1 monoclonal subtype. Several assays are available based on immunochromatographic (IC) card or micro-tube enzyme immunoassay. The IC card is an easy, quick and robust assay but its sensitivity can be up to a 40% lower than the micro-tube assay one.

The antigen detection may be negative on the first day of illness although patients with a severe disease can have a positive test since the onset of symptoms. Following a negative result with compatible symptoms a second sample should be tested 48–72 h after the onset of clinical manifestations.

The detection of the antigen in urine can be prolonged for weeks or months after the patient has completely healed; this is related to the clearance of the pathogen and doesn't evidence a treatment failure.

The sensitivity of the antigen detection varies from 60% to 100% depending on the assay performed, clinical severity and the management of the specimen, sensitivity may decrease if the specimen has been frozen (–20 °C to –80 °C) for several months.

Some studies have shown that the use of ultra-filtration to concentrate the urine can increase sensitivity (Blanco et al., 2004; Domínguez et al., 1998). The specificity of the assays ranges from 95% to 99.9%. False-positive results are usually due to an excess of urinary sediment, rheumatoid arthritis-like factors or melting of frozen urine samples.

Urine antigen detection for diagnosis of community acquired Legionnaires disease in areas where *L. pneumophila* serogroup 1 is more prevalent is highly recommended even so using urine as a single specimen for diagnosis is not recommended.

This method doesn't facilitate identification of linkages between clinical isolates and potential environmental exposures.

Molecular diagnosis

Nucleic-acid amplification techniques (NAATs) for detection of *Legionella* spp. can be performed on lower respiratory tract secretions, sterile fluids, tissues, serum and urine. Sputum digestion is recommended as it helps to eliminate PCR (polymerase chain reaction) inhibitors and produce better results (Avni et al., 2016; Peci et al., 2016; Benitez and Winchell, 2014; Brunel and Charpentier, 2016; Mérault et al., 2011).

The most commonly used primers are:

- 16S rRNA for detection of *Legionella* spp.
- mip (macrophage infectivity protein gene) for detection of *L. pneumophila*.
- ssrA codes for tm-RNA in *L. pneumophila*.
- wzm is part of the lipopolysaccharide (LPS) gene cluster in *L. pneumophila* serogroup 1.

Multiplex assays are usually preferred as in nosocomial infections and immunocompromised patients with Legionnaire's disease; as it is important to test for both *L. pneumophila* and *Legionella* spp. This is also relevant in areas where *L. longbeachae* infection is more prevalent.

Sensitivity of PCR ranges from 80% to 100% in most clinical specimens. This percentage drops to 30% to 50% when the technique is performed on serum or urine specimens. Regarding specificity the value ranges from 95% to 99.9% for all sample types. False positives may be due to contamination of the nuclease-free water and extraction columns in the production process.

Although assays vary depending on the laboratory, PCR is a rapid, sensitive technique that allows identifying different species of *Legionella* from a wide range of clinical specimen.

Serology

Serological diagnosis by indirect fluorescent antibody (IFA) requires a rise in titer of fourfold or $\geq 1:128$ (seroconversion) in paired sera to be considered diagnostic of *L. pneumophila* infection. Approximately 5–10% of the population has titer $1:\geq 256$; therefore single acute phase antibody can't discriminate between Legionnaires disease and other causes of CAP (community-acquired pneumonia).

The presence of IgM in serum should not be used as evidence of acute-phase considering titers of IgM can last up to a year after infection. Total immunoglobulin determination is preferred to avoid misdetection of patients developing only one type of Ig (M, G or A) responses.

Paired serology is recommended and should be performed on a serum sample obtained at acute stage of the disease up to 2 weeks after the onset of symptoms and another one collected 3–6 weeks later. The first sample should be frozen (-20°C) whilst the serum from the convalescent-phase is collected (Yabuuchi et al., 1997; Zimmerman et al., 1982).

Microscopy

Microscopy techniques have the lowest sensitivity and specificity values of all the available tests for Legionnaires disease diagnosis.

- Direct fluorescent antibody (DFA) stain is the most sensitive and specific microscopic method. It detects *L. pneumophila* from tissues and sputum but requires exacting staining methods as well as an experienced microscopist to achieve a good level of identification.
- Acid-fast stain is useful on some *Legionella* spp. especially for *L. micdadei*. Fresh specimens and formalin-fixed tissues can be used. Morphology: small coco-bacilli.
- Silver stain can be used to observe the bacteria in embedded tissues; however this technique produces many artifacts that make difficult identifying the bacteria.
- Gram-stain is not recommended from the clinical specimen due to its difficulty and low sensitivity.

Several studies have been published comparing sensitivity between the different available tests to identify *Legionella* from clinical specimens. The results vary widely depending on the specimen collected, laboratory performance of the test, commercial kit used, patient's condition and severity of the disease, among other test-related factors. Table 3 shows a general resume of sensitivity and specificity of the most commonly used techniques provided by the CDC.

Every available identification method for *Legionella* spp. in clinical specimens has an inherent weakness, therefore more than one test should be performed and ideally more than one specimen collected. For patients with suspected CAP caused by *Legionella* spp. a sample of urine and sputum should be requested for antigen detection and culture, respectively. In case of nosocomial infection or immunocompromised patient with suspected Legionnaire's disease, culture and PCR of the appropriate specimen may be essential for diagnosis. Serologic diagnosis is recommended for epidemiologic studies.

Antimicrobial susceptibilities and treatment

Antimicrobial susceptibility testing is difficult to perform due to *Legionella* fastidious growth requirements; also the complex broth and agar media inactivate many drugs. In addition to this, the correlation between in vitro results and clinical outcome is uncertain; this is because the intracellular location of the bacteria cannot be accessed by some antimicrobials such as β -lactamics or aminoglycosides. Some *Legionella* spp. resides in sub-cellular compartments and may have a different respond to the treatment.

Antimicrobial susceptibility testing

There is no gold standard for antimicrobial susceptibility testing of *L. pneumophila*. The use of media containing charcoal result in elevated MICs for most relevant agents, however this supplementation may be necessary to achieve adequate growth.

Antimicrobial susceptibility testing should be used for detecting resistance mechanisms, although there is no enough data available to establish epidemiological cut off values (ECOFFs). There are published MIC distributions available (Zimmerman et al., 1982) that give an indication of susceptibility for wild type isolates using micro-tube dilution gradient test on BCYE α broth. E-test is not useful for susceptibility in clinical testing.

Table 3 Sensitivity and specificity of recommended diagnostic techniques for detection of *Legionella* spp.

Test	Sensitivity (%)	Specificity (%)
Culture	20–80	100
Antigen detection	60–100	99.9
Polymerase chain reaction (PCR)	95–99	99.9
Direct fluorescent antibody (DFA) stain	25–75	95
Paired serology	80–90	99

Antimicrobial resistance

Acquired antimicrobial resistance in *L. pneumophila* is extremely rare. However, a clinical isolate resistant to ciprofloxacin due to a single point mutation in the *gyrA* gene, has been reported and further fluoroquinolone resistant strains have been isolated from patients. Even so the clinical implications of resistance are uncertain (Edelstein, 2019).

Treatment

The antimicrobial agents successfully used for the treatment of Legionnaire's disease are macrolides, quinolones and tetracyclines.

Pontiac Fever is a self-limited illness; patients usually recover within a week, nevertheless supportive care should be used to relieve symptoms.

To prescribe the correct treatment it is necessary to consult local guidelines. The following treatment regimen (Mensa et al., 2016a) shows some of the most recommended antimicrobial agents and doses, but they must change depending on local guidelines.

Mild to moderate pneumonia is usually treated with Azithromycin 500 mg every 24 h p.o. (orally) during 3–5 days or Clarithromycin 500 mg every 12 h p.o. for 10 days.

In severe Legionnaire's disease including immunocompromised patients:

- Fluoroquinolones: Levofloxacin 500 mg every 12–24 h IV (intravenous) or Ciprofloxacin 400 mg every 8–12 h IV during 10–14 days.
- Azithromycin 500 mg every 24 h IV for 5–10 days.
- Association of Levofloxacin and Azithromycin.

Alternative treatment:

- Doxycycline 100 mg every 12 h IV or p.o.
- Co-trimoxazole (trimethoprim-sulfamethoxazole 160/800 mg) every 8–12 h IV or p.o.
- Association of Azithromycin with Rifampicin.

Chlamydia and Chlamydophila

Taxonomy

The family Chlamydiaceae (order Chlamydiales, phylum Chlamydiae) are important pathogens found throughout the animal kingdom. Although two genera, *Chlamydia* and *Chlamydophila*, were proposed and discussed for decades, recent studies have rejected the existence of *Chlamydophila* (Sachse et al., 2015).

From the diverse number of *Chlamydia* spp. found in nature only five have been recognized as pathogenic for humans (Wolff et al., 2018; Mensa et al., 2016b):

Chlamydia trachomatis, *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Chlamydia abortus* and *Chlamydia felis*.

C. trachomatis is the most prevalent agent in human infection of the genus. It is divided into 15 serovar (A, B, Ba, C-K, and L1-L3) based on analysis of the MOMP (major outer membrane protein).

General characteristics

They are a small obligate intracellular gram-negative bacterium, their size ranges from 0.2 to 1.0 μm .

Chlamydiae cell wall possesses an outer membrane characteristic of that in gram-negative bacteria, but the lack of peptidoglycan distinguishes them from similar intracellular pathogen like Rickettsia.

Through its growth cycle Chlamydiae alternates between two forms (Marrazzo and Suchland, 2014):

- The elementary body (EB) which is extracellular and infectious, but metabolically inactive.
- The reticulate body (RB) which is the intracellular non-infectious form. This is metabolically active and the replicative form (Fig. 1).

Stages of Chlamydiae developmental cycle:

1. The elementary body binds a host cell surface receptor using a specific ligand. This activate a mechanism of endocytosis where the plasma membrane zipper around the bacteria (Cossart and Helenius, 2014).
2. Inside the host cell the bacteria resides in a vacuole (phagosome) and survives by inhibiting lysosome fusion.
3. The elementary body transforms to reticulate body.
4. The reticulate body multiplies by binary fission. Along this phase the phagosome enlarges forming the inclusion body.
5. Reticulate bodies differentiate back into elementary bodies.
6. The inclusion body enlarges more, containing both forms of the bacteria.
7. The elementary bodies are released by lysis or extrusion process, at this stage they are ready to attach the surface of adjacent host cells and initiate new cycles of infection.

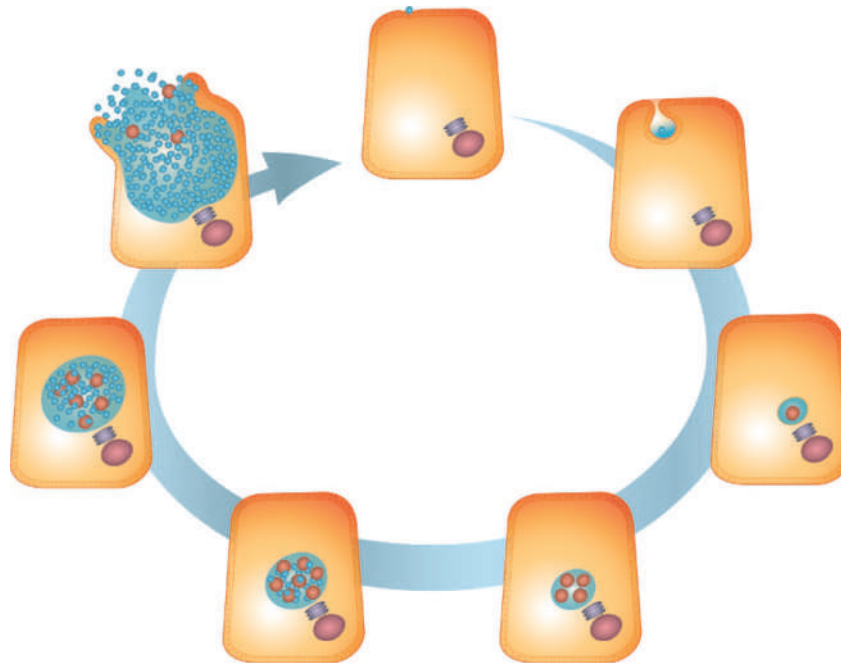


Fig. 1 Chlamydia developmental cycle.

Epidemiology and pathogenesis

Chlamydia trachomatis

Chlamydia trachomatis eye infection

- Trachoma is a chronic ocular disease caused by repeated infections of the conjunctiva and cornea.

It is endemic in many areas of Africa, Asia, Australia, Central and South America and the Middle East. Worldwide this disease affects 500 million people and up to 9 million are reported to be blind from this condition.

The agent spreads by direct contact, contaminated fingers, facecloths, clothing, bed linen, pillows etc. due to infective discharges. The incubation period ranges from 1 to 3 weeks.

Chronic conjunctivitis develops follicles that may necrotize and cause scarring. Over the years the contraction of the scars can turn in the lids leading the abrasion of the cornea by eyelashes. Blindness could be due to conjunctival scarring or secondary bacterial infections (Boyd, 1995b).

- Inclusion conjunctivitis refers to an ocular Chlamydia infection that doesn't progress to blindness. It is usually a self-limited disease; however scarring has been reported in infants without a prompt treatment.
- Ophthalmia neonatorum is the ocular infection of the infant who gets exposed to the pathogen during passage through the infected birth canal. This can be caused as well by *Neisseria gonorrhoeae*. Around a 20% of infants exposed to infected mothers will develop inclusion conjunctivitis and around 15% will develop neonatal pneumonia.

Chlamydia trachomatis genital infection

- Urethritis, cervicitis and proctitis.
- Lymphogranuloma venereum (LGV) is a sexually transmitted disease caused by L1, L2, and L3 serovars of *Chlamydia trachomatis*. Three stages of the disease have been described. Initially the infection may be undetected, in this stage a painless papule or ulceration appears. On a secondary stage unilateral or bilateral lymphadenopathies known as buboes, appear along inguinal and/or femoral areas. On the third stage fibrotic lesions affect the genital and anal area. The incubation period takes around 1–4 weeks.

Asymptomatic infection is common among men and women, this is the reason why CDCs (Centers for Disease Control and prevention) recommend annual screening test on population at increased risk. Having multiple Chlamydia infections can develop serious reproductive health complications in women, including pelvic inflammatory disease and ectopic pregnancy. In men the complication of urethritis progress to severe epididymitis. Women and men should be retested about 3 months after the treatment of an initial infection.

LGV in men that have sex with other men (MSM) is associated with a high rate of co-infection with gonorrhoea, syphilis, herpes simplex virus, hepatitis C virus, and/or HIV. When LGV is suspected, diagnostic tests for other potentially coexisting STDs (sexually transmitted diseases) must be performed (Schachter and Chernesky, 2019; Ceovic and Gulin, 2015).

Chlamydia trachomatis is the world's most common sexually transmitted bacterial pathogen, leading the cause of non-gonococcal urethritis. The number of cases per population is underestimated due to asymptomatic infections, availability of diagnostic tests and lack of universal surveillance protocols. Most recent outbreaks of LGV in Europe and EEUU were caused by L2b affecting HIV men who have sex with men (MSM).

Confirmed cases of *Chlamydia trachomatis* infection should be notified to the reference center of disease control to monitor epidemiology of the disease and activate prevention strategies.

Chlamydia (Chlamydophila) pneumonia

This specie is a common etiologic agent of upper and lower respiratory tract infection. It is one of the pathogens of CAP. Clinical symptoms are similar those found in respiratory infections caused by other pathogens.

The pathogen is transmitted via respiratory droplets and has a long incubation period that may vary from 3 to 4 weeks after exposure. It has also been isolated from some animals like horses, koalas and snakes.

Outbreaks are usually reported from crowded communities like schools, military barracks, nursing homes, prisons or student residences among others. Seroprevalence in adult population reaches around 50–70%, but the real prevalence of this agent is unknown as there is no national reporting or surveillance of *Chlamydia pneumophila* infections (Mensa et al., 2016b; Schachter and Chernesky, 2019).

Chlamydia (Chlamydophila) psittaci

A pandemic of human psittacosis allowed identifying *C. psittaci* for the first time. The spread of the disease was due to infected parrots and other related birds that were exported worldwide from South America between 1929 and 1930.

C. psittaci is a pathogen of birds; in contact with humans it causes a wide range of clinical conditions from asymptomatic infection to fatal pneumonia and systemic infections. The incubation period is 5–14 days. Symptomatic infections usually present with an abrupt onset of fever and chills, headache, myalgia and non-productive cough. The mortality on patients receiving the appropriate treatment is less than 1%.

Psittacosis is classified as category B bioterrorism disease

The bacteria spread by feces and respiratory secretions from infected birds. Human transmission occurs by inhalation of dust and droppings, very rarely from bites or beak-to-mouth contact. Population at higher risk of exposure includes employees of poultry-processing plants, farmers, workers in avian quarantine stations, zoos or pet shops, as well as veterinarian and laboratory staff (CDC).

Chlamydia (Chlamydophila) abortus and Chlamydia (Chlamydophila) felis

These two species of Chlamydia affect mainly mammals but have been proven to cause disease in humans (Mensa et al., 2016b; Schachter and Chernesky, 2019).

- *Chlamydia abortus* is an important cause of abortion in goats and sheep, although cases of abortion have been reported in women. It is considered an occupational infection with a very low prevalence.
- *Chlamydia felis* can cause conjunctivitis in humans

Diagnosis

There are potential risks for laboratory-acquired infections with *Chlamydia* spp.; LGV biovar of *C. trachomatis* and *C. psittaci* are considered a Biosafety level 3 (BSL 3) organisms. The rest of human pathogen species of Chlamydia are considered BSL 2.

The diagnosis of Chlamydia species is usually performed by NAATs as is the most sensitive and easy diagnostic test available; it provides rapid detection and is useful on strain typing. But also antigen detection, culture and serology are useful methods (Schachter and Chernesky, 2019).

Molecular detection

For detection of *C. trachomatis* the PCR can be performed on first-catch urine samples and urethral swabs from men and vaginal or endocervical swabs from women. Rectal and posterior oropharynx swabs can be collected when infection is suspected on either site. Most of the commercially available PCR tests are duplex assays for simultaneous detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, as they are the most prevalent bacteria causing STDs.

For detection of *Chlamydia pneumoniae* and *Chlamydia psittaci* respiratory specimens (sputum, respiratory secretions, nasopharynx and oropharynx swabs) can be processed (Zimmerman et al., 1982).

Antigen detection

The appropriate specimens for these techniques include exudates from conjunctiva on *C. trachomatis* infection; respiratory secretions on *C. pneumoniae* and impression smear of liver and spleen the avian source, for the identification of *C. psittaci*.

- Giemsa stain may be used to detect inclusions as diagnose of conjunctivitis in newborns but is not sensitive for adult conjunctivitis or genital infection. This stain is also used to detect *C. psittaci* from specimens obtained from avian sources of human infection.
- DFA is useful as a rapid diagnosis of inclusion conjunctivitis on infants and probably in adults.

Serology

Serology methods show low specificity and sensitivity rates; even so it may be useful as a complement in the diagnosis of LGV, pneumonia infection on infants, human psittacosis and infections of *C. pneumoniae*. It is a useful tool in clinical epidemiologic studies and research.

The most used techniques are:

- Microimmunofluorescence for detection of specie and serovar specific antibody response as well as the Ig (immunoglobulin) class.
- Enzyme immunoassay (EIAs) as a good technique on epidemiologic studies.

Cell culture

This method is performed at specialized laboratories. Usually the culture needs a transport medium containing antibiotics to prevent other bacterial and fungal contamination. The clinical specimen should be introduced directly in the Chlamydia-specific transport medium.

For isolation of the bacteria the goal is to collect infected cells; for genital infection urethral swabs from men and endocervical swabs are recommended, in case of conjunctival infection the sample must be direct scrapings collected with spatula, but samples obtained by swabs are also acceptable. Tissue samples can be cultured as well. For the isolation of *C. pneumoniae* is indicated to collect respiratory secretions, sputum or throat washes. In the case of *C. psittaci* when avian species are suspected to be the source of human infection the specimens should be collected from liver, spleen or serofibrinous exudates coating those organs.

The inoculated cells (Table 4) may be incubated at $35 \pm 2^\circ\text{C}$ (35°C are optimal for *C. trachomatis*, but 37°C are preferred for *C. psittaci*) in an environment of 5% CO_2 for 48 to 72 h. After this period mono-layers are fixed with methanol and stained with DFA reagents to detect inclusions of the bacteria.

Treatment

Human infections caused by Chlamydia are usually treated with macrolides or tetracyclines; fluoroquinolones are prescribed as alternative. Treatment failures have been reported but the recommendation is to treat again the patient as there is no evidence of antibiotic resistance.

Antimicrobial susceptibility tests are not routinely performed, but there are published protocols (Schachter and Chernesky, 2019). MIC is defined in this case as the level of antibiotic that reduces inclusion counts by 50% (Table 5).

In addition to the treatment of sexually transmitted infections, patients should abstain from sexual activity for 7 days after starting the antibiotic treatment or until this has finished in longer courses, to prevent the spread to sexual partners.

Regarding Trachoma the World Health Organization has targeted it for elimination through a public health strategy known as S.A.F.E.

- Surgery to correct the advanced, blinding stage of the disease.
- Antibiotics to treat active infection.
- Facial cleanliness.
- Environmental improvements in the areas of water and sanitation to reduce disease transmission (Table 6).

Table 4 Shows the most common cell line used for Chlamydia culture.

Chlamydia spp.	Cell line
<i>C. trachomatis</i>	McCoy
	HeLa
	BGM
<i>C. pneumoniae</i>	HL
	Hep2

Table 5 Shows some of the recommended antibiotic regimes for *C. trachomatis*, although local guidelines or up to date recommendations from CDCs or WHO should be consult for an adequate treatment (Mensa et al., 2016b).

	Recommended	Alternative
Lymphogranuloma venereum	• Doxycycline 100 mg p.o. twice daily or 200 mg p.o. once daily for 2–3 weeks or • Azithromycin 1 g p.o. per week for 3 weeks Buboos should be drained or surgically removed Pregnant and lactating women should be treated with Erythromycin or Azithromycin	• Erythromycin 500 mg p.o. every 6 h or • Levofloxacin 500 mg p.o. daily for 21 days
Urethritis, Cervicitis and Trachoma	• Doxycycline 100 mg p.o. twice daily or 200 mg p.o. once daily for 7 days or • Azithromycin 1 g p.o. single dose	• Erythromycin 500 mg p.o. every 6 h or • Levofloxacin 500 mg p.o. daily for 7 days
Eye infection	• Azithromycin eye drops for 3 days. An oral treatment with tetracycline may be used in addition to the eye drops	

Table 6 Shows the recommended antibiotics for *Chlamydia abortus*, *pneumoniae* and *psittaci*.

	Recommended	Alternative
<i>Chlamydia abortus</i>	Doxycycline	
<i>Chlamydia pneumoniae</i>	Doxycycline 2–3 weeks or Azithromycin 3–5 days	Clarithromycin or Levofloxacin 2–3 weeks
<i>Chlamydia psittaci</i>	Doxycycline 10–14 days	Macrolides, Fluoroquinolones or chloramphenicol

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Relevant websites

Legionella

- <https://www.who.int/news-room/fact-sheets/detail/legionellosis>—WHO.
- <https://www.cdc.gov/legionella/clinicians/disease-specifics.html>—CDC.
- <https://www.gov.uk/government/publications/legionnaires-disease-national-surveillance-scheme>—NICE Guidelines.
- <https://www.blf.org.uk/support-for-you/legionnaires-disease>—British Lung Foundation.
- [https://legacy.bd.com/ds/technicalCenter/inserts/L007349\(10\).pdf](https://legacy.bd.com/ds/technicalCenter/inserts/L007349(10).pdf)—BD BBL™ BCYE Agar.
- https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Legionella_guidance_document_20171208.pdf—EUCAST.

Chlamydia

- <https://www.trachoma.org>—International Trachoma Initiative.
- <https://www.health.nsw.gov.au/Infectious/controlguideline/Pages/chlamydia.aspx>—NSW (New South Wales, Australia) Chlamydia control guidelines.
- <https://www.cdc.gov/std/tgd2015/lgv.htm>—CDC Lymphogranuloma venereum.
- <https://www.cdc.gov/pneumonia/atypical/cpneumoniae/index.html>—CDC Chlamydia pneumonia infection index.
- <https://www.cdc.gov/pneumonia/atypical/psittacosis/hcp/diagnosis-treatment-prevention.html>—CDC Psittacosis: Diagnosis, treatment and prevention.

Ehrlichia, Anaplasma and Related Bacteria

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Introduction

Ehrlichia and *Anaplasma* are a group of gram-negative bacteria, belonging to the order Rickettsiales and family Anaplasmataceae (Sneath et al., 1986). The Anaplasmataceae family is composed of five genera: *Ehrlichia*, *Anaplasma*, *Neoehrlichia*, *Wolbachia*, and *Neorickettsia* (Pruneau et al., 2014). These genera can be classified according to their transmission mechanism. The genera *Ehrlichia*, *Anaplasma*, and *Neoehrlichia* are transmitted by ticks, while the genera *Neorickettsia* and *Wolbachia* have different transmission mechanisms that do not require ticks (Pruneau et al., 2014).

Within the *Anaplasmataceae* family we find species that affect a wide multitude of hosts such as horses, cows, goats, etc., therefore having importance in the veterinary field (Dantas-Torres et al., 2018; Sato et al., 2020). The genera *Ehrlichia* and *Anaplasma* include species that can cause disease in humans. The main species are *Ehrlichia chaffeensis* y *Anaplasma phagocytophilum* and, to a lesser extent, the species *Ehrlichia ewingii* and *Ehrlichia muris euclairensis* (Dumler, 2005).

Ehrlichiosis, previously known as human monocytic ehrlichiosis (HME), is caused by *E. chaffeensis*, *E. ewingii*, and *E. muris euclairensis*. In 2008, ehrlichiosis were classified into four categories (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019a): (i) infection caused by *E. chaffeensis*, (ii) infection caused by *E. ewingii*, (iii) infection caused by *A. phagocytophilum*, and (iv) indeterminate ehrlichiosis/anaplasmosis. *E. muris euclairensis* infections are recently discovered and therefore are included in the indeterminate ehrlichiosis/anaplasmosis (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019a). These four categories also include anaplasmosis, previously known as human granulocytic anaplasmosis (HGA), which is caused by *A. phagocytophilum* (Bakken and Dumler, 2015).

The genus *Ehrlichia* and *Anaplasma* are obligate intracellular bacteria, which live inside modified vacuoles of monocytes and granulocytes (Zhang et al., 2007; Troese et al., 2011). They replicate inside the vacuoles and within a few days intracellular inclusions known as morulae can be observed (Zhang et al., 2007). In morulae we can find replicative forms that are not very infective and non-replicative forms that are highly infective (Zhang et al., 2007). They have a very simple cell wall, surface proteins to bind to the target cell and a type IV secretion system that they use to introduce their proteins inside the host cell and modify it: prevents the fusion of lysosomes, modifies the cytoskeleton and alters gene expression (Zhu et al., 2011; Rennoll-Bankert et al., 2015).

They have an aerobic metabolism. They cannot perform glycolysis but can use simpler substrates like glyceraldehyde-3-phosphate and phosphoenolpyruvate for the synthesis of other compounds. They use only proline and glutamine as a carbon source, having to be most of the amino acids exported from the host cell (Dunning Hotopp et al., 2006).

As they are obligate intracellular species, they cannot be grown in cell-free medium. *E. chaffeensis* can be cultured using monocyte and macrophage cell lines of canine, murine, primate, and human origin (Chen et al., 1995). *A. phagocytophilum* can be cultured in vitro using human myeloid leukemia cell lines (Troese et al., 2009). All species can be grown in tick embryonic cell lines.

Transmission

The transmission mechanisms for *Ehrlichia* and *Anaplasma* are the same, showing differences in the vector species. The main route of transmission is through an infected tick bite. *E. chaffeensis* and *E. ewingii* are transmitted by the lone star tick (*Amblyomma americanum*). *E. muris euclairensis* is transmitted by the black-legged tick (*Ixodes scapularis*) (Centers for Disease Control and

Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019a). In the case of *A. phagocytophilum*, we found two vectors: *I. scapularis* in the northwestern and midwestern United States and *Ixodes pacificus* along the west coast (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019b).

Transmission by blood transfusion or transplantation is rare, but even fatal infections have been reported (Annen et al., 2012; Alhumaidan et al., 2013; Regan et al., 2013; Sachdev et al., 2014; Goel et al., 2018). *E. chaffeensis* and *A. phagocytophilum* can be isolated from blood anticoagulated with EDTA and stored at 4 ° C after 11 and 18 days, respectively (Kalantarpour et al., 2000; McKechnie et al., 2000).

The reservoir for *E. chaffeensis* is the white-tailed deer (*Odocoileus virginianus*) (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019a). The *A. phagocytophilum* reservoir is a smaller mammal such as the white-footed mouse (*Peromyscus leucopus*) (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019b).

Epidemiology

Ehrlichiosis is endemic to the southwestern and south-central United States, although cases are also being reported in Europe and Africa (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019a). Recent serology data show infections in South America, as well as China and Korea. Cases tend to increase in summer, especially between the months of June and July. During this time, the number of lone star ticks increased in the adult and nymph stages, which are the two stages that can transmit the disease to humans (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019a).

Anaplasmosis is most frequently reported in the upper Midwest and Northwestern United States (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019b). Cases have also been reported in central Europe (Slovenia and Sweden). Although cases can occur throughout the year, they are also in June and July when more infections are reported, coinciding with the time of the nymphal stage of ticks. In October and November there is a small peak of cases that coincides with the increased activity of adult ticks (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019b).

The number of cases reported by the Centers for Disease Control and Prevention (CDC) of ehrlichiosis caused by *E. chaffeensis* has been increasing gradually, going from 200 cases in 2000 to 2100 in 2019 (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019a). Between 2008 and 2012 the incidence was 3.2 cases per million people (Nichols Heitman et al., 2016). However, these data are not entirely correct since many cases cannot be confirmed serologically, asymptomatic infections are known, etc. However, while cases and incidence increased, largely due to improved diagnosis, fatality has dropped to 1% (Nichols Heitman et al., 2016).

Cases of infection by *E. ewingii* cannot be distinguished from those caused by *E. chaffeensis* if we look only at symptoms. 261 cases were reported to the CDC between 2008 and 2019, with no fatal cases (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019a). The incidence rate between 2008 and 2012 was 0.04 cases per million people and year (Nichols Heitman et al., 2016). It is possible that many *E. ewingii* infections are being diagnosed as *E. chaffeensis* infection because serologic tests do not discriminate (Nichols Heitman et al., 2016). Molecular tests are required to differentiate infection between the two species.

E. muris euclairensis infections have little impact on the population, with only 119 cases reported to the CDC since 2009 (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019a), with no reported deaths. Immunosuppressed patients have been shown to be more prone to the disease.

A. phagocytophilum is the species that causes the most infections. It has been increasing from 348 cases in 2000 to 5655 in 2019, going through a record in 2017 of 5762 cases (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019b). In the United States, during 2008 and 2012, it had an incidence of 6.3 cases per million inhabitants per year (Dahlgren et al., 2015). These data are thought to be incomplete since not all cases are reported, many cases are diagnosed clinically and not serologically, and furthermore, it is thought that due to the large number of tick bites, there must be many cases of asymptomatic disease. The fatality rate is less than 1% (Dahlgren et al., 2015).

Clinical manifestations

Ehrlichiosis and anaplasmosis usually present as acute diseases, but they include subclinical and self-limited cases together with cases of subacute and prolonged disease (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019a,b). Mild disease cases are associated with infection by *A. phagocytophilum*, with severe cases related to ehrlichiosis (Dahlgren et al., 2015; Nichols Heitman et al., 2016).

The incubation period is usually one to two weeks after the tick bite. Results have described that the incubation period can even drop to 5.5 days after the bite (Aguero-Rosenfeld et al., 1996).

Symptoms can range from mild-moderate to severe, depending on the immunocompromised state of the patient, as well as the delay in the administration of treatment.

As mild symptoms we find fever, chills, malaise, headache, nausea, vomiting, diarrhea, loss of appetite, arthralgia, myalgia, cough, confusion, and rash (Dumler et al., 1993; Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2019a,b). The rash appears more frequently in children and is a macular, maculopapular or petechial rash (Schutze et al., 2007; Sigurjonsdottir et al., 2017). The appearance of the rash is seen more frequently in ehrlichiosis (Bakken et al., 1996).

Subacute disease is characterized by long-lasting fever, with one case reported with fever lasting 50 days (Everett et al., 1994; Roland et al., 1995).

Cases of serious diseases are associated with elderly patients, immunocompromised status, transplantation or chronic disease (Bakken and Dumler, 2000; Wormser et al., 2006; Esbenshade et al., 2010). Symptoms of severe disease include meningoencephalitis, respiratory failure, disseminated intravascular coagulation, seizures, coma, multiple organ failure and death. In addition, heart failure and pericardial effusion may occur (Goodman et al., 1996; Vanek et al., 1996). Neurological symptoms are related to ehrlichiosis, being very rare in anaplasmosis. These symptoms can appear in up to 20% of patients and are usually mental changes, neck stiffness and meningoencephalitis (Ratnasamy et al., 1996). Septic shock has also been recorded from this type of infection (Dumler, 2005; Zhang et al., 2008). Finally, cases of hemophagocytic lymphohistiocytosis have been reported (Otrock et al., 2015; Johnson et al., 2017).

Ehrlichiosis infection in children is reported much more frequently than anaplasmosis. Symptoms for ehrlichiosis are fever, headache, myalgia, rash, nausea, vomiting and lymphadenopathy (Schutze et al., 2007), while for anaplasmosis they are usually fever, headache, malaise and intestinal discomfort (Sigurjonsdottir et al., 2017). Only a minority of children have severe ehrlichiosis disease, requiring some admissions to the intensive care unit (UCI) with hemodynamic and ventilatory support (Schutze et al., 2007).

Laboratory findings

In *E. chaffeensis* infections, leukopenia (the most common), thrombocytopenia, as well as elevated transaminases, lactate dehydrogenase, and alkaline phosphatase are frequently observed (Dumler et al., 1993; Otrock et al., 2015; Sigurjonsdottir et al., 2017). Leukopenia and thrombocytopenia also occur in *A. phagocytophilum* infections. Leukopenia due to lymphopenia occurs early in the disease and with the progression of the infection, a lymphocytosis characterized by atypical lymphocytes appears (Aguero-Rosenfeld et al., 1996). In patients with anaplasmosis, thrombocytopenia is more common than neutropenia (Bakken et al., 2001).

In those cases that present neurological symptoms due to *E. chaffeensis*, we can find modifications in the cerebrospinal fluid such as high levels of proteins and lymphocytic pleocytosis (Ratnasamy et al., 1996). In the rare cases in which there are neuronal symptoms in *A. phagocytophilum* infections, the cerebrospinal fluid is slightly altered (Bakken and Dumler, 2008).

Diagnosis

Presumptive diagnosis of HME and HGA is made through clinical and epidemiologic features. Febrile illness with laboratory abnormalities (leukopenia and/or thrombocytopenia), history of tick bite and outdoor activities during the spring or summer months in a resident of an endemic area provides strong evidence for the diagnosis of HME.

Laboratory diagnosis included: culture and isolation, detection of morulae in peripheral blood, detection of specific immune response, detection of specific DNA by polymerase chain reaction (PCR) and direct detection of bacteria in tissue samples by immunohistochemistry.

Culture and immunohistochemistry

Culture and isolation in tissue culture is the gold standard in diagnosis of HME and HGA, however, primary isolation may take more than 2 weeks to obtain results (Thomas et al., 2009).

Isolation of *Erhlichia* spp. from clinical samples is difficult (routine hospital blood cultures cannot detect the organism) and has low diagnostic sensitivity, so it is not used routinely in the laboratory.

Microscopic examination of blood smears or buffy coat preparations during the first week of illness might reveal intracytoplasmic inclusions (morulae) in infected circulating leukocytes. This method is rapid, but it is relatively insensitive compared with other confirmatory tests. Morulae are detected within monocytes in only about 3% of patients with HME. In contrast, they are seen in the cytoplasm of neutrophils in 25–75% of patients with HGA (Ismail and McBride, 2017).

Serology

The most widely used test for diagnosis of HME and HGA is the indirect immunofluorescence (IFA) antibody assay for IgG and IgM. Sera collected during the first 1–3 weeks after onset of illness may be negative (Dawson et al., 1990) and a second serum collected 2–3 weeks later is recommended. False negative results may occur if a patient is immunocompromised or was treated very early in the disease (Paddock and Childs, 2003; Ismail and McBride, 2017).

A single IgG positive result suggests current or previous infection. Specific IgG may remain detectable for months to years after disease has resolved.

A fourfold rise in IgG titer between acute serum and a convalescent serum collected 2–4 weeks later provides the best evidence of active infection. In most laboratories the minimum titer considered to be indicative of current or previous infection ranges from 1:64 to 1:80 (Chapman et al., 2006).

IgM antibodies against ehrlichiae and *A. phagocytophilum* have lower specificity than IgG antibodies (Brouqui et al., 2001) and therefore, IgM antibody titers should be interpreted carefully.

There are high rates of cross-reactivity between antibodies to *A. phagocytophilum* and those to *E. chaffeensis*, so generally, serologic testing for both pathogens should be performed in areas endemic for ehrlichiosis and anaplasmosis. If the titer is much higher for one organism than the other, the one with the higher titer should be considered the likely causative agent. Cross-reactivity has been described with other rickettsias, *Coxiella burnetii*, and infectious mononucleosis (Thomas et al., 2009).

Other techniques that have been used include enzyme-linked immunosorbent assays (ELISAs), but ELISA tests are generally qualitative and are generally not useful to monitor the changes in antibody titers.

PCR

PCR is the most sensitive and specific method for the detection and identification of *E. chaffeensis*, *A. phagocytophilum*, and *E. ewingii*. Whole blood and CSF are the most appropriate samples for PCR testing. The sensitivity of PCR is greatest during the 1 or 2 weeks of infection, decreasing over time. The sensitivity for detecting *A. phagocytophilum* is 67–90% and 60–80% for *E. chaffeensis*, depending on the time of onset of infection (Thomas et al., 2009; Salinas et al., 2010; Ismail and McBride, 2017). Treatment with doxycycline can decrease the sensitivity of PCR, so samples should be collected before initiation of treatment if possible (Dumler et al., 2007).

Treatment

Due to the delay in obtaining confirmatory test results treatment for ehrlichiosis or anaplasmosis is usually started before test results are available. When treatment is started early, most people respond rapidly and well. A delay in treatment may lead to serious complications, including death.

E. chaffeensis, *E. ewingii*, and *A. phagocytophilum* are universally susceptible to doxycycline. In adults, doxycycline can be given either orally or intravenously at a dose of 100 mg twice daily. People take this antibiotic until they improve and have had no fever for 24–48 h, but they must take it for at least 7 days. Children should be treated with doxycycline at a dose of 4.4 mg/kg per day, divided every 12 h, intravenously or orally. Treatment should continue for at least three days after defervescence. In general, the duration of treatment is 7–14 days. Treatment relapses or antibiotic resistance has not been reported.

Rifampicin can be given as an alternative to doxycycline for treatment of ehrlichiosis and anaplasmosis, but clinical experience is limited in anaplasmosis (Krause et al., 2003).

In cases of allergies to doxycycline, and in some pregnant patients for whom the clinical course of ehrlichiosis appears mild, physicians might consider treatment with rifampin. Antibiotics in prophylaxis are not recommended to prevent ehrlichiosis.

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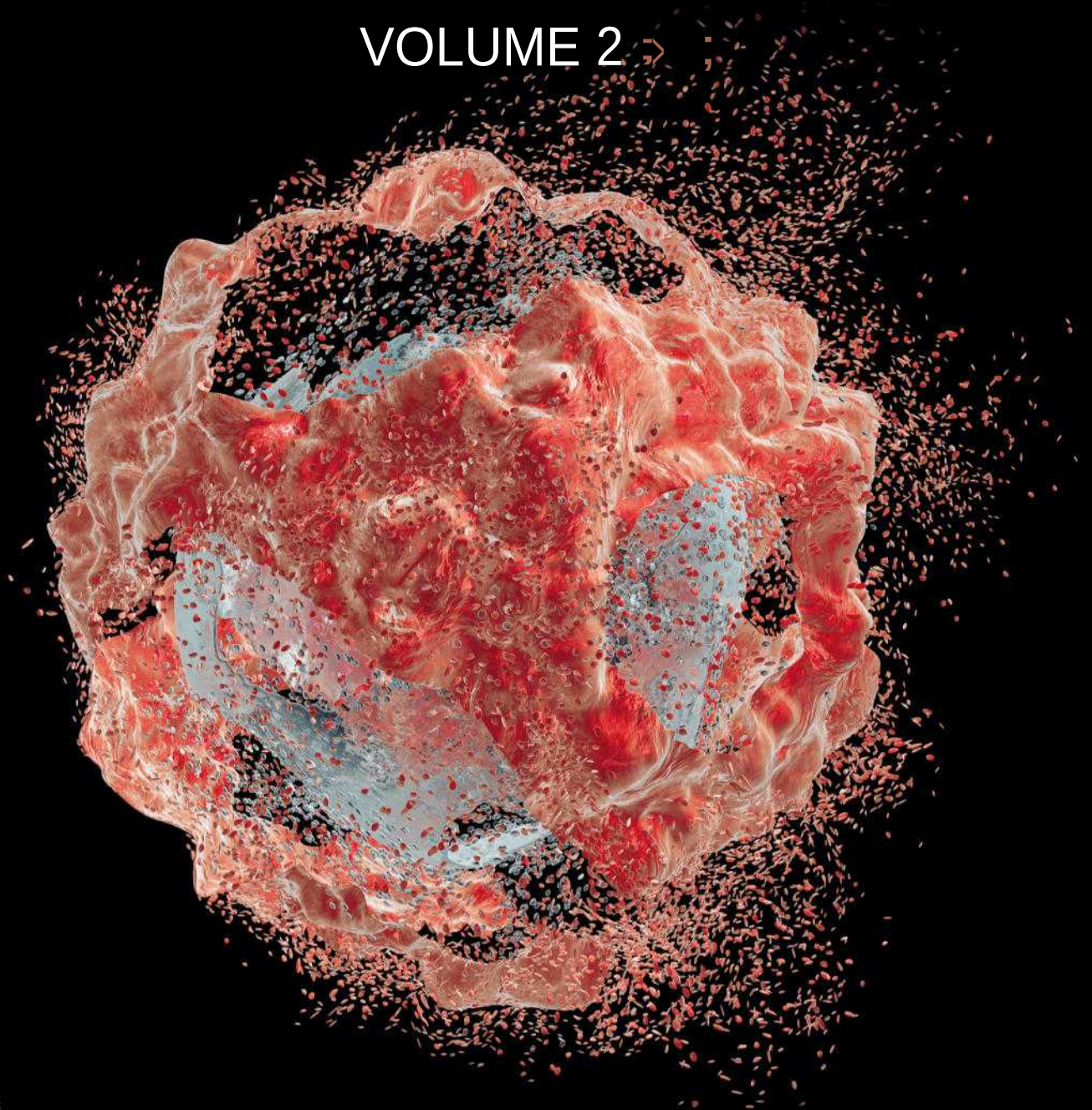


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FOREWORD

It is a great honor to be asked to introduce, with this foreword, the *Encyclopedia of Infection and Immunity*. This work will be read and enjoyed by many students, both graduate and postgraduate, including clinicians, laboratory practitioners, and scientists. Many colleagues have contributed to this important work, with over 230 original chapters assembled in sections and listed alphabetically. I offer my warm congratulations to the editors of this book, for soliciting a wide body of expertise and assembling it in a form that gives so much pleasure and information to the reader.

For those of us who work in the field of infection and immunity, the last two years have been exceptionally challenging. At the current time we are 22 months into a major pandemic caused by a novel coronavirus and its variants, and worldwide, sadly, there have been many hospitalizations and deaths. Clinicians and scientists in our field have been working flat out, caring for patients, generating or utilizing diagnostic tests, generating new knowledge in immunity to coronaviruses, or discovering, testing, and deploying new vaccines. Public Health practitioners have organized our medico-social responses to limit transmission and deal with the consequences of infection. Behavioral scientists have transformed our approach to informing the public, getting the best possible outcomes from effective communication. Governments have learned the danger of ignoring the expertise of scientists, and in many countries, the public look upon clinicians and scientists with gratitude and respect. It is a testament to the advancement of infection and immunity science that the genome of the virus was sequenced within a few weeks of COVID-19 emerging, and that within 12 months, the first trials of novel vaccines had been completed. These vaccines included some with exceptionally novel technologies, including the RNA vaccines and the viral-vectored vaccines.

Underpinning the academic agility of students and practitioners in Infection and Immunity is the universal strength of sound education. Readers of this book will be seeking the knowledge they need for a comprehensive understanding of infectious disease and immunology. We work in a fascinating discipline. Our field is unique in that pathogenesis involves at least two sets of genomes—on the one hand the microbe, and on the other, the host. The microbial genome programs its microenvironment to enable acquisition of and residence in a niche for sufficient time to replicate and further disseminate to other hosts. The human genome has evolved to tolerate and contain a wide range of symbionts and pathobionts, with highly sophisticated immune decision-making at the cellular level, but to respond to invading species with an elaborate coordinated repertoire of innate and acquired immunity. The complex conversation between many different microbes and the human host generates a wide range of disease phenotypes. This makes the job of an infectious disease physician so challenging but at the same time so fulfilling.

Our field is enriched by the many scientists and clinicians who are willing to share their experience with their peers and students. This book reflects the great variety of knowledge that has emerged over centuries of inquiry and endeavor, and I believe you will be rewarded by using it as one of your reference sources.

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PREFACE

Although infections have been one of the most important health threats since centuries ago, the mechanisms of human defense against infections have been unclear until last century. The scientific world has remarkable progresses in the field of immunology during recent decades. Indeed, the novel discovery of genes related to different pathogens has enhanced our knowledge about the immune system. Understanding different pathogens and the immune mechanisms promote diagnostic strategies and design more efficient therapeutic agents.

Encyclopedia of Infection and Immunity is a comprehensive text, which covers topics not only on basic immunology and microbiology but also on clinical immunology and infectious diseases. Section 1 focuses on the immune system, while Section 2 describes the interaction between pathogens and immunity. Bacteria, viruses, fungi, protozoan parasites and helminths, and insect and mites are explained in Sections 3–6, respectively. Section 7 discusses the clinical infectious disease challenges, whereas clinical immunology is covered in Section 8. Laboratory tests and treatment protocols are the main focuses of Sections 9 and 10, respectively.

Encyclopedia of Infection and Immunity is the result of valuable contribution of more than 400 scientists from many well-known universities/institutes worldwide. I would like to hereby acknowledge the expertise of all contributors, for generously devoting their time and considerable effort in preparing their respective chapters. I would also like to express my gratitude to Elsevier for providing us the opportunity to publish this Encyclopedia.

Finally, I hope that this comprehensive book will be of special value for researchers and clinicians who wish to extend their knowledge on infection and immunity.

Nima Rezaei, MD, PhD

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Introduction on Viruses

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What is a virus?

Viruses are very small infectious particles without a cellular structure lacking the ability to reproduce independently. They are all obligate intracellular parasites that can only regenerate by using the resources of an infected host cell. Viruses are comprised of protein packages containing genomic material, which can be either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) (Pellett et al., 2014). Notably, virus and virion are two concepts that may be used interchangeably commonly; however, they have some slight but crucial differences. The virion, also known as a virus particle, is the activated infectious extracellular form of the virus released from an infected cell and can bind to the surface of another cell spreading the virus. In contrast, the virus refers to the nucleoprotein particle at any time.

It is important to study viruses because of several reasons. Viruses have existed since the beginning of life and are critical in evolution. Moreover, they can be found almost everywhere in our surroundings, infecting a wide range of host cells from prokaryotes to eukaryotes. Most importantly, viral infections, such as viral respiratory infections (Monto, 2002) and human immunodeficiency virus (HIV) (Frank et al., 2019), impose an enormous burden on healthcare systems.

It has been more than a century since the discovery of viruses. The tobacco mosaic virus, a plant virus, was the first identified virus in the nineteenth century. In 1901, yellow fever was among the first viruses discovered to affect humans (Woolhouse et al., 2012). While the slope in discovering viruses has relatively decreased in the past decades, many viruses remain yet undiscovered, and there are a number of newly identified viruses each year (Woolhouse et al., 2012). Moreover, novel human viruses, such as the coronavirus SARS-CoV-2 (COVID-19), Ebola virus, and severe acute respiratory syndrome (SARS), can emerge due to variants of already known viruses (Ryu, 2017c). Notably, scientists were not able to actually see a virus until the 1930s, when electron microscopes were introduced (Fig. 1). In addition to electron microscopy, of note, X-ray crystallography is another technique to visualize virion structural organization. With its near-atomic resolution, X-ray crystallography can aid in localizing antigenic sites and further investigation of virion attachment and penetration to the host cells (Burrell et al., 2017a).

It is debatable whether viruses are inanimate or alive. The answer to this question entirely relies on the definition of a living organism. The discovery of exceptionally large and complex viruses with classic cellular genes disputed the notion of considering viruses as inanimate objects. However, due to the following reasons, it is proposed not to consider viruses as life forms. Although viruses contain genetic material similar to cellular organisms, they lack several fundamental features of cellular structures, such as self-maintenance and self-replication. Moreover, having a common ancestry has been observed in living cellular organisms, while this feature is missing in viruses. Lastly, unlike cellular organisms, viruses do not have the required genes for protein synthesis and carbon and energy metabolism independently. Therefore, they are dependent on the host cells for replication (Moreira and Lopez-Garcia, 2009).

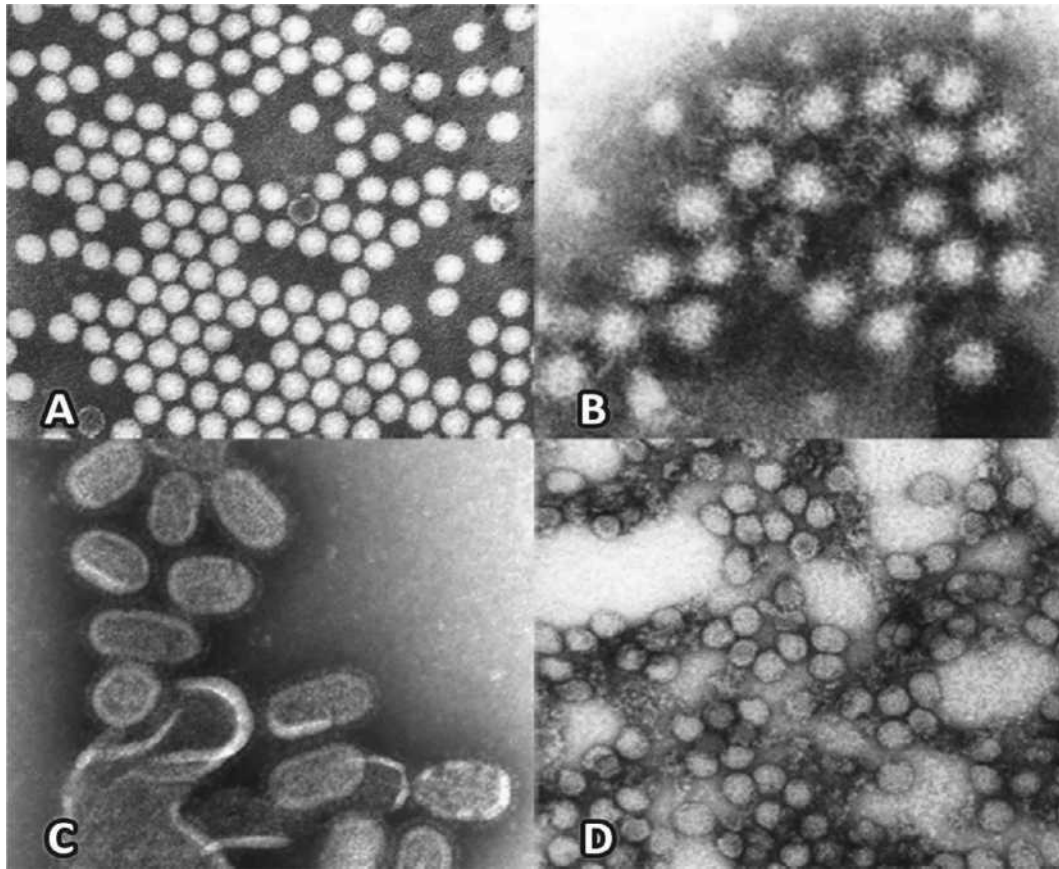


Fig. 1 Electron microscopy images of (A) poliovirus, (B) norovirus, (C) influenza virus, and (D) yellow fever virus. Adapted from Wang-Shick R (2017) Virus structure. In: Wang-Shick R (ed.), *Molecular Virology of Human Pathogenic Viruses*, pp. 21–29, Academic Press, ch. 2, with permission.

The diverse world of viruses

Viruses can be extremely diverse. Their size may vary from 20 nm (Duan, 2006) to 2500 nm (Okamoto et al., 2017). Their genetic material can have a large variety in its structure, with genome sizes ranging from approximately 800 bp (Campillo-Balderas et al., 2015) to 2.7 Mb (Legendre et al., 2019). They have wide-ranging hosts and habitats. They can cause different infection patterns and replication cycles lasting for a few minutes to several days (Payne, 2017). This broad diversity highlights the critical role of viruses in living systems.

The origin of viruses

Three principal hypotheses provide explanations for the origin of viruses, namely “the virus-first”, “the progressive (escape)”, and “the regressive (reduction)” hypotheses.

According to the virus-first theory, viruses existed before the emergence of cellular structures and were the first self-replicating units (Koonin and Martin, 2005). Notably, it is postulated that RNAs (not DNAs) were the first replicating molecules. Based on this notion, these first replicating entities evolved into more complex and organized structures, leading to the formation of cells. Moreover, some have argued that the antecedents of nucleocytoplasmic large DNA viruses (NCLDV) may have resulted in the emergence of eukaryotic organisms (Bell, 2001). NCLDVs comprise a diverse group of more complex viruses, such as mimivirus, a giant virus with a total diameter of approximately 750 nm and containing biosynthetic enzymes that are usually not present in other viruses (Claverie, 2008).

Conversely, the progressive and the regressive hypotheses hold the notion that cellular organisms existed before viruses. The progressive hypothesis states that genetic pieces with the ability to move within the genome attained the capacity of exiting one cell and entering another. The remarkable resemblance between the genetic arrangement in retroviruses and retrotransposons, a major component of the eukaryotic cells, supports this notion. As a result, it can be presumed that gaining a few structural proteins helped the genetic particle to escape the cell and enter a new one.

As opposed to the progressive hypothesis, the regressive hypothesis holds the assumption that viruses were initially free-living entities that began symbiotic relationships with other organisms. Over time, they developed a more parasitic relationship and lost their complexity as they became dependent upon their hosts for replication. The regressive hypothesis mainly hinges upon NCLDVs, which are more complex and larger compared to most other viruses (Wessner, 2010).

Viral structure and anatomy

All viruses are composed of two main structures, namely capsid and nucleic acid. On the other hand, most viruses are enveloped; however, some are non-enveloped or naked. These structures are described below (Harrison, 2013) (Fig. 2).

Capsid

The capsid is a dynamic protein package comprised of an assembly of a large number of capsid protein copies that can either have a single or a few types. The primary role of the capsid is to protect the genome. Additionally, particularly in the non-enveloped viruses, the capsid facilitates the attachment and penetration of the virus to the host cell. The capsid proteins are chiefly bonded by electrostatic interaction (van der Waals interaction), creating capsid protein building blocks. Only a few covalent bonds are developed between the building blocks to allow the capsid to be flexible enough to let the genome release whenever necessary (Mateu, 2013). As a result of the repeated same protein-protein interfaces, the capsid is most commonly symmetrical (Mateu, 2013). There are three main capsid shapes as described in the following.

- Helical:** capsid proteins are assembled in a circular manner resembling a stack of discs that are attached helically, creating a central cavity as a place for the nucleic acid. Tobacco mosaic virus, arenaviruses, and bunyaviruses are some examples of viruses with a helical capsid.
- Icosahedral:** The capsid is comprised of equilateral triangles, commonly composed of an arrangement of three capsid proteins merged together in a spherical shape. The capsid, which is formed in a stepwise manner, has no gaps in between and fully encompasses the genome. Poliovirus, herpesvirus, papillomavirus, rhinovirus, and adenovirus are some examples of viruses with an icosahedral capsid.
- Complex:** A complex capsid is a combination of helical and icosahedral shapes. Viruses with complex capsid may have a head-tail morphology (an icosahedral-shaped head with a helically shaped tail), a distinctive feature of viruses infecting bacteria called bacteriophages. The bacteriophage attaches to the bacterium using the tail. Then they create a hole in the cell wall and insert their DNA into the bacterium using their tail as a channel.

Nucleic acid

Viral genomic material has a wide diversity. It can be DNA or RNA, single- stranded (ss) or double-stranded (ds), circular or linear, and monopartite or multipartite. Of note, in viruses with a monopartite genome, all genes are encoded by a single nucleic acid molecule, while in those with a multipartite genome, genes are encoded by multiple or segmented nucleic acid molecules. Except for retroviruses, which have a diploid genome, all other viruses are haploid. Notably, the capsid and the nucleic acid are collectively called nucleocapsid (O'Carroll and Rein, 2016).

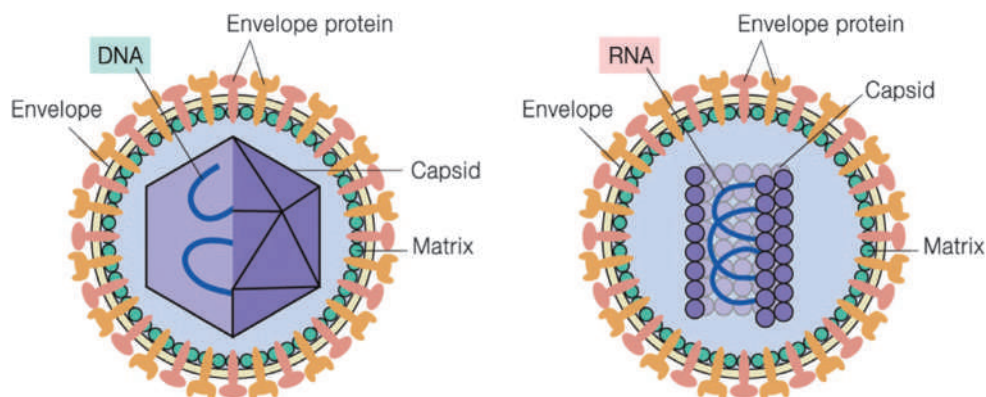


Fig. 2 Two enveloped viral particles with spherical (on the left side), and helical (on the right side) capsids are depicted. Adapted from Wang-Shick R (2017) Virus structure. In: Wang-Shick R (ed.), *Molecular Virology of Human Pathogenic Viruses*, pp. 21–29, Academic Press, ch. 2, with permission.

Envelope

It is a lipid bilayer structure encircling the nucleocapsid. The envelope is derived from either plasma membrane, nuclear membrane, the membrane of the Golgi apparatus, or the endoplasmic reticulum of the host cell. Matrix proteins are structural proteins connecting the envelope with the capsid with a critical role in viral assembly (Burrell et al., 2017c; Ryu, 2017b).

Viral genetics

The vast diversity between viruses is partly owing to the various mechanisms they employ to create genomic changes. The key concepts of viral genetics are described in the following (Fig. 3).

Recombination

Recombination is the exchange of genetic material between at least two viruses when they co-infect the same host cell by crossing over within regions with significant base sequence similarity (Perez-Losada et al., 2015).

Reassortment

Reassortment is exclusively seen in viruses with a segmented genome. It is defined as the exchange of intact genes within the entire segment, which occurs during coinfection. Reassortment has been observed in Bunyaviridae, Reoviruses, arenavirus, and Orthomyxoviruses. Reassortment can result in drastic changes over a short period. For instance, the 2009 novel H1N1 influenza pandemic emerged as a result of a triple reassortment between avian, human, and swine viruses. Antigenic shift, which occurs when a virus has a mixture of surface antigens from two or more viruses, may be a consequence of reassortment (Lowen, 2018; Neumann et al., 2009).

Phenotypic mixing

Phenotypic mixing also occurs during coinfection. In this case, a virus's genome becomes completely or partially coated with surface proteins of another virus (forming a pseudovirion). Notably, the infectivity of the hybrid agent is determined by the surface proteins. Although, the progeny of the hybrid agent will be the same as the primary virus again (Loverdo and Lloyd-Smith, 2013).

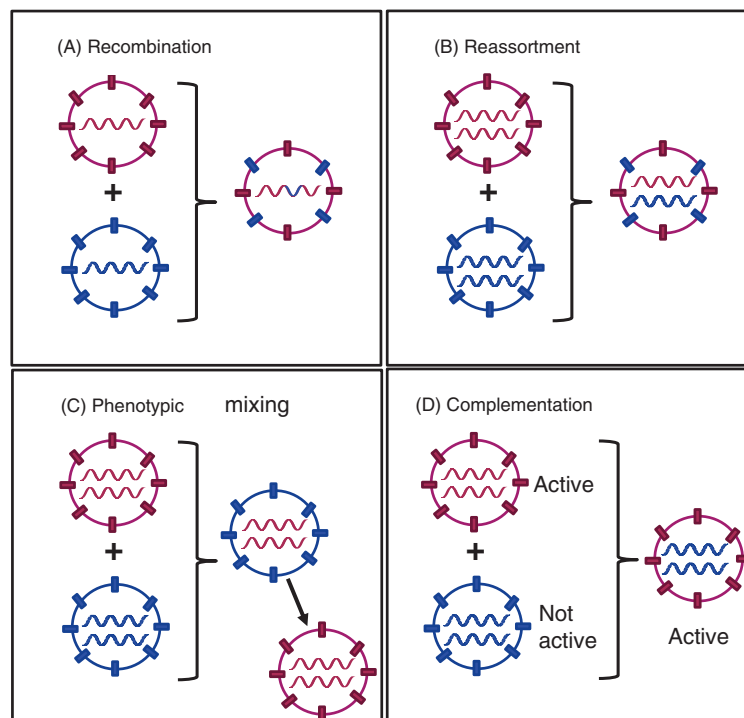


Fig. 3 The four major genomic changes in the viruses: (A) recombination, (B) reassortment, (C) phenotypic mixing, and (D) complementation.

Complementation

When a virus has a mutated gene resulting in a non-functional protein, to infect its host, it requires the presence of another virus with the non-mutated function form of that gene. For example, the hepatitis D virus carries a mutated version of one of the key envelop proteins (hepatitis B surface antigen (HBsAg)). Therefore, replicating hepatitis B virus is necessary for the infectivity of hepatitis D virus.

Classification of viruses

Two main classifications are adopted to organize viruses into different groups, namely the International Committee on Taxonomy of Viruses (ICTV) and the Baltimore classifications.

The ICTV taxonomy, first introduced in 1971, aims to classify the great number of discovered viruses into a single classification system while demonstrating their evolutionary relationships, i.e., their phylogenies (International Committee on Taxonomy of Viruses (ICTV), n.d.; Adams et al., 2017). The current taxonomy database is freely accessible online (<http://ictv.global>) (Lefkowitz et al., 2018). The 2019 version of the ICTV taxonomy encompasses 15 ranks, shown in Fig. 4 (Gorbalenya and Siddell, 2021; Walker et al., 2019). Fig. 5 describes virus taxa infecting vertebrates.

The Baltimore classification, a genome-based classification of animal viruses, was first proposed by David Baltimore in 1971. According to the Baltimore classification, viruses are divided into seven groups based on the way of synthesizing their mRNAs. The

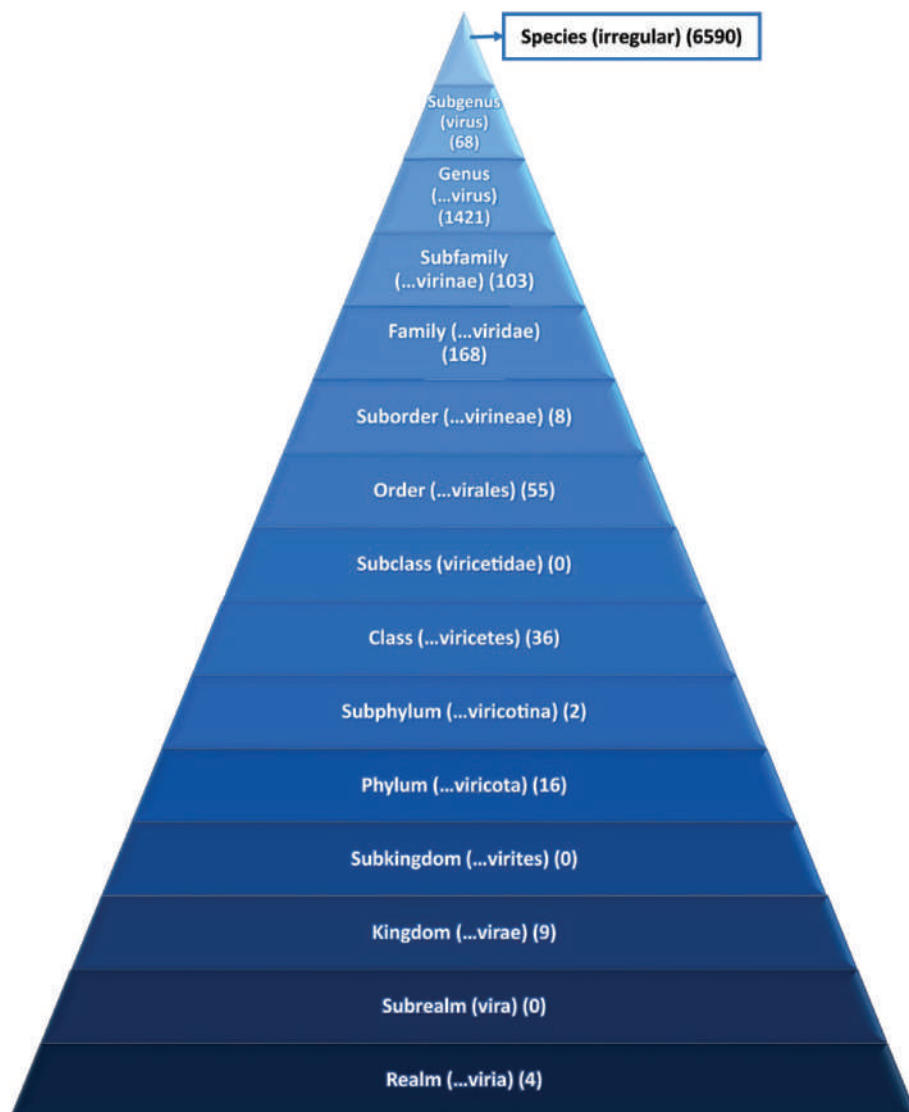


Fig. 4 The 15 ranks included in the 2019 version of the ICTV taxonomy (The nomenclature is shown in the parentheses.)

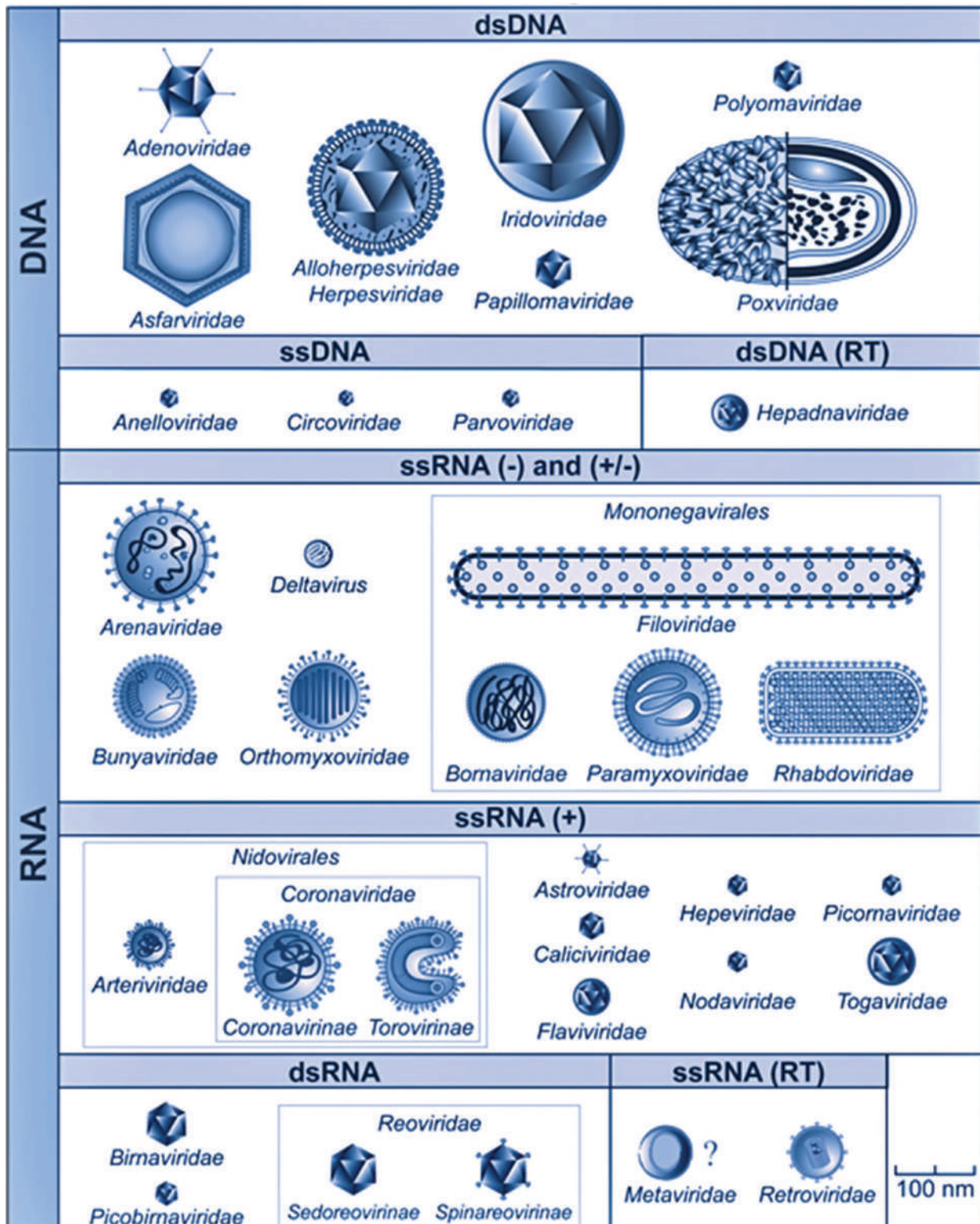


Fig. 5 Illustration of viral taxa infecting vertebrates. Adapted from King AM, Adams MJ, Carstens EB, Lefkowitz EJ (eds.) (2012) *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*. p. 20, Elsevier Academic Press: San Diego, CA, with permission.

following characteristics are considered in the Baltimore classification: (1) whether the genome is composed of DNA or RNA, (2) genome strandedness (either single- or double-stranded), and (3) for single-stranded genome, whether it is positive or negative sense. Overall, DNA viruses are in groups I and II, RNA viruses are in groups III, IV, and V, and reverse transcribing viruses are in groups VI and VII. Viruses with a double-stranded DNA genome are classified under Group I. Viruses with a single-stranded DNA

genome are within Group II. In these viruses, the single-stranded DNA is first converted to the double-stranded form before being used as a mRNA transcription template. Viruses with a double-stranded RNA genome are categorized as Group III. In this group, mRNA is produced from RNA transcription. Viruses with a positive-stranded RNA genome are classified as Group IV. In these viruses, the genomic RNA is directly utilized as mRNA (Baltimore, 1971).

On the other hand, group V is comprised of viruses with a negative-stranded RNA genome. Therefore, in these viruses, transcription of the RNA genome template leads to mRNA synthesis. Even though in groups VI and VII, the genome is either single-stranded RNA (Group VI) or double-stranded DNA (Group VII) but they are classified as reverse transcribing viruses and not as DNA or RNA viruses. This is because there are DNA and RNA intermediates in the life cycle of group VI and VII viruses, respectively. Notably, in all reverse transcribing viruses, the viral DNAs are a product of reverse transcription (Ryu, 2017a; Burrell et al., 2017b).

Viral replication

Overall, the viral replication cycle can be divided into three major phases: viral entry, genome synthesis, and release.

1. Viral entry: this stage comprises four steps, as described below:
 - 1.2. Attachment: The attachment is the first encounter between the virus and the host cell. Two main categories of proteins on the host cell membrane are involved in the attachment stage, including attachment factors and viral receptors. The attachment factors, such as glycoaminoglycans like heparan sulfate proteoglycan (HSPG) and low-density lipoprotein (LDL) receptors, aid in the interaction between the viral receptors and a diverse variety of viral particles. Conversely, viral receptors such as the cluster of differentiation (CD)4 and sialic acid are virus-specific. The interaction of these viruses with the viral particles enhances viral penetration. Notably, the presence of a viral receptor is a key determining factor in the susceptibility of a host cell to a particular virus.
 - 1.3. Penetration: After viral attachment, the viruses penetrate into the cytoplasm via two major mechanisms, mainly based on whether they are enveloped or not. The enveloped viruses can undergo either direct fusion or receptor-mediated endocytosis (Plempner, 2011). In the former mechanism, the nucleocapsid enters the cytoplasm directly after the diffusion of the viral envelope and the host cell plasma membrane. Therefore, the envelope does not enter the cell. Retroviruses are a characteristic example of viruses penetrating the host cell via direct fusion. However, most viruses penetrate into their target cell via receptor-mediated endocytosis. In this mechanism, following the interaction of the virus and its receptor on the plasma membrane of the host cell, a coated pit is formed, and an endosome encompasses the viral particle. At this step, the nucleocapsid should be released into the cytoplasm following an endosomal breakdown. The mechanism of endosomal breakdown differs between enveloped and non-enveloped viruses. In case the virus is enveloped, the low pH of the endosome triggers activation of the fusion peptide, resulting in the diffusion of the envelope with the endosomal membrane. In non-enveloped viruses, one of the capsid proteins induces endosomal lysis. Receptor-mediated endocytosis has two important advantages over the direct fusion mechanism. First, unlike the direct diffusion method, the viral envelope can enter the host cell as well. Second, in direct diffusion, the cell cortex can act as a physical barrier for the virus particle. However, this barrier is bypassed in receptor-mediated endocytosis.
 - 1.4. Intracellular trafficking: Subsequent to penetration, the nucleocapsid moves to the proper site within the cell via intracellular trafficking to initiate the genome replication phase. For most RNA viruses, viral replication occurs in the cytoplasm. On the other hand, most DNA viruses need to enter the nucleus for gene replication. Generally, the viral particles are transported to the appropriate location within the cytoplasm, particularly the perinuclear area, via microtubule-mediated transportation. The interaction between dynein, a molecular motor protein, and either viral capsid or endocytic vesicles mediates the transportation (Robinson et al., 2018).
 - 1.5. Uncoating: In this process, the viral genetic material is released following the removal of the capsid. Viral uncoating is induced by multiple factors and usually occurs during intracellular trafficking. The most common trigger is the gradual decrease of the endosomal pH. The virus is transferred via a nuclear pore using different mechanisms, mainly depending on its genome size in case it requires to enter the nucleus for replication. If the nucleocapsid is small enough to pass the nuclear pore, like polyomavirus, it enters the nucleus directly, and the uncoating process occurs in the nucleus. For larger viruses, the capsid and the nuclear pore interaction results in some structural changes in the capsid, permitting transportation of the DNA genome into the nucleus (Kilcher and Mercer, 2015).
2. Viral genome and protein synthesis: Viral replication includes genome synthesis and transcription of viral proteins. Viruses may exploit various mechanisms to replicate their genome, mainly according to the type of viral nucleic acid. In most viruses, the replication is primarily done by host cells' proteins and enzymes. Most dsDNA viruses enter the nucleus for replication and use cellular DNA polymerase and RNA polymerase.

For dsDNA viruses, transcription occurs in two phases. In the first step, the immediate early and/or early genes are transcribed, most of which facilitate the replication process. Nevertheless, the majority of the proteins involved in the virion structure are called late genes as they are transcribed in the second phase. As an exception, poxviruses contain all of the necessary genes for encoding proteins that are required in the replication process. Therefore, they do not enter the nucleus, and their replication occurs in the cytoplasm.

ssDNA viruses commonly enter the nucleus, and their genome is converted to dsDNA by DNA polymerase during the S phase of the cell cycle. Subsequently, RNA polymerase II leads to transcription of the viral genes, and the genome is replicated by DNA polymerase.

In contrast to most DNA viruses, RNA viruses typically do not enter the nucleus. The cellular RNA polymerase II requires a DNA template and cannot transcribe the RNA. As a result, all RNA viruses encode RNA-dependent RNA polymerase to transcribe their mRNA. The cellular ribosome translates the produced mRNA to form the viral proteins. For viruses with ssRNA, their replication varies depending on whether they are positive or negative sense. The genome of positive-strand RNA viruses can be directly translated, while for negative-strand RNA viruses, it needs to be copied into a positive sense RNA via viral RNA-dependent RNA polymerase before translation (Rampersad and Tennant, 2018).

3. Viral exit: Viruses exit the host cell in three stages: (1) capsid assembly, (2) release, and (3) maturation.
 - 3.2. Capsid assembly: Capsid proteins may come together and form the capsid spontaneously (particularly in viruses with simple icosahedral capsid), or they may also require scaffolding proteins or simultaneous interaction of the viral nucleic acid and capsid proteins. For instance, in picornaviruses, the immature capsid (procapsid) is first formed, and the RNA genome is delivered into the capsid later. On the other hand, in adenoviruses, capsid assembly and genome packaging occur at the same time (Mateu, 2013).
 - 3.3. Release: Most non-enveloped viruses, such as adenoviruses and polyomaviruses, are released subsequent to host cell lysis, which kills the host cell. However, enveloped viruses need to undergo envelopment before their release. Envelopment can occur either after the capsid development, the example of which are herpes viruses, or at the same time as capsid assembly, an example of which are retroviruses. The bilayer envelop may originate from the host cell's plasma membrane or intracellular organelles, including the Golgi apparatus or endoplasmic reticulum. For instance, the envelope of retroviruses and influenza viruses is derived from the plasma membrane, while the envelope of hepatitis B viruses and herpesviruses originate from endosomes. Following the envelopment process, viruses that have traveled through the endoplasmic reticulum and Golgi apparatus are released via exocytosis. In the budding mechanism, viral proteins are recruited together at the budding site, and the host cell's plasma membrane or endosomal membrane composes the viral envelope (Louten, 2016b).
 - 3.4. Maturation: Many viruses need to undergo irreversible changes extracellularly after their release from the host cell to activate their infectiousness. For example, retroviruses require to undergo morphological changes, such as compacting their capsid structure by cleavage of some of their proteins (Ryu, 2017d).

An introduction to disease course in viral infections

Viral infections may have a wide variety of presenting patterns. Even within the same viral family, the disease may be asymptomatic or symptomatic, with a spectrum ranging from mild to severe manifestations. For instance, in infection with COVID-19, approximately 20% of the patients are asymptomatic (He et al., 2021), while near 20% develop severe or critical disease, and most of the patients have mild symptoms (Wu and McGoogan, 2020).

Viral infections may be transient or persistent and local or systemic (Burrell et al., 2017e). Commonly, viral infections are divided into two major groups, namely productive and non-productive infections, based on viral reproduction in the host's body, as explained below.

1. Productive infections: In these infections, the virus successfully infects the host cell resulting in an ongoing viral production (King et al., 2007). Productive infections can be divided into two sub-categories: lytic infection and persistent infection.
 - 1.2. Lytic infection: In viruses causing a lytic infection, viral replication occurs through the host cell's lysis and death. Therefore, these viruses typically cause transient infections, which may be localized or systemic. Like many common respiratory viral diseases, infections caused by adenoviruses and influenza viruses are classic examples of transient localized lytic infections. These viruses typically have an incubation period of 2–5 days, followed by the onset of symptoms. The viral replication peak usually occurs before and after symptom onset, during which the patient has a higher chance of transmitting the infection to others (Greber, 2020; Thangavel and Bouvier, 2014).

In contrast to the mentioned examples, transient infections may be systemic, such as varicella-zoster virus infections. In the first stages of the disease, the virus replicates in the upper respiratory tract. It enters circulation by infecting T cells leading to a systemic spread to the skin and other organs (Gershon et al., 2015).

- 1.3. Persistent infection: The virus has an ongoing replication for a considerable time. These viruses either do not cause cell death or have durable reservoir cells should they result in cell death. Infections with hepatitis B and hepatitis C viruses are examples of the former mechanism, causing systemic diseases. During the acute phase of the disease, virus replication occurs in the liver resulting in the movement of viral antigens into the blood. In this stage, hepatic damage happens due to the immune response mediated by cytotoxic T lymphocytes destroying cells infected with the virus. In most cases, the infection resolves within 3–4 weeks. Although, approximately 5% of the patients develop persistent infection. Persistent hepatitis B infection is a major cause of cirrhosis and a predisposing factor for hepatocellular carcinoma (D'Souza and Foster, 2004). Conversely, most of the patients infected with hepatitis C cannot fully recover and develop chronic infection. (Webster et al., 2015).

On the other hand, while HIV infection can lead to cell death, it causes a persistent infection owing to its reliable reservoir cells. HIV targets CD4⁺ cells, particularly lymphocytes, and upon entrance to the host cell, the ssRNA is transformed to dsDNA by reverse transcriptase enzyme within the cytoplasm. Subsequently, the viral dsDNA integrates into the host cell's genome following entering the nucleus. During the initial phase, there is a considerable amplification of the virus resulting in the destruction of the lymphocyte. However, these lymphocytes are rapidly replaced and the viral reservoir facilitates persistent infection. Nevertheless, after the prolonged asymptomatic phase, approximately 10 years, the number of CD4⁺ lymphocytes gradually decline (Chosn et al., 2018; Chun and Fauci, 2012).

2. Non-productive infections: Opposing to the productive infections, during non-productive infections, the virus either does not have the ability to produce infectious progeny or it only has a limited capacity of replication (Payne, 1999). Non-productive infections can be divided into three sub-categories:
 - 2.2. Latent infection: In these infections, the host cells carry the viral genome for their lifetime; however, the virus does not commonly produce a progeny, and only a limited proportion of their genome might be expressed. Nevertheless, the infection may reactivate spontaneously at any time. All herpes viruses, including herpes simplex virus (HSV), varicella-zoster virus, Epstein Bar virus (EBV), and cytomegaloviruses, can cause latent infection. HSV and varicella-zoster virus reside in neurons, while EBV and cytomegaloviruses persist in lymphocytes that, unlike neurons, proliferate and have a shorter life span. These viruses are typically unintegrated. However, the EBV may integrate into the host's chromosome at low frequency (Morissette and Flamand, 2010). There are two types of HSV, namely HSV1 and HSV2. Both types, particularly HSV2, can cause genital lesions. HSV1 mainly results in oral infections. The majority of the new infections are asymptomatic. Following entering into the epithelial cells via skin or mucosa, HSV begins to replicate. Subsequently, the free sensory nerve endings take up and transport the nucleocapsid from the dermis to the nucleus in the sensory ganglion via retrograde axonal flow. Therefore, HSV becomes a persistent unintegrated infection. At any time, the virus can reactivate and travel back through the sensory nerve to the skin or mucosa. Several factors, such as stress, trauma, and immunosuppression, can trigger reactivation (Gupta et al., 2007). Herpes zoster, also known as shingles, is another unintegrated latent infection caused by reactivation of the dormant varicella-zoster virus in the dorsal horn nerve cells. Almost every individual is at risk of developing shingles due to either having chickenpox during their childhood or receiving the varicella-zoster virus vaccine. It typically occurs at older ages, and the most common trigger is weakened immune responses (Randolph, 2015).
 - 2.3. Transforming infection: It occurs when the viral genome integrates into the host's genetic material without producing progeny. These infections are usually predisposing factors for cancers. Infection with human papillomavirus is an example of this category, which can become persistent in a subset of the patients and can cause cervical cancer (Sudenga and Shrestha, 2013).
 - 2.4. Abortive infection: When the virus fails to produce infectious progeny despite infecting the target cell, it results in an abortive infection (Cohen et al., 2020).

An introduction to viral transmission

Like any other infectious agent, viruses can transmit via two major modes of (1) direct and (2) indirect. Notably, a virus may have several routes of transmission. Moreover, in humans, viruses can have multiple entry routes, including the respiratory tract, alimentary tract, genitourinary tract, skin, and eyes (Burrell et al., 2017d; Van Seventer and Hochberg, 2017).

Direct transmission: The virus is directly transferred from a reservoir to a predisposed host via either direct contact or droplet spread.

1. Direct contact: The infection is transmitted as a result of direct contact of bodily tissues or fluids of an infected person with another individual, such as skin-to-skin contact, kissing, and sexual intercourse. The virus may enter the body of the exposed person through damaged skin barriers or mucosal membranes, like the eyes and mouth. Scratches or bites can lead to direct inoculation. Importantly, blood borne viral infections, such as hepatitis B virus and HIV, can cause direct contact transmission (Beltrami et al., 2000).
 - 1.2. Droplet spread: Droplets are defined as short-range and fairly large (larger than 5–10 µm in diameter) aerosols produced by talking, singing, coughing, or sneezing. Droplets containing the virus can spread the disease via entering through the mouth, nose, or eyes. Additionally, this transmission route can be seen in respiratory viruses like the respiratory syncytial virus (Goldmann, 2000).
 - 1.3. Indirect transmission: In contrast to direct transmission, in indirect transmission, inanimate objects (fomites or vehicles), animate intermediaries (vectors), or air particles transfer the virus from its reservoir to the host. These intermediaries are described in the following.
2. Airborne: Dust or droplet nuclei, which can remain suspended in the air for a prolonged duration and spread over vast distances, transfer the virus. Compared to droplets, the smaller size of droplet nuclei (smaller than 5 µm in diameter) results in its prolonged suspendability. Airborne dust comprises viral materials that are on surfaces and again become suspended via airflow, like the wind. Measles morbillivirus, varicella-zoster virus, SARS virus, Middle East respiratory syndrome (MERS) virus, and SARS-CoV-2 can have airborne spread. These viruses can potentially result in epidemics and pandemics (Morawska et al., 2020; Louten, 2016a).

- 2.2. *Vehicleborne*: The viral infection is transmitted through vehicles, including inanimate objects (fomites), biological products, food, or water. Fomite transmission plays a crucial role in the spread of respiratory and enteric viral agents (Boone and Gerba, 2007). Additionally, adenovirus, astrovirus, hepatitis A, and Norwalk-like caliciviruses are examples of water-transmitted or food-transmitted viruses (Gall et al., 2015; Koopmans et al., 2002). Usually, the vehicle passively carries a pathogen. However, it can also provide a nourishing environment for the infectious agent.
- 2.3. *Vectorborne*: The infectious agent is transmitted from animals to humans or between humans by living organisms called vectors. The vector is commonly a bloodsucking insect. The viral agent enters into the insect after a blood meal from an infected host (either animal or human). The bite of the infected insect can infect another host. Most of the time, once a vector becomes infectious, it can transmit the disease for the rest of its life during each bite or blood meal. Zika virus, West Nile virus, Dengue fever, yellow fever, and chikungunya are mosquito-transmitted viruses (Allison et al., 2019).

The mentioned mechanisms explained the horizontal transmission of viral infections. In horizontal transmission, the infection spreads among the members of the same generation. On the other hand, in vertical transmission, the viral agent is passed from the mother to the offspring.

Three main mechanisms can result in vertical transmission. First, the viral genome embedded in the germ-line genes or encoded as episomes in the gametes can predispose the infant to the viral disease. Nevertheless, this route of transmission has not been reported in any human virus. Second, an infected mother can pass the infection to her fetus via transplacental spread during pregnancy. Rubella, cytomegalovirus disease, and Zika virus encephalitis, and microcephaly can be transmitted during pregnancy via the placenta. Third, contact with infected genitalia secretion or bowel material during delivery can result in perinatal transmission. Coxsackieviruses and HSV 1 and 2 can spread by this route during delivery. Of note, HIV and hepatitis B virus can have both transplacental and perinatal transmission (Burrell et al., 2017d).

Other non-living infectious agents

Viruses are not the only non-living infectious agents. Prions and viroids are infectious agents known as subviral particles that are smaller than viruses. Viroids consist of only a single strand of circular RNA without the ability to code proteins. For a very long time, they were known only to infect plants. However, recently, they have also been found in humans in infection with hepatitis D virus (Flores et al., 2012). Prions are made of proteins without any genetic material. The most common disease caused by prions in humans is Creutzfeldt-Jakob disease (CJD), a fatal disease characterized by rapidly progressive memory loss and neurological symptoms. Prion proteins (PrP^{Sc}) are β -sheet-rich isoforms of cellular prion proteins (PrP^C). It is postulated that the interaction of PrP^{Sc} with the PrP^C changes their conformation of PrP^C, resulting in aggregation of misfolded proteins. The deposition of proteins leads to neurodegeneration, which is mainly immune-mediated (Aguzzi et al., 2008).

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Herpes Simplex Viruses Type 1 and Type 2

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Abbreviations

ACV	Acyclovir
CNS	Central nervous system
DCs	Dendritic cells
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
NK cell	Natural Killer cell
NKT cell	Natural Killer T cell
TLR	Toll-like receptor

Introduction

Herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) are two members of the *Herpesviridae* family, and more specifically, the *Alphaherpesvirinae* subfamily that are highly prevalent in the world population (Boehmer and Nimonkar, 2003). In 2016, the global prevalence of HSV-1 infection was estimated at 66.6% for individuals aged 0–49, with approximately 5042 million people aged 0–99 being infected with this virus worldwide (James et al., 2020). For HSV-2, the global prevalence was estimated at 13.2% for individuals aged 15–49, which corresponds to 3736 million people at the time of the study (James et al., 2020).

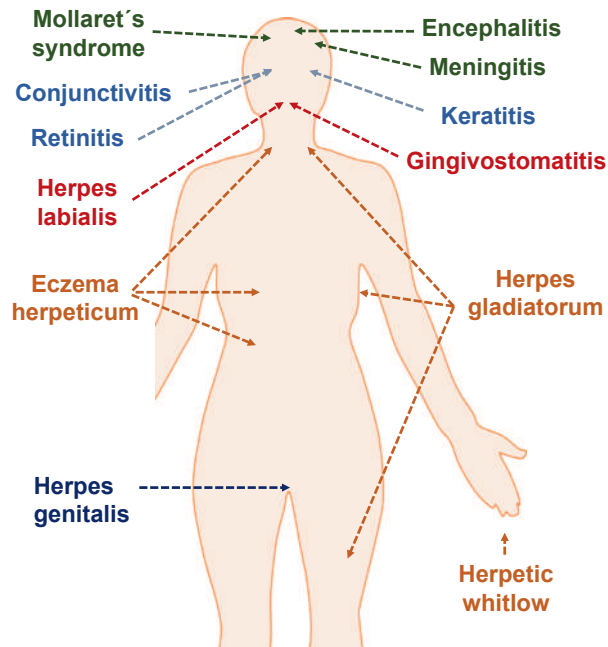


Fig. 1 Tissues affected by HSV infection and HSV-related diseases. Body sites affected by herpes simplex viruses include the facial area (herpes simplex keratitis, conjunctivitis, retinitis, gingivostomatitis and herpes labialis—oral herpes), the brain (meningitis, encephalitis and Mollaret's syndrome), the torso and neck (eczema herpeticum, herpes gladiatorum and herpetic whitlow), and the genitalia (herpes genitalis).

Herpes simplex viruses (HSVs) are known to establish lifelong infections and cause a wide range of clinical manifestations such as *Herpes labialis*, herpes simplex keratitis, genital herpes, and herpes simplex encephalitis, among others (Fig. 1) (Suazo et al., 2015b). Furthermore, genital HSV infections have been reported to increase the risk of HIV infection, which raises concerns about co-infections (Suazo et al., 2015b). Given their importance at the public health level, numerous approaches aimed at treating and preventing infections with these viruses have been investigated over time, with many strategies currently being evaluated in clinical trials (Chung and Sen, 2012; Álvarez et al., 2020). However, currently, there are no vaccines available to prevent infections by HSVs. Nevertheless, numerous attempts for creating a vaccine against these viruses have been made using different schemes, such as subunit approaches, mutant viruses, and replication-deficient viruses, among others (Chung and Sen, 2012).

Importantly, HSVs have evolved different strategies to evade the immune response of the host. After primary infection, HSVs can evade the immediate cellular antiviral response through the interference of signaling pathways related to pathogen recognition by host receptors, by modulating the interferon pathway while also altering differentially the viability of immune and non-immune cells (Suazo et al., 2015a; Retamal-Díaz et al., 2016; Tognarelli et al., 2019). These viruses can also interfere with the innate immune response, such as with the functions of natural killer (NK) and natural killer T (NKT) cells, and by interfering with the activation of the complement (Suazo et al., 2015a; Tognarelli et al., 2019). These viruses have also been reported to interfere with the viability, and T cell-activating capacities of dendritic cells (DCs), which are crucial for mounting and regulating effective antiviral responses (Retamal-Díaz et al., 2016; Carreño et al., 2011). Moreover, HSVs also interfere with adaptive immune components by hampering both, humoral and T cell immunity (Retamal-Díaz et al., 2016).

In this article, we provide an overall perspective of herpes simplex viruses by discussing aspects related to their epidemiology, characteristics of the diseases they produce, current treatments available to counteract their effects, as well as research on new drugs and vaccines. Furthermore, we review aspects related to their structure, lifecycle and, finally, how they interfere with the host antiviral response.

Epidemiology and HSV-related diseases

Incidence and prevalence of HSV infections

Primary infections with HSV-1 mainly occur at a young age. In developing countries, approximately one-third of the population is HSV-1 seropositive by age five; however, in developed countries only one-fifth of the population is estimated to be infected by this age (Whitley and Roizman, 2001). On the other hand, HSV-2 infections usually occur at a more advanced age, with a seroprevalence of 13.2% in people aged between 15 and 49 as determined in 2016 (James et al., 2020). It has also been shown that the prevalence of HSV-2 infections varies depending on the global region, ranging from 5% for countries like Spain and England, approximately 20–30% for countries like Sweden and Germany, and up to 70% for the Sub-Saharan Region in Africa (Suazo et al., 2015b). A world health organization study in 2016 estimated that 3752 million people in the world aged between 0 and 49 years were infected with

HSV-1, with a prevalence of 66.6% (James et al., 2020). It was also estimated that 1290 million people aged 50–99 were infected with HSV-1 (at any site; James et al., 2020). Regarding the individuals affected with oral infections by HSV-1, it was estimated in 2016 that 3583 million people aged 0–49 were infected, with a prevalence of 63.6% (James et al., 2020). The highest number of individuals orally infected with HSV-1 were located in the South-East Asia Region, followed by the Western Pacific Region (James et al., 2020). Genital infection with HSV-1 in 2016 was found to affect globally 192 million people aged 15–49, equivalent to a prevalence of 5.2%. The highest number of individuals affected by this type of infection were located in the Americas Region, followed by the European Region (James et al., 2020).

On the other hand, it was estimated that a total of 491.5 million people aged 15–49 worldwide were living with HSV-2 infection in 2016 (James et al., 2020). The African Region was the most affected by this type of infection, followed by the Western Pacific, South-East Asia, and the Americas Regions. Furthermore, a study estimated that globally 344.5 million people aged 50–99 were infected with HSV-2 that year (James et al., 2020). Overall, the estimated prevalence of HSV-2 in the global population was 13.2%, with 3736 million people aged 15–49 being affected, with those in the Africa Region having the highest number of people infected, followed by the Americas Region (James et al., 2020).

Interestingly, it has been estimated that worldwide 596.0–655.7 million people aged 15–49 have had either HSV-1, HSV-2, or both HSV-1 and HSV-2 genital infections. These numbers are equivalent to 16.0–17.6% of the global population (James et al., 2020). Noteworthy, HSV-1 is more frequently detected during primary genital herpes than HSV-2, yet the latter is more commonly detected in genital herpes recurrences, which is likely due to the different reactivation rates of these viruses from this site of infection (Koren and Decker, 2016; Coyle et al., 2003; Pereira et al., 2012; Samra et al., 2003).

Regarding the global incidences of HSVs, for HSV-1 it has been estimated that 120.4 million people aged 0–49 became infected in 2016, equivalent to a 2.1% of incidence. The incidence of HSV-2 has been estimated at 0.6% for people aged 15–49, equivalent to 23.9 million individuals during 2016 (James et al., 2020). The worldwide incidence of neonatal herpes infection was estimated at 14,257 cases during 2010–15, equivalent to a global rate of 10.3 per 100,000 births when averaged across all regions, with two-thirds of the cases due to HSV-2 (Looker et al., 2017b), and the largest number of cases occurring in Africa. This finding correlates with a high incidence and prevalence of HSV-2 infections in adult women and high birth rates in this region (Looker et al., 2017b; James et al., 2020). Interestingly, in the Americas, Europe, and Western Pacific Regions, HSV neonatal infection was more frequently caused by HSV-1 than HSV-2 (Looker et al., 2017b).

HSV-associated diseases

Herpes simplex viruses cause a wide range of clinical manifestations such as skin-related diseases, ocular complications, central nervous system-related pathologies, and genital infections (Suazo et al., 2015b). While HSV-1 infections are commonly associated with orofacial lesions and HSV-2 with genital ones, infections with either virus may occur at these sites, although HSV-2 infection has been rarely diagnosed in the orofacial area, possibly because of infrequent HSV molecular diagnosis at this site (Crimi et al., 2019; Suazo et al., 2015b).

The transmission of HSV infection usually requires intimate contact with an infected person, and up to 40 close-contact interactions have been estimated to be required to transmit HSV-2 in genital infections (Wald et al., 2006; Suazo et al., 2015b). Infection may be transmitted by direct contact with infected tissues or through the presence of the virus in the secretions of an infected individual in contact with the mucosae or eroded skin of a healthy person (Suazo et al., 2015b; Fatahzadeh and Schwartz, 2007).

It is noteworthy that during herpetic recurrences, only 20–50% or 80–90% of the individuals infected with HSV-1 or HSV-2, respectively, manifest herpes infection-associated clinical symptoms (Álvarez et al., 2020; Johnston and Corey, 2015). Nevertheless, both symptomatic and asymptomatic individuals may shed HSV from infected tissues (Suazo et al., 2015b; Fatahzadeh and Schwartz, 2007). Indeed, HSV-infected persons shed virus from the mouth up to 3–5 days after oral lesions are resolved, whereas viral shedding after genital infections is highest in the first 6–12 months after infection. However, virus shedding can persist for years, thus contributing to the silent infection of other individuals (Gupta et al., 2007; Fatahzadeh and Schwartz, 2007). Additionally, asymptomatic persons may shed virus and transmit it through respiratory droplets or through mucocutaneous secretions in the absence of clinical manifestations (Fatahzadeh and Schwartz, 2007). Importantly, a study estimated that asymptomatic individuals shed HSV-2 3–5% of the days when assessed by cell culture and up to 28% of days when tested by PCR (Wald et al., 1997).

Skin infections

Two of the most common skin-related clinical manifestations caused by herpes simplex viruses are herpes labialis and herpetic gingivostomatitis. Herpes labialis is commonly caused by HSV-1, but HSV-2 infection may also be a cause (Lafferty et al., 1987). Herpes labialis is characterized by the development of a rash in the skin or mucous membranes, primarily in the lips, which causes erythema and blisters frequently accompanied by a burning sensation of pain (Opstelten et al., 2008). A significant number of patients with this type of clinical manifestation elicited by HSV-1 have recurrences, which may repeat more than six times in a year (Celik et al., 2013). Recurrences may last up to 10 days if no treatment is taken (Arduino and Porter, 2008; Álvarez et al., 2020; Fig. 1).

Herpetic gingivostomatitis is commonly observed in children and young adults and consists of painful oral lesions in the buccal and gingival mucosa and the tongue (Amir, 2001). In children, most primary infections with HSV cause clinical manifestations that are either mild or asymptomatic (Arduino and Porter, 2008), and only 13–30% of the infected children manifest gingivostomatitis (Amir, 2001). The disease is mainly self-limiting (Arduino and Porter, 2008). Although most of the cases are caused by HSV-1, some cases due to HSV-2 have been reported (George and Anil, 2014; Fig. 1).

Herpes simplex viruses can also elicit other skin-related diseases, such as Eczema herpeticum (EC), which is characterized by disseminated dome-shaped vesicle eruptions through the upper body, fever, malaise, and lymphadenopathy (Wollenberg, 2012; Fig. 1). Noteworthy, herpes simplex virus-elicited Eczema herpeticum shares some clinical features with shingles produced by the varicella-zoster virus (HHV-3, VZV), and hence may be occasionally mistakenly diagnosed (Freer and Pistello, 2018). Children with severe atopic dermatitis have been reported to be at higher risk of recurrent HSV, suffering EC (Darji et al., 2017). Somewhat related to EC is herpes gladiatorum, which has been defined as herpetic skin lesions produced by HSV in athletes that practice wrestling activities. Infection of the skin in these individuals likely occurs because of skin abrasion or skin breakdown due to wrestling, with the virus from contaminated saliva from the adversary entering in contact with the skin (Anderson, 2003; Anderson, 2008; Johnson, 2004; Fig. 1). On the other hand, another manifestation, herpetic whitlow, is characterized by the formation of a single herpetic vesicle or cluster of vesicles, usually located in the terminal phalanx of the thumb, index, or long finger near the nail and is associated with throbbing pain (Rubright and Shafritz, 2011; Fig. 1).

Ocular infections

Ocular infections by herpes simplex viruses are the most common cause of infectious blindness in developed countries, with herpetic keratitis being estimated to be the cause of approximately 40,000 annual new cases of blindness or severe visual impairment worldwide (Farooq and Shukla, 2012). Herpetic keratitis may result as a consequence of epithelial keratitis or stromal keratitis, compromising the cornea (Rowe et al., 2013). Importantly, this type of clinical manifestation by HSV may occur recurrently, leading in the long-term to irreversible damage of the cornea and the need for cornea transplant (Rowe et al., 2013). Although HSV-1 is more frequently isolated from the eye than HSV-2, a study analyzing corneal buttons of patients with herpes stromal keratitis found that this disease can be caused by either HSV-1 or HSV-2, and in one case, a mixed infection was found (van Gelderen et al., 2000). Ocular infections with HSV-1 may also lead to blepharitis, conjunctivitis, or iridocyclitis (Liesegang, 2001). HSVs have also been reported to cause uveitis (Inoda et al., 2001; Chan and Chee, 2019). Additionally, HSV-2 has been reported to cause retinitis and acute necrotic retinitis (Rowe et al., 2013; Itoh et al., 2000; Grose, 2012; Fig. 1).

Central nervous system infections

Herpes simplex viruses have tropisms for neurons where they can establish latency and from where they can reactivate to produce recurrences (Duarte et al., 2019). Besides infecting neurons of the trigeminal ganglia in the facial area and dorsal root ganglia of the sacral area, HSVs can also reach the brain where they can establish asymptomatic infections with potential contributions to neurodegenerative diseases (Duarte et al., 2019, 2021). However, HSVs can also elicit acute encephalitis with lifelong detrimental effects over this tissue (Duarte et al., 2019; Fig. 1).

Individuals suffering herpetic encephalitis often display lifelong sequelae, despite antiviral treatment, such as with acyclovir (Duarte et al., 2019). Furthermore, neonatal herpetic encephalitis is associated with high morbidity and mortality, with untreated infections reaching case-mortality rates of 60% (Corey and Wald, 2009). Most of the infections in neonates occur due to exposure to virus that was shed by the mother in the genital tract during childbirth (Corey and Wald, 2009). On the other hand, most of the cases in children and young adults have been associated with viral reactivations of HSV-1, likely derived from previously infected neurons (Steiner and Benninger, 2013).

While HSV-1 is the leading cause of sporadic viral encephalitis in adults (Duarte et al., 2019), HSV-2 infections of the brain in neonates are more common than HSV-1 (Kleinschmidt-DeMasters and Gilden, 2001). Encephalitis due to HSV-2 in adults also occurs and has been reported to be the cause of 6% of frontal-temporal encephalitis in immunocompetent patients. It is usually associated with aseptic meningitis, acute and subacute myelitis, and chronic meningitis, among others (Kleinschmidt-DeMasters and Gilden, 2001). This disease is usually characterized as a necrotizing inflammatory process and frequently affects the cortex and underlying white matter of the temporal lobe (Hauer et al., 2019). A comparative analysis has shown that, while HSV-1 mostly elicits intracerebral hemorrhage, cases with infarction were usually associated with HSV-2 infections (Hauer et al., 2019). Brain damage in HSV encephalitis seems to be mainly provoked by neuronal cell death via apoptosis or necrosis, and it is associated with the insular cortex of cerebral hemispheres and with the temporal and front lobes of the brain (Bradshaw and Venkatesan, 2016).

On the other hand, central nervous system (CNS) infection with HSVs can elicit Mollaret's syndrome, which is characterized by aseptic inflammation of the meninges consisting of leukocyte infiltration and the presence of Mollaret's cells in this tissue (Suazo et al., 2015b; Shalabi and Whitley, 2006; Poulikakos et al., 2010; Farazmand et al., 2011). It has been described that one-third of the individuals that undergo recurrent meningitis suffer spasm episodes, cranial nerve paralysis, diplopia, hallucinations, pathological reflexes, and even coma (Suazo et al., 2015b; Shalabi and Whitley, 2006; Poulikakos et al., 2010; Sato et al., 2005; Fig. 1).

Genital infections

Herpes genitalis or genital herpes is a common, sexually transmitted disease. Although it is mostly associated with HSV-2, it is also caused by HSV-1, which is frequently detected during primary genital herpetic infections (Groves, 2016; Löwhagen et al., 2002). Indeed, in the United States, genital herpes caused by HSV-1 is more common than HSV-2 among young women (Bernstein et al., 2013). Nevertheless, HSV-1 seemingly recurs less often from the dorsal root ganglia than HSV-2. Hence, the latter is more frequently found in recurrent herpetic lesions in this tissue, up to 4–5 times a year, and less than 10% of the recurrences are caused by HSV-1 (Retamal-Díaz et al., 2015; Suazo et al., 2015b; Lafferty et al., 1987; Solomon et al., 2003). The differential reactivation of HSV-1 and HSV-2 from neurons linked to the genital area may be related to the preference of these viruses for A5 and KH10 positive neurons, respectively (Retamal-Díaz et al., 2015). Clinical manifestations by HSVs in this area include single or clustered vesicles in the genitalia, perineum, buttocks, upper thighs, or perianal areas (Groves, 2016). Malaise, fever, or localized adenopathy may also occur during primary genital herpetic infection, while recurrences in this area usually cause milder symptoms (Groves, 2016; Fig. 1).

HSV-2 and HIV

Over the last decades, an important association has been made between HSV-2 and the human immunodeficiency virus (HIV; Suazo et al., 2015b; Looker et al., 2017a). Notably, it has been found that infection with HSV-2 increases up to three-fold the risk of acquiring HIV (Freeman et al., 2006). Furthermore, there is evidence that an association between these viruses is somewhat reciprocal, given that infection with HIV also increases the likelihood of HSV-2 reactivations (Mayaud et al., 2008).

The mechanisms by which these two viral infections are related are diverse. While some studies have reported that infections with HSV-2 increase HIV transmission due to genital ulcerations elicited by the former (Corey et al., 2004), other studies suggest that the cytokines secreted by HSV-2-infected DCs contribute to HIV replication (Stefanidou et al., 2013). Additionally, microlesions provoked by HSV-2 may expose HIV to target immune cells that reside in the infection site, such as dendritic cells, macrophages, and Langerhans cells (Duluc et al., 2013). Furthermore, some studies have shown that in HSV-2-infected tissues, receptors for HIV in target cells, such as DC-SIGN and CCR5, are upregulated in CD4⁺ T cells and DCs, respectively, which are present at higher amounts (Johnson et al., 2011; Rebbapragada et al., 2007). In sum, infections with HSV-2 may have a significant impact on the HIV epidemic, and thus, preventing HSV-2 infection may work as an indirect strategy for limiting the HIV pandemic.

Vaccine research and treatment of herpes simplex viruses

Vaccine development against HSVs

There are currently no vaccines available against HSVs. However, there are numerous ongoing studies and vaccine candidates that are being tested in different pre-clinical and clinical settings (Johnston et al., 2016). These vaccines are being developed to prevent or treat (therapeutic vaccines) HSV infections. While most of these studies have prioritized HSV-2 vaccine development over HSV-1, nevertheless, given the high homology and similarity between these two viruses, some HSV-2 vaccine candidates may also prevent HSV-1 infection as observed in animal models and clinical trials (Johnston et al., 2016; Petro et al., 2015; Belshe et al., 2012).

At present, most vaccine candidates have considered targeting the process of HSV entry into target cells through the induction of neutralizing antibodies, yet some clinical studies suggest that these antibodies may not correlate with protection against infection by both HSVs, and their related clinical manifestations (Petro et al., 2015; Belshe et al., 2012; Burn et al., 2018; Chung and Sen, 2012). The different approaches that have been tested include attenuated vaccines against HSVs, replication-limited virus-based vaccines, viral vectors, DNA vaccines, inactivated vaccines, and subunit vaccines, among others (Chung and Sen, 2012; Retamal-Díaz et al., 2016).

Inactivated virus-based vaccines have been tested as a strategy for preventing clinical manifestations by HSV in humans, yet they have been shown to fail in preventing infection and disease (Skinner et al., 1997). For instance, a vaccine candidate that was composed of fractions of HSV-1 proteins obtained from inactivated HSV-1-infected cell lysates was able to induce the stimulation of neutralizing antibodies and lymphocytes, but the reduction in HSV recurrence in patients that were previously infected failed to reach statistical significance when tested in a clinical trial (Skinner et al., 1997; Table 1).

Subunit vaccine strategies are among the most tested approaches against HSV-1 and HSV-2 in human clinical trials (Cairns et al., 2014). Most of these studies were initiated in guinea pigs, in which combinations of purified HSV-1 glycoproteins reduced the morbidity associated with infection after viral challenge with HSV-2, and elicited protection against secondary central nervous system symptoms (Meignier et al., 1987). Later, it was also shown that an HSV-2 glycoprotein D (gD) subunit vaccine protected against both HSV-1 and HSV-2 in the guinea pig model, which promoted clinical trials using this approach (Bourne et al., 2003). A gD2-based subunit vaccine candidate with alum/MPL as an adjuvant was tested in large clinical trials enrolling HSV-1 and HSV-2 seronegative women; regrettably this approach only partially protected against HSV infection (58% protection for HSV-1 genital disease, 35% for HSV-1 infection and no efficacy for HSV-2 infection; Belshe et al., 2012). Surprisingly, high antibody titers against gD-2, induced by the vaccine were found (Belshe et al., 2014; ClinicalTrials.gov Identifier: NCT00057330). Other efforts have also been invested in developing HSV-2 gD-based subunit candidate vaccines in the past and failed at eliciting significant efficacy in clinical trials (Straus et al., 1997, 1994). For instance, although it was shown that a HSV-2 gD- and gB-adjuvanted vaccine candidate with MF59 was immunogenic, it did not prevent recurrent genital herpes and only had mild reactogenicity in humans (Corey et al., 1999; Table 1).

Table 1 Vaccine research on herpes simplex viruses.

<i>Vaccine strategy</i>	<i>Outcome</i>	<i>References</i>
Inactivated virus-based vaccines		
Fractions of HSV-1 proteins	Induced neutralizing antibodies and lymphocytes. Reduced viral recurrence in patients. However, in clinical trials this reduction was not statistically significant.	Skinner et al. (1997)
Subunit-based vaccines		
Mix of purified HSV-1 glycoproteins	Elicited reduced morbidity and protection against secondary central nervous system symptoms upon HSV-2 infection in guinea pigs.	Meignier et al. (1987)
Adjuvanted HSV-2 gD and gB proteins	Immunogenic but results showed no prevention of recurrent genital herpes and only mild reactogenicity in humans.	Corey et al. (1999)
gD-Alum/3-deacylated form of Monophosphoryl Lipid A (MPL)	Phase 3 completed. Results showed partial protection against HSV-1 and no protection against HSV-2 infection.	Belshe et al. (2012) ; Belshe et al. (2014); NCT00057330
HSV-2 recombinant proteins ICP4, gD and matrix M2	Phase 2 completed. No results posted to date.	NCT02114060
32 synthetic 35mer HSV-2 peptides and QS-21 Stimulon	Phase 2 completed. No results posted to date.	NCT01687595
Attenuated virus-based vaccines		
HSV-2 G strain with deletions in both copies of the gamma-34.5 gene and the <i>UL55</i> and <i>UL56</i> open reading frames	Impaired viral recurrences in guinea pigs.	Spector et al. (1998)
HSV-2 with the gene encoding gH (<i>UL22</i>) deleted	Reduces viral replication and the recurrence of genital lesions in guinea pigs, but failed to confer significant protection when tested in humans.	Mc Lean et al. (1994); Aurelian (2004)
HSV-1 with the <i>ICP0</i> open reading frame replaced with a gene encoding a tetracycline repressor and a mutation that replaces the <i>UL9</i> gene with an extra copy of gD (<i>US6</i>)	Reduces latent infections and protects against primary and recurrent HSV-2 infections in guinea pigs.	Brans et al. (2009)
HSV-1 with the gene encoding gK (<i>UL53</i>) deleted	Prevents virus entry into distal axons of ganglionic neurons and protects against intravaginal challenge with HSV-1 and HSV-2 in mice.	Iyer et al. (2013)
HSV-2 with gD deletion in the viral genome complemented in trans with the corresponding glycoprotein	Effective against HSV-1 and HSV-2 genital and skin infections. Prevents the establishment of viral latency.	Petro et al. (2015)
HSV-2 mutant with deletions in the genes encoding for <i>UL5</i> and <i>UL29</i>	Phase 1 completed. No results posted to date.	NCT02571166
Viral vector-based vaccines		
Vaccinia virus expressing HSV-1 gD	Reduces the severity of HSV-1 and HSV-2 infections in a murine model.	Rooney et al. (1991)
Defective adenovirus capable of expressing gD2	Higher protection against vaginal challenge in mice when compared to an FI-HSV2 vaccine.	Liu et al. (2017)
DNA-based vaccines		
DNA encoding HSV-2 gD (<i>US6</i> gene)	Proven to be safe and induce gD-specific cytotoxic T-lymphocyte and lymphoproliferation responses in a phase I clinical trial.	Cattamanchi et al. (2008)
Plasmid DNA vaccine encoding two HSV-2 proteins	Phase 2 completed. No results posted to date.	NCT02837575
Plasmid DNA vaccine encoding one HSV-2 protein	Phase 2 completed. No results posted to date.	NCT02030301
DNA vaccine encoding HSV-2 ICP0, ICP4, ICP22 and ICP27 antigens	Phase 1 completed. No results posted to date.	NCT00274300

Other HSV subunit vaccine candidates that were assessed in clinical trials include a vaccine comprised of the HSV-2 viral proteins ICP4 and gD2 combined with the matrix M2 adjuvant that was tested in a phase II clinical trial ([ClinicalTrials.gov](#) Identifier: NCT02114060), and a peptide-based vaccine candidate composed of 32 HSV-2 synthetic peptides 35mer-long mixed with the QS-21 adjuvant also tested in a phase II clinical trial ([ClinicalTrials.gov](#) Identifier: NCT01687595). However, to our knowledge, the results of these clinical studies have not been reported or published to date (Table 1).

On the other hand, numerous attenuated vaccine candidates against HSV have been developed and tested. One such attenuated vaccine candidate is RAV 9395, which is an HSV-2 G-strain based virus that has deletions in both copies of the gene encoding the viral protein γ 34.5, and the open reading frames of the *UL55* and *UL56* genes. This virus was tested in guinea pigs and was shown to impair viral recurrences (Spector et al., 1998). Furthermore, a vaccine candidate based on HSV-2 with a deletion in the gene that encodes the gH protein was shown to reduce viral replication and the recurrence of genital lesions in guinea pigs, yet it failed to show efficacy when tested therapeutically in humans (Mc Lean et al., 1994; Aurelian, 2004). Another vaccine candidate with replication-limited properties is an HSV-2 mutant with deletions in the genes encoding for *UL5* and *UL29*, which was proven to be safe, protective, and immunogenic when tested in mice and guinea pigs (Hoshino et al., 2009). A vaccine candidate with mutations in these two genes has completed a phase I clinical trial, yet the results have not been posted to date ([ClinicalTrials.gov](#) Identifier: NCT02571166). This vaccine candidate is currently being tested in a phase II clinical trial ([ClinicalTrials.gov](#) Identifier:

NCT04222985; Kim and Lee, 2020). T-REx™ consists of a replication-defective HSV-1 recombinant vaccine reportedly reduces latent infections and produce a protective immune response against both, primary and recurrent HSV-2 infections in guinea pigs (Brans et al., 2009). This vaccine replaces the open reading frame of *ICP0* with a gene encoding for a tetracycline repressor and also has another mutation that replaces the gene encoding for *UL9* with an extra copy of the *US6* gene that encodes for gD (Brans et al., 2009)). There is also evidence that the use of an HSV-1 mutant virus with a deletion of the gene encoding glycoprotein K (gK), which prevents the virus from entering distal axons of ganglionic neurons, protects against intravaginal challenge with the HSV-1 strain McKrae and HSV-2 strain G in mice (Iyer et al., 2013). On the other hand, there is also mounting evidence that a live-attenuated HSV-2 vaccine candidate with a deletion in glycoprotein D, complemented with the corresponding glycoprotein in *trans*, that experiences a single round of replication may be effective against both HSV-1 and HSV-2 genital and skin infections (Petro et al., 2015, 2016; Burn et al., 2018, 2018). Surprisingly, in the mouse model, this approach prevented the establishment of viral latency (Petro et al., 2015; Table 1).

Interestingly, the different outcomes that each HSV mutant yield, particularly at mounting or not protective immune responses may be related to the interaction of these mutant viruses with DCs. Indeed, distinctive mutations in HSV seem to modulate differently the function of these cells, which are key for establishing an effective antiviral immune response (Retamal-Díaz et al., 2017a,b; Table 1).

Additionally, viral vector-based vaccine candidates have also been developed and tested against HSV. For instance, a recombinant vaccinia virus expressing HSV-1 gD has been reported to reduce the severity of HSV-1 and HSV-2 infections in a murine model (Rooney et al., 1991). Also, a vaccine candidate based on adenovirus that consists of a replication-defective adenovirus recombinant for HSV-2 gD (rAd-gD2) has been tested and reported to provide higher protection against vaginal challenge than formaldehyde-inactivated HSV-2 (FI-HSV2) in mice (Liu et al., 2017; Table 1).

DNA vaccines have also been tested as a strategy for developing vaccines against HSV (Cattamanchi et al., 2008; Chung and Sen, 2012). Importantly, an HSV-2 gD-based DNA vaccine was reported to be safe and induce gD-specific cytotoxic T-lymphocyte and lymphoproliferation responses in a phase I clinical trial (Cattamanchi et al., 2008). On the other hand, the therapeutic pPJV7630 HSV-2 DNA vaccine encoding HSV-2 ICP0, ICP4, ICP22 and ICP27 antigens, completed a phase I of clinical trial with no results posted to the date to our knowledge (Stanberry and Rosenthal, 2005) (ClinicalTrials.gov Identifier: NCT00274300, ClinicalTrials.gov Identifier: NCT00310271). Additionally, two plasmid DNA vaccines encoding one or two HSV-2 proteins have completed phase II clinical trials; however, these studies have not reported their results yet (ClinicalTrials.gov Identifier: NCT02030301, ClinicalTrials.gov Identifier: NCT02837575; Table 1).

Some of the HSV vaccine candidates discussed above and currently undergoing advanced stages of testing in animal models or clinical trials are summarized in Table 1.

Current treatments and drugs for HSV infections

At present, several antiviral drugs are available for treating HSV infections and somewhat preventing recurrent, as well as the prolongation of herpetic lesions. The most commonly known and used are acyclovir (ACV), valacyclovir, and penciclovir, which act over the viral polymerase and hamper genome replication of both types of HSVs (Kukhanova et al. (2014)). However, these drugs are somewhat ineffective for some clinical manifestations, such as treating skin lesions (discussed below), and HSV variants that are resistant to these drugs frequently emerge in immunosuppressed individuals (up to 3.5–10% of immunosuppressed individuals), as well as immunocompetent individuals (1% of cases). Therefore, there is an urgent need in finding more effective antivirals and novel approaches for these drug-resistant variants (Reusser, 1996). Noteworthy, at present, there are numerous synthetic and botanical compounds being assessed as potential new antivirals against HSVs (Álvarez et al., 2020; Akram et al., 2018; Gonzalez et al., 2018).

Common first-line drugs used against HSVs

The most common drugs available against HSVs are nucleoside and nucleotide analogs. Acyclovir is an acyclic guanosine nucleoside and is currently the most widely used compound to treat HSV infections around the globe (Hassan et al., 2015; Álvarez et al., 2020). ACV is mostly safe, well-tolerated, and low cost (Hassan et al., 2015). However, this drug has some limitations, as the absorption of ACV through the oral route nears 15–30%, and thus, requires frequent administration (every 4 h). Therefore, the dosing consists in considerable amounts of the drug being taken (Bryson et al., 1983; Bean, 1992; Cortesi and Esposito, 2008). For skin lesions, ACV has been shown to reduce the healing process by only approximately two days if taken as soon as the first clinical symptoms arise, of an estimated total of 8 days (Bean, 1992; Spruance et al., 1990). For ACV to be effective, the drug needs to be phosphorylated to its active form, which is acyclovir triphosphate (Reusser, 1996; De Clercq, 2013). The first phosphorylation is carried out by the viral thymidine kinase (TK, *UL23* gene), and subsequent phosphorylations by cellular kinases (Kukhanova et al., 2014; Reusser, 1996; Table 2).

The nucleic acid analogs valacyclovir, penciclovir and famciclovir are also frequently used to treat HSVs infections. Similar to ACV, these compounds also reduce the duration of the herpetic skin lesions in approximately 1–3 days, as compared to the untreated groups (Emmert, 2000). Valacyclovir is an L-valine ester of acyclovir developed to increase its bioavailability and has an increased absorption at the intestinal level, equivalent to 54% (Soul-Lawton et al., 1995). Penciclovir is more rapidly

Table 2 Current strategies available and under research for treating HSV infection.

Compound	Characteristics	Virus tested	Development stage	Mechanism of action	Reference
Acyclovir	Nucleoside Analogue	HSV-1 and HSV-2	FDA and EMA approved	Inhibitor of viral DNA replication	Bryson et al. (1983) Kukhanova et al. (2014)
Valacyclovir	Nucleoside Analogue	HSV-1 and HSV-2	FDA and EMA approved	Inhibitor of viral DNA replication	Kukhanova et al. (2014); Schuster et al. (2016)
Penciclovir	Nucleoside Analogue	HSV-1 and HSV-2	FDA and EMA approved	Inhibitor of viral DNA replication	Kukhanova et al. (2014); Meira et al. (2019)
Famciclovir	Nucleoside Analogue	HSV-1 and HSV-2	FDA and EMA approved	Inhibitor of viral DNA replication	Emmert (2000); Mondal (2007)
Ganciclovir	Nucleoside Analogue	HSV-1 and HSV-2	FDA and EMA approved	Inhibitor of viral DNA replication	Chou and Hong (2014); Al-Badr and Ajarim (2018)
Cidofovir	Nucleotide Analogue	HSV-1 and HSV-2	FDA and EMA approved	Inhibitor of viral DNA replication	Lea and Bryson (1996); Chilukuri and Rosen (2003)
Brincidofovir	Nucleotide Analogue	HSV-1 and HSV-2	Under FDA Review	Inhibitor of viral DNA replication	Hostettler (2009); Lee et al. (2018)
Pritelivir	Helicase-Primase Inhibitor	HSV-1 and HSV-2	In Clinical Trials	Inhibitor of viral DNA replication	Wald et al. (2014); Poole and James (2018)
Amenamivir	Helicase-Primase Inhibitor	HSV-1 and HSV-2	In Clinical Trials	Inhibitor of viral DNA replication	Kawashima et al. (2017); Poole and James (2018)
Docosanol	Saturated Alcohol	HSV-1 and HSV-2	FDA and EMA approved	Inhibitor of Entry	Treister and Woo (2010); De Clercq and Li (2016)
Idoxuridine	Deoxyuridine Analogue	HSV-1	FDA and EMA approved	Inhibitor of viral DNA replication	Wilhelmus (2015); De Clercq and Li (2016)
Vidarabine	Nucleoside Analogue	HSV-1 and HSV-2	FDA and EMA approved	Inhibitor of viral DNA replication	Chou and Hong (2014); De Clercq and Li (2016)
Trifluridine	Nucleoside Analogue	HSV-1 and HSV-2	FDA and EMA approved	Inhibitor of viral DNA replication	Wilhelmus (2015); De Clercq and Li (2016)
Nelfinavir mesylate	HIV-1 Protease Inhibitor	HSV-1	In use for HIV-1. In clinical trials for HSV treatment	Inhibit the maturation and export of viral particles	Kalu et al. (2014)
Benzalkonium Chloride	Alkylamine	HSV-2	Non reported	Virucidal	Bélec et al. (2000)
<i>Aglaia odorata</i>	Unknown	HSV-1	Evaluated in animals	Inhibition after viral attachment HSV-1	Lipipun et al. (2003)
<i>Moringa oleifera</i>	Unknown	HSV-1	Evaluated in animals	Inhibition after viral attachment HSV-1	Lipipun et al. (2003)
<i>Ventilago denticulata</i>	Unknown	HSV-1	Evaluated in animals	Inhibition after viral attachment HSV-1	Lipipun et al. (2003)
<i>Alternanthera philoxeroides</i>	Chikusetsusaponin IV	HSV-1 and HSV-2	Evaluated in animals	Virucidal	Rattanathongkom et al. (2009)
<i>Melia azedarach</i>	Meliacine (Glycopeptide)	HSV-1 and HSV-2	Evaluated in animals	Unknown or not reported	Petrera and Coto (2009)
<i>Griffithsia</i> sp.	Griffithsin	HSV-2	Evaluated in animals	Inhibition of viral binding, inhibition of viral entry	Derby et al. (2018); Nixon et al. (2013)
<i>Symphyclocladia latiuscula</i>	2,3,6-tribromo-4,5- dihydroxybenzyl methyl ether	HSV-1	Evaluated in animals	Virucidal	Park et al. (2005)
<i>Macrocystis pyrifera</i>	Several bioactive components in the algae extracts	HSV-1 and HSV-2	Evaluated in animals	Unknown or not reported	Castillo et al. (2020)
<i>Durvillaea antarctica</i>	Several bioactive components in the algae extracts	HSV-1 and HSV-2	Evaluated in animals	Unknown or not reported	Castillo et al. (2020)
<i>Agaricus brasiliensis</i>	Polysaccharides	HSV-1 and HSV-2	Evaluated in animals	Inhibition of viral binding, inhibition of viral entry	Cardozo et al. (2013)
<i>Grifola frondosa</i>	Protein	HSV-1	Evaluated in animals	Virucidal	Gu et al. (2007)
<i>Rozites caperata</i>	Peptides	HSV-1	Evaluated in animals	Unknown or not reported	Pradeepet al. (2019)

phosphorylated than ACV and has a higher half-life (Soul-Lawton et al., 1995). While the half-life of ACV against HSV-1 is 0.7 h and against HSV-2 1 h, the half-life of penciclovir for HSV-1 and HSV-2 is 10 and 20 h, respectively (Hodge and Perkins, 1989; Lubber and Flaherty, 1996). Lastly, famciclovir is a prodrug of penciclovir that has been shown to have higher oral bioavailability than penciclovir, approximately 25% vs. 3% for the latter when tested in mice (Hodge et al., 1989; Sutton and Kern, 1993). It is noteworthy to note that resistance to each of these four first-line drugs has been described. Therefore, there is a need for developing new antiviral strategies to treat these drug-resistant variants (Reusser, 1996; Table 2).

Other drugs used to treat HSV skin lesions include a combination of acyclovir (50 mg) with hydrocortisone (10 mg) for topical use, or alternatively docosanol, which consists of an hydrophobic linear chain compound with an alcohol group at one of its ends that is formulated at 10% as a topical cream. Other options, yet seemingly discontinued, are a zinc oxide-based cream that is used to treat HSV-1 skin lesions, and benzalkonium chloride intended for HSV-2 skin lesions (Strand et al., 2012; Woo and Challacombe, 2007; Bélec et al., 2000; Godfrey et al., 2001; Table 2).

On the other hand, trifluridine, a nucleotide analog, is primarily used to treat herpetic keratitis (Chou and Hong, 2014). However, some severe local side effects have been described for this compound (Chou and Hong, 2014; Álvarez et al., 2020). Finally, the anti-HSV compounds idoxuridine and vidarabine, two nucleoside analogs that were the first antivirals identified against HSVs, have been discontinued given that more effective treatments are currently available to treat HSV infections (Álvarez et al., 2020; Table 2).

Second-line drugs against HSVs

Other nucleotide and nucleoside drugs that are considered second-line drugs have emerged in the last decades for treating HSV infections. For instance, ganciclovir is a nucleic acid analog that has antiviral activity against both HSV-1 and HSV-2 (Smee et al., 1983; Chou and Hong, 2014; Matthews and Boehme, 1988). Additionally, cidofovir, an acyclic nucleotide analog, also has antiviral effects against acyclovir-resistant HSV-1 and HSV-2 isolates (Chilukuri and Rosen, 2003). Like acyclovir, this compound also interferes with the activity of the viral DNA polymerase, and once transformed into its nucleotidic form, it has a much higher affinity towards the viral DNA polymerase than the host DNA polymerase. Hence, the inhibition of viral genome replication is more effective than with acyclovir (Ho et al., 1992). Nevertheless, these drugs have been reported to cause some severe side effects such as nephrotoxicity, altered mental status, abnormal levels of liver enzymes in serum, acute tubular necrosis, among others (Arvin et al., 2007; Sauerbrei, 2016; Table 2).

New drugs against HSVs

Currently, numerous new anti-herpetic drugs are being tested in animal models or clinical trials. For instance, 7-chloro-1,3-dihydroxyacridone and 1,3,7-trihydroxyacridon, two DNA topoisomerase II catalytic inhibitors, have been reported to inhibit HSV replication *in vitro* and cell-based experiments (Akanitapichat et al., 2000). Additionally, 5-chloro-1,3-dihydroxyacridone was found to inhibit the cleavage and packaging of viral DNA into capsids, interfering with viral maturation, which produced atypical B-type capsids (B-type capsids are explained in the virus egress section). Interestingly, this compound did not display DNA topoisomerase II inhibitory activity (Akanitapichat and Bastow, 2002; Álvarez et al., 2020; Table 2).

Additionally, new chemical compounds are being assessed in clinical trials as anti-herpetic drugs. For example, brincidofovir an acyclic nucleotide phosphonate has been shown to act as a substrate inhibitor of the viral DNA polymerase *in vitro* studies (Hostetler, 2009; Álvarez et al., 2020). On the other hand, amenamevir and pritelivir are two viral helicase inhibitors that target the viral DNA helicase/primase complex (H/P; Kleymann et al., 2002; Poole and James, 2018). To our knowledge, the results of these clinical trials have not been published yet. Finally, a mesylate salt, nelfinavir mesylate, which is an HIV-1 protease inhibitor, has been reported to have antiviral activity against HSV-1 and inhibit the maturation and export of viral particles (Markowitz et al., 1998; Kalu et al., 2014). This compound is being assessed in a clinical trial to treat Kaposi's sarcoma, but its potential effect against HSV-1 and HSV-2 is also considered a goal in this study (ClinicalTrials.gov Identifier: NCT03077451; Table 2).

On the other hand, many botanical compounds have been reported to have antiviral activity against HSVs, both *in vitro* and animal models. For a comprehensive review on this subject, we suggest referring to Álvarez et al., which revises the antiviral activity of compounds derived from plants, algae, and fungus, among others (Álvarez et al., 2020). Some of the studies reporting such activities are discussed below.

Plant-derived compounds with antiviral effects against HSVs in mice infected with HSV-1 have been reported for *A. odorata*, *M. oleifera*, and *V. denticulata*, which, combined with ACV diminished the development and progression of skin lesions and increased the mean survival time of the animals (Lipipun et al., 2003). Another study reported that an *Alternanthera philoxeroides* extract, namely Chikusetsusaponin IV has antiviral activity against HSV-2 genital infection in mice up to 7 days post-infection (Rattanathongkom et al., 2009). A glycopeptide from *Melia azedarach* glycopeptide has also been reported to have antiviral properties against HSV-2 infections when tested in a mouse model (Petrera and Coto, 2009; Table 2).

Algae extracts and compounds with antiviral potential against HSV include extracts from the algae species *Griffithsia* sp., which was shown to have anti-HSV-2 activity in a murine model, likely through blocking the infection process and cell-to-cell spread of this virus (Nixon et al., 2013). *Griffithsia* sp. extracts have since then been further characterized and tested in different animal models (Derby et al., 2018; Lee, 2019). Extracts of the algae *Symphyocladia latiuscula* have been shown to block viral plaque-forming units *in vitro*, and skin biopsies obtained from HSV-1-infected animals also displayed lesser virus than the controls (Park et al., 2005).

Moreover, recently it was described that algae extracts from *Macrocystis pyrifera* and *Durvillaea antarctica* display potent antiviral activity against acyclovir-sensitive and acyclovir-resistant HSV-1 and HSV-2 and reduced the duration and severity of HSV-1 skin lesions in mice, more than acyclovir (Castillo et al., 2020). Finally, the algae species *L. dendroidea*, *U. fasciata*, *C. decorticatum*, *S. zonale*, *S. cymosum*, *C. acicularis* and *P. capitatus* of the Brazilian coast have been shown to have strong antiviral effects against HSVs, both sensitive and resistant to acyclovir in *in vitro* studies (Soares et al., 2012; Table 2).

Interestingly, fungus-derived compounds have also been identified as potential novel antiviral treatment against HSVs. For instance, *Agaricus brasiliensis* was shown to reduce the severity of HSV-2 disease in a murine genital infection model (Cardozo et al., 2013). Also, mice treated with a protein isolated from *Grifola frondosa* reduced blepharitis, vascularization, and stromal disease, in addition to reduced viral replication in the eye when applied topically at this site (Gu et al., 2007). Interestingly, the *Rozites caperata* RC28 protein has also been shown to reduce the severity of stromal keratitis (Agrawal and Jha, 2020; Table 2).

HSV replication cycle

HSV structure

Herpes simplex viruses share common features with other viruses of the *Herpesviridae* family, and more notoriously with those in the *Alphaherpesvirinae* subfamily (Boehmer and Nimonkar, 2003). These viruses are characterized by four primary features starting from the exterior to their core: namely, they have an envelope with glycoproteins, a tegument, a capsid, and at the center the viral genome (Ibáñez et al., 2018; Fig. 2).

The envelope of HSV virions is composed of a lipid bilayer, which holds numerous of the eleven viral glycoproteins encoded in the viral genome (Suazo et al., 2015a; Fig. 2). These glycoproteins participate in different molecular processes throughout the viral life cycle, such as binding to the cell surface and entry of virion components into the cytoplasm of target cells (described later in this chapter), as well as the exit of infectious particles (Ibáñez et al., 2018). The tegument layer of the virion is composed of a set of approximately 20 different viral proteins (Vittone et al., 2005). The tegument proteins of the virus play relevant roles in disrupting the early host antiviral response, they enhance viral gene transcription, and aid in the transport of the viral capsids towards and out of the nucleus, among others (Ibáñez et al., 2018).

Electron microscopy analyses show that the viral capsids of herpes simplex viruses have an icosahedral shape and measure around 125 nm of diameter (Brown and Newcomb, 2011). Recently, cryo-electron tomography has brought exquisite detail to the structure of the viral capsid revealing details of the portal through which viral DNA is injected into the nucleus of the cells, among others (Cardone et al., 2007; Rochat et al., 2014). The capsid contains a single copy of a linear double-stranded DNA genome of more-less 150 kbp, which encodes approximately 80 genes and has a high GC content (Kieff et al., 1971). The spherical particle in which the tegument and capsid are contained is approximately 186 nm. The overall size of the viral particle, including the extensions of the glycoproteins on the virion surface, nears 225 nm (Grünwald et al., 2003; Fig. 2).

Cell infection

Herpes simplex virus infection of host cells initiates when virions bind to the cell surface, particularly through glycoprotein B (gB) and glycoprotein C (gC) for HSV-1 to heparan-sulfate proteoglycans (HSPGs) on the cell surface. Although gB is required for HSV-2

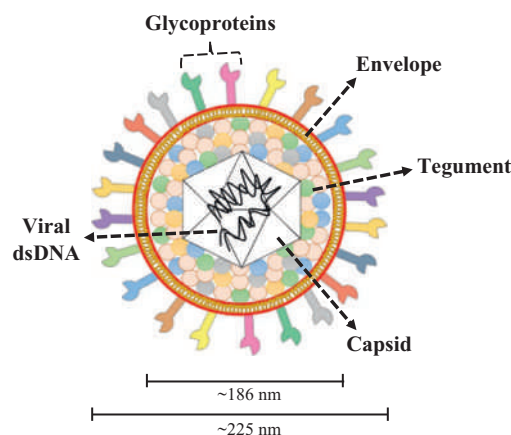


Fig. 2 HSV structure. From the inside-out, HSV virions are composed of a linear double-stranded DNA (~154 kbp) that encodes approximately 80 genes. The viral genome is contained within an icosahedral capsid that is surrounded by a layer of numerous viral proteins (approximately 20) called the tegument. The tegument in turn is enveloped by a bilayer lipid membrane that holds numerous glycoproteins that extend from the surface of the virion. HSV particles can have a diameter up to 225 nm approximately.

binding at this stage, gC of HSV-2 is unessential at this step (Herold et al., 1991; Gerber et al., 1995; Atanasiu et al., 2010; Satoh et al., 2008). Additionally, it has been reported that gB also binds to the paired immunoglobulin-like type 2 receptor (PILR; Satoh et al., 2008). Interestingly, even though HSPGs are the main target for the binding of HSV virions, some compounds acting as HSPG mimics have antiviral properties and prevent viral adhesion to the cell surface via competitive inhibition (Gangji et al., 2018). After viral binding to the host cell, glycoprotein D (gD) interacts with one of its receptors, which will vary depending on the cell type being infected. In non-immune cells, nectin-1 (HveC) or nectin-2 (HveB) will be preferred as receptors, while in immune cells, the herpes virus entry mediator (HVEM, TNFRSF14) seems to be the main target receptor (Martinez and Spear, 2001; Richart et al., 2003; Whitbeck et al., 1997; Kovacs et al., 2009). It has also been reported that 3-O-sulfated heparan sulfates are gD ligands (Shukla et al., 1999; Fig. 3).

Once gD binds to one of its receptors, it will suffer conformational changes that lead to the activation of the viral gH/gL complex present in the viral envelope (Atanasiu et al., 2010). Afterward, the glycoprotein H/ glycoprotein L (gH/gL) complex will interact with gB causing its activation so that the latter can act as the viral fusion protein (Atanasiu et al., 2010). This will allow the fusion of the cell membrane and virus envelope, and consequently, the entry of viral components into the cell cytoplasm (Atanasiu et al., 2010). Alternatively, HSV virions can enter the host cell via a pH-dependent endocytosis mechanism in some cells, by a

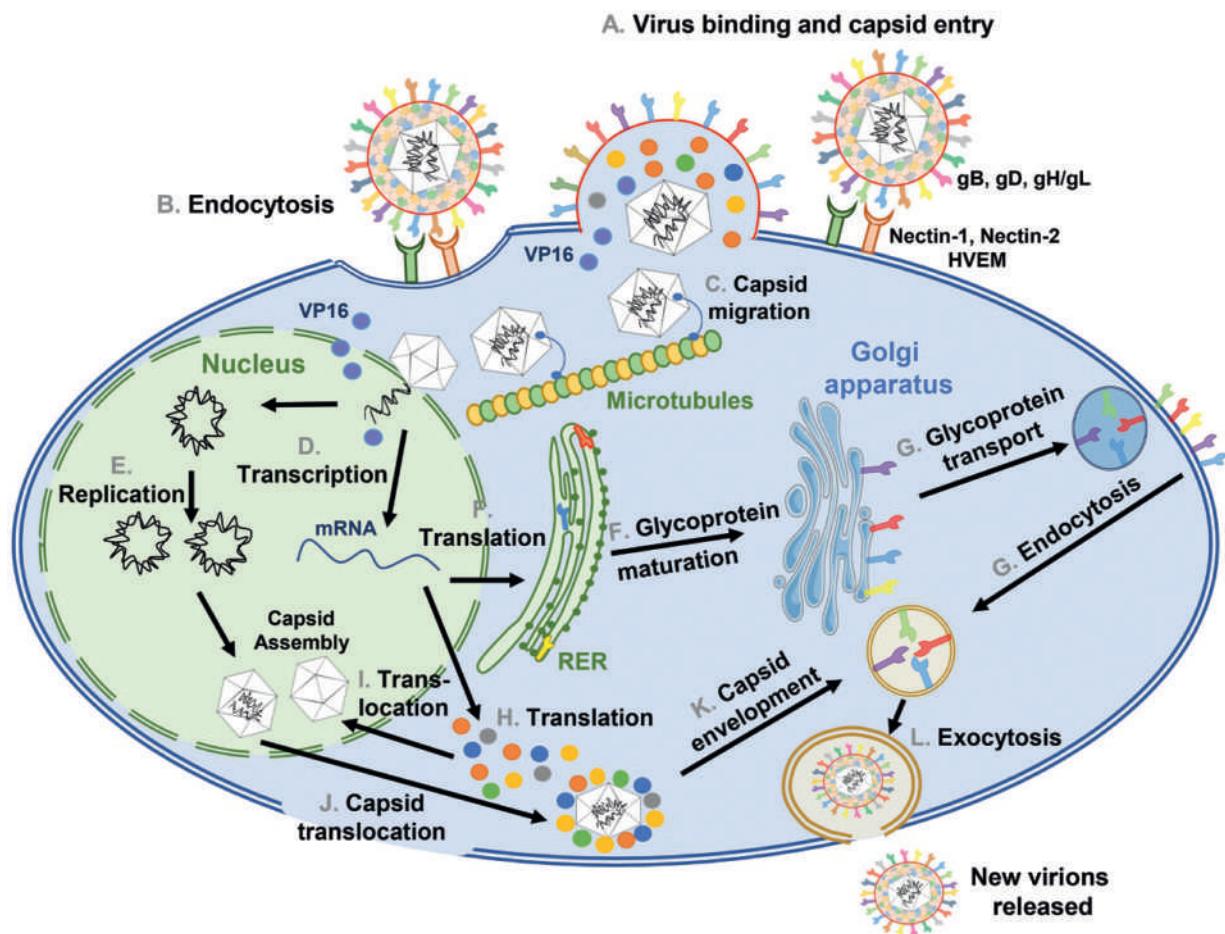


Fig. 3 Replication cycle of herpes simplex viruses. Schematic representations of the lytic infectious cycle of herpes simplex viruses. (A) First, HSV binds to cell surface receptors thanks to its surface glycoproteins. This triggers the fusion of the virus and cellular membranes, or (B) alternatively an endocytic process can occur, which allows the virus and capsids to enter the cell. (C) Once in the cytoplasm, the capsids are generally transported to the outer nuclear membrane through microtubules, where the viral genetic material is delivered into the nucleus through nuclear pores. (D) Some viral proteins within the tegument, such as VP16 (transactivation factor), are translocated to the nucleus of the cell. The viral genome is then transcribed in three consecutive “waves,” with the sequential transcription of alpha, beta and gamma genes. (E) The genome is circularized in the nucleus for its replication which occurs as a rolling-circle. (F) Viral mRNA is exported from the nucleus to the cytoplasm and translated into proteins. The translation of glycoproteins takes place at the rough endoplasmic reticulum (RER), and their glycosylation occurs in the Golgi apparatus. (G) Viral glycoproteins are then exported to the cell surface and later endocytosed in early endosomes. (H) Other viral proteins are translated in free ribosomes in the cytoplasm and some are translocated to subcellular compartments, where needed. (I) Capsid proteins translocate to the nucleus for assembling the viral capsid at this location. (J) The viral genome is inserted into the capsids in the nucleus, which then traverse the nuclear membranes (envelopment and de-envelopment processes) and access the cytoplasm where they acquire additional tegument proteins. (K) Mature capsids are enveloped into endosomes containing the viral glycoproteins, forming virions within exocytic vesicles. (L) These viral particles are then exocytosed to the extracellular space. Alternatively, infection may occur through cell to cell spread (see main text).

phagocytosis-like uptake process or by the independent activation of gH, which promotes virus entry into the cell through acidic endosomes (Yuan et al., 2018; Rahn et al., 2011; Fig. 3).

After virus access to the cytoplasm, the capsids are transported to nuclear pores. Several tegument proteins that are associated with the capsid play important roles at this step (Ibáñez et al., 2018). It has been suggested that VP26, a tegument protein, interacts with dynein by its light chains RP3 and Tctex1 (Douglas et al., 2004). There is also evidence that other tegument proteins, such as VP1/2 and UL37 may interact with dynein or kinesin-1, thus facilitating microtubule-mediated transport (Abaitua et al., 2012). Additionally, simple diffusion of capsids through the cytoplasm has been described to be enough for their accumulation at the nucleus (Garner, 2003). Once the viral capsids reach the nuclear membrane, interactions occur between viral proteins, namely VP1/2 and nucleoporins Nup358 and Nup214, which participate in viral genome delivery into the nucleus (Batterson et al., 1983; Copeland et al., 2009; Ibáñez et al., 2018; Fig. 3).

Modulation of cell viability

HSVs are capable of modulating cell viability differentially depending on the cell type that is infected. Two HSV-1 glycoproteins, namely gI and gD, have been reported as being capable of inhibiting Fas-mediated apoptosis in a neuroblastoma cell line and in Jurkat cells (Zhou et al., 2000; Jerome et al., 2001). As gI is localized at the cell membrane, its mode of interaction might be by signaling via the Fas receptor or antagonizing the activation of caspase-8 (Jerome et al., 2001). It has also been reported that upon HSV infection, the viral proteins ICP6 and ICP10, which are R1 large subunits of a viral ribonucleotide reductase (RR), bind to caspase-8 through specific domains, which leads to the inhibition of apoptosis in human cells induced by TNF- α through the TNF receptor (TNFR1) (Guo et al., 2015b). On the other hand, it has been described that the R1 HSV proteins may inhibit necroptosis of infected human and mouse cells by binding to the host receptor-interacting protein (RIP) 1/3 (Guo et al., 2015a; Huang et al., 2015).

In contrast, HSV induces apoptosis in immune cells. Natural killer cells (NK cells) undergo apoptosis when interacting with HSV-1-infected macrophages expressing Fas/FasL (Iannello et al., 2011). The viability of DCs is also affected by HSV infection (Retamal-Díaz et al., 2016; Jones et al., 2003), yet the specific mechanisms by which HSV induces apoptosis in DCs is somewhat unknown, although some evidence suggests it is mediated by a reduction in the expression of host c-FLIP (Kather et al., 2010; Stefanidou et al., 2013). Furthermore, HSVs induce the apoptosis of T cells by activating caspase-3, -8, and -9, which leads to the apoptosis of these cells (Vanden Oever and Han, 2010).

Genome replication and viral gene transcription

The viral tegument protein VP16 plays an important role in viral gene transcription and viral genome replication. This protein will act as a transactivator, promoting viral gene expression early after infection (Roizman and Zhou, 2015). Once the virion enters the cell, VP16 will translocate from the cytoplasm to the nucleus, where it will bind to the viral genome and facilitate the transcription of viral genes required for virus replication (Roizman and Zhou, 2015). The replication of the genome of HSV requires its circularization in the nucleus (Skaliter and Lehman, 1994) and is copied as a rolling circle, producing concatemers that consist of several copies of the whole genome in a linear form (Skaliter and Lehman, 1994; Fig. 3).

Transcription of HSV genes occurs sequentially, namely in three predominant waves (Kalamvoki and Roizman, 2008). First, a set of genes named immediate-early or alpha genes are transcribed. These genes mostly encode for products with functions related to the impairment of the host immediate antiviral response and proteins that act as transcription factors that promote the transcription of a subsequent set of genes termed early or beta genes (Silva et al., 2012; Ibáñez et al., 2018; Tognarelli et al., 2019). The products of these later transcripts are involved in promoting the replication of viral genomes and additional transcription factors that mediate the transcription of a later set of viral genes (Ibáñez et al., 2018). Late genes, sometimes separated into late early or gamma-1, and late or gamma-2 genes, mainly encode for structural components needed for virion assembly, among other functions (Chen et al., 1992).

Virus egress

After the viral genes are transcribed and then translated into their products, and that the viral genome is replicated in the nucleus, viral capsids are assembled in this compartment and subsequently translocated to the cytoplasm for their exocytosis through vesicles (Ibáñez et al., 2018). During this process, viral capsid proteins such as VP5, VP23, and VP26, that are synthesized in the cytoplasm are translocated to the nucleus for the assembly of empty enclosures that are then filled with viral DNA (Ibáñez et al., 2018; Abaitua et al., 2012; Sankhala et al., 2016). During this process, three types of capsids have been described within infected cells: A- and B-type, which are devoid of viral genomes, and C-type, which contains viral DNA (McNab et al., 1998). Hence, only C-type capsids will contribute to the generation of infectious virions (Taus et al., 1998; Fig. 3).

Following capsid assembly, the maturation of this viral structure is initiated in the nucleus, where numerous tegument proteins are added to the capsid. Some of these tegument proteins are required for the capsids to leave the nucleus, such as VP1/2 and UL17 (Bucks et al., 2007; Owen et al., 2015). During their translocation to the cytoplasm, HSV capsids acquire a primary envelope at the inner nuclear membrane (INM), resulting in the primary envelopment of virions in the perinuclear space (Mettenleiter et al., 2013). In this nuclear compartment, C-type capsids are selected for progressing towards the cytoplasm (Taus et al., 1998). Notably, two

viral proteins are needed for the egress of the capsids from the nucleus to the cytosol, UL31 and UL34, which form the nuclear egress complex (NEC) of the virions (Ott et al., 2011). After these capsids reach the perinuclear space, the primary enveloped capsids fuse with the outer nuclear membrane (ONM), undergoing a de-envelopment process, with capsids being released into the cytoplasm (Mettenleiter et al., 2013; Granzow et al., 2001; Panté and Kann, 2002). Once in the cytosol, the capsids acquire additional tegument proteins (Owen et al., 2015; Fig. 3). The capsids can then be transported separately from the glycoproteins towards the cell membrane in a “separated” manner, which seemingly often occurs in neurons, or transit to the Golgi apparatus to re-envelope in this compartment for “married” exocytosis of viral particles (Snyder et al., 2006). Indeed, the movement of capsids within the cell can be “separated” or “married” (Snyder et al., 2006). In the “married” model, enveloped particles contained in vesicles are transported through axons for virion exit, while in the “separated” model naked capsids travel from the neuron cell body through the axon in parallel with vesicles containing viral glycoproteins with which they will combine at a distal site of the neuron (Ahmad and Wilson, 2020; Wisner et al., 2011; Fig. 4). The separate strategy involves the movement of capsids through microtubules and dynein-mediated transport with capsid components interacting with kinesin-1 proteins, thus enabling anterograde capsid movements through the cytoplasm (Döhner et al., 2002; Radtke et al., 2010).

The glycoproteins that will be present at the surface of the mature virion are translated in the endoplasmic reticulum (ER) and then derived onto the trans-Golgi network (TGN) from which they will be directed to multi-vesicular bodies (Wisner and Johnson, 2004; Turcotte et al., 2005; Calistri et al., 2007). From here, the glycoproteins will be exported to the cell surface from which they will be endocytosed and returned to early endosomes (Alconada et al., 1999; Hollinshead et al., 2012). Once these glycoproteins are reinternalized into the cytoplasm through endosomes, viral capsids will then fuse to them (Albecka et al., 2016) and create enveloped virions contained within exosomal vesicles that will be exported to the cell membrane for exocytosis (Hollinshead et al., 2012; Fig. 3).

Alternatively, HSVs may spread onto neighboring cells through close cell-to-cell contacts (Carmichael et al., 2018). In this case, viral proteins are directed to the boundary of contiguous cells, favoring the occurrence of membrane fusion events with viral components in a process termed virological synapse (Johnson et al., 2001). Cell infection through this mechanism allows the virus to evade the effector functions of immune components, such as antiviral antibodies that otherwise could neutralize the virus (Hook et al., 2006; Lubinski et al., 2011).

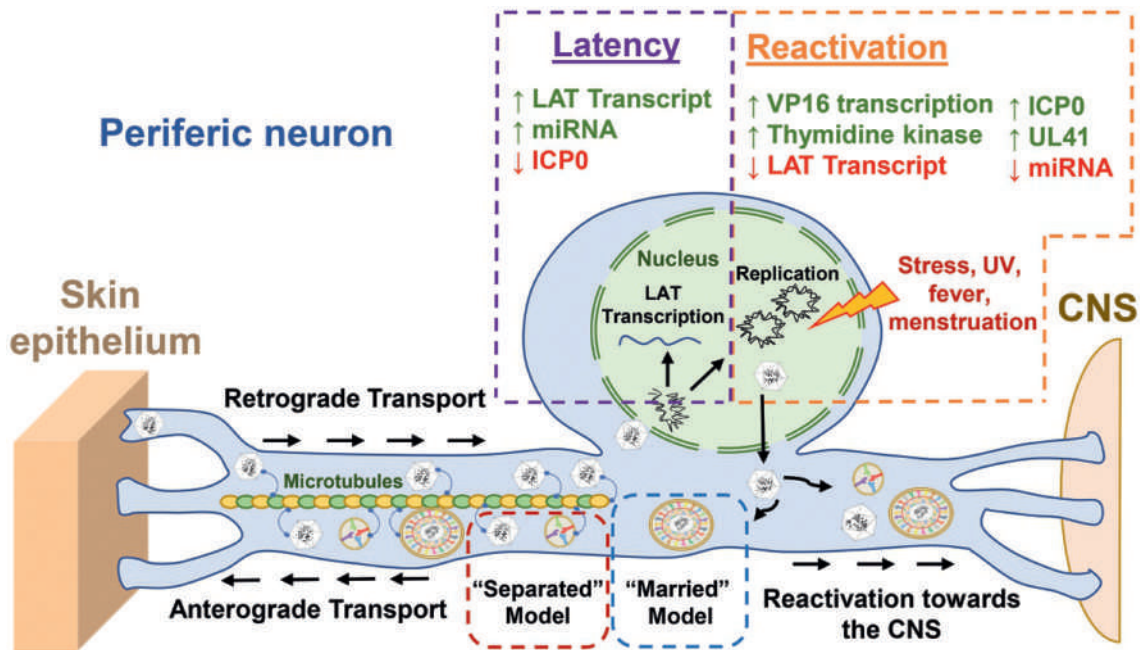


Fig. 4 Herpes simplex virus latency and reactivation. Schematic representation of herpes simplex virus infection in neurons where viral latency and reactivation occur. Neurons innervating the skin are likely infected by virus-infected epithelial cells due to close interactions. Once infected, viral capsids migrate through microtubules to the outer membrane of the cell nucleus. The genetic material is then delivered into the nucleus through nuclear pores, circularized in this compartment and maintained as an episome. During latency, high levels of the latency-associated transcript (LAT) and viral miRNAs are expressed, which leads to transcription repression of viral genes (e.g. ICP0). When the cell is exposed to virus-activating stimuli, such as UV light, fever or menstruation, reactivation of the virus infectious cycle is triggered, producing increased levels of viral proteins, such as VP16, ICP0 and thymidine kinase (TK). At this stage, viral proteins such as UL41 enhance the transcription levels of viral genes. This reactivation process leads to the replication of the virus genome and synthesis of viral proteins. Then, newly synthesized infectious particles can be released in an anterograde manner (towards the initial site of infection) or in a retrograde fashion (towards the CNS). The transport of the virus, or virus components through the axon of neurons can occur according to a “married” model, which consists of virions being transported within vesicles, or “separated” consisting of naked capsids travelling independently of vesicles that carry viral glycoproteins, with both combined at distal regions within the neuron.

Latency

Herpes simplex viruses can establish lifelong infections (Rosato and Leib, 2015). Upon the infection of the epithelium, HSVs may access the termini of sensory neurons that innervate the sites of primary infection and from there, reach the cell body of these cells, mainly neural cells of the trigeminal ganglia during facial infections and the dorsal root ganglia during genital infections (Duarte et al., 2019; Rosato and Leib, 2015). Once these viruses reach the neurons, they can establish latency in these cells, which is characterized by the circularization of the viral genome as an episome in the nucleus and the transcription of a unique RNA transcript, namely the latency-associated transcript (LAT; Margolis et al., 2007; Widener and Whitley, 2014). This transcript controls the expression of lytic genes and is likely involved in providing the virus a stealth mode in neurons which favors immune evasion. However, molecular reactivation of latent HSV has been reported, which is characterized by the expression of a subset of viral genes that yield detectable amounts of viral proteins without the yield of infectious viral particles (Feldman et al., 2002; Duarte et al., 2019; Fig. 4).

Reactivation

Several factors determine the reactivation of latent HSV infections, possibly the virus itself, host factors, and the surrounding environment (Roizman and Whitley, 2013). Some reactivation triggers include fever, exposure to UV radiation, stress and menstruation, among others (Roizman and Whitley, 2013; Fig. 4).

The LAT transcript and viral miRNAs are crucial for the establishment and maintenance of latency (Rosato and Leib, 2015; Roizman and Whitley, 2013). LAT, which is the only or main transcript undergoing transcription during latency encodes several miRNAs that increase exponentially in amount during this stage (Rosato and Leib, 2015; Roizman and Whitley, 2013). The expression of these miRNAs limit the expression of key viral genes such as *ICP0* and this reduction inhibits partially or completely viral reactivation (Roizman and Whitley, 2013; Fig. 4).

Interestingly, it has been suggested that viral reactivation requires either the activation of VP16 and the subsequent de-repression and activation of viral alpha genes, or the de-repression of alpha genes by cellular factors without the need of VP16 (Roizman and Whitley, 2013). A study performed in ganglionic organ cultures showed that the genes encoding for *ICP0* (an alpha gene), thymidine kinase (a beta gene), and VP16 and UL41 (gamma genes) are activated a few hours after explant, which is a known trigger of reactivation (Roizman and Whitley, 2013). Consequently, the transcription of LAT and several viral miRNAs was seen to decrease (Roizman and Whitley, 2013). Hence, it is suggested that viral reactivation may occur due a massive deregulation in viral genome expression that LAT and miRNAs are unable to suppress (Roizman and Whitley, 2013). Consistent with this notion, the expression of viral genes in explanted murine trigeminal ganglia coincides with LAT and miRNA degradation (Du et al., 2012). It is also suggested that viral reactivation may result from a failure of LAT and miRNAs to degrade small amounts of viral messenger RNAs encoding key viral genes (Roizman and Whitley, 2013; Fig. 4).

Once the virus reactivates within neurons in the ganglia, it may engage anterograde movements, which will lead to the spread of infectious viral particles either at peripheral tissues or within the central nervous system (Duarte et al., 2019; Fig. 4).

Evasion of host antiviral responses by HSVs

Evasion of cellular antiviral responses

Immune and non-immune cells can recognize pathogen-associated molecules thanks to numerous surface and intracellular receptors (Tognarelli et al., 2019; Suazo et al., 2015a). Pathogen-associated molecular patterns (PAMPs) consist of a set of microbial elements such as lipids, nucleic acids, carbohydrates, or proteins that may be recognized by pathogen recognition receptors (PRRs). Cell infection with pathogens can also generate damage-associated molecular patterns (DAMPs), which are danger signals released by the cells in response to viral replication and for which cellular receptors also exist (Tang et al., 2012; Johnson et al., 2013). When their corresponding receptors sense these molecules, a cascade of intracellular activating signaling pathways are initiated, which induce the activation of cellular, local and systemic antiviral responses (Mogensen, 2009).

Among the extensively studied PRRs are Toll-like receptors (TLRs), which play an important role in recognizing PAMPs and DAMPs. After binding to viral elements, TLRs elicit intracellular signaling pathways that lead to the early expression of antiviral cellular factors and the expression of soluble and membrane-bound molecules that modulate the activity of infected and non-infected neighboring cells (Alexopoulou et al., 2001; Hemmi et al., 2002; Diebold et al., 2004; Martinez-Martin and Viejo-Borbolla, 2010). Rapid detection of these molecular signals by the host will guarantee an effective antiviral response and promote the establishment of an innate and adaptive immune response (Tang et al., 2012; Johnson et al., 2013; Mogensen and Paludan, 2001).

Upon HSV infection, different TLRs, such as TLR2, TLR3, TLR7, and TLR9 are activated and can mediate antiviral responses against these viruses (Tognarelli et al., 2019). For instance, TLR2 recognizes the viral glycoprotein B and induces the activation of NF- κ B and the secretion of interleukin (IL)-8 (Cai et al., 2013). Furthermore, in response to the HSV-1 gH/gL protein complex, TLR2 in cooperation with the α v β 3 integrin leads to type-I interferon (IFN-I) production and IL-10 secretion in non-immune cells (Gianni et al., 2013, 2012). In DCs, the activation of this TLR leads to NF- κ B activation and the transcription of numerous cytokines, such as IL-6, IL-8, IL-10, IL-12, and TNF- α (Ariza et al., 2014; Fig. 5).

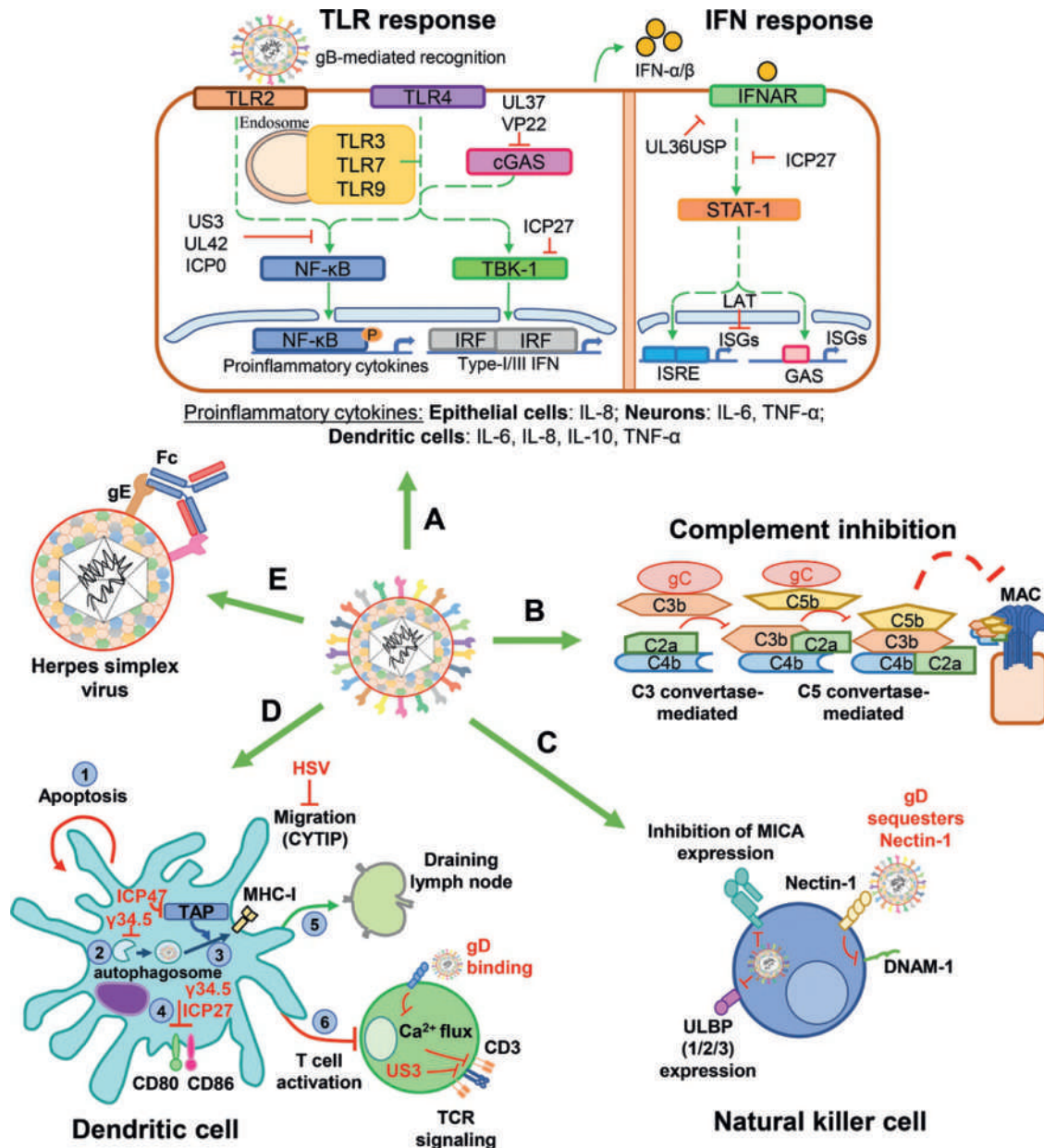


Fig. 5 Immune evasion by herpes simplex viruses. (A) Left panel: TLR responses and cGAS signaling in response to HSV components. TLR2 recognizes glycoprotein B which triggers NF-κB signaling. TLR3, TLR7 and TLR9 are activated by HSV infection, which triggers NF-κB and TANK-binding kinase 1 (TBK-1) signaling pathways. UL37 and VP22 inhibit the cGAS signaling pathway, but nevertheless trigger NF-κB and TBK-1 activation. On the other hand, US3, UL42, and ICP0 inhibit the activation of the NF-κB pathway. ICP27 inhibits the activation of TBK-1. Right panel: Type-I IFN response inhibition by HSV proteins. The IFN-I receptor (IFNAR) recognizes IFN-α and IFN-β and triggers IFN-I signaling pathways. Viral ubiquitin-specific protease UL36 (UL36USP) and ICP27 inhibit the activation of IFN-I responses, and the latency associated transcript LAT inhibits the production of interferon-stimulated genes (ISG). STAT-1: Signal transducer and activator of transcription 1; IRF: interferon regulatory factor; ISRE: interferon-sensitive response element, cGAS: cGMP-AMP synthase; GAS: gamma-interferon activation site. (B) Herpes simplex viruses inhibit complement activation. HSV glycoproteins C (gC) bind to C3b and C5b complement components and inhibits their interaction with other complement elements, which otherwise would lead to the formation of the membrane attack complex (MAC). (C) NK cell function is modulated by HSV. The viral glycoprotein D (gD) sequesters nectin-1, which triggers the suppression of DNAM-1. HSV also block the expression of MICA, ULBP-1, ULBP-2 and ULBP-3 in infected cells, which are NK-activating ligands. (D) HSVs hampers dendritic cell function. (1) HSVs induces apoptosis in DCs. (2) The HSV γ34.5 protein inhibits the maturation of DCs and autophagosome function. (3) the HSV ICP47 protein interferes with the translocation of peptides to the endoplasmic reticulum, mediated by the transporters associated with antigen processing (TAP), which is required for antigen presentation in MHC-I. (4) The HSV γ34.5 and ICP27 proteins inhibit the expression of CD80 and CD86 costimulatory on the DC surface, which are needed for effective T cell activation. (5) HSV infection increases cytohesin-interacting protein (CYTIP) degradation, which induces a reduction in the motility of DCs, and consequently a decrease in the migration of DCs to draining lymph nodes for antigen presentation. (6) HSVs inhibit T cell receptor (TCR) signaling pathways. gD binding to HVEM triggers inhibition of Ca²⁺ fluxes in T cells engaged for activation. The HSV US3 protein also blocks TCR signaling. (E) The HSV glycoprotein gE negatively modulates the binding of the Fc portion of antibodies to Fcγ receptors (FcγRs), thus impairing Fc-mediated functions carried out by antibodies (e.g. complement activation, opsonization and ADCC, among others).

Additionally, TLR3 has been shown to play an important role in HSV disease and is known to sense pathogen and host-derived double-stranded RNA hybrids (Alexopoulou et al., 2001). TLR3 expression in astrocytes enhances the control of HSV infection after viral access to the central nervous system (CNS) and induces type-I interferon responses while favoring the NF- κ B-dependent secretion of IL-6 and TNF- α (Reinert et al., 2012; Liu et al., 2013). Interestingly, individuals who have mutations that negatively modulate the immune response mediated by this TLR are more susceptible to developing HSV encephalitis (Zhang et al., 2007; Guo et al., 2011). Furthermore, *ex-vivo* experiments with differentiated neurons, astrocytes, and oligodendrocytes of individuals with deficiencies associated with TLR3 showed that they were more prone to viral infection and had compromised IFN- β and IFN- γ secretion (Lafaille et al., 2012; Fig. 5).

TLR7 recognizes exogenous nucleic acid molecules and has been reported to induce a response that burdens HSV infection and disease when activated with a specific agonist such as imiquimod in a genital mouse infection model (Miller et al., 1999). These findings led to the evaluation of this agonist as a therapeutic approach to treat herpetic lesions in humans, especially in immunocompromised patients suffering from acyclovir-resistant HSV-2 disease (Hirokawa et al., 2011; Kan et al., 2012; Lascaux et al., 2012). Although a clinical trial assessing the potential of imiquimod against HSV did not show promising results, somewhat similar approaches could eventually contribute to HSV control in immunocompromised patients (Schacker et al., 2002; Bernstein et al., 2005; Fig. 5).

TLR9 is mainly known to sense bacterial and viral DNA and synthetic CpG oligodeoxynucleotides (CpG ODNs), and is expressed in immune and non-immune cells (Tognarelli et al., 2019). Similar to other TLRs, TLR9 agonists can promote antiviral responses against HSV. TLR9 has been shown to sense HSV components and induce the secretion of IL-6, IL-12, and type-I IFNs, among others (Triantafilou et al., 2014; Lund et al., 2003). However, the function of TLR9 in HSV infection may not be essential for host survival upon viral challenge. Indeed, TLR9 knockout mice infected with HSV in the CNS survived an infection, although they showed more susceptibility to viral infection translating into a higher viral load in the brain (Krug et al., 2004). Interestingly, intranasal treatment of mice with CpG ODNs, which act as a TLR9 agonist, before HSV infection, increased their survival rates, reduced viral loads in the brain, and reduced the secretion of the pro-inflammatory cytokines CCL2, CCL5 and IL-6 in the CNS (Boivin et al., 2012; Fig. 5).

Besides TLRs, other host receptors are also capable of recognizing viral nucleic acids present during HSV infection that elicit strong antiviral responses and are frequently associated with stimulation of pro-inflammatory cytokines and interferon production (Diner et al., 2015). For instance, interferon- γ inducible protein 16 (IFI16) has been shown to promote the activation of the transcription factor interferon regulatory factor 3 (IRF3) and NF- κ B, which in turn induce IFN α/β and IL-6 upon HSV recognition (Dawson and Trapani, 1995; Conrady et al., 2012; Triantafilou et al., 2014). Furthermore, this receptor can silence viral gene expression by adding nucleosomes and heterochromatin marks to the viral DNA (Orzalli et al., 2013). However, the ICP0 protein of HSV-1 seems to play an important role in inhibiting IFI16 activation via targeting this protein to proteasome degradation (Johnson et al., 2013).

Additionally, cGMP-AMP synthase (cGAS), a cytosolic DNA sensor, recognizes HSV-1-related genetic material and leads to the secretion of IFN- α and IFN- β in immune and non-immune cells (Cai et al., 2014; Orzalli et al., 2015). It has also been reported that cGAS and IFI16 may act cooperatively, with cGAS promoting IFI16 stabilization (Orzalli et al., 2015). However, it has also been reported that HSV infection may deregulate the functions of these receptors. For instance, the HSV-1 tegument protein UL37 can promote the deamination of an asparagine residue in cGAS, leading to its inactivation. Furthermore, the activation of protein kinase B (PKB, AKT) upon HSV-1 infection suppresses the activity of cGAS via phosphorylation (Zhang et al., 2018; Seo et al., 2015; Fig. 5).

The DNA-dependent activator of interferon (DAI) is another nucleic acid sensor reported to induce IL-6 and IFN- β release in response to HSV-2 infection (Triantafilou et al., 2014). DAI interacts with the HSV-1 ICP0 protein and inhibits viral replication (Pham et al., 2013). Other nucleic acid sensors are retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), which are intracellular and recognize double-stranded RNA (Weber et al., 2006). These sensors have signaling pathways that are modulated by the VHS protein of HSVs early after infection, and results in hampering of signaling events that would otherwise promote IRF3 activation and an IFN- β -mediated antiviral response (Cotter et al., 2010; Yao and Rosenthal, 2011).

The inflammasome, a cellular multiprotein complex, can also sense viral components and initiate an antiviral response (Johnson et al., 2013). This complex promotes the secretion of inflammatory molecules such as IL-1 β (Lopez-Castejon and Brough, 2011). NLRP3, AIM2, and IFI16 are inflammasome cytoplasmatic sensors that can sense HSV components (Johnson et al., 2013). NLRP3 and IFI16 are activated early after HSV infection, which induces IL-1 β release. However, as mentioned before, ICP0 was found to inhibit IFI16 by targeting it to the proteasome (Johnson et al., 2013). On the other hand, AIM2-dependent inflammasome signaling is inhibited by the HSV-1 VP22 protein by preventing its oligomerization (Maruzuru et al., 2018).

To impair the cell capability to sense viral components, HSVs have evolved several mechanisms to modulate the activity of NF- κ B (p65), which participates in the expression of numerous immunomodulatory mediators as a transcription factor (Suazo et al., 2015a). The translocation of NF- κ B to the nucleus can be inhibited by HSVs through the hyperphosphorylation of this factor, thanks to the HSV kinase US3 (Wang et al., 2014). Also, inhibition of NF- κ B translocation to the nucleus can be mediated by the viral protein UL42, which encodes for a DNA polymerase processivity factor, which interacts with p65/RelA and p50/NF- κ B1 (NF- κ B1 forms; Zhang et al., 2013). ICP0 can also interact with p65/RelA and p50/NF- κ B1, leading to the inhibition of NF- κ B activation mediated by TNF- α (Zhang et al., 2013). Another viral protein that inhibits NF- κ B activation is the viral protein VP16, which decreases IL-8 and IFN- β production by HEK 293T HSV-infected cells (Xing et al., 2013; Wang et al., 2014). Despite the negative modulation of NF- κ B by HSV, cells infected with these viruses secrete numerous soluble mediators, such as CCL2, IL-8, IL-6, and TNF- α as seen for primary endometrial genital epithelial cells. HSV has also been reported to induce CXCL9 in the cervical

mucus of infected women (Huang et al., 2012; Fig. 5). Despite several studies showing HSV inhibition of NF- κ B, there is also evidence that activation of NF- κ B seems to be necessary for the infection process (Alvarez et al., 2020; Goodkin et al., 2003; Amici et al., 2006; Liu et al., 2008; Patel et al., 1998). Thus, the dual modulation of NF- κ B inhibition and activation by HSV-1 may be time-dependent during the infection process.

Host cells may restrict viral replication thanks to interferon responses. These cytokines induce antiviral responses in cells that secrete these molecules, as well as neighboring cells (Theofilopoulos et al., 2005). At present, there are different mechanisms by which HSVs negatively modulate the interferon responses. One of these mechanisms involves the viral protein ICP0, which binds to host IRF3, thereby inhibiting its activation and the transcription of IRF3-target genes such as type-I IFNs (Peng et al., 2009; Lin et al., 2004). The tegument protein VP16 also inhibits IRF3, and NF- κ B activation, ultimately leading to a block in IFN- β expression (Xing et al., 2013). IFN- β expression can also be inhibited by the viral ubiquitin-specific protease UL36 (UL36USP), impairing stimuli-mediated IRF3 dimerization through the de-ubiquitination of the TNF receptor-associated factor-3 (TRAF3; Wang et al., 2013; Fig. 5).

Also, HSVs may interfere with signaling events related to interferon receptors. For example, the HSV-1 ICP27 protein interferes with Signal transducer and activator of transcription 1 (STAT-1) activation needed for interferon-stimulated genes (ISG) transcription (Johnson et al., 2008). This protein has also been reported to aid in the secretion of an unknown soluble factor that has antagonizing activity over IFN-I signaling pathways of neighboring uninfected cells (Johnson and Knipe, 2010; Fig. 5).

Evasion of the innate immune response

HSVs are capable of modulating the host's innate immune response, both acellular and cellular components. The complement is an acellular set of host proteins of the innate immune response that can debilitate pathogens by mounting a membrane attack complex (MAC) over the microorganism or infected cells thanks to a cascade of controlled molecular chain reactions (Serna et al., 2016). The glycoprotein C (gC) of HSVs binds to the complement component C3b and blocks the pathways that lead to the formation of the MAC (Friedman et al., 1984; McNearney et al., 1987). This glycoprotein can also bind to the complement component C3 and inhibit complement-mediated virus inactivation or the lysis of virus-infected cells and the binding of gC to the complement component C5, blocking its down-stream activities (Lubinski et al., 2002; Hook et al., 2006; Fig. 5).

On the other hand, HSVs modulate the function of NK and NKT cells by suppressing DNAX accessory molecule-1 (DNAM-1)-dependent NK cell-mediated lysis of virus-infected cells (Grauwet et al., 2014). Importantly, glycoprotein D has been described to sequester nectin-1 from the cell surface, which was shown to impact the expression DNAM-1 on the surface of infected cells (Fig. 5) (Grauwet et al., 2014). It has also been reported that HSV infection blocks the expression of the MHC class I polypeptide-related sequence A (MICA), ULBP1, ULBP2, and ULBP3, which are NK-activating ligands (Schepis et al., 2009; Tomazin et al., 1996; Fig. 5). Additionally, HSVs downregulate the expression of the antigen-presenting molecule CD1d from the surface of HSV-infected cells, which is needed for antigen recognition by NKT cells (Godfrey et al., 2010; Yuan et al., 2006).

Dendritic cells (DCs) are immune cells that play key roles in the intercommunication between innate and adaptive immune cells (Retamal-Díaz et al., 2016). Importantly, their maturation, antiviral activity, viability and migration are affected by HSV infection (Tognarelli et al., 2019; Retamal-Díaz et al., 2016). HSVs have been reported to downregulate autophagosome activity within DCs, affecting antigen processing and presentation (Leib and Gobeil, 2012; Yordy et al., 2012). Furthermore, the ICP47 viral protein may adopt a helical hairpin structure, impairing the activity of transporters associated with antigen processing (TAP) and hence, peptide translocation to the RE (Oldham et al., 2016). HSVs also inhibit the capacity of DCs to activate T cells; the viral proteins ICP22 and γ 34.5 have been shown to downregulate the expression of important T cell co-stimulatory molecules such as CD80 and CD86 from the DC surface (Matundan and Ghiasi, 2018; Jin et al., 2009). Additionally, HSVs can affect the migration of mature DCs from the site of infection to the draining lymph nodes by increasing the degradation of cytohesin-interacting protein (CYTIP) which regulates the motility of these cells (Theodoridis et al., 2011; Tognarelli et al., 2019; Fig. 5). There is also evidence that supports a role for DCs in neuron infection. Indeed some studies have shown that the depletion of DCs (e.g. CD11c⁺/CD8 α ⁺) caused a reduction in HSV latency in neurons after ocular infection (Mott et al., 2009, 2008, 2014; Kassim et al., 2006; Mott and Ghiasi, 2008). Finally, macrophages are also affected by HSV infection with higher levels of pro-inflammatory cytokines secreted by M1 macrophages, as compared to M2 macrophages after HSV-1 infection (Martinez and Gordon, 2014). Additionally, cGAS down-stream events have also been reported to be disrupted by HSV infection, namely through viral inhibition of TANK-binding kinase 1 (TBK1) by ICP27, which interfered with IRF3-dependent type-I IFN secretion by virus-infected cells (Christensen et al., 2016).

Evasion of the adaptative immune response

HSVs encode numerous molecular factors for evading the function of antiviral antibodies and the function of T cells. The viral glycoprotein E (gE) binds to the Fc portion of antibodies thus competing with the capacity of antibodies to bind to the complement component C1q, therefore impairing one of their important functions (Lubinski et al., 2011; Para et al., 1982). Furthermore, by binding to the Fc portion of antibodies, gE inhibits the capacity of IgG antibodies bound to viral antigens to bind Fc γ receptors (Fc γ Rs), and thereby impair the phagocytosis of opsonized virus particles or capture of virus-infected cells by phagocytic immune cells (Para et al., 1982; Armour et al., 2002; Dilillo et al., 2014; Fig. 5). Additionally, HSVs negatively modulate the activation and proliferation of T cells. The binding of gD to the HVEM receptor on the surface of T cells affects intracellular calcium fluxes within these cells which are required for T cell receptor (TCR) activating signaling events after CD3 engagement (La et al., 2002).

Additionally, other HSV determinants have also been suggested to inhibit TCR-related calcium flux in T-cells (Sloan et al., 2006). Also, the HSV protein US3 can affect T cell activation by interfering with TCR signaling through the modulation of the linker for activation of T cells (LAT). The viral protein US3 reduces the ubiquitination of LAT and TNF receptor-associated factor 6 (TRAF6), leading to an overall reduced T cell activation (Yang et al., 2015). Finally, CD8⁺ T cell activation is also hampered by the capacity of HSVs to interfere with the expression of their ligands (MHC-I) on the surface of infected cells (discussed above; Hill et al., 1995; Fig. 5).

Concluding remarks

Herpes simplex viruses are highly prevalent in the human population thanks to their capacity to elicit lifelong infections and establish latency in neurons. These viruses produce a broad spectrum of diseases that range from mild to severe, and most of the individuals infected are asymptomatic. Importantly, HSVs have evolved several strategies to hijack the cellular antiviral response and the immune system. Indeed, HSVs can interfere with different steps of the host antiviral response, modulate cell viability differentially depending on the cell type, hamper the host's capacity to sense viral components, and the function of innate and adaptive immune system elements. At present, multiple drugs for treating HSVs associated diseases exist, however, these drugs are poorly effective for treating some clinical manifestations, such as skin disease and drug-resistance variants may appear. Therefore, new anti-HSVs therapies are needed to treat this type of infection and hopefully a vaccine can be developed to prevent infection.

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Epstein-Barr Virus

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Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that infects at least 90% of the world's adult population (de-Thé et al., 1975). EBV was the first recognized human cancer virus: it was established as the cause of endemic Burkitt lymphoma in 1964 (Epstein et al., 1964). The worldwide burden of diseases due to EBV is enormous. In addition to endemic Burkitt lymphoma, EBV is responsible for infectious mononucleosis. EBV is a risk factor for Hodgkin lymphoma, nasopharyngeal carcinoma, and complications including cancers after solid organ or hematopoietic cell transplantation (Balfour et al., 2020). EBV is also a risk factor for autoimmune diseases, especially multiple sclerosis (Ascherio and Munger, 2010). This article discusses basic virology, epidemiology, clinical manifestations, immune responses, diagnosis, prevention, and treatment of EBV diseases, and suggests priorities for future research.

Epstein-Barr virus

EBV, also known as human herpesvirus 4 (HHV4) is a member of the *Herpesviridae* family and classified within the subfamily of gammaherpesviruses. Like other herpesviruses, the EBV genome is composed of linear, double-stranded DNA approximately 172 kbp in length packaged within an icosahedral nucleocapsid which is surrounded by a protein tegument layer, and an outer envelope with external glycoproteins (Fig. 1). The EBV tegument is composed of several large tegument proteins and tegument binding-protein proteins that are responsible for evasion from host immune surveillance, envelopment, viral morphogenesis, and reprogramming the cell for viral replication (Johannsen et al., 2004). The major envelope glycoproteins, responsible for host cell tropism and viral entry, include gp350, gp220, gH, gB, gp42, gM, gp78, gN, gp150, and gL (Sathiyamoorthy et al., 2016).

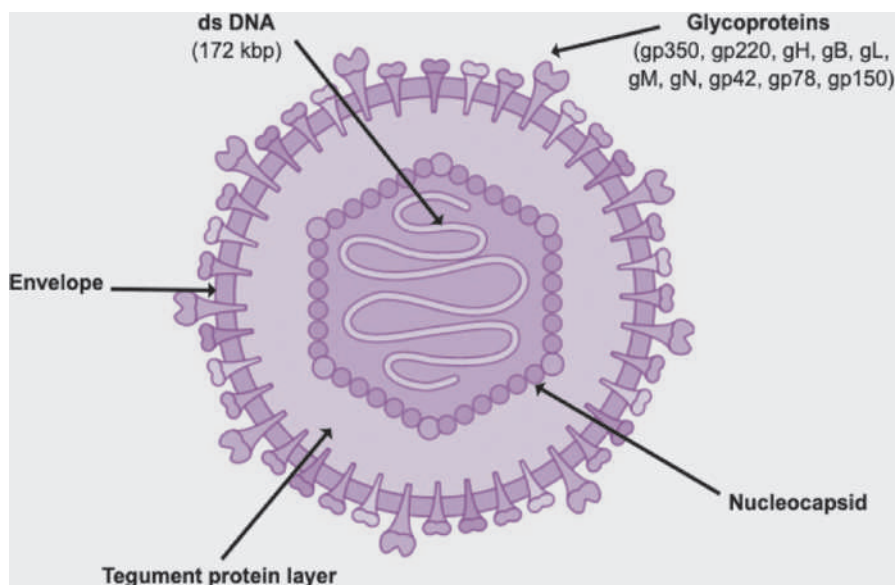


Fig. 1 Schematic diagram of Epstein-Barr virus. The major structural components of the virion are depicted. These include the envelope, which is studded with glycoproteins for binding and fusion of the virus envelope with the host cell, the tegument protein layer, the nucleocapsid, and the viral genome of double-stranded (ds) DNA.

Tropism

The major sites for EBV infection are human B lymphocytes and epithelial cells. EBV has the unique capacity to infect B cells by binding to CD21, also known as complement receptor 2 (CR2), by the EBV glycoproteins gp350 and gp220 (gp350/220) (Nemerow et al., 1989). Binding of gp350/220 triggers capping of CR2 and endocytosis of the virus. Binding of EBV to an epithelial cell is accomplished somewhat differently as CR2 is expressed at low levels on epithelial cells. EBV entry into epithelial cells occurs at the cell surface in the absence of endocytosis. Glycoproteins H and L (gHgL) are essential for viral attachment in coordination with gB to mediate fusion of the virus-cell membrane in epithelial cells (Borza et al., 2004; McShane and Longnecker, 2004; Xiao et al., 2008). Additionally, interactions between BMRF-2 β 1 family of integrins have been shown to be important for infection of polarized oropharyngeal epithelial cells with EBV (Tugizov et al., 2003).

Epidemiology

Incidence and prevalence of EBV infection

EBV is among the most common human viruses and the vast majority of the world's population has been infected by the time they reach adulthood (de-Thé et al., 1975). Seropositivity increases with age, and seropositivity appears to occur at younger ages in resource-limited countries (Piriou et al., 2012; Slyker et al., 2013; reviewed in Winter et al., 2020). The prevalence of EBV infection in children, however, varies from 10% to 90% depending on the child's age, race/ethnicity, and geographic location. Among children surveyed for EBV antibodies in the Minneapolis-St. Paul area, white non-Hispanic children did not reach 50% prevalence until age 18 whereas non-white children reached 50% prevalence by age 10 years (Condon et al., 2014). Data from the National Health and Nutrition Examination Surveys (NHANES) of US children aged 6–19 years old show the overall age-adjusted EBV antibody prevalence declined from 72% in 2003–04 to 65% in 2009–10 ($P = .027$) (Balfour et al., 2013a).

Seroprevalence of EBV has also been found to vary according to socioeconomic factors, as lower household income and education level were significantly associated with a higher prevalence of EBV antibody, that may be associated with crowdedness and lower sanitation (Balfour et al., 2013a; Smatti et al., 2018).

Incidence of primary EBV infection and infectious mononucleosis

Infectious mononucleosis most commonly affects those who have primary EBV infection during adolescence or adulthood. The overall incidence of infectious mononucleosis in the U.S. is about 500 cases per 100,000 persons per year, with the highest incidence among persons aged 15–24 years (reviewed in Luzuriaga and Sullivan, 2010). Interestingly, our prospective studies of students attending the University of Minnesota have shown the incidence of primary EBV infection during the four undergraduate years was 14.4 cases per 100 person-years. Among those with primary infection, nearly 78% (62/80) developed infectious mononucleosis (Balfour et al., 2013b; Grimm et al., 2016). Our findings are consistent to those reported by Crawford et al., who found a rate of 15.2 cases per 100 person-years of primary EBV infection among undergraduates at Edinburgh University, Scotland. However, only 24.5% (27/110) developed infectious mononucleosis (Crawford et al., 2006).

Routes of transmission

The mode of EBV acquisition varies by age and clinical characteristics. While EBV is usually spread by saliva, EBV may also be transmitted through blood, male and female genital secretions, and breastmilk. The routes of transmission are described in greater depth below and depicted graphically in Fig. 2.

Deep kissing

Among adolescents and young adults, EBV infection is acquired primarily by deep kissing, as shown by clinical observations and prospective studies of university undergraduate students, by which primary infection occurred through kissing in the absence of sexual intercourse (Balfour et al., 2013b; Grimm et al., 2016; Hoagland, 1952). EBV DNA in saliva and oral wash fluid has been detected at extremely high quantities relative to blood (median 4.8 $\log_{10}$ copies/mL) (Balfour et al., 2013b; Grimm et al., 2016). Additionally, detection of EBV DNA in the oral cavity has been shown to persist for a median of 5.2 months (Balfour et al., 2013b; Grimm et al., 2016).

Sexual intercourse

While saliva is the main route of transmission, EBV has been detected in male and female genital secretions in both infectious mononucleosis patients and healthy individuals, suggesting direct sexual spread may also contribute to transmission (Israele et al., 1991; Naher et al., 1992; Thomas et al., 2006). However, university students who engaged in deep kissing with or without penetrative sexual intercourse had the same risk of primary EBV infection compared to students who reported no kissing or sexual activity (Balfour et al., 2013b; Grimm et al., 2016).

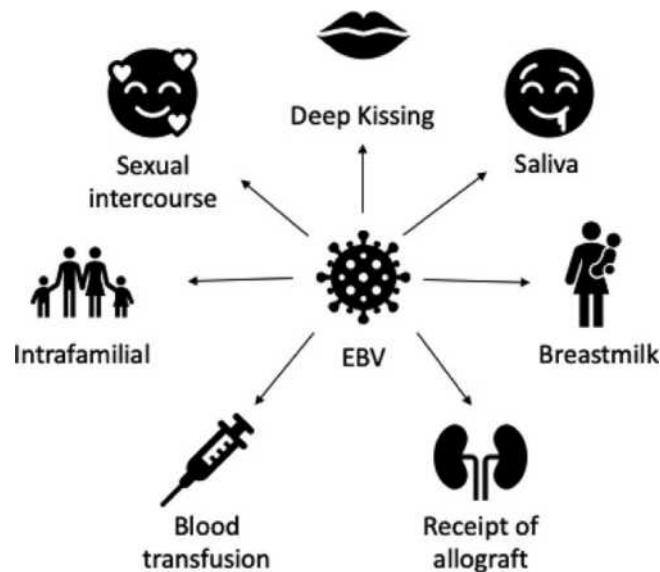


Fig. 2 Modes of EBV transmission. The major modes of transmission are depicted, the most common being through saliva and deep kissing.

Blood

During primary EBV infection, nearly 50% of individuals will have EBV viremia (median 2.60 log₁₀ copies/mL). Therefore, EBV may be transmissible by blood, although the rate is unknown. Transmission of EBV by direct blood transfusion has been reported and resulted in infectious mononucleosis (Trottier et al., 2012).

Solid organ transplantation

EBV infections, especially when transmitted from a seropositive donor to a seronegative recipient, remain a major cause of morbidity and mortality for transplant recipients. Diseases caused by EBV infection range from acute infectious mononucleosis to those as serious as post-transplant lymphoproliferative disorder (PTLD). Transmission has been documented in solid organ transplantation and hematopoietic cell transplantation by comparing sequence variation of EBV strains (Shapiro et al., 1988; Verghese et al., 2015).

Intrafamilial spread

As described previously, EBV infection occurs at younger ages among persons from lower versus higher socioeconomic backgrounds which has been attributed to crowded living conditions (Sumaya et al., 1975). This hypothesis suggests that young children are infected by their parents, siblings, or other caregivers who are healthy carriers of the virus and periodically shed EBV in their oral secretions. Transmission among family groups was demonstrated by Henle and Henle in the Cleveland Family Study, which found evidence of spread in approximately 33% of family groups after a child in the household tested positive for EBV antibodies (Henle and Henle, 1970). Another study examining oral shedding of EBV DNA demonstrated that 28% of parents (221/800) had EBV DNA in oral wash samples, and oral EBV DNA was more prevalent in parents who identified as non-white as compared with white parents, supporting the hypothesis that parents could be a source of virus for their young children (Cederberg et al., 2019).

Breastfeeding

EBV has been detected in breast milk and may be a source of transmission to infants. The prevalence of EBV DNA was reported to be highest at 6 weeks and decreased through 18 weeks postpartum (Daud et al. 2015). Of those with EBV DNA recovered, 60% were shown to be DNAase resistant, suggesting that the viral DNA in the breast milk was encapsidated and therefore infectious (Daud et al. 2015). Indeed, EBV infection in infants <6 months of age has been reported (Piriou et al., 2012). However, a study of Japanese infants indicated that the seroprevalence of babies who were breastfed or exclusively bottle-fed did not differ, suggesting that breast milk was not a source of EBV transmission (Kusuhara et al., 1997).

EBV diseases

Infectious mononucleosis

Establishing EBV as the cause of infectious mononucleosis

The clinical syndrome we now know as infectious mononucleosis was first described in the 1880s by Nil Filatov, a Russian pediatrician, and Emil Pfeiffer, a German pediatrician (reviewed in Balfour et al., 2020). The term “infectious mononucleosis” was

coined in 1920 by Sprunt and Evans, who recognized that this was an acute infectious disease accompanied by the laboratory finding of large numbers of circulating mononuclear cells (Sprunt and Evans, 1920). It was especially important to distinguish these atypical lymphocytes from leukemic cells (Downey and McKinlay, 1923; Sprunt and Evans, 1920), because the etiologic agent had not yet been discovered, so there was no specific diagnostic test for infectious mononucleosis. EBV was established as the major cause of infectious mononucleosis in 1967 when a technologist in the Henle laboratory who had donated blood as a control for experiments involving EBV, came down with the disease and developed antibodies against the virus (reviewed in Balfour et al., 2020; Henle et al., 1968).

Incubation period

The incubation period of infectious mononucleosis, based on careful history taking (Hoagland, 1952, 1964; Svedmyr et al., 1984) and our own prospective studies (Balfour et al., 2015), is 32–49 days. The viral and immunologic events during the incubation period will be described in the “Immune responses” section below.

Clinical features of the acute illness

Eighty undergraduate university students (ages 18–22 years) acquired a primary EBV infection during 2 prospective surveillance studies at the University of Minnesota (Balfour et al., 2013b; Grimm et al., 2016). These studies enabled us to characterize the entire clinical course of infectious mononucleosis in adolescents and young adults. Of the 80 primary EBV infections, 62 (77.5%) manifested as infectious mononucleosis (defined by having at least 2 of the following: sore throat, cervical lymphadenopathy, fever, fatigue), 8 (10%) were symptomatic but did not meet the criteria for infectious mononucleosis, and 10 (12.5%) were entirely asymptomatic. The clinical findings of the 70 participants who were symptomatic are summarized in Table 1. Pharyngitis can be quite severe, as illustrated in Fig. 3, which shows a patient’s sore throat on the tenth day of illness with an enlarged uvula kissing the left tonsil. The median duration of illness was 18 days (mean, 19 days). The only consistent laboratory abnormalities were atypical lymphocytosis and transiently elevated alanine aminotransferase, which was indicative of some degree of hepatocellular damage. Indeed, hepatic involvement is not a complication, but a regularly occurring finding in acute infectious mononucleosis (Hoagland and McCluskey, 1955).

The time course of EBV DNA in oral secretions and blood, and the EBV-specific antibody responses to the viral infection are shown in Fig. 4. EBV DNA is present in oral secretions several days before the host mounts an EBV viral capsid antigen (VCA) IgM antibody response. EBV VCA IgG antibodies become detectable on about the 10th day of illness and persist for life. In contrast, EBV VCA IgM antibodies disappear about 3 months after the onset of illness in most patients, but may linger for up to a year in some individuals. EBV nuclear antigen (EBNA) IgG antibodies are the slowest to appear, but once present persist for life in the majority of cases.

Complications of the acute illness

Complications may be due to tissue-invasive viral infection or immunologic dysfunction. Two of our female participants had complications. One developed acute pancreatitis and the other severe pancytopenia. Both participants recovered completely without sequelae. Table 2 lists the complications whose frequency is estimated to be at least 1%. Complications described in fewer than 1% of patients are: conjunctivitis, hemophagocytic syndrome, myocarditis, neurologic diseases other than meningoencephalitis, pancreatitis, parotitis, pericarditis, pneumonitis, and psychological disorders (Connelly and DeWitt, 1994; Jensen, 2000). Splenic rupture is a rare but feared complication (Hoagland and Henson, 1957), which may be avoided by keeping patients out of contact sports for 3 weeks after the onset of illness or until radiologic imaging shows the spleen to be of relatively normal size.

Table 1 Clinical findings of primary EBV infection in 70 prospectively followed symptomatic students.

Feature	No. positive	Proportion positive	Median duration (days) ^a
Sore throat	66	94%	7.5
Swollen or tender cervical lymphadenopathy	56	80%	15.0
Fatigue	50	71%	15.5
Upper respiratory symptoms(cough, runny nose, nasal stuffiness)	44	63%	8.0
Decreased appetite	37	53%	10.0
Headache	37	53%	8.0
Fever	33	47%	5.5
Body aches	32	46%	8.0
Abdominal pain	9	13%	16.0

^aMedian days for participants with the symptom.



Fig. 3 Marked pharyngitis on the 10th day of symptoms of infectious mononucleosis. The uvula is swollen and nearly touches the swollen right tonsil.

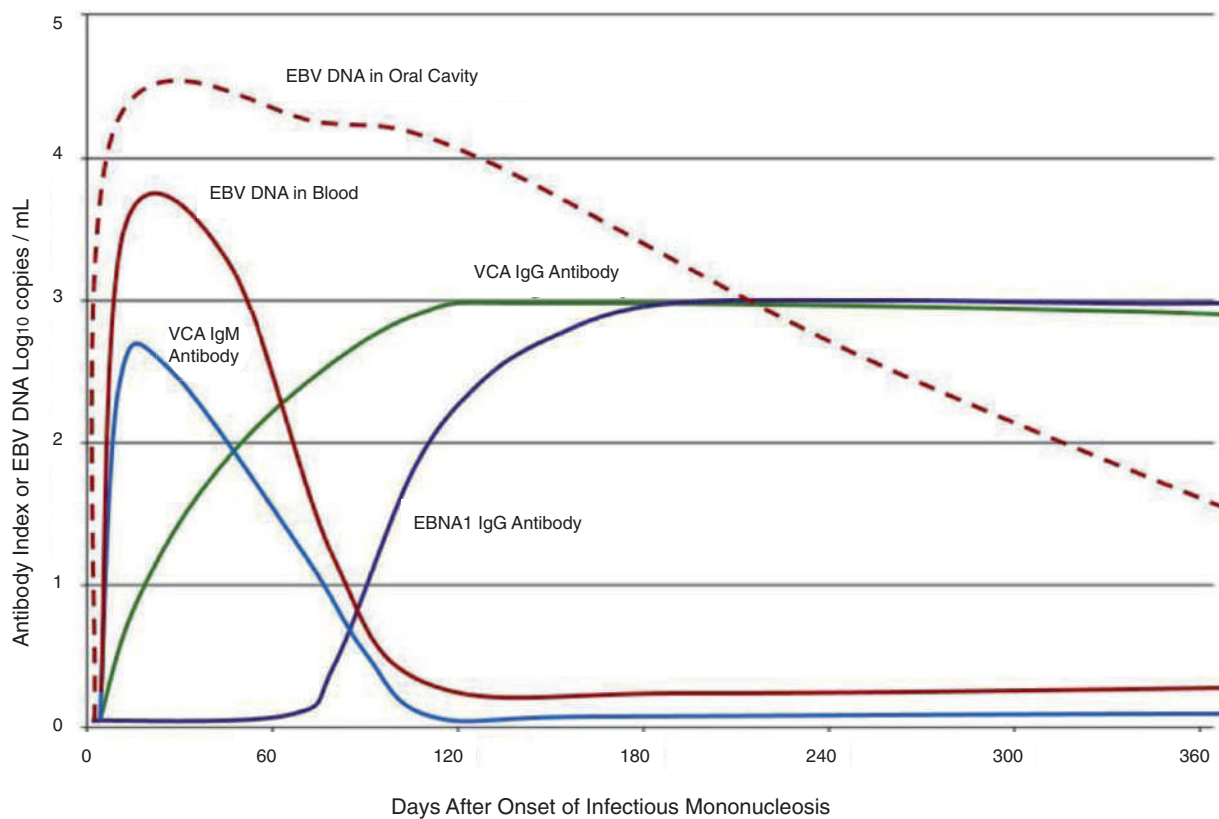


Fig. 4 EBV DNA is present in oral secretions several days before the host mounts an EBV viral capsid antigen (VCA) IgM antibody response. EBV VCA IgG antibodies become detectable on about the 10th day of illness and persist for life. In contrast, EBV VCA IgM antibodies disappear about 3 months after the onset of illness in most patients, but may linger for up to a year in some individuals. EBV nuclear antigen (EBNA) IgG antibodies are the slowest to appear, but once present persist for life in the majority of cases.

Table 2 Complications experienced by at least 1% of patients with infectious mononucleosis.

Complication	Comment
Upper airway obstruction	Due to oropharyngeal swelling especially if tonsils are present. May require anti-inflammatory medication
Meningoencephalitis	Can be fatal
Anemia, thrombocytopenia, and/or leukopenia	Transient; thought to be autoimmune
Maculopapular rash	Usually associated with administration of penicillin or its derivatives

Chronic infectious mononucleosis

A minority of persons who have infectious mononucleosis never recover completely. We have observed two patterns of this, which we call chronic infectious mononucleosis. The most common pattern is recovery from the initial disease, but recurrence of symptoms months to years later. The other pattern is continuation of symptoms that last indefinitely. Symptoms, in order of frequency, are: fatigue, weakness, joint pain, increased susceptibility to infections, diminished cognition, thyroid disorders, hypersomnia, and tender cervical lymphadenopathy. EBV DNA absent in the blood. Patients tend to have very high plasma or serum IgG antibody levels against EBV VCA; modestly elevated plasma or serum antibody levels against EBNA IgG, and the absence of circulating VCA IgM antibodies.

Chronic active EBV

A few patients who become infected with EBV never clear the viral infection (Kimura and Cohen, 2017). This entity is called chronic active EBV disease. Some develop fulminant infectious mononucleosis and die within days or weeks. Others develop a more chronic course with infectious mononucleosis-like symptoms such as fever, persistent lymphadenopathy, splenomegaly, and hepatitis. These patients are unable to control EBV infection and have infiltration of tissues by EBV positive T cells, natural killer (NK) cells, or B cells. The disease is characterized by continuous high levels of EBV in the blood. The only effective treatment is a hematopoietic cell transplant.

EBV and lymphomatous malignancies

EBV has been associated with a farrago of malignant diseases beginning with endemic Burkitt lymphoma. EBV appears necessary but not sufficient by itself to induce these conditions. Genetic, geographic, and environmental factors likely conspire with EBV to produce such malignant outcomes.

Endemic Burkitt lymphoma

EBV was discovered in 1964 by examining Burkitt lymphoma cells under an electron microscope (Epstein et al., 1964). Thus, EBV became the first recognized human cancer virus. Endemic Burkitt lymphoma is still a major cause of childhood cancer in East Africa. A study from Malawi reported that between 2011 and 2013, 74 (65%) of 114 lymphomas in children 2–16 years of age were Burkitt lymphoma (El-Mallawany et al., 2017). Endemic Burkitt lymphoma is a good target for preventive vaccine trials because of its gravity, its relatively short incubation period (as compared with nasopharyngeal carcinoma for example), and the existence of distinct geographic foci with a high incidence of Burkitt lymphoma (Rainey et al., 2007).

Hodgkin lymphoma

The link between infectious mononucleosis and Hodgkin lymphoma was suspected, based on clinical, hematological, and serological characteristics, years before EBV was discovered to be a key player in both (Kenis et al., 1958; Massey et al., 1953). EBV has been associated with ~40% of Hodgkin lymphomas as documented by finding EBV RNA or EBV protein in lymphoma cells using in situ hybridization or immunohistochemistry (Glaser et al., 1997). Infectious mononucleosis has clearly been established as a risk factor for Hodgkin lymphoma with a median time of 2.9 years from onset of infectious mononucleosis to lymphoma (Hjalgrim et al., 2003).

Other B-cell malignancies

Besides endemic Burkitt lymphoma and Hodgkin lymphoma, EBV is implicated in a number of other B-cell malignancies, which include diffuse large B-cell lymphoma, plasmablastic lymphoma, primary effusion lymphoma, and lymphomatoid granulomatosis (reviewed in Ok et al., 2015).

T cell and NK cell malignancies

EBV driven T-cell and NK cell lymphomas are not as common as EBV-spurred B-cell lymphomas. The entities associated with EBV are: extranodal NK/T-cell lymphoma, nasal type, aggressive NK-cell leukemia, peripheral T-cell lymphoma, not otherwise specified, and primary EBV-positive nodal T or NK cell lymphoma (reviewed in Montes-Mojarro et al., 2020).

EBV and epithelioid malignancies

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma has a unique geographical distribution and is a common cancer in China, especially Southern China, the Arctic, Southeast Asia, and Northern Africa (Petersson, 2015). Risk factors include race, family, male sex, and possibly diet (Petersson, 2015). The association of nasopharyngeal carcinoma with EBV was first appreciated by Old et al. who tested 352 patients and found that 64/94 (68%) of those with either nasopharyngeal carcinoma or Burkitt lymphoma had circulating antibodies against an antigen derived from Burkitt lymphoma cells as compared with 30/258 (12%) of persons who were healthy or had other diseases ($P < .0001$, Fisher's exact test, two sided) (Old et al., 1966). Harald zur Hausen et al. clinched the connection between EBV and nasopharyngeal carcinoma by demonstrating EBV DNA in biopsies from Burkitt lymphoma and nasopharyngeal carcinoma (zur Hausen et al., 1970). Nasopharyngeal carcinoma is characterized by relatively high levels of EBV-specific circulating IgA, which increase as the disease progresses (Henle and Henle, 1976; Wara et al., 1975). EBV DNA levels in the plasma or serum can also be used to monitor the clinical course (Lo et al., 1999).

Gastric cancer

Approximately 9% of gastric adenocarcinomas are EBV-positive (Camargo et al., 2014). The histology of EBV-positive gastric carcinoma has been described as very similar to that of undifferentiated nasopharyngeal carcinoma (Shibata et al., 1991). These authors found that EBV was present in epithelial cells but not in the reactive lymphocytes that were invading the tumor. Patients with this entity had a better prognosis than those whose gastric carcinoma was EBV-negative (Camargo et al., 2014).

EBV and immunodeficiency states

EBV is able to establish and maintain persistent infection in memory B lymphocytes (Thorley-Lawson and Gross, 2004). These cells predominantly reside in the germinal centers of lymph nodes, tonsils, and spleen. However, a small number circulate in the peripheral blood (Laichalk et al., 2002) and are capable of initiating an active viral infection under certain circumstances, especially when the host is subject to intrinsic or extrinsic immunosuppression.

Posttransplant lymphoproliferative disorder

Posttransplant lymphoproliferative disorder, a complication of solid organ and hematopoietic cell transplantation, consists of a spectrum of disorders from benign polyclonal B-cell proliferation to malignant lymphoma. EBV is the main pathogenic driver in most but not all cases. We have shown that high levels of EBV DNAemia are a risk factor for developing this condition (Holman et al., 2012). The clinical features include fever, weight loss, fatigue, malaise, and lymph node enlargement anywhere in the body. Extra-nodal involvement is also common (Dharmidharka, 2018).

EBV-positive mucocutaneous ulcer

This benign form of posttransplant lymphoproliferative disorder consists of well-circumscribed mucosal ulcers with patchy necrosis located on the lip, oral cavity or gastrointestinal tract (Hart et al., 2014). Ulcer biopsy specimens are strongly positive for RBV-encoded small RNAs by in situ hybridization, but EBV DNAemia is absent. Patients recover completely when immunosuppression is reduced.

AIDS

Patients with HIV/AIDS are prone to EBV-driven complications, such as oral hairy leukoplakia, smooth muscle tumors, mucosa-associated lymphoid tissue lesions, and a variety of lymphomas with a predilection for the central nervous system (McClain, 2001). Untreated AIDS patients have progressively worse immune function, largely due to depletion of CD4+ T cells. Improved antiretroviral therapies have reduced the incidence of AIDS-related lymphomas, which suggests that an intact immune system is required to keep EBV at bay (Besson et al., 2001).

Hemophagocytic lymphohistiocytosis

This disease, also called hemophagocytosis, has both a genetic and an acquired form (Rosado and Kim, 2013). The genetic form was first described by Farquhar and Claireaux (1952) and the acquired form by Risdall et al. (1979). A number of microbial pathogens have been considered to be the trigger, but EBV is the most prevalent culprit. The disease is characterized by severe hyperinflammation, with the prominent clinical findings being fever and splenomegaly accompanied by numerous laboratory abnormalities including decreased numbers of red cells, platelets, neutrophils, and NK cells, and elevation of triglycerides and ferritin. Hemophagocytosis is present in tissues especially the bone marrow, spleen and lymph nodes. The genetic defects associated with HLH have been summarized by Marsh (2018).

Hypersensitivity to mosquito bites

Hypersensitivity to mosquito bites is a rare but often fatal disease that occurs most frequently in Japanese children (reviewed by Asada, 2007). Patients have intense skin reactions at the sites of mosquito bites and also develop fever, lymphadenopathy and hepatosplenomegaly. The pathogenesis involves EBV infection of NK cells with attendant immune dysregulation.

Primary immunodeficiencies

Numerous primary immunodeficiencies have been associated with uncontrolled EBV proliferation and more certainly will be discovered (Cohen and Meyts, 2020). The best known of these is x-linked lymphoproliferative disease, also known as Duncan's syndrome, which was discovered in the 1970s by Purtilo et al. (1975) and has recently been reviewed (Panchal et al., 2018). Two others involve dysfunction of nuclear factor kappa-light-chain-enhancer of activated B cells (Arjunaraja et al., 2018; Hoeger et al., 2017). A patient with CD70 deficiency has been described who experienced periodic fever, tonsillitis, cervical lymphadenitis, and EBV viremia (Caorsi et al., 2017). Activated PI3K δ syndrome is a combined immunodeficiency resulting in recurrent sinopulmonary infections, lymphoproliferation, and susceptibility to herpesvirus infections especially EBV (Carpier and Lucas, 2017).

Autoimmune diseases

Multiple sclerosis

EBV has been considered a risk factor for a number of autoimmune diseases but the strongest association is EBV and multiple sclerosis (MS). There is convincing evidence that EBV is the major environmental risk factor for developing MS. First, practically all MS patients have been infected by EBV (Ascherio and Munch, 2000). Second, their EBV-specific antibody titers are elevated, especially against EBNA-1 (Banwell et al., 2007; Munger et al., 2011). Third, a history of infectious mononucleosis increases the risk of developing MS (Handel et al., 2010). Fourth, EBV-specific CD8+ T cell responses are elevated in active MS (Angelini et al., 2013). Fifth, EBV antigens that reflect active viral replication are present in the brain tissue of MS patients (Moreno et al., 2018; Serafini et al., 2013). Furthermore, EBV-specific adoptive T cell therapy has shown promise in modifying the severity of MS, which supports the concept that active EBV infection is responsible, at least in part, for disease progression (Pender et al., 2014, 2018).

Other autoimmune diseases

Using a unique genetic computational algorithm, Harley and colleagues recently provided compelling evidence that EBV is a risk factor especially for systemic lupus erythematosus, but also rheumatoid arthritis, inflammatory bowel disease, type 1 diabetes, juvenile idiopathic arthritis and celiac disease (Harley et al., 2018). The basis for this observation was that EBV nuclear antigen 2 shared risk loci with human transcription factors and cofactors for those above mentioned 7 autoimmune diseases.

Immune responses

Incubation period

During primary EBV infection, viral replication is first detected in the oral cavity and then moves to the blood during the incubation period (Balfour et al., 2013b). The incubation period of EBV is 42 days, on average (Balfour et al., 2013b; Grimm et al., 2016). During this time, starting about 2 weeks prior to symptom onset, a systemic type I interferon gene signature can be observed in peripheral blood mononuclear cells, waning by the time symptoms begin due to rapid CD8 T cell expansion (Dunmire et al., 2014, 2015).

Acute phase

During acute infectious mononucleosis, CD8 T cells undergo rapid expansion and comprise both EBV-specific and non-EBV-specific cells (Hislop et al., 2007). Tetramer-positive EBV specific CD8 T cells rapidly increase at the time of symptom onset and exhibit elevated levels of CD38+ for several weeks. CD4 T cells are not significantly affected but the cells are activated and respond to EBV MHC class II tetramers (Dunmire et al., 2015; Long et al., 2013). Meanwhile, as shown in Fig. 4, IgM antibodies to EBV VCA are produced and persist for 4–6 weeks. IgG antibodies against EBV VCA begin to be produced and can be detected during the first month of symptoms (Balfour et al., 2013b; Grimm et al., 2016).

Convalescent phase

T-cell lymphocytosis resolves during convalescence (3–6 months post-infection), in which there is a reduction in the number of T-cells with activation markers and the cytotoxic T-cell response is diminished. The production of antibodies against EBV VCA has been established and anti-EBNA IgG beginning to appear approximately 91 days post-symptom onset (Balfour et al., 2013b; Grimm et al., 2016).

Latency

Latency is the state of persistent viral infection without viral production and is a hallmark of herpesviruses. During latency, EBV persists primarily in memory B-cells and sometimes in epithelial cells and may be stimulated to reactivate EBV (Odumade et al., 2011). This causes virus to be produced that can reinfect new B cells and epithelial cells, becoming a source of viral transmission (described in the “Epidemiology” section above).

Diagnosis of infectious mononucleosis

Infectious mononucleosis due to EBV should be suspected in patients, especially young adults, presenting with at least two of the following findings: sore throat, cervical lymphadenopathy, fever, and fatigue. Clinical signs of pharyngitis with swelling of the tonsils and lymphadenopathy can also be indicative of infectious mononucleosis (Balfour et al., 2013b; Grimm et al., 2016).

A heterophile antibody test utilizing commercially available test kits is a relatively inexpensive and rapid diagnostic tool that supports the clinical diagnosis. Heterophile antibodies by definition are not specific. The presence of heterophile antibodies are detected by broad reactivity with non-human mammalian red blood cells (Balfour et al., 2015). While heterophile antibody tests are most commonly used to diagnose infectious mononucleosis, their results should be interpreted with caution due to their poor sensitivity and specificity. Additionally, heterophile antibodies can be detectable for a year or more and therefore are not always diagnostic of an acute EBV infection (Marshall-Andon and Heinz, 2017). However, if a patient presents with the clinical signs and symptoms of infectious mononucleosis, additional diagnostic procedures are not usually necessary to further confirm diagnosis.

Antibody testing for diagnosis of primary EBV infection

Enzyme immunoassays (EIAs) for EBV VCA IgM and VCA IgG in combination with EBNA IgG can be utilized for confirming the diagnosis of primary EBV infections (Table 3). Individuals who are negative for all three antibodies are considered EBV naïve and susceptible to a primary EBV infection. Everyone who experiences a primary EBV infection develops EBV IgG antibodies to VCA; however, it can take several weeks for them to become detectable depending on assay platform (Balfour et al., 2013b). Therefore, clinicians should order an EBV panel consisting of all three EBV-specific antibodies. A positive VCA IgM response or a positive VCA IgG response coupled with a negative EBNA response is indicative of a current or recent EBV infection (Balfour et al., 2015).

Diagnosis of EBV infection in immunocompromised patients

Monitoring of EBV infection in immunocompromised patients is important to identify complications; specifically, posttransplant lymphoproliferative disorder (PTLD), in patients who have recently undergone a transplantation. Due to an immunocompromised status, a patient might not produce detectable antibodies while experiencing an EBV infection. In this case, the best test for diagnosis and monitoring EBV infections in immunocompromised individuals is a blood viral load or quantitative EBV DNA assay performed using a polymerase chain reaction (PCR) platform (Holman et al., 2012).

Prevention of EBV infection

Prevention of primary EBV infection

Approximately 90% of individuals worldwide are infected with EBV with the majority of primary infections occurring in children as shown in prevalence investigations (Balfour et al., 2013a). Individuals can reduce their risk of infection by utilizing hygienic measures including hand washing and avoiding salivary contamination from infected individuals through intimate or other close contact.

Table 3 Staging EBV infection by enzyme immunoassay antibody results (Balfour et al., 2015).

Stage of infection	Time after onset of illness	VCA IgM	VCA IgG	EBNA-1 IgG
EBV Naïve	–	Negative	Negative	Negative
Acute Primary Infection	0–3 Weeks	Positive	Negative or Positive	Negative
Subacute Infection	4 Weeks–3 months	Positive	Positive	Negative
Convalescent Infection	4–6 Months	Negative or Positive	Positive	Negative or Positive
Past Infection	>6 Months	Negative	Positive	Positive

EBV vaccine

Due to the plethora of diseases associated with EBV, the development of prophylactic vaccine is a priority for researchers. A prophylactic EBV vaccine could potentially prevent infectious mononucleosis, EBV-associated cancers, and multiple sclerosis (MS). A vaccine could also potentially prevent severe illness or even death from EBV infection following transplantation, especially in pediatric patients who have not been exposed to the virus and have no immunity against it. Several prophylactic EBV vaccine candidates have been given to people, but none have been sufficiently studied to establish safety and efficacy for FDA approval (Balfour et al., 2020).

EBV vaccines have been in the works for decades, with the first phase 1 clinical trial published by researchers from Beijing China in 1995 (Gu et al., 1995). The vaccine contained the EBV membrane antigen gp220-340 as the immunogen because antibodies against EBV gp350 are closely related to neutralizing antibodies, which should prevent EBV from infecting B lymphocytes (Pearson et al., 1970). After efficacy was shown in a rabbit model, safety studies were conducted by vaccinating adult and children previously infected by EBV. Following confirmation of safety, the gp220-340 vaccine was further evaluated in EBV-naïve children to test antibody responses during 16 months of follow-up. Results of the prospective study indicated that three out of the nine vaccinees and all ten in the control group became infected with wild-type EBV. These results hint at efficacy; however, no follow-up work on the vaccine has been reported likely because it contains a live vaccinia vector, which has been shown to produce a high rate of adverse events following vaccination (Belongia and Naleway, 2003).

In 1999, researchers produced a recombinant subunit EBV gp350 candidate vaccine in Chinese hamster ovary cells that evoked gp350 and neutralizing antibodies in rabbits (Jackman et al., 1999). A vaccine containing this or a similar immunogen has been subject to multiple clinical trials. EBV-naïve and EBV-experienced young adults, 18–25 years old, were recruited to participate in a phase 1 study that examined the safety and immunogenicity of a three-dose vaccine series given via intramuscular injection (Moutschen et al., 2007). A phase 2 placebo controlled, double-blind randomized control trial was conducted in participants aged 16–25 years old, who were randomized to receive the gp350 recombinant vaccine with adjuvant system 04 (AS04). This study examined the safety, immunogenicity and efficiency of the vaccine. While the results indicated that the vaccine did not prevent primary EBV infection, it did appear that the vaccine had a significant effect on reducing clinical disease (Sokal et al., 2007). Lastly, this recombinant subunit EBV gp350 vaccine was tested in 16 pediatric kidney transplant recipients, a population with a very high risk of developing PTLD. While the study population was too small to provide any statistical evaluation metrics, it did demonstrate the safety of this vaccine in children receiving kidney transplants.

Another proposed EBV vaccine strategy has been to control the expansion of EBV-infected B-cells by generating CD8+ T cell immunity to EBNA5. A phase 1 trial conducted in EBV-naïve individuals showed that none of the vaccinated cohort developed infectious mononucleosis providing evidence that the vaccine might prevent symptomatic primary EBV infection. However, this study was conducted in a very limited cohort, with only nine vaccine recipients and 4 placebo recipients (Elliott et al., 2008).

The introduction of an FDA approved prophylactic EBV vaccine has the potential to reduce the burden associated with many acute and chronic EBV diseases. The National Cancer Institute has recommended more clinical trials be conducted to test the safety and efficacy of a prophylactic EBV vaccine to prevent infectious mononucleosis and associated cancers (Cohen et al. 2011). Further research and funding must be provided to advance the development of an EBV vaccine.

Treatment of EBV infection

Infectious mononucleosis

Nonspecific management

Antipyretics

Acetaminophen is generally prescribed rather than aspirin because of concern that aspirin might increase the risk of splenic hemorrhage (Auwaerter, 2004; Eichner, 1996; Luzuriaga and Sullivan, 2010). Fever usually subsides within a week but has been reported to persist for up to 3 weeks (Hoagland, 1960).

Analgesics

Pain control is important during the acute stage of infectious mononucleosis, especially for patients whose sore throat keeps them up at night. Management strategies include acetaminophen, nonsteroidal anti-inflammatory drugs, saltwater gargles, anesthetic throat lozenges, or viscous lidocaine hydrochloride (Auwaerter, 2004; Eichner, 1996; Luzuriaga and Sullivan, 2010). Codeine sulfate is appropriate for patients who do not respond to nonnarcotic pain control.

Fluids and nutrition

Attention to fluid intake is important, especially for febrile patients. Maintaining adequate nutrition is also important but can be challenging because many patients are anorexic during the first weeks of illness likely due to subclinical hepatitis.

Limitation of activities

Bed rest is unnecessary, but contact sports are contraindicated as mentioned above. Patients generally regulate daily activities to the level of their exercise tolerance.

Corticosteroids

Prescribing corticosteroids for infectious mononucleosis is a relatively common practice. However, an evidence-based literature review of seven studies concluded that there is insufficient evidence to recommend steroids for control of the symptoms of infectious mononucleosis (Candy and Hotopf, 2006). Most clinicians reserve corticosteroids for management of complications, such as impending airway obstruction, autoimmune anemia, and autoimmune thrombocytopenia (Auwaerter, 2004; Eichner, 1996; Luzuriaga and Sullivan, 2010; Putukian et al., 2008).

Specific antiviral therapy

Nucleosides are the only class of antiviral drugs evaluated for treatment of EBV infections in controlled clinical trials. These nucleosides are DNA polymerase inhibitors. They are inactive as given, but when anabolized intracellularly to the active nucleoside triphosphate form, they act as faulty substrates for viral DNA polymerase, disrupting or terminating synthesis of the DNA chain (reviewed in Balfour, 1999). The monophosphorylation step is accomplished more efficiently by virus-encoded enzymes than by host cell nucleoside kinases, thus enhancing the activity of these compounds in herpesvirus-infected cells as compared with uninfected cells (Elion et al., 1977). The enzyme responsible for monophosphorylation in EBV-infected cells is believed to be a virus-encoded protein kinase rather than a thymidine kinase (Gustafson et al., 1998; Meng et al., 2010). To date, no drug has been approved for treatment of EBV diseases by regulatory agencies.

Acyclovir

Intravenous and oral acyclovir have been studied for treatment of infectious mononucleosis (Andersson et al., 1986, 1987; Pagano et al., 1983; van der Horst et al., 1991). One trial evaluated oral acyclovir and prednisolone versus placebo (Tynell et al., 1996). These trials have uniformly shown a reduction of EBV in the oral compartment, but clinical efficacy was not demonstrated.

Valacyclovir

Valacyclovir is an oral prodrug of acyclovir with an oral bioavailability of at least 50%, compared with 10–20% for the parent compound (Weller et al., 2009). Hence, it has the potential to be more effective than acyclovir. We evaluated valacyclovir (3 g/day for 14 days) versus no antiviral drug in 20 University of Minnesota undergraduates with infectious mononucleosis due to laboratory-confirmed primary EBV infection (Balfour et al., 2007). There was a significant reduction in viral load in the oral compartment but not in the blood. The number of reported symptoms and the severity of illness were reduced significantly among the 10 valacyclovir recipients compared with the 10 control subjects. Criticisms of our study are the small number of subjects enrolled and the lack of a placebo. However, the results of this trial warrant further investigation because valacyclovir is very safe (Tyring et al., 2002) and relatively inexpensive now that generic formulations are available.

Ganciclovir and valganciclovir

Ganciclovir is active against EBV in vitro (Ballout et al., 2007; Lin et al., 1984; Romain et al., 2010), and ganciclovir or its oral prodrug, valganciclovir, has been used to prevent posttransplant EBV disease, as discussed below. However, there are no controlled trials to support a clinical benefit of ganciclovir for treatment of EBV diseases.

Tenofovir

Tenofovir and its congeners are nucleoside reverse transcriptase inhibitors approved for prevention and treatment of HIV/AIDS, and for the treatment of chronic hepatitis B infection. An elegant laboratory study has just shown that tenofovir is a potent inhibitor of EBV lytic DNA replication, which encourages investigation of these compounds for potential clinical use (Drosu et al., 2020).

Management of severe EBV disease

Potentially serious EBV disease in transplant patients is managed initially by a reduction of immunosuppression (Paya et al., 1999). If that is insufficient to bring the viral infection under control, humanized monoclonal anti-CD20 antibody (rituximab) may be administered, sometimes in conjunction with chemotherapy (Elstrom et al., 2006). In refractory cases, adoptive immunotherapy with primed CD8+T cells (Haque et al., 2007; Khanna et al., 1999) is occasionally successful.

Future challenges

Prospects for future prophylactic EBV vaccines

An EBV vaccine has the potential to greatly reduce the burden associated with primary EBV infections and associated illnesses. A gp350 subunit vaccine, similar to the one used in the phase 2 trial is currently being developed at the University of Minnesota in collaboration with an industrial partner. Additionally, researchers have also produced a subunit vaccine with a Newcastle disease virus (NDV) virus-like particle containing EBV gp350/220 fused to an NDV-fusion protein. This vaccine product has been shown to produce gp350 and neutralizing antibodies in mouse models (Ogembo et al., 2015). Lastly, the Cohen research group has also

constructed a candidate EBV vaccine that consists of an ectodomain of gp350 fused to ferritin or encapsulin. This vaccine product has been shown to product EBV neutralizing antibodies in monkey and mouse models (Kanekiyo et al., 2015).

With several gp350 based vaccines showing promise, priorities for future research include the development of a plan to determine vaccine efficacy for preventing EBV associated malignancies. Additionally, sufficient funding is required to move the EBV vaccine candidates from their current stage of preclinical research and development to a phase I clinical trial. It is essential that collaborations between academic, industry, and government organizations continue to be established and nurtured to accelerate EBV vaccine development.

Development of EBV-specific cell-mediated immunity using an interferon-gamma assay

In recent years, the roles of T-cell based immunodiagnostic assays have expanded to the diagnosis and risk identification for several infectious diseases including cytomegalovirus (CMV). CMV, a member of the herpesvirus family, is structurally similar to EBV with a relatively large linear double-stranded DNA genome and a highly ordered icosahedral-shaped nucleocapsid (Liu and Zhou, 2007). Researchers have developed a cell-mediated immunity monitoring protocol using an interferon-gamma assay for the CMV. Several studies have shown that the risk of CMV infection after organ and hematopoietic cell transplantation may be predicted by assessing CMV-specific cell-mediated immunity during pretransplant and posttransplant periods (Chemaly et al., 2020; Jarque et al., 2020). Similar laboratory techniques should be developed for EBV to monitor EBV cell-mediated immunity (EBV-CMI) responses during vaccine clinical trials. EBV-CMI may be an important index of protection against symptomatic EBV infections and in predicting the populations at highest risk for EBV-associated diseases and their prognosis (Kim, 2020).

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Cytomegalovirus

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Glossary

Antiviral therapy A medication used to treat viral infection in individuals at risk for severe disease. Usually antiviral agents specifically interfere with aspects of the viral, and not the host, life cycle.

Capsid The innermost, proteinaceous compartment of enveloped viruses such as CMV. It typically contains viral proteins important in virion assembly. The term “nucleocapsid” refers to the viral nucleic acid/capsid protein complexes containing viral genome.

Cytomegalovirus Literally, the word “cytomegaly” means “large cell.” Hence, cytomegalovirus infection of cells characteristically induces cell enlargement, often with accompanying intracellular inclusions.

DNA vaccine Vaccine strategy in which a recombinant DNA molecular encoding a viral gene product (protein) of interest is administered to an individual, toward the goal of inducing a protective immune response.

Envelope The outermost layer of the virion particle, consisting of a prototypical phospholipid bilayer which is studded with viral proteins, in particular viral glycoproteins that serve as ligands for host cell receptors (and are targets of host immune responses that important in convalescence and protection against infection).

HAART Therapy HAART is an acronym for “highly active anti-retroviral therapy.” This is any combination of HIV therapies that promotes control of HIV, reconstitution of cellular (particularly CD4+ cell) immunity, and resolution of HIV-associated opportunistic infections.

mRNA vaccine A novel vaccine strategy, first evaluated in human subjects in the context of the COVID-19 pandemic, in which a messenger RNA (mRNA) encoding a viral gene product (protein) of interest is administered to an individual, toward the goal of inducing a protective immune response.

Subunit vaccine A vaccine designed and manufactured to induce antibody responses against one or more particular proteins or constituents of a pathogenic virus. Subunit vaccines typically utilized cloning technologies to express immunological targets of interest in a variety of expression systems.

Tegument The amorphous, proteinaceous layer of a CMV virion lying between the envelope and the nucleocapsid. It typically contains viral proteins, including many phosphorylated proteins, that are important in viral replication.

Vector vaccine A vaccine approach in which an immunogen of interest (such as SARS-CoV-2 Spike protein) is inserted into another, attenuated viral vector. Immunization of the individual with the vector in turn induces an immune response the gene product of the pathogen, resulting in protective immunity against that virus.

Virion An intact and infectious viral particle, consisting of the envelope, tegument, and nucleocapsid.

Introduction

Long before viruses were discovered, the impact of CMV on human health was recognized by a German pathologist, Ribbert, who noted characteristic cytoplasmic inclusions (sometimes referred to as “Owl’s Eyes” inclusions) in an autopsy of a still born infant with congenital CMV infection in 1881 (Riley, 1997). Three groups independently isolated CMV in cell culture in the mid-1950s, and in 1960 Thomas Weller proposed the term “cytomegalovirus” to describe this agent. The identification of the agent in the urine of infants with generalized disease confirmed the importance of CMV as a congenital pathogen. Subsequent studies revealed that CMV is a ubiquitous agent with diverse clinical manifestations in all age groups (Weller, 1971). As new knowledge about CMV became generated, and as advances in medical care produced the advent of SOT and HSCT, the role of CMV in patients with altered host immune defense became recognized (Steininger, 2007). With the advent of the AIDS pandemic, the impact of CMV on patients with HIV infection also emerged as a major public health issue. CMV has been recognized as the most common viral opportunistic

infection in persons with AIDS, historically producing clinical disease in up to 40% of patients with advanced HIV infection (Chung and Teich, 1999; Fulkerson et al., 2021). Taxonomically, CMV is a member of the *Herpesviridae* family of viruses (Schleiss, 2009). It is considered to be a member of the betaherpesvirus subfamily, which also includes human herpesviruses 6A and 6B (HHV-6A, HHV-6B) and human herpesvirus 7 (HHV-7). CMV continues to stand as a major viral pathogen that compromises human health in diverse clinical settings. The virology, epidemiology, and clinical manifestations of CMV are reviewed below. Prospects for disease control offered by advances in antiviral therapy, as well as potential vaccines currently in clinical trials, are discussed.

Basic biology of CMV

As noted, CMV is a member of the *Herpesviridae* family of viruses. A common feature of these viruses is that they establish lifelong, latent infection in the human host. Episodic reactivations occur during the life-course of the infected individual. Immune suppression, whether it is conferred iatrogenically by the use of chemotherapy and/or immunomodulators in solid organ transplant (SOT) or hematopoietic stem cell transplant (HSCT) patients, or acquired through HIV infection, can lead to reactivation of latent CMV, which may cause symptomatic disease. Interesting, SARS-CoV-2 infection may be a risk factor for reactivation of CMV (Moniz et al., 2021).

CMV, similar to other members of the *Herpesviridae*, has a large, double-stranded DNA genome. CMV has the largest coding content of any Herpesvirus, and the genome encompasses > 230 kbp (Martí-Carreras and Maes, 2019). The CMV genome contains a unique long (UL) and a unique short (US) segment, and each segment is bracketed by terminal repeat sequences, allowing the formation of genome isomers that in turn promotes strain variation among clinical isolates. At least 700 open reading frames, and a number of non-coding RNAs, are transcribed by the CMV genome (Adamson and Nevels, 2020). Many CMV gene products promote evasion of the immune response (Boeckh and Geballe, 2011). As a result, naturally acquired immunity after infection does not confer lifelong protection against re-infection (Britt, 2017), an observation that greatly complicates CMV vaccine design.

The CMV replication cycle utilizes a coordinated, sequential expression of three families of viral transcripts: the so-called immediate early (IE), early, and late genes. IE mRNA is transcribed concomitantly with the initiation of infection, and the encoded IE proteins regulate expression of both host and viral genes. The IE promoter is also of interest, since it is one of the most potent promoters encountered in eucaryotic cell biology (Stinski and Isomura, 2008), and it has been employed in many commercial settings (such as in construction of recombinant plasmids and cell culture expression systems) that require robust transcription. CMV early gene products include DNA replication proteins and structural proteins; this is the stage of the viral life cycle that has been targeted by most licensed antiviral therapies (reviewed below). Finally, the late mRNAs are typically transcribed at more advanced time points post-infection (~72 h) and these gene products include structural proteins and other proteins involved in virion assembly and egress.

The CMV virus particle consists of three components: the capsid; the tegument layer; and the outer lipid bilayer envelope (Fig. 1). The capsid consists of 162 capsomere protein subunits that are oriented in icosahedral symmetry. The viral genomic DNA is surrounded and enveloped by the capsid proteins (the DNA/protein complexes are hence referred to as the nucleocapsid). The nucleocapsid is surrounded by the tegument layer, which contains important structural proteins, including phosphoproteins important in viral pathogenesis and immunity. One such protein, a 65-kilodalton (kDa) phosphoprotein referred to as pp65, is an important target of host cell-mediated immunity, and is a subunit vaccine candidate currently in clinical trials (Schleiss et al., 2017). The envelope surrounds the tegument. It is a typical phospholipid bilayer, as is characteristically found in other enveloped viruses. There are several virally-encoded glycoproteins, including the gB complex, the gM/gN complex, the gH/gL/gO trimer complex, and a pentameric complex consisting of proteins gH/gL/UL128, 130, and 131. These proteins are important in receptor-ligand interactions that mediate binding and entry of CMV into host cells. The proteins also elicit the virus-neutralizing antibodies that are found in CMV-seropositive individuals, and hence are vaccine candidates (discussed below).

Epidemiology of CMV transmission

CMV is transmitted by body fluids (blood, urine, breast milk, saliva, and genitourinary tract secretions) and acquisition of infection typically requires intimate contact. Modes of transmission include sexual transmission, breast-feeding, blood transfusion, and intimate household contact with secretions. CMV infection can also be acquired via solid organ transplantation. Health care providers do not appear to be at enhanced risk for acquisition of CMV (Dworsky et al., 1983). Although most individuals are eventually infected during the life-course, there is considerable variability in the age-specific seroprevalence from population to population globally. Race, ethnicity, and socio-economic factors impact age-specific seroprevalence. In the US, the overall age-adjusted seroprevalence of CMV is 50.4% (assessed for ages 6–49 years), with higher rates noted in non-Hispanic black and Mexican-American children, compared with non-Hispanic white children (Bate et al., 2010). CMV seropositivity in this study was associated with older age, being female, foreign birthplace, low household income, higher rates of household crowding, and low household education level. For children under five in the US, in whom infection is often driven by toddler-to-toddler transmission among attendees in group childcare, the seroprevalence is approximately one-in-three, (Lanzieri et al., 2021). Globally, a CMV seroprevalence rate of approximately 80% has been estimated (Zuhair et al., 2019). This study identified that the highest rates of seropositivity were observed in the developing world, particularly in the Eastern Mediterranean region, whereas lower rates (~65%)

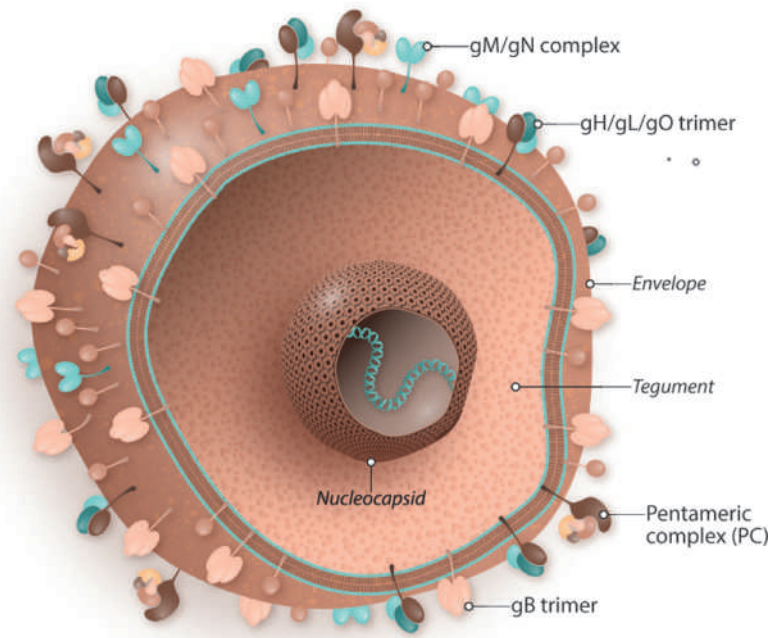


Fig. 1 HCMV virion demonstrating key envelope glycoproteins and glycoprotein complexes important in protective immunity that stand as potential subunit vaccine candidates. Viral envelope, tegument, and nucleocapsid proteins are indicated. Glycoprotein complexes are labeled; see text for additional details.

were noted in Europe. Peaks in CMV transmission and acquisition of infection are noted in young children, particularly in association with group day care attendance, and in adolescence, associated with sexual transmission. Infants often acquire CMV infection, usually asymptotically, from breast-feeding, since CMV seropositive mothers will typically shed virus in breast milk (Schleiss, 2006).

As noted above, the CMV genome has, during the course of co-evolution with the human host, transduced a number of immune modulation and immune evasion genes from the host genome (Boeckh and Geballe, 2011). These gene products interfere with the establishment of permanent immunity following infection. Thus, re-infections with new strains of CMV can occur. Past infection does not protect against maternal re-infection during pregnancy, and, counter-intuitively, the majority of cases of congenital CMV transmission globally appear to occur in the setting of re-infection of women with preconception immunity (Britt, 2017). Primary CMV infection during pregnancy is more likely to lead to transmission to the fetus than maternal reinfection, but fetal transmission with long-term sequelae can occur by either mechanism (Permar et al., 2018). The overall prevalence of congenital CMV transmission in the US is 0.65% (Kenneson and Cannon, 2007).

Clinical manifestations of CMV infection

Most CMV infections are not clinically recognized in immunocompetent individuals. Occasionally, CMV can cause a mononucleosis syndrome clinically indistinguishable from infection with Epstein-Barr virus (Taylor, 2003). In SOT and HSCT patients, CMV can cause a variety of clinical syndromes, including a viral sepsis syndrome characterized by viremia and constitutional symptoms; pneumonia; enteritis; hepatitis; and central nervous system pathologies including encephalitis and retinitis. A risk for CMV disease following SOT can be observed both as a consequence of re-activation of latent infection in the transplant recipient, as well as acquisition of CMV from the transplanted organ itself. In HSCT patients, the greatest risk for CMV disease is in the seropositive transplant recipient. The advent of effective anti-CMV antivirals (reviewed below) has decreased the risk of serious CMV disease in the transplant setting. In addition, highly active anti-retroviral therapy (HAART) against HIV has, by reconstituting cellular immunity, had a major impact on opportunistic CMV infections in HIV-positive patients. However, in regions of the world where HAART therapy is less available, active CMV infection is an important risk factor for adverse outcomes related to other opportunistic infections, including *Cryptococcus neoformans* (Skipper et al., 2020). In the pre-HAART era, CMV was a significant cause of sight-threatening retinal infection (CMV retinitis) in patients with HIV infection (Munro et al., 2019).

In infants with congenital CMV, a wide range of signs and symptoms may be encountered, ranging from completely asymptomatic infection to severe, multi-organ system disease (also known as cytomegalic inclusion disease or CID). The likelihood of symptomatic disease depends both on whether the maternal CMV infection is a primary or recurrent infection (Permar et al., 2018) and on the timing of fetal infection, since most sequelae from congenital CMV occur in the context of first-trimester fetal infections (Chatzakis et al., 2020). Signs and symptoms of symptomatic congenital CMV infection are noted in Table 1. At birth, babies with

Table 1 Clinical manifestations of congenital cytomegalovirus infection.

<i>Clinical manifestations</i>	<i>Potential laboratory and imaging findings</i>
• Small for gestational age • Premature birth • Neurological findings ◦ Seizures ◦ Hypotonia ◦ Microcephaly ◦ Neuronal migration defects ◦ Hearing loss/failed newborn hearing screen ◦ Retinal disease (chorioretinitis) • Hepatomegaly • Splenomegaly • Petechiae • Purpura	• Hematologic abnormalities ◦ Low platelet count ◦ Hemolysis ◦ Low white blood cell count • Hepatic abnormalities ◦ Transaminase elevation (hepatitis) ◦ Hyperbilirubinemia • Cerebral-spinal fluid abnormalities • Brain imaging abnormalities ◦ Enlarged ventricles ◦ Calcifications surrounding the ventricles ◦ Neuronal migration defects • Audiological abnormalities ◦ Brainstem auditory evoked response (ABR) ◦ Otoacoustic emissions (OAE)

CID may manifest with neurological signs and symptoms, rashes (including petechiae and purpura), neurological abnormalities, enlargement of the liver (hepatomegaly) and/or spleen (splenomegaly), and jaundice. Laboratory abnormalities can include low platelet count, elevated bilirubin, and liver function test elevation. The most important long-term sequela of congenital CMV is sensorineural hearing loss. Importantly, this may occur even in otherwise-asymptomatic infants (Rawlinson et al., 2017), and infants with congenital CMV may go on to have hearing loss even if the newborn hearing screen is passed in the nursery at birth. This fact provides impetus for universal newborn screening programs for identification of congenital CMV, even in asymptomatic newborns (Dollard et al., 2021) who could benefit from serial audiological monitoring in infancy and early childhood, as well as neurodevelopmental assessments.

Another patient group at risk for CMV disease is the extremely low birthweight (ELBW) population of premature infants (Osterholm and Schleiss, 2020). Although such infections were formerly acquired from blood transfusions, improvements in leukofiltration in blood from blood banks has made this rare. Almost all such infections are acquired from breast milk. Short-term manifestations include pneumonia, thrombocytopenia, and CMV sepsis syndrome. Whether such infections lead to any long-term disabilities, such as sensorineural hearing loss, requires additional investigation.

CMV diagnostics

For decades, the mainstay of CMV diagnosis was identification of the virus in cell culture. However, culture is laborious and time-consuming, and results were seldom available in a rapid enough turn-around time to impact clinical decision-making. Today, molecular diagnosis through PCR is the preferred approach for identification of virus (Ross et al., 2011). Appropriate substrates for PCR amplification include whole blood, plasma, urine, saliva, and bronchoalveolar lavage fluid. Serologies are also available to evaluate patients for the presence or absence of CMV antibodies. Since the seroprevalence of CMV is very high in most populations, serological assays are seldom of value in the setting of acute CMV disease, where demonstration of virus is preferable. However, serological strategies are useful in guiding and counseling young women of child-bearing age. Serological screening during pregnancy is gaining acceptance (Seror et al., 2021), and may help reduce maternal-fetal transmission of CMV, particularly in CMV seronegative women who can be counseled about exposure sources to avoid during pregnancy (Schaefer et al., 2020). Serological screening is also important for donor/recipient pairs, both for living-related SOT and for HSCT patients, in order to gauge the risk of CMV infection post-transplantation and to engage in appropriate transplant planning.

Antiviral treatments targeting CMV

A number of licensed antivirals are available for treatment of CMV infection (Kotton, 2019). Most of these agents target viral DNA replication (ganciclovir, foscarnet, cidofovir). An oral prodrug of ganciclovir, valganciclovir, is also licensed and available. A recently licensed agent, letermovir, targets a later stage in the viral life-cycle, namely, the cleavage of concatemeric end-to-end viral genomes (generated during DNA replication) into individual unit length genomes for packaging into virions. Antiviral therapy for CMV is reserved for a few clinical scenarios. In the SOT and HSCT setting, antiviral agents are commonly used either as prophylaxis in the post-transplant window, toward the goal of preventing CMV disease, or as a “pre-emptive” therapy, with initiation of treatment triggered (typically) by a positive PCR test on plasma identified during routine post-transplantation laboratory surveillance studies.

For HSCT and SOT patients with CMV disease (pneumonitis, colitis, viral sepsis syndrome), antivirals are also indicated. Antiviral therapy for CMV is also indicated in HIV patients with retinitis and serious end-organ disease (pneumonitis, colitis). For congenital infection, treatment is recommended for infants with symptoms at birth, and consists of 6 months of oral valganciclovir (Rawlinson et al., 2017).

Administration of a polyclonal, high-titer CMV antibody derived from CMV-seropositive donors, Cytogam®, has been used for decades to help ameliorate the severity of CMV disease in SOT, HSCT, and ELBW premature infants. The use of this and other antibodies has been advocated in pregnant women who have evidence of primary CMV infection, toward the goal of preventing fetal transmission. However, a recent randomized controlled trial in pregnant subjects failed to demonstrate efficacy of this intervention in preventing maternal-fetal transmission (Hughes et al., 2021).

CMV vaccines

In principle, a CMV vaccine could be deployed in any of a number of settings: a universal vaccine administered in early childhood; a vaccine targeting young women of child-bearing age (toward the goal of preventing congenital transmission); or a vaccine for patients anticipating SOT or HSCT (aiming at the goal of reducing the risk of CMV disease under the immunosuppressive conditions of transplantation). These potential indications are not mutually exclusive, although the correlates of protective immunity may be different for a HSCT or SOT patient than those for a pregnant woman.

CMV vaccines that have been tested in clinical trials include live, attenuated vaccines; subunit vaccines based on recombinant proteins shown to be important in protective immunity (Fig. 1); and subunit vaccines in which the gene products of interest are expressed either in the context of DNA or RNA vaccination (Anderholm et al., 2016). So-called “vectored” vaccines, in which the immunogen is expressed in the context of a recombinant vector (such as adenovirus or vesicular stomatitis virus), are also under evaluation. Such a “vectored” vaccine approach has been successful in the development of the Ad26.CoV2.S vaccine, in which the COVID Spike protein was expressed via an adenovirus 26 vector (Corchado-Garcia et al., 2021). The Institute of Medicine has identified a CMV vaccine targeting prevention of congenital CMV infection as a “Level 1” priority for new vaccine development. Given that congenital CMV is the single most common infectious disease producing disability in newborns, acceleration of vaccine development is a high priority.

Research priorities and future directions

Much remains to be learned about the biology of CMV as well as its impact on human health. CMV has been linked to human malignancies, such as glioblastoma multiforme (Söderberg-Nauclér, 2006), and although such associations remain unproven, further studies are warranted. CMV has also been implicated in immunosenescence. Thus, the benefits of a putative CMV vaccine may extend far beyond women of reproductive age or SOT/HSCT transplant recipients. More knowledge is needed about the correlates of protective immunity, and this in turn can help drive novel vaccine and immunotherapy strategies. For women of reproductive age and care providers, greater knowledge and awareness is needed about the impact of congenital CMV on neurodevelopment and hearing. Legislative efforts to implement newborn screening are underway in many states in the US (Schleiss, 2018) and these efforts can help promote early intervention and therapies for infants, toward the goal of improving child health.

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Adenoviruses

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Adenoviruses in the context of clinical, applied and basic sciences

Adenoviruses (AdVs) were first isolated from human adenoid tissues in 1953 (Rowe et al., 1953). They were soon thereafter recognized as ubiquitous infectious agents implicated in diseases such as acute respiratory infections, keratoconjunctivitis and gastroenteritis (Ghebremedhin, 2014). Human AdVs (HAdVs) comprise seven species (A-G) and species B, C, D and E are endemic in the general population and cause about 5–7% of acute respiratory diseases in infants. However, these viruses can cause more severe health problems, like pneumonia or even death in immunocompromised patients, and recently severe outbreaks have been reported in Asia, Europe and in the United States (Coleman et al., 2020; Mennechet et al., 2019; Kajon et al., 2019). Adenoviruses are considered DNA tumor viruses. Although HAdV have not been shown to cause cancer in humans, the HAdV type 12 (HAdV12) was shown to induce malignant tumors in laboratory rodents making HAdVs a prominent model of tumor virology (Trentin et al., 1962). Additionally, HAdVD types 36 and 37 have been recently proposed to be etiological factors of obesity, since they have been associated with the altered differentiation and proliferation of pre-adipocytes (Esposito et al., 2012). Although there is an effective vaccine against HAdVB7 and HAdVE4, this vaccine is restricted to US military recruits and not available to the general public (Gray, 2020).

Their ability to infect multiple cellular types (broad tropism), including post-mitotic cells, the relative ease with which their genome can be manipulated and the possibility to generate high viral titers, have made AdVs one of the most commonly used systems for the design and development of candidate vectors for gene therapy and combined anti-cancer therapies (Wold and Toth, 2013; Zhang et al., 2018). Moreover, since adenoviruses induce an effective innate and adaptive immune response, both cellular and humoral, AdVs are also used for vaccine development, the recent SARS-CoV2 and Ebola virus vaccines being two notable examples (Morris et al., 2016; Appaiahgari and Vrati, 2015; Tatsis and Ertl, 2004; Coughlan, 2020; Bliss et al., 2020).

AdVs replication in infected cells induce extensive reorganization of the intranuclear milieu, cause alterations in the host-cell gene expression program, inhibit cellular DNA replication, alter cellular metabolism and can induce cell transformation (Hodge and Scharff, 1969; Beltz and Flint, 1979; Dolph et al., 1988; Huang and Schneider, 1991; Berk, 2005). Hence, adenoviruses have been used as a model system to understand eukaryotic cellular processes, such as transcription, post-transcriptional processing, nuclear organization, cell cycle control and cell death (Bridge and Pettersson, 1995; Miller et al., 2007; Challberg and Kelly, 1979).

Taxonomy

Adenoviruses belong to the *Adenoviridae* family, which comprises more than 130 different types divided in five genera (Fields et al., 2007). The *Mastadenovirus* and *Aviadenovirus* include the types that infect mammals and fowl, respectively. The *Atadenovirus* and *Siadenovirus*, include viruses that can infect a wider spectrum of animal species; the *Atadenovirus* were named for the high content of A + T in their genomes and infect mainly reptiles, fowl and ruminants; the *Siadenovirus* were named because their genome carries a gene that encodes a sialidase and encompass viruses that can infect birds and frogs. The fifth genus, *Ichtadenovirus*, include viruses that infect fish (Davison et al., 2003). Although not yet approved, the genus *Testadenovirus* has been proposed, which includes AdVs that infect testudinoid turtles (Doszpoly et al., 2013).

Within each genus AdVs are also classified into subgroups named according to the host organism they infect and the letters of the alphabet. Human adenoviruses belong to the genus *Mastadenovirus* and are further classified into subgroups or species (A-G) on the

basis of several characteristics such as DNA sequence homology, hemagglutination properties, and oncogenicity in immunosuppressed experimental animals (Harrach et al., 2019). There are more than 100 adenovirus types assigned (Brister et al., 2019), with HAdVC2 and HAdVC5 being the prototypes, as they have been most extensively studied and most commonly used in gene therapy and other applications due to their non-oncogenic properties (Shenk, 2001).

Structure

Adenoviruses form non-enveloped particles characterized by an icosahedral capsid that contains a linear double-stranded DNA genome (dsDNA) of 26–45 kb in length. The human adenovirus species C type 5 (HAdVC5) capsid has a diameter of close to 90 nm and is composed of 11 structural proteins (Fig. 1). The protein II, commonly known as hexon, is organized in 240 trimers forming the most abundant structure of the capsid. The protein III constitutes the penton base, which is associated to protein IV, which forms the fiber spikes that protrude from the surface of each of the 12 vertices of the icosahedral particle. Associated to the inner surface of the capsid are the minor proteins IX, IIIa, VI and VIII. The underlying core is formed by proteins V, VII, Mu and the Terminal Protein (TP). Except for TP, the latter have a high content of Arginine residues and interact with the negative charges of the viral genome. The viral DNA is organized surrounding protein VII, which is the most abundant protein of the viral core (Fields et al., 2007). The TP is covalently bound to the 5' ends of the viral DNA. Additionally, approximately 10 copies of the L3 viral protease 23 K are located inside the viral particles, which are essential for the infectious cycle: the protease is required for maturation of the viral progeny by cleaving the precursors (p) pIIIa, pVI, pVIII, pVII, pMu and pTP; the viral protease also promotes the disassembly and release of the viral particle from the endosome during entry into the infected-cell (Perez-Berna et al., 2009). Moreover, few copies of IVa2, a multifunctional protein that participates both in viral DNA packaging and as a transcription factor, are present in the viral particle (Christensen et al., 2008).

Viral genome organization

The terminal sequences of the genome are inverted-terminal repeats (ITR), where the origins of replication are localized. By convention, the adenovirus genome is represented beginning with the E1A transcription unit (TU) on the left side. The E1A, E1B, IX, ML, VA RNA and E3 TU are encoded on the strand that is read from left to right, while the E4, E2 and IVa2 TU are encoded on the complementary strand and are read from right to left.

The viral genome is organized in four groups of TU that are chronologically regulated (Fig. 2). The early TU are E1A, E1B, E2, E3 and E4, while IVa2 and IX are early-delayed TU. Expression of the latter depends on initiation of viral DNA replication, as it does for the late TU, which is named Major Late (MLTU). These TU are all transcribed by the cellular RNA polymerase II (RNAPII). In addition, two viral TU, VA I and II, are transcribed by the cellular RNAPIII, which produce two small transcripts named virus-associated RNA (VA RNA I and II). Although descriptive, classification of early and late genes is not completely accurate, as early genes continue to be expressed during the late phase and the viral promoter that controls the expression of all late genes, the Major Late Promoter (MLP), is active during the early phase of infection (Bridge and Pettersson, 1995; Fessler and Young, 1998; Shaw and Ziff, 1980).

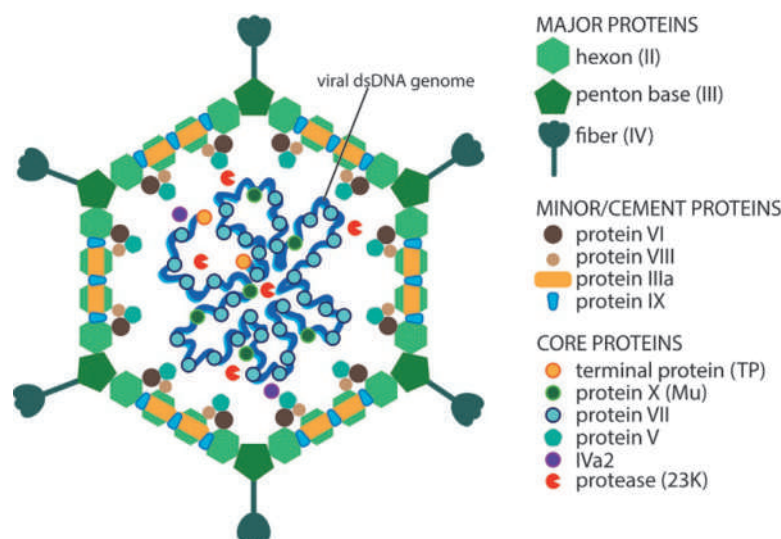


Fig. 1 Structure of a human adenovirus. Schematic representation of major and minor virion proteins within the viral particle, as well as core proteins bound to the viral dsDNA genome in human adenoviruses.

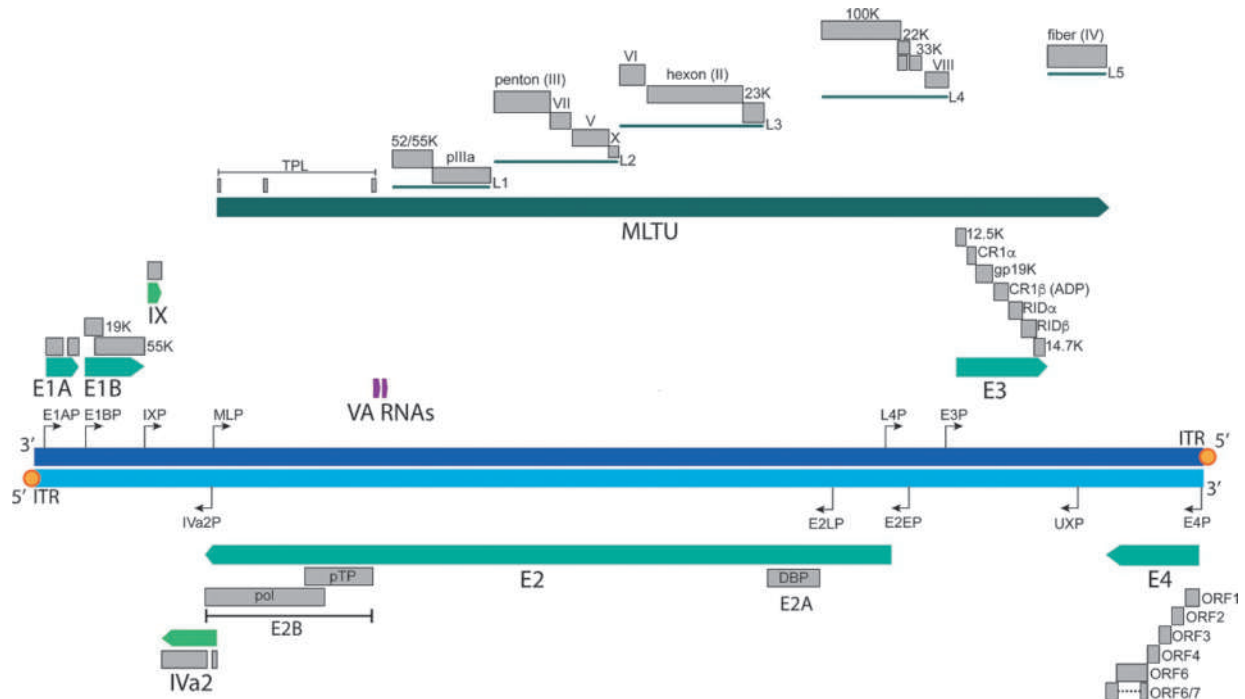


Fig. 2 Diagram of the adenovirus genome. Representation of the adenovirus dsDNA (blue bars) with the E2B-TP protein attached to the 5' ends (orange circles). The viral early (E1-E4), intermediate early (IX and IVa2) and the Major Late (ML) transcription units (TU) are depicted in different shades of green and represented by arrows. The thin dark green arrows represent the L1-L5 viral late mRNA families, which are defined by different poly (A) sites. VA RNAs, transcribed by the cellular RNA polymerase III are depicted in purple. Light gray boxes represent the coding sequences of the tripartite leader (TPL) and early and late mRNAs. The black arrows on the viral genome indicate the viral promoters.

Viral gene products

The proteins encoded in E1A are named according to the sedimentation coefficient of their encoding mRNA (e.g., 12S and 13S), and are transcriptional activators and regulators of cell cycle control mechanisms. E1A proteins interact with pRB proteins inducing the release of the cellular factor E2F, which results in the expression of cellular genes necessary to enter the S phase of the cell cycle. E1A proteins also regulate the infected-cell by regulating transcription factors that control the cell cycle and chromatin remodeling factors such as the histone acetyltransferases (HAT) CBP/p300 and PCAF, the SWI/SNF family member p400, and the histone deacetylase complex HDAC. Hence, E1A products activate transcription of cellular and viral genes and induce the infected-cell to enter S-phase to establish an optimal environment for viral DNA replication and expression.

The E1B and E3 proteins are named according to their molecular mass, e.g., E1B 55K and E3 14.5K. The proteins encoded in these TU modulate a wide variety of antiviral responses that inhibit cellular defense mechanisms, or prevent premature recognition and elimination of the infected cell by the immune system of the host organism, respectively (Fields et al., 2007; Gooding et al., 1990).

The E2 products participate directly in viral DNA replication and are named according to their function: the viral DNA polymerase (pol), the pre-terminal protein (pTP) that works as a primer, and the ssDNA-binding protein (DBP) (Hoeben and Uil, 2013). The E2 TU can be transcribed from two alternative promoters, the early and late promoters (E2E and E2L). Two major classes of transcripts, E2A and E2B, are produced by selection of two different polyadenylation signals (Chow et al., 1979; Stillman et al., 1981; Stillman, 1981). The DBP protein is encoded in E2A, while the viral pol and the pTP proteins are produced from E2B (Stillman et al., 1981; Stillman, 1981; Smart and Stillman, 1982; Tamanoi and Stillman, 1982).

The E4 proteins are designated depending on the open reading frame (ORF) of the corresponding mRNA produced by alternative splicing; unlike all other adenoviral genes, the E4 TU encodes proteins with varied and unrelated activities, such as transcription and translation regulation, splicing, selective export of viral mRNA, modulation of viral DNA replication and apoptosis (Fields et al., 2007).

The IVa2 protein participates in the encapsidation of the viral genome and also activates transcription of viral late promoters (Lutz and Keding, 1996), as does the IX product, pIX, which is a structural protein that stabilizes interactions between hexon protomers (Boulanger et al., 1979), also works as a transcriptional regulator of cellular and viral TATA box-containing promoters (Lutz et al., 1997). All but the IX viral mRNA are processed by splicing (Fields et al., 2007).

The proteins encoded in the MLTU are named by roman numerals, II to VIII, and, as described above are structural proteins or proteins that are required for the assembly of new viral particles and for the expression of viral late genes (Fields et al., 2007). The late genes from the MLTU are under the regulation of two viral promoters, the L4 (L4P) and the ML (MLP) promoters. During the transition to the late phase of infection, when viral DNA molecules begin to accumulate as a consequence of DNA replication, transcription of the early delayed TU IX and IVa2 begins (Winter and D'Halluin, 1991; Venkatesh and Chinnadurai, 1987). At this time, the L4P, localized inside the gene for L4-100K, is activated, and directs the expression of the L4-22K and 33K gene products (Morris et al., 2010). IVa2, IX and the latter two L4 proteins then induce activation of the MLP (Lutz et al., 1997; Morris and Leppard, 2009; Tribouley et al., 1994). However, the MLP is also active, albeit at a low level, early during infection producing exclusively the L1 52/55K mRNA. The main transcript produced from the MLTU is a pre-mRNA processed by selection of one of five different polyadenylation sites that determine each mRNA family (L1-L5, Fig. 2). Additionally, each mRNA is processed by alternative splicing, generating a minimum of 20 different mRNA (Fig. 3). Moreover, all viral late mRNA share a common 5' sequence of 201 bases named the tripartite leader (TPL) formed by the junction of three exons named leader 1, 2 and 3 (Fields et al., 2007).

Viral replication cycle

The adenovirus replication cycle, like other DNA viruses that replicate in the nucleus of the infected-cell, is divided by convention into an early and a late phase, separated by the onset of viral DNA replication (Fig. 3).

Early phase

The early phase commences with the interaction of the fiber protein with the host cell through the receptor CAR (Coxsackie B and Adenovirus Receptor) (Nemerow et al., 2009). CAR is a component of tight junctions of epithelial cells. After this initial interaction, the penton base interacts with integrins $\alpha_v\beta_3$ or $\alpha_v\beta_5$ with lower affinity than that of CAR-fiber (Nemerow et al., 2009). This event induces the disassembly of the fibers from the virion and subsequent endocytosis mediated by clathrins. Inside the endosome, partial disassembly of the capsid continues due to the low pH that activates the viral protease associated to the virion, releasing the penton, IIIa and hexon proteins. It is proposed that protein VI facilitates lysis of the endosome, which releases the viral core to the cytoplasm. The core then is bound to dyneins to be transported through microtubules and directed to the nuclear pore complex (NPC) (Nemerow et al., 2009). The viral DNA is then imported into the nucleus through the NPC, and viral early expression begins (Berk et al., 1979; Jones and Shenk, 1979).

During the early phase of infection, the optimal intracellular conditions necessary for efficient replication and expression of the viral genome are established: (i) induction of the host cell to enter the S phase of the cell cycle in order to provide the precursor dNTP required for viral DNA synthesis, NTP and RNA processing proteins required for viral transcription, and to activate pathways required for protein synthesis (Fields et al., 2007); (ii) inhibition of the immune response, apoptosis and other antiviral mechanisms that may otherwise block viral replication; (iii) and expression of E2 genes responsible for viral DNA replication (Flint, 1977; Kovesdi et al., 1986a,b).

Late phase

Viral genome replication

As described above, viral DNA replication represents the switch to the late phase. Viral DNA synthesis proceeds through an asymmetric strand displacement mechanism. As mentioned before, the viral genome ends contain ITRs that function as DNA replication origins. The ITRs enable viral DNA circularization by base pairing of single strands generated through displacement that is stabilized by cooperative DBP binding and multimerization (Fig. 3, inset). The resulting panhandles function as origins for replication of the displaced strand. The pre terminal protein (pTP) functions as primer for the viral pol to synthesize new DNA molecules (Fields et al., 2007). Additionally, the cellular transcription factors NFI and Oct-1 participate in viral DNA replication. These two cellular proteins bind to sequences inside the ITR, inducing the viral DNA molecule to bend, therefore facilitating displacement of the DNA strands and progression of viral DNA synthesis (Fields et al., 2007).

Selective expression of late genes

With the initial accumulation of viral DNA molecules, expression of IX and IVa2 begins, and activation of the MLP increases (Morris et al., 2010). The L4P is activated, resulting in the production of L4 22K and 33K proteins. These proteins are required to increase MLP activity resulting in the production of the rest of the late transcripts from the L2, L3, L4 and L5 families (Morris et al., 2010). The L4 22K protein promotes transcription and processing of viral late mRNA and the L4 33K protein functions as a splicing factor (Morris and Leppard, 2009). Most of the proteins encoded by viral late mRNA are structural proteins that assemble to form viral progeny (Thomas and Mathews, 1980; Ifode and Flint, 2004). Viral late mRNA are subject to a selective expression program. Synthesis of cellular mRNA continues during the infection, but their export to cytoplasm is inhibited, while viral mRNA are efficiently accumulated in the cytoplasm (Flint, 1986). Viral late mRNA are exported through the cellular Nxf1/Tap-Nxt1/p15 machinery (Yatherajam et al., 2011). In addition to selective export mechanisms, viral late mRNA translation is also selectively

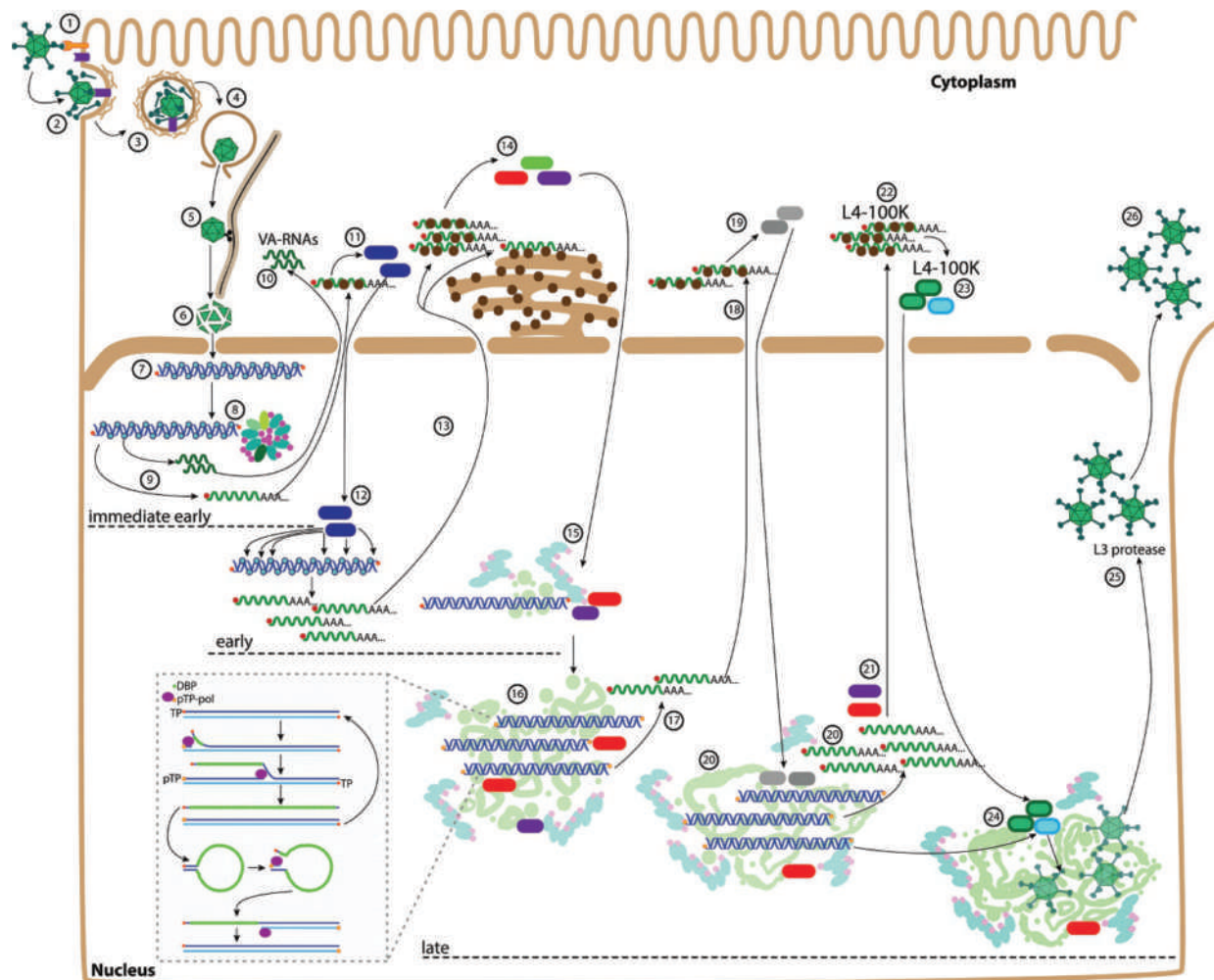


Fig. 3 Schematic representation of the adenovirus replication cycle. Adhesion of adenoviral particles to susceptible and permissive cells is modulated by interaction of (1) the fiber and a receptor protein (the coxsackie-adenovirus receptor, CAR, for most human adenoviruses) and (2) between the penton base and a coreceptor (integrin $\alpha v \beta 3$ or $\alpha v \beta 5$), leading to (3) cell-entry via clathrin-mediated endocytosis. (4) Release of the partially-disassembled virus particle into the cytoplasm is facilitated by a decrease in the pH of the endosome and mediated by the structural protein VI. (5) The virus particle is transported to the nuclear pore-complex (NPC) via microtubules and dynein (6) where it is fully uncoated. (7) The viral genome together with protein VII and possibly other core proteins is imported into the cell-nucleus. Protein VII remains associated with the viral DNA during the early phase of infection (Chatterjee et al., 1986; Spector et al., 2003; Wong and Hsu, 1988). (8) Within the first hours after the viral genome is delivered into the nucleus, it is localized in close proximity to PML-nuclear bodies (PML-NBs) (turquoise and green ovals represent PML and other proteins; magenta circles represent SUMO). (9) The first viral genes to be transcribed are the VA-RNAs by the host RNA polymerase III (RPIII) and the E1A by the host RPII. (10) VA-RNAs are exported to the cytoplasm by the host exportin-5. (11) E1A mRNAs are exported via the exportin-1/CRM1 machinery where they are translated to produce the E1A proteins (dark blue cylinders). (12) E1A proteins are imported to the nucleus where they promote transcription of viral early genes. (13) Viral early mRNAs are exported to the cytoplasm to be (14) translated; some E3 mRNA are translated associated with rough endoplasmic reticulum (RER; brown circles represent the ribosomes). (15) Early viral proteins (red, green and purple cylinders) are imported into the nucleus to modulate virus host-interactions and promote viral genome replication and expression of viral late genes. The cellular nuclear architecture is remodeled, inducing for example the reorganization of PML-NBs into track-like structures by E4Orf3 (E4Orf3-tracks) and formation of early replication compartments (RCs), represented by DBP (green circles) accumulation sites, adjacent to E4Orf3-tracks, where the viral genome is localized. (16) With progression of the viral replication cycle, RCs morphology changes with formation of ring-like structures and other more complex morphologies. The onset of viral genome replication by a strand-displacement mechanism (inset) marks the transition to the late phase of the viral replication cycle. With accumulation of newly synthesized DNA molecules, expression of Iva2 and IX is induced. (17) E1A, E4Orf3, Iva2 and the cellular p53 protein then activate the L4 promoter to transcribe L4-22K and L3-33K genes. (18) L4 22K and 33K mRNAs are exported to the cytoplasm and (19) translated to produce the splicing factor 33K and the transcription activator 22K (gray cylinders). (20) These L4 proteins are imported to the nucleus to activate the expression of all the viral late genes. RCs start to coalesce. (21) Viral late mRNA are selectively and efficiently exported to the cytoplasm while most of the cellular mRNAs are retained in the nucleus. (22) Viral late mRNA-translation is facilitated by the viral L4-100K protein. (23) Viral late proteins include structural proteins (green cylinders) and proteins that help to assemble new viral particles (blue cylinders). The L4-100K acts as a chaperone for the pre-assembly of structural proteins. (24) Viral late proteins enter the nucleus and localize to late RCs and participate in capsid assembly and viral genome encapsidation. (25) Maturation of virus particles is induced by proteolytic cleavage of structural proteins by the L3-23K protease. (26) The E3 adenovirus death protein (ADP) aids in the release of the progeny virus particles via cell lysis by an unknown mechanism.

regulated. This process involves the viral protein L4 100K and the VA RNA I (Hayes et al., 1990; Schneider and Mohr, 2003; Cuesta et al., 2001; Mathews and Shenk, 1991). The L4-100K protein together with the un-translated region (UTR) of the TPL from viral late mRNA, which is complementary to the 3' end of the 18S rRNA, induce a mechanism called “ribosome shunting” which allows the 40S ribosomal subunit to be directed to the start codon (Yueh and Schneider, 1996, 2000) to efficiently and selectively produce viral proteins. Additionally, VA RNA I induce the activation of the cellular kinase PKR, which in turns induces phosphorylation of eIF2 α , thus inhibiting cap-dependent translation of cellular mRNA.

Viral assembly and release

L4 100K also aids in the assembly of hexon trimers. The viral structural proteins and proteins that participate in the assembly of viral capsids and encapsidation of the viral genome are imported to the nucleus. With accumulation of viral proteins required for the assembly and formation of viral capsids, viral progeny is produced. Maturation of viral particles is induced by cleavage of the structural precursor proteins by the viral L3 protease 23K which are then released by cell lysis. Viral replication results in production of up to 10⁵ viral particles per cell, along with an excess of structural proteins and viral DNA that are not assembled into virions.

Virus-host cell interactions

The viral early proteins encoded in the adenoviral genome, E1A, E1B, E3 and E4, as well as the non-coding VA-RNAs, either inhibit antiviral responses of the cell or modulate pathways of cell proliferation and cell death that result in the establishment of conditions that result in very efficient synthesis of viral macromolecules and progeny production.

The E1A proteins are well known to inhibit the interferon response against the infection through several mechanisms, which involve the interaction of E1A with a variety of cellular proteins. The histone acetyltransferases transcriptional co-activators p300/CBP (CREB-binding protein) interact specifically with the Signal Transducer and Activator of Transcription 1 and 2 (STAT-1 and STAT-2). E1A targets p300/CBP, competes for its interaction with STAT-1 and 2 and mediates down-regulation of transactivation by these innate response proteins (Bhattacharya et al., 1996; Zhang et al., 1996). E1A also binds to STAT-1 and impedes formation of the STAT-1-IRF1 complex, leading to inhibition of expression of LMP2 (low molecular mass polypeptide 2), a gene regulated by the heterodimer STAT-1-IRF1 that encodes a protein that participates in antigen processing (Chatterjee-Kishore et al., 2000), and therefore interferes with the presentation of viral antigens. Moreover, E1A is known to downregulate the expression of interferon stimulated genes (ISG) by binding to RuvBL1 (Olanubi et al., 2017) and hBre1 leading to inhibition of the ubiquitylation of the histone H2B at ISG loci (Fonseca et al., 2012). In addition, E1A binds FOXK1/2, DCAF7, and CtBP suppressing the expression of ISGs by IRF3 (Zemke and Berk, 2017). As described before, E1A proteins associate with the pRb protein, releasing the transcription factor E2F and inducing the cell to enter the S phase of the cell cycle. This genotoxic stimulus induces the stabilization and activation of p53 that would have as a consequence the activation of apoptosis dependent on the Bcl-2 protein family (White, 2001; Steegenga et al., 1996); however, the E1B 19K inhibits apoptosis as it interacts with the cellular pro-apoptotic proteins BAX and BAK (Han et al., 1996). The E1B 55K and E4Orf6 proteins associate with and inactivate p53, resulting in the establishment of a proliferative state in which the cell is unable to die or stop DNA synthesis. Moreover, E1B 55K and E4Orf6 associate with Cullin 5-Elonguins B, C-Rbx1 to form a ubiquitin E3 ligase that induces polyubiquitylation and degradation of multiple cellular targets, among which is p53 (Querido et al., 2001; Cardoso et al., 2008; Martin and Berk, 1998; Hidalgo et al., 2019; Ip and Dobner, 2020). Additionally, E1B 55K is a multifunctional protein that also induces the SUMOylation of p53 and its consequent relocalization to viral replication compartments (RCs, described below) in which the tumor suppressor acts as a transcription activator of the L4 promoter (Wright and Leppard, 2013).

E1B 55K is also known to repress transcription of ISGs which in turn promotes efficient formation of RCs and synthesis of viral DNA (Chahal et al., 2012). E1A associates with Ubc9, the only E2 conjugator of SUMO that has been identified, altering the SUMOylation of cellular substrates (Yousef et al., 2010). Furthermore, E4Orf3 induces SUMOylation of components of the DNA damage response (Sohn and Hearing, 2016). These activities of E1 and E4 proteins are necessary for the efficient replication of the virus, but they are also decisive for its oncogenic capacity (detailed reviews can be found in Hidalgo et al., 2019; Ip and Dobner, 2020). The E3 TU encodes seven proteins (in the HAdVC2 and HAdVC5 genomes) whose main functions are to counteract the cellular defense mechanisms, by inhibiting antigen presentation mediated by MHC—I and apoptosis mediated by TNF, TRAIL and FasL signaling (Lichtenstein et al., 2004). Moreover, the viral non-coding VA RNA I and II are known to bind and inhibit effectors of the interferon signaling like the double-stranded RNA-activated protein kinase (PKR) and the 2'-5'-oligoadenylate synthetase 1 (OAS1), and to saturate the exportin 5 pathway interfering with cellular miRNA biogenesis (Vachon and Conn, 2016). A detailed review on adenoviral mechanisms that counteract cellular antiviral mechanisms can be found in Sohn and Hearing (2019).

Like other DNA viruses that replicate in the nucleus, upon infection of the cell AdV DNA is delivered into the cell nucleus where it localizes in close proximity to PML Nuclear Bodies (PML NB) by an uncharacterized mechanism (Ishov and Maul, 1996) (Fig. 3). PML NB are nuclear compartments transiently composed not only of factors involved in DNA replication and transcription, but also in antiviral responses such as epigenetic silencing, DNA repair, cell senescence, apoptosis, and regulation of the interferon-induced antiviral response (Lallemant-Breitenbach and de The, 2010).

Adenovirus infection of permissive cells results in an extensive reorganization of the infected-cell nucleus, including nuclear components that are major constituents of nuclear domains, such as PML NB, interchromatin granules (IG), paraspeckles, Cajal bodies (CB) and nucleoli (reviewed in Ishov and Maul, 1996; Schmid et al., 2014; Hiscox, 2002; Rodrigues et al., 1996; James et al., 2010; Besse and Puvion-Dutilleul, 1995; Bridge et al., 1995) (Fig. 3). The E1A and E4ORF3 proteins induce the disruption of PML NB by

redistributing the PML proteins into “fibrous-like” tracks (Fig. 3) (Leppard and Everett, 1999). These virus-induced nuclear rearrangements lead the formation of viral Replication Compartments (RCs), viral nuclear subdomains where the viral genome replicates and viral late gene expression takes place. These virus-induced nuclear compartments also constitute a hub for regulation of virus-host cell interactions. Besides recruiting molecules necessary for viral DNA replication and expression, cellular anti-viral factors implicated in various cellular activities are also recruited to RC, which are believed to be subverted at these sites, including components of the DNA damage response (ATR, the MRN complex, RPA32, ATRIP, TopBP1 and Spc1), tumor suppressors (p53, BRCA1, PML) and cellular proteins of the innate immune response (STAT-1, PKR) (Cardoso et al., 2008; Evans and Hearing, 2005; Castillo-Villanueva et al., 2014; Blackford et al., 2008; Carson et al., 2009; Maul et al., 1998; Rosa-Calatrava et al., 2003; Sohn and Hearing, 2011; Schreiner et al., 2013). A detailed review of adenovirus RCs can be found in Hidalgo and Gonzalez (2019).

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Influenza Viruses

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Glossary

Antiviral chemoprophylaxis Using antiviral drugs to prevent influenza.

Attenuated virus vaccine A vaccine that contains weakened live virus.

Clade A taxonomic group of organisms classified together based on homologous features traced to a common ancestor.

Inactivated virus vaccine A vaccine that consists of inactivated or “dead” virus.

Negative-strand RNA viruses A group of viruses that their single strand RNA is complementary to mRNA.

Introduction

Influenza viruses (IVs) remain a public health concern since they are very contagious and infect many people annually worldwide. IVs cause respiratory disease in humans, and their clinical presentations range from mild symptoms, such as fatigue and headache, to more severe, like respiratory failure and death (Pleschka, 2013). Influenza is more prevalent in children than adults. According to the world health organization (WHO), influenza epidemics lead to 3–5 million cases of severe illness and approximately 290,000–650,000 respiratory deaths each year (WHO, 2018). More severe strains of the IVs have caused several pandemics. The last pandemic occurred in 2009 by swine flu infection and resulted in 18,631 deaths based on the WHO report; however, the number of deaths may be 10-fold higher than the reports (Simonsen et al., 2013). Several strains of IVs can also infect animals, including pigs, chickens, marine mammals, cats, horses, and ducks (CDC, n.d.). Genetic changes occur in the IVs due to their nature, and this phenomenon may cause the formation of more severe pathogenic strains of the virus leading to the pandemic occurrence (Pleschka, 2013).

This article aims to review the characteristics and epidemiology of IVs, as well as clinical features, laboratory tests, and treatment protocols of influenza disease comprehensively.

Characteristics

Types

IVs belong to the *Orthomyxoviridae* family and are divided into four types: A, B, C, and D (Park and Ryu, 2018). Different IV types have a similar structure but different antigens (Dangi and Jain, 2012). Fig. 1 illustrates the naming system of IVs. The virus type, the place of isolation, strain ID, year of isolation, and its subtypes comprise the name of the virus (CDC, 2019f).

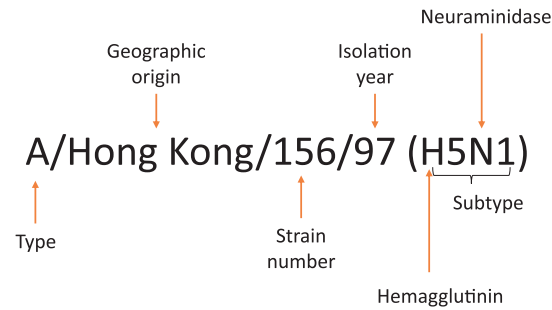


Fig. 1 The naming system of influenza viruses.

Influenza A virus

Influenza A virus (IAV), the most important type of IVs, belongs to *Influenza virus A* genus (MacLachlan and Dubovi, 2017). The first isolation of IAV in swine occurred in 1930, and 3 years later, the first human IAV was isolated (Institute of Medicine Forum on Microbial, 2005). IAVs have caused many epidemics and all of the flu pandemics (CDC, 2019f). They infect both animals and humans, but their natural reservoirs are wild birds. Pigs are also another common zoonotic source of the virus. IAVs are classified into different subtypes based on their two viral glycoproteins: hemagglutinin (HA) and neuraminidase (NA). To date, 18 hemagglutinin types and 11 neuraminidase types have been recognized on the surface of IAVs. Although 198 IAV subtypes are expected to exist, only 131 types have been found in nature. Currently, IAV(H1N1) and IAV(H3N2) are circulating among people and are infecting them. IAV subtypes are further divided into clades (groups) and sub-clades (subgroups). The classification into clades is based on the HA gene sequences. It should be mentioned that although clades and sub-clades differ genetically, they may have the same antigens. Different IAV subtypes have different mortality and infection rate (CDC, 2019f; Park and Ryu, 2018). Overall, IAVs have a higher rate of mutation and recombination in comparison with the other IV types (Dangi and Jain, 2012).

Influenza B virus

Influenza B virus (IBV) was isolated in 1940 for the first time and belongs to *Influenza virus B* genus. IBVs can also cause epidemics worldwide; however, less attention is given to IBVs compared to IAVs. It is believed that there is no animal reservoir for IBV, and it is limited to humans, but few studies have discovered IBV in seals (Zaraket et al., 2021; Krammer et al., 2018; Bodewes et al., 2013). There was no classification for IBVs in the past, but since the 1980s, IBVs have split into two lineages: B/Yamagata and B/Victoria. Like IAVs, IBVs have further subdivisions named clades and sub-clades. It seems that B/Victoria lineage changes more rapidly than B/Yamagata genetically (Zaraket et al., 2021; CDC, 2019f). The potency of IBVs appears to be increasing, and they may even lead to a higher mortality rate in comparison with IAVs in specific populations, such as HIV patients. The importance of IBVs should not be underestimated since they cause high morbidity and mortality (Sharma et al., 2019).

Influenza C virus

Influenza C virus (ICV) belongs to *Influenza virus C* genus and was discovered in 1947. ICVs do not cause epidemics/pandemics, and they are not classified into subtypes, but to date, six lineages have been discovered for ICV: Aichi/1/81, Yamagata/26/81, Mississippi/80, Taylor/1233/47, Sao Paulo/378/82, and Kanagawa/1/76. ICVs are found mostly in humans, but they can also infect animals, such as pigs, cattle, and dogs. ICVs infect mainly children and infants. Unfortunately, ICVs are less known (Sederdahl and Williams, 2020; CDC, 2019f; Speranskaia et al., 2012). It seems that ICVs are spread around the world, but due to their challenging isolation, they are less studied than other IV types (Matsuzaki et al., 2006).

Influenza D virus

Influenza D virus (IDV) belongs to *Influenza virus D* genus and was discovered in 2011 by isolation from pigs. IDVs have three lineages: D/swine/Oklahoma/1334/2011 (D/OK), D/bovine/Oklahoma/660/2013 (D/660), and Japanese. Cattle and pigs (mainly cattle) are reservoirs of the IDVs. IDVs are distant relatives of ICVs. Serum sample screening in humans has shown that seropositivity is more prevalent in people who had exposure to cattle (Su et al., 2017; Murakami et al., 2020). Although IDVs have not led to epidemics/pandemics yet, we should not underestimate its zoonotic potential, and more investigations are required.

Virion structure and function of viral proteins

IVs have segmented single-stranded RNA genome that is negative-sense. IAV and IBV have eight RNA segments, but ICV and IDV have seven RNA segments. The shape of the virus particle (virion) can be spherical with a diameter of 80–120 nm, filamentous with a length of 250 nm–30 µm and width of 80 nm, or pleomorphic. IVs have a lipid envelope that is taken from the host cell. The virus genome encodes for viral glycoproteins (HA and NA), viral nucleoprotein (NP), matrix 1 protein (M1) and matrix 2 protein (M2)–BM2 in IBV and CM2 in ICV–, RNA polymerase subunits, the nonstructural protein 1 (NS1), and nuclear export protein (NEP)/nonstructural protein 2 (NS2). Other proteins, such as PB1-F2 and PA-X, are also produced in IAVs. Ribonucleoprotein

complexes (RNPs) consist of the viral genome (RNA), which is encapsidated with NP, and viral RNA-dependent RNA polymerase complex (RdRp). This RNA polymerase complex has three subunits: basic polymerase 1 (PB1), basic polymerase 2 (PB2), and acidic polymerase (PA)/P3. Due to the similar structure of IBV and IAV, electron microscopy cannot distinguish between them. (Dadonaite et al., 2016; Bouvier and Palese, 2008; Krammer et al., 2018). In IAV and IBV, HA and NA mediate the virus entry and release, but in ICV and IDV, hemagglutinin-esterase-fusion (HEF) glycoprotein fulfills the function of both HA and NA (Sederdahl and Williams, 2020) (see Fig. 2).

The proteins encoded by each segment are shown in Table 1. PB1, a subunit of RdRp, elongates the viral RNA. It seems that the S-D-D motif forms an active site for the protein in its center. PB2 binds to the cap of the cellular pre-mRNA and leads to its cleavage by PA via its endonuclease activity (Fodor, 2013). PB1-F2 has pro-apoptotic activity. HA is one of the main surface glycoproteins and antigens, with the structure of head and stalk, in IAV and IBV that mediates the attachment and entry to the host cell. HA hemagglutinates the red blood cells during incubation with virions. NA is another surface glycoprotein that possesses sialidase activity and mediates the release of virus from the host cell. HA is four times more prevalent than NA in the envelope of the virus (Pleschka, 2013; Krammer et al., 2018). As mentioned before, HEF is the surface glycoprotein of ICV and IDV in which the function of HA and NA is combined (Sederdahl and Williams, 2020; Speranskaia et al., 2012). NP encapsidates the viral RNA and regulates the nuclear import in the host cell. M1 is the matrix protein under the lipid envelope and supports the viral shape. M1 also regulates

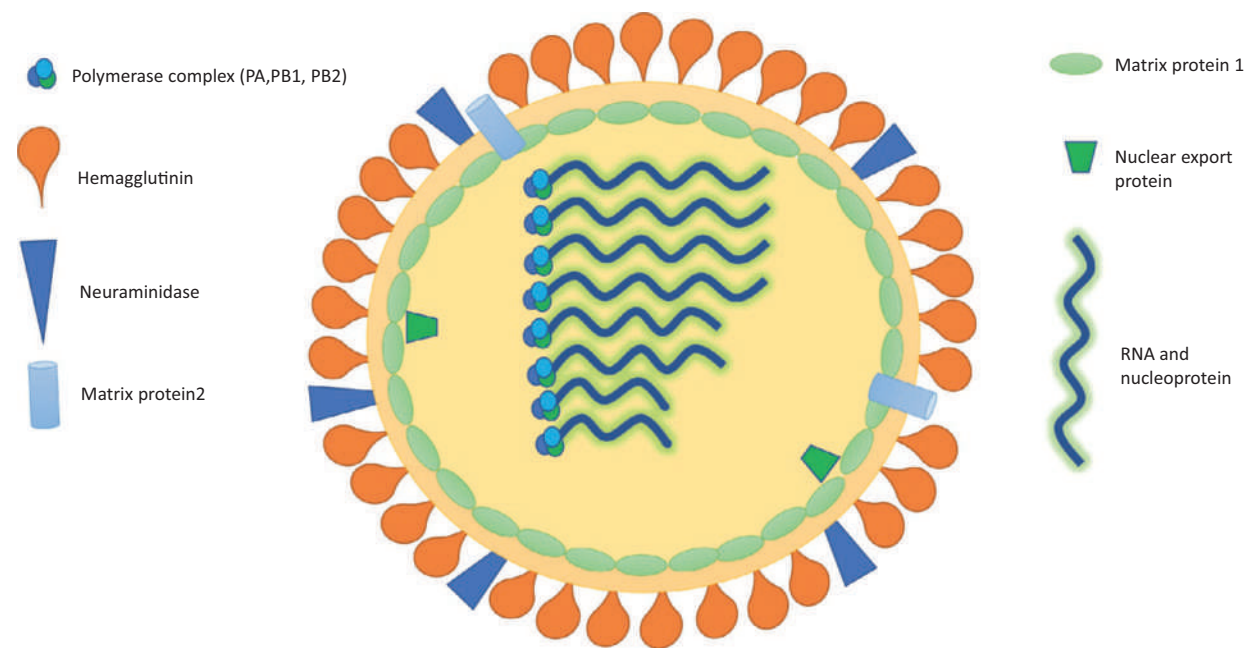


Fig. 2 Structure of influenza virus.

Table 1 Genes on RNA segments.

Segment	IAV	IBV	ICV	IDV
1	PB2	PB2	PB2	PB2
2	PB1	PB1	PB1	PB1
	PB-F2	–	–	–
3	PA	PA	P3	P3
4	HA	HA	HEF	HEF
5	NP	NP	NP	NP
6	NA	NA	M1	M1
	–	NB	CM2	CM2
7	M1	M1	NS1	NS1
	M2	BM2	NS2	NS2
8	NS1	NS1	–	–
	NEP	NEP		

HA, hemagglutinin; HEF, hemagglutinin-esterase-fusion; M1, matrix 1 protein; M2, matrix 2 protein; NP, nucleoprotein; NEP, nuclear export protein; NS1, nonstructural protein 1; NS2, nonstructural protein 2; NA, Neuraminidase; PB1, basic polymerase 1; PB2, basic polymerase 2; PA/P3, acidic polymerase.

the nuclear export of the RNA and interacts with RNP. M2 is an ion channel traversing the lipid membrane of the virus with a pH-dependent activity. NS1 is an interferon antagonist protein that helps to replication of the virus by inhibiting interferons. NS1 also regulates the activity of RdRp. NEP mediates the nuclear export of RNPs (Krammer et al., 2018; Pleschka, 2013; Fodor, 2013; Dangi and Jain, 2012).

Life cycle

As mentioned above, HA mediates the virus entry to the host target cells, such as epithelial cells of the human respiratory tract or epithelial cells of the intestinal tract of birds. HAs, located in the viral envelope, bind to sialic acid (neuraminic acid), which is found in some resident molecules (as a receptor) of the target cell membrane. HA from human influenza has more affinity for sialic acids with $\alpha 2,6$ linkage to the rest of their oligosaccharide, but avian influenza HA shows more affinity to $\alpha 2,3$ -linked sialic acids. After binding to the host cell, the virus becomes surrounded by an endosome, a process called endocytosis. The endosome has low pH (5–6), and M2 protein accelerates the acidification of the endosome. The low pH within the endosome leads to the fusion of the viral envelope with the endosome. This process is mediated by cleavage of the disulfide bond between HA1 and HA2 and the exposure of fusion peptide on HA2, consequently. Eventually, the fusion peptide on HA2 leads to the fusion of the viral envelope and endosomal membrane. In the meantime, M2 protein lowers the pH of the viral particle, leading to dissociation of viral RNPs from matrix proteins (M1) and release of viral RNPs to the host cell cytoplasm (Krammer et al., 2018; Allen and Ross, 2018) (see Fig. 3).

After the release of viral RNPs to the cytoplasm, they become imported to the nucleus of the host cell, mediated by nuclear localization sequences (located in NPs). Transcription and replication of viral RNA to positive-sense RNA occur in the nucleus by RdRp that is bound to the viral RNP complex. PB2 and PA cooperation cause the attachment and cleavage of 5' caps from cellular RNAs. After polyadenylation of 3' end of the RNA, the viral pre-mRNA is ready to be exported from the host cell nucleus. IVs replicate their genetic material (viral RNA) using the complementary ribonucleoprotein (cRNP) complex for transcription of negative-sense RNA. Translation of viral proteins by the host ribosomes occurs in the cytoplasm of the cell, mediated by the viral mRNA (Wu et al., 2007; Krammer et al., 2018; Allen and Ross, 2018). It should be mentioned that NS1 contributes to the translation of viral proteins and the regulation of viral gene expression. Proteins, such as PB1, PB2, PA (proteins of RNA polymerase complex), and viral NP are imported to the cell nucleus from the cytoplasm to accelerate the viral RNA synthesis, and proteins, such as HA (needs a cleavage by cellular proteases for activation), NA, and M2 that eventually take place in the viral lipid envelope, traffic from the rough endoplasmic reticulum and Golgi apparatus to the cell membrane. Specific signals on viral RNA checks the appropriate assembly of all viral parts to form a proper new virus particle. At late stages of the virus replication, M1 and NEP are

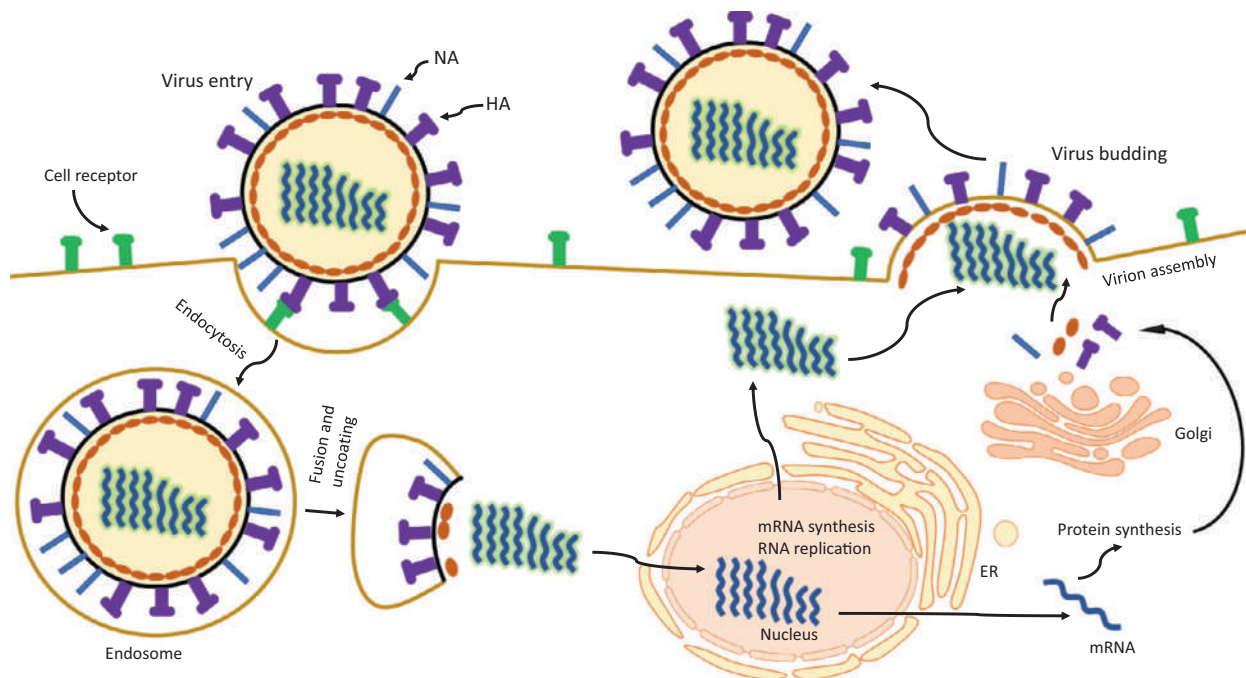


Fig. 3 The life cycle of the influenza virus. HA proteins of the influenza virus bind to cell receptors and mediate the virus entry to the host target cell. The virus becomes surrounded by the endosome. Then the viral envelope fuses with the endosome, and the released RNPs are imported to the nucleus for replication and transcription of mRNA. Viral mRNA encodes viral proteins in the cell cytoplasm. Finally, viral proteins and viral genome are assembled into new virions, and new virions are released by the contribution of NA proteins. NA, neuraminidase; HA, hemagglutinin; ER, endoplasmic reticulum; RNP, ribonucleoprotein; mRNA, messenger RNA.

exported to the cell nucleus to incorporate with viral RNPs and mediate their export to the cytoplasm of the cell with help of recycled endosomes. Finally, the budding of the virus initiates by the accumulation of M1 in the cytoplasmic side of the cell membrane, and NA cleaves the bond of HA with receptors containing sialic acid to release the virions from the cell surface (Krammer et al., 2018; Allen and Ross, 2018; Hao et al., 2020; Pleschka, 2013).

Antigenic drift and shift

Two main mechanisms of antigenic alternation in IVs are antigenic drift and antigenic shift. These events in IVs complicate the vaccine production process; therefore, the vaccines need to be updated each year (Krammer et al., 2018).

Antigenic drift refers to small mutations in the viral genome that alters the epitopes of the virus. Unlike DNA polymerase, the RdRp complex of the virus has no proofreading mechanism, leading to the accumulation of new mutations during each replication cycle. The high rate of replication and mutation accelerates the occurrence of new antigenic changes and adaptation of the virus to the host cell. Even though many of these mutations cause minor changes or disadvantageous alternations to the virus, some of the mutations introduce key conformational changes to the epitopes of HA (in the head, not in stalk) and NA (mainly HA). These changes aid the virus in escaping from the host cell's antibody-mediated immunity (Krammer et al., 2018; Kim et al., 2018; Matsuzaki et al., 2016). Antigenic drift occurs in all the IV types. It mainly occurs in IAVs, but it has a slower rate in IBVs. Antigenic drift rarely occurs in ICBVs and IDVs. It seems that the last antigenic drift in ICBVs occurred 30 years ago (Su et al., 2017; Matsuzaki et al., 2016).

The antigenic shift causes drastic changes in the genome of the IVs, and it is more prevalent in birds. Since the genome of the IVs is segmented, when a host cell is infected with two different strains of the IVs, gene reassortment can occur. The antigenic shift can take place in the host cell when two strains of IAV from the same *genus* co-infect the cell (see Fig. 4). Theoretically, 254 different combinations of segments are expected to happen; however, only a few combinations have occurred and been recognized. Some animals, such as pigs, bats, and quails are called “mixing vessels” for genetic reassortment because they can be infected by both human influenza (possess receptor for $\alpha 2-6$ -linked sialic acids) and avian influenza (possess receptor for $\alpha 2-3$ -linked sialic acids). When these different strains exchange their HA and/or NA genes, a new strain emerges from the host cell that may acquire the ability of human-to-human transmission. The antigenic shift can lead to a pandemic. Although only a limited number of pandemics have happened, no one can anticipate the time of the next pandemic (Kim et al., 2018; Ma et al., 2008; Krammer et al., 2018).

Epidemiology

IAV and IBV co-circulate among the human population and cause seasonal epidemics annually around the world. Children are more prone to infection with IVs, and the severity of the disease is higher in children and elderly individuals (>65 years old) than the rest of the population. Interestingly, children vaccination diminishes the number of the cases in the elderly population. Currently, H1N1 and H3N2 are the main IAVs circulating among the human population. Limited zoonotic transmissions to humans, including avian H5N1 and H7N9, swine H3N2, are being reported in some regions; however, human-to-human

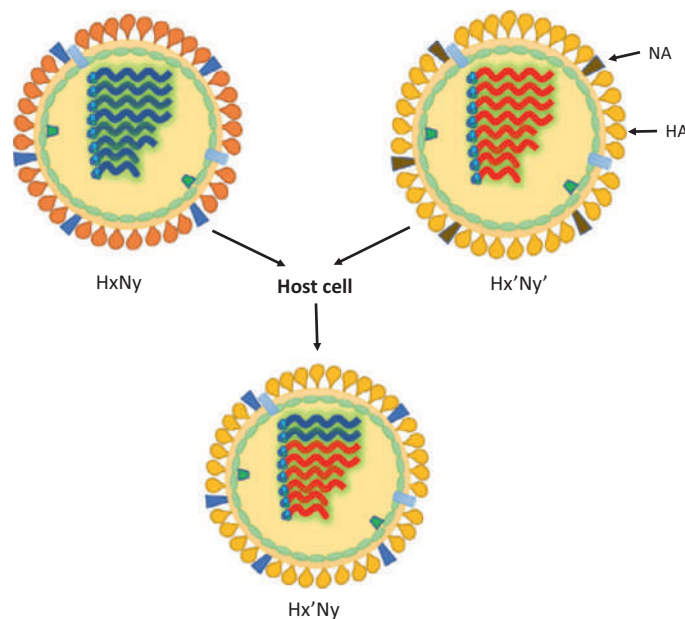


Fig. 4 Process of the antigenic shift in influenza virus.

transmission rarely occurs with these strains (Krammer et al., 2018). Since the 1980s, IBV has spread in seasonal flu epidemics, and it appears to be more prevalent in children aged 1–10. 23% of the IV infections have been caused by IBV between 2000 and 2018 (CDC, 2019e; Zaraket et al., 2021). Most ICV infections occur during early childhood. However, several familial transmissions of ICV and outbreaks have been reported among military recruits, travelers on the Hajj pilgrimage, and healthcare workers (Sederdahl and Williams, 2020). IDV is also circulating among animals, including pigs, bovine, cattle, and small ruminants (Su et al., 2017).

Low temperature and low humidity increase the susceptibility to IV infection. Therefore, the IV epidemics are more prevalent in cold seasons, especially in winter. Approximately 42% of the influenza peaks have occurred in February between 1982 and 2018. Climate factors, such as duration of sunshine and maximum rainfall, are strongly associated with the spread of IVs (CDC, 2019e; Krammer et al., 2018).

According to the WHO, influenza epidemics lead to 3–5 million cases of severe illness and approximately 290,000–650,000 respiratory deaths each year (WHO, 2018). Additionally, based on the Centers for Disease Control and Prevention (CDC) reports, IV has infected 9.2–35.6 million cases and resulted in 140,000–710,000 hospitalizations between 2010 and 2017 in the United States (Krammer et al., 2018). Children and the elderly population, people with co-morbid diseases, pregnant women, and immunosuppressed patients are at higher risk of infection and severe illness (Krammer et al., 2018).

Based on the historical reports, 31 influenza pandemic-like events have occurred since 1580 (Lazzari and Stöhr, 2004). During the 20th century, three pandemics have occurred, and in the 21st century, we have faced one influenza pandemic (see Table 2). The first and the most severe pandemic of the 20th century occurred in 1918–19 by an A H1N1 virus (“Spanish flu”) that had genes with avian origin. A higher mortality rate was seen in under-five children, 20–40 years old population (specific characteristic of this strain), and older adults (>65 years old). H1N1 infected at least one-third of the world population and resulted in more than 50 million deaths. No availability of vaccines and antibiotics complicated control of the “Spanish flu” (CDC, 2019a; Dangi and Jain, 2012). The second pandemic of the 20th century occurred in February 1957–58 by a new IAV, H2N2, that emerged in East Asia. It is believed that “Asian flu” resulted from the genetic shift of human influenza and avian influenza in pigs. H2N2 frequently infected children aged 5–19. “Asian flu” resulted in 1.1 million worldwide. Rapid vaccination decreased the death rate of “Asian flu” in comparison with “Spanish flu” (Lina, 2008; CDC, 2019b; Dangi and Jain, 2012). In July of 1968, the third pandemic of the 20th century started in Hong Kong by a new IAV virus, H3N2 (“Hong Kong flu”). Like the “Asian flu,” H3N2 resulted from gene reassortment of human influenza and avian influenza. Approximately 1 million people died due to the H3N2 virus. Moreover, in this pandemic, rapid vaccination contributed to lower the number of deaths and cases (CDC, 2019c; Lina, 2008). To date, the only pandemic of the 21st century emerged in the United States in 2009 by a novel IAV (H1N1)pdm09, with a swine origin. The gene combination of this new virus was not recognized previously in humans or animals. However, similarities between the H1N1 virus from the 1918 pandemic and (H1N1)pdm09 were detected. Based on the CDC reports, (H1N1)pdm09 resulted in 60.8 million cases (43.3–89.3 million), 274,304 hospitalizations (195,086–402,719), and 12,469 deaths (8868–18,306) in the United States and 151,700–575,400 deaths (in the first year) worldwide. Unlike seasonal influenza, most of the deaths due to (H1N1)pdm09 occurred in people younger than 65 years of age. Due to fundamental differences between seasonal influenza and (H1N1)pdm09, vaccination with seasonal flu vaccines showed only a slight protection against (H1N1)pdm09. After November, large number of vaccines against (H1N1)pdm09 were available in the United States (CDC, 2019d; Dangi and Jain, 2012).

Host immune response and immunopathogenesis

After entering the body, the IV binds to the epithelial cells of the respiratory tract of humans and other mammals; however, in the birds, IV infects the epithelial cells of the intestinal tract (Krammer et al., 2018). It should be mentioned that several other studies have detected infection of the other cells, such as type II pneumocytes, alveolar macrophages, dendritic cells, and retinal epithelial cells with IV (Shim et al., 2017). IAV and IBV infect both the upper and lower respiratory tract, but in contrast, ICV infects only the upper respiratory tract (Su et al., 2017). Lung inflammation is the main reason for illness in influenza. The pathological changes are mainly located in the lungs, including alveolar damage with hyaline membranes, septal edema tracheitis, epithelial hyperplasia, squamous metaplasia of bronchus, necrotizing bronchiolitis, pulmonary vascular congestion, and alveolar hemorrhage (Dangi and Jain, 2012).

IV infection activates the innate immune response, followed by the humoral and cell-mediated response (adaptive immune response). The recognition of viral replication by pattern recognition receptors (PRRs), including toll-like receptors (TLRs), nod-like receptors (NLRs), and Retinoic acid Induced Gene 1 like receptor (RIG-I), initiate the innate immune response. TLR3, 7, 8, and 9

Table 2 Recent influenza pandemics.

Year	Name	Strain	Number of deaths
1918–1919	Spanish flu	H1N1	>50 million
1957–1958	Asian flu	H2N2	1.1 million
1968–1969	Hong Kong flu	H3N2	1 million
2009	Swine flu	(H1N1)pdm09	151,700–575,400 (first year)

recognize the presence of IV in the cell. Other TLRs (TLR 1, 2, 4, 5, and 6) indirectly sense the IV infection by recognizing damage associated molecular patterns (DAMPs). NLRs contribute to the formation of the inflammasome complex, leading to the cleavage of proinflammatory cytokines, s pro-interleukin (IL)1 β and pro-IL-18 and helps to virus clearance. The last member of PRRs, RIG-I is expressed in lung epithelial cells and alveolar macrophages. RIG-I activation by 5'-triphosphate viral ssRNA causes translocation of NF- κ B and/or interferon regulatory factor (IRF)3/7 to the nucleus of the cell and production of type I and III interferons, as well as proinflammatory cytokines, consequently. It seems that the production of type I interferon by the epithelial cells and alveolar macrophages plays an important role in the development of antiviral responses. The activation of PRR pathways leads to the activation of proinflammatory pathways and the production of proinflammatory cytokines, such as IL-1, -6 and -8, tumor necrosis factor (TNF- α), macrophage inflammatory protein-1 α/β (MIP-1 α/β), interferon induced protein (IP-1), etc. The recruitment of immune cells, such as neutrophils, dendritic cells (DC), monocytes, and lymphocytes into the alveolar space is mediated by the proinflammatory cytokines. The excessive activation of inflammatory pathways causes an event called "cytokine storm," which is the excessive response against the IV and increases the patient's mortality. The reason for the poor prognosis in the cytokine storm is the high production of proteases, oxygen, and reactive nitrogen species due to uncontrolled activation of immune cells, leading to tissue damage (Tavares et al., 2017; Sun et al., 2018). Approximately 7 days after IV infection, CD4⁺ and CD8⁺ T cells and B cells become activated. CD4⁺ T cells regulate differentiation of other immune cells and produce cytokines, and CD8⁺ T cells destroy the infected cells with granzymes and perforin. In addition, they express FASL that leads to the apoptosis of the infected cells. B cells secrete antibodies that protect the body against secondary IV infection and neutralize the viruses (Tavares et al., 2017; Zhang et al., 2020). Natural killer (NK) cells also lysis the infected cells via the interaction of NKp46 receptors and HA proteins (Zhang et al., 2020).

The IVs have several ways to neutralize the immune system of their hosts. NS1 protein inhibits type I interferon production as the result of inhibiting IRF3 activation. NS1 protein also binds to the DNA of the host cell and inhibits the transcription of antiviral genes. The failure of interferon responses (due to NS1 protein) and accumulation of viral RNA in the cell activate the apoptotic pathways of the host cell (Shim et al., 2017). Besides, autophagy is another host defense mechanism in response to IV infection (Zhang et al., 2020). NA proteins of IV block the NKp46 receptors and contribute to the escape of HA protein recognition by these receptors. Moreover, HA proteins accelerate the degradation of interferon receptors, and another protein, PB-2, also contributes to interferon antagonism (Chen et al., 2018).

Clinical features

Transmission

The IVs spread among humans mainly through respiratory routes. Coughing, talking, and sneezing of an infected individual generate large droplets (diameter >5 μ m) and small particle aerosols containing virus particles. Large droplets are only able to travel a short distance (approximately 6 ft); therefore, infection through large droplets needs close contact between at least two individuals. But small particle aerosols can be transmitted over longer distances and remains for a longer time in the air. In addition to respiratory routes, contaminated surfaces with respiratory secretions of infected individuals are another route for transmission of IV (Krammer et al., 2018; UpToDate, 2020).

Fecal-fecal, fecal-oral, or fecal-respiratory routes are the main routes for transmitting IV between wild birds. For the transmission of IV in swine, pig-to-pig contact is required. Newly formed IVs in swine (with an avian origin) spread via airborne transmission between animals after establishment in the mammals. Animal-to-human transmission of IVs occurs through direct contact or contaminated environments (Krammer et al., 2018; WHO, n.d.; Webster, 1997).

Each IV types have a specific attack rate. The attack rate of the seasonal influenza is 5–10% in the adults and 20–30% in the children. The attack rate of IAV and IBV in the adults is approximately 2.32% and 0.59%, respectively, but the attack rates for IAV and IBV are higher in the children, 12.27% for IAV and 5.5% for IBV (Park and Ryu, 2018).

Clinical manifestations

The incubation period of IVs is approximately 1–4 days, and the virus has a high rate of transmissibility in its incubation period. The severity of clinical presentations depends on several factors, such as age and previous exposure to the virus. The initial unspecific symptoms have a sudden onset and include fever, chill, myalgia, headache, malaise, and anorexia. Respiratory symptoms are sore throat, cough (usually dry), rhinitis, and nasal obstruction (Krammer et al., 2018; UpToDate, 2020). Infection in elderly patients and patients with weak immunity may have a mild presentation (due to lower secretion of cytokines), but it may result in severe low respiratory tract illnesses. Lack of energy, confusion, and fever without respiratory symptoms may be the clinical manifestations in elderly patients (Krammer et al., 2018). Influenza is usually self-limited, and the patient recovers from the illness within a week (Dangi and Jain, 2012). However, some symptoms, such as cough, may remain for several weeks. The leukocyte count is usually normal or low in the early phase of the disease, but it may become higher in the late stages of the disease (UpToDate, 2020).

As mentioned above, influenza is usually uncomplicated and self-limited, but IV can cause severe respiratory illnesses and complications in the infected patients. Otitis media occur in less than 50% of the infected children 4 days after the symptom onset. Patients with specific medical conditions, such as liver disorders, kidney disorders, blood disorders, heart diseases, etc., may have complicated influenza. Pneumonia is a common complication of influenza in patients with specific medical conditions and

low-risk children under 2 years. Influenza pneumonia is usually mild but has a worse prognosis in hospitalized patients (UpToDate, 2020). Rapid progression of fever, cyanosis and shortness of breath are among the clinical presentations of influenza pneumonia (Krammer et al., 2018). The radiologic findings of influenza pneumonia are ground-glass opacities, centrilobular nodules, branching linear opacities, and consolidation. The infiltration in the lung can be both interstitial and alveolar (Oikonomou et al., 2003; UpToDate, 2020). Other reported complications of influenza are laryngotracheitis or tracheobronchitis, acute myositis (mostly in IBV), myocarditis, pericarditis, febrile seizures, aseptic meningitis, Guillain-Barré syndrome, postinfectious encephalitis, exacerbation of pulmonary diseases (such as asthma), plastic bronchitis, and acute respiratory distress syndrome (ARDS)-one of the leading causes of death in infected patients. ARDS in patients with influenza is the excessive and persistent inflammatory response of the body against the IV (UpToDate, 2020; Khanmohammadi et al., 2021).

Detection methods

Several diagnostic tests can be conducted to recognize IV infection, but they are not performed on all the suspected patients. Viral tissue cell culture, rapid cell culture, reverse transcription-polymerase chain reaction (RT-PCR), rapid antigen test, rapid molecular assay, and immunofluorescence, direct (DFA) or indirect (IFA) fluorescent antibody staining are the available diagnostic tests for IV (CDC, 2020b; Ravina et al., 2020) (see Table 3).

Viral cultures are the most traditional and gold standard test for IV detection. Respiratory specimens, such as nasopharyngeal swabs, throat swabs, and sputum, are used for viral cultures. The result of the test has a high specificity (>95%), and more than 3 days (up to 10 days) are required for the preparation of the results. Viral culture tests are mostly performed in outbreaks to determine the IAV subtypes as well as IAV and IBV strains. They are also used for recognizing the IV strains for the vaccines of the next years. Besides, behavioral and morphological changes, like the cytopathic effect of the IV, can be detected in viral cultures, enabling surveillance of antiviral sensitivity and antigenic drift. Detection of ICV in cell culture is challenging since they have a weak cytopathic effect (Ravina et al., 2020; CDC, 2020b; Dziąbowska et al., 2018; Krammer et al., 2018; Sederdahl and Williams, 2020).

Immunofluorescence assays are used to detect viral antigens. This method has a high specificity and moderate sensitivity, and 2–4 h are needed to prepare the result. Both DFA (easier and shorter response time) and IFA can be used for IAV and IBV detection, but they are unable to recognize the IAV subtype. Epithelial cells from the respiratory specimens are stained with antibodies (produced in animals) that are labeled with fluorescent; then, the epithelial cells are observed under a fluorescent microscope (CDC, 2020b; Dziąbowska et al., 2018).

Rapid influenza diagnostic tests (RIDTs) detect the viral antigens (mostly NP) within 15 min. Different types of RIDTs with the ability to detect only IAV or both IAV and IBV with/without distinguishing between them are available; however, RIDTs cannot distinguish between IAV subtypes. Respiratory specimens, such as nasopharyngeal or throat swabs, are used in RIDTs. RIDTs have low sensitivity, but they have a low rate of false-positive results; however, false-positive and false-negative results should be considered in influenza outbreaks. Different RIDTs have different sensitivities and specificities. The fast response and easy

Table 3 Detection methods for influenza virus.

Method	Test time	Specimens	Advantages	Disadvantages
Viral culture	3–10 days	Respiratory Specimen	- Moderately high sensitivity and highest specificity - Enables public health surveillance, and characterization of virus - Distinguishes between IAV and IBV, and IAV strains	- Slow - Lower sensitivity than RT-PCR - Needs highly skilled staff
Immunofluorescence	2–4 h		- Moderately high sensitivity and high specificity - Distinguishes between IAV and IBV	Prone to false negative results in outbreaks
Rapid influenza diagnostic tests	<15 min		- Fast - Moderate sensitivity and high specificity - Low cost	Prone to false negative results in outbreaks
RT-PCR	1–8 h		- High sensitivity and specificity - Distinguishes between IAV and IBV, and IAV strains - Can be multiplexed	- Expensive - More prone to false positive results than culture
Rapid molecular assays	15–30 min		- High sensitivity and specificity - Distinguishes between IAV and IBV - Fast	Expensive
Serological assays	–	Serum	Public health investigations and research purposes	- Need paired samples - Unsatisfactory sensitivity - Low availability

RT-PCR, reverse transcription polymerase chain reaction; IAV, influenza A virus; IBV, influenza B virus.

conduction are among the advantages of RIDTs. The results of the RIDTs are more accurate in children than adults (Dziąbowska et al., 2018; CDC, 2020b; Ravina et al., 2020).

RT-PCR detects the presence of IV in the respiratory specimens with high sensitivity and specificity, but it is an expensive method. 1–8 h are required for the preparation of the result of the test. In RT-PCR, viral RNA is converted to complementary DNA (cDNA), then the segments are amplified by random hexamers. Matrix gene amplification can be performed to determine the IV types, and RT-PCR containing specific primers for HA gene are also used for IAV subtyping. Several RT-PCR and new real-time PCR is developed for distinguishing between H1 strains. In addition, multiplex RT-PCR can recognize several viruses in the sample by combining multiple primers sets (CDC, 2020b; Dziąbowska et al., 2018; Ravina et al., 2020). RT-PCR is an appropriate method for IDV and ICV detection (Dziąbowska et al., 2018; Sederdahl and Williams, 2020).

Rapid molecular assays are based on isothermal nucleic acid amplification, and their results are ready within 15 min. The results have high specificity and sensitivity. Another method used in the rapid molecular assay is RT-PCR, which prepares the results in 20 min. Some of these tests can distinguish between IAV and IBV and also determine some of the IAV subtypes. It should be mentioned that upper respiratory tract specimens are mostly tested in rapid molecular assays, and they are not approved for lower respiratory tract specimens (CDC, 2020b; Dziąbowska et al., 2018; Ravina et al., 2020).

Serological assays, such as enzyme-linked immunosorbent assay (ELISA), single radial hemolysis (SRH), complement fixation assay, hemagglutination inhibition assay (HAI), western blotting, etc. are based on the detection of antibodies against viral antigens with an unsatisfactory sensitivity. Serological tests are not recommended since they need paired samples (first one at the beginning of infection and second one 2–4 weeks after); therefore, they are only available for public health investigations and research purposes. The serological assays use several techniques, such as hemagglutination inhibition by the increase in the antibody level, the measurement of hemolysis areas (related to antibody level), the ability for virus neutralization, and antigen-antibody interaction to recognize the antibodies against viral antigens (Dziąbowska et al., 2018; CDC, 2020b).

Other methods, such as biosensors, which detect based on recognizing biological analytes, can be used for IV detection (Ravina et al., 2020).

Treatment protocols

Symptomatic treatment and antiviral drugs are available for Influenza treatment. Most of the infected individuals do not require antiviral treatment. In mild illnesses, staying at home, hand washing, minimizing contact with other people and administering over-the-counter medications contribute to the patient's recovery. However, for the hospitalized patients, patients with severe illness, and patients at higher risk of complications, antiviral treatment is required (see Table 4) (CDC, 2021a). People with special medical conditions, elderly patients (≥ 65 years), under-five children, pregnant women, etc., are among the individuals at higher risk of complications. Antiviral drugs are used for both treatment and prevention (UpToDate, 2021b).

Neuraminidase inhibitors, oseltamivir (oral), zanamivir (the first drug in this class, inhaled as powder), and peramivir (intravenous) block the enzyme activity of NA proteins and inhibit the release of progeny influenza virus from the infected host cells. These drugs apply their effect with the contribution of their structure as a sialic acid analog. Both IAV and IBV can be targeted with these drugs. NA inhibitors should be used at the early stages of the disease since they act at the viral replication stages. Reduced viral shedding and duration of symptoms result from the administration of NA inhibitors. Oseltamivir and zanamivir are also recommended for chemoprophylaxis (UpToDate, 2021b; CDC, 2021a).

Baloxavir marboxil (oral) is another antiviral drug that inhibits cap-dependent endonuclease activity of PA protein and subsequent inhibition of mRNA synthesis. Both IAV and IBV are targeted by baloxavir. This drug should be administrated within 2 days after the onset of the disease and can also be used for prevention. It should be mentioned that baloxavir is mainly used in uncomplicated influenza, and data for its efficacy in severe forms of the disease is limited (CDC, 2021a; UpToDate, 2021b). A study examined the efficacy of baloxavir against all IV types, A, B, C, and D, and the results showed that baloxavir could be the first therapeutic option against ICV (Mishin et al., 2019).

Table 4 Influenza medications.

<i>Antiviral agent</i>	<i>Mechanism of action</i>	<i>Affected influenza types</i>	<i>Chemoprophylaxis</i>	<i>Route of administration</i>
Oseltamivir	Neuraminidase inhibitor	IAV and IBV	+	Oral
Zanamivir			+	Inhaled
Peramivir			–	Intravenous
Baloxavir marboxil	cap-dependent endonuclease inhibitor	IAV and IBV	+	Oral
Amantadine	M2 ion channel inhibitor	IAV	N/A	Oral
Rimantadine			N/A	Oral

IAV, influenza A virus; IBV, influenza B virus; N/A, not available.

Adamantanes, amantadine (oral) and rimantadine (oral), inhibit M2 ion channel protein and act only against IAV. They can shorten the duration of symptoms. Adamantanes are no longer recommended for influenza treatment since IAVs are highly resistant to them (UpToDate, 2021b). IAV H3N2 and IAV H1N1 that are currently circulating as seasonal influenza, are also resistant against the adamantanes (UpToDate, 2021b; CDC, 2021a).

Drug resistance in IVs is a major concern of the healthcare system. Although the level of drug resistance is low for baloxavir and NA inhibitors, but drug-resistant viruses can emerge in children, hospitalized patients (due to longer duration of illness), and immunocompromised patients. It seems that using drug cocktails in the future is required to fight against drug resistance in IVs. Besides, new NA inhibitors are being examined in several clinical trials to introduce new antiviral drugs (Krammer et al., 2018).

Vaccination

Effective vaccination against IVs could contribute to overcoming the outbreaks. The first influenza vaccine was inactivated influenza A virus, developed in the 1940s. However, new IV strains were discovered, and this vaccine lost its effectiveness quickly (Krammer et al., 2018). Influenza vaccines need annual updates since the circulating strains of each outbreak are different (Dangi and Jain, 2012). For developing the inactivated viruses, the master high-growth IV (A/Puerto Rico/8/1934) and the circulating strain of IV co-infect the embryonated chicken eggs. It is noticeable that further developments in vaccine production processes enabled using mammalian cell cultures instead of embryonated chicken eggs. After the occurrence of gene reassortment, the new virus that contains NA and HA genes from the circulating strains and internal protein genes of the master high-growth virus is selected and amplified. The virus vaccine becomes inactivated with alkylating reagents. Adding adjuvants to the inactivated vaccines increases the efficiency. It should be mentioned that new technologies have developed new recombinant vaccines with inactivated viruses using DNA-recombinant technology and cell-cultures (IV and egg free) (Krammer et al., 2018; CDC, 2020a, 2021c).

Currently, there are two main types of influenza vaccines: flu shot vaccines and nasal spray vaccines. The flu shot vaccine contains the inactivated viruses and is injected intramuscularly. Trivalent flu vaccines contain IAV H1N1 and H3N2 plus an IBV strain, but quadrivalent flu vaccines contain two IAV strains, H1N1 and H3N2, and two IBV strains. Flu shots are approved for individuals older than 6 months. Severe allergic reaction to the vaccine components is the main contraindication of flu shots (CDC, 2021c; UpToDate, 2021a). The nasal spray vaccine or live attenuated influenza vaccine (LAIV) is another influenza vaccine containing two IAV strains (H1N1 and H3N2) plus two IBV strains. LAIV can be administered in individuals aged 2–49 years. Pregnancy, having specific medical conditions, having an allergy to any vaccine components, etc., are among the contraindications of LAIV (CDC, 2021b; UpToDate, 2021a).

The annual influenza vaccination is recommended for all individuals more than 6 months of age; however, when vaccine supply is inadequate, a specific group of people has a higher priority. Immunocompromised patients, individuals more than 59 years of age, patients with chronic disorders, such as asthma, metabolic, pulmonary, renal, hepatic, or neurologic disorders, native Americans, extremely obese people, pregnant women, and residents of nursing homes have a higher priority for influenza vaccinations (UpToDate, 2021a).

Influenza vaccines have various efficiencies from season to season, depending on the similarity of circulating viruses and vaccine viruses. When the circulating viruses and vaccine viruses have a high similarity, an individual's age and health status are the indicator factors of vaccine efficiency (CDC, 2021c). Common side effects of the influenza vaccine are headache, fever, nausea, muscle aches, pain in the injection site, and fatigue. However, severe side effects, such as serious allergic reactions to vaccines and Guillain-Barre syndrome, rarely occur due to the influenza vaccine (CDC, 2021c).

Conclusion

IVs have been challenging humans for more than 100 years. The ability of IV to change its genetic material and circulate in both human and animals, complicate its management for the healthcare system and government. The epidemiology and burden of influenza show the important effects of this virus on human life. Influenza is a global health concern and needs global preparedness. Despite the progress in managing influenza outbreaks, inadequate attention is given to the population at higher risk of severe illness and even death. No one knows the time of the next influenza pandemic since new strains can emerge at any moment and consequently lead to many deaths. Thus, the world should prepare itself for the management of IV. The efficacy of influenza vaccines currently available is not optimal, and mutations in circulating vaccines can highly affect it. The protection provided by vaccines only lasts for one flu season. Moreover, drug resistance is another concern of the healthcare system for treating influenza. Therefore, proper management frameworks and investigations are needed to prevent and control influenza outbreaks and manage severe forms of the illness. Developing new antiviral drugs and vaccines with higher efficacy could also be a major improvement. We can also learn from the current COVID-19 pandemic and prepare ourselves for the possible influenza pandemics in the future. A better understanding of the animal-to-human transmission process could also prevent emergence at the source. We should not forget that influenza control in humans is not enough, and influenza control in animals should also be considered (Krammer et al., 2018; Santibañez et al., 2009).

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Paramyxoviridae (Paramyxovirus, Measles Virus, Mumps Virus, RSV)

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Introduction

The Paramyxoviridae family belongs to the order Mononegavirales consists, it is one of the largest and fastest growing family causing significant disease burden in human and wildlife (Henrickson, 2003). The Paramyxoviridae family is divided into three genera: Paramyxovirus genera includes the Parainfluenza viruses and mumps virus, Pneumovirus genera includes RSV and Morbillivirus genera includes the measles virus (Baron, 1996). Measles virus, mumps virus, RSV and HPIV are best known because of the greatest burden of disease on humans. Moreover, other viruses of Paramyxoviridae family were also dangerous and have caused diseases in animals such as Canine Distemper virus that causes disease in dogs, Phocine Distemper virus that causes disease in seals, Cetacean Morbillivirus which causes disease in dolphins. . . . This chapter refers primarily to the virus in humans.

In the second to fourth centuries in China and the Roman Empire, scientists documented the first measles outbreaks (Brown, 1997). Measles is a highly contagious and serious disease that causes about 2.6 million deaths a year. Cyclical outbreaks of major epidemics around 2–3 years (as reported by WHO in 1968). Measles is an acute systemic disease, after cured it will create lifelong immunity for patients (Panum, 1939). Two epidemiological parameters of measles have been estimated, the reproductive number (R_0) and the critical community size (CCS). R_0 is defined as the number of secondary cases arising from each primary case and CCS is the minimum number of people needed for the disease to continue to exist. For measles, R_0 is about 15–20 cases and CCS is about 250,000–500,000 people (Bellini and Rota, 1998, Griffin, 2001).

Mumps is a virus of the genus Rubulavirus, which was first discovered through aerosol droplets by Hippocrates in the 5th century BCE. Mumps has a very narrow host range, which is similar to measles (Brown, 1997). Mumps epidemics can occur in both developed and developing countries, mainly caused by vaccination failures (Carbone and Wolinsky, 2001). These epidemics is also cyclical every year, it's estimated that between a few hundred and few thousand people in the United States getting mumps each year (as reported by CDC in 2018). Mumps has two indicators similar to measles, which are $R_0 = 5$ and $CCS = 200,000$ (Gower et al., 2005).

The RSV that causes in human is called human respiratory syncytial virus (HRSV). RSV causes respiratory infections, mucosal cells in the infected respiratory tract combine to form a syncytium. RSV was first discovered in 1956 on chimpanzees infected with respiratory illness by Morris, Blount and Savage, after which they named the virus CCA (Chimpanzee Coryza Agent) (Blount Jr. et al., 1956). It was later realized that the chimpanzees caught the infection from their caretakers. By 1957, Robert M. Chanock had determined that RSV causes pneumonia and laryngitis in children (Chanock et al., 1957). Today, RSV is the main cause of lower respiratory tract infections in infancy and childhood. According to statistics in the United States every year, with only infants, HRSV causes 50% of all pneumonia and 90% of bronchiolitis. 90,000 children in the United States each year hospitalized with RSV, one-third develop lower respiratory tract disease. In addition to children with mild illness, there are cases progressing to severe bronchiolitis and pneumonia, the lower submucosal edema and the bronchioles become blocked, in the worst case, mechanical ventilation may be required (Hall et al., 2009).

HPIV is a virus that causes parainfluenza in humans. It is the second reason that children under 5 years old hospitalized for respiratory disease, just behind HRSV. The nomenclature and classification of HPIV have changed a lot since the discovery of this virus. HPIV is documented in medical literature from the 1950s, however, it was not until 1959 that the virus was officially recognized and called "parainfluenza viruses" (Enders, 1996). HPIV is divided into four categories with names in the order of 1–4, including HPIV-1, HPIV-2, HPIV-3 and HPIV-4. HPIV-1, HPIV-2 and HPIV-3 are the main causes of diseases of the lower respiratory tract in infants, childhood, the elderly, and the immunocompromised (Henrickson, 2003). The results of the surveys show that about 75% of children over 5 years old have antibodies to HPIV-1 (Mizuta et al., 2012). In the United States, it is estimated that 5 million children have lower respiratory infections each year and a third of these cases are caused by HPIV-1, HPIV-1, HPIV-2 and HPIV-3 (Denny and Clyde Jr, 1986). Currently, the scientific literature on HPIV-4 is not popular and clear. In addition to lower respiratory tract infections, HIPV also causes upper respiratory tract infections however at a lower rate.

Biology of paramyxoviridae viruses

General biological characteristics of the Paramyxoviridae family is polymorphic structure, it consists of a helix nucleocapsid core, a lipid envelope and membrane binding proteins. They typically encode 6–10 proteins (5–250 kDa), of which all the viruses of the Paramyxoviridae family consist of three nucleocapsid binding proteins and three membrane-binding proteins. The retinol binding proteins (RBP) and fusion proteins are glycosylated by the N-linked carbohydrate side chain (Rima et al., 2019). The lipid envelope is derived from the plasma membrane of the host cell. In addition, the virions comprise about 6% carbohydrates by weight, composition of which is dependent on host cell. In terms of shape, virions with a spherical shape with a diameter of 100–400 nm are most common, however we can still see filamentous virions. The membrane of the virus of this family have two spines stuffing out 8–20 nm, these are the protein tetramer spines and the trimer or tetramer spines of fusion proteins (Morrison, 1998). About the genome, the axit ribonucleic (RNA) molecules inside the virions are straight, sensory-negative, no single infection but potentially infectious if the ribonucleoprotein (RNP) complex is introduced into the cytoplasm. For effective replication, the genomes of all viruses in the Paramyxoviridae family are required to be multiples of 6. Besides the common biological characteristics of the Paramyxoviridae family, each virus has different characteristics.

Measles virus is a negative, non-segmented, and lipid sheathed virus. The genome of the measles virus is about 16 kilobases long, and it consists of 6 genes that encode 8 proteins. Measles virus RNP complex is made up of nucleoprotein (N), phosphoprotein (P) and large protein (L) and is surrounded by this complex as matrix protein (M). In addition, this virus have two unstructured proteins (proteins V and C), they are expressed from an alternative RNA transcription of the P gene and are primarily effective in preventing interferon-induced immune responses (IFN) type 1. The glycoproteins, hemagglutinin (H) and fusion proteins in the measles virus envelope mediate virus attachment and fusion (Griffin and Oldstone, 2009, Bellini et al., 1985). After the measles virus converts into proteins and is encased in the lipid shell of the cell, it is sent out of the cell as a new virus. Within a few days of exiting a cell, the measles virus spreads through local tissue and is taken up by dendritic cells and alveolar macrophages, then be brought to the local lymph nodes. It continues to spread, eventually reaching the bloodstream, and spread mainly to many lung tissue and other organs such as the gut and brain (Moss and Griffin, 2012). After going to the organs of the body, measles virus reduces dendritic cell function of these organs, thereby causing immunosuppression due to measles (Rota et al., 2016).

The mumps virus contains a non-segmented negative, stranded RNA molecule, which contains 15,384 nucleotides (Davis et al., 2010, Hviid et al., 2008). The mumps genome contains seven parallel linked transcription units, including: nucleo (N), V/P/I, matrix (M), fusion (F) (Griffin), small hydrophobic (SH) (Jajosky et al.), hemagglutinin neuraminidase (Gower et al.) and large proteins (L). Each protein has different roles and functions. The RNP complex is the template for mumps virus replication and transcription, this complex is made up of negative-stranded RNA and surrounded by N protein. The RNA-dependent RNA polymerase is a complex of proteins L and P, it copies the negative sensory RNA to the positive and sensory RNA as a transcription enzyme that produces mRNA from the negative sensory RNP by entering a single promoter at the 3' end of the genome (Elango et al., 1988). When cells are infected, glycoproteins HN and F are transported through the endoplasmic reticulum and the Golgi complex to the cell surface. The M protein then locates RNPs to regions of the cell membrane, where glycoproteins F and HN have passed, thereby helping to infect a virus into a new cell (Li et al., 2009). The HN glycoprotein attaches the newly formed virus to neighboring cells via receptors such as sialic acid, which are abundant on the surface of most animal cells. The SH protein plays an important role in evading the host's antiviral response by blocking the TNF α -mediated path of death (He et al., 1998). Protein V is also involved in evading the host's antiviral response in the same way as the SH protein (Andrejeva et al., 2004). However, the function of protein I has not been clearly published.

The biological structure of the HRSV is spherical and is around 150 nm in diameter. There are fibrous species that have been observed up to several micrometers in length (Gower et al., 2005). Like other viruses, the RNA genome is encapsulated as a non-segmented and photoreceptor molecule. RSV has 10 genes and encodes 11 proteins (Lee et al., 2012). Proteins and functions of each include: internal structural proteins (matrix protein M and nucleoprotein N); proteins required for functional polymerase complexes (phosphoprotein P and polymerase L); non-structural proteins that evade the innate immune response (NS-1 and NS-2); trans membrane glycoproteins (small hydrophobic SH protein, glycoprotein G and F fusion protein) and M2 proteins that regulate transcription or transcription (the anti-inhibitory protein M2-1 and M2-2) (Tawar et al., 2009). About genome, it is approximately 15,000 nucleotides in length and is composed of a single strand of RNA with negative polarity. RSV is divided into two main strains of virus, RSV-A and RSV-B, which are both seasonal circulating in the human population. The RSV-B strain is found in the majority of the population and mainly causes disease without clinical symptoms. The RSV-A strain is known to be associated with most of the clinically important diseases and outbreaks. The HRSV genome was fully explored in 1997. To date, 10 genotypes of HRSV-A have been discovered including GA1 to GA7, SAA1, NA1 and NA2. The HRSV-B strain has 8 genotypes, including GB1 to GB4, SAB1 to SAB3 and BA1 to BA6 (Lee et al., 2012).

Scientists have proved that HPIV has a pleomorphic enveloped. Their envelopes are known to originate from host cells that have been infected with the last time. The size of HPIV is mostly about 150–250 nm, this is one of the medium sized viruses. However, scientists have also discovered much larger viruses (Howe et al., 1967). HPIV usually contains negative single-stranded RNA (in addition to mRNA), in addition, positive virions are said to be non-infectious. Like RSV, the HPIV genome contains about 15,000 nucleotides (Storey et al., 1984). The nucleotides are engineered to encode at least six common structural proteins, such as 3'-NPCMF-HN-L-5' (Tashiro and Homma, 1985). HPIV has different characteristics from other genera of the Paramyxoviridae family, for example that HPIV does not have neuraminidase like morbillivirus genera (measles virus ...), HPIV has thinner nucleocapsid than pneumoviruses genera (RSV, metapneumoviruses) (Henrickson, 2003).

Pathology of paramyxoviridae viruses

The viruses that belong to the Paramyxoviridae family have different pathologies. Instead of describing their general pathology of Paramyxoviridae family, we describe the four most typical viruses: Measles virus, Mumps virus, RSV and HPIV.

The first recognizable clinical sign of measles is usually a high fever, which starts 10–12 days after exposure to the measles virus and usually lasts 4–7 days. In addition, the early stages can also detect symptoms such as a runny nose, red and watery eyes, cough and small white spots inside the cheeks (CDC, 2018). After a few days, a rash called Koplik spots appears, usually on the face and upper neck (Biesbroeck and Sidbury, 2013). The rash spreads and eventually spreads to the hands and feet in about 3 days. After that, the rash lasts for 5–6 days and fades away. Most of us will emerge spots rash after exposure to the virus from 7 to 18 days, but the average of about 14 days (Steichen and Dautherville, 2009). Before disappearing, the rash is said to be “stained” and changes color from red to dark brown. Most measles usually cures after about 3 weeks from the date when symptoms are first discovered (Ludlow et al., 2015). After recovering from the disease, complications of measles ranging from mild to severe such as diarrhea, pneumonia, otitis media, laryngitis, corneal ulcers and acute encephalitis ... are relatively common (O’Brien, 1955, Fisher et al., 2015, Anlar, 2013, Semba and Bloem, 2004). Measles can be fatal, but fatalities are mostly associated with severe complications. Children under 5 years old or adults over 30 years old are at high risk of serious complications. Children with vitamin A deficiency, poor nutrition and people with weakened immune systems from HIV/AIDS or other illnesses are more likely to get measles. According to a 2018 WHO report, an estimated 140,000 people die from measles and the majority of them are children under 5 years old. Now, measles remains one of the highest causes of disease burden and mortality in children in the world (Griffin et al., 2012).

With mumps, the initial symptoms as an important signal include a low-grade fever, headache and feeling generally unwell. The most important symptom is swelling of one or both parotid glands, which will last for about a week. In addition, other symptoms of mumps include dry mouth, facial pain, ear pain, and difficulty speaking (Davis et al., 2010). When infected, the percentage of patients with parotitis is very high, ranging from 31% to 61%. This percentage depends on the age and immunity of each person, it can be as low as 9% or as high as 94% (as the report by CDC). Parotitis can appear in one or both ears, and it can also affect one or more salivary glands. Parotitis usually occurs within 2 days of exposure to the virus and the first symptoms are mostly ear pain and pain when touching the corner of the jaw. After 10 days these symptoms tended to decrease and after a week the symptoms usually resolve. The most common complication of mumps is testicular inflammation (orchitis), but it only occurs in men after puberty. This is a very dangerous complication, with a 15–20% chance of it occurring in men who have completed puberty and have mumps virus infection (Davis et al., 2010). Symptoms of orchitis are usually sudden swelling and pain in the testicles, nausea and vomiting, and fever. Pain and swelling symptoms usually subside after 1 week, but can last for several weeks depending on the case. In addition to orchitis, complications of mumps are usually quite severe and are also relatively common, including encephalitis, ovarian inflammation, deafness and acute pancreatitis (Senanayake, 2008, Di Pietrantonj et al., 2020).

RSV is one of the most common viruses that cause disease in humans, and it often returns, partly due to a lack of immunity long after infection. The incubation period of this disease is about 4–5 days (Bont et al., 2016). RSV usually affects the upper respiratory tract, but has the potential to also affect the lower respiratory tract, depending on the condition and severity of the disease. If the illness only affects the upper respiratory tract, it usually presents itself as flu-like symptoms such as nosebleeds, stuffy nose, cough, sneezing, sometimes fever and muscle aches. RSV may be related to the lower respiratory tract in some patients, especially those with risk factors for severe illness under 2 years of age. When affecting the lower respiratory tract, patients with symptoms similar to the symptoms of bronchiolitis, for example bad breath, rapid breathing, use of accessory muscles, wheezing. Severe complications such as viral pneumonia, hypoxia, acute otitis media, coma, asthma, apnea, and acute respiratory failure can also occur in people with RSV (Omer et al., 2019). Mild symptoms are more common in adults, they are very difficult to distinguish from a common cold because the symptoms are very similar as mentioned above. In the United States, according to CDC statistics has shown that RSV is the most common cause of bronchiolitis and pneumonia in children under 1 year. The rate of children under 2 years old suffering from this disease is up to 90% and the rate in older children and adults is also very high (Schweitzer and Justice, 2020).

Like RSV, HPIV are common respiratory pathogens caused by the community, however this disease occurs without ethnic, socio-economic, sex, age or geographic boundaries. However, scientists have also found a few factors that make HPIV easier to happen such as malnourished children, overcrowded housing, lack of vitamin A, lack of breastfeeding or environmental pollution (Berman, 1991, McIntosh, 1991). The HPIV-1 strain is cyclical and occurs in the fall for two consecutive years (Carballal et al., 2001). In the United States, among pneumonia cases, at least 50% of cases are caused by HPIV. Each outbreak of HPIV-1 in the United States involves 18,000 to 35,000 children under the age of 5 being hospitalized (Marx et al., 1997). HPIV causes infections such as bronchiolitis, tracheitis, pneumonia, fever, and wheezing. Most infections occur in children aged 7–36 months, but the incidence is highest in children aged 2–3 years. The HPIV-2 strain also causes infection every 2 years along with HPIV-1 or it can replace HPIV-1 in each outbreak (Belshe et al., 1983). HPIV-2 usually breaks out in the fall and early winter. The symptoms of this strain are similar to those of a lower respiratory tract infection. According to statistics, about 60% of all HPIV-2 infections occur in children under 5 years old and the highest incidence is from 1 to 2 years old. Particularly, infants under 6 months of age are very susceptible to infection with the HPIV-3 strain, in addition to those with immunodeficiency and chronic diseases. For the HPIV-3 strain, bronchitis and pneumonia are the most common infections. The HPIV-3 strain differs from the previous two strains, causing the annual spring and summer outbreaks. The region of this strain circulates mainly in North America and Europe (Belshe et al., 1983). The strain HPIV-4 has not been fully studied, it is thought to be a common disease, but it is usually mild and rarely detected (Henrickson, 2003). In addition to causing major infections of the respiratory tract, rare cases of HPIV have also been associated with viral meningitis.

Transmission of paramyxoviridae viruses

The paramyxoviridae family has horizontal transmission and the main route is air and direct contact. There are no documents to confirm that these viruses transmit via vectors. Viruses of this family enter the human body and usually start in the respiratory tract, they may not spread to other sites like the HPIV-1 virus and can spread to secondary sites such as lymphoid tissue and endothelium. For measles virus, parotid glands with mumps virus.... Host immunity is a critical condition for infection and reversibility caused by the paramyxovirus family of viruses. Long-term persistent infection can be caused by certain viruses of the genus morbillivirus, for example, the measles virus in subacute sclerosing encephalitis, a serious complication that affects the central system and has a moderate duration is 8 years (Enders, 1996). In addition to the general characteristics of the paramyxoviridae family, the differences in transmission of each virus will be highlighted below (mainly four viruses that cause disease in humans: Measles virus, Mumps virus, RSV and HPIV).

Measles is considered one of the most contagious diseases in the world, as a report by WHO. Measles is transmitted through the air, which is easily spread from person to person through the coughing and sneezing of an infected person. It can also be spread by direct contact with the mouth or nose secretions of an infected person. Measles is so contagious that 9 out of 10 people sharing the same living space with an infected person will become infected. Before and 4 days after the rash appears, the patient is capable of transmitting the measles virus to others, which is difficult to detect and prevent. Measles can easily erupt into an epidemic and cause enormous burden and mortality, especially in malnourished young children. Most measles are eliminated in countries today, but cases imported from other countries remain an important source of infection. Based on the biological properties, the high transmissibility of measles can be explained by three reasons (Laksono et al., 2016). First, measles patients must be effectively eliminated from the measles virus in order to fully recover from the disease and be unable to infect others. Second, the virus must remain infectious until it reaches a new host. Third, the infectious nature of the measles virus is strongly related to the infectious dose of the virus.

Mumps virus is transmitted with respiratory (Davis et al., 2010). When the patient is infected with the mumps virus, it multiplies in the nasopharynx and regional lymph nodes. The patient can then transmit the virus by coughing, sneezing or talking, sharing objects that may have saliva, or by direct or indirect contact with the patient's respiratory secretions. Because it can persist on surfaces and then spread after contact, the mumps virus is highly contagious (Kutty et al., 2010). The virus can spread to many organs of the body, including the meninges and glands such as the saliva, pancreas, testes and ovaries. The tissues of the infected organ will become infected, and characteristic signs of parotid gland inflammation and aseptic meningitis appear. The incubation period for the mumps virus is 12–25 days, but usually 16–18 days. In many cases, people infected with mumps virus do not show symptoms, this rate is about 20–40%, so people can become infected and spread the virus to healthy people without knowing it (Kutty et al., 2010). The mumps virus is quite contagious like flu and rubella, but less contagious than measles virus.

RSV transmission is also the respiratory tract. The incubation period of RSV can last from 2 to 8 days, but an average of 4–6 days. In particular, infants and people with weakened immune systems can continue spreading the virus even after symptoms have stopped, for up to 4 weeks (Schweitzer and Justice, 2020). According to the CDC, the virus spread appears when the sick person coughs or sneezes. Droplets from the cough or sneeze of an infected person shot in the eyes, nose and mouth of an uninfected person or an uninfected person touch surfaces or virus and then touch the face before washing their hands. RSV can be spread through direct contact with the virus by acting like kissing the face of someone with RSV. This virus can persist for half an hour or more on hand or up to 5 h on a table top. After RSV inoculation into the nasopharyngeal mucosa or conjunctiva mucosa, the virus quickly spreads into the respiratory tract and reaches the apical ciliated epithelial cells, which is the preferred growth medium for RSV. According to some studies, HRSV can be transmitted from human to ape and also can be transmitted from ape to human, however this is not common.

In addition, RSV has also been recovered from cattle, goats and sheep but they are not considered the main vectors for disease transmission.

HPIV transmission studies are not common. One study found that only 2 out of 40 children were infected at a distance of 60 cm from the strain HPIV-1 (McLean et al., 1967). Consequently, small particle aerosol transmission can occur. Transmission by close and indirect contact through an RSV surface occurs by misting large droplets. In particular, the surfaces containing RSV can be direct self-inoculation (Hall et al., 1981). Scientists have proven that strains HPIV-1, HPIV-2 and HPIV-3 can last up to 10 h on non-porous surfaces and 4 h on porous surfaces (Brady et al., 1990). However, HPIV-3 is believed to be less capable of living in the external environment. The experiment was conducted on two human fingers, when HPIV-3 was added to the first finger, more than 90% of the virus on that finger lost its ability to infect another finger in just 10 min (Ansari et al., 1991). It was also found from that experiment that person-to-person transmission by direct hand contact did not seem to be a means of transmission. However, the amount of virus excreted from an acutely infected child can be 10 times greater than when tested, so exact conclusions cannot be drawn (Hall et al., 1977). In outdoor environments, HPIV can be easily removed from surfaces with most common detergents or disinfectants (Brady et al., 1990).

Associated conditions of paramyxoviridae viruses

About Measles virus, it is also a locally circulating disease in urban communities and is an epidemic disease with a cycle of 2–3 years or longer depending on each country. Measles occurs seasonally. Measles occurs at different times if in different geographic areas.

Measles occurs at different times if in different geographic areas. In temperate climates, measles usually occurs in late winter and early spring. In the tropics, measles is more common in the dry season (Griffin et al., 2012). Risk factors for measles infection have been specifically studied, including immunodeficiency caused by HIV or AIDS (Gowda et al., 2012), immunosuppression after organ transplant or stem cell transplantation, alkylating agents (Waggoner et al., 2013). Additionally, vitamin A deficiency is an important factor associated with measles in children. A 2017 study showed that vitamin A supplementation significantly reduced mortality and morbidity in children with measles (Imdad et al., 2017). The recommended dose of vitamin A for all children with acute measles is 50,000 IU for infants <6 months, 100,000 IU for children 6 to 11 months, and 200,000 IU for children >12 months. Vitamin A should be taken by mouth once daily for 2 consecutive days with the above dose (Lindberg et al., 2015). In addition to vitamin A, zinc supplementation for children with measles is also mentioned, however, it has not been adequately studied for its effects and dosage. The factor that seems most important is the measles vaccine. Before the popular measles vaccine, over 90% of people before the age of 20 have contracted measles, very few people are not infected with measles. According to WHO statistics, about 100 million people get sick and 6 million people die from measles each year. However, after the dissemination of the measles vaccine, measles has seen very positive improvements, such as an 80% reduction in deaths between 2000 and 2017. By 2017, 85% of children worldwide received the first dose (as the report by WHO).

Mumps occurs worldwide. However, the incidence is higher in densely populated areas with low living standards, and in areas that are often cool or cold. Mumps also has an outbreak time depending on geographic location. It usually occurs year round in the equatorial region and winter and spring in the northern and southern regions of the world (Anon, 2007). The cool, cold, and dry climate allows mumps to spread more strongly. Mumps usually occurs in preschool children, or high school children, can also be found in older children or adolescents and the elderly at a lower rate (Di Pietrantonj et al., 2020). The incidence of mumps is significantly different between men and women, men often suffer more than women. The only known natural host of the mumps virus is humans. Mumps is spread through direct contact or through airborne droplets from the upper respiratory tract of an infected person. Mumps is less susceptible to comorbidities, but is spread mainly by close contact.

RSV is like measles and mumps virus, it varies from season to season but depends on geographic area around the world. The disease usually occurs in winter-spring in temperate climates and occurs in all seasons around the equator. The biggest risk factor for serious illness from RSV is age (Hall et al., 2009). Several epidemiological studies have also identified several factors that can increase the severity of RSV infection, specifically, tobacco smoke and cooking smoke, low socioeconomic status, associated medical conditions. . . (Chen et al., 2014, Wu et al., 2015). The patient's immunity and pathology can affect the patient including premature birth, low birth weight, immune disorders, genetic and chromosomal abnormalities such as Down syndrome and congenital heart defects (Cilla et al., 2006, Fixler, 1996).

HPIV lasts only a few hours and is easily destroyed by soap, detergents, disinfectants and even water. The pH, humidity and temperature of the environment are important factors affecting the survival of HPIV. A pH of 7.4–8.0 causes the infectiousness of HPIV to decrease. In addition, high temperatures above 37 °C and low humidity also reduce the possibility of virus infection (Hambling, 1964). Hospital infection is also one of the causes of the disease. Infection due to chronic medical care or surgery is one of the 'hot spots' of the health sector. In addition, other factors that have been studied that are associated with disease risk include malnutrition, vitamin A deficiency, not breastfeeding, environmental pollution, seasons and crowding (Henrickson, 2003).

Controls of paramyxoviridae viruses

Measles virus vaccine was discovered and widely used. This vaccine is formulated from wild species, then cultured under conditions that render them lethal without losing inherent immunity (Sood et al., 2017). Currently, an important public health strategy for reducing measles deaths is routine measles vaccination for children, combined with mass immunization campaigns, especially in countries with high morbidity and mortality rates. Currently, developed countries often use a combination vaccine for measles, mumps and rubella (MMR), known as the German measles vaccine. Children need their first shot at 12–15 months of age. Then, when the child is 4–6 years old, parents need to give their child a booster shot, or it can also be repeated any time 4 weeks after the first shot. In countries with relatively high rates of measles, the first shot is possible at 6 months of age (Bester, 2016). Common use of the measles vaccine has persisted for nearly 60 years. This vaccine has many advantages such as safety, effectiveness and cost. However, the measles vaccine is a live-attenuated one, so pregnant women, children with primary immunodeficiency, children with tuberculosis, cancer patients, transplant patients, patients used immunosuppressants or with advanced AIDS should not be used for treatment (World Health Organization, 2019). On the other hand, within 6 days of exposure the source of infection, use of globuline can prevent or reduce the severity of measles. This is expensive precautions and not popular. Even in developed countries, only a few cases are recommended to use this method, it is a pregnant woman who is not immune to measles and children were born to mothers do not have immunity against measles. . . So the simplest and most effective method is still vaccination according to the national program.

For mumps, the most common preventive measure is vaccination against mumps. This vaccine was developed by American microbiologist Maurice Hilleman (Buynak et al., 1969). The mumps vaccine can be given alone or as a combination of mumps, measles, rubella or mumps, measles, rubella and varicella (MMRV) vaccine (Di Pietrantonj et al., 2020). The use of the mumps vaccine is recommended by WHO in all countries with childhood immunization programs. In 2015, out of 194 WHO member countries, 121 countries (62%) included mumps vaccine in their national immunization programs, most of which used MMR vaccine. Since then, good results have been achieved in reducing the morbidity burden caused by mumps, for example the incidence

of the disease has decreased significantly in countries where large-scale immunization has been introduced. The most obvious example is in the United States, since the mumps vaccine was widely used in December 1967, the incidence of mumps has steadily declined, falling from 1,51,209 cases in 1968 to 265 cases per year from 2001 to 2008 (Jajosky et al., 2006). In addition to the vaccine, mumps also has therapeutic measures, but it is only supportive. Treatments for symptom relief include applying ice or hot compresses to the affected neck or testicles and using acetaminophen to relieve pain (Davis et al., 2010). A very important measure to prevent mumps is quarantine. Mumps is most contagious 5 days after symptoms first appear, should be quarantined in this time. Standard precautions are needed for hospitalized patients. Medical staff with mumps should be absent for 5 days (Kutty et al., 2010).

As mentioned above, worldwide there are 33 million lower respiratory cases and up to 199,000 child deaths caused by RSV. The burden of disease has made the development of the RSV vaccine a priority for over 50 years. However, there is currently no vaccine available for any of the age groups where RSV normally causes illness including infants under 6 months old, babies 6 months to 24 months old, pregnant women and the elderly (Schmidt and Varga, 2017). There is now a drug to help prevent the serious RSV disease called Palivizumab. This medication is often used for premature infants, congenital heart disease, or chronic lung disease. Palivizumab can help prevent RSV from getting worse, but it cannot cure or treat children who already have RSV, nor can they prevent RSV infection (Andabaka et al., 2013). From there, proactively preventing the spread of RSV is the most important and effective measure. As recommended by the CDC, if you have cold-like symptoms or suspected RSV, don't cover coughs and sneezes with your hands, and instead use a tissue or the upper sleeve of a shirt. In addition, it is advisable to wash your hands frequently with soap for 20 s, avoid direct contact or shake hands and share personal equipment with a suspected person.

Like RSV, HPIV also has no specific vaccine that has been found. The trials found the vaccine strains of HPIV specificity-1, HPIV-2 and HPIV-3 were conducted (Sato and Wright, 2008). There was a trial that used recombinant technology to create vaccines for three strains of HPIV-1, 2 and 3, which resulted in a number of live vaccines attenuated. These vaccines have been shown to have good immunity and resistance to HPIV-3 in phase I trials. However, for strains HPIV-1 and HPIV-2, the result was worse (Durbin and Karron, 2003). However, these vaccines are quite limited and are not an effective one. In addition to vaccines, maternal antibodies may provide some degree of protection against HPIV through colostrum in breast milk in early childhood. The use of drugs to treat HPIV has also been found. There is a drug called Ribavirin, which is a broad spectrum antiviral and is currently being used by people with severe immunodeficiency, but its benefits are not really clear. If a secondary infection occurs, antibiotics may also be used. Antibiotics commonly used are corticosteroids, along with a nebulizer to treat lung cancer if breathing problems occur (Sato and Wright, 2008).

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Papillomaviruses

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Glossary

Carcinogenesis Cancer inducing factors is a multistage phenomenon involving modification of genes by mutagenesis that important in regulation of normal cellular function and cause cell transformation.

Cervical cancer A sexually transmitted viral cancer that occurs in the cells of the cervix with several types of the human papillomavirus.

Papillomaviruses A group of DNA viruses that cause the formation of papillomas or warts.

Persistent infection Persistent infections are characterized as those in which the virus is not cleared in acute phase of infection and last for long periods in the specific cells of infected individuals.

Species-specific A substance or organism that is limited to or found only in one particular species.

Viral clearance A function of the innate immune system, consistent with the rapid kinetics of virus elimination and defined for removal of virus contamination or refers to the time it takes for a virus to no longer be detected in samples or in side tissues.

Virus-like particles (VLPs) The molecules that contain multiprotein structures are closely similar to viruses but are non-infectious.

History

The timeline of virus emergence and evolution have been inspected using ancient DNA, in which evolutionary analysis is performed based on DNA viruses derived from archival materials. This approach has been more successful in the case of human wart-causing papillomaviruses (PVs) as the important examples of ancient DNA virus. Their infectious viral nature was recognized in the early 1900s. Subsequently, PVs were identified in a variety of vertebrate species in addition to humans. The PVs are species-specific in their host ranges, the biology of human PVs must be investigated in their natural host. The first animal PV was identified in the 1930s by Richard Shope. The Shope papillomavirus, now designated the cottontail rabbit papillomavirus (CRPV), that was the first identified DNA tumor virus. Some of the human papillomaviruses causing benign papillomas, but the others undergo to malignant progression. The molecular cloning of PVs in the 1970s, significantly enhanced the ability to study their biological characteristics and biochemical properties. The bovine papillomavirus type 1 (BPV1) represented as a prototype virus in *Papillomaviridae* because the virus induced focal transformation in rodent cell lines (Black et al., 1963). The molecular cloning and sequencing of the cloned PV genomes led to the identification of open reading frames (ORFs) as putative viral genes of the human *papillomaviruses* (HPV) and recognized that they have multiple HPV genotypes, and some types was closely associated with human cancers, including cervical cancer (Fields et al., 2013; Furumoto and Irahara, 2002).

The typical members of *Papillomaviruses* had particular unique characteristics including:

- I. Papillomaviruses are small, non-enveloped, icosahedral DNA viruses and their size, which at 52–55 nm and composed of 72 pentamers.
- II. The genome consists of circular double-stranded DNA (C-dsDNA) molecule of about 8000 base-pairs (bp) that is bound to cellular histones.
- III. The genomes have an average GC content of about 42% (36–59%).
- IV. The nucleus is the site of viral replication and virion assembly.
- V. Papillomaviruses are highly species-specific, tissue-restricted, and do not infect other species even under laboratory conditions; therefore, humans are the only known reservoir for HPVs.
- VI. Papillomaviruses have been characterized by molecular methods.
- VII. About 90% of HPV infections cause no symptoms and resolve spontaneously within 2 years (Ljubojevic and Skerlev, 2014).
- VIII. HPVs cause mucosal and cutaneous lesions in humans, and in some cases, HPV infection persists for years and results in either warts or precancerous lesions.
- IX. Translation of early and late transcripts, alternative splicing, alternative open reading frames.

Classification of papillomaviruses

Taxonomy

Classification of the *Papillomaviridae* is based on sequence identity across the L1 open reading frame (Van Doorslaer et al., 2018; De Villiers et al., 2004). The virus taxonomy (2019 Release) revealed the *Monodnaviria*, *Shotokuvirae*, *Cossaviricota*, *Papovaviricetes*, *Zurhausenvirales*, *Papillomaviridae*, *Firstpapillomavirinae*, *Alphapapillomavirus*: *Alphapapillomavirus* 1–11, 13 and 14. The *Betapapillomavirus* containing *Betapapillomavirus* 1–4, *Gammapapillomavirus* containing *Gammapapillomavirus* 1–5, *Mupapillomavirus* containing *Mupapillomavirus* 1–2 and finally *Nupapillomavirus* containing *Nupapillomavirus* 1 up to final released data (Krupovic and Koonin, 2017). The family includes two subfamilies, *Firstpapillomavirinae*, which includes 53 genera, 133 species, and *Secondpapillomavirinae*, with a single genus and species. This chapter focuses on the human *papillomaviruses* whose member genres include *Alphapapillomavirus*, *Betapapillomavirus*, *Gammapapillomavirus*, and *Deltapapillomavirus*. Genera are named according to the Greek alphabet. The high-risk human papillomaviruses (HR-HPV) which known as HPV 16 and 18, cause more than 99% of cervical carcinomas belong to the subset of mucosotropic HPVs alpha genus (Walboomers et al., 1995). A subset of cutaneous HPV types classified into the beta genus (*Betapapillomaviruses*), called epidermodysplasia verruciformis (EV) which, are consistently found in non-melanoma skin cancers (De Villiers, 1998). These skin lesions mainly arise at sites of the body that are exposed to the sun or during infection with human papillomaviruses (De Villiers et al., 2004).

The Papillomaviruses important genera, are classified based on ICTV2019 release are shown in Table 1.

Virion structure and family characteristics of HPVs

The papillomaviruses comprise a group of small, nonenveloped epitheliotropic icosahedral DNA viruses, that replicate in the nucleus of squamous epithelial cells. The papillomaviruses induce benign lesions of the skin (warts) and mucous membranes (condylomas). Some PVs have been implicated in the development of epithelial malignancies, especially cancer of the uterine cervix, other urogenital tract, and upper airway cancers (Fields et al., 2013).

The epidemiological data determined the more frequent association of specific species at HR-HPV types. Moreover, molecular cloning of the HPV genomes showed multiple HPV genotypes, that high risk types are closely associated with human cancers. PVs are classified principally according to the host species and have been traditionally referred to as types based on their DNA sequence. The PV genomes have been organized phylogenetically on the base of their DNA sequence, according to the relative homology of

Table 1 Papillomavirus classification based on ICTV2019 by focusing on important HPVs.

Family Papillomaviridae		Biological properties
Subfamily	Genus	
First papillomavirinae	53	
Second papillomavirinae	1	
Alpha-papillomavirus		Cause Mucosal and cutaneous lesions in humans with High and low-risk classification based on molecular biological data, immortalize human keratinocytes
Beta-papillomavirus		Cause cutaneous lesions in humans, infections exist in latent form in general population, activated under conditions of immune suppression. Also referred to as EV-HPV types due to close association with EV
Gamma-papillomavirus		Cutaneous lesions in humans, Histologically distinguishable by intracytoplasmic inclusion bodies specific for type species
Mu-papillomavirus		Cutaneous lesions in humans, Histologically distinguishable by intracytoplasmic inclusion bodies specific for type species
Nu-papillomavirus		Human papillomavirus Benign and malignant cutaneous lesions

the L1 ORF (Bernard et al., 2010). All PVs have a similar genetic organization comparable to the L1 DNA sequence between the most divergent genomes. Instead, two very closely related isolates may differ by only a single nucleotide (Bernard et al., 2010). A distinct type is share at least 10% nucleotide sequence different of other HPV types. HPVs are clustered to high-risk and low-risk HPV types based on molecular biological data including high-risk types for pre-cancerous and malignant lesions, and low-risk types containing benign lesions. Low-risk HPV types include types 6, 11, 42, 43, and 44. High-risk HPV types include types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70 (Ahmed et al., 2015).

HPV genome and ORFs

The PV particles are around 60 nm in diameter and consist of a single molecule of double-stranded circular DNA about 8000 base pairs (bp). The genomes of all HPV types contain approximately eight ORFs and all of them are located on one strand of the viral DNA. The ORF can be divided into three functional parts: the early (E) region encodes proteins (E1–E7) that are transcribed from the early promoter and essential for viral replication; the late (L) region encodes the structural proteins (L1–L2) that are transcribed principally from the late promoter and required for virion assembly; and a largely non-coding part which known as the long control region (LCR), contains cis-acting elements that are essential for the replication and transcription of viral DNA (Fig. 1). The viral LCRs contain constitutive enhancer elements that play an important role in the initial expression of the viral genes after virus infection and may also be important in the maintenance of viral latency. The E1 and E2 proteins of HPV act as factors that recognize the origin of replication. E4, is involved in the late stages of the life cycle of the virus and E5 may function during both early and late phases. The E6 and E7 proteins trigger a number of suppressor regulatory proteins of the cell cycle, primarily p53 and p105-Rb, respectively. Although, during the viral life cycle, E6 and E7 enable stable maintenance of viral episomes and induced cells differentiating to entering the S phase. The viral late functions, such as vegetative viral DNA synthesis, L1 and L2 late capsid protein synthesis, and virion assembly, occur exclusively in differentiated keratinocytes. Virion assembly and release takes place in the nuclei of terminally differentiated keratinocytes in which vegetative viral genome replication and expression of the virion proteins has occurred (Fehrmann and Laimins, 2003).

Life cycle and replication pattern of HPVs

The entry of HPVs to keratinocyte stem cells occurs through small wounds or close contact in the skin or mucosal surface. The virion transports to endosomes, the minor capsid protein L2 disrupts the endosomal membranes through a cationic cell-penetrating peptide and finally lead the viral genome to escape and enter to the cell nucleus, along with L2 (Zhang et al., 2018). After successful infection of a keratinocyte, the virus expresses E1 and E2 proteins, which are necessary for replicating and maintaining the viral DNA as a circular episomes. Papillomaviruses are extremely epitheliotropic and able to establish productive infections inside the skin or mucosal epithelia. The viral life cycle is related to the differentiation steps of the infected epithelial cells. As indicated, life cycle is initiated by the infection of basal epithelial cells, probably at the sites of injury by several potential receptors, that have been reported. After entry to the basal cells, the viral genome is transported to the nucleus, where it undergoes a limited amplification to a low copy number, and early genes are expressed preferentially at low levels. The early steps in the virus replication cycle, such as attachment, uptake, endocytosis, transport to the nucleus, and producing uncoated viral DNA, occurred as an early limited event. The early gene transcription is followed by translation of the E proteins and steady state viral DNA replication. The late events in the viral life cycle that lead to the production of virion particles occur in the differentiated keratinocyte, including vegetative viral DNA replication, transcription of the L region, and production of the capsid proteins L1 and L2. Assembly of the virion particles and release of viruses occurred in the nucleus. In the second stage of replication, these genomes are maintained as a stable multicopy plasmid at a constant copy number in the mitotically active basal cells (McBride, 2008; Fields et al., 2013). The ability of HPVs to establish their genome in basal cells depend on the E1, E2, E6 (Hubert and Laimins, 2002), and E7 genes expression (Flores et al., 2000). Generally, during basal cell division, the daughter cell migrates into the suprabasal compartment and initiates terminal

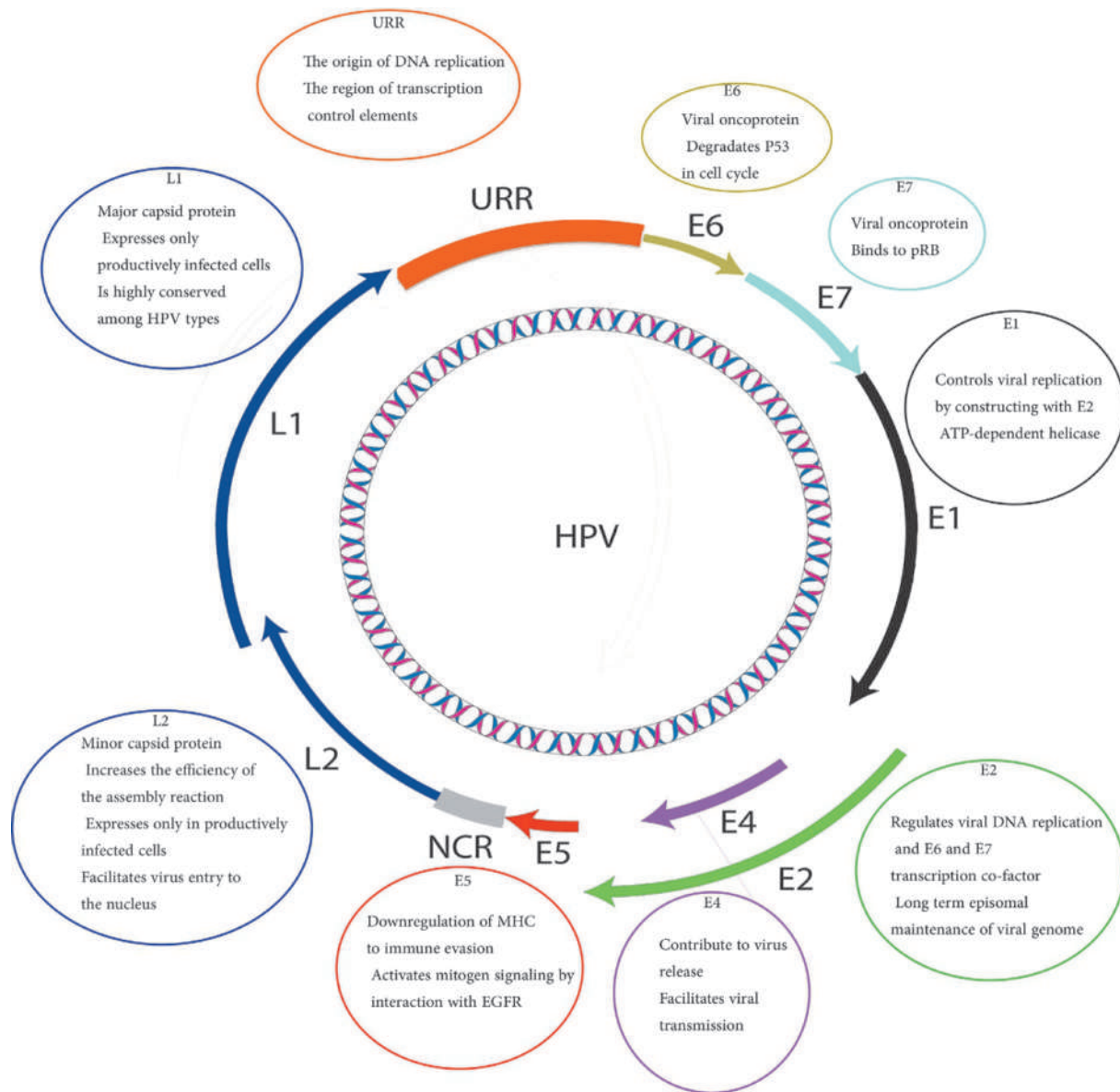


Fig. 1 Schematic representation of the HPV genome organization. The diagram illustrating the position of papillomavirus genes within the genome and their functions. Early regions involved in replication and transcription and late regions participate in viral structural proteins production.

differentiation. In HPV positive human keratinocytes and cervical epithelial cells, the suprabasal cells continue to support DNA synthesis and cell proliferation through HPV 16 E7 protein (Flores et al., 2000). Replication rates of papillomaviruses designated low and occurs concomitantly with the division of host epithelial cells. DNA viruses often replicate more rapidly than do their hosts. The HPVs activate the DNA replication machinery, by overexpression of E6 and E7 proteins to support vegetative viral DNA synthesis for packaging of progeny virions (Flores et al., 2000). This pattern of progeny production seems to be due to a tendency to establish persistent infections.

Pathogenesis

Human papillomavirus causes a wide range of tumors, from benign papilloma lesions (HPV types 6 and 11) to invasive squamous cell carcinoma (HPV types 16 and 18). All papilloma-based lesions can be benign or malignant, such as laryngeal and anogenital papillomas, which are clinically similar to other superficial squamous or invasive cervical squamous lesions (Burd, 2003). There is nowadays sufficient evidence that certain, so-called “high-risk” types of HPV are carcinogenic to humans in the cervix (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66). In developing countries, carcinoma of the cervix uteri is the most frequent type of female (Farahmand et al., 2021).

HPV DNA can be found practically in all patients with cervical cancers, head and neck cancers, recurrent respiratory papillomatosis (laryngeal papillomatosis). The most predominant type in epidermoid carcinomas is HPV16 (Omland et al., 2014; Fields et al., 2013). HPV DNA was demonstrated in more than 50% of the less-prevalent carcinomas of the vulva and penis, and HPV16 is again the most frequent type, followed by HPV18. HPV infection is adequate to induce cancer cells in combination with other spontaneous or virus-induced modifications (Zur Hausen, 1991). The viral genes E6 and E7 are required to trigger these effects. They encode proteins that inactivate tumor suppressors and modulate cell-cycle regulation, DNA repair, and apoptotic processes by interacting with the cellular suppressor proteins of p53, p105-RB, p21, p27, Bak, and PDZ domain proteins. E6 activates the catalytic subunit of the telomerase as an important step in cell immortalization. E6 and E7 induce chromosomal instability, mitotic defects, which will contribute to tumor progression. The E6 and E7 proteins of high-risk viruses display much higher affinities to the cellular proteins than low-risks. High risk HPV DNAs appears integrated into the cellular genome in almost all HPV based cancers, which is a hallmark of cervical cancers.

Persistence and latency in HPVs

Persistence infection (long-duration of detectable HPV infection) is infrequent compared with clearance of infection, but the longer duration of infection with high-risk types of HPV may have implications for the pathogenesis of cervical intraepithelial neoplasia 3 (CIN3) and cancer (Moscicki et al., 2012). Latency for HPV infection has been described in animal models and recently, reactivation of latent infection has been demonstrated among immuno-compromised individuals (Maglennon et al., 2014).

Important oncogenic proteins of HPVs

E5 protein of HPVs

HR-HPV E5 is considered to be tumorigenic protein, because it transforms murine fibroblasts and keratinocytes in tissue culture systems. The E5 protein enhances the immortalization potential of E6 and E7 and, in association with E7, stimulates the proliferation of human cells. The major biochemical activities of HPV 16 E5 have been recognized in vitro by the ability of protein to enhance the activity of the epidermal growth factor receptor (EGFR) in the presence of ligand (Straight et al., 1993; Zhang et al., 2005).

E6 protein of HPVs

The E6 protein of HPV targeted p53 and association of E6 with p53 leads to degradation of p53 via recruitment of a ubiquitin ligase E6-AP. This interaction leads the inhibition of the transcriptional regulatory activities of the p53 protein in tissue culture cells (Li et al., 2019). The transforming gene products of papillomaviruses E6, required for efficient immortalization of primary human fibroblasts as well. Moreover, E7 cooperates with E6 to transform primary rodent cells and also requires for efficient immortalization of primary human fibroblasts or keratinocytes.

E7 protein of HPVs

HR-HPV E7 proteins are best known for their ability to associate with the cellular tumor suppressor protein of pRb (Gage et al., 1990). Association of HR E7 with pRb induced the degradation of pRb, through a proteasome-mediated pathway and disrupts the capacity of pRb to bind and inactivate cellular E2F transcription factors (Gonzalez et al., 2001; Fields et al., 2013). Correspondingly, several researchers showed that HR E7 combines with cyclin A/CDK2 complexes and inactivates p21 and p27. Therefore, E7 can interact with or alter numerous cellular factors that are involved in cell cycle regulation (Berezutskaya and Bagchi, 1997).

Other early and late proteins of HPV

Viral proteins E1 and E2 are assisting in the establishment of 20 to 100 episomal copies per basal cell and responsible for the maintenance of a low copy number of HPV genome. E2 is a DNA-binding protein that recruits E1 to the origin of replication, but also serves to regulate transcription of E6 and E7 from the early promoter. By expressing the early viral genes (E1, E2, E6, and E7), genomes of viruses replicate during host cell replication. The HPV genome migrates from the basal layer and in differentiated cells, production of E4 protein induces viral genome replication. Products of late genes L1 and L2 are needed to form new virions, which reach the cornified layer of the epithelium and are released after assembly (Fields et al., 2013). The main roles of individual produced proteins during the HPV cell cycle described in Table 2.

Incidence, persistence and clearance

Several published studies provide data on incidence of HPV infection and duration of clearance or latent infections. Approximately 5–15% of HPV-negative women are infected each year with one of the high-risk types of HPV. Moreover, the incidence of infection with high-risk HPV types rises to be higher than low-risk types (Muñoz et al., 2004). The most common types of infection occurred

Table 2 Human papillomavirus proteins and their functions.

<i>Protein</i>	<i>Function</i>	<i>Most important biological properties of HPV proteins</i>	<i>Associated cellular/viral proteins</i>
E1	Acts as adenosine triphosphatase (ATPase) and DNA helicase; necessary for replication of viral DNA	Transcription factor & helicase activity, Mediate episomal DNA replication	binding to specific origins of replication of viral DNA
E2	Main regulator of viral gene transcription; DNA replication and viral genomes segregation	Transcription factor & Regulate viral copy number, Regulate early gene promoters	The interaction of E2 with E1/
E3	Not known	Exists only in a few papillomavirus types with unknown function	–
E4	Acts late in the viral life cycle; favors viral genome amplification, regulates virus maturation	Facilitates virion release by destabilizing cyokeratin networks in epithelial cells	E1 E4 arrest cell cycle in the G ₂ phase
E5	Enhances E6 and E7 transforming activity, promotes chromosomal instability	Stimulate cell proliferation & Prevent differentiation, Down regulates surface MHC class I expression. Stimulate mitogenic signals of growth factors	E5 regulates growth signaling pathways & activates EGFR and the downstream Ras-Raf-MAP kinase pathway
E6	Disrupts normal regulation of host cell cycle via p53 degradation in the presence of E6AP, inhibits apoptosis, and activates telomerase	Viral major oncoprotein, Immortalization, Genomic instability, DNA damage responses, Cell proliferation and differentiation in suprabasal DNA synthesis, Induced malignant transformation together with E7	Interact with p53 & reduce p53 degradation potential, Bak, Myc, FADD/ Caspase 8
E7	Degrades tumor-suppressor protein pRb, affects the expression of S phase genes by interaction with transcription factors and histone deacetylases	Immortalization, Genomic instability, DNA damage responses, Cell proliferation and differentiation in suprabasal DNA synthesis, Induced malignant transformation alone or together with E6	E7 with their multiple cellular targets such as pRb and related super family
E8	Not known	The E8 genes are chemically and functionally similar to the E5 genes from some HPVs, and are also called E5/E8	–
L1	Major viral structural protein required for attachment to cell surface receptor	Major capsid protein & is the major component of the HPV prophylactic vaccine	The L1 protein has DNA binding activity and receptor binding protein, Containing nuclear localization signal (NLS)
L2	Minor viral structural protein; interacts with DNA. Contributes to viral localization into the nucleus and helps the packaging of viral DNA into capsids	Minor capsid protein & is an attractive candidate for cross-protective vaccines & disrupts the membrane of the endosome through a cationic cell-penetrating peptide	L2 escorts the genomic DNA into the nucleus, Cellular surface protein binding with L2 affect the cell tropism of mucosal HPVs.

by HPV 16, 18, 31, 33, and 51 among younger women respectively (Harper et al., 2004). Most HPV infections are transient and become undetectable within 1–2 years. On the other hand, the persistence of HPV infection is crucial for the development of cervical lesions and cancer. Undoubtedly, it seems that HPV infections are self-limited and cleared completely by the cell-mediated immune system or are suppressed into long-term latency (Fields et al., 2013).

Integration of HPV sequences

The papillomavirus DNA genome is frequently found integrated into the host chromosome. Integration of HPV genomes affects both viral and host gene expression. Integration of HPV can be found in premalignant lesions, particularly in grade 2/3 cervical intraepithelial neoplasia (CIN2/3). Cervical epithelial cells that anchorage integrated HR-HPV DNA have a selective growth advantage over cells that contain extrachromosomal viral genomes. It is the result of overexpression of E6 and E7 viral genes and continuous expression of these proteins can lead to the accumulation of mutations in the cellular genome (Harper et al., 2004; Fields et al., 2013).

Chromosomal abnormalities in HPV-associated cancers

Several chromosomal changes have been described in HPV-associated cancers. Aneuploidy, loss of heterozygosity are the most frequent events which associated with progression from high-grade lesions to cervical cancer (Nishimura et al., 2000).

Alterations of specific proto-oncogenes

Several studies have addressed the structural or functional alteration of different proto-oncogenes during HPV infection. Most of these alterations were evocative in nature and few were designed to correlate with progression of disease. A straight role for these genetic alterations in HPV-associated carcinogenesis is difficult to establish, but the RAS mutations in the RAS family of oncogenes have been described in premalignant and malignant cervical carcinomas (Mammas et al., 2004).

The c-MYC and HER-2/NEU amplification during HPVs infection

HPVs have been shown to integrate in the proximity of c-MYC, which altered HPV-associated lesions production. Abba et al. (2004) described c-MYC amplification in a high proportion of cervical cancers compared with benign and premalignant cervical lesions. Moreover, a significant association between c-MYC amplification and HPV 16 infection was observed. Although, HER-2/NEU mRNA and protein were detected in a large proportion of cervical adenocarcinomas. Preferential expression of this proto-oncogene was more strongly associated with lesions that contained HPV 16 (Abba et al., 2004).

Epigenetic events

Epigenetic events that involved the methylation of viral genes or LCR of HR-HPVs have been described. The LCR of HPV 16 and 18 (Badal et al., 2004) are hypermethylated in normal and low-grade cervical smears than high-grade smears. This incident correlates with increased transcriptional activity of the early region and consequently greater availability of the E6 and E7 proteins. Hypermethylation of cellular gene promoters has also been observed in cervical carcinomas (Zafari et al., 2018; Dong et al., 2001).

Methods for the detection of HPV infection

Microscopic abnormalities

Microscopic abnormalities are diagnosed in only a minority of women who have detectable HPV by DNA assays. Microscopic diagnoses are disposed to subjectivity and lack of interobserver reproducibility, particularly when mild or confusing changes are involved. The persistence of at least one carcinogenic HPV type is the necessary state for the emergence of precancer. However, other aspects of infection may contribute to the likelihood that an infection will progress, such as type of virus, viral load, and concurrent abnormalities. Among the oncogenic HPV types, HPV 16 is exclusively associated with considerable risk for cancer. Low viral loads, which detectable only by PCR, are associated with normal microscopic view. The value of increasing viral loads with respect to subsequent prediction of lesions, has not been established obviously (Lorincz et al., 2002).

Progression to precancer

The first critical difficult task is to define “precancer” lesion from precancerous ones, on the basis of histology. There is substantial heterogeneity in the microscopic diagnosis and biological meaning of CIN2 lesions in particular. Some acute HPV infections are poorly diagnosed by microscopic examination, while others are preliminary precursors that are supposed to continue at high risk of attack. Some non-carcinogenic HPV infections can cause lesions known as CIN2, indicating that this level of abnormal microscopic appearance is not a sufficient risk factor for cancer. CIN3 should be used as a surrogate for precancer and CIN2 as a buffer zone of ambiguous diagnosis. The attention should be restricted to women with carcinogenic types of HPV. The interval period between the onset of HPV infection and the incidence of precancerous lesions is about 7–10 years in young people. In general, cervical malformations persist longer and are more likely to progress in women with carcinogenic HPV infection than in women with non-carcinogenic infections or no HPV (Schlecht et al., 2003).

The cytology diagnostic methods cannot provide reliable estimates of the extent of the lesion. Because the vast majority of infection in CIN2 grades are transient and regress normally in short time, but some may lead to CIN3 or cancer. The average age of women with invasive cancers are more likely to be frequent in older ages. In several studies, it revealed that HPV DNA integration is associated with invasion. However, it is difficult to demonstrate that it could be causative factor.

Serology test

The effect of serum antibodies to HPV serotypes 6, 11, 16, 18 to risks of subsequent genital HPV infections investigated in several studies. Serology of viral like particles (VLPs) by ELISA methods is a very useful epidemiological tool for defining previous and current exposure to HPV infection. The assays are reasonably type-specific and are usually negative in individuals who have never been infected (Kjaer et al., 2001). The produced antibodies following natural HPV infection are not sufficient to protect against subsequent infection among women, but it is the markers of previous infections; although, some studies showed that antibodies provide partial immunity against subsequent infections and precancerous lesions. Differences in study findings may be due to the use of different antibody assays, serum antibody levels, and time since first exposure to HPV (Wilson et al., 2014).

HPV DNA detection methods

Assessment of previous infections is challenging, especially among individual have been sexually active for a long period of time. Furthermore, current conventional HPV DNA detection methods may not be sensitive enough to detect low viral load of latent infection or reactivations that last for a short time period (Pamnani et al., 2018; Farahmand et al., 2021).

Distribution of the most common types of HPV in women with and without cervical cancer

In some areas, the HR-HPV 16 and 18 positive DNA in cervical cancer is estimated more than 70% (Clifford et al., 2003). The most important types which appear after HPV16 and 18 are HPV45 and other HR-HPVs. There is a possibility of HR-HPV coinfection with two or more HPV genotypes as well, which can lead to cancer (Zeng et al., 2016). The highest incidence of cervical cancer was shown in women aged more than 60 years old in most areas, that the HPV vaccination is currently not a part of the national immunization program (Farahmand et al., 2021).

Innate immune responses against papillomavirus infection

HPV can gain access to the skin through the compromised integrity layers. In fact, epidermidis as an external surface is the first line of defense to protect humans in viral infection warfare and composed of basal germinal, granular, and keratinized layers (Flint et al., 2020).

Keratinocytes act as a non-professional antigen-presenting cell (APC) and participate as a part of the innate immune system to present antigen and provoke type 1T helper (T_{H1}) and type 2T helper (T_{H2}) cells expression through $CD4^+$ and $CD8^+$, respectively. Indeed, keratinocytes play as a cross link cell to improve the adaptive immune response (Nestle et al., 2009; Amador-Molina et al., 2013). Both keratinocytes and innate immune cells are nonprofessional cells that express pattern recognition receptors (PRR) which skilled to recognize pathogens thus named pathogen-associated molecular patterns (PAMPs) and also identify damage signals which known as damage-associated molecular patterns (DAMPs) (Nunes et al., 2018; Barros et al., 2018).

In this regard, the toll-like receptor (TLR) family may locate through expression on the cell surface or in endosomes. TLR 9 is an endosomal immunological receptor which able to recognize unmethylated CpG double-stranded DNA viruses such as HPV. TLR 9 can promote cell signaling to produce cytokines and type I interferon (IFN-I). It reveals that TLR 9 may perform as a key factor in viral clearance during HPV infection (Barros et al., 2018; Zhou et al., 2019; Hibma, 2012). There are some evidence that TLR 9 activation in keratinocytes lead to the production of tumor necrosis factor (TNF)- α , IL-8, CCL2, CCL20, and CXCL9 in addition to IFN-I (Lebre et al., 2007; Miller and Modlin, 2007). TLRs engagement with their ligands leads to activate the nuclear factor κ B (NF κ B) through transducing Toll/IL-1 receptor (TIR) signals into the cells and culminate this transcription factor to regulate inflammatory chemokines and cytokines (Barros et al., 2018; Delves et al., 2017).

Papillomavirus regulate the IFN amplification pathway and innate immunity

Type I interferon which known as antiviral interferon is the part of the innate immune system. They have also immunomodulatory and proliferative properties (Murphy and Weaver, 2016). IFN-1 secretion was climaxed within PRRs signaling transmission and followed by downstream IFN-regulatory factors (IRFs) pathways, the Janus kinase/signal transducers and activators of JAK-STAT transcription factors. Then, the NF- κ B signaling pathway restricts establishment of infection (Fensterl et al., 2015; Schneider et al., 2014). TLR 9, TLR 4, and IFN-inducible protein 16 (IFI16) PRRs can perceive the genome of HPV following initial infection and lead to IFN-1 expression (Cigno et al., 2015; Bahramabadi et al., 2019; Ferreira et al., 2020). Interferons are obviously important factors in innate immunity system to inhibit viral infection and obstruct tumoral effects. Moreover, they have an immune regulatory activity to protect cells around the disturbed region (DeCarlo et al., 2012). Signaling of TLR 9 leads to induction of type 1 IFN or inflammatory cytokines through myeloid differentiation primary response protein (MyD88), MyD88-IRF7 and MyD88-NF- κ B, respectively. MyD88 accomplishes as an adaptor and several TLRs require it to distribute their signals. MyD88 forms mentioned complexes and IRF7 phosphorylated by IL-1 receptor-associated kinase1 (IRAK1), IL-1 receptor-associated kinase4 (IRAK4), or I κ B kinase α (IKK α) and then displaced to the nucleus and result the activation of the type 1 IFNs gene expression (Fig. 2) (Barros et al., 2018; Kawasaki and Kawai, 2014; Zhou et al., 2013).

Different findings indicated that various types of human papillomaviruses which known as “high risk” such as HPV16 and HPV18 and “low risk” such as HPV6 and HPV11, make effects on down-regulation or up-regulation of TLR9 pathway by viral oncoproteins. (Fields et al., 2013; Yeo-Teh et al., 2018). HPV16 E6 and E7 oncoproteins play an important role to interfere with the innate immune response. Indeed, E6 and E7 are able to down-regulate TLR9 and lead to impaired activation of the immune system. It is notable that HPV16 is more significantly capable to promote down-regulation of TLR9 in comparison with HPV18 (Yeo-Teh et al., 2018; Hasan et al., 2007).

In keratinocytes, high risk HPVs augment the expression of IFN related developmental regulator 1 (IFRD1) and reduce RelA subunit at lysine 310 (K310) which cause acetylation of NF- κ B. Subsequently, acetylated NF- κ B lead to down-regulation of proinflammatory downstream cytokines such as IFN- α (Tummers et al., 2015; Westrich et al., 2017). The E7 oncoprotein of HPV may effect on TLR9 promoter through interfering in the NF- κ B pathway. Indeed, suppression of TLR9 expression leads to disturbing of IL-6 and IL-8 synthesis (Barros et al., 2018). In addition of IFN signaling, proapoptotic genes such as TNF-related apoptosis-inducing ligand (TRAIL) and X-linked inhibitor of apoptosis (XIAP)-associated factor 1 (XAF1), other RPRs (Retinoic acid-inducible gene I (RIG-1) and melanoma differentiation-associated protein 5 (MDA5) are also repressed by HR-HPV oncoproteins (Hasan et al., 2013; Reiser et al., 2011).

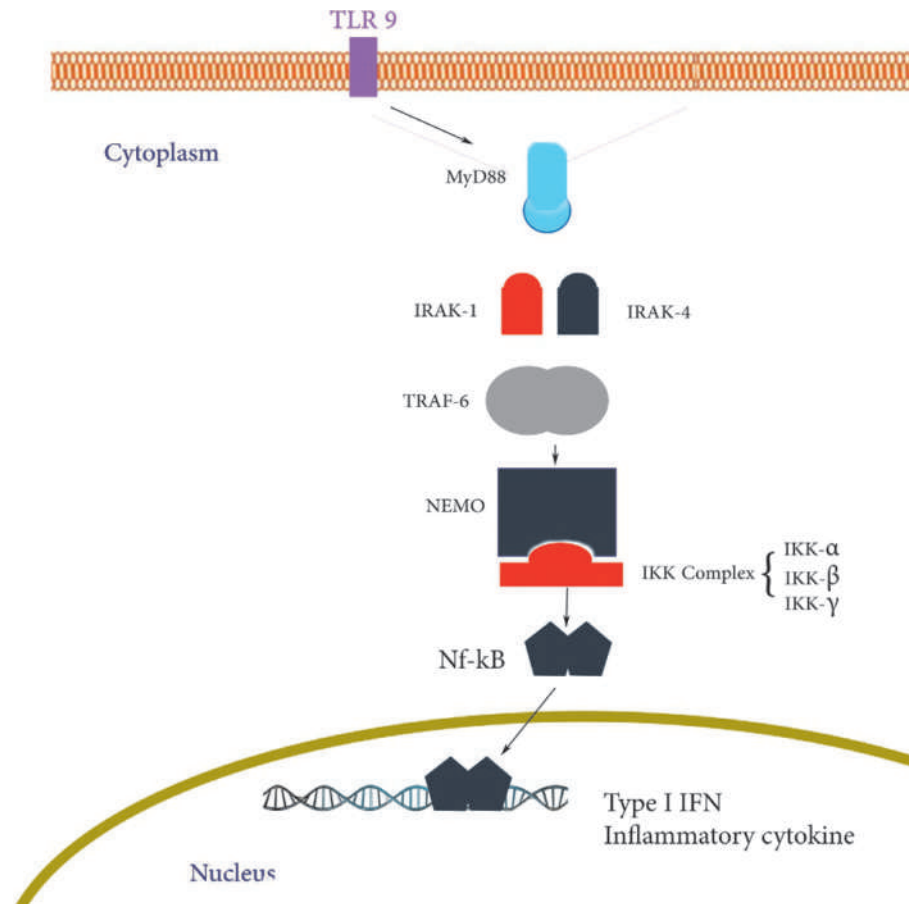


Fig. 2 TLR 9 signaling. TLR 9 is expressed on the surface of cells and recruited MyD88 lead to nuclear translocation of NF-κB in order to induce proinflammatory cytokines and type I interferon.

Adaptive immunity in papillomavirus infection

Both T-helper cells and cytotoxic T-cells (CTL) are representatives of cell-mediated immunity to repress HPV infection. T helper is included of two different phenotypes: T_{H1} and T_{H2} . There are differences in two subsets of T-helper cytokines. Actually, T_{H1} generates immune responses against intracellular infectious agents such as viruses and bacteria. On the contrary, T_{H2} is produced immune responses against extracellular pathogens (Steinbach and Riemer, 2018; Wakabayashi et al., 2019). The majority of T lymphocyte population in the cervical disorders in epithelium belongs to the subgroup of CD8 suppressor/cytotoxic T cells and the minority of them are CD4 helper/inductor subgroup. Initially, viral proteins present through antigen-presenting cells (APCs) by major histocompatibility class 1 (MHC-1) into the CTLs and destroy infected cells in this way (Wakabayashi et al., 2019; Gonçalves and Donadi, 2004). APCs of skin are divided into the Langerhans cells (LC) as the major population of immunity in the epidermis and dendritic cells (DC) in the dermis known as dermal DC (dDC). LCs which have langerin (CD207) as a receptor, are the only APCs which permeate in the dermis and inhabit there in the vicinity of infected keratinocytes (Hibma, 2012; Maarifi et al., 2020). It is obvious that the processed viral antigens in complex with MHC present to T cells receptors through DCs and activate them by multiplication of these cells. The activated $CD8^+$ T cells differentiate into CTLs and become the main source to produce perforin, granzyme, and proteolytic enzymes. Moreover, T_{H1} and T_{H2} are also differentiated from Activated $CD4^+$ helper T cells. T_{H1} generates IL-2, IL-12, IL-15, TNF- α , and λ -IFN whereas T_{H2} produces IL-4, IL-5, IL-6, IL-10, and IL-13 (Amador-Molina et al., 2013; Rosales and Rosales, 2014).

Role of adaptive immunity in the clearance of virus

T cells which express CD8 are known as cytotoxic T cells and predominantly induce perforin/granzyme in order to the contact-dependent mechanism to kill target cells (Litwin et al., 2021; Bhat et al., 2018). Ghosh et al. demonstrated that the predominant immune T cells in the case of cervical cancer were $CD8^+$ lymphocytes (Ghosh and Moore, 1992). It was also the same as in the study by Maskey et al. that showed infiltration of $CD8^+$ lymphocytes are greater than others in the epithelial layer of

positive HPV patients with normal cervical appearance (Maskey et al., 2019). Although, the reactivity of CD8⁺ to E6 as the oncoprotein in progressive stages of the majority of more patients with cervical cancer is predominant, but it is not the main response to viral clearance. In fact, this type of T cells have not been observed as a preventive response in front of tumor progression (Zhou et al., 2019). Nevertheless, a powerful CD4⁺ T cell response is necessary besides of CTL response in order to HPV clearance (Zhou et al., 2019). The relation between special types of papillomavirus and their clinical malignancies have revealed the significance of immune system functions and their ability to defend against viral disease (Steele et al., 2002). T cell responses are the most immunity in order to suppress papillomavirus infectivity because of the severe infection found in immunocompromised patients (Benton et al., 1992). Although keratinocytes of epithelial cells are triggered by HPVs as the main targets of infections, they are nonprofessional APCs. Actually, in normal condition with noninflammatory evidence MHC-II and other adhesions and stimulatory molecules such as CD80, CD8 and CD54 are not expressed by mentioned cells. Nevertheless, the keratinocytes may transfer antigen-specific signals toward T cells. The other cells within epidermal cells which known as LCs that previously mentioned above, are located upper the basal layer of proliferating keratinocytes. They are the professional APCs to capture papillomavirus antigens through macropinocytosis and receptor-mediated endocytosis on the surface of epithelial cells as the primary antiviral immune responses to papillomavirus infection (Steele et al., 2002; Sallusto et al., 1995). In cervical intraepithelial neoplasia (CIN), LCs diminution was found and it relates to the severity of the disease. In the former situation, LCs does not have detectable ability to express adhesion and stimulatory molecules and it suggests a poor condition of antigen presenting status. In contrast, the increased expression of these molecules in keratinocytes during severe disease accompanied with the accumulation of activated leucocytes under the lesion and development of CIN due to weak immune responses (Amador-Molina et al., 2013; Mota et al., 1999).

As a general rule, the main arm of immune response against intracellular agents and tumors is CD8⁺ T cells which activated due to receiving of presented antigens by MHC-I. Moreover, CD4⁺ T cells also interact with presented antigens with MHC-II and causing secretion of cytokines such as IFN γ and employment of macrophages following proliferation and activation of CD8 T cells (Farhood et al., 2019; Subbarayan et al., 2019).

Immune evasion mechanisms of human papillomavirus

Epigenetic modifications play a crucial role in carcinogenesis including deregulation of DNA methylation, histone modification, dysregulation of protein functions. Transcription factors are involved in various means of evading of normal host defense and establishing the infection in human by HPV (Westrich et al., 2017). HPV E5, E6, and E7 proteins account as viral factors to evade of immune responses (Song et al., 2015). The E5 protein is capable to dysregulate the expression of MHC in infected cells and leading to the escape of virus from the immune system. HPV-16 E5 may also down-regulate the ability of two cyclin-dependent protein kinase inhibitors (CKIs), p21 and p27, result in the progression of cell cycle and DNA synthesis (Campo et al., 2010; Venuti et al., 2011). Although expression of E5 protein in 60% of HPV-16 infected patients with cervical carcinoma were detected, its carcinogenesis characteristics are limited to the initial stages. In fact, in the progressive malignant cervical carcinogenesis the E5 protein was deleted when the genome of HPV is integrated (Venuti et al., 2011; DiMaio and Mattoon, 2001). The productions of tumorigenic genes, E6 and E7, are able to interact with p53 protein which known as tumor suppressor protein and promote transformation. Moreover, E7 protein has a high affinity to interact with Rb protein (Fig. 3) (Song et al., 2015; Faridi et al., 2011). E6 and E7 expression are essential for high-risk infection of HPV types and progression. They are capable to down-regulate expression of TLR 9 which is fundamental to present the antigen in cells. Moreover, enhancement of E6 and E7 expression may evade the attack of cytotoxic T cells (Hasan et al., 2013; Kim et al., 2013). Additionally, the E7 protein could down-regulate the expression of TLR 9 by histone modification though histone deacetylase HDAC1 and histone demethylase JARID1B (Hasan et al., 2013; Westrich et al., 2017). Both HPV 16 E6 and E7 oncoproteins contribute in deregulation of DNA methylation of the host DNA. In this manner, the induction of regulatory T (Treg) cells by HPV16 E7 mediates immune regression. Moreover, HPV 16 E6 represses IFN- κ which substantially expresses in keratinocytes and acts as an enhancer for the expression of IFN-stimulated genes (Westrich et al., 2017; Narayan et al., 2009).

HPV vaccination

The case of HPV is more manageable because of the small number of serotypes associated with severe disease and many do not cause problems. Thus, in this case, a significant impact on disease can be made by vaccinating against just a few of the many different genotypes. Both currently available vaccines contain HPV types 16 and 18 to vaccinate against cervical cancer, and the Merck vaccine contains, in addition, HPV types 6 and 11 to vaccinate against genital warts (*condyloma acuminatum*). The absence of efficient cell culture systems for HPVs originates the development of eukaryotic expression systems to produce virus-like particles composed of the L1 capsid protein, which are highly immunogenic. The development of a preventive virus-like particle (VLP)-based vaccine targeted to the human papillomavirus (HPV) types most often found in the cancers (Abdoli et al., 2013; Burd, 2003; Kianmehr et al., 2015). At this time, there are three HPV prophylactic vaccines based on L1 virus-like particles (VLPs) which are approved FDA for the prevention of cervical cancer with some limitations. Primary, HPV vaccines are type-restricted and expressing diverse L1 major capsid proteins. Next, the confirmed current HPV vaccines do not confer therapeutic effect and the third limitation is the relatively high cost of production that limited their broadly use in low-income settings, where about 90% of cervical cancer deaths happens. Hence, there is a vigorous need for an effective therapeutic vaccine through which HPV related neoplasia can be eliminated without

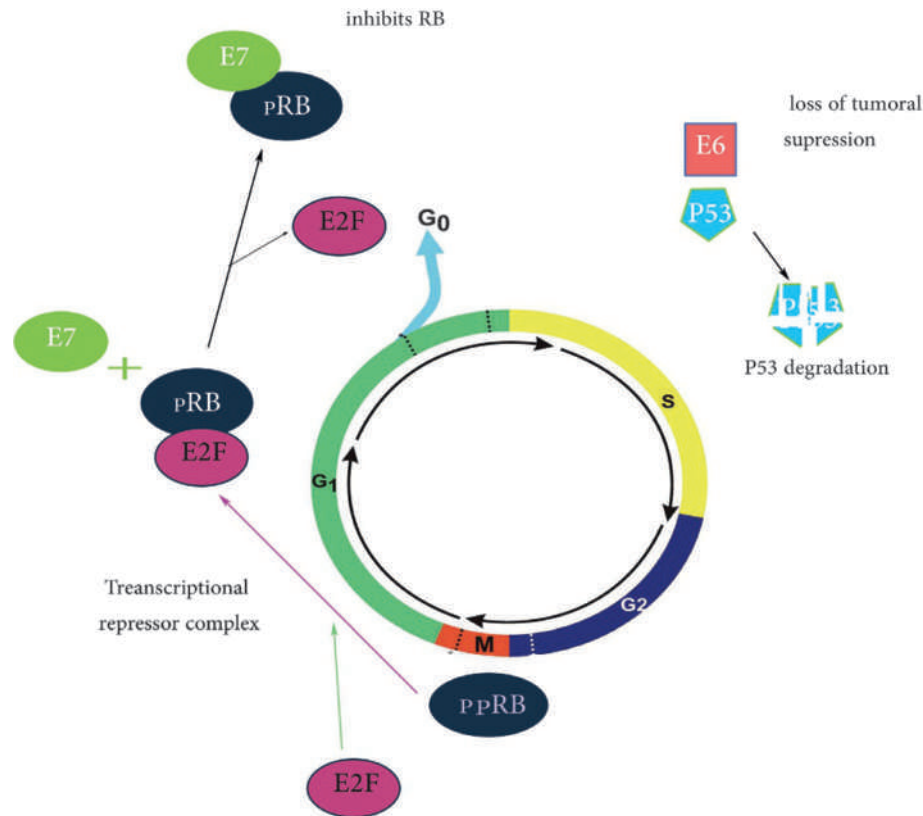


Fig. 3 E6 and E7 oncoproteins override regulation of cell cycle through p53 and pRB, respectively. E2F as a transcriptional factor is inhibited by pRB during the cell cycle. Indeed, phosphorylated pRB as an active form is detectable in G0-G1 phases and regulates cell cycle negatively. On the other hand, E7 leads to release of E2F during papillomavirus infection and permit to translate of cellular genes actively. Moreover, E6 is contributed with proapoptotic protein (p53) in the regulatory complex and degraded p53 to develop loss of tumoral suppression.

any invasive treatment (Karimi et al., 2020). The strategy of somatic gene therapy, the use of DNA as a therapeutic candidate and as a future, second-generation vaccine, is an excellent approach to open new horizon for medical applications. These vaccine will likely incorporate one or more of the additional oncogenic serotypes, such as 31, 33, and 35 (Stanley, 2014). Although, prophylactic vaccines offer an attractive promising vaccine for prevention of cervical cancer, but evidence suggests that the most of sexually active women presently remains neglected. High cost of the current vaccine strategies has encouraged researchers to continue to seek new methods of production and delivery in addition to preventive strategies (Ghaemi et al., 2010). The various therapeutic vaccine strategies remain of interest for majority of women who currently are infected. Several therapeutic strategies have focused on HPV E6 and E7 onco genes lacking the same success as the prevention trials. The most important candidate HPV vaccines are illustrated in Table 3.

Novel insight into the targeted therapies for cervical cancer

Structural and functional information concerning HPV proteins can offer novel insight into the mechanism(s) of cancer progression in the cervical epithelium. Recently, novel structural determinants of the interactions of viral proteins with their targets in keratinocytes have been elucidated. These exciting findings open the way for the development of targeted anti-oncogenic therapies, and may eventually allow the introduction of novel approaches for cervical cancer treatment. Delineation of the interactions of HPV oncoproteins E6 and E7 with their multiple cellular targets can provide the impetus for eventually targeted drug therapy for cervical cancer. The important differences between HPV structural proteins are responsible for different binding partners and offer insight into the mechanisms of HPV-mediated carcinogenesis. In particular, E6 and E7 proteins from high-risk HPV types are responsible for inducing uncontrolled cell proliferation. Only high-risk E6 and E7 bind p53 and of the Rb protein targets for ubiquitination and bypassing the G1/S checkpoint respectively.

These proteins can be used to enable researcher for the development of novel therapeutic targets besides new types of vaccines for prevention and cervical cancer therapy (Pappa et al., 2018).

Table 3 The FDA-approved and in development HPV vaccines.

FDA-approved Vaccines (Prophylactic vaccines)		
<i>Cervarix [2vHPV]</i>	<i>Gardasil [4vHPV]</i>	<i>Gardasil [9vHPV]</i>
HPV16&HPV18	HPV16, HPV18, HPV6&HPV11	HPV6&HPV11 HPV16, HPV18, HPV31&HPV33, HPV45, HPV52&HPV58
<i>Vaccine in development (Therapeutic E6&E7 based vaccines)</i>		
DNA/RNA based vaccine	Viral vector-based vaccine	Cell-based vaccine

In conclusion, insight from the genetic and epigenetic modifications at the regulatory region of HPV has contributed to the clarification of molecular mechanisms associated with HPV-related carcinogenesis that may open the way for the development of innovative therapeutic approaches.

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Togaviridae and Flaviviridae

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Glossary

Arbovirus Arthropod-borne viruses that have replication in both vertebrate and arthropods and transmits between vertebrate animals by the bite of mosquitoes, ticks, etc.

Arthralgia Pain in joints.

Arthritis Inflammation and pain in joints.

Arthritogenic virus Medically important viruses causing inflammatory musculoskeletal disease.

Arthropods Invertebrates having external skeleton, capable of serving as vectors.

ELISA Enzyme-linked assays; immunological assay which is used to measure antibodies, antigens, glycoproteins, and proteins in the biological samples.

Hematophagous Feeding on blood.

Host Vertebrate which harbors the causative agent.

Myalgia Muscle pain.

RT-PCR Reverse transcription polymerase chain reaction; technique used to measure the amount of a specific RNA with an added step of reverse transcription of RNA to DNA.

Vector Living organism capable of transmitting disease to vertebrate hosts.

Virion Viral particle consisting of capsid and nucleic acid.

Introduction

Togaviridae (Chikungunya virus) and Flaviviridae (West Nile virus) arboviruses are among the emerging and re-emerging viruses causing various diseases since past decade. *Arboviruses* are arthropod-borne viruses that have replication in both vertebrate and arthropods and transmit between vertebrate animals through the bite of mosquitoes and ticks. Yellow fever was the first infectious disease transmitted via arbovirus to humans. Earlier, yellow fever virus (YFV) was cultivated in the laboratories and many other related arboviruses were isolated later on. These viruses were categorized into further two groups (Group A and B arboviruses) and were cumulatively named as “togavirus.” The word *toga* in *Latin* means “Roman cloak,” which provided the basis for the adoption of name *Togaviridae* as one of the viral families. Initially, family Togaviridae has two genera i.e. *alphaviruses* and *flaviviruses*. Flavivirus comes from word, *flavus* meaning “yellow” in Latin. The alphaviruses were those formerly known as Group A viruses. Likewise, flaviviruses were those formerly categorized as Group B viruses. However, flaviviruses are no longer categorized into family Togaviridae and are moved to family *Flaviviridae* (Schlesinger and Schlesinger, 2013). This re-classification resulted from the difference in genome, viral structure and morphogenesis of both families. Medically the most important group of arboviruses falls under the family Flaviviridae.

Viruses from both Togaviridae and Flaviviridae families are globally distributed and transmitted via animal vectors to humans. Transmission of arbovirus requires a particular *vector* and a *host* for every viral species. *Arthropods* are the invertebrates having external skeleton, capable of serving as vectors. Arthropods are hematophagous (feeding on blood) vectors carrying the disease causing agents. Host is a vertebrate which harbors the causative agent. Reservoir host is responsible for spreading the virus, and is of two different types i.e. primary and secondary. Primary reservoir host is responsible for indefinitely maintaining the virus in the epidemiologically connected populations. Secondary reservoir host transmits the virus into the target population but cannot maintain the virus in nature in the absence of primary host (Acevedo-Whitehouse et al., 2005; WHO Expert Committee on the Control of the Leishmaniases and World Health Organization, 2010; Artsob et al., 2017; Burrell et al., 2017; Duvall et al., 2018). In response to changes in societal factors such as population growth, tourism, increased commerce and urbanization leads to shift in epidemiology. Global distribution of Dengue virus, Japanese virus, West Nile encephalitis virus and Yellow fever virus is expanding i.e. Japanese encephalitis in Australia. Furthermore, clinical manifestations vary for different viral species.

Togaviridae family

Togaviridae family consists of single genus i.e. alphaviruses. Alphaviruses are highly diversified and globally distributed.

Classification of genera

Viral classification is based on characteristics of virion, nature of nucleic acid, viral envelope, and symmetry of capsid. The Togaviridae family has alphaviruses genus, which further consists of 31 species (ICTV, 2014). Furthermore, the viral species also differ by mode of transmission, antigenicity and ecological information. Alphaviruses can replicate in a variety of vertebrates, invertebrates and cellular cultures (Griffin, 2013, 2016).

Epidemiology and risk factors determining outbreak

Alphaviruses were originally isolated in the tropical regions of Africa, Asia and South America. However, alphaviruses are not detected in Antarctica due to absence of arthropod vectors. Furthermore, different viral species are distributed in different geographical locations.

Eastern equine encephalitis virus (EEEV) is the most virulent specie in United States causing encephalitis in equines. Sign and symptom appears between 3 and 10 days post-infection (Zacks and Paessler, 2010). EEEV is widely distributed in South America, Central America, Eastern coast of Canada, and Caribbean islands. EEV causes severe disease in humans, equines, pheasants, dogs and pigs. However, EEEV in humans is not commonly reported in United States. Furthermore, new cases were reported in Canada and east coast of United States. Similarly, in 2010 new cases were reported in Panama. Strains of EEEV differ in South and North America; with South American strains being more diversified. Furthermore, South American strains effect equines whereas North American strains are pathogenic for both equines and humans (Young et al., 2008; Vander Kelen et al., 2012; Weaver et al., 2012; Lubelczyk et al., 2013; Molaei et al., 2013; Go et al., 2014; Yu et al., 2015).

WEEV strains were initially isolated from equine in California in 1930 and later on from human brain in 1938. Primary host for WEEV are birds, secondary hosts are birds, pheasants and chickens and the dead-end hosts includes humans, equines and other mammals. Later on, viral species were found in South, North America and Caribbean. In Argentina, equine outbreaks were reported from the beginning of twentieth century followed by Brazil and Paraguay (Go et al., 2014; Hubálek et al., 2014).

Other WEEV complex viruses have different geographical distribution. HJV was identified in eastern United States; AURA was initially isolated in Brazil in 1959 and later on found in Argentina in 1966. VEEV was first isolated in Venezuelan followed by South and Central America, Caribbean islands, Mexico, Texas, Colombia, and Argentina. FMV are not pathogenic for humans, and was isolated in Oklahoma and Colorado (Steele and Twenhafel, 2010; Weaver and Reisen, 2010; Hubálek et al., 2014; Allison et al., 2015).

Epidemiological risk factors contributing in the outbreak of chikungunya includes not wearing full body covering clothes, sleeping nets, screening of doors and windows (with net), and uncovered water container which might promote larvae growth. Furthermore, being in contact with people with similar signs and symptoms can also be a risk factor for chikungunya spread (Alayu et al., 2021).

Structure

Alphaviruses are enveloped single-stranded RNA viruses. The lipo-protein viral envelope (host-cell derived structure) surrounds the viral capsid and has structural glycoproteins (E1 and E2) embedded on it. The viral capsid made by C protein contains the viral genome (Jose et al., 2009; Ruiz-Guillen et al., 2017). Genes for the structural protein is located at the 3' and for non-structural protein at 5' end of the genome. All the alphaviruses share their antigenic sites on the capsid (Griffin, 2013; Fig. 1).

Characteristics of members of Togaviridae family

Members of Togaviridae family have a diameter of 65–70 nm and replicates with in the cytoplasm (Table 1). Virus envelope comprises of glycosylated spike proteins (E1 and E2). Genome is enveloped and single stranded RNA having positive polarity. Translation of structural protein and non-structural protein occurs from sub-genomic RNA and genomic RNA, respectively.

Virus genus and disease

Togaviridae family comprises of genus alphaviruses. Earlier, togavirade has four genus with multiple species. In 1996, after genomic sequence analysis, arteriviruses were moved from Togaviridae to another family, Arteriviridae (Chen et al., 2018). In 1980s, due to similarity in genome organization, pestiviruses were also moved out to Flaviviridae family (Collett et al., 1988). In April 2019, Rubiviruses (Rubella virus) were classified into another family called Matonaviridae due to difference in their virion structure and gene expression as well. Currently, Togaviridae family only has alphaviruses (Brinton and Snijder, 2008; Chen et al., 2018).

Alphavirus

All alphaviruses (family Togaviridae) which causes diseases in humans are the arboviruses, being transmitted via hematophagous arthropods (Artsob et al., 2017; Duvallet et al., 2018). Alphavirus has approximately 31 species, out of which 8 species causes disease in both humans and animals (i.e. SINV, SFV, VEEV, EEEV, CHIKV, RRV, WEEV and ONNV) (Atkins, 2013). However, some of them rarely result in death i.e. chikungunya virus, Middelburg virus, Ross River virus, etc. In this chapter, alphaviruses are sub-classified according to the type of disorders they cause i.e. Neurotropic and arthritogenic or polyarthritis alphaviruses (Griffin, 2016; Contigiani and Diaz, 2017). Furthermore, alphaviruses will result in varying clinical manifestations. The viruses of clinical importance will be discussed in detail in this chapter.

Neurotropic alphaviruses

These causes disease typified with by fever, headache, malaise, neurological symptoms and/or encephalitis are Eastern equine encephalitis virus, Western equine encephalitis virus, Highlands J virus, Venezuelan equine encephalitis virus complex, Fort Morgan virus, and AURA virus.

Eastern equine encephalitis virus (EEEV)

EEEV is the most pathogenic and virulent alphavirus in United States. Moreover, highest mortality rate is reported in elderly population. Sign and symptoms appear between 3 and 10 days causing fever, myalgia, followed by encephalitis, coma and death. In equine, EEEV causes encephalitis starting with fever and anorexia. Furthermore, EEV is associated with myelitis, seizures, paralysis and death (Zacks and Paessler, 2010).

Western equine encephalitis virus (WEEV)

WEEV causes neurological diseases but with lesser severity as compared to EEEV in their signs and symptoms. Clinical manifestations of WEEV include fever, headache and meningitis. However, WEEV could be either asymptomatic or lethal too. Neurological signs are reported, including lethargy, tremors, dizziness, stiff neck, altered mental stability and irritability (Griffin, 2013).



Fig. 1 Alphavirus genome showing 5' cap, non-structural proteins (nsP1, nsP2, nsP3, nsP4), capsid, structural proteins (E1 and E2), and 3' end. Information taken from Sherman MB and Weaver SC (2010) Structure of the recombinant alphavirus western equine encephalitis virus revealed by cryoelectron microscopy. *Journal of Virology* 84(19): 9775.

Table 1 Characteristics of Togaviridae family.

Characteristics	Togaviridae
Virion	Spherical virion with single capsid protein and three glycoproteins; 65–70 nm diameter
Genome	Enveloped, single stranded RNA (10–12 kb), positive polarity
Replication	Cytoplasmic
Translation	From sub-genomic RNA (structural protein) and genomic RNA (non-structural)

Information taken from Chen R, Mukhopadhyay S, Merits A, Bolling B, Nasar F, Coffey L.L., Powers, A and Weaver SC (2018). ICTV virus taxonomy profile: Togaviridae. *Journal of General Virology* 99(6): 761–762.

Highlands J virus (HJV)

Highlands J virus (HJV) is pathogenic in ducks, turkeys, pheasants and other birds. HJV may cause equine encephalitis as well. In 1960, HJV was isolated from corvid and is a member of WEE complex distributed in eastern United States. Unlike EEEV, HJV is not pathogenic to humans. Furthermore, migratory birds are the host and *Cs. Melanura* was the vector in United States (Steele and Twenhafel, 2010; Griffin, 2013; Contigiani and Diaz, 2017).

Venezuelan equine encephalitis virus Complex (VEEV)

VEEV is a new world alphavirus found in human and equine. Symptoms of VEEV in human include fever, nausea, vomiting, myalgia and encephalitis. However, equines are presented with fever, depression, tachycardia, and anorexia. It is prevalent in Southern and Central America and has two sub-types (Weaver et al., 2004; Weaver and Reisen, 2010; Zacks and Paessler, 2010; Griffin, 2013):

- (a) Epizootic virus (IAB and IC).
- (b) Enzootic virus (ID, ID and IF).

Epizootic strains causes serious infection whereas enzootic strains cause mild infection. Epizootic strains are found in human and animals (rodents, dogs, sheep, and bats) with mosquitos as vector during out-break. The vectors involved in transmission depends upon the region i.e. *Aedes* and *Psorophora* in United States. Furthermore, epizootic viruses can cause serious infections. These are clinically undistinguishable from influenza and other viral diseases. The early stage (2–5 days) clinical manifestations are chills, fever, severe headache, sore-throat, retro-orbital pain, myalgia, nausea and vomiting. Later on, neurological symptoms are reported, including drowsiness, encephalitis, paralysis, coma and death (Weaver et al., 2004; Weaver and Reisen, 2010; Zacks and Paessler, 2010; Aguilar et al., 2011; Taylor and Paessler, 2013).

Fort Morgan virus (FMV)

Fort Morgan virus (FMV), isolated in North America, are non-pathogenic for both human as well as animals. In Oklahoma and eastern Colorado, these viruses were isolated from sparrows and swallows. Other closely related virus such as Buggy Creek virus (BCV) is similar to the FMV in its antigenicity (Griffin, 2013; Allison et al., 2015).

AURA virus

AURA virus has serological similarity with WEEV and sindbis virus. Aura virus is named after its isolation from a small river in Belém, Brazil. Moreover, AURA has similar sequence identity with the sindbis virus. AURA virus is isolated from *Aedes serratus* in northern Argentina and Brazil. Evidently no pathogenicity has been reported in human (Sabattini et al., 1998; Griffin, 2013).

Arthritogenic alphaviruses

Arthritogenic or polyarthritis alphaviruses are mosquito-borne viruses causing disease with symptoms including fever, rash and joint disorders (arthralgia) in the infected host. These viruses includes Chikungunya virus, Sindbis virus, Ross River virus, Semliki virus, O'nyong-nyong virus, Barmah Forest viruses, and Mayaro virus (Contigiani and Diaz, 2017).

Chikungunya virus (CHIKV)

Chikungunya virus (CHIKV) is the arbovirus first isolated in Tanzania in 1952. The word chikungunya means “which bends up” in Kimakonde/Makonde language and is associated with the distorted posture of patient. CHIKV is found in Asia, Europe, Australia, and Africa. It is one of the old world alphaviruses and is transmitted by mosquito (Contigiani and Diaz, 2017). *Aedes* species such as *Ae. aegypti* and *Ae. albopictus* are the vector mainly involved in transmission of CHIKV. The clinical manifestations of CHIKV in human are non-specific flu like symptoms, fever, rash and arthritis (Bettadapura et al., 2013; Caglioti et al., 2013; Faria et al., 2016).

In the endemic area, insect control and prevention from mosquito bites helps in the prevention of contracting CHIKV (Caglioti et al., 2013). Conventional and time loop-mediated RT-PCR is used for diagnosis purpose in the early days (Pfeffer et al., 2002; Parida et al., 2007). Different serological methods are used to detect the CHIKV-specific immune response (IgM and IgG antibodies) i.e. Enzyme-linked assays (ELISA) and immunofluorescence assays (IFA) (Sam and Abu Bakar, 2006; Litzba et al., 2008). CHIKV is

managed symptomatically with the help of NSAIDs, anti-pyretics and fluids to relieve, inflammation and fever. Faster resolution of arthritis and joint pain were observed after treating the patients with Ribavirin 200 mg (twice a day) for 7 days. Moreover, steroids are occasionally used in the course of treatment. However, effectiveness of chloroquine remains unclear (Ravichandran and Manian, 2008; Taubitz et al., 2007). Passive immunization can be used in humans after its successful results *in vitro* and *in vivo* (Couderc et al., 2009). A vaccine containing EMCV IRES (Encephalomyocarditis virus—internal ribosome entry site) sequence introduced into the CHIKV sub-genomic promoter, is in its pre-clinical trial phase (Plante et al., 2011). Various vaccines are developed but are still not licensed to use in humans (Caglioti et al., 2013).

Sindbis virus (SINV)

Sindbis virus (SINV) is one of the old world alphaviruses named after originally isolated near Sindbis in Egypt. SINV is associated with arthritis and rash (Grywna et al., 2010). SINV is commonly found in Egypt, East and South Africa, Philippines, Israel and Australia. The symptoms of SINV are arthralgia, fever and rash. SINV is the causative agent of Pogosta and Ockelbo disease (Jöst et al., 2010; Sane et al., 2012).

Other arthritogenic viruses

Other arthritogenic viruses are Ross River virus (RRV), Semliki Forest virus (SFV), Barmah Forest viruses, Mayaro virus (MAYV) and O'nyong-nyong virus (ONNV). RRV was first isolated in Ross River in Australia. ONNV in the Nilotic language means "weakening of the joints." SFV is known to cause disease in both human and animals. In 1942, SFV was first isolated from mosquito in a forest in Uganda (Smithburn and Haddow, 1944; Tables 2 and 3).

Transmission and viral life cycle

Alphaviruses are transmitted via mosquitoes and keeps on circulating in the nature via mosquito-vertebrate-mosquito cycle. Virus is contracted usually in the endemic area. Virus is released into the host and later on lysed (Ruiz-Guillen et al., 2017; Lim et al., 2018).

The alphaviruses are multiplied intracellularly via receptor mediated cytoplasmic endocytosis; the diagrammatic representation is presented in Fig. 2. As a result, sub-genomic RNA specie (26S RNA) is formed which codes for structural proteins. However, genomic RNA serves as mRNA for the non-structural protein. The non-structural protein forms the 2/3rd and structural protein makes up the 1/3rd of the genome (Mudiganti, 2007).

Clinical manifestation

Clinical manifestations of alphaviruses are fever, arthralgia, and rash and can also result in neurological disorders. However, immune system eliminates the virus, whereas other symptoms including arthritis prevails for a few weeks (Griffin, 2013, 2016).

Table 2 Important viruses and their characteristics.

<i>Virus</i>	<i>Associated syndrome/disease</i>	<i>Host</i>	<i>Vector</i>	<i>Geographical distribution</i>
Eastern equine encephalitis virus (EEEV)	Eastern equine encephalitis	Birds	Mosquitoes	South America, Central America, Eastern coast of Canada, and Caribbean islands
Western equine encephalitis virus (WEEV)	Western equine encephalitis	Birds	Mosquitoes	South America, North America and Caribbean, Argentina
Venezuelan equine encephalitis virus complex (VEEV)	Venezuelan equine encephalitis	Mammals, horses	Mosquitoes	South America, Central America
Chikungunya virus (CHIKV)	Chikungunya	Monkeys, humans	Mosquitoes	Asia, Europe, parts of Africa, Australia, and Europe
Ross River virus (RRV)	Ross River polyarthritis	Macropods and other mammals	Mosquitoes	Asia, Europe, parts of Africa, Australia, and Europe
Japanese encephalitis virus (JEV)	Japanese encephalitis	Birds, pigs	Mosquitoes	Asia, Australia
St. Louis Encephalitis virus (SLEV)	St. Louis encephalitis	Birds	Mosquitoes	South America, Brazil, Argentina, Panama
West Nile virus (WNV)	West Nile fever/ encephalitis	Birds	Mosquitoes	Africa, North America, Europe, and Asia
Yellow fever virus (YFV)	Yellow fever	Monkeys, humans	Mosquitoes	Africa, Latin America, Brazil
Dengue virus (DENV)	Dengue fever	Humans, monkeys	Mosquitoes	Asia, Africa, United States, Europe
Tick-borne encephalitis virus (TBEVs)	Tick-borne encephalitis	Mammals, birds	Ticks	Europe, Asia, North America, Japan, Siberia, and Saudi Arabia
Sindbis virus (SINV)	Pogosta and Ockelbo disease	Birds	Mosquitoes	Egypt, East and South Africa, Philippines, Israel and Australia

Information taken from Contigiani MS and Diaz LA (2017) Togaviridae. In: *Arthropod Borne Diseases*, pp. 115–135. Springer.

Table 3 Epidemiological features of important viruses and diseases caused by these viruses.

Disease	Causative agent ^a	Mode of transmission ^b	Incubation period (days)
Dengue	Dengue fever virus	Mosquito bite	5–8
Rubella	Rubella virus	Respiratory, congenital	17–20
Alphavirus encephalitis	EEEV, WEEV, VEEV	Mosquito bite	4–10
Hepatitis C	Hepatitis C virus	Parenteral, sexual	40–60

^aDisease causing viruses.

^bUntil the appearance of prodromal set of signs and symptoms.

Information taken from Burrell CJ, Howard CR, and Murphy FA (2017) Epidemiology of viral infections. In: *Fenner and White's Medical Virology*, pp. 185–203.

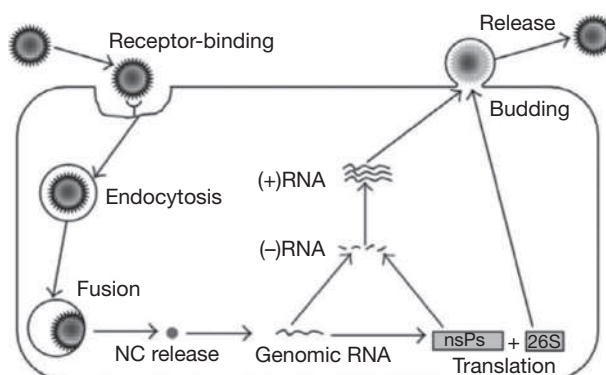


Fig. 2 Life-cycle of alphavirus. Alphavirus binds to the receptor and enters the cell by endocytosis. RNA is released after the nucleocapsid (NC) is transported into cytoplasm. Protein translation and RNA transcription from both genomic and sub-genomic RNA occurs and are eventually release. Adapted from Leung JY-S, Ng MM-L, and Chu JJH (2011) Replication of alphaviruses: A review on the entry process of alphaviruses into cells. *Advances in Virology* 2011: 249640.

Diagnosis

Viral detection and isolation are used to identify the virus and establish a valid diagnosis. Antibody titer of either IgM or IgG also helps in virus determination. Furthermore, reverse transcription polymerase chain reaction (RT-PCR) assay is also used for identification purpose against the panel of alphaviruses available in a geographical region, against the known virus i.e. CHIKV sera (Yap et al., 2010).

Disease management/treatment

Currently, there is no specific antiviral agent for the treatment of alphaviruses induced diseases. However, symptomatic management is indicated to overcome the symptoms. Non-steroidal anti-inflammatory drugs (Naproxen and Ibuprofen) and anti-pyretic agents (Paracetamol) are primarily used for fever and pain (Contigiani and Diaz, 2017).

Prevention

Virological, serological, entomological and epidemiological surveillance should be made a part of preventive program. Available vaccines should be used for passive immunization by the people traveling to endemic areas or the residents. Veterinary vaccines for WEEV and EEEV are being prepared. TC83 strain of VEEV is used successfully in horses as well as in military and laboratory staff. However, its use is still not recommended in general public (Go et al., 2014).

Flaviviridae family

Flaviviruses were originally classified under the Togaviridae family. Initially, there were virological and biological similarities between these two families. However, after years of research flaviviruses were separated from Togaviridae family on the basis of difference in the mode of replication and genetic organization (Chen et al., 2018).

Classification of genera

As per International Committee on Taxonomy of Viruses (ICTV) recognition, Flaviviridae have four genus flavivirus, hepacivirus, pestivirus and pegivirus (Simmonds et al., 2017).

Epidemiology and risk factors determining dispersal

Overall, flaviviruses are globally distributed with individual species restricted to some areas i.e. Dengue fever virus in tropical regions of Africa, Asia, Australia, America, and Oceania; Yellow fever virus in subtropical regions of South America and Africa (Simmonds et al., 2017).

However, dengue is associated with seasonal changes providing favorable breeding environment for the *Aedes* mosquitoes i.e. in optimum temperature, humidity, and rainfall. Over-crowded places and traveling from endemic to the non-endemic areas reportedly increased the spread of dengue virus. Other aforementioned risk factors for chikungunya spread are also applicable for dengue as well (Khan et al., 2018; Alayu et al., 2021).

Structure

Flavivirus are single stranded, positive sense RNA. Morphology of viruses in Flaviviridae family is somewhat similar with some changes in the spike proteins. They are spherical lipid enveloped RNA viruses with single core protein and 2–3 virus-coded glycoproteins. In case of *Flavivirus*, *Hepacivirus* and *Pegivirus* families, 2 envelope proteins are present. However, 3 envelope proteins are present in pestivirus family viruses (Lindenbach et al., 2013; Simmonds et al., 2017).

Characteristics of members of Flaviviridae family

Virions are 50 nm in diameter and are icosahedral in symmetry. Virion is positive sense single-stranded messenger ribonucleic acid (ssmRNA) and has a genome of 9–13 kb (Lindenbach et al., 2013; Simmonds et al., 2017; Table 4).

Virus genus and disease

Flavivirus genus has 53 species out of which commonly causing diseases in humans are YFV, dengue fever virus, West Nile virus, Japanese encephalitis virus, and Tick-borne encephalitis virus. Hepacivirus genus contains single specie hepatitis C virus (HCV) with 17 genotypes and 84 subtypes (Smith et al., 2014).

Flavivirus

Majority of the flaviviruses are transmitted to vertebrates via mosquitos and ticks. They are composed of capsid protein and 2 spike proteins. Flaviviruses are found globally, however, some species are only restricted to the epidemic and endemic areas i.e. dengue virus in tropical regions Asia, Africa, United States and Australia; YFV in subtropical regions of Africa and South America (Simmonds et al., 2017). Important human pathogens in flavivirus genus are Yellow fever virus, Dengue fever virus, West Nile virus, Japanese encephalitis virus, tick-borne encephalitis virus and St. Louis encephalitis virus.

Yellow fever virus

Yellow fever virus (YFV) is one of the initially identified virus which led to the further identification and categorization of the arboviruses (Simmonds et al., 2017). YFV was reported highest in Brazil during the outbreak of 2016–17. YFV is transmitted via *Aedes* spp. Mosquitoes (Faria et al., 2018). YFV is endemic in Latin America, and tropical forests of Africa. When effective control measures are not practiced, Yellow fever (YF) outbreak can occur i.e. in Republic of Congo and Angola in 2015–16. 20–30% patients with mild to moderate (viremic phase) YF presents with high fever, headache, myalgia, nausea, and loss of appetite for 2–4 days. Worsening of the viremic phase symptoms in observed in 15% of the infected patients (toxemic phase) followed by icteric discoloration of skin, bradycardia, neurological impairment, renal dysfunction, bleeding and cardiovascular dysfunction is reported. Furthermore, half of the infected patients are asymptomatic. Differential diagnosis is difficult in case of all the viral

Table 4 Characteristics of Flaviviridae family.

Characteristics	Flaviviridae
Virion	Spherical virion with single core protein and 2–3 enveloped glycoproteins; 40–60 nm diameter
Genome	Non-segmented RNA; positive sense 9–13 kb
Replication	Cytoplasmic
Translation	From genomic RNA containing type cap 1

Information taken from Chen R, Mukhopadhyay S, Merits A, Bolling B, Nasar F, Coffey L.L., Powers, A and Weaver SC (2018). ICTV virus taxonomy profile: Togaviridae. *Journal of General Virology* 99(6): 761–762.

diseases in their initial phase due to presentation of similar clinical manifestations. However, serological testing for IgM and IgG antibodies is recommended at day 5. However, till date no specific antiviral treatment is available for the treatment. Adequate hydration and dipyrone is used for symptomatic relief. There is an ongoing research for the use of sofosbuvir against YFV. YFV is the hemorrhagic fever which was considered a lethal disease before the vaccine was developed. Yellow fever vaccine (Live 17D vaccine; YF-Vax, Stamaril) is available and considered most potent vaccine ever developed. Immunization and vector control helps immensely in the prevention and further transmission of YFV (Johansson et al., 2010; Douam and Ploss, 2018). However, people traveling to endemic areas and those who are unvaccinated can still contract the YFV (Monath, 2001).

Dengue virus

Dengue virus (DENV) is transmitted via mosquitos of *Aedes* spp. Incubation period of DENV is an average of 7 days (3–14 days). There are four different types of serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. DENV infects the human via *Aedes* spp. vector (*A. aegypt* and *A. albopictus*) found originally in Asia later spreading to Africa, United States and Europe. Clinical manifestations varies, commonly including fever, headache, arthralgia, and rash (Kularatne, 2015). Other identification signs are deranged LFTs, thrombocytopenia, leucopenia, hemorrhage and plasma leakage. There are different phases of infection i.e. febrile, critical and convalescent. The early diagnosis is by advising complete blood culture (CBC), thrombocytopenia and leucopenia are characteristics of the day followed by dengue fever. Identification of viral nucleic acid or antigen is evident during the first 5 days of infection (World Health et al., 2009; Kularatne, 2015). No specific anti-viral is developed or recommended for the management of DENV. Oral rehydration, intravenous fluids (i.e. lactated ringer or 0.9% normal saline), colloids (i.e. dextran 70% or 6% starch) are recommended for the management. Only acetaminophen should be used to treat fever and NSAIDs should be avoided to prevent any bleeding (Kularatne, 2015). However, prednisolone is reported to be used and not associated with the prolongation of viremia (Tam et al., 2012). Furthermore, platelet transfusion is only required in case of active bleeding. First vaccine against DENV, a live attenuated recombinant tetravalent dengue vaccine (Dengvaxia®) is under phase III trial. Dengvaxia (CYD-TDV) is a 3-dose vaccine with a 0–6–12 month schedule was registered in Mexico. Other type of vaccines are under development and trial phases including inactivated, DNA, and subunit vaccine (Rajapakse, 2009; Teixeira and Barreto, 2009; World Health Organization. Regional Office for South-East Asia, 2011; Deng et al., 2020).

West Nile virus

West Nile virus (WNV) is neurotropic flavivirus transmitted via mosquito bite to human. WNV is found in Africa, North America, Europe, and Asia. Of the infected patients, 80% remains asymptomatic. WNV cause acute systematic febrile illness common in all age groups i.e. West Nile Fever (WNF). Patient with WNF experiences fever, fatigue, headache, myalgia and gastrointestinal disturbances leading to dehydration. Neurological symptoms are manifested in some patients due to neuro-invasive disease causing infection of meninges mostly in younger age i.e. West Nile Meningitis (WNM). WNM is characterized by fever, headache, nuchal rigidity, photophobia, exacerbating head pain and GI symptoms. Acute polio-like syndrome is also caused by WNV causing the viral infection of horn cells of spinal cord i.e. West Nile Poliomyelitis. Furthermore, encephalitis is also caused by WNV mostly in elder age group, causing infection of brain parenchyma i.e. West Nile Encephalitis (WNE). However, clinical picture of WNM, WNE and WNP might overlap (Granwehr et al., 2004; Sejvar, 2014).

Japanese encephalitis virus

Japanese encephalitis virus (JEV) causes the most important viral encephalitis, Japanese encephalitis (JE) globally. JE has been spread to different parts of Asia and Australia (Unni et al., 2011). However, China still reports the 50% of the total JE cases. About 50% of the JEV infected patients develops neurological sequelae. However, 25% of the patients are expired after contracting JEV (Van den Hurk et al., 2009). Major reservoirs to harbor these viruses are mosquitoes, pigs and water birds. Primary vector for JEV are the mosquitoes i.e. *Culex* genus and *Anopheles* genus in India. Difference in epidemiology is due to genotypic and temperature variations. Majority of the JEV are asymptomatic, reporting mostly in children below 15 years. Clinical course of illness is characterized by fever, headache, backache, myalgia, GI disturbances and anorexia. These symptoms lasts for a week, followed by disturbance in gait, mental status, speech and motor functions. Furthermore, pathological abnormalities are reported in CNS (Fischer et al., 2010). No benefit was observed from the treatment with interferon- α (Solomon et al., 2003).

St. Louis encephalitis virus (SLEV)

St. Louis encephalitis virus (SLEV) causes St. Louis encephalitis (SLE), a viral epidemic in St. Louis, United States. SLEV is transmitted by mosquito species in *Culex* genus. Different genotypes are found in different regions i.e. Genotype III and V in South America, I and II in United States, IV and VI in Panama, VIII in Brazil and VII in Argentina. Clinical symptoms may vary from meningitis and meningococcal encephalitis. Furthermore, disease severity is associated with the patient's age. However, there are no specific treatment and vaccine currently available. Furthermore, management with INF and IVIG in suspected SLEV infected patients is recommended (Reisen, 2003).

Tick-borne encephalitis virus (TBEV)

Tick-borne encephalitis virus (TBEV) is the group of flaviviruses transmitted by the ticks as vectors. These tick-borne viruses results in different diseases in both human and animals, which ranges from fever, body ache, malaise, fatigue, encephalitis, meningitis, myelitis and hemorrhage. TBEV are found in Europe, North America, Japan, China, Russia, Siberia, India, and Saudi Arabia. TBEV is

diagnosed via cerebrospinal fluid (CSF) cell count, IgM and IgG, and enzyme-linked immunosorbent assay (ELISA) of TBEV detection. Furthermore, inactivated vaccine (FSME-IMMUN) is available in Europe but not licensed to be used in United States. No specific TBE treatment is available and only symptomatic treatment is provided with antipyretics, anti-emetics, analgesics, fluid and electrolyte replacement therapy (Hubálek and Rudolf, 2012).

Pestivirus

The word *Pesti* comes from a Latin *Pestis*, meaning “plague.” This genus of Flaviviridae family infects pigs, cattle, goats, sheep and wild ruminants. Pestivirus is transmitted via contaminated secretions. This genus has four species i.e. classical swine fever virus (CSFV), border disease virus (BDV) and bovine viral diarrhea virus 1 & 2 (BVDV). Other diseases associated with pestivirus genus are hemorrhagic syndromes, abortion, and fatal mucosal disease in animals. In this chapter, only CSFV is discussed in detail. Renaming of pestiviruses species are suggested i.e. Pestivirus A (BVDV-1), pestivirus B (BVDV-2), pestivirus C (CSFV), and pestivirus D (BDV). However, pronghorn pestivirus, Aydin-like pestivirus, rat pestivirus, APPV, Bungowannah virus, giraffe pestivirus, and Hobi-like pestivirus are new suggestions to be included into this genus (Simmonds et al., 2017; Kirkland et al., 2019).

Classic swine flu virus

Classical swine fever (CSF) is a viral disease of wild and domestic pigs and boars caused by classical swine fever virus (CSFV) (Luo et al., 2014). CSF occurs either as acute or chronic course of disease. In acute course fever, the typical clinical manifestations 1–2 weeks after contracting infection are fever, weakness, anorexia, gastrointestinal symptoms and conjunctivitis. Neurological disorder occurs 2–4 weeks after the infection. Some atypical symptoms including cyanosis on abdomen, limbs, and ears (Petrov et al., 2014).

Hepacivirus

Hepacivirus name is derived from Greek word *hepatos/hepar* meaning “liver.” These are single stranded RNA viruses having positive polarity, which codes for polyprotein. Human pathogen responsible for liver disease belongs to this genus of family Flaviviridae i.e. Hepatitis C virus (HCV). Hepacivirus causes both chronic and acute hepatitis, carcinoma and liver function failure. The pathogens in this group affect the liver and are widely distributed via rodents, bats, horses and dogs. This genus also includes other viruses which effects rodents, cows, horses and bats (Simmonds et al., 2017). Hepacivirus cause millions of infections presenting with fever, neurological and GI disease and hemorrhage. Chronic hepatitis leads to chronic hepatic fibrosis, cirrhosis and carcinoma. Till date there are no successfully effective vaccination available (Bukh, 2016).

Pegivirus

Initially, pegivirus was found in monkeys and humans. Then equine pegivirus, rodent pegivirus and bat pegivirus have been found since 2010. However, species of this genus are not associated with the disease or infection in mammals (Lu et al., 2016; Simmonds et al., 2017).

Transmission and viral life cycle

Most of the flaviviruses (i.e. Dengue, Yellow, Japanese, Nile, Zika and tick-borne virus) are horizontally transmitted from vector (i.e. mosquitoes and ticks) to vertebrate hosts. Primary vector of flavivirus are birds and mammals. However, some flavivirus are separated exclusively from vertebrates or mosquitoes and are vertically transmitted. Remainder of the virus is transmitted between bats and rodents (Valarcher et al., 2015; Simmonds et al., 2017; Wilder-Smith et al., 2017). Viral infection is initiated by mosquito or tick's bite. Furthermore, virus is dispersed into the host body during lytic infections of the cells (Simmonds et al., 2017).

Clinical manifestations

A variety of the clinical manifestations are presented which can be distinguished by the type of virus. YF is characterized by high grade fever, headache, myalgia, and nausea. However, dengue presents with fever, headache, arthralgia, and rash. Hepacivirus presents with fever, neurological and GI disease and hemorrhage. CSF causes fever, weakness, anorexia, gastrointestinal symptoms and conjunctivitis. JEV is clinically manifested with fever, headache, backache, myalgia, GI disturbances and anorexia. In WNF, fever, fatigue, headache, myalgia and gastrointestinal disturbances leading to dehydration are reported (Kularatne, 2015).

Diagnosis

Early stage differential diagnosis is quite challenging during the diseases caused by these viruses. Serological testing for IgM and IgG antibodies is recommended. However, acute phase of the disease can be identified via RT-PCR. Furthermore, viral infection and encephalitis caused by TBEV is diagnosed via cerebrospinal fluid (CSF) cell count, enzyme-linked immunosorbent assay (ELISA) of TBEV along with IgM and IgG.

Disease management/treatment

There is no specific treatment for the infections caused by viruses from Togaviridae and Flaviviridae family. The symptomatic management is provided to the infected patients through analgesics, anti-pyretics, anti-emetics and rehydration therapy. In some cases of suspected SLEV infected patients, INF and IVIG are used. However, no specific anti-virals against specific viruses are available till date. Management is described in detail under the individual headings of the diseases and causative agents.

Prevention

Detection and containment of viruses (i.e. YFV), requires epidemiological and genomic surveillance (Johansson et al., 2010). Furthermore, vaccination is another preventive measure which provides passive immunization against certain viruses. Table 5 summarizes the type and characteristics of vaccine against viruses of both Togaviridae and Flaviridae family. Vaccines against specific Flaviviridae family viruses are available i.e. YFV vaccine; the most potent vaccine available globally (Johansson et al., 2010; Douam and Ploss, 2018). CSFV vaccine is available and currently in use in China (Luo et al., 2014). An inactivated vaccine (FSME-IMMUN) for TBEV is available in Europe but not licensed in United States (Hubálek and Rudolf, 2012). Attenuated and inactivated vaccines for Japanese encephalitis are also available in certain parts of world (Van den Hurk et al., 2009). Furthermore, vaccine against dengue virus is under trial and development and their safety and efficacy profile will be available soon. However, vaccines against remaining Flaviviridae viruses are not available i.e. Hepacivirus (Rajapakse, 2009; Teixeira and Barreto, 2009; World Health Organization. Regional Office for South-East Asia, 2011).

Table 5 Type of vaccine and availability against Togaviridae and Flaviviridae viruses.

<i>Disease/virus</i>	<i>Vaccine type</i>	<i>Availability</i>
Eastern equine encephalitis virus (EEEV)	N/A	N/A
Western equine encephalitis virus (WEEV)	Inactivated (TSI-GSD 210)	N/A; trials
Highlands J virus (HJV)	N/A	N/A
Venezuelan equine encephalitis virus complex (VEEV)	Inactivated (C-84 strain)	N/A; phase II trials
Fort Morgan virus (FMV)	N/A	N/A
AURA virus	N/A	N/A
Chikungunya virus (CHIKV)	Subgenomic; ChAdOx1 Chik	Clinical trials; not licensed
Sindbis virus	Replicase-based DNA vaccine	N/A
Ross River virus (RRV)	Whole-virus Vero cell culture-derived	Phase III trial, unavailable
Semliki Forest virus (SFV)	N/A	N/A
Barmah Forest viruses	N/A	N/A
Mayaro virus (MAYV)	ChAdOx1 May, AdV-MAYV	Not licensed; unavailable but under trials
O'nyong-nyong virus (ONNV)	N/A	N/A
Yellow fever virus (YFV)	Live 17D (YF-Vax, Stamaril)	Available
Dengue virus (DENV)	Live attenuated recombinant (Dengvaxia)	Mexico
West Nile virus (WNV)	N/A	N/A
Japanese encephalitis virus (JEV)	Inactivated (JE-VAX), Inactivated Vero cell culture-derived (IXIARO), live attenuated (ChimeriVax-JE),	
St. Louis encephalitis virus (SLEV)	N/A	N/A
Tick-borne encephalitis virus (TBEV)	Inactivated vaccine (FSME-IMMUN)	Europe
Classical swine fever virus (CSFV); pigs	Live attenuated; Ingelvac (CSF MLV)	China
Hepatitis C virus (HCV)	N/A	N/A

AdV, adenovirus V; N/A, not available.

Information taken from Plante K, Wang CD, Fau-Partidos E, Partidos J, Fau-Weger C, Weger R, Fau-Gorchakov J, Gorchakov K, Fau-Tsetsarkin R, Tsetsarkin EM, Fau-Borland K, Borland AM, Fau-Powers E, Powers R, Fau-Seymour A, Seymour DT, Fau-Stinchcomb R, Stinchcomb JE, Fau-Orsorio D, Orsorio Je Fau-Frolov I, Frolov SC, Fau-Weaver I, and Weaver SC. Novel chikungunya vaccine candidate with an IRES-based attenuation and host range alteration mechanism (1553–7374 (Electronic)); Dar, PA, Ganesh K, Nagarajan G, Sarika S, Reddy GR, and Suryanarayana VVS (2012) Sindbis virus replicase-based DNA vaccine construct encoding FMDV-specific multivalent epitope gene: Studies on its immune responses in Guinea pigs. *Scandinavian Journal of Immunology* 76(4): 345–353; Monath, TP (2013) 17D yellow fever virus vaccine. *The American Journal of Tropical Medicine and Hygiene* 89(6): 1225; Wressnigg N, van der Velden MVW, Portsmouth D, Draxler W, O'Rourke M, Richmond P, Hall S, McBride WJH, Redfern A, and Askov J (2015) An inactivated Ross River virus vaccine is well tolerated and immunogenic in an adult population in a randomized phase 3 trial. *Clinical and Vaccine Immunology* 22(3): 267–273; Bukh J (2016) The history of hepatitis C virus (HCV): Basic research reveals unique features in phylogeny, evolution and the viral life cycle with new perspectives for epidemic control. *Journal of Hepatology* 65(1): S2–S21; Deng S-Q, Yang X, Wei Y, Chen J-T, Wang X-J and Peng H-J (2020) A review on dengue vaccine development. *Vaccines* 8; Ganges L, Crooke HR, Bohórquez JA, Postel A, Sakoda Y, Becher P, and Ruggli N (2020) Classical swine fever virus: The past, present and future. *Virus Research* 289: 198151; Powers JM, Haese NN, Denton M, Ando T, Kreklywich C, Bonin K, Streblow CE, Kreklywich N, Smith P, and Broeckel R (2021) Non-replicating adenovirus based Mayaro virus vaccine elicits protective immune responses and cross protects against other alphaviruses. *PLoS Neglected Tropical Diseases* 15(4): e0009308.

Conclusion

Flaviviridae and Togaviridae families contain important disease causing agents which contribute to high economic burden across the globe. These viruses cause diseases in both humans (Dengue, YF) and animals (CSF). Spread and re-emergence of alphaviruses is increased due to increased trade and tourism. Lack of specific antiviral treatment and immunization against these are currently the pressing concern. Intensive research is required to evaluate the effectiveness of repurposed drugs or specific antiviral agents in animals as well as humans. However, vaccines for some viruses are available (i.e. YFV, DENV), under clinical trials (i.e. CHIKV, VEEV, RRV) but unavailable, and developed for study purposes but unlicensed to be used (i.e. MAYV). Unavailability of the already developed vaccines (i.e. Dengvaxia®) globally is another pressing concern which needs to be addressed at political and government level. A thorough understanding of epidemiology, biology, and genetic variability will result in improved diagnosis, prevention and treatment of the disease caused by these viruses. Furthermore, vigilant surveillance, epidemiological studies, and updated geographical data should be conducted and readily available to improve the current disease scenario.

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Hepatitis Viruses

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Introduction

Hepatitis or inflammation of the liver is caused by many infectious agents, including viruses and non-infectious factors such as metabolic agents. Although the causes of viral hepatitis are numerous, the five major viruses are known to be the viruses that cause disease exclusively in the liver. Among these, hepatitis B, C and D viruses are known as infectious or serum hepatitis and hepatitis A and E are known as gastrointestinal hepatitis. The criteria for this classification are mainly based on the mode of transmission and their pathogenesis, so that infectious hepatitis is usually caused through blood or sexual transmission and gastrointestinal hepatitis through oral-fecal route. Acute infection with infectious hepatitis is usually asymptomatic and may occur depending on age and host factors, while in gastrointestinal hepatitis the symptoms of acute hepatitis are more common (Khuroo and Sofi, 2020).

Hepatitis B virus (HBV)

Virus character

Hepatitis B virus is a member of the hepadnaviridae family that has a spherical structure with a semi-double stranded circular DNA genome (Fig. 1). The virus genome encodes five proteins called surface protein (S), core (C), polymerase (P), secretory protein (e), and a regulatory protein called X. Of the five virus proteins, three are important in diagnosis. The most important of these proteins is

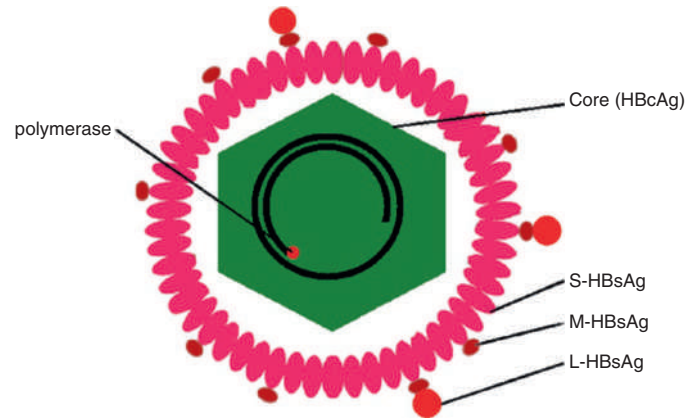


Fig. 1 Schematic structure of HBV.

the surface antigen, called HBsAg, which is produced in large amounts during virus replication. Although this protein is present in three different structural forms called S, preS1 (M) and preS2 (L), it is referred to as HBsAg only in the diagnosis. The second protein is core, which forms the virus capsid and is identified as HBcAg. The diagnostic significance of this protein is that it is produced only during replication and intense activity of the virus. The e protein (HBeAg) is also produced during the replication of the virus and due to its secretory nature, leaves the cell and is able to stimulate the immune system and produces specific antibody (Rybicka and Bielawski, 2020; Knipe and Howley, 2013).

Route of transmission

The main routes of HBV transmission are:

- Exposure to contaminated blood and blood products.
- Sexual ways.
- Vertically (from mother to fetus) through placenta and milk (Hou et al., 2005).

Clinical manifestations

The incubation period of the disease varies from 6 weeks to 6 months. The acute course of the disease is asymptomatic in a high percentage of infected people. The incidence of symptoms increases with age, so that in children 10% and in adults 50% of infections have clinical symptoms. If symptoms occur, acute infection is associated with an increase in ALT of more than 500 IU/L and jaundice. Hepatic complications are generally caused by the destruction of liver cells by the cytotoxic activity of immune T cells. Cases of fulminant hepatitis and death occur in less than 1% of infections. About 95% of adults clear the virus within 6 months, but if the person is unable to clear the virus, the infection will become chronic.

Chronic infection refers to a condition in which the virus has been active in the body for more than 6 months, which can be confirmed by the presence of various viral factors. The most important of these factors is HBsAg, which is considered as a determining factor in infection and chronic disease (Kasper et al., 2015). Depending on the level of virus activity and virologic markers, chronic patients are divided into different groups.

Types of chronic HBV

Inactive carriers

In these individuals, also known as carriers in brief, HBcAb and HBsAg are positive, but the virus DNA level is not detectable or less than 10,000 copies per milliliter or 20,000 international units per milliliter. Liver enzymes are also normal.

Chronic hepatitis

In these people, in addition to HBsAg, HBeAg is also positive and the amount of virus DNA is more than 100,000 copies per milliliter. The liver biopsy shows a score greater than 4 and the level of liver enzymes is also permanent or periodically high (Hadziyannis and Laras, 2018).

Infants born to an infected mother (immune tolerance)

Infection in fetus or at early stages of the life is usually transmitted from an infected mother, especially in cases that the mother is HBeAg positive. This type of chronic infection is called immune tolerance status due to the lack of immune system development (Cryer and Imperial, 2019). In this case, HBeAg is positive and the amount of DNA is more than 20,000 IU/mL, but because of no destruction of liver cells by the cellular immune system, the enzyme alanine aminotransferase is normal. Liver biopsy of these individuals is usually without signs of inflammation and fibrosis. Due to the high amount of serum DNA, these people are highly contagious (Tran, 2011).

Occult hepatitis

Occult hepatitis is a condition that the virus DNA can be detected in liver or serum in the absence of HBsAg. The gold standard for this condition is DNA virus detection in the liver, but for the convenience of clinical cases, blood samples are used. It is important to note that most commercial HBsAg detection kits are able to detect all types and sub-types of virus, but may not be able to detect mutations in the S region of the virus, so it should always be considered that the hepatitis report in cases of a mutated S, may be false positive (Raimondo et al., 2019).

Laboratory diagnosis

HBsAg is the most important serum marker for diagnosing HBV infection. The presence of HBsAg indicates infection and the person is infected as long as HBsAg or HBV DNA can be detected in the blood. In addition to the S antigen, the presence or absence of HBeAg in the serum can determine severe or mild infection. The presence of this antigen indicates a high infection in the individual, while the presence of the e antibody indicates a low or no infection in the blood. However mutations in the virus that lead to the lack of core expression (core mutants) or pre-core mutants, despite the inability to detect HBeAg in some individuals, large amounts of the genome is present in the blood, so the presence of DNA is a more accurate measure of infectivity. Anti-HBc IgM is important in detecting acute infection, although it has also been seen in cases of active chronic infection (Fig. 2). The presence of this antibody in the absence of HBsAg indicates recovery from an acute illness and is one of the criteria for differentiation of immunity caused by infection or vaccination (Coffin et al., 2019; Gerlich, 2013).

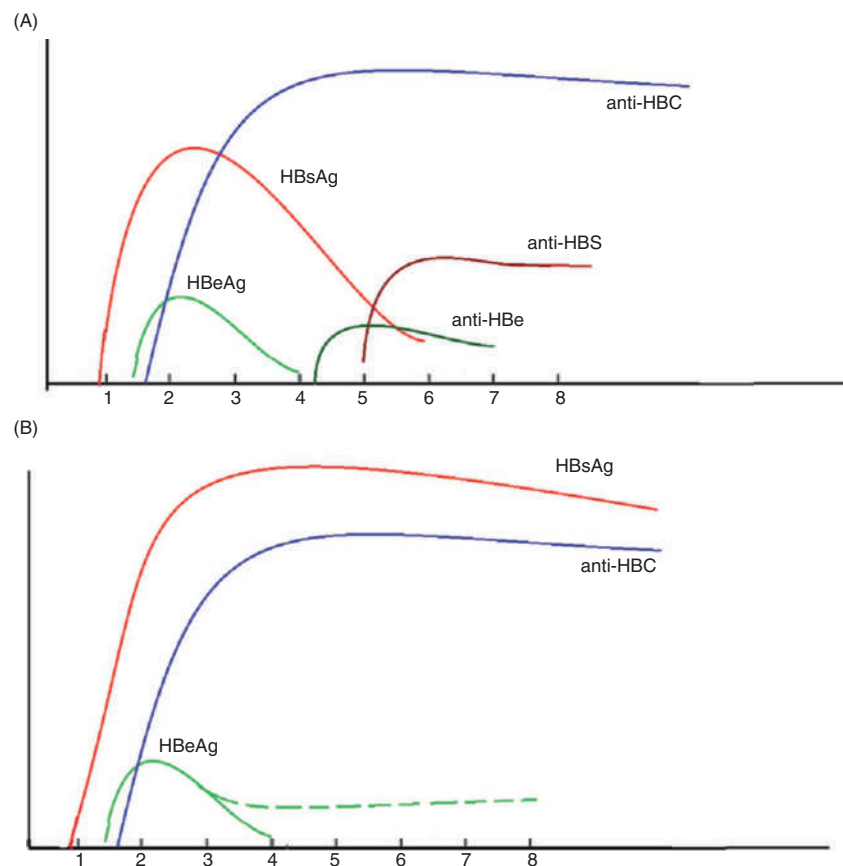


Fig. 2 Serologic markers in acute (A) and chronic (B) HBV infection.

Applications of hepatitis diagnostic tests

Hepatitis B diagnostic tests are used for a variety of reasons:

1. In order to identify the disease in cases of suspected acute hepatitis. In the hepatitis diagnostic panel, along with hepatitis A and C, two tests of HBsAg and IgM against the core antigen (HBcAb) are performed. If HBsAg is positive, the person has hepatitis B. In rare cases, core IgM antibody (Anti-HBc) are the only indicator of infection, depending on the time passed from infection. During the early stages of recovery or the S window period, in which both the antigen and the antibody are negative, this antibody is the only indicator of infection. If HBsAg is positive and HBc IgM is negative, it indicates a chronic infection, and if HBsAg is negative, it indicates a past infection and recovery.
2. To assess the immunity against HBV. Immunity against hepatitis B is obtained through vaccination or recovery from the disease. In 90 to 95% of cases, vaccinated people develop antibodies against the virus that last for 5–10 years. The hallmark of immunity is the presence of antiHBs greater than 10 IU/mL. To distinguish immunity induced by vaccination from infection, antibodies against core virus (antiHBc) are used, which are only positive in people who have been exposed to the virus (Shih et al., 2018).
3. In order to identify chronic forms of the disease and follow the treatment process.

Chronic infection refers to the presence of HBsAg in the blood for more than 6 months, so if the S antigen is negative, the possibility of chronic infection is ruled out. Hepatic alanine aminotransferase (ALT) should be measured frequently if a person is diagnosed with a chronic condition. Permanent or intermittent high level of this enzyme indicates liver disease. It is important to note that the amount of this enzyme varies with age and sex, and in some cases, even with acute liver disease, there is no increase in ALT (EASL, 2017).

Interactions between hepatitis B virus and host immune system

Earlier studies failed to detect innate immune response such as IFNs in the early stage of HBV infection, so HBV was considered as a “stealth pathogen” that could establish persistent infection in hepatocytes by evading the host innate immune system (Kuiper et al., 2020). However, several lines of evidence have been questioned this property of the virus because it has been reported that HBV antigens directly trigger cellular immunity but using some mechanisms avoid recognition by the innate immune system. For instance, it is observed the interferon-stimulated genes (ISGs) expression is induced in the liver of infected chimpanzee model and HBV was unable to interfere with host cellular gene transcription (Megahed et al., 2020).

Furthermore, it has been shown that in the early phase of infection, HBV replication is suppressed in an interferon-independent manner. TLR2 senses HBV particles and leads to production of some inflammatory cytokines such as IL-6, IL1B and TNF. This hepatic innate response in addition to limit HBV replication, organizes adaptive immune responses. On the other hand, some studies indicated that during acute phase before the peak of viremia, HBV infection did not induce strongly the Toll-like receptors, pathogen recognition receptors (PRR) pathways, and production of IFNs but did trigger expression of the anti-inflammatory cytokine IL-10 that may contribute to the establishment of intrahepatic immune tolerance (Broering et al., 2020).

During the activation of the innate immune response, PRRs recognize viral RNAs and Toll-like receptors (TLR3, 7, 8, and 9) detect HBV RNAs in the endosome. After the activation of nuclear factors such as IRFs, NF- κ B and other transcription factors, a large array of genes involved in immune and inflammatory responses are induced and affect HBV replication and assembly. Suppression or defect of the involved receptors and signaling pathway lead to persistence of HBV. The evidence provided that HBV replication can inhibit PRR functions and target the TLR system, therefore, attenuate the anti-HBV innate immune responses. Thus, in the liver microenvironment HBV might has strategies to escape of the host innate immune system and it can counteract the innate responses. For example, HBx protein acts as a deubiquitinating enzyme which targets and disrupts RIG-I pathway and down regulates MAVS. HBV polymerase inhibits IFN-regulatory factors (IRF) activation in response to TLR3 and HBV precore/core proteins inhibit myxovirus resistance A (MxA) gene expression (Tang et al., 2018).

Overall, there are contradictory reports about HBV infection and host innate immunity. Different HBV genotypes induce different levels of innate immunity response. It is critical similarity of the innate immunity response in experimental models and primary human hepatocytes (Broering et al., 2020).

Adaptive immune responses are observed in patients with self-limited HBV infection. The studies showed that T CD8⁺ cells play an important role in the clearance of HBV and T CD4⁺ cells facilitate the induction of T CD8⁺ cells. Additionally, B cell-mediated humoral immune responses limit viral spread from HBV infected hepatocytes that are not eliminated by the T CD8⁺ cells. Nevertheless, patients with chronic hepatitis B (CHB) have defects in immune response which results in persistent viral replication and liver inflammation. Several mechanisms contribute to the weak adaptive immune responses during CHB in which characterized by (1) high HBV DNA, HBsAg, and HBeAg levels which induce HBV-specific immune tolerance, (2) exhaustion and dysfunctions of HBV-specific T cells under sustained HBV antigens stimulation, (3) disorder and decreased numbers of DCs and NK cells, (4) enhanced expression of immune checkpoint proteins, (5) intrahepatic inflammatory reactions can suppress the ability of HBV-specific T cells to access the infected liver parenchyma, (6) some enzymes such as arginase that is released from damaged hepatocytes cause depletion of the arginine which is essential in maintaining T cell functions and TCR-pathway dysfunction due to down-regulating of the CD3z signaling molecule, (7) T regulatory cells, B cells, and stellate cells infiltrating the hepatocytes can

secrete suppressor cytokines, such as TGF- β and IL-10 (Bertoletti and Ferrari, 2016). In general, despite the studies, there are still many complexities regarding immune responses against HBV that remain unknown.

Hepatitis D

Virus character

Hepatitis D or delta is a defective infectious agent that depends on the presence of hepatitis B surface protein for replication. The virus has a negative single-stranded RNA genome that forms a rod-shaped structure due to the complementarity of a high percentage of nucleotides with each other. The virus encodes two proteins called L and S that are different at the carboxyl terminus. The only detectable antigen of the delta virus is the smaller type of antigen (HDVAg or delta Ag) which forms the capsid of the virus (Knipe and Howley, 2013).

Clinical manifestations

Because the virus is unable to reproduce and cause infection without the presence of hepatitis B, infection with the virus is only seen in patients who are infected with HBV. Accordingly, two types of infection may occur:

HBV and HDV Co-infection. In this case, a susceptible person is exposed to both viruses at the same time, and both viruses cause infections. Co-infection with these two viruses generally results in acute hepatitis and clearance of both viruses from the body.

Super-infection with HDV. If a person carrying the hepatitis B virus becomes infected with HDV, it can cause acute hepatitis caused by HDV. Hepatitis D, like hepatitis B, can clear the body after an acute infection or lead to a chronic infection. In the second type of infection, the virus usually stays in the body chronically (Fig. 3). The rate of HDV infection in hepatitis B carriers varies from 70% to less than 1% in different geographical areas. So far, eight different genotypes have been identified for this virus, one of which has a global distribution and is divided into two subgenome "a" and "b". Genotypes 2–8 are limited to more specific regions. Different genotypes are different in terms of pathogenicity, so that genotype 3 is the most common cause of acute hepatitis, but genotype 2 usually does not cause specific pathological changes in the liver (Negro, 2014).

Diagnosis

Infection is detected by measuring IgG against delta antigen. IgM is not always produced in acute infection, but if it is produced, it can confirm acute infection.

IgM is detected during the window phase, the time between the appearance of delta antigen and the detection of IgG. If the infection progresses to a chronic infection, the amount of this antibody increases, so tracking this antibody indicates that the disease is progressing to a chronic disease. Recognition of the virus by molecular methods is the best marker of infection, and by quantitatively measuring the virus genome, the effectiveness of treatment can be assessed so that if the HDV virus is reduced by at least 5 log copies/ml during treatment for hepatitis B, it indicates successful treatment (Botelho-Souza et al., 2017).

Interactions between hepatitis D virus and host immune system

There are only a few models available to study HDV infection in human hepatocytes and the interactions between HDV with the immune responses. Despite the very limited coding capacity of HDV genome, it has mechanisms to escape immunity. Because of the HDV genome replicates in the nucleus and its circular RNA genome, the virus is able to avoid PRR binding and not to activate RIGI (Jung et al., 2020). Although, viral actions trigger immune responses via MDA-5 but HDV immune recognition still needs to be identified and it is not clear if HDV activates IFN signaling pathways in infected patients.

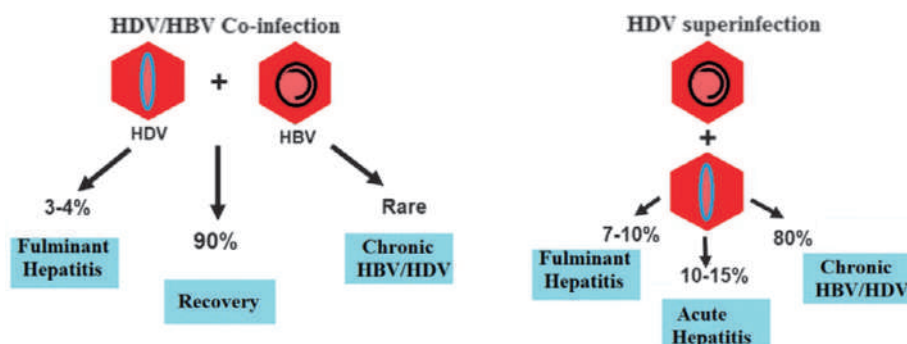


Fig. 3 Consequences of super/co infection of HDV and HBV.

Adaptive immune responses in HDV infections are weak and defective. Since the virus expresses only two proteins, S-HDAg and L-HDAg, with largely overlap in their protein sequence, few epitopes can be identified by T cells. On the other hand, HDV-specific helper T-cell expresses cytokines such as a high frequency of IL-10, IFN- γ , and IL-2 that stimulates cytotoxic T CD8⁺ cells to proliferate. In consequence, cytotoxic T-cells destroy HDV-infected cells. IFN- γ stimulates killing by macrophages and also induces CXCL-10 which recruits NK cells, dendritic cells, T-cells and macrophages to destroy infected cells and causing the more severe course of liver diseases in patients with HDV-chronic infection (Abbas and Afzal, 2013).

It has also been reported that L-HDAg provoke a sustained activation of the innate defense mechanisms. Therefore, the liver damage due to chronic HDV infection seemed to be immune mediated.

Co-infection with HBV must be beneficial for HDV. Indeed, HBX protein down regulates MAVS-induced signaling. Various studies reported HBsAg promotes to activate STAT3 and NF- κ B signaling that causes ER stress and induces the production of reactive oxygen species (ROS), hence, activates DNA methyltransferases, (it can silence the tumor suppressor genes) that might be necessary for induction of L-HDAg translocation from nucleus to cytoplasm in order to viral assembly and persistence of HDV. Thus, it has been proposed HBV prevent the innate immunity and make the cellular conditions favorable for HDV infection (Giersch and Dandri, 2015).

Hepatitis C virus

Virus characters

HCV is an enveloped virus with a positive polarity single-stranded genome from the Flaviviridae family. So far, seven genotypes and a number of subtypes have been identified. Genotypes one to three are globally distributed, while genotypes four and five are found mainly in Africa and limited parts of Asia, and genotype six in Asia. Genotypes are each divided into subtypes like 1a, 1b, 3a and 3b. The genome of HCV encodes 10–11 proteins, of which the core proteins, E1, E2, and possibly P7, are involved in the formation of the virus structure, and the rest are non-structural (NS) regulatory proteins. Protein E, which is produced by changing the reading frame of the core protein, is also considered non-structural. There are non-translating regulatory segments at both ends of the genome that can be used to detect viruses (Knipe and Howley, 2013).

Route of transmission

Transmission of the virus is mainly done through the following ways:

- Transfer of contaminated blood and blood products. Today, with the screening of blood and blood products, transmission through this route has sharply decreased.
- Using shared syringes in drug abusers. This method is one of the most important ways of transmitting the disease.
- Nosocomial infections. If medical equipment is not sterilized properly, it can transmit the disease. Patients undergoing renal dialysis are also prone to infection.
- Accidental infection in case of contact with contaminated blood.
- Tattoos and ear piercings.
- Sexual transmission.
- Vertical transfer from mother to fetus (Alter, 2011).

Clinical manifestations

The incubation period varies from 2 weeks to 6 months. About 60–70% of acute infections are asymptomatic, and in a percentage of people the symptoms include only lethargy, loss of appetite, nausea, and abdominal pain. Symptoms of jaundice are seen in only a few percentage of people. About 20% of patients with acute infection recover, but in 70–80% of cases the virus remains chronically in the body, in which there is no specific symptom of the disease and in some people there are nonspecific symptoms such as lethargy and fatigue. In 20–30% of people, after 20–30 years, the liver progresses to cirrhosis. Age, male gender, alcohol consumption and concomitant infections with other liver infecting agents can contribute to the aggravation of the disease (Wiktor, 2017).

Diagnosis

In general, HCV tests are divided into two main groups: screening and confirmatory tests. Screening tests only detect antibodies and do not distinguish between past, present, or chronic infections. Confirmatory tests diagnose the patients having an active infection.

Despite the high specificity of immunoassay screening tests, false positives are seen especially in pregnant women, people with immunological or blood diseases, and when the test is performed in low-risk communities.

False negatives are also seen in people with suppressed immunity, people with chronic kidney failure, dialysis, and the early stages of HCV infection. In such cases, especially in communities with a low prevalence of the disease, a complementary test is recommended to verify the initial results (EASL, 2020).

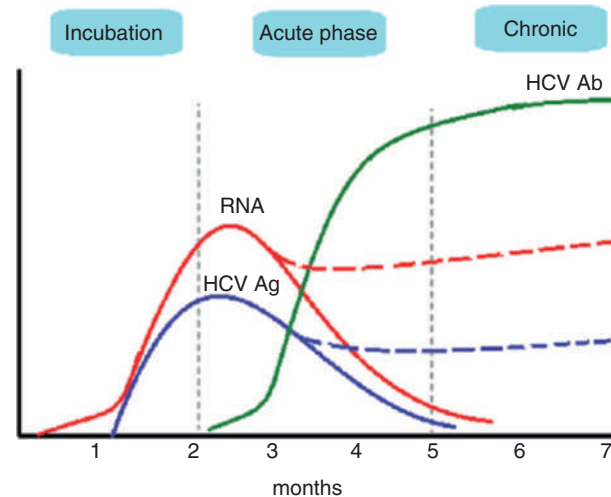


Fig. 4 Serologic markers in HCV infection.

Molecular tests. Serological tests cannot distinguish active infection from past infection. For this reason, a molecular confirmation test is necessary to confirm positive serological results. The viral RNA can be detected in the blood 1–3 weeks after infection and 1 month before the antibody appears (Fig. 4). Virus genome identification is the gold standard of diagnosis in ELISA positive subjects (Fabris et al., 2008).

Tests based on the detection of core antigen have also been designed, the amount of changes in core have shown a good correlation with the load of the virus genome (Chevaliez, 2019; Seme et al., 2005).

Interactions between hepatitis C virus and host immune system

Innate and adaptive immune responses play an important role in determining the viral clearance or progression to chronic infection. Upon HCV infection, the virus immediately activates intracellular pathogen recognition receptors (PRRs) including protein kinase R (PKR), toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I-like receptors (RLRs), RNA helicase retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). These receptors recognize viral pathogen-associated molecular patterns (PAMPs) which trigger different signaling cascades of the intracellular innate immune defense to induce interferons (IFNs) and the pro-inflammatory cytokines genes expression (Ferreira et al., 2020).

HCV also interferes with the adaptive immune system. There has been controversy over the role of humoral immune responses in the outcome of HCV infection. It is demonstrated that HCV clearance was associated with early appearance of neutralizing antibodies (nAbs) in the early phase of infection. In contrast, despite the production of antibody against HCV, during chronic infection, the escape variants of HCV are generated due to the selective pressure exerted by nAb responses (Lapa et al., 2019). It is known that HCV clearance is associated with a strong T-cell CD8⁺ and CD4⁺ response. In patients with persistent infection CD8⁺ cytotoxic T cells are relatively few and these cells recognize fewer epitopes (Tomer and Arora, 2020).

HCV via a number of mechanisms is able to impair the functional cells of immune system or subvert the host immune response resulting in establish a chronic infection. In fact, multiple HCV proteins including core, NS3/4A, NS5A, and NS5B counteract innate immune response by suppression of IFN induction. RIG-I/MDA-5, an intracellular sensor of double-strand RNA, binds to HCV RNA to induce IFNs via a mitochondrial antiviral signaling protein (MAVS) dependent pathway. Another major signaling pathway is mediated by TLR3 resulting in IFN induction. TLR3 is a membrane bound sensor of double-strand RNA which relays the signal downstream to the TRIF adapter. The NS3/4A serine protease of HCV blocks these signaling pathways by cleaving two intermediates, MAVS and TRIF. The result is suppression of IFN induction. NS5A independently interferes interferon action as well in some ways, it acts as antagonist of interferon, binds to PKR during late stages of replication and also induces the IL-8 expression which inhibits the activity of IFN. Beside, E2 glycoprotein directly bind to PKR to regulate its activity. Core protein in monomeric form activates TLR2-MyD88 pathway which it impairs core-TLR interaction. Thus, these particles are not sensed by TLR2. This activation of TLR2 pathway by the monomeric core protein causes the decrease of cytokine responses to TLR ligand and facilitate persistence of HCV infection. Some mechanisms have been suggested for the failure of the adaptive immune responses and persistence of infection. In principle, HCV-specific neutralizing antibodies are not long-lasting even after virus clearance and have a limited role in the control of infection. E1 or E2 glycoproteins-specific antibodies have virus-neutralizing activity. However, the antibodies tend to lose their activity as a result of virus escape mutations (Stuart et al., 2020; Imran et al., 2012).

Interaction of E2 envelope glycoprotein with CD81 leads to polyclonal B-cell activation followed by proliferation of monoclonal antibody (Tomer and Arora, 2020). The expansion of B-cell clones is a major factor in the immunopathogenesis of chronic infection. Interactions of HCV glycoproteins with the scavenger receptor B1 (SCARB1) and high-density lipoprotein (HDL) may protect from neutralizing antibodies. Furthermore, HCV may escape neutralization antibodies by cell to cell direct transmission (Heim and Thimme, 2014).

In acute HCV infections, virus-specific T cells have a crucial role in determining the outcome of virus persistence. Prolonged exposure to virus antigens causes exhaustion of CD8⁺ T cells and functionally the cells are impaired, and characterized by the up-regulation of different inhibitory receptors such as programmed cell death protein (PD1) and cytotoxic T lymphocyte antigen 4 (CTLA4). Under these conditions, the HCV infection progresses to a persistent chronic infection (Mauss et al., 2020).

Hepatitis A

Virus character

The hepatitis A virus is a member of the Picornaviridae that is a nonenveloped virus with positive single-stranded genome. The surface of the virus consists of four different structural proteins called Vp1-Vp4. Hepatitis A is prevalent in all of the world and along with hepatitis E are responsible of foodborne viral hepatitis through the world (Di Cola et al., 2021). In developing countries, 80–90% of children under the age of five have antibodies against the virus while in industrialized countries only 20–5% of children have specific antibodies (Koff, 1992).

Clinical manifestations

The incubation period of the disease is 3–5 weeks. Although the main target of the virus is hepatocytes, due to the gastrointestinal nature of the disease, the gastrointestinal tract appears to be the first site of virus replication. The virus is excreted in the feces from the end of the incubation period and with less intensity than in the middle (Iorio and John, 2020).

Diagnosis

The virus has a serotype and is detected by serological methods. The presence of IgM, which is measured by ELISA, indicates a recent infection, and IgG, indicates past infection. IgM is usually produced up to 10 days after exposure to the virus and peaks after about 4–6 weeks. IgG and IgM can be detected simultaneously 1–2 weeks after the onset of symptoms. If only IgG is positive, it indicates an infection more than 6 months ago because IgM remains in the body for about 4–6 months (average 48–63 days). Virus-specific IgA, which plays an important role in controlling gastrointestinal disease, is also produced following the acute phase.

Viral antigens can be detected in feces, serum, saliva, and cell culture (Abutaleb and Kottilil, 2020).

Molecular methods. Virus genome tracking is the most sensitive method of detecting the virus and appears in the blood before antibody detection and can be checked for a long time during recovery. RNA can also be detected in the stool, although serum is commonly used due to inhibitors such as bile salts, complex polysaccharides, and hemoglobin breakdown products present in the stool. Using PCR, the disease can be detected in the early stages and in the period of the antibody window, especially in the sudden occurrence of the disease.

Interactions between hepatitis A virus and host immune system

Serum HAV RNA level immediately increases after infection. The cytosolic RNA of HAV is sensed by MDA5. Although, the acute HAV infection resolves and does not progress to chronic infection, the studies showed the stimulation of IFN responses and the hepatic expression of ISGs is minimal that can be explained by the high level of viral replication or the disappearance of plasmacytoid dendritic cells (pDCs) (pDCs produce the cytokines including IFN- α by sensing enveloped HAV) from the infected liver after HAV-peak viremia. Indeed, HAV uses several strategies to interfere with the innate immune response. For example, 3ABC viral protein proteolytically cleaves the MAVS and 3CD eliminates TRIF (downstream of TLR3), 3Cpro also cleaves NEMO (NF- κ B essential modulator), thereby attenuates activation and IFN expression (Shin et al., 2016).

In both HBV and chronic HCV infection NK cells have a dual functional, reduced IFN γ production and maintained cytotoxicity, which might contribute to liver injury. It is noteworthy that these functions of NK cells have not been characterized in HAV infection. Also, neutralizing antibodies develop during self-limited infections and provide life-long protective immunity. After convalescence from acute hepatitis A, CD4⁺ T cell responses seem to be important for the resolution of HAV. Overall, acute HAV resolves and does not progress to chronic infection (Walker, 2019).

Hepatitis E

Virus character

Hepatitis E virus is a nonenveloped virus with positive RNA genome that causes a disease similar to hepatitis A. The virus has five main genotypes (HEV1-HEV4 and HEV7) that infect human (Lhomme et al., 2020). Human infection with hepatitis E has two different epidemiological patterns. In areas with lower levels of hygiene, HEV1 and HEV2 circulate between humans and through fecal-oral circulation and is usually transmitted by contaminated water. In developed countries, genotypes 3 and 4 are mainly transmitted by animal reservoirs. In addition to acute infection, chronic infection and sometimes cirrhosis in immunocompromised patients have been reported mainly by genotype 3 (Aggarwal, 2011).

Clinical manifestations

The incubation period is about 6 weeks and jaundice develops in 75% of patients. Other symptoms include nausea, fever, muscle aches, vomiting, diarrhea, and abdominal pain. The amount of aminotransferases (ALT) usually varies between 1000 and 3000 international units per liter (Waqar et al., 2020). If pregnant women are infected, the mortality rate, especially in the third trimester, reaches 20–25% (Terrault et al., 2020).

Diagnosis

Diagnosis of infection can be made either indirectly by measuring specific antibodies or directly by tracking the virus genome in the blood or other body fluids. Following a two to six-week incubation period, IgM is produced for a short period (2 weeks before to 5–7 weeks after the onset of symptoms) and is immediately replaced by IgG antibodies. Commercial immunoassay kits and rapid tests based on ORF1/ORF2 peptides or virus antigens can identify antibodies for all four genotypes. There is no genotype-specific serological test for this virus. Cross-reactivity between the virus and other pathogens has not been reported. IgM decreases rapidly after 8 weeks and reaches an undetectable level within 32 weeks. IgG peaks in 4 weeks and stays high for more than a year. IgM indicates an acute illness.

In patients with acute infection, the RNA reaches an undetectable level about 3 weeks after the onset of symptoms, but can be detected in the stool for up to 2 weeks, so a negative molecular test cannot rule out the disease if patients present with late-stage symptoms.

Due to changes in the results of various laboratories, WHO has recently introduced a standard genome based on genotype 3a. If genomic RNA can be traced for more than 3 months, there is a possibility of latency (Pallerla et al., 2020; Aggarwal, 2011).

Interactions between hepatitis E virus and host immune system

Upon hepatocytes HEV-infection, the virus is recognized by the PRRs, including RIG-I, MDA5, TLRs which these receptors activate downstream cascades such as IRF3/7, MAVS and NF- κ B leading to expression of IFNs and ISGs, and other cytokines. These responses are considered as the first line antiviral immunity in the infected cells (Kang and Myoung, 2017).

Besides hepatitis, HEV also can cause a broad spectrum of extra hepatic manifestations in patients such as renal injuries, acute pancreatitis, neurological and placental diseases. Thus, there is a possibility the innate immune sensors in these non-hepatic cells are also able to detect HEV. The studies showed NK cell prevalence is reduced among pregnant women, thus it would reduce their ability to resolve the virus from the liver and may explain why they are more susceptible to severe hepatitis E. The majority of HEV infections are self-limiting and quickly cleared in healthy persons but in immunocompromised patients, HEV infection may progress to persistent hepatitis and liver injury (Lemon and Walker, 2019). Therefore, the host immune defense plays important role in determinant of outcomes of HEV infection. In turn, it has been reported HEV may encode antagonists of immune responses and also through other multiple strategies able to escape and counteract the host immune defense. HEV-ORF1 encodes a polyprotein including functional regions methyltransferase (Met), papain-like cysteine protease (PCP), HVR, macro domain (X), Y, Hel, and the RNA-dependent RNA polymerase (RdRp). Current data suggest that PCP and X proteins inhibit the activation of RIG-I as well as the phosphorylation of IRF3. Inhibition of the expression of ISGs due to inhibiting nuclear translocation and phosphorylation of STAT1 are attributed to the Met and PCP. Also, RdRp has been shown to interact with miRNA for facilitation of virus replication. ORF2 has inhibitory activity of NF- κ B and can impair the host apoptotic response.

HEV ORF3 protein disrupts IFN α -induced STAT1 phosphorylation and down regulate the expression of ISGs, PKR, and MxA (Li et al., 2019). Eventually, interplay between host immunity and the viral proteins indicates the clinical outcome. Nevertheless, in this regard several knowledge gaps remain that need to further investigate.

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Human Immunodeficiency Virus (HIV)

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Introduction

In the late 1970s and early 1980s, an unusual number of young homosexual men, Haitians, heroin addicts, and hemophiliacs in the United States were noted to be dying of normally benign opportunistic infections (Barre-Sinoussi et al., 1983; Gallo et al., 1983). Their symptoms defined a new disease, the acquired immunodeficiency syndrome (AIDS). However, as is now known, AIDS is not limited to these groups but can occur in anyone exposed to the virus. Now approximately 38 million men, women, and children around the world, including 1.2 million in the United States are living with the virus that causes AIDS. While AIDS was initially believed to be a “lifestyle” disease seen primarily in limited populations, it soon became apparent that this was a blood and body fluid borne disease. Most infections in the United States (66% as of 2019, Centers for Disease Control and Prevention, 2019) have been associated with male-male sexual transmission, however, worldwide, approximately 75% of HIV infections are associated with heterosexual transmission. When HIV was identified as the causative agent, it became possible to track the virus and determine the method(s) of transmission (Levy et al., 1984). The virus is transmitted by IV drug abuse, unmonitored blood and organ transplant but mostly unprotected and promiscuous sex and not by any one group of individuals. AIDS was originally a universally fatal diagnosis; however like diabetes, the advent of anti-retroviral drugs has allowed infected persons to live a normal lifespan (reviews on HIV and AIDS: Deeks et al., 2015; Ghosn et al., 2018; Saag, 2021).

Virology

Montagnier and associates in Paris, and Gallo and colleagues in the United States, reported the isolation of the human immunodeficiency virus (HIV-1) from patients with lymphadenopathy and AIDS (Barre-Sinoussi et al., 1983; Gallo et al., 1983). A closely related, less virulent virus, designated HIV-2, was isolated later and is prevalent in West Africa. HIV appears to have been acquired by humans from chimpanzees as early as the 1800s and then spread through Africa and the world by an increasingly mobile population. Although a devastating disease that cannot be completely cured, the development of antiviral drug cocktails (highly active antiretroviral therapy [HAART]) has allowed many HIV patients to resume a normal life.

HIV, like other retroviruses, is an enveloped positive-strand ribonucleic acid (RNA) virus with a unique morphology, encodes an RNA-dependent deoxyribonucleic acid (DNA) polymerase (reverse transcriptase [RT]) and replicates through a DNA intermediate (Rosenthal, 2021). The DNA copy of the viral genome is then integrated into the host chromosome to become a cellular gene.

HIV is a complex retrovirus based on its genome and mode of replication and within the lentivirus class, based on the nature of its disease. Retroviruses are roughly spherical, enveloped, RNA viruses with a diameter of 80–120 nm. The envelope contains the gp120/gp41 viral glycoprotein complex which is acquired by budding from the plasma membrane. The envelope surrounds a capsid that contains two identical copies of the positive-strand RNA genome inside an electron-dense core. The virion also contains 10 to 50 copies of the reverse transcriptase and integrase enzymes and two cellular transfer RNA (tRNAs). These tRNAs are base-paired to each copy of the genome to be used as a primer for the RT. The morphology of the core for HIV resembles a truncated cone which differs from other retroviruses (Fig. 1).

The genome of HIV includes the three major genes similar to all retroviruses which encode polyproteins for Gag (*group-specific antigen*, capsid, matrix, and nucleic acid-binding proteins), Pol (*polymerase*, protease, and integrase), and Env (*envelope*, glycoproteins). These genes encode polyproteins which are subsequently cleaved into functional proteins by cellular and the viral protease. In addition to these genes, HIV also encodes the tat and rev regulatory proteins and the nef, vif, vpr and vpu (HIV-1)/vpx (HIV-2) accessory proteins. The transcripts for these proteins require more complex transcriptional processing (splicing). These proteins promote the processing or virulence of the virus and will be discussed later. At each end of the genome are long terminal repeat (LTR) sequences. The LTR sequences contain promoters, enhancers, and other gene sequences used for binding different cellular transcription factors. Some of the genes are expressed early in the replicative cycle and others, late in the cycle.

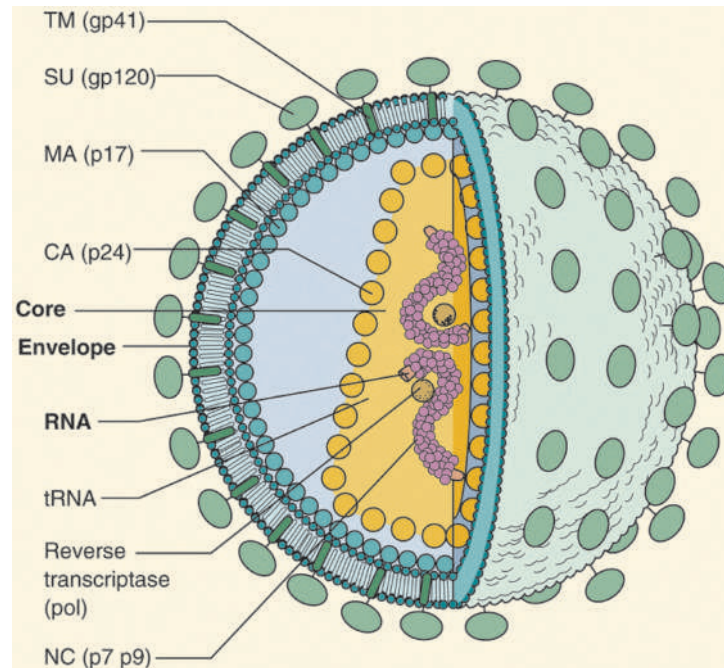


Fig. 1 Cross section of human immunodeficiency virus. The enveloped virion contains two identical ribonucleic acid (RNA) strands, RNA polymerase, integrase, and two transfer RNAs (tRNA) base-paired to the genome within the protein core. This is surrounded by proteins and a lipid bilayer. The envelope spikes are the glycoprotein (gp)120 attachment protein and gp41 fusion protein. CA, Capsid; MA, matrix; NC, nucleocapsid; SU, surface component; TM, transmembrane component of envelope glycoprotein. From Rosenthal KS (2021) Retroviruses. In Murray PR, Rosenthal KS, Pfaller MA. *Medical Microbiology* 9th edn. pp. 533–549. Elsevier.

Replication

HIV infection starts with binding of the viral glycoprotein spikes on the surface of the envelope (trimer of gp120 and gp41 molecules) to the primary receptor, the CD4 protein, and then a second receptor, a 7-transmembrane G-protein-coupled chemokine receptor (Fig. 2) (Rosenthal, 2021). Binding to these receptors determines the tissue tropism and host range of HIV which includes CD4 T cells, myeloid and other cells. The co-receptor used upon initial infection by HIV is CCR5, which is expressed on myeloid and peripheral, activated, central memory, intestinal, and other subsets of CD4 T cells (macrophages, [M]-tropic virus). Later, during chronic infection of a person, the env gene mutates so that the gp120 binds to a different chemokine receptor (CXCR4), which is expressed primarily on T cells (T-tropic virus). Binding to the chemokine receptor activates the cell and brings the viral envelope and cell plasma membrane close together, allowing the gp41 to interact with and promote fusion of the two membranes. Binding to CCR5 and gp41-mediated fusion are both targets for antiviral drugs.

Once the nucleocapsid (genome within the conic protein capsid) is released into the cytoplasm, the early phase of replication begins. As the nucleocapsid traverses the cytoplasm to the nucleus, the RT, encoded by the pol gene, uses the tRNA in the virion as a primer and synthesizes a complementary negative-strand DNA (cDNA). The RT also acts as a ribonuclease H, degrades the RNA genome, and then synthesizes the positive strand of DNA. The RT is a major target for antiviral drugs. During the synthesis of the virion DNA (provirus), sequences from each end of the genome (U3 and U5) are duplicated, thus attaching the LTRs to both ends. This process creates sequences necessary for integration and creates enhancer and promoter sequences within the LTR for regulation of transcription. This creates a DNA copy of the genome which is larger than the original genomic RNA.

RT is very error prone. For example, the error rate for the RT from HIV is one error per 2000 bases, or approximately five errors per genome (HIV, 9000 base pairs). This genetic instability of HIV is responsible for promoting the generation of new strains of virus during a person's disease, a property that switches the tropism of the virus (see glycoprotein above), and can alter the pathogenicity of the virus and promote antiviral resistance or immune escape.

Unlike other retroviruses, the double-stranded cDNA of HIV can enter the nucleus through nuclear pores of resting T cells. However, the genome cannot be utilized until the cell is activated and undergoes division. The genome can remain in the nucleus and cytoplasm in a nonintegrated circular DNA form until the cell is activated. In an activated cell, the cDNA is spliced into the host chromosome with the aid of a virus-encoded, virion-carried enzyme, integrase. Integrase is also a major target for an anti-retroviral drug.

Stimulation of the T cell or macrophages by cytokines, mitogens, in response to other infections, or certain virus infections (e.g. EBV, CMV, HHV6) will generate transcription factors that bind to the LTR to activate the late phase of replication, the transcription

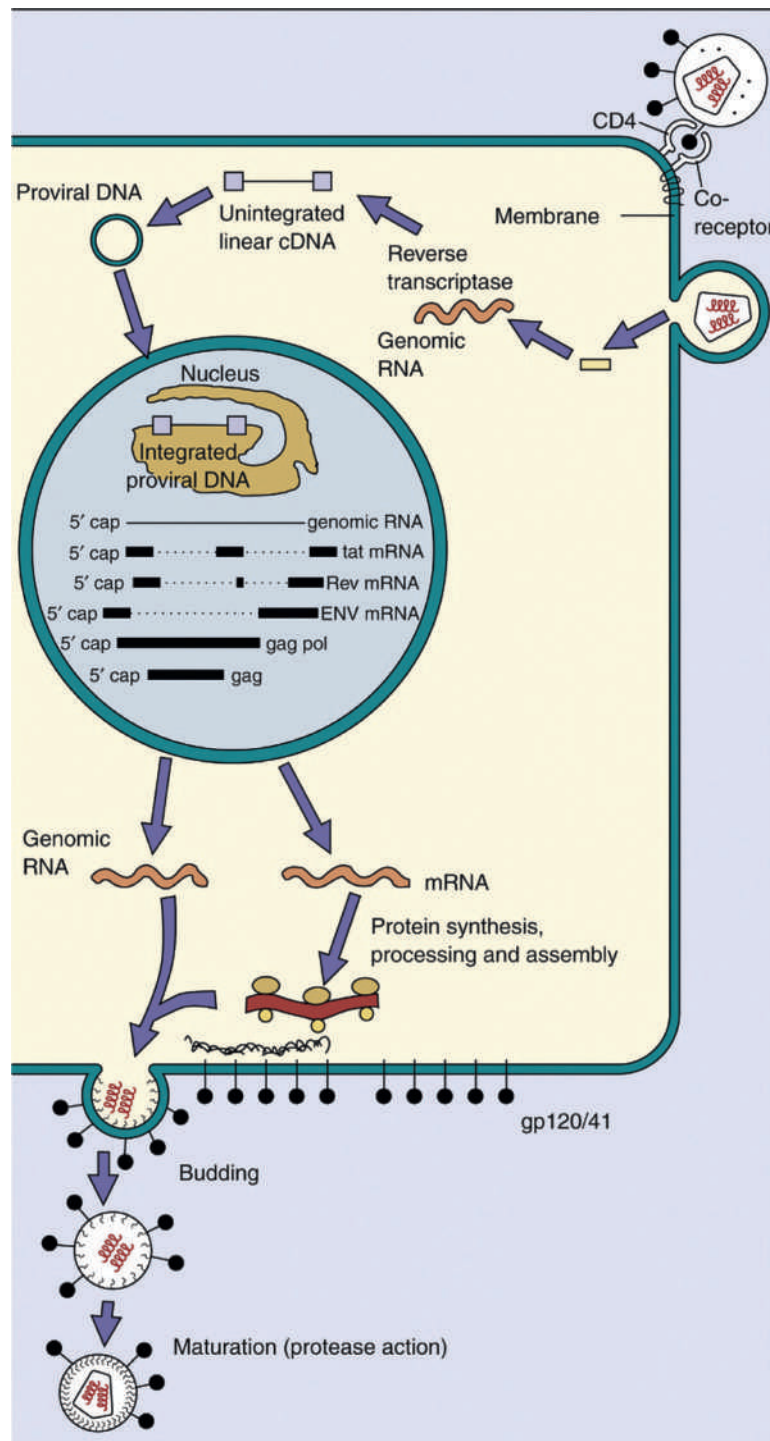


Fig. 2 The life cycle of human immunodeficiency virus (HIV). HIV binds to CD4 and chemokine co-receptors and enters by fusion. The genome is reverse transcribed into deoxyribonucleic acid (DNA) in the cytoplasm, enters the nucleus, and is integrated into the nuclear DNA. Transcription and translation of the genome occur as a cellular gene in a fashion similar to that of human T-cell lymphotropic virus. The virus assembles at the plasma membrane and matures after budding from the cell. *cDNA*, Complementary DNA; *mRNA*, messenger ribonucleic acid. From Rosenthal KS (2021) *Retroviruses*. In Murray PR, Rosenthal KS, Pfaller MA. *Medical Microbiology* 9th edn. pp. 533–549. Elsevier.

of the integrated genome. The viral DNA is transcribed as a cellular gene by the host RNA polymerase II present in activated cells. Transcription of the genome produces a full-length RNA, which like for simple retroviruses is processed to produce several mRNAs that contain the gag, gag-pol, or env gene sequences. The accessory proteins require more complex processing. The full-length transcripts of the genome can also be assembled into new virions.

There are four genotypes of HIV-1, designated M (main), N, O, and P. Most HIV-1 is of the M group, and this group is divided into 11 subtypes, or clades, designated A to K (for HIV-2, A to F). The designations are based on significant differences in the sequence of their env and gag genes and hence the antigenicity and immune recognition of the gp120 and capsid proteins of these viruses. Coinfection of a cell with HIV can allow the two pairs of genomes to recombine and produce hybrids between clades.

Pathogenesis and clinical presentation

As mentioned above, HIV infects cells which express both the CD4 protein and a chemokine receptor resulting in a loss of CD4 positive T cells and monocytes (Fanales-Belasio et al., 2010; Levy, 1993). The loss is most evident in the gut associated lymphoid tissue early in the infection compromising maintenance of barrier and regulatory functions. Chronic stimulation of CD4 and CD8 T cells to HIV and other infections and the HIV induced CD4 T cell death depletes the number of functional cells and causes changes in structure and function to the thymus and lymph nodes. Thus, the infected individual has reduced cell mediated immunity resulting in opportunistic infections.

The original case definition of AIDS, published prior to HIV being identified as the cause, was based on the presence of one or more diseases or conditions indicative of a cell mediated immune deficiency with no known cause for that deficiency. HIV infected individuals are at high risk for viral, fungal, pneumocystis, mycobacterial and parasitic infections (Kerkhoff and Havlir, 2021). CMV retinitis, CNS toxoplasmosis infections, *Pneumocystis jirovecii* and oral candidiasis are common presenting infections. Initially, there was a high incidence of Kaposi's sarcoma seen in late stage AIDS patients, however the incidence of that entity has declined with anti-retroviral treatment. Other blood borne infections also are seen in HIV patients, especially Hepatitis B and C. HIV positive individuals should be screened for each of those entities. In addition to infecting CD4 positive T cells, HIV can infect other CD4 expressing cells, including glial cells in the central nervous system leading to an HIV induced dementia.

The US CDC has staged HIV infection based upon the presence of clinical signs, opportunistic infections and CD4 cell numbers (Fig. 3) (Pahwa et al., 2021). An individual with a negative or indeterminate HIV test who has likely been exposed may be classified as Stage 0. Once an HIV test is positive, an individual may be classified as stage 1. This initial stage of infection may be characterized by a mononucleosis type of syndrome consisting of fatigue, generalized lymphadenopathy and fever. This early retroviral syndrome may last a few weeks. The CD4 count may be normal during this time, however the viral load (see below) will be high. The infection may then enter a quiescent or latent phase. During this period, the virus likely is residing in lymph nodes. The CD4 count may remain stable or slowly decline during this time. In contrast, the viral load may remain at very low levels, or slowly rise during this period. When the CD4 count is below 500 cells/ul, the individual enters Stage 2. This may be an asymptomatic period, or some opportunistic infections, such as CMV, may begin to appear. Stage 3 is entered when the viral load rises, the CD4 count drops below 200 cells/uL and clinically apparent opportunistic infections are present. This is the clinical phase identified as AIDS. In addition to anti-retroviral treatment, individuals in stage 3 may receive anti-pneumocystis prophylaxis and anti-mycobacterial prophylaxis if the CD count drops below 100/ul (Centers for Disease Control, 1981).

Treatment

Initially, an HIV diagnosis was a death sentence. Opportunistic infections might be briefly controlled but there was no effective treatment for the virus. As of this writing (July 2021) there are at least 24 anti-retroviral drugs that may be used in various combinations to treat HIV infected individuals (HIV.gov, 2019; Thompson et al., 2020). Most drugs target the major HIV enzymes, including reverse transcriptase, protease, and integrase. However, there are also cell fusion inhibitors and receptor blockade medications available (Table 1).

The RT inhibitors may be nucleoside (NRTI) or non-nucleoside based (NNRTI). Zidovudine (ZVD), the first anti-retroviral drug used to treat HIV is a nucleoside based anti-RT medication. In an early study, ZVD (known as AZT in its generic form) decreased vertical HIV transmission from 24% to 8%. However, HIV quickly developed mutations rendering it resistant to this medication. This was a harbinger of issues associated with future drugs as the virus is very adept at generating resistant mutations. The biggest advance in anti-retroviral treatment was the use of drug combinations that targeted more than one protein. This highly active anti-retroviral treatment approach, or HAART, initially combined medications that targeted RT and protease (PI). HAART is now referred to as just Anti-Retroviral Treatment (ART). Anti-integrase (INSTI) medications were added and proved effective in keeping viral loads low or even undetectable. Drug combinations also reduce the chance that an individual's virus will develop resistance mutations to multiple drugs simultaneously. If a specific drug combination loses effectiveness, as demonstrated by rising HIV viral loads or decreased CD4 cell counts, laboratories may look for specific resistance mutation in the RT, protease and integrase genes. These methods are detailed below.

While the above-mentioned drug classes work on viral infected cells to inhibit viral replication and assembly, the most recently developed class of anti-retroviral medications prevent viral entry into cells. These medications are not used as front-line treatments but are used when standard ART is ineffective at keeping viral loads low. This class of drugs include a viral gp120-CD4 binding (fostemsavir) inhibitor, a CCR5 antagonist (maraviroc) and monoclonal antibodies targeting the gp120 molecule.

Once a patient is started on anti-retroviral medications, they will be taking those drugs for life. Their medications may need to be changed if their virus develops resistance mutations. The medications are not without side effects, however, and may require an

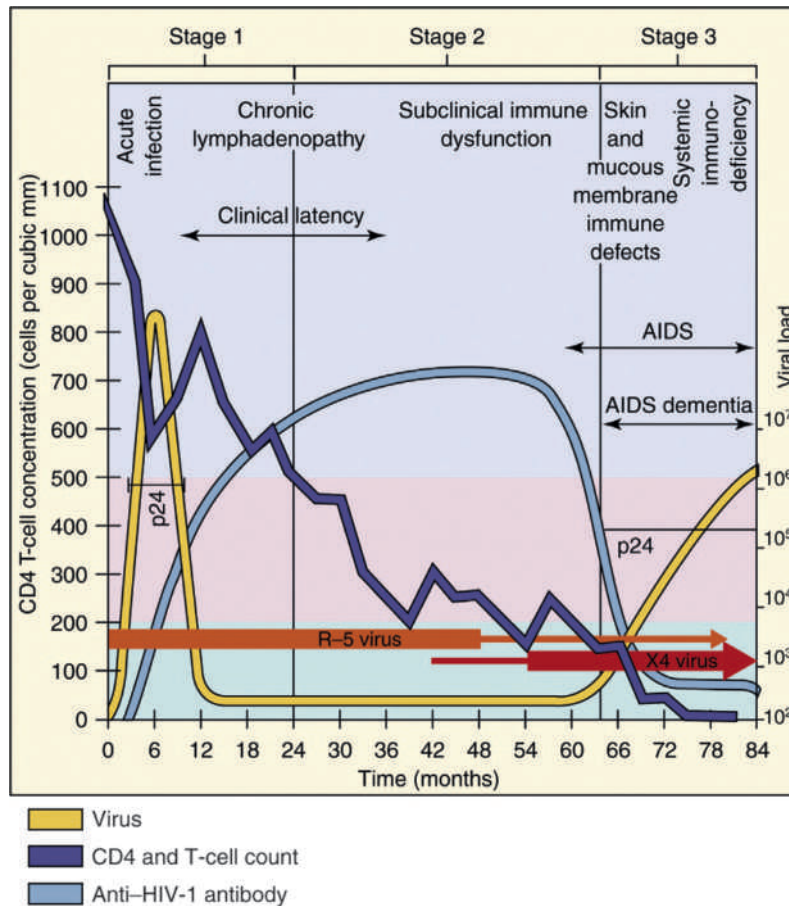


Fig. 3 Time course and stages of human immunodeficiency virus (HIV). A long clinical latency period follows the initial mononucleosis-like symptoms. Initial infection is with the R5-M-tropic virus, and following mutation, the X4-T-tropic virus. The progressive decrease in the number of CD4 T cells, even during the latency period, allows opportunistic infections to occur. The stages in HIV disease are defined by the CD4 T-cell levels and occurrence of opportunistic diseases. HIV can be detected by the presence of p24, HIV genome or antibodies to the virus. From Rosenthal KS (2021) Retroviruses. In Murray PR, Rosenthal KS, Pfaller MA. *Medical Microbiology* 9th edn. pp. 533–549. Elsevier.

Table 1 Anti retroviral medications.

Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTI)

Abacavir, Didanosine, Emtricitabine, Lamivudine, Stavudine, Tenofovir alafenamide, Tenofovir disoproxil fumarate, Zidovudine

Non-nucleoside Reverse Transcriptase Inhibitors (NNRTI)

Cabotegravir, Delavirdine, Efavirenz, Etravirine, Nevirapine, Risperidine

Protease Inhibitors (PI)

Atazanavir, Darunavir, Fosamprenavir, Indinavir, Lopinavir + Ritonavir, Nelfinavir, Ritonavir, Saquinavir, Tipranavir

Integrase Inhibitors (INSTI)

Biktarvy, Dolutegravir, Elvitegravir, Raltegravir

Fusion Inhibitor

Enfuvirtide

CCR5 antagonist

Maraviroc

Adapted from <https://www.webmd.com/hiv-aids/aids-hiv-medication>, accessed 7 August 2021.

alternative ART. Fatigue, gastrointestinal issues, and sleep disorders are known complications. Indinavir, a protease inhibitor, has been associated with kidney stones.

There are two situations where prophylactic anti-retroviral treatment may be given. The first is post exposure prophylaxis. This treatment is used after a single potential exposure to HIV, as in a health care worker receiving a needle stick from an HIV positive patient or a first responder being exposed to blood from an HIV infected individual. The current recommendation is to use a

combination of NNRTI, PI and INSTI medications for 4 weeks post exposure. The medication should be started as soon as at risk exposure is identified, but definitely within 72 h of the exposure (Ford et al., 2018). Rapid HIV testing of the source of the exposure (see below) is routinely used in these situations.

More recently, another form of prophylactic ART, pre-exposure prophylaxis or PrEP, is being used in patients who are not infected with HIV but engage in activities that put them at risk for acquiring HIV. These practices include injection drug use, multiple sexual partners, (heterosexual or homosexual) or a sexual relationship with someone who is known to be HIV infected. Individuals starting PrEP are first confirmed to be HIV negative. They will then take a NRTI based drug combination, Truvada or Descovy, daily. Patients receiving PrEP are tested for HIV at least every 3 months. PrEP has been shown to reduce sexual HIV transmission by over 90% and injection drug use HIV transmission by over 70%. Anecdotally, one of us (TA) has noted a higher-than-expected incidence of syphilis in patients receiving PrEP, possibly due to poorer safe sex practices in those individuals. No formal study has confirmed an association between PrEP and a higher incidence of other sexually transmitted diseases (STDs). However, individuals receiving PrEP are counseled that the treatment is HIV specific and will not protect against syphilis, Chlamydia or other STDs.

Laboratory testing

HIV infection is diagnosed by both antibody and molecular testing (Alexander, 2016; Centers for Disease Control and Prevention and Association of Public Health Laboratories, 2014; Qaseem et al., 2009). The first-generation HIV assays lacked both sensitivity and specificity thus a two-step algorithm was employed. An Enzyme Linked Immunosorbent Assay (ELISA) was used as the first step. Positive specimens were verified by repeat testing followed by a confirming western blot or immunofluorescence assay. These early tests used target antigens obtained from viral culture lysates and detected IgG anti-HIV antibodies. These tests were prone to false positive results and were designed to protect the blood supply, not diagnose an active infection. An infected individual could take 6 weeks or longer to produce a detectable level of antibody, thus a negative result could not be used to rule out an infection. Second and third generation anti-HIV assays used recombinant antigens and detected total antibody (both IgG and IgM) against antigens from both HIV-1 and HIV-2. The third-generation assays reduced the time from infection to diagnosis to 4 weeks. HIV-1 p24 antigen assays, primarily in an ELISA format, were used by blood banks to further reduce the time from infection to detection, as antigenemia precedes detectable levels of antibody by 1–2 weeks. More recently, 4th generation HIV screening assays which detect both antigen and antibody without differentiating them, and 5th generation assays which separately identify p24 antigen and anti-HIV antibody, have been developed (Muthukumar et al., 2015). The current HIV testing algorithm is shown in Fig. 4.

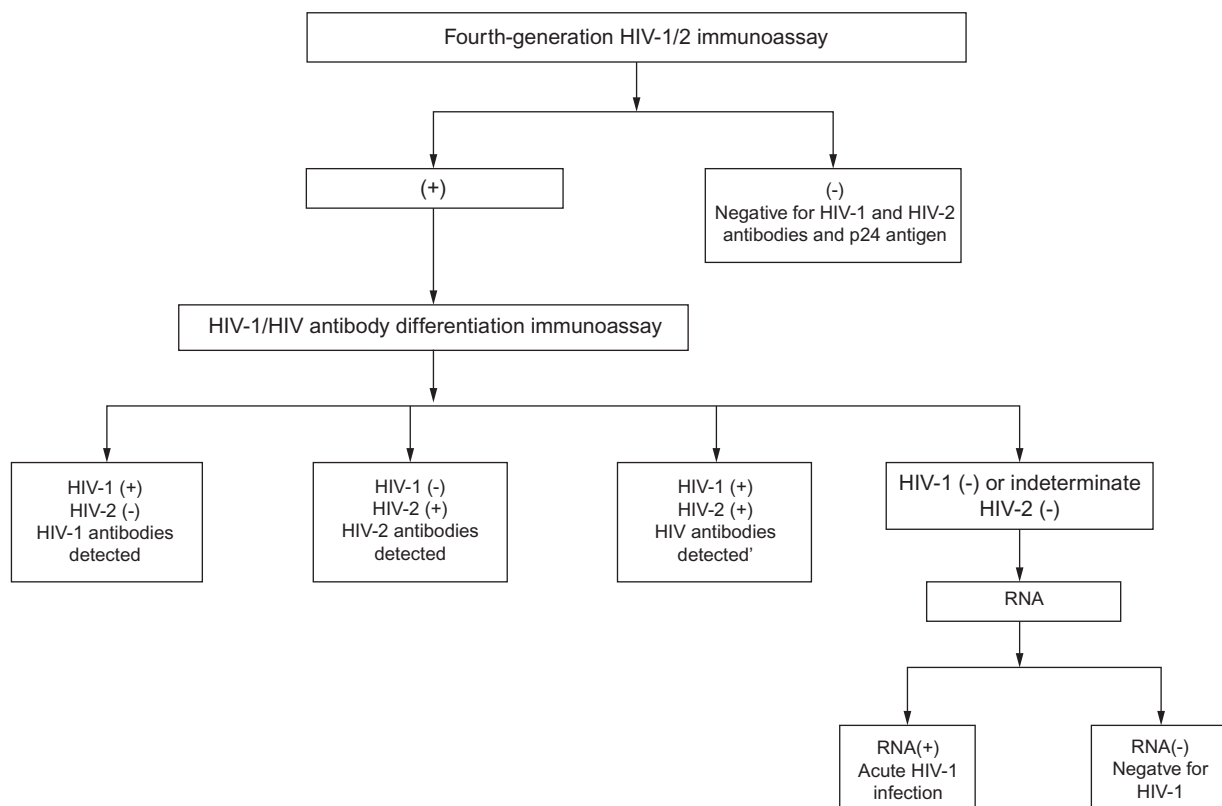


Fig. 4 CDC 4th generation testing algorithm. Centers for Disease Control and Prevention and Association of Public Health Laboratories (2014) Laboratory testing for the diagnosis of HIV infection: updated recommendations. <http://stacks.cdc.gov/view/cdc/23447>, accessed 26 January 2016.

HIV can be transferred from an infected mother to her fetus in utero (Alexander et al., 1999). All serology tests detect an IgG anti-HIV component which can cross the placenta, thus these tests are not useful for identifying HIV infected infants, as maternal antibody would result in a positive result even if the fetus was not infected. Neonatal HIV diagnosis is performed by qualitative PCR testing. The qualitative PCR test is also used to resolve specimens with discrepant results between the first two tests in the current algorithm (Fig. 4). The chances of vertical HIV transmission can be reduced when an infected mother is treated with anti-retroviral therapy.

As mentioned above, HIV infection is a major problem in resource poor areas of the world. These locations may have limited access to facilities that can perform the standard ELISA, chemiluminescent or molecular assays. These areas may use rapid HIV assays, in a card format based on simple ELISA or gold labeled detection methods for HIV screening. These rapid assays are also used in hospitals, emergency rooms and sexually transmitted disease clinics around the world to provide results in as little as 10 min for exposure situations where prophylactic anti-retroviral treatment of an exposed individual may be indicated to prevent a clinical HIV infection. Rapid assays are available for testing serum, whole blood, or oral fluid.

After an individual has been diagnosed with an HIV-1 infection, the amount of virus (viral load) can be monitored with a reverse transcriptase quantitative PCR assay. The immune capabilities of the patient may be followed by determining the % and absolute number of CD4 positive T cells in the peripheral blood. The number of CD4 positive T cells per microliter is used to stage the infection (Fig. 3). Both the viral load and CD4 counts are used to monitor an individual's response to treatment with anti-retroviral drugs.

As mentioned in the treatment section, individuals receiving ART are monitored regularly for viral load levels and CD4 cell counts. The goal of treatment is to reduce the viral load to the lowest or, better still, undetectable levels. Current PCR tests for viral load have a lower limit of detection of 20 HIV copies/ml. This is a vast improvement over the earlier generations of viral load assays that had detection minimums of 75–500 copies/ml. If viral loads do not fall or begin to rise during treatment, it is possible that an individual's virus has developed resistance to one or more of the medications used in their prescribed ART. A laboratory can then subject the individual's virus to an HIV genotype assay which includes genetic sequencing of the RT, protease and integrase genes to determine if there are any mutations known to cause resistance to the medications being used. Alternatively, a more expensive and labor-intensive HIV phenotype assay may be performed. In this procedure, an individual's virus is plated on cells in the presence of specific HIV medications to look for the drug's ability to block the viral replication. There is a combination of the two procedures, an HIV virtual phenotype, which uses the genetic sequence to predict a phenotypic response. In most cases, the HIV genotype assay is sufficient for routine clinical management.

Conclusion

Identifying HIV-1 as the causative agent of AIDS paved the way for developing laboratory testing and treatment modalities. While AIDS remains incurable, viral replication can be controlled by medications and an infected individual's immune response can recover to a point where opportunistic infections may be kept to a minimum. HIV infection remains a serious health problem however, diagnostic, monitoring and treatment regimens continue to improve. One of us (TA) heard Robert A. Good, both a famous and infamous immunologist in the 1960s and 1970s, state at a Medical Laboratory Symposium in Clearwater Florida in 1984 that "AIDS will be a devastating disease but will be the driver of clinical immunology for the foreseeable future." Retrovirology, medical laboratory immunology and clinical infectious disease medicine have certainly been driven greatly by HIV over the past 40 years and the end is not yet in sight.

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Reovirus and Rotaviruses

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Glossary

Reoviruses The name “reovirus” comes from taking the letters R, E, and O from “respiratory enteric orphan virus.”

Icosahedral A regular three-dimensional geometric shape consisting of 20 equilateral triangles and 12 vertices.

ISVP Intermediate (or infectious) subviral particle in an intermediate form of reovirus that removed and modified some proteins by proteolytic enzymes, compared to the infectious virion.

Turreted viruses Designating of the presence of spikes or turrets on the surface of the core particles.

Viroplasms Viroplasms are spotted perinuclear structures that may be thought of as “virus factory” or “virus inclusion.” It is an inclusion body in a cell where viral replication and assembly occurs.

Oncolytic viruses (OVs) The cancer-killing viruses that selectively replicate in the tumor cells and newborn mice.

History

The origins of Reoviruses in the case of virus history and evolution are challenging for defining precisely how and when this virus family was first recognized. It seems that they have been evolving for over about 550 million years. The Reoviruses probably originated a long time ago, and named Reoviruses as an acronym for the respiratory enteric orphan virus in 1959, by Sabin A. B (Sabin, 1959). An orphan virus is a virus that is not associated with a disease but may possess pathogenicity (Knipe and Howley, 2013). *Reoviridae* is a family of viruses, which replicate in a wide host range including invertebrates, plants, protozoa, and fungi (Tenorio et al., 2019).

In 1953, Ramos-Alvarez and Sabin isolated the prototype virus for reovirus serotype 1 (reovirus serotype 1 strain Lang, T1L) from the stool of a baby named Lang. In 1955, Ramos-Alvarez and Sabin also isolated the prototypes for reovirus serotype 2 from the stool of a child named Jones who had a summer diarrheal illness (reovirus serotype 2 strain Jones, T2J) and for reovirus serotype 3 from the stool of a child named Dearing (reovirus serotype 3 strain Dearing, T3D). A second prototype for reovirus serotype 3 was isolated from an anal swab from a baby named Abney (reovirus serotype 3 strain Abney, T3A) by L Rosen in 1957 (Fields et al., 1996; Mahy and Regenmortel, 2010).

The reoviruses had particular unique characteristics including:

- I. Their size, which is 73–75 nm for virion and about 64 for infectious subviral particle (ISVP);
- II. The genome consists of 9, 10–12 discontinuous segments of dsRNA, and with the total Mw of the genome is 0.2 to 3.0×10^6 (Urbano and Urbano, 1994).
- III. The entirely cytoplasmic infectious cycle, therefore, exhibits the capacity to produce cytoplasmic inclusions.
- IV. Gene segment exchange (reassortment) occurs among reovirus strains during mixed infections of cultured cells or during coinfection in animal hosts.
- V. The reoviruses display the capacity to hemagglutinate several mammalian erythrocytes (Knipe and Howley, 2013; Winton et al., 1987).

Taxonomy

There are nine taxonomic families of dsRNA viruses. *Reoviridae* is the most important one, which infect vertebrates, including humans. The virus taxonomy (2019 Release) revealed *Riboviria*, *Orthornavirae*, *Duplornaviricota*, *Resentoviricetes*, *Reovirales*, *Reoviridae*, *Spinareovirinae*, *Orthoreovirus*, *Mammalian orthoreovirus*; there are currently 97 species in this family, divided among 15 genera in two subfamilies (Mäntynen et al., 2019; Igori et al., 2020).

The 15 genera of the *Reoviridae* are classified based on ICTV2019 release and are shown in Table 1.

Sedoreovirinae subfamily includes genera containing “smooth” viruses that do not have large surface projections or spikes on their virions or core particles including *Cardoreovirus*, *Mimoreovirus*, *Orbivirus*, *Phytoreovirus*, *Rotavirus*, and *Seadornavirus*.

Table 1 Reovirus Taxonomy: Classification based on ICTV2019 release by focusing on important human or other important prototype reoviruses.

Realm		<i>Riboviria</i>
Family		<i>Reoviridae</i>
Subfamily		
<i>Sedoreovirinae</i>:	1: <i>Sedoreovirinae</i>	2: <i>Spinareovirinae</i>
Genus1: <i>Cardoreovirus</i> : important Species: <i>Eriocheir sinensis reovirus</i>*		
Genus2: <i>Mimoreovirus</i> : important Species: <i>Micromonas pusilla reovirus</i>*		
Genus3: <i>Orbivirus</i> : important Species:		<i>African horse sickness virus</i> *
		<i>Bluetongue virus</i> *
Genus 4: <i>Phytoreovirus</i> : important Species: <i>Wound tumor virus</i>*		
Genus 5: <i>Rotavirus</i> : important Species:		<i>Rotavirus A</i> *
		<i>Rotavirus B</i>
		<i>Rotavirus C</i>
		<i>Rotavirus D</i>
		<i>Rotavirus F</i>
		<i>Rotavirus G</i>
		<i>Rotavirus H</i>
		<i>Rotavirus I</i>
		<i>Rotavirus J</i>
Genus 6: <i>Seadornavirus</i> : important Species: <i>Banna virus</i>*		
<i>Spinareovirinae</i>:		
Genus 7: <i>Aquareovirus</i> : important Species: <i>Aquareovirus A</i>*-G		
Genus 8: <i>Coltivirus</i> : important Species: <i>Colorado tick fever coltivirus</i>*		
Genus 9: <i>Cypovirus</i> : important Species: <i>Cypovirus 1</i>*-16		
Genus 10: <i>Dinovernavirus</i> : important Species: <i>Aedes pseudoscutellaris reovirus</i>*		
Genus 11: <i>Fijivirus</i> : important Species: <i>Fiji disease virus</i>*		
Genus 12: <i>Idnoreovirus</i> : important Species: <i>Idnoreovirus 1</i>*-5		
Genus 13: <i>Mycoreovirus</i> : important Species: <i>Mycoreovirus 1</i>*-3		
Genus14 <i>Orthoreovirus</i>: Contain 10 Species which Some of them fusogenic and some non-fusogenic <i>Orthoreoviruses</i>		<i>Avian orthoreovirus</i>
		<i>Baboon orthoreovirus</i>
		<i>Broome orthoreovirus</i>
		<i>Mahlapitsi orthoreovirus</i>
		<i>Mammalian orthoreovirus</i>*
		<i>Nelson Bay orthoreovirus</i>
		<i>Neoavian orthoreovirus</i>
		<i>Piscine orthoreovirus</i>
		<i>Reptilian orthoreovirus</i>
		<i>Testudine orthoreovirus</i>
Genus 15: <i>Oryzavirus</i> : important Species: <i>Rice ragged stunt virus</i>*		

• Important prototype reoviruses, which marked by *

The *Spinareovirinae* subfamily containing turreted viruses includes genera—that have relatively large spikes or turrets on the icosahedral vertices or core particle—including *Aquareovirus*, *Coltivirus*, *Cypovirus*, *Dinovernavirus*, *Fijivirus*, *Idnoreovirus*, *Mycoreovirus*, *Orthoreovirus*, and *Oryzavirus*. This chapter focuses on the genus *Orthoreovirus*, which contain important human reoviruses. Some member of the *reoviridae* family encodes an additional FAST (fusion-associated small transmembrane) protein responsible for syncytium formation, which shared sequence and structural similarities in avian *reovirus* (ARV) and Nelson Bay *reovirus* (NBV), but is unrelated to the FAST proteins of Baboon *reovirus* (BRV) and Reptilian *reovirus* (RRV). All of these FAST proteins are small, basic, acylated, transmembrane proteins and they induce fusion in transfected cells in the absence of other viral proteins (Boutillier and Duncan, 2011). The only known examples of nonenveloped viruses that induce cell–cell fusion and syncytium formation in the virus-infected cells are members of the family *Reoviridae*. In the case of fusogenic *orthoreoviruses*, syncytium formation promotes a rapid lytic response and release of progeny virions (Salsman et al., 2005). Some of the *orthoreoviruses* are fusogenic and induce syncytia. Members of the genus *Orthoreovirus* have 10 segments of dsRNA contained in two concentric protein capsids (van den Hengel et al., 2013). The genomes of viruses are grouped into three categories corresponding to their size: L [three larges (L1–L3)], M [three mediums (M1–M3)], and S [four smalls (S1–S4)] segments (Kohl et al., 2012). Proteins of viruses in the *Reoviridae* are denoted by the Greek character corresponding to the segment it was translated from (the L segment encodes for λ proteins, the M segment encodes for μ proteins, and the S segment encodes for σ proteins). Most of the genome segments are mono-cistronic, except for S1, which is bi-cistronic or tri-cistronic in some special species (Urbano and Urbano, 1994). Some segments have in-frame initiation codons or additional ORFs (Kohl et al., 2012) and only one strand of each duplex RNA is coding (Knipe and Howley, 2013).

Diverse morphotypes of reovirus particles

Several different morphotypes of reovirus particles are produced during in vitro culturing of reovirus within the cytoplasm of infected cells. These morphotypes of reovirus particles include genome-containing virions (infectious), intermediate subvirion particles (ISVPs), core particles, genome-lacking particles, and undefined particle types (by the capacity to transcribe the genome in vitro “transcriptase” and “replicase” particles) (Jané-Valbuena et al., 1999).

Virions are infectious stable particles in the environment (Antczak and Joklik, 1992) and they sustain infectivity for years when stored below 4 °C (Knipe and Howley, 2013). Infectious subvirion particles (ISVPs) can be modified by proteases. Treatment of virions with chymotrypsin or trypsin in controlled conditions in vitro or during virion disassembly or in the infected intestine condition generates infectious or intermediate subviral particles (ISVPs). These particles lack sigma3 but retain sigma1 and μ 1. The virion-to-ISVP transition can significantly increase the infectivity of these viruses (Snyder et al., 2019). Core particles can be generated in vitro by treating virions with chymotrypsin or trypsin and remove all of the outer-capsid proteins (μ 1, sigma3, and sigma 1) and expose projecting turrets formed by pentamers of lambda2. Purification of reovirus particles from infected cells yields a considerable proportion of particles that lack genomic dsRNA, with lower buoyant density in CsCl gradients (Knipe and Howley, 2013) empty capsids.

Virion and genome characteristics

Members of the *Reoviridae* form nonenveloped virions composed of 1 to 3 concentric capsid layers, which surround 9, 10, 11, or 12 segments of linear dsRNA segments of the viral genome, depending on the genus. The RNA constitutes about 15–20% of the virion dry weight (Benavente and Martínez-Costas, 2007). The positive strands of each duplex have a 5'-terminal type 1 cap structure and negative strands may have phosphorylated 5' termini. Both RNA strands have a 3'-OH group and viral mRNAs lack 3'-polyA tails (King et al., 2011). The viral dsRNA species are present within virus particles in equimolar, representing one copy of each genome segment per virion. Reovirus RNA is usually regarded as noninfectious. However, recent developments involving the introduction of viral mRNAs into susceptible cells have succeeded in recovering fully viable virus particles, thus providing further research opportunities utilizing reverse genetic technologies (Teimoori et al., 2014; King et al., 2011). Mature virions lack a lipid envelope, but in coltiviruses, rotaviruses, and orbiviruses genus, the particles are associated with membrane fractions of the endoplasmic reticulum (ER) during morphogenesis, acquiring an envelope temporarily in infected cells. This is a transitional event in morphogenesis or release of the virus that is subsequently lost or removed. The dsRNA gene segments and enzymes that catalyze RNA transcription, capping, and replication in reoviruses are contained within the inner capsid. RNA-dependent RNA polymerase (as a transcriptase and replicase of the virus), NTPase, helicase, capping, and transmethylese enzymes are, entirely, active in core particles. A unique feature of reovirus replication cycles is the synthesis of viral mRNAs by virally encoded enzymes within the icosahedral particles (Mahy and Regenmortel, 2010). The virus-produced messenger RNA (mRNA) transcripts during multiplication are capped but not polyadenylated. The viral RNA segments are usually mono-cistronic, although some segments have two or three in-frame initiation codons (Knipe and Howley, 2013). Viral factories, and cytoplasmic inclusion bodies, or viroplasms are produced during replication of the virus (Tao et al., 2002).

Reovirus pathogenesis in human

Reoviruses can affect the gastrointestinal system and respiratory tract. Some of these viruses are not associated with any known disease, but some other viruses in the family cause various known diseases. Reovirus infection occurs often in humans, but most cases are mild or subclinical (Mahy and Regenmortel, 2010; Knipe and Howley, 2013). Rotavirus belonging to *Sedoreovirinae* can cause severe diarrhea. They are a major cause of infant disease and death in developing countries. The virus can be readily detected in feces, and may also be recovered from pharyngeal or nasal secretions, urine, cerebrospinal fluid, and blood. Despite the ease of finding reovirus in clinical specimens, their role in human disease or treatment is still ambiguous (Kapikian and Shope, 1996). A variety of symptoms may be associated with *orthoreovirus* infection in human including respiratory illnesses, diarrhea, and intestinal distress in children. In monkeys, *orthoreoviruses* cause hepatitis and extrahepatic biliary atresia. There are several mechanisms of pathogenesis, including the destruction of enterocytes, leading to malabsorption and triggering osmotic diarrhea. A watery characteristic of rotavirus-induced diarrhea is often seen in children prior to the appearance of histologic changes, which is thought to be induced by the action of the rotavirus enterotoxin NSP4 (Nguyen et al., 2004; Razavinikoo et al., 2015). It has been anticipated that the destruction of enterocytes causes a loss of the permeability barrier, resulting in the osmotic pull of fluid from the circulation into the gut. Rotaviruses infect a wide range of mammalian species but restricted in the adults. *Rotavirus A* is responsible for the majority of seasonal endemic diarrheal disease in young children (Nguyen et al., 2004); *Rotavirus B* causes sporadic epidemic outbreaks of gastroenteritis in adults; and *Rotavirus C* causes self-limiting outbreaks of gastroenteritis in young people (Fields et al., 1996).

Transmission and dissemination

Reoviridae family, including rotaviruses, are the most prevalent cause of diarrhea in infants and children (Parashar et al., 1998). Reovirus infections in humans are usually mild or subclinical. Reovirus entry is through respiratory or fecal-oral route with virus replication and pathology in the intestinal epithelium, bile duct epithelium, lung epithelium, leukocytes, and CNS endothelial cells. Reoviruses spread to their host using bloodborne and neural pathways. Rotavirus entry is through the fecal-oral route, with virus replication and pathology in the proximal small intestine, resulting in diarrhea or vomiting (Greenberg and Estes, 2009; Nguyen et al., 2004).

Entry and replication in the host cell

Viruses need host cells for replication. For most viruses, replication, the production of viral proteins and virus assembly, occurs in the cytoplasmic inclusions. In addition, these neoorganelles are also involved in the sequestration of innate immune response proteins (Miller and Krijnse-Locker, 2008). In rotavirus infection, these structures called viroplasms, which are crucial for virus replication. Coexpression of rotavirus NSP5, with NSP2 or VP2, is essential for the formation of this structure. In contrast to rotavirus, reovirus uses ER fragmentation to construct its viral inclusions. Rotavirus uses ER membranes during particle maturation and assembly (Carreño-Torres et al., 2010). *Reoviridae* family use ER membranes during different steps of infection (Miller and Krijnse-Locker, 2008; Knipe and Howley, 2013).

Life cycle

As told before, reovirus genomes consisted of segmented, double-stranded RNA (dsRNA); therefore, replication occurs exclusively in the cytoplasm and the virus encodes several proteins which are needed for replication in the viroplasms (Carreño-Torres et al., 2010). The sigma 1 protein mediates viral binding to cellular receptors containing sialic acid residue and junctional adhesion molecule-A (JAM-A: a member of the immunoglobulin superfamily) and has the capacity to agglutinate erythrocytes of several mammalian species. Following attachment to cell-surface receptors, reovirus is internalized by clathrin- and caveolin-dependent mediated endocytosis (Sturzenbecker et al., 1987; Guglielmi et al., 2006).

The virus is partially uncoated by proteases in the end lysosome, where the capsid is partially digested to allow further cell entry. Cleavage of sigma3 triggers stepwise reovirus disassembly cascade to form distinct ISVP. The host proteins are required for penetration steps. The sigma protein is found distributed in the cytosol, and viral cores are produced by proteolytic removal of outer capsid proteins, and localized in the cytoplasm of infected cells. Following the entering to the cytoplasm, the genome is transcribed and produce (Knipe and Howley, 2013) RNA positive-sense strands. The ^{m7}G-capped viral mRNAs are synthesized early postinfection. In some cell types, such as mouse L929 cells, alongside with the infection progresses, uncapped viral mRNAs are produced. The same positive-sense RNA is used as mRNA templates to synthesize RNA negative-sense strands. In the late phases of the transcription, process is occurred by not capped RNAs. The different amounts of RNAs and translation step are a controlled step. Assembly of viral particles begins in the cytoplasm 6–7 h postinfection. The diverse translational strategy takes place by leaky

scanning, suppression of termination, and ribosomal skipping (Carreño-Torres et al., 2010). Translation of reovirus mRNAs and viral protein synthesis led to the formation of the viral factory. The virus exists in the infected host cell by cell-to-cell movement in occlusion bodies after cell death, and remains infectious until finding another host.

The μ and σ nonstructural proteins of reovirus and $\mu 2$ structural protein are involved in forming and organizing of the inclusion bodies (Becker et al., 2003; Broering et al., 2002). The $\mu 2$ and μ protein interactions are essential for the formation of inclusions and the use of other viral factors for assembly and replication (Parker et al., 2002; Miller et al., 2010). The $\mu 1$, $\mu 2$, $\lambda 3$, σ , and the viral core proteins are involved in virus RNA replication (Broering et al., 2004; Miller et al., 2003). The small part of the virus core ($\mu 2$ protein) is involved in shaping the morphology of inclusions.

The cycle of rotavirus replication occurs entirely in the host cytoplasm. The virus enters the host cell after attachment via the receptor-mediated endocytosis by VP4 and VP7 proteins. The sialic acid-containing receptors are involved in the rotavirus attachments as well. The transcriptionally active viral core particles are released into the cytoplasm and the transcription process begins.

After penetration, positive-sense ssRNAs are synthesized, which are used as a template for synthesizing negative ssRNAs to produce genomic dsRNA and viral proteins (Ebert et al., 2002; Antczak and Joklik, 1992). Inside core particle within inclusions, viral RNAs are synthesized by the reovirus core proteins. The RNA-dependent RNA polymerase (RdRp) is involved in catalytic activity. The RdRp transcribes using negative-strand RNAs as templates for positive-strand RNA synthesis. The viral 2 and VP3 protein are involved in methylation and capping of positive-strand RNAs during releases from the core respectively by reo and rotaviruses (Furuichi et al., 1975). The parental positive-sense RNA strand reanneals with the negative-sense RNA strand to rehabilitate the dsRNA genome.

The $\sigma 1$ protein and its counterpart VP4 in rotavirus as cell attachment proteins are involved in recruiting translation machinery into viral inclusions (Desmet et al., 2014; Knipe and Howley, 2013). The outer capsid proteins increase viral translation by attaching to dsRNA and prevent the binding of protein kinase RNA-activated (PKR) to dsRNA. In uninfected cells, PKR is an IFN-induced enzyme that inhibits the onset of host translation by inhibiting factor 2 subunit α and thus suppressing host protein synthesis (Lemay, 2018).

The virus uses host cell proteins for virus assembly. For example, several chaperone networks including heat shock protein 70 (Hsp70), heat shock protein 90 (Hsp90), and the cytosolic chaperonin T-complex protein 1-ring complex (TRiC) are involved in the aggregation of virus nascent particles within inclusions for assembly.

After assembly and maturation, the mature virions leave virus inclusions (VIs). The viral proteins are associated with the host machinery to boost replication steps inside VIs and prevent an innate immune response. The viral proteins inhibit the activity of the immune system by breaking down interferon regulatory factor 3, which is involved in inducing antiviral defense, within the inclusion (Stanifer et al., 2017).

Replication

Briefly, the entry of reoviruses into the cells commonly results in loss of outer capsid components. Transcriptionally active particles are released into the cell cytoplasm (Fig. 1). The transcription of full-length viroplasmic mRNA species from each dsRNA segment happens within core particles during infection in inclusion bodies, which is localized in the infected cell cytoplasm (Carreño-Torres et al., 2010). This structure appears to be the site of viral mRNA synthesis, genome replication, and particle assembly. The produced mRNAs are used as templates for a minus-strand synthesis and reforming the dsRNA genome segments of a progeny virus particle. The dsRNA genome segments are packaged in exactly equimolar ratios (one copy of each genome segment per particle). The packaging is highly specific, concerning the recognition signals on each mRNA species for recruitment of other segments.

Reassortment in segmented dsRNA viruses

Reassortment is a process of genetic recombination that is restricted to segmented RNA viruses and plays a vital role in the evolution of the viruses with pandemic potential in some viruses. Genome segment reassortment occurred during natural infection or through coculturing of different viruses in the same species, involving the selection and packaging of mRNAs from different parental strains with the same packaging signals (Zafari et al., 2018). The conserved terminal sequences at both ends of RNA segments may be involved as recognition signals for viral transcription, translation, replication, or packaging. The data providing direct evidence of segment reassortment between isolates are only available for viruses in a few genera (Mahy and Regenmortel, 2010; Knipe and Howley, 2013). Closer relations between certain genera have been identified and it has been realized that the nonturreted viruses may represent an ancestral lineage from which the turreted viruses have evolved. For example, comparisons of the polymerase, capping enzyme, and capsid protein sequences, as well as structural analyses of outer capsid proteins, suggest that an evolutionary jump has occurred between the *Seadornaviruses* and *Rotaviruses*, within the *Sedoreovirinae*. This is supposed that gene duplication and rearrangement may occur and cause the changing of the number of genome segments during infection (King et al., 2011).

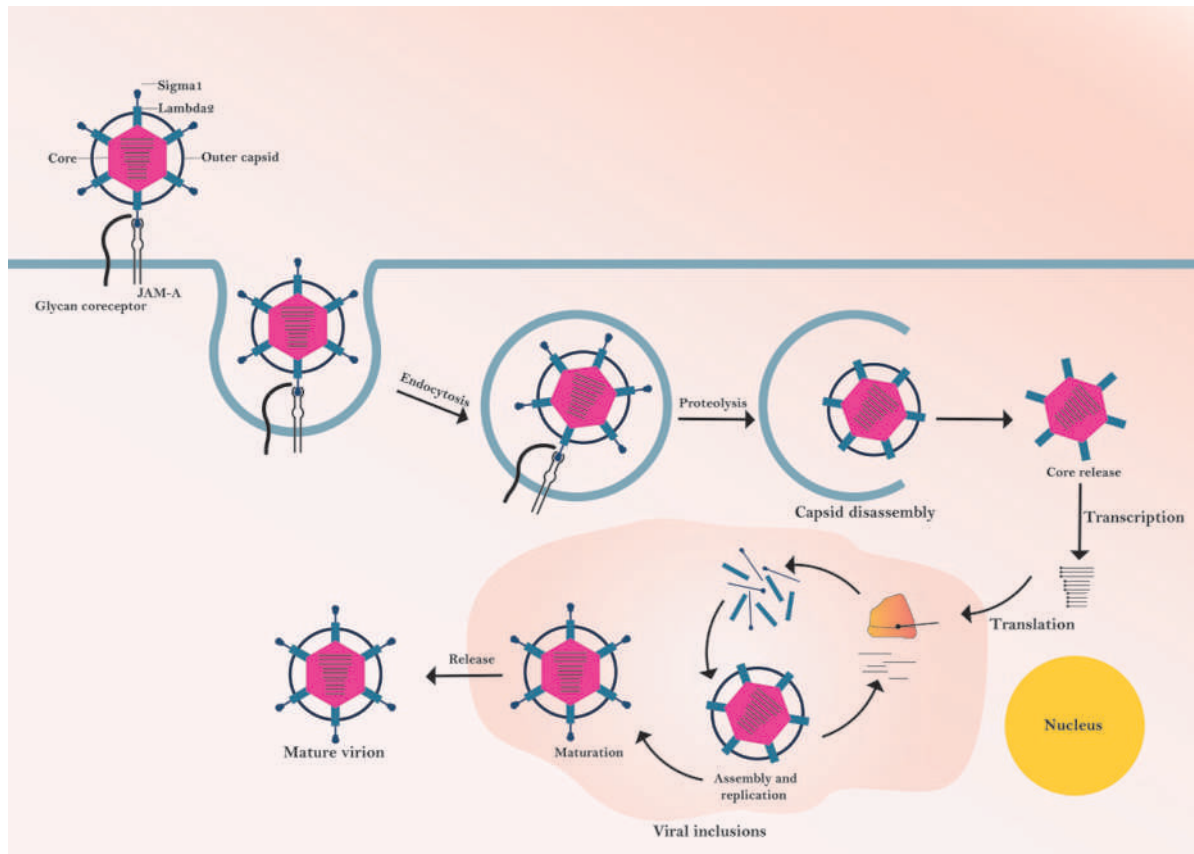


Fig. 1 Reovirus entry and replication. Reovirus attaches to host glycan coreceptor and JAM-A receptor and enters through receptor-mediated endocytosis. After proteolysis and degradation in early and late endosome, viral core particles are released into the cytoplasm. Positive-sense RNA of reovirus is transcribed and virus proteins are expressed step by step. Virus proteins and genomic materials assemble in viral inclusions and mature reovirus release into the cytoplasm.

Genome segments and protein products

The genome segments and protein products of reovirus and rotavirus are noticeable and visible, almost at a glance in [Table 2](#).

Antigenic properties

The serotype-specific antigen of the *orthoreoviruses* is protein $\sigma 1$, which is recognized by neutralizing antibodies. The MRV $\sigma 1$ and $\sigma 1s$ proteins elicit strain-specific and cross-reactive cytotoxic T-cell activities. The MRV proteins $\lambda 2$ and $\sigma 3$ are species-specific antigens.

Biological properties

Ordinarily, human *orthoreoviruses* do not produce symptoms but may cause upper respiratory tract illness and possibly enteritis in infants and children. Reovirus enters the body as an intact virion. The action of proteases in the intestinal lumen cleaves proteins on the surface of the virion, generating a highly infectious form of the virus the intermediate subviral particle, which interacts with the M cells and is transported into the Peyer's patch where the infection is established. Then the virus may enter the bloodstream or enter nerves for spread to organs such as the brain and spinal cord ([Knipe and Howley, 2013](#)).

Rotaviruses can cultivate in vitro, in permissive epithelial cell lines derived from monkey kidneys. The pretreatment of rotavirus with trypsin enhanced the infection properties. The RF strains of Rotavirus propagated easily in confluent monolayers of fetal rhesus monkey kidney cells (MA-104) and consistently gave high yields of virus titer ([Ahmadi et al., 2016](#)). Viruses within species have the ability to reassort their genome segments, whereas, in different species do not happen under normal conditions.

Distinguishing features of genus *Rotavirus*

Rotaviruses infect only vertebrates and are transmitted by a fecal–oral route. The virus particles have a wheel-like appearance by negative contrast electron microscopy. The triple-layered capsid encloses a genome of 11 linear dsRNA segments and is formed in a

Table 2 Genome segments and protein products of mammalian orthoreovirus and its homologous segment in rotavirus.

Gene segment (Orthoreovirus)	Proteins (Reovirus)	Important function
L1	$\lambda 3$	RNA polymerase (Pol), Interferon antagonist
L2	$\lambda 2$	Guanylyl & Methyl transferase (Cap)
L3	$\lambda 1$	Major inner capsid protein, binds dsRNA and zinc, RNA helicase (Hel)
M1	$\mu 2$	Minor capsid protein, required for inclusion body development, probable RNA polymerase subunit, interferon antagonist, NTPase
M2	$\mu 1$ /Cleaved to form $\mu 1N$ and $\mu 1C$	Important for penetration, influences apoptosis
M3	$\mu NS/\mu NSC$	Binds ssRNAs and cytoskeleton, nucleates viral inclusion bodies, phosphoprotein, μNSC (unknown function) from alternate translation start site
S1	$\sigma 1$ (serotype-specific antigen)	Viral attachment protein, The serotype-specific antigen, cell tropism
S1	$\sigma 1s$	Cell cycle arrest, virus dissemination, elicit strain-specific and cross-reactive cytotoxic T-cell activities
S2	$\sigma 2$	Inner capsid structural protein, weak dsRNA-binding
S3	σNS	ssRNAs-binding, inclusion body development, genome packaging?
S4	$\sigma 3$	Major outer capsid protein, inhibits PKR activation and nuclear and cytoplasmic localization
Genome segment (Rotavirus)	Proteins of Rotavirus	Important function
Segment1	VP1	RNA-dependent RNA polymerase
Segment 2	VP2	Required for encapsidation of VP1 and VP3 and has ssRNA- and dsRNA-binding activity
Segment 3	VP3	Methyl and Guanyl transferase
Segment 4	VP4(88 K) VP8* (28 kD) VP5* (60 kD)	Protease sensitive, neutralizing antigen, viral attachment proteins
Segment 5	NSP1	Interferon antagonist
Segment 6	VP6	Subgroup antigen
Segment 7	NSP3	Binds 3' end of viral mRNAs, cellular eIF4G, Hsp90, inhibits host translation
Segment 8	NSP2	RNA binding, NTPase, forms Viroplasms with VP1 and NSP5
Segment 9	VP7	Glycosylated neutralization antigen, glycoprotein calcium-dependent trimer,
Segment 10	NSP4	Enterotoxin, RER transmembrane glycoprotein, Viroporin, Role in morphogenesis of TLPs
Segment11	NSP5(Product of first ORF)	phosphoprotein, RNA binding, protein kinase, forms viroplasms with NSP2, interacts with VP2 and NSP6
Segment11	NSP6 (Product of 2 _{nd} ORF)	Interacts with NSP5, present in viroplasms and most virus strains, RNA binding

unique morphogenic pathway, which involves the attainment of a transient lipid envelope during budding of immature double-layered particles (DLPs) through the endoplasmic reticulum (ER) and results in the transient acquisition of a lipid envelope and addition of VP4 and VP7 to form the outer virion shell.

The simian rotavirus A/SA11 (SiRV-A/SA11) represents as a prototype of the simian rotaviruses within the genus. The innermost layer of the virion is composed of VP2. The VP2 proteins surround the genomic dsRNAs and two structural proteins, the RdRp and VP1, and the capping enzyme VP3. The assembled genomic dsRNAs, along with the enzymatic complexes and VP2 layer, comprise the rotavirus core. The two outer layers of the rotavirus virion have icosahedral symmetry. The intermediate capsid layer is composed of 260 trimeric VP6. The VP6 layer forms the outer surface of the DLPs. Two proteins (VP4 and VP7) form the outer layer of the rotavirus virion and are required for infectivity. The 260 trimers of VP7 glycoprotein make up the surface of the outermost shell, which is stabilized by Ca^{2+} bound in the inter-subunit interfaces. Although, 60 trimeric VP4 spike proteins project from the same layer as VP7 layer as shown in Fig. 2. The amino-terminal (VP8*) and carboxyl-terminal (VP5*) fragments of VP4 were generated by cleaving by trypsin, and primes virions for infectivity. Virus infectivity is dependent upon the presence of the VP4-VP7 outermost protein layer, the integrity of which requires Ca^{2+} (Blutt et al., 2004). Treatment of virions with Ca^{2+} -chelating agents, such as EGTA or EDTA, destabilizes VP7 trimers, leading to loss of the outer capsid. The infectious virions are resistant to pH ranging from 3 to 9; they are reserved for months at 4 °C in the presence of 1.5 mM $CaCl_2$. Infectivity is also relatively thermostable at 50 °C but can be lost by repeated cycles of freezing and thawing. However, infectivity is lost by treatment with sodium dodecyl sulfate (0.1%), and 95% ethanol, which disrupts the outer protein layer of the virion, representing an effective disinfectant. Double-layered particles (DLPs) are noninfectious, and single-layered rotavirus particles (cores) can be produced by treatment of DLPs with chaotropic agents such as sodium thiocyanate or high concentrations of $CaCl_2$.

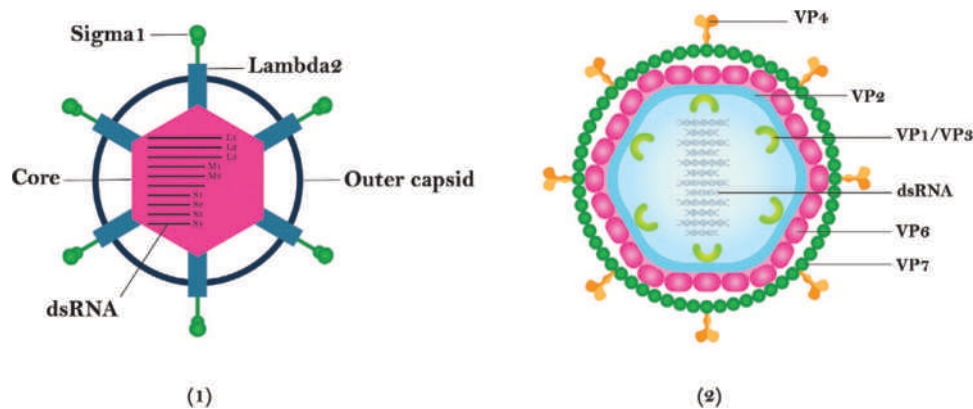


Fig. 2 Reovirus (1) and Rotavirus (2) structures and protein contents.

Rotavirus genomic RNA sequences and its related proteins

The rotavirus genome consists of 11 discrete segments of linear dsRNA. When resolved by gel electrophoresis, the segments of *Rotavirus A* strains typically display a 4:2:3:2 distribution pattern. The genome segments are numbered as segment 1 (Seg1) to segment 11 (Seg11) based on increasing electrophoretic mobility. In some *Rotavirus A* isolates, partial duplication of a genome segment was realized during electrophoresis. Rotavirus genomic nucleic acid sequences are A + U rich (58–67%). The segments are completely base-paired, and the plus-strand contains a 5'-terminal cap structure but lacks a polyadenylation signal near its 3' end. In contrast to members of the genus *orthoreovirus*, there is no evidence for the presence of single-stranded oligonucleotides within the virion. Two levels of terminal sequence conservation are evident in *Rotavirus A* isolates. They may be indicative of *cis*-acting signals that are important for controlling transcription, replication, and segment selection for packaging. Detailed genomic information for *Rotavirus B*, *Rotavirus C*, *Rotavirus D*, and novel adult diarrhea rotaviruses (NADRVs) is sparse. However, complete sequence information for all genome segments of rotavirus isolates is available for each species.

The majority of genome segments encode a single protein, but two segments (Seg9 and Seg11) each encode two primary translation products. In the case of Seg9, two initiation codons in the same reading frame may be used, for producing overlapping forms of the VP7 protein. Although, gene Seg 11 contains two out-of-frame ORFs, which results in the translation of two nonstructural proteins containing NSP5 and NSP6. The rotavirus proteins are numbered according to their migration rates upon SDS-PAGE. The six structural proteins have been identified and are given the prefix "VP," whereas nonstructural proteins are given the prefix "NSP." The viral core is composed of three proteins (VP1, VP2, and VP3), that encapsidates the dsRNA genome. The VP1, the largest viral protein (RdRp), is responsible for both transcription and replication. The VP1 is active only in the presence of VP2. The VP3 core protein (the capping enzyme) exhibits guanylyl transferase and methyltransferase activities bind ssRNA. The amount of VP1 and VP3 in the virion is about 12 molecules/particle, but VP2 is the most abundant protein of the viral core, with 120 molecules per virion. The VP2 is required for encapsidation of VP1 and VP3 and has nonspecific ssRNA- and dsRNA-binding activity. By analogy with other reoviruses, it is likely that VP2 determines both the size of the particle and the structural organization of the outer capsid components, the internal enzymatic components, and the RNAs. This important functional load is reflected in the highly conserved nature of VP2.

The intermediate protein layer of the virion is made up of 260 trimers of VP6. The two remaining structural proteins of the virion, VP4 and VP7, of which there are 60 trimers and 260 trimers per virion, respectively, make up the outermost protein layer (Fig. 2). The spike protein VP4 contains a trypsin cleavage site nearly one-third of the way along its length. Cleavage of the protein *in vitro* by trypsin produces two products, VP5* (60 kDa) and VP8* (28 kDa), enhances virus infectivity, and induces conformational changes. The smaller product (VP8*) has hemagglutinin activity and contains a carbohydrate-binding site. The VP7 outer shell glycoprotein is synthesized on the rough ER (RER) and cotranslationally inserted in the ER membrane.

Six nonstructural proteins are encoded by the viral genome. The NSP1 encoded by Seg5 is the most variable protein of rotavirus with 65% sequence diversity observed between strains of *Rotavirus A*. The total length of this genome segment, as well as the size of the ORF, can vary among strains. Although, NSP1 has a conserved cysteine-rich motif near the amino terminus, which is considered as a zinc-binding RING-domain protein. NSP1 has been shown to bind both the 5' end of viral RNA and zinc and has a role in viral pathogenesis as a virulence determining factor. The NSP1 antagonizes the host innate immune response by inducing the degradation of interferon regulatory factors (IRF3, IRF5, and IRF7) required for the expression of type I interferon. It may be done as an E3 ubiquitin ligase. NSP2 self-assembles protein that produces stable octamer, the functional form of the protein. It binds nonspecifically to ssRNA, has NTPase and RTPase activities, and interacts with NSP5 to form viroplasm (Carreño-Torres et al., 2010). The NSP2 has a direct role in virus replication and functions as a molecular motor that plays an important role in viral RNA packaging.

The NSP3 is a multifunctional protein that forms dimers and has several binding partners such as 3' conserved sequence of viral mRNAs and the eukaryotic translation initiation factor eIF4G. Interaction of NSP3 by eIF4G is expected to inhibit host cellular protein synthesis, in that way promoting viral translation (Flint et al., 2015).

A transmembrane protein (NSP4) is synthesized on the RER as a glycosylated protein, had a role in virion maturation, and functions as a receptor for DLPs supports VP4 and VP7 in budding process through the ER membrane. NSP4 also functions as a viral enterotoxin that leads to Ca²⁺ release from internal stores in the ER. The NSP4 cleavage product is secreted from infected cells and binds to integrin receptors and triggering a signaling pathway that activates phospholipase C, leading to Ca²⁺ release. This pathway is distinct from that of intracellularly expressed NSP4, which caused activation of calcium/calmodulin-dependent kinase II (Razavinikoo et al., 2015).

The two lasting nonstructural proteins, NSP5 and NSP6, are encoded by two ORFs in the same viral gene segment. NSP5 has ssRNA- and dsRNA-binding activities and is essential for viroplasm formation and genome replication. Interactions of NSP2 and NSP5 are responsible for viroplasm formation and localization of core proteins. Interactions of NSP2 and NSP5 with core proteins regulate progeny core formation (Knipe and Howley, 2013).

The majority of rotaviruses are predicted to encode a single protein product per segment. It is clear that other rotavirus species have homologs of the proteins characterized by *Rotavirus A* virus. Further insights into the structure and function of viral proteins from species other than *Rotavirus A* look forward to the establishment of suitable permissive cell lines and wait for the production of the non-*Rotavirus A* recombinant proteins.

Genome organization and replication

Generally, the replication cycle of several animal rotaviruses is completed in 12–15 h at 37 °C, in monkey kidneys' continuous cell cultures. The VP4 is the viral attachment protein, and the N-acetyl-sialic acid residues on the cell surface in some animal viruses serve as a receptor behind several integrins and heat shock protein 70 as coreceptors. Recently, the published data mentioned that rotaviruses may enter cells either by receptor-mediated endocytosis or by direct membrane penetration. In both cases, virus entry primes the loss of the outer VP4 and VP7 protein layer and DLP containing transcriptionally active dsRNA was released into the cytoplasm. Within DLP 5'-capped, nonpolyadenylated mRNAs were generated using the full-length minus strand of each genome segment as a template. The regulation of gene expression was done by differences in the level of transcription, which took place from each genome segment. The viral mRNAs support two steps; primarily, the mRNAs act as templates for translation and producing viral proteins, and furthermore, viral mRNAs act as templates for minus-strand synthesis in order to produce dsRNA genome segments during packaging of progeny. Nowadays, evidence showed that the pools of mRNAs that serve as templates for translation (cytosolic +RNA) and replication (viroplasmic +RNA) are distinct (McDonald and Patton, 2011). Virus assembly begins in cytoplasmic inclusions termed viroplasms, which is mediated by NSP2 and NSP5 (Carreño-Torres et al., 2010). During assembly of progeny, the 11 different viral mRNAs interact with one another and with VP1 and VP3, that is followed by association with VP2, for triggering -RNA synthesis and consequently cause the establishment of cores comprising a complete set of 11 dsRNA segments. The most critical Cis-acting replication elements have been identified for minus-strand synthesis. In the intermediate phase of assembly, VP6 is added to core particles, forming DLPs. The next steps in the morphogenesis of progeny virions are unique to rotaviruses and involve recruitment of DLPs to the ER by the NSP4 (Bugarčić and Taylor, 2006). The VP6 protein of *Rotavirus A*, which forms inner capsid proteins, is highly conserved, immunogenic, and contains virus group and subgroup-specific determinants. Although this protein does not induce neutralizing antibodies, it may play a role in the initiation of protective immunity.

The VP4 and VP7 outer capsid proteins elicit neutralizing antibodies. Rotavirus strains were classified based on VP4 and VP7 proteins into P serotypes (VP4 is protease-sensitive) and G serotypes (VP7 is a glycoprotein). Classification of rotaviruses into P (VP4) or G (VP7) serotypes is performed by cross-neutralization assays. So far, 23 G (VP7) genotypes and 32 P (VP4) genotypes have been identified (Matthijnssens et al., 2009). The P genotype is denoted by a number within square brackets, which immediately follows the P serotype.

Therapeutic applications: Oncolytic activity

Tumor relapses after naturally developed viral infections observed in the early last times ago (Donnelly et al., 2013). Subsequent to these former random annotations, the several studies applied viruses as a targeted anticancer therapeutic strategies (Cao et al., 2020). The oncolytic virus (OV) was introduced for this kind of tumor therapy. OV has tumor-selective replication capabilities and can kill tumor cells directly by natural infection and lysis of cancer cells, as well as indirectly by the initiation of systemic antitumor immune responses (Zainutdinov et al., 2019). Reoviruses are isolated from the respiratory and gastrointestinal tract without any known and severe disease (Antczak and Joklik, 1992; Kim et al., 2007). These viruses have served as very productive experimental models for studies of viral pathogenesis (Morin et al., 1996). Most people have been exposed to reovirus by adulthood; however, the infection does not typically produce symptoms. The reoviruses have been demonstrated to have oncolytic (cancer-killing) properties in various cancer cell lines and lyse these cells, encouraging the development of reovirus-based novel therapies for cancer treatment (Soleimanjahi and Habibian, 2020; Lal et al., 2009).

Human reovirus has three prototype strain viruses (Dearing, Lang, Jones) with antitumor properties, but most clinical trials recently focused on the ReoT3D strain (Reolysin). Recently, many researchers evaluated the potential oncolytic activity of Reolysin (reovirus serotype 3-Dearing strain) that is currently used in preclinical and clinical trials for the treatment of various cancers

(Thirukkumaran and Morris, 2009; Min et al., 2009). Also, the second prototype for reovirus serotype 3 was isolated by L Rosen in 1957, from an anal swab reovirus serotype 3 strain Abney, T3A (Fields et al., 1996; Mahy and Regenmortel, 2010), as indicated in Table 3.

Several studies showed significant antitumor effect of oncolytic reovirus against tumor cells. Therapeutic usage of viruses as a cancer treatment opportunity has been increasingly explored over the past years (Thirukkumaran and Morris, 2009; Babaei et al., 2020). The oncolytic viruses (OVs) selectively replicate in the tumor cells and newborn mice are exquisitely sensitive to reovirus infection and have been used as the experimental systems for studies of reovirus pathogenesis. ReoT3D is wild-type reovirus with innate ability to inhibit KRAS signaling pathway in transformed cells (Babaei et al., 2020; Marcato et al., 2005). KRAS mutant cells endorse viral replication by inhibition of PKR and result in the selective replication of reovirus in the KRAS-transformed cells (Fig. 3) (Shmulevitz et al., 2010). Reolysin significantly induces survival rate, apoptosis, and cell cycle arrests and also reduces proliferation, tumor growth, tumor size, and volume in tumor models. The in vitro and in vivo results suggest that reovirus therapy is effective for treatment of tumor cells and it can be considered as a new antitumor option against different tumor cells with KRAS mutations (Babaei et al., 2020).

Oncolytic viruses are thought not only to cause direct destruction of the tumor cells but also to stimulate host antitumor immune system responses.

Immune responses against RV infection

The innate immune response is the first line of host defense against pathogens and is involved in the early detection of viruses in cells; it is followed by activation of adaptive immunity. Innate immune responses depend on the activation of signaling pathways and produce antiviral molecules such as antiviral cytokines like IFNs. Elevated type I interferon increases the expression of

Table 3 The list of species in the genus of mammalian nonfusogenic *Orthoreovirus* with the oncolytic capability.

Mammalian Orthoreovirus	Abbreviation	Reovirus type
Mammalian <i>Orthoreovirus</i> 1	(MRV-1La)	(Reovirus type 1) Lang
Mammalian <i>Orthoreovirus</i> 2	(MRV-2Jo)	(Reovirus type 2) Jones
Mammalian <i>Orthoreovirus</i> 3	(MRV-3De) (T3D)	(Reovirus type 3) Dearing
Mammalian <i>Orthoreovirus</i> 4	(MRV-4Nd)	(Reovirus type 4) Ndelle

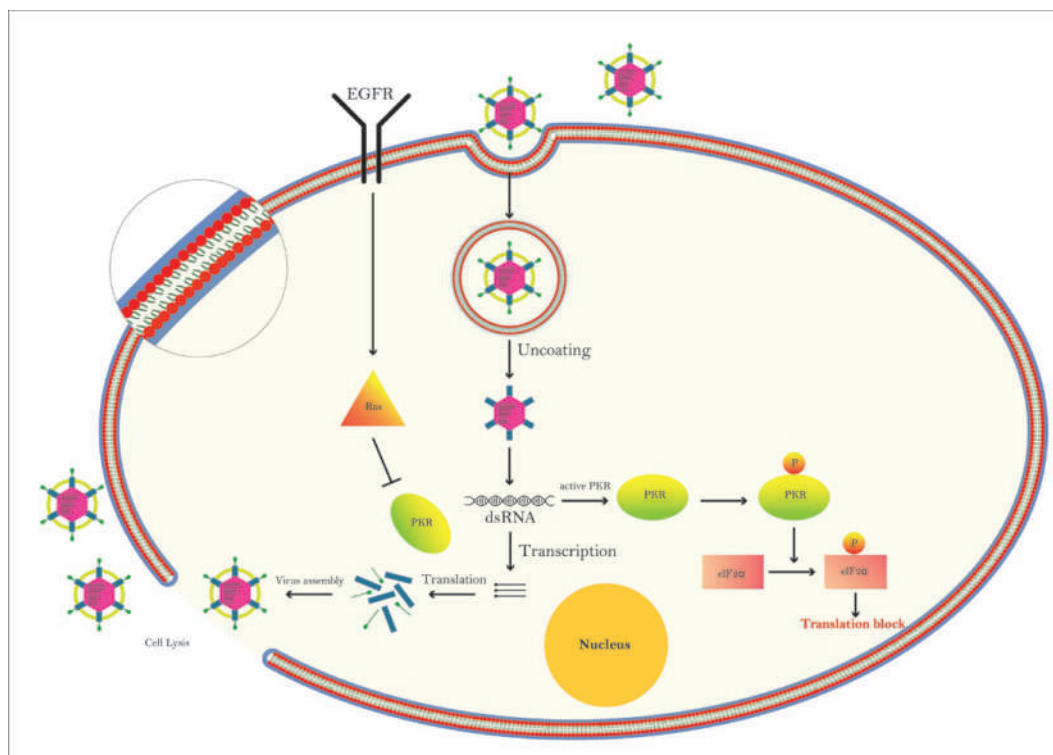


Fig. 3 Reovirus oncolysis mechanism. In normal cells after uncoating, PKR recognizes virus dsRNA; then PKR is phosphorylated and activated. Activated PKR phosphorylates eIF2 α , which leads to protein translation block. In Ras-transformed cells, EGFR signaling pathway activates Ras. Ras inhibits PKR, which leads to increase in viral protein's translation, virus replication, and killing of the cells.

interferon-stimulated genes that produce antiviral proteins like 2'-5' oligoadenylate synthetase (OAS) and pattern recognition receptor (PRR) proteins (Li et al., 2015; Silverman, 2007).

The innate immune system immediately detects pathogen-associated molecular patterns (PAMPs), such as genomic DNA, ssRNA, dsRNA, RNA with 5'-triphosphate ends, and viral proteins via PRRs (Parashar et al., 1998; Torres Pedraza et al., 2010; Jensen and Thomsen, 2012). Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), Toll-like receptors (TLRs), and NOD-like receptors (NLRs) are three different types of PRRs (Parashar et al., 1998; Medzhitov, 2007) that detect viral particles. Other important roles of TLR and RLRs are the production of type I interferons (IFNs) and proinflammatory cytokines and NLRs involved in the production of mature interleukin-1 β by activating caspase-1 (Pétrilli et al., 2007; Kanneganti et al., 2007).

RIG-I and MDA5 discriminate short and long dsRNAs, respectively, and are major sensors of reovirus infection. DExD/H-box helicase 9 (DHX9), DEAD-Box helicase 1 (DDX1), DDX21, and DHX36 are involved in innate immune response in some cell types like myeloid dendritic cells following (Zhang et al., 2011) reovirus infection. Studies indicated that DHX9 knockdown decreases type I IFN secretion after reovirus infection and it is necessary for IRF-3 and NF κ B activation (Zhang et al., 2011).

Another dsRNA sensor is Toll-Like Receptor 3 (TLR3) (Pandey et al., 2015). TLR3 interaction with its ligand leads to signaling through TRIF and boosts IRF3 and NF- κ B activation. TLR3 does not appear to play an important role in the pathogenesis and clearance of reovirus in the central nervous system, and the absence of TLR3 does not increase viral titers (Pandey et al., 2015; Edelmann et al., 2004).

Interferons are proteins in the cytokine family that have antiviral activity (Knipe and Howley, 2013). The interferon is secreted in the cells due to external stimuli, such as the virus infections, and cells that respond to interferon by establishing an antiviral state (Ja et al., 1987). Interferons are divided into three major groups: type I IFN, type II IFN, and type III IFN.

Type I IFNs bind to a specific cell surface receptor; they are often referred to as viral IFNs and are induced by the virus infection; they include four subtypes: IFN- α (α l leukocyte), IFN- β (fibroblast), IFN- ω (ω II), and IFN- τ (trophoblast).

Type II IFN is mostly referred to as immune IFN and induced by mitogenic or antigenic stimuli. Immune cells such as CD4 Th1 cells, CD8 cytotoxic suppressor cells, and natural killer cells secrete IFN-II. All types of IFNs have antiviral activity and regulate the immune system.

Detection of viral components by RLR and TLR in immune cells leads to the onset of intracellular signaling cascades and secretion of type I IFNs. pDCs are the IFN inducers responsible for producing large amounts of interferon. Dendritic cells (DCs) and fibroblasts produce type 1 interferon.

Type I IFNs adjust to a series of genes expression, including protein kinase R and 2'-5'-oligoadenylate synthase that are involved in reducing viral particles in infected cells, apoptosis, and resistance of uninfected cells to viral infection. Reoviruses are induced and are sensitive to interferon. After recognition of virus ssRNA by PRRs such as RIG-1 like receptor, virus-induced Interferon α/β stimulating intracellular signaling cascade which instates the antiviral mode by expression IFN-stimulated genes (ISGs), transcription factor IRF7 and additional secretion of interferon $\alpha\beta$ (Wilkins and Gale, 2010). Reovirus activates both the RIG-I and MDA5 to induce IFN.

Secreted IFN $\alpha\beta$ binds to the receptor, activates the Jak-STAT signal transduction pathway, and leads to the formation of the heterotrimeric IFN-stimulated gene factor 3 (ISGF3) transcription complex, which leads to the expression of IFN-stimulated genes (ISGs) (Kato et al., 2008).

The amount of interferon released as a result of a reovirus infection depends on the reovirus strain and the type of host cells. For example, viral genes such as M1, L2, and S2 genes are involved in the induction of IFN and sensitivity to IFN $\alpha\beta$. M1, which encodes the reovirus μ 2 protein, is a specific strain interferon suppressor, and even single amino acid changes in this protein can affect host IFN β response.

Interferon secretion is one of the first innate immune system responses to most viruses. The Janus kinases (JAKs), JAK1 and Tyk2, phosphorylate signal transducers and activators of transcription (STATs), STAT1 (STAT1 α and STAT1 β) and STAT2 activated by IFN receptor binding (Nan et al., 2017). STAT1/STAT2 heterodimers with p48 interferon regulatory factor 9 (IRF9) construct the heterotrimeric transcription factor complex IFN-stimulated gene factor 3 (ISGF3) (Veals et al., 1992). ISGF3 bind to ISREs (IFN-stimulated response elements) which is a regulatory DNA sequences and IFN-stimulated genes (ISGs) expression begin such as ISG56 (IFIT1) and transcription factor IRF7 and antiviral mood settled. IRF7 forms homo or heterodimer and increases the transcription of type I IFN genes (Basler et al., 2003). Many viruses such as reovirus suppress the interferon pathway by disrupting the innate immune system. Viruses usually do this in two general ways: first by blocking the host cell sensor machinery or downstream signaling molecules such as IRF3, that inhibit interferon induction and second, viruses suppress IFN signaling by modulation of JAK tyrosine phosphorylation activity, ISG promoter activity interfering, ISGF3 complex degradation (Randall and Goodbourn, 2008).

Viruses have mechanisms to inhibit interferon secretion and ISG protein function such as inactivation of JAKs, degradation or sequestration of STATs, and inhibition of karyopherins required for ISGF3 nuclear translocation. Rotavirus NSP1 protein degrades IRF9 and IRF7 which are necessary for IFN amplification responses (Randall and Goodbourn, 2008).

By suppressing the interferon signaling, the virus can inhibit interferon secretion that is initiated by PRRs and inhibits antiviral IFN-induced gene expression.

IFN type I is able to limit the replication of the virus in cell culture (Randall and Goodbourn, 2008). The activation of the transcription factors Interferon Regulatory Factor 3 (IRF-3) and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF κ B) (Pandey et al., 2015) begins after antiviral innate immune response stimulation by nucleic acid recognition. IRF-3 activation depends on the cellular double-stranded RNA sensors RIG-I, Mda-5, and IPS-1 (Holm et al., 2007).

Reovirus μ 2 protein inhibits IFN β -induced gene expression. The μ 2 causes nuclear accumulation of IRF9 and suppresses IFN signaling and subversion innate immune response. IRF9 is a transcriptional factor and exists in the cytoplasm that has an essential

role in the signaling pathway leading to expression of ISGs. Reovirus infection stimulates the activation of IRF9 bound to STAT1 and STAT2 and forms ISGF3 complex. ISGF3 complex translocates to the nucleus and binds to ISREs and initiates the transcription of many IFN-stimulated genes (ISGs). Reovirus IFN β modulation is related to the induction of unusual nuclear accumulation of IRF9 by $\mu 2$ protein and inhibition of IFN-stimulated gene (ISG) expression (Holm et al., 2007; Zurney et al., 2009).

One of the most important responses of the innate immune system to reovirus infection in the central nervous system is microglia and astrocyte activation that produces inflammatory cytokines and chemokines including tumor necrosis factor- α (TNF- α) and chemokine (C-X-C motif) ligand 10 (CXCL10), chemokine (C-C motif) ligand 5 (CCL 5) (Kraft et al., 2009). Activated glia and inflammatory mediators are related to a local microenvironment that is deleterious to neuronal survival and endocytic clearance of apoptotic vesicular material. Glial phagocytic activity leads to virus clearance.

Proinflammatory cytokines and chemokines play a role in eliminating virus infection by stimulating inflammation and recruiting innate and adaptive immune cells.

Costimulatory molecules are necessary for T cell activation and lead to acquired immune reactions.

Natural killer cells, mast cells, eosinophils, basophils, and the phagocytic cells including macrophages, neutrophils, and dendritic cells play important role in innate immune responses. Reovirus dsRNA is an innate immune system stimulator that activates dendritic cells (DCs) (Berger et al., 2017).

DCs are one of the most important cells of the immune system that induce inflammatory responses against pathogens. DCs are also involved in adaptive immunity by activating memory T cells, increasing B cell activation, priming naïve T cells, and linking innate and adaptive immunity system by persistent capturing, processing, and presenting antigens to CD4+ and CD8+ T cells. DC and NK cells agitate bystander T cell activation in reaction to TLR agonists via secretion of type 1 IFNs (Kamath et al., 2005). The virus agitates DC to secrete proinflammatory cytokines like IFN- α , TNF- α , and IL-6 and upregulates IFN- γ production and increased NK cytolytic activity. In response to TLR involvement, different subtypes of DC secrete a wide range of inflammatory cytokines such as IFNs type 1, TNF- α , and IL-12, and then activated DC enters into complex interactions with other immune cells including NK cells. NK cells play role in innate and adaptive immunity regulation (Errington et al., 2008; Kalinski et al., 2005).

Adaptive immune response is active after virus antigen presentation by special cells including DCs and virus antigen sensing by PRRs. PRRs define the origin of the antigens distinguished by the antigen receptors expressed on T and B cells, and the type of infection encountered, and instruct lymphocytes to induce the appropriate effector class of the immune response.

Rotavirus induces the two arms of adaptive immune responses. Antibody secretions against rotavirus VP4 and VP7 neutralize viral infection. RV-specific IgA and cytotoxic T cells (CD8+ T cells) involved in the protection and CD4+ T cells mediate the clearance of primary RV infection. Rotavirus induces types I and III interferons and other cytokines, especially Th1 and Th2. T and B cells involve in both clearance of RV and protection from reinfection.

These double-stranded RNA viruses are identified by the innate immune system and activate signaling pathways for inducing an antiviral interferon response. The most important pathways of host immune response to reovirus infection and IFN induction are summarized in Fig. 4.

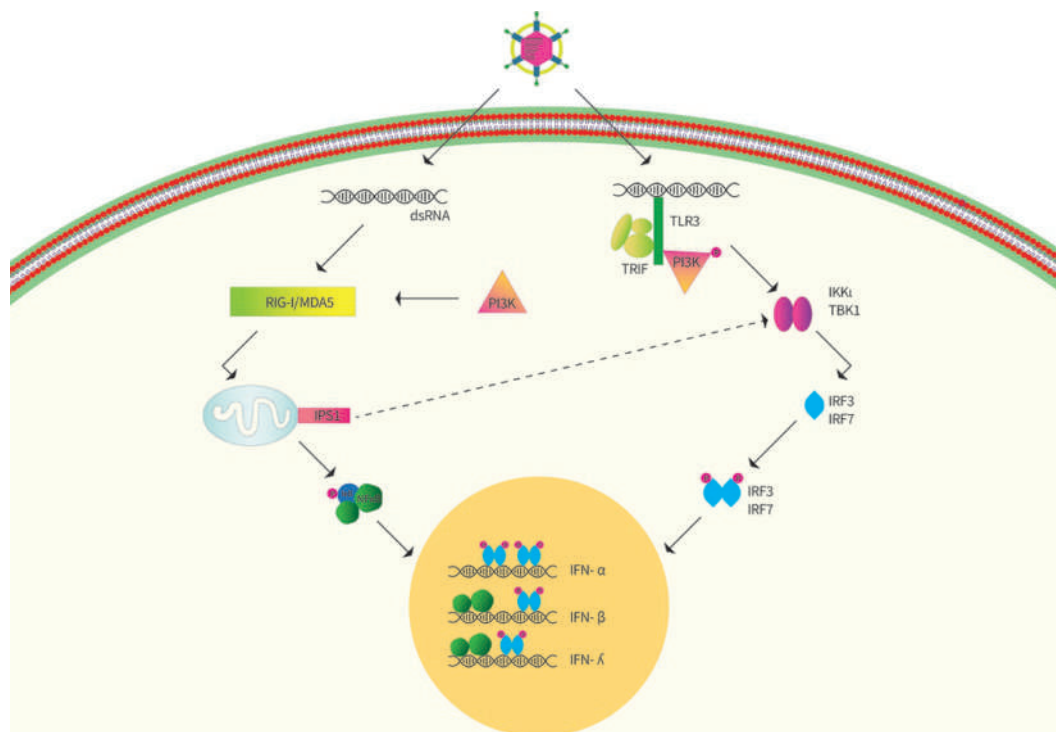


Fig. 4 Host immune response to reovirus and IFN induction. RIG-1/MDA5 is involved in mitochondrial antiviral signaling and IFN and NF- κ B induction. RIG-1/MDA5 senses virus replication leading to IPS1 recruitment and activation of NF- κ B and IRF3. NF- κ B family are transcription factors involved in host immune response induction and lead to IFN gene transcription. Virus dsRNA is recognized by TLR3 or RIG-1/MDA5. TLR3 activation recruitment TRIF induced Interferon regulatory factor (IRF) family (IRF3 and IRF7) phosphorylation and IFN upregulation.

Conclusion remarks and future perspective

Recently, the researchers improved their knowledge on the reoviruses for understanding and optimizing the complex interactions between OV, tumor cells, and the host immune system. Clarifying of relations between factors such as immune status, tumor types and their mutation profiles, as well as patient responses to virotherapy, will encourage in the development of effective, personalized treatments. In the near future, it seems that using targeted OV therapy in combination with immune-modulating therapies will significantly enhance the contribution of OV to the field of oncology. Nevertheless, many questions remain open to be answered. With a completed or ongoing clinical trial, reovirus therapy is well set to transition from “bench” to “bedside” for cancer therapy.

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Conflict of Interests

The authors declare that there are no conflicts of interest.

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Poxvirus

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Introduction

Poxviruses are large, enveloped, double-stranded DNA viruses categorized broadly within the expansive group of Nucleocytoplasmic Large DNA Viruses (NCLDV). Unlike many other eukaryotic DNA viruses that must rely on the nucleus for essential enzymes or metabolites, poxviruses replicate entirely in the cytoplasm of infected cells. Members of the poxvirus family infect a broad range of animal species, from insects to fish to birds to mammals. They pose a significant health threat for animals and humans, most notably exemplified by variola virus (VARV), the causative agent of smallpox.

Poxviridae family taxonomy

The *poxviridae* family is large and encompasses two sub-families: *Entomopoxvirinae* (EPV), which infect insects; and *Chordopoxvirinae* (ChPV), which infect vertebrates. While these two sub-families are highly divergent, they share similar structural and genomic features. Within the EPV sub-family, there are currently four genera recognized by the International Committee on Taxonomy of Viruses (Table 1). Despite an expansion of EPV genomic information in the last 10 years, there have still been relatively few EPV genomes sequenced due to technical difficulties and to date, no GammaEPV genomes have been sequenced. The ChPVs have been far more extensively studied. The number of ChPV genera has been significantly expanded in recent years, reflecting both a boom in the discovery of novel poxviruses and enhanced genomic analysis capabilities. There are now 18 genera within the ChPV sub-family (Table 1), and the genome of at least one member of each genus has been sequenced. ChPV genera can be divided into two clades based on phylogeny (Fig. 1). Clade I represents the evolutionarily more ancient genera including *Orthopoxvirus* and *Avipoxvirus*, whereas clade II represents the more recent genera including *Yatapoxvirus*, *Capripoxvirus*, *Suipoxvirus*, *Cervidpoxvirus* and *Leporipoxvirus*. Salmon gill poxvirus (SGPV) represents the earliest divergence of ChPV, located at the base of the ChPV phylogenetic tree (Gjessing et al., 2015).

Health relevance

The *poxviridae* family has had a powerful impact on both human and animal health. At least five genera (*Centapoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Parapoxvirus*, and *Yatapoxvirus*) have species which are capable of infecting humans. The most well-known of these are the *Molluscipoxvirus mollusum contagiosum virus* (MOCV), and *Orthopoxviruses* VARV and VACV. *Mollusum contagiosum* is a skin disease commonly affecting children. VARV is the causative agent of smallpox and VACV is the closely related species which was used as the smallpox vaccine. Smallpox was one of the world's most devastating diseases, believed to have caused more deaths than any other infectious disease. The mortality rate for smallpox infections was around 30% and many who survived were left with pockmark scars. The pursuit of a protection from smallpox led to one of the greatest medical innovations in history: vaccination (Barquet and Domingo, 1997). Due to a global vaccination campaign instigated by the World Health

Table 1 The *poxviridae* family.

Genus	Type species	Host
<i>Entomopoxvirinae</i> sub-family		
<i>Alphaentomopoxvirus</i>	<i>Melolontha melolontha entomopoxvirus</i>	Coleoptera (beetles)
<i>Betaentomopoxvirus</i>	<i>Amsacta moorei entomopoxvirus</i>	Lepidoptera and orthoptera (butterflies, moths, grasshoppers)
<i>Deltaentomopoxvirus</i>	<i>Melanoplus sanguinipes entomopoxvirus</i>	Grasshoppers
<i>Gammaentomopoxvirus</i>	<i>Chironomus luridus entomopoxvirus</i>	Diptera (flies and midges)
Unclassified	<i>Diachasmimorpha entomopoxvirus</i>	Wasps
<i>Chordopoxvirinae</i> sub-family		
<i>Avipoxvirus</i>	<i>Fowlpox virus</i>	Birds
<i>Capripoxvirus</i>	<i>Sheeppox virus</i>	Sheep, goats, cattle
<i>Centapoxvirus</i>	<i>Yokapox virus</i>	Humans
<i>Cervidpoxvirus</i>	<i>Mule deerpox virus</i>	Deer and meese
<i>Crocodylidpoxvirus</i>	<i>Nile crocodilepox virus</i>	Crocodiles
<i>Leporipoxvirus</i>	<i>Myxoma virus</i>	Rabbits, hares, and squirrels
<i>Macropopoxvirus</i>	<i>Eastern kangaroopoxvirus</i>	Kangaroos
<i>Molluscipoxvirus</i>	<i>Molluscum contagiosum virus</i>	Humans
<i>Mustelpoxvirus</i>	<i>Sea otterpox virus</i>	Sea otters
<i>Orthopoxvirus</i>	<i>Vaccinia virus</i>	Varied; humans, cows, monkeys, rodents, others
<i>Oryzopoxvirus</i>	<i>Cotia virus</i>	Mice
<i>Parapoxvirus</i>	<i>Orf virus</i>	Sheep, goats, cattle, others
<i>Pteropopoxvirus</i>	<i>Pteropox virus</i>	Bats
<i>Salmonpoxvirus</i>	<i>Salmon gillpox virus</i>	Salmon
<i>Sciuripoxvirus</i>	<i>Squirrelpox virus</i>	Squirrels
<i>Suipoxvirus</i>	<i>Swinepox virus</i>	Pigs
<i>Vespertilionpoxvirus</i>	<i>Eptesipox virus</i>	Bats
<i>Yatapoxvirus</i>	<i>Yaba monkey tumor virus</i>	Monkeys and baboons

Listed are all genera within each of the two *poxviridae* sub-families, type species for each genus, and their host species.

Organization (WHO), smallpox was declared eradicated in 1980 and routine vaccination was ceased. Used as the vaccine, the closely related cowpox (CPXV) and VACV were instrumental for this public health achievement.

Poxvirus infections in animals represent major problems for agricultural industries and wildlife alike. *Avipoxvirus* infections cause major economic losses in egg and poultry industries and have devastated wild bird populations (Smits et al., 2005; Alley et al., 2010). *Capripoxvirus* infections are among the most serious infectious diseases of livestock. *Capripoxviruses* have caused an estimated 30–65% production loss for cattle and sheep farmers in some regions and have proven difficult to control, spreading through the Middle East in the last 10 years (Tuppurainen et al., 2017). *Orthopoxvirus* and *Parapoxvirus* infections in animals are also sources of zoonotic poxvirus infections in humans (Tack and Reynolds, 2011).

Aside from the direct effect on human health, VACV has been used extensively in immunology and virology research, greatly expanding our knowledge of viruses and their interactions with cells and the immune system. In more recent years, VACV and other poxviruses have been modified to be used in applications such as vaccines, gene therapy, and cancer treatment.

Genome

The poxvirus genome consists of linear dsDNA with covalently closed terminal hairpin loops that are incompletely base-paired. These terminal hairpin regions are exceptionally difficult to sequence, but progress has been made thanks to innovations in sequencing technology (Mitsuhashi et al., 2014; Gunther et al., 2017). Between 130–380 kilobase pairs (kbp), poxvirus genomes are among the largest of all viruses. EPV genomes tend to be larger than those of ChPVs (225–380 kbp vs. 170–220 kbp).

Approximately 500 poxvirus genomes have now been sequenced, providing a highly detailed picture of poxvirus genomics and evolution. Genes and their order within the genome can vary significantly across genera, especially within the EPV sub-family. Nonetheless, poxvirus genomes consistently follow a similar arrangement; the genome is organized as a central, conserved region flanked by more variable regions containing inverted terminal repeats. The central conserved region generally encodes genes that are essential for core processes like replication and virion assembly. The flanking regions contain genes dedicated to manipulating the host response to the infection, and consequently are tailored to the host species. Genes that the virus likely acquired from the host through horizontal gene transfer are also located within the flanking regions. Among the over 200 genes encoded by poxviruses, approximately 90 genes are conserved among ChPVs and 49 are conserved between ChPVs and EPVs (Upton et al., 2003). The conventional nomenclature for the prototypical VACV genes consists of a letter from A–O representing fragments generated by restriction endonuclease digestion, followed by a number indicating the open reading frame, and L or R to denote left or right orientation, e.g., B18R. The protein product is called the same name as its gene, but without the L or R, e.g., B18.

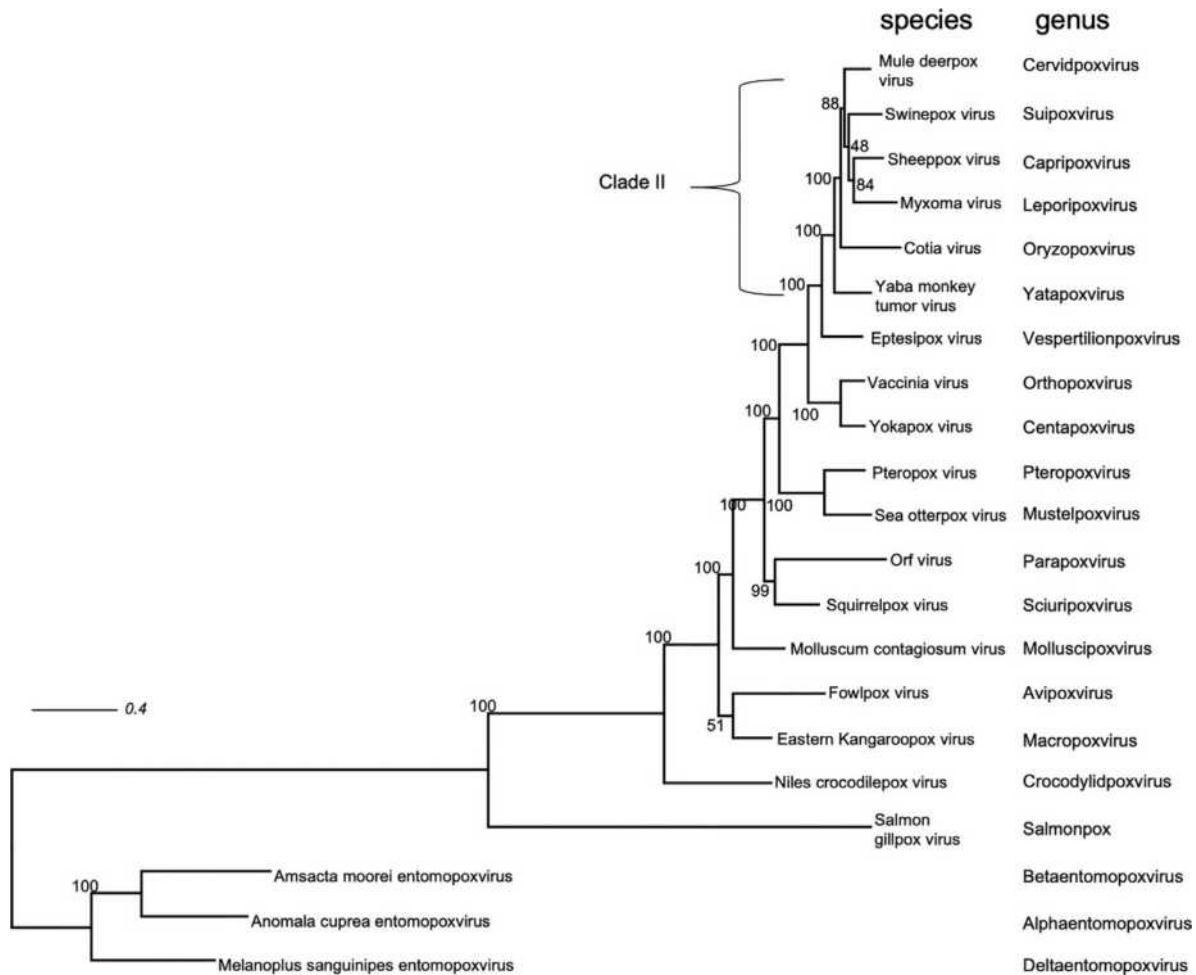


Fig. 1 Phylogenetic tree of poxviruses constructed by the maximum-likelihood method from a multiple sequence alignment of concatenated sequences of seven conserved proteins. The tree is midpoint rooted. Scale bar indicates amino acid substitutions per site. Numbers at internal nodes indicate bootstrap support.

Structure

All ChPVs exhibit similar morphology: a brick-shaped or ovoid virion consisting of a central core and one or two lateral bodies enclosed in a lipid envelope. Some poxviruses, particularly parapoxviruses, have a small spiral coil that wraps around the viral particle, resembling a ball of yarn. Virions are large, approximately 350 nm in length and 250 nm in width. As exemplified by the model poxvirus VACV, ChPVs produce two different forms of infectious virions: the mature virus (MV) and enveloped virus (EV). MV contains one envelope decorated with over 20 different viral proteins, most of which are involved in viral attachment and entry. The distribution of the viral proteins on the envelope appears to be highly ordered. For example, microscopy studies of VACV have shown that viral binding and fusion proteins localize to distinct domains on the VACV particle; attachment machinery is located on the sides of the virion and fusion proteins cluster at the tips (Gray et al., 2019). This polarization is critical for proper function. EV is distinguished from MV by the presence of a second outer membrane which contains several unique viral membrane proteins. MV and EV are generated through two different pathways by which the virus may leave host cells, as described below. EVs, while much less abundant than MVs, are the principal mediators of long-distance spread within the host.

While ChPVs are fairly homologous in morphology, EPV core shapes are distinct among the genera. AlphaEPVs are kidney-shaped, BetaEPVs are cylindrical, the singular DeltaEPV is rectangular, and GammaEPVs exhibit the prototypical biconcave dumbbell shape which the rest of the *Poxviridae* family displays.

Replication cycle

Entry

Since VACV is the best-studied poxvirus, we will describe its life cycle as a representative of the family. The replication cycle begins with virus entry, which can be divided into three steps: attachment, fusion, and core entry. There are distinct viral proteins involved in each step. When an MV encounters a host cell, the four attachment proteins on the viral envelope bind to glycosaminoglycans

(GAGs) or laminins on the cell membrane, allowing the virus to attach to the surface of the cell. Note that the EV form of VACV does not have the same attachment proteins on the outer membrane as the MV. Several EV surface proteins have been identified which mediate binding to the cell and trigger disruption of the outer EV membrane. In effect, this releases an MV particle and entry proceeds accordingly.

Depending on the cell type being infected and the virus strain, fusion can be triggered either at the plasma membrane or within endosomes following micropinocytosis. When entering at the plasma membrane, fusion is pH independent. Entry by the endocytic route involves virus-induced cell signaling to trigger engulfment. The lowering of the pH within the endosome as it acidifies is a necessary trigger for fusion of the viral and endosomal membranes. For most viruses, one viral protein is usually involved in membrane fusion. Poxviruses, however, encode an entry-fusion complex (EFC) made up of approximately 11 small proteins which are mostly conserved across genera (Moss, 2012). Nine proteins make up the core complex while two others are peripherally associated. For fusion through both cell surface and endocytic pathways, the viral EFC is required. The EFC fusion machinery normally exists in an inactive state, suppressed by at least two other viral proteins. Low pH or other signals induce the release of these proteins and de-repress the fusion machinery. After the viral and cellular membrane lipids mix and a pore forms, viral cores enter the cytoplasm (Moss, 2016). The cores are then broken down partially through the action of host proteasomes, and the genomes are released.

Transcription and translation

Viral genes are transcribed in three temporally distinct waves: early, intermediate, and late gene transcription. Early genes code for intermediate transcription factors, intermediate genes code for late transcription factors, and late genes produce early transcription factors which are packaged into the virion. Since virions package a comprehensive set of transcription machinery, early gene transcription can begin almost immediately after core entry before uncoating occurs. This is in contrast with other DNA viruses which rely heavily on cellular factors for gene transcription and a key to the ability of poxviruses to forego a nuclear step in its life cycle. The structures of viral DNA-dependent RNA polymerase (vRNAP) complex at a resting state and in active transcription were recently elucidated with cryo-electron microscopy (cryo-EM) (Hillen et al., 2019; Grimm et al., 2019). The core of the vRNAP shares considerable homology with cellular RNAPs, while the periphery displays virus-specific adaptations. Viral mRNAs possess 5' m7G caps and 3' poly(A) tails like eukaryotic mRNAs, but they can have unique features as well. For example, intermediate and late mRNAs have long heterogeneous 3' ends due to a lack of a specific transcription termination signal, resulting in overlapping transcripts and the formation of dsRNA, a pathogen-associated molecular pattern (PAMP) which can be detected by cellular sensors and trigger an immune response.

Many early genes have the critical function of modulating the host antiviral response. Others encode factors needed for genome replication. Uncoating and genome replication is necessary for the genome to be accessible to intermediate transcription factors and the switch to intermediate and late stages of transcription (collectively called post-replicative gene expression) to occur. Post-replicative genes are mainly involved in virion assembly and maturation.

VACV employs a number of mechanisms such as shutting off host protein synthesis or modulating mammalian target of rapamycin (mTOR) activity to co-opt cellular ribosomes and promote viral protein synthesis (Meade et al., 2019a,b). Post-replicative mRNAs have a unique poly(A) leader at the 5' untranslated region (5'-UTR) which facilitate the preferential translation of the viral transcripts (Jha et al., 2017; Dhungel et al., 2017; Rollins et al., 2019; DiGiuseppe et al., 2020).

DNA replication

DNA replication begins very rapidly (~2 h) after entering the cell. A defining characteristic of poxvirus infection is the formation of cytoplasmic inclusions where genome replication and post-replicative gene expression take place. For EPVs, these are called spheroids, and for ChPVs, these are called viral factories. Each factory arises from a single incoming virion. When a cell is infected with more than one virus, viral factories appear to be a physical barrier to recombination (Kieser et al., 2020).

Remarkably self-sufficient, poxviruses encode a complete set of replication machinery, and many poxviruses even possess enzymes for deoxyribonucleotide synthesis. The viral DNA polymerase has 3'-5' proofreading exonuclease activity. Structural studies on the DNA polymerase have produced a highly detailed understanding of the mechanics of this enzyme and how it associates with other factors like the processivity factor (Czarnecki and Traktman, 2017). Despite these structural insights, the mechanism utilized by poxviruses for DNA replication has continuously been debated. It is unclear whether there is a specific origin of replication used by poxviruses since they can catalyze the replication of any circular piece of DNA transfected into an infected cell. However, deep sequencing has identified putative origins of replication in the terminal hairpin region (Senkevich et al., 2015). One model of replication involves self-priming where a nick is made at the terminal hairpin loop, generating an open 3' end. The polymerase extends the open-end, which folds back on itself to create a hairpin. The hairpin serves as a primer for DNA synthesis, which proceeds by strand displacement. Alternatively, there is evidence that replication may occur by the eukaryotic mechanism of leading and lagging strand synthesis. Ultimately, replication produces head-to-tail concatemers which must be cleaved into unit-length genomes by a viral Holliday junction resolvase. Based on the presence of a concatemer resolution motif in the ITR of one EPV genome, it is thought that EPV genome replication is similar to that of ChPVs.

Assembly and egress

Once the genome has been replicated and post-replicative genes are translated, the components of new virions are ready to be assembled. A unique process of membrane biogenesis first generates the highly unusual, open-ended crescent membranes. This process of viral membrane biogenesis is in contrast with most other enveloped viruses which gain their envelope through the process of budding. Though many details are still unclear, crescent formation is thought to be initiated by the insertion of several viral membrane proteins into the ER membrane. One of the membrane proteins promotes membrane curvature and anchors the assembly of a viral protein scaffold on top of the membrane, shaping the membrane into a sphere. Additional regulatory proteins, collectively known as viral membrane assembly proteins (VMAPs), facilitate the rupture of the ER membranes and stabilize the ends. The crescents grow in length while additional viral components like core proteins are directed to the crescents by a complex of seven viral proteins. The genome is funneled in and the spheres close off, forming the immature virion (IV) (Liu et al., 2014). The IV undergoes a morphological transition involving a series of proteolytic reactions, removal of the scaffold, insertion of additional membrane proteins, and structural transformations. This maturation process converts the spherical IV into the brick-shaped intracellular MV, which is fully infectious. MV envelope proteins are not glycosylated, since they do not pass through the ER, again distinguishing poxviruses from most other viruses. Interestingly, SGPV lacks some conserved membrane biogenesis proteins but still displays the visual characteristics of typical poxvirus crescent formation and virion maturation (Gjessing et al., 2015).

The primary mode of virion egress is by passive release when the cell eventually lyses due to infection. A small fraction of MVs, however, migrate via microtubules to the plasma membrane prior to this and exit the cell by exocytosis. Along the way, the MVs are wrapped with two additional membranes from the Golgi or endosomal vesicles, forming the triple-membraned wrapped virus (WV). Several EV-specific membrane proteins traffic to the trans-Golgi where wrapping occurs. There they are incorporated into the virion (Sivan et al., 2016; Monticelli et al., 2020). Since these do pass through the ER, the EV envelope proteins are glycosylated. During the process of exocytosis, the outermost membrane fuses with the plasma membrane, releasing the double-enveloped EV. These can either remain associated with the membrane as cell-associated EVs (CEVs), or induce actin tail formation to be propelled away from the cell surface, enabling cell-to-cell spread (Pickup, 2015).

Superinfection exclusion

Many viruses implement measures to prevent infected cells from being further infected by another virus, a phenomenon known as superinfection exclusion. For VACV, two sets of protein pairs on the plasma membrane of VACV-infected cells effectively prevent superinfection. One pair repels EVs away from cells via actin tails while another heterodimer blocks EFC function of MVs (Turner and Moyer, 2008; Doceul et al., 2010). These fusion blockers have the additional role of inhibiting interactions between viral fusion machinery left on the surfaces of neighboring infected cells, thereby preventing syncytium formation.

Immune response and evasion

The innate immune response is critical for keeping infections under control until adaptive immunity has been ramped up. Complement, natural killer cells, macrophages, dendritic cells, and innate lymphoid cells are all key effectors for early antiviral defense. Programmed cell death is another important line of defense, terminating infected cells before they can produce progeny virions (Veyer et al., 2017; Nailwal and Chan, 2019). As viruses replicating in the cytoplasm, poxviruses are also vulnerable to the cell's arsenal of pattern recognition receptors (PRRs). Cytoplasmic DNA as well as dsRNA by-products from transcription are major PAMPs which activate nucleic acid sensors. Some of the sensors which respond to poxvirus infection include toll-like receptors (TLRs), cyclic GMP-AMP synthase (cGAS), protein kinase R (PKR), melanoma differentiation-associated protein 5 (MDA5), and retinoic acid inducible gene I (RIG-I). The downstream result of activation of these sensors is production of interferon (IFN). IFNs can then act locally on the cell or be secreted to act distantly and stimulate production of cytokines to induce an antiviral state.

Poxviruses have evolved an extensive set of proteins which block activation of the innate immune response or interfere with its downstream effects. Approximately half of the viral coding capacity is dedicated to modulating the immune response. Species within each genus have different sets of immunomodulatory proteins, reflecting the differences in immune systems of the species they infect. Some of the strategies used by the virus to foil the immune response include sequestering viral nucleic acids to avoid detection, deploying direct inhibitors of sensors and key signaling molecules, secreting decoy receptors to intercept pro-inflammatory molecules, and mimicking cellular proteins to act as sinks for their ligands (Smith et al., 2013, 2018; Agrawal et al., 2017; Nichols et al., 2017). Even now, new viral immunomodulatory proteins and modes of inhibiting the immune response continue to be discovered.

While innate immunity is important to provide early control of infections, the adaptive immune response is key for clearance of infection. Based on studies with VACV and ectromelia virus (ECTV, the mousepox virus), infections can be controlled by antibodies as well as cytotoxic T cells (Kennedy et al., 2009). Many of the envelope proteins are prime targets for neutralizing antibodies (Moss, 2011). Immune responses to VACV can be cross-reactive with other orthopoxviruses such as VARV, CPXV, and MPXV due to antigenic similarity among the viruses (Gilchuk et al., 2016). Importantly, poxvirus infections generally elicit long-lasting immunity. For the smallpox vaccine, memory B and T cell responses can be detected several decades after vaccination.

Host range genes

Viruses often require specific cellular receptors to gain access to host cells. Expression of the receptors on host cells consequently dictates cell and host tropism for many viruses. Poxviruses are somewhat unusual in terms of their entry receptor, or rather, lack thereof. No specific entry receptor has been found for poxviruses and it is believed that they use molecules which are present on a wide variety of cells or that there are redundant receptors. Because of this feature, poxviruses are capable of entering nearly all mammalian cell types and their host ranges are largely determined by post-entry factors such as the ability to evade the immune response. These viral factors are collectively known as host range genes. There are more than 10 different classes of host range genes which are variably expressed among different poxvirus species (Werden et al., 2008; Bratke et al., 2013; Oliveira et al., 2017). The absence of certain host range genes is associated with a defect in the virus' ability to infect certain cell types. Host range genes primarily function to block the host antiviral response. This can include inhibition of complement, inflammation, apoptosis, PRRs, or a combination thereof. Several of these genes are functionally redundant. VACV E3L and K3L are two of the most extensively studied host range genes. They prevent host detection of infection by inhibiting PKR. Additionally, VACV K1L and C7L both inhibit cellular antiviral proteins Sterile Alpha Motif Domain-containing 9 (SAMD9) (Sivan et al., 2015; Liu and McFadden, 2015) and SAMD9L (Meng et al., 2018), which together pose a barrier for poxvirus replication. The T2/cytokine response modifier (Crm) gene family is another notable host range gene family. This family acts as cellular Tumor Necrosis Factor Receptor (TNFR) homologs, interfering with TNF signaling and inhibiting apoptosis.

Pathogenesis and clinical diagnosis

EPVs are transmitted by ingestion of spheroids and cause loss of mobility, flaccidity, and whiteness before ultimately killing the insect host after a few weeks. ChPV infections in humans and animals are commonly transmitted by contact with infectious material, often from a lesion on an infected individual. Infections usually remain localized to the skin, though in some cases they can spread, resulting in much more severe disease. MPXV and VARV, which cause more serious illness, are transmitted via the respiratory route as well as by contact with bodily fluids and consequently spread throughout the body. The incubation period for these two viruses is typically 1–2 weeks. In both humans and animals, the hallmark feature of poxvirus infections is a rash or lesions on the skin. Smallpox and MPXV are associated with a number of other symptoms such as fever, headache and body aches, malaise, vomiting, and diarrhea (Fenner and World Health Organization, 1988). *Orthopoxvirus* infections in animals may also manifest as upper respiratory symptoms.

Clinically, diagnosis is made based on the presence of lesions and a history of contact with infected individuals or animals. Diagnosis may then be confirmed by microscopy of material collected from lesions and observation of virions with characteristic poxvirus morphology. Detection of the specific viral genome by PCR is used to distinguish morphologically similar genera.

Treatment

Vaccination is an effective line of defense against poxvirus diseases. Vaccination originated as inoculation of CPXV into the arms of individuals to prevent smallpox, a procedure brought to practice by Edward Jenner. This strategy was based on the observation that dairymaids who contracted CPXV were protected from smallpox (Barquet and Domingo, 1997). As this practice became widespread, VACV replaced CPXV in the vaccine, though it is not clear how or when this happened. For much of the 20th century, the smallpox vaccine consisted of replication-competent VACV, which is antigenically similar enough to VARV that it generates protective immunity against VARV. The VACV vaccine is arguably the most successful vaccine in history, having eradicated a human disease, but it caused significant complications in immunocompromised individuals or those with allergic skin conditions like atopic dermatitis. In these individuals, the infection can become generalized or systemic, causing conditions such as eczema vaccinatum, progressive vaccinia, generalized vaccinia, and postvaccinal encephalitis. Because of this, there have been efforts to attenuate the virus while retaining immunogenicity to make a safer vaccine (Meyer et al., 2020). One such strain, MVA, has been approved for use in several countries. Vaccines also exist for several animal poxviruses, which can help avoid economic losses caused by infection (Bhanuprakash et al., 2012).

Vaccinia immune globulin (VIG), made from blood of vaccinated donors, was an effective treatment from the time when smallpox was endemic (Hopkins and Lane, 2004). Additionally, there is an antiviral drug licensed in the U.S for treating smallpox disease: tecovirimat. A plethora of other drugs which inhibit poxvirus replication at different stages of the life cycle have been identified though few have been tested in vivo as of yet (Delaune and Iseni, 2020).

Poxvirus vectors

Poxviruses have proven useful for the development of vaccines and cancer therapeutics for a number of reasons: (1) their large coding capacity allows for easy insertion of therapeutic genes or vaccine antigens, (2) the fact that they do not enter the nucleus reduces the risk of integration, and (3) they robustly activate the immune system. The broad tissue tropism of some poxviruses

enables delivery of antigens and other therapeutics throughout the body. VACV, *Avipoxviruses*, and *Capripoxviruses* have been engineered as vaccine vectors for infectious diseases of wildlife like rabies, Newcastle disease, and rinderpest virus (Maki et al., 2017; Bournnell et al., 1990; Teffera and Babiuk, 2019). Avian poxviruses, as well as the attenuated MVA VACV strain, are attractive for safe therapeutic use in humans because they do not productively replicate in human cells (Weli and Tryland, 2011). VACV and other poxvirus vectors have shown success as vaccines for numerous pathogens such as influenza, Zika, chikungunya, human immunodeficiency virus (HIV), *Mycobacterium tuberculosis*, and *Plasmodium falciparum* (Altenburg et al., 2014; Prow et al., 2020; Volz and Sutter, 2017). The large coding capacity and ability to simultaneously express more than one foreign antigen allows poxviruses to be developed as multi-valent vaccines (Prow et al., 2018). VACV has also shown promising results as a cancer vaccine and oncolytic virotherapy for a variety of malignancies (Kim and Gulley, 2012; Yang et al., 2018).

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Picornaviridae: Enterovirus

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Glossary

Cre (Cis-acting replication element) It acts as a template for the viral RNA polymerase 3D^{Pol} for the uridylylation of VPg to form VPg-pU-pU.

IRES (Internal ribosome entry site) IRES is an RNA element that allows initiation of translation in cap-independent manner which helps in the synthesis of proteins.

Polyprotein It is the long protein that forms through the process of translation of open reading frame (ORF) of picornavirus RNA. This polyprotein is processed via proteinases which results in the formation of mature viral proteins.

Positive strand RNA A single molecule of RNA of the PiV helps in the encoding of the functional viral protein through the process of translation in the 5'–3' direction.

Uridylylation The process of the addition of the two uridylyl residues results in the formation of VPg molecule through the PiV RNA dependent RNA polymerase 3D^{Pol} by utilizing a viral RNA template.

VPg It is a virus protein genome linked which acts as a protein primer for the PiV RNA dependent RNA polymerase. This is followed by the uridylylation of VPg-pU-pU which is covalently attached to 5' end RNA of PiV.

Introduction

Picornaviruses (PiVs) are widely distributed across the globe and they are serious threats to human and animal health (i.e., poliomyelitis, encephalitis, meningitis, hepatitis, etc.) PiVs are RNA viruses and differentiated by their biological, biophysical and genetic characteristics (Tuthill et al., 2010a, b). PiVs belong to the family *Picornaviridae* and it consists of 147 species that are grouped into 63 genera (Zell et al., 2017). The major classification of PVs is reported in Table 1. The first virus of this family was identified as foot and mouth diseases virus (FMDV) and the earliest vaccine developed for FMDV and poliovirus (PV) (Loeffler and Frosch, 1898; Enders et al., 1949; Eggers, 1999; Tuthill et al., 2010a, b). PV was the first engineered animal virus in the infection clone and the first virus synthesized outside the cell (Racaniello and Baltimore, 1981a, b; Molla et al., 1991). In 1971, International Committee on Nomenclature of Viruses (ICNV) listed *Picornaviridae* family in their first report (Wildy, 1971). Andre Lwoff (Nobel Laureate, 1965) was elected member of ICNV stated about the name of the family that *Pico* is a prefix of metric system that indicate submultiple unit 10^{-12} and *rna* stands for RNA. Therefore, *picorna* means 10^{-12} ribonucleic acid. It is also stated in the minutes of the subcommittee that the *picorna* refers to *poliomyelitis*, insensitivity to ether, *Coxsackie*, *orphan* and *rhinovirus* (Lwoff and Tournier, 1966; Zell, 2018). The characteristics of *Picornaviridae* family are given in Table 2. The cell entry characteristics for the different PiVs are reported in Table 3. These viruses cause various diseases of the central nervous system (CNS), respiratory system, gastrointestinal tract, heart, liver, pancreas, skin and eye (Grubman and Baxt, 2004; Stanway et al., 2000; Tapparel et al., 2013; Vaughan et al., 2014; Anastasina et al., 2017).

PiVs genome and structure of capsid

PiVs are nonenveloped small RNA viruses (Schwerdt and Schaffer, 1955; Zell et al., 2017; Zell, 2018; Cifuentes and Moratorio, 2019) containing spherical nucleocapsids and they have positive strand polarity in their genomes (Holland et al., 1960; Knipe and Howley, 2013; MacLachlan and Dubovi, 2017). The lengths of the strands are in the range of 6.7–10.1 kilobases for seal picornavirus and goose megavirus, respectively. The 5' end subunit of the RNA virus is covalently bonded with the tyrosine-3 residue of small virus encoded peptide known as 3A or viral peptide genome associated (VPg) (Flanagan et al., 1977; Lee et al., 1977; Ambros and Baltimore, 1978), whereas 3' has a poly tail end (Yogo and Wimmer, 1972; Dorsch-Häsler et al., 1975). Genome codes for a single polyprotein are formed by the process of co- and posttranslationally via virus encoded proteinases to form capsid proteins along with other nonstructural proteins that used in the replication of viral RNA (Maizel Jr, 1963; Jacobson and Baltimore, 1968; Maizel Jr and Summers, 1968; Kiehn and Holland, 1970; Palmenberg, 1990; Strating and van Kuppeveld, 2017). It has been found that dicapiviruses possess a dicistronic genome containing capsid-protein-encoding open reading (ORF) and one ORF coding for nonstructural proteins (Woo et al., 2012a, b).

X-ray diffraction model for PiVs and PV based has been reported, however, a near-atomic three-dimensional structure at a resolution of 2.9 Å of PVs but were not followed till 1985 (Finch and Klug, 1959; Hogle et al., 1985). It has been found that ~200 crystal structures and cryoelectron microscopy structures of PiVs with 7 genera and 17 species were deposited in RCSB Protein

Table 1 Classification of the *Picornaviridae* virus family.

<i>Genus</i>	<i>Species</i>	<i>Genotyping</i>
Enterovirus	Enterovirus-A	Enterovirus-A71
	Enterovirus-B	Coxsackievirus-B3
	Enterovirus-C	Polioviruses
	Enterovirus-D	Enterovirus-D68
	Enterovirus-E	
	Enterovirus-F	
	Enterovirus-G	
	Enterovirus-H	
	Enterovirus-I	
	Enterovirus-J	
	Enterovirus-K	
	Enterovirus-L	
	Rhinovirus-A	Rhinovirus-A2
	Rhinovirus-B	Rhinovirus-B14
	Rhinovirus-C	Rhinovirus-C15
Parechovirus	Parechovirus-A	Human parechovirus 1–16
	Parechovirus-B	
Hepatovirus	Hepatovirus-A	
	Hepatovirus-B	
	Hepatovirus-C	
	Hepatovirus-D	
	Hepatovirus-E	
	Hepatovirus-F	
	Hepatovirus-G	
	Hepatovirus-H	
	Hepatovirus-I	
Aphthovirus	Foot and mouth diseases virus	
	Equine rhinitis A virus	
Cardiovirus	Encephalomyocarditis virus	
	Theilovirus	Saffold virus

Table 2 Characteristics of the family member of *Picornaviridae* (Zell et al., 2017).

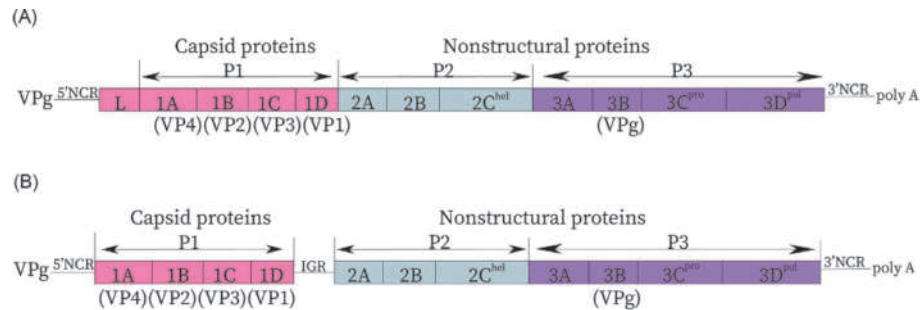
<i>Characteristics</i>	<i>Description</i>
Typical member	Poliovirus 1 Mahoney (V01149), species <i>Enterovirus C</i> , genus <i>Enterovirus</i>
Virion	Nonenveloped, 30–32 nm virions comprising 60 protomers
Genome	6.7–10.1 kb of positive sense, nonsegmented RNA with a poly (A) tail
Replication	RNA synthesis occurs in cytoplasmic replication organelles containing nonstructural proteins derived from 2BC-P3 region of the encoded polyprotein. RNA structures at the 5'- and 3'-ends of the genome direct initiation of RNA synthesis and uridylated 3B serves as primer for the synthesis of the RNA strands
Translation	Occurs directly from genomic RNA containing an internal ribosomal entry site (IRES)
Host range	Vertebrates
Taxonomy	Realm <i>Riboviria</i> , kingdom <i>Orthomavirae</i> , phylum <i>Pisuviricota</i> , class <i>Pisoniviricetes</i> , order <i>Picornavirales</i> ; 63 genera containing 147 species

Databank. Icosahedral $T = 1$ /pseudo $T = 3$ the capsid is made up of 60 protomers and each protomer is composed of one copy of the four capsid proteins 1A (VP4), 1B (VP2), 1C (VP3) and 1D (VP1) (Rossmann and Johnson, 1989; Tuthill et al., 2010a, b; Ren et al., 2013).

PiVs proteins nomenclature was proposed with an idealized genome layout L434 diagram (Fig. 1). In the third meeting of the European Study Group of Molecular Biology of Picornaviruses L434 convention agreed and summarized that idealized polyprotein is found to be cleaved into leader protein (L) and polyproteins (P1, P2, P3) in the processing steps. In the next processing steps that lead to the formation of capsid proteins from P1 (i.e., 1Ab, 1C, 1D) precursor, three nonstructural proteins from polyprotein midpiece P2 (i.e., 2A, 2B, 2C) and four nonstructural proteins from P3 (i.e., 3A, 3B, 3C, 3D). 1AB cut leads to the formation of mature capsid proteins 1A and 1B and this occur in some PiVs after incorporation of RNA. The maturation followed by cleavage is RNA catalyzed step which results in the formation of stable infectious virions (Guttmann and Baltimore, 1977; Basavappa et al., 1994; Hindiyeh et al., 1999; Tuthill et al., 2010a, b; Zell, 2018). The place of VP4, VP2, VP3, VP1, VP0 and VPg for 1A, 1B, 1C, 1D, 1AB and 3B may be maintained (Rueckert and Wimmer, 1984a, b; Hughes, 2004; Norder et al., 2011; Zell, 2018). The systematic

Table 3 PVs entry characteristics in cell (Tuthill et al., 2010a, b).

Genus	Species	Features	Receptors	Endocytosis pathway	Initial trigger	Final product	Membrane penetration
Entero	PV	Canyon circling star shaped	PVR (CD155) binds in canyon	Nonclathrin, noncaveolin	Receptor binding	Empty particle	Membrane pore VP4 are formed in channel formation/genome delivery
	HRV	Canyon circling star shaped	LCLr family binds in shallow pits	Clathrin mediated	Low pH	Empty particle	Bafilomycin blocks the entry Size selective pores are formed in endosome membrane
Aphtho	FMDV	Smooth capsid No canyon No pocket	Integrin binds to flexible exposed loop	Clathrin mediated	Low pH	Acid induced dissociation to pentameric subunits	Dissociation occurs with the formation of transient intact empty particle
Cardio	TMEV	Protursion at fivefold Depression at twofold	Sialic acid, Heparan sulfate		Low pH	Acid induced dissociation to pentameric subunits	

**Fig. 1** Schematic diagram of PiVs genome (A) and of dicistronic Dicipivirus genome.

nomenclature shows that the PiVs proteins are in the order polyprotein and the first position is of capsid proteins (VP1–VP4) which refer to their molecular weights in SDS polyacrylamide gel electrophoresis containing VP1 (largest protein) and VP4 (smallest protein) (Fig. 1). The biological and experimental data for newer PiVs are not present but only sequence data are present. Therefore, it has been found that there is variation in the sizes of proteins as compared to that of reference proteins. So the Study Group of PiVs recommended the use of systematic nomenclature (Minor, 1990; Stanway, 1990; Hughes, 2004).

PiVs genome idealized layout is described as VPg + 5'UTR[(L/1A-1B-1C-1D/2A-2B-2C/3A-3B-3C-3D)]3'UTR-poly(A), where, "[]" indicate the open reading frame (ORF) and "/" shows primary cleavage sites leads to the three polypeptides (P1, P2, P3) and "-" is the final processing sites (Agol, 2002; Paul, 2002; Tuthill et al., 2010a, b; Wimmer and Paul, 2010). The multiple copies of a protein if present is numbered as 2A1, 2A2, 2A3, etc., certain proteins are absent in all members of the genus then the respective genome region is indicated by.

"()". PiVs contains orthologous capsid proteins (1B, 1C, 1D), helicase (2C), chymotrypsin-like cysteine proteinase (3C) and RNA dependent RNA polymerase (3D) (Agol, 2002; Paul, 2002; Tuthill et al., 2010a, b; Wimmer and Paul, 2010). Some proteins (i.e., 1A, 2B, 3A, 3B) which are present in PiVs shows divergence and are not similar but, however, their functions are similar. The processed proteins present in PiVs are presented in Table 4.

18 out of 35 PiVs possess highly modified L proteins and they only show their function for genera Aphthovirus, Cardiovirus and Erbovirus. The L-protein of Aphthovirus and Erbovirus is papain-like cysteine proteinase that releases after breaking from C-terminus from nascent polyprotein (Guarne et al., 1998). In addition to this L_{pro} of FMDV is involved in the breakage of eukaryotic elongation factor 4G which leads to the shutoff of the translation process of the host cell (Devaney et al., 1988; Kirchweiger et al., 1994). The L proteins of the cardiovirus inhibit the stress granules and nucleocytoplasmic transport (Bardina et al., 2009; Porter et al., 2010; Borghese and Michiels, 2011). However, the function of L protein is uncertain. There are different types of 2A that exist which release the P1 polypeptide by proteolytic cleavage (2A_{pro} of Enterovirus, Sapelovirus and Rabovirus) or by the translation elongation arrest followed by the re-initiation of translation at the next in-frame codon (2A_{npgp} of Aphthovirus, Aquamavirus, Avihepatovirus, Avisivirus, Cardiovirus, Cosavirus, Erbovirus, Hunnivirus, Kunsagivirus, Limnipivirus, Mischivirus, Mosavirus, Parechovirus B-D, Pasivirus, Potamipivirus, Rosavirus, Senecavirus, Teschovirus and Torchivirus, etc.) (Donnelly et al., 2001).

Table 4 Proteins present in PiVs and their functions (Zell, 2018).

Protein	Type	Function
L, L ^{pro}	Leader protein	Often its function is unknown and L ^{pro} (papain-like proteinase) release itself from polyprotein and targets host proteins
1A (VP4)	Capsid protein	Present inside on the surface of capsid and short like <i>Hepatovirus</i>
1B (VP2)	Capsid protein	It is like jellyroll fold (8 stranded β -barrel) and safe drug binding site
1AB (VP0)	Capsid protein	Precursor of 1A and 1B, remain uncleaved in various genera <i>Ampivirus</i> , <i>Aquamavirus</i> , <i>Avihepatovirus</i> , <i>Avisivirus</i> , <i>Gallivirus</i> , <i>Kobuvirus</i> , <i>Kunsagivirus</i> , <i>Limnipivirus</i> , <i>Megrivirus</i> , <i>Mischivirus</i> , <i>Oscivirus</i> , <i>Parechovirus</i> , <i>Pasivirus</i> , <i>Passerivirus</i> , <i>Potamipivirus</i> , <i>Rosavirus</i> , <i>Sakobuvirus</i> , <i>Salivirus</i> , and <i>Sicinivirus</i> , etc.
1C (VP3)	Capsid protein	It is like jellyroll fold (8 stranded β -barrel) and safe drug binding site
1D (VP1)	Capsid protein	It is like jellyroll fold (8 stranded β -barrel) and safe drug binding site
2A ^{pro}		It is like Chymotrypsin-like cysteine proteinase present in <i>Enterovirus</i> , <i>Sapelovirus</i> and <i>Rabovirus</i> and cleaves the polyprotein at its N-terminus and targets various host proteins
2A ^{NPP}		It initiates cotranslational processing through translation elongation arrest and mediates at the next in-frame codon in member of the genera i.e., <i>Aphthovirus</i> , <i>Aquamavirus</i> , <i>Avihepatovirus</i> , <i>Avisivirus</i> , <i>Cardiovirus</i> , <i>Cosavirus</i> , <i>Erbovirus</i> , <i>Hunnivirus</i> , <i>Kunsagivirus</i> , <i>Limnipivirus</i> , <i>Mischivirus</i> , <i>Mosavirus</i> , <i>Parechovirus B, C, D</i> , <i>Pasivirus</i> , <i>Potamipivirus</i> , <i>Rosavirus</i> , <i>Senecavirus</i> , <i>Teschovirus</i> , and <i>Torchivirus</i> , etc. The length varies from 17 to >150 amino acids and in single genome 6 genes with NPGP present
2A ^{H-box/NC}		2A protein bind with the H-box/NC proteins founds in the H-rev 107 proteins and nonstructural protein of caliciviruses in the members of genera <i>Avihepatovirus</i> , <i>Avisivirus</i> , <i>Gallivirus</i> , <i>Kobuvirus</i> , <i>Megrivirus</i> , <i>Parechovirus</i> , <i>Passerivirus</i> , <i>Potamipivirus</i> , <i>Sakobuvirus</i> , <i>Salivirus</i> , <i>Sicinivirus</i> and <i>Tremovirus</i> and function is unclear
2A ^{NTPase}		2A protein having similarity to AIG1-type guanine-binding domain (P-loop NTPase with GxxGxGKS) and found in the members of genera <i>Avihepatovirus</i> and <i>Avisivirus</i> and function is unclear
2A		2A protein without any similarity with known proteins and the function is unknown. It is found in the genera <i>Ampivirus</i> , <i>Aquamavirus</i> , <i>Dicipivirus</i> , <i>Harkavirus</i> , <i>Hepatovirus</i> , <i>Kunsagivirus</i> , <i>Megrivirus</i> , <i>Oscivirus</i> and <i>Pasivirus</i>
2B		It act as a plasma membrane permeability enhancer (viroporin) and inhibition of host secretory pathways
2C ^{hel}	Helicase protein	P-loop NTPase with GxxGxKS design associated with membrane
2BC	Membrane-anchored protein	It is precursor of 2B and 2C and involved in the formation of replication organelle
3A	Key protein	It is involved in membrane remodeling and all 3A proteins exhibit a C-terminal hydrophobic domain associated membrane
3B (VPg)	Viral peptide	It is associated with tyrosine-3 residue
3C ^{pro}	Chymotrypsin-cysteine proteinase	It is design with GxCG and involved in polyprotein processing, target numerous host proteins, interacts with a cloverleaf-like structure at the 5' end of the positive strand RNA of <i>coxsackievirus</i> and <i>rhinovirus</i>
3D ^{pol}		RNA dependent with modify with KDE, PSG, YGDD and FLKR
3CD ^{pro}		It is precursor of 3C and 3D, PV 3CD _{pro} initiates different processing steps and interacts with cloverleaf-like structure at the end of 5' end of the positive-strand RNA

There is a third type of 2A, 2A^{H-box/NC} which has similarity with the H-rev107 family proteins and the N-terminus of the viral polyprotein (pfam08405) of caliciviruses (Hughes and Stanway, 2000) but its function in the life cycle of the virus is obscure. This 2A^{H-box/NC} is found in the genera *Avihepatovirus*, *Avisivirus*, *Gallivirus*, *Kobuvirus*, *Megrivirus*, *Parechovirus*, *Passerivirus*, *Potamipivirus*, *Sakobuvirus*, *Salivirus*, *Sicinivirus* and *Tremovirus*, etc. *Avihepatovirus* and *Avisivirus* possess the fourth type of 2A and this protein 2ANTPase is similar to the AIG1 (avrRpt2-induced gene 1)-type guanine binding domain found in P-loop NTPases and binds with GxxGxKS (Ding and Zhang, 2007).

PiVs coding region is beside with 5' and 3' nontranslated regions which lead to the formation RNA structures and these RNA structures initiates cap independent translation (Lozano et al., 2016) for Enterovirus synthesis of negative- and positive strand of RNA (Andino et al., 1990). There are five different types of internal ribosome entry sites (IRESs) which engage ribosomes and other host factor to initiate the translation process in PiVs (Jan et al., 2016). Different types of IRES in their respective PiVs genera are given in Table 5. The only member of the genus *Dicipivirus* has two IRESs and unknown IRES for ampiviruses, passeriviruses, potamipiruses and torchiviruses.

Table 5 Type of IRES present in different genera of PiVs.

Type of IRES	Genera of PiVs
I	Enterovirus and Harkavirus
II	Avisivirus, Cardiovirus, Cosavirus, Erbovirus, Gallivirus, Hunnivirus, Mischivirus, Mosavirus, Parechovirus, Rabovirus, Rosavirus, and Sicinivirus
III	Hepatovirus
IV	Aquamavirus, Avihepatovirus, Kunsagivirus, Limnipivirus, Megrivirus, Pasivirus, Sakobuvirus, Sapelovirus, Senecavirus, Teschovirus and Tremovirus
V	Kobuvirus, Oscivirus and Salivirus

Life cycle of PiVs

There is a genetic and phenotypic difference between the family of *Picornaviridae* but all the group members use the same steps for genome replication and production of virus in the host cell favorable environments (Andino et al., 1999; Lin et al., 2009; Jiang et al., 2014). PiV RNA due to positive-sense RNA genome instantly translated into polyprotein when entered into the cell cytoplasm. This does not require the incorporation of viral proteins i.e., polymerase (present inside capsid) (Smyth and Martin, 2002). All PiVs hinder the cap dependent translation of host cell mRNAs and utilized cap dependent translation mechanisms for genome translation by the reduction of the competition of translation factors in between virus and host cell mRNAs. Different steps involved in the life cycle and replication of PiVs are described in the following sections.

Gene expression

The genomes of PiV are encapsulated into icosahedral virion structures which consist of 60 protomers that are formed by the structural proteins in the coding region of P1 (VP1, VP2, VP3, VP4). VP1, VP2 and VP3 (single copy) are arranged into triangular subunit on the surface of the capsid (Bedard and Semler, 2004; Wimmer and Paul, 2010). These subunits arranged themselves on a fivefold symmetrical axis which results in the formation of pentameric protomer. Total 12 of these pentameric subunits are involved in the formation of icosahedrons with two and threefold axes symmetry that is located at the connection points (Agol, 2002; Ehrenfeld et al., 2010). However, VP4 protein is not found on the exterior of the virion and it is located inside of the capsid structure that interacts with the encapsidated RNA genome. The assembling occurs through the incorporation of the RNA genome coincides with the processing of precursor polypeptide, VP0, into VP2 and VP4. The actual phenomena of the maturation of VP0 to form VP2 and VP4 is unknown but whereas the viral proteases 2A, L and 3C/3D were not found to be involved. Therefore, for the maturation of virion the cellular factor is required or the cleavage is affected by RNA/capsid proteins (Agol, 2002; Ehrenfeld et al., 2010).

VP1, VP2 and VP3 (capsid proteins) of *Enteroviruses* and *Rhinoviruses* move from the surface of capsid structure to form mesa at five and threefold axes at the propeller. X-ray crystallographic studies indicate that there is the impression of a cleft in between axes of symmetry which is termed as canyons and these canyons regions were found to be the site of cell receptors for virion binding before entering into the cytoplasm. The cryoelectron micrograph studies have been confirmed this hypothesis that cell receptors are bind to Rhinovirus and Cocksackievirus virion particles (Lawson and Semler, 1990; Belsham, 2009; Jiang et al., 2014). However, in the case of *Cardioviruses* and *Aphthviruses* the canyon regions are not pronounced as compared to that of *Enteroviruses* and *Rhinoviruses*. The neutralization studies investigations show that the virion capsid of the FMDV indicates that in VP1 capsid protein G-H loop is mandatory for the recognition of receptor. The mechanism of entry of virion RNA of the PV is not fully known, however, it may occur through the modification of receptor mediated endocytosis. The acidification of the endosome is not necessary because the release of the viral RNA is not pH dependent (Lawson and Semler, 1990; Belsham, 2009; Jiang et al., 2014).

Translation of RNA genome

The uncoated virus and the released RNA in the cytoplasm of the host cell resulting in the translation of positive strand RNA. The entered viral mRNA is comprised of cis-acting RNA sequence and the structure is different from the mRNA of the host cell (Jackson et al., 1990; Bedard and Semler, 2004; Chase and Semler, 2012). The RNA processing occurs when in the nucleus 7-methyl guanosine cap is attached to the nascent transcript. The transcript is protected from the degradation via recognizing of the cap structure by the complex eIF-4F which serves as a scaffold for the assembling of initiation factors and 40S ribosomal subunit. Genomes of PiVs do not contain anything that is resembled the cap structure and the 5' end of the RNA is covalently linked with the VPg a viral protein and this protein has not been found to play any role in the translation of the polyprotein (Jackson et al., 1990; Bedard and Semler, 2004; Chase and Semler, 2012).

The translation process of PiV positive strand RNA does not obey the conventional scanning model of the ordinary mRNAs (Lin et al., 2009; Daijogo and Semler, 2011). The analysis of the sequence of PiVs and PV shows that 5'-noncoding region (5'-NCR) consists of disparate AUG start codons which is favorable for the initiation for the process of translation (Jackson et al., 1994; Andino et al., 1999). The biochemical analysis of the 5' NCR shows that the viral RNA consists of several secondary structures of RNA which thermodynamic stability hinders the 40S ribosomal subunit to start the codon. Studies indicate that the IRES is used for the translation process of PV RNA genome and all PiVs consist of IRES in 5' NCR (Meerovitch and Sonenberg, 1993; Martínez-Salas, 2008).

Processing of polyprotein

The identification and initiation of the ribosome in PiV IRES lead to the translation of the polyprotein of 250 kDa that is encoded by the single ORF by PiV genomes. Proteinases L or 2A, 3C and 3CD are involved in the proteolytic cleavage of the viral polyprotein (Meerovitch and Sonenberg, 1993; Martínez-Salas, 2008). The processing of viral protein resulting in the formation of precursor molecules P1, P2 and P3 and P1 zone of the polyprotein which is then further processed to yield 3 or 4 proteins. These proteins are the structural components of viral capsid and in *Cardioviruses*, *Aphthoviruses*, *Erboviruses*, *Teschoviruses* and *Kobuviruses* (Kräusslich et al., 1988; Arnold et al., 1987; Leong et al., 2002; Jiang et al., 2014). The P2 precursor (proteolytically processed) is composed of proteins that are mandatory for remodeling of cytoplasmic membranes and vesicles used in the replication of the virus and for the stopping of translation of cell and viral RNA replication. P3 is processed for the production of important proteins for the replication of RNA i.e., RNA dependent RNA polymerase 3D^{Pol} and the viral proteinases (3C, 3D) (Leong et al., 2002; Jiang et al., 2014). These viral proteinases played an important role in the events of viral polyprotein breakage. The synthesis of polyprotein is instantly stopped with the synthesis of the P3 polyprotein on the carboxy terminus of the 3D^{Pol} sequence.

Translation shut off and protein cleavage

The role of 2A (Enteroviruses, Rhinoviruses) and L (Aphthoviruses) in the maturation of viral proteins also possesses a role in the translation activity of the host cell by breaking factors that are involved in the cap dependent translation initiation. These proteinases inhibit the cell protein synthesis by the breakage of eIF-4G component of eIF-4F initiation factor complex which results in the inability of the amino-terminal domain of eIF-4G to recognize and bind with eIF-4E which is linked with 7-methyl cap structure on 5' end of cellular mRNAs. The protein synthesis in Enterovirus and Rhinovirus is enhanced in response to the increased presence of ribosomes that are available for internal ribosome entry. An alternate method used by encephalomyocarditis virus (EMCV) to hinder the translation of host cell by the activation of a cellular translational repressor (4E-BP1). This translational repressor binds cellular eIF-4E and which in turn hinders the host cap-dependent translation.

Replication of viral RNA

PiVs replication utilizes the viral encoded RNA dependent RNA polymerase for the synthesis of RNA from the viral RNA template. The genomic RNA of PiV is released from the capsid into the cytoplasm without replication proteins (Steil and Barton, 2009a, b; Ogram and Flanagan, 2011; Zell et al., 2017; Zell, 2018). The replication of the viral genome initially followed the translation of its coding region for the generation of 3D^{Pol} and other viral replication proteins. Protein VPg as a primer is used by polymerase to induce RNA synthesis by the addition of two uridylyl residues to form VPg-pU-pU. The negative strand RNA is present in the duplex along with the positive sense RNA template. Double stranded RNA is known as replicative form (RF), whereas, negative strand RNA acts as a template for the synthesis of positive strand RNAs. The positive to negative strand ratio of RNA in the infectious cell is 50:1 which suggests that a single negative strand RNA intermediate is used as a template for the formation of multiple positive strand RNAs. Viral positive sense RNA which is newly synthesized is translated to form additional viral proteins that are used as additional template RNA for the production of negative strand RNA or encapsulated inside the virions to infect the host cell (Chase and Semler, 2012; Zell et al., 2017; Zell, 2018).

Along with IRES, PiVs 5' NCRs consist of other stable RNA secondary structural elements which are important for positive- and negative strand RNAs. PiVs such as Aphthoviruses and Cardioviruses contains RNA structure on their 5' terminal and termed as stem loop A that is analogous in function, however, they are not similar in the structural conformation (Ehrenfeld et al., 2010; Langereis et al., 2014; Flather and Semler, 2015). PiVs replication started when they entered the host cell by receptor-mediated endocytosis. The different mechanism has been reported which leads to the formation of endosome which is followed by the alteration in the structure of the capsid and the penetration of viral RNA in the membrane (Tuthill et al., 2010a, b; Siltz et al., 2014). Viral RNA is translated in the cytoplasm which results in the formation of one of the major polyprotein which is then co- and posttranslationally leads to the formation of L protein, capsid proteins (1AB, 1C, 1D), mature nonstructural proteins and some stable intermediates (2BC, 3AB, 3CD) (Fig. 1). RNA dependent RNA polymerase and nonstructural proteins are important for the replication of viral RNA (Baltimore et al., 1963; Tuthill et al., 2010a, b; Siltz et al., 2014). These proteins modify the environment of the cell and initiate the synthesis of negative strand RNA molecules that act as a template for the production of positive strand RNA. Uridylation at cis-replicative elements of VPg occurs and VPg-pU-pU acts as a primer for the synthesis of both positive- and negative-strand RNA (Paul and Wimmer, 2015). The synthesis of viral RNA takes place at a multimeric replication complex using different nonstructural proteins, their precursors and host factors. The components of replication are gathered on the surface of the viral replication organelle (RO). Viral RNA is protected from host nucleases and cytosolic defense mechanisms of the innate immunity by RO (Belov, 2016; Arakawa and Morita, 2019). Membrane associated viral proteins (2B, 2C, 3A) are responsible for the generation of RO which is resulted from the virus induced rearrangement of intracellular membranes. Golgi components and autophagic mechanisms are involved in membrane rearrangement. These ROs are highly dynamic structures that consist of a network of single-membrane vesicles that leads to the formation of double membrane and at later stages lead to the formation of multilamellar agglomerations (Belov, 2016; Arakawa and Morita, 2019). PiVs employ different mechanisms to collect sterols, glycerophospholipids and sphingolipids at ROs. The collection of sterols occurs with the help of 3A protein, phosphatidylinositol, 4-kinases (PI4Ks), phosphatidylinositol 4-phosphate (PI4P) and oxysterol-binding protein (OSBP) as similar for Enteroviruses, Aichivirus, EMCV and Saffold virus. Other strains of PiVs are independent of OSBP (i.e., Hepatitis A virus, human parechovirus, etc.). Virus induced membrane contact sites (VMCSs) result from the endoplasmic reticulum to viral membranes which then in turn regulate the lipid composition of RO membranes which is important for the various functions (Nagy et al., 2016; van der Schaar et al., 2016; Strating and van Kuppeveld, 2017).

Synthesis of positive and negative strand RNA

As stated previously the synthesis of viral RNA occurs by the process of uridylylation of VPg through the viral polymerase 3D^{Pol} and also there is some involvement of other viral proteins (i.e., 3CD) and precursor (i.e., 3AB) (Banerjee and Dasgupta, 2001; Ogram and Flanagan, 2011). The uridylation of VPg leads to the formation of VPg-pU-pU and 3D^{Pol} which forms negative strand RNA which is complementary to the genomic positive sense RNA. Double stranded RNA intermediate (RI) acts as a template for the initiation of the different processes via RNA polymerase for the synthesis of positive strand RNAs (Barton and Flanagan, 1997; Morasco et al., 2003). The negative strand RNA is observed in the infected cell in duplex with positive strand RNA or with various elongating positive strand RNAs present in a double stranded RNA complex which is known as replicative intermediate (RI). The positive strand RNA synthesis is similar to negative strand RNA synthesis which requires uridylylation of VPg and in vitro studies shows that in the synthesis of positive strand RNAs uridylylation of VPg was found to be more effective by utilizing cre RNA hairpin

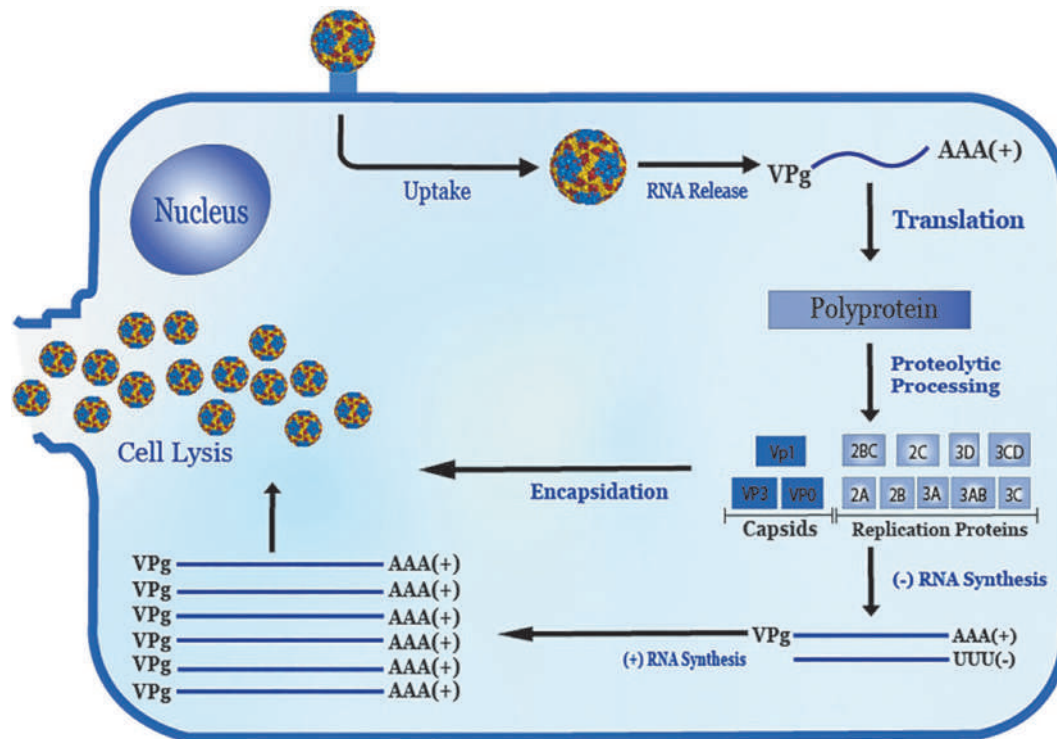


Fig. 2 The illustration of picornaviruses (PiVs) replication cycle from the invasion of virus in the host cell to the lysis of cell and release of PiVs.

which is the source of template (Steil and Barton, 2009a, b; Flather and Semler, 2015). In the synthesis of positive RNA strand, negative strand template are used to initiate multiple positive strand and cre will act as the site of uridylylation which is necessary for the addition of VPg-pU-pU in positive strand RNAs (Steil and Barton, 2009a, b; Flather and Semler, 2015). The secondary structures in 5' and 3' ends of positive- and negative strand RNAs, respectively, destabilizes the base pairing of RNAs to permit 3D^{Pol} to enter the negative strand RNA template. It has been found that 3D^{Pol} of PV possesses duplex unwinding activity and PV 2C protein consists of NTPases activity and similarity in structure of RNA helicases. 3D^{Pol} and 2C formed a complex which simultaneously helps in the unwinding and chain elongation of the RNA (Herold and Andino, 2000; Teterina et al., 2001; Belsham and Normann, 2008). Cellular proteins initiate the RNA replication and virus translation and they have also been found to interact with the intermediates of negative strand RNA. It has also been found that cellular protein hnRNPC could also interact with the secondary structures of RNA that are formed by the 3' end of PV negative strand RNA and also with 3CD and 2C. It may also be speculated that these hnRNPC complexes and PV replication proteins initiate the positive-strand RNA synthesis (Andino et al., 1999; Lyons et al., 2001; Lin et al., 2009). The formed PiVs particles are released through the process of lysis whereas, for Hepatitis A virus, PV and coxsackievirus B₃ an alternative nonlytic mechanism of viral spread has been reported (Feng et al., 2013; Bird et al., 2014; Robinson et al., 2014; Chen et al., 2015). The schematic illustration of the replication cycle of PiVs is given in Fig. 2.

Factors involved in life cycle

Heat shock proteins

Heat shock proteins (HSP) are of two types HSP 70 and 90 and helps in the folding and unfolding of the proteins. In elevated temperatures and other stress conditions, the expression level is increased. Macejak and Sarnow (1992) explain for the first time that there is the interaction between P1 capsid precursor and HSP 70. Geldanamycin (inhibitor of HSP 90) has been used to evaluate the involvement of cellular chaperone HSP 90 for the inhibition of PV growth in culture cells (Geller et al., 2007a, b). The study shows that HSP 70 delivers protein to the HSP 90 in the partially folded state and it has been suggested that HSP 90 helps in the folding of PVP1 capsid precursor to maintain processing-competent conformation and also helps in the inhibition of proteasomal degradation (Pratt and Toft, 2003; Riggs et al., 2004; Geller et al., 2007a, b). The study has also indicated that HSP 90 played a role in FMDV replication which enhances the production of pentamers from protomer (Newman et al., 2014).

Myristic acid (MA)

It is also known as tetradecanoic acid which is saturated fatty acid and cross-linked with N terminal of the P1 precursor (Chow et al., 1987; Paul et al., 1987; Chow and Moscufo, 1995). Myristic acid (MA) helps in the particle assembly in the earlier and later stage of

the process (Marc et al., 1990; Moscufo et al., 1991) and myristoylation involves two functions in the assembly of particles. In the first step, myristoylated VP0 targets the P1 for the membranous replication complex and whereas in the second step myristic moiety initiates the protomer-protomer interaction of pentamer (Ansardi et al., 1992; Lee and Chow, 1992; Martin-Belmonte et al., 2000). The same functions were found to be pentamer formation of FMDV through myristoylation and during virus maturation VP0 myristoylation occurs in the later stage of assembly (Marc et al., 1990; Moscufo et al., 1991; Ansardi et al., 1992; Goodwin et al., 2009).

Glutathione

Chemically glutathione (GSH) is L-glutamyl-L-cysteinylglycine which is an important reducing agent present in human and animal cells. GSH helps in the maintenance of the redox potential of the cell which is important for immune function. It also possesses an extra role in the process of signal transduction, apoptosis, gene expression and the regulation of protein function (Pompella et al., 2003). The biological activity of the cells is due to the thiol group present in GSH and it exists in reduced (GSH) and oxidized (glutathione disulfide, GSSG) state. The synthesis of GSH involves two steps; the first step involves the linking of glutamate and cysteine which forms dipeptide with the help of the enzyme glutamylcysteine synthase. In the second step, glutathione synthase helps in the catalysis of the addition of glycine in the dipeptide. It has been found that L-buthionine sulfoximine (BSO) is the strong inhibitor of glutamylcysteine synthase and the biosynthesis of GSH (Meister et al., 1979). The inhibition of GSH biosynthesis by BSO hinders the growth of Enteroviruses in morphogenesis and is found to be ineffective on the translation or genome replication (Smith and Dawson, 2006; Ma et al., 2014; Jiang et al., 2014).

Cellular membranes

Positive strand RNA in the virus life cycle in eukaryotic cells is responsible for the rearrangement of the cellular membranes which leads to the formation of specific structures that are associated with replication and encapsidation. Various cellular pathways including secretory pathway, autophagy and lipid biosynthesis were found to be involved in the formation of replication organelles (Kirkegaard and Jackson, 2005; Belov et al., 2008; Hsu et al., 2010; Belov and van Kuppeveld, 2012; Jiang et al., 2014). Tubular structures help in the RNA replication and these replication proteins are present locally and on external surfaces of the membrane facing cytoplasm (Bienz et al., 1992; Belov et al., 2008). It has been found that in early infection the tubular structures possess a single membrane whereas in later stage it converted into double membrane vesicles (Belov et al., 2008; Limpens et al., 2011; Belov and van Kuppeveld, 2012; Jiang et al., 2014). It has been found that the blockage of autophagosome formation resulting in the reduction of RNA replication and whereas acidification is necessary for the final maturation cleavage of the virus (Richards and Jackson, 2012, 2013; Jiang et al., 2014).

Inhibitors of life cycle

Hydantoin

Hydantoin ([5(3,4-dichlorophenyl) methylhydantoin]) (HD) is an organic compound that hinders the life cycle by inhibiting the maturation of 110S intermediate (Vance et al., 1997; Jiang et al., 2014). HD effect is reversible due to 110S particles that are chased in mature virions with the removal of the drug. PiVs are resistant to HD and were isolated with the mutations mapped on the 2C^{ATPase} protein. The presence of HD in cell free translation/RNA replication inhibits the protein processing but no 110S particles were observed (Molla et al., 1991; Verlinden et al., 2000; Jiang et al., 2014).

Guanidine hydrochloride

The viral RNA synthesis and encapsidation are found to be reversibly hinder by guanidine hydrochloride (GnHCl) at a concentration where the cellular macromolecular synthesis would not be affected. It has been found that when GnHCl is added to cells halfway via PiVs infection the RNA is trapped in 80S particles known as guanidons (Baltimore, 1969; Jiang et al., 2014). When a drug is removed 80S particles were found to be decreased with an increase in the concentration of provirions and virions. It has also been found that GnHCl inhibits the ATPase effect of 2C^{ATPase} (Pfister and Wimmer, 1999; Jiang et al., 2014).

L-Buthionine sulfoximide

BSO hinders glutamylcysteine synthase in the biosynthesis of glutathione which is a cellular reducing agent (Meister et al., 1979; Smith and Dawson, 2006; Jiang et al., 2014). GSH inhibition by the treatment of BSO has no notable effect on the RNA replication or protein translation of Enteroviruses. BSO has been found to hinder the assembling of 14S particles that affects the formation of mature virus in infected cells. The pulldown assay of GSH shows that it interacts with 14S, 75S and mature virus particles. In the drug resistant mutants, the mutations in VP1 and VP3 has been found at protomer-protomer interfaces that occur during passaging indicating the role of GSH in the life cycle of Enterovirus which stabilizes the pentamers and virus particles (Meister et al., 1979; Smith and Dawson, 2006; Jiang et al., 2014).

Py-11

Py-11 (2-amino-4,6-dichloropyrimidine) is nucleoside analog potent inhibitor of PiVs life cycle during the process of cleavage of capsid precursor P1 which prevents the formation of pentamers through capsid proteins (La Colla et al., 1981; Jiang et al., 2014).

Geldanamycin

Geldanamycin (GM) is benzoquinone ansamycin antibiotic discovered in *Streptomyces hygroscopicus* that binds with ATP/ADP binding pocket of HSP 90 that hinder its function (Schulte et al., 1998; He et al., 2006). A cellular chaperone (HSP 90) plays an important role in the folding and function of protein kinases, steroid hormone receptors and various cellular proteins that regulates the cell cycle and apoptosis. Geller et al. (2007a, b) found GM which inhibits replication of three different PiVs such as PV, HRV and *Coxsackievirus*. It has been found that GM affects the inhibition of HSP 90 aided folding and viral capsid precursor (P1). This folding is necessary for the processing of P1 through the proteinase 3C^{DPro} (Jiang et al., 2014).

Taxonomy of PiVs

Species of PiVs contain different strains of viruses that are related to each other phylogenetically and their proteins are significantly homologous to each other. The genome maps of these viruses are identical to each other and assume to be compatible in the replication, encapsidation, proteolytic processing and genetic recombination. PiVs species are considerably homologs of the orthologous proteins P1 (divergence <0.66), 2C^{hel}, 3C^{Pro} and 3D^{pol} (divergence <0.64) (Zell et al., 2017; Zell, 2018). Therefore, this confirms the International Code of Virus Classification and Nomenclature (ICVCN) rule that a virus genus contains a group species that share some common characters. There are many PiVs genera which show different distinctive features in their genome maps and indicate the difference in the orthologous proteins (Knowles et al., 2008, 2010). Whereas other remaining proteins (i.e., L, 2A, 2B, 3A, 3B) do not shows any detectable similarities. The *Picornaviridae* Study Group suggests that only orthologous genomes regions of PiVs should be compared. It has been found that the amino acid shows a divergence in a genus in the range of 0.40–0.6 depending on the type of protein (Knowles et al., 2008, 2010). However, in intergenic comparison, the divergence is greater than 0.65. In the species where sequence variation is present VP1 gene region would be used to define genetic types. In the case of *Enterovirus*, it has been found that the genetic types represent antigenic serotypes (Oberste et al., 1999; McIntyre et al., 2013). However, in the case of Enterovirus A, B, C the nucleotide sequence divergence is 0.25 whereas the nucleotide divergence threshold of Rhinovirus A, B, C is 0.12–0.13 (Oberste et al., 1999; McIntyre et al., 2013).

The binomial nomenclature of virus names has been used in which *picornavirus* species is composed of the genus name and adjacent capital letters A, B or C [i.e., Ampivirus (genus) and Ampivirus A (species) (Knowles et al., 2008, 2010)]. There are exceptions Dicipivirus (genus), Cadivirus A (species), Enterovirus (genus) and Enterovirus A to J (species), *Rhinovirus* (genus) *Rhinovirus A* to C and *Megivirus* (genus) and Megivirus A (species). However, Aphthovirus is the only PiVs genus that does not confirm International Committee on Taxonomy of Viruses (ICTV) nomenclature rules because the names of four species were derived from the host species or the diseases caused by them (foot and Mouth diseases virus, Equine rhinitis A virus, Bovine rhinitis A virus, Bovine rhinitis B virus) (Knowles et al., 2008, 2010).

With the invention of next generation sequencing techniques, the novel PiVs have been identified in metagenomics studies as compared to that of classical virus isolation methods and subsequent experimental characterization (Knowles et al., 2008, 2010). The typical composition of PiVs including the presence of IRES, three capsid protein domains with drug-binding sequence, helicase with NTP-binding site (GxxGxGKS/T), VPg with a tyrosine three residue, chymotrypsin like proteinase with GxCGx_{10–18}GxH and RNA dependent RNA polymerase with KDE, PSG, YGDD and FLKR could be reliably used to identify based on their sequences when sometimes some of the features are absent. Also, bioinformation techniques also are helpful to identify the new PiVs and metagenomics studies investigate the intestinal viromes of humans and animals which identify various viruses which are of dietary origin (Zhang et al., 2005; Li et al., 2010a, b, c). The nucleotide composition analysis (NCA) or demonstration of characteristic sequence patterns may be helpful to provide the evidence of the identity of PiVs host, however, NCA provides the distinction between the mammal, insect, plant hosts, discrimination of mammal avian and fish viruses (Lange et al., 2014; Yinda et al., 2017).

The advancement of sequencing techniques increases the identification of PiVs which disclose the unexpected degree of diversity. In 2017 total of 35 genera and 80 species of PiVs has been approved by ICTV and more than 500 types are distinguished genetically or by serological techniques. The different genus of PiVs and their characteristics (i.e., genome length and layout) are reported in Table 6 and the types of PiVs viruses with genus are given in Table 7.

Biochemical and physical properties of PiVs

PiVs genus and its species are quite diverse but these species show some common features of structure and gene expression. Virions are of spheroidal shape particles with 22–30 nm of diameter and there is a lacking of an external lipid coat/envelop (Fig. 3). The diameter of wet or hydrated virus particles was found to be 30 nm through nondestructive methods (Rueckert, 1985). The internal core of the virion is surrounded by a protein shell or capsid and X-ray crystallographic studies confirmed that virions show icosahedral symmetry with the exact position of virus proteins. The composition of capsid protein structure is 60 subunits or protomers arranging themselves around axes of five-, three- and twofold symmetry. PiVs genome is a single strand RNA molecule containing 7400 nucleotides and the size may vary from 7389 to 7441 nucleotides for the different strains of PV and coxsackievirus (Kitamura et al., 1981; Racaniello and Baltimore, 1981a, b; Nomoto et al., 1982; Stanway et al., 1984; Toyoda et al., 1984; La Monica et al., 1986; Iizuka et al., 1987; Jenkins et al., 1987; Lindberg et al., 1987; Pevear et al., 1990). The molecular weight of RNA is 2.58×10^6 Da and all PiVs RNAs contain small basic protein (VPg) which is covalently attached to the 5' end of the genome and polyadenylated tail with a variable length 3' end. The percent weight of virion consists of RNA and protein coat around 30% and

Table 6 Types of PiVs Genus and there characteristics.

<i>Genus</i>	<i>Number of species</i>	<i>Name of species</i>	<i>Genome layout</i>	<i>Genome length</i>
Ampivirus	1	Ampivirus A	VPg + 5'UTRIRES-[1AB-1C-1D/2A-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	9246 nt (5'-UTR: 970 nt; ORF: 8142 nt; 3'-UTR: 134 nt), length of polyprotein: 2713 aa
Aphthovirus	4	Bovine rhinitis A B, Equine rhinitis A virus, Foot-and-mouth disease virus	VPg + 5'UTRIRES-II[Lpro/1A-1B-1C-1D-2Anpgp/2B-2C/3A-3B-(3B2-3B3)-3C-3D]3'UTR-poly(A)	7250–8203 nt (5'-UTR: up to 1112 nt with poly(C) tract; ORF: 6657–7020 nt; 3'-UTR: 32–110 nt); length of polyprotein: 2218–2339 aa
Aquamavirus	1	Aquamavirus A	VPg + 5'UTRIRES-IV[1AB-1C-1D-2A1npgp/2A2-2B-2C/3A-3B1-3B2-3C-3D]3'UTR-poly(A)	c. 6700 nt (5'-UTR: 506 nt, ORF: 6154 nt, 3'-UTR: 34 nt); length of polyprotein: 2050 aa
Avihepatovirus	1	Vihepatovirus A	VPg + 5'UTRIRES-IV[1AB-1C-1D 2A1npgp/2A2NTPa s e-2A3H-box/NC-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	Up to 7792 nt (5'-UTR: 625–655 nt, ORF: 6750–6756 nt, 3'-UTR: c. 318 nt); length of polyprotein: 2249–2251 aa
Avisivirus	3	Avisivirus A, B, C	VPg + 5'UTRIRES-II[1AB-1C-1D-(2A 1 n p g p)/2 A 2 n p g p/2 A 3 N T P a s e- 2 A 4 H-box/NC-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	>7167–7532 nt (5'-UTR: > 457–609; ORF: 6441–6720; 3'-UTR: 194–269 nt); length of polyprotein: 2146–2239 aa
Cardiovirus	3	Cardiovirus A, B, C	VPg + 5'UTRIRES-II[L/1A-1B-1C-1D-2Anpgp/2B-2C/3A-3B-3C-3D]3'UTR-poly(A) Despite a significant similarity, the 2A protein of Cardiovirus C lacks the NPGP motif	c. 7730–8530 nt (5'-UTR: up to 1451 nt with poly(C) tract, ORF: 6879–6924 nt, 3'-UTR: 121–144 nt); length of polyprotein: 2293–2308 aa
Cosavirus	5	Cosavirus A,B,C,D,E,F	VPg + 5'UTRIRES-II[1A-1B-1C-1D-2Anpgp/2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	c. 7632–7802 nt (5'-UTR: up to 1361 nt; ORF: 6366–6402 nt; 3'-UTR: 75–93 nt); length of polyprotein: 2113–2133 aa
Dicipivirus	1	Cadicivirus A	VPg + 5'UTRIRES-II[1A-1B-1C-1D]-IGRIRES[2A-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	c. 8785 nt (5'-UTR: at least 982 nt; ORF1: 2535 nt; IGR: 588 nt; ORF2: 4221 nt; 3'-UTR: 549 nt); lengths of polyproteins: 845 aa (P1), 1407 aa (P2-P3)
Enterovirus	13	Enterovirus A, B, C, D, E, F, G, H, I, J, Rhinovirus A, B, C	VPg + 5'UTRIRES-II[1A-1B-1C-1D/2Apro-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	7100–7450 (5'-UTR: 610–822 nt; ORF: 6417–6645 nt; 3'-UTR: 37–99 nt); length of polyprotein: 2138–2214 aa
Erbovirus	1	Erbovirus A	VPg + 5'UTRIRES-II[Lpro/1A-1B-1C-1D-2Anpgp/2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	c. 8828 nt (5'-UTR: up to 905 nt; ORF: 7752–7770 nt; 3'-UTR: 164 nt); length of polyprotein: 2583–2589 aa
Gallivirus	1	Gallivirus A	VPg + 5'UTRIRES-II[L/1AB-1C-1D/2AHbox/NC-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	c. 8500 nt (5'-UTR: 761 nt; ORF: 7425 nt; 3'-UTR: 310 nt); length of polyprotein: 2474 aa
Harkavirus	1	Harkavirus A	VPg + 5'UTRIRES-II[1A-1B-1C-1D/2A-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	8003 nt (5'-UTR: 673 nt; ORF: 7263 nt; 3'-UTR: 67 nt); length of polyprotein: 2420 aa
Hepatovirus	9	Hepatovirus A, B,C, D, E, F, G, H, I	VPg + 5'UTRIRES-III[1A-1B-1C-1D-2A/2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	Up to 7810 nt (5'-UTR: c. >225–773 nt; ORF: 6597–6909, nt; 3'-UTR: 35–257 nt); length of polyprotein: 2199–2303
Hunnivirus	1	Hunnivirus A	VPg + 5'UTRIRES-II[L/1A-1B-1C-1D-2Anpgp/2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	7469–7588 nt (5'-UTR: >600–732 nt; ORF: 7432–6822 nt; 3'-UTR: >14,122 nt); length of polyprotein: 2244–2274 aa
Kobuvirus	6	Aichivirus A, B, C, D, E, F	VPg + 5'UTRIRES-V[L/1AB-1C-1D/2AHbox/NC-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	Up to 8280 nt (5'-UTR: up to 744 nt; ORF: 7299–7467 nt; 3'-UTR: 136–240 nt); length of polyprotein: 2432–2488 aa
Kunsagivirus	1	Kunsagivirus A	VPg + 5'UTRIRES-IV[1AB-1C-1D-2A1npgp/2A2-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	c. 7092–7429 nt (5'-UTR: 393–532 nt; ORF: 6639–6729 nt; 3'-UTR: 25–177 nt); length of polyprotein: 2213–2248 aa
Limnipivirus	3	Limnipivirus A, B, C	VPg + 5'UTRIRES-IV[1AB-1C-1D-2A1npgp/2A2npgp/-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	c. 7700–8050 nt (5'-UTR: > 501–712 nt; ORF: 6810–7011 nt; 3'-UTR: 312363 nt); length of polyprotein: 2270–2337 aa
Megrivirus	1	Melegrivirus A	VPg + 5'UTRIRES-IV[(L)1AB-1C-1D/(2A0-2A1-2A2-)2A3H-box/NC-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	9040–9840 nt (5'-UTR: >461–663 nt; ORF: 8235–8520 nt; 3'-UTR: >175–669 nt); length of polyprotein: 2745–2886 aa
Mischivirus	3	Mischivirus A,B,C	VPg + 5'UTRIRES-II[L/1AB-1C-1D-2Anpgp/2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	c. 8457 nt (5'-UTR: 1407 nt; ORF: 6846 nt; 3'-UTR: 204 nt); length of polyprotein: 2282 aa
Mosavirus	1	Mosavirus A	VPg + 5'UTRIRES-II[L/1A-1B-1C-1D-2Anpgp/2B-2C/3A-3B1-3B2-3C-3D]3'UTR-poly(A)	c. 8385 nt (5'-UTR: >646 nt; ORF: 7653 nt; 3' UTR: 86 nt); length of polyprotein: 2551 aa
Oscivirus	1	Oscivirus A		

Table 6 (Continued)

Genus	Number of species	Name of species	Genome layout	Genome length
Parechovirus	4	Parechovirus A,B,C,D	VPg + 5'UTRIRES-V[L1AB-1C-1D/2A-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	c. 7640–7678 nt (5'-UTR: 636–646 nt; ORF: 6765–6774 nt; 3' UTR: 238–256 nt); length of polyprotein: 2255–2258 aa
Pasivirus	1	Pasivirus A	VPg + 5'UTRIRES-II[L1AB-1C-1D-(2Anpgp)/2AH-box/NC-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	7339–7608 (5'-UTR: 710–730 nt; ORF: 6543–6753; 3'-UTR: 87–111 nt); length of polyprotein: 2180–2250 aa.
Passerivirus	1	Passerivirus A	VPg + 5'UTRIRES[L1AB-1C-1D/2AH-box/NC-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	c. 6938 nt (5'-UTR: c. 420 nt; ORF: c. 6402 nt; 3' UTR: 136 nt); length of polyprotein: 2133 aa
Potamipivirus	1	Potamipivirus A	VPg + 5'UTRIRES[I 1 A B -1C-1D-2 A 1 n p g p/2A 2H-b ox/NC- 2B-2C/3A-3B-3C-3D]3'UTR-poly(A).	c. 8035 nt (partial) (5'-UTR: >415 nt; ORF: 7287 nt; 3' UTR: 334 nt); length of polyprotein: 2428 aa
Rabovirus	1	Rabovirus A	VPg + 5'UTRIRES-II[L1A-1B-1C-1D/2Apro-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	c. 7496 nt (partial) (5'-UTR: > 488 nt; ORF: 6777 nt; 3' UTR: 231 nt); length of polyprotein: 2259 aa
Rosavirus	1	Rosavirus A	VPg + 5'UTRIRES-II[L1AB-1C-1D-2Anpgp/2B-2C/3A-3B-3C-3D]3'UTR-poly(A).	7834 nt (5'-UTR: 751 nt; ORF: 7005 nt; 3'-UTR: 78 nt); length of polyprotein: 2335 aa
Sakobuvirus	1	Sakobuvirus A	VPg + 5'UTRIRES-IV[L1AB-1C-1D/2AHBox/NC-2B-2C/3A-3B-3C-3D]3'UTR-poly(A).	c. 8931 nt (5'-UTR: up to 828 nt; ORF: 7413 nt; 3' UTR: up to 795 nt); length of polyprotein: 2468 aa
Salivirus	1	Salivirus A	VPg + 5'UTRIRES-V[L1AB-1C-1D/2AHBox/NC-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	c. 7800 nt (5' UTR: 591 nt; ORF: 7059 nt; 3'-UTR: 157 nt); length of polyprotein: 2352 aa.
Sapelovirus	3	Avian sapelovirus, Sapelovirus A, B	VPg + 5'UTRIRES-IV[L1A-1B-1C-1D/2Apro-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	7982–8021 nt (5'-UTR: 709–763 nt; ORF: 7110–7125 nt; 3' UTR: 148 nt); length of polyprotein: 2374 aa
Senecavirus	1	Senecavirus A	VPg + 5'UTRIRES-IV[L1A-1B-1C-1D-2Anpgp/2B-2C/3A-3B-3C-3D]3'UTR-poly(A).	c. 7491–8226 nt (5'-UTR: up to 741 nt; ORF: 6969–7566 nt; 3' UTR: 82–235 nt); length of polyprotein: 2322–2521 aa
Sicinivirus	1	Sicinivirus A	VPg + 5'UTRIRES-II[L1AB-1C-1D/2AHbox/NC-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	c. 7310 nt (5'-UTR: 666 nt; ORF: 6546 nt; 3'-UTR: 71 nt); length of polyprotein: 2181 aa.
Teschovirus	1	Teschovirus A	VPg + 5'UTRIRES-IV[L1A-1B-1C-1D-2Anpgp/2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	Up to 9809 nt (5'-UTR: up to 939 nt; ORF: 8595; 3'-UTR: 308 nt); length of polyprotein: 2864 aa
Torchivirus	1	Torchivirus A	VPg + 5'UTRIRES[L1A-1B-1C-1D-2Anpgp/2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	>7110 nt (5'-end missing) (5'-UTR: >335 nt with poly(C) tract; ORF: 6600–6711; 3'-UTR: 67 nt); length of polyprotein: 2199–2236 aa
Tremovirus	1	Tremovirus A	VPg + 5'UTRIRES-IV[L1A-1B-1C-1D/2AHbox/NC-2B-2C/3A-3B-3C-3D]3'UTR-poly(A)	7077 nt (5'-UTR: 192 nt; ORF: 6654 nt; 3'-UTR: 231 nt); length of polyprotein: 2218 aa
				c. 7032 nt (5'-UTR: 494 nt; ORF: 6405; 3'-UTR: 135 nt); length of polyprotein: 2134 aa

70%, respectively. It has been found through RNA hybridization studies that 5% genomic identity in all the human enterovirus (HEV) and 50% identity within groups (i.e., PV, coxsackieviruses A) (Young, 1973). Sequence studies have shown that 70% nucleotide similarity between the three types of PV (Stanway et al., 1983; Toyoda et al., 1984).

The similarity of an amino acid between coxsackieviruses B is found to be around 90% and with PV the similarity is around 60% (Iizuka et al., 1987; Jenkins et al., 1987; Lindberg et al., 1987). The coat of virion consists of four viral proteins (VP) according to the mobility in SDS polyacrylamide gels according to the decreasing molecular weight [i.e., VP1 (1D), VP2 (1B), VP3 (1C) and VP4 (1A)] (Liljas et al., 2002). The parenthesis in the VP position shows the standard nomenclature which is based on their position on the genome (Rueckert and Wimmer, 1984a, b). VP is referred to as structural capsid or coat proteins because of the composition of protein shell encapsulation with RNA genome. In PiVs the trace of fifth protein VPO (1AB) is present that undergoes breakage to yield VP2 (1B) and VP4 (1A) with the insertion of RNA into the capsid (Rueckert, 1976; Liljas et al., 2002). The major 4 virion proteins consist of 60 capsid subunits which termed protomers (Rueckert, 1976; Liljas et al., 2002) and each protomer has some portions of VP1-VP3 which is exposed on the surface whereas VP4 lies beneath the virion surface near RNA (Hogle et al., 1985; Rossmann et al., 1985; Rueckert, 1985). In the case of rhinovirus 14 human PiVs four neutralization antigenic sites have been found on the external surface of VPs. The identification of these sites has been carried out by sequencing viral escape mutants which neutralizes the monoclonal antibodies which lead to the identification of respective positions on the three-dimensional structure of the virus (Rossmann et al., 1985; Sherry et al., 1986; Liljas et al., 2002). It has been found that PVs shows just three neutralizing

Table 7 (Continued)

Genus	Virus	Reference
Sapelo-like	Sapelo-like bovine picornaviruses	Nagai et al., 2015
Sapelo-like	Bat sapelovirus	Yinda et al., 2017
Sapelo-like	California sea lion sapelovirus	Li et al., 2011
Sapelo-like	Canine sapelovirus	Woo et al., 2012a, Woo et al., 2012b
Sapelo-like	Feline sapeloviruses	Lau et al., 2012
Sapelo-like	Phacovirus	Boros et al., 2016
Sapelo-like	Sapelo-like pigeon picornavirus A	Kofstad and Jonassen, 2011
Sapelo-like	Sapelo-like pigeon picornavirus B	Kofstad and Jonassen, 2011
Sapelo-like	Sapelo-like pigeon picornavirus B	Warden et al., 2017
Sapelo-like	Sapelo-like quail picornavirus	Pankovics et al., 2012

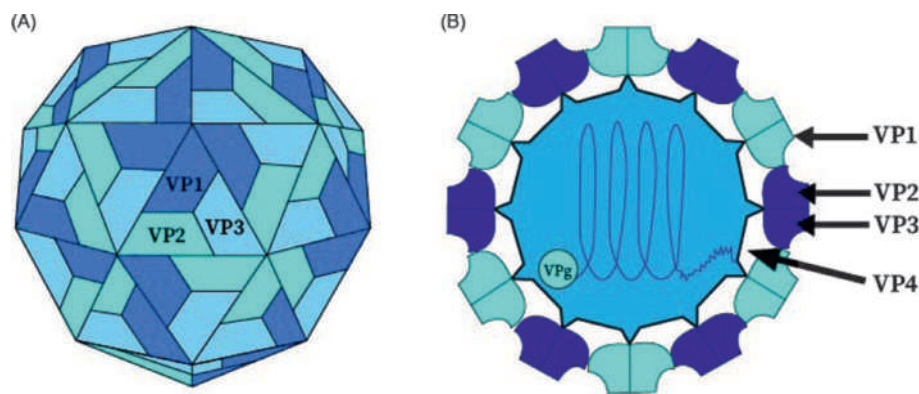


Fig. 3 Nonenveloped and spherical structure of PiVs (A) and capsid (B) structure showing that each protomer containing 4 polypeptides (VP0, VP1, VP2, VP3, VP4) located on the internal side of capsid.

epitopes (Hogle et al., 1985; Page et al., 1988; Liljas et al., 2002) and the viral receptor binding site was identified on the surface of virion and it seems to be like depression or canyon (Hogle et al., 1985; Rossmann et al., 1985). This canyon is found to be 25 Å which is, however, smaller than the diameter (35 Å) Fab fragment of the molecule of antibody. PiVs do not possess any envelope so, therefore, they are resistant to lipid solvents, whereas, human enterovirus (HEV) was found to be acid-stable ($\text{pH} \leq 3.0$). Due to this acid stability, they could easily pass through the stomach to cells lining in the small intestine where they replicate. Enteroviruses are easily inactivated when they are heated at 50 °C, desiccation and treatment with the disinfectants [i.e., formaldehyde (0.3%), HCl (0.1 N)] (Melnick, 1985). When HEV is centrifuged in sucrose gradients the sedimentation and buoyant density were found to be 156S and 1.34 g/mL in CsCl_2 (Rueckert, 1985; Liljas et al., 2002).

Receptors of PiVs

The entry of viruses is controlled by receptors and for PiVs they helped in cell attachment, signaling and endocytosis and the initiation of capsid alterations that are required for entry of infections (Racaniello, 1990; Evans and Almond, 1998; Rieder and Wimmer, 2002). The interaction between virus and receptor has been characterized for different viruses. In the case of the newly identified genera, the receptors have not been identified. Different receptors for PiVs include, i.e., immunoglobulin-like family, low density lipoprotein receptor (LDLR) family, integrin family of cell adhesion molecules, the complement control family and T cell immunoglobulin domain mucin like domain receptors (Rossmann et al., 2002; Tuthill et al., 2010a, b). These receptors and their nature of virus-receptor interactions, the plasticity of receptor usage, and role of receptors in the determination of host, tissue tropism and pathogenesis are described in the following sections.

Immunoglobulin superfamily receptors

Receptors of this group are well characterized for genus *Enterovirus* and adhesion molecule-1 (ICAM-1) is the major receptor for human Rhinoviruses (HRVs) (Greve et al., 1989; Staunton et al., 1989; Tomassini et al., 1989). The CD155 or nectin like molecule-5 (NECL-5) is the receptor of PV and plays an important role in cancer progression and embryonic development (Mendelsohn et al.,

1989; Gromeier et al., 2000; Takai et al., 2008). The receptor, coxsackie and Adenovirus (CAR) is the important part of the tight junction between cells in the epithelium (Coyne and Bergelson, 2005; Freimuth et al., 2008). It has been found that murine vascular cell adhesion molecule-1 (VCAM-1) is the receptor of the cardiovirus encephalomyocarditis virus (EMCV) (Huber, 1994). Type 1 membrane glycoproteins consist of 7 (VCAM-1), 5 (ICAM-1), 3 (PVR) or 2 (CAR) extracellular immunoglobulin like (Ig-like) domains, cytoplasmic and a transmembrane domain. Enterovirus receptors (ICAM-1, PVR, CAR) genetic alteration of molecules shows that the Ig-like domain is important for virus binding. Cryo-electron microscopy has been used to study the interaction of receptors with virus capsids and shows that receptors insert into the canyon (surface depression which encircles fivefold axes of enterovirus and rhinoviruses). This depression results in the greater surface area of interaction between receptor and canyon which increases the energy of the interaction in such a way that receptor binding could act as a trigger for the activation of capsid structural transitions that involved genome release and penetration of membrane. These Ig-like domain receptors help in the binding of the virus on the cell surface, which targets a specific endocytic pathway and activates the process of delivery of the genome to the cytoplasm. The interaction between EMCV and VCAM-1 and has been found that EMCV capsid does not possess a canyon which indicates that it is not necessary for the binding of Ig-like receptors.

Decay accelerating factor

Enteroviruses contain different receptors which are known as decay accelerating factors (DAF, CD55). These receptors are known as complement control family and are used as receptors for microbes and these receptors consist of four extracellular short consensus repeat modules that are attached to the membrane through glycosylphosphatidylinositol (GPI) anchor (Lea, 2002; Lindahl et al., 2000). As compared to the other virus-receptor interactions that are observed in the case of Ig-like receptors the mode of interaction of DAF is not well known. It has been found that different viruses have different binding sites on capsid and receptors (Lea, 2002; Powell et al., 1999). It is not necessary that the canyon is involved in the binding and also not initiate the changes in the capsid. The DAF function as the receptor is to recruit virus to the surface of the cell followed by interaction with co-receptor which is required for the initiation of entry of infectious agent.

Low density lipid receptor (LDLR) family

Low density lipid protein receptor (LDLR), LDLR-related protein (LRP) and very low density lipoprotein receptor (VLDLR) are known as receptors for HRVs (minor group) and possess the ability to hinder the binding of the virus to cultured cells (Hofer et al., 1994; Marlovits et al., 1998a, b, c). They consist of extracellular domains including 7LDLR, 8VLDLR or 31LRP ligand-binding repeats, cytoplasmic domain and transmembrane domain. There are multiple imperfect repeats that improve the capability of these molecules to bind effectively to various serotypes of the virus. The major group HRV/ICAM-1 interaction involves canyon for binding whereas LDLR binding does not involve canyon and also not induced any modifications in the capsid. Five copies of ligand-binding replicate and are thought to bound around the fivefold axis of symmetry to encapsulate the fivefold vertex so, therefore, the various less affinity circumstances merge to give greater affinity (Hewat et al., 2000; Konecsni et al., 2004; Querol-Audí et al., 2009; Rankl et al., 2008; Wruss et al., 2007). PiVs are capable to bind with various copies of a receptor to obtain a high affinity for binding. It has been found through phylogenetic analysis (PA) that 99 conventional HRV serotypes are grouped into two biological groups (HRV-A and HRV-B) (Vlasak et al., 2005; Palmenberg et al., 2009).

Integrins

Integrins are known as cell adhesion receptors that consist of a noncovalent heterodimeric complex in between subunits *a* and *b* (Akiyama, 1996). Subunit is made up of glycoprotein with a large globular extracellular domain, a small cytoplasmic domain and a transmembrane domain. Integrin receptor possesses a classical feature that is the identification of the Arg-Gly-Asp (RGD) tripeptide. Types of integrins i.e., $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 6$, $\alpha v\beta 8$ could perform all the necessary functions as a receptor for the entry of the Aphthovirus FMDV (Jackson et al., 2000, 2004; Duque et al., 2004). Integrin $\alpha v\beta 6$ present on bovine epithelia and they are similar to the sites where lesions are developed during natural infection and it is the natural receptor for FMDV (Monaghan et al., 2005). The binding of integrin required an RGD triplet along with the near modification which is displayed by a flexible exposed loop (VP-1 G-H loop) on the surface of the capsid (DiCara et al., 2008; Logan et al., 1993). These receptor binding do not initiate the structural modification but helps in tethering the virus on the cell surface to facilitate its internalization by the process of endocytosis. Integrins as receptors were also helpful in the cell attachment of the Enteroviruses, Echovirus 1, Echovirus 9, CAV 9, Parechovirus and Human Parechovirus 1 (Bergelson et al., 1997; Triantafilou et al., 1999; Triantafilou and Triantafilou, 2001; Nelsen-Salz et al., 1999; Heikkilä et al., 2009; Roivainen et al., 1994; Williams et al., 2004; Joki-Korpela et al., 2001). These viruses bind integrins through RGD modification near the C-terminus of VP1 which is the exposed flexible site (Hendry et al., 1999). In the case of Echovirus 1 is an exception that binds with RGD independent integrin $\alpha_2\beta_1$ through interaction with canyon (Xing et al., 2004).

HAV cellular receptors

Cellular receptor for HAV (HAVcr-1) and human analog (huHAVcr-1) was found to be a novel class I integral membrane glycoproteins containing extracellular domain, N-terminal membrane distal Ig-like region and mucin like region (Kaplan et al., 1982; Feigelstock et al., 1998; Thompson et al., 1998). It has been found that HAVcr-1 is neutralized HAV and initiates HAV particle modifications (Silberstein et al., 2001, 2003). IgA is a natural ligand of HAVcr-1 and it increases the HAV receptor interactions (Tami et al., 2007). The huHAVcr-1 is classified into prototype members of the T cell immunoglobulin domain, mucin like domain (TIM)

gene family or TIM-1. The study has shown that childhood infection with HAV initiates TIM-1 and protects against allergy in later life that leads to the decreased incidence of HAV in western countries which is responsible for allergy and asthma (McIntire et al., 2004).

EV 71 receptors

EV71 is a type of Enterovirus that causes hand, foot and mouth diseases with normal with relatively mild and self-limiting symptoms. In some cases, the EV71 infection is linked with severe and fatal neurological diseases. There are two receptors reported for EV71 which are human P-selectin glycoprotein ligand-1 (mucin like protein) and serve as EV71 infection of leukocytes and scavenger receptor (class B member 2) which are used for endocytosis of high density lipoprotein (Nishimura et al., 2009; Patel and Bergelson, 2009; Yamayoshi et al., 2009).

Sialic acid

Salic acid (SA) is present on the oligosaccharide chains that trim a variety of glycoproteins of the cell surface. The Cardiovirus Theiler's murine encephalomyelitis virus (TMEV) accepts SA as a receptor and the complex formed between SA and virus has been characterized by crystallography (Zhou et al., 2000; Lipton et al., 2006). EMCV (Cardiovirus) binds through SA with glycoprotein A that is a receptor molecule on virus nonpermissive mammalian erythrocytes (Tavakkol and Burness, 1990). It has been found that EMCV could use the SA mediated entry after cell culture adaptation (Guy et al., 2009). *Aphthovirus* equine rhinitis A virus (ERAV) attach to cells through an interaction with SA and the complex formed between virus and SA is characterized by X-ray crystallography (Warner et al., 2001; Stevenson et al., 2004).

Co-receptors

It is necessary for the productive infection that some DAF binding *Enteroviruses* required co-receptors to affect the modification of capsid structure. It has been found that *Coxsackieviruses*, CAV21 and CBV3 use Ig-like molecules such as ICAM-1 and CAR, respectively, as co-receptors (Shafren et al., 1997a, b, c). DAF (primary receptor) along with co-receptors also bind with the canyon and initiates the capsid modifications. Cultures cell studies have shown that viruses could infect through co-receptors in DAF absence (Shafren et al., 1997a). A study on CBV3 infection of polarized epithelial cells has been carried out and it has been found that CAR is found in its natural location in the tight junctions between cells and not accessible to the virus. The binding of DAF initiates signaling dependent transport of the receptor-virus complex to the tight junctions for the interaction between CAR and cell entry could happen (Powell et al., 1998; Ward et al., 1998; Goodfellow et al., 2000; Coyne and Bergelson, 2006). Integrin-binding enteroviruses also required co-receptors for their entry and studies have been shown that CAV9 required β_2 microglobulin, MHC-I-associated protein GRP78 and MHC-I (Hughes et al., 1995; Triantafyllou et al., 1999, 2002).

Diseases caused by PiVs

PiV family consists of Enteroviruses, Hepatoviruses, Parechoviruses and Rhinoviruses (human pathogens) while Aphthoviruses and Cardioviruses (animal pathogens). On the basis of their pathogenicity these human Enteroviruses are subgrouped into polioviruses (PV, 3 serotypes), Coxsackie A viruses (CAV, 23 serotypes), Coxsackie B viruses (CBV, 6 serotypes), Echoviruses (EV, 28 serotypes) and Enteroviruses 68–71. *Enteroviruses* caused infections in man that varies from minor respiratory illnesses to paralysis, myocarditis, meningoencephalitis, other infections that lead to multiple organ failure in newborns, poliomyelitis, hand foot and mouth diseases (caused by CAV16), acute hemorrhagic conjunctivitis (caused by CAV24, Enterovirus 70), herpangina (caused by CAVs) and pleurodynia (caused by CBVs) (Grist et al., 1978). More than 100 human *Rhinovirus* (HRV) serotypes are known and considered to cause the common cold. HRV mainly caused sinusitis, otitis media and exacerbation of asthma (Minor, 1991; Hyypää et al., 1992; Stanway et al., 1994; Mayo and Pringle, 1998; Pitkäranta and Hayden, 1998). Different disease infections caused by PiVs are given in Table 8.

Detection of PiVs

The standard methods of diagnosis for human PiVs infections are culturing of the virus in infected cell lines which are followed by neutralization typing (Enteroviruses, Parechoviruses) or acid lability testing (HRVs). The indirect evidence of the etiology of the diseases is shown by the serological detection of Enteroviruses specific antibodies. Specific IgM class antibodies are used for the diagnosis of HAV infections. Current methods which are used for the PiVs infections are laborious, insensitive, time consuming and inconvenient. So, therefore, newly developed molecular techniques for PiVs diagnosis were found to be faster, specific and sensitive.

Reverse transcriptase polymerase chain reaction (RT-PCR)

It has been found that sequence analysis and the cDNA clones for the diagnosis of the various Enterovirus serotypes through one single probe had proposed that a few regions of the genome are very similar to the other members of the genus. However, it has also been found that HRVs were found to be similar to the HEVs and based on these examinations the efforts were carried out for the selection of primers and probes that are used to develop the RT-PCR as a diagnosis test.

The genome of PiVs consists of 7000–9000 nucleotides (nt) and single stranded RNA molecules. The PiVs long reading frame coding for the capsid and nonstructural proteins is followed by the NCR. This region also contains several modifications that

Table 8 Diseases caused by PiVs (Santti et al., 1999).

<i>Virus</i>	<i>Diseases</i>
Polioviruses 1, 2, 3	Poliomyelitis
Coxsackievirus A7, Enterovirus 70, 71	Paralytic diseases
Enterovirus serotypes	Meningitis/encephalitis
Coxsackie B viruses	Myocarditis
Coxsackie B viruses, echoviruses	Neonatal infection
Coxsackie B viruses	Pleurodynia
Coxsackie A viruses	Herpangina
Coxsackievirus A16, Enterovirus 70	Hand foot and mouth disease
Coxsackievirus A24, Enterovirus 70	Acute hemorrhagic conjunctivitis
Many enteroviruses, parechoviruses	Respiratory infections
Rhinoviruses	Common cold
Parechovirus 1	Gastroenteritis
Hepatitis A virus	Acute hepatitis

formed the secondary structures that are required for translation and replication of the virus. The RT-PCR test used these homologous regions that are similar in *Enteroviruses* and *Rhinoviruses*. The modification of the products has been found to form a similar length form for which an oligonucleotide probe is required that discriminate between two PiVs groups (Hyypia et al., 1989; Santti et al., 1997). However, a primer is targeted to save the VP2 capsid protein gene that is used along with another primer from NCR (Olive et al., 1990). There is an advantage in the pairing up of these primers because in Enteroviruses and Rhinoviruses amplicons are different in the length. Therefore, it has been found in different studies that all Enterovirus serotypes and HRV isolates could be detected using these formed primers sequences (Fig. 4) (Hyypia et al., 1989, 1998; Horsnell et al., 1995; Pulli et al., 1995; Arola et al., 1996; Huttunen et al., 1996; Pitkäranta et al., 1997; Oberste et al., 1998).

In 1996, with the outbreak of aseptic meningitis (AM) in Finland the effectiveness of this technique has been proven. The specimen collected from the patients include cerebral spinal fluid (CSF), throat swabs, stool samples and sera and it has been found that the *Enterovirus* is the causative agent in CSF by RT-PCR (Arola et al., 1996).

Sequence analysis

It has been found through the analysis of the Enterovirus genome that the members of this genus formed four genetic clusters in the coding region when used for comparison. These genetic clusters are subdivided into four subgroups (A, B, C1, C2 and D). Cluster A includes CAV serotypes and Enterovirus 71 and cluster B contains CAV9, CBVs and EVs. However, cluster C1 and C2 are related to each other containing Polioviruses and CAVs and cluster D consists of Enterovirus 70 (Hyypä et al., 1997). It has been found through partial sequence analysis that all Enterovirus belongs to these four clusters (Pulli et al., 1995; Huttunen et al., 1996) and VP4:VP2 region sequence has been used for the molecular typing of the clinical isolates (Arola et al., 1996). However, Polioviruses could not be differentiated from CAVs in cluster C by this analysis and so, therefore, other genomic regions are required. In NCR (5%) the *Enterovirus* serotypes clustering are not a useful tool for typing but could be used in epidemiological studies. Genetic clustering could also be used in the detection of HRVs and the relation of this group with pathogenicity of the infection is not clear (Horsnell et al., 1995). Partial sequence analysis (PSA) is widely used in the epidemiological studies for PV and helps in the building up of the large sequence database (Rico-Hesse et al., 1987; Zheng et al., 1993; Huovilainen et al., 1995; Kew et al., 1995; Mulders et al., 1995; Chezzi et al., 1997; Morvan et al., 1997; Fiore et al., 1998). 150 nt region in the junction of the structural gene VP1 and the 2A viral protease gene has been used universally for these studies (Rico-Hesse et al., 1987).

Enterovirus

A febrile illness has been developed by Franklin Delano Roosevelt during his summer vacations and this illness then leads to paralysis. This is one of the most commonly recognized cases of paralytic poliomyelitis and polio was discussed in 1580 BCE.

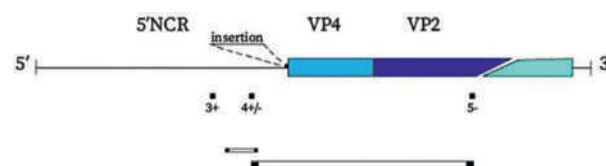


Fig. 4 Representation of PiVs genome and the location of the RT-PCR used for the detection of human enteroviruses (HEVs) and rhinoviruses (HRVs).

In 1949 for the first time, this virus has been grown in tissue culture by John Enders. This isolation is then followed by identification of the prototypic Enterovirus (EV) and then other members of this family have been studied and their properties are characterized. It has been more than 70 years that animal Enteroviruses (EVs) and EV like agents are known for causing diseases. Virulent pig polioencephalomyelitis for the first time was reported in Czechoslovakia in 1929 and in 1932 avian encephalomyelitis was reported in the USA. Tissue and egg culture techniques discovery help in the isolation and biological characterization of these viruses.

EVs are nonenveloped, single-stranded RNA, icosahedral shape and small (30 nm) in size viruses that belong to the family of *Picornaviridae*. It causes a variety of diseases and consists of more than 70 serotypes (Wilfert et al., 1983; Palacios and Oberste, 2005). They are widely distributed around the globe (Rotbart and Hayden, 2000). The infections caused by EVs are mostly mild (i.e., febrile illness, respiratory infections, hand foot mouth diseases, herpangina, etc.) with complete recovery but they can also cause severe and life threatening diseases including encephalitis, myocarditis, neonatal sepsis, polio and meningitis (Table 9) (Lake et al., 1976; Melnick, 1996a, b; Rotbart, 1995a, b; Why, 1995; Sawyer, 1999). EVs include *Polioviruses*, *Coxsackie A* and *B*, *ECHO* viruses and numbered EVs. They are also linked with various other conditions which include chronic autoimmune diseases (i.e., diabetes mellitus I, polymyositis, dermatomyositis, Sjogren's syndrome), cardiovascular diseases (i.e., chronic dilated cardiomyopathy, atherosclerosis, ventricular arrhythmias, transplant rejection) and neurological diseases (i.e., amyotrophic lateral sclerosis) (Palacios and Oberste, 2005). EVs are transmitted from one to another person via air or oral fecal route and the infections caused by them are also possible through contaminated objects.

Classification of EVs

In 2012, the classification of EVs taxonomy was reported by the International Committee on Taxonomy of Viruses (King et al., 2012; Adams et al., 2013, 2014, 2015, 2016). EV belongs to the family of *Picornaviridae* which includes 10 enterovirus species (i.e., Enterovirus A, B, C, D, E, F, G, H, I, J) and 3 rhinovirus species (i.e., Rhinovirus A, B, C). The various serotypes of different EVs are reported in Table 10. There are certain unclassified species of EVs which include monkey EV (SV-47), EV-122 and EV-123 because their genome sequence is not clear and is not matched with existing species (Nikonov et al., 2017).

Serotypes of EVs

Human EVs are subdivided into polioviruses, coxsackieviruses group A, B and echoviruses. However, in 1969 the serotypes of EVs did not previously allocate to the above mentioned groups so, therefore, these serotypes are designated as EV followed by number 68 (Almond and Yin-Murphy, 1999; Sun et al., 2019). There are four serotypes of EVs (EV68–71) which are in the genus EV of the family *Picornaviridae*. These serotypes are described in the following sections.

EV 68 (EV-D68)

Initially, the first isolated prototype of EV68 is called as Fermon virus and in 1962 it was isolated in California from the four children suffering from pneumonia and bronchiolitis (Schieble et al., 1967). This virus was isolated and characterized by the throat swab samples of the children and this result in the detection of four new distinct virus strains i.e., Fermon, Franklin, Robison and Rhyne. The statistics reported by the US National Enterovirus Surveillance System (NESS) 26 cases of EV 68 have been reported from 1970 to 2005 (Midgley et al., 2015). In 2014 the highest number (~1153) of EV-D68 infections has been reported with severe respiratory

Table 9 Diseases caused by different serotypes of EVs.

Serotypes of EVs	Diseases	References
All serotypes	Asymptomatic infection	Kogon et al., 1969; Melnick, 1996a, b
PV 1, PV2, PV3	Paralytic poliomyelitis	Melnick, 1996a, b; Modlin, 1995a, b; Paul, 1971a, b
ENV 70	Paralytic poliomyelitis	Kono et al., 1974, 1977; Wadia et al., 1983a, b
ENV 71	Paralytic poliomyelitis	Chumakov et al., 1979a, b
CAV 7	Paralytic poliomyelitis	Grist et al., 1978; Voroshilova and Chumakov, 1959
PV, CBV, CAV, ECV	Aseptic meningitis	Berlin et al., 1993; Dagan et al., 1988; McIntyre and Keen, 1993; Rotbart, 1995a, b
ENV 71	Meningoencephalitis	Schmidt et al., 1974a, b; Alexander Jr et al., 1994
CBV	Acute myocarditis	Grist, 1977; Lansdown, 1978
CBV	Bornholm diseases	Grist et al., 1978; Chonmaitree and Mann, 1995
CAV 16	Hand, foot and mouth disease	Robinson et al., 1958; Wenner, 1973a, b
ENV 71	Hand, foot and mouth disease	Cao et al., 1985; Hagiwara et al., 1978a, b
CAV, CBV, ECV	Herpangina	Wenner, 1973a, b; Grist et al., 1978; Chonmaitree and Mann, 1995
CAV, CBV	Exanthem	Wenner, 1973a, b
ENV 70	Acute hemorrhagic conjunctivitis	Lim and Yin-Murphy, 1971
CAV 24	Acute hemorrhagic conjunctivitis	Mirkovic et al., 1974
CBV, ECV	Neonatal multisystem disease	Abzug, 1995; Abzug et al., 1993, 1995; Haddad et al., 1993; Kaplan et al., 1983
CAV, CBV, ECV	Nonspecific febrile/respiratory illness	Dagan, 1996; Dagan and Menegus, 1995; Chonmaitree and Mann, 1995

Table 10 List of EVs species and there serotypes (Nikonov et al., 2017).

Species	Number of species	Serotypes
Enterovirus		
A	25	A2 (CV-A2), CV-A3, CV-A4, CV-A5, CV-A6, CV-A7, CV-A8, CV-A10, CV-A12, CV-A14, CV-A16, enterovirus A71 (EV-A71), EV-A76, EV-A89, EV-A90, EV-A91, EV-A92, EV-A114, EV-A119, EV-A120, EV-A121, simian enteroviruses SV19, SV43, SV46, baboon enterovirus A13 (BA13)
B	63	Coxsackievirus B1, CV-B2-B6, CV-A9, echovirus 1 (E-1), E-2-E-7, E-9, E-11-E-21, E-24-E-27, E-29-E-33, enterovirus B69 (EV-B69), EV-B73-EV-B75, EV-B77-EV-B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107, EV-B110 (from chimpanzee), EV-B111, EV-B112 (from chimpanzee), EV-B113 (from mandrill), simian enterovirus SA5
C	23	Poliovirus (PV) 1, PV-2, PV-3, coxsackievirus A1 (CV-A1), CV-A11, CV-A13, CV-A17, CV-A19, CV-A20, CV-A21, CV-A22, CV-A24, EV-C95, EV-C96, EV-C99, EV-C102, EV-C104, EV-C105, EV-C109, EV-C113, EV-C116, EV-C117, EV-C118
D	05	EV-D68, EV-D70, EV-D94, EV-D111 (from human and chimpanzee), EV-D120 (from gorilla)
E	05	EV-E1, EV-E2, EV-E3, EV-E4.
F	07	EV-F1, EV-F2, EV-F3, EV-F4, EV-F5, EV-F6, EV-F7
G	16	EV-G1, EV-G2, EV-G3, EV-G4, EV-G5, EV-G6, EV-G7, EV-G8, EV-G9, EV-G10, EV-G11, EV-G12, EV-G13, EV-G14, EV-G15, EV-G16
H	04	SV4, SV28, SA4, A-2 plaque virus
I	01	<i>Camelus dromedarius enterovirus</i> (camel enterovirus)
J	06	SV6, EV-J103, EV-J108, EV-J112, EV-J115, EV-J121
Rhinovirus		
A	80	A1, A2, A7-A13, A15, A16, A18, A19-A25, A28-A36, A38-A41, A43, A45-A47, A49-A51, A53-A68, A71, A73-A78, A80-A82, A85, A88-A90, A94, A96, A100-A109
B	32	B3-B6, B14, B17, B26, B27, B35, B37, B42, B48, B52, B69, B70, B72, B79, B83, B84, B86, B91-B93, B97, B99-B106
C ^a	01	HRV-A2, HRV-C or HRV-X

^aOn the bases of certain test the authors considered it same like *Rhinovirus A* (Arden et al., 2006; McErlean et al., 2007) and others preferred to mention it as HRV-C (Lamson et al., 2006; Lau et al., 2007; Lee et al., 2007; McErlean et al., 2008) or HRV-X (Kistler et al., 2007).

symptoms (Midgley et al., 2015). Various retrospective studies have been carried out which shows the greater prevalence of EV-D68 infection in Europe in the same period (Poelman et al., 2015). The most common clinical infection caused by EV-D68 is respiratory illness (Oberste et al., 2004).

EV-D68 consists of a positive single stranded RNA which is about 7.6 kb and it contains a single ORF like other picornaviruses. This ORF is associated with untranslated regions (UTR) on each end (Opanda et al., 2014). This association results in the encoding a precursor polypeptide which further autoanalytically breakdown to form structural proteins (VP1 to VP4) and seven other nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, 3D) (Fig. 5). VP1 consists of serotype specific neutralization BC loop site which is used to differentiate between virus serotypes and to identify the new strains (Tokarz et al., 2012). 2C viral protein has been found to play an important role in the infection process and host-viral interaction and this helps in the targeting of anti EV-D68 drugs (Sun et al., 2019). EV-D68 is subdivided into four subtypes and many branches such as original classical subtypes and epidemic related branches (Clade A, B, C, D). These subtypes are widespread in American, Europe and Asia. It is acid labile, favors lower growth temperature and it multiplies in the respiratory tract which is also a typical attribute of human rhinoviruses. Therefore, EV-D68 shows the same important biological and molecular properties as other enteroviruses and rhinoviruses (Oberste et al., 2004).

EV 69

First time in 1969 the EV 69 prototype (Toluca-1 virus) and two other isolates have been found in the rectal swab of healthy 4 year old children in Toluca, Mexico (Rosen et al., 1973; Melnick et al., 1974; Yin-Murphy and Almond, 1996). It is an uncommon virus and seven isolations have been carried out in the countries during the period of 1975–1983. In Australia (Fairfield Hospital, Victoria) one isolation (1979) and WHO collaborating center for Virus Reference and Research, Singapore has identified two strains in 1984 (Almond and Yin-Murphy, 1999; Yin-Murphy and Almond, 1996). EV 69 could be isolated from feces and nasopharyngeal aspirates and produces the EV cytopathic effects (CPEs) in rhesus kidney, Hep-2 and Hela cell cultures. The cross section antigenically with one of these groups (i.e., coxsackie B, coxsackie A9 viruses) has been previously reported. In the case of

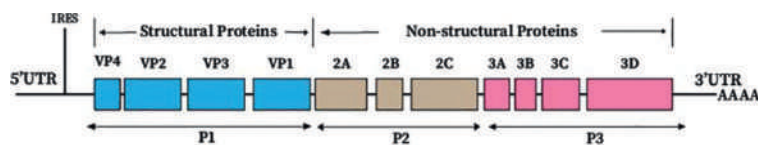


Fig. 5 Representation of the enterovirus 68 (EV D68) genome (Sun et al., 2019).

Toluca-1 antiserum with homologous hemagglutination inhibition (HI) and neutralization (N) shows the titers of 1.320 and 1.500, respectively, whereas in the case of echo 6 D1 the titer was found to be 1.400 and 1.500 for HI and N, respectively (Almond and Yin-Murphy, 1999; Yin-Murphy and Almond, 1996). However, in the case of D1 (cox) antiserum has been found to show the titer of 1.50 for homologous HI and N. The Toluca-1 has been found to show the titer of <1.100 and <1.500 for HI and N, respectively, whereas E6 agglutinates human erythrocytes shows high titers at 37 °C than at lower temperature (4 °C) (Almond and Yin-Murphy, 1999; Yin-Murphy and Almond, 1996). However, Toluca-1 shows the highest titer at a lower temperature than at a higher temperature. The Toluca EV 69 strains have been found in the feces of healthy children and the Australian (1979) and Singapore (1984) strains have been isolated from nasopharyngeal aspirates of infants with respiratory illness. The transmission of this virus is through oral-fecal and respiratory routes (Almond and Yin-Murphy, 1999; Yin-Murphy and Almond, 1996).

EV 70

EV 70 is the causative agent of acute hemorrhagic conjunctivitis (AHC) and was first isolated in 1971 during the wave of AHC in Morocco, Singapore, Hong Kong and Japan. It has been found that EV 70 played an important role in the 1980–1982 AHC pandemic in Africa, Southeast Asian countries, India and the Middle East (Almond and Yin-Murphy, 1999). The outbreak of AHC caused by EV 70 has not been reported in Australia and mostly AHC epidemic occurs in hot and humid areas. The spread of conjunctivitis is mostly occurring due to unhygienic conditions, poor personal hygiene and overcrowding. It possesses similar physicochemical properties to enteroviruses and electron micrographic studies revealed that it is a nonenveloped virion having 22–27 nm diameter with icosahedra symmetry. The buoyant density in cesium chloride and sedimentation coefficient in sucrose was found to be 1.34 g/mL and 160 S, respectively (Almond and Yin-Murphy, 1999). EV 70 has been found to be inactivated through phenol, cresol, formaldehyde, chlorine at the concentration of 1 ppm in distilled water, methanol (50%), phenol (70%), iodine (0.004%) in DIASAN (Almond and Yin-Murphy, 1999). The replication of EV 70 has been found to be inhibited by interferon β , arildone {4-[6–2-chloro-4-methoxyphenoxy]hexyl}-3,5-heptanedione}, RO 09-0179 (4',5-dihydroxy-3,3',7-trimethoxyflavone), benzimidazoles-enviroxime, envirodone and guanidine hydrochloride. However, actinomycin D, benzalkonium chloride, benzethonium chloride, chlorhexidine gluconate and hexachlorophene do not show any inhibitory effect on the replication of EV 70 (Almond and Yin-Murphy, 1999).

EV 70 contains positive sense single stranded RNA having typical EV organization. It possesses 5'-NCR of 726 nucleotides, a long open frame of 6582 nucleotides and 3' NCR of 82 nucleotides earlier to the poly (A) tract. VPg protein is covalently attached to the virion RNA. EV 70 icosahedral virion is formed by the 60 copies of four viruses encoded proteins 1A (Vp4, 9kD), 1B (Vp2, 28kD), 1C (Vp3, 27kD) and 1D (Vp1) (Almond and Yin-Murphy, 1999). The formation and breakdown pathway of virus encoded proteins was found to be following the L434 system with glutamine glycine as the breakdown site. CD55 has been identified as a receptor on Hela cells for EV 70 and GPI-anchored cells surface protein is found to be a natural regulator of complement action. The functions of these GPI-anchored cell is via decay of C3 and C5 convertases in classical and alternative pathways of complement fixation. EV 70 virus has been found to be similar to echoviruses (i.e., E6, E7, E13, E21, E29, E33) (Almond and Yin-Murphy, 1999). The optimal temperature for the growth of EV 70 has been found to be 33 °C whereas at higher temperature (>39 °C) the viral synthesis is hinder and stops the replication (Almond and Yin-Murphy, 1999). The in vitro experiments show that the temperature dependent step is the uridylation of VPg. It has been found that EV 70 from conjunctival swabs and scrapings, throat swabs and feces made cytopathic effects (CPEs) in the tissue cell culture of human and simian origin and Hela cells are in this case are preferred cell lines (Almond and Yin-Murphy, 1999).

EV 71

From 1969 to 1972 in California EV 71 has been found as a causative agent for CNS disease [i.e., aseptic meningitis (AM), encephalitis] (CDC, 1985). The prototype EV 71 strain (BrCr/1970) was isolated from the culture taken from the brain in severe cases of encephalitis. EV 71 is widely spread in the world and has been found to the causative agent of AM, hand foot mouth disease (HFMD), respiratory illness, polyneuritis and encephalitis in New York (1972) and Australia (1972–1973, 1978, 1979, 1982–1983, 1986) (CDC, 1986a, b). In Sweden (1972), Japan (1973, 1978) and Bulgaria (1975) poliomyelitis like paralysis has been caused by EV 71 which results in a high fatality rate in the children (Almond and Yin-Murphy, 1999). EV 71 has been found to be linked with the epidemic of tick borne encephalitis in Hungary (1978), respiratory infections associated with CNS diseases in France (1989), HFMD in China (1989) and Singapore (1984), monoplegia in Hong Kong (1987) and viral encephalitis (1996) (Almond and Yin-Murphy, 1999). The physicochemical properties of the EV 71 viruses are similar to the EV group. The nucleotide sequence of the EV 71 has been determined and found that it is similar to coxsackieviruses A16 and A2. The replication, receptor recognition and 3D structure of the EV 71 are not different than other EVs (Almond and Yin-Murphy, 1999). The presence of EV 71 in throat swabs, spinal cord, tissue from brain, mesenteric lymph nodes, skin vesicles and CSF has been found to produce CPEs in kidney cell cultures of monkey, Hela and HEL cell lines and human fetal kidney. It has been found that an incubation temperature of 35 and 39 °C in patients with encephalitis leads to CPEs production (Chumakov et al., 1979a, b). However, the strains of HFMD grow at 35 °C but not at 39 °C. The strains of EV 71 related to BrCr/71 are different antigenically from other classification of EVs and are evaluated with neutralization and immunodiffusion assays (Almond and Yin-Murphy, 1999).

EV 71 viruses are the type of EVs that are capable of causing various ailments in a pandemic or occasional portion. It majorly causes AM along with bulbar spinal dysfunction and polyneuritis. EV 71 also causes the outbreak of HFMD with or without has been found to be linked with encephalomyocarditis and respiratory illness (Almond and Yin-Murphy, 1999). It has been found that the transmission of EV 71 is mostly through oral fecal, respiratory routes and from lesions on the skin. In response to EV

Table 11 Clinical manifestation associated with EVs infections (McMinn, 2002).

Clinical manifestation	References
Aseptic meningitis (AM)	Kennett et al., 1974; Blomberg et al., 1974; Shindarov et al., 1979; Ishimaru et al., 1980; Melnick, 1984; Gilbert et al., 1988; Alexander Jr et al., 1994; Chang et al., 1999a, b; Takimoto et al., 1998; Komatsu et al., 1999; Ho et al., 1999; Huang et al., 1999; Wang et al., 1999; Merovitz et al., 2000; Liao and Hung, 2001; McMinn et al., 2001a, b; Huang, 2001
Poliomyelitis like paralysis	Chumakov et al., 1979a, b; Shindarov et al., 1979; Ishimaru et al., 1980; Melnick, 1984; Hayward et al., 1989; Alexander Jr et al., 1994; Cardosa et al., 1999; Wang et al., 1999; Shen et al., 2000; Chen et al., 2001; Huang, 2001; Liao and Hung, 2001
Brainstem encephalitis	Shindarov et al., 1979; Alexander Jr et al., 1994; Chang et al., 1998; Lum et al., 1998; Huang et al., 1999; Komatsu et al., 1999; Wang et al., 1999; Huang, 2001; Liao and Hung, 2001; McMinn et al., 2001a, b
Neurogenic pulmonary edema	Lum et al., 1998; Cardosa et al., 1999; Wang et al., 1999; Wong et al., 1999; Chang et al., 1999a, b; Chang, 2000; Chan et al., 2000; Ho, 2000; Huang, 2001; Liao and Hung, 2001
Cerebellar ataxia	Ishimaru et al., 1980; Nagy et al., 1982; Takimoto et al., 1998; Komatsu et al., 1999; Merovitz et al., 2000; McMinn et al., 2001a, b
GBS	Alexander Jr et al., 1994; McMinn et al., 2001a, b; Kennett et al., 1974; da Silva et al., 1996; Takimoto et al., 1998; Chen et al., 2001
Transverse myelitis	McMinn et al., 2001a, b
Opso myoclonus syndrome	McMinn et al., 2001a, b
Benign intracranial hypertension	McMinn et al., 2001a, b
HFMD	Hagiwara et al., 1978a, b; Ishimaru et al., 1980; Miwa et al., 1980; Melnick, 1984; Alexander Jr et al., 1994; Cardosa et al., 1999; Komatsu et al., 1999; Ho et al., 1999; Tagaya and Tachibana, 1975; Tagaya et al., 1981
Herpangina	Hagiwara et al., 1978a, b; Samuda et al., 1987; Chang et al., 1999a, b; Komatsu et al., 1999; Ho et al., 1999; Wang et al., 1999
Acute respiratory disease	Melnick, 1984; Gilbert et al., 1988; Merovitz et al., 2000; Tsai et al., 2001
Intrauterine infection	Chow et al., 2000

71 infections, the neutralizing antibodies are produced and due to high incubation time in CNS disease, the high antibody titers have been observed in neurological disease (Almond and Yin-Murphy, 1999). The clinical manifestations linked with EV 71 are listed Table 11.

Physical, biochemical and biological characteristics of EVs

EVs possess similar properties like *Picornaviridae* such as small in size (22–30 nm), single stranded RNA, naked nucleocapsid (lack of envelope) and intolerance to ether and lipid solvents. Therefore, it indicates that the EVs do not possess essential lipids. Nondestructive methods have been used to evaluate the diameter of hydrated and wet virus particles (Rueckert, 1985; Jubelt and Lipton, 2014). The nucleic acid present in the EVs constituting around 30% of the particle mass with a molecular weight of 2.3–2.8 million. PVs contain 12 genes whereas poxviruses possess 400 genes. This virus mature in the cytoplasm and an ineffective nucleic acid present in EVs and Rhinoviruses is isolated. This isolated nucleic acid is liberated on the surface protein antigen and such RNA could not be neutralized via antiserum with the intact virus. Humans are mostly infected by EVs and *Rhinoviruses* and these genera are found to be different in various properties. However, it has been found that EVs are stable at pH 3–5 (for 1–3 h) but *Rhinoviruses* are found to be acid labile. In infected humans, EVs are found in the alimentary tract (i.e., lower intestine and throat) whereas Rhinoviruses are majorly found in the nose and throat but rarely present in fecal specimens. EVs grew at 36–37 °C in stationary cultures but Rhinoviruses are incubated on roller drums at 33 °C in fetal cell cultures (Hamre and Procknow, 1963; Hamre et al., 1966; Hamre, 1968). It has been found that EVs and *Rhinoviruses* are stabilized by magnesium chloride against thermal inactivation whereas in cesium chloride EVs and *Rhinoviruses* possess the density of 1.34 and 1.40 g/mL, respectively. The physical and biochemical properties of human *Enteroviruses* (HEV) are reported in Table 12.

Replication cycle of EVs

Virions and viral genome organization

The viral genome of EVs possessess a single ORF along with highly structured untranslated regions (UTR) on the position of 5'- and 3'-poly (A) tail. This viral genome is uncapped and 5'- end is covalently bound with 3B viral protein which is called as VPg (viral protein genome linked). Internal ribosomal entry site (IRES) is present in 5'-UTR which helps in the cap independent translation. The ORF is the same in all PiVs, however, there are some differences in each genus. EVs ORF contains polypotein with structural proteins VP1–4 at P1 region. The nonstructural proteins 2A–2C and 3A–3D are present at P2 and P3 regions, respectively (van der Linden et al., 2015).

Table 12 Physical and biochemical properties of HEVs (Jubelt and Lipton, 2014).

Diameter	30 nm
Shape	Spheroidal
Symmetry	Icosahedral
Envelope	None
Virion proteins	VP1, VP2, VP3, VP4
Protein subunits	60
Protomer compositions	One copy of the four virion proteins
Nucleic acid	Single stranded linear RNA
Percent RNA per virion (%)	30
Percent protein per virion (%)	70
Reaction of ether and lipid solvents	Resistant
pH stability	3–9
Buoyant density (g/mL)	1.34 in cesium chloride
Sedimentation coefficient	156S

Translation and processing of protein

The replication cycle of EV is propagated via linkage to a receptor (Tuthill et al., 2010a, b). The receptors bound at depression in the capsid which is known as a canyon and it is surrounded by fivefold axis of symmetry. After this, the virus is then internalized and the viral RNA is released into the cytoplasm. The produced single polypeptide is processed through a proteolytic mechanism by the proteases 2A^{Pro} and 3C^{Pro} which results in the release of structural and nonstructural viral proteins and stable precursors. The viral proteases also help in the cleavage of cellular targets which improve the environment for viral proliferation. Blockage of translation of cellular proteins (host shut off) occurs with the cleavage of eIF4E and poly (A)-binding proteins (PABP) by 2A^{Pro} and 3C^{Pro} (Kräusslich et al., 1987; Lloyd et al., 1988; Joachims et al., 1999; van der Linden et al., 2015). Viral proteases also breakdown various cellular factors that help in the virus reproduction and hinder antiviral responses (Badorff et al., 1999; Barral et al., 2007; Castelló et al., 2011; Mukherjee et al., 2011; Lei et al., 2013; Wang et al., 2013; van der Linden et al., 2015).

EV RNA replication

The nonstructural proteins initiate the replication of the viral genome and uridylation of protein primer VPg begins the RNA genome replication through the viral RNA dependent RNA polymerase 3D^{Pol} with secondary RNA structure in the genome which is called as cis-acting replication element (cre) as a template (Gerber et al., 2001; Paul et al., 2000; Rieder et al., 2000; Yang et al., 2002; van der Linden et al., 2015). 3D^{Pol} helps in the elongation of VPgUpU which produces the negative stranded intermediates that are used as a template for the formation of positive stranded RNA molecules. These positive stranded RNA molecules entered in another process of translation and replication could be wrapped up in the capsids which results in the production of infectious virus particles (van der Linden et al., 2015).

Role of viral proteins and host factors in membrane rearrangements

In the case of positive stranded RNA viruses, the replication of viral RNA occurs at cellular membranes that are recognized in virus infection (Belov and van Kuppeveld, 2012; Paul and Bartenschlager, 2013; van der Linden et al., 2015). It has been found that in infected cells the single and double membranous structures are present (Belov and van Kuppeveld, 2012; Paul and Bartenschlager, 2013; van der Linden et al., 2015). The studies of electron tomography with PV and CB3 shows that in early infection the single membranous tubular structures are present, however, in later stages these structures flatten, curve and fuse to form double membrane vesicles (DMV) (Belov and van Kuppeveld, 2012; Paul and Bartenschlager, 2013; van der Linden et al., 2015). These DMV further wrapped into multilamellar structures by multiple additional cisternae. The formation of these structures has not been clear yet but evidence has been presented that they are either formed in the early secretory pathway or by the autophagy pathway. The electron tomography studies indicate that somehow these theories are correct that early secretory pathway the role of Brefeldin A (BFA) which is an inhibitor of endoplasmic reticulum (ER) to Golgi transport which stop the EV replication (Irrazun et al., 1992; Gazina et al., 2002; Belov et al., 2008; Lanke et al., 2009; van der Linden et al., 2015). Various proteins from the pathway are necessary for the virus replication and could be found on replication organelles. Golgi specific BFA resistance factor 1 (GBF1) stimulated the GTP exchange of the GTPase ADP ribosylation factor 1 (Arf 1). The activation of Arf 1-GTP results in the membrane bound that mediates the effector proteins (i.e., COP-1 coat complex) to initiate the production of the secretory pathway. Thus Arf 1 is the key regulator of protein and lipid transport in the early secretory pathway. During infection viral protein 3A select GBF-1 and indirectly Arf 1 for replication organelles via direct interaction with GBF1 (Belov et al., 2008; Wessels et al., 2006a, b). This leads to the loss of COP-1 from membranes which results in the interference of secretory pathways and hindrance of the secretion of protein (Wessels et al., 2005, a; Doedens and Kirkegaard, 1995; Hsu et al., 2010; van der Linden et al., 2015).

Another Golgi localized protein, phosphatidylinositol-4-kinase III beta (PI4KIII β) is found to be an important host factor for the replication of EV (Hsu et al., 2010). This protein is a kinase that helps in the synthesis of phosphatidylinositol-4-phosphates (PI4P). PI4P lipids as a precursor of PI(4,5)P₂ are part of PI3K and phospholipase C signaling pathways and also select the proteins with PI4P binding pleckstrin homology (pH) domain to membranes. This selection is for the membrane biogenesis, lipid homeostasis and vesicle mediated trafficking on the Golgi complex (D'Angelo et al., 2008; Graham and Burd, 2011; Santiago-Tirado and Bretscher, 2011; Dorobantu et al., 2014). PI4KIII β in infection selected for the replication sites by 3A and which increase in the level of PI4P lipids (Hsu et al., 2010). This selection of PI4KIII β is not dependent on the GBF1, Arf1, and ACBD3 factors (Téoulé et al., 2013; Dorobantu et al., 2014; van der Linden et al., 2015).

Oxysterol-binding protein (OSBP) is PI4P binding protein that transfers PI4P lipids formed by PI4KIII β through Gogi complex in the ER in return of cholesterol which is transported to the other direction (Mesmin et al., 2013). Some other studies carried out show that OSBP bounds to PI4P enriched replication organelles to initiate PI4P/cholesterol exchange between ER and membranes (Arita, 2014; Roulin et al., 2014; Strating et al., 2015; van der Linden et al., 2015). Therefore, this exchange in cholesterol increasing its concentration and endosomal cholesterol accumulate the cholesterol in the membranes of the replication (Illytska et al., 2013; Roulin et al., 2014; Albulescu et al., 2015). The concentration of cholesterol in membranes regulates the fluidity of membranes and leads to the formation of lipid microdomains. The virus initiates the deposition of cholesterol which induces the deformation of membranes which helps in the generation of replication organelles (van der Linden et al., 2015).

Ontogenesis and virus liberation

The synthesized viral RNA with positive polarity encapsulated via capsid proteins leads to the formation of virions. This process combines with active replication to encapsulate the newly synthesized genomes (Molla et al., 1991; Nugent et al., 1999; van der Linden et al., 2015). This process is not controlled by RNA encapsidation signal or RNA protein interaction but through an interlinking between 2C which is located in the replication complex and the capsid VP3 protein (Liu et al., 2010). The new virions assemble with the release of P1 capsid precursor through the polyprotein. These assemble virions are then folded using chaperone protein HSP 90 and refined by 3C^{Pro} which results in the production of VP0, VP1 and VP3 (Ypma-Wong et al., 1988; Geller et al., 2007a, b; van der Linden et al., 2015). This voluntary process results in the formation of protomers by the assembling of the capsid proteins and 5 protomers combine to form pentamer which leads to the formation of provirion. All EVs required glutathione for the production and stability of the pentamers (Mikami et al., 2004; Smith and Dawson, 2006; Thibaut et al., 2014; Ma et al., 2014) and the last step is the virion maturation via RNA induced cleavage of VP0 into VP2 and VP4 which yields infectious viruses. This last step is boosting up by the acidic environment in autophagosome DMVs (Richards and Jackson, 2012; van der Linden et al., 2015). These infectious viruses are then released in the environment through the process of host cell lysis and nonlytic release via DMVs and the release of phosphatidylserine lipid containing vesicles encapsulated with virions (Feng et al., 2013; Robinson et al., 2014; Chen et al., 2015; van der Linden et al., 2015).

Diagnosis of EVs

There are number of laboratories around the globe working to perform the diagnostic test for EVs so, therefore, these tests should be standardized (Harvala et al., 2017). The recommended sample types used for the diagnosis of EVs infections are reported in Table 13 and the details of the molecular assay diagnostic methods are discussed below.

RT-PCR

RT-PCR is a sensitive method used for the detection of EVs in clinical samples and is more sensitive than cell culture (Chonmaitree et al., 1988; Beld et al., 2004; Iturriza-Gómara et al., 2006). The detection of EVs RNA via RT-PCR is based on the targeting of 5' NCR and, therefore, helpful in the detection of all types of EVs (Iturriza-Gómara et al., 2006; Van Doornum et al., 2007; Nijhuis et al., 2002; Janes et al., 2014; Jaramillo-Gutierrez et al., 2013). It is important to prepare the correct sample before the extraction of nucleic acid and the detection of EVs specifically for stool samples that should be disaggregated in buffer and filtered via centrifugation to hinder PCR inhibition. Internal control (IC) is added for the identification of inhibitory compounds in the

Table 13 Clinical samples used for the detection of EVs in different ailments (Harvala et al., 2018).

<i>Clinical samples</i>	<i>Ailments</i>
CSF, blood, stool, and respiratory	Meningitis/meningoencephalitis
CSF, blood, stool, and respiratory	Neonatal sepsis
CSF, blood, stool, and respiratory	Acute flaccid paralysis/myelitis
Vesicle fluid, respiratory and stool	HFMD/other rash
Respiratory and stool	Respiratory diseases
Stool, respiratory, blood or heart biopsy	Myocarditis
Eye swab	Conjunctivitis

CSF = cerebral spinal fluid, HFMD = hand, foot and mouth diseases.

extracted RNA. Although the molecular methods accurate, sensitive and fast but some of the EVs such EV-D68, EV-C15 and EV-109 could not be detected by these methods (Piralla et al., 2013; Jaramillo-Gutierrez et al., 2013; Dunn et al., 2015). Nowadays various real time commercial RT-PCRs are available to detect EVs and additional facts are needed to authenticate the accuracy of the commercially available assay for the detection of EVs with different 5'-NCR sequences (Piralla et al., 2013; Jaramillo-Gutierrez et al., 2013; Dunn et al., 2015). It has been found that PCR of fecal specimens is unsuccessful because different substances are present that hinder the step of polymerization.

Cell culture

Cell culture is no longer used for the diagnosis of EVs and it can be used for the authentication of PCR-negative specimens in case of the new variant, more than one viral strain in specimen detection. The collection of specimens, preparation of sample and methodology in virus isolation can be seen in the WHO guidelines (World Health Organization, 2004; Chen et al., 2014). EVs different strains can be isolated in cell cultures of mammalian cell lines and some other cell lines including monkey kidney cells, human fibroblasts (World Health Organization, 2004; Schmidt et al., 1975). The cell culture from CSF, pericardial fluid, tissue, blood or stool can be used to isolate EVs. The CPEs were found to be in 2–5 days in cell culture after inoculation. After isolation of EVs, the serotype is identified for EVs using RNA sequencing. It has been found that EV 71 is poorly grown on cell cultures (Jaramillo-Gutierrez et al., 2013) whereas EVD68 required lower incubation temperature than normally used for EVs (Zaidi et al., 2016).

Serology

Serological methods (i.e., ELISA, neutralization tests) are not used for the confirmation of acute EV infections. Specific antibodies (i.e., IgG, IgM) assay for the detection of EVs infections are present but their clinical utility is limited because of cross reactivity of the antigens between distinct serotypes. The serological tests are time consuming (2 weeks) and which makes diagnosis slow and clinically irrelevant. In the case of myocarditis and pericarditis or cardiomyopathy serological testing is preferred because direct sampling is not possible so, therefore, antibody detection could be useful for diagnostic purposes. The quantification of neutralizing antibodies testing against selected EVs can be used (Nijhuis et al., 2002).

Diseases caused by EVs

The most recurrent complications of EVs infections are the involvement central nervous system (CNS) (Moore, 1982; Jubelt and Lipton, 2014). The CNS disease syndromes caused by EVs are diverse and each syndrome could be caused by various strains of EVs. The neurological disorders caused by specific strains of EVs are listed in Table 14. EVs associated CNS disease syndromes are discussed below.

Aseptic meningitis (AM)

The most common neurological disorder caused by EVs is aseptic meningitis (AM) (Grist et al., 1978; CDC, 1981; Moore, 1982). However, EVs also cause viral meningitis (VM) (Chonmaitree et al., 1982a, b; Pönkä and Pettersson, 1982; Nicolosi et al., 1986; Kupila et al., 2006). Previously it was believed that AM is a type of nonparalytic poliomyelitis but the majority of the cases are caused by nonpolio EVs (Johnson et al., 1960). Centers of Diseases Control (CDC) gives a surveillance report on AM in 1976 which shows that around 83% of cases are of viral meningitis (CDC, 1979). The primary causative viruses for AM are coxsackievirus, echovirus and EV 71 (Schmidt et al., 1974a, b; Grist et al., 1978; Lerner et al., 1978; Singer et al., 1980; CDC, 1981; Chonmaitree et al., 1981a, b, 1982a, b; Thivierge and Delage, 1982; Moore et al., 1983; Morens et al., 1991; CDCP, 2010; Jeong et al., 2010). The major symptoms associated with AM are fever, headache, meningeal signs, CSF pleocytosis, and sterile bacteriologic cultures. VM is the most common AM and there is an inflammatory reaction in meninges which is due to the replication of the virus in meningeal and ependymal cells. VM results in the nonspecific flu like illness that consists of various symptoms including low grade fever, sore throat, malaise, anorexia, nausea, vomiting, diarrhea, headache and photophobia. Also, there are some specific nonneurological

Table 14 Neurological disorders caused by EVs (Jubelt and Lipton, 2014).

<i>Virus strain</i>	<i>Disorder</i>
PV (1–3), Coxsackieviruses (A1–11, 14, 16–18, 22, 24, B1–6), Echoviruses (1–7, 9, 11–21, 24, 25, 27, 29–33), Enterovirus (EV 71), Parechoviruses (1,2)	Aseptic meningitis
PV (1–3), Coxsackieviruses (A2, 4–9, B1–6), Echoviruses (2–4, 6, 7, 9, 11, 14, 17–19, 25), Enterovirus (EV 71)	Encephalitis
Coxsackieviruses (A16, B1), Enterovirus (EV 71), Echoviruses	Rhombencephalitis/ Brainstem
PV (1–3), Coxsackieviruses (A2–4, 6–11, 14, 16, 21, B1–6), Echoviruses (1–4, 6, 7, 9, 11, 13, 14, 16, 18–20, 30, 31)	Paralytic disease
Enterovirus (EV 70, 71), PV (1,3), Coxsackieviruses (A2, 4, 7, 9, B1–6)	Acute cerebellar ataxia
PV (1–3), Coxsackieviruses (A10, 85), Echovirus (4), Enterovirus (EV 70)	Isolated cranial nerve palsies
PV (1–3), Coxsackieviruses (A15, B3), Echoviruses (2, 3, 5, 7, 9, 11, 15, 17–19, 22, 24, 25, 27, 29, 30, 33)	Chronic infections

syndromes including rashes, pleurodynia or pericarditis that may occur. The fever usually ranges from 38 °C to 40 °C and the meningeal symptoms may vary from neck pain, stiffness to severe nuchal rigidity. The symptoms for AM resolves in 1 week but malaise and fatigue may remain for various weeks. Some bacterial, fungal and parasitic infections may also include in the differential diagnosis of EV meningitis (Klawans and Goetz, 1978). The viruses (i.e., herpes simplex, arboviruses (toga- and bunyaviruses), lymphocytic choriomeningitis) which are causing childhood infections may also cause AM (CDC, 1979; Beghi et al., 1984; Jubelt, 2010a). Sarcoidosis and meningeal carcinomatosis are noninfectious causes that should also be taken into consideration for the diagnosis of AM. Nonviral infectious diseases could also present clinically as meningitis that is associated with a CSF mononuclear pleocytosis. The nonviral infections include less invasive or subacute bacterial infections (i.e., brucellosis, mycoplasma, listeria), bacterial meningitis, parameningeal infections, leptospirosis, secondary syphilis, Lyme disease, tuberculosis (TB), rickettsial infections, fungal meningitis (i.e., cryptococcosis, candidiasis, coccidioidomycosis) and parasitic infections (i.e., cysticercosis, angiostrongylus eosinophilic meningitis) (Editorial, 1978; Johnson, 1998; Jubelt, 2010b, c). The development of these nonviral infections results in the decrease of CSF glucose that helps in the clarification of the diagnosis and this differential diagnosis can be combined with stains, cultures and nucleic acid amplification of the CSF to ensure the disease and causative agent (Lee and Davies, 2007; Archimbaud et al., 2009). Noninfection diseases such as carcinomatous meningitis, collagen vascular meningitis and sarcoidosis could also be present as AM (Johnson, 1998).

Encephalitis

The second most common neurological disease caused by EVs is encephalitis (CDC, 1981; Yang et al., 2005). During the outbreak of EVs infection, it has been found that every fourth case of encephalitis of known cause (CDC, 1982; Frantzidou et al., 2008). The causative agents of encephalitis are coxsackieviruses, echoviruses and EV 71 (Horstmann and Yamada, 1968; Grist et al., 1978). The word encephalitis is used when there is a clinical proof of the involvement of the brain. In EV encephalitis there is a direct invasion of the virus. This invasion results in the damage of parenchyma along with neurons cytolytic infection and is called primary encephalitis. Secondary encephalitis is postinfectious encephalomyelitis and it is the immune mediated process. There is the involvement of meninges in the patients of encephalitis so, therefore, the accurate term to used is meningoencephalitis. There are symptoms of AM (i.e., fever, headache, nuchal rigidity) with proof of the involvement of parenchymal cells. This proof of the involvement of parenchymal cells includes the seizures, focal neurologic signs and alteration of mental status (Howe and Wilson, 1959; Melnick, 1965; Horstmann and Yamada, 1968; Grist et al., 1978; Morens et al., 1991). The changes in mental status are of mild type but strange behavior or coma could occur (Sells et al., 1975; Acharya et al., 2001; Cree et al., 2003). The clinical manifestations occur during EV encephalitis are mild and transient, therefore, it is mild with good recovery.

EV encephalitis differential diagnosis includes various similar viruses that are causative agents for AM (i.e., measles, mumps, varicella, rubella), arboviruses (i.e., toga- and bunya-viruses), and herpes simplex) and other viruses (i.e., adenovirus, cytomegalovirus, Epstein-Barr virus, herpes zoster) (CDC, 1979; Koskiniemi and Vaheri, 1982; Beghi et al., 1984). Various nonviral infections such as mycoplasma, legionnaire's diseases, Lyme disease, leptospirosis, syphilis, brucellosis, subacute bacterial endocarditis, brain abscess Rocky Mountain spotted fever, toxoplasmosis, cysticercosis, amebiasis, schistosomiasis, cysticercosis, amebiasis, schistosomiasis, malaria, trypanosomiasis echinococcus and trichinosis are also included in the differential diagnosis of encephalitis (Ho, 2000; Johnson, 1998; Jubelt, 2010a, b, c). Vasculitis, sarcoid and gliomatosis are noninfectious diseases that have to be considered in the differential diagnosis of encephalitis (Johnson, 1998). EV 71 is a causative agent of rhombencephalitis/brainstem encephalitis in the pandemic of HFMD in the Asia Pacific region for the last 15 years. It has been found that when polioviruses cause encephalitis it involves the brainstem with the reticular formation damage including bulbar polio as compared to that of the cortex (Baker, 1949; Howe and Wilson, 1959; Plum and Swanson, 1959). In the majority of bulbar poliomyelitis cases extremity paralysis has also been found to occur. Few other cases of brainstem encephalitis are caused by echovirus, coxsackieviruses A16 and B1 (Kuntzer et al., 1987; Goto et al., 2009; Brecht et al., 2010).

Paralytic disease

Poliomyelitis is rare in the developed countries with the development of killed (1955) and live vaccines (1961) (Paul, 1971a, b; CDC, 1981). 118 cases of paralytic poliomyelitis have been reported in 1975–1984 and these cases were majorly caused by live vaccines (CDC, 1986a, b; Nkowane et al., 1987). Poliomyelitis is derived from Greek words polios (gray) and myelon (marrow). It is caused by poliovirus that infects anterior horn motor neurons of the spinal cord gray matter. During the preparalytic period (24–48 h) before paralysis, there is the onset of minor illness including fever, headache, nausea, vomiting, malaise and sore throat. The major illness during poliomyelitis comprises of all CNS diseases [i.e., nonparalytic polio, aseptic meningitis (AM), polioencephalitis, bulbar polio, paralytic poliomyelitis] caused by the poliovirus. Before paralysis, polioencephalitis along with tremulousness, agitation and upper motor neuron (UMN) signs (i.e., hyperactive deep tendon reflexes, spasticity, Babinski signs) (Jubelt and Cashman, 1987) may also present. Radicular pain, muscle pain, cramping and fasciculation also occur before the onset of paralysis (Morens et al., 1991; Melnick and Ledinko, 1953; Horstmann, 1963).

Spinal poliomyelitis is more common than the involvement of the brainstem and it has been found that the lumbar region is often more affected than the cervical region. Therefore, the paralysis is asymmetric, flaccid, more proximal than distal and spotty (Howe and Wilson, 1959). It accounts for around 5% of clinical infections and 0.1–2.0% of enterovirus infections (Melnick and Ledinko, 1953; Horstmann, 1963). Paralysis leads to bulbar involvement along with weakening respiration and spinal poliomyelitis may often cause autonomic dysfunction (i.e., hyper or hypohidrosis, low limb temperature), urinary retention, sensory

complaints and rare sensory signs (Plum, 1956; Howe and Wilson, 1959). It has been found that in children bulbar polio without paralysis is more common, however, in adult's bulbar involvement with limb paralysis also present. Cranial nerves i.e., 7, 9, 10th are affected which results in facial weakness and difficulty in swallowing.

There are 10–15% paralytic cases of bulbar poliomyelitis (Howe and Wilson, 1959) and it involves the cranial nerves and the medullary reticular formation (Peart, 1949; Olin, 1952; Weinstein, 1957). Bulbar poliomyelitis is more common in children without paralysis of a limb, however, there may be paralysis of a limb. The common affected cranial nerves are 7, 9 and 10th which lead to facial weakness and difficulty in swallowing (Aycok, 1942; Anderson and Rondeau, 1954; Ogra, 1971). The 5 and 12th cranial nerves are less commonly affected which results in the weakness of muscles of mastication and tongue. The formation of reticular may results in irregularities in breathing, swallowing and cardiovascular system (i.e., hypertension, hypotension, cardiac arrhythmias, etc.) (Levinson et al., 1945; Russell, 1949; Hill and Knowelden, 1950; Horstmann, 1950; Ben-Efraim and Long, 1957; Guyer et al., 1980; Jubelt and Lipton, 2014).

Nonpolio EV has also been found to cause paralytic disease, however, it is not common. In the year from 1976 to 1979, 52 cases of paralytic disease caused by EV in the USA, 25 cases were caused by PV, 18 by echoviruses, 7 by coxsackieviruses and 2 by EV 71 (CDC, 1981). Coxsackievirus, echovirus, EV 70 and 71 are the causative agents of paralytic disease. EV 70 was the causative agent of the acute hemorrhagic conjunctivitis (AHC) epidemic throughout the world. AHC first occur in Asia and Africa but afterward spread to Latin America and the USA (Patriarca et al., 1983). It has been found that in 10,000–15,000 cases there is one case of AHC complicated by neurological disorder (Hung and Kono, 1979; Wadia et al., 1983a, b). The paralytic disorder caused by EV 70 was referred to as radiculomyelitis because of the pain and paresthesias (Hung et al., 1976; Hung and Kono, 1979; Katiyar et al., 1983; Wadia et al., 1983a, b; Vejajiva, 1989). EV 71 was isolated in the USA (1969) is associated with HFMD and AM (Schmidt et al., 1974a, b; Deibel et al., 1975). A severe epidemic caused by EV 71 in Bulgaria and this infection is characterized by poliomyelitis like paralytic disease (Melnick et al., 1979). All EVs should be included in the differential diagnosis of paralytic disease and various other viruses (i.e., rabies, herpes zoster, West Nile virus, flaviviruses, etc.) could also cause acute lower motor neuron paralysis (Thomas and Howard, 1972; Chopra et al., 1980; Jeha et al., 2003).

Cranial nerve palsies

It has been found that cranial nerve palsies (CNP) are part of bulbar poliomyelitis and the CNP always occurs with EV infections. EV 70 has been found to cause CNP along with neurological complications and the patients have also been found to possess limb paralysis (Katiyar et al., 1983; Wadia et al., 1983a, b). It has been found that one fourth of patients have isolated CNP with neurological involvement but in some epidemics, the percentage of isolated CNP is very low (Hung and Kono, 1979; Katiyar et al., 1983; Wadia et al., 1983a, b). Patients suffering from isolated CNP have been found to affect the facial nerve accompanied by motor trigeminal nerve. Cranial nerves such as 2, 3, 4, 6, 8, 9, 10, 11 and 12th are affected in isolated CNP and isolated facial nerve palsies have been found to occur after poliovirus, coxsackievirus and echovirus infections (Weiner, 1978). Rhombencephalitis caused by EV 71 shows the involvement of cranial nerves and the most commonly involved cranial nerves are 3, 4, 6, 7, 9, 10 and 12th (Huang et al., 1999; Ooi et al., 2010).

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The Caliciviridae Family

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Introduction

The *Caliciviridae* family is composed of small non-enveloped icosahedral, positive-sense RNA viruses that infect a wide range of vertebrates including mammals, birds, and fish (Vinjé et al., 2019); moreover, some unclassified caliciviruses have been detected in reptiles and amphibians (Sandoval-Jaime et al., 2012; Smith et al., 1986). Caliciviruses, classified in 11 genera, cause a broad range of species-specific gastrointestinal, respiratory, vesicular, and severe systemic infections.

The majority of the members of this family do not replicate well in cell culture systems, which has caused lags in the knowledge of its life cycle; however, their biology and pathogenesis have been mostly studied using the cultivable Feline calicivirus (FCV) and murine norovirus (MNV) models, and some of the developed reverse genetic approaches. The present chapter aims to provide an overview of the members of the *Caliciviridae*, including animal and human genera.

Taxonomy

The first report of a calicivirus was published in 1957 (Fastier, 1957) when FCV was isolated using tissue cultures, although it was initially classified as a feline picornavirus. Its structure was later defined by transmission electron microscopy (Peterson and Studdert, 1970). However, the first calicivirus identified was Norwalk virus (NV), a human norovirus (HuNoV) first isolated in 1968 from a human stool sample and reported in 1972 as a causative agent of gastroenteritis.

Today, we know that caliciviruses infect a wide variety of vertebrates from fish to humans, causing diverse pathologies. Of the 11 genera that conform the *Caliciviridae* family, the first well established genera were *Norovirus* and *Sapovirus* that contain viruses that infect humans, known as Human caliciviruses (HuCVs); other genera are *Vesivirus*, associated with respiratory and vesicular diseases in wild and domestic cats, as well as vesicular exanthema in swine (Edwards et al., 1987); *Lagovirus* that cause hemorrhagic diseases in lagomorphs such as rabbits and hare (Mahar et al., 2019) and *Nebovirus* that cause gastroenteritis in cattle. The six additional genera recently accepted, were named *Bavovirus*, *Minovirus*, *Nacovirus*, *Recovirus*, *Salovirus*, and *Valovirus* (Vinje et al., 2019) (Table 1). Most of these novel caliciviruses were identified and isolated from poultry and fish farming specimens, but few studies have been done searching for caliciviruses in wild animals (Sandoval-Jaime et al., 2012; Smith et al., 1986), suggesting that there are more possible genera to be discovered and described.

Study of the biology of these novel caliciviruses can have a positive impact in the farming industry, as specific diagnosis protocols, antiviral treatments and/or vaccines can reduce morbidity and mortality in livestock and the subsequent economic losses

Table 1 Representative members of the *Caliciviridae* genera.

Genus	Representative virus	Natural host	Pathology	References
<i>Bavovirus</i>	Bavaria virus	Chicken	Possibly diarrheal disease	Wolf et al. (2011)
<i>Lagovirus</i>	Rabbit hemorrhagic disease virus	Rabbit	Highly lethal liver infection	Liu (1984)
<i>Minovirus</i>	Fathead minnow calicivirus	Fathead minnow	Possible hemorrhage in skin, fin bases and eyes	Mor et al. (2017)
<i>Nacovirus</i>	Turkey calicivirus	Turkey	Gastroenteritis	Wolf et al. (2012)
<i>Nebovirus</i>	Newbury-1 virus	Bovine	Gastrointestinal infection	Kapikian et al. (1972) and Oliver et al. (2006)
<i>Norovirus</i>	Human norovirus, Murine norovirus	Humans, Mouse	Gastrointestinal infection	Karst et al. (2003)
<i>Recovirus</i>	Tulane virus	Rhesus monkeys	Gastrointestinal infection	Smiley et al. (2002)
<i>Salovirus</i>	Atlantic salmon calicivirus	Atlantic salmon	Possible heart and skeletal muscle inflammation	Mikalsen et al. (2014)
<i>Sapovirus</i>	Porcine sapovirus, Human sapovirus	Pigs	Gastrointestinal infection	Guo et al. (2001)
<i>Valovirus</i>	St-Valérien-like virus	Pigs	Apparent asymptomatic	L'Homme et al. (2009)
<i>Vesivirus</i>	Feline calicivirus	Household cat	Upper respiratory system infection/Systemic infection by some strains	Fastier (1957)

and zoonotic risks; moreover, characterization of new caliciviruses can provide new information of their biology and possible new study models to understand HuNoV infection.

For *Norovirus* classification, there are 2 different, complementary systems based on VP1 amino acid or RNA dependent RNA polymerase (RdRp) nucleotide sequences that result in the genogroups and genotypes or P-groups and P-types, respectively. To these days, there are 49 genotypes belonging to 10 genogroups (GI-X) and 60 accepted P-types. Future norovirus nomenclature updates will require the full genome sequences to determine both genotype and P-type (Chhabra et al., 2019).

Molecular biology

Capsid and genome

The *Caliciviridae* family is composed of non-enveloped icosahedral viruses from 27 to 40 nm in diameter. Their capsids consist of 180 copies of the major structural protein VP1 arranged in dimers that form an icosahedral $T = 3$ shell (Prasad et al., 1999; Venkataram Prasad et al., 2000). When expressed in in vitro systems, this protein spontaneously assembles into virus-like particles; however, co-expression with the minor structural protein VP2 has demonstrated that this protein interacts with VP1 for the formation of the viral capsids (Di Martino et al., 2007).

Caliciviruses have a single-stranded and positive-sense genomic RNA (gRNA) of 6.4–8.5 kilobases (Kb) in length, which is covalently linked to the viral genome-linked protein (VPg) to its 5' end; the genome at its 3' end is polyadenylated.

The viral genomes are organized in either two or three open reading frames (ORF) depending on the genus. The non-structural (NS) proteins are always encoded from the first ORF; while the major VP1 and minor VP2 structural proteins from the members of the *Vesivirus*, *Norovirus*, and *Recovirus* genus are encoded from the second and third ORFs; in the members of the *Bavovirus*, *Lagovirus*, *Minovirus*, *Nacovirus*, *Nebovirus*, *Salovirus*, *Sapovirus*, and *Valovirus* genus, VP1 is encoded in-frame and contiguous with the NS proteins, and VP2 is encoded from the second ORF (Vinjé et al., 2019). Particularly in the members of the *Vesivirus* genus, the second ORF encodes the precursor of the VP1 protein that is further cleaved into the mature VP1 and a smaller protein, named leader of the capsid (LC) that is cytopathic and responsible for the induction of apoptosis (Abente et al., 2013; Barrera-Vazquez et al., 2019). A fourth ORF has been reported in the murine norovirus (MNV), a member of the *Norovirus* genus, that encodes for the virulence factor-1 (VF-1) (McFadden et al., 2011); moreover, an equivalent fourth ORF is present in human sapoviruses, although the encoded proteins are yet of unknown function (Chanit et al., 2009) (Fig. 1).

Besides the gRNA, a 5'-end VPg-linked and polyadenylated subgenomic RNA (sgRNA) containing the second and third ORFs is synthesized late in infection and drives the production of VP1 and VP2 structural proteins. However, evidence has published indicating that VP1 can also be produced from the genomic RNA at early times of the viral replication cycle even when the VP1 encoding region is not in the ORF1, as in *Norovirus* and *Vesivirus* (McCormick et al., 2008; Urban and Luttermann, 2020).

The encapsidation of the gRNA and the sgRNA has been documented during RHDV infection (Meyers et al., 1991).

Genomic RNAs

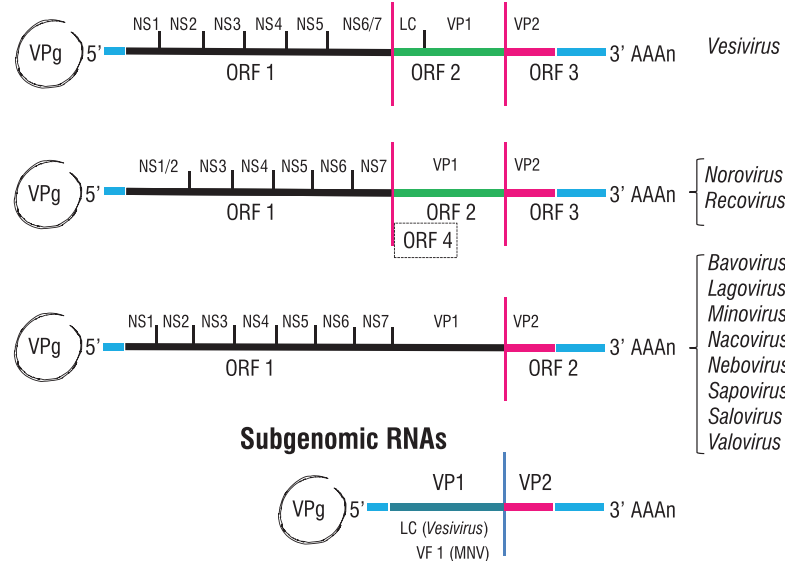


Fig. 1 Calicivirus genome organization. Schematic representation of the genomic organization in the different genera of the *Caliciviridae*. Both the genomic and subgenomic RNAs have the Vpg protein covalently-linked to the 5' ends and are polyadenylated at their 3' ends. Depending on the genus, the genome contains from 2 and up to 4 ORFs flanked by short untranslated regions (blue lines). ORF1 always encodes for a polypeptide that is cleaved into six non-structural (NS) proteins, and in some genus, for the VP1 protein. ORF2 and ORF3 encode for the major and minor structural VP1 and VP2 proteins from the sgRNAs, respectively. A fourth ORF is contained only in the MNV genome and encodes for the VF1 protein from the sgRNA. The members of the *Vesivirus* genus encodes for a precursor of the VP1 protein that is further cleaved to produce the leader of the capsid (LC), and the mature VP1 proteins.

Calicivirus replication cycle

Early events: Binding, entry, and uncoating

The viral replication cycle of caliciviruses, start with the interaction of the viral particle to attachment/binding and entry factors; many of them carbohydrate ligands, glycolipids, and proteins present in the surface of the cell (reviewed in: [Graziano et al., 2019](#)) (see “Calicivirus receptors” section).

Following binding to the cell surface receptors, caliciviruses entry into the cells is mediated by endocytic pathways. FCV entry is both clathrin-dependent and pH-dependent, as capsid uncoating and genome release into the cytoplasm requires the acidic environment of the endosome; in contrast, MNV entry is dependent on cholesterol and dynamin and was reported to be pH-independent ([Gerondopoulos et al., 2010](#); [Perry et al., 2009](#)). Recently, endosomal acidification and cathepsin L activity were reported to be required for FCV, porcine enteric calicivirus (PEC) and MNV entry events ([Shivanna et al., 2014b](#)). If the differences in the endocytic pathway are virus-specific, host species-specific or cell type-specific is still unknown ([Graziano et al., 2019](#)).

Endosomal escape and viral uncoating end in the release of the viral genome into the cytoplasm, a process poorly understood for the *Caliciviridae* members; however, a mechanism of FCV genome release has been recently described ([Conley et al., 2019](#)). The minor FCV capsid protein VP2 forms a portal-like assembly at a threefold axis of symmetry after the feline junctional adhesion molecule (fJAM-1) receptor engagement. This assembly is formed of 12 copies of VP2 arranged with their hydrophobic N termini pointing away from the virion surface, leading to the opening of a pore in the capsid shell that presumably acts as a channel for the delivery of the genome into the cytoplasm ([Conley et al., 2019](#)).

Genome translation and replication

Once the calicivirus genome reaches the cell cytoplasm, the synthesis of a large polyprotein occurs by a VPg-mediated translation initiation process. Both canonical eukaryotic initiation of translation factors (eIFs), as well as non-canonical factors, participate in the initiation and regulation of translation ([Chaudhry et al., 2006](#); [Daughenbaugh et al., 2003, 2006](#); [Goodfellow, 2011](#); [Goodfellow et al., 2005a](#); [Gutierrez-Escolano, 2014](#); [Hernandez et al., 2016](#); [Hosmillo et al., 2014](#); [Karakasiliotis et al., 2010](#)).

The polyprotein is further processed by the only viral protease to produce 6 NS proteins: NS1/2, the helicase NS3, NS4, and NS5 or VPg, and the protease NS6 and the RNA dependent RNA polymerase (RdRp) NS7. Particularly, during *Vesivirus* infection, NS1 and NS2 are excised as separated proteins, while NS6/7 the protease-polymerase does not undergo additional processing ([Sosnovtsev et al., 2002](#)); in the case of the other caliciviruses, NS6 and NS7 are fully processed into independent proteins ([Emmott et al., 2019](#); [Oka et al., 2005](#); [Sosnovtsev et al., 2002, 2006](#)). The function of the NS proteins is partially conserved in the *Caliciviridae* family with, some proteins having a main described function and other possible roles in achieving efficient viral replication (Fig. 2).

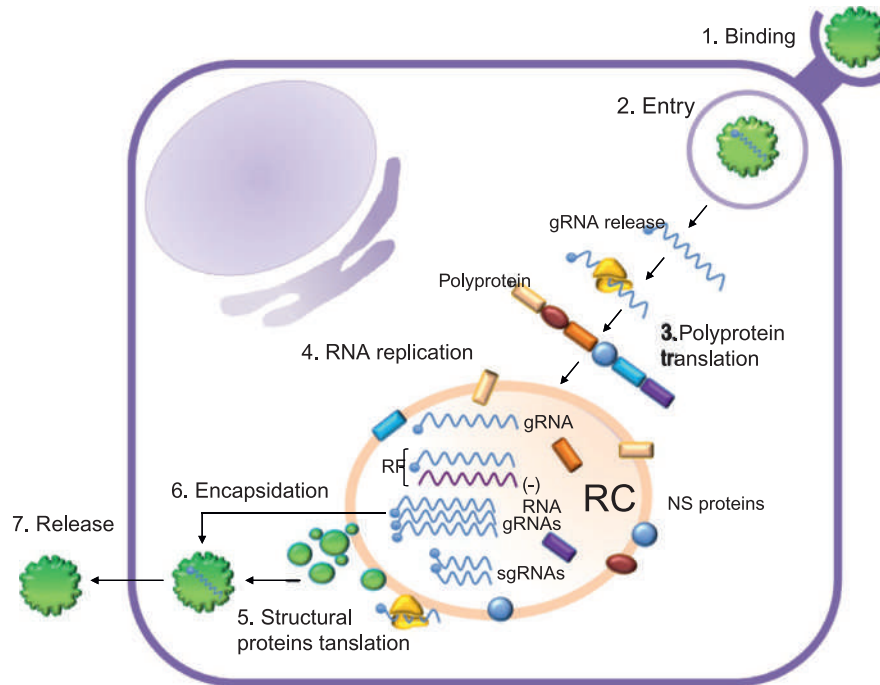


Fig. 2 Calicivirus replication cycle. The viral replication cycle starts with the binding of the viral particles to the cell surface. After the interaction with the cell receptor, the viral particles enter the cells through an endocytic pathway, followed by the genome release into the cytoplasm. Vpg mediated translation of the genomic RNA (gRNA) occurs to produce a polyprotein, which is further cleaved by the viral protease into the individual non-structural (NS) protein. The NS proteins drive the formation of membrane-delimited replication compartments (RC) where the negative sense (–) RNAs are synthesized from the gRNA. The (–) RNAs serve as a template for the synthesis of the sgRNAs and the viral progeny genomes. sgRNAs are immediately translated to produce the structural proteins that auto-assemble together with the gRNAs (encapsidation). Apoptosis induction promotes viral particles dissemination through the host. gRNA (genomic RNA); (–) RNA (negative-sense RNA); sgRNA (subgenomic RNA); RF (replicative forms); NS (non-structural proteins).

Non-structural proteins

NS1/NS2 proteins are fused in *Recovirus* and *Norovirus* genera, and have a viroporin function; the expression of NS1/2 interrupts Ca^{2+} homeostasis in TV and potentiates apoptosis and virus persistence in MNV infection (Nice et al., 2013; Robinson et al., 2019). NV NS1/2 transient expression in cells is involved in the Golgi complex disassembly and membrane rearrangement needed for the replication complexes (RC) formation (Doerflinger et al., 2017; Fernandez-Vega et al., 2004). This Golgi membrane disruption most probably occurs by the interaction of NS1/2 with vesicle-associated membrane protein-associated proteins (VAPs), as demonstrated during NV and MNV infection, resulting in the alteration of vesicular trafficking (Ettayebi and Hardy, 2003; McCune et al., 2017).

On the other hand, during FCV infection, NS2 (p32) is cleaved by the viral protease from NS1 (p5.6), producing two proteins of 32 and 5.6 kDa, respectively. While not much is known about NS1 function, NS2 is involved in membrane alteration and likely in the RC formation, as in NV and MNV infection (McCune et al., 2017).

NS3 is a conserved protein among caliciviruses whose main function is an NTPase- and RNA chaperone that uses Mg^{2+} as a cofactor. NS3 from MNV has an additional helicase activity (Han et al., 2018), whereas in other noroviruses such as the GI HuNoV Southampton, this helicase activity was not detected (Pfister and Wimmer, 2001). The HuNoV GII.4 NTPase has mitochondrial localization due to its C-terminal domain and possesses pro-apoptotic activity. NS3 from FCV, NV, and MNV induces membrane rearrangements (Cotton et al., 2017; Doerflinger et al., 2017); moreover, in both FCV and MNV infection, NS3 impairs the host immune response (Fritzlar et al., 2018; Yumiketa et al., 2016).

NS4, a protein between 22 and 32 kDa, is not highly conserved among caliciviruses, except for a conserved predicted transmembrane domain. This protein has different function(s) in distinct viral genera; NS4 from FCV (p30) and NV (p22) induces membrane rearrangements, (Bailey et al., 2010; Doerflinger et al., 2017) while NS4 (p22) from HuNoV and MNV hamper the cellular secretory pathway (Sharp et al., 2012) likely interrupting the COPII-coated vesicle trafficking that results in the Golgi apparatus disassembly (Sharp et al., 2010). Inhibition of protein secretory pathway or Golgi disruption is not induced by FCV NS4, suggesting that these are only conserved functions among noroviruses.

NS5, also known as VPg (for virion protein, genome-linked), is the genome-linked protein to the 5' of the viral genomic and sgRNAs (Goodfellow et al., 2005b). Its main described function is the recruitment and assembly of the host translation machinery, working as a proteinaceous cap that promotes calicivirus translation. The differential interactions of canonical translation factors with VPg have been demonstrated in *Norovirus* (Chung et al., 2014; Daughenbaugh et al., 2006; Leen et al., 2016) *Vesivirus*, (Goodfellow et al., 2005a), *Sapovirus* (Hosmillo et al., 2014) and *Lagovirus* genera (Chaudhry et al., 2006; Zhu et al., 2015).

Besides participating in calicivirus genome translation, HuNoV VPg has a role in the RNA replication; VPg is nucleotidylated in its tyrosine 27 residue by the action of the RdRp or its precursor, priming the replication of gRNA (Belliot et al., 2008; Rohayem et al., 2006). Nucleotidylation seems to be dependent on NTP binding, appears to be more efficient when the immature protease-polymerase interacts with VPg and involves a 3' unidentified element (Medvedev et al., 2017).

Another reported function of VPg is the cell cycle arrest leading the cells to a G0/G1 stage through its N-terminal domain (Davies and Ward, 2016); this function seems to be both independent of its role in RNA translation and replication and conserved in distinct calicivirus genera (McSweeney et al., 2019). Since this protein is also found in virus particles attached to the genome, it has been suggested to be categorized as a structural protein (Smertina et al., 2019).

The NS6 protein is the only protease codified by caliciviruses. It is also known as the 3C-like proteinase because of the similarity found with the picornaviral 3C protease when it was first characterized. This viral protease has conserved catalytic residues (Oka et al., 2007), and is responsible for the processing of the viral polyprotein into the different mature NS proteins (Liu et al., 1996; Oka et al., 2005; Sosnovtsev et al., 2006; Würblich et al., 1995). In vesiviruses like FCV, where the NS6/7 proteins are fused (Wei et al., 2001), this protease-polymerase also cleaves its capsid protein precursor (Sosnovtsev et al., 1998). The proteolytic processing of the NS proteins follows a particular order determined by the protease enzyme affinity to its cleavage sites (Emmott et al., 2019; May et al., 2014). The structure of the protease NS6 from distinct caliciviruses has been determined, providing insight on 3D conformation of both protease and protease-substrate protein complex, with possible treatment implications (Galasiti Kankana-malage et al., 2015; Leen et al., 2012; Nakamura et al., 2005).

Besides cleaving the viral polyprotein for the generation of the mature NS proteins, the calicivirus proteases cleave cellular proteins to favor the establishment of the infection (Emmott et al., 2017; Kuyumcu-Martinez et al., 2004). For example, FCV NS6/7 protein induces cellular transcription shutoff through its N-terminal domain (Wu et al., 2016), while RHDV NS6 protein induces apoptosis (Chen et al., 2018).

NS7 is the conserved RdRp, involved in the production of all the viral RNAs, such as the negative-sensed RNA replication intermediate, as well as the genomic and the sgRNAs from which the structural proteins VP1 and VP2 are synthesized to encapsidate the genomes of the viral progeny. The initiation of RNA synthesis is dependent on NS7 nucleotidylation of VPg, as mentioned above. The low fidelity of the RdRp may contribute to viral transmission efficiency through an increased genetic diversity (Arias et al., 2016), and it is known that GII HuNoVs RdRp is phosphorylated by the Akt cellular kinase (Eden et al., 2011) and in general, it is known that NS7 proteins from diverse calicivirus form a multiproteic complex with viral and cellular proteins that have a role in viral replication cycle (Cancio-Lonches et al., 2011; Santos-Valencia et al., 2019; Vashist et al., 2015). In RHDV, the NS7 protein uniquely induces the rearrangement of Golgi apparatus membranes (Urakova et al., 2017a) through a partially hidden hydrophobic motif (Urakova et al., 2017b) independent of its polymerase function, possibly contributing to the RC complexes formation. The structural dynamics, biochemical properties, kinetics, and putative interaction partners of the calicivirus RdRps has been recently reviewed (Smertina et al., 2019).

In addition to the role of NS proteins in the RNA translation and replication, several cellular proteins, also known as non-canonical factors, participate in regulating calicivirus replication. The polypyrimidine tract binding protein binds to the 5' end of the FCV genome and functions as a negative regulator of its translation most probably to promote the synthesis of viral RNA (Karakasiliotis et al., 2010). Moreover, nucleolin (NCL) has been reported to bind to the NV and FCV 3' untranslated regions and is most probably part of the FCV translational complex; its N-terminal region is required for an efficient FCV replication (Cancio-Lonches et al., 2011; Hernandez et al., 2016). On the other hand, the poly C binding protein (PCBP)-2 and the heterogeneous nuclear ribonucleoprotein (hnRNP)-A1, bind to the 5' and the 3' ends of the MNV-1 viral RNA and contribute to RNA circularization, playing a role in the virus replicative cycle (López-Manriquez et al., 2013).

The production of the NS proteins contribute in the formation of the replicative complexes (RC), membrane-delimited compartments derived mostly from the Golgi apparatus and the Endoplasmic Reticulum (ER) (reviewed in Penaflor-Tellez et al., 2019), where the synthesis of the genomic and subgenomic RNAs through the production of a negative-sense RNA template occur by the action of the RdRp (Smertina et al., 2019).

While the major function of the gRNAs is to form the genomes of the viral progeny, the sgRNAs are immediately translated to produce the structural proteins VP1 and VP2.

Structural proteins

VP1, known as the major viral protein, is a 58–60 kDa protein divided into three domains: the N-terminal arm (NTA) domain, the highly conserved shell (S) domain that consists of an eight-chain anti-parallel β -sandwich with two α -helical decorations, and the protruding (P) domain. The NTA and S domains are located in the inner surface of the capsid that surrounds the viral RNA. The P domain can be divided into two subdomains (Choi et al., 2008); the P1 subdomain that consists of a single α -helix and eight β -strands and is connected to the S domain by a flexible stem-like hinge, and the P2 subdomain that appears as an insert in P1 (137 amino acids, 278–415) that forms a six-chain anti-parallel β -type antiparallel structure. The P2 subdomain interacts with its respective cell receptor and it is a target for neutralizing antibodies (Choi et al., 2008). The minor capsid protein VP2 is a 8–12.2 kDa basic protein, highly variable in sequence between genera, that is essential for virus infectivity and is incorporated into the virion at a low copy-number providing capsid stability (Bertolotti-Ciarlet et al., 2003; Sosnovtsev et al., 2005) (Fig. 3).

Late steps: Apoptosis induction, encapsidation, and virus release

Increasing evidence indicates that besides their role in the RC formation, viral NS proteins induce intrinsic apoptosis in the infected cells (Chen et al., 2018; Herod et al., 2014; Robinson et al., 2019); a process required for the establishment of a successful infection

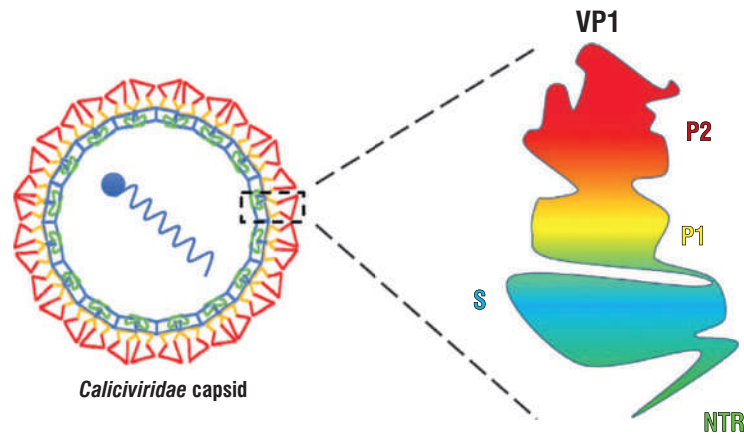


Fig. 3 Calicivirus capsid structure. Schematic representation of the *Caliciviridae* capsid structure. Ninety dimers of VP1 make up the viral capsid, which contains the Vpg-linked genomic RNA (Blue). Schematic representation of the VP1 structure: the hypervariable P2 domain (orange) responsible for the interaction with the cell receptor, the conserved P1 domain (yellow) that surrounds the P2 domain, and the S domain or N-terminal region (NTR) (blue-green) located in the inner side of the viral capsid.

also related to immunopathogenesis (reviewed in: [Penaflor-Tellez et al., 2019](#)). Moreover, recent evidence suggests that apoptosis may also participate in the translation suppression of interferon-stimulated genes (ISG) as a mechanism to evade the innate immune response ([Emmott et al., 2017](#)).

Interestingly, the LC protein, unique in the *Vesivirus* genus and produced later in infection as a consequence of the proteolytic processing of the precursor of the capsid protein VP1, appears to be responsible for apoptosis induction in FCV infected cells ([Abente et al., 2013](#); [Barrera-Vazquez et al., 2019](#)). Furthermore, the unique MNV protein VF1 can also modulate apoptosis ([McFadden et al., 2011](#)). These findings correlate with the previous association of apoptosis with the dissemination of the viral progeny in the host ([Alonso et al., 1998](#); [Bok et al., 2009](#)). Thus, once both the mature structural protein VP1 and VP2 are produced, they are immediately self-assembled together with the genomic RNAs to form the infective viral particles, ready to leave after cell lysis the infected cells and propagate into the host. A non-lytic release of human norovirus particles associated with exosomes into stools has been recently reported, representing another mechanism of viral particles liberation that may contribute to person-to-person transmission ([Santiana et al., 2018](#)).

Calicivirus receptors

Viruses to infect must adhere to the cell surface; thus, identify the cellular proteins that serve as viral receptors used by each virus allows to better understand its target cells, the species barriers, the viral entry mechanisms, and thus, the possible key points to block the viral infection.

While some molecules of different origin have been identified to participate in caliciviruses entry, few molecules of protein nature are known to act as their functional receptors.

Histo-blood group antigens (HBGA) are polymorphic carbohydrate structures that represent terminal exposed portions of larger glucans O- or N bound to proteins or glycolipids, that have been widely reported to participate in the cell binding of several members of the family *Caliciviridae*; such as Tulane virus (TV) ([Farkas et al., 2010](#)), Rabbit hemorrhagic disease virus (RHDV) ([Lopes et al., 2018](#); [Nystrom et al., 2011](#)), Canine norovirus (CaNoV), Bovine nebovirus (BNeV) ([Cho et al., 2018](#)) as well as MNV ([Almand et al., 2019](#)) and HuNoV ([Almand et al., 2017](#)). Bile salts such as glycochenodeoxycholic acid (GCDCA) and lithocholic acid (LCA) are also known to interact with the P domain of the VP1 dimer, stabilizing its binding to cellular receptors. Besides GCDCA, divalent cations like calcium and magnesium can also stabilize the binding of VP1 with the MNV CD300lf receptor ([Nelson et al., 2018](#)), and sialic acid participates in MNV ([Taube et al., 2009](#)) and FCV binding ([Stuart and Brown, 2007](#)).

Four proteic molecules have been identified as receptors for five caliciviruses.

1. The feline Junctional adhesion molecule 1 (fJAM-1), an immunoglobulin-like transmembrane glycoprotein of 40 kDa that regulates tight junctions in epithelial and endothelial cells, and also considerably expressed in platelets and to a lesser extent in leukocytes ([Pesavento et al., 2011](#)), is the main receptor for FCV 1. Transfection of fJAM-1 in non-permissive non-lung hamster cells (HmLu-1), led to binding and infection by different strains of FCV ([Makino et al., 2006](#)). The human junctional adhesion molecule 1 (hJAM1) has been described as the human vesivirus Hom-1 functional receptor ([Sosnovtsev et al., 2017](#)). Other junctional molecules such as occludin have been reported to function as a coreceptor for the Porcine sapovirus (PSaV) entry into LLC-PK cells, while claudin-1 facilitates its entry and infection ([Alfajaro et al., 2019](#)),

2. Coxsackievirus and adenovirus receptor (CAR), is an immunoglobulin-like protein (Heath et al., 1997) of 38–40 kDa, located in the tight junctions (Cohen et al., 2001) that participate in neuronal and cardiac physiological processes (Carson et al., 1997; Freimuth et al., 1999; Honda et al., 2000; Nalbantoglu et al., 1999; Shaw et al., 2004). CAR has been found to be the putative receptor for TV and FT285 ReC (Farkas et al., 2019). CAR knockdown, blockage, and knockout induced resistance of LLC-MK2 permissive cells to ReCV infection. In addition to the expression of CAR, HBGA type B expression into the non-permissive CHO cells is required for ReCV infection (Farkas et al., 2019).
3. CD300ld of 22 kDa and CD300lf of 32 kDa belong to the transmembrane glycoproteins CD300 family that contains an extracellular immunoglobulin variable domain (IgV); both molecules have been found to be the main receptors for MNV. Knockdown or blocking the murine CD300lf (mCD300lf) but not the human (hCD300lf), significantly reduced the entry of MNV to the macrophages cell line Raw 264.7. Even though the induction of mCD300ld or mCD300lf expression in human HEK293T cells allowed the infection with MNV (Haga et al., 2016), breaking the norovirus species barrier (Orchard et al., 2016), the huCD300lf is not essential for the entry of HuNoV into human cells (Graziano et al., 2020). Although CD300ld and CD300lf are the main receptors for MNV infection, other proteins have been suggested to participate in MNV entry, such as the cell surface glycoproteins CD44, CD36, CD968, and the transferrin receptor 1 (TfRc) (Bragazzi Cunha and Wobus, 2016). It is of interest that depending on the cell, each of the above molecules can participate in different degrees in the entry of the MNV.
4. Annexin A2 (ANXA2) is a 36 kDa calcium-dependent phospholipid-binding protein, expressed in some tumor cells, endothelial cells, macrophages, and mononuclear cells. ANXA2 has been reported to play roles in exocytosis, endocytosis, and membrane trafficking (Wang and Lin, 2014). ANXA2 was identified as one of five major proteins that bind to rabbit vesivirus (RaV) virions. Knockdown and blockage of ANXA2 in HEK293T cells caused the reduction of RaV infection and areduction of ~1.4 logs in the viral yield, supporting the role of ANXA2 in RaV attachment and/or internalization (Gonzalez-Reyes et al., 2009).

Calicivirus models of study

Surrogate models

Even though NV was identified as the etiological agent of human gastroenteritis outbreaks nearly 50 years ago, one of the main reasons why up to this date we do not know more about certain aspects of the HuNoVs replicative cycle, pathogeny, and the immune response is due to the lack of a convenient *in vitro* or *in vivo* replication model. It is not known why HuNoVs are incapable of effectively replicate in cell cultures or animal models; although, the use of new 3D cell and organoid cultures has provided promising results for its replication (Ettayebi et al., 2016; Papafragkou et al., 2013).

Due to the lack of convenient replication systems for HuNoV, several surrogate models have been developed over the years and became the principal way to study calicivirus biology and pathogenesis. These models, together with reverse genetic systems, are by far the most useful tools to study these viruses and have different advantages in cost-effectiveness and translation of results to the understanding the HuNoV biology.

RHDV, causes highly lethal liver failure, highlighting its importance in the study of veterinary and livestock (reviewed in: Abrantes et al., 2012). To this date, there is no robust cell culture system for its replication; thus, propagation of viral particles has been done through rabbit inoculation and liver tissue preparation (Teixeira et al., 2012). In addition, certain aspects of RHDV replication cycle have been studied with the use of molecular biology tools like replicons, but without obtaining high viral yields (Wang et al., 2013; Zhu et al., 2015). These study techniques have significant limitations, but over time they have been useful tools for the elucidation of crucial aspects of RHDV biology.

PSaV, one of the causative agents of gastroenteritis outbreaks in pigs, was the first surrogate model developed for calicivirus obtained by passages of the Cowden strain through the primary culture of porcine kidney cells supplemented with an intestinal content filtrate preparation from gnotobiotic pigs (Flynn and Saif, 1988). The primary cultures originally used to propagate the Cowden strain were later replaced by the porcine pig kidney continuous cell line LLC-PK. The viral sequence of VP1 and RdRp from viruses grown in LLC-PK showed that mutations accumulated in the VP1 from the viral Cowden strain resulted important for viral replication (Lu et al., 2016). Later, it was determined that bile salts in the intestinal content filtrate was the factor required for viral replication (Chang et al., 2004), likely by activating de cAMP signaling pathway (Chang et al., 2002) and participating in the virus escape from the endosomes (Shivanna et al., 2014a).

Compared to other study models, the Cowden strain of PSaV produced lower viral yields, probably due to an interferon immune response that hampers viral replication (Hosmillo et al., 2015); however, this model has the advantage of having a reverse genetic system, crucial to the study of the role of viral components in the replication cycle (Chang et al., 2005).

From the late 60s and early 70s and up to now, the most popular model to study calicivirus biology was the FCV; key aspects of calicivirus biology were first described in this model, as it replicates successfully in both primary culture epithelial kidney cells (Adldinger et al., 1969; Crandell et al., 1961) and in the Crandell feline kidney (CrFK) permissive cell line (Crandell et al., 1973), where high viral yields were produced. Another added advantage of FCV is that since 1995, several reverse genetic systems have been developed (Oka et al., 2014; Sandoval-Jaime et al., 2015; Sosnovtsev and Green, 1995).

FCV is, without doubt one of the more useful models to understand many aspects of calicivirus biology. Since vesiviruses and noroviruses have similar genome organization, FCV was initially the best reliable model to understand HuNoV infection. However, the discovery of the MNV provided a better model since it has the additional advantages of sharing cell tropism, pathology, and pathogeny with the HuNoVs.

MNV was first identified in 2003 in immunocompromised mice (Karst et al., 2003), and since then it has been established as the most reliable model for studying the molecular biology and pathogenicity of the members of the *Norovirus* genera. MNV ability to replicate in multiple tissue cultures and cell lines (Ward et al., 2006) as well as in healthy or immunocompromised mice has allowed studying norovirus replication cycle, tropism, immune response evasion, and persistence mechanisms. Moreover, several reverse-genetic systems have been rapidly developed to identify the role of MNV proteins in in vitro and in vivo contexts (Arias et al., 2012; Chaudhry et al., 2007; Ward et al., 2007; Yunus et al., 2010).

All these features make MNV, by far, the most currently used model to study *Norovirus* biology; however, the differences between the immune system of mice and humans, the presence of the unique VF1 protein in MNV, the differences in their functional receptor usage, and the fact that even between HuNoVs genotypes there are significant differences in viral protein functions are important factors to consider before assuming that MNV fully mimics HuNoVs replication cycle, pathology, and pathogeny. With this in mind, even though MNV is a powerful and extremely useful tool, the development of a HuNoV replication system is still needed.

The TV, a member of the recent-accepted *Recovirus* genera, is the most recent HuNoV surrogated model, and represent a useful system to study HuNoVs infection (Farkas et al., 2008). As naturally infects Rhesus monkeys, and cause gastrointestinal symptoms, it is strongly pointed out as a better model to study pathology and pathogeny of HuNoV than MNV. Besides infecting Rhesus monkeys, TV can efficiently replicate in the LLC-MK2 monkey kidney cell line. Phylogenetic analysis of TV strains show that recoviruses are the closest genera to noroviruses (Farkas et al., 2008), and a reverse genetic system is available to study specific viral functions (Wei et al., 2008); however, an important disadvantage of this model is the economic cost and bioethics conflicts of using Rhesus macaques as in vivo models.

HuNoVs growth attempts

Throughout the years, several efforts of growing HuNoVs have been made. Since there are no clear clues regarding why some caliciviruses grow efficiently in culture cells while others do not, attempts to establish an effective HuNoVs replication system has been mostly by trial and error and can be broadly categorized into two groups: cell culture approaches to replicate and harvest HuNoV and infection of distinct animal models with HuNoV.

HuNoV growth in cell cultures

Over several decades a wide variety of primary cultures and cell lines were tested for HuNoVs infection (Murakami et al., 2020). Meanwhile, wild type and engineered HuNoVs reverse systems were also developed, aiming to produce infectious viral progeny (Duizer et al., 2004; Lay et al., 2010). The majority of these systems are limited, but some provided information on necessary elements to achieve an efficient viral replication in cell cultures. Two of the most novel approaches are the use of 3D cell and organoid cultures, pointing to recreate the natural niche of HuNoVs infected cells in its natural host. Recreation of villi-containing 3D cultures did not achieve efficient HuNoV GI or GII viral replication (Takanashi et al., 2014), but Caco-2 cells growing in 3D cultures seemed to efficiently provide an appropriate environment to HuNoV replication (Straub et al., 2011).

Stem cell-derived human intestinal enteroids (HIEs) have served as efficient replication systems for various HuNoVs genotypes (Ettayebi et al., 2016), and has revealed the importance of bile fluids and HBGA in HuNoV infection (Murakami et al., 2020). Moreover, Hosmillo et al. (2020) recently demonstrated that the Janus activated kinase/signal transducer and activation of transcription (JAK/STAT) signaling-induced interferon pathway blocks HuNoV replication in intestinal enteroid cells (IEC) (Hosmillo et al., 2020). Inhibiting both the JAK/STAT pathway and the RNA polymerase II-dependent transcription increased HuNoV replication, resulting in a much more robust model for the study of HuNoV.

HuNoV infection and models

The study of HuNoVs has relied on volunteers (Atmar et al., 2008) and investigation of outbreaks (Fankhauser et al., 1998; Gallimore et al., 2004), and although this methodology is an efficient way to obtain high viral titers and study viral pathogeny, there are obvious ethical and practical reasons that make it problematic to say the least. Attempts to infect the most suitable animal models with HuNoVs through the oral or alternative pathways have been made with variable results (Cheetham et al., 2006; Souza et al., 2008; Wyatt et al., 1978). Recently, the infection of zebrafish *Danio rerio* embryo with HuNoV present in human stool samples represents a promising effective method to produce high viral titers (Van Dycke et al., 2019). Moreover, its tropism in the intestinal tract and hematopoietic lineage cells could signify that larvae can also serve to understand HuNoV pathogeny and pathology.

The immune response induced by caliciviruses

Innate immune response

The innate immune response to calicivirus infection is mainly mediated by the Interferon (IFN) pathway. MNV, HuNoV, and TV trigger this response through the Toll-like receptors (TLRs), melanoma differentiation-associated protein 5 (MDA-5), and the retinoic acid-inducible gene 1 (RIG-I) respectively (Dang et al., 2018; McCartney et al., 2008). During MNV infection, the innate immune response is potentiated by the IFN-induced protein with tetratricopeptide repeats 1 (Ifit1) (Mears et al., 2019), triggering

IFN-I pathway in all cases. Moreover, FCV 2280 strain initiates an IFN- β response and MNV, TV, FCV, HuNoV GII. 3, and NV are sensitive to IFN treatment (Dang et al., 2018; Enosi Tuipulotu et al., 2018; Fulton and Burge, 1985; Qu et al., 2016; Tian et al., 2015), suggesting that recombinant IFN could be used to treat diverse calicivirus infections. In this regard, IFN- ω , has been used to control FCV infections in Japan since 1994 (Minagawa et al., 1999; Yang et al., 2007).

Downstream the IFN pathway, IFN regulatory factor 1 (IRF-1) has been reported to hamper FCV and HuNoV replication in vitro (Dang et al., 2018; Liu et al., 2018).

Interestingly, the study of the IFN role in MNV replication can shed light on the viral mechanisms of spread and persistence in its host. In this regard, STAT-1 limits MNV infection to peripheral tissue in immunocompetent mice's intestine, preventing its dissemination to other organs (Mumphy et al., 2007). It is also known that infection with FCV (Foley et al., 2006), RHDV (Trzeciak-Rydzek et al., 2016) and with both noroviruses HuNoV or MNV induce the production of pro-inflammatory cytokines like Interleukin (IL)-8, tumor necrosis factor (TNF)- α , nitric oxide (NO₂) liberation, and/or IL-1 α . It is suggested that in acute HuNoV infection, a major Th1 and minor Th2 response is mainly established in infected humans (Newman et al., 2015) and gnotobiotic pigs (Souza et al., 2007), as the levels of the regulatory IL-10 cytokine also increase during infection.

As IFN can hamper calicivirus replication, caliciviruses have acquired mechanisms to impair this pathway at different steps. An interesting finding is that the FCV strains F9, Bolin, and HRB-SS, NV, and HuNoV GII.3 genotype do not trigger the IFN immune response (Qu et al., 2016; Tian et al., 2015). On the other hand, FCV F4 strain impairs the IFN response at transcriptional and post-translational levels, through the NS4 (p39) protein that, when transiently expressed, negatively modulates IFN- β and the ISG-15 transcription and prevents the activation of the IFN regulatory transcription factor 3 (IRF-3) by phosphorylation and dimerization, resulting in a deficient IFN-1 type response (Yumiketa et al., 2016).

Stress granules (SG) are ribonucleoprotein complexes that form in the cell cytoplasm as a response to diverse stimuli, including viral infections. These complexes contain stalled non-translated cellular mRNA attached to the translation complex, delaying cell translation rate to avoid viral protein production. Moreover, proteins involved in antiviral responses are recruited to SG, inducing their activation (reviewed in: Protter and Parker, 2016).

The FCV Urbana strain cleaves by the action of the viral protease, the GTPase-activating protein-binding protein (G3BP)-1 a key stress granule protein involved in antiviral response at multiple levels (Reineke and Lloyd, 2015). Similarly, the infection with MNV and HuNoV causes G3BP-1 (Humoud et al., 2016), relocation to the RC (Brocard et al., 2020), impairing SG nucleation while using it as a factor for Vpg-dependent translation (Brocard et al., 2020; Hosmillo et al., 2019).

In MNV infection, the G3BP1 targeting coupled with an impaired cytokine production through cell translational shutoff by eIF2 α phosphorylation (Fritzlar et al., 2019), results in an impaired cellular immune response without affecting viral protein production.

It is also known that PSaV impairs production of NO₂, a well-known antiviral agent, through Cyclooxygenase 2/Prostaglandin E₂ (COX-2) pathway activation likely due to Vpg and the protease/polymerase NS6/7 viral proteins (Alfajaro et al., 2017). Similarly, FCV an MNV activate the same pathway to reduce NO₂ production, although the mechanism is still unknown (Alfajaro et al., 2018).

MNV-induced lysis and pro-inflammatory cytokine release can be beneficial to viral persistence, as recruitment of macrophages and neutrophils to the infection site supplies the virus with an ongoing population of susceptible cells (Van Winkle et al., 2018).

Adaptive immunity

One of the main characteristics of the adaptive immune system is the ability to generate a specific immune memory against foreign agents and, thus to respond more efficiently to a second encounter with the same antigen. The adaptive response can be divided into a cellular and humoral immune response. While the humoral response is given by the antibodies produced by plasma cells, the cellular immune response is given by CD4⁺ and CD8⁺ T lymphocytes. However, viruses have evolved strategies that evade these specialized responses to complete their replication cycle, counteracting the host's immune system at different levels.

Cellular immune response

When a virus infects a cell, it is necessary to produce the cellular context required to generate the viral progeny that disseminate to neighboring cells. To control the expression of non-host proteins, major histocompatibility complex (MHC) class I, expressed in all nucleated cells, processes these proteins, and the resulting peptides are exposed to the outer face of the cell membrane. Cytotoxic CD8⁺ lymphocytes recognize these foreign peptides and destroy the infected cell. The cellular immune response to caliciviruses has not been studied in detail, and much remains to be learned about possible evasion mechanisms. Yet, the presence of specific CD4⁺ T lymphocytes against FCV in the spleen of cats vaccinated with FCV (de Groot-Mijnes et al., 2004). The presence of HuNoV GI.3 and GII.4 specific T lymphocytes in children under 4 years of age has been reported (Malm et al., 2019). Moreover, the stimulation with peptides of NoV GII.4-1999, GII.4-2012 (Sydney), and HuNoV GI.3 VP1 of peripheral blood mononuclear cells of patients with previous exposure to these HuNoV strains generates a good response of CD8⁺ T lymphocytes (Malm et al., 2016), suggesting the importance of the cellular immune response in the clearance of calicivirus infections.

To evade the cellular immune response, viruses have generated different strategies, such as decreasing the expression of MHC-I. During MNV infection, NS3 protein induces the degradation of MHC-I through the proteasome route and vesicular traffic control, impairing the antigenic presentation and activation of cytotoxic TCD8⁺ cells (Enosi Tuipulotu et al., 2017; Fritzlar et al., 2018). Another suggested mechanism for evading the immune response is the location of the infected cells in immune-privileged sites.

Infection with MNV induce robust CD8+ T cell responses, generating virus-specific CD8+ cells that maintain their ability to respond to their antigen, but paradoxically, viral replication is maintained. It is suggested that the virus remains in a place less accessible to these cytotoxic lymphocytes (Tomov et al., 2017), or that some strains generate lymphocytes with suboptimal responses (Tomov et al., 2013).

Humoral immunity

The study of humoral immunity in viral infections is of vital importance, especially for the development of vaccines. Due to the ability of many caliciviruses to re-infect the same host, the notion exists that calicivirus infection do not result in a robust immune response and factors necessary for the generation of efficient immune memory has been an important subject of study. Neutralizing antibodies are known to be key to the clearance of different caliciviruses (Chachu et al., 2008; Ferreira et al., 2008; Wensman et al., 2015), and each subtype of immunoglobulin will confer different efficiencies to eliminate the infection; with IgA having the greatest capacity for neutralization, elimination (Ramani et al., 2015) and cross-reaction against HuNoV (Lindesmith et al., 2015). Another suggested advantage is the great ability of neutralizing antibodies to block norovirus interaction with HBGAs (Sapparapu et al., 2016), although that the anti-HuNoV IgA-producing cells might have a shorter lifespan than IgG-producing cells (Lindesmith et al., 2015).

To avoid the antibody responses, caliciviruses have developed different strategies; one of them is the high mutational rates of the most immunogenic regions of their capsid proteins, which are the target for neutralizing antibodies, such as the P2 domain of VP1 (Iwakiri et al., 2009; Nilsson et al., 2003), which interacts directly with the viral cellular receptors. The virus must maintain a balance to evade the blockade of neutralizing antibodies without losing affinity for their cellular receptors; precisely, in the D epitope of the HuNoV strain GII.4 the amino acid change is generated in such a way that they can cause loss of antibody binding without ablation of the HBGA (Lindesmith et al., 2019). Other mechanisms that rely on small spatial rearrangements of the norovirus GI.1 and GI.4 capsid to reduce exposure time to neutralizing antibodies or steric hindrance by post-translational modifications were recently discussed in detail (Smith and Smith, 2019).

The infection of STAT1-deficient mice with the MNV-4 persistent and systemic strain causes the impairment of B cells development. A significant loss of pro-B/pre-B cells in the bone marrow of the immunodeficient but not in wild-type phenotype mice was observed. Even though the mechanism by which MNV-4 regulates the B cell maturation of STAT1-deficient mice is unknown, it may be related to decreased IL-7 levels, an important cytokine for B cell development, suggesting the role of B cells in the norovirus replication restriction (Hsu et al., 2018).

Concluding remarks

The *Caliciviridae* family includes infectious agents of medical, livestock, and veterinary interest, with HuNoVs nowadays being the leading cause of non-bacterial gastroenteritis outbreaks worldwide, responsible for 200,000 annual deaths in the developing countries. The continuous discovery of new caliciviruses in recent years has led up to the recent acceptance of six new viral genera, and their study might be beneficial to the better understand HuNoVs and the farming industry, as treatments or vaccines could be developed to prevent diseases and the subsequent economic loss.

New calicivirus models, as well as the recent development of effective HuNoV replication systems for the first time in nearly 50 years, are a major breakthrough that will lead to a rapid and better understanding of HuNoV molecular biology and pathogenesis. Yet, the advances made in the understanding of the calicivirus replication cycles in the last years need to be matched with advances in the understanding of the innate and adaptive viral immune responses to calicivirus, areas where much still need to be learned. This may provide the elements to sort out the difficulties in vaccine and treatment research that will help us to overcome the burden that caliciviruses have represented as human and animal pathogens.

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Bunyavirus

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Introduction

Bunyaviruses comprise a large group of RNA viruses that are worldwide pathogens and are mainly transmitted by arthropods, i.e., arthropod-borne viruses (arboviruses) or, in some cases, by aerosols from infected rodent excreta. The term 'Bunyavirus' is derived from the name of the location where the *Bunyamwera virus* was first isolated (Bunyamwera village, Uganda) during yellow fever surveillance in *Aedes* mosquitoes (Smithburn et al., 1946). Serological cross-reactions between this new virus with the *Wyeomyia* (Roca-Garcia, 1944), *Cache Valley* (Holden and Hess, 1959), and *Kairi* (Anderson et al., 1960) viruses led Casals and Whitman (Casals and Whitman, 1960) to sort them into a new antigenic group named "The Bunyamwera serogroup." Due to cross-reactivity between Bunyamwera and other related serogroups, such as Guama and Simbu, they were organized into the "Bunyamwera Supergroup" (Casals, 1963). Combining a total of 11 serogroups, including approximately 90 viruses, the new classification was: Bunyamwera, Bwamba, C serogroup, Capim, California, Guama, Koongol, Patois, Simbu, Tete, and Olifantsvlei. Strong antigen cross-reactions have been reported within each serogroup, as opposed to weak antigen cross-reactions among different serogroups (Murphy et al., 1973; Casals, 1971).

Ultrastructural analyses, at the electron microscope level, allowed researchers to determine that viruses belonging to the Bunyamwera serological supergroup were morphologically indistinguishable (Murphy et al., 1973). However, several other serogroups that are antigenically different, but morphologically similar to the Bunyamwera Supergroup, were also detected (Taylor, 1967). Therefore, the *Bunyaviridae* family name was proposed in order to organize these viruses (Murphy et al., 1973). In 1975, the Bunyamwera serological supergroup was recognized by the International Committee on Taxonomy of Viruses (ICTV) as the *Bunyavirus* genus, within the *Bunyaviridae* family (Fenner and Maurin, 1976). Likewise, unrelated viruses with morphological similarities but antigenic differences from the Bunyamwera serological supergroup were also sorted into the new *Bunyaviridae* family. Nevertheless, they were arranged in other genera (Murphy et al., 1973; Porterfield et al., 1975). Thus, viruses belonging to the *Bunyaviridae* family were characterized as: enveloped and spherical virions, with 90–100 nm in diameter; containing a single-stranded RNA genome, possibly arranged in several segments (generally three segments); membrane-spanning polypeptide spikes at the virus surface; and an internal ribonucleoprotein filament, measuring approximately 2–2.5 nm wide (Fenner and Maurin, 1976).

Several new serogroups were subsequently isolated and, despite presenting antigenic differences from the *Bunyavirus* genus, exhibited morphogenetic and molecular characteristics that were similar enough to warrant their addition to the *Bunyaviridae* family. These newly characterized serogroups were *Phlebovirus*, *Uukuvirus*, and *Nairovirus* (Bishop et al., 1980), which were later reorganized into genera and sorted into the *Bunyaviridae* family (Matthews, 1981). Thus, the *Bunyaviridae* family comprises four genera: *Nairovirus*, transmitted primarily by ticks carrying Crimean-Congo hemorrhagic fever virus (CCHFV) as its type species; *Phlebovirus*, transmitted by sandflies (Phlebotomus), with Sandfly fever virus (SFSV) as its type species; *Uukuvirus*, also transmitted by ticks, with Uukuniemi virus (UUKV) as its type species; and the *Bunyavirus* genus, transmitted mainly by mosquitoes, with Bunyamwera virus (BUNV) as its type species. The *Hantavirus* genus was later sorted into the *Bunyaviridae* family as its fifth genus due to molecular similarities (Schmaljohn and Dalrymple, 1983), and its type species was the Hantaan virus (HNTV), the etiological agent of hemorrhagic fever with renal syndrome (HFRS). Hantaviruses were peculiar in the *Bunyaviridae* family since their transmission is carried out by aerosol particles from small rodent secretions (Lennette et al., 1988). Due to molecular similarities, the *Phlebovirus* and *Uukuvirus* genera were considered related and were grouped into the *Phlebovirus* genus (Francki, 1991). Furthermore, on account of genomic similarities with the *Phlebovirus* genus, the recently established *Tospovirus* genus of plant-related viruses, with tomato spotted wilt virus (TSWV) as its type species, was also sorted into the *Bunyaviridae* family (Francki, 1991). Viruses from this family were generally called "bunyaviruses." Thus, in order to avoid further misstatement, in 2002, the *Bunyavirus* genus was renamed *Orthobunyavirus* (Mayo, 2002; Calisher, 2008). Therefore, the *Bunyaviridae* family was characterized as the largest RNA virus family, classified into five genera: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus* (Elliott, 2009).

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The development of bioinformatics and sequencing techniques enabled the establishment of better classification criteria based on comparative genomics and phylogenetic analyses. Metagenomic tools led to the discovery of new “bunya-like” species, containing two or more than three genome segments (Li et al., 2015; Tokarz et al., 2014; Elbeaino et al., 2009; van Poelwijk et al., 1997), which were reorganized into the *Bunyaviridae* family (Briese et al., 2016). Therefore, in 2016, the ICTV classified the *Bunyaviridae* family as the *Bunyavirales* order and rearranged the *Hantavirus*, *Nairovirus*, and *Tospovirus* genera into families (Briese et al., 2016). The committee also established the *Peribunyaviridae* family from the *Orthobunyavirus* genus and other genera, created the *Phenuiviridae* family, where the *Phlebovirus* and *Tenuivirus* genera were sorted, and the *Fimoviridae* family, where the *Emaravirus* genus was sorted. The *Feraviridae*, *Jonviridae*, and *Phasmaviridae* families were established for newly discovered invertebrate bunyaviruses (Briese et al., 2016). By 2016, the *Bunyavirales* order was divided into nine families (*Feraviridae*, *Fimoviridae*, *Hantaviridae*, *Jonviridae*, *Nairoviridae*, *Peribunyaviridae*, *Phasmaviridae*, *Phenuiviridae*, and *Tospoviridae*), and in 2017, the *Arenaviridae* family was included in this order due to phylogenetic relatedness (Guterres et al., 2017; Kuhn et al., 2017). Additionally, the *Feraviridae* and *Jonviridae* families were classified as genera and sorted into the *Phasmaviridae* family, and the *Cruliviridae*, *Myopoviridae*, and *Wupedeviridae* families were created to sort highly divergent and atypical newly discovered viruses (Kuhn et al., 2017). In 2019, the *Leishbuviridae* family was established to include the new genus *Shilevirus*, which infects trypanosomatid protists (Abudurexiti et al., 2019). Thus, the *Bunyavirales* order is currently divided into 12 families: *Arenaviridae*, *Cruliviridae*, *Fimoviridae*, *Hantaviridae*, *Leishbuviridae*, *Myopoviridae*, *Nairoviridae*, *Peribunyaviridae*, *Phasmaviridae*, *Phenuiviridae*, *Tospoviridae*, and *Wupedeviridae*.

Virus structure and organization

Bunyaviruses are enveloped viruses with spherical conformation that range between 80 and 120 nm in diameter (Ter Horst et al., 2019), with some hantaviruses presenting elongated morphology (Guardado-Calvo and Rey, 2017; Mittler et al., 2019). Arenaviruses are an exception, with pleomorphic virus particles ranging from 50 to 300 nm in diameter (Buchmeier, 2002; Murphy and Whitfield, 1975). Viral particles consist of four major structural proteins: the glycoproteins Gn and Gc; the nucleocapsid protein (N or NP); and the viral RNA-dependent RNA polymerase (RdRp) or L protein (Ter Horst et al., 2019). In addition, arenaviruses contain a zinc-binding matrix protein known as the Z protein (Salvato and Shimomaye, 1989). Also, the ssRNA genome is segmented and associated with the N and L proteins, forming ribonucleoprotein complexes (RNPs) (Olschewski et al., 2020). Structural proteins and virus genome morphology are described in Fig. 1.

N protein: The Bunyavirus N protein structure has been extensively investigated by atomic-resolution analyses (Guo et al., 2012; Olal and Daumke, 2016; Raymond et al., 2012; Reguera et al., 2013), which contributed to the current knowledge of the viral protein's structure and function. The N protein has a core domain where the RNA-binding cleft is located and extended arm or stalk domains, which enable protein oligomerization into several oligomer types, varying according to family and species (Sun et al., 2018). Each protomer's central RNA-binding cleft forms a continuous channel for single-stranded RNA coupling (Reguera et al., 2013). Several studies suggest diversity in N protein conformational changes during RNA binding or oligomerization that are responsible for the formation of RNPs (Sun et al., 2018; Carter et al., 2012; Wang et al., 2012).

L protein: The Bunyavirus L protein, or RdRp, is responsible for both viral genome transcription and replication (Gerlach et al., 2015). The structure of some bunyaviruses' RdRps has been solved by different groups (Gerlach et al., 2015; Peng et al., 2020), thus

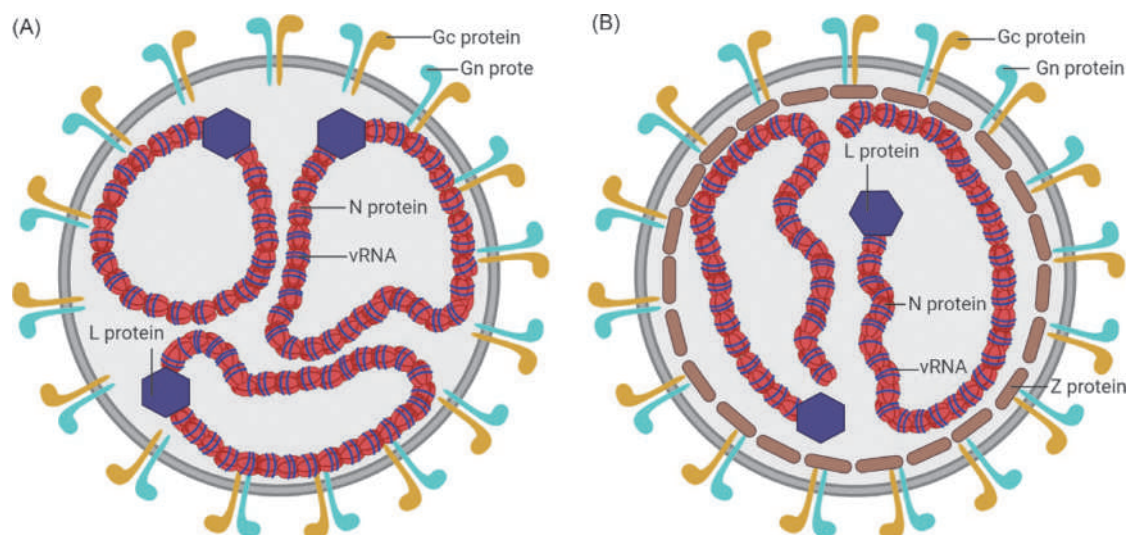


Fig. 1 Bunyavirus particle morphology. (A) Tri-segmented bunyavirus particle structure. (B) Bi-segmented arenavirus particles. All Figures in this Chapter were drawn using BioRender.

contributing to the current knowledge of this viral protein's structure and function. Overall RdRp architecture consists of a large globular core domain and a flexible N-terminal region. The core domain presents polymerase activity and includes palm, thumb, finger, and fingertip domains (Sun et al., 2018). On the other hand, the N-terminal region harbors endonuclease activity (Gerlach et al., 2015) and is connected to the large globular core domain by a flexible linker (Sun et al., 2018).

Glycoproteins: Glycoproteins Gn and Gc (also termed GP1 and GP2 respectively, for Arenaviruses), which are encoded by the virus's M segment as a glycoprotein precursor, are type I integral membrane proteins (Ter Horst et al., 2019; Elliott, 2014) that arrange as heterodimer structures, spanning 5–10 nm projections at the viral surface (Guardado-Calvo and Rey, 2017). The size and structure of Gc and Gn vary considerably among bunyaviruses (Bowden et al., 2013; Elliott, 1996). Thus, the spike structures formed by these glycoproteins change substantially according to the virus family and genus, with hantaviruses possessing tetrameric square-like arrangements (Battisti et al., 2011), phleboviruses having pentamer or hexamer conformations (Huiskonen et al., 2009), and orthobunyaviruses presenting tripodal assembly (Bowden et al., 2013). Gc structural studies suggest that the glycoprotein has a class II fusion protein fold that is crucial for bunyavirus entry into host cells (Guardado-Calvo and Rey, 2017).

Arenavirus Z protein: This zinc-finger protein is the smallest structural protein of arenaviruses, containing 90–99 amino acids, and constitutes the virus matrix protein (Shao et al., 2018). This protein belongs to the RING-finger protein family (Salvato and Shimomaye, 1989) and has an important role in RdRp activity and virus budding regulation (Peng et al., 2020).

Non-structural proteins: Most bunyaviruses, depending on the species, may contain two non-structural proteins encoded by the virus's M and S segments, NSm and NSs, respectively (Ter Horst et al., 2019). Although these proteins are not essential for virus replication, they may be involved in viral assembly, apoptosis, protein synthesis inhibition, and immune evasion (Elliott, 2014; Shi et al., 2006; Soldan and González-Scarano, 2014). In addition, other non-structural proteins, such as VP3 in some phasmaviruses (Li et al., 2015) and p4, p5, and p6 in some fimoviruses (Sun et al., 2018), are less frequently found and do not have a known function.

Genome: The segmented single-stranded RNA genome has a negative-sense polarity, and, according to the family, some viruses may also use an ambisense coding strategy (Olschewski et al., 2020). Each segment has complementary regions at its 5'- and 3'- ends (Elliott, 1996) that are highly conserved within each genus, allowing for a panhandle structure conformation, which contains genome replication and transcription promoters (Wichgers Schreur et al., 2018). The number of genome segments may vary between two and eight, according to virus family and species (Li et al., 2015; Moolla and Weyer, 2020). However, the vast majority of Bunyaviruses possess a tri-segmented genome (Li et al., 2015; Ter Horst et al., 2019). Their genomic structure and coding strategies are detailed in Fig. 2.

Bunyavirus replicative cycle

The life cycle of these viruses has been studied primarily in mammalian cells (Albornoz et al., 2016), and several receptors were identified to be involved in bunyavirus cell entry, with the most well-described being: DC-SIGN, L-SIGN, NMMHC-IIA (non-muscle myosin heavy chain IIA), Heparan sulfate, β 1- β 3 integrins, protocadherin-1, α -DC (alpha-dystroglycan), and hTfR1 (human transferrin receptor 1) (Albornoz et al., 2016; Jangra et al., 2018; Léger et al., 2016; Radoshitzky et al., 2007; Reignier et al., 2006; Riblett et al., 2016; Suda et al., 2016).

Cellular receptors interact with viral proteins to mediate virus entry by endocytosis, in which, for most bunyaviruses, the endocytic pathway involves clathrin-coated vesicles (Garrison et al., 2013; Hollidge et al., 2012; Jin et al., 2002; Santos et al., 2008; Shtanko et al., 2014). However, other bunyaviruses' cell entry may also involve caveolin-dependent mechanisms or micropinocytosis (de Boer et al., 2012; Harmon et al., 2012). Therefore, the diversity of bunyavirus endocytosis pathways highlights their ability to use alternative endocytic routes (Albornoz et al., 2016). When in endocytic vesicles, viral membrane fusion takes place either in early endosomes, late endosomes, or lysosomes, depending on the virus species, in light of the required pH for such event (Albornoz et al., 2016). Membrane fusion is mediated by viral Gc, which exposes hydrophobic domains when in the presence of high endosomal K^+ concentrations and low pH, allowing for virus-cell membrane interactions and fusion, thus releasing RNPs into the cell cytosol (Charlton et al., 2019; Hover et al., 2018), where this complex will be disassembled to enable N and L gene transcription. Seeing that these proteins are crucial for the first steps of virus replication, they are prioritized in virus genome transcription (Fig. 3) (Ferron et al., 2017).

Viral transcription initiation is carried out through a Cap-Snatching mechanism by the L protein endonuclease domain (Olschewski et al., 2020), followed by transcription elongation, which is coupled with viral protein translation (Barr, 2007). The next step, known as transcription termination, is characterized by a specific conserved termination signal sequence that can promote hairpin structure formation (Sun et al., 2018; Ferron et al., 2017). It has been proposed that cytosolic N protein accumulation triggers genome replication by the RdRp polymerase domain (Elliott, 2014; Ferron et al., 2017) in two consecutive steps: complementary RNA (cRNA) is synthesized "de novo" in a primer-independent manner from viral RNA (vRNA), followed by cRNA synthesis into new generation vRNA (Fig. 3) (Sun et al., 2018; Peng et al., 2020; Ferron et al., 2017). Newly synthesized cRNA and vRNA are hijacked by the viral N protein, constituting cRNPs and vRNPs, respectively (Sun et al., 2018). In the cell cytosol, vRNPs derived from the viral M segment are transcribed into mRNA, followed by glycoprotein precursor (GP) translation initiation (Ferron et al., 2017). A signal sequence in the GP targets the Endoplasmic reticulum (ER) for further translation and glycoprotein processing (Cifuentes-Muñoz et al., 2014; Gerrard and Nichol, 2007). ER proteases are thought to cleave GPs into glycoproteins

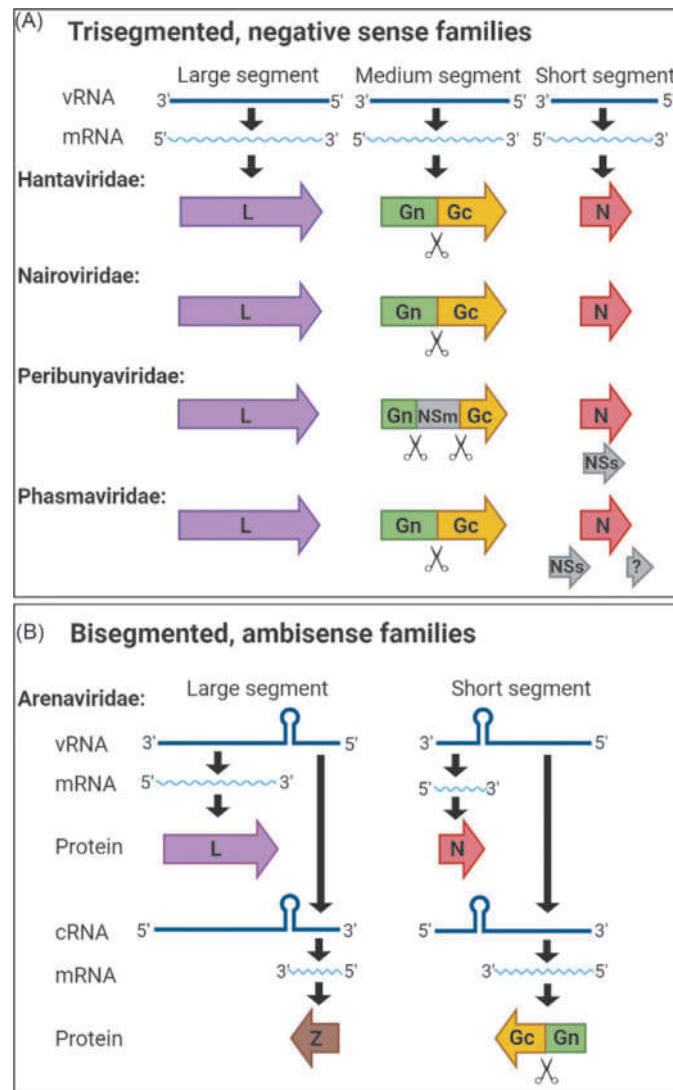


Fig. 2 Bunyavirus coding strategies. (A) Tri-segmented genome coding strategies of negative-sense viruses. (B) Bi-segmented genome coding strategies of ambisense viruses.

Gn and Gc, which will be further processed (Guardado-Calvo and Rey, 2017) and targeted to other organelles after glycoprotein dimerization (Cifuentes-Muñoz et al., 2014; Lappin et al., 1994). Most bunyaviruses are targeted to the Golgi complex for virus assembly and budding, whereas arenaviruses and some hantaviruses are targeted to the plasma membrane for such events (Fig. 3) (Guardado-Calvo and Rey, 2017; Elliott, 2014; Cifuentes-Muñoz et al., 2014; Burri et al., 2012).

At the targeted organelle, glycoprotein heterodimers associate into large complexes, interacting laterally, to induce membrane curvature deformation for viral protein budding (Guardado-Calvo and Rey, 2017). The recruitment of components of the cellular endosomal sorting complex required for transport (ESCRT) machinery has been reported to support such membrane curvature (Cifuentes-Muñoz et al., 2014; Burri et al., 2012; Barbosa et al., 2018). Arenaviruses, for instance, promote membrane deformation by Z protein oligomerization (Burri et al., 2012). It has been suggested that vRNP packaging into viral particles is mediated by glycoprotein cytosolic tail interactions with the N protein (Wichgers Schreur et al., 2018). In arenaviruses, this process is orchestrated by the Z protein (Burri et al., 2012). Although there is some selectivity in vRNP packaging, which appears to be driven by the 5'- and 3'- untranslated regions, evidence showing that vRNP packaging into viral particles is a stochastic process has also been reported (Wichgers Schreur et al., 2018). After virus assembly in Golgi compartments, as occurs in most bunyaviruses, viral particles are targeted to the plasma membrane and are transported through cytoskeleton filaments (Elliott, 2014; Cifuentes-Muñoz et al., 2014; Sanz-Sánchez and Risco, 2013; Shi et al., 2010). The main steps of the bunyavirus replicative cycle are described in Fig. 3.

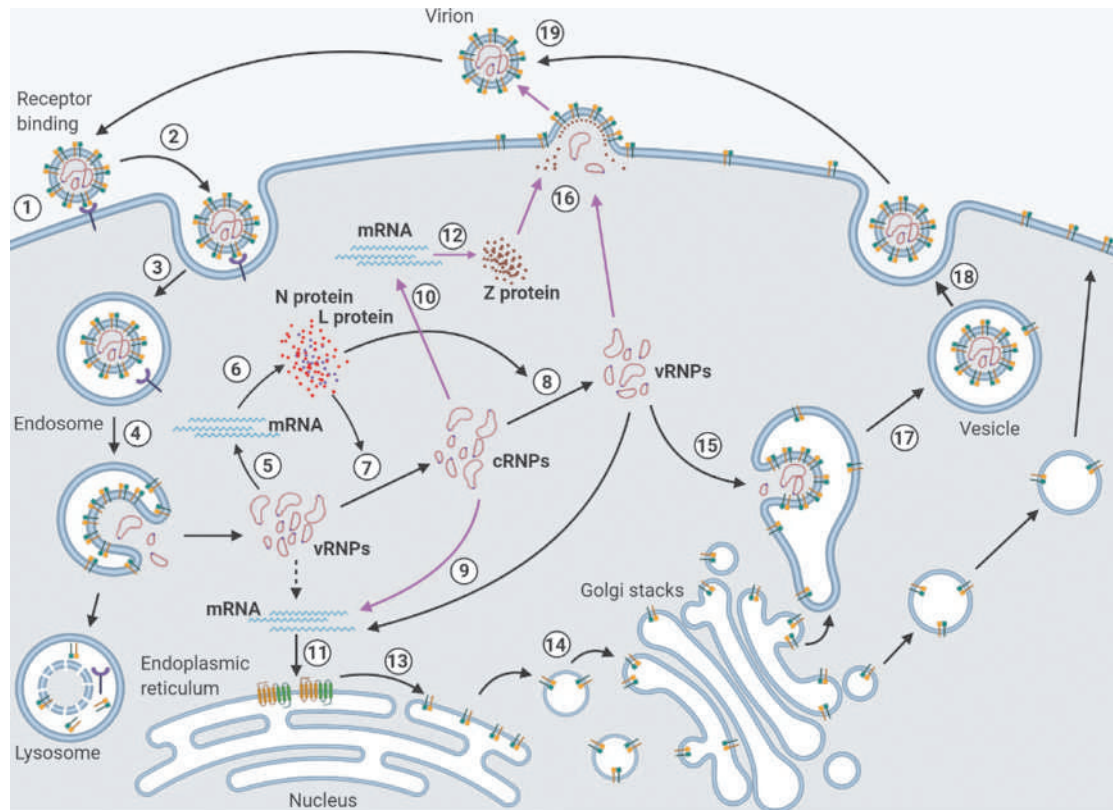


Fig. 3 Bunyavirus replicative cycle. (1) Bunyaviruses bind to specific cellular receptor. (2) Most viral particles enter host cell via endocytic vesicles; (3) Viral particles are trafficked through early endosomes to late endosomes; (4) Membrane fusion takes place either in early endosomes, late endosomes, or lysosomes, depending on virus species, for vRNP release into the cell cytosol; (5) Transcription of the N and L genes are prioritized for further virus replication; (6) N and L protein translation; (7) First step of virus replication into cRNPs; (8) Second step of virus replication into vRNPs; (9–10) Transcription of the M and Z genes, respectively; (11–12) Glycoprotein precursor and Z protein translation, respectively; (13) Glycoprotein precursor processing in the Endoplasmic reticulum; (14) Glycoproteins trafficking within the secretory pathway; (15–16) Virus budding into the Golgi complex and at the plasma membrane, respectively. (17) Viral particle targeting to the plasma membrane. (18–19) Viral particle release. Arenavirus steps are represented by straight pink lines. Even though hantaviruses may also bud in the plasma membrane, they do so without Z protein activity.

Bunyavirus immune evasion

Viral genome released into the cytosol may be detected by the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) family of RNA sensors, such as RIG-I and melanoma differentiation-associated gene 5 (MDA5), leading to the activation of antiviral host responses. Upon viral RNA binding, RLRs activate the mitochondrial antiviral signaling (MAVS) protein, a signaling transduction adaptor. This activates interferon regulatory factors (IRFs) that, together with the nuclear factor κ - β (NF- κ β) transcription factor, induce the expression of type-I interferon (IFN) and other genes to counteract virus infection (Rehwinkel and Gack, 2020). Interferon response is accomplished in two stages, termed initiation and amplification. The initiation stage is when IFN- α/β expression is activated, leading to its secretion. Meanwhile, the amplification stage is when secreted IFN- α/β binds to IFN receptors (IFNARs) at the cell surface, activating the JAK/STAT signaling pathway, resulting in the expression of interferon-stimulated genes (ISGs), thus inducing an antiviral state that provides a crucial defense against viral infection (Eifan et al., 2013; Le May and Bouloy, 2012). Most bunyaviruses are able to evade these cellular immune responses due to NSs activity by shutting down IFN gene expression. Alternatively, some bunyaviruses escape immune recognition by the activity of other viral proteins, as described below (Fig. 4).

Viruses from the *Peribunyaviridae* family were shown to prevent IFN responses via NSs, with this viral protein inhibiting RNA polymerase II (RNA Pol II) activity and thereby blocking host cell mRNA elongation. For instance, BUNV blocks the phosphorylation of the C-terminal domain of RNA Pol II, inhibiting host cell transcription (Léonard et al., 2006). Moreover, infection with this virus specifically prevents IFN- β expression by restraining NF- κ β and IRF-3 activation (Weber et al., 2002). Similarly, LACV inhibits type-I IFN response (Thomas et al., 2004) by inducing the proteasomal degradation of RNA pol II subunit RBP1 (Verbruggen et al., 2011), which shuts down the whole cell's transcription, including IFN and IFN-related genes. Furthermore, Schmallenberg virus (SBV) NSs has an important role in inhibiting IFN expression (Varela et al., 2013), although this mechanism has not yet been fully described. On the other hand, it was shown that OROV NSs is essential to antagonize IFN response, where OROV NSs infection

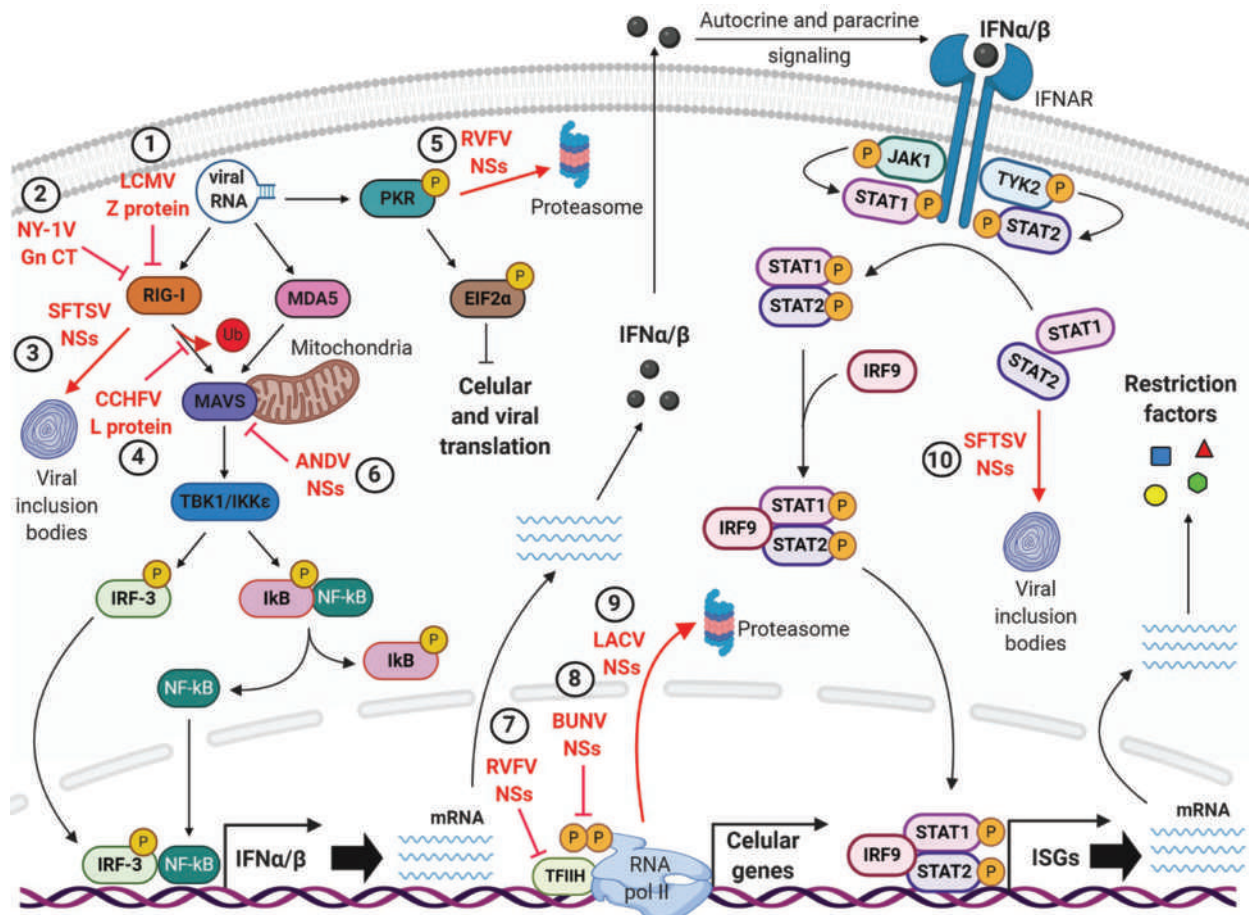


Fig. 4 Bunyavirus cellular immune evasion. Upon viral genome release into the cell cytosol, the exposed foreign genome can be detected by nucleic acid sensors such as RIG-I and MDA5, which will trigger cellular antiviral responses. Bunyaviruses have evolved to inhibit various cellular immune recognition pathways that lead to IFN or IFN related genes expression. (1) The Lymphocytic Choriomeningitis Virus (LCMV) Z protein and (2) the New York-1 hantavirus (NY-1V) GnCT protein inhibit the RIG-I recognition pathway. (3) The Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV) NSs protein relocates RIG-I to viral inclusion bodies. (4) The Crimean-Congo Hemorrhagic Fever Virus (CCHFV) L protein can block RIG-I mediated IFN response through its vOTU domain that removes Ub conjugates from RIG-I, inactivating it. (5) Rift Valley Fever Virus (RVFV) NSs protein can induce proteasome degradation of antiviral PKR protein. (6) The Andes Virus (ANDV) NSs protein inhibits MAVS signaling. (7) The Rift Valley Fever Virus (RVFV) NSs protein inhibits the assembly of transcription factor II, suppressing host cell transcription. (8) Bunyamwera Virus (BUNV) NSs protein inhibits RNA pol II activity through blocking its C-terminal domain phosphorylation, whereas (9) the La Crosse Virus (LACV) NSs protein inhibits RNA pol II activity by inducing the degradation of RNA pol II BDP1 subunit via the proteasome. Finally, (10) SFTSV NSs protein relocates STAT proteins to viral inclusion bodies, thus inhibiting STAT phosphorylation and signaling.

increased IFN production and induced STAT1 phosphorylation and ISG MxA expression (Tilston-Lunel et al., 2015). Thus, viruses from the *Peribunyaviridae* family are very effective in counteracting host cell immune evasion, with the NSs protein playing a significant role in this process. Nevertheless, the effectiveness of viral antagonism to IFN response may vary according to the infection environment. For instance, knock-out (KO) mice for IFNAR, MAVS, IRF-3, and IRF-7, when infected with OROV, presented an increased lethality when compared to wild-type animals (Proenca-Modena et al., 2015). In human peripheral blood cells treated with anti-IFNAR antibodies, which prevent IFN from binding to its receptor, OROV infection was significantly increased compared to non-treated samples (Ribeiro Amorim et al., 2020), indicating that activation of the IFN response was essential to restrict OROV replication in human PBMCs.

Rift Valley Fever virus (RVFV), from the *Phenuiviridae* family, has the ability to evade cell immune recognition in at least three different ways. At early hours (3–6 h) post-infection, RVFV blocks IFN- β gene expression through the action of its NSs protein (Le May et al., 2008), which translocates to the nucleus of infected cells and forms filamentous structures that bind several nuclear proteins, including subunits of an IFN- β gene corepressor complex. As a result, NSs stabilizes the association of the corepressor complex with the IFN- β gene promoter, thus inhibiting recruitment of the transcription factor YY1 and the transcriptional co-activator CBP. Altogether, IFN- β expression is blocked, allowing virus replication to progress in infected cells (Le May et al., 2008).

At later hours post-infection, RVFV suppresses host cell transcription by inhibiting the assembly of transcription factor II (TFIIH), which plays a role in the transcription of several protein-coding genes (Le May et al., 2004). NSs nuclear filaments recruit TFIIH

subunits, inhibiting TFIIF assembly and leading to proteasome degradation of p62, an essential subunit for TFIIF function (Kalveram et al., 2011). In addition to shutting down IFN- α/β and further cellular gene transcription, RVFV is also capable of degrading the antiviral factor double-stranded RNA-dependent protein kinase (PKR). This antiviral factor, which inhibits virus replication by blocking both viral and cellular mRNA translation (García et al., 2007), was shown to be essential for IFN- α/β production in virus-infected cells and the regulation of IFN- α/β mRNA stability (Schulz et al., 2010). RVFV NSs directs PKR to proteasome degradation, thereby displaying an additional anti-IFN property compared to other bunyaviruses (Habjan et al., 2009).

Moreover, another phlebovirus called Severe Fever with Thrombocytopenia Syndrome virus (SFTSV) also suppresses IFN-I and IFN-III signaling via NSs activity. Specifically, this protein can associate with and/or recruit RIG-I, STAT1, and STAT2 to viral inclusion bodies, suppressing IFN and ISG expression by inhibiting RIG-I activation, STAT phosphorylation, and nuclear translocation (Chaudhary et al., 2015; Santiago et al., 2014; Ning et al., 2015). Additionally, it was also shown that SFTSV NSs stabilizes and activates TPL2 (tumor progression locus 2) to induce IL-10 expression, an anti-inflammatory cytokine that limits host immune responses, enabling the progression of viral infection (Choi et al., 2019). Therefore, phleboviruses have efficient strategies for counteracting the host immune response to evade the IFN system to achieve viral replication.

Most viruses from the *Hantaviridae* family do not possess an NSs ORF in their S segment. Thus, some pathogenic hantaviruses evade cellular immune response by inhibiting IFN activation by the action of their glycoproteins and nucleoprotein. The New York-1 hantavirus (NY-1V) Gn cytosolic tail is capable of inhibiting the RIG-I pathway, blocking the transcription of type I IFN genes and, consequently, suppressing the IFN pathway (Alff et al., 2006). On the other hand, hantaviruses that encode an NSs protein, such as the Andes virus (ANDV), can inhibit the IFN pathway by NSs RIG-I and MDA5 downstream signals recognition suppression (Vera-Otarola et al., 2020). The Hantaan virus (HNTV) is able to counteract cell antiviral activation by a different pathway, where the nucleoprotein inhibits the tumor necrosis factor- α (TNF- α) of the NF- κ B pathway to prevent host immune detection (Taylor et al., 2009). Thus, hantaviruses possess different viral factors involved in host cell immune evasion, which appear to be distinct from most of the other bunyaviruses.

Viruses from the *Nairoviridae* family, which also do not present an NSs ORF, use a very peculiar immune evasion mechanism among bunyaviruses involving the cellular ubiquitin-proteasome system. Ubiquitin (Ub) conjugation to proteins plays an important regulatory role in host cell detection and response to virus infection. Upon IFN signaling pathway activation, various cellular proteins are posttranslationally modified by ubiquitination or SGylation, i.e., the conjugation of interferon-stimulated gene 15 (ISG-15) protein, an Ub-like protein. Some viruses have developed an escape mechanism that removes Ub and/or Ub-like moieties from host cell proteins, resulting in IFN pathway suppression (Isaacson and Ploegh, 2009).

In fact, cells express deubiquitinating (DUB) enzymes with an ovarian tumor-like (OTU) domain, which are involved in IFN response suppression by hydrolyzing Ub from cellular proteins (Nijman et al., 2005; Kayagaki et al., 2007). Interestingly, Nairoviruses' L protein contains a viral OTU domain homolog (vOTU) at the N terminus involved in IFN response suppression and immune response evasion. This domain was found to remove Ub and/or ISG-15 from specific substrates. Specifically, CCHFV vOTU deubiquitinase and deISGylase activity were, respectively, shown to be critical for virus-mediated suppression of IFN expression and to inhibit interferon-induced protein ISGylation, allowing CCHFV to evade immune responses (Scholte et al., 2017). Interestingly, there is a diversity among Nairoviruses' vOTU domain activity towards ISG-15 and Ub-conjugated proteins, which is related to the vOTU's primary structure (Capodagli et al., 2013).

Arenaviruses also lack an NSs ORF, as do nairoviruses. Pathogenic Arenaviruses' Z and N proteins are capable of inhibiting IFN response. In order to determine the involvement of arenavirus N protein in IFN inhibition, the Lassa virus (LASV) N protein structure was solved, and when specific mutations at the exonuclease activity domain of this viral protein were inserted, IFN expression was disrupted. Since the arenaviruses' N protein is highly conserved, the study was only performed with LASV (Qi et al., 2010). Arenaviruses' Z protein is able to inhibit RIG-I and MDA5 recognition and disrupt MAVS activation, leading to IFN expression blockage. Cellular immune evasion by Z protein activity was conserved among several pathogenic arenaviruses (Xing et al., 2015).

Therefore, bunyaviruses hold a variety of viral factors capable of evading host cell defenses, thus characterizing them as difficult pathogens to counteract in an infected cell.

Pathogenesis and the need for vaccines

Bunyaviruses are the etiological agents of several human and non-human diseases. Considered emergent viruses, the need for specific and efficient vaccines is crucial to avoid virus spread, followed by severe outbreaks, which may lead to human and/or animal deaths.

The pathogenesis of orthobunyaviruses differs according to virus species. For instance, OROV and LACV, found in South and North America, respectively, may cause febrile illness in humans that, in rare cases, can lead to meningoencephalitis or meningitis (Sakkas et al., 2018; Taylor and Peterson, 2014). On the other hand, Schmallenberg virus (SBV), which is incident in Europe, is a teratogenic virus infecting domestic or wild ruminants (Hoffmann et al., 2012; Wernike and Beer, 2020). BUNV, the prototype of the orthobunyavirus genus, mainly found in the Americas and African countries, causes dengue-like disease, with fever symptoms in humans or neurological deterioration in animals, which may lead to death (Tauro et al., 2012, 2015).

Vaccine strategies to counteract orthobunyavirus infection have not been completely established, although some studies have made substantial efforts to achieve virus neutralization. The SBV Gc N-terminus vaccine, in a regular mammalian vector system, provided some immunization in cattle (Wernike et al., 2017), but when the same SBV domain was constructed in a modified Vaccinia virus Ankara vector, it granted full protection against SBV in those animals (Wernike et al., 2018). Further studies solved this N-terminus Gc structure and showed that it corresponds to glycoprotein head and stalk domains (Hellert et al., 2019). IFNAR KO mice models immunized with SBV Gc head and stalk domains presented sterile immunity when challenged with SBV, and the viral genome load was not detectable in those animals (Hellert et al., 2019). These studies show that the SBV Gc head and stalk domains' peptide and, potentially, equivalent domains of other Bunyaviruses, are strong candidates for future vaccine strategies. Furthermore, LACV studies were conducted in immunized IFNAR KO mice with the DNA-encoding viral glycoproteins Gn and Gc as a vaccine strategy. After the LACV challenge, there was a strong production of neutralizing antibodies against viral glycoproteins. In contrast, when IFNAR KO mice were immunized with the LACV N protein, no neutralizing antibodies were detected (Schuh et al., 1999). Studies on OROV vaccine strategies are unavailable in the literature, although bioinformatics assessments have attempted to predict the immunogenic viral protein and its possible cellular targets. Highly conserved predicted T-cell and B-cell epitopes were found to be promising interaction sites for the OROV M polyprotein, a fact that may indicate specific sites for future vaccine design (Adhikari et al., 2018).

Pathogenic viruses from the *Phenuiviridae* family, such as SFTSV and RVFV, cause significant liver damage in infected patients with increased serum liver enzymes. Mild cases of SFTSV and RVFV present febrile illness; however, in severe cases, SFTSV can lead to thrombocytopenia and multiorgan dysfunction (Yu et al., 2011), while RVFV can cause significant neurological damage (Alrajhi et al., 2004). Efforts for the development of vaccines to counteract either RVFV or SFTSV have been made, but without any strong leading candidates. SFTSV was only recently discovered, and vaccine studies against this virus have been quite challenging due to the lack of animal models that replicate human symptoms (Reece et al., 2018). Fortunately, ferrets proved to be very useful for SFTSV studies since they recapitulate human clinical symptoms (Park et al., 2019) and have been used as research tools to investigate SFTSV vaccines using different DNA vaccine strategies. Gc and Gn vaccines showed higher levels of neutralizing antibodies, with significant immune protection against SFTSV. In contrast, the same phenotype was not observed with the vaccine containing the N, NSs, and RdRp genes (Kwak et al., 2019).

Currently, there are at least two ongoing vaccine investigations for humans against RVFV: formalin-inactivated (TSI-GSD-200) and live-attenuated (MP-12) vaccines. Although TSI-GSD-200 presents mild side effects at tolerable levels, it demands multiple doses in humans to achieve viral protection (Rusnak et al., 2011). MP-12, on the other hand, requires a single dose to provide neutralizing activity and is currently under phase-II clinical trials (Pittman et al., 2016). However, abortion and teratogenic side effects were observed in animals vaccinated during early pregnancy with MP-12 (Hunter et al., 2002). Thus, MP-12 raises a concern for pregnant women who need vaccines against RVFV. An adenovirus-based vector expressing RVFV Gn and Gc was tested in early pregnant animals, which presented high neutralizing antibody titers after a single inoculation dose, without any teratogenic side effects. This vaccine is currently being developed for human testing (Stedman et al., 2019).

Hantaviruses are divided into old world (OW) hantaviruses, mainly located in Europe and Asia, and new world (NW) hantaviruses, found in the Americas. OW hantaviruses are the etiological agents of hemorrhagic fever with renal syndrome (HFRS), with a 1–2% mortality rate, and include HTNV, Seoul virus (SEOV), Puumala virus (PUUV), and Dobrava virus (DOBV). NW hantaviruses are the etiological agents of hantavirus cardiopulmonary syndrome (HCPS), with a 40% mortality rate, and include ANDV and Sin Nombre virus (SNV) (Kruger et al., 2015). The pathogenesis of these viruses involves mainly the endovascular system, where they suppress $\alpha\text{v}\beta 3$ integrin, leading to vascular permeability, plasma leakage, and may even induce hemorrhage (Gavrilovskaya et al., 1999, 1998). Several vaccine strategies have been developed to counteract pathogenic hantaviruses, and the most promising ones are DNA vaccines designed for Gn and Gc glycoprotein expression (Engdahl and Crowe, 2020). The hantavirus DNA vaccine designed using HNTV and PUUV M segment cDNA presented high levels of neutralizing antibodies in hamsters and non-human primates, with promising results for future vaccines (Hooper et al., 2001). Indeed, those vaccines progressed to clinical trials and, to date, are in phase-I trial. HNTV and PUUV vaccines were delivered to individuals by intramuscular electroporation and exhibited significant seroconversion in vaccinated participants (Hooper et al., 2014). Another group showed that the HNTV vaccine constructed with Gc or Gn cDNA fused with the lysosomal-associated membrane protein (LAMP) cytosolic tail resulting in high titers of neutralizing antibodies and long-term immunity in mice (Jiang et al., 2018, 2017). On the other hand, vaccine strategies with inactivated HNTV have also been used, but with little success when compared to DNA-based ones (Yi et al., 2018; Jung et al., 2018). Therefore, among the several hantavirus vaccine strategies that are being developed, so far, DNA vaccines are the most promising ones.

The most studied and well-establishedairovirus is CCHFV due to its 30% mortality rate. Located mainly in Europe, Asia, and Africa, CCHFV infection leads to disseminated intravascular coagulation in patients (Swanepoel et al., 1989); it is still unclear if the virus causes vascular permeability, as previously suggested (Bente et al., 2013). Studies in mild or severely infected patients indicated high serum levels of the inflammatory markers: TNF- α , IL-6, IFN- γ , and IL-10 (Saksida et al., 2010; Ergonul et al., 2006). Necropsy histopathological analyses of CCHFV infected patients revealed massive liver damage, with eosinophilic necrotic hepatocytes and hypertrophic Kupffer cells positive for virus antigen (Bente et al., 2013). Similarly to hantaviruses, CCHFV vaccine strategies were based on DNA and inactivated vaccines. However, mouse models for CCHFV vaccine studies have not rendered promising results due to the lack of vaccine immunogenicity (Spik et al., 2006). Therefore, new studies have been carried out in IFNAR KO mice for such research since they are considered immunocompromised animals. Indeed, when treated with the M segment cDNA-based vaccine, followed by CCHFV infection, 60% of the immunocompromised animals exhibited virus

protection (Garrison et al., 2017). In contrast, the Adenovirus-based vaccine expressing CCHFV-N in IFNAR KO mice, followed by virus infection, presented partial protection against CCHFV, and no viral protein was detected in liver tissue (Zivcec et al., 2018). A plasmid DNA-based vaccine encoding for Ub-linked versions of CCHFV Gn, Gc, and the nucleoprotein (N) has also been developed, as well as another vaccine strategy based on transcriptionally competent virus-like particles (tc-VLPs). The DNA virus-based vaccine provided 100% viral protection in IFNAR KO mice, with Th1 immune response and high levels of neutralizing antibodies. Although tc-VLPs-treated mice also presented high neutralizing antibody levels, a relatively low survival rate (40%) was observed after CCHFV infection. Therefore, the CCHFV DNA virus-based vaccine encoding for viral Ub-linked versions of Gn, Gc, and N showed to be an excellent candidate for further studies on CCHFV vaccine design (Hinkula et al., 2017). Although the CCHFV-inactivated vaccine is the only one currently approved, its use is exclusively for Bulgarian soldiers in endemic areas. Vaccinated individuals were reported to exhibit robust T-cell activity, as measured by serum IFN- γ , albeit with low levels of neutralizing antibodies (Mousavi-Jazi et al., 2012). Therefore, seeing that there are no strong leading vaccine candidates for CCHFV, further studies are required in order to establish a safe vaccine.

Arenaviruses are highly pathogenic viruses that, similarly to hantaviruses, are divided into new world (NW) arenaviruses, located in the Americas, and old world (OW) arenaviruses, found in West African countries. Pathogenic arenaviruses may cause hemorrhagic fever, with symptoms that can vary between NW and OW arenaviruses (vascular damage and liver damage, respectively) (Brisse and Ly, 2019). LASV, for example, is an OW arenavirus that has a 20% mortality rate. Although several LASV vaccine trials have been conducted, no leading candidate has been approved up to date. The LASV VLPs vaccine strategy, which expresses the viral glycoprotein precursor, has been shown to trigger mice T-cell response. However, it did not induce neutralizing antibody production against viral glycoproteins (Wang et al., 2018). A recombinant vaccinia virus vaccine expressing LASV glycoproteins in non-human primates (NHP) prevented animal death after virus challenging (Fisher-Hoch et al., 1989, 2000). Nonetheless, when expressing the LASV N protein, the mortality rate reached up to 80% (Fisher-Hoch et al., 2000). Moreover, inactivated LASV did not induce humoral protection in vaccinated challenged NHP, a fact that led to high levels of animal death after infection (McCormick et al., 1992). Interestingly, a DNA vaccine expressing LASV glycoproteins, introduced by dermal electroporation, provided full protection in NHP without any signs of Lassa fever symptoms (Cashman et al., 2017). Therefore, the DNA vaccine expressing LASV glycoproteins seems to be the most promising vaccine strategy to counteract LASV infection and is currently under further study regarding vaccine safety. Junin virus (JUNV) is a NW arenavirus and is the etiological agent of Argentinian hemorrhagic fever. A leading vaccine candidate is the JUNV strain Candid #1, a virus-attenuated strategy (Grant et al., 2012) that is already in use in endemic regions. This vaccine presents high protection efficacy (Maiztegui et al., 1998) but has not been approved by the Food and Drug Administration. Therefore, it is only used in Argentinian hemorrhagic fever endemic regions.

Several attempts have been made to design vaccines against bunyaviruses. Currently, there are important data showing possible leading vaccine candidates, or viral protein targets, for different bunyaviruses. Meanwhile, antibody-based therapies could represent a viable option in the battle against life-threatening bunyavirus infections. In this regard, a recent study using engineered multimeric single-domain antibody complexes against RVFV and SBV revealed that these molecules were highly effective in reducing and preventing morbidity and mortality in mouse infection models (Wichgers Schreur et al., 2020). Hopefully, in the near future, vaccines and antibody therapies will be developed to avert bunyavirus spread.

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Rhabdoviridae, Rabies Virus

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Introduction

Rabies virus (RABV) causes an acute neurological disease (called rabies) in humans and animals that is almost 100% fatal once infected hosts are symptomatic (reviewed in [Fooks et al., 2014, 2017](#); [Davis et al., 2015](#); [Fisher et al., 2018](#); [Du Pont et al., 2019](#)). Rabies is considered to be as the deadliest neglected tropical disease. RABV invades the central nervous system (CNS) in humans and provokes various symptoms including hydrophobia (fear of water), aggressiveness, agitation, confusion, hallucinations, muscle spasms, convulsions, and/or paralysis within 1–2 weeks prior to death. Rabies is one of the oldest infectious diseases in human history. The Laws of Eshnunna (c.1930 BCE) in ancient Mesopotamia evidenced that this zoonotic deadly disease transmitted from rabid dogs to humans was already recognized 4000 years ago ([Tarantola 2017](#)). Louis Pasteur and Emile Roux demonstrated for the first time in the 1880s that rabies is a vaccine preventable disease in dogs and humans and their achievement thus opened a new era in vaccine development. Although highly effective and safe vaccines and neutralizing antibodies against RABV are currently available (reviewed in [Tantawichien and Rupprecht, 2020](#)), RABV still kills nearly 60,000 people worldwide every year. In most cases, people acquired rabies from dog bites in developing countries with lower vaccination coverage in domestic dogs. In humans, clinical signs and symptoms of rabies are observed after variable asymptomatic periods (1 week to >1 year, average 2–3 months), which may be determined by various factors including initial infection sites, viral loads, viral pathogenicity, and host immunity. RABV is also known to be transmitted to humans from wild animals, such as bats, raccoons, skunks, foxes, mongooses, coyotes, and jackals, although in rare cases. Furthermore, RABV-like lyssaviruses, such as Australian bat lyssavirus, European bat lyssaviruses 1 and 2, Irkut virus, Duvenhage virus, and Mokola virus were reported to cause rabies-like diseases in humans (reviewed in [Johnson et al., 2010](#); [Fisher et al., 2018](#); [Fooks et al., 2017](#)). Therefore, the diseases caused by lyssaviruses remain significant public health threats.

RABV is a member of the *Lyssavirus* genus of the *Rhabdoviridae* family within the order *Mononegavirales* (reviewed in [Lyles et al., 2013](#)). Viruses belonging to the order *Mononegavirales* possess a negative-sense monopartite RNA genome (rarely bipartite genomes) and are called non-segmented negative strand (NNS) RNA viruses or mononegaviruses (reviewed in [Lamb 2013](#)). NNS RNA viruses are highly diversified eukaryotic viruses infecting vertebrates, invertebrates, plants, and/or fungi, and include important human pathogens, such as measles (*Paramyxoviridae*), Nipah (*Paramyxoviridae*), human respiratory syncytial (*Pneumoviridae*), and Ebola (*Filoviridae*) ([Lamb 2013](#); [Maes et al., 2019](#)). The family *Rhabdoviridae* consists of 30 genera that include 191 species as of 2020. Historically, vesicular stomatitis virus (VSV, an arthropod-borne animal rhabdovirus) belonging to the *Vesiculovirus* genus has served as an excellent model to study basic biology of rhabdoviruses and, by extension, other NNS RNA viruses. RABV and VSV share many common mechanisms involved in various aspects of viral replication in host cells, although RABV manifests its pathogenic properties quite differently from VSV. The major breakthrough in NNS RNA viral research was the development of a reverse genetics system for RABV that allows generation of recombinant viruses from cDNAs ([Schnell et al., 1994](#)). Studies using the reverse genetics system have led to deeper understanding of RABV gene functions in viral replication as well as pathogenesis. Furthermore, recent structural, biochemical, and cell biological studies have provided new insights into the roles of individual RABV proteins in each step of the viral life cycle. This chapter describes our current knowledge on various aspects of RABV infection.

Genome and virion structures

The RABV genome with approximately 11,900 nucleotides (nt) in length consists of five genes encoding the nucleocapsid (N), phospho- (P), matrix (M), glyco- (G), and large (L) proteins in the order 3'-N-P-M-G-L-5' similarly to other rhabdoviral genomes, such as the VSV genome ([Fig. 1A](#)) ([Tordo et al., 1986a, b, 1988](#); [Conzelmann et al., 1990](#)). The genome is encapsidated with the N proteins (450 amino acids) ([Iseni et al., 1998](#); [Schoehn et al., 2001](#)) to form a helical nucleocapsid, which serves as a template for

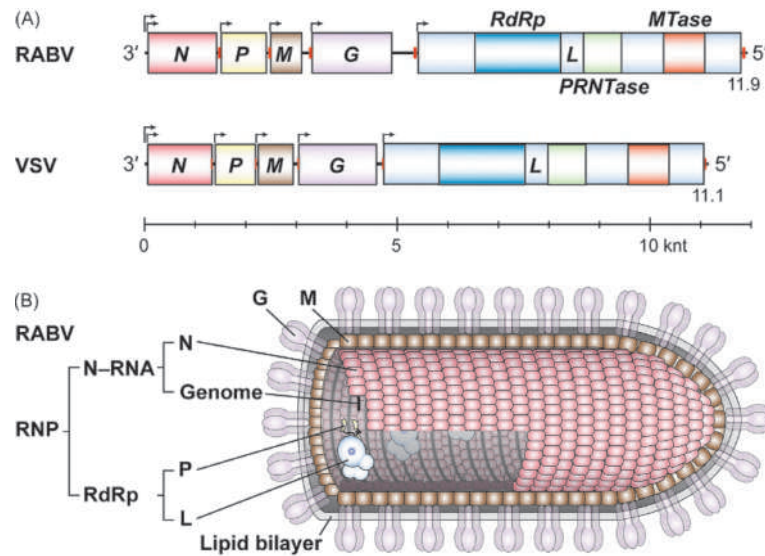


Fig. 1 Schematic diagrams of the RABV genome and particle. (A) The genomic structure of RABV (upper) is compared with that of VSV (lower). The positions of the nucleocapsid (N), phospho- (P), matrix (M), glyco- (G), and large (L) genes in their genomes are shown with a scale bar (knt, kilo nucleotides). The L gene products possess RNA-dependent RNA polymerase (RdRp), GDP polyribonucleotidyltransferase (PRNTase), and methyltransferase (MTase) domains. Bent arrows and red vertical lines indicate transcription initiation and polyadenylation/termination signals, respectively. (B) A RABV particle contains a ribonucleoprotein (RNP), which is composed of the genome and the N, P, and L proteins. The genome is wrapped with the N proteins to form a nucleocapsid (N-RNA), which serves as a template for RNA synthesis. The L protein interacts with its cofactor P protein to form an RdRp complex (L-P). The RNP is covered with the M proteins, and further enveloped by a lipid bilayer, which is studded with the G proteins.

transcription and replication (called N-RNA template) (Fig. 1B). Each N protomer encapsidates nine nucleotides of RNA (Albertini et al., 2006), suggesting that approximately 1300 N proteins are associated with a single RABV genome. RNA-dependent RNA polymerase (RdRp) complexes composed of the L protein (2127 amino acids) and its co-factor P protein (297 amino acids) interact with the N-RNA template to form a ribonucleoprotein (RNP) complex, which acts as a transcriptional machinery. In a virus particle, a bullet-shaped RNP complex is embedded in a matrix layer comprising the M proteins (202 amino acids) to form a skeleton-like structure, and further enclosed by a lipid bilayer derived from host cell plasma membrane (Riedel et al., 2019). The M protein mediates virus assembly and release by its direct interactions with the RNP and envelope (Mebatsion et al., 1999). The viral envelope is studded with the G proteins (505 amino acids, an integral transmembrane glycoprotein), which is critical for virus attachment and cell entry. For VSV, copy numbers of the N, P, M, G, and L proteins within a virion were estimated to be 1200, 500, 1800, 1200, and 50, respectively (Thomas et al., 1985). Typical RABV particles with approximately 80–90 nm in diameter and 180–220 in length represent a bullet-shape with conical and flat ends (Guichard et al., 2011; Riedel et al., 2019).

Transmission, life cycle, and pathogenesis

The major route of RABV transmission to humans is exposure of local tissues (e.g., muscle) to saliva from infected animals through bite wounds (Fig. 2A). After amplification of RABV in muscle cells, RABV infects motor neurons at neuromuscular junctions (Harrison and Murphy, 1978; Lentz et al., 1982; Burrage et al., 1985; Tsiang et al., 1986; Charlton et al., 1997; Yamaoka et al., 2013), where neuronal and muscular cells communicate each other via electrical signals. Alternatively, RABV may directly enter axon terminals of neurons without local replication in muscle cells (Coulon et al., 1989; Shankar et al., 1991). RABV also uses sensory neurons to travel to the CNS (Lycke and Tsiang, 1987; Coulon et al., 1989; Velandia-Romero et al., 2013).

Uptake of RABV into neurons at axon terminals is mediated by receptor binding followed by clathrin-dependent endocytosis (Superti et al., 1984; Lewis et al., 2000; Piccinotti et al., 2013; Piccinotti and Whelan, 2016). The RABV G protein plays critical roles in virus attachment and cell entry. The RABV G protein is composed of an N-terminal signal peptide, ectodomain with three subdomains [central domain (CD), pleckstrin homology domain (PHD), and fusion domain (FD)], transmembrane domain, and C-terminal cytoplasmic domain (Hellert et al., 2020; Yang et al., 2020) (Fig. 3A). A mature form of the G protein lacking the signal peptide is N-glycosylated at aspartate residues (e.g., D37, D319) within the ectodomain (Shakin-Eshleman et al., 1992; Shakin-Eshleman et al., 1993; Yamada et al., 2012, 2013), and is present as a membrane-anchored trimer (Whitt et al., 1991; Gaudin et al., 1992). The RABV G protein serves as not only a major antigen for the host immune system but also a key determinant of viral pathogenesis (reviewed in Lafron, 2016).

RABV may utilize several cell surface molecules as the G-protein-mediated attachment and/or entry receptors to infect muscular or neuronal cells. These cellular receptor candidates include nicotinic acetylcholine receptor (nAChR) (Lentz et al., 1982, 1984,

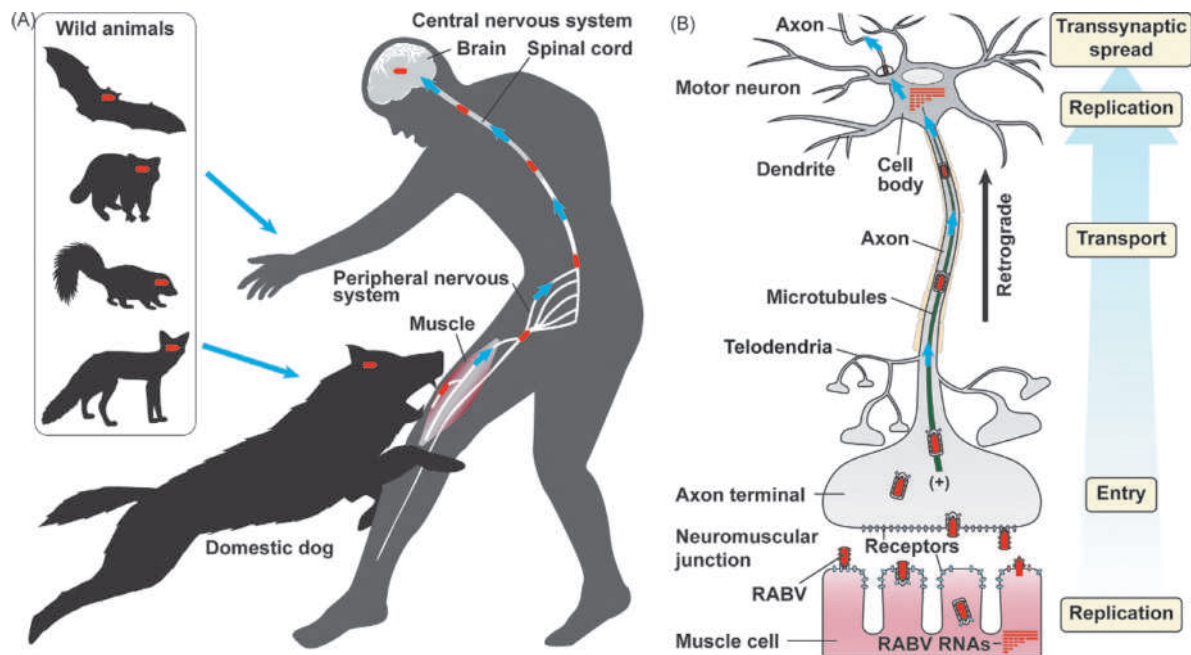


Fig. 2 Invasion of the central nervous system with RABV. (A) RABV is transmitted to humans by bites from rabid dogs or wild animals (e.g., bats, raccoons, skunks, foxes) through their saliva. RABV travels from peripheral muscle tissues to the central nervous system (the spinal cord followed by the brain) through the peripheral nervous system. (B) After replication of RABV in muscle cells, RABV enters motor neurons at neuromuscular junctions, and moves using the microtubule-dependent retrograde axonal transport system to their cell bodies, where viral genome transcription and replication occur. Transsynaptic retrograde transmission of RABV between neurons leads to invasion of the central nervous system.

1987; Lentz, 1990; Baer et al., 1990; Gastka et al., 1996; Kumar et al., 2007), neuronal cell adhesion molecule (NCAM or CD56, a member of the immunoglobulin superfamily) (Thoulouze et al., 1998), p75 neurotrophin receptor (p75NTR or CD27, a member of the tumor necrosis factor receptor superfamily) (Tuffereau et al., 1998; Langevin et al., 2002), metabotropic glutamate receptor subtype 2 (mGluR2, a member of the G protein-coupled receptor family) (Wang et al., 2018), heparan sulfate proteoglycans (Sasaki et al., 2018), integrin $\beta 1$ (Shuai et al., 2020), and carbohydrates (Superti et al., 1984; Conti et al., 1986). Different subtypes of nAChR are expressed on both pre- and post-synaptic membranes at neuromuscular junctions (reviewed in Hurst et al., 2013). Although α -bungarotoxin (a snake venom neurotoxin)-sensitive muscle-type nAChR is believed to serve as a G-receptor on muscle cells at neuromuscular junctions (Lentz et al., 1982; Tsiang et al., 1986), α -bungarotoxin-insensitive neuron-type nAChR may also have an affinity to the G protein (Lentz, 1990). However, the precise roles of these cellular receptor candidates in infection of muscular or neuronal cells with RABV still remain controversial and poorly understood (reviewed in Belot et al., 2019; Guo et al., 2019).

Internalized RABV within endocytic vesicles is transported in axons to their cell bodies via axonal dynein in a microtubule-dependent manner (Tsiang, 1979; Lycke and Tsiang, 1987; Klingenstein et al., 2008; Gluska et al., 2014; Piccinotti and Whelan, 2016) (Fig. 2B). Pseudotyping of lentiviruses or VSV with the RABV G protein confers infectivity to neurons and retrograde axonal transport ability on these viruses (Mazarakis et al., 2001; Beier et al., 2013; Hislop et al., 2014), indicating that the RABV G protein is a determinant of RABV neurotropism. Some of the receptor candidates, such as p75NTR, NCAM, and nAChR, are known to be co-transported with endocytic vesicles containing RABV or HIV pseudotyped with the RABV G protein along axons (Gluska et al., 2014; Hislop et al., 2014). In cell bodies, the endocytic vesicles containing RABV are routed to endosomes (Lewis et al., 1998; Gluska et al., 2014; Piccinotti and Whelan, 2016).

Once the endosomal lumen is acidified ($< \text{pH } 6.2$) during endosomal maturation, low-pH-dependent fusion between the viral and cellular membranes occurs prior to fusion of late endosomes with lysosomes (Fig. 4) (Whitt et al., 1991; Gaudin et al., 1991, 1993). During the fusion process, a structural rearrangement in the ectodomain of the RABV G protein in response to low pH repositions hydrophobic fusion loops in the fusion domain toward target cellular membranes (Fig. 3B), leading to membrane fusion (Gaudin et al., 1993; Hellert et al., 2020; Yang et al., 2020). In VSV, the membrane fusion and RNP release into the cytoplasm are separate events. First, the viral membrane preferentially fuses with intraluminal vesicles in endosomal intermediates, called endosomal carrier vesicles or multivesicular bodies, derived from early endosomes, resulting in uncoating of viral RNPs into the intraluminal vesicles (Le Blanc et al., 2005). Then, viral RNPs in intraluminal vesicles are delivered to late endosomes via endosomal carrier vesicles and subsequently released into the cytoplasm by back-fusion of the intraluminal vesicles with late-endosomal membranes, which is mediated by the late endosomal lipid lysobisphosphatidic acid and its binding protein, Alix (Le Blanc et al., 2005).

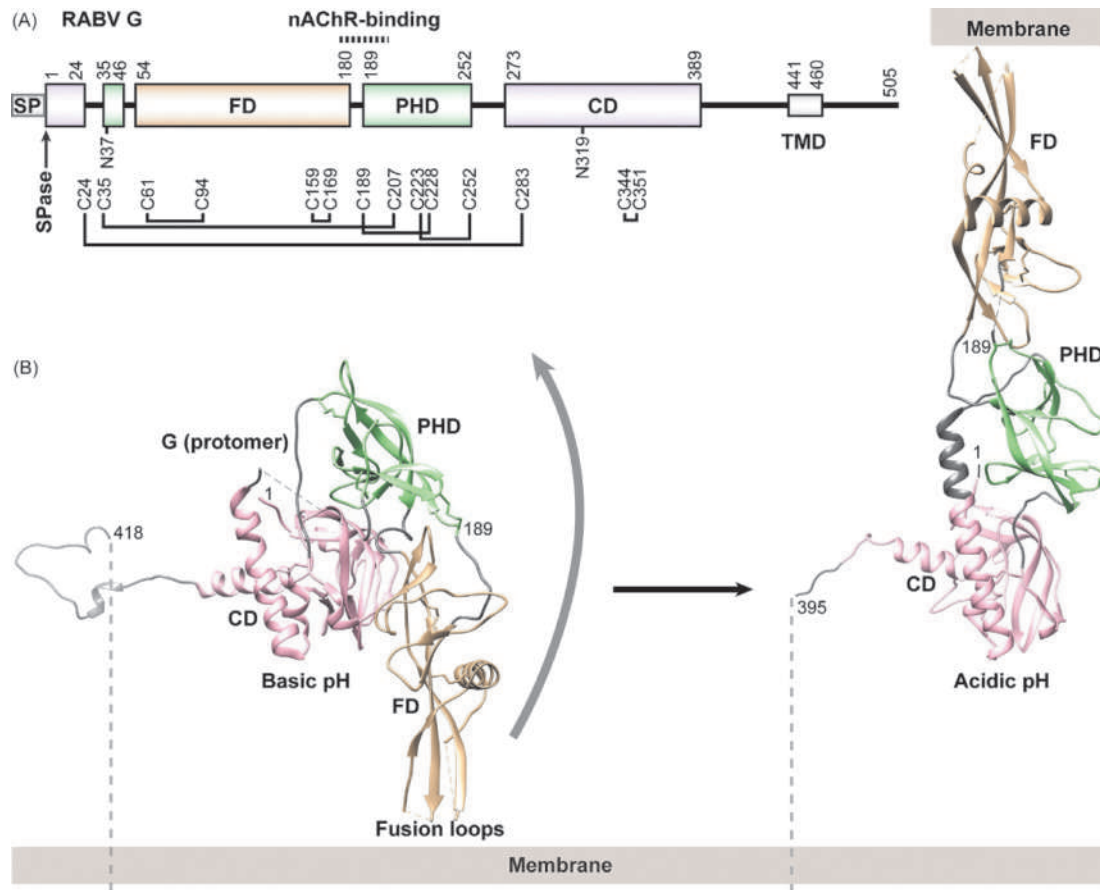


Fig. 3 Structure of the RABV G protein. (A) The domain organization of the RABV G protein is schematically represented. The N-terminal signal peptide (SP) is removed by signal peptidase (SPase). The ectodomain is composed of the central domain (CD), pleckstrin homology domain (PHD), and fusion domain (FD). The C-terminal part consists of the transmembrane domain (TMD) and cytoplasmic domain. The positions of a proposed binding site for nicotinic acetylcholine receptor (nAChR), N-glycosylation sites (N37, N319), and disulfide bridges between cysteine residues are indicated. Numbers indicate the positions of amino acid residues beginning and ending the domains/subdomains. (B) The crystal structures of the ectodomain of the RABV G protein at basic (PDB id: 6LGX) and acidic (PDB id: 6LGW) pH are shown as ribbon models. All structural images were generated with the UCSF Chimera program (Pettersen et al., 2004).

After releasing RNPs into the cytoplasm, RABV genome transcription and replication take place in membrane-less replication compartments (called Negri bodies) (Fig. 4) (Lahaye et al., 2009, 2012; Nikolic et al., 2016, 2017). Negri bodies were originally discovered as cytoplasmic inclusion bodies in neuronal cells of RABV-infected animals by Adelchi Negri in 1903 (reviewed in Kristensson et al., 1996; Nevers et al., 2020). Negri bodies exhibit dynamic liquid-like properties and are formed with multivalent weak interactions between viral replication components (e.g., N, P, and RNA) leading to their liquid-liquid phase separation from the cytosol (Nikolic et al., 2017). Negri bodies contain not only the RABV N, P, L proteins, genome, anti-genome, and mRNAs, but also host proteins, such as HSP70 and focal adhesion kinase (FAK), which positively regulate viral replication (Lahaye et al., 2009, 2012; Fouquet et al., 2015). Newly assembled RNPs are released from Negri bodies and move along microtubules to virus assembly sites (Nikolic et al., 2017).

Transsynaptic retrograde transmission of RABV between neurons leads to RABV invasion of the spinal cord followed by systematic infection of the entire brain leading to the acute progressive neurological disease (Coulon et al., 1989; Astic et al., 1993; Wickersham et al., 2007; Ugolini, 2011). Furthermore, RABV is known to spread in sensory neurons transsynaptically in the anterograde direction (Zampieri et al., 2014), suggesting that the anterograde transport of RABV is involved in centrifugal RABV spread to peripheral tissues. Enveloped RABV particles or viral RNPs are transported in axons of peripheral neurons such as sensory neurons in dorsal root ganglion in both the retrograde and anterograde directions (Lycke and Tsiang, 1987; Tsiang et al., 1989; Bauer et al., 2014). The RABV G protein is essential for transsynaptic transmission in both the directions (Etessami et al., 2000; Yan et al., 2002; Zampieri et al., 2014; Bauer et al., 2014). RABV is transported to salivary glands via the facial nerve and excreted in the saliva to further transmit to other animals or humans (Boonsriroj et al., 2016).

Pathogenic strains of RABV do not cause significant histopathological changes, inflammation, or neuronal cell death in the CNS of infected animals or humans, but induces disappearance of rough endoplasmic reticulum (ER) and ribosomes, mitochondrial dysfunction leading to oxidative stress, and beading/swelling and fragmentation of the dendrites and axons in infected neurons (Li et al., 2005a, b; Scott et al., 2008; Jackson et al., 2010; Alandijany et al., 2013; Kammouni et al., 2017), which may disrupt neuronal

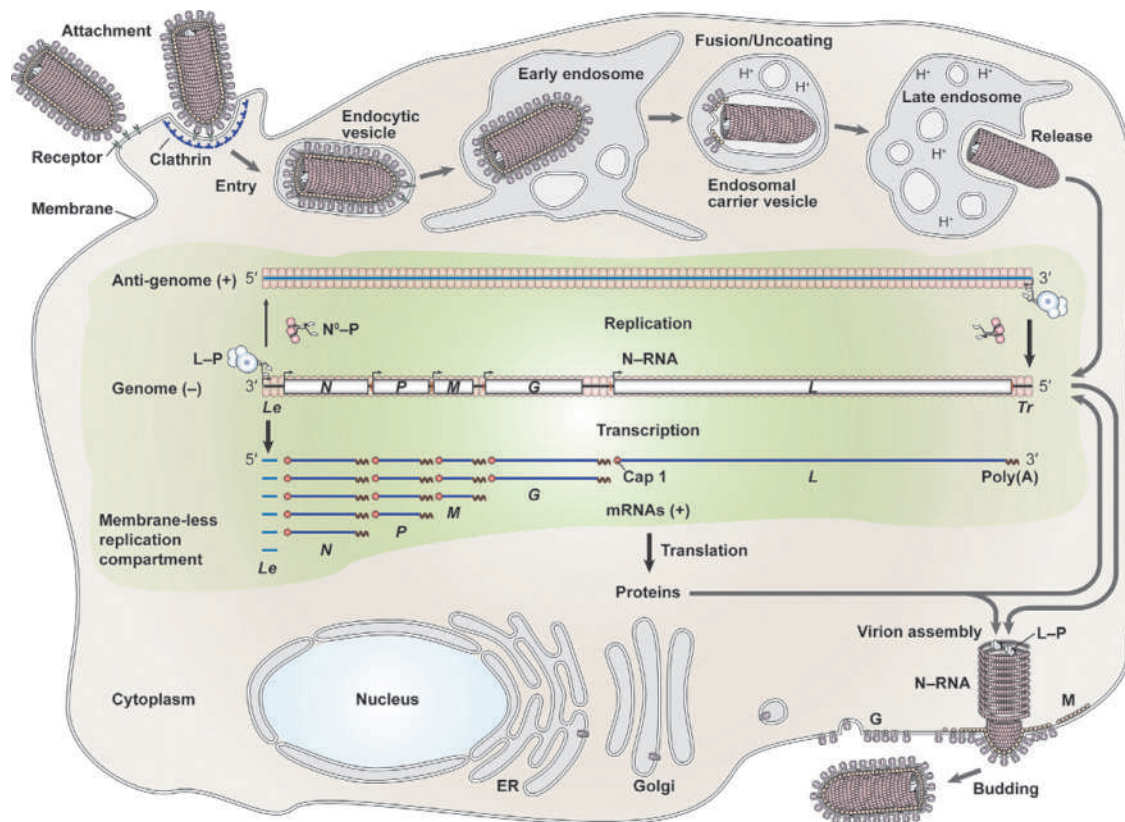


Fig. 4 Life cycle of rhabdoviruses. Rhabdoviruses attach to receptors on the host cell surface by the G protein and enter host cells by clathrin-dependent endocytosis. Viral RNPs are released from late endosomes into the cytoplasm through a stepwise membrane fusion/uncoating process mediated at low pH. Viral transcription and replication occur in membrane-less replication compartments (called Negri bodies for RABV). For transcription, the RdRp (L–P) complex sequentially transcribes the *leader* region (*Le*) and *N*, *P*, *M*, *G*, and *L* genes on the negative-strand RNA genome in the N–RNA complex into the leader RNA (LeRNA) and monocistronic mRNAs with the 5′-cap 1 structure and 3′-poly(A) tail. For replication, the RdRp complex copies the genome into the positive-strand anti-genome, which is in turn used as a template to generate progeny genomes. A complex between the RNA-free N protein and P protein (N⁰–P) is required for encapsidation of these replication products. The M protein mediates the assembly of progeny RNPs and the G proteins into virus particles at the plasma membrane. Finally, viruses are budded from infected cells.

networks and/or other neuronal functions leading to neurological disorders in the late stage of infection. The G proteins of pathogenic and attenuated RABV strains interact with different cellular partners, microtubule-associated serine-threonine kinases 1 and 2 (MAST1 and MAST2) and non-receptor protein tyrosine phosphatase 4 (PTPN4) via slightly different PDZ-binding sites in their C-terminal cytoplasmic domain to promote markedly different outcomes, neuronal survival and apoptosis, respectively (Prehaud et al., 2010; Terrien et al., 2012). The neuronal survival, rather than death, phenotype of RABV strains is associated with their virulence in infected hosts. There are two types of rabies: encephalitic (furious) and paralytic (dumb) (Fooks et al., 2014, 2017). Patients with encephalitic rabies suffer hypersalivation, hydrophobia, aggressiveness, and agitation followed by coma, and die from respiratory failure or cardiac arrest. Patients with paralytic rabies have muscle spasms followed by paralysis and fall into a coma prior to death.

Transcription, replication, and translation

The RABV genome and anti-genome are wrapped with the N proteins during replication to form unique N–RNA templates for RNA synthesis. The N- and C-terminal domains (NTD and CTD) of the N protein are orientated in an angled conformation to form an RNA-binding groove (Fig. 5) (Albertini et al., 2006; Green et al., 2006), in which basic amino acid residues recognizes the phosphate backbone of 9-nt RNA. In the N–RNA complex, each N subunit interacts with neighboring N subunits via N-terminal arm and C-terminal loop structures extended from NTD and CTD, respectively (Albertini et al., 2006; Green et al., 2006). In VSV, the N-arm and C-loop structures and phosphate-binding basic amino acid residues were shown to be required for the formation of the functional N–RNA template (Zhang et al., 2008; Rainsford et al., 2010). In RABV particles, the N–RNA complex is tightly coiled into a bullet-shaped structure with an outer diameter of 56–74 (average 67) nm at the cylindrical trunk (Riedel et al., 2019). The trunk and

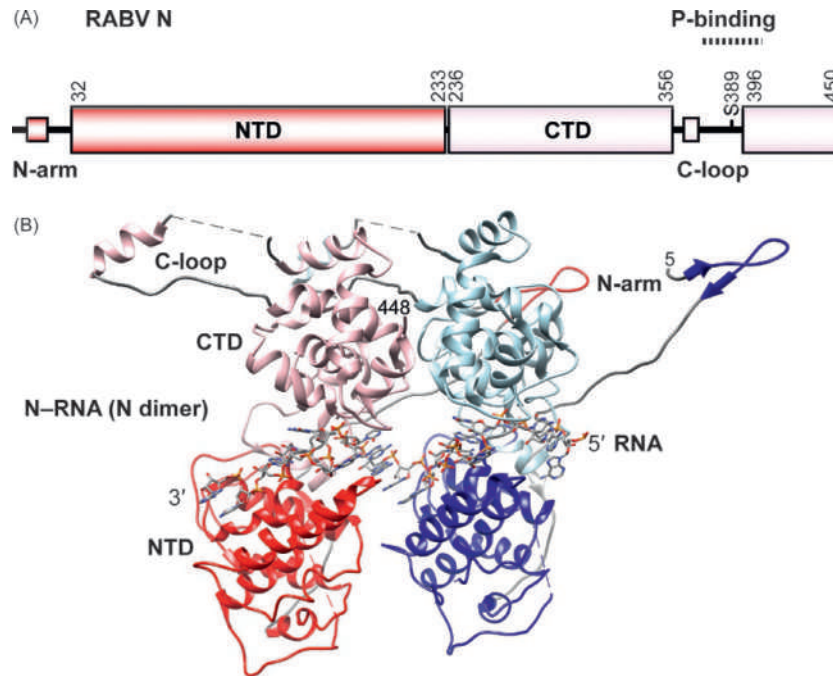


Fig. 5 Structure of the RABV N protein. (A) The RABV N protein is composed of the N- and C-terminal domains (NTD and CTD, respectively) with an arm-like (N-arm) and loop (C-loop) structures, respectively. A proposed binding site for the P protein is indicated by a dashed line. A phosphorylation site (S389) is labeled. (B) The crystal structure of a dimer of the RABV N subunits complexed with 18-mer of RNA (part of the undecameric N-RNA complex, PDB id: 2GTT) is represented as a ribbon model. The NTD and CTD of a subunit (left) are colored red and pink, respectively, whereas those of another subunit (right) are colored blue and light blue, respectively.

conical end of the N-RNA complex consist of 14–24 (average 18) and 1–7 helical turns, respectively (Riedel et al., 2019). A serine residue at position 389 (S389) of the RABV N protein is phosphorylated by cellular casein kinase II, resulting in efficient transcription and replication (Wu et al., 2002, 2003). As in the case of the VSV (Green and Luo, 2009), the C-loop of two adjacent N subunits in the RABV N-RNA complex may provide a binding site for the C-terminal domain of the P protein (Ribeiro Ede Jr. et al., 2009).

The RABV P protein acts as a multifunctional adapter molecule for the L and N proteins during transcription and replication. The RABV P protein is an elongated dimeric protein with non-structured regions (Gerard et al., 2007, 2009) and is phosphorylated at serine residues (e.g., S63, S64, S162, S210, S271) with cellular kinases including protein kinase C (Gupta et al., 2000) (Fig. 6). The P protein is a noncatalytic subunit of the RdRp L protein. Early studies identified two independent regions (residues 1–19 and 40–100) of the RABV P protein as L-binding sites (Chenik et al., 1998; Castel et al., 2009). A recent cryo-electron microscopic (EM) analysis of the RABV L-P complex revealed that a region encompassing residues 51–87 is associated with discontinuous regions of the L protein (Horwitz et al., 2020). Although residues 11–50 of the RABV P protein are sufficient to stimulate an in vitro transcription activity of the L protein (Morin et al., 2017), this region was invisible in the cryo-EM structure (Horwitz et al., 2020). The C-terminal domain (CTD, residues 186–297) of the P protein in the RdRp complex is required to interact with the N-RNA complex (Mavrikis et al., 2004; Ribeiro Ede Jr. et al., 2009). During replication, the P protein forms a complex with an RNA-free N protein (called N⁰) to prevent non-specific binding of the N protein to cellular RNAs and brings the N⁰ protein to the replication site to specifically encapsidate newly synthesized viral genomic and anti-genomic RNAs (Peluso 1988; Peluso and Moyer, 1988; Masters and Banerjee, 1988; Gupta and Banerjee, 1997; Leyrat et al., 2011). Thus, the P protein functions as a chaperone for the N protein. In the RABV P protein, the N-terminal portion (residues 4–40) binds the N⁰ protein to form the N⁰-P complex (Mavrikis et al., 2003, 2006). A central region (residues 90–133) of the P protein serves as a dimerization domain (Ivanov et al., 2010). However, residues 65–175 of the RABV P protein including the dimerization domain and part of the L-binding site are dispensable for RABV transcription in host cells (Jacob et al., 2001). The RABV P protein also interacts with cellular dynein light chain 1 (DLC1, also called LC8, a subunit of the minus-end-directed microtubule motor dynein) (Jacob et al., 2000; Raux et al., 2000; Poisson et al., 2001) and FAK (Fouquet et al., 2015) via residues 139–151 (containing a DLC1-binding motif [K/R]xTQT) and 106–131, respectively. Both the host factors positively regulate RABV transcription and replication in host cells (Tan et al., 2007; Fouquet et al., 2015). The interaction of the P protein with DLC1 does not play any roles in microtubule-dependent axonal transport of RABV (Tan et al., 2007).

As reported for VSV (Emerson and Yu, 1975; De and Banerjee, 1984, 1985; Hunt and Hutchinson, 1993; Hercyk et al., 1988; Grdzlishvili et al., 2005; Li et al., 2005a, b; Ogino and Banerjee, 2007; Galloway and Wertz, 2008; Rahmeh et al., 2009; Morin et al., 2012), the RABV L protein may catalyzes all enzymatic reactions (RNA synthesis, mRNA capping, cap methylation, and

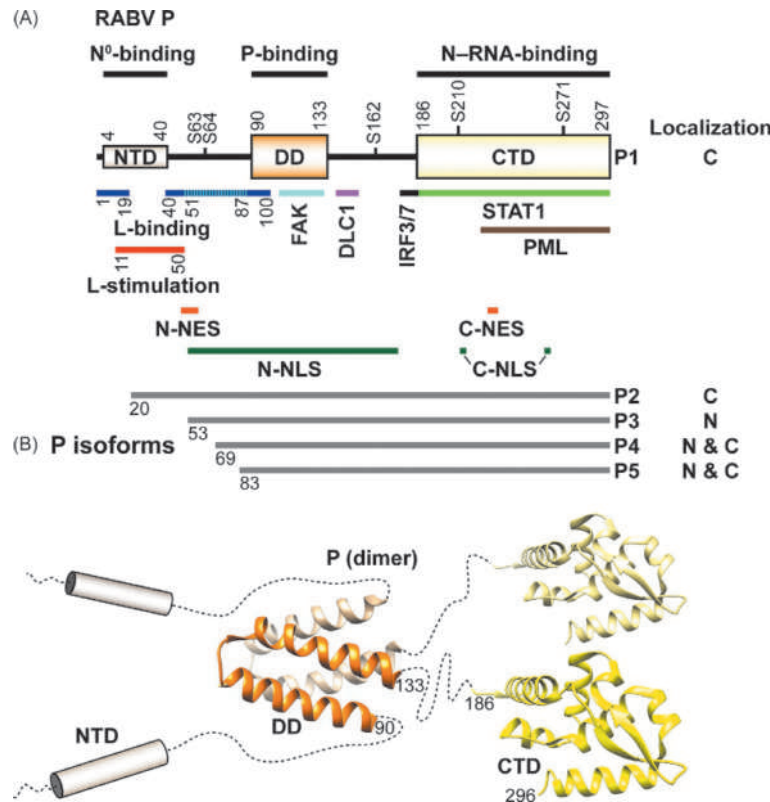


Fig. 6 Structure of the RABV P protein. (A) The RABV P protein possesses the N-terminal domain (NTD), dimerization domain (DD), and C-terminal domain (CTD), which serve as binding sites for the RNA-free N (N^0) protein, P itself, and N-RNA complex, respectively. The reported L-binding sites and L-stimulation domain are located within the N-terminal part of the P protein. Amino acid residues phosphorylated by cellular kinases (S63, S64, S162, S210, and S271) are labeled. The binding sites for focal adhesion kinase (FAK), dynein light chain 1 (DLC1), signal transducer and activator of transcription 1 (STAT1), and promyelocytic leukemia (PML) are marked. The locations of N- and C-terminal nuclear export signals (N-NES and C-NES) and nuclear localization signals (N-NLS and C-NLS) are indicated. P2–P5 are N-terminally truncated isoforms of the P proteins. Nuclear (N) and cytoplasmic (C) localization of the full-length P protein (P1) and isoforms (P2–P5) is indicated. (B) The RABV P protein exists as a dimer with two structured domains: the DD (two subunits colored orange and pale orange, PDB id: 3L32) and CTD (yellow and pale yellow, PDB id: 1VVI). Other regions in the dimer are depicted as cartoons. The structure of residues 51–87 of the P protein in the L–P complex is shown in Fig. 7B.

polyadenylation) for biosynthesis of viral RNAs. However, only its RNA synthesis and capping activities have so far been biochemically demonstrated (Ogino et al., 2016, 2019; Morin et al., 2017). Similar to the VSV L protein (Liang et al., 2015; Qiu et al., 2016; Ogino and Green 2019a), the RABV L protein is composed of multiple domains, such as N-terminal (NTD), RdRp, GDP polyribonucleotidyltransferase (PRNTase), connector (CD), methyltransferase (MTase), and C-terminal (CTD) domains (Fig. 7A and B). The NTD (residues 35–375) comprises two subdomains (NTD_I and NTD_{II}) and topologically resembles domains of segmented negative-strand and double-strand RNA viral RdRps (Qiu et al., 2016), such as the PA C-terminal domain (PA_C) of influenza viruses (He et al., 2008; Obayashi et al., 2008; Pflug et al., 2014; Reich et al., 2014; Hengrung et al., 2015), the PA_C-like domain of La Crosse orthobunyavirus (Gerlach et al., 2015), and λ 3 N-terminal domain of reovirus (Tao et al., 2002). The NTD includes amino acid residues that are conserved among NNS RNA viruses and critical for transcription (Qiu et al., 2016; Ogino and Green, 2019a, b). Similar to other RNA viral RdRps (Poch et al., 1990; O'Reilly and Kao, 1998; Bruenn, 2003; Lang et al., 2013; Ogino and Green, 2019a), the RABV RdRp domain (residues 376–946) is constituted by three subdomains, called fingers, palm, and thumb, and carries structural motifs A–F (Ogino and Green, 2019b). Motifs A and C in the palm subdomain of the RABV L protein contain universally conserved aspartate residues, D618 and D729, respectively, which are thought to be critical for two-metal dependent nucleotide polymerization (Steitz, 1998; Castro et al., 2009; Genna et al., 2016). D729 of the RABV L protein was experimentally confirmed as an essential residue for its RNA synthesis activity (Schnell and Conzelmann, 1995; Ogino et al., 2019).

Between the RdRp and PRNTase domains, there is a functionally unknown region (called “bridge” in this chapter). Although this region as well as the C-terminal part of the thumb subdomain containing a highly conserved GGx(11,12)Rx(3)D motif was originally predicted to be involved in mRNA capping as part of “CAP” domain (residues 880–1352 in the RABV L protein) (Liang et al., 2015; Horwitz et al., 2020), there is no experimental evidence to support this hypothesis. The bridge region may provide a scaffold for the PRNTase domain (Ogino and Green, 2019a) and also form a template exit channel for the RdRp domain similar to the RdRp bridge subdomain of the L protein of La Crosse virus (Arragain et al., 2020). In the RABV L protein, the bridge region ends

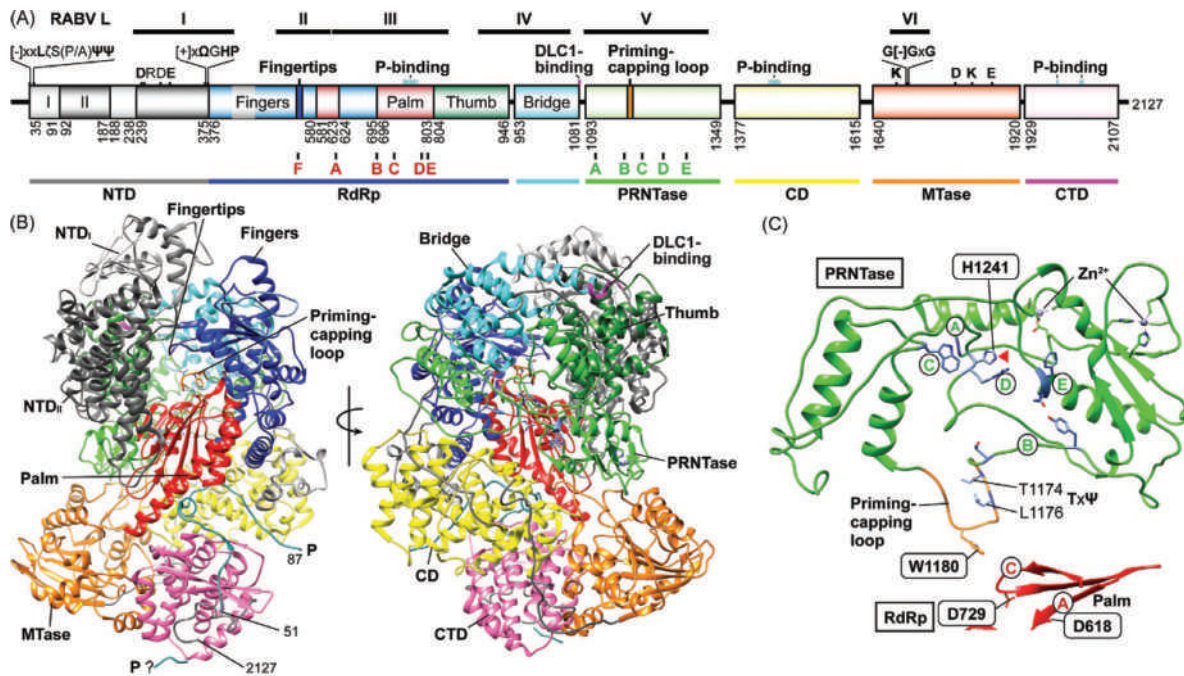


Fig. 7 Structure of the RABV L protein. (A) The RABV L protein is composed of the N-terminal (NTD, subdomains: NTD_I and NTD_{II}), RNA-dependent RNA polymerase (RdRp, subdomains: fingers, palm, and thumb), GDP polyribonucleotidyltransferase (PRNTase), connector (CD), methyltransferase (MTase), and C-terminal (CTD) domains. The RdRp domain is connected to the PRNTase domain via a functionally unknown region (called “Bridge” in this chapter). The originally reported conserved blocks I–VI (Poch et al., 1990) are marked above the diagram. The NTD contains the indicated amino acid sequence motifs ([–], negatively charged; [+], positively charged; ζ, hydrophilic; Ψ, aliphatic; Ω, aromatic; x any amino acids), in which residues essential for transcription are highlighted in bold. The positions of RdRp structural motifs A–F are labeled by red bold letters (Ogino and Green, 2019a). Motif F is located in a loop structure, called fingertips. The PRNTase domain contains PRNTase motifs A–E (green bold letters) and a priming-capping loop (orange). The MTase domain includes an S-adenosyl-L-methionine-binding motif (G[–]GxG) and nucleoside-2′-O-MTase motif (K-D-K-E). Discontinuous P-binding regions are indicated by cyan dashed lines. The binding site for dynein light chain 1 (DLC1) is located at the C-terminal end of the bridge region. (B) Two views of the cryo-EM structure of the RABV L protein complexed with a fragment of the P protein (residues 1–91) (PDB id: 6UEB) are shown. The domains/subdomains of the L protein are colored as in (A). Residues 51–87 and five unassigned residues in the P fragment are colored dark cyan. (C) The structure of the RABV PRNTase domain (green) and part of the RdRp palm subdomain (red) are shown as ribbon models. The positions of PRNTase motifs A–E are labeled by circled green letters. The priming-capping loop is shown in orange with a priming amino acid residue (W1180) and TxΨ motif (T1174–x-L1176). Key amino acid residues (stick models) required for catalyzing the PRNTase activity or maintaining the PRNTase domain structure are colored cornflower blue. The N² position (marked by a red arrowhead) of H1241 in PRNTase motif D serves as a covalent pre-mRNA attachment site during the unconventional capping reaction. There are two binding sites for Zn²⁺ ions (gray spheres). The locations of catalytic aspartate residues, D618 and D729, within RdRp motifs A and C (denoted by circled red letters), respectively, are indicated.

with the DLC1 binding motif (residues 1079–1083) (Bauer et al., 2015) and is connected to the PRNTase domain via a highly diversified loop structure. The DLC1 binding motif in the RABV L protein is required for efficient transcription (Bauer et al., 2015) similar to the DLC1 binding motif in the P protein (Tan et al., 2007). The DLC1 binding motif or similar sequence is present in the L proteins of lyssaviruses, but not other rhabdoviruses, such as VSV. The RABV L protein induces acetylation of α -tubulin with the DLC1 binding motif, leading to microtubule bundling in cells overexpressing the RABV L protein alone (Bauer et al., 2015). At present, the roles of binding of the L protein to dynein and the L-mediated microtubule reorganization in viral replication, transport, and/or pathogenesis in infected cells remain unclear.

The PRNTase domain (residues 1093–1349) is conserved among NNS RNA viruses and includes five collinear sequence elements, Rx(3)Wx(3–8)ΦxGxζx(P/A) (motif A), (Y/W)ΦGSxT (motif B), W (motif C), HR (motif D), and ζxxΦx(F/Y)QxxΦ (motif E) (Φ, hydrophobic; ζ, hydrophilic amino acids) (Fig. 7C) (Ogino and Banerjee, 2011a, b; Neubauer et al., 2016; Ogino and Green, 2019a). In the RABV L protein, G1112 (motif A), T1170 (motif B), W1201 (motif C), H1241 (motif D), R1242 (motif D), F1285 (motif E), and Q1286 (motif E) were identified as critical for mRNA capping (Ogino et al., 2016). The PRNTase domain has a large loop structure, called priming-capping loop (Ogino et al., 2019), which is extended toward the RdRp active site in the apo-structure of the L protein complexed with a fragment of the P protein (Horwitz et al., 2020) and plays dual roles in transcription initiation and mRNA capping (Ogino et al., 2019). The PRNTase domain contains two Zn²⁺ ion binding elements (C1093–E1120–C1317–C1320 and C1132–C1134–H1312–H1314) (Horwitz et al., 2020), which may be essential for the structural integrity of the PRNTase domain.

The MTase domain (residues 1640–1920) includes a typical S-adenosyl-L-methionine (SAM)-dependent MTase fold with a SAM binding motif G[–]GxG ([–], negatively charged amino acids, residues 1704–1708) and also possesses a nucleoside-2′-O-MTase motif (K-D-K-E catalytic tetrad, K1685–D1797–K1829–E1867) (Bujnicki and Rychlewski, 2002). In VSV, the MTase domain was

suggested to catalyze methylation of the mRNA cap structure at the ribose-2'-O and guanine-N⁷ positions (Hercyk et al., 1988; Grdzlishvili et al., 2005; Li et al., 2005a, b, 2006; Rahmeh et al., 2009). The functions of CD (residues 1377–1615) and CTD (residues 1929–2107) are poorly understood. Although previous studies suggested that the RABV P protein is associated with the C-terminal part of the L protein (Chenik et al., 1998; Castel et al., 2009), the recent cryo-EM study (Horwitz et al., 2020) revealed that one of the L-binding regions (residues 51–87) contacts with three discontinuous regions located in the RdRp, CD, and CTD of the L protein. Similarly, the VSV P protein interacts with the RdRp, CD, and CTD of the VSV L protein (Jenni et al., 2020; Gould et al., 2020). The VSV L mutants with a particular amino acid substitution in the CD (F1488S) (Hunt and Hutchinson, 1993; Galloway and Wertz, 2008) or MTase domain (D1762E, K1651A) (Galloway and Wertz, 2008; Li et al., 2009) are known to produce mRNAs with an extra-long poly(A) tail, suggesting that these C-terminal domains are involved in determining the length of poly(A) tails.

The 3'- and 5'-terminal non-coding regions of the genome are called *leader* (*Le*, 58 nt) and *trailer* (*Tr*, 70 nt), respectively (Fig. 8A). The 3'-terminal parts of the genomic *Le* and anti-genomic *Tr* act as promoters for synthesis of the anti-genomic and genomic RNAs, respectively (Conzelmann and Schnell, 1994; Schnell et al., 1994; Finke and Conzelmann, 1997; Morin et al., 2017; Ogino et al., 2019). The promoter strength of anti-genomic *Tr* is approximately 50-fold higher than that of genomic *Le*, resulting in production of a larger amount of the genomic RNA and, thereby, its efficient packaging into virions (Finke and Conzelmann, 1997). The genes of RABV begin and end with conserved sequences, called gene-start (3'-UUUGURRDGA-5'; R = G or A, D = A, G, or U) and gene-end (3'-ACUUUUUUUU-5'), respectively, (Ogino and Green, 2019b) and are tandemly linked by an intergenic region of variable lengths (2–24 nt) (Tordo et al., 1986b; Conzelmann et al., 1990). During transcription, the gene-start and gene-end sequences serve as transcription initiation and polyadenylation/termination signals, respectively (Conzelmann and Schnell, 1994; Schnell et al., 1994; Mebatsion et al., 1996b; Barr et al., 1997; Stillman and Whitt, 1999; Ogino et al., 2019).

According to the “single entry, stop-start transcription” model suggested from in vitro studies on VSV transcription (Abraham and Banerjee, 1976; Ball and White, 1976; Testa et al., 1980; Emerson, 1982), the RABV RdRp complex enters from the 3'-terminal of the genomic *Le* promoter and carries out sequential synthesis of a short non-coding RNA (55–58 nt), called the leader RNA (LeRNA), (Kurilla et al., 1984) followed by monocistronic *N*, *P*, *M*, *G*, and *L* mRNAs (Flamand and Delagneau, 1978; Coslett et al., 1980; Holloway and Obijeski, 1980) (Fig. 8A). As in the case of VSV (Colonno and Banerjee, 1976), the RABV LeRNA synthesis is initiated at the 3'-end of the genome and terminated just before the RdRp reaches the *N* gene-start sequence (Kurilla et al., 1984). LeRNA does not undergo 5'-capping or 3'-polyadenylation. After the LeRNA synthesis, the same RdRp repeats mRNA synthesis cycles including reinitiation and termination steps at each gene junction to sequentially transcribes the internal genes. However, since the efficiency of transcription reinitiation at each gene-start sequence is about 70% or lower, the rhabdoviral RdRps generate a gradient in mRNA abundance in the following order: *N* > *P* > *M* > *G* > *L* (Abraham and Banerjee, 1976; Ball and White, 1976; Iverson and Rose, 1981; Flamand and Delagneau, 1978; Finke et al., 2000). After releasing polyadenylated mRNA at a gene-end sequence, the RdRp scans an intergenic region until a downstream gene-start sequence is encountered. In RABV, longer intergenic regions (*G/L*, 24 nt > *P/M* and *M/G*, 5 nt > *N/P*, 2 nt) are known to significantly downregulate the production of mRNAs from downstream genes (Finke et al., 2000). In contrast, all the intergenic regions of VSV (Indiana) consist of 2 nt (Rose, 1980), achieving nearly constant reinitiation efficiencies (~70%) at the gene junctions.

RABV mRNAs produced in infected cells possess 3'-poly(A) tail of 100–250 nt (Holloway and Obijeski, 1980), and are believed to have a methylated cap structure, such as m⁷G(5')ppp(5')Am- (cap 1), m⁷G(5')ppp(5')AmpAm- (cap 2), and m⁷G(5')ppp(5')m⁶Ampm⁶Am- (m⁷G, N⁷-methylguanosine; Am, 2'-O-methyladenosine; m⁶Am, N⁶,2'-O-dimethyladenosine) at their 5'-termini as observed in VSV mRNAs (Abraham et al., 1975a; Moyer et al., 1975; Moyer and Banerjee, 1976). Detergent disrupted VSV particles, purified RNPs, or reconstituted RNPs with the N-RNA and recombinant RdRp complexes are known to produce mRNAs with the cap 1 structure in the presence of SAM in vitro (Abraham et al., 1975a; Testa and Banerjee, 1977; Ogino, 2013), indicating that VSV particles contain all enzymes required for the formation of the cap 1 structure. However, it remains challenging to characterize RABV-associated mRNA-producing enzymes or identify their products, especially 5'-terminal structures, due to extremely low mRNA synthesis activities of purified RABV particles (Villareal and Holland, 1974; Kawai, 1977; Flamand et al., 1978).

Recent development of in vitro RNA synthesis and capping assay systems with a recombinant form of the RABV L protein (Ogino et al., 2016; Morin et al., 2017; Ogino et al., 2019) allowed us to elucidate the mechanisms of RABV RNA biosynthesis at molecular levels. To synthesize LeRNA and mRNAs from the genome, the RdRp needs to carry out transcription initiation at the 3'-terminal *Le*-start sequence (3'-UGCGAA) and internal gene-start sequences, respectively (Fig. 8A). In vertebrate and/or arthropod rhabdoviruses including RABV and VSV, the priming-capping loop extended from the PRNTase domain contains a highly conserved tryptophan residue (W1180 for RABV) and TxΨ motif (Ψ, aliphatic amino acids, T1174-x-L1176 for RABV) (Ogino et al., 2019). The in vitro transcription system with artificial oligo-RNA templates revealed that the W residue is required for terminal de novo initiation to generate a dinucleotide pppApC RNA at the 3'-terminal U₁G₂ sequence (Ogino et al., 2019). Based on structural models of the VSV and RABV transcription initiation L complexes with ATP (initiator nucleotide), CTP (incoming nucleotide), and a model template (3'-UGCG for RABV or 3'-UGCU for VSV) (Ogino et al., 2019; Ogino and Green, 2019b) (Model Archive id: ma-5 k432 and ma-uqibu), the adenine base of ATP was suggested to be sandwiched between the indole group of the W residue on the loop and the cytosine base of CTP via π -stacking interactions to form a stable initiation complex on the 3'-terminal UG sequence (Fig. 8B). The W residue is not essential for internal de novo initiation from the gene-start sequence to synthesize pppApA-started RNAs, their chain elongation, or RNA capping (Ogino et al., 2019). In contrast, the TxΨ motif is required for RNA capping, but not for transcription initiation either from the 3'-terminal *Le*-start or from the internal gene-start sequence (Ogino et al., 2019). These findings suggest that the priming-capping loop is displaced from the RdRp active site after terminal initiation to conduct LeRNA chain elongation followed by internal de novo initiation for *N* mRNA synthesis and undergoes a further structural rearrangement to

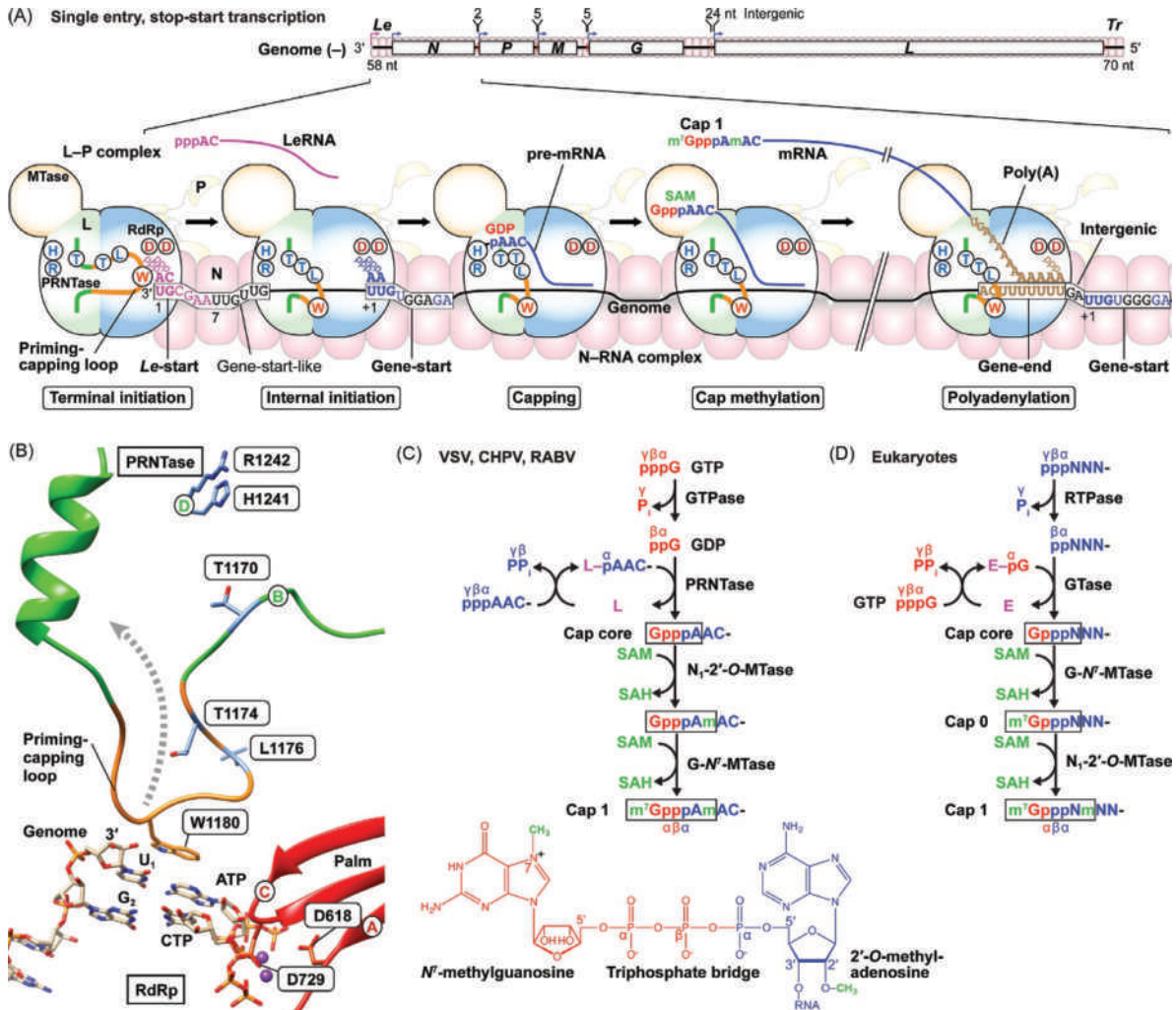


Fig. 8 RNA synthesis and processing by the RABV L protein. (A) The negative-strand RABV genome has the 3'- and 5'-terminal *leader* (Le, 58 nt) and *trailer* (Tr, 70 nt) regions, respectively (upper). The genes are tandemly connected via intergenic regions with the indicated lengths. The 3'-terminal part of the genome encapsidated with the N proteins (pale red) is illustrated with the *Le*-start, *N* gene-start, *N* gene-end, intergenic, and *P* gene-start sequences (lower). The RdRp complex composed of the L protein with the RdRp (pale blue), PRNTase (pale green), and MTase (pale orange) domains and the P protein (pale yellow) is schematically delineated. Circled red "D" letters in the RdRp domain indicate D618 and D729. Circled blue "T", "H", and "R" letters in the PRNTase domain indicate T1170, H1241, and R1242, respectively. Circled blue "T" and "L" (TxY motif) and orange "W" letters on the priming-capping loop (orange) indicate T1174, L1176, and W1180, respectively. pppA, pppC, and SAM indicate ATP, CTP, and S-adenosyl-L-methionine, respectively. The RdRp complex enters from the 3'-end of the genome to synthesize the leader RNA (LeRNA) and then transcribes the *N* gene into *N* mRNA with the 5'-cap 1 structure and 3'-poly(A) tail. The priming-capping loop mediates terminal initiation at the *Le*-start sequence to produce LeRNA and pre-mRNA capping at an early stage of mRNA chain elongation using W1180 and the TxY motif, respectively. In contrast, the loop is not required to initiate mRNA synthesis at the gene-start sequence. The cap structure is methylated with the MTase domain during transcription. Finally, the RdRp adds a poly(A) tail to the 3'-end of *N* mRNA by transcriptional slippage at the U7 sequence in the gene-end sequence. After releasing mature *N* mRNA, the RdRp reinitiates transcription at the *P* gene-start sequence. (B) A predicted conformation of the priming-capping loop in a modeled terminal initiation complex of the RABV L protein (Model Archive id: ma-ujibu) is shown with a 3'-UG-ended model RNA (genome), ATP (initiator nucleotide), CTP (incoming nucleotide), and part of the RdRp palm subdomain. Key amino acid residues are shown as in Fig. 7C. The loop may be retracted after terminal de novo initiation (curved dashed arrow). (C and D) The pathway of the cap formation by rhabdoviruses (C) are compared with that by eukaryotes (D). GTP, RNA, and SAM (substrates) are shown in red, blue, and green, respectively. Moieties of enzymatic products are colored in red, blue, and green based on their origins (GTP, RNA, and SAM, respectively). The positions of phosphate groups in the triphosphate (ppp) on the substrates are labeled by α , β , and γ . P_i, PP_i, and SAH indicate inorganic phosphate, inorganic pyrophosphate, and S-adenosyl-L-homocysteine, respectively. In rhabdoviruses (C), the cap core structure is formed with guanosine 5'-triphosphatase (GTPase) followed by GDP polyribonucleotidyltransferase (PRNTase, shown by "L") and methylated with mRNA (nucleoside₁-2'-*O*)-methyltransferase (N₁-2'-*O*-MTase) followed by mRNA (guanine-*N*⁷)-methyltransferase (G-*N*⁷-MTase) to form the cap 1 structure (m⁷GpppAm, chemical structure shown at the bottom). In higher eukaryotes (D), the cap core structure is generated with RNA 5'-triphosphatase (RTPase) followed by mRNA guanylyltransferase (GTase, shown by "E") and methylated with G-*N*⁷-MTase followed by N₁-2'-*O*-MTase to yield the cap 1 structure (m⁷GpppNm, N: any nucleotide).

mediate pre-mRNA capping during mRNA chain elongation (Ogino et al., 2019). In cryo-EM structures of non-transcribing L proteins of human respiratory syncytial virus (Gilman et al., 2019), human metapneumovirus (*Pneumoviridae*) (Pan et al., 2020), and parainfluenza virus 5 (*Paramyxoviridae*) (Abdella et al., 2020), which are complexed with their cognate P proteins, their counterparts of the rhabdoviral priming-capping loop are associated with or close to their putative PRNTase active sites, but not extended into their RdRp active sites. These observations suggest that the loop structures are flexible and can be rearranged into different conformations during the dynamic stop-start transcription cycle.

Rhabdoviruses, such as VSV (Abraham et al., 1975a, b; Ogino and Banerjee, 2007; Ogino et al., 2010), Chandipura virus (Ogino and Banerjee, 2010), and RABV (Ogino et al., 2016), employ an unconventional mechanism of mRNA capping (Fig. 8C). First, a guanosine 5'-triphosphatase (GTPase) activity associated with the L protein removes the γ -phosphate of GTP to yield GDP, which in turn serves as an acceptor substrate in the subsequent PRNTase reaction (Ogino and Banerjee, 2007, 2008; Ogino et al., 2016). Then, a lone pair of electrons at the $N^{\epsilon 2}$ position of the highly conserved histidine residue (H1227 for VSV, H1241 for RABV) in motif D (also called HR motif) of the L PRNTase domain nucleophilically attacks the α -phosphorus in the 5'-triphosphate group of pre-mRNA, but not LeRNA, to form a covalent enzyme-substrate intermediate composed of the L protein and 5'-monophosphorylated RNA (L-pRNA) (Ogino and Banerjee, 2007; Ogino et al., 2010; Ogino, 2013). In the L-pRNA intermediate, the 5'-monophosphate end of RNA is linked to the $N^{\epsilon 2}$ atom of the histidine residue via a phosphoamide bond (Ogino and Banerjee, 2007; Ogino et al., 2010). The intermediate subsequently transfers pRNA to GDP to form a cap core structure, GpppA, on the 5'-end of pre-mRNA (Ogino et al., 2010; Ogino and Ogino 2017). Therefore, origins of the phosphate groups forming the triphosphate bridge of the rhabdoviral cap structure are the α - and β -phosphates of GTP and the α -phosphate of the initiator ATP. Consistent with conserved lyssaviral (5'-AACABYHCU; B = C, G, or U; Y = U or C; H = A, C, or U) and vesiculoviral (5'-ARCAGNNNUMV; R = A or G, N = any nucleotide; M = A or C; V = A, C, or G) mRNA-start sequences (Ogino and Green, 2019b), the RABV and VSV PRNTase domains specifically cap 5'-triphosphorylated AACNN- and ARCNG-started RNAs (R = A or G), respectively (Ogino and Banerjee, 2007, 2008; Ogino et al., 2016). In contrast, it is well known that eukaryotic mRNA capping enzyme elicits a different set of enzymatic activities to form the cap core structure (GpppN) on cellular pre-mRNAs of any 5'-sequence (reviewed in Furuichi and Shatkin, 2000; Shuman, 2001; Ghosh and Lima, 2010) (Fig. 8D). In the first step, the RNA 5'-triphosphatase (RTPase) activity of eukaryotic mRNA capping enzyme removes the γ -phosphate of 5'-triphosphorylated RNA (pppRNA) to generate 5'-diphorylated RNA (ppRNA). In the second step, mRNA guanylyltransferase (GTase) reacts with GTP to form a covalent enzyme-GMP intermediate (E-pG) using a nucleophilic lysine residue in the conserved KxDG motif, and then transfers GMP from the intermediate to ppRNA to produce the 5'-cap structure on RNA.

In all known eukaryotic cells, the cap structure on cellular mRNAs is methylated by mRNA (guanine- N^7)-methyltransferase (G- N^7 -MTase) in the nucleus to generate the cap 0 structure, m^7 GpppN (Fig. 8D), which is required for mRNA stability and eukaryotic gene expression at various stages, such as mRNA splicing, transport, and translation (reviewed in Banerjee, 1980; Furuichi and Shatkin, 2000; Ramanathan et al., 2016). In higher eukaryotes, the cap 0 structure is further methylated by mRNA (nucleoside₁-2'-O)-methyltransferase (N_1 -2'-O-MTase) followed by N_2 -2'-O-MTase to produce the cap 1 structure, m^7 GpppN₁m, and the cap 2 structure, m^7 GpppN₁mpN₂m. For many viruses, the formation of the cap 1 structure on their mRNAs is critical to avoid anti-viral innate immune reactions triggered by cytosolic pattern recognition receptors [e.g., retinoic acid-inducible gene I (RIG-I)] that recognize RNAs with a 2'-hydroxyl group at the N_1 position as non-self RNAs (reviewed in Hyde and Diamond, 2015; Leung and Amarasinghe, 2016). In VSV, virion-associated MTases catalyze methylation of the cap core structure at the N_1 -2'-O position followed by the G- N^7 position into the cap 1 structure (Fig. 8C) (Testa and Banerjee, 1977, #216; Hammond and Lesnaw, 1987). Although the methylation reactions had been thought to be tightly coupled to mRNA synthesis for a long time (Banerjee, 1980), Rahmeh et al. (2009) reported that a recombinant form of the VSV L protein alone can methylate the cap core structure on exogenously added RNA substrates with the VSV N mRNA-start sequence in the following order: GpppA $\rightarrow$ GpppAm $\rightarrow$ m^7 GpppAm. The single MTase domain in the VSV L protein was suggested to catalyze the two cap methylation reactions (Grdzlishvili et al., 2005; Li et al., 2005a, b, 2006), whereas the two separate host enzymes conduct the individual methylation reactions to form the cap 1 structure on eukaryotic mRNAs. Methylation-defective mutations in the conserved motifs of the VSV MTase domain attenuate VSV (Grdzlishvili et al., 2005; Li et al., 2005a, b, 2006), whereas mutations of the RdRp or PRNTase active site are lethal to VSV (Ogino, 2014). So far, the enzymatic activities of the RABV MTase domain have not been characterized.

During replication, the RABV RdRp copies the genome into the anti-genome, which in turn serves as a template to produce progeny genomes (Fig. 4). Both the genome and anti-genome are encapsidated with newly synthesized N proteins, which may be co-replicationally supplied from the RNA-free N^0 -P complex (Peluso, 1988; Peluso and Moyer, 1988; Masters and Banerjee, 1988; Gupta and Banerjee, 1997). In the presence of the P protein, the N protein may selectively interact with the positive-strand LeRNA, resulting in its elongation into the full-length genome with concomitant nucleocapsid assembly (Blumberg et al., 1981, 1983; Yang et al., 1998). The RdRp ignores the genomic RNA signals for stop-start transcription during replication to synthesize the full-length anti-genome. In RABV, the M protein represses transcription, but enhances replication, thus suggesting that the M protein can switch the RNA synthesis mode from transcription to replication (Finke and Conzelmann, 2003; Finke et al., 2003).

The monocistronic mRNAs of rhabdoviruses are translated into the five proteins in a cap-dependent manner (Both et al., 1975a; Muthukrishnan et al., 1975). The N, P, M, and L proteins are produced on free ribosomes, whereas the G protein is generated on ER-bound ribosomes (Grubman et al., 1974; Both et al., 1975b; Morrison, 1975; Pennica et al., 1980). A precursor of the G protein is co-translationally cleaved after an N-terminal signal peptide sequence by signal peptidase within the ER lumen and further undergoes glycosylation in the ER and Golgi apparatus during its maturation (Hunt and Summers, 1976; Toneguzzo and Ghosh, 1977; Lingappa et al., 1978). In addition to the full-length RABV P protein (also called P1), its N-terminal truncated forms (called

P2–P5) are synthesized from RABV *P* mRNA using alternative in-frame AUG codons by leaky ribosomal scanning (Chenik et al., 1995) (Fig. 6). The M protein of RABV inhibits cap-dependent translation by interacting with eIF3h, a subunit of eukaryotic initiation factor 3 (Komarova et al., 2007). However, the role of the interaction between the RABV M protein and eIF3h in the regulation of cellular and/or viral translation during the course of infection is undefined.

Virus assembly and budding

RABV assembly and budding occur at the plasma membrane of infected cells at the late stage of infection (Fig. 4). The M protein plays central roles in these events. X-ray crystallography revealed that the M protein of rhabdoviruses, such as VSV (vesiculovirus) and Lagos bat virus (LBV, lyssavirus), is a small globular protein with an extended N-terminal segment (Gaudier et al., 2002; Graham et al., 2008). In the crystals, each M protein is associated with neighboring M proteins via their N-terminal segments and globular domains to form non-covalent linear polymers (Graham et al., 2008). Here, based on the structure of the LBV M protein, a structure of the RABV M protein was predicted using the SWISS-MODEL program (Waterhouse et al., 2018) (Fig. 9).

The M proteins cover the RNP by interacting with the N proteins and condense the RNP into a tightly coiled skeleton-like structure (Newcomb and Brown, 1981; Newcomb et al., 1982; Chong and Rose, 1994; Lyles et al., 1996; Mebatsion et al., 1999). The M proteins are self-assembled into higher order structures at lower salt concentrations (Gaudin et al., 1995, 1997). The M protein also interacts with cellular membranes (Chong and Rose, 1993, 1994; Gaudier et al., 2002) and the G protein (Lyles et al., 1992; Mebatsion et al., 1999; Nakahara et al., 1999) to facilitate virion assembly. The cytoplasmic tail of the G protein is required for its efficient incorporation into virus particles by binding to the M protein (Mebatsion et al., 1996a, 1999). Without the M protein, infectious RABV particles containing an RNP and the G proteins cannot be released from infected cells efficiently, and exhibit abnormal shapes, such as long rods (Mebatsion et al., 1999). In VSV-infected cells, the M and G proteins are accumulated in separate plasma membrane microdomains, and are finally assembled with the RNPs at budding sites (Swintek and Lyles 2008).

The VSV M protein has an intrinsic ability to assemble and release virus-like particles composed of the cellular plasma membrane and the M proteins when expressed in cells in the absence of other viral proteins (Li et al., 1993; Justice et al., 1995). The PPxY motif (a late-budding domain motif) in the N-terminal segments of the RABV and VSV M proteins interacts with the WW domains of cellular Nedd4 and/or Nedd4-like ubiquitin E3 ligases and is required for efficient budding (Harty et al., 1999; Craven et al., 1999; Jayakar et al., 2000; Irie et al., 2004; Wirblich et al., 2008), which may be mediated by the cellular ESCRT (endosomal sorting complexes required for transport) machinery (reviewed in Votteler and Sundquist, 2013). However, the detailed mechanisms of rhabdovirus budding are not fully understood.

Subversion of host immune responses

RABV has evolved strategies to subvert host innate immune reactions at various steps. In infected cells, viral RNAs are recognized by various cytosolic pattern recognition receptors, such as RIG-I-like receptors (RLRs, members of the DEAD-box RNA helicase family),

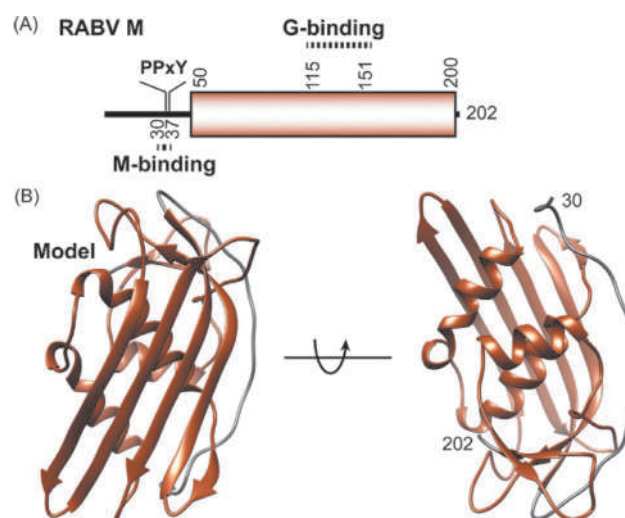


Fig. 9 Structure of the RABV M protein. (A) The schematic diagram of the RABV M protein is shown with putative binding sites for M itself and the G protein. The PPxY motif (a late-budding domain motif) in the N-terminal segment is essential for virus budding. (B) A three-dimensional structure of the RABV M protein (PV strain, GenBank id: M13215) was predicted with SWISS-MODEL (Waterhouse et al., 2018) using the structure of the M protein of Lagos bat virus (PDB id: 2W2S) as a template.

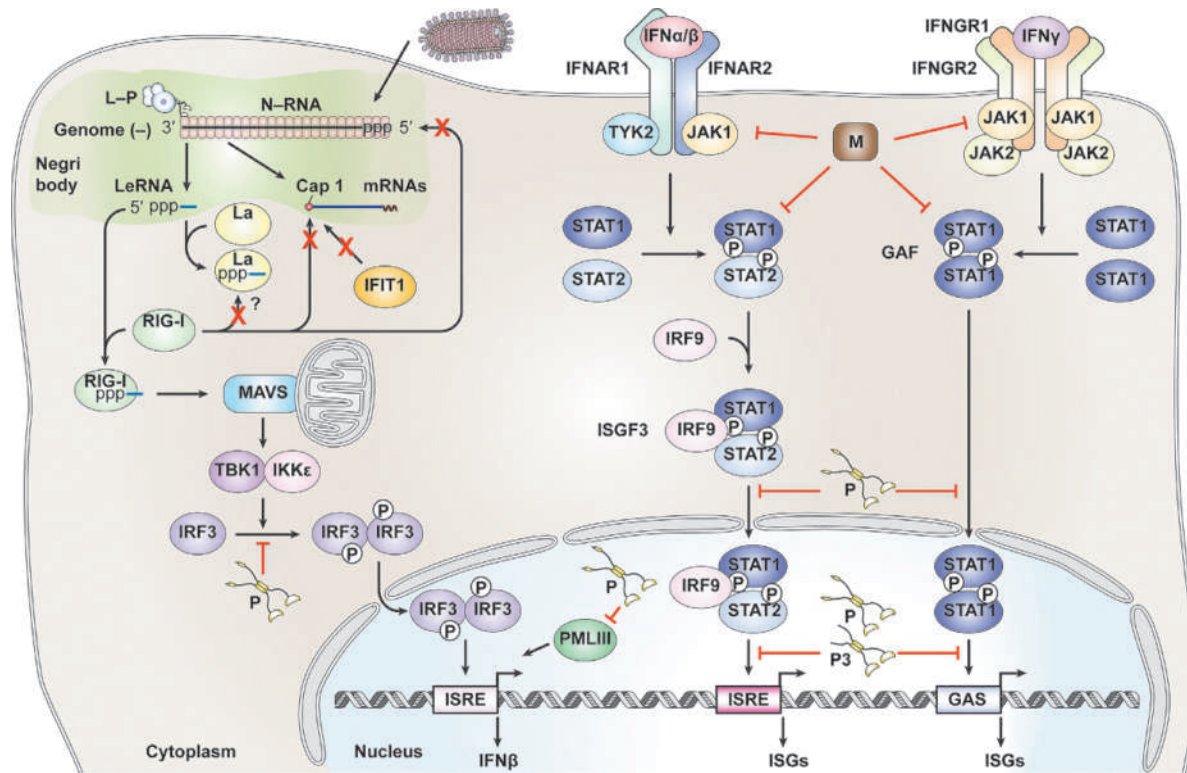


Fig. 10 Subversion of host innate immune responses by RABV. To evade the activation of RIG-I that recognizes viral RNA with a 5'-(p)pp-group or 2'-O-unmethylated cap structure (e.g., m⁷GpppA-, GpppA-), viral genomic and antigenomic RNAs with a 5'-(p)pp-end are wrapped with the N proteins, and the 5'-ends of viral mRNAs are modified into the cap 1 structure (m⁷GpppAm-) with the L protein. Association of 5'-(p)pp-ended LeRNA with cellular La protein may shield it from RIG-I. The RABV P protein inhibits IRF3-dependent RIG-I signaling at the step of IRF3 phosphorylation to abolish expression of type-I IFNs. The RABV P protein also represses both the IFNα/IFNβ- and IFNγ-mediated JAK/STAT signaling by inhibiting nuclear translocation of STAT1 and STAT2 and their target DNA binding. Furthermore, the RABV M protein inhibits JAK1 phosphorylation and nuclear translocation of STAT1. The formation of the cap 1 structure on viral mRNAs is required to avoid translation inhibition with IFIT1. The RABV P protein counteracts antiviral actions of PMLIII. For details and abbreviations, see text.

resulting in initiation of various immune responses (reviewed in [Rehwinkel and Gack, 2020](#)). Although RIG-I is essential to initiate antiviral responses against RABV, RABV efficiently inhibits RIG-I-dependent signaling with the P protein ([Fig. 10](#)) ([Hornung et al., 2006](#)). RIG-I senses N₁-2'-O-unmethylated double-strand and possibly single-strand RNAs bearing a 5'-(p)ppN₁-, GpppN₁-, or m⁷GpppN₁-end as non-self RNAs ([Hornung et al., 2006](#); [Pichlmair et al., 2006](#); [Goubau et al., 2014](#); [Schuberth-Wagner et al., 2015](#); [Devarkar et al., 2016](#); [Linehan et al., 2018](#); [Ren et al., 2019](#)). Thus, the formation of the cap 1 structure (m⁷GpppN₁m) on rhabdoviral mRNAs by the L protein ([Abraham et al., 1975a](#); [Ogino and Banerjee, 2007](#); [Rahmeh et al., 2009](#); [Ogino et al., 2016](#)) is required for their escape from RIG-I. Since the viral genomic/anti-genomic RNA and LeRNA have 5'-(p)pp-ends ([Hefti and Bishop, 1975](#); [Keene et al., 1979](#); [Colonno and Banerjee, 1976](#)), they may serve as ligands for RIG-I ([Hornung et al., 2006](#)). In infected cells, the viral genomic and anti-genomic RNAs are thought to be shielded from RIG-I by the N proteins ([Iseni et al., 1998](#); [Albertini et al., 2006](#); [Masatani et al., 2010, 2011](#)). In human respiratory syncytial virus-infected cells, cellular RNA binding protein La sequesters 5'-triphosphorylated LeRNA from RIG-I, evading RIG-I activation ([Bitko et al., 2008](#)). Similarly, LeRNAs of VSV and RABV are present as complexes with La in infected cells ([Kurilla and Keene, 1983](#); [Kurilla et al., 1984](#)), suggesting that rhabdoviruses use the same strategy as pneumoviruses to mask the potential RIG-I ligands.

Once RIG-I is activated by sensing viral RNAs, it undergoes a structural rearrangement to expose and oligomerize its caspase activation and recruitment domains (CARDs), leading to its interaction with mitochondrial antiviral-signaling protein (MAVS) ([Rehwinkel and Gack, 2020](#)). The activated MAVS signaling complex subsequently activates TANK binding kinase 1 (TBK1) and IκB kinase-ε (IKKε) to phosphorylate IFN regulatory factor 3 (IRF3) and IRF7. Phosphorylation of IRF3 and IRF7 promotes their dimerization and translocation into the nucleus, where these transcription factors induce expression of type I IFNs, such as IFNα and IFNβ. The RABV P protein inhibits the activation of IRF3 and IRF7 with its unstructured region including residues 176–186, thereby preventing the IFN production ([Rieder et al., 2011](#)). Furthermore, the RABV M protein inhibits NF-κB signaling by interacting with RelA/p43 (a p65/RelA variant belonging to the NF-κB family) to reduce IFNβ production ([Luco et al., 2012](#); [Ben Khalifa et al., 2016](#); [Besson et al., 2017](#)).

Type I IFNs are secreted from infected cells and interact with heterodimeric IFNα receptor composed of IFNAR1 and IFNAR2 on the cell surface (reviewed in [Wang et al., 2017](#); [Morris et al., 2018](#)). JAK1 and TYK2, members of the Janus kinase (JAK) family are associated with the cytoplasmic tails of the IFNAR2 and IFNAR1, respectively, and are activated by transphosphorylation upon

binding of type I IFN to the receptor. Activated JAK further phosphorylates the cytoplasmic tails of the IFN receptor, leading to recruitment and phosphorylation of signal transducer and activator of transcription 1 (STAT1) and STAT2. Phosphorylated STAT1-STAT2 heterodimer and STAT2 homodimer interact with IFN regulatory factor 9 (IRF9) to form complexes called IFN-stimulated gene factor 3 (ISGF3) and ISGF3-like complex, respectively, which are translocated into the nucleus to promote expression of interferon-stimulated genes (ISGs) by binding to IFN-stimulated response elements (ISREs) on their promoters. Similarly, type II IFN (IFN γ) is associated with IFN γ receptor composed of IFNGR1 and IFNGR2 to activate STAT1 via JAK1 and JAK2. The resulting phosphorylated STAT1 homodimer, called γ -activated factor (GAF), is transported into the nucleus to stimulate ISG expression by binding to γ -activated sequences (GASs).

The RABV P protein inhibits both the IFN α /IFN β - and IFN γ -mediated JAK/STAT signaling by retaining phosphorylated STAT1 and STAT2 in the cytoplasm and inhibiting their target DNA binding (Vidy et al., 2005; Brzozka et al., 2006; Vidy et al., 2007). The RABV P protein has N- and C-terminal nuclear export signals (NESs, residues 49–58 and 227–232) and nuclear localization signals (NLSS, residues 53–174, 211–214 and 260) to shuttle between the cytoplasm and nucleus (Pasdeloup et al., 2005; Moseley et al., 2007; Oksayan et al., 2012; Rowe et al., 2016). The N-terminal NES of the P protein is critical for its inhibitory activity against STAT1 nuclear translocation (Ito et al., 2010), suggesting that the P protein may form a complex with nuclear STAT1 to exclude it from the nucleus to the cytoplasm by the CRM1 nuclear export receptor. Among the P isoforms, P3–P5 are localized in the nucleus (Pasdeloup et al., 2005; Moseley et al., 2007; Oksayan et al., 2012; Rowe et al., 2016). Both the cytoplasmic (P1 and P2) and nuclear (P3–P5) forms of the P protein were suggested to be involved in its IFN antagonistic activities. Expression of these truncated forms of the RABV P protein is essential for viral IFN antagonism and replication in muscle cells, leading to viral neuroinvasiveness (Okada et al., 2016). The CTD of the RABV P protein contains discontinuous tyrosine-phosphorylated STAT-binding sites (Vidy et al., 2005; Brzozka et al., 2006; Hossain et al., 2019), which are required for viral IFN antagonism in infected cells as well as pathogenesis in brains of infected mice (Wiltzer et al., 2014). In addition, the CTD of P3 was reported to interact with both STAT1 and microtubules in the cytoplasm to inhibit STAT1 nuclear translocation by sequestering STAT1 on microtubules (Moseley et al., 2009; Brice et al., 2016). Furthermore, the RABV M protein binds JAK1 to inhibit its phosphorylation upon IFN stimulation, and also interacts with phosphorylated STAT1 to hamper its nuclear translocation cooperatively with the P protein (Sonthonnax et al., 2019). The RABV P protein also binds activated STAT3-STAT1 heterodimer, but not STAT3 homodimer, resulting in inhibition of gp130 cytokine receptor signaling (Lieu et al., 2013; Harrison et al., 2020).

In response to IFNs, expression of numerous ISG products including double-stranded RNA-activated protein kinase (PKR), RNase L, and Mx1 is induced to combat various viruses (reviewed in Schoggins, 2019). However, antiviral actions of individual ISG products against RABV and RABV strategies to counteract them have not been studied well. Promyelocytic leukemia (PML) protein, a member of the tripartite motif (TRIM) protein family, is one of ISGs and serves as a restriction factor against diverse viruses including VSV (Chelbi-Alix et al., 1998; Bonilla et al., 2002; El Asmi et al., 2014). Together with IFN-inducible Sp100 and other factors, PML forms subnuclear membrane-less structures, called PML nuclear bodies (NBs), which enhance expression of IFN β gene and ISGs and also repress some DNA viral gene expression directly (reviewed in Scherer and Stamminger, 2016). Many nuclear-replicating DNA viruses are known to antagonize one or more of PML-NB components with their viral proteins to counteract PML-NB-mediated anti-viral activities (Scherer and Stamminger, 2016). Although there are isoforms of PML (PML1 to PMLVIIb, produced by alternative splicing), only PMLIII and PMLIV have the ability to repress VSV replication by an as-yet-unknown mechanism (El Asmi et al., 2014). PMLIV also enhances IFN β production in an IRF3-dependent manner in response to VSV infection (El Asmi et al., 2014). For RABV, PMLIV, but not PMLIII, serves as a restriction factor and inhibits viral transcription and replication in an IFN-independent manner (Blondel et al., 2010). The C-terminal part (residues 223–297) of the RABV P protein directly interacts with PMLIII and sequesters it in the cytoplasm (Blondel et al., 2002), conferring resistance of RABV to PMLIII.

In RABV-infected cells, stress granules (SGs), membrane-less structures containing antiviral proteins, such as TIA-1, and RLRs (RIG-I and MDA5) are formed in a PKR-dependent manner. RABV escapes from antiviral actions of SGs by compartmentalizing viral replication machineries into separate membrane-less organelles (Negri bodies) (Nikolic et al., 2016, 2017). Furthermore, the formation of Negri bodies reduces innate immune responses by sequestering Toll-like receptor 3 (TLR3) (Menager et al., 2009), which senses viral double-strand RNAs in endosomes to induce production of type I IFNs and inflammatory cytokines through IRF3 and NF- κ B signaling pathways (reviewed in Lester and Li, 2014). Therefore, Negri bodies play critical roles not only in viral replication but also in innate immune escape in host cells.

Interferon induced protein with tetratricopeptide repeats 1 (IFIT1) interacts with 2'-O-unmethylated cap structures (e.g., GpppN₁, m⁷GpppN₁) on viral mRNAs and thereby abolishes their translation together with its related proteins, such as IFIT2 and IFIT3 (Daffis et al., 2010; Habjan et al., 2013; Kumar et al., 2014; Daugherty et al., 2016; Abbas et al., 2017; Fleith et al., 2018; Johnson et al., 2018). IFIT1 represses replication of positive strand RNA viruses (flavivirus, coronavirus) and DNA viruses (poxvirus) if their N₁-2'-O-MTase activities are defective (Daffis et al., 2010). Therefore, the formation of the fully methylated cap 1 structure on rhabdoviral mRNAs by their L proteins is thought to counteract IFIT1-mediated translational repression. However, IFIT1 was reported to inhibit VSV replication by sequestering 5'-ppp-ended viral RNAs in vitro and in vivo (Pichlmair et al., 2011). On contrary, IFIT2 rather than IFIT1 was shown to act as a restriction factor for replication of VSV specifically in neuronal cells of mice (Fensterl et al., 2012, 2014). Similarly, RABV replication is restricted by IFIT2 rather than IFIT1 in mouse brains (Davis et al., 2017). In addition, some mutations in the 2'-O-MTase motif (e.g., K1685A, K1829A) of the RABV L MTase domain are known to attenuate RABV in vitro and in vivo and cause its high sensitivity to IFIT2 (Tian et al., 2016). However, IFIT2 alone does not exhibit any activities to bind the 2'-O-unmethylated cap structures or 5'-ppp-ends of RNAs (Kumar et al., 2014; Fensterl et al., 2012),

although it can interact with AU-rich RNAs (Yang et al., 2012). Currently, the precise mechanisms of the antiviral actions of IFIT2 and IFIT1 against rhabdoviruses remain unclear.

RABV slowly intrudes the CNS with limited adaptive immune responses. RABV may have immunosuppressive strategies to escape from adaptive immune responses mediated by T and B cells in the CNS, although the underlying mechanisms remain largely elusive. In RABV-infected mice, expression of Fas ligand (FasL, CD95L) is induced in infected neurons, resulting in increased apoptosis of activated T cells expressing Fas (CD95 or Apo-1), which are transiently migrated to CNS tissues, via the Fas-FasL interaction (Baloul et al., 2004). Furthermore, infection of neurons with RABV causes up-regulation of PD-L1 (programmed cell death ligand 1, also called B7-H1) (Lafon et al., 2008), which is known to interact with PD-1 on T cells to repress T cell proliferation and cytokine production (reviewed in Chamoto et al., 2017). RABV infection also triggers neuronal expression of human leukocyte antigen (HLA)-G, a nonclassical immunosuppressive human major histocompatibility complex class I molecule, which binds its receptors on T and NK cells to prevent antiviral responses with these cells (Lafon et al., 2005).

Infection with laboratory adapted, attenuated RABV strains, such as vaccine strains, is known to efficiently induce production of virus-neutralizing antibodies against the G protein in infected hosts. Infection of mice with an attenuated RABV strain prior to secondary infection with a pathogenic strain promotes the clearance of the pathogenic strain from the CNS with neutralizing antibodies produced from B cells by enhancing the permeability of the blood-brain barrier (BBB) leading to the infiltration of immune cells into the CNS (Hooper et al., 2009). In contrast, infection with pathogenic strains does not change the permeability of the BBB in infected hosts, consistent with limited immune responses to these strains (Laothamatas et al., 2003, 2008; Roy et al., 2007; Hooper et al., 2009; Gnanadurai and Fu, 2016). Pathogenic RABV reduces inflammatory responses with the P gene products and, thereby, represses the elevation of the BBB permeability to counteract host CNS immunity (Long et al., 2020). Therefore, increasing the BBB permeability prior to intravenous administration of neutralizing antibodies may offer a new strategy for the future development of rabies treatment to enhance clearance of RABV from the CNS (Huang et al., 2014). Recently, simultaneous intracerebroventricular and intramuscular administration of neutralizing antibodies against the G protein was reported to increase survival rates of symptomatic mice infected with a pathogenic strain of RABV (de Melo et al., 2020), opening up a possibility to develop a new therapeutic intervention for the treatment of rabies.

Concluding remarks

Rabies is 100% preventable if post-exposure prophylaxis (PEP) with a vaccine and human immune globulin against RABV is administered immediately after exposure to RABV (Tantawichien and Rupprecht 2020). However, rabies still remains a persistent global public health threat especially in developing countries, where RABV is transmitted from wild animal reservoirs to unvaccinated domestic dogs followed by humans, but costly PEP is not always available. The World Health Organization set a goal to end human deaths from dog-mediated rabies by 2030 by increasing vaccination coverage in domestic dogs worldwide (Abela-Ridder et al., 2016). Although it is much more challenging to eliminate RABV from wild animals, oral rabies vaccines for wild animals, such as foxes, raccoons, coyotes, and skunks have been used to control RABV infection in wildlife and eventually to reduce the risk of infection of domestic animals and humans in some European countries and the United States (reviewed in Maki et al., 2017; Robardet et al., 2019). Currently, rabies elimination has been achieved only in a small number of countries, particularly island countries. Although there have been numerous studies on RABV in the past decades, the precise mechanisms of RABV spread, replication, immune evasion, and pathogenesis have not been fully understood. Further studies would certainly help in developing advanced strategies for the prevention, diagnosis, and treatment of rabies.

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Coronaviruses

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Nomenclature

3CLpro	3C-like protease
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
C-terminus	Carboxy terminus
CDC	Centers for Disease Control and Prevention
CoV(s)	Coronavirus (es)
CTD	C-terminal domain
E	Envelope protein
EIA(s)	Enzyme immunoassay(s)
EM	Electron microscopy
ER	Endoplasmic reticulum
ERGIC	Endoplasmic reticulum Golgi intermediate compartment
GTIs	Gastrointestinal tract infections
hCoV(s)	Human coronavirus (es)
HCoV-229E	Human coronavirus 229E
HCoV-4408	Human coronavirus 4408
HCoV-HKU1	Human coronavirus HKU1
HCoV-NL63	Human coronavirus NL63
HCoV-OC43	Human coronavirus OC43
ICU(s)	Intensive care unit(s)
IFN(s)	Interferon(s)
kb	Kilobases
kDa	Kilodalton
LRTIs	Lower respiratory tract infections
M	Membrane protein
MERS	Middle East respiratory syndrome
MERS-CoV	Middle East respiratory syndrome coronavirus
N	Nucleocapsid protein

nsps	Non-structural proteins
NTD	N-terminal domain
N-terminus	Amino terminus
ORF	Open reading frame
PBM	PDZ-binding motif
pp1a	Replicase polyprotein 1a
pp1ab	Replicase polyprotein 1ab
RBD	Receptor-binding domain
RBM	Receptor-binding motif
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RTC	Replication-transcription complex
S	Spike protein
SARS	Severe acute respiratory syndrome
SARS-CoV	Severe acute respiratory syndrome coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus-2
sg	Subgenomic
TMDs	Transmembrane domains
URTIs	Upper respiratory tract infections
α	Alpha
β	Beta
γ	Gamma
δ	Delta

Properties of human coronaviruses

Coronaviruses (CoVs) are found worldwide and infect a variety of animals, causing illnesses that range from gastrointestinal tract infections (GTIs), encephalitis and demyelination; and can be fatal (Graham et al., 2013). Traditionally, the human CoVs (hCoVs) have been associated with mostly upper respiratory tract infections (URTIs) and GTIs in humans. In recent years, however, it has become increasingly evident that the hCoVs can cause more severe lower respiratory tract infections (LRTIs) such as bronchitis, pneumonia and even acute respiratory distress syndrome (ARDS) (van der Hoek, 2007), and can be lethal.

Classification

The CoVs belong to the family *Coronaviridae* in the order *Nidovirales*. The subfamily *Coronavirinae* can be further divided into four genera, viz. alpha (α)-, beta (β)-, gamma (γ)- and delta (δ)-CoVs (Table 1). Five of the hCoVs, i.e. HCoV-OC43, HCoV-HKU1, as well as the more pathogenic SARS-CoV, MERS-CoV and SARS-CoV-2, belong to the genus β-CoV. The remaining two hCoVs, HCoV-229E and HCoV-NL63, belong to the genus α-CoV (van Regenmortel et al., 2000; Prades et al., 2014).

Structure and genome

CoVs are spherical-shaped enveloped viruses with a diameter of roughly 100 nm (Davies and Macnaughton, 1979). The CoV particles contain a helical nucleocapsid that is formed by the viral genomic RNA and viral nucleocapsid (N) protein complex. Viewed under an electron microscope, the virus particle is surrounded by a “corona” or halo (Latin for “crown”) (Fig. 1A), from which its name derives (Coronaviruses, 1968). The halo is formed by the three major structural proteins spike, membrane, and envelope (S, M, and E) projecting as spikes from the viral envelope (Fig. 1B).

CoVs have positive sense, single-stranded RNA genomes ranging in size from 26 to 32 kilobases (kb), the largest of the known viral RNA genomes. Common to all CoVs, the genome is organized in the conserved order, 5'-replicase (1a/1b), spike, envelope, membrane and nucleocapsid-3' (Fig. 2). The genome has a 5'-cap and is 3'-polyadenylated (Pyrce et al., 2007). The 5' two-thirds of the CoV genome contain a large 5' frameshifted polyprotein (ORF1a/ORF1ab). The open reading frames (ORFs) produced by this polyprotein encode for proteins essential for viral RNA replication and are called non-structural proteins (nsps) (van der Hoek et al., 2006). The 3' one-third of the genome contain the genes encoding for the major structural proteins. Interspersed among, or even overlapping, these structural ORFs is a number of accessory genes. These subgroup-specific ORFs vary in number, location and size (Liu et al., 2014).

Table 1 Classification of the selected coronaviruses.

Genus	Species
α -CoV	Transmissible gastroenteritis coronavirus (TGEV)
	Canine coronavirus (CCoV)
	Porcine respiratory coronavirus (PRCoV)
	Feline coronavirus (FeCoV)
	Porcine epidemic diarrhea coronavirus (PEDV)
	Human coronavirus 229E (HCoV-229E)
β -CoV	Human coronavirus NL63 (HCoV-NL63)
	Bat coronavirus (BCoV)
	Porcine hemagglutinating encephalomyelitis virus (HEV)
	Murine hepatitis virus (MHV)
	Human coronavirus OC43 (HCoV-OC43)
	Human coronavirus HKU1 (HCoV-HKU1)
	Severe acute respiratory syndrome coronavirus (SARS-CoV)
γ -CoV	Middle East respiratory syndrome coronavirus (MERS-CoV)
	Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)
	Avian infectious bronchitis virus (IBV)
δ -CoV	Turkey coronavirus (TCoV)
	Porcine Deltacoronavirus (PDV)

hCoVs in bold.

Jaiswal NK and Saxena SK (2020). Classical coronaviruses. *Coronavirus Disease 2019 (COVID-19): Epidemiology, Pathogenesis, Diagnosis, and Therapeutics*, pp. 141–150. https://doi.org/10.1007/978-981-15-4814-7_12.

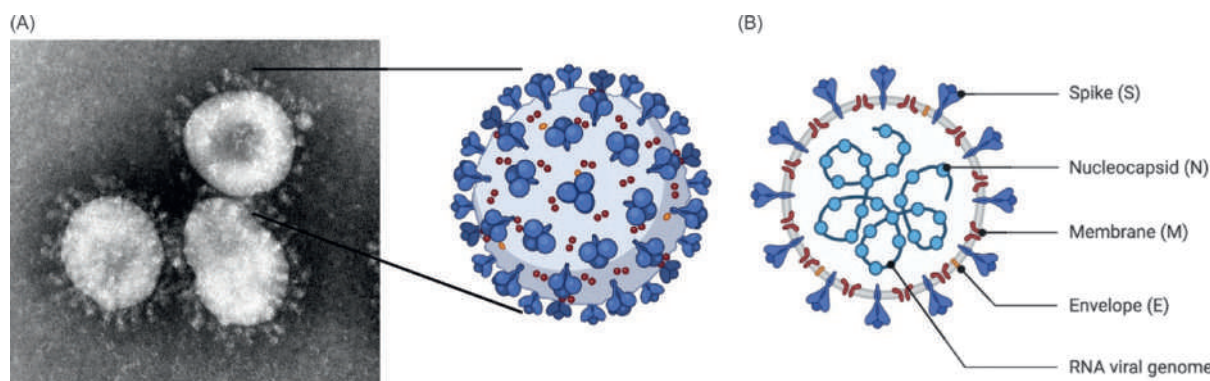


Fig. 1 Coronavirus morphology and structure. (A) Negative contrast electron microscopy of SARS-CoV particles, showing the large spikes and characteristic halo. (CDC/Dr. Fred Murphy—This media comes from the Centers for Disease Control and Prevention's Public Health Image Library (PHIL), with identification number #4814. This image is in the public domain and thus free of any copyright restrictions). (B) Representation of the CoV virion structure, showing the single-stranded, positive-sense RNA genome in complex with the nucleocapsid (N) to form ribonucleoprotein. The (N) is packaged into the core of the virus particle, with the spike (S), envelope (E), and membrane (M) proteins embedded into the envelope (created with BioRender.com). (A) Centers for Disease Control and Prevention's Public Health Image Library (PHIL), (B) Reprinted from *Human Coronavirus Structure*, by BioRender.com (2020). Retrieved from <https://app.biorender.com/biorender-templates>.

Genes and proteins

Spike protein (S)

S is a large protein (about 150 kDa) that trimerizes to form the distinctive spike structures on the surface of the virion. This protein facilitates viral entry into the host cell by mediating attachment of the virus to the host cell surface receptors. This is then followed by fusion between the viral and host cell membranes (Siu et al., 2008; Kirchdoerfer et al., 2016; Song et al., 2004). Attachment and fusion are facilitated by host proteases processing the S into S1- and S2-subunits (Millet and Whittaker, 2015). Next, the two subunits trimerize and fold into a metastable pre-fusion conformation. Research now shows that it's the S1-subunit that mediates receptor binding, while the S2-subunit is responsible for membrane fusion. The S1-subunit typically possesses two domains capable of binding to host cell receptors: an amino (N)-terminal domain (NTD) and a carboxy (C)-terminal domain (CTD) (Fig. 3) (Mou et al., 2013; Li et al., 2003).

For some CoVs, the expression of S at the cell membrane stimulates cell-to-cell fusion between infected and neighboring, uninfected cells. This formation of syncytia (i.e. giant, multinucleated cells) could possibly be a viral strategy that allows direct

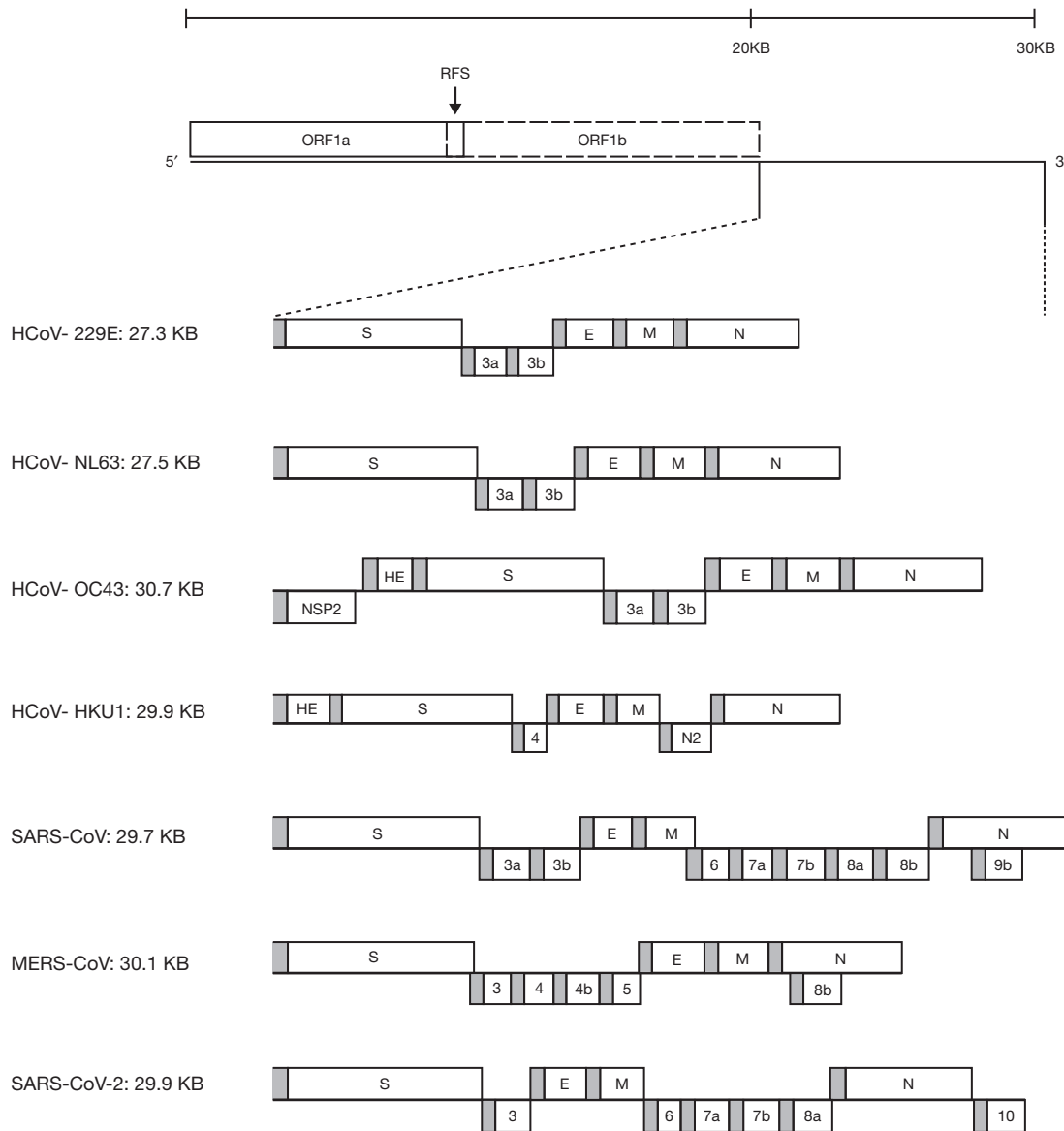


Fig. 2 Schematic organization of human coronavirus (α and β CoVs) genomes. hCoVs contain large genomes about 26–32 kb in size. The 5'-end, two-thirds of the genome contain the overlapping open reading frames 1a and 1b. The remaining 3'-end one-third of the genome (expanded region) encodes for the structural spike (S), nucleocapsid (N), membrane (M), envelope (E), and accessory proteins (vary depending on the specific CoV).

spread of the virus between cells, thereby avoiding virus-neutralizing antibodies in the host serum (Glowacka et al., 2011; Qian et al., 2013).

Nucleocapsid protein (N)

The CoV N contains three conserved and highly distinct domains; two structural and independently folded structural regions, the NTD and a CTD, separated by an intrinsically disordered central region called the RNA-binding domain (Fig. 4). All three regions are capable of binding RNA, allowing N to bind the viral genome in a beads-on-a-string type conformation (McBride et al., 2014; Fehr and Perlman, 2015). N is a multifunctional protein and is abundantly produced within infected cells. Important functions of N include, binding to viral RNA to form the ribonucleocapsid and it has been proposed to play key roles in virus replication, transcription and translation. In host cells, N has been shown to target and interact with many host proteins, potentially leading to deregulation of the cell-cycle and inhibition of interferon (IFN) production, which probably plays a role in viral pathology (McBride et al., 2014).

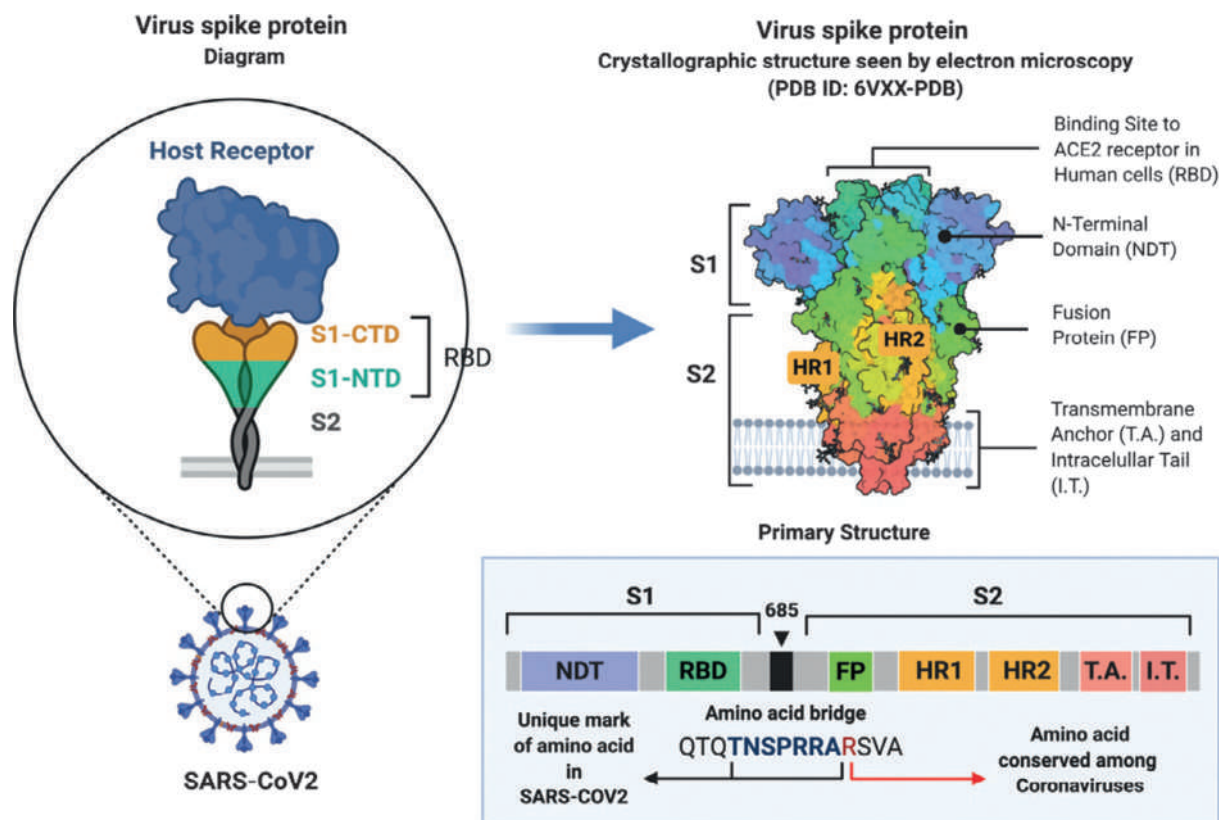


Fig. 3 The S glycoprotein of the newly discovered SARS-CoV-2. Representation of the three-dimensional structure of coronavirus S. S1, receptor-binding subunit; S2, membrane fusion subunit; TA (or TM), transmembrane anchor; IT (or IC), intracellular tail. The one-dimensional structure of coronavirus S shows the NTD (N-terminal domain). FP (fusion protein or peptide), HR1 (heptad repeat 1), and HR2 (heptad repeat 2) are structural units in coronavirus S2 that function in-membrane. S1 and S2 are linked together by a polybasic amino acid bridge, which may be important in studying viral targeting (created with BioRender.com). Based on *An In-Depth Look Into the Structure of the SARS-CoV2 Spike Glycoprotein and Schematic of Spike-Receptor Binding Mechanism of SARS-CoV-2*, by BioRender.com (2020). Retrieved from <https://app.biorender.com/biorender-templates>.

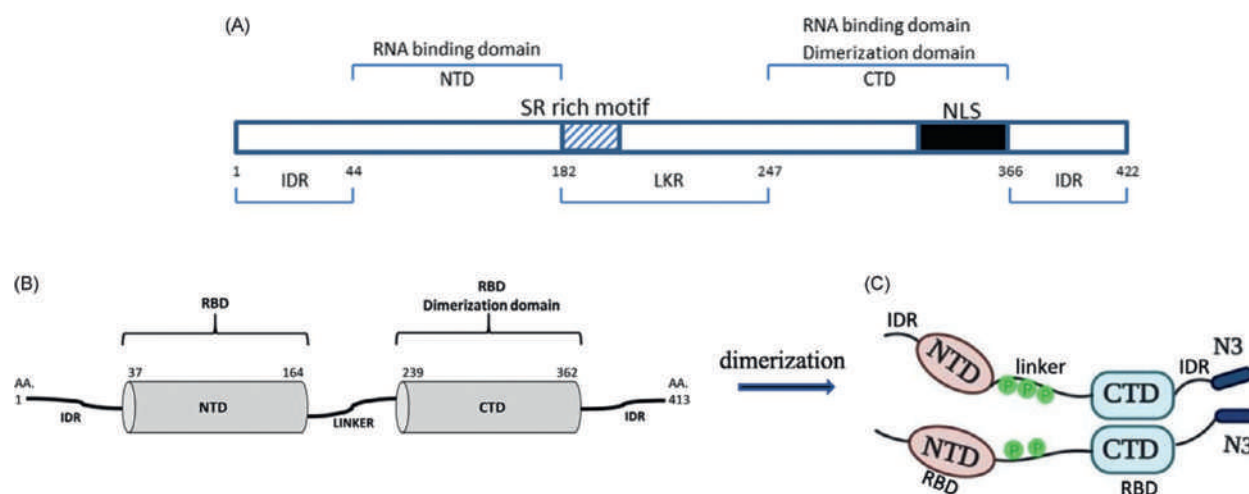


Fig. 4 The coronavirus nucleocapsid (N). (A), (B) Domain organization of the SARS-CoV N protein with the intrinsically disordered regions (IDR), N terminal domain (NTD), linker region (LKR), and C-terminal domain (CTD) shown. In (A) the charged SR rich (striated box) and the nuclear localization signal (NLS, solid box) motifs are shown (McBride et al., 2014). (C) Phosphorylation (P, green) of the SR/RS dipeptides by cellular kinases prevents aggregation of N and leads to the assembly of soluble dimers with the acidic carboxyl-tail domains (N3) exposed (McBride et al., 2014; Nikolakaki and Giannakouros, 2020). (A) and (B) McBride R, Van Zyl M, and Fielding BC (2014). The coronavirus nucleocapsid is a multifunctional protein. *Viruses* 6: 2991–3018, (C) Nikolakaki E and Giannakouros T (2020). SR/RS motifs as critical determinants of coronavirus life cycle. *Frontiers in Molecular Biosciences* 7: 219. <https://doi.org/10.3389/fmolb.2020.00219>.

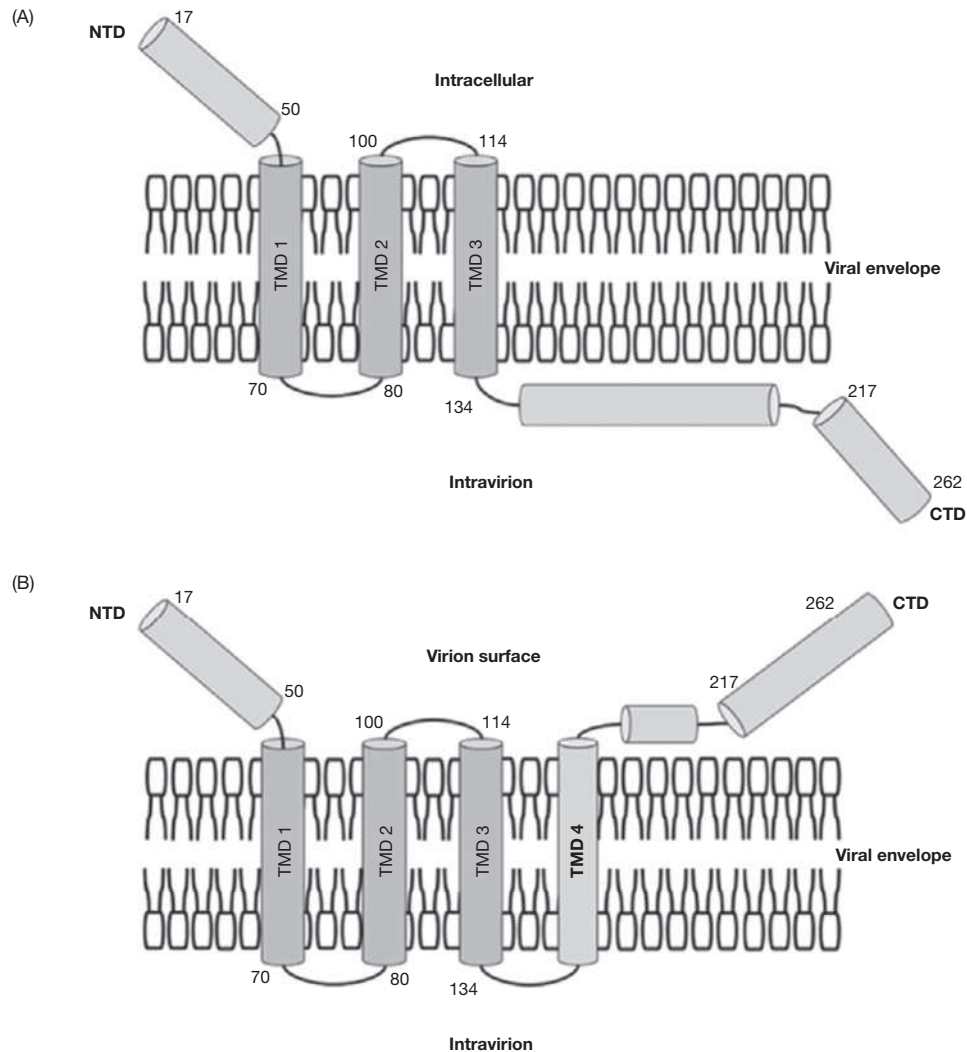


Fig. 5 The two predicted topologies of the coronavirus membrane (M). (A) In the first prediction, the M has three transmembrane domains (TMDs) located within the viral envelope. In this topology of the coronavirus M, the N-terminus domain (NTD) is found on the outside of the virion envelope and the C-terminus domain (CTD) on the inside of the virion. (B) In the second prediction, the M is presented with both the NTD and CTD at the outer surface of the virion. The TMDs are labeled 1–4.

Membrane protein (M)

M is the most abundant structural protein and defines the shape of the viral envelope (Neuman et al., 2011). This is a small (about 25–30 kDa) protein and contains three or four transmembrane domains (TMDs) (Fig. 5). Moreover, the protein has a small N-terminal glycosylated ectodomain and a larger C-terminal endodomain that extends into the virus particle (Fehr and Perlman, 2015). Homotypic interactions between the M proteins are the major driving force behind virion envelope formation but, on its own, is not sufficient for virion formation (de Haan et al., 2000). M needs to interact with all the major CoV structural proteins to drive the process of viral assembly (Masters, 2006). Critical for the incorporation of S into new virions, the interaction between S and M facilitates the retention of S in the endoplasmic reticulum (ER)-Golgi compartment (ERGIC)/Golgi complex where this incorporation can proceed (Mortola and Roy, 2004). M also interacts with N to stabilize the nucleocapsid (N-viral RNA complex), as well as the internal core of virions, thereby promoting completion of viral assembly (Narayanan et al., 2000). Together, M and E make up the viral envelope and their interaction is enough for the assembly and release of virus-like particles (Corse and Machamer, 2003).

Envelope protein (E)

E is a small protein (~8–12 kDa) present in low quantity within the viral structure. Interestingly, the sequence of CoV E proteins is not well conserved, but all have a common architecture (Fehr and Perlman, 2015). The E protein is a transmembrane protein, containing an N-terminal ectodomain and a C-terminal endodomain (Fig. 6). More recent studies have shown that the CoV E likely

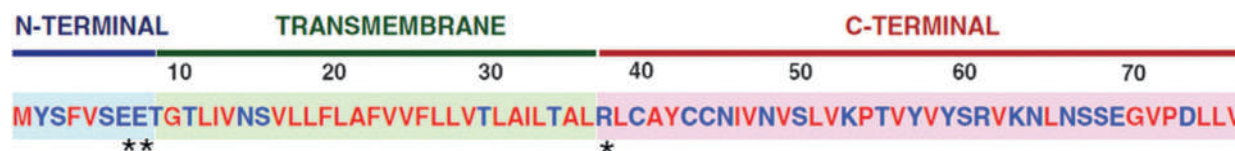


Fig. 6 The primary structure of the coronavirus envelope (E). The primary amino acid sequence and predicted structural domains of the SARS-CoV E, which consists of the N-terminal domain (NTD), the transmembrane domain (TMD), and the C-terminal domain (CTD). The NTD is predicted to be on the external surface of the virion, with the CTD on the inside. The amino acid properties shown: hydrophobic (red), hydrophilic (blue), polar, charged (asterisks) (Schoeman and Fielding, 2019). Schoeman D and Fielding BC (2019). Coronavirus envelope protein: Current knowledge. *Virology Journal* 16: 69.

plays crucial roles in the viral replication cycle and immunopathology, including virion assembly, budding and release, apoptosis, inflammation and even autophagy (Schoeman and Fielding, 2019, 2020).

Accessory proteins

The accessory proteins are subgroup-specific ORFs that vary in number, location and size in the CoV genome (Liu et al., 2014). These ORFs share no similarity with accessory genes of CoVs belonging to other subgroups (Lai and Cavanagh, 1997), nor to any other known viral or cellular proteins. Even though CoV accessory proteins are believed to be mostly dispensable for growth in vitro (Oostra et al., 2007), these genes are maintained in the virus genomes under selective pressures. This is evidence that they likely confer some biological advantage to the virus in the natural environment, i.e. these proteins have a function in vivo in the natural host. These accessory proteins normally act by interfering with cellular processes, or by modulating virus-host interactions at the organism level. In fact, recent research proposes a wide range of possible functions for CoV accessory proteins, including modulation of viral pathogenicity and replication, acting as cell death inducers, and IFN antagonists, to name a few. Some of these accessory proteins have also been identified as minor viral structural components (McBride and Fielding, 2012; Oostra et al., 2007).

Viral replication

After binding to the cellular receptor, the CoV S undergoes conformational changes that lead to fusion between the viral envelope and the cell membrane (Fig. 7). The CoV then deposits its RNA genome into the host cytoplasm through endocytosis or direct membrane fusion. To synthesize viral subgenomic (sg) mRNAs, CoVs employ a discontinuous replication strategy (Pyrce et al., 2004). During the first stage of virus replication, the positive strand viral RNA serves as the mRNA for translation of two large proteins—ORF 1a and 1b—each encoding units of the RNA-dependent RNA polymerase (RdRp). The synthesis of the 1ab polyprotein involves a -1 ribosomal frameshift during translation of the 1a gene (Sawicki et al., 2007; Pyrc et al., 2004). The 1a and 1ab polyproteins (pp1a and pp1ab) are then cleaved by viral-encoded proteases to produce, on average 15–16, nsps. Typically, the 1a polyprotein is cleaved to form nsp 1–11, and the 1ab polyprotein gives rise to nsp 12–16. These nsps form the replicase-transcriptase proteins of CoVs and assemble, along with host and other viral proteins, into a membrane-bound replication-transcription complex (RTC). The active RNA polymerase transcribes full-length complementary (negative-sense) RNA, which is then copied into a positive sense sg mRNA. The subsequent transcription of the positive sense sg mRNA produces six distinct mRNAs, which includes the full length RNA genome, as well as a nested set of 3'-coterminal overlapping sg mRNAs. Each of the sgRNAs serves as a code for one of the ORFs downstream of the replicase gene. With the exception of ORF-E, the 5'-end of each ORF contains a transcription regulatory sequence of AACUAAA, which is essential for sg mRNA synthesis. Also, all the positive sense mRNAs share a leader sequence of approximately 70 nucleotides which is identical to the leader sequence of the genomic RNA. The viral membrane proteins insert into the ERGIC. As this happens, the full length viral RNA genome associates with the N. Next, the newly formed ribonucleoprotein complex interacts with the M in the ERGIC membrane and viral particles form as the nucleocapsid complex buds into the lumen of the ERGIC. The virus then moves through the ERGIC and exits the cell, most likely via exocytosis (Marra et al., 2003).

Pathogenic coronaviruses

Before 2003, only two CoVs, HCoV-OC43 and HCoV-229E (Tyrrell and Bynoe, 1965; Hamre and Procknow, 1966), were known to infect humans. In the years following the severe acute respiratory syndrome (SARS) outbreak, two additional HCoV were identified, viz. HCoV-NL63 (van der Hoek et al., 2004) and HKU1 (Woo et al., 2005). As far as we know, only HCoV-OC43, HCoV-229E, HCoV-NL63 and HKU1 continuously circulate in the global human population, resulting in infections throughout the year (Fielding, 2011). The first pathogenic CoV to cause severe acute respiratory tract disease in humans was identified in early 2003 (Drosten et al., 2003; Peiris et al., 2003b; Ksiazek et al., 2003). This novel CoV was eventually named severe acute respiratory syndrome CoV (SARS-CoV). In September 2012, the second pathogenic novel CoV was identified in two patients, of which one died

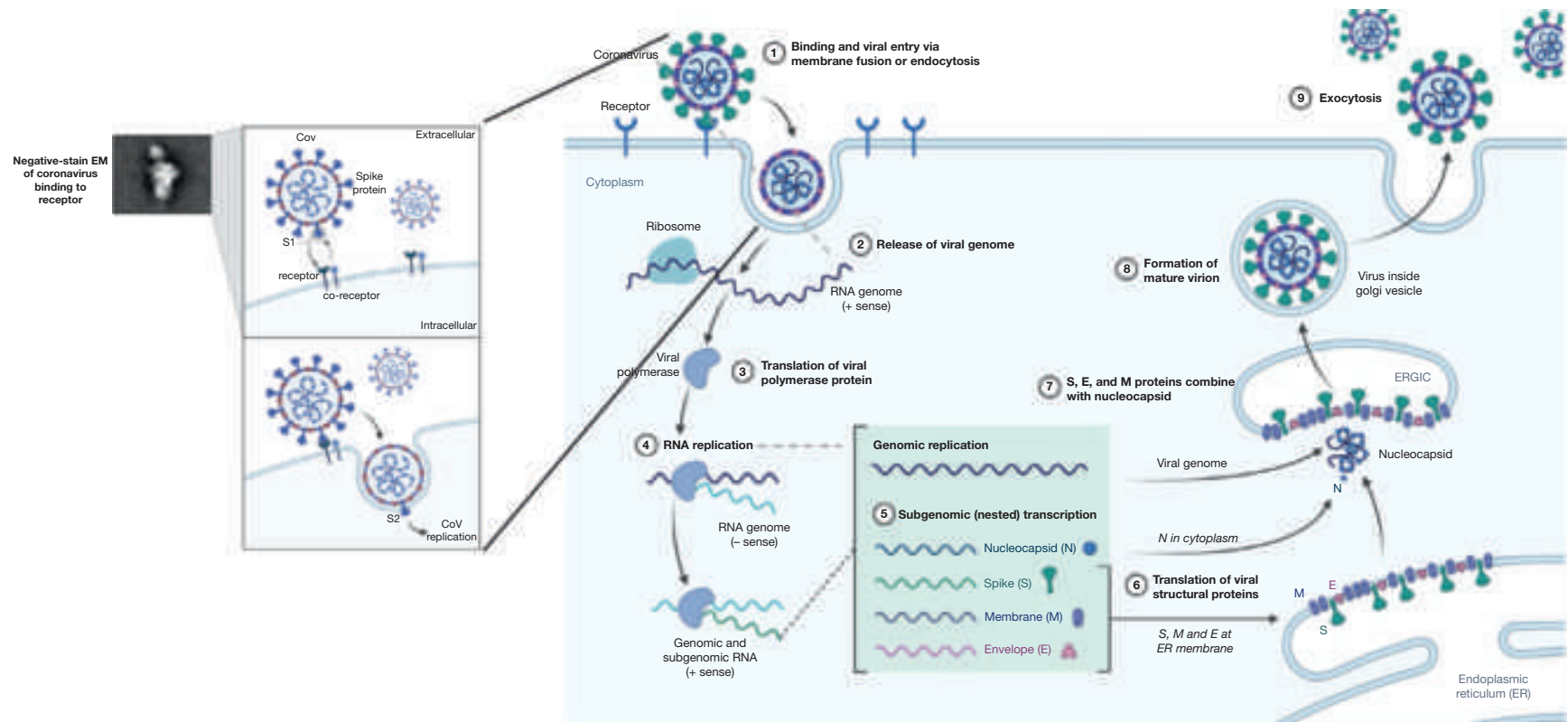


Fig. 7 Overview of the coronavirus (CoV) replicative cycle. After the CoV binds to its specific host cell receptor, the virus enters the cell by receptor-mediated endocytosis (1), and the genomic RNA is released into the cytosol (2); the latter is a result of the fusion of the endosomal and viral membranes. Next, translation of the viral genome yields the pp1a and pp1ab replicase polyproteins 1a and 1ab (pp1a and pp1ab) (3). Numerous internal proteases then cleave the polyproteins, and the resultant viral non-structural proteins (nsps) assemble into a replication-transcription complexes (RTCs) that engages in minus-strand RNA synthesis (4). During this step, both full-length and subgenomic (sg)-minus strand RNA are produced; the sg-minus strand RNA is then used as templates in the synthesis of the sg mRNAs which are needed to express the CoV structural and accessory protein genes (5,6). Ultimately, the (full-length) genomic RNA is encapsidated by multiple copies of the N protein and meets the structural proteins at the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC) and become enveloped by budding from smooth intracellular membranes (7,8). Finally, the new virions leave the cell by following the exocytic pathway (9) (de Wilde et al., 2018) (created with BioRender.com). Based on *SARS-CoV-2 Targeting of ACE2 Receptor and Entry in Infected Cell and Coronavirus Replication Cycle*, by BioRender.com (2020). Retrieved from <https://app.biorender.com/biorender-templates>.

(Zaki et al., 2012); this virus was eventually named Middle East respiratory syndrome CoV (MERS-CoV). Not until early 2020, was the third pathogenic hCoV—and only seventh CoV known to infect humans—identified and eventually named SARS-CoV-2 (Huang et al., 2020b).

Severe acute respiratory coronavirus (SARS-CoV)

SARS-CoV eventually spread to more than 30 countries, infecting ~8000 people with a mortality rate of ~10% (Fielding and Tan, 2007). The spread of the virus was eventually controlled by an unprecedented global health response and the virus has not been detected in the human population since April 2004.

Clinical features and pathogenesis

Even though SARS affects people of all ages, the majority of infections occur among adults with the median age 42–57 years. The elderly and individuals with a history of chronic comorbidities, including diabetes mellitus or chronic kidney failure, may have atypical presentations, such as lack of fever. Infections among children and adolescents, especially those younger than 12 years of age, are not common, and even if they do become infected, the disease is considerably less severe; the outcome is also much more favorable (Stockman et al., 2007).

The severity of SARS varies from asymptomatic infection to fatal acute ARDS, with asymptomatic or mild illness very uncommon. The initial, prodrome of symptoms of the disease are nonspecific and similar to a flu-like illness. One to two days after exposure to the virus, the patient develops early symptoms that could include fever, headache, chills, rigors, malaise, and myalgias (Pormohammad et al., 2020). Fever develops in all patients and is typically the first symptom, but it can also develop after the other initial symptoms. The lower respiratory tract phase of the illness follows after a further 3 or more days—but typically appears in week two—comprising of non-productive cough, shortness of breath and increasing respiratory distress sometimes requiring mechanical ventilation (Coleman and Frieman, 2014). Gastrointestinal symptoms, including diarrhea, vomiting and nausea have been recorded, but are not very common, with vomiting the least common; the diarrhea is often self-limiting. SARS-CoV has also been detected in other organs, including the kidneys, liver and spleen (Peiris et al., 2003a).

The most disturbing clinical feature of SARS is how fast many patients develop symptoms of acute ARDS. This is also the most common reason for admission to an intensive care unit (ICU). As soon as the second week after the onset of symptoms, pneumonia and hypoxemia linked to a rapid deterioration in the clinical condition of the patients may occur. Between 20% to 30% of patients admitted to hospital with SARS-CoV infection were admitted to an ICU, and about 75% of these patients required mechanical ventilation. Mechanical ventilation was required in approximately 16% of all SARS patients, and was associated with a mortality rate of about 50% (Fowler et al., 2003; Poutanen et al., 2003).

Epidemiology

SARS-CoV was originally believed to originate in civet cats, but these mammals were later shown to serve as intermediary hosts providing a source of infection to humans (Guan et al., 2003). Subsequent field epidemiological and molecular screening studies revealed that the most likely natural reservoir of SARS-CoV is horseshoe bats. Not only do many wild populations of horseshoe bats harbor several distinct SARS-like CoVs, many others have developed antibodies, indicating previous infection by the these CoVs (Lau et al., 2005; Wang and Eaton, 2007). Serological studies have now shown that SARS-CoV is a new human pathogen and had not previously circulated to any significant extent in humans prior to the outbreak in 2003 (Guan et al., 2003). The transmissibility of SARS-CoV between individuals is high and the virus appears to be transmitted primarily by large droplet spread and close contact. Transmission through fomites likely occurs as the virus remains viable for long periods on contaminated surfaces. Airborne spread, via droplets, is also likely to play a role in transmission between individuals (Low, 2004).

Middle East respiratory syndrome coronavirus (MERS-CoV)

MERS-CoV was the first pathogenic hCoV to be identified following the 2003 SARS-CoV outbreak. This CoV was originally isolated in June 2012 from a patient presenting with severe respiratory illness. Eleven days after admission to a hospital in Jeddah, Saudi Arabia, the patient—a 60-year-old man—died from renal and respiratory failure (Zaki et al., 2012). Since then, MERS-CoV infections were reported in more than 27 countries across the Middle East, Europe, North Africa and Asia, with the virus currently posing a potential threat in the Middle East where it is still sporadically detected in the human population (Chafekar and Fielding, 2018).

Clinical features and pathogenesis

MERS-CoV infections disproportionally affect adults, especially males, with children rarely affected (Arabi et al., 2014; Al-Tawfiq et al., 2016). Moreover, the virus typically causes more severe clinical manifestations in older people and, based on some evidence,

people with weakened immune systems. Those with chronic co-morbidities such as hypertension, obesity, diabetes, chronic lung disease, cardiovascular diseases or end-stage renal disease are also much more likely to be hospitalized when infected with MERS-CoV (Kim et al., 2017). In actuality, about 75% of MERS patients have at least one co-morbidity and MERS-associated fatalities are more likely to have other underlying medical conditions (Zumla et al., 2015).

Similar to SARS-CoV, MERS-CoV infection causes a clinical spectrum that ranges from asymptomatic (Drosten et al., 2014) to rapidly progressive, ARDS, septic shock and multi-organ failure, which can result in death (Al-Dorzi et al., 2016). In symptomatic individuals, the early symptoms are typically nonspecific, with patients reporting anything from general malaise, to a sore throat, low-grade fever, non-productive cough, chills, headache, shortness of breath and/or muscle pain. Less frequently, MERS-CoV patients present with gastrointestinal symptoms such as nausea, vomiting, anorexia, abdominal pain and diarrhea (Kapoor et al., 2014). Radiographs and computed tomography (CT) findings of the patient's chest are generally consistent with viral pneumonitis and ARDS (Zumla et al., 2015). Laboratory findings also include elevated lactate dehydrogenase levels, lymphopenia and thrombocytopenia (Arabi et al., 2014; Senga et al., 2017), with some cases resulting in elevated levels of creatinine, lactate dehydrogenase and liver enzymes (Zumla et al., 2015; Senga et al., 2017).

Patients who require hospitalization are typically admitted approximately 4 days after the onset of symptoms and the mean time spent in hospital is 41 days (Arabi et al., 2014). Worryingly, as much as 50% of adult symptomatic patients are admitted to ICUs, followed by between 40% and 70% then requiring mechanical ventilation within 7 days (Obobo et al., 2015). Reports indicate that between 22% and 70% of critically ill patients require renal replacement therapy (Arabi et al., 2014; Senga et al., 2017), but this needs to be confirmed since the higher end of this range could also be a result of hospital-acquired infections in patients with pre-existing kidney disease. In the end, MERS has a case mortality rate of about 36%, with the median time from the onset of symptoms to death about 11.5 days (Senga et al., 2017).

Epidemiology

Even though there is no conclusive evidence supporting bats as a source for transmission of MERS-CoV to humans, bats are hosts to MERS-CoV-like viruses (Corman et al., 2014). So, even though these viruses are genetically distinct from MERS-CoV, the possibility that bats serve as a primary animal host for MERS-CoV cannot be excluded (Memish et al., 2013). Importantly, the jump of MERS-CoV from bats directly to humans is currently purely speculative. There is some, although weak, evidence that the virus could have been transmitted to humans from dromedary camels (Reusken et al., 2013; Chan et al., 2015). This is primarily based on the presence of MERS-CoV-specific neutralizing antibodies specific in dromedary camels (Reusken et al., 2013), and one incidence of MERS-CoV infection in humans that could be linked to camels (Haagmans and Osterhaus, 2014). The transmissibility of MERS-CoV between individuals is low, and human-to-human transmission has only been observed in situations with prolonged close contact, such as health care settings, or in immunocompromised individuals with co-morbidities (Sharif-Yakan and Kanj, 2014).

Severe acute respiratory coronavirus-2 (SARS-CoV-2)

Clinical features and pathogenesis

The incubation period of COVID-19 is typically between 1 and 14 days, with most people showing signs of disease around a median time of 5–8 days. However, recent reports suggest that symptoms might even manifest up to 24 days after infection (Lauer et al., 2020; Bai et al., 2020; Guan et al., 2020). Following the incubation period, infected persons develop fever, dry cough, dyspnea, myalgia, and fatigue, features resembling pneumonia. Studies in China revealed that less common symptoms could include headache, diarrhea, hemoptysis, nausea, vomiting, sore throat, chest pain, chills, and a productive cough. ARDS is a feature seen in more severe cases (Chen et al., 2020a; Guan et al., 2020; Huang et al., 2020a; Wang et al., 2020b). Olfactory and taste disorders were also self-reported by patients from Italy (Giacomelli et al., 2020).

From a report in China, involving 72,314 cases, the majority of patients (~81%) only develop a mild form of pneumonia, or not at all; more severe pneumonia, characterized by shortness of breath, low oxygen saturation, and involvement of at least 50% of lung parenchyma in chest imaging, was seen in 14% of patients; and only 5% of patients became critically ill, developing shock or respiratory failure that required medical ventilation, or multi-organ dysfunction (Wu and McGoogan, 2020). Similar to SARS and MERS, the leucocyte count in majority of infected persons revealed lymphopenia, while the total white blood cell count may either be elevated or lower than normal. Additional biochemical factors such as procalcitonin, hepatic transamines, D-dimer, and prothrombin time were elevated in critical patients. The levels of plasma cytokines were reportedly higher in ICU patients than in non-ICU patients, suggesting that the cytokine storm is the basis for immunopathology (Huang et al., 2020a; Chen et al., 2020b; Yang et al., 2020). Almost all patients also exhibited abnormal chest radiographic features (Huang et al., 2020a). Chest X-rays frequently showed bilateral patchy shadows and ground-glass opacifications were seen in ARDS patients. Multiple ground-glass opacifications with or without reticular pattern, and parenchymal consolidations that involve both lungs were among the typical CT findings (Chung et al., 2020; Zhou et al., 2020b). It is worth noting that a report noted changes in CT scans from patients before the onset of clinical symptoms, even before viral RNA was detected in specimens from the upper respiratory tract (Shi et al., 2020). Recovery time varied depending on the extent of the infection; most patients recovering from mild infections were discharged after 2 weeks, whereas more severe cases recovered between 3 and 6 weeks, and approximately 2.3% of patients died from the infection

with a median time of 16 days after illness onset (Wu and McGoogan, 2020; Chen et al., 2020b; Wang et al., 2020b; Guan et al., 2020).

The pathogenesis of SARS-CoV-2 can range from merely manifesting as mild symptoms to more severe manifestations, such as respiratory failure. Pathogenesis begins after SARS-CoV-2 binds to epithelial cells in the respiratory tract and initiates replication, permitting migration of the virus deeper into the lungs to reach the alveolar epithelial cells. In the lungs SARS-CoV-2 replicates rapidly and can induce a strong immune response. This response is brought on by a cytokine storm syndrome, which culminates in ARDS and can be accompanied by respiratory failure. This is considered to be the main cause of death in patients with severe COVID-19 (Huang et al., 2020a; Mehta et al., 2020). Multiple organ failure, or dysfunction, is another serious sequelae reported in some cases of COVID-19 (Wu and McGoogan, 2020; Chen et al., 2020a; Yao et al., 2020). Although the exact nature of the SARS-CoV-2 pathophysiology is not yet clear, the immune system clearly plays an integral role as a combination of the cytokine storm and viral evasion of the cell-mediated immune response play very important roles in the progression and severity of the disease (Channappanavar and Perlman, 2017). Elevated levels of inflammatory cytokines and chemokines have been reported to result in lung injury and required patients to be urgently administered to the ICU (Huang et al., 2020a).

Given the target tissue of SARS-CoV-2, it stands to reason that the histopathological changes seen in patients with COVID-19 occur mainly in the lungs. Common histopathological features include bilateral diffused alveolar damage, formation of hyaline membranes, desquamation of pneumocytes, and in severe COVID-19 patients, fibrin deposits in the lungs. In some cases, exudative inflammation was also seen. Antigens of SARS-CoV-2 were detected in several tissue sites: the upper airway, submucosal gland epithelium, bronchiolar epithelium, as well as pneumocytes (type I and II), alveolar macrophages, and hyaline membranes in the lungs (Huang et al., 2020a; Chen et al., 2020a; Martinez et al., 2020; Zeng et al., 2020).

Epidemiology

The SARS-CoV-2 outbreak occurred in China and was traced to Huanan Wholesale Seafood Market, citing the market as the source of the outbreak (Ji et al., 2020). Based on the genetic evidence, SARS-CoV-2 was likely to have originated in animals, although when and where humans were first exposed to the virus is not known for certain. With some of the first reported cases having no epidemiological link to the Huanan seafood market, it is possible that the market may not have been the initial source of human exposure to SARS-CoV-2 (Li et al., 2020a). Horseshoe bats, however, are a particularly important source of both α - and β -CoVs (Zhou et al., 2020a; Fan et al., 2019; Li et al., 2005). The *Rhinolophus affinis* bat carries a CoV designated “RaTG13,” which is 96.2% identical to the full-length genome of SARS-CoV-2. All SARS-CoV-2 ORFs, including the hypervariable S protein and ORF8, are more than 90% similar to those of RaTG13 (Zhou et al., 2020a). More recently, *R. malayanus* was also reported to carry a CoV designated “RmYN02” which shares 93.3% of its genome with SARS-CoV-2. Both the clustering and the high genetic similarity between these bat CoVs and SARS-CoV-2 suggests that SARS-CoV-2 originated in bats (Paraskevis et al., 2020). The diverse number of bat CoVs found to be closely related to SARS-CoV-2 suggests that bats are the likely reservoir of SARS-CoV-2 (Lau et al., 2020).

Pangolins were initially the suspected source of the SARS-CoV-2 outbreak. Comparison of pangolin CoV genomes from the Guangxi and Guangdong provinces to the SARS-CoV-2 genome revealed that their sequences were 85.5% and 92.4% similar, respectively, to SARS-CoV-2 (Lam et al., 2020). The RBD of the Guangdong pangolin CoV is also highly similar to that of SARS-CoV-2, with only one variation in the RBM while still containing the five residues critical for ACE2 receptor binding (Xiao et al., 2020b). However, despite these similarities and having found SARS-CoV-2-related CoVs in pangolins, pangolins cannot be regarded as reservoirs for these CoVs (Xiao et al., 2020b; Lam et al., 2020). Bats, for one, can maintain a healthy physiological state while carrying CoVs. Pangolins infected with CoVs, however, exhibit clinical signs and histopathological changes indicative of CoV infection, suggesting that they are not natural hosts or reservoirs of SARS-CoV-2 (Xiao et al., 2020b).

Transmission

Although animals are the original source of the pathogenic hCoVs—transfer between the animal host or reservoir and humans after a spill-over event—global spread is driven by human-to-human transmission. The primary route of transmission for the pathogenic hCoVs is via the respiratory tract—i.e. pathogenic hCoVs are present in respiratory tract secretions, with tracheal secretions and bronchoalveolar lavage (BAL) specimens containing higher viral loads than nasopharyngeal swabs. Transmission typically occurs through close contact with an infected person, where infectious droplets and, in some cases, aerosols may come into contact with mucous membranes (Low, 2004; Deng and Peng, 2020). The spread by small droplet aerosols and droplet nuclei can also occur when much smaller and more numerous aerosol particles linger in the air and, when inhaled, can reach deep inside the lungs of a person; this has also been confirmed in recent studies for SARS-CoV-2 (Meselson, 2020; van Doremalen et al., 2020). This is thought to be a particularly important transmission route in enclosed rooms where large gatherings tend to take place (Stadnytskyi et al., 2020). Interestingly, the efficiency of transmission between individuals vary greatly between studies, and is likely associated with the degree of severity of illness and with the virulence of viral strains. Transmission through inanimate objects contaminated with infectious secretions occurs as CoVs remain viable for long periods on surfaces (Kampf et al., 2020), and has been a source of infection in healthcare settings (Bin et al., 2016; Gopalakrishna et al., 2004). These CoVs has also been detected in serum, feces and urine (Poissy et al., 2014). Increasing evidence also points to the possibility of, at least for SARS-CoV-2, transmission through contaminated feces. As an example, SARS-CoV-2 infected ferrets were found to have shed the virus in feces for up to 8 days after infection and is somewhat consistent with what was reported for some human studies (Wang et al., 2020d; Xiao et al., 2020a; Kim et al., 2020).

Laboratory diagnosis

Laboratory confirmation of pathogenic CoV infections has broadly included (i) molecular detection of CoV RNA; (ii) CoV antigen detection; or (iii) identification of a humoral response to previous infection (Mackay and Arden, 2015), often with varying degrees of success in terms of sensitivity and specificity.

During the SARS-CoV outbreak, a combination of serological and reverse transcription polymerase chain reaction (RT-PCR) assays was frequently used to confirm infection. Rapid diagnosis was most frequently made by RT-PCR using primers specific to the viral N-gene sequence. Throat, nasal, pharyngeal or serum samples were used to detect viral RNA by RT-PCR, as early as the first week of illness. During the second week of illness, stool and respiratory samples were also suitable for viral RNA assays. In rural areas, where expensive and complex molecular tests could not routinely be performed, enzyme immunoassays (EIAs) for viral N and S antigens were developed to screen suspected SARS cases (Bermingham et al., 2004; Chan et al., 2004). A positive real-time RT-PCR assay, targeting at least two different genomic regions of the MERS-CoV, can be used to confirm MERS. Probe and primer sets targeting the ORF1a and upstream of the E gene show the highest sensitivity and remain the most widely used for MERS-CoV confirmation assays (Corman et al., 2012). A single positive real-time RT-PCR test result, confirmed by gene sequencing, can also be considered positive for MERS-CoV infection (Chafekar and Fielding, 2018).

For a definitive COVID-19 diagnosis a combination of clinical manifestations, radiographical evaluations, and laboratory diagnosis, such as the detection of SARS-CoV-2 RNA (Zu et al., 2020), is required. SARS-CoV-2 viral RNA can be detected in a variety of samples, but it should be cautioned that not all samples are equally representative of the viral load, which could affect the diagnosis. Although SARS-CoV-2 can be detected in different parts along the respiratory tract, including from throat swabs, posterior oropharyngeal saliva, nasopharyngeal swabs, sputum, and bronchial fluid, samples from the lower respiratory tract contain a higher viral load since it is generally the target site of the virus (Zhou et al., 2020a; Pan et al., 2020; To et al., 2020b; Wang et al., 2020d; Han et al., 2020). Recommendations provided by the United States (US) Centers for Disease Control and Prevention (CDC) stipulate that BAL fluids, nasopharyngeal swabs (not throat swabs), and blood are all accepted clinical samples (CDC, 2020). However, when respiratory samples test negative, viral RNA can also be detected from the intestinal tract (Zhang et al., 2020). Since oral swabs commonly produce false positives, it is advised that multiple detection methods be used to confirm a COVID-19 diagnosis (Li, 2020; Xie et al., 2020). CT can be used in conjunction with laboratory testing to confirm a positive COVID-19 diagnosis. In Wuhan, when molecular detection services were overwhelmed, patients were screened for radiographic features of COVID-19. Features included bilateral multilobar ground-glass opacities along with peripheral or posterior distribution (Xie et al., 2020; Kanne, 2020). It is, therefore, recommended that patients who initially tested negative for viral RNA, but are strongly suspected of having COVID-19, receive chest CTs in conjunction with continued swab tests (Xie et al., 2020). Serological testing for the presence of pathogenic CoV-specific antibodies, such as those specific for the SARS-CoV-2 N or S protein, is another diagnostic method that proves useful for determining previous exposure to the virus. These tests can be used to quickly triage suspected cases of pathogenic CoV infection by confirming or ruling out infections *ex post facto*. It is, however, only useful in the later stages of the disease or even retrospective studies, after sufficient time has elapsed to mount an adaptive immune response (Zhang et al., 2020; Guo et al., 2020; To et al., 2020a). Of concern is that the extent and duration of the immune response can differ from patient to patient, and tests can vary in specificity and sensitivity (Bastos et al., 2020), possibly affecting the diagnosis. Nevertheless, good progress has been made regarding COVID-19 laboratory diagnostic methods, but the limitations warrant a combination of diagnostic methods to increase the reliability of a confirmed diagnosis. This, in turn, can help to accurately identify infected persons and consequently mitigate or limit the spread of SARS-CoV-2 (Lin et al., 2020).

Treatment and prevention

For the vast majority of pathogenic hCoV infections, supportive care, which includes rest, taking in fluids and analgesics are used, and mainly depends on the provision of organ support and management of complications. At times, broad-spectrum antibiotics, antivirals, IFNs and/or antifungals are prescribed to minimize the risk of co-infection with opportunistic pathogens (Momattin et al., 2013).

Ribavirin and corticosteroid treatment of SARS-CoV proved largely ineffective, since corticosteroids suppress the humoral and cellular components of the immune system (Feron et al., 2018). Other drugs, like pentoxifylline, were promising therapeutic candidates for treating SARS-CoV on account of its anti-inflammatory, antiviral, immunomodulatory, and bronchodilatory effects, but it proved largely unsuccessful in the clinical treatment of SARS-CoV infection (Bermejo Martin et al., 2003). Many antioxidant compounds demonstrate anti-SARS-CoV activity by inhibiting 3C-like protease (3CLpro), an enzyme vital to the replication of SARS-CoV (Jo et al., 2020). Accordingly, several studies of antioxidant compounds, including quercetin and quercetin-related compounds, reported the efficient inhibition of 3CLpro, in some cases reducing SARS-CoV replication (Ryu et al., 2010).

For MERS-CoV, monitoring epidemic patterns and investigating the spread of infection allows efficient identification, control, and prevention of possible pandemics. Combined treatment of MERS-CoV with ribavirin and IFN- α 2b was proposed as an early treatment since a combination of ribavirin and IFNs could inhibit MERS-CoV replication in vitro and improve clinical outcomes in MERS-CoV-infected non-human primates (Falzarano et al., 2013). The limited effective therapeutic window of MERS-CoV infections might also limit the efficacy of broad-spectrum antivirals when treating severe MERS-CoV patients (Widagdo et al., 2017). Alisporivir, a non-immunosuppressive cyclosporin A-analogue, inhibited MERS-CoV replication in vitro and could be

further potentiated by ribavirin, underpinning the potential use of cyclophilin inhibitors as host-directed, broad-spectrum inhibitors of CoV replication (de Wilde et al., 2017). However, since none of these potential anti-MERS-CoV candidates have demonstrated a significant benefit to treating acute MERS-CoV infections in a consistent and controlled manner, supportive management adapted from SARS-CoV guidelines has formed the basis for MERS-CoV treatment (Modjarrad, 2016). Various MERS-CoV vaccines have been designed, of which one has been progressed to clinical trials. Current MERS-CoV vaccines have been shown to induce effective protection in a few animal models (see review by Chafekar and Fielding, 2018).

Both researchers and pharmaceutical manufacturers are conducting large-scale clinical trials to evaluate a variety of COVID-19 treatment options. Some drugs, such as umifenovir (Arbidol), aim to block the binding of SARS-CoV-2 to the ACE2-receptor to prevent entry, but its efficacy is not yet established (Wang et al., 2020e; Zhu et al., 2020c; Li et al., 2020b; Lian et al., 2020). Camostat mesylate can also block the entry of SARS-CoV-2 into human lung cells (Hoffmann et al., 2020), but clinical data to support its efficacy is insufficient (Kawase et al., 2012; Zhou et al., 2015). Specific monoclonal antibodies (mAbs), recombinant human ACE2 receptors, or fusion inhibitors targeting the S protein have also been considered, but lack the required safety and efficacy parameters for clinical trials (Monteil et al., 2020; Tian et al., 2020; Xia et al., 2020b). SARS-CoV-2 viral replication inhibitors lopinavir and ritonavir (3CLpro inhibitors), and remdesivir, favilavir, and ribavirin (targets RdRp) have also been investigated (Wang et al., 2020c; Tahir ul Qamar et al., 2020; Williamson et al., 2020; Grein et al., 2020; Cai et al., 2020; Cao et al., 2020; Hung et al., 2020).

Immunomodulatory agents are used to mitigate the excessive inflammatory response induced by SARS-CoV-2. Dexamethasone reduced the mortality of COVID-19 hospitalized patients who received invasive mechanical ventilation by 1/3 and by 1/5 for patients who received oxygen support, but no benefit was found in patients who did not require oxygen support for COVID-19 treatment (RECOVERY, 2020; The Recovery Collaborative Group, 2020). Tocilizumab and sarilumab, antibodies (Abs) specific for the interleukin-6 receptor (IL-6R), were effective in treating severe COVID-19 by attenuating the cytokine storm (Xu et al., 2020). Preliminary results using eculizumab, a mAb which inhibits the proinflammatory complement protein C5, showed potential for treating severe COVID-19 cases by decreasing inflammatory markers and C-reactive protein (CRP) levels (Diurno et al., 2020). SARS-CoV-2 is reportedly more sensitive to type I IFN than SARS-CoV, making it a promising candidate in the early treatment of COVID-19 (Mantlo et al., 2020).

The Food and Drug Administration (FDA) approved convalescent plasma treatment as an adjunctive therapy for COVID-19 and preliminary results suggest that patients improved after treatment, but there are possible adverse effects (Duan et al., 2020; Shen et al., 2020). Conversely, mAbs can neutralize the SARS-CoV-2 infection both in vitro and in vivo and has an advantage over convalescent plasma therapy since it can be produced in much larger quantities to meet clinical demands (Wang et al., 2020a; Wu et al., 2020; Zost et al., 2020).

Several vaccines are currently being developed against SARS-CoV-2—recombinant vectors, DNA vaccines, mRNA in lipid nanoparticles, live-attenuated viruses, and protein subunit-based vaccines (Smith et al., 2020; Zhu et al., 2020b; Gao et al., 2020). Several of these are being tested in phase II trials and some have already advanced to phase III trials (Hu et al., 2020). An adenovirus type 5-vectored vaccine expressing the SARS-CoV-2 S protein produces a considerable humoral and cellular immune response in most recipients after a single immunization and has proven safe (Zhu et al., 2020a). An initial dose of the lipid nanoparticle-formulated mRNA vaccine candidate encoding the pre-fused SARS-CoV-2 S protein (mRNA-1273) induces a robust neutralizing antibody response in a dose-dependent manner, increasing after the second dose (Jackson et al., 2020). A whole virus, inactivated vaccine induces the production of neutralizing antibodies effectively and reported a low rate of adverse reactions (Xia et al., 2020a). These vaccine candidates show promise toward preventing and controlling the spread of SARS-CoV-2, but their safety and immunogenicity will need to be evaluated first with the aim of protecting healthy populations from the SARS-CoV-2 infection.

Until a successful, effective vaccine is produced and distributed, public health measures are the most effective at limiting the spread of SARS-CoV-2 and, to some extent, MERS-CoV. The ability of SARS-CoV-2 to remain viable on inanimate surfaces validates the need for proper sanitation through regular hand washing or the use of hand sanitisers with at least 70% ethanol. Despite some controversy over the efficacy of masks in reducing SARS-CoV-2 transmission, their mere presence acts as a physical barrier that can, in the least, minimize the spread of potentially virus-laden respiratory droplets from one person to another or onto surfaces.

Conclusions

Since the intermediary hosts for the pathogenic hCoVs have not been conclusively identified, there is always a likelihood that these CoVs could pose a problem, even after they are removed from the human population. Also, with no effective, ubiquitous therapeutic available for infections caused by the pathogenic hCoVs, the development of a vaccine remains a priority. Some of the challenges faced when developing a safe and effective CoV vaccine, include (1) host immunity to CoVs often wanes rapidly; (2) individuals requiring protection include the elderly and those with chronic co-morbidities; and (3) vaccines could instead of protect, worsen lung immunopathology (Enjuanes et al., 2008).

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Parvoviridae

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Taxonomy

The family *Parvoviridae* includes a large number of viruses infecting animals, sharing two main characteristics: (i) a linear, single-stranded DNA (ssDNA) genome, and (ii) a capsid formed by an icosahedral protein shell made of 60 protein subunits arranged in $T = 1$ symmetry, without envelope. Both genome and capsid have small dimensions, in the range 3.9–6.0 kb for ssDNA, and 20–28 nm for capsids, justifying the prefix name “Parvo-” (Cotmore et al., 2014).

Within the family, a major distinction is the separation in two subfamilies, the *Parvovirinae* infecting vertebrates, and the *Densovirinae* infecting invertebrate hosts. More recently, a third subfamily, *Hamaparvovirinae*, has been created to accommodate viruses of a possible ancient lineage, found to infect both vertebrates and invertebrate hosts (Penzes et al., 2020).

The criteria to assign a virus to the *Parvoviridae* family are mostly based on genome sequence information, obtained from culture-adapted isolates or directly from biological specimens. Genomes must conform to the general size and organization scheme characteristic of the family. The left side of the genome encodes for at least one large nonstructural (NS) protein, which must possess an SF3 helicase protein domain, while the right side contains the capsid protein (VP) coding region. Subdivision in genera and species is operated on the basis of sequence homology within the NS coding region, with a level of amino acid homology about 35–40% for viruses in the same genus, and >85% for viruses in the same species.

The current scheme approved by ICTV recognizes the three subfamilies, and further ten genera within the *Parvovirinae*, eight within the *Densovirinae*, and five within the *Hamaparvovirinae* (Table 1). Virus species in a same genus should be monophyletic. Species are identified with a binomial denomination, stating the main host taxon, the genus, and a progressive numeral. Within each species, different viruses and viral isolates can maintain their traditional name established in the literature. This classification scheme is regularly updated based on novel findings, brought also by the enormous potential for virus discovery of NGS technology and bioinformatic analysis, and in the future will probably undergo further refinement.

Table 1 Taxonomy of Parvoviridae.

Family	Parvoviridae		
Subfamily	Genus	No. of species	Type species
Parvovirinae	<i>Amdoparvovirus</i>	5	<i>Carnivore amdoparvovirus 1</i>
	<i>Artiparvovirus</i>	1	<i>Chiropteran artiparvovirus 1</i>
	<i>Aveparvovirus</i>	2	<i>Galliform aveparvovirus 1</i>
	<i>Bocaparvovirus</i>	25	<i>Ungulate bocaparvovirus 1</i>
	<i>Copiparvovirus</i>	7	<i>Ungulate copiparvovirus 1</i>
	<i>Dependoparvovirus</i>	10	<i>Adeno-associated dependoparvovirus A</i>
	<i>Erythroparvovirus</i>	7	<i>Primate erythroparvovirus 1</i>
	<i>Loriparvovirus</i>	1	<i>Primate loriparvovirus 1</i>
	<i>Protoparvovirus</i>	13	<i>Rodent protoparvovirus 1</i>
	<i>Tetraparvovirus</i>	6	<i>Primate tetraparvovirus 1</i>
Densovirinae	<i>Aquambidensovirus</i>	2	<i>Decapod aquambidensovirus 1</i>
	<i>Blattambidensovirus</i>	1	<i>Blattodean blattambidensovirus 1</i>
	<i>Hemiambidensovirus</i>	2	<i>Hemipteran hemiambidensovirus 1</i>
	<i>Iteradensovirus</i>	5	<i>Lepidopteran iteradensovirus 1</i>
	<i>Miniambidensovirus</i>	1	<i>Orthopteran miniambidensovirus 1</i>
	<i>Pefuambidensovirus</i>	1	<i>Blattodean pefuambidensovirus 1</i>
	<i>Protoambidensovirus</i>	2	<i>Lepidopteran protoambidensovirus 1</i>
	<i>Scindoambidensovirus</i>	3	<i>Othopteran scindoambidensovirus 1</i>
Hamaparvovirinae	<i>Brevihamaparvovirus</i>	2	<i>Dipteran brevihamaparvovirus 1</i>
	<i>Chaphamaparvovirus</i>	8	<i>Rodent chaphamaparvovirus 1</i>
	<i>Hepanhamaparvovirus</i>	1	<i>Decapod hepanhamaparvovirus 1</i>
	<i>Ichthamaparvovirus</i>	1	<i>Syngnathid ichthamaparvovirus 1</i>
	<i>Penstylhamaparvovirus</i>	1	<i>Decapod penstylhamaparvovirus 1</i>

Taxonomic scheme within the family Parvoviridae, from ICTV [<https://talk.ictvonline.org/>]. In bold the genera or type species containing viruses known to infect humans.

General characteristics

Genome

A key feature of viruses in the family is a linear ssDNA genome, but it should be considered that the ssDNA is the form in which the genome is encapsidated, while it is converted in a double-stranded form (dsDNA) during its replication in cells. Depending on the different species, genomes of either polarity can be encapsidated at about the same frequency, or the negative polarity might be encapsidated at higher frequency. All members in the family present a similar organization of the genome, such that two terminal regions acting as replicative origins flank an internal region containing all reading frames and coding sequences for viral proteins.

The terminal regions can be inverted repeats (ITRs) or different between the two genomic ends, thus 'homotelomeric' or 'heterotelomeric', respectively. In both cases, they are composed of palindromic sequences around a site of dyad symmetry, allowing for fold back into a stem and loop configuration, a so-called 'terminal hairpin'. Mostly, the palindromes are imperfect, and can be present in two different arrangements, one the inverted complement of the other, usually referred to as 'flip-flop'. The hairpin structures are relevant for replication of the viral genome, providing priming for second-strand synthesis, and containing the cis-signals required to drive genome replication by a strand-displacement mechanism, according to a rolling hairpin model.

The internal region (IR) contains all open reading frames and the cis-signals that regulate the expression of the viral genome. Most viruses in the family have unisense genomes, such that ORFs are on a same strand (positive sense by definition), but viruses in the Densovirinae subfamily can show ambisense genomes, with ORFs distributed in the two strands. A common organization of the genomes is the division in two gene cassettes, the one (by convention, left side) coding for the non-structural protein(s) of the virus (NS), and the second (right side) coding for the virion structural proteins (VP). Within this common scheme, viruses differ in the actual number and distribution of coding sequences, and in the strategies adopted for the regulation of genome expression. At the transcriptional and post-transcriptional level, these include differences in the number and position of promoters, cleavage-polyadenylation signals, splicing signals, giving rise to an ample number of viral mRNAs on a genomic template of small dimensions. Further regulation can occur at the translational or post-translational level to expand the viral proteome or determine relative protein abundances.

In all viruses within the family, at least one major non-structural protein is produced (NS1), necessary for replication and regulation of expression of the genome, and a major player in the interaction with cellular proteome. Depending on the virus, a small set of other non-structural proteins can be produced, possessing accessory functions to replication, regulation of expression and interaction with the cellular environment. Concerning the structural VP proteins, at least two different proteins, and up to four, can be produced because of different mRNA processing, translational regulation or post-translational processing. All VP proteins are colinear and share common C-terminal sequences, differing mainly on the extension of the N-termini.

Structure

The capsid structure of many viruses in the family, at least one member per each genus, has been determined to atomic level, by studies exploiting X-ray crystallography and more recently cryo-electron microscopy (Mietzsch et al., 2019). The basic structure is a very simple icosahedral shell made of 60 VP proteins in a $T = 1$ arrangement. In all cases, a major VP protein, which is by norm the shorter form, provides ~90% of the capsid subunits, the remaining being contributed by the other longer forms. The common part of VP proteins provide the basic structure of the capsid shell. Each subunit consists of a core, formed by an eight-stranded antiparallel β -barrel motif, providing the capsid scaffold, while the amino acid insertions between the β -strands form long loops shaping the surface of the capsid. Typical is a cylinder at the 5-fold axis of symmetry, forming a channel to the interior, while the surface topology can alternate spikes, usually around the 3-fold axis, and depressions, around the 5-fold or at the 2-fold axis. Thus, the surface topology of viruses in the family can differ widely, with implications to the interaction with cellular receptors or immune system recognition.

The N-termini of VP proteins, especially those unique to the longer and less abundant forms, are not resolved in their topology because of intrinsic disorder and submolar abundance. These domains are normally buried within the capsid shell, but become exposed by extrusion through the 5-axis channel following interaction with cellular receptors or subsequent internalization steps. The N-termini of the smaller proteins are known to play essential functions in the viral lifecycle. In particular, they have a domain provided with enzymatic activity, a viral PLA2 phospholipase (vPLA2) which is essential for infectivity (Zadori et al., 2001), promoting endosomal escape following internalization.

Replicative cycle

The tropism of parvoviruses can be very selective, and results as a combination of receptor interaction and dependence on a permissive cellular environment (Mantyla et al., 2017; Ros et al., 2017; Qiu et al., 2017). Depending on the virus, a first interaction can occur with a glycan moiety on the surface of target cells, followed by a more specific recognition of a cellular receptor. Following attachment, internalization normally occurs via receptor-mediated endocytosis, in clathrin-coated vesicle or by other mechanisms depending on the virus. Externalization of the N-termini of minor capsid proteins with the enzymatic vPLA2 domain allows endosomal escape followed by nuclear localization. Final uncoating and extrusion of the viral genome occur in the nucleus, with the 3' end of the genome becoming accessible to cellular enzymes. This event may constitute the start of macromolecular synthesis, whose occurrence and outcomes are largely dependent on the interaction with the cellular replicative and transcriptional machinery.

The macromolecular synthesis at first involve the conversion of the incoming ssDNA in a dsDNA replicative intermediate, an event primed by the 3' end of terminal hairpins and operated by cellular enzymatic machinery. The dsDNA thus serves as a template for transcription to generate the set of mRNAs. Depending on the different viruses and genome organization, regulation of expression may depend on the use of different promoters, or on post-transcriptional processing of the pre-mRNA by a combination of regulated events. A biphasic pattern of expression can be recognized, with mRNAs encoding for non-structural and capsid proteins that may be produced in a sequential series of early and later events, the first leading to genome replication and the others to capsid proteins production and assembly.

Replication of the viral genome proceeds through intermediate dsDNA replicative forms (rfDNA). The terminal regions with their hairpin structure are the viral origin of replication, which are relevant for reinitiating DNA synthesis through site-specific recognition, single-strand DNA nicking and strand unwinding operated by the NS1 protein, in the so-called terminal resolution event. Primed by the 3' DNA end produced by the terminal resolution event, DNA synthesis proceeds through a continuous strand-displacement mechanism dubbed as 'rolling hairpin'. Thus, on a double-stranded template, novel single-stranded DNA molecules are produced, that can be converted to rfDNA to amplify the replication process, or finally packaged within preassembled capsid shells, before final maturation and egress of progeny virions from infected cells.

Virus-cell interaction and pathogenesis

Viruses in the family largely depend on functions provided by cellular environment for productive replication. Classically, parvoviruses have been divided as 'autonomous', capable of productive replication mainly exploiting S-phase cellular factors, or 'helper-dependent', typically the AAV, requiring coinfection with a helper virus to complete a productive replicative cycle (Weitzman and Linden, 2011; Meier et al., 2020). This distinction is blurred, since most viruses in the family are capable of modulating the cell cycle to their own advantage. The autonomous parvoviruses may establish long-term non-productive infections with long-term persistence of DNA in tissues, or their replication can be enhanced in normally non-permissive cells in the presence of a coinfecting helper. Dependent viruses on the other hand can undergo autonomous replication if the cellular environment becomes permissive in particular instances. The key characteristic of dependent viruses is their ability to integrate their genome into the host genome and establish true latent infections, which can be rescued by helper viruses with viral genome replication as autonomous genetic units. This property, coupled to advantage of absence of pathological effects, is successfully exploited by AAV-based transduction vectors (Li and Samulski, 2020).

Interaction with the cell environment is complex, and effects of infection, either productive or not, are multiple (Kailasan et al., 2015). Parvoviruses can interfere with the regulation of cell cycle, interact with the cellular DNA damage repair mechanisms, can induce apoptosis and trans-activate the expression of proinflammatory cytokines. Consequences can be a cytolytic effect on cells,

mostly by induction of apoptosis, or through induction of inflammation. Given the selective tropism of parvoviruses and the large dependence on actively replicating cells to achieve a productive infection, most infections are usually restricted to specific cell types or subpopulations, and the pathological effect on the host depend on the type and extent of virus-induced cell death. Association of parvoviruses with neoplastic conditions have been reported, but caution should be exerted in attributing a causal role and standby mechanisms are more likely. Because of the cytolytic effects, parvoviruses are regarded as oncolytic viruses and this property offers the opportunity to exploit these viruses for the development of oncolytic virotherapy (Angelova and Rommelaere, 2019; Bretscher and Marchini, 2019).

Human viruses

Viruses able to infect humans are found in the *Parvovirinae* subfamily, and are distributed in five out of ten genera. The adeno-associated viruses (AAV) in the *Dependoparvovirus* genus are considered non-pathogenic and are developed as safe and effective gene transfer vectors. Others belong to the *Erythroparvovirus*, *Bocaparvovirus*, *Tetraparvovirus* and *Protoparvovirus* genera. Among these, the most relevant human pathogenic virus is Parvovirus B19 (Qiu et al., 2017).

Parvovirus B19

Formally belonging to the species *Primate Erythroparvovirus 1*, Parvovirus B19 (B19V) was discovered in 1975 while screening blood donations against hepatitis B virus (Cossart et al., 1975). The virus was soon associated to a diverse group of clinical manifestations: acute anemic aplastic crises, mainly observed in the course of sickle cell disease (Pattison et al., 1981; Serjeant et al., 1981); chronic anemias in immunodeficient individuals (Kurtzman et al., 1987); the exanthematous fifth disease typical in children (Anderson et al., 1983, 1984); arthralgias observed mainly in adult patients with a tendency to chronicization (White et al., 1985; Reid et al., 1985); fetal hydrops or death when transmitted from pregnant women (Anand et al., 1987). Thereafter, the virus has been continuously implicated in an increasing number of diseases, involving to some extent and causal relevance a wide spectrum of different target organs and pathogenetic mechanisms.

Epidemiology and genotypes

B19V is widely circulating on a global scale, without any relevant geographical restriction. The virus is normally transmitted through the respiratory airway, favored by close family and community contacts. Circulation is seasonal, more prevalent in late spring/early summer, and can show local epidemic cycles every 4–5 years. Seroprevalence studies indicate that in general, about 50% of population can be infected within 25–30 years of age, reaching >90% in elder people (Mosson et al., 2008). Due to the spread of virus in bloodstream, there is a risk, although low, of transmission through blood, blood products or transplantation (Marano et al., 2015). The virus has the property of crossing the placental barrier, thus infection in pregnant women pose a risk of fetal infections with potentially severe consequences.

Three different genotypes are recognized for B19V (Servant et al., 2002; Gallinella et al., 2003a). The genotype 1 is the prototype and the main circulating genotype, prevalent in all geographic areas (Hubschen et al., 2009). Genotype 2 is considered an ancient genotype, mostly found as persistent genomes in tissue of elder people (Norja et al., 2006), or as DNA remnants from archived (Toppinen et al., 2015b) or even archeological specimens (Muhlemann et al., 2018), while is only sporadically found in patients as an actively circulating virus (Eis-Hubinger et al., 2014). Genotype 3 is the lesser circulating variant, more prevalent in geographic areas such as tropical Africa or Southern America, although with an expanding record (Hubschen et al., 2009). On a phylogenetic basis, the three genotypes possibly share a common ancestor, but presently form clearly separate clusters. The intertypic genetic distance is about 10%. Within each genotype, genetic diversity is lower for genotype 1, about 2–3%, and higher for genotypes 2 and 3, 5–8%. Further divisions in sub-genotypes (e.g., 1a, 1b, 3a, 3b) have been proposed, based on genetic distance and construction of phylogenetic trees (Parsyan et al., 2007). However, to our present knowledge, all genotypes share a same biological, immunological and pathogenetic profile (Ekman et al., 2007), and the main interest of reconstructing genome sequences is for molecular epidemiology evaluation.

Genome and structure

The genome is a 5.6 kb linear ssDNA molecule; either polarity is encapsidated at equal frequency (Zhi et al., 2004; Manaresi et al., 2017). The genome is homotelomeric, the terminal regions are constituted by inverted terminal repeats, 383 nts long whose distal 365 can form the hairpin structures. The internal unique region is 4830 nts long, and presents the typical organization with the two gene cassettes, for NS and VP proteins, at the left and right hand side respectively (Fig. 1). The hairpin terminal structures serve as origin of replication (Guan et al., 2009; Manaresi et al., 2017), and include cis-elements recognized by the viral NS1 protein (NSBE) (Tewary et al., 2014) and a binding site for the cellular STAT5 protein (Ganaie et al., 2017), all required for a functional replicative complex. A single promoter is located at the left end of the genome, at map position 6 (P₆), which requires cellular transcription factors such as Sp1 and Sp3 proteins, and is trans-activated by the viral NS1 protein (Raab et al., 2001, 2002). Other cis-elements

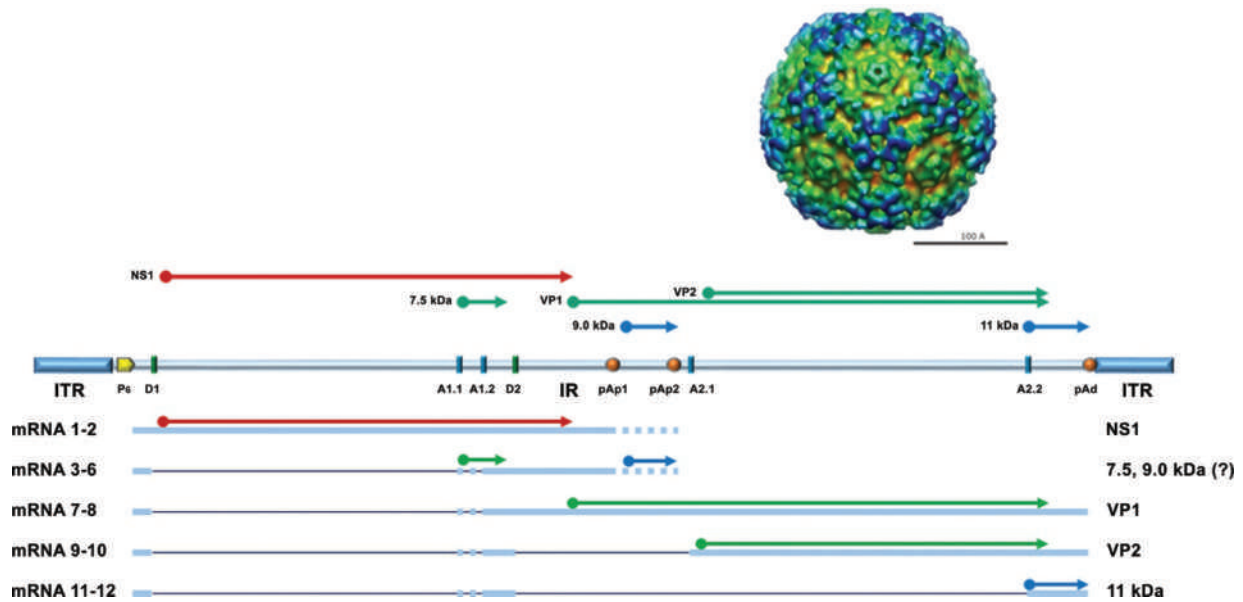


Fig. 1 B19V genome organization. Top: Major open reading frames identified in the positive strand; arrows indicate the coding sequences for the viral proteins. NS, non-structural protein; VP, structural proteins, colinear VP1 and VP2, assembled in a $T = 1$ icosahedral capsid (above); 7.5 kDa, 9.0 kDa, 11 kDa: minor non-structural proteins. Center: Schematic diagram of B19V genome. ITR, inverted terminal regions; IR, internal region; cis-acting functional sites: P₆, promoter; pAp1, pAp2, proximal cleavage-polyadenylation sites; pAd, distal cleavage-polyadenylation site; D1, D2, splice donor sites; A1.1, A1.2, A2.1, A2.2, splice acceptor sites. Bottom: Simplified transcription map of B19V genome, indicating the diverse mRNAs (mRNA 1–12) with introns (thin lines) and alternative splicing/cleavage forms (dashed), and their coding potential. Modified from Manaresi and Gallinella, *Advances in the Development of Antiviral Strategies against Parvovirus B19, Viruses* (2019). Capsid shell image from PDBJ (www.pdbj.org; EMD-1467) © Protein Data Bank Japan (PDBj) licensed under CC-BY-4.0 International.

have been mapped within the internal region, to promote pre-mRNA processing; in particular a pair of cleavage-polyadenylation signal in the middle of the genome (pAp1/2); a cleavage-polyadenylation signal at the distal (pAd) (Yoto et al., 2006); two different splicing donor sites, each with two possible acceptor sites; several regulatory elements such as intronic or exonic splice enhancers (Guan et al., 2011a,b). The combination in the use of these regulatory elements allows that on a genome of small dimensions, up to 12 mature mRNAs can be produced, with the potential to encode for a major NS1 protein, the two capsid proteins VP1 and VP2, and additional minor non-structural proteins, among which an 11 kDa protein has a demonstrated essential functional role (Zhi et al., 2006).

The icosahedral virion is constituted by VP1 and VP2 proteins, assembled in a ratio $\sim 1:10$. The C-terminal region common to both conforms to the general eight-stranded antiparallel β -barrel motif with intervening loops, providing the capsid shell and shaping the shell surface. The general appearance of the virion is rather smooth, prominent 3-fold axis spikes are absent, while smooth depression are present around the 5-fold and 2-fold axis (Kaufmann et al., 2004). The N-terminal portion of the VP1 protein, dubbed as VP1 unique region (VP1u), is possibly an exception to parvoviruses because it does not appear to be contained within the capsid, although it is not freely exposed in the mature virions (Kaufmann et al., 2008). Possibly, the VP1u region is maintained in a buried conformation proximal to the 5-fold axis, becoming fully exposed and accessible upon interaction of the capsid with the docking receptor, the neutral membrane glycolipid globoside (Bonsch et al., 2010). Typically, it has critical functions because of its associated vPLA2 enzymatic activity (Dorsch et al., 2002), and of a specific receptor-binding domain (RBD) at its extreme N-terminus (Leisi et al., 2013, 2016a).

Lifecycle

A striking property of B19V is its selective although not exclusive tropism for cells of the erythroid lineage in the bone marrow, in particular at their intermediate stage of differentiation. This selectivity is due to a double adaptation, the one due to binding of capsids to a cell-specific receptor with restricted distribution, the other linked to the requirement for replication of intracellular erythroid-specific factors, dependent on the erythropoietin pathway activation. Study of the biology of B19V has been favored by availability of primary cultures of erythroid progenitor cells, differentiated from peripheral blood and able to sustain B19V replication (Wong et al., 2008).

A first role in virus-cell interaction is due to globoside, a neutral membrane glycolipid that is also present on mature erythrocytes as antigen of blood group P (Brown et al., 1993). Docking of virions on globoside induces conformational modifications of the capsid shell, thus leading to extrusion of the VP1u region (Bonsch et al., 2010). This is followed by irreversible binding of its N-terminal domain to a specific binding partner on cells, which acts as the specific attachment receptor (Leisi et al., 2013, 2016a,b). The presence of globoside is necessary to achieve a productive infection, and individuals lacking globoside (a blood group antigen

polymorphism) are not susceptible to B19V (Brown et al., 1994), but it is not sufficient, and the internalization step depends on the role exerted by the VP1u region and the RBD (Quattrocchi et al., 2012; Bieri and Ros, 2019; Caliaro et al., 2019).

This process is highly specific and plays a key role in determining viral internalization within a specialized cell type. A wide distribution of globoside may support the capacity of B19V to bind other diverse cell types, but the VP1u receptor is present mainly on erythroid progenitor cells at the intermediate stage of differentiation (Leisi et al., 2015). This restricts infection to a very specific cell subpopulation characterized by a potentially permissive cellular environment apt to support viral replication. Other cell types, included endothelial or connective tissue cells, can be infected by mechanisms different from receptor-mediated endocytosis, mostly resulting non-permissive to viral replication. A relevant phenomenon observed for B19V is the antibody dependent enhancement of infectivity (ADE), that has been shown relevant for the infection of endothelial cells (through C1q receptor) (Von Kietzell et al., 2014), and for B lymphocytes from tonsil tissue (Pyoria et al., 2017).

As a further specific adaptation, the erythroid progenitor cells are permissive to viral replication only when stimulated by the lineage-specific growth factor erythropoietin (Chen et al., 2010a), while hypoxic conditions that are typical of the bone marrow milieu can enhance cell permissiveness to virus (Pillet et al., 2004; Chen et al., 2011). The signal transduction events triggered by Epo receptor activation promote binding of an activator protein, STAT5 in its phosphorylated form, to the viral DNA close to origin of replication, and this binding is necessary to replicate the genome in coordination with the activity of viral NS1 protein. Hypoxia converge on the same transduction pathways enhancing permissiveness to viral replication (Ganaie et al., 2017).

Initial phases of the replicative cycle follow the general scheme common to parvoviruses (Fig. 2). After surface docking and receptor recognition, they involve in sequence internalization by receptor-mediated endocytosis, escape from endosomal

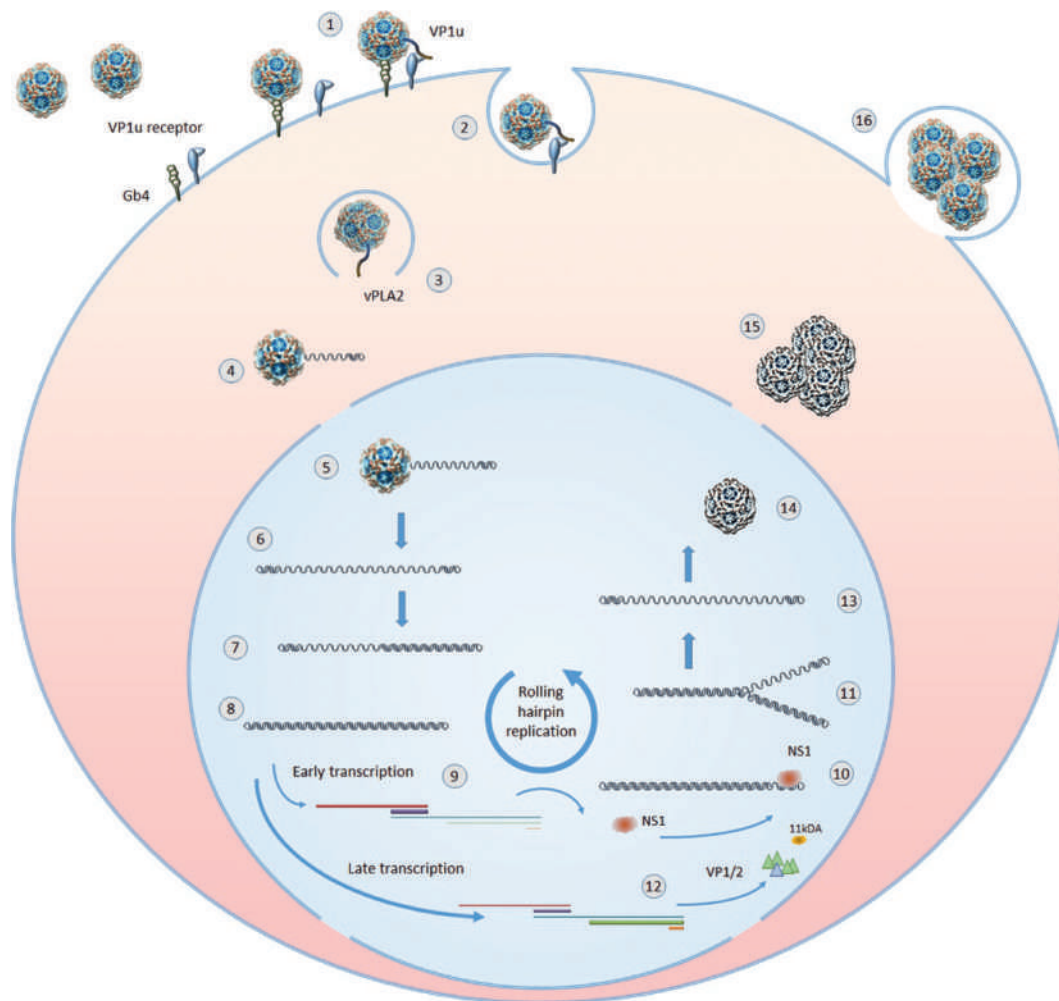


Fig. 2 Outline of B19V replicative cycle in erythroid progenitor cells. 1: Virion binding to globoside, extrusion of VP1u region and binding to an erythroid specific receptor. 2: Clathrin-mediated endocytosis. 3: Virions in endosomal vesicles and vPLA2 mediated escape from endosomes. 4: Partial uncoating and externalization of viral ssDNA. 5: Translocation in the nucleus and complete uncoating. 6: Parental ssDNA and onset of macromolecular syntheses. 7: Hairpin-primed second strand synthesis. 8: Formation of a dsDNA replicative intermediate. 9: Early phase of transcription on the parental template, mainly yielding mRNAs for NS protein. 10: dsDNA terminal resolution by NS and priming of replication. 11: Replication by a rolling hairpin mechanism, via self-primed single-strand displacement mechanisms. 12: Late phase of transcription on the replicative intermediates, mainly yielding mRNAs for VP and 11 kDa proteins. 13: Progeny ssDNA released from the replicative intermediates. 14: Encapsidation of progeny ssDNA molecules in newly formed virions. 15: Accumulation of virions. 16: Release via cell lysis or apoptosis. Modified from Manaresi and Gallinella, *Advances in the Development of Antiviral Strategies against Parvovirus B19*, Viruses (2019).

degradation by vPLA2 activity, transport of nucleocapsids within the nucleus, and final uncoating of the genome in the nuclear environment. The initial phases of the replicative cycle are characterized by a low efficiency, and this can contribute to restriction of viral infectivity (Quattrocchi et al., 2012). Within the nucleus, macromolecular synthesis starts with second-strand synthesis primed by the 3' genomic end extruded from the capsid shell (Caliaro et al., 2019), also a step that can be restrictive to a productive replicative cycle (Gallinella et al., 2000). On the double-stranded template, transcription and replication can occur to achieve a productive replication (Bonvicini et al., 2006a, 2008).

For transcription, a single promoter is present at the left end of the genome, with a basal activity that is enhanced by the same viral NS1 protein in a positive feedback loop. Regulation of expression is obtained by the different usage of the cleavage-polyadenylation and splicing signals in the pre-mRNA, which can yield up to 12 different mature mRNAs, with differentiated coding potential. Expression of the genome is biphasic, with an early phase mainly producing the NS1 protein, followed after onset of genome replication by a late phase mainly producing the structural VP1 and VP2 proteins, and the 11 kDa as well (Bua et al., 2016). The switch in the expression profile is tightly linked to replication of the genome (Guan et al., 2008), which is exerted by the cell machinery redirected by the NS1 protein on the viral origin of replication, located within the terminal regions (Zou et al., 2018). Mechanistic details of replication conform to the rolling hairpin model, with the NS1 protein providing the endonuclease and helicase functions necessary for genome replication, unwinding and packaging into preassembled capsids (Luo and Qiu, 2015; Ganaie and Qiu, 2018).

Effects on cells

The effect of a productive replicative cycle in permissive cells, typically the erythroid progenitor cells, is mainly cytolytic due to the role of NS1 and 11 kDa proteins (Morita et al., 2001; Chen et al., 2010c). These contribute to dysregulation of the cell cycle, inducing a block in G2 phase, which is functional to the accumulation of cell factors needed for replication of the virus (Wan et al., 2010), and to induction of apoptosis by caspase cascade activation (Luo et al., 2013; Xu et al., 2017). A block in G1 induced by B19V has also been reported, due to the activity of NS1 protein and possibly by sensing of the incoming ssDNA and PRR activation (Morita et al., 2003). Intracellular sensing of virus and induction of an antiviral state, or induction of the innate immune response effector mechanism, are likely to occur but poorly investigated for B19V. The NS1 protein has trans-activating activity on several cellular genes including TNF α and IL-6, which may promote inflammation (Moffatt et al., 1996).

The tropism of B19V is not limited to erythroid progenitors. Other cell types can be infected by B19V, including endothelial cells (Duechting et al., 2008; Von Kietzell et al., 2014), connective tissue cells (Ferri et al., 2002; Zakrzewska et al., 2005), and synovia (Ray et al., 2001; Mehraein et al., 2003). In these cells, infection is normally non-productive but the interaction with virus can induce dysregulation of cell physiology and/or trigger inflammatory processes (Duechting et al., 2008; Ray et al., 2001). Uptake of virus in these cells may not follow the same pathway as in erythroid progenitors, and can be favored by immune complex recognition (Von Kietzell et al., 2014). The cell environment is normally non-permissive, and following internalization the viral genome may be degraded, or maintained within cells in a silenced state, or be able to express only early genes without replication. Expression of early genes might not lead to replication of the genome, but the NS1 protein can induce alterations to the cell environment, including apoptosis, inflammation and necrosis, or induction of cell physiology dysfunction. The VP1u region with its associated vPLA2 activity may contribute to the development of an inflammatory process and cell dysfunction. In these cell types, only sporadically the virus has been shown able to complete a productive replication with production of viral capsid proteins (Adamson-Small et al., 2014).

A peculiarity of B19V is its tendency to persistence in tissues following acute infection (Bua and Gallinella, 2017). When sought by molecular methods, viral DNA can be detected although at low levels in 25–50% samples from tissues as disparate as bone marrow, lymphoid organs, liver, kidney, joint and bones, skin, thyroid, testis (Manning et al., 2007). Endothelia and connective tissue cells are very likely a diffuse reservoir for persistence (Norja et al., 2006), and B-lymphocytes in tonsils have been shown to harbor virus (Pyoria et al., 2017). Association is long-term, possibly lifelong, providing a permanent record of the infection and being a marker for molecular epidemiology. Discrimination of a persistent active infection, with possibly a low expression of viral genes and pathological consequences, from the mere presence of silenced DNA is a difficult task, but is crucial to distinguish conditions of clinical relevance. Reactivation of persistent, silenced viral genome has been hypothesized, but systematic evidence is lacking and a general assumption should be avoided.

Infection and pathogenesis

Initial studies on B19V pathogenesis involved experimental infection of volunteers (Anderson et al., 1985; Potter et al., 1987). B19V is normally transmitted through the respiratory airway. Whereas a primary site of replication has not been identified, tonsils can be a possible portal of entry. Then, the virus gains access to the bloodstream and following a primary viremic phase that is clinically unapparent, reaches its primary target organ constituted by the bone marrow. Within the bone marrow, the virus can infect erythroid progenitor cells, achieving a productive infection and exerting cytotoxic effects. The cytolytic effects exerted mainly at the proerythroblast stage of differentiation can induce a block in the erythropoiesis process. In this phase, the bone marrow can show erythroid aplasia and the presence of characteristic giant erythroblasts, which are considered typical of B19V infection (Koduri, 1998). The effects on bone marrow are derived from cell-cycle arrest, block of erythroid differentiation and eventually apoptosis of susceptible and infected cells on one side, and from the dimension and turnover rate of the erythroid compartment on the other.

The fact that the more undifferentiated precursors are resistant to infection ensures that the block in erythropoiesis is temporary, and can be relieved by the development of a neutralizing immune response (Young and Brown, 2004).

A balance is reached between cell population dynamics and viral replication, depending on the dynamics of erythropoietic compartments and the immune response (Brown, 2000; Kerr, 2015). At the peripheral level, reticulocytes can be low or absent, while erythrocytes and hemoglobin level are only marginally decreased because of the long lifespan of mature red cells. Anemia becomes evident and can be severe if there are concurrent defects in the erythropoiesis process, expanding the erythroid compartment or limiting the lifespan of erythrocytes, or immune system reactivity, with delayed viral clearance. Thus, from the hematological side, the possible clinical manifestations depend on the combination of infection and the physiological and immune status of the individuals (Fig. 3).

In a normal individual, with physiological erythropoiesis and normal immune response, infection is limited in extent and temporal frame, usually asymptomatic from the hematological point of view, and is controlled by the development of neutralizing antibodies. The productive infection supported by bone marrow leads to a secondary viremia that in the initial phase, before the development of an effective immune response, can reach exceedingly high viremic levels (10^{12} virus/mL). Clearance of infection, determined by detection of virus in the blood, takes usually 3–4 months with constantly decreasing levels, while sustained persistence of viral DNA in peripheral blood implicates active chronic infection (Musiani et al., 1995; Lindblom et al., 2005; Lefrere et al., 2005). Very low levels of DNA can be detected for years in peripheral blood, with the caveat of discriminating virus from circulating DNA (Molenaar-De Backer et al., 2016).

Infection becomes manifest as acute or chronic pure red-cell aplasia (PRCA) with peripheral anemia when defects in the erythropoiesis process or in the immune response alter the balance between viral replication and cellular turnover (Young and Brown, 2004). In case of stressed and expanded erythropoietic compartment, because of a reduced lifespan of erythrocytes or increased need, such as observed in hemoglobinopathies, typically sickle cell disease, or thalassemia, or other cellular or enzymatic deficits, infection by B19V can lead to an acute episode of profound anemia, that presents as the classical aplastic crisis in patients with underlying hematological disorders. This acute phase is normally relieved by the development of a specific immune response. On the opposite, when the immune system has not the capability to control, neutralize and clear viral infection, infection can become persistent and lead to chronic anemia. This last situation does not require underlying deficits in the erythropoiesis, and can occur in case of congenital or acquired immunodeficiency, in cases of concurrent malignancies, in the course of chemotherapy, or immunosuppressive treatments, such as in bone marrow or solid organ transplant recipients.

The secondary viremia leads to the systemic distribution of the virus and involvement of secondary target organs, by direct or indirect pathogenetic mechanisms. Thus, in this second phase, infection may become clinically apparent with diverse manifestations, some common, some rarer. Among the commonest, B19V is responsible of the erythema infectiosum (fifth disease), typical of children, and arthritis, typical of adults. Involvement of the skin normally takes the clinical feature of an erythematous rash with typical distribution, from cheeks to trunk and limbs, and appearance. Typical in childhood, it can also be present in adults, and its appearance may be more varied with respect to the classical picture and include necrotic or vasculitic lesions. Arthritis is mainly but

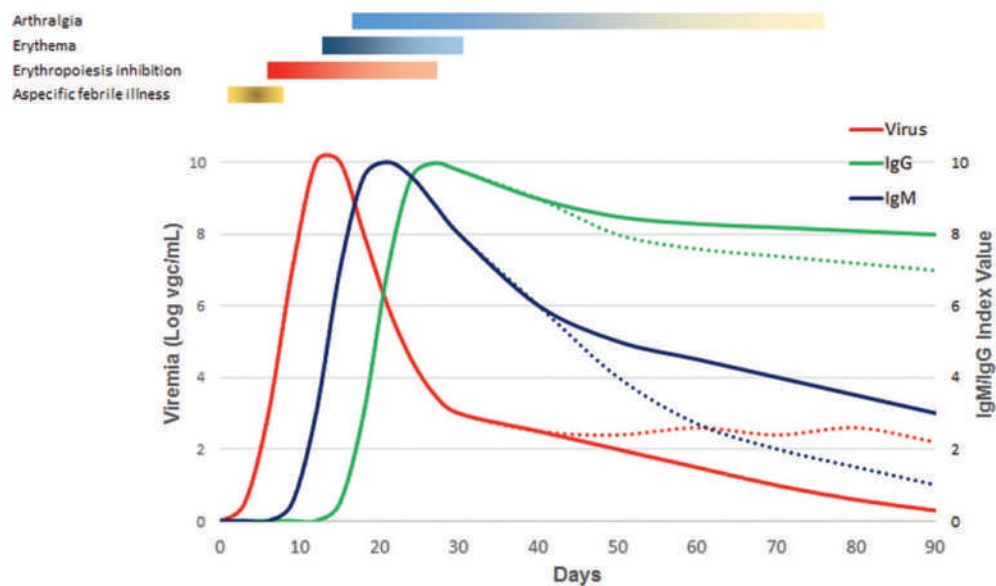


Fig. 3 Course of B19B infection in normal individuals. Viremia is determined by qPCR in peripheral blood, while class-specific antibody response (IgG, IgM) can be determined by EIA and expressed as index value (ratio sample to cut-off). Values are indicative but representative of a normal course of infection in otherwise healthy individuals. Resolution of infection, clearance of viremia, persistence of IgM and IgG have a variable outcome, with a delayed course (dashed lines). In case of ineffective immune response, the infection can assume a chronic course with virus persistence, different levels of detectable antibodies, and possible persistent clinical manifestations.

not exclusively observed in adulthood and is more subject to chronicization. In this case, macrophage-like synovial cells appear to be involved in a local inflammatory infiltrate, with tendency to regress without long-term sequelae.

The range of clinical manifestations associated to B19V infection has been constantly increasing, to involve almost all organs and tissues and including systemic syndromes, and descriptions of clinical presentations have progressively stressed atypical aspects (Table 2). Categories broadly include: (i) hematological disorders other than those involving the erythroid series, rare and in some case severe, often on an immunopathological basis; (ii) organ specific involvement (myocarditis, hepatitis, encephalitis, glomerulonephritis, etc.) on a sporadic basis; (iii) association to systemic disease, possibly by triggering autoimmune mechanisms; (iv) infection in the immunodeficient patient; (v) association to malignancies (e.g., pediatric leukemia/lymphoma, thyroid cancers). The sporadic nature of these associations makes problematic both a definite assessment of a causal relationship, and the individuation of pathogenetic mechanisms, and calls for accurate diagnostic evaluation.

In the systemic phase of infection, endothelial cells may play a relevant role. Endothelia constitute a diffuse tissue that can account for the wide distribution of virus in disparate organs. Endothelial cells can be infected by B19V and, normally non-permissive, can be a site of persistence of the viral genome (Zakrzewska et al., 2005). In the systemic phase, the pathogenic process may depend upon direct cytotoxic or pro-apoptotic effects or via stimulation of an inflammatory response by viral proteins, such as the NS protein or the VP1u vPLA2 (Duechting et al., 2008). The interplay with the immune systems, by its innate and adaptive recognition mechanisms, may lead to the development of immunopathological mechanisms, for example linked to immune complex deposition, or to the onset of autoimmune processes. These may contribute to local effects, for example in the development of glomerulopathies (Chandra and Kopp, 2013), or present as systemic, linked to connective tissue diseases or with features reminiscent of systemic lupus (Lunardi et al., 2008; Kerr, 2016).

In recent years, B19V has been detected at increasing frequencies in endomyocardial tissues. B19V has been directly involved as an etiologic agent in sporadic acute myocarditis both in pediatric and adult populations (Bock et al., 2010; Molina et al., 2013). Active B19V infection has been shown in myocardial endothelial cells, so B19V probably acts by inducing endothelial dysfunction, which in turn triggers inflammatory responses in the cardiac tissue (Kuhl et al., 2003; Bultmann et al., 2003). The definition of a possible role of B19V in the development of chronic cardiomyopathies and cardiac dysfunction is instead matter of ongoing debate (Verdonshot et al., 2016; Rigopoulos et al., 2019). The frequency of B19V DNA detected in cardiac bioptic samples is constantly

Table 2 Clinical manifestations of parvovirus B19 infection.

Category	Frequent	Sporadic
Hematological	Transient anemia	Bone marrow necrosis and fat embolism
	Aplastic crisis	Myelodysplastic syndrome
	Chronic anemia	Thrombocytopenia
	Chronic pure red cell aplasia	Granulocytopenia
Specific tissue/organ disease		Pancytopenia
		Idiopathic Thrombocytopenic purpura
		Hemophagocytic lymphohistiocytosis
		Petechial purpura
	Erythema infectiosum	Henoch-Schonlein purpura
	Mono or poly-arthritis	Papular-purpuric glove-and-socks syndrome
	Chronic arthralgias	Acute myocarditis/pericarditis
		Chronic inflammatory myocarditis
		Myositis
		Hepatitis
		Glomerulonephritis
		Meningitis
		Encephalitis
		Peripheral neuropathy
Systemic	Aspecific febrile illness	Thyroiditis and thyroid neoplasia (?)
		Chronic fatigue syndrome
		Vasculitis
		Scleroderma
Infection in pregnancy	Intrauterine infection (30–50%)	Lupus erythematosus
		Mirror syndrome
		Meconium peritonitis
		Fetal malformations
		Congenital anemia
		Neurodevelopmental delay (?)

A compilation of diseases and clinical manifestations associated to parvovirus infection. Frequent clinical manifestations are typical of B19 infection, although the presentation and course of disease can vary depending on the physiological and immune status of the individuals. Sporadic clinical manifestation include diseases that have been more rarely associated to B19 infection, occurring at a low frequency and without any predictable concurrent factor. The list of sporadic clinical associations is not exhaustive. (?) denotes uncertain association.

high, and a marked cardiotropism should be mentioned as one of the main characteristics of B19V. A positive association and a role of B19V in the development of inflammatory chronic cardiomyopathies has been proposed because of significant higher mean viral loads or the presence of viral mRNAs, suggesting active viral replication and expression.

The role of B19V in transplantation has been investigated for solid organ and bone marrow transplant recipients (Eid et al., 2019). In solid organ, a higher relevance has been found for kidney transplants (Ki et al., 2005; Barzon et al., 2009) while for bone marrow it is a sporadic but clinically relevant event (Broliden, 2001; Plentz et al., 2004). Other immunodeficient patients at higher risk are pediatric leukemia patients (Kerr and Matthey, 2015), where B19V infection affects to a significant level on the clinical course and response to treatments (Lindblom et al., 2008). The possibility that chronic B19V infections may act as a trigger in preneoplastic situations has been hypothesized but needs sounder evidence (Vasconcelos et al., 2011). A possible link to oncogenic processes has also been proposed for thyroid malignancies (Hobbs and Adamson-Small, 2015).

A relevant property of B19V is its ability to cross the placental barrier and infect the fetus. Placental trophoblasts are not permissive to the virus but may consent access of virus to the fetal circulation (Jordan and Deloia, 1999; Jordan and Butchko, 2002; Wegner and Jordan, 2004). Endothelial placental cells can be productively infected (Pasquinelli et al., 2009), facilitating the establishment of fetal infection and contributing to placental damage. In the fetus, the virus can infect erythroid progenitor cells, in liver and/or bone marrow depending on the gestational age, and exert direct cytolytic effects (Morey et al., 1992a). The ensuing block in fetal erythropoiesis can be severe, because of the physiologically expanded erythropoietic compartment combined to an immature immune response, and lead to profound fetal anemia, fetal hydrops and/or fetal death. The immune status of the mother plays a protective role, and intrauterine infections are confined to primary infections in non-immune pregnant women. The rate of transmission, 30–50%, will depend on the gestational age, being higher in the first two trimesters, while the effect on the fetus, ~10% severe effects, will depend on its developmental stage, the rate of expansion of its erythroid compartment and maturity of fetal immune response. Infections occurring at earlier stages carry a higher risk of leading to fetal deaths, while the development of hydrops is more frequent in the central part of pregnancy. Hydrops may lead to fetal death, or the fetus may more frequently recover without persistent damage. In the third trimester, the overall risk of fetal damage decreases so that fetal death is a rare event (Bascietto et al., 2018; Xiong et al., 2019; Bonvicini et al., 2017b). The virus is not teratogen, and only sporadic B19V-related fetal complications are described in documented intrauterine infections. Long-term neurological effects in congenitally infected children are reported and attributed to the grade of fetal anemia rather than to a direct effect of virus (Lindenburg et al., 2013).

Immune recognition

The pattern of innate immune recognition is not known to detail, although general symptoms related to cytokine production are typical of a prodromal phase of infection (Isa et al., 2007; Wu et al., 2016). In case of primary infection, IgM are produced first and are detectable starting a few days after the viremic peak, then decreasing and lasting for about 3–6 months. IgG follow soon and are generally detectable lifelong. Due to a slow viral clearance, viral DNA can still be detected for months or longer periods even in the presence of specific IgG, as it can happen in the case of chronic infections with production of non-neutralizing antibodies (Young and Brown, 2004).

Antibody reactivity is directed mainly against the viral capsid shell and can be neutralizing. Epitope mapping on B19V virions is incomplete. The capsid shell is known to present conformational epitopes, and an atomic structure of Fab binding to capsid has been resolved (Sun et al., 2019). Additional linear epitopes have been mapped, many on the VP1–2 common region (Sato et al., 1991; Yoshimoto et al., 1991), others and most relevant for neutralization of infectivity being located on the VP1u region (Saikawa et al., 1993; Anderson et al., 1995), and especially in the extreme N-terminus, coincident with the RBD (Zuffi et al., 2001).

IgM mainly recognize conformational epitopes in the VP1–2 common region (Manaresi et al., 2001). IgG also recognize conformational epitopes in the common region, and in addition they can be directed against linear epitopes mainly in the VP1u region (Manaresi et al., 1999; Musiani et al., 2000). A maturation of the immune response includes recognition of an acute-phase epitope on VP common region (Kaikkonen et al., 1999, 2001), then an expansion of the repertoire by progressive selection of high-avidity clones producing antibodies recognizing VP1–2 conformational epitopes and VP1u linear epitopes (Soderlund et al., 1995a,b). Antibodies against NS1 protein can also be detected in about 30–40% of seropositive individuals, without any significant association to duration, severity or presentation of disease (Venturoli et al., 1998; Heegaard et al., 2002). Cell-mediated immunity is less characterized. B cell reactivity (Tolfvenstam et al., 2000), T cell CD4 and CD8 reactive clones can be detected (Tolfvenstam et al., 2001; Isa et al., 2005), but very few antigenic determinants have been identified so far (Kasprowicz et al., 2006a,b).

Immune deficits favor persistence of active, chronic infections (Isa et al., 2006). Deficits range from the inability to produce detectable antibodies, to the production of a transient immature response, with low-level IgM without the switch to IgG, to the production of IgG that are inefficient in neutralizing the virus, as unable to recognize mature phase neutralizing epitopes. Except in cases of profound deficits, a chronic infection usually evolves and lastly an immune response is mounted, able to control infection, although the course of events can be prolonged and unpredictable.

Diagnosis

A laboratory diagnosis is required to confirm or exclude a clinical suspect, can be included as part of larger diagnostic panel, or be carried out for screening purposes (Gallinella, 2018). The mainstay is detection of a specific antibody response in serum/plasma from peripheral blood, with coupled detection of IgM and IgG. Automated or semi-automated methods make use of EIA/CLIA

assays, using as antigens the capsid proteins, produced as recombinant proteins in heterologous expression systems. VP proteins are able to self-assemble in viral-like particles (VLPs), composed of VP2 only or VP2 + VP1 antigens (Brown et al., 1990; Kajigaya et al., 1991); or VP2 plus VP1u segment produced as isolated fragment (Soderlund et al., 1992). Depending on the assay used a diverse set of epitopes are recognized, but use of VP2 only can be sufficient to obtain correct discrimination of reactive samples (Manaresi et al., 2004). Line blot assays are also available to better dissect the antibody repertoire against a combination of conformational and linear epitopes, also including the NS1 protein (Kerr et al., 1999). More recently, singleplex and multiplex microsphere-based Suspension Immune Assays (SIAs) are being developed (Wang et al., 2016). An intrinsic limit of immunological diagnosis is due to the evenience of an acute phase infection before the development of antibodies (virus positive, antibodies negative), or of a chronic infection with or without presence of IgG (virus positive, antibodies either positive or negative). The presence of IgM is suggestive of acute infection, but false reactivity linked to polyclonal activation or technical issues should be considered. The presence of IgG without IgM is normally considered as a sign of past infection and immunity, but chronic infections can go undetected if virus is not sought.

The use of molecular assays to detect viral DNA is the necessary complement to immunological diagnosis to discriminate active infections in case of relevant clinical suspect, and if the subject has known immune deficiencies (Gallinella et al., 2003b). Molecular detection should also be recommended for screening, for example in pregnancy, or as pre- or post-transplantation protocols. The presence of viral DNA in peripheral blood is a marker of active infection, but due to the long-term persistence and slow clearance following the initial phase of infection, a quantitative assay is necessary to determine viral load as an essential parameter in evaluation of the clinical course. Standardized qPCR assays have been developed to this purpose, with the additional requirement of equal detection of all genotypes (Gallinella et al., 2004; Bonvicini et al., 2013; Toppinen et al., 2015a). Detection of virus in tissues, including bone marrow or fetal samples, can be carried out by qPCR, but in situ hybridization and/or immunocytochemistry may better serve as assays discriminating active infections from mere persistence (Morey et al., 1992b; Bonvicini et al., 2006b).

Prevention and treatment

To prevent iatrogenic transmission by blood or blood products, screening by molecular assay has been introduced, in particular as a voluntary standard adopted by manufacturing companies (Marano et al., 2015). A threshold of 10^4 IU/mL (IU are NIBSC International Units (Baylis et al., 2012)) has been proposed for limit of acceptable contamination without risk of transmission to susceptible individuals. For protection in the general population, or at least at-risk subjects, a vaccine would be useful. In fact, a vaccine against B19V is technically feasible by use of VLPs, but after a long history it is still under development and not in any priority line for introduction on a general basis (Bansal et al., 1993; Ballou et al., 2003; Bernstein et al., 2011; Chandramouli et al., 2013; Penkert et al., 2017).

Concerning treatment, the infection in most case provokes mild and self-limiting diseases, but in serious cases, supportive or symptomatic treatment is required. Transfusions are necessary to support anemic patients in acute aplastic crisis or in case of chronic infections. Intrauterine transfusions can be carried out to improve favorable outcome of infected and anemic fetuses, justifying antenatal screening and non-invasive follow-up for B19V in pregnancies (Oepkes and Adama Van Scheltema, 2007; Lindenburg et al., 2014). The only treatment currently used for B19V are IVIG, which normally contain high levels of antibodies (Onodera et al., 2018) and can be used for replacement treatment in case of immune deficiencies and chronic infections (Crabol et al., 2013). Anti-inflammatory drugs are used in cases of arthralgias, although relief may not be effective. None of these treatment options is specific to B19V, and resolution of infection will eventually depend on the ability of the immune system to control and clear infection.

Development of specific antiviral strategies should be considered a major topic in research on B19V (Manaresi and Gallinella, 2019). Antiviral compounds that showed efficacy in *in vitro* assays include hydroxyurea and Cidofovir/Brincidofovir. Hydroxyurea is an antiproliferative drug used in the treatment of sickle-cell disease that also showed inhibitory activity against B19V (Bonvicini et al., 2017a). Its use in SCD pediatric patients correlates with milder clinical presentation of B19-induced aplastic crisis (Hankins et al., 2016), but whether this effect might be due to the antiviral activity is not determined. The nucleotide analogs Cidofovir and its lipid conjugate Brincidofovir are broad-range antivirals active against dsDNA viruses, which showed an antiviral activity also against B19V (Bonvicini et al., 2015, 2016; Bua et al., 2019). Unfortunately, their use is limited by severe toxic side effects, and systematic clinical evaluation of their efficacy against B19 has not been carried out yet. Continuing effort in the field is required to find specific, effective and safe treatments for B19V infection.

Human Bocaviruses

Bocaviruses are a large group of viruses found in several vertebrate hosts. Human Bocavirus was first discovered in 2005 (Allander et al., 2005) by unbiased direct molecular investigation in respiratory secretions. Identification of the first species, HBoV1, was then followed by HBoV2–4 (Kapoor et al., 2010) found in feces. Bocaviruses are able to infect the respiratory or intestinal mucosa, with a limited propensity to systemic spread and tissue persistence. They are recognized human pathogens, causing respiratory and gastrointestinal infections, although their relevance as such is blurred by their wide distribution, mainly subclinical course or association to mild diseases, and by the presence of other viruses coinfecting a same tissue in more severe cases.

Epidemiology

There are four Human Bocaviruses species, HBoV1–4, which are similar but not identical in biology and capacity to cause diseases (Jartti et al., 2012). HBoV1 is mainly a respiratory virus, while HBoV2–4 are mainly found as associated to gastrointestinal infections. Circulation is widespread and, by seroprevalence, most infections occur in the first years of life. HBoV1 is the more diffuse species in the population, transmitted through the respiratory route and reaching a prevalence of 80% by 6 years of age. HBoV2–4 are not found in respiratory secretions, but can be found in stool samples, indicating a fecal-oral transmission. Seroprevalence is about 50% at the age of six for HBoV2, 20% for HBoV3 and very low for HBoV4. Molecular epidemiology suggests a wide circulation of viruses but fails to indicate any association with different biological or clinical features.

Genome and structure

All Bocavirus species share a similar genetic organization and structural features, but most studies have been carried out for HBoV1 (Huang et al., 2012). The genome is ~5.5 kb linear ssDNA molecule; 95% of encapsidated genomes are of negative sense polarity (Fig. 4). The terminal regions are heterotelomeric, constituted by an imperfect palindromic hairpin of 140 nt at the left-end and a perfect palindromic hairpin of 190 nt at the right-end, both being required for replication of the genome. The internal unique region is ~5200 nts long, encoding on the left hand side a group of non-structural proteins, NS1–4, a viral nucleoprotein, NP1, in the middle of the genome, and the colinear viral capsid proteins, VP1–3, on the right hand. A single promoter is located at the left end of the genome, at map position 5 (P_5). *cis*-elements mapped within the internal region to promote pre-mRNA processing include two cleavage-polyadenylation signal in the middle (pAp) and at the distal end of the genome (pAd), and four different splicing donor/acceptor sites. The three VP proteins originate from a same mRNA by different regulation of translation initiation. The icosahedral virion is constituted by VP1, VP2 and VP3 in a ratio ~1:1:10; as for other viruses in the family, the common region constitutes the shell, while the N-terminal VP1 unique region (VP1u) includes a domain with vPLA2 enzymatic activity.

Lifecycle

Bocaviruses infect terminally differentiated epithelial cells in the airway (HBoV1) and intestinal (HBoV2–4) mucosae. The replicative cycle has been studied in detail in polarized airway epithelial cell cultures for HBoV1 (Deng et al., 2013), which can

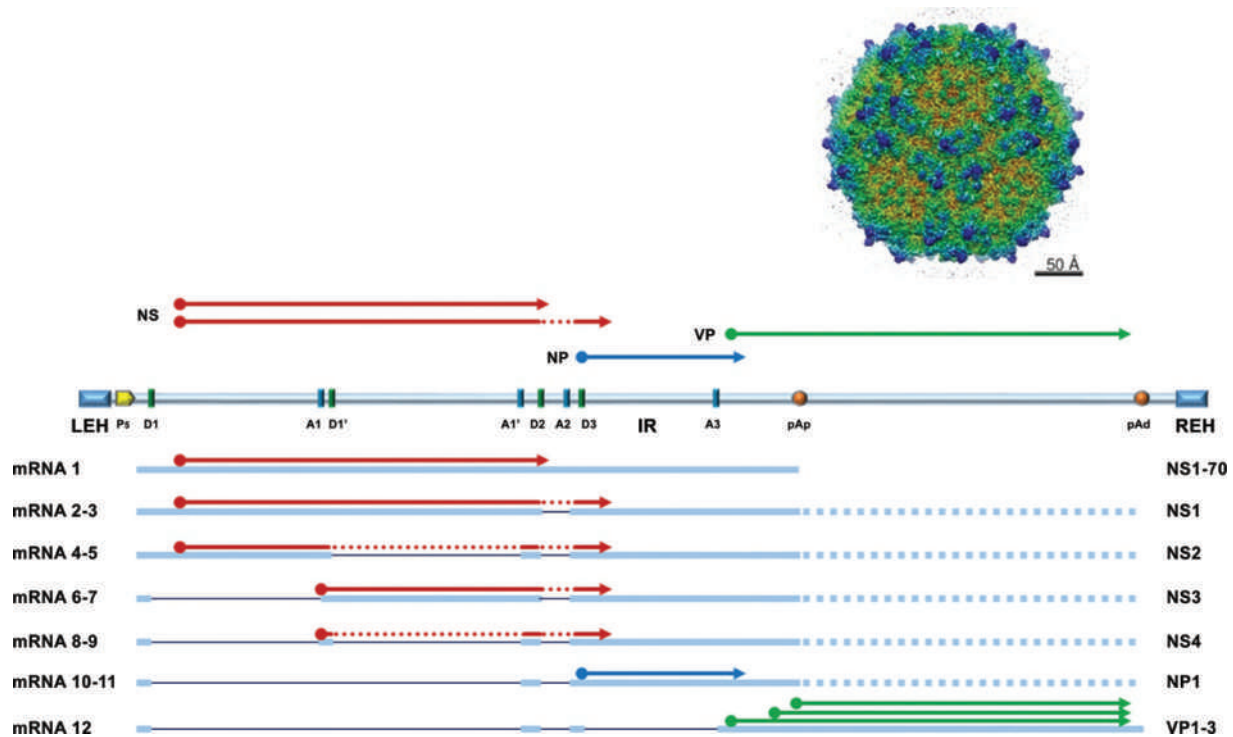


Fig. 4 HBoV1 genome organization. Top: Major open reading frames identified in the positive strand; arrows indicate the coding sequences for the viral proteins. NS, non-structural protein; NP, nucleoprotein; VP, structural proteins, colinear VP1–3, assembled in a $T = 1$ icosahedral capsid (above). Center: Schematic diagram of HBoV1 genome. LEH, left-end hairpin; IR, internal region; REH, right-end hairpin; *cis*-acting functional sites: P_5 , promoter; pAp, proximal cleavage-polyadenylation site; pAd, distal cleavage-polyadenylation site; D1–3, splice donor sites; 1–3, splice acceptor sites. Bottom: Simplified transcription map of HBoV1 genome, indicating the diverse mRNAs (mRNA 1–12) with introns (thin lines) and alternative cleavage forms (dashed), and their coding potential. Capsid shell image from PDBJ (www.pdbj.org; EMD-8598) © Protein Data Bank Japan (PDBJ) licensed under CC-BY-4.0 International.

be infected and release virus for long periods, establishing a model of persistent productive infection that may correlate with what happens in vivo. In these cell cultures, the virus can infect cells by the apical and basolateral surfaces, while it is released mainly by the apical surface.

The virus does not require actively dividing cells to achieve a productive replication. In fact, replication is cell cycle independent and the virus recruits the cellular DNA damage repair machinery to replicate the genome, including cellular DNA repair DNA polymerases, in viral DNA replication centers (Chen et al., 2010b; Luo et al., 2011; Deng et al., 2017). For replication of the genome, both a rolling hairpin and a rolling circle mechanism have been proposed (Schildgen et al., 2012). Transcription of the viral genome occurs starting from a single promoter, and by use of different cleavage-polyadenylation and splicing signals, gives rise to at least 12 mature mRNAs translated in non-structural proteins NS1–4, the nucleoprotein NP1 and the three viral capsid proteins VP1–3 (Zou et al., 2019).

Pathogenesis and infection

In cell cultures, HBoV1 infection induces damage of the epithelial layer and airway tract injury, including disruption of the tight-junction barrier, loss of cilia, and epithelial cell hypertrophy. Infection is at last cytolytic, but airway epithelium is regenerated by basal stem cells, which can maintain integrity and sustain prolonged virus release. Infection also upregulates release of cytokines, which may contribute to disease.

As human pathogenic viruses, a distinction can be made between HBoV1, which is mainly a respiratory virus, and HBoV2–4, which are mainly gastrointestinal viruses (Moesker et al., 2015; Christensen et al., 2019). HBoV1 can be detected in nasopharyngeal secretions by molecular detection techniques, in a large proportions of cases of upper and lower respiratory tract infections, from 2% to 20%, thus indicating a pathogenic potential. However, in a large part of cases, up to 80%, HBoV1 is not the only virus detected, being found in association to one or more other potential pathogenic agents. A high viral load or other marker of active virus replication is suggestive of a more direct role of HBoV1, but its tendency to give long-term persistent infections explains its frequent detection and questions its pathogenetic relevance (Martin et al., 2015). Thus, HBoV1 may cause upper or lower respiratory tract infections, which may have different degrees of clinical relevance, while in its detection in association with other viruses may be due to a standby phenomenon, favored by a same route of transmission and the tendency of HBoV to persist for prolonged times in infected tissues. Alternatively, a coinfection may be more clinically relevant because of concurrent pathogenetic activity. Similarly, HBoV2–4 have a wide circulation but their pathogenic potential is even more unclear (Paloniemi et al., 2014). They have been associated to pediatric gastroenteritis, but also in this case they are often been detected with other viral pathogens, especially rotavirus and norovirus. Infection has mild clinical consequences, if any, and coinfection does not exacerbate clinical conditions. As it happens for Parvovirus B19, also for HBoV there are increasing reports on atypical clinical presentations or involvement in other diseases that require further confirmatory studies.

Immunity

The capsid shell constitutes the main target for a specific immune response, with production of antibodies with neutralizing activity (Kantola et al., 2011). The four different viruses share a structure similarity of about 80–90% in the major VP3 protein. The presence of a common antigenic repertoire, explains for an extended immune cross-reactivity, which can be a confounding factor when using serology as a diagnostic tool. Moreover, the phenomenon of ‘original antigenic sin’ seems to take place, as an anamnestic cross-reactive activation upon infection with different HBoV types (Li et al., 2015). A strong cellular immunity is induced, and a CD4 specific response has been characterized for HBoV1 (Lindner et al., 2008), with a sustained release of cytokines in blood and secretions (Chung et al., 2008; Hirose et al., 2014).

Diagnosis

For a diagnosis of clinical relevance, a combination of molecular and antibody detection is advisable. Detection of viral DNA by qPCR is the method of choice for detection of virus in nasopharyngeal secretions or stools, and assays able to distinguish and quantitate the different HBoV types is required (Kantola et al., 2010). A high viral load can be suggestive of acute infection and causal association with clinical manifestations, while a lower load can be more indicative of a delayed viral clearance. Detection of specific antibodies is possible by using VLPs as antigens in EIA/CLIA assays (Kantola et al., 2008). In addition to prevalence studies, utility of antibody detection is important. Seroconversion, increase in IgG titer or IgM detection is strongly suggestive of a recent infection, and can be a valid support to discriminate clinically relevant episodes (Hedman et al., 2010). The cross-reactivity between HBoV species require a cautious approach to interpreting results and possibly the introduction of technical steps to discriminate a species-specific response (Kantola et al., 2011; Li et al., 2015).

Treatment

No specific treatment is available, and its need can be questionable as long as a clear pathogenetic role is established.

Human Tetraparvovirus

The so-called Human Parvovirus 4, now *Primate Tetraparvovirus 1*, was discovered in 2005 in the serum of a HBV infected person with acute febrile disease. Now, three different genotypes are known (Matthews et al., 2017). The virus has not been adapted to cell culture and information on its genome sequence is incomplete, so knowledge about its biology and lifecycle is limited. The internal coding region is known for its full length, so its transcription map and coding potential, which include at least two non-structural proteins (NS1–2) and two capsid proteins (VP1–2) forming the capsid shell.

The virus, with its three genotypes, can sporadically be detected in blood, but the clinical relevance is unclear. Its frequent association with viruses such as HIV, HBV, HCV, and in subjects such as injected drug users, suggest a parenteral route of transmission. In most cases, infection as determined by high viremic loads appears asymptomatic, and does not alter the clinical course of underlying infections. Symptomatic infections manifest as a febrile illness are rare and mainly linked to immunodeficient states. Diagnostic is rarely performed and it relies on quantitative molecular detection techniques, while detection of specific antibodies by EIA assays is possible by the availability of recombinant VLP. Seroprevalence studies indicate a diverse prevalence in the general population, not exceeding 40% in sub-Saharan Africa and close to zero in northern countries.

Human Protoparvoviruses

More recently, novel parvoviruses have been found in human stool samples (Vaisanen et al., 2017). Such viruses have not been isolated, but relevant genome sequence information recovered from clinical specimens allowed the definition of new viral species and their inclusion in the Protoparvovirus genus. Among these viruses, the Human Bufavirus (BuV), with three different genotypes, has been detected more frequently and an association with gastroenteritis has been proposed. The Human Cutavirus (CuV), also detected in stool samples, has been detected in skin tissue, and appears highly associated to cutaneous T-cell lymphoma. Seroprevalence studies indicate a wide circulation of BuV with a differential geographical distribution, higher in African and middle/central-east countries, lower for European countries and United States, while circulation of CuV is more limited. Discovery of these new viruses poses interesting challenges to the characterization of biology and definition of clinical relevance (Soderlund-Venermo, 2019).

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Arenaviruses

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Introduction

The *Arenaviridae* family was established in 1976 predominantly harboring rodent-associated viruses, made up of the single genus *Arenavirus* with species distributed in different regions around the world. After the discovery of different arenaviruses in *Alethinophidian* snakes, the family taxonomy was substantially modified and amended including the reptilian viruses in the genus *Reptarenavirus* and renaming the original *Arenavirus* genus as *Mammarenavirus* (Radoshitzky et al., 2015). A new genus *Hartmanivirus* was later added which owes its name to the Haartman Institute snake virus (HISV) isolated from a boa constrictor in captivity. Finally, in 2018 the last genus *Anntenavirus* was included, made up of the Wénling frogfish arenaviruses 1 and 2 (WIFAV-1/2), found in toad fish, and at the International Committee on Taxonomy of Viruses Report Consortium 2019 the taxonomy was restructured including the family in the Bunyavirales order (Abudurexiti et al., 2019).

The family includes more than 40 species, some reptarenaviruses cause boid inclusion body disease in captive snakes (Hetzel et al., 2013), whereas some mammarenaviruses can infect humans and other primates, causing severe and sometimes fatal diseases. Mammarenaviruses have been divided into two groups based on antigenic properties and geographical location: Old World (OW) or Lassa-lymphocytic choriomeningitis serocomplex include viruses indigenous to Africa, Asia and the worldwide ubiquitous lymphocytic choriomeningitis virus (LCMV), whereas New World (NW) or Tacaribe serocomplex include viruses from the Americas (Wulff et al., 1978; Charrel et al., 2008). NW arenaviruses are further subdivided into four clades named A, B, C, and A/Rec (Clade D), on basis of the nucleoprotein (NP) sequences. Clade A/Rec (Clade D) is hypothesized to be a recombination of clades A and B that includes North American viruses such as Whitewater Arroyo virus (WWAV), Bear Canyon virus (BCNV) and Tamiami virus (TAMV) (Radoshitzky et al., 2015).

Virions are spherical or pleomorphic particles, ranging from 50–300 nm in diameter, composed of helical nucleocapsids within a dense lipid envelope. A variable number of cellular ribosomes, which are not required for virus multiplication, are also found inside the particle and the family name originated from this peculiar granular structure (arenosus, from the Latin arena) observed in electron-microscopic thin sections (Rowe et al., 1970). *Arenaviridae* was traditionally thought to be a family of bi-segmented RNA viruses with two single-stranded RNA molecules called L (large, 7.1 kb average) and S (small, 3.4 kb average), both with an ambisense coding strategy. This is true for mammarenaviruses, but there is genome diversity in other genera, such as the antennarenavirus genome comprising three segments termed small, medium and large (Shi et al., 2018). Each RNA fragment is closely associated to the L protein, the viral RNA dependent RNA polymerase, and covered by the NP, forming the nucleocapsid or ribonucleoprotein (RNP) in the interior of the virion. The external envelope has a lipid membrane where the viral spikes or glycoprotein (GP) complex are inserted forming tetramers, composed of three proteins that arise from proteolytic cleavage of the

precursor GPC by the cellular subtilase S1P/SKI: the surface exposed GP1 protein, the transmembrane GP2 protein and the stable signal peptide SSP, that is unusually retained in the mature GP complex. Immediately below the membrane and closely associated with it, it is located the matrix protein Z, a small protein with an important RING-finger motif able to bind zinc that is highly conserved. The structural proteins in fish and reptile arenaviruses may exhibit some differences in comparison to the structure of mammarenaviruses above described, particularly about the presence in the virion of Z or SSP.

Given their importance as human pathogens, the focus of this article is on OW and NW mammarenaviruses, the more characterized members of the family.

Pathogenic arenaviruses

Epidemiology

The *Mammarenavirus* genus contains the highest number of viruses that cause hemorrhagic fever (HF) in humans, most of them located in Clade B of the NW group. However, the most important pathogen is Lassa virus (LASV), a member of the OW that causes approximately 100,000–300,000 annual infections and about 5000 deaths, primarily in West Africa (McCormick et al., 1987). Infections with arenaviruses in South America are seasonal and significantly lower, with 100–1000 cases reported per year but with higher mortality rates of 15–35%, in the absence of treatment. Junin virus (JUNV) is the most recognizable pathogen among this NW group and is the causative agent of Argentine HF (AHF). Machupo (MACV), Guanarito (GTOV), and Sabiá (SBAV) viruses are the causative agents of Bolivian HF, Venezuelan HF, and Brazilian HF, respectively. Other arenaviruses pathogenic to humans have emerged more recently. These include Chapare virus (CHAPV) in Bolivia, and Lujo virus (LUJV) in Southern Africa (Delgado et al., 2008; Paweska et al., 2009). Additionally, another NW arenavirus, WWAV, was implicated in human disease in North America (Fulhorst et al., 1996).

With more than 40 members, the weight of the genus *Mammarenavirus* accounts by including many important, deadly-human pathogens. Some of them such as LASV, JUNV and MACV have been reported to recurrently produce severe disease whereas others have appeared with rare (CHAPV), small clustered (LUJV, WWA) or unique occurrence (SBAV). Here we will focus on those that pose biggest threat to human populations either by the severity of the disease or the size of the population affected. Fig. 1 geographically locates OW and NW arenaviruses, highlighting the pathogenic members and its animal reservoir.

OW human pathogens

Lassa fever (LF), caused by LASV, is mainly endemic to the West African countries of Guinea, Liberia, Nigeria, and Sierra Leone (Owolabi et al., 2016). About 80% of people who become infected with LASV are asymptomatic or have mild symptoms. This is supported by the moderate to high seroprevalence of LASV specific antibodies in endemic regions, along with an annual rate of up to 20% for LASV specific seroconversion within nonimmune populations (McCormick et al., 1987; Lukashevich et al., 1993; Tomori et al., 1988). Therefore, newer estimates claim LASV more likely affects 2–3 million people annually (Richmond and Baglole, 2003).

LF can affect all age groups and both genders (Akpede et al., 2018). From those who develop severe disease and get hospitalized fatality rate raises up to 10%. Pregnant women, children under 5 years of age, and individuals infected with human immunodeficiency virus or other immunosuppressive conditions have an increased risk of death. Pediatric LF is known to occur more commonly in male children for as yet unknown reasons. The mortality rate can be as high as 87% among women infected with LASV during the third trimester of pregnancy (Price et al., 1988) and vertical transmission has proved to occur. Also, prolonged detectable Lassa viral RNA in blood and semen suggests the possibility of sexual transmission (Raabe et al., 2017).

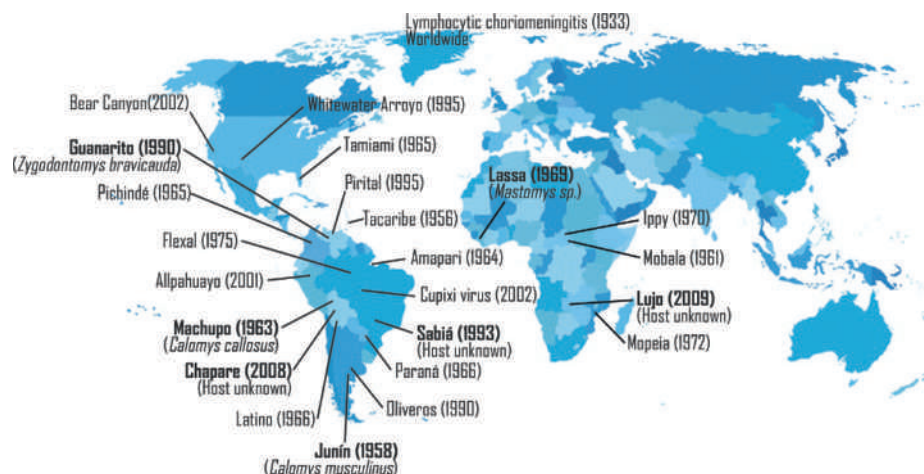


Fig. 1 Geographical distribution of OW and NW mammarenaviruses. The location for each arenavirus and the year of isolation is indicated. For HF-causing viruses, designated in bold, the animal reservoir is shown.

Fifty years after its discovery in 1969 in Nigeria, LF outbreaks have continued in West Africa with an enlarged number of cases in 2016, 2018 and 2019 outbreaks (Ilori et al., 2019; Iseri et al., 2018; Owolabi et al., 2016). LASV is now on the World Health Organization Blueprint list of priority pathogens for which there is an urgent need for accelerated research and development.

The genus prototype species LCMV produces subclinical, often mild human infections with a mortality rate lower than 1%, and the recovery from even severe disease occurs without sequelae in most cases. Approximately 5% of humans show evidence of previous infection with LCMV (Jamieson et al., 2006; Peters, 2006). On the other hand, given that LCMV is a neurotropic virus, congenital infection can produce a spectrum of pathologic effects from minimal to severe, depending on the developmental stage of the fetus at the time of infection (Bonthius et al., 2007). In some cases, infection may result in abortion (Jamieson et al., 2006) and the mortality rate of infants diagnosed with congenital LCMV is approximately 35% (Wright et al., 1997). Moreover, LCMV infection has been reported in immunocompromised patients, often proved to be fatal.

NW human pathogens

JUNV causes AHF in agricultural lands and at an increasingly large area of the country. The seasonal distribution of the illness and the prevalence in male rural workers reveals the risk of occupational exposure to humans and the habits of the host-rodents. Annual incidence within the endemic area may be as low as 1 in 100,000 but in the areas of highest activity it reaches 140 per 100,000 population and 355 per 100,000 adult males. Most infections with JUNV result in mild clinical disease (Enríe et al., 2008; Weissenbacher et al., 1987). About 70–80% of infected humans will self-resolve with no consequences while the remaining 20–30% will develop hemorrhagic or/and neurological manifestations. When untreated, the case fatality for AHF is 10–30%, which is lowered to 1% when treated with serum from patients who have recovered (Enríe et al., 2008). AHF is the only arenavirus related condition for which both efficient therapy and vaccination have been stabilized. These topics will be addressed on the corresponding sections.

Bolivian HF (BHF) was first described in human patients in the Beni district of Northeast Bolivia near the city of San Joaquin during an outbreak which lasted from 1959 to 1963. BHF, as other South American arenavirus HF, resembles AHF symptomatology, with a total mortality rate of around 25% (Webb, 1965). With the reemergence of BHF in 2006, an increase in the number of cases has been reported annually with a peak in 2008 (Aguilar et al., 2009). Patterson et al. (2014) extensively reviewed the knowledge on this disease highlighting that there has been minimal research for the development of countermeasures or treatment for BHF. Although more studies have been done on the biology of MACV, limited in-field information is available. Lack of accessible material or data from clinical new samples, the difficulty and remoteness of the region, and the geopolitical environment within Bolivia, makes this disease overlooked.

Reservoirs and transmission

The main groups at risk of infection by arenaviruses are laboratory workers who perform virus purification procedures, people in contact with rodents in endemic areas, and healthcare providers working with infected patients. In areas where these viruses are present, field workers are particularly at risk because of their close contact with infectious rodent excrete.

As zoonotic agents capable of transmitting diseases from animal to human, mammarenavirus circulation follows its natural host reservoir distribution. With exception of the nonpathogenic Tacaribe virus (TCRV) that circulates in bats, all identified mammarenaviruses have rodent species as natural reservoirs (Childs and Peters, 1993; Salazar-Bravo et al., 2002). Of note, TCRV was recently isolated from *Amblyomma americanum* ticks in Florida (United States), opening the possibility of viral evolution through adaptation to arthropod hosts (Sayler et al., 2014).

OW members, other than the prototype LCMV, are associated with reservoir rodents of the genus *Praomys*, instead NW viruses associate with rodents from the subfamilies *Murinae* and *Neotominae*. LCMV, which reservoir corresponds to the urban worldwide-spread mouse *Mus musculus*, is the only member found globally. All other members of the genus are geographically restricted, due to the wild to rural features of the rodents acting as reservoirs. Then, the incidence rate of the human disease produced by arenaviruses is highest when local population densities of the reservoir increases, rising the human to rodent contact likelihood. Arenaviruses cause acute or persistent infections in rodents without any major life-threatening associated symptoms. Infected animals show chronic viremia and spreading of the virus through urine, saliva, and feces to other rodents, and accidentally to humans (Childs and Peters, 1993; Martínez Peralta et al., 1979). Thus, it is assumed that humans become primarily infected with arenaviruses through material contaminated with excretions or in aerosolized droplets of secretions from infected rodents.

Human vertical transmission from the infected mother to her fetus during pregnancy is not rare for LCMV and LASV (Barton et al., 2002). LCMV transmission through solid organ transplantation from infected donors has been also documented (Fischer et al., 2006; MacNeil et al., 2012). Person-to-person transmissions are infrequent and restricted to LASV, MACV and SBAV. These cases have been particularly well documented in LASV endemic region by intra-family or nosocomial exposure, via contact with body fluids of ill persons or the use of contaminated instruments in medical procedures (Fisher-Hoch et al., 1995; Kernéis et al., 2009). Human infection with LCMV may also occur from pet hamsters and serious infections acquired by laboratory workers were also reported. Despite all before mentioned and because person-to-person spread has occurred in very specific settings, like hospitals or research laboratories, interpersonal transmissibility is considered more an exception than a primary route of infection.

In addition to the high human risk of contagion in endemic regions, the increase in international air travel has contributed to the transport of LASV to other geographic areas such as Europe, the United States, and Japan. (Amorosa et al., 2010; Choi et al., 2018; Ehlikes et al., 2017) Importantly, in January 2020, AHF was diagnosed in a woman in Brussels (Belgium) who had traveled from

Argentina to Europe (Veliziotis et al., 2020). This case demonstrates also the real risk of imported NW HF outside South America, as another unexpected infectious agent with threatening features. Finally, arenaviruses also have the potential to be used as agents of bioterrorism outside of their endemic regions.

The arenavirus multiplication cycle

Entry

Virus entry into the host cell is mediated by the envelope glycoprotein complex that conform the trimeric spike: the receptor binding protein GP1, the transmembrane fusion protein GP2 and the small SSP. Arenavirus entry comprises the initial virus attachment to a cellular receptor, then internalization through an endocytic process which includes virion uptake into vesicles followed by transport to late endosomes where a low pH-dependent fusion of viral and endosome membranes is triggered by GP2, and finally, the release of viral nucleocapsids to the cytoplasm. Different cellular receptors and entry pathways are used by distinct arenaviruses. OW viruses, such as LASV, LCMV, Mopeia virus (MPOV) and Mobala virus (MOBV), and clade C NW viruses bind to α -dystroglycan (α DG), an extracellular glycoprotein expressed in the matrix of a variety of tissues and cells (Cao et al., 1998; Spiropoulou et al., 2002). For the more recently emerged pathogenic OW LUIJV, another initial receptor was reported, the neuropilin (NRP)-2 receptor (Raaben et al., 2017). GP1 of clade B NW arenaviruses binds to transferrin receptor 1 (TfR1) to initiate infection, with recognition of the human protein for pathogenic viruses and TfR1 orthologs from other host species for the nonpathogenic ones (Radoshitzky and Abraham, 2007; Radoshitzky et al., 2008) whereas the receptor for clades A and D is not well elucidated. More recent studies have reported the involvement of other host components, like the C-type lectins DC-SIGN/L-SIGN (Goncalves et al., 2013; Martinez et al., 2013), the phosphatidylserine receptors Axl (Fedeli et al., 2018) and TIM-1 (Brouillette et al., 2018), suggesting the existence of multiple co-receptors or alternative receptors when the main primary receptor, α DG or TfR1 for OW or NW viruses, respectively, is absent.

After binding, NW viruses are endocytosed through a clathrin-mediated pathway and OW members enter through a nonclassical clathrin-independent route guided by the endosomal sorting complex required for transport (ESCRT) proteins (Martinez et al., 2007; Rojek et al., 2008; Pasqual et al., 2011). Recent studies also indicated a macropinocytosis pathway as possible entry route for OW arenaviruses (Oppliger et al., 2016). Additionally, a crucial host factor has been demonstrated for LASV infective entry: at the endosomal acidic pH LASV undergoes a receptor switch leaving α DG and binding to lysosomal associated membrane protein 1 (LAMP-1), a protein interaction that is essential for membrane fusion (Cohen-Dvashi et al., 2016; Jae et al., 2014). In OW and NW arenaviruses, the pH-activated fusion between virion and endosome membranes is triggered by GP2, a class I fusion protein, and modulated by the interaction of GP2 with SSP, an essential requirement to complete the viral penetration process (Nunberg and York, 2012).

Transcription and replication

The presence in the cytoplasm of the arenavirus genome closely associated to NP and L proteins, in a replicative complex designed RNP, is the only requirement to start macromolecular biosynthesis (Hass et al., 2004; Lee et al., 2000; López et al., 2001). The hallmark of transcription and replication in arenaviruses is the ambisense coding strategy of both RNA fragments. The S RNA encodes NP protein at its 3' portion in the genome complementary sense and the glycoprotein precursor GPC at the 5' portion in the genome sense whereas the L RNA encodes the L protein at the 3' portion and the Z protein at the 5' end in opposite orientations (Fig. 2). Although S and L genome segments contain protein coding sense sequences at their 5' regions, they are not directly translated and thus, arenaviruses behave as true negative strand viruses with transcription as the first biosynthetic process after uncoating.

The ambisense arrangement and the structural characteristics of genomes provide a mechanism for temporal regulation of transcription and replication. Primary transcription of the mRNAs for NP and L proteins from S and L genomes is the initial event followed by translation of both proteins (Fig. 2). The mRNAs of the arenaviruses have cap at the 5' end but are not polyadenylated at 3' end and the 5' cap is derived from cellular mRNAs. L protein has the RNA dependent RNA polymerase activity as well as an endonuclease activity required for cap-snatching from 5' end of host mRNAs to prime the initiation of viral transcription (Morin et al., 2010). The intergenic region (IGR) in both fragments folds into a hairpin configuration that is thought to serve as a transcription termination signal, leading to the release of the viral polymerase (López and Franze-Fernández, 2007; Pinschewer et al., 2005). Furthermore, the 3' and 5' untranslated regions (UTR) are complementary in each genome fragment generating a panhandle structure, important to regulate transcription, replication and translation (Foscaldi et al., 2017; Perez and de la Torre, 2003). After NP synthesis has occurred, replication would proceed via the synthesis of full-length antigenomic RNAs and then genomic RNAs (Fig. 2), both tightly recovered by NP as RNPs. The switch from transcription to replication involves the bypass of the hairpin-dependent termination signal and is mediated at least by the intracellular level of NP that functions as a transcriptional antiterminator through its interaction with the IGR sequence (Tortorici et al., 2001). The later event in arenavirus macromolecular biosynthesis is the transcription of subgenomic mRNAs for GPC and Z, respectively, from the antigenomic S and L RNAs, respectively, and their translation (Fig. 2).

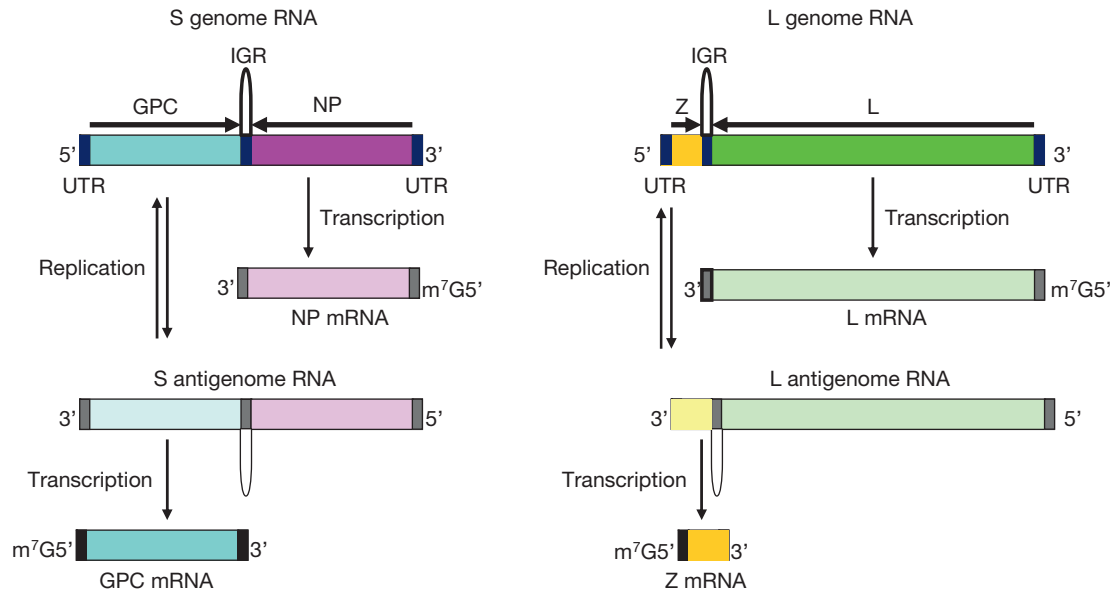


Fig. 2 Ambisense genome strategy for transcription and replication of the two RNA fragments of arenaviruses. Each RNA fragment, L and S, encodes the genetic information for the synthesis of two polypeptides in opposite sense.

Assembly and budding

GPC glycosylation and cleavage take place in the endoplasmic reticulum and the trans-Golgi network by cellular enzymes to generate the mature GP1, GP2 and SSP proteins that are transported and inserted into the cell plasma membrane (Burri et al., 2012). As it is usual for enveloped negative stranded viruses, the assembly and budding of the viral particles is totally dependent on the matrix Z protein that directs the recruiting of the viral components at the plasma membrane regions enriched in viral GP. To this end, Z interactions with L, NP and GP have been demonstrated to assess their colocalization for virion assembly as well as the interaction of Z with the ESCRT proteins to drive the last step of the viral cycle: the exit of virions from the cell by budding at the plasma membrane (Loureiro et al., 2012). The only expression of Z in a cell, without the presence of either the viral genome or any other viral protein, was able to produce the budding of viral-like particles (Strecker et al., 2003; Urata et al., 2009). The formation of these particles empty of genome and packaged by a lipid cell membrane containing Z, confirms the key function of the matrix protein in arenavirus morphogenesis.

The human disease: Pathogenesis and immune response

Pathogenesis

LASV and JUNV cause the prototypical manifestations of human disease for the OW and NW mammarenaviruses, respectively. With an incubation period of 2–21 days for LASV and 6–14 days for JUNV, about 80% of the infections with these viruses turn out to be either asymptomatic or include flu-like symptoms, such as fever, malaise, headache, conjunctivitis, nausea, vomiting, and diarrhea. This makes it difficult to provide an accurate diagnosis unless there is evidence of a known outbreak (Kerber et al., 2015). These symptoms last from 7 to 10 days, after which approximately 75% of the patients start showing signs of improvement; while the remaining 25–30% shows a progression of the illness with the development of hemorrhages and neurological disorders in addition to fever (McCormick and Fisher-Hoch, 2002). Studies in rodent and human survivors of mammarenavirus infection indicate that the proper functioning of innate and T-cell mediated immune responses is critical to minimize viral growth rates, symptoms appearance, and mortality rates (Meyer and Ly, 2016). Monocytic cells such as alveolar macrophages and dendritic cells are the first viral targets; however they are not activated after viral entry as is reflected by the lack of increase in levels of activation markers and cytokines (Baize et al., 2004). This is consistent with the generalized immunosuppression seen in individuals with severe and fatal infection. These cells would be acting as a “reservoir” and promoters of viral spread while entering the draining lymph nodes.

It is estimated that 20–30% of patients infected with LASV get hospitalized and recover, while about 2% of them pass away, presenting a very high viral load from the initial stages of the disease and maintaining an uncontrolled high viremia throughout the entire period of illness. The reason they cannot limit the spread of the virus is believed to be a marked immunosuppression and lack of adaptive immune response. Patients who progress to severe cases of LF can develop very prominent liver damage with platelet factors and heme degradation products such as biomarkers, bleeding, and neurologic involvement that can be fatal (Gale et al., 2017). Survivors present a high rate of neurological sequelae of which sensorineural deafness is the most frequent (Okokhere et al.,

2009). In LASV case as so as LCMV the neutralizing antibody response is very delayed, it is complement dependent and of poor quality.

The AHF has three well defined phases: prodromal, neurological-hemorrhagic, and convalescence. Without treatment, patients would develop severe symptoms characterized by bleeding, vascular damage, petechiae in the oral mucosa, chest, arms, and axillary region, gingival bleeding, leukopenia, thrombocytopenia, ataxia, mental confusion, increased irritability, tremors that can progress to seizures, shock, bacterial infections, and coma (Enríá et al., 2008). Bleeding seems to be the result of the simultaneous occurrence of thrombocytopenia, abnormal platelet function induced by a plasma component, and alterations in blood coagulation with activation of fibrinolysis (Enríá et al., 2011). At the immunological level, AHF is characterized by an acute transitory immunodeficiency: cell-mediated immunity is depressed as evidenced by the decreased levels of delayed-type hypersensitivity to nonviral recall antigens and of lymphocyte proliferation stimulated by mitogens, as well as by marked changes in the T-cell subpopulations. There is also a lag in the humoral immune response, with antibodies appearing during the second week of illness, coincident with recovery. In contrast with LASV and LCMV, patient survival is characterized by a robust and persistent neutralizing antibody production which ultimately results in viral clearance.

On the other hand, a typical LCMV infection is characterized by two phases, a period of fever and viremia which is followed by a central nervous system (CNS) phase without virus circulation, but antibodies can be found in serum (Lehmann-Grube, 1972). The majority of the infections are subclinical, some of them consist of fever alone, and others show both the initial febrile state and the CNS disease, often with a short afebrile remission between both episodes. On those patients presenting clinical manifestations the disease may begin with fever, malaise, myalgia, gastrointestinal symptoms, and occasionally sore throat or cough. Arthralgia, testicular pain, parotid pain, may occur as well. During this phase, which may last from a few days to 2–3 weeks, leukopenia, lymphopenia, thrombocytopenia, and elevation of lactate dehydrogenase and aspartate aminotransferase levels are also observed. The neurologic stage begins with aseptic meningitis. Headache may be severe and disturbances of consciousness may be seen. Signs of nervous system involvement may last 1–4 weeks and resolve without sequelae. Rarely, encephalitis may occur with a very occasional fatal outcome (Enríá et al., 2011). The neurologic disease is thought to be immunopathologic and mediated by CD8+T cells because immunosuppressed patients infected with LCMV did not develop CNS symptomatology (Kang and McGavern, 2008). Convalescence may last several weeks with asthenia, febrile sensations, memory difficulties, poor cognitive function, headaches, or arthralgia.

Immune response

Virus-host interactions have evolved to enable viruses to resist or avoid host antiviral responses. Viral infection usually produces pathogen-associated molecular patterns (PAMPs) during viral replication that are easily recognized by immune cells. These PAMPs can be detected by host pattern recognition receptors, including retinoic acid inducible gene 1 (RIG-1)-like receptors, Toll-like receptors (TLR), and protein kinase receptors (PKR), activating downstream signaling pathways that stimulate an antiviral response in the form of the up-regulation or expression of type I interferons (IFN- α and IFN- β), cytokines, proapoptotic factors and the activation and maturation of innate immune cellular arms such as dendritic cells, T cells and macrophages (Jensen and Thomsen, 2012).

Although OW and NW viruses generate a few similar clinical symptoms, both groups show many differences at an immune response level. Moderate to severe symptoms in LASV infected patients are associated with inhibition of the innate immune response with decreased levels of IFN-I and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and a lack of neutralizing antibody presence, leading to the subsequent deficiency in stimulation of T cells, dendritic cells and macrophages (Brisse and Ly, 2019). Many of the mediators activated in septic shock are involved in the pathogenesis of LASV such as sulfidopeptide leukotrienes, catecholamines, platelet-activating factor and endorphins (Mahanty et al., 2003). In contrast, NW mammarenaviruses and LCMV tend to produce increased levels of IFN-I and cytokines during the acute period. JUNV and MACV do not suppress the innate immune mechanisms but shift towards a pro-inflammatory response involving an up-regulation of IFN- α and TNF- α , probably correlated with the severity and fatal outcome in NW infections (Enríá et al., 2011). For LCMV, clearance of acute infection is due to the robustness of the IFN response early during infection, prior to a peak in viral titer. The more substantial an IFN response is in the early stages of infection, the more likely it is that virus-specific CD8+ cytotoxic T cells will be induced and virus clearance will occur, whereas a weaker IFN response leads to the observed chronic and persistent LCMV infections in mice (Meyer and Ly, 2016).

Immune responses to arenavirus infections are modulated by the arenavirus multifunctional proteins NP and Z that control the antiviral response to infection by targeting multiple cellular pathways. The most important role of NP in pathogenesis is the inhibition of IFN-I and this role is largely mediated by its C-terminal exonuclease domain that was reported to prevent the potentiation of RIG-I function (Shao et al., 2018) and the activation of interferon regulatory factor 3 (IRF-3) and downstream genes (Hastie et al., 2011). The 3′–5′ exonuclease function of NP also possess high specificity for degradation of small dsRNAs triggering host defenses as the PKR pathway to halt viral replication and the downstream phosphorylation of the eukaryotic translation initiation factor 2 α (eIF2 α) to inhibit host and viral protein translation (García et al., 2006; King et al., 2017). Furthermore, immunosuppression mediated by NP-exonuclease activity also was shown to prevent the activation of natural killer cells (Wauquier et al., 2019). Other NP host interaction partner is the DEAD (Asp-Glu-Ala-Asp)-box ATP-dependent RNA helicase (DDX3), a protein that exhibits a proviral role for arenaviruses and acts as a transcriptional regulator of IFN- β promoter. NP may therefore promote viral spread by sequestering DDX3 from macromolecular complexes, and so suppress IFN-I induction subverting

immunity (Loureiro et al., 2018). An additional role of NP in infected cells is the inhibition of apoptosis, a cell process to induce their own death in response to cellular stress and damage caused by invading pathogens (Wolff et al., 2013).

Z is the other viral protein involved in arenavirus innate immune suppression and virulence through various protein-protein interactions. Z protein of the human pathogenic OW and NW strains can interact directly with RIG-1 to inhibit IFN-I expression, but Z of nonpathogenic viruses cannot (Fan et al., 2010; Xing et al., 2015). The proline-rich homeodomain (PRH), a cellular transcription factor with antiproliferative effects, is downregulated by Z protein of pathogenic viruses (Djavani et al., 2005). Z also binds to the translation initiation factor 4E (eIF4E), crucial for mRNA transport, transcript assembly onto polysomes and initiation of translation, affecting protein production (Campbell Dwyer et al., 2000). Z may also have a role in abrogating apoptosis induced upon infection through its interaction with the promyelocytic leukemia protein (PML), a regulator of cell growth involved in a number of apoptosis related pathways. After binding, Z produces PML redistribution from nuclear bodies to cytoplasmic bodies (Meyer and Groseth, 2018). Further, PML and Z bind to ribosomal P proteins in the nucleus thereby implying a role in regulating protein translation (Borden et al., 1998).

Another way in which arenaviruses may activate the innate immune system and promote an IFN-I response is through the activation of various TLRs (Vidya et al., 2018). NW and OW seem to have also diverged in this mechanism as JUNV induces activation of TLR 2 (Cuevas and Ross, 2014) while LCMV produces a TLR 7 and TLR 9 mediated response (Buechler et al., 2015).

Host restriction factors represent cellular proteins that interfere with specific steps of the viral multiplication cycle to inhibit infection. Many of these factors are IFN inducible and few of them have been shown to limit arenavirus entry and exit processes, such as gamma-interferon-inducible lysosomal thiol reductase (GILT), viperin and tetherin. GILT is a soluble enzyme that is highly expressed in the endosomal and lysosomal compartments blocking viral fusion and genome release (Chen et al., 2019) while viperin and tetherin have been shown to negatively affect arenaviral assembly and budding (Peña Cárcamo et al., 2018; Zadeh et al., 2020).

Lastly, the autophagy pathway is an important component of host antiviral defense, functioning to degrade pathogens and trigger both innate and adaptive immunity. During arenavirus infection, both OW and NW arenaviruses may utilize components of the autophagy pathway to aid in replication (Baillet et al., 2019; Perez Vidakovics et al., 2019; Roldán et al., 2019). However, the biological significance of this interaction of viral proteins with components of autophagy remains unknown.

Treatment and antiviral development

Treatments are very limited for the HF-causing arenaviruses (Fig. 3). Current antiviral therapies are restricted to the administration of either passive antibodies through transfusion of convalescent plasma for AHF patients (Enríá et al., 2008), or the off label use of the nucleoside analog ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) which has proven to be effective in treating LF patients (McCormick et al., 1986). More recently, favipiravir has also been used under compassionate use program to treat LF as well as AHF (Raabe et al., 2017; Veliziotis et al., 2020).

Convalescent plasma therapy has been in use for over a century as both post exposure prophylaxis and treatment, and is one of the best therapies currently available to treat COVID-19 (Bloch et al., 2020). However, critical parameters such as the timing of the treatment and the administered dose (volume and antibody titer levels) need to be adjusted. A successful example of this approach is the standard dose of JUNV-neutralizing antibodies provided by the National Institute of Human Viral Diseases of Argentina to treat infected patients which has shown to decrease the mortality rate from 30% to 1% if initiated within the first 8 days of disease (Enríá et al., 2008). An important drawback of this therapeutic approach is the exposure to transfusion-associated diseases and the development of late neurological complications in 10% of treated patients. This post-AHF neurologic syndrome occurs after a symptom-free period and is characterized by fever, cerebellar signs, and cranial nerve palsies. Finally, this therapy is highly specific for AHF since treatment of LF with convalescent serum was not effective in a clinical trial (McCormick et al., 1986).

As the availability of immune plasma from convalescent AHF patients may be limited, researchers have been putting their effort into alternative immunotherapeutic agents. The use of anti-TfR1 antibodies has been explored (Helguera et al., 2012), and recently Cohen-Dvashi et al. (2020) proposed the use of immunoadhesins as an attractive approach for fighting infections by TfR1-tropic mammarenaviruses. Moreover, Pan et al. (2018) have reported the generation of mouse monoclonal JUNV neutralizing antibodies. Recently, by vaccinating horses with vesicular stomatitis virus pseudotypes bearing JUNV GP, IgG antibodies were obtained that showed some cross-neutralization against pathogenic NW arenaviruses (Pan et al., 2020). Also in this context, other groups have explored different strategies (Amanat et al., 2020); however, further *in vivo* evaluation of all these immunotherapeutic agents is necessary.

On the other hand, arenaviral therapies using synthetic compounds have been historically ignored by pharmaceutical companies due to their limited profitability. Ribavirin is a broad spectrum antiviral approved for the treatment of hepatitis C virus and respiratory syncytial virus. It is a guanosine analog that directly inhibits RNA-dependent RNA polymerase, host inosine monophosphate dehydrogenase and viral capping enzymes (Graci and Cameron, 2006). In addition, there is also evidence that ribavirin causes viral mutagenesis as well as inhibition of viral replication (Moreno et al., 2011). Remarkably, ribavirin is the only drug shown to be effective for the treatment of LF in clinical trials decreasing mortality from 55% to 5% but only if its administration started within 6 days of symptoms onset (Attinsounon et al., 2018; McCormick et al., 1986). However, subsequent observational studies have shown mortality rates of 45% among treated patients (Shaffer et al., 2014). It must be noted that ribavirin is currently recommended for high risk exposures only, as it can induce adverse side effects such as thrombocytopenia, anemia, and birth

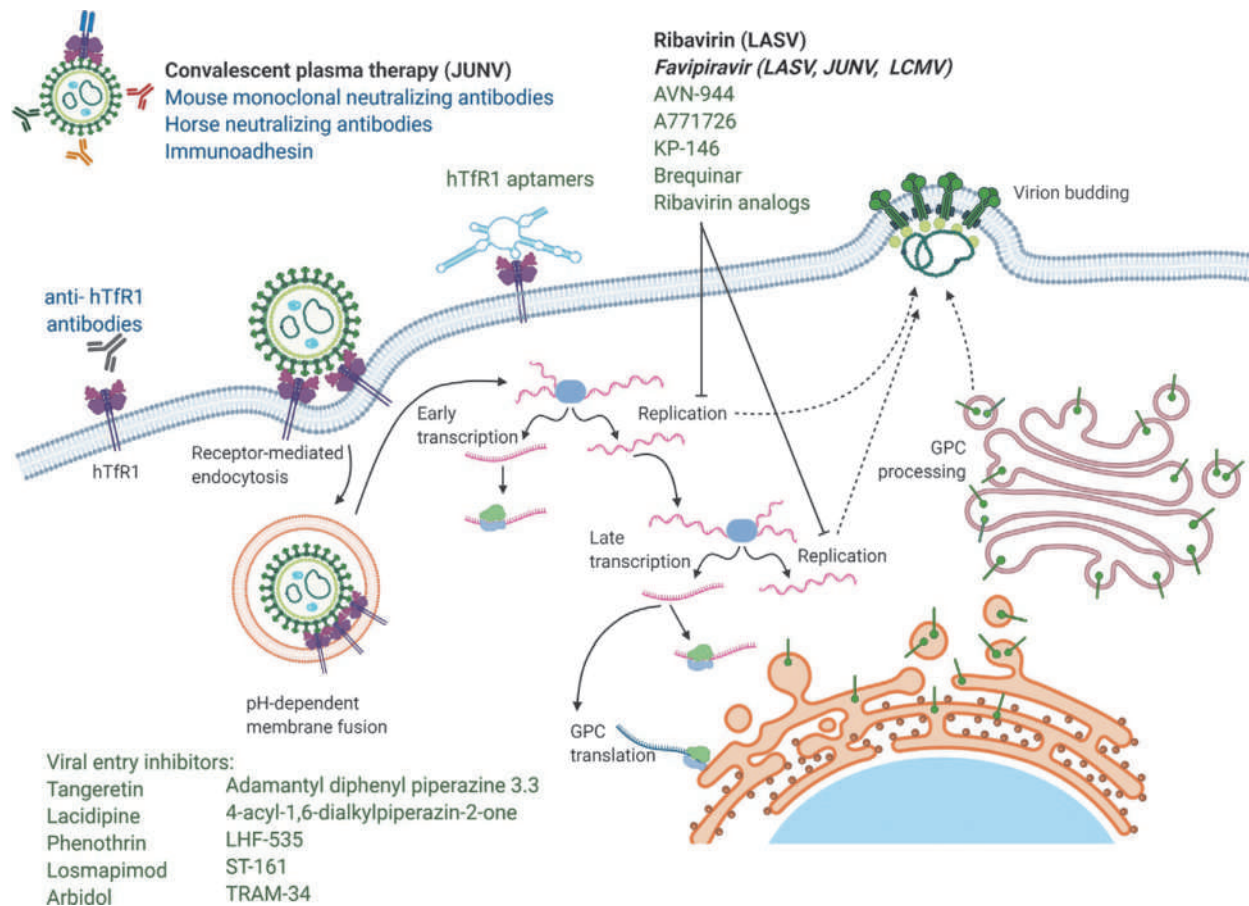


Fig. 3 Mammarenavirus multiplication cycle and antiviral targets. The steps of the viral cycle are depicted together with the corresponding therapies in clinical use and the antiviral agents under research: currently used therapies (letters in black and bold) and drug under compassionate use (letters in black and italics), experimental immunotherapeutic agents (letters in blue), and experimental natural and synthetic compounds (letters in green).

defects. Although treatment of LF with convalescent serum was not effective in a clinical trial, the efficacy of ribavirin when given as post-exposure prophylaxis was enhanced by concurrent treatment with convalescent serum (Jahriling et al., 1984). Unfortunately, ribavirin has been shown to be only marginally effective for the treatment of AHF (Enríá et al., 2008). Later reports have described the efficacy of different doses and treatment schedules of ribavirin tested in a guinea pig lethal model of JUNV infection (Salazar et al., 2012), and recently, modified ribavirin analogs were reported to have comparable *in vitro* effective concentration against JUNV, but with a higher selectivity index (Contin et al., 2019). However, no trials have been performed due to difficult recruitment of patients, inadequate supplies and the high drug cost.

The limited target market represented by arenaviruses restricts commercial interest and makes drug repurposing and/or the identification of broadly acting inhibitors targeting common mechanistic features of these viruses (or their interactions with the host) highly attractive approaches for therapeutic development. In addition, these strategies would simplify therapy in resource limited areas by allowing treatment initiation for clinically similar diseases that occur frequently within the same geographic region in advance of a detailed molecular diagnosis, which indeed may not always be available. Also, compounds with such a broad activity spectrum would in theory also be expected to target newly emerging viruses, such as the latest arenavirus isolates identified in recent years. In this sense, the era of large-scale screening made possible the random exploration of a broader number of compounds which brought the discovery of some interesting active molecules (Sudhakar et al., 2020). A screening of 11,968 compounds from a drug repurposing library (Repurposing, Focused Rescue and Accelerated Medchem [ReFRAME]) identified several potent inhibitors of LCMV, LASV and JUNV. The findings indicated that enzymes of the rate-limiting steps of pyrimidine and purine biosynthesis play critical roles in the completion of the mammarenavirus multiplication cycle, suggesting they represent potential druggable targets to counter human pathogenic infections (Kim et al., 2019). These results are in concordance of previous and recent studies reported from different groups, where some interesting broad compound candidates such as the inosine monophosphate dehydrogenase inhibitor AVN-944 (Dunham et al., 2018) and the dihydroorotate dehydrogenase inhibitors A771726 (Sepúlveda et al., 2018), KP-146 (Miranda et al., 2018), brequinar and other new investigational drugs (Kim et al., 2020) demonstrated promising anti-arenaviral activity.

An example of success of a broad antiviral compound is the anti-influenza drug favipiravir (T-705) which proved to be also efficacious against many other RNA viruses, including LASV and other OW arenaviruses in animal models (Mendenhall et al., 2011; Rosenke et al., 2018). Hickerson et al. (2018) have demonstrated complete protection against mortality and dramatic reduction in viral load in a mouse model of acute and disseminated LCMV infection after favipiravir treatment whereas ribavirin was mostly ineffective. These findings support the consideration of favipiravir as a first-line therapeutic option in organ transplant recipients, a target group at substantial risk to LCMV infection. Moreover, favipiravir significantly decreased mortality if given intraperitoneally in a guinea pig model of JUNV (Gowen et al., 2013). One of the mechanisms of action proposed for this drug is its incorporation into nascent single-strand RNA with the consequence loss of the RNA-NP interaction, leading the viral genome more accessible to cellular ribonuclease (Omotuyi et al., 2019). As previously mentioned, in January 2020 a case of imported AHF was reported in Belgium in a woman not engaged in agricultural activities, but somehow she got infected a few days before her trip from Argentina to Europe (Veliziotis et al., 2020). After confirmation of JUNV infection by real-time PCR, ribavirin was given first orally and then intravenously on day 6 after symptoms onset. The next day, due to its proven synergistic antiviral activity against JUNV in animal models (Westover et al., 2016) and previous experience with LF (Raabe et al., 2017), oral favipiravir was given through a nasogastric tube. Ribavirin therapy was discontinued on day 9 due to secondary effects and the dose of favipiravir was progressively increased during 14 days. Fortunately, the patient was discharged from the hospital after 50 days without sequelae. Although it was only one case report, it has demonstrated the importance of academics studies in the advance of the arenavirus antiviral field.

Regarding basic antiviral research, several groups have been focused on novel strategies during the last few years, targeting the viral entry with natural products like tangeretin (Tang et al., 2018) or different types of synthetic compounds like aptamers (Maier et al., 2016), the calcium channel blocker lacidipine, the synthetic pyrethroid pesticide phenothrin (Wang et al., 2018), the enzymes p38 α / β mitogen-activated protein kinases inhibitor losmapimod (Zhang et al., 2019a,b) and the anti-influenza drug arbidol (Hulseberg et al., 2019). Other type of promising compounds are blockers of the low-pH-induced membrane fusion as the adamantyl diphenyl piperazine 3.3 (Wang et al., 2018) and the 4-acyl-1,6-dialkylpiperazin-2-one (Plewe et al., 2019), and small molecules like LHF-535 (Madu et al., 2018), ST-161 (Zhang et al., 2019a,b) and clotrimazole derivatives like TRAM34 (Torriani et al., 2019). Some of these compounds are under phase I human safety studies. Compounds targeting the endonuclease domain of the L protein, required for arenavirus transcription, were also recently described as a new type of arenavirus inhibitors (Saez-Ayala et al., 2019). Hopefully, follow up of these studies (Fig. 3) will be reported in a near future.

Vaccines

Candid#1 vaccine

Currently, there are no vaccines licensed by the Food and Drug Administration (FDA) in USA to protect against arenavirus infection. The only clinically successful anti-arenaviral vaccine is the anti-JUNV Candid#1 strain, which is manufactured at a dedicated facility fully supported by the Argentinian government (Ambrosio et al., 2018). This vaccine developed through a joint international effort is a live attenuated vaccine derived from JUNV XJ44 parental strain, so designated to define the XJ prototype strain, the first JUNV clinical isolate from a human fatal case (Parodi et al., 1958), after two passages in guinea pig and 44 passages in mouse brain (Ambrosio et al., 2011). The XJ44 strain was serially passaged in fetal rhesus lung cells to get Candid #1, the first vaccine produced and registered under Argentina regulations since 2003.

Following a single intramuscular injection, Candid#1 proved to induce JUNV specific antibodies in 91.1% of the vaccinated population and a protective response of 95% against AHF. Its administration had a remarkable impact on the magnitude of JUNV epidemics in Argentina reducing the number of annual cases (Ambrosio et al., 2011; Maiztegui et al., 1998). The efficacy of Candid#1 vaccination is in accordance with the main role of JUNV neutralizing antibodies against GPC for clearance of the virus (Seregin et al., 2010), as also commented in the previous section. Neutralizing antibodies effectively target GP1 pocket which accepts the tyrosine residue of the cellular receptor TfR1 (Mahmutovic et al., 2015).

Whereas a number of unique mutations in Candid #1 were originally thought to attenuate the virulence of its parental strain (Chiringhelli et al., 1997; Goñi et al., 2006, 2010), further studies have identified a single mutation (F427I) in the transmembrane domain of GP2 to be the cause of reduced infectivity (Albariño et al., 2011). While the mechanism for this attenuation has yet to be elucidated, it is thought that the mutation may affect viral fusion or maturation efficiency. On the other hand, additional evidence suggests that at least one additional mutation in GP likely contributes to the attenuation in dissemination and virulence during infection (Seregin et al., 2015). It has also been suggested that the loss of an N-glycan in GP1 of Candid #1 could also contribute to *in vivo* attenuation, turning the vaccine strain more antigenic and therefore more susceptible to neutralizing antibodies (Sommerstein et al., 2015).

Other vaccine developments

Over the years, diverse strategies have been used in order to design vaccines for HF causing arenaviruses. Even though some showed protection in animal models, most of them have not been tested in humans. The earlier studies intended to get inactivated or live attenuated vaccines using classical protocols. Among inactivated vaccines, it was reported that the inoculation with formaldehyde-treated or gamma-irradiated LASV as well as formaldehyde-inactivated JUNV lead to an increase in specific antibodies but without conferring protection against viral challenge in animal models (Peters et al., 1987; Videla et al., 1989). In an attempt to obtain an

attenuated MACV strain to control BHF, as it was performed with JUNV, 60 serial virus passages were done in chick embryo cell cultures. Unfortunately, attenuation resulted insufficient and unstable, so those studies were abandoned (Peters et al., 1987). Furthermore, a preliminary report demonstrated some efficacy for Candid#1 against MACV infection in nonhuman primates, but the research has not continued to human trials.

Currently, various different approaches with new technologies and platforms are being implemented to obtain attenuated vaccinal strains of arenaviruses. Among the candidates showing efficacy in preclinical animal models, a live attenuated reassortment between LASV and MOPV, named clone ML29, was selected and tested as a vaccine in guinea pigs and rhesus macaques. ML29 has the S genome of LASV and the L genome of MOPV, a nonpathogenic OW arenavirus closely related to LASV (Lukashevich et al., 2005). Inoculation with this vaccine was followed by low levels of replication and transient viremia. Furthermore, small nonhuman primates showed protection against challenge with LASV and prevention of hepatic pathology (Lukashevich et al., 2008).

Reverse engineering was other strategy used to obtain an attenuated LASV strain. Cai et al. (2020) cloned the IGR of LASV S segment in the LASV L segment and co-transfected cells with this plasmid together with plasmids encoding LASV S segment, NP and L genes to produce recombinant viral particles. Guinea pigs inoculated with this virus, designed rLASV(IGR/S-S), showed no signs of infection and produced detectable levels of antibodies against LASV. Importantly, when challenged with wild-type LASV, animals were protected against the otherwise lethal virus.

Virus-like particles have also been used as vectors to develop vaccines. A recombinant vaccine based on vesicular stomatitis virus (VSV) vector that express the GPC of LASV was successfully assayed in two animal models of LF (Safronetz et al., 2015). Morbidity and mortality were fully prevented in guinea pigs and macaques vaccinated with this VSV-based strain and challenged with homologous and heterologous challenges of LASV. Recently, Johnson et al. (2020) used the human Venezuelan equine encephalitis vaccine (VEEV) as a basis to generate a vector that expressed both JUNV and MACV GPCs. While nonreplicative, this vector also guaranteed high immunogenicity due to high levels of expression of the viral GPCs specifically targeted. Guinea pigs inoculated with this vector developed specific neutralizing antibodies and showed protection when challenged with either virus.

During the writing of this manuscript, there were two vaccines against LASV registered in WHO's International Clinical Trials Registry Platform. One of them, MV-LASV is based on a proprietary replication-competent, live attenuated measles vector platform expressing LASV NP and GP (Purushotham et al., 2019). Developed by Themis Bioscience and the Institut Pasteur, it has shown protection in macaques after a single dose (Mateo et al., 2019) and since September 2019 its optimal dose, safety, tolerability and immunogenicity has been under evaluation in healthy volunteers. The second registered clinical trial aims to determine the safety, tolerability and immunological profile of the DNA vaccine INO-4500. This vaccine encodes the codon-optimized sequence of LASV GPC and it is administered by intradermal injection and posterior electroporation. Prior studies showed that INO-4500 provided protection and induced the production of neutralizing antibodies both in guinea pigs and macaques (Cashman et al., 2013, 2017) while research in humans has been planned to begin in August 2020.

Concluding remarks

Mammarenaviruses remain as a group of RNA viruses causing endemic diseases that represent a serious threat to human public health. Several factors are responsible of this challenging situation: the high case-fatality rate in patients with LF in Africa during the occurrence of annual outbreaks; the increase in the emergence of new viruses around the world in recent years as consequence of a regular search for the detection of virus, genome or antibodies, in rodents reservoirs; the risk of importation of HF arenaviruses outside of the endemic regions through increasing air travels. In fact, the isolation of new agents from severe human cases has been reported not long ago, underlining the urgent need of effective tools to combat these still neglected viral infections.

In spite of this critical situation, at present no specific safe chemotherapy or globally approved vaccines are available for most HF arenaviruses. Recent progress in the knowledge of virus-host interaction, at the molecular level of cell biology as well as at the multiple modulators of the complex arenavirus pathogenesis, will be helpful to improve the achievement of prophylactic or treatment alternatives. This effort will be further supported by the recent inclusion of LASV in the list of the WHO priority pathogens that require compelling clinical solution.

As with other viral diseases, vaccination was considered the most effective control strategy. A preventive vaccine, the live attenuated Candid#1, has been successful to control JUNV and reduce the incidence of HF in Argentina. A great effort has also been devoted to the development of a vaccine for LASV, with the implementation of diverse platforms based on recombinant arenaviruses, reverse engineering, plasmid DNA and vector viruses. Although several vaccinal strains have shown efficacy in the preclinical testing in animal models during the last 20 years, the initial phase I clinical trials in human volunteers for two LASV vaccine candidates has been just recently scheduled.

The only current treatment options for HF arenaviruses are the use of immune plasma or the nucleoside analog ribavirin, both presenting important limitations and undesirable side effects. Viral and host components essential for virus infection are the targets of several agents ongoing research. Hopefully, advances in understanding the detailed molecular biology of HF virus replication, coupled with determination of three-dimensional structures of viral molecules, could lead to the development of highly effective antiviral drugs against arenaviruses in a near future. The availability of arenavirus chemotherapy is mandatory since, even if successful vaccines against HF-viruses will be at hand, ecological changes in the habits of the rodent reservoirs as well as the appearance of new virus variants through genetic alterations cannot be discarded.

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Ebolavirus and Other Filoviruses

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Glossary

Negative-stranded RNA virus A virus that uses negative sense, single-stranded RNA as its genome. The negative viral RNA is complementary to mRNA (positive sense).

Viral envelope The outermost layer of many kinds of viruses, typically derived from portions of the host cell membranes including some viral glycoproteins.

RNA-dependent RNA polymerase An enzyme that catalyzes the synthesis of RNA from an RNA template.

Neutralizing antibody An antibody that inhibits infection through binding to a pathogen.

Ribonucleoprotein complex (RNP complex) A complex of RNA and proteins that plays an integral part in regulating gene transcription and replication.

Niemann-Pick C1 (NPC1) protein The cholesterol transporter that has been shown to be a receptor that is essential for filovirus infection. Physiological function of NPC1 is involved in intracellular lipid transport and mutations in the NPC1 gene are known to cause Niemann-Pick disease, type C.

Innate immune system One of the two main immunity strategies found in vertebrates. It plays an important role in activation of the adaptive immune system through antigen presentation and cytokine production.

Natural host The living organisms in which an infectious agent naturally lives and reproduces.

Porcine reproductive and respiratory syndrome A swine disease caused by infection with an arterivirus (Porcine reproductive and respiratory syndrome virus).

Vesicular stomatitis virus (VSV) A virus in the family *Rhabdoviridae* that causes vesicular stomatitis in cattle, horses and pigs.

Antibody-dependent enhancement of infection A phenomenon in which a virus particle bound to antibodies enhances its entry into host cells.

Filovirus classification

Filoviruses are a family of non-segmented negative-stranded RNA viruses. Viruses in the family *Filoviridae* are phylogenetically divided into six genera: *Marburgvirus*, *Ebolavirus*, *Cuevavirus*, *Dianlovirus*, *Striavirus*, and *Thamnovirus* (Amarasinghe et al., 2019) (Table 1). The genus *Marburgvirus* has a single species with two known viruses (Marburg virus and Ravn virus). Six distinct species are currently known in the genus *Ebolavirus*: *Zaire ebolavirus*, *Sudan ebolavirus*, *Tai Forest ebolavirus*, *Bundibugyo ebolavirus*, *Reston ebolavirus*, and *Bombali ebolavirus*, represented by Ebola virus, Sudan virus, Tai Forest virus, Bundibugyo virus, Reston virus, and Bombali virus, respectively. The other genera in the family *Filoviridae* each have only one known species with one virus. Novel filoviruses (Bombali virus and Mênglà virus) were recently discovered and classified as a new ebolavirus species (*Bombali ebolavirus*) and new filovirus genus (*Dianlovirus*), respectively. The majority of ebolaviruses and marburgviruses are known to cause severe hemorrhagic fever (Ebola virus disease and Marburg virus disease) in humans and nonhuman primates, whereas Reston virus is believed to induce only subclinical infection in humans. Although infectious forms of the viruses in the *Cuevavirus* and *Dianlovirus* genera and the *Bombali ebolavirus* species have not been isolated, their complete genomes have been detected in bats. RNA genomes of the viruses in the *Striavirus* and *Thamnovirus* genera have been detected in fish. Due to their highly pathogenic characteristics, ebolaviruses and marburgviruses are classified as requiring biosafety level 4, the highest level of biocontainment for handling infectious viruses.

Table 1 Members of the family *Filoviridae*.

Genus	Species	Virus name	Known host (isolation or genetic detection)
<i>Ebolavirus</i>	<i>Zaire ebolavirus</i>	Ebola virus	Human, monkey, bat, duiker
	<i>Sudan ebolavirus</i>	Sudan virus	Human
	<i>Tai Forest ebolavirus</i>	Tai Forest virus	Human, monkey
	<i>Bundibugyo ebolavirus</i>	Bundibugyo virus	Human
	<i>Reston ebolavirus</i>	Reston virus	Monkey, pig, bat
	<i>Bombali ebolavirus</i>	Bombali virus	Bat
<i>Marburgvirus</i>	<i>Marburg marburgvirus</i>	Marburg virus	Human, monkey, bat
	<i>Marburg marburgvirus</i>	Ravn virus	Human, bat
<i>Cuevavirus</i>	<i>Lloviu cuevavirus</i>	Lloviu virus	Bat
<i>Dianlovirus</i>	<i>Mengla dianlovirus</i>	Měnglà virus	Bat
<i>Striavirus</i>	<i>Xilang striavirus</i>	Xīlāng virus	Fish
<i>Thamnovirus</i>	<i>Huangjiao thamnovirus</i>	Huángjiāo virus	Fish

Structure and viral protein functions

Filovirus particles are filamentous, enveloped by a host-derived lipid bilayer, and have an almost uniform diameter of approximately 80 nm. The filaments can be shaped like a “6,” a “U,” or a circle. The name “filovirus” is derived from this unique filiform structure (Fig. 1A). The filovirus RNA genome encodes at least seven structural proteins that comprise virus particles: a nucleoprotein (NP), polymerase cofactor (VP35), matrix protein (VP40), glycoprotein (GP), transcription activator (VP30), minor matrix protein (VP24), and an RNA-dependent RNA polymerase (L) (Fig. 1B and C) (Feldmann et al., 2013). Ebolaviruses produce nonstructural secretory GPs (sGP and ssGP), which are not produced by marburgviruses. The viral surface GP, which is responsible for the viral entry into cells (attachment to cell surface receptors and membrane fusion between the viral envelope and cellular

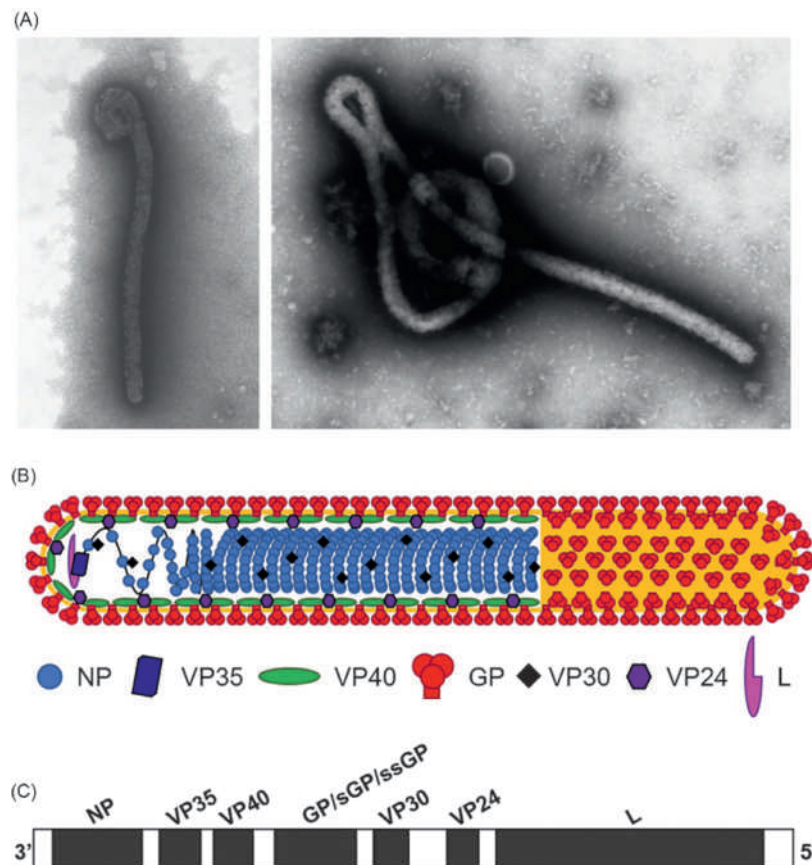


Fig. 1 Ebola virus particles, genes, and proteins. (A) Electron micrographs of Ebola virus particles. (B) Schematic diagrams of a filovirus particle. (C) Negative-sense genome organization.

membrane), is the only target for neutralizing antibodies. Although the function of the nonstructural GPs remains unclear, it has been suggested that they may act as decoys to interfere with neutralizing antibodies or as inhibitory factors against macrophage activation. The viral RNA, NP, VP35, VP30, and L form the ribonucleoprotein complex (RNP complex), which is involved in the transcription and replication of the viral genome. NP molecules form a helical nucleocapsid that contains the viral genome. VP40 is the key driving force for virus particle formation and budding from infected cells. Ebolavirus VP35 and VP24, and Marburgvirus VP35 and VP40 have been shown to be interferon antagonists (Messaoudi et al., 2015).

The entry of filoviruses into host cells is mediated by viral surface GP and initiated by the attachment of GP to cell surfaces (Takada et al., 1997). Virus particles are then delivered to endosomes by micropinocytosis, a mode of endocytosis (Nanbo et al., 2010; Saeed et al., 2010). During the entry into cells, filovirus GP interacts with several host cell receptors such as C-type lectins, T-cell immunoglobulin and mucin domain 1, along with Niemann-Pick C1 (NPC1) protein (Carette et al., 2011; Côté et al., 2011; Kondratowicz et al., 2011; Marzi et al., 2004; Simmons et al., 2003; Takada et al., 2004). Of these receptor molecules, NPC1 is known to be a critical factor for determining the host tropism of filoviruses (Ng et al., 2015; Takadate et al., 2020). The interaction between GP and NPC1 triggers fusion between the viral envelope and the endosomal membrane, leading to the release of the RNP complex into the cytoplasm. In the cytoplasm, each filovirus gene is transcribed into mRNA by the L protein (RNA-dependent RNA polymerase) and other viral proteins, such as VP35, and viral mRNAs are translated by the machinery of the host cell. The negative-sense RNA genome is also used to synthesize full-length complementary and antigenomic RNAs, which in turn serve as templates for viral genomic RNA synthesis. Newly synthesized viral proteins and genomic RNA are transferred to the cell surface for incorporation into viral particles. During the budding process, membrane-associated VP40 accompanying the viral surface GP extrudes from the cell membrane as a viral envelope and incorporates the RNP complex. Viral particles are then released by pinching off the plasma membrane.

Epidemics of filovirus diseases

Marburg virus was first discovered at the Philipps University of Marburg in Germany in 1967. Researchers and laboratory technicians showed symptoms of a hemorrhagic fever after handling samples from African green monkeys that had been imported from Uganda to Germany and the former Yugoslavia for research purposes. The virus was then isolated from the infected patients (Malherbe and Strickland-Cholmley, 1968). Ebola virus was discovered in 1976. In that year, outbreaks of hemorrhagic fevers with high mortality rates were confirmed in the Democratic Republic of the Congo (named Zaire at the time) and Sudan at around the same time. These hemorrhagic fevers were initially suspected to have been caused by Marburg virus. However, it was subsequently discovered that, although the shapes of the virus particles were highly similar, the virus was of a new genus. This genus was later given the name *Ebolavirus* (Johnson et al., 1977). Two species of the *Ebolavirus* genus were discovered independently in the Democratic Republic of the Congo and Sudan, currently named *Zaire ebolavirus* and *Sudan ebolavirus*, including Ebola virus and Sudan virus, respectively. Sporadic reports of outbreaks of infections caused by marburgviruses and ebolaviruses have continued in African countries such as the Democratic Republic of the Congo, Sudan, Gabon, the Republic of the Congo, Uganda, Cote d'Ivoire, Kenya, and Angola, alongside the discovery of new species in the genus *Ebolavirus*: *Tai Forest ebolavirus* and *Bundibugyo ebolavirus* found in 1994 and 2007, respectively (Fig. 2). In particular, the outbreak of Ebola virus disease that occurred in West Africa (primarily Guinea, Liberia, and Sierra Leone) in 2014 grew into an unprecedented epidemic that spread into neighboring African countries. In addition, a large number of infections were reported among medical personnel who had been involved in medical treatment in the affected region. Some of these personnel exhibited symptoms and were hospitalized after returning to Europe or to the United States of America, leading to the virus becoming a worldwide issue. Over the course of the epidemic, approximately 30,000 cases and more than 10,000 deaths were reported. The Ebola virus disease outbreak that occurred in the Democratic Republic of the Congo in 2018 also spread widely (but was mostly limited to that country) and became the second-largest epidemic of Ebola virus disease in history, with at least 3000 cases and 2000 deaths. Reasons for the spread of infection included delays in definitive diagnosis, outbreaks in densely populated areas, and political instability. These epidemics allowed research and development into prevention, treatment, and diagnostic methods relating to Ebola virus disease to be accelerated and unapproved vaccines and therapeutic agents to be clinically tested and advanced towards implementation.

Pathogenesis of filovirus diseases

Ebolaviruses and marburgviruses are highly pathogenic for humans and nonhuman primates. However, the pathogenicity differs depending on the virus species and variant. For example, Ebola virus has the highest mortality rate among ebolaviruses, sometimes close to 90%, whereas Reston virus is believed to induce only subclinical infection in humans (Feldmann et al., 2013). Clinical symptoms during the early stages of infection include fever, chills, fatigue, loss of appetite, nausea, diarrhea, and muscle pain, making it difficult to distinguish filovirus diseases from other febrile diseases such as malaria and influenza. Symptoms during the later stages of infection include those accompanying blood coagulation abnormalities, such as petechial hemorrhages and bleeding from mucous membranes. Pathobiological events during Ebola virus infection are summarized in Fig. 3. After entering the body from mucous membranes or wounds through a medium such as blood, mucus, or vomit, ebolaviruses primarily target antigen-presenting cells such as dendritic cells and macrophages, which are one of the main components of the innate immune system.

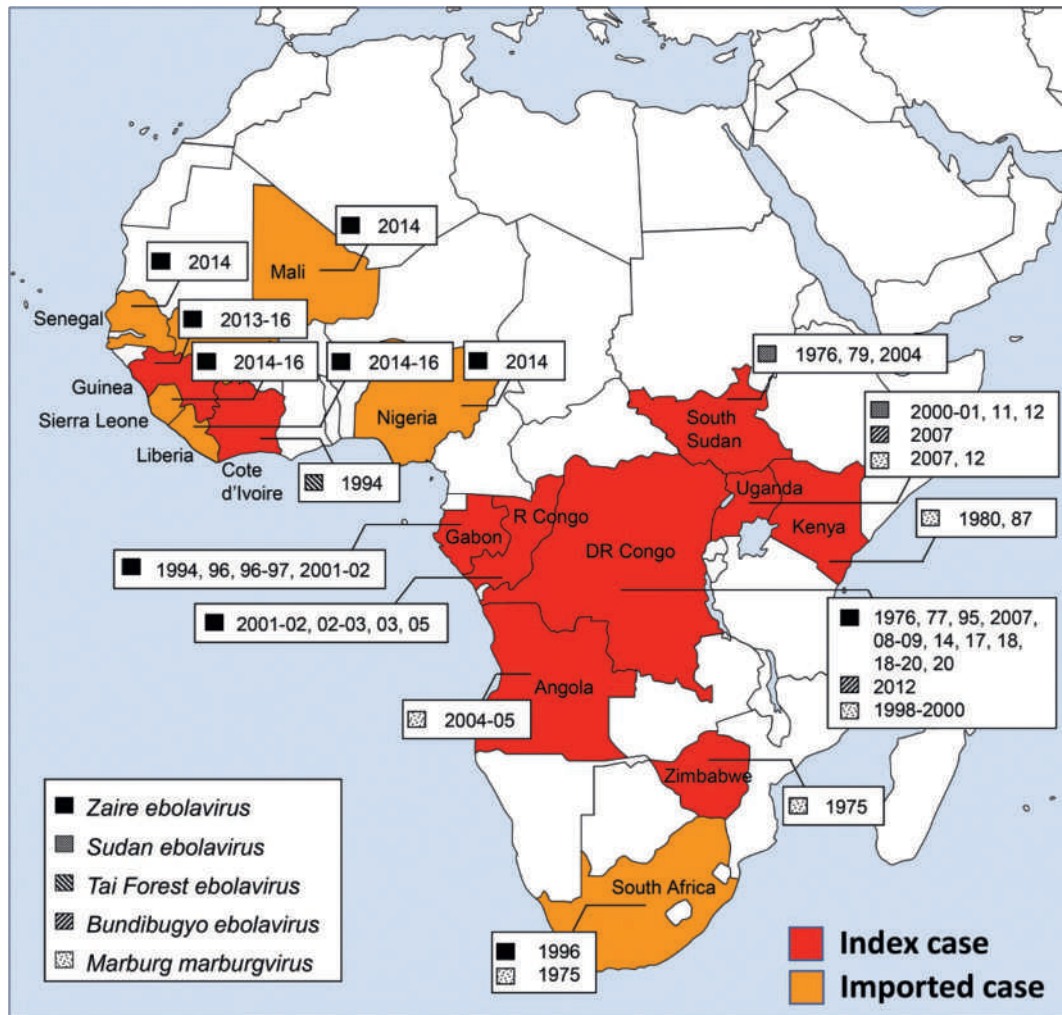


Fig. 2 Outbreaks of filovirus diseases in Africa. Affected countries, filovirus species, and years of outbreaks are shown.

Impeded function and/or hyperreaction of these cells plays an important role in the pathogenicity of the viruses (Geisbert and Hensley, 2004). Bystander apoptosis of lymphocytes is also observed, and the induction of adaptive immunity is also impaired by a significant reduction in their numbers (Baize et al., 1999). Lymphocytes themselves are insensitive to ebolaviruses and marburgviruses, suggesting that direct infection of lymphocytes may not be the cause. However, the detailed molecular mechanisms of the pathobiological events induced by filoviruses are largely unknown (Dolnik et al., 2008).

Hepatocytes are also known to be highly susceptible to the viruses and impairment of liver function results in elevated levels of liver enzymes in the blood (Feldmann et al., 2013). In the later stages of infection, the virus spreads throughout the body and disrupts the functions of many tissues. At the same time, hemorrhagic diathesis and multiple organ failure occur due to thrombocytopenia, cytokine storms (dysregulated cytokine production from large numbers of white blood cells), and vascular endothelial cell dysfunction. The variant of Ebola virus that caused the epidemics in West Africa from 2013 through 2016 belongs to the species *Zaire ebolavirus*; however, it was less pathogenic in experimentally infected animals than other Ebola viruses isolated previously and the hemorrhagic manifestation was not typically seen in many cases of human infection during the outbreak (Marzi et al., 2015; Schieffelin et al., 2014). The disease caused by ebolaviruses was originally known as Ebola hemorrhagic fever; however, in recent years it has come to be called Ebola virus disease, reflecting the fact that ebolavirus infection does not necessarily lead to a hemorrhagic symptom.

Ebolaviruses usually cause acute and fatal infections as described above, but there are some reports suggesting that persistent infections might also occur. Multiple studies carried out on patients who had recovered during the epidemics in the Democratic Republic of the Congo in 1995 and in West Africa between 2013 and 2016 suggested that the viral RNA genome could remain in the mucous tissues of the eye or in semen for up to 9 months (Deen et al., 2015; Varkey et al., 2015). However, given that only viral RNA was found and an infectious form of the virus was not isolated, it cannot be concluded that these recovered patients carried infectious forms of the virus over long periods or that they could infect others. Despite this, a case was reported in which an infectious form of the virus was detected in the cerebrospinal fluid of a nurse from the United Kingdom who recovered from Ebola

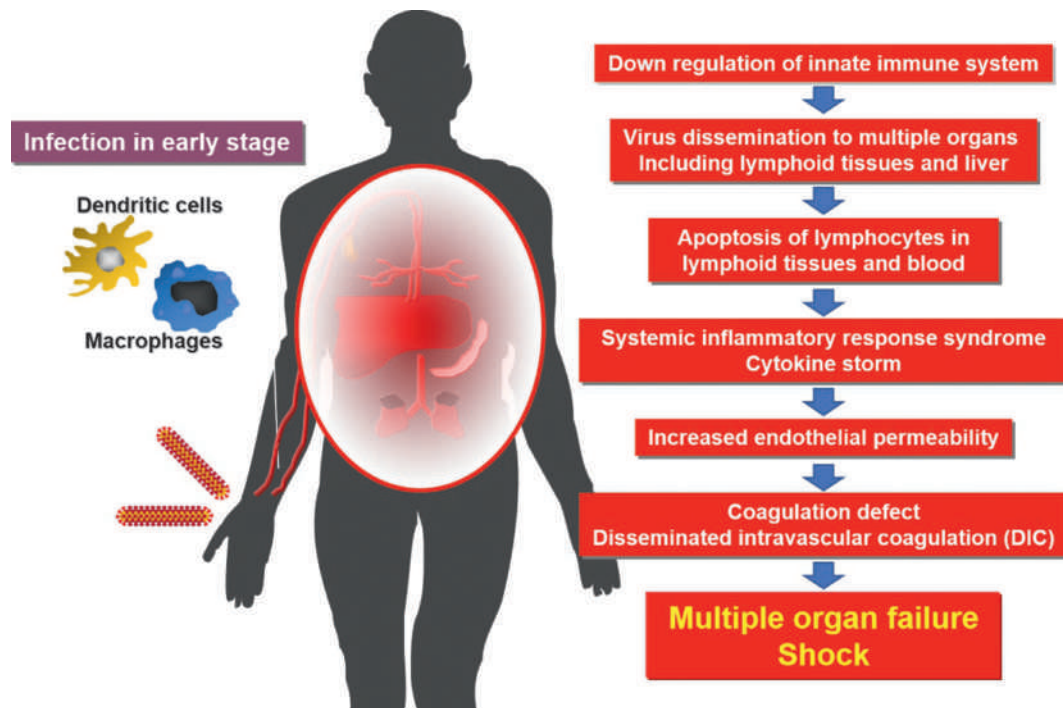


Fig. 3 Pathogenesis of Ebola hemorrhagic fever. Multiple detrimental pathobiological events occur during the infection.

virus infection and developed meningoencephalitis several months later. This could potentially be a case of persistent infection and reactivation of the virus (Jacobs et al., 2016). However, there is currently limited knowledge of the pathogenesis and mechanisms of its persistent infection due to the near 100% mortality rate for animal models of Ebola virus infection, and further research is required.

Susceptible hosts and transmission routes

For viruses such as ebolaviruses and marburgviruses, which cause infectious diseases with high mortality rates in some animal species, the existence of other animals that can maintain the virus for prolonged periods without acute or fatal disease is generally essential in order for them to survive in nature. Such animals are referred to as “natural hosts” or “natural reservoirs” of the virus. The virus is maintained within an individual or population of natural hosts over a long period of time. This is considered the natural form of virus perpetuation in nature. Such viruses maintained by their own natural hosts occasionally infect humans, in some cases fatally.

Some species of fruit bats have been considered to be the most likely natural hosts for ebolaviruses since the viral genomic RNA fragment (*Zaire ebolavirus*) and ebolavirus-specific antibodies were detected in fruit bats captured in the affected area during the large outbreak of Ebola virus disease among humans and great apes that occurred in Gabon and the Republic of the Congo between 2001 and 2003 (Leroy et al., 2005). Ebola virus-specific antibodies have also been detected in multiple species of fruit bats in subsequent studies (Ogawa et al., 2015; Pourrut et al., 2007), and Reston virus RNA fragment and/or antibodies that reacted to Reston virus were detected in insectivorous bats living in Asia (Jayme et al., 2015; Taniguchi et al., 2011). Bombali virus, a recently discovered species of ebolavirus, was detected in insectivorous bats in West Africa in 2018 (Goldstein et al., 2018). These observations clearly indicate that bats are susceptible to ebolavirus infection. However, in the past Ebola virus disease outbreaks, there has not been any compelling evidence showing that humans were infected with an ebolavirus by direct contact with bats. In addition to the fact that an infectious form of an ebolavirus has never been isolated from bats, almost all studies conducted so far have failed to even detect ebolavirus genomes in bats. Based on these observations, it cannot be proven that bats are a natural host for ebolaviruses. To provide epidemiological proof that bats are a natural host for ebolaviruses and are the source of infection for other animals, including humans, viruses that are genetically similar to those isolated from humans need to be detected or isolated from bats continuously over a prolonged period of time.

In the case of marburgviruses, it is highly likely that fruit bats living in caves retain the virus continuously and are a direct source of infection to humans. This is supported by the fact that multiple marburgviruses were continuously isolated from the tissue samples of Egyptian fruit bats (*Rousettus aegyptiacus*) (Fig. 4) living in a cave during the outbreak of Marburg virus disease among miners in Uganda in 2007 (Towner et al., 2009). The virus isolated from the bats was a strain that was almost genetically identical to the virus isolated from the infected people, demonstrating that a marburgvirus carried by the bats had been transmitted to humans.



Fig. 4 A fruit bat (*Rousettus aegyptiacus*) captured in Africa. Marburgviruses are frequently found in these cave-dwelling fruit bats.

In 2007 and 2008, travelers from the United States of America and the Netherlands who had been visiting a cave in Uganda began to show symptoms of Marburg virus disease after returning to their home countries, and Marburg viruses were isolated from bats captured in the cave (Amman et al., 2012).

There have been several cases so far of humans contracting Ebola virus disease or Marburg virus disease through direct contact with infected monkeys. However, nonhuman primates are not considered natural hosts for filoviruses because they are thought to develop severe infectious diseases with high mortality rates when infected with ebolaviruses or marburgviruses as indicated by natural and experimental infections of them with these viruses. Nevertheless, seroepidemiological studies have demonstrated filovirus-specific antibodies in primates, including humans (Becker et al., 1992; Leroy et al., 2004), and shown that susceptibility to ebolaviruses varies among species and among individual monkeys. This means that there is a possibility of frequent occurrences in nature among certain species of monkeys that experience subclinical or non-lethal filovirus infection. Clinical cases of persistent infection in humans have also been observed (Jacobs et al., 2016). If filoviruses with low pathogenicity among primates exist in nature and only viruses that have acquired a certain mutation exhibit high pathogenicity, or if a known virus with high pathogenicity frequently results in non-fatal infections of a particular species of monkey or individual monkeys, the possibility that monkeys could be a natural host for filoviruses cannot be ruled out.

There have also been reports of tetrapods being naturally infected with filoviruses. Reston virus was unintentionally isolated from pigs with porcine reproductive and respiratory syndrome in the Philippines in 2008 and 2009 (Barrette et al., 2009). This virus was subsequently detected in pigs with the same disease in China. However, although Reston virus has been shown to be highly pathogenic among monkeys, its pathogenicity for humans and livestock has not been established. Previous reports have suggested that Reston virus only induces subclinical infections in humans and pigs. On the other hand, it has been confirmed that duikers, of the Bovidae family, were infected with Ebola virus during the epidemic in Gabon and the Republic of the Congo between 2001 and 2003, where large numbers of great apes such as gorillas and chimpanzees succumbed to the infection in the forest. There have also been reports that about 25% of dogs living in the villages that had experienced an epidemic of Ebola virus disease had Ebola virus-specific antibodies (Allela et al., 2005). Since various animal species are considered to be susceptible to filoviruses, as described above, livestock and wild animals could be intermediate hosts, and direct sources for filovirus transmission to humans.

Distribution of filoviruses

Ebola virus disease and Marburg virus disease mainly occur in Central Africa. However, marburgviruses have been detected in fruit bats (*R. aegyptiacus*) in African countries other than those where outbreaks and/or patients with the diseases have been reported (Amman et al., 2020; Kajihara et al., 2019; Pawęska et al., 2018). Indeed, it has been predicted that filoviruses may exist across the Afrotropics (Peterson et al., 2004). More interestingly, the presence of Reston virus has also been confirmed in Asia (Philippines and China). In addition, Mënglà virus, a filovirus of the recently-recognized genus *Dianlovirus*, has been detected in bats in southern China (Yang et al., 2019). With that being said, the presence of filoviruses has also been suggested or confirmed in countries outside of Africa, the Philippines, and China. For example, filovirus-specific antibodies have been detected in the sera of orangutans in Indonesia, humans (bat hunters) in India, and bats in India, Singapore, and Bangladesh (Dovih et al., 2019; Laing et al., 2018; Nidom et al., 2012; Olival et al., 2013). Reston virus is the only filovirus to have been confirmed in Asia; however, in many cases the

detected antibodies were specific to other ebolaviruses that have not been discovered outside of Africa to date. Antibodies against various filoviruses have also been detected in bats in China (Zhang et al., 2020). In Europe, Lloviu virus, of the genus *Cuevavirus*, was discovered in bats that died as part of mass fatalities that occurred among insectivorous bats in caves in France, Spain, and Portugal (Negredo et al., 2011). The discovery of Xīlǎng virus and Huángjiāo virus (*Striavirus* and *Thamnovirus* genera, respectively) in fish (frogfish and filefish) in China is also of interest (Shi et al., 2018). These reports suggest that filoviruses are distributed over a wide area that includes Asia and Europe, and that multiple filovirus species, including unknown varieties, infect various animal species. However, it is unclear whether filoviruses other than ebolaviruses and marburgviruses are pathogenic to humans and nonhuman primates.

Animal models of filovirus diseases

Nonhuman primates such as rhesus and cynomolgus macaques and rodents such as mice and guinea pigs have been used as animal models for hemorrhagic fevers caused by filoviruses (Nakayama and Saijo, 2013). Nonhuman primates experimentally infected with ebolaviruses and marburgviruses show high susceptibility to filoviruses isolated from humans and exhibit clinical signs and pathologies similar to hemorrhagic fevers in humans. Ferrets are also used as experimental animals that exhibit pathologies similar to Ebola virus disease (Cross et al., 2016). However, infection of rodents such as guinea pigs and mice with virus strains isolated from humans does not cause lethal disease though they are susceptible to the viruses. The human-derived virus strains must be adapted through multiple passages in these animals, which accumulate several genetic mutations, before they can be used as an adapted virus strain to cause a lethal infection in these rodents. In addition, although the rodent-adapted filoviruses cause lethal infections of mice and guinea pigs, the blood coagulation abnormalities differ from those seen in primates, and these animal studies could thus be considered suboptimal as a method to elucidate the mechanism of the pathogenesis and determine the efficacy of vaccines and therapeutic agents. However, infection of hamsters with mouse-adapted viruses has been shown to cause a lethal disease more similar to Ebola virus disease in primates (Ebihara et al., 2013). At the same time, it is important to note that, even before adaptation to these rodents, filoviruses isolated from humans can replicate inside the body to some extent without causing disease, and pathogenicity to these animals is acquired with only a few genetic mutations that are most likely involved in interference with the innate immune system. The difference between lethal and subclinical infections is determined by a limited number of amino acid substitutions, and viral pathogenesis is generally controlled by the delicate balance between the proliferative capacity of the virus and the host immune response.

Vaccines and treatment

Following two large outbreaks in West Africa between 2013 and 2016 and in the Democratic Republic of the Congo between 2018 and 2020, research and development into prevention, treatment and diagnostic methods for Ebola virus disease were accelerated and various unapproved vaccines and therapeutic agents were clinically tested and advanced towards the practical use (Hoenen et al., 2019). The vaccines that underwent clinical testing during these outbreaks were based on recombinant viral vectors in which the gene of Ebola virus glycoprotein (GP), the target of neutralizing antibodies, was incorporated into other safe viruses. Although not approved at the time, vaccines using vesicular stomatitis virus (VSV) and combination of chimpanzee adenovirus and vaccinia virus as viral vectors were tested in humans (Ewer et al., 2016; Henao-Restrepo et al., 2015). The recombinant vesicular stomatitis virus-based vaccine was confirmed to be effective and safe, and was subsequently approved by the Food and Drug Administration in the United States of America. In general, a vaccine that uses a viral vector replicates in target cells and newly expresses exogenous viral antigens. For this reason, they are considered to be important for protective immunity because they efficiently activate the innate immune system and induce both neutralizing antibodies and cellular (e.g., cytotoxic T cell) immune responses.

Antibody therapy is the most effective therapeutic method in nonhuman primate models. However, the effectiveness of traditional passive immunization methods (administration of blood serum or plasma from humans who have recovered from the disease) was unclear in the past outbreaks in the Democratic Republic of the Congo. Passive immunization using plasma from patients who had recovered was also attempted during the epidemic in West Africa between 2013 and 2016, but beneficial effects were not evident. This might imply undesirable effects due to the presence of antibodies that cause antibody-dependent enhancement of infection (Takada and Kawaoka, 2003). Meanwhile, the efficacy of neutralizing monoclonal antibody cocktails has been proven in monkey models (Takada, 2013). During the Ebola virus disease epidemic in West Africa, a monoclonal antibody cocktail was used for the first time as an unapproved drug, and it was confirmed to be somewhat effective. Other antibodies were also used during the epidemic in the Democratic Republic of the Congo in 2018 and antibodies with even greater therapeutic effects have been found. However, the fact that the antigenicity of GPs varies substantially among ebolavirus species is a challenge that needs to be overcome in the development of vaccines and antibody therapies. The vaccines and therapeutic antibodies that have been developed so far are specific to Ebola viruses in the species *Zaire ebolavirus* and cannot be expected to be effective against other ebolaviruses. However, several antibodies exhibiting cross-neutralizing activities against multiple species of ebolaviruses have been discovered, and therapeutic effects have been confirmed in animal models (Flyak et al., 2016; Furuyama et al., 2016; Holtsberg et al., 2016). In the future, it is expected that a highly versatile therapeutic agent for Ebola virus disease will be developed using a cocktail of cross-reactive neutralizing antibodies. In addition to antibody drugs, several nucleic acid analog compounds, including those

developed as therapeutic agents for other viral infections, were used as emergency treatments during the Ebola virus disease epidemics in West Africa and the Democratic Republic of the Congo (Kupferschmidt and Cohen, 2015). Some of these compounds have potential as therapeutic agents and may be effective against multiple RNA viruses as broad spectrum inhibitors of RNA-dependent RNA polymerases (Furuta et al., 2005; Warren et al., 2016), although none of them has been approved for treatment of Ebola virus disease (as of June 2020).

Conclusion

For some time after their discovery, filoviruses were considered to be unique to Africa. However, the confirmed presence of filoviruses in Europe and Asia and the detection of filovirus genomes and virus-specific antibodies in some animals in areas where there have been no reports of outbreaks of filovirus infections suggest that unknown filoviruses may be present in a wider area, including in Asia. Alongside the development of effective vaccines and therapeutic agents, the elucidation of the ecology of the viruses in nature and the dissemination of simple and stable rapid diagnostic methods are essential to establish control measures against filovirus diseases. The former will prevent the virus transmission to humans, and the latter will enable appropriate containment measures through early diagnosis.

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Main Acquired Risk Factors of Different Fungal Diseases

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Introduction

Invasive fungal disease (IFD) is associated to high morbidity and mortality. Prolonged survival of patients with malignant diseases, as well as the increased number of patients undergoing solid-organ transplantation (SOT) and hematopoietic stem cell transplantation (HSCT) have significantly heightened the burden of patients “susceptible” to opportunistic infections. Similarly, the appearance and rapid development of new therapies like monoclonal antibodies and small-molecule inhibitors for autoimmune diseases and B-cell malignancies have further increased the pool of patients who might be susceptible to fungal infections. Finally, improvements in diagnostic tests and microbiology laboratories have resulted in a higher likelihood of diagnosing patients with fungal infection.

Over the last, few years, costs associated with fungal infections have increased significantly due to high costs in radiological techniques, microbiology labs and antifungal agents, especially. The widespread use of antifungal agents as both prophylaxis and treatment, and in agriculture has led to an increase in antifungal resistance. To add another element of complexity, antifungal agents are associated with significant short- and long-term toxicity; drug-drug interactions among antifungals, chemotherapy and immunosuppressive agents are a major concern. In this setting, a thorough evaluation of the risk of fungal infection in different populations is essential in assessing the requirement of antifungal prophylaxis, initiating prompt antifungal therapy in high-risk patients with suggestive clinical presentations, avoiding detrimental effects, costs, toxicities, and increasing antifungal resistance associated with the misuse of such agents.

Table 1 At-risk populations and overall risk factors for invasive candidiasis and invasive aspergillosis.

	<i>Invasive candidiasis</i>	<i>Invasive aspergillosis</i>
Risk factors	• Severity of disease • Age: <1 year or >65 years • Comorbidities: diabetes mellitus, chronic hepatopathy, malnutrition, etc. • Prior surgery (mainly abdominal) • Prolonged ICU stay • Invasive devices (urinary catheter, central vascular catheter, arterial catheter, etc.) • Total parenteral nutrition • Mechanical ventilation	• Neutropenia • Deficit of phagocytic function • Cellular immunity alteration • Corticosteroids use • Immunosuppressive drugs • Breach of mucocutaneous barriers • Environmental exposure
At-risk populations and interindividual conditions	• Long-term central vascular catheter • Broad-spectrum antibiotic • Prior <i>Candida</i> spp. colonization • Renal insufficiency and hemodialysis • Neutropenia • Chemotherapy, corticosteroids, and other immunosuppressants • Pancreatitis, visceral perforation, etc. • Critical care patients • Polytraumatic patients • Large burns • Newborns with a low gestational age, use of anti-H2, congenital malformations, gastrointestinal disease or shock	• Profound (<100/mm³) and prolonged (>10 days) neutropenia • Acute leukemia and myelodysplastic syndromes receiving induction or salvage chemotherapy • Allogenic HSCT presenting graft-vs-host disease requiring corticosteroids or suffering CMV infection • Solid organ transplant recipients: lung > heart > bowel > liver > kidney • HIV/AIDS without HAART and a CD4 count below 100/mm³ • Use of “biological therapies”: anti-CD52, Jak-kinase inhibitors, tyrosine-kinase inhibitors, anti-tumor necrosis factor, etc. • Critical care patients, especially patients with viral infections, such as influenza or COVID-19 requiring ICU admission • Patients with solid tumor and predisposing pulmonary lesions • Patients with COPD receiving corticosteroids

Abbreviations: ICU, intensive care unit; HSCT, hematopoietic stem-cell transplant; HIV, human immunodeficiency virus; AIDS, acquired immune deficiency syndrome; HAART, highly-active antiretroviral therapy.

The specific risk of a patient is determined by several different factors, including those associated with the baseline disease and treatment, factors intrinsic to the patient, and the environment. In this chapter, we will analyze risk factors for fungal infections per these factors and grouped in accordance with different high-risk populations.

In the last decade, we have overseen the emergence of fungi, such as *Cryptococcus*, *Trichosporon*, *Fusarium*, Mucorales and dematiaceous fungi. However, this chapter will mainly focus on invasive candidiasis and invasive aspergillosis, as these are the most frequent IFDs worldwide. Table 1 shows overall risk factors and populations at risk for invasive candidiasis and invasive aspergillosis.

Intrinsic and environmental factors associated with fungal infection

Age and comorbidities

Advanced age (with different cut-offs) has been associated with an increased risk of IFD and higher secondary mortality in various studies, particularly in hematological patients. Diabetes mellitus and hyperglycemia hinder innate immunity and have been linked with an elevated risk of IFDs, particularly for rhinoorbital mucormycosis. Deep hypoalbuminemia has also been found to be associated with a higher risk of IFDs in some studies and might be especially important for patients in the intensive care unit (ICU). Smoking was an independent risk factor for fungal infection in some studies, although some confounders may have existed. Patients with previously disrupted lung parenchyma, such as patients with previous tuberculosis or atypical mycobacterial infection, chronic obstructive pulmonary disease (COPD), bronchiectasis, sarcoidosis or lung cancer have a significantly increased risk of aspergillosis, more specifically with chronic and subacute forms. In these series of patients, there are prominent challenges, such as biomarkers are commonly negative, signs and symptoms are quite unspecific, and radiological imaging is quite different from that obtained from immunosuppressed patients.

In the last, few years, some prognostic scores in HSCT patients have been shown to correlate with the risk of fungal infection (Busca et al., 2018). However, performance of these scores in a non-transplant setting remains unknown.

Environmental factors

Molds are found in soil and decaying plant matter, contaminated water and building materials. As molds are usually airborne spread with inhalation and pulmonary involvement as the first compromise step, environmental factors that increase the number of spores in the environment have been related to a higher risk of IFD in susceptible hosts (Garcia-Vidal et al., 2014). Dry weather, high temperatures and some high-exposure activities such as gardening, construction or farming could also increase the risk of IFD caused by molds.

Within the context of a hospital setting, construction work in the hospitalization ward or even beside hospital premises could be responsible for an unusual increase in the incidence of IFD. Additionally, patients undergoing bone marrow transplantation who are hospitalized in rooms without laminar flow or high-efficiency filters are 5.6 times likelier to develop an IFD than patients hospitalized in rooms with such systems in place (Wald et al., 1997). That stated, a thorough anamnesis regarding potential exposures is paramount.

Treatments and manipulations

Chemotherapeutic agents and other new treatments for onco-hematological or autoimmune diseases will be commented upon in the following section. However, we will first present common risk factors that are mainly independent of baseline disease.

Most yeasts causing IFD in humans are commensals, residing in the oral cavity, gastrointestinal tract, and vaginal mucosa. The main risk factors for disseminated yeast infection (mainly candidemia) are as follows: (i) disruption of the cutaneous barrier mostly due to intravascular access devices (which may lead to catheter-related fungemia) but also as a result of deep wounds, ulcerations or burns; (ii) disruption of the mucosal barrier due to gastrointestinal tract surgery; (iii) alteration of normal bacterial flora due to broad-spectrum antibiotics (which leads to an overgrowth and predominance of yeast) and; (iv) total parenteral nutrition (lipid formulations favor fungal invasion).

Glucocorticoids inhibit neutrophil chemotaxis and oxidative bursts and debilitate macrophages' capacity to remove conidia (Stuehler et al., 2015). Consequently, glucocorticoids have been long recognized as an important risk factor for several different IFD, although it is not well established what exact dose and timing place patients at a high risk of infection. A dose of 0.3 mg/kg for at least 3 weeks is generally recognized as posing a significant risk of opportunistic infections. Nevertheless, significantly lower doses may suffice and present such a risk, too, if patients are also neutropenic or lymphopenic or are receiving other predisposing treatments.

Many fungal species, such as Mucorales, use iron as a growth factor. Some studies, mainly performed in hematological patients, have shown iron overload to be associated with an elevated risk of IFD. However, as ferritin is also an acute-phase reactant, these results are controversial.

Respiratory viral infections

Respiratory viruses, such as influenza virus, respiratory syncytial virus, adenovirus and, parainfluenza, are associated with a higher risk of IFD. Within the last, few years, this association has become more evident in patients with severe influenza requiring ICU admission and more recently, with SARS-CoV-2 (Garcia-Vidal et al., 2011; Marr et al., 2020). Respiratory viruses impair mucociliary activity and bronchoalveolar mucosa, favoring the development of IFD (Garcia-Vidal et al., 2014, 2013). Furthermore, respiratory viruses have been hypothesized as being capable of disrupting the local innate immune system and systemic host defenses.

Main groups associated with fungal infection

Hematological patients and hematopoietic stem cell transplant recipients

Patients with a hematological malignancy and those undergoing allogeneic hematopoietic stem cell transplants (HSCT) have been generally considered as facing the highest risk of developing an IFD. This series of patients are extremely susceptible to fungal infections due to the strongly toxic chemotherapies associated with profound and prolonged aplasia, and the increased survival rate in both adult and pediatric populations. However, this risk is highly dependent on factors related to underlying malignancy status and treatment, factors intrinsic to the patient and environment, and the use of prophylaxis.

Risk factors for yeast and mold infections are significantly different in general. The epidemiology of IFD in hematological patients is continuously changing, with a significant decrease in *Candida* spp. in favor of mold infections and particularly *Aspergillus* spp. (Pagano et al., 2007; Kontoyiannis et al., 2010). Tables 2 and 3 summarize the main risk factors for IFD in hematological patients and HSCT recipients, respectively.

Baseline disease and treatment-related factors in hematological malignancies and HSCT

Baseline disease is an extremely important factor when determining the risk of IFD. Patients with acute myeloid leukemia (AML) and more specifically, those receiving induction chemotherapy, have been considered as having the highest risk of developing IFD. Incidence ranging from 7% to 11% for proven/probable IFD and from 24% to 41% for possible IFD have been described in patients not receiving prophylaxis (Douglas and Slavin, 2016). However, some important factors could impact the likelihood of an IFD in these patients. Leukemias with worse prognosis, such as secondary leukemias, refractory leukemias to multiple previous lines, or those with unfavorable cytogenetics, may experience a significantly higher risk of IFD. Acute lymphoblastic leukemia (ALL) or myelodysplastic syndromes (MDS) also present high rates of fungal infections, ranging from 8% to 21% in the absence of prophylaxis. As in AML, baseline disease status, the intensity of chemotherapy regimens, and host factors can highly influence such rates. Other lymphoproliferative diseases, such as chronic lymphocytic leukemia, multiple myeloma, large B-cell lymphoma, or Hodgkin and non-Hodgkin's lymphoma have been classically considered as presenting with low risk for IFD onset. Nonetheless, an increase in incidence has been reported in the last, few years, mainly related to patients treated with multiple lines of treatment.

Table 2 Risk factors for invasive fungal disease in hematological patients.

Net state of immunosuppression
• Neutropenia: intensity, duration, and dynamics
• Lymphopenia: intensity and duration
• Cellular and humoral immunosuppression
• Malnutrition
• Advanced age
• Concomitant immunosuppressor condition
• Use of high doses of cytarabine
• Use of high doses of corticosteroids
• Use of alemtuzumab, anti-thymocyte globulin or infliximab
• Infection due to immunomodulatory virus: CMV, EBV, HHV-6, HHV-7, HHV-8
Organ and system dysfunction
• Mucosa alteration (mucositis) due to radiotherapy, chemotherapy, graft-vs-host disease, herpes viruses, etc.
• Skin and mucosa alteration due to broken barriers, secondary to graft-vs-host disease, vascular catheters, urinary catheters, chemotherapy, radiotherapy
• Renal insufficiency, hepatic insufficiency
• Obstructive and surgical events in the gastrointestinal tract
• Hyposplenias/asplenia
• Lung parenchyma insult due to viral respiratory infection
Microbial colonization and reactivation of latent infections
• Use of broad-spectrum antibiotics
• Antacid use
• Prolonged hospital stay
• Presence of foreign bodies and instrumentalization
• Ciliary function disruption
• Reactivation of latent infections: mycobacteria, toxoplasmosis, CMV, EBV, HSV, VZV, HBV, HCV
• Prior invasive fungal disease
• Increased iron deposits

Table 3 Risk factors for invasive fungal disease in hematopoietic stem-cell transplant recipients.

	<i>Invasive candidiasis</i>	<i>Mold infections</i>
Host factors	• Advanced age • Hematological malignancy • Gastrointestinal tract colonization • Multiple-site colonization prior to transplant	• Baseline disease: acute leukemia, myelodysplastic syndrome, aplastic anemia, multiple myeloma • Advanced age • State of underlying malignancy: refractory > relapsed > complete response • CMV seropositivity status (high/intermediate risk vs low risk) • Iron overload • Diabetes mellitus • Malnutrition • Prior invasive mold disease • Colonization prior to transplant
Hematopoietic stem-cell transplant treatment and complications	• Long-term central vascular catheter • Broad-spectrum antibiotic use and other infectious complications • Severe gastrointestinal mucositis due to conditioning regimen or graft-vs-host disease • High-dose total body irradiation • Prolonged neutropenia	• Type of transplant: allogenic >> autologous • Type of donor: unrelated > related • HLA relatedness: haploidentical > matched siblings > matched unrelated • Source of stem cell: cordon blood > bone marrow > peripheral blood • Conditioning regimen: full intensity > reduced intensity • T-cell depleted transplants, CD-34 selected transplants • Duration of pre-engraftment • Severe (Grade > II) graft-vs-host disease (acute or chronic) with systemic immunosuppression • Corticosteroids use • CMV reactivation (especially CMV disease) • Environmental factors: buildings, high-efficiency filters • Respiratory virus infections • Graft loss

Allogenic HSCT recipients are the other classic group of patients who face the highest risk of fungal infection. Most studies report incidence rates of IFD at around 10–15%, while other studies describe rates reaching up to 50%. Several factors influence the risk of fungal infection in these patients. Active or refractory disease at the time of the transplant is a major risk determinant. Graft source and donor relatedness impact transplant-related toxicity and risk of IFD. That stated, when compared to peripheral blood stem cells, bone marrow and umbilical cord transplants are associated with the highest risk due to delayed immune reconstitution. Similarly, haploidentical transplants and, to a lesser extent, stem cell donors who are not matched siblings are associated with longer immunosuppression and an increased risk of IFD (Marr et al., 2002; Garcia-Vidal et al., 2008). Within the post-transplant setting, graft-vs-host disease (GVHD) confers an extremely high risk of IFD on these patients due to its own immunosuppressive effect, but also due to the required immunosuppressive treatment typically with high-dose glucocorticoids (Teshima et al., 2016). CMV is also associated with an increased risk of IFD in the post-transplant setting probably due to its harming effect on innate immunity. Lastly, autologous HSCT is associated with a significantly lower risk of fungal infection due to a short duration of neutropenia and the absence of risk of GVHD.

Neutropenia is one of the main risk factors for IFD, especially in those patients with profound (<100 neutrophils/mm³) and prolonged (>7 days) neutropenia. However, several recent studies have outlined the importance and relevance of lymphopenia and lymphocyte dysfunction in IFD as well. The degree and duration of these cytopenia will impact the risk of IFD as well as the associated mortality.

Breakthrough fungal infections and patients undergoing solid organ or hematopoietic stem cell transplants after presenting with an IFD are on the rise. Such clinical scenarios pose a great challenge for treating physicians and require a multidisciplinary approach (Lionakis et al., 2018; Puerta-Alcalde et al., 2020).

Risk factors for specific fungi in hematological malignancies and HSCT

Candida spp. and other yeasts

Candida spp. continues to be the main microorganism responsible for opportunistic fungal infections within a hospital environment. Furthermore, it is the fourth leading cause of nosocomial bloodstream infections, which are associated with significant costs and attributable mortality ranging from 10% to 49%. Table 4 outlines the main risk factors and characteristics of IFD caused by *Candida* spp. and other yeasts. Patients with an increased risk of invasive candidiasis, or candidemia in its most common manifestation, are those with hematological malignancy and neutropenia who do not receive prophylaxis. Other factors typically involved in a higher risk of disseminated candidiasis are mainly iatrogenic and/or nosocomial. Chemotherapy-induced mucositis, prior *Candida* spp. colonization, antibacterial therapy (especially antibiotics with activity against anaerobes), central venous catheters, parenteral nutrition, and abdominal surgery are some of the most common risk factors. All these factors facilitate the introduction of *Candida* isolates into the bloodstream and its later dissemination, by which the CNS, eyes and heart will most likely be involved. Chronic disseminated candidiasis or hepatosplenic candidiasis occurred following neutropenia recovery; however, it is rarely seen nowadays due to the widespread use of prophylaxis.

There is great geographical variability in the distribution of species causing invasive candidiasis, according to the epidemiological characteristics and the environment, as well as the specific center. Furthermore, there are different common and specific risk factors per species involved, being associated with different rates of mortality. *Candida albicans* has commonly been the most common species to cause invasive candidiasis. Yet, the widespread use of antifungal prophylaxis within the last, few years have led to a significant increase in non-*albicans* species. This increase is challenging since these species are commonly azole resistant. In this scenario, implementing a bundle of measures have been proven to reduce mortality (Cardozo et al., 2020).

Aspergillus spp.

In the last decade, the incidence of IFD caused by filamentous fungi in hematological patients has increased. Currently, species of the genus *Aspergillus* are the main cause of invasive mold disease in such patients, causing high morbidity and mortality. *A. fumigatus* is the most frequently involved species, responsible for approximately 90% of the infections by this genus. This is mainly secondary to the relatively small size that characterizes *A. fumigatus* conidia, and which allows its penetration deep into the alveolar space. Additionally, *A. fumigatus* can grow in high temperatures (37–50 °C), being more resistant and thermotolerant than other *Aspergillus* species. Depending on geographical factors, host type or antifungal prescription, other species such as *A. flavus*, *A. nidulans* and *A. terreus* are increasingly isolated. This is important, as differences in virulence, response to treatment and prognosis of invasive aspergillosis may arise depending on the species of *Aspergillus* involved.

In relation to the host, the most frequent risk factors are profound and prolonged neutropenia, the presence of organic dysfunctions (mainly renal and hepatic) and/or an underlying pulmonary pathology that facilitates the development of invasive pulmonary aspergillosis. Risk factors associated with treatment include induction or salvage chemotherapy of acute leukemias and myelodysplastic syndromes, HSCT presenting GVHD, and prolonged and/or high-dose corticoid therapy. With respect to environmental conditions, invasive aspergillosis is of external origin (healthcare-related or extrahospitalary) in most cases, unlike most other infections that occur in a hematological patient. Hospital stay in rooms without high-efficiency filters or in close proximity to construction sites or institutional remodeling therefore increase the risk significantly.

Table 4 Risk factors and characteristics of primary *Candida* species and other yeasts that cause invasive fungal disease.

Genus/species	Frequency and characteristics	Risk factors	Mortality
<i>Candida</i> spp. (mainly <i>C. albicans</i>)	Fungemia, endocarditis, endophthalmitis, osteomyelitis, hepato-splenic candidiasis, brain abscess, meningitis, close relation to foreign bodies and endovascular devices	Abdominal surgery, central vascular catheters, total parenteral nutrition, broad-spectrum antibiotics, immunosuppressants, corticosteroids, diabetes mellitus, kidney failure, hemodialysis, transplants, pancreatitis	Overall, around 30–65%, depending on severity scores (APACHE II, SOFA), species, type of host, septic shock, etc.
<i>C. krusei</i>	2–25% of all fungemia 13% in patients with cancer 13–25% in patients with leukemia Breakthrough candidemia	Leukemia, hematopoietic stem-cell transplant, neutropenia, prophylaxis, prior use of azole	Overall 30–70%, with attributable mortality around 40%
<i>C. glabrata</i>	8–37% of candidemia 4.5–13% in patients with cancer Breakthrough candidemia	Solid tumors, abdominal surgery, central vascular catheter, advanced age, hematological malignancy, extensive burns, prophylaxis, prior use of azoles	Overall 45%
<i>C. parapsilosis</i>	12–15% of all candidemia in patients with cancer	Endovascular devices, central vascular catheters, parenteral nutrition, newborns	Overall 8%
<i>C. tropicalis</i>	4–25% of all candidemia 11–25% in HSCT 18% in hematological malignancies 4–9% in solid tumors	Acute leukemia, neutropenia, mucositis	Overall 33–90%
<i>C. lusitanae</i>	2–9% of all candidemia Breakthrough candidemia	HSCT, neutropenia, high-dose chemotherapy, hematological malignancy, prior use of polyenes	Overall mortality of 35%, with attributable mortality around 25%
<i>C. guilliermondii</i>	0.7–5.5% of all fungemia	Malignancies, HSCT, neutropenia, prior use of polyenes	Attributable mortality around 24%
<i>C. dubliniensis</i>	3–25% of oropharyngeal candidiasis in patients with HIV	Neutropenia, mucositis, oropharyngeal in HIV patients	No data
<i>Trichosporon</i> spp.	Fungemia, endocarditis, hepato-splenic forms, purple papules, chorioretinitis	Leukemia, central vascular catheter, intravenous drug user, prosthetic valves	80% in neutropenic patients
<i>Saprochaete capitata</i>	Fungemia, pneumonia, hepato-splenic forms, red skin nodules	Leukemia, central vascular catheter, prior antibiotic use, contaminated milk products	>50% in neutropenic patients
<i>Malassezia</i> spp.	Related to parenteral lipid formulations (requires lipids for growth)	Low-weight newborns, leukemia, central vascular catheter, large burns, solid organ transplant	Low, except for lung involvement
<i>Hansenula</i> spp.	Great spectrum of disease (from asymptomatic to endocarditis)	Low-weight newborns, leukemia, central vascular catheter, intravenous drug use	Low, <10%
<i>Saccharomyces</i> spp.	Fungemia, endocarditis, 0.5% of all fungemia in the ICU	Prolonged ICU stay, newborns, large burns, corticosteroid use, probiotics, central vascular catheter	Overall 30%
<i>Cryptococcus</i> spp.	Meningitis, pneumonia, fungemia, and cutaneous nodes and papules	HIV, leukemia, lymphomas, solid organ transplant, corticosteroid, cirrhosis	20–30% in patients with HIV; 30–40% in patients with malignancy

Abbreviations: HSCT, hematopoietic stem-cell transplant; HIV, human immunodeficiency virus; ICU, intensive care unit.

Emerging fungi

The last two decades have seen an increase in the frequency and severity of IFD caused by Mucorales (*Mucor* spp., *Rhizopus* spp. and *Lichtheimia* spp.), *Fusarium* spp., *Scedosporium* spp., *Lomentospora prolificans* and others. These infections have been closely related with the wide use of antifungals as both prophylaxis and treatment (Lamoth et al., 2017; Imhof et al., 2004). Although IFD by emerging fungi are rare, the importance of these types of infections lies in the aggressive behavior of the IFD, degree of host immunosuppression and broad profile of resistance to most antifungals available. All of this is associated with high morbidity and mortality rates around 65–90%.

Solid organ transplant

IFD poses a significant threat to patients undergoing a solid organ transplant (SOT). IFD caused by opportunistic fungi have a universal distribution and are mainly caused by *Candida* spp., *Aspergillus* spp. and, to a lesser extent, *Cryptococcus* spp., fungi pertaining to the order Mucorales, and other molds. IFD features vary widely depending on the organ transplanted, surgery performed, baseline disease and comorbidities, and type of immunosuppression. The most important determinants of IFD include epidemiological exposure and the net state of immunosuppression.

Table 5 Most common IFD in SOT and prophylaxis recommendations.

Transplant	Most common invasive fungal disease			Prophylaxis recommendation	Length of prophylaxis
	First	Second	Third		
Kidney	Candidiasis	Cryptococcosis	Aspergillosis	Not recommended	–
Liver	Candidiasis	Aspergillosis	Cryptococcosis	Fluconazole	1–4 weeks
				High-risk: liposomal amphotericin B or echinocandin	
Pancreas	Candidiasis	Endemic mycoses	Aspergillosis	Fluconazole	1–4 weeks
Lung	Aspergillosis	Other molds	Candidiasis	Voriconazole or nebulized amphotericin B	3–6 months
Heart	Candidiasis	Aspergillosis	Other molds	Not recommended	–
Bowel	Candidiasis	Other yeasts	Aspergillosis	Fluconazole or liposomal amphotericin B	1–4 weeks

The timing of various IFD after SOT is highly dependent on the different fungi pathogenesis. For example, within the first month of a transplant, invasive candidiasis is clearly predominant due to surgical complications. Later, secondary to the immunosuppression due to transplant or acute rejection, other IFDs become more frequent.

IFD diagnosis is challenging in SOT recipients. A nuanced approach, including the consideration of the type of transplantation, immunosuppression and time since transplant, is paramount. The role of fungal biomarkers within this setting of non-neutropenic patients is low, and the culture remains the most helpful technique. Table 5 summarizes the most common fungal diseases and prophylactic recommendations in SOT.

Risk factors for specific fungi in SOT

Candida spp. and other yeasts

Candida spp. is the main agent responsible for IFD in SOT recipients and is particularly frequent following small bowel transplants due to long-lasting abdominal surgery. In recent years, a reduction in the incidence of invasive candidiasis has been observed in patients with SOT, being reported at around 2% and in contrast to the 4–6% described in other decades. However, this incidence varies according to the organ transplanted, and is especially high in liver, pancreatic, and intestinal transplants. For these patients, invasive candidiasis implies high morbidity and mortality between 30% and 40%. In liver transplants, the main risk factors for invasive candidiasis are renal failure (especially in patients undergoing dialysis) and re-transplantation. An increase in risk has also been described in the following situations: fulminant hepatitis, need for high transfusion requirements, biliary anastomosis by Roux-en-Y technique, and need for surgical reintervention during the immediate post-transplant period. In pancreatic transplant, risk factors for invasive candidiasis include bladder drainage vs enteric drainage, graft thrombosis, the presence of pancreatic fluid fistulas in the peritoneum, cytomegalovirus infection and the age of the donor being older than the recipient. *Candida* infections can present as candidemia, peritonitis, urinary tract infection, empyema, surgical wound, or anastomosis infection.

Aspergillus spp.

The most common clinical form of invasive aspergillosis is pulmonary, with subsequent dissemination. Incidence of invasive aspergillosis varies from less than 1% in kidney and liver transplants to around 2% in pancreas and heart transplants, and 4% in lung transplants. Although invasive aspergillosis has been generally considered as a complication arising soon after the transplant, different studies have shown that incidence of such infection remains high even once this period passes. Invasive aspergillosis is particularly concentrated in specific subpopulations among SOT recipients. These high-risk patients differ according to each of the transplanted organs (Table 6). However, there are some common factors: re-transplantation, CMV disease, and hemodialysis significantly increase the risk of early invasive aspergillosis (<3 months after transplantation). For late aspergillosis (>3 months post-transplantation), risk factors include renal failure or a higher degree of immunosuppression.

In liver transplant recipients, fulminant hepatitis, and chronic graft dysfunction due to hepatitis C virus recurrence have been described as specific factors that increase the risk of invasive aspergillosis. Mortality due to invasive aspergillosis in liver transplant ranges from 50% to 65%.

Incidence of invasive aspergillosis in lung transplant is the highest among SOT recipients, ranging from 4% to 23.3% without prophylaxis. Currently, more than half of episodes occur after the first 6 months of transplantation. Airway colonization by *Aspergillus* spp. during the first 6 months after transplantation increases the risk of invasive aspergillosis by 11 times. Other factors that confer a greater risk onto this population are ischemia of the bronchial anastomosis, unilateral pulmonary transplantation, hypogammaglobulinemia, placement of a bronchial prosthesis and CMV infection. Mortality of invasive aspergillosis in lung transplant depends on clinical presentation, with those patients suffering from invasive lung disease, presenting facing a mortality rate as high as 80%.

Table 6 Risk factors for invasive aspergillosis following solid organ transplant.

	Early invasive aspergillosis	Late (>90 days after transplant) invasive aspergillosis
Hepatic transplant	Re-transplant Kidney failure, especially post-transplant hemodialysis Transplant for fulminant liver failure Complicated surgery or surgical re-operation CMV disease	Over 6 g of prednisone in the 3 months after transplant Post-transplant hemodialysis Post-transplant kidney failure Post-transplant leukopenia ($<500/\text{mm}^3$) Chronic graft failure due to hepatitis C virus recurrence
Lung transplant	Bronchial Anastomosis Ischemia or bronchial stent placement CMV Infection Acute rejection Single-pulmonary transplant Colonization by <i>Aspergillus</i> spp. pre-transplant or within the first year Hypogammaglobulinemia (IgG <400 mg/dL) CMV disease	Unilateral pulmonary transplantation Chronic rejection
Heart transplant	Isolation of <i>Aspergillus</i> spp. in respiratory tract cultures Surgical re-intervention CMV disease Post-transplant hemodialysis CMV disease	ICU re-admission Post-transplant kidney failure Levels FK > 15 or CyA > 500 after month 3 > 2 episodes of acute rejection
Renal transplant	Graft loss Hemodialysis High and prolonged doses of steroids CMV disease	—

Abbreviations: CMV, cytomegalovirus; ICU, intensive care unit; FK, tacrolimus; CyA, cyclosporine A.

Cryptococcus spp.

The incidence of cryptococcosis in the American TRANSNET cohort accounted for 8% of all IFD, being the third most frequent causal agent after candidiasis and invasive aspergillosis, and is particularly frequent among kidney and heart recipients (Pappas et al., 2010). Evidence suggests that most cryptococcoses in SOT recipients result from reactivation of subclinical infection and cause infections with late clinical presentation (over 12–24 months after transplant). Patients receiving high doses of corticosteroids or monoclonal antibodies, such as alemtuzumab and infliximab, appear to face the highest risk of disseminated cryptococcosis.

Emerging fungi

Over the last, few years, mucormycosis has been reported as increasingly important in SOT, occurring late after transplant in general (Park et al., 2011). The most common clinical presentation forms are fungal pneumonia and rhinosinus infection. A high degree of clinical suspicion is necessary, as a delayed initiation in administering appropriate therapy is associated with poor prognosis (Chamilos et al., 2008). However, the frequent lack of profound neutropenia results in a more favorable prognosis for these patients when compared to hematological patients, with mortality rates around 20%. Classical risk factors for invasive mucormycosis are uncontrolled diabetes mellitus, corticosteroids, neutropenia, renal failure, reactivation of herpes viruses, malnutrition, and prior use of either voriconazole or caspofungin (Singh et al., 2009).

Critical patients

In the last decades, intensive care units have grown in complexity for various reasons: admission of older and more comorbid patients, rising admission of immunosuppressed patients, increased survival in situations of extreme frailty, advancement and novelty of surgical techniques, and multiple instrumentations. As a result, incidence of IFD has risen in critically ill patients, with patients admitted to intensive care units constituting the second group in frequency of invasive mycosis (40%) only after surgical patients. This is of the utmost importance since the impact of IFD on the prognosis of these patients is huge. Table 7 shows the main risk factors for IFD in ICU patients.

Invasive candidiasis is the most frequent fungal infection in this setting, followed less commonly by invasive aspergillosis. The percentage of fungal isolates in severe infections depends on patient characteristics, patient's individual risk factors, and the type of ICU. In a general ICU, approximately 15–20% of severe infections are IFD.

There are three fundamental factors in managing fungal infections in critically ill patients: (i) those related to the host, such as the presence of comorbidities or clinical severity; (ii) those related to the pathogen, such as antifungal sensitivity or fungal virulence; (iii) those related to treatment, such as the spectrum of activity, PK/PD characteristics, safety and interactions.

Table 7 Risk factors for invasive fungal disease in intensive care unit patients.

Non-neutropenic	Neutropenic
• Disease severity • Major surgery (mainly abdominal) • Central venous catheters • Prior use of antibiotics • Total parenteral nutrition • Renal or hepatic insufficiency • Extreme ages (old patients and low-weight newborns) • Corticosteroid use • Multifocal colonization • Need for mechanical ventilation • Comorbidities • Cellular immunity dysfunction due to severe inflammatory response syndrome	• Baseline disease • Solid organ transplant and hematopoietic stem cell transplant • Prior use of antibiotics • Hemodialysis • Need for mechanical ventilation • Chemotherapy and other immunosuppressants • Pancreatitis • Disease severity

Risk factors for specific fungi in critical care units

Candida spp.

Candida spp. is the fungal genus that most frequently causes IFD in critical care patients. Incidence of invasive candidiasis is 10 times higher in critical patients than when compared to patients in general wards from the same hospital. *Candida albicans* is the fifth most frequent pathogen isolated as a cause of major intra-ICU infections. *Candida* spp. is the second leading cause of urinary tract infection, and *C. albicans* the second causal pathogen, only after *Escherichia coli*. In several series, different species of *Candida* spp. constitute the third or fourth most frequent cause of bloodstream infection (Kett et al., 2011). Around 10% of these infections are candidemia, with *C. albicans* being the fourth etiological agent globally (Wisplinghoff et al., 2004).

In ICU patients, overall mortality stands at approximately 45–70% with an attributable mortality of 25–45%. The following factors are associated with increased mortality in critically ill patients with invasive candidiasis: (i) the type of *Candida* species involved, as some species have a higher degree of virulence; (ii) inappropriate antifungal treatment; (iii) delay in starting appropriate antifungal treatment; (iv) degree of involvement, as measured by the APACHE scale; (v) prolonged ICU stay; (vi) secondary renal failure; (vii) hematological malignancies; (viii) need for mechanical ventilation; (ix) need for vasoactive support.

Although almost 80% of ICU patients can be colonized by *Candida* spp., only around 10% of these patients will present with invasive candidiasis. Critical patients with a higher risk of invasive candidiasis are generally those with a history of major intestinal surgery, especially those on total parenteral nutrition, receiving broad-spectrum antibiotics, with central venous catheters, with any type of immunosuppression, on dialysis or in the most severe condition.

Identifying risk factors early for invasive candidiasis is fundamental in order to initiate prompt treatment and thereby reduce the mortality rate associated with such infections. There are different rules to identify patients at risk of invasive candidiasis. One of the most recognized rules is the “*Candida* score” (León et al., 2006). This score allows for the identification of critical, non-neutropenic patients with suspected candidemia. It is based on the predictive value of different risk factors previously demonstrated by logistic regression. Estimated scores obtained for each risk factor are as follows: parenteral nutrition—1, previous surgery—1, multifocal colonization by *Candida*—1, and severe sepsis—2. A value of a “*Candida* score” equal or higher than three allows clinicians to select those patients who may benefit from early antifungal treatment under the clinical suspicion of invasive candidiasis, with a sensitivity of 81% and a specificity of 74%. This score has been prospectively validated, demonstrating that <5% of patients with a “*Candida* score” below three developed invasive candidiasis (Leroy et al., 2011). Conversely, of patients with a score higher than 3, 30% presented with invasive candidiasis. These scores may therefore be useful during the decision-making process about whether empirical treatment should be initiated in a febrile patient. Similarly, these scores may even help to assess the use of antifungal prophylaxis in patients who have high score values.

Aspergillus spp.

In recent years, an increase in the incidence of invasive aspergillosis in critical patients without a diagnosis of neutropenia or a hematological disease has been documented. Such incidence ranges from 0.3% to 5.8% with overall mortality close to 80% and attributed mortality of 20%. *Aspergillus fumigatus* is the main species involved, followed by *A. niger* and *A. flavus*.

Critically ill patients present with an alteration in the immune response and depression of the phagocytic mononuclear system function, occurring in late-phase multiorgan dysfunction, primarily. Such immunosuppression especially affects alveolar macrophages and can explain the increased risk of pulmonary aspergillosis. Predominant risk factors for invasive aspergillosis in the ICU are chronic obstructive pulmonary disease (COPD) and steroid treatment. Chronic liver disease, malnutrition and diabetes mellitus are also considered risk factors.

Association of severe influenza viruses, especially influenza A (H1N1), with invasive aspergillosis has been recently described. One hypothesis for such association is that influenza infection may cause diffuse injury to the respiratory mucosa and facilitate

fungus invasion. A recent review on this topic showed that the great majority of invasive aspergillosis cases were caused by influenza A (only 10% of cases were described as being caused by influenza B) and median time from influenza to invasive aspergillosis was only 5 days (interquartile range 2–11 days) (Schauwvlieghe et al., 2018). Twenty-eight percent of cases had no comorbidity and only 25% had immunosuppression. Furthermore, 21-day mortality was 57%. Most importantly, performing a diagnosis of such pathogenic entity remains challenging, as around 50% of cases did not meet classical criteria for invasive aspergillosis, 65% presented with atypical CT lesions and galactomannan was positive in less than a third of all cases.

The coronavirus disease 2019 (COVID-19) pandemic has become a great health challenge, with tremendous impact on our social, economic and health livelihoods. Aspergillosis complicating COVID-19 has been sporadically reported since the beginning of this devastating pandemic; however, exact incidence of this severe complication is unknown and commonly underreported (Marr et al., 2020). Most importantly, most patients who experience this complication do not present any of the classic risk factors, e.g., immunosuppression or neutropenia, even though corticosteroid use is common in the different series (Apostolopoulou et al., 2020). Patients with a higher risk are those requiring ICU admission and mechanical ventilation due to moderate to severe to acute respiratory distress syndrome (ARDS) (Dupont et al., 2020).

Other fungal infections

Other types of yeasts, such as *Cryptococcus*, *Rhodotorula* or *Trichosporon*, are seldomly reported in the ICU context. Like *Candida* spp., these types of yeast are mainly related to surgical procedures and long-term central vascular catheters. Similarly, invasive mold disease caused by either Mucorales, *Fusarium* spp. or *Scedosporium* spp. is very rare in this group of patients.

Pediatric patients

The burden of pediatric patients at risk of IFD has also been on a rise in the last, few years. Fungal infections in this population are associated with significant morbidity and mortality. Characteristics and clinical manifestations of pediatric patients are different from those observed in adults. Table 8 shows the main risk factors for IFD in pediatric patients. In order to assess the risks of these patients, the following items should be borne in mind: (i) etiological agents involved may be different from those seen in the adult population; (ii) there is a greater frequency of focal infection in pediatric patients following a fungemia, even though clinical findings and manifestations may be unspecific; (iii) the sensitivity and specificity of available diagnostic resources are lower and there is less evidence to apply early treatment; (iv) the use of antifungals in pediatrics is very limited due to the both lack of studies on pharmacokinetics and toxicity in this population and challenges presented in monitoring such patients; (v) with the exception of the neonatal population, few studies consider the risk by age group.

The most common risk factors in this population are the following: (i) extremely premature infants (particularly those with a weight <1500 g); (ii) children with cancer; (iii) those undergoing HSCT or SOT; (iv) neutropenic children; (v) those with long ICU stay; (vi) children undergoing invasive procedures and major surgeries; (vii) children with primary or acquired immunodeficiency, congenital cardiopathies or pulmonary diseases.

Table 8 Special-risk populations and risk factors for invasive fungal disease in the pediatric population.

Pediatric populations at high risk of invasive fungal disease
• Immunosuppressed patients
• Newborns, especially those with low birthweight (<1500 g)
• Patients admitted to the ICU
• Onco-hematological patients and HSCT recipients
• Solid organ transplant recipients
• Children infected with HIV
• Congenital immunodeficiencies
Main risk factors for invasive fungal disease (mainly <i>Candida</i> spp.)
• Age: newborns and breastfeeding babies (microbiota and local immunity have not sufficiently developed)
• Use of corticosteroids: due to immunity alteration
• Broad-spectrum antibiotics: due to alterations in bacterial microbiota
• Skin and mucosa alteration: vascular catheters, urinary catheters, peritoneal catheter, valvular prosthesis or any other foreign device
• Malnutrition and immunosuppression: hypovitaminosis, hypogammaglobulinemia, cellular immunity dysfunction
• Total parenteral nutrition
• Prior abdominal surgery
• Respiratory viral infections
• Mechanical ventilation
• <i>Candida</i> spp. multiple-site colonization

Abbreviations: ICU, intensive care unit; HSCT, hematopoietic stem-cell transplant; HIV, human immunodeficiency virus.

***Candida* spp. and other types of yeast in pediatric patients**

Candida spp. is the main agent responsible for IFD in the pediatric patient. In this population, most cases of invasive candidiasis are produced by *C. albicans*. However, given the increasing use of azoles in recent years, an increase in other species with decreased sensitivity to azoles, such as *C. glabrata* or *C. krusei*, has been observed.

There are certain risk factors that predispose the pediatric population to invasive candidiasis:

- 1) Skin colonization: 5–10% of newborns admitted to the ICU are colonized by *Candida* spp. In low-birth-weight infants, the percentage seems to be slightly higher. At 1 week of admission, colonization increases to 50%; at 1 month, it is 64%.
- 2) Broad-spectrum antibiotic therapy over a 5-day period: associated with the destruction of intestinal bacterial flora and colonization by *Candida* spp. with subsequent invasion of the mucosa.
- 3) The presence of a central venous catheter: especially in critical and immunosuppressed children, or pediatric patients undergoing chemotherapy, transplants, or with parenteral nutrition, etc. Some species, such as *Candida parapsilosis* and *Candida tropicalis*, can produce biofilms making it difficult to eliminate them effectively.
- 4) Hyperglycemia: this can favor fungal growth and decrease the host's defense ability by altering the regulation of protein adhesion genes and the capacity of the complement to opsonization.
- 5) The loss of protective barriers: this may include damage to skin barriers caused by wounds or burns and a decrease in gastric acidity due to the use of proton pump inhibitors. The latter can favor fungal growth in the gastrointestinal tract.
- 6) Other factors that directly alter a patient's defense mechanisms: mechanical ventilation, major abdominal surgery, acute pancreatitis, diabetes mellitus, corticosteroid therapy, neutropenia, neutrophilic dysfunction and other immunological changes.

In premature infants, especially those who weigh little (1000–1500 g), invasive candidiasis associated risk factors include those previously mentioned, with emphasis on malnutrition and invasive devices. In preterm infants, the main risk factor associated with invasive candidiasis is an immature immune system, especially with respect to low levels of maternally transmitted immunoglobulin G, decreased opsonization and decreased complement functions. In older pediatric patients, risk factors resemble more closely those found in adults.

IFD caused by emergent yeasts, such as *Cryptococcus* or *Trichosporon*, are seldom and generally affect immunosuppressed children secondary to HIV, SOT, HSCT, primary immunodeficiencies or hematological malignancies.

***Aspergillus* spp. and other molds in pediatric patients**

Incidence of invasive aspergillosis and other molds in pediatric patients has increased in the last decades. Around 75% of all mold infections in pediatric patients are observed in those with onco-hematological conditions. In such populations, cumulative incidences are described at being around 2%, with related mortality at 55%. Other populations at risk of invasive mold disease are SOT recipients and patients with primary or acquired immunodeficiency. These infections are also seen in critical care patients, especially in premature infants with a low birth weight.

Similar to adults, profound and prolonged neutropenia (<500 neutrophils/mm³ and >10 days) is the main risk factor for invasive mold disease in these populations. Some other factors are the following: (i) prior chemotherapy; (ii) broad-spectrum antibiotics; (iii) high-dose corticosteroids; and (iv) some specific immunosuppressants, such as purine analogs, monoclonal antibodies, cyclosporine, or tacrolimus.

Epidemiology and clinical profiles of patients with invasive mold disease other than *Aspergillus* spp., such as mucormycosis or hyalohyphomycosis, are less well characterized in pediatric patients.

Solid tumor patients

In the last years, the number and mechanisms of oncological treatments have increased. For many of these treatments it is still too early to know if they will be related to a significantly heightened risk of IFD. Some of these treatments, such as tumor necrosis factor inhibitors, anti-lymphocyte antibodies, interleukin inhibitors, tyrosine kinase inhibitors or immune checkpoint inhibitors have been reported to be associated with cases of fungal diseases (Davis et al., 2020). However, attributing the specific role of these agents in the likelihood of fungal infections is challenging, as this series of patients often receive multiple lines of therapies, present with advanced malignancies and commonly receive other immunosuppressive agents, such as corticosteroids. Finally, toxicities secondary to these agents usually require high doses of corticosteroids, being the final cause of infection in many cases.

***Candida* spp. and other yeast in oncological patients**

While candidemia in patients with hematological malignancies is mainly related to profound and prolonged neutropenia and to important mucositis, these factors are rather uncommon in patients with solid tumors. The main risk factors for yeast infection (mainly *Candida* spp.) in solid tumors are oncological surgery and concomitant complications, especially abdominal surgery; ICU admission and need for mechanical ventilation; parenteral nutrition and the frequent need for long-term central vascular catheters. As fluconazole is not systematically used in these patients (in contrast to hematological patients), the prevalence of non-*albicans* species of *Candida* and other yeasts (such as *Trichosporon* spp.) is expected to be lower.

***Aspergillus* spp. and other molds in oncological patients**

The diagnosis of invasive aspergillosis is commonly not considered in oncological patients, leading to significant delays in proper diagnosis and treatment, as well as worse outcomes more frequently. The aforementioned EORTC/MSG definitions for fungal infections are focused on hematological patients (De Pauw et al., 2008). Oncological patients do not usually meet host criteria and radiological signs of pulmonary fungal disease can vary significantly in these individuals. Similarly, the yield of fungal biomarkers, such as galactomannan, has not been well established in these patients. Despite these difficulties, IFD and predominantly invasive pulmonary aspergillosis, are being increasingly reported in patients with solid tumors, with mortality rates similar to those observed in hematological patients. Main risk factors associated with invasive pulmonary aspergillosis in patients with solid tumors are primary pulmonary compromise due to neoplasia, use of glucocorticoids, prior chemotherapy, prior radiotherapy, critical illness and previous pulmonary disease, such as chronic obstructive pulmonary disease or asthma (Dib et al., 2020). Additionally, mold disease in oncological patients can commonly present in more saprophytic or chronic forms in lung areas previously affected by baseline disease or treatment, such as cavitation, scars, or bronchiectasis.

***Pneumocystis jirovecii* and others**

The emergence of new-generation immunosuppressive agents like rituximab and other cytotoxic agents have been associated with an increased number of *Pneumocystis jirovecii* pneumonia (PJP) cases in patients with solid tumors. Apart from the direct immunosuppressive effect of these agents, toxicity requiring glucocorticoids is the main factor evidenced in many cases. Profound and prolonged lymphopenia predisposing to PJP can also develop after biological and immunomodulatory treatments. Although primary prophylaxis with cotrimoxazole could help diminish the risk of PJP, its value in patients without HIV has barely been tested, and clear indications for treatment initiation are lacking. Similarly, the value of corticosteroids in these patients is less proven, although it seems to be beneficial (Mundo et al., 2020).

Most importantly, the mortality of patients without HIV and with PJP seems to be higher than in patients with HIV (Avino et al., 2016). Several reasons may explain this fact: (i) less awareness of this disease among oncologists, leading to delays in diagnosis and treatment; (ii) difficulties in differentiating pulmonary involvement in PJP from that secondary to lung toxicity secondary to cancer treatments, or from radiotherapy pneumonitis; (iii) patients with oncological diseases and clinical suspicion of PJP frequently present with an advanced tumor state and thereby limit the possibility of performing a bronchoscopy and obtaining a firm diagnosis.

Sporadic case reports exist of different rare fungal infections, such as cryptococcosis in patients with solid tumors. However, these cases are highly uncommon and usually due to a sum of factors, such as multiple lines of therapy for a progressive disease, corticosteroids, and ICU admission.

Other groups of at-risk patients

Patients with HIV

Patients infected with HIV have been the paradigm of the immunosuppressed patient for a long time. In fact, for reasons unbeknownst, patients with low CD4 present with higher rates of certain IFD (oropharyngeal candidiasis, meningeal cryptococcosis, etc.) than, for example, hematological patients with similar lymphocyte counts. However, since the development of highly active antiretroviral therapy (HAART), frequency and severity of IFD have been widely improved. In the beginning years of the HIV pandemic, rates of fungal infection were reported at around 25%, but rapidly decreased after the introduction of HAART (Antinori et al., 2009). Alongside HAART, better knowledge of such infections and improved therapies have resulted in a significant decline in morbidity and mortality. However, in low-income countries, IFD continues to be a huge problem for patients with HIV/AIDS, particularly cryptococcosis and histoplasmosis. Over 200,000 new cases of cryptococcal meningitis occur each year worldwide, resulting in 181,000 deaths; the majority of which occur in sub-Saharan Africa (Rajasingham et al., 2017). In Latin America, histoplasmosis is one of the most common opportunistic infections in patients with HIV, with rates of mortality around 30%.

When evaluating IFD in patients with HIV, environmental, structural, and immunological factors should be considered. One of the main risk factors in this population is a CD4 count below 200/mm³. Risk factors for developing IFD depends mainly on the following aspects: (i) degree of cellular immunity impairment; (ii) use of antifungal therapy; (iii) risk of environmental exposure; (iv) neutropenia; (v) use of prophylactic regimens.

Pneumocystis jirovecii pneumonia or PJP is the most common invasive fungal disease in patients with AIDS and is also the most common opportunistic respiratory infection in this population. It typically occurs in patients with HIV and a CD4 count <200 cells/mm³ who are not receiving appropriate prophylaxis. Depending on the geographical context and degree of immunosuppression, the other, more frequently seen IFD in patients with HIV are invasive aspergillosis and cryptococcosis. Almost all cases of cryptococcal-disseminated disease (mainly with meningitis +/- encephalitis) are seen among patients with AIDS and a CD4 count <100 cells/mm³.

In some high-income countries with high adherence of patients to HAART, the incidence of both pneumocystosis and cryptococcosis has sharply decreased. In some such areas, these IFD have been replaced by endemic fungi, such as *Histoplasma* spp. and *Coccidioides* spp., or endogenous IFD, such as invasive candidiasis.

Chronic pneumopathy patients

Pulmonary aspergillosis is the most common fungal disease in patients with chronic pneumopathy (mainly chronic obstructive pulmonary disease [COPD]), causing different entities, such as invasive aspergillosis, chronic pulmonary aspergillosis (including aspergilloma), hypersensitivity pneumonitis, and allergic bronchopulmonary aspergillosis. Invasive pulmonary aspergillosis in patients with COPD is an extremely severe entity, with a high mortality rate of around 90% (Bulpa et al., 2020). A relevant factor for this poor prognosis seems to be the difficulties in diagnosis, arising from a non-specific clinical presentation and the absence of complementary, non-invasive tests. In addition, as these patients are commonly considered as non-immunosuppressed, diagnosis may be further delayed due to the lack of awareness regarding diagnostic possibility of invasive pulmonary aspergillosis. Further, the absence of an agreed definition and infection surveillance measures, as well as the challenge in differentiating colonization from infection add further obstacles.

The main factors associated with an increased risk of invasive aspergillosis are the administration of corticosteroids (mainly systemic but also inhaled), advanced stages of the disease (Gold III-IV), administration of broad-spectrum antibiotics in the previous months, and ICU admission, especially.

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Candida and Candidiasis

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Epidemiology

Candidiasis is a cosmopolitan fungal infection caused by fungi of the genus *Candida*. These yeasts exist in single-cell form and multiply by budding. In human samples, *Candida* spp. is observed as round yeasts 4–6 μ m in diameter with a thin wall, which can emit pseudo-hyphae, particularly *C. albicans*. *Candida* spp. is saprophyte of skin, vagina and intestinal flora, and is found in 30% of individuals (Sardi et al., 2013). The majority of disseminated diseases is of endogenous origin with digestive, skin, or genitourinary portal of entry. Exogenous transmission is rare, generally secondary to human-to-human transmission by manual, oral, or sexual, or vertical route during the perinatal period.

Ten main species are pathogenic to humans, more than 90% of invasive candidiasis being attributed to five species: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* (Falagas et al., 2010; Guinea, 2014; Pfaller et al., 2005). *C. albicans* is the most widespread and ubiquitous yeast. Until the 1990s, it was responsible for about two-third of candidiasis with a decreasing incidence in time (Guinea, 2014). Increased use of antifungal prophylaxis, and other factors such as age, use of broad spectrum antibiotics, increased life expectancy with cancer have substantially changed the epidemiology over the past two decades (Farmakiotis et al., 2014; Guinea, 2014; Leroy et al., 2009; Tan et al., 2015). Species with lower susceptibility to antifungals have emerged. These strains are associated with intrinsic resistance (*C. krusei*) or reduced susceptibility to azoles (*C. glabrata*) or echinocandins (*C. parapsilosis*). Epidemiological changes are largely linked to the use of antifungal drugs, particularly azoles. Geographic location and age of the patient are two factors influencing the distribution of species. *C. glabrata* infections are more particularly present in the northern hemisphere, northern Europe, the United States, and Canada, and their frequency increase with age and number of cancer patients previously exposed to antifungal treatments (Farmakiotis et al., 2014; Milazzo et al., 2014). In contrast, *C. parapsilosis* infections are more common in the southern hemisphere, especially Asia, and in southern Europe. They typically affect newborns and patients with intravenous catheters (Arnold et al., 2010; Gamaletsou et al., 2012; Pappas et al., 2018). *Candida tropicalis* is more common in patients with leukemia and neutropenia (Silva et al., 2012). Common risk factors for *Candida* infections are age, colonization, length of stay in intensive care, broad spectrum antibiotic therapy, intravascular devices, diabetes, parenteral nutrition, renal failure, hemodialysis and hemofiltration, antifungal prophylaxis, surgery, acute necrotizing pancreatitis and corticosteroid therapy.

A new *Candida* species has emerged and is currently present in all continents. *Candida auris* was first described in 2009 in Japanese and Korean patients. Prevalence and mortality are largely underestimated due to its being a difficult-to-identify species (Navalkele et al., 2017). In a prospective study conducted in intensive care in India, *C. auris* was the fifth cause of candidemia, with a prevalence of 5.3% (Rudramurthy et al., 2017). Analysis was even more worrisome in South America, between March 2012 and July 2013, where *C. auris* represented the sixth pathogen found in blood stream infections.

The risk factors for *C. auris* infection seemed quite similar to those of other *Candida* spp. A study comparing patients with *C. auris* candidiasis versus those with candidemia from another *Candida* spp. showed that patients with respiratory disease, vascular surgery, and 30-day exposure to antifungal drugs were at high risk of developing an infection with this new (Rudramurthy et al., 2017). Regarding resistance to usual antifungal treatments, more than 60–80% of *C. auris* isolates are resistant to fluconazole, 10–30% to amphotericin B, and approximately 10% are resistant to echinocandins (Chakrabarti et al., 2015; Chowdhary et al., 2013; Colombo et al., 2014).

The worldwide incidence of candidemia is difficult to ascertain. It depends on hospital admissions or ICU admissions, rate of cancer, rate of transplant recipients, patient age, and the overall health of the population. In the United States from 2008 to 2011, candidemia had an annual incidence rate of 13.3–26.2/100,000 population associated with a 30-day mortality rate of 28–29% (Cleveland et al., 2012; Fagan et al., 2013). Candidemia is the fourth most common cause of death and represents more than 85% of all fungemia (Sardi et al., 2013; Silva et al., 2012). To compare, in France from 2001 to 2010, the annual incidence rate was 3.6/100,000 population (Bitar et al., 2014). In the US, candidemia represents 10% of blood stream infections versus 2–3% in Europe (Asmundsdottir et al., 2013; Cleveland et al., 2012, 2015; Meyer et al., 2013; Pfaller and Diekema, 2007; Puig-Asensio et al., 2014).

A large prospective population-based cohort study carried out between May 2010 and April 2011 in 29 Spanish hospitals described the epidemiology of candidemia according to main comorbidities (Cuervo et al., 2015). In older patients >75 years, a urinary origin of candidemia was more often found; risk factors such as presence of central venous catheter, parenteral nutrition, neutropenia, immunosuppressive therapy were less often encountered. In neutropenic patients, candidemia was less often linked to long-term catheter infection and was more frequently caused by *C. tropicalis*, *C. krusei*, and *C. glabrata*. 30-Day mortality was the same as in the general population, with a rate approximating 30%. In surgical wards, mortality attributable to candidemia was significantly lower than in medical wards, hematology or ICU. This may be due to less severe underlying diseases. In solid organ transplant recipients, a high rate of species resistant to fluconazole (>50%) and non-albicans species, particularly *C. glabrata*, has been described. In this setting, early catheter withdrawal and early initiation of appropriate antifungal therapy were protective with regard to 30-day mortality (Fernández-Ruiz et al., 2019; Fortún and Gioia, 2017; Ramos-Martínez et al., 2017; Vena et al., 2017).

Invasive candidiasis, accounting for 70–90% of all invasive fungal diseases, remains the most common cause of sepsis or septic shock of fungal origin. Invasive candidiasis is associated with high mortality, particularly in ICUs, even though this rate has varied among studies from 5% to 71%. Factors such as *Candida* species, age, underlying chronic diseases and hospitalization in ICUs influence the mortality rate (36–40). It should be noted that the candidiasis increase in ICU has been reported worldwide (Colombo et al., 2014; Lortholary et al., 2014). When compared with candidemia outside of ICUs, ICU candidemia is characterized by higher pre-exposure to azoles and echinocandins, a lower incidence of *C. parapsilosis*, and higher mortality (Leroy et al., 2009; Lortholary et al., 2014). In patients with hematological malignancies, the incidence of invasive candidiasis has decreased with the use of antifungal prophylaxis (Gamaletsou et al., 2014; Sipsas et al., 2009). Studies have shown that the proportion of *C. krusei* and *C. glabrata* is higher in this population, probably due to prolonged exposure to azoles (Bodey et al., 2002; Cleveland et al., 2015; Hachem et al., 2008; Horn et al., 2009; Lockhart et al., 2011; Schuster et al., 2013).

In the general population, attributable mortality is mainly due to delayed administration of an appropriate antifungal treatment (Arnold et al., 2010; Pfaller and Diekema, 2007; Puig-Asensio et al., 2014).

Despite improvement in antifungal treatments, the associated mortality has remained unchanged for 10 years, posing a considerable public health problem. Invasive candidiasis is responsible for a high cost of health care due mainly to prolonged hospitalization, especially in ICUs, and to protracted courses of expensive antifungal treatments. Several sources estimate the total median cost per person to be more than US \$ 50,000 (Asmundsdottir et al., 2013; Craver et al., 2010; Lortholary et al., 2014; Moran et al., 2009, 2010; Pfaller and Castanheira, 2016; Pfaller and Diekema, 2007).

Definition

Candidiasis brings together three distinct entities: asymptomatic colonization of a non-sterile site, i.e., skin, digestive and respiratory tracts; superficial skin and mucosal infections; and invasive tissue infections. The European Organization for Research and Treatment of Cancer (EORTC) and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium reviewed and updated the criteria for invasive disease diagnosis in 2019 (Ascioglu et al., 2002; Donnelly et al., 2019):

- proven: For all patients, regardless of their immunological status, histopathologic, cytopathologic, or direct microscopic examination of a specimen obtained by needle aspiration or biopsy from a normally sterile site (other than mucous membranes) showing *Candida*; recovery of *Candida* spp. by culture of a sample obtained by a sterile procedure from a normally sterile site; blood cultures growing *Candida* spp.
- probable: The patient must meet at least one criterion from each of these categories: risk factors intrinsic to the host (recent neutropenia, hematologic malignancy, allogeneic stem cell transplantation, prolonged corticosteroid therapy, recognized T-cell immunosuppressants, inherited severe immunodeficiency, acute graft-versus-host disease), clinical criteria and mycological criteria (two consecutive positive β -D-glucan or positive T2Candida);
- possible: Presence of host risk factor and clinical criteria but no mycological evidence.

Physiopathology

Candida spp. becomes pathogenic only when the host microbiota or antifungal immune defenses (phagocytes, T lymphocytes) are disturbed. Antibiotics, especially broad spectrum antibiotics active on anaerobes, consequently permit growth of yeasts by modifying digestive or vaginal microbiota. The integrity of the mucocutaneous barrier can be affected by surgical wounds, chemo- or radiation-induced mucositis, or central venous catheters. Once the barrier is altered, the yeasts pass into the bloodstream. Skin and mucosal damage occurs mainly in patients with an acquired immune deficiency, as shown by their high prevalence in patients with HIV infection. However, deep-seated *Candida* infections are often linked to a deficit in innate immunity or to an impairment of phagocytic functions (Sardi et al., 2013).

Candida spp. has several virulence factors: membrane and cell wall barriers, dimorphism, proteins related to stress tolerance, hydrolytic enzymes and toxin production. *Candida* is able to form biofilm, an extracellular matrix secreted by different bacteria and yeasts. It allows host colonization, adhesion to foreign materials (catheters, prostheses, etc.), and protection against antifungal treatments.

Surface receptors on innate immunity cells recognize the conserved structures of *Candida*, essentially components of the yeast wall, such as chitin, β -glucans, or mannans. This recognition leads to an inflammatory reaction characterized by macrophage activation and neutrophil influx. After which, Th1/Th17 immune response is initiated, allowing the production of cytokines such as gamma interferon and interleukins 17 and 19.

In chronic disseminated candidiasis (CDC), deregulation of the Th1 immune response explains the pathophysiology of CDC. Chemotherapy-induced neutropenia allows establishment of an anti-inflammatory environment with a dominant Th2/Treg response. Neutropenia combined with inflammation of the mucous membranes and ulcers along the digestive tract promoted by chemotherapy facilitates *Candida* translocation in the portal venous system through liver and spleen. After neutrophil recovery, specific anti-*Candida* immunity characterized by a pro-inflammatory Th1 response is restored promoting the occurrence of clinical and radiological symptoms. The pro-inflammatory response can quickly become excessive with an imbalance in the Th1-Th2/Treg immunological balance leading to an inflammatory immune reconstitution syndrome (IRIS) (Candon et al., 2020; Dellièrre et al., 2018).

General information on diagnostic methods

Direct examination and culture: The gold standard methods

The diagnosis of certainty for *Candida* infection is based on yeast isolation from blood or pathological products (tissues or urine). Despite relatively low sensitivity of around 21–71%, blood cultures remain an essential diagnostic tool (Clancy and Nguyen, 2013). Their sensitivity varies according to species, culture media, and underlying comorbidity. It is lower in neutropenic patients and in those receiving prophylactic antifungal therapy (Jordana-Lluch et al., 2014; Neely et al., 2013). Blood cultures should be taken daily from different sites. Growth times vary from one *Candida* species to another (1–14 days). For instance, *C. tropicalis* grow in an average of 19 h compared to 75 h for *C. glabrata*. Incubation time is approximately 5 days, to which approximately 24–48 h for species identification must be added. A negative culture does not rule out *Candida* infection. However, due to its high specificity, a yeast in a blood culture is very likely to be *Candida*, other yeasts such as *Cryptococcus* spp. being rare (Griffin and Hanson, 2014; Pappas et al., 2018).

Since *Candida* is a saprophyte of skin and mucous membranes, interpretation of unprotected samples (throat sample, endoscopic sample, skin biopsy, stool and urine samples, sputum ...) must be carried out with care. Their positivity is most often linked to colonization, which is nevertheless important to identify in patients at risk of invasive infection. On the other hand, the presence of *Candida* in bronchoalveolar lavage almost always signals colonization.

In addition, the gold standard for diagnosing deep-seated candidiasis without associated candidemia is fungal culture in normally sterile tissues or biological fluids. As with blood cultures, sensitivity is lacking. Moreover, it is often difficult to perform deep-seated biopsies due to fragile population, which complicates the performance of invasive procedures (Clancy and Nguyen, 2013, 2018; Pappas et al., 2018).

Indirect diagnostic tools

1,3- β -D-Glucans

1,3- β -D-Glucans (BDG) are sugar components of the cell wall of most fungal species. The positivity of the BDG assay is therefore not specific to invasive candidiasis. There are currently four commercial kits (fungitell[®], fingitec-G[®], Wako[®], and Maruha[®]) with similar performances. The Fungitell[®] assay was approved by the United States Food and Drug Administration in 2004 to improve the sensitivity of invasive candidiasis diagnosis and is the most widely used test in the world.

For diagnosis of an invasive fungal disease, the European Society of Clinical Microbiology and Infection Diseases (ESCMID) guidelines recommend repeating the sample: two consecutive positive samples have very high specificity (96–98%), good positive predictive value (79–83%), and good negative predictive value (91–94%). Due to low sensitivity (49–63%), this test should be combined with clinical, radiological, and microbiological proofs.

There are several limitations to the use of BDG. It does not distinguish an invasive fungal infection by *Candida* spp. from another fungus (Pfaller and Castanheira, 2016). There are false positives, especially with previous administration of albumin or immunoglobulins, during hemodialysis, and during some systemic bacterial infections or severe mucositis. Administration of antibiotics such as intravenous amoxicillin-clavulanic acid or piperacillin-tazobactam is less and less an issue. The test is also better for patients without hematological malignancy. While it is recommended to perform it twice a week when an invasive infection is suspected in at-risk patients, it has not yet been validated for children (Clancy and Nguyen, 2013; Karageorgopoulos et al., 2011; Lamoth et al., 2012; Pappas et al., 2018; Tran and Beal, 2016; Wheat, 2009).

Candida serology

The only serological test currently useful for the diagnosis of invasive candidiasis is the Platelia® test, a sandwich enzyme-linked immunosorbent assay (ELISA), which detects mannan antigens, other sugars from *Candida* cell wall. However, this test has low sensitivity for the diagnosis of invasive candidiasis (Mikulska et al., 2010). To overcome this caveat, diagnostic accuracy can be improved by the use of another indirect ELISA test which detects circulating anti-mannan antibodies. The combination of the two tests' mannan antigens and anti-mannan antibodies gives sensitivity of 83% and specificity of 86% in acute disseminated candidiasis. Sensitivity is better for *C. albicans* compared to *C. glabrata* and *C. tropicalis*. The combined test has the advantage of being positive about 5 days before blood cultures. It is recommended by ESCMID for the diagnosis of candidemia and CDC, while BDG can be used for any type of invasive candidiasis (Cuenca-Estrella et al., 2012).

Other diagnostic tools

Polymerase chain reactions (PCR) are under evaluation. Sensitivity and specificity are variable depending on the studies, type of PCR (standard, nested, quantitative), target (rRNA, cytochrome P450 ...), and type of sample (serum, tissue, etc.). Results are obtained earlier than with other diagnostic tools. There is a risk of false positives due to contamination with saprophytic fungal species or to similarities between human and fungal DNA (Pappas et al., 2018). There is also a risk of false negatives due to the usual low number of fungal cells in the blood. In addition, the presence of a fungal cell wall limits access to DNA (Clancy and Nguyen, 2018; Cleveland et al., 2015; Moran et al., 2009; Wang et al., 2015). Despite the promising results demonstrated in a meta-analysis on 54 studies (Avni et al., 2011), due to lack of standardization the use of PCRs remains limited today in diagnosis of invasive candidiasis. Only positive results are reliable, negative results do not rule out infection.

Two other tests exist based on detection of human antibodies in serum or plasma: indirect immunofluorescent assay kit to test specific IgG antibodies against antigens located on the cell wall surface of the mycelium of *Candida albicans* CAGTA (*Candida albicans* germ tube antibody) IFA IgG, Vircell Microbiologist® and indirect chemiluminescent immunoassay (CAGTA VirClia®). These tests have low sensitivity, which limits their use alone despite their high specificity. Some studies have shown that the association of CAGTA tests with the detection of BDG or anti-mannan antigen could improve diagnostic performance for invasive candidiasis (León et al., 2012; Martínez-Jiménez et al., 2014, 2016; Pini et al., 2019; Zaragoza et al., 2009).

Antifungal susceptibility testing (AFST)

Although the first role of antifungal susceptibility testing (AFST) is to provide information on minimal inhibitory concentration to antifungal drugs and the chances of success of initiated treatment, clinical breakpoints are not well-established for every *Candida* species. Epidemiological breakpoints may be relevant to track antifungal resistance and changes in epidemiology. Currently two methods are standardized for *Candida* spp., broth dilution and disk diffusion. ESCMID guidelines published in 2012 recommend the testing of all *Candida* isolates found in the blood stream and other deep sites (Cuenca-Estrella et al., 2012). The Infectious Diseases Society of America (IDSA) made the same statement in 2016, and recommended the testing of azoles, at least (Lortholary et al., 2012; Pappas et al., 2016). An echinocandin susceptibility test is also recommended for *C. glabrata* and *C. parapsilosis* (Bassetti et al., 2020; Berkow et al., 2020).

Promising technologies for the diagnosis of invasive candidiasis

With the same idea of reaching a faster and more precise diagnosis, diagnostic methods using immunohistochemistry, in situ hybridization and mass spectrometry are being developed and some have already been validated by the FDA.

MALDI-TOF-MS is a technique using mass spectrometry allowing rapid identification (10–30 min) of pathogens from a vial of positive blood culture (Griffin and Hanson, 2014; Bassetti et al., 2020). Despite the high cost of this technique, it could replace conventional species identification techniques thanks to its rapidity and accuracy. PNA-FISH uses fluorescent in situ hybridization (FISH) on specific *Candida* rRNA. It allows the detection of five *Candida* species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*) from a positive blood culture bottle. This test does not have 100% sensitivity o, in fact if the species found is not one of the five mentioned, the test will be falsely negative.

The T2 candida panel is a molecular diagnostic test combining whole blood PCR and T2 magnetic resonance detection. This tool is independent of blood culture, providing a major advantage compared to other techniques. Almost all of the process is automated: after collecting three milliliters of whole blood, red blood cells and *Candida* cells are lysed to amplify *Candida* DNA, which is then detected by magnetic resonance. The assay identifies and results in *C. albicans* and/or *C. tropicalis* or *C. parapsilosis* or *C. glabrata*

and/or *C. krusei*; not allowing a distinction between *C. albicans* and *C. tropicalis*, or between *C. glabrata* and *C. krusei*. Its main limitations are the cost and the decrease in sensitivity if yeast cells are not intact in blood samples (Ibáñez-Martínez et al., 2017; Neely et al., 2013). This assay also should be validated in case of deep-seated candidiasis, especially when there is no candidemia (Zacharioudakis et al., 2018).

Use of clinical score to help in the diagnosis of invasive candidiasis

Clinical prediction scores have been implemented to advance the diagnostic process. They are mainly based on combination of risk factors for IFD as mentioned above. These algorithms allow early and appropriate medical intervention to lower IFD mortality. The *Candida* Score uses four risk factors for invasive candidiasis (*Candida* colonization, surgery on intensive care unit admission, sepsis, and total parenteral nutrition) (León et al., 2006). In a prospective study it has shown good negative predictive value of invasive candidiasis under certain conditions: if the score is less than three, if the patient is non-neutropenic, had been admitted to ICU for at least 7 days and has not received antifungal treatment (León et al., 2009). Due to high negative predictive values and low positive predictive values, these clinical prediction scores would be more useful to stop unnecessary antifungal treatment than to diagnose invasive candidiasis (Eggimann et al., 2011; Pfaller and Diekema, 2007).

General information on antifungal treatments

The main antifungal drugs active on *Candida* spp. and their special features are detailed in Table 1.

Oropharyngeal candidiasis (OPC)

Also called thrush, OPC is observed in infants, elderly, denture wearers, and HIV seropositive patients, of which it is one of the first and most frequent clinical manifestations. Some treatments favor the occurrence of OPC in particular antibiotic therapy, immunosuppressive treatments, and inhaled corticosteroid therapy. It results in diffuse erythematous inflammation of the oropharyngeal mucosa and of the tongue which appears stripped. Whitish deposits, which can be removed by scraping, appear secondarily. A perleche can be associated. In chronic forms, leukoplakia or mucous atrophy (black tongue) can be observed. The clinical symptoms are often mild (dry mouth, ageusia, burning sensation) but can have serious consequences in infants and malnourished patients with severe dehydration or malnutrition.

The diagnosis is essentially clinical. Identification of species and resistance screening are required during recurrences in patients who had previously been exposed to azoles. Deposit scraping samples are seeded on a specific culture medium. In non-immunocompromised patients, discreet lesions can be treated with topical treatments (AmB suspension, miconazole buccal gel). For larger lesions, oral fluconazole 7–14 days is recommended. Treatment for HIV seropositive patients is indicated in Table 2 (Lortholary et al., 2012).

Esophageal candidiasis (EC)

Rarely observed in immunocompetent patients, EC is more frequent during chemotherapy or mediastinal irradiation for malignant diseases and during AIDS. Although it may be diagnosed in the absence of OPC, EC is often due to locoregional OPC dissemination. The main symptom is dysphagia, sometimes associated with retrosternal pain. Coexistence of these symptoms and thrush should be enough to start anti-*Candida* systemic treatment and to look for immunosuppression.

The only way to confirm diagnosis is upper digestive endoscopy with targeted biopsies. It is necessary if symptoms persist under appropriate treatment. Esophageal endoscopy has two other indications:

- screening for an associated infection, in particular during AIDS (herpes simplex virus, cytomegalovirus);
- identification of the responsible strain in the event of suspected resistance to common antifungals, or of recurrence.

Endoscopic exploration reveals lesions similar to those observed in thrush with mucosal inflammation and whitish pseudo-membranes that can partially obstruct the esophageal lumen. Ulcerated lesions are scarce. The infection can spread contiguously to the stomach. Treatment indicated in Table 2 is similar for both HIV and non-HIV patients.

Vulvovaginal candidiasis

Vulvovaginal candidiasis is a very frequent infection, 75% of women having at least one episode in their life. The diagnosis is suggested in cases of intense vulvar pruritus accompanied by dyspareunia, burning sensation or dysuria, and white leukorrhea with a curdled appearance (slatted secretions). Vulvovaginal candidiasis is hormone dependent. Rare before puberty and after menopause, it can be triggered by pregnancy, antibiotic therapy, or immunosuppression. Vulvovaginitis can be treated with imidazole prolonged-release intravaginal capsules and local cleansing with alkaline soap. It is necessary to seek and treat a genital candidiasis in the partner. Recurrent candidiasis (more than four episodes per year) can be treated with cyclical fluconazole or itraconazole (Table 2).

Table 1 Principal systemic antifungals used in the treatment of adult candidiasis and their particularities.

<i>FAMILY NAME site of action</i>	<i>DCI</i>	<i>Particularities</i>	<i>Administration</i>	<i>Toxicity</i>	<i>Specific diffusion sites</i>
AZOLES inhibit synthesis of fungal membrane by inhibiting the enzyme responsible for the transformation of lanosterol into ergosterol	Fluconazole	- fungistatic on yeasts - Few drug interactions - Renal excretion - Half-life: 25 h, allowing a once-a-day dosing regimen - <i>C. glabrata</i>: decreased sensibility - <i>C. krusei</i>: resistant	PO = IV First day loading dose: twice maintenance dose Maintenance dose: • 200 mg/day for urinary candidiasis for 14 days • 200–400 mg/day for esophageal for 14–21 days • 400 mg/day for systemic candidiasis Maximum dose: 1200 mg/day	Excellent tolerance	Brain, CSF, eyes, bone, kidneys
	Voriconazole	- fungistatic on yeasts - Hepatic metabolism by cytochrome P450 (drug interactions) - half-life: 6–9 h - Plasma level monitoring recommended: therapeutic value between 1 and 4 mg/L; if residual plasma concentration >5 mg/L, risk of hepatic and neurologic toxicity	IV, PO (empty stomach) First day loading dose IV: 6 mg/kg/12 h PO: 200–400 mg/kg/12 h Maintenance dose: IV 4 mg/kg/12 h PO 100–200 mg/kg/12 h	Renal toxicity with IV administration, reversible visual disturbances Photosensibility; risk of epidermoid carcinoma during prolonged treatments Cholestatic and cytolytic hepatitis Few side effects (neutropenia, neurosensory disturbances, QT prolongation, cholestatic hepatitis)	CSF, eyes
	Posaconazole	- fungistatic on yeasts - Hepatic metabolism by cytochrome P450 (drug interactions) Plasma level monitoring. . . - Indicated in oropharyngeal candidiasis (OPC) including OPC refractory (rOPC) to itraconazole and/or fluconazole - prophylaxis of invasive candida infections in patients who are at high risk (HSCT recipients with GVHD or those with hematologic malignancies with prolonged neutropenia from chemotherapy) - Not indicated in esophageal candidiasis and candidemia	- IV - 400 mg × 2/day during fatty meals, after a loading dose divided in 4 doses/day (200 mg × 4) during 48 h - gastro-resistant tablets: loading dose day 1 300 mg × 2/then 300 mg/day		
	Itraconazole	- erratic absorption (capsules to be taken during meals, oral solution on empty stomach) - Hepatic metabolism by cytochrome P450 (drug interactions) Plasma level monitoring. . .	- Loading dose IV 200 mg/12 h or PO 400–600 mg during 48 h - maintenance dose: IV 200 mg/day PO 400 mg/day	- severe liver disease - worsening of congestive cardiomyopathy	- poor dissemination in LCS
	Isavuconazole	- lower interactions than other azoles but be careful with rifampicin and tyrosine-kinase inhibitors - indicated for invasive aspergillosis as an alternative to voriconazole - indicated mucormycosis in case of intolerance to AmB	- same dosage if intravenous or oral form - loading dose 200 mg/8 h on D1 and D2 - maintenance dose: 200 mg/day	- some headache, drowsiness - lower liver and skin toxicity than voriconazole - caution in patients with hepatic failure: risk of overdose	- good dissemination in cerebral parenchyma but poor in CSF

POLYENES alter membrane permeability by binding to membrane ergosterol	AmB	- weak oral absorption (>5%)	- IV: 0.7–1 mg/kg	- renal insufficiency	Brain, kidneys, eyes, bone
	deoxycholate	- Half-life: 24–48 h	- Duration: ≥ 4 h	- Anemia	
	AmB liposomal ^a	- not dialyzable	IV: 3–5 mg/kg	- Electrolyte disturbances (hypokalemia and hypomagnesemia)	
		- ineffective on <i>C. lusitanae</i>		- Test dose: 1 mg in 20 min	
		- Renal excretion	- Duration: 1–2 h but more if intolerance		
		- good tissue diffusion			
ECHINOCANDINS alteration of fungal wall by inhibiting beta 1-3-glucan synthase	Caspofungin	- Fungicidal on yeasts	over 1 h	for AmB liposomal: better renal tolerance	Renal parenchyma, ocular involvement
		- IV only	First day loading dose	Common:	
		- Hepatic elimination	70 mg	disturbance of liver function	
		- Risk of resistance in <i>C. guilliermondii</i> and <i>C. parapsilosis</i>	Maintenance dose 50 mg/day	(augmentation of AST and ALT, -GT, alkaline phosphatase)	
		- not dialyzable	<80kg		
			70mg/day ≥ 80 kg		
			- Dose adjustment for hepatic insufficiency		
			- absence of adjustment if renal insufficiency		
	Micafungine		No loading dose		
			max 150 mg/j		
	Anidulafungine		Loading dose 200 mg		
			Maintenance dose		
			100 mg/day		
PYRIMIDINE inhibits nucleus DNA and RNA synthesis	Flucytosine	- Inactive on <i>C. krusei</i>	PO	- Hematological	Brain, CSF, kidney, eyes, bone
		- High risk of secondary resistance: always prescribed in association with other drugs	100–200mg/kg/day	- Hepatic (cytolysis)	
		Plasma level monitoring. . .		- Gastrointestinal	
		- Renal excretion		- (ulcerative membranous enterocolitis)	

AmB: amphotericine B; IV: intravenous; PO: per os.

^aThe lipid forms of amB are generally indicated if the patient has pre-existing renal failure or has developed one under treatment. (Creatinine >220 μ mol or clearance less than 25 mL/min), except for special indications.

Table 2 Treatment of oro-pharyngeal candidiasis (OPC), esophageal candidiasis (EC) and vulvo-vaginal candidiasis in HIV patients.

Primary prophylaxis in mucosal involvement	Treatment
Treatment of first episodes of OPC (with or without identification of an azole susceptible strain)	• Introduction or monitoring of ARV therapy • Fluconazole (100 mg/day) • or Miconazole gingival muco-adhesive pill (50 mg/day) • or Itraconazole oral solution after fasting (200 mg/day) • Response within 72 h • Treatment duration 7 days, to be renewed 7 days in case of failure • Therapeutic response in 80% of cases
Treatment of EC ^a	• Initiation of treatment without prior endoscopy • Endoscopy not necessary if patients have esophageal symptoms and lesions of oropharyngeal candidiasis • Fluconazole per os (200 mg/day for 14–21 days) possibly increased to 400 mg/day in the case of clinical failure • or Itraconazole oral solution after fasting (200 mg/day, 1 or 2 doses)
Treatment of resistant OPC/EC Symptoms persisting after 2 weeks of Fluconazole treatment with a dose higher than 200 mg/day	• A sample must be taken to identify the responsible species and test its fluconazole susceptibility in vitro • Itraconazole oral solution after fasting (200–600 mg/day during 2 weeks; in the absence of clinical improvement, treatment can be continued for 2 weeks) • Or Posaconazole suspension (100 mg b.i.d) to be administered with a fatty food • Or Echinocandin • Or Voriconazole
Treatment of vulvo-vaginal candidiasis ^a	• Topical agents (azole vaginal capsule: econazole LP 150 mg 1 capsule at night) • Or short course of azole per os: single 150 mg oral dose of fluconazole • Disease recurrence^b: 10–14 days of induction therapy with a topical agent, then fluconazole per os 150 mg/week for 6 months

^aDoes not differ whether the patient is immunosuppressed or not.^bMore than 4 episodes per year.

Mucosal candidiasis in HIV-seropositive patients

OPC or EC are very common and considered to be markers of immunosuppression. Prior to the arrival of highly active anti-retroviral treatments (HAART), about 90% of HIV-positive patients had OPC. OPC and EC are favored by CD4 T cells less than 200/mm³. *C. albicans* is the most common species involved. Acquired resistance to fluconazole is observed after exposure to large or frequent doses of fluconazole or by direct transmission of a resistant strain; 93% of patients still remain susceptible to fluconazole despite repeated use of azoles. In parallel with *C. albicans* resistance, new less-susceptible-to-fluconazole species are found on the oral mucosa and vaginal mucosa (*C. krusei* and *C. glabrata*). *C. glabrata* is responsible for frequent relapses, especially in patients with advanced immunosuppression.

Occurrence of genital candidiasis is not linked to the severity of immune deficiency in HIV patients. Management is therefore the same as in the general population.

Although some studies have shown the benefit of preventive OPC treatment with fluconazole, primary prophylaxis is not recommended in Europe. Risks of drug interactions with HAART, development of resistance, cost of long-term courses of therapy and potential toxicity of the drugs are not in favor of primary prophylaxis (Lortholary et al., 2012).

Secondary prophylaxis is recommended only when relapses are frequent or severe after initiating optimal HAART. Maintaining treatments after immune reconstitution (i.e., CD4 count $\geq 200/\text{mm}^3$) is not recommended (Blanc et al., 2018; Lortholary et al., 2012).

Skin and nail candidiasis

Intertrigo due to *Candida* spp. appears as yellowish white deposits on erythematous skin. There may also be a “crumbled” aspect of the lesions at the periphery (small erythematous papules). The diagnosis is essentially clinical but samples for mycological confirmation can be made. Treatment involves the prevention of risk factors such as maceration and topical imidazole treatments.

Onychia or perionyxis begins with inflammation around the nail and with moderate pain, followed by a transparent exudate. The nail is then invaded through the lateral edge by a yellow spot that reaches the free edge. The diagnosis is made by direct examination and culture of nail samples. After cutting the sick nail, the treatment consists in application of an imidazole cream on the perionyxis and the lateral furrows. Treatment with film-forming solution can speed up healing. Local treatments are effective only on mild lesions of a single finger in immunocompetent patients. In other cases, systemic treatment is associated with local treatment after *Candida* identification.

Peritonitis

Peritonitis frequency is increasing. It can complicate peritoneal dialysis, digestive surgery, intestinal perforation or advanced cirrhosis. Dissemination is most often hematogenous, except during peritoneal dialysis. It is associated with significant morbidity and mortality between 20% and 30%. Isolation of *Candida* spp. from a postoperative peritoneal drain does not necessarily require treatment but strengthened clinical and microbiological monitoring is desirable.

The diagnosis requires direct examination and culture of the peritoneal fluid. There are four predictive factors of mortality due to polymicrobial peritonitis with *Candida* isolation in peritoneal fluid in critically ill patients: APACHE II (Acute Physiology and Chronic Health Evaluation II) score on admission of at least 17, respiratory failure on admission, upper gastrointestinal tract site of peritonitis, and results of direct examination of peritoneal fluid that were positive for *Candida* spp. (Dupont et al., 2002).

For treatment of *Candida* peritonitis, several learned societies have attempted to give clear guidelines for treatment. The presence of live yeast in peritoneal fluid is associated with excess mortality (Montravers et al., 2015). Clinical features suggestive of yeast infection are hemodynamic failure, upper gastrointestinal perforation, female gender, and antibiotic therapy during the previous 48 h. When 3 of these 4 criteria are present, the probability of isolating *Candida* in peritoneal fluid is 71%: in this case empirical antifungal therapy should be introduced. In healthcare-associated intra-abdominal infections, an echinocandin treatment should be preferred if yeasts are observed at direct examination and if peritoneal fluid culture is positive. Introducing antifungal therapy is not necessary in community peritonitis in the absence of severity signs except in immunocompromised patients, transplant recipients or patients with an inflammatory disease (Montravers et al., 2015).

IDSA guidelines suggest considering empiric antifungal therapy for patients with clinical evidence of intra-abdominal infection and significant risk factors for candidiasis, including recent abdominal surgery, anastomotic leaks, or necrotizing pancreatitis, who are doing poorly despite treatment for bacterial infections. The recommended treatment is echinocandin (Caspofungin: loading dose of 70 mg, then 50 mg daily; Micafungin: 100 mg daily; Anidulafungin: loading dose of 200 mg, then 100 mg daily) (Pappas et al., 2016; Sartelli et al., 2017).

Candiduria

Candiduria is defined by isolation in the urine of *Candida* spp. colonies whatever the threshold (Pappas et al., 2016). Candiduria may reflect candidemia or an isolated infection of the urinary system. Most of the time, it is a simple colonization of the bladder mucosa. It can occur in patients with a urinary catheter, in diabetics, at extreme ages (premature, elderly), during the extension of a vaginal infection and when using broad-spectrum antibiotics. The other known risk factors are urinary tree abnormalities and abdominal surgeries. Candiduria is also more frequent in intensive care units (Achkar and Fries, 2010).

The infection can be asymptomatic or revealed by classic signs of cystitis. Sometimes it spread to kidneys with the formation of abscesses or obstruction of the urinary tract with fungus ball. The risk of ascending kidney damage is more frequent in the event of obstacle, intra-ureteral device or in diabetics. Nevertheless, hematogenous spread still remains the cause number one of *Candida* kidney abscesses.

Clinical examination to search for signs of disseminated infection is mandatory once candiduria is detected (Pappas et al., 2016). In women, prescription of antifungal vaginal capsules or a bladder catheter for urine sampling may be necessary to confirm candiduria and avoid false positives due to vulvovaginal candidiasis. Leukocyturia or positivity of other mycological samples can help to differentiate colonization and true infection. There is no quantitative threshold in the urine to define the infection (Table 3).

Table 3 ESCMID and IDSA recommendations for the treatment of *Candida* spp. urinary tract infections.

Patient	Treatment ^a
Asymptomatic	None, except for patients with high risk of dissemination (neutropenia, infants born hypotrophic <1500 g, planned urologic intervention) - If urologic intervention: Oral fluconazole 400 mg/day or AmB deoxycolate 0.3–0.6 mg/kg/day for several days before and after the procedure - If neutropenic patients or very-low-birth-weight infants: candidemia treatment (echinocandin at initial therapy) Removal indwelling bladder catheters
Pyelonephritis	Fluconazole 200–400 mg/day for 2 weeks if fluconazole susceptible strain If fluconazole-resistant strain <i>C. glabrata</i> : AmB-d 0.3–0.6 mg/kg/day or oral flucytosine 25 mg/kg 4 times per day for 2 weeks ^b <i>C. krusei</i> : AmB-d 0.3–0.6 mg/kg/day Elimination of urinary tract obstruction if present
Cystitis	Fluconazole 200 mg/day for 2 weeks if fluconazole susceptible strain If fluconazole-resistant strain (<i>C. glabrata</i> or <i>C. krusei</i>) AmB-d 0.3–0.6 mg/kg/day for 1–7 days Or oral flucytosine ^b 25 mg/kg × 4/day during 7–10 days
Fungus ball	- Irrigation through nephrostomy tubes, if present, with AmB-d, 25–50 mg in 200–500 mL sterile water ^c

AmB-d = amphotericin B deoxycolate.

^aMaintenance doses are indicated in the table, loading dose of azoles should be administered the first day or during the first 2–3 days depending on azole chosen.

^bThe excellent urinary diffusion of flucytosine allows it to be used as monotherapy in this case.

^cLipid forms of AmB have low urinary diffusion at standard doses, but may have higher efficacy at higher doses.

Systemic candidiasis

The indications and treatments are detailed in Table 4.

Table 4 ESCMID and IDSA recommendations for the treatment of systemic candidiasis depending on the affected organ.

TYPE OF INFECTION		TREATMENT ^a
Candidemia without organ involvement		• 1 blood culture per day until obtaining negative results • 14 days of treatment after the end of candidemia and resolution of symptoms attributable to candidemia • oral relay possible with fluconazole (loading dose 800 mg (12 mg/kg) then 400 mg (6 mg/kg) per day) as soon as patient is clinically stable, the species is susceptible, and repeated blood cultures are negative • testing for azole susceptibility is recommended for all blood stream infection and other clinically relevant <i>Candida</i> isolates
Ocular involvement	Central catheter can be removed	Removal of catheter
	Central catheter cannot be removed	Use an antibiofilm antifungal drug: Echinocandin, liposomal AmB or lipid complex AmB
	Chorioretinitis without vitritis with isolated resistant strain to fluconazole/voriconazole	IV L-AmB 3–5 mg/kg daily ± flucytosine oral 25 mg/kg 4 times daily
	Chorioretinitis without vitritis with isolated susceptible strain	- Fluconazole, loading dose, 800 mg then 400–800 mg once a day OR Voriconazole loading dose of 6 mg/kg IV twice daily for 2 doses, followed by 4 mg/kg IV or oral twice daily - Duration should be at least 4–6 weeks, depending on resolution of the lesions - Same IV treatment plus intravitreal injection of AmB-d 5–10 µg or voriconazole 100 µg - Vitrectomy should be considered to decrease fungal burden and to remove fungal abscess
	Chorioretinitis with vitritis	
Meningitis		- L-AmB 5 mg/kg daily ± flucytosine 25 mg/kg 4 times daily followed by fluconazole 6–12 mg/kg daily after initial response to treatment - therapy should be continue until all signs and symptoms, and CSF and radiological abnormalities have resolved. Generally, L-AmB ± flucytosine for 10 weeks followed by fluconazole for 5 weeks
Endocarditis	Native valve	- Initial therapy: AmB lipid formulation 3–5 mg/kg daily ± flucytosine 25 mg/kg 4 times daily at initial therapy OR high dose echinocandin (casprofungin 150 mg daily, micafungin 150 mg daily or anidulafungin 200 mg daily) - Followed by fluconazole (6–12 mg/kg daily) for patients who have susceptible <i>Candida</i> isolates, were clinically stable, and have negative blood cultures - Surgery within the week is recommended - duration of 6 weeks after surgery
	Valvular prosthesis	- Urgent surgery (in the following days) and same antifungal treatment as native valve - chronic suppressive antifungal therapy with fluconazole (6–12 mg/kg daily) to prevent recurrence
	Valvular prosthesis with contraindication for excision	- long term suppression with fluconazole (6–12 mg/kg) daily if the isolate is susceptible
Osteomyelitis (including vertebral)		- Surgical debridement in selected cases - Fluconazole 400 mg (6 mg/kg) daily for 6–12 months OR an echinocandin (casprofungin 50–70 mg daily, micafungin 100 mg daily, anidulafungin 100 mg daily) for at least 2 weeks followed by fluconazole 400 mg per day for 6–12 months - Another less attractive alternative due to renal toxicity: AmB lipid formulation 3–5 mg/kg for at least 2 weeks followed by fluconazole 400 mg (6 mg/kg) for 6–12 months
Arthritis		- Surgical drainage in all cases - Fluconazole 400 mg (6 mg/kg) daily for 6 weeks OR an echinocandin (casprofungin 50–70 mg daily, micafungin 100 mg daily, anidulafungin 100 mg daily) for 2 weeks followed by fluconazole 400 mg per day for at least 4 weeks - Another less attractive alternative: lipid formulation AmB 3–5 mg/kg for 2 weeks, followed by fluconazole 400 mg for ≥ 4 weeks
Joint prosthesis infection		Prosthesis removal If prosthesis left in place: chronic suppression by fluconazole 400 mg/day

AmB, amphotericin B; Amb-d, amphotericin B deoxycholate; L-AmB, liposomal amphotericin B.

^aMaintenance doses are indicated in the table, loading dose of azoles should be administered the first day or during the first 2–3 days depending on azole chosen.

Candidemia

Candidemia is defined by one or more blood cultures positive for *Candida* spp. without clinical signs of dissemination. To confirm the absence of secondary localizations, retinal examination and transthoracic echocardiography should systematically be performed. A study has shown that transthoracic echocardiography alone can miss one third of clinically unsuspected endocarditis, suggesting systematic transesophageal echocardiography (Fernández-Cruz et al., 2015). Another study found a much lower incidence of endocarditis, suggesting that transthoracic ultrasound should be done only if risk factors such as prosthetic heart valves, injecting drug users, prolonged candidemia, new onset heart failure or embolic events are present (Cleveland et al., 2015; Cornely et al., 2012).

If the patient is immunocompromised, the risk of systemic infection is very high and treatment should be started immediately. Disseminated candidiasis should be carefully searched, removal of all catheters should be discussed, and blood cultures repeated daily until negative.

It is now widely accepted in the general population as well as in the hematological population that the withdrawal of a central venous catheter and early establishment of an adequate antifungal treatment are two factors that could reduce mortality (Garnacho-Montero et al., 2013). It is recommended to use a treatment with anti-biofilm activity (echinocandin or a lipid formulation of amphotericin B) if the central catheter cannot be removed (Seidler et al., 2010; Shuford et al., 2006).

Disseminated candidiasis

Acute disseminated candidiasis

Acute disseminated *Candida* infections are generally associated with sepsis or septic shock. Symptoms are unspecific and can lead in several days to shock with multiple organ failure, especially in neutropenic patients. In that case, mortality rate exceeds 50%. Blood cultures being negative in more than half of the cases, the diagnosis must therefore be suggested, and preemptive antifungal treatment should always be considered in neutropenic patients with persistent fever under broad spectrum antibiotic therapy. Oropharyngeal and digestive colonization with *Candida* spp. is an additional argument to start antifungal treatment. Biopsies (oral, ocular or osteoarticular candidiasis) can provide diagnosis of certainty by allowing yeast isolation.

Septic metastases are common. Secondary locations can be:

1. ocular, especially endophthalmitis, present in 28% of cases. A dilated eye exam reveals chorioretinitis with cottony nodules and hyalite and it can also reveal an extension to the vitreous body.
2. cutaneous: erythematous nodules and macules, sometimes necrotic (ecthyma gangrenosum).
3. renal: the most frequent after cutaneous and ocular localizations;
4. osteoarticular: mainly in drug addicts. Osteomyelitis and vertebral osteomyelitis often require surgical management associated with antifungal treatment (48%) (Gamaletsou et al., 2012);
5. central neurological: brain abscess, meningitis;
6. myocardial: often discovered at autopsy, can result in arrhythmia or conduction disturbances, or even heart failure.

The lungs and adrenal glands are more rarely affected.

Endocarditis

Candida endocarditis is rare with increasing incidence around 2–4% due to the use of endovascular prostheses (Antinori et al., 2014). The two main risk factors are prosthetic valves and short-term indwelling catheters. Patients with prosthetic valves may be at risk of postoperative transient candidemia. *Candida* colonizes the valve prosthesis thanks to biofilm production, which reduces susceptibility to antifungal agents (Falcone et al., 2009; Nasser et al., 1997). Symptoms are similar to those of bacterial endocarditis, while vegetations are often larger with high embolic risk requiring surgical management. Even with adequate care, mortality is high (around 56%). The two major causes of death are cardiac failure and uncontrolled sepsis (Rivoisy et al., 2018).

Osteomyelitis and arthritis

Being rare, these infections are sparsely studied, and little is known about their pathophysiological mechanisms. For *Candida* osteomyelitis, more than half of the patients are immunocompetent with an average age around 30 years (Gamaletsou et al., 2012). Hematogenous dissemination seems to be the first cause of osteoarticular involvement (67%) followed by direct inoculation (24%) and infection contiguous (9%), associated candidemia being found in less than 20% of the cases. The most frequent localizations are vertebrae (>50%), femur, ribs and sternum with discrepancies according to age: adults have predominant vertebral involvements whereas children have long bone involvements (Gamaletsou et al., 2012; Slenker et al., 2012).

Over 90% of osteoarticular infections are due to a single species of *Candida*. The most commonly found species is *C. albicans* (>50%), followed by *C. tropicalis* (16%), *C. glabrata* (8%), and *C. parapsilosis* (7%), reflecting the overall epidemiology of *Candida* spp. (Gamaletsou et al., 2012).

Few patients remain asymptomatic. The most commonly reported symptoms are localizing pain, tenderness and/or edema. Fever has been reported in less than a third of patients. Infection may evolve with few signs delaying the diagnosis for several weeks to several months.

Regarding treatment management, no antifungal drug has been proven superior. Based on expert experience (B-III, level of evidence), the 2016 IDSA guidelines for *Candida* osteomyelitis recommend fluconazole 400 mg (6 mg/kg) daily for 6–12 months or

an echinocandin (caspofungin 50–70 mg daily, micafungin 100 mg daily, or anidulafungin 100 mg daily) for at least 2 weeks followed by fluconazole, 400 mg (6 mg/kg) daily for 6–12 months. (Cornely et al., 2012; Pappas et al., 2016) Use of Lipid formulation AmB, 3–5 mg/kg daily, instead of echinocandin is less attractive.

Surgical management seems beneficial especially in cases of functional impairment such as neurological deficit, or vertical instability, or when the antifungal treatment alone seems ineffective.

A literature review evaluated 112 cases of *Candida* arthritis between 1967 and 2014 (Gamaletsou et al., 2016). Surprisingly, more than half of the patients (65%) were non-immunocompromised. *Candida* septic arthritis most commonly occurred post-surgically (35%). Almost two-thirds of the patients (63%) had initial candidemia or another form of candidiasis (ocular, central nervous system involvement or an infected central venous catheter).

The clinical manifestations most often found are pain, tenderness, edema; and the most affected joints are knees, hips, and shoulders. The two *Candida* species most often found are *C. albicans* and *C. tropicalis*. The goal of early and appropriate treatment is to limit bone destruction and joint effusion. The latest recommendations from IDSA 2016 recommend using fluconazole, 400 mg (6 mg/kg) daily, for 6 weeks or echinocandin (caspofungin 50–70 mg daily, micafungin 100 mg daily or anidulafungin 100 mg daily) for 2 weeks followed by fluconazole, 400 mg (6 mg/kg) daily, for at least 4 weeks. Surgical drainage is indicated in all cases of septic arthritis (Pappas et al., 2016).

Chronic disseminated candidiasis

Chronic disseminated candidiasis (CDC), improperly called hepatosplenic candidiasis, affects liver and/or spleen, and also lungs, kidneys, and CNS. It occurs exclusively after a period of deep and prolonged neutropenia lasting more than 10 days. The incidence of CDC in acute leukemia is probably around 3–5%. The most common symptom is isolated fever that does not respond to broad spectrum antibiotic therapy while the patient is recovering from neutropenia. Other signs may be right upper quadrant pain, hepatomegaly, splenomegaly, or dry cough. Liver enzymes are generally disturbed with cholestasis; inflammation markers are high. Blood cultures are positive in approximately 15–20% of patients. (Candon et al., 2020) Ultrasound or CT guided liver biopsies sometimes reveal yeast and granuloma in histopathology. They generally remain sterile in culture. They allow differential diagnosis such as mycobacteriosis. Most of the CDCs whose agents have been identified are due to *C. albicans*. Serum biomarkers such as BDG, mannan antigens/anti-mannan antibodies may be helpful in CDC diagnosis. Currently, MRI is considered to be the most sensitive exam to detect less than one-centimeter abscesses in liver and/or spleen before CT and ultrasound. It takes several weeks/months for lesions to disappear from conventional imaging. 18F-fluorodeoxyglucose positron emission tomography-computed tomography could be used for initial diagnosis and monitoring of patients.

A recent study has demonstrated that CDC is associated with IRIS, mainly due to the presence of a significant biological inflammatory syndrome, the very common absence of associated candidemia, the presence of epithelioid granulomas in liver tissue, and a good clinical-biological response to high doses of corticosteroids. *Candida* specific T cells producing interferon-gamma (IFN- γ) at a high rate have been shown to be useful in CDC diagnosis (Candon et al., 2020; Sallah et al., 1999).

The IDSA and the ESCMID recommend a protracted course of antifungal treatment (Table 4). (Pappas et al., 2016) Corticosteroids may be used to improve symptoms but not biological and radiological signs.

Candidiasis in organ transplantation

One-year incidence for invasive candidiasis in the US cohort named Transplant - Associated Surveillance Network (TRANSNET) was 1.95% (Pappas et al., 2010). Invasive candidiasis represents 53% of IFD (Pappas et al., 2010). In this cohort, one-year incidence of IFD was highest in small bowel transplant recipients, whereas prevalence of *Candida* infection was highest in liver transplant recipients, followed by kidney and pancreas transplant recipients. All-cause mortality at 90 days was 26.5% for all species in solid organ transplant recipients (SOT) (Andes et al., 2016).

The diagnostic methods and treatments for candidemia and invasive candidiasis in SOT follow the 2016 IDSA recommendations. However, special features should be noted, notably in lung transplant recipients, where tracheobronchitis caused by *Candida* spp. must be treated.

In transplant recipients, antifungal prophylaxis is guided according to at-risk patients (Table 5) (Giannella et al., 2018). This approach seeks to reduce the number of adverse events and drug-drug interactions, and to limit costs. During liver transplantation, prophylaxis in at-risk patients only has been shown to be an effective strategy to reduce the risk of IFD, thereby reducing exposure to azoles (Lavezzo et al., 2014; Pappas et al., 2006). It is not recommended to introduce antifungal prophylaxis either in heart transplant recipients or in renal transplant recipients. In pancreatic transplantation, prophylaxis is controversial. According to the American Society of Transplantation Infectious Disease Community of Practice, it is recommended to use an antifungal prophylaxis only if at least one risk factor is present (Table 5).

In hematopoietic stem cell transplant (HSCT) patients, *Candida* prophylaxis is recommended during the pre-engraftment period with Fluconazole (400 mg/day) for centers with a low incidence of mold infections (<5%). Centers with a higher incidence of mold infections should prefer anti-mold prophylaxis. During the post-engraftment period, anti-mold prophylaxis is advised if GVH disease occurs. The fifth European conference on leukemic infections (ECIL-5) recommended using echinocandin as first-line curative treatment in the event of candidemia/invasive candidiasis followed by a step-down approach in clinically stable patients upon receipt of species identification and antifungal susceptibility testing results (Tissot et al., 2017). The three echinocandins (caspofungin, micafungin and anidulafungin) now have the same grade (A II) recommendation in hematological patients (Krishnan-Natesan et al., 2010).

Table 5 Candidiasis primary chemoprophylaxis in transplant recipients.

Indication	Primary prophylaxis	Doses	Duration post-transplant
High risk in liver transplantation ^a	Fluconazole	100–400 mg/day	At least 14–28 day
Pancreatic ^b	<i>Alternative:</i> Echinocandin		At least 14–28 day
	Fluconazole	400 mg/day	≥ 4 weeks
	OR		
	Liposomal AmB or echinocandin ^c		
Small bowel ^d	Fluconazole	400 mg/day	≥ 4 weeks (until the anastomosis has completely healed and rejection is not present)
	OR		
	Liposomal AmB or echinocandin ^c		
Hematopoietic stem cell	Fluconazole in centers with incidence <5% of mold infections	400 mg/day	During pre-engraftment until neutrophil recovery

^aDefined by re-transplantation, re-operation, renal failure requiring hemodialysis, transfusion of ≥ 40 units of cellular blood products, choledochojejunostomy, and Candida colonization in the perioperative period. MELD (Model for End-stage Liver Disease) score ≥ 30, biliary leaks, and living liver transplantation.

^bRisk factors: enteric drainage, vascular thrombosis, and post-perfusion pancreatitis.

^cIn centers with high prevalence of non-albicans Candida. Echinocandin dosage (Caspofungin: 70 mg then 50 mg/day or Micafungin 100–150 mg/day) Liposomal AmB dosage: 3–5 mg/kg/day.

^dRisk factors: Graft rejection or dysfunction, enhanced immunosuppression, anastomotic disruption, abdominal re-operation, or multivisceral transplantation.

Conclusion

Candidiasis remains a severe disease with a poor prognosis despite therapeutic and diagnostic advances. Technology advances will allow faster and more targeted diagnosis for better treatment. This step forward is crucial due to emergence of low susceptibility species with regard to current antifungal therapies. Judicious use of antifungal drugs and better knowledge of risk factors may limit the expansion of these threatening species.

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Aspergillus and Aspergillosis

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Aspergillus and aspergillosis

Aspergillus species are omnipresent saprophytes that can thrive on different organic matter owing to their capacity to adapt with a range of environmental conditions (Cray et al., 2013) (Fig. 1). Asexual spores (conidia) are formed by *Aspergillus* species as propagates that are airborne and highly tolerant to environmental stress, while some species are also known to have sexual life-cycle forming ascospores (Stevenson et al., 2015; Wyatt et al., 2015). Being an opportunistic pathogen, depending on the immunosuppression host factor and underlying lung disease, *Aspergillus* species can cause a spectrum of diseases of the upper and lower respiratory tract from benign maxillary sinus aspergilloma to severe invasive pulmonary aspergillosis: (i) individuals severely immunocompromised will develop invasive pulmonary and/or sinus aspergillosis that rarely disseminates, but can involve the central nervous system (CNS), leading to CNS aspergillosis, the most severe form of extrapulmonary aspergillosis, (ii) those with underlying lung disease with structural modification and/or chronic inflammation may develop chronic pulmonary aspergillosis, and (iii) those with hypersensitivity and allergic background are prone to develop allergic bronchopulmonary aspergillosis (ABPA) or severe asthma with fungal sensitization. Regardless of the forms of aspergillosis, the most prevalent *Aspergillus* species is *A. fumigatus* (responsible for >90% infection), followed by *A. flavus*, *A. niger*, and less commonly, *A. terreus*, *A. nidulans* and *A. versicolor* [reviewed in (Paulussen et al., 2017)].

The spectrum of aspergillosis

Invasive pulmonary aspergillosis (IPA) is a well-known complication in immunocompromised individuals, specifically in neutropenic patients with acute leukemia, stem cell and solid organ transplant recipients as well as patient receiving high dose

*Contributed equally.

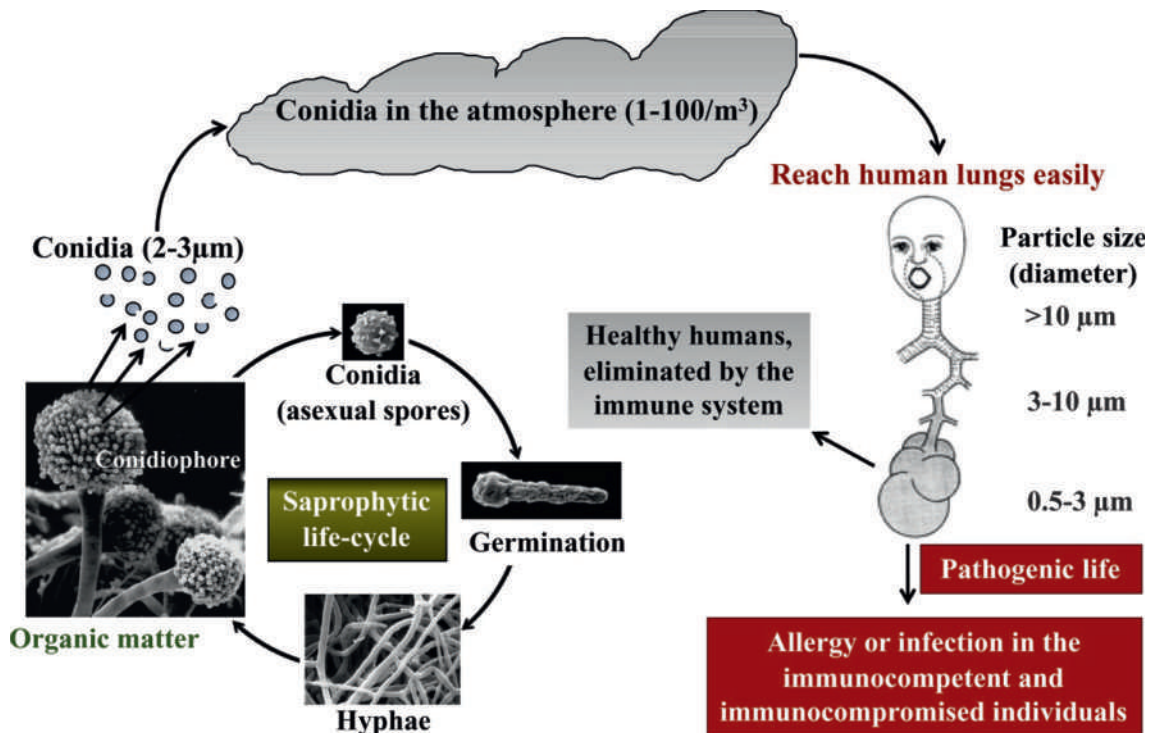


Fig. 1 *Aspergillus* species inhabit different organic matter. Asexual spores (conidia) produced by them are dispersed through air-current, which allow them to exploit new environment wherein they continue with saprophytic life cycle, or access to the human lungs through breathing in whom they may be eliminated by the immune system, or lead to allergy or infectious diseases.

corticosteroids (Donnelly et al., 2019). Chronic lymphoproliferative diseases has emerged as a new high-risk group (Lortholary et al., 2011), especially when patients were treated with ibrutinib (Ghez et al., 2018). It may also develop in critically ill patients without classical risk factors, most likely when the patient enters a stage of protracted immunosuppression due to various immune dysfunctions (Monneret et al., 2011). This includes patients with influenza (Schauwvlieghe et al., 2018; Shah et al., 2018; van de Veerdonk et al., 2017) and more recently, COVID-19 (Alanio et al., 2020; Bartoletti et al., 2020; Rutsaert et al., 2020; van Arkel et al., 2020). IPA has also been described in patients with severe chronic obstructive broncho-pulmonary aspergillosis (COPD) likely due to several predisposing factors including prolonged use of corticosteroid therapy, structural change in the lung architecture, broad spectrum antibiotic treatment and comorbid illnesses (Lionakis and Kontoyiannis, 2003). Global incidence of IPA has recently been updated to >300,000 cases and is likely underdiagnosed (Bongomin et al., 2017). Associated mortality remains high ranging from 30-80% (Table 1) (Bongomin et al., 2017). Environmental exposure affects incidence of the disease and nosocomial clusters have been previously reported, often linked to hospital construction and *Chronic pulmonary aspergillosis* (CPA) is a problematic pulmonary disease that complicate many other respiratory disorders and affect over 3,000,000 patients worldwide (Bongomin et al., 2017). Prior tuberculosis may be the most common underlying condition for CPA but COPD, sarcoidosis, ABPA, atypical mycobacteria lung disease are also responsible for structural lung changes and mucociliary impairment responsible for the development of CPA. The term CPA includes various entities: aspergilloma (also called fungus-ball), *Aspergillus* nodule, chronic cavitary pulmonary aspergillosis (CCPA) and chronic fibrosing pulmonary aspergillosis (CFPA), which affect non-immunocompromised patients (Denning et al., 2016). The most common form is CCPA, which may progress to CFPA if untreated. Despite milder symptoms than IPA, patients are at high risk of disease progression and hemoptysis, and mortality rate of CPA is 80% within 5 years if untreated (Kosmidis and Denning, 2015). Subacute invasive pulmonary aspergillosis affect patients with mild immune disorder including long term corticosteroid use, diabetes, malnutrition, alcohol abuse, and is a more rapidly progressive infection (Denning et al., 2016).

Allergic bronchopulmonary aspergillosis (ABPA) is a progressive allergic lung disease due to sensitization to *Aspergillus* antigens and commonly complicating asthma or cystic fibrosis. Both condition affect mucociliary clearance of fungal elements leading to fungal growth, mucosal colonization and higher exposure to fungal antigens and persisting airway inflammatory response (Tracy et al., 2016). ABPA may lead to severe lung damage, respiratory failure and increased premature mortality. Prevalence rate in asthma patient is 2.5% translating to over 4.8 million cases of ABPA in adults worldwide (Bongomin et al., 2017). Severe asthma with fungal sensitization (SAFS) describes patient with evidence of sensitization to aspergillus among other fungi but who do not meet ABPA diagnostic criteria. It is estimated that 3% of asthmatic adult will develop SAFS leading to increased severity and more frequent exacerbation (Denning et al., 2006).

Table 1 Clinical, radiological and microbiological characteristics of pulmonary aspergillosis.

	<i>Invasive pulmonary aspergillosis</i>	<i>Chronic pulmonary aspergillosis</i>	<i>Allergic bronchopulmonary aspergillosis</i>
Host factors	Neutropenia <500 cells/mm ³ HSCT Chronic lymphoproliferation Ibrutinib SOT CGD (hereditary neutrophil dysfunction) Critically ill Severe COPD	Tuberculosis COPD ABPA Sarcoidosis Bronchiectasis NTM infection Treated lung cancer	Asthma Cystic fibrosis
Incidence	>300,000 cases worldwide	>3,000,000 cases worldwide	>4,800,000 cases worldwide
Clinical features	Cough Sputum production Dyspnea Fever Thoracic pain Hemoptysis	Aspergilloma: asymptomatic CCPA: Cough ++ Fatigue, weight loss, night sweats, shortness of breath, fever, hemoptysis	Recurrent pulmonary exacerbations: cough, wheeze, shortness of breath +/- systemic features (fever, anorexia)
Radiological features on computed tomography scanning	Neutropenic Nodules with halo sign Angio-invasive aspergillosis Non-neutropenic Bronchiolitis Airway-invasive aspergillosis +/- CNS dissemination Non-specific in critically ill and COPD patients	Underlying lung disease features + Aspergilloma: intracavitary mass partially surrounded by a crescent of air ("air crescent" sign) CCPA: areas of consolidation associated with multiple expanding usually thick-walled cavities CFPA: severe fibrotic destruction of at least two lobes of lung complicating CCPA Aspergillus nodules: nodules <3 cm	Central bronchiectasis (upper lobe predominance) Bronchial wall thickening Hyper-attenuating mucoid impaction (pathognomonic ++, 20% of cases)
Microbiological criteria	Direct examination/culture from sputum, BAL or aspirate Galactomannan (serum, BAL or CSF) Aspergillus PCR (serum, BAL)	Direct examination/culture Aspergillus IgG antibodies Aspergillus precipitins	Aspergillus IgE antibodies Total IgE > 1000 IU/mL Aspergillus precipitins or IgG antibodies
Diagnostic algorithms and classification	1. EORTC/MSG if immunocompromised (Donnelly et al., 2019) 2. AspiCU if patient in ICU ^a (Blot et al., 2012) 3. In COVID-19 patients (Verweij et al., 2020)	ESCMID/ECCM/ERS rational and clinical guidelines (Denning et al., 2016)	ISHAM working group diagnostic and classification criteria (Agarwal et al., 2013)
Treatment guidelines	ESCMID/ECMM/ERS guidelines (Ullmann et al., 2018) IDSA guidelines (Patterson et al., 2016) ECIL guidelines (Tissot et al., 2017)	ESCMID/ECCM/ERS rational and clinical guidelines (Denning et al., 2016)	No consensual guidelines. Regimen proposed by ISHAM working group (Agarwal et al., 2013)
First line therapy	Voriconazole 2 × 6 mg/kg IV (oral 400 mg twice daily) on day 1, then 2–4 mg/kg IV (200–300 mg twice daily) or Isavuconazole 200 mg IV thrice daily on day 1–2 then 200 mg daily	Aspergilloma: observation or surgical resection if symptomatic CCPA: Itraconazole 200 mg twice daily	Oral corticosteroids (0.5 mg/kg/day) +/- itraconazole regimen

HSCT, hematopoietic stem cell transplant; SOT, solid organ transplant; BAL, bronchoalveolar lavage; CGD, chronic granulomatous disease; COPD, chronic obstructive pulmonary disease; CSF, cerebral spinal fluid; BAL, bronchoalveolar lavage; NTM, non-tuberculous mycobacteria.

^aSpecific algorithm for patients with influenza associated pulmonary aspergillosis by Verweij et al. (2020).

Fungal rhinosinusitis due to *Aspergillus* species present as different entities showing similarities with pulmonary aspergillosis. Acute invasive aspergillosis sinusitis, occurring in severely immunocompromised hosts (see section "The spectrum of aspergillosis"), is characterized by bone lysis on CT-scan, and bone and sinus tissue invasion by hyphae on biopsy. In non-immunocompromised patients, simple aspergilloma from odontogenic origin may develop in the maxillary sinus cavity. Allergic fungal sinusitis, reported in patients with atopy, is characterized by chronic pansinusitis resistant to antibiotic therapy, nasal polyposis, without bone involvement on CT-scan, and without bone hyphae invasion on biopsy (deShazo et al., 1997; Lafont et al., 2017).

Biology, pathogenesis and virulence factors of *Aspergillus* species

Aspergillus conidia have an outer layer composed of hydrophobins (forming a rodlet layer) and melanin (Aimanianda et al., 2009; Paris et al., 2003; Valsecchi et al., 2019). The rodlet layer contributes to the hydrophobicity of the conidia, which in turn contribute to their airborne nature (Grünbacher et al., 2014). In *A. fumigatus*, there are seven hydrophobins (RodA-G), but only the most expressed one, RodA, was shown to be responsible for rodlet formation (Valsecchi et al., 2017). In *A. nidulans*, there are six hydrophobins (RodA and DewA-E), but only the deletion of RODA results in the loss of rodlet layer (Grünbacher et al., 2014). Among other *Aspergillus* species, several putative hydrophobin genes have been reported (Littlejohn et al., 2012; Pel et al., 2007), but are not well characterized.

The melanin pigment, synthesized by a cluster of genes, gives the conidia the color of bluish green, and protects the cell from environmental stresses, such as ultraviolet light (Geib et al., 2016). The color of the conidia of different *Aspergillus* conidia varies due to the different melanin composition resulting from corresponding melanin biosynthetic pathways (Fig. 2). For instance, 1,8-dihydroxynaphthalene-melanin (DHN-melanin) is present in *A. fumigatus* and *A. terreus*, while 3,4-dihydroxyphenylalanine-melanin (DOPA-melanin) is found in *A. flavus*, *A. niger* and *A. nidulans* (Pal et al., 2014).

Upon swelling, outer rodlet-melanin layers disappear and the inner cell wall, composed of different polysaccharides (chitin, β -1,3-glucan, α -1,3-glucan and galactomannan), is exposed. Upon germination, a polysaccharide (galactosaminogalactan) specific to *Aspergillus* mycelia is synthesized (Fontaine et al., 2011). Galactomannan (GM) and galactosaminogalactan (GAG) are heteropolysaccharides; GM is composed of mannan backbone with galactofuranose side-chains (Latge et al., 1994), while GAG is composed of galactose, galactosamine (GalN) and *N*-acetyl-galactosamine (GalNAc) (Fontaine et al., 2011; Lee et al., 2016).

Aspergillus species possess various virulence factors which facilitate its pathogenesis which will be described in the following.

Hydrophobins and other surface proteins

The dormant conidia of *Aspergillus* species are covered by a rodlet layer, which are immunologically inert (Aimanianda et al., 2009). Its masking of the underlying immunogenic polysaccharides in the inner cell wall immunologically silence the dormant conidia, and thus, enhances fungal survival in vivo (Carrion Sde et al., 2013). Distortion or absence of rodlet layer facilitate the conidial phagocytosis by the immune cells, confirming its role in evasion from immune recognition (Aimanianda et al., 2009; Dagenais et al., 2010). Proteomic analysis revealed that, besides the highly abundant hydrophobins, the conidial surface includes various proteins. For example, the CcpA protein has also been described to minimize innate immune response (Voltersen et al., 2018). Some conidial surface proteins act as allergens, such as Asf 3 (Asif et al., 2006), that are associated to the allergic form of aspergillosis (ABPA or SAFS).

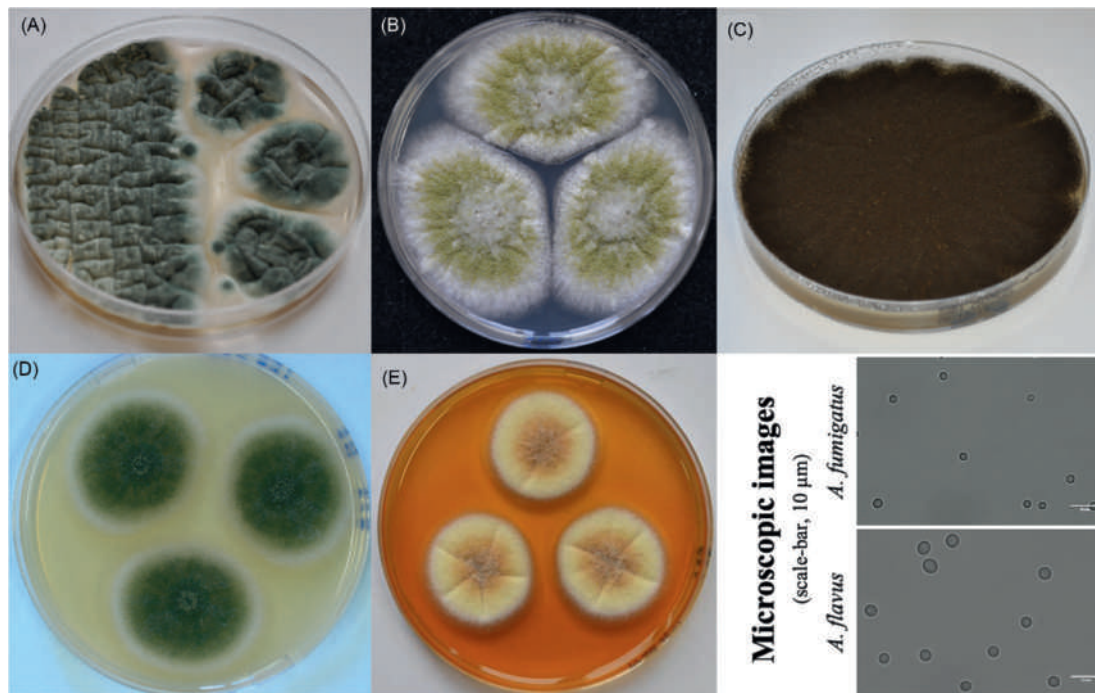


Fig. 2 Macroscopic and microscopic images of different pathogenic *Aspergillus* species. (A) *A. fumigatus*, (B) *A. flavus*, (C) *Aspergillus* section *Nigri*, (D) *A. nidulans*, (E) *A. terreus*. The species-specific conidial melanins contribute to the varying color across the *Aspergillus* species. Additionally, the conidial size also varies; In diameter, *A. fumigatus* 2–3.5 μm , *A. flavus* 3–6 μm , *A. niger* 4–5 μm , *A. terreus* 1.5–2 μm (Bengyella et al., 2017; Koch et al., 2019; Pasqualotto, 2009). Courtesy of the French National Reference Center for Invasive Mycoses and Antifungals (CNRMA).

Melanin

Fungal melanin has been considered as an important virulence factor. Of note, different melanin (predominantly DHN-melanin and DOPA-melanin) has been found across various *Aspergillus* species (Pal et al., 2014). DHN-melanin has been reported to interfere with the intracellular killing mechanisms by inhibiting the acidification of phagolysosome (Amin et al., 2014; Thywissen et al., 2011) and quenching reactive oxygen species (Jahn et al., 2000). In addition, melanin also inhibits LC3-associated phagocytosis by calcium sequestration (Akoumianaki et al., 2016; Kyrmizi et al., 2018). Melanin-deficient conidia induce significantly more proinflammatory cytokines as compared with the wild-type conidia in a lectin receptors dependent manner, suggesting that, like hydrophobins, melanin also participates in masking the immunogenic polysaccharide in the inner cell wall (Chai et al., 2010a). DOPA-melanin of *A. nidulans* was shown to inhibit nitric oxide and TNF- α production (Gonçalves Rde et al., 2013), suggesting that DOPA-melanin of pathogenic *Aspergillus* species could modulate the host immune response. On the other hand, *A. terreus* lacks naphthopyrone synthase, which is highly conserved among *Aspergillus* species (Gressler et al., 2015; Zaehle et al., 2014); the cinnamon-brownish pigment formed in *A. terreus* is independent of tyrosine or L-DOPA supplementation (Eisenman and Casadevall, 2012). In contrast to other *Aspergillus* species, *A. terreus* pigment is of 4-hydroxyphenylpyruvate origin (Geib et al., 2016). Nevertheless, *A. terreus* conidia were shown to persist inside macrophages and dendritic cells without inhibiting phagolysosome acidification, contributing to its reported higher dissemination rate and lower response to antifungal treatment than *A. fumigatus* (Hsieh et al., 2017).

Galactosaminogalactan

Galactosaminogalactan (GAG) is an *Aspergillus*-specific polysaccharide, which is a linear heterogenous polysaccharide composed of randomly distributed units of α -1,4-linked galactose, galactosamine (GalN) and *N*-acetyl-galactosamine (GalNAc) (Fontaine et al., 2011; Lee et al., 2016). GAG has been considered as virulence factor of *Aspergillus* species due to its anti-inflammatory properties, by inducing the cytokine IL-1Ra (Fontaine et al., 2011; Gresnigt et al., 2014), and by masking mycelial pro-inflammatory polysaccharide β -1,3-glucan (Gravelat et al., 2013). GAG also mediates virulence by resisting the antifungal activity of neutrophil extracellular traps (Lee et al., 2015). Furthermore, soluble GAG (with less amount of GalNAc) has been shown to promote neutrophil apoptosis via natural killer cells (Robinet et al., 2014). Besides being associated to the mycelial surface, GAG is also secreted by *Aspergillus* mycelia. Mycelial surface GAG or GAG secreted from *A. fumigatus* and *A. flavus* are capable of activating platelet, and thus, causing excessive inflammation, thrombosis and thrombocytopenia (Deshmukh et al., 2020; Rambach et al., 2015).

Iron acquisition

Iron is essential for microbial growth, and pathogenesis depends on the sequestration of iron from the host. For *Aspergillus*, iron has been shown to be a major determinant for its invasive growth in the host (Hsu et al., 2018). The siderophore-mediate iron uptake is essential in maintaining fungal growth in iron-limited environment in the host, and thus, is crucial for the virulence of *Aspergillus* (Hissen et al., 2004; Schrettl et al., 2004). Deleting the siderophore biosynthetic gene *SidA* and bZIP-type regulator (Schrettl et al., 2010) (required for transcriptional remodeling under iron starvation in *A. fumigatus*) rendered the fungi avirulent in a mouse infection model (Hissen et al., 2005).

Secreted proteases/allergens

Aspergillus secrete proteases to degrade factors involved in host antifungal immunity. The proteins Alp1 (Behnsen et al., 2010) and Mep1 (Shende et al., 2018) secreted by *A. fumigatus* degrade complement proteins C3, C4 and C5 (see section “Complement activation” for the importance of complement system against *Aspergillus*). Alp1 is found oversecreted in the exoproteome of *A. flavus* isolates from cornea, suggesting its possible role in the establishment of keratitis (Selvam et al., 2015). Another secreted allergen of *A. fumigatus*, enolase (Eno1), recruits complement regulators and plasmin, thus controlling complement activation and resulting in epithelial cell damage, respectively (Dasari et al., 2019).

Mycotoxins

Mycotoxins are secondary metabolites produced by fungi that are capable in causing diseases. Aflatoxin produced by *A. flavus* and *A. parasiticus* is among the mycotoxins that have received attention due to its impact on agricultural foodstuff (El Khoury et al., 2011; Sarma et al., 2017). Intake of food that is contaminated by aflatoxin, which is carcinogenic and mutagenic, could cause stunted growth in children, liver damage or cancer. Nevertheless, aflatoxin has not been shown to be associated to the pathogenesis of aspergillosis, and thus, is not considered a virulence factor. Gliotoxin is an immunosuppressive mycotoxin found in *A. fumigatus*, demonstrating cytotoxic effects to macrophage-like cells and T cells (Kupfahl et al., 2006), and is associated with apoptosis of leukocytes at the foci of infection in murine model of aspergillosis (Liu et al., 2018). The deletion of the gliotoxin gene *gliP* attenuates virulence in non-neutropenic murine infection model, but not the neutropenic model (Spikes et al., 2008; Sugui et al., 2007).

Host-immunology against *Aspergillus*

Upon inhalation, the *Aspergillus* conidia are instantly encountered by the host innate immunity. The two major arms of innate immunity, cellular and humoral immunity, synergistically elicits antifungal properties for efficient clearance of these fungal

pathogens. The absence or malfunctions of one or more components in this host-defense, as observed in immunocompromised population, could lead to aspergillosis.

Innate immunity

Cellular-mediated immunity

Cellular-mediated immunity is crucial in eliminating the fungal pathogen. The inhaled conidia are cleared by intracellular killing mechanisms, while the hyphae are generally managed extracellularly. The intracellular killing in phagocytes depends on oxidative burst triggered by conidial swelling. Chronic granulomatous disease (CDG) patients are prone to aspergillosis due to the defective oxidative burst in their phagocytes caused by mutation in NADPH oxidase, resulting in the inability to kill *Aspergillus* conidia. Iron-sequestration by secreted lactoferrin is an alternate to check fungal growth, as iron is essential for fungal growth. In response to *Aspergillus*, the immune cells also secrete various cytokines and chemokines to recruit and enhance the immune effector functions.

Macrophages and neutrophils

Upon inhalation and reaching the distal alveoli, the conidia are likely to encounter the resident alveolar macrophages. As a primary phagocyte, macrophages readily phagocytose the inhaled conidia, traffic the conidia to phagolysosomal compartment through endocytic pathway and then intracellularly through respiratory oxidative burst (Philippe et al., 2003; Sun et al., 2014). The alveolar macrophages also secrete cytokines to mediate an inflammatory response and chemokines to recruit other immune effector cells, most importantly, neutrophils (Grazziutti et al., 1997; Pylkkanen et al., 2004). Neutrophils are important immune cells for host defense against aspergillosis, as neutropenia is one of the main risk factors for invasive aspergillosis. Beside oxidative intracellular killing of conidia, neutrophils are also capable in inhibiting conidial growth, through oxidative burst-independent mechanism, by lactoferrin-mediated iron depletion as iron is essential for *Aspergillus* mycelial growth (Zarembek et al., 2007).

In the case where the phagocytes are unable to kill the intracellular conidia, germination will take place. *Aspergillus* mycelia are too large to be phagocytosed. Extracellular antifungal strategies against mycelia are mainly imposed by neutrophils, through neutrophil extracellular traps (NETs). While NETs are fungicidal against other medically important fungal pathogens, they impose fungistatic activity on *Aspergillus* species instead (Bruns et al., 2010; McCormick et al., 2010). The extracellular destruction of mycelia by neutrophils requires mycelial opsonization by antibody and mediation of Fc- γ receptors, triggering the production of toxic reactive oxygen species (Gazendam et al., 2016). The constituents in *Aspergillus*-induced NETs responsible for fungal growth inhibition are not fully elucidated. Calprotectin, a zinc ion chelator that is present in NETs, was demonstrated to be responsible for NETs-mediated fungal growth inhibition (McCormick et al., 2010). Importantly, NETs also have contradictory role in which it could obstruct fungal clearance in vivo. Inhibiting NETosis in vivo results in less lung injury in IPA mouse model (Alflen et al., 2020).

Dendritic cells

Dendritic cells (DCs) contributed to host defense through internalization of conidia or hyphae via pattern recognition receptors, secretion of cytokines, migration to draining lymph nodes after activation, and regulate T cells polarization (Bozza et al., 2002). DCs interact and internalize *A. fumigatus* conidia through the C-type lectin receptor dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) through the galactofuranose moiety of galactomannan, resulting in the induction of pro-inflammatory cytokines (Chiodo et al., 2014; Serrano-Gomez et al., 2004, 2005). DCs can also be activated by α -1,3-glucan partly through TLR2 (Stephen-Victor et al., 2017) and by β -1,3-glucan through Dectin-1 (Mezger et al., 2008). The cross-talk between neutrophils and DCs in response to *Aspergillus* germ tubes promotes the activation and efflux of DCs (Park et al., 2012). Upon *Aspergillus* infection, DCs release CXCL8, responsible for recruitment of neutrophils (Gafa et al., 2007). *Aspergillus*-infected DCs express CCR7 receptor (Gafa et al., 2006), release soluble factors that induce the expression of CD11b and CD18 (complement receptor 3, see section "Membrane-bound PRRs") on neutrophils, express cytokines for migration of memory T cells (CCL3, CCL4, CXCL10 and CCL20) and for migration of CCR7+ naïve T cells and mature DCs in the lymph nodes (CCL19) (Gafa et al., 2007).

Notably, different subsets of DCs display distinct effector functions against *A. fumigatus*. Myeloid DCs and monocyte-derived DCs phagocytose *A. fumigatus* conidia and germ tubes by the receptors TLR2, TLR4 and Dectin-1, while plasmacytoid DCs (pDCs) do not mediate phagocytosis of the fungus and do not express Dectin-1 (Lothar et al., 2014). Instead, pDCs recognize and bind to *A. fumigatus* hypha through the receptor Dectin-2, which then mediates the expression of cytokines TNF- α and IFN- α , and the formation of pDC extracellular traps (Loures et al., 2015), partly through gliotoxin production (*A. fumigatus*-specific mycotoxin) (Ramirez-Ortiz et al., 2011). The importance of pDC against IA is also validated in the higher susceptibility in pDC-depleted mouse infection model (Loures et al., 2015; Ramirez-Ortiz et al., 2011).

Natural killer cells

Patients with severe invasive aspergillosis have lower natural killer (NK) cell counts than those with well-controlled invasive aspergillosis (Stuehler et al., 2015). Depleting NK cells contributed to higher mortality and fungal burden in murine model of invasive aspergillosis (Morrison et al., 2003). *A. fumigatus* hypha, but not resting conidia, activate NK cells (Schmidt et al., 2011). In turn, NK cells are capable of killing the hypha, but not the resting conidia, through perforin-mediated cytotoxicity (Schmidt et al., 2011). NK cells also mediate anti-*Aspergillus* activity by releasing cytokines IFN- γ and GM-CSF (Bouzani et al., 2011; Park et al., 2009; Ziegler et al., 2017). IFN- γ and GM-CSF are important cytokines contributing to resistance to aspergillosis (see details in

section “Adaptive immunity”). CD56 (specific receptor on NK cells) has been shown to interact with *Aspergillus* mycelia (Ziegler et al., 2017), for which the ligand(s) remain unknown. Neutropenic mice infected with *A. niger* showed NK cell proliferation, inhibiting fungal growth (Benedetto et al., 1988).

Platelets

Although *Aspergillus* species is rarely found to be circulating in the bloodstream, thrombocytopenia (low platelet counts), which often coincides with neutropenia, has been suggested to be a risk factor for invasive aspergillosis (Bae et al., 2020). In vitro, platelets do not internalize conidia, but surround and aggregate around them (Perkhofer et al., 2008). Platelet activation by conidia is suggested to be mediated by the surface melanin, in the case of *A. fumigatus* (Rambach et al., 2015), which in turn attenuates germination and induces conidial phagocytosis and killing by neutrophil (Perkhofer et al., 2008; Tischler et al., 2020). On the other hand, platelets adhere to the hypha of *Aspergillus* species and are activated by the cell wall polysaccharide, galactosaminogalactan (Deshmukh et al., 2020; Rambach et al., 2015). Activated platelets damage the *Aspergillus* hypha via granule-dependent mechanisms, which attenuate hyphal elongation and viability (Christin et al., 1998; Perkhofer et al., 2008, 2015). In thrombocytopenic mice intranasally infected by *Aspergillus*, the increased mortality is contributed by excessive inflammation and severe lung hemorrhage, without significant differences in neutrophil phagocytosis or hyphal growth, suggesting that although platelets play a minor role in anti-*Aspergillus* defense, it is crucial for survival by maintaining lung homeostasis in response to extensive inflammation during aspergillosis (Tischler et al., 2020).

Respiratory epithelial cells

The respiratory epithelial cells are not professional phagocytes, but instead, assist in the host antifungal defense. Airway epithelial cells are capable to internalize conidia and bind to *Aspergillus* mycelia, only low levels of cytokines are induced, in the absence of serum (Zhang et al., 2005). But they release antimicrobial peptide like β -defensin upon contact with *Aspergillus* (Alekseeva et al., 2009). Meanwhile, in the presence of serum, bronchial epithelial cells release IL-8, a chemokine recruiting neutrophils, upon contact with mycelia but not conidia (Balloy et al., 2008).

Mast cells, basophils and eosinophils

The immune effectors contributing to allergic bronchopulmonary aspergillosis (ABPA) are predominantly mast cells, basophils and eosinophils, which otherwise do not contribute any protective role against aspergillosis (Agarwal et al., 2011; Gernez et al., 2016). *Aspergillus* allergen-specific IgE binds to mast cells and basophils, causing the release of inflammatory mediators, such as histamine, that contribute to allergic reactions (Madan et al., 1997b); nevertheless, circulating basophils are not professional antigen presenting cells (Sharma et al., 2013). Beside its role in allergic reactions to *Aspergillus*, mast cells could also mediate detrimental effect on lung function. Comparing to *A. flavus*, *A. niger* and *A. nidulans*, the *A. fumigatus* hypha, but not the resting conidia, are significantly more capable in activating the degranulation of mast cells in an IgE-independent manner, resulting in decline in lung function due to aggravation of inflammation and airway obstruction (Urb et al., 2009). Repeated exposure of *Aspergillus* conidia leads to influx of eosinophils into the lung (Murdock et al., 2011). Eosinophilia (elevated count of eosinophils) is observed in murine model of ABPA, as well as in human ABPA patients (Agarwal et al., 2011; Kurup et al., 1992). In response to *Aspergillus*, eosinophils release extracellular traps that have neither killing nor fungistatic activity against the fungus (Muniz et al., 2018).

Humoral immunity

The anti-*Aspergillus* activity of humoral immunity has always been overshadowed by the cellular immunity. In fact, it constitutes an essential arm of host immune defense against aspergillosis. A myriad of humoral factors has been found to interact with *Aspergillus* conidia or mycelia. Examples include the complement system, C-type lectins, antimicrobial peptide and antibodies.

Complement activation

The complement system comprises of three activation pathways (classical, lectin and alternative) regarding the different starting complexes. The three activation pathways all lead to the production of C3 convertase that activates C3 into C3b, which is an opsonin that binds to pathogen surface. C3b also participates in the production of C5 convertase, transforming C5 into C5b, which then polymerize with C6, C7, C8 and C9 to form membrane attack complex (MAC). The formation of MAC on microbial surface causes death of the microbe through lysis. In case of *A. fumigatus*, the formation of MAC on the dormant conidial surface was not observed (Wong et al., 2020a). Instead, C3b opsonizes *A. fumigatus* dormant conidia and mycelia (Kozel et al., 1989; Sturtevant and Latgé, 1992), and facilitates phagocytosis through CR3 and CR4 (Braem et al., 2015; Wong et al., 2020a). In *A. fumigatus*, RodA in the conidial surface rodlet layer has been shown to be the ligand for C3b binding (Wong et al., 2020a), while C3-fragments have also been shown to be deposited on the melanin-ghosts obtained from *A. niger* conidia (Rosas et al., 2002).

Antimicrobial peptides

Antimicrobial peptides (AMPs) are naturally occurring peptides, often cationic and amphipathic, that control infections and modulate inflammation. Direct antifungal effect of AMPs on *Aspergillus* has not been clearly demonstrated. However, peptides of human lactoferrin and histamine-5 demonstrated activity in damaging hypha of *A. fumigatus* (Lupetti et al., 2008). Human β -defensins secreted by airway epithelial cells in response to *A. fumigatus* are associated to the activation-maturation of DCs, as well as stimulation of pro-inflammatory cytokines from DCs (Alekseeva et al., 2009; Presicce et al., 2009). In chronic rhinosinusitis

patients, lysozyme has been reported to show fungicidal activity against different fungal species (Woods et al., 2011). LL37, a human cathelicidin antimicrobial peptide, could directly bind to *A. fumigatus* surface showing mycelial growth inhibition in vitro (Luo et al., 2019).

Pattern recognition receptors (PRRs) of *Aspergillus*

Pattern recognition receptors (PRRs) recognize the structurally conserved pathogen associated molecular patterns (PAMPs) associated with microbes, and thus, are crucial in mediating the proper clearance of the invading pathogens through the corresponding downstream signaling. PRRs are present in immune cells as well as various other cell types not typically involved in immunity, and are membrane-bound or secreted in soluble forms.

Membrane-bound PRRs

Membrane-bound PRRs are mainly composed of two classes: Toll-like receptors (TLRs) and C-type lectins receptors (CLRs). Both TLRs and CLRs are involved in the immune recognition of *Aspergillus*. Other membrane-bound PRRs, such as complement receptors, are also crucial for host defense against *Aspergillus*.

TLRs: Among the TLR family, the crucial role of TLR2 and TLR4 in immune response against *Aspergillus* has been implicated for *A. fumigatus*, *A. flavus*, *A. niger*. Knockout of TLR2 rendered higher mortality in murine model of invasive aspergillosis (Balloy et al., 2005). In human, the polymorphisms in TLR2, TLR4 or TLR9 are associated to the susceptibility to severe asthma with fungal sensitization (Carvalho et al., 2008). TLR2 is associated to conidial phagocytosis, macrophage activation, neutrophil recruitment and production of proinflammatory cytokine (particularly, TNF- α) in response to *A. fumigatus* and *A. niger* (Balloy et al., 2005; Luther et al., 2007; Mambula et al., 2002; Meier et al., 2003), whereas transcriptional expressions of PRRs including TLR2, TLR4 and TLR9, are increased in corneal ulcers by *A. flavus* (Karthikeyan et al., 2011). Zymosan (repeating units of glucose connected by β -1,3-glycosidic linkage) is one of the ligands for TLR2, suggesting that *Aspergillus* β -1,3-glucan could be recognized by TLR2. By experimental data, β -1,3-glucan and galactomannan are suggested to be recognized by TLR4, while α -1,3-glucan is recognized by both TLR2 and TLR4 (Chai et al., 2011; Stephen-Victor et al., 2017).

The TLRs activate the MyD88-dependent pathway, thus, activating the nuclear factor kappa B (NF- κ B)-mediated anti-*Aspergillus* inflammatory responses (Bretz et al., 2008; Mambula et al., 2002). Additionally, in response to *Aspergillus*, the release of IL-12 and IL-6 (the anti-*Aspergillus* cytokines) by dendritic cells are also shown to be dependent on TLR2 and TLR4, respectively (Braedel et al., 2004).

CLRs: Through their carbohydrate recognition domains, the CLRs play a central role in immune recognition of fungal pathogens that are covered by polysaccharide-rich cell wall. Several membrane-bound CLRs have been reported to associate with *Aspergillus* species, including Dectin-1, Dectin-2, mannose receptor, DC-SIGN, and MelLec.

The β -1,3-glucan receptor Dectin-1 plays an important role in recognizing fungal pathogens, including *Aspergillus* species (Steele et al., 2005). The level of Dectin-2, that recognizes mannan on *A. fumigatus* hypha, is elevated during *Aspergillus* invasion (Loures et al., 2015; Sun et al., 2013). Dectin-2 is present on immune cells that also express mannose receptor (Sun et al., 2013). Both mannose receptor and DC-SIGN recognize *Aspergillus* galactomannan, mediate phagocytosis of pathogens and promote antigen presentation (Bennett et al., 1987; Chiodo et al., 2014; Serrano-Gomez et al., 2004, 2005). Mincle, expressed by immune cells of myeloid origin interacts with *A. fumigatus* causing keratitis (Lin et al., 2017; Zhao et al., 2017), but its role against invasive infection need to be studied. Exceptionally, the CLR MelLec has been discovered to recognize, not cell wall polysaccharide, but the DHN-melanin on the cell wall of *A. fumigatus* (Stappers et al., 2018). CD23, another CLR, interacts with *A. fumigatus* through α -mannan and β -glucan (Guo et al., 2018).

Complement receptors (CRs): As mentioned in section “Complement activation”, complement activation leads to opsonization of *Aspergillus* conidia and hypha by C3b and iC3b (the inactivated form). CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are heterodimers that share a common subunit of CD18, recognize the opsonized *Aspergillus* and facilitate phagocytosis (Braem et al., 2015; Wong et al., 2020a). Opsonization by iC3b is mainly recognized by CR4, while CR3 recognize a range of ligands, including iC3b, β -1,3-glucan and pentraxin-3 (a soluble PRR, refer to section “Soluble PRRs”) (Bose et al., 2013; Moalli et al., 2010; Myones et al., 1988). Meanwhile, CR1 recognizes C3b, C1q and mannose binding lectin (soluble PRRs, please refer to section “Soluble PRRs”) (Fallman et al., 1993; Ghiran et al., 2000; Klickstein et al., 1997).

Fc- γ receptors: Fc γ receptors demonstrate protective role against *Aspergillus* opsonized by pentraxin-3, or *Aspergillus*-specific antibodies, through facilitation of phagocytosis and oxidative burst (Becker et al., 2016; Moalli et al., 2010; Zhou and Brown, 1994).

Others: CD56 (specific receptor on NK cells) has been shown to interact with unknown ligand(s) on *Aspergillus* mycelia, and is responsible for activation of NK cells (Ziegler et al., 2017). Calreticulin (CALR) in association with CD91 (CALR-CD91 complex) has been reported to mediate *A. fumigatus* conidial phagocytosis through surfactant protein D (SP-D) (Vandivier et al., 2002). NOD2 has been reported to interact with *A. fumigatus*, and their interaction results in the NF- κ B activation; nevertheless, NOD2 deficiency do not increase the susceptibility to invasive aspergillosis (Li et al., 2012; Ogura et al., 2001).

Soluble PRRs

Collectins: Collectins are soluble C-type lectins with a collagenous region and a carbohydrate recognition domain. Collectins contributing to protective role of aspergillosis include surfactant proteins, and mannose binding lectin.

Surfactant proteins SP-A and SP-D are produced in the lungs and are involved in innate immune system. Both SP-A and SP-D have been demonstrated to bind to *A. fumigatus* conidia and mycelia and enhance the effector functions of neutrophils and

macrophages (Geunes-Boyer et al., 2010; Madan et al., 1997a, 2001; Wong et al., 2018). SP-D binds to the melanin of *A. fumigatus* conidia and to galactomannan and galactosaminogalactan on *A. fumigatus* mycelia (Wong et al., 2018), while the ligands of SP-A is unidentified.

Mannose-binding lectin (MBL), a starting complex of the lectin pathway in the complement system, binds to *A. fumigatus* and provides a protective role in murine model of invasive pulmonary aspergillosis (Kaur et al., 2007). Deficiency in MBL are demonstrated to be associated to acute invasive aspergillosis (Lambourne et al., 2009).

Others: The long pentraxin-3 (PTX3) is an important humoral factor against *A. fumigatus*. PTX3 binds to *A. fumigatus* dormant conidia and mycelia (Garlanda et al., 2002). Polymorphism in the PTX3 gene leads to impaired neutrophil antifungal functions and thus, higher susceptibility to invasive aspergillosis (Garlanda et al., 2002). Galectin-3, a β -galactoside binding lectin, binds to the conidia and hypha of *A. fumigatus* and is associated to neutrophil recruitment to the site of infection (Snarr et al., 2020).

Adaptive immunity

After phagocytosis of pathogens, antigen presenting cells (APCs) migrate to the draining lymph nodes where they present antigens to naïve T cells through major histocompatibility complex (MHC) molecules. The PAMPs of *Aspergillus* shape the differentiation of T cells subsets and B cells to mediate host inflammation and antibody response.

T cells

The susceptibility to aspergillosis is suggested to correlate with the preferential induction of the subsets of CD4⁺ helper T cell (Th), Th1 versus Th2, through pattern of induced cytokines. The mechanisms driving the differentiation of *Aspergillus*-specific T helper cells into Th1 or Th2 cells depend on multiple factors from the host and the pathogens. In general, live conidia induce Th1 response, while inactivated conidia or non-viable *Aspergillus* antigens (culture filtrate or crude mycelial extract) promote Th2 response (Grazziutti et al., 1997; Koth et al., 2004; Rivera et al., 2005). The signature cytokines of Th1 cells (IFN- γ , TNF- α , GM-CSF, IL-2, IL-12, etc.) are crucial in anti-*Aspergillus* activity by activating macrophages. Adoptive transfer of IFN- γ producing *Aspergillus*-specific T helper (Th) cells have been suggested to provide protective role for the host against aspergillosis (Beck et al., 2006; Cenci et al., 2000). Inhibiting or lower levels of IFN- γ or TNF- α results in increased mortality associated with higher fungal burden in immunocompetent and immunocompromised murine model of aspergillosis (Cenci et al., 1998; Mehrad et al., 1999; Nagai et al., 1995). In human, lower secretion of IFN- γ has been shown to be associated with higher risk of chronic aspergillosis in the specific context of STAT3 deficient patients (Danion et al., 2020).

In contrast, the Th2 response cytokines (IL-4, IL-5 and IL-10, etc.) have detrimental effect on the host against invasive aspergillosis, and support the pathogenesis of allergic bronchopulmonary aspergillosis (ABPA) (Cenci et al., 1998; Skov et al., 1999). IL-10 is associated with down-regulation of Th1 response and macrophage activation, which then contribute to higher susceptibility for invasive aspergillosis in mice (Clemons et al., 2000; Roilides et al., 2001). Gene polymorphisms of IL-10 predispose cystic fibrosis patients to *Aspergillus* colonization and ABPA (Brouard et al., 2005; Casaulta et al., 2003). Cystic fibrosis patients with ABPA have elevated levels of IL-4 and IL-5, which are responsible for eosinophilia (Kurup et al., 1997; Murali et al., 1993; Skov et al., 1999).

Even though studies suggested that Th17 cells either do not play any role against *Aspergillus* or may have detrimental role (Chai et al., 2010b; Zelante et al., 2007), they are involved in the migration, recruitment and activation of neutrophil granulocytes to the site of infection, and therefore suspected to play a role against *Aspergillus*. *Aspergillus* is also known to drive Th17 response via dendritic cell stimulation and IL-23 production (Wong et al., 2019), and in turn this Th17 activation depends upon recognition of *Aspergillus* cell wall polysaccharide β -glucan by Dectin-1 (Armstrong-James et al., 2017). Both in infectious aspergillosis and allergic conditions due to *Aspergillus*, a higher expression of IL-17 has been reported (De Luca et al., 2017; Fukahori et al., 2014; Zelante et al., 2015).

Regulatory T cells (Treg), another distinct subset of T cells, are important in regulating the inflammatory response induced by *Aspergillus* through the expression of indoleamine 2,3-dioxygenase (Bacher et al., 2014; Montagnoli et al., 2006; Paveglia et al., 2011). Dendritic cells primed by *A. fumigatus* α -1,3-glucan stimulates the differentiation of naïve T cells into Treg cells through programmed death-ligand 1 (PD-L1) (Stephen-Victor et al., 2017).

CD8⁺ cytotoxic T cells are responsible in direct killing of host cells infected with intracellular pathogens. Although such direct killing is not described, *Aspergillus*-specific CD8⁺ T cells are found in human, with unclear roles (Chaudhary et al., 2010; Cui et al., 2013).

B cells and antibody

B cells are not directly involved in orchestrating anti-*Aspergillus* immune response, as shown in the resistance to aspergillosis in B cell-deficient mice with increased Th1-mediated response (Montagnoli et al., 2003). However, B cells are essential in generating regulatory T cells and *Aspergillus*-specific antibodies (Montagnoli et al., 2003).

Owing to the immunocompromised status, antibody response to *Aspergillus* is generally undetectable in the sera of patients during the course of invasive aspergillosis (Young and Bennett, 1971). However, patients recovering from invasive aspergillosis have increased titer of *Aspergillus*-specific antibodies that is associated with good prognosis. In other forms of aspergillosis in otherwise immunocompetent patients, such as aspergilloma, ABPA and CPA, *Aspergillus*-specific antibodies are present in the patient sera.

These antibodies are specific to *Aspergillus* proteins or polysaccharides, and have been studied for their diagnostic potentials (Kazakova et al., 2020; Komarova et al., 2018; Sarfati et al., 2006; Wong et al., 2020b).

Clinical aspects of aspergillosis

Diagnosis and diagnostic tools available

Clinical and radiological features

IPA usually presents as non-specific bronchopneumonia with cough, sputum production, dyspnea and fever unresponsive to antibiotics. Pleuritic chest pain and hemoptysis are important clinical signs of IPA that may be observed due to pulmonary infarction. Computed tomography (CT)-scan identifies early stages of the disease which includes nodules, consolidative lesions and wedge-shaped infarcts. The main radiological characteristic of IPA is the "halo sign": a central nodule surrounded by ground-glass opacity representing hemorrhage (Fig. 3). It is mainly seen in neutropenic patients early in the course of infection (first week). However, the halo sign is not specific of IPA and can be observed in all invasive mold infection as well as some bacterial pneumonitis. A later radiological sign is the air crescent sign, secondary to nodule necrosis and cavitation (Kousha et al., 2011). Bergeron et al. described two different radiological patterns depending on the type of immunosuppression; (1) Nodule with a halo sign revealed angioinvasive aspergillosis in neutropenic patients with acute leukemia, (2) Centrilobular nodules, bronchiolitis with tree-in-bud opacities revealed airway-invasive aspergillosis in non-neutropenic with chronic lymphoproliferative disorders, stem cell and solid organ transplant recipients receiving steroids (Bergeron et al., 2012).

Patients with CPA usually present with constitutional symptoms including weight loss, malaise, sweats and non-specific pulmonary symptoms (chronic productive cough, breathlessness, chest discomfort and sometimes hemoptysis). Radiological findings depend on the subtype of CPA (Godet et al., 2016). Aspergilloma is the morphological appearance of a fungus-ball composed entirely of fungal hyphae and extracellular matrix in a preexisting pulmonary cavity or an ectatic bronchus (Denning et al., 2016). It may be associated with all form of CPA. For CCPA, CT-scan shows single or multiple cavities, with enlargement of cavity wall thickness. Extensive fibrosis with fibrotic destruction of at least two lobes of lung characterize CFPA and leads to a major loss of lung function. *Aspergillus* nodules (<3 cm) are an unusual form of CPA, frequently misdiagnosed as it mimics many differential diagnosis (i.e., lung cancer and metastases, rheumatoid nodules, etc.). Subacute invasive pulmonary aspergillosis is characterized on CT-scan by nodules with cavitation, consolidation, and lung tissue invasion by hyphae on biopsy (Denning et al., 2016).

Clinical symptoms of ABPA include poorly controlled cortico-dependent asthma with recurrent pulmonary exacerbation (cough, wheeze, shortness of breath), sub-acute deterioration of lung function, and expectoration of mucus plugs with or without

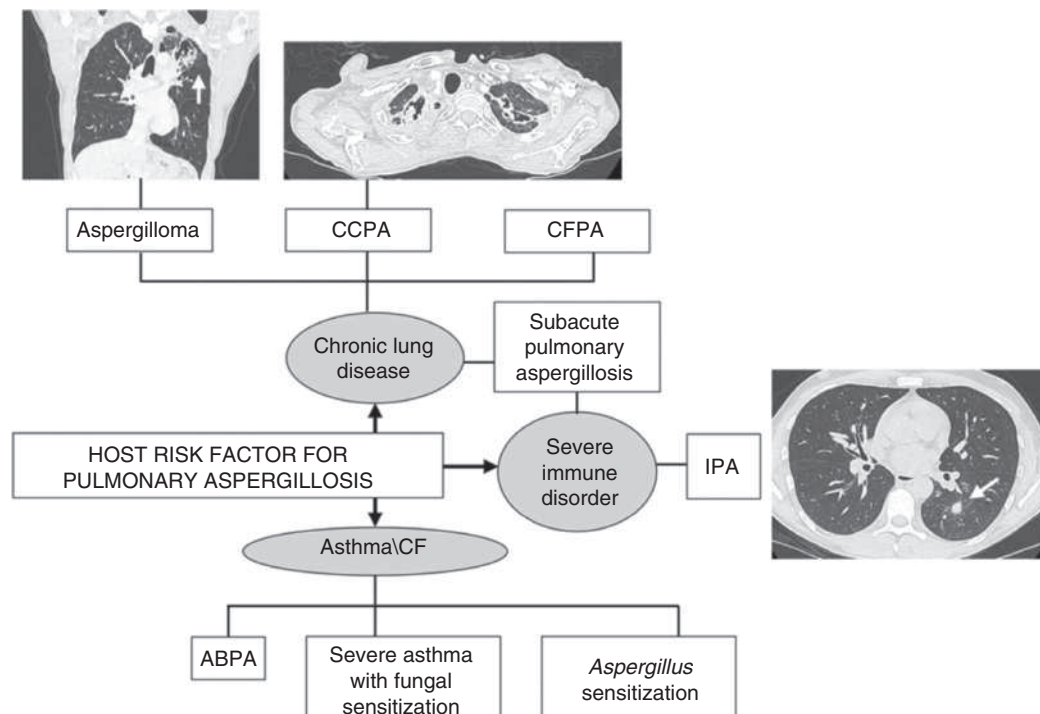


Fig. 3 Categories of pulmonary aspergillosis. ABPA, allergic bronchopulmonary aspergillosis; CCPA, chronic cavitary pulmonary aspergillosis; CF, cystic fibrosis; CFPA, chronic fibrosing pulmonary aspergillosis; IPA, invasive pulmonary aspergillosis.

fever (Greenberger et al., 2014; Tracy et al., 2016). CT-scan usually display central bronchiectasis and bronchial wall thickening. Excessive mucus production appears as mucoid impaction. Hyper-attenuating mucoid impaction on high-resolution CT-scan may be considered as a pathognomonic radiographic finding and observed in 20% of patients (Agarwal et al., 2007) (Fig. 3).

New imaging based-technique may have the potential to make the diagnosis and locate the site of infection. Positron emission tomography (PET)-scan either combined to monoclonal antibody JF5 that binds to a secreted glycoprotein during hyphal invasion or coupled with Gallium-68 fungal siderophore taken by the fungus if present may be of great help and overcome the problem of specificity faced by CT and PET scans (Herrera and Husain, 2018; Savelieff and Pappalardo, 2017).

Microbiological diagnosis

Microbiological tools available to evidence *Aspergillus* in clinical samples are divided into (i) direct examination and histopathology of fresh and fixed sample, (ii) culture-based method, (iii) antigen detection (galactomannan and β -D-glucan), (iv) nucleic acid detection, and (v) serology (antibody detection). Although all may be useful for the diagnosis of aspergillosis, their sensitivity and specificity are highly variable depending on the type of aspergillosis.

For IPA, histopathologic, cytopathologic or direct examination of a specimen obtained by needle aspiration or biopsy in which hyphae are seen associated with tissue invasion constitute the gold standard mycological criteria (Donnelly et al., 2017). Antigen detection in blood, such as galactomannan, a cell wall constituent of *Aspergillus*, is considered a strong mycological criterion. However, sensitivity of this assay vary greatly among at-risk patients. Galactomannan has poor sensitivity in patients receiving mold-active prophylaxis and non-neutropenic patients (Zhou et al., 2017). Furthermore, galactomannan testing gives a number of false positive may be observed in patients receiving antibiotic, which production was contaminated with *Aspergillus* or in patient receiving enteral nutrition. False positive results may also be due to erroneous operational use of the kit or due to crossed reactions with some bacteria from the human microbiota. This can be explained by a poorer specificity of the monoclonal antibody design as it recognize much shorter oligosaccharide than initially thought (Krylov et al., 2019). Galactomannan may also be measured in bronchoalveolar lavage with a value of >1.0 optical density index for the diagnosis of IPA (Eigl et al., 2017). Although detection of *Aspergillus* DNA in blood samples is now included in consensual diagnostic criteria, results vary greatly among centers due to different sampling, extraction and PCR protocols (i.e., different target used, different primer efficiency, platforms and master mixes various efficacy) (White et al., 2011). β -D-Glucan test may be used to diagnose invasive aspergillosis but is less sensitive and specific owing to pan-fungal nature of this polysaccharide (Hoenigl et al., 2014). Accepted criteria are presented in Table 1 according to the latest updated consensus definitions of invasive fungal disease (Donnelly et al., 2019).

To diagnose CPA, an immunological response to *Aspergillus* (*A. fumigatus* IgG or precipitins) is a key diagnostic feature by allowing to differentiate infected from colonized patients. Isolation of *A. fumigatus* in sputum is not enough to make the diagnosis because it may only reflect colonization. Bronchoscopic specimens are more specific to diagnose infection but culture-positive rates are 56–81% (Denning et al., 2003; Nam et al., 2010). High level of galactomannan or *Aspergillus* DNA in respiratory fluids indicate high fungal burden and are complementary markers, however, no cut-offs value have been determined and *Aspergillus* PCR is highly variable among centers as stated previously (White et al., 2011). Antigens and nucleic acids detection in serum have poor sensitivity in immunocompetent patients.

Hypersensitivity to *A. fumigatus* is documented by a positive skin-test or high levels of *Aspergillus* specific IgE and IgG. Diagnosis of ABPA is made by satisfying a set of non-specific clinical, radiographic and serologic criteria (Agarwal et al., 2013).

Although diagnosis and management of aspergillosis has been improved in the last decades by biomarker assays, their limited accessibility and requirement for batch testing restrict their impact. The recent development of lateral flow assay will allow outside specialist center testing, provided performance is acceptable. Two commercial tests are currently available; to detect galactomannan, and the other, an extracellular glycoprotein (Pan et al., 2015; White et al., 2020). Research in novel biomarkers for aspergillosis diagnosis focus on metabolite-based detection. Indeed, fungal pathogens possess a rich metabolism resulting in a vast array of secondary metabolites, many species-specific. Latest emerging metabolite-based methods for aspergillosis detection include gliotoxin, siderophore and volatile compound in the breath of patients (Savelieff and Pappalardo, 2017). Other new methods propose the use of soluble mediators detection (pentraxin-3) and specific signature of cytokines for IPA that could both support diagnosis and predict outcome (Herrera and Husain, 2018).

Classifications and algorithm

Diagnosis of aspergillosis remains difficult, therefore classifications and algorithm have been developed by experts to ease and homogenize diagnostic practices. Invasive aspergillosis in immunocompromised patients is classified into proven, probable or possible aspergillosis depending on the strength of different clinical, radiological and microbiological criteria that may be seen in patients. These criteria were recently revised by the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (EORTC/MSG) (Donnelly et al., 2019). This classification requires host factors that are most often absent in critically ill patients. For this reason, specific algorithm were developed to diagnose IPA in critically ill patient (Blot et al., 2012) and modified to suit specific predisposing diseases such as severe influenza (Verweij et al., 2020). Consensual definition and diagnosis of the various forms of CPA were proposed by the European Society for Clinical Microbiology and Infectious Disease (ESCMID), European Confederation of Medical Mycology and European Respiratory Society (ERS) (Denning et al., 2016). This classification requires a combination of radiological evidence, direct evidence of *Aspergillus* infection or an immunological response to *Aspergillus* spp. and exclusion of alternative diagnoses (i.e., other fungal diseases, mycobacterial infection, lung carcinoma, etc.), all present for at least 3-months (Denning et al., 2016). Regarding ABPA and the spectrum of

disease that derives from *Aspergillus* hypersensitivity, most recent diagnostic and classification criteria were proposed by the ISHAM (international society for human and animal mycology) dedicated working group (Agarwal et al., 2013). These criteria include (i) a predisposing condition (asthma or cystic fibrosis), (ii) two mandatory criteria (elevated total IgE levels and specific IgE against *A. fumigatus*) and (iii) at least two other criteria among radiographic findings, eosinophilia and precipitins or IgG antibodies against *A. fumigatus* (Agarwal et al., 2013).

Treatment guidelines

Treatment of IPA should not be delayed for the establishment of diagnosis due to the rapid progression and high mortality rate of the disease. It relies on both antifungal agent prescription and improvement of immune status when possible. Voriconazole or isavuconazole are recommended first line treatment (Table 1) (Patterson et al., 2016; Ullmann et al., 2018). At this time there is no clear consensus about duration of therapy; however, a minimum of 6–12 weeks is recommended, depending on clinical severity. Prophylaxis against IPA is strongly suggested for high-risk patients with posaconazole or voriconazole (Maertens et al., 2018; Ullmann et al., 2018). Echinocandins should not be used as first line IPA treatment as randomized clinical trials comparing their efficacy with other antifungals are lacking (Aruanno et al., 2019). Also, *A. fumigatus* isolates failing echinocandin therapy have been reported (Arendrup et al., 2008).

Treatment of CPA mostly aims at improving quality of life, preventing hemoptysis and disease progression. The lack of proper clinical trials and the difficulty to clearly separate the various subset of CPA impact the ability to offer strong evidence-based recommendations (Denning et al., 2016). Most forms of CPA require long-term medical therapy using either itraconazole or voriconazole. The use of oral triazole therapy depends on the type of CPA, clinical phenotype and eligibility for surgical treatment. Long term oral itraconazole treatment for CCPA is considered the standard of care. First line therapy with dosage are presented in Table 1. Alternative therapy and dosage are available in dedicated guidelines (Denning et al., 2016). Optimal duration of therapy in CPA is unknown, indefinite suppressive therapy may be appropriate in selected patients. Symptomatic hemoptysis therapy may also be part of therapeutic options. Surgery may be considered in single aspergilloma as a definitive treatment option, especially in case of severe hemoptysis. However, to be eligible for surgery a careful selection and assessment of respiratory status of patient is needed as many patients with CPA have comorbidities contributing to a higher death and peri- and post-operative complications.

Treatment of ABPA aims at reducing asthma or CF symptoms, pulmonary inflammation and prevent progression to end-stage fibrotic lung disease. Only oral corticosteroids have proven to improve ABPA symptoms and have been used for decades (Tracy et al., 2016). A recent randomized controlled trial in patients with asthma and ABPA found 0.5 mg/kg/day was effective and safer than higher doses (Agarwal et al., 2016). Duration of treatment is not clearly defined. Although having minimal systemic effects and achieving high concentration in tracheobronchial tree, inhaled corticosteroids have failed to show a benefit in ABPA (Tracy et al., 2016). The adjunction of an antifungal agent to the regimen may have a steroid sparing effect helping control inflammation (Moreira et al., 2014). Triazole such as itraconazole or voriconazole are advised but no consensual approach exists to this date. Anti-IgE (omalizumab) may be a potential immunotherapy, however there is a lack of evidence for the efficacy and safety of this drug in APBA so far (Jat et al., 2018).

Of note, regardless of the aspergillosis subtype, the prescription of triazole antifungal agent requires adjustment with therapeutic drug monitoring (Stott and Hope, 2017). Pharmacokinetics in patients with aspergillosis may vary greatly because of frequent associated comorbidities and drug interactions.

Emergence of resistance and the need for new therapeutic strategy

Azoles are the most important class of antifungal agents used for the management of aspergillosis and include itraconazole, voriconazole, posaconazole and isavuconazole. However, the use of these molecules and successful treatment of aspergillosis have been threatened by emergence of resistance. Triazole resistance has been shown to worsen prognosis in treated patients, demonstrating correlation between microbiological resistance and clinical resistance (Lestrade et al., 2019). Although resistance frequencies vary considerably between geographic regions. While France observes low rate of resistance (Alanio et al., 2016), the prevalence may be up to 10% in the Netherlands and significantly complicates the management of aspergillosis (Verweij et al., 2015). Triazole resistance is largely attributed to mutations of *cyp51A* gene, encoding an enzyme involved in the ergosterol biosynthesis. Two routes are mainly involved in triazole resistance. The first one, known as “the patient route”, occurs mainly in patients with chronic pulmonary aspergillosis or inherited immunodeficiencies who were given azoles for long term course and are the consequence of point mutations in the *cyp51A* (Verweij et al., 2015). The second one, known as “the environmental route”, is usually acquired by azole-naïve patients directly from the environment. The combination of a mutation in the *cyp51A* gene with a tandem repeat (TR) in the promoter region (e.g., TR34/L98H) is usually observed and may cause pan-azole resistance in *A. fumigatus* isolates. Emergence of this resistance mechanism are related to the widely used azole fungicides in agriculture and has been shown to be the most important route for resistance selection (Snelders et al., 2008). In Europe, wood from sawmill, wood chippings, flower bulb waste, green waste materials and soil from marker garden were described as environmental hotspots for triazole resistance selection of *A. fumigatus* (Schoustra et al., 2019). In the light of this upcoming challenge, international expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus* were published and depends on the local epidemiology (Verweij et al., 2015). Furthermore, emergence of resistance calls for an urgent need for in depth research and characterization of new therapeutic targets and strategies against this opportunistic pathogen.

Conclusion

The ubiquitous fungal pathogen *Aspergillus* causes a wide spectrum of infections of the respiratory system, which depends heavily on the complex interaction between the host immune system and the pathogen. Collaborations between basic and clinical research on *Aspergillus* and aspergillosis would certainly allow us to overcome the current shortcomings in diagnosis and therapeutic strategies.

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Mucorales and Mucormycosis

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Mucorales

Mucorales consist of a group of filamentous molds with aseptate or minimally septate hyphae and evolving taxonomy. The term “mucormycosis” originally belonged to infections caused by agents under two distinct orders, Mucorales and Entomophthorales (Mendoza et al., 2015). Recently scientists prefer to restrict the term to infections caused by Mucorales only, as members of the order Entomophthorales cause chronic subcutaneous infections without any angioinvasion, in contrast to acute course of Mucorales. The epidemiology, pathogenesis, pathology and clinical courses of the diseases caused by fungi under two orders differ considerably (Mendoza et al., 2015; Kwon-chung, 2012). Mucorales inhabit various environmental niches like decaying vegetable matter, soil, dust, etc. They are thermotolerant or thermophilic in nature, a property that imparts contrasting implications. On one hand, they are implicated as rot and spoilage organisms initiating degradation of wet organic material and also fermenting traditional foods and cheese (Reid et al., 2020). On the other hand, their thermotolerant nature enables their growth and survival at human body temperature, thus leading to severe human infection, mucormycosis. Mucormycosis is more prevalent in tropical regions possibly due to the thermotolerance of the agents (Walther et al., 2019b). Being ubiquitously present in nature, Mucorales continue to thrive on various environmental ecosystems, releasing large number of infectious particles as spores. Man acquires the infection by either inhalation/ingestion or inoculation of those spores through the breach of intact skin. In a healthy individual, those spores usually do not get past the host-pathogen interface of effective immune surveillance. However, in patients with compromised immunity or co-morbidities, those spores overcome host defense and grow to produce the disease (Mendoza et al., 2015). In developed countries, severely immunocompromised patients especially with hematological malignancies and transplant recipients are at risk of mucormycosis, but in developing countries uncontrolled diabetes mellitus overshadows other risk factors (Petrikos et al., 2012; Chakrabarti and Singh, 2014). Once the Mucorales get inside the host, the organisms tend to invade the blood vessels. This angioinvasion causes occlusion of blood supply leading to thrombosis, infarction and necrosis of the affected area, and further embolic dissemination leading to substantial morbidity and mortality.

Taxonomy of Mucorales

The taxonomy of Mucorales evolved over the years with expanding knowledge. The earliest taxonomical classification was based solely on morphological characters and sexual reproductive structures. When the sexual structure could be identified, hyphal fungi were placed under *Phycomycetes*, *Ascomycetes*, and *Basidiomycetes* depending on the structure, otherwise they were placed under class *Deuteromycetes* (Fungi imperfecti). The fungi of those four classes were classified under subdivision *Thallophyta* in the plant kingdom (Emmons et al., 1977). Mucorales, owing to coenocytic (aseptate) hyphae and zygosporangia, were categorized under *Phycomycetes*. With the increase in knowledge regarding the habitat, ecology, nutritional requirements and ultrastructure of fungi, a separate kingdom (kingdom Fungi) was proposed by Whittaker in 1969 (Whittaker, 1969). Class *Phycomycetes*, containing the maximum number of evolutionarily unrelated organisms was abolished and four phyla under kingdom Fungi were formed: *Zygomycota*, *Chytridiomycota*, *Ascomycota* and *Basidiomycota* (Kwon-chung, 2012). Phylum *Zygomycota* was further divided into two classes:

Zygomycetes containing mammalian pathogens and *Trichomycetes* containing obligate parasites of arthropods. Order Mucorales was classified under phylum *Zygomycota* and class *Zygomycetes* (along with nine other orders including Entomophthorales) (Kwon-chung, 2012). The spectrum of diseases caused by them were named as “zygomycosis.” The *Zygomycetes* were defined as those coenocytic molds that reproduce sexually with hyphal anastomosis between compatible mating types of the same species having opposite alleles at the mating type locus (Emmons et al., 1977). The mating causes fusion of haploid nucleus with subsequent meiosis and one mitotic division lead to formation of a zygospore. The zygospores are thick-walled, pigmented and often ornamented zygotes. In the presence of appropriate growth conditions, this zygospore germinates to form a sporangiophore (broad aseptate stem-like structure) ending in a sporangium (a spherical receptacle) containing numerous asexual spores, the sporangiospores (Fig. 1). The sporangiospores germinate to form vegetative haploid hyphae (Emmons et al., 1977). The placement of Mucorales under *Zygomycetes* was the accepted classification till the turn of the century.

The advancements in molecular biology enabled genomic relatedness to form the basis of evolutionary relationships. To resolve the lineages under the phylum *Zygomycota*, sequences from more than nine genes were evaluated: three sequences from ribosomal RNA—large subunit, small subunit, 5.8S; two RNA polymerase subunits RPB1 and RPB2; mitochondrial subunit DNA, elongation factor, α - and β -tubulins (Kwon-chung, 2012). The individual analysis of RNA polymerase subunits indicated *Zygomycota* to be monophyletic in origin (Liu et al., 2006). But, all other gene in isolation as well as in combination with RNA polymerase subunits clearly indicated a polyphyletic origin of *Zygomycota*. Consequent to this, a new phylogenetic tree was constructed using small ribosomal subunit. A distinct monophyletic phylum *Glomeromycota* (containing arbuscular mycorrhizal fungi that are plant symbionts) was separated out from *Zygomycota* in 2001 (Schubler et al., 2001). Subsequently, another comprehensive evaluation was carried out in 2007 (Hibbett et al., 2007) on the findings of multiple phylogenetic studies. It was reiterated that *Zygomycota* was polyphyletic and the phylum was divided into four sub-phyla: *Mucoromycotina*, *Entomophthoromycotina*, *Kickxellomycotina*, and *Zoopagomycotina*. However, the placement was left uncertain pending future studies that could better resolve the lineages (Hibbett et al., 2007). Even six-gene phylogenetic tree echoed similar findings, but a reliable outgroup was not identified (Kwon-chung, 2012). Analysis of more than 130 proteins indicated that *Nucleariids* (amoeboids of enigmatic origin) are closely related to Fungi, and could serve as reasonable outgroup (Steenkamp et al., 2006), and the reconstruction of phylogenetic tree using *Nucleariids* as the outgroup led to a separate subkingdom *Dikarya* (containing *Ascomycota* and *Basidiomycota*). However, the previous classification of *Glomeromycota* and four sub-phyla was retained (Liu et al., 2009). With the availability of increasing number of sequences under each taxon, there remains the possibility of further revisions of phylogenetic classification.

In 2016, a phylum-level phylogenetic classification of zygomycete fungi was proposed on the basis of genome-scale data (Spatafora et al., 2016). Genomes of 25 zygomycetes taxa and 192 proteins were combinedly used for phylogenetic analysis. Two clades were defined with paraphyletic relationship, and two phyla were identified under them—*Mucoromycota* and *Zoopagomycota*. Both of these phyla contained three sub-phyla each; Mucorales were placed under phylum *Mucoromycota* and sub-phylum *Mucoromycotina*, while Entomophthorales were placed under phylum *Zoopagomycota* and sub-phylum *Entomophthoromycotina* (Fig. 2) (Spatafora et al., 2016). With this classification, the term “zygomycosis” was abandoned and the terms “mucormycosis” and “entomophthoromycosis” were defined for human infections caused by Mucorales and Entomophthorales, respectively (Kwon-chung, 2012).

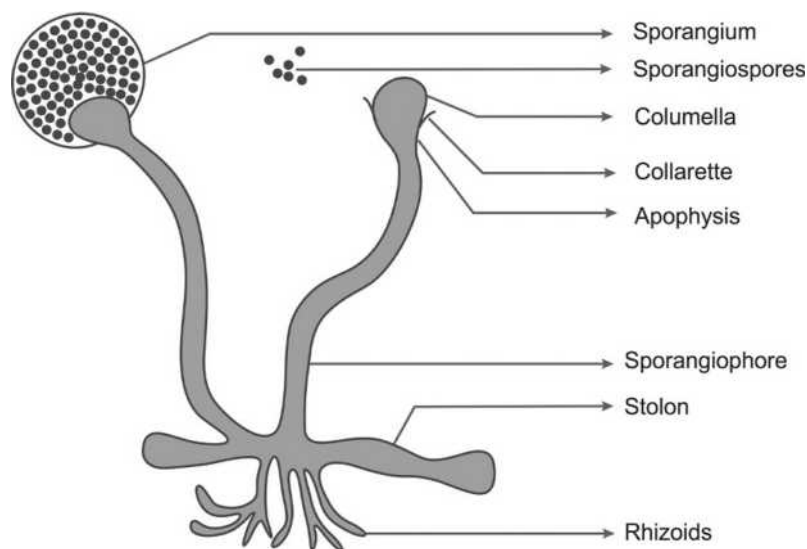


Fig. 1 Schematic representation of morphological features of *Rhizopus* spp. showing stem-like sporangiophores arising from branch-like stolons having root-like rhizoids and terminating into sac-like sporangium filled with numerous seed-like sporangiospores. The sub-sporangial structures of apophysis, columella and collarete are also seen.

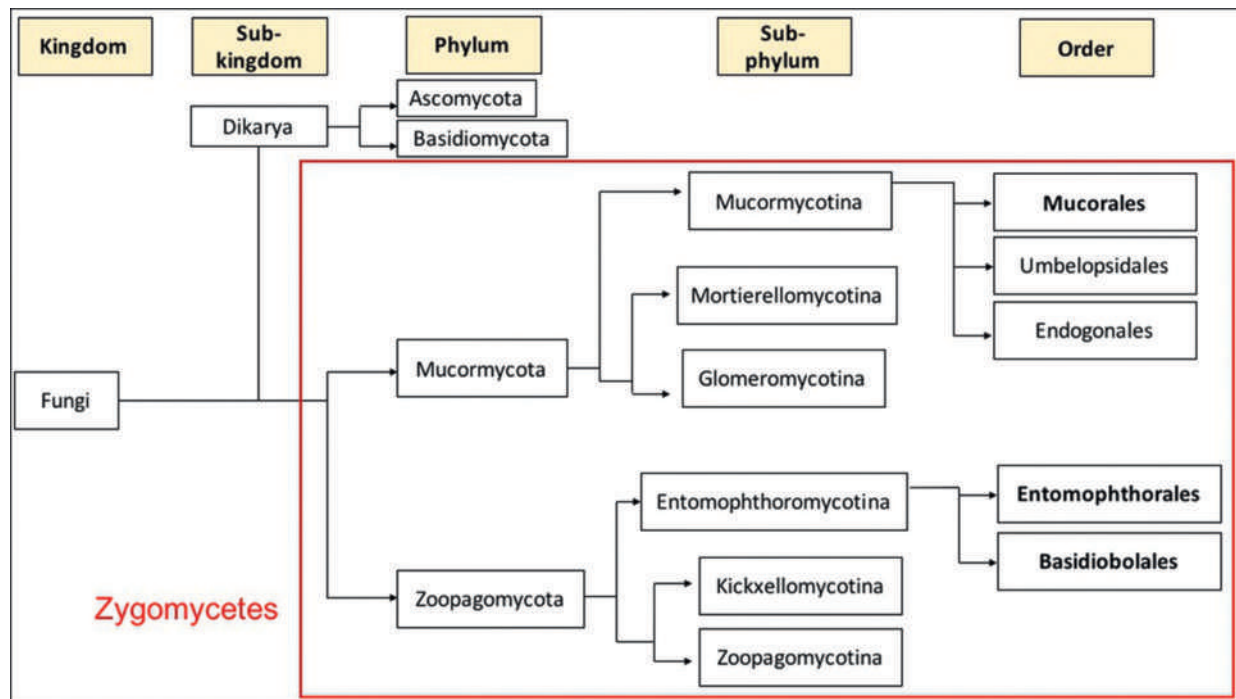


Fig. 2 Current taxonomical placement of *Mucorales*. Phyla *Mucormycota* and *Zoopagomycota* collectively constitute the “zygomycetes” and human infections are caused by agents under three orders—*Mucorales*, *Entomophthorales* and *Basidiobolales* (seen in bold).

Classification of Mucorales

Based on the phylogenetic analysis, Mucorales currently consist of 261 species under 55 genera (Benny et al., 2016). Human infections have been reported by 38 species under 11 genera—*Actinomucor*, *Apophysomyces*, *Cokeromyces*, *Cunninghamella*, *Lichtheimia*, *Mucor*, *Rhizomucor*, *Rhizopus*, *Saksenaia*, *Syncephalastrum*, and *Thamnostylum*. Members of *Rhizopus*, *Mucor* and *Lichtheimia* are responsible for 70–80% of human mucormycosis cases. Infections with *Apophysomyces*, *Cunninghamella*, *Saksenaia*, *Rhizomucor*, *Cokeromyces*, *Actinomucor* and *Syncephalastrum* spp. are rarely reported (Gomes et al., 2011). The prevalence of species isolated from patients varies geographically, as depicted in Fig. 3.

The medically important genera can be identified on the basis of morphological and physiological characteristics, as shown in Fig. 4.

Rhizopus arrhizus among the members under the genus *Rhizopus* (*R. arrhizus* or *R. oryzae*, *R. microsporus*, *R. homothallicus*) is the most common agent causing mucormycosis globally (Jeong et al., 2019). Genus *Rhizopus* is characterized by unbranched sporangioophores arising singly or in whorls bearing sporangia with apophysis, the rhizoids arising opposite the sporangioophores. The three medically important species are differentiated on the basis of size, shape and ornamentation of sporangiospore. The issue of the correct name of its type species, *R. arrhizus* vs *R. oryzae*, has not been fully resolved. The term *R. arrhizus* was suggested by Fischer et al. (1892) and *R. oryzae* was suggested by Went and Prinsen Geerligs (1895). As *R. arrhizus* had a short description without any figure and type strain, mycologists preferred the term *R. oryzae* for years. However, Ellis (1985) legitimized the name *R. arrhizus* by describing an ex-neotype strain for *R. arrhizus*, which is also clustered phylogenetically with *R. oryzae*.

The genus *Mucor* has 76 accepted species, 12 out of which are pathogenic to man. They commonly cause cutaneous infections or rarely disseminated and gastrointestinal disease. Members of this genera are characterized by formation of sporangia and similar shaped suspensors; the apophysis, sporangiola, and rhizoids are usually absent (Wagner et al., 2019). However, recent studies have shown that certain species under this genera may have rhizoids (Walther et al., 2013).

Genus *Rhizomucor* can withstand high temperature (55°C) and consists of short ellipsoidal sporangiospores. They predominantly cause pulmonary infections. Recently, all mesophilic species of *Rhizomucor* were transferred to *Mucor* on the basis of molecular studies (Walther et al., 2019b).

All thermotolerant members under the genus *Absidia* were separated to form the genus *Lichtheimia*, as they could be differentiated by growth at 43°C and absence of sub-sporangial septum (Garcia-Hermoso et al., 2009; Alastruey-Izquierdo et al., 2010). Members of genus *Lichtheimia* have pyriform sporangia and distinct apophysis over branched sporangioophores. Three pathogenic species, *L. corymbifera*, *L. ramosa* and *L. ornata*, can be differentiated by physiological properties. *L. ramosa* has higher growth rate at 43°C, and *L. ornata* has densely packed giant cells during growth on yeast extract agar (Alastruey-Izquierdo et al., 2010). *Lichtheimia* spp. commonly cause cutaneous and pulmonary infections, and occasionally rhino-orbital form (Alastruey-Izquierdo et al., 2010). *Lichtheimia* spp. are the second most common agents of mucormycosis, after *Rhizopus* spp., in Europe and Africa (constituting 18–24% cases) (Lanternier et al., 2012).

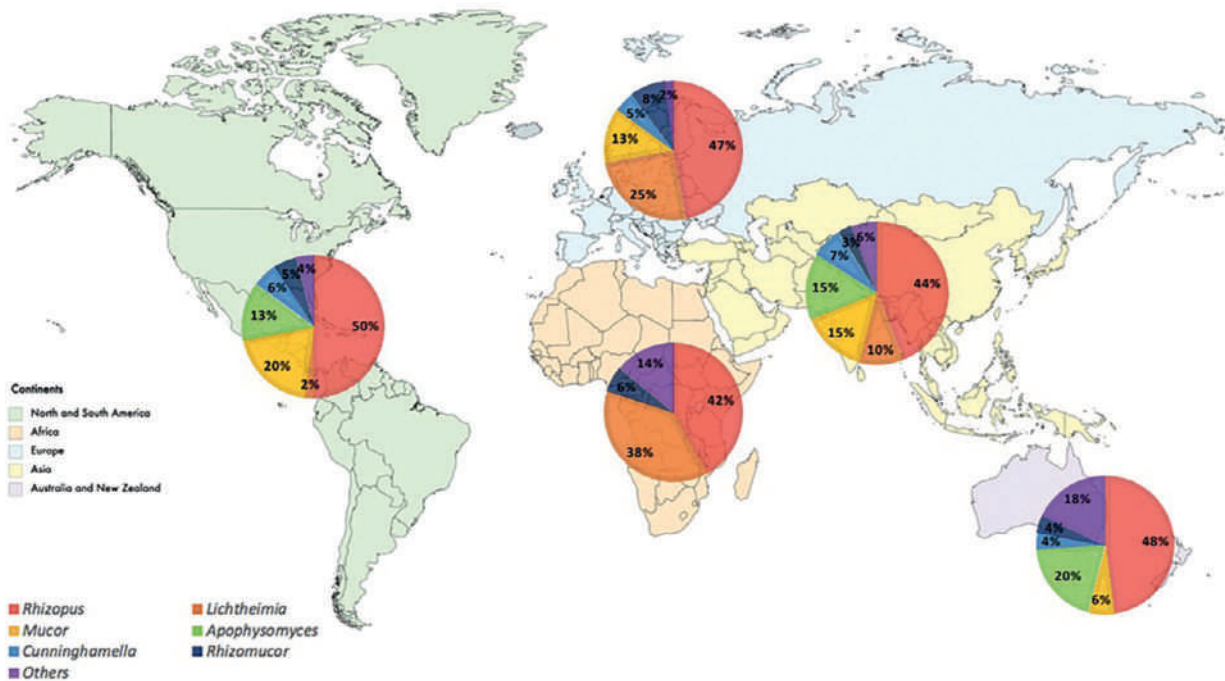


Fig. 3 Inter-continental differences seen in the geographical distribution of agents of mucormycosis.

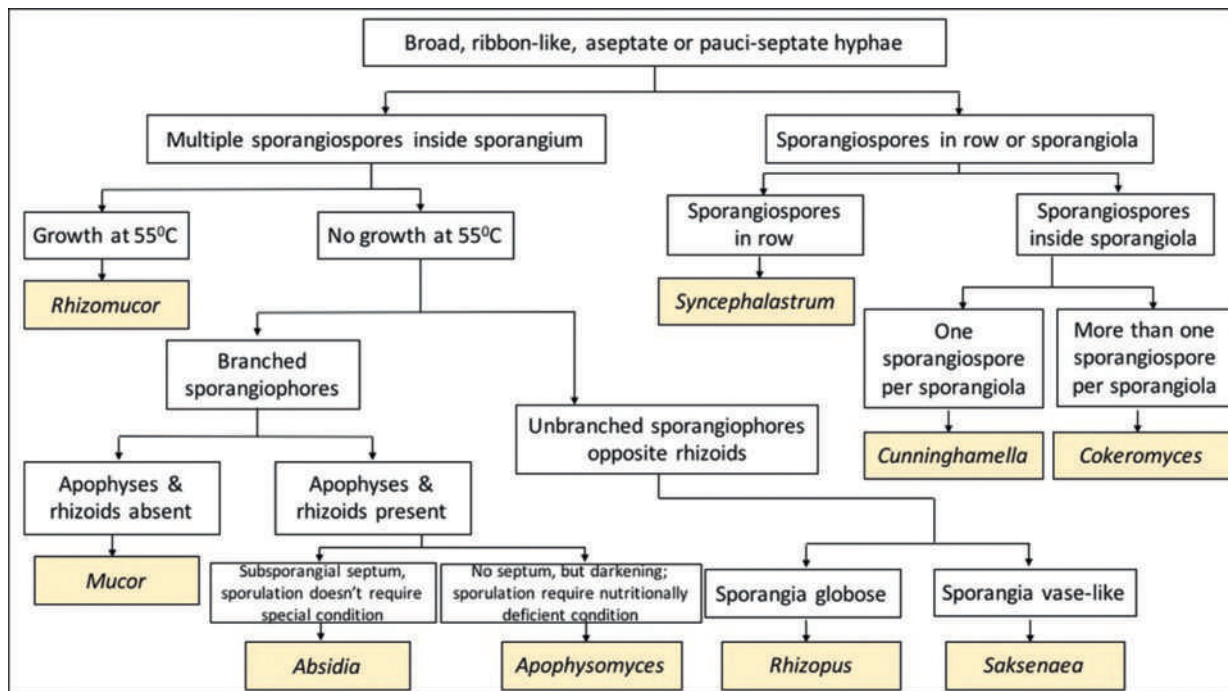


Fig. 4 A scheme of identification of common agents of mucormycosis on the basis of their morphological and physiological characteristics.

The genus *Apophysomyces* contains six pathogenic species. The organism was first described from the soil of a mango orchard in North India in 1979 (Misra et al., 1979). Subsequently *Apophysomyces* spp. were isolated from soil and decaying matter of the tropical and subtropical regions. The low nitrogen content and alkaline pH of the soil were found to be significantly associated with isolation of *Apophysomyces* species (Prakash et al., 2016). The fungi are more commonly implicated in causing cutaneous mucormycosis especially post-traumatic and post-natural disaster in tropical and sub-tropical regions. *Apophysomyces variabilis* is the second most common agent of mucormycosis in India, after *Rhizopus arrhizus* (Chakrabarti and Singh, 2014; Gomes et al., 2011).

Colonies are brownish grey in color. Sporangiophores are unbranched, arising perpendicularly from aerial hyphae ending in pyriform sporangia with funnel-shaped columella ("Champagne glass" appearance) (Gomes et al., 2011).

There are seven species under genus *Cunninghamella* but only *C. bertholletiae* is pathogenic to humans. It is also a soil fungus found in Mediterranean and subtropical zones. The sporangiophore is straight and branched, ending in globose or pyriform-shaped vesicle from which several sporangia develop. The sporangia are ovoid, single-celled and smooth-walled (Gomes et al., 2011). Mucormycosis due to *Cunninghamella* spp. have highest mortality rates among all Mucorales (Jeong et al., 2019).

The genus *Saksena* is characterized by flask-shaped sporangia with columella and darkly pigmented rhizoids. Three species are recognized to be clinically relevant—*S. vasiformis*, *S. oblongispora* and *S. erythrospora*. The three species can be differentiated by the shape of their sporangiospores—cylindrical in *S. vasiformis*, oblong in *S. oblongispora* and biconcave in *S. erythrospora* (Alvarez et al., 2010). *S. vasiformis* has been associated with cutaneous mucormycosis in tropical and sub-tropical regions (Gomes et al., 2011) and at least 10 case reports of *S. erythrospora* causing cutaneous mucormycosis have been reported (Mukherjee and Kundu, 2018).

The genus *Syncephalastrum* is characterized by cylindrical merosporangium inside which sporangiospores are arranged in rows, giving an *Aspergillus*-like resemblance. *S. racemosum* occasionally produces human infection. The species is usually found in soil and dung, and may be isolated as laboratory contaminant (Gomes et al., 2011). A rare case of rhino-orbito-cerebral mucormycosis due to *Thamnostylum lucknowense* has been reported from India (Xess et al., 2012).

Mucormycosis

Mucormycosis is caused by the fungi under the orders Mucorales and Entomophthorales, and *Mucormycetes*. The diseases caused by Mucorales are different from Entomophthorales, and the fungi under first order produce acute and aggressive disease needing prompt diagnosis and management. The present chapter discusses the diseases caused by Mucorales only.

Magnitude of the problem

Incidence

Mucormycosis has long been considered as a rare disease, however, the incidence has increased manifold in the last two decades (Reid et al., 2020; Ambrosioni et al., 2010). Fig. 5 depicts the density of cases of mucormycosis from different countries across the world. The rise in number of cases has been reported from Belgium (Saegeman et al., 2010), France (Bitar et al., 2009), India (Prakash et al., 2019) Middle East and North Africa (Stemler et al., 2020), Spain (Torres-Narbona et al., 2008; Guinea et al., 2017), Switzerland (Ambrosioni et al., 2010), and United States (Vallabhaneni et al., 2017). A population-based study from France reported a rise in mucormycosis cases from 0.7 per million in 1997 to 1.2 per million in 2006 (Bitar et al., 2009). Belgium reported a eightfold increase in the number of cases per 10,000 patient days over a period of 9 years from 2000 to 2009 (Saegeman et al., 2010). Switzerland reported a sharp rise from 0.57 cases/100,000 population/year to 6.3 cases/100,000 population/year after 2003 (Ambrosioni et al., 2010). The highest burden of mucormycosis is borne by India and Pakistan with 140 cases/million population each, followed by Portugal at 95 cases/million population and Qatar at 12.5 cases/million population (Prakash and Chakrabarti, 2019; Jabeen et al., 2017). The burden of mucormycosis is estimated at four continents having 3 cases/million for North America, 1–3 cases/million for South America and Europe (excluding Portugal) and 0.6 cases/million for Australia (Prakash and Chakrabarti, 2019).

Economic burden

Mucormycosis levies a considerable economic burden as well, much higher than other invasive fungal diseases. The mean treatment cost for mucormycosis in a German healthcare facility was estimated at €53,261 per patient, more than two times that of €28,432 per patient for candidiasis and €21,063 for invasive aspergillosis (Heimann et al., 2018). Further, the patients of mucormycosis required an additional 26.5 days of hospital stay compared to candidiasis or aspergillosis (Heimann et al., 2018). A USA study estimated that there was an additional cost of \$64,526 and length of stay of 16.5 days per patient for invasive mucormycosis (Zilberberg et al., 2014). The cost-of-illness analysis reported the mean daily and overall hospitalization cost per patient of invasive mucormycosis were \$4096 and \$112,419, respectively (Kontoyannis et al., 2016). The main drivers for the high cost of management of mucormycosis were the antifungal drugs and need for specialized critical care units.

Mortality

The all-cause mortality for mucormycosis range from 40% to 80% depending upon the underlying risk factors and the site of mucormycosis (Reid et al., 2020). While healthy non-immunocompromised patients without comorbidities have the highest rate of survival, those with hematological malignancies, stem-cell transplant and extensive burns have the lowest rate of survival. Disseminated forms of the disease, especially those reaching to the central nervous system, generally have mortality rate higher than 80%. Localized sinus disease or limited skin infection have much lower mortality as they are amenable to early diagnosis and surgical resection. Gastrointestinal mucormycosis has also higher mortality due to delay in diagnosis and polymicrobial sepsis ensuing with it (Prakash and Chakrabarti, 2019).

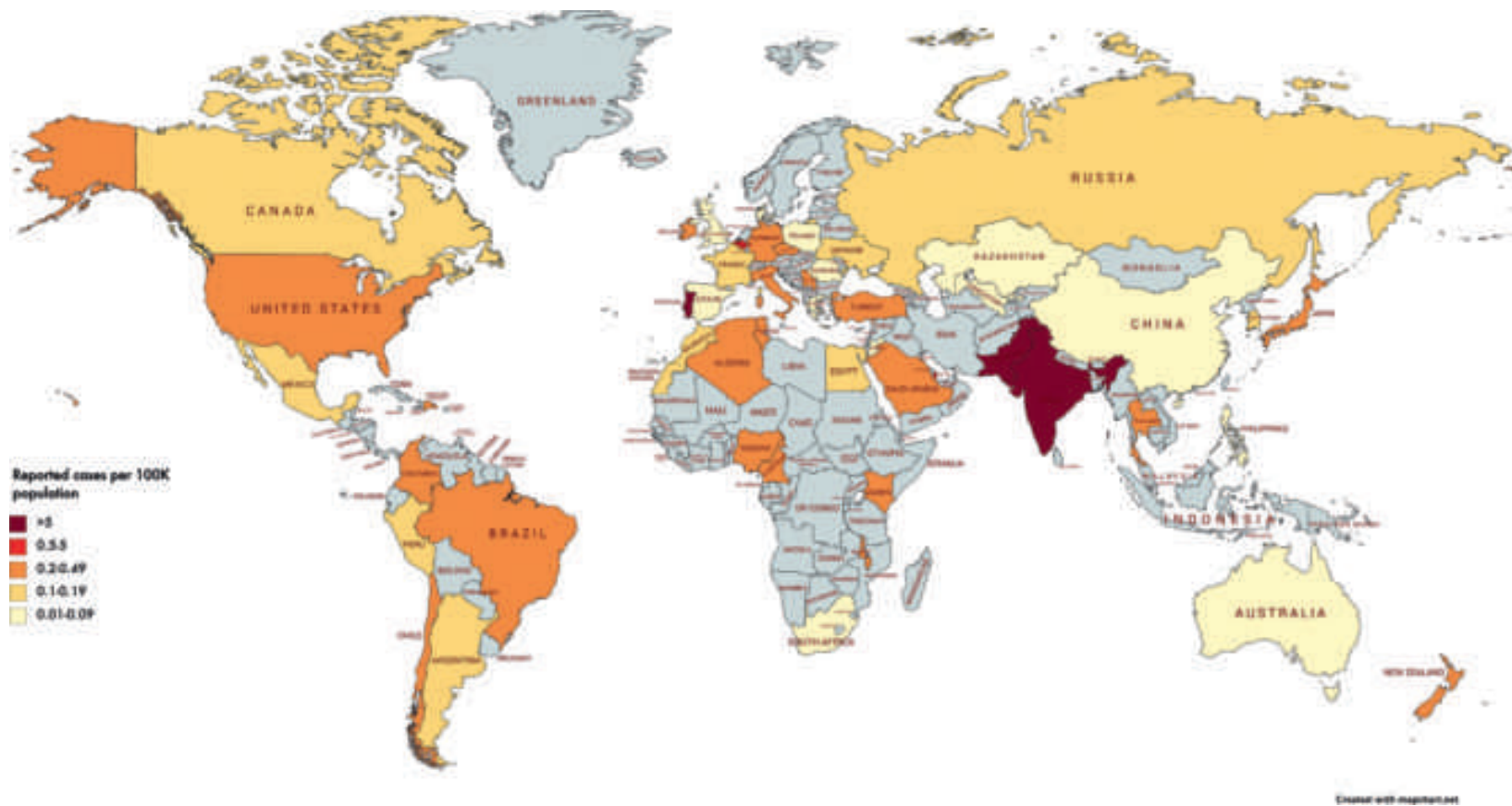


Fig. 5 World map showing the density of cases of mucormycosis across different countries of the world.

Epidemiology

Though the agents of mucormycosis are ubiquitous throughout the world, the epidemiology of mucormycosis varies between the developed and developing countries. This difference is attributed to the difference in risk factors among the patient populations. The important risk factors associated with increased risk of mucormycosis are uncontrolled diabetes mellitus (DM), hematological malignancy (HM) undergoing chemotherapy, hematopoietic stem cell (HSCT) and solid organ transplant (SOT) recipients undergoing immunosuppressive therapy, other immunocompromised hosts (immunosuppression due to HIV, chemotherapy, autoimmune disease therapy), peritoneal dialysis, iron overload, extensive skin injury (burns, trauma) and previous voriconazole therapy (Reid et al., 2020). A cumulative worldwide analysis of 600 publications between 2000 and 2017 revealed that DM was the commonest predisposing factor being present in 40% patients with ketoacidosis in half of the subjects, followed by trauma and HM (32.5% each), solid organ transplant (14%), burns (11%). In 18% patients of mucormycosis, no predisposing factor was identified (Jeong et al., 2019).

On analyzing the risk factors specific to geographical locations, DM was found to be the commonest risk factor in China (Jiang et al., 2016; Chen et al., 2018), India (Chakrabarti et al., 2006; Chakrabarti and Singh, 2014), Iran (Dolatabadi et al., 2018), and Mexico (Corzo-León et al., 2018); while HM and transplant recipients were the major predisposing factors for mucormycosis in Europe and North America (Skiada et al., 2011; Kontoyiannis et al., 2016). Though HM and transplant associated mucormycosis rate have gone up in developing countries as well, DM still overshadows those risk factors (Meis and Chakrabarti, 2009). Globally, the contribution of DM as a predisposing factor for mucormycosis varies from 17% to 88% (Skiada et al., 2011). The prevalence of mucormycosis among Indian diabetics was estimated at 1.6 cases/1000 diabetics (Bhansali et al., 2004). In Iran and Mexico diabetes was reported at 74% and 72% cases of mucormycosis respectively (Corzo-León et al., 2018; Dolatabadi et al., 2018). Though it is claimed that due to the regular use of statins in diabetics, a downward trend of mucormycosis is noted in United States, DM is still a major risk factor (52%) among mucormycosis cases (Kontoyiannis et al., 2016). However, the frequency of DM as risk factor is low (<30%) in European countries, with Italy reporting at 18%, France reporting at 23% and Greece reporting at 29%. The Mucormycosis case registry under European Confederation of Medical Mycology reported DM in 17% of mucormycosis patients (Skiada et al., 2011).

HM is considered the most common predisposing factor for mucormycosis in Europe and the United States, being present in 38% to 62% of the patients (Prakash and Chakrabarti, 2019). Contrary to those high figures, HM was identified as a risk factor for mucormycosis in 13.6% patients in Mexico (Corzo-León et al., 2018), 6.4% patients in India (Prakash et al., 2019) and 3.4% patients in Iran (Dolatabadi et al., 2018). In France 0.4% patients of allogeneic HSCT recipients were reported to develop mucormycosis (Xhaard et al., 2012) while the annual cumulative prevalence of mucormycosis was 0.29% among HSCT patients of the USA (Kontoyiannis et al., 2010).

SOT has been reported as a predisposing factor for mucormycosis in 2–15% cases (Kontoyiannis et al., 2016; Jeong et al., 2019; Prakash et al., 2019). It varies with the type of SOT and the underlying comorbidities. The reported incidences of mucormycosis are 4–16/1000 liver transplants, 13–14/1000 lung transplants, 8/1000 heart transplants and 0.4–0.5/1000 renal transplants (Almyroudis et al., 2006). SOT recipients with additional factors like DM, renal failure, voriconazole prophylaxis and prolonged steroid therapy (>600 mg prednisolone) are at increased risk of developing mucormycosis.

Invasive mould infections in intensive care units (ICUs) are gaining importance in recent years. In a multi-center ICUs study in India, 24% of all invasive fungal diseases were reported to be of mucormycosis (Chakrabarti et al., 2019). The gastrointestinal mucormycosis was seen to occur majorly in premature neonates and adults admitted to the intensive care units (Rammaert et al., 2012).

Mucormycosis has also been reported to occur as breakthrough invasive fungal infection while on antifungal prophylaxis, especially azoles and echinocandins (Lerolle et al., 2014; Fung et al., 2018; Lionakis et al., 2018; Louis-Auguste et al., 2018). In a meta-analysis of 92 breakthrough mucormycosis cases, the patients were either on voriconazole (52%) or fluconazole (25%) or caspofungin (9.8%) (Jeong et al., 2019). Breakthrough mucormycosis had also been reported even when the patient was on Mucorales-active antifungal prophylaxis like posaconazole (Lerolle et al., 2014) or isavuconazole (Fung et al., 2018).

Contrary to the belief that mucormycosis is primarily community-acquired disease, the healthcare-associated mucormycosis is gaining importance. Cases of mucormycosis have been reported from contaminated umbilical catheter, wooden tongue depressors and adhesive bandages and also from hospital air (Richardson and Rautemaa-Richardson, 2020). Mucorales were isolated from both indoor and outdoor environments of a North Indian hospital; with *R. arrhizus*, *Cunninghamella* spp., and *A. variabilis* as common isolates (Prakash et al., 2020). As indicated by the types of exposure, the most common site of involvement was skin (57%) followed by gastrointestinal tract (15%). In a study analyzing 169 cases of healthcare associated mucormycosis over three decades, Rammaert et al. mentioned 69 (41%) cases were at surgical site infections happening commonly after cardiovascular surgery, followed by gastrointestinal, ocular and orthopedic surgery. In 57% cases, the mucormycosis was limited to the skin (Rammaert et al., 2012).

Mucormycosis has also been reported to occur as outbreaks (Antoniadou, 2009). Walther et al. (2019a) have reviewed 32 outbreaks due to Mucorales; 28 occurred in healthcare settings and four in community. The bandages, adhesive tapes, tongue depressor and linen contaminated with Mucorales, were incriminated in the outbreaks of healthcare settings. Among seven outbreaks from contaminated adhesive tapes, five were due to *Rhizopus* spp. and two due to *Lichtheimia* spp. (Patterson et al., 1986; Fréalle et al., 2018). Wooden tongue depressors used as splints for cannulation of neonates or for preparing oral medications were the source of two outbreaks, and *Rhizopus microsporus* was isolated (Walther et al., 2019a). Hospital linen were also implicated in two outbreaks

involving eleven patients infected with *Rhizopus* spp. (Duffy et al., 2014). Contaminated air was the source of nine outbreaks; air conditioners, construction activities and proximity to helipad were indicated as the source, and *Rhizomucor* spp. were isolated in four of those outbreaks (Walther et al., 2019a). In China, 12 cases of intestinal mucormycosis due to *Rhizopus microsporus* were reported over six months in hematology ward. The contaminated corn-starch in allopurinol tablet or ready-to-eat food was considered as the possible source of that outbreak (Cheng et al., 2009). Mucormycosis outbreaks due to *Apophysomyces elegans*, *A. trapeziformis*, and *Lichtheimia* spp. following natural disasters like tsunami, tornado and volcanic eruptions have also been documented (Etienne et al., 2012). An outbreak from contaminated yogurt involving more than 200 consumers occurred in 2013 wherein *Mucor circinelloides* was identified as the causative agent (Lee et al., 2014).

Clinical types

Mucormycosis has varied presentation depending on site of involvement and usually classified as rhino-orbito-cerebral mucormycosis (ROCM), pulmonary, cutaneous, gastrointestinal, and disseminated form. Other less commonly involved sites have been heart, bone, ear, parotid gland, urinary bladder, uterus and lymph nodes (Serris et al., 2019). Isolated renal in immunocompetent host is a unique entity recognized in China and India (Chakrabarti et al., 2001; Yu and Yu Li, 2006; Chakrabarti and Singh, 2014). Fig. 6 depicts the prevalence of each clinical type of mucormycosis and associated major predisposing factors with those types.

ROCM is the most common clinical type of mucormycosis and uncontrolled DM is the most common risk factor (69, 70). Other risk factors identified are SOT, corticosteroid therapy, intravenous drug abuse and renal failure. The patients present with facial swelling, facial pain, nasal ulceration and discharge, epistaxis, sinusitis and cranial nerve palsy in addition to fever and headache. Extension to orbit causes painful eye, blurred vision, proptosis, orbital cellulitis, discoloration and necrosis. Spread to cerebral hemisphere occurs via the superior orbital fissure or the cribriform plate. Massive necrosis can result due to the angio-invasive property of Mucorales. The agents are also notorious for bony erosion and destruction. Cerebral invasion can cause altered mental status, hemiplegia and is associated with high mortality (Lanternier et al., 2012). The overall mortality of ROCM was 41.5%: reaching up to 56% in cerebral involvement, 20% for sino-orbital form and almost nil in disease limited to sinus only (Serris et al., 2019).

Pulmonary mucormycosis, the second most common clinical type of mucormycosis is associated commonly with patients of HM and transplant recipients, though diabetes may also be an associated factor in some cases. HM was identified as the most important risk factor (in nearly 40% patients) by Roden et al. (2005) possibly due to chemotherapy-induced defect in innate pulmonary defenses. In another series including 92 cases of pulmonary mucormycosis, HM was found as risk factor in 32-50% of patients; renal disease in 13-18% cases and transplant in up to 18% cases (9% each in HSCT and SOT) (Feng and Sun, 2018). One study from India reported that 21% cases of pulmonary mucormycosis had history of pulmonary tuberculosis in the past (Prakash et al., 2019). Pulmonary mucormycosis is usually unilateral and upper lobe is the most commonly involved site. The presenting

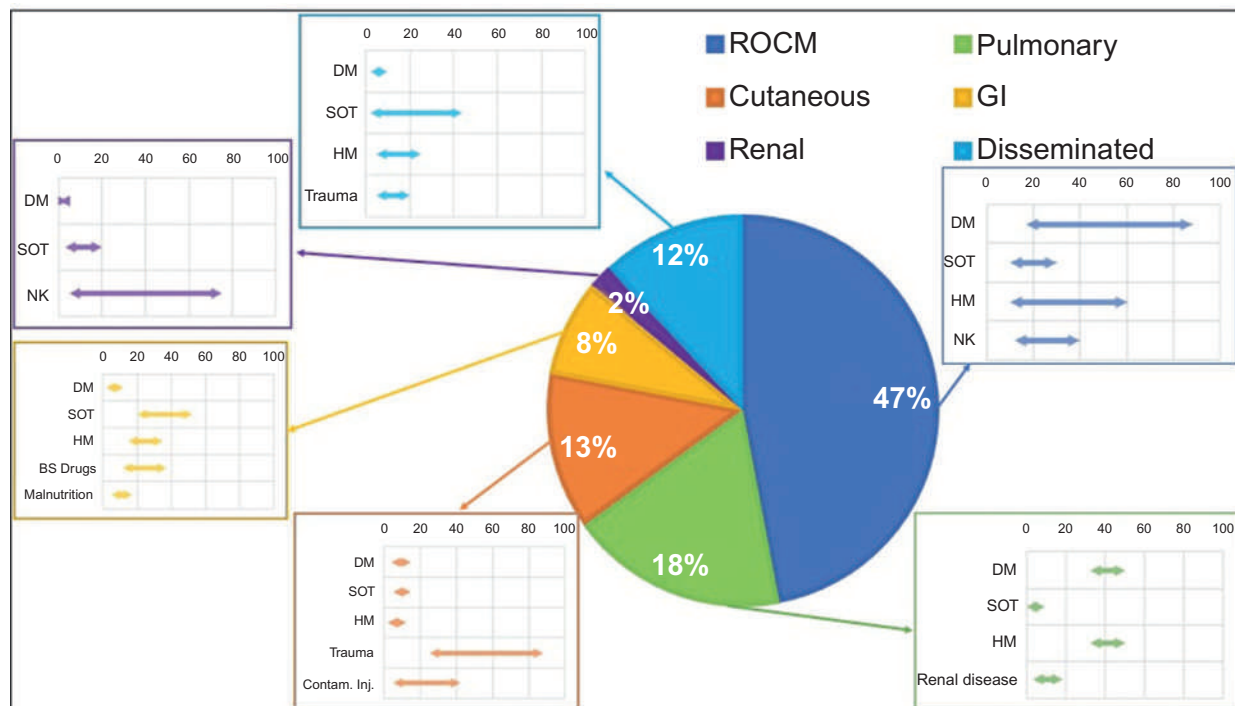


Fig. 6 Prevalence of different clinical types of mucormycosis and contribution of different predisposing factors for each.

symptoms include high grade fever, cough, pleuritic chest pain, dyspnea and hemoptysis. The 90-day mortality of pulmonary mucormycosis was 48% (Lanternier et al., 2012); and among different species of Mucorales, *Cunninghamella* spp., though rare in frequency, was commonly associated with high mortality (Jeong et al., 2019).

Cutaneous mucormycosis occurs when Mucorales gain entry through the breached skin after trauma or burns. In nearly 44–68% cases, the host is immunocompetent; DM and SOT are reported in few (5–15%) patients only (Skiada et al., 2012). The single most important predisposing factor is penetrating trauma, seen in 25–88% cases followed by contaminated intramuscular injection (42%), open wound injury (21%) (Rodén et al., 2005; Skiada et al., 2012). Other risk factors that predispose to cutaneous mucormycosis are road traffic accidents, surgical procedures, contaminated dressings, burns, natural disasters, animal bites. Cutaneous mucormycosis can be divided into three types on the basis of extent of invasion: (a) localized infection (32–56%) wherein the Mucorales remain restricted to the cutaneous and subcutaneous tissue without further invasion; (b) deep infection (24–52%), wherein deep tissue, muscles, fascia, tendons and even bones may be involved leading to massive necrosis of tissue and blackish eschar; (c) the third type (16–20%) is the one occurring as part of disseminated mucormycosis (Rodén et al., 2005; Feng and Sun, 2018; Skiada et al., 2012). The outcome is better in cutaneous mucormycosis owing primarily to early diagnosis and liberal excision of infected tissue. The 90-day mortality varies between 13 and 23% (Lelievre et al., 2014).

Gastrointestinal mucormycosis (GIM) is rarely reported ante-mortem due to non-specific symptoms or signs and difficulty in collecting samples for diagnosis; majority of the cases are diagnosed accidentally during surgery. The classical risk factors for developing GIM among non-classical immunosuppressed patients were very low birth or premature babies (64%) and malnutrition (17%) in pediatric population; and use of broad-spectrum antimicrobials (27%) diabetes (23%) and dialysis (15%) in adult population (Kaur et al., 2018). Among immunocompromised adults, SOT (52%), neutropenia (38%) and HM (35%) are important predisposing factors (Rodén et al., 2005). The common presenting symptoms are abdominal pain, bleeding and abdominal distension. The most common site of involvement is large intestine followed by stomach and then small intestine. The mortality remains high at 57% owing primarily to delay in diagnosis and management (Dioverti et al., 2015).

Isolated renal mucormycosis (IRM) in apparently healthy individuals is a distinct clinical form with highest number of cases reported from India (Chakrabarti et al., 2001, 2006; Chakrabarti and Singh, 2014) followed by China (Yu and Yu Li, 2006; Lin et al., 2003). Kidney may be involved as part of dissemination in 22% cases of disseminated mucormycosis. Contrary to this observation, in IRM; kidney is the sole organ involved in majority of cases (Chakrabarti and Singh, 2014). In nearly 75% of the Indian cases of IRM, no predisposing factor was identified (Chakrabarti et al., 2001; Chakrabarti et al., 2006). The patients usually present with fever, flank pain, hematuria and anuria. The classical radiological features include enlarged non-hydronephrotic kidneys with hypo-densities, perinephric stranding (Dhaliwal et al., 2015). Over 75% patients are diagnosed ante-mortem due to characteristic clinical presentations and clinical features. However, the disease mortality still remains high (>50%) (Chakrabarti et al., 2001, 2006; Chakrabarti and Singh, 2014). The exact route of acquiring IRM remains unknown and it is speculated that lungs could be a possible source. Though ascending infection could occur, as foci of infection have been documented in both the lungs and urinary bladder in few patients of IRM (Gupta et al., 1998, 2010).

Disseminated mucormycosis is the most severe form and occurs due to the angio-invasive property of the Mucorales. The entity is mostly seen in immunocompromised individuals; SOT and HM serve as the most important predisposing factors. In a meta-analysis of different clinical forms of mucormycosis, disseminated form was found in 13% cases (Skiada et al., 2011). The common site of involvement is lung (>90%) followed by central nervous system (53%). The sinus, liver and kidney involvement may also be part of dissemination. The mortality of disseminated form varies from 58 to 96% (Skiada et al., 2011; Lanternier et al., 2012).

Diagnosis of mucormycosis

The diagnosis of mucormycosis depends on multi-pronged approach involving clinical, radiological, microbiological and histopathological modalities. A high index of clinical suspicion should be kept in mind especially when handling patients with various predisposing factors. Angio-invasion and tissue necrosis are the hallmark of the disease, but waiting for these features to develop may substantially worsen the overall prognosis of the patient. A good clinical judgement, like development of diplopia in a diabetic patient or pleuritic chest pain in a neutropenic patient should alert the clinician of the possible mucormycosis. In premature neonate triad of shock requiring vasoactive drugs, metabolic acidosis, and abdominal distension may alert a neonatologist of possible gastrointestinal mucormycosis (Gupta et al., 2018). Think about mucormycosis in any patient suspected of invasive fungal infection where biomarkers galactomannan or beta-D-glucan are negative. Occasional indolent presentation may cause dilemma in the diagnosis (Celis-Aguilar et al., 2019).

Imaging is one of the earliest modalities ordered to diagnose mucormycosis. Computed tomography may help to delineate the extent of disease in case of ROCM. In case of pulmonary mucormycosis, though the reverse halo sign, multiple nodule are commonly associated with mucormycosis (Chamilos et al., 2005), the patient may present with non-specific infiltrates, pneumonic patches and peripheral ground glass halo (Hammer et al., 2018). The radiological diagnosis is often difficult when the host has coinfection with *Aspergillus* spp. or tuberculosis. Imaging has proven useful in differentiating isolated renal mucormycosis from acute pyelonephritis, glomerulonephritis and renal tuberculosis as enlarged non-functioning kidney with low attenuation areas is suggestive of renal mucormycosis (Chakrabarti and Singh, 2014). 18F-fluorodeoxyglucose (FDG) positron-emission tomography (PET) has also been used to delineate the extent of mucormycosis (Liu et al., 2013).

Microbiological diagnosis involves examination of smear and culture of the offending Mucorales. The most important sample for isolating the agent is the necrotic tissue. Direct microscopic examination of clinical samples with 10% KOH and a fluorescent dye like Calcofluor and Blankophor allow rapid presumptive diagnosis (Cornely et al., 2019; Skiada et al., 2018). The characteristic appearance of Mucorales is described as broad (6–25 µm) ribbon-shaped aseptate or sparsely-septate hyphae with wide-angle branching. The hyphae are friable and hence teasing of tissue should be avoided. Mucorales are non-fastidious in their growth requirements and culture on Sabouraud's dextrose agar usually yield growth in 2–5 days (Ribes et al., 2000). The incorporation of antibacterial agents in SDA would prevent bacterial contamination, but chlorheximide (actidione) should be avoided as it is inhibitory for Mucorales. The fungi have a typical wooly growth and most species sporulate on ordinary media. *Apophysomyces* and *Saksenaea* species, however, do not sporulate easily and may require special media like potato dextrose agar, oat meal agar, cornmeal-glucose-sucrose yeast extract agar, water agar with yeast extract (Chakrabarti et al., 2006). The macroscopic and microscopic examination of the isolate enables identification owing to their distinct morphological features. Once sporulated, the identification, even up to species level, can be made for majority of the Mucorales at the hands of an expert mycologist. Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS) can also be used for species identification in a relatively short span of time by comparing the mass spectra of the isolate with either the in-built database of the machine or in-house database of the individual laboratory (Schwarz et al., 2019). Other commercially-available platforms that can be used for identifying species of Mucorales are ID32C and API 50CH, both from BioMérieux, Marcy l'Etoile, France (Ramani et al., 1998). Once obtained in culture, the Mucorales can be subjected to drug susceptibility testing also, though breakpoints of antifungal drugs are not available for this group of fungi.

As the yield of culture is low (maximum 50%), histopathology is an important tool for diagnosis (Walsh et al., 2012). The most commonly employed stains are hematoxylin and eosin, periodic acid Schiff, and Gomori's methanamine silver wherein the broad aseptate hyphae of Mucorales can easily be visualized (Cornely et al., 2019). The histological feature is the necrosis of the surrounding tissue seen as acute infiltrates involving the blood vessels. The infiltrated blood vessel can be seen having thrombi and infarction. At sites where enough aeration is available, like those involving sinus or a pulmonary cavity communicating with airways, sporulating hyphae can also be seen. The diagnosis of Mucorales on the basis of histopathology is occasionally challenging as characteristic ribbon shaped broad hyphae may not be always present (Kung et al., 2018). For better differentiation, immuno-histochemical stain may be used wherein the specific monoclonal antibody helps differentiate Mucorales from other fungi (Jung et al., 2015).

Molecular assays have greatly revolutionized the identification of the offending Mucorales in culture or in tissue. DNA extraction from paraffin-embedded tissues may pose some difficulty, as paraffin degrades the DNA of the fungi (Millon et al., 2019). Several gene targets have been used for the identification of Mucorales like internal transcribed spacer (ITS), ribosomal targets 18S, 23S, *cytochrome b* and *FTR1* (Millon et al., 2019). Mucorales-specific primers targeting 18S region in semi-nested form are commonly being used for identification of species (Dannaoui, 2009). Other molecular formats with increased sensitivity like real-time PCR followed by high resolution melt curve analysis can also be used (Hrncirova et al., 2010). The sequence so obtained is matched with the available public databases and identification is made on the basis of best match. These molecular assays provide confirmatory identification in cases with overlapping clinical-radiological-histopathological features and help to differentiate Mucorales from *Aspergillus* spp.

Whole genome sequencing and next generation sequencing have been used to identify outbreaks due to Mucorales and define individual properties like those conferring virulence or resistance (Garcia-Hermoso et al., 2018). According to a recent meta-analysis, there has been an increase in the use of molecular diagnosis from 10% in 2005 to 64% in 2017 contributing significantly to our understanding of the epidemiology of Mucorales (Jeong et al., 2019). Though detection of Mucorales specific DNA in body fluid requires standardization, encouraging results have been observed in few studies (Millon et al., 2016; Legrand et al., 2016). The detection of Mucorales specific T-cell may also help in diagnosis (Potenza et al., 2016). Recently, conservation of α 1,6-mannan in Mucorales was exploited to develop a monoclonal antibody (2DA6) based lateral flow immunoassay for diagnosis (Burnham-Marusich et al., 2018). The detection of sesquiterpene, a volatile metabolite in the breath may help in distinguishing mucormycosis from invasive aspergillosis (Acharige et al., 2018). However, all the above molecular or point-of-care tests require standardization before its use in routine laboratory.

Management of mucormycosis

Optimal management requires simultaneous efforts at different fronts—prompt and liberal surgical resection of the necrotic tissue, antifungal administration, correction of immunosuppression and treatment of the underlying disease condition. Untreated cases are universally fatal and a delay in initiation of antifungal therapy by 6 days causes a two-fold increase in mortality (from 48% to 83%) especially in neutropenic patients (Chamilos et al., 2008). An analysis of 929 published cases of mucormycosis revealed that prompt initiation of antifungal therapy and surgical intervention significantly improved survival by 69%, whereas absence of treatment was fatal in 97% (Roden et al., 2005).

Medical management

Among the antifungal agents, lipid formulations (liposomal preparations and lipid complexes) of amphotericin B (AMB), and recently introduced isavuconazole, have been used as the first-line therapy for mucormycosis; while posaconazole or isavuconazole serves as the most effective salvage therapy. Lipid formulations of amphotericin B, have better therapeutic index than conventional

amphotericin B deoxycholate (Gamaletsou et al., 2012). The *in vitro* activity of AMB against Mucorales is quite variable and the optimal dosage for therapeutic use is yet to be determined. Daily dosage ranging from 5 mg/kg/day to 10 mg/kg/day have been recommended (Cornely et al., 2019). However, no significant improvement in outcome was observed by raising the dose to 10 mg/kg/day in the AmBisyo study (Lanternier et al., 2015), rather increased renal toxicity and electrolyte derangements are seen with higher doses (Walsh et al., 2001). Generally 10 mg/kg/day is recommended in central nervous system and bones involvement (Cornely et al., 2019).

Among the triazole antifungal agents, the drugs that act by inhibiting ergosterol synthesis in fungal cell wall, posaconazole and isavuconazole have been found to be effective against Mucorales. The *in-vitro* activity of posaconazole against different species of Mucorales varies with an MIC ranging from 1 µg/mL to 8 µg/mL (Cornely et al., 2017). Posaconazole has been used as salvage therapy in cases refractory to AMB or where AMB therapy is not possible. A response rate of 60% was observed when 91 patients of mucormycosis refractory to AMB were put on salvage therapy using oral suspension of posaconazole (VanBurik et al., 2006). The oral suspension is administered as 800 mg/day in 3–4 doses preferably with high-fat meal or carbonated drinks to increase bioavailability. However, this choice of meal may not be possible for critically-ill patients leading to therapeutic failure (Dolton et al., 2012). To overcome this, delayed release tablet of posaconazole is used that are resistant to gastric acidity and allow steady plasma concentrations with once-daily dosage (Krishna et al., 2012). Intravenous formulation of posaconazole with the addition of β-cyclodextrin has also become available. However, cyclodextrin may increase the chance of nephrotoxicity (Cornely et al., 2017).

Isavuconazole is a broad-spectrum antifungal agent given as prodrug isavuconazonium sulfate. It is recommended as first line therapy for the treatment of mucormycosis in United States, and as salvage therapy both in United States and Europe (Cornely et al., 2019). Both intravenous and oral formulations are available. With linear pharmacokinetics, minimal toxicity and less drug interaction, isavuconazole can be safely given without therapeutic drug monitoring. Though the MIC values of isavuconazole are two- to fourfold higher than those of posaconazole, its excellent bioavailability without any dependence on food makes it a better choice for critically-ill patients (Chitasombat and Kontoyiannis, 2015). One important concern, however, is the reported breakthrough mucormycosis with isavuconazole treatment. Two studies reported breakthrough fungal infection at 18% (Cornely et al., 2015) and 4.6% (Dadwal et al., 2016) while using isavuconazole prophylaxis in leukemia patients. Though therapeutic drug monitoring is not recommended routinely during isavuconazole therapy, long-term administration may require drug level monitoring in patients with sepsis, low or high body weight, hepatic impairment, renal replacement therapy or other extracorporeal devices (Gómez-López, 2020).

The duration of antifungal therapy is not clear and varies according to the response in the patient. The aim is to achieve clinical, radiological and microbiological cure and permanent reversal of immunosuppression. The radiological response is slow in mucormycosis compared to invasive aspergillosis (Legouge et al., 2014). Oral posaconazole or isavuconazole serves as important link to shift the patient from intravenous therapy to oral therapy.

Combination therapy has been used to achieve synergism and broader range especially in immunocompromised patient populations, however, contrasting results have been noticed. While *in vitro* studies showed synergism against Mucorales when combination of AMB and posaconazole was used, *in vivo* murine model showed no benefit (Sipsas et al., 2018; Rodriguez et al., 2008). The synergy of liposomal amphotericin B and echinocandin therapy has been reported both *in-vitro* and *in-vivo* in animal models (Ibrahim et al., 2008; Spellberg et al., 2005). Human studies on combination therapy are limited. In a cohort of 32 patients with HM not responding to first-line AMB monotherapy, favorable response was reported in 56% patients after three months of combination therapy with polyene and posaconazole (Pagano et al., 2013). Currently, the global guideline for management of mucormycosis does not provide discrete recommendation for the use of combination therapy (Cornely et al., 2019). The guideline further highlights the concern on downsides of added toxicity, drug interaction and overall cost (Cornely et al., 2019). Newer agents in antifungal pipeline are few. VT-1161, an inhibitor of fungal CYP51, has good activity against Mucorales and has shown improved survival in neutropenic mice model (Gebremariam et al., 2015). APX001A, inhibiting post-translational protein modification of fungi, has also been tried successfully in mice model (Gebremariam et al., 2017).

Surgical management

Resection of the necrotic tissue in mucormycosis, if possible, improves patient outcome. In cases where the disease is limited, as in cutaneous mucormycosis or ROCM or in localized lesion in any organ, surgical resection can contribute in cure of the disease. In other forms like pulmonary, gastrointestinal and CNS mucormycosis, surgery may be attempted to control further spread of infection (Cornely et al., 2019). Radical or repeated surgeries could control local spread of ROCM in 90% of the patients compared to only 41% patients who has limited surgery (Vironneau et al., 2013). However, a 10-year study on outcome of 44 patients with HM, 13 of whom had mucormycosis, showed no improved outcome with early endoscopic surgery (Davoudi et al., 2015). Even early extensive surgical debridement in immunosuppressed patients, carried a high rate of morbidity and mortality in few studies (Saraiya, 2012; Konigsberg et al., 2020).

Adjunctive therapy

Correction of the underlying risk factor is an important pillar of optimal management of mucormycosis in addition to antifungals and surgical interventions (Sipsas et al., 2018). The neutropenia in patients of HM should be corrected by hematopoietic growth factors and transfusions, administration of corticosteroids should be tapered in patients of autoimmune disorders, anti-retroviral therapy should be started in HIV patients. For patients with uncontrolled diabetes or ketoacidosis, reversal of metabolic acidosis should be corrected by aggressive glycemic control and use of sodium bicarbonate (Cornely et al., 2019). Iron chelators like

deferasirox have been used to scavenge the reduced iron and make it unavailable for Mucorales. Iron is required for fungal growth and its absence helps in inhibiting growth of Mucorales. The beneficial role of iron chelators is best described in patients with ketoacidosis, where continuous acidity causes generation of free iron promoting fungal growth (Cornely et al., 2019). In other patient populations, like those of HM, combination of AMB and iron chelator was counterproductive (Spellberg et al., 2012). Hyperbaric oxygen is occasionally used as adjunctive therapy in cutaneous and rhino-orbital mucormycosis. The hyperbaric oxygen improves the functioning of neutrophils, promotes action of AMB by decreasing acidosis and favors wound healing (Tragiannidis and Groll, 2009). This beneficial effect was noticed more in diabetic patients of mucormycosis than those with HM (John et al., 2005). Augmentation of the immune system has also been tried as useful adjunctive therapy in few case reports. Granulocyte colony stimulating factor, interferon- γ and monoclonal antibody nivolumab that inhibits T-cell apoptosis have been used as rescue therapy in few patients (Gil-lamaignere et al., 2005; Grimaldi et al., 2017). However, all these observations regarding adjunctive therapy require confirmation by randomized clinical trial before recommendation in management of mucormycosis.

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Fusarium and Fusariosis

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Introduction

Fusarium species are filamentous fungi widely encountered in nature, including soil, plants and water biofilms (Kauffmann-Lacroix et al., 2016; LeBlanc et al., 2017), and are important plant pathogens (Raza et al., 2017; Wang et al., 2019). In humans, the most frequent diseases caused by *Fusarium* species are superficial: onychomycosis and keratitis (Homa et al., 2013; Ranawaka et al., 2015). In addition, individuals with severe immunosuppression, particularly patients with acute leukemia and hematopoietic cell transplant (HCT) recipients may develop invasive fusariosis, with typical disseminated skin lesions, positive blood cultures and a frequently fatal outcome (Boutati and Anaissie, 1997). In the text that follows, we will review the characteristics of *Fusarium* spp. and the epidemiology of infection both in immunocompetent and immunocompromised patients. We will also review the clinical presentation, evolution and the management of infection caused by these molds, including treatment and prevention.

Characteristics and taxonomy

Fusarium species (Hypocreales, Ascomycota) are ubiquitous fungi mainly related to disease in plants, causing a great economic concern in agriculture (Daughtrey, 2019). *Fusarium* is also recognized as a human pathogen, with a broad spectrum of clinical manifestations. *Fusarium* is encountered in plants, soil, organic matter, and water biofilms. The disease is acquired both in the community and in the hospital, through direct inoculation or inhalation (Nelson et al., 1994).

More than 300 phylogenetically distinct species have been described, but only a minority is of medical interest (Al-Hatmi et al., 2016; Daughtrey, 2019). Changes in taxonomy are very frequent; tools like GenBank, MycoBank, and Fusarium-ID are excellent sources to keep people updated (Tupaki-Sreepurna and Kindo, 2018). *Fusarium* species are grouped into various phylogenetic species complexes (more than 20), seven of which have been reported to cause significant disease in humans (Table 1): *Fusarium solani* species complex (FSSC), *Fusarium oxysporum* species complex (FOSC), *Fusarium fujikuroi* species complex (FFSC), *Fusarium incarnatum-equiseti* species complex (FIESC), *Fusarium chlamydosporum* species complex (FCSC), *Fusarium dimerum* species complex (FDSC), and *Fusarium sporotrichioides* species complex (FSAMSC) (van Diepeningen et al., 2014). Approximately half of all cases of invasive diseases are caused by FSSC, followed by FOSC (Nucci and Anaissie, 2007). The distribution of species causing disease in humans suffers geographic variations. For instance, while in Brazil and in France FSSC predominates (Herkert et al., 2019; Lortholary et al., 2010), a study evaluating isolates from various European countries found that the most frequent species complex causing human infection was FFSC, especially *F. verticillioides*, and *F. proliferatum* (both belonging to FFSC) (Tortorano et al., 2014).

Clinical spectrum of disease in humans

Fusarium species cause a broad spectrum of infections in humans, including superficial, locally invasive, and disseminated infection (Table 2). The immune status of the host and the portal of entry of infection are the key determinants of the clinical form of fusariosis.

Table 1 Species complex of the genus *Fusarium* causing significant human infections and their respective species within each complex.

FSSC	FOSC	FFSC	FIESC	FCSC	FDSC	FSAMSC
<i>F. falciforme</i>	<i>F. oxysporum</i>	<i>F. acutatum</i>	<i>F. incarnatum</i>	<i>F. chlamydosporum</i>	<i>F. dimerum</i>	<i>F. aereniacum</i>
<i>F. keratoplasticum</i>	Unnamed	<i>F. anthophilum</i>	<i>F. equiseti</i>		<i>F. delphinoides</i>	<i>F. brachygibbosum</i>
<i>F. lichenicola</i>		<i>F. andiyazi</i>	Unnamed		<i>F. penzigii</i>	<i>F. langsethiae</i>
<i>F. petrophilum</i>		<i>F. fujikuroi</i>				<i>F. sporotrichioides</i>
<i>F. pseudensiforme</i>		<i>F. nygamai</i>				
		<i>F. proliferatum</i>				
		<i>F. verticillioides</i>				

Table 2 Clinical spectrum of fusariosis.

<i>Immunocompetent</i>
Eye infection
Keratitis
Endophthalmitis
Skin infection
Onychomycosis
Interdigital intertrigo
Tinea pedis
Cellulitis and soft tissue infection
Burn
Trauma
Peritonitis in patients receiving peritoneal dialysis
Arthritis
Sinusitis and pneumonia
Allergic
Chronic invasive
<i>Immunocompromised</i>
Endophthalmitis
Cellulitis at sites of skin breakdown, onychomycosis or interdigital intertrigo
Fungemia
Disseminated disease
Sinusitis
Pneumonia
Arthritis

Epidemiology and clinical manifestations of fusariosis in non-immunocompromised patients

The most frequent infections caused by *Fusarium* species in immunocompetent individuals are keratitis and onychomycosis. Other than these infections fusariosis is rare in immunocompetent patients, and occurs usually as a result of skin breakdowns or trauma.

Eye infection

Keratitis was the first clinical disease caused by *Fusarium* species described in humans (Forster and Rebell, 1975). The infection usually develops after trauma, including surgery, or in the presence of other risk factors, such as climate, agricultural work, use of contact lenses and topical corticosteroids (Guarro, 2013). Among the etiologies of corneal ulcer, fungi are responsible for half of the cases, especially *Fusarium* species, being one of the leading causes of blindness in Asian countries (Mahmoudi et al., 2018). The outcome of such infections is usually poor, and many cases of keratitis evolve to endophthalmitis, as show in a recently German case series (Walther et al., 2017).

Outbreaks of keratitis caused by *Fusarium* species have been reported. During 1 year, 164 confirmed cases occurred in 33 US States, with no history of eye trauma. 100 and 54 (94%) patients were users of soft contact lenses; a case-control analysis identified that a specific solution (ReNu with MoistureLock) was related to the outbreak. At least 10 different *Fusarium* species were identified. One-third of the patients required corneal transplantation (Chang et al., 2006). Other outbreaks were subsequently reported, occurring in Singapore and France (Gaujoux et al., 2008; Khor et al., 2006; Saw et al., 2007). In addition to the use of specific contact lens solutions, risk factors for keratitis due to *Fusarium* species include prior topical antibiotics, steroid use for a long time, diabetes and ocular surface problems (Mahmoudi et al., 2018; Mosquera Gordillo et al., 2018).

Fusarium keratitis presents clinically with reduced visual acuity, redness, pain, photophobia and corneal opacity, suggestive of an ulcer (Fig. 1A). The symptoms are milder and more insidious than in keratitis caused by bacteria. The serrated edge finding on ophthalmic examination is frequent (up to 79%), but not pathognomonic. In addition, dry corneal surfaces with feathered margins and satellite lesions have been described (Thomas and Kaliyamurthy, 2013).

Endophthalmitis may occur as a complication of keratitis, and is associated with a poor visual prognosis (Barrios Andres et al., 2018; Dursun et al., 2003). By contrast, endophthalmitis in the immunosuppressed host results from hematogenous seeding, in the setting of disseminated infection (Rizzello et al., 2018).

Superficial skin infection

While the large majority of cases of onychomycosis are caused by dermatophytes, non-dermatophyte molds, including *Fusarium* species, have been increasingly reported as causes of onychomycosis (Gupta et al., 2003; Ng et al., 1999; Ramani et al., 1993; Ranawaka et al., 2012). The typical clinical presentation is that of distal subungual lesion in the toe nails, occurring more frequently in females (Fig. 1B) (Calado et al., 2006; Ranawaka et al., 2015). In addition to onychomycosis, *Fusarium* species may occasionally cause interdigital intertrigo (Fig. 1C), tinea pedis and hyperkeratotic plantar lesions (Calado et al., 2006; Hay, 2007). In severely immunocompromised patients, onychomycosis or intertrigo caused by *Fusarium* species may serve as portal of entry for cellulitis with or without disseminated infection (Nucci et al., 2013b).

Other infections

In addition to superficial skin infections, patients who suffer deep trauma, burns, or have predisposing conditions such as diabetes mellitus, may develop cellulitis and soft tissue infection, with necrosis, abscess formation, plaques, ulcers, nodules and eumycetomas, characterized by tumefaction, draining secretions with grains (Al-Hatmi et al., 2017; Dutta et al., 2013; Muhammed et al., 2013; Nucci and Anaissie, 2002). In addition, there are a few reports of infection in non-immunocompromised patients, including peritonitis in patients receiving continuous ambulatory peritoneal dialysis (Gaur et al., 2010), septic arthritis (Jakle et al., 1983), pneumonia (Nucci et al., 2015), sinusitis (Gamba and Lombardi, 2017; Hashemi et al., 2015; Lee et al., 2012), thrombophlebitis (Murray et al., 2003), and osteomyelitis (Schade and England, 2019). Sinusitis in immunocompetent individuals may manifest as allergic and chronic non-invasive disease (Wickern, 1993). An allergic form of pneumonia has also been described in immunocompetent patients (Backman et al., 1995).

Epidemiology of fusariosis in immunocompromised patients

Invasive fusariosis occurs predominantly in patients with acute leukemia and in HCT recipients. A prospective study conducted in eight Hematology centers in Brazil evaluated 237 patients with acute myeloid leukemia (AML) or myelodysplasia (MDS) receiving intensive induction chemotherapy and 700 HCT recipients (378 allogeneic and 322 autologous). The overall 1-year cumulative incidence of invasive fungal disease (IFD) was 18.7%, 11.3% and 1.9% in AML/MDS, allogeneic and autologous HCT, respectively. Invasive fusariosis (23 episodes) was the most frequent IFD. The incidence of fusariosis was 5.2% in AML/MDS, 3.8% in allogeneic HCT, and 0.6% in autologous HCT. The 6-week probability of survival of patients with invasive fusariosis was 41% (Nucci et al., 2013a).

In a previous study describing the epidemiology of invasive fusariosis in HCT recipients, the overall incidence was ~6 cases per 1000 HCT, being lowest in autologous (1.5-2/1000 HCT), intermediate in HLA matched donor allogeneic HCT (2.5-5/1000) and highest (20/1000 HCT) in mismatched related donor HCT, with a trimodal distribution: the first peak in the pre-engraftment

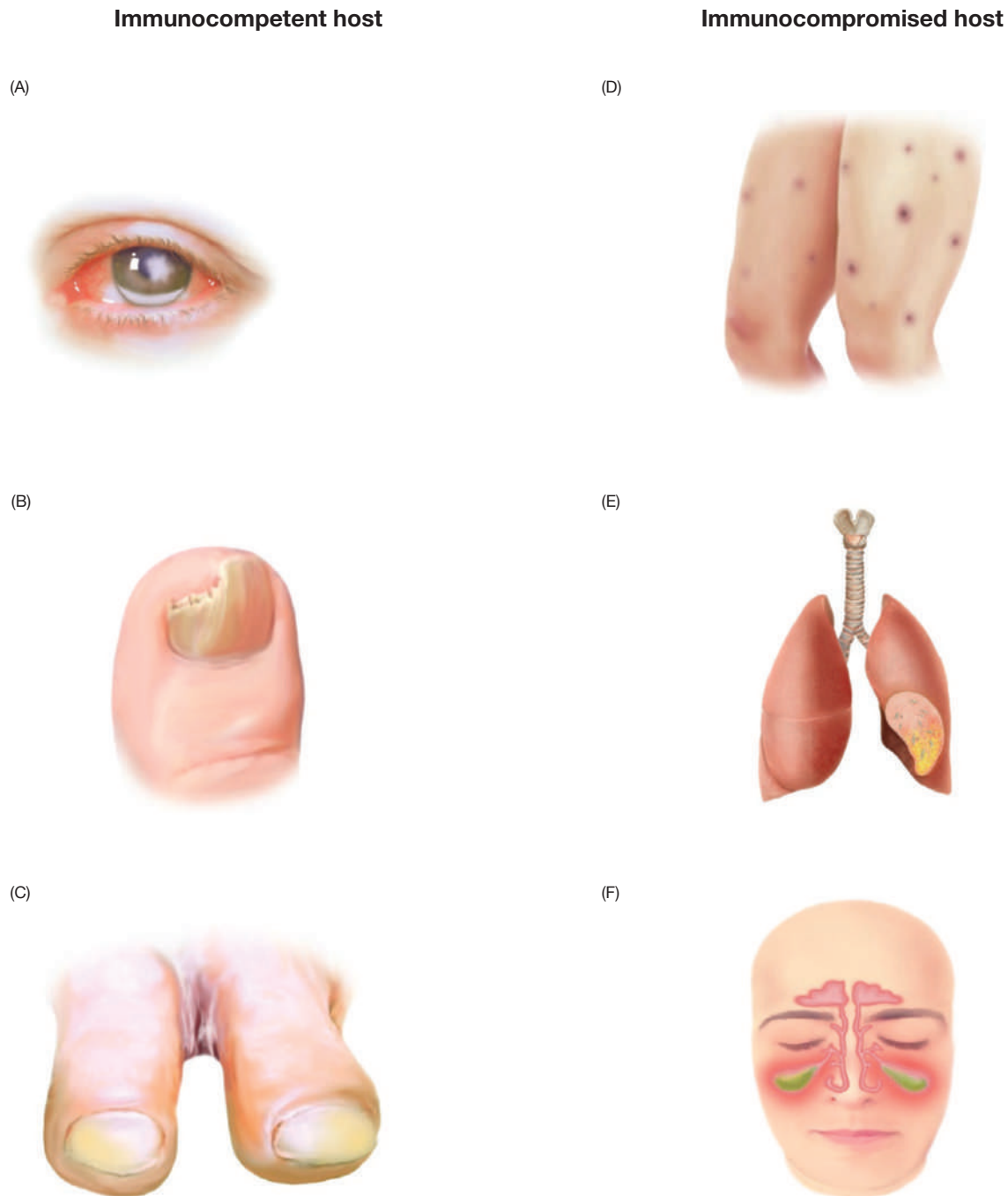


Fig. 1 Clinical manifestations of fusariosis in immunocompetent and immunocompromised patients. (A) Redness and corneal opacity of *Fusarium* keratitis, (B) distal subungueal lesion in the toe nails of *Fusarium* onychomycosis, (C) interdigital intertrigo caused by *Fusarium* species, (D) disseminated skin lesions of invasive fusariosis, (E) Pneumonia caused by *Fusarium* species, (F) Paranasal cellulitis of *Fusarium* sinusitis..

period, the second occurring ~day + 70, in the context of acute graft versus host disease (GVHD), and the third occurring >1 year, in the context of chronic GVHD (Nucci et al., 2004).

A study evaluated risk factors for invasive fusariosis in hematologic patients. Among patients with AML/MDS, active smoking was the only significant variable. In allogeneic HCT, receipt of antithymocyte globulin, hyperglycemia and AML were risk factors in the pre-engraftment period, and non-myeloablative conditioning regimen, grade III-IV GVHD and previous invasive mold disease were risk factors in the post-engraftment period (Garnica et al., 2015).

Immunocompromised patients may be exposed to *Fusarium* species both in the community and in the hospital. In 2007 we observed an increase in the incidence of invasive fusariosis with a cutaneous portal of entry (onychomycosis and interdigital

intertrigo), and suspected that the patients might have been admitted with skin lesions that became colonized and subsequently infected by *Fusarium* in the hospital, since *Fusarium* had been recovered from the hospital water system (Nucci et al., 2013b). However, cases of invasive fusariosis were caused by FSSC whereas most environmental isolates were FOSC, and among concordant species, most did not exhibit genotypic similarities (Scheel et al., 2013). Furthermore, in the same period we observed an increase in the recovery of *Fusarium* species from skin lesions of immunocompetent patients from the Dermatology outpatient unit (Nucci et al., 2013b). Taken together, these data indicated a community origin of invasive fusariosis. On the other hand, cases of invasive fusariosis associated with a hospital source of infection have been reported both from the air (Moretti et al., 2018) and from the hospital water system (Anaissie et al., 2001).

Invasive fusariosis has been occasionally reported in solid organ transplant recipients, but the frequency is much lower than in patients with hematologic malignancies and HCT recipients (Carneiro et al., 2011).

Clinical manifestations of invasive fusariosis in immunocompromised patients

The top four clinical presentations (Table 3)

Invasive fusariosis in severely immunocompromised patients manifests in four major clinical presentations: fever and disseminated skin lesions, pneumonia, fever and positive blood culture for a mold, and superficial infection in the feet with subsequent lymphangitis.

The most frequent clinical presentation is that of disseminated disease. This form is particularly frequent in patients with severe neutropenia, and starts with the sudden appearance of one or (more frequently) various skin lesions (see detailed description below) in the context of persistent or recurrent fever (Boutati and Anaissie, 1997). This picture is usually accompanied by generalized myalgia, and a toxemic appearance.

Skin involvement

In a review of skin lesions in 167 immunocompromised patients, we found localized lesions in 20 patients only. Among 147 patients with disseminated disease, the most frequent skin lesions were multiple painful erythematous popular or nodular lesions, with or without central necrosis, with an ecthyma gangrenosum-like appearance (Fig. 1D). The lesions involved all parts of the body, with predominance to the extremities, and evolved over a few days. Myalgia was a frequent symptom (Nucci and Anaissie, 2002).

The evolution of the skin lesions in cases of disseminated fusariosis is important to assess treatment response. Once the treatment is started, nodular lesions begin to flatten, reduce in size and no new lesions appear. On the other hand, the continuous appearance of new skin lesions in a patient under treatment is a clear sign of therapeutic failure.

A cutaneous portal of entry of fusariosis may occur. In a series of 21 cases of invasive fusariosis occurring in our hospital between 2000 and 2010, 14 (66.7%) had a cutaneous portal of entry: onychomycosis with periungueal cellulitis in six, onychomycosis with interdigital intertrigo in one, intertrigo with lymphangitis in two, interdigital intertrigo in four, and ulcer in one case (Nucci et al., 2013b). Patients with skin portal of entry may evolve to localized skin infection only, or (more frequently), subsequently present with disseminated disease.

Pneumonia

Pneumonia is a frequent manifestation of fusariosis in immunocompromised patients, occurring in 145 of 317 (46%) patients (Fig. 1E), with 73% of patients presenting with bilateral lung involvement (Nucci et al., 2015). The pathogenesis of fusarial pneumonia in immunocompromised patients is similar to aspergillosis, with a first bronchoalveolar phase, followed by angioinvasion (in neutropenic patients) and lung infarction. We have compared the clinical characteristics of 26 cases of invasive fusariosis with 36 cases of invasive aspergillosis occurring in hematologic patients (Nucci et al., 2018). Pneumonia occurred in 88.9% and 50% of cases of aspergillosis and fusariosis, respectively. The most frequent radiologic lesions in fusariosis were macro (>1 cm) nodules (61.5%), centrilobular micro (<1 cm) nodules and ground-glass infiltrates (53.8% each). The only significant difference between aspergillosis and fusariosis lung infection was that macronodules with halo sign were more frequent in cases of

Table 3 Clinical presentations of invasive fusariosis in immunocompromised patients.

Clinical presentation	Characteristic	Comments
Fever + skin lesions	Persistent or recurrent fever in neutropenic patients + sudden onset of nodules and papules	Most frequent clinical presentation of disseminated disease
Pneumonia	New images in chest CT scan	Nodules with or without halo sign, alveolar consolidations, tree in bud, ground-grass infiltrates
Fever + positive blood culture for a mold	Persistent or recurrent fever in neutropenic patients + positive blood culture for a mold	Most frequent mold recovered in blood cultures in neutropenic patients
Superficial skin lesions with subsequent lymphangitis	Onychomycosis or interdigital intertrigo on admission with sudden appearance of lymphangitis	Primary skin lesions frequently overlooked in routine physical examination

CT, computed tomography.

aspergillosis (62.5% vs. 23.1%, $P = .02$). Another important finding in this study was that in 31% of patients with fusariosis and lung involvement, the disease presented with pneumonia as the sole manifestation. Considering that patients with invasive fusariosis may have positive galactomannan test (Nucci et al., 2014a), the distinction between fusariosis and aspergillosis may be difficult.

An important difference between fusariosis and aspergillosis is that while in aspergillosis the disease is acquired by inhalation of conidia, in fusariosis pneumonia may occur either by inhalation of conidia or by hematogenous route from a primary skin lesion. Interestingly, compared with patients with an airborne acquisition, those with a cutaneous portal of entry are more likely to have bilateral lung involvement (Nucci et al., 2015).

Sinusitis

Sinusitis has been reported in patients with invasive fusariosis. In a literature review, 52 of 262 immunocompromised patients (20%) had sinusitis (Nucci and Anaissie, 2006). In our comparison between cases of aspergillosis and fusariosis occurring in patients with hematologic diseases, sinusitis was diagnosed more frequently in aspergillosis (63.9% vs. 38.5%, $P = .048$) (Nucci et al., 2018). The clinical manifestations of fusarial sinusitis may vary from slight headache and nasal discharge to mucosal necrosis and periorbital and paranasal cellulitis (Fig. 1F).

Fungemia

The growth of molds from blood cultures is not frequent. A few organisms produce yeast-like structures, called aleuroconidia, that invade the bloodstream causing fungemia (Nucci and Anaissie, 2007). The most frequent mold causing bloodstream infection is *Fusarium* species. In a series of 233 cases of invasive fusariosis in hematologic patients, fungemia occurred in 37% of cases (Nucci et al., 2014b). Occasionally fungemia is the sole manifestation of invasive fusariosis, sometimes in the absence of neutropenia, in the context of catheter-related infection. This form of invasive fusariosis is usually associated with good prognosis (Carlesse et al., 2017; Velasco et al., 1995).

Disseminated infection

Disseminated infection is the most frequent clinical presentation of invasive fusariosis in immunocompromised patients. Typically the patient presents with disseminated skin lesions, fungemia and pneumonia (Nucci and Anaissie, 2007). This form of invasive fusariosis is more likely to occur in severely neutropenic patients, and is associated with poor outcome (Nucci et al., 2014b). Occasionally patients develop endophthalmitis in the context of disseminated disease that may complicate with blindness (Tiribelli et al., 2002).

Diagnosis of fusariosis

The diagnosis of fusariosis depends on the clinical form. Superficial infections caused by *Fusarium* species usually do not have typical clinical manifestations that per se suggest or indicate the diagnosis of fusariosis. By contrast, in severely immunocompromised patients, two clinical presentations are highly suggestive of invasive fusariosis: positive blood culture for a mold, and the appearance of multiple skin lesions.

Apart from clinical presentation, the diagnosis of fusariosis (as occur with other fungi) relies on direct exam, culture and histopathology. In addition, antigen detection and molecular diagnostic tests may be of help.

The diagnosis of fusariosis is based on the identification of the fungus in culture. The specimen to be examined depends on the clinical presentation, and is mostly represented by the skin, nails, cornea, blood, bronchoalveolar lavage and sinus drainage (Fig. 2) (Nucci and Anaissie, 2002). A special attention is needed in the interpretation of culture from non-sterile specimens, since environmental contamination may occur. With this regard, a direct exam of the biologic material showing acute-branching septate hyaline hyphae is of great help to interpret a positive culture as indicating infection and not contamination.

In culture, *Fusarium* species grow easily and rapidly in most media without cycloheximide. On dextrose potato agar, colonies have a velvety or cotton surface and have different colors (white, yellow, pink, purple, or gray salmon) (Nelson et al., 1994). Although the genus *Fusarium* can be identified by the production of hyaline, banana-shaped, multicellular macroconidia with a foot cell at the base, species identification is difficult and requires molecular methods. In tissue, the hyphae are septate, hyaline with dichotomization at an acute angle, a common characteristic of the agents of hyalophycomycoses and of *Aspergillus* species. However, adventitious sporulation that is characteristic of *Fusarium* may be present in tissue, and the finding of hyphae and yeast-like structures together is highly suggestive of fusariosis in the high-risk population.

A study evaluated the performance of a specific fungal medium and a standard aerobic medium of Bactec™, and found that fungal medium should be preferably used in patients with suspected invasive fusariosis (Hennequin et al., 2002). In another study, bacteria and molds (including *Fusarium*) are inoculated in the same blood culture bottles of Bactec™. Bottles with selective fungal media had a better performance than aerobic media (Oz et al., 2020). In the experience of the authors with BacTAlert™ aerobic bottles, the median time to positivity of blood cultures was 3 days (range 1–4) (Nucci et al., 2014a).

As stated before, the identification of *Fusarium* at species level requires molecular techniques. Two different regions have been used for DNA amplification: the internal transcribed spacer (ITS) and the elongation factor 1 alpha (EF-1alpha), with primers with high specificity (Boch et al., 2016; de Souza et al., 2017; Wehrle-Wieland et al., 2018). Unfortunately, this technique is expensive, required better extraction protocols, and is not available in most laboratories. More recently, matrix-assisted laser desorption/

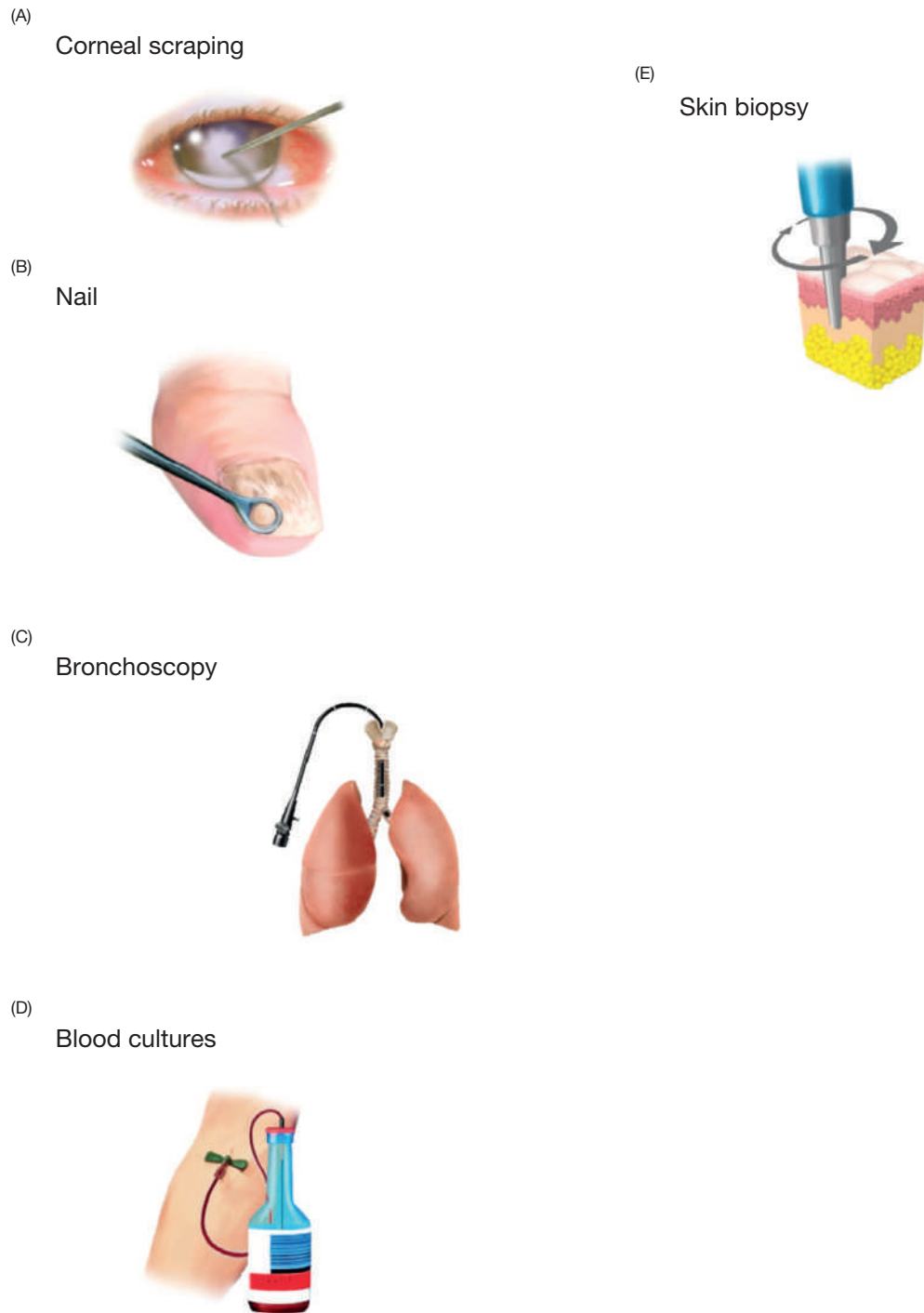


Fig. 2 Tools often required for specimen collection to fusariosis diagnosis. (A) Corneal scraping for the diagnosis of *Fusarium* keratitis, (B) nail scraping for the diagnosis of *Fusarium* onychomycosis, (C) bronchoscopy with bronchoalveolar lavage for the diagnosis of *Fusarium* pneumonia, (D) blood cultures are frequently positive in invasive fusariosis, (E) skin biopsy with culture and histopathology for the diagnosis of disseminated fusariosis.

ionization flight time (MALDI-TOF) has been evaluated in species identification, both in pure colonies and directly from bottles of positive blood cultures (De Carolis et al., 2012; Walsh and McCarthy, 2019).

In addition to conventional mycology, another approach to establish the diagnosis of invasive fusariosis is the use of fungal biomarkers. The two main targets are polysaccharides present in the fungal cell wall: galactomannan (GMI) and 1,3- β -D-glucan (BDG). While GMI is a backbone in the diagnosis of invasive aspergillosis and initially was thought to be specific for *Aspergillus*, cross reactivity may occur with some fungi, including *Fusarium* (Boch et al., 2016; Tortorano et al., 2012). In a multicenter study, we found that 15 of 18 patients (73%) with invasive fusariosis had at least one positive serum GMI test. The test was positive before the

diagnosis of invasive fusariosis in 11 of the 15 cases (73%), at a median of 10 days (range 3–39) (Nucci et al., 2014a). In another study, we compared the characteristics of 36 hematologic patients with invasive aspergillosis and 26 with invasive fusariosis. Serum galactomannan was positive in 88.6% of patients with aspergillosis and in 73.3% with fusariosis (Nucci et al., 2018). While there are important differences in the clinical presentation of aspergillosis and fusariosis, such as skin lesions and positive blood cultures, which are much more frequent in fusariosis, in our study, positive serum galactomannan plus lung infiltrates was the clinical presentation of 4 of 13 patients with invasive fusariosis who had pneumonia. In these cases, it is difficult to differentiate between fusariosis and aspergillosis.

Unlike GMI, which is considered a reasonably specific fungal biomarker for *Aspergillus*, BDG is a component of various fungi, including *Candida*, *Aspergillus* and *Fusarium* (Odabasi et al., 2004). We tested serum samples of 13 patients with invasive fusariosis, analyzing the temporal relationship between the first positive BDG test and the date of the diagnosis of fusariosis, and evaluated the performance of the test in the diagnosis of fusariosis. Twelve of the 13 patients had at least one positive serum BDG test, and it was positive before the diagnosis of fusariosis in 11 of the 12 patients (91.6%), at a median of 10 days (range 1–32). However, as BDG is associated with various false positive tests (Mikulska et al., 2015), the positive predictive value was very low (7%, considering two consecutive positive BDG tests). Therefore, serum BDG is more useful at ruling out rather than confirming the diagnosis of invasive fusariosis.

Management

The treatment of invasive fusariosis varies according to the immune status of the host and the site(s) of infection. The treatment of the two most frequent infections occurring in immunocompetent individuals, keratitis and onychomycosis, is challenging. By contrast, the sporadic cases of localized infection in immunocompetent individuals can be treated with surgical debridement. On the other hand, infection in immunocompromised patients requires systemic therapy, and the main determinant of the outcome is the immune status of the host.

Treatment of fusariosis in immunocompetent patients

Keratitis

The treatment of keratitis caused by *Fusarium* species is challenging because of the poor tissue penetration of antifungal agents. The treatment usually consists of using a topical antifungal agent alone, or in combination with systemic therapy. Natamycin, a polyene amphoteric molecule, has been used for more than 50 years and continues to be the first line treatment (Ansari et al., 2013). In a non-randomized, comparative prospective study in patients with fungal keratitis, patients treated with natamycin 5% eye drops hourly showed better success outcomes than patients receiving topical itraconazole 1% eye drops (Kalavathy et al., 2005). A randomized study compared topical 5% natamycin with 1% voriconazole. The proportion of patients with healed or resolved ulcer was significantly higher in natamycin recipients (89.2% vs. 66.6%) (Sharma et al., 2015). Another randomized controlled trial comparing natamycin with voriconazole showed a benefit of topical natamycin over topical voriconazole, while adding oral voriconazole resulted in a trend towards a decreased rate of ulcer perforation (Prajna et al., 2016, 2017). Topical amphotericin B is an option if natamycin is not available. Surgical intervention is reserved for patients with refractory disease. While corneal scraping should be limited to obtaining diagnostic samples, the most common therapeutic surgery is penetrating keratoplasty, followed by lamellar keratoplasty (Ansari et al., 2013).

Onychomycosis

The management of onychomycosis caused by *Fusarium* species is challenging, with a long duration of treatment, and high failure rates with both systemic and topical therapy. For topical treatment, amphotericin B solution (2 mg/mL) is the preferred agent (Monod and Mehul, 2019). Laser therapy may occasionally work (Khurana et al., 2018), as well as a combination of topical and oral terbinafine (Shah et al., 2016). Among systemic therapies, terbinafine and itraconazole have been used more frequently, with variable response rates (Hattori et al., 2005; Ranawaka et al., 2008, 2015). In hematologic patients who present with onychomycosis or interdigital intertrigo before receiving chemotherapy or transplant, systemic voriconazole or posaconazole reduce the risk of dissemination during periods of severe immunosuppression (Varon et al., 2014, 2016).

Management of fusariosis in immunocompromised patients (Table 4)

Prognostic factors

In neutropenic patients, neutrophil recovery is the most important prognostic factor in invasive fusariosis. In addition, T-cell immunodeficiency associated with the use of corticosteroids impacts the outcome. In an analysis of 84 hematologic patients with invasive fusariosis, the 90-day survival was 21%. By multivariate analysis, factors associated with death were persistent neutropenia (hazard ratio 5.43) and receipt of corticosteroids (hazard ratio 2.18). The actuarial survival was 0% for patients with persistent neutropenia and on corticosteroids, 4% for those with persistent neutropenia only, 30% in patients on corticosteroids but no neutropenia, and 67% in patients without any of these two factors (Nucci et al., 2003). In another study analyzing the outcome of 206 patients with invasive fusariosis who received treatment, variables associated with death were receipt of corticosteroids (hazard

Table 4 Recommendations for the management of invasive fusariosis in severely immunosuppressed patients.

Action	Drug	Comments
Primary prophylaxis	Voriconazole or posaconazole	High-risk hematologic patients who present on admission with superficial skin lesions with positive cultures for <i>Fusarium</i> spp.
Secondary prophylaxis	Voriconazole, posaconazole or lipid amphotericin B	Patients with prior invasive fusariosis who will be exposed to subsequent periods of immunosuppression
Primary therapy	Lipid amphotericin B or voriconazole or combination therapy	Outcome largely dependent on recovery of host defenses

ratio 2.11), persistent neutropenia (hazard ratio 2.70), and receipt of deoxycholate amphotericin B (hazard ratio 1.83) (Nucci et al., 2014b).

In vitro susceptibility

Fusarium species exhibit high minimal inhibitory concentrations (MICs) to most antifungal agents including the voriconazole, posaconazole, isavuconazole and amphotericin B. Epidemiologic cutoff values of different antifungal agents tested in 1150 *Fusarium* isolates belonging to different species complexes (Espinel-Ingroff et al., 2016). For FSSC (608 isolates tested), MIC ranges and mode ($\mu\text{g/mL}$) were ≤ 0.25 –16 and 2 for amphotericin B, 0.5 — >16 and 16 for itraconazole, 0.5 — >16 and 8 for voriconazole, and 1 — >16 and 8 for posaconazole. For FOSC, MIC ranges and mode ($\mu\text{g/mL}$) were ≤ 0.25 –16 and 2 for amphotericin B, 1 — >16 and 16 for itraconazole, 0.5 — >16 and 4 for voriconazole, and 0.5 — >16 and 2 for posaconazole. Preliminary data on the in vitro activity of isavuconazole also indicate poor activity. A study evaluating 75 isolates of *Fusarium* of different species complexes showed a MIC₅₀ of >16 $\mu\text{g/mL}$, without great variations between species (Broutin et al., 2020).

A very important clinical question regarding MICs for *Fusarium* species is whether results of in vitro susceptibility tests are of help to select the appropriate treatment. Indirect clinical experience suggests that this might not be the case, as shown by the good clinical response to voriconazole in patients with invasive fusariosis, with rates similar to those achieved by lipid amphotericin B, despite the high MICs exhibited in the susceptibility test studies (Lortholary et al., 2010; Nucci et al., 2014b). More recently, a multicenter study analyzed the correlation between MIC and the outcome in 88 cases of fusariosis, 74 with hematologic diseases. There was no difference in MIC₅₀ and MIC distribution among survivors and patients who died. By contrast, persistent neutropenia and receipt of corticosteroids were strong predictors of 6-week mortality (Nucci et al., 2020).

Primary prophylaxis

Primary anti-mold azole prophylaxis (posaconazole or voriconazole) is considered standard of care in high-risk hematologic patients, including patients with AML receiving intensive chemotherapy as remission induction and allogeneic HCT (Maertens et al., 2018). In this scenario, breakthrough infection by *Fusarium* species have been reported (Cudillo et al., 2005; Wu et al., 2014). Primary prophylaxis for invasive fusariosis was evaluated in one study, in a subset of patients. We reported an increased incidence of invasive fusariosis with a cutaneous portal of entry, with most patients presenting onychomycosis and interdigital intertrigo (Nucci et al., 2013b). Subsequently, we conducted a prospective study to evaluate the frequency of such lesions in hematologic patients, and observed that patients with superficial skin lesions in the feet (interdigital intertrigo and/or onychomycosis) on hospital admission with positive culture for *Fusarium* spp. were at an increased risk to develop invasive fusariosis (Varon et al., 2014). Then, in a non-randomized trial, we administered anti-mold prophylaxis (voriconazole or posaconazole) in 20 episodes at risk (neutropenia or graft versus host disease), and compared with 219 episodes where fluconazole or no prophylaxis was given. Invasive fusariosis occurred in 5.9% without and in 5% with anti-mold prophylaxis. However, 4 of 5 patients with superficial skin lesions with positive cultures for *Fusarium* species who did not received anti-mold prophylaxis developed invasive fusariosis vs. none of ($P = .01$) (Varon et al., 2016). On the basis of these data, we recommend primary prophylaxis with an anti-mold azole (voriconazole or posaconazole) in high-risk hematologic patients who present on admission with superficial skin lesions with positive cultures for *Fusarium* spp.

Secondary prophylaxis

Secondary prophylaxis refers to the use of an antifungal agent to patients with prior story of invasive fusariosis who will be submitted to subsequent periods of immunosuppression. Secondary prophylaxis was evaluated in a multicenter retrospective study of 40 patients who were successfully treated for invasive fusariosis and were exposed to subsequent periods of immunosuppression (neutropenia in 35 and graft versus host disease in 5). Relapse of invasive fusariosis occurred in 2 of 8 patients (25%) who were not on prophylaxis and in 3 of 32 (9.4%) who received secondary prophylaxis (voriconazole in 24, posaconazole in 2 and a lipid formulation of amphotericin B in 6). Considering only patients who had disseminated fusariosis, relapse occurred in 2 of 2 (100%) not on secondary prophylaxis and in 3 of 26 (11.5%) who received secondary prophylaxis ($P = .03$) (Nucci et al., 2019). Therefore, secondary prophylaxis with a mold-active azole or a lipid formulation of amphotericin B should be strongly considered in patients

with prior invasive fusariosis who will be exposed to subsequent periods of immunosuppression, especially if the disease was disseminated.

Other preventive measures

Other preventive measures in immunocompromised patients at high risk to develop invasive fusariosis include preventing patients' exposure by treating these patients in rooms with HEPA filter and positive pressure and avoiding contact with reservoirs of *Fusarium* (Anaissie et al., 2001). In addition, decreasing immunosuppression (if possible) should be attempted in patients with prior invasive fusariosis (e.g., use of colony-stimulating factors in patients who will become neutropenic).

Primary therapy

It is difficult to define the best regimen for the treatment of invasive fusariosis because there are no randomized trials and because the outcome is largely dependent on immune reconstitution. The largest series ever published is a multicenter retrospective study of 236 patients diagnosed between 1985 and 2011 in 44 centers from 11 countries. Among 206 patients who received treatment, deoxycholate amphotericin B was given to 110 patients, 38 received voriconazole, 34 were treated with a lipid formulation of amphotericin B (liposomal 20, lipid complex 8, colloidal dispersion 6), 21 received combination therapy (mainly voriconazole plus amphotericin B), and 3 received other therapies. The 90-day probability of survival was 53% for patients receiving voriconazole, 48% for those receiving a lipid formulation of amphotericin B, and 27% for patients treated with deoxycholate amphotericin B. The response rate was similar among patients receiving combination therapy and monotherapy (Nucci et al., 2014b).

Posaconazole and isavuconazole have some in vitro activity against *Fusarium* species, but the experience with these agents in the primary treatment of invasive fusariosis is very scarce. For example, among 206 patients with fusariosis treated in 44 centers worldwide, only two patients were treated with posaconazole (Nucci et al., 2014b).

Many clinicians start treatment with a combination of voriconazole and a lipid formulation of amphotericin B, with the argument that the disease is very severe, with a poor outcome, and because MICs for voriconazole are high. However, there are no data indicating that combination therapy is better than monotherapy. Furthermore, the similar response rates in patients receiving monotherapy with voriconazole or a lipid formulation of amphotericin B are against in vitro data that shows higher MICs for voriconazole. These data suggest that results of in vitro susceptibility tests are of little help in establishing the optimal treatment for invasive fusariosis.

Assessment of treatment response

As stated above, the outcome of invasive fusariosis is largely dependent on immune reconstitution. In patients with metastatic skin lesions, daily examination and counting of lesions is very helpful to assess treatment response. Responding patients should not present new skin lesions, and the existing lesions should flatten. In addition, myalgia, which is very frequent, should improve, and blood cultures should become negative. In patients with isolated lung involvement the assessment of treatment response is more complicated because as in aspergillosis (Caillot et al., 2001), lesions can increase in size after the first week of treatment.

Salvage therapy

In practice, changes in the primary therapy usually include either switch from one drug to another (from voriconazole to lipid amphotericin B or vice versa) or to give combination therapy. However, we have to keep in mind that patients who fail to respond to treatment and have persistent neutropenia are very unlikely to respond to any treatment regimen.

Adjunctive therapies

Adjunctive therapies include removal of central venous catheters in the occasional cases of catheter-related fungemia (Velasco et al., 1995), the use of granulocyte or granulocyte-monocyte colony-stimulating factors (G-CSF and GM-CSF), interferon gamma and granulocyte transfusions to persistently neutropenic patients. While many patients receive these adjunctive therapies, their effectiveness have not been proven.

A single center study reported 11 patients with invasive fusariosis who received granulocyte transfusions as adjunctive therapy. With a median of 7 transfusions per patient, neutrophil counts remained $>500/\text{mm}^3$ for 36 h in 61% of transfusion events. Clinical response was observed in 10 of the 11 patients, and 8 patients were alive 3 months after the diagnosis of fusariosis. In the same article, a literature review of 23 published cases showed a response rate of 30% only (Kadri et al., 2015).

Conclusions

Infections by *Fusarium* species may occur in immunocompetent and in immunocompromised patients. Onychomycosis and keratitis are the most frequent infections in humans. Other than these two infections, immunocompetent patients may occasionally develop deep-seated infection that is limited to single organs, and tend to respond well to therapy. By contrast, immunocompromised patients, especially HCT recipients and patients with severe and prolonged neutropenia, frequently develop disseminated disease, which is frequently fatal. In such patients, successful outcome is largely determined by the degree and persistence of immunosuppression and the extent of infection.

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***Sporothrix* and Sporotrichosis**

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Introduction

Implantation mycoses include a diverse group of fungal diseases with subacute to chronic manifestations that start at the site of transcutaneous trauma. These diseases are also known as “subcutaneous mycoses,” but this term is inadequate, as involvement may go beyond the skin and subcutaneous tissues to involve deeper structures like the lymphatic vessels, osteoarticular system, and bones (Arenas, 2005; Errol Reiss et al., 2012; Queiroz-Telles et al., 2011).

Sporotrichosis is the most prevalent and globally distributed of the implantation mycoses (Chakrabarti et al., 2015a; Queiroz-Telles et al., 2017). It is caused by several *Sporothrix* species, a genus of thermally dimorphic fungi that infect humans and animals. This disease usually develops after inoculation with contaminated organic material or via zoonotic transmission (de Lima Barros et al., 2011a,b; Bonifaz and Vazquez-Gonzalez, 2010; Lopes-Bezerra, 2018). The most common sites of infection are the skin and lymph nodes, and infection typically results in ulcers, nodules, and abscesses. Less frequently, the mucous membranes, bones, and joints may be involved and rarely, dissemination to the lungs, the central nervous system (CNS), and other organs may occur. Classically, sporotrichosis is known as “rose gardener’s disease” because it is typically spread by sapronotic transmission, the inoculation of tissue by contaminated plant materials. However, an emerging species of *Sporothrix*, *S. brasiliensis*, is transmitted to humans and animals by cats and is expanding through Brazil and into neighboring South American countries (Cordoba et al., 2018; Etchecopaz et al., 2019; García Duarte et al., 2017; Orofino-Costa et al., 2017; Rodrigues et al., 2020).

A brief history of sporotrichosis

The first confirmed report of human sporotrichosis is attributed to Benjamin R. Schenck, in 1898, when he was a medical student at Johns Hopkins University in Baltimore, USA (Schenck, 1898). Previously, in 1809 and in 1889, Link and Lutz reported probable cases of the disease but did not provide microbiologic evidence (Link, 1809; Rippon, 1988). Hektoens and Perkins used the name *Sporothrix schenckii* for the first time when they reported the second case, also in the United States, in 1900 (Hektoen and Perkins, 1900). In 1903, Beurmann and Ramond reported the same disease in France and the organism was named *Sporotrichum beurmanni*, by Matruchot and Ramond (De Beurmann and Ramond, 1903; Matruchot, 1910). Over time, hundreds of cases were described in France and the resulting literature serves as the classic characterization of sporotrichosis (de Beurmann and Gougerot, 1912). The first zoonotic cases of sporotrichosis were described in Brazil in 1907 following rat bites, and in 1909, zoonotic cases caused by horses were documented in Madagascar, caused by the equine isolate *S. equi* (Carougeau, 1909; Lutz and Splendore, 1907). Zoonotic cases of sporotrichosis were infrequently reported throughout the 20th century, until the emergence of cat-transmitted sporotrichosis caused by *S. brasiliensis* in the 1990s (Marques et al., 1993; Schubach et al., 2001).

The pathogenic species of the genus sporothrix

Taxonomic studies

Sporothrix is not considered a complex of cryptic fungal species, but rather a single genus with genetic, ecological, and biological diversity (Lopes-Bezerra et al., 2018). The fungi that cause sporotrichosis are currently classified in the Kingdom Fungi, Subkingdom Dikarya, Phylum Ascomycota, Class Euascomycetes, Order Ophiostomatales, Family Ophiostomataceae, Genus *Sporothrix*, and includes around 53 species (de Meyer et al., 2008; Rodrigues et al., 2020). This fungus is widely distributed in nature, including in soil, water, and decomposing organic material such as sphagnum moss, thorns, wood, and dry leaves (de Lima Barros et al., 2011a, b; Chakrabarti et al., 2015a). Human and animal sporotrichosis is caused by *S. schenckii*, *S. globosa*, *S. brasiliensis* and *S. luriei*. These form the “pathogenic or clinical clade” of medical and veterinary importance, while several non-pathogenic species are grouped in an “environmental clade” (Lopes-Bezerra et al., 2018; Rodrigues et al., 2020). Notably, sapronotic species from diverse environmental clades, such as *S. mexicana*, *S. pallida*, *S. humicola* and *S. chilensis*, may infect mammals, but these infections are uncommon and have little epidemiological relevance for this disease (Table 1).

Table 1 The clinical and environmental clades of *Sporothrix* species.

Clinical clade		Environmental clade	
Clade I	<i>S. brasiliensis</i>	Clade V	<i>S. pallida</i>
Clade II	<i>S. schenckii</i>	Clade IV	<i>S. mexicana</i>
Clade III	<i>S. globosa</i>		
Clade VI	<i>S. luriei</i>		

S. schenckii is the most extensively distributed of the species. It has been found in North, Central, and South America, Southern Africa, Australia, and Europe. *S. brasiliensis* is confined to Brazil and Argentina but has the potential to spread to other South American countries (Chakrabarti et al., 2015a; Cordoba et al., 2018; Etchecopaz et al., 2019; Gremião et al., 2017). This fungal species is unique among those that cause endemic mycoses because it has the biological capacity for transmission while in the yeast phase. Unlike other *Sporothrix* spp., *S. brasiliensis* does not undergo a dimorphic transformation phase during mammal infections (Gremião et al., 2017; Queiroz-Telles et al., 2017). *S. globosa* has a more limited geographic distribution, with cases occurring predominantly in Asia, particularly in China and Japan. The least common etiology of sporotrichosis among the “pathogenic clade” is *S. luriei*. Isolated cases of *S. luriei* have occurred in South Africa, India, Italy, and Brazil (Ajello and Kaplan, 1969; Alberici et al., 1989; Oliveira et al., 2011; Padhye et al., 1992).

Molecular phylogeny

Several publications have reported the relationship between *Sporothrix* genotypes and their geographical distribution. *Sporothrix globosa* from Brazil, China, Japan, Spain, and the United Kingdom are closely genetically related, suggesting that they have a common ancestor (Rodrigues et al., 2013b; Zhang et al., 2015). Plant debris is hypothesized to be a major source of the pathogen, and sporotrichosis has been called “reed toxin” in regions with extensive sapronotic transmission (Yu et al., 2013). *Sporothrix* cannot be classified to the species or clade level using only macroscopic and microscopic characteristics. Distinguishing these species by phenotypic similarities has led to misidentification of *Sporothrix* species in the past. However, the development of novel molecular techniques has led to advances in fungal epidemiological studies. These novel approaches led to the discovery that *S. schenckii* was actually composed of several distinct species with a common ancestor (Rangel-Gamboa et al., 2016; Rodrigues et al., 2013b).

Over decades, two common techniques have been used for phylogenetic study: amplified fragment length polymorphisms (AFLP) and restriction fragment length polymorphisms (RFLP) (Ishizaki et al., 2009; Neyra et al., 2005). Internal transcribed spacers (ITS), chitin synthase, β -tubulin, and calmodulin genes are the loci most commonly used in these studies. Based on the sequence of calmodulin (a highly conserved protein, important as an enzymatic cofactor in fungal calcium-calcineurin signaling pathways), *S. schenckii* is considered a complex of phylogenetically related, cryptic species (Marimon et al., 2006, 2007). Two groups of *S. schenckii*, *sensu lato* (*s. l.*) and *sensu stricto* (*s. str.*), were differentiated, with *S. schenckii sensu lato* (*s. l.*) signifying the species complex, while *sensu stricto* (*s. str.*) specifies the exact species (Rangel-Gamboa et al., 2016).

It is noteworthy that phylogenetic analysis is accompanied by different conidia morphology, biochemical characteristics, and ecological behaviors. *S. schenckii* and *S. globosa* have been isolated from humans, various animals, and soil (Marimon et al., 2006, 2007), whereas *S. brasiliensis* has been isolated primarily from humans, cats, and dogs (Rodrigues et al., 2013b).

Epidemiology

Geographical distribution, incidence and prevalence

Sporotrichosis is the most widespread implantation mycosis in the world. It has been reported on all continents, but the principal endemic areas for this disease are Latin America, India, South Africa, Japan, China, and Australia. The incidence and prevalence of this condition are difficult to determine because it is not mandatory to report. However, the prevalence has been estimated to range from 0.1 to 0.5% (Bongomin et al., 2017; Chakrabarti et al., 2015a; Queiroz-Telles et al., 2017). Sporotrichosis is hyperendemic in some geographic regions, like Albacay, Peru, with an incidence rate ranging from 48 to 98 cases/100,000 (Lyon et al., 2003; Pappas et al., 2000; Ramirez-Soto et al., 2012), and in Jalisco and Puebla, Mexico with a prevalence of 25 cases/100,000 (Bonifaz et al., 2007; Estrada-Castañón et al., 2018; García Vargas et al., 2008). Other hyperendemic areas can be found in Colombia, Venezuela, Brazil, China, and Japan (Bustamante and Campos, 2001). There are few reports of sporotrichosis from Europe, and most are from people who traveled or immigrated to endemic areas. The European countries with most reported cases are France, Italy, and Spain (Alberici et al., 1989; Criseo et al., 2008; Magand et al., 2009; Ojeda et al., 2011; de Oliveira et al., 2014). Other scattered case reports have been published from Asia and the Pacific Islands, including Thailand (Kwangsukstith et al., 1990; Tovikkai et al., 2020), Indonesia (Campbell, 1965), Korea (Kim et al., 2019), Laos (Newton et al., 2005), and New Zealand (Black et al., 1968) (Fig. 1).

Clusters and outbreaks

Although most of the *Sporothrix* infections are isolated and scattered around the globe, several clustered cases and outbreaks have been reported in diverse regions over time (Hay and Morris-Jones, 2008). The largest ever recorded sapronotic sporotrichosis outbreak occurred in the gold mine of Witwatersrand, in South Africa, when approximately 3300 miners were infected via fungus-infested wood timbers between 1938 and 1947 (Brown et al., 1947). Interestingly, a more recent investigation in 2011 found 81 new cases in another South African gold mine (Govender et al., 2015).

Currently, the largest and longest zoonotic sporotrichosis outbreak is related to transmission from cats in several Brazilian geographic regions, and it has recently expanded into neighboring countries in South America (de Lima Barros et al., 2004; Cordoba et al., 2018; Etchecopaz et al., 2019; Gremião et al., 2017; Rodrigues et al., 2020).

This outbreak involves cat-to-human transmission (zoonotic transmission) and animal transmission, mainly cat-to-cat and cat-to-dog. The zoonotic and epizootic transmission of *S. brasiliensis* started at the end of the 1990s in the state of Rio de Janeiro, Brazil

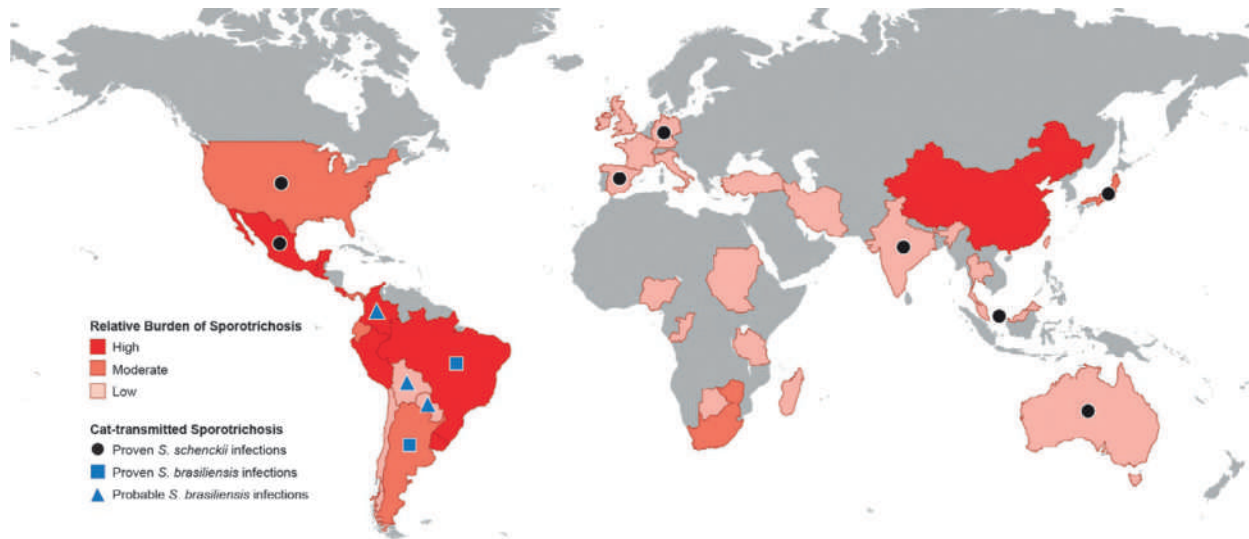


Fig. 1 Global distribution of sapronotic and zoonotic sporotrichosis. Proven zoonotic infections caused by *S. brasiliensis* and *S. schenckii* were defined as clinical isolates obtained from human and/or feline patients identified to species by molecular methods. Probable *S. brasiliensis* infections were based on clinical and epidemiological evidence, including feline microbiologic identification, but lacking species identification by molecular methods.

and is estimated to involve about 5000 humans, 8000 cats, and nearly 300 dogs (Gremião et al., 2020). This data is certainly underestimated because reporting sporotrichosis cases is not compulsory in Brazil.

Interestingly, small outbreaks and isolated cases of cat-transmitted sporotrichosis have been reported from several other countries since 1952. Unlike the outbreak in Brazil, zoonotic transmission was associated with *S. schenckii* in these other countries, including the United States, Mexico, Argentina, Spain, Germany, India, Australia, Japan and Malaysia (Gremião et al., 2017) (Fig. 1)

Source of infection and routes of transmission

The natural habitat of *Sporothrix* species appears to be soil and plant material, as cellulose, vegetable detritus, wood, and leaves can serve as an important nutritional substrate for fungal growth (Bongomin, 2018; de Lima Barros et al., 2011a,b). Occasionally, environmental strains have been isolated from pastures and other environmental sources as well (McGuinness et al., 2016).

Many isolates have been reported in the United States from sphagnum moss, which is used in gardening, particularly with bonsai trees and pine crops. Most of the environmental and clinical isolates have been identified in temperate and humid climates, with an average temperature of 20–25°C and relative humidity above 90% (Dixon et al., 1991; Hajjeh et al., 1997). *Sporothrix* spp. are often thermotolerant, with some species being found in temperatures from 2°C to 6°C (Jilin, China), while others have been adapting to warmer climates, such as in western Mexico and parts of southern Brazil (Almeida-Paes et al., 2009; Moussa et al., 2017). *Sporothrix* can grow in a pH range of 3.5–9.6. Sporotrichosis occurs at all times of the year, but in the northern hemisphere cases are seen more often in late autumn and early winter. This is thought to be due to the timing of the rainy season and harvest for certain crops, particularly corn. *Sporothrix* spp. have been isolated from the roots and stems of corn crops in China (Song et al., 2013).

Sporotrichosis can also be acquired from animals that act as indirect or passive vectors, the most common source being the domestic cat (*Felis catus*). There are several reports of sporotrichosis associated with injuries caused by other animals, such as rodents (rats, mice, gophers and squirrels), horses, mules, donkeys, cattle, camels, chimpanzees, boars, dogs, foxes, dolphins, fishes, parrots, other birds, and the nine-banded armadillo (Alves et al., 2010; Kwon-Chung and Bennett, 1992; Rodrigues et al., 2016b; Saravanakumar et al., 1996; Seyedmousavi et al., 2018).

However, there are also recorded cases attributed to insect bites, animal scratches, and the bites of reptiles, spiders, and bats (Rodrigues et al., 2016b; Zhang et al., 2015). *S. brasiliensis* is the only species in the *Sporothrix* genus that is saprozoönotic—existing in environmental reservoirs while easily infecting a mammalian host. *S. brasiliensis* is the only dimorphic fungus with the biological capacity for transmission directly from the yeast phase, and the high fungal load of yeasts harbored in lesions of infected cats facilitates transmission (Gremião et al., 2017; Macedo-Sales et al., 2018; Queiroz-Telles et al., 2017). Human infection may occur after traumatic, transcutaneous inoculation caused by feline scratches and bites, but also via non-traumatic routes, such as direct contact between the mucocutaneous secretions of sick animals and a host's mucous membranes or damaged skin (Gremião et al., 2017; de Miranda et al., 2018).

Most cases of sporotrichosis are the result of cutaneous inoculation, through trauma with contaminated material. It has also been proven that in individuals living in highly endemic areas, the fungus can enter the airways via inhalation, leading to pulmonary manifestations and many asymptomatic infections, or even penetrate through the various mucous membranes, including through the tarsal conjunctiva. It is also important to note that the zoonotic transmission of *S. brasiliensis* may result

from direct fungal contact with the host's integumental barriers, even without evidence of trauma (Almeida-Paes et al., 2014; Schubach et al., 2005a; Yamagata et al., 2017).

Occupation

Sporotrichosis is considered an occupational disease, with most cases seen among agricultural workers, horticulturists, gardeners, forestry workers, carpenters, hunters, miners, and fishermen (Chakrabarti et al., 2015b). However, cases have also been seen among housewives and school-age children (Lyon et al., 2003; Pappas et al., 2000). For cat-transmitted sporotrichosis, veterinarians, veterinary technicians, and cat owners (especially those who own many cats) are at increased risk of *S. brasiliensis* infection (Brandolt et al., 2018; de Carvalho Aguinaga et al., 2014; Gremião et al., 2017).

Sex and age

The distribution by sex may vary by geographic region. Most authors give a sex ratio of 1:1. However, during the Brazilian zoonotic epidemic, a few reports noted a marked predominance of sporotrichosis among women, while in another series, more cases were seen among men (de Lima Barros et al., 2011a,b). The age distribution is broad, but two incidence peaks are frequently reported: in school-age children (5–15 years, 30% of cases) and young adults (16–35 years, 50% of cases). In the hyperendemic area of Peru, 50% of cases occur in children under 15 years old. However, the disease can be seen at all ages, from newborns to the elderly (de Lima Barros et al., 2008; de Lima Barros et al., 2011a,b).

Incubation period

The length of the incubation time is multifactorial. The inoculum dose, isolate's virulence, and the integrity and capability of the host's defenses all contribute to the incubation period. For cutaneous and lymphocutaneous forms of sporotrichosis, the time from inoculation to clinical manifestation may vary from 1 to 4 weeks. For the extracutaneous clinical forms, the incubation period is uncertain (Bonifaz and Tirado-Sanchez, 2017; Tirado-Sánchez and Bonifaz, 2018).

Risk factors

For sapronotic sporotrichosis, the risk factors for acquiring this disease are related to occupation, and in the case of zoonotic infections, risk factors relate to contact with feral and domestic cats. However, the more severe and disseminated clinical forms of sporotrichosis are also associated with malnutrition, chronic alcoholism, agricultural pesticide exposure, uncontrolled diabetes, TNF- α blockers, steroid therapy, hematologic malignancies, solid organ transplant, and HIV-AIDS (Gewehr et al., 2013; Moreira et al., 2015; Queiroz-Telles et al., 2019).

Pathogenesis and host defenses

Virulence factors

To survive in the hostile environment of a host organism, microbes developed structures and factors to evade the host's defense mechanisms. Most of the virulence-related factors observed in sporotrichotic infections are similar to those studied for other pathogenic fungi.

Thermotolerance

The cutaneous and systemic infectivity of yeast forms of *S. schenckii* were characterized in 5–10-week-old male BALB/c mice by inoculation via their hind footpads and intraperitoneal routes. The study showed strong infectivity in the subcutaneous layer but not via hematological routes. The inoculation of mice with lung isolates via the intraperitoneal route was found to result in death within 117 days. The less virulent isolate demonstrated slow growth of *S. schenckii* at 37°C. Regarding thermotolerance, this study also agreed with the previous evidence that infection with isolates grown at 37°C could cause multiple lesions on internal organs. However, it is still uncertain if heat susceptibility is a virulence factor. Later, the thermotolerant mutant of the lung isolate demonstrated a slow growth rate of ≥ 4 days at 37°C, compared with the parent strain lung isolate, by repeat plating to verify the virulence factor. These mutant isolates were capable of infecting the skin but did not lead to disseminated infections in mice (Tachibana et al., 2001). There was additional evidence to show that the growth of *S. schenckii* was inhibited at $\geq 37^\circ\text{C}$ and halted at $\geq 39^\circ\text{C}$ in vitro during susceptibility testing (Castro et al., 2013).

Cell wall

The cell wall is a pathogen's first line of defense, but the cell wall can also trigger the innate immune system. Novel cell wall structures from two common pathogenic *Sporothrix* spp., *S. schenckii* and *S. brasiliensis*, were analyzed using high-pressure freezing

transmission electron microscopy (HPF-TEM). In a murine model, *S. brasiliensis* showed more virulence than *S. schenckii* (Castro et al., 2013; Lopes-Bezerra, 2018). Glycoconjugates, β -1-3 and β -1-6 glucans, and chitin are presented and functioned as pathogen-associated molecular patterns (PAMPs) and triggered secretion of specific cytokines. The α -glucan polymer, which blocks β -glucans from dectin-1 recognition in other dimorphic fungi, is absent in *Sporothrix* spp. Both *S. schenckii* and *S. brasiliensis* have a bilayered cell wall. However, the cell wall of *S. brasiliensis* has 30% more chitin and 100% more rhamnose and rhamnomannan than *S. schenckii*, making the outermost fibrillar layer in *S. brasiliensis* significantly thicker than that of *S. schenckii*. The fibrillar layer can adhere to other yeast cells to build cellular network, whereas the fibrillar layer of *S. schenckii* detaches from the cell wall as it ages. After these isolates were assessed for their interaction with human macrophages, greater phagocytic activity was shown for *S. brasiliensis* than *S. schenckii*, and both species demonstrated a significantly greater uptake of 4-day-old cells over 10-day-old cells (Lopes-Bezerra, 2018). The lipid component of the *Sporothrix* cell wall is also reported to inhibit phagocytosis, as well as limit the stimulation of NO and TNF- α release (Lima et al., 2001).

Adhesins

The ability of the fungal cell to adhere to endothelial and epithelial cells is crucial to the invasion of host tissues. Adhesins support the binding of yeast cells to the dermal matrix, especially fibronectin. Moreover, adhesin has also been reported to have a major role in host immuno-modulation, which is explained in the immune response section (Castro et al., 2013). In *Sporothrix*, both the conidia and yeast forms are able to recognize three major glycoproteins in extracellular matrix: fibronectin, laminin, and collagen type II (Lima et al., 1999, 2001). Glycoprotein of 70 kDa (Gp70) is the main adhesin detected in all mice with sporotrichosis (Ruiz-Baca et al., 2009). By Western blot analysis, Gp70 was recognized together with the positive bands of fibronectin in the range of 37–92 kDa. This finding was confirmed by the co-localization of fibronectin and Gp70 on the yeast cell surface (Castro et al., 2013). There is some evidence that the fungus also has integrins or adhesin molecules with lectin-like domains that recognize human fibronectin at several points on the molecule (Lima et al., 2004). Expression of these molecules in *S. schenckii* is likely related to their virulence, because a higher level of expression was observed in the infective mycelial phase than in the parasitic yeast form. These adhesins can allow fungi to attach to human endothelial cells causing infection without tissue injury in vitro. Moreover, fungi are able to cross the intercellular space, resulting in bloodstream infections and hematogenous dissemination (Figueiredo et al., 2004).

Melanin

This pigment is broadly found in nature and is a common virulence factor in many pathogenic fungi, including *Sporothrix*. The major melanin, DHN-melanin in hyphae, can be synthesized without precursors, whereas eumelanin in hyphae and pheomelanin synthesis require precursors, such as 3,4-dihydroxy-L-phenylalanine (L-DOPA) or L-tyrosine (Jacobson, 2000; Langfelder et al., 2003; Nosanchuk and Casadevall, 2003). The role of melanins on minimum inhibitory concentration (MIC) of antifungal agents (terbinafine, itraconazole, ketoconazole, and amphotericin B) in type strains and clinical isolates of *S. brasiliensis* and *S. schenckii* was evaluated using the precursors 3,4-dihydroxy-L-phenylalanine (L-DOPA) and L-tyrosine, and the three melanin inhibitors—tricyclazole, glyphosate, and sulcotrione, according to CLSI protocol M38-A2. A twofold decreasing MIC for ketoconazole was found in each strain of *S. brasiliensis* and *S. schenckii* in the presence of L-tyrosine. A twofold decreasing MIC for terbinafine in the presence of tricyclazole was found in *S. brasiliensis* and type strains of *S. schenckii*. Regarding the Minimum Fungicidal Concentration (MFC), twofold decreasing MIC for amphotericin B in the presence of all inhibitors was observed in *S. brasiliensis* but not in *S. schenckii* or for other antifungal agents. These results indicated that melanin is protective for *S. brasiliensis* and *S. schenckii*. However, more studies should investigate these interactions in other *Sporothrix* species (Almeida-Paes et al., 2009; Madrid et al., 2010; Romero-Martinez et al., 2000).

Ergosterol

This crystalline steroid alcohol is a major compound in fungal cell membranes (Schroeder, 1984). The putative product of the H₂O₂-dependent enzymatic oxidation of ergosterol, known as ergosterol peroxide, can be found in *S. schenckii* (Sgarbi et al., 1997). This compound can be converted to ergosterol when in contact with an enzyme extract from the fungus. This molecule is able to inactivate or scavenge toxic oxygen products found in phagocytic cells (Basaga, 1990; Sgarbi et al., 1997). This finding indicates that ergosterol peroxide in *S. schenckii* plays a role as a protective mechanism to evade reactive oxygen species (ROS) during phagocytosis (Carnero et al., 2018). However, the ergosterol peroxide activity of *S. schenckii* after phagocytosis also relies on other detoxification strategies (Carlos et al., 2009).

Proteins

Several types of proteins are involved in fungal virulence. In *Sporothrix*, extracellular cell wall glucanase, a vesicle-associated protein, promotes fungal virulence by inducing the lysis of macrophages and other host cells (Tamez-Castrellón et al., 2020). Superoxide dismutase (SOD), a cell wall protein, promotes the growth and survival of pathogens under oxidative stress conditions, such as those inside macrophages and other cells (Rossato et al., 2018; Téllez et al., 2014). Certain proteinases, such as Proteinase I, are

associated with the ability of *Sporothrix* to invade the dermis by damaging skin (Rossato et al., 2018). Differences in protein expression are the primary factors contributing to the differing virulence among *Sporothrix* species. A study of 60 proteins found that higher levels of 9 specific proteins were associated with evasion of the host immune system in *S. brasiliensis* and *S. schenckii* when compared to *S. globosa*: aminopeptidase I, extracellular cell wall glucanase, Mn-superoxide dismutase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), heat shock 70-kDa protein 1/8, hydroxymethyl-glutaryl-coenzyme A (HMG-CoA) lyase, acetyl-CoA hydrolase, progesterone binding protein, and rhamnolipid biosynthesis 3-oxoacyl-(acyl-carrier-protein) reductase (Rossato et al., 2018). In contrast, non-virulence exoantigens such as secreted proteins of 46, 60, 90, and 110 kDa are commonly secreted at similar levels by *S. schenckii*, *S. brasiliensis*, and *S. globosa* (Fernandes et al., 2013).

Biofilms

Similar to other pathogenic fungi, *Sporothrix* is able to form biofilms in vitro and in vivo, overwhelming the host's defenses and decreasing the effectiveness of antifungal therapy (Mitchell et al., 2016; Tournu and Van Dijck, 2012). In general, fungal biofilms may promote adhesion on biotic or abiotic surfaces, helping the community of fungal cells to survive in hostile environments. Regarding the immune response, biofilms assist cells in resisting neutrophil attacks and avoiding ROS triggering (Hernandez-Chavez et al., 2017).

In a study involving *S. schenckii*, *S. brasiliensis*, *S. globosa* and *S. mexicana*, Brillhante et al demonstrated that all species are able to form well-structured biofilms in vitro in the mycelial phase (Brilhante et al., 2018). The formation of biofilm-like structures is believed to occur in the yeast-like form as well. Human and feline patients with severe and disseminated clinical forms of this disease may have a slow response to therapy or develop refractory disease due to *Sporothrix* biofilm formation (Brilhante et al., 2018; Mialski et al., 2018).

Innate host defense

To infect the host, fungi need to break down the host's first line of defense, such as skin or mucous membrane. The innate host defense provides the rapid recognition of a broad spectrum of pathogens through pattern recognition receptors (PRRs) and PAMPs (Janeway, 2002). The innate immune response plays a key role in the response to fungi, including *Sporothrix* (Carlos et al., 2009). So far, little is known about the PAMPs of *Sporothrix*.

Phagocytosis by macrophages and neutrophils, together with the production of ROS, are the major innate host defenses in response to *Sporothrix* spp. (Martinez-Alvarez et al., 2014; Mora-Montes et al., 2015). It is of note that each form of *S. schenckii* and *S. brasiliensis* (conidia, germings, and yeasts) are recognized by different receptors on the surface of host immune cells. The complement system is another immune defense triggered by *S. schenckii*. The alternative complement pathway is the most effective pathway for activating fungal phagocytic activity, via C3b component deposition on the fungal cell wall. Membrane attack complex (MAC) also plays a role in fungal cell lysis (Scott et al., 1986; Torinuki and Tagami, 1985).

To date, several publications emphasized the significance of toll-like receptors, especially TLR-2 and TLR-4, in sporotrichosis. In mice models in vivo, the absence of TLR-2 enhanced the dissemination of both *S. schenckii* and *S. brasiliensis* after 14 and 28 days of infection and stimulated the Th-17 response to control the infection (Rossato et al., 2019b). The in vitro results matched observations in vivo and supports the hypothesis that the impairment of TLR-2 after *Sporothrix* infection leads to insufficient macrophage activation. This also decreases nitric oxide synthesis, which is an essential micro-biocide molecule in the fungicidal activity of macrophages (Godaly et al., 2001). Similar to TLR-2, a deficiency of TLR-4 leads to a weakened immune response to both *S. schenckii* and *S. brasiliensis* infections. Moreover, the oxidative burst in response to *Sporothrix* spp. is not activated without TLR-4, further inhibiting the host immune response (Rossato et al., 2019a).

Cellular response

The components of a pathogen's cell wall play a major role in immune activation. In *Sporothrix* spp. infections, T-helper 1 and T-helper 17 responses are triggered by the secretion of certain cytokines, including IFN- γ , TNF- α , and IL-17A. These cytokines can stimulate both the innate and adaptive immune systems. IFN- β is considered the most important cytokine in the Th1 response against sporotrichosis because it is responsible for macrophage activation. In the Th17 response, IL-17A is the major cytokine secreted, as it activates macrophages and helps maintain epithelial barriers. The Th17 response is also essential for recruiting natural killer (NK) cells and mounting antifungal defenses (Bozza et al., 2002; Kajiwaru et al., 2004; Uenotsuchi et al., 2006).

To date, several antigenic peptides have been reported as immunodominant molecules in *Sporothrix* infections. Of the antigenic peptides, ZR3, ZR4, and ZR8 are all able to induce cellular proliferation, whereas only ZR3 and ZR8 have the capacity to induce IL-17A and IFN- γ . ZR8 can also induce the secretion of IFN- β , and IL-1 β , neutrophil proliferation in the lesion, and increased CD4⁺ T cells in the lymph nodes (with increased levels of IFN- β) and spleen (de Almeida et al., 2018). At this stage, Fc gamma receptor (Fc γ R) pathogen opsonization by antibodies also mediates macrophage functions to stimulate cytokine secretion and respiratory burst activation (Franco Dde et al., 2012). In the presence of antibodies against Gp70 glycoprotein, the fungicidal ability of macrophages is increased, and there are increased levels of TNF- α , IL-1 β , IL-6, and the anti-inflammatory cytokine IL-10 (Franco Dde et al., 2012). This leads to improved phagocytosis of the *Sporothrix* cells.

Dectin-1 is a receptor involved in cytokine production in sporotrichosis. This receptor must be triggered by 1,3- β -glucans, which are present on the fungal cell wall. Even though all morphological forms of *Sporothrix* can induce cytokine production, the strongest cytokine response is demonstrated in response to conidia, rather than yeasts or germlings (Guzman-Beltran et al., 2012; Martinez-Alvarez et al., 2017).

Humoral response

In the past, the cell-mediated immune response (CMI) was believed to be the major mechanism for fungal infection control, whereas humoral immunity (HMI) was not thought to play a role in combatting pathogenic fungi. However, HMI is now understood to play a critical role. The involvement of antibodies in a host's defense against fungal infection has been reported, although several factors affect its efficiency, such as antibody concentration and isotype (Almeida-Paes et al., 2007; Portuondo et al., 2016). Several mechanisms of HMI have been described: agglutination of fungal cells, opsonization and enhancement of phagocytosis, inhibition of fungal cell attachment, complement activation and mediated lysis, neutralization of immunoregulatory molecules, Fc-mediated cytokine release, and antibody-dependent cellular cytotoxicity (ADCC) (Portuondo et al., 2016). In sporotrichosis, little is currently known about the importance of HMI (Almeida-Paes et al., 2007; Rodrigues et al., 2015). However, an antibody-mediated response may provide some measure of protective immunity, based on murine models (Almeida, 2012; Almeida-Paes et al., 2007; Rodrigues et al., 2015).

Both CMI and HMI play an important role in protecting the host. In short, cytokines IL-4 and IL-13 induce T-cell differentiation from Th0 to Th2, and they decrease the Th1 response at 5–6 weeks after infection (Maia et al., 2006). In parallel, antibody production is triggered (Alba-Fierro et al., 2016; de Lima Barros et al., 2011a,b). This finding indicates that HMI plays a role in the advanced stages of sporotrichosis (Carlos et al., 2009). Based on an in vitro study, antibodies were also shown to have an effect on *S. schenckii* growth and differentiation (Toledo et al., 2010). These results were also found in vivo; a monoclonal antibody against 70 kDa adhesin induced an immune response against *Sporothrix* in mice (Nascimento et al., 2008). Moreover, it has been reported that murine models of *S. schenckii* infection can produce specific IgG1 and IgG3 antibodies against 70 kDa protein. This finding could explain the role of the antigen neutralization mechanism, because IgG1 and IgG3 are involved in opsonization and neutralization of *Sporothrix* spp. by enhancing macrophage efficiency. The administration of monoclonal antibodies also led to a significant decrease in colony forming units (CFUs) in mouse organs (Nascimento and Almeida, 2005). The same finding was demonstrated in T-cell deficient mice (nude mice). This confirmed that antibodies are able to control an infection in the absence of a T-cell response. The antibodies seem to function through two mechanisms: (1) increasing the CMI and IFN- γ production and (2) inhibition of yeast adhesion to host tissues and extracellular matrix (Alba-Fierro et al., 2016; Nascimento et al., 2008; Toledo et al., 2010).

In humans, IgG, IgM, and IgA antibodies have been demonstrated in patients with sporotrichosis. The antibody levels were reported to decrease during the treatment process, and differences between antibody level and clinical manifestations have been described. Lower optical density (OD) values for IgM and IgA were detected between cutaneous and extracutaneous forms of sporotrichosis, whereas no significant difference was present between disseminated cutaneous and extracutaneous forms. The presence of these antibodies affects the process of pathogen elimination. While IgM can activate complement by the classical pathway, IgA can prevent mucosal infections (Almeida-Paes et al., 2007).

Clinical aspects

The clinical presentations of sporotrichosis may be divided into two main categories: cutaneous and extracutaneous. However, in patients with zoonotic sporotrichosis, a wider and more diverse spectrum of clinical manifestations can be found.

Cutaneous sporotrichosis

Lymphocutaneous

Lymphocutaneous infections are the most common form of sporotrichosis, observed in 50%–70% of cases. The most common sites of infection are the upper and lower limbs and face, and lesions typically appear within days to weeks of infection. Initially, a papular skin lesion develops at the site of inoculation, which can evolve into a nodule or ulcer, known as the “sporotrichosis chancre.” This is characterized by swelling, erythema, and nodular lesions with erythematous halos, accompanied by itching. Subsequently, the lesions spread in a linear and staggered fashion along the regional lymph vessels towards the lymph nodes, which is described as “sporotrichoid spreading”. Nodular or fluctuant lesions tend to ulcerate. In chronic cases, it is observed that some lesions can self-involute. Occasionally and through multiple inoculations, it can form a mycetoma-like lesion, characterized by increased volume due to lymph stasis. Among children, this form is more commonly located on the face (up to 40% of cases). It may occur unilaterally or bilaterally, depending on the inoculation zone (Bonifaz and Tirado-Sanchez, 2017; Bonifaz and Vazquez-Gonzalez, 2010) (Fig. 2).

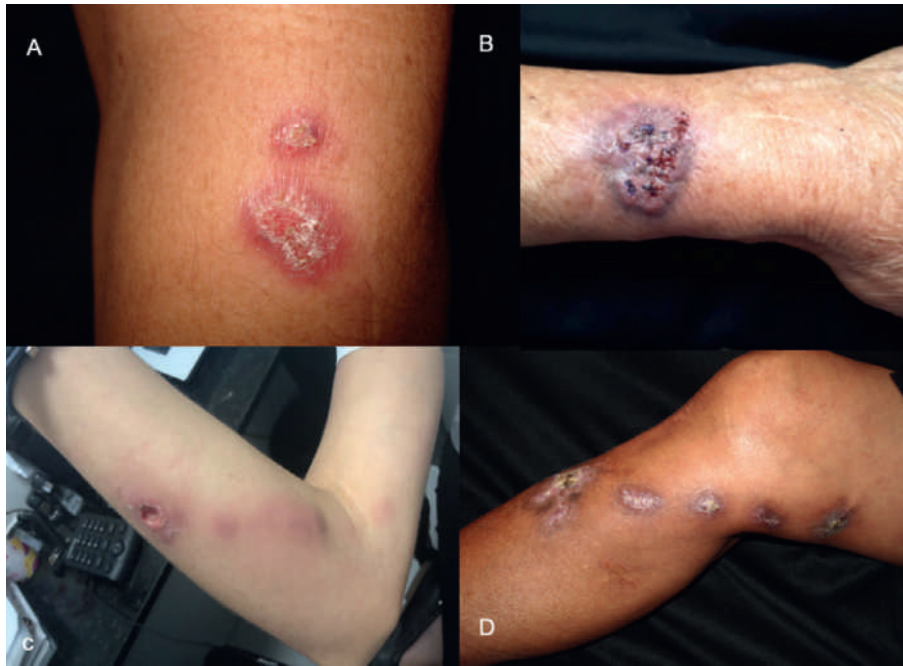


Fig. 2 Fixed cutaneous (A and B) and lymphocutaneous with ascending lymphangitis (C and D) clinical forms

Fixed cutaneous

The fixed cutaneous form is a chronic manifestation which occurs in 20%–40% of cases. It may be a single lesion or multiple lesions, depending on the number of implanted sites. It begins similarly to the lymphocutaneous form: with a single vegetative or warty lesion with defined edges and an erythematous-violet halo, and it is usually covered with flakes and meliceric scabs. Other than these isolated lesions, this form is often considered asymptomatic, and there is no lymphatic spread of the fungus (de Lima Barros et al., 2011a,b; Bonifaz and Vazquez-Gonzalez, 2010) (Fig. 2).

Disseminated cutaneous

The disseminated cutaneous manifestation (also known as cutaneous-hematogenous sporotrichosis) is rare (1%–5%). It is generally considered a unique entity and is associated with host risk factors and several types of immunosuppression, including HIV. The morphology is similar to the previously described forms, with a predominance of nodular, suppurative lesions, ulcers, and warty plaques located anywhere on the skin and mucous membranes of the nose, mouth, pharynx, and glans. Disseminated forms tend to spread to the bones and joints (monoarthritic), as well as to other organs, and may include fungemia and CNS involvement. Patients co-infected by HIV are more likely to develop disseminated forms of sporotrichosis (Bonifaz et al., 2018a; Bonifaz and Tirado-Sanchez, 2017) (Fig. 3).

Extracutaneous sporotrichosis

Ocular involvement

Although manifestations involving the eye and adnexa are rarely reported, the number of cases with ocular involvement has increased in Brazil since the start of the zoonotic outbreak of *S. brasiliensis*. It typically arises from fungal implantation in external ocular adnexa, like the conjunctive, cornea, and palpebra, rather than endogenous dissemination. Infection may occur after bites or scratches, or even after contact with mucocutaneous secretions from cats infected with *S. brasiliensis* yeast-like cells (de Lima Barros et al., 2004). The most commonly reported clinical manifestations are granulomatous conjunctivitis, but a wide spectrum of ophthalmologic manifestations have been reported. They include dacryocystitis, keratitis, granulomatous uveitis and retinitis, choroiditis, scleritis, necrotizing retinochoroiditis endophthalmitis and Parinaud's oculoglandular syndrome (Biancardi et al., 2017; Freitas et al., 2014; Kashima et al., 2010). Less frequently, blindness and eye enucleation are sequelae to choroiditis and endophthalmitis, respectively (Cartwright et al., 1993; Ramirez-Soto et al., 2018). Ocular involvement is also frequently reported in HIV patients with associated sporotrichosis (Moreira et al., 2015; Silva-Vergara et al., 2012) (Fig. 4).

Osteoarticular involvement

Manifestations of this disease in the bones and joints may follow systemic dissemination or direct implantation of the fungus. This may result in isolated osteomyelitis, with or without arthritis. Bone involvement has been observed in the knee, hands, and feet, as



Fig. 3 Cutaneous disseminated sporotrichosis in immunocompromised hosts. Multiple erythematous, papular and ulcerative necrotic lesions in an uncontrolled diabetic patient (A). Erythematous plaques and verrucous lesions in both thighs of a patient with alcohol use disorder (B). Ulcerative necrotic lesion in the face of a patient with medullar aplasia due to organophosphate pesticide exposure (C). Disseminated cutaneous ulcerated lesions (D and E) and noduloulcerative skin lesions in patients with HIV (F).

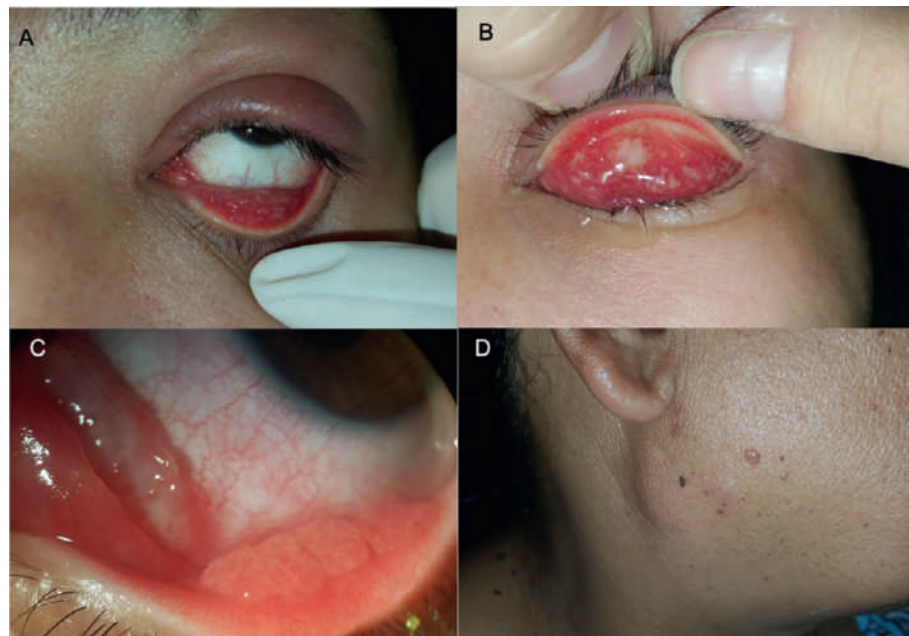


Fig. 4 Eye involvement related to *Sporothrix brasiliensis* infections in Brazilian patients. Granulomatous tarsal conjunctivitis in the left eye (A and B) and granulomatous conjunctivitis with preauricular adenopathy (C and D), also known as Parinaud oculoglandular syndrome.

well as isolated to the tibia, radius, and ulna. Lesions are painful with localized erythema and swelling. Radiologic images may present findings similar to bacterial osteomyelitis, with lytic lesions, bone erosion, osteopenia, and periosteal reaction (de Lima Barros et al., 2011a,b; McLawhorn et al., 2019; Orofino-Costa et al., 2010; Saeed et al., 2019).

Pulmonary involvement

This is a rare, although possibly underdiagnosed, clinical manifestation. Most pulmonary cases are primary infections, and all have been reported among people living in highly endemic areas (Pluss and Opal, 1986; Tiwari and Malani, 2012). There are two forms of pulmonary involvement: chronic and acute. The most common form is chronic, which is typically observed in patients with obstructive pulmonary disease or chronic alcoholism. This form manifests similarly to tuberculosis, with persistent productive cough, fever, night sweats, chills, and weight loss. Oligosymptomatic patients present with a self-limited disease including pulmonary cavitory lesions very similar to tuberculosis, while symptomatic patients present with a pneumonia-like illness with discrete coughing and little expectoration. Radiologic findings include areas of condensation or miliary opacities (Comstock and Wolson, 1975). The second form of pulmonary involvement is acute and progressive (associated with immunosuppression), presenting with a ring-like lymph node (tracheobronchial disease), and large adenopathies that cause bronchial obstruction. This form may be accompanied by general malaise, weight loss, a cough with abundant expectoration, hemoptysis, dyspnea, and fatigue. Radiologic findings may include adenopathy and mediastinal widening (Aung et al., 2013; Comstock and Wolson, 1975).

Neurological involvement

The CNS may be primarily or secondarily affected from systemic dissemination. Primary CNS involvement is more likely to occur in immunocompetent hosts, while hematogenous dissemination is associated with immunosuppression, as with HIV co-infection (Freitas et al., 2015; Galhardo et al., 2010). Neurologic involvement in sporotrichosis is rare, but the number of cases has increased since the zoonotic outbreak of *S. brasiliensis* in Brazil (Almeida-Paes et al., 2014). Chronic meningitis is the most common clinical manifestation and is easily confused with tuberculosis, which can lead to delayed diagnosis and treatment for many patients. The patients may complain of long duration of symptoms, insidious and refractory headaches, seizures, neurologic focal signs, ataxia, and mental confusion. Late diagnosis can result in the development of intracranial hypertension and hydrocephalus (Kauffman, 2019; Mialski et al., 2018).

Immunoreactive clinical forms

Some patients with cutaneous sporotrichosis may present clinical dermatologic and/or rheumatologic immunoallergic manifestations, resulting from a hypersensitivity reaction to circulating antigens. Up to 30% of the patients with zoonotic *S. brasiliensis* infections may present immunoreactive forms of sporotrichosis, including cutaneous rash, papular eruptions, erythema nodosum, erythema multiforme, reactive arthritis, and other dermatologic presentations (de Lima Barros et al., 2011a,b; Papaioordanou et al., 2015; Queiroz-Telles et al., 2017) (Fig. 5).

Opportunistic disease

The clinical manifestations of endemic mycoses in immunocompromised hosts can be quite remarkable. The weakened inflammatory and immune responses of immunosuppressed patients typically result in high fungal burdens, greater frequency of disseminated and severe clinical manifestations, and higher mortality rates. Endemic fungi in immunocompromised hosts often show false negative results by serology and typically require prolonged periods of antifungal therapy (Carvalho et al., 2002; Queiroz-Telles et al., 2019). Patients with several risk factors or underlying conditions are likely to present with more severe clinical forms of sporotrichosis with different varying degrees of severity. Over 100 sporotrichosis cases associated with AIDS have been reported, and most of them have been caused by *S. brasiliensis* in Brazil (Freitas et al., 2012; Mialski et al., 2018). Patients with low CD4+ levels and high viral loads may present cutaneous disseminated disease, fungemia, meningitis, pneumonia, or

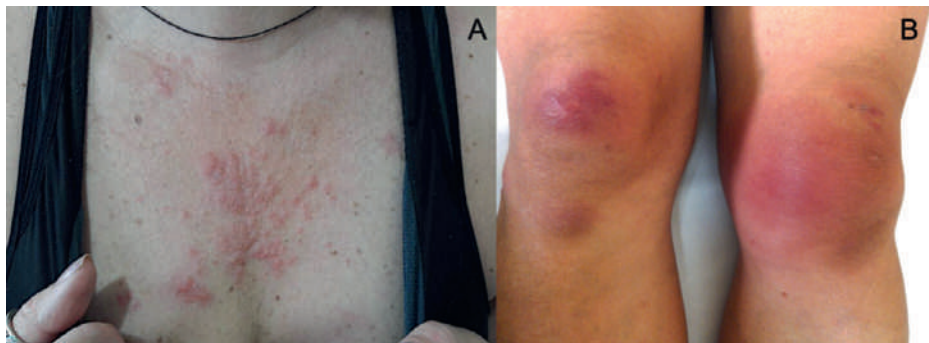


Fig. 5 Immunoreactive clinical forms. Erythematous confluent papular lesions causing Sweet's syndrome (A) and reactive arthritis (B), in patients with *Sporothrix brasiliensis* infections.

endophthalmitis, with mortality rates of up to 30% (Moreira et al., 2015). Like some other fungal infections, refractory sporotrichosis may be associated with CARD9 deficiency (Bao et al., 2019) (Fig. 3).

Feline sporotrichosis

While sporotrichosis has been reported in a variety of animal species, the most significant and frequent animal infections occur in cats (Pereira et al., 2014). The epidemiology of feline sporotrichosis differs by the infecting *Sporothrix* spp. Feline infections caused by *S. schenckii* have occurred on five continents, and cases are typically isolated or limited to small outbreaks (Gremião et al., 2017; Hirano et al., 2006; Mackay et al., 1986; Pereira et al., 2014; Scheufen et al., 2015; Singer and Muncie, 1952). However, the emergence of *S. brasiliensis* has led to long-lasting epizootics of feline sporotrichosis through the South and Southeast regions of Brazil, as well as smaller outbreaks in other regions of Brazil and Argentina (Etchecopaz et al., 2019; Gremião et al., 2017; Schubach et al., 2005b). There has also been a documented case of travel-associated feline sporotrichosis in Paraguay (García Duarte et al., 2017). The ongoing outbreak of feline sporotrichosis in Brazil presents a serious threat for zoonotic transmission; accurate and rapid identification of feline cases is necessary to limit human exposures.

Feline sporotrichosis occurs most often among unneutered, male cats (Pereira et al., 2014), and infection typically occurs following traumatic inoculation via the scratch or bite of an infected cat (Rodrigues et al., 2016b). The clinical manifestations of feline sporotrichosis can range from subclinical to fatal disseminated infection (Schubach et al., 2004). Cutaneous manifestations are most commonly seen with multiple distinct nodular or ulcerated lesions occurring across the body. The most common sites of infection include the head and limbs, with frequent involvement of the nasal mucosa (Pereira et al., 2010; Schubach et al., 2004). In addition to cutaneous involvement, respiratory signs such as coughing and sneezing are frequently reported, and the presence of respiratory signs or nasal mucosal lesions are associated with treatment failure (Boechat et al., 2018; Pereira et al., 2010) (Fig. 6).

The most commonly used treatment for sporotrichosis in cats is itraconazole at 50–100 mg/cat/day (de Souza et al., 2018). In cases of therapeutic failure or recrudescence, the addition of potassium iodide at 2.5–20 mg/kg/day as a combination therapy can be effective (Gremião et al., 2015; Reis et al., 2016; da Rocha et al., 2018). To prevent cases of feline sporotrichosis among domestic pets, cat owners should prevent their cats from interacting with feral cats by keeping them indoors.

Differential diagnosis

Sporotrichosis must be differentiated from several infectious and non-infectious conditions. Patients presenting with cutaneous and extracutaneous clinical disease, as described above, should be carefully interviewed about previous injury from or contact with

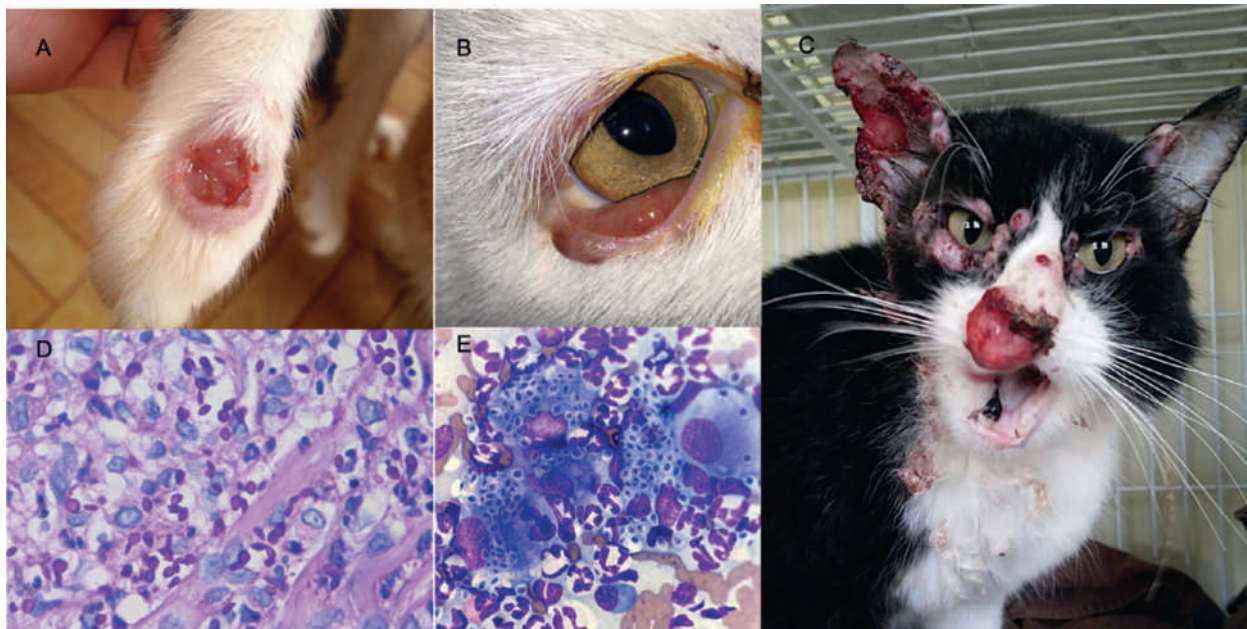


Fig. 6 Clinical and microscopic characteristics of *Sporothrix brasiliensis* in cats. (A) Cutaneous ulcerated lesion in a cat paw. (B) Granulomatous palpebral conjunctivitis in the right eye. (C) Disseminated cutaneous ulcers and mucosal lesions with nasal involvement ("Clown-nose" lesion) causing open-mouthed breathing. (D) Several yeast cells in a skin biopsy. Periodic Acid-Schiff stain $\times 400$. (E) Cytopathologic exam showing the high burden of yeast cells in secretions swabbed from an ulcerated lesion. Giemsa stain $\times 400$.

plants and animals. In endemic areas where *S. brasiliensis* infections are prevalent, a detailed history about cat contact should be collected. According to the clinical form or organ involvement, sporotrichosis must be differentiated from the following conditions:

Lymphocutaneous sporotrichosis

Cutaneous lymphatic leishmaniasis (Pian-bois), phaeohyphomycosis, ganglionic tuberculosis, syphilis, pyogenic infections, tuberculoid leprosy, and non-tuberculous mycobacterial infections (*M. marinum*), cat-scratch disease, filariasis, and others (Tirado-Sánchez and Bonifaz, 2018).

Fixed cutaneous sporotrichosis

Warty tuberculosis, chromoblastomycosis, eumycetoma and actinomycetoma, granulomatous dermatophytosis and candidiasis, cutaneous leishmaniasis, papilloma and verrucae, orf, yaws, botryomycosis, impetigo, infections by mycobacteriosis (*M. fortuitum* and *M. marinum*), sarcoidosis, psoriasis, systemic lupus erythematosus, mossy foot, squamous cell carcinoma, Bowen's disease, mycosis fungoides and others (de Lima Barros et al., 2011a,b; Bonifaz et al., 2007).

Disseminated cutaneous sporotrichosis

Cutaneous spreading of systemic mycoses (paracoccidioidomycosis, coccidioidomycosis, histoplasmosis, cryptococcosis, talaromycosis, and emergomycosis), tuberculosis and non-tuberculous mycobacteria, syphilitic gums, and others (Bonifaz et al., 2018a; Bonifaz and Tirado-Sanchez, 2017).

Pulmonary involvement

Endemic pulmonary mycosis, chronic aspergillosis tuberculosis and other mycobacteriosis, bacterial and viral pneumonia (Aung et al., 2013).

Ocular involvement

There are several bacterial, viral and fungal infections that may mimic ocular and eye adnexa sporotrichosis, including *Fusarium* and *Aspergillus* keratitis, hordeolum, chalazion, and others.

Neurologic involvement

All causes of chronic meningitis, including CNS tuberculosis and CNS mycosis (Kauffman, 2019).

Laboratory diagnosis

Direct examination and stains

Direct observation and conventional stains are of little use, because yeasts are not typically observed in lymphatic and cutaneous fixed sporotrichosis. With immunofluorescence techniques, fungi can be better identified. In general, mycological structures are noted in 5%–10% of cases. The yeasts appear as round to oval “cigar” or “boat-like” shapes. They are almost always isolated, so it is difficult to find them. However, in disseminated disease, immunosuppressed patients, and sick cats, multiple clusters of yeast are often seen (Orofino-Costa et al., 2017; Rudramurthy and Chakrabarti, 2017) (Fig. 6).

Culture

Fungal culture is the gold standard for diagnosis. Cultures can be made with samples from the exudate of lesions, abscess aspirates, scales, tissue fragments, synovial fluid, and sputum. The samples can be plated on Sabouraud dextrose agar with antibiotics and incubated at 28°C. Colonies appear in an average time of 5–8 days. All species of *Sporothrix* are dimorphic fungi, so yeast colonies can be obtained in rich culture media (blood agar, BHI agar, etc.) and incubated at 37°C. Colonies of the various species of *Sporothrix* develop rapidly (3–5 days), and they are membranous, radiated, and whitish or beige. The colonies later produce air mycelium-type coremium, maintain a mucoid appearance on the periphery, and turn dark brown in color due to melanization. The species *S. pallida* and *S. chilensis* are unique in that they do not generate melanic pigment (Cruz Choappa et al., 2014; Rodrigues et al., 2013a, 2016a) (Fig. 7).

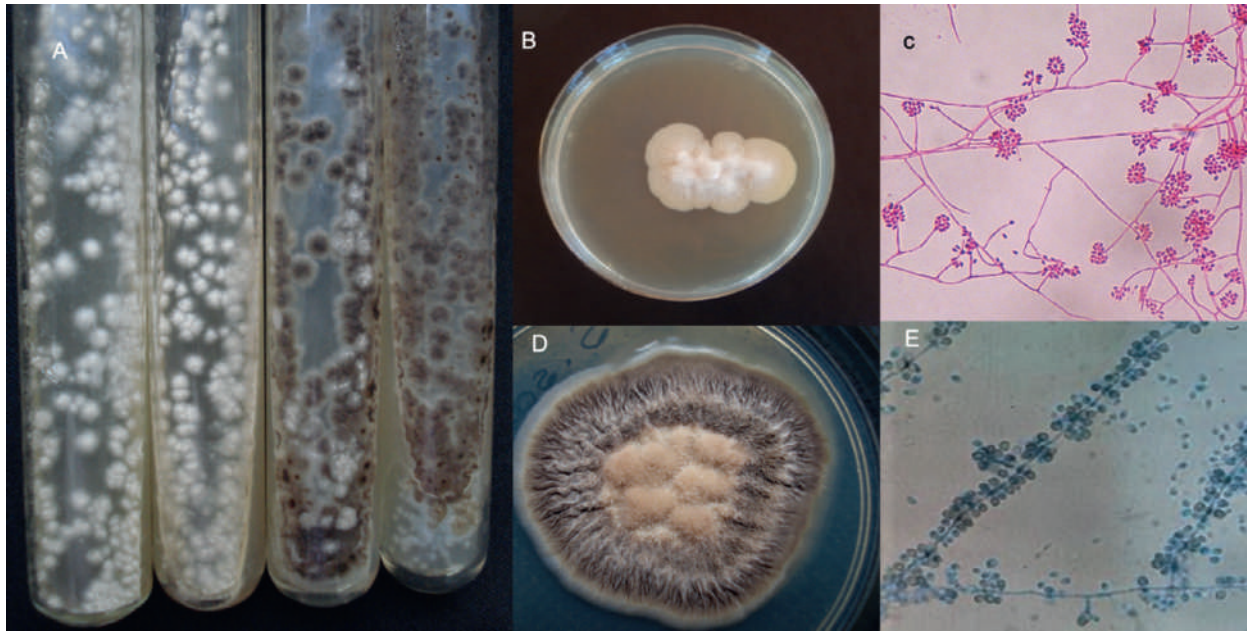


Fig. 7 Mycological diagnosis. Macroscopic characteristics of *Sporothrix* spp. cultures in the mycelial phase (A and B). Some isolates may be melanized with time and become brown to black pigmented like the two rightmost tubes in (A) and the plaque colony in (D). The micromorphology shows thin hyaline septate hyphae with sympodial conidiophores (bouquet shape) forming right angles with the mycelium. Erytosin stain X 200 (C). In some isolates, conidia may become brownish due to melanization. Cotton blue X 400 (E).

Histopathology

Similar to direct mycologic exam, the histopathologic findings are not pathognomonic. The tissue response is non-specific and usually depicts a granulomatous, suppurative, and pyogenic reaction, consisting of micro-abscesses of polymorphonuclear cells and sometimes tuberculoid granulomas with enbibed histiocytes, lymphocytes, and Langhans giant multinucleated cells. In a study of 119 sporotrichosis cases, 84% had suppurative granulomas, while the rest were tuberculoid, foreign body-type, caseous, and fibrinoid granulomas. Yeasts and asteroid bodies were observed in 35% of cases (Quintella et al., 2011; Zhang et al., 2011). With unique stains of PAS and Gomori-Grocott, a few scattered growing yeasts can be observed. Rarely, typical “asteroid bodies” can be observed. This eosinophilic “spike like” structure results from the “Splendore-Hoeppli” immune-reaction, consisting of antigen-antibody immuno-complexes and cellular debris deposits surrounding the yeast cell wall (Daniel Da Rosa et al., 2008; Rodríguez and Sarmiento, 1998) (Fig. 8).

Immune tests

Serological screening has been improved to detect *Sporothrix*-specific IgG. Detection of circulating antibody is useful to differentiate sporotrichosis from other similar cutaneous diseases and to monitor the success of treatment. The most sensitive tests are the immunoenzymatics (ELISA) and Western blot methods, but neither are commercialized (Bernardes-Engemann et al., 2015; Orofino-Costa et al., 2017). Due to the common antigenic properties of *S. schenckii*, *S. brasiliensis*, and *S. globosa*, cross reactivity is seen when performing these tests (Rodrigues et al., 2020). A latex agglutination test to diagnose sporotrichosis meningitis has been developed and evaluated. The sensitivity and specificity of the test is high, but the availability of the test is still limited (Blumer et al., 1973).

An intradermal reaction test with sporotrichin was employed in the past to evaluate the endemicity of sporotrichosis (Bonifaz et al., 2018b). The test uses a polysaccharide metabolic fraction (peptide-rhamnomannan) of *S. schenckii* and is cross reactive with *S. brasiliensis* and *S. globosa*. Although it is highly specific (94.5%), it is neither standardized nor commercially available (Bonifaz and Vazquez-Gonzalez, 2013). It should be emphasized that this method is not authorized in some European countries or the United States.

Molecular diagnosis

This rapid DNA detection provides high sensitivity (83%–92%) and specificity (95%). Molecular diagnostics overcome the limitations of direct and histopathological exams, and they can facilitate surveillance efforts in outbreak scenarios. Different PCR

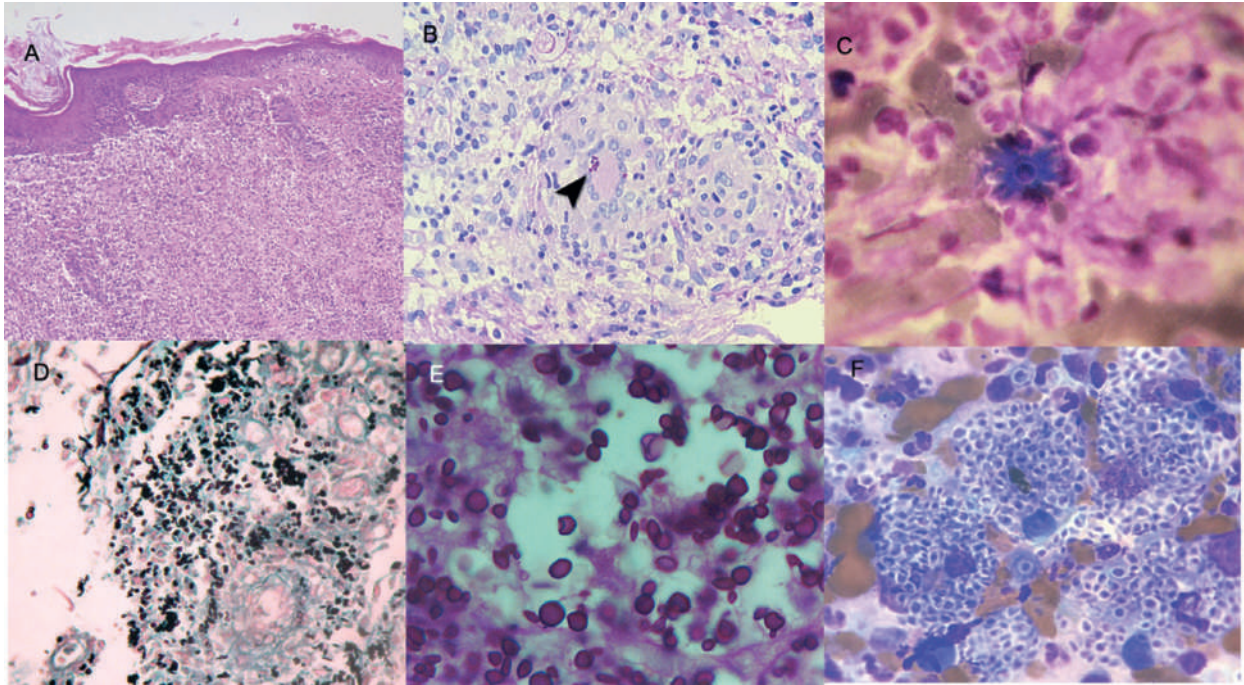


Fig. 8 Histopathologic and cytopathologic diagnosis. A skin section with pseudoepitheliomatous hyperplasia, parakeratosis and hyperkeratosis, covering a mixed suppurative and granulomatous inflammatory dermal and subcutaneous tissue reaction. Hematoxylin eosin X 100 (A). Yeast cells with round and cigar shapes (arrowhead) in the cytoplasm of a multinucleate Langerhans giant cell. Periodic Acid-Schiff stain X 400 (B). Typical asteroid body made up of a spiked crown formed by a round yeast cell surrounded by deposits of antigen-antibody immune complexes. X 1000 (C). Cutaneous biopsy showing several round to cigar-shaped yeast cells from a diabetic patient (Fig. 4A). Grocott-Gomori stain X 200 (D). Round to elongated budding and non-budding yeast cells from a skin biopsy of an AIDS patient (Fig. 4D). Periodic Acid-Schiff stain X 1000 (E). Impression smear from an ulcerated cutaneous feline lesion, showing a high burden of yeast cells from *Sporothrix brasiliensis*. Giemsa stain X 400 (F).

tests have been developed for major targets such as the ITS region, calmodulin, β -tubulin, and EF-1 α (Rodrigues et al., 2020). No commercial kit is currently available. This molecular diagnosis approach can be applied in the samples collected from any lesion, such as skin scrapings, tissue biopsies, pus, lymph node aspirates, conjunctival swabs, bronchoalveolar lavages, central nervous fluid, blood, etc. In addition, molecular methods based on the calmodulin gene sequencing are fundamental for the species identification (Rodrigues et al., 2014).

With the advent of matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS), rapid user-friendly tests have been developed to aid in the identification of fungi to the genus and species level. However, more work is needed to build the database and improve identification of *Sporothrix* spp. (Oliveira et al., 2015).

Antifungal susceptibility tests

To date, standard guidelines for antifungal susceptibility testing of the genus *Sporothrix* have not been established. By the in vitro test based on the Clinical and Laboratory Standards Institute (CLSI) reference protocol (CLSI, 2008), terbinafine was claimed to be the most effective drug for both yeast and mold forms of *Sporothrix* spp., whereas voriconazole and fluconazole displayed very low activity (Alvarado-Ramírez and Torres-Rodríguez, 2007; Rodríguez-Tudela et al., 2008). The frequency of *S. brasiliensis* with high MICs of itraconazole and amphotericin B has been reported over the time (Alvarado-Ramírez and Torres-Rodríguez, 2007; de Lima Barros et al., 2011a,b). Similar to *S. schenckii*, the MICs of amphotericin B are varied when compared to *S. brasiliensis* isolates (Borba-Santos et al., 2015; Espinel-Ingroff et al., 2017). The breakpoints for *Sporothrix* have not yet been established. To set up the epidemiological cut-off value, meta-analysis of antifungal susceptibility profiles among the genus *Sporothrix* are required.

Treatment

Although molecular identification is crucial for accurate identification of the species causing sporotrichosis, it is not needed to initiate therapy. Despite recent data suggesting different in vitro susceptibility of *Sporothrix* spp. to antifungals and higher MICs for *S. brasiliensis*, these findings have not been confirmed in animal models or in human patients (Bonifaz and Vazquez-Gonzalez, 2013; Kauffman et al., 2007; de Lima Barros et al., 2011a,b; Queiroz-Telles et al., 2017).

Itraconazole is the treatment of choice for sporotrichosis, particularly in lymphocutaneous and fixed cutaneous clinical forms. The clinical response rate of patients treated with itraconazole ranges from 80% to 100% (de Lima Barros et al., 2011a,b). Although several regimens of oral itraconazole have been proposed, including 100 mg/day or intermittent administration (400 mg/day for 1 week and 3 week break) as pulse therapy, we recommend itraconazole for fixed cutaneous or lymphocutaneous forms at doses of 200 mg/day, continuously for an average time of 3–6 months or for 2–4 weeks after clinical resolution. For the disseminated cutaneous form, the itraconazole dose may be increased up to 400 mg/day in two doses. Children are treated with 6–10 mg/kg/day (Bonifaz et al., 2007).

The best alternative therapy to itraconazole is terbinafine. This allylamine derivative shows better *in vitro* activity against *Sporothrix* spp., but there is less literature on its efficacy when compared to itraconazole. Terbinafine therapy should be used at 500 mg twice daily. Children may receive 125–250 mg/day in two doses (Francesconi et al., 2009). Another therapeutic option is a saturated solution of potassium iodide (SSKI), which was the standard therapy for sporotrichosis in the past (Macedo et al., 2015). Despite its effectiveness, SSKI is less tolerated than itraconazole or terbinafine, and its main side effects include gastritis, rhinitis, bronchitis, hives, erythema, metallic taste, and swelling of the salivary glands (Kauffman et al., 2007). The dosage of SSKI in adults is 3–6 g/day, and for children it is 1–2 g/day. SSKI is administered at an initial dose of 5 drops, three times a day and is increased daily by 5 drops, up to a maximum dose of 30–40 drops. Due to the bitter taste, it can be given with milk or fruit juice. The duration of therapy in the most common cases of sporotrichosis (lymphatic and fixed) is three months on average. It is advisable to continue treatment for an additional one or two months to avoid relapse (Sterling and Heymann, 2000).

Fluconazole at the dose of 400–800 mg/day is also indicated, but only as a second line therapy or when itraconazole and terbinafine are not available (Kauffman et al., 1996). Posaconazole is effective for treating sporotrichosis, but it has only been used in a small number of cases (Fernandez-Silva et al., 2012). Voriconazole and isavuconazole have high MICs against *Sporothrix* spp., so these triazoles are not indicated for sporotrichosis (Thompson and Wiederhold, 2010).

Amphotericin B formulations can be used for disseminated and severe forms of sporotrichosis, such as neurologic, pulmonary, osteoarticular, and systemic infections. Unlike the triazoles, amphotericin B can be used in pregnant patients requiring systemic therapy. The deoxycholate form should be used at a dosage of 0.7–1 mg/kg/day and the lipid formulations at 3–5 mg/kg/day. Once the acute course is finished, itraconazole can be continued at a maintenance dose (Kauffman et al., 2007).

Some patients with mild forms of sporotrichosis, especially the fixed cutaneous form, can be treated with local heat or thermotherapy. This treatment is recommended for minor cases of sporotrichosis as concomitant therapy, especially in pregnant patients (Kauffman et al., 1996).

For patients with immunoreactive forms, nonsteroidal anti-inflammatory drugs (NSAIDs) can be added to antifungal therapy. If patients are unresponsive to therapy or have immune reconstitution inflammatory syndrome (IRIS), prednisone at 20 mg/day should be given for limited period of time (de Lima Barros et al., 2011a,b; Papaioordanou et al., 2015; Queiroz-Telles et al., 2017).

Prevention

Similar to other mycotic diseases, there is no vaccine for sporotrichosis. As with other implantation mycoses, the best way to prevent this disease is to use protective measures to avoid transcutaneous trauma, especially in endemic areas. The use of shoes, gloves, and clothes may diminish the risk for infection. In the case of cat-transmitted sporotrichosis, individuals should avoid contact with sick cats. The control of zoonotic sporotrichosis involves antifungal therapy for infected animals and people, and isolation or euthanasia of infected animals. Castration has been suggested to control the spread of this disease among cats in hyperendemic zones.

Regarding vaccine development in sporotrichosis, it has been proposed that after infection or active immunization with whole cell or cell wall of *Sporothrix* spp., a specific immune response can be developed (Portuondo et al., 2016). In mouse models, specific IgG1 and IgG2a antibodies against cell wall proteins of *Sporothrix* spp. were produced after inoculation with aluminum hydroxide-adsorbed *S. schenckii* cell wall protein. The IgG2 antibodies are involved in several immune mechanisms, including complement fixation and binding to Fcγ receptors to stimulate phagocytosis and ADCC in mice (Portuondo et al., 2016).

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Dermatophytes and Dermatophytosis

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Introduction

Dermatophytes are specialized filamentous fungi that are able to degrade keratin. They are the cause of most fungal infections of the skin, hair and nails. Three ecological groups of dermatophytes are distinguished: (1) the anthropophilic species whose natural habitat is man; (2) zoophilic species whose natural habitat is an animal, and which are responsible for the most frequent zoonoses and (3) the geophilic species whose natural habitat is the soil (Weitzman and Summerbell, 1995). Dermatophytoses vary depending on the body site and the causative agent. The disease is often described with the word “tinea” followed by a term for the particular infected body site (Degreef, 2008). The anthropophilic species are generally not inflammatory and have a chronic character. On the contrary, zoophilic and geophilic species cause inflammatory fungal infections in humans (Fig. 1). Dermatophytosis is one of the most common zoonotic diseases. The main dermatophytes infecting humans are listed in Table 1 under anthropophilic, zoophilic and geophilic species.

The diagnosis of dermatophytosis cannot usually be made on the basis of clinical signs alone, but requires both direct microscopic examination of dermatological specimens and identification of the infectious species. Direct microscopic examination using fluorescence techniques is by far the most sensitive technique for detecting rare hyphae and spores in dermatological samples (Fig. 2) (Monod et al., 1989). However, non-dermatophyte fungi can be the infectious agent in onychomycosis (Monod et al., 2006; Verrier et al., 2012). A positive direct examination in a nail does not automatically signify that the fungus is a dermatophyte.

Early clinical description of dermatophytes to species identification by PCR

The first dermatophyte species were described solely by the clinical appearance of the lesions and microscopic observation of the fungal nature of the infection. Schoenlein (1839) first reported the mycological nature of a ringworm caused by a dermatophyte. The fungus was named *Achorion schoenleinii* 6 years later by Remak (1845) and *Trichophyton schoenleinii* by Langeron and Milochevitch in 1930. Three other species were described by Gruby between 1841 and 1844. (1) *Trichophyton mentagrophytes*, which has been briefly described under the name of “mentagre,” with a Greek and Latin etymology meaning “plant caught on the chin” (mentum: chin; αἴρω: to catch; φυτόν: plant) for a beard mycosis (Gruby, 1842). (2) *Microsporum audouinii* for which the name was given in reference to small spores located along the hair in beard and scalp ringworms (Gruby, 1843). (3) *Herpes tonsurans* for scalp mycosis causing tonsures (Gruby, 1844). This third species was named *Trichophyton tonsurans* by Malmsten in 1845 and 1848. It results that *M. audouinii* and *T. tonsurans* are the type species of the genus *Microsporum* and *Trichophyton*, respectively, in the current dermatophyte nomenclature (de Hoog et al., 2017).

The first systematic study of dermatophytes was “Les teignes” published by Sabouraud (1910). Based on the clinical aspects of the lesions, direct mycological microscopy and culture examinations, Sabouraud classified dermatophytes into four genera: *Achorion*, *Trichophyton*, *Microsporum* and *Epidermophyton*. Emmons (1934) modernized the taxonomy proposed by Sabouraud and other authors. He classified the dermatophytes in three genera (*Trichophyton*, *Microsporum*, and *Epidermophyton*) based on the morphology of spores produced by the fungi in cultures, and eliminated the *Achorion* genus.



Fig. 1 Human dermatophytoses. (A) *Tinea barbae* with *T. mentagrophytes*. (B) *Tinea capitis* (kerion) with *T. benhamiae*, (C) *Tinea manuum* with *T. erinacei*. (D) *Tinea unguium* with *T. rubrum*.

Table 1 Ecological classification of the main dermatophytes infecting humans into anthropophilic, zoophilic and geophilic species.

Anthropophilic	Zoophilic ^a	Geophilic
<i>Trichophyton rubrum</i>	<i>Trichophyton mentagrophytes</i> (Cats, dogs, rodents (rabbits, mice, chinchilla), horses)	
<i>T. interdigitale</i>	<i>T. benhamiae</i> (Guinea pigs, dogs)	
<i>T. violaceum</i>	<i>T. verrucosum</i> (mainly cattle, but occasionally horses, pigs)	
<i>T. soudanense</i>	<i>T. erinacei</i> (hedgehogs)	
<i>T. tonsurans</i>	<i>T. quinckeanum</i> (Mice (favus))	
<i>T. schoenleinii</i>	<i>T. equinum</i> (Horses)	
<i>T. concentricum</i>	<i>T. simii</i> (Monkeys, poultry)	
	<i>T. bulbosum</i> (Horses, donkeys)	
<i>Epidermophyton floccosum</i>		
<i>Microsporum audouinii</i>	<i>M. canis</i> (Cats and other felines, dogs)	
<i>M. ferrugineum</i>	<i>Nannizzia persicolor</i> (Voles, shrewmouse, dogs)	
		<i>N. gypsea</i>
		<i>N. fulva</i>
		<i>N. incurvata</i>

^aHost reservoirs are in brackets.

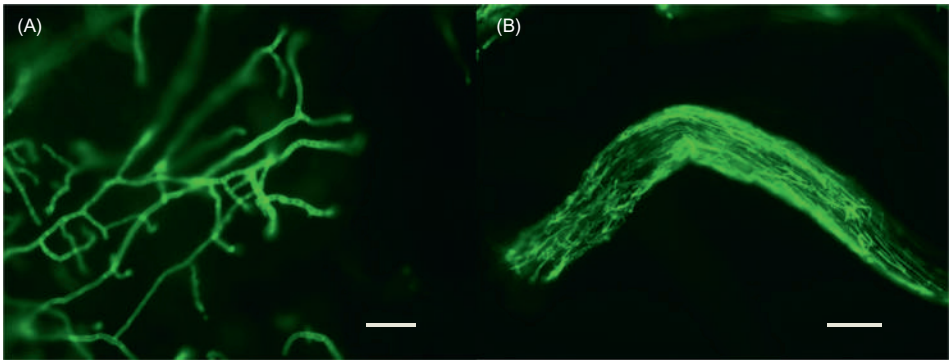


Fig. 2 Direct mycological examinations of clinical and animal samples. (A) Hyphae in tinea corporis (bar = 10 μ m). (B) *Tinea capitis*, infected hair (bar = 40 μ m). One portion of each sample was examined in a dissolving solution containing a fluorochrome that specifically binds to vegetal and fungal cell wall polysaccharides (Monod et al., 1989). The sample preparations were observed using a fluorescence microscope using excitation between 400 and 440 nm.

Until the 1990s, macroscopic and microscopic characters of the fungus in culture identified the different species of dermatophytes. However, their identification in culture often remained uncertain due to the variations of morphological features within species. In addition, isolates of different species may have the same morphology. There was also a profusion of species and varieties for which no reference material (culture or strain) was deposited in a collection, creating great confusion in the delimitation and nomenclature of species. As early as the 1990s, the synonymy and distinction of species was made by DNA sequences (Gräser et al., 1999a,b). The polymorphism of the internal transcribed spacer sequences of the ribosome-coding DNA (ITS1 and ITS2) was first used to differentiate the species.

Last review of dermatophyte species and their nomenclature in 2016

de Hoog et al. (2017) have revised the taxonomy of dermatophytes based on the analysis of DNA sequences. A phylogenetic tree has made possible to clearly define the genera to which anthropophilic, zoophilic and exclusively geophilic species belong. The species of dermatophytes have been defined based on DNA sequences, on phenotypic characters observed in culture, on interfertility between strains and on their anthropophilic, zoophilic or geophilic character. A species is either anthropophilic or zoophilic, but not both. Geophilic species include many species that are not pathogenic to humans. The nomenclature was revised according to the newly defined genus, and according to the rules of the new convention adopted for the nomenclature of the fungi, where it was decided that a species could have only one name (de Hoog et al., 2015). The genus *Trichophyton* has been reduced to about 15 species, which are anthropophilic or zoophilic pathogens. *Epidermophyton* contains a single anthropophilic species, *Epidermophyton floccosum*. The genus *Microsporum* now contains only three species: *Microsporum canis* (zoophilic), *M. audouinii* and *Microsporum ferrugineum* (anthropophilic). Pathogenic geophilic species are in other distinct genera (*Nannizzia* and *Arthroderma*).

Identification of species in the laboratory

Dermatophyte species are generally identified by observing under the microscope: (1) the growth rate of the fungus in culture; (2) the appearance and color of the mycelium (Fig. 3); (3) the pigmentation of the agar and (4) the presence of microspores, microscopic macrospores and chlamydospores (Fig. 4). Anamnesis (age, ethnicity, housing, travel, contact with people of other nationalities, contact with animals, occupation) and clinical aspect of the lesions are essential to guide or confirm species identification.

In cases of uncertainty, strain identification can be performed by PCR using universal (panfungal) primers targeting ribosomal DNA (Ninet et al., 2003; Gräser et al., 1999a,b; Symoens et al., 2011, 2013). The most commonly used DNA sequences are those of the ITS1 and ITS2. The DNA sequence coding for the large 28S subunit of the ribosomes is also often used, but is less discriminatory. Species identification by PCR can be problematic using the public database of the National Center for Biotechnology Information (NCBI) (<http://www.nlm.nih.gov/>), which has weaknesses including identical sequences under different names, and different sequences under the same name. Specialized database is available in Mycobank (<http://www.mycobank.org/>).

It is also possible to identify dermatophytes as well as yeasts and molds directly in situ in a dermatological sample by PCR (Verrier and Monod, 2017; Verrier et al., 2012). In situ PCR techniques are particularly useful when the diagnosis of a mycosis has been made by a positive direct mycological examination or when the fungus does not grow (35% of onychomycosis cases). It is important to identify molds responsible of nail infections. Indeed, *Fusarium*, *Acremonium*, *Scytalidium* or *Aspergillus* onychomycosis are naturally insensitive to terbinafine and/or azole standard treatments (Baudraz-Rosselet et al., 2010; Lurati et al., 2011).

Dermatophyte species in culture can also be identified by MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) (Nenoff et al., 2013; De Respinis et al., 2013, 2014). This technique allows the distinction between closely related species such as *Nannizzia gypsea*, *Nannizzia fulva* and *Nannizzia incurvata*. Unfortunately, it is not possible to identify infectious agents in situ in a clinical lesion using MALDI-TOF MS.

Change in prevalence of dermatophytes

The prevalence of dermatophyte species in populations varies with geography and continuously evolved from the middle of the 19th century until nowadays (Seebacher et al., 2008; Zhan and Liu, 2017). The evolution of urban and rural populations, migration from different continents, changes in the human lifestyle with the increasing number of pets and the emphasis on healing dermatophytosis in different countries are factors that make the reported frequency of dermatophyte species varies in a given location.

Trichophyton rubrum, which was only described in 1910 (as *Epidermophyton rubrum*) is now the dominant species in the world. This dermatophyte developed during the second half of this century. *T. rubrum* has supplanted *M. audouinii*, *E. floccosum* and *T. schoenleinii*, which were the main dermatophytes in the 18th and early 20th centuries. The two former species are now uncommon. The frequency of *M. audouinii* and *E. floccosum* is now in the range of 1–2% in Western Europe (Seebacher et al., 2008; Zhan and Liu, 2017) while *T. schoenleinii* has almost completely disappeared. The prevalence of *T. tonsurans* varies greatly with geography. The species is widespread in America (33–45%) in the United States (Seebacher et al., 2008; Zhan and Liu, 2017),

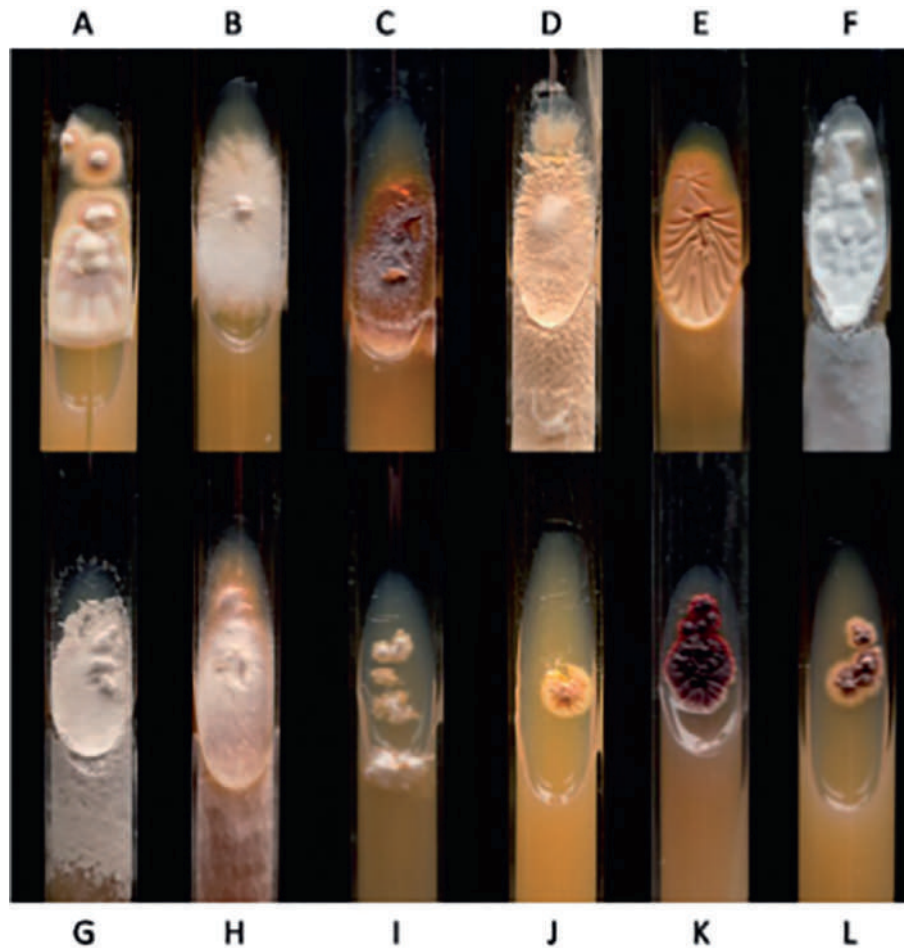


Fig. 3 Most frequently isolated dermatophytes species. (A): *T. rubrum*, (B) *T. interdigitale*, (C) *T. tonsurans*, (D) *N. gypsea*, (E) *T. benhamiae* (yellow phenotype), (F) *T. benhamiae* (white phenotype), (G) *T. mentagrophytes*, (H) *M. canis*, (I) *T. verrucosum*, (J) *T. soudanense*, (K and L) *T. violaceum*.

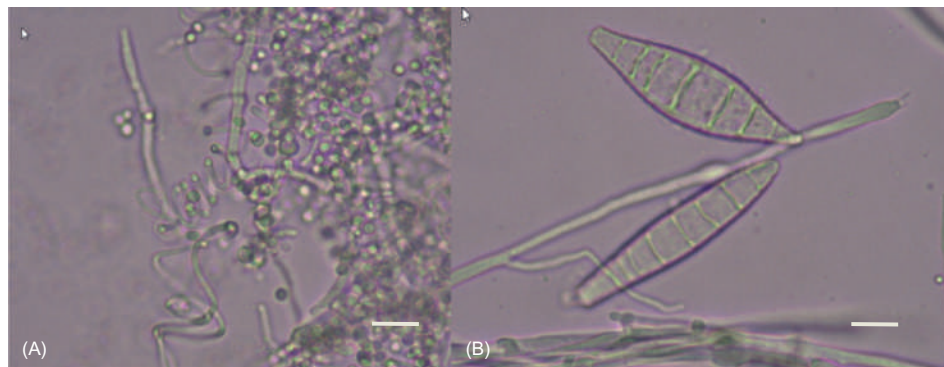


Fig. 4 Microscopic characteristics of various dermatophytes. (A) *T. mentagrophytes* microspores. (B) *M. canis* spindle-shaped, thick-walled macroconidia (bar in A and B = 10 μ m).

whereas, for example, it is infrequent in Switzerland with an incidence of about 1%. *Trichophyton violaceum* and *Trichophyton soudanense* are now frequently isolated in Europe from tinea capitis in patients of African and Mediterranean origin.

M. canis, *T. mentagrophytes*, *Trichophyton benhamiae* and *Trichophyton verrucosum* are the major zoophilic species. *M. canis* is the main zoophilic species and causal agent of tinea capitis and tinea corporis in Southern Europe (Seebacher et al., 2008; del Boz et al., 2011; Tsoumani et al., 2011; Otašević et al., 2019) like in many countries where stray cats and dogs are numerous. *T. benhamiae* is an emerging species in Europe with the increasing number of Guinea pigs as pets. The incidence of *T. verrucosum* has decreased significantly in many countries as the rural population has declined.

Dermatophyte teleomorphs

Only dermatophytes anamorphs (asexual fructifications) are isolated from clinical samples of infected patients, animals or soil. However, like *Aspergillus* spp., dermatophytes produce teleomorphs (sexual fructification), which are cleistothecia with prototunicate asci (Weitzman and Summerbell, 1995) (Fig. 5). Cleistothecia can be obtained by inoculating two isolates of different mating type (designated as either “+” or “–”) at a short distance (3 cm) at the center of a Petri plate containing an agar medium poor in substrate, for instance Sabouraud’s medium 1/10 or MAT (for mating) agar (Takashio, 1972; Kröber et al., 2017). All dermatophyte pathogenic species are heterothallic meaning that conjugation for sexual reproduction is only possible through the interaction of individual isolates of different mating type.

A bias of one particular mating type was observed in anthropophilic species where sexual reproduction has not been observed, as well as in zoophilic species. All isolates of the anthropophilic species *T. rubrum*, *T. tonsurans* and *T. interdigitale* were found of the—mating type. The latter was determined by sexual stimulation and pseudocleistothecia formation using an *Arthroderma simii* (now *Trichophyton simii*) tester strain of the opposite mating type (Stockdale, 1981). In the zoophilic species *T. benhamiae*, both mating types were isolated within a group of strains with a particular white phenotype, but only one mating type (–) was detected in a second group of strains that were characteristic by a yellow phenotype (Symoens et al., 2013).

Mating experiments and observation of fertile cleistothecia was a particularly helpful tool to delineate species of the *M. gypseum* (now *Nannizzia gypsea*) and of the *T. mentagrophytes* species complexes (Stockdale, 1961, 1963; Ajello and Cheng 1967; Takashio 1973, Demange et al., 1992; Symoens et al., 2011; Symoens et al., 2013). In this way, *T. interdigitale*, which is anthropophilic and generally isolated from tinea pedis and tinea unguium, was distinguished from *T. mentagrophytes*, which is zoophilic. Fertile cleistothecia could never be obtained from confrontations between isolates of *T. interdigitale* and tester strains of *T. mentagrophytes* (Symoens et al., 2011).

Dermatophytes with a + mating type were found to contain an *Hmg* gene coding for the mating type protein Mat1-2, while dermatophytes with a—mating type were found to contain an alpha box gene coding for the mating type protein MAT1-1 (Symoens et al., 2011, 2013). However, in other ascomycetes, the + mating type and the – mating type design strains with an alpha box gene and an *Hmg* gene, respectively. Li et al. (2010) adopted this designation when these authors characterized and compared the Mat-1 and Mat-2 locus of five dermatophyte species. During sexual development, two transcription factors, SteA and StuA, also play important functions. *T. benhamiae* mutated in one of these regulators makes only pseudocleistothecia (non-fertile cleistothecia) in mating experiments (Kröber et al., 2017).

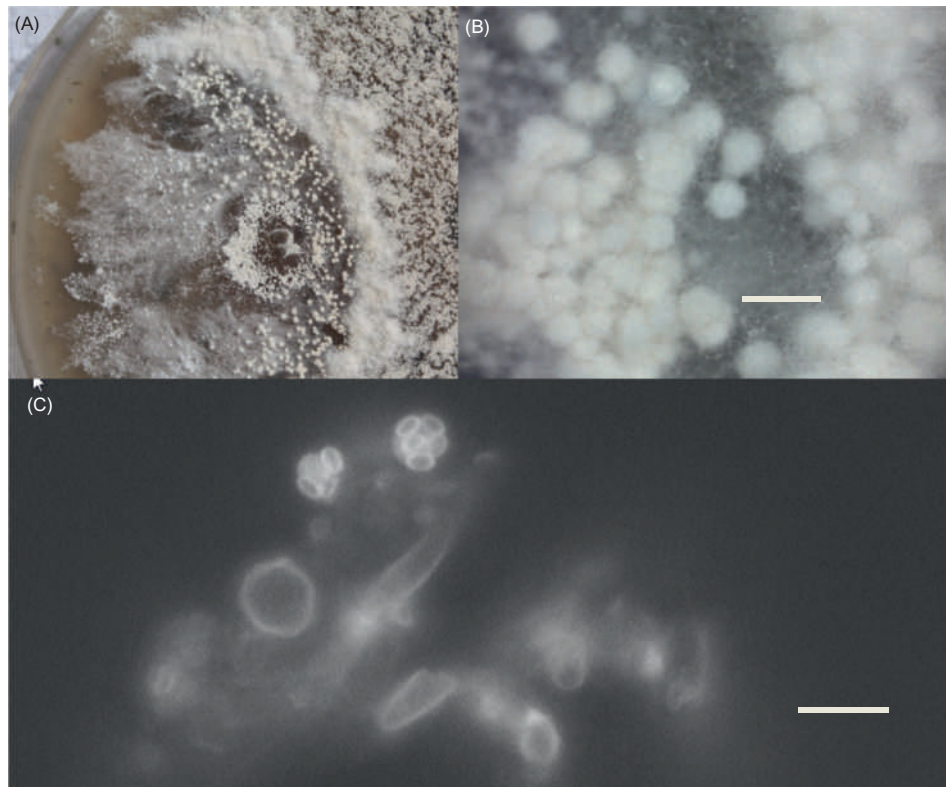


Fig. 5 *T. benhamiae* teleomorphs. (A) Mating of a + strain with a – strain of *T. benhamiae*. (B) Binocular microscope observation of cleistothecia (bar = 400 μ m). (C) Asci and ascospores from squashed cleistothecia (bar = 10 μ m).

Characteristics of the dermatophyte genomes

Pathogenic dermatophytes are closely related and their genomes are highly collinear (Martinez et al., 2012). The sizes of the genomes are between 22 and 24 Mb, which is small in comparison to the size of the genomes of saprophytic fungi such as *Aspergilli* (from 28–38 Mb) and *Neurospora crassa* (41 Mb) (de Vries et al., 2017). The number of predicted protein-coding genes varies from 7980 in *T. benhamiae* to 8915 in *M. canis* (Burmester et al., 2011; Martinez et al., 2012). Several distinguishing features characterize the genomes of the dermatophytes:

1. The presence of large gene families encoding secreted endoproteases of the subtilisin, deuterolisin and fungalisin families. Enrichment of families of secreted protease attests for the ability to better adapt to different environmental conditions such infection (parasite phase of the fungus) and hard keratin degradation (saprophyte phase of the fungus).
2. The depletion of genes coding for enzymes involved in the metabolism of sugars and the degradation of plant cell walls. For instance, there are no genes encoding glycoside hydrolases and alpha amylases.
3. The enrichment of genes encoding proteins containing one or several LysM domains known to be specific for binding chitin and N-linked oligosaccharides in fungal glycoproteins and in human skin. Protein expansion with a LysM domain could be related to the ability of the fungus to escape the immune system during infection.
4. The conservation of the genes involved in mating and meiosis in all dermatophyte species, suggesting interfertility of strains in same species in the environment.

Proteolytic activity of dermatophytes as saprophyte fungi

Pathogenic species of dermatophytes evolved from soil saprophyte fungi able to efficiently degrade hard keratin in the environment into amino acids and short peptides in the process of recycling soil nitrogen (Fig. 6). Microorganisms via membrane transporters can assimilate these products of proteolysis, but not large peptides or full-length protein (Payne and Smith, 1994). At neutral or alkaline pH in a medium containing protein as the sole source of carbon and nitrogen, dermatophytes secrete two major subtilisins (Sub3 and Sub4) and two major fungalisins (Mep3 and Mep4), which are endopeptidases (Jousson et al., 2004a,b; Sriranganadane et al., 2011). In addition, dermatophytes secrete aminopeptidases, e.g., leucine aminopeptidases (Lap1 and Lap2) and dipeptidyl peptidases (DppIV and DppV). In a protein medium at acidic pH, dermatophytes secrete an aspartic protease of the pepsin family (Pep1) as an endoprotease, as well as tripeptidyl peptidases of the sedolisin family, prolyl peptidases and carboxypeptidases of the MEROPS S10 family, as exoproteases (Sriranganadane et al., 2011). Dermatophytes secreted proteases show activities similar to those of orthologues enzymes secreted by *Aspergillus fumigatus* (Monod et al., 2005). This suggests mechanisms of extracellular proteolysis at acidic and neutral pH by dermatophytes comparable to those known in *Aspergillus* species (Byun et al., 2001; Sriranganadane et al., 2010). Endoproteases produce large free-end peptides from keratin, on which exoproteases can act to produce oligopeptides and free amino acids.

Fungal secreted proteases are incapable of digest by themselves hard keratin structures such the stratum corneum of skin, hair and nails. Proteases operate only after the reduction of cystine disulfide bridges, which are responsible for the resistant nature of hard keratin (Kunert, 1992, 2000). During the degradation of keratin, dermatophytes and filamentous fungi secrete sulfite as a reducing agent using a sulfite efflux pump (Ssu1) (Léchenne et al., 2007). Sulfite is a product of the metabolism of cysteine. In the presence of sulfite, disulfide bonds of the keratin substrate are directly cleaved into cysteine and S-sulphocysteine (Kunert, 1972, 1976, 2000; Ruffin et al., 1976) and after sulfitolysis, reduced proteins become accessible to further digestion by various *endo*- and *exo*proteases secreted by the fungi. *T. benhamiae* mutants in *Ssu1* were defective for growth on hair and nails in vitro (Grumbt et al., 2013). The same was also observed for mutants in the gene *Cdo1* encoding the cytoplasmic cysteine dioxygenase, which is involved in the intracellular conversion of cysteine into sulfite. Therefore, sulfitolysis is mandatory for the digestion of hard compact keratin by dermatophytes.

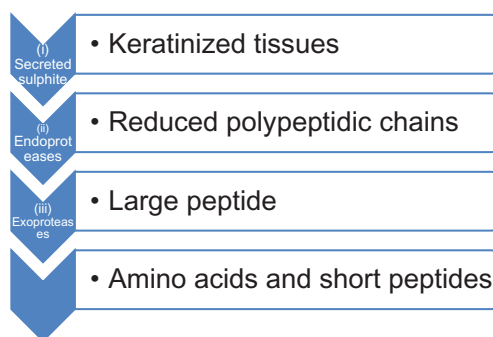


Fig. 6 The different steps of keratin degradation by dermatophytes.

Potential virulence factors of dermatophytes as parasitic fungi

Secreted proteases

Unexpectedly, most of the proteases secreted in vitro during keratin digestion appeared to be not secreted in onychomycosis and infection of guinea-pigs (Staib et al., 2010; Tran et al., 2016; Méhul et al., 2016). Therefore, one cannot consider these proteases as virulence factors by which dermatophytes penetrate keratinized tissues. During infection, dermatophytes secrete proteases other than those secreted in vitro in a protein medium. Transcriptome analysis performed with RNA from guinea pigs infected by *T. benhamiae* and proteomic analyzes of proteins extracted from infected nail beds of patients with tinea unguium revealed that a particular subtilisin (Sub6) was the major protease secreted during infection (Staib et al. 2010; Tran et al., 2016; Méhul et al., 2016, 2019). Sub6 was found to be a marker of *Trichophyton* onychomycosis by proteomics and Western blot analysis of proteins in nail samples. It is important to note that Sub6 has never been detected among the proteases secreted by *T. rubrum* and *T. benhamiae* in vitro. Additional proteins secreted by *Trichophyton* during nail infection were the closely related Sub7 and the dipeptidyl peptidase DppV. Sub6 and DppV were the major Tri r 2 and Tri r 4 dermatophyte allergens, respectively, that had previously been identified in patients with asthma (Woodfolk, et al., 1998).

Potential non-protease secreted proteins as possible virulence factors

Transcriptomics revealed in vivo overexpression of genes encoding proteins with functions other than proteolysis. ARB_01183 encoding a presumed antigenic protein of the thaumatin domain was the most highly expressed secreted protein-encoding gene in vivo (Tran et al., 2016). Thaumatin-like proteins (TLPs) are present in many eukaryotes and have been studied in plants, where they are involved in defense against fungi (Palacín et al., 2012). The role of thaumatin during infection is not known.

Two 1,3-beta-glucanotransferases encoded by ARB_07487 and ARB_05770 were also found up regulated during infection. 1,3-beta-glucanotransferases play an important role in fungal cell wall morphology and pathogenicity (Tran et al., 2016). The deletion of the gene *Gel2* encoding a 1,3-beta-glucanotransferase in *Aspergillus fumigatus* leads to a change in cell wall composition and a reduction in virulence in a mouse model of invasive aspergillosis (Mouyna et al., 2005).

Like the gene encoding Sub6, the genes encoding thaumatin and 1,3-beta-glucanotransferases were found to be highly upregulated during infection but not under in vitro conditions (Tran et al., 2016). Dermatophyte infections after a saprophytic lifestyle is achieved through extensive reprogramming of the expression of the genes encoding secreted proteins.

Proteins harboring LysM domains

A LysM (for Lysin Motif) domain is a small globular domain of approximately 40–65 amino acids with a structure composed of a pair of antiparallel beta strands separated by a pair of short alpha helices ($\beta\alpha\alpha\beta$ secondary structure) (Bateman and Bycroft, 2000; Buist et al., 2008). The LysM domains are found in proteins of bacteriophages, bacteria and eukaryotes allowing binding to peptidoglycan, chitin and carbohydrate ligands. They were first discovered as tandem repeats in the lysozyme of *Bacillus* phage $\phi 29$ (Garvey et al., 1986) and subsequently found in the peptidoglycan hydrolase of *Streptococcus faecalis* (Béliveau et al., 1991).

Two *T. rubrum* proteins of with three LysM domains (TERG_05627 and TERG_01873, respectively) were characterized after overexpression of their encoding genes in *Neurospora crassa* and in *E. coli* (Kar et al., 2019). Both proteins through their LysM domains bind to chitin, to N-linked oligosaccharides on fungal glycoproteins and to N-linked oligosaccharides in human skin glycoproteins. Therefore, the LysM dermatophyte proteins could be important in virulence by masking cell wall components of the pathogen's (chitin and/or glucans) on the surface of hyphae like in plant pathogenic fungi (de Jonge and Thomma, 2009) and helping the fungus to escape the innate immune system (Martinez et al., 2012). By binding to human skin glycoproteins, the LysM proteins could also function as adhesion proteins.

The expansion of LysM binding domains is a trademark of the dermatophyte genomes (Martinez et al., 2012). The *T. rubrum* genome contains six proteins containing two or more LysM domains (Kar et al., 2019). A role for LysM proteins in pathogenicity has also been suggested because members of gene families are a priori considered to be virulence factors in pathogenic microorganisms. The emergence of multigenic families is most often due to processes of gene duplication allowing organisms to better adapt to different environmental conditions. However, the role of LysM proteins as virulence factors remains hypothetical, as none has yet been identified during infection.

Clinical manifestations distant from dermatophyte infections: Asthma and skin dermatophytides

Dermatophyte infections may provoke secondary immune reactions such as eczematous skin reactions (dermatophytids) and severe asthma. Common dermatophytids are dyshidrotic and vesicular eczemas on the hands (palms and/or fingers) associated with tinea pedis and/or tinea unguium in adults, most caused by *T. interdigitale*, rarely by *T. rubrum* (Veien et al., 1994). Eczema on the chest, trunk and back (elsewhere on the body) has also been reported concomitantly with tinea capitis and tinea corporis caused by zoophilic and anthropophilic dermatophytes (Gianni et al., 1996; Liu et al., 2011; Cheng et al., 2011). Four diagnostic criteria were retained to conclude to a dermatophytid reaction (i) there is a proven dermatophyte infection in a body site other than the locus of the eczematous skin reaction; (ii) the eczema appeared after the dermatophyte infection and the fungus is not present in the

site of the cutaneous eruption. From this site, culture assays remain sterile and direct mycological examination is negative; (iii) the dermatophytid symptoms only resolve after eradication of the primary focus of fungal infection; (iv) the patients are sensitized to dermatophyte antigens and show a positive skin test response to fungal extracts (termed “Trichophytin”). Individual antigens involved in dermatophytids have not been identified.

Patients with *Trichophyton* asthma are also sensitized to dermatophyte antigens (Ward et al., 1989) and asthma improved after specific antifungal treatment (Platts-Mills and Woodfolk, 2009). Woodfolk et al. (1998, 2000) discovered and characterized two main allergens, Tri r2 and Tri r4, inducing both either immediate (IH) and delayed type (DTH) hypersensitivity skin test reactions in patients. These antigens are two proteases secreted during infection (see above). Tri r2 and Tri r4 were later renamed Sub6 and DppV, respectively, in the protease nomenclature of *T. rubrum* (Jousson et al., 2004b; Monod et al., 2005; Sriranganadane et al., 2011).

Immunity

Dermatophytosis is frequent and usually limited to the keratinized tissues: skin stratum corneum, nails and hairs. Anti-dermatophytes immunity knowledge has gained insight thanks to recent in vivo data and knowledge of acquired or inherited immunodeficiency predisposing to invasive dermatophytosis.

By contrast with superficial dermatophytosis limited to stratum corneum some individuals develop invasive dermatophytosis where dermatophytes invade dermis. Patients prone to develop invasive dermatophytosis have acquired immunodeficiencies including oral corticosteroids for solid organ transplantation (SOT), other immunosuppressive therapies, HIV infection, atopy, diabetes mellitus, Cushing's disease, congenital adrenal hyperplasia, hematologic malignancy, autoimmune diseases, liver disease, or trauma such as shaving (Rouzaud et al., 2015, 2018). Other patients at risk of developing invasive dermatophytosis have primary immunodeficiencies, including CARD9 deficiencies. (Lanternier et al., 2013). Defective pathways in SOT patients and CARD9 pathway should have a key role in anti-dermatophyte immunity. CARD9 deficient patients with deep dermatophytosis were evidenced to have a reduced number of IL-17 producing T cells, a reduced pro inflammatory cytokine production by dendritic cells and neutrophils recruitment to the site of infection. It was also evidenced in a recent animal model that Th1 and Th17 response were complementary to control superficial dermatophytosis (Heinen et al., 2019).

A recent publication evidenced that patients with chronic superficial dermatophytosis had an elevated IgE level, elevated Th17 and Treg cells, increased IFN- γ and IL-10 levels by comparison with controls (Rai et al., 2020). It was also shown recently in skin biopsies of patients with superficial dermatophytosis that the number of Langerhans cells were significantly decreased in the epidermis (Reis et al., 2019). Therefore, both innate and adaptative immunity have a major role in dermatophytosis control with involvement of Langerhans cells, Th17 and Th1 immunity.

Antifungal drugs against dermatophytes and antifungal resistance

The antifungals most commonly used to treat dermatophytosis are terbinafine, azole compounds, such as itraconazole and fluconazole, and griseofulvin (Hay, 2018). Terbinafine and azole compounds target the biosynthetic pathway of ergosterol (Leyden, 1998). A lack of ergosterol results in an alteration of the structure of the plasma membrane and its function (Leyden 1998; Odds et al., 2003). Griseofulvin inhibits fungal mitosis by disrupting the mitotic spindle through interacting with polymerized microtubules (Gull and Trinci, 1973). Although potentially toxic, griseofulvin remains the treatment of choice in many cases of tinea capitis (Hay, 2017, 2018) insensitive to terbinafine and azole compounds.

Increased exposure to drugs favors the generation of resistant microbes, and following this trend, dermatophytes resistant to antifungal drugs have emerged in several countries. Osborne et al. (2005, 2006) reported the two first cases of terbinafine resistance in *T. rubrum*. Since 2017, several cases of terbinafine-resistant onychomycosis, tinea pedis and tinea corporis with either *T. rubrum* or *T. interdigitale* were reported in both Europe and Asia (Yamada et al., 2017; Saunte et al., 2019). Such resistance was associated with a non-synonymous point mutation in the squalene epoxidase (SQLE) gene of *T. rubrum* and *T. interdigitale* (L393, F397, F415 and H440), the first two being predominant.

The occurrence of terbinafine-resistant *T. rubrum* mainly from nails and tinea pedis was estimated around 1.0% in Switzerland (Yamada et al., 2017). This contrasts with the prevalence of *T. mentagrophytes* isolates with a particular genotype (*T. mentagrophytes* type VIII), which are responsible for an ongoing epidemic of tinea cruris and tinea corporis in India (Nenoff et al., 2019). Depending on the studies, the prevalence of terbinafine-resistant isolates was comprised between 30% and 70% (Rudramurthy et al., 2018; Singh et al., 2018; Ebert et al., 2020). Most resistant Indian strains of *T. mentagrophytes* revealed SQLE with a point mutation identical to one of the mutations previously recorded in *T. rubrum* at positions 393, 397, 415 and 440. The amino acid substitution F397L was highly prevalent, till 91% among resistant isolates (Ebert et al., 2020). Three new substitutions, S395P, Q408L and S443P, were also recorded in the SQLE of resistant *T. mentagrophytes* isolates (Ebert et al., 2020).

One case of *T. rubrum* with reduced susceptibility to azoles has been found caused by overexpression of two genes (*MDR2* and *MDR3*) encoding a multidrug transporter of the ABC family (Monod et al., 2019). Among 39 ATP-binding cassette (ABC) transporters and 170 major facilitator superfamily (MFS) transporters identified in silico within the *T. rubrum* genome, 4 ABC transporters (TruMDR1, TruMDR2, TruMDR3 and TruMDR5) and 2 MFS transporters (TruMFS1 and TruMFS2) were able to operate

as azole efflux pumps when their encoding genes were overexpressed in *Saccharomyces cerevisiae*. TruMDR3 and TruMFS1 were able to act as an efflux pump toward fluconazole, itraconazole, ketoconazole, miconazole and voriconazole. TruMFS1 was also able to transport cycloheximide suggesting that acts as a pleiotropic drug transporter with a broad substrate spectrum. In contrast, TruMDR1 and TruMFS2 were found specific for fluconazole and voriconazole, which have a similar molecular structure. While resistance to azoles remains exceptional in *T. rubrum*, so far, about 15% of *T. mentagrophytes* type VIII isolates were less sensitive to itraconazole and voriconazole (Ebert et al., 2020). To date, we have no data on the possible azole resistance mechanisms in these isolates. The emergence of *T. mentagrophytes* type VIII strains resistant to both terbinafine and azole antifungal compounds is of concern.

The acquired dermatophyte resistances are not anymore exceptional. However, the identification of the infectious agent remains important in cases of nail and hair infections for the prescription of appropriate treatment because of the possible intrinsic resistance of the fungal infectious agent. For instance, standard treatments with terbinafine and azoles are inefficient in cases of onychomycosis with a non-dermatophyte mold, in particular *Fusarium* spp., *Acremonium* spp. and *Aspergillus* spp. (Baudraz-Rosselet et al., 2010; Lurati et al., 2011). Regarding dermatophyte species, many cases of tinea capitis with *M. audouinii* and zoophilic species such as *M. canis*, *T. benhamiae* and *T. mentagrophytes* do not respond to terbinafine and azole treatments (Baudraz-Rosselet et al., 1996; Hay, 2017, 2018). In such cases, the use of griseofulvin is required.

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Introduction

The term “anisakiasis” or “anisakiosis” refers to the accidental human infection with the anisakid nematodes belonging to the genus *Anisakis*. This genus, giving the root name to the family Anisakidae, includes the most distributed nematodes in Boreal, Austral and Tropical marine ecosystems, around the globe. They are parasites at their larval stages of several fish species worldwide, even of commercial importance. As a consequence, the zoonotic disease is acquired by humans upon the ingestion of raw, undercooked or lightly processed fishery products. In the same family, other nematodes are considered as zoonotic to humans, i.e., some species of the genera *Pseudoterranova* and *Contracaecum*; the latter regarding only those species maturing in pinniped hosts. In the case of human infection with “anisakid nematodes” the term “anisakidosis” should be instead used. Due to the high distribution of *Anisakis* spp. in the marine realm and the number of human cases so far described due to *Anisakis*, we will focus on the species so far included in the genus *Anisakis* and the zoonotic disease due to the species *A. simplex* (s.s.) and *A. pegreffii*, which are those, at the time of this chapter writing, recognized as responsible for the human disease.

Taxonomy, morphology, life cycle of *Anisakis* spp.

Life cycle

Anisakis species have complex and heteroxenous life cycle which, generally speaking, involves various marine organisms at different levels of the trophic web in the marine ecosystem (Fig. 1). The adults live in the gastric chamber of marine mammals, mainly cetaceans (definitive hosts). Fish and squids are intermediate or paratenic hosts, while planktonic or semi-planktonic crustaceans act as first intermediate hosts. Female worms release fertilized eggs which are passed in the stools of their definitive hosts into the sea and lead to free swimming unsheathed larvae in the marine environment (Fig. 2). It has been generally accepted that the larvae undergo one or two molts before being ingested by the first intermediate invertebrate host (mostly small crustaceans, krill). These intermediate hosts (i.e., *Euphausia similis*, *Euphausia pacifica*, *Thysanoessa raschii*, *Nyctiophanes couchii*) (Gregori et al., 2015) (Fig. 2) harboring the third stage larvae (L₃) are then predated by a wide variety of paratenic hosts, such as squids and fishes, where the L₃ stage does not molt. Indeed, most of *Anisakis* L₃ stages remain in the visceral cavity of the fish encapsulated outside the visceral organs, whereas in other cases, parasites can migrate to the musculature of the fish (Roepstorff et al., 1993; Karl and Levsen, 2011; Cipriani et al., 2016) (Fig. 1). To this step, the infected fish may be directly eaten by the definitive host when the L₃ stage molts to L₄ stage and then matures to the adult form of the parasite. Alternatively, the fish may be eaten by another fish: the larvae re-encapsulate in this new host. This phenomenon may occur several times, inducing a potential accumulation of *Anisakis* spp.

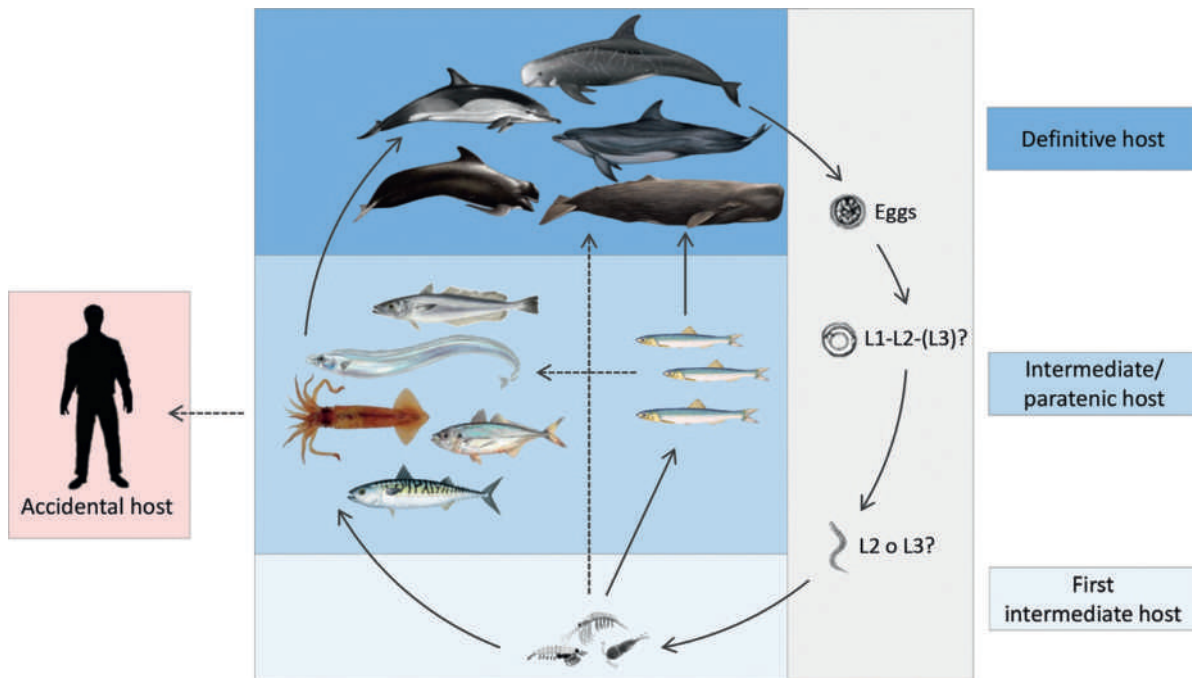


Fig. 1 General life-cycle of *Anisakis* spp.

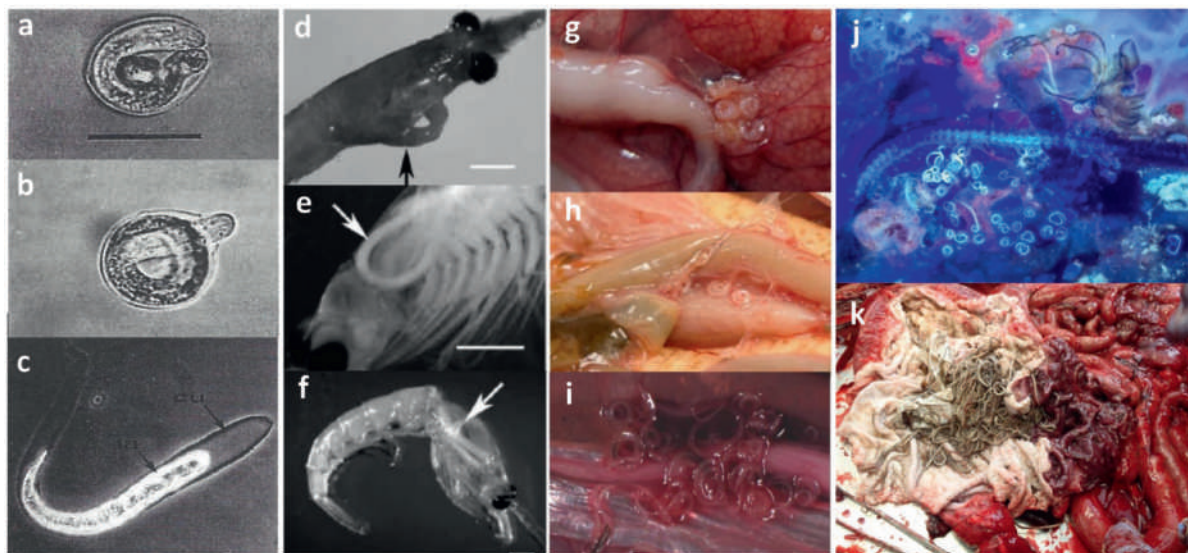


Fig. 2 Developmental stages of *Anisakis*. (A) Eggs with a L₁ stage larva; (B) Emerging 2nd stage larva from the egg; (C) Newly hatched L₂ stage larva; (D–F) 3rd stage larvae in the celomic cavity of first intermediate hosts; (G–I) 3rd stage larvae coiled outside the fish organs and free in the visceral body cavity of the fish; (J) muscle sections of a fish under the UV-Press system allowing to detect *Anisakis* larvae as bright fluorescent spots; (K) adults of *Anisakis pegreffii* in the first gastric chamber of a striped dolphin, *Stenella coeruleoalba*. (A–F) From Gregori M, Roura A, Abollo E, Gonzalez AF and Pascual S (2015) *Anisakis simplex* complex (Nematoda: Anisakidae) in zooplankton communities from temperate NE Atlantic waters. *Journal of Natural History* 49: 755–773.

larvae, in predatory fish, along the trophic web (Zuo et al., 2016; Munster et al., 2015). Despite the general pathway of the life cycle speculated by the species of the genus *Anisakis* is that reported in Fig. 1, some differences in ecological aspects regarding both host preference and geographical distribution of the so far recognized species in the genus, do exist (detailed in Mattiucci et al., 2018).

Molecular systematics and current taxonomy of *Anisakis* spp.

Molecular/genetic methodologies have revolutionized the taxonomy of the species of the genus *Anisakis* during the last 20–30 years (Mattiucci and Nascetti, 2008). The molecular/genetic markers so far available in *Anisakis* parasites, and based on the existing

literature, have aimed to: (1) disentangling sibling (cryptic) species; (2) establishing phylogenetic relationships between related taxa; (3) providing an intraspecific population analysis; (4) estimating their genetic diversity; (5) providing suitable markers for species identification at any stage of their life cycle; (6) study hybridization and/or introgression phenomena between closely related species.

Among the nuclear gene loci, the multilocus allozymes analysis allowed the discovery of all the so far genetically characterized species of *Anisakis* (see next paragraph) (reviewed in Mattiucci and Nascetti, 2008; Mattiucci et al., 2018). Later on, the direct sequencing of the ITS region of the rDNA and the RFLPs of the same gene loci, allowed their recognition by PCR-DNA methodologies (D'Amelio et al., 2000). Further, the recognition of all the species of *Anisakis*, as based on nucleotide diagnostic positions, as well as their phylogenetic relationship was inferred from the direct sequencing of the mitochondrial gene locus mtDNA *cox2* (Valentini et al., 2006). In recent years, other nuclear loci have been used in the identification of the *A. simplex* (s.l.) species complex. They are: the sequence analysis of the EF1 α -1 nDNA (Mattiucci et al., 2016); the *nas10* nDNA and the development of ARMS-PCR based on the same gene locus (Palomba et al., 2020b), as well the development of DNA microsatellite markers (SSRs) (Mladineo et al., 2017; Mattiucci et al., 2019), permitting to find additional diagnostic markers between those *Anisakis* species (Mattiucci et al., 2019; Bello et al., 2021). Several among the developed SSR loci resulted to be sex-linked in the three species of the *A. simplex* (s.l.) complex (Mattiucci et al., 2019; Bello et al., 2021).

As based on genetic/molecular systematics approach, starting from the Davey (1971) revision of the species of *Anisakis*, according to which only three species were considered as valid taxa: i.e., *A. simplex*, *A. typica* and *A. physeteris*, to date nine nominal species and two undescribed taxa are recognized in the genus *Anisakis*.

Indeed, the existence of nine species as distinct phylogenetic units was also demonstrated by various phylogenetic analyses, as inferred from nuclear and mitochondrial genes (Valentini et al., 2006; Mattiucci and Nascetti, 2008; Cavallero et al., 2011; Mattiucci et al., 2014a). According to those analyses, four distinct major clades within the genus *Anisakis* clearly exist. The clade 1 includes the three sibling species of the *A. simplex* (s.l.) complex: *A. simplex* (s.s.), *A. pegreffii* and *A. berlandi* (Fig. 3). A second clade is that formed by *A. ziphidarum* and *A. nascetti*; the two species are sister taxa. The species *A. physeteris* and its related genotype *Anisakis* sp. 2 (Mattiucci et al., 2018; Aco Alburquerque et al., 2020), *A. brevispiculata*, and *A. paggiae* (Fig. 3) are forming a third highly supported clade (Mattiucci et al., 2014a,b, 2018; Murata et al., 2011). Finally, phylogenetic inference obtained from the combined nuclear and mitochondrial sequences identified *A. typica* and its related *Anisakis* sp. 1 (Mattiucci and Nascetti, 2008; Mattiucci et al., 2014a; Mattiucci et al., 2014b; Mattiucci et al., 2018) as a separate lineage with respect to the other species (Mattiucci et al., 2018). However, the precise positioning of the *A. typica* clade is not yet clearly resolved from a phylogenetic point of view. Finally, phylogenetic hypotheses attempted for the species of the genus *Anisakis* with respect to the phylogeny so far proposed for their definitive cetacean hosts, have shown that co-divergence and host-switching events could have accompanied the evolution of these group of anisakid nematodes (Mattiucci and Nascetti, 2008) (Fig. 2).

Geographical distribution and host specificity of *Anisakis* spp.

Anisakis simplex (s.s.). This parasite species occurs in subarctic, and temperate waters of the Northern hemisphere approximately from 35°N to the Arctic Seas (Fig. 4). It has been recorded in both the Western and Eastern Atlantic and Pacific Oceans. Actually, *A. simplex* (s.s.) is a parasite, at the adult stage, of dolphin species belonging to the family Delphinidae, Monodontidae, Phocoenidae and whales in Balenopteridae. It is a parasite at the third stage of more than 50 pelagic, benthopelagic and demersal teleost fish and squid species mainly belonging to the family Ommastrephidae (Mattiucci et al., 2018). Its southern limit of distribution in the North-Eastern Atlantic waters is the Spanish-Portuguese Atlantic coast. The species occurs at a rare prevalence of infection in some fish species from the Morocco-Mauritania Atlantic coast. The southern limit of *A. simplex* (s.s.) distribution in the Pacific Ocean would be the coast of Japan, while in the eastern part, along the North American coast (Mattiucci et al., 2018), up to California waters (Baldwin et al., 2011). The species has been identified, at the larval stage, in the Mediterranean Sea, from the Alboran Sea, which is a basin considered as part of the Atlantic oceanographic waters rather than the Mediterranean Sea (Tintore et al., 1988; Mattiucci et al., 2014a,b). Spot occurrence of *A. simplex* (s.s.) in the eastern part of the Mediterranean Sea has been also reported (Blazekovic et al., 2015; Mladineo et al., 2017) as likely related to the large migratory route of those intermediate and definitive host species.

Anisakis pegreffii Campana-Rouget and Biocca (1955). Previously indicated as *A. simplex* A (see: Nascetti et al., 1986), *A. pegreffii* is a parasite, at the adult stage, in the oceanic dolphins belonging to the families Delphinidae, porpoises in Phocoenidae and whales in Neobalenidae. While, at its third larval stage, it occurs in pelagic and benthopelagic fish species (Mattiucci et al., 2018; Cipriani et al., 2021). *A. pegreffii* is the dominant species of *Anisakis* in the Mediterranean Sea (reviewed in Mattiucci et al., 2018). In the Atlantic waters, the northerly limit of its geographical range is represented by the Iberian coast, from where the species has been frequently identified in sympatry with *A. simplex* (s.s.) in intermediate/paratenic and definitive hosts (reviewed in Mattiucci et al., 2018). *A. pegreffii* is also widespread in the Austral Region, between 30°S and 60°S where the species was identified, at both adult and larval stages (Mattiucci et al., 2018, 2019; Irigoitia et al., 2018, 2021) (Fig. 4).

Anisakis berlandi (see Mattiucci et al., 2014a) previously indicated as *A. simplex* C in Mattiucci et al. (1997) is a parasite with a geographical range including mainly the Austral Region: Chilean Pacific Ocean waters, the South Shetland Islands, New Zealand waters and the South African Atlantic coast (Mattiucci and Nascetti, 2008; Klimpel et al., 2010; Mattiucci et al., 2018). It is a parasite, at the adult stage, mainly in cetaceans species belonging to the families Delphinidae, while its L₃ stage infects fish species from Austral waters from off New Zealand (Mattiucci et al., 2014a, 2019; Bello et al., 2020), South African, Southern Shetland Islands

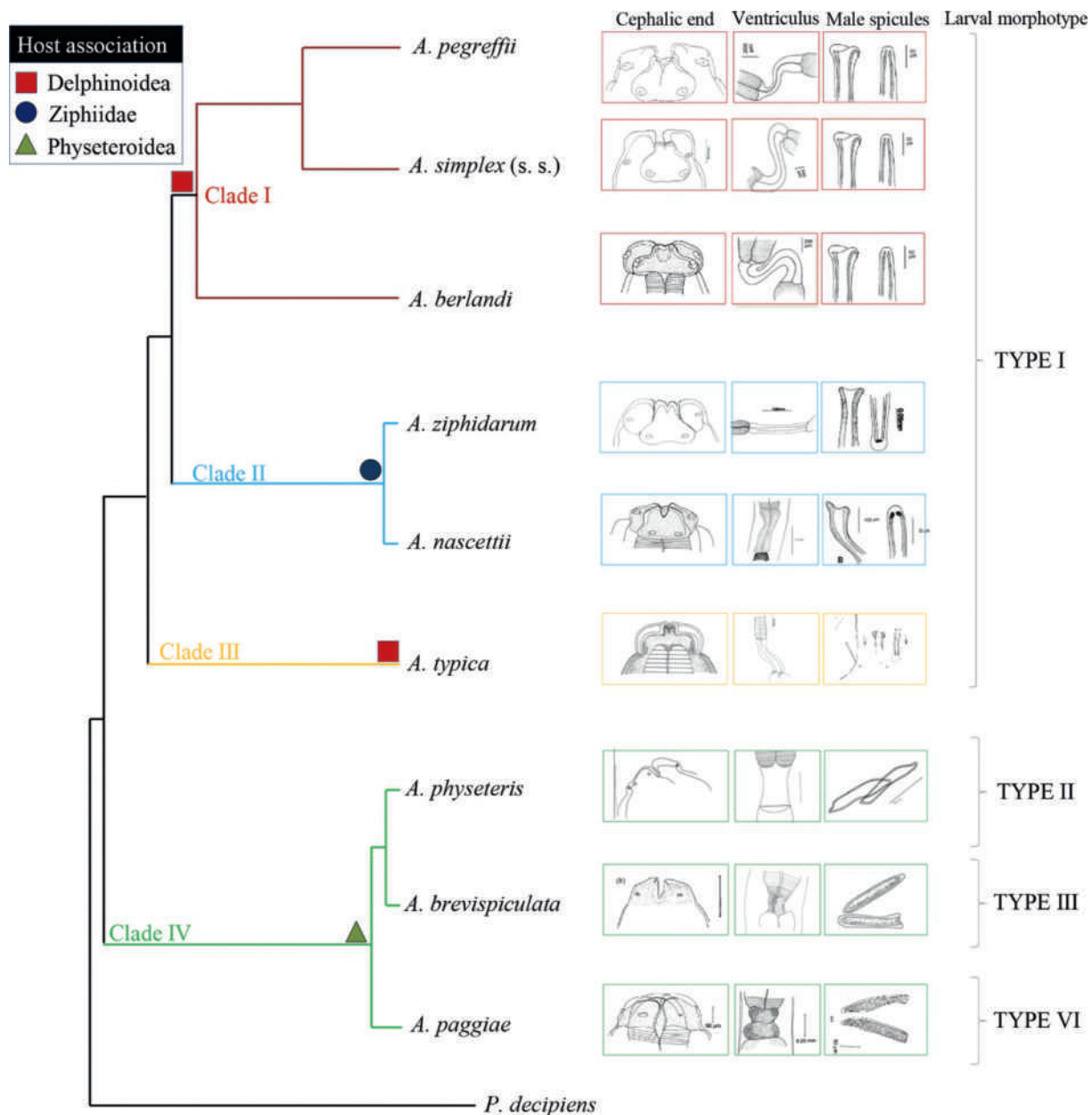


Fig. 3 Phylogenetic inference (BI) of the *Anisakis* spp. Definitive hosts (cetaceans)—parasite association is also shown at the nodes of major clades. Morphological diagnostic features among the genetically characterized species of *Anisakis*, mapped into the major clades of the phylogenetic tree are reported.

(Klimpel et al., 2010), and the Southern Chilean coasts (Fig. 4). A benthopelagic life cycle of these parasites has been supposed (Irigoitia et al., 2021).

Anisakis ziphidarum Paggi et al. (1998). It is a parasite species at the adult stage of the beaked whales *Mesoplodon layardii*, *M. mirus*, *M. densirostris* and *M. grayi* from the South Atlantic waters (off South Africa coast), from *M. europaeus* of Caribbean sea (Colon-Llavina et al., 2009; Cavallero et al., 2011), from *M. bowdoini* of New Zealand waters (Mattiucci et al., 2009), *Ziphius cavirostris* from the South Atlantic Ocean (off the South African coast), from Chilean waters, and occasionally reported in *Kogia sima* from Philippine waters (Quiazon et al., 2013). Its geographical range is mainly related to the distribution of those definitive hosts (Fig. 4). It is rarely occurring, at the larval stage, in fish species (Mattiucci and Nascetti, 2008; Di Azevedo et al., 2016; Quiazon et al., 2011; Mattiucci et al., 2018).

Anisakis nascettii Mattiucci et al. (2009). It is a parasite species of the beaked whales, sympatrically and syntopically occurring with *A. ziphidarum*. Its larval stages were found in the deep squid species *Moroteuthis ingens* (Mattiucci et al., 2009). This appears to support the hypothesis that this species, such as the sister taxa *A. ziphidarum*, involves mainly squids rather than fish, in the life-cycle.

Anisakis physeteris Baylis (1920). This parasite has been reported, at the adult stage, from the sperm whale, *Physeter macrocephalus* from the Mediterranean Sea (Mattiucci et al., 1986, 2001; Blazekevic et al., 2015), and the Caribbean Sea (Colon-Llavina et al.,



Fig. 4 World map showing the current range of distribution of *Anisakis* spp.

2009; Cavallero et al., 2011). The infection has been also recorded in the pygmy sperm whale, *Kogia* spp., from the Caribbean Sea, Gulf of Mexico (Cavallero et al., 2011) and in the Mediterranean Sea (Santoro et al., 2018). Its Type II larvae have been rarely occurring in pelagic and demersal fish species (Mattiucci et al., 2018); while squid species belonging to the family Histioteuthidae from deep waters of the Mediterranean Sea have been found infected by larvae of *A. physeteris* (Palomba et al., 2021).

Anisakis brevispiculata Dollfus (1966). The species, previously synonymized with *A. physeteris* by Davey (1971), was recognized as valid species by molecular systematics (Mattiucci et al., 2001). Its adult stage occurs in the pigmy sperm whale, *Kogia breviceps*; its L₃ stage Type III (sensu Shiraki, 1974) were identified in the stomach lumen of the swordfish *Xiphias gladius* (Mattiucci et al., 2011, 2014b), in the fish *Beryx splendens* from Japanese waters (Murata et al., 2011) in fish from Taiwan (Chen and Shih, 2015) and Japanese waters (Quiazon et al., 2011).

Anisakis paggiae Mattiucci et al. (2005). It is a parasite species of pygmy sperm whales, *Kogia* spp. often found in sympatry and syntopy with *A. brevispiculata*. Its Type IV larvae (sensu Shiraki, 1974) have been identified in fish from Atlantic waters (Fig. 4), i.e., *Merluccius merluccius* and *Xiphias gladius* (Mattiucci et al., 2014a,b). The finding of its Type IV larvae in deep-sea fish seems to support an oceanic deep-water life cycle for *A. paggiae* in the North-East Atlantic Ocean (Klimpel et al., 2011).

Anisakis typica Diesing (1860). This species has a geographical range extending from 35° to 40° N to 36° S in warmer temperate and tropical waters (Fig. 4). In these areas, it was found as an adult in oceanic dolphins (Mattiucci and Nascetti, 2008; Colon-Llavina et al., 2009; Íñiguez et al., 2009, 2010; Kleinertz et al., 2014) and as larva in several fish species. It was also found in fish captured along the North African coast of the Mediterranean Sea (Farjallah et al., 2008), in central Portuguese waters of the NE Atlantic Ocean (Marques et al., 2006; Hermida et al., 2012), from the Atlantic Ocean off Madeira (Mattiucci et al., 2002; Hermida et al., 2012), and Morocco-Mauritania Atlantic coast (Farjallah et al., 2008). The species is reported also from the Central Pacific Ocean (Kuhn et al., 2013; Palm and Bray, 2014), the Red Sea (Kleinertz et al., 2014) and the Persian Gulf (Shamsi et al., 2017), East China Sea and Taiwan Sea (Zhu et al., 2007; Kong et al., 2015; Umehara et al., 2010; Quiazon et al., 2011; Chou et al., 2011; Chen and Shih, 2015), Yellow Sea (Du et al., 2010), Indonesia (Palm et al., 2008; Anshary et al., 2014) and from Papua New Guinea (Koinari et al., 2013). It seems that this species mainly involves pelagic fish hosts in its life-cycle.

Anisakis sp. 1. A gene pool, provisionally indicated as *Anisakis* sp. 1, genetically closely related to *A. typica*, was genetically detected by both allozyme and mtDNA analysis, at the larval stage, as a parasite of the fish from off the Malaysian coast (Mattiucci and Nascetti, 2008; Mattiucci et al., 2018). Likely, the same taxon was found by Palm et al. (2008), Koinari et al. (2013), Anshary et al. (2014), Quiazon et al. (2020), from Indonesia, Papua New Guinea and Philippines waters, respectively. The formal description of this provisional taxon, as a possible sibling species of *A. typica* was based on both genetic and morphological features, has not yet been performed.

Anisakis sp. 2 (see Mattiucci et al., 2014b). It seems to represent a further taxon, genetically closely related to the species *A. physeteris*. Adult stages of this taxon have been so far found in *Physeter macrocephalus* and swordfish from central Atlantic waters (Mattiucci and Nascetti, 2008; Garcia et al., 2011), and from fish from Central Pacific waters (Aco Alburquerque et al., 2020). Further genetic/molecular analyses are needed to clarify the taxonomic status of this genotype.

Morphological features of *Anisakis* species

The existence of distinct clades in the phylogeny of the species of the genus *Anisakis* seems to be also supported by some differential morphological features detected in the species clustering in those clades (Fig. 3). Similar morphological characters at the adult stage are quite maintained by the *Anisakis* species included in the same Clade, such as in the ratio between spicules length, as well as spicule shape, ventriculus length (see Mattiucci et al., 2018) (Fig. 3). Indeed, the four major clusters defined by the phylogenetic analysis correspond and can be also morphologically characterized with distinctive morphological traits in adult worms (for details, Mattiucci et al., 2018).

As to the morphological traits of third stage larvae (L_3) of the genetically characterized species of *Anisakis*, four different morphotypes, i.e., Type I, Type II (Berland, 1961), Type III and Type IV (Shiraki, 1974), have been found; however molecular/genetic markers are necessary for their specific identification. The L_3 indicated as a Type I morphology (sensu Berland, 1961) are shared by species of three species of the *A. simplex* (s.l.) complex, *A. ziphidarum*, *A. nascettii* and *A. typica* (Fig. 3). While the morphotypes indicated as Type II, Type III and Type IV morphology (sensu Berland, 1961; Shiraki, 1974) are corresponding to *A. physeteris*, *A. brevispiculata*, *A. paggiae*, respectively (Murata et al., 2011); these last morphotypes would represent an ancestral character of this group of species (Mattiucci et al., 2018).

Zoonotic role of third-stage larvae of *Anisakis* spp.

The L_3 , known as Type I (sensu Berland, 1961) of the two species *A. simplex* (s.s.) and *A. pegreffii*, are those so far recognized having a zoonotic role to humans. These larval species are most frequently found in the edible parts of commercially important fish and squid species. The musculature as site of infection in the fish can be reached by the L_3 both as an *intra vitam* (for a review, Mattiucci et al., 2018; Levsen et al., 2018) and post mortem event; the last phenomenon has been found to be related to the temperature (Cipriani et al., 2016).

In any case, the ventral flesh (belly flap) of the fish with respect to the dorsal one has been frequently reported as the most infected part of the fish species (Mattiucci et al., 2018) (Fig. 2). As a consequence, those *Anisakis* species are also the species with the highest risk to infect humans. Indeed, live larvae of those two species infecting the fillets of fish and the edible parts of squid to be consumed raw, undercooked, or in various traditional dishes, may cause anisakiasis. Several fish dishes are considered at high risk for consumers to contract anisakiasis. They include, among the others, Spanish boquerones, Italian marinated anchovies, the Scandinavian gravlax, South American ceviche, dutch salted and marinated herring, Japanese sushi and sashimi.

As for the *Anisakis* species included in Clade 2, i.e., *A. ziphidarum* and *A. nascettii* are parasites only rarely found in commercially important fish species and so far the larvae were found on the viscera of the fish rather than the flesh. Further, a higher level of infection was found in deep-sea squid species which, however, are usually not used as “sea-food.” Therefore, it seems that their role in the transmission of the infection to humans is neglectable.

Finally, the larvae of Type I of *A. typica* are only very rarely reported to occur in the fish muscle. Palm et al. (2008) reported the occurrence of one larva of *A. typica* penetrating the muscle of a single fish specimen. Anshary et al. (2014) reported a positive seroprevalence for anisakiasis in humans in Indonesia; however, the etiological agent possibly responsible for that positive serodiagnosis was not identified. No current data exist as regards the occurrence of human anisakiasis from those geographic areas, where *A. typica* has been generally found infecting natural hosts (Fig. 5).

Similarly, no human cases related to the larvae of Type II, Type III and Type IV i.e., *A. physeteris*, *A. brevispiculata* and *A. paggiae* have been so far reported. These species have never been found, to infect the muscle of fish and squids, at least those of commercial importance. Thus, it seems that the risk of infection to humans due to those species could be low. However, Romero et al. (2013) carried out experimental infection of Wistar rats with *Anisakis* larvae, which, according to the authors, were belonging to the species *A. physeteris* and *A. paggiae*. They found, after the necropsy, very few larvae attached to the gastrointestinal wall or penetrating it, thus suggesting that those two species would be able to provoke “invasive anisakiasis” even in humans.

The human disease

Humans become accidental hosts of the L_3 of the parasite species *A. pegreffii* and *A. simplex* (s.s.) by eating parasitized raw or undercooked fish containing viable L_3 of those parasite species in their muscles. These larvae do not mature in humans.

Within few hours of being ingested by humans, L_3 *Anisakis*, alive, penetrates the mucosal layers of both gastric or the intestinal tract, causing direct tissue damage that may lead to the zoonotic disease known as “anisakiasis” or “anisakiosis.” This acute gastrointestinal form of *Anisakis* infection can be transient, with the larval worm also surviving, alive, for some days found trying to penetrate in the submucosa of stomach and intestine (Mattiucci pers. obs.). Clinical symptoms of anisakiasis are not pathognomonic; thus, the acute form could be misdiagnosed. In this case, the infection could become chronic by inducing the formation of



Fig. 5 World map of human anisakiasis, based on the 1960–2020 published literature.

granuloma by the larva. The symptoms could range from nausea to vomiting, from mild to severe abdominal pain, and intestinal obstruction, even mimicking other common gastrointestinal diseases, such as acute appendicitis, peptic ulcer. As a consequence, the anamnestic analysis of the symptomatic patient is mandatory to verify the ingestion of a putative infected raw or improperly treated fish during the previous days before the occurrence of the symptoms. Early removal and identification of the worm seems to be the best preventive measure to avoid the chronic infection evolving in eosinophilic granulomatosis.

Epidemiology of human anisakiasis

Starting with the first case reported from the Netherlands by Van Thiel et al. (1960) and by Smith and Wootten (1978, and ref. therein), human anisakiasis has become increasingly more relevant as human health risk, in particular in countries or regions where the consumption of raw or only lightly processed fish and squid is frequent and/or has become increasingly popular. Consequently, cases of the disease are increasingly reported from Asia (mainly Japan, Korea, China, Taiwan), representing more than 50% of the cases, while the remaining are from European countries, especially Italy, Spain, France and Croatia. While fewer cases are from Germany, Netherlands, United Kingdom and Norway despite comparatively high per capita fish consumption rates in these countries, as well (reviewed in Chai et al., 2005; Audicana and Kennedy, 2008; Bao et al., 2017; Mattiucci et al., 2018; Daschner et al., 2021) (Fig. 5). A number of factors are responsible for the increase of the anisakiasis cases: (i) increased awareness of the disease among physicians, clinicians and biologists; (ii) the improved methods for the detection of the parasites by endoscopy, gastric and intestinal tract, which have facilitated early diagnosis and removal of *Anisakis* spp. larvae; (iii) the molecular identification of the etiological agent; (iv) increased seafood consumption leading to higher per capita fish demand, in parallel with the increased popularity of raw fish.

Most of the human reports (72.0%) describe the occurrence of *Anisakis* spp. larvae associated with gastric anisakiasis (GA), while 26% were invasive and not invasive intestinal cases of anisakiasis (IA), with only 2% being extra-gastrointestinal (Reviewed in Mattiucci et al., 2018).

Actually, the species *A. pegreffii* was reported to cause gastric, intestinal and gastro-allergic anisakiasis (GAA); it was identified in cases of “invasive” anisakiasis, after observing viable larvae during endoscopy (D’Amelio et al., 1999; Umehara et al., 2007; Mattiucci et al., 2013, 2017b; Arai et al., 2014; Lim et al., 2015), colonoscopy (Mattiucci et al., 2017b), or in surgically removed eosinophilic granulomas (Mattiucci et al., 2011, 2017b; Mladineo et al., 2016). Those cases, molecularly identified as caused by *A. pegreffii*, were mostly from Italy (D’Amelio et al., 1999; Mattiucci et al., 2011, 2013), Croatia (Mladineo et al., 2016), from South Korea (Lim et al., 2015) and, more rarely, from Japan (Umehara et al., 2007). These findings are in accordance with the geographic distribution of the parasite species and its infection in those fishes collected within the parasite’s range of distribution.

Anisakis simplex (s.s.) has been identified (by molecular methods) to cause “invasive” gastric and intestinal anisakiasis in Japan (Umeshara et al., 2007; Auer et al., 2007; Arai et al., 2014). But in some European countries, such as Spain, despite widely reported cases of “allergic anisakiasis,” the etiological agent associated with those cases is not being identified at species level by molecular identification (Audicana and Kennedy, 2008 and ref. therein) (Daschner et al., 2021).

It should be underlined that the notification of anisakiasis in humans is not yet mandatory so that, in general, the disease is likely still underreported and misidentified all over the world, especially from those areas where the zoonotic species are widespread (Fig. 5).

Immune response to *Anisakis*

During infection with *Anisakis simplex* (s.l.) larvae, the pathological changes, occurring within the gastrointestinal tract, are the combined result of the direct action of the larva during tissue invasion and the complex interaction between the host immune system and the substances released by, or contained within, the parasite (Audicana and Kennedy, 2008). Indeed, accidentally ingested L₃ of *Anisakis* have to penetrate the gastrointestinal wall, through degradation of the mucosa and submucosa, causing tissue damage and inflammation.

As with other helminth infections, immune responses against internal and cuticular antigens (CE) and excreted/secreted products (ESPs) of *A. simplex* (s.l.) are characterized by an early innate immune response, the first line of defense, rapidly activated, followed by an adaptive immune response, which develops more slowly and provides a tailored response directed against specific antigens (Langrish et al., 2004; Nieuwenhuizen et al., 2013). Innate response against *Anisakis simplex* (s.l.) may involve tissue macrophages, neutrophils, naïve dendritic cells, basophils eosinophils attracted from the chemotactic molecules and cytokines secreted by epithelial cells (Fig. 6).

In the adaptive immune response, through contact with the antigen, the dendritic cells become activated (ADC) and allowing them to act as antigen-presenting cells (APC) after the migration to the adjacent lymph nodes. The recognition of parasitic pathogen-associated molecular patterns (PAMPs) is known to be mediated by certain pattern recognition receptors (PRRs) that are expressed on the APC of the host, so they process and present the antigens to lymphocytes T naïve cells. Napolitano et al. (2018) reported that *Anisakis pegreffii* larvae and their ESPs can inhibit *in vitro* generated DCs, preventing their differentiation and maturation and inducing their apoptosis with a probable immune escape effect.

T naïve cells induce effector cell generation consisting of Th1, Th2, Th17 and T regulatory cells (Treg). In the blood of *Anisakis* spp.-sensitized patients, both Th1 and Th2 cytokines have been recorded (Hrabar et al., 2019). The helminth-induced host immune

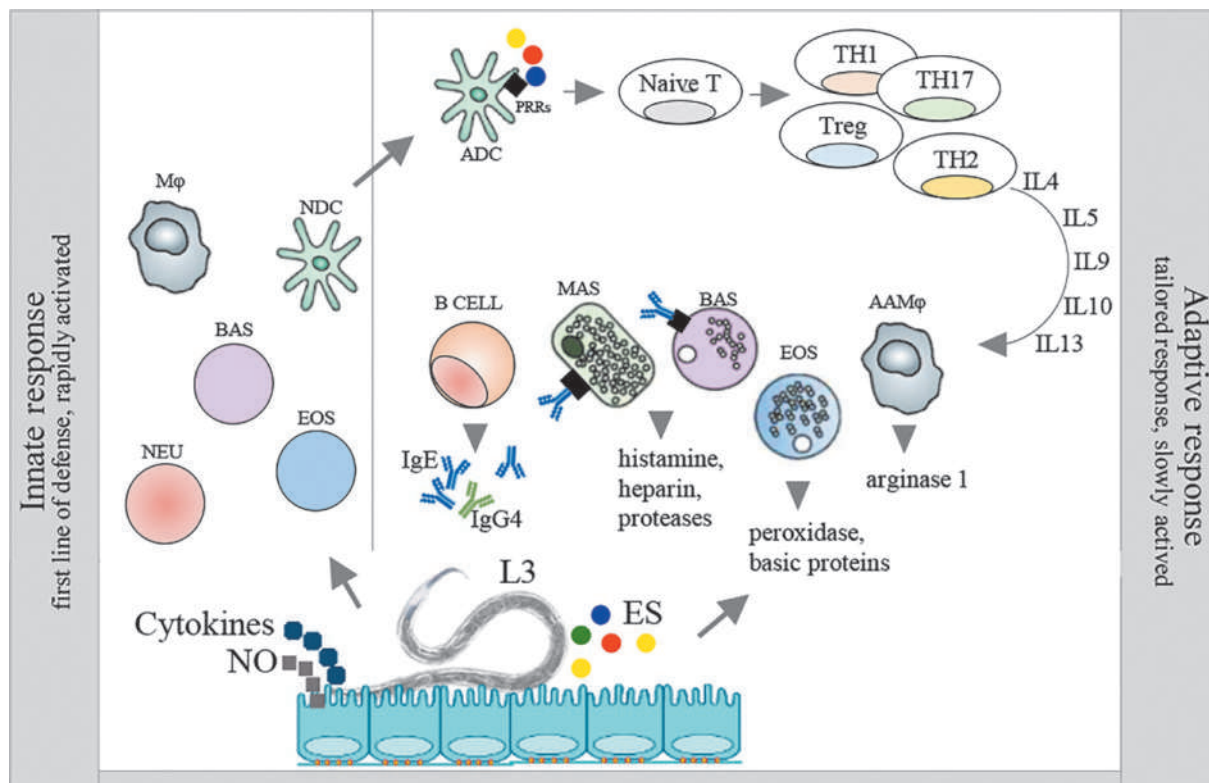


Fig. 6 Functional scheme of host defense response against zoonotic species of *Anisakis*. Nitric oxide (NO), excreted/secreted products (ES), macrophages (M^φ), neutrophils (NEU), naïve dendritic cells (NDC), activated dendritic cells (ADC), basophils (BAS), eosinophils (EOS), alternatively activated macrophages (AAM^φ).

response is focused on the protection of the host organism and it is mediated basically by Th2 cells. *A. simplex* induces either a Th1 or a Th2-type immune response, and as this parasite cannot continue its life cycle in humans, it is not possible to determine which type of response could favor the resolution of infection (Nieuwenhuizen, 2016). The alternatively activated macrophages (AAMs), induced by Th2 cytokines such as IL4 and IL-13, are characterized by the production of several enzymes such as arginase-1, which is involved in collagen deposition (Kreider et al., 2007). The activation of AAMs can inhibit the proliferation of other cells like Th1, Th2, and Th17 cells. The Th2 response includes IL-4, IL-5, IL-9, IL-10 and IL-13 secretion, the production of IgG4 and IgE by B cells, as well as the activation of effector cells such as eosinophils, mast cells, and basophils. The Th2 cytokines driving these responses are the same as those that drive allergic diseases and are responsible for the symptoms of Gastro-Allergic Anisakiasis, such as vomiting, itching, angioedema, urticaria and anaphylaxis, as well as *Anisakis* allergy, which can have symptoms such as asthma, rhino-conjunctivitis, urticaria and atopic dermatitis, primarily driven by the mediators released from mast cells. So, after a primary tissue damage (within the first week) marked by edema, massive eosinophil and neutrophil infiltration but also histiocytes or epithelioid histiocytes, lymphocytes, monocytes and plasma cells, of an acute infection, 2 weeks after the infection, a large number of fibroblasts are recruited and deposit connective tissue to form a granuloma and formation of foreign body multinucleated giant cells occurs. Larval remains are broken down and unrecognizable. The generation of T and B cells memory during primary infection sensitizes the host, who may experience allergic reactions upon subsequent exposure to *Anisakis* (Nieuwenhuizen, 2016; Nieuwenhuizen et al., 2013; Caldara et al., 2014).

Molecules involved in the pathogenesis of anisakiasis

The combined result of the direct invasive capacity of the *A. simplex* (s.s.) and *A. pegreffii* larvae and their ESPs and the interaction with the host's immune response during tissue invasion, causes the pathological findings associated with the infection. Although it is likely that the pathogenic effect of the two species is similar, the molecular mechanisms which underlie the human host-parasite species interactions, have not yet been fully elucidated. The "omic" approaches (i.e., transcriptomics and proteomics) of the species of *A. simplex* (s.l.) complex (Baird et al., 2016; Cavallero et al., 2018, 2020; Palomba et al., 2019, 2020a; Łopieńska-Biernat et al., 2020; Stryński et al., 2019; Kochanowski et al., 2020; Marzano et al., 2020; Robertson et al., 2020) has improved the knowledge about some proteins and their transcripts likely having a putative role in the parasite's invasion, infection and allergy.

ESPs are responsible for a multitude of functions during the parasite's infection, such as penetration of host tissues and evasion of host immune responses and, at the same time, are known to elicit immune responses. Therefore, ES proteins are hypothesized to be the major contributors in the clinical manifestation of the disease in humans. Among them, there is ES *Ani s 4* allergen, a cysteine-type endopeptidase with inhibitor activity, present both in excretory glands and below the cuticle. It has been shown that *Ani s 4*, is able to cross the epithelial barrier. These findings could explain the increased intestinal permeability observed in *Anisakis*-IgE sensitized patients (Polimeno et al., 2010; Carballeda-Sangiao et al., 2020).

Proteolytic enzymes, such as peptidases, would be responsible for *Anisakis* pathogenicity likely due to their role in biological pathways linked to the fundamental host-pathogen invasion mechanism of the host tissues. Among the peptidases, the metalloendopeptidase belonging to the Astacin peptidase family and carboxypeptidase were recently identified among the upregulated transcripts in the pharynx of *A. simplex* (s.s.) and *A. pegreffii* (Cavallero et al., 2018). Moreover, high transcripts of metalloendopeptidase were found to be higher in *A. pegreffii* L₃ found in the naturally infected fish musculature compared to the larvae infecting the viscera, enforcing the role of this enzyme as a significant player in host tissues invasion (Palomba et al., 2020a). High transcripts of the gene coding for the Kunitz-type trypsin inhibitor protein (Palomba et al., 2020a) would aid *A. pegreffii* to counteract the host proteolytic enzyme and coagulation factors, as well as high transcripts of hemoglobin. This last protein has been suggested to aid the parasite in maintaining a locally anaerobic environment, while their ability to break down nitric oxide (NO) and hydrogen peroxide produced by innate immune cells which would also help the parasite's survival (Palomba et al., 2020a). The *Anisakis* glycoprotein, GP, which was found highly expressed, would be able to interact with both innate and adaptive branches of the immune system in intermediate and human hosts (Palomba et al., 2020a). Indeed, glycoprotein molecules are involved in the regulation of the IgM response of the intermediate/paratenic fish host (Bahlool et al., 2013).

In order to evaluate molecular markers related to stress response, oxidative stress, inflammation and apoptosis, it was observed that fibroblast cell line HS-68 cells exposed to ESPs and CE products of *A. pegreffii* products led to increased production of reactive oxygen species (ROS) (Messina et al., 2016). In addition, the elevated induction of fibroblasts treated with those products, suggests a significantly negative effect on the human host DNA (Messina et al., 2016). It was also found that *A. simplex* induces an early and reversible alteration of integrity and permeability of epithelial cell monolayer and that an underlying mechanism of this effect would involve the oxidative stress and disruption of the proteins maintaining the barrier function (Carballeda-Sangiao et al., 2020).

It has been also observed that the exposure to *A. pegreffii* CE and ES impaired DC viability, their differentiation and maturation, modulating the ERK1, 2 signaling. These retard DCs at an immature stage, reducing their ability as APCs to trigger an efficacious Th1 response. The interaction between *A. pegreffii* and DC precursors seems to be relevant for inducing a "crippled" immune response, thus suggesting the DC impairment induced by *A. pegreffii* as a possible evasion mechanism of the host immune system, evolved by this parasite (Napoleatano et al., 2018).

It has been also supposed that *Anisakis* CE is able to increase cell proliferation, decreasing apoptosis and inducing changes in the expression of serum cancer-related miRNA in rats. This would envisage a tumorigenic potential for *Anisakis* (Corcuera et al., 2018).

Some of those ESPs of *A. simplex* (s.s.) and *A. pegreffii* are antigenic in the human host immune response, being recognized by specific IgE, IgG and IgG4 in humans and have been so far isolated and characterized (Moneo et al., 2000; Shimakura et al., 2004;

Table 1 Characterized antigens/allergens of *Anisakis* sp. larvae.

A. simplex (s.l.)/A. pegreffii allergen	Function	MW (kDa)	Localization	References
Ani s 1/A. peg 1	Kunitz-type Trypsin inhibitor	24	ES	Moneo et al. (2000) and Palomba et al. (2019)
Ani s 2	Paramyosin	97	Somatic	Perez-Perez et al. (2000)
Ani s 3	Tropomyosin	41	Somatic	Asturias et al. (2000)
Ani s 4	Cystatin	9	ES	Rodríguez-Mahillo et al. (2007)
Ani s 5	SXP/RAL protein	15	ES	Kobayashi et al. (2007)
Ani s 6	Serpin	7	ES	Kobayashi et al. (2007)
Ani s 7/A. peg 7	Glycoprotein	139	ES	Anadon et al. (2009) and Palomba et al. (2019)
Ani s 8	SXP/RAL protein	15	ES	Kobayashi et al. (2007)
Ani s 9	SXP/RAL protein	14	ES	Rodríguez-Perez et al. (2008)
Ani s 10	—	22	Somatic?	Caballero et al. (2011)
Ani s 11	—	55	Somatic?	Kobayashi et al. (2011)
Ani s 11 like	—	?	Somatic?	Kobayashi et al. (2011)
Ani s 12	—	—	—	Kobayashi et al. (2011)
Ani s 13 A. peg 7	Hemoglobin	37	ES	Gonzalez-Fernandez et al. (2015) and Palomba et al. (2019)
Ani s 14	—	27	—	Kobayashi et al. (2015)

Rodríguez-Perez et al., 2008; Caballero et al., 2008; Kobayashi et al., 2011; Daschner et al., 2012) (Table 1). Among them, *A. pegreffii* shows the antigens *A. peg 1*, *A. peg 7* and *A. peg 13*, which are recognized by IgE antibody response in cases of GA or IA (Gonzalez-Fernandez et al., 2015; Mattiucci et al., 2017a; Palomba et al., 2019; de Las Vecillas et al., 2020). Furthermore, parasite' proteins (Table 1) such as *Anis 2* and *Anis 3* are those mainly reported as associated to *Anisakis*-allergic manifestations, such as, for instance, chronic urticaria (CU+) being considered as panallergens (Mattiucci et al., 2017a).

Some *Anisakis* proteins are present in the Allergome database; they have been registered as *Anisakis* allergenic proteins by WHO/IUIS.

Clinical manifestation of anisakiasis

Gastric anisakiasis (GA) and intestinal anisakiasis (IA)

Clinical symptoms of GA as due to *A. simplex* (s.s.) and *A. pegreffii* are similar: epigastric pain, nausea, vomiting, abdominal fullness or distension, anorexia and chest pain (Mattiucci et al., 2013; Guardone et al., 2018). In most of the GA cases, the larvae were mainly located in the lumen of the stomach penetrating the submucosal layer of the gastric wall (Fig. 7). As for the localization in the gastric

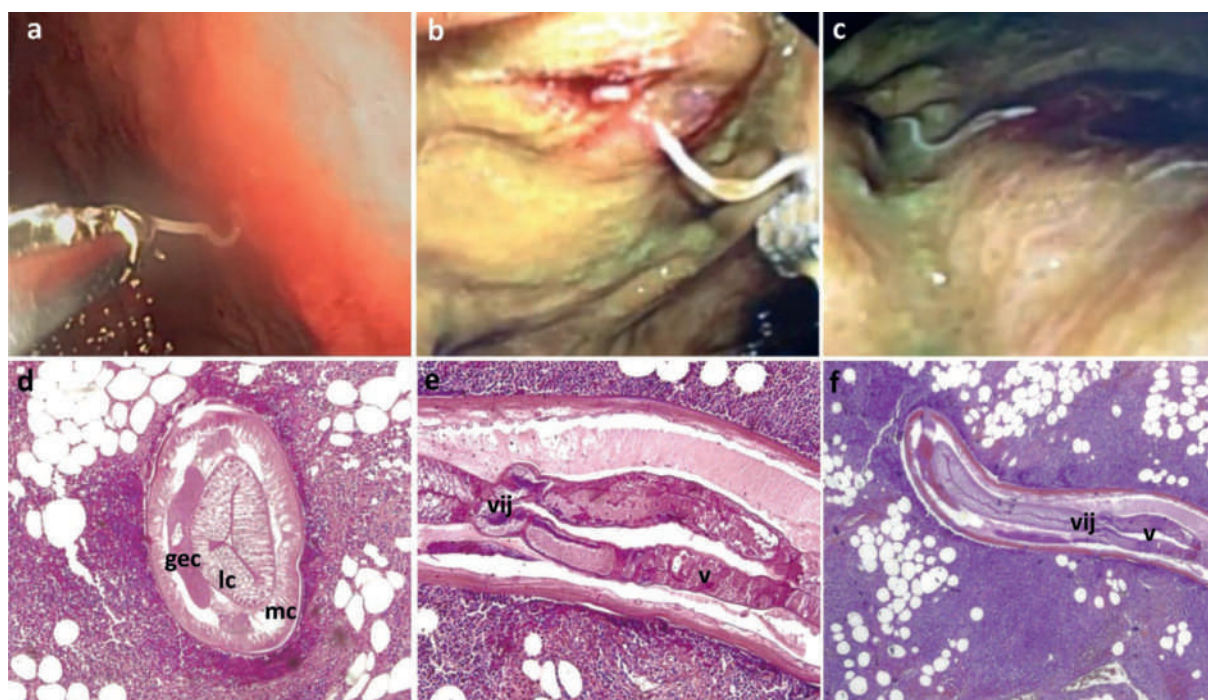


Fig. 7 L₃ of *Anisakis pegreffii* in humans provoking: Gastric Anisakiasis (GA) (A–C) with larva extracted by endoscopy (A); and Intestinal Anisakiasis (IA) (D–F) with granuloma formed around L₃ stage, in a transversal (D), and sagittal (E and F) section. *m*, polymyarian muscular cells; *lc*, lateral cords; *i*, intestine; *gec*, glandular excretory cell; *v*, ventriculus; *vij*, ventriculus-intestine oblique junction. D and E: From Moschella CM, Mattiucci S, Mingazzini P, De Angelis G, Assenza M, Lombardo F, Monaco S, Paggi L and Modini C (2004) Intestinal anisakiasis in Italy: Case report. *Journal of Helminthology* **78**: 271–273.

mucosa, the majority of larvae has been reported found in the greater curvature, followed by the posterior wall. Endoscopic findings such as edematous hypertrophic gastric folds, increase in gastric secretion and mucosal lesions, including edema, redness, fibrin deposits, hemorrhage and ulceration have been generally described.

In an experimental model of infection of Wistar rats using the two species, *A. pegreffii* and *A. simplex* (s.s.) (Romero et al., 2013), it was observed that the penetration rate of *A. pegreffii* was lower than that observed for *A. simplex* (s.s.). It could be that *A. simplex* (s.s.) may prefer to invade and penetrate the normal intact gastric mucosa, instead of an atrophic mucosa. This finding can be related to the fact that pH value is lower in the normal mucosa than in atrophic mucosa and that *A. simplex* (s.s.) (Arai et al., 2014) seems to possess a higher penetration rate in agar at low pH (Arizono et al., 2012). In vitro studies have demonstrated that at pH 8.5, proteolytic activity was significantly higher in *A. simplex* (s.s.) than in *A. pegreffii*. At this pH, the majority of activity was due to matrix metalloproteases whose activity was significantly higher in *A. simplex* (s.s.) than in *A. pegreffii*; this phenomenon could be related to the greater invasive capacity of the former species (Molina-Fernández et al., 2019). However, in spite of those findings, it cannot be definitely stated that *A. simplex* (s.s.) is a more pathogenic species with respect to *A. pegreffii*.

Cases of IA due to *A. pegreffii* and *A. simplex* (s.s.) include a “mild form” characterized by eosinophilic granulomas forming “tumor-like” formations in the intestinal wall, and a “fulminant form” with the symptoms of acute ileus, acute appendicitis, acute abdomen or regional ileitis (Fig. 7). Cases of IA reported from Japan, Korea, and Italy (reviewed in Chai et al., 2005; Audicana and Kennedy, 2008; Mattiucci et al., 2018; Guardone et al., 2018), were characterized by a clinical picture of acute appendicitis or acute abdomen. Risk factor differences between human GA and IA have been also observed as regards human anisakiasis, with the male gender in older patients having a major risk of IA (Yamamoto et al., 2020).

Gastro-Allergic-Anisakiasis (GAA)

The term Gastro-allergic Anisakiasis (GAA) (Daschner et al., 2000) indicates an acute allergic reaction in the context of the presence of an alive *Anisakis* sp. larva on the gastric mucosa when attempting to invade the submucosal layer of the stomach wall. GAA is characterized by different manifestations such as urticaria, angioedema and generally consists of an acute IgE-mediated, generalized reaction, starting from few hours to few days after the ingestion of infected fish (Daschner et al., 2011; Mattiucci et al., 2013). Daschner and Pascual (2005) suggested that the allergic reaction following the invasion of an *Anisakis* sp. larva is a protective immunological reaction by the human host to avoid the progressive evolution in the chronic disease, i.e., the granuloma formation.

GAA cases, due to *A. pegreffii*, characterized by urticaria and edema of the oral mucosa, were recognized by molecular methods in patients after they had consumed “marinated anchovies” (Mattiucci et al., 2013). In those cases, endoscope observations showed *Anisakis* larvae invading the submucosa layer of the gastric wall. In addition, serum samples from the patients showed IgE reactivity in western blotting analysis against *A. peg 1*, *A. peg 7* and *A. peg 13* antigenic proteins of *A. pegreffii* (Table 1) (Mattiucci et al., 2017a). In accordance with those findings, *Anisakis* hemoglobin, namely *Anis 13* antigen, was found to induce a strong and prolonged immunoreaction in rats. Similarly, it was shown that *Anis 1* and *Anis 7* antigens/allergens are biomarkers of allergy severity in human anisakiasis (de Las Vecillas et al., 2020).

Anisakis IgE-hypersensitization

Starting since the 1990s, an intense IgE sensitization to *Anisakis* IgE response has been suggested to occur in patients from Japan, Spain, and Italy suffering from GAA, or showing chronic urticaria (CU+) with or without fish consumption. *Anisakis* sensitization can occur by exposure to species-specific allergenic molecules such as *Anis 1*, *Anis 4* and *Anis 7* (de Las Vecillas et al., 2020) or other proteins such as tropomyosin (*Anis 2*) and paramyosin (*Anis 3*), having a strong molecular and immunological cross-reactivity with similar binding proteins from other invertebrates, including crustaceans and dust mites (Mattiucci et al., 2017a; Aibinu et al., 2019). Further, cross-reactive molecules belonging to the SXP/RAL family proteins, similar to those from other nematodes (i.e., *Ascaris*) were identified (Brusca et al., 2020). It has been also reported an “occupational allergy” (dermatitis, asthma, conjunctivitis) caused by *A. simplex* (s.l.) in fishermen and other industry workers (Armentia et al., 1998; Nieuwenhuizen et al., 2006; Jeroncic et al., 2020; Mazzucco et al., 2018). Additionally, it should be taken into account that *Anisakis*-IgE hypersensitivity could vary according to the geographical areas, human populations’ characteristics and genetics, as well the laboratory assays used for its diagnosis (Mattiucci et al., 2018; Daschner and Cuéllar, 2020).

Diagnosis of human anisakiasis

Diagnosis is generally carried out by considering anamnestic data, endoscopy, radiography, serum specific anti-*Anisakis* IgE determination; mast cell degranulation test; the molecular identification of the *Anisakis* larva removed by endoscopy or surgery or expelled by patients is the conclusive assessment. To date, the high number of false positives in the serodiagnosis of *Anisakis*-IgE-hypersensitivity due to cross-reactivity with numerous panallergens have underlined the need to improve the diagnostic approaches.

Histological diagnosis

At the histopathological level, three stages have been defined in the granuloma evolution in both GA and IA, according to Kojima et al. (1996) (Fig. 7). The first stage is recognized as the “phlegmon formation,” and the second one is the “abscess formation.” The last is rather frequent in GA and is characterized by abundant necrotic tissue around the larvae and by a rich population of eosinophils. The third stage is the “abscess-granuloma formation,” which corresponds to the flogistic evolution of the disease from one to several months, after the accidental ingestion of the larva. During the formation of granuloma, the tissue damage created by the *Anisakis*

larva secreting proteins induces chemotactic response for eosinophils and neutrophil recruitment. The larva is then surrounded by eosinophils, neutrophils, lymphocytes and monocytes producing effector molecules possibly capable to kill the parasite. In the meanwhile, fibroblasts are also recruited, which collaborate to the granuloma formation. At this stage, the larva is reduced to few remnants which are invaded by eosinophils, surrounded by giant cells and abundant inflammatory parvicellular infiltrate. Finally, the most advanced stage is that of “granuloma formed” characterized by a further decreased infiltration of eosinophils, but with abundance of lymphocytes, giant cells and significant collagen accumulation (Kikuchi et al., 1990).

When an eosinophilic granuloma has been removed surgically from gastric and intestinal sites, histological preparations (H&E) may reveal relevant features it can reveal the presence of characteristic morphological features of *Anisakis* sp. larvae. In histological transverse sections, the following characters can be noted: a thin cuticle lacking lateral alae; polymyarian muscle cells, separated into four quadrants by chords with two wing-like distal lobes; circular intestine with a triangular lumen and around 50–70 µm tall columnar epithelial cells; banana-shaped excretory cells (renette cells), situated ventrally to the intestine. In the histological sagittal section (Fig. 7), the following features are typical of *Anisakis* sp. larvae: the muscular part of the oesophagus followed by the glandular part (ventriculus) and absence of a ventricular appendix and/or intestinal caecum. However, this can only be achieved when the larva in the nodule is in a good state of preservation. Additionally, those morphological features do not allow identification to species level. This can be, instead, obtained from surgically removed granulomas, by a validated RT-PCR method (Paoletti et al., 2017) which has been proved to be efficient in the identification of the etiological agent, even if the larva in the granuloma is spoiled and/or present at very low quantity (Mattiucci et al., 2017b) (see paragraph below).

Molecular diagnosis

Molecular diagnosis of anisakiasis in humans allows the detection of *Anisakis* DNA in GA and IA cases, by using conventional PCR-DNA direct sequences analysis of target genes (D’Amelio et al., 1999; Fumarola et al., 2009; Umehara et al., 2007; Mattiucci et al., 2011, 2013, 2017b; Mladineo et al., 2016; Kim et al., 2013; Arai et al., 2014). The RT-PCR hydrolysis probe (Mattiucci et al., 2017b) has overcome some limitations related to DNA quantity with respect to the conventional PCR-DNA direct sequencing, allowing the identification of the parasite species even when present in low quantities, in GA and IA cases (Mattiucci et al., 2011, 2017a,b). In the last cases, the etiological agent would have been undetectable as inferred by direct DNA sequencing, due to the low quantity of DNA available.

Serodiagnosis of human anisakiasis and IgE-sensitization

Currently, serodiagnostic tests for *Anisakis* reactivity include the most conventional use of ImmunoCAP (iCAP) assay. However, in recent years other methods have been developed, such as western blotting (WB) and ELISA (reviewed in Mattiucci et al., 2018). Among these, it should especially be noted that specific IgE detection using iCAP assay can overestimate the number of human hypersensitive patients to *Anisakis* allergens. In other words, the sensitivity of these tests can be exaggerated and may yield false-positive results. In recent years, some authors have preferred to use IgE and IgG detection by WB assay, mostly using E/S antigens to differentiate, between GAA and hypersensitive patients (Daschner et al., 2012). Among the antigens related to infection due to *Anisakis*, *Ani s 1*, *Ani s 7* and *Ani s 13* could be considered the major antigens in the diagnosis of anisakiasis (Daschner et al., 2012; Mattiucci et al., 2017b). Nevertheless, the iCAP assay is still used in routine laboratory diagnosis for “*Anisakis*-allergy.” However, some of the apparently positive sera in iCAP assay, can be false-positives, due to: (i) cross-reactive antibodies directed against non-parasitic epitopes; in this case, the iCAP usually shows a pronounced reactivity against panallergenic protein (e.g., tropomyosin); and (ii) an underlying atopic state of the patients who may have some of their polyclonal IgE (Mattiucci et al., 2017b).

Treatment

As stated above, the endoscopically removal of larvae in GA and the surgical intervention of granulomas in IA represent a remedy to the disease and its symptoms in humans. However, despite the fact that there is not a large clinical evidence for a significant success of the use of chemical treatment in the clinical manifestation, it should be mentioned that the use of Albendazole, Prednisolone and Olopatadine hydrochloride have found to improve symptoms in cases of IA (Toyoda and Tanaka, 2016). Furthermore, treatment with corticosteroid and an anti-allergic agent could be an option for patients with IA and GA (Toyoda and Tanaka, 2016).

Preventive measures for raw fish treatment before its human consumption includes deep-freezing at –20 °C for at least 24–48 h, following the Reg. UE n. 853/2004, Annex III, Chapter III).

Conclusion

The disease provoked by *Anisakis* seems to represent in humans an emerging infectious disease where the culinary preference of raw fish consumption is widespread. Not all the species of the genus *Anisakis* are zoonotic; however, data on the fish muscle infection by other *Anisakis* species (i.e., *A. berlandi*, *A. typica*) have been not yet fully investigated. Reports of allergic features associated with the zoonotic species of *Anisakis* are expected to increase in the next decades. In this contest, research aimed to investigate the immunoregulatory features of molecules derived from those parasite species, even to distinguish between a true “*Anisakis*-induced allergy” from “Food allergy.”

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Blastocystis

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Taxonomy, morphology, and life cycle

Blastocystis has a complicated taxonomic history. It was described by Alexieff (1911) and despite the efforts of numerous researchers there are still many unknowns surrounding this organism. It has been viewed as a fungus, a sporozoan and even the cyst of another organism until 25 years ago (Silberman et al., 1996) when it was placed among the Stramenopiles, which is a heterogeneous group of eukaryotes (Adl et al., 2019). The classification of *Blastocystis* was complicated and for so long controversial due to its anaerobic nature and the lack of flagella. Moreover, it is the only human enteric organism within this subgroup, while most of Stramenopiles are free-living and aerobes (Denoeud et al., 2011).

The typical morphology of *Blastocystis* is the spherical “vacuolar” form, ranging in size from 5 to 40 µm (usual range in human stool, 8–10 µm), comprising a large central vacuole with the cytoplasm containing multiple nuclei and mitochondria-like organelles pushed to the periphery (Tan, 2008) (Fig. 1). Moreover, further morphologies have been described such as the granular, the ameboid, and the cystic form. Avacuolar and multivacuolar forms have also been reported, but the vacuolar and the granular ones are the more commonly described in fecal samples and cultures (Tan, 2008).

Blastocystis is characterized by the presence of mitochondria-like organelles (MLOs) (Nasirudeen and Tan, 2004) (Fig. 2), which, like mitochondria, are limited by a double membrane. Sequencing of the complete circular DNA in the MLOs of these parasites by different authors (Pérez-Brocal and Clark, 2008; Wawrzyniak et al., 2008) have showed that the cellular compartments have the metabolic properties of both aerobic and anaerobic mitochondria.

The life-cycle of *Blastocystis* remains incompletely known. Experimental infectivity studies in animals have demonstrated that the water-resistant and environmentally resistant infective cysts are the transmission stage (Suresh et al., 1993; Yoshikawa et al., 2004). Upon ingestion, excystment takes place in the host intestine, giving rise to vacuolar forms. These forms divide by binary fission and may develop further into amoeboid or granular forms. Later, encystment may occur during passage down the colon before cyst excretion in the feces (Tan, 2008).

Molecular characterization of *Blastocystis* subtypes and host specificity

Molecular studies based on the small subunit rRNA gene (SSU-rDNA) have allowed the identification in mammalian and avian hosts of at possibly 22 distinct ribosomal lineages, termed subtypes (STs), which could be considered separate species (Stensvold and Clark, 2020).

A minimum of 5% sequence divergence from the SSU-rDNA is the recommendation for establishing new subtypes, but some (e.g. ST6 and ST9) with respect to other STs, have less than 5% divergence (Stensvold and Clark, 2016).

Several surveys conducted to evaluate the potential risk of zoonotic transmission of *Blastocystis* detected the protist in a wide range of animal hosts, including non-human primates and other mammals such as artiodactyls, perissodactyls, proboscideans, rodents, and marsupials, as well as birds, reptiles, amphibians, fish, annelids, and insects (Yoshikawa et al., 2016; Cian et al., 2017).

Among the 22 *Blastocystis* ribosomal lineages, 10 subtypes have been so far identified in humans. In detail, STs 1–4 are frequently found in humans (Alfellani et al., 2013a), but they have also been detected in hoofed mammals, primates, pigs, cattle, rodents and even birds (Alfellani et al., 2013b). Conversely, STs 5–8 are rarely reported in humans and more commonly found in animals. For instance, ST5 is detected typically in hoofed animals, ST6 and ST7 in birds, and ST8 in non-human primates. When detected in humans, it has been suggested that these subtypes are of zoonotic origin. For instance, ST5 is prevalent in piggery workers in

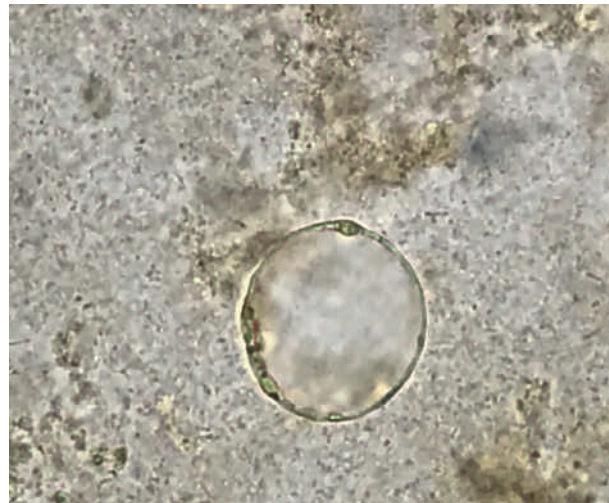


Fig. 1 *Blastocystis* in a wet mount stained with iodine (40 $\times$ magnification).

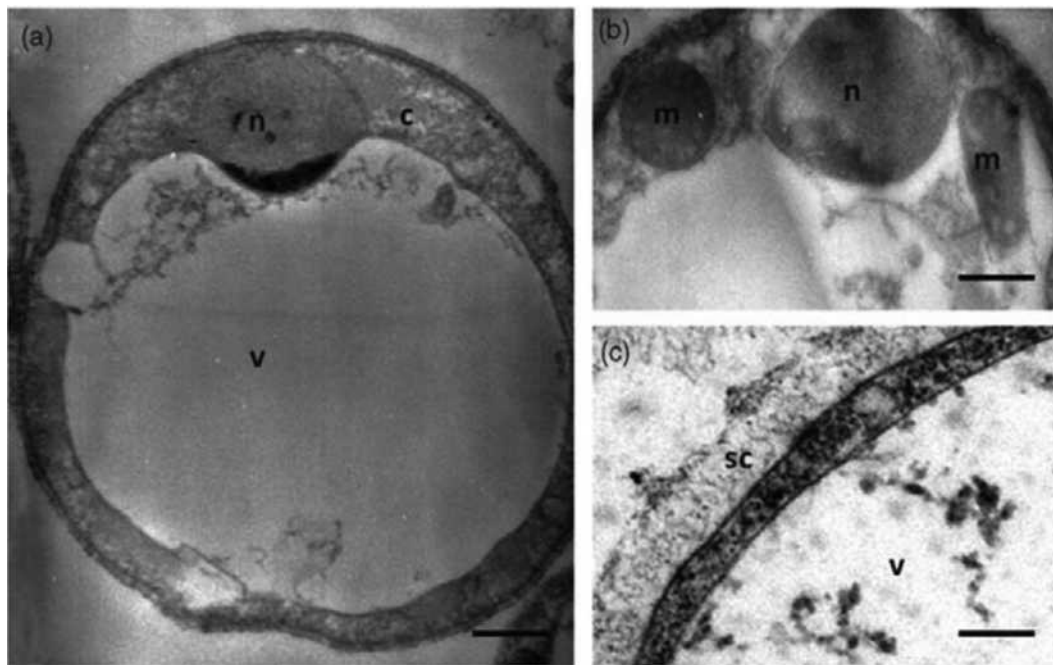


Fig. 2 Vacuolar form of an *in-vitro* cultivated *Blastocystis* as viewed by transmission electron microscopy (a). This form is spherical with a large central vacuole (v) and a thin peripheral band of cytoplasm (c) around the vacuole. (b) The cytoplasm contains nuclei (n) and mitochondrion-like organelles (m). (c) *Blastocystis* is surrounded by a surface coat (sc). Bars, 2 μ m for (a) and 500 nm for (b) and (c). From Wawrzyniak I, Poirier P, Viscogliosi E, Dionigia M, Texier C, Delbac F, and Alaoui HE (2013) *Blastocystis*, an unrecognized parasite: An overview of pathogenesis and diagnosis. *Therapeutic Advances in Infectious Disease* 1(5): 167–178.

Australia (Wang et al., 2014a), ST6 is frequently isolated from poultry and slaughterhouse staff (Greige et al., 2018) while ST8 has been frequently found in zookeepers who work with non-human primates (Alfellani et al., 2013c). ST9 is the only subtype found in humans today, and even rarely so (Stensvold and Clark, 2016). ST12 was found in humans ($n = 3$) in a study by Ramírez et al. (2016).

The subtype distribution in animals had been investigated in several surveys conducted in animal groups mainly from zoological gardens (Abe et al., 2002; Stensvold et al., 2009a; Alfellani et al., 2013a,c; Cian et al., 2017), national parks (Petrášová et al., 2011), and farms (Wang et al., 2014a) or studies focusing on specific populations (Spanakos et al., 2011; Ruaux and Stang, 2014; Gazzonis et al., 2019; Jiménez et al., 2019).

In general, a large predominance of ST1 to ST3 was identified in primates in Europe (Cian et al., 2017) while in South America monkeys were frequently infected by ST4 and ST8 (Jiménez et al., 2019).

Regarding artiodactyls, Bovidae were mainly positive with *Blastocystis* ST5, ST10 and ST14 while Suidae were shown to be frequently infected by ST5. Birds usually harbor ST6 and ST7, which are considered “avian STs” because of their predominance in this host group (Cian et al., 2017).

Carnivores such as dogs and foxes were not infected by *Blastocystis* (Abe et al., 2002; Spanakos et al., 2011; Paulos et al., 2018) or infected with a low or moderate prevalence (Calero-Bernal et al., 2020), with the exception of stray or sheltered dogs, which showed higher prevalence values (Ruaux and Stang, 2014; Gazzonis et al., 2019).

Potential additional STs were also proposed in non-mammalian and avian hosts including amphibians, reptiles, and insects by Yoshikawa et al. (2016).

In general, most of the vertebrate hosts harbor a single subtype. Mixed infections (i.e. infections by at least two different STs) could result from multiple sources of *Blastocystis*. The prevalence of mixed infections is similar in different countries and roughly ranges between 2.6% and 14.3%. However, their true distribution remains difficult to ascertain and is likely underestimated as it depends on the molecular method employed for subtyping (Meloni et al., 2012).

Studies on the distribution of STs in human populations have revealed geographic variation in the prevalence of STs, which might reflect different epidemiological and demographic characteristics including climate, geography, cultural habits, exposure to reservoir hosts, and mode of transmission (Alfellani et al., 2013a). In general ST3 is the most widespread subtypes followed by ST1 and ST2. The subtype ST4 is the second most common ST in the United Kingdom and it is generally found across Europe but it is less frequently detected or absent in the Far East, South America and Africa. The subtype 5–9 were detected with low frequency in all the major geographic regions (Fig. 3).

However, as the molecular identification of *Blastocystis* was not yet performed from many countries, this epidemiological scenario could change.

Blastocystis genomics

Despite the unanswered questions regarding its potential clinical relevance, studies of complete genomes of *Blastocystis* STs are still scant. Currently, nuclear genomes sequenced so far belong to ST1 (Gentekaki et al., 2017), ST4 (Wawrzyniak et al., 2015), and ST7 (Denoëud et al., 2011).

The ST7 *Blastocystis* nuclear genome, obtained using a culture established from stool of a symptomatic human, was the first complete nuclear genome to be sequenced (Denoëud et al., 2011). The study reported a genome size of 18.8 Mb with 6020 protein-coding genes, which in part appear to have been acquired by horizontal gene transfer. Introns are numerous and small, but elements of repetitive DNA are rare. Individual ribosomal RNA cistrons are sometimes present in sub-telomeric regions of the genome rather than being exclusively found in long tandem arrays as in many other eukaryotes.

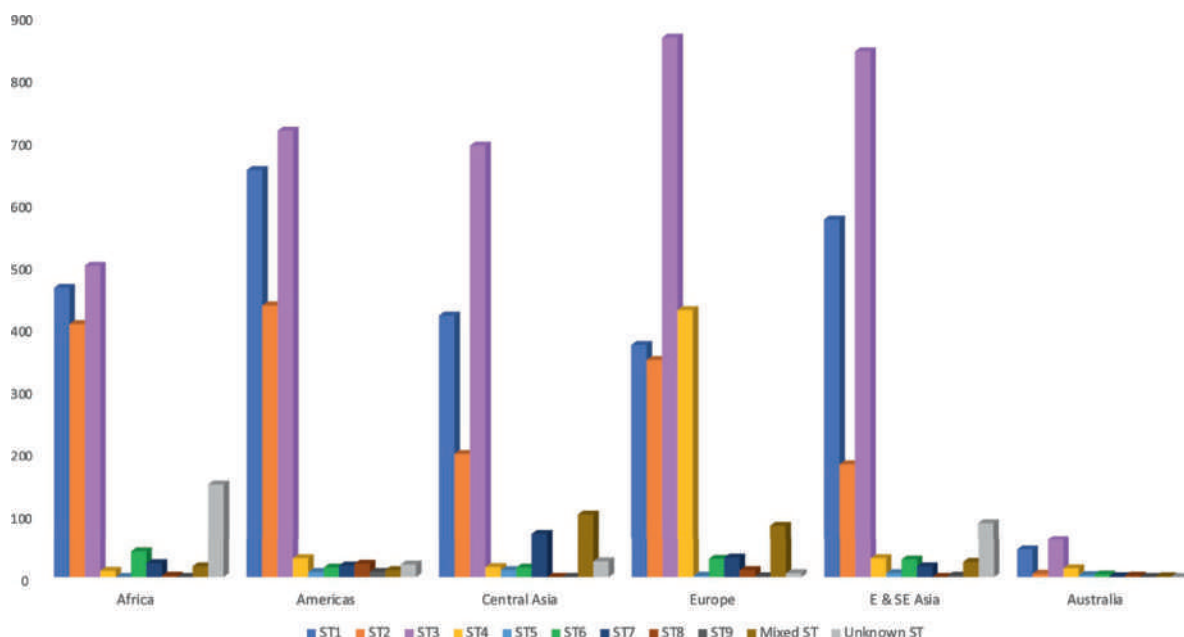


Fig. 3 Distribution of *Blastocystis* STs in humans from major geographic regions. Data modified and updated from Alfellani MA, Stensvold CR, Vidal-Lapiedra A, Onuoha ES, Fagbenro-Beyioku AF, and Clark CG (2013) Variable geographic distribution of *Blastocystis* subtypes and its potential implications. *Acta Tropica* 126(1): 11–18.

The ST4 genome was obtained from a laboratory rodent (Wawrzyniak et al., 2015); its size was 12.91 Mb and a set of 5713 protein-coding genes was predicted. There were no detailed analyses of the genome structure or of the genes themselves. The study of the whole genome from these two STs (subtypes 4 and 7) revealed protein-coding genes (aspartic, cysteine, metallopeptidases, serine and threonine proteases) suggesting their role as virulence factors (Gentekaki et al., 2017).

Recently the ST1 genome sequence was also described, highlighting an extreme genomic diversity between the subtypes (Gentekaki et al., 2017). Indeed, ST1 has 6544 protein-coding genes, which is several hundred more than reported for ST4 and ST7. The percentage of proteins unique to each ST ranges from 6.2% to 20.5%, greatly exceeding the differences observed within parasitic protistan genera. Orthologous proteins also display extreme divergence in amino acid sequence identity between STs (i.e. 59–61% median identity), on par with observations of the most distantly related species pairs of parasite genera. The STs also display substantial variation in gene family distributions and sizes, especially for protein kinase and protease gene families, which could reflect differences in pathogenetic potential. As many as 26% of the genes in ST1 have stop codons that are created on the mRNA level by a novel polyadenylation mechanism found only in *Blastocystis*. Reconstructions of pathways and organellar systems revealed that ST1 has a relatively complete membrane-trafficking system and a near-complete meiotic toolkit, possibly indicating a sexual cycle. Unlike some intestinal protistan parasites, *Blastocystis* ST1 has near-complete *de novo* pyrimidine, purine, and thiamine biosynthesis pathways and is unique amongst studied stramenopiles by being able to metabolize α -glucans rather than β -glucans. It lacks all genes encoding heme-containing cytochrome P450 proteins.

However, in addition to the nuclear genome, *Blastocystis* also has mitochondrion-like organelles (Fig. 2), which contain their organellar genome. Indeed, based on staining properties, it was known for many years that these organelles contained DNA, but it was not until 2007 that the coding potential of these molecules was uncovered. Two groups published sequences of the genomes present in the mitochondrion-like organelle in the subtypes ST1, 4 and 7 (Pérez-Brocal and Clark, 2008; Wawrzyniak et al., 2008). The gene content and gene order of the 27–29 kilobasepair circular molecules was identical, although the sequence divergence was considerable. Predictions of the MLO proteome from ST1 has revealed an expanded repertoire of functions, including lipid, cofactor, and vitamin biosynthesis, as well as proteins that may be involved in regulating mitochondrial morphology and MLO/endoplasmic reticulum interactions (Gentekaki et al., 2017).

Subsequently, mitochondrion-like organelle genomes from additional subtypes have been obtained (Jacob et al., 2016). The gene content of the genome of the *Blastocystis* mitochondrion-like organelle is distinct from the more familiar ones from mammals and yeast. Of particular note is the absence of any genes encoding cytochrome and ATPase subunits and the presence of a number of genes encoding ribosomal proteins. In common are the genes encoding ribosomal RNAs and several tRNAs plus NADH dehydrogenase (Complex I) subunits. The nuclear genomes and expressed sequence tag (EST) surveys that are available confirm that the *Blastocystis* mitochondrion-like organelle has only retained complexes I and II of the electron transport chain, a characteristic shared with certain other anaerobic eukaryotes. However, many other features of mitochondrial metabolism are also present (Denoëud et al., 2011). This is in contrast to, for example, *Giardia* and *Entamoeba*, where the mitochondrial genome has been lost completely, and the function of the resulting organelles has become highly reduced. Whether the *Blastocystis* organelle would follow a similar path given enough time is impossible to predict (Pérez-Brocal and Clark, 2008).

Diagnosis

The most common approach to detecting *Blastocystis* is direct microscopic examination of fecal material, with or without addition of Lugol's solution (Suresh and Smith, 2004). Permanent smears stained with trichrome, iron hematoxylin, Giemsa, Gram and Wright's stains have also been recommended (Stenzel and Boreham, 1996). Concentration methods such as zinc sulfate flotation or gravity sedimentation technique are unsuitable for the detection of *Blastocystis* because water, as well as several other solutions, can lyse the non-cyst forms of the protist (Stenzel and Boreham, 1996). Despite the use of microscopy for routine diagnosis of *Blastocystis*, it continues to be one of the more difficult enteric organisms to identify in clinical samples based on morphology. Challenges in its detection pertain to its small size, the occurrence of different forms (especially the difficult-to-recognize cystic form), its degradation caused by environmental conditions or drug treatment, or its occurrence in small numbers. Moreover, vegetative stages can be mistaken for fat globules, other contaminants, or yeast in the stool. *In vitro* culture may help overcome these problems by amplifying the protist number and making them more conspicuous. Other benefits include the possibility of maintaining and expanding isolates of *Blastocystis* in culture for further studies or for their cryopreservation. Various media are available for cultivating of *Blastocystis* such as Locke-egg medium, Iscove's modified Dulbecco's medium, Robinson's medium, TYSGM-9, and Jones' medium. The differences in composition among media suited for growing *Blastocystis*, and the protocols applied for its cultivation, may contribute to variation in sensitivity and specificity of the culture technique (Clark and Stensvold, 2016).

Moreover, culturing the protist may be time consuming and can bias subsequent genotyping due to the differential ability of isolates to grow in selective medium (Roberts et al., 2011). Therefore, to overcome these limitations, several PCR-based diagnostic protocols using DNA extracted from feces directly or after culture of fecal specimens, have been described (Santín et al., 2011; Abe et al., 2003; Scicluna et al., 2006). The SSU rDNA is the marker most frequently used followed by sequencing (Scicluna et al., 2006), which allows identification and analysis of ST genetic diversity. Recently, highly sensitive real-time quantitative PCR assays have been developed to detect and identify *Blastocystis* STs in stool samples by using specific probes or melting curve analysis (Jones et al., 2008; Poirier et al., 2011; Stensvold et al., 2012b).

Immunological approaches are not commonly used in the diagnosis of *Blastocystis*, although the usefulness of a commercially available immunofluorescence assay (Blasto-Fluor, Antibodies Inc.) and ELISA (CoproELISA™ *Blastocystis*, Savyon Diagnostics) were recently evaluated against standard methods for the detection of *Blastocystis* in stool samples (Dogruman-Al et al., 2010, 2015).

Clinical presentation

Blastocystis can be found in both symptomatic and asymptomatic patients (Eroglu et al., 2009). This has stimulated debate in the literature as to whether this microorganism is a pathogen or a commensal—or both.

Typically, *Blastocystis* has not been found to cause macroscopic changes in gut mucosa at upper or lower endoscopy (Zuckerman et al., 1994) although case reports exist where children with high burden of *Blastocystis* in the stools have presented with an acute abdomen suggestive of mucosal invasion and irritation of the peritoneal surface of the bowel (Andiran et al., 2006). Studies conducted in naturally infected pigs evidenced *Blastocystis* primarily in the lumen usually associated with digested food debris, sometimes in close proximity or appearing to adhere to the epithelium, but no stages were found to penetrate the epithelium or the lamina propria (Fayer et al., 2014).

In this context, there is not yet a consensus about the term to describe the host-protist interaction, which could be considered an “infection” or a “colonization”.

Anyway, blastocystosis, i.e. to simplify the presence of *Blastocystis* in the gut, is associated with a variety of non-specific gastrointestinal symptoms e.g. diarrhea, abdominal pain, flatulence, nausea, vomiting, constipation, weight loss or fatigue (Wawrzyniak et al., 2013). Furthermore, it has also been linked to cutaneous disorders and chronic or acute urticaria (Bahrami et al., 2020).

Several studies linked the severity of disease with the protist density. The presence of greater than five cysts per high-power field ($\times 400$) for wet mounts or under oil immersion ($\times 1000$) in permanent-stained smears was frequently associated with an acute presentation of gastrointestinal symptoms (Moghaddam et al., 2005). On the other hand, it has been also suggested that the illness could be related to the different STs (zoonotic or not) involved, to the impact of the protist on the gut microbiota (Andersen et al., 2015) or to the synergic effect of more factors (see the section “Pathogenetic mechanisms”).

Blastocystis was suspected to be involved in irritable bowel syndrome (IBS) (Tan et al., 2010; Poirier et al., 2012; Scanlan, 2012) as a higher prevalence of the protist, in particular of *Blastocystis*-ST1 and ST3, was reported in patients with IBS compared with healthy populations (Yakoob et al., 2010; Jimenez-Gonzalez et al., 2012). However, these studies did not conclude that *Blastocystis* was the sole etiologic agent. Conversely, recent studies demonstrated that *Blastocystis* was more common in healthy individuals than in patients with IBS (Krogsgaard et al., 2015) and, especially, with inflammatory bowel disease (IBD) (Petersen et al., 2013). Furthermore, studies showing the concomitant eradication of *Blastocystis* with the disappearance of symptoms in patients with IBS are needed to clarify this critical issue.

As for as extraintestinal symptoms linked to *Blastocystis*, including arthritis or skin disorders, like urticaria (Bahrami et al., 2020), it has been suggested that the amoeboid form adheres efficiently to the intestinal epithelium, affecting gut immune homeostasis and causing an inflammatory response against the parasite that leads to urticaria (Valsecchi et al., 2004).

In addition, a possible link between *Blastocystis* and allergic disorders was found, but few studies have been so far conducted to elucidate the immunological pathways and antigens that act as allergens. Just one study proposed that the protist induces allergy by increasing complement 3 serum levels in a ST-dependent manner, this being higher in ST3 with a wide absorption of intestinal allergens that indirectly elevate IgE serum levels (El Saftawy et al., 2019). Be careful on this article, probably *Blastocystis* molecules could trigger mast cells in an IgE-independent way.

Furthermore, although the protist was never considered as an opportunistic organism, it has also been frequently found in immunocompromised individuals presenting diarrhea, with prevalence ranging from 15% to 72.4% (Tan et al., 2009; Fontanelli Sulekova et al., 2019), and in patients with HIV/AIDS or cancer (Kurniawan et al., 2009; Tan et al., 2010).

Children from low income countries are also more susceptible due to their socioeconomic status, the quality of drinking water, the consumption of contaminated food and personal hygiene habits (Abdulsalam et al., 2012; Daryani et al., 2012). Also, people in close contact with animals are considered at risk of *Blastocystis*, emphasizing the potentially zoonotic nature of the protist (Yoshikawa et al., 2009; Parkar et al., 2010).

Pathogenic mechanisms

The pathogenic potential of *Blastocystis* remains a controversial issue, primarily due to (i) the high prevalence of asymptomatic carriers (as detailed in the previous section), (ii) the lack of remarkable phenotypic virulence properties showed by the protist relative to other pathogenic protozoa, such as the presence of flagella, lectins, or rhoptries etc., and (iii) the evidence suggesting its pathogenicity has been supposed mainly from *in vitro* models while few experiments have been so far conducted *in vivo*.

However, studies involving co-culture of intestinal epithelial cells with *Blastocystis*, either lysate or excretory/secretory products, in culture filtrates have led to the identification of several potential pathogenic mechanisms (Fig. 4). These include apoptosis (Puthia et al., 2006), the degradation of tight junction proteins resulting in increased intestinal permeability (Mirza et al., 2012), the

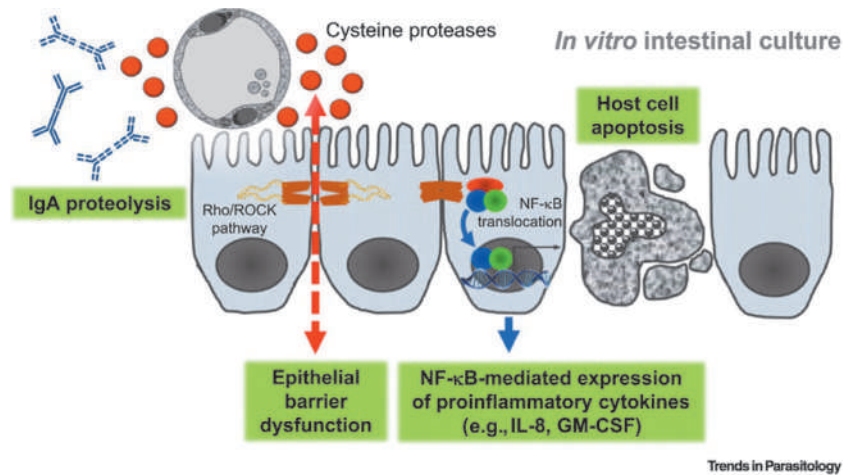


Fig. 4 Pathogenic mechanisms of *Blastocystis* described in *in vitro* cultures. From Stensvold CR, Tan K, and Clark CG (2020). *Blastocystis*. *Trends in Parasitology* 36(3): 315–316.

induction of pro-inflammatory cytokines (Puthia et al., 2008), and downregulation of iNOS (Mirza et al., 2011a). In addition, a central role for cysteine proteases in virulence of *Blastocystis* has been demonstrated (Puthia et al., 2008; Nourrisson et al., 2016).

In detail, studies involving co-culture of human colon epithelial cell-line Caco-2 with axenic ST4 and ST7 isolates demonstrated a ST-dependent induction of apoptosis by ST7 mediated by caspase 3 and caspase 9. This effect was not observed in Caco-2 cells infected with the ST4 isolate (Wu et al., 2014). Earlier studies on non-transformed rat intestinal epithelial cells showed that both live protist isolates and lysate of ST4 increased intestinal permeability in a time-dependent manner (Puthia et al., 2006). This study also showed that treatment with lysate and live cells resulted in F-actin disruption and formation of actin stress fibers. In Caco-2 cells, ST7 isolates (both live cells and lysate) were also found to mediate an increase in intestinal permeability in a time- and dose-dependent manner (Mirza et al., 2011a). *In silico* analysis of the *Blastocystis* ST7 genome revealed the presence of a large number of secreted proteases, including 29 cysteine proteases (Denoeud et al., 2011). Among these, a legumain and a cathepsin B, might play a role in pathogenesis of *Blastocystis* by increasing intestinal cell permeability (Nourrisson et al., 2016).

It has been suggested that cysteine proteases of *Blastocystis* induce rho kinase (ROCK)-dependent disruption of intestinal epithelial barrier function with reorganization of cytoskeletal F-actin and tight junctional ZO-1 (Mirza et al., 2012). Caspase 3- and 9-dependent enterocyte apoptosis could also play a role in *Blastocystis*-mediated epithelial barrier compromise (Wu et al., 2014).

In addition to the direct effects on the host intestinal epithelium, *Blastocystis* might also have immunomodulatory properties. Indeed, *Blastocystis* cysteine proteases in lysates as well as in conditioned medium are able to downregulate the product of humoral response by cleaving human secretory immunoglobulin A in a pH- and time-dependent manner (Puthia et al., 2005). Protease activity was found to peak at 24–48 h following culture and also showed inter-subtype variation with higher levels observed in ST7 than ST4 (Mirza and Tan, 2009).

As a pro-survival mechanism, metronidazole-resistant isolates highly susceptible to nitrosative stress were able to avoid cell death by downregulating the expression of iNOS in intestinal epithelial cells, even in the presence of pro-inflammatory cytokines and also by increasing the uptake of arginine (Mirza et al., 2011a). Cysteine proteases from ST4 isolates have been found to induce NFκB-mediated upregulation of IL-8 in human intestinal epithelial cells (Puthia et al., 2008). In murine macrophages, both live isolates and lysates induced MAP kinase mediated upregulation of IL-1β, IL-6 and TNF-α. Serine proteases in ST7 lysate were found to act through ERK and JNK pathways while cysteine proteases were able to induce these pro-inflammatory cytokines in a MAP kinase independent pathway (Lim et al., 2014).

Moreover, clinical studies reported some isolates of *Blastocystis* associated with IBS showed a resistance to metronidazole due to a decrease in cellular immune function (Mirza et al., 2011b). Low levels of CD3+, CD4+, and CD4+/CD8+ ratio counts were observed in *Blastocystis*-carriers compared with non-carriers (Wang et al., 2002). A similar study showed that dysregulation in inflammatory cytokine production (IL-12, IL-10, and TNF-α, IL-8) in colonic mucosa was associated with *Blastocystis* carriage in IBS patients (Yakoob et al., 2014).

In addition to *in vitro* investigations, recent studies have been carried out on laboratory animals including rats, mice, guinea pigs and chickens to understand and confirm the virulence factors of *Blastocystis* using *in vivo* models. During the course of infection, the presence of inflammatory infiltrates in the sub-mucosa (lymphocytic and eosinophilic), mucosal sloughing, and edematous lamina propria have been observed (Abou El Naga and Negm, 2001). Severity of inflammation has been associated with intensity of infection and subtype. Rats infected with ST1 showed more severe pathology while those infected with ST2 (Hussein et al., 2008). In another study, unlike *in vitro* studies, histological examination revealed neither mucosal sloughing nor inflammatory cell infiltration but showed a slight but significant increase in goblet cell numbers in the cecal mucosa 1–3 weeks post-infection with

Blastocystis laboratory strain RN94-9, assigned to ST4 (Iguchi et al., 2009). Upregulation of pro-inflammatory cytokines in intestinal tissue such as IFN, IL-12, and TNF- α , was also reported, suggesting that *Blastocystis* strain RN94-9 is a weakly pathogenic organism that could elicit pro-inflammatory as well as protective responses in local tissues (Iguchi et al., 2009).

Studies on serological response to *Blastocystis* in animal models have been limited with one study describing elevation of anti-IgM and IgG in serum and anti-IgA in intestinal secretions (Santos and Rivera, 2009). A range of potentially immunodominant proteins were identified in western blots arranged with protist lysates but they remain to be further characterized. In pigs, fecal IgA response was demonstrated in ~80% of naturally infected pigs. In addition, an increased response to a high molecular weight antigen (250 kDa) was seen among piglets and pigs that had been immunosuppressed (Wang et al., 2014b).

In addition to the potential virulence factors mentioned, it has been proposed that pathogenicity could be related to genetic differences on the subtype- or strain-level, correlating the ST-1, 4, and ST-7 with pathological alterations in humans, while ST2 and ST3 has been identified as “non-pathogenic” STs (Stensvold et al., 2009b; Eroglu et al., 2009). Also, the presence of both pathogenic and non-pathogenic strains within one subtype has been reported (Hussein et al., 2008). A recent multi-locus sequence typing analysis of *Blastocystis* ST3 and ST4 has provided valuable insight into genetic variation within and between the two subtypes, showing high or low level of genetic diversity for ST3 and ST4, respectively (Stensvold et al., 2012a; Mattiucci et al., 2016). In the latter study, an intra-subtype variability was further evidenced as three haplotypes (H1, H3, H7) were identified in ST3 isolates, while a single haplotype (H2) was observed in ST4 symptomatic patients (Fig. 5), suggesting that the intra-subtype diversity showed by ST3 and ST4 could be linked to the evolutionary history of *Blastocystis* subtypes and ST3 may have co-evolved with human hosts over a longer period than ST4, which may have extended its range to humans more recently.

Impact of *Blastocystis* on the gut microbiota

Over the past few years, several authors linked the impact of the protist on the gut microbiota with its pathogenetic role. Analysis of the microbial communities in healthy individuals or affected by intestinal disorders evidenced three main microbiota patterns, named enterotypes, characterized by three dominant bacteria clusters: *Bacteroides* (enterotype I), *Prevotella* (enterotype II), and *Ruminococcus* (enterotype III) (Arumugam et al., 2011). Each enterotype harbors different bacteria genera, defines a distinctive way of generating energy, and appear to be linked to dietary habits (Rinninella et al., 2019). Next generation sequencing (NGS)-based

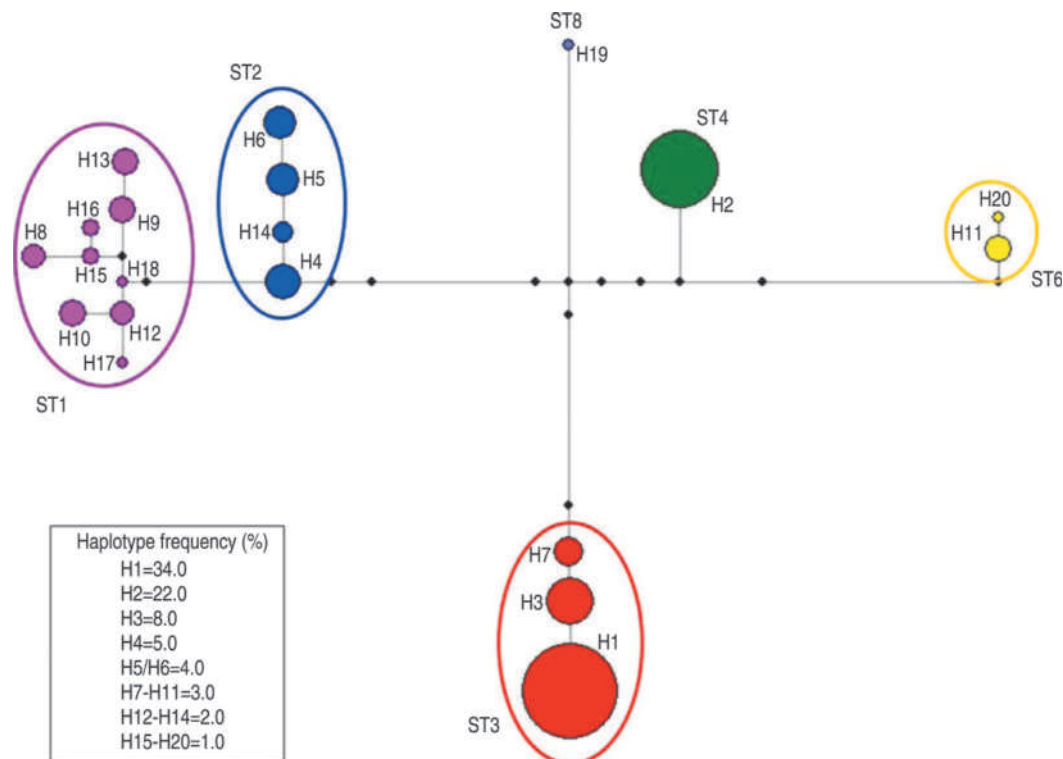


Fig. 5 Median joining network analysis of the SSU rDNA sequences obtained in *Blastocystis* subtypes detected in Mattiucci et al. (2016). It was estimated using maximum parsimony (MP) analysis as the optional post-processing calculation using Network 4.6.1.3 software (<http://www.fluxus-engineering.com>) with the default settings and assumptions. Each haplogroup represents a distinct subtype to which a different color is given. The number of the haplotype connection represents a single base difference; the missing haplotypes are indicated by black circles. From Mattiucci S, Crisafi B, Gabrielli S, Paoletti M, and Cancrini G (2016) Molecular epidemiology and genetic diversity of *Blastocystis* infection in humans in Italy. *Epidemiology and Infection* 144(3): 635–646.

studies associated *Blastocystis* with higher diversity of gut microbiota, low body mass index (BMI), and the *Ruminococcus* or *Prevotella*-driven enterotype rather than the *Bacteroides*-driven enterotype (Andersen et al., 2015). These findings were validated using a modified real-time quantitative PCR-based analysis platform, termed ‘Gut Low-Density Array’ (GILDA), including *Dientamoeba fragilis* as well as a control parasite (Andersen et al., 2016). The platform was designed for simultaneous analysis of the change in the abundance of 31 different microbial 16S rRNA gene targets in fecal samples obtained from individuals at various points in time. Both *D. fragilis* and *Blastocystis* were studied in relation to the six bacterial taxa such as *Bacteroides*, *Prevotella*, the butyrate-producing clostridial clusters IV and XIVa, the mucin-degrading *Akkermansia muciniphila*, and the indigenous group of *Bifidobacterium*. In this study, it was observed that *Blastocystis*-carriers alone or along with *D. fragilis* typically had gut microbiota characterized by low relative abundances of *Bacteroides* and clostridial cluster XIVa and high levels of *Prevotella*, confirming the colonization with *Blastocystis* being significantly linked to a low relative abundance of *Bacteroides* (Andersen et al., 2016). Further studies have corroborated these observations and evidenced *Blastocystis* associated with a healthy gut microbiota, characterized by an abundance of bacterial species and, in particular, of potentially beneficial species (Audebert et al., 2016; Nieves-Ramírez et al., 2018). In a study comparing the microbiota in individuals affected by pathogenic parasites such as *Giardia*, with commensal parasites such as *Entamoeba coli*, *E. hartmanni* and *E. dispar* and with *Blastocystis*, dysbiosis, as evidenced by a low *Faecalibacterium prausnitzii*–*Escherichia coli* ratio, was identified in individuals with *Giardia*, while those with *Blastocystis* and *Entamoeba* spp. appeared to be characterized by eubiosis, characterized primarily by a high *F. prausnitzii*–*E. coli* ratio (Iebba et al., 2016).

Conversely, Nourrisson and colleagues noted a decrease of fecal microbiota protective bacteria *F. prausnitzii* in *Blastocystis*-colonized individuals and that levels of *Bifidobacterium* were decreased in males with IBS type C (Nourrisson et al., 2016).

Recent investigations in Italy, focused on *Blastocystis* ST3, confirm and support the link between the protist and an eubiotic state showing a higher bacterial diversity in *Blastocystis* ST3 carriers compared with that identified in *Blastocystis*-negative individuals (Gabrielli et al., 2020). Moreover, at the family level, Prevotellaceae, Methanobacteriaceae, Clostridiaceae were also more abundant, whereas Bacteroidaceae were enriched in *Blastocystis*-free subjects (Fig. 6).

Findings from this and previous studies, suggested that specific subtypes of *Blastocystis* might be considered ‘old friends’, as they may be required for intestinal homeostasis and/or play a role as a marker of intestinal homeostasis (Stensvold and van der Giezen, 2018; Mattiucci et al., 2016). This hypothesis, known as “Hygiene hypothesis” (Rook, 2010), based on Darwinian medicine, incorporates the concept of ‘old friends’, which states that that certain parasitic infections regulate intestinal homeostasis. A lack of childhood exposure to these microorganisms and/or parasites may increase the likelihood of autoimmune diseases in predisposed individuals later in life (Scanlan and Stensvold, 2013). This lack of exposure of the immune system to antigens leads to an imbalance in immune response or dysregulation that facilitates the development of chronic inflammatory conditions. This hypothesis has been recently reappraised to incorporate the concept of ‘Old Friends’ (Rook, 2012). The ‘Old Friends’ hypothesis moreover states that our shared co-evolutionary history with microbes has led to a state of host-evolved dependence on this microbiota. This evolved dependence is such that in a host of particular genetic background, the presence of a particular microbial

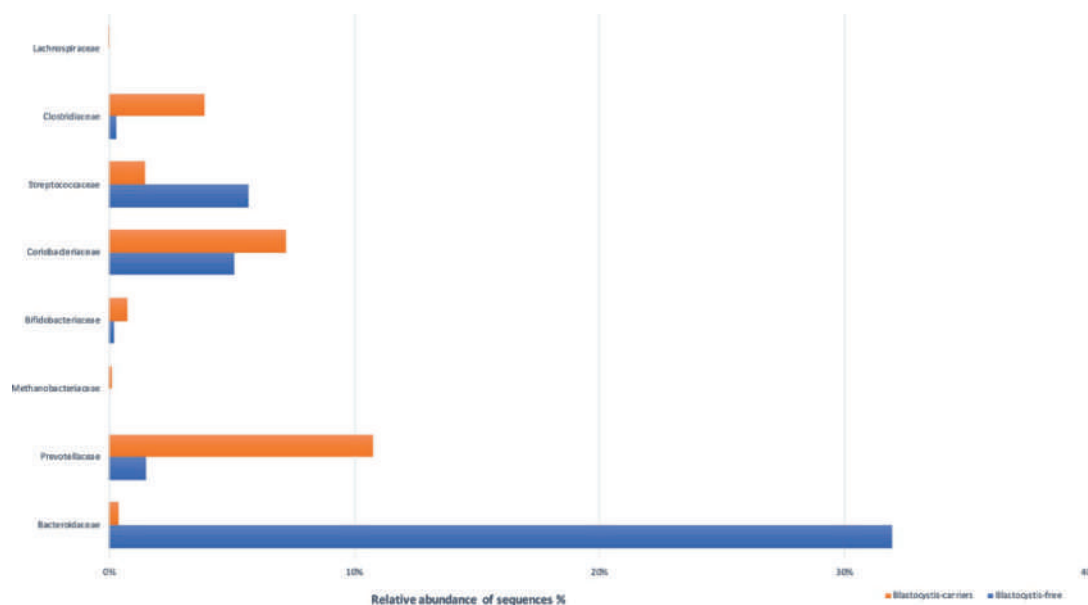


Fig. 6 Comparisons of the median values of the relative abundance at the level of bacterial family (level 5) for Bacteroidaceae, Prevotellaceae, Methanobacteriaceae, Clostridiaceae, Lachnospiraceae, Veillonellaceae, Erysipelotrichaceae, Pasteurellaceae in *Blastocystis*-carriers and *Blastocystis*-free subjects (orange and blue bars, respectively). The Mann–Whitney test was used to evaluate the differences between the two groups. From Gabrielli S, Furzi F, Fontanelli S, Sulekova L, Taliani G, and Mattiucci S (2020) Occurrence of *Blastocystis*-subtypes in patients from Italy revealed association of ST3 with a healthy gut microbiota. *Parasite Epidemiology and Control* 9: e00134.

community and/or specific components of the microbiota is required to induce and/or maintain normal immunological responses (Bach, 2018).

Having this concept in mind, it has been suggested that certain STs of *Blastocystis* may actually be part of a normal human intestinal microbiota since the protist has been detected in healthy and asymptomatic hosts for extensive periods of time. In this regard, Krogsgaard et al. (2015) suggested that *Blastocystis* contributes to the diversity of microbiota, and their absence could be a potential indicator of intestinal dysbiosis. In order to persist for such an extended period of time, *Blastocystis* must either evade the immune response, or be recognized as a 'friend'. Furthermore, the predominance of ST1–ST3 (the subtypes most commonly associated with humans) in non-human hosts, such as cattle and pigs (Alfellani et al., 2013c), would suggest that these subtypes have shared co-evolutionary history with humans and their closest living relatives. Conversely, ST4, uncommon in non-human primates, is apparently usual in humans in Europe; however, contrary to ST1–ST3, this subtype appears fairly genetically conserved (Stensvold et al., 2012a), indicating a recent entry in the human population. Consequently, the lack of exposure to intestinal helminths in young generations living in industrialized countries, compared with previous generations (which would have been exposed to several helminths during their youth, as is the case in Italy), may negatively affect the development of the immune system, predisposing them to the colonization even by an 'old friend' protist, such as *Blastocystis* ST3 (Mattiucci et al., 2016).

Despite the above described evidences about *Blastocystis* colonization and eubiotic gut, the presence of the protist in fecal samples is still considered an exclusion criterion for fecal microbiota transplantation (FMT), which is now widely accepted as the most effective therapy in the treatment of recurrent *Clostridium difficile* infection (Paramsothy et al., 2015). Now there is also interest in exploring FMT in the treatment of a range of other gastrointestinal and even systemic conditions, including inflammatory bowel diseases, irritable bowel syndrome, and insulin resistance/metabolic syndrome with clinical trials in progress (Paramsothy et al., 2015). Recently the first transmission of *Blastocystis* ST1 and ST3 by fecal microbiota transplantation was evidenced from two donors with prior negative microscopies (Silva et al., 2019). Molecular analyses on stool samples after donation evidenced the two donors as carriers of ST1 and ST3. Such STs were further detected in patients transferred with *Blastocystis*-positive donor feces, which did not report any significant differences in bowel complaints in the first week, after 3 weeks, or in the months following FMT. Moreover the presence of *Blastocystis* didn't have any significant effect on rCDI treatment outcome.

As previously discussed (section "Clinical presentation"), *Blastocystis* is also speculated to be a direct or indirect cause of gastrointestinal disorders such as IBD, but variation in its relative presence (including ST distribution) between case and control groups and between different studies has led to further confusion. Microbial profiling studies on gut microbiota of patients with IBD showed a decrease in strict anaerobic bacteria and a shift toward facultative anaerobes, such as members of the family Enterobacteriaceae, compared with healthy controls, indicating a role for oxygen in intestinal dysbiosis (Ni et al., 2017). Although it was known that oxygen concentrations increase in a disturbed gut ecosystem, the molecular mechanism was only recently elucidated (Byndloss et al., 2017). Indeed, in a healthy gut, bacteria produce butyrate, which is the preferred metabolic substrate used by colonic epithelial cells in mitochondrial β -oxidation producing ATP, resulting in a decrease of available oxygen (Vital et al., 2014). In addition, the produced butyrate is sensed via a human nuclear receptor and subsequently represses the iNOS (Byndloss et al., 2017), which leads to a reduction in nitrate (Lundberg et al., 2008), inducing a decrease in the proliferation of facultative anaerobes (Byndloss et al., 2017). Butyrate is required to activate oxidative metabolism (Donohoe et al., 2012). A shift away, caused by antibiotics for example, from obligate anaerobic bacteria among the Firmicutes and Bacteroides phyla toward members of the Enterobacteriaceae disrupts the host's butyrate-linked control mechanism and represses oxidative metabolism, increasing luminal oxygen in the human gut (Byndloss et al., 2017; Donohoe et al., 2012). This might partly explain why *Blastocystis* is rare in patients with intestinal dysbiosis-linked diseases: *Blastocystis* being a strict anaerobe (Zierdt, 1986), it is expected that its ability to establish or maintain itself in a microaerophilic environment is limited. This mechanism provides also a possible explanation for the negative correlation between *Blastocystis* and Bacteroides, the latter which, unlike the Firmicutes, contributes little to butyrate production in the gut (Vital et al., 2014) and therefore does not provide an environment where *Blastocystis* thrives (Stensvold and van der Giezen, 2018) (Fig. 7).

The association between gut microbiota and *Blastocystis* also provides a possible explanation for some human populations being more susceptible to *Blastocystis* colonization. Indeed, it has also been frequently found in immunocompromised individuals suffering diarrhea, with prevalence ranging from 15% to 72.4% (Tan et al., 2009). In the survey performed by Fontanelli Sulekova et al. (2019), *Blastocystis* was more frequently identified in fecal samples from HIV-positive patients (22%) than in immunocompetent subjects (7%). This was in agreement with findings from previous surveys (Piranshahi et al., 2018; Tan et al., 2009). However, no significant differences in fecal microbiota composition was found between HIV-positive and immunocompetent subjects, despite immunocompromised individuals showing low bacterial variability and burden. Several authors have evaluated the intestinal microbiome in HIV-positive subjects, with somewhat inconsistent or controversial results (Williams et al., 2016). This may be because of small sample sizes, lack of appropriate controls, and/or regional differences in dietary and environmental factors. Regardless, many authors noted a change in the ratio *Bacteroides*:*Prevotella*; this finding may be linked to the antiretroviral therapy (Vesterbacka et al., 2017), and/or to the sexual practice and lifestyle (Noguera-Julian et al., 2016) of the patients.

Fecal samples from HIV-positive patients enrolled in the aforementioned study (Fontanelli Sulekova et al., 2019) exhibited the so-called *Prevotella* enterotype (or enterotype II), which is characteristic of HIV-positive treated patients (Lozupone et al., 2013; Vujkovic-Cvijin et al., 2013) and, as above described, favors *Blastocystis* spp. colonization. Taking these observations into account, it appears that the high prevalence of *Blastocystis* observed in these patients is a consequence of the microbiota composition, probably induced by the antiretroviral therapy, which highly supports the colonization by the protist, rather opportunism by *Blastocystis* (Gabielli et al., 2020).

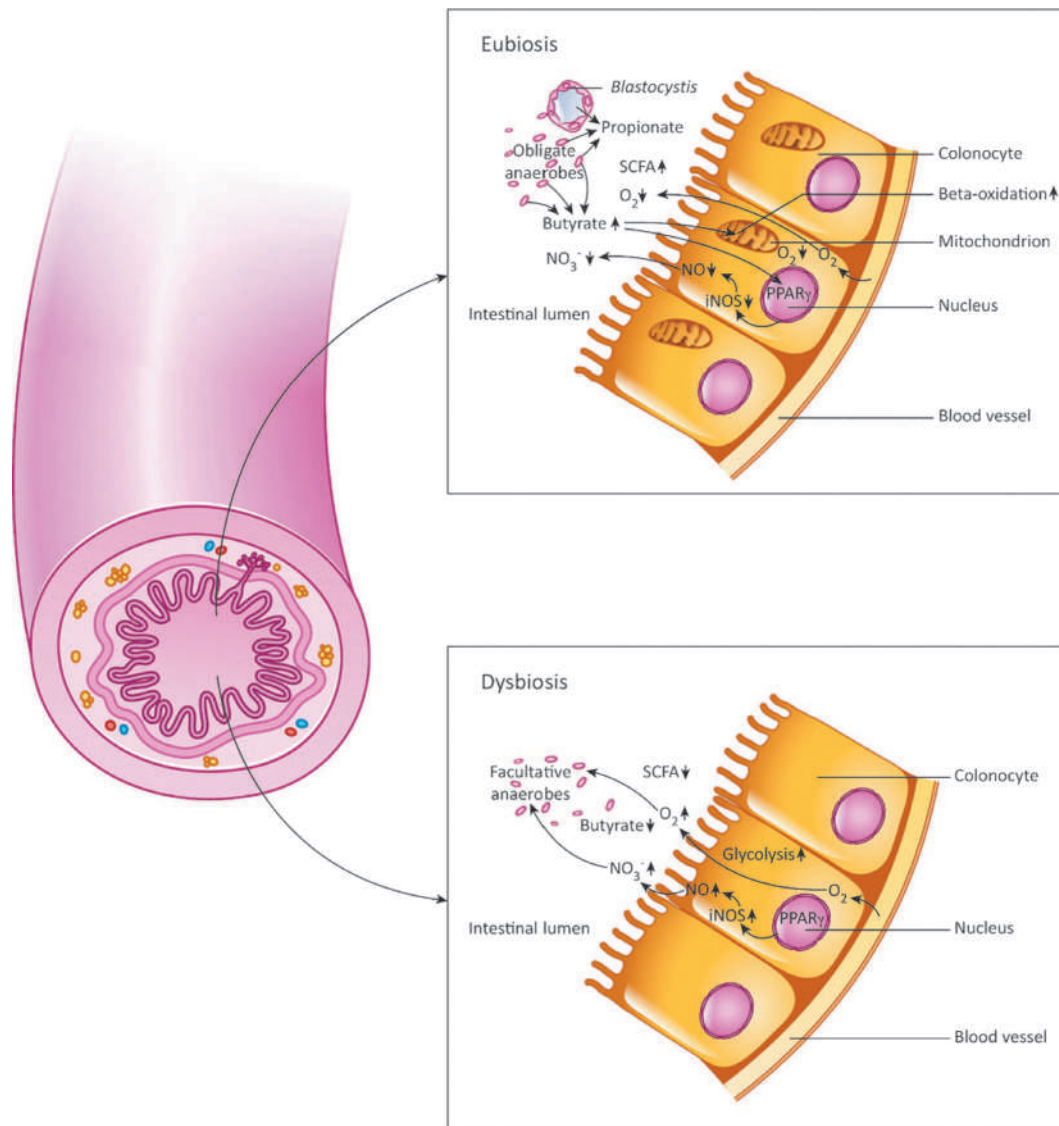


Fig. 7 Dynamic interplay between gut flora and human colonocytes. From Stensvold CR and van der Giezen M (2018) Associations between gut microbiota and common luminal intestinal parasites. *Trends in Parasitology* 34(5): 369–377.

Moreover, it has been suggested that microbiota composition in *Blastocystis*-carriers may be dependent on the organism's ST and that certain isolates of *Blastocystis*, as the ST7, potentially lead to an imbalance of the gut microbiota (Yason et al., 2019; Tito et al., 2019). *In vivo* and *in vitro* studies showed that *Blastocystis* ST7 exerts a positive effect on the viability of representative gut bacteria except for *Bifidobacterium longum*. Gene expression analysis and flow cytometry indicate that the bacterium may be undergoing oxidative stress in the presence of *Blastocystis*. *In vitro* assays demonstrated that *Blastocystis*-induced host responses are able to decrease *Bifidobacterium* counts. Mice infected with *Blastocystis* also reveal a decrease in the beneficial bacteria *Bifidobacterium* and *Lactobacillus*. Therefore, these results suggest that certain isolates of *Blastocystis* cause changes in microbiota population and exert their pathogenic effects through disruption of the gut microbiota, providing a counterpoint to the increasing reports indicating the commensal nature of this ubiquitous protist (Yason et al., 2019).

Treatment

Because the pathogenesis is controversial and symptoms are self-limiting, treatment might generally be considered only when other pathogens have been excluded (Stensvold et al., 2010). Several trials have been attempted to evaluate the efficacy of different compounds (Kordestani Shargh et al., 2020) but the eradication of *Blastocystis* is difficult and not straightforward to standardize.

In persistent symptomatic cases, metronidazole and cotrimoxazole (TMP/SMX) have often been the drugs of choice, despite the fact that they are not always effective (Stensvold et al., 2010). Other drugs have been trialed with variable success, including iodoquinol, inidazole, nitazoxanide, paromomycin, ketoconazole, and ornidazole (Roberts et al., 2015). It has been speculated that any resolution of symptoms may actually be secondary to eradication of some unrecognized microorganism(s) rather than to be a result of *Blastocystis* eradication (Markell and Udkow, 1986).

However, several factors depending on both protist and host can affect the therapy outcomes and the persistence of *Blastocystis* in the stool. Indeed, while in some cases the infection spontaneously resolves (Nigro et al., 2003; Rossignol et al., 2005), other individuals remain non-responsive to the treatment (Haresh et al., 1999). It has been speculated that treatment failure could reflect the acquisition of drug resistance by *Blastocystis* (Moghaddam et al., 2005); however, genes potentially responsible for activation/inactivation of metronidazole have not so far been identified (Roberts et al., 2014). Indeed, the success of *Blastocystis* elimination also depends on whether sufficient amount of drug can be delivered to the colon (Kumar et al., 2008). Moreover, inactivation of the drug by the normal bacterial flora has been suggested to explain treatment failure with metronidazole since healthy people receiving metronidazole exhibit almost no change in their fecal microbiota profile (Stensvold et al., 2010). Finally, particularly in highly endemic areas, reinfection may occur frequently, which could be interpreted as treatment failure. Here, longitudinal detailed strain characterization could be useful, and the use of highly sensitive and discriminatory molecular diagnostic source and disease-tracking tools are required to provide further insight into reinfection, and transmission dynamics of *Blastocystis* and to clarify the effectiveness of the therapy.

Furthermore, since the link between gut microbiota composition and *Blastocystis* was elucidated, the potential of probiotic bacteria as alternative therapy in *Blastocystis*-carriers was investigated (Dinleyici et al., 2011). Recently, a study aimed to evaluate the efficacy of the lactic acid bacteria *Lactobacillus rhamnosus*, *Lactococcus lactis*, and *Enterococcus faecium* in *Blastocystis*-ST3 eradication and showed the potential of using these bacteria as a prophylactic treatment against *Blastocystis* colonization. The ability of these probiotic bacterial strains to disrupt the cell cycle of *Blastocystis* shows a promising future in the use of probiotics for prophylaxis, or as an additional treatment regimen in combination with standard drugs (Lepczyńska and Dzika, 2019).

Conclusion

Recently, there has been increasing interest by the scientific and medical community in *Blastocystis* coupled with new data on epidemiology, potential pathogenicity, and, more recently, the first whole genome investigation of some *Blastocystis* STs. In 2008 the protist was included in the Water Sanitation and Health programs of the World Health Organization (WHO, 2008). Accumulating *in vivo* and *in vitro* data appear to indicate some importance of *Blastocystis* to human health, but its role in human and non-human disease remain to be identified in greater detail, and the development and standardization of axenization, diagnosis tools, transfection and animal models, microarrays and genotyping markers would be useful in this process. Sequencing of genomes from different STs and comparative genomic projects are in progress in several laboratories and the results will be useful to identify specific virulence factors. Multicenter studies and networking are also required to further our comprehension of the clinical implications of *Blastocystis* in IBS and other conditions. Finally, the interaction of *Blastocystis* with gut microbiota should be studied because of the increasing awareness of the role played by microbiota disturbances in the development of various gastrointestinal dysfunctions.

To conclude, as much remains to be learned about this parasite, a multidisciplinary approach is required to increase our knowledge of key aspects of the *Blastocystis* biology and further our understanding of its clinical relevance and public health significance.

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Clonorchis and Opisthorchis

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Abbreviations

Caco 2	Cell line of human colorectal adenocarcinoma cells
CCA	Cholangiocarcinoma
EFSA	European Food Safety Authority
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial mesenchymal transition
ESPs	Excretory and secretory products
EVs	Extracellular vesicles
FVB	Derived from an outbred Swiss colony, this mice are susceptible to liver flukes infection
GNR	Granulin
GST	Glutathione S-transferase
H69	Human cholangiocyte cell line
HSC	Hepatic stellate cells
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
MMNK	Highly differentiated immortalized human cholangiocyte cell line
NF_κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PCR	Polymerase chain reaction
s.l.	Sensu lato
TGF	Transforming growth factor
Th	T helper cells
TLR	Toll like receptor
Treg	Regulatory T cells
TSPs	Tetraspanins, a family of membrane proteins

Introduction

Clonorchis sinensis, *Opisthorchis viverrini* sensu lato (s.l.) and *Opisthorchis felinus* are liver fluke parasites that humans can acquire by consuming raw or partially cooked freshwater fish infected with the larval stage metacercariae.

Besides being the etiological agents of helminthic diseases, *C. sinensis* and *O. viverrini* s.l. have been classified as class I carcinogens, since they are the causative agents of cholangiocarcinoma (CCA) in chronically infected people, whereas *O. felinus* as class III (IARC, 1994; Bouvard et al., 2009). It is estimated that 35 million people are infected with *C. sinensis* mainly in China, 10 million with *O. viverrini* s.l. in Southeast Asia, in Lower Mekong Basin (Thailand, Lao PDR, Cambodia, Myanmar and Southern Vietnam). In Northeastern Thailand the prevalence of human opisthorchiasis (16.6%) is the highest in the world, and it is coincident with the highest incidence of CCA (Sithithaworn et al., 2012; Sripa et al., 2012a). *O. felinus* infects 1.2 million people in Eastern Europe and Russia (WHO, 1995; Sithithaworn et al., 2012; Saijuntha et al., 2021) and some hundreds of cases have been documented in Europe (Pozio et al., 2013). For 2018, an estimated 2.2 million infection-attributable cancer cases were diagnosed worldwide, from them, 3500 new cases of CCA were attributable to *C. sinensis* and *O. viverrini* s.l. infections (De Martel et al., 2020).

Systematics

Clonorchis and *Opisthorchis* belong to the phylum Platyhelminthes, class Trematoda, subclass Digenea, order Plagiorchiida, family Opisthorchiidae (Mordvinov and Furman, 2010). Studies of the population genetic structure of these liver flukes have uncovered sub-structure patterns that are related to geographical isolates in defined catchment/wetland systems (Petney et al., 2018). Molecular genetic investigations of *O. viverrini* in Southeast Asia showed that it is a species complex '*O. viverrini sensu lato*' containing two evolutionary lineages with many cryptic species (morphologically similar but genetically distinct species) occurring in and within different catchment (wetland) systems in Thailand and Lao PDR (Saijuntha et al., 2007; Sithithaworn et al., 2012).

Life cycle

The adult hermaphrodite worms (*C. sinensis*: 10–25 × 3–5 mm; *O. felinus*: 7–12 × 1.5–2.5 mm; *O. viverrini*: 5.5–10 × 0.8–1.6 mm) are dorso-ventrally flattened with an anterior oral sucker, a centrally located ventral sucker, and a uterine pore. The natural life cycle of these trematodes is similar among them and complex (Fig. 1). Freshwater species of snails belonging to the families Assimineidae, Bithyniidae, Hydrobiidae, Melaniidae and Thiaridae are the first intermediate hosts for *C. sinensis* (Lun et al., 2005). *Bithynia funiculata* and two subspecies of *B. siamensis* (*B. siamensis siamensis* and *B. siamensis gomiomphalos*) are the first intermediate host for *O. viverrini* (Petney et al., 2018; Sithithaworn et al., 2012). *O. felinus* has been found in the species *B. inflata*,

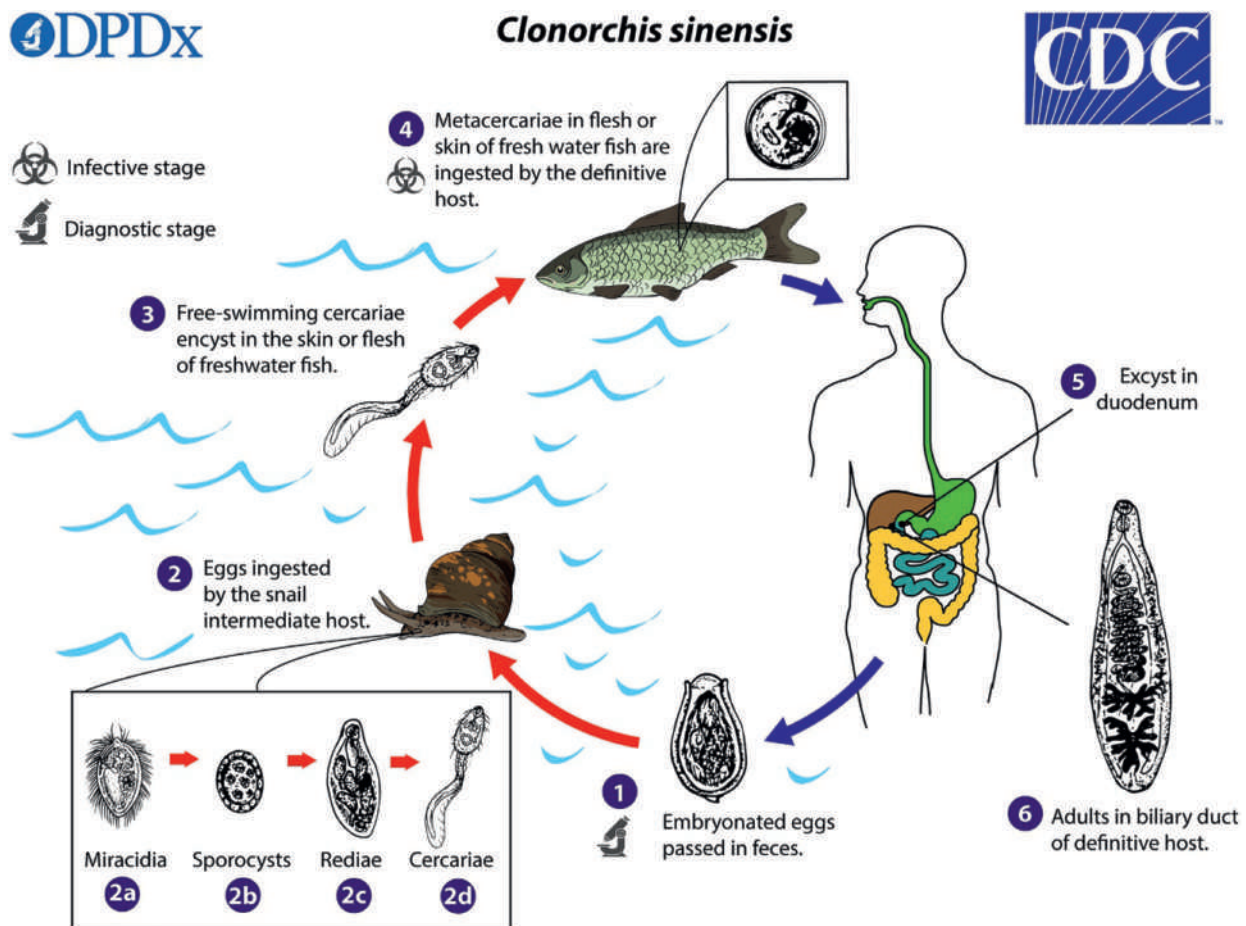


Fig. 1 The natural life cycle of *Clonorchis sinensis*.

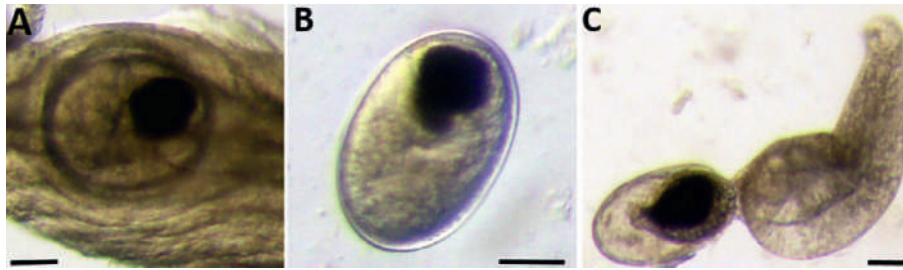


Fig. 2 (A) Metacercariae of *Opisthorchis felineus* in muscle of a tench (*Tincatinca*) from Lake Bolsena (central Italy). (B) Metacercariae of *O. felineus* collected from a tench filet after digestion with 1% pepsin and 1% HCl in tap water. (C) Excystation of an *Opisthorchis felineus* metacercariae after artificial digestion with 1% pepsin and 1% HCl in tap water. Scale bars = 100 μ m. Courtesy of Gianluca Marucci, Department of Infectious Diseases, Istituto Superiore di Sanità, Italy.

B. leachii, and *B. troscheli* (Erhardt et al., 1962; Hering-Hagenbeck and Schuster, 1996; Mordvinov et al., 2012). The second intermediate hosts in which metacercariae develop are freshwater fish, commonly of the Cyprinidae family, however other fish families and crustaceans have been reported to host *C. sinensis* larval stages with high prevalences (Lun et al., 2005). In Europe, *O. felineus* metacercariae have been detected in *Alburnus alburnus*, *Abramis brama*, *A. ballerus*, *Blicca bjoerkna*, *Idus idus*, *Rutilus rutilus*, *Scardinius erythrophthalmus*, and *Tinca tinca* with prevalences up to 95% (De Liberato et al., 2011; Pozio et al., 2013). The most important second intermediate hosts of *O. viverrini* are cyprinoid fish of the genera *Cyclocheilichthys*, *Hampala*, and *Puntius* (Wykoff et al., 1965). The number of metacercariae in fish varies by season, species, and physical and biological parameters of the water bodies (Sithithaworn et al., 2007). The metacercarial burden of *C. sinensis*, *O. felineus*, and *O. viverrini*, peaks in spring and summer, summer and autumn, and winter, respectively (Sithithaworn et al., 2007; De Liberato et al., 2011).

After ingestion of the infected fish, the metacercariae (Fig. 2) excyst in the duodenum of the definitive hosts (mammals) and within 30 min the juvenile flukes migrate up through the Ampulla of Vater and the common bile duct into the intra-hepatic bile ducts where they attach to the bile-duct epithelium using their suckers. Then, flukes develop into adults after at least 1 month (Sripa et al., 2007) and operculate eggs ($22\text{--}35 \times 10\text{--}22 \mu\text{m}$) containing the miracidium, are shed with feces (Fig. 1). An adult worm can produce up to 4000 eggs per day (Wykoff et al., 1965; Fig. 3). Fluke metabolic pathways are highly adapted to a lipid-rich diet from bile and/or cholangiocytes as showed by the studies based on the draft genome and transcriptomes of *O. viverrini* and *C. sinensis* (Wang et al., 2011; Huang et al., 2012; Young et al., 2014). When eggs reach the freshwater and are ingested by the first intermediate host (snails), they hatch in the gastrointestinal tract of the snail, and the miracidium develops into a sporocyst in the intestinal wall or in other organs to undergo asexual reproduction. The sporocyst produces rediae which mature in the hepatopancreas within about 17 days. The cercariae, about 5–50 per redia, leave the snail via the siphon into freshwater during the day, when it is warm and sunny, approximately 1–2 months after the snail is infected; however, the duration of development in the snail body is strongly influenced by the water temperature. The free-swimming cercariae, which are characterized by a positive photo- and geo-tropism, have a long tail with a long dorsal and some shorter ventral fins, a finely spined tegument, penetration and cystogenous glands, and a pair of eyespots (Fig. 1). They shed their tail, penetrate fish tissue between the scales (mainly near the fins), and encyst as metacercariae under the skin or in the musculature approximately 3 weeks later. In doing so, the cercariae lose their eyespots and develop a sac-like excretory bladder filled with coarse, refractile granules. The metacercarial stage is usually ovoid ($140 \times 120 \mu\text{m}$)

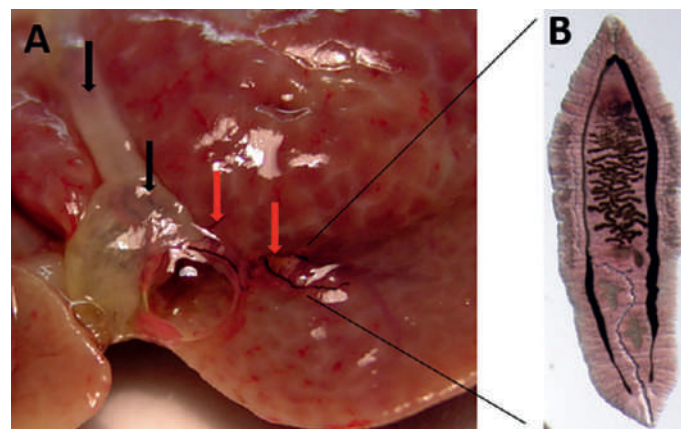


Fig. 3 (A) Liver from an *Opisthorchis felineus* infected hamster in which parasites are visible inside the main bile duct (black arrows) and coming out from it (red arrows). (B) *Opisthorchis felineus* adult kept in culture ($12 \times 2.5 \text{ mm}$) once isolated from the liver of an infected hamster. Photographs are at original magnification $10\times$. Courtesy of Alessandra Ludovisi, Department of Infectious Diseases, Istituto Superiore di Sanità, Italy.

with a thin wall (Fig. 1). The definitive host acquires the infection by eating fermented, raw or undercooked freshwater cyprinid fish containing the infective metacercaria. In humans, flukes can survive for more than 20 years (Attwood and Chou, 1978; Kaewpitoon et al., 2008).

As an example, the life cycle of *C. sinensis* is shown in Fig. 1.

Pathogenesis

The magnitude of the pathology caused by these parasites depends on their number, duration of the infection and susceptibility of the host. The intrahepatic flukes move up to and clog biliary branches causing a mechanical injury by their feeding and migrating activities, both oral and ventral suckers of the fluke hook up the biliary epithelium. Moreover, the excretory/secretory products (ESPs) released by the parasites induce inflammation and stimulate cell proliferation of the biliary epithelium, causing immunopathological responses (Sripa et al., 2018b).

In light *C. sinensis* human infections, the liver appears normal, in massive infections, a localized dilation of the thickened peripheral bile ducts can be seen on the surface beneath the Glisson's capsule. At late stages of infections, *C. sinensis* adults block the biliary passages and bile flow. The congested bile increases hydrostatic pressure, presses on the ductal wall, and causes dilatation (Na et al., 2020). The damaged tissue present edema followed by tissue desquamation mainly in the areas close to the flukes. At later infection stages, there is epithelial hyperplasia that advances to adenomatous hyperplasia in the biliary epithelium with a high number of goblet cells. In chronic infections, epithelial cells proliferate with metaplasias of the biliary epithelial cells into mucin-producing cells. Goblet cells can proliferate to produce many small gland-like structures in the mucosa (adenomatous hyperplasia), leading to an excessively high mucus content in the bile, which in combination with the presence of eggs and worm fragments, causes cholestasis and serves as support for bacterial superinfection and cholelithiasis (Lim, 2011). Secondary bacterial infections, mainly of enteric origin, may occur, and *Escherichia coli* is the pathogen most frequently identified. These alterations may progress to a pyogenic cholangitis, liver abscess, and hepatitis (Na et al., 2020). Cholecystitis may appear associated to the infection, consequently, a poor function of the gallbladder causes precipitation of bilirubinate, calcium carbonate crystals, and mucin on the eggs, which causes stone formation in the gallbladder that can lead to biliary obstruction (Sithithaworn et al., 2007; Qiao et al., 2014). In severe infections, periductal fibrosis extends up to bile capillaries and down to the portal area. The area around the affected bile duct is infiltrated by lymphocytes, plasma cells and eosinophils. As the fibrosis proceeds, the epithelial proliferation appears milder and fecal egg production drops markedly. In some chronic cases, hepatobiliary fibrosis progresses to cirrhosis (Na et al., 2020). Pancreatitis associated to *C. sinensis* infection have been documented (Kim, 1999). Developmental retardation has been reported in *C. sinensis* infected children with heavy infection. These children often present inappetence, diarrhea, malnutrition, anemia, and hepatomegaly (Zhu et al., 1983).

In *O. viverrini* infections followed by ultrasonography and cholecystography in Northeast Thailand, gall bladder wall irregularity, enlargement, and bile sludge has been reported among apparently healthy individuals with moderate infections (Elkins et al., 1996; Mairiang et al., 1992). In heavy *O. viverrini* infections, the liver may be enlarged, its weight may be more than double the normal (3000–3500 g or more), the predominant changes documented included desquamation of the biliary epithelium, epithelial hyperplasia, bile duct hyperplasia, and periductal fibrosis (Riganti et al., 1989; Sripa et al., 2012a). In the large and medium-size bile ducts, the flukes can cause chronic cholecystitis. In case of a superimposed bacterial infection, empyema of the gall bladder may exist. Cholelithiasis is not particularly frequent in *O. viverrini* infections; however, biliary sludge is often seen in heavy infections. The enlargement of the gall bladder is commonly found in ultrasonographic studies (Mairiang et al., 1992, 2012). In chronic infection, periductal fibrosis developed along the infected bile ducts is the most prominent pathological feature and it is implicated, in its advanced form, i.e., as advanced periductal fibrosis (APF), as one of the risk factors for CCA development (Sripa et al., 2012a). Nonfibrotic presentations, such as gallbladder morphology and function, recover after removal of parasites by drug treatment; however, AFP appears to be permanent (Mairiang et al., 1993). Granulomatous inflammation around the parasite eggs is occasionally seen in the gall bladder wall during infection (Riganti et al., 1989). *O. viverrini* infection modulates the microbiota of the biliary tract since there is a documented increased of microbial diversity in *O. viverrini* associated tissues versus non-*O. viverrini* associated tissues, whether tumoral or normal (Chng et al., 2016).

In *O. felinus* human infections, pathological changes seem to be like those induced by the other liver flukes; however, the information is scarce. In patients with heavy or prolonged infections or superinvasion 84% develop duodenal hypertension, 94% gastric hypertension, and 75% duodenogastric reflux that can lead to chronic gastritis and chronic esophagitis (Suvorov et al., 2004). Moreover, regurgitation of intestinal contents into the pancreatic duct can be cause of chronic indurative pancreatitis of the head of the gland. Sonographic studies carried out in *O. felinus* infected people have evidenced disturbances in the gall bladder ranging from dyskinesia to cholestatic syndrome, mainly in the early phase of the disease (Bronshtein et al., 1989). In a patient who underwent liver biopsy, acute inflammatory signs with dilatation of portal spaces and eosinophilic infiltration with lymphocytes and monocytes, were found (Traverso et al., 2012). *O. felinus* infection may cause complications, such as cholangitis, cholecystitis, cholangiofibrosis, hepatic cysts, hepatic abscesses, and pancreatitis. In studies conducted in Western Siberia (Russia), out of 4756 people chronically infected with *O. felinus*, 1170 (24.6%) required surgical intervention for complications associated to the infection (cited by Pakharukova and Mordvinov, 2016). Renal alterations have been described in human infected with *O. viverrini* (Boonpucknavig and Soontornniyomkij, 2003) and *O. felinus* (Lapteva, 1990). Analyses of microbial communities did not reveal differences in bacterial richness between participants infected with *O. felinus* and noninfected individuals (Saltykova et al., 2016).

From liver fluke infections to cancer

Epidemiological studies revealed that current or past *O. viverrini* and *C. sinensis* infections are the major risk factor of intrahepatic CCA (Honjo et al., 2005; Choi et al., 2006). Moreover, coinfections with other carcinogenic microbes and local and systemic chronic inflammation influence the process (Brindley et al., 2015). However, the highest risk factor is the elevated serological positivity associated to the host genetic polymorphism of glutathione S-transferase mu 1 (GSTM1) gene. Other risk factors have been specified as area of residence, alcohol consumption, age (older than 60), sex (male), smoking and consumption of fermented raw fish (Fried et al., 2011). Sripa and coworkers associated elevated plasma IL-6 with increased risk of advanced fibrosis and cholangiocarcinoma in individuals infected with *O. viverrini* (Sripa et al., 2012b).

Even if the understanding of the mechanisms leading from liver fluke infection to CCA is not complete, the general mechanisms proposed to contribute to CCA through chronic infection include mechanical damages of the parasites, toxic effects of their excretory/secretory products (ESPs) to the biliary epithelia and the immunopathology due to infection-related inflammation.

Differential transcriptional and proteomic profiles in human CCA cells treated with *C. sinensis* ESPs showed upregulation of carcinogenesis and cell proliferation related genes and down regulation for genes related to apoptosis (Pak et al., 2009a,b, 2014; Kim et al., 2010; reviewed by Qian et al., 2016). As *O. viverrini* ESPs, *C. sinensis* ESPs also trigger enzymatic free-radical generation through the activation of TLR isoforms, which in turn lead to NF- κ B-mediated inflammatory processes, inducing the increase in mRNA and protein expression of the pro-inflammatory cytokines IL-1 β and IL-6, suggesting that continuous disruption of host redox homeostasis during liver fluke infection may create an environment for the development of an advanced hepatobiliary disease, including chronic-inflammation-associated CCA (Kim et al., 2012; Nam et al., 2012; Bahk and Pak, 2016; Maeng et al., 2016). Recent studies show that *C. sinensis* ES enhance the activation of hepatic stellate cells (HSC) by a cross-talk of TLR4 and TGF- β /Smads signaling pathway, which turns in secretion of TGF- β 1 from activated HSCs and other cells. TGF- β 1 is an essential mediator of fibrogenesis (Li et al., 2020). These studies indicate that *C. sinensis* ESPs may function as inducers of multiple oncogenic pathways (Pak et al., 2014).

Experimental studies demonstrated that *O. viverrini* ESPs can be internalized into liver cell lines by endocytosis, driving IL-6 secretion and inducing proliferation of liver cells (H69 and CCA lines) but not intestinal (Caco-2) cells (Chaiyadet et al., 2015a). Within *O. viverrini* ESPs, extracellular vesicles (EVs) are released as for communication with neighboring host cells. EVs promote inflammation yet simultaneously act as modulator, since cholangiocyte protein expression (H69) undergoes dysregulation after coculture with EVs (Chaiyadet et al., 2015b). EVs are highly enriched in tetraspanins (TSPs), proteins that interact with transmembrane and cytosolic signaling proteins (Hemler, 2005). Antibodies to a TSP located on the surface of *O. viverrini* EVs blocked the ability of fluke EVs to be internalized by cholangiocytes and suppress the cell proliferation and secretion of IL-6. *O. viverrini* ES products comprise a complex mixture of proteins, some of which have homologs in the human host that are associated with cancer (Mulvenna et al., 2010). Among them, the Ov-GRN-1 (Smout et al., 2009) has a sequence like that of the mammalian growth factor granulins and may mimic the action of IL-33 as epithelial mitogen for cholangiocytes in the development of CCA, priming type 2 innate lymphoid cells to induce proliferation of neighboring cholangiocytes by the release of IL-13 (Li et al., 2014; Sripa et al., 2018a). When internalized by human cholangiocytes, Ov-GRN-1 can induce gene and protein expression changes associated with cancer pathways, as the epithelial mesenchymal transition (EMT) phenotypes (Smout et al., 2015). A glutathione S-transferase (OvGST) has been also identified in ESPs, which had a dose-dependent proliferative effect on murine fibroblasts (NIH-3T3) and on the non-tumorigenic human bile duct epithelial cells MMNK1 cells (Daorueang et al., 2012).

Proteomic identification of the ESPs released by *O. felineus* allowed the identification of 37 proteins, among them, the major component is a glutathione-dependent prostaglandin synthase (Lvova et al., 2014), which seems involved in *O. felineus* pathogenesis, including the precancerous lesions (Pakharukova et al., 2019). Even if no recognized association has been reported for *O. felineus* and CCA, epidemiological and clinical data in humans and animals suggest that *O. felineus* can be the cause of neoplasia. Thus, in Russia, the highest incidence of bile duct cancer in humans was documented in the same area (i.e., Tyumen oblast) with the highest prevalence of *O. felineus* infection in humans (Mordvinov et al., 2012). In Italy, pre or cancerous lesions have been found during autopsy in *O. felineus* naturally infected animals, in particular cats and dogs (Pozio et al., 2013). In addition, there is an increase of experimental data supporting information for a role of *O. felineus* infection as a risk for CCA (Lvova et al., 2012; Maksimova et al., 2017; Kovner et al., 2019).

Clinical manifestations

Most of the reported liver fluke infections are dormant and the infected people are asymptomatic, except for the patients with very heavy infections and for those presenting complications.

Clonorchis sinensis

Patients with mild infections, i.e., with fewer than 100 flukes, have few symptoms as general malaise, abdominal discomfort, and diarrhea. In 10–40% of patients, peripheral eosinophilia accompanies a fluctuating jaundice that is usually obstructive. Moderate infection (generally fewer than 1000 flukes) presents with fever and chills, as well as fatigue, anorexia, diarrhea, weight loss, discomfort, and abdominal distension. Up to 20,000 flukes may be present in patients with severe disease, who present with acute

right upper quadrant pain, often superimposed on the signs and symptoms seen in moderate infections. In the late stage of severe cases, jaundice, diarrhea, ascites, and edema, can occur. Differential diagnoses of clonorchiasis include acute or chronic hepatitis, cancer along the bile ducts, hepatolithiasis with recurrent pyogenic cholangitis, sclerosing cholangitis, Caroli's disease, and *Fasciola hepatica* infection (Choi et al., 2005, 2006; Keiser and Utzinger, 2009).

Opisthorchis viverrini

In patients with a severe infection, the clinical signs and symptoms include lassitude, and nonspecific abdominal complaints such as anorexia, nausea, vomiting, abdominal discomfort, diarrhea, indigestion, weight loss, ascites, and edema (Furst et al., 2012). There is no report of acute *O. viverrini* infections. Most subjects with opisthorchiasis have non-specific symptoms or no symptoms at all. Intrahepatic duct stones and recurrent suppurative cholangitis is not a common manifestation of opisthorchiasis caused by *O. viverrini* but can be present. Obstructive jaundice can be presented alone, with fever, or with acute abdominal complications. Since jaundice is the main clinical manifestation of the CCA, whenever jaundice and ascending cholangitis are detected in endemic areas, the fluke-related CCA is suggested (Uttaravichien et al., 1999). Non-jaundiced patients may present dyspeptic pain, anorexia, weight loss, right upper abdominal mass (Chunlertrith et al., 1992).

Opisthorchis felineus

The clinical manifestations during the acute stages of the infection in humans are characterized by fever, abdominal pain, headache, asthenia, arthralgia, lymphadenopathy, skin rash, diarrhea, nausea, hepatitis-like symptoms, eosinophilia, and increased liver enzymes (Mairiang and Mairiang, 2003; Mairiang, 2017; Mordvinov and Furman, 2010; Traverso et al., 2012; Pozio et al., 2013). These clinical features may lead to misdiagnosis as acute viral hepatitis (Belova et al., 1981) and rheumatic disease (Gordon et al., 1984). Acute opisthorchiasis occurs early in infection and may be associated with primary exposure to a large dose of metacercariae (Furst et al., 2012). In endemic regions such as Ukraine, Russia and Siberia, where people frequently consume raw fish, the number of worms in the bile ducts can be very high, inducing chronic infection, which is characterized by anorexia, dyspepsia, dryness and bitter taste in the mouth, fatigue, intolerance to greasy foods, nausea, and pain in the right hypochondrium (Mordvinov and Furman, 2010). In non-endemic areas, such as EU countries, most infected persons show pauci-symptomatic or asymptomatic forms and in some cases, clinical disease and the seroconversion can develop up to 2 months after infection. In Italy, about 1/3 of infections was asymptomatic (Pozio et al., 2013). In symptomatic persons, during the acute stage, the more frequently observed signs and symptoms were asthenia, headache, abdominal pain, and fever, which started about 2–3 weeks after the infection (Armignacco et al., 2008; Traverso et al., 2012; Pozio et al., 2013). Jaundice was not observed. The main laboratory findings were leukocytosis, eosinophilia, increased transaminases (Armignacco et al., 2008; Traverso et al., 2012). In an outbreak in Italy, in persons not diagnosed during the acute phase and then not treated, there was a spontaneous remission of the clinical symptoms within 2–3 months of infection, although the liver flukes were still present and produced eggs (Armignacco et al., 2013).

Immune response

During infection, liver flukes are exposed to the biliary epithelium and neither mucosal nor tissue immune responses appear to cause parasite death or protect the host against new flukes, as evidenced by the persistence of human infections for decades in the body and rapid reinfection following treatment (Sripa et al., 2018a).

Humans are susceptible to infection, re-infection and superinfection by *C. sinensis* (Hong and Fang, 2012). However, rats, mice, and rabbits resist against re-infection and super-infection of *C. sinensis* (Sohn et al., 2006). Only a few worms survive as small and immature forms in the bile duct of re-infected or super-infected rats; however, the rat resistance has not been observed in immune-suppressed or nude (athymic) rats (Zhang et al., 2008a,b). In FVB mice, which are susceptible to *C. sinensis*, primary infection induced elevated levels of Th2 cytokines (IL-4, IL-5) and by 70-fold increase of Kupffer cells in the liver compared to non-infected controls. These animals were resistant to re-infection and presented an increase of B cells in biliary lymph nodes as well as increased serum levels of *C. sinensis*-specific IgG1 and IgG2a (Kim et al., 2017). Using the same strain of mice, Yu et al., reported an enhanced IgE's binding capacity for B cells after infection, which suggested the roles of IgE as a Th2 response amplifier and its protective role in parasite infection (Yu et al., 2019). These studies are in line with previous investigations, in which specific antibodies to *C. sinensis* were produced in serum and bile of experimentally infected rats, mainly IgE in serum and IgA in bile, and their levels correlated with resistance (Wang et al., 2009; Zhang et al., 2008a,b). Splenectomized rats show resistance to *C. sinensis*, which suggests that the spleen is not a key organ in the development of resistance (Zhang et al., 2008a). Zhao et al., showed that *C. sinensis* adult-derived proteins inhibited LPS-induced maturation of bone marrow-derived dendritic cells and induced the differentiation of naive T cells into Th2 cells though the production of IL-10. They also confirmed in the same study that *C. sinensis* proteins initiated Th2-cell skewing immune responses to markedly elevate the production of IL-13 and IL-4, which are closely associated with the generation of liver fibrosis (Zhao et al., 2018). Previously, Zhang and colleagues reported an association between increased hepatic Th2 and Treg cell subsets and biliary fibrosis in *C. sinensis*-infected mice (Zhang et al., 2017).

O. viverrini induces a strong antibody response in the human host, with IgG being the predominant subclass followed by IgA and IgE. The specific immune response is detectable in serum and bile, and it comes along with a marked increase of total IgE in serum

(Wongratanacheewin et al., 1988). Antibodies recognized mainly proteins of 45, 52, 56, 59, 65, 69, 75 and 81 kDa (Wongratanacheewin et al., 1991), which are associated to the surface tegument, reproductive organs, eggs and ESPs (Boonpucknavig et al., 1986; Wongratanacheewin and Sirisinha, 1987). The protein profiles and immunogenicity of *O. viverrini* and *Clonorchis sinensis* are similar except for a 16-kDa protein found in *O. viverrini* that seems to lack in *C. sinensis* (Sirisinha et al., 1990).

In humans, there is a significant positive correlation of the IgG antibody titer, the intensity of the infection and the severity of disease, whereas serum IgA levels correlate more closely with age than with intensity of infection (Elkins et al., 1991; Haswell-Elkins et al., 1991; Pinlaor et al., 2012). In infected individuals, the level of serum antibodies decreased slowly but remained elevated for several months after the praziquantel treatment (Ruangkunaporn et al., 1994).

The fact that prevalence and intensity of infection in humans do not decline with age in the absence of treatment, arguing against the existence of exposure-related resistance (Upatham et al., 1984; Haswell-Elkins et al., 1991), suggests that reinfection occurs, despite the strong cellular and humoral immune response elicited by the parasites as it has been observed in human infections (Wongratanacheewin et al., 1988) and immunized mice (Wongratanacheewin et al., 1991). However, experimental studies in *O. viverrini* infected hamsters have shown a limited protection of the animals, which was conferred by previous infection or immunization (Reviewed by Sripa et al., 2018a).

The involvement of T cells in pathogenesis is supported by the fact that T-cell-deprived *O. viverrini* infected hamsters show less severe damage of the bile duct tract (Flavell and Flavell, 1986). In these animals, the parasite stimulates the expression of the Th1-inducing cytokine, IL-12, in the early stage of infection (2 weeks post infection), whereas the expression of the Th2-inducing cytokine, IL-4, and the regulatory cytokines, TGF- β and IL-10, are significantly increased in chronic and/or heavy infections, in particular at mucosal level (Jittimanee et al., 2007). This may indicate their role in disease progression as they may inhibit T-cell proliferation, leading to prolonged worm survival (Maizels and Yazdanbakhsh, 2003), and thus blocking an effective immunity (Jittimanee et al., 2012; Kaewraemruaen et al., 2016).

Haswell-Elkins et al. reported increased level of urinary nitrates and salivary nitrites in *O. viverrini*-infected subjects in Northeast Thailand that decreased following treatment with praziquantel (Haswell-Elkins et al., 1994). They assumed that specific T cells synthesize nitric oxide (NO) that reacts with superoxide anion (O_2^-) to form peroxynitrite, a highly reactive species that causes nitrative and oxidative DNA damage to the cells. Further, a 10-fold greater potential for endogenous nitrosation among people living in endemic areas with positive antibody titers for *O. viverrini* as compared to uninfected controls has been demonstrated (Pinlaor et al., 2005). Nitrosamines play a very important role in inflammation-associated carcinogenesis, especially if they are generated in situ and their production is both chronic and located in close proximity to cells containing P450 enzymes which can metabolize the nitrosamine to DNA methylating agents (Sripa et al., 2012a,b). As other liver flukes, *O. felinus* elicits a humoral immune response already detectable from the third week after infection (Armignacco et al., 2008, 2013; Traverso et al., 2012). The main antigens recognized by human antibodies have been associated to the tegument, muscles, uterus, gonads, intestine and eggs of the liver fluke, as showed by immune electron microscopy. These findings have led to the conclusion that the surface structures of liver flukes stimulate a low B-cell immune response, whereas the structures linked to the ES system of the parasite and their products contain main antigens able to induce B-immune response in man (Kotelkin et al., 2001).

Diagnosis

In non-endemic areas, liver fluke infections are very difficult to diagnose due to the lack of pathognomonic signs and symptoms and the decreasing number of professionals able to identify opisthorchiid eggs in stool samples (Yossepowitch et al., 2004; Pozio et al., 2013). In endemic areas, the presence of signs revealing injury of the bile ducts by ultrasound or other imaging techniques is suggestive of infection (Sithithaworn et al., 2007). The clinical diagnosis of liver fluke infections should be confirmed by the detection of eggs in stools.

Parasitological diagnosis

Fecal examinations by the Kato-Katz (KK) and the formalin–ether concentration technique (FECT) have been frequently used for diagnosis of liver fluke infections. At a worm burden >20 , the rate of egg detection by both methods is comparable with the worm recovery (sensitivity 100%), as it was assessed in autopsied subjects grouped according to the number of worms recovered from their liver (Sithithaworn et al., 1991). However, FECT seems more sensitive than KK for the diagnosis of extremely low burden infections and for the follow-up after treatment (Sithithaworn et al., 2007). Multiple stool sampling or the combination of different diagnostic tests should be considered to enhance diagnostic accuracy (Bergquist et al., 2009; Johansen et al., 2010). Promising results have been obtained with the multivalent flotation method (FLOTAC), which allows considerably larger amounts of feces to be examined and showed a considerably higher sensitivity than the classical methods (Cringoli et al., 2010). It is necessary to consider that there are several species of foodborne trematodes which have similar egg morphology (as Opisthorchiidae, Heterophyidae and Lecithodendriidae families), consequently, recognition of the egg morphology is essential for a correct diagnosis (Chai and Lee, 2002).

Detection of parasite antigens in stools

ELISAs for antigen detection in stools and urine from infected persons have been developed using monoclonal antibodies (MAb) to different recombinant or native proteins from *O. viverrini* (Chaicumpa et al., 1992; Sirisinha et al., 1995; Worasith et al., 2015) and *C. sinensis* (Nie et al., 2014). A sandwich ELISA using recombinant *O. viverrini* cathepsin F chicken IgY in combination with rabbit IgG antibody to the somatic *O. viverrini* antigens for coproantigen detection was applied for the diagnosis, exhibiting sensitivity and specificity of 93.3% and 76.7%, respectively (Teimoori et al., 2015).

Detection of parasite DNA in stools

The detection of parasite DNA, whether by PCR and sequencing or by Real Time PCR is very useful to identify cryptic infections. There are species specific PCR tests to identify *O. viverrini* (Ando et al., 2001; Wongratanacheewin et al., 2002), *O. viverrini* and *C. sinensis* (Le et al., 2006; Sato et al., 2009; Kim et al., 2009; Huang et al., 2012), and *O. felineus* (Pauly et al., 2003; Müller et al., 2007) from the different parasite stages. The sensitivity can vary depending on the test, reaching 100% in moderate to severe infections (eggs per gram > 1000); whereas in light infections (eggs per gram < 200), the sensitivity drops to 68.2% (Wongratanacheewin et al., 2002). The presence of PCR inhibitors in human fecal specimens can strongly reduce PCR sensitivity.

Serological diagnosis

The time between infection and the detection of antibodies in serum ranges from 3 to 8 weeks for *O. felineus* infections (Armignacco et al., 2008, 2013; Traverso et al., 2012; Pozio et al., 2013), and it is reported from 2 to 4 weeks for *O. viverrini* infection in hamsters, therefore, the detection of specific antibodies, mainly IgG, has been considered as a complementary tool to establish a definitive diagnosis of the infection (Sripa and Kaewkes, 2000).

The serodiagnosis of liver flukes infections has been attempted by different immunodiagnostic assays, producing results of varying degrees of sensitivity (Pinlaor et al., 2012). However, the specificity is limited by the cross-reactive nature of the antigens (Wongratanacheewin et al., 1988; Haswell-Elkins et al., 1991; Sirisinha et al., 1995; Sakolvaree et al., 1997; Sawangsoda et al., 2012). Among infected people, serum titers have been found to be higher in CCA than in with cholangitis cases. The detection of IgG and IgG4 in serum yielded good sensitivities (99.2% and 93%, respectively) but poor specificity (23.1% and 29.6%, respectively); whereas the detection of IgG and IgG4 in urine had much lower sensitivity (43% and 45.9%, respectively) but better specificity (64.5% and 67.2%, respectively) (Tesana et al., 2007).

ELISA is widely used in Korea for *C. sinensis* infections (Choi et al., 2003; Lee et al., 2010), with 93.1% sensitivity when ESPs are used as antigens and 87.8% when crude extract is used (Choi et al., 2003). Several recombinant proteins from *C. sinensis* have been produced and identified (Shen et al., 2009) and shown to be sensitive and specific for serodiagnosis of clonorchiasis, but not enough to replace crude extract (Hong and Fang, 2012).

According to Meniavtseva et al. (1996), immunoenzymatic tests show the best performance among the serological tests for the diagnosis of opisthorchiasis. An ELISA based on ESPs has been validated for *O. felineus* infection in humans from low endemic areas (Gómez-Morales et al., 2013).

Human treatment

Currently, the drug of choice to treat people with clonorchiasis or opisthorchiasis is a pyrazinoisoquinoline named praziquantel (2-[cyclohexyl(oxo)methyl]-3,6,7,11b-tetrahydro-1H-pyrazino[2,1-a]isoquinolin-4-one). Praziquantel increases the permeability of the worm tegument, inducing a muscle contraction and a vacuolization of the tegumental syncytium of the flukes. This drug is rapidly absorbed and peak serum levels occur 1–3 h after administration; then, it is excreted with bile and urine within 24 h. According to WHO, the recommended dosage of praziquantel is 25 mg per kg of body weight, 3 times a day on 2 consecutive days or 40 mg per kg of body weight in a single administration with a repeat interval of 6 or 12 months (WHO, 2011). This treatment gives 100% and 80–85% cure rate for *O. viverrini* and *C. sinensis* infection, respectively (WHO, 1995).

The second drugs of choice are the benzimidazole-derivatives mebendazole and albendazole. However, these two drugs are effective only when given over a long period or at high doses (Armignacco et al., 2008). The treatment of *O. felineus* infections with albendazole (10 mg/kg body weight daily in two doses for 7 days) failed to eradicate all the flukes from one patient involved in an outbreak in Italy (Armignacco et al., 2013). Tribendimidine is another candidate for liver fluke infections treatment. It is an L-subtype nicotinic acetylcholine receptor antagonist (Hu et al., 2009; Xiao et al., 2013). Clinical studies of tribendimidine conducted in Lao PDR showed efficacy similar to that of praziquantel, since the egg reduction rates of tribendimidine and praziquantel are 99.3% and 98.4%, respectively (Soukhathammavong et al., 2011).

Prevention and control

Prevention and control measures should be proportionate to the epidemiological situation. In high endemic areas, morbidity can be prevented or controlled by treatment, health education, improved sanitary conditions, and implementation of food safety measures (WHO, 1995; Sithithaworn et al., 2007). Successful results were obtained only when a mass praziquantel treatment was combined to an extensive improvement of the health care system and to socioeconomic development (Jongsuksuntigul and Imsomboon, 2003). The limited level of natural protective immunity generated after infection makes the development of a vaccine for the prevention of these zoonoses challenging. Major recent advances in post-genomic technologies have aided new antigen discovery, but liver flukes have largely resisted successful vaccine development efforts. Successful vaccination of hamsters has, nevertheless, been reported using *O. viverrini* exosome-like EVs and recombinant tetraspanin proteins derived from the surface of EVs (revised by McManus, 2020).

To prevent clonorchiasis and opisthorchiasis, freshwater fish should be cooked until the core reaches 65 °C for at least 1 min (EFSA, 2010). According to Lloyd and Soulsby (1998), metacercariae may be killed by freezing at −10 °C for 5–70 days, depending on the size of the fish. Metacercariae of *O. felinus* can survive in smoked fish causing human infections (Yossepowitch et al., 2004). Marinating does not kill *O. felinus* metacercariae present in tench muscles (Armignacco et al., 2008, 2013; Traverso et al., 2012).

Closing remarks

Diseases caused by liver flukes remain important threats to public health due to the high morbidity they cause in many parts of the world, mainly in developing areas. National sampling surveys calculate prevalence rates in which counties and small villages are absent, thus hampering control activities. More emphasis should be placed on surveillance, so that control interventions can be tailored to specific settings. Currently, treatment is entirely dependent on a small number of effective drugs. Whereas vaccination is recognized as one of the most sustainable control options, development of protective and therapeutic vaccines against these pathogens has proven exceptionally difficult due to the complexity of the life cycles of flukes and their capacity to evade host immunity. The strategy for control of foodborne infection clearly requires collaboration among all sectors of public health. Consumer education is crucial and once it is recognized, the infected person should be monitored for the development of bile duct cancer.

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Cryptosporidium

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Introduction

The protozoan parasite *Cryptosporidium* causes a disease of remarkable global socioeconomic significance. The parasite affects both humans and animals causing acute gastroenteritis, abdominal pain, and diarrhea (Dillingham et al., 2002). Though the exact location of parasite varies between species, it most frequently abodes the gastrointestinal (GI) tract (Noordeen et al., 2002). The infection mainly affects children, resulting in chronic diarrhea which leads to child development impediment (Khalil et al., 2018; Korpe et al., 2018). Although the condition only symptomizes as a self-limited diarrhea in healthy adults, it manifests as a prolonged and persistent diarrhea in high risk associated patients such as immunocompromised, including HIV/AIDS patients (Kurniawan et al., 2013; Janssen and Snowden, 2020).

Even though Edward Tyzzer in 1907 discovered the genus *Cryptosporidium* spp., by first detecting *Cryptosporidium muris* in the gastric glands of laboratory mice, only by early 1980s the organism was observed as a pathogen. The parasite was named *Cryptosporidium* because of the lack of presence of sporocysts in the oocysts, a characteristic present in other Coccidians (Tzipori and Widmer, 2008). Also, *Cryptosporidium* organisms infect only the brush border of the intestinal epithelium, as opposed to other Coccidian parasites which infect deeper tissue (Feng et al., 2018; Janssen and Snowden, 2020).

Cryptosporidium spp. are categorized under the phylum Apicomplexa which also includes other well-known genera such as *Plasmodium* and *Toxoplasma*. Currently more than 40 species of *Cryptosporidium* have been found and are categorized as most and less frequently detected ones. The ones belonging to first category are *Cryptosporidium hominis* and *Cryptosporidium parvum*, and ones in the latter are *Cryptosporidium meleagridis*, *Cryptosporidium felis*, *Cryptosporidium canis*, *Cryptosporidium ubiquitum*, *Cryptosporidium cuniculus*, *Cryptosporidium muris*, and *Cryptosporidium viatorum* (Feng et al., 2018; Innes et al., 2020). Though so many species have been identified, more than 90% of cryptosporidiosis cases in humans is caused by *C. parvum* and *C. hominis*, and a few cases of *C. meleagridis* infections have also been reported (Xiao and Ryan, 2004). However, in immunocompromised individuals, *C. canis*, *C. felis*, *C. meleagridis*, *C. suis*, *C. muris*, and *C. andersoni* species have been detected (Fayer, 2010; Pumipuntu and Piratae, 2018). Most of the zoonotic infections in humans from cattle show the presence of *C. parvum* (Ryan et al., 2014). Absence of host specificity of *Cryptosporidium* spp. is responsible for infection in multiple hosts, such as mammals, marsupials, birds, reptiles, and fish (Zahedi et al., 2015; Leitch and He, 2012; Gerace et al., 2019).

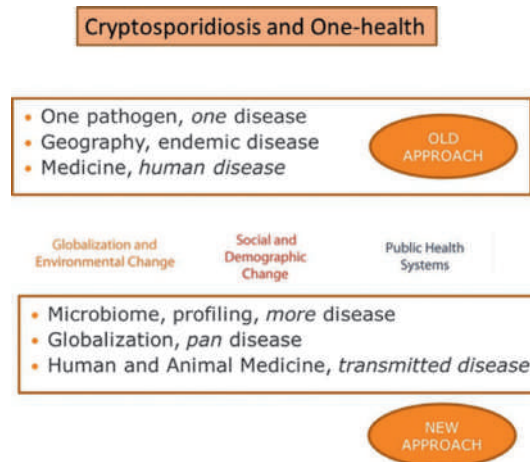


Fig. 1 The new holistic approach of the systems medicine places cryptosporidiosis within the framework of the one health medicine considering the protozoan *Cryptosporidium* as member of the gut microbiota ecosystem, namely parasitome.

Cryptosporidiosis is primarily transmitted through the fecal-oral route by means of a zoonotic or anthroponotic modality, via contaminated water or food (Putignani and Menichella, 2010). Waterborne cryptosporidiosis outbreaks are the outcome of contaminated municipal water and recreational water (swimming pool, lakes, ponds). This occurs as *Cryptosporidium* in the oocyst stage of its life cycle can withstand disinfection which includes chlorination, aiding the parasite to sustain for extended duration in the environment. Foodborne outbreaks are also observed but with less frequency (Ryan et al., 2018). Apart from water and food borne outbreaks, transmission of the parasite can happen through hospital personnel, daycare centers, contaminated soil and carrier insects such as cockroaches and houseflies (Alpert et al., 1986; Dillingham et al., 2002). Inhalation of oocysts has been described as another mode of transmission, especially in children and immunocompromised patients (Tzipori and Ward, 2002). Oocysts have been detected in sputum and bronchial aspirates (Clavel et al., 1996; Mor et al., 2010). Considering the modes of transmission and travel hygiene parameters, *Cryptosporidium* is also described as the cause of traveler's diarrhea (Vanathy et al., 2017).

Nitazoxanide is currently the only FDA, EMA and PMDA approved drug for the treatment of cryptosporidiosis in most immunocompetent people, it helps to resolve diarrhea from the third or fourth day of treatment. However, the drug does not portray promising results in young children and immunocompromised individuals (Rossignol et al., 2001; Amadi et al., 2002; Bones et al., 2019).

Nowadays, the holistic approach of system medicine in infectious diseases can assist also in discovering new clinical traits of cryptosporidiosis, considering the pathogen *Cryptosporidium* spp. not as *single pathogen* but as a *pathogen within the complexity of the gut microbiota ecology*. This novel approach may provide new insights on the role of host response and environment in the physiopathology of the disease, contributing to its understanding and clinical management. The cryptosporidiosis can therefore be reinterpreted in term of the *one health medicine* perspective rather than in term of classical *human medicine* (Fig. 1).

Epidemiology

In the early spring of 1993, a widespread outbreak of acute watery diarrhea was registered among the residents of Milwaukee (Mac Kenzie et al., 1994). Some residents had confirmed *Cryptosporidium* infection and a sample of those presented with acute watery diarrhea consistent with *Cryptosporidium* infection. Authors investigated the Milwaukee water-treatment plants to perform tests for enteric pathogens, including *Cryptosporidium* oocysts that were identified in water from ice made in the Southern Milwaukee plant in the same period. The rates of isolation of other enteric pathogens remained stable, but there was more than a 100-fold increase in the rate of isolation of *Cryptosporidium*, associated to clinical manifestations included watery diarrhea, abdominal cramps, fever, and vomiting. Remarkably, up to 403,000 people had watery diarrhea attributable to this massive outbreak caused by *Cryptosporidium* oocysts that passed through the filtration system of one of the city's water-treatment plants. This report actually opened the avenue to the molecular studies on cryptosporidiosis linked to water- and food-borne transmission.

Nowadays, during the last couple of years in the global scenario, the number of reported cases of cryptosporidiosis has increased to three cases per 100,000. However, clinical symptoms estimate the actual frequency of infection to be 100 times higher than the number of reported cases (Shrivastava et al., 2017). Among children from underdeveloped countries such as Brazil, Venezuela, Indonesia, Thailand, South Africa, Ghana, India, and Bangladesh, who suffer from diarrhea, cryptosporidiosis accounts for 3–13% of cases. Whereas in children from industrialized countries such as Britain, United States, Canada, Australia, and Denmark who experience diarrhea, the *Cryptosporidium*-related diarrhea falls between 1% and 4%. Children are more prone to the infection as their immune system is not well developed. Early age and malnutrition contribute to immune system impairment state, hence favoring cryptosporidiosis (Vohra et al., 2012). Indeed, incidence of the GI cryptosporidiosis is significantly lower in industrialized countries

compared to low income countries. This occurs because there still exists many people in the latter countries, who are not privileged to basic prerequisites such drinking water and sanitation facilities (Shoultz et al., 2016; Gerace et al., 2019).

Generally, cryptosporidiosis is associated with combined primary immunodeficiency disorders which are inherited or *de novo*. Only a few studies on the association are available to understand the dangers of the infection and they do not portray the risk and prognosis of the disease in the primary immunodeficient individuals (Wolska-Kusnierz et al., 2007; Jo et al., 2002; Gomez Morales et al., 1996; Hayward et al., 1997; Jacyna et al., 1990; Kocoshis et al., 1984; Levy et al., 1997). Prevalence of cryptosporidiosis in the case of HIV infected in Africa is 3.8–73.6% (Tumwine et al., 2005). Whereas in the South Asia India has a prevalence ranging from 4.7% to 56.5% and Nepal has 10.7%, in Southeast Asia, Thailand has a prevalence rate of 8.8–34.4%, Cambodia 45%, Malaysia of 3–23%, and Indonesia 52.5%. In the middle Eastern Asia, Iran has a prevalence of 0.9–26.7% while in the Eastern Asia Korea has 10.5% (Iqbal et al., 2012). The prevalence is reported from 8% to 39% in European countries, 3.5% to 11.9% in North America, and 4% to 22.8% in South America (Iqbal et al., 2012).

In 2016, reports in the EU/EEA by 21/32 countries showed 3.8 confirmed cases per 100,000 population (ECDC, 2016). A study conducted in UK reports that for every reported case there were approximately 8.2 undiagnosed cases in the community (Tam et al., 2012). The occurrence of *Cryptosporidium* infection was also found to be 76.66% in malignant and 15% in nonmalignant patients. Immunosuppressive therapy in malignant patients contribute to the increase in infection (Al-Warid et al., 2012). Organ transplant recipients are also at risk because *Cryptosporidium* was observed as one of the most common infections among them (Hunter and Nichols, 2002; Vanathy et al., 2017). Interestingly, in a recent meta-analysis on parasitic diseases affecting solid organ transplantation recipients, intestinal protozoan parasites were detected in the 38.6% of cases published in the period 1996–2016, with *Cryptosporidium* spp. the most reported (Fabiani et al., 2018).

Cryptosporidium are omnipresent, except in Antarctica and infection occurrence is found to be high during the warm and moist months (White, 2020; Painter et al., 2016). In the United States, the infection commonly occurs from July through September. In England, the incidence occurs in separate peaks, in the spring where the disease is associated with *C. parvum* and farm animals, and the fall where *C. hominis* plays a role with a mode of transmission via recreational water (Chalmers et al., 2011, 2019). Factors such as differences in access to health care, sample processing, diagnostic practice and variation in reporting requirements, gives rise to a marked variability to the prevalence of cryptosporidiosis, thus making interpretation of surveillance data within and between countries difficult (Cacciò and Chalmers, 2016; Innes et al., 2020).

The *Cryptosporidium* infective stage

The thick walled oocyst is the infective agent in *Cryptosporidium* spp. The resistance to extreme environmental conditions can be attributed to the complex double layer glycocalix-lipid-protein matrix of the oocyst wall (Templeton et al., 2004). The outer glycocalix comprises of heteropolymers, followed by a middle lipid layer and an inner protein layer. The lipid layer has been earlier described to contain phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin, and cardiolipin (Jenkins et al., 2010). The protein layer consists of multiple *Cryptosporidium* oocyst wall proteins (COWPs) with cysteine-rich domains, giving rise to extensive disulfide bonds that provide rigidity to the structure (Spano et al., 1997). To top it all, a double layer is formed during oocyst maturation, where the outer wall is constructed first, and then the inner wall (Templeton et al., 2004).

Life cycle

The life cycle of *Cryptosporidium* has several developmental phases, completed within the GI tract of a single host (monoxenous life cycle) (Current and Garcia, 1991). The phases begin with asexual multiplication, followed by sexual reproduction.

Upon ingestion of oocysts by a susceptible host, excystation occurs, after forming sutures on the oocyst wall thereby releasing four infective sporozoites, usually in the upper small intestine. Excystation is known to be triggered by various factors including temperature, pH, bile salts and pancreatic enzymes (Bouazid et al., 2013). The motile sporozoites glide over the host enterocytes, and the organelles (rhoptries and micronemes) help attach it to the plasma membrane via mucin-like glycoproteins and thrombospondin-related adhesive proteins (Wetzel et al., 2005; Wanyiri and Ward, 2006; Chatterjee et al., 2010). This facilitates internalization resulting in the recruitment of vacuoles within the cell, forming an intracellular but extra-cytoplasmic parasitophorous vacuole at the apical surface of the enterocyte (Huang et al., 2004). Triggered by certain host and parasite driven factors, the sporozoite within the vacuole de-differentiates, and then differentiates undergoing morphological changes, finally transforming into a replicative trophozoite (Smith et al., 2005; O'Hara and Chen, 2011; Edwinson et al., 2016). The parasite is supplied with energy and nutrients via a tubular “feeder organelle” that connects it to the host cytoplasm (Huang et al., 2004; Umemiya et al., 2005).

The trophozoites then undergo asexual multiplication, known as merogony, to generate a type I meront containing 6–8 type I merozoites. The type I merozoites are similar to sporozoites and escape the parasitophorous vacuole to infect nearby host cells and develop into trophozoites, thus continuing the asexual life cycle and propagating the infection.

Alternatively, some of the type I merozoites can develop into type II meronts, containing 4 type II merozoites. These type II merozoites invade cells and undergo gametogony, differentiating into either microgamonts or macrogamonts. The microgamont produces 16 non-flagellated microgametes by nuclear division, which bud-off from the microgamont and fertilize an adjacent

macrogamont, forming a diploid zygote. Recent studies show that the class II membrane fusion protein hapless2 (HAP2) is essential for gamete fusion (Tandel et al., 2019). The zygote further multiplies twice in an asexual manner, termed as sporogony, to produce an oocyst containing four sporozoites. The resulting oocyst can be of two types: thin-walled or thick-walled (Thompson et al., 2008). The thin-walled oocyst excysts and autoinfects the host, capable of causing persistent and chronic infection. Whereas, the thick-walled oocysts are released into the environment through feces, allowing it to infect other susceptible hosts.

Molecular pathogenesis

The parasite life cycle within the host can result in tissue damage and loss of function of the intestinal barrier. In vitro studies report disruption of tight cell junctions, increased permeability, mal-absorption, release of lactate dehydrogenase, and increased rates of cell death (Adams et al., 1994). Moreover, villus atrophy, crypt hyperplasia, infiltration of the lamina propria have also been described. *C. parvum* attaches to intestinal and biliary epithelial cells via specific molecules presenting on host-cell surface membranes including Gal/GalNAc-associated glycoproteins. Subsequent cellular entry of the parasite depends on host-cell membrane alterations to form a parasitophorous vacuole via activation of phosphatidylinositol 3-kinase (PI-3K)/Cdc42-associated actin remodeling. How *C. parvum* exploits these host-cell processes to facilitate its infection of target epithelia is unclear. Sphingolipid-enriched membrane microdomains (SEMs) were detected in aggregations of host-cell SEM components at infection sites during *C. parvum* infection of cultured human biliary epithelial cells (i.e., cholangiocytes), with a membrane-targeted translocation of the acid-sphingomyelinase (ASM). Pharmacological disruption of SEMs and knockdown of ASM via a specific small interfering RNA (siRNA) significantly decreased *C. parvum* attachment and cellular invasion. Importantly, knockdown of ASM and disruption of SEMs significantly blocked *C. parvum*-induced accumulation of Gal/GalNAc-associated glycoproteins at infection sites by ~90%. Disruption of SEMs and knockdown of ASM also significantly blocked *C. parvum*-induced activation of host-cell and subsequent accumulation of Cdc42 and actin (Nelson et al., 2006). These pioneer results suggested an important role of SEMs for *C. parvum* attachment to and entry of host cells, likely via clustering of membrane-binding molecules and facilitating of *C. parvum*-induced actin remodeling at infection sites through activation of the PI-3K/Cdc42 signaling pathway (Nelson et al., 2006). SEMs are involved in cellular entry of several pathogens, including viruses, bacteria and parasites, and associated toxins. A well-known example is the cholera toxin which enters cells via its binding to ganglioside GM1, a common component in SEMs (Lafont and van der Goot, 2005) and that has been hypothesized also for *C. parvum* (Guarino et al., 1994).

The parasitic proteins, such as phospholipases, proteases, and hemolysins can directly cause cell damage and are associated with virulence (Bouazid et al., 2013). The involvement of Hemolysin H4 in membrane lysis allows pathogenic invasion of host cells (Okhuysen and Chappell, 2002). Moreover, the parasite has evolved various molecular mechanisms to regulate the parasite-host interactions and evade the host immune response. Certain parasitic proteins such as cysteine proteases (for example, cryptopain-1) and patatin-like phospholipases have been identified as modulators of the host immune response (Bouazid et al., 2013; Wilson and Knoll, 2018). Also, several studies on *C. parvum* show that it can suppress apoptotic pathways in the host cell by various mechanisms, including BCL2 and survivin signaling, in order to complete its life cycle (Karczuk et al., 2020). Additionally, Galluzzi et al., states that *C. parvum* is able to induce the ER stress response or unfolded protein response through PERK2/NRF2 signaling to prolong host cell survival (Galluzzi et al., 2017). However, during the later stages of infection pro-apoptotic factors are activated inducing death of host cell and surrounding uninfected cells (Laurent and Lacroix-Lamandé, 2017). Another mechanism of host cell-biology modulation involves microRNAs (miRs) and long non-coding RNAs (lncRNAs). One example is to induce over-expression of miR21 in the infected cell, which in turn downregulates expression of the anti-microbial chemokine CCL20 (Guesdon et al., 2015). Interestingly, the parasite is also capable of delivering gene transcripts into the host cell nucleus to regulate gene expression (Wang et al., 2017). Emerging evidences further suggest that parasitic lncRNAs are transported into the host cell to suppress host genes involved in several pathways including cell migration and adherence (Li et al., 2020).

Cytokines play an important role in host immune response, but in the case of disease progression, they can contribute to pathogenesis. The cellular and tissue damage by the parasite result in increased permeability of the intestinal epithelium leading to infiltration of immune cells. Higher levels of pro-inflammatory cytokines, including interferon gamma (IFN- γ), tumor necrosis factor (TNF- α) and interleukin 8 (IL8) have been observed, contributing to the pathophysiology. These cytokines liberate soluble factors thereby increasing the secretion of water and chloride ions, and decreasing the sodium absorption, causing osmotic diarrhea. Moreover, Halliez and Buret point out that neutrophil influx into the mucosa results in elevation of prostaglandin (PGE2) and prostacyclin (PGI2), that can alter ion transportation and enterocyte secretion in an enteric nervous system (ENS)-dependent or independent manner (Halliez and Buret, 2015). Ultimately, all these factors combined contribute to impairment of intestinal function and architecture, leading to loss of absorptive surface area, malabsorption and fluid loss in patients.

Host immune response

Both innate and adaptive immune responses are essential in combating *Cryptosporidium* infection. The immune capacity of the host organisms plays a decisive role in the progression and severity of pathogenesis. In general, cells of the innate immune system initiate the first response and subsequently evoke the adaptive response via B and T cells to clear the infection.

Innate immune response to the parasite

The infected intestinal epithelial cells (IECs) express pattern-recognition receptors as well as major histocompatibility complex (MHC) required for activation of innate immune system by means of pro-inflammatory chemokines (via MyD88 and NF- κ B signaling cascade) and anti-microbial peptides (Leitch and He, 2012). Subsequently, dendritic cells, macrophages and natural killer (NK) cells are recruited to the site of infection and mediate significant immune responses.

Interferons (IFNs) produced by these cells have been identified as major factors that confer protection against the pathogen in the early phases of infection. Type I IFNs, namely IFN- α and - β , have been found to be elevated soon after *Cryptosporidium* infection suggesting a protective role (Barakat et al., 2009). IFN- β is responsible for activating the stress signaling pathway by activating macrophages to produce nitric oxide (Vanathy et al., 2017). Moving to type II IFNs, IFN- γ has been stipulated as one of the main links between the innate and adaptive immune response in apicomplexan infections (Ivanova et al., 2019). IFN- γ produced by NK cells and other innate lymphoid cells (ILCs) in response to Th1 inducing cytokines IL-12, IL-15, and IL-18 is critical in activating the adaptive immune response. However, the exact mechanisms and signaling pathways involved in *Cryptosporidium* infection are yet to be delineated (Ivanova et al., 2019). Recently, Ferguson et al. investigated the role of type III interferons IFN- λ in the epithelial defense against *C. parvum* (Ferguson et al., 2019). IFN- λ is well-known to protect mucosal sites against viral pathogens (Mahlakoiv et al., 2015). In cryptosporidiosis, IFN- λ 3 was found to be significantly upregulated during the peak of the infection, suggesting their involvement in epithelial defense mechanism (Ferguson et al., 2019).

Adaptive immune response to the parasite

The specific adaptive immune response is the dominant factor in ultimately resolving the infection. Pathogen clearance mainly happens in a cell-mediated response fashion. In response to cytokine cues and antigen presentation by innate cells, the naïve CD4⁺ T cells differentiate into effector cells to mount an adequate immune response.

IL-6 and TGF β produced by dendritic cells help in the differentiation into Th17 cells. Th17 cells secrete IL17 recruiting neutrophils to the site of infection. Oftentimes increased IL-17 production can lead to pathogenesis and tissue damage. Furthermore, differentiation into Th1 cells is stimulated by IL-12 (secreted by macrophages and DCs) and IFN- γ (secreted by NK cells). Th1 cells promote phagocytosis, neutrophil degranulation, differentiation of cytotoxic CD8⁺ cells and IgG2 production. In addition, Th1 cells also secrete IFN- γ creating a positive feedback loop and have inhibitory effect on Th2 differentiation, thereby creating an initial Th1 dominant environment during cryptosporidiosis (Leitch and He, 2012). On the other hand, IL4 inhibits Th1 differentiation but stimulates differentiation of naïve CD4⁺ into CD4⁺ Th2 cells which induce eosinophil activation, production of IgG1, and secretion of IL-5, IL-4, and IL-10. Experiments demonstrate that Th2 cells are essential in eliminating the parasite, thus suggesting a balance between Th1 and Th2 responses (Lemieux et al., 2017). Although CD8⁺ T cells have been speculated to help clear infection, studies on mice show that they are not the foremost actors in adaptive immune responses against infection by *Cryptosporidium* spp. (Chen et al., 1993). Studies further reveal that humoral response through systemic circulation of pathogen specific antibodies (IgM, IgA and IgG) are not essential in clearing the parasite (Lemieux et al., 2017). However, they might have a supportive role in conferring overall protection.

To sum up, it can be said that the cytokine milieu at the site of infection will direct the host immune response. The Th1 and Th17 ratios can tip the balance towards pathogen clearance or pathogenesis. Correspondingly, a decline in CD4 population due to disease conditions can be directly associated with disease progression. AIDS patients with CD4 count < 200 cells/mm³ have been observed to exhibit severe infection, although they express high IgG/IgA and mucosal IgA. Other vulnerable groups include malnourished children, patients with hyper-IgM syndrome and patients under immunosuppressive drugs.

Clinical symptoms of cryptosporidiosis

The main mechanisms put forward for the underlying cause of symptoms are (i) increased permeability of epithelial cells, parasite induced cell death and villous atrophy; (ii) breach in the lamina propria and influx of inflammatory cells; and (iii) malabsorption and malnutrition due to loss of intestinal architecture.

The intensity of the infection and subsequent symptoms differ in children, immunocompetent and immunocompromised individuals. The disease may appear asymptomatic or manifest as a fulminant one. There occur cases where the infections may not manifest any symptoms in both immunocompetent and immunocompromised individuals (Vanathy et al., 2017).

In healthy individuals

Symptoms which appear in healthy individuals are self-limiting and mostly 5–10 episodes of watery diarrhea per day with mucus flecks. Nausea, abdominal cramps, low-grade fever, and anorexia are the other symptoms and these generally resolve within 2 weeks. But when chronic cryptosporidiosis develops in infants, they fail to thrive. Stunted growth and respiratory tract involvement also results in the case of malnourished children (Current and Garcia 1991; Vanathy et al., 2017).

In immunocompromised individuals

Incompetent individuals turn immunocompromised due to a virus infection, organ transplantation, malignancy, primary immunodeficiency, and long-term steroids usage. Hence, immunosuppressive patients can be categorized into HIV individuals and non-HIV individuals. In HIV individuals, the presentation varies from asymptomatic to fulminant condition. The symptoms may persist for days and patients tend to pass 3–6 L of persistent watery stools per day. Such patients show a CD4 count <200 cells/mm³ and as much as 17 L of the watery stool has been reported. The symptoms are found to range from chronic diarrhea to intermittent diarrheal illness (Manabe et al., 1998). With time the symptoms worsen, dwindling the survival chances of the affected.

In addition, in such patients, extra-intestinal manifestations involving several organs such as biliary tract with the right upper quadrant nonradiating pain are observed. The typical symptoms involve primary acalculous cholecystitis and sometimes sclerosing cholangitis, pancreatitis with elevated amylase level, and abnormal radiographic findings. Moreover, respiratory tract disease with symptoms such as cough, shortness of breath, wheezing, croup, hoarseness, respiratory failure, and hypoxemia are also recorded. From the sputum, tracheal aspirates, bronchioalveolar lavage, and brush biopsy specimen of infected individuals, oocyst can be collected (Current and Garcia, 1991). Other clinical presentations such as gastric outlet obstruction and esophagus causing dysphagia are also reported in such cases (Vanathy et al., 2017).

Whereas in non-HIV individuals, symptoms that appear are profuse watery diarrhea, anorexia, and weight loss. Reports also show that the infection can spread to the respiratory tract in these patients (Current and Garcia, 1991; Vanathy et al., 2017).

Diagnostics

Since none of the symptoms of cryptosporidiosis have been identified as pathognomonic, differential diagnosis is often required to differentiate from viral, bacterial and other enteric parasites. Laboratory verification includes microscopic examination, immunological methods, histology and molecular techniques. The routinely examined sample is feces for detection of oocysts, but sometimes small bowel aspirates, biopsies or tissue samples may be available. (Khurana and Chaudhary, 2018; Bouzid et al., 2013). Ideally three samples are collected because the oocysts are shed intermittently and the sample should be processed as per the guidelines in a safety cabinet since *Cryptosporidium* spp. are classified as biosafety level II organism (Smith, 2007; Khurana and Chaudhary, 2018).

Microscopic examination

Currently, microscopic examination utilizes either wet mount preparation or the smear stains with modified acid-fast stain or fluorescent stains. Preferably, the stool sample is concentrated by centrifugation or formalin-ether sedimentation. Under light microscopy the oocysts appear as smooth, colorless, spherical or slightly ovoid bodies of 3–8 µm diameter (Smith, 2007). To increase specificity and sensitivity several stains have been standardized, including acid-fast modified Ziehl-Neelsen (mZN) stain, safranin-methylene blue and fluorogenic auramine-phenol stains (Khurana and Chaudhary, 2018). Rapid test using auramine-phenol followed by a confirmatory test by mZN or Giemsa stain is often considered the gold standard method as it offers the highest combination of sensitivity and specificity. The advantage auramine-phenol or ZN stain provides is that stained oocyst can be scraped off from the slide for subsequent DNA extraction for speciation (Amar et al., 2001). Apart from the different approaches, the results are very subjective as the specificity relies on the skill of the microscopist. Moreover, other factors including turnaround time, performance in batch mode, hands-on time, consumables cost per test or batch, and specialist equipment determine efficiency of microscopy platform (Chalmers et al., 2011; Bouzid et al., 2013).

Immunological detection

Both antigen and antibody detection assays have been developed with sensitivity and specificity varying from 93% to 100%. While antigen detection tests are useful for diagnosis of acute infection, antibody detection tests are useful in seroepidemiological surveys.

Several antigen detection assays are available that use labeled antibodies. Fluorescence-labeled C-mAbs, monoclonal antibodies against oocyst wall antigens, are commercially available to detect the epitopes present on the surface of the *C. parvum* oocysts. However, fluorescence intensity will differ across other species and genotypes of *Cryptosporidium* spp. Thus it is advised to confirm the negative samples by using conventional methods or polymerase chain reaction (PCR) methods (Smith, 2007; Khurana and Chaudhary, 2018).

Another method is the use of antibodies that are labeled with enzymes to detect antigens. These techniques may have low sensitivity but does exhibit high specificity (98–100%) and also can be used to process a large number of samples in a short time (Smith, 2007; Kaushik et al., 2008; Khurana et al., 2012). Commercial antibodies are available for enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) or immunochromatographic (IC) protocols. Since IC format produces rapid results, it is a more preferred technique among others in laboratories. EIA and IC kits can be used to detect pathogens individually or multiplexed with other pathogens such as *Giardia* and/or *Entamoeba histolytica*. These tests are suggested to be performed on fresh, frozen, or formalin-preserved samples and can be done in a short time and obtain multiple results in one reaction device (Khurana and Chaudhary, 2018).

Unlike antigen detection for routine diagnostics, antibody detection methods are mostly recommended for seroepidemiological surveys since it can detect elevation in titer, or antibody isotype switching. Different assays exist for antibody detection in which native or recombinant *Cryptosporidium* antigens are utilized and the latter is reported to exhibit higher specificity. Recombinant Cp41 are used to determine human seropositivity (Kjos et al., 2005) whereas, Cp23 helps detect longitudinal infection trends (Ong et al., 2005) and age-specific seroprevalence (Cox et al., 2005). Although antigen/antibody detection is highly specific, commercially available assays have varying sensitivities and most of them fail to detect pathogen load below a certain threshold.

Histology

Histological diagnostics on biopsies can detect parasite in the brush border of intestinal mucosa. *Cryptosporidium* is found to be small, basophilic, spherical structures ranging from 3 to 5 µm and appearing in rows or clusters (Fig. 2). Also, techniques such as PCR and immunohistochemistry may be performed on paraffin-embedded tissue sections (Scallan et al., 2011; Morgan et al., 2000). However, this procedure present various disadvantages such as requirement of invasive procedures and careful processing of the sample thus making it an expensive, time consuming technique not recommended for routine diagnosis (Khurana and Chaudhary, 2018).

Molecular methods

Molecular methods offer better accuracy, high specificity and increased sensitivity ranging from 1 to 106 oocysts (Smith, 2007). They are also rapid and reliable for speciation and finding transmission routes. Nucleic acid detection methods such as PCR-restriction fragment length polymorphism (PCR-RFLP), multiplex allele-specific-PCR (MAS-PCR), and quantitative real-time PCR are available. Gene targets such as 18S rRNA, TRAP C1, COWP, Hsp 70, and DHFR genes help in species identification (Smith, 2007). Whereas, GP60 and microsatellite markers are useful for subtype identification. The highly sensitive 18S rRNA based PCR-RFLP is one of the most widely accepted molecular technique for cryptosporidium diagnosis. During outbreaks, MAS-PCR is considered a valuable tool to differentiate between *C. hominis* and *C. parvum* using dihydrofolate reductase gene sequence (Gile et al., 2002).

A commercial test developed and validated by Milwaukee Health Department Laboratory, 19-plex Gastrointestinal Pathogen Panel using Luminex xTAG analyte-specific reagents (ASRs) can be implemented for differential diagnosis of diarrhea-causing pathogens which includes 9 bacteria (*Campylobacter jejuni*, *Salmonella* spp., *Shigella* spp., enterotoxigenic *E. coli*, Shiga toxin-producing *E. coli*, *E. coli* O157:H7, *Vibrio cholerae*, *Yersinia enterocolitica*, and toxigenic *Campylobacter difficile*), three parasites (*Giardia lamblia*, *Cryptosporidium* spp., and *E. histolytica*), and four viruses (norovirus GI and GII, adenovirus 40/41, and rotavirus A) straight from fecal specimens (Zhang et al., 2015). Recently, telomeric Chos-1 gene was targeted by a unique real-time PCR assay to identify subtelomeric regions of *C. hominis* and *C. parvum* and exhibited improved genomic analysis (Bouazid et al., 2016). Some studies suggest Loop-mediated isothermal amplification (LAMP) to be a useful diagnosis of genes of *Cryptosporidium* spp. (Bakheit et al., 2008; Khurana and Chaudhary, 2018).

Presently, during the diagnosis of cryptosporidiosis various technical and practical difficulties are posed since skilled technicians are obligatory. In addition, there is an increasing requirement for cost-effective, point-of-care diagnostics, especially in low resource settings and low income countries.

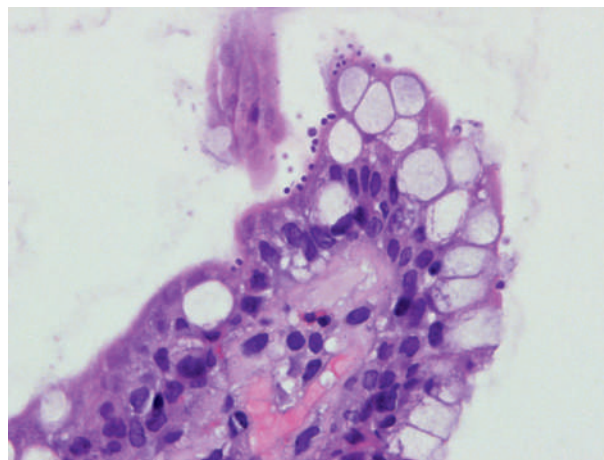


Fig. 2 *Cryptosporidium parvum* oocysts surrounding the brush border of ileum mucosa. Magnification 100×, stained by Giemsa. We thank the Laboratory of Pathology of Bambino Gesù Children's Hospital and Research Institute Rome, Italy, Dott. ssa Paola Francalanci and Prof. ssa Rita Alaggio.

Therapeutic landscape

The current treatment and management regime is mainly supportive care which include (i) replacement of fluids and electrolytes, (ii) nutritional support, and (iii) restoration of immune function. The only approved anti-parasitic drug till date is Nitazoxanide. The new drug development pipelines for cryptosporidiosis have been slow owing to the lack of animal models and in vitro culturing difficulties. Another hurdle is the presence of parasitophorous vacuole membrane (PVM) and feeder organelle that acts as a protective barrier and blocks uptake of drugs from the host cell (Mukerjee et al., 2015). Of late, technical advances in genetic engineering, cell culture, 3D organoid modeling, transgenic mice models and biocomputation methods have led to a better understanding of the parasite at a molecular level, paving way for numerous studies on new therapeutic targets and drugs (Heo et al., 2018; Bhalchandra et al., 2018; Bones et al., 2019).

Currently available drugs

Nitazoxanide, a broad spectrum anti-parasitic drug, has been shown to be effective against *Cryptosporidium* in immunocompetent individuals. However, the efficacy is lower in malnourished children and negligible in AIDS patients (Sparks et al., 2015). In transplant patients on immunosuppressive drugs, nitazoxanide and fluoroquinolone combination therapy have been demonstrated as a better approach (Bhadauria et al., 2015). Another case study in France reports that withdrawal of immunosuppressant and prescription of nitazoxanide in multiple sclerosis patients can help resolve severe diarrhea and clear the pathogen completely (Martinot et al., 2020).

Passive immunity using hyperimmune sera is another therapeutic intervention that has seen partial success. Reports say that treatment with hyperimmune bovine colostrum (HBC) in AIDS patients have not yielded high success rates (Mead, 2014). Since AIDS patients are one of the most vulnerable to this parasite with life-threatening manifestations, most of the research and clinical trials have been focused on management in AIDS patients. Several drugs including paromomycin, azithromycin, spiramycin, rifamycin, and bovine anti-cryptosporidium immunoglobulin have been tested for therapeutic efficacy. Out of these, paromomycin has shown notable activity, though comparatively lesser than nitazoxanide. Recently, a publication by Debnath and Cheriya states that the FDA approved drug octreotide, can be used as a first-line therapy for diarrhea in AIDS patients infected by *Cryptosporidium* spp. (Debnath and Cheriya, 2020).

Molecular targets for drug development

Technical advances in genomics and proteomics have expanded the therapeutic possibilities in cryptosporidiosis. Parasitic enzymes are significantly different from human enzymes making them reliable drug targets. Some of the notable strategies that are under investigation are described below.

Inhibitors of host-cell invasion

Cryptosporidium calcium-dependent protein kinases (CpCDPK), namely CpCDPK1, is one of the most described targets to block microneme secretion and prevent host cell invasion. CpCDPK1 is known to express throughout the life cycle implying a role in cell invasion and growth (Castellanos-Gonzalez et al., 2016; Kuhlenschmidt et al., 2016; Huang et al., 2017). In vitro experiments show bumped kinase inhibitors (BKI), including Pyrazolopyrimidine (PP-BKIs) and 5-aminopyrazole-4-carboxamide (AC-BKIs) to be effective in arresting CpCDPK1 activity (Castellanos-Gonzalez et al., 2016; Huang et al., 2017). Clan CA cysteine proteases, such as cryptopain-1, expressed in sporozoites have also been identified to participate in host cell invasion (Siqueira-Neto et al., 2018). Oral dosage of small molecule inhibitors of cryptopain-1, such as K11777, was successful in reducing infection in animal models (Ndao et al., 2013).

Inhibiting growth and metabolism

Several metabolic pathways and growth related enzymes have been recognized as promising targets for pharmaceutical intervention. Targeting thymidylate synthase dihydrofolate reductase (TS-DHFR), an important enzyme in the folate synthesis pathway, has been portrayed as an effective strategy in *C. hominis* and *C. parvum* (Mukerjee et al., 2015). The enzyme Inosine 5'-monophosphate dehydrogenase (IMPDH) involved in guanine synthesis is another target under investigation (Omolabi et al., 2020). Furthermore, the lipid kinase phosphatidylinositol-4-OH kinase (PI(4)K) has been reported as a potential drug target. Manjunatha et al. report successful treatment of infected immunocompromised mice using pyrazolopyridine KDU731 as a PI(4)K inhibitor (Manjunatha et al., 2017). Recently, Zhang and team has published a study demonstrating that blocking CpCDPK3 (expressed in sporozoite and merozoite stages) inhibits growth in vitro (Zhang et al., 2020).

Access to the Malaria Box compounds has enabled screening of several drug candidates that could be repurposed for cryptosporidiosis. Some of these notable compounds are inhibitors of parasite phenylalanine-tRNA synthetase (PheRS) enzyme and macrogamont development. Targeting prenyl synthases (Artz et al., 2008), histone deacetylase (Guo et al., 2018) and HAP2 (Tandel et al., 2019) have also been described as potential anti-cryptosporidium mechanisms.

Drug-resistance

While drug-resistance is a very well phenomenon for *Giardia* spp. treatments (Yukiko and Lars, 2015) limited evidences are reported for anti-cryptosporidiosis drugs. Indeed, there is currently no fully effective treatment for cryptosporidiosis, which has stimulated interest in anticryptosporidial development over the last 10 years with numerous lead compounds. In a recent study (Hasan et al., 2021), the results of a dairy calf efficacy trial of the methionyl-tRNA (CpMetRS) synthetase inhibitor 2093 and the spontaneous emergence of drug resistance was reported. Dairy calves experimentally infected with *Cryptosporidium parvum* initially improved with 2093 treatment, but parasite shedding resumed in two of three calves on treatment day five. Parasites shed by each recrudescence calf had different amino acid altering CpMetRS mutations, coding either an aspartate 243 to glutamate (D243E) or a threonine 246 to isoleucine (T246I) mutation. This is the first report of naturally emerging *Cryptosporidium* drug resistance, highlighting the need to address the potential for anticryptosporidial resistance and establish strategies to limit its occurrence (Hasan et al., 2021).

Vaccine development

An ideal vaccine against *Cryptosporidium* should be able to confer protection against both *C. parvum* and *C. hominis*. Many different types of vaccines are being explored for cryptosporidiosis.

Live attenuated vaccines are the most tried and tested type of vaccine that is able to provide long lasting immune memory. However, in the case of *Cryptosporidium* spp., replication is host dependent, therefore, large scale culturing of oocysts is challenging. Consequently, peptides, DNA vaccines and vaccine vectors pose as more reliable options. In addition, “prime-pull” vaccine strategy developed by Shin and Iwasaki can be a suitable choice for eliciting tissue specific T-cell memory (Shin and Iwasaki, 2012).

Several surface proteins of sporozoites and merozoites have been selected for the development of vaccines. Some the potent surface antigens that have shown promising outcomes include gp40/15, Cp12, Cp15/60, Cp21, p23 and COWPs (Checkley et al., 2015; Lemieux et al., 2017). DNA vaccines encoding Cp12, Cp21 and Cp15, among others, have demonstrated increased Th1 response in animal models (Yu et al., 2010; Wang et al., 2010). Studies in cattle reveal that immunization of pregnant cattle with recombinant p23 subunit vaccine confers passive immunity to calves via antibodies in colostrum (Askari et al., 2016). Several bacterial vector vaccines, for example *C. parvum* antigen-expressing *Salmonella* spp., are under evaluation for cryptosporidiosis. Experiments for prime-pull vaccine approach utilize DNA or bacterial vector vaccine to prime mice models, followed by intraperitoneal delivery of recombinant protein to boost the immune response (Lemieux et al., 2017).

Overall, there has been remarkable advancements over the last decade with in vitro and in vivo experiments presenting promising results for the development of potent drugs and vaccines in the near future.

Concluding remarks

One of the primary objectives of *Cryptosporidium* research is to reduce morbidity and mortality due to infection, especially in children and immunocompromised patients. Despite great progress, cryptosporidiosis still remains as one of the main human and veterinary health concerns worldwide. Some of the major milestones in this area of research is undoubtedly the establishment of long term in vitro culturing techniques and development of relevant in vivo disease models. This has enabled scientists to probe deeper into the molecular basis of parasite biology and parasite-host interaction. These findings in turn allow for development of better diagnostics and precise pharmaceutical intervention strategies.

Although great advances have been made in discovering promising drug compounds, none have moved to clinical trials yet. In this scenario practice of basic hygiene to prevent disease transmission is essential. Governments must ensure better management of waste treatment, plant and water treatment in order to minimize contamination. Precautionary measures and availability of reliable diagnostic are necessities, especially in low-resource settings and areas with livestock and poor sanitization. A vaccine for humans and livestock is the need of the hour, and is critical to lessen the global disease burden.

However, the current approach of system medicine in handling infectious disease can assist also in the clinical management of cryptosporidiosis, considering the *single pathogen* in the ecosystem of the gut microbiota ecology, host response and environment, within the framework of the one-health strategy.

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Cyclospora and Cystoisospora

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Introduction

Cyclospora and *Cystoisospora* are both unicellular parasites from the Chromalveolata supergroup within eukaryotes belonging to the Coccidiasina protist group, known as Apicomplexa (Levine et al., 1980; Cox, 1993; Adl et al., 2005). They cause gastrointestinal disease mainly in the tropical and subtropical climates, especially in Central and South America, India, Africa, and Asia, although cases have also been reported in Europe and North America (Lindsay et al., 1997; Almeria et al., 2019). Thus, the two parasites have a worldwide distribution in both developed and low income countries. As suggested by the names, these genera belong to the class Sporozoasida (Almeria et al., 2019). Ingestion of sporulated oocysts in fecal contaminated food or water or other transmission vehicle causes infection in susceptible subjects, typically a diarrhea, with abdominal cramping, and possible anorexia, nausea, fatigue and weight loss, among others (Shields and Olson, 2003; Ortega and Sanchez, 2010; Legua and Seas, 2013; Li et al., 2020a). This is due to the fact that in the gut lumen the sporocysts excyst and released sporozoites invade enterocytes of the duodenum and jejunum, where undergo multiplication (Figs. 1 and 2). They present two types of multiplication, asexual and sexual, the last one generating microgamonts and macrogamonts and after fertilization the formed zygotes differentiate as environmentally resistant unsporulated oocysts which are excreted in the feces. Hence, parasite transmission is via the feco-oral route. An external stage is required for the oocysts to mature and become infectious (sporulation), with *Cyclospora* requiring more time than *Cystoisospora* (7–14 against 1–3 days) (Lindsay et al., 1997; Almeria et al., 2019).

The species infecting humans are *Cyclospora cayetanensis* and *Cystoisospora belli* (formerly *Isospora belli*). They are along with *Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium* spp. and microsporidia, among parasites causing “traveler’s diarrhea” although *C. belli* is reported as one the least common intestinal coccidia that can infect humans (Vassalos and Vassalou, 2012; Rodriguez-Morales and Castaneda-Hernandez, 2014). To these, *Toxoplasma gondii*, *Blastocystis* sp. and *Balantoides coli* must be added, and other protist, such as *Acanthamoeba* spp. and *Naegleria* spp., can be transmitted with contaminated water (Marshall et al., 1997; Lv et al., 2013; Plutzer and Karanis, 2016; Pozio, 2020).

C. cayetanensis and *C. belli* sporocysts have different size with the first one being smaller (Figs. 3 and 4) (Ortega et al., 1994; Lindsay et al., 1997). *Cyclospora* and *Cystoisospora* belong to the order Eimeriorina, although are classified in different families, based on genetic and morphological differences. *Cyclospora* is within the family Eimeriidae along with *Eimeria* and *Isospora*, while *Cystoisospora* in the family Sarcocystidae along with *Toxoplasma*, *Neospora* and *Sarcocystis* (Samarasinghe et al., 2008).

Cyclosporiasis and cystoisosporiasis (formerly isosporiasis) are definitely foodborne and waterborne illnesses related to poor sanitation areas, affecting young children, the elderly, and immunocompromised people, albeit in most of the cases infections are self-limiting (Shields and Olson, 2003; Giangaspero et al., 2015; Li et al., 2020a). Cases of cyclosporiasis in North America and Europe were also associated to importation of contaminated fresh food (Almeria et al., 2019; Li et al., 2020b). Both infections are more severe in immunosuppressed persons, notably in HIV-infected patients, being considered as opportunistic (Masur, 2015; Laksemi et al., 2020; Dubey et al., 2020). *C. belli* and *C. cayetanensis*, together with *Cryptosporidium*, may also be found in the biliary system (Noor et al., 2019; Dubey et al., 2020). For both *C. belli* and *C. cayetanensis* first line treatment is based on

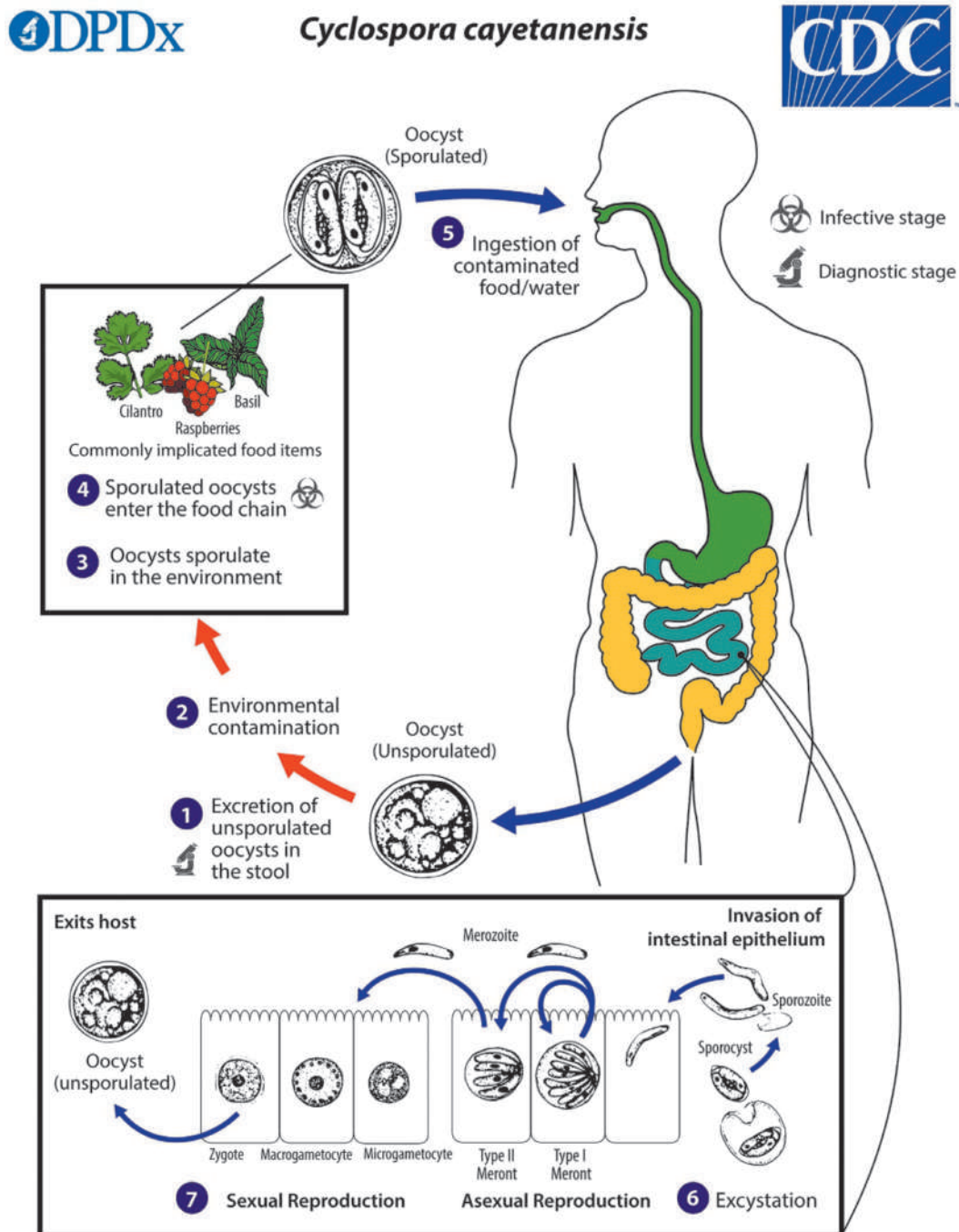


Fig. 1 The life cycle of *Cyclospora cayetanensis*. Image from the Centers for Disease Control and prevention Image Library. <https://www.cdc.gov/parasites/cyclosporiasis/biology.html>.

trimethoprim-sulfamethoxazole (TMP-SMX) (Cama and Mathison, 2015; Suh et al., 2017). Here we have introduced features common to the two parasites and peculiar traits. In the following sections after a brief historical picture, for each of the two genera we report sections on (i) biology and epidemiology, (ii) clinical symptoms and histopathology, (iii) diagnosis and (iv) treatment and prevention.

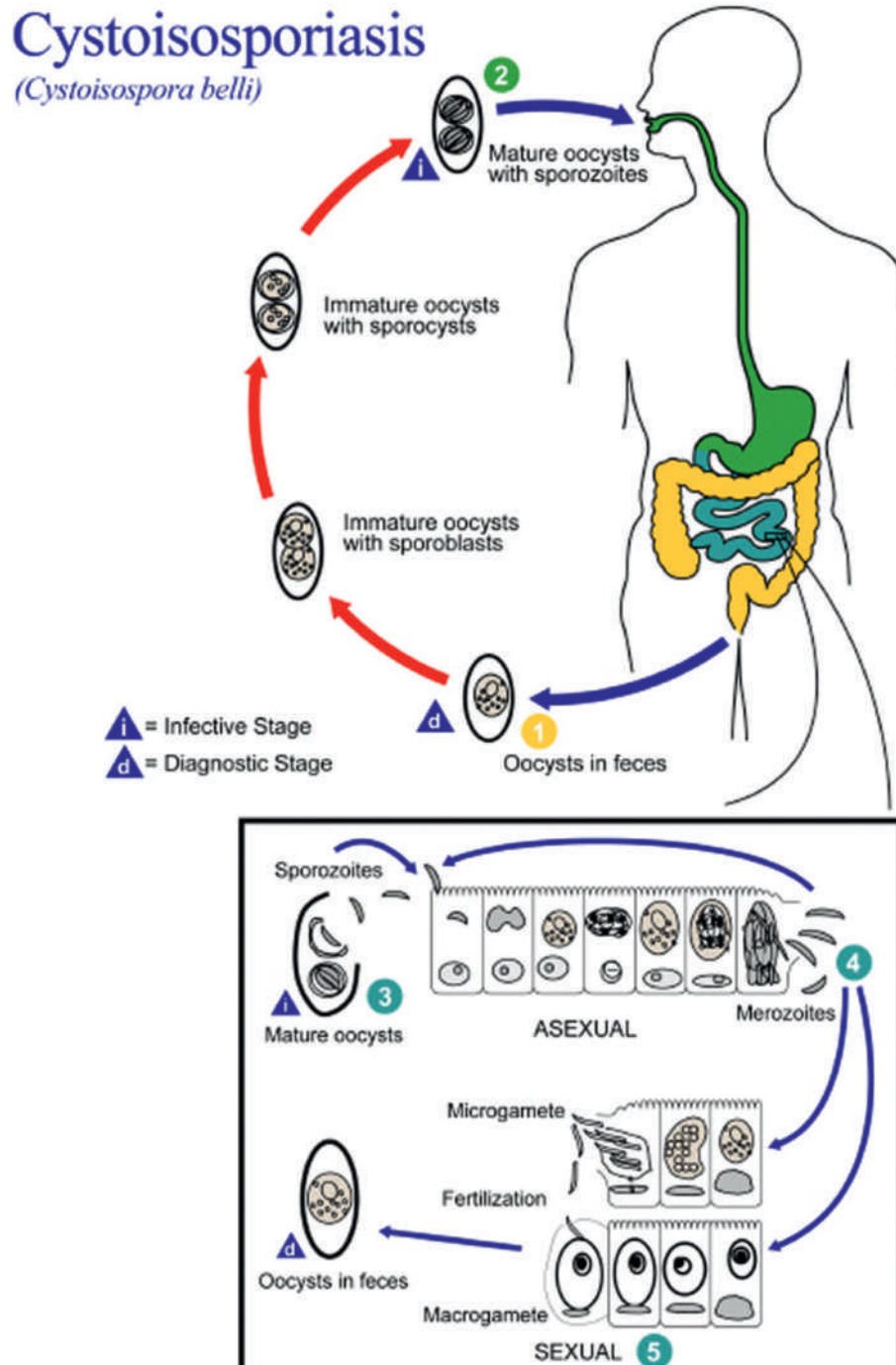


Fig. 2 The life cycle of *Cystoisospora belli*. Image from the Centers for Disease Control and prevention Image Library. <https://www.msmanuals.com/professional/infectious-diseases/intestinal-protozoa-and-microsporidia/cystoisosporiasis>.

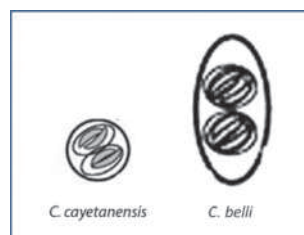
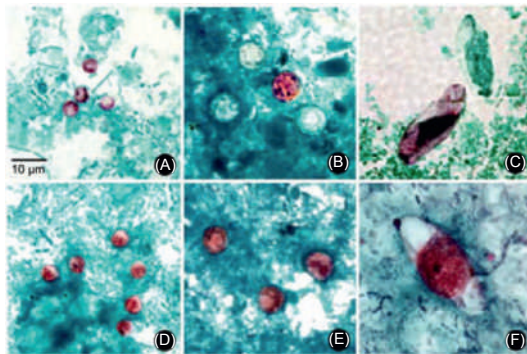


Fig. 3 Scheme of fully sporulated oocysts of *Cyclospora cayetanensis* and *Cystoisospora belli*.

Key points for laboratory identification of

Cyclospora, *Cryptosporidium*, and *Cystoisospora belli***Oocysts in stool smears stained with modified acid-fast stain:**

- A *Cryptosporidium* sp.
- B *Cyclospora cayetanensis*
- C *Cystoisospora belli*

Oocysts in stool smears stained with safranin stain:

- D *Cryptosporidium* sp.
- E *Cyclospora cayetanensis*
- F *Cystoisospora belli*

**Wet mount preparations**

G *Cyclospora cayetanensis* sequence showing sporulation event from unsporulated oocyst (far left) to sporulated oocyst containing 2 sporocysts (far right) under differential interference contrast (DIC)

H *Cystoisospora belli* (UV fluorescence microscopy)
I *Cyclospora cayetanensis* (UV fluorescence microscopy)



Fig. 4 Oocysts of *Cryptosporidium* sp., *C. cayetanensis* and *C. belli*. Image from the Centers for Disease Control and prevention Image Library https://www.cdc.gov/dpdx/resources/pdf/benchAids/coccidia_benchaid.pdf.

Cyclospora

Among the first *Cyclospora* species, there are *Cyclospora glomericola* found in 1881 in an invertebrate host and *Cyclospora caryolitica* at the beginning of last century in the mole (Lainson, 2005). Ortega and collaborators in the 1990s demonstrated that *C. cayetanensis* infects humans causing gastrointestinal disease (Ortega et al., 1993, 1994); this is the only species reported to infect humans, and humans are the only known host for this species (Eberhard et al., 2000; Ortega and Sanchez, 2010). Twenty-two *Cyclospora* species are identified at the beginning of 2020 infecting myriapodes, reptiles, insectivores, rodents and primates (Shields and Olson, 2003; Ortega and Sanchez, 2010; Giangaspero and Gasser, 2019; Li et al., 2020a). Recently identified *Cyclospora* species are from rhesus monkeys *Macaca mulatta* and North American mole *Scalopus aquaticus* (Li et al., 2015; McAllister et al., 2018). Classification traditionally was by morphological characterization of the oocysts in the feces and now mainly based on molecular methods (Shields and Olson, 2003; Lainson, 2005; Ortega and Robertson, 2017). For *C. cayetanensis* nuclear, mitochondrial and apicoplast genome sequencing were performed (Qvarnstrom et al., 2015; Cinar et al., 2015, 2016; Almeria et al., 2019). In epidemiological studies mitochondrial genome comparison by combined use of PCR and next-generation sequencing has recently been proposed for speciation and genotyping of the parasite (Cinar et al., 2020).

Cyclospora cayetanensis

Biology and epidemiology

The oocyst is spheroidal with a bilayered wall, which typically autofluoresces under UV light, as for other coccidia. *Cyclospora* and *Cryptosporidium* appear green when exposed to 450–490 nm light while under 365 nm UV *Cyclospora* looks blue (Fig. 4I) and *Cryptosporidium* violet (Ortega and Sanchez, 2010; Almeria et al., 2019). Sporocysts contain both Stieda and substieda bodies at a localized region of the wall, which are places where breakdown by the action of bile salts and trypsin begins (Cox, 1993; Ortega et al., 1994). Most recently, Dubey et al. showed all the developmental stages of the parasite in the gallbladder surgically removed

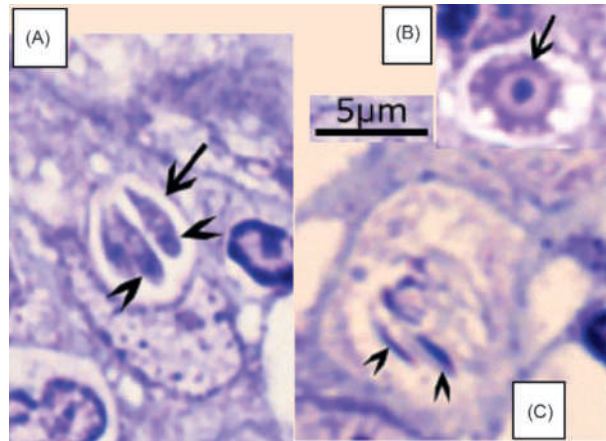


Fig. 5 Stages of *C. cayetanensis* in 0.5-µm sections of gallbladder. Toluidine blue stain. Schizont with three merozoites (A), a macrogamont (B), microgametes (C). Adapted from Dubey JP, Almeria S, Mowery J, and Fortes J (2020) Endogenous developmental cycle of the human coccidian *Cyclospora cayetanensis*. *Journal of Parasitology* 106: 295–307, with permission.

from an HIV-infected patient with cholecystitis (De Gorgolas et al., 2001; Dubey et al., 2020). After epithelial cells invasion by the excysted sporozoites, these within parasitophorous vacuoles (PV) round up and subsequently form schizonts, where multiple merozoites develop by schizogony (asexual multiplication in which the nucleus divides into four or more nuclei before merozoites are formed). Merozoites are pear-shaped or slender (banana-shaped) (Fig. 5A). Besides, sexual multiplication occurs when in some merozoite-invaded cell microgamonts (male) and macrogamonts (female) form (Ortega et al., 1997). Uninucleated macrogamonts contain several wall-forming bodies while microgamonts seat many flagellated microgametes (Fig. 5B and C) (Dubey et al., 2020).

Only sporulated oocyst is infective. Sporulation occurs into the environment within 7–14 days at temperatures above 22 °C and consists of differentiation into two sporocysts each containing two sporozoites (Figs. 1, 3 and 4G) (Ortega et al., 1993; Sathyanarayanan and Ortega, 2006; Almeria et al., 2019). Fungicides, insecticides and common disinfectants do not stop it while is inactivated by cooking at 70 °C (Sathyanarayanan and Ortega, 2004; Ortega and Sanchez., 2010; Almeria et al., 2019; Giangaspero and Gasser, 2019). Cyclosporiasis outbreaks in cruise, in Europe and mainly in North America were reported, and associated with the consumption of contaminated fresh produce such as berries and leafy greens, often imported from endemic regions (Almeria et al., 2019; Giangaspero and Gasser, 2019; Li et al., 2020a,b). Contamination has been found in pre-packaged vegetable products too, as well as in wastewater, chlorinated water and coastal waters not only in endemic countries. Nepal, Vietnam, Haiti, Guatemala, Perú, Ghana, Egypt, among others, are indicated as endemic for *C. cayetanensis*, nevertheless contaminated water has also been reported in Spain, Italy, Turkey and United States (Arizona) (Kitajima et al., 2014; Giangaspero et al., 2015; Almeria et al., 2019). Shellfish contamination has been reported in the Mediterranean Coast near Turkey and Tunisia (Ghozzi et al., 2017; Almeria et al., 2019). Parasite diffusion is certainly underestimated since many countries do not include *Cyclospora* in diagnostic protocols, furthermore climate change is expected to increase parasites spread, especially in the world poorest regions (Pozio, 2020). There is also a seasonality in cyclosporiasis, which depends in great part on the temperature (Shields and Olson, 2003; Kaminsky et al., 2016; Almeria et al., 2019). Some outbreaks and most of sporadic cases are due to travelling of persons, never exposed to the parasite before, to endemic countries, in which most of infections are self-limiting and followed by the acquisition of immunity to reinfection (Shields and Olson, 2003).

Clinical symptoms and histopathology

Prodromal stage is reported with flu-like symptoms although the onset is voluminous watery diarrhea, fatigue, and abdominal cramps. Other symptoms can be abdominal bloating, headache, constipation, loss of appetite, nausea, low-grade fever, chills and vomiting. Dehydration, malabsorption and weight loss can follow (Ortega et al., 1997; Lv et al., 2013). Duration can consist in few days as longer periods, even more than 1 month, mainly in people with deficits in their immune system (Li et al., 2020a). In cases of persisting symptoms which include dehydration, malaise, fatigue and sometimes fever, patients must be treated, otherwise extraintestinal complications can rise such as Guillain-Barré and Reiter syndromes, the last one involving more dysfunctions including oligoarthritis, urethritis and ocular inflammatory diseases (Richardson et al., 1998; Connor et al., 2001). Thus, chronic forms are not only in immunosuppressed and immunocompromised people, which anyway present the most severe form of cyclosporiasis, with complications such as a calculous cholecystitis (De Gorgolas et al., 2001; Ortega and Sanchez, 2010).

Jejunal biopsies show inflammation with altered mucosal architecture, edema, shortening of villi and hyperplasia of the crypts, disruption of the surface epithelium with extensive infiltration of lymphocytes (Ortega et al., 1997; Connor et al., 1999). HIV patients can show cholecystitis and removed gallbladder presented parasite infiltration in all parts (Fig. 5) (Ortega and Sanchez,

2010; Dubey et al., 2020). Presence of oocysts in patients suffering of tuberculosis has also been reported (Hussein et al., 2005). If not correctly treated, mainly in immunosuppressed patients especially those transplanted, severe dehydration due to cyclosporiasis as to other types of infectious enteritis may produce acute kidney injury, which can lead to chronic kidney disease (Lugo et al., 2021).

Diagnosis

Microscopic ova-and-parasite examination and molecular testing for DNA are used for diagnosis of *Cyclospora* infections (Almeria et al., 2019; Li et al., 2020a). Oocysts can be detected by microscopic analysis in multiple fecal specimens collected at 2–3 day intervals from a patient. *Cyclospora* oocysts are spherical and approximately 8–10 µm in diameter (Figs. 1, 3 and 4), which is the most important finding in the differential diagnosis of *Cryptosporidium*, whose oocysts are approximately half the size (4–6 µm, Fig. 4). Epifluorescence microscopy allows to exploit *Cyclospora* autofluorescence (as reported in the biology section, Fig. 4I) while conventional light microscopy is usually employed on stained smears. The modified Ziehl-Neelsen stain (acid fast) is recommended, where oocysts appear pink to red, although heated safranin staining is sensitive as well (Fig. 4B and E, respectively). Other staining methods are also used including Kinyoun, lacto-phenol cotton blue and Giemsa (Ortega and Sanchez, 2010; Giangaspero and Gasser, 2019). Concentrated samples by means for instance of sucrose or Percoll gradient centrifugation or formalin-ethyl acetate sedimentation (Ortega and Sanchez, 2010; Giangaspero and Gasser, 2019) enhance detection efficiency. Flow cytometry combined with autofluorescence was also efficiently used to detect *C. cayetanensis* on concentrated samples (Li et al., 2020a). In fresh stool specimens or more probably, in concentrates, *Cyclospora* oocysts can be observed by phase contrast or bright field microscopy; even better with differential interference contrast microscopy appearing as spherical bodies with a central or membrane-bound cluster of globules enclosed within a membrane (Fig. 4G) (Ortega and Sanchez, 2010; Cama and Mathison, 2015). Since these structures are similar to blue-green algal morula they were described in the first reports as alga-like and *Cyanobacterium*-like bodies, then associated to Coccidian-like bodies (Shlim et al., 1991; Ebrahimzadeh and Rogers, 1995).

Another method to identify *C. cayetanensis* is to allow sporulation in culture at 25 or 32 °C in 2.5% potassium dichromate, which occurs between the seventh and thirteenth day after excretion (Eberhard et al., 2000; Sathyanarayanan and Ortega, 2006; Almeria et al., 2019). Microscopic analysis reveals the difference in the structures of the oocysts, which only after sporulation present the two developed ovoid sporocysts with each the two sporozoites inside (Figs. 1, 3 and 4G). Excystation of sporozoites is possible after mechanical disruption of the sporocysts and exposure to trypsin (0.5%) and sodium taurocholate (1.5%) (Ortega et al., 1993). Parasite stages can be detected in microscopic examination of stained tissue biopsy sections, even kept fixed and paraffinated for very long periods (Fig. 5) (Dubey et al., 2020).

Molecular methods are the most sensitive; once even few oocysts are purified, quantitative PCR (qPCR) can be used for *C. cayetanensis* identification (Li et al., 2020a). Multiplex real-time PCR can detect with high sensitivity simultaneously *C. cayetanensis*, *Giardia lamblia*, *Cryptosporidium parvum* and other gastrointestinal pathogens (e.g. Biofire FilmArray Gastrointestinal Panel; Seegene Allplex™ Gastrointestinal Panel-Parasite Assay) (Buss et al., 2015; Ryan et al., 2017; Hannet et al., 2019; Yoo et al., 2019). *Cyclospora* DNA sequences amplified by PCR-based techniques are within the 18S small subunit ribosomal DNA (ssrDNA); the second internal transcribed spacer (ITS2) is a ribosomal DNA sequence more specific while another used locus is the 70 kDa heat shock protein gene (Shields and Olson, 2003; Lalonde and Gajadhar, 2008; Sulaiman et al., 2013; Giangaspero and Gasser, 2019). Commercial serological tests are not available although potential oocyst antigens have been identified (Hussein et al., 2020).

Treatment and prevention

Treatment is highly effective and consists of the systemic antiprotozoal drug trimethoprim-sulfamethoxazole (TMP-SMX, co-trimoxazole). In the presence of symptomatic infection TMP-SMX given orally (160 mg TMP and 800 mg SMX twice per day for 7–10 days or, in more severe cases as immunocompromised patients with relapse, four times per day for 10 days, and then twice per day for 3 weeks) (Shields and Olson, 2003; Giangaspero and Gasser, 2019). TMP-SMX is sold under the trade names Bactrim, Septra, Cotrim, Eusaprim and others. This treatment is important, mainly in immunodeficient individuals, and can also be used in patients with biliary disease (Verdier et al., 2000; Li et al., 2020a). In children, TMP-SMX at 5–25 mg/kg body weight (bwt) for 7 days is suggested, although a 3-day course has been shown sufficient in Peruvian children (Madico et al., 1997; Ortega and Sanchez, 2010). Alternative treatments in patients who have received transplantation or who are sulphonamide-intolerant or with sulfa allergy are the fluoroquinolone antibiotic ciprofloxacin at 500 mg twice daily for 7 days, and the thiazolide nitazoxanide at 9.5 mg/kg bwt for 3 days for failing with ciprofloxacin. These alternatives are not as effective as TMP-SMX, and may cause antibiotic resistance especially fluoroquinolones (Verdier et al., 2000; Li et al., 2020a). In patients with AIDS, it is also very important to treat the HIV infection as effectively as possible with antiretroviral drugs. Such treatment can strengthen the compromised immune system, and usually helps to control diarrhea and other symptoms.

Prevention is strictly linked to good hygiene conditions, which can exclude fecal-oral transmission of infective oocysts. Hence, endemic regions are mainly in underprivileged and low income countries, with problems in water and food sanitation. Risk factors are the lack of toilet facilities even in developed countries, the extreme population density as in megacities, difficulty in water supply, especially for drinking water, lack in treating human sewage and improper use of that, for instance in agriculture (Pozio, 2020). Thus, when travelling in endemic countries it is recommended drinking bottled or boiled water and not eating raw produce.

Some outbreaks in developed countries were related to consumption of imported contaminated fresh produce that were difficult to clean thoroughly (including berries, leafy greens, cilantro, basil) or to ready to eat-packaged vegetables. Hence, also globalization of the food supply has increased the risk of exposure to this type of parasites (Ortega and Sanchez, 2010). Oocysts are not affected by commonly used chlorine concentrations and most other chemical disinfectants while sodium dichloroisocyanurate solution reduces parasite number (El Zawawy et al., 2010). Also, membrane filtration, treatment with magnesium oxide nanoparticles, boiling and microwave heating are effective in cyclosporiasis control (Ortega and Sanchez, 2010). Gamma-irradiation, treatment with either ozone or hydrostatic pressure or chlorine dioxide showed to be effective with similar parasites (Almeria et al., 2019; Li et al., 2020a). Furthermore, when well assessed, UV light could be the most practical and safe control method (Giangaspero and Gasser, 2019).

Finding the source of *C. cayetanensis* infection in outbreaks in both non-endemic and endemic countries is essential to stop infection propagation. Beshearse et al. (2021) reported that in United States *C. cayetanensis* is estimated to have majority transmission through the foodborne pathway. Oocysts detection in vehicles such as raw vegetables and fruits is usually performed after specific washing protocol using a filter bag whose filtrate is then concentrated (Shields et al., 2012) although efficient concentration will be possible only when antibodies against *C. cayetanensis* can be available (Almeria et al., 2019). Several molecular methods have been investigated to identify different strains or geographic origin, such as sequencing the polymorphic region of mitochondrial genome and multilocus sequence typing (MLST) in nuclear DNA (Guo et al., 2016, 2019; Nascimento et al., 2019; Hofstetter et al., 2019). Mitochondrial DNA amplification is more efficient allowing to genotype almost all the samples, most probably due to the high copy numbers of the mitochondrial genome in *C. cayetanensis* (more than 500 copies estimated) (Tang et al., 2015). These procedures have allowed to distinguish at least two major groups; one including the American isolates (both North and South) and the other the Chinese specimens. Some similarity was found for China and Nepal specimens and for certain aspects for Nepalese and Indonesian ones (Guo et al., 2019). The worldwide diffusion of *C. cayetanensis* diagnostic tests, along with the monitoring of water sources, bathing and sewage waters, are recommended. Coastal waters may be easily monitored analyzing wild shellfish samples for parasite DNA since they retain and concentrate parasites while filtering large volumes of water (Ghozzi et al., 2017). Melt curves and gel electrophoresis of quantitative PCR products allow differentiating very rapidly the parasite isolates (Guo et al., 2019). In addition, it is mandatory to continuously check imported food and, above all, to spread health education worldwide, including the importance of proper hand washing.

Cystoisospora (previously Isospora)

The genus *Cystoisospora* includes species infecting mammals which were reclassified in 2005, previously belonging to the genus *Isospora* (Barta et al., 2005). Now the last genus is also called *Atoxoplasma*, and contains only species infecting birds (Schrenzel et al., 2005; Samarasinghe et al., 2008). In 1977, Frenkel proposed the new genus *Cystoisospora* for the two facultative two-host, feline species *Isospora felis* and *Isospora rivolta*, having monozoic tissue cysts (MZTC, containing a single asexual stage called zoite) in the rodent intermediate host (better considered paratenic since it is only a transport host where the parasite does not develop) (Frenkel, 1977; Lindsay et al., 1997). Analogous structures are formed by *C. belli* in human tissues (Fig. 6) (Lindsay et al., 1997). Then, genetic differences were found between species from non-mammalian and mammalian hosts (Samarasinghe et al., 2008). In addition, the oocysts from birds possess Stieda bodies, which conversely are absent in those from mammals (Barta et al., 2005).

Many studies have been performed on *Cystoisospora* species in cell cultures (Lindsay, 2019). After entering cultured cells some species, including *C. belli*, divide repeatedly by endodyogeny (asexual division with internal budding of two daughter cells from the common cytoplasmic mass of the parental cell), and *Cystoisospora suis* in in vitro models produces all development stages, comprehending the sexual ones (Lindsay et al., 1998). *C. suis* and *C. belli* cause serious disease in nursing pigs and in humans, respectively, while seldom *Cystoisospora* species do the same in nonhumans primates, dogs, or cats (Lindsay et al., 1997). *C. belli* was first described by Virchow in 1860, and identified as a human pathogen in 1915 among military personnel stationed in the Middle East during the World War I (Klassen-Fischer et al., 2011; Legua and Seas, 2013). Humans are the only known host for this species (Noor et al., 2019).

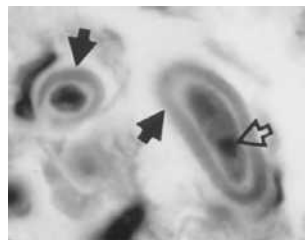


Fig. 6 Tissue cysts of *C. belli* in the spleen of an AIDS patient. A longitudinal view and a cross-section of tissue cysts are present. Note the tissue cyst wall (arrows) and the nucleus (open arrow) of one zoite. Magnification, $\times 1000$. Adapted from Lindsay DS, Dubey JP, and Blagburn BL (1997) Biology of *Isospora* spp. from humans, nonhumans primates, and domestic animals. *Clinical Microbiology Reviews* 10: 19–34, with permission.

Cystoisospora belli (previous *I. belli*)**Biology and epidemiology**

Oocysts are ellipsoidal with one end being blunt and the other tapered or both ends slightly tapered (spindle-shape) (Lindsay et al., 1997; Sherman, 2018) (Figs. 2, 3, 4 and 7). The oocyst has a bilayered wall, and has a large size as all the *Cystoisospora* stages, compared to *Cyclospora* and *Cryptosporidium* (Fig. 4). Mature sporulated oocyst measure on average $30 \times 12 \mu\text{m}$ (Minnaganti, 2018; Serrano-Batista et al., 2019; Dubey et al., 2019). When oocyst is excreted with feces it is unsporulated or partially sporulated (usually one sporoblast stage, Fig. 2). Then in appropriate conditions of temperature ($25\text{--}30^\circ\text{C}$) and humidity the sporoblast divides in two (Fig. 2), and after secretion of a cyst wall the two sporoblasts become sporocysts. These divide twice to produce four infective sporozoites each (sporogony, Figs. 2, 3, 4H, and 7) (Skinner, 1972; Lindsay et al., 1997). This oocyst maturation can range from 24 h to 10 days (Liu, 2010). At a low frequency *Caryospora*-like oocysts were also observed, characterized by oocysts containing one sporocyst with enclosed eight sporozoites (Jongwutiwes et al., 2007). The mature oocyst can remain infective in the environment for months (Sherman, 2018).

After ingestion of contaminated material, the sporocysts excyst in the small intestine and release sporozoites, which, after activation by bile and trypsin, become motile (McKenna and Charleston, 1982; Lindsay et al., 1997). Sporozoites invade epithelial cells lining the villi, and initiate schizogony (asexual multiplication) by repeated endodyogeny events (Fig. 2). Schizont and meront are terms for immature and mature asexual division stages, respectively, within PV in host cytoplasm. When the schizont and its host cell break, merozoites move into the gut lumen and penetrate uninfected enterocytes, where other replication rounds occur. After about 1 week some merozoites can shift to gametogony, the sexual phase with macrogamonts and microgamonts generating female and male gametocytes, respectively (Dubey et al., 2019). In patients with HIV asexual and sexual stages of *C. belli* have also been found in the epithelium of bile ducts and gallbladder, as for *C. cayetanensis* (Lindsay et al., 1997, 2014; Dubey and Almeria, 2019).

As other Sarcocystidae, *Cystoisospora* presents extraintestinal (EIN) tissue cyst stages, which are typically MZTC, that is a single centrally located zoite surrounded by a granular tissue cyst wall (Fig. 6) within a PV. That is, some sporozoites and/or merozoites leave the intestine and form dormant stages in EIN tissues. For *C. belli* they were mainly observed in AIDS patients, most frequently in *lamina propria* of the small and large intestine, lymph nodes, spleen, and liver (Dubey and Almeria, 2019). Recurrent cystoisosporiasis is believed to be due to reactivation of the zoites present in the MZTC and migration to the human intestinal tract (Lindsay et al., 2014). In an article published in 2019, Noor et al. in United States showed *C. belli* (inside intraepithelial PVs) in a great number of cholecystectomy specimens, and on a cohort of 22 more recent resections from patients with functional gallbladder disorder (biliary pain in the absence of gallstones or sludge) reported a prevalence of 27%. They suggested that *C. belli* is more prevalent among immunocompetent humans than previously recognized and may reside in a relatively latent state in the gallbladder (as a commensal organism), only giving rise to symptomatic infection in the setting of pronounced immunodeficiency and/or immunosuppression (Noor et al., 2019).

C. belli is mainly found in the Caribbean, Central and South America, Africa, the Middle East, India and Southeast Asia (Lindsay et al., 1997). As *C. cayetanensis*, in endemic countries it is more prevalent in children, and immunodeficiency, malnutrition and poor hygiene are risk factors for cystoisosporiasis, although in immunocompetent subjects it is usually a transient, self-limited illness (Sasaki et al., 2004; Rodriguez-Morales and Castaneda-Hernandez, 2014; Laksemi et al., 2020). It has been found in patients with colorectal cancers and it has been implicated in traveler's diarrhea (Vassalos and Vassalou, 2012; Mahmoudvand et al., 2019; Caner et al., 2020). Nevertheless, also in the rest of the world it is an opportunistic parasite of patients with HIV/AIDS, with lymphoma and leukemia, and recipients of renal and liver transplants, thus receiving immunosuppressive therapy, although the prevalence was higher in AIDS patients from endemic regions (Sorvillo et al., 1995; Guiguet et al., 2007; Stark et al., 2009; Akateh et al., 2018; Noor et al., 2019; Tsutsui et al., 2021). It has also been reported to cause severe diarrhea in a French patient affected by rheumatoid arthritis on corticosteroid and immunosuppressive therapy (Post et al., 2018), and in an Italian case of Good syndrome, characterized by the association of thymoma, hypogammaglobulinemia, low or absent B cells in the peripheral blood and defects in cell-mediated immunity, which differently from HIV has higher CD4 T-cell count (Esvan et al., 2018). *C. belli* has also been reported as the cause of outbreaks of diarrheal illness in daycare centers and psychiatric institutions in United States in past century (Rodriguez-Morales and Castaneda-Hernandez, 2014).



Fig. 7 Oocysts of *C. belli* in the stool smear. Magnification, $\times 1000$. Adapted from Tsutsui T, Kakizaki Y, and Miyashita Y (2021) *Cystoisospora belli* associated persistent diarrhea in an AIDS patient. *Journal of Infection and Chemotherapy* 27(2): 348–349. <https://doi.org/10.1016/j.jiac.2020.08.010>, with permission.

Clinical symptoms and histopathology

In immunocompetent patients cystoisosporiasis usually presents as mild forms in adults, characterized by acute, nonbloody diarrhea, crampy abdominal pain, vomiting, fever. Disease is more severe in infants. Diarrhea lasts 6–10 days, with infection self-resolving in 2–3 weeks, sometimes continuing for other 2–3 weeks (Cama and Mathison, 2015). Protracted diarrhea with malabsorption, steatorrhea, dehydration and weight loss may occur and sometimes the syndrome can be fatal (Lindsay et al., 1997; Sasaki et al., 2004). In some patients, peripheral eosinophilia is observed, which is a distinctive feature of this pathogen (Legua and Seas, 2013). Infection may be chronic with recurrences. Immunocompromised hosts present more severe and prolonged diarrhea and extra-intestinal manifestations involving *lamina propria* of the intestinal mucosa, gallbladder and biliary tract, mediastinal and mesenteric lymph nodes, liver, pancreas and spleen (Masur, 2015; Akateh et al., 2018; Noor et al., 2019). As for cyclosporiasis and other intestinal parasites, there may follow in some patients a rheumatic syndrome as a result of direct infiltration of musculoskeletal structures or an immune mediated mechanism (Hussein et al., 2019). Reactive arthritis has been described in a patient with AIDS and thrombotic thrombocytopenic purpura and hemolytic uremic syndrome both were described in association with this infection (Gonzalez-Dominguez et al., 1994; Auwaerter, 2016). Recurrent infection may require, after therapy, indefinite prophylaxis (Legua and Seas, 2013).

Villous atrophy and crypt hyperplasia can be present, also large numbers of eosinophils in *lamina propria* and other increased inflammatory cells, mainly in severe infections. Widespread mucosal changes such as a granular appearance of duodenum and proximal jejunum are even associated with severe symptoms, and the lymphatics may be dilated (Lindsay et al., 1997; Klassen-Fischer et al., 2011).

Diagnosis

Because of the large size, unsporulated oocysts of *C. belli* can be visible unstained by light microscopy on direct fecal smears (Fig. 7), although repeated stool examinations and concentration procedures are recommended (Lindsay et al., 1997). Modified Ziehl-Neelsen stain (acid-fast, Fig. 4C) or autofluorescence (Fig. 4H) can help the identification. Other staining procedures can also be useful (e.g. Fig. 4F) (Cama and Mathison, 2015). Extraintestinal cysts have been highlighted with Periodic Acid-Schiff (PAS) with predigestion with the polysaccharide digesting diastase (PASD) (Akateh et al., 2018) although Swanson reported that this last staining procedure needs confirmation by alternative methods or expert parasitologist consultation since can give false positives (Swanson et al., 2018). Charcot-Leyden crystals and high fat content are often observed in stool specimens (Minnaganti, 2018). Molecular methods are more sensitive; multiplex real-time PCR were used for the detection of DNA of opportunistic protozoa in HIV patients, targeting for *C. belli* the ITS2 sequence (Ten Hove et al., 2008), and for detection of *Cyclospora*, *Cystoisospora* and Microsporidia, targeting for *C. belli* ITS2 and 5.8 s rRNA (Taniuchi et al., 2011). In case of suspected cystoisosporiasis with stool examination negative, parasites can be sought in duodenal aspirates, string tests or intestinal biopsies after hematoxylin and eosin staining, typical vacuolated epithelium may also be a clue (Lindsay et al., 1997). The fact that *C. belli* can elicit blood eosinophilia, in contrast to most of intestinal protozoa, is an important clue for its diagnosis (Marques et al., 2019). Moreover, diagnosis of cystoisosporiasis should suggest investigation for HIV infection (Minnaganti, 2018). Also, HIV-infected persons with low CD4⁺ cell counts appear at greater risk of acquiring this parasitic infection (Certad et al., 2003).

Treatment and prevention

Apart hydration therapy in case of severe diarrhea, treatment highly effective is oral TMP-SMX, as for cyclosporiasis (Cama and Mathison, 2015; Vassalos and Vassalou, 2012; Suh et al., 2017). Immunosuppressed patients and those with AIDS may require long-term prophylaxis (Legua and Seas, 2013). As alternative, pyrimethamine with sulfadiazine (or sulfadoxine) or, for patients who cannot tolerate sulfonamides, ciprofloxacin, or pyrimethamine plus folinic acid. The veterinary agent diclazuril, doxycycline, roxithromycin, nitazoxanide, albendazole, bismuth subsalicylate, furazolidone, metronidazole, and quinacrine have also been reported to have some efficacy (Lindsay et al., 1997; Vassalos and Vassalou, 2012; Minnaganti, 2018). Recently a report of a kidney transplant patient underlined as diagnosis can be sometimes very difficult and delayed, resulting in poor prognosis. The authors suggested their screening protocol for transplant candidates, including *Mycobacterium tuberculosis*, *Strongyloides stercoralis*, Hepatitis B and C virus, HIV, Cytomegalovirus, Human Herpesvirus 6 and 8 and Epstein-Barr virus, might be extended to *C. belli* (Marques et al., 2019; Akateh et al., 2018).

For prevention, epidemiological studies, and surveillance on potential infection vehicles such as food and water sources are basic. Contamination of fruit and vegetables has been reported in Ethiopia and Ghana (Li et al., 2020b). Isolates from HIV-infected patients showed after DNA amplification distinct restriction fragment length polymorphism (PCR-RFLP) patterns in the 18S small subunit ribosomal DNA (SSU rDNA) (Resende et al., 2011), which could be useful not only to characterize different clinical presentations but also for epidemiological trace-back investigations on cystoisosporiasis. Both *Cystoisospora* and *Cyclospora* are considered neglected waterborne protozoa due to few information on its frequency in the global water sources (Plutzer and Karanis, 2016). In this setting, low cost and sensitive test for these parasites are needed, to allow wide monitoring, able to avoid outbreaks of diarrheal diseases, that in the endemic Countries are the second-leading cause of death in children younger than 5 years (Giangaspero and Gasser, 2019).

Conclusions

Cyclospora and *Cystoisospora* infections are considered emerging infective diseases for several reasons (Legua and Seas, 2013; Hadjilouka and Tsaltas, 2020). Global warming is bringing more of either drought or rainfall intensity, which both effect increase of these parasites in endemic countries. Drought causes oocysts concentration in water while rainfalls and floods increase their spread (Semenza et al., 2012; Pozio, 2020). In non-endemic countries cases increase, due to rise of both, travelling to endemic countries and importation of fresh foods from those regions. Among industrialized countries, in United States and Canada many *C. cayetanensis* outbreaks have been reported in the last 25 years (Almeria et al., 2019; Legua and Seas, 2013; Hadjilouka and Tsaltas, 2020). Several cases both in United States and Europe were domestically acquired, occurring mainly during spring and summer months (Almeria et al., 2019; Legua and Seas, 2013). Moreover, tropicalization of climate could favor establishment of these parasites in new regions (Semenza et al., 2012; Pozio, 2020). Hence, it really is important physicians know *C. cayetanensis* and *C. belli*, in all aspects we have discussed.

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Echinococcus

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Introduction

Cystic echinococcosis (CE) and Alveolar echinococcosis (AE) are two zoonotic diseases caused by tapeworms belonging to the *Echinococcus* genus. CE is a cosmopolitan zoonosis present in countries across all continents excluding Antarctica, whereas AE is limited to the northern hemisphere (Deplazes et al., 2017). Human CE affects an estimated 1.2 million people worldwide, with 1–3 million disability-adjusted life years (DALYs) lost globally every year, although these figures are likely to be underestimated (Deplazes et al., 2017; Budke et al., 2006; Craig et al., 2007a). AE affects a lower number of individuals, but carries a significantly higher mortality burden, with a 90% mortality rate when untreated. Burden estimates for AE calculate the amount of lost DALYs to be around 660,000 (Torgerson et al., 2010). *E. granulosus sensu lato* (sl), the causal agent of CE, develops its cycle between dogs and other canids (definitive hosts) and livestock, especially sheep (intermediate hosts), with humans as an accidental intermediate hosts. In humans, the parasite develops to its metacestode stage, forming cysts in organs and tissues, mainly in the liver. CE is mostly endemic in rural areas where sheep (and other livestock) raising is practiced, such as central Asia and China, South America and Mediterranean countries (Deplazes et al., 2017). In 2012 a joint FAO/World Health Organization (WHO) expert group classified *E. granulosus* second among the top eight ranked food-borne parasites of global public health concern (FAO/WHO, 2012).

E. multilocularis develops its life cycle between small rodents (intermediate hosts) and foxes or dogs (definitive hosts). Its major areas of diffusion include the Tibetan plateau, northern China, central Asia and northern Europe. As such, AE is considerably less widespread than CE. However, even though both diseases have a significant impact on health systems, they are still scarcely studied in endemic areas and research is mainly carried out in referral centers (Brunetti et al., 2011).

This chapter highlights the main parasitological features of CE, while also covering the main diagnostic and therapeutic aspects of the disease.

The agents

Both CE and AE are zoonotic diseases caused by cestodes. The life cycle of both parasites, is shown in Fig. 1. The parasite is dependent on a predator-prey association involving mammalian hosts. This most frequently includes dogs and ungulates in the case of *E. granulosus* and small rodents and foxes or dogs in the case of *E. multilocularis*. However, it should be noted that both parasites are able to infect a range of hosts: in the case of CE, several ungulates species including pigs, horses, donkeys, buffaloes and cows can act as intermediate hosts, albeit with a reduced fertility rate of CE cysts. In the case of AE, a wide range of small rodents as well as raccoons can act as intermediate hosts, and reports of cats being infected have also been published. Other canids such as wolves can also act as definitive hosts (Romig et al., 2017a; CDC, n.d.).

The cycle starts when eggs are expelled with fecal material of infected dogs or other definitive hosts. These animals can be infected with a high load of worms and produce hundreds of eggs per day. Eggs are sensitive to exsiccation and to exposition to direct sunlight, but they can withstand high temperature provided that humidity is high enough, as well as low temperatures (Agudelo Higueta et al., 2016; Romig et al., 2017a).

Embrionated eggs are then ingested by intermediate hosts and hatch into oncospheres before penetrating the intestinal wall. From there, they are carried to the rest of the body via initial venous dissemination (Romig et al., 2017a; Tamarozzi et al., 2016a). Once they reach their target site, the oncospheres encyst, giving rise to the formation of the “classical” metacestode or hydatid cyst (Fig. 2). In fact, in all organs save for the bone, the cysts develop with the formation of three layers. The endocyst, which is the metabolically active part of the parasite, charged with producing brood capsules, protoscolexes and daughter vesicles. The parasite also produces a laminated layer (LL), an acellular membrane, composed by proteins, synthesized by the hexacanth larva as it develops and before synthesis for the germinal layer starts. This “protein wall” plays a major role in immune evasion. The adventitial part of the cyst (pericyst) is host-derived and is composed of fibrous connective tissue. Its presence is useful during surgical procedures as it provides a clear surgical reference for the operating team. While developing, the cyst becomes fluid filled and brood capsules containing protoscolexes (PSC) are released in the cyst fluid. PSCs are thought to develop within 10 months of infection, even though the exact timing has never been demonstrated and these data derive from animal studies (Tamarozzi et al., 2016a).

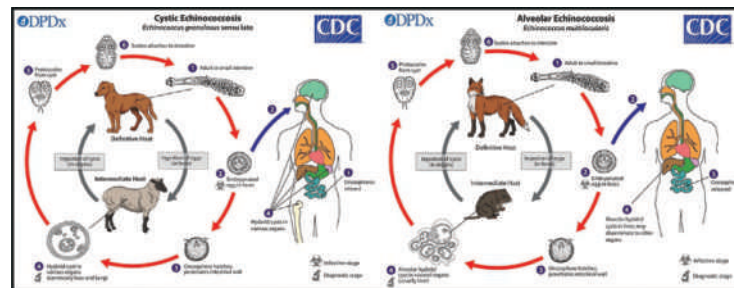


Fig. 1 Parasite life cycle. From Centers for Disease Control and Prevention (CDC). *Echinococcosis—Biology*. <https://www.cdc.gov/parasites/echinococcosis/biology.html>. Accessed September 9, 2019.

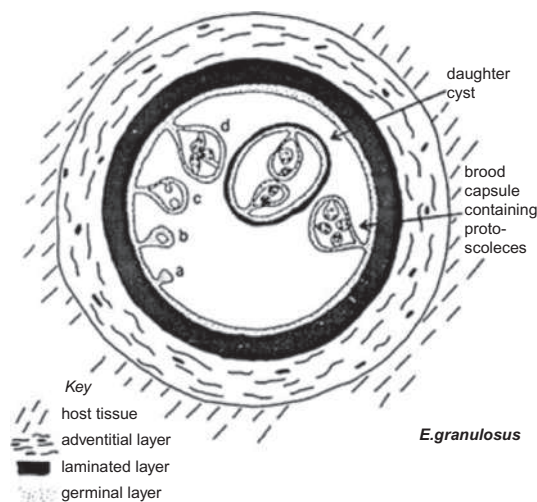


Fig. 2 Schematic representation of a CE cyst. From Brunetti E, Kern P and Vuitton DA (2010) Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. *Acta Tropica* 114(1):1–16. doi:10.1016/j.actatropica.2009.11.001.

The cycle continues when a definitive host has access to the viscera of an infected intermediate host. Once ingested, PSC attach to the intestinal walls of the hosts and develop into adult worms. The cycle then starts again. PSCs cannot pass the intestinal walls of mammals. For this reason, the consumption of infected organs by intermediate hosts is not infective (Agudelo Higueta et al., 2016; Romig et al., 2017a; Tamarozzi et al., 2016a).

Humans are “accidental” or “dead-end” intermediate hosts, as they very rarely perpetuate the parasite life cycle. Humans get infected with the ingestion of eggs present on food or contaminated, unwashed hands. After infection, most of the times the cysts develop in the liver or lungs (50–70 and 10–20% of cases respectively), due to the initial hematogenous spreading via the portal veins and inferior vena cava. The spleen is the third anatomical location by frequency (10–15%) while other organs such as the bone, heart, brain are more rarely involved (Agudelo Higueta et al., 2016; Romig et al., 2017a; Eckert and Deplazes, 2004).

The structure of AE and CE cysts shares these baseline characteristics, however the way the lesions induced by the two parasites are is profoundly different: while in the case of CE parasitic growth leads to the formation of a single cyst compressing and displacing the surrounding tissues, AE develops with the formation of multiple lesions that slowly erode target organ tissues. The different development of the two lesions is thought to be an adaptive response to the different kinds of intermediate hosts colonized by the two parasites: while in the case of CE larger hosts are usually infected, allowing for the growth of a uniloculated lesion, AE prefers smaller intermediate hosts with reduced space for growth and a reduced life span: a more aggressive growth and faster lesion development would be needed to ensure parasite survival (Romig et al., 2017b). Another key difference is the localization of the cysts, which are in AE located in the liver in 98% of cases, with extra-hepatic localization very rarely described (Wen et al., 2019).

CE classical and molecular epidemiology

As previously stated, CE is particularly present in areas where animal husbandry is practiced and is a cosmopolitan zoonosis, present in all continents except Antarctica (Deplazes et al., 2017; Wen et al., 2019). The only territories that have been declared free from CE are small island nations or regions, namely Finland, Iceland and New Zealand (Fig. 3).

The epidemiology of CE has not been well investigated in several countries that are thought to be endemic on the basis of the presence of previously reported human cases and of animal surveys (Deplazes et al., 2017). However, a complete evaluation of the epidemiology of CE would require the collaboration of several parties, including public health authorities, medical and veterinary experts (Brunetti et al., 2011). This often proves impossible, hindering control efforts. For this reason, the exact epidemiology of CE in most of Africa is not clearly known, as well as in parts of Central Asia and South America (Deplazes et al., 2017; Angheben et al., 2017; Gestal et al., 2015). While efforts to clarify the animal epidemiology of CE rely on animal studies including surveys on

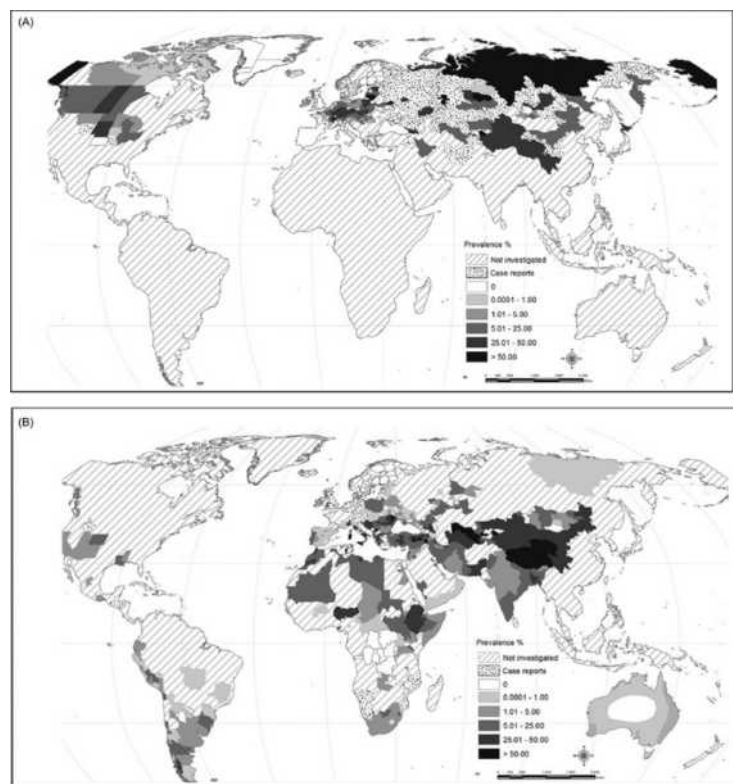


Fig. 3 Map showing the distribution of *E. alveolaris* (Panel A) and *E. granulosus* (Panel B) in the world. From Deplazes P, Rinaldi L, Alvarez Rojas CA, Torgerson PR, Harandi MF, Romig T, Antolova D, Schurer JM, Lahmar S, Cringoli G, Magambo J, Thompson RCA and Jenkins EJ (2017) Global distribution of alveolar and cystic echinococcosis. *Advances in Parasitology* 95: 315–493. doi:10.1016/bs.apar.2016.11.001.

slaughtered intermediate hosts and on dogs (Scala et al., 2017; Conchedda et al., 2016; Craig et al., 2007b; Lahmar et al., 2013; Boufana et al., 2015), a complete evaluation of CE animal epidemiology requires enormous commitment from public health authorities. In fact, in many countries where CE is hyperendemic, animals are often slaughtered at home and the offals are then left on the street for stray dogs to feed upon, or given to house dogs (El Berbri et al., 2015; Benchikh ElFegoun et al., 2016). In several countries this practice is partly due to religious reasons as the ritual killing of animals in the house is a practice diffused in prevalently Islamic countries (El Berbri et al., 2015). However, the lack of abattoirs designated for the slaughtering and inspections of animal carcasses may represent an aggravating factor (Lahmar et al., 2013; Ducrotoy et al., 2015; Torgerson et al., 2003). Studies have also shown that when abattoirs are present, inspections by veterinarians are often badly conducted due to the scarce commercial value of offals from sheep (El Berbri et al., 2015). Protocols for the appropriate disposals of offals from abattoirs are often lacking as well (Benchikh ElFegoun et al., 2016).

The epidemiology of human CE has been assessed in research projects and by public health authorities (Tamarozzi et al., 2017, 2018; Chebli et al., 2017; Macpherson et al., 2004). China, one of the most endemic countries worldwide, currently manages the widest public health surveillance network in the world. However, in many instances human surveillance programs are often lacking or non-existent. For instance, in Italy CE is not a notifiable disease (Brundu et al., 2014), and public health estimates have been conducted on the basis of Hospital Discharge Records, which are an imperfect tool as they leave out outpatients and asymptomatic cases (Tamarozzi et al., 2015). Recently, results from ultrasound (US)-based surveys carried in Bulgaria, Turkey and Romania show that a consistent proportion of patients with CE were not aware to carry the infection, which was expected. Active cysts were found in all ages and both sexes. The presence of active cysts in young patients may indicate continuing transmission. Most cysts were inactive, which is an expected result considering that they tend to spontaneously progress towards inactivation (Tamarozzi et al., 2018). However, the most relevant result of Tamarozzi and colleagues was showing that surveillance systems for CE are often lacking information. In fact, the survey carried out in Romania looked at around 1% of the country's rural population and found approximately the same number of cases notified by hospital records in the whole country in that same year (Tamarozzi et al., 2018). The study shows well that official notification systems often underestimate the number of cases, as in Romania the projections obtained by the researcher showed that around 151,000 people could be affected by the disease and be asymptomatic, and that 40% of the patients may carry active cysts (Tamarozzi et al., 2018). The result is even more impressive considering that estimates for the global burden of CE until now gave the number of patients infected at one to three million people (Budke et al., 2006).

Human studies include seroprevalence surveys (Fomda et al., 2015; Cetinkol et al., 2017; Akalin et al., 2014; Himsawi et al., 2019; Manciuilli et al., 2017). These studies aim to estimate the prevalence of CE based on the number of subjects with a positive serology for CE. However, the immunology of CE is complex and false positives in seroassays may be cross-reactions or reflect environmental exposure to parasitic antigens without the establishment of an infection (Tamarozzi et al., 2016a). Subjects with positive serology but negative imaging often revert to seronegative after a relatively short time, hence seropositivity should not be a marker for early infection (Hernández et al., 2005). However, although McPherson and colleagues demonstrated the superiority of US over serology in the assessment of CE epidemiology over 20 years ago, serosurveys continue to be employed (Macpherson and Milner, 2003; MacPherson et al., 1987).

Initial distinctions among different strains of *E. granulosus* were made on a morphological basis by spotting differences in adult worms in the definitive hosts or metacystodes and protoscoleces in intermediate hosts. Currently, *E. granulosus* is considered a species complex comprising nine genotypes (Romig et al., 2017a; Kinkar et al., 2016). Since the advent of molecular methods, morphological differences have been matched with genomic data and initially 10 genotypes were proposed (Moro et al., 2009). However, the G9 genotype has recently been assimilated to the G7 strain. The genotypes are grouped in different species and show a certain specificity for some species of intermediate hosts. The most prevalent genotype is G1, which accounts for 85% of CE cases worldwide and mainly parasites sheep in its cycle (Kinkar et al., 2016). Initial studies were conducted using molecular techniques ranging from RFLP-PCR to sequencing using two mitochondrial genes (nad1 and cox1) (Nikmanesh et al., 2014). The molecular resolution from these two genes has proven to be insufficient for in-depth analysis of phylogenetical relations between genotypes. For this reason, the use of nuclear genes has been explored in the last years (Kinkar et al., 2016, 2017). After new data were obtained from nuclear markers, the elimination of the G2 genotype has been proposed (Kinkar et al., 2017). However, a consensus still must be reached on this point. All known genotypes but G4 have conclusively been shown to be able to infect humans (Agudelo Higuaita et al., 2016).

AE classical and molecular epidemiology

AE has a significantly narrower distribution area compared to CE (Fig. 3) as its main endemicity area is located in some Asiatic regions, although it is also found in northern Europe with parts of France, Germany and other northern countries being affected. Human cases have also been reported in Hungary, and wolves infected by *E. multilocularis* have been found in northern Italy. In the Americas, while northern American regions including parts of the US and Canada are known endemic regions, recent surveys have confirmed an expansion of areas where the parasite is present, although this might just be due to increased sampling efforts and the impact of these findings on the epidemiology of human AE cases is still uncertain. Molecular markers for the characterization of AE include sequencing studies and the use of microsatellite markers. More specifically the EmsB marker, has proven useful in the execution of phylogenetic studies (Umhang et al., 2014, 2016, 2021).

Immune response in intermediate hosts

CE immune response in intermediate hosts

CE infection generates a complex immune response, as the parasite passes through several stages while in the body of the intermediate host. Briefly, studies on the immune response to infective oncospheres have shown that infection by oncospheres elicits a strong immune response, which is able to kill most parasites within days (Tamarozzi et al., 2016a). Oncospheres elicit a strong antibody response, acting together with complement to mediate protection against oncospheres, with the help of neutrophils. This strong immune response has been harnessed to develop the EG95 vaccine, that has proved highly efficacious in trials in several geographic regions (Tamarozzi et al., 2016a). The protection elicited by the vaccine can also be transmitted to the offspring of vaccinated animals via colostrum. The vaccine has been developed for the parasite G1 genotype and its efficacy against other genotypes is still unknown. This same initial immune response may be the basis of the high seroprevalence rates found in several studies, but no experimental data so far were produced to substantiate this hypothesis (Tamarozzi et al., 2016a; Fomda et al., 2015).

The immune response has been frequently investigated by using a mouse model where PSCs are injected in the peritoneum, mimicking secondary echinococcosis deriving from spillage of hydatid from ruptured cysts. The initial immune response has been shown to be of a Th1/Th2 phenotype, with an increased expression of interleukin (IL-) 4, IL-5, IL-10 and interferon (IFN-) γ (Petrone et al., 2015a,b; Wang et al., 2014). Studies have shown that the parasite is then able to downregulate both arms of this mixed immune response, which allows for parasite survival. Inflammation around the cysts (i.e., in host derived tissues) has been shown to be less prominent in live cysts than in degenerating cysts (Tamarozzi et al., 2016a). This may suggest that with the parasite proceeding towards inactivation, the mechanisms that allow for parasite tolerance are also less efficacious, allowing for a renewed immune response (Petrone et al., 2015a,b). However, the relationship of causality between cyst inactivation and inflammation in the host tissue still must be clarified, as cyst inactivation may well be the consequence of the renewed inflammation rather than its cause (Tamarozzi et al., 2016a). Parasite survival in the host does not rely solely on the isolation of the parasite from the host through the barrier effect of the LL. Actually, the LL has been shown to allow the exchange of molecules between the metacestode and the host (Tamarozzi et al., 2016a). This is proven by the high level of anti-hydatid cyst fluid (HCF) antibodies produced in intermediate hosts, and more recently by the detection of exosomes (vesicles that contain a variable parasite-derived and host-derived cargo and that may represent a future target for diagnostic or therapeutic approaches) in HCF and plasma of hosts (Cwiklinski et al., 2015; Siles-Lucas et al., 2017a; Fratini et al., 2020). Furthermore, host immunoglobulins are found in HCF (Tamarozzi et al., 2016a). While the LL does allow for the exchange of molecules between host and parasite, it also has immunomodulatory properties as shown by its ability to inhibit activation of antigen presenting cells and complement activation (Tamarozzi et al., 2016a; Gottstein et al., 2017).

HCF contains parasite molecules with immuno-active properties, involved in the complex immune-mediated interplay between the parasite and the host (Siles-Lucas et al., 2017b). Antibody, and more recently cytokine, in response to components of the HCF are explored and used for the immunodiagnosis of CE (Carmena et al., 2006).

AE immune response in intermediate hosts

Opposite to what is observed in CE, AE elicits a strong granulomatous response. Cellular immunity plays a major role in the immune response to AE infection and genetic background also has an influence in determining the efficacy of immune response (Vuitton et al., 1989). An initial Th1 response changes gradually to a mixed Th1/Th2 during the chronic phase of AE. Various cytokines (including IL-4, IL-5, IL-13, and IFN- γ) are produced. However, the most relevant are regulatory (IL-10 and transforming growth factor (TGF-) β), whose level has been shown to be increased in advanced disease stages (Zhang et al., 2012).

The Th2 to Th1 shift is observed in liver tissues in the early phases of infection (Wang et al., 2014). As in CE, enhancing Th1 immune responses, especially by acting on the innate immunity using IL-12, IFN- α , and TNF- α , increases resistance to *E. multilocularis* both in experimental mice and in humans (Liance et al., 2000).

Fibrosis formation, also induced by TGF- β seems to also have a role in host protection. However, fibrosis explains several clinical complication of AE. *In vitro* interactions have been shown between *E. multilocularis* vesicular fluid and some immune pathways involving PD-1/PD-L1, CTLA-4, LAG-3 and TIM-3 (Bellanger et al., 2017). The PD-1/PD-L1 pathway inhibits lymphocyte proliferation in the development of tumors and is over-expressed at the chronic stage of experimental AE (Jebbawi et al., 2021). Another promising checkpoint is the T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) and its CD 155 ligand. TIGIT expression is increased and correlated with lesion activity in animal models and AE patients (Zhang et al., 2021). Studies have shown that TIGIT has a role in the exhaustion and functional impairment of natural killer (NK) cells in AE models (Zhang et al., 2021).

In *E. multilocularis* infection the LL has an important role in immune modulation. Immune-modulatory molecules are polysaccharide-containing antigens such as Em2 (G11), antigen C, EmP2, and Em492, a neutral glycosphingolipid, and novel mucin-type glycoforms. EmAP, a protein antigen, induces antibodies associated with disease severity and resistance to treatment in AE patients and causes the production of Th2-type cytokines (Vuitton and Gottstein, 2010).

Immunological applications in the diagnosis of CE

Diagnostic assays based on the detection of immune responses to parasitic antigens have shown considerable variability in results; this surely partially due to heterogeneity of antigenic preparations and lack of standardization in preparation of single antigens (Pagnozzi et al., 2016). Furthermore, the influence of different genotypes on the diagnostic efficacy has been scarcely explored. Native or purified versions of HCF and of the two main antigens of HCF, AgB and Ag5, have been investigated and used from the serodiagnosis of CE (Siles-Lucas et al., 2017b). Both molecules are expressed in all parasitic life stages have been shown to have immunomodulatory properties, but their biological role still has to be completely explored (Tamarozzi et al., 2016a). Other antigens such as EgTeg, EgTPx have been isolated but their role in *E. granulosus* immunity is not clear and they are not used in current serological assays (Tamarozzi et al., 2016a).

Recombinant antigens have been proposed to overcome the lack of antigen standardization. Some of these antigens (2B2t, rAgB/2) (Manzano-Román et al., 2015; Hernández-González et al., 2012, 2018; Li et al., 2003) have been tested in the diagnosis of CE (see below) with promising results (Manzano-Román et al., 2015; Hernández-González et al., 2012, 2018; Li et al., 2003). However, most studies assessing the diagnostic performance of new serological tests have been carried on small cohorts of patients and without including a crucial piece of information, cyst stage, which is one of the key variables influencing serology responses, together with cysts number, localization, size, and previous treatment (Li et al., 2003; Manterola et al., 2005, 2007; Baraquin et al., 2017). Seroassays have been shown to be falsely negative in a high proportion of both active (particularly CE1) and inactive cysts.

Besides antibodies, other host-derived molecules like interleukins, for example, are involved in the immune response to *E. granulosus* infection. As mentioned before, *E. granulosus* seems to be able to cause a switch in the immunophenotype from a Th1 or mixed Th1/Th2 phenotype to a down-modulated environment (Petrone et al., 2015b). This observation has been used to study changes in the expression of different IL levels during infection (Petrone et al., 2015a,b). In particular, IL-4 produced by whole blood stimulated with purified AgB has been proposed both as a diagnostic marker and as a marker of cyst activity (Petrone et al., 2015a, 2020, 2021).

Another recent advancement in the understanding of parasite biology, with potential diagnostic exploitation is represented by microRNAs (miRNAs), another class of molecules that have been intensively studied recently in both infectious and non-infectious diseases, as alterations in the composition of a host or parasite miRNome or in miRNA expression levels of these small molecules may give information on the status of such processes (Coakley et al., 2015; Tsitsiou and Lindsay, 2009). For instance, they have been studied in infections by *Schistosoma japonicum*, *Schistosoma mansoni* and *Fasciola hepatica* (Gantier, 2010; Lu and Rothenberg, 2013; Silakit et al., 2014; Arora et al., 2017). In the case of *E. granulosus*, miRNAs from infected sheep have been shown to be differentially expressed in strains that had MHC profiles resistant or susceptible to *E. granulosus* infection (Macchiaroli et al., 2015; Cucher et al., 2015). miRNA levels were also altered in humans with active cysts (Mariconti et al., 2019; Örsten et al., 2021). Further studies are needed to assess the potential of miRNAs for the diagnosis CE in general and specifically for active infections.

Immunological applications in the diagnosis of AE

In the case of AE, the role of cytokine assays has also been studied recently, with preliminary results showing a potential role for the use of IL-4 as a marker to differentiate AE and CE. Several cytokines have been shown to differ in expression between AE infected patients and healthy controls. However, further studies are needed to assess the applicability of cytokine assays in the diagnosis of AE (Petrone et al., 2020; Ma et al., 2020). Cell-free DNA has also been proposed as a marker for the diagnosis of AE as well as for the follow-up of treated patients (Baraquin et al., 2018; Fan et al., 2021), but its limited availability outside of referral centers is likely to impair its widespread use.

CE—Clinical presentation

Primary infection

In most cases, CE infection in humans is asymptomatic, and is diagnosed while the patient is undergoing imaging for other reasons (Agudelo Higuaita et al., 2016; Brunetti et al., 2010). Some patients have symptoms related to CE cysts, which vary depending on their location. While CE can theoretically affect all human organs, most patients carry a CE cyst in the abdomen, either in the liver (50–70%) or the spleen (5–15%) (Brunetti et al., 2010). In this instances symptoms are fairly non-specific and include abdominal pain, discomfort and the perception of a mass in the abdomen if the cyst is very large. Spleen CE has practically the same symptoms as liver CE, while other locations in the abdomen are less frequently observed.

If CE cysts are in the lungs, which are the second localization by frequency, patients can experience cough or thoracic pain in the case of uncomplicated cysts (Cappello et al., 2013).

Brain and cardiac CE cysts tend to be symptomatic since the limited space for the expansion of the cysts (Polat et al., 2003; Hamamci et al., 2004). This also applies to bone CE, where cysts grow with a branching pattern and do not develop the classical cyst structure (Papanikolaou, 2008; Neumayr et al., 2013).

Complicated cysts in the abdomen or lungs are usually symptomatic. A classic complication of lung CE is the elimination of cyst fragments via the airways after the formation of a cysto-bronchial fistula (Brunetti et al., 2010). Mechanical complications can also interest the biliary and urinary tract, as well as blood vessels. For instance, cysts near the Inferior Vena Cava (IVC) occasionally may leak into this blood vessel, with the release of parasitic material causing disease dissemination, potentially including pulmonary

embolism (Akbulut et al., 2013; Jaussi, 2009; Poyraz et al., 2016). Cyst rupture occurs (Neumayr et al., 2011) but no study has assessed risk factors for cyst rupture, though experts report cases of rupture of cysts located under the liver capsule, while in lung, large cysts can rupture after the initiation of ABZ administration (Brunetti et al., 2010).

Anaphylaxis is one of the most feared complications of CE, but is a rare phenomenon (Neumayr et al., 2011). In general, mechanical complications are also associated with IgE elevation and hypereosinophilia, due to the sudden release of parasitic antigens from the cyst (Agudelo Higuaita et al., 2016). Cysts can also become infected with the formation of an abscess.

Secondary infection

Secondary CE is defined as the appearance of cysts in anatomical sites that are distant from the primary localization, following spillage of cystic fluid and seeding of protoscoleces from the primary cyst. Due to the long natural history of CE and the fact that infections are diagnosed years after the initial contact with the parasite, it can be hard to establish whether patients have two primary localizations or if there has been disease spreading (Neumayr et al., 2013). This is particularly evident in the case of patients with bone localizations. Most of the cases of secondary CE are, however, due to the diffusion of scoleces in the abdomen following the rupture of cysts. Other localizations include cerebral, spleen, musculoskeletal CE following hematogenous spreading and lung CE due to embolization (Neumayr et al., 2013; Prati et al., 2016). Patients with peritoneal CE are often asymptomatic. Musculoskeletal CE tends to be more symptomatic, because of mass effect with compression of vascular or nervous structures (Agudelo Higuaita et al., 2016; Cattaneo et al., 2019).

CE diagnosis and follow-up

Imaging

It is the main tool for CE diagnosis. In particular US has a key role in the diagnosis of CE as a portable, non-invasive imaging method. A US-based diagnosis of CE requires the detection of pathognomonic signs of CE cysts. The first attempts at a US classification were made by Gharbi. Later, Caremani and colleagues also proposed their own classification (Macpherson et al., 2003). At the start of the 2000s, the WHO-IWGE produced a standardized classification (Brunetti et al. 2010). This classification essentially made some modifications to that proposed by Gharbi. The classification distinguishes six cyst stages and three groups of cysts (Fig. 4). CE1, CE2 and CE3b cysts are considered active, CE3a are considered transitional and CE4 and CE5 are considered inactive. These distinctions have led the WHO-IWGE to endorse a stage-specific approach to the treatment of uncomplicated CE cysts, which will be reviewed in more detail below.

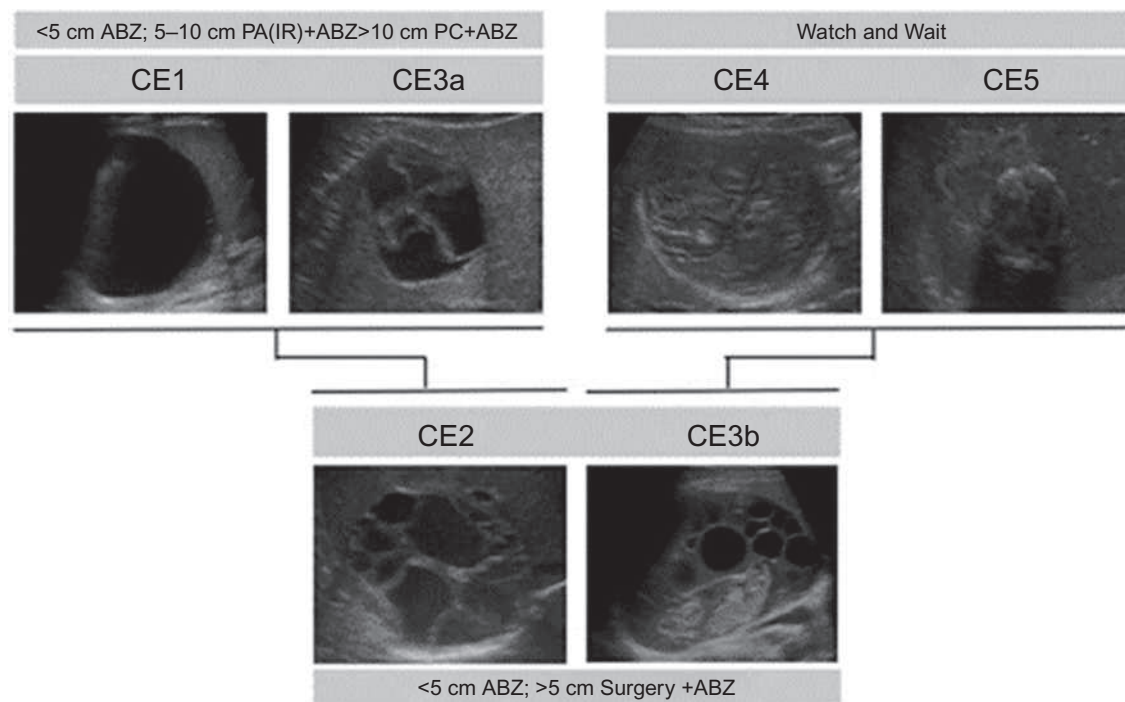


Fig. 4 Stage-specific clinical management of hepatic uncomplicated CE cysts, in line with Expert Consensus recommendation (Brunetti et al., 2010). From Firpo G, Vola A, Lissandrin R, Tamarozzi F and Brunetti E (2017) Preliminary evaluation of percutaneous treatment of echinococcal cysts without injection of Scolicidal agent. *The American Journal of Tropical Medicine and Hygiene* 97(6):1818–1826. doi:10.4269/ajtmh.17-0468.

The classification and the stage-specific approach proposed by the WHO-IWGE are still far from being universally accepted although the situation has recently improved (Mirabile et al., 2019). Experience from the CE control program in the Rio Negro province of Argentina has shown that, given enough time and tutorship, general physicians with no prior education on US or CE can learn to use the classification and the stage-specific approach endorsed by the WHO-IWGE (Larrieu et al., 2011). Such programs are not widespread, however, as they require political and economic commitment from authorities, something which is rarely available for CE.

US allows for the diagnosis of CE cysts in the muscles and in lung CE if the cyst is adjacent to the pleura, as well as cardiac CE (Polat et al., 2003; Richter et al., 2003). Other anatomical locations that are not accessible to US can be explored by either CT scan or MRI, and X-ray in the case of lung CE, although this should be followed by a second level exam if surgery is deemed necessary (Stojkovic et al., 2012). CE cysts explored via contrast-enhanced radiological examinations do not show contrastographic impregnation. This can be detected around the cyst if pericystic inflammation is present (Polat et al., 2003). Stojkovic and colleagues showed that MRI is superior to CT for the diagnosis and staging of hepatic CE (Stojkovic et al., 2012). Currently, experts from the WHO-IWGE also recommend the use of MRI for extra-hepatic localizations (Tamarozzi F, personal communication). This is more complex for lung as lung MRI is often not available, even in industrialized countries.

US and other imaging methods also allow for the non-invasive follow-up of CE patients. While many cysts tend to evolve towards inactivity in their natural history, in diagnosed humans with active cysts they should do so in response to treatment (Tamarozzi et al., 2016a). In fact, sometimes the administration of treatment can help clarify the nature of a suspect CE lesion, as the administration of ABZ for as little as 1 month can cause the detachment of the endocyst in CL lesions of parasitic nature. A course of ABZ has also been shown to cause temporary cyst solidification, in CE2 and CE3b cysts (Rinaldi et al., 2014a).

Serology

Serological assays are widely used for the diagnosis of CE. Several techniques (ELISA, Western Blot—WB, IHA, EIA and Rapid Diagnostic Tests—RDT) have been developed for the diagnosis of CE. The first antigen used for the development of tests was native Hydatid Cystic Fluid (HCF), followed by native and recombinant antigens (Carmena et al., 2006; Manzano-Román et al., 2015). Problems regarding these different preparations have been illustrated in the Immunology section of this chapter. To date no study has rigorously assessed the role of serology in the differential diagnosis of CE. The current consensus on the use of serology is that it is complementary to imaging in the diagnosis of CE. Moreover, some studies have attempted to establish if these tests could be used in the follow-up of patients with CE, especially in surgically treated patients (Boubaker et al., 2014; Stojkovic et al., 2017). Surgical treatment of a CE cyst should entail the elimination of all antigenic stimuli on the immune system, and patients should ideally revert to seronegativity if no relapse or new infection occurs. This hypothesis is hard to study and verify, as several surgical techniques are used in the surgical management of CE, and there is no absolute guarantee that all the parasitic tissue is eliminated during surgery (Wen et al., 2019). Tests with native, purified and recombinant tests have been used and they have all shown that surgical patients cannot be followed-up by serology to detect relapses with the currently available tests (Stojkovic et al., 2017).

RDTs have also been developed for the diagnosis of CE and tested in a few studies. Most of these assessed the performance of serological assays in a hospital setting, some did not include information on cyst stage (Baraquín et al., 2017; Tamarozzi et al., 2016b; Gao et al., 2018). A recent study on the use of RDTs in resource-limited settings as shown a potential role as confirmatory tests in the presence of suggestive imaging (Manciulli et al., 2021). The detection of antibodies belonging to the IgG4 subclass has been proposed to be more specific than other serological methods, as well as to correlate with the degree of activity of CE cysts (Tamarozzi et al., 2016a). However, the study that came to this conclusion used HCF from equine cysts with a possible bias due to differences in the infecting genotypes. Moreover, the study did not report the stages of the involved cysts (Lawn et al., 2004).

For practical purposes, serology should never be used alone in the diagnosis of CE, as the presence of a lesion with suggestive characteristics is needed to justify the execution of serological tests. Furthermore, single center experiences, testing of multiple commercial assays and a systematic review of the literature have shown that the execution of a single test suffers from the fact that results of CE serology are influenced by several factors, both test-dependent (antigen choice, antigen batch variability, preparation method, test technique) and laboratory-dependent. Parasite-dependent factor (cyst number, stage and size) also play a role (Tamarozzi et al., 2016b, 2021a,b; Vola et al., 2019; Hernández-González et al., 2008). Ideally, two first line tests are initially employed, of which one is ideally WB (Tamarozzi et al., 2021a; Vola et al., 2019). As discussed above, no reliable serological marker for disease activity exists, as antibodies can persist for years after treatment (Hernández-González et al., 2008, 2018; Piccoli et al., 2014).

Cross reactions with other parasitic infections are also possible in CE, with *Theridion solium* and *E. multilocularis* cross-reactions being quite frequently present (Manzano-Román et al., 2015; Hernández-González et al., 2008).

Molecular and immunological methods

Molecular methods are not widely used in the diagnosis of CE in humans. PCR protocols have been developed for the diagnosis of CE in animals, using cyst samples from slaughtered animals (Piccoli et al., 2013), but parasitic DNA cannot be found in human serum, unless a cyst ruptures. In this case, DNA has also been found in the urine of patients. However, this is a rare instance and has little clinical utility in most patients (Chaya and Parija, 2014). The potential role of cytokine-based assays and exosome detection has been discussed above.

CE treatment

Surgery has been traditionally considered the first line treatment for CE. Several techniques have been proposed, either radical or conservative. From the end of the 1980s, a new class of drugs became available and showed activity against both CE and AE infections. Benzimidazoles are a class comprising several compounds, two of which are approved for human use and used in the treatment of CE: Mebendazole (MBZ) and ABZ. It soon became clear that medical treatment alone could not treat all CE cysts. The need for other treatments led to the development of percutaneous procedures, the most widely used being the Puncture, Aspiration, Injection, Respiration (PAIR) procedure. This technique was developed roughly at the same time by a group of researchers in Pavia (Filice and Brunetti, 1997) and in Tunisia (Gargouri et al., 1990). A second percutaneous procedure with percutaneous catheters was also developed for cysts larger than 10 cm (Brunetti et al., 2010). Over time, it also became clear that inactive cysts very rarely reactivate. As such, a Watch and Wait (WW) approach was also proposed for uncomplicated inactive cysts. All these approaches were taken into account when formulating the Expert consensus for the diagnosis and treatment of CE and AE, whose recommendations are summarized in Fig. 4 (Brunetti et al., 2010).

Medical treatment

MBZ and ABZ exert their anti-parasitic activity by blocking the formation of β -tubulin polymers within the staminal cells of the parasite. They have also been shown to interfere with glucose uptake from the parasite, which proves damaging to *E. granulosus* due to the key role of glucose in this parasite metabolism (Hemphill et al., 2014; Siles-Lucas et al., 2018). However, these drugs only have a parasitostatic activity in the treatment of CE, and it is thought that their effect is to impede the biological activity of the parasite while the immune system also fights the infection (Siles-Lucas et al., 2018). ABZ and MBZ are commonly used to treat other parasitic infections such as soil transmitted helminthiasis or neurocysticercosis (Horton, 2002). However, the duration of treatment for these infections is considerably shorter than the one required by CE and AE (Horton, 2002). Currently, drugs are administered over the course of months, as experts believe that this is required to exert an effect on cysts (Brunetti et al., 2010). ABZ and MBZ were initially administered in courses of 28 days with 1 week of suspension, due to concern on the presence of side effects such as hepatotoxicity and bone marrow toxicity. This was based on animal studies, but subsequent studies have shown the safety profile of both drugs with an acceptable risk/benefit ratio for patients undergoing prolonged administration (Horton, 1997, 2002). However, most national pharmacovigilance authorities have not updated the recommendations for the use of ABZ and MBZ in the treatment of CE. Currently, France is the only country to accept the administration of ABZ in a continuous fashion (Manciulli et al., 2018a). ABZ has now become the drug of choice in the treatment of CE, with MBZ playing a secondary role. This is largely due to the better bioavailability of ABZ which can be translated in a lower dose needed to attain clinical efficacy (Siles-Lucas et al., 2018).

Another problem with ABZ and less frequently MBZ is that the drugs can sometimes become unavailable, or are not available at all in some countries. In Italy ABZ shortages that have impacted on the treatment of patients (Manciulli et al., 2018a).

Other candidate molecules for medical treatment

Several compounds have been tested in vitro or in vivo to determine their possible anti-echinococcal activity. These include other benzimidazoles such as oxfendazole, flubendazole, fenbendazole, antimalarials such as mefloquine and artemisinin derivatives, anti-cancer drugs and antibiotics or antivirals (Siles-Lucas et al., 2018). Most trials outside the benzimidazole class in vivo have been carried out to assess combination therapies with benzimidazoles, not single therapy protocols (Siles-Lucas et al., 2018). Another interesting point is the search for new formulations of existing benzimidazoles to increase the drug penetration into the cyst, something which affects ABZ efficacy. Liposomal formulations have been tested, as well as tablets containing nanopolymers (Siles-Lucas et al., 2018). Sodium salts of ricobendazole have also recently been used and have been tested in a mouse model. Several compounds derived from medicinal plants have also been tested in both experimental and animal models, with none reaching human clinical experimentation thus far. However, the largest obstacle to assessing new medical treatments is their long duration (Siles-Lucas et al., 2018).

Surgical treatment

Radical and conservative techniques can be distinguished. In the former, CE cysts are completely removed from the liver parenchyma, with or without hepatic resection (Wen et al., 2019; Rinaldi et al., 2014b). Different techniques can also be distinguished based on an open or closed cyst approach. One of the most diffused is the “total cystectomy” or pericystectomy approach, which uses the natural cleavage plane formed by the pericyst to remove the entire cyst without opening it. In conservative approaches, only the parasitic material is removed, while the resulting residual cavity is managed by capitonnage, omentoplasty or other techniques (Rinaldi et al., 2014b). Recently, a standardized endocystectomy approach has been described by the group at the Heidelberg University Hospital (Al-saeedi et al., 2019). All open techniques should be executed by experienced teams and extreme caution should be used to avoid protoscoleces spillage from the cyst, as this is the most relevant risk factor for recurrence (Al-saeedi et al., 2019). The use of surgical sponges soaked with 20% hypertonic saline has been recommended since the establishment of the WHO-IWGE expert consensus (Brunetti et al., 2010). Since this solution is toxic for the peritoneum and

the biliary tract and can cause hyperosmolar coma, the surgical field should also be covered by sponges soaked in 0.9% NaCl solution (Al-saeedi et al., 2019). Currently, studies on surgical patients suffer from the same limitations of those focusing on medical therapy, as it is impossible to effectively generate evidence on the best treatment of uncomplicated cysts without randomized controlled trials (Rinaldi et al., 2014b). The same applies to the management of surgical complications, as one recent review found that questions are many and answers are lacking.

Cysts complicated by fistulization or rupture are generally treated by surgery, general conditions of the patient allowing. Infected cysts may be treated by surgery if percutaneous procedures are not available (Brunetti et al., 2010; Rinaldi et al., 2014b).

Percutaneous procedures

Percutaneous procedures were developed for the treatment of predominantly liquid CE cysts (CE1 and CE3a). The first technique to be developed was PAIR, and some other techniques have been proposed over time, all variations of the PAIR protocol, which sees a first step in the US-guided percutaneous puncture of the cyst, followed by aspiration of the cyst content, usually presenting as a clear, transparent fluid in young untreated cysts, while it becomes yellow and turbid in cysts that are spontaneously evolving towards inactivation or have been treated by ABZ (Rinaldi et al., 2014b; Brunetti et al., 2018; Örmeci, 2014). After aspiration, a scolicidal agent (hypertonic saline or alcohol) is injected in the cavity, and is left there for 20 min to attain cyst sterilization. After this, the fluid is re-aspirated and the patient is kept in observation for 24 h. The whole procedure is performed in the presence of an intensive care specialist, ready to intervene in the case of collateral effects, mainly anaphylactic reactions. It should be noted that these are possible but quite rare (Neumayr et al., 2011; Rinaldi et al., 2014b).

Variations of the PAIR technique have been proposed, and recently a procedure without the use of scolicidal agents has been proposed by our group (Firpo et al., 2017).

PAIR is recommended to treat cysts that are large more than 5 cm, while another kind of procedure using large bore catheters has gained ground as an alternative to surgical treatment in patients with large cysts (more than 10 cms) (Brunetti et al., 2010; Akhan et al., 2016). In this procedure, a catheter is inserted under US guidance in the cyst cavity, and is left in place draining the cyst content over days, while the patient is hospitalized. Scolicidal agents can be used in a similar fashion to what is done for the PAIR procedure (Brunetti et al., 2010).

Cysts that are larger than 10 cms can also be treated with PAIR, however this requires the use of a large bore catheter to progressively drain cyst content until a drainage of less than 75 ml is obtained on a daily basis (Brunetti et al., 2010).

More recently, a procedure relying on cyst aspiration has been applied in CE2 and CE3b cysts in the liver with excellent results due to the absence of relapses (Akhan et al., 2017), but its general applicability would require validation through the execution of a clinical trial.

Due to the use of scolicidal agents, patients undergoing percutaneous procedures should always be evaluated for the presence of a biliary fistula, especially if the cyst is larger than 7,5 cm. This has been shown to be a clear risk factor for the presence of fistulas (Rinaldi et al., 2014b). The fact that this evaluation ideally requires access to or to colangio-MRI has limited the use of percutaneous procedures in resource-limited settings.

All patients treated by a percutaneous procedure must undergo ABZ administration before the procedure and for the subsequent month, according to the WHO-IWGE (Brunetti et al., 2010). This recommendation is also valid for patients undergoing surgery and is based on data from animal models showing that in case of peritoneal dissemination of cyst content, 1 month of ABZ could prevent secondary CE. However, this recommendation is followed inconsistently in endemic countries (Manciulli et al., 2018b).

The last recorded clinical trial to date has regarded the use of ABZ post-operatively in percutaneously treated patients, conducted by colleagues from the University of Ankara. The group concluded that 1 month of ABZ is enough to reduce recurrence rates (Akhan et al., 2017).

Watch and wait

Although not strictly a “treatment”, watch and wait (WW) is recognized as a clinical management option for inactive cysts. Its rationale derives from the demonstration of the scarce reactivation rate of cysts that are found to be spontaneously inactive, or that have been treated according to the WHO-IWGE recommendations (Piccoli et al., 2014; Solomon et al., 2017; Stojkovic et al., 2016; Lissandrini et al., 2018). A limitation of currently published studies is that these are based on patients seen at referral centers for CE and the number of patients is not high enough to generate conclusive evidence. Generating data from hyperendemic countries is also hard, as in these settings surgeons tend to dominate the treatment of CE (Manciulli et al., 2018b). However, WW has been shown to avoid unnecessary treatment for patients, reducing risks and saving precious resources for health systems, as surgery for CE is costly (Narra et al., 2016).

Hints for a watch and wait approach have been suggested by a work considering CE3b cysts. In this paper, the authors concluded that CE3b cysts had an indolent behavior after receiving medical treatment, with the development of a low number of complications. The authors then suggested that a WW approach for CE3b cyst can be implemented in selected cases in which surgery is not strictly indicated and that are compliant with medical therapy and a subsequent long-term follow-up.

AE clinical presentation

As in the case of CE, the clinical presentation of AE can be subtle due to the slow natural history of the disease, with lesions growing during the course of years. Due to its radiological features, AE is often misdiagnosed initially, since symptoms are unspecific and radiological appearance can be quite similar to that of a malignant tumor. Jaundice is the most frequent symptom at presentation in acutely ill cases. However, the vast majority of cases are paucisymptomatic or asymptomatic at first diagnosis. Immunocompromised patients can present without symptoms more frequently, but also tend to show a faster progression towards end organ damage (Wen et al., 2019). Complications of AE include cholangitis due to biliary infection, abscess formation, and metastatic spreading either by contiguity or hematogenous dissemination (Vuitton et al., 2016; Brunet et al., 2015; Meinel et al., 2018). AE lesion progression has been associated with the presence of HLA-DR3 DQ2 haplotype as patients showed a more severe disease and a more pronounced Th2-type immune response, associated with a deeper tolerance status. Moreover, AIDS has been associated with an accelerated course of the disease, and the initiation of antiretroviral therapy usually corrects the immune defects and slows disease progression (Ran et al., 2016). Transplant patients are another category at higher risk for a faster disease progression, due to immunosuppressive drugs (Wen et al., 2019).

AE diagnosis

Imaging

As in CE, the main tool for the diagnosis of AE is imaging. Given that AE lesions tend to develop in a different fashion than CE cysts, the WHO-IWGE has introduced a staging system based on the TNM classification used for tumors. Classifications based on US, MRI and CT appearance have been proposed and are currently being used in clinical practice at referral centers (Kratzer, 2015; Kodama et al., 2003; Graeter et al., 2020a). The CT classification divides cysts into five subtypes based on morphological characteristics and it has recently been evaluated in the context of an international collaboration involving AE referral centers in China and Europe (Graeter et al., 2020b). In a recent paper, an update of the Kodama classification based on the use of MRI has been proposed, introducing two subgroups for lesions considered to be type III (IIIa and IIIb, based on the absence or presence of microcysts). Interestingly, the authors have advanced an hypothesis that would allow to establish a radiological progression of lesions through the different stages of the Kodama classification, as it was noted that type I and II lesions were more frequently present in Europe, where disease is diagnosed early, while type IV and V lesions were more frequently present in China, where in endemic area patients seek care late in the course of the disease. The authors of the proposed modification compared the introduction of these two subtypes to the introduction of the CE3a/3b categories used for CE. Further studies using PET-CT scans and MRI spectroscopy will be needed to establish the relationship between biologic viability of the various cyst subtypes and imaging features (Brumpton et al., 2021).

Based on overall imaging evaluation, AE can be staged with a staging system that follows the same structure as NTM staging used for tumors:

- PX Primary tumor cannot be assessed
- P0 No detectable tumor in the liver
- P1 Peripheral lesions without proximal vascular and/or biliary involvement
- P2 Central lesions with proximal vascular and/or biliary involvement of one lobe
- P3 Central lesions with hilar vascular or biliary involvement of both lobes and/or with involvement of two hepatic veins
- P4 Any liver lesion with extension along the vessels and the biliary tree
- N Extrahepatic involvement of neighboring organs [diaphragm, lung, pleura, pericardium, heart, gastric and duodenal wall, adrenal glands, peritoneum, retroperitoneum, parietal wall (muscles, skin, bone), pancreas, regional lymph nodes, liver ligaments, kidney]
- NX Not evaluable
- N1 Regional involvement of contiguous organs or tissues
- M The absence or presence of distant metastasis [lung, distant lymph nodes, spleen, CNS, orbital, bone, skin, muscle, kidney, distant peritoneum, and retroperitoneum]
- MX Not completely evaluated M 0 No metastasis
- M1 Metastasis

Scoring system: Stage I, P1 N0 M0; stage II, P2 N0 M0; stage IIIa, P3 N0 M0; stage IIIb, P1–3 N1 M0 or P4 N0 M0; stage IV, P4 N1 M0 or any P, any N, and/or M1.

Serology

Serological assays for AE suffer from the same limitations as the ones for CE. However, serological markers are more reliable for follow-up in the case of AE, since antibodies directed against the Em18 antigen have been shown to have good performances and can also be used to monitor response to therapy, given that imaging changes might occur over a longer time interval (Wen et al., 2019). A commercial ELISA using the recombinant Em18 antigen is available (*E. multilocularis* recEm-18, Bordier, Crissier, Switzerland).

AE treatment

AE treatment is challenging given the invasiveness of the disease, and it requires a multidisciplinary approach (Brunetti et al., 2010). The above-mentioned PNM staging system helps clinicians caring for AE patients choose an appropriate treatment strategy. Surgery is considered an option only in cases where radical resection of AE lesions is feasible, a situation which occurs only in one third of patients (Wen et al., 2019). Partial resection is currently not recommended due to poor results, while perendoscopic procedures carried out to manage complications such as biliary endoprosthesis or multiple stent insertions are accepted means. These procedures are currently an alternative to percutaneous procedures previously used together with medical therapy. Transplantation and *ex-vivo* liver resection followed by autotransplantation (ELRA) have also been used. However, the latter is scarcely used in Europe despite studies showing its efficacy on Chinese patients. Transplantation has been employed in AE patients, with good survival rates even in the presence of residual parasitic tissue in grafted livers. However, it is not frequently used in Europe due to the demographic characteristics of patients, who tend to present at a later age than Asian patients.

Medical treatment constitutes the other pillar of AE management. Prompt initiation of benzimidazole therapy is required in all cases, with the possibility to use continuous treatment, often required for the entire lifetime of the patient.

In patients with small and calcified lesions, with no FDG uptake on PET/CT, a “watch-and-wait” attitude can be followed. It is also applied when ABZ or MBZ withdrawal has been decided on the basis of serological and PET/CT negative results and in patients with radical resection after 2 years of ABZ or MBZ.

Echinococcosis control and surveillance

The control of echinococcosis has been hard to implement due to the very heterogeneous epidemiology of both AE and CE. For CE, efforts until now were mainly based on the use of dog deworming with praziquantel at regular intervals with frequencies dependent on the control program (from once monthly to twice yearly) (Larrieu et al., 2019a). Most control programs were carried out in continental south America and were able to achieve relative success with a reduction in the population of parasitized dogs and infected sheep, although some programs were discontinued due to the reemergence of the disease (Larrieu et al., 2000, 2019a). Other deployed tools include the removal of older sheep which are more likely to be infected and perpetuate transmission and post-mortem examinations to remove offals from infected sheep (Larrieu et al., 2019a). A vaccine for *E. granulosus* has been introduced by some control programmes in the last few years, with initial successes regarding vaccine efficacy (Larrieu et al., 2019b; Larrieu and Zanini, 2012). However, studies focusing on cost-effectiveness and integration with other measures of control are still pending (Larrieu et al., 2019a).

Surveillance measures include farm-based surveillance for the periodic detection and treatment of infected dogs, surveillance in humans through US-based surveys (especially in school-aged children given that the presence of active cysts indicates recent transmission) and training of primary care physicians in the use of US to stage CE (Larrieu et al., 2019a). This approach has been shown to be effective in the Rio Negro Province of Argentina. In contrast (Larrieu et al., 2011; Del Carpio et al., 2012), AE control has been less studied and implemented also due to the limited geographic distribution of the disease. Control can target foxes with periodic deworming through the use of medicated baits, and can involve surveillance in rodents to monitor for disease prevalence. Environmental surveillance using molecular tools has been proposed by some authors, however this requires further studying (Wen et al., 2019).

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Introduction

Entamoeba is a tiny protozoan parasite of man and animals. The genus *Entamoeba* includes different species that infect different animals as reptiles, birds and amphibians and others. About seven species have been recovered from the gut of man. They are *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. coli*, *E. polecki* and *E. hartmanni*. *E. gingivalis* is recovered from the buccal cavity of man. *E. histolytica* is the only known pathogenic species of the human intestinal amoebae. However, some members have tendency to behave in an aggressive way and these have been incriminated in the development of pathological reactions. *E. invadens* is a parasite of reptiles and is used as a model for in vitro studies on pathogenic Entamoeba.

The pathogenic species *Entamoeba histolytica* has been identified as the cause of different health problems ranging from mild diarrhoea to invasive and extraintestinal infections. Up to 50 million persons are infected by *E. histolytica* in the world, with almost over 100,000 deaths per year (Bercu et al., 2007; Ximenez et al., 2010), especially in developing countries as Africa, India, Central and South America (Haupt et al., 2016). *E. dispar* and *E. moshkovskii* were identified as non-pathogenic amoebae. The great resemblance of the three species in morphology, makes it difficult to differentiate between the pathogenic and non-pathogenic Entamoeba by microscopic examination and adds to the complexity of the epidemiology of the disease. Previous statistics reported that 90% of cases infected with *E. histolytica* were asymptomatic, and only 10% had some symptoms (Jackson et al., 1985; Haque et al., 1999) with an overestimation of the prevalence of human intestinal amoebiasis (Diamond and Clark, 1993).

The exact identification of different species needed the use of some more sophisticated methods as Polymerase chain reaction-based assays (PCR). The studies of DNA of different organisms was the corner stone in the identification of different species within the genus *Entamoeba* (Verweij et al., 2001, 2003; Ayed et al., 2008; Ahmed et al., 2019).

Historical notes

The history of Amoeba dates back to the prehistoric era. In May 18, 1981, Kapoor published his book about Amoebic liver abscess and included a detailed description of the history of *Entamoeba* as one of the first forms of life on earth, before the evolution of man (Kapoor, 1979).

Sushruta, one of the oldest medical authority in India, mentioned the word “Antisaar” as a description of Dysentery. However, there was no clear description of any hepatic involvement (Rajasuriya and Nagaratnam, 1962).

Hippocrates (460–377 BC) was the first to introduce the medical practice based on observation and study and described dysentery as a medical problem (Americana Corporation, 1970a).

One of the famous patients in history was Alexander the Great. He was born in 356 BC and shortly after, he became an Emperor of a great and extensive Empire. During his travels to the Eastern countries with endemic disease, he became seriously ill and died later probably due to an amoebic abscess of the liver (Americana Corporation, 1970b).

Galenus (129–210 AC), observed some association between dysentery and hepatic disease (Napier, 1946) and later in the 18th century, Joaquin Pio Eguia y Lugo in Mexico, was able to connect deaths due to liver disease to dysentery (Kapoor, 1979).

The famous French leader Napoleon Bonaparte contracted bloody dysentery “thought to be amoebic in nature” in his Exile in St. Helena. The infection was common in the island. Later he was diagnosed to have a condition known as tropical hepatitis (O’Meara, 1882). The physicians who diagnosed and managed his disease were arrested and dismissed from their jobs. In the year 1821, Bonaparte’s family appointed another physician: Antomarchi to look after Bonaparte’s health. This doctor was confused of the cause of his death, if it was due to stomach cancer or as a result of liver abscess that ruptured into his stomach, as was suggested after autopsy (Chaplin, 1913). In India, Ballingall reported that there were two different types of dysentery, the acute colonitis and hepatic flux. He mentioned the method of draining liver abscesses (Ballingall, 1818; Imperato, 1980).

A post-mortem study was carried out by Annesley (1828) on 51 cases that died as a result of severe dysentery in India. He reported that 26 of them had tropical liver disease.

It is noteworthy to mention that the first parasitic amoeba (*Entamoeba gingivalis*), was discovered by Gros in 1849 (Favoreto and Machado, 1995).

Later, Lambl reported some intestinal parasites during his study on the characteristics of stools, however he didn’t mention amoeba (Lambl, 1859).

The first identification of intestinal amoebae as a cause of disease was reported by Fedor Aleksandrovich Loch in the year 1875, when he detected a moving trophozoite in the stools of a Russian patient who was suffering from dysentery (Elsdon-Dew, 1969). Later in Berlin, Robert Koch and his assistant (Koch and Gaffky, 1887) reported the presence of amoebae in ulcers of the colon of dysenteric patients. Kartulis confirmed their findings, after he reported the presence of amoebae in tissue sections of 150 dysenteric patients by microscope in Egypt (Kartulis, 1886). The same author mentioned that the amoebic liver abscess could be a complication of dysentery caused by amoebae, a condition that was called tropical dysentery at this time.

In the year 1889, Councilman and Lafleur detected amoebae in the pus from liver abscesses, and also detected that red blood cells were engulfed by amoebic trophozoites, which they called *E. dysenteriae* (Elsdon-Dew, 1969).

Experimental infections of animals were performed successfully. Kovacs infected kittens with fecal material isolated from cases with amoebic dysentery (Kovacs, 1892). His findings were confirmed later by Kruse and Pascale (Kruse and Pasquale, 1894).

The genus *Entamoeba* was established by Cosagrandi and Barbagallo in 1895 and the Latin name “*Entamoeba histolytica*” was invented by Schaudin in 1903, who suggested giving the name to the hematophagous form. He named the quadrinucleated cysts as *E. tetragena* and imagined that *Entamoeba* multiplies by some sort of budding or schizogony (Zoology, n.d.).

Walker (1908) suggested that *E. histolytica* has phases including the *tetragena* and *minuta* forms and that the development of *E. histolytica* is similar to that of *E. coli*. Elmassian called the trophozoites that come out of the quadrinucleated cysts, *E. minuta* (Elmassian, 1909).

Wenyon and O’connor and Dobell considered that the ideal condition for amoebae, is the “carrier state” in which they live in harmony with the host, feeding, growing and multiplying on the expense of the tissue of the colon (Wenyon and O’Connor, 1917; Dobell, 1919). Meanwhile, human tissue can regenerate every day, compensating any damage caused by the parasite. This was after Walker and Sellers who reported the pathogenic effect of amoebae after the deterioration of host’s natural immunity (Walker and Sellards, 1913).

The differentiation between the pathogenic *E. dysenteriae* and non-pathogenic *E. dispar*, diagnosed in asymptomatic carriers, came after studies of Brumpt (1928). This postulation was supported later by Diamond and Clark (1993).

E. moshkovskii is another species of *Entamoeba*, which has been isolated from polluted water and sewage in Moscow (Tshalaia, 1941). Later, it was called “Laredo strain” and was reported to be non-pathogenic, although morphologically identical to *E. histolytica*/*dispar*.

The differentiation between *E. histolytica*/*E. dispar*/*E. moshkovskii* is relevant by the detection of their DNA by different PCR-based tests, e.g., ssrRNA gene and other genes. Also, the Luminex PCR and multiplex PCR are considered very appropriate methods that

can differentiate more than one organism in the same test (Santos et al., 2013; Zebardat et al., 2014; Fallah et al., 2014; Gachuhi et al., 2014).

Biology and life cycle

Entamoeba is an anaerobic organism which doesn't have mitochondria (Sodeman, 1996). It has a relatively simple life cycle that has been described previously by Dobell and Laidlaw (1926), in which it alternates between two main stages, trophozoite and cyst stages. The route of transmission of *Entamoeba* species occurs initially after ingestion of mature cysts via feco-oral route and contaminated food or drink. Upon entering the host, most of these species pass through sequential developmental stages; mature cyst, metacystic trophozoites, motile pleomorphic trophozoites, precyst, cyst and metacyst stages.

When cysts are swallowed with contaminated food or drinks, they excyst, and begin to divide into small trophozoites in the gut. The pathogenic strains of amoebae may engulf RBCs into their cytoplasm, while the non-pathogenic strains feed on bacteria and the surrounding contents of the colon. The trophozoites grow and multiply by binary fission in the intestinal wall. Trophozoites are capable to adhere and lyse the epithelium of the colon and spread hematologically through the portal vein system to distant sites as the peritoneum, liver, lungs or brain, where they can initiate amoebic abscesses (Wuerz et al., 2012; Prakash and Bhimji, 2017).

Adherence to the colonic mucus layer and colonization is through the Gal/GalNAc lectin, which targets galactose and *N*-acetyl-D-galactosamine residues found on O-linked sugar side chains of mucins (Haque et al., 2003; Cornick and Chadee, 2017). Mammals that do not carry N-terminal galactose or *N*-acetyl-D-galactosamine are resistant to amoebic trophozoite adherence, providing some degree of immunity against invasive disease (Wuerz et al., 2012).

Once the parasites invade the intestinal wall, they reach the submucosa and the underlying blood vessels. From there, trophozoites travel to distant sites such as the liver, lungs or skin. These parasites are now considered to be dead-end course since they cannot leave the host and cause infection in others. It can reach different vital organs of the human body, usually the liver, but sometimes the lungs, brain, spleen, etc. However, some of trophozoites stop growing, round up and begin encystation in the lumen of the gut, a process called "precyst formation." The precyst is a spherical non-pathogenic form which is smaller than trophozoites. The precysts only undergo encystation in the colon. The signal that leads to this cyst formation is not very clear. Studies on *E. invadens* of reptiles, suggest that there may be a trigger manifested by ligation of a surface galactose-binding lectin on the surface of the parasite (Eichinger, 2001a; Stanley Jr, 2003). Also, studies showed that some Rab genes/proteins are involved in stress response, pathogenesis and virulence (Picazarri et al., 2008; Chatterjee et al., 2009; Novick and Zerial, 1997; Stenmark, 2009; Nozaki and Nakada-Tsukui, 2006). *E. histolytica* Rab gene 11B (EhRab11B) and its over expression are involved in the secretion of cysteine protease (Nozaki and Nakada-Tsukui, 2006; Mitra et al., 2007). EhRab11A is another gene which is recruited to the surface of cells and was suggested to have a role in encystation (McGugan and Temesvari, 2003).

After the organisms round up, they get rid of food vacuoles, meanwhile, their glycogen is accumulated in a vacuole besides the formation of one or more black rod-like cigar-shaped ribosomes called chromatoid bodies. Cysts also secrete thin resistant, colourless, transparent and protective cyst wall. *Entamoeba* cysts differ in size, with an average range between 12 and 20 µm (Katz et al., 1989; García and Bruckner, 1997; Clark et al., 2000). The nucleus of *E. histolytica* cyst divides twice ending in four nuclei. Cysts with one nucleus, two nuclei or four nuclei, can pass in stool, together with trophozoite stages and can contaminate food or drink. The only infective stage of them is the cyst with four nuclei.

In a study done by Chatterjee and colleagues on *E. invadens* as a model for the in vitro study of encystation of other *Entamoeba*, they reported the expression of "wattle and daub" model of formation of *E. invadens* cyst wall formation (Chatterjee et al., 2009). They described the chitin and chitin-binding lectins in the cyst wall and confirmed the presence of chitin by the use of chitin-binding stain calcofluor (Chávez-Munguía et al., 2003). They also reported that chitin only is synthesized in plasma membrane, while chitin synthases of *saccharomyces* are stored in chitosomes, which act as reservoir for its transport (Ziman et al., 1996; Cabib et al., 2001).

Cysts passed in feces can survive outside the human body in soil and in water for up to few months in favorable conditions of heat and moisture. Cysts are killed by very high as well as by freezing temperatures (American Water Works Association, 2006). Patients can catch infection in case of swallowing viable cysts in food or drink contaminated with feces of infected persons (El-Dib, 2017). In the small intestine excystation takes place in response to alkaline surroundings and intestinal enzymes forming the metacystic uni-nucleate motile trophozoites. These trophozoites finally migrate to the large intestine where they multiply, feed on mucus, bacteria and cell debris and encyst.

According to Levin's grouping for species differentiation, *Entamoeba* species can be categorized as per the number of nuclei in their fully mature cyst (Table 1) concurring with molecular techniques such as full-length 16S-like rDNA sequences (Levine, 1985; Clark and Diamond, 1997; Silberman et al., 1999).

Morphology and ultrastructure

Trophozoite

The trophozoite is the active stage of amoeba. Its size differs from one type of amoeba to another depending mainly upon the number of cytoplasmic food vacuoles and the size of phagocytosed grains. The average size of the trophozoite cell is from 20 to 30 µm with occasional diversity such as "minute form" (10 µm) or "magna form" (60 µm). Actively motile amoebae are elongated

Table 1 Members of genus *Entamoeba* inhabiting wide range of vertebrate and invertebrate species.

Category	Name of <i>Entamoeba</i> species	Other names	Geographical range	Potential hosts	Habitat	Pathogenic tendency
Species with no cyst stage	1- <i>Entamoeba gingivalis</i> (Gros, 1849)	<i>Amoeba buccalis</i> (Steinberg, 1862) <i>Amoeba dentalis</i> (Grassi, 1879) <i>Amoeba kartulis</i> (Doflein, 1901) <i>Entamoeba buccalis</i> (Prowazek, 1904) <i>Entamoeba maxillaris</i> (Kartulis, 1906) <i>Amoeba pyogenes</i> (Verdun and Bruyant, 1907) <i>Entamoeba canibuccalis</i> (Smitch, 1938) <i>Entamoeba equibuccalis</i> (Simitch, 1938) <i>Entamoeba suigingivalis</i> (Tumka, 1959)	Worldwide	Human Monkey Horse Dog Cat Pig	Oral cavity	
	2- <i>Entamoeba barreti</i> (Taliaferro and Holmes, 1924)			Snapping turtle	Colon	
	3- <i>Entamoeba gedoelsti</i> (Husing, 1930)	<i>Entamoeba intestinalis</i>		Horse	Large intestine	
	4- <i>Entamoeba caprae</i> (Fantham, 1923)			Goat	Large intestine	
	5- <i>Entamoeba mola</i> (Noble and Noble, 1966)		Southern California	Fish (Ocean sunfish)	Hindgut	
Species with single-nucleated mature cyst	1- <i>Entamoeba polecki</i> (Von Prowazek, 1912)	<i>Entamoeba deblicieki</i>	Southeast Asia, France, United States, Venezuela, Guinea, Iran	Pig Human Monkey	Colon and caecum, large intestine	
	2- <i>Entamoeba chattoni</i> (Swellengrebel, 1914)		Africa	Monkey Human	Large intestine	
	3- <i>Entamoeba bovis</i> (Liebetanz, 1905)		Africa	Cattle Buffalo	Large intestine	
	4- <i>Entamoeba antilocapra</i> (Noble, 1953)		America	Antelope	Large intestine	Pathogen of Intestinal lesion, Bowel inflammation, Necrosis
	5- <i>Entamoeba ovis</i> (Swellengrebel, 1914)	<i>Entamoeba deblicieki</i>	World Wide	Sheep Goat	Large intestine	
	6- <i>Entamoeba dilimani</i> (Noble, 1954)	<i>Entamoeba deblicieki</i>	Philippines	Goat	Large intestine	
	7- <i>Entamoeba struthionis</i> (Martínez-Díaz et al., 2000)		Spain	Ostrich	Large intestine	
	8- <i>Entamoeba suis</i> (Hartmann, 1913)	<i>Entamoeba deblicieki</i>	China, Bulgaria, France, Yugoslavia, England, United States	Pig	Large intestine	
	9- <i>Entamoeba bubalis</i> (Noble, 1955)		Philippines	Buffalo	Large intestine	
	10- <i>Entamoeba paulista</i> (Carini, 1933)	<i>Brumptina paulista</i>	United States, Africa, Chili, Uruguay	Opalinata	Cytoplasm of Opalinata	
	11- <i>Entamoeba gadi</i> (Bullock, 1966)		North America	Pollock fish	Rectum	

(Continued)

Table 1 (Continued)

Category	Name of Entamoeba species	Other names	Geographical range	Potential hosts	Habitat	Pathogenic tendency
Species with quadri-nucleated mature cyst	12- <i>Entamoeba nezumia</i> (Orias and Noble, 1971)		North Atlantic	Macrourid fish	Stomach, Intestine	
	13- <i>Entamoeba chiangraiensis</i> (Jinatham et al., 2019)		Northern Thailand	Asian swamp eel (<i>Monopterus albus</i>)	Gut	
	1- <i>Entamoeba histolytica</i> (Schaudinn, 1903)	<i>Amoeba coli</i> (Losch, 1875) <i>Amoeba dysenteriae</i> (Councilman and Lafleur, 1891) <i>Amoeba lobosa var.coli</i> (Celli and Fiocca, 1894) <i>Entamoeba africana</i> (Hartmann and Prowazek 1907) <i>Entamoeba tetragena</i> (Viereck, 1907) <i>Entamoeba schaudinni</i> (Lesage, 1908) <i>Ponerauiocba histolytica</i> (Liihe, 1908), <i>Entamoeba minuta</i> (Elmassian, 1909) <i>Entamoeba nipponica</i> (Koizumi, 1909) <i>Entamoeba brasiliensis</i> (Aragao, 1912) <i>Loschia histolytica</i> (Mathis, 1913) <i>Entamoeba venaticum</i> (Darling, 1915), <i>Entamoeba caudata</i> (Carini and Reichenow 1949)	Worldwide	Human	Large intestine	Intestinal (human amoebic dysentery) and extra-intestinal amoebiasis
	2- <i>Entamoeba dispar</i> (Brumpt, 1925)		Worldwide	Human Chimpanzees Baboon Macaques	Large intestine	
	3- <i>Entamoeba hartmanni</i> (Von Prowazek, 1912)	<i>Entamoeba minuta</i> (Woodeock and Penfold, 1916) <i>Entamoeba minutissima</i> (Brug, 1918) <i>Entamoeba tenuis</i> (Kuenen and Swellengrebel, 1917)	Worldwide	Human	Large intestine	
	4- <i>Entamoeba moshkovskii</i> (Tshalaia, 1941)	Laredo strain/Huff strain of <i>E. histolytica</i>	World wide	Present in sewage as free living amoeba May infect human	Large intestine	

Table 1 (Continued)

Category	Name of <i>Entamoeba</i> species	Other names	Geographical range	Potential hosts	Habitat	Pathogenic tendency
	5- <i>Entamoeba ecuadoriensis</i> (Clark and Diamond, 1997)		Ecuador			
	6- <i>Entamoeba bangladeshi</i> (Royer et al., 2012)		Bangladesh	Human	Large intestine	
	7- <i>Entamoeba invadens</i> (Rodhaim, 1934)	<i>Entamoeba serpentis</i> (Cunha and Fonseca, 1917)	Worldwide	Captive reptiles; Snake Lizard Turtle Crocodile	Large intestine	Intestinal and extra intestinal amoebiasis
	8- <i>Entamoeba insolita</i> (Geiman and Wichterman, 1937)			Turtle	Large intestine	Potentially pathogenic
	9- <i>Entamoeba terrapinae</i> (Sanders and Cleveland, 1930)		World-wide	Turtle	Colon	
	10- <i>Entamoeba knowlesi</i> (Rodhain and Hoof, 1947)			Turtle	Large intestine	
	11- <i>Entamoeba ranarum</i> (Grassi, 1879)		World-wide	Frog, Toad	Large intestine	Intestinal and extra intestinal amoebiasis
	12- <i>Entamoeba pyrrhogaster</i> (Lobeck, 1940)			Frog Toad Salamander	Large intestine	
	13- <i>Entamoeba aulastomi</i> (Noller, 1919)			Leech especially Haemopsis sanguisuga	Intestine	
	14- <i>Entamoeba ctenopharyngodoni</i> (Chen, 1955)		China	Carp Fish	Rectum	
	15- <i>Entamoeba anatis</i> (Fantham, 1921)		South Africa Asia United States	Duck Bustard	Caecum	Intestinal amoebiasis
	16- <i>Entamoeba lagopodis</i> (Fantham, 1910)			Duck Lagopus	Caecum	
	17- <i>Entamoeba equi</i> (Fantham, 1921)		South America	Horse	Large intestine	Potential pathogen, intestinal amoebiasis
	18- <i>Entamoeba nuttalli</i> (Castellani, 1908)	<i>Entamoeba duboscqi</i> (Mathis 1913), <i>Entamoeba cynomolgi</i> (Brug, 1923) <i>Entamoeba ateles</i> (Eichhorn and Gallagher, 1916)	Japan Nepal Southwest China	Baboon Macaques Chimpanzees	Large intestine	Potential pathogen, intestinal and extra intestinal amoebiasis
	19- <i>Entamoeba philippinensis</i> (Kidder, 1937)			Termite, Cockroaches	Hindgut	
Species with eight-nucleated mature cyst	1- <i>Entamoeba coli</i> (Grassi, 1879)	<i>Entamoeba hominis</i> (Casagrandi and Barbagallo, 1897) <i>Entamoeba Loeschi</i> (Lesage, 1908)	Worldwide	Human	Large intestine	

(Continued)

Table 1 (Continued)

Category	Name of Entamoeba species	Other names	Geographical range	Potential hosts	Habitat	Pathogenic tendency
Species of uncertain data	2- <i>Entamoeba muris</i> (Grassi, 1879)	<i>Loschia coli</i> (Chatton and Lalung-Bonnaire, 1912) <i>Councilmania decumani</i> (Rudovsky, 1921) <i>Entamoeba coli Varratti</i> (Kessel, 1923) <i>Amoeba muris</i> ,	Worldwide	Rats Mice Hamster Wild and domestic rodents	Lumen of the caecum, and occasionally colon	
	3- <i>Entamoeba citelli</i> (Becker, 1926)	<i>Entamoeba caviae</i> (Chatton, 1918)	Worldwide	Ground squirrel	Large intestine	
	4- <i>Entamoeba cobayae</i> (Walker, 1908)			Guinea pig	Cecum	
	5- <i>Entamoeba criceti</i> (Starkoff, 1942)			Hamster	Large intestine	
	6- <i>Entamoeba cuniculi</i> (Brug, 1918)			Rabbits	Large intestine	
	7- <i>Entamoeba dipodomysi</i> (Hegner, 1926)			Kangaroo rats	Large intestine	
	8- <i>Entamoeba funambulae</i> (Ray and Bunik 1966)	India, Bengal	India	Indian palm squirrel	Large intestine	
	9- <i>Entamoeba marmotae</i> (Crouch, 1936)			Marmot	Large intestine	
	10- <i>Entamoeba chiropteris</i> (Mandal and Choudhury, 1988)			Bats	Large bowel	
	11- <i>Entamoeba gallinarum</i> (Tyzzer, 1920)	Worldwide	Sudan	Fowl	Caecum	
	12- <i>Entamoeba wenyoni</i> (Galli-Valerio, 1935)			Goat Camel	Large intestine	
	13- <i>Entamoeba flaviviridis</i> (Knowles and Das Gupta, 1935)			Lizard	Intestine	
	14- <i>Entamoeba apis</i> (Fantham and Porter, 1911)			Bee (<i>Apis mellifica</i>)	Intestine	
	15- <i>Entamoeba polypodia</i> (Schultze, 1954)			Bug (<i>Leptocoris trivittatus</i>)	Ventricle, Intestine and rectum	
	<i>Entamoeba testudinis</i> (Hartmann, 1910), <i>Entamoeba varani</i> (Lavier, 1928), <i>Entamoeba michini</i> <i>Entamoeba phallusae</i> <i>Entamoeba cervum</i> (Jian Han and Yang, 1989) <i>Entamoeba celestini</i> (Froilano de Mello, 1946) <i>Entamoeba bobaci</i> (Li Yuan Po, 1928) <i>Entamoeba blostomae</i> (Brug, 1922)					



Fig. 1 *E. histolytica* trophozoite, with irregular outline, numerous food vacuoles and single nucleus with central karyosome “arrow” $\times 1000$ original magnification.

with advancing pseudopodia (Fig. 1). A pseudopodium is a tongue-like protrusion composed mainly of ectoplasm. The trophozoite changes its direction of motion according to the microenvironmental conditions, while resting trophozoites tend to be spherical (Fig. 2) (Ludvik and Shipstone, 1970; Bansal et al., 2009).

The trophozoite's body is confined by typical plasma membrane with numerous circular openings that correspond to the mouths of micro-pinocytic vesicles, in addition to transient surface differentiations such as pseudopodia, phagocytic mouths, and a caudal uroid. Actin filaments are massed below the plasma membrane and in the pseudopodial extensions and phagocytic stoma (Gonzalez-Robles and Martinez-Palomo, 1992).

Its cytoplasm is differentiated into thin rim of clear ectoplasm and inner granular endoplasm. The granular part comprises nucleus, ribosomes, osmiophilic lipid granules and extensive vacuolar system. Electron-dense granules (EDG) were detected in trophozoites re-covered from tissue lesions. Those EDG are circular discs located near the surface of the amoebic plasma membrane and formed of a dense complex of proteins with proteolytic activities, as well as actin (Leon et al., 1997).

The cytoplasm contains great number of ribosomes and polysomes, which are dense, adjoining, ribosome-like bodies. They are arranged in the form of scattered groups of osmiophilic particles and helical bodies. Food vacuoles (from 0.5 μm in diameter to 12 μm) and lysosomes (minute vacuoles, varies from 50 to 200 nm) form the main part of the characteristic highly vacuolated system of *Entamoeba* trophozoites. Lysosomes of the amoebae differ from those of other eukaryotes in that the contained enzymes are bound to the lysosomal membrane rather than being free in the vesicular compartment, and this may give an explanation of the unique ability of *Entamoeba* lysosomal enzymes to work extracellularly (Chavez-Munguia et al., 2004).

Another number of small vacuoles is observed inside the cytoplasm of the uroid. The vacuoles are emptied by a sudden bursting of the surface or by the expulsion of the entire vacuoles into the swellings on the surface of the uroid. In actively motile trophozoites, a trail of micro-exudate is deposited on the substrate, which has cytochemical properties similar to those of the surface coat. This occurs due to the dramatic redistribution of surface components that accumulate at the uroid and are later released into the medium (Nakada-Tsukui and Nozaki, 2016).

Entamoeba species were known to have a very primitive cytoplasm, apparently lacking important organelles such as mitochondria, peroxisomes, Golgi complex and endoplasmic reticulum. In 1997, Mazzuco and his team has suggested that some of the dispersed vacuolar elements may contain the macromolecules involved in the several functions performed by the Golgi complex,

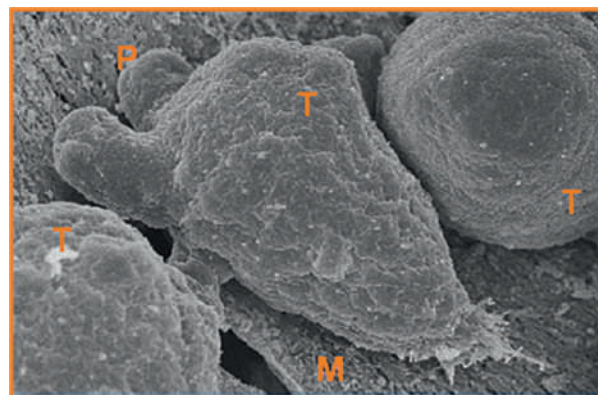


Fig. 2 Scanning electron micrograph of *Entamoeba histolytica* trophozoites; motile and resting, adhering to the human mucus covering the intestinal epithelium. (T: trophozoite; M: mucus; P: pseudopodia) (Bansal et al., 2009).

and raised the possibility that *Entamoeba* may lack molecules involved in the association of the cisternae and vesicles to form the Golgi stacks. Later on, it has been recently demonstrated the presence of a continuous endoplasmic reticulum compartment in living *E. histolytica* trophozoites (Teixeira and Huston, 2008; Perdomo et al., 2015). Moreover, the amoebic mitochondrion-derived organelle is called “mitosome” or “crypton”. The crypton lacks enzymes of oxidative phosphorylation and lacks fermentation proteins, yet it contains 2.2% of the amount of amoebic DNA. Another amoebic cytosolic structure named *E. histolytica* kinetoplastid organelle was found to contain circular DNA (Makiuchi and Nozaki, 2014).

The nucleus is about one sixth of the size of the cell body. It is bounded by double-layered membrane with numerous pores. The chromatin material is peripherally organized, and the nucleolus “endosome” is situated in the center of the nucleus. The size and distribution of chromatin is variable from cell to cell and is not a reliable diagnostic feature. Delicate achromatic fibrils radiate from the endosome to the inner surface of the nuclear membrane (Chavez-Munguia et al., 2006). *E. histolytica* trophozoites have the ability to attack the host’s cells, dissolving, killing and eating them (Ralston, 2015).

Mature cyst

Mature cysts are rounded or slightly oval bodies 8–20 µm in diameter. They are uni/multinucleated with condensed chromatin attached to nuclear membrane, contain big vacuoles, ribonucleoprotein helices and densely packed chromatoid bodies in the cytoplasm (Fig. 3). An apparently smooth wall surrounds it, 125–150 nm thick. The cyst wall is composed of fibrillar material, which forms a tight mesh (Aguilar-Diaz et al., 2010).

In early cystic stages, the chromatoid bodies arise by aggregation of ribosomes forming polycrystalline masses with blunt, rounded, irregular or splinter like ends (Fig. 3). In maturing cysts, these crystalline masses fragment into separate particles, and the classical chromatoid body tends to disappear. This phenomenon hypothesizes that the large chromatoid bodies formation is a manifestation of special parasite-host adaptive mechanism; ribonucleoprotein is synthesized under favorable conditions, crystallized in the resistant cyst stage, and dispersed in the newly excysted amoebae, thereby enabling them to establish themselves in a new host by a period of quick growth (Barker and Svihla, 1964). Large carbohydrate reserves in the form of glycogen are present in the precyst stages, and then disappear gradually as the cyst goes to maturity. Similarly, the cyst wall precursors appear in the cytoplasm as vesicles with filamentous hyaline content of intermediate density. Rapidly the secreted material forms tough mesh-like cyst wall around the cell (Fig. 4), the nucleus starts to divide, and the rest of organelles disappear while the cyst proceeds to maturity (Chávez-Munguía et al., 2003; Mehlhorn, 2005).

Taxonomy and important species

Taxonomic hierarchy:

Kingdom: Protozoa

Phylum: Protozoa

Subphylum: Sarcodina

Superclass: Rhizopoda

Class: Lobosa

Order: Amoebida

Family: Entamoebidae

Genus: *Entamoeba*

Species: *Entamoeba histolytica* (Schaudinn, 1903)

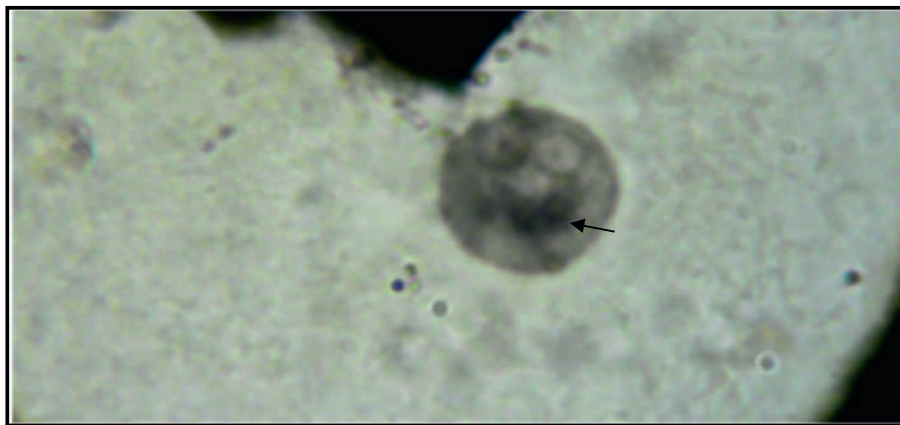


Fig. 3 *E. histolytica* pre-cyst, with rounded boundaries, two nuclei and thick chromatoid body “arrow”. ×1000 original magnification.

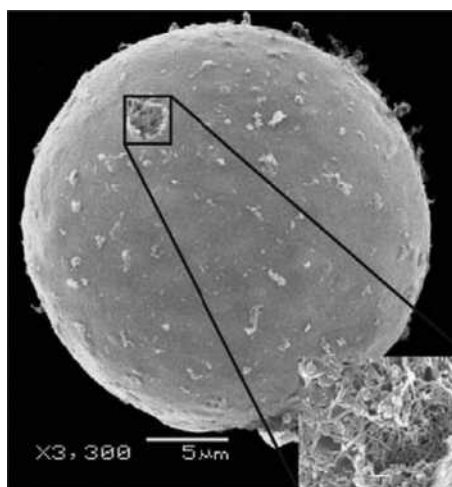


Fig. 4 SEM showing the smooth surface, spherical shape and small size of the cyst. Insert shows a magnification of a hole in the surface, where overlapping fibers that resemble chitin-fibers in other organisms are observed (Aguilar-Diaz et al., 2010).

Entamoeba species of medical importance to man

Entamoeba dispar

Entamoeba dispar gets its importance from its morphological resemblance to *E. histolytica*, however, it has its famous reputation as a non-pathogenic species of *Entamoeba*. Recent studies showed that it can produce some pathological lesions (Espinosa-Cantellano et al., 1997, 1998). *E. dispar* was reported as a cause of non-dysenteric colitis (Costa et al., 2006) or amoebic liver abscess (Ximenez et al., 2010). Graffeo et al., reported a case of enteritis caused by *E. dispar*, in a patient that lives in a non-endemic area. Diagnosis was done by the identification of DNA (Graffeo et al., 2014). After studying many samples from amoebic liver abscesses, Olivera et al., reported that *E. dispar* began to show a different facet (Oliveira et al., 2015). *E. histolytica* was believed to show two different groups by isoenzyme electrophoresis; a pathogenic and a non-pathogenic form of the parasite. This fact was used to explain why infection could be asymptomatic in some cases in the past (Sargeant et al., 1978). DNA analysis of the two different species was the proper method for their identification into *E. histolytica* and *E. dispar* (Diamond and Clark, 1993). It was estimated by WHO that *E. histolytica/dispar* infects around 12% of world populations, putting into consideration that *E. histolytica* accounts for about 1% of infections (World Health Organization, 1997). Hematophagy is always associated with *E. histolytica* infection. Although *E. dispar* is sometimes pathogenic, but without tissue invasion (Van Den Broucke et al., 2018).

Entamoeba moshkovskii

E. moshkovskii was recovered from wastewater in the year 1941 in Moscow, Russia by Tshalaria (Van Den Broucke et al., 2018; Scaglia et al., 1983) and thought to be free living. The first human isolate of this parasite was known as “Laredo strain” of *E. moshkovskii* when identified in Laredo, Texas in the year 1991 from a case who complained of diarrhoea, epigastric pain and weight loss (Heredia et al., 2012). The identified strain was called *E. histolytica* Laredo strain and it was found to share some features similar to *E. moshkovskii*, as both organisms can grow in culture at room temperature, resist the osmotic shock and could be resistant to the anti-amoebic drug emetine (Fotedar et al., 2007a). *E. moshkovskii* resembles *E. histolytica/dispar* in morphology (Fotedar et al., 2008). Human cases have been detected by the identification of DNA of the organism (Ali et al., 2003). Scientists examined 109 preschool children in Bangladesh and could detect 15.6% positive for *E. histolytica*, 35.8% positive for *E. dispar* and 21.1% positive for *E. moshkovskii*. This result shows that man acts as a host for this parasite (Ali et al., 2003).

E. moshkovskii was reported in Malaysia, India, Bangladesh, Turkey, Tanzania, Australia and North America, affecting all ages and genders (Shimokawa et al., 2012).

Experimental studies in Bangladesh, showed that *E. moshkovskii* was pathogenic to the mice, and was manifested with diarrhoea in children. Scientists reported the association of human infection with diarrhoea, weight loss, abdominal colic and bloody stool (Heredia et al., 2012).

Entamoeba coli

E. coli is a common non-pathogenic, commensal species that lives and multiplies in the colon and have a similar life cycle as *E. histolytica*. It is considered the largest member of the genus. The trophozoite is about 15–50 μm, with an average of 20–30 μm. *E. coli* has a sluggish movement, with slow formation of 3–5 pseudopodia that moves the parasite in its place with non-progressive motility. However, trophozoites stay immobile mostly with an almost rounded shape. Its cytoplasm is hyaline and granular, when

seen with microscope in fresh mount, it may have a number of short blunt pseudopodia. It contains many food vacuoles containing bacteria, starch and yeast. Sometimes the trophozoite may have food vacuoles that contain some smaller parasites e.g., *Giardia*, or other small protozoa, besides fungi e.g., *Sphaerita* species (Hamad et al., 2017). Scientists claim that *E. coli* has the potential to fight other organisms for nutrition by phagocytosing them, however they never invade the mucosal layers or produce any lesion. Nucleus of *E. coli* is 5–7 µm, lined with irregular coarse chromatin granules, eccentric karyosome which gets attached to the nuclear membrane with fine threads. Cyst of *E. coli* is globular, measures from 10 to 35 µm with a thick, double layered cyst wall. Usually the mature cysts are smaller in size and contains 8 nuclei, however sometimes it is possible to see a cyst with 16 or even 32 nuclei “supernucleate cysts” (Atlas, n.d.). Some immature cysts, with one nucleus may have a large glycogen vacuole, that pushes the nucleus to one side of the cyst. This glycogen vacuole disappears later with maturation of the vacuole. Thin chromatoid bodies with tapering ends could be seen inside the cyst (Atlas, n.d.).

Entamoeba hartmanni

Entamoeba hartmanni belongs to the four-nucleated cyst-forming amoebae, formerly known as “the small race of *E. histolytica* or *E. histolytica sensu stricto*” (Von Prowazek, 1912). It has been detected in many stool specimens worldwide, possessing a similar life cycle to that of *E. histolytica* but with no histolytic or hematophagous characteristics (Burrows, 1957).

The main feature to differentiate *E. hartmanni* from pathogenic *E. histolytica* is the small-sized trophozoites, which may reach (3–10 µm) in diameter. However, the distribution of peripheral chromatin on the inner nuclear membrane has added another significant difference. Since *E. hartmanni* can show many patterns of chromatin aggregation such as uniform distribution in ~2/3 of samples, thick large or small masses in ~1/6 or mostly discrete compact areas of chromatin aggregation, closer to that of *E. coli*. Accordingly, it forms small-sized cyst (3.8–8 µm) with one-to-four nuclei of relatively small diameter. They have characteristic chromatoid bodies with smooth, rounded edges, varying in number and diffuse glycogen stores (Burrows, 1964).

Laboratory diagnosis is achieved by looking for the above morphological features of trophozoite and cyst in wet/or iodine stained stool preparations. *E. hartmanni* has been successfully cultivated and surprisingly, it was indistinguishable from *E. histolytica* on both morphology and size (~13.2–15.6 µm) (Sarkisian, 1957).

The high endemicity of *E. hartmanni* in tropical and sub-tropical rural areas plays an important role in the misdiagnosis of *E. histolytica*, which lead to increased false positive results (Matey et al., 2016).

Though all the data mentioned *E. hartmanni* as non-pathogenic commensal, yet it has proven recently that it may cause limited to mild level of pathogenicity. It was incriminated as a cause of diarrhoea in AIDS cases (Rolston et al., 1986) and as a causal agent in reactive arthritis individuals, which was dramatically improved after the application of metronidazole (Schirmer et al., 1998). Finally, *E. hartmanni* colonization was associated with mild to severe diarrhoea, which was diagnosed by molecular assays (Matsumura et al., 2019).

Entamoeba gingivalis

E. gingivalis was the first identified human amoeba (Favoreto Jr. and Machado, 1995). It inhabits the buccal cavity, gingiva, and the periodontal pockets (Prieto-Prieto and Calvo, 2004; Bonner et al., 2014) as an opportunistic protozoon (Lyons et al., 1980). It is common in about 95% of cases with gum disease, but not in healthy gums (Kofoed et al., 1929; Trim et al., 2011). This type of *Entamoeba* has a trophozoite stage only (20–150 µ), and no cyst forms, therefore the mode of infection is direct from mouth to mouth during kissing or the use of the same contaminated food utensils. Trophozoites have active pseudopodia and numerous food vacuoles containing nuclei of polymorphonuclear leucocytes, RBCs and bacteria (Bonner, 2013). The organism could be visualized by light microscope after preparing a direct smear from the gingiva at room temperature.

Entamoeba polecki

This intestinal amoeba acts as a parasite of pigs and monkeys. In the year 1912, Von Prowazek detected the parasite in Czechoslovakia in the stools of two cases from Kampuchea. Although human infection has been reported, clinical symptoms are not known due to rarity of cases (Smith, 2019).

Entamoeba polecki is world-wide in distribution, however most of the reported cases came from developing countries and sporadic cases occur in developed countries. *E. polecki* was reported in 19% of the population of Papua New Guinea and was also reported as endemic in Cambodia, Venezuela and Vietnam. However, there were infections in refugees from South East Asia living in France, Minnesota and Venezuela (Smith, 2019).

Zoonotic Transmission could be the source of infection probably from pigs or monkeys (Wiwanitkit, 2004). Human infection with this parasite is mostly asymptomatic, but some non-specific symptoms as diarrhoea, nausea, vomiting, fever, abdominal pain and weight loss may be accompanied with this infection. It is recommended to treat cases with *E. polecki* with metronidazole (Smith, 2019).

The cyst is between 12 and 15 µm and are spherical or subspherical and the nuclear peripheral chromatin is generally distributed non-uniformly. As the morphology of *E. polecki* resembles those of *E. coli* and *E. histolytica*, detection of mature cysts is required for proper diagnosis of *E. polecki*. DNA was extracted from infected feces and used successfully to amplify part of the amoeba ssrRNA (Verweij et al., 2001). There are four subtypes of *E. polecki* from “ST1 to ST4”, all have been recovered from human cases. Although

they are exclusively parasites of primates, ST4 is the most common subtype in man denoting a possible potential zoonotic transmission (Stensvold et al., 2011).

Metronidazole has been used to treat cases known to be infected with *E. polecki* to avoid its possible pathogenicity (Lyons et al., 1980; Wiwanitkit, 2004). Combination of Metronidazole and diloxanide furate is also recommended in treatment of infections (Solaymani-Mohammadi and Petri, 2006).

Entamoeba invadens

This species of *Entamoeba* infects reptiles e.g., snakes and lizards with severe mortality that may reach up to 100%, although turtles and crocodiles tend to be asymptomatic carriers (Segovia-Gamboa et al., 2010). Transmission occurs by feco-oral route. Its life cycle resembles that of *E. histolytica*. The resistant cyst stage rich in carbohydrates is the infective stage (Eichinger, 2001b), and the motile active trophozoite stage, causes the pathologic effect of the parasite (Chia et al., 2009).

Because of similarity in their life cycles, this parasite, as previously stated, is used as a model for studying the development and encystation in vitro in order to understand some missing points in *E. histolytica* (Ehrenkauser et al., 2013), particularly *E. invadens* is the only *Entamoeba* species that can encyst in vitro (Welter et al., 2017). Trophozoites of *E. invadens* move in amoeboid movements, but they round up under stress. Cysts, are smaller, rounded and quadrinucleated. The cyst has a homogenous unlayered cyst wall (Chatterjee et al., 2009), which resists extreme environmental conditions such as desiccation, heat and detergents (Smith, 2019).

Pathophysiology and clinical picture

The reason why *E. histolytica* acts in a virulent way is still a matter of debate. Some patients may have asymptomatic infection, while others develop more serious attacks as acute colitis or even extraintestinal disease. The environment, the host's immune status as well as the genetic susceptibility, age and gender, all were thought to have roles in the severity of the disease (Ximenez et al., 2010; Jackson et al., 1985; Bernin et al., 2014).

Recent studies have been done on how the intestinal flora can influence the pathogenicity of *E. histolytica* (Dolabella et al., 2012; Furst et al., 2002) and how they can influence the adhesion capacity and the cytopathic effects of *E. histolytica* trophozoites (Galván-Moroyoqui et al., 2008). These trophozoites have more power of invasion of the colonic epithelium as a result of the proinflammatory cytokines produced in the presence of bacterial flora (Galván-Moroyoqui et al., 2008). They subsequently spread hematologically through the portal vein system to distant sites such as the peritoneum, liver, lung, or brain (Wuerz et al., 2012; Prakash and Bhimji, 2017).

Certain enzymes have been incriminated in the process of virulence and invasiveness. Glucosidase, *N*-acetylgalactosaminidase and *N*-acetylglucosaminidase are enzymes that can help to remove branched polysaccharides from mucin cells. This process allows the trophozoites to penetrate the epithelial wall of the colon after degradation of the protective mucous barrier (Ximenez et al., 2010; Prakash and Bhimji, 2017; Haque et al., 2003; Cornick and Chadee, 2017; Bernin et al., 2014; Thibeaux et al., 2013). Trophozoites that can't adhere to the mucosa of the gut of the hosts are those which do not have N-terminal galactose or *N*-acetyl-D-galactosamine. They are eventually incapable of causing invasive disease (Prakash and Bhimji, 2017).

Secretory mucosal immunoglobulins have essential role in the immune response, especially IgA produced in the lamina propria as a protective element of the gut mucosa. Mucosal anti-Gal/GalNAc lectin IgA was reported to prevent the amoebic colonization and penetration of the gut mucosa (Haque et al., 2003, 1990; Quach et al., 2014).

The pathophysiology caused by amoebic trophozoites in tissue of the host is not very clear, however host cells are killed by the amoebic trophozoites using two main mechanisms, trophocytosis and phagocytosis. Trophocytosis is a process during which *Entamoeba* bites distinct fragments of the host cell. This biting process is called "amoebic trophocytosis or nibbling." After attaching to host cells, amoebae bite off and ingest distinct host cell fragments, and this contributes to cell killing (Ralston, 2015).

Phagocytosis usually depends on three consecutive actions; adherence, lysis of host cell, and phagocytosis. The contact-dependent attachment is moderated by the amoebic Gal/GalNAc adherence lectin. *Entamoeba* host cell is thought to induce host cell apoptosis, followed by phagocytic clearance of human C1q protein-labeled apoptotic cell. Limitation of the inflammation and evasion of the immune system are important outcomes from these processes (Cornick and Chadee, 2017; El-Dib, 2017). Host cell viability is an important factor for the occurrence of trophocytosis and phagocytosis. Ralston (2015) incubated amoebic trophozoites with living host cells and reported that the amoebae ingested the living cells by trophocytosis, while the dead cells were ingested by phagocytosis (Ralston, 2015).

Asymptomatic infection

Some infections are not manifested by symptoms and these are diagnosed accidentally.

Approximately 90% of *Entamoeba* infections are asymptomatic (Haque et al., 2003; Stenmark, 2009; Cheepsattayakorn and Cheepsattayakorn, 2014).

Acute diarrhoea

Acute diarrhoea is the second cause of death due to parasitic disease in low income countries (Kotloff et al., 2017). It may cause death among children under 5 years (Liu et al., 2016). Acute diarrhoea is sometimes associated with vomiting, colic and fever. It is considered a mild symptom of amoebic colitis.

Amoebic colitis

Amoebic colitis is usually mild in most of the cases with subacute onset and mild diarrhoea. However, it could be manifested by severe dysentery i.e., with mucous and blood in stool, colic, fever, and tenesmus (Haque et al., 2003). The incubation period after ingestion of the infective stage also differs from 7 to 28 days. The mild infection may be abdominal colic, diarrhoea "3–8 motions/day", or dysentery, flatulence and tenesmus. In severe infection there may be fever with tenderness of the abdomen, vomiting, diarrhoea "10–20 motions/day" and loss of weight (El-Dib, 2017). If diagnosis is delayed, the case may develop to a fulminating necrotizing colitis, which is fatal in 40% of patients (Haque et al., 2003; Bradly et al., 2015; Misra et al., 2006). Fortunately, this complication is not common. In those with concomitant liver abscess, mortality increases to 89% (Ortiz-Castillo et al., 2012; Takahashi et al., 1997). Patients look toxic, with fever, bloody diarrhoea, and signs of peritoneal irritation (Bradly et al., 2015; Moorchung et al., 2014).

A condition as toxic megacolon could develop as a result of corticosteroid use and the patients need surgical intervention when they don't respond to antiamoebic therapy (Moorchung et al., 2014; Shirley et al., 2016).

Toxic megacolon

This rare complication occurs in about 0.5% of infections. It is defined as segmental or total dilatation of the external diameter of the colon to more than 6 cm, accompanied by systemic toxicity. This type of infection may end by fatal colon perforation (McGuiness and Leder, 2019).

Ameboma formation

The formation of an ameboma is another uncommon manifestation that may occur in amoebic colitis. Ameboma is a localized hyperplastic granulation tissue that occurs in the cecum or ascending colon. It tends to present with pain and swelling in the right iliac fossa, or with symptoms of bowel obstruction (Misra et al., 2006). It could be misdiagnosed with neoplasms, tuberculosis or abscess and could be properly diagnosed by colonoscopy and histopathology (Fig. 5A and B) (Misra et al., 2006; Moorchung et al., 2014). Amebomas may be single or multiple. The common locations are in the coecum, caecal appendix and sigmoido-rectal areas. However, may also occur in other sites, although very rare. Lomeli et al. (2016), reported an ameboma in the ampulla of Vater, extending to the pancreas as well as in the colon. The lesion shows extensive chronic reaction with the presence of *E. histolytica* trophozoites, necrotic tissue and fibrosis. Ameboma formation has been generally associated with untreated or partially treated amoebic colitis (Misra et al., 2006).

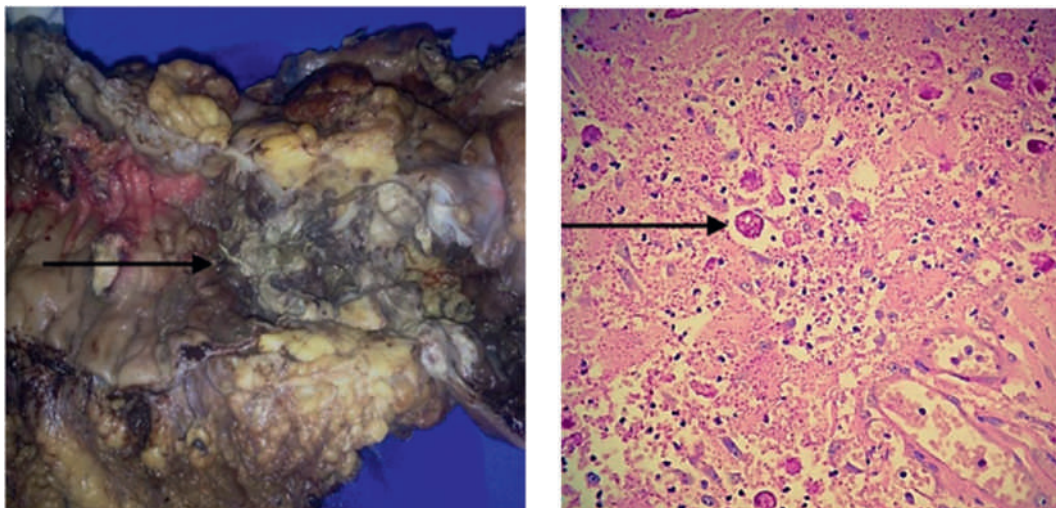


Fig. 5 (A) Colon with an indurated grey lesion of 4 × 5 cm (Black arrow), and an intense inflammatory reaction. (B). Microphotography (10 ×) of a PAS stained tissue showing *E. histolytica* trophozoites (Black arrow) (Lomeli et al., 2016).

Extraintestinal infection

Amoebic liver abscess

After penetrating the colonic wall and reaching the portal veins, *E. histolytica* trophozoites find accessibility to the liver parenchyma. They take 2–4 weeks to develop amoebic liver abscess. Patients usually present with fever and pain in the upper abdominal quadrant (Wuerz et al., 2012; Prakash and Bhimji, 2017; Haque et al., 2003). Around 50% of cases may complain of chronic diarrhoea, abdominal pain and weight loss. In some cases where the abscess is near the diaphragmatic surface, the patient may develop right pleural effusion with subsequent cough and right chest pain (Haque et al., 2003). Around 40% complain of dysentery, leukocytosis and elevation in the liver enzyme alkaline phosphatase (Wuerz et al., 2012; Prakash and Bhimji, 2017). Usually amoebic abscess is solitary, however multiple abscesses have been reported (Lomeli et al., 2016; Nattakom et al., 2001).

Amoebic liver abscess is an important cause of space-occupying lesions of the liver, mainly in tropical regions. Prompt recognition and appropriate treatment lead to improved morbidity and mortality (Brallita, 2019).

Hemolytic uremic syndrome (HUS)

Another severe complication of extraintestinal invasion by *E. histolytica*, is manifested by kidney injury which could lead to kidney failure, anemia and thrombocytopenia (Exeni et al., 2018). The kidneys are affected by microangiopathic thrombi resulting in long-term pathology. Other organs such as the intestine, central nervous system, and heart may also be affected.

E. histolytica and metronidazole are incriminated in developing a HUS in a 9-year old child (Mele et al., 2014) and *E. histolytica* was identified as a cause of HUS in a 11-year-old child that necessitates hemodialysis (Malaki, 2014).

Diagnosis of amoebic infection

Laboratory diagnosis of Amoebiasis

Entamoeba species are diagnosed directly either by identifying cysts or trophozoites in fecal specimens or by visualizing parasitic stages in biopsy specimens of intestinal mucosa (Fig. 5B). Immunological and molecular-based diagnostic methods should also be used simultaneously (Heredia et al., 2012; Cavagnaro et al., 2006; Sheehan et al., 1979).

Direct coproscopic examination

For over 100 years, microscopy remained the most frequently performed laboratory procedure worldwide, by which almost all intestinal parasites can be detected at the same time. It is considered as a screening method for *Entamoeba* species when performed by an experienced microscopist, especially in low-income settings (Nunez et al., 2001; Tanyuksel and Petri, 2003).

Grossly, formed stool is likely to contain cysts, whereas watery or dysenteric stool usually harbors trophozoites. Direct wet mount preparations help to demonstrate the motility of trophozoites and the internal structure of cysts, if temporary stains are used. However, it is well known that trophozoites are fragile and can rapidly disintegrate soon after passage unless examined or immediately preserved, which is believed to outweigh the limited mobility information, gained by examining the fresh specimens (Garcia and Bruckner, 1997).

Various preservatives are available. They should be paired with the appropriate concentration procedure and permanent staining to give the best results (Lebbad and Svard, 2005; Jensen et al., 2000). Laboratories worldwide used numerous successful staining methods such as Wheatley's trichrome, iron hematoxylin, Giemsa, Wright's, Chlorazol Black E and iodine-trichrome stains. Even though they are tedious and time-consuming, yet they increase sensitivity of microscopy, provide a permanent record, and allow for subsequent consultation if unusual morphologic characteristics are detected (Tan et al., 2010).

The principal limitation of this method is its inability to differentiate closely related *Entamoeba* species and heterogeneity within species (Petri et al., 2000). In addition, the intermittent shedding of intestinal stages, which necessitate examination of three successive stool samples to increase the sensitivity by 23%, and low detection threshold in light infections all add to the limitation of microscopy (Nunez et al., 2001; Hiatt et al., 1995).

Immunodiagnosis

Serology-based diagnosis tools can be divided into two categories: antibody-detection assays and antigen-detection assays.

Antibody detection

Variety of assays are commercially available for detection of *Entamoeba* species antibodies in serum to detect cases of chronic carriers and extra-intestinal invasive species infected patients. They include indirect hem-agglutination and latex agglutination (least sensitivity and specificity), immuno-electrophoresis, counter immuno-electrophoresis, the amoebic gel diffusion test, immuno-diffusion (time-consuming), indirect immunofluorescence assay, while enzyme linked immunosorbent assay (ELISA), proves to be the best way for antibody detection (Fotedar et al., 2007a; Caballero-Salcedo et al., 1994).

Serological diagnosis of *Entamoeba* infection is not beneficial to distinguish past from current infection especially in endemic areas. However, it may be helpful in industrialized nations, where feco-oral infections are not common (Ohnishi and Murata, 1997). In addition, it is still subjected to false positive results due to cross reactivity and negative reactions because of delayed

appearance of antibodies in blood, which may take around 7–10 days to be detected (El-Dib, 2017; Zengzhu et al., 1999; Blessmann et al., 2002; Haque et al., 2010).

Antigen detection

Antigen detection assays in sera have proved to be very useful in the diagnosis of *Entamoeba* species. More specific detection can be achieved using commercially available antigen detection kits, which use monoclonal antibodies against soluble Gal/GalNAc-specific lectin (adhesin molecule) released in the whole blood/serum/stool samples from suspected patients. Although antigen testing is both rapid and technically simple, yet it is more sensitive in areas with high prevalence of *E. histolytica* rather than non-endemic setting (Stark et al., 2008). No specific antigen tests are available for the differentiation (Haque et al., 2000).

One of the available assays is immunochromatographic card test (ICT), which plays an important role in the diagnosis, as it is fast, cost-effective, sensitive and does not need special equipment or experienced personnel. Triple stool immunochromatographic assays is the first convenient alternative enzyme immunoassay for the simultaneous detection of *Entamoeba histolytica*/*E. dispar*, *Giardia lamblia* and *Cryptosporidium parvum* in fresh or frozen fecal samples (Fig. 6) (Garcia et al., 2000; Banisch et al., 2015; Swierczewski et al., 2012).

It is not a method of choice for the final diagnosis due to its inability to differentiate between pathogenic and non-pathogenic species of *Entamoeba*. However, it provides adequate sensitivity and specificity in outbreaks, screening proposals and for massive assay in endemic areas where a large number of samples must be analyzed or as complementary test for individual diagnosis (Goñi et al., 2012).

Molecular diagnostic techniques

Several recent protocols for detection of *Entamoeba* species are now available, which include conventional PCR, nested PCR, and real-time PCR. *Entamoeba* PCR assays target different genetic loci, but the subunit ribosomal RNA gene has been used most often for all *Entamoeba* species due to its multi-copy nature and high analytical sensitivity (Fotedar et al., 2007b).

In an effort to improve the PCR protocol, molecular assays were developed for specific discrimination between different *Entamoeba* species and/or simultaneous detection of other protozoan species in a single assay, such as Duplex PCR, Multiplex real-time PCR (Fig. 7) and Luminex PCR assay. In addition, these closed-tube assays minimize the potential for cross-



Fig. 6 RIDA® QUICK *Cryptosporidium*/*Giardia*/*Entamoeba* Combi ICT showing three bands. From left to right: crimson band “control”, green band “*Entamoeba* positive”, red band “*Giardia* positive” and blue band “*Cryptosporidium* positive”.

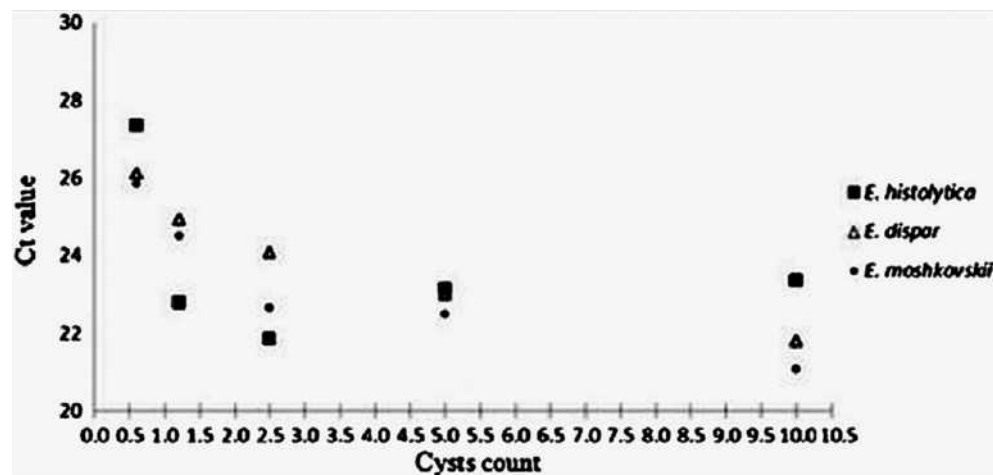


Fig. 7 Detection limits of *Entamoeba* sp. using Real-time PCR. Real-time PCR showed the detection limits of each *Entamoeba* sp. to be as low as 0.625 cysts (Lau et al., 2013).

contamination, reduce the time needed and provide highly accurate quantitative data (Blessmann et al., 2002; Verweij et al., 2004). On the other hand, these techniques are still of high cost, need experience and have been postulated that they show more sensitivity in detection of samples with trophozoites than cysts (Fotedar et al., 2007b; Haque et al., 1998).

Diagnosis of amoeboma

Suspected cases are diagnosed after histopathological examination of specimens taken by endoscopy. As stated before, amoebomas could be single or multiple, appears tumour-like with irregular friable surface and necrotic bleeding tissue.

Diagnosis of extraintestinal lesions

Diagnosis is often needed after suspected clinical picture. It is initiated with imaging techniques to determine the presence of abscess in the liver, lungs, brain, etc. If indicated, aspiration preferentially at the periphery of the lesion is performed under US or CT scan for culture, DNA and/or antigen detection. Aspiration is also recommended in cases where the abscess is located in the left lobe of the liver, or in cases with severe pain or elevated diaphragm (Kapoor, 1979).

Treatment

At the days of Hippocrates, curing an abscess was done by draining its contents (Americana Corporation, 1970a). Hippocrates stated that "Desperate diseases need desperate remedies" (Anon, 1959) and advised physicians to assist Nature's ways to heal the sick.

Bloodletting by leeches was a common method for treatment of many diseases. However, laxatives and mercurial purgatives were also commonly used. Meanwhile some surgical practices as trocar aspiration or open drainage were also known in the 19th century (Ballingall, 1818). This technique was adopted when the author noticed a case of an officer in Madras, India who had been shot in his liver, which was infected with an abscess. This shot drained the abscess leading to complete cure (Ballingall, 1818).

The use of quinine was also tried in few such cases (Morehead, 1856). Ipecacuanha was a famous herbal therapy for the "bloody flux, or dysentery" since the 16th century among natives in Brazil. Rogers got the use of Ipecacuanha back replacing other unsatisfactory methods. He also reported the isolation of alkaline emetine from Ipecacuanha and mentioned its amazing effect on amoebic dysentery and liver abscess (Rogers, 1912).

It is recommended that all infections with *E. histolytica* including the carriers should be treated. This is going to prevent the development of invasive disease, but also will decrease the spread of infection (Bradly et al., 2015).

Paromomycin, a luminal antibiotic can eliminate cysts of *E. histolytica* from the gut lumen, however metronidazole and tinidazole which mainly affect the trophozoite stages, are important agents against invasive amoebiasis (Haque et al., 2003; Bradley et al., 2015).

Nitroimidazoles e.g., Metronidazole and those with longer half-lives, such as tinidazole, secnidazole, and ornidazole, allow for shorter treatment periods and tend to be better tolerated. After a 10-day course of a metronidazole, paromomycin should be given to assure that the luminal parasites are cleared and prevent relapse (El-Dib, 2017; Liu et al., 2016). It has been shown that 40–60% of patients have persistent intestinal parasites after treatment with only nitroimidazole. Second line luminal agents include diiodohydroxyquin and diloxanide furoate (Haque et al., 2003; Liu et al., 2016).

Treatment of asymptomatic infection constitutes a real medical conflict, to treat or not to treat. McGuinness and Leder as well as the current US treatment guidelines recommend treatment of asymptomatic infection, as there is always a potential risk of causing an invasive disease or spread to family members (McGuinness and Leder, 2019).

The recommended protocol approves the administration of Iodoquinol 650 mg PO three times a day for 20 days in adults, while children are given: 30–40 mg/kg/day PO in three divided doses for 20 days OR paromomycin 25–35 mg/kg/day PO in three divided doses for 7 days OR diloxanide furoate 500 mg PO tds. For 10 days in adults and 20 mg/kg/day PO in three divided doses for 10 days in children (McGuinness and Leder, 2019).

Treatment of cases infected with other species of *Entamoeba* represents a real challenge. It could be better to treat cases for the potential risk of development to an invasive disease as well as spread to the community (McGuinness and Leder, 2019).

As stated before, *E. dispar* has the potential to behave in an invasive way, so infected cases are treated with luminal amoebicide e.g., Paromomycin 30 mg/kg/day for 10 days in three divided doses (Tengku and Norhayati, 2011).

Control of *E. polecki* infection as a newly emerging zoonosis is important. Although cases may be asymptomatic, yet treatment with metronidazole is necessary to avoid possible pathogenicity (Smith, 2019).

Cases with *E. moshkovskii* in Tropical areas are treated with antiparasitic therapy (Fotedar et al., 2007a). Nitroimidazole and luminal drugs are paired together when gastrointestinal symptoms are present (Fotedar et al., 2007b).

Epidemiology

The global statistics on the prevalence of *E. histolytica* infection indicates that 90% of infected cases are asymptomatic while the other 10% develop clinical manifestations. It has been estimated that up to 50 million people in the world, particularly in developing countries are affected by *E. histolytica*. It is estimated that infection may reach up to 50% in developing countries e.g., Africa, Central

and South America and some Asian countries (WHO, 2009). WHO stated that factors as poverty, bad sanitation, lack of clean water and lack of knowledge can affect spread of infection (Lo et al., 2014). In industrialized countries, infection is detected among travelers, institutions, homosexual males and HIV infected persons (Khairdar et al., 2007).

The disease could be responsible for over 100,000 deaths per year (Bercu et al., 2007; Ximenez et al., 2010). Although all the deaths could be due to invasive *E. histolytica* infections, Recently *E. dispar* and *E. moshkovskii*, morphologically identical species, have been detected in individuals inhabiting endemic areas of amoebiasis and could be contributing to the prevalence percentages. Thus, the reclassification of *E. histolytica* into the three morphologically identical yet genetically different species has further added to the complexity of the epidemiology of amoebiasis since they cannot be differentiated by microscopy most commonly used for diagnosis particularly in tropical countries where resources are limited (Ali et al., 2003; Shimokawa et al., 2012).

However sometimes infection is severe due to invasion of the colonic epithelium or extraintestinal tissues by virulent species *E. histolytica*. Patients may exhibit abdominal pain with cramps, diarrhoea which could contain blood or mucous (Haque et al., 2003).

Amoebic liver abscess is the most common extraintestinal or invasive amoebiasis, that occurs due to transmission of the organism by hematogenous route, followed by pulmonary, pericardial and brain abscesses (Haque et al., 2003).

Epidemiology of amoebiasis is affected by some factors, the most important of which is that man is the only host of infection with no animal reservoir hosts. Asymptomatic carriers constitute the main source of infection, passing cysts in feces. Cysts are directly infective and survive long periods in the environment resisting chlorination. Disease transmission occurs due to ingestion of contaminated food and/or water. Sexual transmission has been mentioned among male homosexuals as another mode of infection (Stark et al., 2008).

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Fascioliasis in Humans and Animals

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The parasite

Morphological and anatomical characterization

Fascioliasis is a freshwater snail-borne zoonotic disease caused by two species of *Fasciola* (Trematoda: Fasciolidae): *Fasciola hepatica* distributed almost worldwide and *F. gigantica* restricted to parts of Africa and Asia (Mas-Coma et al., 2009a).

These species are leaf-shaped, with two suckers in a conelike anterior extension of the body and a broadly pointed posterior end. In size, *F. gigantica* (slightly more than 50 mm) is almost the double length of *F. hepatica* (up to 29 mm), and in shape it is more elongate and narrower, with lateral walls tending to be parallel, and with nonexistent or less marked shoulders of the cephalic cone (Fig. 1A and B).

Their digestive system includes a patent pharynx and two long highly ramified intestinal caeca reaching the posterior end. The genital hermaphroditic system comprises (i) a male system with two branched testes in longitudinal tandem within the second and third fourths of the body, and a prominent cirrus pouch, containing a spined cirrus protrusible through a post-bifurcal genital pore, and (ii) a female system with a pretesticular, dextral branched ovary, numerous small vitelline gland follicles extending bilaterally up to the hind body, and a short uterus between the ovary and caecal bifurcation.

The eggs are ovoid, yellowish, operculated, and nonembryonated when laid. Egg length/width in *F. hepatica* measure 73.8–156.8/58.1–98.1 µm in animals and 100.6–162.2/65.9–104.6 µm in humans, whereas in *F. gigantica* they are of 130.3–182.8/74.0–123.6 µm in animals and 150.9–182.2/85.1–106.2 µm in humans (Fig. 1C and D) (Valero et al., 2009; Mas-Coma et al., 2014a).

The measurements of the two species overlap, although given features allow to classify specimens in populational analyses (Periago et al., 2006). Given that fasciolids grow inside the biliary canals and morphometrics vary according to age and host species, studies and comparisons should be made only with other fasciolid populations infecting the same host species (Valero et al., 2005). Moreover, intermediate forms appear in overlap areas of Africa and Asia, due to hybridization of both *Fasciola* species (Periago et al., 2008; Afshan et al., 2014a; Ahasan et al., 2016).

Molecular and genetic characterization

A study on the origin of *F. hepatica* and *F. gigantica* and their subsequent worldwide evolution was made by Mas-Coma et al. (2009a), based on palaeobiogeographical and evolutionary data of their mammal host species and lymnaeid vector species, by considering parasite-host relationships in crucial aspects as specificity, pathogenicity and immunity, developmental requirements



Fig. 1 Fasciolid adults and eggs: (A) adult stage of *Fasciola hepatica* from cattle in the Northern Bolivian Altiplano; (B) adult stage of *F. gigantica* from zebu of Bobo Dioulasso in Burkina Faso; (C) egg of *F. hepatica* from a child in the Northern Bolivian Altiplano; (D) egg of *F. gigantica* from zebu of Bobo Dioulasso in Burkina Faso. For measurements, see text. Photographs S. Mas-Coma.

(e.g. temperature thresholds) and present geographical distribution and ecological requirements of the respective specific lymnaeid vector species of both fasciolids in the five continents. The long-time evolutionary scenario of fasciolid global spread up to the present was defined by distinguishing two periods: (i) a long pre-domestication million-year period from their respective palaeobiogeographical origins and subsequent evolution until human arrival; and (ii) a shorter post-domestication 12,000-year period from the beginning of the domestication of herbivore mammal species, along the archaeological knowledge on year thousands during which these domesticated animals were moved or transported by humans throughout until defining the present day scenario (Mas-Coma et al., 2009a). This evolutionary scenario offers a framework for the interpretation of the results of genetic studies.

According to this framework (Mas-Coma et al., 2009a), the fasciolid ancestor may be found in an ancient liver fluke form infecting old Artiodactyla in Africa during the early Oligocene when the first pecoran radiation occurred. The origin of *F. gigantica* was probably the result of an adaptation to bovids (Alcelaphinae, Reduncinae, Bovinae), resulting in an explosive radiation during the early Miocene, in the warm, eastern Africa, where the lymnaeid snail *Radix natalensis* assured the transmission. The origin of *F. hepatica* was probably in the Eurasian Near East, as a derivation from the same ancient fasciolid or a *F. gigantica*-close old form introduced with ruminants from Africa during a major sea level lowering in the early Miocene. The *F. hepatica* origin was likely the result of colonization and adaptation to a northern temperate-colder region, including two host capture phenomena to smaller lymnaeid species as *Galba* and to mid-sized ruminants (Mas-Coma et al., 2009a).

Fasciolids show a pronounced variability which is the consequence of thousands of years of animal movements and livestock exchange between areas, countries and continents during the livestock post-domestication period (Mas-Coma et al., 2009a). The result is the existence of local mixture puzzles whose origin interpretation is usually very difficult or highly speculative, in front of many accumulative waves of selfing and outcrossing flukes.

Genetic characteristics of fasciolids have been assessed by different techniques. In *F. hepatica*, the same isoenzymes were found regardless of the host species, but densities of some isoenzyme bands showed differences according to the host (Blair, 1993). Attempts by means of whole-body protein profiles and excretory/secretory products obtained with isoelectric focusing proved to be useless because fasciolids evidenced differences according to host species (Lee et al., 1992).

The aforementioned local mixture puzzles were evidenced when applying random amplified polymorphic DNA (RAPD), which showed that the majority of genetic diversity occurred within, rather than between animal hosts and was also greater within than

between populations, suggesting the influence of animal migrations and transportation (Semyenova et al., 2003). Similar results were later obtained by using a microsatellite approach (Beesley et al., 2017).

At present, fasciolid genotyping mainly relies on DNA sequencing of markers of the ribosomal DNA operon (rDNA) and the mitochondrial DNA genome (mtDNA). Sequencing of rDNA and mtDNA is useful to distinguish between *F. hepatica* and *F. gigantica* in overlap areas of Africa and Asia. In rDNA, the ITS-2 spacer is the best species marker (Mas-Coma and Bargues, 2009). In an analysis of “pure” *F. hepatica* from numerous countries and continents, only two 364-bp-long ITS-2 haplotypes and only one 432-bp-long ITS-1 were found. In “pure” *F. gigantica*, only one ITS-2 and one ITS-1 were found. The two species differ by five polymorphic sites at ITS-2 and other five at ITS-1 (Mas-Coma et al., 2009a). A wider comparison showed four ITS-2 haplotypes in *F. hepatica* and five in *F. gigantica*, whereas the ITS-1 was uniform in both species everywhere (Mas-Coma et al., 2009a).

In the mtDNA, the codifying genes *cox1* and *nad1* are the markers most used, mostly short fragments of them but in their complete length in a few studies. In *F. hepatica*, a total of 69 1533-bp-long *cox1* haplotypes and 23 510-aa-long COX1 protein haplotypes, and 51 903-bp-long *nad1* haplotypes and 15 300-aa-long NAD1 protein haplotypes were found in different continents (Mas-Coma et al., 2009a). In *F. gigantica*, a total of 11 1533-bp-long mtDNA *cox1* haplotypes and 5 510-aa-long COX1 protein haplotypes, and 15 903-bp-long mtDNA *nad1* haplotypes and 10 300-aa-long NAD1 protein haplotypes were found (Mas-Coma et al., 2009a).

Two single locus nuclear protein-coding genes, phosphoenolpyruvate carboxykinase (pepck) and DNA polymerase delta (pold) were proposed for fasciolid discrimination (Shoriki et al., 2016; Hayashi et al., 2018). Unfortunately, pepck revealed different heterozygosity and homozygosity levels with high length polymorphism and pepck multiplex PCR patterns could not differentiate between *Fasciola* species (Amor et al., 2020). The evaluation of the usefulness of pold is still pending.

The complete mitochondrial genomes of *F. hepatica* and *F. gigantica*, as well as that of an intermediate fasciolid form, have been already sequenced (Le et al., 2001; Liu et al., 2014). The *F. hepatica* genome has proved to be among the largest known pathogen genomes at 1.3 Gb. The substantial polymorphism levels found have tentatively been linked to the evolutionary potential for rapid adaptation to changes in host availability, climate change or to drug or vaccine interventions (Cwiklinski et al., 2015). Intriguingly, the *F. hepatica* genome from sheep of North America showed a markedly higher repeat content (55.29%) than the *F. hepatica* genome from cattle of United Kingdom (32.0%) (McNulty et al., 2017).

Biology

Life cycle

Both species follow a freshwater two host life cycle (Mas-Coma and Bargues, 1997). The adult stage lives inside the biliary canals and gallbladder, where it grows, matures and fecundates, by selfing or cross fertilization. Eggs are excreted outside with faeces via bile and intestinal lumen and those reaching freshwater will embryonate, giving rise to an inner miracidium.

The miracidium, of 130/28 µm, hatches under light stimulation and swims until contacting a freshwater snail of the family Lymnaeidae. The miracidium penetrates the snail and evolves into a 150–500-µm-long saccular sporocyst, which develops in the mantle, mantle collar or peri-esophageal area, and produces the subsequent stage of rediae. Rediae come out of the sporocyst and migrate mainly to the digestive gland. The first mother rediae in turn produce cercariogenous daughter rediae. Mature 250–750-µm long rediae are cylindrical, presenting a rudimentary digestive system (pharynx and a short caecum). Up to four redial generations have been found, although 2–3 generations are usual and follow the same developmental pattern in different lymnaeid species (Rondelaud and Barthe, 1986, 1987) according to a complex larval process (Rondelaud et al., 2009a).

Cercariae originate inside the rediae, escape through a birth pore, and are shed into water. The cercaria has a rounded spinose body of 28–320/250 µm, and a motile 700-µm-long tail. The prepatent period is dependent on temperature (15 °C: 56–86 days; 25 °C: 38 days). The shedding process in *F. hepatica* takes place between 9 °C and 26 °C. Cercariae swim for a short time (1 h) until contacting a solid support, mostly plants above or below the water line. They then lose their tails and quickly encyst, changing into metacercariae.

Metacercarial cysts are round, 200 µm in diameter, and infective within 24 h after encystment. Floating metacercarial cysts are also originated at the water surface line. Metacercarial cysts are resistant and viable for a long period, but are killed by excessive heat and dryness. Metacercariae infect the definitive host after ingestion, excyst in the small intestine within an hour, penetrate the intestine wall, and reach the abdominal cavity by about 2 h after ingestion. Most reach the liver within 6 days after excystment. In the liver they migrate for 5–6 weeks, and penetrate into bile ducts where they become sexually mature. It has been also speculated that juvenile flukes may (i) enter the blood stream and so reach various parts of the body, (ii) reach the liver by moving up the bile duct, or (iii) follow a very short intrahepatic migration (Chen and Mott, 1990; Moazeni and Ahmadi, 2016).

The prepatent period (from metacercariae ingestion to first egg appearance in faeces) varies according to the host: 35–42 days in mice; 55 days in guinea pigs; 63 days or 13–15 weeks in sheep infected with 200 or 2000 metacercariae, respectively; 56–61 days in cattle. In humans, the prepatent period is of at least 3–4 months (Mas-Coma and Bargues, 1997).

Intermediate hosts or transmission vectors

Freshwater Lymnaeidae snails are the vectors of the two *Fasciola* species. Each *Fasciola* species shows a different specificity regarding different lymnaeids. There are lymnaeid species which cannot transmit fasciolids, others transmit *F. hepatica*, others transmit

F. gigantica and a very few which have been reported to transmit both fasciolids. The lymnaeid species proved to be involved in the transmission of both *Fasciola* species in all continents have been reviewed in detail (Bargues et al., 2001).

Fasciola hepatica is mainly transmitted by small-sized *Galba/Fossaria* group species (Bargues et al., 2007, 2011a), including *Galba truncatula* as the most efficient vector (Bargues et al., 2017) and the only one in Europe, but also present in Africa, Asia and South America (Fig. 2A). The wide knowledge and literature on *G. truncatula* have been appropriately reviewed (Rondelaud et al., 2009b). Other vector species of this group include: *Lymnaea humilis*, *L. bulimoides* and *L. cubensis* in North America; *L. cubensis* in the Caribbean (Fig. 2C); *L. neotropica*, *L. cousini* and *L. viator* in South America; and *L. tomentosa* in Australia. The discovery of the nontransmitting species *L. schirazensis*, always confused with *G. truncatula* and other similar vector species throughout, highlighted specimen classification problems distorting fasciolid–snail specificity/susceptibility and fascioliasis geographical distribution data (Bargues et al., 2011a).

Species of the genus *Radix* transmit *F. gigantica*, mainly *R. natalensis* in Africa and varieties of *R. auricularia* (Fig. 2B) and *R. viridis* in Asia (Bargues et al., 2001; Ashrafi and Mas-Coma, 2014). *Pseudosuccinea* only includes the species *P. columella* which appears to be able to transmit both fasciolids (Bargues et al., 2011c) and has recently colonized all continents (Bargues et al., 2011c; Lounnas et al., 2017).

A few lymnaeid species among the stagnicoline group have proved their capacity to transmit *F. hepatica* under exceptional or local natural conditions in a few areas, such as *L. (Stagnicola) palustris*, *L. (S.) fuscus*, and *Omphiscola glabra* (Bargues et al., 2003).

The geographical distribution of the lymnaeid vectors species defines not only the distribution of fascioliasis, but may also explain the distribution of human infection within a country (Fig. 3A–D), as has been recently observed in Venezuela (Bargues et al., 2011b) and Chile (Artigas et al., 2011), and, within an endemic area, its seasonality or permanent transmission (Mas-Coma et al., 1999c; Bargues et al., 2020, 2021).

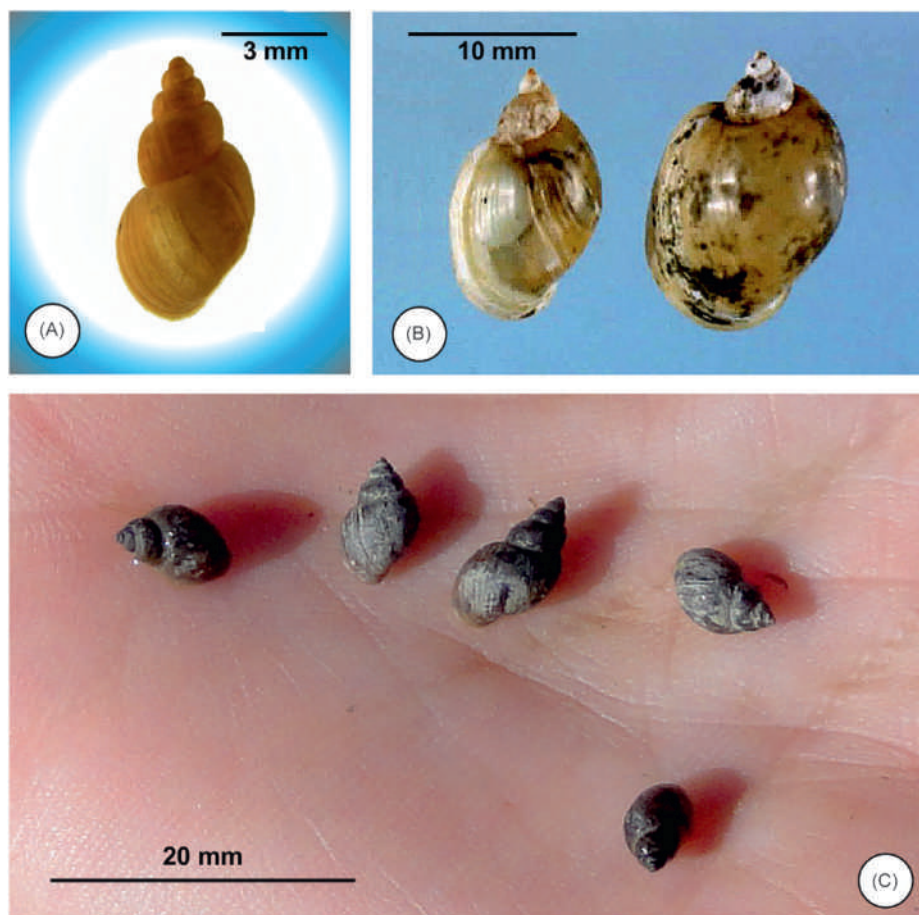


Fig. 2 Lymnaeid vector species transmitting fascioliasis: (A) *Galba truncatula* from Spain, the most efficient vector of *Fasciola hepatica*; (B) specimens of *Radix auricularia* transmitting *F. gigantica* in Guilan, Iran, showing shell variability; (C) specimens of *Lymnaea cubensis* from Cuba. Photographs S. Mas-Coma.



Fig. 3 Eco-epidemiology of main species of lymnaeid snail vectors of fascioliasis: (A) most small-sized lymnaeid vectors of the *Galba/Fossaria* group transmitting *Fasciola hepatica* show an evident amphibious trend, as shown by *Galba truncatula* on mud in Europe; (B) the *Fasciola gigantica* transmitting lymnaeid species *Radix viridis* is also a small-sized amphibious snail in South East Asia; (C) the *F. gigantica* transmitting *Radix auricularia* is a large-sized lymnaeid pronouncedly more aquatic in the Near East; (D) in the Nile Delta in Egypt, *Galba truncatula* and *Radix natalensis* coexist in the same irrigation canals surrounding vegetable cultivated fields and villages where inhabitants are co-infected by *F. hepatica*, *F. gigantica* and intermediate hybrid fasciolids. Photographs S. Mas-Coma.

Definitive host Spectrum and animal reservoirs

Adult fasciolids infect herbivorous mammals, mainly ruminants as sheep, goats, cattle and domestic buffaloes. Cattle and sheep are usually the most important animal reservoirs regarding human infection (Fig. 4A) (Mas-Coma et al., 2020a). Goats react to fasciolosis similarly to sheep, although their trend to more dry areas make them to be a secondary reservoir. Buffaloes are also of epidemiological importance in human endemic areas, mainly in Asia and Africa, where it is a preferential host for *F. gigantica* (Fig. 4B). Grazing domestic pigs may also be infected, but this host usually shows a higher natural resistance (Mas-Coma and Bargues, 1997).

Equids are alternate hosts which may play a role in the epidemiology of the disease and have been recently in the focus (Howell et al., 2020; Mas-Coma et al., 2020b). Horses appear to be more resistant to liver fluke infection than donkeys (Dorchies, 2010), with mules showing an intermediate susceptibility (Mera y Sierra et al., 2020).

Many other herbivorous domestic and wild animals may also be infected. Infection in Old and New World camelids has been repeatedly reported. Wild herbivorous mammals such as buffalo, deer, wild sheep, wild boar, various marsupials, rabbit and hare are also susceptible hosts. Many African wild animals have been also found naturally infected by *F. gigantica* (Losos, 1986). Additional to capybara and nutria, several rodent species have been found naturally infected by *F. hepatica* and others are usually used for experimental purposes.

Epidemiology

Geographical distribution

Livestock fascioliasis has been reported from almost all countries, except the most extreme northern latitudes. Fascioliasis is a worldwide veterinary problem. It is the vector-borne parasitic disease presenting the widest latitudinal, longitudinal and altitudinal distribution known at present (Mas-Coma et al., 2003, 2005). In the last three decades, it proved to be an important public health problem as well. The increasing human case and community reports throughout and results on pathogenicity and immunity studies

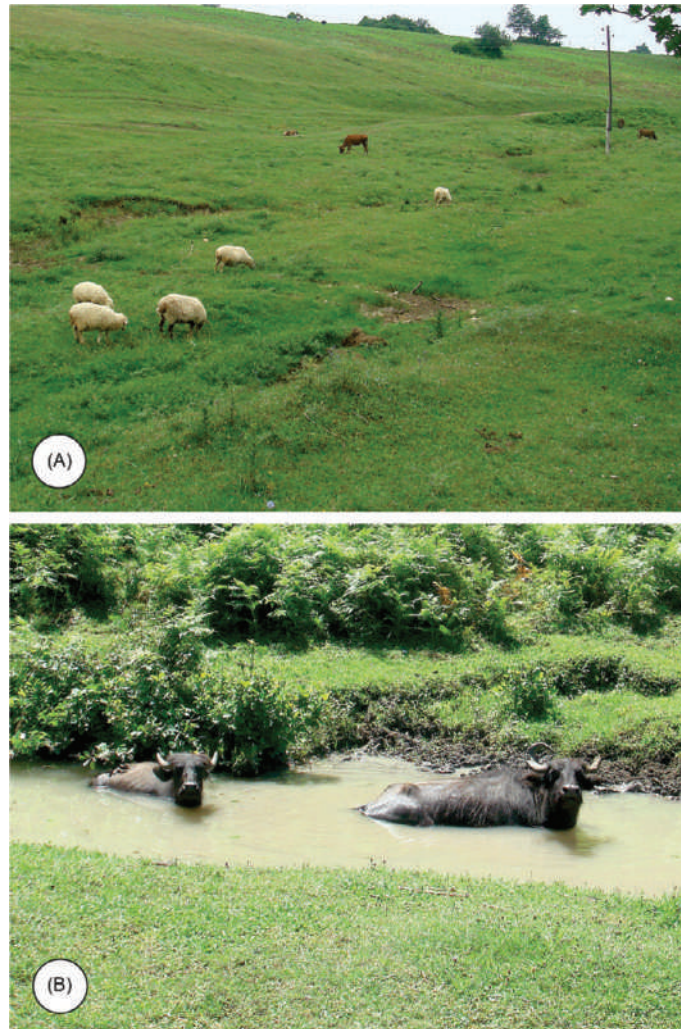


Fig. 4 Main domestic animal reservoirs of fascioliasis: (A) sheep and cattle grazing around small creek inhabited by amphibious *Galba truncatula* snails in a typical *Fasciola hepatica* transmission focus in the Southern Caucasus; (B) in Near East Asia, buffaloes tend to refresh inside deep freshwater collections inhabited by *Radix auricularia*, where they become infected by *Fasciola gigantica*. Photographs S. Mas-Coma.

were the reasons why it was decided to no longer consider fascioliasis a secondary human disease but an important one, not only in rural areas of developing countries (Mas-Coma et al., 1999a), but also concerning from individual patients to small epidemics in developed countries (Mas-Coma, 2020). It was therefore included as a food-borne trematode disease priority within the World Health Organization agenda (WHO, 2013).

Present estimations on human fascioliasis reach from 2.4 million up to 17 million people, or even higher depending from the hitherto unknown situations in Asia and Africa (Mas-Coma, 2004). A global analysis shows that the expected correlation between animal and human fascioliasis only appears at a basic level. High prevalences in humans do not seem to be necessarily related to high prevalences in livestock. Within a human endemic area, human infection appears irregularly distributed, with patchy transmission foci linked to lymnaeid-inhabited water collections (Fig. 5A and B) (Mas-Coma et al., 1999c).

Although human infection may occur in all animal endemic areas, the major health problems have been reported from Andean countries (Bolivia, Peru, Chile, Argentina), Central America (Mexico, Costa Rica), the Caribbean area (mainly Cuba but also Haiti), northern Africa (Egypt), the Near East (Iran and neighboring countries), southern Asia (Pakistan), and Southeast Asia (mainly Vietnam) (Mas-Coma et al., 2005, 2009a). The highest prevalences and intensities in humans have been reported in the Northern Altiplano in Bolivia and Peru (Esteban et al., 1999, 2002), the Cajamarca province in Peru (Gonzalez et al., 2011), and the region of the Nile Delta in Egypt (Esteban et al., 2003). Areas presenting lower human endemics and/or human reports concern: Venezuela, Ecuador, Brazil and Uruguay in the Americas; Ethiopia, Tanzania and South Africa in Africa; and India, Laos and southern China in Asia. Sporadic cases are being diagnosed throughout Europe, although mainly in western Europe (France, Portugal, Spain, Italy and Switzerland) (Mas-Coma, 2020). The impact of this disease in all kind of travelers has recently been reviewed (Ashrafi et al., 2014).

The different temperature thresholds of the two fasciolids underlie different preferences in seasonal, latitudinal and altitudinal distribution (Mas-Coma et al., 2009a). Thus, *F. hepatica* is mainly transmitted during the colder months, prefers temperate latitudes



Fig. 5 The anthropophilic behavior of given lymnaeid vector species assures fascioliasis transmission close to human dwellings and therefore increases human infection risk: (A) *Galba truncatula* transmission focus of *Fasciola hepatica* inside village in the Northern Bolivian Altiplano; (B) *Radix viridis* transmission focus of *Fasciola gigantica* in irrigated fields surrounding village in Vietnam. Photographs S. Mas-Coma.

and appears markedly confined to highlands in tropical and subtropical regions. Contrarily, *F. gigantica* is mainly transmitted during the warmer months, prefers warmer latitudes and appears markedly confined to lowlands in tropical and subtropical regions.

The two fasciolids overlap in several regions of Africa and Asia. Two new epidemiological terms were proposed for these areas (Mas-Coma et al., 2009a): (i) local overlap, where the two species are transmitted in neighboring foci or the same focus when the respective specific lymnaeid vectors share the same freshwater collection, and (ii) zonal overlap, where the two species are transmitted within the same area but *F. gigantica* foci are in the lowlands and *F. hepatica* foci in the nearby highlands (Fig. 6). Both overlaps furnish hybridization opportunities and consequent appearance of intermediate forms, as in the local overlap in the Nile Delta, Egypt (Periago et al., 2008), and the zonal overlap in Guilan, Iran, respectively (Ashrafi et al., 2015).

Human infection sources

A worldwide analysis has clarified all human fascioliasis infection sources, their diversity in the different areas and countries, the related incidence factors, and the methods for the study and analysis of these sources (Mas-Coma et al., 2018). The diversity of infection sources in different countries underlies the large epidemiological heterogeneity of human fascioliasis throughout (Mas-Coma et al., 2018):

- Ingestion of freshwater wild plants: main aspects to be considered are the plant markers of transmission foci, watercress, other freshwater wild plants, and wild plants sold in urban markets
- Ingestion of freshwater cultivated plants, mainly watercress

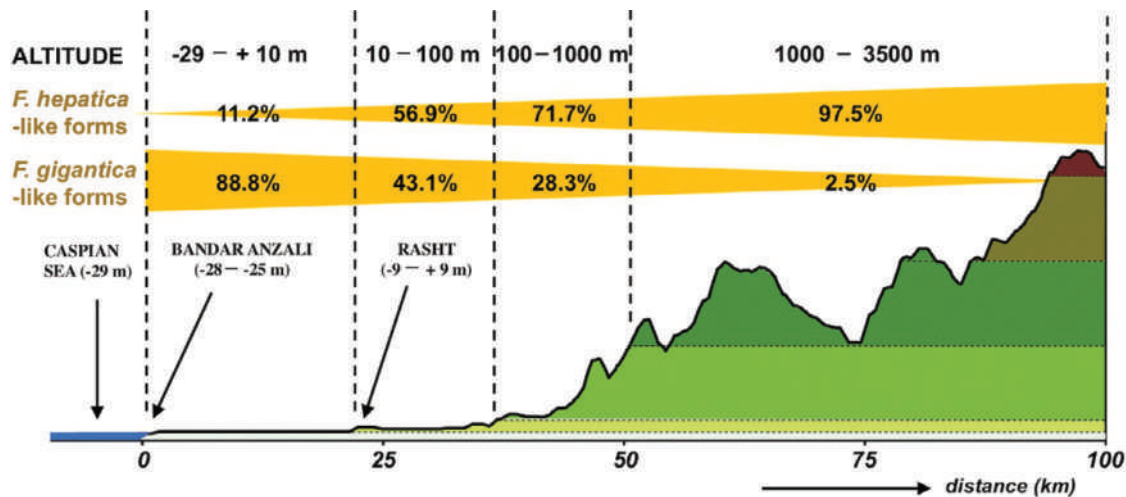


Fig. 6 Altitude is a crucial factor in the distribution of lymnaeid species of the *Galba/Fossaria* and *Radix* groups and in the temperature thresholds of development of the fasciolid species: *Fasciola gigantica* and *F. gigantica*-like forms predominate in the *Radix auricularia* inhabited lowlands, whereas *Fasciola hepatica* and *F. hepatica*-like forms predominate in the *Galba truncatula* inhabited highlands, in the zonal overlap in Guilan, Iran. Animal movements and transhumance underlie the exchange of livestock between lowlands and highlands. Schema S. Mas-Coma.

- Ingestion of terrestrial cultivated plants needing frequent irrigation
- Ingestion of terrestrial wild plants: collected in dry habitats but which were submerged in water a few weeks or months before
- Ingestion of traditional local dishes made with contaminated sylvatic plants
- Ingestion of raw liver infected with migrating metacercariae which may keep the capacity to restart migration
- Drinking of contaminated water
- Drinking of beverages and juices made from local plants
- Ingestion of dishes and soups made with contaminated water
- Washing of vegetables, fruits, tubercles, kitchen utensils or other objects with contaminated water.

Among the incidence factors, disease transmission seasonality, the infectivity of metacercariae under field conditions, as well as several community, familial and social factors in infection risk appear in the forefront. The methods to assess human infection sources include: (i) detection of metacercariae attached to plants or floating in freshwater; (ii) anamnesis in individual patients, and (iii) questionnaire surveys in endemic areas (Mas-Coma et al., 2018).

Metacercarial infectivity is dependent upon storage time, being lower when metacercariae are older, and do not differ between isolates from different reservoir species (Valero and Mas-Coma, 2000).

Transmission patterns and epidemiological situations

Fascioliasis presents a very wide spectrum of transmission and epidemiological patterns in human hypo- to hyperendemic areas. A classification of transmission patterns has been proposed (Mas-Coma, 2005) and is progressively updated to offer a baseline for future research (Mas-Coma et al., 2009a; Valero et al., 2012b; Bargaes et al., 2016).

A useful epidemiological classification of human infection situations has been proposed (Mas-Coma et al., 1999a, 2009a), including:

- (1) Autochthonous, isolated, nonconstant cases;
- (2) Imported cases;
- (3) Hypoendemic (prevalence less than 1%);
- (4) Mesoendemic or holoendemic (prevalence between 1% and 10%);
- (5) Hyperendemic (prevalence higher than 10%);
- (6) Epidemics in nonhuman endemic but animal endemic areas (family or small group reports);
- (7) Epidemics in human endemic areas (higher number of subjects may be concerned).

In the human hyperendemic areas, 5–15-year-old school children are the group most affected (Fig. 7A and B). However, in other human endemic areas, infection peaks at an adult age (usually between 30 and 40 years old). Fascioliasis appears to affect more females than males, this preference for females being detected in prevalences or intensities according to areas. However, infection in humans may occur earlier than previously expected, from early postnatally infected lactants to pre-school age and, in such small children, males instead of females are the most infected (De et al., 2020).



Fig. 7 Children are the age group most infected by fascioliasis in human hyperendemic rural areas of developing countries: (A) 5–15-year-old schoolchildren have a high risk of fascioliasis infection along the ways from home to school and back in the Northern Bolivian Altiplano; (B) the closeness of livestock grazing, activities of children, and cloth and utensil washing by women underlie their high infection rates, as in the Mantaro valley in Peru. Photographs S. Mas-Coma.

Influences of climate change and global change

Climatic factors are decisive in fascioliasis transmission. Seasonal variation of rainfall and temperature gives rise to different fascioliasis seasonality. In Europe, the transmission is typically bi-seasonal, due to the lymnaeid vector activity in spring and autumn. In the Bolivian Altiplano, the transmission takes place along the year, lymnaeids inhabiting permanent water bodies instead of temporary ones due to the high altitude evapotranspiration (Mas-Coma et al., 1999c; Bargues et al., 2021). In other areas, the transmission is mono-seasonal, due to only a season with surface water availability.

Global warming is a crucial factor. In the Bolivian Altiplano, at 3820–4100 m a.s.l., recent increased temperatures have allowed the lymnaeid vector to colonize zones of higher altitudes outside the previously established borders of the endemic area, with the consequent spread of the human infection risk area (Bargues et al., 2020).

Climate change overlaps anthropogenic and environmental modifications which give rise to suitable new environments for the lymnaeid vectors and influence transmission seasonality (Mas-Coma et al., 2009b). Thus, artificial field irrigation is sufficient by its own to allow for fascioliasis transmission in Cambodia (Tum et al., 2007). In Pakistan, transmission follows bi-seasonality with a peak related to rainfall and another related to man-made irrigation (Afshan et al., 2014b). Establishing fascioliasis emergence causality in such an influencing factor overlap may sometimes be difficult.

Three types of approaches are useful for the study of climate and environment influences on fascioliasis, including the assessment from geographical distribution and seasonality up to human and animal infection risk and forecasting methods, namely mathematical modelling based on climate factors, remote sensing (RS) based on images and information furnished mainly by space satellites, and geographic information systems (GIS) based on computer mapping by geo-positioning of different abiotic and biotic

factors and characteristics. These methods allow for developments from low to very high resolution adapted to endemic areas of different characteristics (Malone et al., 2019).

The disease

Phases, pathology, symptomatology and impact

Fascioliasis is a highly pathogenic disease in both humans and animals. Fascioliasis has a large economic and trade impact on husbandry and import/export of livestock, globally (Mehmood et al., 2017) and in different countries (Schweizer et al., 2005; Mazeri et al., 2017), including countries where human fascioliasis is a wide health problem (Espinoza et al., 2010). An estimation of total world economic losses in only cattle and buffalo reached US\$ 3.2 billion (Copeman and Copland, 2008).

In humans, fascioliasis causes community underdevelopment in rural areas of low income countries. There is much clinical variability related to (i) the burden (flake number infecting the patient), (ii) disease phase or period, and (iii) host individual susceptibility. Four periods may be distinguished (Chen and Mott, 1990; Mas-Coma and Bargues, 1997; Mas-Coma et al., 1999b, 2000, 2014a):

- Incubation phase: From the ingestion of metacercariae to symptom onset.
- Acute, invasive or migratory phase: It corresponds to the fluke migration up to the bile ducts. The symptomatology is due mainly to mechanical destruction of liver tissue and abdominal peritoneum by the migrating larvae causing localized or generalized toxic and allergic reactions lasting 2–4 months. This phase includes fever, abdominal pain usually in the right hypochondrium or below the xyphoid, gastrointestinal disturbances such as loss of appetite, abdominal flatulence, nausea and diarrhea, respiratory symptoms such as cough, dyspnea, hemoptysis, urticaria and chest pain.
- Latent phase: From arrival to biliary canal until starting of oviposition. It can last from days to years and show eosinophilia, gastrointestinal complaints or one or more acute symptom relapses.
- Chronic, biliary or obstructive phase: This period may develop after months to years of infection. Adult flukes in the bile ducts cause inflammation, hyperplasia of the epithelium, and thickening and dilatation of duct and gall bladder walls. Cholangitis and cholecystitis, combined with the large body of the flukes, may be sufficient to cause obstruction. This period includes biliary colic, epigastric pain, fatty food intolerance, nausea, jaundice, pruritus, right upper-quadrant abdominal tenderness and Murphy's sign, among others. Lithiasis of the bile duct or the gall bladder is frequent, whereas cirrhosis does not appear to be so. The bile duct and the gall bladder may contain blood mixed with bile (haemobilia), blood clots and fibrinous plugs.

The comparison of the development of *F. hepatica* and *F. gigantica*, followed for the first time in the same animal host, demonstrated a higher pathogenicity by *F. gigantica*. This result was opposite to previous studies indicating a higher *F. hepatica* pathogenicity and which in fact was only reflecting the faster development of *F. hepatica* observed in short-term experiments (Valero et al., 2016).

The fasciolid life span in humans is between 9 and 13.5 years, which suggests acute lesions by re-infections to occur superimposed on both latent and obstructive phases in human endemic areas (Chen and Mott, 1990; Valero et al., 2017). This very long life span underlies complications and sequelae in long-term chronicity (Mera y Sierra et al., 2011), even after successful treatment (Rondelaud et al., 2006).

Before the 1990s, clinical studies had mainly focused on the acute phase because human infection was mainly detected in industrialized countries in which patients attended health centers usually after symptom onset. The scenario changed in recent years because in rural areas of low income countries quick health care is not always available and most children infections are diagnosed in the chronic phase in surveys. Therefore, increasing studies are now focusing on the chronic phase, whether in animal models or infected patients. The long-term impact of chronicity has shown to lead to gallstone appearance, bacteriobilia (Valero et al., 2003, 2006), long-term liver function alterations (Valero et al., 2016) and also cirrhosis (Marcos et al., 2009). Worth mentioning results demonstrated that a high risk of anemia is to be expected in subjects with a heavy fluke burden, which is frequent in children in human hyperendemic areas (Valero et al., 2008).

During the intraorganic migration, immature flukes may deviate, enter other organs and cause ectopic fascioliasis. The most frequent ectopic lesions are those of the gastrointestinal tract. Other ectopic lesions are in: abdominal wall, pancreas, spleen, subcutaneous tissue, heart, blood vessels, the lung and pleural cavity, brain, skeletal muscle, appendix and epididymis. Such ectopic flukes almost never achieve maturity. The usual pathological effects of ectopic lesions are due to the migratory tracks causing tissue damage with inflammation and fibrosis (Mas-Coma et al., 2014a).

Neurofascioliasis (= intracranial infection by *Fasciola*) and ophthalmofascioliasis (=ocular affection by migrating flukes) may be rare, although not sporadic as previously believed. However, manifestations including many neurological symptoms, signs and syndromes, together with meningeal, psychiatric or neuropsychic manifestations, and ocular disorders caused at distance by flukes infecting the liver may be frequent but underestimated due to misdiagnosis (Mas-Coma et al., 2014a). Impressive clinical pictures include from hemiplegia and paraplegia to disturbances and difficulties of walking capacity, speech disorders, convulsions, epilepsy and coma, amnesia, or visual hallucinations and permanent blindness, among other manifestations. Moreover, there is a clinical complexity of puzzling polymorphisms, a disconcerting multifocality of the manifestations, and their changes along the evolution of the disease in a same patient, as well as differences between the clinical pictures shown by different patients. Additionally, these studies emphasized post-treatment sequelae and mortality in neurological patients (Mas-Coma et al., 2014a).

Numerous plasminogen-binding proteins enhancing plasmin generation, most of which cathepsins of well-known immunomodulating effects, were identified in the *F. hepatica* excretome/secretome (Gonzalez-Miguel et al., 2019). This was suggested to underlie blood-brain barrier leakage whether by many simultaneously migrating, small-sized juvenile flukes in the acute phase, or by breakage of encapsulating formations triggered by single worm in the chronic phase. Blood brain barrier leakages may subsequently occur due to a fibrinolytic-contact system-dependent mechanism involving plasmin-dependent generation of the proinflammatory peptide bradykinin, after different plasminogen-binding protein agglomeration waves, and in that way explain the complexity, heterogeneity and timely variations of neurological disorders caused in humans by fasciolids located in the liver (Gonzalez-Miguel et al., 2019).

Immunity

Fasciolids develop immune-modulation mechanisms which underlie their wide mammal host spectrum specificity.

In cattle the disease is self-limiting, most of the flukes are eliminated within 9–12 months, the duration of egg production is short and high egg output lasts for only a few weeks, and resistance is acquired during primary infection (Mas-Coma et al., 2020a). Nucleotide polymorphisms in given regions of the bovine genome have been recently found in cattle presenting resistance to *F. hepatica* (Twomey et al., 2019). In cattle, a mixed response with high levels of IL-10, TGF- β , IL-4 and IFN- γ occurs during the acute phase. Subsequently, Th2/Treg immune responses become progressively dominant. In the chronic phase, these Treg cells produce cytokines which cause inflammatory Th1/Th2 cytokine inhibition (Flynn et al., 2007). In sheep, the immune response is similar, including a mixed Th1/Th2 cytokine profile in the acute phase which later leads to increased Th2 cytokines instead of Th1 ones (Alvarez Rojas et al., 2015), with IL-10 dominating at spleen level and IL-5 in hepatic lymph nodes (Hacariz et al., 2009a; Pleasance et al., 2011a).

In sheep, there is no evidence of acquired immunity against *F. hepatica*, although resistance to *F. gigantica* is reported in Indonesian Thin Tail sheep (Pleasance et al., 2011b). The only breed in which similar susceptibility to both fasciolids has been reported is Guirra sheep (Valero et al., 2016). In sheep, egg output is relatively high (4000–50,000 eggs per fluke/day and 8800–25,100 eggs per host in weeks 13–19 post-infection). Thus, sheep may play a more important role in pasture contamination and consequently in disease transmission (Mas-Coma et al., 2020a).

In ruminants as in rodent model hosts, a potent regulatory/Th2-type response has been described to underlie Th1 immune suppression (Brady et al., 1999; O'Neill et al., 2000; Flynn and Mulcahy, 2008; Hacariz et al., 2009b; Pleasance et al., 2011b). The immune response in advanced chronic fascioliasis in the Wistar rat, in which experimental results proved to be reasonably extrapolable to humans, showed serum cytokine quantification to be at basal levels, with IgG1 as the predominant immunoglobulin isotype. There were no significant differences in T cell (CD3+, CD4+, and CD8a+), B cell (CD45R+), and macrophage (CD11b+) populations in the spleen between infected and noninfected rats, and the proliferation of splenic mononuclear cells in response to T and B mitogens was strongly inhibited. The predominant Th2 response in the early chronic infection subsequently decreased giving rise to a persistent immune suppression in the advanced chronic infection (Girones et al., 2007). It should be considered that, in human hyperendemic areas, children are always or almost always diagnosed in the advanced chronic phase of fascioliasis, and reinfection is frequent.

In the chronic phase characterized by a mixed Th2/Treg expression together with IFN- γ and associated Th17, reinfection is able to activate a mixed Th1/Th2/Th17/Treg immune response. However, T and B proliferation to mitogens is strongly suppressed in the advanced chronic phase independently of reinfection. Results suggested that suppression is mediated by different mechanisms, i.e. due to the parasite in primo-infected rats and rats reinfected at 8 weeks, and to the induction of suppressive cells such as Treg in rats reinfected at 12 weeks. In reinfection, two phenotypic patterns were detected: (i) one correlating with severe anemia which includes only increased splenic IFN- γ expression levels but no Treg expression; (ii) another correlating with a less severe anemia which includes increased splenic IFN- γ and Treg expression levels (Valero et al., 2017). In rats reinfected at 8 weeks and at 12 weeks, transiently higher averages of epg and epg/worm were detected in the weeks following reinfection. Such egg increases correlated with transient increases of IgG1 levels, systemic IL10 and thymic IFN- γ , and IL10 expression levels. This suggests that egg production depends on the Th1 and Treg phenotype, and means that fluke burden estimation by epg may likely be an overestimation in recent reinfection cases (Valero et al., 2020).

Fasciolids interact with several cells involved in innate immunity. CD11c⁺ dendritic cells have been seen to increase, with lower expression of co-stimulatory markers, MHC class II, increased expression of CCR5, and are hyporesponsive to TLR activation (Dalton et al., 2013). These dendritic cells increase intracellular IL-10, and the secretion of antigen specific IL-17 and IFN- γ becomes suppressed (Walsh et al., 2009). Regulatory/M2 phenotype macrophages are induced early in infection (Donnelly et al., 2008; Flynn et al., 2007). These macrophages are TLR ligand hyporesponsive and therefore suggest a low capacity to promote the differentiation of host Th1 immunity, and have proved to promote the polarization of Th2 cells (Donnelly et al., 2008). A mast cell number increase occurs at the infection site and gut mucosa (van Milligen et al., 1998; Vukman et al., 2013), which is thought to be involved in the reparation of the tissue damage caused by migratory fasciolid juveniles (Robinson et al., 2010). T cells are induced to enter an anergic state characterized by a decreased cytokine responses and reduced proliferative activity (Aldridge and O'Neill, 2016) and may explain why these cells become hyporesponsive to antigen stimulation in the chronic phase.

In coinfection with other pathogens, the synergistic capacity of fasciolids is well known, immune responses to pathogen antigens being markedly suppressed and concomitant infection being exacerbated. Protozoan and helminthic species, multi-parasitisms, and associations between infection by fasciolids and other parasites, are similar in inhabitants of the different human hyperendemic

areas (Esteban et al., 1997a,b, 2002, 2003; Gonzalez et al., 2011). These synergistic associations of fascioliasis with other pathogens may induce higher morbidity and even mortality rates (Mas-Coma, 2004).

Diagnosis

Alterations in biochemical markers, mainly liver function tests, and markers such as eosinophilia, are helpful initial indicators of an helminth infecting the liver. Radiology, radioisotope scanning, ultrasound, computed tomography and magnetic resonance may be additionally helpful (see reviews in Esteban et al., 1998 and Hillyer, 1999). Fluke adults and eggs may be found by invasive techniques, such as surgical interventions (laparotomy, cholecystectomy, sphincterotomy) or histological examination of liver and/or other organ biopsy materials (Mas-Coma et al., 1999b).

For the differential diagnosis of *F. hepatica* and *F. gigantica*, clinical, pathological, or immunological methods are useless. This is a problem in overlapping areas because of the different pathological, transmission and epidemiological characteristics of the two fasciolids, as well as due to intermediate forms in which egg measurements may overlap. Despite the recent development of many molecular tests, DNA marker sequencing still remains the best method for both pure fasciolid species haplotyping and detection of hybridization in intermediate forms (Mas-Coma et al., 2009a).

Stool and blood techniques, the main diagnostic tools, have been improved in the last two decades. Present availabilities for human diagnosis have recently been reviewed exhaustively, focusing on advantages and weaknesses, sample management, egg differentiation, qualitative and quantitative diagnosis, antibody and antigen detection, post-treatment monitoring, and post-control surveillance (Mas-Coma et al., 2014b). Main conclusions referred to the pronounced difficulties of diagnosing fascioliasis given the different infection phases and parasite migration capacities, clinical heterogeneity, immunological complexity, different epidemiological situations and transmission patterns, and the lack of a diagnostic technique covering all needs and situations (Mas-Coma et al., 2014b).

Direct and indirect techniques for human diagnosis

Fasciolid egg finding in human stool samples, but also endoscopic aspirates of duodenal or bile contents, is the most usual diagnostic strategy to qualitatively and quantitatively assess infection, despite egg absence during the acute period and sensitivity problems in chronic cases of very low intensity. Coprological techniques from a simple direct smear to different concentration methods may be used. Egg concentration has been achieved by flotation and sedimentation techniques, the latter being more accurate and sensitive (Esteban et al., 1998; Mas-Coma et al., 1999b, 2014b).

Quantitative coprological analyses are important in epidemiological surveys, post-treatment monitoring, and to decide the appropriate treatment dose to avoid colic risk in patients presenting with high burdens (WHO, 2007; Valero et al., 2012a). The Kato-Katz technique appears to be appropriate, because of its simplicity, very low cost, and reproducibility (Mas-Coma et al., 1999b). Its low sensitivity may be solved by repeated application.

Immunological techniques include mainly serological tests and a few whole blood tests based on immunological reactions. Numerous serological, intradermal and stool antigen detection tests have been developed. Immunological techniques present several advantages which solve problems posed by the parasitological diagnosis, such as: (i) its uselessness in the acute period because coprological examination is positive only after 3–4 months post-infection, (ii) intermittent egg output dynamics, (iii) very low or even absence of egg shedding in cases of only one or a few fluke adults and old, chronic infections, (iv) ectopic infections, (v) “false” fascioliasis related to eggs in transit after ingestion of infected liver from domestic animals, or (vi) flukes unable to attain maturity in human subjects in nonhuman endemic areas (Mas-Coma et al., 2014b).

However, immunological techniques are not able to differentiate between *F. hepatica* and *F. gigantica*. The negativization delay of serological antibody-detecting techniques also pose problems in post-treatment monitoring. Moreover, they are useless to assess fluke burden and consequently are not helpful in establishing the appropriate treatment dose, above all in human hyperendemic areas where high intensities are frequent. Additionally, several immunological techniques offer other types of problems related mainly to sensitivity and specificity (Valero et al., 2012c).

Cysteine proteinases offer highly sensitive and specific markers for human fascioliasis serodiagnosis (Fig. 8) (Mas-Coma et al., 2014b). *Fasciola hepatica* recombinant cysteine proteinases produced in yeast (O'Neill et al., 1999) or in *Escherichia coli* (Carnevale et al., 2001) have been used as antigens in ELISA methods.

The MM3 coproantigen-detection test allows for high sensitivity and specificity, fast large mass screening capacity, detection in the chronic period, early detection of treatment failure or reinfection in post-treated subjects, and usefulness for surveillance programs (Valero et al., 2012a). The use of a new preservative/diluent CoproGuard™, developed for preservation of *Fasciola* coproantigens, proved to enhance coproantigen extraction and antigenicity throughout the complete observation period (Ubeira et al., 2009). However, this technique falls short when evaluating the fluke burden on its own, and failed in a few cases of children shedding eggs (Valero et al., 2012a). This MM3 coproantigen-detection test has therefore recently been improved to increase its sensitivity (Martínez-Sernández et al., 2016).

A new lateral flow test (SeroFluke) for human diagnosis appears to be a useful step forward (Martínez-Sernández et al., 2011). In comparison with an ELISA test (MM3-SERO), SeroFluke showed maximal specificity and sensitivity and the advantage of being applicable to both serum or whole blood samples.

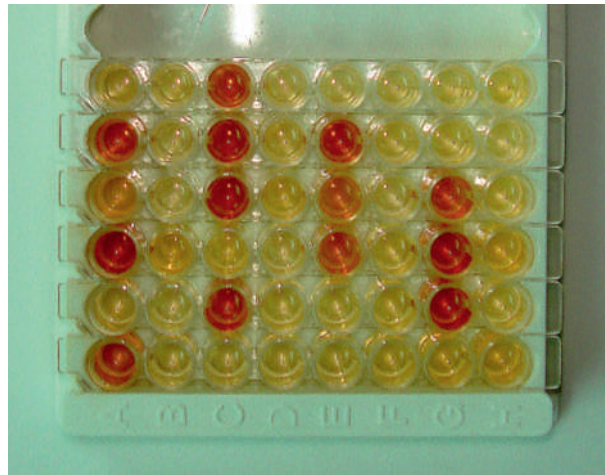


Fig. 8 Fascioliasis seropositive samples from hospital patients in Quy Nhon, central Vietnam. Photographs S. Mas-Coma.

In the exhaustive review of human fascioliasis diagnosis, it was concluded that there is at present no diagnostic technique covering all needs and situations and therefore an evident advisability for a combined use of different techniques, at least including a stool technique and a blood technique (Mas-Coma et al., 2014b).

Diagnosis in livestock

In livestock, diagnosis of animal infection, evaluation of drug efficacy and of drug resistance should be distinguished. A number of tests are available, including a few tests allowing for burden estimation by quantification by fecal egg counts, serological and coprological methods, egg hatch assays, molecular techniques, and even histological methods (Charlier et al., 2008; Fairweather et al., 2020). Specific reviews on diagnosis in sheep (Rojo-Vazquez et al., 2012) and cattle (Charlier et al., 2008) have recently been made.

A bulk tank milk ELISA has been used to survey for fluke prevalence in dairy herds across regions and across seasons (Byrne et al., 2018).

The coproantigen detection test is suitable for the diagnosis in sheep, cattle and deer, but not horses (Palmer et al., 2014).

Egg formation, production, development and viability are crucial in fascioliasis transmission and sensitive to drug action. A number of protocols or egg hatch tests have been developed to study drug action impact on the development and hatching of fasciolid eggs, but there is no standardized method (Fairweather et al., 2020).

Molecular methods, mainly targeting the mtDNA *cox1* gene or the nuclear rDNA ITS-2, have been proposed for the detection of *F. hepatica* in fecal samples from sheep and cattle, including the polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA). Such methods are however not easy to be applied in remote rural areas, mainly in front of simple and less expensive coprological egg detection.

Treatment

From the first half of last century, many drugs have been used to treat human fascioliasis. Emetine derivatives were used widely. Dehydroemetine, at a dose of 1 mg/kg daily for 10–14 days, was even considered the therapy of choice a few decades ago (Esteban et al., 1998).

Bithionol was proposed as the drug of choice during the last three decades of last century. It was applied at a dose of 30–50 mg/kg daily, divided into three oral doses on alternate days for 20–30 days. In cases of fascioliasis resistant to emetine and praziquantel (PZQ) treatment, bithionol achieved cure in dosages of 50 mg/kg daily for 10 alternate days or 40 mg/kg daily for 14–15 alternate days. Occasionally, the patients required a second course to obtain a complete cure (Chen and Mott, 1990; Esteban et al., 1998).

PZQ was widely applied during the 1980s and 1990s, because it is the drug of choice for human trematodiasis. However, controversial results were found in fascioliasis patients, including treatment failures even at high doses. Today, it is generally accepted that *Fasciola* may be the only trematode genus that has practically no response to PZQ (Esteban et al., 1998).

Among imidazole derivatives, cure rates of up to around 70% have been reported with albendazole, but triclabendazole (Egaten®) has become the drug of choice for human fascioliasis (Savioli et al., 1999; Gandhi et al., 2019). This drug is better adsorbed if administered after meals. The recommended regime is two doses of 10 mg/kg separated 12 or 24 h, with cure rates of around 80% after a first dose and higher than 95% after two doses. In patients shedding more than 400 eggs, absence of secondary

effects should be verified before second dose administration (WHO, 2007; Valero et al., 2012a). The need for a third dose has been reported sporadically.

The risk of triclabendazole resistance cannot be overlooked considering the veterinary use of triclabendazole (Fasinex®) for livestock treatment since long ago, the tradition of human self-treatments with Fasinex® owing to the very general availability of this drug, and the appearance of triclabendazole resistance in animals in different countries. So far, triclabendazole resistance only concerned a few patients infected in areas where resistance in animals was previously detected. Additionally, our understanding of the triclabendazole resistance mechanism remains far from complete, so that there is even a knowledge gap regarding its spreading capacity. A multigenic resistance origin has been suggested to underlie this resistance. Strategies to minimize the development of resistance include the use of synergistic drug combinations (Fairweather et al., 2020).

Nitazoxanide, a drug of broad parasitological spectrum, may be considered an alternative to triclabendazole, at least for chronic and low burden infections, in countries where Egaten® is still not registered but nitazoxanide is since several years (Zumaquero-Rios et al., 2013). Nitazoxanide demonstrated its efficacy in trials in Egypt and Peru. Its long 7-day treatment course is nevertheless a problem. However, its usefulness for human cases not responding to triclabendazole may be of additional value. Differences in fasciolid susceptibility to nitazoxanide may exist depending on geographical strains. Thus, no response to nitazoxanide treatment was reported in Cuba, and a triclabendazole-resistant *F. hepatica* infected patient not responding to nitazoxanide was reported in the Netherlands. Nitazoxanide proved, however, to be effective for the treatment of children in a human endemic area of Mexico where intensities are low (Zumaquero et al., 2013).

A number of existing flukicides for veterinary use are active against triclabendazole-resistant fasciolids, including albendazole, clorsulon, closantel, nitroxynil and oxiclozanide, although these alternatives do not act against migrating juveniles. Combinations of flukicides, anthelmintics and other drugs may be also useful (Vercruysse and Claerebout, 2001; Fairweather et al., 2020).

Prevention and control

Animal fascioliasis control efficiency depends on the correct and integrated application of: (i) reduction of parasite load of animal hosts and pasture contamination by regular strategic drug use (preventive treatment in appropriate year periods according to different regions); (ii) reduction of snail number by physical, chemical and biological means; (iii) reduction of infection risk through correct farm management practices (rotational system through fluke-infected and fluke-free paddocks, combined with effective treatment) (Fig. 9A and B) (Mas-Coma and Bargues, 1997; Fairweather et al., 2020).

Highly specific environmental DNA (eDNA) assays have been developed for the detection of *F. hepatica* and lymnaeid vectors in freshwater habitats (Jones et al., 2018; Rathinasamy et al., 2018). Such assays may be useful in farms, but their usefulness in open endemic areas pose many question marks, mainly where the lymnaeid vectors show a marked amphibious behavior.

Human infection was always related to animal endemics, so that prevention measures were traditionally the same to be applied at the levels of domestic animals, snails and field (Roberts and Suhardono, 1996; Torgerson and Claxton, 1999; Spithill et al., 1999). However, recent studies on human endemic areas have shown that epidemiological patterns of animal fascioliasis not always explain human infection characteristics.

The prevention of human infection may be achieved by strict control of the human infection sources in each place, mainly with regard to watercress and other metacercariae-carrying plants for human consumption (Mas-Coma et al., 2018). The possibility of human infection in urban areas should also be considered. Thanks to transport of vegetables (both aquatic and terrestrial) from rural zones to cities, plants carrying metacercariae can be sold in noncontrolled city markets (Fig. 10) (Mas-Coma et al., 2018). Additionally, commercial growing of watercress should be carried out under completely controlled conditions.

Education should always be included within general control measures in human endemic areas. The community should be appropriately informed about the disease, its transmission and danger, with emphasis on the human infection sources. The construction and utilization of the so-called “washing units,” in which water was appropriately filtered, gave rise to a marked decrease of human infection in an Egyptian locality (Mas-Coma, 2004; Mas-Coma et al., 2018).

In recent years, the availability of triclabendazole prompted WHO to launch a worldwide initiative against human fascioliasis (WHO, 2007, 2008). This initiative includes action in human endemic areas presenting different epidemiological situations and transmission patterns (Mas-Coma, 2005; Mas-Coma et al., 2009a). Pilot schemes were designed for the best control strategies according to local characteristics in Vietnam and Egypt. In Bolivia and Peru, pilot interventions demonstrated side effect absence in triclabendazole treatments of schoolchildren (Villegas et al., 2012), which subsequently allowed for preventive chemotherapy by annual mono-dose mass treatment campaigns. Many other countries are nowadays receiving yearly triclabendazole donations through WHO for the treatment of their patients.

In the Bolivian Altiplano, inter-annual treatment campaign monitorings detected infection and reinfection of children due to a high infection risk linked to the endemic maintained by livestock. A wide One Health action has therefore been launched to complement preventive chemotherapy, including control activities on: (i) environment and climate (Bargues et al., 2021), (ii) snail vector (Bargues et al., 2020), (iii) animal reservoirs (Mas-Coma et al., 2020a,b), (iv) human infection, and (v) social, tradition, diet, behavior and education factors (Mas-Coma et al., 2018). The ulterior purpose of this One Health action is to provide a guideline protocol applicable to other human endemic areas.

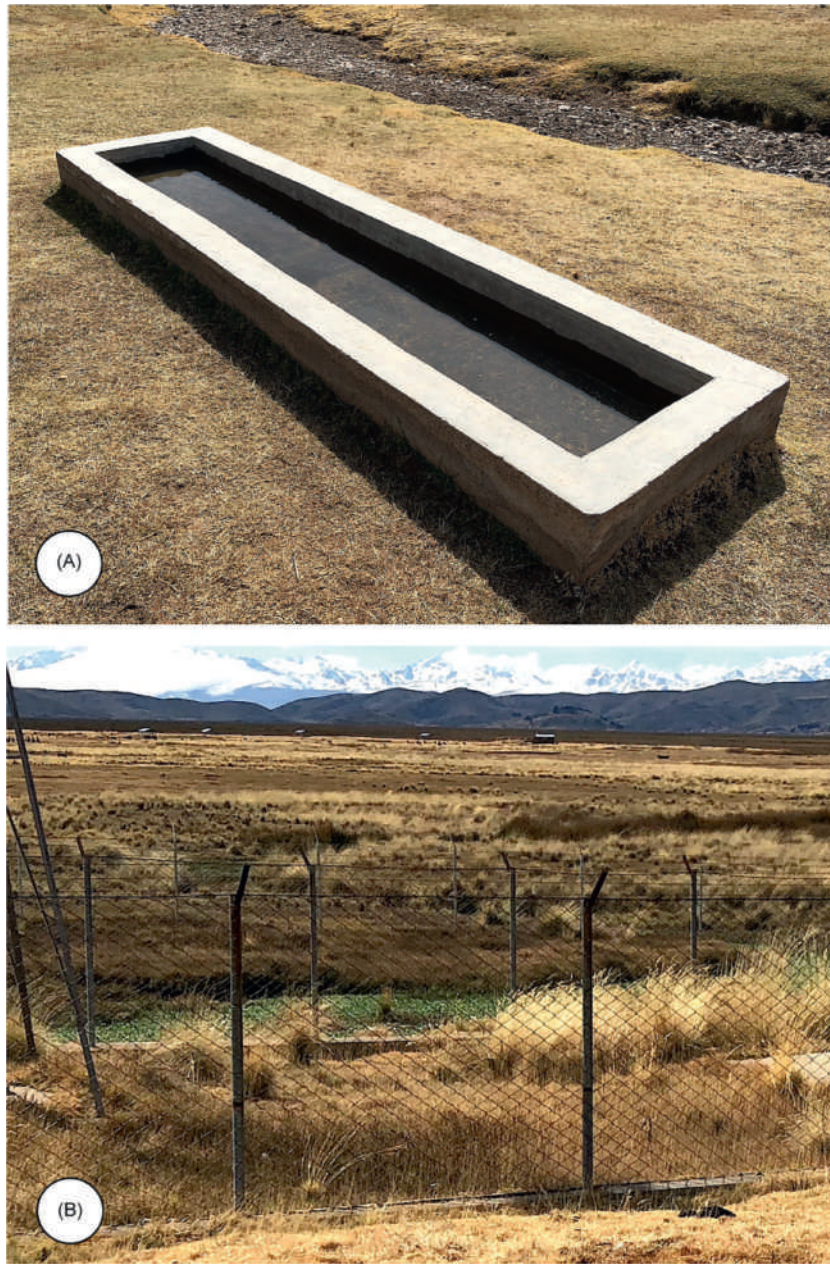


Fig. 9 Fascioliasis control measures affordable in poor-income rural areas of developing countries: (A) livestock artificial watering construction besides small stream in the Northern Bolivian Altiplano; (B) fences installed around *Fasciola hepatica* transmission focus inhabited by *Galba truncatula* besides hyperendemic village in the Northern Bolivian Altiplano. Photographs S. Mas-Coma.



Fig. 10 Edible vegetables sold in uncontrolled city market in Quy Nhon, Vietnam. Transport of sylvatic and cultivated vegetables carrying attached fasciolid metacercariae from the rural areas to cities facilitates urban fascioliasis to occur. Photographs S. Mas-Coma.

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Free Living Amoebas

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Introduction

Free living amoebae (FLA) are aerobic, mitochondriate, eukaryotic protists that are ubiquitous in nature and potentially infectious to humans (Schuster and Visvesvara, 2004; Visvesvara et al., 2007). The FLA are amphizoic in nature being able to reside as parasites within the host tissue (Page, 1988). They are commonly found in natural environment including air, water and soil (Edagawa et al., 2009; Huang and Hsu, 2010). They have also been isolated from drinking water, swimming pools, sewage, surgical instruments, dialysis units, eyewash solutions and contact lenses (Siddiqui and Khan, 2012a). Four genera of FLA including *Acanthamoeba*, *Naegleria*, *Balamuthia* and *Sappinia* are known to be pathogenic to humans (Schuster and Visvesvara, 2004; Visvesvara et al., 2007). These parasites have the potential to cause opportunistic infections among humans. The *Acanthamoeba* spp. is the causative agent of granulomatous amebic encephalitis (GAE) primarily among immunocompromised patients and *Acanthamoeba* keratitis (AK) among immunocompetent individuals (Alizadeh et al., 2014; Siddiqui and Khan, 2012a). *N. fowleri* causes primary amebic encephalitis (PAM) among healthy children and young adults (Cope and Ali, 2016). *Balamuthia mandrillaris* often causes fatal GAE and skin lesions among immunocompetent as well as immunocompromised patients (Matin et al., 2008; Bravo and Seas, 2012a,b). *Sappinia diploidea* has been reported in brain infection of a healthy man (Gelman et al., 2001).

Historical perspective

Amoebae are among the earliest eukaryotes that gained attention due to their role in causing fatal infections among humans. *Acanthamoeba* was discovered as a contaminant of yeast culture, *Cryptococcus parvulus* in Castellani (1930). In 1958, C. G. Culbertson demonstrated the concept that *Acanthamoeba* can cause disease among humans (Culbertson et al., 1958). While growing

poliomyelitis virus in monkey kidney cell cultures, Culbertson and his colleagues observed clear plaques in the control cell cultures. They inoculated experimental animals with the culture supernatant. They observed that the animals died with symptoms of meningoencephalitis. Culbertson isolated *Acanthamoeba* Lilly A-1 strain from the brains of dead animals. They later named this isolate as *Acanthamoeba culbertsoni*. Later, *Acanthamoeba* was described as the causative agent of GAE and keratitis infections in 1960s and 1970s, respectively (Castellani, 1930). In 1899, Schardinger discovered *Naegleria* and named it as "*Amoeba gruberi*." In 1912, Alexeieff renamed the genus as *Naegleria*. In later years, Rodney F. Carter and Malcolm Fowler reported first human case of PAM due to *N. fowleri* (De Jonckheere, 2002; Carter, 1972; Fowler and Carter, 1965). After that another amoeba *Balamuthia mandrillaris* was added to the list of free living amoebas. In the year 1986, free living amoeba *B. mandrillaris* was discovered from the brain tissue of a pregnant mandrill baboon (*Papio sphinx*) that died due to meningoencephalitis (Visvesvara et al., 1990, 1993). *Sappinia diploidea* is another free living amoeba that was identified as the causative agent of GAE in Gelman et al. (2001).

Taxonomic classification

The classical taxonomic classification divided Protozoa into four groups namely, Sarcodina (amoeba), Mastigophora (flagellates), Sporozoa (parasitic protozoa) and Infusoria (ciliates) (Visvesvara et al., 2007). However, later a new taxonomic classification based on the morphologic, biochemical and molecular approaches was put forth by the International Society of Protozoologists (Adl et al., 2005). As per this new proposal, the eukaryotes were placed under six clusters or "Super Groups," namely Amoebozoa, Opisthokonta, Rhizaria, Archaeplastida, Chromalveolata and Excavata. The four FLA were classified under two Super Groups, Amoebozoa and Excavata. *Acanthamoeba* and *Balamuthia* were classified under Super Group Amoebozoa: Acanthamoebidae. *N. fowleri* was classified under Super Group Excavata: Heterolobosia: Vahlkampfiidae. *Sappinia* was classified under Super Group Amoebozoa: Flabellinea: Thecamoebidae (Adl et al., 2005).

Acanthamoeba

Morphology

Acanthamoeba exists in two distinct morphological forms, viz., active trophozoites and dormant cysts. Trophozoites are actively feeding, dividing forms and their size ranges between 15 and 50 µm. They are uninucleate with a large, centrally placed nucleolus. The cytoplasm contains food vacuole, a contractile vacuole, mitochondria and ribosomes. They possess fine, tapering, surface structures called acanthopodia that are responsible for amoeboid movement (Khan, 2006). In the presence of unfavorable environmental conditions including food scarcity, desiccation or other environmental stresses amoeba transforms into a cyst i.e., encystation occurs (Khan and Tareen, 2003; Mazur et al., 1995). Cysts are double walled structures that can range from 10 to 25 µm. The outer cyst wall i.e., ectocyst is majorly made up of protein and lipid and gives wrinkled appearance. The inner cyst wall i.e., endocyst mostly contains cellulose that makes it periodic-acid Schiff positive and have smooth appearance (Visvesvara et al., 2007). Endocyst can be star, polygonal, oval or spherical in shape. Pores covered by opercula are present at the junction of ectocyst and endocyst. Cysts are also uninucleate and have a centrally placed nucleolus. Cysts are resistant to drying out, ultraviolet radiation, variable osmotic pressure, changes in humidity and can also survive at low temperatures (Khan, 2006). Upon favorable growth conditions, amoebae leave the cyst by dislodging the operculum and return to trophozoite form Fig. 1.

Genotypes of *Acanthamoeba* species

Earlier, *Acanthamoeba* spp. was identified based on their morphological characteristics such as shape and size of ecto- and endocyst walls as well as temperature required for their growth. But now-a-days, identification is primarily based on the use of molecular techniques (Khan, 2006). Till date, about 22 genotypes of *Acanthamoeba* (T1–T22) have been reported worldwide (Taher et al., 2018). The genotypes of *Acanthamoeba* are identified based on variation in the nucleotide sequences of the 18S rRNA gene using PCR assays (Behera et al., 2016; Schroeder et al., 2001; Booton et al., 2005). Some genotypes of *Acanthamoeba* have been associated with human infections. Genotypes T2–T6, T10, T11 and T15 have been associated with AK, while genotypes T1, T2, T4, T5, T10 and T12 have been associated with GAE (Behera et al., 2016). Various studies have shown that genotypes T3, T4, and T5 are highly

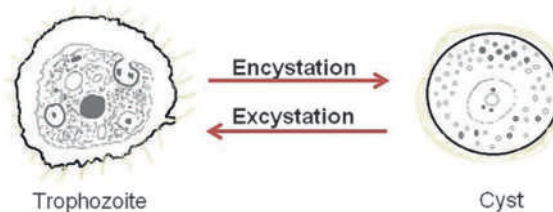


Fig. 1 Morphological structures of *Acanthamoeba*.

pathogenic to humans (Walochnik et al., 2000). Genotype T4 is the most common causative agent of AK and GAE worldwide that could be due to its greater virulence and enhanced transmissibility as well as its reduced susceptibility to chemotherapeutic agents (Ledee et al., 2009; Adamska et al., 2014). *Acanthamoeba* spp. has been commonly identified in natural environment as well. The pathogenic genotypes T4 and T12 have been frequently isolated from water samples (Haniloo et al., 2017; Lass et al., 2014; Gavarane et al., 2018). Genotype T15 has been isolated from moist, dry and aquatic samples (Basher et al., 2018). Recently identified genotypes are environmental genotype T19 (*Acanthamoeba micheli*), pathogenic genotype T20 and T21–T22 (Corsaro et al., 2015; Fuerst et al., 2015).

Human infections caused by *Acanthamoeba*

Acanthamoeba spp. causes infection among humans, with high mortality in spite of low incidence (Diaz, 2010). *Acanthamoeba* is a well-known causative agent of AK and GAE among immunocompetent as well as immunocompromised individuals (Kot et al., 2018a). AK is a severe vision threatening infection of the cornea (Lorenzo-Morales et al., 2015). It mostly occurs in contact lens wearers; however, infections have also been reported in non-contact lens wearers (Lorenzo-Morales et al., 2015). Common clinical symptoms associated with AK include redness, massive pain, tearing, photophobia, ring-like stromal infiltrate, epithelial defect and lid edema (Lorenzo-Morales et al., 2015). Lack of timely treatment of AK can lead to loss of vision (Clarke and Niederkorn, 2006; Marciano-Cabral and Cabral, 2003; Khan, 2009, 2006; Rocha-Azevedo et al., 2009; Visvesvara et al., 2007). The risk factors associated with AK include use of contact lens for extended period of time, inappropriate cleaning of contact lens, lack of personal hygiene, biofilm formation on contact lenses and exposure to contaminated water.

Acanthamoeba causes rare yet lethal infections among immunocompromised individuals known as GAE with mortality rate up to 98% (Abd et al., 2009). It usually occurs among patients with human immunodeficiency virus (HIV) infection, or among those who are on immunosuppressive drugs, antibiotics or chemotherapeutic treatment (Gonzalez et al., 1986; Carter et al., 2004; Vernon et al., 2005; Duarte et al., 2006). It has also been reported among immunocompetent individuals (Kalra et al., 2020). The symptoms of GAE usually start with headache, nausea, irritability, dizziness and low grade fever. Other neurological symptoms include, altered mental state, focal neurologic signs, diplopia, cranial nerve palsies, ataxia, high grade flaccid paralysis of right lower limb, lethargy, stiff neck and personality changes (Coven et al., 2017; Ghadage et al., 2017; Sutcu et al., 2018; Harrison et al., 2018).

Pathogenesis

As regards AK, the pathogenesis of infection starts with adhesion of pathogen to the surface of the host tissue (Panjwani, 2010). Several adhesins including mannose-binding protein, 28.2 and 55 kDa laminin-binding proteins have been identified in *Acanthamoeba* (Garate et al., 2004; Hong et al., 2004; Rocha-Azevedo et al., 2009). Various studies have revealed that the mannose-binding protein (400 kDa) of *Acanthamoeba* mediates its adhesion to the surface of the cornea (Garate et al., 2006a, 2004, 2005, 2006b). *Acanthamoeba* adheres to the mannose-containing glycoproteins of corneal epithelial cells that produce amoeba-induced cytopathic effects in mannose dependent manner (Cao et al., 1998). After mannose mediated adhesion, amoeba secretes enzymes such as neuraminidase and proteases that lead to colonization of the parasite and degradation of basal membranes, respectively (Siddiqui and Khan, 2012a; Lorenzo-Morales et al., 2015). Serine proteases induce degradation of keratocytes, retinal pigment epithelium, corneal epithelium and endothelial cells (Lorenzo-Morales et al., 2015). Proteases cause damage in the cornea stroma with inflammatory reactions, oedema and necrosis (Kot et al., 2018b).

The pathogenesis of GAE is not fully understood. Trophozoites of *Acanthamoeba* migrate to the CNS through the nasal mucous membrane, endothelium of the brain capillaries and the ethmoid bone along the olfactory nerves (Khan and Siddiqui, 2011).

Host immune response

Infection due to *Acanthamoeba* induces both innate and adaptive immunity in the host (Cano et al., 2017). Toll like receptors (TLRs) play an important role in recognizing *Acanthamoeba* spp. infection and inducing cytokine production in the host (Ren and Wu, 2011). Studies have reported increased mRNA expression of TLR2 and TLR4 in the lungs and brain of mice and cornea of hamsters infected with *Acanthamoeba* (Derda et al., 2016; Wojtkowiak-Giera et al., 2016; Alizadeh et al., 2014). The complement cascade shows activity against protozoa; however, it is not fully understood in *Acanthamoeba*. The in vitro studies have demonstrated lytic activity against *Acanthamoeba* spp. in the presence of phagocytes (Ferrante and Rowan-Kelly, 1983). However, trophozoites of *Acanthamoeba* express regulatory proteins which inhibit the complement cascade (Toney and Marciano-Cabral, 1998). The secretions of pro-inflammatory cytokines like TNF- α , IL6 from monocytes and macrophages activate neutrophils and vascular endothelial cells (van Klink et al., 1993; Benedetto et al., 2003; Stewart et al., 1994). It has been shown that TNF- α can induce the encystation of *Acanthamoeba* leading to the resistance towards phagocytosis (Mattana et al., 2016). In vivo studies demonstrated the presence of neutrophils in the brain and retina of the eye, which may inhibit the spread of invasion to other organs of the body (Harrison et al., 2010). Significant increase of natural killer cells has also been observed in mice infected with *Acanthamoeba* (Kim et al., 2012). Animal studies have been conducted to study the adaptive immune response against *Acanthamoeba*. T helper lymphocytes and regulatory T lymphocytes were observed in the cornea and atopic lymph nodes of AK mouse model. A study demonstrated that *Acanthamoeba* spp. also induces IL-17A, which relieves the symptoms of ocular infection (Suryawanshi et al., 2015). Recent study demonstrated that *Acanthamoeba* spp. induced expression of mediators such as cyclooxygenase-1 (COX-1) and

COX-2 proteins in the lungs of immunocompetent mice but a decrease in immunocompromised mice (Lanocha-Arendarczyk et al., 2018).

Diagnosis

Diagnosis of AK is often challenging as it can be easily confused with viral or fungal keratitis (Lorenzo-Morales et al., 2015; Page and Mathers, 2013; He et al., 2016). Early diagnosis is the key to a good prognosis. Samples for diagnosis of AK include corneal scraping or biopsy and contact lenses, which are stored in sterile saline (PBS or 0.9% NaCl) in order to prevent its desiccation (Lorenzo-Morales et al., 2015). *Acanthamoeba* trophozoites and cysts can be stained using silver staining, lacto phenol cotton blue, acridine orange, calcofluor white, haematoxylin and eosin (HE) and Immuno stains (Lorenzo-Morales et al., 2015). Samples with high density of *Acanthamoeba* can be detected by direct microscopy (Winchester et al., 1995). However, noninvasive and highly sensitive confocal microscopy (CF) is the preferred diagnostic technique with specificity and sensitivity higher than 90% (Winchester et al., 1995). The advantage of CF is that it can image layers within the cornea (Winchester et al., 1995; Vaddavalli et al., 2011). The trophozoites or cysts can also be readily recognized using phase contrast microscopy in which cysts appear as hyper-reflective, spherical structures of double wall (Lorenzo-Morales et al., 2015). CF and clinical suspicion can aid in presumptive diagnosis; however, definitive diagnosis of AK is based on in vitro culture, histology or molecular methods. Culture based identification of *Acanthamoeba* involves inoculation of sample on 1.5% non-nutrient agar (NNA) medium seeded with heat inactivated *Escherichia coli*. Inoculated plates are incubated at 30 °C and screened daily for amoebae using inverted microscopy. In high density samples, amoebae can be easily visible after 24–48 h of incubation. Samples should be observed for at least 1 week to give a negative result. Sub-culturing of positive samples can be done by transferring small piece of agar with few cysts on it upside down to a fresh plate (Lorenzo-Morales et al., 2015). *Acanthamoeba* can also be cultured axenically in peptone-yeast extract glucose (PYG) media. Molecular methods including polymerase chain reaction (PCR) and multiplex real-time PCR methods have been extensively used for the diagnosis of AK (Lehmann et al., 1998; Schroeder et al., 2001; Khan, 2006; Siddiqui and Khan, 2012a). In addition, matrix assisted laser desorption-ionization time-of-flight mass spectrometry and ¹H NMR (Nuclear magnetic resonance) spectroscopy has the potential to identify *Acanthamoeba* in the clinical samples (Siddiqui and Khan, 2012a).

Brain image analysis based on computed tomography (CT) and magnetic resonance imaging (MRI) is often adopted for diagnosis of GAE (Schumacher et al., 1995). Microscopic examination of cerebrospinal fluid (CSF) can be done; however, trophozoites can be easily confused with monocytes, polymorphonuclear leucocytes and macrophages (Alkhunaizi et al., 2013). *Acanthamoeba* can be cultured axenically as well as xenically by inoculating CSF/brain biopsy sample in PYG and 1.5% NNA media, respectively (Duggal et al., 2017). Histopathological examination of frozen and paraffin-embedded brain biopsy tissue can be done using Giemsa-Wright, HE, acridine orange or calcofluor white stain to identify trophozoites or cysts of *Acanthamoeba* (Kalra et al., 2020). Molecular techniques are being used for confirmative diagnosis of GAE due to *Acanthamoeba* spp. (Walochnik et al., 2015; Qvarnstrom et al., 2006).

Naegleria

Morphology

Naegleria is a free living, pathogenic amoeba. It exists in three distinct phenotypes namely, the resting cyst, a swimming flagellate, and the active multiplying trophozoites depending on the environmental conditions (Siddiqui et al., 2016). Under favorable conditions, reproductively active trophozoites of size 22 µm in length and 7 µm exists (Siddiqui et al., 2016). The trophozoites have a large nucleus with nucleolus, mitochondria, food vacuoles, contractile vacuole, endoplasmic reticulum, ribosome and membrane-bound cytoplasmic organelles. The locomotory rate of trophozoites is 45 µm/min at 37 °C (Carter, 1970). The flagellate stage exists in non-nutrient stage; but water is present (Siddiqui et al., 2016). The flagellates are pear-shaped and possess two flagella of equal length, a nucleus in the narrower anterior region and cytostome is absent. The cysts are formed in the presence of adverse or unfavorable environmental conditions. The size of cyst usually ranges between 7 and 15 µm in diameter (Maciver et al., 2020). Each cyst possess one to two mucoid-plugged pores, through which trophozoites emerge (John, 1982). *Naegleria* feeds on bacteria, algae and yeast, and can tolerate temperatures up to 45 °C (Yoder et al., 2012; Visvesvara et al., 2007).

Genotypes of *Naegleria*

The genus *Naegleria* consists of 47 pathogenic as well as non-pathogenic species that are often found in aquatic environment and soil habitats (De Jonckheere, 2002). Internal transcribed spacer (ITS) sequence of ribosomal RNA (rRNA) gene has been used to classify species of *Naegleria* (De Jonckheere, 2002, 2004). Among all the species, *N. fowleri* is pathogenic to humans (Siddiqui et al., 2016). Genome of *N. fowleri* is diploid (66 MB) and the haploid genome size is 29.62 MB (Zysset-Burri et al., 2014). The nuclear DNA of *Naegleria* is about 0.2–0.3 pg/amoeba (Byers, 1986).

Primary amebic meningoencephalitis (PAM)

The causative agent of this fatal infection of brain is *N. fowleri*. PAM is a rare yet fatal human disease with mortality rate of 95–97% (Baig and Khan, 2014). It occurs among immunocompetent children, young adults as well as immunocompromised patients and causes lethal, necrotizing, fulminant and hemorrhagic meningoencephalitis among them (Gupta et al., 2015). The incubation period after exposure to the parasite varies from 1 to 16 days. The symptoms include sore throat, fever, blocked nose, headache, stiff neck, altered taste and smell perception, changed mental status, seizures and coma that results in fatality (Siddiqui et al., 2016; Siddiqui and Khan, 2012b; Capewell et al., 2015). Clinically, it can be easily confused with bacterial meningitis due to similarity in their symptoms (Jain et al., 2002). Till date, around 431 cases of PAM have been reported worldwide (Maciver et al., 2020). *N. fowleri* is commonly present in hot springs, ponds, freshwater lakes, rivers, untreated and under treated water supplies and untreated swimming pools (Ong et al., 2017; Miller et al., 2018). PAM occurs in individuals who have been exposed to contaminated water through recreational activities such as swimming, diving or water skiing in recent past (Siddiqui et al., 2016; Grace et al., 2015).

Pathogenesis

N. fowleri enters the human host through the nose after exposure to contaminated water. The parasite is splashed or forced through the nasal cavity. It attaches to the nasal mucosa, followed by locomotion through the olfactory nerve and cribriform plate to gain entry to the brain (Siddiqui et al., 2016). The protist reaches the olfactory bulb within the CNS and causes hemorrhage and inflammation (Jarolim et al., 2000). Leptomeninges become congested, diffusely hyperaemic with limited infiltration and focal demyelination in spinal cord and white matter of the brain has been observed (Viriyavejakul et al., 1997). Virulence factors associated with the pathogenesis of *N. fowleri* are broadly categorized into contact dependent and contact independent mechanisms (Siddiqui et al., 2016). Contact dependent mechanisms include a fibronectin binding protein (60 kDa) and protein kinase C, which have been reported in *N. fowleri*. These play a role in binding to and cytotoxicity of host cells (Han et al., 2004). After binding to host cells, sucker-like surface structure protruding from the surface of *N. fowleri* aids in gradual consumption of target cells. Phagocytosis and amoebastome lead to *N. fowleri* mediated host cell damage (Tiewcharoen et al., 2014). Contact-independent mechanisms based on pore forming proteins (N-PFP) and hydrolytic enzymes contribute to the pathogenicity of *N. fowleri*. *N. fowleri* releases phospholipases, sphingomyelinase, lysophospholipase which cause damage to lipid-rich cytoplasmic membrane of the cell and demyelinate nerve tissue (Ferrante and Bates, 1988).

Host immune response

Upon exposure to *N. fowleri*, activation of innate defence mechanism occurs in the respiratory epithelial cells. This activation broadly includes the inflammation (IL-8 and IL-1 β) and mucin secretion by the production of reactive oxygen species (Cervantes-Sandoval et al., 2009). Mucin inhibits the binding of *N. fowleri* to cells and cytotoxicity in vitro and in vivo. Neutrophils and eosinophils have been observed in later stages of infection. Knockout mice experiments have demonstrated that host polymorphonuclear (PMN) cell lysis action and inflammatory response leads to the damage of CNS tissues (Siddiqui et al., 2016). In vitro study on interaction of PMNs with trophozoites of *N. fowleri* demonstrated that trophozoites induced formation of neutrophils extracellular traps that inactivate and catch the pathogen (Contis-Montes de Oca et al., 2016). The activated macrophages such as Microglial cells act against the invasion of CNS by *N. fowleri* (Vyas et al., 2015). They induce production of nitric oxide and arginine-dependent cytolytic mechanism to kill *N. fowleri*.

Diagnosis

The diagnosis of PAM is difficult as it follows a rapid course. Usually diagnosis is made on histopathological examination of tissue obtained post-mortem. A high suspicion of PAM is deemed after CSF culture is negative for bacteria. CSF sample collection may cause brain herniation due to high intracranial pressure in patients with PAM (Visvesvara, 2013). Nasal secretions from the primary site of infection can be collected for the identification of *N. fowleri* (Baig and Khan, 2015). Wet mount preparations may show motile trophozoites. Trophozoites of *N. fowleri* have also been identified in the CSF using trichrome or Giemsa staining (Visvesvara and Maguire, 2006). *N. fowleri* has been identified in the culture of CSF and nasal secretions of PAM patients (Singh et al., 1998). Molecular techniques such as PCR and nested PCR have been used to identify *N. fowleri* in cultured amoebae from patients (Marciano-Cabral and Cabral, 2003; Zhou et al., 2003). Real time multiplex PCR has been used to identify *N. fowleri* in the CSF and brain tissue samples from PAM patients ante mortem (Qvarnstrom et al., 2006).

Balamuthia mandrillaris

Morphology

B. mandrillaris exists in two distinct morphological structures including a vegetative trophozoite and a dormant cyst. The size of trophozoites varies from 12 to 60 μ m in diameter. They are uninucleate; however, binucleate forms have also been reported

(Visvesvara et al., 2007). The nucleus contains two or three large, centrally placed nucleoli and cytoplasm contains numerous mitochondria, ribosomes and endoplasmic reticulum. Trophozoites reproduce asexually by binary fission and also form pseudopodia (Jayasekera et al., 2004, 2005; Schuster and Visvesvara, 2004). Under hard conditions such as extremes in pH, temperatures and lack of food, trophozoites transform into a cyst stage. The cysts are uninucleate, more or less spherical and their size varies between 12 and 30 μm . Cysts of *B. mandrillaris* have three layers including an outer, irregular ectocyst, a thick endocyst and a middle nebulous mesocyst (Visvesvara et al., 2007). Both forms of *B. mandrillaris* can enter the human body through nasal passages to the lower respiratory tract and wounded skin Fig. 2.

Granulomatous amebic encephalitis

B. mandrillaris is one of the possible causative agents of GAE among both immunocompetent and immunocompromised patients (Siddiqui and Khan, 2008). GAE due to *B. mandrillaris* clinically resembles viral or bacterial meningitis, leptomeningitis and tuberculous meningitis (Siddiqui and Khan, 2008). The clinical symptoms include fever, headache, skin lesions, nausea, vomiting, acute confused state, photophobia, cranial nerve palsies, increased intracranial pressure, seizures, brain edema and death (Jayasekera et al., 2004; Schuster and Visvesvara, 2004). Brain images of patients with GAE caused by *B. mandrillaris* demonstrate ring enhancing lesions (Siddiqui and Khan, 2008). GAE due to *B. mandrillaris* has a slow onset, which takes months and years to develop into a sub-acute and chronic disease. However, organ transplant recipients have a rapid clinical course (Farnon et al., 2016). Till date, approximately 200 cases of *B. mandrillaris* amebic encephalitis have been reported worldwide (Takei et al., 2018). Males are 2.5 times more susceptible to this infection than females as they are more exposed to soil (Diaz, 2011). Patients with HIV/AIDS, diabetes mellitus, liver disease or cancer and those on immunosuppressive drugs, and alcoholics are at high risk of infection (Takei et al., 2018; Siddiqui and Khan, 2008). Contaminated soil is the major risk factor for contracting GAE (Dunnebacke et al., 2004; Schuster et al., 2003).

Pathogenesis of GAE caused by *Balamuthia mandrillaris*

It is not completely understood. *B. mandrillaris* colonizes the skin lesions or nasopharynx and enter into the intravascular space. These parasites then survive within the blood stream and invade the CNS. This leads to brain tissue damage and ultimately meningoencephalitis (Siddiqui and Khan, 2008). Amoeba can invade the nasal mucosa and migrate along the nerve fibers, followed by invasion of the olfactory bulb (Kiderlen and Laube, 2004). Amoebae invade the CNS at the site of blood brain barrier (Schuster and Visvesvara, 2004). Therefore, *B. mandrillaris* adhesions, protease activity, blood brain barrier injury or host inflammatory response can disrupt the blood-brain barrier. *B. mandrillaris* through galactose binding protein on their surface binds to the human brain microvascular endothelial cells (Matin et al., 2006). The pro-inflammatory cytokines like TNF- α , IL-1 along with IL-6, IL-8 and granulocyte/macrophage colony stimulating factor increase the permeability of blood-brain barrier (Leib and Tauber, 1999). Studies have reported that *B. mandrillaris* exhibits proteolytic activities to degrade extracellular matrix that leads to the invasion of and growth in the brain tissue (Matin et al., 2006; Rocha-Azevedo et al., 2007).

Immune response

Antibodies (IgG and IgM) with titres ranging from 1:64 to 1:256 against *Balamuthia* have been identified in the sera of healthy individuals as well those with *Balamuthia* GAE (Visvesvara, 2010; Visvesvara et al., 2007). The presence of antibody in humans could be due to the exposure to these ubiquitous amoebas in the environment (Visvesvara, 2010).

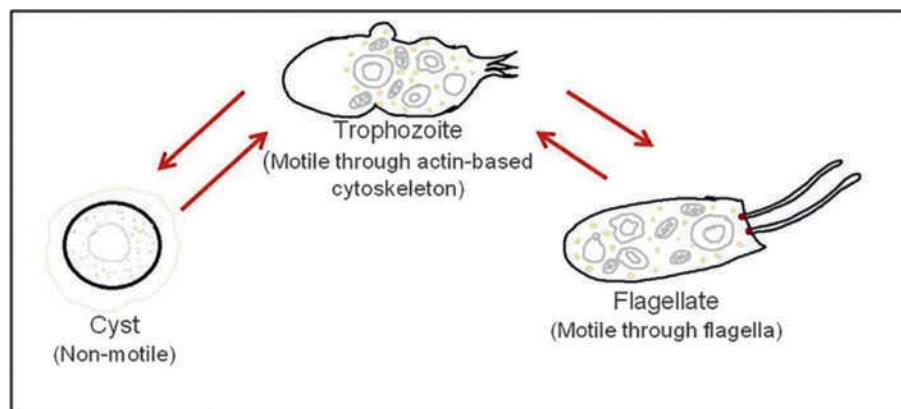


Fig. 2 Life cycle of *Balamuthia mandrillaris*.

Diagnosis

Diagnosis of GAE caused by *B. mandrillaris* is challenging as the symptoms of the infection may be non-specific. In patients from Peru, skin lesions especially the central face plaque (nose lesion) is the best clue for diagnosing *Balamuthia* GAE (Bravo et al., 2011). Neuroimaging findings based on CT and MRI are often non-specific and reflect lesions with surrounding edema (Lorenzo-Morales et al., 2013a). However, evidence of intralésional hemorrhage is an important clue for diagnosis (Visvesvara et al., 2007; Kiderlen et al., 2008). *B. mandrillaris* can be detected in brain or skin biopsies either by immunofluorescence or immunoperoxidase staining with rabbit anti-*Balamuthia* sera in paraffin-fixed tissue (Visvesvara et al., 2007; Guarner et al., 2007). However, these tests are available at CDC, United States. Other serological tests such as ELISA and flow cytometry assays are there; however, it requires careful interpretation as healthy individuals may have positive serology. Molecular techniques including PCR, real-time multiplex PCR have been used to identify *Balamuthia* DNA in human CSF and brain tissue samples (Tavares et al., 2006; Yagi et al., 2005; Qvarnstrom et al., 2006; Schuster et al., 2003; Niyiyati et al., 2009).

Sappinia pedata

Sappinia are coprozoic amoebas that exist as a feeding trophozoite and a dormant cyst form. The trophozoites are ovoid or oblong with occasional wrinkles on their surface. Their size varies from 40 to 80 µm. Trophozoites are binucleate with two tightly opposed nuclei and its cytoplasm contains food vacuoles and a contractile vacuole (Visvesvara, 2013). The cysts are round in shape and size measures 15 to 30 µm in diameter. They are also binucleate with two tightly opposed nuclei. The first and only case of amebic encephalitis caused by *S. pedata* (earlier identified as *S. diploidea*) was diagnosed based on the nuclear morphology of the amoeba (Gelman et al., 2001). GAE due to *S. pedata* occurred in an immunocompetent individual who presented with nausea, vomiting, bifrontal headache, photophobia, and blurry vision that lasted for 2–3 days. MRI findings revealed a 2 cm mass in the posterior left temporal lobe with ring enhancement. Microscopic examination of the excised mass showed necrotizing hemorrhagic inflammation containing amebic trophozoites with double nuclei (Gelman et al., 2001). *Sappinia* spp. can be identified by molecular techniques including PCR and real-time PCR.

Treatment

Treatment of *Acanthamoeba* keratitis

As of now, no single drug can be used to eradicate both cystic or trophozoite forms. Trophozoites are more susceptible to elimination by drugs (Lorenzo-Morales et al., 2013b). Treatment of AK relies upon topical agents like biguanides and chlorhexidine in combination with aromatic diamidines. Both biguanides and chlorhexidine increase the cytoplasmic membrane permeability. These drugs are positively charged and bind negatively charged phospholipids of cellular membrane leading to cell lysis. Biguanides like polyhexamethylene biguanide or PHMB, though effective at low concentrations (0.02%), may cause toxic keratopathy. Chlorhexidine (0.02%) with diamidines like 0.1% propamidine isethionate, 0.15% dibromopropamidine, hexamidine 0.1% have also shown good results (Lorenzo-Morales et al., 2015; Roberts and Henriquez, 2010). These topical agents are given after corneal debridement for several days. Treatment duration can extend up to 3–4 weeks. Corneal transplantation may be done if treatment fails and cornea has been damaged in acute phase of infection (Lorenzo-Morales et al., 2013b, 2015). Therapeutic penetrating keratoplasty may be considered when infection involves paracentral cornea despite antiamebic therapy (Cohen et al., 1987; Hamburg and De Jonckheere, 1980). Medical treatment should be continued even after surgery for several months to ensure successful elimination of *Acanthamoeba*. In such cases, prognosis is poor (Lorenzo-Morales et al., 2015). In addition to the current therapeutic approach, various researchers are also investigating the effectiveness of nanoparticle conjugated drug against *Acanthamoeba* (Sharma et al., 2020).

Treatment of Granulomatous amebic encephalitis due to *Acanthamoeba* species, *Balamuthia mandrillaris* and *Sappinia pedata*

The successful treatment relies upon an early diagnosis. Usually, skin lesions precede CNS symptoms by weeks to months. The combination of surgical resection of lesions and multiple antibiotics like pentamidine, cotrimoxazole, propamidine isethionate, azoles like fluconazole, itraconazole and voriconazole, amphotericin B, flucytosine, rifampin, azithromycin, amikacin have been tried. In >90% of successfully treated cases of GAE, miltefosine, azoles, pentamidine, and cotrimoxazole were used. A major concern is side effects of pentamidine and low permeability of pentamidine among HIV infected patients (Orozco et al., 2011; Parija et al., 2015).

Treatment of primary amebic meningoencephalitis due to *Naegleria fowleri*

No clinical trials have been conducted to assess the treatment options among humans. The most commonly used agent for treatment of PAM is Amphotericin B. Other agents mentioned in literature are fluconazole, miconazole, azithromycin, miltefosine and rifampin (Kim et al., 2008; Goswick and Brenner, 2003). Amphotericin B is given intravenously or intrathecally. Centers for

Table 1 Therapeutic management of patients diagnosed with *Acanthamoeba* keratitis, primary amebic meningoencephalitis and granulomatous amebic encephalitis.

Disease entity	Medical treatment	Dose	Duration
<i>Acanthamoeba</i> keratitis			
<i>Acanthamoeba</i> species	Biguanides like polyhexamethylene biguanide (0.02%) and chlorhexidine (0.02%) in combination with diamidines like propamidine isethionate (0.1%) and hexamidine isethionate Antifungals like ketoconazole given topically in a concentration of (1%) in combination with biguanides If topical therapy fails, keratoplasty or corneal transplantation Novel agents like alkylphosphocholines e.g., Miltefosine	Topical therapy after corneal debridement: Chlorhexidine and propamidine Given hourly for initial 3 days than 3 hourly	For a total of 3–4 weeks to 6–12 months
Primary amebic meningoencephalitis			
<i>Naegleria fowleri</i>	Conventional Amphotericin B	Given intravenously in a dose of 0.25–1.5 mg/kg/day in adults and 0.5–0.7 mg/kg/day in children in two divided doses for 3 days followed by 1 mg/kg/day once daily for 11 days OR Intrathecal in a dose of 1.5 mg/kg/day for 2 days followed by 1 mg/kg/day for 8 days	Intravenous regimen for 14 days and intrathecally for 10 days
	Miltefosine	50 mg orally two to three times daily with maximum dose of 1.5 mg/kg/day	28 days
	Adjunctive therapy with Fluconazole	Given in combination with Amphotericin B Intravenously in a dose of 10 mg/kg/day once daily	28 days
Granulomatous amebic encephalitis			References
<i>Acanthamoeba</i> spp.	Multiple antibiotics given in combination	Successful treatment has been done with combination of drugs like rifampicin, cotrimoxazole, fluconazole, albendazole and ceftriaxone	Khanna et al. (2014)
<i>Balamuthia mandrillaris</i>		Prolonged therapy (upto years) with combination of agents like Pentamidine, Miltefosine, fluconazole, flucytosine and albendazole, sulfadiazine has been tried successfully	Vollmer and Glaser (2016)
<i>Sappinia diploidea</i>		Azithromycin, Pentamidine, Itraconazole and Flucytosine have been used successfully	Gelman et al. (2001)

Disease Control and Prevention (CDC) recommends use of conventional amphotericin B 1.5 mg/kg/day in two divided doses intravenously for 3 days that is followed by 1 mg/kg/day once daily for next 11 days. Intrathecal regimen consists of 10 days of treatment with conventional amphotericin B (CDC, 2014). Another drug recommended by CDC for treatment of infections caused by FLA including *N. fowleri*, *Acanthamoeba* species and *B. mandrillaris* is miltefosine. The recommended dose is 50 mg orally two to three times daily for a total of 28 days (CDC, 2013) Table 1.

Prevention

The predominant risk factors associated with AK infection includes contact lenses and lens care solution. Therefore, contact lenses and lens care solutions should be kept clean. Individuals should remove contact lenses while swimming and performing other water activities. Soap based hand washing should be performed before handling contact lenses. Periodic inspection of hot water tanks, Jacuzzis, filters used in heating, ventilating and air conditioning units is strongly recommended as *Acanthamoeba* can easily grow and colonize them. *N. fowleri* proliferates in less chlorinated water at ambient temperature above 30 °C. Therefore, chlorine levels should be maintained in the water of swimming pools especially in summers. Untreated water should be avoided for nasal rinsing. Cases of *Balamuthia* have been associated with contamination of wound after contact with soil.

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General Aspects of Helminths

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Introduction

The term helminths (from the Greek helmins that simply means worm) includes Annelida, Acanthocephala, Platyhelminthes, Nematoda, and Pentastomida, parasites extremely varied as far as anatomical organization, and very different from the phylogenetic point of view (Melhorn, 2001). However the term, nevertheless bit aspecific, is very useful and used as it encloses in a single word many species of medical and veterinary interest.

Differently from Protozoa (unicellular eukaryotes in which each cell has to be able of survive, live and reproduce), helminths are pluricellular eukaryotes (Metazoa) in which the many cells are well organized in a highly integrated functioning, with the development of fully specialized organs. Differently from Protozoa and like all macroparasites, helminths are characterized by the following features: have long time of sexual maturation; reproduce giving progeny not similar to itself, but disseminative elements often inactive; their number in the host do not increase (except in opportunistic species), being each individual the result of a new infection; remain in the host many years but, as they do not elicit permanent immunity, reinfections are possible; tend to be overdistributed in the population: most subjects have a low parasitic burden, and few individuals harbor many specimens, as effect of environmental, behavioral, and parasitic factors, but also a feature partially under genetic control and related to specific chromosomal regions (Williams-Blangero et al., 2002).

As proven by paleoparasitology, systematics, biology, biochemical and molecular studies, the mankind faces helminths from prehistory to present age. Therefore, the knowledge of helminth fauna throughout the years, largely recorded around the world, is allowing progress toward understanding changes occurred during the late Pliocene and Quaternary (as episodic shifts in climate, habitat, and ecological structure), and parasite relationships to global public health (Hoberg et al., 2012; Zarlenga, 2014). For example, a recent review about the origin of human parasites (Araújo et al., 2013) denied the common knowledge that intestinal helminths have been introduced to America by African slaves: they already existed in American prehistoric populations (Fornaciari and Gaeta, 2014).

The medically most important species belong to two phyla: Platyhelminthes (flatworms) and Nemathelminthes (roundworms), which infect approximately 25% of the world population mostly living in developing areas of sub-Saharan Africa, Asia, and the Americas. Here, one-third of the almost 3 billion people are parasitized by one or more species, which are responsible for substantial morbidity to 100–150 million people, and for more than 14 million of disability-adjusted life years due to disability plus mortality (Global Burden of Disease 2010 Project) (GBD; WHO, 2008). Therefore, helminths have an enormous impact on the life quality, mainly considering the many chronic and disabling infections by them induced. Finally, they affect also livestock, worsening malnutrition in poor countries and allowing the establishment of further parasitic diseases. Notwithstanding their impact on the health, helminth diseases continue to be among the neglected tropical diseases and, despite all medical and veterinary interventions, they remain a substantial problem for people, their domestic food animals, and wild vertebrates (Fenwick, 2012).

Among the 75,000–300,000 species of helminths that globally infect terrestrial and aquatic vertebrates (Dobson et al., 2008), about 300 are reported in humans (Cleaveland et al., 2001; Ashford and Crewe 2003; Kuris 2012), with 83 as prevalent. Apart from

many species that require further elucidation, only 39 species are considered anthropoparasites (totally dependent on humans for their transmission), whereas about 20 are considered zoo-anthropoparasites (with vertebrates, humans included, as final hosts, and people possibly involved in the parasite circulation but not required for its transmission) as occurs in *Trichuris trichiura*, *Paragonimus westermani*, *Diphylobothrium latum*, or species of the genus *Trichostrongylus* (Cancrini et al., 1982; Ashrafi et al., 2020). Finally, about 160 species are considered zooparasites as they have vertebrates different from humans as final host, and humans are not involved in their transmission or circulation. People, accidentally infected by eggs or larval stages of these zoonotic species, being unsuitable hosts, mostly block the full development. It happens in some tapeworms (as *Dipylidium caninum*, and species of the genus *Echinococcus*), and flukes (as *Schistosoma bovis*, and species of the genus *Trichobilharzia*) (Polley, 2005; Kuris, 2012). As far as roundworms, larval stages went into humans may have three different outcomes: they can be blocked in tissues if the cycle of the species includes paratenic (=transport) hosts, that is vertebrates in which the parasite merely remains viable without develop or multiply (as happens in *Parastrongylus cantonensis* and in species of the genus *Gnathostoma*, *Gongylonema*, *Anisakis*, *Pseudoterranova*, and probably also *Contracoecum* and *Hysterothylacium*); in absence of usual paratenic hosts, they long and actively migrate at cutaneous (species of the genus *Ancylostoma*, *Afrancylostoma*, *Uncinaria*, *Dirofilaria*), or visceral (*Toxocara*, *Parascaris*, *Toxascaris*, *Baylisascaris*), ocular (*Toxocara*, *Dirofilaria*), or encephalic (*Toxocara*, *Setaria*, *Dirofilaria*) levels; they become not reproducing adults (as happens in *Parastrongylus costaricensis*, and species of the genus *Spirocerca*, *Calodium*, *Dirofilaria*). *Dirofilaria repens* is an example of the tangible biological event regarding possible changes in host adaptation, as in few cases not only adult females, but also circulating microfilariae have been reported (Nozais et al., 1994; Petrocheilou et al., 1998; Supriaga et al., 2004; Fontanelli Sulekova et al., 2016). That means not only the presence of a male worm (often not detected because too small) but also that both worms survive long enough to reach sexual maturity and mate. The same has been reported in *Brugia* infections (Greene et al., 1978; Baird et al., 1986) and in a *Dipetalonema*-like infection (Simmons et al., 1984).

From these data we can infer that human infection by helminths often means the presence of parasitized animals and, consequently, attendance of habitats devoted to livestock or used by wild/company animals or, finally, poor food security/hygiene, and poor hygienic habits (Daszak et al., 2000; Lafferty et al., 2008). Of course these elements act as opportunity (not cause) of infection, however the complexity of biological cycles primarily suggests a more strict correlation with all components of any ecosystem, geographic, climatic and anthropic included. Actually, helminth diseases go with humans throughout the history, as their geographic expansion starts with hominid expansion, and become a major problem with changes then occurred: rapid increasing of sedentary human population, development of agriculture, animal domestication, slave trade, colonial occupations. Currently parasitic diseases are an emergence mostly due to anthropogenic drivers as environmental modifications, urbanization and industrial revolution (Daszak et al., 2000; Patz et al., 2008; Rosenthal, 2009; Hoberg, 2010; Kuris, 2012). These challenges between people and the biosphere have also upset the climate: temperature and humidity, important limits for any possible cycle, are now favoring the spreading of soil-transmitted and vector-borne diseases in areas to date inapt to the development of geohelminths or filarial worms (Cancrini and Gabrielli, 2009). The geographic pattern of helminth diseases may be also influenced by the disruption of socioeconomic controls on infections due to wars, by movement of refugees (Patz et al., 2008; Weaver et al., 2010; Zarlenga et al., 2014) and, more recently, by the application of medical interventions that require administration of immunosuppressive drugs (Muñoz et al., 2011; Chokkalingam Mani et al., 2013; Iori et al., 2014). Improved diagnostics and therapeutics, and preventive strategies started since 1909 (a hookworm eradication campaign, applied to 53 countries on six continents) (Fosdick, 1952) have affected only partially distribution and outcomes of helminth diseases (Savioli et al., 2004; Macchioni et al., 2015; Spinicci et al., 2018; Errea et al., 2019; Della Bella et al., 2020). In general poverty limits effective implementation of control strategies (Macchioni et al., 2016); it is a true primary social barrier to health, expressed as low- and middle-income, lack of safe drinking water, overcrowding, hygienic-sanitary precarious conditions of people and, at personal level, working activity and hygienically erroneous behaviors (as utilization of feces as fertilizer, and their environmental dispersal), all factors particularly influent in the helminth spreading.

Although the most important helminths (flatworms and roundworms) evolutionarily diverged many hundreds of millions years ago, their patterns of infection and transmission are similar, as also pathogenesis and host immune responsiveness, which is dominated by a Type 2 (Th2) profile with a significant regulatory (Treg) function (Bruschi et al., 2013). However helminth species considerably vary in virulence, prevalence, abundance, and pathogenicity (Kuris, 2012), and are etiological agent of many major tropical diseases.

As far as the helminth classification, the number and types of characters and methodologies (historical, phylogenetic, biological, biochemical and genetic) used to define the concept of species is now more convoluted than in the past (Brooks and McLennan, 2002; Wiley and Lieberman, 2011), thus the establishment and validation of flatworms and roundworms is still a matter of controversy (Zarlenga, 2014). The only groups that are today fairly stable are the phyla, the families, and the species (Melhorn, 2008), therefore they are the only taxa reported in this chapter.

As regards drugs used against helminths, the treatment of specific parasitic infections is addressed in detail in other chapters of this Encyclopedia, therefore this chapter will set only very basic information related to possible damages induces by each group of drugs.

Platyhelminthes

The Platyhelminthes include, in addition to free-living turbellarian, to ectoparasites and endoparasites mostly of fishes and turtles, endoparasites of medical interest (Eucestoda and Digenea) that will be here described as cestodes and digeneans.

This phylum, which collects a total of about 25,000 species, is of strategic phylogenetic importance as it includes the first bilaterally symmetrical organisms in evolution. From here the cephalization took place and the early metazoan brain began to develop; therefore Platyhelminthes constitute the basal stock from which all higher animal phyla are thought to have evolved (Melhorn, 2001).

The body of these dorsoventrally flattened worms is soft, circumscribed by a plasma membrane or tegument, bilaterally symmetrical, provided of holdfast organs. Flatworms are lacking a body cavity (coelom) and specialized organs for circulatory or respiratory systems. In their absence the permeable tegument allows the diffusion of oxygen and other nutrients. All species are oviparous, and most of them are hermaphroditic (monoecious animals). The life cycle is usually indirect (heteroxenous development): it involves a vertebrate (called final host, parasitized by sexually mature adults) and one or more species of invertebrate/usually vertebrate animals (called intermediate hosts, in which larval stages develop).

Although cestodes and digeneans share many biological and developmental characters, faunal and morphological diversity overflows.

Cestodes

Cestodes, commonly named tapeworms, are among the oldest helminths that colonized our ancestors about 2 million years ago (Hoberg et al., 2001). The earlier fossil records are in Egypt: eggs of *Taenia* dated 1198 years BC (Horne and Lewin, 1977; Harter et al., 2003), and cysticerci detected in a mummy of the II-I century BC (Bruschi et al., 2006).

A total of about 6,000 species have been to date described. Species of medical interest, grouped in families and presented as usual/possible human parasites or zoonotic species, are listed in Table 1.

Morphological and functional characteristics

Adult worms colonize, often for many years, the intestine of a vertebrate host (man included), whereas larval stages (globally called metacestodes) develop in different tissues of invertebrate hosts (exceptions: species of the genus *Taenia* and *Echinococcus*).

The body of these worms is 3 mm–12 m long, segmented, and is firmly anchored to intestinal wall by means of the acetabulate scolex supplemented with a rostellum (carrying hooks or apical gland), suckers, or bothria (wing-like sucking grooves) that make possible a very close contact with the host. The outer surface is covered by a syncytial cytoplasmic tegument that preserves the body shape, allows the uptake of nutrients (and drugs), and protects the worm against digestive enzymes and immune response of the host. The absorptive surface of the tegument is amplified by the formation of many cytoplasmic extensions (microtriches) similar to microvilli of enterocytes.

The body includes: the scolex (head, bearing specific holdfast organs well supplied with nerve plexuses), a neck (area continuously differentiating in new segments named proglottids) and the strobilus (chain of 3–4,000 proglottids: the first immature, followed by the mature ones and, at the hind end, by the gravid segments, all provided of smooth muscle fibers, nerves and excretory vessels). The complex scolex-neck is the target of therapy, inasmuch as in case of drug failure the entire worm will regrow (Fig. 1).

Tapeworms are protandric hermaphrodites, therefore each mature proglottid contains one or two sets of the complete male and female sexual apparatus that, however, do not mature at the same time (male gonads mature before those of female). Size, number and structures associated to sexual organs vary depending on the species. Testes converge in a vas deferens and cirrus (copulatory structure); the ovary releases oocytes that reach the ootype where meet spermatozoa and, with yolk cells produced by the Mehlis's and vitellary glands, become shelled eggs and move to uterus and vagina (both may also have the function of storing injected spermatozoa). Sexual organs, merged in a parenchymal tissue, converge with a genital porus; fertilization may occur either within each segment or between contiguous segments (autogamy), or between proglottids of different strobila. Last proglottids, after the obliteration of all other elements of the sexual apparatus, only present the branched uterus packed with eggs (Fig. 1). In species belonging to the order traditionally named Cyclophyllidea, groups of gravid segments from time to time actively detach (apolysis) and are voided with feces. In the environment proglottids disintegrate on impact with the weathering, and the eggs are liberated. Instead, in species belonging to the order traditionally named Pseudophyllidea each segment presents a uterine porus from which the eggs are continuously eliminated.

Table 1 Cestodes of medical interest, grouped as usual/possible pathogens for humans or zoonotic species, and their possible classification in families.

Family	Anthropoparasites and Anthro-Zooparasites	Zooparasites
Anoplocephalidae	<i>Inermicapsifer cubensis</i>	<i>Bertiella studeri</i> , <i>B. mucronata</i>
Davaineidae		<i>Raillietina demerariensis</i> , <i>R. asiatica</i> , <i>R. formsana</i>
Dilepididae		<i>Dipylidium caninum</i>
Diphyllobothriidae	<i>Diphyllobothrium latum</i>	24 species: gen. <i>Diphyllobothrium</i> , <i>Spirometra</i> , <i>Diplogonoporus</i> , <i>Schistocephalus</i> , <i>Pyramicocephalus</i>
Hymenolepididae	<i>Rodentolepis nana</i>	<i>Hymenolepis diminuta</i>
Mesocestoididae		<i>Mesocestoides lineatus</i> , <i>M. corti</i>
Taeniidae	<i>Taenia asiatica</i> , <i>T. saginata</i> , <i>T. solium</i>	13 species: gen. <i>Taenia</i> , <i>Echinococcus</i>

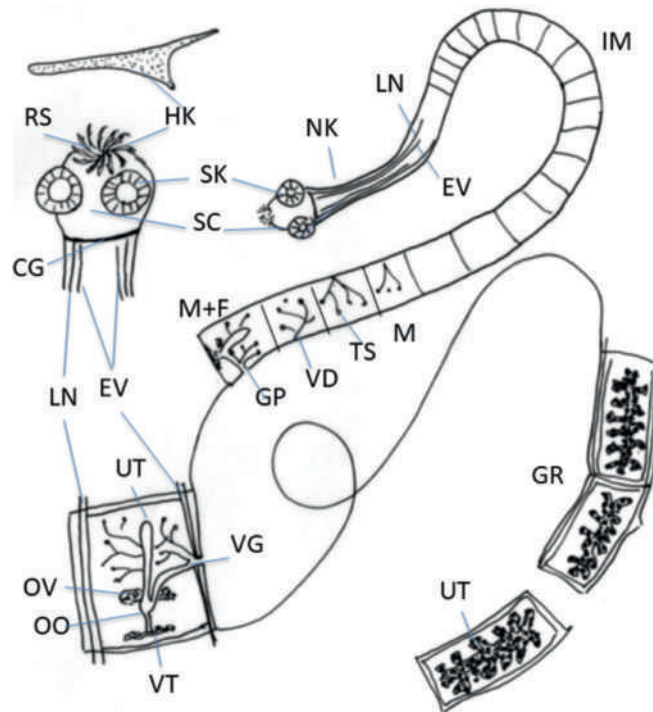


Fig. 1 Diagrammatic representation of a tapeworm: flattened, segmented, extremely long (3 mm–12 m) and hermaphrodite helminth. Scolex (SC) provided of suckers (SK) and rostellum (RS) armed with hooks (HK) to adhere to intestinal wall. The scolex is followed by a neck (NK) and by a chain of proglottids (continuously produced by the neck) named strobilus. The neoproducted proglottids are immature (IM), and are followed by that provided with only male reproductive organs (M), then by that with both sets of sexual apparatuses (M + F, a bit magnified) and, at the bottom, that gravid ones with a branching uterus filled of eggs (GR). Common systems: reproductive, simple nervous and excretory. Testes (TS) confluent in a vas deferens (VD); ovary (OV), ootype (OO), vitellarium (VT), uterus (UT), vagina (VG), and genital porus (GP). Cerebral ganglion (CG), from which depart longitudinal nerves (LN) present along all the body, with transverse minor ring commissures, all parallel to excretory vessels (EV) present in each segment.

Cestodes are lacking a coelom, but also digestive, respiratory, and circulatory systems. Nervous system is simple (cerebral bilobed ganglionic structures, two main longitudinal nerve cords and minor longitudinal cords connected, at intervals, by transverse minor ring commissures, well developed plexuses/nerve nets, and secretory inclusions) but very efficient. Indeed, to assure the maintenance of contact with the host, attachment organs are equipped with nerve plexuses reactive for cholinesterase, serotonin, and several neuropeptides. Moreover, in absence of coelom and circulatory system, by releasing neurosecretory or peptidergic components (as catecholamines, histamine, nitric oxide, neuropeptides, growth factors) into the intercellular space, the nervous system accomplishes any long-distance control of processes such as growth and development. In this way it also functions as an endocrine system (Melhorn, 2001). Parallel to longitudinal nerve cords and to transverse minor ring commissures, in each segment excretory vessels are sited.

Their anaerobium metabolism depends on carbohydrates that are adsorbed through the many microtriches present on the outer surface of the body. In some cases the development is conditioned by the presence of vitamins of the B group or sexual hormones.

Eggs produced contain a hooked embryo that develops into a larval stage generally named metacestode: cysticercus, or cysticercoid, or coenurus, or hydatid, or proceroid and plerocercoid (Fig. 2).

Cysticercus and cysticercoid are monosomatic larvae (they are vesicles containing only one early scolex, called protoscolex), whereas coenurus and hydatid are polysomatic (undergo a budding process that produces, respectively, many or a very large number of protoscolices invaginated in the vesicle).

Basic life cycles (Fig. 3)

The life cycle of almost all cestodes involves two or more host species, and may be very different. In detail:

- Species having a terrestrial cycle

The proglottids excreted by people/vertebrates on the soil release eggs bordered by a thick embryophore, which contain an embryo named oncosphaera or hexacanth (because six-hooked). The cycle may go on only when an intermediate suitable host ingests the eggs from the contaminated environment. The ontogenesis proceeds as metamorphosis in larval stages sometime asexually reproducing (Fig. 3A). In detail, depending on species and intermediate host, the hexacanth may develop into: cysticercus, or coenurus or hydatid (in tissues of a wide variety of terrestrial domestic or wild animals, but also humans), or cysticercoid (usually in arthropods; however, the cysticercoid development is also possible in the intestinal wall of humans

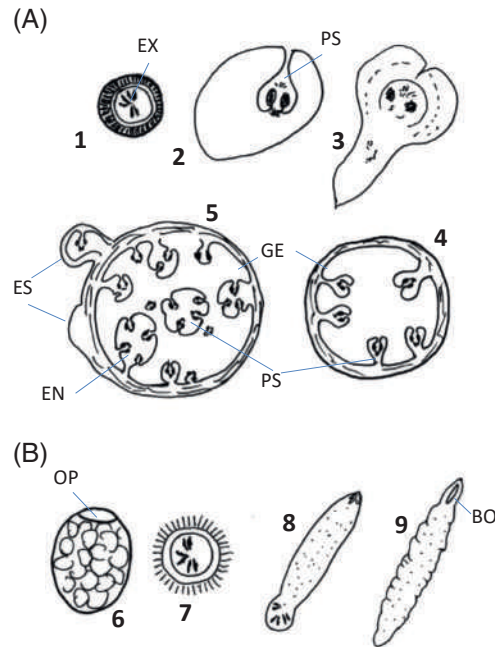


Fig. 2 Eggs and metacystodes of species having terrestrial (A) or aquatic (B) cycle. (A) (1) Egg enclosing the hexacanth (EX); (2) cysticercus, fluid-filled cyst containing a single invaginated protoscolex (PS); (3) cysticercoid, a single evaginated protoscolex embedded in a small solid cyst; (4) coenurus, fluid-filled cyst with germinal epithelium (GE) producing many protoscolices; (5) hydatid, large and growing fluid-filled cyst with germinal epithelium producing thousand of brood capsules and protoscolices, daughter and granddaughter, endogenous (EN) or exogenous (ES) cysts, very invasive. (B) (6) Egg with operculum (OP); (7) ciliated coracidium went out from the egg and supplied with 6 hooks; (8) proceroid larva, endowed with characteristic terminal rosette; (9) plerocercoid, solid-bodied larva starting the body segmentation, and showing the characteristic scolex with bothria (BO) instead of suckers.

parasitized by the adult worms) (Fig. 3B). The life cycle is completed when people/another vertebrate eats raw or undercooked meat of terrestrial domestic/wild animals parasitized by metacystodes, so allowing the development of adult intestinal worms. Monosomatic larvae will give birth to one adult worm, whereas polysomatic ones will mature in many (even a very large number) of adults. If people accidentally ingest eggs (auto- or hetero-infection), as any intermediate host will allow their development into metacystodes that will grow up and expand in any tissue.

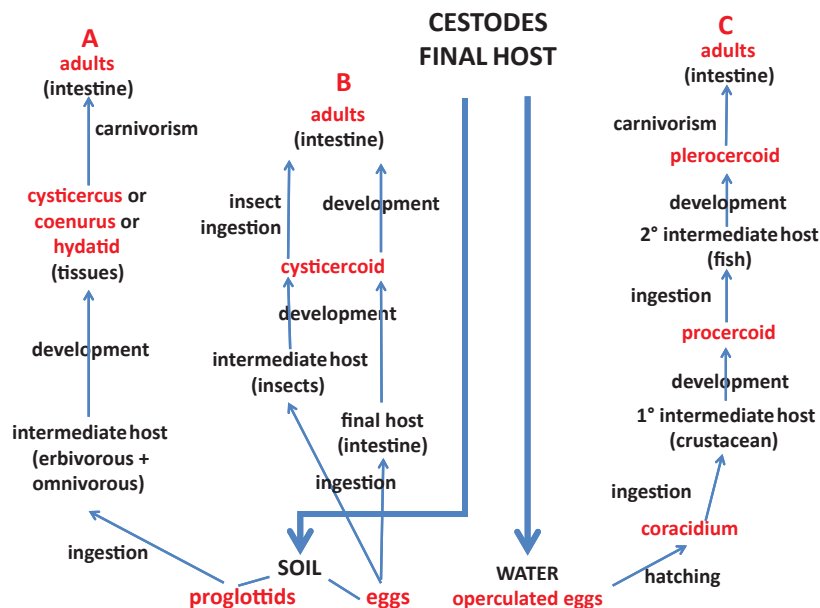


Fig. 3 Life cycles of cestodes pathogen for humans. Development and transmission patterns of species releasing proglottids (A) or eggs (B) on the soil, and that of species having an aquatic cycle (C).

- Species having an aquatic cycle (Fig. 3C)

Proglottids produce huge number of eggs that leave the vertebrate host with the feces. Eggs are endowed of an operculum (cap like) that, only in presence of water, opens up and let out a ciliated hexacanth (coracidium). Coracidium, eaten by an aquatic arthropod, develops into proceroid and then, in the same/a second intermediate host (usually a fish), into plerocercoid larva (named sparganum). The cycle is completed if people/another vertebrate eats raw or undercooked fishes containing encysted sparganum, so developing an intestinal infection by adult worm. In case of accidental drink of water containing parasitized very small crustaceans, or following ingestion of amphibians/reptiles raw/undercooked, in vertebrate hosts proceroid or plerocercoid larvae reach tissue locations with severe consequences.

Infection, prevention, transmission

Intestinal infections are mostly due to eating habits, whereas metacestodoses have as predisposing factors poor hygienic/socio-economic conditions or the profession.

Cooking (or freezing before eating) edible meats, washing accurately vegetables before eating, drinking uncontaminated water, and accurately observing hygiene rules (washing hands before eating) are useful to prevent the infection.

People affected by adult tapeworms are source of infection for intermediate hosts and, in some cases, for himself/other people, whereas people affected by larval stages are a dead end for the parasite. To break the parasite transmission, apart the appropriate drug taking, infected people must avoid the environmental dispersal of feces, and edible meats should be controlled at public level or cooked/frozen at personal level.

Pathogenicity and drugs

In general adult cestodes deprive the host of nutrients (sometime specific substances), exert a toxic action and may cause diarrhea, decreased appetite or voraciousness, and weight loss. Moreover, they induce physical damages, both little by holdfast organs, and possibly severe in case of bowel obstruction. Adulticidal drugs used may: damage the parasite tegument exposing it to the defenses of the host (aminoglycosides as paromomycin); cause spastic paralysis of the parasite muscle cells (pyrazino-isoquinolines as praziquantel); induce energetic depletion responsible for the scolex detachment (halogenated salicylanilides as niclosamide), or starvation of the parasite (benzimidazole carbamates as albendazole and mebendazole).

Larval stages developed in tissues are more dangerous, mainly if located in the brain, lungs, liver, eye or muscles, where they can grow up and, even, rupture releasing their allergenic content. Therefore their presence exerts mechanical and allergenic actions, and may cause anaphylactic shock. Drugs used are corticosteroids associated to albendazole or praziquantel; however, may be required surgical removal of the metacestode or, depending on the developed larva, the percutaneous aspiration-injection-reaspiration (PAIR) of the content guided by ultrasound, plus albendazole treatment.

Digeneans

The oldest demonstration of digeneans in human tissues are referred to eggs of *Schistosoma*, detected in Egyptian mummies dated 1184–1087 BC (Ruffer, 1910), followed by that of *Clonorchis*, widespread in Korea and China already 2300 years ago.

Usually known as flukes, digeneans are perhaps the largest group of endoparasitic metazoan, whose species (more than 6,000) parasitize all class of vertebrates, humans included. Species of medical interest, grouped in families and subdivided as usual/possible human parasites or zoonotic species, are listed in Table 2.

Few species are intestinal parasites, whereas most are systemic: the adults colonize liver, bile ducts, gallbladder, circulatory system, and respiratory shaft. Larval stages develop in tissues of intermediate hosts (mostly aquatic).

Table 2 Digeneans of medical interest, grouped as usual/possible pathogens for humans or zoonotic species, and their possible classification in families.

Family	Anthropoparasites and Anthro-Zooparasites	Zooparasites
Dicrocoeliidae		<i>Dicrocoelium dendriticum</i> , <i>Eurytrema pancreaticum</i>
Diplostomidae		<i>Alaria americana</i>
Echinostomatidae	<i>Echinostoma echinatum</i>	21 species: gen. <i>Echinostoma</i> , <i>Echinocasmus</i> , <i>Acanthoparyphium</i> , <i>Artytechinostomum</i> , <i>Hypoderaeum</i>
Fasciolidae	<i>Fasciola hepatica</i> , <i>Fasciolopsis buski</i>	<i>Fasciola gigantica</i>
Gastrodiscidae	<i>Gastrodiscus hominis</i>	
Gymnophallidae		<i>Gymnophalloides seoi</i>
Heterophyidae	<i>Heterophyes heterophyes</i> , <i>H. nicens</i>	30 species: gen. <i>Heterophyes</i> , <i>Metagonimus</i> , <i>Haplorchis</i> , <i>Centrocestus</i> , <i>Apophallus</i> , <i>Cryptocotyle</i> , <i>Stictodora</i> , <i>Heterophyopsis</i> , <i>Pygidioopsis</i> , <i>Stellantchasmus</i>
Lecithodendriidae		<i>Phaneropsulus bonnei</i> , <i>P. spinicirrus</i> , <i>Prosthodendrium molenkamp</i>
Opisthorchiidae	<i>Opisthorchis sinensis</i> , <i>O. felineus</i>	<i>Metorchis albidus</i> , <i>Opisthorchis viverrini</i> , <i>Pseudoamphistomum truncatum</i>
Paragonimidae	<i>Paragonimus westermani</i>	8 species: gen. <i>Paragonimus</i>
Paramphistomidae	<i>Watsonius watsoni</i>	
Plagiorchiidae	<i>Plagiorchis elegans</i>	<i>Plagiorchis muris</i>
Schistosomatidae	<i>Schistosoma haematobium</i> , <i>S. intercalatum</i> , <i>S. japonicum</i> , <i>S. mansoni</i>	16 species: gen. <i>Schistosoma</i> , <i>Austrobilharzia</i> , <i>Trichobilharzia</i> , <i>Gigantobilharzia</i> , <i>Bilharziella</i> , <i>Orientobilharzia</i>
Troglorematidae		<i>Nanophyetus salmincola</i>

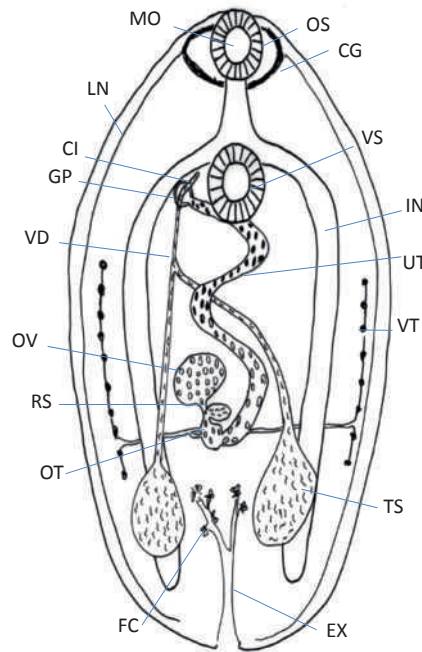


Fig. 4 Diagrammatic representation of a fluke: flattened, unsegmented, ovoid-elongate, hermaphrodite helminth, equipped to move and adhere to host surfaces by means of firm muscular structures: oral (OS) and ventral (VS) suckers. Common systems: reproductive, simple nervous and excretory, incomplete digestive. Testes (TS) converge in a vas deferens (VD), followed by the cirrus (CI); ovary (OV), receptaculum seminis (RS), vitellarium (VT), ootype (OT), uterus (UT), and genital porus (GP). Cerebral ganglion (CG) connected to longitudinal nerve cords (LN) and to transverse minor ring commissures. Ciliated flame cells (FC) and excretory bladder (EX) sited posteriorly. Mouth (MO) followed by an esophagus and a blind ending intestine (IN).

Morphological and functional characteristics

The body of these worms is 1 mm - few cm long, ovoid-elongate, unsegmented. Flukes move and adhere to inner walls of epatobiliary ducts, blood vessels, bronchial tree, or gut, mostly by means of firm muscular suckers (species specific for shape and location; usually two, oral and ventral, respectively; only sometimes three, one or none) (Fig. 4). The outer surface is covered by a syncytial cytoplasmic tegument supplied of microtriches (with or without spines) that protects the worm, preserves the body shape, allows the uptake of nutrients, and is the site of the attacks on the host's defense system. Like tapeworms, flukes are lacking coelom, respiratory and circulatory system, have a nervous system simple, arranged in the same way, and are hermaphrodites (with testes different in size and number: from 2 to more than 100, depending on the species) both self- and cross-fertilizing. The only exception is represented by species of the genus *Schistosoma*, in which firstly the dioecism appears. Instead, unlike tapeworms, flukes have a digestive system (with a mouth, surrounded by the oral sucker, followed by a gut branched and blind ending), and a simple excretory system (ciliated flame cells that carry waste metabolic products along a system of tubules, which ultimately join and open to the exterior).

The metabolism mostly depends on carbohydrates and amino acids that, per pinocytosis, pass through the many microtriches present on the outer surface of the body. High molecular weight substances, instead, are ingested.

Flukes release operculated/bearing a spine eggs containing a ciliate embryo named miracidium that leave the mammal host with feces, urine, or sputum, and develops progressively into miracidium, sporocyst, redia, cercaria, and metacercaria (Fig. 5).

Basic life cycles (Fig. 6)

Evolved as primarily permanent parasite of molluscs, all species maintain a snail in their biological cycle. The life of almost all flukes involves two or more host species (heteroxenous development): humans/other mammals in which adult worms develop and reproduce sexually, and intermediate hosts in which larval stages develop and asexually multiply. They can develop in aquatic or in terrestrial environment. In detail:

- Species having an aquatic cycle

Most flukes of medical importance develop in fresh or brackish water. Their life cycle is characterized by very high intermediate host specificity, even at geographic strain level for each species. Therefore, the free-swimming stages have many kinds of sensory receptors and reach their hosts responding to environmental stimuli, such as light, gravity, temperature, magnetic field, chemo-orientation. The host-finding strategies used are greatly different at level of dispersal from the site of origin, microhabitat of preferred host selection, host-directed orientation, host contact and invasion.

In presence of water, the miracidium escapes from the egg, actively swims, searches and infects the specific gastropod mollusc. In this host, through larval stages of sporocyst, redia (sometimes two generations), it gives origin to many cercariae (about 4,000

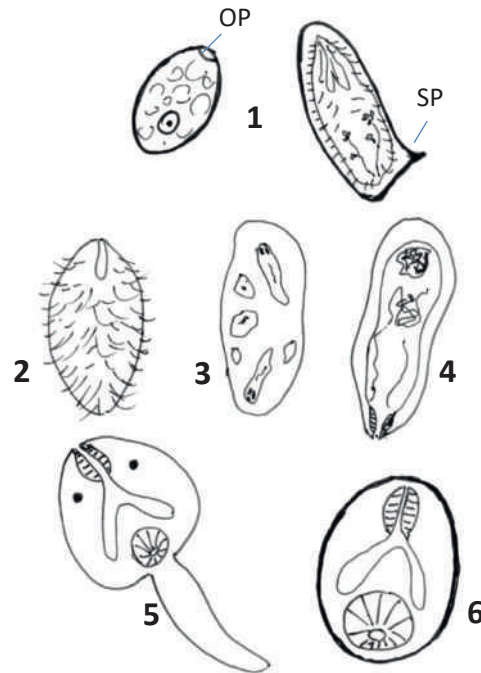


Fig. 5 Eggs and larval stages of flukes. (1) egg with operculum (OP) or armed with a spine (SP); (2) ciliated miracidium went out from the egg; (3) sporocyst asexually reproducing; (4) redia; (5) cercaria showing the digestive system and a sucker; (6) encysted metacercaria.

from one miracidium, more than 500,000 from one snail). Developed cercariae leave the snail and, free-swimming, within 1–3 days depending on the species, encyst on aquatic plants (Fig. 6A) or recognize and penetrate their final host (Fig. 6C), or invade a second intermediate host (Fig. 6B). Cercariae arrived on plants or in a second intermediate host (mollusc, shellfish, fish) encyst and develop into metacercariae that complete their life cycle when people/another vertebrate eat these raw or undercooked plants/animals. The ingestion of metacercariae allows the development of adult hermaphrodite worms in the final location (gut, liver, bile ducts, respiratory tree, reached more or less directly), and the following release of their eggs in the environment.

Conversely, cercariae directly penetrated in the skin of the competent final host (Fig. 6C), pass to circulatory system where will develop into dioecious adults. Female worms produce eggs provided of a spine, useful to open the way through the walls (intestinal, bladder, blood vessels), pass through tissues and reach/join feces or urine to start a new cycle in the environment. In case of accidental penetration in the human skin by cercariae parasites of other animals, their development is stopped.

- Species having a terrestrial cycle (Fig. 6D)

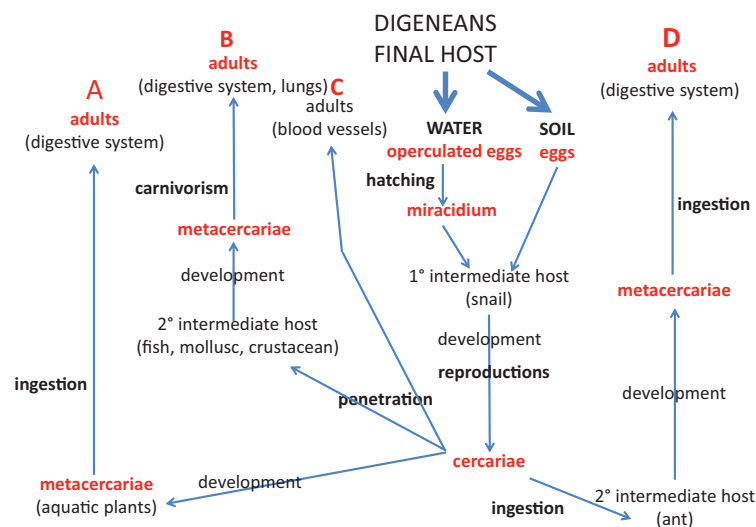


Fig. 6 Life cycles of digeneans pathogen for humans. Development and transmission patterns of species releasing operculated eggs (A–C) that develop only in aquatic environments, and that of species having a terrestrial cycle (D).

Few species of medical importance develop on the ground. In this case the eggs, mostly excreted by vertebrates different from humans, are swallowed by land-living snails. In this host a polyembryonic mitotic reproduction occurs involving larval stages of sporocyst, redia (sometimes two generations), and cercaria. Developed cercariae leave the snail and, ingested by the second host (ant) when eating slime balls, encyst as metacercariae. The accidental ingestion of the ant cause in the final host the development of adult worms that reach intestine or bile/pancreatic ducts, and release eggs then excreted with feces.

Infection, prevention, transmission

All infections are due to eating habits, except that due to immersion in fresh/brackish contaminated water.

Washing accurately vegetables before eating, cooking (or freezing before eating) edible fishes, molluscs, crustaceans, and keep from diving in possibly contaminated water are the measures to prevent the infection.

People affected by adult flukes are source of infection only for intermediate hosts, and people penetrated by cercariae of species that usually parasitize other animals are a dead end for the parasite. To break the parasite transmission, apart the appropriate drug taking, infected people must avoid the environmental dispersal of feces, urine, and sputum. At public level is crucial to prevent dams from providing ecological conditions to support snails, and edible aquatic animals should be controlled (or coked/frozen at personal level).

Pathogenicity and drugs

Intestinal adult flukes in general induce mucosal lesions by suckers, and possibly severe damages in case of bowel mechanical obstruction, usually treated with drugs that cause spastic paralysis of muscle cells in the parasite (as praziquantel).

On the contrary, the many systemic species induce severe damages such as obstructive, hemorrhagic, fibrotic and neoplastic type, due initially to the action of the migrating (possibly also in aberrant locations) pre-adults, and then, more severely, to crossing and often trapped eggs and their spines. Drug currently used is praziquantel, or drugs that irreversibly inhibit nucleic acid synthesis (oxamniquine, artemether), or induce starvation of the parasite (albendazole, triclabendazole), or decrease the ATP synthesis (halogenated bisphenols as bithionol).

Nemathelminthes

The phylum encloses only one class of worms of parasitic significance: that of Nematoda, commonly named roundworms due to their appearance in cross-section.

Nematodes

Many intestinal nematodes are very old (9,000–10,000 years), as they were found in prehistoric populations of the Americas, probably brought from the Old World to the Americas by the prehistoric migrants (Fornaciari and Gaeta, 2014). The oldest dating is for hookworm eggs ($7,230 \pm 80$ years old) from archaeological sites of Brazil (Ferreira et al., 1983), whereas that for *Ancylostoma duodenale* adult is 900 BC, specimen found in a Peruvian mummy (Allison et al., 1974).

The nematodes are the most abundant animal group (about 500,000 species), widespread from the chill polar regions to the scorching equatorial ones. They include, in addition to many free-living ground, marine and freshwater species, parasites of plants and animals. Animal parasites occur in vertebrates, man included, and in arthropods. A possible and simplified classification of those of medical importance is reported in Table 3, in which zoonotic species are reported separately from anthrozo/anthropo-ones.

The nematodes significant for humans may be located in the digestive system or may be found in various tissues, lymphatics, and body cavities. All are very impactful or disabling.

According to WHO soil-transmitted intestinal nematodes are among the 10 most important agents of human diseases (together filarial nematodes, schistosomes, plasmodia, amoebae, and leishmaniae), as they cause more death than anything else (except HIV/AIDS, tuberculosis, COVID). It is noteworthy the presence, among them, of opportunistic species. In low- and middle-income tropical and subtropical countries they represent a significant public health problem reinforced by poor socio-economic conditions. Moreover, nematode group encloses the pinworm, an intestinal worm as much numerous but not soil-transmitted and not as pathogenic.

Morphological and functional characteristics

The body of these cylindrical worms, bilaterally symmetrical, is 1 mm-more than 1 m long, colorless and unsegmented; the most long are filiform and are named filariae. The body is covered by an outer layer of cuticle, underlied by the hypodermis, multi-nucleate cells that forms thick lateral, ventral, and dorsal chords protruding into the body cavity (pseudocoel) and filled by muscle cells. The cuticle is thick (reach 5–10% of the diameter of the worm), and forms pseudometameric annulations. It is greatly different in the various nematode groups and during their life, due to the presence of plaques, bosses, cordons, longitudinal ridges, lateral hooklet, and cervical or caudal alae (flattened wing-like expansions of the cuticle in the oesophageal and tail regions). It provides protection from environmental agents, keeps the shape, actively uptakes nutrients, allows the movement (undulating waves) of the worm and actively transports the water and all organic substances, some drugs included. Often, an additional coat (epicuticle)

Table 3 Nematodes of medical interest, grouped as usual/possible pathogens for humans or zoonotic species, and their possible classification in families.

Family	Anthropoparasites and Anthro-Zooparasites	Zooparasites
Ancylostomatidae	<i>Ancylostoma duodenale</i> , <i>Necator americanus</i>	6 species: gen. <i>Ancylostoma</i> , <i>Uncinaria</i> , <i>Bunostomum</i>
Anisakidae		8 species: gen. <i>Anisakis</i> , <i>Pseudoterranova</i> , <i>Contracoecum</i>
Ascarididae	<i>Ascaris lumbricoides</i>	6 species: gen. <i>Toxocara</i> , <i>Parascaris</i> , <i>Toxascaris</i> , <i>Lagochilascaris</i> , <i>Baylisascaris</i>
Chabertiidae	<i>Oesophagostomum bifurcum</i> , <i>Ternidens deminutus</i>	<i>Oesophagostomum aculeatum</i> , <i>Oe. stephanostomum</i>
Diectophymatidae		<i>Diectophyma renale</i>
Dracunculidae	<i>Dracunculus medinensis</i>	
Gnathostomatidae		6 species: gen. <i>Gnathostoma</i>
Gongylonematidae		<i>Gongylonema pulchrum</i>
Metastrongylidae		<i>Parastrongylus cantonensis</i> , <i>P. costaricensis</i>
Onchocercidae	<i>Brugia timori</i> , <i>B. malayi</i> , <i>Loa loa</i> , <i>Mansonella ozzardi</i> , <i>M. perstans</i> , <i>M. streptocerca</i> , <i>Onchocerca volvulus</i> , <i>Wuchereria bancrofti</i>	9 species: gen. <i>Dirofilaria</i> , <i>Onchocerca</i>
Oxyuridae	<i>Enterobius vermicularis</i>	
Panagrolaimidae	free-living <i>Halicephalobus gingivalis</i>	
Physalopteridae	<i>Physaloptera caucasica</i>	
Rhabditidae		9 free-living species: gen. <i>Rhabditis</i> , <i>Pelodera strongyloides</i> , <i>Micronema deletrix</i> , <i>Diploscapter coronata</i> , <i>Spirocerca lupi</i>
Spiruridae		<i>Mammomonogamus laryngeus</i>
Strongyloididae	<i>Strongyloides stercoralis</i> , <i>S. fuelleborni kellyi</i> , <i>S.f. fuelleborni</i>	<i>Thelazia callipeda</i> , <i>T. californiensis</i> , <i>T. rhodesi</i>
Syngamidae		
Thelaziidae		8 species: gen. <i>Trichinella</i>
Trichinellidae		5 species: gen. <i>Trichostrongylus</i> , <i>Haemonchus</i> , <i>Marshallagia</i>
Trichostrongylidae	<i>Trichostrongylus orientalis</i> , <i>T. colubriformis</i> , <i>T. brevis</i> , <i>Ostertagia</i> spp., <i>Marshallagia</i> spp.	
Trichuridae	<i>Trichuris trichiura</i>	<i>Eucoleus aerophilus</i> , <i>Paracapillaria philippinensis</i> , <i>Calodium hepaticum</i>

envelops the outer layer of the cuticle. Sensorial buds and pits (amphids and phasmids) are present at cephalic and caudal ends, respectively.

The anterior pole may present, around the buccal cavity, papillae, cuticular hooks, teeth, cutting plates, leaf crowns or lips (Fig. 7). Papillae (occur in the oesophageal region, are spine-like or finger-like processes, usually diametrically placed, and may have sensory or supportive function) are typical of species moving in tissues, whereas the cuticular structures, which help the specimen to maintain its position and, in some cases, to draw blood as nourishment, are present in species that choose various different intestinal areas to locate. So the worms may be more or less anchored to the mucosal wall or, when provided of a very sharp cephalic end, may even be partially crept in the mucosa and directly draw nutrients, blood included. Contrary, species with lips, little useful as attachment structure, move continuously along the gut or live in the lumen pointing cephalic and caudal end to the intestinal wall.

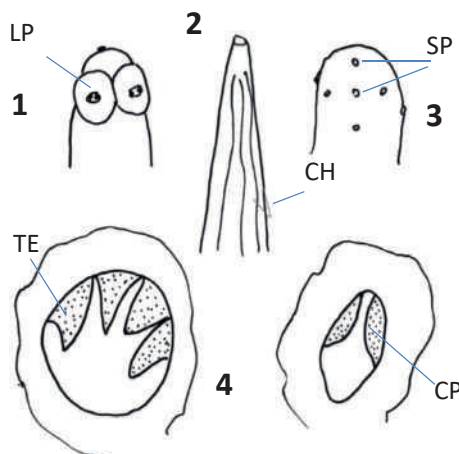


Fig. 7 Diagrammatic representation of the anterior pole of some nematodes showing possible cephalic peculiarities. (1) Lips (LP) surrounding the mouth; (2) cuticular hooks (CH); (3) sensorial papillae (SP); (4) mouth endowed of teeth (TE) or cutting plates.

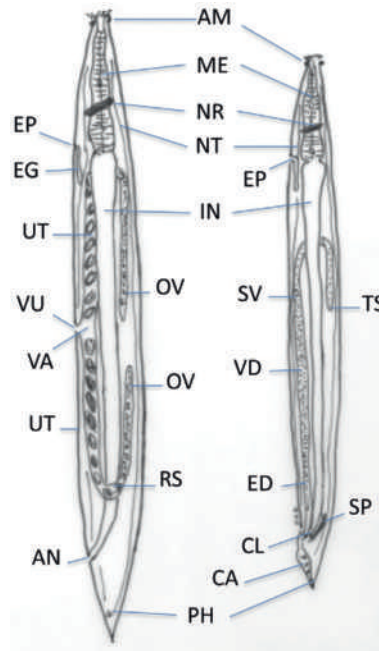


Fig. 8 Diagrammatic representation of female and male nematodes: bilaterally symmetrical, unsegmented, cylindrical, 1 mm-more than 1 m long, sometimes filiform, pseudocoelomatic, oviparous or ovoviviparous or viviparous, dioecious. Males (on the right) usually smaller than females. Common systems: digestive, excretory, nervous, reproductive (with tubular gonads blind ending on one end). Straight digestive tract: mouth followed by muscular esophagus (ME) and intestine (IN) ending with an anus (AN) in females and with a cloaca (CL) converging with the reproductive system, in males. Excretory gland (EG), with excretory porus (EP). Nerve ring (NR) from which originate nervous trunks (NT); sensorial amphids (AM) and phasmids (PH) at cephalic and caudal ends, respectively. Female specimen: ovaries (OV), each followed by a receptaculum seminis (RS) and by an uterus (UT), both converging in a vagina (VA) and vulva (VU). Male specimen: testis (TS), seminal vesicle (SV), vas deferens (VD), ejaculatory duct (ED), spiculum (SP), caudal alae (CA).

Nematodes are dioecious, sexually dimorphic, with males usually smaller than females (Fig. 8). Gonads, freely floating in the fluid that fills the pseudocoel, are tubes blind ending on one end. The female gonads usually consist of two tubes (each subdivided into ovary, oviduct, receptaculum seminis, and uterus) that join in a single vagina communicating with the exterior through the vulva. The male genital tube is subdivided in testis, seminal vesicle, vas deferens, and ejaculatory duct that opens into the rectum intestine (cloaca). Copulatory structures are the spicula (usually two needle-shaped thick cuticular elements, sometimes only one), whose protrusion is stabilized by the gubernaculum. For a better attachment with the female, male specimens may be supplied with a bursa copulatrix (cuticular expansion that envelops the vulvular region of the female), or may twist its caudal end around the female (Fig. 9).

The pseudocoel contains, along with the reproductive system, a straight digestive tract (mouth, buccal cavity, muscular esophagus sucking in nutrients, intestine, rectum) completed by a subterminal anus. A simple excretory system (glands whose function is osmoregulatory, ion regulatory and secretory rather than excretory) is present in most species, and empties its content

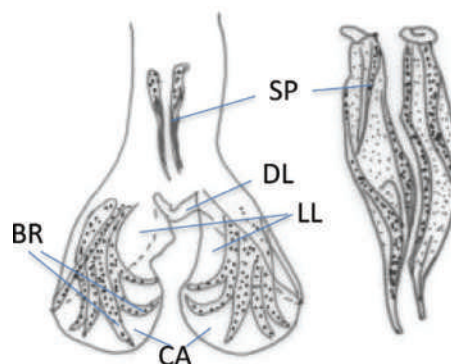


Fig. 9 Diagrammatic representation of the caudal end of a male specimen showing the copulatory bursa, an expansion of the caudal alae (CA) that embraces the female during copulation. It is composed of two lateral lobes (LL) and a single small dorsal lobe (DL) that are supported by elongated caudal papillae called bursal rays (BR). On the right, detail of the needle-shaped spicula (SP), copulatory chitinous organs that can be protruded to copulate.

through an anterior excretory porus. Often in the pseudocoel cells named ceolomocytes are present, regarded as phagocytes able to purify the body fluid. The nervous system is simple, being a nerve ring from which originate nervous trunk anteriorly and posteriorly directed, whereas respiratory and circulatory apparatuses are absent. Food (intestinal content, body fluids, mucus, blood, etc.) is usually taken up by means of the species-specific mouth, but filarial nematodes are able to feed also through the body wall.

Nematodes living in the digestive system are almost always oviparous, whereas those living in tissues are ovoviviparous or viviparous. The number of eggs/larvae daily released by each female is very high, because the continuity of the species only relies on the sexual reproduction of the adults. Indeed, unlike what happens in platyhelminths, larval stages do not asexually reproduce.

The ontogeny runs as metamorphosis involving four larval stages (L1, L2, L3, L4) that differ first at all as far their esophagus: muscular bulb in L1s and L2s, and capillary tube in L3s (Fig. 10); in each of them the cuticle must be shed.

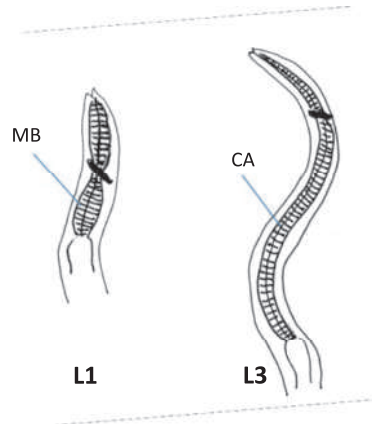


Fig. 10 Diagrammatic representation of the cephalic end of nematode larval stages. L1 showing a sturdy esophagus muscular bulb shaped (MB), and L3 equipped with a long capillary esophagus (CA).

Basic life cycles (Figs. 11 and 12)

The life cycle may be direct (monoxenous development) or indirect (with vertebrates/arthropods as intermediate/vector hosts). In detail:

- Direct life cycle

It's the cycle typical of nematodes (oviparous and viviparous) located in the gut. It may be very fast, as happens in pinworms: the eggs produced are already embryonated and within few hours contain infectious L3 that, if ingested, complete their development to L4s and adults in the intestine of the new/same host (Fig. 11A). However, in most cases the cycle is less fast as females lay eggs not yet embryonated. They need oxygen to complete the embryogenesis that, therefore, will occur in the environment, on the soil, in long times (3–4 weeks) and optimal temperature and humidity. Species belonging to this group are therefore called geo-helminths, i.e., they are soil-transmitted and are the etiological agents of the most prevalent neglected tropical diseases worldwide. Embryogenesis may occur inside the eggshell or outside. In the first case (Fig. 11B) the eggs containing the developing larvae, swallowed by a new host (with vegetables, soil or, seldom, eating intermediate hosts of zoonotic species), develop into L4s and, then, in adults, in the digestive system directly or after doing a pulmonary migration. That is: hatched larvae pass through the gut wall, enter the blood stream, reach heart, pulmonary alveoli, respiratory tree, pharynx, esophagus, digestive system where develop into L4s and adult worms. This peculiar migration is considered as a phylogenetic reminiscence of a cycle in past completed in two different hosts. In other species embryogenesis occurs outside the egg since the L1s and L2s, having a muscular esophagus, may take food from the environment and free live (Fig. 11C). However they, developed into L3s, change the esophagus in a capillary tube unable to digest available nutrients and are, therefore, forced to find a host (they are the only infectious stage). Host finding is performed thanks to the presence of amphids that detects different environmental stimuli such as temperature, chemicals, touch, light, and electric potential. The L3s actively burrow into the skin (sometimes into the oral mucosa) and, as above described, through the circulatory, respiratory and finally digestive systems develop into L4s and adult worms.

Less often, intestinal females, instead of oviparous, are ovoviviparous/viviparous (Fig. 12) and deliver actively moving L1s that have three options. The first is: to be excreted and complete on the soil the development to L3s, infectious via skin (Fig. 12A) or, in case of immunosuppression, to pierce directly the intestinal wall and migrate in different tissues of the same host starting a cycle of internal autoinfections, often lethal. The second option is: to develop on the soil even till to the adult stage, free-living and here again and again reproducing (amphizoic species) (Fig. 12B). The last one is: to pierce directly the intestinal wall of the host, via blood pass through heart, lungs, heart, arrive to all cells and encyst in muscular ones of the same host, from which they may pass to a new carnivorous host that eats raw meat, and there complete the adulthood (Fig. 12C). In this case, obviously, each subject acts at the same time as final and intermediate host.

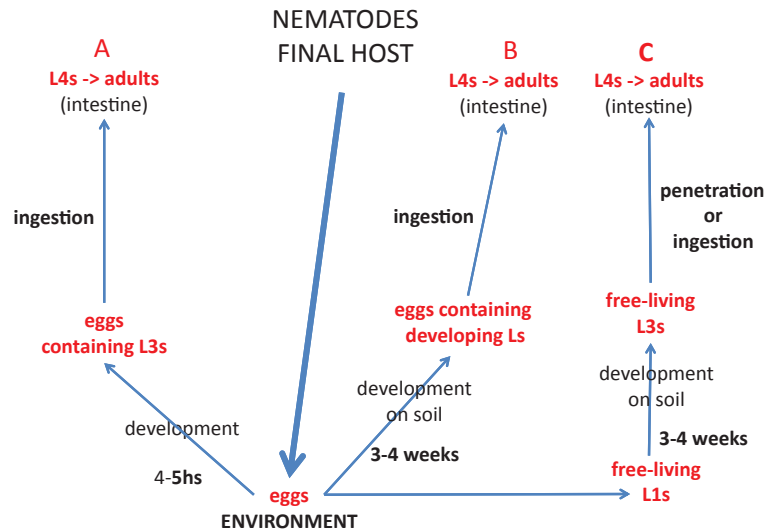


Fig. 11 Life cycles of nematodes pathogen for humans. Development and transmission patterns of oviparous species, whose biological cycle, more (A) or less (B and C) fast, takes place without intermediate hosts.

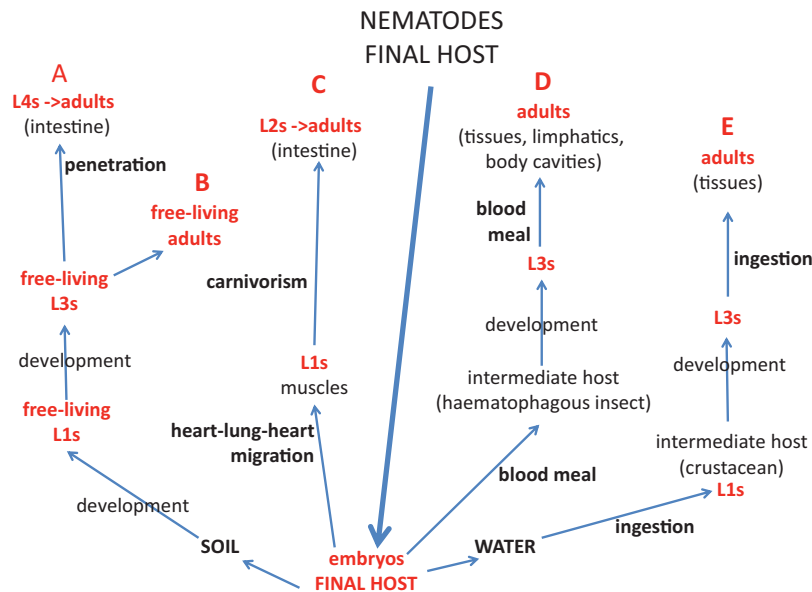


Fig. 12 Life cycles of nematodes pathogen for humans. Development and transmission patterns of viviparous species whose biological cycle takes place without (A–C) and with (D, E) intermediate hosts.

In species that perform the pulmonary migration the passage of L3s to placenta or to milk may occur, therefore further ways of transmission (mother to child) are possible, whereas in case of in tissues migrating larvae the solid organ and hemopoietic stem cells transplant transmission is possible.

- Indirect life cycle

This cycle is forced to species living, as adults, in subcutaneous/deep tissues, connective tissue, lymph nodes/ducts or body cavities. Females are viviparous and the progeny, deeply produced, needs an intermediate host to leave the host. This group of adult worms, filiform and reaching the greatest length among nematodes, is called filariae (better macrofilariae). Embryos delivered (microfilariae) move to blood and here wait for the arrival of a hematophagous insect (mosquito, horsefly, blackfly) in which they will develop into L3s. During a following blood meal of the insect on humans/vertebrate the L3s actively pass to a new final host in which reach the specific location and develop into adult worms (Fig. 12D). Finally, females of the most long filarial species, located in the subcutaneous tissue of people, with fresh water contact protrude the uterus through an ulcer-like

sore and release L1s. Microscopic crustaceans present in the water ingest the L1s and allow the development to infectious L3s that, when accidentally ingested inside the parasitized crustaceans with water, develop in man to adult worms (Fig. 12E).

Infection, prevention, transmission

Infections may be due to hygienic and socio-economic conditions, behavioral habits, direct contacts with soil, eating habits, potable water supply, or presence of insect/crustacean vector species. With regard to very few species, additional risks may be due to transmission via placenta, milk, or transplants.

Infection may be prevented by: appropriate hygienic habits; agricultural hygiene; protection of foot and hands by direct contact with contaminated soil; washing the hands before eating; washing accurately vegetables before eating; cooking (or freezing before the eating) edible meats, fishes, and crustaceans; boiling possibly contaminated water before drinking; protection by insect bites.

People affected by adult nematodes usually are source of infection for the environment or for intermediate hosts; however, they may infect themselves (in case of infection by pinworm and *Strongyloides*) and living-in (pinworm), or, rarely, the newborn (hookworms) and people recipient infected organs (*Strongyloides*). People penetrated by L3s of species that usually parasitize other animals are mostly a dead end for the parasite.

To break the parasite transmission, apart the appropriate drug taking, infected people should: make ordinary tasks accurate personal hygiene (mainly in the morning), avoid the environmental dispersal of feces and, in at risk areas, avoid insect bites and contact of the cutaneous lesions with the water. Finally, edible meats should be controlled at public level or cooked/frozen at personal level. Solid organ donors should be serologically controlled before donation, like hemopoietic stem cells that may contain infective larvae.

Pathogenicity and drugs

Intestinal adult nematodes mostly exert toxic or allergenic action, or cause severe blood loss inducing permanent intellectual and cognitive deficits from iron deficiency; moreover, they can produce severe damages in case of bowel perforation or mechanical obstruction. Opportunistic species are potentially lethal in hyperinfections. Drugs currently used cause starvation of the parasite (benzimidazoles as mebendazole and albendazole), or its immobilization/paralysis (tetrahydropyrimidine and imidathiazole groups, with drugs as pyrantel pamoate and levamisole, and macrocyclic lactones as ivermectin); they have replaced older anthelmintics as piperazine salts.

Systemic species lead to severe damages of allergenic, toxic, obstructive, and fibrotic type, sometimes heavily disabling. Migrating L3s, pre-adults, and adults of intestinal and tissue zoonotic species originate severe and difficult to be diagnosed *larva migrans* syndromes. Currently, drugs are not addressed to macrofilariae that, like pre-adult worms, may require surgical removal; however, the antibiotic therapy recently introduced and addressed to endosymbiotic *Wolbachia* bacteria present in most filarial species, proved very effective as it is indirectly macrofilaricidal and has long-term sterilizing effects. Moreover, in endemic areas the once a year administration of macrocyclic lactones as ivermectin, associated or not to diethylcarbamazine (damages the parasite tegument, reduces muscular activity and immobilizes the microfilariae), eliminate embryonic stages and circulating microfilariae for several months leading to a rapid reduction of disease transmission.

Conclusions

According to WHO, on this hearth 1/10 people suffers from 1 or more helminths, mostly associated with overcrowding, undernutrition, poor sanitation, contaminated water and food, and other poverty-related factors, worsened by conflicts that damage poor existing public health infrastructures. These parasitic infections are of concern in developing countries, however it is useful to remember that rich nations are not exempt, as demonstrated by the pinworm, which is more prevalent in the West where it represent a super-annoying problem difficult to wear out, rather increasing due to increasingly more intense social life.

Control programs had been carried out against some impacting helminths, such as schistosomes, filariae and geo-helminths, with varying degrees of success. However, it is difficult to maintain the benefits gained, as slackening of control measures and drug resistance in the parasite or its vector impends (Lustigman et al., 2017).

Therefore, despite progresses in some cases achieved, a lot rests with improvements related to socioeconomic conditions of the population at risk. Based on available general data, the sanitary problem should be faced taking into account the concrete intersection of human health, animal health, and environmental health. This implies that, beside health education and socioeconomic improvement, "any collaborative effort of multiple disciplines working locally, nationally, and globally, should be made to attain optimal health for people, animals and our environment," as defined by the One Health Initiative Task Force. An interdisciplinary approach to planetary health.

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Geohelminths

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Introduction

With the term “geohelminths” and “geohelminthiases” it is usually meant several species of nematodes and associated diseases that are ecologically related although taxonomically and phylogenetically heterogeneous (Holland and Kennedy, 2002). The name comes from the Greek. γεω “soil, terrain” ἑλμινς -ινθος “worm”, thus indicating that these worms are strictly depending from soil environment for a critical developmental stage of their life cycles. Geohelminths, also known as soil transmitted helminths (STHs), belong to several orders of nematodes that are responsible of specifically human diseases as well as of zoonotic infections.

The nematodes specific to humans are mostly responsible for intestinal diseases at their adult stage while most of the zoonotic species exert their pathogenic effects through larval aberrant migrations that can be cutaneous, visceral or cerebral.

The life cycles of soil transmitted nematodes may include a free-living stage in soil where larvae undertake usually two moults to reach the third larval stage (L3), that is infective to the definitive host. The infections are therefore associated to parasitic eggs present in human feces, which may contaminate soil especially in areas where sanitation and hygienic conditions are poor. Such diseases are widespread in very low socio-economic conditions. Climatic factors can also affect the distributions of geohelminthiases, as larval development is favored by appropriate temperature and humidity. Therefore, the burden of the diseases is definitively higher in the tropical or sub-tropical regions, as sub-Saharan Africa, the Americas, China and East Asia.

Geohelminthiases represent the most widespread parasitic diseases in the world, accounting for over 1.5 billion infections (around 24% of the entire world's human population), being probably more frequent than any other infectious disease, including those from bacteria and viruses. Many mammalian-parasitic nematodes infect only one or a limited number of host species, but zoonotic transmissions and coinfections may also occur.

Despite the outstanding incidence of STHs, in particular in tropical and sub-tropical conditions, only recently efforts have been focused in limiting the impact of these diseases. Such initial scarce level of interest forced the WHO to establish a new sector called “Neglected tropical diseases” (NTDs).

General aspects of the life cycles

Despite some striking differences among species, the life cycle of geohelminths shows several shared features (also with other nematodes). All geohelminths are transmitted by eggs (or larvae) with feces of infected people or in some cases animals. All nematodes go through four moults (cuticle substitution) during larval development, shifting from the first to the fourth larval stage (L1 → L2 → L3 → L4), up to the adult stage. L1 and L2 are typically free-living stages and are known as rhabditoid (or rhabditi-form) larvae; they can survive both free in the soil or within the egg. Rhabditoid larvae are characterized by the so-called rhabditoid

esophagus, (i.e., a bulb-like esophagus, armed with robust tricuspid valves, able to grind solid substances). The other two stages L3 and L4 are always parasitic forms and are known as strongyloid (or filariform) larvae. They show a filariform thin and straight esophagus, adapted to pre-digested food intake.

In the case of *Ascaris lumbricoides* and *Trichuris trichiura*, larvae reach their infective stage still within the eggs, parasitizing the definitive hosts following their ingestion of vegetables not carefully washed or contaminated water or directly by poor hand hygiene, particularly for children.

Other mechanisms of infections are represented by the skin-penetrating nematodes such as the human hookworms *Ancylostoma duodenale* and *Necator americanus*, and the human threadworm *Strongyloides stercoralis*. They show similar life cycles inside the host, but eggs are the developmental stage that reach the environment for hookworms, while *S. stercoralis* larval form can be observed in stool as the eggs with thin walls hatches directly into the host intestine. Infective larvae commonly infect a new host through the feet skin penetration. The two kind of skin-penetrating parasitic nematodes vary in their host-seeking strategies. The dog hookworm *Ancylostoma caninum* and the human hookworms *A. duodenale* and *N. americanus* are ambushers that remain relatively motionless in the absence of stimulation, starting to crawl following the contact with the host (Haas et al., 2005). By contrast, *Strongyloides* species appear to be more motile (Gang and Hallem, 2017).

Excluding the members of the species *T. trichiura*, that reach directly the intestine where they develop to adults, all the remaining species migrate through circulatory system using the portal vein to reach the heart and then the pulmonary capillaries. The larvae grow up in size, break the capillary and fall down in the alveoli. Subsequently, larvae are coughed up reaching the glottis and swallowed, and then pass through the stomach into the intestine, where they resume development into adults. This route is mandatory for the skin penetrating nematodes as this is the only pathway to arrive to the final site of infection. The same pathway is observed for *A. lumbricoides*, with a difference: this migration is more intriguing as it arrives to the intestine following ingestion of the eggs, then the larva penetrates the intestinal barrier, reaches the circulatory system and then undertakes the hematic migration as described above. The reason for this unusual behavior is still under debate and explanation by Fülleborn in the first years of 1900 suggested the loss of one of the hosts during the course of evolution and a return to direct transmission (Anderson, 2000).

Intestinal geohelminths

Five nematode species are parasitic at their adult stage of human intestinal tract, namely *A. lumbricoides*, *T. trichiura*, *A. duodenale*, *N. americanus* and *S. stercoralis* (the latter specifically treated in a separate chapter of this Encyclopedia). Many parasitic nematode species are co-endemic and mixed infections are frequently observed in a single individual, due to similarities in the transmission routes, environmental, socio-economic conditions and geographical distribution. Such parasitic infections can cause chronic gastrointestinal diseases, anorexia, anemia, and impairment of physical and cognitive development in children.

Ascaris lumbricoides

General aspects

The large intestinal roundworms of the genus *Ascaris* belong to the order Ascaridida (Nematoda: Secernentea). This order is classified into families that occur in a broad range of vertebrate hosts world-wide, such as fishes, reptiles, birds, and mammals including humans. The human disease caused by *Ascaris* spp. is well known from the past. The first detailed morphological description is due to Edward Tyson (1683), followed by the observations of Francesco Redi (1684). However parasitic eggs have been found in ancient remains and mummies from Peru, Brazil and Egypt. As for paleoparasitological records, iguanodont coprolites dated from 100 million years ago were found positive for ascarid eggs (Poinar and Boucot, 2006). Chinese, Egyptian, Roman and Arabic writings have also recorded the infection of *A. lumbricoides*, including citation from Hippocrates in the 5th century.

A. lumbricoides belongs to the superfamily Ascaridoidea, a taxonomic rank characterized by medium-sized to large nematodes with three lips sometimes separated by interlabia, usually inhabiting the stomach and intestine of the definitive host and generally consuming food ingested by the host.

At present the genus contains several species, of which a large number is now placed in synonymy (see Crompton, 2001), but only two have been extensively investigated as they are related to humans and to economically relevant domesticated animals as pigs, namely *A. lumbricoides* Linnaeus 1758, a parasite of humans and *Ascaris suum* Goeze 1782, which infects pigs. Although mainly found in different hosts, the two species are morphologically very conservative, with little or no variation in morphological traits.

A. suum is prevalent worldwide in both intensive and extensive pig production systems (Fig. 1), having negative impact on weight gain and marketability (Skallerup et al., 2012) representing probably the most common nematode infection (Katakam et al., 2016).

A. lumbricoides infects 1.2 billion people globally and even if DALYs (disability-adjusted life years) is around 10.5 million, ascariasis is still considered a neglected tropical disease (Dold and Holland, 2011), causing approximately 60,000 deaths in humans each year. The infection is widespread worldwide and in particular in warm and humid climates, including temperate zones during warmer months and is found in association with poor personal hygiene, poor sanitation, and/or in areas where human feces are still used as fertilizer.



Fig. 1 Adult specimens of *Ascaris suum* from the intestine of a young pig.

Similarities between the two nematodes are present also in the life cycle: adult worms live in the lumen of the small intestine. When a female reaches maturity may produce around 200,000 eggs per day. Eggs measure are $65\text{--}75 \times 35\text{--}50 \mu\text{m}$ in *A. lumbricoides* and $50\text{--}70 \times 40\text{--}60 \mu\text{m}$ in *A. suum*; the outer surface is coarsely mammillated and sticky. Fertile eggs may embryonate and become infective after several days in the soil depending on environmental conditions (13–18 days at $30\text{--}33^\circ\text{C}$). They do not hatch spontaneously and are markedly resistant and long-lived. Humans and pigs become infected upon ingestion of embryonated eggs. The larvae invade the intestinal mucosa, they reach the lungs where they migrate for 5–6 days being $0.98\text{--}1.68 \text{ mm}$ in length in 6–7 days and there they further mature in order to arrive in the small intestine, ready to develop into adult worms. These can live 1–2 years. Fully embryonated eggs can remain viable in the environment for up to 5 years under favorable conditions. Eggs of both *Ascaris* spp. can hatch in the gut of several animals like mice, rats, guinea pigs, rabbits, cattle, sheep and goats, but they are unable to reach maturity in these hosts.

Given the absence of evident diagnostic morphological features, *A. lumbricoides* and *A. suum* can be considered to be cryptic/sibling species. Nadler and De Leon (2011) defined cryptic species as morphologically indistinguishable forms that show high genetic differentiation, while sibling species are defined as cryptic sister species in a phylogenetic context. Since we have no clear information on the phylogenetic relationships with other *Ascaris* species (provided that they exist), the term cryptic rather than sibling will be used hereafter for the given taxa.

A. lumbricoides and *A. suum* are considered two distinct species (Betson et al., 2014). This issue is still under debate since their validity as separated species or the existence of a single species is not only an academic exercise. Species definition has obvious influences on the epidemiology and transmission patterns of these nematodes, and in general is crucially linked to the comprehension of biodiversity and evolution (de Meeûs et al., 2003). Species delimitation has also relevance with respect to host specificity, or to the ability of the worm to survive, develop and reproduce within its favorite or accidental hosts. To date, molecular markers are used to assist a correct species identification and the combination of a nuclear and a mitochondrial marker seem to be a reliable approach. The diagnostic key based on RFLP of nuclear ribosomal ITS allows to identify the two species and their hybrid, while mitochondrial *cox1* and *nad1* are suitable for haplotype studies according to different geographical regions or hosts, in order to evaluate the amount and nature of genetic diversity of *Ascaris* spp. circulating in humans and pigs, and to explore their zoonotic potential and cross-transmission (Zhou et al., 2011; Cavallero et al., 2013). Evidences from these molecular studies suggested the presence of gene flow between the two species and their different occurrence on the base of endemicity of the human species: a large environmental contamination from both species correspond to separated transmission cycles; on the contrary, where *A. lumbricoides* is not endemic, most of the human infections are zoonotic and *A. suum* is the causative agent of ascariasis.

Pathology and clinical aspects

The adverse pathogenic effects of ascariasis is mostly due to the host response to parasitic adults, larvae and eggs in different tissues or sites. During larval migration, molecules involved in the invasion of intestinal barrier, such as peptidases, are likely to be overexpressed, causing tissue damages and cell disruption and subsequent inflammatory response. Then, during the typical migration through the circulatory system to the lungs, at pulmonary level they penetrate the alveoli and cause an allergic reaction characterized by hypersecretion of mucus, inflammation due to eosinophils and bronchial spasm, irritation and a dry cough. Rarely, it has been recovered through pleural biopsy needle (Elhadidy et al., 2017). This pulmonary phase is usually characterized by the so-called Loeffler's pneumonia. Although the infection by *A. lumbricoides* determines a shift to the Th2 immune reaction, the protective effect to allergies is uncertain. Given their large size, the severe outcomes may be due to massive infections, especially in children, and intestinal obstruction is frequently observed. These cases usually require surgical removal of parasites. *Ascaris* can also cause malabsorption, malnutrition and Vitamin A deficiency. Additionally, as these nematodes do not attach to the intestinal mucosa, they depend on continuous movements to counteract peristalsis. So that they can accidentally migrate to the pancreatic and bile ducts and provoke obstruction and jaundice or gallbladder rupture or again migrate to the appendix.

Diagnosis

Ascariasis should be suspected in patients from endemic areas, in travelers from tropical and sub-tropical regions or in farmers working with pig farming, displaying cough, fever and eosinophilia as well as abdominal pain and vomiting. Sputum can contain Charcot-Leyden crystals. Laboratory diagnosis is based on the detection of eggs in the sediment of concentrated stools (coprodiagnosis). Both fertilized and unfertilized *Ascaris* eggs may be found at the microscopical observation (Fig. 2A and B, respectively). Imaging techniques may also be used to visualize the worms in the intestine, and cholangiograms often show their presence in the biliary tract of the liver. Pulmonary stage may be diagnosed by the collection of larvae in sputum. One or few worms can be also found in stools or in children's diapers (Fig. 3).

Trichuris trichiura

General aspects

The members of the genus *Trichuris* (Order Trichinellida) are commonly known as whipworms as they are shaped as a whip. In fact, the anterior part corresponding to the structure of the esophagus consists of a narrow tube with one to three rows of large gland cells called stichocytes and the entire glandular structure is known as a stichosome. This anterior part is usually much narrower than the posterior part of the body, that is thick and containing the reproductive organs (Figs. 4 and 5).



Fig. 2 *Ascaris* fertilized (A) and unfertilized (B) egg, visualized after coprological analysis and microscope magnification. Bars indicate dimensions in micrometers.



Fig. 3 Adult specimen of female *Ascaris* found in a child's diaper.

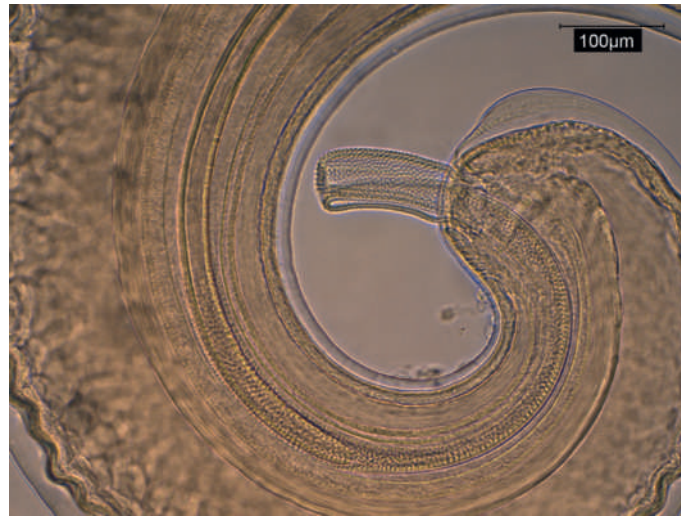


Fig. 4 Male specimen of *Trichuris trichiura*: caudal end.



Fig. 5 Female specimen of *Trichuris trichiura*: caudal end.

Similarly to *A. lumbricoides*, *T. trichiura* has worldwide distribution, and its occurrence and incidence are directly correlated with poor sanitation and the use of human feces as fertilizer. In fact, whipworms are listed in the NTDs with the other nematodes and usually co-occur with roundworms in the same host, but with a different and peculiar gastrointestinal localization.

It displays a simple, direct life cycle. The infection starts with the ingestion with contaminated food or water of eggs containing a third-stage larva that hatch into the small intestine. Eggs are generally lemon or barrel shaped with thick-shelled with polar, plug-like structures (Fig. 6).

The larvae migrate to the intestinal caecum where they molt and mature to adult stage. They embed into the mucosa and after 3 months the females release up to 10,000 eggs per day. Eggs are generally oviposited in a not embryonated state and become infective after a period of 20–30 days into the soil (Fig. 7).

A broad range of mammalian hosts including ruminants, marsupials, rodents, and primates can be infected by whipworms. To date, around 100 species have been described and synonymized, showing a high biodiversity in the genus. Humans are commonly infected as well, mainly by the medically-relevant species *T. trichiura* and, occasionally, by zoonotic species such as *Trichuris vulpis* and *Trichuris suis*. *Trichuris* spp. usually infect a particular host or a group of related host species and parasitic species designation is often based on an integrative approach based on morphological and molecular methodologies. The parasitic species identification is usually performed using information on host-specificity and on general morphology of eggs and adults, in combination with evidences from some diagnostic nuclear and mitochondrial molecular markers. In the case of humans and other primates, whipworms are automatically identified as *T. trichiura*, but recently its taxonomic status has been largely debated.



Fig. 6 Eggs of *Trichuris trichiura*.



Fig. 7 Female specimen of *Trichuris trichiura*: detail on vagina with eggs.

Molecular studies have revealed the existence of more than one taxon able to infect humans and other primates, including captive individuals, and new species have been described thus suggesting the status of *T. trichiura* as a complex of species, counting different cryptic taxonomic units with different ability to infect one particular or several species.

Trichuris infections in humans have been described since the 18th century, but ancient paleoparasitological evidences report the recovery of *Trichuris* eggs in Korea, dating 2000 years ago as well as findings of this species in South America in pre-Columbian times. *Trichuris* eggs were also found in the intestine of a glacier mummy from the Copper Age (about 5000 years ago), known as Ötzi or the Similaun mummy, who was recovered in an astonishingly good state of preservation on the Alps at the border between Italy and Austria.

Pathology and clinical features

Trichuriasis show mild to severe clinical outcomes, mostly related to the presence of adult worms, of approximately 4 cm in length, feeding on intestinal host tissues. Diarrhea and dysentery are common symptoms. Light whipworm infections are usually asymptomatic. The pathogenic effect of trichuriasis is mainly at gastrointestinal level, as the larval stages do not migrate as other STHs larvae do. The infection site of adults is in the large intestine, mostly caecum and colon, although they may reach the appendix and the rectum. This happens usually in heavy infections, while, when a low number of worms are present, they are usually confined

to cecum and ascending colon. Re-infection occurs as a result of contact with infective stages in the environment, most frequent in areas where sanitation is poor. The consequences of infections are related to an impairment of the nutritional status of the infected people, in particular for iron and protein loss caused by parasitic blood absorption. Iron deficiency and poor nutrition caused by parasitic competition may cause in children anemia, growth retardation, and even impaired cognitive development.

In fact, the presence of whipworms causes the typical symptoms of rectal bleeding and abdominal pain because of cell destruction and activation of the host immune system, recruiting eosinophils, lymphocytes and plasma cells. At intestinal level, an increase of macrophages, TNF α and mast cell degranulation are observed, suggesting that colitis can be regarded as a sort of localized anaphylactic reaction. Long lasting infections may produce chronic effects that are similar to IBD (intestinal bowel disease) and stools of infected people may contain a mixture of mucus, water, and blood. Heavy *Trichuris* infections may result in chronic dysentery and rectum prolapse. The inflammation is mainly produced by the intestinal tissue damage caused by the intimate embedding of the long, thin anterior end of the worm in the mucosa. Rectal prolapse, one of the most serious damage due to these nematodes, seems to occur with an intussusception mechanism, i.e., when the rectum invaginates into itself.

On the other hand, the host immune response to *Trichuris* seems to be mostly characterized by the T helper (Th) 2 cells, which prevent worm survival and facilitate their expulsion. Th2 response attenuates the Th1-driven inflammatory response. This may have the effect to impair the protection versus viral and bacterial infections. Moreover, a partial protective immunity that develops with age was observed (Truscott et al., 2015). Such features imply the potential use of parasites for therapy of inflammatory diseases. In fact, intentional exposure to intact helminths or helminth-derived products have been investigated and by now, therapy with *T. suis* eggs showed no real benefit for IBD patients, suggesting a careful evaluation is needed before its use into clinical practice. Several studies described the mechanisms by which helminths manipulate the host immune system, but little consideration has been given to the actual tested helminths and in particular to their zoonotic potential. For *T. suis*, the porcine species has the ability to infect humans, too, and its use should be carefully evaluated.

Diagnosis

Trichuriasis should be suspected in patients from endemic areas. Many cases may be asymptomatic, but severe infections display abdominal pain, blood in diarrhetic feces and rectal prolapse. Stool examination following concentration reveals the presence of the characteristic oval-shaped eggs with polar plugs. Abdominal pain and general gastrointestinal symptoms are shared with other infective agents or typical of certain human diseases, a differential diagnosis should be accounted in order to correctly identify the cause of disease.

Ancylostoma duodenale* and *Necator americanus

General aspects

Important in animal and human health, these two nematodes are also known as hookworm. *Ancylostoma duodenale* (Dubini, 1843) is the so-called “Old World hookworm” and *Necator americanus* Stiles, 1902 the “New World hookworm,” distributed in all tropical and subtropical regions, in particular after the humans first long-distance movements and the late globalization. Both belonging to the Order Strongylida, called also the bursate nematodes, they differ for geographic distribution, mouth morphology, size and pathogenic impact but they both do not occur in the northern hemisphere above 52 degrees latitude as the free-living stages require temperatures in excess of 22 °C.

A large, highly cuticularized buccal capsules provided with teeth or cutting plates is a shared features used to attach themselves to the intestinal mucosa of the host. In particular, the hook-shaped anterior end observed in *Necator* accounts for the vernacular name of these species. *A. duodenale* has a mouthpart armed with sharp teeth while *N. americanus* display two plates; both of them are chitinous and adapted for sucking blood.

It is estimated that more than 900 million individuals worldwide are infected with hookworms. The transmission is favored in warm and humid climates, but is frequently reported in temperate regions, too. Direct contact with contaminated soil is a risk factor.

Contrary to what has been observed for *Ascaris* and *Trichuris*, the infection in humans initiates by the skin penetration by L3 (filariform) larvae, even if they can also infect orally and in this case they exsheath in the gut and develop directly to adulthood without a migration to the lungs (Haas et al., 2005). The first and second stages are rhabditiform and the third stage strongyliform; the tail in all three stages is conical and pointed and the infective larva retains the cuticle of the second stage. Through blood circulation, the larvae arrive to the lungs and then they follow the same route as described in *A. lumbricoides*, that is, they penetrate the pulmonary alveoli, climbing up from the bronchial tree to the pharynx, where it can be coughed up and swallowed. So, the larvae arrive in the small intestine, where they develop to adult worms. The bloodsucking mouth allows to the worms to be firmly anchored to the mucosa. Adult females are able to lay up to 20,000 thin-shelled eggs per day for *A. duodenale* and around 10,000 eggs for *N. americanus*, which are released with human feces. Eggs are generally in the four- to six-cell stage when passed in the stool of the host. In the soil, the eggs hatch a rhabditiform larva (still not infective) that moults to a filariform L3 stage, able to penetrate intact skin and to restart a new cycle. Larvae survive in moist, shaded areas with vegetation and their life span varied from 2 to 12 months, according to environmental conditions.

Pathology and clinical features

The first phase of the infection is represented by skin penetration of the filariform larva after shedding their sheaths. This may provoke a cutaneous rash and an allergic reaction. From the subcutaneous tissues, they invade lymphatic capillaries and in turn the

larger lymph channels leading to the thoracic duct; once reached the general circulation, they arrive to the heart and lungs. After larvae enter alveoli, the development to the fourth stage begins. The lung migration phase can be the cause of pneumonia, cough and sore throat, accompanied by the already mentioned Löffler syndrome along frequently with eosinophilia.

The adult worms produce gastro-intestinal symptoms including nausea and diarrhea. At intestinal level, adults start feeding on blood, causing losses that are 10-fold more severe in *Ancylostoma* infections rather than in those caused by *Necator*, resulting in a severe anemia. In fact, a long prepatent period varying from 44 to 100 days was reported in humans infected with *N. americanus*, and adults worms may live for 5–6 years. Massive chronic infections can give rise to iron-deficiency anemia and to mental and physical retardation, especially if accompanied by other nutritional depletions.

Some species infective for others mammals can be zoonotic, as *Ancylostoma braziliense* and *Ancylostoma ceylanicum* parasites of cats and dogs (and humans for *A. ceylanicum* in Asia) may be a common cause of cutaneous *larva migrans* defined “creeping eruption,” as result from the reaction to third-stage larvae migrating in the skin of humans (Fig. 8). Larval morphology is identical in the two species of medical interest, *A. duodenale* and *A. ceylanicum*, while larvae of *N. americanus* can be distinguished from that of *A. duodenale* for the prominent walls of the buccal cavity, the anterior and smaller genital primordium and the broader esophagus. Members of the genus *Necator* occur in several animals as apes, swine and, rarely, rhinoceroses, pangolins and dogs; *N. americanus* shows also the ability to infect not only humans but also other primates.

Diagnosis

Conventional stool examinations, such as formalin-ether concentration technique, is the gold-standard of diagnosing such infection, based on the detection of the eggs. The eggs are $50\text{--}80 \times 36\text{--}42$ μm in size, segmented, clearly showing 4–8 blastomeres. If the slide is left wet and unfixed, we can observe the multiplication of blastomeres, and if the stools are left in suitable conditions, larvae will hatch out of the egg.

Larvae migrans

Several species of geohelminths that are parasitic on domestic and wild animals have been recorded to accidentally infect humans, becoming responsible of zoonotic infections generally known as “larvae migrans.”

Different syndromes have been so far described, basically differentiated in visceral (VLM), ocular (OLM), cutaneous (CLM) and neuronal larvae migrans (NLM).

Visceral (VLM) and ocular (OLM) larvae migrans

In this paragraph, basic information on the etiological agents of VLM and OLM syndromes are reported; the biology of these species is available in a specific chapter. Larvae of *Toxocara* species, and specifically the dog nematode, *T. canis* and, to a much lesser extent, the *T. cati* from cats, are causative agents of visceral (VLM) and ocular (OLM) larvae migrans syndromes. The clinical outlook of toxocariasis is classified in accordance with the affected organ. VLM includes organ diseases while OLM displays limited symptoms and findings related to ophthalmological findings. Human cases by *T. cati* are rather rare, therefore hereafter we will refer only to *T. canis* infections. A third form of disease called Covert Toxocariasis (CT) has been described mainly in children with positive



Fig. 8 Cutaneous *larva migrans* due to *Ancylostoma ceylanicum*.

Toxocara serology and the combination of several clinical signs as abdominal pain, headache, behavior disturbance and cough (Zheng et al., 2020).

Cutaneous (CLM) larvae migrans

Ancylostoma spp. are responsible for the CLM infections, mainly due to parasitic species of dogs and cats that accidentally penetrate human skin. The infection is typical of warm climate regions and may be determined by *A. braziliense*, *A. ceylanicum*, *Ancylostoma caninum* and by members of the genus *Uncinaria*. The dermatitis associated with these zoonotic infections are known as “creeping eruption” or “serpiginous rash”: after the penetration, larvae cannot disseminate through the blood circulation, and remaining under the skin, their slow migration is clearly visible as a serpiginous line, depicting the route travelled by the nematode. Pruritus is often intense and scratching may provoke bacterial hyper-infections and impetigo.

Risk factors are a close contact with soil and barefoot walking on the beach in tropical areas.

Neuronal (NLM) larvae migrans

NLM larvae migrans are due to accidental infection with the ascarid *Baylisascaris procyonis*. The raccoon roundworm is increasingly recognized as a cause of often fatal NLM especially in children and of OLM in adults. Humans become infected by accidental ingestion of infective *B. procyonis* eggs from raccoon fecal areas, contaminating food consumed fresh and not properly cleaned. The infection in human is characterized by somatic tissues migration and invasion of the central nervous system. The larvae can reach a large size in the central nervous system, causing an acute fulminant eosinophilic meningoencephalitis. Usually, the prognosis is severe despite of treatment. To date, even after anthelmintic treatment, neurological damages seem unavoidable.

Conversely to other geohelminths, the life cycle of *B. procyonis* can be monoxenic but can accidentally involve intermediate hosts, such as rodents, rabbits or birds, where the larvae provoke as well the formation of eosinophilic granulomas in several tissues.

The clinical picture associated to this infection includes VLM, NLM and OLV. VLM often involves damages in heart, lungs, intestinal wall and lymph nodes, among others. NLM usually develops after 2–4 weeks after infection.

Treatment of geohelminthiases

Intestinal infections due to soil transmitted nematodes are treated using anthelmintic drugs. Among the most commonly used are benzimidazoles as albendazole and mebendazole. Albendazole is a vermicide that act in several different manners, by causing degenerative alterations in the intestine of the worm by inhibiting tubulin assemblage, depleting the glycogen stores, blocking egg production and inhibiting the Krebs cycle. Mebendazole mechanisms of action is similar to albendazole. Both drugs cause ATP depletion and nematode paralysis.

Ivermectin derives from the avermectin family of compounds and is routinely used to control parasitic worms in the gastrointestinal tract of ruminant animals worldwide. The wide and successful use of this treatment for the control of river blindness and lymphatic filariasis makes their inventors as worthy to be awarded of the Nobel Prize in Medicine in 2015. Ivermectin is a macrocyclic lactone which interfere with nervous system and muscles of the nematodes by inhibiting the neurotransmission through an alteration of ion chloride channels, thus causing the nematode paralysis as benzimidazoles.

The above compounds are routinely used not only for the specific treatment of human cases of helminthic infections, but also in massive deworming programs that have been implemented in the last decade, known as Massive Drug Administration (MDA), with the commitment of pharmaceutical companies to donating medicines specifically targeted to NTDs.

Nowadays, self-treatment for intestinal worm infection is a common public health issue, and the delivery of donated drugs through mass drug programs for soil-transmitted helminths exceeds 1 billion doses annually (Bundy et al., 2017). Specific donations are targeted at school-age children and comprise 400 million treatments of albendazole, 200 million treatments of mebendazole as well doses of ivermectin, the last intended to be used against onchocerciasis and filariasis but with an effect also on STHs.

In this context, monitor plans to detect the potential emergence of drug resistance in treated populations are mandatory. Resistance to benzimidazoles is in fact a relevant issue and it has already been observed in livestock (Morgan et al., 2019). Excluding a portion of the community from treatment is a strategy that can slow the emergence of drug resistance, although it may have an adverse effect in the success of elimination programs.

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Relevant websites

<https://www.cdc.gov/parasites/sth/index.html>—CDC.

<https://www.who.int/news-room/fact-sheets/detail/soil-transmitted-helminth-infections>—WHO.

Giardia duodenalis

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Introduction

Giardia is a one-celled protozoan that can cause diarrheal illness in humans and animals. Giardiasis is often reported in locations with low socio-economical conditions, but it can be acquired worldwide. Repeated *Giardia* infections in children can affect their growth, nutritional status, and cognitive functions. In most instances transmission is waterborne, but giardiasis can also be acquired via ingestion of contaminated foods and by exposure to infected persons or animals. Metronidazole is the most frequently used antibiotic for treatment of giardiasis, but prevention can be achieved by following good sanitary practices and avoiding ingestion of untreated water. Giardiasis has been a notifiable disease to the Centers for Disease Control and Prevention (CDC) in the U.S. since 1993. In 2018, 15,579 cases were reported. The incidence of giardiasis in the U.S. is less than seven cases per 100,000 population since 2011 (CDC, 2018).

In this article we address aspects of *Giardia* and giardiasis that will provide a better understanding of their biology, epidemiology, and control. Large strides in the molecular biology of this parasite have been made recently but will not be described here.

Biology

The first account of the description of *Giardia* occurred in 1681 when Anton van Leeuwenhoek observed his own diarrheal stool. *Giardia* is a one-celled protozoan that belongs to the Phylum Metamonada in the Order Giardiida subclass Diplozoa (Thompson and Monis, 2012). There are seven species of *Giardia* (*G. agilis* in amphibians, *G. psittaci* and *G. ardeae* in birds, *G. muris* and *G. microti* in rodents, *G. peramelis* in marsupials, and *G. duodenalis* in mammals). *Giardia duodenalis* is grouped based on its genome and host specificity and species names have been proposed. *G. duodenalis* assemblages A and B infect humans and other mammals, whereas C and D are found in canids (*G. canis*), E in hoofed animals (*G. bovis*), F in cats (*G. cati*) and G in rodents (*G. simondi*), and H in pinnipeds (Thompson and Monis, 2012).

The life cycle of *G. duodenalis* (also called *G. intestinalis* and *G. lamblia*) consists of two stages: the trophozoite and the cyst. Susceptible individuals and animals acquire the infection when water or foods containing cysts are ingested (Fig. 1). The cysts excyst in the small intestine when exposed to gastric acid and bile salts. Two trophozoites are released from each cyst, multiply by binary fission, and colonize the intestinal epithelia of the duodenum and jejunum. *Giardia duodenalis* trophozoites are pyriform in shape, measuring 12–15 µm long. They have eight flagella, a ventral adhesive disk that is used for attachment, two axonemes and two median bodies. Trophozoites feed and multiply in the lumen of the small bowel or they attach to the intestinal mucosa with a ventral sucking disk. Although there is no cell invasion, massive infections result in malabsorption by reducing the surface area for absorption of nutrients. Trophozoites also elicit metabolites that are responsible for the development of diarrhea. They are environmentally sensitive and briefly survive outside the host. Cysts are considered as the main stage of infection. As a result, infection can occur upon exposure to surfaces on which *Giardia* cysts are present or by accidental ingestion of contaminated foods.

Cyst are formed in the colon and is excreted in the feces. The cysts are ovoid, measuring 10–14 µm (wide-long). Each cyst contains four nuclei. The axonemes and median bodies are prominent (Fig. 2). Cysts are the environmentally resistant form of the parasite. They can survive at 0–8 °C for about 2 months and are often associated with waterborne outbreaks. Infections may be caused by consumption of as few as 10–100 viable *Giardia* cysts (Acheson, 2009).

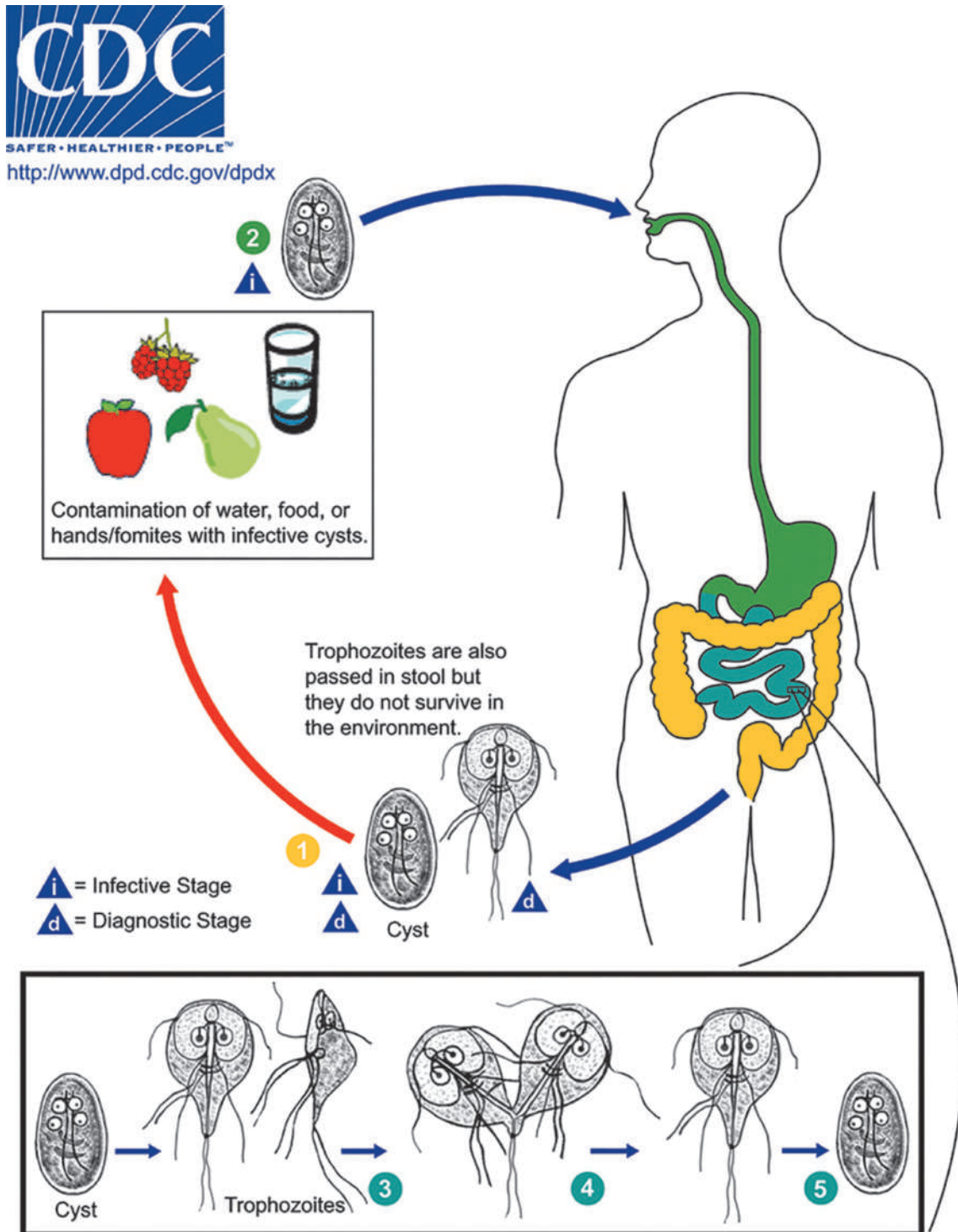


Fig. 1 Life cycle of *Giardia duodenalis*. Image courtesy of DPDx, Centers for Disease Control and Prevention (<https://www.cdc.gov/dpdx>).

Giardiasis is characterized by foul-smelling watery diarrhea, gas, abdominal pain, vomiting, nausea, steatorrhea, and dehydration. The prepatent period is usually 3 days to 3 weeks (Thompson 2008) and infection can last from a few days to several months. Although most cases can resolve quickly, in some instances prolonged giardiasis can result in weight loss, retarded growth, and delayed cognitive development.

The symptoms and oocyst shedding can vary with time and recurrent episodes of diarrhea are frequent in individuals with giardiasis (Robertson et al., 2010). Infection rates have a tendency increase in late summer and early fall (Naumova et al., 2007; Noradilah et al., 2019).

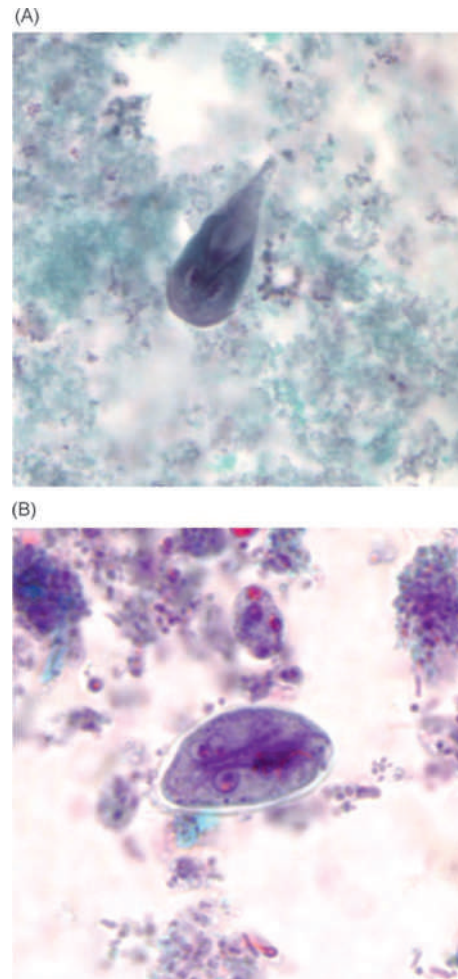


Fig. 2 *Giardia duodenalis* stained with trichrome. (A) Trophozoite, (B) Cyst. Images courtesy of DPDx, Centers for Disease Control and Prevention (<https://www.cdc.gov/dpdx>).

Epidemiology

Giardiasis is the most common protozoal diarrheal disease in humans worldwide (Ankarklev et al., 2010; Caccio et al., 2005; Torgerson et al., 2015). Along with *Cryptosporidium*, *Giardia* species can lead to a significant morbidity and mortality rates in low income as well as industrialized countries (Caccio et al., 2005). *Giardia* has a higher prevalence in areas where sanitation is poor and adequate water treatment facilities are lacking (Ortega and Adam, 1997). It causes an estimated 8×10^8 global cases of giardiasis per year (FAO/WHO, 2014).

Giardia duodenalis is prevalent in children in low income countries (Gilman et al., 1985; Quihui-Cota et al., 2017). This prevalence has been estimated to be 15–20% in children younger than 10 years of age (Quihui-Cota et al., 2017). A cross-sectional study done from February 2013 to September 2014 in northwest Mexico found that 23% of 173 children tested were infected with *G. intestinalis* (Caccio et al., 2005). In a seroprevalence study, 40% of Mexican children younger than 10 years of age and 70% of adults older than 25 years of age had a past or present infection (Cedillo-Rivera et al., 2009). Examinations of stools from children in Bangladesh, Zimbabwe, and Nepal showed prevalence rates of *Giardia* in approximately 20% of the children (Gilman et al., 1985; Tandukar et al., 2018). A recent study reported that almost 64% of 97 individuals infected with *Giardia* in Algeria were children 1–10 years of age (Belkessa et al., 2021). Most patients positive for giardiasis are asymptomatic while others may suffer severe abdominal pain, chronic diarrhea, and/or weight loss (Belkessa et al., 2021; Quihui-Cota et al., 2017; Tandukar et al., 2018; Thomas Iv et al., 2014).

Giardia duodenalis infections are estimated to cause 2–5% of gastrointestinal infections in industrialized countries (Ortega and Adam, 1997). According to the New Zealand Public Health Surveillance committee, more than 30 cases of giardiasis per 100,000 population occur every year in that country. In 2018, children 1–4 years of age (106.0 per 100,000) and adults 30–39 years of age (51.7 per 100,000) had the highest infection rates. Southwest England has been reported to have the highest rate of *Giardia* infections per 100,000 population in England and Wales, with 20.1 cases per 100,000 population. Infections were highest for males

45 to 49 years of age in 2017 (*Giardia* data 2008 to 2017, 2021). Waldram et al. (2017) reported that the prevalence of *Giardia* infection in Lancashire households was 30%. Highest *Giardia* infection rates were associated with households having a child under 5 years of age and/or attending a daycare facility (Waldram et al., 2017). A 3-year study in Canada found a *Giardia* sp. infection rate of 19.6 per 100,000 population. A significant seasonal variation was observed, with a peak in late summer or early fall (Laupland and Church, 2005; Rose et al., 1998). Almost 1.2 million cases of giardiasis and 3581 hospitalizations are estimated to occur annually in the U.S. (Adam et al., 2016). In 2018, there were 15,579 confirmed cases of giardiasis in the U.S. The highest incidence of giardiasis was among children (age 1–4 years) for both males and females: 10.7 and 8.1 cases per 100,000 population, respectively (CDC, 2021).

Ingestion of contaminated water and direct fecal-oral transmission are the most prevalent routes of infection. *Giardia duodenalis* is considered to be a common cause of endemic waterborne diarrheal disease. Giardiasis often infects travelers to areas of endemicity. Studies of travelers to St. Petersburg in the former Soviet Union have demonstrated giardiasis-related symptoms (Brodsky et al., 1974; Ortega and Adam, 1997). Hikers, hunters, and campers are also at risk for *Giardia* infection (Mosites et al., 2018; Ortega and Adam, 1997). For instance, during the summer of 2007, a giardiasis outbreak affected attendees at a private recreational camp in California. Two days before this outbreak began, the camp had installed a slow-sand water filtration system that was filled with sand that contained substantially elevated total coliform and turbidity levels (Karon et al., 2011). Transmission can also occur among oral-anal sexual practices.

Prevalence

Water

Depending on the year, *Giardia* spp. are often the leading cause of parasite-linked outbreaks of gastroenteritis associated with drinking and recreation waters (Atwill et al., 2012; Wolfe, 1992; Schets et al., 2004; Shields et al., 2008). Several reported outbreaks of giardiasis have been associated with these waters (Adam et al., 2016; Ligda et al., 2020; Ngowi, 2020). Among 381 reported outbreaks attributed to waterborne parasitic protozoa between 2011 and 2016, *Giardia* spp. were the etiological agent in 37% (Efstratiou et al., 2017). Contaminated water sources include unfiltered surface and household water, recreational water and rivers. *Giardia duodenalis* has been detected in river water in the U.S. (Reses et al., 2018; Rose et al., 1998; Rosenthal et al., 2017), Romania (Imre et al., 2017), Germany (Strathmann et al., 2016), and Greece (Ligda et al., 2020), among other countries.

Recreational and drinking water-related outbreaks have been linked to *Giardia* in Canada. *Giardia* cysts were found in water from 53 of 72 municipalities; 18.2% of the positive samples were from treated water (Wallis et al., 1996). Portugal has experienced *Giardia* contamination in drinking water. The source of contamination was determined to be river water (Almeida et al., 2010). A South Korean province had its first documented parasitic waterborne outbreak in 2010. The incident was linked to use of a provisional water supply (Cheun et al., 2013). Others have reported *Giardia* positive samples of water taken from recreational lakes, splash parks, pools, and ponds (Ehsan et al., 2015; Srisuphanunt et al., 2010).

Hamilton et al. (2018) reported that 77% of untreated sewage water samples and 61% of treated effluent samples were positive for *Giardia* (Hamilton et al., 2018). Other studies reported that viability of *Giardia* cysts is retained for almost 9 weeks in 4 °C water (Olson et al., 1999) and that a prolonged survival of *Giardia* cysts is consistent with low temperature (<10 °C) in various types of ecological waters (deRegnier et al., 1989). Waterborne outbreaks of giardiasis can occur throughout the entire year (Wolfe, 1992), suggesting that *Giardia* cysts are resistant to environmental degradation.

Food

Although bacteria are generally associated with foodborne disease outbreaks, many cases of gastroenteritis caused by protozoa have occurred. Raw vegetables and fruits, chicken salad, clams, and oysters have been linked to giardiasis outbreaks (Adam et al., 2016). Of 15 foodborne giardiasis outbreaks, 20% implicated vegetables while 13.3% implicated fruits. Adam et al. (2016) determined that the most common sources of infection for such outbreaks are restaurants and delicatessens (44.4%) followed by private homes (19.4%). In another study, out of 642 vegetable samples, including spinach, wild cabbage, lettuce, and others obtained from street vendors in China, 73 samples were found to be positive for *Giardia*, with a prevalence of 11.4% (Li et al., 2020). *Giardia* cysts have been found in raw vegetables and fruits in India (Utaaker et al., 2017), Spain (Amoros et al., 2010), Ghana (Duedu et al., 2014), Jordan (Ismail, 2016), the U.S. (Mintz et al., 1993; Porter et al., 1990), and other countries. Outbreaks have been linked *Giardia* infections to consumption of contaminated shellfish and clams (Manore et al., 2020; Schets et al., 2007).

Human pathogenic parasites in surface and untreated waters used for irrigation can result in contamination of food crops that are minimally processed (Mota et al., 2009; Thurston-Enriquez et al., 2002). An increase in consumption of raw fruits and vegetables can increase the risk of infection. In fact, the majority of fruit intake in the U.S. is fresh fruit and juices, and related sales accounted for about half of the total produce segment sales in 2020 (Shahbandeh, 2021). The U.S. per capita consumption of fresh vegetables was 153.32 pounds in 2019 (Shahbandeh, 2019). An increase in domestic and international trade can result in a higher exposure to produce contaminated with foodborne pathogens. Produce arrives at destination within hours or days after harvest, and agricultural practices in countries where production and harvest occur directly affect the microbial safety and health of consumers at final destination. Oysters are preferentially consumed raw, which can increase the risk of infection. Transmission may also result from cross contamination by infected, asymptomatic food handlers (Figgatt et al., 2017).

Soil

As *Giardia* is ingested, it reproduces in the host and the infective cysts are then shed in the environment, including various types of soil. Risk of soil contamination occurs when effluents such as water runoff and wastewater (Dai and Boll, 2003) carrying fecal matter are applied. Soil can consequently become a potential vehicle for *Giardia* infections. Several studies have reported the presence of *Giardia* cysts in soil (Barwick et al., 2003; Dib et al., 2008; Ferreira et al., 2018; Olson et al., 1999). Olson et al. (1999) reported that *Giardia* cysts can remain viable in soil for up to 9 weeks at 4 °C. The calculated daily risk for acquiring giardiasis is 2.3×10^{-1} when 0.02 g of soil containing *Giardia* cysts is ingested (Balderrama-Carmona et al., 2014). The risks for *Giardia* and *Cryptosporidium* infections were reported to be considerably higher in children than in adults (Balderrama-Carmona et al., 2014). Inadequate sanitary conditions such as the absence of proper water and sewage services increase the risk of soil contamination. Higher cyst concentrations are detected in soil with high moisture (Armon et al., 2002). Children and farmers who play in or work with soil, are more likely to have intestinal protozoal parasitic infections than those who do not (Shrestha et al., 2012; Tegen et al., 2020).

Soil type, pH, temperature, and plant roots, as well as water content and water flow within the soil, play a major role in the motility and survival of *Giardia*. Active motility as well as surface properties and their association with the soil particles are also considered major factors affecting survival of microorganisms in various types of soil (Mawdsley et al., 1995).

Animals

Although humans are the major reservoir for *Giardia*, several studies have shown that some animals are also capable of carrying this pathogen. Different species of *Giardia* naturally infect beavers (Dunlap and Thies, 2002; Fayer et al., 2006) and coyotes (Trout et al., 2006). Dunlap and Thies (2002) reported the presence of *Giardia* sp. in 30% of samples from beaver and 67% of samples from nutria in Texas, U.S. Samples positive for *Giardia* have also been reported in dogs (Leonhard et al., 2007; Palmer et al., 2008; Smith et al., 2020) and cats (Saleh et al., 2019; Tangtrongsup et al., 2020), as well as sheep and cattle (Godinez-Galaz et al., 2019; Santin et al., 2012). A meta-analysis study found that prevalence of *Giardia* in cattle ranges from 9% to 73% and prevalence ranges from 45% to 100% of farms (Geurden et al., 2010b). Wade et al. (2000) reported that out of 109 dairy cattle farms in the U.S., 70% were positive for *Giardia* spp. Animals with ages between 5 days through adults have been found to be positive for *Giardia* sp. cysts (Wade et al., 2000). Out of 278 fecal samples from pre-weaned dairy calves, 14.4% were positive for *G. duodenalis* (Zhong et al., 2018a). The occurrence of *G. duodenalis* in adult goats has been reported to be 14.9% (Zhong et al., 2018b). The frequency of *G. duodenalis* in cattle can lead to economic losses, as this parasite causes intermittent diarrhea and weight loss (Geurden et al., 2010a). Several *Giardia* spp. have also been found in rodents (Helmy et al., 2018; Ma et al., 2018). *Giardia duodenalis* was detected in approximately 11% of bamboo rats in China (Ma et al., 2018).

Although many farm animals and pets can be infected with the same species of *Giardia* that infect humans, these species may still differ at the molecular level (Cooper et al., 2010). It is important to detect the genotype of the species causing infection to properly determine whether farm animals actually play a role in human giardiasis. Nevertheless, the species detected in rodents by Ma et al. (2018) was later identified as assemblage B for the species also identified in human samples (Cooper et al., 2010), indicating that rodents are a potential source of zoonotic infections. Several studies have reported that animals carrying *Giardia* species are potential sources of zoonotic and anthroponotic disease (Godinez-Galaz et al., 2019; Tumova et al., 2018).

The reader interested in immunological aspects of host-*Giardia* relationship are referred to (Argüello-García et al., 2020).

Diagnosis of giardiasis

Diagnosis of giardiasis in humans and animals is done by the detection of cysts in feces. This can be accomplished by conventional coprological tests. Examination of clinical samples (stools) can be achieved by direct examination of feces using bright field microscopy. If a sample of watery diarrhea is being examined, trophozoites in motion and cysts can be clearly observed. In asymptomatic cases, cysts are more likely to be present in stool samples. Morphological features can be clearly observed if iodine is used in a wet mount. Routinely, fecal samples are collected and preserved using a fixative such as sodium acetate-acetic acid-formalin (SAF), polyvinyl alcohol (PVA), 10% buffered formalin, or merthiolate-iodine-formalin. Repeated testing may be required for an accurate diagnosis, as cyst shedding is not consistent (Hoosyar et al., 2019).

Fecal concentration methods are especially useful when a low number of cysts is being excreted. Flotation and sedimentation methods are described in most parasitology manuals. Procedures often used worldwide involve flotation in a zinc sulfate solution and sedimentation, e.g., formalin-ether sedimentation. In research laboratories, sucrose density gradients have been used to collect large numbers of cysts. *Giardia* in stool smears can also be detected with stains such as trichrome and iron hematoxylin.

A variety of commercial kits are available for detecting *Giardia*. Platforms for these kits can be based on enzyme immunoassays, rapid immunochromatographic cartridge assays, and direct fluorescent assays. Molecular assays (qPCR and PCR) are also used for examination of clinical samples (human and animal) and for research purposes. One of the latest platforms for analysis of human fecal samples is the BioFire FilmArray Gastrointestinal Panel where 22 target microorganisms (bacteria, parasites, and viruses), including *Giardia*, can be tested simultaneously (Biomerieux, Inc., Durham, NC).

Patients with symptoms consistent with giardiasis and negative parasitological results, endoscopic aspiration or the string test (entero-test) can be used to determine the presence of *Giardia* trophozoites. For the entero-test, the patient swallows a capsule

containing a nylon string of 90–140 cm. The capsule dissolves and the string is released reaching the duodenum. Four hours later or overnight the string is removed and examined for the presence of *Giardia* trophozoites. The entero-test, duodenal aspirate, intestinal biopsy and intestinal impression have been used for many years; however, they are invasive and expensive tests that don't give much benefit in diagnosis of giardiasis (Jahan et al., 2014).

Detection of *Giardia* in large volumes of drinking or irrigation water can be accomplished by filtration using filters with less than 1 μm porosity (EPA, 2012, 2014). Methods for testing water samples use filters to trap cysts and other small particles. The concentrated samples are then removed from the filters using an elution solution containing a detergent and antifoam. The eluted material is concentrated by centrifugation and the pellet is examined using immunofluorescent assays or molecular assays specific for *Giardia*.

Sanitation

Food processors use various treatments to inactivate foodborne pathogens, including *Giardia*, in food, water, and the environment. *Giardia* can survive at ambient temperature and in cold storage environments. Consequently, inactivation of *Giardia* and other parasites under these conditions is critical to public health because survival may result in sources to contaminate food. Physical, chemical, and biological treatments, alone and in combination, have been used in the food industry to inactivate *Giardia* species.

The Centers for Disease Control and Prevention (CDC) recommends boiling water for 1–3 min to kill *Giardia*. Water would then need to be stored in clean and sanitized refrigerators. In addition, cooking food will render *Giardia* cysts non-viable (Wolfe, 1992). Water, however, is consumed as an ingredient and used as a transport medium and sanitation aid (Kirby et al., 2003), which makes heat treatment inefficient for use in processing plants. Water from municipal suppliers and private wells, as well as treated wastewater and treated surface water, are various sources used in the food industry (Kirby et al., 2003). Because *Giardia* is a recognized waterborne parasite and most of the time giardiasis outbreaks are linked to its presence in water, the food industry constantly focuses on finding reliable treatment for its inactivation.

Conventional water treatment plants use coagulation, sedimentation, and filtration methods to remove *Giardia* (Swertfeger et al., 1999; Wolfe, 1992). A full-scale conventional water treatment process reduces the number of *Giardia* cysts (States et al., 1997). However, treated water can still contain low numbers of cysts so recycling of backwash water is a potential source for contamination of the treatment plant intake. A combination of physical and chemical treatments to remove *Giardia* in water sources is considered to be more efficient (Finch and Belosevic, 2001).

Chlorine based sanitizers have been used to reduce the number of *Giardia* and other microorganisms in water. A study conducted on wastewater reported that *Giardia* is inactivated when with chlorine at 0.5 mg/L for approximately 1 h (Adeyemo et al., 2019). Others have reported that along with exposure to 1.5 mg/L or higher of chlorine, temperature (25 °C) and pH (6–8) are important factors contributing to inactivation of the parasite. Statistical models for predicting survival and inactivation of *Giardia* as affected by exposure to chlorine have been developed (Haas, 2004; Haas and Heller, 1990). *Giardia* cysts are fairly resistant to treatment with chlorinated water. It is recommended that other treatments besides chlorine be used for its inactivation (Moorehead et al., 1990; Ortega and Adam, 1997).

The relative ineffectiveness of chlorine-based sanitizers for inactivation of *Giardia* cysts has led to the evaluation and adoption of alternative chemicals for water treatment. One alternative sanitizer is treatment with molecular ozone as a biocide against protozoan parasites. Ozone is a strong oxidant used in water treatment plants to enhance the removal of some naturally occurring organic and inorganic particles, algae, and other microorganisms (Can and Gurol, 2003). Treatment of surface water with 5 mg/L ozone at 23 °C and pH 7.1 inactivates 78.5% of *Giardia* cysts (Nakada et al., 2019). Kondo Nakada et al. (2020) reported that non-viable cysts of *Giardia* are more frequently detected in ozonated water (80%) than in chlorinated (68.2%) or raw water (37.7%). The reduction of calculated risk of acquiring *Giardia* infections from ozonated water is 27.5-fold and for chlorinated water is 13-fold compared to raw water (Kondo Nakada et al., 2020).

Another alternative for the removal of *Giardia* from water and wastewater is the use of ultraviolet (UV) light in treatment plants. Adeyemo et al. (2019) reported that the lethality of UV irradiation of *Giardia* cysts is higher than that of chlorine. Other studies have shown that 99.9% of *Giardia* cysts are killed by UV irradiation at 20–40 mJ/cm² (Campbell and Wallis, 2002). A UV radiation treatment of 50 mJ/cm² inactivated 75% of *Giardia* cysts (Rahdar and Daylami, 2016). *Giardia* cysts are less resistant than trophozoites to UV light treatment at 10 mJ/cm². Treatments exceeding 20 mJ/cm² prevent the excystation of *Giardia* cysts (Einarsson et al., 2015). One of the main advantages of UV treatment in water facilities is efficiency in inactivating cysts. UV treatment is a physical process that does not rely on chemicals. The formation of by-products resulting from treatment has not been reported. One limitation of the treatment is its dependence on a secure power supply, which is not always readily available in low income countries.

Other methods, including Thermophilic Windrow Composting (Van Herk et al., 2004) as well as various new technologies such as the hot bubble pilot plant (Garrido Sanchis and Jin, 2020) and the advancement of membrane technology (Betancourt and Rose, 2004), are being used to inactivate *Giardia*.

In summary, management measures are essential to improve cyst inactivation in water and wastewater, particularly when water is used in food and beverage processing and preparation. To prevent contamination, proper management and filtration systems may contribute to decreasing the infection rate and break the transmission cycle and spread of *Giardia*. Good manufacturing and agricultural practices as well as robust cleaning and disinfection of facilities and clinical settings are essential to reduce the risk of contamination and, thus, risk of infection.

Treatment and control of Giardiasis

The common antibiotic regimen for the treatment of giardiasis has been metronidazole for 7–10 days. Tinidazole, albendazole, mebendazole, and nitazoxanide have also been used to treat giardiasis. Refractory infections have been reported in various studies. A study in 95 Spanish travelers returning from Asian countries revealed that 22% had refractory giardiasis. Another similar study in Tropical Disease Units in Spain reported that 20% of infected individuals were refractory to metronidazole or tinidazole (Lalle and Hanevik, 2018).

Inactivation or removal of *Giardia* cysts from water and wastewater significantly reduce the risk of acquiring giardiasis in communities where these interventions are applied. The U.S. Environmental Protection Agency (EPA) Surface Water Treatment Rule requires that water from public water systems be filtered and that surface and well water should be treated to remove/kill 99.9% of *Giardia* present. The EPA has developed methods for detecting *Giardia* in water (1623.1 for drinking water and 1693 for wastewater) (EPA, 2012, 2014).

Common hygienic practices such as washing hands and avoidance of drinking untreated water from rivers or streams can help reduce the risk of infection. Use of adequate protective equipment in hospitals, nursing homes, hospices, and daycare centers minimizes cross contamination. Caretakers of domestic and farm animals also should take precautions to avoid cross contamination.

Development of vaccines for *Giardia* has been an important area of research in recent years. Various target proteins have been evaluated. The *Giardia* cyst wall protein 2 (CWP) and α 1-giardin are among them. The α 1-giardin is a more conserved antigen between isolates and assemblages (Radunovi et al., 2017). *Giardia* undergoes antigenic variation with about 200 surface protein variants (VSP) that results in avoidance of the host immune defenses. A vaccine using a composite of all of these VSP has been developed and tested in cats and dogs. This vaccine prevented infections and reduced chronic giardiasis in animals. When evaluated in animals of a peri-urban community in Argentina, the vaccine not only protected the vaccinated animals roaming in the area, but also was noted that the prevalence of children with *Giardia* living in this community had decreased (Serradell et al., 2016).

Conclusions

Giardia has coexisted with humans and animals since antiquity. In humans, giardiasis is frequently associated with poor socio-economical conditions (Einarsson et al., 2016). In children, repeated *Giardia* infections can result in impaired growth, nutritional status, and cognitive functions (Rogawski et al., 2017). In animals, infection can result in weight loss, poor growth, and death (Ryan and Cacciò, 2013). The zoonotic potential of giardiasis opens the possibility of acquiring the infection while practicing outdoor activities such as contact with infected animals and drinking untreated water.

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Host Immune Responses Against Intestinal Unicellular Parasites and Their Role in Pathogenesis and Protection

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Key intestinal unicellular parasites and their public health significance

A number of intestinal parasites that infect humans are multicellular, soil-transmitted helminths (*Ascaris*, *Ancylostoma/Necator*, and *Trichuris*) together with intestinal unicellular parasites (IUP), mainly protozoa, which have been included in the WHO's Neglected Diseases Initiative (Horton, 2003), cause the highest burden worldwide, requiring massive drug administration for treatment/control (Bundy et al., 2017; Horton, 2003). In this chapter, we review the current state of knowledge of the immune responses and evasion mechanisms pertaining to four major IUP and the diseases these cause in humans and other hosts, and discuss the potential of vaccines to protect against infection and transmission.

Giardia duodenalis (syn. *G. lamblia*, *G. intestinalis*) causes water- and food-borne diarrhoea in humans and a wide range of mammals. The life cycle involves vegetative trophozoites and infective cysts that following ingestion excyst and generates four trophozoites with an adhesive disk that allows its attachment to epithelial cells at duodenum and jejunum. At distal small intestine encystment proceeds and after nuclear replication mature, infective cysts are excreted in faeces and survive outside of the host for several weeks contaminating water and food. Giardiasis can be acute or chronic, and can be self-limiting. Some hosts can carry asymptomatic infections and giardiasis causes ~280 million clinical cases annually and it is more frequent in children (Kirk et al., 2015; Einarsson et al., 2016).

Entamoeba histolytica inhabits the large intestine of humans and other primates. In amebiasis that affects 500 million people worldwide a 10% of infections relate to *E. histolytica*, causing 40,000–100,000 deaths per year, and 90% to other species, particularly *E. dispar* (WHO and OMS, 1998). When the infective cyst is ingested and excysts, trophozoites can colonize without causing clinical signs (asymptomatic) or cause epithelial and mucosal ulceration (dysentery) and eventually invade intestinal tissues, reaching the portal vein causing tissue destruction and abscess formation at the liver. Amebiasis can be fatal if untreated (Ackers and Mirelman, 2006). Symptoms and signs from intestinal amebiasis can include bloody diarrhoea and fulminating dysentery, weight loss and abdominal pain whereas the extra-intestinal outcome is hallmarked by multifocal hepatic granulomata (amoebomata).

Cryptosporidium is a group of apicomplexan alveolate protists causing intestinal alterations (moderate to watery diarrhea, abdominal cramps) with or without respiratory signs. Cryptosporidiosis affects a range of animals including livestock (e.g., calves), dogs, cats, rabbits and birds. Two species cause most human infections: *C. hominis* (formerly *C. parvum* genotype 1) with human-to-human transmission and *C. parvum* with animal-to-human transmission (Ryan et al., 2016; Sow et al., 2016) causing up to >200,000 deaths per year (Guerrant et al., 1999). In its life cycle, infective oocysts in water or food are ingested and excyst in the small intestine or associated ducts, after which sporozoites are released and penetrate epithelial cells, transform briefly to trophozoites and then asexually replicate, as type I meronts, and merozoites and following maturation (type II meronts) enter a

sexual cycle, in which thin- and thick-walled oocysts of which the former re-enter in the infective cycle and the latter are released in faeces (Ryan et al., 2016). Cryptosporidiosis is endemic in developing countries of Sub-Sahara and South Asia and causes >200,000 deaths or can lead to a failure to thrive and/or impaired cognition in young children (Guerrant et al., 1999; Sow et al., 2016).

The non-flagellated, stramenophile *Blastocystis* is an emerging pathogen that infects humans, domestic (e.g., pigs) and companion (e.g., dogs) animals worldwide. Vacuolar and/or granular stages predominate in stools, amoeboid forms are rare but associated with symptomatic cases, infective cysts remain viable for 2–3 weeks outside the host and contaminate water and food. Its life cycle likely involves faecal-oral transmission and blastocystosis' symptoms include diarrhoea, abdominal pain, bloating, vomiting, nausea and/or anorexia and less frequently dermatological conditions such as urticaria, intense itching and skin redness (Stensvold et al., 2009; Verma and Delfanian, 2013). As with most intestinal protozoa, *Blastocystis* infection can be asymptomatic (Stensvold et al., 2009) and 1 billion people is likely affected worldwide (Dogruman-Al et al., 2010).

On the basis of the relatively similar life cycle but distinct pathogenic potentials displayed by these IUPs, unravelling pathological and protective immune responses is a hallmark task to design and exploit novel control strategies for the diseases they cause.

Giardia duodenalis

Giardia duodenalis is one of the major causes of protistan infections worldwide, causing giardiasis. This infection affects predominantly children in low income countries, leading to malnutrition and decreased growth/development. Giardiasis is often self-limiting, since host defence mechanisms, which include innate and adaptive immunological components, are activated upon infection. The final outcome of this infection involves the interplay between activation of immune mechanisms, the development of immunopathology and changes in the composition of the microbiota in the infected host (Fink and Singer, 2017; Barash et al., 2017). Giardiasis also constitutes a significant risk factor for post-infection irritable bowel (PI-IBS) and chronic fatigue syndromes (Chen et al., 2013; Allain et al., 2017; Hanevik et al., 2017). Advances on understanding how the innate response to *Giardia* infection is initiated and how it contributes to the development of adaptive immunity have been reviewed in detail elsewhere (Allain and Buret, 2020; Lopez-Romero et al., 2015; Roxstrom-Lindquist et al., 2006; Singer et al., 2019; Solaymani-Mohammadi and Singer, 2010). Here, a brief description of the immune mechanisms activated by this parasite is given in the next sections.

Innate immune mechanisms against *Giardia duodenalis*—Cells and effector molecules

Knowledge of the immune responses to *Giardia* have been based on in vitro investigations, in vivo studies of zoonotic *G. duodenalis* assemblages (A and B) or with *Giardia muris* in mice and gerbils and studies of cohorts of humans infected with *G. duodenalis* (Faubert, 2000). In mice, immunity to *Giardia* occurs in two major phases: a B-cell-independent phase early after infection followed by a T-cell-dependent antibody response and activation of CD8+ cells (Eckmann 2003; Muller and von Allmen, 2005). Initially, several defense mechanisms which involve innate immune cells and other molecules play a role in immunity to *Giardia*. These include intestinal epithelial cells (IECs), mucus, nitric oxide (NO) production, antimicrobial peptides, Complement, dendritic cells, mast cells and/or macrophages (Fig. 1) (Aley et al., 1994; Andersen et al., 2006; Eckmann et al., 2000; Kamda et al., 2012; Li et al., 2004, 2018; Stadelmann et al., 2013). As *Giardia* infection progresses, hosts develop mucosal humoral and cellular immune responses, which include the production of specific antibodies particularly IgA against parasite antigens, a balanced response of antigen-specific CD4+ T cells, the release of cytokines, and the activation of CD8+ cells (Fig. 1) (Faubert, 2000; Jimenez et al., 2014; Kamda et al., 2012; Roxstrom-Lindquist et al., 2006; Singer and Nash, 2000a; Zhou et al., 2007).

A first line of defense by the immune system include the natural surface barriers. In giardiasis, a major component of these are IECs, which express pattern-recognition receptors (PRRs) and initiate innate immune responses through the production of chemokines. Microarray analysis revealed that exposure of human colon carcinoma, CaCo2 cells, to *Giardia* trophozoites resulted in an increased expression of chemokines, including CXCL1-3, CCL2, and CCL20 (Roxstrom-Lindquist et al., 2005). In addition, antimicrobial peptides produced by specialised IECs have been detected, specifically the α -defensins cryptdin-2 and -3, which reduced *Giardia* viability in vitro (Aley et al., 1994) (Fig. 1A). IEC exposed to *Giardia* produce also matrix metalloprotease 7, a mediator known to activate α -defensin (Tako et al., 2013). Additionally, a brief exposure of *Giardia* to human epithelial cell cultures triggers the release of three enzymes: arginine deiminase (ADI), ornithine carbamoyltransferase and enolase which participate in host immunoregulation and metabolism in *Giardia* (Ringqvist et al., 2008). All of these studies emphasize the importance of IEC in the induction of immune responses and in the outcome of the infection.

The mucus layer protects the luminal surface of the entire gastrointestinal tract, acting as both physical and chemical barriers that *Giardia* must overcome to reach and attach to the epithelium. Some studies have suggested that intestinal mucus may interfere with trophozoite attachment and colonization (Muller and von Allmen, 2005; Roskens and Erlandsen, 2002). Although mucus protects the host from *Giardia*, it has been shown that, during the acute stage of infection, *Giardia* disrupts the triple intestinal barrier constituted by the microbiome, the mucus and the epithelial lining, which in turn initiate the pathophysiological processes ultimately responsible for disease (Allain et al., 2017) (Fig. 1B).

Recent studies in which human intestinal biopsy tissues, mouse models and human goblet cell lines were used show that the mucus barrier could be bypassed in giardiasis (Amat et al., 2017). *Giardia* cysteine proteases can cleave human mucin-2 and trigger mucus hypersecretion from goblet cells, which in turn leads to a depletion of mucin stores, both in the small and large intestines in a

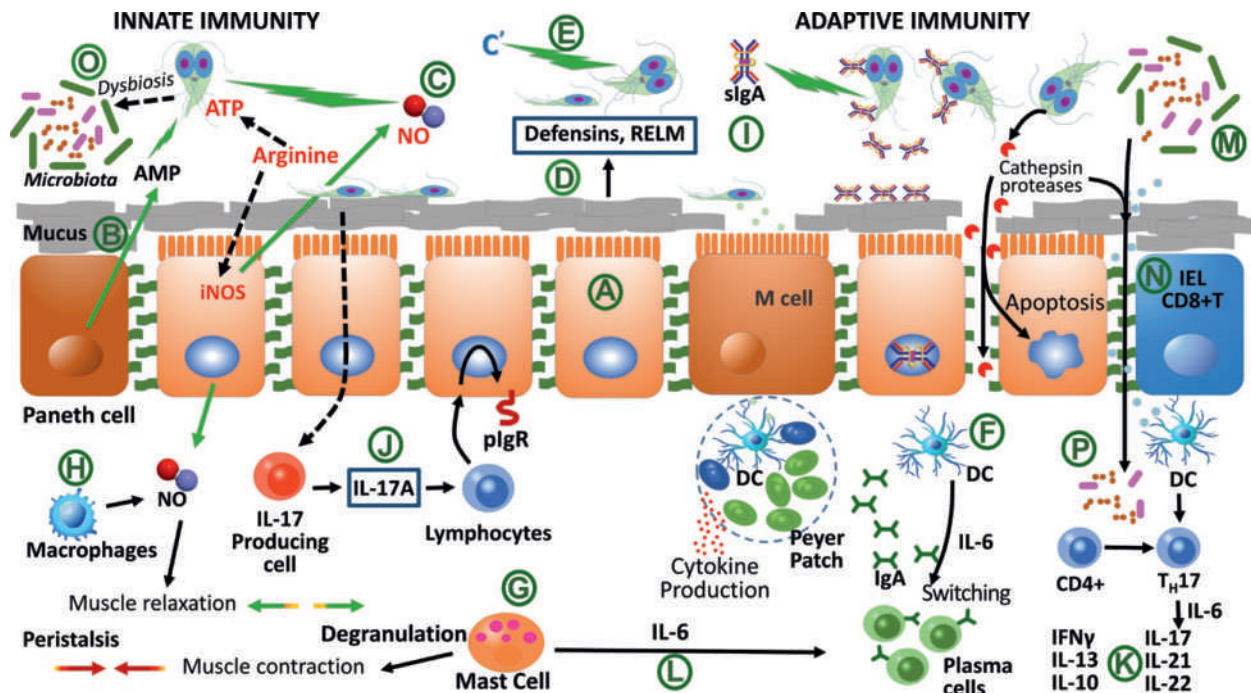


Fig. 1 Immune response mechanisms against *Giardia duodenalis*. IEC cells are a first line of defense of the immune system in *Giardia* infections. These cells initiate innate immune responses through the production of chemokines. Also antimicrobial peptides like α -defensins cryptdin-2 and -3 are produced by IECs and other cells. These reduce *Giardia* viability in vitro (A and D). Mucus may interfere with trophozoite attachment and colonization but *Giardia* cysteine proteases can cleave human mucin-2 disrupting the intestinal barrier initiating the pathophysiology of giardiasis (B). NO inhibits replication and encystment of *G. duodenalis* but the parasite can inhibit the production of epithelial NO by taking up and consuming arginine as a source of energy producing ornithine that competitively inhibit arginine uptake by the intestinal epithelium blocking NO production (C). Complement can kill the parasite (E). Dendritic cells (DCs) can clear *Giardia*, present antigens to T cells and produce several cytokines and participate in the protective immune response against *Giardia* (F). Mast cells are effector cells, release pro-inflammatory cytokines such as IL-6 and degranulation of mast cell promotes peristalsis (G). Macrophages may participate in control of *Giardia* infections and present an increased expression of both nitric oxide synthase 2(NOS2) and arginase 1 (ARG1) (H). Activated T cells produce cytokines such as IL-6 that induces production of IgA which plays a role in *Giardia* clearance in rodent models of giardiasis (I). IL-17 produced by Th-17 CD4+ T cells orchestrate several immune responses that ultimately eliminate the parasite (J). Other cytokines also participate in the control of *Giardia* infections (K). In particular IL-6 participates in parasite clearance (L). Commensal intestinal microbiota participates in colonization and establishment of *Giardia* in the intestine (M), modulates pathogenesis, influences CD8+ T cell dependent-disaccharidases deficiencies (N). *Giardia* induces microbial dysbiosis (O) and secreted cathepsin proteases may aid epithelial translocation of luminal intestinal microbiota (P).

Giardia assemblage-dependent manner (Amat et al., 2017). Interestingly, a cysteine protease-dependent increase in the expression of the MUC2 gene in human goblet cells was observed due to an intracellular depletion of mucin (Amat et al., 2017). Indeed, *Giardia* cysteine proteases can cleave and activate protease-activated receptor-2 (PAR2) on intestinal goblet cells, leading to intracellular calcium release, which leads to an alteration of mucin expression and secretion (reviewed by Allain and Buret, 2020). This information suggests that goblet cell mucin production may be regulated following PAR2 activation. These alterations were associated with facilitated bacterial translocation, indicating that *G. duodenalis* evades and changes host innate defences in the mucous layer, preventing a definite immune response (Amat et al., 2017).

Gut epithelial and immune cells can synthesize NO, which has immunomodulatory and cytotoxic activities (Bogdan, 2001). Initial studies showed that NO inhibited the replication and encystment of *G. duodenalis*; however, the parasite can inhibit the production of epithelial NO by taking up and consuming arginine as a source of energy producing ornithine (Eckmann et al., 2000; Ringqvist et al., 2008) (Fig. 1C) which can competitively inhibit arginine uptake by the intestinal epithelium, thus blocking NO production (Mokrzycka et al., 2010). This block of NO production may have an effect on the proliferation of IEC (Stadelmann et al., 2012). Further, the depletion of arginine from growing CaCo2 cells decreased the number of cells when compared with medium supplemented with arginine or citrulline (Stadelmann et al., 2012), suggesting the induction of apoptosis in these cells. Interestingly, human giardiasis exhibits an increased rate of apoptosis in intestinal epithelial cells, which is a pathogenic mechanism of giardiasis (Troeger et al., 2007). A recent study has shown that host macrophages expressing arginase (Arg1) accumulate in the lamina propria of mice infected with *G. duodenalis* (Maloney et al., 2015). Thus, there is a competition for arginine during in vivo infection by at least three enzymes: ADI in the parasite, arginase (Arg1) and inducible NO synthase (NOS2) in the host.

The potential role of antimicrobial peptides in killing *Giardia* trophozoites was analyzed in vitro (Aley et al., 1994). Human neutrophil peptide (HNP-1), rabbit neutrophil peptide NP-2, bovine indolicidin, and the murine Paneth cell α -defensins, cryptdins 2 and 3, all significantly decreased trophozoite viability in vitro, whereas cryptdins 1 and 6 did not kill the parasites, suggesting a

high degree of specificity for killing trophozoites, despite the extensive sequence similarities between cryptdins (Aley et al., 1994) (Fig. 1D). Similar to its ability to inhibit production of NO, *Giardia* can protect itself from the actions of antimicrobial peptides. Studies of CaCo2 cells co-incubated with trophozoites showed increased human β -defensin 2 (HBD-2) and trefoil factor 3 (TFF3) levels compared with control cells (Manko et al., 2017). This effect could be blocked using inhibitors of cysteine proteases, suggesting that parasite proteases might be involved in this response (Manko et al., 2017). In a recent study, a dose-dependent cleavage of human β -defensin 1 (β -HD1) and α -human defensin 6 (α -HD6) was shown to be triggered by *Giardia*-released cysteine proteases in vitro (Liu et al., 2019). Due to the large numbers of antimicrobial peptides in the intestinal mucosa, further study is required to define the role of these defensins during *Giardia* infection.

The role of complement in the protective immune response against *Giardia* was initially suggested based on parasite killing induced by human sera from individuals with no history of *Giardia* infection, and this was prevented by blocking the complement pathway (Hill et al., 1984) (Fig. 1E). Studies have shown that the activation of complement takes place via both the alternative and the lectin pathways (Hill et al., 1984; Evans-Osses et al., 2010). It was shown that a mannose binding lectin (MBL) binds to trophozoites in vitro, causing lysis via the complement system (Evans-Osses et al., 2010). Other work has shown that MBL is expressed in the murine small intestine during *Giardia* infection (Tako et al., 2013) and that MBL- or complement component C3a receptor (C3aR)-deficient mice have a delayed clearance of *Giardia* and recruitment of mast cells to the small intestine (Li et al., 2016). Furthermore, more recent work showed that mice that lack C3aR have a decrease in T cell responses against *Giardia* antigens following infection, although IgA levels within the small intestine remained unaffected (Li et al., 2016). Taken together, these results demonstrate that the complement system activates immune mechanisms and mediates “downstream” immune responses during infection with *Giardia*.

Several studies have shown that DCs play a role in the clearance of *Giardia*, since IL-6-deficient mice are defective in control the parasite, and that DC are a significant source of IL-6 during infection (Bienz et al., 2003; Kamda et al., 2012; Zhou et al., 2003). On the other hand, in vitro studies have demonstrated that a modulation of DCs cytokine response can be induced by exposure of DCs to *Giardia* protein extract and lipopolysaccharide (LPS). In these studies, a reduction of IL-6, IL-12 and TNF α was observed, while IL-10 production was enhanced compared with LPS-treated control DCs (Kamda and Singer, 2009). Further, the blocking of IL-10R and phosphoinositide-3 kinase (PI-3K) restored the production of IL-12, which suggested that *Giardia* might activate PI-3K in DCs and that *Giardia* may restrict the development of Th-1 cells via IL-12 inhibition (Kamda and Singer, 2009). The reduction of pro-inflammatory cytokines was shown also in human monocyte-derived DCs (mo-DCs) stimulated with *Giardia* extracts. In these reports, a reduction in IL-12/23p40, IL-12p70, and IL-23 levels was observed (Obendorf et al., 2013; Summan et al., 2018). Moreover, a reduction in human mo-DCs survival upon exposure to *Giardia* extracts or live parasites was established, concomitant with an increase in apoptotic DCs. The results of these studies suggest that DCs participate in the protective immune response against *Giardia* (Fig. 1F).

Currently, mast cells are recognized as effector cells of the immune response against *G. duodenalis* in mast cell-deficient mice (c-kitw/wv) (Li et al., 2004, 2007). These mice failed to produce parasite-specific IgA and could not eliminate *G. duodenalis* infection, whereas wild-type mice effectively cleared the infection (Li et al., 2004, 2006, 2007). *Giardia* infections in gerbils (*Meriones unguiculatus*) and mice showed an accumulation of mast cells in the small intestine following infection where mast cell degranulation occurs (Hardin et al., 1997; Leitch et al., 1993; Venkatesan et al., 1997). Given that MBL binds to *G. duodenalis* in vitro, it has been proposed that mast cells are recruited through the activation of complement via the lectin pathway (Li et al., 2016). Other studies have shown an upregulation of protease gene transcripts in mast cells within tissues of the small intestines of mice infected with *G. duodenalis* (Tako et al., 2013).

The contribution of mast cells in resistance to reinfection with *Giardia* was further investigated in studies in which mice infected with *Giardia*, treated with metronidazole and then re-infected exhibited a rapid recruitment of mast cells to the mucosa, along with a substantially reduced intensity of infection compared with mice challenged with *Giardia* but not pre-treated with metronidazole (Li et al., 2014). Mast cells and NO may act together to induce peristalsis (Li et al., 2006, 2007) (Fig. 1G), suggesting a role of mast cells in the immunopathology of giardiasis, particularly in some patients with intestinal motility issues, such as severe cramps (Solaymani-Mohammadi and Singer, 2010). These results suggest that mast cells play a significant role in the protective immune response against *Giardia*, and in the immunopathology of giardiasis.

The role of macrophages in controlling *Giardia* infections was suggested based on the observation of an increase of intestinal macrophages in *G. muris*-infected mice. Increased expression of both NOS2 and ARG1 were recorded (Maloney et al., 2015) (Fig. 1H). Other results showed that IL-10-deficient mice infected with *G. muris* had increased cell numbers expressing CD11b+, CD11c- in the colon but not in the small intestine. Concomitant with the expansion of these cells, an inflammatory response was observed in the deficient mice infected with *G. muris* but not in wild-type mice, suggesting that these cells may regulate the inflammatory process in the infected mice (Dann et al., 2018).

Adaptive immune responses to/against *Giardia* in mammalian hosts—Role of immunoglobulin A, CD4+ and CD8+ cells, and interleukins

It has been established that antibodies contribute to the maintenance of protective immunity against giardiasis (Langford et al., 2002). The production of anti-*Giardia* antibodies has been detected in serum of and secretions from humans infected with *G. duodenalis* or mice with *G. muris* (Faubert, 2000; Nash et al., 1987; Palm et al., 2003; Tellez et al., 2005). Further, in humans with antibody-production related immunodeficiencies chronic giardiasis can develop (Eckmann, 2003; Faubert, 2000).

The main antibody involved in *Giardia* infections is IgA, a primary antibody on mucosal surfaces including those of the intestine (Fig. 1I). Anti-*Giardia* secretory IgA can be detected in human saliva and breast milk (El-Gebaly et al., 2012; Tellez et al., 2003). The role of this antibody in *Giardia*-clearance has been studied in mice infected with *G. duodenalis* or *G. muris*. Mice deficient in the production of IgA displayed a significantly delayed clearance of primary and secondary *G. muris* infections compared with control mice (Langford et al., 2002). Further, mice deficient in the poly-Ig receptor (pIgR) were shown to fail to transport secretory IgA into the intestinal lumen. Such mice develop chronic infection when inoculated orally with *G. muris*. In contrast, a lack of pIgR had no significant effect on *G. duodenalis* infection, suggesting a differential effect of secretory IgA antibodies on these two *Giardia* species (Davids, et al., 2006). Another study (Li et al., 2014), using mice with a deletion in the μ exons of the immunoglobulin heavy chain locus (μ MT mice) and lacking mature B cells, showed that when such mice were infected and re-infected with *G. duodenalis*, parasite burdens were lower soon after infection as compared with control mice that had not been primed with *G. duodenalis*, despite that these animals lack B cells. Collectively, these studies demonstrate that, although IgA-producing B cells are important for the clearance of *Giardia* infections, other immune mechanisms are activated in the infected host to counter *Giardia* infections.

Studies of humans and animals have shown that T-cell-mediated immune responses are crucial for the clearance of *Giardia* infections, since a decrease in CD4⁺ T-cell populations in both human and animals contributes to the development of chronic giardiasis (Singer and Nash, 2000a). In particular, adoptive transfer of CD4⁺ T cells to T-cell deficient mice restores their resistance to infection with *G. muris* or *G. duodenalis* (Singer and Nash, 2000a). Other studies have shown that humans infected during an outbreak of giardiasis caused by *Giardia* assemblage B had *Giardia*-reactive CD4⁺ T cells in peripheral blood, suggesting immunological memory to *Giardia* (Hanevik et al., 2011). Furthermore, the role of T cells in clearing *Giardia* was demonstrated since T-cell-deficient mice were not able to control *G. duodenalis* or *G. muris* infection, whereas B-cell-deficient mice could eliminate the parasites (Singer and Nash, 2000a).

These results suggest a B-cell-independent mechanism of CD4⁺ T cell-control during giardiasis. Interestingly, a mechanism involved in a B-cell-independent T cell activation was suggested in studies of calves infected with *G. duodenalis* that exhibited a significantly increased proliferation of CD4⁺ T cells with an increased expression of interleukin 17 (IL-17). Indeed, a central role of this cytokine in controlling infection in mice infected with *G. duodenalis* has been suggested in several studies (Solaymani-Mohammadi and Singer, 2011; Dann, et al., 2015) (Fig. 1J). Some studies of mice have reported that IL-17 is produced by splenocytes in mice early during *G. duodenalis* infection (Solaymani-Mohammadi and Singer, 2011), and IL-17 mRNA levels in the small intestine early in *G. muris* infection (Dann et al., 2015). Interestingly, other work showed that mice lacking the IL-17A receptor exhibit increased *G. muris* cyst shedding in faeces compared with wild-type mice, suggesting that IL-17A is critical for parasite elimination (Dreesen et al., 2014). IL-17A has been detected in mice infected with *G. duodenalis*, whereas IL-17A deficient mice did not clear the infection with either *G. duodenalis* or *G. muris* (Dann et al., 2015) (Fig. 1K). It has been observed that this cytokine is required for the transport of IgA into the intestinal lumen, since IL-17A deficiency led to marked reduction of faecal IgA levels, as well as an increased intestinal expression of several other potential effectors, including β -defensin 1 and resistin-like molecule β (Dann et al., 2015). These studies clearly demonstrate that IL-17 plays an important role in controlling *Giardia* infections in mice.

The role of IL-17 was also explored in humans. Study of peripheral blood mononuclear cells isolated from people with giardiasis and incubated with *G. duodenalis* extracts showed that IL-17 and TNF- α were upregulated in individuals previously exposed to *Giardia* (Saghaug et al., 2016); the accelerated elimination of *Giardia* from these individuals correlated with high levels of IL-17A and TNF- α , strongly suggesting that IL-17 has an immunoprotective role in humans against giardiasis, as it was observed in mice.

Cytokines other than IL-17 also play important roles during *Giardia* infections (Fig. 1K). Recent studies have shown that IFN- γ levels are increased in patients with symptomatic giardiasis compared with uninfected people (Babaei et al., 2016). IFN- γ and IL-4 appear to play a role because these are elevated during giardiasis in mice (Jimenez et al., 2014; Solaymani-Mohammadi and Singer, 2011). The role of IFN- γ is also supported by the results of a study in Ecuadorian children infected with the soil-transmitted helminth, *Ascaris lumbricoides*, *G. duodenalis*, or both (Weatherhead et al., 2017). In this, co-infection of children with *Ascaris* and *Giardia* induced decreased serum levels of IL-2, IL-12 and TNF- α compared with children infected with only *Giardia* (Weatherhead et al., 2017). In each group, the level of IFN- γ was not found to be reduced. Another study in a cohort of children in Venezuelan children infected with *Giardia* with a light concurrent *Ascaris* infection showed increased serum IFN- γ levels compared with children with *Ascaris*-only infection (Hagel et al., 2011).

Interleukin-6 was demonstrated to have a significant role in parasite clearance, since IL-6^{-/-} mice take a significantly longer time to clear infection compared with wild-type controls (Bienz et al., 2003; Zhou et al., 2003). Bone marrow chimera experiments have demonstrated that IL-6 from bone marrow-derived cells is necessary and sufficient for the control of *Giardia* (Kamda et al., 2012). Interestingly, dendritic cells were identified as a potent source of IL-6 (Kamda et al., 2012). Furthermore, IL-6 in combination with TGF- β , is able to drive the differentiation of naïve T cells into IL-17 producing cells (Weaver et al., 2006) suggesting that IL-6 from dendritic cells helps to drive the development of IL-17 producing CD4⁺ T cells, leading to the control of *Giardia* infection (Fig. 1L).

An increased production of IL-13 was reported in spleen cells from mice infected with either GS or WB strain *Giardia* after in vitro stimulation with parasite extracts (Solaymani-Mohammadi and Singer, 2011). Also an elevated IL-13 mRNA in mice infected with cysts of strain H3 has been reported (Bartelt et al., 2017).

In mice infected with *G. muris* mucosal IL-10 mRNA levels were shown to increase between weeks 2–4 post infection (Dann et al., 2018). IL-10 mRNA was also increased in mesenteric lymph nodes of gerbils infected with *G. duodenalis* (Serradell et al., 2018) and spleen cells from mice infected with this parasite produced IL-10 after in vitro-stimulation with parasite extracts (Solaymani-Mohammadi and Singer, 2011).

IL-8 is the key cytokine that induces inflammation during bacterial intestinal infections (Maaser and Kagnoff, 2002) and its low level during *Giardia* infection partially explains the mild inflammation. It is possible that *Giardia* can actively downregulate the inflammatory response.

It has been shown that CD8+ T cells do not play a role in controlling the infection since $\beta 2m$ -deficient mice which lack class I MHC expression and are devoid of CD8+ T cells maintain such ability. (Singer and Nash, 2000a). However, it has been reported that CD8+ T cells are required for reduction of intestinal disaccharidase activity (Scott et al., 2004) and that this is related to shortening of microvilli lining the gut (Buret et al., 1990; Scott et al., 2000) (Fig. 1N). This pathological condition has been reported in both human infections and in animal infection models and leads to reduced nutrient absorption (Buret et al., 1990, 1992).

Interactions between *Giardia* and the gut microbiota

Some recent studies have highlighted the interactions between *Giardia* and intestinal microbes (Allain et al., 2017; Fink and Singer, 2017) and suggested that a synergy occurs in a two-way mode (Allain and Buret, 2020). In this cooperative interaction, commensal microbiota appear to play a key role in the colonization and establishment of the parasite in the host (Singer and Nash, 2000b), (Fig. 1M) while the transfection of protective anti-*Giardia* microbiota into different mice prevents the establishment of the *Giardia* (Singer and Nash, 2000b), although the mechanisms involved remain to be unravelled. It has also been reported that microbiota influence the CD8+ T cell-dependent disaccharidase deficiencies observed in giardiasis (Keselman et al., 2016; Scott et al., 2004).

Other studies have shown an alteration in the composition of intestinal microbiota in acute giardiasis in mice, such as a decrease in major phyla (e.g., Firmicutes), while other minor taxa were enriched (Barash et al., 2017).

Studies have demonstrated that *Giardia* induces microbial dysbiosis in co-cultures with human mucosal microbial biofilms and increases virulence of commensal microbiota that affect human epithelial cells in vitro (Fig. 1O); these *Giardia*-altered biofilms were capable to induce immunopathology (intestinal permeability) that was dependent on microbial community disruption caused by *Giardia* secretory proteases (Beatty et al., 2017) (Fig. 1P). Further, it was suggested that these changes may be linked to symptoms present during the infection with this parasite (Allain et al., 2017; Amat, et al., 2017). Interestingly, in humans with symptomatic giardiasis an overgrowth of duodenal commensals has been observed (Chen et al., 2013; Torres et al., 2000), and it has been reported that the commensal microbiota content can modulate the pathogenesis of giardiasis (Bar et al., 2015).

All these studies indicate that intestinal microbiota impact the colonization by *Giardia* and the pathogenesis of giardiasis and likely influence the anti-*Giardia* immune response. Further considering that infections with *Giardia* may be involved into post PI-IBS and CFS, it will be interesting to understand whether disbiosis induced by *Giardia* might induce complications during or following infection.

Vaccines against *Giardia*

Up to now, several studies have been reported in relation to vaccines against *Giardia*. Of these, the *Giardia*Vax™ is the only vaccine approved against this parasite and it is only for veterinary use, specifically for canines and consists of a total lysate of trophozoites from *Giardia* isolates (Fort Dodge Animal Health, Overland Park, Kansas, United States) (Olson et al., 2000). Although it induces good IgA production and reduces the number of cysts, the induced immunity is not so efficient, therefore its use may be restricted.

Among *Giardia* proteins that have been identified as potential immunogens are the variant surface proteins (VSP) which are the dominant protein expressed on the surface of *Giardia* parasites (Prucca et al., 2011). In this context, expression of multiple surface variant proteins (VSPs) has been achieved by disrupting the RNA interference mechanism using a siRNA (Prucca et al., 2008). Thus when gerbils were infected with *Giardia* expressing multiple VSPs simultaneously they excreted fewer cysts in a secondary infection, the animals were also resistant to infection when the same *Giardia* clone was used with a single VSP, but resistance was lost when a clone with a different VSP was used (Rivero et al., 2010). When *Giardia* trophozoites expressing multiple VSPs were employed to infect dogs and cats most of the animals infected with these trophozoites were able to self-cure by day 40 post infection, whereas the animals infected with a clonal population expressing a unique VSP remained chronically infected. Importantly, most animals that were initially infected with trophozoite populations expressing the entire repertoire of VSPs were protected from future infections with clonal populations expressing a particular VSP on their surface. Furthermore, an oral vaccine formulation comprising VSPs immunopurified from trophozoites of the *Giardia* WB isolate expressing the full repertoire of their VSPs was tested in experimental infections in dogs inhabiting a *Giardia* endemic. These animals showed a reduced number of cysts that were released to the environment and, remarkably, the vaccination ceased the symptoms of the disease, suggesting that it provides protection from infection as well as parasite clearance post establishment of an infection (Serradell et al., 2016).

Further studies on immunization of gerbils with the oral vaccine comprising the entire repertoire of VSPs specifically showed high levels of IL-17, IL-6, IL-4, and IL-5, without obvious signs of inflammation. Both immunized and infected animals developed local intestinal secretory IgA and systemic (serum IgG) humoral immune responses against VSPs providing evidences on the involvement of the immune system during both symptomatic infections and protective vaccination (Serradell, et al., 2018).

Other *Giardia* antigens including $\alpha 1$ -giardin, ornithine carbamoyl transferase (OCT) and α -enolase have been tested for the induction of protection against this parasite. These proteins have been expressed using live recombinant attenuated *Salmonella enterica* serovar Typhimurium. Oral administration of the vaccine strains induced antigen specific serum IgG, particularly IgG2A, and mucosal IgA for $\alpha 1$ -giardin and α -enolase, but not for OCT. Immunization with the $\alpha 1$ -giardin vaccine induced significant

protection against subsequent *G. lamblia* challenge, which was further enhanced by boosting with cholera toxin or sublingual α 1-giardin administration. The enolase vaccine did not induce protection (Jenikova et al., 2011).

In other studies, the generation of a bivalent DNA vaccine that included CWP2 and α 1-giardin based on the attenuated strain *S. enterica* serovar Typhimurium SL7207 was reported using mice as experimental model. In these studies, it was observed that after immunization a reduction of 79% in trophozoite burden and 93% in cysts were obtained as well as an increase in IgA and IgG2 antibodies. In contrast, when CWP2 and α 1-giardin were used as univalent vaccines, a lower reduction was observed; immunization with α 1-giardin showed a 74.2% reduction in trophozoites and a 28% reduction in cysts, whereas when CWP2 was used, an 89% reduction in cysts was observed (Feng et al., 2016).

In a recent study, using a proteomic analysis, 23 *Giardia* surface antigens were identified and four of these proteins were selected. These included uridine phosphorylase-like protein-1, protein 21.1 (GL50803_2 7925), α 1-giardin, and 11-giardin. These proteins were subsequently produced in recombinant form and were administered intranasally to mice as individual purified antigens together with the prototype mucosal adjuvant cholera toxin. This immunization protocol showed that all four tested antigens reduced infection by 98% of peak parasite burden and completely prevented infection in several mice, and one antigen also shortened the infection duration; yet it is unclear how much reduction in infectious load would be required to attenuate or prevent potential clinical symptoms (Davids et al., 2019).

In the context of *Giardia* infections, vaccines should be beneficial to both animals and humans by eliminating the parasite or reducing the parasite load in the host, thus preventing shedding of cysts. This should reduce oral-fecal transmission and environmental contamination of water and food which is important in locations where giardiasis is endemic. Further, vaccination of companion and farm animals will have an impact in zoonotic transmission, thus reducing inter- and intraspecies transmission. In particular, this is important in relation to wildlife infections, where control of exposure is very difficult to achieve. Another aspect that needs to be considered is the safety of vaccines thus avoiding side effects. This together with the elimination of clinical signs or infections with *Giardia* strains that are resistant to chemotherapeutic agents should be considered in the effectiveness of a vaccine to prevent *Giardia* infections in susceptible hosts (Olson et al., 2000).

Entamoeba histolytica

Due to its great cytolytic capacity, *E. histolytica* trophozoites are considered one of the most pro-inflammatory protozoans affecting humans. Once trophozoites overcome the mucus barrier and adhere to the surface of the intestinal epithelium, an intense inflammatory response ensues, which persists throughout the invasive process. The inflammatory response is important for parasite control; however, there is also evidence that it plays a very important role in the immuno-pathogenesis of intestinal and extra intestinal amebiasis. Since *E. histolytica* trophozoites are potent inducers of inflammation, the fine-tuning of cellular infiltration levels into the tissues is extremely important in defining the outcome of infection. The main mechanisms of amoeba-induced innate and acquired immunity in intestine and liver compartments as well as their role(s) in disease control are described below.

Innate and acquired immune responses against *Entamoeba histolytica* infection

E. histolytica trophozoites relies on adhesion to the mucus layer and to the intestinal epithelium as a critical first step for colonization and establishment of infection (Fig. 2A). The mucus gel and its anti-microbial components (α -defensins, antibodies and others) constitute the first barrier of innate defense mechanisms. However, *E. histolytica* trophozoites possess potent secretagogue activity in goblet cells as a result of a signal cascade triggered by the cleavage of surface α v β 3-integrin by the parasite's cysteine protease 5 (Chadee et al., 1991; Cornick et al., 2016). The amoeba adheres to the mucus, which is generally bypassed by glycosidases and proteases from trophozoites. Once the mucus is overcome, the trophozoites interact with the intestinal epithelium through the recognition of an amoebic surface Gal-lectin adhesin by TLR2 and TLR4 in colonic cells (Fig. 2B) (Galvan-Moroyoqui et al., 2011). Canonical signaling pathways are activated, resulting in the expression of pro-inflammatory cytokines and anti-microbial peptides, such as HBD2-defensin 2 from Paneth cells (Ayala-Summano et al., 2013). A possible protective role for this peptide, as well as for peptides derived from human cathelicidin LL-37 and lactoferrin, has been suggested from in vitro studies in which membrane damage of *E. histolytica* trophozoites was observed (Lopez-Soto et al., 2010; Ayala-Summano et al., 2013; Rico-Mata et al., 2013). Moreover, high clearance rates of amoebic colitis were observed in a C3H/HeJ mouse model after oral treatment with lactoferrin and lactoferrin-derived peptides, highlighting the role of these innate molecules present in milk and in neutrophil granules (Leon-Sicairos et al., 2012; Diaz-Godinez et al., 2019).

Studies of macrophages have shown that the pro-inflammatory profile of cytokines induced by the amoebic Gal-lectin acting as PAMP via α 5 β 1-integrin is shaped by gasdermin D and caspase 1-dependent activation of the NLRP3 inflammasome and gasdermin D and caspase 1-independent activation of HMGB1 alarmin (Fig. 2B) (Mortimer et al., 2015; Quach et al., 2019; Begum et al., 2020). Amoebic calreticulin has also been shown to shape the expression of cytokine profile in peripheral blood mononuclear cells (Gonzalez Rivas et al., 2018). Among the amoeba-induced pro-inflammatory mediators, IL-8, IL-1 α , IL-1 β , IL-6, IL-12, TNF- α , IFN- γ and CXCL1 have been reported (Guo et al., 2007; Bansal et al., 2009). Once attached, the amoeba releases PGE2 which disrupts tight junctions allowing the parasite to penetrate; furthermore, the parasite induces death in the target cells by trogocytosis or apoptosis (Fig. 2C) (Lejeune et al., 2011; Ralston et al., 2014). Neutrophils are mainly recruited during the intestinal and hepatic invasion by amoeba (Fig. 2B and D). In vitro studies have shown that amoebic trophozoites activate human neutrophils, inducing

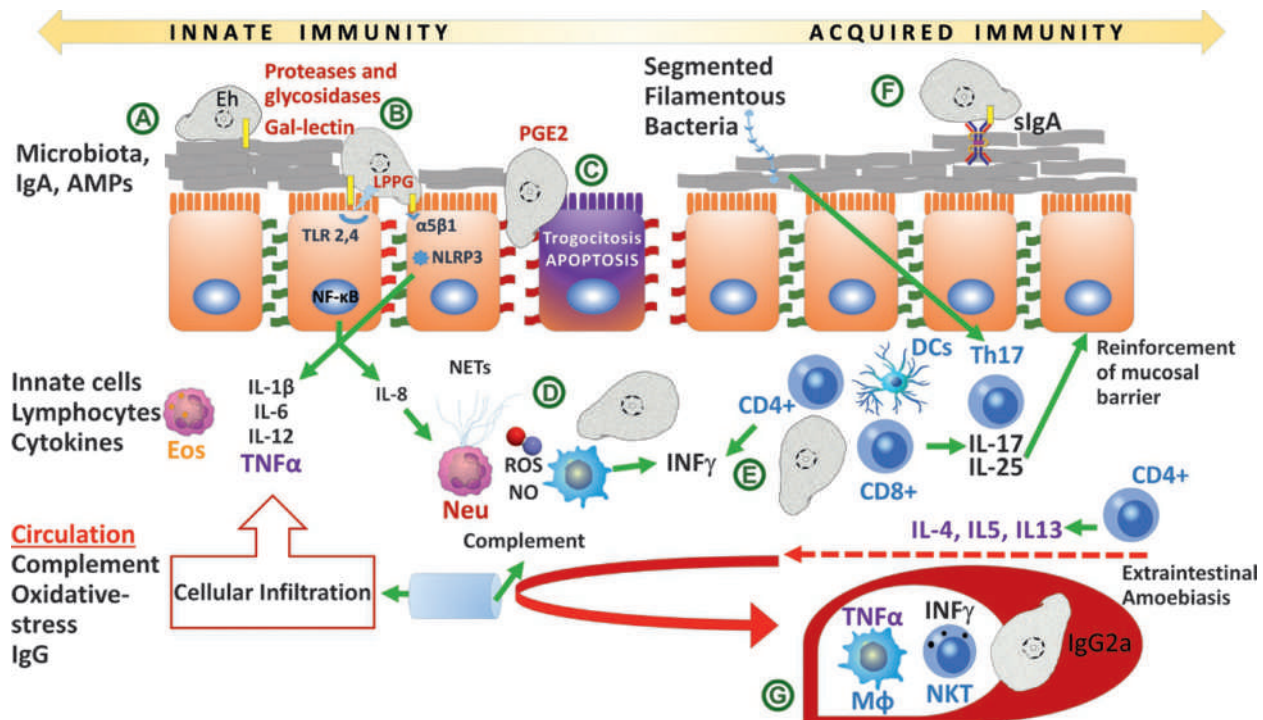


Fig. 2 Mechanisms of innate and acquired immunity during infection with *E. histolytica*. Trophozoite are exposed to microbiota, sIgA antibodies and AMPs which are degraded by parasite proteases and glucosidases (A). In the epithelium amoeba activates TLR2- and TLR4-canonical signal pathways inducing expression of pro-inflammatory cytokines. Activation of the NLRP3 inflammasome via $\alpha 5\beta 1$ -integrin also contributes to inflammation (B). Tight junctions are disrupted by trophozoite's PGE2 inducing death by trogocytosis or apoptosis, allowing the parasite to penetrate the mucosa (C). Neutrophils macrophages and eosinophils are recruited into the submucosa. Neutrophils produce ROS and release NETs and macrophages produce NO inducing protection (D). $\text{INF-}\gamma$ produced by activated neutrophils, macrophages and TCD4+ cells induce protection in intestinal amebiasis. IL-17 produced by TCD8+ cells and segmented filamentous bacteria-driven Th-17 cells, as well as IL-25, promote increase mucus secretion and immunoglobulin switching to IgA. $\text{TNF}\alpha$ and Th-2-cytokines IL-4, IL-5 and IL-13 are associated with susceptibility to intestinal amebiasis (E). Anti-amoeba secretory IgA antibodies against-Gal-lectin inhibit trophozoite adherence and protect against intestinal reinfection in children and in adults cured of ALA (F). When amoeba migrates to the liver complement, intense oxidative stress and specific IgG2a antibodies damage trophozoites. $\text{INF-}\gamma$ from iNKT cells protects the host while $\text{TNF}\alpha$ produced by macrophages induces tissue damage (G). Cytokines associated with susceptibility are in purple. Gal-lectin: Galactose/N-acetyl-galactosamine lectin; LPPG: lipopeptidophosphoglycan; PGE2: prostaglandin E2.

degranulation, ROS production and the release of pro-inflammatory cytokines and neutrophil extracellular DNA traps (NETs) (Fig. 2D) (Guerrant et al., 1981; Avila et al., 2016; Villalobos-Gomez et al., 2018). It has been reported that NETs kill *E. histolytica* trophozoites in vitro but their role in vivo is not yet known. Interestingly, trophozoites of the non-pathogenic amoeba, *E. dispar*, did not trigger NETosis, suggesting a relationship between pathogenicity and NETs formation (Fonseca et al., 2019).

The role of neutrophils in host's protection has been reported in studies in which depletion of neutrophils results in the development of larger amoebic liver abscesses (ALA) in early stages of infection in SCID mice, and in a greater percentage of intracecal infection in partially resistant (CBA strain) or susceptible (C3H/HeJ strain) mice (Seydel et al., 1997; Asgharpour et al., 2005). Nevertheless, a role for the products released by neutrophils, and by other immune innate cells, in the pathogenicity of amebiasis has been suggested (Tsutsumi and Shibayama, 2006; Pacheco-Yepes et al., 2011). For example, the inhibition of neutrophils recruitment by immunosuppression with cyclosporine A or N-acetylcysteine was shown to result in the inhibition of ALA development in hamsters even in the presence of numerous foci of aggregated amoebas (Olivos-Garcia et al., 2007).

On the other hand, macrophages, eosinophils and basophils also are recruited in amoebic infection but in lower numbers, and in vitro studies suggest that these two cell types may have a protective role in the host against this parasite. In vitro activation of $\text{INF-}\gamma$ -primed murine macrophages with a fraction of the amoebic Gal-lectin induced the release of NO that kills *E. histolytica* trophozoites (Seguin et al., 1997). In in vivo studies, macrophages and neutrophils have been associated with protection against hepatic amebiasis through the production of reactive nitrogen species (Fig. 2D) (Jarillo-Luna et al., 2002). However, protection in intestine and liver is highly dependent on the presence of $\text{INF-}\gamma$, a cytokine that activates inflammatory cells with amoebicidal activity (Fig. 2E and G). $\text{INF-}\gamma$ may be released from activated neutrophils and macrophages, but it is mainly produced early in the immune response by NKT cells, and later by TCD4+ cells. In this context, $\text{INF-}\gamma$ from TCD4+ cells have been shown to confer protection against intracaeal infection in a CBA/J mouse model, whereas $\text{INF-}\gamma$ from iNKT cells protects against ALA in a golden hamster model (Guo et al., 2009; Lotter et al., 2009; Meneses-Ruiz et al., 2015). Other cytokines related to the protection induced at the intestinal level are IL-17 and IL-25 (Fig. 2E). IL-17, produced by CD8+ T cells at the intestinal mucosa, was associated with protection against *E. histolytica* in vaccination experiments using the amoebic Gal-lectin (Guo et al., 2011a). However, a recent study

of the natural course of intestinal infection using IL-17A knockout CBA/J mice suggests that this cytokine may contribute to the persistence of the amoebic infection by modulating the ratio IFN- γ /IL-4 (Deloer et al., 2017). Detailed participation of IL-17 and IL-25 will be discussed in relation to the role of microbiota in amebiasis.

A role for mucosal eosinophils in protection (Fig. 2D) has also been recently reported in CBA/J mice following depletion of these cells using anti-Siglec-F antibodies (Noor et al., 2017). Eosinophil depletion resulted in a loss of IL-25-mediated protection and an increase in TNF- α -mediated susceptibility, a combination that resulted in an impaired gut barrier. Repletion of IL-25 in this mouse strain reversed the effects, restoring the integrity of the intestinal barrier and resistance to amoebic infection (Noor et al., 2017). In another study, eosinophilia induced by injection of a *Toxocara canis* antigen into gerbils resulted in protection against ALA, suggesting that eosinophils may also contribute to protection against extra-intestinal amebiasis (Lopez-Osuna et al., 1997). The role of basophils and mast cells has not been explored in detail, but IL-4 and IL-13 produced by these cells and by CD4⁺ T cells may be involved in disease manifestation by increasing epithelial permeability (Fig. 2E) (Houpt et al., 2002).

Interestingly, a novel mechanism of innate defense, involving STAT-3 signaling by the adipose hormone leptin, was shown to prevent amoeba-induced apoptosis of target cells (Marie et al., 2012). A 9 years follow-up study in Bangladeshi children showed a correlation between the rate of intestinal infection with *E. histolytica* and leptin suppression in malnourished children due to a leptin-receptor polymorphism (223R vs. ancestral 223Q) (Duggal et al., 2011; Guo et al., 2011b). A higher probability to develop a liver infection was also seen in adult subjects bearing the same polymorphism (Duggal et al., 2011). In general, reports based in animal models suggest that cellular-mediated innate immunity and acquired Th-1 responses may be protective against amebiasis, in contrast to a Th-2 response which is responsible for chronic infection (Fig. 2E). However, the protection observed also depends on the susceptibility of the host, since in susceptible animals (hamsters and gerbils) inflammatory cells seem to contribute to tissue damage, whereas in resistant animals (mice), inflammatory cells seem to contribute to protection by killing the parasite (Tsutsumi and Shibayama, 2006).

The acquired humoral immune response can be considered as a second line of defense in the intestinal infection by *E. histolytica*. Specific secretory IgA antibodies (sIgA) are induced when amoebic antigens released from trophozoites are presented in the Peyer's patches and the draining mesenteric lymph nodes (Fig. 2F). Anti-amoebic sIgA contained in the saliva of patients with intestinal amebiasis and in human breast milk inhibited the adherence of *E. histolytica* trophozoites to epithelial cells, preventing their destruction, and showed amoebicidal effect (Carrero et al., 1994; Leon-Sicaire et al., 2006). Evidence for the protective role of anti-amoebic sIgA antibodies in intestinal amebiasis is shown by data obtained from several field studies. Thus, a correlation between the presence of anti-Gal-lectin sIgA antibodies in stool or breast milk and reduced re-infection rates were observed in the multi-year follow-up study of the susceptible population of Bangladeshi children (Haque et al., 2001, 2006; Korpe et al., 2013). A similar association was found in patients recovered from ALA who controlled subsequent *E. histolytica* intestinal infection and developed immune memory (Ravdin et al., 2003). Moreover, in a subsequent prospective study, the clearance of *E. histolytica* and *E. dispar* infections in subjects cured from ALA correlated strongly with anti-gal-lectin sIgA peaks (Abd-Alla et al., 2006). Therefore, the stimulation of intestinal secretory response via a mucosal delivery of amoebic antigens has been proposed as a promising strategy for vaccination against human amebiasis. Once trophozoites reach the blood stream, specific IgG antibodies are produced (Fig. 2G) and can either protect or increase susceptibility to the infection, depending on the main isotype induced by the infection. A high level of anti-Gal-lectin IgG1 (Th-2 response) has been associated with active ALA and intestinal amebiasis cases, whereas anti-Gal-lectin IgG2a (Th-1 response) has been correlated with follow-up cases of ALA and asymptomatic cyst carriers (Kaur et al., 2004; Bernin et al., 2014).

Although classical and alternative pathways of human complement are activated in the presence of *E. histolytica* trophozoites causing in vitro lysis of the parasite (Calderon and Schreiber, 1985), studies on complement activation in patients with ALA or in golden hamster with ALA suggest that the complement system could be involved in the immunopathogenesis of ALA (Fig. 2D and G) (Munoz and Salazar, 1987; Olivos-Garcia et al., 2004). However, complement seems to be an important factor in the natural resistance to ALA in rats (Olivos-Garcia et al., 2018). Variations in complement susceptibility have been related to the amoeba strain used as well as with the susceptibility of the host studied.

Role of the intestinal microbiome in the immune response to amoeba

The establishment of amoebic intestinal infection has long been reported to be dependent on the presence of microbiota as germ-free animals are not colonized by *E. histolytica* trophozoites (Phillips et al., 1955; Leon-Coria et al., 2018). Thus, it has been suggested that there is bacterial-dependent expression of virulence factors in amoeba (Galvan-Moroyoqui et al., 2011). However, the interaction networks between amoeba and the microbiota are very complex and these also include microbiota-activated immune mechanisms that contribute to protection (Guo et al., 2011b).

The pioneering studies of Dr. William Petri's group on the role of microbiota in the protection against amoebic colitis have been of great importance. Initial study by this group indicated that the protection in a mouse model using the LecA fragment of Gal-lectin was dependent on the production of IL-17A, as the blockade of this cytokine abrogated the protection (Guo et al., 2011b). Since colonization of mice with the commensal *Clostridia*-related segmented filamentous bacteria (SFB) had been previously shown to promote a potent Th-17 response (Farkas et al., 2015), then they demonstrated that SFB colonization also provides protection against intestinal amebiasis (Fig. 2E and F) (Burgess et al., 2014). Moreover, protection was also obtained by adoptive transfer of bone marrow-derived dendritic cells (BMDs) from mice colonized with SFB, suggesting a cross-talk between microbiota and the bone marrow that regulates intestinal immunity and, thus, the outcome of gut infection diseases (Burgess et al., 2014). Further

studies of the effect of SBF on BMDCs showed that this protection is mediated by an increase in serum amyloid that increased the expression of Jmjd3 (a H3K27 histone demethylase), colony stimulating factor 2 receptor subunit alpha (CsF2ra) and granulocyte monocyte precursors (GMPs), promoting myelopoiesis which, in turn, increases the number of neutrophils, and probably macrophages which are recruited into the gut (Burgess et al., 2014). As a result, the innate protection against amoeba infection is activated via SFB-bone marrow cross-talk through epigenetic mechanisms.

On the other hand, the expansion of the usually commensal bacteria *Prevotella copri* in faeces of a population of Bangladeshi children was associated with diarrhoea and a high intensity of *E. histolytica* infection (Gilchrist et al., 2016), suggesting that an alteration in the microbiota could affect the course of the infection. In two recent reports, it was shown that the antibiotic-induced dysbiosis of C57BL/6 mice rendered the animals susceptible to the intracaecal infection with *E. histolytica* (Leon-Coria et al., 2018; Watanabe et al., 2017). In the first study, the susceptibility was associated with low levels of gut IL-25 and mucus protein Muc2 as well as a deficient recruitment of neutrophils to the caeca. Interestingly, a decrease in the surface expression of the chemokine receptor CXCR2 was identified in the neutrophils from mice with dysbiosis; the blockage of this receptor increased the rate of infection in control mice (Watanabe et al., 2017). In the second study, the effect of dysbiosis in the susceptibility of mice to *E. histolytica* infection was confirmed in Muc2^{+/+} and Muc2^{-/-} littermates, an effect that was only reversed in the antibiotic-treated Muc2^{+/+} mice by homologous fecal microbial transplants (Leon-Coria et al., 2018). These results highlight the link between the microbiota and the outcome of amoeba infection.

Cryptosporidium spp

As in other intestinal protozoosis, cryptosporidiosis may be asymptomatic, acute and chronic, depending largely on the host immune status or response. Immunocompetent individuals harbour parasites in the distal small intestine and may recover from acute infection within 2 weeks. However, in patients who are immunocompromised (e.g., HIV/AIDS), under immunosuppressive treatment (cancer, organ transplantation) or with inherited immunodeficiency (SCID), the infection is severe, chronic and life-threatening since it disseminates to the liver, pancreas, upper small intestine and gall bladder, thereby causing cholecystitis, cholangitis, sclerosis and pancreatitis (Cabada et al., 2015). The role of innate and adaptive immune responses in protection and development of vaccination strategies against *Cryptosporidium* spp. have been detailed elsewhere (Lemieux et al., 2018), and a brief description of immune responses to this parasite is given in the next sections.

Innate and acquired immunity in control, resolution and prophylaxis of cryptosporidiosis

Innate epithelial mechanisms activated against *C. parvum* include IECs which secrete chemokines (CXCL1, CXCL8, CXCL10, CCL2, CCL5) which attract, among others, myeloid (CD11c⁺ CD11b⁺) and double negative (CD11c⁺ CD11b⁻ CD8 α ⁻) dendritic cells (DC) in the ileum and draining lymph nodes involving IFN- γ (Auray et al., 2007) (Fig. 3A). In infected human IECs, Toll-like receptors TLR4 and TLR2 mediate MyD88, and NF- κ B signaling cascades are activated, thus leading to the secretion of free or exosome-containing antimicrobial peptides, such as LL37 and HBD2, which contribute to parasite clearance (Chen et al., 2005; Zaalouk et al., 2004) (Fig. 3B). IECs can also be induced to apoptosis, thus preventing parasite development (Laurent and Lacroix-Lamande, 2017) (Fig. 3C). Also, recruited monocytes secrete TNF α and IL-1 β , which increase the permeability and decrease integrity of epithelial barrier (de Sablet et al., 2016). Importantly, the antimicrobial effector NO (Fig. 3B) is produced independently of IFN- γ and depends on NOSII activity, as observed in *C. parvum*-infected mice and piglets. In these animal models, the inhibition of NOSII increases oocyst shedding, whereas L-arginine supplementation decreases it (Gookin et al., 2006; Leitch and He, 1994).

In relation to innate mucosal immunity, NK cells (non-T, non-B lymphocytes) act as both a source of IFN- γ , promoted by IL-12 and IL-18, and as pro-apoptotic effectors against *C. parvum*-infected IECs, releasing cytotoxic granules through an activation by IL-15. Therefore, NK cells are considered crucial contributors to the control of infection in mice (Barakat et al., 2009; Dann et al., 2005). The activation of NK requires a synergistic action of IL-18, TNF α and IL-12, the latter produced by macrophages and DC in the acute phase of infection (Bedi and Mead, 2012; McDonald et al., 2006; Urban et al., 1996). However, experiments with gene knock-down mice have shown a NK-independent mechanism, in which IL-18 along with IL-12 probably activate macrophages to produce IFN- γ because NK, T- and B-cell-deficient mice (Rag2^{-/-} γ C^{-/-}) can clear the infection (Choudhry et al., 2009). Accordingly, mice lacking IFN- γ receptor (IFN- γ R-KO) or IL-12 (IL-12KO) are highly susceptible to infection by *Cryptosporidium* spp. (Jakobi and Petry, 2008; von Oettingen et al., 2008).

Adaptive cellular immune responses are crucial for the resolution of cryptosporidiosis; indeed, individuals with defective adaptive responses have potentially fatal and long lasting courses (Hunter and Nichols, 2002). When DCs are recruited by chemokines from IECs at the early stage of the infection, they secrete IL-6 and TGF β , which induce the differentiation of intraepithelial naïve CD4⁺ T cells into regulatory Th-17 cells (Fig. 3D) which, in turn, are stimulated by IL-23 to produce IL-17. An increase of the subset of Th-17 cytokines was observed in gut-associated lymphoid tissue (GALT) and spleen of immunosuppressed, *C. parvum*-infected mice (Zhao et al., 2016). In the same infection model, IL-12 produced by macrophages and DC as well as IFN- γ produced by NK cells promote differentiation of intraepithelial naïve CD4⁺ cells into Th-1 cells (Fig. 3E). These latter cells, in turn, secrete IFN- γ , promote IgG2b production, favour conversion of naïve CD8⁺ cells into cytotoxic T cells, stimulate phagocytosis and neutrophil degranulation and release of ROS that contribute to killing intracellular *Cryptosporidium* spp. (Lantier et al., 2013; Lean et al., 2002). Despite the predominance of Th-1 cytokines, a contributory role of Th-2 cytokines as IL-4

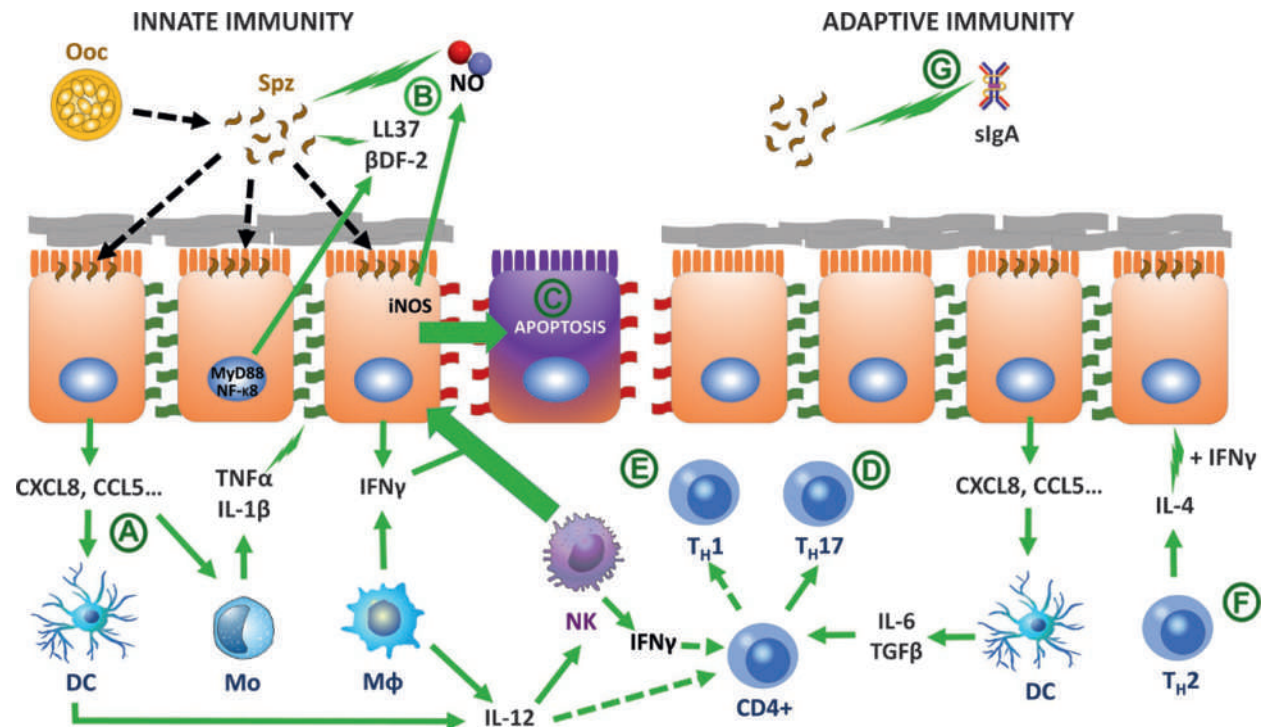


Fig. 3 Immune response mechanisms against *Cryptosporidium* spp. During the onset of infection oocysts (Ooc) release sporozoites (Spz) that infect intestinal epithelial cells (IECs) (broken black arrows). During activation of innate immune responses, infected IECs respond by releasing cytokines for intraepithelial recruitment of monocytes (Mo) and dendritic cells (DC) (A), production of antimicrobial peptides and nitric oxide (NO) (B) as well as apoptosis induction by IL-12 activated cytotoxic natural killer (NK) cells in the presence of IFN- γ (C). At later stages of infection, adaptive immunity mechanisms include differentiation of Th-1 cells from naïve CD4+ T cells by IL-12 produced by DCs and macrophages (M ϕ) along with IFN- γ produced by NK cells (broken green lines) (D). On the other hand, DC secrete IL-6 and TGF β that promote differentiation of naïve CD4+ T cells into regulatory TH-17 cells that secrete IL-17 (E). In spite of a strong Th-1 environment, a complementary action of TH-2 cytokines as IL-4 is needed to clear parasites (F). The effector role of humoral immunity during infection or re-infection is still unclear but experimental evidence supports the protective role of sIgA in hyperimmune bovine colostrum (HBC) that blocks invasion of IECs by Spz (G). For legibility, only the infective Spz is represented attached to IECs.

is needed for *C. parvum* clearance because IL-4 accelerates resolution of infection; indeed, IL-4 displays an IFN- γ -dependent protective role (Aguirre et al., 1998; McDonald et al., 2004) (Fig. 3F). On the other hand, experiments with anti-CD4/CD8-treated mice have shown that CD4+ T cells have a predominant role over CD8+ T cells for resolving *Cryptosporidium* infection (Chen et al., 1993; McDonald et al., 1994).

The role of humoral response against *Cryptosporidium* spp. that involves systemic IgM, IgA and IgG specific antibodies (Borad et al., 2012; Kassa et al., 1991) seems neither lead to host recovery nor parasite clearance (Mead, 2014). In this context, anti-*Cryptosporidium* secretory IgA (Fig. 3G) present in AIDS patients does not protect them against cryptosporidiosis without the participation of CD4+ T cells (Cozon et al., 1994; Kassa et al., 1991). However, antibodies have a complementary role in protection, as exemplified by the prophylactic and “therapeutic” effects of hyperimmune ovine/bovine colostrum (HBC) against ovine cryptosporidiosis (Naciri et al., 1994). It is now a matter of debate as to whether anti-*Cryptosporidium* effects conferred by HBC are similar to those in humans because variable therapeutic successes were observed in HBC to HIV-immunocompromised patients (Abubakar et al., 2007; Plettenberg et al., 1993). Using immunodeficient mice models, a promising therapeutic strategy is the use of monoclonal antibodies directed against parasite antigens, such as SA-1, CSL and P23 that reduce parasite loads and oocyst shedding (Enriquez and Riggs, 1998; Perryman et al., 1993; Riggs et al., 2002). A simplified scheme depicting major mechanisms involved in innate and adaptive immune responses against *Cryptosporidium* spp. is shown in Fig. 3.

Immune evasion by *Cryptosporidium* spp

Effector mechanisms that control *Cryptosporidium* infection of IECs involve key processes, such as antimicrobial peptide production, IFN- γ signaling and apoptosis induction. The pathways that enable parasite survival and multiplication at the expense of these processes have been mainly studied in experimental rodents infected with *C. parvum*.

In the initial stages of infection, IECs produce antimicrobial peptides, such as the β -defensins mBD-1 and HBD-1, which expression is reduced by the parasite in an unknown way (Zaalouk et al., 2004). Further, CCL20 is a chemokine with anti-*Cryptosporidium* activity that is down-regulated during infection by a concomitant up-regulation of miR21 that targets CCL20 (Guesdon et al., 2015).

In the IFN- γ signalling cascade, STAT1, is an essential transcription factor that promotes the expression of multiple genes, including indolamine 2,3-dioxygenase (IDO), an enzyme that catabolizes tryptophan, an aminoacid required for *C. parvum* growth. During the infection of enterocytes, the parasite interferes with STAT1-induced IDO expression, thus restoring tryptophan pools to favour its survival (Choudhry et al., 2009).

In order to limit apoptosis of IECs, *C. parvum* has evolved some strategies. One is via the production of anti-apoptotic factors, such as BCL-2, which blocks mitochondrial cytochrome *c* release (Mele et al., 2004) as well as survivin which inhibits caspases (Liu et al., 2008). Also *C. parvum* induces the expression of osteoprotegerin, which “operates” as a decoy TNF receptor besides its ability to bind the TNF-related apoptosis-inducing ligand (TRAIL), thereby avoiding pro-apoptotic signals in IECs (Castellanos-Gonzalez et al., 2008).

Vaccines and protection against *Cryptosporidium* infection

The importance of cryptosporidiosis in the clinical and veterinary contexts, along with the complexity of *Cryptosporidium* life cycle and the multiplicity of its cell stages, impose a challenge for the development of a vaccine. Vaccination has been addressed using a variety of strategies, which include DNA-encoding proteins, subunits of defined antigens, bacterial vectors expressing parasite antigens, inactivated live parasites, and some other approaches such as antigen priming-vector boost regimes (Lemieux et al., 2018).

Initially, some DNA vaccines were designed to target selected sporozoite surface proteins of *C. parvum* (Cp), and the mechanisms leading to protective immune responses were defined in mouse models. A divalent vaccine encoding Cp12 and Cp21 displays protection (77.5% reduction of oocyst shedding by nasal route) through induction of high IgG levels and increased numbers of CD4+ and CD8 cells (Yu et al., 2010). The use of DNA encoding Cp15 and Cp23 showed a similar protection with higher IgG titers and elevated Th-1 cytokines (Wang et al., 2010). Using the sporozoite surface protein Cp15/60, mice were protected against an inoculum of up to 1×10^6 *C. parvum* oocysts (He et al., 2004). In pregnant cows, immunization with subunit vaccines using immunodominant proteins has been carried out and the corresponding HBC have been evaluated to induce protection in calves. Of these, anti-P23 HBC was reported to induce protection or at least limited symptoms and oocyst shedding (Askari et al., 2016; Perryman et al., 1999). In mice, a Cp15-Cp23 vaccine induces greater CD4+ and comparable CD8+ numbers concomitant with a significant Th-1 and antibody responses, leading to partial protection as compared with crude extracts and monovalent Cp23 vaccines (Liu et al., 2010).

In the case of live-attenuated vaccines, these may induce a long lasting, strong Th-1 and protective cell-mediated immunity, as has been shown against some bacteria, viruses and protistan pathogens (Bhattacharya et al., 2015; Buxton et al., 1993; Jenkins et al., 1997). Limited vaccination trials have been carried out, but some protection in calves has been achieved after 3 weeks of challenge using *C. parvum* oocysts inactivated by γ -irradiation at 400-Gy (Jenkins et al., 2004).

Vaccines based on attenuated viruses, bacteria or parasites carrying *Cryptosporidium* antigens have been designed for intestinal delivery to microfold cells to allow their processing in Peyer patches to stimulate mucosal immune responses (revised by Lemieux et al., 2018). In particular, live bacterial vectors such as attenuated *Salmonella enterica*, have been reported (Benitez et al., 2009; Manque et al., 2011; Roche et al., 2013). This strategy mainly involves the fusion of the parasite protein to a signal peptide (for type III secretion) and a chaperone-binding domain, providing a low-cost and readily administrable vaccine (Hegazy et al., 2012). Initial trials using attenuated *S. enterica* serovar Typhimurium SL3261 as the delivery system employing a vector (pTECH1) expressing *C. parvum* Cp23 or Cp40 fused to fragment C of the tetanus toxin under the control of the anaerobic *nir* promoter were tested for oral immunization of mice. Serum anti-Cp23 and -Cp40 IgG titers were detected after 35 days, with serum and faecal IgA being detected in 30% of Cp23-immunized mice (Benitez et al., 2009). Using reverse vaccinology, the strain *S. enterica* serovar Typhi CVD-908-*htrA*, expressing either *C. hominis* apyrase, profilin or Cp15 fused to cytolysin A and administered as heterologous prime boost regime in mice, induced strong humoral (IgG1 and IgG2b) and cellular (IFN- γ , IL-2, IL-6 and IL-12) immune responses (Manque et al., 2011). Further experiments with the same vaccine strain, but expressing the Cp15 protein by intranasal administration, in spite of inducing strong secretion of IL-6 and IFN- γ and robust anti-Cp15 IgG titers, after oral challenge with *C. parvum* oocysts, achieved only a transient reduction in oocyst-shedding without protection against disease (Roche et al., 2013). Interestingly, these studies have shown that the “Prime-Pull” vaccine approach, in which the oral dose of *Salmonella* spp. vector (“boost”) following subcutaneous immunization with Cp23 or Cp40 DNA as “prime” (Benitez et al., 2009) or *vice versa* [i.e., priming with *Salmonella* spp. vector then boosting with intraperitoneal administration of recombinant proteins (Manque et al., 2011; Roche et al., 2013)] enhances immune responses against individual immunogens.

Other studies have analyzed the *C. parvum* P23 antigen as an immunogen expressed by *Toxoplasma gondii* and the probiotic bacteria *Lactobacillus casei* in mice. Using the first vector, high titers of neutralizing IgG1, a typical subtype of Th-2 response, were achieved (Shirafuji et al., 2005), whereas the use of the latter vector induced increased levels of serum IgG, faecal IgA, IL-6 and IFN- γ (Geriletu et al., 2011), although their protective effects against *C. parvum* challenge were not further evaluated.

In a general view, protective responses against *Cryptosporidium* spp. involve specific mucosal responses in a context of a predominant Th-1 cellular immunity, supported by a Th-2 humoral response. Some vaccine candidates, such as Cp12, Cp15, Cp21, Cp40 and P23, display epitopes that need to be evaluated in analyses employing combined vaccine approaches, such as those involving systemic priming and mucosal boosting, for which ongoing work might pave the way to an effective vaccine.

Blastocystis spp

The involvement of host immune responses in the acquisition, development and outcome of *Blastocystis* infection was suggested based on the observation of the higher susceptibility of HIV(+) or cancer patients, children and tourists to this infection (Kurniawan et al., 2009; Tasova et al., 2000). In addition, spontaneous remission of the infection (22%) has been observed in population cohorts shedding *Blastocystis* in stools (van Hellemond et al., 2013). Likewise, an epidemiological link between *Blastocystis* and IBS and inflammatory bowel disease (IBD) has been reportedly seen in patients (Jimenez-Gonzalez et al., 2012; Kaser et al., 2010; Yakooob et al., 2010). However the symptomatology caused by *Blastocystis* is still contentious; hence, this may be considered as the result of interactions among parasite, host immune response and possibly microbiota-related factors, as discussed in the following sections.

Subtypes, pathogenicity, immune response and immunomodulation

This microorganism was historically referred as *Blastocystis hominis* when isolated from humans, but humans can be infected with other species (e.g., *B. ratti*). Molecular studies using the small subunit ribosomal RNA gene (SSU) revealed at least 17 *Blastocystis* subtypes (STs), all of which are morphologically similar but genetically distinct (Alfellani et al., 2013; Stensvold et al., 2007). Thereafter, the designation "*Blastocystis* spp. STnn" covers all subtypes, of which ST1 to ST9 and ST12 have been detected in humans, and ST1 to ST4 are involved in 90% of human cases (Stensvold and Clark, 2016). Further, subtypes ST18 to ST26 have been proposed to infect humans, but this finding is still controversial (Maloney et al., 2019; Stensvold and Clark, 2020).

To date, there is no proven relationship between *Blastocystis* STs and virulence, although there appears to be an association. However, key processes involved in intestinal pathogenicity have been identified. In vitro interaction experiments have shown that *Blastocystis* is able to (a) attach intestinal mucin; (b) promote tight junction alteration mediated by Rho/ROCK, which, in turn, disrupts epithelial barrier function, hence increasing permeability; (c) NF- κ B-mediated secretion of pro-inflammatory cytokines (GM-CSF, IL-1 β , IL-6, IL-8 and TNF- α) (Fig. 4A); and (d) enterocyte apoptosis by contact-independent, caspase 3-dependent

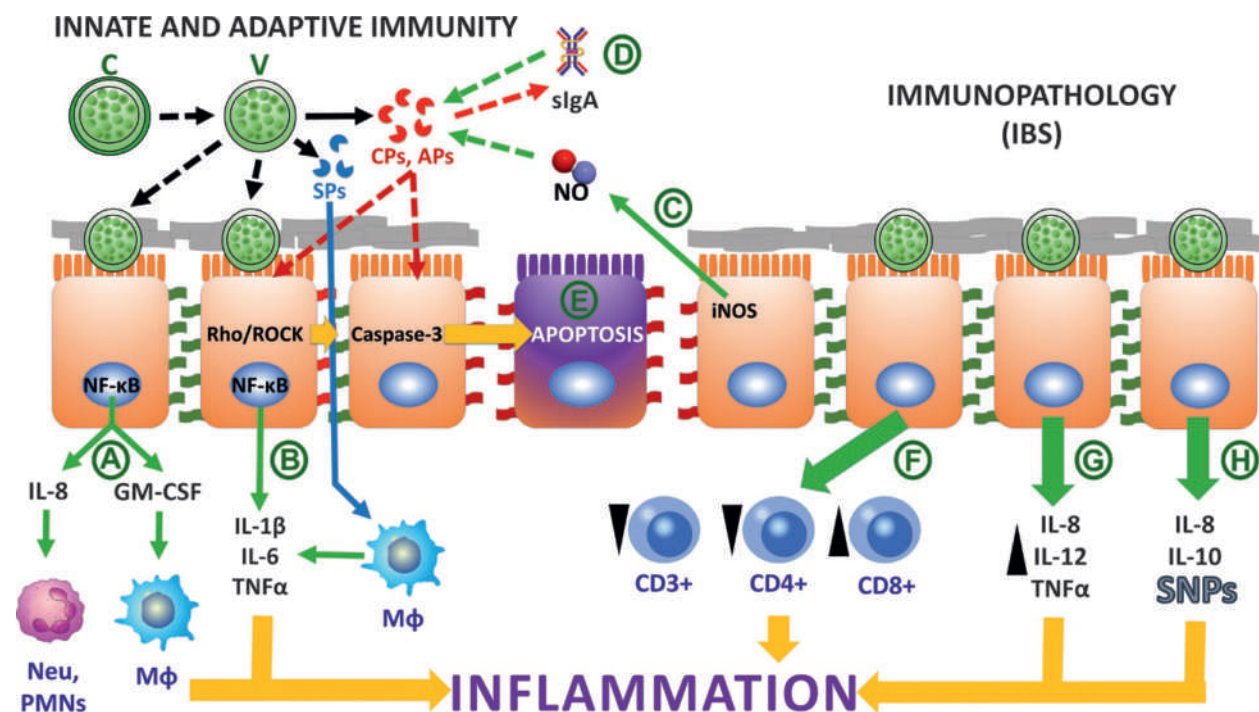


Fig. 4 Immune response mechanisms against infection by *Blastocystis* spp. and immunopathological patterns. The infective *Blastocystis* cyst (C) releases pathogenic forms that develops into vacuolar (V), ameboid or granular forms that attach to mucin and/or surface of intestinal epithelial cells (IECs). These latter activate NF- κ B signaling to produce chemoattracting chemokines for proinflammatory cells as neutrophils (Neu), polymorphonuclear cells (PMNs) and macrophages (M ϕ) (A) as well as proinflammatory cytokines (B). Two important antiparasitic effectors are nitric oxide (NO) produced from iNOS activity (C) and secretory IgA (sIgA) as predominant isotype at luminal compartment (D). *Blastocystis* counteracts some of these mechanisms by secreting proteases of the cysteine (CPs), aspartic (APs) and serine (SPs) types: CPs and APs elicit ruptures of intercellular junctions through the Rho/ROCK pathway, degrade secretory antibodies and induce contact-independent, caspase-3 dependent apoptosis in IECs (E); on the other hand SPs at the mucosal level induce production of proinflammatory cytokines from resident or recruited M ϕ . In patients with inflammatory bowel disease, particularly IBS, a decrease of T lymphocytes (CD3+) and an imbalanced proportion of CD4+ and CD8+ cells is present (F: increased, $\downarrow$: decreased) (F). Interestingly an increased production of inflammatory cytokines (IL-8, IL-12 and TNF- α) (G) and even single nucleotide polymorphisms (SNPs) in sequences of cytokines as IL-8 and IL-10 (H) have been associated to these inflammatory outcomes.

mechanisms (Lim et al., 2014; Puthia et al., 2006) (Fig. 4B). The high genetic variability among *Blastocystis* isolates is a major contributing factor, not only to explain the distinct inter-ST and intra-ST abilities to induce the alterations mentioned above (Gentekaki et al., 2017; Wu et al., 2014), but also to define likely virulence factors and immune evasion mechanisms used by this parasite.

In spite of the non-invasive nature and controvert pathogenicity of *Blastocystis* spp., local-intestinal and systemic immune responses have been observed, but are somewhat conflicting. In symptomatic humans, higher levels of serum IgG have been found than in asymptomatic individuals, with variable levels of IgA in serum (Mahmoud and Saleh, 2003; Zierdt et al., 1995). On the other hand, mucosal antibody response in the intestine, mediated by secretory IgA (Fig. 4C), seems to be associated with latent infection, because this isotype is more abundant both in symptomatic patients and in immunized BALB/c mice, acting as a first-line defense against attachment and invasiveness by *Blastocystis* (Mahmoud and Saleh, 2003; Santos and Rivera, 2009). Further, in pigs, where ST1, ST3 and mainly ST5 predominate, a study testing IgA by immunoblot in 233 fecal samples from asymptomatic, PCR-positive pigs revealed 81% reactivity against different protein bands of a *Blastocystis* extract from isolate WR-1 (ST4), particularly against a 250 kDa component (Wang et al., 2014). In this context, recent experiments have identified an important role for secreted proteases as major candidate for virulence factors based on their ability to degrade secreted antibodies, disrupt epithelial barrier function and promote the production of pro-inflammatory cytokines (Ajjampur and Tan, 2016; Nourrisson et al., 2016). Particularly cysteine proteases of *Blastocystis* from symptomatic human cases displayed a higher enzyme activity than those from asymptomatic carriers (Mirza and Tan, 2009). Indeed, secretory dimeric IgA was degraded by cellular and secretory *Blastocystis* proteases of the cysteine (isolate B, ST7) and a aspartic protease (isolate WR-1, ST4) (Puthia et al., 2005). Interestingly, a variant repertoire and activity of cysteine proteases within and among STs have been reported (Gentekaki et al., 2017; Mirza and Tan, 2009), supporting the notion of variability in pathogenicity and in immune evasion ability among STs.

NO is an important component of the innate immune system, which is produced in large quantities from L-arginine through the activation of NOSII, and is a free radical causing nitrosative stress-mediated death in microbial pathogens. In *Blastocystis*, necrosis as well as apoptosis-like processes might be induced by NO (Eida et al., 2008; Mirza et al., 2011b) (Fig. 4D). Interestingly, *Blastocystis* isolate B (ST7) is more prone to apoptosis (i.e., more susceptible to NO) than isolate WR-1 (ST4) (Eida et al., 2008); nevertheless, the ST7 isolate is able to downregulate NOSII mRNA levels in colonic epithelial cell monolayers (CaCo-2) (Mirza et al., 2011b). Although the ST7 isolate contains much higher arginase activity than ST4 isolate, this feature is thought to be secondary to the decrease in NOSII expression in evasion (Mirza et al., 2011a).

Cellular immune response elicited by *Blastocystis* spp. has been studied in vitro and in vivo. The ability of this parasite to disrupt intestinal barrier integrity allows its secreted antigens to cross epithelium and reach the lamina propria, thus reaching effector cells. In this context, cysteine proteases released by *Blastocystis* negatively affect enterocyte junctions through Rho kinase activation, F-actin rearrangement and ZO-1 distribution increasing epithelial permeability (Mirza et al., 2012; Puthia et al., 2006). Experimental infections with cysts in BALB/c mice (human isolate) and rats (isolate RN94-9, ST4) showed, by histological examination, an intense infiltration of pro-inflammatory cells in colonic and caecal mucosa, with a moderate increase in goblet cell numbers (Iguchi et al., 2009; Moe et al., 1997). This recruitment is, in part, mediated by soluble mediators acting on inducing granulocytes/macrophages (GM-CSF) and neutrophil recruitment (IL-8), both released by colonic epithelial cell lines (HT-29 and T84) in response to infection by *Blastocystis* (Long et al., 2001). Regarding mucus production by goblet cells that alleviate colitis-induced symptoms, this production is triggered via IL-22 from CD4⁺ cells (Leung et al., 2014). In addition, macrophages initially resident in the lamina propria or recruited to the inflamed bowel may be induced by serine proteases from *Blastocystis*, which may activate protease-activated receptor 2 (PAR2), which, in turn, triggers mitogen-activated protein kinase (MAPK) pathways involving ERK1/2 and JNK, thereby promoting the expression of pro-inflammatory cytokines as IL-1 β , IL-6 and TNF α (Lim et al., 2014) (Fig. 4E). Interestingly, this response was found to be more intense when macrophages and even mice with colitis or mouse colon explants were exposed to lysates from isolate B (ST7) as compared with isolate WR-1 (ST4), indicating a differential pro-inflammatory potential among STs (Lim et al., 2014). Further, some IBS cases with concomitant *Blastocystis* infection are refractory to metronidazole treatment, as parasite isolates (e.g., isolate B) display resistance to this drug (Mirza et al., 2011a). Moreover, isolate B has a higher virulence-arsenal than isolate WR-1, as it contains more cysteine and serine proteases, and arginase activities that appear to explain isolate B's higher IgA degradation capacity, higher phosphorylation of MAPKs leading to higher pro-inflammatory responses and lower NO production in host cells to allow parasite survival.

The relationship between cellular immune responses to *Blastocystis* and development of intestinal inflammation, i.e., IBS/IBD, is unclear, but there are significant insights into the activation of the immune response, loss of tolerance to the parasite and immunosuppression (Kaser et al., 2010; Strober et al., 2002). In IBS patients with concomitant *Blastocystis* infection, lower levels of CD3⁺ and CD4⁺ cells and CD4⁺/CD8⁺ ratios were observed (Wang et al., 2002) (Fig. 4F). In the same context, IBS patients infected with subtypes ST1 or ST3 produced higher levels of pro-inflammatory cytokines as IL-8, IL-12 and TNF α compared to patients with only IBS (Yakoob et al., 2014) (Fig. 4G). In addition, *Blastocystis* carriers harbouring polymorphisms in the alleles of pro-inflammatory IL-8 + 396 (GG) and anti-inflammatory IL-10-592 (C) cytokines were at significant risk of developing IBS (Olivo-Diaz et al., 2012) (Fig. 4H).

Besides pro-inflammatory cytokines, there is limited information regarding other soluble parasite mediators. Interestingly, colorectal cancer cell line HCT116 exposed to antigen extracts of *Blastocystis* from symptomatic patients significantly up-regulated transcription factor NF- κ B, Th-1 (IFN- γ and TNF α) and Th-2 (IL-6, IL-8 and TGF β) mRNA responses with dominance of Th-2 pattern in comparison with the same cells exposed to extracts from asymptomatic patients (Chan et al., 2012). Considering this aspect, a

comparison among subtypes ST1 to ST5 showed that ST3 evoked a higher up-regulation of Th-2 cytokines, particularly TGF β (Kumarasamy et al., 2013). Altogether, these observations provide evidence of host immunomodulation induced by this parasite.

Immune responses in immunocompromised hosts

It is recognised that infection by *Blastocystis* spp. is more effectively counteracted by mixed Th-1/Th-2 cytokine responses, since this parasite is classically extracellular (Daugelat and Kaufmann, 1996). Nevertheless immunomodulation may occur at multiple levels in immunocompetent (IC) hosts (i.e., sIgA destruction, proinflammatory cytokine production, NOSII down-regulation, Th-2 dominance), raising questions about the outcome of immune response in infected immunocompromised hosts. In this context, experiments in BALB/c mice treated with dexamethasone-induced immunosuppression (IS) have provided interesting evidence. Here, the survival rate of IS mice infected with cysts from human carriers (presumably ST3) decreased by 50% from 5 days post-infection and, remarkably, parasite vacuolar forms infiltrated more severely the epithelial, lamina propria, submucosa and mucosa layers of IS-mice, possibly by an invasive mechanism (Abdel-Hafeez et al., 2016). These mice also showed an up-regulation of pro-inflammatory cytokines (IL-12 and TNF α) and downregulation of anti-inflammatory cytokines (IL4 and IL-10) as compared with IC-mice, suggesting a likely influx of macrophages, T cells and/or NK cells and an exacerbated inflammatory response (Abdel-Hafeez et al., 2016; Iguchi et al., 2009). On the contrary, sIgA levels in the gut were downregulated in IS mice as compared to IC-mice, allowing the permanence of parasites at intestinal tract (Abdel-Hafeez et al., 2016). In other studies, the expression of regulatory cytokines, such as IL-17 and IL-23, was higher in the small and large intestines of *Blastocystis*-infected IS-mice than in non-infected IS- and IC-mice (Wu et al., 2012). These studies indicate a more severe pathology and inflammatory responses in immunocompromised mice, but it remains to be established whether this is the case in humans. An integrative scheme summarising current knowledge of immune response mechanisms in immune and immunopathological responses to *Blastocystis* infection is shown in Fig. 4.

Microbiota changes and *Blastocystis* infection, a role for host immunity?

The inventory and diversity of prokaryotic gut microbiota in humans infected by *Blastocystis* spp. vary depending on the outcome of infection. In infected carriers, when compared to non-infected ones, a more diverse microbiota with higher abundance of *Clostridia* and lower abundance of *Enterobacteriaceae* was observed (Audebert et al., 2016) as well as a reduced frequency of *Blastocystis* in stools from patients with *Bacteroides*-rich microbiota (Andersen et al., 2015). In infected patients with IBS and constipation a decrease in protective and anti-inflammatory flora, i.e., *Bifidobacterium* spp. and *Faecalibacterium prausnitzii*, was observed (Nourrisson et al., 2014). Interestingly, the carriage of *Blastocystis* in patients with IBS and diarrhoea was not associated to altered fecal microbiota (Nagel et al., 2016).

From these observations, there is likely a complex interplay of immunomodulatory processes as a consequence of the coexistence of *Blastocystis* and bacterial communities in the gut. On one side, a Th-2-dominant response in asymptomatic carriers decreases cellular responses, such that more bacterial species remain in the host gut. On the other, infected individuals with concomitant IBS-constipation might display a dominant pro-inflammatory response and an inhibition of NO production that affect intestinal peristalsis and ultimately hinder the survival of anti-inflammatory flora. Nevertheless, unaltered microbiota in IBS-diarrhoea spectrum is a challenging issue that deserves serious attention. Further, effects of resident microbiota on the expression of possible virulence factors of *Blastocystis*, e.g., protease secretion, warrant future studies.

Concluding remarks

In this century, efforts to improve the control for neglected tropical diseases, such as soil-transmitted helminths and gastrointestinal protozoa inhabiting the intestinal tracts of humans and other animals (i.e., IUP), have been enabled through the use of “next-generation” tools, including sophisticated immunological methods, genome sequencing and molecular engineering strategies, applied at the in vitro- and in vivo-levels and employing valuable experimental models to assess host-pathogen interactions, immunity and the pathogenesis of disease. Importantly, these tools have been used to gain basic knowledge and understanding of mechanisms involved in host innate and adaptive immune responses, immunopathological outcomes and intricate interactions between pathogens and bacterial communities in the host gut. This framework has offered a solid foundation for gaining deep insights into fundamental aspects of the immunobiology of protistan pathogens and for applied aspects, such as the design of new strategies aimed at prevention or protection against related diseases.

In spite of the current absence of prophylactic biologicals against human giardiasis, amebiasis, cryptosporidiosis and blastocystosis, immunological investigations are providing new insights into immunoprotective responses. Thus, a paradigm regarding “predominant Th-1 responses supported by Th-2-dependent processes” to eliminate unicellular intestinal pathogens is now a consensus. Novel candidate molecules are emerging for the development as vaccines against selected unicellular intestinal parasites; these include the secreted proteins of *G. duodenalis* (e.g., giardins, VSPs, giardipain-1), secreted proteases from *Blastocystis* spp., sporozoite surface proteins of *Cryptosporidium* and surface lectins (galectins) of *E. histolytica*, although progress differs depending on parasite. However, an ample repertoire of strategies for vaccine design and delivery, from DNA-based vaccines to live-attenuated

bacterial vectors, is now available to make significant progress. These promising elements point towards an acceleration of progress to develop effective methods and reagents for the prevention, treatment and/or control of some of these insidious protistan diseases.

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Human Filariasis

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Glossary

Calabar swelling A transient subcutaneous swelling marking the migratory course through the tissues of *Loa loa* adult worms.

Hydrocele Accumulation of lymph fluid in the scrotum of men during lymphatic filariasis.

Macrofilaricide A drug that kills adult filariae.

Microfilariae Progeny of the filarial nematodes.

Microfilaricide A drug that kills microfilariae.

Onchocercoma Subcutaneous nodule that contains the adult *Onchocerca volvulus* worms.

Sheath Microfilariae cover of some filarial species.

Sowda Severe, unilateral form of dermatitis in onchocerciasis patients.

Wolbachia Endosymbiotic bacteria of some filarial species.

Onchocerciasis

Short overview

Onchocerciasis is listed by the WHO as a neglected tropical disease (NTD) and is caused through infection with the filarial nematode *Onchocerca volvulus* which is transmitted by bites of *Simulium* blackflies. Although the majority of infections show few or light symptoms, the disease can lead to severe clinical symptoms like vision loss, blindness and dermatitis including the unilateral, severe form sowda. Since breeding sites of the black flies are usually found in fast-flowing oxygen-rich rivers and creeks, transmission and disease prevalence stretch along these rivers and thus onchocerciasis is often referred to as river blindness.

Biology/Etiology

The life cycle of *O. volvulus* consists of two hosts, the human as definite host and *Simulium* black flies as intermediate host. Thus, the life cycle of *O. volvulus* is comparable to other human-pathogenic filariae, with humans that harbor the adult worms and transmitting blood-feeding insect vectors (Fig. 1). Female *Simulium* blackflies can transmit infective larvae (L3 stage) during the blood meal into the human body. Until now little is known about the fate of L3 in the human skin but L3 molt once to become L4 within 10 days. The L4 persist in the host and develop into adult worms within 6–12 months. Female (33–50 cm long and 270–400 µm wide) and male adult worms (19–42 mm length and 130–210 µm wide) reside in subcutaneous nodules, also called onchocercomata, that are commonly located around the iliac crest, but also the head and torso (Brattig et al., 2020; Murdoch, 2021). Adult worms are also able to migrate freely in the subcutaneous tissue. Females and males reside in a ratio of approximately 3:1 in the onchocercoma and mate. Consequently, gravid female worms produce thousands of first stage unsheathed larvae (L1), the so-called microfilariae (MF; “offspring”; 220–360 µm long and 5–9 µm wide) that are released into the subcutaneous tissue (Schulz-Key, 1990). They can sometimes be found in blood, urine or other fluids such as lymphatics (Mackenzie et al., 2010), especially in individuals with high worm burdens. It is estimated that the reproductive life span of female worms can achieve 9–11, sometimes 15 years, and even MF may live for several years in dermal tissue (Specht et al., 2009; Udall, 2007) i.e., the dermal papillae just below the epidermis. The migrating MF can be taken up and ingested by black flies during a subsequent blood meal. In the black fly the MF penetrate the peritrophic membrane of the midgut and migrate through the haemolymph where they settle in the syncytial cells of the thoracic longitudinal flight muscles. Within 1–2 weeks, the MF molt twice to become infective L3, which can be transmitted via another blood meal by the blackfly into the human body (Brattig et al., 2020; Murdoch, 2021). As most human pathogenic filariae, *O. volvulus* contain endosymbiotic *Wolbachia* bacteria that are found in the hypodermis and embryonal stages of the filariae. *Wolbachia* are essential for filarial development, embryogenesis and survival (Taylor et al., 2005).

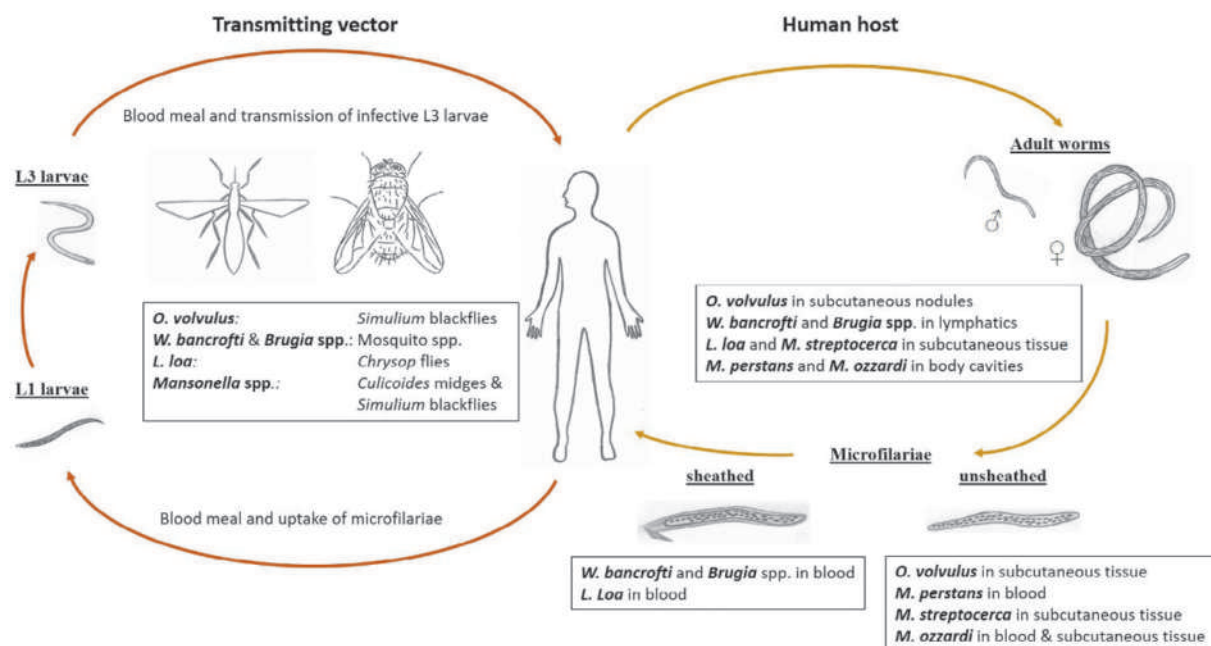


Fig. 1 Generalized life cycle of human pathogenic filariae.

Epidemiology

As mentioned above, onchocerciasis predominantly affects people living close (12 km) to rivers that serve as breeding grounds for the *Simulium* blackflies. Several subspecies serve as vector but *S. damnosum* is the predominant vector in Africa and *S. neavei* blackflies also drive transmission in East and Central Africa (Fischer et al., 1993, 1997b; Makunde et al., 2000). According to the Global Burden of Disease study 2017, approximately 21 million people are infected worldwide (Global Burden of Disease Study 2017, 2018) and more than 218 million individuals live in endemic areas and are at risk of infection. Moreover, approximately 15 million of the infected people suffer from skin disease and more than 1.2 million from vision loss leading to approximately 205 million disability-adjusted life years (DALY's) (Global Burden of Disease Study 2017, 2018). Onchocerciasis is prevalent in tropical areas but 99% of infected people live in 31 Sub-Saharan African countries named Angola, Benin, Burkina Faso, Burundi, Cameroon, Central African Republic, Chad, Republic of Congo, Côte d'Ivoire, Democratic Republic of the Congo, Equatorial Guinea, Ethiopia, Gabon, Ghana, Guinea, Guinea-Bissau, Kenya, Liberia, Malawi, Mali, Mozambique, Niger, Nigeria, Rwanda, Senegal, Sierra Leone, South Sudan, Sudan, Togo, Uganda, United Republic of Tanzania. Detailed data and maps about the prevalence of onchocerciasis and elimination programmes can be obtained from the Expanded Special Project for Elimination of Neglected Tropical Diseases (ESPEN; <https://espen.afro.who.int/>; Rebollo et al., 2021). Due to mass drug administration (MDA) programmes, onchocerciasis transmission could be interrupted in Ecuador, Colombia, Mexico and Guatemala. Onchocerciasis transmission areas in America only remain in a remote cross-border region between Venezuela and northern Brazil including approximately 34,000 people (WHO, 2020c).

Epidemiology of onchocerciasis depends on vector abundance, competence and feeding characteristics, parasite strains as well as host ethnicity, heritability and immune responses as well as socio-economic factors like occupation, seasonal migration, income and social standing. Although susceptibility of men and women is comparable, there is evidence that children from *O. volvulus*-infected mothers are more susceptible to infection since they get infected in earlier life and have higher MF in the skin compared to children from non-infected mothers (Kirch et al., 2003). It is suggested that during pregnancy of infected women the filariae-induced immunosuppression towards *O. volvulus* is passed to the fetus and shapes its susceptibility to *O. volvulus* infection.

Onchocerciasis endemicity is classified in hypoendemic, mesoendemic or hyperendemic areas according to the MF prevalence rates of <40%, 40–59% and >60%, respectively. Based on transmission infection and disease data sets, mathematical models can characterize transmission dynamics (Basanez et al., 2016; NTD Modeling Consortium Onchocerciasis Group, 2019) and are a useful tool to predict the effects of treatment and vector control programmes.

Pathology

The majority of *O. volvulus*-infected individuals remain with few symptoms such as mild dermatitis and are referred to as generalized onchocerciasis (GEO); this status includes onchocercomata formation under the skin (Fig. 2A and B), high parasite burden and weak skin inflammation. Onchocercal nodules are normally 0.5–3 cm in diameter (Fig. 2C) and constitute granulomatous reactions that can be separated in chambers with thick fibrous walls and a variable degree of cellular infiltration around the adult worms (Fig. 2D), consisting of macrophages, neutrophils and eosinophils (Brattig et al., 2001; Korten et al., 1998). These nodules can degenerate, calcify and cause scarring and atrophy (Albiez et al., 1988; Burchard et al., 1979; Büttner et al., 1988; Lupi et al., 2015) (Fig. 2E). Cutaneous onchocerciasis can induce various skin pathology manifestations like acute and chronic papular dermatitis that can lead to skin hyperpigmentation (Fig. 3A) or depigmentation (leopard skin) (Fig. 3B) and atrophy with loss of elasticity (hanging groin) (Murdoch et al., 1993). Early symptoms of onchocercal dermatitis are itching and rash due to dying or dead MF that can disappear within a couple of days. However, the rash can persist and result in intense pruritus accompanied with secondary bacterial or fungal infections due to scratching. Consequently, repeated acute inflammation due to dying MF can lead to chronic onchodermatitis including large papules. In severe cases this leads to lichenification and thickening of skin, so called lizard skin, and depigmentation and hyperpigmentation, so called leopard skin (Fig. 3A and B), that can be used as epidemiological marker for onchocerciasis-endemic areas. However, the severest form of symptoms and skin diseases including dermatitis is sowda (Fig. 3C), which is characterized by pruritic hyperpigmented hyperkeratotic plaques, often asymmetrical and localized, associated with local lymphadenopathy (Edungbola et al., 1987; Katawa et al., 2015; Murdoch et al., 1993; Njim et al., 2015). This disease manifestation is called hyperreactive onchocerciasis (HO) and individuals suffering from HO normally present low parasite burden.

In addition, *O. volvulus* infection can cause vision loss due to immune responses against MF in the anterior and posterior segments of the eye (Abiose, 1998; Tamarozzi et al., 2011) (Fig. 4). Until now, the entry route of the MF into the eye is not clear, but it is suggested that blood sheaths of the posterior ciliary arteries and nerves, cerebrospinal fluid, as well as the sheath of the optic nerve serve as entry point (Basak et al., 2007; Budden, 1976). Dying MF release *Wolbachia* endosymbionts causing the infiltration and activation of fibroblasts, dendritic cells and macrophages that induce chemokine-based recruitment of neutrophils. Neutrophil-driven immune responses degrade the corneal matrix, causing corneal haze and visual impairment, edema and opacity (Tamarozzi et al., 2011). With regards to the posterior segment, the mechanisms leading to optic nerve atrophy and chorioretinitis are not fully understood. Interestingly, persistent deterioration in the posterior segment was also shown in individuals despite repeated ivermectin treatment and clearance of MF (Banla et al., 2014; Semba et al., 1990). It is suggested that autoimmune responses, i.e., cross-reactive antibodies to retinal pigment epithelial antigen, or persistence of MF or their products may play a role for posterior segment diseases (Braun et al., 1991; Chandrashekar et al., 1990).

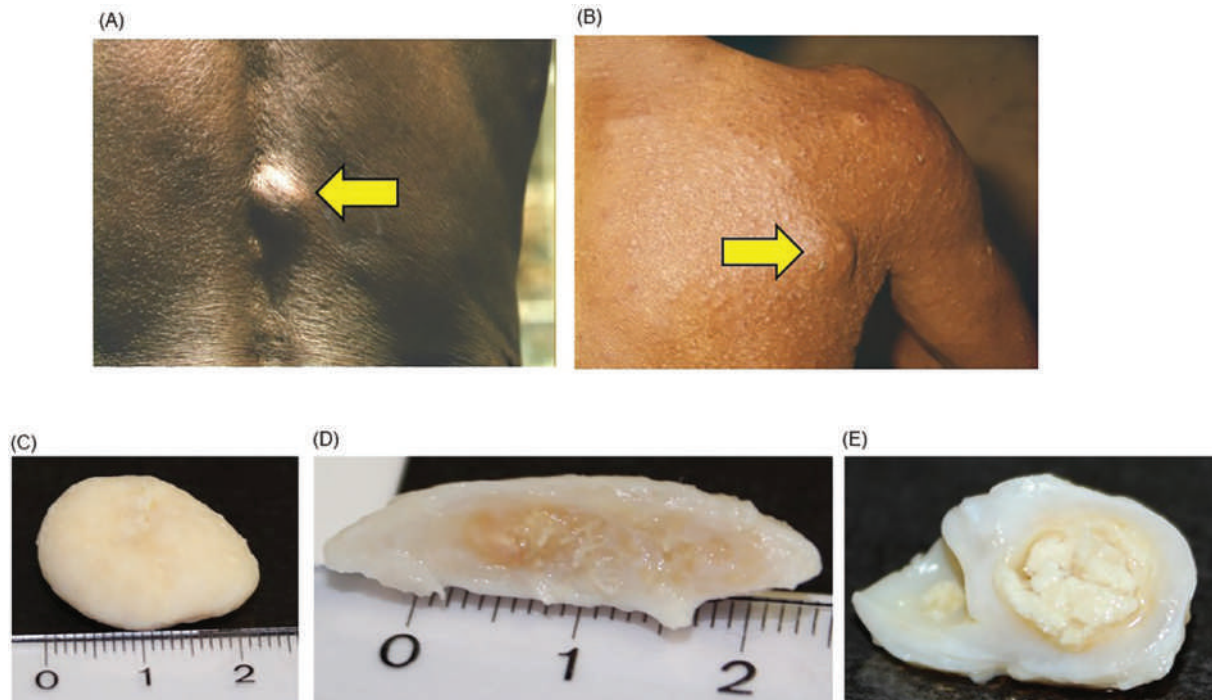


Fig. 2 Onchocercomata. (A and B) Arrows mark onchocercomata under the skin including pustular dermatitis in (B). (C) Isolated intact onchocercoma and (D) cut open onchocercoma showing adult *O. volvulus* filariae and (E) calcified adult worms. The scale bar shows cm. (C–E) Nodules were obtained during the MoRiOn study (ISRCTN43697583).

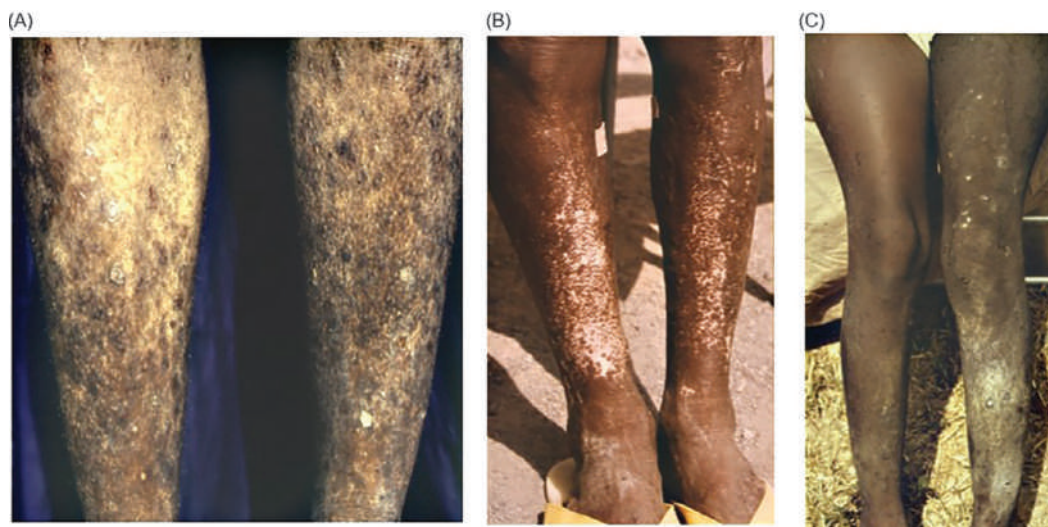


Fig. 3 Papular dermatitis during onchocerciasis. (A) Hyperpigmentation and lichenification, (B) leopard skin and (C) unilateral hyperreactive onchodermatitis (left leg), termed sowda.

Finally, onchocerciasis is associated with two forms of epilepsy, called onchocerciasis-associated epilepsy and nodding syndrome characterized by paroxysmal head nodding (Colebunders et al., 2019; Vieri et al., 2021), as well as with Nakalanga syndrome that is characterized by growth arrest and delayed sexual development (Kipp et al., 1996; Newell et al., 1997); the mechanisms how *O. volvulus* infection induces these diseases remains unclear.

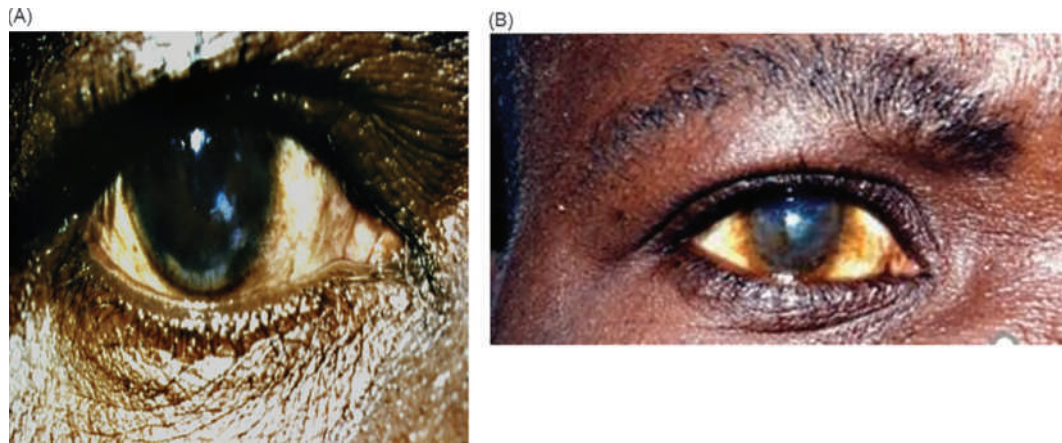


Fig. 4 Onchocerciasis-driven sclerosis of the cornea. (A) Onset of cornea sclerosis and (B) advanced sclerosing keratitis leading to vision loss.

Immunology and immunomodulation

As mentioned above, the majority of *O. volvulus*-infected individuals, i.e., those with generalized onchocerciasis (GEO), have few and mild symptoms; they tend to have high skin MF with low tissue reaction accompanied with the absence of clinical symptoms. Host immune responses of GEO individuals are regulated through the induction of regulatory T cell subsets, immunomodulatory IgG4 as well as IL-10 and TGF- β leading to suppression of effector mechanisms associated with parasite survival and MF release (Arndts et al., 2014; Doetze et al., 2000; Hoerauf and Brattig, 2002; Satoguina et al., 2002). It has been shown that the down-regulation of host immune responses impairs *Mycobacterium tuberculosis* (BCG) and tetanus vaccination efficacy and reduces immune response to allergens (Cooper et al., 1999; Kilian and Nielsen, 1989a,b). However, as mentioned above *O. volvulus*-infected individuals can suffer from severe disease; especially hyperreactive onchocerciasis (HO) individuals are characterized by increased effector mechanisms such as induction of IgE and Th1, Th2 and Th17 immune responses and suppression of regulatory cell subsets and immune responses (Hoerauf and Brattig, 2002; Katawa et al., 2015). The effector immune responses successfully eliminate MF from the skin but simultaneously elicit severe dermatitis and sowda, which is suggested to depend on genetic predisposition and the IL-13 gene (Hoerauf et al., 2002).

Diagnosis

Clinical manifestations of onchocerciasis are subcutaneous nodules, dermatitis, skin and eye lesions. The adult-worm containing nodules are usually mobile and painless. In sub-Saharan Africa their location is mainly around the hip region, calves and thorax (Hoerauf, 2011). For parasitological diagnosis MF can be detected from skin snips and adult worms from nodules. Diagnosis of *O. volvulus* adult worms via ultrasound (Mand et al., 2005) or histology from extracted nodules (Hoerauf et al., 2008) requires expertise and therefore MF diagnosis is the gold standard. In general, the iliac crest is the preferred site to diagnose MF. For MF diagnosis, 2–4 skin snips are taken with a 2 mm Holth corneoscleral punch or razor and transferred to phosphate buffered saline containing wells of a 96-well plate, where MF migrate out of the tissue within 24 h and can be microscopically quantified (Nutman et al., 1996). The unsheathed *O. volvulus* MF have to be distinguished from *M. streptocerca* in co-endemic areas in Sub-Saharan Africa and *M. ozzardi* in co-endemic areas in South-America. Contamination of skin snips with blood has to be avoided to exclude filarial species with blood-dwelling MF (e.g., *W. bancrofti*, *Brugia* species, *L. loa*). *O. volvulus* MF can also be detected inside the eye using a split lamp. Bending the head forward for 10 min increases the chances to locate the MF in the eye (Hoerauf, 2011). In general, *O. volvulus* MF are most often found in residents from endemic areas that are not under MDA, while non-resident travelers often develop amicrofilaridemic infections. For serological analysis a commercially available rapid-format antibody card test is available that detects IgG4 antibodies against the *O. volvulus* Ov-16 recombinant antigen (Lipner et al., 2006). Furthermore, several molecular tests are available to detect DNA from *O. volvulus* in skin snips and *Simulium* blackflies via either PCR-based methods (Lloyd et al., 2015; Prince-Guerra et al., 2018) or field-applicable Loop-mediated isothermal amplification (LAMP) (Abong et al., 2020, 2021; Poole et al., 2017).

Intervention/Therapy

In accordance with the United Nations Sustainable Development Goals on health, the WHO Neglected Tropical Disease roadmap 2021–30 stated as target for onchocerciasis the confirmed elimination of transmission by 2030 (WHO, 2020a, c). The current intervention relies on the distribution of MDA with ivermectin in endemic areas (WHO, 2020c).

Ivermectin is a macrocyclic lactone that is given as a single oral dose of 150 µg/kg. Ivermectin clears the majority of MF within a few days after treatment and temporarily inhibits the embryogenesis of the adult *O. volvulus* female worms (Awadzi et al., 1989; Gardon et al., 2002). Thus, the release of MF and the transmission of onchocerciasis is interrupted for several months (Duke et al., 1991). However, since ivermectin is not macrofilaricidal—does not kill the adult worms—MDA has to be repeated every 6–12 months for the reproductive life-span of *O. volvulus*, i.e., 10 years and more, to eliminate the transmission of the disease. Contraindications of ivermectin treatment in pregnant or breast-feeding women with children less than 1 week of age as well as children below 90 cm of height, migrating populations, discontinuous or sub-coverage MDA and areas that showed suboptimal ivermectin efficacy further hamper the efforts to eliminate onchocerciasis by ivermectin MDA. Most common adverse events following ivermectin treatment are caused by the dying MF and consist of inflammatory immune responses leading to itching skin, rash, fever, and/or headache (Whitworth et al., 1988). Importantly, in comparison to diethylcarbamazine (DEC), adverse events caused by ivermectin treatment are less severe, and while permanent vision impairment can occur following DEC treatment in onchocerciasis patients, this is not the case with ivermectin (Greene et al., 1985). Thus, DEC is contraindicated for treatment of onchocerciasis patients. More recently, moxidectin, another macrocyclic lactone, was introduced for onchocerciasis treatment. Similar to ivermectin, moxidectin clears MF, temporarily inhibits filarial embryogenesis and has no macrofilaricidal efficacy (Awadzi et al., 2014; Opoku et al., 2018). However, in comparison to ivermectin, moxidectin leads to an extended absence of microfilaridermia, which lasts for up to 1 year (Opoku et al., 2018).

The only well-tolerated macrofilaricidal drug for onchocerciasis so far is doxycycline (Hoerauf et al., 2000, 2001; Walker et al., 2015). Doxycycline targets the *Wolbachia* endosymbionts of *O. volvulus* and by depleting these endosymbionts doxycycline therapy leads to a permanent inhibition of filarial embryogenesis and death of the adult worms after 1.5–2 years (Hoerauf et al., 2003, 2008, 2009). Doxycycline treatment does not cause MF-induced adverse events, as it has no or only little effect on the MF itself, and MF clearance over time is only due to the natural removal of MF and the inhibition of filarial embryogenesis. To achieve the macrofilaricidal efficacy, doxycycline has to be given for 6 weeks with 200 mg/day (Hoerauf et al., 2008; Turner et al., 2010; Walker et al., 2015). To achieve permanent sterility and permanent amicrofilaridermia, doxycycline treatment with 200 mg/day for 4 weeks or 100 mg/day for 5 weeks are sufficient (Hoerauf et al., 2008, 2009; Walker et al., 2015). In order to accelerate the clearance of MF, doxycycline treatment can be combined with a single dose of ivermectin (Hoerauf et al., 2001; Turner et al., 2010). Doxycycline is now recommended by the WHO as individual therapy and in mopping up remaining foci in the Americas (WHO, 2019). Although the extended treatment with doxycycline over several weeks prevents its broader use for MDA and its use is contraindicated in pregnant, breast-feeding women and children below the age of 8, doxycycline treatment confirmed the efficacy of anti-wolbachial drugs.

Currently, research is performed to identify anti-wolbachials that allow shorter treatment regimens. The Tylosin analog ABBV-4083 is such an anti-wolbachial candidate (Taylor et al., 2019) that was recently tested in phase 1 clinical trials, and phase 2 clinical studies in onchocerciasis patients are ongoing. Similarly, high dose rifampicin treatments with 30–35 mg/kg/day are predicted to be superior to doxycycline treatment (Aljayyousi et al., 2017; Specht et al., 2008) and are currently tested in phase 2 clinical studies. Two additional macrofilaricidal candidates are the direct-acting cyclooctadepsipeptide emodepside (Krücken et al., 2021) and the benzimidazole oxfendazole (Hübner et al., 2020), which both passed phase 1 clinical studies. Emodepside is currently evaluated in phase 2 clinical studies in onchocerciasis patients.

A summary of the key facts for onchocerciasis covered in this chapter is presented in Table 1.

Lymphatic filariasis

Short overview

Lymphatic filariasis (LF) is listed by the WHO as neglected tropical disease (NTD) and is caused by infection with the filarial nematodes *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*. LF is the second leading infectious cause of disability worldwide after leprosy. The disease impairs the lymphatic system and can lead to severe clinical manifestations such as hydrocele, lymphedema and elephantiasis, causing a major public health problem and an overall increase in disability-adjusted life years (DALYs) in endemic countries.

Biology/Etiology

Three species of the lymph-dwelling filarial nematodes, named *W. bancrofti*, *B. malayi* and *B. timori* cause LF in humans and so far, no major reservoir host is known, except for a zoonotic habitat in South Thailand. The disease is transmitted by mosquito species including the genera *Aedes*, *Anopheles*, *Culex* and *Mansonia* (Manguin et al., 2010). *B. timori* is transmitted by *Anopheles barbirostris* (Atmosoedjono et al., 1977), *B. malayi* by *Mansonia* and *Anopheles* spp. (Bizhani et al., 2021). 90% of LF infections are caused by *W. bancrofti* during a bite of all mentioned mosquito spp. (Fig. 1). During the blood meal of the mosquito, infective L3 (1.5 mm in length and 18–23 µm in diameter) are transmitted and migrate to the lymphatics, where they molt and become adult worms within a period of 5–18 months. Female *W. bancrofti* worms are 8–10 cm long and 0.24–0.30 mm in diameter, while males are 4 cm long and 1 mm in diameter. In contrast, *Brugia* adult worms are smaller and female worms measure 4.3–5.5 cm in length and

130–170 μm in diameter, while males are 1.3–2.3 cm long and 70–80 μm width. Gravid female worms release sheathed MF (*W. bancrofti*: 244–296 μm long and 7.5–10 μm wide; *Brugia spp.*: 177–230 μm long and 5–7 μm wide) in the lymphatic vessels that drain into veins entering the blood stream. Interestingly, MF release in the blood stream follows a nocturnal or diurnal periodicity that corresponds to the biting behavior of the vector in the endemic area. In most areas endemic for *W. bancrofti* and *B. malayi* a nocturnal periodicity and peak in MF numbers in the peripheral blood around midnight are prevalent (Simonsen et al., 1997), however, transmission by day biting (diurnal) has been shown in the Pacific region (Moullia-Pelat et al., 1993), with mainly *Aedes* mosquitoes, especially *Ae. polynesiensis*. Female worms live for 5–10 years and produce MF that have a lifespan of 6–24 months (Stolk et al., 2006). These MF are ingested by female mosquitoes during a blood meal. When MF reach the mosquito's stomach, they lose their sheaths and migrate to the midgut to reach the thoracic muscles. Consequently, they molt twice to become infective L3 that migrate through the haemocoel to the mosquito's proboscis where they are transmitted to the human host during a blood meal of female mosquitoes (Bizhani et al., 2021; Edeson, 1972; Nutman and Kazura, 2011; Srividya et al., 2019).

Epidemiology

Since the launch of the Global Programme to Eliminate Lymphatic Filariasis (GPELF) more than 8.2 billion treatments have been distributed between 2000 and 2019. This decreased the population at risk for LF to approximately 858 million people in 49 countries worldwide and led to a reduction of infections and even elimination in Togo, Egypt, Maldives, Sri Lanka, Thailand, Cambodia, Cook Island, Marshall Islands, Niue, Palau, Tonga, Vanuatu, Vietnam, Wallis, Futuna, Japan, Korea and China (Cheun et al., 2009; De-Jian et al., 2013; WHO, 2020b). Currently it is estimated that 65 million individuals are infected with LF and that 19 million hydrocele and 17 million lymphedema cases exist, leading to 1.3 million DALYs (Global Burden of Disease Study 2017, 2018; Ramaiah and Ottesen, 2014; WHO, 2020b). Approximately 90% of LF infections are caused by *W. bancrofti* that occurs in tropical regions of South and Southeast Asia, Africa, the Americas, Middle East and the Pacific, whereas *B. malayi* is found in India, Indonesia, Thailand, Malaysia, and the Philippines. *B. timori* is restricted to islands in Eastern Indonesia (Local Burden of Disease Neglected Tropical Diseases Collaborators, 2020; Michael and Bundy, 1997; Rebollo and Bockarie, 2013). Nevertheless, epidemiology of LF strongly depends on the available habitat, vector capacity and mosquito density, and distribution is often focal so that transmission rates can vary strongly between the communities and even households (Bockarie et al., 2009; Rwegoshora et al., 2007).

Pathology

Although the majority of infected individuals remain asymptomatic and do not develop clinical symptoms, LF can lead to severe clinical symptoms. The most common clinical symptoms of LF are hydrocele (only *W. bancrofti*), lymphedema of the legs, lymphangitis, and elephantiasis (Rebollo and Bockarie, 2017; WHO, 2001). The presence of worms (including *Wolbachia* endosymbiotic bacteria) and their products in the lymphatic system induce endothelial cell proliferation and dilatation of lymphatic vessels (lymphangiectasia) that is mediated by host proteins such as vascular endothelial growth factors, angiopoietin and matrix metalloproteinases. The development of lymphedema is associated with genetic risk factors, and nucleotide polymorphisms for the above genes in the human host have been identified, explaining, at least in part, the clustering in families (Bennuru and Nutman, 2009; Debrah et al., 2006, 2017; Taylor, 2003). Due to the impaired lymphatic function of the dilated lymph vessels, lymph is no longer normally pumped against gravity (similar to venous stasis), resulting in elevated tissue pressure. This can lead to lesions at the skin of the affected leg, which allow secondary bacterial and fungal infections facilitating acute dermatolymphangioadenitis (ADLA) attacks that accelerate the progression of chronic lymphedema development and elephantiasis of the lower extremities (Dreyer et al., 1999a, b, 2006; Olszewski et al., 1999). Progression of lymphedema occurs over periods of years during which leg skin thickens and loses elasticity, develops deep folds accompanied with development of dermatosclerosis and papillomatous “mossy” skin lesions. Lymph fluid may leak from chronic ulcerations in the affected part leading to foul smell of the affected individuals. Moreover, due to rupture of dilated abdominal lymphatic vessels into the upper urinary tract, chyle (and sometimes blood) can be present in the urine (chyluria), leading to weight loss, hypoproteinaemia, lymphopenia and anemia (Gulati et al., 2007). In general, lymphedema is subdivided into seven stages depending on swellings (reversible or non-reversible), leg volume, presence of skin folds, nodules, knobs and lesions (Dreyer et al., 1999b; WHO, 2001) (Fig. 5). Besides lymphedema, hydrocele is a common clinical symptom that affects *W. bancrofti*-infected men due to fluid accumulation inside the tunica vaginalis and swelling of scrotal and groin area (Fig. 6). Acute hydrocele is a result of natural or anti-filarial treatment-induced worm death, leading to acute filarial lymphangitis (AFL) followed by temporally clogging of the lymphatics due to disintegrating worms (Noroes et al., 2003), whereas chronic hydrocele can develop after years of infection due to impaired lymph transport. Migration of adult worms into the scrotal lymphatics and formation of scrotal nodules is regularly observed, with and without hydrocele (Pfarr et al., 2009). Finally, a rare clinical symptom of LF is called tropical pulmonary eosinophilia (TPE). TPE is caused by immunological hyperresponsiveness to MF in the lungs accompanied with paroxysmal coughing and wheezing, extremely high blood eosinophil counts (>3000 cells/ mm^3 of blood) and serum levels of total IgE and antifilarial antibodies that can lead to extrapulmonary manifestations such as splenomegaly, lymphadenopathy and hepatomegaly (Ottesen and Nutman, 1992) or inguinal lymphadenopathy, monoarticular arthritis with pain, warmth and tenderness as well as haematuria and proteinuria (Dreyer et al., 1999a; Sarker et al., 2007).

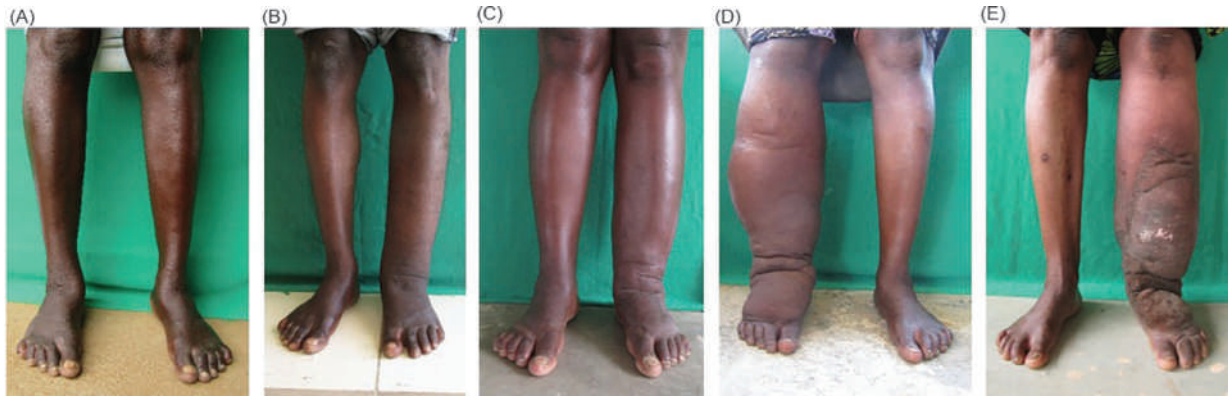


Fig. 5 Lymphedema stages of lymphatic filariasis. Staging was performed according to Dreyer et al. (2002), based on the presence of swelling, skin folds, knobs and mossy lesions. (A) Stage 2 right leg, (B) stage 3 left leg, (C) stage 4 left leg, (D) stage 5 right leg and (E) stage 6 left leg. Pictures were obtained from the LEDoxy trial (ISRCTN14042737) within the TAKEOFF project (<https://www.gesundheitsforschung-bmbf.de/en/takeoff-filariasis-and-podoconiosis-disease-control-7700.php>).



Fig. 6 Hydrocele from *W. bancrofti*-infected man being prepared for hydrocelectomy.

Immunology and immunomodulation

Despite the fact that individuals in endemic regions are constantly exposed and live with a high risk of infection, a wide range of clinical manifestations and infections status, such as infected MF-positive and infected but MF-negative, as well as uninfected individuals, can be observed. For example, individuals with asymptomatic infections are relatively tolerant to filarial worms and are characterized by strongly regulated and dampened immune responses via the induction of regulatory B and T cell subsets, alternatively activated macrophages and increased secretion of regulatory molecules IL-10, TGF- β and IgG4 that are essential for the long-term survival of filariae and prevent the onset of severe pathology (Adjomey and Hoerauf, 2010; Arndts et al., 2012; Babu et al., 2006; Hoerauf et al., 2005; Metenou et al., 2010; Pfarr et al., 2009; Ritter et al., 2019). Host immune regulation also leads to decreased immune responses to other pathogens and impaired vaccination efficacy (Kroidl et al., 2016; Metenou et al., 2011; Michael et al., 1994; Nookala et al., 2004; Simonsen et al., 2002), but was also suggested to protect against diabetes and controlling autoimmunity and inflammation (Aravindhana et al., 2010a,b; Babu et al., 2006). Indeed, loss of regulation and immune tolerance to filarial worms and activation of host immune responses such as increased Th17 responses and proinflammatory immune responses leads to death of the parasites and collateral damage to lymphatic vessels, showing that the host immune status strongly influences the infection status (Arndts et al., 2012; Babu and Nutman, 2014; Freedman, 1998; Jaoko et al., 2006; Maizels et al., 2009; Ritter et al., 2019). Indeed, most chronic lymphedema patients are no longer infected and accelerated immune responses like increased IgE levels and eosinophils are associated with TPE (Chitkara and Krishna, 2006; Hussain et al., 1981).

Diagnosis

Acute filarial lymphangitis (AFL; reversible after death of adult worms), lymphedema, hydrocele, and the other abnormalities mentioned above are the most prominent pathologies occurring during LF. In order to differentiate them from non-filariae-caused pathologies, parasitological, serological and molecular tests are available.

Classical parasitological diagnosis relies on the detection of MF in peripheral blood. However, detection of MF alone is not a reliable test for the diagnosis of LF, as especially patients with clinical filariasis, i.e., lymphedema or hydrocele, are often amicrofilaremic, whereas MF positive patients are often asymptomatic. Furthermore, the predominant nocturnal periodicity of the MF requires that MF diagnosis is performed from blood collected during the peak of microfilaremia between 21:00–3:00 at night (Simonsen et al., 1997). Microscopic evaluation further requires the differentiation of the sheathed MF of LF from other filariae with blood-dwelling MF such as *M. perstans*, *M. ozzardi* and *L. loa*; this can be achieved via the filarial-species specific arrangement of the nuclei. Besides the routine thick blood smear, concentration methods such as Knott's and membrane filtration are used to increase the sensitivity for MF detection. For example, if MF density is <100 MF/mL blood, a smear using only 10 µL of blood may turn out false negative.

Adult *W. bancrofti* infections can be also detected in the male scrotum via ultrasonography (Mand et al., 2003). The specific movement pattern of the adult filariae is referred to as filarial dance sign within the dilated lymphatic vessels. Ultrasonography further allows the assessment of hydroceles. Detection of adult worms in other locations such as the female breast or inguinal regions as well as *B. malayi* adult worms is not regularly achieved by ultrasonography (Mand et al., 2006).

A big step forward in LF diagnosis has been the introduction of card tests that detect Circulating Filarial Antigen (CFA) released by adult *W. bancrofti* (but not *Brugia*) antigens. Results appear to correlate with adult worm burden (Weil et al., 2013). Importantly, the CFA test is field-applicable, has a hand-on time of approx. 15 min. and does not require blood that is taken at night. Recent data suggest that the CFA test can cause false-positive results in heavily infected *L. loa* patients (Pion et al., 2016). This presents a problem for MDAs, as the CFA test is most commonly used for the mapping of LF endemic areas (WHO, 2011); however, more recent research has found that endemic areas for LF and loiasis do not grossly overlap, due to different habitats of the transmitting vectors. Similar to onchocerciasis, IgG4-detecting antibody tests are also available for LF and they have an increased specificity in comparison to IgG based antibody tests. Recombinant antigens used in those assays are e.g., Bm14 and Wb123 (Steel et al., 2013; Weil et al., 2011). However, while those antibody tests are valid for the diagnosis of expatriates, they are not recommended for people from LF endemic regions, as these individuals have been multiply infected and often possess specific antibodies in the absence of active infection or clinical filariasis.

Several PCR and LAMP assays are also established to diagnose MF DNA from peripheral blood (Poole et al., 2017; Rao et al., 2006). Similar to the microscopic detection of MF, the molecular techniques detecting MF DNA require blood samples that were taken during night. The sensitivity of those molecular tests is comparable to the microscopic techniques.

Intervention/Therapy

The WHO NTD roadmap 2021–30 stated the goal to achieve for LF in 80% of endemic countries the elimination as public health problem by 2030.

Already in the 1990s, annual MDA with ivermectin plus albendazole or DEC plus albendazole were used in combination with mosquito control programs. In 2000, the WHO launched the GPELF with the goal to eliminate LF by 2020. Within GPELF a total of over 8 billion doses were distributed to more than 923 million people by annual MDA treatments with ivermectin plus albendazole or DEC plus albendazole and 17 countries are currently under surveillance for verifying the elimination the LF transmission. While GPELF did not reach the goal of global LF elimination by 2020, it provides a platform that countries can use to sustain their efforts to eliminate LF as public health problem by 2030 (Hooper et al., 2014; Ichimori et al., 2014; Ottesen and Horton, 2020; Ramaiah and Ottesen, 2014; Turner et al., 2016).

Thus, in endemic areas, the main intervention for LF consists of annual, single-dose mass administration of drugs (DEC, ivermectin and albendazole) that are primarily targeting the MF stage. Single-dose oral DEC or ivermectin treatment removes MF from the peripheral blood and inhibits MF release by the adult worms for a few months, but does not efficiently kill the adult worms (Cao et al., 1997; Eberhard et al., 1997). Similarly, a single oral dose of albendazole reduces *W. bancrofti* microfilaremia for several months (Ismail et al., 1998). Over the past decades, combination therapies with DEC plus albendazole (in areas non-endemic for onchocerciasis) or ivermectin plus albendazole (in areas co-endemic for onchocerciasis) were performed as annual MDA. Today, for LF in areas that are not co-endemic for onchocerciasis and loiasis, WHO recommends MDA using “IDA” triple therapy (WHO, 2017) of a single dose of ivermectin (200 µg/kg), DEC (6 mg/kg) and albendazole (400 mg), which reduces microfilaremia for more than 2 years (King et al., 2018; Weil et al., 2019). Furthermore, it is suggested that IDA also has some macrofilaricidal efficacy (Thomsen et al., 2016). The implementation of the triple therapy may be a game changer, as it is expected to reduce MDA rounds. IDA is not recommended in areas that are co-endemic for onchocerciasis or loiasis, since DEC can lead to severe adverse ocular events in onchocerciasis patients and ivermectin caused life-threatening severe adverse events in *L. loa* patients with very high MF loads (Gardon et al., 1997; Gentilini and Carme, 1981; Greene et al., 1985; WHO, 2017), which may even be aggravated by the additional microfilaricidal activity of DEC. Thus, in onchocerciasis co-endemic areas a combination of ivermectin and albendazole is used, whereas in loiasis co-endemic areas people with high microfilariae loads have to be identified and excluded from treatment, or be pre-treated with albendazole. For TPE, a 21-day treatment with 6 mg/kg DEC is recommended.

Similar to onchocerciasis, doxycycline was also shown in LF patients to be the first well-tolerated macrofilaricidal treatment that can be used as individual therapy. Four weeks of 200 mg/kg doxycycline treatment are sufficient to clear the filarial *Wolbachia* endosymbionts, which permanently inhibits filarial embryogenesis thereby clearing MF without having a direct microfilaricidal effect and slowly killing the adult worms (Debrah et al., 2011). This treatment can also be safely used against LF in patients co-infected with *L. loa* (even high) infections, since *L. loa* do not contain *Wolbachia* and are therefore not affected. It is expected that novel macrofilaricidal drugs (e.g., ABBV-4083) that are currently developed for onchocerciasis are also active against filariae causing

LF, which could help to clear remaining infections. Importantly, it is suggested that doxycycline treatment also improves lymphedema pathology, which is thought to be due to the immunomodulatory and anti-inflammatory properties of doxycycline. For the treatment of lymphedema, doxycycline should be given daily at 200 mg/kg for 6 weeks, with intervals every 12–24 months (Mand et al., 2012). In order to include this regimen into the recommendations for lymphedema management by the WHO, a multicentre study in five endemic countries (Ghana, Mali, Sri Lanka, India and Tanzania) is currently ongoing to evaluate the efficacy of doxycycline treatment (<https://doi.org/10.1186/ISRCTN14042737>) (Horton et al., 2020). The current basic standard care for lymphedema consists of hygiene measurements involving the washing of the affected limbs with soap and water, the use of topical antibiotics and antifungals and wearing shoes, to reduce bacterial and fungal infections which otherwise promote the progression of lymphedema (Dreyer et al., 2002). For large hydroceles, surgery is the only option (Betts et al., 2020; Johnson and King, 2019).

A summary of the key facts for LF covered in this chapter is presented in Table 1.

Loiasis

Short overview

Loiasis is caused by the filarial nematode *L. loa*, commonly known as “eye worm”, and is restricted to tropical rain forest areas in Central and West Africa (Benin to Gabon). In contrast to onchocerciasis and LF, loiasis has not yet been listed as NTD. It is however considered of public health relevance on its own, and because ivermectin or DEC treatment during MDA programmes against onchocerciasis and LF revealed severe adverse events including encephalitis in co-infected individuals with high *L. loa* MF loads, MDA programmes in co-endemic regions in sub-Saharan Africa are hindered.

Biology/Etiology

As shown in Fig. 1, loiasis is caused by the tissue-dwelling nematode *L. loa* that is transmitted through a bite of infected *Chrysops silacea* and *C. dimidiata* flies (Padgett and Jacobsen, 2008; Wanji et al., 2002). These flies have a diurnal biting behavior and transmit L3 during a blood meal into the bite wound. L3 migrate through the subcutaneous tissue and mature to adult female (40–70 mm in length and 0.5 mm wide) and male worms (30–34 mm in length and 0.35–0.43 mm wide). Gravid female worms release sheathed MF (250–300 µm in length and 6–8 µm wide) that can be recovered in peripheral blood, spinal fluid, urine, lung and even sputum. During another blood meal, *Chrysops* flies ingest the MF, which lose their sheaths and migrate from the midgut into the thoracic muscles of the arthropod. The L1 develop to L3 which migrate to the proboscis of the fly to get transmitted during another blood meal (Padgett and Jacobsen, 2008; Wanji et al., 2002; Zoure et al., 2011).

Epidemiology

L. loa infections are restricted to the rainforest and some savannah areas of 11 Western and Central African countries, namely Angola, Cameroon, Central African Republic, Chad, Congo, Democratic Republic of the Congo, Equatorial Guinea, Ethiopia, Gabon, Nigeria and Sudan. Approximately 14.4 million people live in high risk areas where a history of “eye worm” is present in more than 40% of individuals and 15.2 million people live in intermediate risk areas with an estimated loiasis prevalence of 20–40% (Zoure et al., 2011). *Chrysops* flies are more common during the rainy season and usually bite during the day and are attracted by the movement of people as well as smoke from fire (Noireau et al., 1990). Besides the rainforest, *Chrysops* are also prevalent in rubber plantations, palm oil groves and fringes of mangrove swamps, where they can transmit the disease (Davey and O'Rourke, 1951). Transmission of *L. loa* depends on infected vector abundance, time of exposure and number of bites.

Pathology

Due to the migration of adult worms the common clinical symptoms of loiasis are oedemas, Calabar swelling (transient subcutaneous swelling marking the migratory course of the parasite) (Fig. 7A), pruritis, arthralgia and eye worm (Fig. 7B), although the majority of infected individuals remain asymptomatic (Boussinesq, 2006; Lukiana et al., 2006). However, reports about serious adverse events including fatal cases of encephalopathy after ivermectin or DEC administration raised attention to the disease (Carme et al., 1991; Duke, 2003; Nieves-Moreno et al., 2017; Twum-Danso and Meredith, 2003). It has been shown that serious adverse events are associated with high MF loads (>30,000 MF/mL) in the peripheral blood and the inflammatory responses to the dying MF are associated with serious adverse events (Boussinesq et al., 1998, 2001; Gardon et al., 1997) causing wide-spread concern on the sustainability of community-directed treatment strategies applied for onchocerciasis and LF elimination programmes (Addiss, 2010; Amazigo et al., 2002).

Immunology and immunomodulation

Knowledge about the *L. loa*-driven immunology is limited to few studies in humans and experimental infections in mandrills, rhesus monkeys and recently developed mouse models. Those studies revealed that non-specific polyclonal IgE and elevated levels of antigen-specific IgG4 are associated with *L. loa* infection (Akue et al., 1994, 1996; Njambe Priso et al., 2018; Pinder et al., 1994) as well as distinct cytokine and chemokine patterns, especially Th1 and Th2 immune responses and eosinophil-associated cytokines (Baize et al., 1997; Chunda et al., 2020; Herrick et al., 2015; Leroy et al., 1997; Tendongfor et al., 2012).

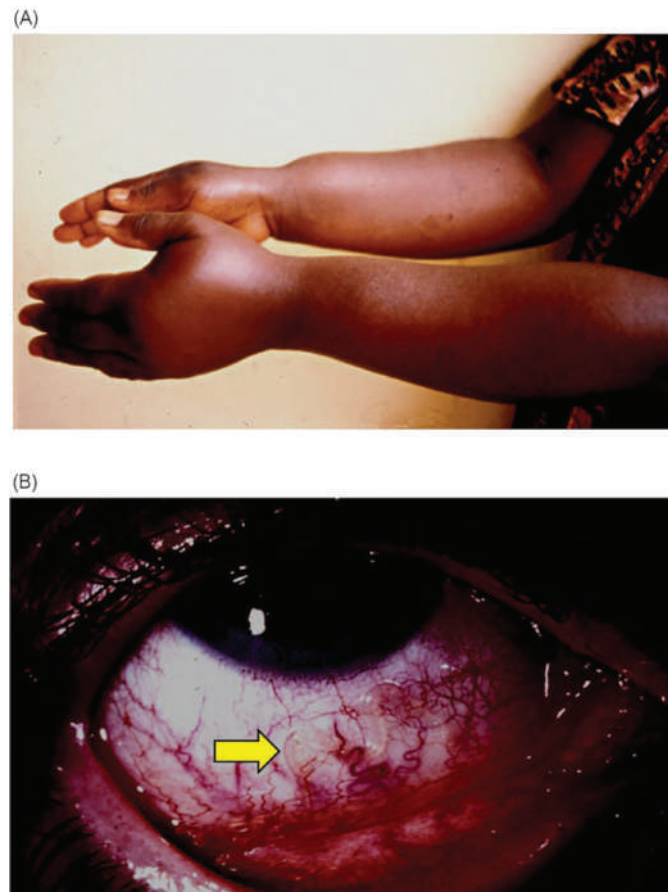


Fig. 7 Clinical pictures caused by migrating adult *Loa loa* worms. (A) Calabar swelling and (B) eye worm.

Diagnosis

A history of Calabar swellings, which are caused by immune responses to the subcutaneous migration of the adult *L. loa* filariae, or their passage through the eye are indications of loiasis. Symptoms that can be mistaken with Calabar swellings are lesions induced by *M. perstans* or skin reactions to migrating larvae of *Strongyloides stercoralis* or hookworms from dogs or cats. The primary parasitological diagnosis is the detection of MF from the peripheral blood, although MF-negative infections, especially in patients with clinical symptoms, do exist (Dupont et al., 1988). Antigen detection tests, such as with *W. bancrofti*, are not available. Since *L. loa* MF are diurnal, blood should be taken for MF analysis during noon. Similar to the microscopic analysis of MF from *W. bancrofti* and *Brugia* species, Knott or filtration concentration methods can be used to increase the sensitivity to detect MF. Furthermore, microscopic analysis requires the differentiation of *L. loa* MF from MF of other filarial species based on morphological characteristics such as the presence of a sheath and the species-specific arrangement of the nuclei. Since a “test and not-treat” strategy is implemented in areas co-endemic for onchocerciasis (Kamgno et al., 2017), the LoaScope, a cell phone based method detecting moving MF in a fresh blood smear, was developed to identify *L. loa* patients with high MF counts that have an increased risk for severe adverse events following ivermectin treatment (Pion et al., 2020). Molecular tests to identify *L. loa* in peripheral blood and *Chrysops* flies using PCR and LAMP techniques have been established (Amambo et al., 2021; Drame et al., 2014; Fink et al., 2011). Previous serological tests were limited due to their cross-reactivity to other filarial species (Akue et al., 1996), but recent serological tests showed an increased sensitivity and specificity (Gobbi et al., 2020; Pedram et al., 2017). However, serological tests are not able to differentiate between active infections and previous exposures/infections.

Intervention/Therapy

Adverse events following drug treatment in loiasis patients is related to the MF count (summarized in Gobbi et al., 2018). DEC given at 5–10 mg/kg daily for 2–4 weeks is the standard treatment that quickly clears microfilaremia and has some macrofilaricidal efficacy. Most common adverse events following DEC treatment include fever, nausea and itching. A single oral treatment with ivermectin (200 mg/kg) also reliably clears microfilaremia, but harbors the risk of causing life-threatening serious adverse events in patients with high MF loads (as would DEC). To avoid those serious adverse events consisting of neurological symptoms, encephalopathy, coma and death, patients with more than 20,000 MF/mL blood should not be treated with ivermectin. Those patients with high MF loads can be treated twice daily with 200 mg albendazole for a total of 21 days, which has only a minor microfilaricidal efficacy and leads to a well-tolerated slow decline in MF levels (Klion et al., 1993). The risk of serious adverse events following ivermectin treatment presents a challenge for onchocerciasis elimination programs in central Africa, as it requires test-and-not-treat measurements in *O. volvulus* and *L. loa* co-endemic areas (Pion et al., 2020). However, MDA activities continue in *L. loa*

co-endemic areas, as the Mectizan Expert Committee/Technical Consultative Committee (MEC/TCC) based their recommendations on the health benefits of preventing blindness due to onchocerciasis rather than the risk of *L. loa* encephalopathy following ivermectin MDA (The Mectizan Expert Committee and The Technical Consultative Committee, 2004). Given that *L. loa* does not harbor endosymbiotic *Wolbachia* bacteria (Büttner et al., 2003; Desjardins et al., 2013; Hoerauf et al., 2008), treatments with anti-wolbachials such as doxycycline are no option.

A summary of the key facts for loiasis covered in this chapter is presented in Table 1.

Mansonellosis

Short overview

Based on the worldwide distribution of mansonellosis, but the limited knowledge about the epidemiology, biology and immunology accompanied with the lack of a distinct clinical picture of the disease, mansonellosis can be considered as the most neglected human filarial infection.

Biology/Etiology

Mansonellosis is caused by four *Mansonella* species, named *Mansonella perstans*, *M. ozzardi*, *M. streptocerca* and a newly discovered molecular clade of *M. perstans* named *Mansonella* sp. “DEUX” (Mourembou et al., 2015; Simonsen et al., 2011). Moreover, zoonotic species like *M. rodhaini* and unidentified *Mansonella* sp. have been described, but until now knowledge about mansonellosis is mainly based on *M. perstans*. Thus, transmission of *M. perstans* was associated with *Culicoides* midges (Mediannikov and Ranque, 2018; Simonsen et al., 2011; Ta-Tang et al., 2018) and recently a study in Cameroon revealed the night-biting *C. milnei* transmitting *M. perstans* in equatorial rainforest areas in Cameroon (Wanji et al., 2019). In addition, *M. streptocerca* and *M. ozzardi* are also transmitted by *Culicoides* midges, but studies also revealed that blackflies from the genus *Simulium* and non-*Culicoides* Ceratopogonidae biting midges transmit *M. ozzardi* in South America and Haiti (Crosskey, 1990; Linley et al., 1983; Shelley and Coscarón, 2001). During a blood meal, an infected arthropod transmits L3 onto the skin of the human host where they penetrate into the bite wound. Then, they develop into adult worms. *M. perstans* female (70–80 mm in length and 120 µm wide) and male (45 mm long and 60 µm wide) reside in body cavities like peritoneum, pleura and pericardium, whereas the smaller *M. ozzardi* adult worms (female: 32–61 mm long and 130–150 µm wide; male 24–28 mm long and 70–80 µm wide) reside in subcutaneous tissue that are comparable in size to *M. streptocerca* adult worms. Gravid female worms produce unsheathed subperiodic MF (MF are present at all times but reach a peak at distinct time points; *M. perstans*: 200 µm long and 4.5 µm wide) and non-periodic MF (*M. ozzardi*: 207–232 µm long and 3–4 µm wide and *M. streptocerca*: 180–240 µm long and 3–5 µm wide) that reach the peripheral blood and skin (Fig. 8). MF can be taken up during another blood meal of the vector where they migrate from the midgut into the thoracic muscles and develop from L1 to L3 that can be transmitted again to the human host (Lima et al., 2016; Mediannikov and Ranque, 2018; Orihel and Eberhard, 1982; Simonsen et al., 2011; Ta-Tang et al., 2018). An overview of the life cycle of *Mansonella* spp. is shown in Fig. 1.

Epidemiology

It is estimated that 114 million individuals are infected with *M. perstans* in West, East, and Central Africa, and in some neotropical regions of Central and South America, and that 600 million people live at high risk in 33 African countries. *M. ozzardi* is found in Central America, Argentina, Bolivia, Brazil, Colombia, Guyana, Suriname and Venezuela and several Caribbean islands. *M. streptocerca* is only prevalent in tropical regions of West, Central and East Africa. Recently *Mansonella* sp. “DEUX” have been determined in the Gabonese population (Mediannikov and Ranque, 2018; Mourembou et al., 2015; Sandri et al., 2021; Simonsen et al., 2011; Ta-Tang et al., 2018), but it remains unclear if *Mansonella* sp. “DEUX” corresponds to other zoonotic *Mansonella* species. Transmission of mansonellosis is strongly associated to distribution, abundance and seasonal occurrence of the vectors, which depends on the availability of aquatic or semi-aquatic habitats (e.g., mud or moist soil close to streams, ponds and marshes), dung of various animals, rotting fruit, cacti, banana stems and leaf detritus that serve as breeding site and are essential for the development of immature stages (Meiswinkel et al., 2004; Mellor et al., 2000; Wanji et al., 2019).

Pathology

Interestingly, mansonellosis is not associated with distinct severe clinical symptoms and thus often not considered as a public health problem. Nevertheless, there are clinical reports that associate symptoms to *Mansonella* spp. infection, including eosinophilia, subcutaneous swellings, pericarditis and pleuritis, keratitis, urticaria, arthralgia, pain or ache in bursae, joint synovia, serous cavities and liver region, fatigue and skin rashes accompanied with itching (Asio et al., 2009; Downes and Jacobsen, 2010; Fischer et al., 1999; Hoerauf, 2009; Mediannikov and Ranque, 2018; Simonsen et al., 2011; Ta-Tang et al., 2018).

Immunology and immunomodulation

The absence of distinct clinical conditions and the lack of animal infection models has resulted in a shortfall about mansonellosis immunology and immunomodulation. However, recently a study showed that *M. perstans* strongly modulates the host immune responses by dampening systemic cytokine and chemokine secretion accompanied with increased Th2 and regulatory B and T cell subsets (Ritter et al., 2018). This systematic modulation might explain the increased susceptibility and worsened disease course of tuberculosis, HIV and malaria as well as the impaired vaccination efficacy of Calmette-Guerin vaccination against tuberculosis (Elliott et al., 2010; Hillier et al., 2008; Mhimbira et al., 2017; Muhangi et al., 2007; Stensgaard et al., 2016) and suggests mansonellosis as public health concern as well as the implementation of treatment strategies against this neglected filarial nematode.

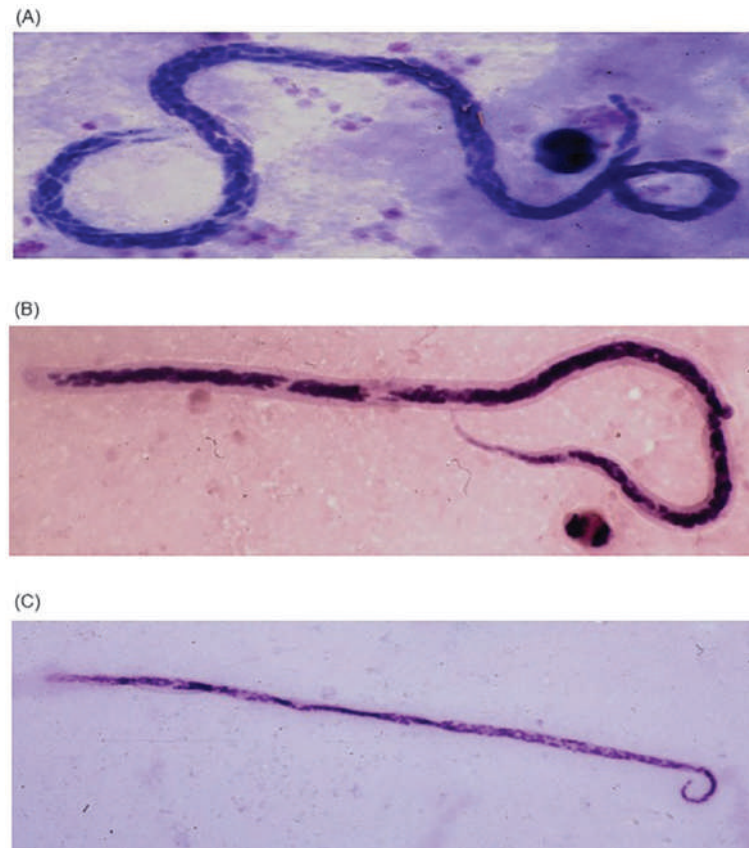


Fig. 8 Microfilariae of *Mansonella* spp. (A) Giemsa staining of *M. perstans* and hematoxylin staining of (B) *M. ozzardi* and (C) *M. streptocerca* microfilariae.

Diagnosis

Clinical indications for *Mansonella* infections are the development of pruritus, rash and dermatitis as well as eosinophilia and high IgG4 levels. As the adult worms reside in the serous cavities (*M. perstans* and *M. ozzardi*) or dermis (*M. streptocerca*), they are rarely detected. Diagnosis of *Mansonella* infections is usually based on the microscopic detection of MF in blood (*M. perstans* and *M. ozzardi*) or skin (*M. streptocerca* and *M. ozzardi*). Blood concentration techniques (Knott's or filtration technique) can be used for *M. perstans* and *M. ozzardi*, and collection of skin snips for *M. streptocerca* and *M. ozzardi* are analog to the procedure used for *O. volvulus*. In contrast to *W. bancrofti*, *Brugia* spec. and *L. loa*, MF of all three *Mansonella* species do not have a periodicity and are unsheathed. Another characteristic of *Mansonella* MF is their smaller size and in case of *M. streptocerca* MF the shepherd's crook' tail (Orihel, 1984). While serological tests are not yet available for *Mansonella* infections, PCR tests to detect MF DNA for all three species (Drame et al., 2016; Fischer et al., 1998; Morales-Hojas et al., 2001) and LAMP assays specific for *M. perstans* and *M. ozzardi* detection in peripheral blood and *Culicoides* midges do exist (Poole et al., 2019).

Intervention/Therapy

Treatment and treatment success differs among the three *Mansonella* species. Single combination treatment of ivermectin and albendazole has little to no effect on *M. perstans* microfilaremia (Asio et al., 2009; Wanji et al., 2016) and therefore *M. perstans* transmission is not interrupted by the current MDA treatments for onchocerciasis or LF. Extended treatments with DEC (200 mg twice daily) or albendazole (100 mg twice daily) alone for 21 and 28 days, respectively, reduces the MF load, and combination treatment further enhances MF clearance (Bregani et al., 2006). *Wolbachia* are present in *M. perstans* from West and Central Africa (potentially also from East Africa) and clinical trials demonstrated that daily 200 mg doxycycline treatment for 6 weeks clears microfilaremia (Batsa Debrah et al., 2019; Coulibaly et al., 2009).

For *M. streptocerca*, a 21-day treatment with DEC (2–6 g/kg body weight) was shown to eliminate both adult filariae as well as microfilariae. This treatment can cause severe pruritus and urticaria, but severe adverse events such as vision loss in onchocerciasis were not reported (Meyers et al., 1972, 1978). Similar adverse events occur following a single treatment with 150 mg/kg ivermectin, which leads to a long-lasting reduction of the *M. streptocerca* MF load (Fischer et al., 1997a).

In contrast, *M. ozzardi* MF are not susceptible for DEC treatment, but a single dose of ivermectin (150 µg/kg) mediates a maintained reduction of MF counts (Ferreira et al., 2021). As *M. ozzardi* harbors endosymbiotic *Wolbachia* bacteria (Casiraghi et al., 2001), it can be expected that doxycycline can be used as therapy.

A summary of the key facts for mansonellosis covered in this chapter is presented in Table 1.

Table 1 Summary of the key facts about the filariae *Onchocerca volvulus*, *Wuchereria bancrofti*, *Brugia spp.*, *Loa loa* and *Mansonella spp.*

<i>Filarial species</i>	<i>Endemic areas</i>	<i>Estimated infections</i>	<i>Major forms of pathology</i>	<i>Microfilariae characteristics</i>	<i>Residency of adult worms</i>	<i>Presence of Wolbachia</i>	<i>Treatment</i>	<i>Vector</i>
<i>Onchocerca volvulus</i>	Sub-Saharan Africa, Yemen, foci in Latin America	21 million	Blindness, dermatitis, Sowda	Skin, unsheathed	Subcutaneous nodules	Yes	Ivermectin, Doxycycline	<i>Simulium</i> blackflies
<i>Wuchereria bancrofti</i>	Sub-Saharan Africa, South & Southeast Asia, the Americas, Middle East, Pacific Islands	59 million	Lymphangitis, elephantiasis, hydrocele	Blood, nocturnal, sheathed	Lymphatic vessels and lymph nodes; Scrotum of men	Yes	DEC, Doxycycline, Albendazole, Ivermectin	<i>Aedes</i> , <i>Anopheles</i> , <i>Culex</i> , <i>Mansonia</i> mosquitoes
<i>Brugia malayi</i>	India, SE-Asia	6 million	Lymphangitis, elephantiasis	Blood, nocturnal, sheathed	Lymphatic vessels and lymph nodes	Yes	DEC, Doxycycline, Albendazole, Ivermectin	<i>Mansonia</i> , <i>Anopheles</i> mosquitoes
<i>Brugia timori</i>	Eastern Indonesia	?	Lymphangitis, elephantiasis	Blood, nocturnal, sheathed	Lymphatic vessels and lymph nodes	Yes	DEC, Doxycycline, Albendazole, Ivermectin	<i>Anopheles barbirostris</i> mosquitoes
<i>Loa loa</i>	Western and Central Africa	3–13 million	Eye worm, Calabar swelling	Blood, diurnal, sheathed	Subcutaneous tissue	No	DEC, Albendazole	<i>Chrysops</i> flies
<i>Mansonella perstans</i>	Sub-Saharan Africa, Central and South America	114 million	Mainly asymptomatic	Blood, unsheathed	Serous cavities	Yes	DEC, Albendazole, Doxycycline	<i>Culicoides</i> midges
<i>Mansonella streptocerca</i>	West and Central Africa	?	Mild dermatitis (mainly following treatment)	Skin, unsheathed	Dermal skin layer	Not yet verified	DEC, Ivermectin	<i>Culicoides</i> midges
<i>Mansonella ozzardi</i>	Central and South America and the Caribbean	?	Mainly asymptomatic	Blood and skin, unsheathed	Serous cavities	Yes	Ivermectin, Doxycycline?	<i>Culicoides</i> midges, <i>Simulium amazonicum</i>
<i>Mansonella Spec. "DEUX"</i>	Gabon	?	Mainly asymptomatic	Blood, unsheathed	Serous cavities	Yes	DEC?, Albendazole?, Doxycycline?	<i>Culicoides</i> ?

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Leishmaniasis

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Introduction

The term Leishmaniasis refers to a broad range of clinical diseases caused by different obligate intracellular protozoan parasites of the genus *Leishmania* transmitted by phlebotomine sand flies.

Traditionally, according to the geographical distribution the *Leishmania* infections are indicated as Old World leishmaniasis and New World leishmaniasis defined by different clinical presentation due to the tissue tropisms of the species involved as well as the immune status of the host (Akhoundi et al., 2016; Mejia, 2018). At least five different clinical forms can be recognized: cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (MCL), post-kala-azar leishmaniasis (PKDL) and visceral leishmaniasis (VL).

Historic perspective

Records of cutaneous lesions that can be assimilated to the actual description of CL of the Old World dates back some 4500 years ago with typical ulcers localized on the face reported in the tablets of the court of King Ashurbanipal (650 BCE) (Manson-Bahr, 1996; Akhoundi et al., 2016; Steverding, 2017). However, no other references are recorded until Avicenna (10th century AD) described the Balkh sore in people living in the actual northern Afghanistan. On the other hand, old ceramics (4th to 9th century AD) of human figures (called huacos) from Peru and Ecuador shows face disfigurements compatible with MCL of the New World (Lainson, 1996). In the middle of 19th century Dr. Russell detailed the clinical description of “Aleppo evil” (oriental sore) and Professor Julian Bravo suggested the similarity with the Peruvian “uta” (Manson-Bahr, 1996; Lainson, 1996).

As far as VL, it is more difficult to find ancient accurate descriptions considering the wide etiologies of fever in the tropics until the outbreak of fever reported during the year 1824 in Jessore (Bengal) that was unresponsive to quinine therapy and characterized by anemia and severe cachexia (Manson-Bahr, 1996). In 1862 a similar disease was observed in Burdwan where it was responsible of a huge number of deaths and became known as “Burdwan fever” or “kala azar” (Manson-Bahr, 1996). A disease affecting mainly young children and causing anemia and splenomegaly was described in Italy in 1880 by Antonio Cardarelli and it was identified for many years (until it was recognized as Mediterranean kala-azar) as “anemia splenica infantile.”

The first accurate description of the causative agent of CL as a protozoan is by a Russian military surgeon, Pjotr Fokich Borowsky (1863–1932) who reported in 1898 the presence of the parasites in patients affected by “Sart sore” (Hoare, 1938; Manson-Bahr, 1996). However, since its discovery was published in a Russian journal it was overlooked at his time and 5 years later James Homer Wright (1869–1928) a pathologist from Massachusetts General Hospital (Boston), described and illustrated the protozoan responsible of “Delhi boil” and named it *Helicosoma tropicum* (Wright, 1903; Wyers, 2016). In the same year (1903), William

Boog Leishman (1865–1926) a Scottish army pathologist published in the *British Medical Journal* his observation made 3 year previous of “small round or oval bodies, 2–3 μm of diameter” in the spleen of a soldier who died of “Dum Dum fever” (Leishman, 1903; Wyers, 2016). However, he initially misinterpreted his findings as “degenerated trypanosomes” and raised the possibility that trypanosomiasis occurred also in India. Two months later following the article by Leishman, Charles Donovan (1863–1951) an Irish physician working at Madras Medical College in India published his post-mortem observations of similar bodies found in the spleen of three consecutive patients (Donovan, 1903; Cook, 2007). Donovan sent his slides to both the future awarded Nobel Prize Ronald Ross and Alphonse Laveran who gave different interpretation: the latter being convinced that the organisms were members of Piroplasmida (Gibson, 1983). Ross on the contrary correctly interpreted the bodies as belonging to a new genus of Sporozoa that he named *Leishmania* in honor of the discoverer and the forms observed in the spleen were referred as Leishman-Donovan bodies (Ross, 1903, 1904). In 1904 Leonard Rogers (1868–1962) was able to culture the parasite and showed under certain circumstances the appearance of a flagellate phase (resembling a trypanosome but without the undulating membrane) which suggested the possibility of a vectorial transmission (Rogers, 1904; Scott, 1939). The role of the sand fly as the possible vector of kala-azar initially suggested by Mackie should be ascribed to the epidemiological and entomological work conducted at the Calcutta School of Medicine during the years 1924–26 by Robert Knowles, LE Napier and RO Smith (Mackie, 1914; Knowles et al., 1924; Napier, 1926) which finally culminated, 16 years later, in the proof of transmission by Swaminath et al. (1942).

The parasite

The genus *Leishmania* (Ross, 1903) belongs to the Kingdom Protozoa, Infrakingdom Excavata, Phylum Euglenozoa, Class Kinetoplastea, Order Trypanosomatida, Family Trypanosomatidae (Mauricio, 2018). According to the proposed classification based on molecular data *Leishmania* species are divided into two major phylogenetic lineages: *Euleishmania* and *Paraleishmania* (Cupolillo et al., 2000; Akhoundi et al., 2016, 2017). Within the *Euleishmania* lineage there are four subgenera: *Leishmania*, *Viannia*, *Sauroleishmania* and *Mundinia* whereas the *Paraleishmania* lineage includes two subgenera: *Porcisia* (proposed to include the neotropical porcupine parasites) and *Endotrypanum*. At least 54 *Leishmania* species (without synonyms) are actually known 21 of which are able to infect humans (Maroli et al., 2012; Akhoundi et al., 2017; Cotton, 2017) (Table 1). The widespread use of different molecular typing has been responsible of the identification of several described species as synonymous: *L. infantum* (syn. *L. chagasi*), *L. donovani* (syn. *L. archibaldi*), *L. tropica* (syn. *L. killicki*), *L. mexicana* (syn. *L. pifanoi*), *L. amazonensis* (syn. *L. garnhami*) (Schonian et al., 2001, 2010; Mauricio et al., 2000; Kuhls et al., 2011). *Leishmania* are dimorphic protozoa with two distinct morphology: the amastigote (round to oval of 1.5–3 μm $\times$ 3–5 μm) found in the mammalian cells and the promastigote (elongate and flagellated form, 1.5–3.5 μm $\times$ 15–200 μm) present within the digestive tract and proboscis of the sand fly. The amastigote multiply by binary division eventually rupturing the macrophage and invading other mononuclear phagocytes. It contains a specialized mitochondrial structure that is known as kinetoplast. The promastigote possess a flagellum positioned at the anterior end of the body responsible of the movement of the parasite in the environment (Bates, 2008).

The vectors and reservoirs

Leishmaniasis is a vector-borne disease transmitted to humans by the female sand fly that belongs to the genera *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Kamhawi, 2007). There are about 100 phlebotomine species identified as proven or suspected vectors of leishmaniasis (Maroli et al., 2012). The sand flies are 2–3 mm long arthropods which characteristically, in the rest position, maintain the wings at an angle to the abdomen (V configuration) (Killick-Kendric, 1999). They had limited fly capacity with hopping movement in short distance (no more than 1 m); once infected sand flies are capable of infection transmission seven to 10 days later and remain infective throughout their adult life (Dvorak et al., 2018). Phlebotomine vectors interacts with their reservoir hosts as well as the environment in a complex way that is responsible of the survival of *Leishmania* parasites (Bourdeau et al., 2020). A zoonotic transmission occurs either in domestic cycles (with the dog as the major reservoir) or in sylvatic cycles (Americas and Central Asia) where enzootic transmission between wild animals and humans (who acts as dead end hosts) is observed (Bern et al., 2008). Anthroponotic transmission (sand fly-human-sand fly) with humans as the main reservoir (Indian subcontinent and East Africa) is particularly involved for *L. donovani*. However, in East Africa it is supposed that also gerbils, rats, other rodents, small carnivores and also dogs can act as reservoir of the infection (Ashford, 1996; Sadlova et al., 2019).

In the Mediterranean basin *L. infantum* is responsible of VL and CL with nine proven or potential *Phlebotomus* vectors of the subgenus *Larrousius*: *P. perniciosus* (distributed in all countries of the Mediterranean basin), *P. ariasi* (found in Portugal, Spain, France, Italy and from Morocco to Tunisia), *P. perfiliewi*, *P. neglectus*, *P. tobbi* (identified in Italy, Greece and the Balkan peninsula), *P. longicuspis* (North Africa and Spain), *P. langeroni* (Egypt, North Africa, Spain). Dogs are highly susceptible to *L. infantum* and are considered the main reservoir but other wild canids (red foxes, jackals and wolves) can contribute to maintain the infection in nature (Lainson et al., 1969; Ashford, 1996; Dipineto et al., 2007). However, as illustrated by the “Fuenlabrada outbreak” observed between 2009 and 2013 in the Southwestern Madrid region, hares emerged as the predominant reservoir suggesting the existence of a complex rural/sylvatic transmission cycle (Molina et al., 2012; Jimenez et al., 2014). Moreover, in the Mediterranean Basin, apart the dogs the feline population remains the most affected by leishmaniosis and their possible role as primary and/or secondary reservoir hosts is increasingly considered (Asfaram et al., 2019; Cardoso et al., 2021).

Table 1 *Leishmania* species affecting humans, geographic distribution, vectors and clinical diseases.

Species	Species	Distribution	Clinical disease	Reservoir	Sand fly vector
<i>Euleishmania</i>	<i>Leishmania aethiopica</i>	OW (Ethiopia, Kenya)	CL; DCL	Mammals; humans	<i>P. (Lar.) longipes</i> ; <i>P. (Lar.) pedifer</i> ; <i>P. (Par.) sergenti</i>
	<i>L. amazonensis</i> (syn <i>L. garnhami</i>)	NW (Bolivia, Brazil, Venezuela)	CL; DCL; MCL	Mammals; humans	<i>Lu. (N.) flaviscutellata</i> , <i>Lu. (Lu.) longipalpis</i> , <i>Lu. (Lu.) nuneztovari anglesi</i> , <i>Lu. (N.) olmeca novica</i> , <i>Lu. (N.) olmeca reducta</i>
	<i>L. donovani</i> (syn <i>L. archibaldi</i>)	OW (Central Africa; China; India; Middle east; South Asia)	VL; PKDL	Mammals; humans	<i>P. (Pa.) alexandri</i> , <i>P. (Eu.) argentipes</i> , <i>P. (Syn.) celiae</i> , <i>P. (Syn.) martini</i> , <i>P. (La.) orientalis</i>
	<i>L. infantum</i> (syn <i>L. chagasi</i>)	OW (Mediterranean basin; North Africa; Middle East; North, central and South America) (Brazil, Bolivia, Venezuela, Mexico)	CL; VL	Mammals; humans	<i>Lu. (Lu.) almeriois</i> , <i>P. (La.) ariasi</i> , <i>P. (Ad.) balcanicus</i> , <i>P. (Ad.) chinensis</i> , <i>Lu. (Lu.) cruzi</i> , <i>Lu. (Pf.) evansi</i> , <i>P. (La.) kandelakii</i> , <i>P. (La.) langeroni</i> , <i>P. (Ad.) longiductus</i> , <i>Lu. (Lu.) longipalpis</i> , <i>P. (La.) major</i> , <i>P. (La.) perfiliewi s.l.</i> , <i>P. (La.) perniciosus</i> , <i>P. (Ad.) sichuanensis</i> , <i>P. (La.) smirnovi</i> , <i>P. (La.) tobbi</i> , <i>P. (Ad.) turanicus</i> , <i>P. (La.) wui</i> , <i>P. (Syn.) caucasicus</i> , <i>P. (P.) duboscqi</i> , <i>P. (P.) papatasi</i> , <i>P. (P.) salehi</i>
	<i>L. major</i>	OW (Central and North Africa; Middle east; Central Asia)	CL	Mammals; humans	<i>P. (Syn.) caucasicus</i> , <i>P. (P.) duboscqi</i> , <i>P. (P.) papatasi</i> , <i>P. (P.) salehi</i>
	<i>L. mexicana</i> (syn <i>L. pifanoi</i>)	NW (United States; Ecuador, Perú, Venezuela)	CL; DCL	Mammals; humans	<i>L. (Hel.) ayacuchensis</i> , <i>Lu. (N.) olmeca olmeca</i> , <i>Lu. (Lu.) ovallesi</i>
	<i>L. tropica</i> (syn. <i>L. killicki</i>)	OW (Central and North Africa; Middle east; Central Asia; India)	CL; VL	Mammals; humans	<i>P. (Ad.) arabicus</i> , <i>P. (La.) guggisbergi</i> , <i>P. (Syn.) rossi</i> , <i>P. (Pa.) saevus</i> , <i>P. (Par.) sergenti</i> ,
	<i>L. venezuelensis</i>	NW (Northern South America)	CL	Mammals; humans	
<i>Viannia</i>	<i>L. braziliensis</i>	NW (Western Amazon basin; South America) (Brazil, Bolivia, Peru, Guatemala, Venezuela)	CL; MCL	Mammals; humans	<i>Lu. (Ps.) carrerai</i> , <i>Lu. (Ps.) complexa</i> , <i>Lu. (Pi.) fishceri</i> , <i>Lu. (Lu.) gomezi</i> , <i>Lu. (Ps.) llnosmartinsi</i> , <i>Lu. (Lu.) migonei</i> , <i>Lu. (N.) neivai</i> , <i>Lu. (Lu.) nuneztovari anglesi</i> , <i>Lu. (V.) ovallesi</i> , <i>Lu. (Psy.) panamensis</i> , <i>Lu. (X.) shawi</i> , <i>Lu. (V.) spinicrassa</i> , <i>Lu. (N.) whitmani</i> , <i>Lu. (Ps.) wellcomei</i> , <i>Lu. (N.) ylephiletor</i> , <i>Lu. (Psy.) yucumensis</i>
	<i>L. guyanensis</i>	NW (Brazil, Bolivia, French Guiana, Suriname)	CL; MCL	Mammals; humans	<i>Lu. (N.) anduzei</i> , <i>Lu. (Hel.) ayacuchensis</i> , <i>Lu. (N.) shawi</i> , <i>Lu. (N.) umbratilis</i> , <i>Lu. (N.) whitmani</i>
	<i>L. lainsoni</i>	NW (Brazil, Bolivia, Perú)	CL	Mammals; humans	<i>Lu. (N.) nuneztovari anglesi</i> , <i>Lu. (T.) ubiquitalis</i>
	<i>L. lindbergi</i>	NW (Brazil)	CL	Mammals; humans	<i>L. (Lu.) atunesi</i>
	<i>L. naiffi</i>	NW (Brazil, French Guyana)	CL	Mammals; humans	<i>Lu. (Ps.) ayrozai</i> , <i>Lu. (Ps.) squamiventris</i>
	<i>L. panamensis</i>	NW (Central and South America, Brazil, Panama, Venezuela, Colombia)	CL; MCL	Mammals; humans	<i>Lu. (Lu.) gomezi</i> , <i>Lu. (H.) hartmanni</i> , <i>Lu. (Psy.) panamensis</i> , <i>Lu. (N.) trapidosi</i> , <i>Lu. (N.) yuilli</i>
	<i>L. peruviana</i>	NW (Perú, Bolivia)	CL; MCL	Mammals; humans	<i>Lu. (Hel.) ayacuchensis</i> , <i>Lu. (Hel.) peruensis</i> , <i>Lu. (V.) verrucarum</i>
	<i>L. shawi</i>	NW (Brazil)	CL	Mammals; humans	<i>Lu. (N.) whitmani</i>
<i>Mondinia</i>	<i>L. martiniquensis</i>	NW, OW (Martinique; Thailand)	CL; DCL; VL	Mammals; humans	Unknown
	<i>L. siamensis</i>	OW, NW (Central Europe, Thailand, United States)	VL; CL	Mammals; humans	<i>S. (Ne.) gemmea</i>
<i>Paraleishmania</i>	<i>L. colombiense</i>	NW (Colombia)	CL; VL	Mammals; humans	<i>Lu. (Hel.) hartmanni</i>

OW, Old World; NW, New World; CL, cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; VL, visceral leishmaniasis; MCL, mucocutaneous leishmaniasis; PKDL, post-kala-azar dermal leishmaniasis; Hel, *Helococyrtomyia*; La, *Larroussius*; Lu, *Lutzomyia*; N, *Nyssomyia*; Ne, *Neophlebotomus*; P, *Phlebotomus*; Pa, *Paraphlebotomus*; Ps, *Psathyromyia*; Psy, *Psychodopygus*; S, *Sergentomyia*; Sy, *Synphlebotomus*; V, *Verrucarum*.

Life cycle

Following a blood meal on an infected vertebrate, ingested *Leishmania* amastigotes will transform within the abdominal midgut of the sand fly into procyclic promastigotes (with short flagella) and, after the disintegration of the peritrophic matrix that surround and protect it, they develop into nectomonads that bind through the flagella to the microvilli of the midgut wall. Once attached these parasite forms (now leptomonads) replicate and migrate into the thoracic midgut that will be obstructed by the parasite mass embedded in the gel produced by promastigotes itself (Rogers et al., 2002). The next stage is the formation of highly motile metacyclic promastigotes (the infective stage) that concentrate in the anterior part of the midgut and sometimes in the pharynx, proboscis and salivary glands of the sand fly (Killick-Kendric, 1999; Rogers et al., 2002; Dvorak et al., 2018). It is estimated that the time needed for *Leishmania* parasites to complete the development in the sand fly is approximately 6–9 days. The mode of transmission of metacyclic promastigotes is believed to be by either deposition into the skin during feeding or blood regurgitation from the midgut (Bates, 2007, 2008).

Non-vectorial transmission

Besides the classic vector-borne transmission, leishmaniasis can be transmitted, although rarely, with blood through blood transfusion or shared syringes by intravenous drug users and following organ transplantation (Campino et al., 1994; Singh et al., 1996; Cruz et al., 2002a,b; Singh and Sivakumar, 2004; Dey and Singh, 2006; Antinori et al., 2007a,b; Mestra et al., 2011; Jimenez-Marco et al., 2016). A systematic review and meta-analysis of prevalence of *Leishmania* infection among blood donors showed, using molecular tests, a pooled prevalence of 2% with the highest prevalence in Spain (7%) and the lowest in Iran (0.05%) (Asfaram et al., 2017). Congenital transmission has been rarely reported with few well documented cases outside endemic areas (Eltoum et al., 1992; Meinecke et al., 1999; Boehme et al., 2006). Sexual transmission well demonstrated among dogs with shedding of *Leishmania* in the semen was documented in a single case in a human (Symmers, 1960; Silva et al., 2009; Naucke and Lorentz, 2012; Turchetti et al., 2014; Guedes et al., 2020).

Epidemiology

Leishmaniasis is endemic in 97 countries of which 88 are considered endemic for CL and 78 for VL in the six WHO regions (Africa, Americas, Eastern Mediterranean, Europe, South East Asia and Western Pacific) (Ruiz-Postigo et al., 2020). According to the last report of WHO in 2018, 253,435 new cases of CL (99% autochthonous) and 17,223 new VL cases (99% autochthonous) were reported (Ruiz-Postigo et al., 2020). Seven countries (Afghanistan, Algeria, Brazil, Iran, Iraq, Pakistan and the Syrian Arab Republic) report more than 70% of all CL cases; five countries (Brazil, Ethiopia, India, South Sudan and Sudan) reported more than 1000 VL cases a figure responsible of 83% of all cases worldwide.

The Global Burden of Disease Study 2013 using different sources (i.e., hospital records, national and global registries and literature) quantified for the first time the global burden of CL in 152 countries (Karimkhani et al., 2016). The highest incidence among males was observed in Palestine (616.2 per 100,000 people) followed by Afghanistan (566.4), Syria (357.1) and Nicaragua (354.8); among females, Afghanistan showed the highest incidence rates (623.9) followed by Syria (406.3), Palestine (222.1) and Nicaragua (180.8). However, CL is an emerging health problem also outside endemic regions as a consequence of war conflicts, high human mobility with migration and adventurous tourism as recognized important risk factors (Saroufim et al., 2014; Lederman et al., 2008; Sobirk et al., 2018; Kaufer et al., 2019; Vandeputte et al., 2020). Indeed, CL was diagnosed in 3.3% of travelers observed in the GeoSentinel Surveillance Network over a 10-year period (1997–2006) (Lederman et al., 2008) but imported CL is also increasingly diagnosed in endemic countries with the possible introduction of *Leishmania* species not circulating in such areas (Di Muccio et al., 2015; Ozbilgin et al., 2020). It should be highlighted that cases of imported MCL are increasingly diagnosed among young male tourists travelling in South America (especially Bolivia and Costa Rica) even after short term travel (Boggild et al., 2019).

Pathogenesis

The immune response and the pathogenesis of *Leishmania* infection is complex and not yet fully understood but the different extent of observed clinical manifestations is driven by both genetic factors of the host and the ability of the parasite to escape the innate and adaptive immune response against it. A simple model based mainly on experimental *L. major* in mice favors the description of a polarized immune response similar to what it is observed in leprosy where at one extreme of the spectrum predominate a Th2 response with polyparasitic infection (DCL and VL) and on the opposite extreme a Th1 response is responsible of a oligoparasitic infection (MCL, latent VL) (Heinzel et al., 1991; Guler et al., 1996; Launois et al., 1999; Wilson et al., 2005; Teufel et al., 2021).

However, as actually known the various contribution to susceptibility vs resistance is considered only a partial consequence of the different pleiotropic action of cytokines and their interactions with cells of the immune system (Osero et al., 2020; Carneiro et al., 2020; Teufel et al., 2021). The initial stage of the infection is characterized by recognition of parasites by macrophages, dendritic cells (DCs) and natural killer (NK) cells through pattern recognition receptors (PRRs) via Toll-like receptors (TLRs)-2,

TLR4 and TLR9. NK cells recruited by alarmins released by the damaged epithelial cells following the sand fly bite stimulates interferon-gamma (IFN- γ) production which in turn activates macrophages and up-regulates antigen processing and presentation pathways (Alexander and Brombacher, 2012; Lieke et al., 2011; Teufel et al., 2021); moreover, NK cells are able to directly kill promastigotes by lysis and their role is crucial in controlling *L. amazonensis* infection (Sanabria et al., 2008; Lieke et al., 2011). Neutrophils play, as well, a crucial role in the initial defense against *Leishmania* promastigotes by secreting neutrophil elastase (NE) which enhances the production of macrophages inflammatory cytokine and by the formation of neutrophil extracellular traps (NETs) (Kupani et al., 2021). However, neutrophils serve also as a “Trojan horse” by delayed apoptosis and intra macrophagic entry of parasites or as a “Trojan rabbit” when the parasites directly jump to macrophages without a carrier (apoptotic bodies) (Hurrell et al., 2016). Macrophages and monocytes are key cells in the defense against *Leishmania* parasites in the first hours of infection by internalization of promastigotes; within the phagolysosome of macrophages promastigotes transform in amastigotes which can replicate and destroy the macrophages causing the infection of the neighboring cells or be destroyed by killing through production of reactive oxygen species and nitric oxide through inducible nitric oxide synthase activation. (Glennie et al., 2017; Romano et al., 2017; Loria-Cervera and Andrade-Narvaez, 2020). The adaptive immune responses against *Leishmania* parasites are orchestrated by DCs through the four different cell subtypes: monocyte-derived DCs (MoDCs) and Langerhans cells (LCs) which act as antigen-presenting cells; dermal plasmacytoid DCs responsible of the synthesis of IFN- γ and dermal myeloid DCs contributing to antigen capture and presentation (Feijó et al., 2016). The interplay of different cytokines in the immunopathogenesis of *Leishmania* infection remains an unsolved paradox with the same cytokine that can be either selectively host-protective or disease-inducer according to parasite strains (Osero et al., 2020). For instance, interleukin (IL)-17A acts in synergy with IFN- γ to control *L. infantum* or *L. donovani* but on the contrary enhances the production of pro-inflammatory mediators (including IL-6 and TNF- α) in MCL caused by *L. braziliensis* and CL caused by *L. major* (Bacellar et al., 2009; Pitta et al., 2009; Terrazas et al., 2016; Gonzalez-Lombana et al., 2013). IL-12 is involved in the induction of IFN- γ that is responsible of the control of *L. major* and *L. donovani* infection but is not required for control of *L. mexicana* infection (Hurdal and Brombacher, 2017; Buxbaum et al., 2002; Engwerda et al., 1998). IL-10 produced by Th2 cells blocks directly the synthesis of Th1 cells and of IL-12 induced by macrophages impairing IFN- γ development with onset of VL and some form of CL (Anderson et al., 2007; Murphy et al., 2001; Murray et al., 2002).

Genetic studies employed with genome-wide association have been generally hampered by difficult to replicate results and by the underpower of studied population but recently robust genetic data emerged either for VL and CL resistance and susceptibility (Blackwell et al., 2009; Da Silva et al., 2020; Castellucci et al., 2020). A study by Fakiola et al. (2013) conducted in India and Brazil showed that the HLA-DRB1-HLA-DQA1 HLA class II region (rs9271858) was the single major genetic determinant of VL susceptibility (OR 1.41, 95% CI = 1.30–1.52). The studies on CL showed a more complex disease inheritance with at least six genetic risk loci identified for *L. braziliensis*: SERPINB10, CRLF3, KRT80, STX7, LAMP3 and IFNG-AS1 (Castellucci et al., 2020). Another recent study conducted in Manaus (Brazil) found that the rs2069705TT genotype was associated with a 70% risk of developing CL by *L. guyanensis* and the haplotype 1 (H1) associated with low level of IFN- γ presents a 60% risk of developing CL (Da Silva et al., 2020).

To further highlight the complex pathogenesis of leishmaniasis it should be also considered the role of *Leishmania* RNA virus (LRVs) which affects some species responsible of CL and MCL (Rossi and Fasel, 2018; Olivier and Zamboni, 2020). LRVs are members of Totiviridae, ds-RNA virus infecting a wide range of organisms with LRV1 subgenus found among New World *Leishmania* species (*L. braziliensis* and *L. guyanensis*) and LVR2 subgenus in Old World *Leishmania* (*L. major*, *L. aethiopica* and possibly *L. infantum*) (Zangger et al., 2013, 2014; Hajjarian et al., 2016). Coinfection with LRV1 and LRV2 has been associated with increased risk of developing MCL in the New World and treatment failure both in the Old and New World (Hajjarian et al., 2016; Rossi et al., 2017; Bourreau et al., 2016; Cantanhede et al., 2015).

Clinical presentation

The diagnosis of different forms of leishmaniasis (i.e., CL, MCL and VL) can be challenging because they can mimic several infectious and malignant conditions. To further complicate the picture there are atypical clinical presentations described both in immunocompetent and immunocompromised hosts (Horrillo et al., 2015; Santos-Oliveira et al., 2011; Vassallo et al., 2014; van Griensven et al., 2014a,b; Thakur et al., 2018; Neumayr et al., 2013).

Visceral leishmaniasis

The incubation period of VL ranges from as few as 10 days to as long as >1 year with a median time of 2–6 months. Asymptomatic *Leishmania* infection has been described in different geographical areas with a ratio ranging from 4 to 8.1 in the Indian subcontinent to 50:1 in Spain (Moral et al., 2002; Bern et al., 2007; Ostin et al., 2011; Singh et al., 2014). A recent study conducted in India showed that approximately 24% of asymptomatic cases (with rk-39, DAT and qPCR positivity) will develop clinical symptoms of active VL (Das et al., 2020). In such subjects high levels of Adenosine deaminase (ADA) and IL-10 were early predictors of progression from asymptomatic infection to symptomatic VL. Genetic factors (common variants in the HLA-DRB1-HLAQA1 HLA class II region), malnutrition and any conditions associated with immunosuppression contributed to the development of VL (Blackwell et al., 2009; Fakiola et al., 2013; Anstead et al., 2001).



Fig. 1 Visceral leishmaniasis with massive hepatosplenomegaly.

The presentation of VL includes the onset of fever with different clinical patterns (intermittent, remittent with double quotidian peaks or continuous), weight loss, asthenia and progressively enlarged liver and spleen (Herwaldt, 1999; Pintado et al., 2001; Cascio et al., 2002a,b; Levy et al., 2009; van Griensven and Diro, 2012). Massive splenomegaly (Fig. 1) is an important hallmark of VL and can predispose to complications such as subcapsular bleeding, infarction or even spontaneous rupture. Lymphadenopathy described in East Africa is rarely observed in the Mediterranean basin (Zijlstra and El-Hassan, 2001; Cascio et al., 2002a,b). Cutaneous papules at the site of inoculation can be observed rarely among patients with VL (Cascio et al., 2002a,b) and skin darkening (black fever or Kala-azar as indicated in Hindi language) is mainly described in South Asia.

Typical laboratory alterations in VL include anemia (almost always present) variably associated with leukopenia and thrombocytopenia as a consequence of bone marrow suppression and splenic sequestration. Impaired albumin synthesis by the liver and concomitant polyclonal hypergammaglobulinemia (5–10 g/dL) are responsible of the characteristic electrophoretic pattern known as “donkey ears” (Fig. 2). A mild-to moderate increase of liver enzymes as well as of bilirubin is frequently observed. Secondary hemophagocytic lymphohistiocytosis (HLH) associated with VL is observed especially among pediatric patients with the diagnosis complicated by the paucity of *Leishmania* amastigotes in the bone marrow or even their absence as described in 22% of cases in a study from France (Gagnaire et al., 2000; Levy et al., 2009; Minodier et al., 1998). In such cases the typical laboratory findings include hypertriglyceridemia, coagulopathy with hypofibrinogenemia and hyperferritinemia.

An atypical presentation, localized leishmanial lymphadenopathy, caused by *L. infantum*, has been recently observed among immunocompetent subjects diagnosed during the outbreak in Fuenlabrada (Spain) (Horrillo et al., 2015). This clinical entity has been rarely observed previously with only few case reports (Ignatius et al., 2011; Vera-Alvarez et al., 1999; Harms et al., 2001). A viscerotropic form caused by *L. tropica* (typically associated with CL) characterized by a milder visceral disease described among US veterans of Operation Desert Storm in Saudi Arabia, has been reported also in other regions (Iran, Kenya, Israel, India) (Magill et al., 1993; Thakur et al., 2018). Also *L. amazonensis* (in the New World) and *L. major* (in the Old World) have been reported to be responsible of VL in Bahia (Brazil) and Bushehr province (Iran), respectively (Barral et al., 1991; Karamian et al., 2007).

Undiagnosed or untreated VL progress to severe cachexia, ascites, bleeding and secondary bacterial infections responsible of death (Herwaldt, 1999; Murray et al., 2005). A scoring system based on risk factors for death developed in Brazil showed that patients with a score of 4 had a probability of death of 4.5% with a sensitivity of 89.4%, a specificity of 51.2% and an accuracy of 53.5% (Coura-Vital et al., 2014).

Visceral leishmaniasis in HIV infection

The association between VL (caused by *L. infantum*) and HIV infection was first reported in Southern Europe, mainly in Spain, France, Italy and Portugal, affecting in the majority of cases intravenous drug-users (IDU) or former IDU (Alvar et al., 1997, 2008; De la Loma et al., 1985; Vigevani et al., 1989; Monge-Maillo et al., 2014). Subsequently this coinfection has been increasingly reported in countries of Latin America and East Africa (especially Brazil, Ethiopia, Sudan, South-Sudan) where actually the burden

Serum protein electrophoresis	Results	Reference range
Albumin	25.8 %	55.8-66.1 %
α -1 globulins	4 %	2.9-4.9%
α -2 globulins	5.1 %	7.1-11.8 %
β -1 globulins	3.4 %	4.7-7.2 %
β -2 globulins	3.5 %	3.2-6.5 %
γ globulins	58.2 %	11.1-18.8 %

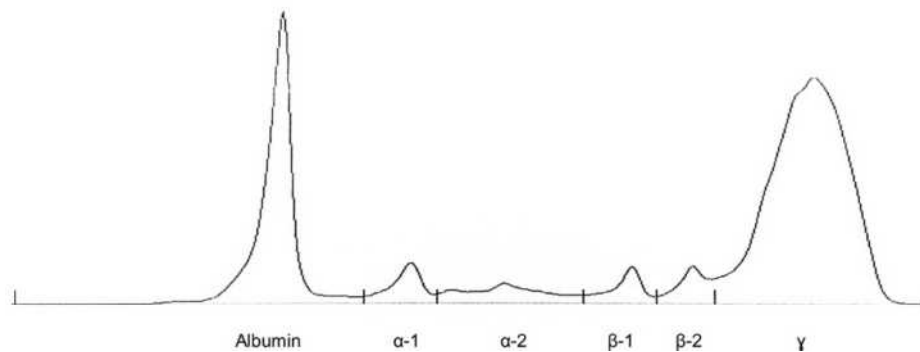


Fig. 2 Electrophoresis of a patient affected by visceral leishmaniasis showing the characteristic “donkey’s hear” (hypoalbuminemia and polyclonal hypergammaglobulinemia).

of the disease is highest (van Griensven et al., 2014a,b; Lindoso et al., 2014). Clinical manifestations of VL in HIV-coinfected patients mirror those observed among HIV-negative subjects (i.e., fever, weight loss, hepato-splenomegaly, pancytopenia) but several differences have also been described (Pintado et al., 2001; Romeu et al., 1991; Lopez-Velez et al., 1998; Jarvis and Lockwood, 2013). Involvement of esophagus and stomach with severe dysphagia or of the small intestine with chronic diarrhea and malabsorption or lung and pleural localization with pleural effusion or renal involvement with nephrotic/nephritic syndrome and acute renal failure are among some of the atypical manifestations described in HIV/*Leishmania* coinfection (Dattay et al., 1990; Cenoweth et al., 1993; Sollima et al., 1999; Rosenthal et al., 2000; Vassallo et al., 2014; Diro et al., 2015). Moreover, an heterogeneous number of cutaneous manifestations associated with VL occurring while the patients are still on treatment (para-kala-azar dermal leishmaniasis) or as the consequence of immune reconstitution (post-kala-azar dermal leishmaniasis-PKDL) have been described among HIV-infected patients (Ridolfo et al., 2000; Antinori et al., 2007a,b; Zijlstra, 2014). Interestingly, HIV-associated PKDL appears to be more common and more severe in this setting and caused not only by *L. donovani* but also by *L. infantum* (Ridolfo et al., 2000; Stark et al., 2006; Antinori et al., 2007a,b; Celesia et al., 2014). However, the most relevant differences regarding VL between HIV-infected and HIV-uninfected patients are about natural history and outcomes with higher mortality rates (13–18% vs 1–5%) and an high relapse rates (30–56%) observed in the former group even among patients receiving antiretroviral therapy (Antinori et al., 2007a,b; Ritmeijer et al., 2011; Sinha et al., 2011; De Araujo et al., 2012; Jarvis and Lockwood, 2013). An active chronic VL characterized by several episodes of clinical oligosymptomatic VL spared by asymptomatic periods but with the inability to clear the parasites has been described as a possible novel nosological entity in HIV-positive subjects (Bourgeois et al., 2010). A systematic review indicates that antiretroviral therapy seems to be insufficient to prevent VL-relapse with secondary prophylaxis and a CD4⁺ lymphocyte count greater than 100 cells/ μ L at the time of diagnosis as possible protective factors (Cota et al., 2011).

Visceral leishmaniasis in other immunocompromised patients

Beside HIV-positive subjects, other immunocompromised hosts such as solid organ transplant (SOT) recipients, allogenic hematopoietic stem cell recipients, rheumatologic patients treated with immunosuppressive or biologic drugs and patients with other cause of immunodeficiency are increasingly recognized as affected by VL (Antinori et al., 2008; De Leonadis et al., 2009; Boqdan, 2012; Zanger et al., 2012; Akuffo et al., 2018; Fletcher et al., 2015; van Griensven et al., 2014a,b; Komitopoulou et al., 2014; Harrador et al., 2015; Kalmi et al., 2020). SOT recipients show similar signs and symptoms of VL although splenomegaly is reported less frequently than in immunocompetent HIV-negative patients (Antinori et al., 2008). However, as observed among HIV-positive patients atypical presentation and low awareness by physicians caring for these patients may be responsible for delayed diagnosis or misdiagnosis due to overlapping symptoms and signs with other opportunistic infections (Antinori et al., 2008; Komitopoulou

et al., 2014; van Griensven et al., 2014a,b). As for HIV-positive patients, multiple relapsing course has been described, especially in kidney transplant recipients and patients treated with tumor necrosis alpha (TNF- α) antagonists (Simon et al., 2011; Bosch-Nicolau et al., 2019).

Cutaneous and mucocutaneous leishmaniasis

CL is the most frequent clinical syndrome caused by *Leishmania* infection and can present with different aspects (single or multiple lesions; localized, diffuse, disseminated lesions; dry or ulcerated lesions) according to the species involved and the host immune response (Figs. 3–5) (Goto and Lindoso, 2012; Handler et al., 2015a; Neumayr et al., 2013; Barkati et al., 2019). In the Old World, CL, known with popular name such as *Baghdad boil*, *Aleppo evil*, *bouton d'orient*, *Dehli boil*, is caused by *L. tropica*, *L. major*, *L. infantum* and *L. aethiopica*. CL is increasingly diagnosed also in non-endemic areas as the consequence of travels and population migration due to war and famine (Schwartz et al., 2006; Sharara and Kanj, 2014; Antinori et al., 2004; Wall et al., 2012; Saroufim et al., 2014; Showler and Boggild, 2015; Sobirk et al., 2018). CL can be anthroponotic (in the case of *L. tropica*) or zoonotic (when *L. major*, *L. aethiopica* and all the New World species are involved). CL caused by all *Leishmania* species presents initially with a macular lesion at the site of the vector bite that evolve over weeks into a papule or a nodule that ulcerates. The lesion does not cause pain although sometimes is responsible of itch and can heal spontaneously over several months (2–6 in the case of *L. major*; 6–15 if *L. tropica*, *L. braziliensis* or *L. panamensis* is involved) leaving a depressed hypopigmented scar (Reithinger et al., 2007; Dowlati, 1996). In the case of *L. tropica* more than 60% of affected subjects have one lesion and 95% have less than three lesions (Sang et al., 1994). Ulcerative lesions shows an oval shape with well-defined indurated borders and a central area with granulation tissue. Hyperkeratosis upon the ulcer is typical of Old World CL (Fig. 6). Sometimes a lymphangitic dissemination is observed with a lymphangitic cord detected at palpation and a “sporotrichoid” appearance (Fig. 7). Slow growing ulcers that take longer period to heal (2–5 years) are observed with *L. aethiopica*. DCL is the anergic form of CL, initially described in Africa, that can be caused by *L. aethiopica* in the Old World and by *L. amazonensis* and *L. mexicana* in the New World. After the appearance of a localized papule or nodule the disease spreads to the entire body with a predilection of the face, ears, elbows and knees resembling lepromatous leprosy (Convit et al., 1957). Systemic symptoms such as fever, weight loss, lymphedema can be present and the disease is characterized by long evolution and resistance to treatment.



Fig. 3 New World cutaneous leishmaniasis caused by *Leishmania (Viannia) panamensis* in a traveller.



Fig. 4 Old World cutaneous leishmaniasis caused by *Leishmania major* MON25.



Fig. 5 New World cutaneous leishmaniasis with several lesions localized on the face caused by *Leishmania (Viannia) panamensis*.

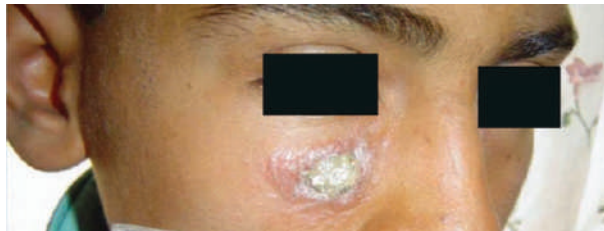


Fig. 6 Old World cutaneous leishmaniasis with hyperkeratotic appearance.



Fig. 7 Old World cutaneous leishmaniasis localized on the leg with sporotrichoid appearance.

Leishmania recidivans (LR) is a rare variant associated with *L. tropica* characterized by small papules (usually on the face) at the edge of a healed or healing lesion; it is a “relapsing tuberculoid form” with an intense cell mediated response and scanty parasites. LR has been observed also in the New World (Brazil and Ecuador) (Bittencourt et al., 1993; Calvopina et al., 2006).

Several forms of LCL in the New World have been described: *Chiclero’s ulcer* caused by *L. mexicana* presents with a slowly evolving ulcer usually localized on the face or pinna of the ear; *ulcera de Bejuco* is the form induced by *L. panamensis* with shallow ulcers metastatizing to lymphatic channels and with possible mucosal destruction of nasopharynx in 2–5% of patients (Koff and Rosen, 1994); *Uta*, caused by *L. peruviana*, is observed in Perú usually among children with few lesions that usually tend to heal spontaneously; CL associated with *L. braziliensis* is characterized by lymphatic spread with nodular lymphangitis (sporotrichoid distribution) and lymph node involvement (Schwartz et al., 2006). DCL is generally observed in Latin America where it was



Fig. 8 Old World mucocutaneous leishmaniasis observed in an immunocompromised host treated with Infliximab.

originally described in 1920 with *L. braziliensis* as the main species causing the disease (Torres, 1920; Turetz et al., 2002; Machado et al., 2019); however, also *L. panamensis* and *L. guyanensis* can be responsible of this tegumentary syndrome in the New World (Calvopina et al., 2005). DCL has been rarely observed also in Europe, in a patient treated with TNF- α antagonists, and in the Middle East (Neumayr et al., 2013; Alborzi et al., 2008; Alkover et al., 2018). A single case caused by *L. siamensis* has been described in a Thai patient treated with systemic steroids (Noppakun et al., 2014). DCL is defined by multiple pleomorphic lesions (more than 10, frequently hundreds to thousands) in two or more non-contiguous areas of the body; The lesions are localized on the face, the trunk and the limbs with acneiform, papular, nodular or ulcerated appearance and an high rate (>50%) of mucosal (especially of the nose) involvement (Machado et al., 2019).

MCL is traditionally caused by *L. braziliensis* in Latin America although other species (*L. amazonensis*, *L. guyanensis*, *L. panamensis*) have also been implicated (Goto and Lindoso, 2010). Although MCL can present simultaneously with cutaneous lesions it generally appears several months or years following CL. Initially symptoms are nonspecific with itching to the nose, mucosal swelling with stuffy nose, crust formation with bleeding; following the initial involvement of the nasal mucosa the disease spreads to oral mucosa, soft palate, pharynx and larynx. MCL has been observed, although rarely, also in the Old World, especially among patients treated with immunosuppressive drugs (Cobo et al., 2016; Patel et al., 2017). (Fig. 8).

Post-kala-azar dermal leishmaniasis (PKDL)

Post-kala-azar dermal leishmaniasis is a possible complication of VL caused by *L. donovani* observed mainly in Sudan and India (Zijlstra, 2014); however, in recent years cases of PKDL have been reported also in the Mediterranean basin and Latin America following infection with *L. infantum/chagasi* in HIV-positive patients (Bittencourt et al., 2002; Antinori et al., 2007a,b; Ramos et al., 2008). A difference regarding the prevalence rate, clinical presentation and time onset from the episode of VL is observed in Asia and Africa (Zijlstra, 2014; Burza et al., 2018). In Africa, PKDL develop in 50% of individuals after a short interval from the episode of VL, affects mainly children and papulo-nodular lesions predominate; in Asia, the rash is more frequently macular, symmetric and hypopigmented, the time lag is longer (2–3 years) and the prevalence rate is 5–10% (Zijlstra et al., 2003). In one study conducted in Ethiopia, PKDL was observed more frequently among HIV-positive patients in comparison with HIV-negative (27% vs 13%) and the clinical course was more severe (Rijtmeyer et al., 2006). Moreover, PKDL lesions in HIV-positive subjects contain an high parasite burden (Zijlstra, 2014).

Diagnosis of leishmaniasis

The diagnosis of the different clinical syndromes associated with *Leishmania* infection traditionally relies on the direct microscopy observation of the parasite on suitable samples (i.e., skin, bone marrow, spleen, liver, lymph node biopsies or aspirates) and/or culture (Kumar et al., 2007; Srivastava et al., 2011; Gramiccia and Di Muccio, 2018; Burza et al., 2018; Reimao et al., 2020). However, besides histopathology and *in vitro* culture the diagnostic armamentarium for leishmaniasis is actually based on several serologic and antigen detection assays and molecular tools (Cunningham et al., 2012; De Ruiter et al., 2014; Srivastava et al., 2011; Hossain et al., 2017). Molecular tests, especially polymerase chain reaction (PCR) have been increasingly used not only for diagnosis and species identification but also for post-treatment assessment of cure (Kip et al., 2015; Hossain et al., 2017). More recently, mass spectrometry has been proposed as a possible application for rapid and reliable identification of *Leishmania* species (Cassagne et al., 2014; Mouri et al., 2014; Lachaud et al., 2017).

Diagnosis of visceral leishmaniasis

The differential diagnosis of VL must take into account several infectious and non-infectious diseases characterized by febrile pancytopenia and splenomegaly. Bone marrow, spleen or less frequently lymph nodes are the main sources of samples from which microscopy examination and culture are performed (Sundar and Rai, 2002a; Srivastava et al., 2011). Spleen aspirate is characterized by the highest sensitivity (93–99%) but it is routinely done only in the Indian subcontinent and East Africa (Zijlstra et al., 1992; Sundar and Rai, 2002a); in Europe, Brazil and the United States the bone marrow is the preferred sample but its sensitivity is around 60–85% (Zijlstra et al., 1992; Siddig et al., 1988; Ho et al., 1948). Several bone marrow findings associated with VL have been described with hypercellularity being the most frequently observed (Kumar et al., 2007; Dhingra et al., 2010; Al-Jurayyan et al., 1995; Wickramasinghe et al., 1987); in such cases, the abundance of Leishman Donovan (LD) bodies is a distinct features that allow to discriminate from hematologic and storage diseases (Kumar et al., 2007). Noncaseating granuloma is another frequently observed picture among patients with VL characterized by paucity of LD bodies and need to employ PCR to obtain the correct diagnosis (Kumar et al., 2007). Ultrastructural studies showed the presence of a high proportion of immature erythroblasts with giant lysosomes suggesting a role of dyserythropoiesis in the pathogenesis of anemia (Wickramasinghe et al., 1987).

In highly endemic regions rapid diagnostic tests (RDTs) such as the direct agglutination test (DAT) and the lateral flow immunochromatographic test (ICT) are extensively used for the diagnosis of VL (Singla et al., 1993; De Assis et al., 2011; Chappuis et al., 2006; Cunningham et al., 2012; Bhattacharyya et al., 2013; Boelaert et al., 2014; van Griensven and Diro, 2019). The DAT showed a good sensitivity and specificity for the diagnosis of VL in both the Indian subcontinent and Eastern Africa with the major disadvantages being the time needed for the response (hours), the persistence of positive results even among cured subjects and a high rate of positivity among patients with asymptomatic infection (Chappuis et al., 2006). Moreover, since the test is based on whole parasite antigen in endemic areas cross-reactivity with other parasites can be a problem (Kohanteb and Ardheali, 2005). The k39 antigen that contains 39 amino acids encoded by the highly conserved kinesin region of *L. infantum*/*L. chagasi* is the basis of the ICT test widely used especially in the Indian subcontinent as a part of the kala-azar elimination program (Sundar et al., 1998; Boelaert et al., 2014; Maia et al., 2012). The rK39 tests shows a high sensitivity and specificity in study conducted in the Indian subcontinent whereas its performance is lower in East Africa and among HIV-coinfected patients (Boelaert et al., 2014; Bangert et al., 2018; Kassa et al., 2020). Although this test is considered a reliable point-of-care method, as for DAT, it is unable to discriminate between current, subclinical and past leishmania infections (Sundar and Singh, 2018). Another ICT adopting the rK28 antigen demonstrated similar high performance in the Indian Subcontinent but improved sensitivity in East Africa (Pattabhi et al., 2010; Vaish et al., 2012; Bezuneh et al., 2014; Mukhtar et al., 2018). Molecular diagnosis of VL by means of PCR can be considered a real breakthrough for the detection and typing of different *Leishmania* species (De Ruiter et al., 2014). The PCR-based approach using different primers targeting the nuclear and mitochondrial genome of the parasite (i.e., small subunit ribosomal RNA, kinetoplast DNA, multicopy mini-exon RNA) has been increasingly adopted because of several advantages in comparison with standard microbiologic methods: high sensitivity (>95%) even when peripheral blood samples are employed; rapid response; direct applicability on clinical samples not requiring the parasite cultivation; usefulness for therapeutic monitoring with low invasiveness especially in pediatric population; similar performance with *L. donovani* and *L. infantum* (Nuzum et al., 1995; Osman et al., 1997; Singh et al., 1999; Pizzuto et al., 2001; Cruz et al., 2002a,b; Cascio et al., 2002a,b; Fisa et al., 2002; Wortmann et al., 2005; Antinori et al., 2007a,b; Boelaert et al., 2014). A quantitative real-time PCR has been suggested to be useful as an early predictor of VL relapse especially among HIV-positive subjects although there is no agreement about the cycle threshold value to be used (Bossolasco et al., 2003; Mary et al., 2006; Antinori et al., 2009; Hossain et al., 2017). A further achievement in the diagnosis of VL has been the development of a loop-mediated isothermal amplification (LAMP) assay to be used especially in the field setting (Abbasi et al., 2016; Mukhtar et al., 2018; Silva Nunes Bezerra et al., 2020). For a more extensive dissertation about the molecular diagnosis of VL the readers are referred to several updated reviews (Boelaert et al., 2014; Sundar and Singh, 2018; Gramiccia and Di Muccio, 2018; van Griensven and Diro, 2019; Reimao et al., 2020). Serological diagnosis of VL has been extensively used in the past but its indirect nature and variable sensitivity and specificity together with the availability of more specific methods is responsible of their actual limited use (Sundar and Rai, 2002a,b; Aronson et al., 2016). Moreover, in the context of low endemicity or absence of the disease is not infrequent that such tests are requested when a diagnosis of VL has been obtained with a direct method. The available serological tests are based on immunofluorescence (indirect fluorescent antibody test-IFAT), enzyme linked immunosorbent assay (ELISA) and western blotting and their performance is variable and dependent to antigens used and the epidemiological setting (Gari-Toussaint et al., 1994; Kumar et al., 2001).

Diagnosis of cutaneous and mucocutaneous leishmaniasis

CL presents with lesions that can mimic several infectious (ecthyma, sporotrichosis, tuberculosis, leprosy, blastomycosis, paracoccidioidomycosis) and non-infectious (basal cell carcinoma, squamous cell carcinoma, lymphoma, sarcoidosis, pyoderma gangrenosum) disease (Handler et al., 2015b; Goto and Lindoso, 2012; Singh and Ramesh, 2013). Diagnosis should be suspected in any afebrile subjects with a history of residence or travel in a leishmaniasis-endemic area presenting with a non-healing cutaneous lesion (Aronson et al., 2016; Goto and Lindoso, 2012). Histologic examination of a sample obtained by skin scraping or by biopsy (incisional/excisional) of ulcer or nodule border is the first step to accomplish the diagnosis of CL although sensitivity is around 50–60% (Singh et al., 2014; Viana et al., 2013). Since identification of the species is of paramount importance, especially in the New World, for species-directed therapy the use of PCR is highly recommended (Aronson et al., 2016; Marfurt et al., 2003a,b; Jara et al., 2013).

MCL needs to be differentiated from syphilis, yaws, rhinoscleroma and oral squamous cell carcinoma; mucosal samples for histopathologic and molecular diagnosis are generally obtained by an otolaryngologist (Aronson et al., 2016).

Treatment

Traditionally pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) have been used as the drugs of choice for the treatment of VL all over the world. However, in some endemic areas such as the state of Bihar (India) the resistance rate to pentavalent antimonials (PA) reached a 60% value which required the adoption of different therapeutic regimens (Sundar and Rai, 2002b; Hefnawy et al., 2017). Several clinical trials showed the usefulness of different drugs such as amphotericin B deoxycholate (AMB-d), pentamidine and paromomycin for the treatment of VL (Thakur et al., 1993, 2000; Sundar et al., 2007). Major breakthrough for the treatment of VL were the approval in 1997 by the Food and Drug Administration of liposomal amphotericin B (L-AMB) and in 2002 the clinical trial that conducted to the approval in India of miltefosine, the first and only oral drug with such an indication (Davidson et al., 1994; Meyerhoff, 1999; Sundar and Rai, 2002b). Since then, several randomized clinical trials have been conducted especially in India and East Africa which are elegantly summarized in two reviews (Monge-Maillo and Lopez-Velez, 2013; Berman, 2015). Up to now options for the treatment of VL include parenteral monotherapy (with L-AMB, PA, pentamidine or AMB-d) or combination therapy (PA plus paromomycin), use of high dosage of L-AMB for short course (10 mg/kg over 2 days), oral therapy with miltefosine.

PA have been used for VL for over 80 years and except in the Indian subcontinent they maintain a role as first line therapy in eastern Africa, administered either alone (20 mg Sb^{V+}/mL/kg for 30 days) or in association with paromomycin (15 mg/kg intramuscularly) for 17 days (Table 2). The two available formulations have different pentavalent antimony concentration: 100 mg Sb^{V+}/mL sodium stibogluconate and 85 mg^{V+}/mL meglumine antimoniate. Both drugs show good efficacy but the main concern is about cardiotoxicity (T-wave flattening or inversion, QT interval prolongation) and pancreatic damage; therefore an electrocardiogram and pancreatic enzyme monitoring are mandatory during treatment. AMB-d is an option used for the treatment of VL and MCL characterized by good efficacy but undermined by the need of long hospitalization and high rate of nephrotoxicity. L-AMB with a total dose of 18–21 mg/kg is currently the treatment of choice for VL in high-income countries but is adopted also in the Indian subcontinent with different schedules of administration (Table 2). Paromomycin at a dosage of 15 mg (11 mg base)/kg/day emerged as a good options for the treatment of VL caused by *L. donovani* in India; however, its efficacy as monotherapy is lower in East Africa where prolonged regimen (from 21 to 28 days) or higher dosage (20 mg (15 mg base)/kg/day) proved more effective than the regimen adopted in India. Pentamidine isethionate (4 mg/kg three times weekly for 12–21 days) was used in India when PA became less effective due to emerged resistance but its actual role is limited to secondary prophylaxis for VL in HIV/coinfected patients (Thakur, 1984; Mishra et al., 1992; Diro et al., 2019). Miltefosine is registered in India, Germany and other countries of South America for treatment of VL and CL (Monge-Maillo and Lopez-Velez, 2015) at a dosage of 2.5 mg/kg/day for children and 150 mg/day for adults for 4 weeks. Main toxicity observed for miltefosine is on the gastrointestinal tract (nausea, vomiting, diarrhea) and the possible teratogenicity that precludes its use in pregnant women.

For more exhaustive information about treatment of VL the readers should refer to guidelines of IDSA/ASTMH (Aronson et al., 2016), WHO (Gradoni et al., 2017), Organizacion Panamericana de la Salud (2013), India (National Vector Borne Disease Control Programme, 2015) and to several comprehensive reviews (Monge-Maillo and Lopez-Velez, 2013, 2015; Copeland and Aronson, 2015; van Griensven and Diro, 2012, 2019; Burza et al., 2018).

Treatment of LCL is complicated by several factors including a high number of involved species with different susceptibility to available drugs, lack of well conducted clinical trials, different host populations (immunocompromised, travelers, children) and different possible options (local treatment, systemic treatment, intralesional treatment, physical treatment). As a general rule systemic treatment is recommended for all patients with CL acquired in the New World to prevent the development of MCL (Blum et al., 2012). Moreover, other factors that need to be considered when choosing a treatment regimen for CL are the infecting *Leishmania* species, the number and size of lesions and their location (face, hands, joints) and the host immunological status (i.e., immunocompromised patients) (WHO, 2014; Hodiamont et al., 2014; Showler and Boggild, 2015; Aronson et al., 2016; Blum et al., 2018). In Table 3 (near here) are summarized the treatment options for CL according to the different *Leishmania* species involved. Disseminated cutaneous leishmaniasis (DCL) and PKDL are tegumentary form more difficult to be treated. DCL is a hard to treat syndrome generally relapsing once therapy is discontinued with options represented by the combination of paromomycin and antimonials or pentamidine isethionate (van Griensven et al., 2016); prolonged treatment with miltefosine has been reported to be successful in several case reports (Zerpa et al., 2007; Ordaz-Farias et al., 2013). For DCL caused by *L. braziliensis* a high rate of therapeutic failure up to 76% has been observed with the use of pentavalent antimony (Machado et al., 2011). More recently, the use of L-AMB with a total cumulative dose above 30 mg/kg showed a cure rate of 75% (Machado et al., 2015). PKDL is considered an obstacle of VL elimination in the Indian subcontinent and therefore it is indicated as treatment although in the majority of cases skin lesions are self-healing (Le Rutte et al., 2019; Gedda et al., 2020). Sodium stibogluconate (20 mg/kg/day for 20 days per month for 6 months) was considered the treatment of choice but it has been replaced by other drugs because of several limitations (long hospitalization needed, high toxicity) (Singh and Sivakumar, 2004). Miltefosine (100 mg/day for 12–16 weeks) is associated with high cure rates but its use is sometimes limited by gastrointestinal toxicity (Ramesh et al., 2011; Pijpers et al., 2019). L-AMB using different treatment schedules (2.5 mg/kg for 20 days in East Africa; 5 mg/kg dose twice per week for 3 weeks in the Indian subcontinent) is suggested by some expert as a first-line treatment (Gedda et al., 2020; Blum et al., 2018). In East Africa, another option is the combination of sodium stibogluconate (20 mg Sb^{V+}/kg/day) plus paromomycin (15 mg (11 mg base)/kg/day) for 17 days (Burza et al., 2018).

Table 2 Drugs and treatment schedules for visceral leishmaniasis.

Leishmania species/Geographic area	Regimen and dose	Major side effects	Comments and grade
<i>L. donovani</i> /East Africa	Sodium stibogluconate 20 mg/kg Sb ^{V+} /day i.m. or i.v. for 30 days	Cardiotoxicity, pancreatitis, hepatotoxicity	Grade A ^a Cure rate 94% (Burza et al., 2018)
	Sodium stibogluconate 20 mg/kg Sb ^{V+} /day i.m. or i.v. plus paromomycin 15 mg (11 mg base)/kg/day i.m. for 17 days	Cardiotoxicity, pancreatitis, hepatotoxicity (SSG); ototoxicity, nephrotoxicity (PM)	Grade A ^a Cure rate 91% (Burza et al., 2018)
	Liposomal amphotericin B (L-AMB) 3–5 mg/kg/day i.v. for 6–10 days (total dose 30 mg/kg)	Fever, back pain, nephrotoxicity	Grade B ^a STRONG recommendation, HIGH quality of evidence (Aronson et al., 2016) (Doses of up to 40 mg/kg may be necessary in person with VL acquired in East Africa) Cure rate >91% (Burza et al., 2018) Not recommended ^a
Coinfection HIV/ <i>L. donovani</i> /East Africa	Pentamidine isethionate 4 mg/kg/day i.v. on alternate days for 15–20 doses	Hypotension, pancreatitis, diabetes mellitus	
	L-AMB 3–5 mg/kg/day i.v. (days 1–5, 10, 17, 24, 31, 38) up to a total dose of 40 mg/kg	Fever, back pain, hypokalemia, nephrotoxicity	Cure rate 50% (Burza et al., 2018)
	L-AMB 5 mg/kg/day i.v. (days 1, 3, 5, 7, 9, 11) plus miltefosine 2.5 mg/kg/day (children) or 100 mg/day os for 28 days	Fever, back pain, hypokalemia, nephrotoxicity (L-AMB)	Not recommended ^a Cure rate 67% (Burza et al., 2018)
<i>L. donovani</i> /Indian subcontinent	L-AMB 10 mg/kg i.v. for 1 or 2 doses	Fever, back pain, hypokalemia, nephrotoxicity	Grade A ^a STRONG recommendation, HIGH quality of evidence (Aronson et al., 2016) Cure rate 96% (Burza et al., 2018)
	L-AMB 3–5 mg/kg/day i.v. for 3–5 days	Fever, back pain, hypokalemia, nephrotoxicity	
	L-AMB 5 mg/kg as a single dose plus miltefosine 150 mg/day for 7 days		Grade A ^a Cure rate 98% (Burza et al., 2018)
	AMB-d 0.75–1 mg/kg i.v. daily or on alternate days for 15–20 doses	Fever, hypokalemia, nephrotoxicity (frequent)	Grade A ^a Cure rate 93% (Burza et al., 2018)
	Miltefosine 150 mg/day os for 28 days	Gastrointestinal side effect (nausea, diarrhea, vomiting); hepatotoxicity (MF)	Grade A ^a STRONG recommendation, MODERATE quality of evidence (Aronson et al., 2016) Cure rate 90% (Burza et al., 2018)
Coinfection HIV/ <i>L. donovani</i> /Indian subcontinent	L-AMB 3–5 mg/kg i.v. for 10 doses (days 1–5, 10, 17, 24, 31, 38)	Fever, back pain, hypokalemia, nephrotoxicity (rare)	STRONG recommendation, LOW quality of evidence (Aronson et al., 2016)
	L-AMB 5 mg/kg/day i.v. for 6 days plus miltefosine 100 mg/day for 14 days os	Fever, back pain, hypokalemia, nephrotoxicity (rare) (L-AMB); Gastrointestinal side effects (nausea, diarrhea, vomiting); hepatotoxicity (MF)	WEAK recommendation, VERY LOW quality of evidence (Aronson et al., 2016)
<i>L. infantum</i> /chagasi/Mediterranean basin; Latin America	L-AMB 3–5 mg/kg/day i.v. (days 1–5, 10)	Fever, back pain, hypokalemia, nephrotoxicity (rare) (L-AMB)	Grade B ^a ; Grade C ^a (Latin America) STRONG recommendation, HIGH quality of evidence (Gradoni et al., 2017) Cure rate 90–98% (Burza et al., 2018)
	Meglumine antimoniate 20 mg Sb ^{V+} /kg/day i.m. or i.v. for 28 days	Cardiotoxicity, hepatotoxicity, pancreatitis	Grade B ^a STRONG recommendation, MODERATE quality of evidence (Gradoni et al., 2017) Cure rate 97% (Burza et al., 2018)
Coinfection HIV/ <i>L. infantum</i> /chagasi/Mediterranean basin; Latin America	L-AMB 3–5 mg/kg i.v. for 10 doses (days 1–5, 10, 17, 24, 31, 38)	Fever, back pain, hypokalemia, nephrotoxicity (rare) (L-AMB)	STRONG recommendation, VERY LOW quality of evidence (Gradoni et al., 2017) Cure rate 80% (Burza et al., 2018)
	Miltefosine 150 mg/day for 28 days os	Gastrointestinal side effects (nausea, diarrhea, vomiting); hepatotoxicity	WEAK recommendation, LOW quality of evidence (Gradoni et al., 2017)

i.v., intravenous; i.m., intramuscularly; L-AMB, liposomal amphotericin B; MF, miltefosine.

^aOxford evidence grading system: (A): randomized controlled trials in representative patient groups; (B): randomized controlled trials in less homogenous patient group; (C): cohort trials or case control studies in less homogenous patient group, as well as case series of representative patient groups; (D): case series of less homogenous patient groups and expert opinion.

Table 3 Treatment regimens suggested for cutaneous (CL) and mucocutaneous leishmaniasis (MCL).

Old World CL	Local treatment	Grade	Systemic treatment	Grade
<i>Leishmania major</i>	Cryotherapy plus intralesional antimonials	A (Burza et al., 2018); A (Blum et al., 2014)	Fluconazole 200 mg bid for 6 weeks	A (Burza et al., 2018); C (Blum et al., 2014) Fact, no grade (Aronson et al., 2016)
	15% paromomycin plus 12% methylbenzethonium chloride bid for 10–20 days	A (Burza et al., 2018); A (Blum et al., 2014)	Miltefosine 50 mg every 8 h for 4 weeks	B (Blum et al., 2014)
	Heat therapy (50 °C for 30 s): 1–2 sessions	A (Blum et al., 2014)	Pentavalent antimonials (Sb ^{v+} 20 mg/kg/day plus pentoxifylline 400 mg three times daily for 20 days)	A (Burza et al., 2018); A (Blum et al., 2014) STRONG recommendation, MODERATE quality of evidence (Aronson et al., 2016) (Systemic treatment recommended for persons with complex CL)
<i>Leishmania tropica</i> and <i>L. infantum</i>			L-AMB 3 mg/kg/day (days 1–5 and 10)	D (Blum et al., 2014)
	Pentavalent antimonials (Sbv + 20 mg/kg/day)	D (Burza et al., 2018); A (Blum et al., 2014)	Pentavalent antimonials (Sb ^{v+} 20 mg/kg/day for 10–20 days)	D (Burza et al., 2018); D (Blum et al., 2014)
	Heat therapy (50 °C for 30 s): 1–2 sessions	A (Burza et al., 2018); A (Blum et al., 2014)	L-AMB 3 mg/kg/day (days 1–5 and 10)	C (Blum et al., 2014) STRONG recommendation, MODERATE quality of evidence (Aronson et al., 2016) (Systemic treatment recommended for persons with complex CL)
	15% paromomycin plus 12% methylbenzethonium chloride bid for 10–20 days	D (Blum et al., 2014)	Miltefosine 50 mg every 8 h for 4 weeks	D (Blum et al., 2014)
			Pentavalent antimonials (Sb ^{v+} 20 mg/kg/day for 10–20 days)	C (Blum et al., 2014)
New World CL	Local treatment	Grade	Systemic treatment	Grade
<i>Leishmania braziliensis</i>	Heat therapy (50 °C for 30 s): 1–2 sessions (not from Bolivia)	A (Burza et al., 2018)	Pentavalent antimonials (Sb ^{v+} 20 mg/kg/day for 20 days)	C (Burza et al., 2018) STRONG recommendation, MODERATE quality of evidence (Aronson et al., 2016) (All species: choice of agent, dose, and duration of therapy should be individualized)
<i>Leishmania guyanensis</i>			L-AMB 3 mg/kg/day (days 1–5 and 10)	C (Burza et al., 2018)
	No data		Miltefosine 50 mg every 8 h for 4 weeks	
			Pentamidine isethionate 4 mg/kg on alternate day for 3 infusions	C (Burza et al., 2018)
<i>Leishmania mexicana</i>			Miltefosine 50 mg every 8 h for 4 weeks	C (Burza et al., 2018)
	15% paromomycin plus 12% methylbenzethonium chloride bid for 10–20 days	B (Burza et al., 2018)	Pentavalent antimonials (Sb ^{v+} 20 mg/kg/day for 10–20 days)	C (Burza et al., 2018)
			Ketoconazole 600 mg/day for 4 weeks	B (Burza et al., 2018)
<i>Leishmania panamensis</i>	Heat therapy (50 °C for 30 s): 1–2 sessions	B (Burza et al., 2018)	Miltefosine 50 mg every 8 h for 4 weeks	B (Burza et al., 2018)
	15% paromomycin plus 12% methylbenzethonium chloride bid for 10–20 days		Pentamidine isethionate 4 mg/kg on alternate day for 3 infusions	C (Burza et al., 2018)
			Pentavalent antimonials (Sb ^{v+} 20 mg/kg/day for 10–20 days)	C (Burza et al., 2018)
			Ketoconazole 600 mg/day for 4 weeks	
New World MCL	Local treatment	Grade	Systemic treatment	Grade
Any species	Not applicable		Pentavalent antimonials (Sb ^{v+} 20 mg/kg/day plus pentoxifylline 400 mg three times daily for 4 weeks)	A (Burza et al., 2018) STRONG recommendation, MODERATE quality of evidence (Aronson et al., 2016) (Drug, dose and duration therapy should be individualized)
			L-AMB 3 mg/kg/day up to 40–60 mg/kg as total dose	C (Burza et al., 2018) STRONG recommendation, MODERATE quality of evidence (Aronson et al., 2016) (Drug, dose and duration therapy should be individualized)
			Pentamidine isethionate 4 mg/kg on alternate day for 3 infusions	C (Burza et al., 2018) STRONG recommendation, MODERATE quality of evidence (Aronson et al., 2016) (Drug, dose and duration therapy should be individualized)

MCL requires the use of systemic antileishmanial therapy to prevent complications such as disfigurement and mortality (Aronson et al., 2016); PA in Latin America still stand as the treatment of choice (20 mg Sb^{V+}/kg/day for 28–30 days) alone or in combination with pentoxifylline 400 mg every 8 h for 30 days (Lessa et al., 2001; Machado et al., 2007). AMB-d at the dose of 0.5–1.0 mg/kg/daily or every other day up to a cumulative total dose of 20–45 mg/kg or L-AMB with a cumulative total dose of 20–60 mg/kg are possible alternative drug therapies (Amato et al., 2007, 2008).

Perspectives of control

Leishmanization, i.e., the practice of inoculation of live *Leishmania* parasites to prevent CL has been used for centuries in the Middle East and former Soviet Union countries with reported efficacy of near 100% (Nadim et al., 1983; Khamesipour et al., 2005; Mohebbi et al., 2019). Several first-generation vaccines (using dead parasites), second-generation vaccines (using genetically modified parasites expressing *Leishmania* genes encoding for recombinant proteins) and third-generation vaccines (plasmid-DNA-based) are currently studied to control human leishmaniasis (Pandey et al., 2020). Interestingly, the demonstration that pre-exposure to sand fly saliva confers protection against vector-transmitted leishmaniasis (Kamhawi et al., 2000; Oliveira et al., 2015) raised the possibility to develop a pan-leishmania vaccine through a reverse vaccinology approach (Cecilio et al., 2020).

Currently, a third-generation vaccine (ChAd63-KH) based on a simian adenovirus expressing a synthetic gene (KH) encoding two *Leishmania* proteins (KMP-11 and HASPB) has been evaluated in phase 1 and 2 clinical trials in humans (Osman et al., 2017; Younis et al., 2021). In this regard a recent simulation showed that a possible vaccine against leishmaniasis able to reduce infectivity towards sand flies or to reduce the risk of developing symptoms after infection can greatly reduce VL incidence on the Indian subcontinent (Le Rutte et al., 2020).

In the context of One Health approach, the practice of culling infected dogs as a measure to control the domestic reservoir of *L. infantum* and consequently reduce the risk for VL has been recommended to no longer be used (Dantas-Torres et al., 2019). Three vaccines are commercially available for canine leishmaniosis prevention (LetiFend[®], Leish-Tec[®] and CaniLeish[®]) showing different efficacy in their capacity to prevent infection (from 68.4% to 80%) but it appears difficult to make any attempt to compare their efficacy and effectiveness (Velez and Gallego, 2020).

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Paleoparasitology

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The existence and the status of parasitic infections in living beings of the past periods of time can be demonstrated by taking advantage of parasitological investigations. The study of biological remains obtained from the archeological sites, reveals that diseases have accompanied humans and animals on the earth since their emergence, which proves that no paradise free of pathogens has existed in the world (Souza et al., 2003). Besides detecting the parasites of ancient times, paleoparasitology sheds light on many other angles for a better understanding of the lives of human ancestors. The pattern of exchanging diseases in human societies beyond the geographical territories with different cultural beliefs can be also be discussed in this line of research. Specimens for studying in paleoparasitology include soft tissues of mummies, human and animal excrement, burial soils, and many other sediment materials that may have retained many biological agents including parasite particles such as helminth eggs from the past up to the current times. The samples submitted to the laboratory of paleoparasitology are invaluable biological remains that have been preserved in archeological sites over time and should be carefully managed so that no further sampling would be necessary. Some of these materials are unique enough which are not accessible on ordinary occasions. Although all owners of these samples have been previously traveled elsewhere and or died, a wide range of biological samples is left for us around their residing areas. On occasions, food remains and animal excrements nearby humans can be found during the excavation projects that may help researchers to confirm the initial results of being investigated. Apart from the bioarcheological specimen, there are some remarkable old historical documents on parasitic infections of which the Ebers papyrus of 1500 BCE, along with more accurate descriptions of parasitic diseases by the recent Iranian physicians, Rhazes AD 850 to 923 and Avicenna 980 CE to 1037 that are more exemplary herein (Cox, 2002) are available. Based on signs and symptoms described in historical narratives, the possible role of certain parasites as causative pathological agents can be imagined in some of those known characters. This is while tracking to find the mentioned individual's samples would not be possible for the most heard scenarios. There are some pathognomonic clinical manifestations that are appeared in fiction that declare public awareness concerning certain diseases in the far past. The "devil snake" that Iranian poet Abul-Qâsem Ferdowsi Tusi mentions as the legend of "Zahhak the Snake Shoulder" in Shahnameh (Book of Kings 977–1010 CE), and the metaphor "the disease of Reshteh" by Saadi Shīrāzī in (1210 CE), are both presumably referring to *Dracunculus medinensis* "Little Dragon of Medina" or "fiery serpent" that has also been described in the Bible.

Since the recovery of *Schistosoma haematobium* eggs in an Egyptian mummy by Ruffer (1910) various perspectives have been put forward to describe the importance of paleoparasitological findings, like what is stated in "Foundations of Paleoparasitology" by the pioneers, Luiz Fernando Ferreira, Karl Jan Reinhard, and Aduino Araujo. In this new emerging line of research, many historical events can also be tracked and re-documented. Finding a parasite at a specific time in a geographical region indicates the establishment of that life cycle and the presence of its various related biotic and abiotic elements. Information like the route of ancient human migration on the earth, predominant diets among human beings as well as lifestyles and behaviors could be indicated by the identification of a specific parasite. In studying the parasite of ancient times, coprolites are known as the most valuable enriched specimens of the past people or their infected animals, provided by the archaeologists to increase the understanding of the health status and diseases in that time (Chin, 2002). The coprolite specimen represents the intestinal contents that were firstly used by Buckland (1829) in paleontology to describe the mineralized dinosaur feces (Reinhard and Bryant, 1992). Of course not as much as coprolites, the ambers however are also regarded as occasional appropriate natural preservatives for the insects, and the biological agents they carry over time (Poinar Jr and Poinar, 2005). The revealing of mermithid nematode and gordiid hairworm parasites of 100–110 million years ago in amber from Burma and *Paleoleishmania neotropicumis* in the extinct sand fly from 20–30 million-year-old amber are unique examples to define appropriate preservation conditions (Poinar Jr and Buckley, 2006; Steverding, 2017). Through the analysis of digestive tract contents, the Behavioral Nutrition that might have been addressed by the other researchers can be supported or rejected. The definition of Veganism that stated for the Tyrolean Iceman "Ötzi" of 5000 years ago using Stable isotope analysis (SIA) and then has been discussed, was revised later by illustrating the fragments of meat fibers recovered from its coprolites (Dickson et al., 2000). Paleoparasitology may also unfold the habit of consuming raw or undercooked fish in human communities like in the Joseon Dynasty of medieval Korea, following the finding of *Clonorchis sinensis* eggs (Seo et al., 2007). Regarding the details in the lifecycle of certain identified parasites in archeological sites, the specific human behaviors and/or an exotic diet can be predicted within that community. In the same way, observing a lizard's parasitic agent in human coprolites, the feeding on the squamate reptiles can be imagined among those people (Araújo et al., 2013). The existence of some socio-political practices like the human slavery in 1450–1550 AD in France could be also described by tracking the occurrence of *Schistosoma mansoni* in biological remains of that time and region since the parasite has been known endemic to Southeastern Asia (Bouchet et al., 2002). In the same way, traveling to remote geographical regions or neighboring countries will be certified by the identification of parasites known endemic in passengers that have traveled to that given territory. To attain a reliable diagnosis in studying coprolites and their containing materials, however, polynology discipline looks like an advantageous in distinguishing pollen resembling helminth eggs from those real ones (Jiménez et al., 2016). In coprolites, the identifying of *Cistus ladanifer* shrub as the historical aromatic product that might have been used to conceal the unpleasant smelling around the latrines describes a beneficial role of polynology in paleoparasitological investigations (Deforce, 2006). From the other perspectives of the study on

ectoparasites such as human lice, the co-evolution process can be explained based on evidence (Araújo et al., 2000). It is quite clear that the life subsystems and the human operation patterns on the earth have undergone many changes from the time of our earliest ancestors in Paleolithic times to the Neolithic threshold and until recently. In this context, paleoparasitological findings help us to realize how the various biological items involved in the parasite's life cycle have initially emerged, adopted, or possibly eliminated from the environment. Domestication and the beginning of agriculture following the permanent settlement of humans can evidently justify the emergence of new diseases that had not been prevalent before the Neolithic period. For instance, retrieving of ascarid and taeniid eggs in a collected soil sample from a dog's pelvic bones in East Chia Sabz archeological site in Iran dated back to the Neolithic period (8100 BC) provides insights into the transmission of toxocariasis and echinococcosis in Fertile Crescent (Paknezhad et al., 2017). In addition, the human parasitic fauna, however, might have been altered during the Neolithic revolution due to an increase of contact between humans and their nearby animals (Bouchet et al., 2003). Proving the existence of a specific parasite along with its intermediate host in the same region exhibits the natural climatic and environmental conditions of the given region. As an example the finding of Fasciolid eggs in the paleofeces, and recovering of *Lymnaea* (*Galba*) *truncatula* the freshwater snail describes the appropriate natural condition and the existence of biological possibilities for this trematode's intermediate host in that site (Dittmar and Teegen, 2003). To explain the meaningful events over the past, the capability of microscopical detection should be supported by more advanced techniques like aDNA analysis technique. Trying to implement immunological tools such as enzyme-linked immunosorbent assay (ELISA) in paleoparasitology has provided the determination of the past status of protozoan parasitic infections of public health importance, mainly *Entamoeba histolytica* in ancient human populations (Le Bailly et al., 2014). Nowadays scientists are confidently enabled to predict the health status of the past, through identifying the existence of the possible pathogenic agents within the biological remains to some extent (Anastasiou and Mitchell, 2013). In addition, the Next Generation Sequencing (NGS) has also offered another new powerful means of detecting a wide range of biotic elements, by which reconstructing the entire ancient macrobiotic environment of humans and animals is possible (Cano et al., 2014). The current access to this valuable biological banking system has enabled researchers to describe the various biological behaviors such as predator-prey in the wildlife (Chin, 2002), along with the needed potentials in occurrences of zoonotic infections in the far past supported by documents. Traits like carnivory, herbivory, omnivory, and cannibalism can be also described for animals and humans on occasions, through the metagenomic analysis. Revealing bones, fragments, and striated muscles in coprolite of a Cretaceous dinosaur illustrating the fate of a carnivore victim using preferred and selective techniques is also worth mentioning here (Poinar and Boucot, 2006). The achievements in this field of research may also indicate the existence of vector-borne diseases 100 million years ago on the earth through the tracking of the stages of trypanosomes or Early Cretaceous sand (Poinar Jr and Poinar, 2005). This is however interesting that geophagy as a clinical sign (Shinondo and Mwikuma, 2008) that is elaborated in patients suffering from a heavy hookworm infection and trichuriasis can be described by the implementation of the NGS precise technique, illustrating the elements that indicate the presence of soil in the human coprolites. Analyzing ancient intestinal contents has also expanded our knowledge to track the changes that happened during the developmental stages in human life. Taking advantage of these golden specimens, the predominant diets of carnivores, herbivores, and omnivores have been confidently demonstrated through the study on their sterol profiles and bile acids at a Neolithic settlement in the main-land Near East (Ledger et al., 2019). From the perspective of paleopathology, the finding of helminth eggs, known to be involved in human anemia of patients suffering from a heavy worm burden, in the coprolites of mummified skeletons with pathological bone lesions "porotic hyperostosis," demonstrates a unique interdisciplinary medical interpretation (Elleboudy, 2015). Moreover, describing some other phenomena like the differentiation between real parasitic infections and false parasitism "pseudo parasitism" cannot be attained by merely ordinary techniques such as common rehydration. In these cases, the consumption of animal parasitized organs like their infected livers may exhibit the parasite eggs in the consumer's coprolite that should not be regarded as a real infection (Reinhard, 1992). Since the finding of the eggs of few helminths like *Dicrocoelium* eggs in human stool samples cannot be initially attributed to a real process of infection, repeated stool examination is recommended even for the present patients that the point should be also regarded precisely in paleoparasitology to exclude any probable false parasitoses (Le Bailly and Bouchet, 2010). The finding of the oldest *Dicrocoelium* eggs of Near East in a cemetery of Bronze Age (2600–2200 BCE) close to Yasuj city, southwestern Iran indicates the presence of this zoonotic worm at that time that might have infected those people through hunting (Mowlavi et al., 2015a). Paleoparasitological findings can also explain more accurately the real emerging and re-emerging infections that are a topic of discussion in the World health organization (Aufderheide et al., 2004). Meanwhile, the other benefit of interdisciplinary researches like paleoparasitology is practicing techniques that are used in a specific discipline routinely, can be practiced in another field of science. The petrographic thin section which is utilized in geology to study fossilized object's structures was successfully experienced in studying two recovered calcified objects from a grave of an adolescent of 3rd to 4th-century in Amiens (Northern France) (Mowlavi et al., 2014a). Taking advantage of this technique, the capillariid eggs around the hydatid cyst laminated layer were also detected in one slide out of 15 thin-section prepared slides. Meanwhile, a comprehensive study of biological remains attainable within the settlement region in ancient populations can assist the scientists to find a possible medical answer in understanding the reasons related to the vanishing of the past human societies and cultures. This also should be considered that the existence of some biological elements such as nematophagous fungi is another possibility that may overshadow the real status of helminth infections in ancient times. In paleoparasitological investigations in a wide ancient cemetery, the existence of these fungi as natural predators feeding on helminth eggs should be regarded since the real number of positive cases might be overlooked in that archeological site (Leles et al., 2010). *Physaloptera* spp. eggs as the only tracked parasite in one burial site among 320 skeletons studied in Shahr-e Sukhteh archeological site of Bronze Age (2800–2500 BCE) in eastern Iran, can imply the possibility of false-negative results due to nematophagous fungi that have been detected in the same skeleton (Makki et al., 2017). The very important role of appropriate

preservation condition as a key factor in preventing destructive taphonomic processes on biological remains should not be underestimated as it has been demonstrated in sheltered sites (Reinhard et al., 2019). However, based on available evidence in the Hallstatt salt mines archeological site, the salty environments are known as the most appropriate factor in coprolites preservation over time (Bouchet et al., 2003). Meanwhile and referring to the effective role of salt, our previous studies in Chehrabad salt mine archeological site (Sassanid Era, 224–651 CE) in Iran, the intact appearances of the eggs of *Trichosomoides crassicauda*, *Syphacia* sp. and *Trichuris* sp. in rats, *Macracanthorhynchus hirudinaceus* eggs of canine, as well as the eggs of Strongylidae and Anoplocephalidae helminths of herbivore which have been observed in animal coprolites is worth mentioning (Mowlavi et al., 2014b, 2015b; Meigouni et al., 2020). Describing the reasons for the shift from the prevalence of *Trichuris* eggs in the early decades of the 19th century and even earlier times to *Ascaris* eggs half a century later until nowadays has been justified by the biology of helminth eggs and the changes raised in environmental and socio-cultural conditions following urbanization (Trigg et al., 2017). Many other historical realities could be however documented or even rejected based on approved paleoparasitological findings. From the perspective of the human lifestyle, it is expected that in hunter-gatherer societies, uncooked meat should have been the cause of most of the parasitic infections, while the sedentary life could have increased amoebic dysentery owing to crowdedness and more human contacts with the water resources (Reinhard, 2000). In the case of human pinworm infection due to *Enterobius vermicularis* a directly transmitted helminth that is believed to have been originated from Africa and its existence was documented in the North American archeological sites about 10,000 BP, might have been intermittently occurred among ancient hunter-gatherers (Araújo et al., 2015). Jean-Pierre Hugot and colleagues have explained the significant phenomenon by the description of the air-borne enterobiasis in humans when they selected indoor life in caves and left outdoor habits on the route of evolution (Hugot et al., 1999). This interindividual direct transmitted helminthiasis in ancient societies is another subject of interest to be discussed in paleoparasitology (Formaciari and Gaeta, 2014). Parallel to this parasitological fact, tracking of enterobiasis in the 7000-year-old Tehrani woman, following the identification of the *E. vermicularis* egg in her pelvic soil sample has extended the antiquity of human residency in Tehran to 5th millennium BCE while the previous documents in Gheytaieh Cemetery archeological site in the northeast of the city had shown the human settlement background to a closer time in the region (Paknazhad et al., 2016). It is valuable for the interested researchers to understand more about the origin of the parasites. It is also important to note that the identification of the parasites of ancient times that are still current on the Earth is easier than those extinct parasites. However, the identification of those parasites that have been extinct along with their natural hosts together, is a more challenging issue in paleoparasitology (Strona, 2015). Today, looking at the approximately 100,000 museums in the world, it can be expected that a valuable and variant amount of ancient materials obtained from archeological excavations are available to be precisely investigated. These great archeological items however do not certainly include all of the world's apparent and hidden heritage (Statista Research Department, 2020).

In conclusion, the study on these valuable resources and ongoing new biological remains excavating from archeological sites as a unique topic of research can incredibly increase and improve the knowledge of researchers about the emerging parasites, diseases, and the health status among the earliest ancestors of human beings on earth.

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Pinworm

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Epidemiology

Pinworm infection, also called enterobiasis is caused by the parasitic nematode *Enterobius vermicularis*. The worm is also known as threadworm or seatworm. Enterobiasis occurs worldwide and affects persons of all ages, socioeconomic levels and race, although it is primarily a pediatric condition being most frequent in children. It affects also people living in crowded environments, including caregivers, and within families (household members). In institutions, transmission is facilitated by communal living and possibly by the residents' difficulties in maintaining good personal hygiene which contributes to create a large reservoir of infection (Lohiya et al., 2000).

The relationship between the prevalence of pinworm and gender is controversial. It is generally reported to occur with equal frequency in both males and females. However, some studies indicate that the risk of *E. vermicularis* infection could be higher among male children with a male to female infection frequency of 2 to 1. In contrast to these studies, females sometimes resulted to be affected more often (Wendt et al., 2019) than males (Al-Daoudy, 2005; Al-Daoudy and Al-Bazzaz, 2020).

The highest rate of infection has been observed in younger age group (3–6 years old) with children aged 5–10 years old accounting for over 30% of cases (Kucik et al., 2004) and can probably reflect a lower attention to personal hygiene in small children compared to older ones as well as by the anal scratching being more frequent among small children, facilitating the autoinfection. Educational level among children, overcrowding, socioeconomic condition, and climate could be also related to pinworm infection. Among risk factors, scratching the perianal region, nail biting, putting contaminated toys or utensils in the mouth, as well as close person-to-person contact seem to play an important role. Wang and colleague found that no hand washing before eating, sucking fingers or toys, and scratching of the anus were all factors associated with the infection (Wang et al., 2016).

Pinworm is mainly spread by fecal-oral route and occurs via self-inoculation or through exposure to eggs in a contaminated environment. However, transmission via inhalation is also reported (Rawla and Sharma, 2019). Eggs may be directly transferred from the perianal area to the mouth when hands scratch the perianal region (autoinfection) or may be ingested indirectly, through contaminated objects or surfaces. Occasionally, the hatched larvae can also enter the anus retrogradely migrating to rectum and the small intestine, causing retrograde infection (Schuffner and Swellengrebel, 1949). Reinfection is frequent.

Risk factors

Risk factors for pinworm infection include poor hygiene with particular regard to hand-washing prior to eating, scratching in the perianal region, nail biting (onychophagia), living with an infected individual, being a child and taking care of infected people, especially individuals who are institutionalized. Pets like cats and dogs cannot become infected. Transmission is also possible sexually (Abdolrasouli and Hart, 2009; Waugh, 1974; Baker and Peppercorn, 1982).

Geographic distribution and prevalence

E. vermicularis is the most prevalent gastrointestinal nematode in humans. Pinworm is an exclusive obligate parasite of humans, directly transmitted from person to person with a worldwide distribution, nearly ubiquitous, being very common in temperate and cool climate regions (Ariyathenam et al., 2010; Powell et al., 2013), although the infection is more common in temperate than in tropical districts (Fan et al., 2019).

Climate and soil temperature are limiting factors for some human helminths such as whipworms and hookworms, that are host-specific and without intermediate hosts (Araujo et al., 2008). By contrast, *Enterobius* egg survival is not dependent on climate conditions (Hugot et al., 1999).

In Europe, prevalence rates of 37.0% up to 50% have been reported (Herrström et al., 2001; Burkhart and Burkhart, 2005) although they are generally near 20% (Wendt et al., 2019).

Estimated prevalence among kindergarten and primary school pupils in Europe are generally near 20%. Infants (<2 years of age), adolescents (>14 years of age), and adults are only sporadically infected (Wendt et al., 2019).

In a recent epidemiological study on the occurrence of enterobiasis in Germany, *E. vermicularis* was retrospectively detected in 17.4% of samples between 2007 and 2017. The detection rate increased significantly within the period of investigation and was higher in male than in female patients (20.0% vs. 15.4%). Children 4–10 years old (and their relatives) were most frequently affected. Frequency of detection in children <6 years was evenly distributed throughout the year, whereas in older patients it showed a seasonality with peaks during the winter months (Friesen et al., 2019).

In Italy, a study showed a prevalence in children during the years 2002–03 of 13.4% (Crotti and D'Annibale, 2006).

In Eastern Slovakia, pinworm prevalence was 4.07% in boys and 3.21% in girls. The highest prevalence was recorded in the group of children aged 3–6 years (5.03%). Most of the samples were positive at age 4 and 5 (Dudlová et al., 2018).

Similar results (3% prevalence) were found in children and adolescents from a North-Eastern area of Poland (Zukiewicz et al., 2011).

A Norwegian study found that the parasite is widespread in children. A total of 18% of children tested positive for *Enterobius* eggs, with the highest prevalence (34%) among 6–11 years old (Bøås et al., 2012).

Enterobiasis is the most common helminth infection in the USA, with an estimated rate up to 42 million people (14% of the population) affected by this parasite (Burkhart and Burkhart, 2005), with the highest prevalence in children, institutional populations, and families (Lohiya et al., 2000).

Investigators in other parts of the world report variable estimates of prevalence according to the country. In Korea, the overall egg positive rate (EPR) in children was 10.5% (range 3.3–18.6%), higher in boys than in girls (adjusted odds ratio, AOR: 1.5), and in rural compared to urban area (AOR: 1.2) (Lee et al., 2011).

Prevalence of *Enterobius* infection among preschool children in Henan province, China, showed a significant decrease over a 10-year period. The overall EPR for *E. vermicularis* was indeed, 12.75% in 2003 and 5.13% in 2013, respectively. In both years, the EPR for 5 to 6-year-old children was significantly higher than that of 2 to 4-year-old children. However, positive rates were not significantly dependent on gender or area (Wang et al., 2016).

In Sri Lanka, the highest prevalence rate in children was 37.5% (Suraweera et al., 2015).

A meta-analysis reported high differences in prevalence of *E. vermicularis* among Iranian children, ranging from 1.2% to 66.1%. In particular, boys, and girls showed wide ranges from 2.3% to 65.5% and 1.7% to 65.5%, respectively. When adjusted for the random effect model, the overall prevalence of oxyuriasis among all children, boys, and girls were 17.2% (12.6%–21.8%), 17.2% (12.6%–21.8%), and 16.9% (9.03%–24.8%), respectively (Moosazadeh et al., 2017).

Prehistoric findings

Host-specific human parasites have been used to track ancient migrations (Araujo et al., 2008; Fornaciari and Gaeta, 2014; Reinhard et al., 2016). Evidence of pinworm in ancient populations has been demonstrated in different archaeological sites located in the Americas, Europe, and Asia (Gonçalves et al., 2003). The oldest evidence of a pinworm in Asia has been revealed by the presence of one *E. vermicularis* egg attached to the skeleton sacral region of a female adolescent discovered in the area of Tehran (Iran) and dated to approximately 7000 years ago (Paknazhad et al., 2016).

Enterobius is considered one of the most ancient human parasites (Iñiguez et al., 2003). According to paleoparasitological findings of *Enterobius* eggs from different parts of the world (Fry and Moore, 1969; Hugot et al., 1999; Araujo et al., 2008), the prevalence of pinworm infection seems to be higher in primitive populations of Neolithic period (Paknazhad et al., 2016).

Eggs of *E. vermicularis* were recently identified in coprolites from one pre-Columbian mummy. This is the first evidence of ancient intestinal parasites in Bolivian mummies, being dated from 1150 to 1450 CE (Valverde et al., 2020).

E. vermicularis eggs were also found in human coprolites from archaeological sites in western Utah, USA (Fry and Moore, 1969). These samples, dated at 7837 BCE, are considered the oldest reports of human parasitic infection (Iñiguez et al., 2003).

The discovery of an egg of a parasite of the order Oxyurida in a coprolite from the remains of species of Cynodontia, indicates that the pinworm lineage extends at least as far back as 240 million years (Hugot et al., 2014). This is the oldest record of an oxyurid nematode yet discovered, and is particularly meaningful since the cynodonts are considered a stem-group of the mammals.

Four parasite species were found in the Atacama Desert in coprolites of the Inca period, including pinworm (*E. vermicularis*) (Santoro et al., 2003). *Enterobius vermicularis* eggs were also found in coprolite samples of Salmon Ruins, New Mexico, USA, dating from 1140 to 1280 CE (Reinhard and Camacho, 2019) (Fig. 1).

Biology - Characteristics of the organism

E. vermicularis worms are tiny, thread-like and whitish. At maturity, adult females measure 9–12 mm and have a pointed tail, whereas males are 2–5 mm in length. Gravid adult females migrate to the anal area mostly during the night and deposit thousands of eggs on perianal area. Migration and egg hatching causes pruritus.

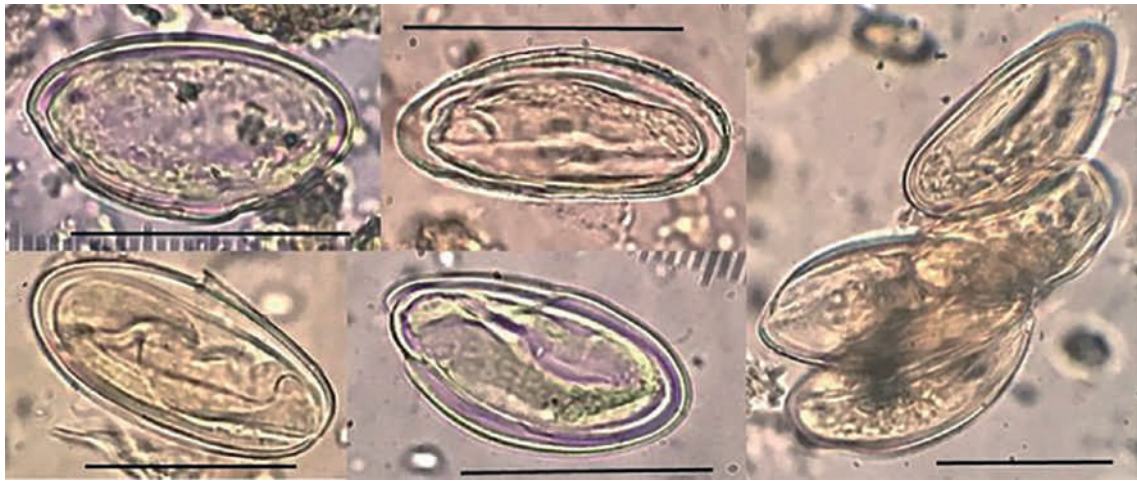


Fig. 1 *E. vermicularis* eggs found in coprolite samples of one of the two latrines of Aztec Ruins, New Mexico, USA. With permission of Reinhard KJ and Camacho M (2019) Pinworm infection at Salmon ruins and Aztec ruins: Relation to Pueblo III regional violence. *The Korean Journal of Parasitology* 57(6), 627–633.

Eggs $50\text{--}54 \times 20\text{--}27 \mu\text{m}$ in size, are translucent, with an asymmetric shape and their shell is constituted by two parts. The outer layer facilitates the adhesion of the eggs to the perianal region where they develop in infective larvae in about 4–6 h following oviposition and can survive for 2 or 3 weeks in the domestic environment, mainly on clothing, bedding and other objects or surfaces. Differently from other helminth, eggs of *E. vermicularis* are indeed infective as soon as they are expelled from female worms.

E. vermicularis life cycle takes place in the gastrointestinal lumen. Ingested eggs hatch in the small intestine (duodenum). Larvae mature during their migration to the large intestine. The third larval stadium (L3) represents the infective form of the parasite.

After molting twice, the adult worms copulate and then migrate downwards to the large intestine, where they can be found in large numbers particularly in lumen of caecum, appendix, or ascending colon. The males die soon after copulation; the gravid females, on the other hand, have an overall life span up to 100 days (3 months), reaching the anal canal by means of active migration (Wendt et al., 2019). An adult female can lay more than 10,000 eggs in her lifetime. Oviposition occurs mainly at night, when the host is sleeping. The time interval (incubation period) from ingestion of infective eggs to oviposition by the adult females is of 1–2 months (Fig. 2).

Pinworms generally exhibit high host specificity with humans being the only known host for *E. vermicularis* although occasional infections have been reported in non human primates such as captive chimpanzees (CDC, 2019, <https://www.cdc.gov/parasites/pinworm/biology.html>).

Despite the wide, cosmopolitan, distribution and the high prevalence of enterobiasis in the world, molecular studies for the genotype characterization of *E. vermicularis* are limited. Molecular studies have showed three genotypes (A, B, and C) in *E. vermicularis* from humans and chimpanzees (Nakano et al., 2006; Ferrero et al., 2013). Genotype B is the predominant genotype in human (HayatiHagh et al., 2014). In a work on children in North-Eastern Poland by Kubiak and colleagues, the isolates of the parasite studied were different in terms of the sequences within the cytochrome *c* oxidase subunit 1 (cox1) gene (Kubiak et al., 2017).

Another pinworm geographically distributed in Europe, Africa, and Asia, has been described, namely *E. gregorii*. However, new morphologic and molecular evidence suggests *E. gregorii* likely represents an immature form of *E. vermicularis* (CDC, 2019, <https://www.cdc.gov/parasites/pinworm/biology.html>).

Clinical manifestations

Infection is usually asymptomatic, when the worm is confined in the ileocaecum tract. The most common symptom is perianal itching (pruritus), due to female migration to the anal area.

The larvae and, at a minor extent, adult worms, may penetrate with their cephalic extremity into the mucosa, thus causing ulcerations that may occasionally give origin to hemorrhages and bacterial superinfections. Rarely, diarrhea has been reported in some patients. Other symptoms include insomnia and irritability, particularly in children, loss of appetite, emotional instability, and enuresis in up to 53% of cases (Wendt et al., 2019).

Adult worms reside in the intestine, primarily the ileum and caecum, ascending colon and rectum. Occasionally, infection may involve extraintestinal sites. The parasites can migrate to organs such as appendix, prostate, lungs, and liver. Female genitourinary infections have also been reported in the literature, although rare, with worms migrating to the vaginal area causing vulvovaginitis or urinary tract infection in women (Burkhart and Burkhart, 2005; Rawla and Sharma, 2019). Although of low pathogenicity, serious complications such as infertility or peritonitis may arise (Powell et al., 2013). *E. vermicularis* has also been isolated from the

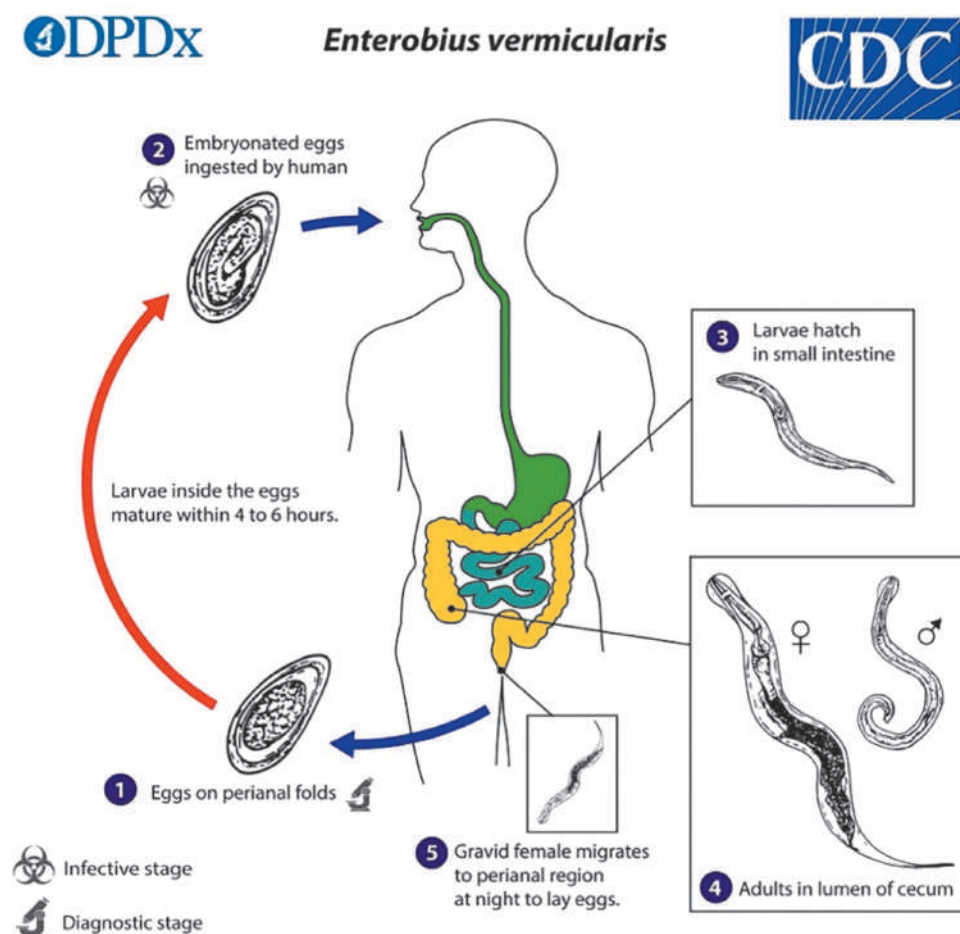


Fig. 2 Life cycle of *E. vermicularis*. From: https://www.cdc.gov/dpdx/enterobiasis/modules/Enterobius_LifeCycl_Ig.jpg.

male urinary tract (Zahariou et al., 2007). Atypical manifestation of the disease mostly with mental symptoms has also been reported (Karamitros et al., 2017).

Host immune response

The host immune response to helminths is regulated mainly by Th2 cells, which undergo an expansion, and activation which stimulates the production of eosinophils (EO), mast cells, basophils, and specific IgE, by the release of IL-4, IL-5 and IL-13, through the signal transducer and activator of transcription (STAT) 6 activation (Anthony et al., 2007).

In a study by Patsantara et al. (2016), EO, eosinophil cationic protein (ECP), and serum immunoglobulin E (IgE) were found to be higher in pinworm infected than in uninfected children, suggesting a Th2-type oriented immune response activation during this helminth infection. However, EO and ECP were lower in atopic infected children than in those non atopic. These children also exhibited higher total IgE levels compared to non-atopic ones, although they were completely asymptomatic (Patsantara et al., 2016).

Human and animal studies have shown a relationship between allergy and parasitic infections (Reddy and Fried, 2008). A negative correlation between pinworm infection and allergic airway diseases was suggested by Huang and colleagues (Huang et al., 2002). Similarly, enterobiasis has been found to be associated with a decreased risk of allergic wheezing in Turkish school-aged children (Bahceciler et al., 2007).

A change in microbiota composition and in secretory IgA (sIgA) levels after two weeks mebendazole treatment has been observed (Yang et al., 2017). The sIgA provide an important defense against enteric agents. This antibody can bind to adhesion molecules expressed on the surface of by numerous pathogens. Gut sIgA level was low in pinworm infected children, but it was increased in about 50% of the subjects after mebendazole deworming treatment, and it was associated to a higher proportion of probiotic bacteria (Yang et al., 2017).

Diagnosis

The diagnosis of *E. vermicularis* can be confirmed by identifying the female worms or the eggs (Fig. 3).

The diagnostic method of choice is the Scotch-tape test, where a clear adhesive cellulose tape is applied to the anal area early in the morning and then affixed to a slide. Microscopic detection of the characteristic worm eggs confirms pinworm infection. The tape method should be performed in the morning prior to defecation on three consecutive days in order to increase its sensitivity. This varies from around 50% in the case of a single tape test, to about 90% or even more when performed on 3 days (Jeandron et al., 2010).

Stool microscopy examination is not a helpful diagnostic tool since eggs can be detected in only about 5% of fecal samples (Fan et al., 2019). For this reason, pinworm infection is generally thought underestimated. However, stool examination can show adults worms in case of severe infection (Wendt et al., 2019).

A diagnosis of a pinworm infection can also be made by examining the area around an infected person's anus at night with a flashlight to find a female worm. Occasionally, adult worms may be seen on proctoscopy or colonoscopy (Wendt et al., 2019). Serological methods are of no diagnostic relevance and are not commercially available (Fig. 4).



Fig. 3 Eggs of *E. vermicularis* in a wet mount (<https://www.cdc.gov/dpdx/enterobiasis/index.html>).

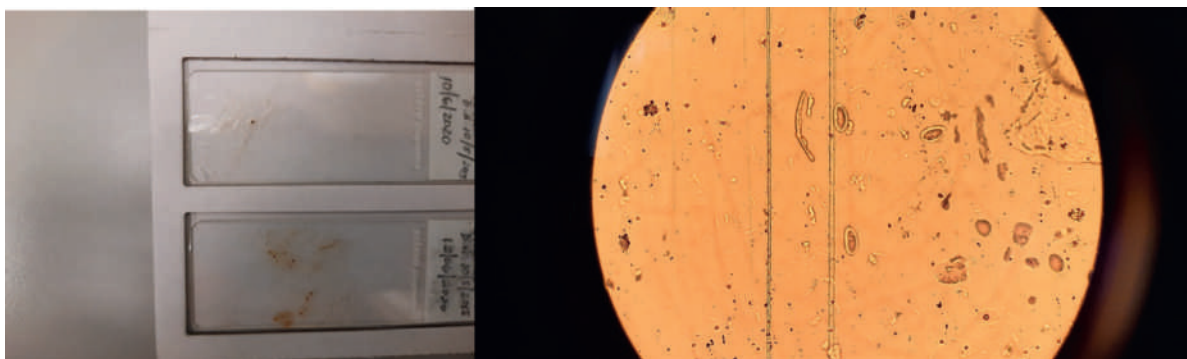


Fig. 4 Collection of *E. vermicularis* eggs by the cellulose tape method. Left, scotch test. Right, a positive scotch test.

Treatment

The most commonly used drugs are mebendazole (Vermox®), albendazole (Zentel®, Albenza, Eskazole), pyrantelpamoate (Combantrin®), and piperazine. Mebendazole and albendazole are both adulticidal and ovcidal. The first is usually administered at a single dose of 100 mg whereas the albendazole and pyrantel are administered at 10–14 mg/Kg and 10 mg/Kg, respectively. Treatment must be repeated after 2 or 3 weeks because the larval stages are not affected by antihelminth drugs. The following doses are, therefore, essential to achieve the complete destruction of adult worms. Albendazole is not recommended in children under 2 years of age, whereas mebendazole can kill only adults, but not eggs nor larvae (Dudlova et al., 2018).

All the antihelminthic drugs are generally well tolerated causing harmless and short-term side effects, most commonly headache, abdominal pain, and diarrhea. Unlike albendazole, only 7% of mebendazole is absorbed when administered orally as the drug largely remains in the intestinal lumen. This results in fewer systemic side effects and acts effectively when the infection is confined to intestine (St Georgiev, 2001). The safety of drugs used to treat pinworm have not been studied for pregnant women (CDC, 2019).

Since the eggs can be easily spread, all the member of a family or community should be treated with the drug, although they are asymptomatic.

In murine experimental models of infection, a genetic susceptibility to infection has been proved. (Moulia et al., 1993). Indeed, the strain (i.e. genetic) effect seems to be the main parameter which determines the levels of infection (Derothe et al., 1997). Egg counts were significantly higher in some strains compared to other ones. Differences in susceptibility between various species and subspecies of *Mus* have been recently confirmed (Curtis et al., 2017). Differently from previous studies, in this new study, egg counts did not show any difference in regard to age or sex.

Prevention

Hand washing and nail care using soap prior to eating and following defecation is the main preventive measure. Underwear and bedclothes should be changed daily and washed at temperatures of at least 55–60 °C. Household, community members, and sexual partners should also be treated with antiparasitic drugs and the treatment should be repeated after 2 weeks.

Incomplete treatment associated to easy transmission of the parasites may frequently cause reinfection. Indeed, this is common because live eggs are excreted in stool up to a week after the treatment.

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<https://www.cdc.gov/dpdx/enterobiasis/index.html>—Centers for Disease Control and Prevention (CDC), Page last reviewed: 2019.

Plasmodium

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Introduction

Named *Oscillaria malariae* by Alfonse Laveran, the Frenchman posted in Algeria who first described this parasite using a basic microscope without staining in October 1880 (Note to the Academy of Medicine, 8 November 1880), the causative agents of fevers, headaches, chills and enlarged spleen were associated with environment, food and drinking water for centuries. Eighteen years later this first description, an Englishman posted in India demonstrated the role of mosquitoes in transmission of this parasite to birds (Ross, 1898). *Plasmodium* is a genus of unicellular parasites that infects mammals, reptiles, birds and most of vertebrates. Few species of *Plasmodium* parasites are responsible for human diseases, but their public health impact is considerable since centuries. While progresses have been obtained from a highly engaged research and political involvement worldwide, Malaria, the disease caused by these parasites in humans, is still one of the main serial killer.

Among the five species that regularly infect human, *P. falciparum* is the most devastating and by far the most studied. Others species have long been considered as less pathogenic, but recent evidence is arising from many studies indicating that *P. vivax* has also a considerable death toll. Studying *P. vivax* has gained more interest among researchers and funders during the last decade, but it is still wrongly considered as a second issue. With the same rational, *P. ovale curtisi*, *P. ovale wallikeri* and *P. malariae* are very poorly considered by the scientific and economic communities. This may be a culpable negligence and the future will tell more about the health impact of these more chronic but still debilitating parasites.

The life cycle is based on a host (mammals, birds, reptiles etc.) where the symptomatic (clinical and/or biological) phase takes place, and a vector (mosquitoes, mostly anopheles) allowing transmission. The host phase of the cycle is the asexual part of the parasite multiplication, while the sexual multiplication occurs within the mosquito's vector. Most of the textbooks are using this sexual/asexual dichotomy to characterize the parasite life cycle and all the steps of the parasite cycle have been perfectly and extensively described in thousands of papers. The *Plasmodium* cycle is more than a complex description of a parasite adaptation to different conditions with the goal of being transmitted. It is a significant timetable for clinic, diagnosis, treatment and public health (Bray and Garnham, 1982).

Plasmodium transmission stages: Gametocytes and sporozoites

The transmission stages of *Plasmodium* are gametocytes (from human to mosquito) and sporozoites (from mosquito to human). Gametocytes are the first step of sexual cycle which occurs inside the anopheline vector. The maturation of gametocytes is done in bone marrow and spleen, depending on species. Mature gametocytes are the only stages circulating in peripheral blood. The

production rate and timing of gametocytes is highly regulated by internal (genetic and epigenetic) and external factors (host, nutrients, lipids, environment). When ingested by mosquitoes, gametocytes are exposed to a major drop in temperature and to xanthurenic acid, triggering their maturation into gametes (Josling and Llinás, 2015). Male gametocytes produce 8 flagellated gametes in the mosquito mid-gut in less than 20 min (exflagellation step) and the female gametocytes evolve into a single macrogametes (Ngotho et al., 2019).

Sporozoites are the infectious stage injected to the dermis during the infected mosquito's bite. Using gliding motility properties, a proportion of these sporozoites can reach the blood-stream in 1–3 h, while others are destroyed by host response (Cowman et al., 2016). The next step is traversal through the sinusoidal barrier, including endothelial cells and Kupffer cells, to liver cells, where sporozoites establish and develop in exo-erythrocytic forms in 2–10 days. All these steps are driven by a complex organization of surface proteins. The central role of circumsporozoite protein in these processes lead to its extensive study as a vaccine target. The evolution of liver stages is different depending to species particularities, with a special interest to *Plasmodium vivax* hypnozoites described below. It was recently stressed that we should clearly define the respective timings between each phase of the parasite cycle (Pinkevych et al., 2015). Time-to-infection, which could be different according to the immune status of the patient, is different from time-to-detection, which highly dependent on the biological methods (microscopy versus PCR). Both timings may be affected by the burden of injected sporozoites and the growth rates of parasites. Thus this information should be taken into account with caution and a clarification is probably needed in the future.

Plasmodium human stages

Up to 40,000 merozoites could be produced in a single liver cell. The budding of merozoite inside vessels lead to the release of these parasites inside the blood flux. Free merozoites invade red blood cells in less than 2 min. The penetration is a very active mechanism requiring deformation of red blood cell, irreversible attachment, and reorientation of the parasite. The merozoite is then propelled inside the red blood cell by the actinomyosin motor, and the membrane fusion entrapped the parasite in the digestive vacuole. Schizogony occurs in 48 h, leading to the egress of 16–32 merozoites in the case of *Plasmodium falciparum*. The intraerythrocytic cycle of *Plasmodium* species is responsible for the occurrence of major symptoms such as fever and anemia due to the regular disruption on infected red blood cells and to the liberation of the parasite pigment (hemozoin). The early trophozoite stage (ring form) is probably poorly involved in the pathology, while late trophozoites and schizontes will sequester in the deep microvasculature, mainly in the brain and the lungs, triggering the endothelial damages responsible for the more severe symptoms. The pathophysiological mechanisms were fully described in details elsewhere (Erice and Kain, 2019; Jensen et al., 2020).

Parasite metabolism

Plasmodium parasites were qualified as voracious consumers of glucose (Olszewski and Llinás, 2011). Parasite increase up to 100-fold the infected red blood cell glucose consumption rate (Qureshi et al., 2020). The energetics needs are primarily provided by glucose fermentation, and this resource is abundant in the serum. The membranes required for the parasite proliferation are produced via a complex mechanism of lipid metabolism, including scavenging of preformed fatty acids from the serum and incorporation into membrane glycerides (Olszewski and Llinás, 2011). The *clag* multigene family encodes the Rhop H1 proteins involved in the formation of channels at the surface of red blood cell. These channels are needed for the acquisition of nutrients (Duraisingh and Skillman, 2018).

The *Plasmodium* genome

The *Plasmodium* genome is haploid except in the mosquito midgut, with fourteen chromosomes, a circular plastid genome and multiple copies of mitochondrial DNA (Su et al., 2019). The genome size of *P. falciparum* is lower (23.2 Mb) than those of *P. vivax* (29.1 Mb), *P. ovale* (33.5 Mb) and *P. malariae* (33.6 Mb). The adenine-thymine contents (AT) are very different among *Plasmodium* species infecting humans, from 80% in *P. falciparum* to 60% in *P. vivax*. The *P. falciparum* genome is highly polymorphic near the subtelomeric regions of the 14 chromosomes where multigene families are encoded. Extensive transcriptomic studies shown that most of the multigene families have clonal variation, leading to the definition of the “variantome” of the parasite (Duraisingh and Skillman, 2018). The epigenetic control of gene expression is under the control of vast array of histone-modifying enzymes including histone deacetylases, histone acetyltransferases and demethylases. *Plasmodium* also shows a large number of single nucleotide polymorphisms and copy number variation. Comprehensive reviews of genomic analysis of *Plasmodium* parasites were published and reader should refer to these papers (Abel and Le Roch, 2019; Escalante and Pacheco, 2019; Galinski, 2019; Ross and Fidock, 2019; Saralamba et al., 2019; Su et al., 2019).

Antigenic and gene expression variation

The parasite has developed a smoke-screen system to escape the host's adaptive immunity. One of this main mechanism is the controlled alternating expression of adhesive surface protein variants (Deitsch and Dzikowski, 2017) The *var* gene family encodes surface antigens named *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). These surface proteins, as well as the repetitive interspersed families of polypeptides (RIFINs) and subtelomeric variant open reading frame (STEVAR), are highly

involved in the pathogenesis mechanisms of malaria and their role in sequestration of parasites is crucial during cerebral malaria and placental malaria (Wahlgren et al., 2017). These surface antigens are considered as candidates for a vaccine development. Among the sixty *var* genes present in the genome of a single parasite, only one encodes a variant of PfEMP1 at a time. When this surface antigen is detected by the immune response of the host, the parasite switches its expression to another variant. How the parasite turn on or off the expression of these genes has been extensively reviewed by authors (Deitsch and Dzikowski, 2017). RIFINS and STEVOR subvert the host immune system via a still obscure mechanism, probably different from the mutually exclusive manner of *var* genes (Duraisingh and Skillman, 2018). Much less information is available concerning the antigenic variations and immune evasion of the other *Plasmodium* species infecting humans while the immunology of vivax malaria has been extensively studied (Antonelli et al., 2020).

Plasmodium infecting humans

Five *Plasmodium* species are regularly infecting humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale curtisi*, *Plasmodium ovale wallikeri*, *Plasmodium malariae*. Among the zoonotic species, *Plasmodium knowlesi* is the most frequently observed in humans.

Plasmodium malariae (Fig. 1)

Plasmodium malariae was named by Grassi and Feletti in 1890. *P. malariae* is the only human parasite (with the zoonotic parasite *P. brasilianum*) presenting an erythrocytic cycle that last for 72 h, releasing 6–14 (average 8) merozoites in the blood stream (Collins and Jeffery, 2007). This regular increase in parasite count was associated with fever peak occurring on the fourth day defining the quartan malaria. The prepatent period after mosquito bites varies between 16 and 59 days, but a recent study demonstrated that the delay to onset of symptoms in travelers could be from 1 day to 1 year (Sutherland, 2016; Teo et al., 2015).

The low parasitemia (around 10,000 parasites/ μ L = 0.25%) observed in patients infected by *P. malariae* is linked to these parameters: slow and low multiplication rates and infection of older erythrocytes (Collins and Jeffery, 2007). This mild infection may last for 60 days without treatment, and it is supposed that the blood stages may persist for years or decades (Vinetz et al., 1998), without identified dormant liver stages. This chronic infection may results in a chronic nephrotic syndrome responsible for poor outcome (Hedelius et al., 2011; Mueller et al., 2007).

P. malariae transmission is present in most of the malaria endemic word, including sub-Saharan Africa, South-East Asia, South America and western Pacific. The seasonal transmission of *P. malariae* seems to be opposite to that of *P. falciparum* in co-endemic areas leading to the hypothesis of specie specific suppression by *P. falciparum* (Bichara et al., 2017; Molineaux et al., 1980).

At least 30 different species of anopheles' mosquitos were involved in *P. malariae* transmission experimentally, and 8 of them were proven to infect humans.

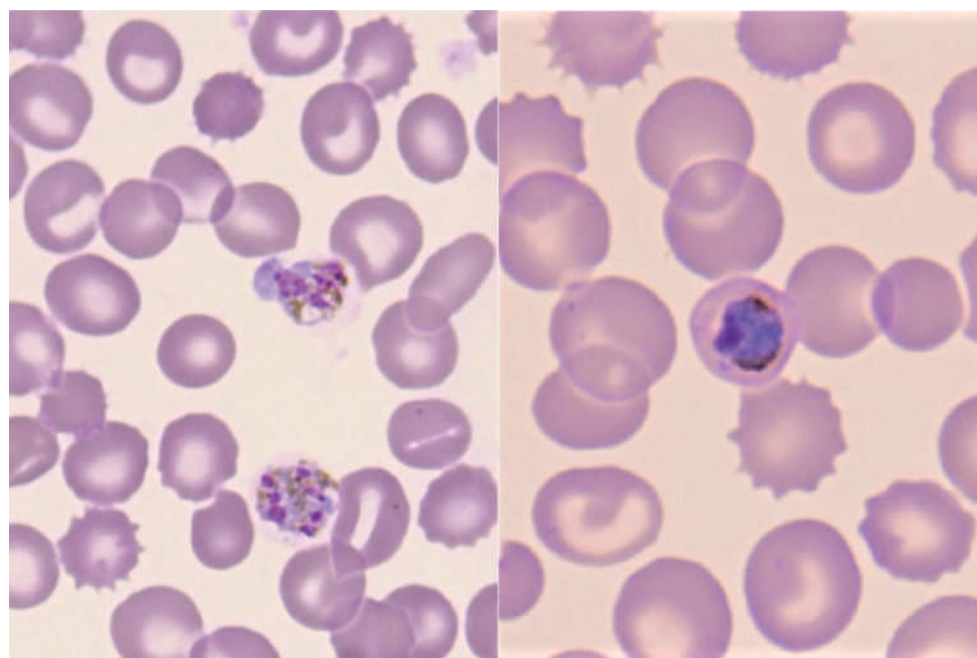


Fig. 1 *Plasmodium malariae* schizontes and trophozoites.

P. malariae is morphologically similar to monkey's parasites *P. knowlesi* (quotidian cycle) and *P. inui* (quartan cycle) from South-East Asia, and to *P. brasilianum* from South America.

A new reference genome for *P. malariae* was recently produced from clinically isolated parasites, allowing extensive *Plasmodium* phylogeny (Rutledge et al., 2017). This study provided evidence that the split between *P. malariae* and *P. malariae*-like parasites (monkeys) occurred 3.5 million years ago, meaning more recently than *P. falciparum*/*P. reichenowi* split (3.0–5.5 million years ago). The genome size of *P. malariae* is 33.6 Mb, with 6540 genes.

Plasmodium ovale* (Fig. 2): *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri

Plasmodium ovale, first described in 1922 (Stephens, 1922) is transmitted to humans mostly in Sub-Saharan Africa, and infrequently in Western Pacific, Sri Lanka (Wickremasinghe et al., 2008), Vietnam (Gleason et al., 1970), Myanmar (Win et al., 2002), Lao PDR (Toma et al., 1999; Win et al., 2002) and Timor (Baird et al., 1990; Kotepui et al., 2020; Lysenko and Beljaev, 1969). Its prevalence is highly variable according to areas and seasons, but probably underestimates due to low parasitemias, frequent co-infections (Bichara et al., 2017), poor performance of diagnosis methods including light microscopy and rapid diagnosis tests, and mild infection in humans leading to low interest of the scientific and medical community.

Plasmodium ovale prepatent period is 12–20 days, followed by a tertian cycle with a development in erythrocytes that last 49 h for asexual and sexual forms, and to 8–20 merozoites (Collins and Jeffery, 2005). The parasitemias remain at a low level with a peak at 10–12 days.

P. ovale is usually described as a relapsing species due to the existence of hypnozoites in the liver (Collins and Jeffery, 2005). However the evidence for this characteristic is lower than for *P. vivax* (Richter et al., 2010).

A decade ago, Sutherland and colleagues demonstrated that *P. ovale* consists of 2 non recombining species: *P. ovale curtisi* (classic type) and *P. ovale wallikeri* (variant type) (Sutherland et al., 2010) as suspected previously (Win et al., 2004). The new names of these two different species were proposed to honor Christopher Curtis and David Walliker. These two species are sympatric in time and space and were probably separated more than 1 MY apart, leading to a genetic drift preventing fertilization and recombination (Sutherland, 2016). Most of the knowledge on *P. ovale* was obtained prior to this new classification, and evidence for differences in biological, clinical and epidemiological traits between the two *P. ovale* are scarce. One of the difference was the latency period measured at 85.7 days for *P. ovale curtisi* and 40.6 days for *P. ovale wallikeri* (Nolder et al., 2013).

These parasites have gained a renewed interest during the last decade, while most of the cases are uncomplicated mild malaria with favorable outcome. A recent metaanalysis of severity and mortality of *P. ovale* infection included only eight studies (Kotepui et al., 2020). The mortality rate of severe *P. ovale* infections was 0.15%, but this was based on only 2 deaths among 1365 cases. It is thus impossible to compare the mortality rate of *P. ovale* species to those of *P. falciparum* or *P. vivax*, suggesting the fact that we should pay more attention to this species to improve our knowledge which is mainly based on the use of the Donaldson strain for general paralysis therapy during decades.

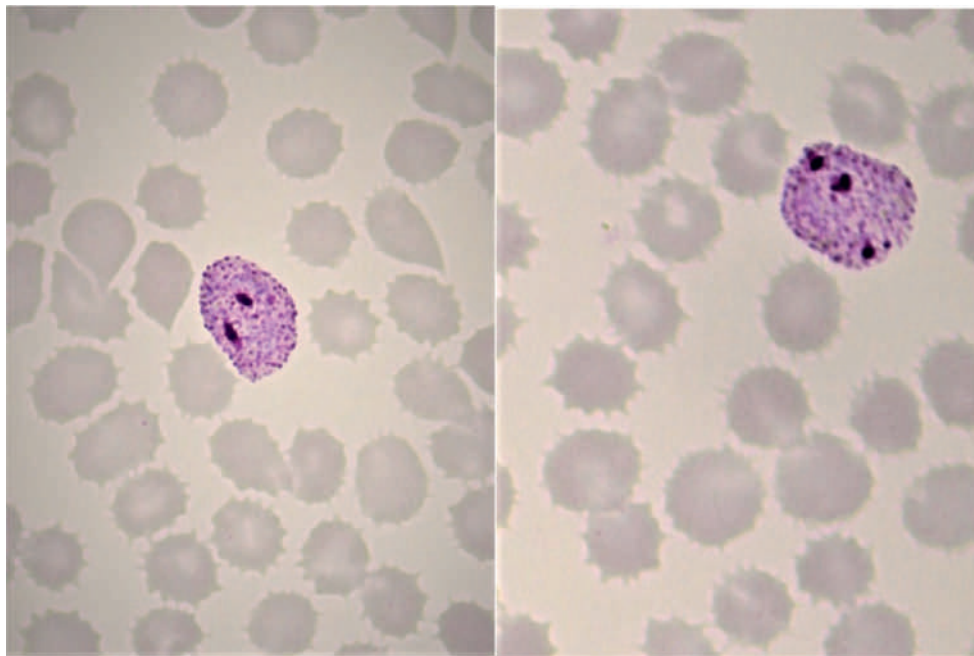


Fig. 2 *Plasmodium ovale* trophozoites.

Plasmodium vivax (Fig. 3)

Plasmodium vivax was long a neglected malaria parasite compared to *P. falciparum* (Carlton et al., 2011). The recent progresses made during the last decade has highlighted the remaining role in malaria transmission of *P. vivax* in areas where *P. falciparum* incidence were significantly reduced. Vivax malaria was classically designed as “benign tertian fever”, but the availability of more powerful methods for species diagnosis showed that vivax malaria can be responsible for severe disease, including fatal outcomes (Picot, 2006; Picot and Bienvenu, 2009; Price et al., 2007). A recent review on its biology indicated that four key words are linked to future challenges on the way toward malaria elimination: relapse, gametocyte, infectivity, short cycle (Adams et al., 2018).

A list of three cells can be used to stratify the *P. vivax* issues: reticulocytes (no culture), hypnozoites (relapse) and gametocytes (transmission). These issues have knowledge and therapeutic consequences that should be addressed.

The prepatent period of *P. vivax* is 7–8 days, followed by erythrocytic cycle of 48 h, leading to the release of 12–16 merozoites (Galinski et al., 2013). Those merozoites invades young red blood cells for both asexual and sexual development. The preferential invasion of reticulocytes or young red blood cells is a factor limiting parasitemia. The parasite create a network of caveola-vesicle complexes (Aikawa et al., 1977; Barnwell et al., 1990) open to the exterior of the red blood cell. It is suspected to uptake or release toxic metabolites such as lactic acid (de Koning-Ward et al., 2016) and to play a major role in the selection of reticulocytes for parasite growth (Galinski et al., 2013). These complexes can be observed by light microscopy after giemsa staining and are named Schüffner's dots (Schüffner, 1899). *Plasmodium* spp. have the capacity to export proteins beyond the plasma membrane (de Koning-Ward et al., 2016).

Gametocytes circulate early in the infection, leading to the possible vivax malaria transmission by mosquito's bite prior to symptoms and treatment (Bousema and Drakeley, 2011). A gametocyte is fully developed in 48 h and remain only 3 days in circulation. But the production of *P. vivax* gametocytes is continuous, by waves at 5 days intervals and last as long as the infection (Galinski et al., 2013).

The hypnozoites stage of *P. vivax* is one of the most intriguing and challenging stage of malaria parasite (Krotoski, 1989; Krotoski et al., 1982; Markus, 2011). After inoculation of sporozoites by the mosquito, trophozoites penetrate in liver cells. Some of these small (4–5 µm) trophozoites will become dormant for long periods of time, up to two years (Galinski et al., 2013; Petersen et al., 2013). The documented knowledge on the determinants and the biology of these hypnozoites is scarce because they are small, almost inert for the host cell, located inside the liver and difficult to reproduce using in vitro models of hepatocytes culture. Hypnozoites are responsible for relapses of *P. vivax*, which are a major challenge for both complete treatment of individual patient and control of vivax malaria transmission (Olliaro et al., 2016). The risk of early relapse is more associated with tropical climate than temperate zones, and it is supposed that the periodicity is triggered by the seasonality of mosquitoes' bites. If mosquitoes are present during the whole year, the periodicity is short. But if mosquitoes are present only during a season, the occurrence of relapse could be less frequent. It seems that parasites are waiting for the availability of mosquitoes to wake up from hypnozoites in the liver, produce gametocyte, and be transmitted. The number of relapses is higher in tropical areas for *P. vivax* because such adaptation to seasonality didn't drive this evolution required elsewhere (Shanks, 2012).

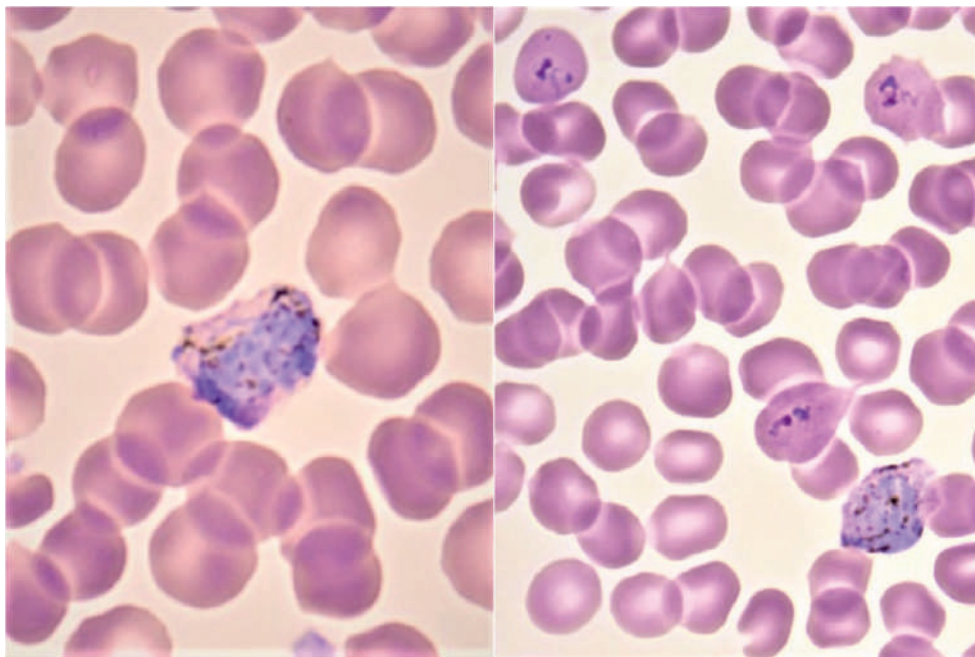


Fig. 3 *Plasmodium vivax* gametocytes and trophozoites.

Plasmodium vivax is preferentially infecting reticulocytes which represents 0.5–2% of circulating red blood cells. Several proteins have been described as essential for merozoites invasion in reticulocytes, including reticulocyte-binding proteins (RBP) and Duffy binding proteins (DBP). But other mechanisms may be involved since Duffy negative red blood cell phenotypes are permissible for *P. vivax* infection (Cavasini et al., 2007; Popovici et al., 2020). *Plasmodium vivax* infected red blood cells have an increased deformability allowing circulation through small capillary vessels and avoiding splenic entrapment (Suwanarusk et al., 2004).

Plasmodium vivax genome was first sequenced in 2008 (Carlton et al., 2008) from the monkey-adapted lab strain Salvador 1. Then samples from patients were also sequenced providing a fantastic material for further analysis. The evidence of the high genetic diversity of *P. vivax* is a matter of concern in the toward malaria elimination (Neafsey et al., 2012).

The drug sensitivity of *Plasmodium vivax* is decreasing in many parts of the endemic areas, especially in tropical areas where chloroquine is easily circulating and is responsible for a pressure of selection. The Pvmdr1 gene has been described (Brega et al., 2005) and its link with drug resistance and therapeutic failure was documented (Barnadas et al., 2008; Marfurt et al., 2008; Suwanarusk et al., 2008).

***Plasmodium (Laverania) falciparum* (Fig. 4)**

Plasmodium falciparum belongs to a different subgenus because of the shape of its gametocytes and their lengthy development (Bray, 1958; Bray and Garnham, 1982). It is clearly the most impactful *Plasmodium* parasite for humans, and thus the most studied. *P. falciparum* schizogony is complete in 5–6 days after sporozoites injection. Only the youngest forms of erythrocytic stages circulate in the peripheral blood, while the development to late trophozoites and schizonts occurs in the microvasculature of the various organs such as spleen, liver, lung, brain and placenta. *Plasmodium falciparum* is responsible for an increased rigidity of infected red blood cells during the maturation process, resulting in sequestration in small vessels and avoiding splenic clearance.

While the export pathways are easily visible in *Plasmodium ovale* and *vivax* species as Schüffner's dots, it does exist in all the *Plasmodium* species, including Maurer's cleft in *P. falciparum*. These structures are membranous organelles involved in the sorting of parasites proteins outside the parasitophorous vacuole (De Niz et al., 2017).

The maturation of *P. falciparum* gametocytes requires 9–12 days for the development of five morphologically distinguishable stages (Hawking et al., 1971). This maturation time is longer than for other species (mainly 24–60 h) and is associated with a longer survival in the blood circulation (several days compared to 12–24 h). Finally, the morphology of *P. falciparum* gametocytes is also exceptional compared to the almost round forms of gametocytes from other species, giving the name of this specie: *falciparum* comes from “falx” and “parere”. The *P. falciparum* gametocytes stages 2, 3, and 4 are sequestered in the bone marrow. Early and late stages (1 and 5) are circulating in the blood.

Interestingly, the first evidence of regulated cell death of malaria parasite was provided from the analysis of dying parasites during in vitro culture (crisis forms) (Meslin et al., 2007; Picot et al., 1997). Accumulative evidence that the parasite is able to regulate its own growth via a selection of the most viable parasites have been obtained (Al-Olayan et al., 2002; Hurd et al., 2006;

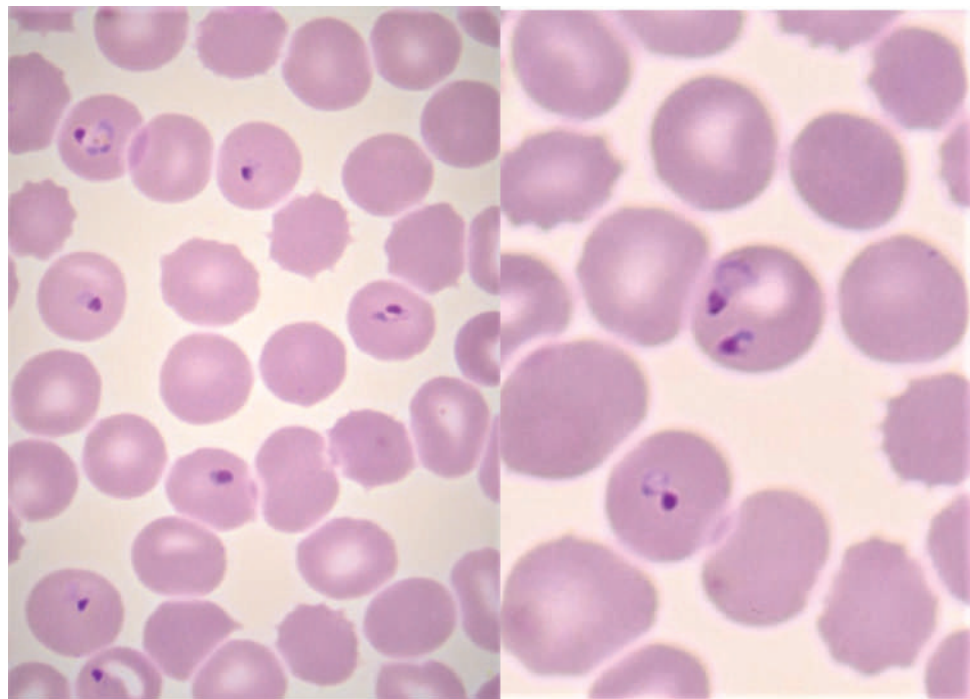


Fig. 4 *Plasmodium falciparum*: ring stage trophozoites.

Meslin et al., 2011a,b; Sow et al., 2015). These observations opened the way to innovative approaches for killing this parasite (Raj et al., 2020).

Plasmodium falciparum is by far the most frequent and deadliest human parasite. It was extensively studied since its first description and a huge number of references is available for reader (Althoff and Abramson, 2020; Gibbons et al., 2018; Jabeena and Rajavelu, 2019; Julien and Wardemann, 2019; Snow et al., 2017).

Plasmodium infecting other animals

Non-human primates

At least 18 *Plasmodium* species (Collins and Aikawa, 1993) were described in non-human primates such as *Macaca* and *Presbytis*, *Hylobates* (gibbons) and *Pongo* (orangutans) (Galinski and Barnwell, 2009). Most of these species show a tertian periodicity except *P. knowlesi* (quotidian) and *P. brasilianum* (quartan). The rhesus monkey (*Macaca mulatta*) is highly susceptible to infection with Asian species, presenting very high parasitemias and a fatality rate over 70%. Aotus and Saimiri monkeys are also very susceptible to *P. knowlesi*, *P. cynomolgi* and *P. fragile*. Among the leucosphyrus group of mosquitoes, *Anopheles dirus* is the most potent vectors of many of non-human primate *Plasmodium*. The link between non-human primate malaria and zoonotic malaria was extensively described (Baird, 2009). More recently, evidence of ape-to-human *Plasmodium* transmission aroused from work conducted in different areas of Central Africa.

Zoonotic malaria

Plasmodium knowlesi

Plasmodium knowlesi may infect humans as shown experimentally in 1932 (Knowles and Gupta, 1932) and observed after natural infection in 1965 (Chin et al., 1965, 1968). The real impact of natural infection in humans has been probably underestimate due to the resemblance of *P. knowlesi* with *P. malariae* under microscope and its proximity with *P. vivax* genome. There is now an increasing knowledge on the role of *P. knowlesi* as a malaria reservoir in Malaysia and in some areas of Southeast Asia and Western Pacific islands (Cox-Singh and Singh, 2008; Divis et al., 2020; Lee et al., 2011). *Plasmodium knowlesi* is the only species responsible for malaria in Brunei and Singapore (Anstey and Grigg, 2019). Surprisingly, *P. knowlesi* was associated with asymptomatic malaria cases in Cambodia, as well as *P. cynomolgi*. But *P. knowlesi* is mainly responsible for mild malaria and severe cases may occur in less than 10% of these cases, leading sometimes to fatal outcome.

Other zoonotic malaria parasites

P. cynomolgi, *P. brasilianum*, *P. inui* and *P. schwezi* were shown to be infectious to humans, inducing severe cases with relatively low parasitemias. Those cases are unfrequently detected, probably because the diagnosis is not suspected or the morphology of parasites is too close: *P. cynomolgi* resemble *P. vivax* and *P. knowlesi* resemble *P. malariae*, for example. A genetic similarity may also be an obstacle to accurate specie diagnosis even using molecular methods. Recently, *Plasmodium simium* was identified in patients living in Rio de Janeiro and infected in the nearby Atlantic forest (Abreu et al., 2019; Brasil et al., 2017).

Avian Plasmodium parasites

The number of bird species is approximately 10,000 in the world, and 55 *Plasmodium* species were reported in 20% of them (Nourani et al., 2020). Four genera of avian hemosporidians have been described: *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, *Fallisia* (Fecchio et al., 2020). *Plasmodium* genus shown 5 sub-genera. *Plasmodium gallinaceum* and *P. relictum* are among the well-known species of avian malaria. The life cycle of birds *Plasmodium* is almost the same than in mammals, while pre-erythrocytic cycle may occur in bone marrow or reticuloendothelial system and erythrocytic cycle in immature or mature red blood cells. The morphological characteristics of avian *Plasmodium* have been extensively reviewed by authors (Fecchio et al., 2020). Culicidae mosquitos (including *Aedes*, *Anopheles* and *Culex*) are frequent vectors to birds. Migrating birds are exposed to different pathogenic species and may spread these parasites to resident hosts, and favor apparition of new zoonotic agents.

Murine Plasmodium

Four *Plasmodium* species naturally infect African rodents: *Plasmodium berghei*, *P. vinckei*, *P. yoelii*, and *P. chabaudi*. These parasites were widely used in experimental infections and were considered as models for biological and therapeutic studies (Yoeli, 1976). However, the relevance of these models for human malaria were subject of debates (White et al., 2010). The distribution of these parasites is limited to the Congo Basin (Boundenga et al., 2019; Carter and Walliker, 1976). Two species, *Plasmodium yoelii yoelii* and *Plasmodium berghei*, developed mainly in reticulocytes, while the other two, *P. chabaudi* and *P. vinckei* invaded mature erythrocytes.

Plasmodium berghei (Vincke and Lips, 1948) (named in honor of Professor L. van den Berghe) was described as a natural infection in the blood of Congo tree rat (*Thamnomys surdaster*). This parasite was then transmitted to laboratory rats and mice. The erythrocytic cycle requires 22–24 h with a preferential invasion of reticulocytes and the schizonts released 6–20 merozoites. The experimental

infection of Congo tree rats led to 20% mortality in 15–19 days, while 80% of animal maintained a fluctuating parasitemia. The mortality after experimental infection in mice is higher and faster, depending on the number of parasites in the inoculum. In 1968, Bafort isolated the ANKA strain *P. b. berghei* (ANKA came from ANtwerpen and KAtanga) which shows an erythrocytic cycle of 43 h, earlier than do other strains (Landau et al., 1970). This strain was widely used as a model of cerebral malaria (Bienvenu and Picot, 2013).

Plasmodium vinckei was first isolated by Vincke, by the River Kinga (Katanga, Democratic Republic of the Congo). *Plasmodium chabaudi* was first described by Landau near Bangui (Central African Republic) in 1965 as well as *P. b. yoelii* isolated in 1965 near the field station of La Maboke in the Central African Republic (Landau and Cbabaud, 1965). *Plasmodium berghei yoelii* was later elevated to a status of a species by Killick-Kendrick in 1975: *Plasmodium yoelii*.

Conclusion

The aim of this review was to up-to-date the basic knowledge on *Plasmodium* parasites biology. It was not to detail malaria as a disease, including epidemiology, symptoms, immunology, treatment and vaccination, neither entomological aspects, which may require a full text-book. Important knowledge gaps are still to be filled with the objective of malaria elimination and eradication. The exact mechanisms used by the parasite to adapt itself to different hosts, to different biological environment, to therapeutic and immunological pressures are not fully depicted. It is clear from the fight against *Plasmodium falciparum* malaria that the “test-treat-track” strategy will not be sufficient to achieve complete elimination, while the disease is under control in many endemic areas (Bienvenu et al., 2019). Basic researches on the metabolism of *Plasmodium*, the regulation of parasite growth, the parasite cell death (Meslin et al., 2007; Picot et al., 1997), the active interplay between parasites and the host in a dynamic perspective, the transmission bottleneck (Meibalan and Marti, 2017) may open new opportunities to fight against the incredible plasticity of this well adapted parasite.

As regards malaria the reader is referred to the review published by White et al. (2014) and for severe malaria to Mer et al. (2019).

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Schistosomiasis

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Background

Schistosomiasis is a neglected tropical disease caused by blood flukes (trematode parasites) belonging to the genus *Schistosoma*. It is a vector-borne disease transmitted by several species of freshwater snails, and is associated with two main clinical forms: intestinal schistosomiasis and urinary (or urogenital) schistosomiasis.

The species responsible for intestinal schistosomiasis in humans are six: *S. mansoni*, transmitted by *Biomphalaria* spp. snails; *S. japonicum*, transmitted by *Oncomelania* spp. snails; *S. mekongi*, transmitted by *Neotricula* spp. snails; *S. malayensis* transmitted by *Robertsiella* spp.; *S. guineensis* and the related *S. intercalatum*, transmitted by *Bulinus* spp. (McManus et al., 2018).

The only species responsible for urinary (or urogenital) schistosomiasis in humans is *S. haematobium*, transmitted by *Bulinus* spp. snails.

S. mansoni, *S. mekongi*, *S. japonicum* and *S. malayensis* affect other mammals in addition to humans. *S. mansoni* is found in rodents and primates, and *S. mekongi* in dogs and domestic pigs, although the role of animal reservoir in sustaining transmission is limited. *S. japonicum* infects a wide range of animals including bovines, dogs, pigs, horses, goats and rodents (more than 40 animal species have been shown to be susceptible); it is considered a true zoonosis as animal reservoir significantly sustain transmission in endemic areas, as in the case of water buffaloes in China. *S. malayensis* mainly infects rodents but can occasionally infect humans (Gordon et al., 2019).

More than 10 other schistosome species are animal parasites; they may occasionally infect humans, but parasitic larvae rarely reach adult stage in the human host.

The causal agent of schistosomiasis was first identified and described by the German physician Theodor Bilharz in 1851, while performing an autopsy at Qasr el-Ayni hospital in Cairo (McManus et al., 2018). The new parasite was initially called *Distomum haematobium* by Bilharz himself, but was renamed *Bilharzia haematobium* in his honor in 1856. Eventually the International Commission on Zoological Nomenclature decided to name the genus *Schistosoma*, in 1858. In reason of the above, schistosomiasis is also known as bilharziasis or, although incorrectly, bilharzia.

Occurrence and epidemiology

Schistosomiasis is a global disease, whose transmission is still reported from a large number of countries. Among the species affecting humans, only *S. haematobium* and *S. mansoni* have a wide geographical distribution (WHO, 2012).

- *S. haematobium* is found in sub-Saharan Africa, northern Africa, the Middle East and Europe (Corsica, France) (Kincaid-Smith et al., 2017; Rothe et al., 2021).
- *S. mansoni* is found in large portions of Africa (mainly sub-Saharan Africa and Nile valley), in the Arabian peninsula (Saudi Arabia, Oman, Yemen), in some South American countries (Brazil, Venezuela, Suriname), and in the Caribbean.
- *S. guineensis* is found in West and Central Africa.
- *S. intercalatum* is found only in the Democratic Republic of the Congo.
- *S. japonicum* is found in China, the Philippines, and in the island of Sulawesi (Indonesia). Despite its name, it is no longer transmitted in Japan, where disease control efforts led to its elimination in the 1970s.
- *S. malayensis* is found only in Malaysia (Chuah et al., 2019).
- *S. mekongi* occurs in limited areas of Cambodia and Laos, along the Mekong river.

Overall, transmission of human schistosomiasis is still reported from or presumed to occur in 78 countries (WHO, 2012, 2021). Of them, 27 countries have low levels of transmission, while the other 51 have moderate or high transmission levels still requiring preventive chemotherapy through mass treatment interventions.

Recently *S. mansoni*, *S. haematobium*, *S. mekongi* and *S. japonicum* cases have been reported from Myanmar (Gordon et al., 2019), and *S. haematobium* (Sah et al., 2020) and *S. japonicum* (Bajracharya et al., 2020) from Nepal. However, these findings require further investigation before being confirmed.

Focal distribution of schistosomiasis even within small geographical areas is well-known. Focal distribution implies that prevalence and intensity of infection may vary significantly, even between two contiguous villages. This is because the cycle of schistosomiasis is highly dependent on the environmental variables such as existence of freshwater bodies, presence of suitable intermediate host snails and human behavioral patterns of contact with water. Such patterns may be related to domestic (e.g. cloth-washing), recreational (e.g. playing and swimming in ponds, lakes and rivers) or professional activities (e.g. fishing). Lack of access to clean water as well as poor sanitation and limited hygiene practices also play a role in shaping epidemiology of schistosomiasis (WHO, 2012; Gordon et al., 2019; Kulinkina et al., 2019).

As a reflection of the above factors, people infected with schistosomiasis may belong to diverse age, gender and professional groups; nevertheless, children of school age, adolescents and adults are usually those harboring the highest levels of prevalence and intensity of infection.

Various indicators are alternatively used to quantify the relevance of schistosomiasis as a public-health problem. Population at risk of infection (i.e. living in endemic areas) is estimated at 750–800 million, while people infected are estimated at more than 200 million (Steinmann et al., 2006). Mortality is estimated at about 24,000 deaths per year (WHO, 2021). The Global Burden of Disease Study 2016 estimated the global burden of schistosomiasis at 2.5 million disability-adjusted life years (DALYs) (WHO, 2018, 2021). Nevertheless, consensus is generally lacking on calculations applied to schistosomiasis because of the limited reliability of available epidemiological information.

To overcome the problem, WHO rather applies an operational definition, i.e. number of people requiring preventive chemotherapy with praziquantel (PZQ). Estimates indicate that in 2019 approximately 236.6 million people required preventive chemotherapy globally, of which 128.3 million school-age children and 108.3 million adults (WHO, 2020). Of them, the vast majority (211.5 million; 89.4%) were living in the WHO African region; 20.6 million (8.7%) in the Eastern Mediterranean region; 2.9 million (1.24%) in the Western Pacific region, 1.6 million (0.7%) in the region of the Americas; only 22,000 in the South-East Asia region; and none in the European region.

Causative agents

The parasites

Schistosomes life cycle implies transformation of the parasite in successive forms: egg, miracidium, cercaria, schistosomulum and adult worm (Sobhon and Upatham, 1990).

Eggs differ in shape and size. They measure $50\text{--}240 \times 35\text{--}85 \mu\text{m}$ and are characterized by a spine whose position and shape varies according to the species and can guide its identification (terminal spine in *S. haematobium*, *S. guineensis* and *S. intercalatum*; lateral in other species). The number of eggs released daily by a female worm varies between 1500 and 3000 according to the species. Each egg contains a miracidium. The egg hatches in the environment and releases the miracidium when optimum temperature and illumination are present.

The miracidium is a ciliated larval stage. Miracidia differ in size and shape according to the schistosome species but not in behavior and morphology. The general morphological feature of the miracidia comprise a hemispheric structure named *terebratorium*, and epidermal plates covered by cilia. The miracidia have a primitive gut, a pair of cephalic penetration glands, a nervous system and an excretory system. Miracidia swim with the cilia to find and penetrate a suitable snail in 8–12 h or die.

Penetration is initiated by the mechanical action of the miracidium's apical papilla which breaks the snail epithelium. It is facilitated by histolytic secretions from the glandular cell. When penetration is complete, the ciliated membrane of the miracidium disappears. Few days after, the miracidium develops into a sporocyst, which subsequently gives rise to thousands of cercariae by asexual reproduction. The period of development from the miracidial penetration to the production of mature cercariae varies according to the species (6–9 weeks).

Cercariae are the freely swimming, short-lived stage of *Schistosoma* spp. They measure approximately 500 µm in length and their emergence from infected snails is characterized by a periodicity which is linked to the water contact habit of the natural host. The peak of cercarial shedding of *S. haematobium* occurs at noon between 10:00 am and 4:00 pm while for *S. japonicum*, it happens at night. The number of cercariae daily produced varies: it is about 200 for *S. haematobium* and 250–600 for *S. mansoni*. In oriental species, it is usually lower, e.g. 15–160 for *S. japonicum*. Cercariae may survive in fresh water up to 72 h (Nelwan, 2019). Cercariae have a forked tail that facilitates their movements. After penetration of the skin of the definitive host, they transform into schistosomula.

After developing to mature worms and pairing, schistosomes migrate to relevant venous districts where eggs laying begins. The period from penetration of the cercaria to the shedding of eggs may range from 6 to 10 weeks. Adult schistosomes measure 6.5–20 mm. The adult male is thicker and shorter than the slender and longer female, and the male has the gynaecophoral canal in which the female is held during copulation.

The snails

Two snail families are involved as intermediate snail host for human schistosomiasis (*Pomatiopsidae* and *Planorbidae*). *Littorinidae*, *Thiaridae*, *Pachychilidae*, *Cerithidae*, *Potamididae* and *Planaxidae* families are involved in the transmission of other schistosomes.

Each of the schistosome species has its characteristic spectrum of intermediate snail hosts. The intermediate hosts of *S. mansoni*, *S. haematobium* and *S. intercalatum* belong to the family *Planorbidae* (Brown, 1994), while those of *S. japonicum*, *S. mekongi* and *S. malayensis* belong to the family *Pomatiopsidae* (Sobhon and Upatham, 1990).

Among the *Planorbidae*, various species of *Biomphalaria* serve as intermediate hosts for *S. mansoni* and certain *Bulinus* species are intermediate hosts for *S. haematobium* and *S. intercalatum* (Brown, 1994). Among the *Pomatiopsidae*, certain subspecies of *Oncomelania hupensis* are known to transmit *S. japonicum*; *Neotricula* spp. transmit *S. mekongi* and *Robertsiella* spp. transmit *S. malayensis* (Sobhon and Upatham, 1990).

Biomphalaria spp. and *Bulinus* spp. are aquatic and hermaphroditic snails which can be found in almost all types of water bodies, ranging from small temporary ponds or streams to large lakes and rivers (Brown, 1994). Artificial water bodies such as irrigation canals and dams are particularly suitable habitats and, because of the intense human–water contact, may play a very important role in transmission of schistosomiasis. Both species are usually found in shallow waters and often in association with aquatic plants; in some of the large lakes they may be found also at considerable depth, e.g. *Biomphalaria* spp. in the East African lakes and *Bulinus nyassanus* in Lake Malawi.

Oncomelania spp. snails are small, amphibious and dioecious. Females tend to be larger than males. Eggs are laid singly on solid objects. Hatching occurs after 10–25 days, depending on temperature, and newly-hatched snails pass through an aquatic stage of 1–2 weeks. Snails reach sexual maturity after 10–16 weeks and may live for 24–35 weeks (Leonardo et al., 2020). *Oncomelania* spp. inhabit flood plains, especially artificial habitats resulting from agricultural development, such as drainage and irrigation channels, roadside ditches, and rice paddies. Snails are found primarily on the banks of flood plains, but more rarely in very shallow waters (i.e. depth less than approximately 20 cm). Habitats preferred by *O. hupensis* are shaded by vegetation, and the temperature is relatively constant and cool. Water currents of above 0.14 m/s are generally unfavorable for *O. hupensis* (Leonardo et al., 2020).

Neotricula aperta snails transmit *S. mekongi*, which primarily infects humans across the central Lao People's Democratic Republic and eastern Cambodia. Three strains of *N. aperta* have been identified on the basis of body size and mantle pigmentation. All three strains are suitable hosts for *S. mekongi* but in nature only the γ -strain is known to be epidemiologically significant. *N. aperta* is found in large rivers in central Laos, Thailand and eastern Cambodia.

The intermediate hosts of *S. malayensis* are species of *Robertsiella* (*Pomatiopsidae*: *Triculinae*), that are very small snails. *R. kaporensis* may be found in small streams attached to rocks, leaves and twigs. *R. silvicola* lives in seepages and springs (Sobhon and Upatham, 1990; Chuah et al., 2019).

Several schistosomes of birds and mammals (e.g. *Trichobilharzia ocellata* (La Valette), *Gigantobilharzia* spp., *Ornithobilharzia* spp., as well as *S. bovis*, *S. margrebowiei* and *S. rhodhaini*) may penetrate human skin; cercariae can cause an allergic reaction (cercarial dermatitis or *swimmers' itch*), but do not progress into further development stages. Exposure of man to such schistosomes mainly occurs in water bodies of temperate climate zones, but may also occur in some areas endemic for human schistosomiasis, thus confusing serodiagnostic techniques.

Several factors determine the distribution and the abundance of the snails. Snail presence is dependent on physical and chemical factors such as hydrogen ion, dissolved oxygen in water, light, the geology (type of soil), temperatures, rain fall and water level, water chemistry (pH, concentration of mineral), presence of food for the snails (algae) (Sobhon and Upatham, 1990; Leonardo et al., 2020).

Mode of transmission and life cycle

Schistosomiasis is transmitted to humans when cercariae shed by infected snail intermediate hosts in freshwater bodies penetrate through the skin; this is the result of direct contact with infested water. Cercariae are attracted to the human host by chemical and physical factors (e.g. temperature). Once inside the host, cercariae lose their tail and develop into immature schistosomula, which enter the venous circulation and reach subsequently the right heart, the lungs, the left heart, the arterial circulation and the venous

portal system, where they mature into separate-sex adults (length ranges from 6 to 28 mm, depending on sex and species). Here adult worms form a male-female pair and migrate to the bladder venous plexus in the case of *S. haematobium* and to the mesenteric veins of the bowel and rectum in the case of all other species. Approximately 5 weeks after infection, female worms start producing eggs in the bloodstream. Eggs eventually reach the bladder lumen (in the case of *S. haematobium*) and the intestinal lumen (all other species) and are excreted in urine and faeces, respectively. When in contact with freshwater, as in the case of defecation or urination in proximity of a pond or stream, eggs hatch and release miracidia (free-swimming ciliated larvae). Miracidia then penetrate a suitable intermediate snail host, develop into sporocysts and further multiply by two asexual reproduction cycles, eventually giving rise to numerous cercariae. Cercariae subsequently emerge from the snail in response to sunlight and contaminate freshwater bodies, thus creating the conditions for contact with the human host and closing the *Schistosoma* spp. life cycle (Fig. 1).

Host–parasite interrelationships

S. japonicum and *S. mekongi* are highly zoonotic species, involving intense transmission between human and animals (Sobhon and Upatham, 1990; McManus et al., 2018; Budiono et al., 2019; Gordon et al., 2019; Leonardo et al., 2020). Rodents and non-human primates are also involved in the transmission of *S. mansoni* (Catalano et al., 2020; Kebede et al., 2020). *S. malayensis* is mainly an animal infection affecting rodents (*Rattus muelleri* and *R. tiomanicus*), but its eggs have been documented in humans, indicating that

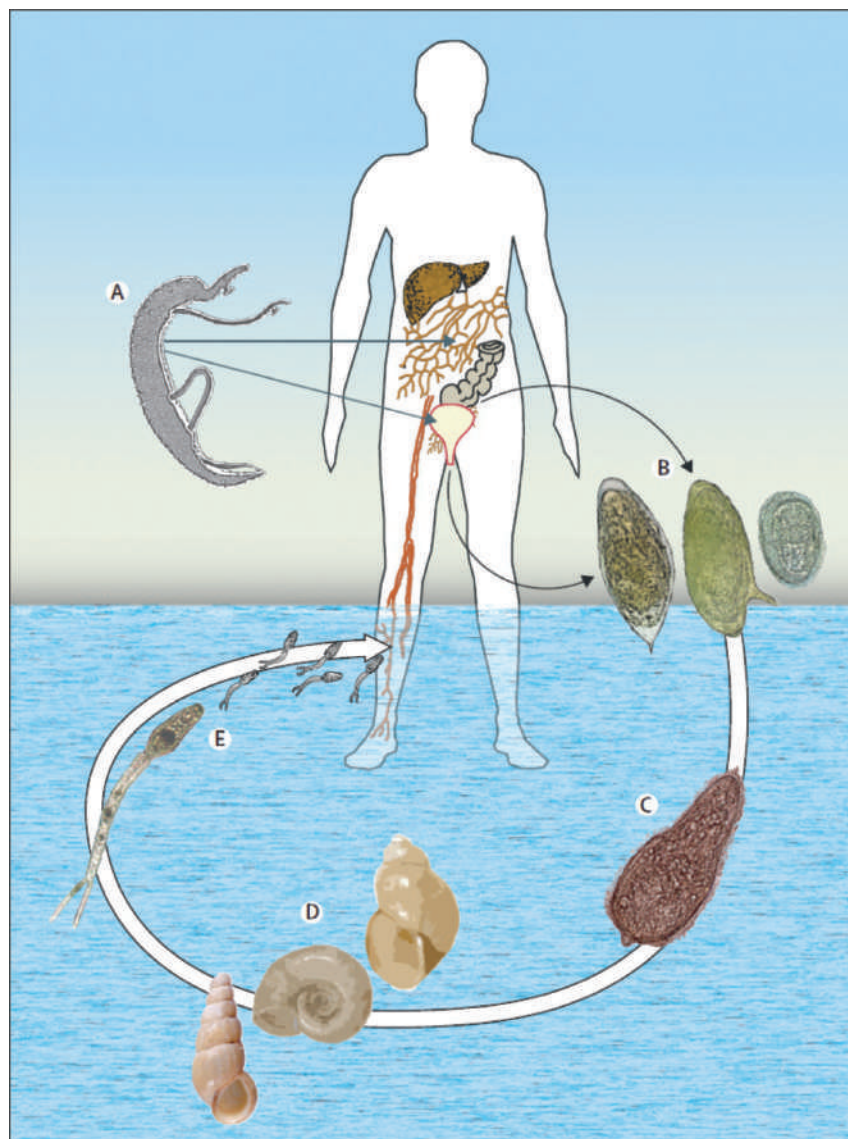


Fig. 1 Life cycles of *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*. (A) Paired adult worms (larger male enfolded slender female). (B) Eggs (left to right, *S. haematobium*, *S. mansoni*, *S. japonicum*). (C) Ciliated miracidium. (D) Intermediate host snails (left to right, *Oncomelania*, *Biomphalaria*, *Bulinus*). (E) Cercariae. (Colley et al., 2014).

the worm can reach sexual maturity in the human host (Chuah et al., 2019). Hybridization between human species such as *S. haematobium* and *S. guineensis* (Moné et al., 2012), and also between human and animal species has been suspected (Brémond et al., 1993), and documented recently in Africa (Webster et al., 2013; Moné et al., 2015; Léger and Webster, 2017; Catalano et al., 2018; Sene-Wade et al., 2018). *S. haematobium* can also hybridize successfully with livestock schistosome species and maintain its capacity to infect humans. Likewise, other hybridized livestock *Schistosoma* species can also infect humans (Moné et al., 2015; Léger et al., 2016; Sene-Wade et al., 2018).

Pathology, clinical manifestations, and phases of development of the diseases

In the human host, adult worms of *Schistosoma* spp. reside in mesenteric and pelvic venules where they copulate. The usual location may vary from species to species: mesenteric and hepatic veins for *S. japonicum*, *S. mansoni*, *S. malayensis*, *S. intercalatum* and *S. guineensis*, the perivesical venous plexus for *S. haematobium*.

Eggs are released by the female worms in the bloodstream and get trapped in contiguous organs, especially the intestine, bladder and genitourinary tract; they may also be carried away by the bloodstream and embolize different organs, typically the liver in the case of intestinal schistosomiasis. Once lodged in the organ's tissue, eggs evoke a granulomatous reaction resulting in inflammation and eventually tissue fibrosis. The chronic inflammation of the organ tissues leads to the progressive translocation of the eggs through the organ walls toward the lumen (intestinal or bladder) where they are finally released, thus being expelled from the human host with urine or faeces and contaminating the environment (Gryseels, 2012).

Granulomatous reactions around eggs trapped in organ tissues are the key events leading to schistosomiasis-induced morbidity. Granulomas are formed by inflammatory cells (e.g. eosinophils, neutrophils, lymphocytes and macrophages) that accumulate around eggs as a response to their presence; active granulomas are progressively replaced by fibrous tissue (Schwartz and Fallon, 2018). The development of morbidity entails therefore the presence of mature adult worms and well-established egg production. The severity of morbidity is proportionate to intensity of infection, i.e. the number of worms infecting a person, that can be measured by proxy in terms of excretion of eggs in stools and urines.

In intestinal schistosomiasis, especially in the case of *S. mansoni*, *S. japonicum* and *S. mekongi*, lesions such as ulcerations, pseudopolyps and microabscesses can be detected in the bowel. From a clinical perspective, this may translate into abdominal pain, blood in stools and altered bowel habits.

Embolization of eggs to the liver determines typical granulomatous reactions and fibrosis along the branches of the portal venous system (periportal fibrosis), eventually leading to portal branch occlusion and consequently portal hypertension and ascites, possibly associated with spleen hardening and enlargement; liver functions however usually remain normal. Severe, advanced cases are characterized by superficial abdominal blood vessel dilatation, spleen enlargement, and esophageal varices that are bleeding-prone and may rupture, with risk of hematemesis and death. No association between the agents of intestinal schistosomiasis and cancer has been demonstrated.

In urinary schistosomiasis caused by *S. haematobium*, polyps and masses protruding in the lumen, and fibrosis of the wall, are common finding. The opening of the ureter in the bladder may be affected by chronic inflammation, thus leading to reflux of urine and eventually to hydronephrosis and hydronephrosis. The main sign associated with urinary schistosomiasis is hematuria, although vaginal bleeding and dyspareunia (pain during sexual intercourse) may be present when the female genital tract is affected, and be caused by lesions in the vulva (e.g. nodules) and the Fallopian tubes; other complications may include early abortion, ectopic pregnancy and infertility. Damage to prostate and seminal vesicles may lead to painful ejaculation and infertility in males. In both sexes, urogenital schistosomiasis is a risk factor for Human Immunodeficiency Virus (HIV) infection, mainly because of physical damage to genital mucosae. A causal relationship has been established between *S. haematobium* infection and the squamous cell carcinoma of the urinary bladder, and in 2009 *S. haematobium* was included in Group 1 (extensively proven carcinogens) by the WHO International Agency for Research on Cancer (IARC) (McManus et al., 2018).

More rarely, eggs may reach other organs, causing ectopic schistosomiasis (Schwartz and Fallon, 2018).

Eggs reaching the cerebral or spinal venous plexus cause inflammatory reactions in the central nervous system. This may give rise to neurological complications, whose clinical manifestations may vary depending on the site and include transverse myelitis, epileptic seizures, encephalopathy with headache, visual impairment, motor deficits and ataxia (reduced coordination of voluntary muscle movements). The overall clinical picture is usually referred to as neuroschistosomiasis (Carod-Artal, 2010).

In pulmonary schistosomiasis eggs reach the lung capillaries and cause granulomas in the perialveolar area. The resulting fibrosis may lead to pulmonary hypertension and *cor pulmonale* (an enlargement of the right ventricle of the heart due to increased pressure in the lung capillaries) (Gryseels, 2012; Knafl et al., 2020).

In addition to chronic pathology as described above, schistosomiasis is also associated with acute pathology. The associated clinical picture is also referred to as "acute schistosomiasis".

A transient cutaneous rash may persist from hours to days at the site of cercarial penetration, especially in the case of people not previously infected, as in the case of tourists to endemic areas. Such cercarial dermatitis is comparable to the *swimmers' itch* occurring in human exposed to animal (usually avian) cercariae.

Another form of acute pathology is the Katayama fever (also called Katayama syndrome or disease), which is a systemic hypersensitivity reaction to schistosome (migrating larvae) and circulating immune complexes, usually occurring weeks to months after a primary infection (infection in non-immune people). Symptoms may include fever (usually nocturnal), cough,

headache, fatigue and myalgia; eosinophilia and transient pulmonary infiltrates (visible on chest radiography) are also common findings. As in the case of the cutaneous rash, Katayama fever is common in tourists and travelers and is infrequently reported in residents of endemic areas (Ross et al., 2007). This is because the conditions determining its pathogenesis almost exclusively occur in individuals exposed to *Schistosoma* spp. infection for the first time, probably in reason of the lack of early-life sensitization, either in utero or during the first years of life, which on the contrary are common in populations living in endemic areas (Gobbi et al., 2020).

In the case of infections by *S. japonicum*, however, Katayama fever may occur in people living in endemic areas and with a history of previous infections, as in the case of the endemic area located in Katayama district, Hiroshima prefecture (Japan), where it was first described in the 19th century (Ishii et al., 2003). In *S. japonicum* infections, Katayama fever may be associated with severe manifestations including persistent fever, diarrhoea, abdominal pain, hepatomegaly, and generalized rash (Gryseels, 2012; McManus et al., 2018).

In acute schistosomiasis, eggs are usually not found in urine or stool samples, and serological tests or PCR may help in establishing a diagnosis. As worms are still juvenile, treatment with praziquantel will have no effect; symptomatic treatment including use of corticosteroids should be considered. Artemisinin appears to be effective against schistosomula (Utzinger et al., 2000). 4–5 weeks later the patient should be examined for schistosome eggs and, if found positive, treated with praziquantel.

Immune response, immunopathology, and immunity

The interaction between the host's immune system and schistosomes is complex. It involves the different stages of the life cycle of the parasite, which may be present in the body of the host (cercaria, schistosomula, adult worms and the parasite eggs). Immune responses against cercariae and possibly other parasite stages are important for development of resistance to reinfection, whereas responses toward egg antigens are responsible for the pathology in schistosomiasis (McManus et al., 2018).

Protective immune responses against schistosomes develop slowly over a period of 10–15 years, and children of <10 years of age in schistosomiasis-endemic areas are susceptible to reinfection after treatment of the schistosome infection, whereas adults are usually resistant. This observation may also explain the characteristic age-prevalence and age-intensity curves observed in schistosomiasis endemic areas (McManus et al., 2018).

Following infection, cercariae develop into schistosomula which migrate through the host body, eliciting a type 1 (cell-mediated) immune response characterized by increased release of interleukin (IL)-12 and interferon (IFN)- γ . This response persists for a few weeks, and is mainly targeted at worm antigens. However, as soon as schistosomula mature into adult worms and start to produce eggs (5–6 weeks after infection), the immune response shifts toward type 2 (antibody-mediated). Type 2 immune response is characterized by decreased production of IFN- γ , expansion of CD4+ T helper (Th) 2 cells, eosinophils, and basophils, increased production of IL-4, IL-5, and IL-13, and an isotype switch toward IgG1 and IgE (Schwartz and Fallon, 2018). Unregulated production of IL-13 is specifically responsible for liver fibrosis (Colley and Secor, 2014). During the chronic phase of infection (> 3 months), the magnitude of the Th2 response decreases, which coincides with a reduction in granulomatous inflammation around eggs, while regulatory T and B cells emerge leading to a state of immune hyporesponsiveness (Schwartz and Fallon, 2018).

Chemotherapy with PZQ affects the development of individual protective immunity. PZQ kills adult schistosome worms, thereby providing passive immunization through an antigenic stimulus delivered as adult worms die (Oettle and Wilson, 2017). Treatment with PZQ has also been shown to alter the relationship between human host's age and antibody response (which reflects age-related exposure to parasite antigens and the age-related acquisition of resistance to infection) (Mutapi et al., 2003).

Antigens released by schistosomes damaged by PZQ treatment accelerate the acquisition of an immune response which confers resistance to reinfection (Mutapi et al., 1998). This protective immunity may also lead to reduced egg production in infected individuals, a phenomenon observed in *S. haematobium* (Wilson et al., 2014), but not seen for *S. mansoni* infections (Agnew et al., 1996).

Control: Diagnoses, prevention, and treatment

Diagnosis

Detection of parasite eggs in the stool and urine is the gold standard for diagnosis of schistosomiasis.

For diagnosis of infection with *Schistosoma* spp. responsible for intestinal schistosomiasis, stool samples can be examined for the presence of parasite eggs. The Kato-Katz thick smear is the most frequently employed technique, especially in population-based surveys implemented for mapping or monitoring and evaluation purposes. Although the Kato-Katz has low sensitivity if only one stool sample from each subject is examined, its sensitivity can be increased by multiple stool sample examinations from the same individual (Utzinger et al., 2015; McManus et al., 2018). However, multiple examinations are not often carried out as this makes the technique impractical for wide-scale field diagnosis.

Concentration techniques for stool samples have been developed and evaluated. Examples of these tests are the Merthiolate-Iodine-Formaldehyde concentration technique (MIFCT), the Merthiolate-Formaldehyde concentration technique (MFCT), and the Formaldehyde-Ether concentration technique (FECT, formol-ether). Sedimentation techniques such as McMaster, or flotation techniques such as FLOTAC and Mini-FLOTAC (Barda et al., 2014; Allam et al., 2020) can also be employed. These tests, however, are time consuming, laborious to perform, and also impractical for field-based screening.

For urogenital schistosomiasis, examination of urine samples using a filtration technique and microscopy to detect *S. haematobium* eggs was the basis of early studies on this species of schistosome and is still widely used for population-based surveys implemented in endemic countries for mapping or monitoring and evaluation. Various urine concentration techniques have been developed to increase sensitivity of the test.

For people from non-endemic areas or living in low transmission areas, serological and immunological tests may be useful to show exposure to infection and highlight the need for thorough examination and treatment. In an elimination scenario, these tests may also have a role in showing decline of transmission and eventually demonstrating its interruption (Utzinger et al., 2015; Hinz et al., 2017; McManus et al., 2018).

Methods to detect schistosome antigens in the body fluids of infected individuals have been developed. An example of these methods is the use of monoclonal antibodies to detect schistosome antigens that are circulating in the blood and excreted in the urine. The most promising and extensively studied are tests that can detect two proteoglycans regurgitated by adult worms in the bloodstream, the Circulating Anodic Antigen (CAA) and the Circulating Cathodic Antigen (CCA) (Utzinger et al., 2015; Danso-Appiah et al., 2016; Bärenbold et al., 2018). Detection of CAAs or CCAs is an indication of active infection. Commercially available CAA/CCA kits based on enzyme-linked immunosorbent assay (ELISA) or monoclonal antibody techniques have shown excellent sensitivity and specificity, and have been used to detect both *S. mansoni* and *S. haematobium* infections in urine and serum samples. A point-of-care (POC) urine assay test for detection of *S. mansoni* CCA in urine sample is also commercially available.

In high-resource settings, PCR-based detection of parasite DNA in stool or urine samples is also employed (Utzinger et al., 2015; McManus et al., 2018).

Tests for the detection of antibodies (e.g. Circumoval Precipitin Test [COPT] and Indirect Hemagglutination Assay [IHA]) are available but their use in regular practice is nowadays limited, notably as they cannot distinguish present and past infections, although they may still have a role in the case of travelers returning from endemic areas. In addition, they have been used to demonstrate absence of exposure to *Schistosoma* spp. in younger age groups living in formerly endemic areas, as a proxy for interruption of *Schistosoma* spp. transmission.

For detection of organ pathology, chest radiography can reveal pulmonary infiltrates in acute schistosomiasis, while imaging methods, such as ultrasound, computed tomography scan (CT scan), and Magnetic Resonance Imaging (MRI), can demonstrate evidence of chronic pathology such as liver fibrosis and portal hypertension in intestinal schistosomiasis, and urinary tract fibrosis, polyps, and ulcers in urinary schistosomiasis.

In endemic settings, ultrasound is often the only imaging resource and can be used both in clinical settings and in the context of population-based screening exercises, thanks to the availability of portable ultrasound machines; the use of abdominal and pelvic ultrasonography for the assessment of schistosomiasis-related morbidity of the liver and the urinary tract, respectively, has been standardized by WHO through the so-called Niamey protocol (Richter et al., 2016).

Testing for microhematuria (invisible, abnormal presence of blood in urine) using a reagent strip test is also commonly used in population-based surveys to estimate prevalence of *S. haematobium* infection or burden of morbidity in endemic areas. These surveys have been especially carried out among school-aged children, as in this population other causes of hematuria can usually be excluded, and therefore a correlation can be established between presence of blood in urine and *S. haematobium* infection (Utzinger et al., 2015).

Malacological techniques have largely been employed to detect *Schistosoma* spp. infection in the intermediate host snail, as a mean to demonstrate presence or absence of transmission in a given freshwater body. Traditionally, collection of intermediate host snails was followed by incubation or crushing of the snails to detect shedding of schistosome cercariae or presence of sporocysts, respectively. Such techniques are gradually being replaced by PCR-based methods or loop-mediated isothermal amplification (LAMP) assays that can detect prepatent or patent infection by demonstrating presence of sporocysts and cercariae in intermediate host snails, respectively. Recently, detection of DNA in the environment (eDNA; Alzaylaee et al., 2020) has been evaluated in some countries, and proven to be a promising technique for determining absence of schistosomes or their emergence. Malacological techniques are playing a growing role in demonstrating interruption of *Schistosoma* spp. transmission and therefore elimination of schistosomiasis from an endemic area.

Treatment

PZQ is an anthelmintic introduced in 1979; it is currently the treatment of choice against all the species of schistosomes affecting humans. WHO's recommendations are as follows: for adults and children over 4 years, by mouth, 40–60 mg/kg (Olliaro et al., 2011) as a single dose or using a dose pole, which calculates number of tablets according to the height of the individual (Montresor et al., 2005). Adverse events following treatment are usually minor and transient, although relatively frequent, especially in individuals harboring infections of heavy intensity. PZQ is marketed under different commercial names and the usual formulation is 600-mg tablets.

In the past, several antischistosomal pharmacological agents have been employed. Their use is nowadays rare because of lower efficacy and/or higher toxicity as compared to PZQ. Examples include hycanthone, metrifonate and oxamniquine against *S. haematobium* or *S. mansoni*, and niridazole and antimonials against *S. japonicum* (Kramer et al., 2014; Siqueira et al., 2017).

Chemotherapy aims at reducing and preventing burden of infection and associated morbidity, but does not prevent reinfection, although it has the potential to affect transmission at the community level through reduced egg output.

By decreasing the number of worms harbored by an individual (intensity of infection), chemotherapy reduces organ inflammation and pathology, and improves associated conditions such as anemia by limiting loss of blood in urine and stool

(WHO, 2006; Mduluzi et al., 2020). Through the same mechanism, chemotherapy can prevent the further development of chronic morbidity. Some authors have claimed that administration of PZQ is able to reverse or resolve existing organ fibrosis, but this finding requires further investigation (Nono et al., 2020).

Advanced stages of the disease cannot be treated by PZQ. Surgery may bring relief in severe cases of intestinal schistosomiasis, notably in case of hepatosplenic complications, through procedures such as ligation of esophageal varices, and porta-caval shunt surgeries. In urinary schistosomiasis, reconstructive surgery may be required in case of affected ureters. Ablative surgery, antineoplastic chemotherapy and radiotherapy are employed in the management of bladder cancer caused by schistosomiasis.

Prevention and control strategies

Preventive chemotherapy

Mass treatment with PZQ is the mainstay of WHO's strategy for public health control of schistosomiasis (WHO, 2006, 2012). Single-dose PZQ is administered at regular intervals to populations living in endemic areas, with a focus on children and young adults, among whom the highest levels of prevalence and intensity of *Schistosoma* spp. infections are usually found. Treatment, extended to wider population groups, should the program aim at decreasing and interrupting transmission. Frequency of treatment varies according to transmission levels and is usually 12–24 months.

The rationale of preventive chemotherapy lies in the fact that although a single administration would not kill 100% of the worms (especially in the case of individuals who have high-intensity infections), treatment at regular intervals will keep intensity of infection at low levels, thus preventing the development of morbidity; in addition, reducing the average number of worms harbored by infected individuals would consequently decrease the amount of eggs discharged in faeces and urines, thus reducing environmental contamination and impacting on overall transmission rates in the target area (Gabrielli et al., 2011).

Mass administration of PZQ to children under 4 years is challenging because of the lack of safety data in younger population groups and of the large size and bitter flavor of the tablet which may increase risk of choking (Kernell et al., 2018). Pediatric formulations of PZQ (Coulibaly et al., 2017) are under development to fill this gap and enable extending the target of mass treatment interventions, also considering that evidence has shown that very young children may already be infected and contribute to sustaining transmission (Montresor and Garba, 2017). Another challenge is associated with the continuous and extensive use of the drug, which may lead to PZQ-resistant strains of schistosomes (Crellen et al., 2016).

Snail control, access to water and sanitation and environmental management

Before the introduction of PZQ, snail control was widely used in schistosomiasis programs, with the aim of decreasing the population of snail intermediate hosts and the number of cercariae in water bodies, thus preventing the completion of the parasite's cycle, and limiting the chances of transmission to humans.

The release of chemicals, such as niclosamide, directly into water, is still the most commonly used approach to snail control and has been implemented in many endemic areas (King et al., 2015). Other approaches include the use of biological and ecological measures, including snail predators such as fish and crustacea (Lardans and Dissous, 1998).

Over the past 40 years, in many parts of the world, snail control programs have been gradually discontinued and competencies faded away, while the strategic focus moved toward preventive chemotherapy. Recently, however, WHO has recommended a multi-pronged approach and reintroduced snail control as an important public-health measure to complement mass treatment and sustain the public health impact of this core intervention. Snail control is specifically recommended as an essential intervention where the impact of preventive chemotherapy alone is less substantial than expected, and where low levels of transmission have been achieved and the public health goal moves from control of morbidity to interruption of transmission (WHO, 2017). This reflects the global commitment to elimination of schistosomiasis affirmed through the adoption of World Health Assembly resolution WHA65.21 (2012).

Snail control is part of wider efforts aimed at modifying the environmental conditions in which schistosomiasis thrives, namely through water supply and adequate sanitation, and environmental management such as removal of vegetation on which snails feed, lining canals with cement, or draining water bodies.

Health education

Health education and health promotion messages have an important role in prevention of schistosomiasis, notably in endemic countries (Price et al., 2015; Person et al., 2016). Primary preventive messages focus on discouraging unsafe water practices (e.g. bathing in contaminated ponds) and avoiding indiscriminate urination and defecation (to decrease environmental contamination). Secondary prevention encourages compliance with disease control interventions, notably mass treatment with PZQ. Social mobilization and sensitization on the public-health impact of schistosomiasis, its transmission cycle and the ways and means to tackle its burden are key activities that are regularly implemented in endemic communities.

Vaccines

Several vaccine candidates against schistosomiasis have been the subject of investigations over the past 40 years and have reached different stages of development; some of them are currently in various phases of human clinical trials (Tebeje et al., 2016). Candidate vaccines can be aimed at preventing morbidity (e.g. by eliciting protective immune responses that would lead to reduced worm burdens and decreased egg production) and/or infection (e.g. by limiting transcutaneous penetration of the cercariae).

The limited understanding of the complex interactions between the human host and parasites, including the mechanisms behind protective immunity has so far hampered the development of an effective vaccine for schistosomiasis. In principle, availability of a vaccine for animals would accelerate the reduction of the zoonotic transmission of the diseases (You et al., 2018).

The two most advanced vaccines which have undergone trials in humans are the Sh28GST and the Sm14. Both vaccines aim first to reduce morbidity due to schistosomiasis by decreasing fecundity of the worms, and secondarily to prevent reinfection. Administration of PZQ at the same time as the vaccine is recommended to boost the immune system and increase the production of antibodies. The Sh28GST vaccine went through human trials (Phase I to III). The phase III trial was conducted in an area endemic for *S. haematobium* in Senegal, and has shown no efficacy of the vaccine in protecting from reinfection (Riveau et al., 2018). Phase II trials of the Sm14 vaccine were conducted in Senegal in 2017 and 2018, and have shown a good tolerance and immunogenicity to both *S. haematobium* and *S. mansoni* (Tendler et al., 2018).

Due to the lack of an effective vaccine, vaccination is not currently included among the strategic approaches recommended by WHO to control and eliminate schistosomiasis.

Chemoprophylaxis

PZQ cannot be used for chemoprophylaxis because of its short half-life (1–1.5 h) and because it cannot kill schistosomula, the migrating larvae that are 3–21 days old. Artemether, a derivative of artemisinin which comes from the leaves of the medicinal plant *Artemisia annua*, is commonly used for the treatment of malaria, and has been shown to be effective against juvenile schistosomes during the first 21 days of infection in animals and humans (Elmorshedy et al., 2016). Accordingly, it has been used as a chemoprophylactic for high-risk groups, such as flood relief workers and fishermen, in areas where schistosomiasis is endemic. Although tested in several countries and against various *Schistosoma* spp. (Pérez del Villar et al., 2012), chemoprophylaxis with artemether is not commonly used outside China.

Treatment of animal reservoir

A wide range of mammals can be infected with schistosomiasis (Sobhon and Upatham, 1990; McManus et al., 2018; Gordon et al., 2019). The role of animals in sustaining transmission in endemic areas is debated. Bovines, however, and particularly water buffaloes (*Bubalus bubalis*), have been shown to play a major role in the transmission of *S. japonicum* in China. Periodic treatment of bovines with PZQ, managed by local veterinarians, is therefore often included in control programs for *S. japonicum* in that country (Li et al., 2015).

Progress and perspectives

In 2019, 105.4 million people received preventive chemotherapy with PZQ globally. Of them, 86.3 million were school-age children, and 19.1 million adults. Coverage among school-age children was 67.2%, while among adults it was 17.7%. Overall coverage was 44.5% (105.4 million out of 236.6 million). Treatments in the African region represented 86% of all those delivered globally (WHO, 2020).

In addition to scaling up treatment for populations in need, progress has also been made in several countries toward elimination as a public-health problem and elimination (interruption of transmission).

Elimination of schistosomiasis as a public health problem is defined by WHO as <1% proportion of heavy-intensity schistosomiasis infections in the population of the geographical under consideration, and has been achieved in several countries and areas (WHO, 2012, 2021).

Elimination of schistosomiasis is equivalent to its interruption of transmission, i.e. reduction of incidence of infection to zero. This achievement has been claimed by some formerly endemic countries on the basis of the absence of detection of cases by the surveillance system and of the results of population-based surveys in areas previously known to be endemic.

As of 2021, WHO lists several countries and territories in the categories “Interruption of transmission to be confirmed” and “Status of transmission to be determined” (Fig. 2; WHO, 2021): the health authorities of these countries usually consider their nations as free of transmission.

The 19 countries where interruption of transmission has to be confirmed include Algeria and Mauritius in the African region; Antigua and Barbuda, Dominican Republic, Guadeloupe, Martinique, Montserrat and Puerto Rico in the region of the Americas; Djibouti, Islamic Republic of Iran, Jordan, Lebanon, Morocco and Tunisia in the Eastern Mediterranean region; Turkey in the European region; India and Thailand in the South-East Asia region; Japan and Malaysia in the Western Pacific region.

The 7 countries where the status of transmission has to be determined include Saint Lucia and Suriname in the region of the Americas; and Iraq, Libya, Oman, Saudi Arabia and the Syrian Arab Republic in the Eastern Mediterranean region.

The list is closed by China (in the Western Pacific region), which is currently listed as endemic but not requiring preventive chemotherapy, bringing the number of countries with low levels of transmission to 27.

The new road map for neglected tropical diseases 2021–30 was endorsed by the Seventy-third World Health Assembly on 13 November 2020, through the adoption of decision WHA73(33), and officially launched on 28 January 2021. The road map lists schistosomiasis among the diseases targeted for elimination as a public health problem. The aim is to have all 78 countries still reporting schistosomiasis or where transmission is presumed to occur validated as having achieved this target by 2030 (WHO, 2021). WHO is currently in the process of establishing a formal acknowledgment process, in line with similar initiatives for other neglected tropical diseases.

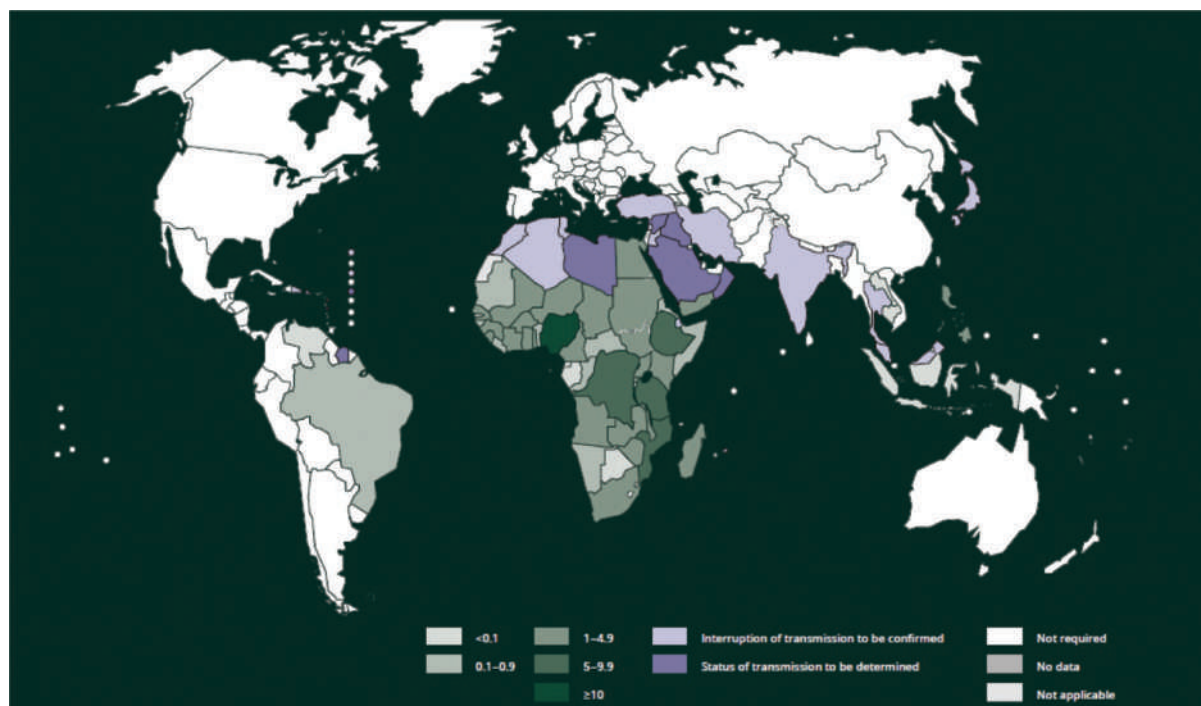


Fig. 2 Status of countries and territories endemic for schistosomiasis, and proportion (%) of global population requiring preventive chemotherapy, 2019 (WHO, 2021).

Critical actions to achieve the above target set by the road map include implementing effective interventions, such as extending preventive chemotherapy to all populations in need by securing access to the necessary medicines; improving the arsenal of diagnostic tests, especially those suitable to low-transmission areas; creating cross-sectoral governance mechanisms to coordinate the fully-fledged implementation of all strategic approaches; and ensuring availability of sufficient resources to enable the above steps (WHO, 2021).

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Strongyloides

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Introduction

Soil-transmitted helminths belonging to the genus *Strongyloides* (*Strongyloides fuelleborni* and the more prevalent *Strongyloides stercoralis*) are currently believed to infect an estimated 30–100 million people worldwide.

Infection caused by *Strongyloides* is the most neglected of neglected tropical diseases, despite that the global burden is significant (Olsen et al., 2009).

The disability-adjusted life year (DALY) estimation is complicated for *Strongyloides* infection, because of the absence of any clinical markers that can be easily monitored during the follow-up and the evident limitations in identifying the uninfected control groups with the poor sensitive direct diagnostic tools available (Krolewiecki et al., 2013).

The most severe effects of strongyloidosis occur in patients receiving immunosuppressive therapy with corticosteroids; disseminated infection may lead to life-threatening conditions with pneumonia, septicemia and intestinal obstruction with a mortality rate of 98% (Olsen et al., 2009).

Systematics

The genus *Strongyloides* (nematoda: Rhabditida) comprises more than 50 gastrointestinal parasitic nematode species. In humans, *S. stercoralis* is the major causative agent of strongyloidosis. Rarer human-infecting species of *Strongyloides* are the zoonotic.

S. fuelleborni (*fuelleborni*) subsp. *fuelleborni* and *S. fuelleborni* subsp. *kellyi*, for which the only currently known host is humans (CDC, DPDx homepage). While *S. stercoralis* has a cosmopolitan distribution, mainly in tropical and sub-tropical regions, human infection with *S. fuelleborni fuelleborni* only occurs sporadically in Africa and the Southeast Asian country of Papua New Guinea (Korenaga and Bruschi, 2014). Recently, a molecular phylogenetic analysis using nuclear and mitochondrial markers indicated the presence of two distinct lineages of *S. stercoralis* (referred to as type A and type B), respectively clade I and clade II, subdivided in different haplotypes groups. While type A parasites were isolated both from humans and dogs in different endemic countries, type B parasites were found exclusively in dogs. The 18S rDNA hyper-variable region I (HVR-I) analysis differentiated human and dog strains (Nagayasu et al., 2017).

Life cycle

The life cycle of *S. stercoralis* is unique, alternating between free-living and parasitic cycles and involving autoinfection (CDC, DPDx homepage). A range of vertebrate hosts, including humans and dogs, become infected when free-living infective larvae (L3i) penetrate the skin, facilitated by their dispersal and nictation behaviors (Little, 1966). The larvae migrate into the lungs via the bloodstream, pass the capillary walls, reach the alveoli, bronchus and trachea, where they are swallowed and end up in the small intestine. The larvae molt twice and become adult female worms. The sizes are 2.1–2.7 (2.4 in average) mm in length and 30.0–40.0 (37.0) mm in width. In the small intestine submucosa the females produce eggs via parthenogenesis, from which rhabditiform larvae (L1i) hatch, pass through the gut of the host with feces and develop in the external environment.

Streit (2008) reviewed the literature on the modes of reproduction and development of *Strongyloides*. Parthenogenesis, probably, is the most common mode of reproduction in parasitic females of *Strongyloides*. Cytological studies revealed that no meiosis occurred during oogenesis in the parasitic females; Viney (1994) demonstrated that all individuals of the progeny were genetically identical, resulting in a mitotic reproduction.

The development of the L1i can take two alternative life cycles: indirect (heterogonic) and direct (homogonic). In the indirect cycle L1 larvae develop via L2–L4 to free-living male and female adults, reproduce offspring and release L1i which molt into filariform L3 (infective stages). Infective L3 stages are long lived and persist in the environment until they encounter a suitable host (Little, 1966). In the direct cycle rhabditiform larvae (female) have the alternative fate to molt twice via an L2 into infective L3i. L3i are threadlike in shape (filariform), 490–630 (563) mm in length, and 15–16 (15.8) mm in width in *S. stercoralis*.

Genetic background of *Strongyloides* isolates and host factors affect the sex ratio and the homogonic versus heterogonic switch. Temperature and host immune response affect the two alternative developmental switches. It has been demonstrated that the increase of the host immune response induces females to undergo the heterogonic development. Matoff (1936) and Graham (1939) demonstrated also a seasonal dependent variation in the life-cycle 'choice,' such that in summer both development cycles occurred, whereas in winter only the homogonic cycle occur. The ratio of homogonically to heterogonically developing females was demonstrated to be altered during experimental transfer to different host species in *S. stercoralis* and *S. fuelleborni*. Interestingly, Crook et al. (2005) showed a reduction of the proportion of free-living adults of both sexes during the transfer of *S. ratti* into the atypical murine host, acting as non-immunological stressor.

Autoinfection is possible when L1 larvae transform within the intestine into L3i (Korenaga and Bruschi, 2014). They penetrate either the intestinal mucosa (internal autoinfection) or perineal skin area (external autoinfection), complete their maturation in adults and give rise to a generation of worm females.

Interestingly, *S. fuelleborni* produces eggs which are shed in the feces, although hatched rhabditiform larvae may be found if fixation and processing are delayed. They are slightly ovoid (50–60 × 30–40 mm) with a thin, colorless shell, and are passed partially embryonated.

Strongyloidosis has been considered potentially zoonotic because dogs may also serve as definitive hosts; some authors supported the idea of a possible cross-transmission of *Strongyloides* between humans and dogs (Shoop et al., 2002; Thamsborg et al., 2017), whereas studies from Takano and collaborators excluded any relationship between human and canine strongyloidosis in endemic areas. In dogs, an eventual transmammary transmission has been demonstrated for several species of *Strongyloides*, except for *S. stercoralis* (Takano et al., 2009).

The life cycle of *S. stercoralis* is shown in Fig. 1.

Immune response and pathogenesis

S. stercoralis L3i (infective larvae) are shown to find and infect hosts using specific host-emitted olfactory, thermosensory and gustatory cues. The most attractive component is urocanic acid, a histidine metabolite particularly abundant in mammalian skin, whose activity may be inhibited by metal ions (Safer et al., 2007). A variety of metalloproteinases play important role in tissue penetration, immune evasion, disruption of host blood coagulation cascades (Rajamanickam et al., 2016). Hystolytic metalloprotease secreted by L3 larvae catalyze the degradation of dermal extracellular matrix facilitating the rapid migration (McKerrow et al., 1990). An astacin-like metalloproteinase, strongylastacin, is demonstrated to elicit specific IgE antibody in *S. stercoralis* infected humans (Varatharajulu et al., 2011). Recently, significant advances in studies of host seeking have been made possible by the application of CRISPR-associated protein 9 (CRISPR/Cas9) technology to molecular components of sensory neuronal signaling; the knockout of Ss-TAX-4 gene, highly conserved in sensory neurons of nematode, was shown to affect the normal thermotaxis in *S. stercoralis* (Bryant et al., 2018). The host innate and adaptive immune response play a critical role in the maintenance of chronic strongyloidosis and the prevention of hyperinfection syndrome and dissemination (Weatherhead and Mejia, 2014). During primary infection, the recruitment of neutrophils, eosinophils and macrophages to the parasite microenvironment result in release of myeloperoxidase (MPO) from neutrophils and major basic protein (MBP) from eosinophils, which are toxic to larvae, and activation of complement component C3b to kill the pathogen (Bonne-Année et al., 2011). The transition from the innate to the adaptive response requires the capacity of eosinophils to act as APC (antigen presenting cell), presenting parasite antigens to T cells, resulting in the activation of Th-2 response, which is protective against helminth infections. Th-2 cells produce cytokines including IL-4 and IL-5. IL-4 activates B lymphocytes to class-switch for production of IgE and IgG4 antibodies, whereas IL-5 is essential for differentiation, proliferation of eosinophils.

Thymic stromal lymphopoietin (TSLP) has been considered important for initiating innate and adaptive responses (Ziegler and Artis, 2010). TSLP is one of the tissue factors mainly expressed by epithelial cells at barrier surface. After helminth infection and disruption of colonic epithelium caused by penetration and migration of the *Strongyloides* larvae through the dermis, TSLP acts promoting and regulating the Th-2 cytokine response, dendritic cells and the effector functions of CD4 + Th-2 cells, basophils and granulocyte populations. TSLP was shown to promote protective immunity to *Trichuris muris*, *Nippostrongylus brasiliensis*, *Heligmosomoides polygyrus*, and *Schistosoma mansoni* in mice, but the role in protective immunity to *S. stercoralis* still remains uncertain (Ziegler and Artis, 2010). Further investigations have demonstrated that TSLP play an important parasite specific role in the worm expulsion during *T. muris* and *T. spiralis* infection, whereas its deficiency does not impair type 2 cytokine response following infection with *S. mansoni* (Inclan-Rico and Siracusa, 2018).

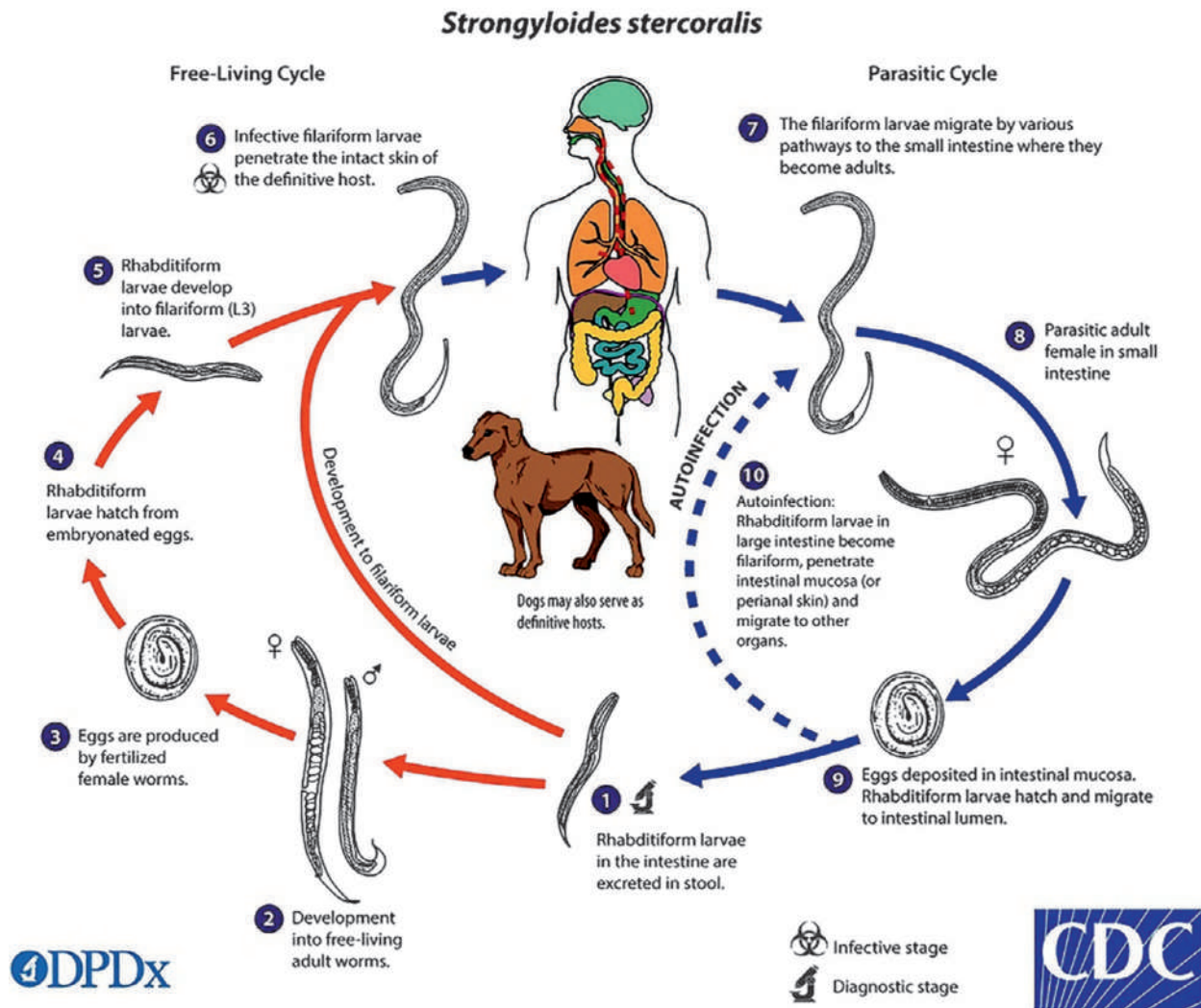


Fig. 1 The life cycle of *S. stercoralis*: parasitic and free-living cycle. From <https://www.cdc.gov/dpdx/strongyloidiasis/index.html>.

The molecular and cellular mechanism remain topics of considerable debate and controversy. Most of the evidence suggests that epithelial cells make important contributions toward Th-2 differentiation (Artis et al., 2008; Zhu et al., 2010).

Trefoil factor 2 (TFF2), produced by epithelial cells, has a critical role during larval migration through the lungs (Wills-Karp et al., 2012). In the lungs, TFF2 triggers the release of IL-33, a type 2 inflammation regulator, from lung epithelia within 24 h after infection. IL-33 activates innate cells including lymphoid cells, eosinophils and DCs. The DC stimulation of TH2 cells induces an amplified production of IL-4, IL-5 and IL-13, which drive alternatively the activation of macrophages involved in the attack of the lung migrating larvae (Maizels et al., 2012), adding to the eosinophils contribution. In mice infected with *Strongyloides venezuelensis*, IL-33 production stimulates IL-5 release, resulting in eosinophilia, contributing to worm expulsion (Yasuda et al., 2012).

Basophils are also functional for the host's protective Th2 response. Their number expand in blood and mesenteric lymph for the control of early intestinal parasite expulsion, during intestinal infection with *Strongyloides ratti* in mice. By contrast, in basophils-deficient mice, the absence of these cells as intestinal effectors, clearly impairs the efficient control of parasite burden (Reitz et al., 2018). Although basophils and mucosal mast cells have overlapping functions in contributing to the early intestinal immunity, the expulsion of the *S. ratti* nematodes has been proven to be selectively dependent on mast cells presence, alone or in synergy with basophils (Reitz et al., 2017).

Granulocytes are crucial for the host's defense against migrating larvae. The histological examination of skin, lungs and small intestine of mice infected with *S. ratti* revealed an inflammatory reaction due to neutrophils and eosinophils surrounding larvae 12 h after infection, whereas mononuclear cells were around the worms more than 24 h later (Dawkins et al., 1981).

Toll-like receptors (TLRs) are involved in recognition of pathogens via pathogen-associated molecular patterns (PAMPs) and in development of protective immunity against larval *S. stercoralis* in murine model. Interestingly, TLR4 is capable to induce the Th2-mediated allergic asthma in mice and DC2 phenotype acquisition by dendritic cells in case of helminth infection (Kerepesi et al., 2007). By means of an innovative method based on diffusion chambers containing L3 larvae and implanted in naïve mice,

TLR4 has been shown to be required for activating neutrophils in mediating parasites killing during the adaptive immune response. Neutrophils require both MPO and TLR4 to kill the larvae of *S. stercoralis* in the adaptive immune response (O'Connell et al., 2011). Animal in vitro models are essential for maintaining *Strongyloides* spp. in the laboratory and clarifying the interactions between adult worms and host immune response (Korenaga and Bruschi, 2014). The Mongolian gerbil was the first animal model of uncomplicated and hyperinfective *S. stercoralis* infection studied by Nolan et al. (1993) and a significant amelioration was obtained by Charuchaibovorn et al. (2019) as regards the potential applicability of gerbils infected with a human isolate of *S. stercoralis* as a good model for further immunological studies.

Helminth infections are highly prevalent in many low income countries where autoimmune disease are uncommon. For example, an inverse relation between autoimmune liver disease and *S. stercoralis* infection is the result of a disequilibrium of Th1 and Th2 cells, which are reciprocally cross-inhibitory and play a crucial role in downregulation of autoimmune processes. The pathogenesis of autoimmune liver diseases is modulated by *S. stercoralis* infection through Th1–Th2 cross-inhibitory process and/or induction of CD4 + regulatory T cell, which produce IL-10 and transforming growth factor- β (Aoyama et al., 2007).

Interestingly, in patients co-infected with Human T-lymphotropic virus 1 (HTLV-1) and *S. stercoralis*, the immunological shift to a Th-1 dominated immune response, induces a Th2 response blockade and leads to increased susceptibility to helminth infection with significantly lower eosinophil counts and serum IgE levels (Hirata et al., 2006). When *S. stercoralis* is present alone, Th-2 response induces IL-4 increase, which primes the B cells switching and the IgE production. Conversely, in case of co-infection with HTLV-1, an increased production of IFN γ , typical of a Th-1 response, occurs (Iriemenam et al., 2010). However, other mechanisms seem to be involved. Several studies have shown an association between ϵ chain expression and T-reg cells, which may contribute to the pathogenic mechanisms of HTLV-1 coinfection. An additional observation was done by Malpica et al. (2019), who studied the role of T-regs on the intestinal immune response, demonstrating the increased CD3 +, CD8 + and T-reg cell activities in duodenal biopsies of co-infected patients compared non-specific duodenitis or normal patients.

Clinical manifestations

Strongyloidosis has variable clinical pictures ranging from asymptomatic or mild (more frequent) to hyperinfection, or disseminated infection (Norsarwany et al., 2012). The initial signs of acute strongyloidosis may give a characteristic cutaneous reaction at the skin penetration site, commonly at the foot level, from which an urticarial tract with severe pruritus starts, lasting for several days. Cough and tracheal irritation are associated to the larvae migration through the lungs. Abdominal cramping and bloating, watery diarrhea are due to the lodging and maturation into adult of larvae at the host intestinal level (Keiser and Nutman, 2004). Chronic infections with *S. stercoralis* are sustained by a relatively low and stable number of adult worms; a well-regulated autoinfection, generally asymptomatic, either can lead to cutaneous, gastrointestinal or pulmonary symptoms. Skin involvement is characterized by a migratory, *larva currens* serpiginous maculopapular or urticarial rash along the buttocks, perineum and thighs due to repeated auto-infection (CDC, DPDx homepage). Gastrointestinal symptoms include diarrhea, abdominal discomfort, nausea and anorexia. The symptoms of pulmonary strongyloidosis include cough and shortness of breath (Woodring et al., 1994). Up to 75% of people with chronic strongyloidosis have mild peripheral eosinophilia or elevated total IgE levels.

The term “hyperinfection” denotes an unbalanced autoinfection, due to the increased number of worms and their detection at the extraintestinal areas, especially the lungs (Woodring et al., 1996). Hyperinfection affects immunosuppressed patients, in particular, who have a depression of cell-mediated immunity, malignant clinical conditions or chronic illness, also who receive a renal transplantation or with systemic lupus erythematosus, nephritic syndrome (Grove, 1989), rheumatoid and bronchial asthma (Altintop et al., 2010), treated with high-dose and long-term corticosteroids and other immunosuppressants can suffer of it.

Bacterial and fungal infections can occur as a severe consequence because of bowel damage, being frequently the immediate cause of death in patients with hyperinfection and disseminated infection.

Disseminated infection is associated to the migration of larvae beyond the range of normal migratory route, invading numerous organs and including various clinical manifestations, with a possible fatal complication of gram-negative bacteremia (*Streptococcus bovis*, *Escherichia coli*, *Streptococcus faecalis*, *Klebsiella pneumonia*, *Enterobacter* sp.), leading to septicemia, pneumonia or meningitis (Link and Orenstein, 1999; Concha et al., 2005; Cremades et al., 1997).

Several studies have shown a relation between human T-lymphotropic virus-1 (HTLV-1) infection and *S. stercoralis* hyperinfection (Nakada et al., 1984; Newton et al., 1992; Satoh et al., 2002). HTLV-1 patients seem to have an increased risk of acquiring strongyloidosis, preparing to recurrent and severe helminth infections. In patients co-infected with HTLV-1, the high production of IFN γ induces downregulation of IL-4, IL-5, IL-13 and IgE, promoting a switching of immune response from Th-2 to Th-1. The early and effective treatment as well as the *S. stercoralis* infection surveillance in patients with HTLV-1 are important for the prognosis of the coinfection and to prevent recurrent and disseminated strongyloidosis. Furthermore, after treatment, an increase of TNF α and a decrease in sIL-2R levels seem to empower the role of helminth in modulation of HTLV-1 immune response (Salles et al., 2013). Coinfection with HIV and *S. stercoralis* is common in endemic areas. Interestingly, the direct development (homogonic) of *S. stercoralis* is more frequent in HIV patients with normal immune function compared to those with impaired (lower CD4 + cell counts). The homogonic development is fundamental for disseminated infection (Viney et al., 2004). However, the treatment of HIV-infected patients with corticosteroids is an important additional risk factor for *Strongyloides* hyperinfection (Mascarello et al., 2011).

Diagnosis

Diagnosis is made by microscopic examination or via serologic methods.

Microscopic examination and histopathology

The microscopic identification of *S. stercoralis* larvae is done in feces, duodenal aspirates. Biopsy specimens, bronchoalveolar lavage and possibly in the sputum in disseminated infections (CDC). A number of methods has been used to detect larvae in samples, including direct smear of feces in saline-Lugol iodine stain (DS), Baermann (Fig. 2) and formalin-ethyl acetate concentration techniques (FECT), filter paper and agar plate culture (Fig. 3).

The formalin-ether concentration technique (FECT), described by Ritchie, is the most widely used in the clinical practice. The Harada-Mori technique is a filter paper culture technique that uses the water tropism of *Strongyloides* larvae to concentrate them. Compared to FECT and DS, it seems to be more sensitive, but less than Baermann and agar plate methods (Arakaki et al., 1990; Blatt and Cantos, 2003). In the agar culture method, the stool sample is placed on a nutrient agar plate and incubated for at least 2 days. As the larvae crawl over the agar, they carry bacteria with them, creating visible tracks (Arakaki et al., 1990). This method is laborious and time-consuming, but more sensitive than the other procedures (Zaha et al., 2000).

Unfortunately, although the examination of several sample improve the microscopic performance (Sato et al., 1995; Siddiqui and Berk, 2001), most of these methods have low sensitivity, because of the intermittent excretion of larvae and, mainly, the number of larvae in the stool, lower than the detection threshold of the diagnostic tests, especially in chronic infections (Knopp et al., 2001).



Fig. 2 Baermann technique: nematodes migrate downward in the water through the paper tissue and settle in the tubing above the clamp. Courtesy of Simona Gatti, University of Pavia, Italy.



Fig. 3 Agar plate culture method. Courtesy of Simona Gatti, University of Pavia, Italy.

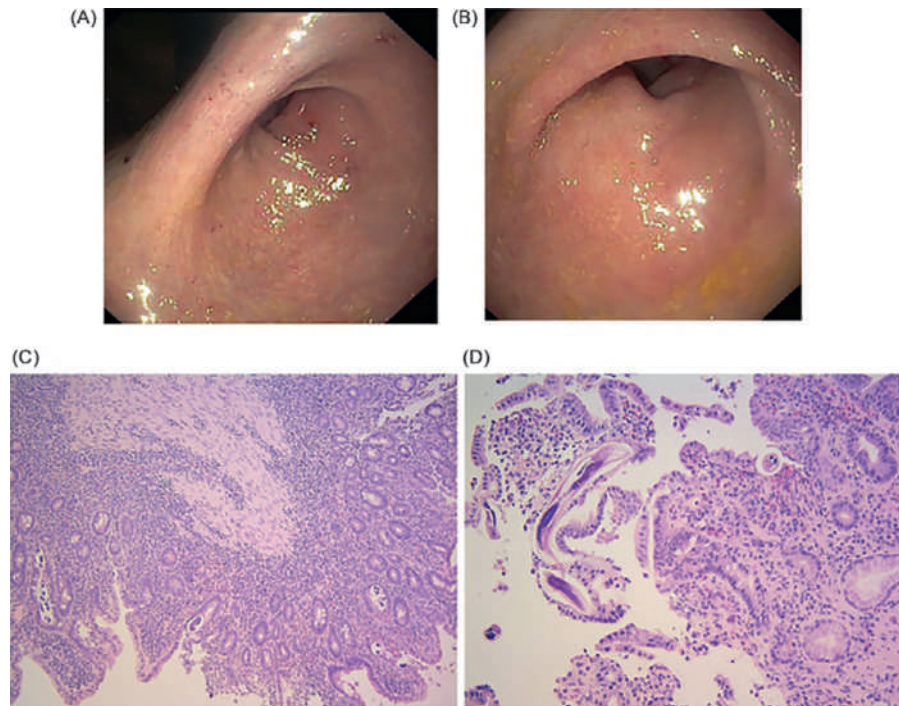


Fig. 4 Endoscopic and histopathological findings in a patient with chronic strongyloidosis. Esophagogastroduodenoscopy shows signs of petechial lesions and gastric (A) and duodenal atrophy (B). Histological assessment (H&E; X 200) shows chronic active inflammation with villous atrophy (C) and a large amount of eosinophils around numerous longitudinal and cross-sectioned larvae of *S. stercoralis* (D). From Beltrame A, Bortesi L, Benini M, Bisoffi Z (2018) A case of chronic strongyloidiasis diagnosed by histopathological study. *International Journal of Infectious Diseases* 77: 1–2, with permission.

A cross-sectional study (Khieu et al., 2013) conducted in a semirural village in Cambodia using the Agar plate and Baermann methods on three stool samples, demonstrated that the examination of multiple stool sample is fundamental to achieve a satisfactory level of sensitivity, in absence of a “gold standard.” The sensitivity of Agar plate culture and Baermann was 88.4% and 75.0%, respectively. The negative predictive values were 96.4% and 92.5%, respectively.

According to recent literature, the accuracy of fecal-based methods in terms of sensitivity and specificity was evaluated in a meta-analysis work conducted between 1980 and 2013 (Polanco et al., 2014), which showed that the Agar plate culture and Baermann methods are the most sensitive, comparing to the others conventional diagnostic techniques.

The histological investigation can confirm the diagnosis showing sections of larvae, eggs, some adult worms embedded in the gastric or duodenal crypts and eosinophils infiltrating into the lamina (Fig. 4) (Beltrame et al., 2018).

S. stercoralis infection induces an imbalance between cell death and proliferation, resulting in a disruption of the epithelial kinetics, but the mechanism is still unclear (Werneck-Silva et al., 2006).

Duodenal edematous mucosa, white villi, and erythematous mucosa are reported as abnormal endoscopic findings, whereas thickened folds and mucosal erosions may be seen in the stomach (Thompson et al., 2004).

The study of Kishimoto et al. (2008) demonstrated a major severity of the endoscopic findings in patients with positive biopsies for larvae compared to those with negative biopsies; thus, endoscopic findings are suitable and fundamental markers to establish or support diagnosis, avoiding a fatal outcome.

Serological examination

Serological methods are the most sensitive diagnostic tools (Requena-Méndez et al., 2013). A variety of antigens (crude, purified or recombinant) have been used to detect antibodies against *S. stercoralis*.

Although serological assay have the best potential for point-of-care solution, development of a sensitive serologic tool still remain an important challenge, especially for the cross-reactions with other helminth infections such as filariasis (Norsyahida et al., 2019) and difficult to discriminate chronic from acute infections.

Three tests have shown high sensitivity and specificity; immunofluorescence antibody test (IFAT), the enzyme-linked immunoabsorbent assay (ELISA) and immunoblotting.

IFAT is technically more complex than ELISA and requires a sophisticated equipment for the larval *S. stercoralis* antigen preparation and results interpretation. Recent studies documented decreased antibody titers after a successful treatment, making IFAT test particularly useful for follow-up. IFAT has a 92% positivity for IgG antibodies with no cross-reactivity with other parasitic infections (Korenaga and Bruschi, 2014).

ELISA is the easiest test and is the most widely used technique for the serological diagnosis of strongyloidosis, with sensitivity rates of between 73% and 100% (Requena-Méndez et al., 2013). Many ELISA tests have been used over the years; several in-house ELISAs use crude antigenic extracts of filariform larvae of *S. stercoralis*, *S. venezuelensis* and *S. ratti*. In addition, two commercial kits (Bordier-ELISA and IVD-ELISA) use *S. ratti* and *S. stercoralis*, respectively.

In recent years other two serological tests have been introduced: an ELISA assay based on 31-kDa candidate antigen (NIE), and a luciferase immunoprecipitation test (NIE-LIPS).

NIE is a recombinant 31 kDa antigen derived from L3s of *S. stercoralis*, resulting in a specificity of 87.5% of 48 sera from *Strongyloides*-infected, with no-cross reactivity to *Onchocerca volvulus*, *Loa loa*, and *Mansonella perstans*. In tropical pulmonary, eosinophilia presumably caused by *Wuchereria bancrofti* and false-positive results obtained. NIE could have a potential use as sensitivity skin antigen, because of its capacity to trigger the histamine release from blood of *Strongyloides*-infected patients (Ravi et al., 2002).

LIPS assays, similar to the optimized NIE-ELISA, demonstrated to well-distinguish between *S. stercoralis* and other infections that normally cross-react in standard ELISAs and accurately differentiate between *S. stercoralis*-infected patients from uninfected controls (Ramanathan and Nutman, 2008).

A LIPS assay that uses the antigenic combination of NIE with *S. stercoralis* immunoreactive antigen (SsIR), resulted to have a greater degree of sensitivity (100%) and specificity, compared to the LIPS format using either NIE or SsIR as antigens, with an optimal negative (NPV) and positive predictive values (PPV).

An excellent study (Krolewiecki et al., 2010) reported a comparison between parasitological (agar plate, Baermann, sedimentation concentration, and Harada-Mori) and serological methods (CrAg-ELISA, NIE-ELISA, NIE-LIPS, and NIE-SsIR-LIPS), operating the characteristic receiver operating characteristic curve (ROC) curves to determine the optimal cut-off for each immunoassay based on plotting the sensitivity and specificity. NIE-LIPS showed to be the highest sensitivity and specificity for IgG detection.

Further, a recent meta-analysis demonstrated the accuracy of different serological assays and recommended them for the screening of *S. stercoralis* infection throughout the world. The overall sensitivity, in comparison with stool examination, was 71.7%, ranging from 49.0% to 81.0%, based on different types of *Strongyloides* larvae antigens and dissimilar methods, whereas the specificity ranges from 83% with *S. stercoralis* to 95% with *S. ratti* larvae antigens, respectively (Kalantari et al., 2020).

The diagnostic accuracy of five different serological tests (IFAT, IVD ELISA, Bordier ELISA, LIPS, NIE LIPS) was recently investigated using a composite reference standard (Bisoffi et al., 2014). IFAT was shown to be the most sensitive test, followed by IVD ELISA with a sensitivity of 93.9% and 91.2%, respectively. LIPS was the most specific test (specificity 100%) followed by IVD ELISA and Bordier ELISA.

In general, serological diagnosis have the evident limit of the diminished sensitivity of serum antibody detection in immunosuppressed patients; in case of hyperinfection, due to the immunosuppression, larval output is higher and fecal-based methods are fundamental (Requena-Méndez et al., 2013).

Molecular diagnosis

The molecular biology techniques for the diagnosis are based on the detection of parasite-specific DNA from stool, urine and sputum samples using conventional the polymerase chain reaction (PCR), nested-PCR and real-time PCR techniques (Lodh et al., 2016; Kramme et al., 2011). PCR restriction fragment length polymorphism (PCR-RFLP) analysis has been utilized for phylogenetic and epidemiological studies. The amplification of the ITS1 region in the rDNA gene revealed that the four human isolates from different geographic areas of the world had identical patterns (Nilforoushan et al., 2007).

Other molecular marker is the hypervariable regions in 18S rDNA, which is highly conserved in *Strongyloides* spp. and widely used for molecular taxonomy (Blaxter et al., 1998).

Hasegawa et al. (2009) reported several nucleotide polymorphisms among different *Strongyloides* in the four of the HVRs of the 18S rDNA, resulting in species-specific arrangements.

Moghaddassani et al. (2011) established a single-PCR and nested-PCR for the DNA detection of *S. stercoralis* in fecal samples, previously concentrated in acid/ether solution before the extraction process.

S. stercoralis real-time PCR is another application and important contribution to the accuracy of the molecular PCR-based techniques. A real-time PCR method targeting the small subunit of the rRNA gene was developed for the detection of *S. stercoralis* DNA in fecal samples from northern Ghana, resulting in a higher sensitivity and specificity compared to Baermann and cultural methods (Verweij et al., 2009). However, this PCR design was applied to asymptomatic schoolchildren in Cambodia and showed a sensitivity of 88.9% and specificity of 92.7%, compared to the studies on symptomatic patients (Schär et al., 2013).

The rRNA primes and probe set has been incorporated into multiplex assays on variety of different platforms (Taniuchi et al., 2011). A multiplex real time PCR, focusing on four target parasites (*E. histolytica*, *Giardia lamblia*, *Cryptosporidium* and *S. stercoralis*), proved to be a highly sensitive and specific technique compared to traditional diagnostic methods (Moghaddassani et al., 2011). However, a considerable challenge involved in these methods is the extraction of DNA from larvae of *Strongyloides*. To improve the performance, Repetto et al. (2013) developed an in-house method, which consists in a pre-incubation of the samples with glycine-SDS buffer and subsequent disruption mechanism.

The use of the molecular methods is still limited to the reference laboratories; only a few commercial kits are available and the protocols used are not standardized.

Treatment

Main targets for therapy of *S. stercoralis* infection focus on the complete cleaning of the organism, treatment of symptomatic infections and prevention of the complications related to asymptomatic infections (Nutman, 2017).

According to Center of disease control and prevention (CDC) (www.cdc.gov/parasites/strongyloides/health_professionals/index.html), oral ivermectin (200 mg/kg for 2 days) remains the treatment of choice for uncomplicated infections; in many different clinical trials, conducted between 1994 and 2011, comparing ivermectin with albendazole, ivermectin seemed to be better tolerated with no reports of serious adverse events (Henriquez-Camacho et al., 2016). Potential contraindications to the use of ivermectin include pregnancy, weight <15 kg and suspected or confirmed *Loa loa* infection, which may cause serious side effects.

In case of immunosuppressive patients or with disseminated strongyloidosis, the treatment with ivermectin (200 mg/kg per day) should be recommended until stool or sputum exam is negative for 2 weeks.

Albendazole (400 mg for 3–7 days) is considered an alternative therapy, but is contraindicated in patients with a history of hypersensitivity to benzimidazole and should be avoided in the 1st trimester of pregnancy. The most common adverse effects are headache and elevated liver enzymes. Some other side effects include abdominal pain, nausea, vomiting and fever (Veraldi et al., 2012).

Prevention and control

The best way to prevent strongyloidosis is to cure the personal hygiene, wear protective shoes and avoid contact with fecal matter or sewage (CDC; www.cdc.gov/parasites/strongyloides/health_professionals/index.html).

To minimize the risk of de novo infection and reinfections with *Strongyloides*, serological screening is recommended for immigrants and short-term travelers coming from endemic area, immunosuppressed patients in chronic treatment with corticosteroids and chemotherapy and candidates (donors and recipients) for solid and or bone-marrow transplant.

The updated guidelines from the Infectious Disease Community of Practice of the American Society of Transplantation (La Hoz and Morris, 2019) review the diagnosis, prevention and management of intestinal parasites in the pre- and post-transplantation period. The screening approach is different between endemic and non-endemic areas. In high prevalence endemic area, where laboratories resources are limited, a reasonable approach is to treat all transplant candidates. On the other side, in non-endemic countries, with available diagnostic tools, a targeted screening approach based on epidemiological risk factors for *Strongyloides* infection has been used (Fitzpatrick et al., 2010). Serology is considered the best screening test for living and deceased donors (Abanyie et al., 2015; Requena-Méndez et al., 2017).

Unfortunately, no vaccine has been put into practical use. However, vaccine and passive immunization have been developed and tested on animal model and different antigenic preparations derived from *S. stercoralis*, *S. venezuelensis* and *S. ratti* have been used for vaccine strategies.

Deoxycholate (DOC) soluble proteins, extracted from *S. stercoralis* and combined with aluminum hydroxide adjuvant have been found to induce protective immunity in mice 5 days after infection (Herbert et al., 2002).

Kerepesi et al. (2005) demonstrated for the first time the possible transfer of human protective immunity to mice against *S. stercoralis*. The human IgG were able to bound antigens on the surface and internal in the esophagus of *S. stercoralis* L3s. Combinations of antigens recognized by human IgG and mouse protective antibodies could result potential candidates for a multivalent vaccine. In particular, three antigens (tropomyosin, Na⁺ -K⁺ ATPase and LEC-5) of *S. stercoralis* were identified and the genes were cloned into plasmids for intradermal immunization in the mouse.

Abraham et al. (2011) demonstrated the potential role for a vaccine of a recombinant antigen Ss-IR, which is highly immunogenic in humans and able to generate a protective immunity.

Furthermore, the excretory and secretory component HSP60 produced by all stages of *S. ratti* was investigated as a promising target for the vaccine. Passive immunization of mice with monoclonal IgM anti-HSP60 resulted in a successful immune protection (Nouir et al., 2012).

Closing remarks

Strongyloidosis is an underestimated parasitic public health problem in developing countries.

Although the large number of affected people worldwide, the volume and growth of literature in strongyloidosis are still relatively poor (Waleed, 2019).

Low socioeconomic status and poor sanitation infrastructures are strictly associated with the transmission of the infection. Developing strategies such as the implementation of a preventive healthcare system in low income countries, health education and the adoption of proper environmental controls are fundamental measures to prevent *Strongyloides* infections. Cambodia, for example, benefits from a well-established STH control program and is the first country to reach the 75% national coverage target (Forrer et al., 2019).

Strongyloidosis is considered a silent chronic disease and may be misdiagnosed because of its low parasitic load and uncertain clinical symptoms. The availability of more accurate diagnostic tools is a key element for the performance of more reliable surveys, case control studies and even monitoring for the emergence of drug resistance (Cimino and Krolewiecki, 2014).

A more complete understanding on strongyloidosis and the development of an efficient vaccine are important goal for managing this disease and protecting the communities at risk.

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Taeniasis and Cysticercosis

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Glossary

Anthelmintic Any chemotherapeutic substance directed against a helminth infection.

Carrier A host that harbors a particular pathogen without manifestations of disease.

Cysticercus The hollow fluid-filled larval stage of *Taenia*, containing a single invaginated scolex.

Definitive host The animal in which a parasite passes its adult existence and/or sexual reproductive phase.

Diheteroxenous Parasite requiring two hosts to complete its life cycle.

Intermediate host The animal that harbors the larval stage of the parasite.

Metacestode A collective term for any of the various larval stages of cestodes which are found in the intermediate host.

Oncosphere The motile, six-hooked embryo (hexacanth) of a tapeworm that is contained inside the egg membranes.
Proglottid One complete unit of a tapeworm, also called segment.
Scolex Attachment end of a tapeworm, commonly referred to as the anterior end which can bear suckers and hooks.
Strobila The chain of proglottids or segments of the tapeworm formed by budding.
Strobilization Process to produce proglottids from the neck region of tapeworms.
Zoonosis Infection or disease naturally transmitted between humans and other animals.

Agents and host involved

Both, taeniasis and cysticercosis are parasitic diseases caused by flatworms belonging to the class Cestoda (tapeworms), family Taeniidae, genus *Taenia*. The adult stages of *Taenia* species cause intestinal taeniasis in the definitive host whilst their larval stages or metacestodes (cysticerci) cause extra-intestinal cysticercosis in the intermediate host.

Humans are the only definitive hosts for the intestinal adults of three *Taenia* species, namely *Taenia solium* Linnaeus, 1758 (the pork tapeworm), *T. saginata* Goeze, 1782 (the beef tapeworm) and *T. asiaticus* n. n. Eom et al., 2020 (syn. *Taenia asiatica* Eom and Rim, 1993) (the Asian *Taenia*). In addition to the adult stage, humans are also intermediate hosts for *T. solium*, consequently suffering not only taeniasis but also cysticercosis. *T. solium* was included in the World Health Organization (WHO) neglected tropical disease (NTD) roadmap in WHO (2012) and this species is also the first on the list of the top 10 foodborne parasites elaborated by the Food and Agriculture Organization (FAO) in FAO (2014).

Other mammals involved in the three *Taenia* life cycles are pigs as intermediate hosts of *T. solium* and *T. asiaticus*, and cattle in the case of *T. saginata*. Cysticerci of *T. solium* have also been molecularly confirmed in dogs and bears (Ito et al., 2002; Theis et al., 1996). A strain of *T. saginata* uses reindeer (*Rangifer tarandus*) instead of bovids as intermediate hosts in northern Russia (Konyaev et al., 2017). There are other *Taenia* species also causing human cysticercosis (*T. crassiceps* or *T. martis* for instance), although these are only sporadically reported (Ntoukas et al., 2013; Eberwein et al., 2013). It has been suggested that *T. asiaticus* could also cause human cysticercosis, but there is no clear evidence so far (Galán-Puchades and Fuentes, 2013a).

Historical background

It is generally estimated that the origin of the association between humans and *Taenia* arose no more than 10,000 years ago, coincidental with the domestication of major food animals such as cattle and pigs and the advent of agriculture (Cox, 2002). Supposedly, at these early stages of animal husbandry, tapeworms circulating in synanthropic cycles between dogs and domesticated ruminants which were the source of *Taenia* parasites that first colonized and then diversified in humans. Another contrasting hypothesis, based on phylogenetic studies using mitochondrial genes, whole mitochondrial genomes, and select nuclear DNA sequences from modern specimens of *Taenia* species from humans and mammalian hosts, revealed that *T. solium* is most closely related to *Taenia* species infecting hyaenids, while *T. saginata* and *T. asiaticus* are most closely related to the species that infects lions (Terefe et al., 2014). This indicates that host switching that allowed *Taenia* spp. to infect humans was likely to be a result of hominids scavenging on the same animals as hyenas, in the case of *T. solium*, and large cats, in the case of *T. saginata*.

Phylogenetic evidence suggests that *Taenia* tapeworms began infecting hominids in sub-Saharan Africa 1–2.5 myr ago, prior to the domestication of animals (Ledger and Mitchell, 2019). Phylogenetic analyses indicate that *T. saginata* and *T. asiaticus* are putative sister species, unrelated to *T. solium*. The divergence between *T. saginata* and *T. asiaticus* is estimated to have taken place 0.78–1.71 myr ago in Africa or Eurasia (Hoberg et al., 2001). The biotic expansion from Africa into Eurasia may have resulted in the isolation and later divergence of *T. asiaticus* in Asia. Eight extant boar species have been identified in South-East Asia. This diversity of potential intermediate hosts in this area may have influenced the intermediate host switch in *T. asiaticus* from herbivore to swine. Therefore, the association of *T. asiaticus* with swine may have been established in Asia following divergence of host populations that later expanded into Europe, being consistent with the dominant distribution of this species in Asian countries (Hoberg, 2006; Michelet and Dauga, 2012).

Taenia spp. have been identified in human remains (pelvic soil and mummified remains) in several locations of Europe, Middle East, Africa and Asia, dated from 8300 BCE to 167 BCE (Ledger and Mitchell, 2019). Particularly in Egypt, the species *T. solium* was immunohistochemically diagnosed in a mummy being dated from the second to the first century BCE. This finding constitutes the oldest on record of the antiquity of this parasite (Bruschi et al., 2006). Likewise, one of the oldest and most famous possible patients suffering from neurocysticercosis (NCC), pathology due to the presence of the cysticerci of *T. solium* in the central nervous system (CNS) that can cause epilepsy, was Julius Caesars (100–44 BCE), who used to suffer headaches and epileptic crises (Bruschi, 2011).

Historically, until *T. asiaticus* was described in Eom and Rim (1993), only two *Taenia* species were considered to parasitize humans, *T. solium* and *T. saginata*. The reason is due to the fact that *T. asiaticus* was confounded with *T. saginata* for more than 200 years in Asia (Eom, 2006); an issue that will be addressed later.

In absence of any available molecular tool, scientists, two centuries ago, were finally able, after failed conclusions, to distinguish between *T. solium* and *T. saginata*. Authors, among others, Grove (1990), Cox (2002) or Wadia and Singh (2002), described the history of human *Taenia* infections in detail.

As already reviewed by Galán-Puchades and Fuentes (2013b), it was not until the mid-19th century that Küchenmeister recognized the differences between *T. solium* and *T. saginata* based on the morphology of the scolex. Leuckart was the first who experimentally showed that proglottids of *T. saginata* fed to cattle developed into cysticerci in calf muscles. Finally, Oliver was the first who discovered that “bladder worms” developed into adult *T. saginata* when ingested by humans.

The first person who demonstrated that pigs are the intermediate hosts of *T. solium* was the Belgian, van Beneden. In 1853, he gave *T. solium* eggs to a pig. When the pig was slaughtered at the abattoir four and a half months later, he found a large number of *T. solium* larvae (named *Cysticercus cellulosae*) in the muscles. Cysticercosis in humans due to *T. solium* was described by Rumler in 1558; however, the connection between tapeworms and cysticercosis had not been made at that time. Around 1850, Küchenmeister, largely criticized for his unethical practices, fed pork meat containing cysticerci of *T. solium* to humans awaiting execution in prison, and after they had been executed, he recovered developing and adult tapeworms in their intestines. By the mid-19th century, it was established that cysticercosis was caused by the ingestion of *T. solium* eggs. Another experimental infection was carried out in the 1930s by the Japanese scientist, Kozen Yoshino (1898–1965), and three more people, who voluntarily swallowed cysticerci from pigs in order to describe the outcomes of the infection (Ito et al., 2020).

The existence of the third *Taenia* species was not considered until the middle of the 20th century. In 1993, the South Korean scientist Keeseon Eom and colleagues provided descriptors of the new species, and have also recently published a complete historical overview of the species in which they proposed a new name for *T. asiatica*, specifically, *T. asiaticus*, according to the Code of Zoonotic Nomenclature (Eom et al., 2020). The origin of the discovery was an epidemiological incongruity observed in Taiwanese aborigines since, although the morphology of the expelled proglottids indicated *T. saginata*, the patients had not eaten beef—cattle being the natural intermediate host of *T. saginata*—but their diet was exclusively based on wild boars and pigs, the intermediate hosts for *T. solium* (Huang et al., 1966; Huang, 1967).

Morphology

Just as the others of the tapeworms belonging to the family Taeniidae, the species of the *Taenia* genus have three stages, two of them are parasite forms, i.e., the adult and the larval stage or metacystode, and the other one, the egg, is a free-living stage. In the definitive host, the intestinal adult stage produces eggs that leave the host and live in the environment for months. When ingested by the intermediate host, the egg turns into a cysticercus-type larva that, in turn, turns into the adult stage when the larva is ingested by the definitive host.

Adults

An adult *Taenia* tapeworm is made up of three parts: a fourth-sucker *scolex* at the anterior smaller part of the worm, behind a short *neck* followed by the *strobila*, a kind of chain comprising hundreds or thousands of hermaphroditic proglottids or segments. Macroscopically, the three species look alike. They are long whitish flat worms looking like tapes (hence tapeworms) (Fig. 1). *T. saginata* is the longest, measuring between 4 and 12 m. Table 1 shows some of the most relevant morphological characteristics of the adult stages of the three human *Taenia* species.

The only way to specifically distinguish them by their external morphology is by means of observing the 1–2 mm sized scolex under a stereomicroscope or a microscope, since only *T. solium* has hooks inserted in the rostellum, a cone-like muscular structure of the scolex that can protrude in some cestodes, but not in the case of *Taenia* species. The presence of hooks in *T. solium* made this species known as the “armed *Taenia*.” Neither *T. saginata* nor *T. asiaticus* have hooks. Therefore, only *T. solium* could be differentiated from the two other unarmed species by the mere observation of a complete adult stage.

Fig. 2 illustrates the morphology of the three scolices; in *T. solium*, the hooks are arranged in two rows of 22–36 characteristically shaped hooks (like a cat claw), including small and large ones, measuring from 11 to 176 µm.

The neck, the part immediately behind the scolex, is the region of growth from where the proglottids of the body, that constitute the strobila, are continuously generated. Actually, a strobila is a kind of factory aimed at the production of eggs. According to the age of the proglottid, the production still has not started (young and immature segments near the neck), the production is in operation (sexually mature proglottids in the middle of the chain) and, finally, at the end of the strobila, the gravid and oldest proglottids, are full of already formed eggs. These latter proglottids are expelled on a daily basis and can be found in human feces. Fig. 3. shows the general internal morphology of a sexually mature proglottid as well as that of the three gravid segments which, once processed at the laboratory (see “Diagnosis” section), by means of counting the number of lateral branches, would allow to distinguish between *T. solium* and *T. saginata*/*T. asiaticus* [these two species present a similar number of branches (Table 1)].

Eggs

The eggs of the three human *Taenia* species have the same morphology (in fact, the eggs of any tapeworm of the family Taeniidae) and, therefore, do not permit species identification. In gravid proglottids, the uterus is full of spherical eggs, measuring 31–43 µm in



Fig. 1 Detail of a fragment of the strobila of *Taenia saginata*.

Table 1 Morphological characteristics of the adult stages of *Taenia* species.

	<i>Taenia solium</i>	<i>Taenia saginata</i>	<i>Taenia asiaticus</i>
Length (m)	1–5	4–12	4–8
Scolex	Armed rostellum	Rostellum absent	Unarmed rostellum
Number of Proglottids	≤1000	>1000	<1000
Number of testes	350–600	800–1200	320–1200
Ovary	Three lobes	Two lobes	Two lobes
Vaginal sphincter	Absent	Present	Present
Number of lateral branches in uterus	7–13	14–32	16–21
Branching pattern	Dendritic	Dichotomous	Dichotomous

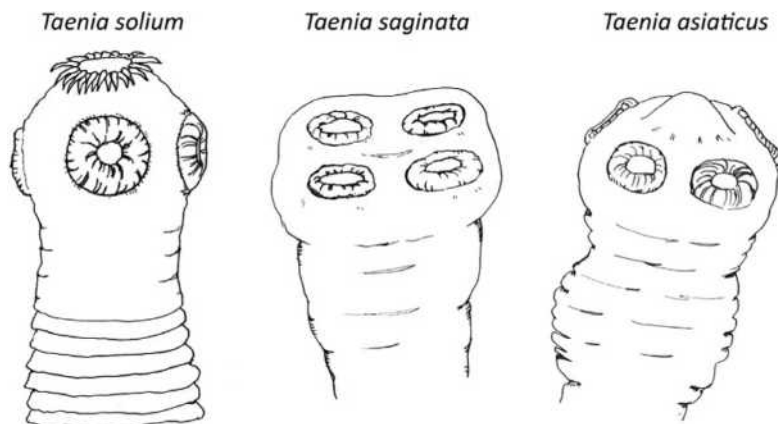


Fig. 2 Scolex morphology of the three human *Taenia* species (illustration by Angela DeBenedetti, PhD).

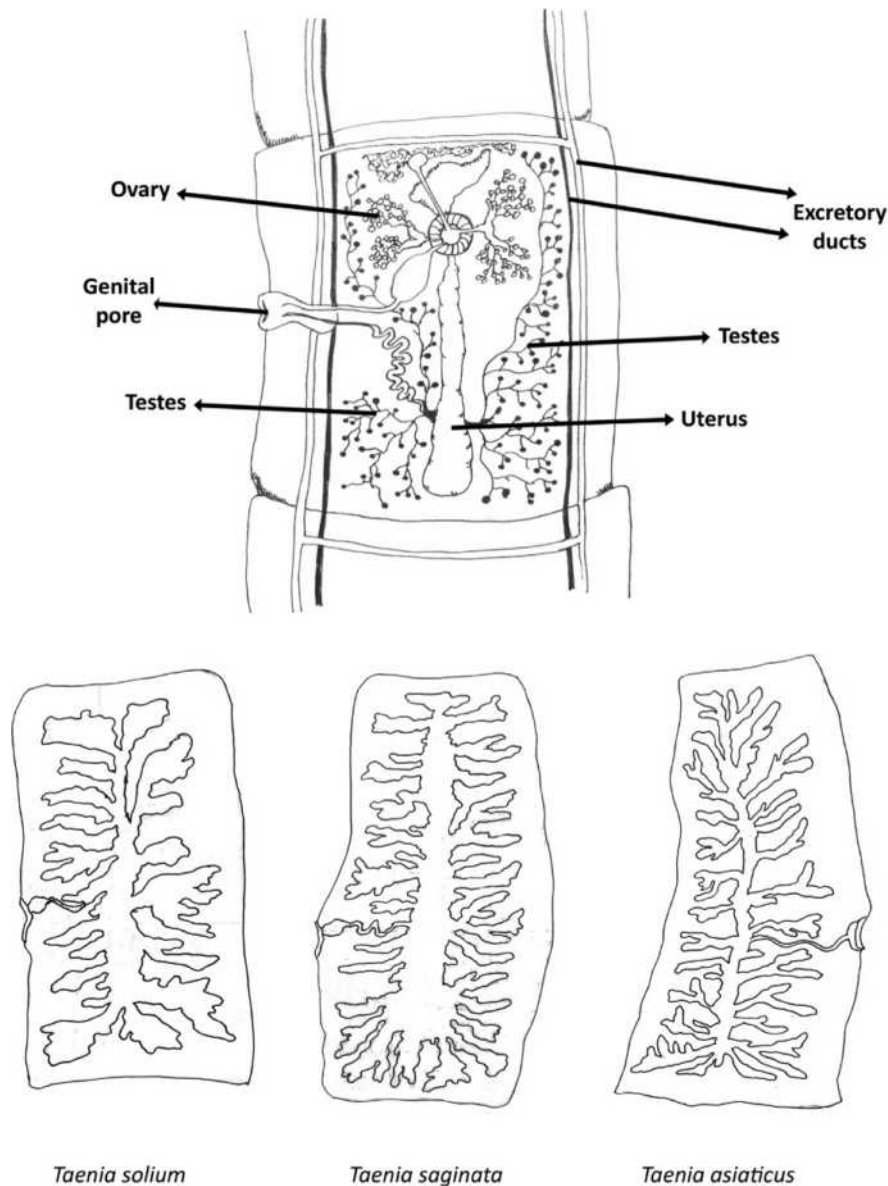


Fig. 3 Up: General morphology of a sexually mature proglottid of *Taenia* spp.; below: uterus morphology of the three human *Taenia* species in the gravid proglottids (illustrations by Angela Debenedetti, PhD).

diameter, presenting an outer embryophore, made up of keratin blocks cemented together, which is thick and radially striated protecting a fully-developed embryo, the oncosphere. This embryo is surrounded by several membranes and possesses three pairs of hooklets, the reason why the oncosphere is also known as hexacanth embryo (Fig. 4, left). The color of the eggs varies between pale yellow and brown.

The eggs of *T. asiaticus* were analyzed by means of electron microscopy and TEM (transmission electron microscopy) as well as SEM (scanning electron microscopy) images, which showed the presence of small vesicles consistent with extracellular vesicles (exosomes and ectosomes) (Galán-Puchades et al., 2016).

The eggs of *T. saginata* (Fig. 4, right) only infect cattle, causing bovine cysticercosis, those of *T. asiaticus* apparently only cause pig cysticercosis. However, the eggs of *T. solium* are infective to pigs as well as to humans, causing not only pig but also human cysticercosis.

Metacestodes

Cysticerci are rounded or oval extra-intestinal metacestodes visible to the naked eye. They are whitish bladders measuring, depending on the *Taenia* species, between 0.2 and 1.5 cm in diameter (Fig. 5). The fluid-filled vesicle has an invaginated scolex which can be seen as a kind of small eccentric and solid granule. In the case of *T. solium*, the vesicular fluid consists of water but also contains calcium, glycoproteins, cholinesterase, and coproporphyrin (Cervantes et al., 1986).

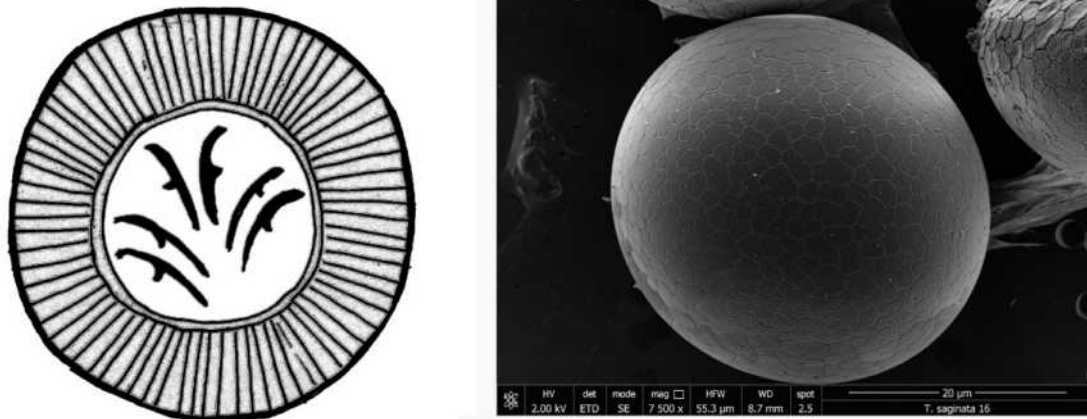


Fig. 4 Left: *Taenia* egg (illustration by Angela DeBenedetti, PhD); right: SEM image of a *T. saginata* egg.

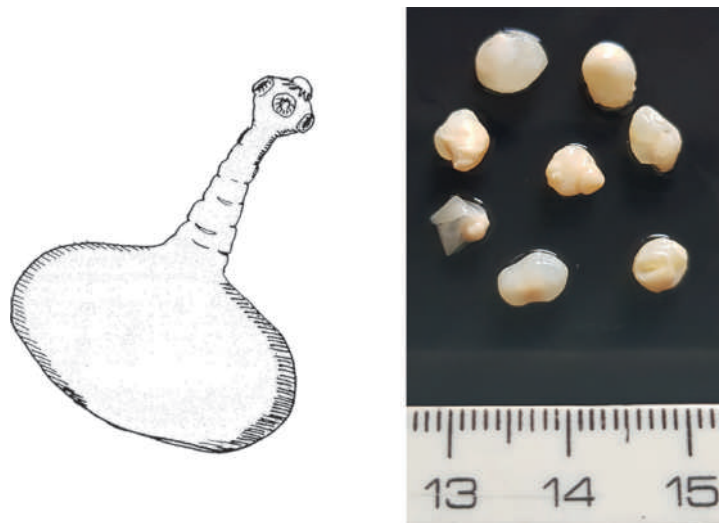


Fig. 5 Left: Morphology of a *Cysticercus cellulosae* with the scolex evaginated (illustration by Angela DeBenedetti, PhD); right: Cysticerci of *Taenia solium* from a pig brain.

Having different specific names for the cysticercus and for the adult stage of the human *Taenia* species is an established habit. The cysticercus of *T. solium* is known as *Cysticercus cellulosae*, *C. bovis* that of *T. saginata* and *C. viscerotropica* for *T. asiaticus*. In the case of *T. solium* and *T. saginata*, when the cysticerci in the intermediate hosts were matched with the adult stages in humans, both had already been differently named, which has remained the same ever since. In the case of *T. asiaticus*, when the species was described, the cysticercus was named differently to keep this habit.

The nature of the intermediate host distinguishes *C. bovis* (cattle) from the porcine cysticerci (*C. cellulosae* and *C. viscerotropica*). *C. bovis*, with a size of 7–10 mm, contains the unarmed scolex of the future adult stage. In the case of cysticerci from pigs, the organ in which the metacestode is found as well as the size and the internal morphology are noticeable. *C. viscerotropica* shows a clear liver tropism, although other less frequent locations should not be ruled out (Singh et al., 2016). The cysticercus of *T. asiaticus* is the smallest, measuring 1.9–3.0 mm in maximum length. Up to 37 rudimentary hooklets on the rostellum can be observed in about 17% of the larval stage of (Eom et al., 2020). *C. cellulosae*, measuring 0.5–1.5 cm, exhibits no defined tropism (Fan et al., 1994), thus developing in muscles and other organs in pigs. Contrary to *C. viscerotropica*, *C. cellulosae* has an armed scolex with the two rows of hooks of the future adult stage in the interior of the cysticercus.

Life cycle

Fig. 6 illustrates the life cycles of the three *Taenia* species and Table 2 shows certain peculiarities of each one of these three cycles.

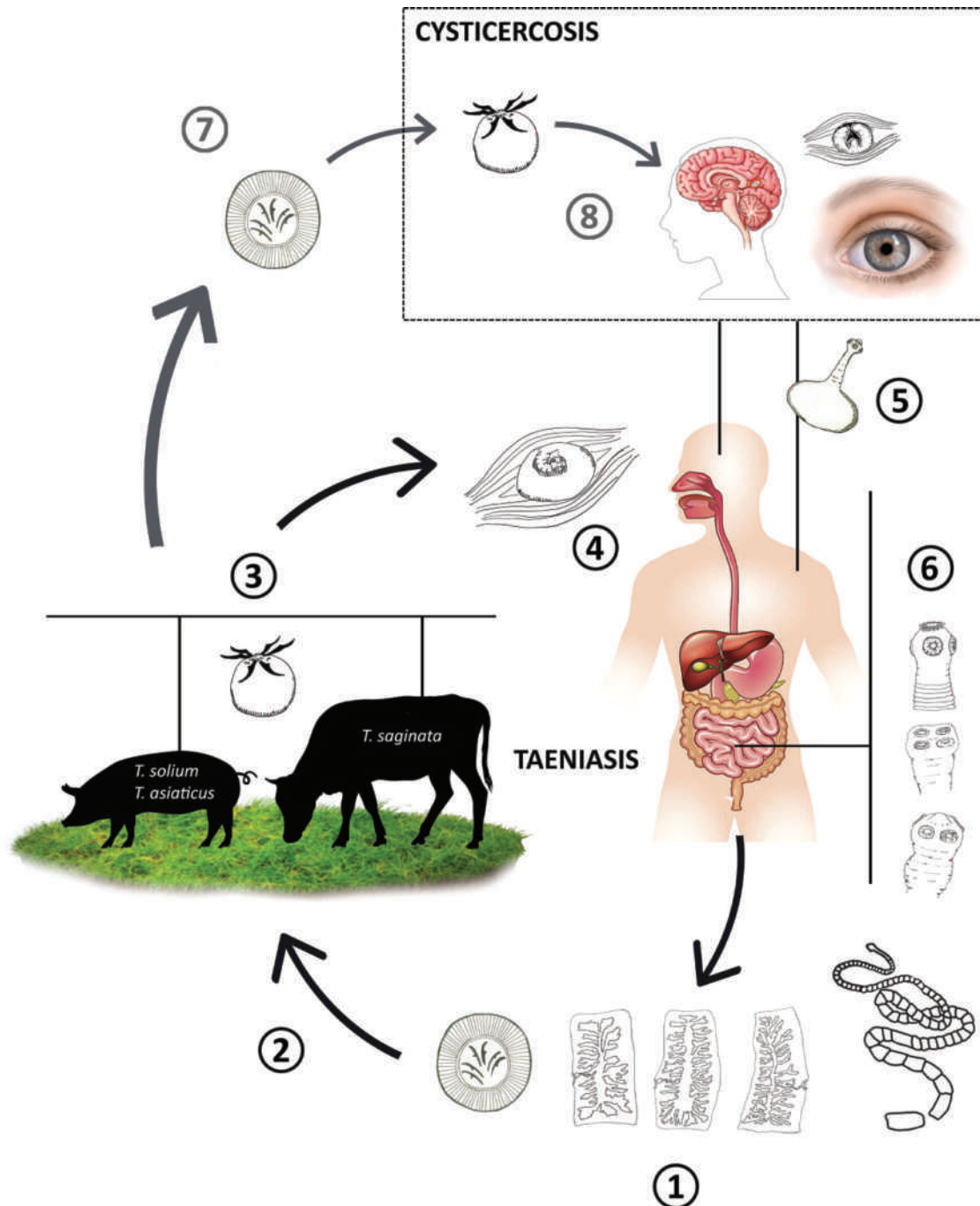


Fig. 6 Human *Taenia* life cycle: (1) gravid proglottids are shed in feces; (2) pigs (*T. solium*, *T. asiaticus*) and cattle (*T. saginata*) become infected by ingesting the eggs which contaminate water and food; (3) oncospheres from eggs turn into cysticerci in the intermediate hosts (IH); (4) humans acquire taeniasis ingesting raw/undercooked infected meat and/or viscera of the IH with cysticerci; (5) cysticerci turn into adult tapeworms in the human intestine; (6) the adult stage remains in the small intestine where the scolex attaches itself; (7) humans develop cysticercosis through the accidental ingestion of *T. solium* eggs; (8) cysticerci can develop in any organ but most commonly in subcutaneous tissue, the brain (NCC) and eye (ophthalmocysticercosis) (illustration by Angela DeBenedetti, PhD).

The three species have a similar diheteroxenous life cycle. They require two mammalian hosts in order to complete them. Humans are the only known definitive host harboring the intestinal adult stage. In general, on a daily basis or two or three times per week, eggs are shed in the feces of the taeniasis individual, either free or in the interior of gravid proglottids. Gravid proglottids of *T. saginata* and *T. asiaticus* are motile, able to actively force their way out through the anal sphincter, independent of human defecation.

Table 2 Features of human *Taenia* life-cycles.

	<i>Taenia solium</i>	<i>Taenia saginata</i>	<i>Taenia asiaticus</i>
Gravid proglottids release	In short chains of 3–5 segments	Isolated segments	Isolated segments
Movement of the proglottids	No	Yes	Yes
Alternative intermediate hosts	Wild boar, dogs, bears	Reindeer	Wild boar
Larval development (in weeks)	9–10	10–12	4–8
Larval survival	3–6 years	1 year	1–2 months

The eggs can survive in the environment, water or soil, for months, depending on temperature and moisture (see section “Epidemiology and distribution”). A survival of 413 days was reported for *T. saginata* eggs on pasture in Kenya (Duthy and van Someren, 1948).

Once the eggs are ingested by the intermediate host, cattle in the *T. saginata* life cycle and pigs in the case of *T. solium* and *T. asiaticus*, and in contact with hydrochloric acid, digestive enzymes and bile, the blocks of the embryophore become unglued, thus liberating the oncosphere which penetrates the intestinal wall reaching the blood or lymphatic vessels of the mesentery. Subsequently, these post-oncospheres are passively transported to several host tissues in which they will develop into the larval or metacystode stage, the cysticerci, in several weeks (Table 2). When humans eat raw or undercooked infected meat or viscera of intermediate hosts (beef and pork), in the upper part of the small intestine, the scolex evaginates out of the cysticercus, becomes attached to the mucosa with the aid of the suckers (and hooks in *T. solium*), and by gradual strobilization develops into adults in several weeks.

The adult life span of *Taenia* species is not clearly known. The life span of *T. solium* is controversial. Although longevity of up to 25 years was accepted for years (Hoberg, 2002), a shorter life span of less than 5 years has also been suggested (Lightowlers, 2010; García et al., 2014). In the case of *T. saginata* and *T. asiaticus*, cases in which infections persisted for decades have been recorded (Wright and Jain, 1984; Eom et al., 2020).

In addition to taeniasis, humans may develop cysticercosis by means of the ingestion of *T. solium* eggs, acting then as intermediate hosts. Unfortunately, the CNS is one of the main target organs of cysticerci in humans; this infection produces a complex disease, NCC.

Epidemiology and distribution

There are numerous articles in the literature that describe a great variety of aspects of the epidemiology of human taeniasis/cysticercosis. Only considering those most generalist published in the last 20 years, is worth mentioning the papers by Eom and Rim (2001), Flisser (2002), Hoberg (2002), Pawlowski (2002), Ito et al. (2003), Murrell (2005), Devleesschauwer et al. (2012), Zammarchi et al. (2013), Coral-Almeida et al. (2015), Ito et al. (2016), Laranjo-Gonzalez et al. (2017), Wu et al. (2017), Braae et al. (2018), Trevisan et al. (2018), Bucur et al. (2019), Dixon et al. (2019), Abraham et al. (2020), Eichenberger et al. (2020), among others, are noteworthy.

The epidemiology of the human *Taenia* species depends on two basic factors: the natural ecology of the life cycle and the artificial changes introduced by humans in that ecology. The high biotic potential of the adult stages (great ability to produce eggs), the increase of host populations (humans, pigs and cattle), changes in livestock production, high consumption rates of undercooked meat, as well as the inherent consequences of globalization, i.e., progressively more intensified contacts between hosts due to human migratory movements, in addition to the role of international trade, make taeniasis a progressive zoonosis.

Parasite populations of these three species comprise three subpopulations: The adult stage in humans, the released eggs in the environment and the metacystodes in the intermediate hosts. Therefore, three epidemiological factors determine the geographical distribution of taeniasis/cysticercosis: (a) the human factor; (b) the environment; and (c) the type of livestock.

Taenia solium distribution

The pork tapeworm has a cosmopolitan distribution being infrequent in those regions where pork is not consumed. *T. solium* is an endemic parasitic disease in many countries of Central and South America, South Africa and Asia. Its prevalence varies according to the epidemiological factors, i.e., the level of sanitation, pig husbandry practices (pigs scavenging for food), limited veterinary meat inspection and eating habits. In endemic regions, large portions of the population may be infected. In these areas up to 6% of people may harbor intestinal worms at a given time and serve as a source of infectious eggs that cause cysticercosis (Allan et al., 1996).

Out of more than 190 countries analyzed by WHO, only 20% were non-endemic (there is no data from 47% of the countries) (<https://apps.who.int/gho/data/node.main.NTDT SOL1?lang=es>; last updated 2018-12-12). Worldwide about 66 million people are estimated to be infected with *T. solium*, mainly in Sub-Saharan Africa, Latin America and South and South-East Asia (Pal et al.,

2018). According to a systematic review carried out in endemic countries in 2015, the estimates for the prevalence of circulating *T. solium* antigens or antibodies for Africa, Latin America and Asia were: 7.30%, 4.08% and 3.98%, respectively. Seroprevalence estimates of *T. solium* antibodies were 17.37%, 13.03% and 15.68%, respectively. Taeniasis reported prevalences ranged from 0% to 17.25% (Coral-Almeida et al., 2015).

Since routine diagnostic methods to identify human taeniasis lack specificity and usually cannot differentiate among the three species (see “Diagnosis” section), the true burden of *T. solium* taeniasis estimated by those techniques can only be an approximation. On the contrary, if serodiagnostic methods are employed, its prevalence may be an overestimate of its real prevalence due to transient antibodies. In fact, and due to its public health importance, the NCC burden has been analyzed by two different research groups. NCC-associated epilepsy Disability Adjusted Life Years (DALYs) were estimated at 0.5 million by The Global Burden Disease. However, the WHO’s Foodborne Disease Burden Epidemiology Reference Group obtained a much larger estimate at 2.8 million (Carabin et al., 2017). The total number of people suffering from NCC, including symptomatic and asymptomatic cases, is estimated to be between 2.56 and 8.30 million (OPS, 2019). These contrasting results reveal uncertainties regarding the epidemiology of *T. solium*.

Improved pig rearing conditions seem to have eliminated the parasite in most Western European countries. However, little is known about the current true endemicity status of *T. solium* throughout Europe. Although autochthonous *T. solium* taeniasis/cysticercosis may be possible in Europe, peer-reviewed literature does not provide sufficient information on the current endemicity status of *T. solium* in Europe (Devleeschauwer et al., 2017). Indeed, only a few case reports were available from Eastern European countries.

According to Cantey et al. (2014), data on the prevalence of taeniasis, or adult tapeworm infection, in the United States is scarce. The few population-based taeniasis studies suggest prevalence may be 0.5–3% in selected populations. Moreover, there is also a lack of data on the seroprevalence of cysticercosis in the United States. The limited understanding of the NCC burden is caused by multiple factors, including the fact that the disease is only reportable in Arizona, California, New Mexico, Oregon, and Texas.

Although *T. solium* is no longer endemic in industrialized nations, where livestock is raised commercially with regulations to ensure minimal contact with human feces, the prevalence there has been increasing in recent years due to immigration. Also, specific identification has increased due to improved diagnostic techniques.

Taenia solium epidemiology

The human factor

The tapeworm life cycle is propagated via foodborne and fecal-oral transmission (Fig. 7). Consumption of uninspected pork meat is the major source of human *T. solium* taeniasis. To circumvent the requisition of infected pigs, owners of a small number of pigs, especially in rural areas, use them for their own consumption or they are illegally sold, thereby escaping control (Murrell, 2005). In addition to an inadequate inspection of pork, other risk factors for infection include age, open sewers and improper disposal of human feces, poor education, and the inability to recognize infected pigs. Local techniques for food preparation can also influence the spread of the disease.

Although humans usually harbor only one intestinal adult, multiple infections can be higher than 40%, reinfection being a common phenomenon in meat eaters. Although it has been accepted that taeniasis is more frequent among individuals between

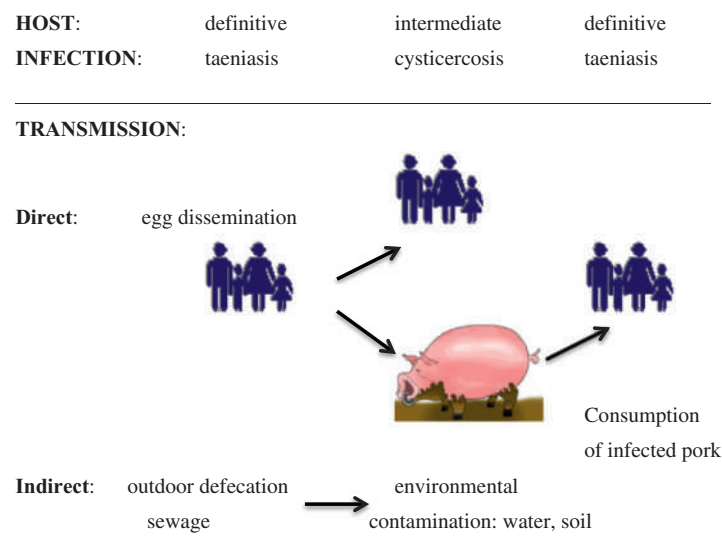


Fig. 7 Graphical scheme of *Taenia solium* transmission.

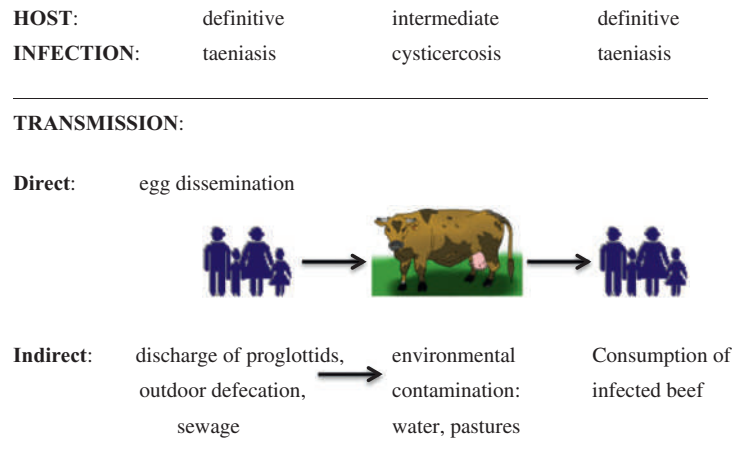


Fig. 8 Graphical scheme of *Taenia saginata* transmission.

20 and 40 years of age, a study in the Democratic Republic of Congo revealed that the highest positivity was found in the 5–10 year age group (Madinga et al., 2017).

Human travel and migration undoubtedly favor the spread of the parasite considering that human taeniasis usually occurs asymptotically. Infected individuals may even be unaware of their condition, since gravid proglottids are shed only by defecation, therefore, probably being unnoticed by the patient.

Cysticercosis, in pigs as well as in humans, occurs following the ingestion of eggs. The very high fecundity of the tapeworms facilitates this transmission, that in the case of human-to-human (Fig. 8), can be caused by the accidental ingestion of eggs contaminating food and water as well as by eggs directly ingested via dirty hands. This person-to-person transmission explains the presence of human cysticercosis cases among people who, for religious or dietary reasons, do not consume pork (Schantz et al., 1992).

Regarding NCC, the majority of infected individuals is between 20 and 50 years of age (Hoberg, 2002). People do not need to harbor an intestinal worm to acquire cysticercosis since it is caused by the ingestion of eggs, but about 15% of patients with NCC do harbor a tapeworm at the time of diagnosis (García et al., 1999; Gilman et al., 2000) and 25% report having had one at some point (Dixon and Lipscomb, 1961). External auto-infection (anus-hand-mouth) seems to be more probable than internal auto-infection by reverse peristalsis of the intestine (Murrell, 2005), although prevalence of antibodies to oncospheres suggests that this may occur more frequently than previously thought (Bowman et al., 2006). Extraordinarily, cysticercosis can even be acquired by bizarre medical practices that ignore the infective ability of the eggs (Bourke and Petana, 1994).

The environment

The uterus of a gravid proglottid of *T. solium* can harbor up to 55,000 eggs. The segments are detached from the strobila in groups (Table 2). Thereby, *T. solium* can shed up to 300,000 eggs per day into the environment (Pawlowski, 2002).

According to the Environmental Classification of excreta-related infections elaborated by WHO (2004), *T. solium* and *T. saginata* belong to Category IV, i.e., latent and persistent infection; pigs and cattle as intermediate hosts and successful transmission.

The survival of parasites outside the gastrointestinal tract of the host depends on the environmental conditions. There are many abiotic and biotic variables that affect the eggs. Among them there are physical factors (temperature, sunlight, humidity, etc.), chemical ones (ammonia, salt, acids, etc.) as well as biological ones (fungi, protozoa, and invertebrates) (WHO, 2004). Factors influencing egg survival and infectivity have been studied in several members of the genus *Taenia*. Infective *Taenia* eggs can survive for up to 9 months in the environment. However, temperatures above 60 °C, pH 12 and desiccation lead to rapid death of the eggs. Conversely, humidity and temperatures above 10 °C and room temperature favor egg survival. A number of agents such as wind, water and some invertebrates and birds are believed to aid taeniid egg dispersal (Pawlowski, 2002). *Taenia* eggs are able to survive in sludge. As a consequence, a potential route of transmission is the irrigation of pasture with wastewater or using domestic sludge as fertilizer for soil.

Livestock: Pigs

The third link of the epidemiological chain is livestock as reservoir of the disease. Pig cysticercosis considerably affects pork meat quality and entails substantial economic losses in the affected areas where *T. solium* is endemic. In these endemic areas, *T. solium* porcine infections have been associated with poverty, absence of latrines and free access of scavenging pigs to human feces. In many low income countries, pigs are usually poorly fed, are not confined and are allowed, and even encouraged, to roam about free in search of food. These free roaming pigs, in combination with the generalized practice of open-air fecalism that prevails, end up ingesting a significant amount of human feces, some of which are contaminated with *T. solium* eggs. In the worst social conditions, pigs are deliberately placed to graze in terrains used for public defecation or near rural out-houses. In rural areas, piglets become

infected at the age of 4 weeks, i.e., immediately after weaning. In tropical areas, infection in piglets is higher during the dry and hot season; this paradox arises from adult animals being reluctant to move at high temperatures, whilst piglets remain active (De Aluja et al., 1998), and, as a consequence, have easy access to fecal material during the hot season.

The prevalence of porcine cysticercosis in endemic countries usually varies between 0% and 56.6%, 1% and 61%, and 0.02% and 77% in Africa, Latin America and Asia, respectively (Murrell, 2005; Wu et al., 2017). *T. solium* has been eradicated in the European Union (EU), primarily due to the change toward industrial pig farming, in which the animals are raised in controlled housing conditions with no outside access. Thereby, the probability of pigs feeding on human feces is very low. However, since 2007, according to the European Food Safety Authority (EFSA), cysticerci consistent with *T. solium* have been detected in pigs slaughtered in five countries of the EU (Austria, Estonia, Lithuania, Poland and Romania) (EFSA, 2010). The entry of tapeworm carriers into the EU seems a lot more plausible than the import of infected pork (Gabriel et al., 2015).

Taenia saginata distribution

The most common and widely distributed human *Taenia* tapeworm is *T. saginata*. Infection in humans has been reported in 45 countries and 45–60 million people are affected (Pal et al., 2018). *T. saginata* is common in cattle-breeding areas of the world. The areas with the highest prevalence (up to 27%) are found in Central Asia, the Near East, and Central and East Africa. Areas of lower prevalence (<1%) are found in Europe, Southeast Asia, Central America, and South America. Consumption of “measly” (cyst-infected) raw or undercooked beef is the usual means of transmission. Rare steak or kebabs and steak tartare are dishes typically associated with *T. saginata* infection.

Data published between 1990 and 2017 on *T. saginata* taeniasis and cysticercosis occurrence and prevalence in Europe, America, Asia and Africa has been gathered by The European Network on Taeniosis/Cysticercosis (CYSTINET) (<https://www.biomedcentral.com/collections/GDTS>).

Taenia saginata epidemiology

The human factor

As in the case of *T. solium*, the tapeworm life cycle is propagated via foodborne and fecal-oral transmission (Fig. 8). Unlike *T. solium*, the eggs of *T. saginata* are only infective for cattle and not for humans. Therefore, taeniasis *saginata* in humans is not the serious public health threat that taeniasis *solium* is, although the economic burden of bovine cysticercosis should not be underestimated.

Human taeniasis results from the consumption of contaminated beef that has not been frozen or thoroughly cooked containing viable *Cysticercus bovis*. In pastoral societies raw or rare meat is part of the normal diet, however, *T. saginata* taeniasis is, in many developed countries, also associated with the increased consumption of raw and rare beef.

Taenia saginata has been reported in children below 1 year of age as well as in adults over 80, although infection is most common in the 20–40-year age group.

There are three main patterns of transmission of *T. saginata*: (i) hyperendemic, characterized by pastoral farming in areas with a high prevalence of human *T. saginata* taeniosis and bovine cysticercosis; (ii) endemic, characterized, by the existence of a small number of human carriers, a wide dispersal of eggs in the environment and a moderate prevalence of bovine cysticercosis mostly of low intensity; and (iii) epidemic, characterized by feedlot situations in which a single human carrier, whose close contact with a herd of susceptible cattle can result in a massive outbreak of bovine cysticercosis (Murrell, 2005).

The environment

In the epidemiology of *T. saginata*, the presence of eggs in perianal zones, clothes, hands, as well as the shedding of gravid proglottids independent of defecation (up to five to 15 per day) cause humans to contaminate the environment to a greater extent than in the case of *T. solium* taeniasis.

T. saginata eggs have been found to survive in the environment for several months. In a damp temperate climate, the eggs of *T. saginata* can survive in the environment for about 10 months, remaining viable for 130 days in water, which represents a major way of dissemination of the eggs (Murrell, 2005). Eggs could be activated after 4 months of being kept in a stream of water at temperatures ranging from -10°C to 17°C , as well as after 1 week of constant freezing at -18°C , or repeated freezing and thawing from -6°C to 5°C . Therefore, *T. saginata* eggs can survive in cold environments, such as the Northern European winter, and pose a significant risk of transmission (Bucur et al., 2019).

There are several ways in which the eggs of *T. saginata* can become available to cattle through sewage. In systems which use primary settling tanks, grit tanks, sedimentation tanks or aeration tanks, eggs in the effluent will pass through these systems into rivers or farms using sewage. Flooding of the rivers will ensure that the eggs are dispersed onto livestock pastures. If the outlet falls directly into the sea from a raw sewage collection plant, segments will be present and the eggs can then be disseminated onto pastures by birds.

Livestock: Cattle

According to Taylor et al. (2007), in cattle, there are two quite distinct epidemiological patterns found in low income and industrialized countries, respectively. In certain parts of Africa, Asia and South America cattle are reared on an extensive scale, frequently human sanitation is poorly developed and cooking fuel is expensive, which leads to a high level of human infection with

T. saginata (up to 20%). As a consequence, cattle are usually infected in early life, often within the first few days after birth. Based on routine carcass inspection, the infection rate is often around 30–60%, although the real prevalence is considerably higher.

In industrialized countries (mainly countries of Europe, North America, Australia and New Zealand), the standards of sanitation are high and meat is carefully inspected and generally thoroughly cooked before consumption. In such countries, the prevalence of bovine cysticercosis is low, being less than 1% of carcasses inspected. Occasionally however, a cysticercosis “storm,” in which a large proportion of cattle is infected, has been reported on certain farms. In Britain and Australia, this has been associated with the use of human sewage on pasture as a fertilizer in the form of sludge, i.e., sedimented or bacterial-digested feces. Other causes of a sudden high incidence of infection on certain farms are due to a tapeworm infection in a stockman occurring either as a random event or as the result of the presence of migrant labor from a country with a high prevalence of infection.

***Taenia asiaticus* distribution**

So far, *Taenia asiaticus*, also known as the Asian *Taenia*, shows a geographical distribution exclusively linked to the Asian continent, and was primarily found only in South-East Asian countries. However, with the introduction of molecular methods in epidemiological studies, its far more extensive geographical distribution was laid bare. Hitherto, the species has been found in Korea, Taiwan, the Philippines, China, Thailand, Indonesia, Vietnam, Japan, Lao PDR, Nepal and India (Eom et al., 2020). In Bangladesh, its presence is suspected but it would require molecular confirmation. *T. asiaticus* prevalence can reach up to 21% and is even becoming the dominant human *Taenia* tapeworm in several countries (Eom et al., 2009).

Considering that *T. asiaticus* has cosmopolitan hosts (humans and pigs), it seems strange that globalization has not affected it (Galán-Puchades and Fuentes, 2014). The lack of identifying this parasite outside Asia is probably due to misdiagnosis since eggs as well as gravid proglottids, have the same morphology as *T. saginata*. Therefore, unless molecular techniques are applied in diagnosis, *T. asiaticus* can easily be confounded with *T. saginata* outside Asia as, aforementioned, was the case in Asia for more than 200 years (Eom, 2006).

***Taenia asiaticus* epidemiology**

The human factor

According to the clear hepatic tropism of the cysticercus of *T. asiaticus* in pigs, the main risk factor for humans is the consumption of raw or undercooked infected pork liver or foods containing it. Although the consumption of pork liver is generally less popular than that of pork meat, this liver tropism does not seem to be the reason of its, so far, exclusively Asian distribution, since pork liver is a cheap and ubiquitous food consumed in whatever non-Islamic country and Israel (Fig. 9). The possibility of becoming infected when consuming raw or undercooked pork meat also exists since cysticerci of *T. asiaticus* have also been found in the muscles of naturally infected pigs (Singh et al., 2016).



Fig. 9 Pork liver trays in a Spanish supermarket.

In Asian countries, the transmission of *T. asiaticus* is often related to social, cultural, and religious practices that entail the consumption of raw pork liver. Ale et al. (2014) reviewed the different ethno-geographical foci where *T. asiaticus* has been studied. The consumption patterns could explain the fact that prevalence increases with age and its apparent predominance in males.

For more than 30 years, the passing of motile proglottids from carriers has been reported in 3% of all cases (Eom et al., 2020). It is still not known whether *T. asiaticus* eggs are able to produce human cysticercosis (Galán-Puchades and Fuentes, 2013a).

The environment

No specific study has been carried out on the survival or dispersal agents of *T. asiaticus* eggs, although the same results obtained with other taeniid eggs could be expected for this species.

Livestock: Pigs

T. asiaticus grows to young cysticercus in the visceral organs of pigs within 1–2 months after the ingestion of the eggs. The metacestodes are mainly found in the liver parenchyma and to a lesser extent on the liver surface. Other viscera, such as lungs, omentum, serosa and mesentery, may also harbor cysts (Eom et al., 2020). *C. viscerotropica*, as already mentioned, has also been found in the muscles of pigs, which increases the likelihood of human infection.

Pork is a staple of the diet in South-East Asia. In fact, it is a common practice in many rural parts to house pigs and other animals under the floor of the house, creating an ideal environment for its transmission. Consumption of pork meat and viscera was far more common than that of beef, yet *T. solium* had a surprisingly low prevalence compared to *T. saginata* in certain Asian countries. This epidemiological paradox led investigators to hypothesize that another *Taenia* parasite might be present, closely resembling the *T. saginata* morphology but the *T. solium* life cycle, and, hence, *T. asiaticus* was subsequently classified (Eom et al., 2020).

In spite of the presence of *T. asiaticus* human taeniasis in 11 Asian countries, according to the literature search carried out by Braae et al. (2018) in certain countries of East and South-East Asia, the presence of *T. asiaticus* porcine cysticercosis was only confirmed in one country (Lao PDR). However, Simanjuntak et al. (1997) found that approximately 23% of pork liver samples in Bali, Indonesia, harbored *T. asiaticus* metacestodes and 1.01% was found in South Korea (Eom et al., 2020).

The scarcity of data indicates that *T. asiaticus* porcine cysticercosis is vastly underreported, and that more epidemiological survey data are required in order to determine the burden of this parasite within Asia as well as in the rest of the world. Due to the small size of the cysticerci and their location in pigs, routine post-mortem inspection lacks sensitivity, making the disease practically undetectable. In experimental infections, 88.9% of the small cysticerci were located in the hepatic parenchyma. Consequently, unless slices of 2–3 mm were made, cysticerci could not be found in livers, and this procedure is not carried out in any routine inspection. This scenario makes it currently almost impossible to determine if the parasite is present or not on a worldwide scale (Galán-Puchades and Fuentes, 2014).

Clinical aspects and human response to the parasite

Taeniasis

Taeniasis is generally a silent disease. In spite of the size of human *Taenia* spp. and regardless of the species involved, intestinal taeniasis is usually asymptomatic. Only mild and non-specific symptoms are described as associated to intestinal taeniasis. Among those infected with *T. saginata*, the most common sign of the disease is the unpleasant sensation due to the discharge of proglottids, independent of defecation. Abdominal pain, nausea, diarrhea or constipation, changes in appetite, weakness, weight loss, pruritus at the perianal area, and general malaise are other symptoms that a small number of patients experience. On occasions, neuropsychiatric disturbances usually appear after diagnosis, when the patient is aware that a several-meter long parasite inhabits his/her intestine.

Exceptional symptoms gathered from literature include toxic effects due to metabolites of the parasite, Binge Eating Disorder (Fernández-Aranda et al., 2000) and iron deficiency anemia (Shorbagi et al., 2012). Wani et al. (2018) and Karanikas et al. (2007) described, respectively, two cases of bowel obstruction due to *T. saginata* and the latter also reviewed other rare complications such as acute obstruction of the Wirsung duct and pancreatitis, gangrenous cholecystitis, Meckel's diverticulitis, acute abdomen following rupture of a liver abscess caused by the presence of *T. saginata* in the right lobe, acute appendicitis and cholangitis.

There are only few studies on the immune response in patients suffering from taeniasis. By contrast, numerous studies have been made on cysticercosis to determine the mechanisms of immune response directed against the cysticerci of *T. solium*. Recently, Prodjinotho et al. (2020) reviewed the host immune responses during NCC infection. This response to the cyst is remarkably complex and diverse. The immune response to the parasite is of type 2 as observed in the majority of affected individuals with asymptomatic infection, however, it can change during symptomatic diseases. Regarding humoral responses in NCC, several antibody classes are produced during the clinical course of NCC, with the most prominent being IgG in serum and CSF. In extraparenchymal infection, increased levels of Ig G, IgM, and IgE were observed in symptomatic patients (Prodjinotho et al., 2020).

Cellular immune responses during NCC are diverse and distinct with regard to the localization and stages of parasite in the brain and mostly limit the progression of the disease. After months to years in the brain tissue, the cysticerci start to degenerate, either naturally or due to anthelmintic treatment, and lose their ability to regulate the host response. Thus, an inflammatory immune

response characterized by an increase in interleukin 1 beta, tumor necrosis factor alpha, and interferon gamma production is instigated (Prodjinotho et al., 2020).

Regarding the interaction with the host, *T. solium* is the species that deserves more attention considering that its adult stage produces the eggs, i.e., the source of human and pig cysticercosis infection. Due to the scolex fixation by means of the suckers and the hooks, there is local damage and an inflammatory response in the upper small intestine that involves mast cells and goblet cells and varied cell populations including plasma cells, lymphocytes, neutrophils and eosinophils (Willms, 2008). Stage-specific antibody responses have been identified in serum from *T. solium* tapeworm carriers. However, considering that humans can suffer from taeniasis and cysticercosis at the same time, it is difficult to determine whether the antibodies detected could be due to the adult or the larval stage (Correa and Medina, 1999).

Human tapeworm carriers are the main risk factor for acquiring cysticercosis. Since humans are the only definitive hosts, different experimental models of *T. solium* taeniasis have been explored in order to study humoral and cellular immune responses in the intestinal mucosa, mesenteric lymph nodes, spleen, serum, saliva and feces of experimental hosts for immunodiagnosis and vaccine development (Flisser et al., 2010).

Cysticercosis

When humans accidentally ingest *T. solium* eggs, within 2 h of liberation, oncospheres enter submucosal blood and/or lymphatic vessels and migrate to a number of internal organs. *T. solium* cysticercosis can occur in almost any part of the body. In humans, cysticerci are most commonly found in skeletal muscles and in the CNS (NCC), mainly in the brain although they can also be found in the skin, subcutaneous tissue, eye (ophthalmocysticercosis), and heart. Although embryos are distributed to all tissues, they are commonly destroyed by the human immune response, thus surviving, preferentially, in the brain and eye (García et al., 2020).

Cysticerci in humans (and pigs) can be found in different morphological states; some are clearly cystic with intact structures (vesicular) while others seem coagulated and their structures somewhat disassembled (colloidal), or, they may be necrotic and sometimes calcified, partially or totally (calcified) (Sciutto et al., 2000). All these forms of the cysticerci possess the potential of causing functional disturbances due to space occupation and/or local inflammation. Viable cysticerci have been shown to use multiple active mechanisms of immune evasion even covering themselves with host immunoglobulins (García et al., 2020).

When located in the tissues, cysticerci are rarely symptomatic unless they encyst in the eye or in the CNS.

Cysticercosis out of the CNS

Cysticercosis can cause subcutaneous nodules in the skin that, occasionally, become swollen and painful. In muscles or viscera, they do not seem to cause any apparent clinical manifestation beyond detectable masses, although muscular pseudohypertrophy when there is a very high worm burden in skeletal muscles has occasionally also been reported. Cysts in the heart are apparently asymptomatic in most cases and are mostly found in patients with disseminated cysticercal infections (Vaidya et al., 2013). Disseminated cysticercosis, a rare complication of the disease with the involvement of almost any organ of the body, has more frequently been reported in immunocompromised than in immunocompetent patients (Banu and Veena, 2011; Gnanamoorthy and Suthakaran, 2019).

Conversely, ophthalmic cysticercosis is frequently a clinical problem. Ophthalmocysticercosis is the most common intra-orbital parasitic infection. Ocular and orbital cysticercosis has varied clinical manifestations depending upon the site of involvement, stage of the cyst and the host-immune responses. The infection may involve the sub-retinal space, extra-ocular muscles, eyelid and/or lachrymal glands surrounding the eye. The most frequent symptoms are pain in the eyes, blurriness, partial or complete loss of vision and, in extreme cases, complete detachment of the retina. A study, in which 171 patients suffering from orbital cysticercosis were involved, revealed periocular swelling, proptosis, ptosis and diplopia as the main symptoms (Rath et al., 2010).

With the advent of the new imaging techniques, ocular and orbital cysticercosis is now increasingly diagnosed even in non-endemic zones (Dhiman et al., 2017).

Neurocysticercosis

NCC, the infection of the CNS by the metacestode of *T. solium*, is the most common helminthic neurological infection and a major public health problem in most parts of the world, still being a major cause of acquired epilepsy in most low-income countries. In endemic settings, approximately 30% of all epilepsy is due to NCC (WHO, 2016). This causes 50,000 deaths/year, and many of the people affected suffer from active epilepsy, with all the social and economic consequences that this implies (García et al., 2005).

In addition to the cysticerci with the same morphology as those from pigs (cellulose cysticercus), a racemose cysticercus can grow exclusively in the human brain. These peculiar cysticerci show a larger size (10–20 cm), being rounded or lobulated and, importantly, without scolex. These racemose cysticerci usually occur in extraparenchymal sites (e.g., subarachnoid space, meninges). The absence of the scolex is believed to be the result of hydropic degeneration caused by the continuous adsorption of cerebrospinal fluid (Del Brutto, 2002). Between the cellulose and the racemose forms, intermediate forms of cysticerci can also be found in human brains (Rabiela et al., 1989).

NCC is highly pleomorphic and there is no possibility to define a pathognomonic clinical syndrome (Del Brutto, 2013). The clinical presentation of NCC is varied and depends on the stage, number, size, form and location of the larval stages within the CNS and the host's immune response to the parasite. Thus, NCC can be classified following different criteria (Mahanty and García, 2010; Ferrer and Gárate, 2014): (i) cysticerci location: *intraparenchymal* (the most common form) and *extraparenchymal* (subarachnoid

space, ventricles, or spinal cord); (ii) viability of the cysticerci: *viable or active* (single, multiple, massive), *transitional or degenerating* (single, multiple, massive), and *calcified or inactive* (single, multiple); (iii) the evolutionary stages of parenchymal lesions as evidenced by neuroimaging techniques: *viable or vesicular form* (small single or multiple lesions), hypodense rounded images, with a hyperdense nodule inside (scolex), lacking surrounding edema. *Colloidal*, lesions surrounded by edema representing the acute encephalitis phase in which the host's immune system reacts against the parasite. *Nodular-granular*, hyperdense lesions surrounded by edema, referred to as "cysticercus granuloma." *Calcified lesions*: small hyperdense nodules without perilesional edema, being the most common finding in NCC.

Therefore, depending on the location, number and type of cysticerci in the CNS, clinical manifestations can vary from completely asymptomatic infection to severe disease and death. Generally, most of patients with parenchymal brain cysticercosis develop seizures as the main or sole manifestation of the disease. Viable cysts are able to survive for years or even decades with intermittent neurological symptoms. Conversely, extra parenchymal NCC has a direr prognosis. Those patients presenting the subarachnoid and ventricular forms of the disease often display focal neurological deficits (paralysis of extraocular muscles, hearing loss, facial nerve palsy or trigeminal neuralgia, and focal neurological symptoms related to brainstem impairment), acute intracranial hypertension secondary to hydrocephalus, ischemic cerebrovascular complications and cognitive decline.

Racemose cysts usually are encountered in the basal cisterns and can cause an intense inflammatory reaction, fibrosis, and progressive thickening of the leptomeninges at the base of the brain; it is also usually an obstruction of the cerebrospinal fluid circulation, resulting in hydrocephalus and intracranial hypertension as a consequence of the unrestricted growth of the racemose parasite. Mortality rates can reach up to 20% in those who do not receive optimum treatment (Ferrer and Gárate, 2014; García et al., 2014). However, NCC prognosis has apparently improved thanks to better diagnostic techniques and improved disease management.

Other presentations of NCC are subdural and spinal. Radicular pain, weakness, and sensory deficits are common in patients with cysticercosis of the spinal cord (García et al., 2014).

NCC might, in fact, present any neurological symptom, but other manifestations more frequently seen are headache, nausea, vomiting, ataxia, confusion, associated stroke, or involuntary movements (Ferrer and Gárate, 2014; García et al., 2014).

Diagnosis

Taeniasis

Specific diagnosis of human taeniasis is mainly required in order to detect *T. solium* carriers, the source of infection of human cysticercosis. Thomas (2015) analyzed the different diagnostic assays for *T. solium* pointing out their benefits as well as their barriers.

Historically, taeniasis has been diagnosed by microscopic examination of human feces with the aim of finding parasite eggs and/or proglottids. Considering the intermittence in eggs/proglottids excretion, this microscopic method lacks sensitivity. In addition, the finding of eggs only reveals the presence of a tapeworm of *Taenia* genus since the morphology of the eggs of the three human species is the same. Concentration methods, particularly those using sedimentation, increase the diagnostic yield; however, the overall sensitivity of serial stool exams using concentration methods is still suboptimal (García et al., 2020). In fact, the reported sensitivity of microscopy ranges from 0% to 59% (Zammarchi et al., 2017).

The study of well-preserved gravid proglottids allows distinguishing between *T. solium* and the complex *T. saginata/asiaticus*. Proglottids can be pressed between glass slides to count the number of lateral branches (7–13 branches for *T. solium* and 14–32 for *T. saginata/asiaticus*) (Table 2) after India ink injection staining through the genital pore or some other clearing method. If the scolex were retrieved, the presence of the rostellar hooks could also enable the differentiation of *T. solium* from *T. saginata/asiaticus*, but the scolex is rarely available. There are some other morphological differences in mature proglottids to distinguish *T. solium* but the morphological identification of mature proglottids requires laboratory facilities and trained personnel.

Specific techniques to differentiate *Taenia* species include enzyme electrophoresis (glucose phosphate isomerase zymograms), molecular methods [different types of polymerase chain reaction (PCR) (Yamasaki et al., 2004; Mayta et al., 2008; Ng-Nguyen et al., 2017), loop mediated amplification method (LAMP) (Nkouawa et al., 2010), LAMP in combination with dot enzyme linked immunosorbent assay (dot-ELISA) (Nkouawa et al., 2016)] and coproantigen ELISA (CoAg-ELISA). A *T. solium* species-specific CoAg-ELISA (Guezala et al., 2009) showed high sensitivity and specificity. Unfortunately, it is experimental, and rarely available except to those working in this field. Similarly, neither enzyme electrophoresis nor molecular methods are available in most laboratories.

Summarizing, the following tools are recommended for the diagnosis of *T. solium* taeniasis (Zammarchi et al., 2017): (i) investigating the history of proglottid expulsion within the past year, with the support of visual materials to help the subject identify the proglottids; (ii) microscopic and macroscopic examination of stool samples collected on three different days; (iii) *Taenia* species identification should always be performed, especially in subjects exposed to *T. solium* in highly endemic countries (Latin America, Asia and Africa).

Cysticercosis

Cysticercosis out of the CNS

Palpable subcutaneous nodules can sometimes be felt and diagnosis is confirmed by biopsy. X-rays will only detect old cysts that have calcified. Ultrasonography may enable the visualization of the echogenic scolex in viable cysts (Satya et al., 2016).

Imaging studies are the most helpful in establishing the diagnosis of ophthalmocysticercosis. High resolution ultrasonography (USG), computed tomography (CT) and magnetic resonance imaging (MRI) help in the detection of the orbital cyst. The clinical diagnosis of live intraocular cysticercosis is based on the morphology of the parasite as visualized with the ophthalmoscope or slit-lamp biomicroscope (Dhiman et al., 2017).

Neurocysticercosis

Currently, a combination of neuroimaging tools and immunodiagnosis is used to identify cysticerci in the SNC. CT and MRI are safe and precise techniques (providing data on the number, localization, stage of the lesions and inflammation status). However, as they are also expensive ways of diagnosing NCC, they are usually scarce in many impoverished endemic areas of the world. Routine hematological tests are of poor use and, to date, molecular tests are not sensitive enough to be directly applied for routine case assessment (García et al., 2020).

When neuroimaging is inconclusive, the diagnosis can be confirmed with serology. Antibody detection is more frequently used given its higher sensitivity. The assay of choice for antibody detection is the enzyme-linked immunoelectrotransfer blot (EITB) assay, using lentil lectin purified parasite glycoprotein antigens (LLGP), which is available through the CDC Parasitic Disease Reference Laboratory (García et al., 2018). EITB sensitivity is around 98% for patients with two or more live parasites in the nervous system. However, the sensitivity is low (50–60%) in patients with only one intracranial cysticercus. The sensitivity is, in turn, higher in serum than in CSF (100% vs. 90%) (García et al., 2014). EITB specificity seems to be 100%, although pigs infected with *T. asiaticus* showed positive bands when tested with the EITB assay (Pilcher et al., 1991). The antibody detection in CSF by ELISAs in patients with more than one viable cysticercus is 89% sensitive and 93% specific. ELISAs are used when EITB is not available (García et al., 2014).

The detection of parasite antigens confirms the presence of viable parasites. Circulating antigens are only present as long as the infection is active. That is why the detection of antigens by ELISA with monoclonal antibodies in serum (the duration of antigens in CSF is unknown) is used to monitor the effectiveness of treatment or surgery (García et al., 2014).

Considering that tuberculosis and NCC show similar clinical and radiological features, a panel of diagnostic criteria for NCC was elaborated by Del Brutto et al. (2001), which has recently been updated by García et al. (2020). These diagnostic criteria are organized into absolute, neuroimaging, and clinical/exposure criteria. Likewise, proper interpretation of these criteria allows two degrees of diagnostic certainty, definitive and probable. These criteria have been widely used for the diagnosis of NCC in both hospital and field settings and have proven useful in areas of endemicity and non-endemicity.

Treatment

Taeniasis

Two drugs are the most effective against the three species of human *Taenia*, niclosamide and praziquantel. Niclosamide has little absorption, and therefore, no effect on NCC. However, praziquantel is well absorbed after oral administration and it is active not only against the adult stages of *Taenia* spp. but also against the larval stages. In the case of human taeniasis caused by *T. solium*, the patient could probably be infected by the adult stage as well as by the cysticercus. Therefore, to avoid that possible previously silent brain cysts can trigger seizures or other neurological symptoms as a consequence of the treatment with praziquantel (Flisser et al., 1993), niclosamide is recommended in *T. solium* taeniasis cases.

According to the systematic review conducted by Haby et al. (2020), the treatment with niclosamide 2 g for adults, praziquantel also in a single oral dose of 10 mg and triple-dose albendazole 400 mg, seem to be effective as taenicides in mass chemotherapy for *T. solium* taeniasis. However, White et al. (2020) are critical of the systematic review elaborated by Haby et al. (2020), since with respect to the analysis of efficacy, the authors did not account for differences in the methods used to ascertain the outcomes in the studies analyzed.

Neurological side effects in *T. solium* carriers are a potential danger in albendazole treatment that, like praziquantel, also crosses the blood-brain barrier (Sotelo and Jung, 1998).

Cysticercosis

Cysticercosis outside of the central nervous system and the eye is usually asymptomatic and does not require therapy. Untreated intraocular cysticercosis incites severe ocular inflammation, more so when the cyst dies. Anthelmintic therapy can lead, in turn, to severe inflammation in the event of a live cyst degenerating, hence, surgery is the treatment of choice.

In NCC cases, a single therapeutic approach does not exist. Depending on the viability, size and location of the cysts as well as the severity of the host immune response, a rational treatment has to be planned (García and Del Brutto, 2000). The treatment of NCC involves (Nash, 2003; Quintana, 2013): (i) prevention and health monitoring; (ii) anthelmintic drugs, which induce the death of

the larva and tapeworm; (iii) corticosteroids and other agents, which reduce or prevent the inflammatory phenomena related to the involution of the cyst, either spontaneous or induced; (iv) antiepileptic drugs, which reduce the frequency of or suppress seizures; (v) osmotic diuretics to treat increased intracranial pressure; and (vi) surgical procedures to treat increased intracranial pressure, hydrocephalus, and the mass effect of some lesions.

Related to the use of anthelmintic treatment in NCC, unfortunately, the efficacy of the two available cysticidal drugs, albendazole and praziquantel, is suboptimal, with cure rates between 40% and 50% at the doses usually recommended (Nash et al., 2013). The main point of the debate on the treatment of NCC is centered on whether the destruction of parasites by the antiparasitic drugs has clinical benefits to patients. There are arguments in favor (disappearance of the cysts, better clinical evolution of the treated patients, less severe cases), as well as against (antiparasitic treatment can cause severe and unnecessary brain inflammation or stroke which can be fatal, NCC becomes symptomatic only when the parasite naturally dies years after) (García et al., 2017). In 2002, a relative consensus was adopted among the specialists involved in the disease, and guidelines for the use of antiparasitic treatment in NCC were published (García et al., 2002).

Surgery is reserved for selected patients with NCC and plays a minor role in the management of the disease. The commonest indication is for the excision of intraventricular cysts, with endoscopy surgery being the procedure of choice as it is minimally invasive (Rajshekhar, 2010).

Control

Transmission of taeniasis has been largely eliminated in industrialized countries by improving sanitation and meat inspection. The intervention measures in the three *Taenia* species to interrupt the life cycle can be targeted at human level (tapeworm carriers as the infective source of infection of human, porcine and cattle cysticercosis), and at pig and cattle level (infected pigs and cattle as reservoirs of human taeniasis). Most of the control interventions target *T. solium* in order to prevent human NCC.

At human level

Ignorance is a major obstacle for the effective control of whatever disease. Even small changes in the behavior of people living in endemic areas by means of public education can affect parasite transmissions. In this sense, Johansen et al. (2014) developed an inexpensive, locally-adaptable, and implementable computer-based education tool, "The Vicious Worm," to improve the knowledge concerning *T. solium*.

By not eating raw or poorly cooked pork (*T. solium*, *T. asiaticus*) or beef (*T. saginata*), the likelihood that a person becomes infected is considerably reduced. Meat should be cooked at a temperature of between 60 °C and 65 °C, or until it loses its pink color, to ensure that cysts are killed (Okello and Thomas, 2017).

Improvements in personal sanitation (personal hygiene, washing hands after defecation) and safe disposal of feces (avoiding open defecation) could prevent human and porcine/bovine cysticercosis.

A limiting factor of the widespread application of health education is, on one hand, the high cost of such programs (Pawlowski et al., 2005), which also impacts the sustainability of this measure of this control measure. On the other hand, in cases in which health education has been included together with other intervention measures against *T. solium*, education appears to have had a limited impact (Lightowers, 2013; Thomas, 2015).

Preventive chemotherapy is defined as the use of anthelmintic drugs, either alone or in combination, as a public health tool against helminth infections (WHO, 2006). Treatment of *Taenia* spp. carriers can be implemented in three ways (Gabrielli et al., 2011): mass drug administration (MDA) (the whole population is treated), targeted chemotherapy (only specific risk groups are treated), and selective chemotherapy (only patients, according to clinical status, are treated). Both, MDA and selective chemotherapy have been recommended for the control of *T. solium* and some evidence is available on their relative efficacy and cost-effectiveness.

The multiple control initiatives implemented in Ecuador, Guatemala, Mexico, and China were reviewed by Thomas (2015), concluding that (sic). "Despite inconclusive evidence on the efficacy and cost-effectiveness of identification and treatment of taeniasis as a control strategy, the epidemiological basis of removing carriers from the population is undeniable. We would strongly recommend that all in-contacts of NCC cases are treated (2 g niclosamide for safety) as standard (Lian F. Thomas and Andrea Winkler)".

At porcine/bovine level

A key point in the propagation of the *Taenia* spp. lifecycle is the contact between pigs/cattle and human fecal material containing infective eggs. Hygiene measures such as best practices in animal husbandry and a correct management of sewage sludge and wastewater contribute to the prevention of the intermediate host becoming infected.

To prevent infection, chemoprophylaxis in pigs has been widely suggested as a control strategy. Besides, several vaccines against *T. solium* pig cysticercosis have been developed, of which two have progressed a great deal and have shown a high degree of potential for use in control strategies, namely SP3Vac and TSOL18 (Cysvax® produced by India Immunological Limited). Vaccination of

cattle against *T. saginata* with the TSA9/TSA18 vaccine has been attempted with some success. However, it does not seem that this vaccine is going to be commercialized any time soon (Thomas, 2015).

Once infected, porcine cysticercosis can be treated with a series of anthelmintics (Lightowlers, 2013; Thomas, 2015) of which oxfendazole (one single dose of 30 mg/kg) has demonstrated the best efficacy with a rapid reduction in cyst viability with 50% of cysts found to be nonviable at 1 week post-treatment and after 12 weeks all muscle cysts appear to be nonviable. Bovine cysticercosis also responds to anthelmintic treatment with praziquantel, and protection against reinfection appears to last for at least 12 weeks. Despite its efficacy, praziquantel has not yet been formulated for cattle and is, therefore, not commercially available as a control measure for *T. saginata* (Okello and Thomas, 2017).

Once dead, post-mortem detection of cysticerci in pigs and cattle by means of visual inspection of the carcasses is fundamental to prevent human taeniasis. However, meat inspection lacks sensitivity, especially in cases of low cost burden. In addition, in many *T. solium* endemic low income countries infected pigs are diagnosed ante-mortem (tongue inspection) and sold in clandestine markets, thus escaping the condemnation of carcasses (Murrell, 2005).

Cold treatment has been proven to kill the cysticerci of *T. solium* and *T. saginata*, within 6–10 days at temperatures below -10°C and at -24°C in just 24 h (Sotelo et al., 1986; Hilwig et al., 1978). In addition to freezing, gamma-radiation, cooking and salt pickling have been shown to successfully reduce the viability of cysticerci.

Combining methods in the one health approach

Available data suggests that choosing a single approach to control human *Taenia* is not sufficient. The three *Taenia* species cause zoonotic diseases, in which not only humans but also animals are involved. Therefore, to tackle the problem, strategies that target both, the human and animal hosts, should be adopted (Okello and Thomas, 2017).

In this sense, WHO is promoting prevention and control of *T. solium* infection through animals with the One Health approach (<https://www.who.int/activities/promoting-prevention-and-control-of-taenia-solium-infection-through-animals-with-the-one-health-approach>). Specific control measures in the pig population include the vaccination of pigs with the TSOL18 vaccine and the treatment with oxfendazole.

Multiple control initiatives can be found in the literature with different results. One successful example is Peru: a large integrated program combining human and porcine mass chemotherapy, pig vaccination, and coproantigen detection-based case confirmation over a one-year period has proven effective in achieving focal elimination of *T. solium* transmission in a large area on the northern coast of the country (García et al., 2016). Likewise, more recently, the integrated interventions in humans and pigs carried out in a 2-year study in Zambia (Africa) (CYSTSTOP), eliminated viable infection in pigs and significantly reduced the prevalence of taeniasis caused by *T. solium* in the intervened villages (Gabriel et al., 2020).

Future perspectives

Taenia solium: The 2012 WHO roadmap for *T. solium* control and elimination by 2015 or 2020, depending on the countries, has not been met, although notable progress has been made (De Coster et al., 2018). *T. solium* has been included in the consultation process for the new 2030 goals proposed for priority NTDs (CystiTeam Group for Epidemiology and Modelling of *Taenia solium* Taeniasis/Cysticercosis, 2019). According to Dixon et al. (2020), the 2030 target is technically feasible with the current intervention options and tools with the implementation of computational transmission models such as cystiSim (Braae et al., 2016) and EPICYST (Winskill et al., 2017). However, current tools, like diagnostic methods or necropsy of pigs to measure the infection, present many limitations to effectively achieve the target. Existing *T. solium* models can be improved through collaboration between modeling groups, field epidemiologists, program stakeholders and policymakers, and used jointly to support the design, implementation and assessment of interventions in endemic countries (Dixon et al., 2020).

Taenia saginata: Since the eggs of *T. saginata* do not infect humans, NCC is not related to this species, and therefore, the human burden of disease related to this species is assessed as low, although no precise studies have been undertaken. In this sense, the FERG (WHO Foodborne Disease Burden Epidemiology Reference Group) report excluded *T. saginata* from their priority list (FERG, 2015). *T. saginata*, therefore, is mainly of economic interest in the cattle industry due to carcass condemnation. In this sense, *T. saginata* has recently been included in a risk-based surveillance for meat-borne parasites by means of a design tool called SURVTOOLS (Alban et al., 2020).

Taenia asiaticus: An international forum called *Taenia asiatica* Research Group was created in 2011 (TaRG international, [targint.net](http://taeniarsearchgroup.net)) to raise awareness of the existence of this third *Taenia* species, *T. asiaticus*, among veterinarians, health workers and researchers. TaRG is made up of scientists from countries of different continents with the aim of cooperating in the molecular identification of human and pig material (adult and larval stages) around the world. This cooperation is essential to definitely elucidate whether *T. asiaticus* is an exclusively Asian parasite or if it tends to be misdiagnosed around the world. Another gap in the pathogenicity of this species is to ascertain whether *T. asiaticus* is able to cause human cysticercosis (Galán-Puchades and Fuentes, 2004, 2013a,b). Currently, only molecular methods are able to identify *T. asiaticus* in humans and pigs, since morphology and immunology are useless, but unfortunately, although largely used in research, those methods are not employed in routine diagnosis.

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Toxocara

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Introduction

Human *Toxocara* spp. were first described by Wilder (1950). He identified a nematode larva of unknown species in eosinophilic granulomas within the enucleated eyes of children with suspected retinoblastoma, providing the first description of the condition currently known as ocular *larva migrans* (OLM). In 1952, Beaver and colleagues described *Toxocara* larvae in eosinophilic granulomas of the livers of young children with extreme eosinophilia, hepatomegaly, respiratory symptoms, anemia, and geophagia, and introduced the term visceral *larva migrans* (VLM) to describe this clinical syndrome (Beaver et al., 1952). They correctly classified, in histopathological sections of tissues obtained at biopsy, the causative agents as the larva of either *Toxocara canis* or *Toxocara cati*.

Systematics

Among the Nematodes belonging to the genus *Toxocara*, currently 26 species have been described.

The most frequently reported and wide spread zoonotic species is *T. canis*. Zoonotic infection with *T. cati* remains controversial and only a few infections, mostly in children with OLM, have been reported (Beaver et al., 1952; Fisher, 2003). Only a single-suspected case of human infection with *T. pteropodis* of bats has been reported and this species has yet to be confirmed as zoonotic. Of the non-zoonotic species *Toxocara vitulorum* of cattle is of major economic importance and there are other 13 species with minor economic impact (*Toxocara alienata*, *Toxocara apodemi*, *Toxocara genettae*, *Toxocara indica*, *Toxocara leonina*, *Toxocara mackerrasae*, *Toxocara malaysiensis*, *Toxocara paradoxura*, *Toxocara pearsei*, *Toxocara sprengi*, *Toxocara surkattae*, *Toxocara tanuki* and *Toxocara vajrasthira*). Further nine species have been reported only once and only in animals: *Toxocara canis*, *Toxocara cynonycteridis*, *Toxocara elephantis*, *Toxocara herpestum*, *Toxocara hippopotami*, *Toxocara lynx*, *Toxocara manzadiensis*, *Toxocara vincenti* and *Toxocara warren*.

Toxocara spp. have different definitive host species, such as canids, felids, civets, mongoose, cattle, raccoons, bats and rodents (Ziegler and Macpherson, 2019).

Canids and felids (puppies under 6 months of age and kittens between 2 and 6 months old, more frequently) are the definitive hosts of the only 2 species recognized as causal agents of human toxocariasis, *T. canis* and *T. cati*, respectively (Lightner et al., 1978; Roddie et al., 2008).

Epidemiology

T. canis and *T. cati* are ubiquitous parasites of dogs and cats, respectively, worldwide.

For both *T. canis* and *T. cati*, paratenic hosts can act as important reservoirs of infection for the circulation and maintenance of the parasites in the environment (Dubinsky et al., 1995).

Infection rates are defined by the presence of eggs in feces of dogs and cats contaminating the environment.

The remarkable fecundity of *Toxocara* spp. worms explains the high level of environment contamination with *Toxocara* eggs (Roddie et al., 2008; Amaral et al., 2010; Kłapeć and Borecka, 2012).

Female worms may produce up to 200,000 eggs per day. Eggs passed in the feces are not infective and must remain in the soil to become embryonated, then infective (Glickman and Schantz, 1981), depending on the soil type and environmental conditions such as humidity and temperature, and potentially remaining viable in the soil for several years posing an infection risk to humans (Overgaauw, 1997a; Mizgajska-Wiktor and Uga, 2006).

Table 1 lists the positivity rates deriving from the analysis of approximately 43,000 fecal samples, collected in different geographical areas of the World, in 40 Countries (Fakhri et al., 2018).

Humans acquire the infection as accidental hosts ingesting eggs of *Toxocara* spp. via contaminated hands or food (Overgaauw, 1997b).

Since humans are accidental hosts of *T. canis* and *T. cati* and do not develop patent infections, exposure must be estimated using seroprevalence studies, although these have many limitations (first of all the underestimation due to the low sensitivity of the methods used). Seroprevalence of *Toxocara* spp. varies widely worldwide (Rostami et al., 2019).

Seroprevalence surveys carried out in Western countries showed that the positivity rate was between 2% and 5% among apparently healthy adults from urban settlements, whereas it ranged from 14.2% to 37% in rural areas (Holland et al., 1995; Magnaval et al., 2001). When a tropical humid climate is combined with a poor socio-economic status, the transmission level of toxocarasis appears to be significantly higher, as the seroprevalence rate among children was found to be 47% in Salvador, Brazil (Mendonça et al., 2012), 63.2% in Bali (Chomel et al., 1993) and 86.8% in the Marshall Islands (Fu et al., 2014), respectively. The rate was shown to peak at 92.8% in teenagers and adults in La Réunion island (French Overseas Territories, Indian Ocean) (Magnaval et al., 1994).

There are several factors that have been associated with higher rates of infection with *Toxocara*:

- geophagia (common to find in neuropsychiatric conditions such as autism spectrum disorders) (Holland et al., 1995; Won et al., 2008; Pieroni et al., 2021);
- ingestions of raw vegetables or fruits grown in contaminated kitchen gardens /soil (Yoshikawa et al., 2008; Kłapeć and Borecka, 2012; Noh et al., 2012);

Table 1 Globally and regional environmental contamination by eggs of *Toxocara* spp., found in stool samples (in bold the extreme values) (Fakhri et al., 2018).

	Average prevalence (%)	Range
Overall	21	16–27
Europe		
Italy	15	12–19
France	38	21–58
United Kingdom	6	4–9
Ireland	13	9–17
Spain	7	5–8
Germany	23	21–28
Poland	20	14–26
Africa		
Sudan	75	65–83
Nigeria	24	4–47
Kenya	25	16–38
Egypt	13	7–20
Middle east		
Iran	20	13–29
Turkey	16	11–23
America		
United States	12	4–23
Canada	33	19–49
Mexico	25	23–27
Brazil	30	21–39
Asia and Oceania		
Japan	46	5–91
China	75	74–75
Australia	0	0–1

- ingestion of raw liver or other undercooked organs from an infected paratenic host (a host that is not required for the parasite life cycle but nonetheless can serve to maintain the life cycle) like rabbit, chicken, cattle, or swine, containing encapsulated larvae, see below (Nagakura et al., 1989; Lim, 2008; Yoshikawa et al., 2008; Kłapeć and Borecka, 2012; Noh et al., 2012; Yoshida et al., 2016);
- having a dog as a pet or close contact with pets or exposure to playgrounds and sandboxes contaminated by dog feces (Holland et al., 1995; Wolfe and Wright, 2003; Roddie et al., 2008; Hotez and Wilkins, 2009; Amaral et al., 2010; Keegan and Holland, 2010, 2013);
- younger age (Centers for Disease Control and Prevention, CDC, studies have shown that toxocariasis is predominantly a disease of children, typically those aged 2–7 years) (Holland et al., 1995; Centers for Disease Control and Prevention, 2011);
- male > female risk exposition (Holland et al., 1995; Won et al., 2008; Yoshida et al., 2016);
- low sanitation levels (Kroten et al., 2018);
- socio-economic status (this infection is more common in people living in poverty) (Hotez and Wilkins, 2009; Congdon and Lloyd, 2011);
- tropical regions (Azam et al., 2012) and rural areas (Holland et al., 1995; Magnaval et al., 2001);
- ethnicity (studies by the US CDC have found that, among all age groups, toxocariasis is more common in non-Hispanic blacks than in Mexican Americans and non-Hispanic whites) (Centers for Disease Control and Prevention, 2011; Congdon and Lloyd, 2011).

Toxocariasis is currently an important preventable zoonosis occurring worldwide (Won et al., 2008), even if, despite the high levels of seroprevalence that can be found in various geographical regions, it remains relatively unknown in public opinion and it is considered a neglected parasitic infection, that has been targeted by CDC for public health action.

Fig. 1 (1–11) summarizes *T. canis* and *T. cati* life cycle accomplish their life cycle in dogs and cats, respectively, with human acquiring the infection as accidental hosts (Fig. 1).

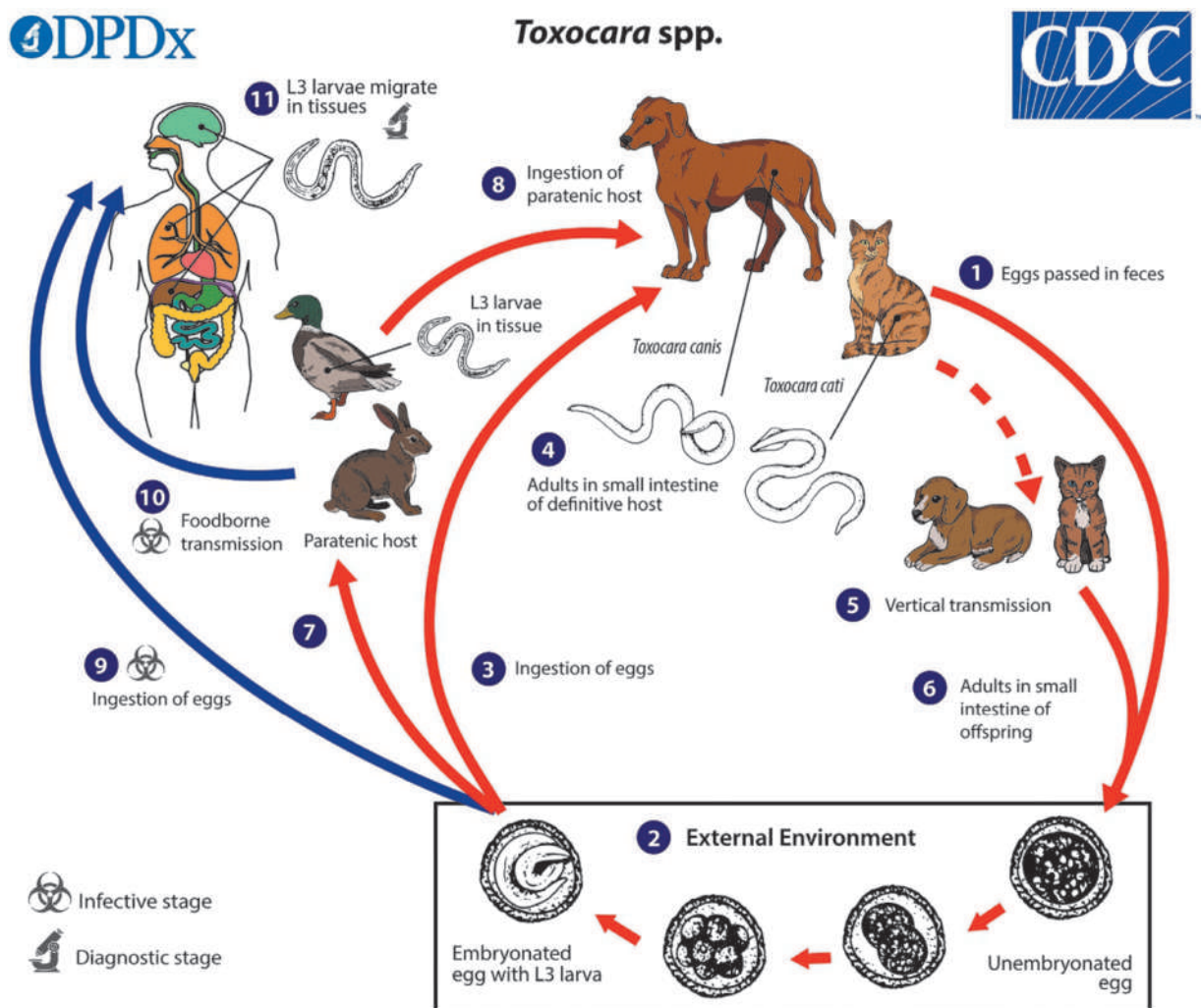


Fig. 1 The life cycle of *Toxocara* spp. (<https://www.cdc.gov/parasites/toxocariasis/biology.html>).

Adult worms reside in the small intestine of dogs or cats where females produce unembryonated eggs which are passed into the environment in the feces, 4–5 weeks after initial infection by *T. canis*, 8 weeks after initial infection by *T. cati* (Overgaauw, 1997a).

In the environment (1) (the numbers in parentheses are referred to those in Fig. 1), eggs embryonate and become infective within 3–6 weeks (2).

Following ingestion of infective eggs by younger dogs or cats (3), the eggs hatch and larvae (0.5 mm in length) penetrate the gut wall (4), enter the venous vessels and migrate via the liver to the lungs where they can pass the capillary blood vessel wall to reach the alveoli (Overgaauw, 1997a); from here, the larvae either migrate up to the trachea, oral pharynx where they are swallowed and returned to the small intestine to develop into adult worms, which then copulate and the females become gravid, producing eggs which will be excreted with feces (1), or undergo visceral migration (Glickman and Schantz, 1981).

Tracheal migration and the development of a patent infection are more common in young dogs (Greve, 1971); in contrast to the dog, tracheal migration and the development of a patent infection can remain high in older cats although it is still less frequent than in younger cats (O'Lorcain, 1994).

Where somatic migration occurs, the larvae do not develop into adult worms and they can remain in a state of arrested development in the tissues of the host for many years (Overgaauw, 1997a). This phenomenon offers two further possible ways of transmission of *T. canis* if the dog becomes pregnant: (a) the larvae can be mobilized from the tissues and migrate across the placenta infecting puppies in utero, leading to tracheal migration in the pup and eggs being shed in the feces 2–3 weeks after birth, or (b) they can migrate to the mammary glands and infect puppies during lactation in which case there is no tracheal migration and the larvae develop to adults in the intestines (Overgaauw, 1997a).

Infection during lactation is possible for *T. cati*, too (Coati et al., 2004), but there is no transplacental transmission.

Toxocara spp. can also be transmitted through ingestion of paratenic host organs. Eggs ingested by small non canine mammals (such as rabbits) can hatch and larvae can penetrate the gut wall with subsequent migration into various tissues where they encyst (7). The life cycle is completed when dogs or cats eat larvae encysted in the tissues of these paratenic hosts (8) and the larvae develop into egg-laying adult worms in the small intestine. It is probably a route of transmission more significant in cats than in dogs, given the predatory nature of cats.

Humans are accidental hosts who become infected by ingesting infective eggs in contaminated soil or food (9) or via ingestion of encysted larvae in the tissues of infected paratenic hosts (11), direct contact with infected puppies and kittens as sometimes pets carry embryonated eggs in their fur (see section on risk factors for human toxocariasis); following ingestion, the eggs hatch and larvae penetrate the intestinal wall and are carried by the circulation to a variety of tissues (liver, heart, lungs, brain, muscle, eyes) (11). The larvae do not undergo any further development in these sites, but the host inflammatory response against the migrating larvae can cause both mechanical and immunopathologic damage to tissues, which leads to local reactions that are the basis of clinical toxocariasis.

Immune response and pathogenesis

Disease severity depends not only on the number of larvae ingested but also on the degree of inflammation caused by the immune response directed against the excretory-secretory (ES) antigens of *Toxocara* spp. larvae (Fig. 2). These antigens are a mixture of glycoproteins, including a potent allergenic component named TBA-1 (Zibaei et al., 2019).

ES antigens stimulate CD4⁺ T helper-type 2 (Th2) lymphocytes (Maizels, 2013; Zibaei et al., 2019). *Toxocara* spp. infection also causes a concomitant reduction in the number of Th1 cells (Zibaei et al., 2019). It is worthy of note that Th2 cells were described for the first time in humans using lymphocytes from *Toxocara*-infected patients, stimulated with ES (Del Prete et al., 1991).

The Th2 responses are specially recognized by the production of the type 2 cytokines interleukins (IL) -4, IL-5, IL-9, IL-10 and IL-13, during infection and each one individually begins different immunological pathways. IL-4 induces B lymphocyte differentiation and antibody class-switching versus IgG1 subclass, with significant levels of IgE (Fillaux and Magnaval, 2013; Zibaei et al., 2019). IL-5 causes the differentiation of eosinophils, and their activation which leads to the production of superoxide anion and other damaging free radicals, major basic protein, etc. (Maizels, 2013; Zibaei et al., 2019).

Apart Th2 responses significant increase in IFN- γ levels have been found in VLM, as evidence of a complex cytokine response (Maizels, 2013).

The granulomatous inflammatory response, characterized by aggregates of eosinophils, neutrophils and monocytes, leads to the encapsulation of larvae and blocks their migration elsewhere (Kayes, 2006). Some larvae are able to escape the confines of these granulomas; indeed, serial sections of an entire granuloma may yield no evidence of the larva which initiated the response (Kayes, 2006).

The granulomatous inflammatory response and the raised antibody titer and eosinophilia appear incapable to control or eliminate *Toxocara* spp. larvae, which has given rise to the idea that the parasite is able to evade host immunity (Maizels et al., 2006). Studies have shown that *T. canis* larvae have a mucin-rich, highly labile surface coat which is loosely attached to the parasite epicuticle, and is shed when antibodies and/or eosinophils bind, allowing the parasite to escape (Maizels et al., 2006).

Toxocara spp. related immunopathological reactions have prompted researchers to investigate the association between *Toxocara* spp. infection and allergic asthma.

Certainly, patients with atopy may experience more severe toxocariasis or vice versa.

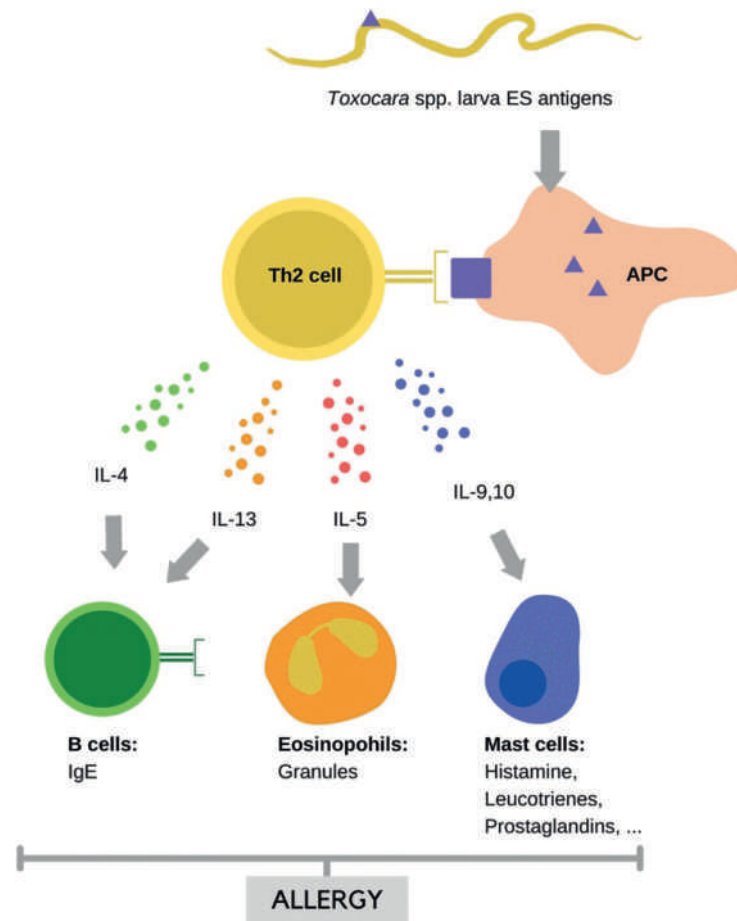


Fig. 2 Allergy and immune responses in toxocariasis.

Clinical manifestations

The degree of host damage and concomitant manifestation of clinical signs and symptoms are determined by the size of the inoculum, the location of the invading larvae and the host immune response (Pawlowski, 2001; Despommier, 2003).

Because the *Toxocara* seropositive population is much common than that with clinical toxocariasis, most patients are thought to be asymptomatic.

The most common characteristic of *Toxocara* spp. infection is chronic eosinophilia, while most of the clinical manifestations vary depending on the organs infected.

Nowadays, four different syndromes have been described: VLM, OLM, neurotoxocariasis (NT) and covert toxocariasis (CT) (Hamilton et al., 2014; Ma et al., 2018).

Visceral larva migrans

VLM occurs most commonly in young children and it is thought to be associated with a larger infectious dose and multi-systemic disorder, resulting in hepatitis and pneumonitis as the larvae migrate through the liver and lungs, respectively. Heavy infection may result in fever, anorexia, malaise, irritability, hepatomegaly, respiratory symptoms, pruritic urticaria-like cutaneous lesions, or other cutaneous manifestations such as transient rash, hypodermic nodules, chronic *prurigo*, miscellaneous eczema and vasculitis (Fig. 3A–C) (Gavignet et al., 2008), very often accompanied by eosinophilia.

Larvae frequently localize in the liver; hepatic manifestations may include hepatomegaly or nodular lesions (Laroia et al., 2012).

Pulmonary involvement may cause dyspnea, wheezing, and a chronic nonproductive cough in 20–80% of patients (Snyder, 1961; Huntley et al., 1965). Rales are common on physical examination.

Severe respiratory tract involvement is an uncommon complication of heavy infection.

Some studies have found a positive association between *Toxocara* spp. seropositivity and asthma in a large cohort of patients (over 11,000 adults with an age ranging between 18 and 65 years), suggesting a diminished lung function in the presence of *Toxocara* infection (Walsh, 2011).



Fig. 3 Cutaneous manifestations associated with toxocariasis. (A) urticaria; (B) chronic prurigo; (C) acquired disseminated eczema (Gavignet et al., 2008).

Granulomatous lesions, induced by the immune response to secreted larval antigens, both in the liver and lungs of infected patients can be mistaken for metastatic cancer (Anderson et al., 2006; Ota et al., 2009); the presence of eosinophilia may be helpful differentiating features.

Larvae can also migrate via the systemic blood circulation to muscles, the heart with myocarditis, pericarditis or Loeffler endocarditis potentially leading to heart failure or cardiac tamponade (Kuenzli et al., 2016), the eye, or the central nervous system (CNS) (Marx et al., 2007; Enko et al., 2009).

Ocular larva migrans

OLM involvement may occur as the sole manifestation; it often presents in individuals without antecedent history of symptomatic VLM (Good et al., 2004). OLM occurs most commonly among older children and adolescents (Hotez and Wilkins, 2009). In one review of ocular toxocariasis in the United States, the median patient age was 8.5 years (range 1–60 years) (Centers for Disease Control and Prevention, 2011).

The ocular lesion is due to larval localization in the eye and the granulomatous response around the larva. Common symptoms are unilateral visual impairment, causing failing vision and subsequent strabismus, leukocoria. The typical lesion is a whitish elevated granuloma. Occasionally, OLM may present as uveitis (often intermediate and posterior uveitis), papillitis, endophthalmitis, scleritis, or chronic endophthalmitis (Stewart et al., 2005). Ocular lesions may resemble retinoblastoma (Chuah et al., 2006). The most serious consequence of infection is invasion of the retina with granuloma formation in the periphery or posterior pole, leading to dragging of the retina and eventual retinal detachment, which can lead to blindness (Suh et al., 2006).

Neurotoxocariasis

Although humans are known to harbor *Toxocara* spp. larvae in the brain, as some studies describe the presence of larvae within granulomas in the CNS, discovered accidentally at autopsy when the patient had died from another cause (Dent et al., 1956; Hill et al., 1985; Nelson et al., 1990), possible clinical pictures of cerebral toxocariasis include eosinophilic meningo-encephalitis, space-occupying lesions, myelitis, and cerebral vasculitis (Deshayes et al., 2016; Dzikowiec et al., 2017). The degree of neurological symptoms is likely to depend on the number and location of the larvae in the brain, the immune response directed against them, and the resulting pathology (Despommier, 2003).

In a meta-analysis including 11 case-control studies and more than 4700 patients with toxocariasis, an association between epilepsy and *Toxocara* spp. seropositivity was observed (pooled odds ratio [OR] 1.69, 95% CI 1.42–2.01) (Luna et al., 2018).

Death due to CNS involvement has been described but is rare.

The pathogenetic connection between toxocariasis and impaired cognitive function is still under investigation (Richartz and Buchkremer, 2002).

Manifestations of the peripheral nervous system include radiculitis, affection of cranial nerves, or musculoskeletal involvement (Jabbour et al., 2011).

Covert toxocariasis

CT was first put forward by Taylor et al. (1987) to describe a series of mild, non-specific symptoms which did not fall within the categories of VLM or OLM yet were recognizable as *Toxocara* spp. infection.

Non-specific symptoms and signs to which CT refers include fever, abdominal pain, anorexia, nausea, vomiting, hepatomegaly, splenomegaly, cough, headache, lethargy, behavioral and/or sleep disturbances, skin symptoms, limb pains, and lymphadenitis associated with high titers of anti-*Toxocara* antibodies, with or without eosinophilia (Taylor et al., 1987).

CT mainly affects children beyond the toddler stage (Taylor et al., 1987).

A similar syndrome, probably variations of the same clinical entity, named “common toxocariasis” has been described by Glickman and Shofer (1987) among adults.

Such a variant of CT refers to a syndrome comprised of chronic weakness, shortness of breath, abdominal pain, rash, itch, urticarial and arthralgia, often with eosinophilia, high Ig E levels, and high titers of *Toxocara*-specific antibodies (Glickman and Shofer, 1987).

Diagnosis

The broad spectrum of clinical manifestations in toxocariasis (VLM, OLM, NT and CT) varies from asymptomatic to non-specific clinical signs which make it difficult to directly identify clinical cases of toxocariasis. High index of suspicion based on positive history for risk factors of toxocariasis and a set of symptoms and clinical signs induces clinicians to elaborate a diagnostic workup based on medical imaging techniques, hematological and biochemical assessment, serological tests, molecular methods, microscopic examination and histopathology to confirm diagnosis (Table 2).

Imaging techniques

Hepatic lesions may be observed with ultrasonography, computed tomography (CoT), and magnetic resonance imaging (MRI) (Jain et al., 1994; Zachariah et al., 1994; Laroia et al., 2012). Lesions in the liver appear as small multiple hypoechoic areas with abdominal US (Baldisserotto et al., 1999; Lim, 2008) and areas of low density with a CoT scan (Dupas et al., 1986; Lim, 2008), characteristically hyperintense lesions in T1 sequence without intravenous contrast (Bernardo-Cofiño et al., 2020) (Fig. 4).

Table 2 Diagnostic criteria for toxocariasis.

History	- Presence of risk factors of toxocariasis (e.g., geophagia, consumption of contaminated vegetables and raw or undercooked meat, exposure to dogs or playgrounds and sandboxes contaminated by dog feces, younger age, male gender, poverty, tropical and rural residence, ethnicity); - Presence of following symptoms: constitutional (fever, malaise, fatigue, etc.), cutaneous, gastrointestinal, respiratory, ocular, nervous system, cardiac, etc.
Physical examination	- Evidence of organ involvement (see clinical scenarios in VLM, OLM, NT, CT) supported by medical imaging techniques (e.g., US, CoT, MRI, ophthalmoscopy, bronchoscopy, echocardiography, etc.)
Routine laboratory tests	- Eosinophilia on peripheral blood and/or other specimens (e.g., bronchoalveolar lavage, cerebrospinal fluid.); - Hypergammaglobulinemia IgE and IgG; - Liver enzymes and function tests (plasma protein and cholinesterases, bilirubin, coagulation, etc.) alteration (possible if severe liver involvement)
Serological examination	- ELISA, western blot (need to confirm suspected cases)
Molecular diagnosis and microscopic examination and histopathology	- Not routinely performed
Differential diagnosis	- <i>Eosinophilia</i>: infectious diseases (helminths, such as strongyloidiasis, trichinellosis, filariasis, schistosomiasis, hookworm, ectoparasites, such as scabies and myiasis, protozoans, such as isosporiasis, sarcocystis myositis, fungi such as coccidiomycosis, allergic bronchopulmonary aspergillosis, histoplasmosis, viral such as HIV), allergic disorders (asthma, allergic rhinitis, atopic dermatitis), drug hypersensitivity (drug reaction with eosinophilia and systemic symptoms, eosinophilia-myalgia syndrome, interstitial nephritis, eosinophilic hepatitis), immunologic disorders (immunodeficiencies, such as hyper-IgE syndrome, autoimmune and idiopathic disorders, such as sarcoidosis, inflammatory bowel disease, IgG4 disease, other connective tissue disorders), eosinophilic disorders (idiopathic hypereosinophilic syndrome, eosinophilic granulomatosis with polyangiitis, formerly Churg-Strauss Syndrome, eosinophilic gastrointestinal disorders), neoplastic disorders (primary hypereosinophilic syndrome, acute or chronic eosinophilic leukemia, other myeloid neoplasms, such as chronic myeloid leukemia, systemic mastocytosis, lymphoid malignancies, such as B cell lymphoma, B or T leukemia/lymphoma, cutaneous T cell lymphoma/Sézary syndrome, solid tumor such as adenocarcinoma, squamous carcinoma), etc.; - <i>VLM</i>: strongyloidiasis, schistosomiasis, capillariasis, ascariasis, echinococcosis, allergic bronchopulmonary aspergillosis, cancer and metastatic cancer; - <i>OLM</i>: retinoblastoma, toxoplasmosis, tuberculosis; - <i>NT</i>: gnathostomiasis, toxoplasmosis, tuberculosis, cancer and metastatic cancer

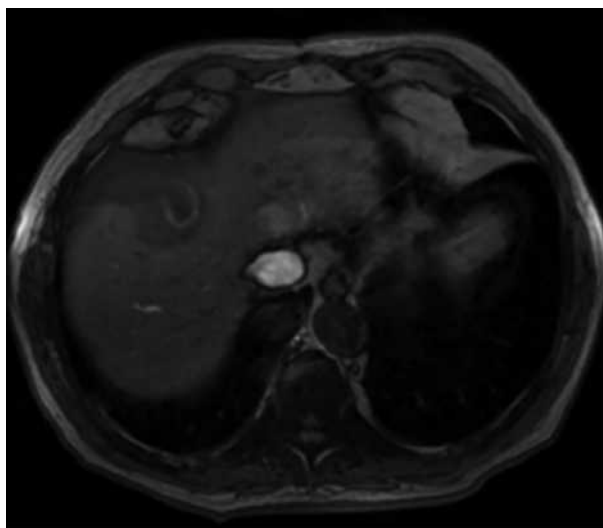


Fig. 4 Liver lesion due to *Toxocara* infection in MRI: a characteristically hyperintense lesion in a T1 sequence without intravenous contrast (Bernardo-Cofiño et al., 2020).

Pulmonary involvement in VLM appear on chest radiograph as bilateral peribronchial infiltration; parenchymal infiltrates can also occur (Snyder, 1961; Huntley et al., 1965; Ribeiro and Fischer, 2002) (Fig. 5A).

CoT may demonstrate multifocal subpleural nodules with halo or ground-glass opacities and ill-defined margins (Ribeiro and Fischer, 2002; Sakai et al., 2006) (Fig. 5B).

In one review of radiographic changes associated with pulmonary VLM, four patterns were observed: ground-glass opacities, solid nodules, consolidation, and linear opacities; lower lung involvement was predominant (Lee et al., 2015).

Globally radiographic techniques demonstrate abnormalities in $\geq 40\%$ of patients with symptomatic illness.

Ophthalmoscopy, ocular US and CoT has been used as diagnostic techniques for the evaluation of patients with ocular toxocariasis (Liu et al., 2017). The *fundus oculi* typical lesion is a whitish elevated granuloma (Molina et al., 2019) (Fig. 6).

CoT and MRI can help to evaluate CNS involvement, revealing granulomas appearing on CoT as hypodense lesions (Fig. 7A) and on MRI as hyper-intense lesions on T2-weighted images (Fig. 7B), primarily located cortically or subcortically in brain areas (Ruttinger and Hadidi, 1991; Jabbour et al., 2011; Abir et al., 2017).

Laboratory tests

Peripheral blood eosinophilia is the most important finding; eosinophilia occurs in more than 30% of cases of VLM (Rubinsky-Elefant et al., 2010), however, it may be absent in patients with OLM or CT, presumably because of the low larval burden (Pawlowski, 2001). Eosinophilia may also be absent in patients with long-standing infection and in those with cutaneous symptoms only (McGuinness and Leder, 2014).

NT patients often show eosinophilia not in the peripheral blood but in the cerebrospinal fluid (Jabbour et al., 2011).

Pulmonary involvement may result in eosinophilia, detectable in bronchoalveolar lavage fluid (Roig et al., 1992).

An eosinophilic granulomatous hepatitis may develop, leading to abnormalities in liver function tests including elevated transaminases and/or alkaline phosphatase.

Patients with toxocariasis have often hypergammaglobulinemia with a marked increase in serum levels of total IgE and IgG.

Serological examination

Serodiagnostic tests should always be performed using at least two consecutive serum samples taken approximately 2 weeks apart.

The most commonly used serological tests for confirming toxocariasis are an indirect enzyme linked immunosorbent assay (ELISA) and western blot (WB) based on *Toxocara* ES antigens of the third-stage larvae (MagnaVal et al., 2001; Fillaux and MagnaVal, 2013; Yoshida et al., 2016).

This test cannot differentiate between *T. canis* and *T. cati* infections.

The reliability varies by time of infection and clinical presentation.

The sensitivity of the *Toxocara* ES-based ELISA for the detection of IgG and diagnosis of VLM has been estimated to be 91%, with a specificity of 86% (Jacquier et al., 1991).

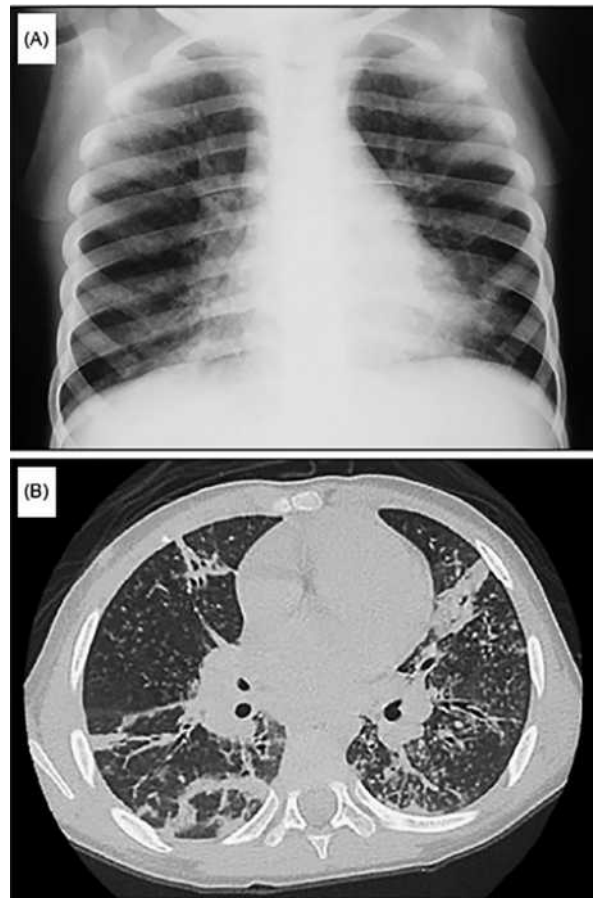


Fig. 5 Toxocariasis chest images. (A) Radiograph of a three-year-old girl with toxocariasis; (B) CoT scan of the same 3-year-old girl with toxocariasis (Ribeiro and Fischer, 2002).

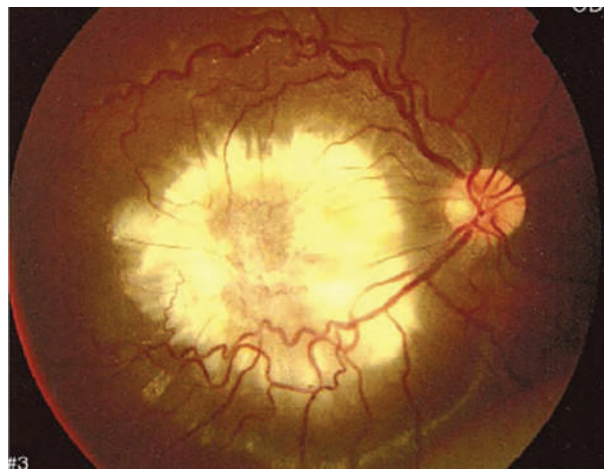


Fig. 6 Ocular toxocariasis: a white mass in the posterior pole of the right eye extending toward the macula suggestive of granuloma (Molina et al., 2019).

However, a positive ELISA result does not necessarily demonstrate the presence of active *Toxocara* infection or prove that clinical symptoms are attributable to toxocariasis, as IgG response elicited after *Toxocara* spp. infection may persist for many years (Cypess et al., 1977); the result must be interpreted in the setting of compatible clinical symptoms and epidemiologic exposure. However, a negative test can help to rule out VLM, although OLM and NT can occur with negative serology.

In OLM, the sensitivity of ELISA is considerably lower than for VLM, and even if it is positive an IgG or IgE titer is generally lower.

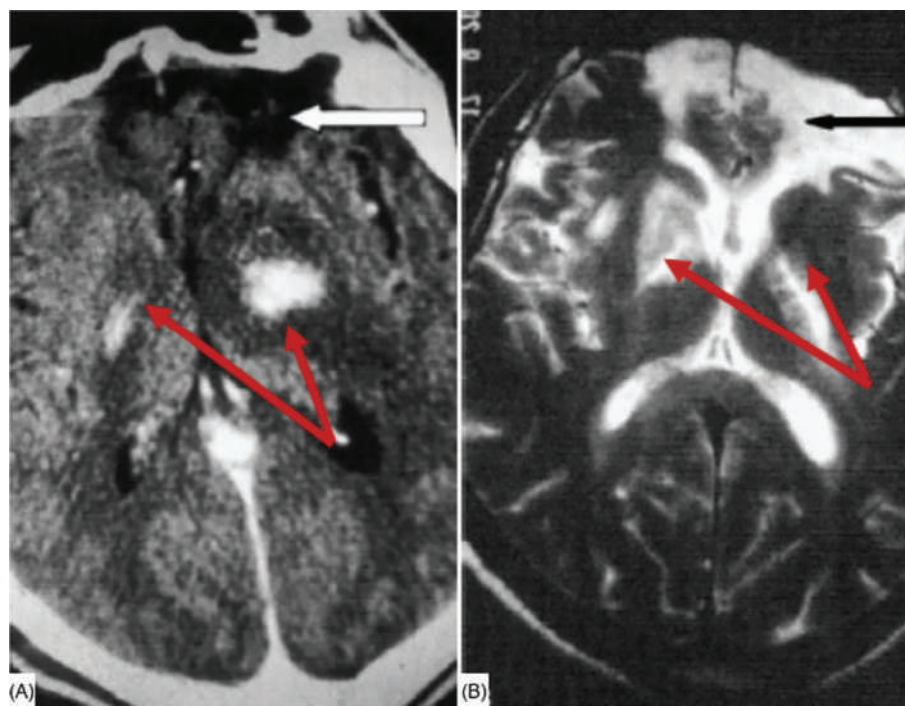


Fig. 7 Neurotoxocariasis: (A) brain CoT scan shows bilateral hypodense capsulo-lenticular lesions with focal nodular contrast enhancement (red arrows); (B) brain T2-weighted MRI shows the hyperintense lesions (red arrows). NB: The frontal lesions are post-traumatic sequelae (white and black arrows) (Abir et al., 2017).

ELISA with aqueous fluid (af) is therefore useful when OLM is suspected, and it is possible to compare the antibody levels between the serum and af; if the (level of specific IgG in af/serum level of specific IgG)/(total IgG in aqueous humor/total IgG in serum) is greater than 3.0, this can be considered diagnostic (de Visser et al., 2008). Negative serology can also occur with a positive af titer (Inchauspe et al., 2018).

However, for the diagnosis of OLM generally the findings on ophthalmologic examination are quite suggestive for etiology.

Cross-reactivity of the ELISA assay with other parasite antigens (e.g., ascariasis, anisakidosis, strongyloidiasis, trichinellosis, paragonimiasis, and fasciolosis) is common, and the test may remain positive for several years even following treatment (Elefant et al., 2006). In some settings, positive ELISA results can be confirmed by WB (MagnaVal et al., 1991), which has better sensitivity and specificity compared with ELISA (Roig et al., 1992), but it is also more expensive and labor intensive (MagnaVal et al., 1991; Fillaux and MagnaVal, 2013).

Other antibody isotypes, such as IgE, can be more specific and useful for identifying acute infection or progressive inflammation caused by toxocariasis and for monitoring therapy, but are less sensitive than IgG for the diagnosis (Elefant et al., 2006). Thus, more sensitive assays such as radioimmunoassay or fluoro-enzyme immunoassay are needed to detect *Toxocara*-specific IgE (MagnaVal et al., 2006). Unlike most other infections, IgM levels are present in both the acute and chronic phase of infection and are therefore not useful to distinguish between these two phases (Smith, 1993). Measuring the avidity of *Toxocara*-specific IgG antibodies may aid in distinguishing acute or chronic infections but not utilized on clinical practice (Dziemian et al., 2008; Rudzinska et al., 2017).

Molecular diagnosis

Methods for detecting circulating antigen are being developed (Rodríguez-Caballero et al., 2015).

Polymerase chain reaction-based methods for detecting *Toxocara* in clinical samples have been described but are not commercially available (Fogt-Wyrwas et al., 2007). Extraction of DNA is inconvenient for routine diagnosis of human infection since most patients have a very low worm burden and larvae are often not present in tissue samples. In an experimental murine model for toxocariasis, *Toxocara* larvae DNA was detected in bronchoalveolar lavage of infected animals using polymerase chain reaction assay (Pinelli et al., 2013). This finding indicated the possibility of using molecular tools and a less invasive method (bronchoalveolar lavage) for the direct diagnosis of toxocariasis particularly for patients with pulmonary disease. Future studies are needed to evaluate the sensitivity and specificity of these molecular diagnostic methods in human cases.

Microscopic examination and histopathology

Stool examinations are not applicable since the parasite does not complete a full life cycle involving the human gastrointestinal tract.

Definitive diagnosis of VLM may also be established via detection of larvae in biopsy tissue, which will show *Toxocara* larvae within eosinophilic granulomatous lesions. However, direct diagnosis of *Toxocara* infection through biopsies is not recommended since the larvae continuously migrate and biopsies are usually negative.

Furthermore, it remains difficult, or even impossible, to distinguish larvae of different *Toxocara* spp. as well as from larvae of other ascarid nematodes such as that of *Ascaris* spp. based only on their morphology (Nichols, 1956).

Treatment

Data on treatment approach are limited and often subject of debate due to the self-limiting nature of the disease, the lack of pathognomonic signs, and the possible development of allergic responses, particularly in critical sites such as the eye (Othman, 2012).

Thus, therapy varies according to symptoms and location of the larvae.

Most individuals with mild symptoms due to toxocariasis do not require anthelmintic therapy; symptoms are usually self-limited and resolve within a few weeks (Schantz and Glickman, 1978). Eosinophilia may resolve much more slowly over many months, likely due to ongoing antigenic stimulation from dead larvae. In the setting of protracted symptoms, the possibility of reinfection (such as from continued ingestion of contaminated soil) should be considered.

Treatment is usually recommended for individuals with moderate to severe symptoms due to VLM, significant peripheral blood eosinophilia (e.g., >1500/ μ L), inflammation foci in one or several organs (e.g., lungs, liver, heart, CNS) demonstrated in medical imaging, such as CoT, MRI, and US, active uveitis. Some are in favor of a treatment in the setting of moderate eosinophilia and positive serology even when only minimal symptoms are present, given risk of larval localization to the brain during the course of infection (Pawlowski, 2001; McGuinness and Leder, 2014).

Treatment consists of albendazole (ABZ) (400 mg orally with fatty meal twice daily for 5 days). In cases of severe respiratory, myocardial, or CNS involvement, OLM with sight-threatening ocular inflammation, concomitant prednisone (0.5–1 mg/kg/day) is warranted and ABZ treatment prolonged for 2 weeks or even up to 8 weeks (Pawlowski, 2001; Caumes, 2003; Kuenzli et al., 2016; Kakimoto et al., 2019; Hombu et al., 2019).

Optimal doses and duration of treatments are largely undefined.

The most frequent adverse events in the long-term treatment were liver dysfunction, which was reversible and well-tolerated in most cases (Hombu et al., 2019).

In complicated cases of OLM, surgical intervention may be warranted (Jee et al., 2016; Giuliani et al., 2011).

Mebendazole (MBZ) (100–200 mg orally twice daily for 5 days) is an alternative to ABZ (Magnaval, 1995), but ABZ is preferred (particularly in OLM and neurologic disease) since it crosses the blood-brain barrier. Ivermectin does not appear to be effective for toxocariasis as showed only moderate larvicidal activity in mice (Magnaval, 1998). Diethylcarbamazine (3–4 mg/kg/day for 21 days, starting at 25 mg/day for adults) has resulted effective in a small number of cases but with greater side effects than ABZ, for this it is rarely used.

Follow-up consists of monitoring clinical manifestations up to the resolution and of the eosinophil count, which usually decreases within 1 month of treatment (Magnaval et al., 2001). Serology is not useful for the limitation seen before.

Prognosis

Toxocariasis is generally a self-limited disease. The prognosis is good when adequately treated, except in some patients with severe VML, ocular or cerebral involvement.

Prevention and control

Toxocara spp. can be infective to a very wide range of accidental and paratenic hosts such as pigs, cattle, sheep, chickens and humans. Human toxocariasis is presumed to be acquired after the accidental ingestion of embryonated eggs or infective larva. No vaccines are available.

Prevention for minimizing the risk of infection is the best strategy.

Good hygiene practices, timely disposal of pet feces, and routine deworming of pets are important strategies for prevention of toxocariasis in humans (Centers for Disease Control and Prevention, 2011). Hand washing should be encouraged after contact with pets or areas at high risk for soil contamination, such as playgrounds and sandboxes (Centers for Disease Control and Prevention, 2011). Education regarding behaviors that increase risk for infection is also important, including geophagia and consumption of raw or undercooked meat (particularly liver).

Collaboration between veterinary and public health professionals plays a significant role in the control of toxocariasis.

Acknowledgment

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Toxoplasmosis

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Overview and background

Toxoplasma gondii is a coccidian parasite with a global distribution. The definitive host is the cat (and other felids) and all warm-blooded animals can act as intermediate hosts, including humans. Sexual reproduction (gametogony) takes place in the definitive host and oocysts are released in the environment, where they then sporulate to become infective. In intermediate hosts the cycle is extra-intestinal and results in the formation of tachyzoites and bradyzoites. Tachyzoites represent the invasive and proliferative stage and on entering a cell it multiplies asexually by endodyogeny. Bradyzoites within tissue cysts are the latent form.

T. gondii is a food-borne parasite causing toxoplasmosis, which can occur in both animals and humans. Infection in humans is asymptomatic in more than 80% of cases in Europe and North-America. Nevertheless, toxoplasmosis is life threatening if it occurs in immunocompromised subjects. Another aspect is congenital toxoplasmosis that may occur in pregnant women and the severity of the consequences depends on the stage of pregnancy when maternal infection occurs. Acute toxoplasmosis in developing fetuses may result in blindness, deformation, mental retardation or even death. Virulence and genotypes hardly influenced the clinical course of infection, in fact atypical genotypes are commonly associated to severe cases of congenital, ocular and disseminated toxoplasmosis also in healthy subjects.

The European Food Safety Authority (EFSA), in recent reports on zoonoses, highlighted that an increasing numbers of animals resulted infected with *T. gondii* in EU, high prevalence values have been detected in cats, cattle and dogs, as well as several other animal species, indicating the wide distribution of the parasite among different animal and wildlife species.

The main route of transmission is consumption of food and water contaminated with sporulated oocysts. However, infection through the ingestion of meat contaminated with tissue cysts is frequent. Finally, although less frequent, other food products contaminated with tachyzoites such as milk, may also pose a risk. Indeed, it has been reported that tachyzoites can resist gastric acidity for up to 30 minutes at a pH value of 4 (Koethe et al., 2017).

The importance of this parasite as a risk for human health was recently highlighted also by EFSA's opinion on modernization of meat inspection, where *T. gondii* was identified as a relevant hazard to be addressed in revised meat inspection systems for pigs, sheep, goats, farmed wild boar and farmed deer.

Taxonomical status

Phylum	Apicomplexa	Levine, 1980
Class	Sporozoa	Leukart, 1879
Subclass	Coccidiasina	Leukart, 1879
Order	Eimeriorina	Leger, 1911
Family	Toxoplasmatidae	Biocca, 1956
Genus	Toxoplasma	Nicolle and Manceaux, 1909
Species	Only one, <i>Toxoplasma gondii</i>	Named "gondii" after the African rodent (<i>Ctenodactylus gundl</i>) from which it was first isolated in the early 1900s. Nicolle and Manceaux, 1909

Toxoplasma gondii is a coccidian parasite, coccidia are among the most important parasites of animals and they were the first protozoa discovered (Dubey, 2010). The oocyst is the key stage of all coccidian and their classification was based on the structure of oocyst. Oocysts with four sporocysts, each with two sporozoites (total: eight sporozoites), are classified as *Eimeria*. Oocysts containing two sporocysts, each with four sporozoites, historically were classified as *Isospora* (Dubey et al., 2011). Unlike *Eimeria*, the life cycle of which has been known for many years, little was known of the complete life cycle of most *Isospora* species until 1970, when the life cycle of *T. gondii* was discovered and described. In that year, *T. gondii*, a parasite previously known as able to parasitize extra-intestinal tissues of virtually all warm-blooded hosts, was found to be an intestinal coccidium of cats and to have an isosporan-like oocyst (Dubey and Frenkel, 1972). This finding was a major breakthrough in medical and veterinary science and also led to the recognition of several new taxa of economically important *Toxoplasma*-like parasites (*Neospora*, *Sarcocystis*) and discovery of their cycles.

Historically, *T. gondii* originated probably as a coccidian parasite of cats with a fecal-oral cycle. With domestication, it adapted its transmission by several modes, including transmission by carnivorous, and transplacentally. There are three infectious stages of *T. gondii*: the tachyzoites, the bradyzoites, and oocysts, all linked in a complex life cycle.

Structure and life cycle

The tachyzoite is often crescent-shaped and is approximately the size of a red blood cell (2 μm $\times$ 6 μm). The anterior end of the tachyzoite is pointed (conoidal), and the posterior end is round. It has a pellicle (outer covering), several organelles including subpellicular microtubules, mitochondrion, smooth and rough endoplasmic reticula, a Golgi apparatus, apicoplast, ribosomes, micropore, and a well-defined nucleus which is usually situated toward the posterior end or in the central area of the cell.

T. gondii is able to penetrate various types of cells into innumerable hosts, indicating that the biochemical receptors recognized by the parasite for "attack" and therefore entry are quite common and widespread in most animal cells.

The penetration of the host's cell plasmalemma occurs in 26 s and the process involves two dependent Ca^{++} channels for the protrusion of the conoid and the secretion of the content of microneme and rhoptries for the formation of a moving junction. Once entered, the parasite is surrounded by a membrane derived from the plasmalemma which will then become the membrane of the parasitophorous vacuole where *T. gondii* is protected from host defense mechanisms. The tachyzoite multiplies asexually within the host cell by repeated divisions in which two progenies form within the parent parasite, consuming it. Tachyzoites continue to divide until the host cell is filled with parasites. The degree of replication is strictly dependent on the genotype and the host cell: more virulent genotypes replicate earlier and faster. The tachyzoites, despite the absence of eyelashes or flagella, move by wave and rotational movement. This is thanks to the activation of an actin-myosin system present at the level of the internal membrane.

After a few divisions, *T. gondii* forms tissue cysts and remains intracellular. Tissue cysts may vary in size: young ones may be small as 5 μm and contain only two bradyzoites, while older ones may contain thousands of organisms. Brain cysts usually reach 50–70 μm in size while those at the skeletal muscle level can reach 100 μm in diameter. The tissue cyst wall is elastic, thin (<0.5 μm), and may enclose hundreds of the crescent-shaped, slender *T. gondii* stage known as bradyzoites with dimensions of 5 $\times$ 8.5 μm . In the bradyzoites the nucleus is not central, as for the tachyzoites, but located in the back bottom. After inoculation (in experimental infections) the tissue cysts begin to form already after 3 days and constitute a fundamental part of the life cycle of *T. gondii*. Their location is dependent on the host, in fact, cysts have been found in lungs, liver, kidneys but they are more prevalent in muscular and neural tissues, including the brain, eye, skeletal, and cardiac muscle. Bradyzoites represent the cystic stage of the parasite. They live inside tissue cysts and replicate very slowly by endodyogeny. Inside the cyst they remain alive and vital and, when the host's immune defenses get down for any reason, the replication starts again in a more marked way until it determines the cyst rupture and the escape of infectious bradyzoites that can reach the circulatory stream and infect other cells and tissues after transformation into tachyzoites. Bradyzoites are slenderer and less susceptible to destruction by proteolytic enzymes than tachyzoites. After the ingestion of tissue cysts by cats, the tissue cyst wall is dissolved by proteolytic enzymes in the stomach and small intestine. The released bradyzoites penetrate the epithelial cells of the small intestine and initiate development of numerous generations of asexual and sexual cycles of *T. gondii*.

Toxoplasma gondii multiplies profusely in intestinal epithelial cells of cats (enteroepithelial cycle) and these stages are known as schizonts. Male and female gametes might derive from the merozoites generated by type D and E schizonts. The formation of

gametes occurs in the ileum, from 3 to 15 days after the inoculation or ingestion of tissue cysts, and we distinguish female and male gametes. With the two flagella the male gamete swims and enters the female gamete. After the female gamete is fertilized by the male gamete, the zygote formed will be surrounded by a wall which will contain the cytoplasm and the large central nucleus. At this point the epithelial cells break down, release the oocysts into the intestinal lumen which will then be eliminated in the environment with the feces. Shed oocysts are unsporulated and depending on external climatic conditions (temperature, humidity, oxygenation) need 1–5 days to sporulate and therefore become mature and infectious. The shape changes slightly becoming elliptical, the large nucleus of the zygote will divide giving birth to two sporoblasts with one nucleus each and a double lamina. As sporulation proceeds, the sporoblasts become longer, they become two sporocysts each containing four sporozoites formed by endoduo-geny, so that each oocyst contains eight sporozoites (Dubey, 2010).

Life cycle in the definitive host: The cat

Cats, both domestic and nearly all the species of felids, can shed *T. gondii* oocysts. Cats shed oocysts after ingesting any of the three infectious stages of *T. gondii*. Prepatent periods (time to the shedding of oocysts after initial infection) and frequency of oocyst shedding vary according to the stage of *T. gondii* ingested. They can go from 3 to 10 days after ingesting tissue cysts, and more than 18 days after ingesting oocysts, irrespective of the dose. The prepatent period after ingesting tachyzoites may vary. Fewer than 50% of cats shed oocysts after ingesting tachyzoites or oocysts, whereas nearly all cats shed oocysts after ingesting tissue cysts. *T. gondii* is adapted to be transmitted biologically by carnivorous cats and transmission by the oocysts is more efficient in non-feline hosts; bradyzoites are more infective to cats and oocysts are more infective to mice. After the ingestion of tissue cysts the tissue cyst wall is dissolved by proteolytic enzymes in the stomach and small intestine and initiates the development of numerous generations of *T. gondii*. Five morphologically distinct types of *T. gondii* develop in intestinal epithelial cells before gametogony begins. These asexual stages in the feline intestine are structurally distinct from tachyzoites that also develop in the lamina propria. The enteroepithelial stages (A-E gamonts) are formed in the intestinal epithelium and the development of types B-E schizonts in enterocytes. Tachyzoites occur exclusively within the lamina propria. Schizonts and gamonts develop exclusively in enterocytes. Types C, D, E multiply by schizogony; in this process the nucleus divides two or more times without cytoplasmic division. Before or simultaneous with the last nuclear division, merozoite formation is initiated near the center of the schizont. The merozoites eventually move towards the periphery of the schizont and the schizont plasmalemma invaginates around each merozoite forming the plasmalemma of the merozoite. The merozoites separate from the schizont at their posterior ends. The sexual cycle starts 2 days after ingestion of tissue cysts by the cat. The origin of gamonts has not been determined, but the merozoites released from schizont type D and E probably initiate gamete formation. Gamonts occur throughout the small intestine but most commonly in the ileum, 3–15 days after inoculation. They occur above the nucleus of the host epithelial cell near the tips of the villi of the small intestine. Female (macro) gamonts are subspherical and each contains a single centrally located nucleus and several PAS-positive granules. Mature male gamonts (micro) are ovoid to ellipsoidal in shape. During microgametogenesis, the nucleus of the microgamont divides and after several nuclear division, it migrates to the periphery of the gamont. Microgametes are biflagellate and contain a *perforatorium*; they use their flagella to swim to penetrate and fertilize mature macrogametes to form zygotes. After fertilization, an oocyst wall is formed around the parasite. Infected epithelial cells rupture and discharge oocyst into the intestinal lumen. Unsporulated oocysts are subspherical to spherical and are $10 \times 12 \mu\text{m}$ in diameter. Sporulation occurs in the environment, within 1–5 days depending upon aeration and temperature. Sporulated oocysts are subspherical to ellipsoidal and are $11 \times 13 \mu\text{m}$ in diameter. Each sporulated oocyst contains two ellipsoidal sporocysts measuring $6 \times 8 \mu\text{m}$. Each sporocyst contains four sporozoites. During sporulation the nucleus divides twice giving rise to four nuclei that are situated at the periphery of the zygote; at this stage, a second limiting membrane is formed. After the cytoplasm divides, two spherical sporoblasts are formed, each with two nuclei. As sporulation continues, the sporoblasts elongate and sporocysts are formed. Ultrastructurally, the sporozoite is similar to a tachyzoite except that there is an abundance of micronemes, rhoptries and amylopectin granules with a subterminal nucleus (Dubey, 2010).

Population structure of *Toxoplasma gondii*

The modern *T. gondii* was born in the Amazonian forest 1.5 million years ago (Bertranpetit et al., 2017).

The parasite is characterized by a clonal population structure and the genotypes are historically divided into types I, II, III. This distinction was originally made based on the different virulence patterns observed in mice. Despite the presence of a sexual stage in the life cycle of the parasite and a worldwide distribution, the population structure, in isolates genotyped and sequenced in United States and Europe, appears highly clonal with low genetic diversity. However, multi-locus and multi-chromosome genotyping of isolates from other continents have revealed a much more complex population structure. The majority of isolates in South America, Africa or Asia do not fit into the three major lineages and they are also considered the more virulent ones, responsible of important outbreaks and severe cases of ocular toxoplasmosis in humans (Carme et al., 2009; de-la-Torre et al., 2013).

Genotypes

Several molecular techniques including polymerase chain reaction, restriction fragment length polymorphism analysis (PCR-RFLP) (Su et al. 2006, 2010), microsatellite DNA analysis (MS) (Ajzenberg et al., 2010), multilocus DNA sequence typing of introns

(MLST) (Khan et al., 2011) and Whole Genome Sequencing have been used to study the genetic makeup of *T. gondii* isolates. PCR-RFLP genotyping is a relatively inexpensive, not needing sophisticated equipment technique, based on the analysis of 10 markers distributed over 8 chromosomes and one apicoplastic marker (Su et al., 2006). Microsatellite (MS) markers on the other side are more polymorphic and in this way more helpful to trace outbreaks and recent events of the evolutionary history of living organisms. The most recent and used technique is based on analysis of 15 MS markers distributed over 11 different chromosomes (Ajzenberg et al., 2010). With MLST it is possible to study the nucleotide sequences of several loci coding for housekeeping genes.

Up to now, 16 different haplogroups originating from 6 ancestral populations or clades have been described (including the initially 3 lineage I, II, III), based on DNA sequence typing and Whole Genome Sequencing. They are not completely homogeneous, in fact some isolates are defined as “atypical” for the presence of unique polymorphisms which cannot be clustered into one of the 16 haplogroups (Su et al., 2012; Lorenzi et al., 2016).

There is currently no precise nomenclature for *T. gondii* genotypes. Conventional designation defined type I, II, III and lumped together all the others as atypical or exotic. The subsequently identified major genotypes were added to the list including Type *BrI*, *BrII*, *BrIII* and *BrIV*, Type 12, *Africa 1* and *Chinese 1* (Pena et al., 2008; Mercier et al., 2010; Chen et al., 2011; Khan et al., 2011). However, this scheme of genotype designation is complicated to define the hundreds of genotypes identified by the multilocus PCR-RFLP method. To overcome this cumbersome designation, a scheme has been adopted in which each genotype is designated as a “ToxoDB PCR-RFLP genotype” followed by a specific numeral. The 10 most frequently identified are, in order, genotypes #2, #3, #1, #5, #4, #9, #6, #7, #8 and #10. Genotypes #1 and #3 differ only at the Apico locus, together compose the conventional Type II lineage. Genotype #1 is also referred to Type II clonal, whereas #3 as Type II variant. Genotype #2 is also known as Type III while genotypes #4 and #5, which differ only at the SAG1 locus, are collectively known as Type 12. Different studies reported that genotype #1, #2 and #3 (Type II clonal, Type III and Type II variant) are identified worldwide. These three genotypes are highly prevalent in Europe. Genotypes #1, #2, #3, #4 and #5 dominate in North America. Genotypes #2 and #3 (Types III and II variant) dominate in Africa, and genotypes #9 and #10 (*Chinese 1* and Type I) are prevalent in East Asia (Lorenzi et al., 2016).

Genotypes and their geographic distribution

From Northern to Southern Europe the population structure of *T. gondii* is highly clonal, with a predominance of strains belonging to the type II lineage. Type II and III predominate in Europe and atypical strains have been rarely isolated. In North America the population structure is very similar to that one described for Europe, but also a fourth clonal lineage (haplogroup 12) and atypical strains isolated both from domestic and wild animals showed an important prevalence (Dubey et al., 2011). A high level of diversity for *T. gondii* was recorded in Central and South America; in Brazil in particular numerous studies have highlighted the presence of atypical and new strains isolated from animals and also from humans with acute toxoplasmosis. Eighty-eight genotypes (defined with 11 genetic PCR-RFLP markers) have already been identified in Brazil, and new genotypes are continuously being identified in different animal species (Pena et al., 2011). The high level of genetic diversity was recorded in many species in the Amazonian area (Ajzenberg et al., 2004) and prevalence, incidence, severity of acquired and congenital ocular toxoplasmosis (OT) in some areas of Brazil, Colombia, and Argentina are considerably higher than anywhere else (de-la-Torre et al., 2008; Rudzinski et al., 2016).

Looking at African countries less information are available and most samples identified and genotyped (118/141) were from Egypt. Overall, genotype #3 and #2 are the two dominant types. Genotype #6, known as *Africa 1* microsatellite marker, and *Africa 3* were identified in Western and Central Africa and in Gabon, respectively (Mercier et al., 2010). The limited data that are available from sampling in Western Africa seem to suggest a high level of diversity, with a relatively low frequency of common genotypes (Shwab et al., 2014).

In Asia, there also appears to be a high degree of genetic uniformity. Genotype #9 (*Chinese 1*) is by far the most commonly found, and it is present in China, Vietnam and Sri Lanka, indicating a widespread distribution in Eastern Asia. Genotype #10 (Type I) is also common in China, unlike in most other countries. Genotypes #4, #18 and #20 have relatively high frequencies in Asia together with ToxoDB#1 and #3 (Type II and II variant) and #2 (Type III). Genotype #20 has been identified in a wide range of areas, from Sri Lanka in south Asia to Egypt in North Africa. Thus far, most of the sampling from Asia has been from the more populous eastern regions (Shwab et al., 2014).

Genotypes and virulence

Experimental virulence is defined by the mouse reaction after the inoculation of the parasite intraperitoneally. Type I isolates are highly virulent, leading to the death of mice in less than 10 days after the inoculation of less than 10 tachyzoites, while type II and III are considered avirulent strains, allowing survival after the inoculation of $>10^3$ tachyzoites. Also genotypes with a majority of type I alleles are usually more virulent (Mercier et al., 2010). The virulent strains display several characteristics that may explain the rapid dissemination and virulence. Type I strains are more rapid in destroying a cell monolayer than type II or III because they multiply rapidly and show a lower rate of tachyzoite-to-bradyzoite interconversion (Saeij et al., 2006).

In vivo infection with type I strains display a higher ability to penetrate the epithelia, lamina propria and submucosa (Barragan and Sibley, 2002).

It has been demonstrated that the rhoptry protein family, in particular the association between ROP5/ROP18, plays a role in the infection process. This allele association is always present in virulent strains and the deletion of ROP18 made the strains completely avirulent (Jensen et al., 2015).

As already reported, atypical strains mainly diffused in south America are responsible of serious clinical manifestations in humans. For example, many cases of multivisceral toxoplasmosis, commonly called "Amazonian toxoplasmosis," in immunocompetent people have been reported in French Guiana caused by highly pathogenic strains of the parasite from the wild (Carme et al., 2002). Similarly, in South America, in particular in Brazil, Colombia and northern Argentina, there is a high prevalence of severe ocular forms, representing a significant public health problem (de-la-Torre et al., 2013; Rudzinski et al., 2016). Because South America is also the hotspot of *T. gondii*'s genetic diversity, it has been hypothesized that severe forms of toxoplasmosis may be the consequence of poor adaptation of the human host to the unusual diversity of strains in this part of the world, resulting in impaired immune response and, thus, a more aggressive disease (Khan et al., 2006; Gilbert et al., 2008a). Also in North America, an association between the atypical strains and the incidence of severe ocular and systemic disease in immunocompetent patients has been recently found (Pomares et al., 2018).

Virulence factors of *Toxoplasma gondii* and host-parasite relationship

Virulence loci

During invasion of host cells, secretory organelles known as rhoptries discharge their contents into the host cell, making these primary candidates for modulating host signaling (Hakansson et al., 2001). ROP18 is an active serine/threonine (S/T) protein kinase that is secreted into the host cell where it decorates the surface of the parasitophorous vacuole membrane (PVM). It is a polymorphic protein and it was identified as a major factor that contributed to strain-specific differences in virulence (Taylor et al., 2006; Saeij et al., 2006). Transgenic expression of ROP18 from type I or type II strains in the type III background greatly enhanced virulence confirming that this locus was responsible for the observed differences in virulence between strains. The role of ROP16 in altering host gene transcription was initially identified by analyzing the differences in host gene expression induced by different parasite strains. These studies led to a focus on genes involved in IL-4 and IL-6 responses and implicated changes in the activity of the transcription factors STAT3 and STAT6 (Saeij et al., 2007). Although all three strains initially induce STAT3 and STAT6 activity, only the type I or III strains (which share the same ROP16 variant, ROP16I/III) sustain this response. Prolonged STAT3/6 activation by ROP16I/III down-regulates the induction of IL-12, thus limiting the protective TH1 cytokine responses (Saeij et al., 2007), which might lead to less inflammation, reduced pathology and also enhanced parasite survival.

Genetic studies show that the pseudokinase ROP5 is essential for acute virulence. ROP5 in fact is an inactive member of protein kinase family that controls virulence by blocking IFN- γ mediated clearance in activated macrophages. It is able to regulate the active protein kinase ROP18, which normally prevents clearance of the parasite in interferon-activated macrophages phosphorylating host immunity related GTPases (IRGs). Additionally, ROP5 has other functions indicating that it plays a general role in governing virulence factors that block immunity (Behnke et al., 2012, 2015).

Another virulence factor that stimulates a high production of IL-12, is a dense granule protein named GRA15. The molecular mechanism of GRA15 function is uncertain (Rosowski et al., 2011) but deletion of GRA15 in the type II strain ME49 prevents nuclear translocation of NF κ B and consequently the release of IL-12. This was demonstrated in particular for Type II GRA15 and not for Type I and III. The activation of NF κ B pathway by type II GRA15 results in high levels of IL-12 production, enhancing the classical activation pathway in cells infected by type II parasites. High production levels of IL-12 are also modulated by ROP16 since the allele expressed in type II strains is incapable of sustained phosphorylation of STAT3, and the down-regulation of the IL-12 induction.

Mechanism of cell invasion

Toxoplasma gondii usually parasitizes the host, definitive and intermediate, without producing clinical disease. Only rarely it produces severe clinical manifestations. The bradyzoites from the tissue cysts or sporozoites from the oocyst penetrate intestinal epithelial cells and multiply in the intestine as tachyzoites within 24 h of infection. The parasite may spread first to mesenteric lymph nodes and then to distant organs by invasion of lymphatic system and blood, and can multiply in virtually any cell in the body. All extracellular forms of the parasite are directly affected by antibody but intracellular forms are not. In fact, immunity does not eradicate infection and tissue cysts can persist for several years after acute infection, potentially for all the life of the subject. Whether bradyzoites can form new tissue cysts directly without transforming into tachyzoites is not known. It has been proposed that tissue cysts may break during the life of the host and the release of bradyzoites may induce their own destruction by the host's immune responses or alternatively, new tissue cysts may be formed.

In immunocompromised patients a rupture of a tissue cyst may result in transformation of bradyzoites into tachyzoites and renewed multiplication. This may lead to serious clinical complications till the death of the patient unless treated.

Pathogenicity of *T. gondii* is determined by the virulence of the strain and the susceptibility of the host species. Certain strains of mice are more susceptible than others and the severity of infection in individual mice within the same strain may vary. Mice of any age are susceptible to clinical *T. gondii* infection. Instead, if adult rats do not become ill, young rats can die of toxoplasmosis. Adult dogs, like adult rats, are resistant, whereas puppies are fully susceptible to clinical toxoplasmosis. Certain species are genetically

resistant to clinical toxoplasmosis, cattle and horses, for example, are among the hosts more resistant to clinical toxoplasmosis, whereas certain marsupials and New World monkeys are highly susceptible to *T. gondii* infection (Dubey and Beattie, 1988).

The immune response to *Toxoplasma gondii*

Innate immune response is mainly supported by a cell-mediated response through the production of high levels of IL-12, interferon γ , and the intervention of the implementation of B lymphocytes and T lymphocytes CD4⁺ and CD8⁺, necessary to “observe” the reactivation of the cysts and for the development of a protective immune response against a possible reinfection (Hunter and Sibley, 2012; Yarovinsky, 2014).

Humoral response is also important as it leads to the synthesis of IgA, IgM, IgE and IgG antibodies that act by preventing the parasite from attacking the surface receptors of the cells, blocking the cell invasion process, activating the complement pathway and driving the opsonization of the parasite itself (Sayles et al., 2000).

Most laboratory studies have used type II strains (intermediate virulence), which has facilitated the study of the immune response in mice during the acute and chronic phases of infection. They have shown that control of *T. gondii* requires the early production of the pro-inflammatory cytokine IL-12, which stimulates natural killer (NK) and CD4⁺ and CD8⁺ T cells to release IFN- γ (Johnson, 1992; Khan et al., 1994).

The importance of IL-12 induction in early infection is evident from studies reporting that monocytes (Robben et al., 2004) CD8 α ⁺ dendritic cells (DCs) (Reis e Sousa et al., 1997), plasmacytoid DCs (Bierly et al., 2008), and neutrophils (Bliss et al., 2000) all contribute to the production of this activation signal. IL-12 is also essential for controlling parasite growth.

IFN- γ is the major mediator of resistance to *T. gondii* and is crucial for the activation of a variety of antimicrobial mechanisms in hematopoietic and non-hematopoietic cells that limit parasite replication (Suzuki et al., 1988), altering cell metabolism. This cytokine also stimulates professional phagocytes to produce reactive oxygen and nitrogen intermediates, which can lead to parasite damage and growth inhibition in macrophages. This pathway relies on IRGs that are induced by IFN- γ and contribute to clearance of *T. gondii* in multiple cell types (Taylor et al., 2006). Although innate immune responses to *T. gondii* have been examined in detail, how these processes lead to the stimulation of adaptive immunity, including the ability of DCs to access antigens for priming of CD4⁺ and CD8⁺ T cells, are less well understood. Moreover, infection of host cells is associated with reduced expression of major histocompatibility complex (MHC) molecules (Lüder et al., 1998). Despite these mechanisms of avoidance, infection with type II strains of *T. gondii* leads to the activation and expansion of DCs and a strong CD8⁺ T cell response, while infection with virulent type I strains induces a weaker response (Tait et al., 2010). Several prominent endogenous antigens that are presented on class I MHC molecules include the dense granule proteins GRA6 and GRA4, and the rhoptry protein ROP7 (Frickel et al., 2008). These antigens are polymorphic between strains, suggesting that they may be involved in strain-dependent evasion mechanisms that also influence adaptive immunity. The importance of highly immunogenic surface antigens (SAGs) and SAG-related surface antigen (SRS) in stimulating the adaptive immune response have been also highlighted (Lekutis et al., 2001).

Alterations in host signaling and immune evasion

Many innate immune effectors are under the control of transcription factors that enhance or regulate the overall immune response to invading microorganisms. Infection of mammalian cells with *T. gondii* induces many changes in host cell gene transcription, including those genes involved in energy metabolism, immune responses and signaling (Blader et al., 2001). Infection with *T. gondii* inhibits host cell signaling pathways involved in protective immunity, for example, by blocking the transcription factors signal transducer and activator of transcription 1 (STAT1) (Lüder et al., 2001) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (Butcher et al., 2001). Infection with *T. gondii* also upregulates anti-inflammatory pathways including those involving the suppressor of cytokine signaling protein (SOCS) 1 (Zimmermann et al., 2006), SOCS3 (Whitmarsh et al., 2011) and STAT3 (Butcher et al., 2011), potentially compromising host mechanisms of parasite control. Although IFN- γ promotes antimicrobial activities in many cell types, in macrophages it is not sufficient for the control of *T. gondii* and a second signal is required to fully activate killing. The best-characterized second signals that promote control of *T. gondii* in vitro are provided by TNF- α or signals through CD40 (Andrade et al., 2006; Reichmann et al., 2000), both of which utilize the NF- κ B signaling pathway. Notably, the activation of NF κ B by type II strains is associated with higher levels of IL-12 production (Robben et al., 2004), an effect that contributes to early control of infection.

Toxoplasmosis: Sources of infection

The majority of horizontal transmission in human is caused by the ingestion of tissue cysts in infected meat or by the ingestion of soil, water, or food contaminated with sporulated oocysts. The relative contribution of oocysts and tissue cysts to human infections is not well known, but experts assessed that 50% of infections are foodborne (EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2018).

Infection through tissue cysts

In Europe and in developed countries meat consumption was estimated to be responsible for about 50% of cases of infection (Scallan et al., 2011), while soil contact represented 6–17% of cases (Cook et al., 2000). Outbreaks due to the consumption of raw or undercooked meat have been described but they usually involve only a few patients (EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2018). In United States pork meat was estimated to be the main infection source for humans causing about 41% of foodborne human toxoplasmosis (Batz et al., 2012).

Regarding meatborne infections, it is known that the prevalence in livestock increases when the animals have outdoor access and increases with age. This underlines the importance of correct biohazard measures in farms and at the same time pose a question in relation to animal health. Organic and open access breeding assures best animal health conditions but the risk of infection increases.

Tissue cysts remain infectious in refrigerated carcasses (1 °C–6 °C) or minced meat for up to 3 weeks. However, the deep-freezing of meat at –12 °C or lower for at least 3 days is usually efficacious to kill cysts, although the effect may depend on the thickness of the piece of meat (Dubey, 1988). On the other side, tissue cysts are killed immediately when heated at 67 °C. Cooking for a prolonged period of time may be necessary under household conditions to achieve the temperature necessary to kill the cysts in all parts of the meat.

Infection through oocysts

A single cat may shed more than 100 million oocysts, which are non-sporulated. These need between 1 and 5 days to become infective for other hosts, depending on temperature, humidity and oxygenation, which explains why the direct contact with cats is not thought to be a major risk for human infection. Unsporulated oocysts lose the ability to sporulate and to become infective, after freezing at –6 °C for 7 days or after exposure to 37 °C for 1 day. Once sporulated they remain viable in a moist environment for more than a year. Under laboratory conditions, they resist at 4 °C for up to 54 months. They survive freezing at –10 °C for 106 days and heating at 35 °C and 40 °C for 32 days and 9 days, respectively. However, they are killed within 1–2 min by heating at 55–60 °C (when cooking vegetables for example). They are also high impermeable and very resistant to disinfectants (Dumètre and Dardé, 2003).

All these characteristics makes oocysts very resistant and durable; the reason why we can find them in water and in soil and from there they can reach vegetables and fruits and finally infect humans and intermediate hosts. Oocyst ingestion constitutes a significant proportion of *T. gondii* infections and the water-borne route of infection is one of the most common route of transmission of toxoplasmosis in human, mainly in countries with precarious infrastructure for water and sewage treatment. Lots of outbreaks have been described in Brazil for example with water or products implicated as the common source of exposure (Ferreira et al., 2018).

Infection through tachyzoites

Outside its host cell the tachyzoite is a fragile stage, and easily destroyed by digestive enzymes (10 min survival in pepsin-HCl). It is also very sensitive to environmental conditions and it usually dies rapidly outside the host. Therefore, tachyzoites are not thought to be very important for horizontal transmission from an epidemiological point of view. However, there is evidence that infected goats may release tachyzoites in milk and in rare cases subjects who drink unpasteurized goat's milk may acquire toxoplasmosis (Tenter et al., 2000). Similarly, also sheep can release tachyzoites with milk, as reported in Italy (Vismarra et al., 2017b). Tachyzoites are, however, responsible for congenital infection (mother-fetus) if the mother acquires primary toxoplasmosis during gestation. A real estimation of the incidence of congenital toxoplasmosis is difficult because in the majority of countries there are not mandatory monitoring programs, and neither pre- nor postnatal screening programs have been implemented in many countries. In France, where universal systematic screening of pregnant women exists from 1978 the incidence of seroconversion during pregnancy in 2010 was estimated at 2 per 1000 susceptible pregnant women but has decreased markedly during the last 30 years (Nogareda et al., 2014; Villena et al., 2010). The last report EFSA on zoonoses reported for 2017, 194 confirmed cases of congenital toxoplasmosis in the EU/EEA, with France accounting for 78.9% of all confirmed cases due to the active screening of pregnant women (EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2019). In the United States, it is estimated that the incidence of congenital toxoplasmosis ranges from 500 to 5000 cases annually (Roberts and Frenkel, 1990). In Colombia a multicentric national screening of 15,000 newborns found an incidence of 1 case per 1000 newborns, with important regional variations related to mean annual rainfall (Gómez-Marín et al., 2011). One of the most important estimation about the global incidence of congenital toxoplasmosis was a meta-analysis published in 2013 by World Health Organization that described a global annual incidence of congenital toxoplasmosis of 190,100 cases (Torgerson and Mastroiacovo, 2013).

Tachyzoites may also be responsible for toxoplasmosis if blood or bone marrow, deriving from infected donors, are injected during transfusion or transplant, but it is rare (Gangneux and Dardé, 2012).

Toxoplasmosis in animals and humans

Infection in domestic animals

Toxoplasma gondii is capable of causing infection and severe disease in animals other than humans. Natural infection in cats can occur and the severity of the symptoms is correlated with the age, the stage of the parasite and the route of inoculation (in the case of

experimental infections). Pneumonitis is the most common finding in cats and it can be rapidly fatal. Infected cats can also show high fever, which is unresponsive to penicillin and streptomycin. Even if toxoplasmosis is often included in the differential diagnosis of neurologic disease, cats rarely have obvious neurologic signs. Other organs affected by toxoplasmosis include the eyes (multifocal iridocyclochoroiditis) and pancreas (pancreatitis).

In livestock animals, toxoplasmosis causes marked losses in sheep and goats, and may cause embryonic death and resorption, fetal death and mummification, abortion, stillbirth, and neonatal death in these animals (Innes et al., 2009).

Outbreaks of toxoplasmosis in pigs have been reported from several countries, especially Japan (Dubey et al., 1986), China (Li et al., 2010), Korea (Kim et al., 2009). Mortality is more common in young pigs than in adult pigs. Pneumonia, myocarditis, encephalitis, and placental necrosis have been reported to occur in infected pigs. Sporadic and widespread outbreaks of toxoplasmosis occur in rabbits, mink, birds, and other domesticated and wild animals. Animals that survive infection harbor tissue cysts, and can therefore transmit *T. gondii* infection to human consumers (Dubey et al., 2011).

The role of *T. gondii* infection in cattle is still under investigation. In fact, it does not have a relevant impact from a clinical point apart from some described cases of abortion, but in any case in a lower percentage compared to those caused by *Neospora caninum*. Worldwide seroprevalence ranged from 1% to 92% while the number of tissue cysts is extremely lower compared to pigs or sheep. Compatibly a metanalyses study identified a pooled *T. gondii* prevalence of 2.6% in bovine meat and cattle compared to pigs (12.3%) and sheep (14.7%) (Belluco et al., 2016). From one side we can confirm the low prevalence in cattle can be used to support the theory of limited bovine role in *T. gondii* epidemiology. However, the role of beef in *T. gondii* human epidemiology cannot be ruled out considering that this meat is often eaten raw or undercooked in several countries with a consequent high probability of infection with *T. gondii* (Belluco et al., 2016).

Chickens do not usually present clinical signs. Confirmed severe toxoplasmosis was first reported in a flock of 40 hens from Norway (Erichsen and Harboe, 1953). In some rare cases it was recorded a neurologic symptomatology; Goodwin et al. (1994), reported a peripheral neuritis in three chickens in United States diagnosed with immunohistochemistry (IHC), but also the Marek's disease could not be run out. One case of systemic toxoplasmosis (apathy, dyspnea and diarrhea and death 18 h after the onset of illness) in a quail from Brazil was recorded recently (Casagrande et al., 2015) with the presence of tachyzoites highlighted in a lot of organs through IHC and histology.

Infection in wild animals

Clinical and subclinical toxoplasmosis has been reported in many host species. Wild animals can be a source of *T. gondii* infection in humans, cats, and other carnivores. An Italian study conducted on red foxes, roe deers, wild boars, alpine chamois, red deers in Piedmont region (Northern Italy) revealed a higher prevalence of infection in carnivores and omnivores than in herbivores (no positive ones in this study). This reflects the higher probability of a carnivore or omnivore to consume tissues infected with *T. gondii* than the probability of an herbivore to ingest *T. gondii* oocysts from the environment (Ferroglia et al., 2014). The idea that tissue cysts are important as oocysts in the transmission of *T. gondii* and that wild animals (especially wild boar and roe deer) could be an important source of foodborne toxoplasmosis in humans was the same expressed by Smith and colleagues in United States (Smith and Frenkel, 1995).

Sea mammals have also been found positive for *T. gondii*. The parasite is considered a significant cause of encephalitis in sea otters (Cole et al., 2000). In a study conducted in 2002 from Cole et al. (2000) in 67 sea otters found dead or killed because of their inability to survive due to advanced disease or trauma, 15 (22.4%) were positive by bioassay. Miller et al. (2002) has documented a high prevalence (42–62%) of infection of southern sea otters with *T. gondii* in areas along the Pacific coast of United States resulting from surface water run-off contaminated with *T. gondii* oocysts. Several cases of *T. gondii* infection were confirmed in striped dolphins affected by Morbillivirus from the 1990–92 morbilliviral epidemic (Domingo et al., 1992), with lethal cases of *T. gondii* meningo-encephalitis being subsequently reported in striped dolphins stranded along the Ligurian Sea coast of Italy between 2007 and 2008 (Di Guardo et al., 2010).

Toxoplasmosis in humans

It is generally assumed that approximately 25–30% of the world's population is infected by *T. gondii* (Elbez-Rubinstein et al., 2009). The prevalence varies between countries and low seroprevalences (10–30%) are recorded in North-America, South East Asia, Northern Europe, Sahelian countries of Africa. Moderate prevalence (30–50%) have been found in countries of Central and Southern Europe, and higher prevalence in Latin America and in tropical African countries (Gangneux and Dardé, 2012).

Climate factors such as temperature and humidity influence the resistance of oocysts and as a consequence the level of infection. High prevalence are classically recorded in tropical countries (warm and humid) and lower ones in arid or colder countries. Other factors such as economic, social and cultural habits can explain the wide variations in human seroprevalence (dietary habits, methods of cooking meat, hand washing, water quality, risk knowledge associated with food consumption).

In a recent assessment of foodborne illnesses in the United States, toxoplasmosis was identified as the second leading cause of foodborne illness-related deaths and fourth leading cause of foodborne illness-related hospitalizations (an estimated 327 deaths, and 4428 hospitalizations annually) (Scallan et al., 2011; www.cdc.gov/parasites/toxoplasmosis). In Europe the WHO reported that 20% of foodborne-diseases is caused by *T. gondii* with about 1 million people infected every year (<http://www.eurosurveillance.org/12; www.who.int/en>).

Toxoplasma gondii and human health: Pathogenesis

Primary acquired infection in immunocompetent subjects is asymptomatic in more than 80% of cases in Europe and North-America. In the remaining cases patients show fever, cervical lymphadenopathy sometimes associated with myalgia or other non-specific clinical signs, very similar to a common flu (Montoya and Liesenfeld, 2004). Nevertheless, toxoplasmosis is life threatening if it occurs in immunocompromised patients. Patients with HIV/AIDS, transplants, those with cancer or with other diseases affecting the immune system, or treated with the so-called biological drugs are at risk for severe toxoplasmosis. A reactivation of a chronic infection may occur and this risk is related to the duration and degree of immunosuppression. HIV patients often develop encephalitis that leads to lethargy, ataxia, loss of memory, dementia, but the heart (myocarditis), lungs, eyes and pancreas may also be involved and parasite can be isolated from these tissues. Pulmonary or disseminated toxoplasmosis is seen mostly in transplant patients (Pereira-Chioccia et al., 2009; Gangneux and Dardé, 2012; Wang et al., 2017).

Congenital toxoplasmosis may occur in pregnant women and the severity of the consequences depends on the stage of pregnancy when maternal infection occurs. When a primary infection is acquired by a pregnant woman, tachyzoites can colonize placental tissue during the dissemination process and from there gain the access to the fetal compartment. Acute toxoplasmosis in developing foetuses may result in blindness, deformation, mental retardation or even death (Montoya and Liesenfeld, 2004). The frequency of vertical transmission increases with the gestational age at maternal infection. If the infection occurs in the first trimester of pregnancy the risk of abortion or severe abnormalities in the brain and eyes is high. During the second trimester, fetal infection can be of variable severity. Ultrasound echography (US) reveals areas of hepatosplenomegaly, cerebral calcification or areas of hyper-echogenic mesentery. At birth the baby may show epilepsy, anemia, thrombocytopenia-induced petechiae, rash, hepatic disorders, pneumonitis, or retinochoroiditis (Remington et al., 2001). This last clinical manifestation is a common feature that can be observed whatever the time of maternal infection. Although the vast majority of congenital infections result from primary acquired infection during pregnancy, parasite transmission can occur in rare instances in immunocompetent, previously immunized women who are re-infected with *T. gondii* during gestation. One such clinical case has been described due to an atypical strain of *T. gondii* (Elbez-Rubinstein et al., 2009).

Data from all over Europe, United States and South America (mainly from Brazil), identify *T. gondii* as a risk for human health. It is known that the majority of the population at risk of complications derived from toxoplasmosis, are mainly people with concurrent health issues (organ transplant, patients with cancer, HIV, etc.) and pregnant women who are at risk of abortion or severe congenital defects (Cenci-Goga et al., 2011; Gangneux and Dardé, 2012; Campos et al., 2014). However, a high incidence and severity of ocular toxoplasmosis in immunocompetent subjects and in congenitally infected babies, caused mainly by atypical strains, have been reported from Africa, Brazil and Colombia (Gilbert et al., 2008a,b; Huang et al., 2012, 2018). The prevalence of ocular toxoplasmosis varies from less than 1% to approximately 18% of persons, depending on geographical location. For the majority, ocular toxoplasmosis involves the posterior eye and is characterized by recurrent necrotizing retinitis, with frequent extension of the inflammation and tissue destruction into the choroid, which encapsulates the retina (Furtado et al., 2013).

Moreover, chronic toxoplasmosis has been shown to cause behavioral changes in mice, making them attracted rather than repelled by the scent of cat urine, due to inhibition of neuronal function and alteration of neurotransmitter levels (Gatkowska et al., 2012; Haroon et al., 2012). In humans, the condition has also been shown to correlate with schizophrenia and bipolar disorders (Sutterland et al., 2015).

Foodborne toxoplasmosis: The main food products at risk

As said before, the consumption of contaminated food is the major route of infection with *T. gondii* for humans. The potential risk associated with the consumption of products derived from infected animals including raw or undercooked meat products and in a very low percentage, milk, cheese made with unpasteurized milk, has been shown in numerous studies. Meat consumption is certainly one of the main transmission routes for the infection with *T. gondii*. Currently, there are no identification systems for the parasite at slaughter, in any animal species, neither in United States nor in Europe. Even if the recorded data about infection in retail meat are limited, one study showed a prevalence of only 0.33% in over 2000 samples analyzed (Dubey et al., 2005). This may lead to think that the risk for the consumers related to meat consumption is very low. On the contrary, the consumption of sausages, typical meat products such as raw ham, or undercooked meat which has not been frozen before eating, is strongly associated with food-borne infection in humans (Vitale et al., 2014; Bonametti et al., 1996; Choi et al., 1997). A quantitative microbial risk assessments (QMRA) based on data available in literature on *T. gondii* viability and ability to survive in certain conditions (i.e., salt concentration, temperature, humidity, etc.), on data about type and quantity of meat consumed in a certain country, economic conditions, etc. allowed the identification of beef as the most important type of meat responsible of *T. gondii* transmission, followed by pork and sheep meat (Opsteegh et al., 2011). Pork, poultry, beef and sheep (in lower percentage) are the main meats consumed in Italy, Europe and the United States (www.assocarni.it; Daniel et al., 2011).

All these species have been identified as intermediate hosts of *T. gondii* and parasite DNA or antibodies against a specific antigen have been recorded all over the world. The presence of DNA or antibodies against *T. gondii* indicate a previous contact with the parasite and, in the former case, certainly the presence of tissue cysts.

The importance of *T. gondii* as a risk for human health was recently highlighted by European Food Safety Authority's (EFSA) opinion on modernization of meat inspection, where the parasite was identified as a relevant hazard to be addressed in revised meat inspection systems for pigs, sheep, goats, farmed wild boar and farmed deer (Opsteegh et al., 2016).

Toxoplasma gondii was reported by the European Member States (MSs) from pigs, sheep, goats, cattle, wild animals and pets (cats and dogs) in 2017 and 2018. The highest overall prevalence of *Toxoplasma* infections in animals was detected in small ruminants (sheep and goats; 18.3%; 12 MS reported data) and in cattle (27.8%; 6 MS reported). Due to different diagnostic methods used (indirect methods detecting antibodies vs direct methods), different sampling schemes in the MS and the lack of information on the animals' age and rearing conditions it is not possible to make a real risk evaluation and to estimate the real prevalence of *T. gondii* in animals in EU (EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2019).

The growing consumer demand for biological, free-range products in order to guarantee animal welfare and to have healthy food derived from them is another point to consider. Europe, together with North-America, is the main economic market for biological products, with an annual growth rate of 10–15% (Willer and Kilcher, 2009). Organic program standards (Anonymous (n.d.-a): Italian regulation DM n. 18354/09; Anonymous (n.d.-b): European Regulation CE 834/2007; Anonymous (n.d.-c): American legislation, USDA) require that all organically-raised animals must have access to the outdoors, including access to pasture for ruminants. Access to grass, soil, feed, or water contaminated with cat feces, or to rodents and wildlife infected with *T. gondii*, during outdoor pasturage substantially increases the risk of exposure of pigs, sheep, cows and chickens to the parasite. Kijlstra et al. (2004) found that none of the 621 conventionally raised pigs studied were seropositive for *T. gondii*, while 38 out of 1295 (2.9%) pigs raised in "animal friendly" management systems were seropositive for *T. gondii*, with a farm positive rate of 39.0%.

All that have access to the outdoors, including livestock and wild animals, have a potentially higher risk of coming into contact with the coccidian. This has already been demonstrated in several studies in the United States (Dubey et al., 2011, 2012) and Europe (Bacci et al., 2015; Vismarra et al., 2017a) in pigs, sheep, chickens, wildlife and also in derived products as milk.

Correct behaviours would lead to reducing the risk naturally associated with products derived from animals raised outdoors and at the same time all this would allow the development of a greater number of organic-organic farms in compliance with the dictates of animal welfare imposed by the European Commission.

Free-range chickens are a good indicator of soil contamination with oocysts because they feed from the ground and they rarely become ill from *T. gondii* (Ruiz and Frenkel, 1980; Dubey et al., 2011). Infected chickens are also an important source of infection for cats that in turn shed oocysts after eating tissues of this intermediate host. The ingestion of undercooked poultry may be a source of infection for humans. Freezing and cooking by conventional methods will inactivate tissue stages in meat. While meat is considered to be a major source of human exposure to *T. gondii* (WHO/FAO, 2016), little risk is attributed to chicken because it is typically well cooked (Kijlstra and Jongert, 2008). Pork, on the other hand, is considered a major source of human exposure (Dubey and Jones, 2008). Results of a recent study indicate that, while the density of *T. gondii* in poultry muscle is low, the ingestion of undercooked poultry muscle remains a potential source of infection for humans. The results showed that hearts of serologically positive chickens were used to infect cats and they all shed oocysts. Isolation of viable *T. gondii* from these free-range chickens is definitive evidence of the public health importance of the parasite (Dubey et al., 2015).

This risk can be significantly reduced if meat, milk and other products of animal origin are cooked (or pasteurized) adequately (minimum 67 °C for meat) and avoiding raw-cooked cross-contamination during the preparation of food in the kitchen. Commercial procedures of curing with salt, sucrose, or low-temperature smoking may kill tissue cysts, but the effect is strictly dependent on the combination of concentration of the solution with the temperature of storage. Authors reported for example that solutions containing 2% sodium chloride or 1.4% potassium or sodium lactate are effective within 8 h of injection in pork loin for the killing of *T. gondii* tissue cysts (Hill et al., 2006). On the other side, it was proved for example that Parma ham (from Italy) processing is able to kill the parasite in 12 months seasoning (Genchi et al., 2017), while Serrano ham (from Spain) was found positive for *T. gondii* in 9 and 12 months curing hams (Herrero et al., 2017). Recently, it was proposed that a High Pressure Processing treatment for at least 20 min at 600 MPa has to be applied on Spanish hams for a complete neutralisation (Gracia et al., 2020). Similarly, irradiation was also found to be effective against tissue cysts in meat (Lindsay et al., 2006). Finally, particular attention must be paid to vegetables, berries and fruits that can be contaminated by sporulated oocysts. In this case, an adequate washing under abundant running water is recommended.

Diagnosis in animals and humans

Diagnosis in animals

Diagnosis of toxoplasmosis is essentially based on the identification of a specific immune response against *T. gondii* through serology. We can choose among different types of serological tests to use as Enzyme-Linked Immunosorbent Assay (ELISA), agglutination (MAT), immunofluorescence (i.e., IFAT), Western Blot commercially available for humans, domestic animals and livestock animals. They allow the identification of IgM, IgG and also IgA. We can use serum or also meat juice (on muscle tissues of animal carcasses at slaughterhouse), depending on manufacturers' instructions and also oral fluids as recently reported in pigs (Campero et al., 2020).

IgM are commonly produced 2–4 weeks after infection and tended to disappear within 12–16 weeks in cats. On the other side IgG are expression of a non-recent infection. They could be found in the blood already 3 weeks after the contact and since *T. gondii*

infection tends to remain latent rather than resolve, IgG remain throughout the life of the animal. It is also important to remember that a high IgG titer is neither symptom of an acute infection, nor of a reactivation of the parasite. They have been described cases of healthy cats with recorded IFAT values higher than 1:10,000 many years after the first contact with *T. gondii*. Similarly, also in case of re-activation of infection by the encysted parasite no increase in the antibody titer was reported, both in animals and humans (Lappin et al., 1989). At the same time, low IgG values should be due to phenomena of analytical variability. It is also important to remember that during the shedding cats are usually negative to serological tests because the synthesis of antibodies required at least 2–3 weeks and at that point cats have already finished to shed oocysts. This lead that a seropositive cat will no longer eliminate oocysts with feces, except in rare cases of re-shedding.

A visual fecal exam should be made on cat feces through flotation for the *identification of oocysts* (Dubey et al., 2011). It is a *standard fecal flotation* using sucrose solution 1.15 gravity made with 53 g of sugar, 100 mL of water, 0.8 mL of liquid phenol or 2% sulfuric acid. However, this type of diagnosis requires qualified personnel and also in this case the chance to find oocysts is low. In fact, two studies made in United States reported that less than 1% of the analyzed cats (about 600) shed oocysts (Dabritz et al., 2007; Spain et al., 2011). This could be explained because after the first contact with the parasite, cats, especially young cats, shed oocysts for only 1–2 weeks and in this period they usually did not show any clinical sign.

The *identification of tachyzoites* is not so easy to do because they are present in blood or other body fluids only during active stage of infection, so for only short periods of time. On the other side, their identification allows a clear and certain diagnosis of toxoplasmosis. Materials such as cerebrospinal fluid, aqueous humor, or obtained from needle-aspiration from numerous tissues (e.g., lymph nodes) and body fluids (broncho-alveolar or trans-tracheal lavage or fluids from peritoneal or thoracic effusion) can allow the detection of tachyzoites through cytological examination. Similarly, histology and immunohistochemistry should be applied on biopsies from different body districts.

Polymerase chain reaction can be used for *T. gondii* identification and it is applicable on biopsies, on aqueous humor samples, on cerebrospinal fluid (CSF), on broncho-alveolar lavage (BAL), in blood (even if it is rare to find tachyzoites as described before), on muscle tissues of slaughtered animals for food safety purposes for example and in cat's feces positive for oocysts. In this case PCR allows a differential diagnosis with respect to *Hammondia* or *Besnoitia*. In the other cases, PCR is able to identify and differentiate the parasite but it is important to remember that the presence of DNA does not necessarily correlate with a viable and infectious parasite (also for this reason the gold standard to prove the presence and viability of the parasite is, still today, the bioassay). The genetic markers most commonly used are the B1 gene, the 529 bp repeat marker, but there are also others as SAG1, SAG6, APICO, etc. A PCR targeting the 529 bp repeat element has been reported to be 10–100 times more sensitive than the B1 gene (Homan et al., 2000), even if B1 is more specific. There are several PCR methods available and applicable for identification of *T. gondii* as nested PCR, used to increase the specificity of DNA amplification as well as to detect very few amounts of parasite. Real-time PCR (Rt-PCR) based methods allowed both the identification and quantification of the parasite and have a high degree of complex-specific diagnostic accuracy for diagnosis of pathogens in clinical samples. These assays are more rapid, sensitive and reproducible than conventional PCR, in fact when the target DNA is in low concentration, Rt-PCR is the most sensitive molecular assay for the detection of pathogens. Different protocols have been proposed and the majority of the studies reported that it is more sensitive compared to other molecular and biological methods (Calderaro et al., 2006; Juránková et al., 2013).

Loop-mediated isothermal amplification (LAMP) is a one-step PCR characterized by high sensitivity, specificity, efficiency. Moreover, it is also easy to perform, feasible, rapid and does not require expensive instruments (as Rt-PCR needs for example). LAMP amplifies DNA under isothermal conditions (60–65 °C) using a DNA polymerase (*Bst*) with strand settlement activity and 4–6 specific primers, which are able to recognize a total of 6–8 distinct regions within the target DNA, increasing significantly the specificity of the method (Abbasi et al., 2010). LAMP is used for *T. gondii* detection in several biological and environmental samples amplifying different targets as B1, 18S rRNA, SAG1 (Lalle et al., 2018; Wang et al., 2013).

Bioassay is still now the “gold standard” to demonstrate the presence of live and infectious *T. gondii* in animals and food products. Their use has been strongly limited in recent years after the development of a specific legislation on animal welfare, the so-called “3R” rules (replacement-reduction-refinement). Mice are commonly used and infected with intraperitoneal injection for increasing the number of tachyzoites, useful for lab experiments (i.e., drugs test in vitro) for example, or to check the presence of *T. gondii* in meat or food samples (i.e., after a treatment with UV, high pressure, different time-temperature conditions, etc.). Also cats might be used, in this case they are infected orally and the presence of the parasite is demonstrated if infected cats shed oocysts.

Currently, bioassay using either cats or mice is the standard method to demonstrate *T. gondii* viability but, for ethical reasons, this is not preferred for large scale testing as has, for example, become evident from the recent closure of these facilities at the United States Department of Agriculture (USA) (USDA Press Release No. 0042.19) (Opsteegh et al., 2020).

Alternative methods as *cell culture* combined with a Rt-PCR (Vismarra et al., 2017a; Zintl et al., 2009) have been proposed and the method seems promising. Anyway, critical points are still present as tissue digestion, since the presence of digest affected sensitivity, and issues with PCR inhibition and fungal contamination were encountered (Opsteegh et al., 2020).

As already cited meat is probably the principal food product responsible for *T. gondii* transmission to humans (Scallan et al., 2011). The problem is that the sole gold standard for identification of *T. gondii* in meat is the bioassay in cats or mice, with obvious bioethical implications. Serological tests are useful to prove if contact with the parasite has occurred, but cannot certify the presence of tissue cysts. Moreover, various PCR methods have been developed, but the sensitivity was low because of small amount of DNA, low amount of meat that can be tested, together with a low tissue cyst density. For all these reasons researchers tried to develop an alternative method enough sensitive and specific to use instead of bioassay: *the magnetic capture Rt-PCR*. The method combined the hybridization of specific, biotinylated probes with the capture of those probes with streptavidin-coated paramagnetic beads together

with a Rt-PCR reaction. In this way *T. gondii* DNA can selectively be “fished out” from a large volume of meat lysate. The last version of the method was validated as ISO 17025 with a 99% detection limit of 65.4 tachyzoites per 100 g of meat, so more sensitive and economic (Algaba et al., 2017).

Diagnosis in humans

Diagnosis in humans is mainly based on *serology*. Depending on countries and relative monitoring control programs, generally pregnant women are subjected to serological test for *T. gondii* at the beginning of pregnancy and if they are seronegative, every month till the birth (at least in developed countries). Tests include the research of IgM and IgG antibodies with ELISA test, Sabin-Feldman dye test or other lab methodologies. The newest techniques for the research of anti *T. gondii* immunoglobulins are chemiluminescence assays (CLIA), enzyme-linked fluorescence assay (ELFA), immunochromatographic test (ICT), serum IgG avidity test and immunosorbent agglutination assays (ISAGA) (Rostami et al., 2018). In ISAGA, anti-human IgM antibodies are used for diagnosis of acute or congenital toxoplasmosis. A disadvantage for this method is the requirement of a large numbers of *T. gondii* tachyzoites. Although, this problem was resolved by replacing *T. gondii* tachyzoites with latex beads coated with soluble antigens (Liu et al., 2015). The last developed serological tests exploited the recombinant antigen technology, in this way sensitivity, specificity, repeatability of the tests are guaranteed without the need of a huge amount of tachyzoites and the need for standardization at each laboratory. Moreover, different antigens, specific of acute and chronic phase of infection, could be tested simultaneously, discriminating between the two stages of *T. gondii* (typically SAG1 for tachyzoites and BAG1 for bradyzoites) (Holec-Gąsior, 2013). In addition, more recent studies using chimeric antigens and multiepitope peptides reported very promising results. In fact, these chimeric and/or synthetic peptide antigens, including different immunoreactive epitopes from various strains of *T. gondii*, that are also hydrophobic and generally well exposed on the antigen surface, seem to be strongly able of discriminating recently acquired infections from chronic one (Dai et al., 2012). New recombinant proteins, as TgERP (Hill et al., 2011), have been designed with the aim to develop diagnostic systems able to discriminate between a *T. gondii* infection due to ingestion of oocysts or tissue cysts. In 2015, for example, researchers created the CCp5A recombinant protein from oocyst/sporozoite of *T. gondii*. It was reported to be a potential new tool for diagnosis and epidemiological studies since the CCp5A protein reacted only with serum of pigs, chickens and mice infected with oocysts and it is able to detect both IgM and IgG in serum of people from an outbreak of toxoplasmosis and also in serum of pregnant woman (Santana et al., 2015).

IgG, IgA and IgM specific for *T. gondii* could also be found in saliva and oral fluid at different concentrations depending on several factors with results comparable to serum (Hajeer et al., 1994; Mangiavacchi et al., 2016). IgG in particular are considered good markers for acute toxoplasmosis (Hajeer et al., 1994; Sampaio et al., 2020) and used for diagnosis and monitoring of infants with suspected congenital toxoplasmosis (Chapey et al., 2015a). Moreover, also the interferon gamma release after T-cell stimulation by *T. gondii* antigens has been proven to be useful for the diagnosis of congenital toxoplasmosis (Chapey et al., 2015b). Interferon-gamma release assay (IGRA) accurately distinguished infected from uninfected individuals, showing strong lymphocyte activation after *in vitro* stimulation with *T. gondii* antigens, even during the first days of life. New tools following this principle have been developed as for example the ELISA-based IGRA test, reported as an easy-operation and low-cost method to measure cell mediated immunity against *T. gondii* (Mahmoudi et al., 2017).

To overcome the limitations of the serological tests, different *molecular methods* have been developed to detect *T. gondii* DNA in biological samples.

To diagnose congenital and ocular toxoplasmosis, the PCR has been successfully used with amniotic fluid, placental and brain tissues, aqueous humor and vitreous fluid. Moreover, whole blood, urine, CSF, BAL, pleural and peritoneal fluids have been effectively used to diagnose toxoplasmosis in immunocompromised patients. The most used target also in clinical setting are the B1 gene as well as the 529 bp repeat element.

Imaging techniques are useful for diagnosis of cerebral and ocular toxoplasmosis.

A variety of techniques as computed tomography (CT), magnetic resonance imaging (MRI), nuclear imaging and US could be applied to diagnose cerebral toxoplasmosis (encephalitis) (Rostami et al., 2018).

The ocular toxoplasmosis can be diagnosed by clinical findings such as visual acuity, intraocular pressure, biomicroscopy and indirect ophthalmoscopy but also with the help of imaging techniques such as fundus autofluorescence (FAF), confocal scanning laser ophthalmoscopy (CSLO), optical coherent tomography (OCT), fluorescent angiography (FA), ultrasound, and indocyanine green angiography (ICG) (Rostami et al., 2018).

Congenital toxoplasmosis on the other side might be diagnosed with US to identify brain abnormalities. Similarly, US can be used postnatally also to monitor ventricular size in infants till 18 months of age (Khan and Smirniotopoulos, 2015).

In adult peoples hepatosplenomegaly and abdominal lymphadenopathy due to toxoplasmosis might be identified with an abdominal US.

To conclude, for a correct diagnosis of congenital toxoplasmosis several medical protocols suggest to apply IgG/IgM ELISA in combination with Rt-PCR/LAMP on amniotic fluid and prenatal US for detection of acute infection in pregnant women. Serology is also the “gold standard” in newborns, although Rt-PCR/LAMP on cord blood or whole blood can be useful for definitive diagnosis.

Moreover, combining of ELISA (using TLA, recombinant or chimeric antigens), Rt-PCR/LAMP on CSF and imaging findings by MRI or CT could result in definitive diagnosis in immunocompromised patients.

Drug treatment

Regarding drug therapy, the treatment of cats with clinical toxoplasmosis is mainly based on the use of clindamycin, with dosages of 20–25 mg / kg for 1–2 weeks in the case of intestinal forms and of 20–40 mg / kg for 2–4 weeks in the case of systemic forms. Clinical signs generally improve 24–48 hours after the start of treatment, however the side effects can be important and manifest themselves with gastroenteritis and diarrhea (Davidson, 2000). Generally, most of the clinical signs related to toxoplasmosis tend to strongly decrease after 7 days of treatment, if this does not happen, prolonged use of the same molecule will hardly produce the desired effects and for this reason it is advisable to change the drug (Lappin, 2010).

In the case of systemic pathology and uveitis, it is recommended to administer clindamycin with glucocorticoids topically, parenteral or oral, to avoid glaucoma or dislocation of the lens as a secondary outcome of the infection. In case of systemic manifestations, the use of pyrimethamine and sulfonamides in combination has also been described. Also in this case, side effects can be important, in particular for the myelosuppressive action of these drugs. In the case of neurological forms, the administration of anti-convulsants may be necessary (Dubey et al., 2009). In addition, treatment with clindamycin, sulfonamides and pyrimethamine can reduce the elimination of oocysts with faeces in cats infected for the first time with *T. gondii* (Dubey and Lappin, 2006).

It is important to remember that the drugs against *T. gondii* do not eliminate the parasite but only stop its replication. As a defense mechanism against the host's immune response, coccidium "hides" in the musculoskeletal and cerebral tissues and the formation of cysts containing bradyzoites protects the parasite from attack by pharmacological molecules and blocks the action of monocytes, macrophages and other immune cells (Dunay et al., 2018). In fact, the main problem in finding effective treatments for toxoplasmosis is the inability of lots of molecules to reach tissue cysts and acting against bradyzoites. Actually, the two drugs mainly used for treating human toxoplasmosis (active against the "free form" of the parasite) are Pyrimethamine and Sulfadiazine. The first one, typically used for malaria, is a folinic acid antagonist and other potential side effects include bone marrow suppression and liver toxicity, while Sulfadiazine is an antibiotic (Class: sulfonamides).

Actually, in case of seroconversion which occurred after the 18th week, the guidelines generally foresaw a treatment with Spiramycin or a protocol with Pyrimethamine, Sulfadiazine and Folinic Acid with the aim of avoiding the manifestation of congenital toxoplasmosis. On the other hand, in case of congenital toxoplasmosis the therapeutic protocol foresaw the administration of Pyrimethamine + Sulfadiazine + Folinic Acid (Pyrimethamine 1 mg / kg / day for 2 months, then 0.5 mg / kg / day; Sulfadiazine 50 mg / kg 2 times a day; Folinic Acid 1 tablet of 25 mg 2 times a week) or Sulfadiazine + Pyrimethamine + Folinic Acid (Sulfadiazine 17.5 mg / kg once a week; Pyrimethamine 0.875 mg / kg once a week; Folinic Acid 1 tablet of 25 mg twice a week) (Peyron et al., 2019).

About vaccination, to date, there is no anti-*T. gondii* vaccine effective in blocking the vertical transmission of the parasite or in eliminating brain and tissue cysts, despite the fact that numerous antigenic determinants capable of inducing a humoral immune reaction have been identified. Currently, the only anti-*T. gondii* vaccine is intended for sheep and is only authorized in some countries such as New Zealand, France and Great Britain, excluding Italy. This is due to the fact that it is a live attenuated vaccine containing tachyzoites and therefore potentially risky. Furthermore, not everyone agrees on its real effectiveness.

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- <http://www.fao.org/news/story/en/item/237323/icode/>—Food and Agriculture Organization of United States.
- <http://www.eurosurveillance.org/>—European Centre for Disease Prevention and Control.
- www.cdc.gov/parasites/toxoplasmosis—Centre for Disease Control and Prevention.
- www.who.int/en—World Health Organization.

Trichinella

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History

The parasite was fortuitously discovered in 1835 by Richard Owen and James Paget in the muscles of a dead patient. It was diagnosed as a “microscopic entozoon infecting the muscles of the human body.” The pathogenic role of the parasite for humans was revealed by Friedrich Albert von Zenker in 1860 after the finding of the parasite in the muscles of a young woman (who died of trichinellosis) and also in the eaten meat. The parasite was first called *Trichina spiralis*, then *Trichinella spiralis* in 1896.

Epidemiology

Trichinella spp. are widespread in wildlife on all continents (except probably Antarctica) and in domestic pigs of many countries (Murrell and Pozio, 2011). The natural cycle is maintained by a large number of infected vertebrates (≈ 100 animals species), divided into three classes: mammals, birds, and reptiles. Man, an accidental host, is a parasitic dead end. Ten species and three genotypes are recognized by molecular analyses in the genus *Trichinella* (Korhonen et al., 2016), namely, *Trichinella spiralis* (T1), *Trichinella nativa* (T2), and its related genotype *Trichinella* T6, *Trichinella britovi* (T3), and its related genotype *Trichinella* T8, *Trichinella pseudospiralis* (T4), *Trichinella murrelli* (T5), and its related genotype *Trichinella* T9, *Trichinella nelsoni* (T7), *Trichinella papuae* (T10), *Trichinella zimbabwensis* (T11), *Trichinella patagoniensis* (T12), and the last described *Trichinella chanchalensis* (T13) (Pozio and Zarlenga, 2013; Sharma et al., 2020). All species can develop in mammals, but *T. pseudospiralis* can also develop in birds, and *T. papuae* and *T. zimbabwensis* also occur in some reptile species (Pozio, 2007) (Fig. 1).

There is a wild cycle and a domestic cycle, both are more or less intertwined. The wild cycle is maintained by a large number of invertebrates that become contaminated by the ingestion of live prey or infested carrion. *T. spiralis* and *T. pseudospiralis* are globally distributed (La Rosa et al., 2003; Rosenthal et al., 2008), whereas the other species are geographically restricted. In temperate regions, *T. spiralis* and *T. britovi* infect wild boars, canids, viverrids, mustelids, and marsupials. In the tropics, *T. nelsoni* infects large carnivores, scavengers, and warthogs. *T. papuae* infects pigs in New Guinea and Southeast Asia (Caron et al., 2020). In the polar regions, *T. nativa* and *Trichinella* T6 infect bears, canids, and walruses. The domestic cycle is maintained by pigs, rodents, and accidentally horses. Pigs are responsible for the majority of epidemics around the world. It becomes infected by ingesting rodents or by tail-biting. Herbivores, such as horses, sheep, or goats, would become contaminated because of the accidental presence of animal waste in their food (Soule et al., 1989; Rostami et al., 2017).

The most common source of human trichinellosis is meat from pig or wild pig (*Sus scrofa*) (Gottstein et al., 2009; Murrell and Pozio, 2011). The disease is endemic in regions where pig farming is traditional, veterinary controls are absent or insufficient and this meat is consumed raw or undercooked. Moreover, large outbreaks linked to the consumption of horse meat are reported; and small outbreaks occur due to the consumption of game meat (wild boar, bear, warthog, reptile, etc.). Travel in regions with high incidences is a classical driver for acquiring trichinellosis (Dupouy-Camet, 2014; Barluet et al., 2020).

Approximately 11 million persons worldwide might be infected by *Trichinella* spp. (Dupouy-Camet, 2000). A total of 65,818 human cases of trichinellosis and 42 deaths have been reported in 41 countries from 1986 to 2009 (Murrell and Pozio, 2011): In Africa, Asia and America, parasitosis is rarely reported (0.04, 2.37, and 10.90% of cases, respectively). In Africa, outbreaks have been described in natives from Christian regions of Ethiopia or in European travelers to Algeria and Senegal. In America, most of the cases are reported in Argentina. In Canada and Greenland, a few outbreaks occurred in native people and foreign hunters. In Asia, most of the cases are reported in China; but the parasitosis could be underestimated in some regions, for example in Vietnam (Caron et al., 2020). Europe matters the higher number of cases (86.47%). Eastern European countries reported particularly most cases (Romania, Serbia, Bulgaria, and Lithuania). Since the early 1990s, the incidence of the disease has decreased in this region.

Parasitology

Cycle

The adults are a few millimeters long. The microscopic larvae, sources of contamination, are most often present in the striated skeletal muscle fibers of the infected animals. After eating parasitized meat, larvae are released by stomach digestion and become adults within 48 h in the small intestine. The adults die in less than 4 weeks; after mating, the females lay, 5 to 15 days after the contamination, thousands of larvae. The newborn larvae (NBL) migrate, through the lymphatic-blood route, into the general circulation before moving to the muscle fibers. There, the NBL diverted the metabolism of the host cell, which will become a nurse cell (NC), they will encyst 3 weeks after contamination and will survive for several years. Although the parasite is transmitted by carnivorous, herbivores can be sources of contamination (Rostami et al., 2017). Currently, the 13 lineages of *Trichinella* are grouped into encapsulated and non encapsulated clades based on the presence or absence of a collagen layer surrounding the NC containing the infective larva stage 1 (L1) (Zarlenga et al., 2006; Pozio and Zarlenga, 2013). Interestingly, the three non encapsulated species are those that can develop in other animals than mammals, *T. pseudospiralis* (T4) in birds, *T. papuae* (T10) and *T. zimbabwensis* (T11) in some reptile species (Pozio, 2007). Seven *Trichinella* species have been found pathogenic for humans, *T. spiralis*, *T. nativa*, *T. britovi*, *T. murrelli*, *T. nelsoni*, and *T. pseudospiralis* and *T. papuae* (Fig. 2).

Pathogenicity

Parasite factors

Adult females play a role in the pathogenesis by mechanical and irritant action on the intestinal mucosa leading to its penetration, or by toxic and antigenic action in releasing of excreted secreted (ES) products, and by limited bactericidal action. The larvae have also a pathogenic role through their toxic and irritant action during encystment in muscle, and an antigenic action leading to the activation of the host's immune system.

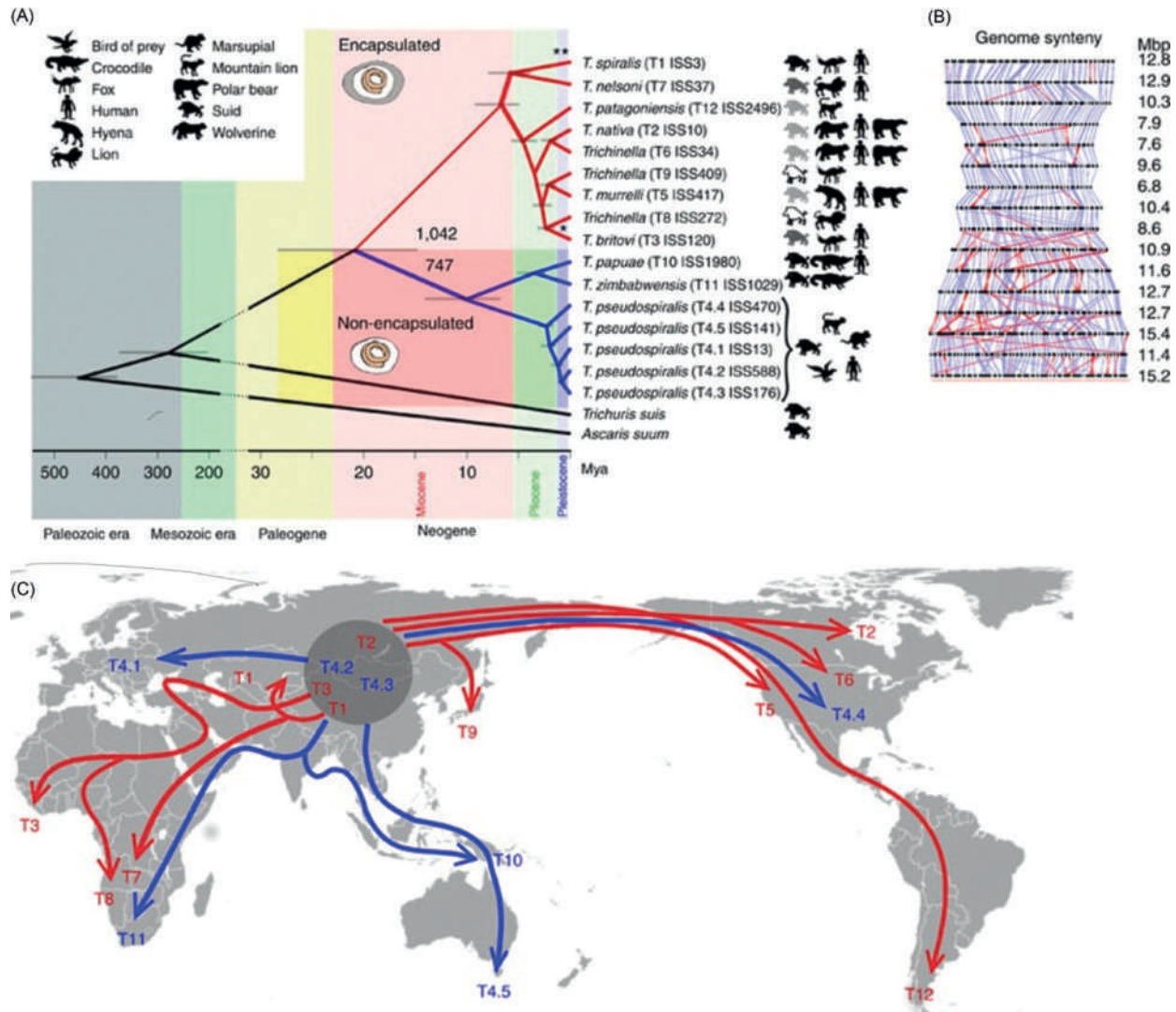


Fig. 1 The evolution and biogeography of *Trichinella* taxa. (A) The phylogeny of all 12 currently recognized taxa of *Trichinella* based on analyses of amino-acid sequence data from shared single-copy orthologous genes SCOs ($n = 1284$) employing Bayesian inference, ML and MP methods, with *Trichuris suis* and *A. suum* as outgroups; 1042 and 747 are the numbers of orthologous gene groups, which are unique to encapsulated (red) and non-encapsulated (blue) *Trichinella* taxa, respectively. The topology of the trees constructed using each of these methods was the same; all nodes have absolute statistical support (1.00 or 100%), except for one node (*) in the ML analysis, where it was 99%. The grey bars on the nodes represent 95% confidence intervals for the estimate of species branching time. *T. spiralis* (ISS195) shares the same phylogenetic position (**) as *T. spiralis* (ISS3). Host animals: suids (*Sus scrofa*) represent both the sylvatic and domestic porcine hosts (left); the reproductive potential of a particular *Trichinella* taxon in *S. scrofa* is indicated by the color scale: white: not assessed; light grey: low; dark grey: medium; black: high. Other animals (right) represent examples of carnivorous sylvatic hosts in different geographic regions, including fox, lion, mountain lion, marsupial, crocodile and bird of prey, and the accidental human host. (B) Representation of genome-wide synteny among *Trichinella* taxa (same order as listed on the right in A). Genomic scaffolds (black) sharing at least 10 SCOs between *Trichinella* taxa are displayed. A purple line indicates a single SCO and a red line an inverted SCO. The numbers on the right indicate the genomic length in megabases (Mb). (C) Biogeography of *Trichinella* taxa proposed on the basis of known global (climate, extinctions and/or tectonic) events and diversification times (mya) for *Trichinella* taxa, estimated using a molecular clock approach. Encapsulated taxa: *T. spiralis*, T1; *T. nativa*, T2; *T. britovi*, T3; *T. murrelli*, T5; *T. nelsoni*, T7; *T. patagoniensis*, T12; and *Trichinella* genotypes T6, T8 and T9. Non-encapsulated taxa (infecting mammals, reptiles and/or birds): *T. pseudospiralis*, T4; *T. papuae*, T10; *T. zimbabwensis*, T11. Geographic distributions of *Trichinella* taxa were reported by Pozio and Zarlenga. The embedded public domain world map image (<https://commons.wikimedia.org/wiki/File:BlankMap-World6.svg>) has been modified using the programs World map tool v.1.16 (<http://law.nagoya-u.ac.jp/en/appendix/software/worldmap>) and GIMP v.2.8 (<https://www.gimp.org/>), <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4740406/bin/ncomms10513-f1.jpg>. From Korhonen PK, Pozio E, La Rosa G, Chang BC, Koehler AV, Hoberg EP, Boag PR, Tan P, Jex AR, Hofmann A, Sternberg PW, Young ND, and Gasser RB (2016) Phylogenomic and biogeographic reconstruction of the *Trichinella* complex. *Nature Communications* 7: 10513.

The degree of pathogenicity of *Trichinella* is directly related to the species as the females have different fertility rates (Sadaow et al., 2013) and to the initial infecting dose (Pozio et al., 1992; Murrell et al., 2000; Gottstein et al., 2009). *Trichinella spiralis* is considered the most pathogenic species. One larva per gram of meat is enough to cause clinical signs in humans, corresponding to an infectious dose of around 150 larvae in a usual ration of meat (Zarlenga et al., 2006).

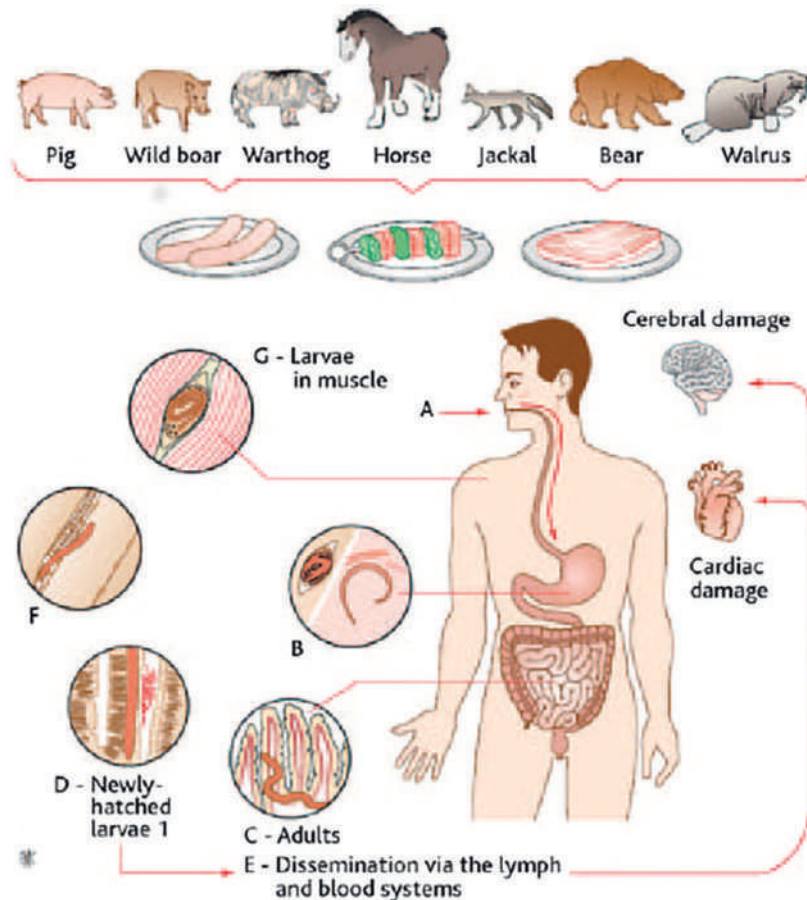


Fig. 2 Life cycle of *Trichinella* in humans and main sources of contamination. From Dupouy-Camet J, Lacour S, Vallée I, Yera H, Boireau P. (2015) Trichinelloses. *Encyclopédie Médico-Chirurgicale - Maladies infectieuses* 12(2): 1–13. DOI 10.1016/S2211-9698(15)57764-9.

Host susceptibility

The nature of the host influences the parasite infectivity. *T. spiralis*, *T. pseudospiralis* and *T. papuae* appear to be more infectious (i.e. greater reproductive capacity) in hamsters and gerbils than in mice and rats (Sadaow et al., 2013). Age influences the severity of the disease. Old people have more risk of complications (Barruet et al., 2020; Dupouy-Camet and Bruschi, 2007). The immune status also influences the severity of the disease. People, that had previous contact with *Trichinella* parasites, may develop an immunity, leading to a longer incubation phase and milder symptomatology in case of reinfections. Such immunity has been observed in Inuit populations having frequent contact with infected meat (Viallet et al., 1986) and during small outbreaks in France and Serbia (Dupouy-Camet et al., 2016; Barruet et al., 2020).

Type of meal

The quantity of alcohol consumed during the meal can affect the virulence of the parasite (Steven et al., 1990; Na et al., 1997). The type and duration of meat treatment, or the cooking mode of the meat, as well as the quantity of treated or prepared meat influence the survival of larvae. The survival of *Trichinella* in dried meat depends on the moisture content (Gamble et al., 2007).

Physiopathology

Infection is clinically manifest in humans when the number of larvae per gram of muscle biopsy is about 10 and can become severe when this number is greater than 100 (Pawlowski, 1983). Complications are linked to the toxic effect of eosinophils in large numbers in the tissues (Durack et al., 1979; Katz et al., 1989; Mawhorter and Kazura, 1993) and the migration of larvae in the tissues. So, heart and brain lesions are often associated and could result from the simultaneous intervention of local prothrombotic effects of eosinophil activation and vascular injury caused by the migrating larvae (Fourestie et al., 1993).

Immunity

Trichinella adults and larvae are responsible for an immune response at intestinal, systemic, and tissue levels, respectively.

During the intestinal phase

The infection causes infiltration of inflammatory cells (lymphocytes, mast cells, and eosinophils) in the lamina propria of the intestinal mucosa. The CD4 + T lymphocytes (TL) recognize the parasite antigens and are activated within 2–4 days post-infection. A Th1-type immune response sets in with the release of interferon γ and interleukins (IL) 2 and 3 in the mesenteric ganglia. Interferon γ allows the activation of macrophages. IL 2 stimulates the proliferation of T and B lymphocytes. IL 3 allows mast cells to release mediators (proteinases with chymotryptic activity, “platelet activating factor” PAF, “eosinophil chemotactic factor” ECF-A, leukotrienes, histamine and, serotonin) which modify intestinal motility allowing the expulsion of adult worms from the intestine. The goblet cells prevent contact between the parasite and the intestinal epithelium by hypersecretion of mucus (Bell, 1998; Bruschi and Chiumiento, 2012).

During the migratory phase

Eosinophils appear during NBL migration and allow their elimination, at least in a secondary infection (Bruschi, 2002; Bruschi and Chiumiento, 2011; Huang and Appleton, 2016).

During the muscle phase

The entry of the parasite produces chronic inflammation in the muscle of the host. A Th2-type immune response sets in with the release of IL 4, 5, 6, 10, and 13. This lasts throughout the chronic phase of the infection. Specific IgG and IgE antibodies are produced in high quantities, the levels of IgM and IgA are lower.

IL 4 and 10 stimulate the synthesis of IgE. IL 4 also participates in the transport of IgE in the intestine and stimulates the proliferation of mast cells in the intestinal mucosa. This cytokine plays a role in the expulsion of worms from the intestine. IL 5 and 6 promote the development of eosinophils.

In vitro eosinophils and neutrophils can kill NBL by antibody-dependent cell-mediated cytotoxicity (ADCC), whereas in vivo their role is not yet clear (Bruschi and Chiumiento, 2012). The cellular infiltrate around the NC is composed of macrophages, CD4 +, CD8 + TL, and some B lymphocytes (Finkelman et al., 1997; Bell, 1998; Bruschi, 2002; Bruschi and Chiumiento, 2011).

Variability according to parasite species

Pig model studies showed that host immune response differs according to the parasite species. Seroconversion occurred slowly with *T. pseudospiralis*, as with *T. britovi* compared to *T. spiralis*. The highest infectivity and immunogenicity, and the longest larvae survival in muscles were found with *T. spiralis* (Pozio et al., 2020).

Variability of host's response

The host's response to *Trichinella* infection is highly variable, believed to be related to the major histocompatibility complex (MHC).

Host's immune system escape strategies

Animal model studies showed that *T. spiralis* and its products induce an immunomodulatory network (which encompasses Th2- and Treg-type responses) (Sofronic-Milosavljevic et al., 2015).

Antigen dependent mechanisms

In muscle, *Trichinella* will seclude itself from the host's immune system through the formation of the NC and the collagen capsule. From this immunologically privileged place, the parasite orchestrates a long-lasting molecular cross-talk with the host. The maintenance of the NC is possible thanks to the exchanges between the parasite and its host. These exchanges depend essentially on secreted signal molecules such as muscle larvae ES products and cytokines. ES products originate from stichocyte granules in the stichosome, secretory organelle of the *T. spiralis* muscular larvae (Despommier, 1983; Cvetkovic et al., 2016). Host cells respond to molecular signals by participating in the formation of the NC (Despommier, 1998; Bruschi, 2002; Bruschi and Chiumiento, 2012). The *T. spiralis* cuticle is a dynamic organ that undergoes protein changes during the molting process and growth. The modification of the parasitic surface antigens allows *Trichinella* to escape the action of effector cells of the host's immunity (Phillipp et al., 1980).

Modulations of the host's immune system

Trichinella can modulate the production and functions of the host's immune cells.

At the central level

During the production of NBL, immunosuppression occurs, which suggests that NBL release lymphocytotoxic factors. The parasite also stimulates the production of eosinophils decreasing the Th1-type immune response, which is harmful to the parasite. The parasite stimulates the polyclonal activation of lymphocytes, which may contribute to the increase of non-specific IgE in favor of specific IgE.

At the peripheral level

Antigens secreted by the parasite may also interfere with the maturation of antigen-presenting dendritic cells, thereby promoting a Th2-type response (Ashour, 2013). They could also stimulate the production of regulatory TL which inhibit the production of other effectors TL (Aranzamendi et al., 2012).

L1 ES proteins inhibit the chemotaxis of neutrophils. ES products of *T. spiralis* could modulate the functions of macrophages, in particular by reducing their capacity to express pro-inflammatory cytokines (TNF α , IL 1, IL 6, and IL 12) in the presence of lipopolysaccharide (LPS) (Bai et al., 2012; Zawistowska-Deniziak et al., 2021). *Trichinella* is thought to induce specific antibodies capable of blocking the ADCC of eosinophils and neutrophils against NBL. The parasite may produce compounds able to inhibit the assembly and polymerization of the membrane attack complex (MAC), preventing damage to the complement system. *T. spiralis* and *T. nativa* can activate the complement system and escape its attack (Nareaho et al., 2000).

Clinics of trichinellosis

Clinical signs are related to the infected dose, the species of *Trichinella*, and the host (see Parasitology section).

Acute trichinellosis

Classically, trichinellosis manifests itself in three phases: an incubation phase; an acute phase or invasion phase, and a convalescence phase (Dupouy-Camet and Bruschi, 2007) (Table 1).

During incubation (1 to 4 weeks), diarrhea is most often observed (but in less than 50% of cases), as well as vomiting or abdominal pain. These first digestive symptoms correspond to the presence of adult worms in the intestine (2 days to 4 weeks after contamination). They last an average of 10 days. During the acute phase (1 to 4 weeks), the patient has a fever, swelling of the face and eyelids, severe muscle pain, asthenia. These general symptoms are present in more than three-quarters of the cases. They correspond to the NBL circulation (5–15 days after the contamination); then to their migration into the muscles. Fever could be high ($>39^{\circ}\text{C}$) and associated with pruritus and urticarial or morbilliform rash on the trunk, the abdomen, and limbs. Facial edema may be palpebral and/or conjunctival. It appears between 7 and 25 days after the contamination. Fever and edema last an average of 10 days. Conjunctivitis or subconjunctival bleeding are sometimes present. Peripheral edemas have been described. Distal subungual hemorrhages, specific to disseminated vasculitis, are sometimes observed. Myalgia and asthenia persist for 2–4 weeks. At this phase, there is an increase in eosinophils and muscle enzymes in the blood. During the convalescence phase, myalgia and asthenia persist longer.

Main complications

Complications could sometimes occur during the acute phase and mainly in the elderly. Neurological, cardiac, and vascular damages are responsible for the severity of trichinellosis. Their frequencies are variable according to epidemics and can affect up to 30% of cases for neurological complications and 4 up to 20% for cardiac and vascular complications, respectively (Ancelle et al., 1998; Dupouy-Camet and Bruschi, 2007). They can be life-threatening. The reported mortality rates of trichinellosis in humans are 0.2 to 0.5%, but a high rate of 24% was observed in an outbreak in Cambodia where the infective doses were certainly very high (Caron et al., 2020).

Neurotrichinellosis represents the major complication of trichinellosis in humans. It is caused by vasculitis, small vessel thrombosis, punctiform hemorrhages, ischemia, and granulomatous inflammatory reactions often involving eosinophils (Cortez et al., 1986; Soulié et al., 1986). Neurological involvement may present as meningoencephalitis or focal neurological signs or coma.

Table 1 Clinics and diagnosis of trichinellosis.

Phase	Duration	Symptomatology	Blood biology
Invasion phase	1–4 weeks	Unapparent or digestive disorders – Diarrhea – Abdominal pain	Eosinophilia
Acute phase	3–4 weeks	Fever, myalgia, edema (face), bleeding (rare) – subconjunctival – subungual, and complications	Eosinophilia, muscle enzymes increase, positive serology
Convalescence and chronic phases	Months, years	Myalgia, asthenia	Positive serology

Therefore, the symptoms are very polymorphic: disorientation, frontal syndrome, hemi- or quadriplegia, oculomotor deficit, central visual deficit, aphasia, cerebellar syndrome, facial nerve paralysis, seizures, etc. . .). Symptoms last from 1 to 2 months, but motor or neuropsychological sequelae may persist after 6 months.

Cardiac involvement manifests in myocarditis. Pericarditis, endocarditis, wall thrombosis, or coronary artery can rarely be observed. These complications must be systematically sought by EKG. The symptoms include pain in the heart region, tachycardia, and EKG abnormalities. Vascular complications are sometimes associated. They consist of thromboembolic disease, specifically, deep thrombophlebitis, intraventricular thrombi, and/or pulmonary embolism. Cardiovascular complications may evolve during 1 month and engage the vital prognosis. Death may result from embolism of the pulmonary artery or paroxysmal tachycardia. Definitive sequelae are possible.

Other complications

Digestive complications (acute necrotizing enterocolitis, massive protein exudation with hypoalbuminemia, and systemic edema) are possible (Dupouy-Camet and Bruschi, 2007). Respiratory complications (pneumonia, obstructive bronchitis, or Löffler-type infiltrates, or respiratory failures) are rare. They can occur both in the early and late stages of trichinellosis (Compton et al., 1993). Ocular complications (edema and vascular lesions within the conjunctiva, the uvea, the retina, and the optic nerve, pain when moving the eyeballs, muscle paralysis, diplopia, or a disturbed accommodation) can occur (Kociecki et al., 2014).

Clinical forms

Different clinical forms have been described in acute trichinellosis according to the severity of symptomatology and the presence or absence of complications: severe, moderately severe, benign, abortive, or asymptomatic forms (Dupouy-Camet et al., 2021).

In children, the signs and symptoms of trichinellosis are less pronounced and regress more quickly than in adults. Moreover, the frequency of complications is lower. The clinical picture is milder possibly because of lower infecting doses and a less intense allergic reaction to the larvae invasion (Ozdemir et al., 2005; Dupouy-Camet and Bruschi, 2007; Neghina et al., 2011a, b).

In pregnant women, trichinellosis can cause abortion or premature delivery (Ancelle et al., 1998). Congenital trichinellosis has not been established (Kociecka, 2000; Taybouavone et al., 2009; Bruschi and Carlier, 2013).

In immunocompromised patients, trichinellosis has rarely been described with asymptomatic, benign, and severe forms. Hypereosinophilia could be absent in these patients (Dupouy-Camet et al., 2021).

In patients with a history of previous *Trichinella* infection, a longer incubation period and milder symptomatology have been reported (Dupouy-Camet and Vallee, 2006; Dupouy-camet et al., 2016; Barluet et al., 2020). In persons who regularly eat infected meat (i.e., Inuit populations), a particular chronic diarrheal syndrome has been described and is due to the strong intestinal immune reaction; that consists of rapid expulsion of adult worms from the intestine, thus preventing the muscular phase (MacLean et al., 1989).

Chronic trichinellosis

The existence of a chronic form of trichinellosis is debated. It should not be confused with definitive neurologic and cardiovascular sequelae of trichinellosis due to complications (Dupouy-Camet and Bruschi, 2007; Dupouy-Camet et al., 2021). Chronic trichinellosis has been described as persistent symptoms (myalgia, muscle fatigue, formication, numbness, coordination disorders, conjunctivitis) 10 years after acute infection (Harms et al., 1993; Kociecka, 2000; Pielok, 2001; Kociecka et al., 2001). Tongue pain or lesion associated with the presence of *Trichinella* sp. larvae and inflammation have been reported in patients infected for at least 1 year or up to 32 years (Tande and Pritt, 2012; Ait-Ammar et al., 2021).

Diagnosis

Circumstance of diagnosis

The diagnosis is suggested by the combination of fever, periorbital or facial edema, myalgia, and high blood eosinophilia, especially if there are grouped cases. It will be confirmed on the positivity of serology (or muscle biopsy). Serology allows a more sensitive and earlier diagnosis than a biopsy (approximately 10 days after infection for western blot (wb) versus 1 month for muscle biopsy). Parasitological examination of the remains of the eaten suspected meat would confirm the diagnosis and avoid muscle biopsy.

In countries where trichinellosis is infrequent, the disease may be undiagnosed by physicians not familiar with it. Therefore, the use of specialized services is necessary to make an early specific diagnosis and to give an appropriate treatment that will make it possible to avoid complications and sequelae.

Indirect diagnostic methods

Unspecific biological markers

Unspecific biological markers can suggest the diagnosis of trichinellosis. High blood eosinophils and elevated muscle enzymes are indicative of acute infection as early as 15 days ingestion of infected meat. The blood eosinophilia is very high (often > 1 G/L) and corresponds to the circulation of the larvae. It increases up to 5 weeks and can reach 30 G/L, then it gradually decreases over

2 months. Eosinophilia is associated with hyperleukocytosis. The increase in serum muscle enzymes (creatine phosphokinase CPK, lactate dehydrogenase LDH, and aldolase) is the witness of muscle lysis caused by the presence of larvae. The serum level of these enzymes will normalize in 2 months. A discrete biological inflammatory syndrome (increased C reactive protein CRP, or rate of erythro sedimentation), and lymphopenia can be found (Dupouy-Camet and Bruschi, 2007).

A moderate hyperproteinorachia can be observed during neurotrichinellosis. High levels of troponin could be associated or not with myocarditis during trichinellosis (Lachkar et al., 2008; Barruet et al., 2020).

Serology

Serology is important for the diagnosis because it differentiates trichinellosis from influenza-like illnesses, polymyositis, or other parasitosis. Several techniques have been proposed (ELISA, indirect immunofluorescence, indirect hemagglutination, latex agglutination, wb, etc.) but few have been marketed (ELISA, wb) (Bruschi et al., 2019). In practice, ELISA is widely used in screening, and wb is used as a confirmation technique (Yera et al., 2003; Dupouy-Camet and Bruschi, 2007). ELISA using soluble or ES antigens (Ag) has good performances: sensitivity is close to 100% and specificity is around 80% (Andiva et al., 2002). Wb using the same antigens has also good performances: sensitivity and specificity are close to 100% (Yera et al., 2003). It eliminates, when ES Ag is used, ELISA cross-reactions observed with other parasitosis (anisakidosis, toxocariasis, filariasis, hepatic distomatosis, schistosomiasis...) and systemic diseases (Bruschi et al., 2019). Seroconversion usually occurs between 2 and 5 weeks after infection (Dupouy-Camet and Bruschi, 2007). But, serology can be positive 10 days after infection and a few days after the onset of clinical signs. The delay of antibody appearance in serum depends on the *Trichinella* species and the infectious dose (the lower the dose, the later the antibodies appear). Wb maybe earlier than ELISA (lower detection threshold) (Yera et al., 2003). IgG are usually detected. IgM or IgA, appearing earlier in the serum, can also be tested but not routinely. IgM and IgA evaluations are used only in research laboratories.

If negative, serology should be repeated a week later. Seroconversion or increasing serum antibody levels confirm acute infection.

Direct diagnostic methods

Direct diagnosis of trichinellosis is rarely made in humans. It consists of performing and analyzing a muscle biopsy. The biopsy is done in the deltoid, triceps, or quadriceps, then stored and transported in physiological saline. The larvae can be observed on direct examination in the fresh state after crushing between 2 glass slides (trichinoscopy) or after artificial cholorhydropeptic digestion.

Artificial digestion is the more sensitive and the reference method recommended by the European Union (European Commission, 2015). This is a microscopic examination of the digested muscle tissues obtained by the combined action of heat, hydrochloric acid, and pepsin. This last method is required to determine the parasite burden (number of larvae per gram of muscle), to highlight larvae not (yet) encapsulated, and to isolate them alive (Dupouy-Camet and Bruschi, 2007). The histological examination allows the observation of larvae within the hypertrophied striated skeletal muscle fibers, which have often lost their structure. Although muscle biopsy has an absolute specificity; it is invasive and has low sensitivity especially in the absence of digestion. Besides, its prescription poses an ethical problem, because it does not help the diagnosis until the patient has recovered (3–4 weeks after the infection) and does not provide benefit to the patient.

Viability and infectivity of the larvae can be evaluated by mouse bioassay, consisting of the inoculation of a mouse immunosuppressed by injection of cyclophosphamide with the isolated larvae and analysis of the presence of larvae in muscle 5 weeks after infection (Kapel et al., 2003).

Molecular biology allows the identification of species from isolated larvae or directly from fresh or fixed samples (Caron et al., 2020). PCR can target *Trichinella* ribosomal DNA intergenic spacer region and is followed by DNA sequencing (De Bruyne et al., 2005). Multiplex PCR targets *Trichinella* expansion segment V (ESV), and internal transcribed spacer 1 and 2 (ITS1 and ITS2) (Zarlenga et al., 1999; Pozio and La Rosa, 2003).

The parasitological examination of the stool is usually negative, but Charcot-Leyden crystals (witnesses of local hypereosinophilia) can be found and, exceptionally, adults in the first days of infection (Dupouy-Camet and Bruschi, 2007).

Artificial digestion, and molecular identification, are reserved for specialized laboratories.

Human trichinellosis diagnostic criteria

The diagnosis is based on the combination of clinical or biological signs, associated with epidemiological criteria. An algorithm can help to diagnose acute trichinellosis (Dupouy-Camet and Bruschi, 2007) (Table 2).

The presented algorithm is useful to judge the validity of an individual clinical case. European Centre for Disease Prevention and Control (ECDC) established similar criteria for case definition; however, they are more appropriate in an epidemic context (Commission Implementing Decision, 2018).

Animal testing

Parasitological examination of the remains of eaten suspected meat can confirm the diagnosis and avoid muscle biopsy. Meat should be sent for analysis in authorized veterinary services or reference laboratories on animal trichinellosis. Meat analysis makes a species diagnosis and quantifies the inoculum, which are two prognostic elements of the severity of the disease.

Table 2 Algorithm for diagnosing the probability of being infected with acute *Trichinella* in humans (Dupouy-Camet and Bruschi, 2007).

Group	Clinical or biological signs
A	• Fever • Face and periorbital edema • Myalgia
B	• Neurological signs • Cardiac signs • Conjunctivitis • Subungueal hemorrhage • Skin rashes (maculopapular rash) • Diarrhea
C	• Eosinophilia (>1G/L) and/or increased total IgE • Elevations of muscle enzymes (CPK, LDH, Aldolase)
D	• Positive serology (with a highly specific test) • Seroconversion • • Positive muscle biopsy

CPK: creatine phosphokinase, LDH: lactate dehydrogenase.

The diagnosis of trichinellosis is:

- unlikely: association of a sign A or a sign B or a sign C.
- suspect: a combination of an A sign or two B signs and a C sign.
- highly probable: a combination of three A signs and two C signs.
- certain: association of 3 signs A, two C, and a D or association of a few signs A or B, a C and a D.

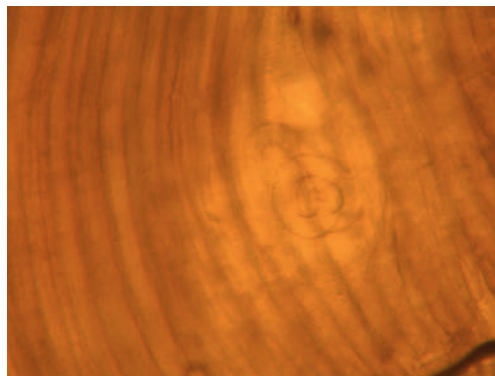


Fig. 3 Muscle larva of *Trichinella* observed after compressing a muscle fragment from wild boar between two glass slides (trichinoscopy) From A. Debruyne collection, X400.

Meat analysis consists to detect the larvae by trichinoscopy (Fig. 3) or isolating the larvae after artificial digestion.

As previously mentioned (see [Direct diagnostic methods](#) section), artificial digestion is the more sensitive and the reference method recommended by the European Union (European Commission, 2015). The parasitic burden is quantified in the different animal parts (number of larvae/g of muscle). The detection threshold is 0.1–1 larva/g depending on the quantity of meat analyzed. Species identification of the isolated *Trichinella* larvae is performed after DNA extraction and multiplex PCR (Zarlenga et al., 1999; Pozio and La Rosa, 2003). The viability and infectivity of the larvae can be evaluated by mouse bioassay (Kapel et al., 2003).

Treatment

The treatment combines a benzimidazole anthelmintic, albendazole, and a systemic corticosteroid. The possibility of complications requires the rapid initiation of treatment. Therefore, it should not be delayed by waiting for serological results.

The corticosteroid is highly recommended, it prevents the occurrence of complications. Anthelmintic treatment is more effective when it is prescribed early. It is active during the intestinal phase in adult females to stop the release of larvae.

Albendazole is prescribed at 15 mg/kg/day for 10–15 days. Its intestinal absorption is favored during a fat meal. The molecule cannot be administrated during pregnancy and in children under 2 years old. Prednisone is given at 1 mg/kg/day for 1 week.

Prevention

Globally, the main source of human cases remains pork, particularly in areas where family farming is practiced without sanitary controls. Other sources of infection are game meat (wild boar, bear, warthog, reptiles, etc.) and horse meat.

Like raw meat, undercooked meat (smoked or dried meat) is at risk of transmission. Curing (salting), drying, smoking, or using the oven microwave are not efficient methods to kill the larvae.

Monitoring system

Trichinellosis is one of the zoonoses monitored by the World Organization for Animal Health (OIE). An annual report allows estimating the incidence of animal trichinellosis country by country (OIE site http://www.oie.int/fr/fr_index.htm).

In Europe, human trichinellosis is a reportable disease obligatory in the context of toxic-infections collective food (Commission Implementing Decision, 2018). During outbreaks, the search for incriminated food is essential. It allows to subtract the source of the food circuit and prevents new contaminations.

Collective prophylaxis

Pigs raised in the open air present a risk of contamination by eating the corpses of wild animals. Therefore, in Europe, they are subjected to a mandatory diagnosis of trichinellosis during slaughter (Council of the European Communities, 2004). If the above-ground pig farms do not meet obligations of controlled housing conditions, i.e., ensuring control of the risk of contamination by *Trichinella* spp., the animals must undergo a compulsory diagnosis at the slaughterhouse. All horses are diagnosed at the slaughterhouse, as well as wild boars. Game meat intended for consumption outside the private family circle must also be analyzed by a certified laboratory.

For wild boar meat, information from contamination risk is disseminated to hunting federations. Travelers should be informed of the risks of eating raw meat products bought outside of the official market (uninspected) in countries with a high prevalence of trichinellosis (Romania, Serbia, Bulgaria, China, Southeast Asia).

Individual prophylaxis

Individual prophylaxis is based on sufficient cooking of the meat. *Trichinella* larvae are destroyed by cooking at least 65 °C for 5 min. Freezing for a long time can be an alternative but temperature depends on the thickness of the meat and the *Trichinella* species.

When applied, the prevention is effective. In the United States, a significant decrease in cases was observed and was attributed to the improvement of breeding conditions for pigs (confinement) and information on consumers regarding the risks (cooking, freezing) (Wilson et al., 2015). Western European countries had a strong decrease in their numbers of cases after applying control of meat before processing and marketing (Murrell and Pozio, 2011). In 2017, 15 EU/EEA countries reported only 224 cases of trichinellosis, of which 168 cases were confirmed (European Centre for Disease Prevention and Control (ECDC), 2019).

Conclusion

In countries with efficient veterinary authorities, autochthonous trichinellosis has become a rare occurrence, usually associated with hunting or traveling. However, trichinellosis could be underdiagnosed in low-income countries with limited access to health infrastructures and even in industrialized countries where physicians are not familiar with this infrequent disease.

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Trichomonas

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Introduction

Trichomonas is a genus of amitochondriate metamonads, flagellated protozoans of the Parabasilia lineage of eukaryotes, within the clade Excavata and family Trichomonadidae (Guiliano and Lamb, 2012). The flagellates typically possess four free flagella, and a fifth along the outer margin of the undulating membrane. No cysts are present (Burgess, 2005). Species that infect humans include: *Trichomonas vaginalis*, in the genitourinary tract, and *Trichomonas tenax*, in the oral cavity. Other trichomonad species that have been identified in the human lungs include: *Pentatrichomonas hominis*, a commensal in the human intestinal tract, *Tetratrichomonas gallinarum*, a parasite of the avian intestinal tract, *Tritrichomonas foetus*, a pathogen of the bovine reproductive tract. This may highlight the zoonotic potentials and the unrecognized pathogenicity of trichomonads (Duboucher et al., 2008; Kamaruddin et al., 2014).

Trichomonas vaginalis

The parasite

Historical trajectory

The parasite was discovered by Alfred François Donné, a French medical doctor and microbiologist in 1836. As the parasite combined features of the flagellate protozoan genera Tricodes and Monas, he named it Trico-monas with the species name vaginalis, but he designated that the parasite was to be found in the secretions of both male and female genitalia. In collaboration with the Parisian physicist Leon Foucault, Donné made a photomicrograph showing several protozoan parasites lying among vaginal epithelial cells (VECs). His publication of an engraved image of the photomicrograph in 1945 was a landmark in the history of photography and microbiology (Hajdu, 1998; Campbell, 2001). For long, the pathogenicity of *T. vaginalis* was questioned, and the parasite has been considered a self-cleaning female nuisance agent. It was not until the early 1930s, that attention towards *T. vaginalis* as a common cause of urogenital morbidity was redirected (Forgan, 1972).

Forms and structure

T. vaginalis exists in different cellular forms, the best characterized being the highly motile ovoid or pyriform trophozoites. The trophozoites are about $9.7 \times 7 \mu\text{m}$ and typically possess four anterior flagella, and a fifth recurrent flagellum along the edge of the undulating membrane. The flagella arise from the parabasal bodies, at the most anterior region, and emerge from the cell through the flagellar canal. The costa is a slender, striated structure at the base of the undulating membrane, that originates from the second basal body (Lee et al., 2009). The pelta-axostyle system, cytoskeletal structures, consists of microtubules that overlap anteriorly

(Benchimol, 2004). The pelta is comma shaped, surrounding the basal bodies, and reinforcing the wall of the flagellar canal. The axostyle runs along the longitudinal axis of the body, from the area of the basal bodies to the posterior end, where it protrudes as a thin tip (Ribeiro et al., 2000). The Golgi complex, the parabasal apparatus, comprises the Golgi cisternae, and the parabasal filaments, two microfibrillar striated strands linked to the basal bodies (Fig. 1) (Honigberg and Brugerolle, 1990; Benchimol et al., 2001; Lee et al., 2009). The cytostome is not conspicuous (Beaver et al., 1984). A nucleus, with one nucleolus, is at its anterior portion (Honigberg and King, 1964; Benchimol, 2004). The rough endoplasmic reticulum is clearly seen around the nucleus, and also in close association with the axostyle and the hydrogenosomes (Benchimol et al., 2000). The cytoplasm is glycogen rich, has different vacuoles including lysosomes (Costamagna and Figueroa, 2001). The hydrogenosomes, spherical double membrane organelles, are seen as paracostal and paraxostylar sets of granules (Nielsen and Nielsen, 1975; Petrin et al., 1998). Trophozoites can bind together to form large aggregates, and this clumping is displayed only by some parasite strains (Hirt, 2013; Coceres et al., 2015).

An amoeboid form, characterized by an increase in surface contact, is induced upon trophozoite contact with epithelial cells and extracellular matrix proteins (Hirt, 2013). Morphological transformation is associated with cytoskeletal protein rearrangement, that connects the tubulin based flagellar swimming with actin based amoeboid gliding (Kusdian et al., 2013). Upon unfavorable conditions; as chemical and temperature perturbations, a third cellular form, pseudocysts, have been described as rounded structures having internalized flagella (Pereira-Neves et al., 2003). In addition, cyst like structures have been demonstrated in the stationary phase of *T. vaginalis* axenic culture and in vaginal swabs from cervical neoplasia patients (Afzan and Suresh, 2012; Dias-Lopes et al., 2017). These forms have been recently reported to exhibit features similar to other parasitic protozoan cysts (Beri et al., 2020).

Genome

T. vaginalis genome is the largest of any protozoan genome currently available, with about 160 Mbp organized into 6 haploid chromosomes (Carlton et al., 2007). The original genome identified approximately 60,000 protein coding genes, which were reduced to about 46,000 after clustering identical sequences, but the genome was further estimated to reach 175 Mbp (Smith and Johnson, 2011). Differences in estimation of genome size and number of coding genes are mainly due to its repetitive nature, up to 65% appeared to consist of repetitive sequences, and an unknown amount of genome duplication events (Gould et al., 2013). Despite the large size of the genome, *T. vaginalis* lacks the entire repertoire of genes involved in glycosylphosphatidyl inositol (GPI) anchors synthesis (Carlton et al., 2007; Hirt et al., 2011). GPI is a lipid anchor for many cell surface proteins, and is used ubiquitously in eukaryotes (Kinoshita, 2016). The repetitive nature, and the large array of transposable elements, hinder a complete assembly of the genome, which still consists of thousands of individual scaffolds listed at TrichDB (Aurrecoechea et al., 2009; Kusdian and Gould, 2014).

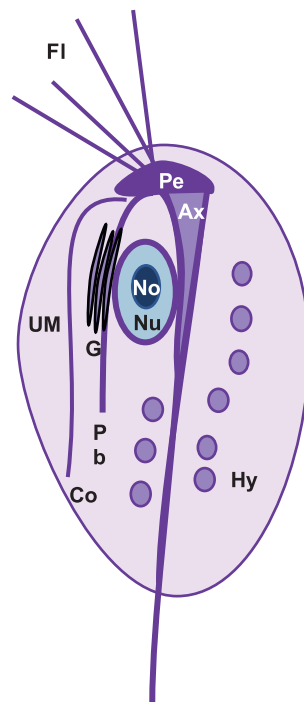


Fig. 1 Diagrammatic presentation of TV structure. Ax, axostyle; Co, costa; FI, flagella; G, golgi apparatus; Hy, hydrogenosomes; No, nucleolus; Nu, nucleus; Pb, parabasal filament; Pe, pelta; UM, undulating membrane.

Biology

T. vaginalis is an extracellular protozoon, with high tissue and host specificity, that resides in the complex human genitourinary environment. The parasite reproduces every 8–12 h by longitudinal binary fission. Trichomonads have one of the most primitive mitosis methods, as the spindle is extranuclear (paradesmosis) and the nuclear envelop does not fragment during the cell cycle; closed mitosis (Benchimol, 2004). The presence of meiotic specific genes in *T. vaginalis* points at the possibility of sexual reproduction (Malik et al., 2008).

T. vaginalis is an obligate parasite that lacks the ability to synthesize many macromolecules de novo, particularly purines, pyrimidines and many lipids. The parasite can phagocytose human cells including VECs and immune cells (Midlej and Benchimol, 2010). It can also phagocytose host microbiota (Pereira-Neves and Benchimol, 2007; Hirt et al., 2011). Phagocytosis provides the parasite with important sources of nutrients. Iron is an important nutrient for the parasite and a key factor in its gene regulation, and *T. vaginalis* has evolved multiple mechanisms for acquiring iron from specific iron binding, and iron containing proteins (Lehker and Alderete, 1992; Hernández et al., 2014). Iron may be obtained by hemoglobin degradation after lysis of erythrocytes (Lehker et al., 1990; Petrin et al., 1998). The parasite can also internalize lactoferrin and hemoglobin through receptor mediated endocytosis (Sutak et al., 2008).

Trichomonas is a typical anaerobe, with no oxygen requirements, but sometimes is considered as microaerophile as it grows slightly better at very low oxygen tension (Paget and Lloyd, 1990). Hydrogenosomes contain enzymes that engage in the metabolism of pyruvate formed during glycolysis and are the site of molecular hydrogen production. The process is associated with ATP synthesis (Muller et al., 2012). In the urogenital tract, *T. vaginalis* is regularly exposed to low oxygen concentration, and therefore must possess mechanisms to protect its organelles from the detrimental effects of molecular oxygen and reactive oxygen species. The parasite seems to utilize a sophisticated system to buffer oxygen stress; a combined cytosolic—hydrogenosomal antioxidant system (Coombs et al., 2004; Mentel et al., 2008).

T. vaginalis was found to establish symbiotic relationships with different microorganisms, which may affect its metabolic biochemical pathways and/or have important consequences for its virulence and pathogenicity (Fichorova et al., 2017). The parasite can harbor up to four DS-RNA viruses, members of the Totiviridae named TV virus (TVV1, TVV2, TVV3 and TVV4), closely associated with the Golgi complex (Goodman et al., 2011a). TVV may modulate parasite virulence by influencing gene expression (Orujzadeh et al., 2019). Moreover, a symbiotic association between *T. vaginalis* and *Mycoplasma hominis* has been described (Hirt et al., 2011; Dessi et al., 2019). There is also a recent discovery of a new bacterium named *Candidatus M. girerdii*, a mycoplasma within *Trichomonas* (Fettweis et al., 2014; Margarita et al., 2020).

Epidemiology

T. vaginalis is the causative agent of trichomoniasis, a disease with a high global burden being the most common non-viral sexually transmitted infection (STI) (Menezes et al., 2016; Van Gerwen and Muzny, 2019). WHO estimates of the disease rates globally in 2008, were an incidence of 276 million new cases each year, and a prevalence of 187 million infected individuals with ages between 15 and 49 years old. Data collected during the 2013–14 National Health and Nutrition Examination Survey (NHANES) in US, revealed a prevalence of 1.8% in females and 0.5% in males, aged 18–59 years (Patel et al., 2018). In 2016, WHO estimated 156 million cases of *T. vaginalis* worldwide, accounting for almost half of the global STIs incidence that year (Fig. 2) (Rowley et al., 2019).

The incidence of infection depends on several factors including age, gender, ethnicity, sexual activity, number of sexual partners, other sexually transmitted diseases, menstrual phase, hormonal contraception, diagnostic technique, social and economic conditions (Meites, 2013; Poole and McClelland, 2013). Unlike other STIs, significantly higher rates of *T. vaginalis* infections are found in older men and women compared with adolescents and younger adults (Sutton et al., 2007; Meites et al., 2015). Many sexually transmitted infections are more prevalent in women than men. Biological factors include large surface area of the female genital tract, alterations in physiology of reproductive tissues during different phases of the menstrual cycle, influence of sex hormones and hormonal contraception, and effect of indigenous microbiota (Wessels et al., 2018). Sex hormones may contribute to the variation of *T. vaginalis* acquisition and persistence and may influence the mucosal immune defense. Moreover, trichomonad movement and attachment to cells appear to be affected by the presence of estrogen, and protein containing estrogen binding sites have been identified in *T. vaginalis* (Ali and Abdul Khaliq, 2015). The prevalence in pregnant women was found to range from 3.9–24% in low to middle income countries, in Latin America and Southern Africa (Davey et al., 2016).

Infections are more common among certain racial and ethnic groups; Africans or persons of African descent have higher rates. High prevalence of infection has been also detected among incarcerated men and women, and patients at STD clinics. In US, higher rates of *T. vaginalis* infection have been reported among black, compared to man and women of other ethnicities (Sutton et al., 2007; Gaydos et al., 2013; Muzny et al., 2013). In South Africa and Australia, the parasite is more prevalent among indigenous populations (Bygott and Robson, 2013; Hillier, 2013; Naidoo and Wand, 2013). The ethnic disparity concerning *T. vaginalis* may be multifactorial, involving differences in sexual risk behaviors, and inadequate access to health care resources (Ford and Browning, 2011; Van Gerwen and Muzny, 2019). *T. vaginalis* was found significantly associated with STIs (Chlamydia, gonorrhea, Herpes simplex virus types 1&2, syphilis, and HIV), in the 2001–04 NHANES among women aging 14–49 years in the civilian, non-institutionalized US population (Allsworth et al., 2009). Among high risk women, rates range from 5% in female sex workers in Lahore in Pakistan, 9% in Yunnan Province in China, to 53% amid incarcerated women in US (Khan et al., 2011; Roth et al., 2011;

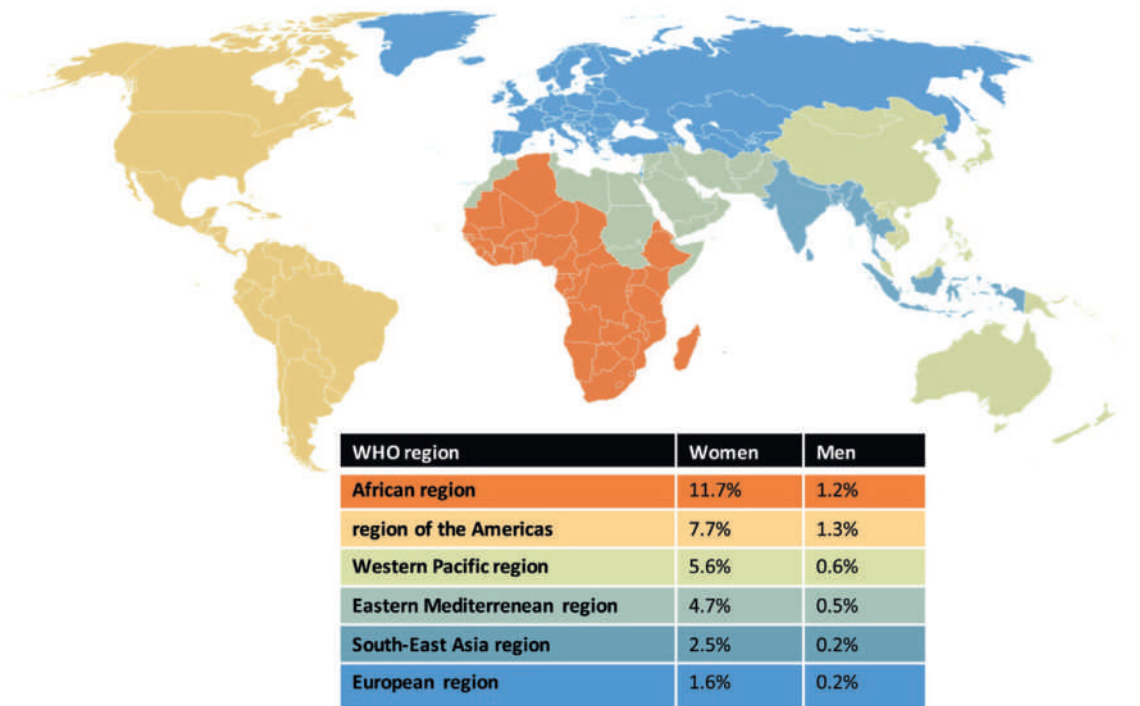


Fig. 2 Prevalence of TV infections in women and men distributed over WHO regions, 2016.

Luo et al., 2016). Among high risk men, rates range from 2% amid jail inmates in California in US, 3–17% in STI clinic attendees, to 75% among male partners of women with *T. vaginalis* in Southeast US (Schwebke and Hook, 2003; Hobbs et al., 2006; Freeman et al., 2010).

The parasite is a highly contagious protozoan with an apparently simple life cycle, consisting mainly of the trophozoite, no true cyst stage is known and contribution of pseudocysts in the parasite life cycle is not well investigated. Trophozoites are transmitted from host to host through sexual intercourse (Conrad et al., 2012). The parasite can survive outside the body for 6–24 h in urine, semen, and swimming pool water, but only up to 30 min when exposed to air (Pereira-Neves and Benchimol, 2008). *T. vaginalis* can grow over a wide range of pH values, with an optimum level between pH 6 and 6.3 (Petrin et al., 1998). Nonsexual transmission is rare but had been observed in cases involving contaminated showers, moist wash clothes, toilet seats or specula (Adu-Sarkodie, 1995; Mielczarek and Blaszkowska, 2016). Transmission is also possible via artificial insemination of infected cryobanked semen (Sherman et al., 1991; Habib et al., 2004). Neonatal infection with *T. vaginalis* is infrequently reported but has been associated with urinary tract infections and vaginitis, infection being acquired from the maternal genital tract (Trintis et al., 2010). The parasite may also play a role in multiple sites neonatal infection, including the respiratory and urinary tract, and colonization of the latter may increase the risk of developing concomitant bacterial urinary tract infection (Carter and Whithaus, 2008; Bruins et al., 2013; Kusdian and Gould, 2014).

Investigations in the molecular epidemiology of *T. vaginalis* infections have identified two distinct genome structure types, associated with clinically relevant unique phenotypes (Goodman et al., 2011a; Conrad et al., 2012). Type 1 *T. vaginalis* isolates have a higher prevalence of infection with TVV, while type 2 *T. vaginalis* isolates are marked by a higher prevalence of resistance to metronidazole (MNZ). The global distribution of Types 1 and 2 *T. vaginalis* infections has been outlined by analysis of clinical isolates from US, Mexico, Chile, Italy, South Africa, Mozambique, Australia, Papua New Guinea, and India. Types 1 and 2 *T. vaginalis* infections were found of global distribution, being detected with equal frequency in most regions, but with two notable exceptions: in isolates from South Africa and Mozambique, all samples were type 1 *T. vaginalis*, while samples from Mexico had significant higher prevalence of type 2 *T. vaginalis* infection (Conrad et al., 2012; Poole and McClelland, 2013).

Still, trichomoniasis is classified as a neglected disease. *T. vaginalis* is not a reportable infection, and there is a lack of case reporting data at national global levels (Poole and McClelland, 2013). Moreover, prevalence data are underestimated due to failure in diagnosis, as consequence of insensitive methods, or lack of testing in asymptomatic patients, and limited knowledge related to infection duration (Secor et al., 2014; Menezes et al., 2016).

Virulence factors and pathogenesis

Establishment and maintenance of infection are mediated through several mechanisms including: binding to, and degradation of elements from the mucus and extracellular matrix (ECM) proteins, binding to host cells, including VECs and immune cells, leading

to cytotoxic effects, phagocytosis of vaginal bacteria and host cells (VECs, erythrocytes, and immune cells), endocytosis of host proteins, immune evasion strategies, and interactions with the microbial community of the vagina (Fig. 3) (Hirt et al., 2007; Kusdian and Gould, 2014).

Surface membrane proteins

Surface proteins of mucosal pathogens play essential roles in initiating and sustaining the colonization of the highly defended mucosa (Hirt et al., 2007). The surface of *T. vaginalis* is covered by a highly variable coat of proteins, some proteins are found more frequently exposed on the surface of the more virulent strains than others (De Miguel et al., 2010; Noël et al., 2010; Ryan et al., 2011). A major lipid anchored phosphosaccharide, known as lipophosphoglycan of *T. vaginalis* (TVLPG), is perhaps the most abundant component of the parasite surface glycocalyx, and is an important virulence factor (Bastida-Corcuera et al., 2005; Singh et al., 2009).

The largest gene family in *T. vaginalis* encoding candidate surface proteins, is the Bacteroides surface protein A-like proteins (BspA) (Carlton et al., 2007; Hirt et al., 2007). They share a specific type of leucine rich repeats (LRRs), known as *Treponema pallidum* LRRs, and have been shown to mediate binding to host epithelial cells, and/or ECM proteins (Kobe and Kajava, 2001; Hirt et al., 2011). A group of potential surface proteins, that are classified as transmembrane proteases, includes four families: GP36-like metalloproteases, subtilisin-like, serine proteases, and calpain-like cysteine proteases (Kusdian and Gould, 2014). Among other identified parasite surface proteins are: P270, a prominent, and phenotypically variable protein (Khoshnan et al. 1994; Alderete, 1999a), Chlamydial polymorphic membrane proteins (TVPmp) (De Miguel et al., 2010), Legume-like surface lectins, may be involved in binding and endocytosis (Rendon-Maldonado et al., 2003), and tetraspanins (TVTSPs), are localized on the surface and in intracellular vesicles, have been implicated in cell adhesion, parasite migration and sensory reception during infection (Hemler, 2003; Coceres et al., 2015).

Secretion of soluble factors

T. vaginalis contact independent pathologic mechanisms comprise the secretion of a number of soluble biologically active molecules. A soluble factor released by the parasite into the medium, is a glycoprotein of 200 kDa known as cell detaching factor (CDF) (Arab-mazar and Niyiyati, 2015). CDF helps the parasite to traverse the mucus barrier of the host epithelium and promotes the release of host cells from the tissue and mucosal desquamation (Sood and Kapil, 2008; Harp and Chowdhury, 2011). The parasite secretes a wide range of hydrolases, including cysteine proteinases (CPs), that appear to be associated with parasite virulence. *Trichomonas* CPs are involved in various pathogenic processes including invasion of the mucus layer, cell adhesion, cytotoxicity, cytoskeleton disruption of erythrocytes, apoptosis of VECs, and evasion of the host immune response (Hernández

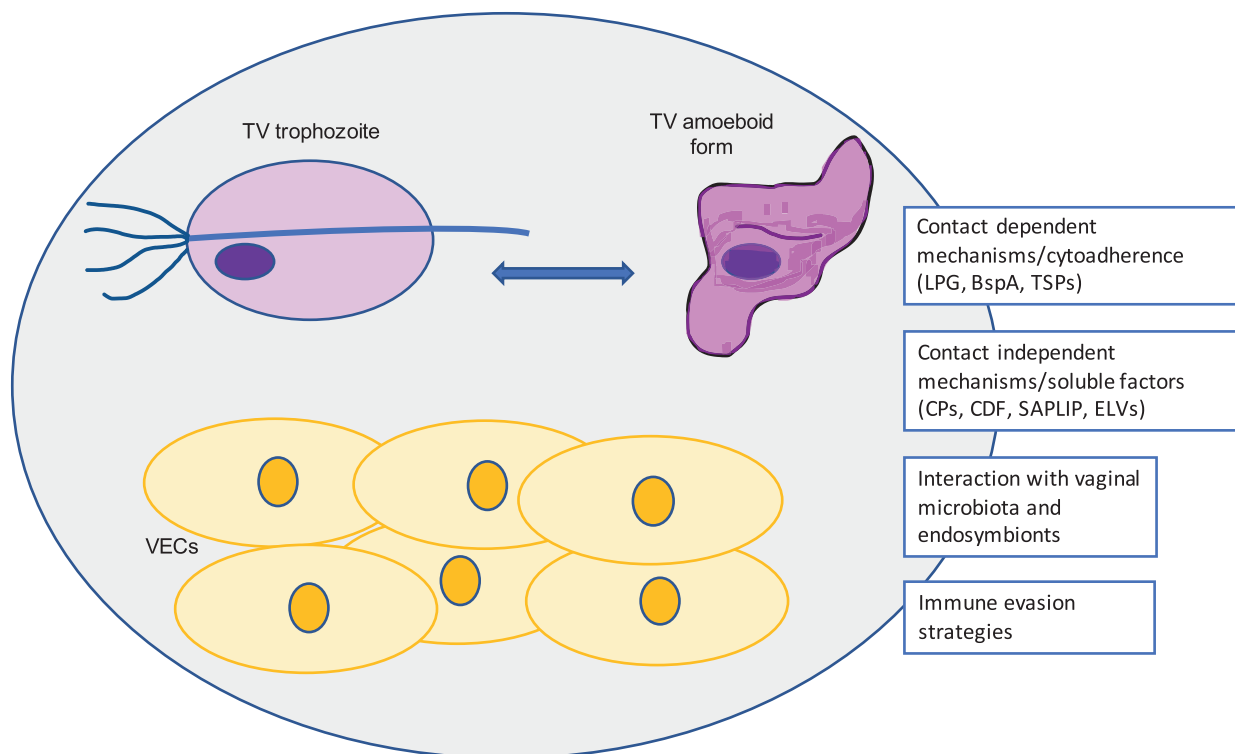


Fig. 3 TV virulence mechanisms involved in the establishment of infection and pathogenesis. BspA, bacteroides surface protein A-like; ELVs, exosome-like vesicles; LPG, lipophosphoglycan; SALIPs, saposin-like proteins; TSPs, tetraspanins; VECs, vaginal epithelial cells.

et al., 2014). The synthesis and proteolytic activity of certain CPs are regulated by environmental factors, such as iron, pH, temperature, and polyamines (Carvajal-Gamez et al., 2014).

A set of soluble proteins, that are reported to be secreted and act as adhesion molecules, include heat shock proteins (HSP), and metabolizing enzymes of the hydrogenosome (Kusdian and Gould, 2014). *T. vaginalis* produces several cytotoxic molecules, including pore forming proteins, members of a conserved family of saposin-like proteins (SAPLIPs). *T. vaginalis* SAPLIPs (trichopores) contribute to cell lysis, and death by forming transmembrane channels, in the lipid membrane of target cells, leading to osmotic lysis (Ojcius and Young, 1991; Fiori et al., 1996; Menezes et al., 2016). Moreover, lytic factors with phospholipase A-like activity, that destroy nucleated cells and erythrocytes, have been described (Hirt, 2013). *T. vaginalis* was found to secrete exosome-like microvesicles. *T. vaginalis* exosomes are about 50–100 nm in diameter and contain a large diversity of proteins and RNA (Twu et al., 2013). Exosomes fuse with, and deliver their contents to the host cell, and can modulate the host immune reactions. The proteome of the exosomes appears to differ according to the strain, as exosomes of a highly infectious strain increased the binding efficiency to host tissues of a less adherent strain (Kusdian and Gould, 2014).

Trichomonas morphogenesis and cytoadherence

An essential step of the infection process is the rapid transformation of *T. vaginalis*, from the flagellated to an adherent amoeboid cell. Upregulation of actin and actin associated genes occurs, while α -actinin is distributed throughout the cytoplasm in the flagellate, the protein localizes at the parasite periphery in the amoeboid form (Gould et al., 2013; Menezes et al., 2016). The speed with which the transformation occurs, few minutes upon exposure to VECs, is unique. The flagella and costa are not degraded during morphogenesis and attached cells can rapidly detach again and change location. Parasite morphogenesis comprises at least two distinct, but simultaneous processes: shape transition, and adherence to host cells (Kusdian and Gould, 2014).

Contact dependent mechanisms play a crucial role in host cell destruction. The parasite can cross the mucus layer of the genital tract by binding to mucin, followed by its proteolytic degradation by the parasite mucinase; thus, reaching the underlying epithelium (Hernández et al., 2014). Adhesion of *T. vaginalis* to epithelial cells is mediated by numerous adhesions. The parasite expresses LPG at several million copies per parasite, anchored on the surface by an inositol phosphoceramide (Singh, 1993; Singh et al., 1994). TVLPG binds to galectin-1 and -3 receptors in the host cells, playing a role in adherence, and cytotoxicity to VECs. It can also modulate the inflammatory responses of epithelial cells and macrophages (Fichorova et al., 2006, 2016; Okumura et al., 2008). Although the binding of LPG-receptor may be central in establishing the infection, *T. vaginalis* involves other adhesion factors to implement the host-parasite interactions (Hirt et al., 2007).

Parasite interaction with vaginal microbiota and endosymbionts

T. vaginalis interaction with the genital microflora is complex. The parasite may modulate and induce imbalance in the vaginal microbial community, leading to dysbiosis with reduction of the protective Lactobacilli (Brotman et al., 2012). TVLPG ceramide phosphoinositol glycan core, cooperates with pathogenic vaginal bacteria to alter bacterial colonization patterns, and the local immune environment in support of bacterial vaginosis, which in turn facilitates parasite survival (Fichorova et al., 2013). The vagina of the reproductive age women is characterized by the presence of a complex population of aerobic and anaerobic microorganisms that has been classified into five community state types (CSTs); four of these CSTs are dominated by the protective Lactobacilli. While one type (CST-IV) has anaerobic bacteria and no Lactobacilli. CST-IV are the causative agents of bacterial vaginosis, as they induce physical, immunological, and biochemical changes in the cervicovaginal mucosa, promoting a greater proinflammatory response in the vagina than the Lactobacilli-dominant communities (Mitchell and Marrazzo, 2014; Amabebe and Anumba, 2018). Lactobacilli exert its protective effect by lowering vaginal pH through lactic acid and by competing for nutrients. Moreover, the presence of Lactobacilli was found to inhibit *T. vaginalis* adhesion (Phukan et al., 2013). The low abundance of Lactobacilli associated with trichomoniasis creates a more basic milieu, more favorable for parasite growth and pathogenicity (Petrin et al., 1998). Parasite active feeding of the host microbiota may cause in addition the acquisition of a number of bacterial genes through gene transfer, that modulates its virulence. A complete set of bacterial enzymes capable of degrading glycans, and mostly acquired from the Bacteroides group, have been identified (Alsmark et al., 2013; Hirt, 2013). *T. vaginalis* cooperation with pathogenic vaginal bacteria, was found to enhance the paracellular permeability of the genital tract epithelium by disturbing the integrity of the tight junction complex (Hinderfeld et al., 2019).

The presence of *Trichomonas* endosymbionts may enhance its pathogenic effect and modify the host immune response (Margarita et al., 2020). In Europe, up to 90% of *T. vaginalis* strains are naturally infected with *M. hominis* (Fürnkranz et al., 2018). The parasite-Mycoplasma symbiosis could interfere with production of the antimicrobial NO by human macrophages, through outcompeting for the key substrate arginine (Das et al., 2010; Gogoi et al., 2016; Margarita et al., 2016). The role of TVV in the pathogenesis of trichomoniasis is still not well clarified. The virus has been observed to alter cell surface expression of P270, a highly immunogenic protein, and CP expression profile. This may suggest that the virus impairs the clearance of the infection, and predisposes to its longer persistence (Alderete et al., 1986; Provenzano et al., 1997; Goodman et al., 2011b).

Clinical features

T. vaginalis infection results in a variety of clinical manifestations. Studies have revealed that around 80% of infections are asymptomatic in both men and women (Sutton et al., 2007; Allsworth et al., 2009; Menezes et al., 2016). However, even asymptomatic infections are a public health concern, due to the risk of transmission to sex partners, and the pathologic sequelae of the infection (Poole and McClelland, 2013).

In women, symptoms may appear after an incubation period of 4–28 days, however, this period is not clearly known, and about one third of women become symptomatic within 6 months (Petrin et al., 1998; Menezes et al., 2016). The main complaints are vaginal discharge, odor, pruritis, dysuria and dyspareunia (Muzny and Schwebke, 2013). Although the discharge is typically described as being frothy yellow or green in color; the aspect and consistency are widely variable among patients (Schwebke and Burgess, 2004; Swygard et al., 2004). The vagina and cervix may be erythematous and edematous, and punctate hemorrhagic spots may be found on the mucosa; strawberry cervix. This clinical sign is the most indicative of the disease, still it is clinically diagnosed only in 2–5% of women (Lehker and Alderete, 2000).

The extent of the inflammatory response to the parasite may determine the severity of symptoms. Factors influencing the host inflammatory responses include hormonal loads, coexisting vaginal flora, and the strain and relative concentration of the parasite in the vagina (Schwebke and Burgess, 2004). Trichomoniasis symptoms are cyclic and more intense around the menstrual period; as it has been suggested that the iron rich environment of the vagina in menstruating women enhances *T. vaginalis* growth and persistence (Petrin et al., 1998).

In men, the natural history and the spectrum of the disease are less well characterized as the infection is commonly self-limited and transient (Hirt and Sherrad, 2015; Menezes et al., 2016). The oxidative nature of the male genital fluid and the zinc rich environment of the prostate may inhibit persistent infection (Figuerola-Angulo et al., 2012; Gimenes et al., 2014). The mean incubation period in men is approximately 10 days (Poole and McClelland, 2013). Manifestations include urethritis accompanied by scanty, clear to mucopurulent discharge, dysuria and mild pruritis or burning sensation immediately after sexual intercourse (Petrin et al., 1998; Menezes et al., 2016).

Pathologic sequelae

In women, the parasite can persist for long periods in the urogenital tract. Untreated or persistent *T. vaginalis* infection has been implicated in adverse birth outcomes and infertility (Van Gerwen and Muzny, 2019). In pregnant women, infection has been associated with preterm rupture of membranes, preterm delivery, low birth weight neonates, and cases of neonatal vaginal and respiratory infections (Cram et al., 2002; Simhan et al., 2005; Carter and Whithaus, 2008; Silver et al., 2014). Preterm birth may be caused by the parasite modulating the human innate immune responses including proinflammatory cascades and mucosal inflammation (Mercer and Johnson, 2018). A compromised cervical epithelial barrier can promote cervical remodeling which is an initial step towards preterm birth (Timmons et al., 2010; Hinderfeld et al., 2019). Moreover, cytokines (IL-1 β , IL-6) and prostaglandins (PGE2, PGE2a) which are released in response to *T. vaginalis* infection can induce uterine contractions increasing the risk of preterm birth (Cram et al., 2002).

T. vaginalis has been linked to infertility, pelvic inflammatory disease (PID) and cervical neoplasia. It is more often reported in infertile women than in control subjects and is probably associated with cervical and tubal factor of infertility (El-Shazly et al., 2001; Soper, 2004; Roh et al., 2015). PID is the infection and inflammation of upper genital tract structures caused by ascending microorganisms from the lower genital tract. PID can lead to infertility, ectopic pregnancy or chronic pelvic pain (Wiringa et al., 2020). There is a growing body of evidence that *T. vaginalis* may play a role in the pathogenesis of PID (Moodley et al., 2002; Cherpès et al., 2006; Reighard et al., 2011; Wiringa et al., 2020). Different studies have demonstrated that infected individuals have a higher risk of cervical cancer; especially in cases co-infected with Human Papilloma virus (Verteramo et al., 2009; Lazenby et al., 2014; Yang et al., 2018). A remarkable health outcome of *T. vaginalis* infection is its positive relation with both transmission and acquisition of HIV (Menezes et al., 2016). *T. vaginalis* screening among HIV infected women in US revealed high prevalence (17.4–20%), and repeated infection rates up to 22.7% over a median of 16 months (Muzny et al., 2019). The main mechanisms by which the parasite may enhance HIV acquisition include mucosal microhemorrhages that compromise the epithelial barrier, the local inflammatory response and recruitment of immune cells including HIV target cells as CD4⁺ and macrophages to which the virus can bind and gain access, and by increasing susceptibility to bacterial vaginosis or persistence of abnormal vaginal flora (Moodley et al., 2002; Shafir et al., 2009; Quinlivan et al., 2012; Kissinger and Adamski, 2013). Endocytosis of the virus and its accumulation in endosomal-like structures, might contribute to transmission when the parasite is transferred from dually infected patients to new hosts (Pindak et al., 1989; Rendon-Maldonado et al., 2003; Lal et al., 2006; Hirt et al., 2011).

In men, sequelae of infection include prostatitis, balanoposthitis, epididymo-orchitis and infertility, possibly through chronic inflammation and cell lysis (Menezes et al., 2016). Moreover, the parasite can decrease both sperm motility and viability (Martinez-Garcia et al., 1996; Kissinger, 2014). *T. vaginalis* has been associated with a risk of cancer prostate development; possible mechanisms include its prostatic tropism, ability to elicit inflammation and damage to the prostatic epithelium, its tendency to cause chronic subclinical infections, altering polyamine levels, and upregulation of expression of anti-apoptotic and other proto-oncogenes (Sutcliffe, 2010; Marous et al., 2017). A *T. vaginalis* homologue of the inflammatory cytokine, macrophage migration inhibitory factor (TVMIF), has been suggested as a contributor to prostate cell growth and invasiveness (Twu et al., 2014; Menezes et al., 2016).

Immunity in trichomoniasis

Trichomoniasis does not produce an effective permanent immunity; this may lead to recurrent infections. Consequently, the innate immune responses become crucial for infection control (Menezes et al., 2016). Moreover, the quality of the adaptive immune responses, is probably influenced by concomitant microbial infections and symbionts and may be further complicated by parasite lysis of effector immune cells (Mercer et al., 2016).

The non-specific defense mechanisms against the parasite include activation of the alternative pathway of the complement system, which provides C3 opsonin that promotes neutrophil mediated phagocytosis of *T. vaginalis* (Shaio et al., 1991). Cervical mucus is deficient in complement, the menstrual blood is the only source of complement in the vagina. Although its activity is about half that of the venous blood, menstrual blood has considerable complement mediated cytotoxicity towards *T. vaginalis*. Consequences of iron level increase during menses, upregulation of CPs expression occurs; and the proteolytic activity assists in trichomonal infection persistence after menses (Demes et al., 1988; Alderete et al., 1995; Arab-mazar and Niyiyati, 2015).

Upon binding of the parasite to host cells, activation of Toll-like receptors (TLR2, TLR4, TLR9) in epithelial cells occurs, and proinflammatory cytokines and chemokines (IL-6, IL-8, and macrophage inflammatory protein MIP-3 α) become upregulated triggering inflammation (Behzadi and Behzadi, 2016; Nemati et al., 2018). Neutrophils are the predominant immune cells recruited at the site of infection, mediating the initial inflammatory response to an acute *T. vaginalis* infection. IL-8 acts as a powerful chemoattractant for neutrophils (Escario et al., 2010; Nemati et al., 2018). Neutrophils participate in parasite killing through production of reactive oxygen species (ROS) and antimicrobial peptides (AMPs) as defensins and the lipid mediator leukotriene LTB4. *T. vaginalis* can secrete LTB4 (TVLTB4) that contributes to neutrophil chemotaxis; the TVLTB4—neutrophil/mast cells—IL-8 axis is thought to share in the mucosal inflammation that occurs during trichomoniasis (Nam et al., 2011, 2012; Le Bel et al., 2014). Monocyte derived macrophages also generate the proinflammatory cytokines (IL-1 β , IL-6, IL-8, TNF- α) in response to infection. Macrophages enrollment is an important constituent of host defense against genitourinary trichomoniasis (Han et al., 2009). Parasite stimulation of macrophages leads to expression of inducible nitric oxide (NO) synthase and NO production (Park et al., 1997).

In trichomoniasis, CD4⁺ T-helper cells interplay may lead to parasite elimination, persistence or pathological events (Nemati et al., 2018). The infection was found to induce vaginal CD4⁺ T cell infiltration in experimental infection (Smith and Garber, 2015). Upon stimulation and activation, naïve CD4⁺ T cells differentiate into separate subsets including T helper cells Th1, Th2, Th17, Th22 (regulatory T cells); each with its own cytokine profile (Zhang et al., 2014; Schmitt and Ueno, 2015). Th1 cells trigger cell mediated immunity through production of cytokines; such as INF- γ , TNF- α and IL-2, that lead to activation of the effector leucocytes; macrophages, cytotoxic lymphocytes, and natural killer cells (Nemati et al., 2018). Th1 cell related cytokines (INF- γ , IL-2) have been reported to be significantly high in the serum and vaginal secretion of mice infected with isolates from asymptomatic women as compared to mice infected with isolates from symptomatic patients. Th1 cytokines have been suggested to promote low burden infection (Malla et al., 2007). Th17 cells secrete a number of cytokines and chemokines including IL-17, with controversial reports regarding their role in trichomoniasis (Makinde et al., 2013; Masson et al., 2015). A prominent function of IL-17, is the induction of neutrophil chemotaxis at the inflammation site (Isailovic et al., 2015; Nemati et al., 2018). Th22 cells release IL-22 and TNF- α , together with IL-17 they synergistically upregulate production of AMPs, defensins and LL-37; the sole member of the cathelicidin family (Kahlenberg and Kaplan, 2013; Dempsey, 2017; Eyerich et al., 2017). Elevated levels of IL-22 and LL-37 were reported in vaginal secretions from *T. vaginalis* infected women as compared to uninfected, and IL-22 has been assumed as a protective immune factor against the infection (Makinde et al., 2013).

Antibody mediated immunity in trichomoniasis has been reported to be short lived and its role in parasite elimination and protection is unclear. Parasite specific IgG, IgM, IgA antibodies have been demonstrated by several techniques (Menezes and Tasca, 2016). LPG is one of the most commonly recognized antigens by serum samples of *T. vaginalis* infected women, in which levels of LPG specific IgA in vaginal secretions were higher than in control subjects. Also, LPG specific IgG was higher in infected women with normal pregnancy than in those with adverse pregnancy sequelae. The vaginal LPG specific IgG may be protective against adverse pregnancy outcome (Bastida-Corcuera et al., 2013; Menezes and Tasca, 2016; Nemati et al., 2018).

T. vaginalis has acquired several strategies to evade the host immune response. Parasite CPs with their high proteolytic activity can degrade host immunoglobulins, helping the parasite to survive the antibody response (Hernández et al., 2014). They can degrade C3 portion of complement on the surface of the parasite, evading complement mediated lysis. *T. vaginalis* association with the host protein CD59, a complement lysis restricting factor, may be another mechanism by which the parasite resist complement attack (Ibáñez-Escribano et al., 2015). CPs can also break down the secretory leucocyte protease inhibitor (SLPI), a factor protecting the mucosal surface of the vagina (Al-Mohammed and Hussein, 2006). Moreover, TVLPG was found to suppress the expression of SLPI by vaginal and cervical epithelial cells (Huppert et al., 2013).

Molecular mimicry is another mechanism by which the parasite can escape the host immune response. *T. vaginalis* adhesins (AP33, AP51, AP65) share immunological similarities with host metabolic enzymes and plasma proteins. The parasite can also coat its surface with molecules homologous to host proteins; avoiding being recognized by the host immune system (Figueroa-Angulo et al., 2012). The parasite challenges the immune system by displaying strain variation; including antigenic phenotypic and genetic variation (Nemati et al., 2018). P230 and P270 surface antigens are involved in parasite phenotypic variation; the former goes through conformational changes, preventing its accessibility and antibody binding, while the later surface expression is dependent on TVV endosymbiosis and iron concentration (Alderete, 1999b). A high degree of genetic diversity in *T. vaginalis* isolates has been reported using different genotyping techniques (Meade and Carlton, 2013).

Zinc assists in antimicrobial defense in the male lower genital tract. Studies have revealed the variation in zinc sensitivity between different parasite isolates; as well as variation in the zinc content of the male prostatic secretions (Krieger and Rein, 1982). These variations can induce differential expression of the parasitic proteins and affect parasite survival in the male genitourinary tract (Vazquez-Carrillo et al., 2011; Menezes and Tasca, 2016).

T. vaginalis can modulate the host immune response by stimulating the production of anti-inflammatory cytokines (IL-10, TGF- β) from macrophages and dendritic cells, which can contribute to infection persistence. *T. vaginalis* exosome-like vesicles (TV-ELVs)

increase the expression of IL-10 in macrophages (Olmos-Ortiz et al., 2017). Moreover, the parasite can reduce the expression of Th1 proinflammatory cytokines (TNF- α , IL-12) by suppressing the transcription factor NF- κ B activation; which is found to be dependent on parasite burden. Hence, interfering with cell differentiation, inflammation and apoptosis and subsequently effective Th1 cell mediated response (Chang et al., 2004; Quan et al., 2017).

Diagnosis

Laboratory diagnosis is required when trichomoniasis is clinically suspected; the diagnostic yield is mostly dependent on sample collection and type of test used (Garber, 2005). Specimens are mainly obtained from vaginal discharge, swabs from posterior fornix, and cervical smear in females, while in males from urethral discharge ideally collected before first-voided morning urine or prostatic secretions (Garber, 2005; Fernando et al., 2011). In settings where clinical genital examination is limited or unwanted, first catch urine sample and self-collected vaginal swabs could be used as well but are proved to be less effective (Mohamed et al., 2001; Karaman et al., 2008; Domeika et al., 2010; Hobbs and Seña, 2013).

Traditionally, direct wet mount preparation and microscopic observation of motile trophozoites is the widely used method, especially in field as well as in low and middle income countries (Darani et al., 2010; Adjei et al., 2019). Wet mount technique is simple, rapid, inexpensive, specific but suffers from a major drawback of being of low sensitivity, which was described to range from 30%–80%; where negative examination cannot exclude *T. vaginalis* infection (Darani et al., 2010; Fernando et al., 2011; Adjei et al., 2019). This limitation is attributed to various factors; the size of inoculum as $<10^4$ organisms/mL will not be detected, the viability of the protozoa and the need to remain moist in sample, in addition to the experience of the microscopist. Therefore, sample should be immediately examined after collection otherwise the motility will be significantly reduced or lost, making it difficult to distinguish the trophozoites from the nuclei of vaginal epithelial cells (Garber, 2005; Adjei et al., 2019). In an attempt to overcome these problems, transport media containing 5% glucose solution as energy source were used to prolong the organism viability for up to 24 h (Fernando et al., 2011). Staining techniques; such as Giemsa, acridine orange and periodic acid-Schiff, were introduced as well to improve the sensitivity of direct microscopic examination. However, their usefulness was questioned as reports revealed no increase in sensitivity in comparison to unstained wet mount smears (Garber, 2005; Adjei et al., 2019). Also, Papanicolaou smear was considered as it is routinely used in screening of variety of cervical lesions. However, the technique was indicated to be ineffective for *T. vaginalis* detection, showing poor sensitivity and specificity (Aslan et al., 2005; Hezarjaribi et al., 2015). Leukorrhea, raised vaginal pH often >6 in florid infection, and positive whiff test are findings in vaginal discharge that could be correlated to trichomoniasis (Garber, 2005; Muzny et al., 2014).

Culture is another parasitological technique; it is considered the “gold standard” for the diagnosis of *T. vaginalis*, with higher sensitivity and specificity when compared to wet mount (Darani et al., 2010; Muzny et al., 2014). Culture is especially valuable when the inoculum amount is small, depending on the type of the medium used, counts as few as 10 organisms/mL can be detected (Fernando et al., 2011; Patil et al., 2012; Adjei et al., 2019). The commonly used culture media are the standard broth Diamond’s TYI medium and the InPouch *T. vaginalis* culture. The latter offers many advantages over other culture media; it is of lower cost, stable with shelf-life of 6 months, used for sample transport and culture, furthermore the microscopic examination is done directly on the pouch itself; saving time and reducing contamination (Gallion et al., 2009; Patil et al., 2012). However, culture procedure was categorized as moderately complex, time consuming requiring 2–7 days of incubation, expensive, not readily available in field laboratories, and reliant on presence of viable *T. vaginalis* and maintenance in microaerophilic condition (Darani et al., 2010; Patil et al., 2012; Muzny et al., 2014).

Immunodiagnostic techniques based on antigen detection are alternatives to microscopy for detection of *T. vaginalis*, including rapid antigen tests; as latex agglutination and lateral flow, in addition to ELISA and immunofluorescence. Each test differs in the type of targeted antigen and has its own advantages and limitations regarding performance, cost and ease of use. The overall sensitivity and specificity of these tests were described to be higher than wet mount and comparable to culture (Darani et al., 2010; Domeika et al., 2010; Adjei et al., 2019). A number of antibody detection tests were described in the past; yet they have low sensitivity and specificity and subject to cross-reactivity (Domeika et al., 2010).

Nucleic acid amplification tests (NAATs) emerged as powerful diagnostic tools of high sensitivity and specificity surpassing those of culture techniques, plus providing the ability to detect nonviable organisms. A variety of amplification technologies and sets of primers were validated for use in *T. vaginalis* infection on multiple specimen types. These procedures can be conducted on the same testing platforms for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (Wendel et al., 2002; Patil et al., 2012; Muzny et al., 2014). NAATs are useful and crucial in situations where low parasite density is expected as in asymptomatic individuals and those at risk of infection. Also, they perform well with non-invasive specimens as urine samples and self-collected vaginal swabs (Van Der Pol, 2016).

Treatment and prevention schemes

Trichomoniasis in females is not a self-clearing infection, and chronic infections may be maintained indefinitely. While in males, infection is usually resolved without treatment, but there is argument concerning the duration of infection (Cudmore and Garber, 2010).

Oral MNZ (α - β -hydroxyethyl-2-methyl-5 nitroimidazole; Flagyl) remains the standard treatment for trichomoniasis (Bouchemal et al., 2017). In the case of drug resistance and allergy, treatment alternatives are limited (Muzny and Schwabke,

2013). MNZ and tinidazole; 5-nitroimidazole agents, can cure *T. vaginalis*. Systemic treatment is preferred over topical applications to reach proper drug concentrations in non vaginal sites, as urethra, Bartholin's, and periurethral glands. Cure rate for single oral 2 g MNZ dose, is 90% to 95%, while tinidazole goes towards 100% cure rate (Coleman et al., 2013). Tinidazole has better pharmacokinetics for *T. vaginalis*; lower minimum lethal concentration (MLC), long half-life and higher tissue concentration (Wood and Monro, 1975; Sobel et al., 2001). MNZ targets the metabolic pathway of the hydrogenosome, where it is incorporated by passive infusion and subsequently activated. The activation generates nitro-radicals that are toxic to the parasite, by interfering with proteins and protein trafficking (Kusdian and Gould, 2014).

MNZ resistance is estimated to occur in 2.5–5% of *T. vaginalis* cases (Muzny and Schwebke, 2013). The mechanism of MNZ resistance is assumed to be due to multiple mutations (Paulish-Miller et al., 2014). In anaerobic resistance, there is a decrease in the activity of the pyruvate-ferredoxin oxidoreductase (PFOR) activity, whereas in aerobic resistance the transcription of the ferredoxin gene is reduced (Yarlett et al., 1987; Kulda, 1999; Leitsch et al., 2014; Bouchemal et al., 2017). MNZ resistance has been reported to be relative, increasing the dose or shifting to tinidazole can usually overcome the resistance (Coleman et al., 2013). However, higher doses may be associated with significant gastrointestinal adverse effects and challenging in patients who do not respond (Muzny and Schwebke, 2013).

Drugs that have been investigated as alternatives to 5-nitroimidazoles; albendazole/mebendazole (Sears and O'Hare, 1988; Oxberry et al., 1994), nitazoxanide (Dan and Sobel, 2007), have limited antiparasitic outcome (Muzny and Schwebke, 2013). Topically applied treatments are restricted to adjunctive therapy or particular cases of allergy or resistance. The use of vaginally applied anti-*T. vaginalis* thermosensitive and mucoadhesive formulations was recently described with improved residence time (Pradines et al., 2015; Bouchemal et al., 2017; Grisin et al., 2017).

Problems with current treatment and prevention strategies lead to limited success in controlling the incidence of the disease especially among the disadvantaged. *T. vaginalis* infection is usually asymptomatic and so is not treated, creating and maintaining reservoirs of infection. Moreover, most cases occur in developing countries, where healthcare facilities are restricted, allowing infection to spread within the community (Cudmore and Garber, 2010). Education concerning STIs and healthy sexual lifestyles can reduce infection incidence. Simultaneous treatment of sexual partners is recommended to prevent reinfection. Condoms use is still the best and most reliable protection means against STIs. Discomfort, religious and cultural rationales may hinder condoms use (Bouchemal et al., 2017). Female partners of circumcised males are reported to be at reduced risk of *T. vaginalis* infection (Sobngwi-Tambekou et al., 2009). However, different conclusions have been declared regarding its protective effect (Turner et al., 2008; Mehta et al., 2009). Another means of prevention is self-administration of vaginal microbicides before intercourse, which may limit parasite interaction with host cells (Lushbaugh et al., 2000).

Vaccination against *T. vaginalis* could provide long term protection, prevent health consequences, reduce medical costs and disease rates. The presence of a good animal model of *T. vaginalis* infection can help clarifying the specific elements needed to induce a strong and protective immune response required for an efficient vaccine (Cudmore and Garber, 2010). Vaccination could target high risk individuals, to protect themselves and their partners. However, the main challenges for the development of a vaccine are strain variation, parasite immune evasion strategies and transient specific humoral immune responses (Nemati et al., 2018).

Trichomonas tenax

Trichomonas tenax (*T. tenax*) was discovered and first described by the Danish naturalist Otto Friedrich Müller in 1773, who observed a flagellate in a culture of tartar from the teeth and named it *Cercaria tenax*. Thereafter, uncertainty and disagreement existed concerning the proper name of the parasite found in the human mouth. It is the English protozoologist, Clifford Dobell, in 1939, who has contributed the parasite discovery to O.F. Müller. Dobell reported that Müller's description of a pear-shaped organism in the human mouth, was the first record of the infection, and that instead of the name *Trichomonas buccalis*, *T. tenax* is the correct name (Dobell, 1939; Théodoridès and Rousset, 1980).

T. tenax is a pyriform flagellate, smaller and more slender than *T. vaginalis*, with average length of 7 µm. Morphologic differentiation from *T. vaginalis* includes: the undulating membrane is relatively longer over 2/3 of the length of the body, a thicker axostyle that extends as a sharp structure a considerable distance behind the body, a cytostome near the anterior end of the side opposite the undulating membrane (Beaver et al., 1984). *T. tenax* is an anaerobic commensal of the human oral cavity that is also colonized by other specific bacteria, fungi and protozoa (Mehr et al., 2015). The occurrence is depending on oral health status, being highly reported in those with coated tongue, dental calculus, and poorly maintained periodontal tissue (Bracamonte-Wolf et al., 2019). The level of oral contamination ranges from 4% to 53%. It survives in the mouth feeding predominantly on local microorganisms (Mallat et al., 2004).

The parasite is transmitted mainly by saliva through kissing and droplet spray and use of contaminated dishes and drinking glasses (Osborne et al., 1984; Hersh, 1985; Mallat et al., 2004). Although *T. tenax* is regarded as a human specific parasite, it has been reported to inhabit the oral cavity of different domesticated animals, raising its importance as a potential zoonotic infection (Maritz et al., 2014; Ribeiro et al., 2015; Dybicz et al., 2018a).

The flagellate has been linked to the occurrence and/or severity of periodontitis, a multifactorial inflammatory dental disease, affecting the soft tissue and bone of the periodontium (El Sibaei et al., 2012; Benabdelkader et al., 2019). Dysbiosis of the oral microbial communities takes place, with degradation of the tissue supporting the teeth, which may lead to teeth loss (Bisson et al., 2019; Dubar et al., 2020). The role of *T. tenax* in the pathogenesis of the oral disease, and the mechanisms involved in tissue damage

are still obscure (Bracamonte-Wolf et al., 2019). High parasite proteolytic activity, especially collagenolytic activity, has been demonstrated (El Sibaei et al., 2012; Dybicz, et al., 2018b). Genotyping revealed the presence of strain variability, and different strains with diverse pathogenic potential may exist (El Sibaei et al., 2012; Benabdelkader et al., 2019).

T. tenax has been detected in infections outside the oral cavity; in salivary duct infection of the submaxillary gland (Duboucher et al., 1995), in cervical adenopathy (Duboucher et al., 2000), in cerebrospinal fluid samples of patients with polymicrobial meningitis (Masur et al., 1976), in sputum samples of immunocompromised patients with chronic lung disease and in pleural empyema samples (Mahmoud and Rahman, 2004). Pulmonary *T. tenax* infection is usually caused by aspiration from the oropharynx and usually reported in patients with underlying lung disease (Hersh, 1985; Mallat et al., 2004).

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Trypanosoma cruzi

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Glossary

Achalasia A neuromuscular dysfunction on the esophagus characterized by swallowing difficulties.

Chagoma A hardened skin lesion, nodule or swelling at the inoculation (bite) area of the parasite (*T. cruzi*)

Enzyme immunoassay (EIA) An *in vitro* technique that applies a passive agglutination process to observe and quantify antigens.

Enzyme Linked Immunosorbent Assays (ELISA) An enzyme immunoassay used to quantify antibodies, antigens, and proteins.

Fecaloma A hardened fecal mass in the rectum or sigmoid colon.

Immunoblot (TESA) A technique used in molecular biology to identify certain proteins in a process that consist in denaturation and electrophoresis.

Indirect hemagglutination (IHA) A serological analysis that detects specific antibodies raised in response to an infection

Reservoir The reservoir is the habitat (can be humans, animals, or the environment) in which the infectious agent is conserved (can live, grow, and multiply), enabling the persistence of the transmission cycle.

Romana's sign Unilateral periorbital edema resulting observed after *T. cruzi* inoculation (1-2 weeks).

Volvulus A bowel obstruction that results from intestine twisting around itself.

Introduction

The *Trypanosoma cruzi*, the causative agent of a neglected tropical disease, the Chagas disease (CD) was first uncovered by the Brazilian physician and researcher Carlos Chagas in April 14th, 1909, in the city of Lassance, state of Minas Gerais in Brazil (Kropf and Sá, 2009). It is currently estimated that CD affects about 6-7 million people (WHO, 2020), representing a major global public health problem with significant socioeconomic impact (Lee et al., 2013; Lidani et al., 2019). In the last decades, many cases of CD have been identified in classically non-endemic regions including Europe, North America, and the Western Pacific area, where the transmission mainly occurs via blood transfusions or organ transplantation (Tarleton et al., 2014; Angheben et al., 2015).

More than a century since its discovery, CD still represents a life-threatening illness with a broad range of symptoms. The acute phase usually is asymptomatic, however, individuals chronically infected may present lower quality of life (Sousa et al., 2015), loss of productivity and even sudden death (Nunes et al., 2018) due to the CD symptomatic forms and/or associated comorbidities (Teixeira et al., 2011; Lee et al., 2013). To call international attention to CD, in 2019, the World Health Organization established the April 14th as the World Day for Chagas Disease (WHO, 2019).

Biology of *Trypanosoma cruzi*

Morphological aspects

T. cruzi, a hemoflagellate protozoan parasite, belongs to the Kinetoplastida Order, Family Trypanosomatidae (Fig. 1A). The presence of a flagellum and a particular region in mitochondria, the kinetoplast, which localizes a complex and unique mitochondrial DNA (kDNA), determine systematic morphological characteristics (Kaufer et al., 2017). The life cycle of *T. cruzi* involves invertebrate and vertebrate hosts, where different morphological forms occur during development (Contreras et al., 2002; de Souza, 2009).

In the **epimastigotes** (Fig. 1B and C), the kinetoplast is located in the central region of the body, anterior to the nucleus. The flagellum emerges from the central portion of the parasite and forms a short undulating membrane. Epimastigotes have elongated shape (20-40 µm long, 2-5 µm wide) and are less mobile than trypomastigotes. When vectors feed on the blood of the infected vertebrate, they ingest blood with trypomastigotes. These trypomastigotes pass from the salivary glands to the insect intestine, where they differ into epimastigotes by metacyclogenesis, multiplying through binary fission. After replication, they migrate to the terminal portion of the intestine, where they differentiate into metacyclic trypomastigotes.

In the **trypomastigotes** (Fig. 1D and E), the kinetoplast is in the posterior position in relation to the nucleus, usually in the terminal portion of the body. The flagellum emerges from the posterior portion and runs through the entire body of the parasite. The adhesion of the flagellum to the parasite's surface forms an undulating membrane until the anterior portion where the flagellum is free. The undulating membrane improves movement and facilitates the mobility of the protozoan. Trypomastigotes can be found in the digestive tract of the vector insect and human blood. They are infective life forms and present high resistance to different environments. The **metacyclic trypomastigotes** are those from the gut of vectors and responsible for human infection. Its body extends about 25 µm long and 2 µm wide. After the metacyclic trypomastigotes cross the skin, they travel briefly through the bloodstream and then localize in different tissues, but preferably muscle, hepatic, and nerve tissues.

T. cruzi **amastigotes** (Fig. 1F and G) are spherical (2-7 µm) and do not have prominent flagella outside of the body. The kinetoplast is close to the nucleus. Amastigotes are not mobile and are replicative/proliferative forms in the intracellular environment of the vertebrate host. The process of cell division of the amastigote begins after 24 h of the invasion of the trypomastigote form. The binary division starts with a gradual increase in the parasite body size, where two basal bodies arise, initiating the division of the kinetoplast and modification of nuclear chromatin, which condenses. A new flagellum begins to form. The nucleus acquires an elongated aspect, then occurring a constriction in the middle, giving rise to a cell with two nuclei and having two kinetoplasts. A cytoplasmic constriction then occurs that leads to the separation of the new forms of the parasite. The division cycle occurs every 14 h, varying according to the strain, nature of the host cell, and temperature. After a certain number of divisions (approximately 9), the amastigote forms undergo differentiation into trypomastigotes. This differentiation is known as **metacyclogenesis** and begins with the growth of the flagellum and structural change of the kinetoplast. Metacyclogenesis is synchronous, however, the molecular basis of this kinetics is not known. At the end of the intracellular cycle, a large number of extremely mobile trypomastigotes are formed. These new forms' intense movement leads to the disruption of the host cell and release of the trypomastigote forms (de Souza, 2009; Tonelli et al., 2010; Martins et al., 2012; Gonçalves et al., 2018).

Ultrastructure

T. cruzi has eukaryotic cell structures, but some specializations are found in its morphological stages and related to vital functions and success in host infection (Fig. 1).

The parasite's **cell surface** has a particular molecular association between different glycoconjugates and structural components of the plasma membrane. This association is essential in the initial interactions of parasite-host cell and host immune response (de Souza, 2009; de Souza et al., 2010a; Ferreira Cura Das Neves et al., 2020).

Below the plasma membrane of *T. cruzi* there is a helical distribution of microtubules—**subpellicular microtubules**—fundamental for the shape and locomotion of this protozoan (de Souza, 2009).

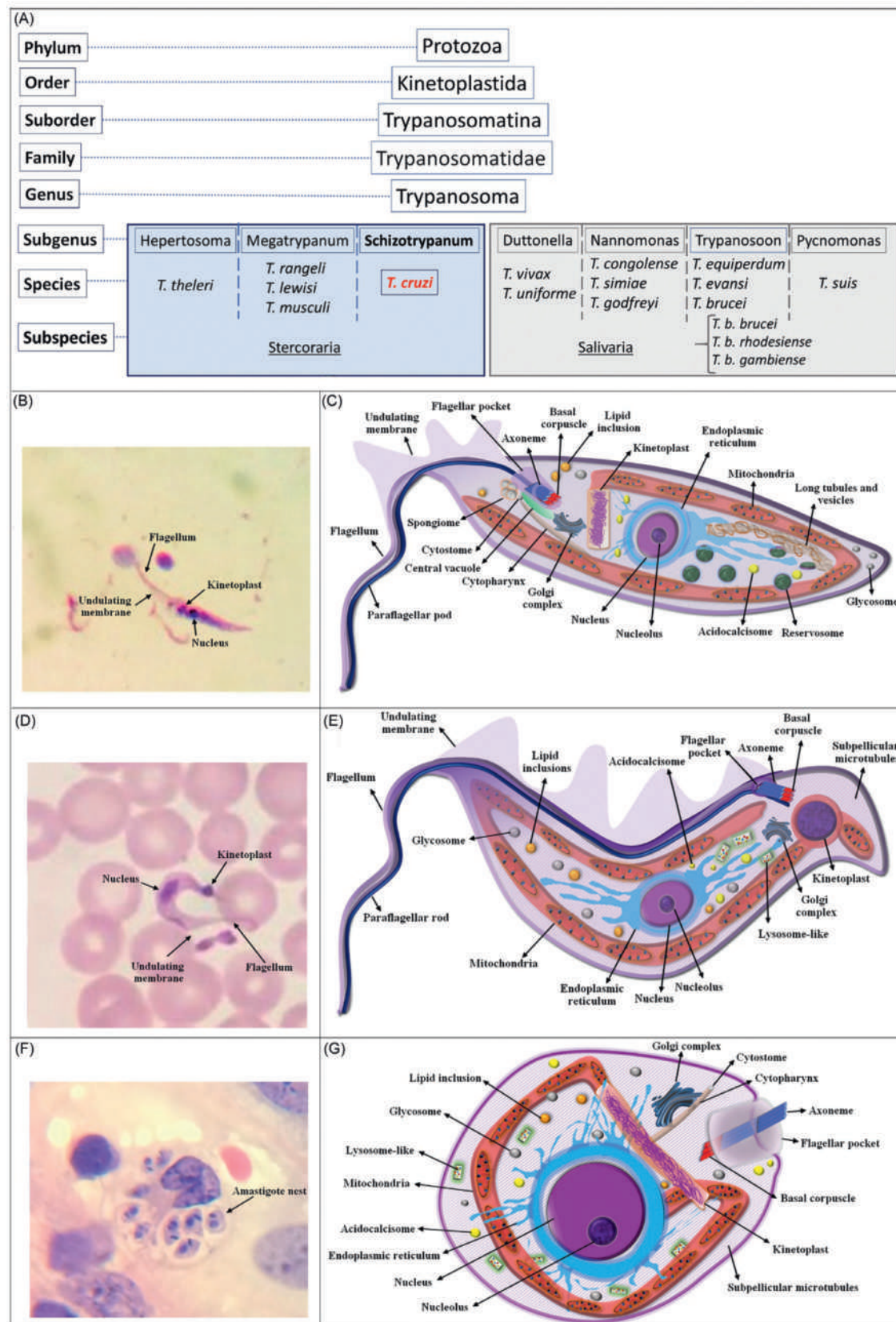


Fig. 1 Taxonomic classification and the ultrastructure of the different stages of *T. cruzi*. (A) Taxonomic classification of *T. cruzi*. (B) Giemsa-stained *T. cruzi* epimastigotes. (C) Schematic illustration of the epimastigote architecture of *T. cruzi* and organelles. (D) *T. cruzi* trypomastigote in a thin blood smear stained with Giemsa. (E) Schematic illustration of the trypomastigote architecture of *T. cruzi* and organelles. (F) Nest of *T. cruzi* amastigote in a cardiac tissue section stained with hematoxylin and eosin. (G) Schematic illustration of the amastigote architecture of *T. cruzi* and organelles. The images (B, D and F) were kindly provided by Prof. Dr. Ivan Neira Cortés.

From the flagellar pocket, the flagellum originates with variable length through the different stages of the parasite that confers locomotion and helps in the initial adhesion of the parasite to host cells.

In the cytoplasm of *T. cruzi* some structures are special and aligned to the metabolism of the parasite. In epimastigotes and amastigotes forms, near the flagellum, there is the **cytostome**, a specialized portion of the plasma membrane that invaginates through the flagellar pocket. This structure internalizes and metabolizes macromolecules for parasite survival (de Souza, 2009). Besides, the **reservosome**, a pre-lysosomal organelle, stores macromolecules from the cytostome. The reservosome is vital during the metacyclogenesis of the *T. cruzi*, especially from epimastigote to trypomastigote, when nutrients become scarce and the stored molecules are then used by the parasite (de Souza, 2009; de Souza et al., 2010a).

The **glycosomes** are organelles related to glycolytic and metabolic pathways such as peroxide metabolism, β -oxidation of fatty acids, phospholipid synthesis, carbon dioxide fixation, purine recycling, pyrimidine biosynthesis, fatty acid elongation, isogenoids, and sterol biosynthesis (de Souza, 2009). Many of these metabolism stages are potential targets for new chemotherapy and have been extensively studied.

The **acidocalcisome** is an acidic vacuolar organelle with high phosphorus concentration in the form of pyrophosphate or polyphosphate and calcium (Docampo et al., 2011). Cytochemical studies have shown the presence of H^+ ATPase, Ca^{2+} ATPase, and pyrophosphatase in the acidocalcisome membrane. The amount of these organelles varies according to the form of the parasite, with a relatively high amount in amastigotes when compared to epimastigotes and trypomastigotes (Benchimol et al., 1998). Ions such as sodium, potassium, zinc, and iron can be found in acidocalcisomes (Docampo et al., 2011). Among the functions described for this organelle are the maintenance of cytoplasmic pH, osmoregulation, storage of ions, and phosphate compounds such as pyrophosphates and polyphosphates (Docampo et al., 2005).

The **kinetoplast** is a unique structure composed of circular extranuclear DNA whose rings are concatenated in maxicircles and minicircles, known as **kinetoplast DNA (kDNA)** (de Souza, 2003; Cavalcanti et al., 2009). The ultrastructural morphology of the kinetoplast and its location in the cytoplasm determine the morphological stage of the parasite (Riou and Yot, 1977). In epimastigote and amastigote forms, the kinetoplast presents as a compacted rod located in the posterior portion of the nucleus and anterior to the flagellum, while in trypomastigotes, kinetoplast has a rounded shape with less compacted DNA and is located in the anterior portion of both the flagellum and nucleus (Riou and Yot, 1977). The kDNA cycle is similar to the bacterial system replication, which leads to the use of antibiotics as a therapeutic option against parasite infection.

Recently, multivesicular structures, called **exosomes**, were reported in *T. cruzi*. These membrane-based vesicles contain proteins, enzymes, RNAs, nucleic acid, and other molecules, and are secreted from the surface of the parasite. **Exosomes** are present in several eukaryotic cells, but in the case of *T. cruzi* may be related to signal transmission between parasites and induction of inflammatory responses in the infected host (Colombo et al., 2014; Lovo-Martins et al., 2018; de Souza and Barrias, 2020).

Biological diversity of *Trypanosoma cruzi*

Genome organization

T. cruzi presents a complex genome, where aneuploidies in hybrid strains and repetitive clusters of different gene families were found. This genomic variety may result from the degree of adaptation of the protozoan to different environments within the two hosts in its life cycle.

Size, ploidy, and chromosomes

The complete genome of *T. cruzi* is predicted to be around 105 Mb in length, distributed across 20 to 46 chromosomes (Henriksson et al., 1996; Lewis et al., 2009; Souza et al., 2011; Reis-Cunha et al., 2018). A difference of up to 47.5% (equivalent to ~73 Mb of DNA) is found in the genome size of different strains, which is attributed to both nuclear and kinetoplast DNAs (Dvorak and Howe, 1976; Castro et al., 1981; Lanar et al., 1981; Dvorak et al., 1982; McDaniel and Dvorak, 1993; Lewis et al., 2009). The amplitude found in the number of chromosomes is due to the frequency of aneuploidies (gain or loss of chromosomal copies) between and within strains, despite being predominantly a diploid organism. This fact may be associated with the degree of genome plasticity regarding parasite fitness (Reis-Cunha et al., 2018).

T. cruzi's genome is organized in small homologous pairs of chromosomes that present a significant variance in number and size among the strains (Henriksson et al., 1996, 2002; Brisse et al., 2003; Pedroso et al., 2003; Branche et al., 2006). This variance may probably be due to the location of surface molecule genes in subtelomeric regions that suffered rearrangements for immune modulation and evasion overtime (Souza et al., 2011). Moreover, up to 50% difference in certain genetically equivalent chromosomes and size differences reaching up to 1.9 Mb in some strains suggest that larger chromosome rearrangements and fusions occurred through the evolutionary process (Gaunt et al., 2003; Heitman, 2006; Messenger and Miles, 2015). Interestingly, during cell division (mitosis), *T. cruzi* chromosomes poorly condense precluding classical cytogenetic analysis (Santos et al., 1997).

T. cruzi has been considered a clonal organism that replicates by binary fission (asexual process) (Tibayrenc et al., 1990; Tibayrenc and Ayala, 2002). Recently, recombination events were found to be frequent in *T. cruzi* populations (Ramírez et al., 2012; Baptista et al., 2014; Ramírez and Llewellyn, 2014; Messenger et al., 2015b; Tomasini and Diosque, 2015). Independent exchange of DNA content in the nucleus and kinetoplast has been reported in natural populations (Messenger and Miles, 2015), indicating that hybridization has contributed to the present parasite population structures and the evolution of distinct *T. cruzi* subgroups.

Trypanosoma cruzi genome

The *T. cruzi* genome contains ~12,000 genes (CL Brener strain) with a minimum of ~10,800 for Sylvio strain (Franzén et al., 2011) and up to a maximum of ~15,300 for Dm28c (Berná et al., 2018). Approximately 50% of the genome is repetitive and presented as tandem arrays or pseudogene copies (El-Sayed et al., 2005). Recently, these repetitive genes were divided into well-conserved core genes presenting a lower GC content (48%) and variable genes, mainly coding for the multigenic surface families with a higher GC content (53%) (Berná et al., 2018).

Repetitive DNA

The most representative multigene families (e.g., TS, MASP, Mucins, DGF-1, and gp63) are related to host-pathogen interaction at the cellular level (adhesion, invasion, infection) and in immune modulation responses and thus to the success and pathogenicity of infection in the vertebrate host (Reis-Cunha et al., 2015).

The *trans*-sialidase (TS) superfamily comprises proteins that can be secreted or anchored to the surface membrane via glycosyl-phosphatidylinositol (GPI). TS is encoded by 1430 genes that are found usually near telomeric regions and, for this reason, are related to the expansion and size variability in the genome due to the exposure to the immune system pressure (Hansson et al., 2005). TS family proteins present four main functions: (i) the transfer of the sialic acid from host's cell glycoconjugates (sialylation) to parasite's β -galactosidase (β -Gal) mucins via *trans*-sialidase enzyme, which negatively charges the trypomastigote's membrane therefore protecting from the attack of anti- α -galactosyl antibodies (Pereira-Chioccola et al., 2000; Buschiazzi et al., 2002); (ii) binding and stabilization of the contact between the host cell receptors and the parasite, which determines the tissue tropism as by the conserved motif FLY domain, present in 371 members of this family, which shows tropism to heart, esophagus, and bladder endothelium (Magdesian et al., 2001a; El-Sayed et al., 2005; Tonelli et al., 2010); (iii) act as parasite-derived neurotrophic factor, together with tyrosine kinase receptors, TS proteins work as an antiapoptotic factor in mammalian cells, facilitating the persistence of the parasite in infected tissues (Chuenkova and Pereira, 2000; de Melo-Jorge and PereiraPerrin, 2007); (iv) complement regulatory proteins (CRPs) that restricts the activation of the complement pathway and therefore the lysis of the parasite (Norris et al., 1991).

Mucins (TcMUCs) are the most commonly expressed glycoproteins on the surface of *T. cruzi* ($\sim 2 \times 10^6$ copies/parasite) during different life stages. Mucins are the third most widely expanded gene family with approximately 863 genes (being 201 pseudogenes). TcMUCs have two main functions: to protect the parasite from both the vector and the host immune mechanisms and to ensure the anchorage point and invasion of specific cells and tissues (Buscaglia et al., 2006).

The MASP-encoding is the most recently discovered gene family in the *T. cruzi* genome and comprises 1377 genes and 433 pseudogenes distributed in arrays of up to 600 kb (Arner et al., 2007). It is named mucin associated surface protein (MASP) due to their proximity to mucin genes. MASP family is characterized by conserved *N*-terminal signal peptide, a conserved *C*-terminal domain containing a GPI anchor addition site, and a variable central region (De Pablos and Osuna, 2012). The distribution of the genes for these proteins among those for other surface proteins may help maintain genetic diversity (Bartholomeu et al., 2009). Also, a role in the immune evasion during the acute phase of Chagas Disease was proposed for this gene family (Villalta et al., 2001).

Dispersed gene family 1 (DGF-1) contains 565 genes and 136 pseudogenes (El-Sayed et al., 2005), which are located in subtelomeric chromosome regions where the variability in their sequences may be favored (Kim et al., 2005; Kawashita et al., 2009). The DGF-1 family members stimulate genetic diversity by gene duplication, recombination, or hybridization (Kawashita et al., 2009). They are present with greater abundance in amastigotes than in trypomastigotes and epimastigotes. The variation in those protein location during parasite differentiation and the high amount of members found in different parasite life stages (Lander et al., 2010) reveals the importance of the DGF-1 family in the biology of *T. cruzi* (Ulrich et al., 2011).

Gp63 family is GPI anchored metalloproteases that are found widely expanded in *T. cruzi* (400–700 genes or pseudogenes) (El-Sayed et al., 2005). More than 60% of those genes are assigned as pseudogenes and, although the function of this family is not elucidated yet in *T. cruzi*, in *Leishmania* they seem to have a role in invasion and innate immune evasion (Weinkauff and Pereiraperrin, 2009; Weinkauff et al., 2011). Transcriptomic analysis showed that most of those proteins are highly expressed in trypomastigotes while few expressed exclusively in amastigotes. Phylogenetic analysis of the 3'UTR region of gp63 family members revealed three distinct groups: one with genes highly expressed in trypomastigotes, another one with genes highly expressed in amastigotes, and the third group of genes with almost no expression in any stage of the parasite (Tonelli et al., 2010).

In addition to the multicopy gene families, retrotransposons and satellite DNA (tandem repeat sequences) also contribute to the repetitive nature of the *T. cruzi* genome. Retrotransposons (or class I transposable elements) are repeated DNA sequences with the ability to move from one to another *locus* in the genome by reverse transcription of an intermediate RNA into DNA upon insertion (Thomas et al., 2010). These elements were related to the parasite evolution by reshaping genomes through genetic rearrangements as creating, disrupting, shuffling genes, and even modulating the expression pattern (Thomas et al., 2010). Tandem repeats are classified according to their monomer or cluster length in micro (monomers size from 2 to 5 bp), mini (from 15 to 100 bp), and macro-satellite or satellite (greater than 100 bp) (Charlesworth et al., 1994). Around 5–10% of the genome is composed by the 195 bp satellite (TcSAT1) (Sloof et al., 1983; El-Sayed et al., 2005), being variable among strains (differences of up to six-fold), but organized similarly throughout the genome (Hoft et al., 1989). A part of the 195 bp satellite, other several tandem repeats are composed of protein-coding genes organized in tandem where the intergenic regions are highly conserved. The broad distribution of tandem repeats along the *T. cruzi*'s genome and the antigenicity indicate that they work as a mechanism triggering a polyclonal immune response, deceiving the host immune response and allowing parasite immune evasion (Berná et al., 2018).

Single copy genes

Recently, Berná et al. (2018) found 5000 single-copy genes in Dm28c, of which only 1377 have an assigned function. Some genes, previously considered to be single-copy, form a cluster of a few copies, leading to copy number miscalculations, as the lipamide dehydrogenase gene (dos Santos et al., 2016) which exhibits seven copies in TCC strain and six in Dm28c strain. This may be why *T. cruzi* is traditionally considered a refractory organism to genetic manipulation.

kDNA

Trypanosomatids present another genome in their single mitochondria (kinetoplast DNA—kDNA), composed of ~30 copies of 20–50 kb maxicircles and thousands of copies of ~1 kb minicircles (Lukeš et al., 2002; Messenger et al., 2012). The kDNA accounts for 20% of the parasite's total DNA (Baptista et al., 2014; Messenger et al., 2015a) and comprises two types of circular DNA molecules called maxicircles (the longer and functional equivalent of eukaryotic mitochondrial DNA) and minicircles (the shorter—max 2 kb—and encoding gRNAs). gRNAs are short RNA molecules responsible for guiding RNA editing, a process of post-transcriptional modifications (the addition and deletion of uridines). Maxicircle coding regions (~63% of the *T. cruzi* maxicircle genome) are homogeneous (Messenger et al., 2012), while minicircle sequences are highly heterogeneous even within a single clone (Telleria et al., 2006).

Gene expression

Genes in trypanosomatids are organized into non-overlapping clusters on the same DNA strand with unrelated predicted functions. Genes are transcribed mostly by RNA polymerase II into long polycistronic primary transcripts composed of clusters of unrelated genes (10 to 100) that are processed through coupled *trans*-splicing (a mechanism described 30 years ago in trypanosomatids, it is the processing of polycistronic RNA to mature monocistronic mRNA through two consecutive transesterification reactions that utilize two RNA molecules, the spliced leader RNA and the pre-mRNA) and polyadenylation (the addition of a tail of several adenosines poly(A) to a messenger RNA's 3' end to mature the mRNA for translation) to obtain mature individual mRNAs (Souza et al., 2011). This fast processing of the primary transcript causes the loss of the 5' start site, making the finding of promoters in this parasite difficult. It seems that the typical RNA polymerase II promoters are not present in *T. cruzi*'s genome (Souza et al., 2011) and, therefore, it is not clear how transcription is initiated. In *T. cruzi*, gene clusters can range from ~30 to 500 kb separated by divergent or convergent strand-switch regions (SSR) (Myler et al., 1999). Epigenetic mechanisms also may regulate gene expression as histone modifications and chromatin-modifying enzymes/variants (Hanke et al., 1996; Elias et al., 2001; da Cunha et al., 2006; Respuela et al., 2008). Recently, the enrichment of histone modifications associated with transcription initiation at SSR was demonstrated in *T. cruzi* (Respuela et al., 2008). Gene expression in *T. cruzi* is mainly regulated post-transcriptionally, i.e., at the level of *trans*-splicing efficiency, mRNA stability and decay, translation efficiency, and post-translational modifications (Martínez-Calvillo et al., 2010). Additionally, gene duplication and the clustering of various genes in multi-cistronic transcription units have been suggested as common means by which trypanosomes regulate protein levels (Santos et al., 1997).

Molecular genotypes

Historically, the population structure of *T. cruzi* was characterized to define the relevant subgroups using different approaches as zymodemes (Miles et al., 1977, 1978, 1981), schizodemes (Morel et al., 1980), biotemes (Andrade and Magalhães, 1996), clonets (Tibayrenc and Ayala, 1991), lineages (Souto et al., 1996), clades (Kawashita et al., 2001), discrete typing units (DTUs) (Tibayrenc, 1998), and haplotypes (de Freitas et al., 2006; Herrera et al., 2007). First, Miles and collaborators classified the *T. cruzi* subpopulations using a biochemical tool (enzyme electrophoresis) in three zymodeme groups (Miles et al., 1977). Zymodeme 1 and 3 groups were associated with sylvatic transmission cycles and Zymodeme 2 with domestic transmission cycle. In 1999, for the first time, an expert committee revised all the current knowledge on the biological and biochemical features of *T. cruzi* strains (biotemes and zymodemes) characterized by molecular techniques and recommended the division of the parasites in two major groups: *T. cruzi* I and *T. cruzi* II (Anonymous, 1999). With the advance of the knowledge on *T. cruzi* diversity, multilocus genotyping revealed six distinct DTUs with two major subdivisions: DTU I and DTU II (Brisse et al., 2001). This first DTU classification was based predominantly on clonal evolution (Tibayrenc et al., 1986), then the report of field hybrid strains in *T. cruzi* populations (Sturm and Campbell, 2010) and also experimentally generated hybrid strains (Gaunt et al., 2003) together with the gained knowledge on *T. cruzi* evolution made necessary a new revision of the *T. cruzi*'s nomenclature. A second expert meeting occurred in 2009 to standardize the nomenclature based on the characterization of *T. cruzi* eco-epidemiological features, pathogenicity, and questions of basic biology (Zingales et al., 2009). Since then, *T. cruzi* strains are subdivided into seven discrete typing units (DTUs): TcI to TcVI and Tcbat, being associated with different geographic distribution and transmission cycles (Miles et al., 2009; Zingales et al., 2009, 2012; Pinto et al., 2012; Lima et al., 2015). The term discrete typing unit is defined as “sets of stocks that are genetically more similar to each other than to any other stock and that are identifiable by common genetic, molecular or immunological markers” (Tibayrenc, 1998). In Table 1, a summary containing *T. cruzi*'s genotype classifications (actual and former) and epidemiological features is displayed.

Different evolutionary models have been proposed to explain the origin of *T. cruzi* hybrid lineages: the “Two Hybridization” (Westenberger et al., 2005), the “Three Ancestor” (de Freitas et al., 2006), the “*Trans*-sialidase” (Burgos et al., 2013) and most recently the “Hybridization and Mitochondrial Introgression” (Tomasini and Diosque, 2015) models (Fig. 2). Even though these

Table 1 Summary of former genotype classification, geographic distribution, vector, transmission cycle, host, and disease associations of *T. cruzi*'s DTUs.

DTU genotype	Abbreviation	Former <i>T. cruzi</i> genotype	Geographic distribution	Vectors	Transmission cycle	Chagas Disease pathophysiology	Sylvatic hosts
<i>T. cruzi</i> I	TcI	<i>T. cruzi</i> I and DTU I	Latin America and North America (South): more frequent in the North of the Amazon and sporadic in the Southern Cone	Primary: <i>Rhodnius</i> spp. Secondary: <i>Panstrongylus</i> spp. <i>Triatoma</i> spp. <i>Eratyrus</i> spp.	Domestic and Sylvatic	Cardiomyopathy	<i>Didelphis</i> , rodents, primates, <i>Tamandua</i>
<i>T. cruzi</i> II	TcII	<i>T. cruzi</i> II and DTU IIb	Southern Cone (mainly Atlantic and Central Brazil), sporadic further North	Not completely known (Triatominae)	Domestic (rarely in Sylvatic)	Cardiomyopathy, megasyndromes	Atlantic forest primates, <i>Didelphis</i> , <i>Euphractus</i> (Paraguay)
<i>T. cruzi</i> III	TcIII	Z3/Z1 ASAT, Z3-A, DTU IIc and <i>T. cruzi</i> III	South America	<i>Panstrongylus geniculatus</i>	Sylvatic (rare in humans and dogs)	Acute cases in Amazonian Brazil. Clinical presentation poorly known	Armadillos (especially <i>Dasyus</i> , <i>Chaetophractus</i> , <i>Euphractus</i>); <i>Didephids</i> , <i>Monodelphis</i>
<i>T. cruzi</i> IV	TcIV	Z3, Z3-B and DTU IIa	North America (South) and South America (North)	<i>Rhodnius</i> spp., <i>Panstrongylus</i> spp., <i>Triatoma</i> spp.	Predominantly sylvatic with hotspots of domestic transmission in Venezuela and North of Brazil	Secondary cause of CD in Venezuela, sporadic elsewhere in South America	Primates, <i>Nasua nasua</i>
<i>T. cruzi</i> V	TcV	Bolivian Z2, rDNA 1/2, clonet 39 and DTU IIId	Southern Cone (greater Gran Chaco and Extreme South of Brazil)	Not completely known	Domestic (rare in Sylvatic)	Cardiomyopathy, megasyndromes	<i>Dasyus</i> , <i>Euphractus</i> , <i>Octodon</i>
<i>T. cruzi</i> VI	TcVI	Paraguayan Z2, Zymodeme B and DTU IIe	Southern Cone (greater Gran Chaco and Extreme South of Brazil)	Not completely known	Domestic (rare in Sylvatic)	Cardiomyopathy, megasyndromes	Not completely known
<i>T. cruzi</i> bat	Tcbat	Not applicable	Central and South America	Not known	Not known (one human case reported in Colombia)	Apparently asymptomatic (TcBat is able to invade mammalian cells but don't cause tissue damage)	Bats (mainly insectivorous, frugivorous and nectarivorous)

models disagree about the ancestral strains and the number of hybridization events during *T. cruzi* evolution, they all agree that TcV and TcVI are hybrids, originated from parental TcII and TcIII strains. It is still unknown if these hybrids arose from a single hybridization (Messenger et al., 2012) or multiple independent recombination events (Virreira et al., 2003; Piron et al., 2007; Tomasini and Diosque, 2015). Molecular data suggest that these two hybrid lineages evolved recently, reinforcing the assumption that genetic exchange could still drive the emergence of *T. cruzi* recombinant isolates (Virreira et al., 2003; Messenger et al., 2012).

Among the DTUs, TcI is the lineage that shows the highest genetic heterogeneity, possibly due to its dispersion throughout the Americas, which is compatible with a long-term evolution. In the sylvatic cycle, TcI infects about 50 mammalian genera and a significant number of triatominae genera turning the sylvatic TcI populations extremely diverse (Llewellyn et al., 2009). In contrast, human infection with TcI is prevalent in the north of South America, Central America, and Mexico, thus presenting a considerable reduction in genetic diversity. Genetically, five intra-DTU TcI genotypes (Ia, Ib, Ic, Id, Ie) were described based on sequence polymorphisms in the mini-exon intergenic region (Fallá et al., 2009; Cura et al., 2010).

From the six DTUs, TcI, TcII, TcV, and TcVI are usually involved with the domestic cycle of Chagas disease, accounting for most human infections (Zingales et al., 2012). Human infections caused by TcI strains are more prevalent from Central America to Bolivia, while TcII, TcV, and TcVI human infections are more common in the Southern cone of South America, encompassing countries like Argentina, Chile, Paraguay, Bolivia and Brazil (Barnabé et al., 2011; Zingales et al., 2012; Fernández et al., 2014; Lima

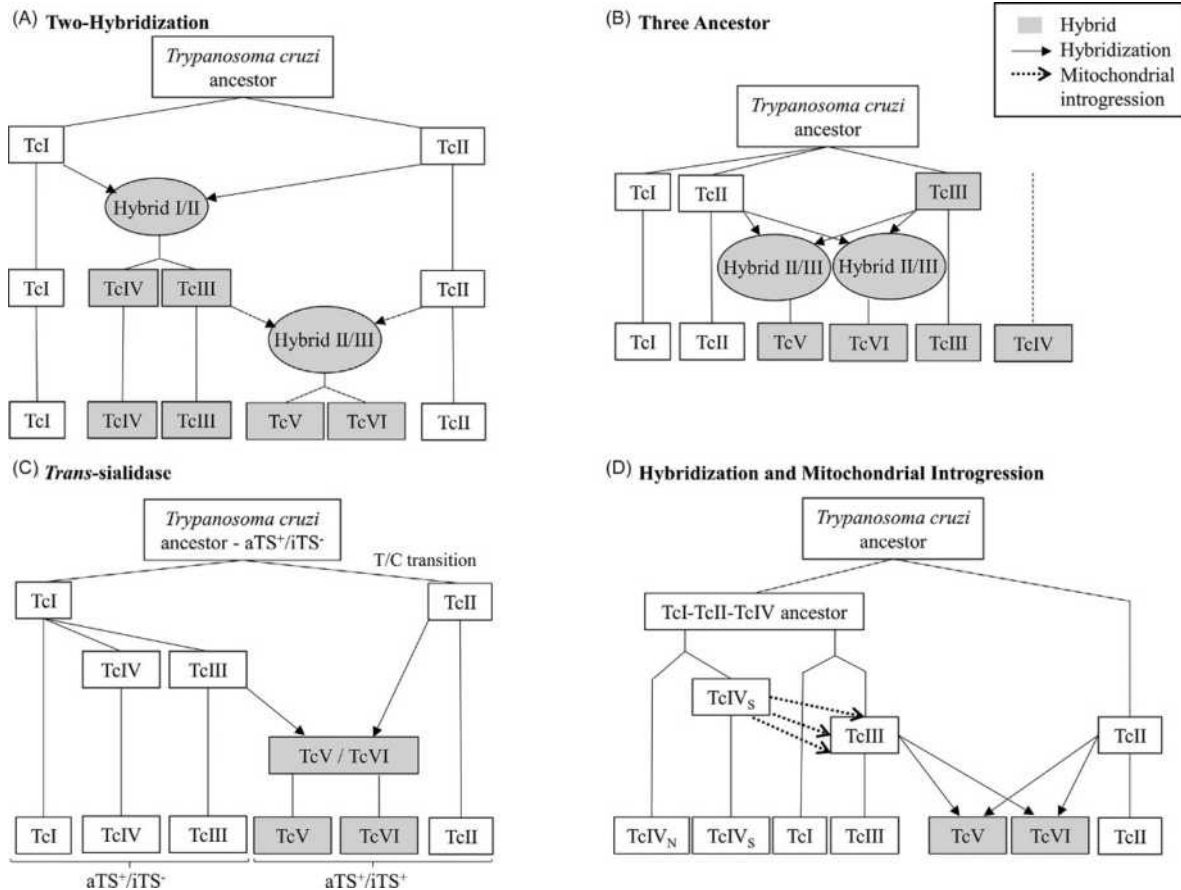


Fig. 2 *T. cruzi* evolutionary models.

et al., 2014). Recently, several TcVI parasites were isolated from humans and vectors in Colombia, suggesting that the distribution of this DTU could be broader than previously speculated (Messenger et al., 2016).

kDNA clades

Three mitochondrial clades were identified by *T. cruzi* maxicircles phylogenetic analyses: clade A comprising TcI maxicircles; clade B comprising TcIII, TcIV, TcV, and TcVI maxicircles; and clade C comprising TcII maxicircles (de Freitas et al., 2006; Messenger et al., 2012). Although maxicircle sequences are usually conserved within a DTU, evidence of intra-lineage mitochondrial introgression (when the maxicircle genome from a DTU is associated with a non-recombinant nuclear genome of a different DTU) was found (Machado and Ayala, 2001; de Freitas et al., 2006; Carranza et al., 2009; Messenger et al., 2012; Messenger and Miles, 2015). Besides introgression, the occurrence of minor heteroplasmy (the presence of heterogeneous mitochondrial genomes in an individual cell) has also been reported (Messenger et al., 2012; Messenger and Miles, 2015). The mechanism and the role of both processes in *T. cruzi* evolution are still unclear, however, they may be important to avoid the accumulation of deleterious mutations resulting from clonal reproduction (Ramírez and Llewellyn, 2014; Messenger and Miles, 2015).

Life cycle of *Trypanosoma cruzi*

Cycle in the vertebrate and invertebrate hosts

T. cruzi is a heteroxenic protozoan which presents distinct stages of development in invertebrate and vertebrate hosts. Throughout its life cycle in the different hosts, *T. cruzi* endures several microenvironmental factors such as pH and temperature changes, nutrient availability, and osmotic and oxidative stress that threatens its survival (Wheeler et al., 2013; Melo et al., 2020). To ensure complete its life cycle successfully and colonize a host efficiently, the parasite uses different strategies by undergoing complex changes in morphology, metabolism, and gene expression profile (Jimenez, 2014).

The life cycle scheme of *T. cruzi* is displayed in Fig. 3. During a blood meal, the infected triatominae insect eliminates metacyclic trypomastigotes along with its urine/feces on the host's skin (López-Vélez et al., 2019). These parasite forms enter into the host through the bite wound, injured skin, or mucosal surface. Triatominae insect excreta induce a cytokine storm, including tumor necrosis factor alpha (TNF- α) and chemokines ligand-2 (CXCL2) and -ligand 3 (CXCL3) at the inoculation site followed by

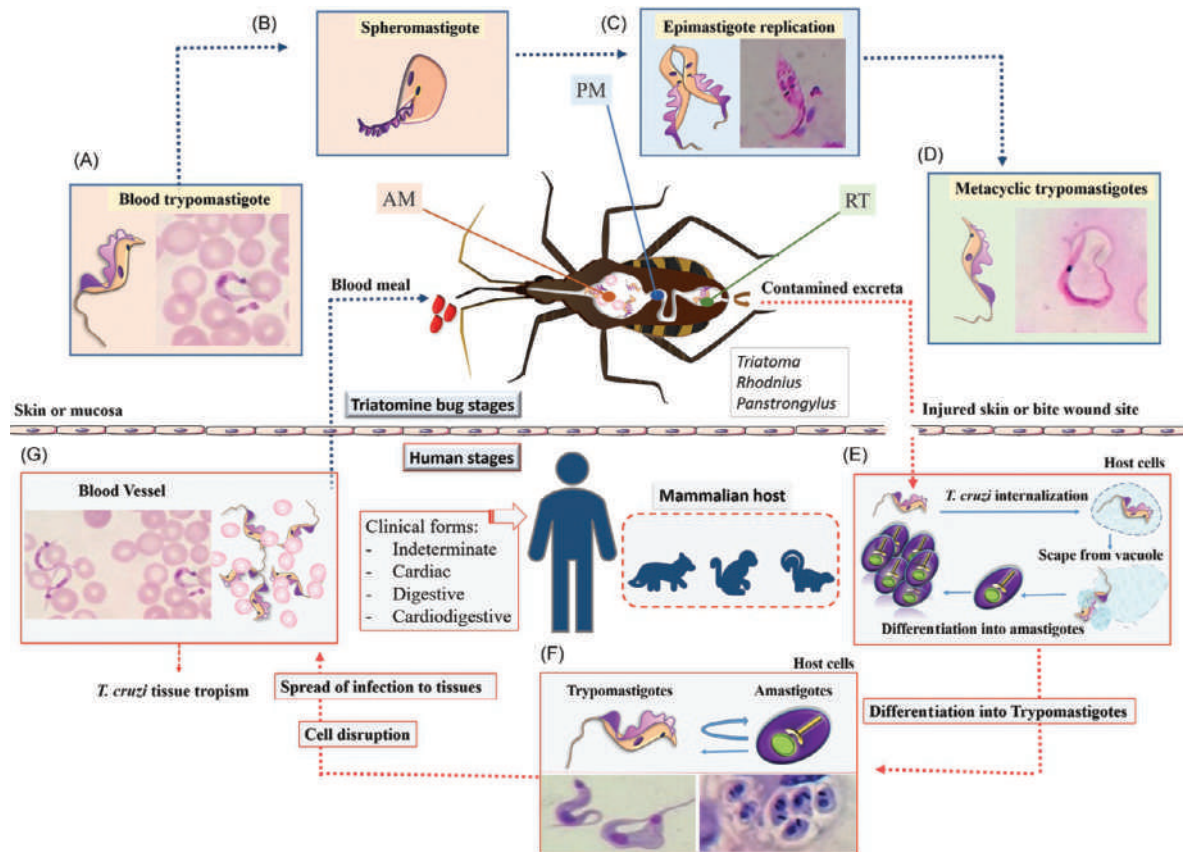


Fig. 3 Life cycle of *Trypanosoma cruzi* in triatomine insect and mammalian host. Note: After feeding on blood containing trypomastigotes (A), the parasites migrate to the vector midgut, where they differentiate into spheromastigotes—intermediate form—(B) and epimastigotes in the anterior midgut (AM). In the posterior midgut (PM) epimastigotes multiply and adhere to the perimicrovillar membranes of the intestinal cells (C). In the rectum (RT) the epimastigotes multiply intensely, attach to the rectal wall and turn into infectious metacyclic trypomastigotes that are eliminated in vector's feces and/or urine (D). In the following blood meal, the triatomine releases the infective metacyclic trypomastigotes, which penetrate the mammalian host (human, opossums, armadillos, raccoons, among others) by scratching/rubbing or through injured skin near to the bite site. Trypomastigotes invade cells and are internalized inside the parasitophorous vacuole (0–4 h) from which they escape, and differentiate into the dividing amastigote (12–24 h) that multiplies freely in the cytoplasm by binary fission (24–72 h) (E). After about 9 cycles, these intracellular forms differentiate back into trypomastigotes (72–120 h). Alternatively, amastigotes prematurely released from infected cells, or derived from the extracellular differentiation of trypomastigotes, may also invade cells and continue an infective cycle (F). After the cell disruption, the bloodstream forms can reach a variety of tissues through blood/lymphatic circulation or be taken by the insect vector (G). The most well-characterized parasite reservoirs are represented in the box named *T. cruzi* tissue tropism. Microscopic images were kindly provided by Prof. Dr. Ivan Neira Cortés. *Epimastigotes and amastigotes are the replicative forms found in the insect midgut and mammalian cells, respectively. Meanwhile, the metacyclic trypomastigote (in the insect vector) and the bloodstream trypomastigote (in the vertebrate host) are the non-proliferative and infectious stages of the parasite.

recruitment of neutrophils and monocytes. This strong inflammatory reaction induces edema favoring the escape of metacyclic trypomastigotes into lymph/blood vessels (López-Monteón et al., 2019). The establishment of the infection depends on the parasite's ability to invade and differentiate into a replicative stage inside mammalian host cells (Lidani et al., 2017; Melo et al., 2020). The parasite invasion leads to the formation of an endocytic vacuole (called parasitophorous vacuole) which subsequently merges with a lysosome – an endocytic pathway present in all eukaryotic cells. Apparently, the trypomastigote stimulates, by exposing itself to a low pH from the lysosomal enzymes, the secretion of lytic enzymes that pierces the vacuolar membrane and enables the parasite to be adapted to the cytoplasmic environment. Then, the parasite is differentiated in the form of intracellular amastigote (De Souza et al., 2010b; Horta et al., 2020). The amastigotes multiply asexually (by binary fission), forming pseudocysts in many host tissues mainly in cardiac, smooth, and skeletal muscles and reticuloendothelial cells in the liver, and spleen. Inside pseudocysts, after many cycles of multiplication, amastigotes differentiate into trypomastigotes and are released into the extracellular environment, either infecting adjacent cells to initiate new replicative cycles or reaching the bloodstream and lymph to spread to other tissues (Lidani et al., 2017; Melo et al., 2020).

Triatomine insects feeding on an infected host may ingest trypomastigotes circulating in the blood, which pass to the midgut where they differentiate into spheromastigotes, an intermediate form. The differentiation of spheromastigotes into epimastigotes is a response to decreasing glucose levels as the blood meal is digested (Tyler and Engman, 2001). The epimastigotes multiply by longitudinal binary fission in the hindgut and migrate to the rectum, where they attach to the intestinal cuticle by their flagella and differentiate into infectious metacyclic trypomastigotes, thereby completing the parasite life cycle (Fig. 3).

Transmission forms

Although the vectorial transmission has been controlled or interrupted in many countries after governmental efforts, *T. cruzi* is still mainly transmitted in endemic areas by contact with feces/urine of infected blood-sucking triatominae insects (subfamily Triatominae) (de Lana and de Menezes Machado, 2017). They usually bite an unprotected skin area as the face (hence its common name 'kissing bug') and during blood feeding, the insect excretes parasites that enter the body through scratching at the bite area (WHO, 2020). Around 140 species of Triatominae insects widely distributed in the Americas are known to transmit *T. cruzi* (Schofield and Galvão, 2009; López-Monteón et al., 2019).

T. cruzi can also be transmitted orally by consumption of contaminated food/drinks; by blood transfusion or organ transplantation from infected donors; and congenitally (from an infected mother to her newborn); as well as by laboratory accidents (Liu and Zhou, 2015). More recently, these transmission routes have gained attention, especially in developed countries, in which up to 25% of immigrants may be infected with *T. cruzi* (Schmunis, 2007). The oral transmission has also raised attention in the last decades, due to recurring outbreaks of parasite-contaminated food and drink products consumption, usually associated with severe symptoms and high fatality rates (Shikanai-Yasuda and Carvalho, 2012; Cielo et al., 2017).

Mechanisms of infection

Adhesion of *Trypanosoma cruzi* to vertebrate cells

In the vertebrate host, the first invasion stages require adhesion of metacyclic trypomastigote's flagellum to the cell's membrane. The subsequent adhesion of parasites to vertebrate cells depends on the interaction of surface molecules between host cells and parasites, acting as receptors-ligands. The variety of these molecules involved in adhesion demonstrates the tropism and versatility of the type of tissue that can be invaded by the parasite (Pech-Canul et al., 2017). The amount and type of surface molecules vary according to the strain and morphological stage of the parasite and the type of host cell. Either way, adhesion triggers cascades of different signals that stimulate various cellular events. Despite the breadth of adhesion molecules involved, signaling will trigger the same secondary messengers and transcription factors to ensure invasion (De Souza et al., 2010b) (Fig. 4A).

The metacyclic trypomastigote presents a varied number of glycoconjugates (glycoproteins and glycolipids) on its surface, forming a layer called the glycocalyx. Among those glycoconjugates, mucins and glycoinositolphospholipids (GILPs) are the most frequent (Romano et al., 2012).

To support the process of attachment and invasion of host cells, these glycoconjugates and other groups of molecules seem to be important in modulating the immune response of the host (Pech-Canul et al., 2017). For example, the gp85/*trans*-sialidase family contains members of Tc85, gp82, gp35/50, *trans*-sialidases, among other surface glycoproteins. Tc85 molecules can bind to different receptors on the cell surface or extracellular matrix, such as cytokeratin, laminin, and fibronectin (Giordano et al., 1999; Tonelli et al., 2010). While gp82 mediates tyrosine phosphorylation and initiates phospholipase C and IP3 in host cells, resulting in calcium mobilization necessary for parasite invasion (Ramirez et al., 1993; Ruiz et al., 1998; Magdesian et al., 2001b). While gp82, which triggers a dicalcium signaling, is present in most virulent strains such as the G strain, gp35/50 that poorly induces calcium release is more concentrated in less virulent strains such as CL Brenner. *Trans*-sialidases transfer sialic acid from the host cell to the glycoconjugates of the parasite, which provide resistance to the human complement system (Zingales et al., 1987; Tomlinson et al., 1994). Another important group of molecules is proteases, such as oligopeptidase B, a soluble peptidase that indirectly induces the release of calcium during invasion (Burleigh and Andrews, 1995) and Tc80 that hydrolyzes fibronectin and collagen type I and IV present in the extracellular matrix to facilitate the penetration process (Santana et al., 1997; Grellier et al., 2001). In addition, other molecules present on the surface of the parasite, such as gp83 and penetrin, also act as ligands to extracellular matrix elements that promote cell adhesion and penetration (Ortega-Barria and Pereira, 1991; Villalta et al., 1992). The recognition and adhesion phases trigger different signaling pathways, resulting in a dependent and independent lysosome invasion processes and vary according to the type of host cell.

Cell invasion

The invasion process dependent on the presence of lysosomes occurs in non-phagocytic cells (such as epithelial and fibroblast cells) and phagocytic cells (Romano et al., 2012) (Fig. 4A). During the recognition phase, signaling cascades of cyclic adenosine monophosphates (cyclic AMPs) are triggered, inducing a bidirectional increase in calcium concentration in both parasite and host cell cytoplasm. This calcium promotes the reorganization of the cell microtubules causing the migration of lysosomes to the invasion site and the depolymerization of actin filaments at the site of parasite adhesion to facilitate invasion (Low et al., 1992; Rodriguez et al., 1995, 1999; Woolsey and Burleigh, 2004). The fusion of lysosomes with the plasma membrane is a natural mechanism of mammalian cells to repair the damaged membrane. The release of the lysosomal sphingomyelinase induces deformation of the membrane, while lysosomal membranes partially reconstitute the plasma membrane of the host cell (Reddy et al., 2001; Andrews, 2002). Additionally, it was shown that the parasite takes advantage of this membrane repair mechanism to invade the cells (Fernandes et al., 2011).

The invasion processes independent of lysosomes also occur, resulting in endocytosis in nonphagocytic cells and phagocytosis in professional phagocytic cells (De Souza et al., 2010b) (Fig. 4A). In these cases, the parasite recognition triggers kinase proteins such as tyrosine kinase and phosphatidylinositol (PI)-3-kinase in phagocytes or cyclic AMPs and PI-3-kinase in a nonprofessional

phagocytic cell, leading to the accumulation of PI-(3,4,5)-triphosphate in the host cell membrane. The high concentration of PI-(3,4,5)-triphosphate in the cell membrane causes a reorganization of the actin filaments. This reorganization induces the formation of pseudopods (in the case of phagocytosis) or invagination of the cell's plasma membrane (endocytosis) to internalize the parasite (Burleigh and Woolsey, 2002; Burleigh, 2005).

All invasion mechanisms (lysosome mediated invasion, endocytosis, and phagocytosis) culminate in an intracellular endocytic vacuole or parasitophorous vacuole where the parasite will stay obligatory and temporarily (Fig. 4A). This vacuole membrane is composed mainly of the host cell membrane and endoplasmic reticulum (ER) membrane, but the parasite can also modulate its composition. Besides, the parasitophorous vacuole membrane contains β -1 integrin (Hall et al., 1991), glycoconjugates (Barbosa et al., 1992), early endosome markers (EEA1—early endosome antigen 1, Rab 5) (Wilkowsky et al., 2002), secondary endosome protein (Rab 7) (Wilkowsky et al., 2002), autophagic protein (LC3) (Romano et al., 2009), and lysosomal membrane proteins (LAMP1 and LAMP 2) (Wilkowsky et al., 2002). These molecules indicate that the vacuole containing the parasite belongs to the traditional endocytic pathways when intracellular digestion of endocytosed material occurs through lysosomal enzymes. These events suggest that the parasite utilizes the same mechanisms as mammalian cells use for membrane repair to invade and subvert the host cell microbicidal defense to establish the intracellular infection. Moreover, the high capacity of skeletal muscles and cardiomyocytes to repair their damaged membranes could explain the tropism of *T. cruzi* for these cells (Fernandes and Andrews, 2012).

Escape of the parasite to the cytoplasm

Shortly after internalization, the vacuoles containing parasites fuse with lysosomes as they do in the endocytic pathway. However, unlike other intracellular pathogens that prevent lysosome fusion, this event is crucial for establishing *T. cruzi* infection (Andrade and Andrews, 2004). After fusion, lysosomes release enzymes that acidify the intravacuolar environment to digest the parasite, but the parasite uses this low pH to secrete its activated enzymes that help it to escape to the host cytoplasm (Andrews and Whitlow, 1989; Andrews et al., 1990). The combined action of *trans*-sialidase and neuraminidase removes sialic acid from the parasitophorous vacuole membrane, making it more vulnerable. Then, the enzymes Tc-Tox and perforin cause the rupture of pores in the vacuole membrane, which facilitates the outflow of parasites to the host cytoplasm (Andrews and Whitlow, 1989; de Carvalho and De Souza, 1989) (Fig. 4B).

Parasite multiplication and evasion of the host cell

Approximately 24 h after invasion, the parasite reaches the cytoplasmic environment and differentiates into amastigote form due to the low pH (Tomlinson et al., 1995) to initiate its intracellular replicative cycle and establish the infection (Fig. 4C). After approximately 5–6 days of cell infection and many multiplication cycles, the movements of trypomastigotes in the cytoplasm burst the host cell membrane, releasing the trypomastigotes in the extracellular environment from where they can infect new adjacent cells or reach the bloodstream (Osorio et al., 2012) (Fig. 4D).

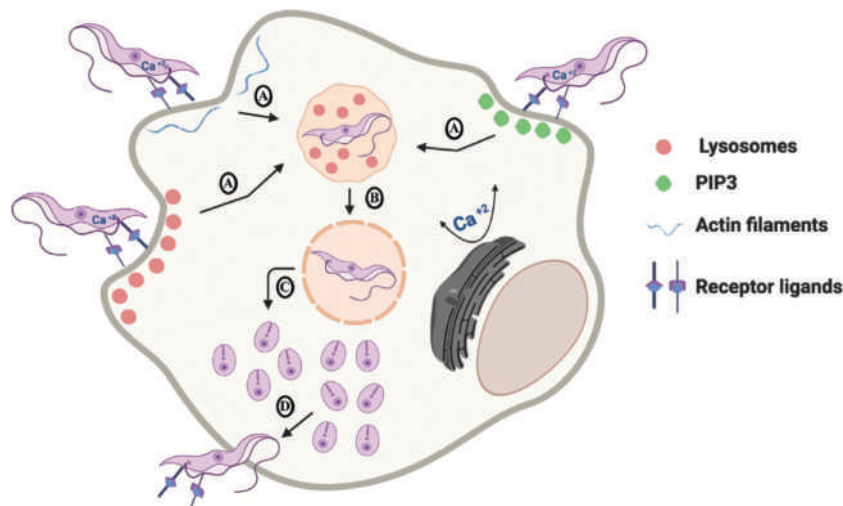


Fig. 4 Metacyclic trypomastigote during the processes of invasion and establishment of intracellular infection. (A) The recognition of surface molecules found in parasite and host cells leads to attachment and triggers signaling pathways to allow parasite invasion. The invasion is mediated by three different processes depending on the cell type, including lysosome or phosphatidylinositol-(3,4,5)-triphosphate accumulation in the plasma membrane of the host cell or the reorganization of actin filaments. All internalization events culminate in an endocytic or parasitophorous vacuole, containing parasite that will fuse with lysosomes to degrade the internalized parasite through lysosomal enzymes. (B) However, the low pH activates parasite enzymes that disturb and rupture the vacuole membrane, allowing the parasite to escape to the host cytoplasm. (C) In the cytoplasm, the metacyclic trypomastigote differentiates into amastigote, the replicative form. (D) After several cycles of multiplication, the amastigotes evolve to trypomastigotes and rupture the cell, infecting new adjacent cells or reaching the bloodstream.

Mechanisms of immune evasion

The long interaction of *T. cruzi* with vertebrate hosts has provided this protozoan a sophisticated mechanism to evade the host immune system (Aufderheide et al., 2004; Lidani et al., 2017). Although both innate and adaptive immune responses are induced against *T. cruzi* infection, this response is not totally effective in the clearance of the parasite, driving the infection towards the chronic phase (Ramírez-Tolosa and Ferreira, 2017; Acevedo et al., 2018).

Trypanosoma cruzi resistance to the complement system

An essential immune evasion strategy used by *T. cruzi* seconds after the infection resides in its capacity to inhibit or disrupt different steps of the activation of complement (the first line immune response in the vertebrate host), avoiding opsonization, immune stimulation, and its lytic effects (Lidani et al., 2017). The complement system is a crucial component of innate immunity comprising more than 50 circulating proteins and cell surface receptors/regulators, which acts in the host defense against infections and recognition and removal of damaged or self-altered components (Ricklin et al., 2010; Andrade et al., 2017). In fact, *T. cruzi* holds a range of molecules that interfere, particularly, in the assembly/decay of C3/C5 convertases and thereby inhibits important complement-mediated processes, such as opsonization, phagocytosis, generation of anaphylatoxins C3a and C5a and lysis, among others. Following, the main *T. cruzi* factors that interfere in complement activation will be covered in more detail.

T. cruzi calreticulin (TcCRT): is a multifunctional ER protein that is translocated to parasite surface, where it mediates infection (Ferreira et al., 2004; González et al., 2015). TcCRT can bind to different pattern recognition molecules, such as C1q, MBL, and ficolin-2, thereby interfering at the initial stage of classical and lectin pathways (Table 2) (Ferreira et al., 2004; Sosoniuk et al., 2014). In addition, C1q on the parasite surface may facilitate *T. cruzi* internalization into mammalian cells contributing to the survival strategy of *T. cruzi* (Rimoldi et al., 1988; Malhotra, 1993).

Trypomastigote decay acceleration factor (T-DAF): is a glycoprotein analogous to human DAF, a membrane protein that prevents complement activation on autologous cells. T-DAF is expressed in the surface of *T. cruzi* trypomastigotes (metacyclic and tissue-culture-derived) but not epimastigotes (Kipnis et al., 1986, 1988; Tambourgi et al., 1993). T-DAF acts as a negative complement regulator (Tambourgi et al., 1993), favoring *T. cruzi* survival in the host.

T. cruzi complement C2 receptor inhibitor trispanning (TcCRIT): a protein expressed by distinct parasite strains that acts as a receptor for C2 inhibiting its cleavage by C1s (Inal and Schifferli, 2002). TcCRIT is expressed in metacyclic trypomastigotes, but not in epimastigotes (Cestari et al., 2008, 2009) and its overexpression in transgenic parasites confers high resistance against complement-mediated lysis (Cestari et al., 2009), contributing to *T. cruzi* survival enabling the infection to progress (Table 2) (Cestari et al., 2008, 2009).

T. cruzi complement regulatory protein (TcCRP): also known as gp160, is a surface-anchored glycoprotein that presents genetic and functional similarities to human DAF (Norris et al., 1991). It is expressed in trypomastigotes (metacyclic and tissue-culture-derived), but not in epimastigotes or intracellular amastigotes (Norris et al., 1989, 1991). TcCRP has the ability to inhibit the assembly of classical and alternative C3 convertase, enabling *T. cruzi* to evade the lytic effect of complement (Table 2) (Norris et al., 1991; Norris and Schimpf, 1994; Norris, 1998). TcCRP expression varies among *T. cruzi* strains, with most virulent strains having higher TcCRP expression levels (Henrique et al., 2016).

T. cruzi complement regulatory gp58/68 (gp58/68): this protein prevents the interaction of factor B with parasite-bound C3b to form the C3 convertase (Table 2) (Fischer et al., 1988). By this means, *T. cruzi* gp58/68 favors evasion from the alternative pathway supporting parasite survival.

In addition to the surface regulatory molecules, *T. cruzi* releases microvesicles during infection as another strategy to circumvent the effect of complement (Ming et al., 1995; Cestari et al., 2012; Ramirez et al., 2017). During *T. cruzi* infection both parasite and host cells may release microvesicles (Cestari et al., 2012; Torrecilhas et al., 2012; Ramirez et al., 2017) that can confer protection against complement attack and enhance *T. cruzi* cell invasion (Cestari et al., 2012; Torrecilhas et al., 2012) (Table 2). Moreover, monocytes and lymphocytes-derived microvesicles may carry TGF- β (Ming et al., 1995) and thereby enhance *T. cruzi* cell invasion (Cestari et al., 2012). In addition, microvesicles released by *T. cruzi* may carry important virulence factors that affect the parasite--host interaction promoting infection (Torrecilhas et al., 2012; Garcia-Silva et al., 2014b, a; Fernandez-Calero et al., 2015).

Trypanosoma cruzi evasion from phagolysosome oxidative response

Macrophages at the site of infection are among the first host cells to be invaded by *T. cruzi*, and to establish an infection, metacyclic trypomastigotes must endure the highly oxidative conditions generated inside the phagolysosome (David Sibley, 2011). The parasite relies on a sophisticated system of antioxidant proteins that acts synergistically to inactivate the macrophage oxidative process, which includes antioxidant enzymes and iron superoxide dismutase (TcSOD) (Table 2) (Wilkinson et al., 2000, 2002a, b; Piacenza et al., 2008, 2013; Mateo et al., 2008). The antioxidant metabolic network used by *T. cruzi* provides resistance against macrophage killing and consequently increases its virulence.

Table 2 *T. cruzi* molecules involved in the immune evasion mechanisms.

Parasite molecule	Expression site	Developmental stage of protein synthesis ^a	Evasion mechanisms	Reference
<i>T. cruzi</i> calreticulin (TcCRT) [also termed TcCalr, Tc45]	Parasite surface	Trypomastigotes (metacyclic)	Binds to MBL and Ficolin-2 collagen-like domain inhibiting the lectin pathway; Binds to C1q collagen-like domain inhibiting the classical pathway	Rimoldi et al., 1988; Malhotra, 1993; Sosoniuk et al., 2014
Trypomastigote decay acceleration factor (T-DAF)	Parasite surface	Trypomastigotes (metacyclic, tissue-culture-derived)	Interferes with factor B binding to C3b on the parasite surface and C4b2a formation inhibiting C3 convertase of both classical and alternative pathways of complement	Kipnis et al., 1988; Rimoldi et al., 1988; Tambourgi et al., 1993
<i>T. cruzi</i> complement C2 receptor inhibitor (TcCRIT)	Parasite surface	Trypomastigotes (metacyclic)	Binds to C2 preventing its cleavage by C1s and MASP-2, avoiding C3 convertase formation and blocking classical and lectin complement pathways	Cestari et al., 2008, 2009
<i>T. cruzi</i> complement regulatory protein (TcCRP) [also termed gp160, CRP-160, CRP-10]	Parasite surface	Trypomastigotes (metacyclic, tissue-culture-derived)	Binds to C3b and C4b inhibiting the classical and alternative C3 convertase formation	Norris et al., 1991; Norris and Schrimpf, 1994; Norris, 1998
<i>T. cruzi</i> complement regulatory gp58/68 (gp58/68)	Parasite surface or released	Trypomastigotes (metacyclic)	Interacts with factor B preventing its binding to parasite-bound C3b inhibiting the formation of C3 convertase of the alternative pathway of complement	Fischer et al., 1988
<i>T. cruzi</i> -induced membrane-derived vesicles from host cells (microvesicles)	Release during parasite infection	Trypomastigote, (metacyclic, tissue-culture-derived) and epimastigote	Binds to the C3 convertase of the <i>classical</i> and <i>lectin</i> pathways (C4b2a) on the parasite surface, inhibiting C3 cleavage	Cestari et al., 2012; Torrecilhas et al., 2012; Ramirez et al., 2017
<i>T. cruzi</i> cytosolic trypanodioxin peroxidase (TcCPX)	Parasite cytosol	Trypomastigotes (metacyclic)	Inactivates peroxynitrites, hydrogen peroxide and short chain organic hydroperoxides in phagolysosome	Wilkinson et al., 2000
<i>T. cruzi</i> mitochondrial trypanodioxin peroxidase (TcMPX)	Parasite mitochondria	Trypomastigotes (metacyclic)	Inactivates peroxynitrites, hydrogen peroxide and short chain organic hydroperoxides in phagolysosome	Piacenza et al., 2008
Glutathione peroxidases I (TcGPXI)	Parasite glycosome	Trypomastigotes (metacyclic)	Inactivates hydroperoxides in phagolysosome	Wilkinson et al., 2002a; Wilkinson et al., 2002b
Glutathione peroxidases II (TcGPXII)	Endoplasmic reticulum	Trypomastigotes (metacyclic)	Inactivates lipid hydroperoxides in phagolysosome	Wilkinson et al., 2002a; Wilkinson et al., 2002b
Ascorbate-dependent heme peroxidase (TcAPX)	endoplasmic reticulum	Trypomastigotes (metacyclic)	Inactivates hydrogen peroxide in phagolysosome	Wilkinson et al., 2002a; Wilkinson et al., 2002b; Piacenza et al., 2008
<i>T. cruzi</i> superoxide dismutase A (TcSODA)	Parasite mitochondria	Trypomastigotes (metacyclic)	Inactivates peroxynitrites and superoxide anion on phagolysosome	Mateo et al., 2008; Piacenza et al., 2013
<i>T. cruzi</i> superoxide dismutase B1 (TcSODB1)	Parasite cytosol	Trypomastigotes (metacyclic)	Inactivates peroxynitrites and superoxide anion on phagolysosome	Mateo et al., 2008; Piacenza et al., 2013
<i>T. cruzi</i> superoxide dismutase B1-2 (TcSODB1-2)	Parasite glycosome	Trypomastigotes (metacyclic)	Inactivates peroxynitrites and superoxide anion on phagolysosome	Mateo et al., 2008; Piacenza et al., 2013
<i>T. cruzi</i> superoxide dismutase C (TcSODC)	Parasite mitochondria	Trypomastigotes (metacyclic)	Inactivates peroxynitrites and superoxide anion in phagolysosome	Mateo et al., 2008; Piacenza et al., 2013
Glycoinositolphospholipids (GIPLs)	Parasite surface	Most abundant in epimastigote and metacyclic trypomastigotes	Immunomodulatory role in host cell response, including inhibition of proinflammatory cytokines and costimulatory immune molecules expression in human DCs and macrophage as well as inhibition of T cells activation	Gomes et al., 1996; De Lederkremer and Bertello, 2001; Brodsky et al., 2002; Previato et al., 2004

(Continued)

Table 2 (Continued)

Parasite molecule	Expression site	Developmental stage of protein synthesis	Evasion mechanisms	Reference
<i>T. cruzi</i> trans-sialidase (TcTS) [GPI-anchored mucins]	Parasite surface and released	Primarily expressed on metacyclic trypomastigote	Transfers sialic acid from host glycoconjugates to <i>T. cruzi</i> surface masking the parasite; Mediates immunomodulation of DCs response; Induces the production of non-neutralizing antibodies by B-cells and non-specific polyclonal B cells activation; Immunosuppressive role by inducing T cells apoptosis and reducing the magnitude of CD8+ T cells response due to immunodominant epitopes	Leguizamón et al., 1999; Buschiazzi et al., 2002; Gao et al., 2002; Pitcovsky et al., 2002; Martin et al., 2006; Erdmann et al., 2009
AgC10 [GPI-anchored mucins]	Parasite surface and released	Metacyclic trypomastigotes, epimastigotes and amastigotes	Immunosuppressive role by inhibiting T cells activation and IL-2 secretion	Alcaide and Fresno, 2004
Mucin-associated surface proteins (MASP) [GPI-anchored mucins]	Parasite surface and released	Primarily expressed on metacyclic trypomastigote	Immunomodulatory role on B cells by inducing the production of spurious and non-neutralizing antibodies	Bartholomeu et al., 2009; dos Santos et al., 2012
<i>T. cruzi</i> glutamate dehydrogenase (TcGDH)	Parasite cytosol	Epimastigotes	Induces B cells mitogenesis irrespective of their specificity	Montes et al., 2006
<i>T. cruzi</i> polyclonal activator of 45 kDa (TcPA45)	Cytoplasmic and membrane-associated protein	Metacyclic trypomastigote and epimastigotes	Induces B cells mitogenesis irrespective of their specificity	Reina-San-Martín et al., 2000
Heat shock protein 70 (HSP70)	Cell membrane, cytoplasmic and kinetoplast	Metacyclic trypomastigotes cultured at 29 °C	Immunosuppressive role by inducing T cells apoptosis	Marañón et al., 2000
Cruzipain (Cz)	Lysosome and parasite surface	Epimastigotes and amastigotes	Immunosuppressive role by reducing the macrophage trypanocide activity	Cazzulo et al., 1997; Stempin et al., 2002

^aEvolutive form associated to the described evasion mechanisms.

Trypanosoma cruzi immunomodulates host cell response

During *T. cruzi* infection, several immunomodulatory molecules are released and expressed by the parasite which interfere in the host responses mediated by dendritic cells (DCs), B and T lymphocytes (B and T cells), allowing parasite survival and spreading in the host (Buscaglia et al., 2006; Rodrigues et al., 2009; DosReis, 2011; Gravina et al., 2013; Acevedo et al., 2018; Ross and Cantrell, 2018).

Some virulence molecules, such as the glycoinositolphospholipids (GIPLs), and Glycosylphosphatidylinositol-anchored mucins (GPI-mucins) which, among others mechanisms inhibit DC maturation, induce the secretion of immunosuppressive cytokines and prevent the expression of proinflammatory molecules, inducing host tolerance to *T. cruzi* (Table 2) (De Lederkremer and Bertello, 2001; Buschiazzi et al., 2002; Previato et al., 2004; Poncini et al., 2008; Erdmann et al., 2009; DosReis, 2011). Factors secreted by the parasite may also play an immunosuppressive role by altering the maturation of human DCs, impairing antigen presentation and host capacity to fight *T. cruzi* infection (Van Overtvelt et al., 1999, 2002; Chaussabel et al., 2003; Poncini et al., 2008). However, these secreted *T. cruzi* factors are still not known.

Other factors belonging to the highly polymorphic multigenic protein family, such as GPI-mucins [*trans*-sialidases (TcTS) and mucin-associated surface proteins (MASPs)] (Table 2) inhibit effective adaptive immunity response by disturbing B cells activities (Pitcovsky et al., 2002; Buscaglia et al., 2006; Bartholomeu et al., 2009; dos Santos et al., 2012). Among other mechanisms, the simultaneous expression broad range of antigenic motifs delays the activation of specific B cells, driving the immune system into a series of spurious and non-neutralizing antibody responses, delaying the production of high-affinity anti-*T. cruzi* antibodies (Pitcovsky et al., 2002; Buscaglia et al., 2006; Bermejo et al., 2011).

Moreover, *T. cruzi* acute infection was associated with the subversion of signals required for T cells activation, such as IL-2 secretion, resulting in anergic T response (Gomes et al., 1996; Alcaide and Fresno, 2004; Ross and Cantrell, 2018). Anergy to T cells response allows parasite replication and spread, contributing to the establishment of chronic infection. It was also proposed that

T. cruzi may induce T cells apoptosis, affecting memory processing, and generation of specific T cells to combat the intruder in the long term infection (Leguizamón et al., 1999; Marañón et al., 2000).

In order to survive in the host and establish a life-long chronic infection, *T. cruzi* can benefit from a phenomenon known as immunodominance (DosReis, 2011; Acevedo et al., 2018). In this context, pathogen-specific T cells appear to preferentially recognize a small number of *T. cruzi* epitopes, preventing efficient pathogen elimination (Rodrigues et al., 2009). Immunodominant antigens can be selected by several factors, including stable MHC-peptide complexes on antigen-presenting cells, the affinity of T-cell receptors, as well as the abundance of parasite epitopes (Yewdell and Bennink, 1999; Goto et al., 2008; Rodrigues et al., 2009). Parasite *trans*-sialidases are among the significant known immunodominant targets for CD8⁺ T cells (Table 2), (Martin et al., 2006). Taken together, *T. cruzi* immunodominant epitopes may impair immunity to a range of other epitopes, reducing the potential of an effective response and eventually favoring parasite persistence in the chronic stage (Martin et al., 2006; Tzelepis et al., 2008; Rodrigues et al., 2009).

Trypanosoma cruzi immune evasion and latency in adipose tissue

Another escape mechanism used by *T. cruzi* to avoid the host immune defense allowing its continued persistence consists in the establishment of latency sites. In this regard, studies have shown that the adipose tissue may be an important target for *T. cruzi* during acute infection and a reservoir in the chronic phase (Combs et al., 2005; Ferreira et al., 2011; Nagajyothi et al., 2012; Morrot et al., 2016). Studies have also demonstrated that *T. cruzi* may induce deep changes in the adipocyte homeostasis, affecting the plasma levels of adipokines (Combs et al., 2005) and allowing the establishment of an inflammatory phenotype, which possibly affects parasite control contributing to disease evolution (Manarin et al., 2013; Morrot et al., 2016; González et al., 2019). The reason why *T. cruzi* persists in the adipose tissue is not completely understood, but it is possibly related to the metabolic conditions that the parasites find inside the adipocyte as well as the extremely slow turnover of these cells (Combs et al., 2005; Nagajyothi et al., 2012). Thus, adipose tissue may be a long-term reservoir for *T. cruzi* where the parasite might persist in a dormant state avoiding host defense, and from which recrudescence may occur, causing disease decades later.

Trypanosoma cruzi and disease

T. cruzi is the causative agent of CD, also known as American trypanosomiasis, that presents itself in acute and chronic phases. CD is characterized by a sophisticated and immune-driven interaction of host and parasite factors, which results in multiple clinical forms of the disease. In this context, the host-parasite relationship is key to understanding the pathophysiology of *T. cruzi* infection and its clinical progression.

Acute and chronic CD phases

The acute phase starts after the contact with *T. cruzi* and is characterized by extensive parasite replication, making parasites easily found in the bloodstream. In this phase, nonspecific symptoms, such as headaches, somnolence, pallor, fever, enlarged lymph glands, muscle pain, difficulty in breathing, swelling, and abdominal or chest pain can be observed (Prata, 2001; Lidani et al., 2019). First visible characteristic clinical signs can be observed in around 50% of the people and consist of either a skin lesion (chagoma) or a purplish swelling of unilateral eyelids (Romaña sign) resulting from *T. cruzi* inoculation (WHO, 2020). Severe CD, including myocarditis, pericardial effusion, and/or meningoencephalitis in acute phase is rare and associated with high mortality risk (Acquatella et al., 2018). Moreover, orally transmitted *T. cruzi* infection has been reported to cause more severe acute morbidity and higher mortality than vector-borne infection, possibly due to alterations in parasite surface glycoproteins caused by exposure to gastric acid which leads to increased parasite infectivity (Covarrubias et al., 2007; Yoshida, 2008).

After the acute phase (4 to 8 weeks after infection), the parasitemia decreases markedly in 90% of the cases and the infection may remain clinically silent for years or throughout life in a chronic asymptomatic (or indeterminate) form (Nunes et al., 2013). The parasite tropism for different tissues varies according to host and parasite factors, but frequently includes heart, stomach, small intestine, gut mesenteric, adipose tissue, and central nervous system (Lewis et al., 2018; Pereira et al., 2019). Over a period of years to decades, around 20 to 30% of patients will present some cardiac disorders and up to 10% will develop digestive (typically enlargement of the esophagus or colon), neurological or mixed alterations (Bern, 2015).

People with asymptomatic form present reactive serology and/or positive parasitological tests for *T. cruzi* but no evidence of organ dysfunction (mainly cardiac or digestive). The evaluation consists of nonspecific signals or symptoms related to CD; and negative tests to detect early organ damage, such as electrocardiogram, echocardiogram, chest X-ray, esophagography and barium enema (Lidani et al., 2019). Even without any evidence of abnormalities, a silent progressive process might be taking place due to the presence of *T. cruzi* in the blood or infected tissues where replication occurs (Sánchez-Valdéz et al., 2018; Alvar et al., 2020). Most infected individuals, about 60–70%, can remain asymptomatic for their entire lives.

During the infection process, the parasites can induce damage directly and by microvascular immune alterations response that leads to an imbalance in the host favoring the disease establishment (Marin-Neto et al., 2007). Consequently, an extensive infiltration of immune cells and the production of proinflammatory cytokines takes place (Bonney and Engman, 2015; Lidani et al., 2017), leading to chronic inflammation and organ damage as seen in patients with symptomatic CD forms. In this scenario,

inflammatory and fibrotic events can overlap, resulting in the cardiac form, which ranges from asymptomatic electrocardiogram abnormalities to congestive heart failure, arrhythmias, and/or thromboembolic events (Biolo et al., 2010). The typical dilated cardiomyopathy observed in most patients with heart failure related to CD consists of chronic diffuse myocarditis with tissue substitution by fibrosis, causing different types and grades of cardiac block and progressive dysfunction (Atié and Steinberg, n.d.; Marin-Neto et al., 2007). CD has also been associated with sudden death due to cardiac arrhythmias or heart failure caused by the selective destruction of the heart muscle and its neurons (Rassi et al., 2001; Dyer, 2018).

In the gastrointestinal tract, chronic infection affects mainly the lower esophageal sphincter and colon, but also salivary glands, stomach, small intestine, gallbladder, and biliary tree (Matsuda et al., 1995). The chronic injuries of the enteric nervous system lead to long-term waves, hypercontraction of muscle fibers, resulting ultimately in hypomotility of the digestive system and dilatation of the organs (Matsuda et al., 1995; De Oliveira et al., 2008; Pinazo et al., 2010). Whereas esophageal manifestations range from asymptomatic motility disorders through mild achalasia to megaesophagus; colonic involvement results in prolonged constipation, fecaloma, volvulus, bowel ischemia, or megacolon (Bern et al., 2020). The concomitant manifestation of digestive syndromes with cardiomyopathy characterizes the cardiodigestive form.

The exact molecular mechanisms underlying the pathogeny of the chronic forms of CD are still unknown. So far, critical elements related to the host genetic factors influencing the development of different clinical forms, as well as parasite polymorphism (DTUs) and tissue-tropism have been described (Villarreal et al., 2004; Nogueira-Paiva et al., 2015). It is conceivable that all these factors could influence the different outcomes of CD. The finding of specific molecular biomarkers for early cardiac and digestive damage that could be useful in defining prognosis would be a significant advance in the clinical management of CD patients.

Laboratory diagnosis

Diagnostic methods for *T. cruzi* encompass parasitological, serological, and molecular approaches. Parasitological methods aim to visualize the presence of fresh-blood parasites by microscopic examination, and their sensitivity varies according to the stage of infection and parasitemia grade (Becerril, 2011). The serological methods search for anti-*T. cruzi* antibodies in patient's serum using parasite antigens in distinct immunoassays (Guhl and Vallejo, 2003). The molecular approach consists in the detection of parasite DNA by PCR-based techniques. Although molecular methods could increment the diagnostic, its implementation in endemic regions remains limited, due to the complexity, lack of standardization, and high cost (Picado et al., 2018). It is also important to note that careful anamnesis considering the patient's history may help to identify possible risks for *T. cruzi* infection.

The main methods of determining *T. cruzi* infection according to the phases or clinical forms of CD are shown in Table 3. In the acute phase, IgM is usually positive and circulating trypomastigotes may be visualized in blood or detected by PCR techniques. In the chronic stage, due to the low parasitemia, conventional parasitological methods such as blood culture and xenodiagnosis are of low sensitivity, being serologic tests more suitable. The standard approach for diagnosing CD recommended by the WHO (WHO, 2002) requires at least two different techniques using distinct antigens, and when the results diverges, then a third assay is needed.

Table 3 Laboratorial diagnostic tests for the detection of *T. cruzi* according to the phase and clinical form of CD.

CD phase	CD form	Clinical characteristics	Laboratorial Diagnosis of infection			
			Blood smear ⁺⁺	Serology		PCR-based
				IgM	IgG	
Acute ⁺	NA	Usually mild, nonspecific symptoms	✓	✓	–	✓
Acute congenital infection ^a	NA	Asymptomatic or mild, nonspecific symptoms	✓	–	✓	✓
Early chronic	Indeterminate	Asymptomatic; ECG usually shows no abnormalities suggestive of CCC	–	✓	✓	✓
Chronic ^b	Indeterminate	Asymptomatic; ECG shows no abnormalities suggestive of CCC	–	✓	✓	✓
	CCC	ECG abnormalities suggestive of CCC, arrhythmias, syncope, left-ventricular dysfunction, heart failure	–	✓	✓	✓
	Digestive	Dysfunction followed by dilatation of esophagus, colon, or both	–	✓	✓	✓
	Cardiodigestive	ECG abnormalities suggestive of CCC, arrhythmias, syncope, left-ventricular dysfunction, heart failure. Dysfunction followed by dilatation of esophagus, colon, or both	–	✓	✓	✓

Note: The sensitivity and specificity of these tests are variable. CCC, chronic Chagas cardiomyopathy; ECG, electrocardiogram; PCR, polymerase chain reaction; IgM, Immunoglobulin M; IgG, Immunoglobulin G.

+ Positive IgM test should be recommended in cases of CD symptoms related to suggestive epidemiological history.

++ Microscopy of blood smear or buffy coat films stained with Giemsa or other appropriate stains.

^aPositive IgG serologic test for 9 months old of age or later.

^bIn cases of suspected central nervous system involvement, cerebral spinal fluid (CSF) specimens could be evaluated.

Modified from Bern C (2015) Chagas' disease. *New England Journal of Medicine* 373: 456–466. <https://doi.org/10.1056/NEJMra1410150>.

The available tests vary considerably in their performances due to the source of antigens employed, genetic diversity of the parasite, host immune responses, and disease prevalence (Umezawa et al., 1999; Leeftang et al., 2013; Balouz et al., 2017; Zingales, 2018; Díaz et al., 2019). Among the available serological tests are enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) (Afonso et al., 2012) recombinant *T. cruzi* antigens; immunoblot (TESA) (Umezawa et al., 1996; Furuchó et al., 2008); indirect immunofluorescence (IFI) (López-Monteón et al., 2019); and indirect hemagglutination (IHA) (López-Monteón et al., 2019). Although serology is generally more appropriate in the chronic CD, a molecular test may be useful in immunosuppressed patients with re-activation of CD. Moreover, not so commonly used, heart or gastrointestinal tissue biopsy can show amastigotes (López-Monteón et al., 2019).

Molecular diagnosis is indicated in cases of suspected acute infection (including transfusion or transplant transmission), congenital CD, and for monitoring accidental laboratory exposures. The molecular detection of *T. cruzi* DNA targets mainly the kinetoplast genome (kDNA), a nuclear mini-satellite (SatDNA) region designated TCZ (Piron et al., 2007; Duffy et al., 2009; Schijman et al., 2011; Cura et al., 2017), and a small ribosomal RNA subunit (18S rRNA) (Schijman et al., 2011). Due to the high genetic variability of *T. cruzi* strains (DTUs) a multi-target PCR test is essential for more accurate results.

Treatment

Benznidazole (N-benzyl-2-nitroimidazole-1-acetamide) and nifurtimox (5-nitrofurantoin derivative) are the only options for effective treatment of *T. cruzi* infection. Benznidazole is a prodrug metabolized by parasite enzymes to become active; metabolites seem to act through several mechanisms to block *T. cruzi* glutathione and trypanothione pathways (Müller Kratz et al., 2018). Nifurtimox impedes *T. cruzi* carbohydrate metabolism through the inhibition of pyruvic acid synthesis (Forsyth et al., 2016; Bern et al., 2020). Both antiparasitic drugs must be administered at the onset of the acute phase, including congenital transmission and reactivation due to immunosuppression. During early chronic phase, in adults, regardless symptoms, some guidelines recommend antiparasitic treatment in order to prevent or cure disease progression and in pregnant women (Dias et al., 2016).

Benznidazole is the first-choice drug in most Latin American countries due to the high toxicity of nifurtimox. Even so, treatment has a suspension rate of around 20% due to poorly tolerated side effects (Jackson et al., 2010; Pinazo et al., 2013). Unfortunately, less than 10% of CD patients in the Americas have been diagnosed and only around 1% of them have access to antiparasitic treatment (DNDI, 2018). Importantly, the potential benefits of medication should be weighed against the duration of treatment (up to 2 months), contraindications (such as pregnant women), and possible adverse reactions which occurs in up to 40% of adult patients (Bern et al., 2007; Torrico et al., 2018). Thus, these drugs have been effective mainly during early infection and their benefits in the chronic CD phase remain questionable.

Important concerns associated with the use of benznidazole and nifurtimox need to be addressed such as the severe collateral effects that lead to treatment interruption; *T. cruzi* resistance to the drugs; effectiveness in the chronic phase; and specific formulations for children and pregnant women are still an emerging need (Menezes et al., 2011; Morillo et al., 2015; Ribeiro et al., 2020). In this context, development of new drugs against *T. cruzi* targeting novel mechanisms of action in order to minimize toxicity and adverse effects have been carried out by several research groups (Bernatchez et al., 2020; Ribeiro et al., 2020). Additionally, specific supportive therapy for cardiac, digestive or neurological manifestations may be required.

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Insects and Mites of Medical and Veterinary Importance: A Broad Overview

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Introduction

Arthropods constitute the animal phylum including the largest number of species on earth (Zhang, 2011), with at least 40,000 species feeding on/in mammals and birds (Balashov, 2006; Di Giovanni et al., 2020). They are of primary importance in medical and veterinary entomology, playing both a direct and indirect role in several of the most widespread human, pet and livestock diseases. Most of the arthropods affecting humans are bloodsucking insects, which are responsible for the transmission of some of the deadliest human and animal pathogens, both in tropical and temperate areas (Benelli and Duggan, 2018) (Fig. 1). Among arachnids, the subclass Acarina is the most important, including hundreds of species of mites and ticks, which act as dangerous pests and/or are vectors of pathogens. More than 700,000 deaths yearly are caused by pathogens transmitted by arthropod vectors (WHO, 2020). Vector-borne diseases (VBDs) include some of the most important human diseases, like malaria, dengue, African trypanosomiasis, onchocerciasis, leishmaniasis, Chagas disease, typhus, plague, tularaemia, Lyme disease, and babesiosis, just to cite some examples (Raoult and Roux, 1999; Becker et al., 2010; Brun et al., 2010; Taylor et al., 2010; Bhatt et al., 2013; Cucunubá et al., 2016; Musso and Gubler, 2016; Petersen et al., 2016; Benelli and Beier, 2017; de Fuentes-Vicente et al., 2018; Kennedy, 2019; WHO, 2019, 2020). Crustaceans are usually less important as human parasites, while they can act as intermediate or paratenic hosts of other parasites, including those that cause widespread diseases such as dracunculosis, diphyllorhynchiasis, sparganosis, and paragomiasis (Cairncross et al., 2002; Hayunga, 2013; Keiser and Utzinger, 2005; Scholz et al., 2009).

Arthropod-borne diseases played a crucial role in shaping human history. Acting as vectors of the bacterium *Yersinia pestis*, the human flea (*Pulex irritans* Linnaeus, 1758) and the human body louse (*Pediculus humanus humanus* Linnaeus, 1758) might have been the cause behind the spreading of recurrent epidemics of plague in Europe, including the Black Death (1346–1353) (Ayyadurai et al., 2010; Dean et al., 2018) (Fig. 1). Louse-borne infectious diseases, such as epidemic typhus, might have been a major factor in the Napoleon's Grand Army retreat from Russia in 1812 (Raoult et al., 2006). Mosquito-borne diseases, such as malaria and yellow fever, mined the construction of the Panama Canal by French companies, causing thousands of deaths amongst workers, costing millions of dollars and eventually forcing French to cease the project in 1889 (Lechuga and Castro, 2017); United States purchased building rights from French and were able to complete the Canal only thanks to a massive vector eradication program (Sutter, 2007).

In many developing countries, VBDs have a significant impact on health, economy and society, preventing the achievement of a sustainable development (Ehrenberg and Ault, 2005; Stolk et al., 2016). Affecting mostly children, VBDs also become a crucial factor in determining a reduction in schooling (Alonso and Alvar, 2010; Hofstra and van Brakel, 2016). Several of them may also cause severe disabilities (like blindness because of onchocerciasis, an infectious disease caused by the filarial nematode *Onchocerca volvulus* Bickel, 1982 transmitted by blackflies) or deformities (like elephantiasis caused by lymphatic filariasis agents transmitted by mosquitoes), which eventually led to social stigmatization of affected people (Molyneux, 2013; Wilke et al., 2020a). In addition, increased transcontinental mobility of men and goods, rapid urbanization and global climate changes led to the range expansion of many vectors and their related pathogens (Wilke et al., 2020b), and to the emergence or re-emergence of several VBDs also in temperate and urbanized areas in which they had never been recorded before, or from areas where they have been eradicated since long time (Barker and Reisen, 2019; Barmak et al., 2016; Becker et al., 2012; Caminade et al., 2018; Colwell et al., 2011; Dantas-Torres, 2015; Massaro et al., 2019; Wilke et al., 2019).

Arthropod parasites of humans

Only relatively few species of arthropods have evolved as specific human parasites, i.e., their development is strictly dependent on humans to complete. Follicle mites belonging to the family Demodicidae and the human lice (Phthiraptera) are ones of the few parasites specific to humans. Among Demodicidae, *Demodex folliculorum* (Simon, 1842) and *Demodex brevis* Akbulatova, 1963 are the only species that live on humans. The two species closely resemble one another but occupy different ecological niches. The first,

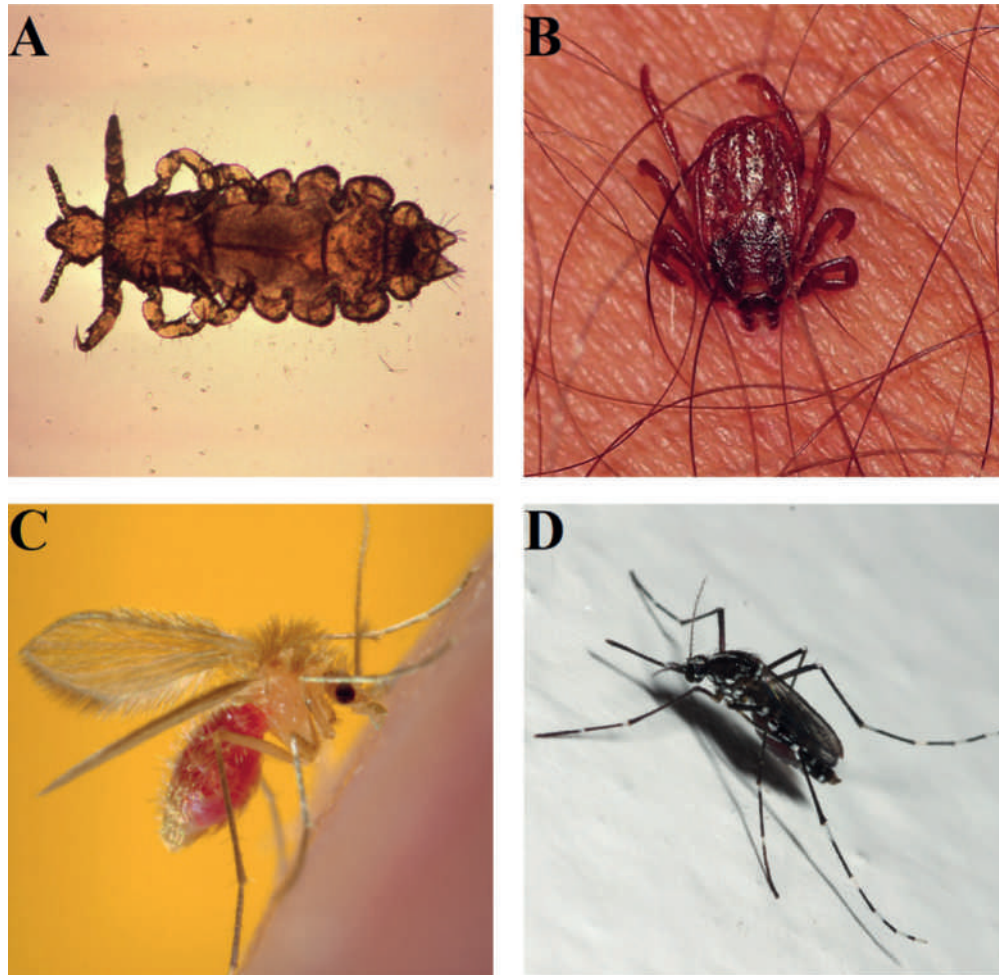


Fig. 1 (A) Female of the head louse, *Pediculus humanus* Linnaeus, 1758 var. *capitis*, a parasite strictly associated with humans. (B) *Ixodes* sp. tick, intent on sucking blood. Ticks are responsible of the transmission of several diseases such as tick-borne encephalitis, Crimean-Congo hemorrhagic fever, Lyme borreliosis, tularaemia, ehrlichiosis, relapsing fever, spotted fevers, Q fever, and babesiosis. (C) The sandfly *Phlebotomus papatasi* (Scopoli, 1786), responsible for the spread of visceral and cutaneous leishmaniasis. (D) Female of the Asian tiger mosquito, *Aedes albopictus* (Skuse, 1894), a potential vector of chikungunya, dengue and Zika among other arboviruses; this highly invasive mosquito species has spread to many countries worldwide through international trading routes. (A) Photo credit D. Juranek, CDC 1979, (B) photo credit L. Lenzini, (C) photo credit F. Collins, CDC 2006, and (D) photo credit L. Barraco.

D. folliculorum, occurs primarily in hair follicles, while the second, *D. brevis*, lives in sebaceous glands. Both species can infest the same host, feeding on host cells by piercing them with their styletiform chelicerae to suck cell contents (Mullen and O'Connor, 2019). They usually cause no major health problems, but their presence has been associated with minor skin issues, like rosacea, seborrheic dermatitis, and blepharitis (Sędzikowska et al., 2018; Wesolowska et al., 2014). Another skin mite that commonly infests humans is the itch mite, *Sarcoptes scabiei* (Linnaeus, 1758) (Sarcoptidae). This species is represented by a complex of physiological varieties, each with different biochemical and ecological characteristics and associated with different mammals, even if monospecificity of these host-specific varieties is still debated (Andriantsoanirina et al., 2015; Mullen and O'Connor, 2019). Sarcoptic mange constitute a significant problem for livestock in many countries (Currier et al., 2011), while the worldwide prevalence of the human-adapted variety has been estimated in more than 100 million cases in 2010 (Hay et al., 2014). *S. scabiei* var. *hominis* usually produces intense pruritus, dermatitis or allergic reactions in humans, due to the mites burrowing into hair follicles and the adult females digging burrows in the lower stratum corneum of the skin. In chronic infestations, the number of mites tends to decrease thanks to the host immune responsiveness. However, in immunosuppressed subjects, mites can multiply causing a crusted scabies (also called Norwegian scabies) and increasing risk for secondary bacterial infections (Hengge et al., 2006; McCarthy et al., 2004; Orion et al., 2006). Despite scabies mite varieties show some degree of host preference, cases have been reported of humans temporary infested with varieties associated with other animals; such infestations typically result in localized papules and temporary itchy rashes, which self-limit within few weeks, as the mite does not burrow nor survive to reproduce (Andriantsoanirina et al., 2015; Arlian et al., 1984; Mullen and O'Connor, 2019).

As for skin mites, human lice, *Pediculus humanus humanus* Linnaeus, 1758, *Pediculus humanus capitis* De Geer, 1767, and *Phthirus pubis* (Linnaeus, 1758) are strictly associated with humans. Different species are specialized on different parts of the human body.

Lice are obliged permanent ectoparasites and transmission occurs by direct contact between individuals or occasionally through contaminated clothing, bedding, and furniture (Orion et al., 2006; Ruffi and Mumcuoglu, 1981). Infestations cause symptoms like itching, erythema, papules, fever, and inflammation (Orion et al., 2006). Differently from skin mites, lice may be responsible of transmitting pathogens; *P. humanus humanus* can be vector of bacteria like *Rickettsia prowazekii* (the etiologic agent of epidemic typhus), *Bartonella quintana* (the agent of trench fever), and *Borrelia recurrentis* (a bacterium associated with relapsing fever) (Brouqui, 2011; Raoult and Roux, 1999; Schaub et al., 2012), and even a role as additional vector of plague, *Y. pestis*, from human to human has been suggested during the Black Death epidemic (Ayyadurai et al., 2010). The vector competence of *P. humanus capitis* remains controversial (Mumcuoglu, 2012; Robinson et al., 2003), whereas *P. pubis* is not known to transmit pathogens (Schaub et al., 2012).

Arthropod micropredators and vector-borne diseases

The great majority of arthropods of public health importance affecting humans are micropredators (Di Giovanni et al., 2020). Differently from parasites in a narrow sense, micropredators are free-living organisms that prey on host blood or other tissues, usually for a limited time and without forming a permanent association with their host (Di Giovanni et al., 2020; Klowden, 2019; Lafferty and Kuris, 2002; Poulin, 2011; Poulin and Randhawa, 2015). Consequently, micropredators usually show a scarce specificity, and differently from “true” parasites, they feed on multiple host individuals per generation, with hosts potentially belonging to one or more species (Di Giovanni et al., 2020; Lafferty and Kuris, 2002; Poulin, 2011). This category includes all the blood-sucking Diptera that attack humans (e.g., mosquitoes, sandflies, biting midges, blackflies, tumbu flies, tsetse flies, louse flies, stable flies), as well as insects belonging to the orders Hemiptera (kissing bugs, bed bugs, and bat bugs) and Siphonaptera (fleas), and arachnids of the subclass Acarina (mites and ticks). Blood is the typical resource of micropredators; vessel-feeders like mosquitoes, kissing bugs, bed bugs, and fleas have modified mouthparts for cannulating host dermal blood vessels (Benelli and Mehlhorn, 2018), while pool-feeders like blackflies, biting midges, horse flies, stable flies, tsetse flies, and sandflies, suck blood through micro-lacerations opened on the host skin (Lane and Crosskey, 1993). The adaptation to hematophagy combined with a general scarce host specificity, makes them perfect candidates as carriers of different pathogens, which can reproduce into the parasite body and be easily conveyed from one host to another by entering directly into the host bloodstream. A peculiar case of blood-sucking insect-mediated transmission of pathogens is represented by kissing bugs of subfamily Triatominae; kissing bugs are vector of *Trypanosoma cruzi*, the agent of Chagas disease or American trypanosomiasis (de Fuentes-Vicente et al., 2018). Instead of being passed to the host directly during the insect blood meal, the pathogen completes the development in the triatomine intestinal tract and it is excreted along with feces and urine, eventually entering the host through the wound caused by the insect bite or other lacerations of the skin (Garcia and Azambuja, 1991).

According to WHO (2020), VBDs account for about 17% of all infectious diseases and cause more than 700,000 deaths annually. Except for schistosomiasis, all the vector-borne diseases listed by WHO are transmitted by arthropods (WHO, 2020). Mosquitoes alone are responsible of transmitting some of the deadliest diseases known to man, such as malaria, yellow fever, dengue, Zika, chikungunya, yellow fever, West Nile fever and lymphatic filariasis (Becker et al., 2010; WHO, 2019, 2020). WHO estimated that 247 million people became infected from mosquito-borne diseases in 2006, with about one million people dying (Becker et al., 2010). In 2018, 228 million cases of malaria were reported and 405,000 deaths occurred worldwide; 93% of the cases were recorded in the African region and 67% of the death affected children under 5 years of age (WHO, 2019).

Some of the arthropod-borne diseases, such as dengue, chikungunya, Chagas disease, African trypanosomiasis, visceral and cutaneous leishmaniasis, lymphatic filariasis, and onchocerciasis, have been included in the WHO list of neglected tropical diseases (NTDs) (WHO, 2012), a group of tropical or subtropical diseases that affect more than one million people in the developing countries. Dengue transmitted by *Aedes* mosquitoes is considered ubiquitous throughout the tropics, with an estimate of 390 million people infected per year, 96 millions of which manifest symptoms (Bhatt et al., 2013). Chagas disease is a major public health problem in Latin America, causing nearly 22,000 deaths yearly, with about 7–8 million infected people estimated in the continent (Cucunubá et al., 2016; de Fuentes-Vicente et al., 2018). Lymphatic filariasis chiefly due to *Wuchereria bancrofti* (Cobbold, 1877) and *Brugia malayi* (Brug, 1927), accounts for 120–130 million cases, with more than 90% in African and South-East Asian regions (Mathew et al., 2020; Taylor et al., 2010), while river blindness caused by *O. volvulus* transmitted by blackflies affects nearly 37 million people in Africa and Central and South America (Taylor et al., 2010). African trypanosomiasis (sleeping sickness) occurs in 36 sub-Saharan countries, with 70 million people at risk (Kennedy, 2019). Zika outbreaks, such as the Polynesian one in 2013–2014 and the South American one in 2015–2016, were responsible for an increase in case of severe neurological complications or congenital malformations in affected countries (Musso and Gubler, 2016; Petersen et al., 2016).

Several micropredators exploit food sources other than blood. For example, mites of the family Trombiculidae primarily feed on lymph and partially digested skin cells, eventually transmitting pathogens, as for the genus *Leptotrombidium* spp. transmitting *Orientia tsutsugamushi*, the causative agent of tsutsugamushi disease (Mullen and OConnor, 2019); species of the genus *Musca* in the tropics swarm around humans and cattle to feed on suppurating sores or body secretions (Crosskey and Lane, 1993); Steganinae drosophilids of genus *Phortica* and *Amiota* feed on lachrymal secretions of animals and humans and can transmit eyeworms of the genus *Thelazia*, which cause lacrimation, ocular discharge, epiphora, conjunctivitis, keratitis, corneal opacity and ulcers (Otranto and Traversa, 2005).

Most micropredators establish a temporary relationship with their hosts, usually limited to the few seconds it takes them to complete the blood meal, but in some cases the time spent on the host can be highly variable. Several species of argasid and ixodid ticks, as well as chigoe fleas, establish a prolonged association with their host, which is no longer regarded only as a food reserve but also as a suitable environment for the development (Di Giovanni et al., 2020). In some cases, the degree of dependence between the micropredator and its host became so strict that the parasite may spend all his life on a single individual host and even require direct contact for transmission to occur efficiently. For example, females of the chigoe fleas *Tunga* burrow into the skin of a single individual host where they remain for the rest of their life. Argasid ticks are a particular group of parasitic arthropods, which evolved different parasitic strategies in terms of time spent on their hosts and time spent actually feeding on their hosts. In general, some species may be considered short-term micropredators (e.g., *Argas cucumerinus* Neumann, 1901), whereas others are long-term micropredators (e.g., *Argas transversus* Banks, 1902, *Otobius* spp., and *Ornithodoros lahorensis* Neumann, 1908). It is also worth mentioning that some larva and nymphs of argasid ticks can moult to the next stage without feeding, whereas adults of some species (e.g., *Antricola* spp. and *Otobius* spp.) do not feed at all.

Arthropods include also species of medical importance as potential mechanical vectors of human diseases. Adults of some of the most common species of calyptate flies are synanthropic, and because of their habit of feeding on human food as well as rubbish and excrement can be involved in the mechanical transmission of pathogenic organisms (Crosskey and Lane, 1993; Gestmann et al., 2012). Pathogens can be transported through hairs and microtrichia of the fly and contaminate food or can be transmitted by the fly to a substrate through defecation or partial regurgitation of the food during a meal (Crosskey and Lane, 1993). More than 100 different pathogens have been isolated from the common house fly *Musca domestica* Linnaeus, 1758, including polio, hepatitis, cholera, tuberculosis, *Salmonella*, *Shigella*, hemolytic streptococcus, or pathogenic *Escherichia coli* (Greenberg, 1971), although they do not necessarily play a significant role in the transmission of these agents from an epidemiological perspective. Flies inhabiting human settlements can potentially convey also several species of fungi, like *Aspergillus* spp. or *Candida* spp., and even helminths (Gestmann et al., 2012; Sulaiman et al., 1988). Although the number of pathogens carried by the flies is often too small to induce infection, pathogens may easily multiply when transported on suitable substrates like food or wounds (Gestmann et al., 2012).

Endoparasites of humans

Maggots of several fly families cause subcutaneous or visceral myiasis, i.e., enter live tissues and organs of vertebrate animals, and feed on host's dead or live tissues, body fluids or ingested food for a certain period of their life (Zumpt, 1965). Myiasis-causing flies may be considered as a particular type of micropredator (Di Giovanni et al., 2020), as they are still temporary parasites and do not need a direct contact between hosts to be transmitted. However, differently from blood-sucking arthropods and other micropredators, they live and feed on the host until the development is completed, and then leave it to pupate in the external environment (Balashov, 2006). No species are restricted to humans, but a few species, like *Dermatobia hominis* (Linnaeus Jr., 1781), are a plague for humans and cause significant economic losses (Sancho, 1988; Hall and Wall, 1995). In obligatory myiasis flies, the parasite depends on the host for part of its life cycle (Francesconi and Lupi, 2012). In relation to the body part attacked by the maggot, several classifications have been proposed (Francesconi and Lupi, 2012; James, 1947; Zumpt, 1965). If the larva is localized in areas such as the eyes or neck, sporadically myiasis evolve in severe complications, such as blindness or even brain damage (Edwards et al., 1984; Kalelioğlu et al., 1989).

Other flies do not need a host to complete their development but their larvae can infect humans as well, causing ailments. Several free-living flies can cause facultative myiasis in humans and other animals, or are unable to initiate a myiasis unless the host is previously infested by other species or the host is almost dead (Francesconi and Lupi, 2012). Eggs or larvae of several dipterans can be accidentally eaten and, passing through the digestive tract unharmed by digestive enzymes may cause accidental enteric myiasis (Hall and Smith, 1993). Rarely, accidental myiasis can result from larvae that enter the rectal or urogenital tract, hatching from eggs laid in the perianal area, like in the case of myiasis caused by the cosmopolitan lesser house fly *Fannia canicularis* (Linnaeus, 1761) and the latrine fly, *Fannia scalaris* (Fabricius, 1794) (Hall and Smith, 1993; Zumpt, 1965).

Maggots of myiasis flies are one of the few endoparasitic arthropods. Another exception is represented by the enigmatic clade of tongue worms (Pentastomida), a group of bizarre crustaceans that reproduce into nasopharyngeal region of carnivores or respiratory tract of large snakes (Mendoza-Roldan et al., 2020; Tappe and Büttner, 2009). The sporadic human cases are related to larvae of two species, *Linguatula serrata* Frölich, 1789 and *Armillifer armillatus* (Wyman, 1845), that cause visceral pentastomiasis when humans accidentally ingest parasite eggs from respiratory secretions or faeces from their natural definitive hosts.

Other arthropods of medical and veterinary importance

The importance of arthropods in human parasitology is also given by the role they play as intermediate or paratenic hosts in the life cycle of other parasites. Copepods are intermediate hosts of the broad fish tapeworm *Dibothriocephalus latus* (Linnaeus, 1758) (= *Diphyllobothrium latum*), the cestod genus *Spirometra* spp., and of the nematodes *Gnathostoma spinigerum* Owen, 1836 and *Dracunculus medinensis* Linnaeus, 1758 (Cairncross et al., 2002; Hayunga, 2013; Herman and Chiodini, 2009; Scholz et al., 2009). In the marine environment, members of the parasites taxa Digenea, Cestoda, Nematoda, and Acanthocephala, are common parasites frequently including pelagic and benthic crustaceans in their life cycle (Busch et al., 2012). First larval instars of the

nematode *Anisakis* spp. and *Pseudoterranova* spp. usually infect euphausiids (“krill”), but also copepods, skeleton shrimps, and Decapoda can act as intermediate hosts (Busch et al., 2012; Kagei et al., 1978; Uspenskaja, 1960). Mites of order Oribatida are intermediate hosts of primate tapeworms of the genera *Bertiella* and *Mesocestoides*, that occasionally affect humans (Mullen and OConnor, 2019).

Several human infections can be acquired through the consumption of raw or undercooked infected crabs or crayfish, which act as intermediate hosts in the cycle of helminths. The paragonimiasis, caused by the lung fluke *Paragonimus westermani* Kerbert, 1878 (Hayunga, 2013; Yokogawa, 1969), is one of the most widespread food-borne disease due to raw crustacean consumption, interesting an estimate of 293,8 million people in Asia, Africa and America (Keiser and Utzinger, 2005; Liu et al., 2008). In some cases, crustaceans act as paratenic hosts, i.e., harbor the sexually immature stages of a parasite even if they are not necessary for the parasite to complete its cycle; this is the case of crayfish hosting the larvae of the Chinese liver fluke *Clonorchis sinensis* Loos, 1907, or the nematode *Angiostrongylus cantonensis* (Chen, 1935), which are responsible of clonorchiasis and angiostrongyliasis in humans, respectively (Dooley and Neafie, 1976; Hayunga, 2013; Wang et al., 2012).

Furthermore, the accidental ingestion of arthropods can be the cause of infestations by several parasites; the intake of non-purified water containing infected copepods is the basis of the life cycle of the guinea-worm, *D. medinensis*. Dracunculiasis is one of the most ancient known human disease, so that it is believed the “fiery serpents” of the Israelites or symbolic serpents in the Greek caduceus are actually guinea-worm representations (Biswas et al., 2013; Hayunga, 2013). In 1986, its annual incidence was estimated in 332 million people, with 120 million people at-risk (Watts, 1987), and it has been dramatically reduced thanks to eradication campaigns that have led to a reduction of cases up to 99% (Ruiz-Tiben and Hopkins, 2006). Accidental ingestion of copepods may be also the cause of infestations by *G. spinigerum* or *Spirometra* spp. (Hayunga, 2013).

More rarely, humans can be infested by parasites after accidentally swallowing insects like ants harboring *Dicrocoelium dendriticum* (Rudolphi, 1819), fleas with *Dipylidium caninum* (Linnaeus, 1758) or *Hymenolepis diminuta* (Rudolphi, 1819), and beetles and cockroaches which may carry the acanthocephalans *Moniliformis moniliformis* (Bremser, 1811) and *Macracanthorhynchus hirudinaceus* (Pallas, 1781) (Hayunga, 2013). Transmission of the apicomplexan *Hepatozoon* spp. to an intermediate vertebrate host may take place by the accidental ingestion of infected ticks (Dantas-Torres et al., 2012; Giannelli et al., 2013).

A wide range of other arthropods are of medical importance, even though they cannot be considered parasites of humans, and do not take part in the life cycles of other human parasites. These species cause direct harm to humans, because of the presence of offense/defence mechanisms, or indirectly, in attending the same places inhabited by man. Most aculeate Hymenoptera and even few parasitic wasps (i.e., Ichneumonoidea) can sting humans for self-defence, causing from a moderate and short (usually characterizing solitary species) to very intense and persistent pain (usually characterizing social species that live in colonies), occasionally leading to systemic reactions (Schmidt, 2009, 2016). The venom of several spiders and scorpions can cause transitory to systemic reactions that range from mild to life-threatening symptoms (Mullen and Sissom, 2019; Mullen and Vetter, 2019). Other arthropods are capable of remarkable and harmful defence mechanisms, as for the explosive exothermic secretions of some carabid beetles (Di Giulio et al., 2015), or produce highly toxic molecules such as pederine and cantharidin (Frank and Kanamitsu, 1987; Iserson and Walton, 2012; Moed et al., 2001). Larvae of many insects are armed with toxic setae or spines, which may cause burning sensation or urticaria on skin by contact or induce allergic reactions if ingested or inhaled (Mullen and Zaspel, 2019). Members of several families of mites can cause allergic responses in humans by direct contact of mites with the skin, inhalation of mites or mite parts, or ingestion of mite-contaminated food (Arlian, 2002; Colloff, 2009). House dust mites or mold mites (e.g., members of the families Pyroglyphidae, Acaridae, Glycyphagidae, and Carpoglyphidae), which commonly live in human dwellings, feeding on stored products or skin detritus, may be responsible for respiratory allergies and asthma (Calderón et al., 2015). Under certain conditions of exposure, mites may even enter natural body orifices, leading to cases of temporary pulmonary, enteric or urinary acariasis (Mullen and OConnor, 2019).

Conclusions and challenges for future research

Overall, arthropods and arthropod-borne diseases have a considerable impact on public health and livestock production both in tropical and temperate areas. Many of those diseases can have tremendous social and economic consequences in countries where large outbreaks occur (Gopalan and Das, 2009; Egdenbewe-Mondzozo et al., 2011; Gubler, 2012). Over the years, WHO (2012) produced evidences that the burden caused by several infectious diseases can be effectively controlled and, in several cases, eliminated or eradicated. The WHO Assembly issued several resolutions on the control of selected diseases. In 2011, a roadmap was drafted for the control, elimination and eradication of NTDs, which affect more than 1 billion people in the world; the resolution considered arthropod-borne diseases such as dengue, Chagas disease, sleeping sickness, leishmaniasis, onchocerciasis, and lymphatic filariasis, and set ambitious targets to be achieved during the period 2012–2020 (WHO, 2012). WHO recommendations include preventive mass-administration of drugs and enhanced access to care on a large scale, intensified disease management and improved case-detection, effective vector control, including the proper management of intermediate hosts or host reservoirs, veterinary public health, and access to safe water, sanitation and hygiene. These goals require a background made by an increase in health awareness and education (WHO, 2012). Despite considerable progresses, many of these diseases are far from being defeated. Global control and eradication programs achieved a clear reduction in number of infected people and in the resulting economic losses (Mathew et al., 2020). The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD, 2017) estimated a reduction in percentage of all-age deaths due to malaria and NTDs by about 30% from 2007 to 2017. Unfortunately, the

application of control measures is often neglected during periods of political instability, especially in tropical areas, thus leading to resurgence (Brun et al., 2010).

In addition, during the last years, several VBDs became an emerging global scale problem, due to increasing in transcontinental migrations, international trade, rapid urbanization, deforestation and climate change (Barker and Reisen, 2019; Barmak et al., 2016; Basile et al., 2011; Becker et al., 2012; Caminade et al., 2018; Connors et al., 2016; Dantas-Torres, 2015; Martins-Melo et al., 2014; Massaro et al., 2019; Parham et al., 2015; Walsh et al., 1993). Changing environmental conditions may also enhance the possibility of arising of novel host-virus interactions, as for recent outbreak of chikungunya in Northern Italy, resulting from the contact between an infected person coming back from tropical areas and a previously introduced vector, the Asian tiger mosquito, *Aedes albopictus* (Skuse, 1894) (Becker et al., 2010; Sambri et al., 2008).

The control and eradication of arthropod vector populations is a crucial and timely challenge (Benelli and Duggan, 2018), which can be won only through a multidisciplinary approach integrating human, animal, and environmental health (Benelli et al., 2016; Benelli and Beier, 2017; Benelli and Duggan, 2018; Di Giovanni et al., 2020; Mackenzie et al., 2013). Past disease management approaches, such as the frequent and massive use of synthetic insecticides to fight vectors, can no longer be sustained. The large use of synthetic insecticides gave rise to the spread of insecticide resistance in vectors, and caused concern regarding potential negative effects on human health and environment (Hemingway et al., 2016; Benelli et al., 2016). New management programs must rely on Integrated Vector Management strategies, more respectful of the environment and people's health, such as the release of natural enemies of arthropod parasites and vectors or of sterile vectors. At the same time, a more in-depth investigation of ethological aspects and population dynamics of parasites and vectors are needed (Benelli et al., 2016; Benelli and Duggan, 2018). To enhance interdisciplinary collaborations and communications amongst all subjects involved in VBD management, new worldwide strategies, like One Health and EcoHealth, have been proposed (Benelli and Duggan, 2018; Bergquist et al., 2017; Mackenzie et al., 2013). In this scenario, the collection of entomological and ecological data, monitoring of vector populations, studies on the biology and ecology of vectors and transmitted pathogens, all require global concerted actions, that involve experts and data from multiple research fields as well as education and active community participation.

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Mosquitoes (Culicidae)

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Introduction

Mosquitoes (Diptera, Nematocera: Culicidae) belong to a monophyletic group containing about 3600 species (Wilkerson et al., 2015) with cosmopolitan distribution, to which belong 44 genera and 145 sub-genera (Wood and Borkent, 1989; Miller et al., 1997; Harbach and Kitching, 1998; Harbach, 2007). It is estimated that they originated in the Jurassic period of the Mesozoic Era, between 200 and 150 million years ago (Reidenbach et al., 2009). They are distributed from the coldest areas, close to the Poles, to the tropical belts and equator, including the desert zones. They are considered absent only in Antarctica and in few islands.

Mosquitoes historically influenced human activities more than any other insect group, not only for their constant nuisance, but more importantly for their role in the transmission of pathogens to humans and animals (Ayala and Coluzzi, 2005; White et al., 2011). The pathogens transmitted by mosquitoes include viruses, protozoa, helminths (Becker et al., 2010) and possibly at least a bacterial species (*Rickettsia felis*), for which mosquito vector competence has been recently demonstrated (Laroche et al., 2018). Despite less than 5% of mosquito species are involved in disease transmission (Harbach, 2007), they are by far the most important cause of morbidity and mortality among humans than any other known animal species, particularly in tropical areas (“WHO Mosquito-borne diseases” www.who.int/neglected_diseases/vector_ecology/mosquito-borne-diseases/en). In recent years, moreover, the increasing global trade and the gradual change of climate patterns favored the spread of invasive species in several countries (Kraemer et al., 2019; Wilke et al., 2019, 2020; Benelli et al., 2020), particularly in temperate areas, posing the basis for new transmission routes of pathogens and unexpected epidemiological scenarios. Despite the huge efforts made in recent years, mosquito control is still a global public health problem far from being resolved.

Generalities on morphological characteristics

Mosquitoes measure about 0.5–1 cm with a slender structure and the typical subdivision of the insect body of in head, thorax and abdomen. As all dipterans, they have one pair of mesothoracic wings, with the second pair modified to halteres, structures that serve to stabilize the flight. A distinctive element of the family is the presence of the proboscis, a piercing-sucking mouth structure suitable to exploit blood and sugar substances of vegetable origin. The only exception is the subfamily Toxorhynchitini, a non-hematophagous group in which the proboscis is sclerotized and unable to pierce host skin, becoming a mouth apparatus specialized in nectar feeding.

The sexes are easily distinguished by the presence of distinctive phenotypic characters; the females have filiform antennae and usually short maxillary palps (except for the subfamily Anophelinae, in which the palps have the same length as the proboscis), while the males have feathery antennae, palps as long as the proboscis (the latter is not structured as a “channel” for sucking) and the external copulatory apparatus (*hypopygium*) structured with claws at the extremity of the abdomen, which serves to hold the female during the copulation. The adult body is covered by thin scales of variable color which, depending on their disposition and abundance, give typical colorations to the species and are useful for the taxonomic determination, together with other less evident characters such as the distribution of bristles and the morphology of the *hypopygium*.

The mosquito larvae are aquatic filter feeders, apod, eucephalic. One of the distinctive elements of the mosquito larvae is the dilated thorax, larger than the abdomen and the distinct head without raptatory antennae (which are instead typical of the family Chaoboridae, the taxonomically closest group). The abdomen ends with a respiratory siphon or spiracles with which mosquito larvae breathe atmospheric air at the water surface. The taxonomic features of the species in the larvae are the antennae, the bristles present on the head and body, a series of bristles and spicules on the VIII abdominal segment and the respiratory siphon, when

present (subfamily Culicinae). The shape and structure of the latter, as well as the anal segment, also have typical structures useful for identification.

Life cycle

Like all holometabolous insects, mosquitoes have a life cycle that allows larvae and adults to exploit completely different ecological niches. Following the laying of eggs (on or near the surface of the water) by a female, which are usually matured thanks to a blood meal, the aquatic and filtrator larva completes four stages of development. Then, it pupates into a mobile pupa from which, once the metamorphosis is completed, a flying adult emerges on the water surface (Fig. 1). Each time interval of the above-mentioned phases changes according to the species, with a large variability. Eggs normally need a few days to complete embryonic development. However, several adaptive strategies involve this stage, particularly in the *Aedes* genus, leading to an interruption of egg maturation until water submerge the eggs oviposited on a potential breeding site or the climatic conditions become suitable for mosquito survival. Larvae can take form a few days up to some weeks to complete their development, while pupae usually complete the metamorphosis within 1–2 days. Once emerged, adults mate as soon as possible, usually with male swarms which attract virgin females with complex behavioral mechanisms including visual markers, circadian time synchronization, flight patterns, pheromones and wingbeat frequency (Clements, 1999; Gibson and Russell, 2006; Gibson et al., 2010; Cator et al., 2009; Manoukis et al., 2009; Fawaz et al., 2014). Females mate once, usually with a single male, preserving the sperms in spermathecae for all life. After copula, the male of several species usually releases a mating plug on the female gonoduct to avoid other copulation events, thus preventing polyandric fertilization. This process alters both female physiology and behavior, inducing refractoriness of the female to mate (Degner and Harrington, 2016). At each gonotrophic cycle, eggs will be fertilized by the sperms before oviposition. Several oviposition events can occur in the female lifetime, producing at each event a variable number of eggs, ranging from few dozens to few hundreds depending on the species. Mosquitoes can survive from a few weeks up to several months, according to the species and survival strategies. Adults are often the phase of the life cycle that overcomes the adverse season (winter or dry season) through an overwintering/aestivation strategy. The diapausing adults tend to hide in sheltered microhabitats with stable climatic conditions, such as sewerage networks, natural cavities, basements, etc., and then reactivate during the favorable season. The last blood meal taken before diapause has the function of overcome hibernation phase through gonotrophic dissociation and development of energetic resources on fat bodies useful to survive during the period of inactivity (Clements, 1992).

Larval ecology

Mosquito larval development is strongly dependent from the nature of the breeding site, both in terms of trophic web and water composition. The breeding sites exploited by mosquito larvae are stagnant water collections that can be of different sizes, from small containers or crevices to lagoons or rice fields (Fig. 1; Becker et al., 2010). Many species of health interest are associated to synanthropic environments, from rural to urbanized areas, developing in small and medium water collections (e.g., *Aedes aegypti*, *Aedes albopictus*, *Culex quinquefasciatus*, *Culex pipiens* and *Culex coronator*, Soghigian et al., 2017; Wilke et al., 2020). The major urban breeding sites used by these mosquitoes are cisterns, purification tanks, roadside drain pipes, ornamental tanks and fountains, used tyres, bins and buckets used to collect rainwater in vegetable gardens, vases in cemeteries and under racking. In rural and agricultural areas, on the other hand, the main breeding sites are the network of canals and ditches used for irrigation. Finally, rice paddies represent a type of environment highly suitable to the development of other species of public health interest, such as several species

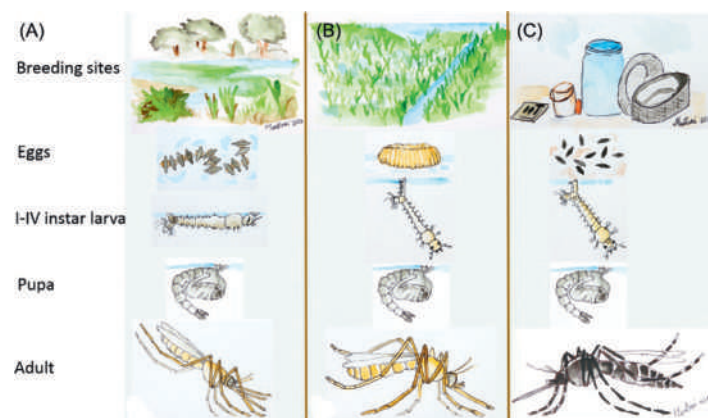


Fig. 1 Life cycle of three representative mosquito genera: (A) *Anopheles* sp., (B) *Culex* sp., (C) *Aedes* sp. The major morphological characteristics of the development phases are shown, as well as the breeding habitats typically exploited by these genera.

belonging to *Culex pipiens* complex and several *Anopheles* species (e.g., *Anopheles maculipennis* complex, *Anopheles gambiae* complex, *Anopheles freeborni*, *Anopheles albimanus*, Smith et al., 1988).

According to species, gravid mosquito females distribute the eggs of a single gonotrophic cycle in several breeding sites (scatter oviposition), as it frequently happens in container-breeding mosquitoes such as those belonging to *Aedes* genus, or they lay the whole egg batch in a single site, as it often happens in *Culex* and *Anopheles* species (Day, 2016). These two strategies are strongly dependent to how larvae are adapted to different water habitats: large stable water collections are often rich in organic matter, with a structured and complex biocenosis in which predators are abundant, while small ephemeral habitats can have lower biodiversity but also fewer nutrients. The rapidity of the larval development, as well as their behavioral strategies for nutrition intake and predator avoidance typical of each mosquito species, affect the oviposition site choice and larval phase completion. The duration of the larval development is also influenced by environmental factors, such as water temperature (higher temperatures reduce the time to complete the larval phase).

An emblematic example of these two opposite strategies is given from the afro-tropical species complex *Anopheles gambiae* sensu lato, to which belong the major malaria vectors in the world (Coluzzi et al., 1985; Toure et al., 1998; Coluzzi et al., 2002). Three of the sibling species of this complex, *Anopheles gambiae* sensu stricto, *Anopheles coluzzii* and *Anopheles arabiensis*, differentially exploit larval habitats ranging from rain-filled temporary water sources (mostly *A. gambiae* s.s. and *A. arabiensis*) up to large permanent man-made water collections (*A. coluzzii*). These species also show a different larval tolerance to salinity and pollution (Gimonneau et al., 2012; Kamdem et al., 2012; Tene Fossog et al., 2013, 2015). The ecological factors involved in the larval habitat segregation of these species are not completely known but a different ability to escape predators or tolerate abiotic stress have been identified (Lehmann and Diabate, 2008; Gimonneau et al., 2012; Roux et al., 2014).

Hematophagy and host-seeking behavior

The trophic regime of mosquitoes varies according to the sex and age of the individual: males feed throughout their lives exclusively on sugary plant substances, obtained from flowers, fruits and honeydew. The females feed on sugary liquids as well (Barredo and De Gennaro, 2020), but after mating they need blood meals to mature their eggs (Clements, 1992). Blood is a nutrient source with high protein content, and as such it can provide the large amounts of amino acids necessary for the development of eggs. The blood meal takes place at different times of the circadian clock, depending on mosquito species. Most species take a single blood meal for egg maturation of a gonotrophic cycle, while some species need multiple meals. The female can uptake from 3 to 6 μL of blood in a single meal, requiring from 1 to 3 min for its completion (Becker et al., 2010). To complete the blood meal, mosquitoes, as all hematophagous arthropods, release saliva into the host before and during the blood intake process. The biological purpose of saliva is to allow the blood intake, minimizing the time of feeding and avoiding the reaction by the host. Salivary secretions have specific biochemical properties and contain dozens of secretory proteins, whose functions are still only partially known, in particular anticoagulants, vasodilators, platelet aggregation inhibitors and immunomodulators (Ribeiro and Arcà, 2009). These proteins have the function of keeping the blood fluid during sucking and preventing the formation of clots that could occlude the mouthparts. Besides, they promote meal intake by limiting the inflammatory response that may increase the host's perception of mosquito presence. Already during the probing phase preceding blood intake, mosquitoes release their saliva superficially into the host skin to prepare the environment for subsequent perforation by the mouthparts. Mosquitoes have a piercing-sucking apparatus that during blood intake is inserted through the epidermis to reach the dermis. The perforation of the skin is done until a blood capillary is reached. At this point, the mosquito introduces into the wound all the fascicle, except for the sheath (labium), which is folded and used as a guide to facilitate the sliding of the stilets (labrum, hypopharynx, maxillae and mandibles). The sharp maxillae represent the main penetrative element, while labrum and hypopharynx delimit respectively the salivary channel (from which saliva is introduced) and alimentary channel (from which blood is taken) separated from each other by the mandibular stylets.

Mosquitoes are temporary ectoparasites that are associated with the host only for blood meal intake and in response to specific physiological signals. Long-distance localization of the host is ensured by sophisticated neurosensory systems, adapted according to biting behavior, that can occur during daytime or the night (Fig. 2). Specific receptors (thermal, olfactory, chemo- and mechanoreceptors) located on the antennae and/or the palps, facilitate the recognition of these different stimuli and orientate the mosquito towards the host, favoring the choice of the optimal site of the body where to take the blood meal (Takken, 1991; Dekker and Carde, 2011; Montell and Zwiebel, 2016). In diurnal mosquitoes (mainly *Aedes* but it has been observed also in night-biting *Anopheles coluzzii*) a combination of visual and olfactory stimuli play an important role (Kennedy, 1940; Van Breugel et al., 2015; Hawkes and Gibson, 2016). In nocturnal mosquitoes (e.g., *Anopheles* and *Culex*), which feed when the ambient temperature is low, an important stimulus can be the orientation towards heat sources, in addition to the ability to perceive water vapor, carbon dioxide produced by breathing and other volatile organic components (such as lactic acid, octenol, acetone) emanated by tetrapods through breathing and muscle activity and present in sweat and sebum. To these are added substances produced by the bacterial microflora of the skin, whose catabolites are an integral part of the complex odor blend characteristic of a specific host (Wooding et al., 2020). The long-distance identification of the host by the mosquito female is done flying upwind following a source of carbon dioxide emitted by the host (Rebollar Téllez, 2005; Takken and Verhulst, 2013). Once near the potential host, other substances that direct the mosquito to the point of biting, such as water vapor, organic compounds and body temperature, come into play (Fig. 2). Some species of mosquitoes feed on a wide variety of hosts, while others feed exclusively on certain species (Lehane, 2005; McBride et al., 2014). Differences in host preference exist not only among different species but also among populations of the same mosquito species and

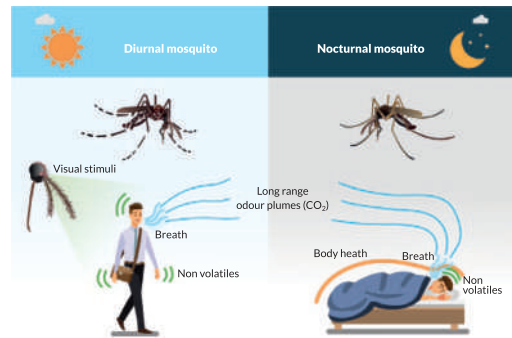


Fig. 2 Schematic diagram describing the different stimuli followed by a diurnal mosquito (e.g., *Aedes* sp.), on the left, and a nocturnal mosquito (e.g., *Culex* sp.), on the right.

even within the same population (Lefèvre et al., 2009; Vantaux et al., 2014; Takken and Verhulst, 2017; Busula et al., 2017). Host choice is also influenced by factors such as abundance and availability of hosts as well as mosquito genetic background (Takken and Verhulst, 2013).

Vector role

As already mentioned, mosquitoes play a primary role as vectors of pathogens for human and animal diseases, in particular protozoa of the genus *Plasmodium*, aetiological agents of malaria, and several arboviruses (arthropod-borne viruses) such as West Nile, dengue, chikungunya and Zika viruses (Table 1).

The capacity of a mosquito to be a good vector is a combination of factors: physiological, behavioral and ecological. The interplay among the biological characteristics of vector, host and pathogen defines the prerequisite for a successful transmission cycle of a vector-borne disease. After this, aspects related to the capacity of a mosquito to successfully enter in contact with a specific host, its density, survival and time needed to be infectant for the pathogen are the parameters that transform in a specific context a biologically competent vector in a good vector (Kramer and Ebel, 2003). These conditions change according to the ecology and climate of different habitats in which the mosquito populations are present.

A paradigmatic example is malaria, a parasitic disease caused by protozoan Apicomplexa belonging to genus *Plasmodium* affecting many vertebrate species. Human malaria is caused by five species, four of which are anthroponotic (*P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale*) and one zoonotic (*P. knowlesi*). Despite the enormous efforts in tackling this disease, in the world 228 million cases and 405,000 deaths still occur each year, particularly in sub-Saharan Africa (World Health Organization, 2019a). The human *Plasmodium* parasites are successfully transmitted by only 15% of *Anopheles* mosquito species among the about 460 species described in this genus (Sinka et al., 2012) even if the proportion of competent vectors is much larger. In the most important vectorial system, the *A. gambiae* complex, only some of them are major vectors (*A. coluzzii*, *A. gambiae* s.s. and *A. arabiensis*) due to their large synanthropy, distribution and high anthropophily (Davidson, 1964; White, 1974; Coetzee et al., 2000; Coluzzi et al., 2002). The other species of the complex may have a local role in malaria transmission due to their specific ecological requirements (e.g., *A. merus*, *A. melas*) or even they do not have any epidemiological role (e.g., *A. quadriannulatus*) because of their marked zoophily and a lower degree of competence to the deadliest pathogen *Plasmodium falciparum* (Takken et al., 1999; Habtewold et al., 2008).

Also, mosquito-borne virus threats, which were originally restricted to specific regions, have globally increased in the past decades, with a recent burst in epidemiology and geographic distribution worldwide (Kraemer et al., 2015; Wilder-Smith et al., 2017). The causes of this expansion are multifactorial and strongly associated to the conditions favoring the increasing distribution of the major vectors *A. aegypti* and *A. albopictus*: global trade, uncontrolled urbanization, increase of human population density and movement, inefficient vector control programs (Gubler, 2011; Roiz et al., 2018). Among all the mosquito-borne viruses menacing human health, dengue, yellow fever, chikungunya, Zika and West Nile are the major ones ("WHO Mosquito-borne diseases" www.who.int/neglected_diseases/vector_ecology/mosquito-borne-diseases/en).

Dengue virus (DENV) is nowadays the most common arbovirus, with 390 million cases per year with more than 4000 deaths occurring mostly in central and south America, Indian peninsula and south-east Asia (Bhatt et al., 2013).

Contrarily to DENV, for which the only available vaccine is partially protective (Villar et al., 2015), Yellow fever (YFV) is a virus that has a highly protective vaccine but its availability is limited (Barrett, 2016). This will be the major issue for the control of this virus that is endemic in 33 countries in Africa and 11 countries in South America. YFV has recently demonstrated its capacity to quickly become a resurgent arbovirus, as shown by the outbreak occurred in Angola in 2016 that suddenly requested more than 28 million doses of vaccines, exhausting the existing global vaccine supply (Monath et al., 2016; World Health Organization, 2018).

Table 1 List of major mosquito-borne virus diseases and their major vectors.

Disease	Pathogen(s)	Major mosquito vector(s)	Major reservoir species	Other vertebrate hosts	Geographic distribution	References
Dengue	DENV	<i>Aedes aegypti</i> , <i>Aedes albopictus</i> , <i>Aedes furcifer-taylori</i> , <i>Aedes luteocephalus</i> , <i>Aedes mediovittatus</i> , <i>Aedes niveus s.l.</i> , <i>Aedes opok</i> , <i>Aedes polynesiensis</i> , <i>Aedes scutellaris</i> , <i>Aedes vittatus</i>	Apes, monkeys, humans	Unknown	Africa, South Asia, Central and South America, Oceania	Vasilakis and Weaver (2008)
Zika virus disease	ZIKV	<i>Aedes aegypti</i> , <i>Aedes africanus</i> , <i>Aedes albopictus</i> , <i>Aedes dalzielii</i> , <i>Aedes furcifer-taylori</i> , <i>Aedes hensilli</i> , <i>Aedes luteocephalus</i> , <i>Aedes polynesiensis</i> , <i>Aedes vexans</i> , <i>Aedes vittatus</i> , <i>Culex coronator</i> , <i>Culex quinquefasciatus</i> , <i>Culex tarsalis</i>	Apes, monkeys, humans	Mammals, birds, reptiles, amphibians	Africa, South Asia, Central and South America, Oceania	Gutiérrez-Bugallo et al. (2019) and Sharma et al. (2020)
Yellow fever	YFV	<i>Aedes aegypti</i> , <i>Aedes africanus</i> , <i>Aedes furcifer-taylori</i> , <i>Aedes simpsonii</i> , <i>Haemagogus janthinomys</i> , <i>Haemagogus leucocelaenus</i> , <i>Haemagogus spegazzinii</i> , <i>Sabethes chloropterus</i>	Apes, monkeys	Humans	Africa, Central and South America	Hubálek et al. (2014) and Childs et al. (2019)
Chikungunya infection	CHIKV	<i>Aedes albopictus</i> , <i>Aedes aegypti</i> , <i>Aedes vexans</i>	Apes, monkeys, humans	Squirrels, bushbabies, Bats, rats, Buffalos, elephants	South America, Africa South and East Asia	Guo et al. (2015), Weaver et al. (2018), Mascarenhas et al. (2018)
West Nile infection	WNV, WNVkun	<i>Culex annulirostris</i> , <i>Culex australicus</i> , <i>Culex globocoxitus</i> , <i>Culex modestus</i> , <i>Culex pipiens s. l.</i> , <i>Culex salinarius</i> , <i>Culex tarsalis</i> , <i>Culex univittatus</i> , <i>Aedes albopictus</i> , <i>Aedes annulipes</i> , <i>Aedes cantans</i> , <i>Aedes caspius</i> , <i>Aedes cinereus</i> , <i>Aedes dorsalis</i> , <i>Aedes excrucians</i> , <i>Aedes flavescens</i> , <i>Aedes japonicus</i> , <i>Aedes rossicus</i> , <i>Aedes sticticus</i> , <i>Aedes vexans</i> , <i>Anopheles hyrcanus</i> , <i>Anopheles maculipennis s.l.</i> , <i>Coquillettidia richiardii</i> , <i>Culex territans</i> , <i>Culex theileri</i> , <i>Culex torrentium</i> , <i>Culiseta morsitans</i> , <i>Uranotaenia unguiculata</i>	Birds and rodents	Humans, horses, rabbits, cats, dogs, camelids, amphibians	Cosmopolitan	Prow (2013), Hubálek et al. (2014) and Ciota (2017)
Rift valley fever	RVF	<i>Aedes vexans</i> , <i>Aedes mcintoshi</i> , <i>Culex theileri</i> , <i>Aedes caballus</i> , <i>Aedes circumluteolus</i> , <i>Aedes dentatus</i> , <i>Aedes lineatopennis</i> , <i>Aedes palpalis</i> , <i>Aedes tarsalis</i> , <i>Culex antennatus</i> , <i>Culex pipiens</i> , <i>Culex poicilipes</i> , <i>Culex tritaeniorhynchus</i> , <i>Eretmapodites chrysogaster</i>	Wild and domestic ruminants, rats, bats	Humans, dogs, cats, monkeys	Africa, Yemen, Saudi Arabia	Hubálek et al. (2014) and Linthicum et al. (2016)
Japanese encephalitis	JEV	<i>Culex gelidus</i> , <i>Culex tritaeniorhynchus</i> , <i>Culex vishnui</i> , <i>Culex annulirostris</i> , <i>Culex fuscocephala</i>	Ardeid birds, pigs	Humans, bats, fruit bats, cattle, dogs, goats, rodents	South and East Asia	van den Hurk et al. (2009), Hubálek et al. (2014) and Sudeep (2014)
Usutu infection	USUV	<i>Culex modestus</i> , <i>Culex perexiguus</i> , <i>Culex perfuscus</i> , <i>Culex pipiens</i> , <i>Culex univittatus</i> , <i>Aedes albopictus</i> , <i>Aedes japonicus</i> , <i>Aedes rossicus</i> , <i>Aedes vexans</i> , <i>Coquillettidia aurites</i> , <i>Culex hortensis</i> , <i>Culex territans</i> , <i>Culiseta annulata</i> , <i>Mansonia africana</i>	Birds	Humans, horses, dogs, bats, reed deer	Africa, Europe	Hubálek et al. (2014), Gaibani and Rossini (2017) and Vilibic-Cavlek et al. (2019)

(Continued)

Table 1 (Continued)

<i>Disease</i>	<i>Pathogen(s)</i>	<i>Major mosquito vector(s)</i>	<i>Major reservoir species</i>	<i>Other vertebrate hosts</i>	<i>Geographic distribution</i>	<i>References</i>
Western Equine Encephalitis	WEEV	<i>Culex tarsalis</i> , <i>Aedes melanimon</i> , <i>Aedes vexans</i> , <i>Culiseta inornata</i>	Passerine birds, squirrels, jackrabbits	Humans, equines, snakes, frogs	North, Central and South America	Hubálek et al. (2014)
Sindbis fever	SINV	<i>Culex univittatus</i> , <i>Aedes cinereus</i> , <i>Aedes communis</i> , <i>Aedes excrucians</i> , <i>Anopheles hyrcanus</i> , <i>Culex modestus</i> , <i>Culex pipiens</i> , <i>Culex theileri</i> , <i>Culex torrentium</i> , <i>Coquillettidia richiardii</i> , <i>Culiseta morsitans</i> , <i>Mansonia africana</i>	Passeriform birds	Humans, rodents, bats, amphibians	Europe, Asia, Africa, Australia, New Zealand	Hubálek (2008), Hubálek et al. (2014), Hesson et al. (2015) and Calzolari (2016)
Eastern Equine Encephalitis	EEEV	<i>Culiseta melanura</i> , <i>Aedes sollicitans</i> , <i>Aedes taeniorhynchus</i> , <i>Culex erraticus</i> , <i>Culex pedroi</i> , <i>Culex taeniopus</i> , <i>Coquillettidia perturbans</i> , <i>Uranotaenia sapphirina</i>	Passerine birds	Humans, equines, small mammals, reptiles, amphibians	North, Central and South America	Hubálek et al. (2014), Molaei et al. (2016) and Morens et al. (2019)
La Crosse encephalitis	LACV	<i>Aedes triseriatus</i> , <i>Aedes albopictus</i> , <i>Aedes canadensis</i> , <i>Aedes communis</i> , <i>Aedes dorsalis</i> , <i>Aedes japonicus</i> , <i>Aedes informatus</i> , <i>Culex restuans</i> , <i>Orthopodomyia signifera</i>	Chipmunks, squirrels, lagomorphs	Humans, red foxes, woodchucks, raccoons, horses, cows, pigs, dogs	North America	Hubálek et al. (2014) and Harding et al. (2018)
St. Louis encephalitis	SLEV	<i>Culex nigripalpus</i> , <i>Culex pipiens</i> , <i>Culex tarsalis</i> , <i>Aedes dorsalis</i> , <i>Aedes vexans</i> , <i>Culex quinquefasciatus</i>	Passeriform and columbiform birds	Humans, bats, rodents	North, Central and South America	Tsai et al. (1988), Kopp et al. (2013) and Diaz et al. (2018)
Murray Valley encephalitis	MVEV	<i>Culex annulirostris</i>	Ardeid birds	Humans, horses, monkeys, kangaroos, rabbits	Australia, Papua New Guinea	Hubálek et al. (2014) and Mackenzie et al. (2017)
Ross river infection	RRV	<i>Culex annulirostris</i> , <i>Aedes camptorhynchus</i> , <i>Aedes notoscriptus</i> , <i>Aedes vigilax</i>	Kangaroos, wallabies	Humans, possums, horses	Australia	Mackenzie et al. (2017)
Barmah forest infection	BFV	<i>Culex annulirostris</i> , <i>Aedes camptorhynchus</i> , <i>Aedes notoscriptus</i>	Unknown	Humans, possums, horses	Australia	Jacups et al. (2008)
Jamestown Canyon infection	JCV	<i>Aedes abstratus</i> , <i>Aedes canadensis</i> , <i>Aedes cantator</i> , <i>Anopheles punctipennis</i> , <i>Coquillettidia perturbans</i> , <i>Aedes cinereus</i> , <i>Aedes dorsalis</i> , <i>Aedes sticticus</i> , <i>Aedes vexans</i>	Wild ungulates	Horses and cattle	North America	Andreadis et al. (2008) and Fulhorst et al. (1996)
Turlock orthobunyavirus (Lednice virus) infection	TURV (LEDV)	<i>Culex modestus</i>	Migratory birds	Unknown	Eastern Europe	Napp et al. (2018) and Berčić et al. (2019)
California encephalitis and Valtice fever	CEV, SSHV, TAHV	<i>Aedes cataphylla</i> , <i>Aedes cinereus</i> , <i>Aedes communis</i> , <i>Aedes punctor</i> , <i>Aedes vexans</i> , <i>Culiseta impatiens</i> , <i>Culiseta inornata</i> , <i>Aedes cantans</i> , <i>Aedes caspius</i> , <i>Aedes detritus</i> , <i>Aedes dorsalis</i> , <i>Aedes excrucians</i> , <i>Aedes flavescens</i> , <i>Aedes hexodontus</i> , <i>Aedes hyrcanus</i> , <i>Aedes intrudens</i> , <i>Aedes sticticus</i> , <i>Anopheles maculipennis</i> s.l., <i>Coquillettidia richiardii</i> , <i>Culex modestus</i> , <i>Culex pipiens</i> , <i>Culiseta annulata</i>	Lagomorphs, hedgehogs, rodents, wild ungulates	Humans, horses, gulls, terns, bald-coots	Eastern Europe, Russia, China, Iraq, West Africa, United States	Hubálek (2008), Hubálek et al. (2010), Hubálek et al. (2014), and Napp et al. (2018)

Table 1 (Continued)

Disease	Pathogen(s)	Major mosquito vector(s)	Major reservoir species	Other vertebrate hosts	Geographic distribution	References
Inkoo virus infection	INKV	<i>Aedes communis</i>, <i>Aedes punctor</i>, <i>Aedes hexodontus</i>	Hares	Humans	Northern Europe	Hubalek (2008)
Bunyamwera orthobunyavirus (Batai virus) infection	BUNV	<i>Anopheles claviger s.l.</i>, <i>Anopheles messeae s.l.</i>, <i>Coquillettidia richiardii</i>, <i>Aedes communis</i>, <i>Aedes punctor</i>, <i>Aedes vexans</i>, <i>Culex pipiens</i>, <i>Culiseta annulata</i>	Pigs, horses, ruminants	Humans, birds	Eastern Europe, Asia, Africa	Lvov et al. (2004), Hubalek (2008), Calzolari et al. (2016), and Napp et al. (2018)

In “Major mosquito vector(s)” column the primary vector species are highlighted in bold.

Chikungunya virus (CHIKV) spread out from its original African distribution in 2004, causing epidemics in several islands of Indian Ocean, India, Southeast Asia, China, reaching Europe in 2007 and finally Caribbean and Latin America in 2013–14 (Rezza et al., 2007; Kariuki et al., 2008; Grandadam et al., 2011; Khan et al., 2014; Weaver and Lecuit, 2015; Riccardo et al., 2019).

Zika virus (ZIKV) is the most recent example of global spreading: isolated for the first time about 70 years ago in Uganda on a *Rhesus* monkey and subsequently on *Aedes africanus* mosquito (Dick et al., 1952; Macnamara, 1954). The occurrence of the virus was limited in African continent until 2007, when an outbreak was recorded in the Federated States of Micronesia (Hayes, 2009). In 2013–15 the virus reached Brazil, subsequently spreading throughout central and south America (Musso et al., 2014; Brito and Cordeiro, 2016; Brasil et al., 2016; Baud et al., 2017).

All the above-mentioned viruses are transmitted in urban or periurban areas mainly by *A. aegypti* and *A. albopictus*, while in sylvatic conditions are transmitted in zoonotic cycles involving non-human primates and forest-associated *Aedes* mosquitoes (Kramer and Ebel, 2003; Hanley et al., 2013; Benelli and Romano, 2017; Braack et al., 2018).

The last example, West Nile virus (WNV), has a life cycle different from the above-mentioned arboviruses. WNV is a cosmopolitan arbovirus, isolated for the first time in Uganda in 1937 (Smithburn et al., 1940), which from early 1990s rapidly increased its distribution in the Europe and Asia (Hayes, 2001) and subsequently throughout North and South America (Lanciotti et al., 1999; Ebel et al., 2001; Hayes et al., 2005). WNV is maintained in nature by ornithophilic mosquitoes, mostly belonging to *Culex* genus, and wild birds (Hubalek, 2008; Rizzoli et al., 2015). Only in North America, about 60 and more than 280 mosquito and bird species, respectively, have been found infected from the virus (Hayes et al., 2005; Kain and Bolker, 2019). Other than birds and mosquitoes, in which the natural cycle of WNV occurs, it can infect a broad range of vertebrates (including amphibians, reptiles and mammals), showing to be a virus ecologically more generalist than other arboviruses (Kramer et al., 2008). This occurs when, during late summer, the avian preferred hosts of vector mosquitoes become less abundant. Then, ornithophilic mosquitoes shift their feeding behavior toward other species, mammals in particular, as observed for *C. pipiens* in the United States (Kilpatrick et al., 2006). However, despite the virus is particularly pathogenic for horses and humans, these are “dead-end hosts” for WNV, as the viraemia does not reach levels sufficiently high to infect mosquito vectors (Pérez-Ramírez et al., 2017).

Dispersal and invasiveness

The adult mosquitoes move around the environment for different needs, trophic and related to the resting and oviposition sites, with marked differences among species. Some mosquitoes have a very narrow range of action, tens or hundreds of meters, while others can move even over distances of kilometers (Service, 1997; Clements, 1999). This intrinsic characteristic is then modulated by extrinsic factors, both ecological and climatic, such as distribution patterns of vegetation and potential hosts, infective status, seasonal conditions affecting temperature, rainfall, etc. (Reisen et al., 2003; LaPointe, 2008; Gu and Novak, 2009; Hemme et al., 2010; Newman et al., 2016). Complementarily to the above mentioned active dispersal, some mosquito species can successfully exploit (intentionally or not) passive means of dispersal, essential for long-range distributions. Increased global trade, transports and mobility of people, together with concomitant climate and environmental change, have increased the chances of mosquito species to spread and adapt to new areas (Laird, 1984; Daszak et al., 2000; Lounibos, 2002; Juliano and Lounibos, 2005; Jones et al., 2008). According to Juliano and Lounibos (2005), “invasive” mosquitoes are species introduced to new environments that have increased and spread, creating the potential for impacts on native species and ecosystems or on human activities (agriculture, conservation). These mosquitoes have a large ecological niche breadth, which allows them to quickly adapt to a wide range of new environments, from natural to highly anthropized habitats. They may expand into new geographic contexts by filling an empty or unsaturated habitat or exploiting a previously unused resource (Williamson, 1996).

Their major ecological characteristics are: a wide range of temperature tolerance, diapausing strategies, often through drought-resistant eggs, allowing the exploitation human transportation channels (Hawley et al., 1989; Lounibos, 2002; Benedict et al., 2007; Eritja et al., 2017; Maynard et al., 2017; Sherpa et al., 2019; Wilke et al., 2020), and the capacity to breed in small, artificial water habitats (Vezzani, 2007). These features are common in invasive mosquitoes, despite not essentially present all together (Juliano

Table 2 List of invasive species that successfully established in countries out from their geographic distribution of origin.

Species (origin)	Invaded/colonized region	Macrohabitat preference	Larval habitats	Desiccation-resistant eggs	Diapause	References
<i>Aedes aegypti</i> (Africa)	Cosmotropical	Urban, domestic	Man-made containers	Yes	None	Kraemer et al. (2015)
<i>Aedes albopictus</i> (temperate and tropical Asia)	Americas, Europe, Africa, Oceania	Urban, suburban	Phytotelmata, man-made containers	Yes	Egg	Kraemer et al. (2015)
<i>Aedes atropalpus</i> (N. America)	Europe	Riparian	Rock pools, man-made containers	Yes	Egg	Scholte et al. (2009)
<i>Aedes j. japonicus</i> (temperate Asia)	North America, Europe	Rural, sylvan	Rock pools, man-made containers	Yes	Egg	Kaufman and Fonseca (2014)
<i>Aedes notoscriptus</i> (Australia)	New Zealand, United States	Urban, suburban, rural	Tree holes, rock pools	Yes	Egg, larva	Peterson and Campbell (2015)
<i>Aedes koreicus</i> (temperate Asia)	Europe	Suburban, rural	Man-made containers	Yes	Egg	Medlock et al. (2015)
<i>Aedes togoi</i> (temperate Asia)	North America	Urban, suburban	Coastal rock pools, artificial containers	Yes	Egg, larva	Belton (1980)
<i>Culex pipiens</i> (Old World)	North America	Urban, domestic, suburban	Man-made containers, subterranean, small groundwater pools	No	Adult	Vinogradova (2000)
<i>Culex quinquefasciatus</i> (Africa)	Americas, Asia, New Zealand, southern Europe	Urban, domestic, suburban	Man-made containers, small groundwater pools	No	None	Vinogradova (2000)
<i>Culex panocossa</i> (Central and South America)	United States	Suburban, rural	Natural water bodies	No	None	Blosser and Burkett-Cadena (2017)
<i>Culex coronator</i> (Central America)	South America, United States	Urban, domestic, suburban	Rock pools, tree holes, man-made containers	No	None	Akaratovic and Kiser (2017) and Wilke et al. (2020)
<i>Anopheles stephensi</i> (tropical Asia and Middle East)	Djibouti, Ethiopia	Urban, domestic, suburban	Man-made containers, small groundwater pools	No	None	Carter et al. (2018) and Seyfarth et al. (2019)

The criterion of establishment is based on subsequent observations in a new environment for at least two reproductive seasons.

and Lounibos, 2005). Among these mosquitoes, species belonging to the genus *Aedes* have the ecological characteristics that make them particularly suitable for spreading and colonizing new areas (Table 2).

The spreading success of the two major invasive mosquitoes, *A. albopictus* and the closely related *Stegomyia* species *Aedes aegypti* (major vectors of DENV, ZIKV, CHIKV, and YFV), is an example of how niche expansion of these two mosquitoes allowed the geographic spread from their respective native ranges of Asia and Africa to a worldwide diffusion (Kraemer et al., 2019). Their trophic behavioral change from zoophily to anthropophily and larval adaptation from natural small water sources to artificial containers in domestic habitats is the key feature that allowed this spread (Powell and Tabachnick, 2013; Powell, 2016). Recent models show that these two species will not stop their diffusion to new areas, particularly in urban settings, filling unoccupied suitable habitats and increasing the risk to human health (Kraemer et al., 2019).

In particular, the world's most invasive mosquito, *A. albopictus*, is a species that is able to actively disperse only a few hundred meters (Marini et al., 2010; Marini et al., 2019a,b; Gouagna et al., 2015; Medeiros et al., 2017; Vavassori et al., 2019) but, despite its not exceptional flying capacity, this mosquito has been capable to reach all continents. It is a species native to South-East Asia which, given its extreme aggressiveness, opportunistic feeding behavior (Savage et al., 1993; Delatte et al., 2010; Valerio et al., 2010) and ability to transmit several pathogens, is one of the species of greatest interest to the scientific community. Its capacity of diffusion and colonization of different anthropized habitats exploiting trade in items such as tyres and potted plants (Paupy et al., 2009), coupled with the capacity to establish in temperate areas thanks to a diapause strategy at egg stage (Vega-Rua et al., 2013; Armbruster, 2016), led *A. albopictus* to become the second most abundant species after *Culex pipiens*, particularly in urban areas of European and Mediterranean countries ("ECDC Mosquito maps," www.ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-maps).

Another important invasive mosquito is *Aedes japonicus*, a container-breeding species endemic of temperate Eastern countries (Tanaka et al., 1979) and now present in several areas of Europe, North America and New Zealand (Medlock et al., 2015). As already observed for *A. albopictus*, *A. japonicus* is spreading by continuous importation of goods and a subsequent dispersal through active

flight and possibly passive transports by human activities (Kampen and Werner, 2014; Koban et al., 2019). It can reproduce in a wide variety of environments, both artificial and natural (Crans and McNelly, 1999; Schaffner et al., 2009; Kampen and Werner, 2014; Sáringier-Kenyeres et al., 2020). Compared to other invasive species, it is even more tolerant to low temperatures and its resistant eggs can survive to very cold winters. This characteristic led *A. japonicus*, together with another invasive mosquito in Europe with similar ecology, *Aedes koreicus* (Montarsi et al., 2013), to be a species ecologically complementary to *A. albopictus* in temperate areas (Cunze et al., 2016; Koban et al., 2019; Marini et al., 2019a,b). Moreover, *A. japonicus* is a competent vector of several arboviruses (Veronesi et al., 2018; Abbo et al., 2020), increasing the list of invasive vectors threatening the health of human population worldwide.

The species described so far are morphologically very similar to each other (Fig. 3), then difficult to identify if not by specialized personnel. The continuous arrival of new mosquito species in a specific context is therefore, a problem of great importance in terms of surveillance of disease vectors, in which appropriate sampling approaches and the constant and capillary monitoring of the territory are fundamental to detect their presence at an early invasion stage.

The invasion of new territories by mosquitoes is possible not only by the involvement of human activities but also exploiting natural phenomena. A spreading strategy that is receiving more attention among medical entomologist is the wind-assisted long-distance dispersal. According to Service (1997), wind-assisted strategy in mosquitoes was not considered as migration, because there is no convincing evidence that this taxonomic group purposely fly downwind to colonize new areas and subsequently they, or their progeny, return. However, new information is raising on this topic and some new evidence of migratory events are now available also for mosquitoes. A seminal study recently published by (Huestis et al., 2019) describes for the first time that wind-assisted dispersion can occur in the major malaria mosquitoes of the *A. gambiae* complex. Through the usage of helium balloons, mosquitoes were collected by sticky nets between 40 and 290 m a.s.l. for about 2 years of sampling. Among all the 461,000 insects collected, 0.6% were mosquitoes, including blood-fed *Anopheles* females. The model built from these data indicates that malaria-bearing mosquitoes can travel for several kilometers in a single night, with an estimate of about 6 million *A. coluzzii* and 81,000 *A. gambiae* s.s. able to travel each year for 100 km across regions. The long-distance travel strategy described in this study suggests that malaria transmission dynamics can be more complex than expected, considering the potential genetic exchange of *Anopheles* and *Plasmodium* populations between distant areas carrying, respectively, insecticide and drug resistant genes, with potentially dramatic consequences for malaria control strategies.

Surveillance, monitoring and control

Mosquito-borne pathogens are increasing their distribution, with consequent worsening of their impact on public health. Therefore, surveillance systems are mandatory to prevent and tackle mosquito-borne diseases. Entomological surveillance follows a path in which the identification of an arthropod-borne pathogen, the presence and abundance of its potential vectors through standardized monitoring and characterization, and finally the realization of control campaigns are integrated and harmonized according to the different ecological contexts (Jourdain et al., 2019):

- (1) Risk assessment: prioritization of public health threats
- (2) Early warning systems
- (3) Identification of the vector species involved in a transmission event

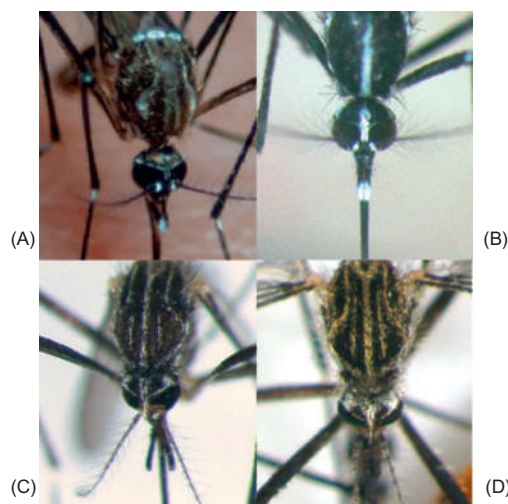


Fig. 3 Example of morphological details of head and thorax of four morphologically similar *Aedes* mosquitoes that can be collected in sympatry: (A) *Aedes aegypti*, (B) *Aedes albopictus*, (C) *Aedes koreicus*, and (D) *Aedes japonicus*.

- (4) Identification of the circulating pathogen strain
- (5) Optimizing vector control in time and space
- (6) Guidance for source reduction campaigns
- (7) Insecticide resistance monitoring
- (8) Evaluating the efficacy of vector control

Integrated Vector Management (IVM) is often the only means available to reduce the incidence of mosquito-borne diseases for which effective treatments or vaccines are not available yet. This imposes the use of all tools available and requires research efforts to develop novel technologies, of which the appropriate use depends on the eco-epidemiological contexts to maximize the impact on disease transmission (Benelli and Beier, 2017; Wilder-Smith et al., 2017; Achee et al., 2019).

The first aspect to be addressed is the estimation of mosquito presence and density. This is achieved through specific monitoring approaches, aiming to collect eggs, larvae or adults according to the different target species. Eggs and adults, in particular, are the mosquito stages that can be collected with trapping approaches, while larvae are usually collected by sampling in breeding sites. The choice of the sampling approach (e.g., type of trap, numbers, location etc.) must be carefully evaluated because it has an important influence on the success of the catches and therefore the affordability of the monitoring system. It is also important to standardize the sampling approach to have geographically and temporally comparable data.

Among the several trapping methods available (Silver, 2008), we here cite just a few examples. The ovitrap is the method traditionally used to sample container-breeding mosquitoes belonging to *Aedes* genus. It consists of a black plastic pot, filled with water, in which is placed a wooden stick. The trap exploits the propensity of *Aedes* mosquitoes to lay their eggs on wet substrates (the wooden stick) in a water container. A limitation of this approach is that the number of eggs laid by a single female can be highly variable, thus affecting population density estimates. Some variants of this approach, based on sticky surfaces, have been developed to collect gravid females instead of eggs (e.g., Facchinelli et al., 2007; Lee et al., 2013). Another method to collect ovipositing mosquitoes, in particular *Culex spp.*, is the gravid trap (Di Menna et al., 2006), which actively aspirate mosquitoes attracted by the water container present in the trap. The attractiveness of all these traps can be increased using plant infusions that simulate a suitable oviposition site. Among the sampling approaches for the monitoring of resting mosquitoes, traps have been only recently developed. This is particularly useful for *Anopheles* mosquitoes, where indoor-outdoor sampling based on active aspirations are affected in terms of standardization by the ability of the operator and by the characteristics of the site. Some examples for this genus are the clay pots (Odiere et al., 2007) and the resting box and bucket (Kweka et al., 2009; Pombi et al., 2014; Kreppel et al., 2015). However, the most targeted fraction of mosquito populations are the host-seeking females. This because they are the closest proxy to the actual numbers of mosquitoes biting a host and, consequently, to the risk of disease transmission. Apart from landing catches, which consists in catching mosquitoes that try to land on the collector and are consequently the better estimate of the biting pressure (Service, 1977), several traps are available. Among these, which show different performances on the several species targets, the CDC light trap and the BG-Sentinel are by far the most used. Both traps actively aspirate the mosquitoes that are attracted, respectively, by a light source or a dark-white pattern coupled with an odor blend. The number of mosquitoes and species collected by both traps increases using a source of CO₂ as attractant. Despite the large number of species that CDC light trap and BG-Sentinel can collect (Drago et al., 2012; Pezzin et al., 2016), the CDC light trap is considered a gold standard for nocturnal species, *Culex* and *Anopheles* in particular, while the BG-Sentinel for daytime biting mosquitoes, *Aedes* above all.

Of note, monitoring mosquito vectors is an essential activity to not only evaluate the presence, abundance and diffusion of a target species as part of an entomological surveillance system but also to assess the effectiveness of vector control actions in terms of reduction of mosquito density, host-vector contact and pathogen infection levels.

For more than a century vector control has been the major preventing strategy of vector-borne diseases and continue to be in several cases the only option available (Wilson et al., 2020). To date, many tools of mosquito control have been developed, which can be classified as: environmental, biological, mechanical, chemical and genetic.

Environmental control methods are those activities that are part of good prevention practices, tailored on ecology and behavior of the species target. Larval habitat removal is one of the major environmental intervention. As an example, in urban areas where the major source of container-breeding mosquitoes are the peri-domestic habitats, removal/emptying of containers that could retain water and covering large containers with nets or lids are particularly useful in reducing mosquito density.

One of the simplest biological control methods is the introduction of natural predators such as fish (e.g., Poeciliidae) or copepods (e.g., *Macrocyclus*, *Mesocyclops*) in the larval breeding site (Benelli et al., 2016). This is possible in medium-large permanent water collections, where pollution is at low levels. When this is not feasible, biological larvicides can be used, which are harmless to other non-target animals, particularly vertebrates. The most common biological larvicides, highly specific for mosquito larvae, are bacteria of the genus *Bacillus* such as *B. thuringiensis* var. *israelensis* and *B. sphaericus*. However, their persistence is limited and require repeated treatments. Another biological control approach is based on the usage of entomopathogenic fungi (Kanzok and Jacobs-Lorena, 2006). These moulds, such as those belonging to the species *Beauveria bassiana* (Clark et al., 1968; Achonduh and Tondje, 2008; García-Munguía et al., 2011) and *Metarhizium anisopliae* (Scholte et al., 2004, 2007), have shown in laboratory conditions to be selective and versatile mosquito control tools that can be added on surfaces where mosquitoes lay eggs, on clothes, fabrics or mosquito nets (Lovett et al., 2019).

Mechanical control methods are represented using monomolecular films. This approach is particularly useful if the size of the larval site is rather large and the larvicidal treatment would require considerable amounts of product or the effectiveness of the larvicide/predator is limited by the organic water pollution. The monomolecular films are apolar products that are poured in drops

onto the water surface to create a thin film that reduces surface tension. This prevents the larvae from breathing properly and the mosquito adults (e.g., *Culex*, *Anopheles*) from laying their eggs on the surface (Nayar and Ali 2003; Ngrenngarmlet et al., 2016).

The most used mosquito control approaches are chemical insecticides. Five major classes of insecticides are used against vector mosquitoes: pyrethroids, organochlorines, organophosphates, carbamates, neonicotinoids (World Health Organization, 2019b). To the above-mentioned classes, insect growth regulators (IGR) must be added to the list as larvicides only, even if several effects also on adults, particularly on fecundity, have been described (Mbare et al., 2014; Yadav et al., 2019). While for *Anopheles* malaria vectors adulticides are recommended in terms of epidemiological impact (World Health Organization, 2019b), in the case of *Culex* and *Aedes* arbovirus vectors the usage of chemical larvicides, IGR in particular, is far more common (World Health Organization, 2013).

When used outdoors, chemical adulticide interventions are aimed at drastically lowering the density of adult mosquitoes, but it should always be kept in mind that the knockdown and lethal effects of the treatment, even if carried out applying good practices, are limited in time (a few days). Among the several insecticide formulations for the control of adult mosquitoes, the most common are pyrethroids. Particularly in urban areas, adulticide treatments are conducted outdoors using large-scale sprayers/nebulizers/foggers able to produce a mist of microdroplets with a high dispersal capability. On the other hand, the indoor usage of chemical adulticides for vector control is split into two major approaches: Insecticide Treated Nets (ITNs) and Indoor-Residual Spraying (IRS): ITNs in particular have demonstrated to be highly effective in reducing the malaria burden at global scale (Bhatt et al., 2015; Wangdi et al., 2018). Several ITNs are available on the market, most of them are Long-Lasting Insecticidal Nets (LLINs) impregnated with pyrethroids that can contain or not the synergist piperonyl butoxide (PBO) ("WHO Vector Control," www.who.int/vector-control/vcag/new-interventions/en/index1.html). According to WHO indications, recommended ITN are bednets manufactured in compliance with standards of efficacy, safety, and quality ("WHO New types of insecticide-treated nets," www.who.int/news-room/q-a-detail/new-types-of-insecticide-treated-nets; "WHO Prequalification Team: Vector Control Products," www.who.int/pq-vector-control/en). They are expected to be effective in terms of insecticidal content and durability for at least 3 years or 20 washes. These are most effective against nocturnal, indoor-biting species such as *Anopheles* malaria vectors. The IRS strategy is based on the aspersions of a residual insecticide to mosquito resting surfaces (e.g., house walls and ceilings, animal shelters). The efficacy of this treatment is expected to last several months, according to the product used. In both cases, the insecticidal activity occurs when mosquitoes come into contact with the insecticide-impregnated surface, knocking-down or killing them in a short time-frame.

IGRs act on larvae by altering their development, preventing metamorphosis into adult. Contrarily to the other chemical insecticide classes, these are slow-acting, leading to the death of the insect at the end of the larval stage. Among the active ingredients available, one of the most used is pyriproxyfen (Maoz et al., 2017).

The major threat to the efficacy of chemical compounds is insecticide resistance, which raised in most of the mosquito vectors and currently spreading worldwide (Fig. 4; World Health Organization, 2012; Liu, 2015; Hemingway et al., 2016; Ranson and Lissenden, 2016; Riveron et al., 2018). Multiple resistance mechanisms are known to undermine insecticide-based control approaches in mosquito species (Liu, 2015; Ingham et al., 2018; Riveron et al., 2018), ranging from mutations conferring target site insensitivity (e.g., sodium channel, acetylcholinesterase, GABA receptors) to overexpression of metabolic detoxification systems (cytochrome P450s, esterases, glutathione S-transferases) or cuticular hydrocarbon alterations (Balabanidou et al., 2019) and chemosensory proteins overexpression (Ingham et al., 2020). To these genetic and morpho-physiological factors are also included behavioral mechanisms of resistance to insecticides, which concur to reduce lethal contact to insecticides and thus their effectiveness (e.g., increased exophily, opportunistic biting activity and/or shift in biting activity; Killeen, 2014; Carrasco et al., 2019).

Several alternative control approaches based on combinations of the above mentioned methods have been developed, e.g., autocidal gravid ovitraps (Mackay et al., 2013; Barrera et al., 2017; Cilek et al., 2017; Liu et al., 2019), autodissemination (Devine et al., 2009; Caputo et al., 2012; Gaugler et al., 2012; Swale et al., 2018; Brelsfoard et al., 2019; Lwetoijera et al., 2019), ultrasonics against larvae (Britch et al., 2016; Kalimuthu et al., 2020), spatial repellents (Grieco et al., 2007; Choi et al., 2011; Ogoma et al., 2014; Norris and Coats, 2017; Mwanga et al., 2019), attractive toxic sugar baits (Fiorenzano et al., 2017; Traore et al., 2020). However, further optimizations of these approaches are ongoing and their potential impact in vector control should be considered complementary to the existing strategies (Achee et al., 2019).

Finally, the last frontier of vector control is represented by modified mosquitoes, which consist of manipulation techniques of mosquito populations based on different approaches that can be grouped in two major categories: population suppression and population replacement (Flores and O'Neill, 2018; Wilke et al., 2018).

Sterile Insect Technique (population suppression): Through radiation or chemical treatment, male mosquitoes are rendered sterile. These males are released in the field to compete with wild-type males for mating with natural females. If a large number of females mate with the sterile males, they will be unable to produce viable offspring, thus reducing mosquito population density. This is not a novel approach (Dame et al., 1974; Weidhaas et al., 1974) and has the limit to oblige continuous release of males in the field. However, SIT continues to be developed as it still has promising potential, mostly thanks to the optimization of mosquito irradiation approaches and mass-rearing protocols (Balestrino et al., 2010; Bellini et al., 2013a,b).

Dominant Lethal Transgene (population suppression): Males carrying a dominant transgene that causes lethality at the larval stage are released in the field. When these males mate with wild-type females, the resulting progeny, which is heterozygote for the transgene, die before reaching the pupal stage.

Incompatible Insect Technique (both population suppression and replacement): It is based on the usage of *Wolbachia*, a genus of intracellular gram-negative bacteria that naturally infect some insect species, including some mosquitoes, and transmits vertically to the progeny by infecting eggs. Its presence modulates fitness in the population as confers a frequency-dependent fitness through a

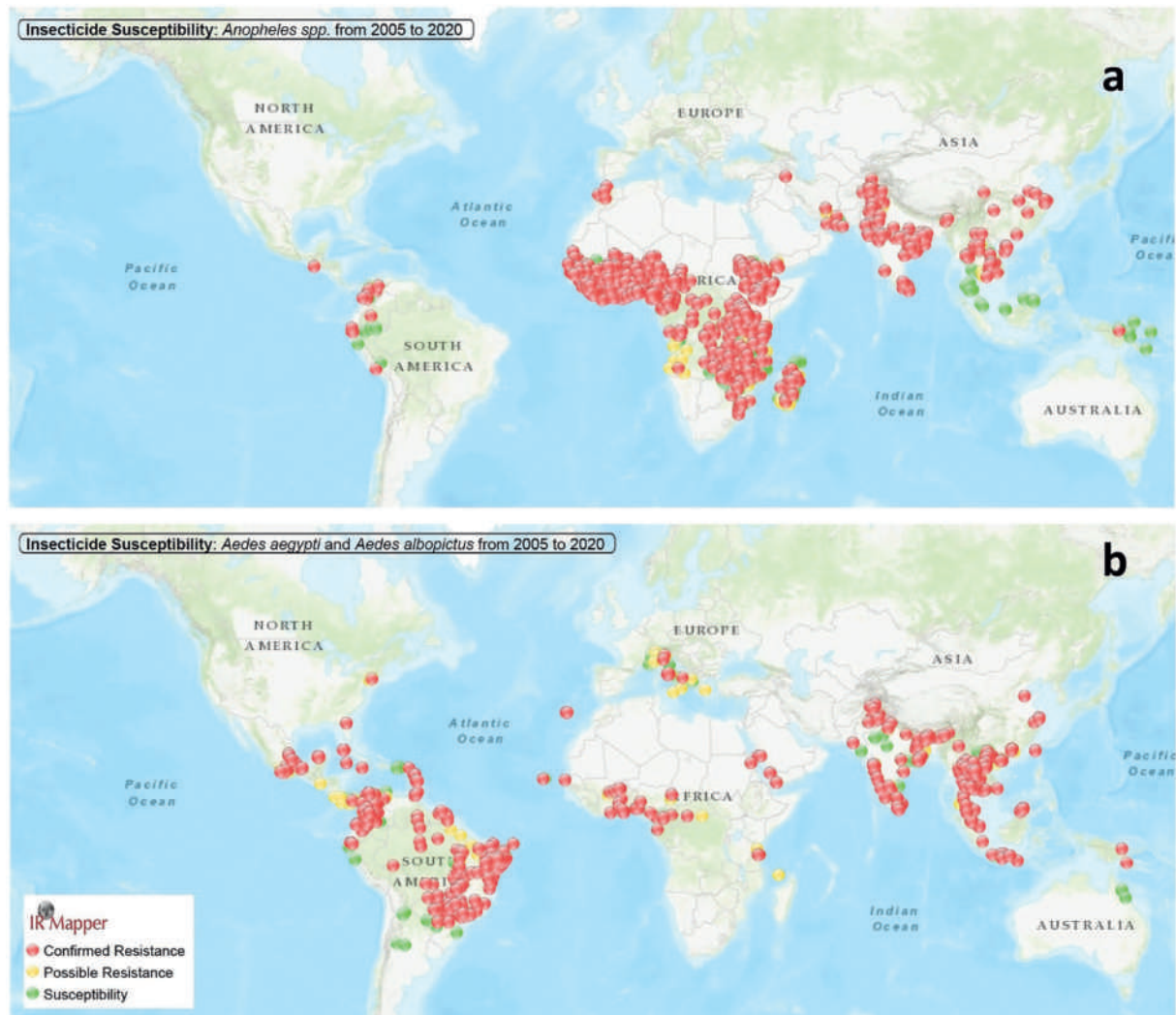


Fig. 4 Worldwide geographic distribution of pyrethroid insecticide susceptibility of (A) *Aedes albopictus* and *Aedes aegypti*, (B) *Anopheles* spp. Red dots: confirmed insecticide resistance; Yellow dots: possible insecticide resistance; Green dots: insecticide susceptibility.

mechanism of cytoplasmic incompatibility: crosses between *Wolbachia*-infected males and wild-type females are unviable. The exploitation of this mechanism is at the base of *Wolbachia*-release strategies. The sterility can occur also when infected males mate with wild-type females carrying a different, incompatible strain of *Wolbachia*. Moreover, the introduction of specific *Wolbachia* strains in a natural population can lead to refractoriness of the mosquitoes for a pathogen (e.g., *A. aegypti* and dengue virus) (Moreira et al., 2009; Walker et al., 2011). Thanks to the characteristic of *Wolbachia* to guarantee the progeny only of individuals carrying the bacteria, the whole mosquito population become infected by *Wolbachia* and contemporarily unable to transmit the pathogen.

Gene drive (both population suppression and replacement): is a method of genetic modification that can be used to spread a desired phenotype through the mating of the natural population with released modified mosquitoes (Gabrieli et al., 2014; Adelman, 2015; Hammond and Galizi, 2017). The gene drive technologies have the advantage to potentially introduce modified mosquitoes in wild-type populations through a limited number of releases and quickly rise to high frequencies. This allows desired transgenes to spread through populations in a self-sustaining manner, even if they confer a fitness cost. Several gene drive systems have been described and laboratory tested (Marshall and Taylor, 2009; Adelman and Tu, 2016), but the two most developed technologies are homing endonuclease genes (HEG) and CRISPR–Cas9. These approaches are based on an endonuclease that recognizes a specific DNA sequence and catalyses a break. The DNA is then naturally repaired through homology-directed repair, using as a template the sequence present in the other chromatid. This occurs during mitosis of germline cells or meiosis, leading to a non-Mendelian inheritance of the desired character. This technique theoretically allows a quick, complete replacement of the natural population with the new phenotype. However, the epidemiological impact of GM-based mosquito control tools is still debated (Wilke et al., 2018).

Wolbachia and gene drive vector control approaches have the potential to be game-changer in mosquito-borne disease epidemiology, giving long-term reductions in pathogen transmission after only a single implementation period, by stable reductions in mosquito density or vector competence (Ferguson, 2018). However, most of the above mentioned new approaches are still in the early phases, and only *Wolbachia*-based approaches are being utilized at operational scales in medium-sized cities (Flores and O'Neill, 2018).

Concluding remarks

The control of mosquito-borne diseases is a far-off goal that we will never be able to achieve unless we adopt a holistic view of the problem, through a One Health approach. Human and animal hosts, pathogens and their vectors are interconnected elements of a single ecological system that needs to be fully known in relation to each of these diseases. Current and future vector control strategies, to be effective, must necessarily identify all the actors involved, acting on multiple levels through an IVM approach. In this long arms race between humans and mosquitoes, we must not forget the importance of being part of a complex ecological network in which the introduction of a disturbing element can have unexpected consequences that must be evaluated in as much detail as possible. The application of each control tool could have advantages in terms of immediate effectiveness but may present threats that need to be considered through a weighted analysis of risk assessment. The current resistance to insecticides is an example of how the resilience of several mosquito species to this selective factor has allowed them to react in many ways, undermining our ability to control the diseases they transmit, malaria above all. There is no doubt that we will never stop cohabiting with this ancient enemy, precisely because we have always shared the same house and neither of us wants to be kicked out.

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Phlebotomine Sand Flies (Diptera, Psychodidae, Phlebotominae)

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Glossary

Autogeny Production of batch(es) of eggs before first blood meal.

Bartonellosis Biphasic disease caused by *Bartonella bacilliformis*, causing anemia and warts, potentially mortal.

Cibarium A dilatation of alimentary tube, visible in the head, visible from venter of clarified flies, containing teeth and dark areas.

Gonotrophic concordance Synchronization between a blood meal and production of a batch of eggs.

Kairomone Substance from any source that influences animals of a different specie at a distance.

Leishmanization Injection of live *Leishmania* in a hidden part of the body, to prevent lesions in an exposed part, mostly the face.

Palpus Maxillary palpus, with five subsegments, the second one fused to first, in sand flies.

Pheromone Substance produced by and animal that influences a member of the same specie at a distance.

Solenophages Insects that suck blood direct from capillary, having long and well developed mouth parts.

Spermatheca A reservoir for spermatozoa, found in females of sand flies and very variable in form.

Telmophages Insects that suck blood from subcutaneous pools, caused by lesions made by mouthparts, which are short or for other reasons non-adapted to suck from capillaries.

Introduction and history

Phlebotomine sand flies (sandflies in British English) are small Diptera widely distributed, mostly in tropical and subtropical areas, but also in temperate regions, like the South of France and some areas in Germany and Switzerland.

The first known reference to leishmaniasis was in a clay tablet in the library of the Assyrian king Ashurbanipal in Nineveh (now Mossul, Iraq). Ibn Sīnā (Avicenna, 980–1037) described cutaneous leishmaniasis. Gorgani (1042–1136) called it in Persian “pashé gazidegui” (“bitten by the mosquito”), and *saalak* (“one-year sore”), the last still utilized in Iran. Also, the transmission of

Leishmania by sand flies was suspected by some Andean people several centuries ago, naming the flies and leishmaniasis by *uta*, and Cosme Bueno cited the transmission of (agents of) Carrión disease and leishmaniasis in 1764 (Herrer and Christensen, 1975).

In the beginning of 20th century, first species of *Leishmania* were described, and many blood feeding animals (except leeches and vampire bats) were tested, finally concentrating on sand flies. Researches were developed mostly in India, and also in Brazil, where H. B. Aragão transmitted *L. braziliensis* to dogs by injection of infected *Nyssomyia intermedia*¹ in 1922, and other countries. Efficient transmission by bite only was obtained after feeding infected insects on sugar from raisins, in 1940s (Killick-Kendrick, 2013).

An outbreak of a biphasic disease in the Andes region, during the building of the railway Lima-Oroya, in 1871, caused at least 4000 deaths among workers, and it was called Oroya fever. A medicine student, Daniel Carrión, injected himself with exudate from a wart ("verruca" in Spanish), developed Oroya fever and died. The common etiology of both diseases was established and the infection was called Carrión disease, in homage to the heroic Peruvian researcher. In 1905, the causing bacterium was described by Alberto Barton from blood smears of patients. Transmission by sand flies was first demonstrated in 1913 by C. H. T. Townsend, which described its major vector, *Pintomyia verrucarum* (= *Phlebotomus verrucarum*).

The exclusive observation of external characters, like scales and dimensions of appendices, produced several incomplete descriptions, like those of *Lutzomyia longipalpis*, *Ny. intermedia* and *Psychodopygus squamiventris* (all in *Phlebotomus*) based mostly on the length of palpi and the presence of scales on the abdomen (Lutz and Neiva, 1912). The study of internal structures, after Adler and Theodor's (1926) observation of spermathecae and their ducts and cibarium of females, gave big boost for classification and description of new species, and there are now at least 1000 described species in the world. Description of new species in previously unstudied areas, like the Highlands of Madagascar (Randrianambinintsoa et al., 2019) will certainly enlarge this number.

Recently, new and potentially useful structures have been added to descriptions (Galati et al., 2017; Galati, 2018), and molecular biology has been much utilized for characterization and differentiation of species. Recent description of *Trichophoromyia velezbernalei* (Posada-López et al., 2018) is an example of utilization of those structures for the differentiation of species.

Other ca. 2000 species of Psychodidae are distributed among five subfamilies (Pape et al., 2011). Some species of these so-called moth flies (or owl flies) may be annoying, mostly near sewage treatment sites, and contribute to contamination in hospitals, cause allergy or opportunistic myiasis. Several psychodids of Sycoracinae (*Sycorax* - 45 spp.) (Santos et al., 2013) have bloodsucking mouthparts, some of them biting Amphibians and even transmitting filariae.

Morphology and taxonomy

Adults

Sand flies are small (length — 2–3 mm) hairy Diptera, constituting Phlebotominae in Psychodidae, which belongs to suborder Psychodomorpha, one of eight orders in Diptera; due mostly to their long and multiarticulate antennae,² this family used to be included in Nematocera, now considered as a paraphyletic group.

Legs are long, lanceolate wings are maintained erect over the body, when landing, and head and thorax are in 90° angle (Figs. 1 and 2). Wing transversal veins are restricted to basal half and all ramifications of vein R_s (R₂-R₄) reach the border of the wing.



Fig. 1 Adults of *Phlebotomus papatasi* in copula. Right – male. Courtesy of CDC (photo – Edgard Rowton).

¹They could be *Ny. whitmani*, described in 1939 and present in forested areas of the city (N. A. Souza – pers. commun.), one of them near that locality ("Águas Ferreas") (Souza et al., 2015). The locality had in 1922 many trees and was humid, and cases of cutaneous leishmaniasis were widely distributed.

²Antennae of all insects have three segments, and the most distal (flagellum) may be divided in subsegments (or annuli), which have no implantation of musculature (Snodgrass, 1993).



Fig. 2 Adults of *Lutzomyia longipalpis*. In black rectangle: couple in copula, male below. Courtesy of Nataly A. Souza.

For males, the study of genitalia, including the length of sperm pump and length, shape and tips of aedeagal ducts, number and localization of spines on gonostyle, non-deciduous hairs and shape of gonocoxite, parameres and epandrial lobes (=lateral lobes) are important for identification (Galati, 2018).

For females, shape of spermathecae and ducts (individual and common) and cibarial teeth are very important. Other structures potentially useful were listed by Galati et al. (2017) and thoroughly described by Galati (2018).

Immature forms. Eggs are oval or ellipsoid (0.3 to 0.5 mm in length/0.07 to 0.15 mm width); when laid, they are whitened or yellowed, and in hours become dark brown (Brazil and Brazil, 2015). Their exochorium present structures not yet well studied in many species, probably due to fragility of eggs (J. Santos-Mallet – pers. commun.).

Larvae are wormlike and have distinct black head, three thoracic and nine abdominal segments; first instar larvae have two posterior long setae, and four are (in most species) present in the other three instars³ (Figs. 3 and 4). External structures are potentially useful for identification and deserve additional studies; detailed description of larvae and pupae of the important species *Ny. umbratilis* (Alencar et al., 2018) is an example for these studies. Pupae are fixed to the substrate by larval exuvia (Fig. 4) and have flexion and extension movements when disturbed (Brazil and Brazil, 2015).



Fig. 3 Larva of *Lutzomyia longipalpis*. Courtesy of Reginaldo P. Brazil.

³The only known reference to the function of these long setae was done by M. P. Barretto in a thesis in 1942, reporting their utilization to expel mites from their bodies.



Fig. 4 Larvae and pupae (indicated by red arrows) of *Lutzomyia longipalpis*. Courtesy of Reginaldo P. Brazil.

Taxonomy. They have been organized in 31 genera and 35 subgenera (Galati, 1995, 2018), most of them in the American continent. Special abbreviations for genera and subgenera were proposed (Marcondes et al., 2007) and have been widely utilized. Akhoundi et al. (2016) revised the history of classification, evolution and dispersal of *Leishmania* and sand flies.

Although there is not a strict association between phylogeny and biology and vectorial role, which are influenced by several factors (Ready, 2011), this classification has been shown to be reliable and useful. A review of molecular taxonomy studies on sand flies, comparing isoenzymes, cuticular hydrocarbons and DNA sequences (Depaquit, 2014) emphasized the concentration on medically important species; those techniques were indicated as less laborious and probably more productive and useful than morphology, mostly if utilized with due care. The only limitation would be the availability of material and equipment, usually expensive.

Evidence of genetic divergence and cryptic speciation by reproductive, morphological, isoenzyme, biochemical, and genetic traits (Soto et al., 2001; Arrivillaga et al., 2002, 2003; Hamilton et al., 2005; Pech-May et al., 2018) have shown that *Lu. longipalpis* is a species complex, whose components should be formally described (Brandão-Filho et al., 2009).

Mitochondrial DNA of several vectors of *L. major* around the Mediterranean was studied to understand phylogeography and coevolution of protozoa and sand flies (Esseghir et al., 1997).

Illustrated keys for both sexes of American species were provided by Galati (2018), and a digital key for Brazilian species, adapted to cell phones, was built by Rocha et al. (2019).

Biology and ecology

Biology

Based on laboratory studies, blood-fed females lay ca. 30–70 eggs (Lawyer et al., 2017). They are laid on wet and dark places on the soil, in a great variety of habitats (34 types): under stones and decomposing leaves, near tree roots, in caves, rodent burrows and bird nests etc. (Feliciangeli, 2004).

Breeding places for sand flies may also be in human-modified places, like the contaminated soil of animal shelters for medically important *Lu. longipalpis* in the New World, *Ph. argentipes* in India, *Ph. chinensis* in China and *Ph. ariasi*, *Ph. perfiliewi* and *Ph. perniciosus* in Europe (Feliciangeli, 2004). Some species are associated to specialized niches, like termite hills in Kenia (*Ph. celiae*) and caves and among rocks in East Africa (*Ph. longipes* and *Ph. pedifer*) (El-naïem, 2011).

Larvae of four instars feed on organic matter on the soil and molt to pupae, with an egg-adult cycle of about 40 days. Localization of immature forms is very difficult, due to low knowledge or to their scattered distribution, and productivity of searches is usually small. Sixty-one larvae and 91 pupae were collected in Sebastopol, in former Soviet Union, after processing six tons of soil, in 965 samples, and in the Brazilian state of Paraná, in a locality where more than 200,000 insects could be collected per year, 0.8 insects/m² (total – 21 insects), and six from emergence traps were obtained (Reinhold-Castro et al., 2015), illustrating the difficulty of such search. In a rare example of success, more than 1500 immature forms were collected inside an abandoned cement building in Sardinia (Bettini et al., 1986).

Male adults have an 180° rotation of external genitalia in the first 24 h, and the collection of non-rotated males indicates the probable proximity of locality of collection to breeding places. Both sexes feed on carbohydrates from vegetal sap and secretions of aphids. Ingestion of sugars is fundamental for flight and other activities, and very important for development of *Leishmania* in the digestive tract of females. Louradour et al. (2017) indicated the importance of microbiota in the midgut of *Ph. duboscqui* on the development of *L. major*, emphasizing the role of glucose on the osmotic conditions for the survival of infective promastigotes.

Females also feed on blood by laceration of skin and capillaries, due to their short proboscis (0.2–0.4 mm), being pool-feeders (or telmophages) (Lavoipierre, 1965). Saliva is very important for preventing hemostasis. As an incidental result, saliva also influences the development and transmission of *Leishmania*, and intensive studies have been developed on their constitution and function, with possible usefulness for development of tests and vaccines. For example, it has been suggested that Costa Rican *Lu. longipalpis* containing little maxadilan, a potent erythema-inducing peptide, promotes cutaneous proliferation, while those from Brazil, richer on this peptide, promote visceralization (Warburg et al., 1994).

For most species, it is assumed a gonotrophic concordance, with some exceptions. So, it is possible to evaluate the quantity of blood meals from the analysis of internal structures related to egg-laying. Small size of insects makes difficult this task.

However, autogeny has been observed in some Old and New World species: *Ph. papatasi*, *Lu. gomezi*, *Lu. cruciata* and possibly *Psychodopygus davisi* and *Pintomyia damascenoi* (Forattini, 1973). In the laboratory, *Pi. mamedei* is autogenous and parthenogenetic, just producing females (Brazil and Oliveira, 1999). *Deanemyia maruaga* (Alves et al., 2011a), whose larvae have a visible reserve of fat in the abdomen, unusual in such flies and attributable to richness of guano in the cave they live, also is apparently autogenous and parthenogenetic (Alves et al., 2011b).

Blood feeding is mostly nocturnal, but some species may feed during the day, mostly in shaded areas in caves and forest areas. *Nyssomyia umbratilis*, when disturbed on tree trunks, may bite during the day, sometimes causing multiple lesions by *Leishmania guyanensis*. Most species are eclectic and opportunistic in their feeding choices, and may present different preferences according to region, as observed for *Ny. whitmani*. *Psychodopygus wellcomei*, an important vector of *L. braziliensis* in the Amazon forest, also bites during the day, and *Ps. complexus*, a very similar species and a vector in some areas, bites mostly between 6 and 8 a.m. (Godoy et al., 2016).

They usually disperse for short distances, but *Lutzomyia longipalpis* and some other New World species associated to human modified environments and several Old World *Phlebotomus* seem to disperse for some hundreds of meters. In a coffee plantation at Colombia, flights of up to 100 m for *Psathyromyia shannoni* were observed, but marked flies of *Pi. spinicrassa*, a much more plentiful species in the area, were rarely recaptured (Alexander, 1987). Another study in similar environment showed dispersion of *Pa. shannoni* to 200 m, and a female of *Lu. gomezi* was found at 960 m from the point of release (Alexander and Young, 1992). Ready et al. (1986) noticed continuous flights of sand flies of several species for at least 20 m in the first 5 minutes after releasing, and J. D. Andrade (pers. commun.) collected sand flies in a boat at 80 m from the edge of a river in the Brazilian state of Minas Gerais, showing the possibility of long flights without landing.

Influence of wind on sand flies has been studied for some species, and results are variable. Wind speeds between 1 m/s (=3.6 km/h) and 4 m/s (=14.4 km/h) were considered as prejudicial, and >4 m/s as prohibitive (Lane, 1993), but this seems to be variable according to interaction to other factors, as shown by a study in Saudi Arabia (Roberts, 1994). Since they have a predominantly hopping flight, it is necessary to check if wind can transport them to large distances, like biting midges.

A difference between the movement of *Ph. argentipes* and *Sergentomyia* sp. to the palm tree canopy in Bihar (India) was noticed. One-hundred and four from 105 flies collected by sticky traps around the trunk were *Sergentomyia*, but *Ph. argentipes* was the only species on CDC traps (130 flies in 29 trap days) in the canopy, an indication of direct flights (Poché et al., 2012).

Males arrive in the vicinity of host animals before females; they compete with each other for females in lekking displays (Bray and Hamilton, 2007) where their hopping and wing fanning are believed to distribute pheromones that encourage female choice (Jones and Hamilton, 1998). Males spend longer on and around hosts than the more discrete females, and this may contribute to the higher male: female ratio (typically >3:1) caught in both CDC mini light traps and pheromone traps in the proximity of breeding sites.

Ecology

Most species are associated to habitats not modified by human activity, but several species are adaptable to modified environments. There is a gradient of adaptation to different environments. For example, in the New World, *Psychodopygus* spp. are well adapted to forest, becoming very rare after deforestation. They are replaced by species of *Nyssomyia*, like *Ny. whitmani*, and those of *Ny. intermedia* complex (*Ny. intermedia* and *Ny. neivai*), and very modified places may be populated by *Lutzomyia longipalpis*. The last four species may invade houses and annexes. Deforestation around henhouses and utilization of insecticides on them in the Brazilian state of Paraná caused the replacement of *Migonemyia migonei* by *Ny. whitmani* as the predominant species (Teodoro et al., 1999).

Type of soil may influence predominance of species. For example, in the Brazilian state of Paraná, *Ny. neivai* is predominant in areas with sandier soils, while *Ny. whitmani* is more frequent in areas with red and red-yellow latosol or nitosol soils (Teodoro et al., 2006).

In desert areas in Iran (Yaghoobi-Ershadi, 2012) and the South of former USSR, *Ph. papatasi*, a vector of *L. major*, is associated to burrows of great gerbils, good reservoirs of the parasite. In Tunisia, they have a strong association to rabbit burrows (Chelbi et al., 2008).

Medical and veterinary importance

Damage by biting

Bites of sand flies can cause skin irritation and have been compared to the touch of a red hot pin. They are referred as *quemador* and *pringador* in some areas of Colombia, referring to this sensation. The hypersensitivity reaction to bites of *Ph. papatasi* is called *harara*

in Palestine, and the Author observed a measles-like skin in some children frequently bitten by *Lu. longipalpis* in the East of Brazilian state of Paraíba.

Pemphigus foliaceus is an autoimmune disease present in several South American countries, and also in Tunisia, called *Fogo Selvagem* (FS, wild fire) in Brazil. It is characterized by skin blisters and exfoliation, and can assume several forms and become very serious (Aoki et al., 2015). It has been associated with bites of sand flies and black flies (Vernal et al., 2017), and *Lu. longipalpis* salivary antigens can initiate a cross-reactive IgG4 response in genetically susceptible individuals, leading to FS (Ye et al., 2012).

Transmission of pathogenic agents

Leishmaniasis is usually divided in visceral and cutaneous forms, according to predominant localization of parasites. However, the localization and evolution of disease is influenced by several factors, like the species and strain of parasite, the immunity, and even the sand fly injecting it in the host. Visceral leishmaniasis (VL) can be mortal, mostly when associated to malnutrition or AIDS, and cutaneous leishmaniasis (CL) can cause serious deformities, mostly when affecting mucosae or disseminating in the body.

In 2016, VL caused 707.9 Disability-Adjusted Life Years (DALY) and CL 273.1 DALY, with a reduction of 70.6% in VL and an increase of 117.9% in CL from 1990 incidence in the world (Hay et al., 2017).

Leishmania has been divided in four subgenera: *Leishmania*, *Viannia*, *Mundinia* and *Sauroleishmania* (Espinosa et al., 2018). The last subgenus occurs in lizards, and parasites, only present in the hindgut of flies, need to be ingested by the vertebrate host. Subgenus *Leishmania* is usually restricted to midgut of flies, and *Viannia* also has parasites in the hindgut, and in both subgenera, they need to migrate to proboscis to be transmitted. *Mundinia* includes some badly known parasites of *L. enrietti* clade from South and Central America, Southeast Asia and Australia. Some *Leishmania* (*Mundinia*) of horses in Europe (Müller et al., 2009) need to be more carefully studied.

Leishmania of the first two subgenera, with 16 species already reported in humans (Ready, 2013) are vectored by sand flies, from more than 30 known species. Sand flies acquire amastigote forms, usually contained in macrophages, when blood feeding on mammals. In their guts, they suffer a series of modifications, finally producing metacyclic promastigotes (Kamhawi, 2006), which may be injected in the following bites.

Leishmania (*Mundinia*) *siamensis*, causing infection in humans in Thailand, is a *nomem nudum* and a synonym of *L. martiniquensis* (Espinosa et al., 2018), and in Australia, biting midges have been incriminated as vectors of *Leishmania* (*Mundinia*). A review of this group is mandatory.

Although flies of many species have been found infected by *Leishmania*, no more than 70 species have been implicated in transmission. The actual role of a species of sand fly as vector is determined by seven conditions (Ready, 2013), the last two added to five previously proposed by Killick-Kendrick (1990): (1) on more than an occasion, promastigotes were isolated or typed from wild females not containing blood meals; (2) many forms of *Leishmania* are found in the anterior midgut or stomodeal valve; (3) flies are attracted to and bite humans and reservoir hosts; (4) strong ecological association, including seasonality, is shown between fly, humans and reservoir hosts; (5) experimental transmission is achieved after infection from a natural host or laboratory model. Mathematical modeling demonstrates: (6) that the vector is essential for maintenance of infection with or without the involvement of other vectors and (7) that a significant decrease in the incidence of disease following a significant decrease in the biting density of the specific vector.

Incriminated and suspected vectors of all species of *Leishmania*, respectively meeting criteria 1–4 and only some of them, were listed, but no fly satisfied criteria 6 and 7 yet, and in reality, none is actually a proven vector (Ready, 2013). Maybe the implantation of evidence-based control (Wilson et al., 2015) could obtain better results, besides defining the role of each species in the transmission.

Some vectors (*Ph. papatasi*, *Ph. sergenti*) support the development of only their specific parasites, while *Lu. longipalpis* is susceptible to several parasites. *Leishmania*-host adaptation may be influenced by several factors, including the parasite lipophosphoglican (LPGs) (Kamhawi et al., 2000) and proteophosphoglicans (PPGs) (Secundino et al., 2010), respectively responsible for mediation of midgut binding and for prevention of digestion by midgut enzymes. Galectins, associated to LPGs, also have a function on binding to midgut surface and could be targeted in vaccines (Kamhawi et al., 2004). Some parasites have been shown to secrete a promastigote secretory gel (PSG), with filamentous PPGs, in the anterior midgut, which enhances transmission of *L. infantum* and *L. mexicana* by *Lu. longipalpis* (Rogers and Bates, 2007; Ready and Rogers, 2012).

This does not mean that no other species will be incriminated in the future, mostly due to ecological modifications, like deforestation and global warming. For example, in a study at Peruvian Amazonia, sand flies of 14 species in several genera were found infected by *L. braziliensis*, *L. guyanensis* or *Leishmania* sp. (Zorrilla et al., 2017), and infection of *Warileya rotundipennis* by *Leishmania* (*Viannia*) was reported in Colombia (Moreno et al., 2015).

Epidemiology of leishmaniasis is influenced by several factors, including ecology of vectors, reservoir hosts and species and strains involved. It is extremely diversified according to species of parasite and region, with some examples below.

The influence of several factors on the epidemiology of leishmaniasis must be carefully studied. A comparison between different areas (natural, urbanized, with or without some vegetation or reservoir etc.) is essential for understanding the transmission.

In Palestine, five transects were set between a rocky area with hyrax reservoirs of *L. tropica* and that containing houses (Salah et al., 2020). The risk of transmission to humans by *Ph. sergenti* increased with higher density and closer proximity to reservoir hosts and higher densities of vector near houses.

Leishmania guyanensis, widely distributed in the East of Andes, is associated to tree trunks at North of Brazil. *Nyssomyia umbratilis* bites sloths and anteaters in the canopy, becoming infected, and after going to the soil to oviposit, may bite people near the trunks; there are several other suspected vectors (Ready, 2013).⁴ *Leishmania braziliensis* has a wide distribution from Belize to Argentina, with several vectors and reservoir hosts, transmission being done in several environments, varying from primary forests to houses.

Leishmania major is transmitted in Sahel dry savannas in Sub-Saharan Africa and Yemen by *Phlebotomus duboscqui*, with rodents as reservoir hosts, while *Ph. papatasi* is its vector, with gerbils and other rodents as reservoir hosts in areas of North Africa, Middle East and South of Asia (Ready, 2013).

Leishmania infantum is mostly transmitted by *Ph. ariasi*, *Ph. perniciosus* and other species of *Phlebotomus* (Larroussius) and by *Ph. chinensis* in Eurasian areas, and mostly by *Lu. longipalpis* in Neotropical region. It has been reported in areas at southern and center-western Brazilian states, without the presence of *Lu. longipalpis*, and alternative vectors have been proposed for the above regions. Dogs and humans are reservoirs, and some wild dogs and possibly opossums are sylvatic reservoirs of the parasite.

Leishmania donovani, causing VL and PKDL (Post Kala-azar Dermal Leishmaniasis), is transmitted by *Ph. argentipes* in the Indian subcontinent and by *Ph. orientalis* and *Ph. martini* in East Africa; humans are the only reservoirs, except in some African savanna areas (Ready, 2013).

As an example of diversity of epidemiology of leishmaniasis, in China *L. infantum*⁵ has different vectors, reservoirs and life cycles in different regions of the country. It can cause CL in a small focus of Karamay, VL in anthroponotic cycle (AVL) in Kashi in the north-west (Xinjiang) and zoonotic cycle (Mountain Type – MT-ZVL) in mountainous areas in the western provinces of Sichuan and Gansu, with dogs as reservoirs, and in oases in the north-west (Desert Type – DT-ZCL), with *Ph. wui* as the vector. For AVL, no reservoir besides humans has been found, and vectors are *Ph. chinensis* in most places and, in Xinjiang, *Ph. longiductus*. For MT-ZVL, dogs are frequently infected and *Ph. chinensis* is the vector, while for DT-ZVL several vectors and a species of hare (*Lepus yarkandensis*) has been found infected (Lun et al., 2015). Ecology and distribution of four (three above plus *Ph. alexandri*) major vectors of VL in China was recently reviewed (Guan et al., 2016).

Human behavior is very important for infection. In sylvatic areas, men are usually more exposed to infection by *Leishmania* spp., due to their activity, than women and children, unless these also frequent those areas. On the contrary, if transmission is in or near the house, the chance of infection is similar for both sexes, if transmission is effected at night, but more women will become infected if this transmission is during the daytime, when men are probably out. So, the epidemiological investigation must include a careful survey of human activities.

In some areas of French Guiana, risk of transmission of *Leishmania* (probably *L. guyanensis*) to humans is higher in the short time between two rainy seasons, in the first half of November, when the quantity of infected *Ny. umbratilis* is greater (Le Pont and Pajot, 1980).

Flies that have already fed on blood and laid eggs (parous), which may be infective, may bite in different hours of the day, compared to those that have not yet produced eggs, and so have not blood fed (nulliparous). For example, a significantly greater proportion of parous females of *Psychodopygus wellcomei* bite in the morning than in the evening, indicating a higher risk of infection in that time (Wilkes et al., 1984).

Since some areas of Amazonia may have more than 60 species of sand flies (e.g., Galardo et al., 2015) and sometimes several species of *Leishmania*, the epidemiological study may be very laborious.

Environmental modifications through the building of dams for electricity (Galardo et al., 2015) or global warming (Moo-Llanes et al., 2013) may influence the predominance of fly species and their role in transmission of *Leishmania*.

Trypanosomatids of *Endotrypanum* (*E. schaudinni* and *E. monterogeii*), also are transmitted by sand flies, with sloths as vertebrate hosts. These protozoa are phylogenetically near *Porcisia hertigi* and *P. deanei*, from porcupines, also transmitted by sand flies (Espinosa et al., 2018).

Bartonellosis, caused by *B. bacilliformis*, one of 17 species in the genus, occurs in Andean valleys in Peru, Ecuador and Colombia, with possibility of cases in Brazil and Bolivia, due to its occurrence also in the Amazonian region. *Pintomyia verrucarum* and *Lu. peruensis* are usually the major vectors, but *Pi. maranonensis* and *Pi. robusta* were also found infected, among several suspected vectors (Garcia-Quintanilla et al., 2019).

This is a neglected disease, mostly in isolated (and poor) small villages, and more studies must be developed. *Lutzomyia peruensis* and *Pintomyia verrucarum* are considered as primary vectors, and *Pi. maranonensis* has been recently found infected in Peru (Ulloa et al., 2018). Natural infection of ticks and experimental transmission has been demonstrated, but their role has also been considered doubtful (Telford III and Wormser, 2010). A *Bartonella* sp. near *B. grabhami* and *B. elizabethae*, emerging pathogens that can cause endocarditis in dogs and humans, was isolated from 8.69% of sand flies (2/26) in Mexico (Lozano-Sardaneta et al., 2019).

Several *arboviruses* are transmitted by sand flies. They belong to Rhabdoviridae (mostly *Vesiculovirus* – VSV group), Bunyaviridae (*Phlebovirus*) and Rheoviridae (*Orbivirus*). Some of them may cause febrile diseases, meningitis and even death.

⁴In a short visit to a forest in Recife, in the northeast of Brazil, Bruce Alexander collected some *Ny umbratilis*, the predominant species (96%) in the area, on a tree trunk (Balbino et al., 2001).

⁵Although Lun et al. (2015) called all protozoa *L. infantum*, *Leishmania donovani/infantum* complex is very diversified in China, sometimes with several different strains in the same locality (e.g., Xinjiang) (Alam et al., 2014), and even including some undescribed *L. (Sauroleishmania)* (Yang et al., 2013).

Vesiculovirus includes several human and veterinary pathogens as Chandipura (CHAV), in India, and Vesicular Stomatitis Indiana (VSIV) and Vesicular Stomatitis New Jersey (VSNEJV), in the New World. Ten of them have been associated to sand flies (Comer and Tesh, 1991).

Infection of salivary glands of *Ph. argentipes* (Mavale et al., 2007) and venereal transmission by *Ph. papatasi* of Chandipura virus were obtained. In the American continent, VSNEJV may be experimentally transmitted by sand flies, biting midges and horse flies, but transovarial transmission has been demonstrated only in sand flies (Rozo-Lopez et al., 2018).

Phlebovirus is one of five genera in Bunyaviridae, and includes 70 species, classified into two broad groups: the sandfly (or phlebotomus) fever group, which includes Rift Valley virus, mostly transmitted by mosquitoes, and Toscana viruses, with sand flies as vectors, and Uukuniemi group, with three species transmitted by ticks, besides some newly described mosquito-specific viruses (Palacios et al., 2015). Toscana viruses, usually isolated from *Ph. perniciosus* and *Ph. perfiliewi*, have wide distribution in Central and Eastern Europe, North Africa and Turkey, probably transmitted by several species of sand flies (Aihan et al., 2020).

Orbivirus has been found only in tropical New World, mostly in the Amazon region, where 52 arboviruses of Changuinola antigenic group were isolated from sand flies between 1961 and 1995 (Shaw et al., 2018).

Transovarial transmission of arbovirus seems to be frequent in several species of sand flies, but at variable rates. This probably compensates short parasitemia of several of these arboviruses (Tesh and Chaniotis, 1975).

Sand flies have been found infected by several species of Trypanosomatidae from Amphibia, Reptilia and Mammalia, and the study of infection and interaction between several of these parasites in sand flies must be developed (Shaw et al., 2018). *Plasmodium* of reptiles also are transmitted by sand flies, with more than 50 known species (Ayala, 1978), and may influence biology and competition of animals (Bower et al., 2018).

Methods for sampling, rearing and research activity

Detection of adults

Sand flies may be collected when trying to bite humans and domestic animals. Baits need to be protected to prevent the risk of infection. Traps have been designed to collect these flies.

Adult flies may be sucked by mouth-aspirated tubes, with adequate filters to prevent suction of setae, scales and mold. On walls, light from flashlights must preferably be projected in parallel to surface. According to future processing, they may be blown on in 70° GL ethanol or in a small plastic cage (Marcondes et al., 2007) or other cage types (Lawyer et al., 2017), utilizing voile instead of screen.

Taking advantage of the hopping flight of flies, a trap with a metal tray, supporting a small cage with an animal and having a layer of Castor oil was produced (Disney, 1966), being improved by Dorval et al. (2007), mostly adding a protection from predation, stealing and rain. This trap is very productive for rodent-attracted flies, like *Bichromomyia flaviscutellata* and *Ny. olmeca*, respectively vectors of *L. amazonensis* and *L. mexicana*, but also collected other 10 species, including *Lu. longipalpis* (Dorval et al., 2007).

Miniature light traps have been very useful to collect sand flies, preferably utilizing LED (Silva et al., 2016). Shannon trap, composed by a 3 × 2 × 2 m box of cloth with eaves, preferably utilizing a lampoon with batteries,⁶ fed by photoelectric cells, to attract insects, is very useful (Figs. 5 and 6). Since composition of samples is different with black or white cloth (Brilhante et al., 2017), both types should be used. Due to limitations on the utilization of light traps to evaluate the risk of transmission of pathogens, other methods should be utilized in parallel.

The method of collection is very important for the composition of samples. Several methods must be utilized in the same area, for a more complete knowledge of the fauna, and results from different types of traps cannot be statistically compared. Sampling endophagic species in the Peruvian Andes, the proportion of *Pi. verrucarum* was higher in light trap than in human bait catches, an indication that *Lu. peruensis* is more anthropophilic or less phototropic than *Pi. verrucarum* (Davies et al., 1995). Methods for collecting and processing sand flies were recently reviewed (Alten et al., 2015; Vilela et al., 2018).

Observation of meteorological conditions during the collections is fundamental for the evaluation of biology and ecology of sand flies (Fig. 6).

The identification of blood feeding source is very important, and precipitin, ELISA and Real-Time PCR techniques have been utilized for the identification. The last, for its sensitivity (10 pg to 100 fg) and specificity (Sales et al., 2015) should be chosen if adequate material and equipment are available.

Detection of immature forms

Land may be examined or processed with NaCl or sugar solutions to get the immature forms. The first substance is noxious for the forms and the second may attract house flies, mostly if utilized in open environments (L. M. Deane, 1974 - pers. commun.).

⁶Utilization of electric generators is cumbersome, and gas or kerosene lampoons are too dangerous, I remember to have carried in the beginning of 1974 a heavy Honda generator on the mountains of the primary forest in the southeast of the Brazilian state of São Paulo, and some of our Shannon traps were damaged by contact to lampoons.



Fig. 5 Shannon trap.



Fig. 6 Bench to wait for sundown for the collection by Shannon trap (ST), to collect with human bait and to sustain light source, with a thermo-hygro-anemometer, a GPS and a light source with photoelectric charge. The box (or a back pack) is utilized to transport the ST.

Immature forms may be localized by the utilization of funnels or similar structures on the soil to collect the adults emerging from it, like that of [Casanova et al. \(2013\)](#), derived from the one described by [Ferro et al. \(1997\)](#).

[Casanova et al. \(2013\)](#) obtained good results for the collection of *Lu. longipalpis* (119 of 131 flies) and other sand flies with a soil emergence trap utilizing PVC tubes, with adhesive tape in the inner surface of superior part, covered by voile ([Fig. 7](#)). They collected 57 flies/m² in chicken sheds, with an estimative of production of 121 flies/m² per day.

A detailed study on habitats in Amazon forest, utilizing three techniques (floatation-sieving, Berlese–Tullgren funnels, and direct examination), examined 370 l of material, of which positive samples constituted 59 l. One-hundred eighteen sand flies of 18 species were obtained, mostly from tree bases ([Alencar et al., 2011](#)).



Fig. 7 Emergence trap for sand flies. (A) Fixing the trap to the ground; the serrated bottom facilitates its fixation in the soil. (B) Placing the adhesive paper. (C) Covering the trap with a sand fly-proof fine mesh to prevent sand flies escaping. (D) Traps covered with a small tent. Courtesy of Cláudio Casanova. From Casanova C, Andrighetti MTM, Sampaio SMP, et al. (2013) Larval breeding sites of *Lutzomyia longipalpis* (Diptera: Psychodidae) in visceral leishmaniasis endemic urban areas in southeastern Brazil. *PLoS Neglected Tropical Diseases* 7: e2443.

Proteins specific for immature forms of four laboratory-bred species of sand flies were detected by matrix-assisted laser desorption/ionization time of light mass spectrometry (MALDI-TOF MS), much more efficiently than for biting midges (Halada et al., 2018). By Real Time-PCR, the presence of 1 larva/40 ml of soil could successfully be detected (Giantsis and Chaskopoulou, 2019).

Natural infection

Dissection of flies for the finding of infection, isolating and studying the parasites, has been widely utilized. Briefly, head is cut in saline, and the posterior tip of abdomen is partially separated and pulled in zigzag movement, examining under microscope with a coverslip. Positive material may be inoculated in hamsters and/or cultivated in adequate media. Localization of parasites in the gut can be checked, but there is risk of contamination and loss of samples, and the analysis of thousands of flies is very tiring.

Molecular methods have been utilized. The head and the last two abdominal segments of each fly are dissected for identification, and the rest is processed by several techniques. Material must be preserved at -20°C or in absolute ethanol, and slides and pins utilized for dissection must be washed in potassium hydroxide between flies. After identification of flies, they may be grouped in pools of the same species and origin. If available, techniques for molecular simultaneous identification of flies may be utilized.

A DNA sequencing based multi detection test for the detection and identification of *Leishmania*, blood sources, plant meals and intestinal microbiome was developed (Abbasi et al., 2019).

Tests to evaluate the presence of metacyclics and of other forms of *Leishmania* in sand flies have been recently developed (Giraud et al., 2019; Coutinho-Abreu et al., 2020) and are very useful for the evaluation of their role as vectors. More research to get a marker of gene expression exclusive for these forms is necessary.

Laboratory rearing

For starting colonies, fed females must be put in vials or cloth cages, maintained in dark and very wet environments. Adults are fed on several animals, like mice and hamsters, and even utilizing artificial membranes, according to species and experience of laboratories; chicken may be utilized, but they are rather messy. In the beginning of colonies, flies can refuse to feed on anesthetized rodents, and nude guinea pigs may be utilized. Sugar in cotton balls must be furnished and daily replaced.

A Petri dish with a layer of plaster of Paris must be exposed and saturated by placing the pots in a tray with water 5 days after blood feeding of females, immediately stimulating egg-laying. Dead and living insects must be removed with a vacuum-powered pipette after egg-laying, reducing the chance of larvae eating dead adults and getting infection by gregarines and other pathogens. Eggs must be removed and washed in a 1% sodium hypochlorite solution, rinsed with tap water and placed back into oviposition/rearing pots.

Larvae are fed by a 1:1 mixture of rabbit chow and rabbit feces, preventing proliferation of fungi. These and other techniques, including “tricks” to rear some species, like *Pi. youngi*, are extensively described by [Lawyer et al. \(2017\)](#), which must be consulted.

Although colonies are very important, mostly for insecticide susceptibility tests and studies of biology and experimental infection, this activity is very time-consuming, and there is a high risk of contamination and loss of colonies, probably a factor for so few species having been mass reared.

Control

Factors to help control of leishmaniosis and to worsen it were respectively called *yang* (=clarity: access to funds, collaboration, commitment, effective vaccines, rapid case detection, diagnosis and treatment) and *yin* (darkness: conflict, drug and insecticide resistance, global warming, new parasites/vectors/reservoirs, unknown or inaccessible reservoirs) ([Kamhawi, 2017](#)).

[Alexander and Maroli \(2003\)](#) reviewed several types of transmission, defining vectors, hosts and effect of seasonal variation and human behavior. Methods for the control of leishmaniasis through the control of vectors and reservoirs were recently reviewed ([González et al., 2015](#)).

For the control of sand flies and transmission of *Leishmania* and other pathogenic agents, it is necessary a deep knowledge of the epidemiological situation and biology of vectors and reservoirs, and non-effective interventions that reduce transmission but not eliminate it may worsen the situation due to reduction of bites and immunization by the flies' saliva ([Courtenay et al., 2017](#)). Although several methods have been utilized for the prevention of transmission of *Leishmania*, 35% have not included human-specific outcomes and are difficult to generalize to different geographic locations, being advisable to know more on the epidemiology ([Stockdale and Newton, 2014](#)).

For example, if the cycle is partially or totally anthroponotic, treatment and personal protection, associated to control of vectors, will probably be effective, but if it is zoonotic, the destruction or isolation of reservoirs or breeding sites (e.g. rodent burrows) is useful. If transmission is in or around houses, several methods cited below would be effective, but since no sand fly is exclusively adapted to houses, it is not possible to eradicate any of them, and protection will be effective only when maintained.

Since Carrion's disease has a restricted geographical distribution, lacks non-human reservoirs and may be cured by antibiotics, it is a potentially eradicable disease. However, the invisibility of this disease of very poor populations in isolated rural areas of South America is the Achilles heel of commitment to eradication ([Gomes and Ruiz, 2018](#)).

Insecticides

Adults

For sand flies adapted to domiciles and annexes, the application of several insecticides has produced good results in some conditions. However, the efficacy has been affected by the existence of populations of the same species out of those environments, resistance to insecticides and low knowledge of their biology.

The utilization of DDT, associated to treatment of patients produced a reduction of incidence of VL in the Indian subcontinent. Because of application of DDT for the control of malaria in India, in 1958–1970, no case of VL were reported on Bihar during this period ([Alexander and Maroli, 2003](#)). However, the surging of cases in other areas, difficulty of treatment with new and efficient drug milfetosine ([Selvapandiyan et al., 2019](#)), and symptomatic PKDL (Post Kala-azar Dermal Leishmaniasis) and HIV co-infected patients complicate the control ([Singh et al., 2016](#)). Although pyrethroids have shown to be efficient in the control of the vector *Ph. argentipes*, the persistence of outdoor populations, associated to sleeping outside in part of the year complicates control ([Singh et al., 2016](#)).

DDT was also successfully utilized to control the transmission of *Leishmania* in several countries, also simultaneously controlling bartonellosis in Peru, since the 1940s. Lambda-cyhalothrin also was efficient to control *Pi. verrucarum*, vector of *L. peruviana* and *B. bacilliformis* ([Davies et al., 2000](#)).

Resistance to insecticides has been reported in the Indian subcontinent for *Ph. argentipes*, *Ph. papatasi* and *Sergentomyia shortii*, and some tolerance have been observed ([Alexander and Maroli, 2003](#)).

Deltamethrin and other synthetic pyrethroids have shown some effect on *Lu. longipalpis*, as observed in a test in Bolivia ([Le Pont et al., 1989](#)); it was not effective for *Pi. nuneztovari anglesi*, probably due to its more exophilic behavior. However, a test has indicated

the presence of adults of *Lu. longipalpis* hopping on walls pulverized 2–3 weeks before (Marcondes and Nascimento, 1993), and R. P. Brazil (2009 – pers. commun.) cited the finding of living flies on a surface pulverized 1 week before.

Susceptibility of *Lu. longipalpis* to alpha-cypermethrin varied according to locality in the Brazilian state of Minas Gerais, from susceptibility in a population from a cave (Lapinha) to variable resistance in other localities.

Due to small size of sand flies, non-impregnated bed nets may be useless for protection against bites, but *impregnated bed nets* and *impregnated curtains* are useful for the control of VL and CL (Alexander and Maroli, 2003).

Nyssomyia ovallesi and other species in Venezuela passed through a 6-mm mesh net treated by deltamethrin at 15 mg/m² and could bite volunteers (Felicangeli et al., 1995). Alexander et al. (1995) observed a significant reduction of bites of *Lu. lichyi* and the predominant *Pi. youngi*, under treated nets (64 holes/cm² = 412 holes/in²), and impregnated curtains significantly influenced collections of both species and also of them plus *Pi. columbiana*.

Bed nets impregnated by deltamethrin reduced transmission of *L. major* and *L. tropica* in Iran, while non-impregnated ones did not provide any protection (Yaghoobi-Ershadi et al., 2005; Moosa-Kazemi et al., 2007). In Turkey, Olyset® Plus nets incorporated with 2% permethrin and 1% pyperonyl butoxide (PBO, a synergist) reduced incidence of CL caused by a hybrid *L. infantum/L. donovani*, with *Ph. tobbi* as a proven vector, by 92.2%, and the nets retained full insecticide strength for 12 months (Gunay et al., 2014).

Tests of Permanet® bed nets acquired impregnated by deltamethrin (Long Lasting Insecticidal Nets – LLIN) with three mesh sizes (196 mesh/in. with and without a sand fly-proof 75 cm border and 156 mesh/in.) indicated absence of statistical difference between them; the last was preferred, due to the and smaller reduction on ventilation (Singh and Kumar, 2016).

For *Ph. argentipes*, reducing the size of holes from 156 holes/in² to 625 holes/in² (1 in² = 6.4516 cm²) of non-impregnated nets increased the barrier effect by 60% (56–88%), while impregnating 156 holes/in² nets by alpha-cypermethrin (200 mg/m²) reduced the number of flies under the net by 77% (27–93%) (Das et al., 2014).

Impregnated curtains (permethrin at 50 g/m²) were tested in henhouses in Argentina, reducing the density of *Ny. whitmani* (Acosta et al., 2017), with potential use for the control of *Lu. longipalpis*, which is very attracted by chickens (Alexander et al., 2002). The utilization of curtains impregnated by permethrin (0.5, 1.0 and 1.5 g/m²) in rooms significantly reduced the density of *Ph. papatasi* in Sudan (El-naïem et al., 1999).

Cotton wall cloths impregnated with 0.5 mg/m² permethrin inside houses retained insecticidal effect for 6 months against *Ph. martini* and *Ph. dubosqui* in Kenya, reducing the quantities collected in houses by 76 and 85%, respectively (Mutinga et al., 1992, 1993). These curtains and bednets have been useful for the control of VL by *L. donovani* in East Africa, mostly transmitted by *Ph. orientalis* (El-naïem, 2011).

In visceral leishmaniasis caused by *L. infantum*, mostly in Brazil, it was recommended in the 1950s the association of DDT pulverization in houses and annexes, killing of positive and suspect dogs and treatment of humans. However, positive dogs, which were difficult to find, spend a time infecting flies before being eliminated and were rapidly replaced by young (and susceptible) ones, besides the resistance of owners and associations for protection of animals and ethical problems (Dantas-Torres et al., 2019). The failure of pyrethroids to control *Lu. longipalpis* and the difficulties on diagnosis and elimination of infective dogs stimulated the audacious proposal of reevaluate DDT for the control of VL in Brazil (Marcondes and Costa, 2014).

Collars impregnated by insecticides, direct application and *vaccines* have been successfully tested to prevent infection of dogs, which would not become reservoirs of parasite.

Since Killick-Kendrick et al. (1997) first demonstrated collars impregnated by deltamethrin had a potent antifeeding effect on *Ph. perniciosus* and killed 60% of the insects, many successful tests of these collars were developed. Collars impregnated by 4% deltamethrin (Albuquerque e Silva et al., 2018; Kazimoto et al., 2018) or 10% Imidacloprid/4.5% Flumethrin (Otranto et al., 2013) have been utilized for protection of dogs against infection by *L. infantum* and the control of visceral leishmaniasis, and the last also got a 75% protection of felines (Brianti et al., 2017).

Pyrethroids applied in shampoos have also been utilized for protection of dogs and cats against several biting arthropods, but they need frequent reapplications (Otranto and Wall, 2008). Flumethrin applied *pour on* to dogs was effective to reduce biting by sand flies (no specified species), inhibiting biting and killing 90–100% of exposed flies until 2 months after application (Jalilnavaz et al., 2016). Imidacloprid plus permethrin (Advantix®) applied *pour on* in dogs protect against bites of *Ph. perniciosus* for at least 4 weeks (Bouhsira et al., 2018).

In conclusion, impregnated bed nets and curtains have been shown to be useful in the control of several forms of leishmaniasis, and the association to protection of dogs against bites, where they are reservoirs, may get a good control.

Repellents and clothes. Substances which can be applied on potential sources of blood and cause a reduction on the quantity of bites could be very useful for the control of sand flies and diseases. Several repellents have been shown to be efficacious against their bites. DEET, the less toxic Icaridin and several other substances, also useful for mosquitoes, are efficient to repel sand flies (Islam et al., 2017; Tavares et al., 2018). The above impregnated collars and insecticides exert a role on repellency of sand flies, being recommended for the protection of reservoirs of leishmaniasis (Dantas-Torres et al., 2019).

Sand flies are telmophages, needing to have direct contact to skin, unlike solenophages insects like mosquitoes and triatomine bugs. They will only bite exposed parts of the body, possibly insinuating into sleeves and collars.

Resting sand flies. Chanotis et al. (1982) treated the forest in Panama by a ULV mist of Malathion or tree trunks until the height of 1 m, reporting a reduction on quantities of flies in the second treatment, without defining the affected species.

Since *Ny. umbratilis* is associated to the bases of the larger forest trees and most contact between them and humans occurs when humans are near these trees, with possible infection by *L. guyanensis*, a trial was developed to control the insects in these places. DDT

was applied on the lower 4 m of all large trees (girth > 1 m) in the test area and, although there was a temporary reduction on quantities of flies on the treated trunks, no influence was noticed on collections by CDC traps (Ready et al., 1985).

Such methods obviously would be useful only in very special conditions, and care on pollution of forested areas would be mandatory.⁷

Control of infection by *L. major* in Iran by the control of sand flies utilizing insecticides in rodent (*Rhombomys opimus*) burrows has not been efficacious, but in some trials the elimination of rodents around houses has reduced transmission (Yaghoobi-Ershadi, 2016).

Wolbachia pipientis has been reported in some species of *Phlebotomus* (Benlarbi and Ready, 2003; Bordbar et al., 2014) and in lower proportions of *Lu. longipalpis* (Rocha et al., 2018), being a potential tool for the control of these insects and related diseases.

Immature forms

Insecticides

Since larvae and pupae of sand flies are very difficult to find, it has been difficult to apply methods of control for them.

Ivermectin at 2–100 ppm was added to food of hamsters, and larvae of *Ph. papatasi* eating feces of animals fed with all concentrations had significantly reduced survival, and all eating on feces of 20–60 ppm died (Mascari et al., 2008). The chitin synthesis inhibitors diflubenzuron (Mascari et al., 2007) and novaluron (Mascari and Foil, 2010) also impaired respectively the larva-pupa and larval molts. The insecticide fipronil killed all flies 28 days after ingestion by hamsters, also being efficacious against larvae (Mascari et al., 2013b). Baits with these and other toxic substances could be useful for the control of rodent-associated sand flies.

A study in a village at Bihar (North of India) showed that fipronil orally administered to cattle, affecting adults feeding on cattle and larvae feeding on their feces, would be successful to control *Ph. argentipes* and visceral leishmaniasis (Poché et al., 2016).

Due to the importance of *Ph. papatasi* as vector of *L. major* and the availability of several laboratory colonies (Lawyer et al., 2017), several tests like the above cited have been developed on this sand fly, but their effect on other important rodentophilic species, like *Bi. flaviscutellata* and *Ny. olmeca*, should also be developed.

Alternative methods

Environmental modification can be useful to control certain sand flies. In French Guiana, a village (Cacao) with *Ny. umbratilis* infected by *L. guyanensis* had a 11-ha area around it deforested, and insecticides were applied by fog in the village, causing a great reduction of incidence (Esterre et al., 1986).

The cleaning of the area around houses and henhouses, associated to the cutting of trees, reducing the shadow and drying the soil, associated to the utilization of insecticides in the houses and annexes, in the Brazilian state of Paraná reduced in 3.4 times the hourly means of collected sand flies (Teodoro et al., 2003). Due to the above cited association of *Ph. papatasi* to burrows of rabbits, not susceptible to infection by *L. major*, the promotion of their breeding near houses could be useful to control infection (Chelbi et al., 2008).

Feeding of sand flies of four species on *Cannabis sativa* in five regions was reported (Abbasi et al., 2018). Since the breeding of this plant is forbidden in most countries and it is certainly rare and hidden, this finding indicates a preference for it. They could even be utilized for localization of plantations, and the smell of the plants should be tested for attraction and killing of sand flies.

Ecological techniques for the association between adults and immature forms and for the control of rodent-associated sand flies, mostly utilizing isotopes, were studied by Mascari et al. (2013a).

Bacillus thuringiensis var. *israelensis* (Bti) added to sugar baits at concentrations of 4.4×10^2 – 10^4 killed 100% of *Ph. papatasi*, *Ph. argentipes* and *Lu. longipalpis* (Yuval and Warburg, 1989). Bti serotype 14 killed 100% of larvae of *Ph. perniciosus* after 6 days of exposition (Majori and Maroli, 1893). Also *B. sphaericus*, sprayed at the entrances of animal burrows in Kenya, or, diluted in sugar baits, onto the vegetation, caused great mortality to sand flies (Robert et al., 1997).

Attractive Toxic Sugar Baits (ATSB), when applied on vegetation and barrier fences in an arid region (Jordan Valley) caused respectively a reduction on samples to 6 and 12% of those in control areas (Müller and Schlein, 2011). In Morocco, ATSB caused a significant reduction on populations of *Ph. papatasi* and *Ph. sergenti* (Qualls et al., 2015). This method would be, like in other insects, promising for the control of sand flies, but should be tested in more rich and diversified environments, like tropical forests.

Some plants were shown to be toxic to *Ph. papatasi* (Schlein et al., 2001): *Solanum jasminoides* (Solanaceae), *Ricinus communis* (Euphorbiaceae) and *Bougainvillea glabra* (Capparaceae). The last is the most promising and could be cultivated around houses. More research is needed on this approach in other regions.

Different members of the *L. longipalpis* species complex in Brazil produce different sex/aggregation pheromones (Hamilton et al., 2005). Synthetic copies of these pheromones present potential as tools for survey and monitoring, if associated with insecticides. This lure-and-kill method can be used for their control and reduction in *Leishmania* transmission (Bray et al., 2010, 2014; Courtenay et al., 2019).

⁷In the 1980s, an undergraduate student of mine proposed in an examination to apply insecticides by plane on forests to control leishmaniasis, and in the 2000s a man from Florianópolis proposed the same on mangrove swamp, to control biting midges, not noxious because “the area was small.”

The kairomone lactic acid was useful for surveying sand flies in the Brazilian state of Maranhão, when associated to incandescent lamps, but not to diode lights, while octenol did not produce significant increases on quantities of flies collected (Silva et al., 2019).

Vaccination

This tool may be useful, mostly to protect humans and reservoirs from infection. Leishmanization has been utilized for several centuries in Middle East and from the beginning of 20th century also in former Soviet Union for the protection against cutaneous lesions caused by *Leishmania*. Exudate from a lesion was inoculated in a covered part of the body, to prevent lesions in the face. *Leishmania major* has been utilized, providing crossed protection against *L. tropica* (Mohebbi et al., 2019). Promastigotes from *L. tropica* were inoculated from 1910 on, just after the production of NNN medium in 1908. Although leishmanization has been shown as useful for the protection against infection, a selection of less pathogenic strains would be fundamental.

From these old techniques, vaccines utilizing selected epitopes of *Leishmania* and recombinant vaccines are being searched in several laboratories (Gillespie et al., 2016).

Vaccines have been intensely searched, and the saliva from several species of sand fly (*Phlebotomus* and American species) has an immunomodulatory effect on infection and may be useful as part of a vaccine (Kamhawi et al., 2014). Other adjuvants have been analyzed (Gonçalves et al., 2019).

For visceral leishmaniasis caused by *L. infantum*, vaccines Leishtec® and Leishmune® for the immunization of dogs have got 60–80% of protection of dogs (Oliva et al., 2014; Wylie et al., 2014), and Canileish® produced a significant reduction on the quantity of *Ph. perniciosus* fed on vaccinated dogs (Bongiorno et al., 2013). Preliminary tests on new vaccines (Leish-F1 and ChAd63-KH DNA) produced good results to protect humans against infection by *Leishmania* (Moafi et al., 2020). Specific serological biomarkers are needed to differentiate immunity against infection and that induced by Canileish® (Lima et al., 2019).

Conclusion

Due to the importance of diseases related to sand flies, which are very diversified and adaptable, these flies deserve greater attention from researchers and public health authorities. Breeding places need to be localized and delimited, and movement of adults needs to be studied, to improve barriers and control. Leishmaniasis are complex diseases, caused by several parasites and influenced by many environmental factors, by the reaction of affected hosts and the biology and physiology of vectors. Other poorly known diseases, like Carrión disease and arboviroses need to be more attentively studied and controlled. Their control can only be obtained if the epidemiology of diseases is well known in each locality and situation. New methods for the control of sand flies and related diseases, like the association of pheromones to insecticides and a better understanding of the relationship between gut microbiota and *Leishmania* would be very useful.

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Tsetse Flies (Glossinidae)

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Glossary

Animal African trypanosomiasis (AAT) Also known as nagana or cattle wasting disease. AAT is caused by *Trypanosoma B. brucei*, *T. congolense*, *T. vivax*, *T. evansi*, and *T. equiperdum* in Africa. The former four types are transmitted by tsetse flies.

Adenotrophic viviparity Specific reproductive mechanism of *Glossina* and closely related species where larvae are nourished with secretions produced by a modified accessory gland, and live larvae develop within a common oviduct (or uterus). This reproductive strategy is a type of matrotrophic viviparity, where nutrients are provided by the mother beyond those in the oocyte before live birth.

Hippoboscoidea A dipteran superfamily that includes Glossinidae (tsetse flies), Hippoboscidae (louse flies, sheep keds), and Streblidae and Nycteribiidae (bat flies). All members reproduce by adenotrophic viviparity and feed solely on blood.

Human African trypanosomiasis (HAT) The disease caused by *Trypanosoma brucei gambiense* (Gambiense HAT, represent 98%) and *T. b. rhodesiense* (Rhodesiense HAT).

Milk gland In all Hippoboscoidea, a modified female accessory gland that provides nutritional products to the developing intrauterine larvae. All nutritional products required for larval and pupal development, beyond those in the oocyte, are provided by this gland.

Obligate hematophagy (=sanguinivory) The acquisition of nourishment by feeding exclusively on blood (hematophagy or sanguinivory).

Wigglesworthia spp. A gram-negative, obligate bacterial endosymbiont of tsetse flies. This symbiont provides specific B vitamins critical to tsetse growth and development, and stimulates the maturation and function of the fly's immune system.

Introduction

Tsetse flies (Fig. 1) represent peculiar dipteran species, and their competence as vectors of African trypanosomes make them a critical pest for human health and for agricultural development. In this article, we provide a general synopsis of tsetse fly biology covering (1) their basic ecology and physiology, (2) their ability to serve as a vector for African trypanosomes, (3) interactions with their indigenous microbiota (Weiss and Aksoy, 2011; Wang et al., 2013; Rio et al., 2016), and (4) unique aspects of their reproductive biology (Tobe, 1978; Benoit et al., 2015). These topics are followed by a discussion of strategies currently utilized to control trypanosomiasis, with a focus on methods that aim to reduce tsetse population size. This article provides an encompassing synopsis of *Glossina* biology. Throughout the article, we highlight review papers and primary literature that cover specific topics of *Glossina* biology in more detail.

Tsetse flies (Glossinidae) consist of 33 extant taxa composed of 22 distinct species (five subspecies groups) and three subgenera; *Fusca*, *Palpalis*, and *Morsitans* (Krafsur, 2009). A fourth subgenera, *Machadomia*, includes *Glossina austeni*, which based on recent studies, likely falls within the *Morsitans* group (Gooding and Krafsur, 2005; Attardo et al., 2019). *Morsitans* flies are largely savanna



Fig. 1 Female tsetse fly, *Glossina morsitans*. Photo by Geoffrey M. Attardo.

and woodland inhabitants, while *Palpalis* flies commonly reside in riverine and lacustrine habitats (Rogers and Robinson, 2004). Individuals within the *Fusca* subgenera inhabit the moist forests of West Africa with the exception of *G. brevipalpis* that has discontinuous distribution in East Africa, Democratic Republic of the Congo and Mozambique. Host specificity varies greatly between groups, and *Palpalis* group flies are anthropophilic while *Morsitans* and *Fusca* are more zoophilic. The genomes of six tsetse species, including members of all four subgenera, have been sequenced (International Glossina Genome Initiative, 2014; Attardo et al., 2019). These tsetse species exhibit significant differences in geographical distribution, habitat preferences, host choice, and in their ability to act as disease vectors (Fig. 2).

Tsetse flies are obligate blood feeders that serve as the primary cyclic vectors for old world trypanosomes, the causative agents of human and animal African trypanosomiasis (HAT and AAT, respectively) in sub-Saharan Africa. These diseases inflict profound socio-economic hardships on indigenous communities by compromising the health of humans and domesticated animals and thus hampering development across large geographic regions (Sutherland et al., 2015; Aksoy et al., 2017). HAT is caused by the protozoan parasites *Trypanosoma brucei gambiense* (Gambiense HAT, represent 98% of cases) and *T. b. rhodesiense* (Rhodesiense HAT, represents 2% of cases). *T. b. gambiense* causes a chronic form of HAT in West Africa, while *T. b. rhodesiense*, localized to eastern and southern Africa, is responsible for an acute form of the disease (Simarro et al., 2012). Both of these diseases, which are fatal if left untreated, are associated with rural areas where individuals are exposed during outdoor activities. At least 70 million people across 36 countries are at risk for HAT (Büscher et al., 2017). HAT infection progresses through two stages, the early hemolymphatic stage, during which parasites multiply and spread in the bloodstream, lymphatic system, and peripheral organs, and the late encephalitic phase when the parasites cross the blood-brain barrier and proliferate in the cerebral spinal fluid (MacLean et al., 2012; Kennedy, 2013). A HAT epidemic beginning in the 1990s has to date resulted in about 500,000 cases (Barrett, 1999; Aksoy et al., 2017). Coordinated control efforts by the World Health Organization, African governments, and other groups has reduced the number of new cases to 2000–4000 per year (Aksoy et al., 2017; Büscher et al., 2017). A current goal is the elimination of HAT by 2030, through a combination of control protocols that range from infection treatment to vector population suppression (discussed later in the article). Many more targeted reviews of HAT are available (Büscher et al., 2017; Baker and Welburn, 2018; Bottieau and Clerinx, 2019; Kennedy, 2019).

Along with the impact on human health, AAT, or nagana, is a chronic wasting disease that significantly suppresses livestock production and thus food security in sub-Saharan Africa. The two main causative agents of AAT are *T. b. brucei* and *T. congolense* (Namangala and Odongo, 2014; Morrison et al., 2016; Radwanska et al., 2018). Beyond those parasites limited to sub-Saharan Africa, others such as *T. evansi* (Desquesnes et al., 2013), *T. equiperdum* (Claes et al., 2005) and *T. vivax* (Jones and Dávila, 2001) exhibit more expansive distributions across tropical regions (Jones and Dávila, 2001). Outside of Africa, these parasites are either sexually transmitted (usually between livestock) or mechanically vectored (no biological development in the fly) by hematophagous flies other than *Glossina* (e.g. stable flies, *Stomoxys* or tabanid flies). Within Africa, *T. evansi* and *T. vivax* can be mechanically or cyclically (developmental stages occur in the fly) transmitted by tsetse flies. The economic and social burden of AAT is substantial as sustainable livestock production is suppressed, which could yield losses of US\$ 4–5 billion per year (Budd, 1999; Kristjanson et al., 1999).

***Glossina* as a vector of African trypanosomes**

To successfully transmit between vertebrate hosts, biologically transmitted African trypanosomes must complete a rigorous journey (which takes between 15 and 30 days depending on genetic and ecological factors) through their obligate tsetse vector (Rotureau and Van Den Abbeele, 2013). Specifically, upon ingestion of an infectious blood meal, “stumpy” bloodstream form (BSF) parasites pre-adapted for survival in tsetse must differentiate into insect stage procyclic form (PCF) cells and infect and replicate within the

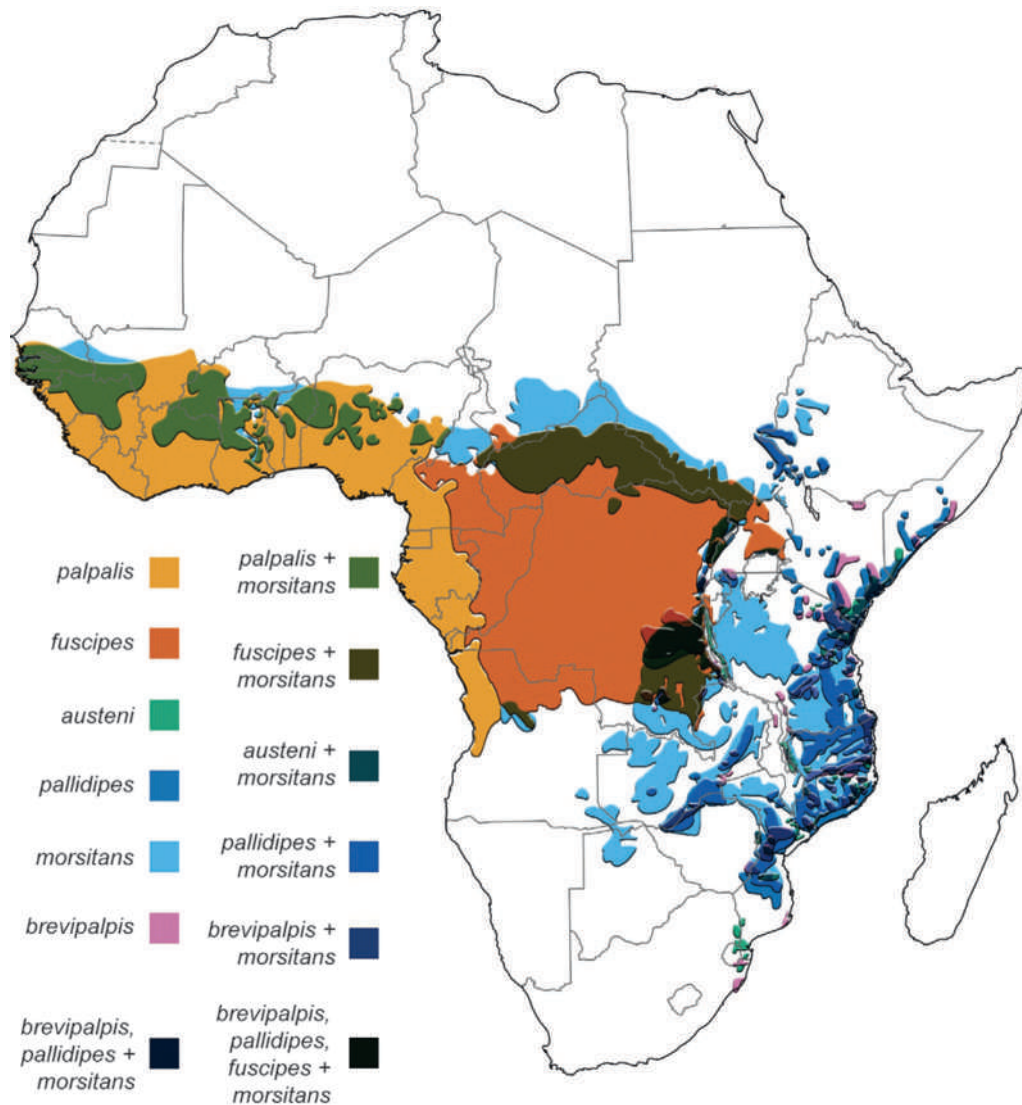


Fig. 2 Geographical distribution of specific *Glossina* species across sub-Saharan Africa that have genomics-based resources (Attardo et al., 2019).

fly's midgut. PCF parasites then move into the tsetse's immunologically hostile cardia organ (also known as proventriculus), where they differentiate into epimastigote forms (EMF). In some flies, EMF cells are unable to advance past this stage (Vigneron et al., 2018), in which case that lineage is not transmitted to another vertebrate host and thus lost. EMF parasites that make it through tsetse's cardia migrate back into the fly's foregut and then salivary glands (SGs) where they replicate and complete their final differentiation into vertebrate-infectious metacyclics (MCF).

Despite feeding regularly on infected vertebrate reservoirs, only between 1% and 10% of tsetse harbor transmissible trypanosome infections (Aksoy et al., 2003; Abdi et al., 2017). This low infection prevalence is reflective of robust passive and active gut-associated innate immune mechanisms. Active antimicrobial immunity in tsetse's midgut involves the production of reactive oxygen intermediates (ROIs) (MacLeod et al., 2007; Vigneron et al., 2018), immune deficiency (IMD) pathway-associated antimicrobial peptides (AMPs) (Hao et al., 2003; Hu and Aksoy, 2005, 2006), peptidoglycan recognition protein LB (PGRP-LB) (Wang et al., 2009; Wang and Aksoy, 2012) and tsetse EP protein (Haines et al., 2010). Passive immunity in this environment is provided by the fly's peritrophic matrix (PM). This sleeve-like structure, components of which are constitutively secreted by tsetse's cardia organ and midgut (Lehane et al., 1996), lines the fly's midgut and separates epithelial cells from ingested products and pathogens (Hegedus et al., 2009). Mature adult tsetse with a fully formed PM are more refractory to trypanosome infection than are young adults that present an immature PM structure (Walshe et al., 2011; Haines, 2013). Furthermore, experimental depletion of PM integrity through the use of RNAi results in a significant increase in infection prevalence in otherwise resistant adults (Weiss et al., 2014). Tsetse's PM thus serves as a physical barrier that mediates the ability of trypanosomes to establish an infection in their insect vector's midgut. This structure also regulates the fly's ability to immunologically detect and respond to exogenous bacteria (Weiss et al., 2014).

Tsetse and its associated bacterial microbiota

While only a small percentage of tsetse are infected with trypanosomes, all flies house a population of indigenous bacterial endosymbionts that are intimately associated with their host's biology (Fig. 3). All tsetse harbor the obligate mutualist *Wigglesworthia*, which has been associated with the fly for 50–80 million years (Chen et al., 1999). This bacterium is found intracellularly within bacteriocytes that collectively form tsetse's bacteriome organ (located immediately adjacent to the fly's anterior midgut), and extracellularly within maternal milk (see below). *Wigglesworthia*'s chromosome encodes several complete B vitamin synthesis pathways, the products of which are likely synthesized to supplement the fly's vertebrate blood specific diet (Akman et al., 2002; Rio et al., 2012, 2019). Furthermore, *Wigglesworthia* exhibits immuno-stimulatory properties in that the bacterium must be present in developing intrauterine larvae in order for subsequent adults to present functional passive and active immune barriers (Weiss et al., 2011, 2012, 2013; Benoit et al., 2017). In addition to *Wigglesworthia*, all laboratory colonized tsetse, and most natural fly populations (Mbewe et al., 2015; Wamwiri et al., 2014; Channumsin et al., 2018; Kame-Ngasse et al., 2018 REFS), harbor a secondary commensal symbiont from the genus *Sodalis*. *Sodalis*' acquisition by tsetse is relatively recent (~1 million years; Aksoy et al., 1997), and strains from different tsetse species are closely related, suggesting horizontal transmission or recent independent acquisition of the symbiont (Aksoy et al., 1997; Toh, 2005). The bacterium resides both intracellularly and extracellularly within tsetse's midgut, hemolymph and salivary and milk glands. *Sodalis*' function within tsetse is largely unknown, although it has been associated with fly longevity (Dale and Welburn, 2001) and susceptibility to trypanosome infection (Welburn et al., 1993; Dale and Welburn, 2001). Finally, some tsetse populations house parasitic *Wolbachia* and *Spiroplasma* (Doudoumis et al., 2017; Schneider et al., 2019), both of which likely induce host reproductive abnormalities.

Field-captured tsetse house a midgut-associated population of environmentally acquired microbes (Geiger et al., 2009, 2010; Lindh and Lehane, 2011; Aksoy et al., 2014; Griffith et al., 2018). This microbial population is transient and comprises only a small percentage of the fly's total microbiota. The functional role of tsetse's environmentally acquired microbes is currently unknown.

Tsetse fly ecology, feeding, and metabolism

General characteristics

Although tsetse flies have overlapping distribution across sub-Saharan Africa, different species often reside within distinct ecological niches. Tsetse are divided into three major ecological groups that consist of savannah (*Morsitans* sub-genus), riverine (*Palpalis* sub-genus), and forest (*Fusca* sub-genus)-associated species (Fig. 4). The general trend is that those of the *Palpalis* and *Fusca* groups prefer relatively moist regions with denser vegetation cover, while the *Morsitans* group prefers more open areas. The specific ecological

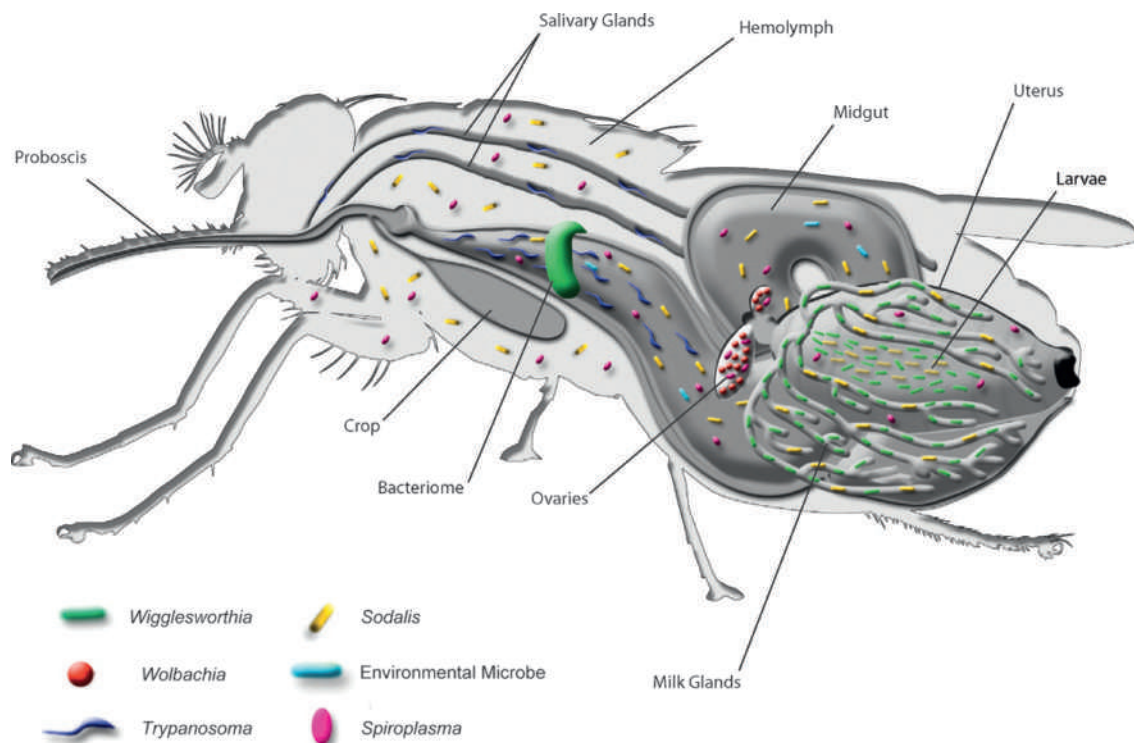


Fig. 3 Distribution of microbial agents within tsetse flies.



Savannah Flies (*Morsitans* sub-genus)

Glossina austeni
Glossina morsitans morsitans
Glossina morsitans centralis
Glossina pallidipes
Glossina swynnertoni



Riverine Flies (*Palpalis* sub-genus)

Glossina fuscipes fuscipes
Glossina palpalis gambiensis
Glossina caliginea
Glossina fuscipes martini
Glossina fuscipes quanzensis
Glossina pallicera pallicera
Glossina pallicera newsteadi
Glossina palpalis palpalis
Glossina tachinoides



Forest Flies (*Fusca* sub-genus)

<i>Glossina brevipalpis</i>	<i>Glossina nashi</i>
<i>Glossina fusca fusca</i>	<i>Glossina nigrofusca nigrofusca</i>
<i>Glossina fuscipleuris</i>	<i>Glossina severini</i>
<i>Glossina frezili</i>	<i>Glossina schwetzi</i>
<i>Glossina haningtoni</i>	<i>Glossina tabaniformis</i>
<i>Glossina longipennis</i>	<i>Glossina vanhoofi</i>
<i>Glossina medicorum</i>	

Fig. 4 Ecological characteristics associated with the habitats for savannah (*Morsitans* sub-genus)-, riverine (*Palpalis* sub-genus)-, and forest (*Fusca* sub-genus)-associated species.

zones have also been divided based on levels of aridity, where *Morsitans* flies prefer arid to semi-arid regions, *Fusca* flies in sub-humid to humid areas, and *Palpalis* flies from semi-arid to humid regions (Jahnke and Jahnke, 1982; Leak, 1999). Specific vegetation types are associated with each species, which they utilize mostly as resting sites (Challier, 1982; Leak, 1999). Choice of resting site varies significantly on both daily and seasonal cycles, and a major contributing factor is thermoregulation and maintenance of water balance (MacLennan et al., 1972; Turner, 1980). Specifically, tsetse will reside on the sunny side of vegetation during cool periods and retreat into lower, shaded areas during warmer times (Laveissière et al., 1978; Challier, 1982). The preferred height of vegetation resting sites varies between species but does show a general trend that as plant height increases the numbers of resting tsetse decrease. In addition, resting sites are at lower heights during the day and the dry season (Challier, 1982; Leak, 1999). Larviposition sites vary between species within the three sub-genera, but most females deposit their larvae in shaded areas with soil qualities that facilitate larval burrowing (Challier, 1982; Leak, 1999). Extensive details on tsetse fly ecology can be found in reviews by Challier (1982) and Leak (1999).

Circadian biology and seasonality

Daily rhythms and seasonality are critical to tsetse fly biology. Multiple aspects related to tsetse's behavior and physiology have been examined in relation to circadian rhythms (Brady, 1972a, 1972b; Nash and Trewern, 1972; Crump and Brady, 1979; Van der Goes van Naters et al., 1998). An excellent review of tsetse fly circadian biology examined key rhythmic aspects among multiple *Glossina* species (Brady, 1988). Most aspects of tsetse biology exhibit a U-shaped pattern, with increased general activity, such as host seeking and probing, occurring more frequently during dawn and dusk (Fig. 5). The impact of starvation will increase the peaks of activity, while feeding and pregnancy status will suppress daily changes (Brady, 1988). Importantly, not all biological characteristics of tsetse flies have the U-shaped pattern. For example, larviposition and eclosion occur more frequently in the evening (Fig. 5). Seasonal changes in rainfall and temperature exert significant behavioral and physiological changes across *Glossina* species (Challier, 1982). Additionally, microclimates, local vegetation, and the movement/availability of potential hosts have a significant impact on local tsetse population density during each season (Hargrove and Vale, 1980; Diallo, 1981). Climate change is predicted to have a significant impact on the survival and distribution of *Glossina* across Africa (Kleynhans and Terblanche, 2011; Messina et al., 2012; Lord et al., 2018).

Host location

Tsetse flies locate and decide to feed on their host through a combination of visual, thermal, olfactory, and tactile cues (Torr, 1989, 1990; Torr et al., 1995; Tirados et al., 2011; Soni et al., 2019). Vision and olfaction are the two most well studied aspects of tsetse host seeking. Recent genome sequencing has allowed the identification of the chemosensory genes and significant process has been made in tsetse chemosensation (Obiero et al., 2014; Macharia et al., 2016). Many specific host derived chemicals have been examined for their ability to attract tsetse flies (Vale, 1980; Vale and Hall, 1985; Torr and Vale, 2015). Most of these studies focused on the identification of artificial baits for traps or repellents for animals or humans (Wachira et al., 2016). The specific odorants that have attractant properties can be divided into those derived from animal breath (acetone, octenal, CO₂), skin (sebum-based), and urine (phenols). Recent studies have examined the response of *Glossina* to chemical cues at the molecular and sensilla levels (Chahda et al., 2019; Soni et al., 2019), where these analyses have linked specific sensilla and molecular receptors to both chemical cues and repellants (e.g. CO₂-sensitive basiconic sensilla were identified). These combinatory analyses are likely to improve traps to increase tsetse capture rate and be able to target multiple *Glossina* species. As an example, 2-propanol was identified as a target that would effectively trap two tsetse fly species, *G. morsitans* and *G. fuscipes* (Chahda et al., 2019).

Visual and thermal cues are critical for tsetse flies to locate a host (Torr, 1989; Vale, 1993). Black and blue colored targets, as opposed to those of lighter color (red, yellow, and white), are preferred tsetse landing sites. Specific shapes, patterns, and the movement of targets are more or less desirable depending on the specific characteristics (Torr, 1989; Tirados et al., 2011; Torr et al., 2011; Lindh et al., 2012). A lack of UV reflectance from the materials is likely a critical aspect in relation to attraction (Lindh et al., 2012). Thermal cues, along with CO₂, likely act as close range cues prompting *Glossina* landing (Willemse and Takken, 1994; Lazzari, 2009).

Host preference

The combination of the above-described host cues, along with animal availability, likely allows each tsetse fly species to feed on their preferred hosts. Studies on tsetse host preference have used observation of feeding patterns, serological assays (such as an enzyme-linked immunosorbent assay, ELISA), and more recently, molecular analysis of bloodmeals (Clausen et al., 1998; Leak,

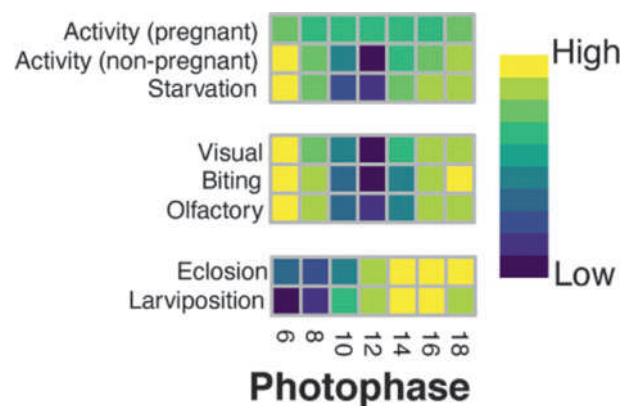


Fig. 5 Circadian rhythm changes in tsetse flies in relation to the biological status of these flies. Data is based upon previous studies that have assessed daily changes in tsetse fly behavior (Crump and Brady, 1979; Brady, 1988). Photophase is in hours since midnight. *Visual*, response to moving stimuli; *Biting*, probing by tethered male on warm sponge ball; *Olfactory*, response to human body odor; *Starvation*, increases general activity without altering the higher levels at dawn and dusk; *Eclosion*, adult emergence; *Larviposition*, female birth.

1999; Njiokou et al., 2004; Steuber et al., 2005; Auty et al., 2016; Gaithuma et al., 2020). Importantly, ingestion of blood from multiple host types serves as an adequate source of nutrients for tsetse (Moloo et al., 1988). Thus, the nutritional value of blood does not appear to mediate tsetse host selection. Early findings based upon precipitin ring or haemagglutination inhibition tests established five *Glossina* groups based upon feeding profiles (Weitz, 1963). Group 1: Species that feed mainly on Suidae (*G. swynnertoni*, *G. austeni*, *G. tabaniformis*, *G. fuscipleuris*), Group 2: Species that feed on both Suidae and Bovidae (*G. morsitans morsitans*, *G. m. submorsitans*), Group 3: Species that mainly feed on Bovidae (*G. pallidipes*, *G. longipalpis*, *G. fusca*), Group 4: Species that feed on mammals other than pigs and bovids (*G. longipennis*, *G. brevipalpis*), and Group 5: Species that feed on the most available species, including humans (*G. palpalis palpalis*, *G. fuscipes fuscipes*, *G. tachinoides*). Local variations do occur, where the main host species for a specific tsetse fly species is replaced by another more abundant or other preferred host species (Moloo, 1993; Clausen et al., 1998). Interestingly, some abundant animals, such as zebra, are not used by tsetse as hosts (Moloo, 1993; Clausen et al., 1998). This is likely due to specific host cues (Olaide et al., 2019) and striped colors that disrupt landing of flies (Caro et al., 2019). More details on tsetse host preference has been reviewed by Moloo (1993), Leak (1999), and Clausen et al. (1998).

***Glossina* feeding, digestion, and metabolism**

Following host location, heat and humidity cues induce tsetse flies to commence skin probing behaviors (Chappuis et al., 2013). Tsetse may probe multiple sites on a host before beginning to feed (Leak, 1999). Chemical stimuli detected by the fly's mouthparts, such as ATP (Margalit et al., 1972; Rice et al., 1973), are also likely to stimulate feeding. While probing and feeding, *Glossina* will discharge saliva into the host. Early studies identified that this saliva is important to the process of feeding, and recent projects have identified most of the major components of tsetse saliva (Telleria et al., 2014; Matetovici et al., 2016). Salivary gland enriched genes, which consist predominantly of highly expressed secreted peptides, were identified through the use of RNA-seq (Telleria et al., 2014; Matetovici et al., 2016). Multiple proteinaceous components of tsetse saliva have been assigned functions, including anticoagulant activity, DNA/RNA nucleic acid binding, suppression of platelet aggregation, and allergenic activities (Mant and Parker, 1981; Cappello et al., 1996; Li and Aksoy, 2000; Li et al., 2001; Alves-Silva et al., 2010). When tsetse flies have salivarian trypanosome infections, many, if not all, of the salivary proteins are reduced at the transcript, and likely protein, level (Telleria et al., 2014; Matetovici et al., 2016). This decrease in saliva proteins is likely due to trypanosome-induced immune and stress responses within the tsetse fly SGs (Telleria et al., 2014; Matetovici et al., 2016). The reduced levels of saliva proteins underlie inefficient and more frequent feeding (Van Den Abbeele et al., 2010), which is likely to increase parasite transmission.

Tsetse flies feed exclusively on vertebrate blood, which contains abundant proteins and lipids but few carbohydrates. Recent observations suggest that tsetse flies may also utilize plant-derived sugars as a nutrient resource (Solano et al., 2015). However, the ecological importance of this finding has yet to be established and it has yet to be validated by other research groups. The size of tsetse blood meals is species and sex specific (Moloo and Kutuza, 1970; Taylor, 1976), and the large volume of warm blood ingested can cause thermal stress (Lahondère and Lazzari, 2015; Benoit et al., 2019). When the blood is ingested, most flows past the cardia into the crop, causing the fly to become reddish immediately after feeding. As blood passes back through the cardia it becomes encased within the fly's PM. This structure acts to prevent direct interaction between components of the blood meal and the fly's midgut epithelium (Hegedus et al., 2009). Importantly, tsetse's PM also plays a critical role in the ability of trypanosomes to establish an infection in tsetse's midgut and migrate to the fly's SGs from which they transmit to a new vertebrate host (Weiss et al., 2014; Aksoy, 2019; Rose et al., 2020; Vigneron et al., 2020). While in the gut, the blood meal will become a darker brown as digestion advances. The bloodmeal consists mostly of water, which is rapidly excreted (a few hours) via diuresis after the termination of feeding (Benoit et al., 2014b). This is a common physiological strategy among blood feeding insects to reduce excess water from blood feeding (Benoit et al., 2014c).

Following elimination of excess water, digestion occurs through a suite of specific digestive enzymes that break down lipids and proteins (Langley, 1967; Leak, 1999). Early studies on this process were conducted by Sir Vincent B. Wigglesworth (1929) and a synopsis of tsetse digestive processes can be found in Leak (1999). The full suite of proteins involved in tsetse digestion have now been thoroughly characterized by transcriptome studies (Bateta et al., 2017). The initial process involves the breakdown of red blood cells by hemolysin (Gooding, 1977). This process is followed by the secretion of multiple peptidases and proteinases, which act to induce rapid digestion of the proteinaceous components of the blood. Free amino acids and lipids generated from the breakdown of blood are used to accumulate lipid reserves. The process of lipid accumulation is critical to success of tsetse's pregnancy cycle, as lipids are a prominent component of the milk that pregnant female flies generate to feed their intrauterine larvae (more information provided below, Moloo, 1976a, 1976b; Benoit et al., 2015). Intervals between feeding events vary from 36 h to nearly 5 days depending on environmental conditions (Leak, 1999). The process of digestion occurs most rapidly at higher temperatures (McCue et al., 2016), but more efficient digestion decreases the ability of the flies to tolerate starvation and dehydration between each blood meal. Waste products generated include excess ions, specific amino acids, and nitrogenous waste that is excreted in the form of uric acid (Leak, 1999).

An interesting physiological consequence of tsetse's reliance on vertebrate blood as its sole nutrient source is the fly's transition to utilizing proline as energy to fuel metabolically expensive reproductive processes and flight (Bursell, 1977, 1981; Michalkova et al., 2014b). Specifically, tsetse converts proline to alanine within muscles (and other organs), which provides biochemical products that can be utilized in the tricarboxylic acid cycle. This process represents a substantial variation from other dipteran systems, which rely on carbohydrate-based factors (mainly trehalose) as a rapid source of energy necessary for activities such as flight (Wyatt and Kale, 1957; Bursell, 1981; Thompson, 2003). Tsetse catabolize lipids to produce acetyl-CoA in their fat body, which

facilitates the regeneration of proline from alanine (McCabe and Bursell, 1975; Bursell, 1977; Norden and Matanganyidze, 1977). The process of proline metabolism is controlled by the release of adipokinetic hormone (AKH) from the corpora cardiaca (Pimley and Langley, 1982; Attardo et al., 2012; Caers et al., 2016). Proline homeostasis is critical to the survival of procyclic trypanosomes that reside in tsetse's midgut, and interference with the metabolism of this amino acid prevents *T. brucei* from establishing an infection in the fly's midgut. Specific B vitamins generated by obligate *Wigglesworthia* are required to maintain proline homeostasis (Michalkova et al., 2014b), and when this symbiont is eliminated via antibiotic treatment, homeostasis of tsetse nutrient reserves and circulating proline becomes impaired. The critical metabolic roles of proline in tsetse and trypanosome biology highlight the importance of this amino acid in relation to nutritional homeostasis of this vector-pathogen system.

Tsetse fly life cycle

Basic characteristics of *Glossina* viviparity

Tsetse flies reproduce via a process called "matrotrophic viviparity." In this case nutrients are provided by the mother to her developing intrauterine larvae (Tobe, 1978; Benoit et al., 2015). This form of reproduction has also been described as "adenotrophic viviparity," which is defined as 'glandular fed before live birth' and is a more tsetse-specific designation. This reproductive strategy represents a marked deviation from oviparity (egg laying) used by most insect systems that do not provide nourishment beyond the embryonic (egg) stage. Although rare, evolution of live birth systems across insects has occurred multiple times (Kalinka, 2015). In utero larvae are provided nourishment in the form of milk that is secreted from specialized glandular organs (Hagan, 1951; Benoit et al., 2015). Adenotrophic viviparity is a common characteristic among the superfamily Hippoboscoidea, which includes bat flies and sheep keds (Lenoble and Denlinger, 1982; Meier et al., 1999).

Tsetse have undergone three significant morphological adaptations that facilitate their reproduction via adenotrophic viviparity (Fig. 6). First, tsetse ovaries only produce a single oocyte per gonotrophic cycle. Second, the reproductive tract has been greatly expanded and surrounded by tracheated, muscle-lined epithelial cells that collectively compose the uterus (Tobe, 1978; Pellegrini et al., 2011; Benoit et al., 2015). Within the uterus, cuticular projections form horns, known as the choriothete, that serve to remove the egg chorion and larval cuticle and anchor the larvae in the correct position to ensure it can properly feed (Saunders, 1960; Tobe and Davey, 1971; Pellegrini et al., 2011). The last modification is that the female accessory gland is expanded from a few cells common in other fly systems (Attardo et al., 2014) into a large, branched organ consisting of thousands to millions of cells (Hagan, 1951; Tobe, 1978; Benoit et al., 2015).

Females deposit a single larva each gonotrophic cycle (GC) (Tobe, 1978; Benoit et al., 2015). Mating, which usually occurs within a week of adult emergence, involves the male-to-female transfer of a spermatophore that contains sperm and a multitude of *Glossina*-specific proteins (Scolari et al., 2016; Attardo et al., 2019). Many of these proteins are derived from the male accessory gland (Scolari et al., 2016) and are likely to impact female receptivity to males and sperm viability. Oogenesis and ovulation usually occur within the first 10 days following adult emergence, details of these processes have been described in (Benoit et al., 2015). Embryogenesis (3–4 days) occurs within the uterus followed by larvigenesis (5–6 days). During embryogenesis and larvigenesis, development of the second oocyte progresses in the ovary so ovulation can occur within 30–35 min after birth of the fully developed larva (Denlinger and Ma, 1974; Benoit et al., 2018). The first larvae are deposited after 20 days and immediately burrow into the surrounding substrate and pupate (Tobe, 1978). Subsequent larvae are deposited every 9–10 days, with the duration between births extending and the quality of the offspring declining as the female ages (Michalkova et al., 2014a; McIntyre and Gooding, 1998). Oogenesis alternates between the two ovaries during each GC. The period of each reproductive cycle is influenced by factors such as blood quality, time between meals, and exposure to stress that can slightly reduce or significantly extend each reproductive cycle (Tobe, 1978; Hu et al., 2008). Additionally, the length of tsetse's GC significantly increases if the fly is infected with trypanosomes (Hu et al., 2008). Specific details on *Glossina* reproductive biology have been reviewed by Tobe (1978) and Benoit et al. (2015).

Glossina milk production

One of the more unique aspects of tsetse biology is the provisioning to nutritive secretions (fly milk) to developing intrauterine larvae. Tsetse larvae grow over 100-fold (dry mass) during each cycle of larvigenesis (Denlinger and Ma, 1974; Langley and Pimley, 1975). The fat body and milk gland are both critical to this process, as lipid reserves are mobilized from the fat body and milk proteins and other factors are produced by the milk gland (Langley et al., 1981; Attardo et al., 2012; Baumann et al., 2013). Tsetse milk is mostly water (80–85%), and the caloric portion consists of proteins and lipids (Cmelik et al., 1969). The protein components of tsetse milk consists of a lipocalin (Attardo et al., 2006), eight or nine *Glossina*-specific novel milk proteins (Benoit et al., 2014a), transferrin (Guz et al., 2007), acid sphingomyelinase (Benoit et al., 2012), and other minor constituents (Wang and Aksoy, 2012; Benoit et al., 2014a; Attardo et al., 2019). The 12 genes encoding the primary tsetse milk proteins account for nearly 50% of all mRNAs transcribed by lactating female flies (Benoit et al., 2014a). Similar levels of significant transcriptional investment have been noted in other matrotrophic insects (Jennings et al., 2020). Water and other smaller ions are incorporated into the milk via channel proteins, such as aquaporins (Benoit et al., 2014b). *Wigglesworthia* and *Sodalis* are found extracellularly in maternal milk secretions, which serve as the route through which these microbes are trans-generationally transmitted (Wang et al., 2013). *Wolbachia* and *Spiroplasma* are transmitted via the germline (Balmand et al., 2013; Doudoumis et al., 2017).

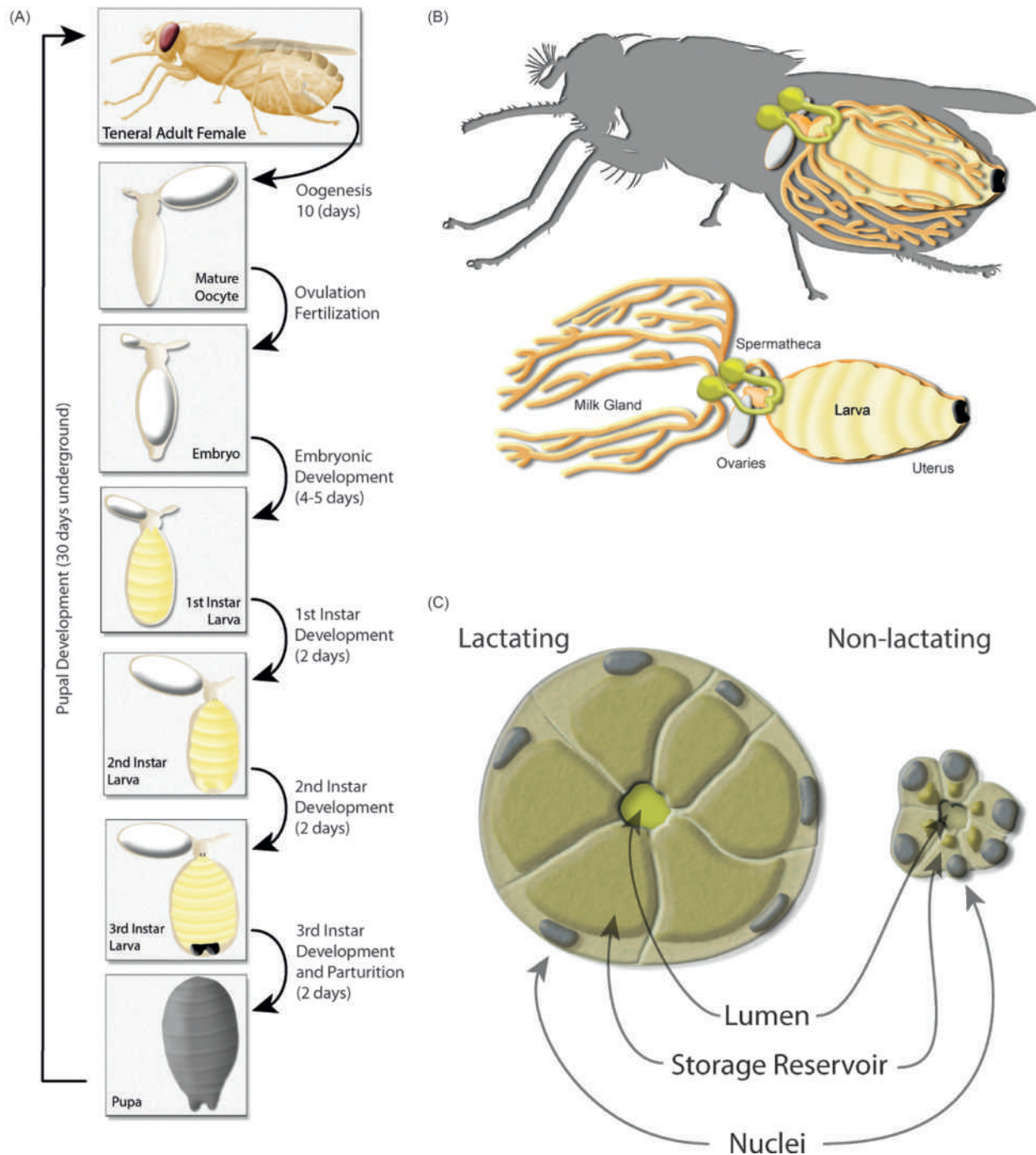


Fig. 6 Female reproductive biology of *Glossina*. (A) First gonotrophic cycle of a *Glossina morsitans* female under optimal environmental and nutritional conditions. Oogenesis, embryogenesis, and larvigenesis within the *Glossina* reproductive system (ovaries and uterus) are shown. (B) Diagram of a reproductive tract during lactation. (C) Cross section of milk gland during lactation and following involution during the dry (non-lactating) period.

The process of milk production is regulated by the dynamics between insulin signaling, juvenile hormones (JH) and ecdysteroids, where higher JH levels are associated with a non-pregnant/lower milk production status (Baumann et al., 2013). A reduction in JH levels may be a convergent mechanism used during extended viviparity, as similar hormonal dynamics are observed in distantly related viviparous insect species (Jennings et al., 2020). The process of birth (=parturition) is likely prompted by input from the mother and intrauterine larvae and regulated by an increase in ecdysteroids and parturition hormone that prompt birth (Denlinger et al., 1983; Robert et al., 1986; Zdárek and Denlinger, 1997). A rapid autophagic breakdown of the milk gland (=involution) occurs immediately after birth, which is critical to allow for recovery between pregnancy that allows the replenishment of nutrient

reserves (Benoit et al., 2018). As with the termination of pregnancy, the involution period is triggered by increased ecdysteroids that precedes parturition.

Prevention and control of tsetse flies and trypanosomiasis

The control of African trypanosomiasis involves the integration of multiple strategies, and this is complicated by the fact that AAT and HAT are very different from an epidemiological perspective. Specifically, a large diversity of wild and domesticated animals can serve as a reservoir for AAT-causing trypanosomes as well as those that cause HAT in east Africa (i.e. *Trypanosoma brucei rhodesiense*). Conversely, parasites that cause west African HAT (i.e. *T. b. gambiense*) circulate only through humans, and to the best of our knowledge no other animals serve as reservoirs (Franco et al., 2014). Independent of the trypanosome species, HAT and AAT are best controlled via three specific routes: (1) reducing disease incidence and burden in vertebrate hosts, (2) controlling populations of the tsetse fly vector, and (3) inhibiting trypanosome development and subsequent transmission by tsetse flies (Fig. 7) (Aksoy et al., 2017). Limited progress has been made regarding reducing/eliminating and/or treating vertebrate disease. This largely reflects the lack of anti-trypanosomal vaccines, due to the phenomenon of trypanosome antigenic variation (Bangs, 2018). Anti-trypanosomal chemotherapeutics present limited effectiveness. Encouragingly, progress is being made on the development of both anti-trypanosomal vaccines (Vigneron et al., 2020) and drugs (Field et al., 2017; Pollastri, 2018). Detection of the parasite within vertebrate hosts is expensive and logistically difficult due to the lack of testing capabilities, the reliance on clinical manifestation of trypanosome infection, and the large number of potentially infected livestock (Mumba Ngoyi et al., 2014; Jamonneau et al., 2015; Koffi et al., 2016; Aksoy et al., 2017). The importance of detection is critical, as current treatments are toxic to vertebrates and parasites quickly develop resistance (Babokhov et al., 2013; Aksoy et al., 2017; Fairlamb and Horn, 2018). The development of novel treatments, such as nifurtimox combination therapy (NECT) (Priotto et al., 2009; Babokhov et al., 2013), is likely to improve the safety and effectiveness of treatment. Chemotherapy is predominantly used to control AAT (Giordani et al., 2016), but resistance due to limited chemical options is becoming more common. *Trypanosoma*-resistant livestock breeds have been noted and represent a potential solution for AAT (Bocoum et al., 2012; Berthier et al., 2016; Yaro et al., 2016), but asymptomatic infections could allow these resistant animals to serve as reservoirs.

Tsetse control is an effective means of controlling disease transmission, largely because female tsetse produce so few offspring over the course of their lifespan (10–12 offspring compared to thousands of eggs produced by other vector systems; Benoit et al., 2015). Vector control is essential for both AAT and Rhodesiense HAT, but has not been used as significantly for Gambiense HAT (Solano et al., 2013; Aksoy et al., 2017). Even though not uniformly used, modeling indicates that tsetse population control should be included as a critical component for the elimination of African trypanosomiasis (Hargrove et al., 2012; Aksoy et al., 2017; Rock et al., 2018). Sterile insect technique (SIT) is under consideration for continent-wide control, but the cost due to the diversity of species that transmit trypanosomes, along with the necessary infrastructure and logistics, make feasibility difficult (Kabayo, 2002; Rogers and Randolph, 2002; Aksoy et al., 2017; Sutherland et al., 2019). Despite these challenges, SIT was successfully used to eradicate *G. austeni* from Unguja Island, Zanzibar (Vreysen et al., 2000). Trapping and insecticide treatments have proven to be much more cost effective on mainland Africa. Recent progress has been made on the development of smaller, more effective insecticide-impregnated targets that are effective and easier to deploy when compared to large targets that have been used in previous control programs (Courtin et al., 2015; Shaw et al., 2015). Habitat manipulation (= bush clearing) was used in early control programs for the reduction of tsetse fly presence, which included the removal of specific vegetation near villages or farms

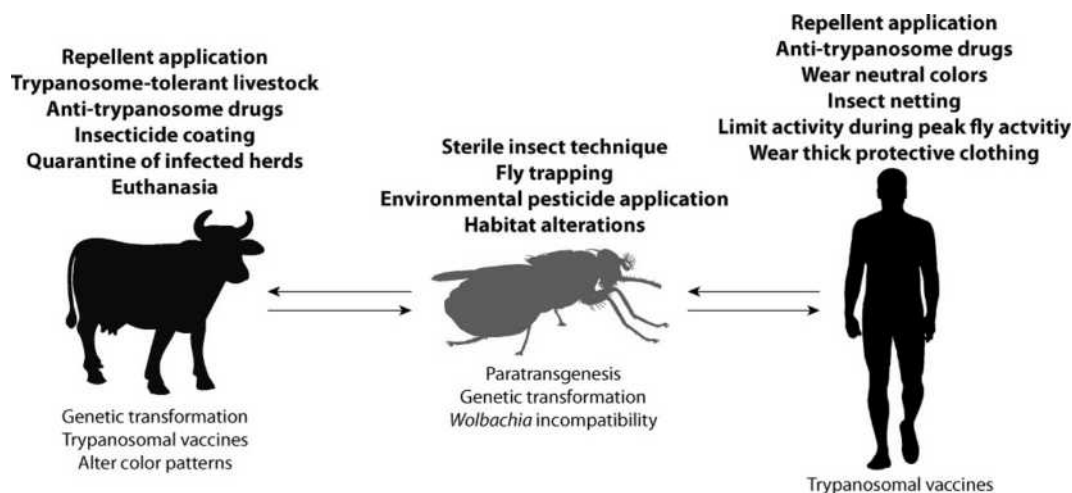


Fig. 7 Prevention on trypanosomiasis and suppression of tsetse populations in relation to human and livestock hosts. Each point in bold (top) represents a method that has been used to interfere with the transmission cycle. Points on bottom represent potential control methods.

(Challier, 1982; Leak, 1999; Keating et al., 2015). This was only minimally successful and re-invasion occurred fairly quickly. Also, bush clearing to reduce tsetse population density did not outweigh the negative side effects such as soil erosion (Lloyd et al., 1933). The use of fly repellent, wearing thick clothes that prevent the proboscis from reaching the skin, insect barriers (e.g. netting), and reduced activity outside of peak periods of tsetse activity (dawn and dusk) are other mechanisms that help to suppress trypanosomiasis by reducing *Glossina* feeding (Challier, 1982; Uilenberg and Boyt, 1998; Leak, 1999; World Health Organization, 2013; Keating et al., 2015).

Of importance is that following reduction/elimination, tsetse populations should be monitored to ensure that re-establishment does not occur (Gooding and Krafur, 2005; Davis et al., 2019), specifically since tsetse migration and population differences are likely to have significant impact on suppression of African trypanosomiasis by *Glossina* control (Aksoy et al., 2013; Ndeffo-Mbah et al., 2019). The sequencing of six tsetse species genomes provides insight into tsetse's olfactory biology, thus leading to the identification of targets for the development of novel attractants and/or repellents (International *Glossina* Genome Initiative, 2014; Obiero et al., 2014; Macharia et al., 2016; Attardo et al., 2019). Additionally, this genomic information will facilitate the development of novel control strategies aimed at reducing tsetse fecundity (Benoit et al., 2014a, 2015; Scolari et al., 2016; Attardo et al., 2019) and/or interfering with the interwoven tsetse/endosymbiont relationship for the purpose of enhancing the fly's innate anti-trypanosomal immunity (Weiss et al., 2011; Michalkova et al., 2014b; Benoit et al., 2017; Bing et al., 2017; Attardo et al., 2019). These newly developed resources are likely to provide novel, *Glossina* specific targets for vector control programs.

Genetic manipulation could be used to control tsetse or inhibit trypanosome development within the fly. Direct genetic manipulation of *Glossina*, as has been used successfully in a multitude of other insect systems (Shaw and Catteruccia, 2019; Raban et al., 2020), is unlikely to work in tsetse because the process would involve removal and injection of eggs found in the maternal uterus. Treated eggs would require implantation into another female at the same pregnancy stage. An alternative that could prove successful is injection of genetic materials into the female that are incorporated into the germline, which has been successful in other insects (Chaverra-Rodriguez et al., 2018; Macias et al., 2020). A major issue for producing genetically modified *Glossina* is that any reduction in reproductive output would make prolonged rearing of a specific line nearly impossible due to the reduced number of larvae produced by a single female under optimal rearing conditions (Gooding et al., 1997).

Two microbial-based methods have been suggested to control tsetse. First, *Wolbachia*-based cytoplasmic incompatibility (CI) has been noted (Alam et al., 2011), and this method could be used to suppress tsetse fly populations. However, this control strategy may be inefficient for *Glossina*, as population genetics-based studies suggest that *Wolbachia* infections are low density and CI has little influence on population structure (Symula et al., 2013). Second, paratransgenesis through the genetic modification of tsetse's secondary symbiont, *Sodalis*, has been suggested as a mechanism to suppress *Glossina* populations and/or interfere with tsetse-trypanosome interactions (Aksoy et al., 2008; Weiss and Aksoy, 2011; Medlock et al., 2013; Kariithi et al., 2018). Proof-of-concept paratransgenesis studies have been conducted with *Sodalis* that produce anti-trypanosome factors (De Vooght et al., 2012, 2014).

Concluding remarks

Tsetse flies are unlike many other flies because they feed solely on vertebrate blood and reproduce by adenotrophic viviparity. Their slow reproductive rate suggests that insect control protocols should be more successful than in other systems. This has not been the case as most tsetse fly control programs have failed in the long term. Prolonged suppression of flies and the prevention of African trypanosomiasis will require a multi-targeted approach that relies heavily on fly control along with treatment of vertebrate hosts followed by population monitoring to ensure elimination of both flies and the trypanosomes. These targeted approaches will require continual research on this pest to ensure an increasing toolbox of techniques to suppress tsetse flies if resistance to current control methods develop.

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Biting Midges (Ceratopogonidae, Culicoides)

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Taxonomy and Identification

The Ceratopogonidae are a family of small flies (generally 3–4 mm in length) belonging to the order Diptera, Suborder Nematocera, infraorder Culicomorpha and superfamily Chironomoidea. They are closely related to the Chironomidae (commonly known as non-biting midges) but can be easily distinguished from them by the presence of biting mouthparts, short forelegs, wings kept flat on the abdomen and folded like a closed scissor when the insect is resting, characteristic wing venation and by the lack of the two thoracic and abdominal pseudopods in the larvae. Ceratopogonids are commonly known as biting midges, but are also called biting gnat, punkies, no-see-ums or sandflies, although this last name is best reserved for Phlebotominae.

The Ceratopogonidae counts over 6200 species comprised in 112 extant genera (Borkent, 2016a,b). The Ceratopogonidae family is divided into four subfamilies: Leptoconopinae (2 genera), Forcipomyiinae (2 genera), Dasyheleinae (1 genus) and Ceratopogoninae (107 genera). Adults have multi-segmented antennae (14–15 articles), wings with reduced venation and concentration of the radial ones in the coastal area, which allow the distinction of the Ceratopogonidae from the other Diptera. Taxonomic keys, at genus level, for adult ceratopogonids are provided by Wirth et al. (1974), Downes and Wirth (1981), de Meillon and Wirth (1991), and Borkent and Spinelli (2007). The immature stages of Ceratopogonidae are poorly known, but the larvae of many genera, including *Culicoides*, have been described by Kettle and Lawson (1952), Blanton and Wirth (1979), and Glukhova (1979, 1989).

The most important genus in this family is *Culicoides*, which includes species that are vectors of well-known animal diseases. The first description of a species of *Culicoides* dates back to 1713 by W. Derham, Rector of the University of Upminster in Essex in England, in his work entitled “Physico-Theology: or a demonstration of the being and attributes of God, from His Work of Creation” and dedicated to the Archbishop of Canterbury. In his work, Derham described the species *Culex minimus nigricans maculatus sanguisuga* as “...it is about one tenth of an inch, or somewhat more, long, with short antennae. It is spotted with blackish spots especially on the wings. ... It comes from a little slender eel-like worm of a dirty white colour, swimming in stagnating waters by a wriggling motion.” The genus *Culicoides* was later created by Latreille in 1809. Several small characteristics distinguish the members of this genus from the other ones of the Ceratopogonidae family: (i) presence of two small humeral pits near the front edge of the thorax, (ii) empodium vestigial or extremely small between tarsal claws, which are the same size and toothless, (iii) two small and narrow radial cells at the end of radial vein in the wings. However, the most important feature is the presence of spots on the wings, which represent one of the main morphological characters used in the species identification.

To date, the genus *Culicoides* counts over 1340 species (Borkent, 2016a) of which about 60 are implicated as vectors of pathogens and parasites to humans and livestock (Borkent, 2004). The subgeneric classification is based almost exclusively on the morphology of adults and counts 31 subgenera (Harrup et al., 2015). The most abundant group is subgenus *Oecacta* followed by the subgenus *Avaritia* which contains a wide proportion of vector species of viruses affecting livestock. As reported by Borkent (2004), the *Culicoides* fauna of some regions of the world has been poorly studied, so that many species remain to be described. Furthermore, although a number of very good systematic studies exist, a refined taxonomy using morphological and genetic techniques is essential to understand the relationships between *Culicoides* species. In the last decades, molecular analyses and phylogenetic studies have helped to discriminate the morphologically cryptic species belonging to a complex of similar species, such as *Culicoides variipennis* complex, *Culicoides obsoletus* complex and *Culicoides pulicaris* complex (Holbrook et al., 2000; Meiswinkel et al., 2004; Nolan et al., 2007; Nielsen and Kristensen, 2015). These studies will provide a more accurate framework to elucidate distribution, ecology, phenology, biting habits, vector capacity and other characteristics of harmful species.

Some useful works for the *Culicoides* identification at species level are provided by Khamala and Kettle (1971), Boorman and Dipeolu (1979), Glick (1990), and Meiswinkel (1995) for Afrotropical fauna; by Tokunaga (1962), Dyce et al. (2007), Bellis

(2013), and Bellis et al. (2015) for Australasian fauna; by Blanton and Wirth (1979), Downes and Wirth (1981), Wirth et al. (1985), and Holbrook et al. (2000) for Nearctic fauna; by Aitken et al. (1975), Wirth et al. (1988), and Felipe-Bauer et al. (2003) for Neotropical fauna; by Kitaoka (1985a,b), Wirth and Hubert (1989), and Bellis (2014), for Oriental fauna; by Campbell and Pelham-Clinton (1960), Kremer (1965), Delécolle (1985), and Glukhova (2005) for Palaearctic fauna. Generic keys for larvae are published by Jamnback (1965), Glukhova (1977, 1979), and Murphree and Mullen (1991). Useful indications for taxonomic identification of *Culicoides* pupae were provided by Borkent (2014).

Morphology

Culicoides eggs are tiny (0.3–0.5 mm in length) and banana shaped (Fig. 1). As soon as laid they are white in color, and subsequently turn dark brown. The membrane surface is covered with minute projections used for adhering the eggs to the substrate.

Culicoides larvae are translucent white and approximately 2–5 mm long (Fig. 2). They are worm-like and move resembling small eels. Larvae have a well chitinized yellow-brown head, a thorax consisting of three segments and an abdomen with nine segments. Two maxillae and two mandibles, normally retained into cephalic capsule and everted to scrape and/or rip the substrate during feeding, are evident in the anterior position of the head (Fig. 3). The pharyngeal apparatus, a sclerotized structure formed by epipharynx and hypopharynx, is observed inside the buccal cavity (Fig. 4). These structures bear combs used to crush and strain ingested food. The shape and number of combs is related to the feeding behaviors and varies according to the species. At the caudal end, setae are present to enhance the larval mobility. Bifid anal papillae are retracted in the anus and can be everted to help osmoregulation and larval movement (Fig. 5).

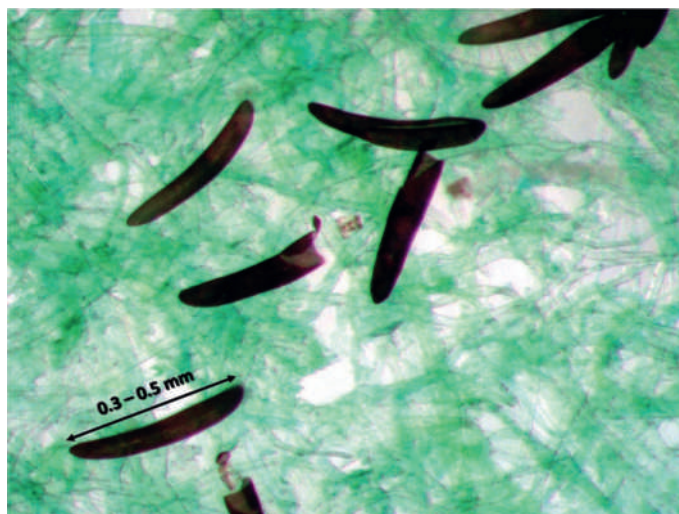


Fig. 1 Eggs of *Culicoides* biting midges obtained from field collected gravid females. Some eggs are hatching and young larvae are visible. Photo credit: Cipriano Foxi.



Fig. 2 *Culicoides* biting midge larva, fourth instar. Photo credit: Cipriano Foxi.

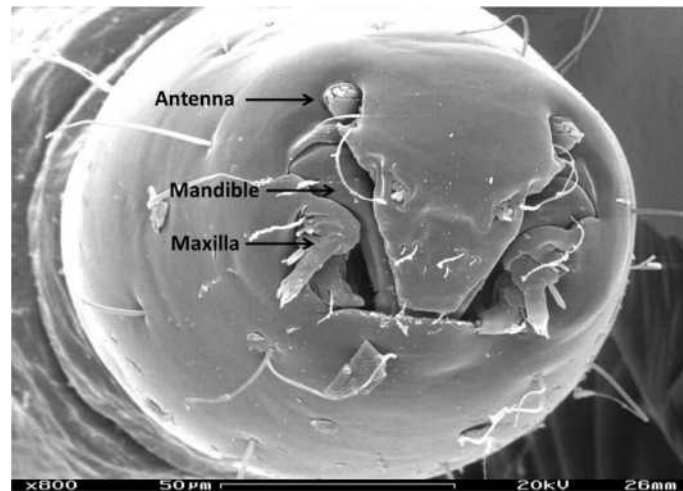


Fig. 3 *Culicoides* biting midge larva, fourth instar; frontal view of head capsule (SEM). Photo credit: Cipriano Foxi.

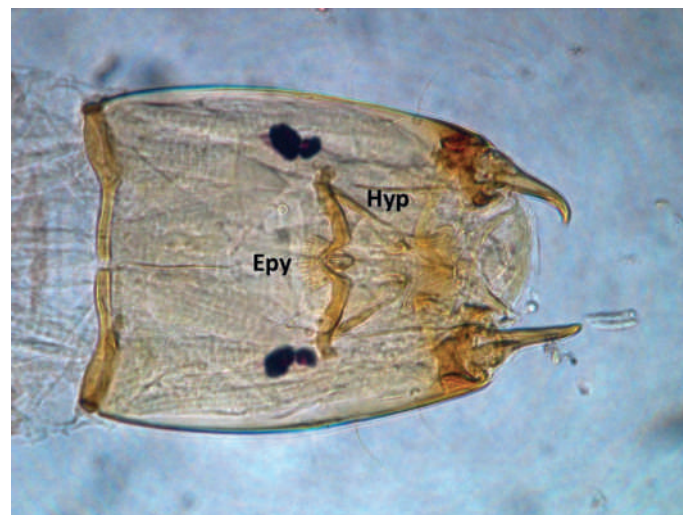


Fig. 4 *Culicoides* biting midge larva, fourth instar; epipharynx (**Epy**) and hypopharynx (**Hyp**) observed in cleared, slide-mounted specimen. Photo credit: Cipriano Foxi.

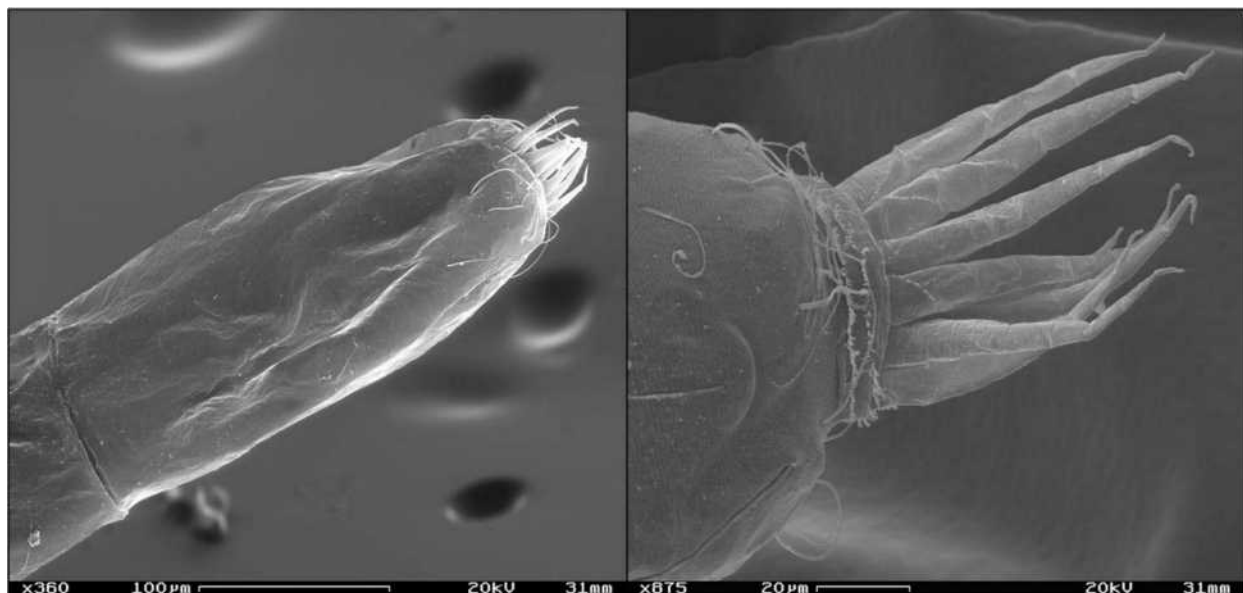


Fig. 5 *Culicoides* biting midge larva, caudal segment of fourth instar (SEM). Anal papillae, partially retracted (left) and totally extruded (right). Photo credit: Cipriano Foxi.

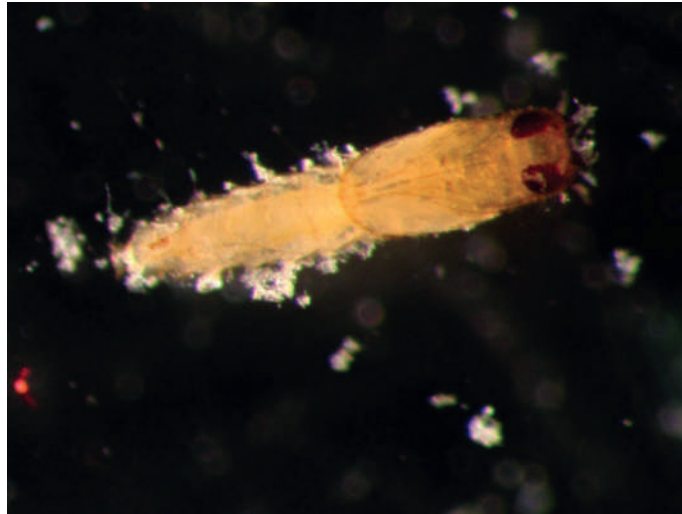


Fig. 6 *Culicoides* biting midge, pupa. Photo credit: Cipriano Foxi.

The pupae, 2–4 mm long, are brown in color, with a pair of prothoracic horns used for breathing (Fig. 6). In aquatic forms, thin spiral openings at the ends of these respiratory tubes, allow the pupae to breathe on the water surface.

Culicoides adults are small flies with a body measuring 0.8–3 mm in length (Fig. 7). Most of the head is occupied by compound eyes made up of numerous ommatidae separated by small spaces, which in some species are covered by a fine pubescence. The 15-segmented adult antennae show a basic structure with scape, pedicel and flagellum consisting of 13 segments. In the female antennae, the flagellum has the first height segments ovoid and the last five elongated. The male antennae are more plumose and only the last three flagellomeres are elongated. The antennae bear five types of sensilla (Fig. 8):

- ampullacea: small deep dimples internally hosting a bulb-shaped sensillum in the center;
- trichodea: transparent, long or short bristles; they have no articulated insertion;
- chaetica: long and dark bristles characterized by a flexible membrane at the insertion point with the antenna. In females these sensilla are abundant in short articles (3–10 segments). In males they form the plume;
- basiconica: short and transparent bristles, numerous in long segments;
- coeloconica: small depression with a rounded protuberance in the center surrounded by several microtrichi. They probably function as hygroreceptors or olfactoreceptors.

The number and the distribution of these sensory pits in the antennal segments, particularly the coeloconic sensilla, are useful taxonomic characters. Furthermore, it seems that the number of antennal flagellomeres bearing sensilla coeloconica correlates with



Fig. 7 *Culicoides imicola*, adult females. Photo credit: Cipriano Foxi.

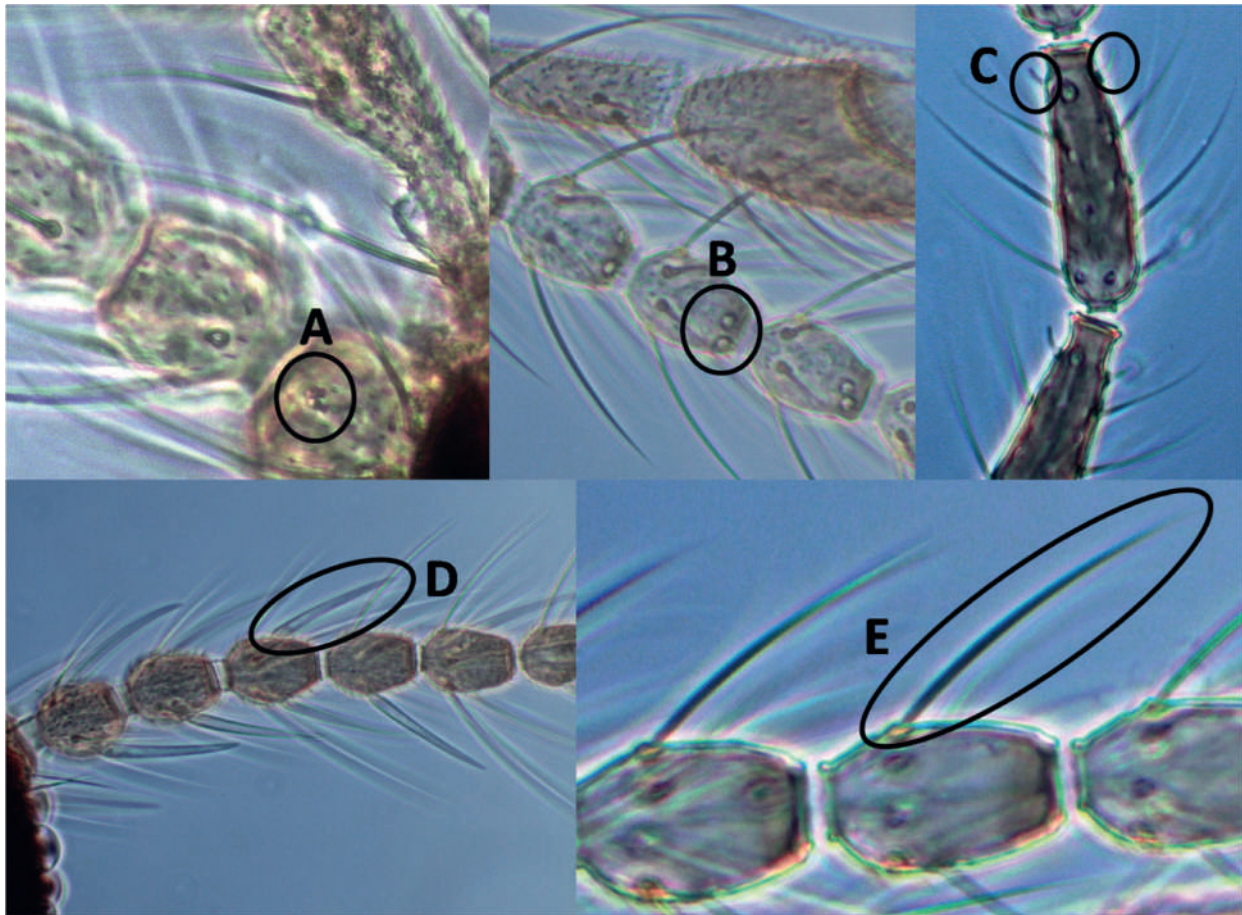


Fig. 8 *Culicoides* biting midge, antennal sensilla. (A) Ampullacea, (B) coeloconica, (C) basiconica, (D) trichodea long, (E) chaetica. Photo credit: Cipriano Foxi.

the host preference. Indeed, *Culicoides* that feed on birds have more sensilla distributed in almost all segments while mammalophilic species tend to have fewer flagellomeres with sensilla coeloconica.

The mouthparts, located below the antennae, consist of an upper labrum or epipharynx, a pair of mandibles, a pair of maxillary laciniae, a hypopharynx and a labium (Fig.9). In the females, mandibles and lacinia bear a row of teeth near the tip which are used to cutting skin. The hypopharynx is covered for its entire length by the salivary groove, while, distally, it is rounded with teeth to facilitate penetration in the skin. A pair of maxillary palps is connected with the mouthparts. The maxillary palps are 5-segmented with the third segment bearing a sensory pit rich of sensilla (Fig. 9). The males have mouthparts reduced to feed on sugar sources.

The thorax is moderately broad and hooped above, extending very slightly over the head. The wings are short, relatively broad, and covered by minute hairs with a density which gives rise to pigmentation patterns. The presence/absence of clear and dark spots as well as their shape, size and distribution in the wings are morphological characters used to discriminate the different species. The 9-segmented abdomen is yellowish brown in color, and slightly tapered at the end. In females, the abdomen ends with two cerci covered by short hairs. The spermathecae, ranging one to three, are often joined to a common duct through a small sclerotized ring. The male abdomen ends with the hypopygium (male genitalia), which consists of an aedeagus, two parameres and, laterally, of two triangular apicolateral expansions of the 9th segment, basistyle and dististyle (Fig.10). Shape and size of the aedeagus and parameres are the most important taxonomic characters for species identification.

Life cycle

Biting midges grow through the typical development cycle of nematoceros flies consisting of egg, four larval instars, pupa and adult. Females begin to lay up to 400 eggs at 3–4 days post blood feeding. Mostly of the midge species produce 2–4 clutches with an average of 80–100 eggs at each oviposition. The eggs hatch at 2–7 days post spawning. The larvae through four instars before pupation. Larval development time is extremely variable depending on the species, seasonal trend and latitude. This period varies from 2 weeks to 8 months, reaching 2 years in some arctic species. The pupal stage is very short, lasting 2–10 days. The pupae float

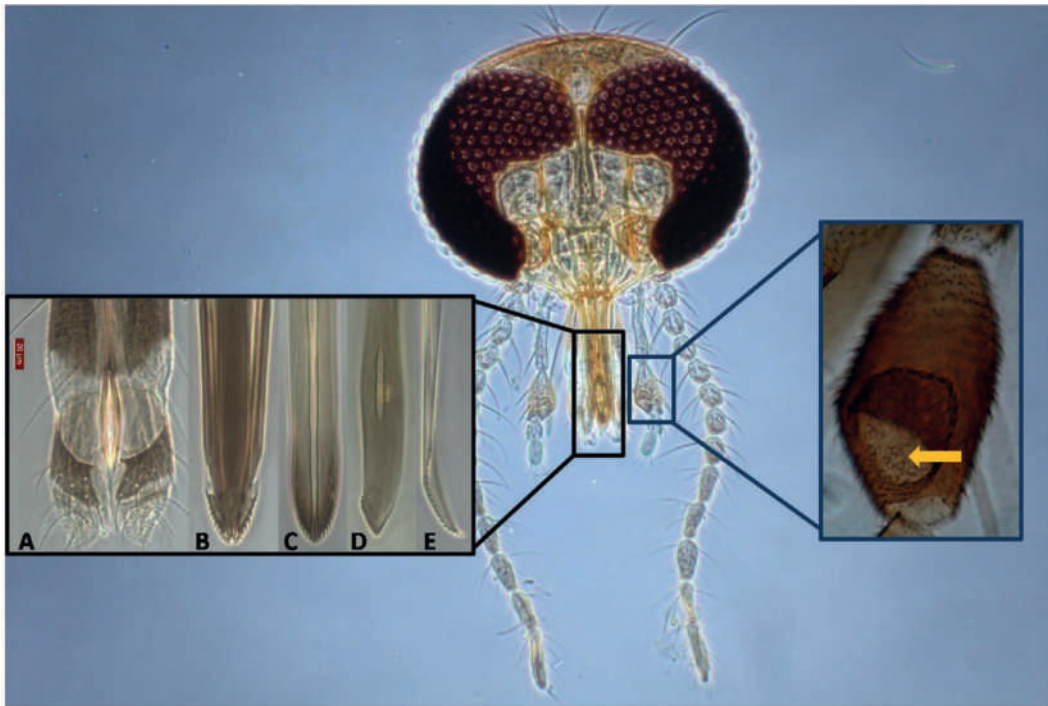


Fig. 9 *Culicoides* biting midge, head with dissected and enlarged mouthparts (left) and enlarged 3rd palpal segment (right). (A) Labium, (B) upper labrum or epipharynx, (C) hypopharynx, (D) mandible; (E) maxillary lacinia. The yellow arrow indicates 3rd palpal segment bearing a sensory pit rich of sensilla. Photo credit: Cipriano Foxi.



Fig. 10 *Culicoides* biting midge, male hypopygium. Photo credit: Cipriano Foxi.

on the water surface and breathe with the prothoracic respiratory horns. Most species overwinter as fourth-instar larvae, although, cases of summer diapause are also documented. During the spring, the wintering larvae pupate and give rise to the first generation. Each generation can last from 2 weeks to over a year depending on the species, latitude and climatic conditions. Generally, the emerging *Culicoides* adults, stay close to the breeding sites and form swarms to mate. After mating, females disperse in search of a food source.

The multivoltine species complete two or more generations per year while univoltine produce only one generation per year. The presence and abundance of adults varies greatly throughout the year and each species shows a typical seasonal trend with characteristic peaks of abundance.

Ecology

Adult ceratopogonids have a wide range of feeding habits. Both sexes regularly feed on plant sugar, as nectar of flowers. Only the females of the genera *Austroconops*, *Culicoides*, *Leptoconops* and those belonging to the subgenus *Lasiohelea* of the genus *Forcipomyia* are hematophagous by sucking vertebrate blood. The blood meal is required to have protein reserves for egg maturation. Some species are autogenous by absorbing at the larval stage enough nutrients to bring the first egg deposition to maturity. However, these species require a blood meal to complete additional gonotrophic cycles. The autogeny is documented for several species belonging to the genera *Culicoides* (e.g., *Culicoides circumscriptus*, *Culicoides impunctatus*), *Leptoconops* (e.g., *Leptoconops kerteszi*, *Leptoconops carteri*), *Forcipomyia* (e.g., *Forcipomyia inornatipennis*, *Forcipomyia squamipennis*) and *Dasyhelea*, in which all species appear to be autogenous for the first cycle.

Culicoides females feed on vertebrate hosts, primarily on mammals, birds, and reptiles. While several species are host-specific, showing a preference for a group of animals, others are generalist and feed on a wide range of animals, depending on host availability. There are also species that exploit other insects, such as the *Culicoides anophelis* which feeds on the ingested blood from the abdomen of the *Anopheles* mosquitoes.

Visual stimuli and olfactory systems are involved in midge-host interactions by highly sensitive receptors located on the antennae and maxillary palps. Lactic acid, acetone, butanone, 1-octen-3-ol, CO₂ are the most important semiochemicals involved in host-location and host-selection. *Culicoides* are telmophages that use toothed tip of mandibles and maxillae to lacerate the skin causing the leakage of small drops of blood which are sucked through the pharyngeal pump. Following the bite the diuresis process begins, determining the concentration of the ingested blood. Females remain hidden in the vegetation or in other protected environments until eggs maturation.

Mating of *Culicoides* usually takes place in flight, with males swarming adjacent to hosts on which females are feeding or in proximity of moist environments where females lay the eggs, depending on the species. Swarming and mating are modulated by sex pheromones. Most species mate only once and the sperm stored in the spermathecae is sufficient to fertilize all eggs laid throughout midge life.

Most *Culicoides* species have crepuscular and nocturnal activity while some species (e.g., *Culicoides phlebotomus*, *Culicoides kingi*, *Culicoides paraensis*) as well as *Leptoconops* are more active during the day. Among the first, some species show a bimodal pattern of flight activity with peaks during sunrise and sunset (e.g., *Culicoides brunnicans*, *Culicoides impunctatus*), while others are more active only at dawn time (e.g., *Culicoides debilipalpis*) or dusk time (e.g., *Culicoides furens*, *Culicoides obsoletus*).

Several climatic variables affect flight activity of adult *Culicoides*, especially temperature, light intensity, wind speed, relative humidity, rainfall, barometric pressure, and lunar cycles. Among them temperature and wind are of fundamental importance. Temperatures out of range steadily restrict the activity of midges. In this regard, adults of *C. variipennis* and *C. brevitarsis* became inactive at temperatures lower than 10 °C and 18 °C, respectively. Conversely, temperatures higher than 32 °C affect the flight activity of *C. variipennis* while *Culicoides barbosai* is much less active with temperature greater than 24 °C.

After emergence, *Culicoides* adults remain mainly in proximity of breeding sites, flying for short distance to seek shelters, hosts, females to mate, or other oviposition sites. Because of their small size, midges are not very strong fliers and the flight are limited when wind speed over 2–2.5 m/s. Generally, all activities are suppressed at wind speeds higher than 2.7 m/s for *C. furens* in the Caribbean regions or 3 m/s for *C. imicola* in Africa. In the last decades, several studies are carried out to estimate the short-range dispersal and evaluate the mean distance travelled by *Culicoides* adults within and between farms. One of the most important methods used is the Mark-Release-Recapture (MRR) technique which estimates the ratio of recaptured specimens to the total of marked individuals released in a defined area. The mean flight distance for females is relatively similar between species. Adults of *Culicoides mississippiensis* were recaptured after 24 h between 2 and 2.5 km from the release point. *Culicoides mohave* midges were found 12 h post release at 1.2 km. The maximum distance travelled by marked specimens of *C. variipennis* and *Obsoletus* group was 1.93 km and 1.75 km, respectively. The mean flight distance for males is estimated in less than half of that for females. It is of relevant epidemiological importance the fact that midges can passively be carried by the wind over considerable distances, even over 700 km, explaining the introduction and spread of *Culicoides*-borne diseases in new free areas.

The larvae of the Ceratopogonidae present a cutaneous respiration and develop in aquatic or semi-aquatic environments. *Leptoconops* larvae are found in sandy soils and along the beaches and marshes near the coasts while larvae of the genus *Dasyhelea* can develop in bums, but also in the roots of umbrella plants. The larvae of *Culicoides* genus develop in a wide range of moist environments rich in decaying organic matter (Fig. 11): marshy soils, low margins of brackish ponds, streams and rivers, puddles, irrigation pipe leaks, leaking watering troughs, marshes, peat bogs, tree holes with standing water, rotting wood, cacti and fungi, waterlogged pastures, animal manure, mangroves. In muddy environment, the vertical distribution of the larvae is very homogeneous and they are rarely found below 5–7 cm depth. Many habitats can simultaneously host larvae belonging to different genera. As regards the diet, the larvae of family Ceratopogonidae are primarily predators of eggs and larvae of chironomids, mosquitoes, other midges, nematodes, annelids and protozoa. *Culicoides* larvae can have a phytophagous, zoophagous and saprophic regime. Many species are generalists and omnivorous feeding on algae, diatoms, fungi, bacteria, and organic substances. The ecology of



Fig. 11 Some typical *Culicoides* larval habitats. (A) Permanent puddle, (B) temporary puddle, (C) mud close to leaking water trough, (D) manure heap, (E) dung puddle, (F) marsh. Photo credit: Cipriano Foxi.

Culicoides larvae is better known for salt marsh species, some of which are important vector of viruses, and tree-hole species while for most species feeding habits remain unknown.

Medical importance

Culicoides biting midges with their bites cause, in some regions, annoyance and discomfort to humans, especially in outdoor recreation and work activities. The bites can be painful causing a slight temporary burning and mild swelling lasting up to a few hours. Secondary infection may occur if bite is scratched vigorously. In this regards, *C. impunctatus* is particularly important, being responsible for 70–95% of the attacks on humans in some European countries. This biting midge causes serious damage to human

activities, preventing the development of tourism during the summer days. In the United States, some species, such as *C. furens*, *C. mississippiensis*, *C. melleus* and *C. barbosai*, represent the most troublesome midges biting humans, especially in coastal zones.

Culicoides can transmit viruses and filarial nematodes to humans (Table 1). Although several viruses have been isolated from *Culicoides* biting midges, it is only a few viruses that seriously affect humans. The most important is Oropouche virus, which belongs to the family Peribunyaviridae. Oropouche virus is responsible for Oropouche Fever, a widespread disease in tropical areas of Central and South America, especially in Trinidad and Tobago, Perú, Colombia and Brazil. This virus produces acute flu-like symptoms with fever, headaches, vomiting and muscular and joint pains. Although it is generally nonfatal, the symptoms can last up to 2 weeks and in rare cases evolves into meningo-encephalitis. Oropouche virus is maintained in nature in a complex cycle involving both an urban and a sylvatic cycle. In the urban cycle humans are the sole vertebrate host, while in the sylvatic cycle, mammals and birds represent the natural reservoir hosts. The predominant vector is *Culicoides paraensis*, but some mosquitoes belonging to the genera *Aedes*, *Culex* and *Coquillettidia* can also contribute to the virus transmission, with low efficiency of transmission.

While no protozoan blood parasites are transmitted to man by *Culicoides*, some species of this hematophagous flies are vectors of filarial nematodes of the genus *Mansonella*. In particular, three species are pathogenic to man, *M. ozzardi*, *M. streptocerca* and *M. perstans*, causing mansonellosis. Infestations evolve asymptotically or, rarely, with mild clinical symptoms. Humans are the only known vertebrate hosts for *M. ozzardi*, while others primates are involved in the cycle of *M. streptocerca* and *M. perstans*. *Mansonella ozzardi* is widely present in South America and Caribbean regions, where *C. furens* and *C. phlebotomus* are the main vectors. Other species that have been found to support the development of the parasite are *C. barbosai* and *Leptoconops bequaerti*. Also blackflies of the genus *Simulium* appears to be competent vectors for *M. ozzardi*. *Mansonella perstans* occurs mainly in Central and West Africa where *C. austeni*, *C. inornatipennis*, *C. grahamii* and some species belonging to *C. milnei* group are the main vector species. This nematode was introduced to Central and South America but the vectors have not yet been identified. *Mansonella streptocerca* is restricted to the tropical Africa, where *C. grahamii* is considered its main vector.

Veterinary importance

In domestic and wild animals, *Culicoides* can transmit almost 60 viruses, 40 protozoa species and 24 filarial nematodes species (Table 1). In recent years, also a bacterium has been detected in adults of *C. brevitarsis*, thus hypothesizing its possible role as a vector. In some areas, midges can occur in abundant numbers and their persistent bites can lead to allergic reactions in the animal skin, including the so-called “Sweet itch” or “Queensland itch” in horses.

Certainly, biting midges are well known as vectors of a number of viruses causing economically important livestock diseases. Among them, Bluetongue virus, African horse sickness virus, Epizootic hemorrhagic disease of deer virus and Schmallenberg virus are considered to be some of the most important, as causing mortality, abortions, sterility, reduced milk yields, and other serious losses in the affected animals. These arboviruses have a defined geographical distribution correlated with their vector species and hosts. However, especially in recent decades, disease incursions into areas previously immune to outbreaks have been observed. This expansion can mainly be related to intense international trades and more favorable climatic conditions for the vectors in these new areas. An explanatory example is the spreading of Bluetongue in previous unaffected European countries and the discovery of Schmallenberg virus, which has never been detected in Europe before.

Bluetongue virus was widespread in the world and has been found before the 2000s affecting ruminants in Australia, Middle/Far East in Asia, Africa and Americas, restricted to the geographical areas included between 40° and 50° North latitude and to the 20° and 30° South latitude (Kundlacz et al., 2019). Sporadic incursion for BTV has been observed for restricted period in the European countries of the Mediterranean Basin until 1998. This distribution radically changed in the period 1998–2005, when five different BTV serotypes, namely 1, 2, 4, 9, and 16, were diagnosed in the countries of Mediterranean Basin, with Greece and Italy being the first involved countries. After 2006, BTV incursion started in Northern European countries, with severe consequences determined by the serotype 8 (Saegerman et al., 2008). While the incursion of BTV in the European Mediterranean countries was connected with Northern African countries, after 2006 the circulation of BTV in the Nord Europe was of unknown origin. Globally, 9 BTV conventional serotypes, namely 1, 2, 3, 4, 6, 8, 9, 11, and 16, have been characterized in numerous outbreaks in Europe, so far. The factors that could have influenced this northward spread of the Bluetongue are the well-known climatic changes, the northward expansion of *C. imicola* and the vector competence of autochthonous European *Culicoides* species. In the last years a significant increase in temperatures has been observed in Europe (Purse et al., 2005). Undoubtedly, the temperature greatly influences the *Culicoides* life cycle and the BTV transmission cycle. Warmer temperatures increase development, activity and survival rate of *Culicoides*, viral replication rates and could enhance vectorial capacity of European *Culicoides* species.

African horse sickness

African horse sickness (AHS) is a viral insect-borne disease affecting equine species caused by AHS virus (AHSV), which belongs to the Orbivirus genus. Severe and often lethal clinical signs are observed most frequently in horse than in mule and African donkey (Mellor and Hamblin, 2004). Zebra species are considered asymptomatic reservoir playing an important role in the epidemiology of AHSV in Africa, (Becker et al., 2018). With regards to other species, in dogs AHSV can be fatal after eating contaminated

Table 1 Pathogens transmitted by *Culicoides* biting midges.

Pathogens ^a	Demonstrated or putative vectors	Hosts	Geographic area
Bacteria			
<i>Elizabethkingia</i> sp.	<i>C. brevitarsis</i> (also mosquitoes)	–	Africa, Asia, Australia, Europe, North America
Filarial nematodes			
<i>Chandlerella chitwoodae</i>	<i>C. stilobezzioides</i> , <i>C. travisi</i> , <i>Culicoides</i> spp.	Birds	Canada, Indonesia, Spain
<i>Chandlerella quiscali</i>	<i>C. crepuscularis</i> , <i>C. haematopotus</i> , <i>C. travisi</i>	Birds	North America
<i>Chandlerella striatospicula</i>	<i>C. haematopotus</i>	Birds	North America
<i>Dipetalonema caudispina</i>	<i>C. hollensis</i>	Monkeys	South America
<i>Dipetalonema gracile</i>	<i>C. hollensis</i>	Monkeys	South America
<i>Eufilaria bartlettiae</i>	<i>C. nubeculosus</i>	Birds	Europe
<i>Eufilaria delicata</i>	<i>C. nubeculosus</i>	Birds	Europe
<i>Eufilaria kalifai</i>	<i>C. nubeculosus</i>	Birds	Europe
<i>Eufilaria longicaudata</i>	<i>C. crepuscularis</i> , <i>C. haematopotus</i> , <i>Culicoides</i> spp.	Birds	North America
<i>Macacanema formosana</i>	<i>C. amamiensis</i>	Monkeys	Taiwan
<i>Mansonella llewellyni</i>	<i>C. hollensis</i>	Raccoons	North and South America
<i>Mansonella marmosetae</i>	<i>C. hollensis</i> , <i>C. furens</i>	Monkeys	South America
<i>Mansonella ozzardi</i>	<i>C. barbosai</i> , <i>C. furens</i> , <i>C. paraensis</i> , <i>C. phlebotomus</i> (also <i>Leptoconops</i> spp. and Blackflies <i>Simulium</i> spp.)	Humans	South and Central America
<i>Mansonella perstans</i>	<i>C. austeni</i> , <i>C. grahami</i> , <i>C. inornatipennis</i> , <i>C. milnei</i> group	Humans	Africa, South and Central America
<i>Mansonella streptocerca</i>	<i>C. grahami</i>	Humans, monkeys	Africa
<i>Onchocerca cebei</i>	<i>Culicoides</i> spp.	Water buffaloes	Asia, Australia
<i>Onchocerca cervicalis</i>	<i>C. nubeculosus</i> , <i>C. parroti</i> , <i>C. obsoletus</i> , <i>C. variipennis</i>	Equids	Africa, Asia, Australia, Europe, North and South America
<i>Onchocerca flexuosa</i>	<i>C. nubeculosus</i>	Cervidae	Europe, Asia
<i>Onchocerca gibsoni</i>	<i>C. buckleyi</i> , <i>C. orientalis</i> , <i>C. oxystoma</i> , <i>C. pungens</i> , <i>C. shortti</i> , <i>C. marksii</i> (also <i>Forcipomyia</i> spp.)	Cattle	Africa, Asia, Australia
<i>Onchocerca gutturosa</i>	<i>C. nubeculosus</i> (but primarily blackflies <i>Simulium</i> spp.)	Cattle	Europe, North and South America
<i>Onchocerca reticulata</i>	<i>C. nubeculosus</i>	Horses	Australia
<i>Ornithofilaria fallisensis</i>	<i>Culicoides</i> spp.	Ducks	Asia, North America
<i>Splendidofilaria californiensis</i>	<i>C. multidentatus</i>	Birds	North America
<i>Splendidofilaria picacardina</i>	<i>C. crepuscularis</i>	Birds	North America
Protozoa			
<i>Haemoproteus attenuatus</i>	<i>C. festivipennis</i> , <i>C. nubeculosus</i> , <i>C. obsoletus</i>	Birds	Europe
<i>Haemoproteus balmorali</i>	<i>C. impunctatus</i> , <i>C. scoticus</i>	Birds	Africa, Europe
<i>Haemoproteus belopolskyi</i>	<i>C. impunctatus</i>	Birds	Europe
<i>Haemoproteus danilewskii</i>	<i>C. arboricola</i> , <i>C. crepuscularis</i> , <i>C. edeni</i> , <i>C. knowltoni</i> , <i>C. sphagnumensis</i> , <i>C. stilobezzioides</i>	Birds	North America
<i>Haemoproteus dolniki</i>	<i>C. impunctatus</i>	Birds	Europe
<i>Haemoproteus fringillae</i>	<i>C. crepuscularis</i> , <i>C. impunctatus</i> , <i>C. sphagnumensis</i> , <i>C. stilobezzioides</i>	Birds	North America
<i>Haemoproteus handai</i>	<i>C. nubeculosus</i>	Birds	Europe
<i>Haemoproteus lanii</i>	<i>C. impunctatus</i> , <i>C. obsoletus</i>	Birds	Europe
<i>Haemoproteus majoris</i>	<i>C. impunctatus</i> , <i>C. punctatus</i>	Birds	Europe, Asia
<i>Haemoproteus mansonii</i>	<i>C. arboricola</i> , <i>C. edeni</i> , <i>C. haematopotus</i> , <i>C. hinmani</i> , <i>C. sphagnumensis</i>	Birds	North America
<i>Haemoproteus meleagridis</i>	<i>C. edeni</i> , <i>C. hinmani</i> , <i>C. arboricola</i> , <i>C. haematopotus</i> , <i>C. knowltoni</i>	Chickens, turkeys	North America
<i>Haemoproteus minutus</i>	<i>C. impunctatus</i> , <i>C. nubeculosus</i> , <i>C. kibunensis</i> , <i>C. obsoletus</i> , <i>C. scoticus</i> , <i>C. pictipennis</i> , <i>C. punctatus</i>	Birds	Europe
<i>Haemoproteus motacillae</i>	<i>C. impunctatus</i> , <i>C. nubeculosus</i>	Birds	Europe
<i>Haemoproteus nettionis</i>	<i>C. downesi</i>	Ducks	Worldwide
<i>Haemoproteus noctuae</i>	<i>C. impunctatus</i> , <i>C. nubeculosus</i>	Birds	Europe, Asia
<i>Haemoproteus pallidus</i>	<i>C. impunctatus</i> , <i>C. kibunensis</i> , <i>C. punctatus</i>	Birds	Europe
<i>Haemoproteus parabelopolskyi</i>	<i>C. impunctatus</i> , <i>C. pictipennis</i>	Birds	Europe
<i>Haemoproteus symii</i>	<i>C. nubeculosus</i>	Birds	America, Asia, Europe
<i>Haemoproteus tartakovskyi</i>	<i>C. impunctatus</i> , <i>C. nubeculosus</i> , <i>C. kibunensis</i> , <i>C. obsoletus</i> , <i>C. scoticus</i> , <i>C. punctatus</i>	Birds	Europe
<i>Haemoproteus velans</i>	<i>C. sphagnumensis</i> , <i>C. stilobezzioides</i>	Birds	India, North America
<i>Hepatocystis bouillezi</i>	<i>Culicoides</i> spp.	Monkeys	Africa

(Continued)

Table 1 (Continued)

Pathogens	Demonstrated or putative vectors	Hosts	Geographic area
<i>Hepaticystis brayi</i>	<i>Culicoides</i> spp.	Squirrels (Sciuridae)	Malaysia
<i>Hepaticystis cercopithecii</i>	<i>Culicoides</i> spp.	Monkeys	Africa
<i>Hepaticystis foleyii</i>	<i>Culicoides</i> spp.	Monkeys	Africa
<i>Hepaticystis kochi</i>	<i>C. adersi</i> , <i>C. fulvithorax</i> , <i>Culicoides</i> spp.	Monkeys	Africa
<i>Hepaticystis semnopithecii</i>	<i>Culicoides</i> spp.	Monkeys	Africa, Old World
<i>Hepaticystis taiwanensis</i>	<i>Culicoides</i> spp.	Monkeys	Taiwan
<i>Leishmania donovani</i>	<i>Culicoides</i> spp.	Humans, dogs	Africa, Europe, Russia, India, Nepal, China, South America
<i>Leishmania enriettii</i>	<i>C. sonorensis</i> (primarily phlebotomine sand flies, but also <i>Forcipomyia</i> spp.)	Guinea pig	Brazil
<i>Leishmania infantum</i>	<i>C. nubeculosus</i> , <i>Culicoides</i> spp. (but primarily phlebotomine sand flies)	Humans, dogs	Worldwide
<i>Leishmania orientalis</i>	<i>C. sonorensis</i>	Not known	Thailand
<i>Leucocytozoon caulleryi</i>	<i>C. arakawe</i> , <i>C. circumscriptus</i> , <i>C. odibilis</i> , <i>C. schultzei</i>	Chickens	Asia
<i>Leucocytozoon neavei</i>	<i>Culicoides</i> spp.	Birds	Africa
<i>Leucocytozoon sabraezii</i>	<i>Culicoides</i> spp.	Chickens	Asia, Indonesia
<i>Leucocytozoon toddi</i>	<i>Culicoides</i> spp.	Chickens	Europe
<i>Trypanosoma avium</i>	<i>C. nubeculosus</i>	Birds	Worldwide
<i>Trypanosoma bennetti</i>	<i>Culicoides</i> spp.	Birds	North America
<i>Trypanosoma evansi</i>	<i>Culicoides</i> spp.	Wild and domestic mammals	Africa, Asia, Central and South America, Europe
<i>Trypanosoma vivax</i>	<i>C. furens</i> , <i>Culicoides</i> spp. (but primarily tsetse flies <i>Glossina</i> spp.)	Wild and domestic mammals	Africa, Central and South America, the Caribbean
<i>Vavraia</i> sp.	<i>C. edeni</i>	Probably insects	North America
Viruses			
African horse sickness	<i>C. imicola</i> , <i>C. bolitinos</i> , <i>C. pulicaris</i> , <i>C. obsoletus</i> complex <i>Culicoides</i> spp.	Horses, donkeys, mules, zebras, dogs	Africa, Asia, Europe, the Middle East
Aino	<i>C. brevitarsis</i> , <i>C. oxystoma</i>	Cattle, sheep (antibodies detected in goats, horses, pigs humans)	Asia (mainly Japan), Australia
Akabane	<i>C. brevitarsis</i> , <i>C. imicola</i> , <i>C. milnei</i> , <i>C. oxystoma</i> , <i>C. wadai</i>	Cattle, sheep, goats	Asia, Africa, the Middle East, Australia
Ananindeua	<i>C. paraensis</i> (also mosquitoes)	Birds, rodents, marsupials	Brazil
Barmah Forest	<i>C. marksi</i>	Birds, marsupials, monkeys, humans	Australia, Papua New Guinea
Barur	<i>C. punctatus</i>	Cattle (antibodies detected in cattle, goats and rats)	India, Japan, Kenya, Somalia
Beatrice Hill	<i>C. peregrinus</i>	Not known	Australia
Belmont	<i>C. bundyensis</i> , <i>C. marksi</i>	Antibodies detected in cattle, wallabies and kangaroo	Australia
Bivens Arm	<i>C. insignis</i>	Antibodies detected in cattle, buffaloes, horses, deer	North and Central America
Bluetongue	<i>C. sonorensis</i> , <i>C. brevitarsis</i> , <i>C. imicola</i> , <i>C. bolitinos</i> , <i>C. insignis</i> , <i>C. brevitarsis</i> , <i>C. wadai</i> , <i>C. actoni</i> , <i>C. fulvus</i> , <i>C. pusillus</i> , <i>C. pulicaris</i> complex, <i>C. obsoletus</i> complex	Wild and domestic ruminants	Africa, Asia, Europe, the Middle East, North and South America, Australia, the Caribbean
Bovine ephemeral fever	<i>C. imicola</i> , <i>C. kingi</i> , <i>C. oxystoma</i> , <i>C. punctatus</i> , <i>C. schultzei</i> , <i>C. brevitarsis</i> , <i>C. coarctus</i> , <i>C. bedfordi</i> , <i>C. puncticollis</i>	Cattle, yaks, water buffaloes (antibodies detected in many wild and domestic animals)	Africa, Asia, Australia, the Middle East
Bunyip Creek	<i>C. brevitarsis</i> , <i>C. oxystoma</i> , <i>C. schultzei</i>	Cattle (antibodies detected also in buffalo, sheep, deer)	Australia, Japan
Buttonwillow	<i>C. variipennis</i> , <i>Culicoides</i> (<i>Selfia</i>) spp.	Leporids (antibodies detected also in humans)	North America
Crimean-Congo hemorrhagic fever	<i>Culicoides</i> spp. (but primarily ticks)	Humans, wild and domestic animals	Africa, Asia, Europe, the Middle East
CSIRO Village	<i>C. brevitarsis</i> , <i>Culicoides</i> spp.	Cattle (antibodies detected also in buffalo, sheep, deer)	Australia
D'Aguilar	<i>C. brevitarsis</i> , <i>C. oxystoma</i> , <i>Culicoides</i> spp.	Cattle (antibodies detected also in sheep and other vertebrates)	Australia, Japan
Douglas	<i>C. brevitarsis</i>	Ruminants	Australia
Dugbe	<i>Culicoides</i> spp. (but primarily ticks)	Humans, cattle	Africa

Table 1 (Continued)

Pathogens	Demonstrated or putative vectors	Hosts	Geographic area
Eastern Equine Encephalomyelitis	<i>Culicoides</i> spp. (but primarily mosquitoes)	Birds, horses and other equids, humans	North and South America, Caribbean Basin
Epizootic haemorrhagic disease	<i>C. sonorensis</i> , <i>C. brevitarsis</i> , <i>C. kingi</i> , <i>C. stellifer</i> , <i>C. venustus</i> , <i>Culicoides</i> spp.	Cervids	North and South America, the Caribbean, Australia, Asia, Africa and the Middle East
Equine encephalosis	<i>C. imicola</i> , <i>C. bolitinos</i> , <i>Culicoides</i> spp.	Horses	Africa, Israel
Eubenangee	<i>C. marksii</i> , <i>C. variipennis</i> (but primarily mosquitoes)	Antibodies detected in cattle and marsupials	Australia
Gweru	<i>Culicoides</i> spp.	Cattle, goats, sheep	South Africa
Humpty Doo	<i>C. marksii</i> (also in <i>Lasiohelea</i> spp.)	Antibodies detected in cattle	Australia
Israel-Turkey meningoencephalitis	<i>Culicoides</i> spp. (also mosquitoes)	Turkeys	Israel, South Africa
Itacaiunas	<i>Culicoides</i> spp.	Not known	Brazil
Kasba	<i>C. oxystoma</i> , <i>C. schultzei</i>	Cattle, goats	Africa, Asia
Keterah	<i>C. schultzei</i> (primarily ticks)	Birds, bats, humans	Malaysia
Kimberley	<i>C. brevitarsis</i>	Cattle (antibodies detected in water buffaloes, goats, horses)	Australia, Indonesia, Papua New Guinea, China
Kotonkan	<i>Culicoides</i> spp.	Antibodies detected in cattle, rodents, humans	Nigeria
Leanyer	<i>C. marksii</i>	Not known	Australia
Lokeren	<i>C. variipennis</i> , <i>Culicoides</i> (<i>Selfia</i>) spp.	Rabbits (antibodies detected also in domestic ruminants)	North America
Main Drain	<i>C. variipennis</i> , <i>C. nubeculosus</i>	Leporids	North America
Marrakai	<i>C. schultzei</i> , <i>C. peregrinus</i>	Cattle	Australia
Mitchell River	<i>Culicoides</i> spp.	Antibodies detected in various vertebrates	Australia
Mudjinbarry	<i>C. marksii</i>	Antibodies detected in wallabies, dingoes, domestic fowl and human	Australia
Nairobi sheep disease	<i>C. tororoensis</i> (but primarily ticks)	Sheep, goats	Asia, East and Central Africa
Ngaingan	<i>C. brevitarsis</i> , <i>C. actoni</i>	Antibodies detected in cattle, wallabies, kangaroos	Australia
Nyabira	<i>C. imicola</i> , <i>C. zuluensis</i>	Virus isolations from aborted bovine fetuses	South Africa, Zimbabwe
Oropouche	<i>C. paraensis</i> (also mosquitoes)	Humans, monkeys, wild and domestic birds	Central and South America
Parker's Farm	<i>C. marksii</i>	Not known	Australia
Peaton	<i>C. brevitarsis</i>	Cattle	Australia
Rift Valley Fever	<i>Culicoides</i> spp. (but primarily mosquitoes)	Humans, wild and domestic animals, rodents	Africa, Saudi Arabia and Yemen
Sabo	<i>C. imicola</i> , <i>Culicoides</i> spp.	Cattle, goats	Nigeria
Sango	<i>Culicoides</i> spp.	Cattle	Nigeria, Kenya
Sathuperi	<i>C. imicola</i> , <i>C. oxystoma</i> , <i>C. puncticollis</i>	Cattle	India, Israel, Nigeria
Schmallenberg	<i>C. obsoletus</i> complex, <i>C. pulicaris</i> complex, <i>C. imicola</i>	Wild and domestic ruminants	Europe
Shamonda	<i>C. imicola</i> , <i>Culicoides</i> spp.	Cattle	Nigeria, Japan
Shuni	<i>Culicoides</i> spp.	Humans, cattle, sheep	Nigeria, South Africa
Tahyna	<i>Culicoides</i> spp. (but primarily mosquitoes)	Lagomorphs	Europe, Africa, Asia
Thimiri	<i>C. histrio</i>	Birds	Africa, Australia, India
Tibrogargan	<i>C. brevitarsis</i>	Antibodies detected in cattle, water buffaloes	Australia
Tinaroo	<i>C. brevitarsis</i>	Antibodies detected in cattle, buffalo, sheep, goats	Australia
Vesicular stomatitis-New Jersey	<i>C. variipennis</i> , <i>C. stellifer</i> , <i>Culicoides</i> (<i>Selfia</i>) spp.	Horses, cattle, swine, humans	North, Central and South America
Wallal	<i>C. dycei</i> , <i>C. marksii</i> , <i>Culicoides</i> spp.	Marsupials (antibodies also detected in various vertebrates)	Australia
Warrego	<i>C. dycei</i> , <i>C. marksii</i> , <i>C. actoni</i> , <i>Culicoides</i> spp.	Antibodies detected in various vertebrates	Australia
Weldona	<i>Culicoides</i> spp.	Birds	North America
Wongorr	<i>C. pallidothorax</i> (also mosquitoes)	Antibodies detected in humans and other vertebrates	Australia

^aPathogens in each group are listed in alphabetical order.

horsemeat (Van Rensburg et al., 1981) and camels are also reported as susceptible, however both species should be considered accidental hosts (Mellor and Hamblin, 2004).

AHS is endemic in Africa with the most affected countries being in the Sub-Saharan territory, particularly in South Africa. In this country, epidemic waves appear around every 10–15 years associated with climate conditions connected to different phase of El Niño (Baylis et al., 1999). However, outbreaks of AHS has been also reported outside of Africa causing relevant costs to the equine husbandry. AHS was diagnosed in 1944 in Israel, between 1959 and 1966 in Turkey, Middle East, and South West Asia (Howell, 1960; Dennis et al., 2019). More recently, in the period 1987–91 the disease was reported in Morocco, Portugal and Spain (Lubroth, 1988), thus raising concerns on the risk that could be constituted by the international movements of competition horses (Herholz et al., 2008). In addition, on the basis of the unexpected wide spreading in Europe in the early 2000s of bluetongue virus, which is closely related to AHSV, it remains the significant risk for the introduction the AHS to the European countries.

It has been suggested that the first sites of virus replication are the regional lymphoid tissues. After a first viremia, a subsequent invasion in several organs is observed, with a particular tropism to lung, spleen and heart (Mellor and Hamblin, 2004). By using electron microscopy AHSV has been localized in the endothelial cells (Laegreid et al., 1992). Further studies, based on immunohistochemistry and in situ hybridization analysis, have demonstrated that AHSV antigen mostly accumulates in the endothelial cells of the micro vessels, in the infiltrating macrophages and circulating monocytes of the lung, heart and spleen (Brown et al., 1994; Clift and Penrith, 2010), thus suggesting that sampling from these organs is the most appropriate for diagnostic purposes. In addition, after experimental intranasal inoculation, a neurotropic strain of AHSV was immunohistochemically found associated with damaged neuron and astrocytes in the gray matter of the brain (Clift and Penrith, 2010).

The viremic phase in AHS is very short compared to other related arbovirosis such as Bluetongue, ranging 4–8 days post-infection, (Coetzer and Erasmus, 1994). In the blood of the infected animal, AHSV is associated to the red cells component, probably engulfed in the cellular membrane, miming what was demonstrated for BTV in cattle.

The clinical outcomes of AHSV infection in horses is very variable, ranging from subclinical to fatally severe, with the mortality rate estimated around 95%–100% (Clift and Penrith, 2010). Clinical aspects of the disease have been summed up in four different forms, namely horse sickness fever, mixed, cardiac and pulmonary (Mellor and Hamblin, 2004). The pulmonary form is commonly considered to be the most fatal acute form while the mixed form is the most common. The mortality rate in the pulmonary form achieves the 100% in many outbreaks. The fever form is the mildest which only displays a transitory rise of fever. However the different clinical courses of the disease are not easy to distinguish in practical conditions (Scacchia et al., 2015). Frequent clinical signs include marked depression, dyspnea, coughing, as well as oedema of the head, neck and orbital fosses (Fig. 12). Petechial



Fig. 12 Horse affected by AHS. Edema of the orbital fosses (A), pulmonary edema (B) and gelatinous edema of the inter-muscular fascia (C). Photo credit: Massimo Scacchia, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise.

haemorrhages are frequently found in the conjunctival mucosae and in the ventral surface of the tongue. At the necroscopy, gross changes are hydropericardium, ascite in the dominal and pleuric cavity, oedema of the subcutaneous tissues and of the muscular fascea (Fig. 12).

Bluetongue disease

Bluetongue (BT) is an arbovirosis affecting ruminants and caused by Bluetongue virus (BTV), which belongs to the family Reoviridae, genus Orbivirus. BTV genome consists in 10 segments of linear double stranded RNA (Seg-1 to Seg-10), encoding 7 structural proteins (VP1-Vp7) and five non-structural proteins (NS1 to NS4 and S10-ORF2) involved in viral replication, morphogenesis and assembly processes. The intermediate capsid protein layer VP7 expresses group antigens common to all BTV strains, VP2 and VP5 outer capsid proteins are involved in the virus binding and entering into the mammalian and, probably, insect vector cells (Mertens and Diprose, 2004; Lourenco and Roy, 2011; Jacquot et al., 2019). Sequence variability of VP2 forms the basis for grouping/classifying BTV into different serotypes (Patel and Roy, 2014).

Based on variations in their nucleotide sequences genome segments, two major geographic BTV groups are defined and named as western or eastern topotypes. The western topotype includes viruses given source from Africa and the Americas, the eastern includes viruses from the Middle East, India, China and Australia (Mertens et al., 1987; Gould and Pritchard, 1990; Pritchard et al., 1995, 2004; Maan et al., 2008).

Up to now there are 24 conventional BTV serotypes able to cause BT. Recently, plus novel serotypes in goats have been identified as atypical because the infected animals are generally asymptomatic and the virus can be transmitted by direct contact (Maan et al., 2011; Zientara et al., 2014; Schulz et al., 2016; Sun et al., 2016; Savini et al., 2017; Marcacci et al., 2018).

The clinical outcomes of BTV infection are significantly different among the species. In sheep they can be severe with high rate of mortality (MacLachlan et al., 2008), whereas in cattle, goats and wild ruminants symptoms they usually are mild, if not, completely absents. By using controlled experimental conditions it was demonstrated that the species of the host and the single isolates of the BTV serotype play a key role in modulating the clinical aspects (Caporale et al., 2014). In clinically susceptible ruminants, most common symptoms are different grades of fever, conjunctivitis, hyperaemia of the periorbital areas, nasal discharge, anorexia, lameness, prostration, and subcutaneous oedema in the inter-mandibular space, hemorrhagic-necrotic erosions on the dorsal mucosal surface of the tongue, which in the severe cases exhibited a bluish staining (Fig. 13). In experimental BTV infection the body temperature increases at 6 days post intra-dermal inoculation together with other early clinical (Puggioni et al., 2018). One the morpho-pathological feature, which results in the clinical presentation of BTV infection, is the localization of the virus in the



Fig. 13 Ram affected by BT. Representative photos showing characteristic clinical signs, including inter-mandibular edema (A), conjunctivitis (B), mucopurulent nasal discharge (C), erythema with ulcerations in the buccal mucosae (D), coronitis (E), erythema of the scrotum and inguinal skin areas (F). Photo credit: Davide Pintus, Istituto Zooprofilattico Sperimentale della Sardegna.

endothelial cell of vessel (MacLachlan et al., 2008). Indeed, BTV is detected in sheep, during 24 h after experimental infection, particularly associated with the endothelial cells of the lymphatic, rather than hematic vessels, in the dermis of the skin close to the inoculation sites. Within 24 h after infection, BTV is also found in the sinus of the draining lymph nodes of the inoculation point, inside the macrophages and the endothelial cells and in the cortical follicles associated to the B lymphocytes. Later, from 3 to 11 days post infection, replication of BTV is mostly observed by immunohistological staining in the endothelial cells of the smaller vessel in numerous organs, particularly skin, lung, lymphoid tissues nodes far from the side of inoculation, and testicles (Melzi et al., 2016; Puggioni et al., 2018). At 15 days after inoculum BTV is not detected in the endothelial cells by immunohistochemistry (Puggioni et al., 2018). In the blood of the host, BTV is associated with platelets, but considering the short lifespan of these cells, it is mostly engulfed into the cell membrane invagination of erythrocytes, remaining infectious despite the presence of neutralizing antibodies (Brewer and MacLachlan, 1992; Parsonson and McColl, 1995). The viremic status has been considered, by using PCR assay or virus isolation in culture cells, long lasting until several weeks in cow (Singer et al., 2001; MacLachlan, 2004).

Serological diagnosis of BTV infection is carried out using enzyme-linked immunosorbent assay (ELISA) tests that detect anti-VP7 antibodies in the serum and milk of animals infected with BTV (Gumm and Newman, 1982; Hamblin, 2004). Serum neutralisation test is the only serological method that is able to identify and quantify BTV serotype antibodies specific and determine viral serotype responsible for the infection. In order to confirm clinical cases, to establish uninfected animals before moving and for surveillance purposes, OIE recommends the use of a real-time RT-PCR (RT-PCRNS3) assay (OIE, 2019). The method real-time RT-PCRNS3 described by Hofmann et al. (2008) is suitable to identify the serogroup by retro-transcription and amplification of a region of segment 10 of the viral RNA, coding for a non-structural NS3 protein. Real time RT-PCR targeting in Seg-2 coding for a least conserved outer virion capsid protein (VP2) recognizes the specific BTV serotype (Huismans and Erasmus, 1981; Shaw et al., 2013).

Epizootic haemorrhagic disease

Epizootic haemorrhagic disease (EHD) is an insect-born disease caused by a virus (EHDV) belonging to the genus Orbivirus and affecting wild and domestic ruminant species. The disease was first recognized during the 50s years (Shope et al., 1960) in white tailed deer (*Odocoileus virginianus*). By molecular and serological data based on the reactions between the structural proteins V2 and V7 and the specific neutralizing antibodies, seven different serotypes of EHDV have been recognized and conventionally accepted (Anthony et al., 2009; King et al., 2012).

Whole genome sequencing has demonstrated that some EHD isolates were reassortant between different serotypes, suggesting that these seven serotypes are not stable and multiple strains could come out annually in different outbreaks (Allison et al., 2010; Wang et al., 2019). These serotypes have different geographical distribution, EHDV-1, EHDV-2 and EHDV-6 are endemic in USA (Allison et al., 2010), while EHDV-6, previously indicated as EHDV-318, and EHDV-2 are found in the Arabic peninsula (Mohammed et al., 1996; Savini et al., 2011). Moreover, EHDV serotypes 2, 3 and 4 have been identified in the African countries (Savini et al., 2011), whereas EHDV serotypes 1, 2, 5, and 7 have been characterized in Australian sentinel cattle (Maan et al., 2010; Savini et al., 2011). A particular EHDV-2 strain, named Ibaraki, which is phylogenetically considered to be as an Asian topotype (Maan et al., 2010), was isolated in Japan causing severe clinical disease in cattle (Ohashi et al., 1999). Beyond Japan, others EHDV outbreaks in cattle have been reported in the Mediterranean basin due to the EHD serotypes 6 and 7 (Yadin et al., 2008; Temizel et al., 2009).

Clinical signs following EHDV infection have been mostly observed in white tailed deer and much less in cattle, in which clinical disease remains sporadically associated to EHDV-2, EHDV-6, EHDV-7 and Ibaraki virus. In cattle cases, clinical signs included serous-purulent nasal discharge, scialorrea, muzzle redness, and tongue swelling with cyanosis and erosions (Yadin et al., 2008; Temizel et al., 2009). A wide range of wild ruminants is found to be susceptible to EHDV, including captive yaks (*Bos grunniens*) (Van Campen et al., 2013), bighorn sheep (*Ovis canadensis*) (Clark et al., 1985) in USA, and gray brocket deer (*Mazama gouazoubira*) in Brazil (Favero et al., 2013). In these outbreaks, clinical manifestations of disease were reported, however the symptomatic disease is a rare event in these wild ruminants, despite that seroprevalence in the endemic areas is usually found relatively high.

In white tailed deer, peculiar clinical signs during the course of the EHD are fever, redness of the orbital areas, perineum and ear pinna, hyperemia of the conjunctiva, facial oedema, erythema of buccal mucosae with ulcerations and bleeding (Fig. 14).

In white tailed deer experimentally inoculated with EHDV-2 and EHDV-7 serotypes, clinical signs started between 4 and 5 days post-infection (Quist et al., 1997; Ruder et al., 2012). In these experiments, lesions were minimal to severe and included petechial or ecchymotic haemorrhages in the pulmonary artery and peritoneal serosa and necrosis secondary to bacterial infections. In addition, pulmonary edema was observed with different individual severity. The length of the viremic phase was different among the infected animals and ranged 4–56 days post infection (Quist et al., 1997) by using the EHDV-2 serotype or between 3 and 39 days after inoculum of EHDV-7 (Ruder et al., 2012). The haematochemical examinations showed leukopenia, lymphopenia, hypoproteinaemia and diminished platelet count (Ruder et al., 2012). In cattle, experimentally infected with EHDV-6 serotype, high levels of viral RNA were demonstrated in the blood, although they displayed very mild, if any, clinical signs, thus suggesting that this specie can be a reservoir of the virus (Breard et al., 2013; Batten et al., 2011). During the autopsy of experimental EHDV inoculated cattle, virus RNA was isolated from various tissues by RT-qPCR, such as spleen, liver, skin, kidney, and lymph-nodes (Breard et al., 2013). These clinical and pathological findings, together with many other field observations, suggest a manifested species and individual variability of the clinical outcomes in deer and cattle with EHD, as observed in sheep affected by BT (Caporale et al., 2014).



Fig. 14 Deer affected by EHD. Mild clinical signs after experimental infection. Redness of the orbital areas, ear pinna, muzzle, and peri-orbital skin. Photo credit: Juergen Richt, Kansas State University and William Wilson, USDA-ARS.

Currently, there are no experimental studies aimed to define the kinetic of EHDV infection in the host body. However, on the basis of the clinical-pathological patterns which have been observed so far in field and experimental conditions, EHDV infection share many aspects with better studied Orbivirus infections, such as BT in ruminants. Indeed, as in BTV, EHDV early localizes in the endothelium of the smaller vessels causing disseminated intravascular coagulation (MacLachlan et al., 2009). Considering the long viremic phase in deer and cattle (Quist et al., 1997; Batten et al., 2011; Breard et al., 2013), it is also conceivable that this virus remains strictly adherent to the cell membrane of the erythrocytes, thus surviving despite the presence of neutralizing antibodies, as observed in bovine bluetongue (MacLachlan et al., 2009).

Schmallenberg disease

Schmallenberg disease (SBD) is an arbovirosis causing abortion, congenital malformations in newborn lambs, calves and kids, whilst in the adult ruminants, mild clinical signs such as drop of milk production, hyperthermia, and diarrhea are occasionally reported (Hoffmann et al., 2012; Lievaart-Peterson et al., 2012). The etiological agent is the recent discovered Schmallenberg virus (SBV). By metagenomic analysis, SBV was isolated in the blood of cows hold in a dairy farm located in the village of Schmallenberg (North Rhine-Westphalia, Germany), at the end of the summer 2011 (Hoffmann et al., 2012).

SBD has been described in many other European countries including Italy, Spain, Belgium, France, United Kingdom, with a total of 29 countries being involved (European Food Safety Authority (EFSA), 2012; Afonso et al., 2014). As consequence of this epidemic wave, SBD caused productive performance losses in the affected farms and a negative impact on the well-being of the farmer dealing with this new emerging disease (Häsler et al., 2015; Pythian and Glover, 2019).

Specific antibodies against SBV or viraemia are reported in numerous wild ruminant species in natural and experimental conditions, including alpacas, water buffalo, elk, bison, deer and chamois (European Food Safety Authority (EFSA), 2014). However, the failure to observe clinical signs and the low seroprevalence suggests that the role of the wild animals in the epidemiology of SBV requires further investigations (Collins et al., 2019). So far, the data available for domestic dog are conflicting. While in one dog showing neurological signs, SBV genome was found in the cerebellum (Sailleau et al., 2013), in a serological survey no dogs seropositive for SBV were found (Garigliany et al., 2013). No antibodies or viraemia has been found in human exposed to SBV in the affected farm (European Food Safety Authority (EFSA), 2012).

The source of SBV remains unknown. This gap is firstly due to the little knowledge on the phylogeny of SBV and of the other closely related Simbu serogroup virus, which is widespread in other continents (Collins et al., 2019). Interestingly, antibodies against SBV were found in 56.6% of dairy cattle sampled in Ethiopia during the period September 2011–12 (Sibhat et al., 2018), as well as, between 2012 and 2014, SBV seropositivity rates for cattle and sheep were found of 91.2% and 65.4%, respectively, in Nigeria (Oluwayelu et al., 2018).

Taken together these reports demonstrated that SBV was circulating even in Africa, soon after its earlier appearance in Europe. Nowadays, circulation of SBV in the ruminant population could be considered worldwide (Rasekh et al., 2018; Zhai et al., 2018).

SBV viraemia is short lasting both in sheep and calves and RNA is detected until 5–6 days post inoculum (Hoffmann et al., 2012; Poskin et al., 2014). In calves experimentally infected, neutralizing antibodies in blood were detected firstly at 21 days after inoculum (Hoffmann et al., 2012) and remained present until 63 days (Wernike et al., 2013). Seroprevalence of SBV in the affected bovine herd can be very high, reaching 75% of rate (King et al., 2015), while in sheep and goat are 43.8% and 58.7%, respectively (Helmer et al., 2015). The presence of neutralizing antibodies may be not sufficient to prevent re-infection, as infected sheep with

neutralizing antibodies had viraemia (Jones et al., 2019). However, in a previous experimental study SBV seropositive calves, which were re-challenged, did not develop testable viraemia (Wernike et al., 2013).

Considering that diffusion of SBV is strictly related to the seasonality of the *Culicoides* vectors, the infection occurs in the summer and in the autumn, with the outcomes on the fetus being evident at the end of gestation period. In this regard, a long surveillance period in France revealed that congenital malformations occurred mostly during the months of January-February in cattle and November-December in ewes (Cache et al., 2018). However, some SBV infection may occur even in the winter (Cache et al., 2018).

The characteristic signs of SBD are the malformations of the offspring, which are the outcomes of the infection of the dams at determined stage of gestation, the transplacental crossing capacity of the virus and the susceptibility of the embryo tissues to the virus. An overview of the studies based on challenging pregnant ruminants with SBV suggested that this virus has a limited capacity to cause malformations in the foetus (Collins et al., 2019). Depending on the breeding strategy applied in the different farms, the within flock sheep prevalence of malformed stillborn was estimated between 3% and 19% (Dei Giudici et al., 2013).

For understanding the pathogenesis of SBV, data from experimental study on Akabane (AKA), a closely related Simbu serogroup virus, has been used (Konno et al., 1982). It is assumed that, the outcomes of AKA virus (AKAv) infection on embryos are strictly determined by the gestation period at which dams are infected. In pregnant cow, AKAv is able to affect the embryo after the first month of gestation, with the critical period ranging from the 3rd to 6th month, causing fetal malformation in the central nervous system and skeletal muscle (Konno et al., 1982; Kirkland et al., 1988). By challenging pregnant sheep with intravenous inoculations, AKAv was demonstrated after 24 h in the placenta, however, only in sheep at 46–53 days of gestation fetus malformation occurred (Parsonson et al., 1988).

In naturally infected pregnant cows, the embryo seems to be susceptible for the SBV between 47 and 162 days of age (Wernike et al., 2014). The gross morphological examination of the SBV malformed stillborn lambs, kids and calves mostly found hydrocephalus, cerebellar hypoplasia and spinal cord hypoplasia in the central nervous system as well as brachygnathia inferior, curvature of the vertebral spine (kyphosis, scoliosis and torticollis) and arthrogryposis in the musculoskeletal system (Fig. 15). Histologically, moderate focal of lymphocytic meningo-encephalomyelitis are observed in some malformed brains with the lymphocytes T being the most represented inflammatory cells (Herder et al., 2012, 2013). Interestingly, a significantly higher occurrence of malformations interests only one of the twin lamb pregnancy.

Control

Culicoides control programs are generally implemented to reduce biting nuisance of livestock and/or humans and arbovirus transmission. Control measures can be applied against both larvae and adults. In the framework of Integrated Pest/Vector Management (IPM/IVM) and One Health criteria, habitat manipulation and larvicidal treatments with chemical and/or biorational insecticides have been the mostly used strategies against *Culicoides* larvae. Habitat management, such as alteration and/or removal and drainage of breeding sites, could be useful for reducing larval development sites and *Culicoides* larval density, particularly, for those species that live in specific and well know breeding habitats. An essential factor that affects the effectiveness of a larval control is the perfect knowledge of the immature habitats. *Culicoides* midges lay eggs in a wide range of moist environments with the larvae being generally semi-aquatic and susceptible to desiccation. However, removing the breeding sites for reduce the abundance of *C. sonorensis*, the North American Bluetongue virus vector, from a farm, did not result in a reduction of vector population compared with an untreated farm (Mayo et al., 2014). Habitat alteration covering dung heaps and mechanical disrupting cowpats have a negligible effect in reducing population density for European species such as *C. scoticus*, *C. chiopterus* and *C. dewulfi* (Lühken et al., 2014a,b).

Several studies have been carried out to evaluate the efficacy of larvicidal application on larval habitats, mainly on species responsible for biting nuisance. A significant larvicidal efficacy of organophosphates, pyrethroids, organochlorines and Insect Growth Regulators was reported against *C. furens*, *C. melleus*, *C. mississippiensis*, *C. sonorensis*, and *C. circumscriptus* in laboratory and field trials. More recent biorational larvicides showed a promising efficacy against biting midges. These active products include plant extracts, like neem-based products (Mulla and Su, 1999; Benelli et al., 2017), entomopathogenic fungi, namely *Metarhizium anisopliae*, *Beauveria bassiana*, and *Paecilomyces fumosoroseus* (Ansari et al., 2010, 2011; Nicholas and McCorkell, 2014; Narladkar et al., 2015), and mermithid nematodes such as *Heleidomeris* species (Mullens et al., 2008). The efficacy of bacterial agents *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus*, widely employed in the field control of mosquitoes, were tested mainly in laboratory trials. Several studies showed *B. t. israelensis* to be ineffective against field collected *Culicoides* larvae (Kelson et al., 1980; Larget and De Barjiac, 1981). By contrast, in another study *B. t. israelensis* and *Bacillus sphaericus* showed a lethal effect on Indian *Culicoides* species (Narladkar et al., 2006). A significant biocidal activity was also exhibited by *Brevibacillus laterosporus* in laboratory and field trials against some European *Culicoides* species (Foxi et al., 2019).

The IPM/IVM of *Culicoides* adults can be implemented with physical and/or chemical methods. Physical control strategies include housing livestock in stables during seasonal activity period of *Culicoides* midges to reduce biting activity. Nets on the windows are useful to limit the entry of midges inside the shelters. For a more successful strategy, these window nets can be treated with insecticides or repellents. Chemical control of *Culicoides* adults can be achieved by treating animal body with insecticides or repellents, mainly pyrethroids, with pour-on or dip-wash formulations. However, the real efficacy of this method in reducing biting rate has not been proved (Carpenter et al., 2008). Although the broad scale treatments on the whole farm area have not reduced the



Fig. 15 Stillborn lambs affected by SB. Malformations include arthrogryposis and torticollis (A), brachygnathia (B), spider-like arthrogryposis (C), hydrocephalus (D), skull malformation (E), macroscopic and histological feature of the spinal cord hypoplasia compared with a control (F). Photo credit: Davide Pintus, Istituto Zooprofilattico Sperimentale della Sardegna.

number of *Culicoides* adults (Satta et al., 2004), the treatments restricted to the walls and roofs of animal shelters combined with larval treatments on breeding sites has determined a significant reduction of *Culicoides* adult density (Meloni et al., 2018).

As a conclusion, the control of *Culicoides* is not easy feasible and should be conducted according to the guidelines of IPM/IVM, combining the different techniques available. Indeed, a single strategy as a stand-alone technique usually show limited effects in reducing the abundance of *Culicoides* vector species and consequently on pathogens transmission (Carpenter et al., 2008; European Food Safety Authority (EFSA), 2008; Mullens et al., 2015; Pfannenstiel et al., 2015; Harrup et al., 2016; Benelli et al., 2017).

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Blackflies (Simuliidae)

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Introduction

The small size of flies in the family Simuliidae, with wingspans of 3–15 mm, belies the magnitude of their importance in medical, veterinary, and economic sectors of society. The nearly 200 vernacular names applied to these widespread insects (Davies, 2006) attest to their global significance. Numbering about 2330 species worldwide (Adler, 2020), or roughly 1.5% of all species of flies, they are most often referred to as “black flies” (or “blackflies”).

Driven by the quest for vertebrate blood to mature their eggs, female blackflies are responsible for withering attacks on humans, domesticated animals, and wildlife, often with significant economic losses and transmission of the agents of disease. Yet, only about 10–20% of the world’s species actually hold status as pests or vectors of disease agents of consequence to humans and their animals (Adler and McCreadie, 2018). Simuliid-borne human diseases include onchocerciasis and mansonellosis, the former debilitating and the latter mild. The most prominent simuliid-borne animal diseases include avian trypanosomiasis and leucocytozoonosis, onchocerciasis, and vesicular stomatitis.

Adult blackflies are compact insects characterized by an arched thorax—inspiring the common name “buffalo gnats”—anteriorly concentrated wing veins, and short beadlike or conical antennae (Fig. 1). But, not all blackflies are black. Many are gray, orange, iridescent, or patterned. Male blackflies have large, contiguous compound eyes (holoptic), whereas females have smaller, separated eyes (dichoptic). Species of the family can be notoriously difficult to identify, a problem compounded by the high frequency of cryptic species that show little to no structural differentiation and must be identified by chromosomal or molecular criteria (Adler et al., 2010; Adler and Crosskey, 2015).

The immature stages are confined to freshwater streams and rivers where the eggs (Fig. 2) are deposited. Upon hatching larvae lead a largely sedentary life as filter-feeders attached to in-stream substrates (Fig. 3). Pupation takes place in a silken cocoon spun from the enormous larval silk glands; the cocoon anchors the pupa to an object in the flow (Fig. 4). The adults are terrestrial and diurnal. Only the females feed on blood, whereas both sexes take water and sugars, such as floral nectar and insect honeydew (Stanfield and Hunter, 2010).

Biology and ecology

Blackflies occupy two profoundly different environments—the aquatic and the terrestrial. The dichotomy sets up a paradox whereby the immature stages are ecosystem benefactors but the adults, more specifically the females, are often in direct conflict with humans and their enterprises. Society is, therefore, faced with safeguarding its citizens and economic interests while conserving the ecological benefits of blackflies.

Blackflies are found everywhere that freshwater flows, including desert oases and remote oceanic islands. They are remarkably common insects. In any given area of the world, they are typically found in 90% or more of flowing water habitats, from the tiniest streams to the largest rivers. Although they are generally associated with clean waters, some species are quite pollution-tolerant. Mountainous areas of the globe, which offer a wide range of habitats along an elevation gradient, typically have the greatest number of species. Blackflies are often the most abundant macroinvertebrates in running water, reaching densities of 1 million larvae per square meter in extreme cases (Wotton, 1988).

The larvae attach to objects in the current, such as stones, trailing vegetation, and refuse (e.g., plastic), by enmeshing tiny abdominal hooks in a silk pad spun from the large silk glands. About 30 highly specialized species are phoretic, attaching to, and developing on, larval mayflies (Ephemeroptera) in Africa and Central Asia and to freshwater crabs and prawns in tropical Africa (Crosskey, 1990). Larval life concludes with spinning a silk cocoon in which molting to the pupa occurs. The compact pupa, with its conspicuous pair of respiratory organs (gills), is adapted for gas exchange in water or in air if decreasing water levels strand it.

The larvae of all but 1% of the world’s species are filter feeders, capturing fine particulate matter (0.09–350 µm in diameter) with their labral fans and processing it into fecal pellets rich in carbon, nitrogen, and bacterial films, which become available as food to



Fig. 1 Female of a blackfly, *Simulium vittatum*. Photo by Jena Johnson.



Fig. 2 Eggs of a blackfly, *Simulium vittatum*. Photo by Jena Johnson.

other aquatic organisms (Malmqvist et al., 2004). Fecal production by larval black flies can reach astonishing levels—429 metric tons of dry mass passing a river site per day—prompting researchers to refer to black flies as “ecosystem engineers” (Wotton et al., 1998; Malmqvist et al., 2001). Larvae also feed by scraping adherent material from the substrate; the 25 species that lack labral fans do so exclusively. Some species, especially those living in nutrient-poor streams, ingest small prey items. Larvae disperse or relocate by moving inchworm fashion or drifting on silk lifelines that they spin. Peak drifting of larval black flies occurs around dusk.

The egg stage can last a few days to years, depending on environmental conditions such as temperature and water availability. Some species undergo an obligatory egg diapause through the winter or the summer. Larvae can develop in as few as 4 days to as long as 6–9 months, depending largely on temperature. They typically pass through six or seven instars, although more if parasitized or starved. The pupal stage usually lasts a few days to a few weeks. The entire life cycle from egg to adult can be completed in 2 weeks or less, with some species in tropical environments completing as many as 15 or more generations per year. Univoltine species, those that complete a single generation annually, are associated with northern environments and high elevations. Adults emerge from the pupa in a partial covering of air and fly to a nearby location to tan and harden the body and wings.

The essentials of adult life are completed within a typical lifespan of a month or less: mating, sugar feeding, and for the females, blood feeding and egg laying. Mating takes place shortly after emergence, usually when males form swarms into which entering females are intercepted. These swarms typically form over landmarks, such as waterfalls, tips of tree branches, and bare ground. Less frequently, males and females locate one another and mate on the ground near the emergence site. About 15 species are parthenogenetic; males do not exist.

About 97.5% of all species have mouthparts fitted for cutting animal tissue. The other 2.5% of species, all in far-northern or extreme high-elevation environments, have mouthparts incapable of cutting animal tissue; they mature their eggs without blood (obligate autogeny). In cooler environments, larvae with high-quality diets produce females that can mature their eggs without benefit of a blood meal for the first cycle of egg production but require blood for subsequent egg batches (facultative autogeny). Most species of blackflies, however, require blood for all cycles of egg maturation (anautogeny). Multiple gonotrophic cycles of blood feeding and oviposition are possible and are prerequisites for parasite acquisition and transmission. Females of some species,



Fig. 3 Larvae of a blackfly, *Simulium vittatum*, filter-feeding. Photo by Jena Johnson.



Fig. 4 Pupae (ventral view) of a blackfly, *Simulium vittatum*. Photo by Jena Johnson.

particularly certain vectors in the *Simulium damnosum* complex, can disperse up to 500 km before a blood meal, although dispersal distances of fewer than 20 km are typical for most species (Crosskey, 1990). The female flies locate their blood hosts—exclusively birds and mammals—by a series of cues, such as host color, size, shape, and odor, especially carbon dioxide (Sutcliffe, 1986), which provides an effective method of sampling and trapping female flies.

Female flies deposit their eggs directly into the water during flight, or they lay them in strings and masses while walking on wetted surfaces or vegetation trailing in the current. Communal egg laying on substrates is mediated in some species by pheromones emitted from the freshly deposited eggs (McCall et al., 1997). The number of eggs matured in one ovarian cycle varies from 30 to more than 800, depending on the species (Crosskey, 1990).

Although the blood hosts of most species of blackflies are unknown, the general feeding habits can be inferred from the structure of the claws. Females that feed on mammals have curved claws, with or without a small basal tooth, whereas females that feed on birds have curved talons with a variably sized thumblike lobe that aids purchase as the flies move through feathers. Thus, roughly 51% of all bloodsucking species of blackflies feed predominantly on mammals and the other 49% on birds. At times, however, some species feed indiscriminately on birds and mammals, regardless of claw structure. Mammalophilic blackflies, including anthropophilic species, typically do not venture into enclosures to acquire blood meals. At least some ornithophilic species, however, frequently enter avian nest cavities and other enclosures (e.g., bird houses). Host tissue is cut by the serrated mandibles, which operate as micro-scissors to produce a pool of blood that is imbibed (Sutcliffe and McIver, 1984). Blackflies are determined feeders, typically completing a blood meal on a single host animal, acquiring about 2 μ l or more of blood per meal. The numerous molecules in simuliid saliva play a variety of roles: localized anesthesia, prevention of clotting, modulation of the host immune response, inhibition of platelet aggregation, enhanced vasodilation, and direction of microfilarial parasites to the feeding site (Cupp and Cupp, 1997; Stallings et al., 2002).

The various life-history attributes of blackflies, such as requirements for flowing water, egg diapause, aerial swarming for mate finding, and blood feeding, can complicate sustained laboratory colonization. Few species, consequently, have been colonized for more than one generation. The most prominent exception is the abundant North American species *Simulium vittatum*, which has been in perpetual laboratory colonization for nearly 40 years without introduction of wild material since its inception (Gray and Noblet, 2014).

Medical and veterinary importance

The negative effects of blackflies on humans, their animals, and wildlife can be categorized as nuisance problems, reactions to bites, and disease. These categories are not mutually exclusive; individual species of blackflies can be both pests and vectors.

The effects of blackflies on outdoor activities, such as agriculture, forestry, and tourism, are legendary. But only about 10% of species worldwide are anthropophilic, and no species feeds exclusively on humans. Human variation in the attraction of black flies depends on a number of factors, most importantly the individual's production rate of carbon dioxide (Schofield and Sutcliffe, 1996). Swarms of flies crawling on the body and entering the eyes and other openings, whether biting or not, can be a terrific nuisance. A single persistent blackfly darting about the face and into the eyes can be a nuisance to a golf enthusiast, and one bite can cause an eye to swell shut. The bite is often manifested by a small, purplish, subcutaneous hemorrhagic spot that may or may not ooze blood, depending on the species of blackfly and the components of its saliva. Localized inflammation, swelling, and intense itching can occur. Dozens, hundreds, or thousands of bites can cause blackfly fever, a response to the saliva of the flies, resulting in nausea, fever, headaches, and swollen lymph nodes. Greater fly densities at a host prompt increased feeding rates, at least in some species, suggesting an "invitation effect" (McCall and Lemoh, 1997). More than 6700 bites per person per day have been recorded in Brazil's northern Amazon Basin (Shelley et al., 2010), and more than 13,000 in the Democratic Republic of the Congo (Makenga Bof et al., 2019). In severe cases, allergic reactions and hospitalizations can occur (Gudgel and Grauer, 1954). No authenticated human deaths have been attributed to blackflies, historical anecdotes notwithstanding.

Domesticated animals are frequent targets of blackflies. The attacks are often directed at unfeathered areas of birds, such as around the eyes, or thinly haired areas of mammals, such as the belly and face and in the ears. Reactions of domesticated animals under siege can be severe and include weight loss, reduced egg and milk production, delayed pregnancies and abortions, malnutrition of young animals, unruly behavior and stampedes, dermatitis, stress-related diseases such as pneumonia, and death (Fredeen, 1977). A handful of species, notably *Cnephia pecuarum*, *Simulium colombaschense*, and *S. vampirum*, are responsible for a large number of animal deaths from direct blood-feeding attacks (Adler et al., 2004, 2016). The worst recorded outbreak of blackflies occurred along Romania's Danube River in 1923 when more than 22,000 animals died (Ciurea and Dinulescu, 1924). A debilitating, often fatal, condition known as "simuliotoxicosis" involves acute toxemia and anaphylactic shock resulting from introduction of excessive salivary components during blood feeding (Adler and McCreadie, 2018). The distinction between death from simuliotoxicosis versus exsanguination is not always clear.

The effects of blackflies on wildlife are less conspicuous because of the difficulty of observing host animals in the wild. Nonetheless, many cases of attacks have been documented, and deaths are not uncommon, particularly among nestling songbirds and raptors (Adler et al., 2004). These attacks are especially worrisome when the fitness of endangered wildlife is severely reduced (Adler et al., 2019).

An abundance of flies that can cause pest problems generally occurs by one of two pathways: developing in the majority of flowing-water habitats in an area (habitat generalists), or developing in the world's largest rivers (habitat specialization) (Adler, 2016). Many of the worst pest species breed in large rivers, some of which produce nearly a billion flies per kilometer of riverbed per day (Amrine, 1982; Adler et al., 2016).

Epidemiology: Humans

Two human diseases are simuliid-borne, and several additional human diseases or syndromes have been associated with, but not definitively linked to, blackflies. The two bona fide simuliid-borne human diseases are onchocerciasis (river blindness) and

mansonellosis (or mansonelliasis). The filarial nematodes *Onchocerca volvulus* and *Mansonella ozzardi* are the causal agents that provoke the two diseases, respectively. At least 26 species of blackflies serve as vectors of *O. volvulus* and at least five as vectors of *M. ozzardi* (Adler and McCreddie, 2018). Other than humans, no animal reservoir is known for either of the two filarial species.

All investigated blackflies responsible for transmitting these filarial worms are complexes of cryptic species with differential capacities as vectors (Post et al., 2007; Adler et al., 2010, 2017). The most important vectors involved in onchocerciasis are in the African *Simulium damnosum* complex, which includes about 13 species with vector competency; the majority of species in the *S. damnosum* complex are not vectors. Different strains of *O. volvulus* are associated with different members of the *S. damnosum* complex, forming parasite–vector complexes, such as the forest and savanna complexes in West Africa (Basáñez et al., 2009; Cheke and Gams, 2013). Only about one-quarter of the 28 members of the *S. damnosum* complex in East Africa are anthropophilic (Krueger, 2006). Removal of alternative hosts of the *S. damnosum* complex, such as cattle, in onchocerciasis foci can increase anthropophily and, hence, the risk of human onchocerciasis (Krüger et al., 2005). The *S. neavei* group, which includes the species obligatorily phoretic on freshwater crustaceans, and *S. albivirgulatum* are additional vectors of *O. volvulus* in East Africa. The *S. guianense* complex is a major vector in the few remaining New World foci.

According to the Global Burden of Disease Study in 2017, an estimated 20.9 million people are infected with *O. volvulus*, of which 14.6 million have skin disease and 1.15 million have vision loss (Makenga Bof et al., 2019). More than 99% of the cases are in 31 countries of Africa's central belt, with scattered foci in Yemen and the New World. The disease, which was introduced to the New World, presumably with the slave trade, at one time was endemic in two countries of Central America (Guatemala and Mexico) and four of South America (Brazil, Colombia, Ecuador, and Venezuela). Onchocerciasis has been eliminated from all New World onchocerciasis foci except a few that straddle the Brazil–Venezuela Amazonian region (WHO, 2016). In Africa, some of the greatest success in conquering onchocerciasis has been achieved in Sudan and Uganda.

Female blackflies acquire microfilariae (275–300 µm long) from humans during blood feeding. The peritrophic matrix in the fly's midgut and, in some species, the toothed cibarial armature, serve as barriers to microfilariae, thereby reducing injury to the vector. The tiny worms make their way from the fly's midgut to its flight muscles where they transform to first-stage larvae (L1) and then second-stage larvae (L2). Another molt produces the infective third-stage larvae (L3) that travel to the head and mouthparts of the flies where they are ready for delivery to a human, the definitive host, during blood feeding. The infective larvae can be transmitted to another human when the fly is about 7–10 days old, corresponding to when the fly would take a third blood meal. Once in humans, the third-stage larvae molt to incipient adult worms that grow and, within a year to a year and a half, begin reproducing. Adult male and female worms become encapsulated in relatively benign subcutaneous nodules (Fig. 5) where they mate. Nodules typically form where the infective larvae enter the body, which is determined by where the particular vector species blood feed, for example, on the lower body in Africa, but on the head and shoulders in Latin America. Each long, slender female worm (35–70 cm long) subsequently releases millions of microfilariae over the next 14 years of its potential lifespan. Temperature determines when parasites can become infective and when vectors can transmit them, suggesting that climate change could have profound effects on transmission dynamics (Cheke et al., 2015).

The familiar name “river blindness” or, in the New World, “Robles disease” masks the range of manifestations of the disease. The release of enormous numbers of microfilariae by adult female worms, and the movements of the tiny worms through the dermis



Fig. 5 Nodule encapsulating adult filarial worms of *Onchocerca volvulus*, and rough skin typical of onchocerciasis in Nigeria. Photo by Roger Crosskey.

and the inflammatory responses when they die, can cause insufferable itching with various sequelae, including secondary infections, loss of sleep, and suicide. Social stigma can be associated with the disease. Chronic heavy infections with microfilariae also cause changes to the skin (Fig. 6): lesions, depigmentation (leopard skin), loss of elasticity (elephant knees and, combined with lymphatic challenges, hanging groin), fibrosis, dry wrinkling (lizard skin), and swollen dark areas (sowda in Yemen, Fig. 7; Abdul-Ghani et al., 2016). Two additional skin conditions are known from Central America, involving rashes on the face (erisipela de la costa) and on the torso and arms (mal morado) (Vargas, 1974).

Visual impairment by the microfilariae, particularly the inflammatory response when they die, and co-involvement of their obligate endosymbiotic *Wolbachia* bacteria (Tamarozzi et al., 2011) is the most serious consequence of infection, especially when it leads to total, irreversible blindness. Ocular issues, in addition to blindness, include, inter alia, corneal inflammation and opacities, retinal hemorrhages, and secondary glaucoma. Frequency of blindness is greatest in the West African savanna, whereas it is minimal or absent in areas such as the East African highlands, Latin America, and Yemen.

Mansonellosis is caused by the simuliid-borne filarial nematode *Mansonella ozzardi* (Shelley and Coscarón, 2001); additional species of *Mansonella* are transmitted by ceratopogonid midges (*Culicoides*). Blackflies, especially certain members of the *S. amazonicum* species group, in northern Argentina, Brazil, Colombia, Guyana, Panama, and Venezuela transmit the filarial worms. Infection rates in human populations can be high, generally 20–47%, and the disease is spreading into uninfected areas (Adami et al., 2014). The life cycle of the worms is comparable to that of *O. volvulus*. The microfilariae typically circulate in the blood of humans, and sometimes can be found in the skin. Ocular pathology and dermal issues are not associated with the disease, and it has historically been considered of little concern. Some recent evaluations, however, indicate associated headaches, joint pains, and other complications (Adami et al., 2014; Ta-Tang et al., 2018). Like *O. volvulus*, *M. ozzardi* is associated with symbiotic *Wolbachia* bacteria (Casiraghi et al., 2001). Of the vector-borne diseases of humans, mansonellosis remains one of the most neglected (Mediannikov and Ranque, 2018).

Additional, albeit rare examples, of parasite transmission to humans by blackflies are known. A total of 37 cases of zoonotic onchocerciasis involving at least 11 species of *Onchocerca* have been reported (Saeung et al., 2020). Female blackflies seeking a blood meal can serve as hosts for transmitting the eggs of the New World human bot fly (*Dermatobia hominis*), which hatch upon contacting the vertebrate host (Guimarães and Papavero, 1966). This simuliid–bot dynamic is probably underreported.

Several additional diseases and syndromes of humans—fogo selvagem (pemphigus foliaceus), thrombocytopenia, and nodding syndrome—have been associated with blackflies, although the connections remain nebulous. Fogo selvagem is a rare blistering cutaneous disease that has been associated with the presence of blackflies in rural areas of Brazil (Aoki et al., 2004). Thrombocytopenia, a condition of lowered platelets, has been associated with the bites of blackflies, also in Brazil (Pinheiro et al., 1974). Nodding syndrome is a neurological condition of unknown etiology, which involves epileptic seizures, head dropping, and staring. It primarily affects children in parts of South Sudan, Tanzania, and Uganda, especially in areas associated with onchocerciasis, and can progressively worsen to include death (Johnson et al., 2017; Abd-Elfarg and Boele van Hensbroek, 2019). Effective treatment is not available. Human pathogens detected in or on blackflies, but without compelling evidence of field transmission, include hepatitis B virus (Chanteau et al., 1993) and *Mycobacterium leprae*, the causal agent of leprosy (de Souza-Araujo, 1961).



Fig. 6 Paired nodules encapsulating adult filarial worms of *Onchocerca volvulus*, and onchocerciasis skin (including depigmentation) in Nigeria. Photo by Roger Crosskey.



Fig. 7 Onchocerciasis skin condition known as “sowda” in Yemen. Photo by Abdul Samad.

Epidemiology: Domesticated animals and wildlife

Four general diseases of domesticated animals and wildlife are simuliid-borne: avian trypanosomiasis, filariasis (e.g., bovine onchocerciasis and canine ocular onchocerciasis), leucocytozoonosis, and vesicular stomatitis.

Simuliid-borne avian trypanosomiasis is caused by protozoans in the genus *Trypanosoma*. The most common species of *Trypanosoma* associated with blackflies is *T. avium*, which infects numerous species of vectors and hosts (Bennett, 1961). Pathways of infection include contamination of the bite by fecal droplets from blackflies and consumption of other birds or blackflies that are infected (Votýpka and Svobodová, 2004). Infections are typically subclinical.

A total of 32 species and one subspecies of *Onchocerca* have been reported as parasites of mammals worldwide (Uni et al., 2020), although the presence of cryptic species underestimates this figure (Verocai et al., 2018). Blackflies (and ceratopogonid midges) are vectors. Among the more prominent hosts are cattle, with blackflies the vectors of *O. dukei*, *O. gutturosa*, *O. lienalis*, and *O. ochengi*. The microfilariae typically concentrate in different body regions, for instance those of *O. lienalis* in the umbilical region and those of *O. gutturosa* in the skin of the neck and back. The effects on bovid hosts are typically minor or subclinical, although the quality of hides and milk might be reduced (Beytut et al., 2005). *Onchocerca volvulus*, the causal agent of human onchocerciasis, is hypothesized to have been derived from an ancestral onchocercid parasite of bovids during their domestication (Lefoulon et al., 2017). Nonbovid hosts of simuliid-borne onchocercids include canids, cervids, and suids. Canine ocular onchocerciasis caused by *O. lupi*, with occasional infections in felids and humans, is an emerging ophthalmic disease; simuliids have been implicated as vectors in some cases (Hassan et al., 2015). In cervids, the worms are found in subcutaneous connective tissues, especially of the legs, giving rise to the vernacular names “footworms” and “legworms”; heavy infections can cause hoof damage (Verocai et al., 2012). A few species of simuliid-borne *Onchocerca* infect the subcutaneous tissues in the feet of wild boars and warthogs (Uni et al., 2001). Like *O. volvulus* of humans, simuliid-borne onchocercids of domesticated and wild animals are hosts of *Wolbachia* bacteria (Uni et al., 2020).

Additional filarial worms transmitted by blackflies include *Splendidofilaria fallisensis*, a parasite of ducks (Anderson, 1968) and *Dirofilaria ursi* a parasite of black bears (Addison, 1980), both of which infect the subcutaneous tissues. Tiny nematodes in the family Robertdollfusidae have been recovered from African blackflies and are possibly transmitted to wildlife (Bain and Renz, 1993). Given the diversity of filarial worms in wildlife, simuliid transmission of additional parasitic species can be expected.

Leucocytozoonosis is a malaria-like disease of birds caused by protozoans of the genus *Leucocytozoon* (O’Roke, 1934). The vectors are blackflies, such as *S. anatinum* and *S. rugglesi* of *Leucocytozoon simondi* in ducks and geese and *S. meridionale* and *S. slossonae* of *Leucocytozoon smithi* in turkeys. A wealth of new *Leucocytozoon* species and host–vector relationships continue to be resolved with molecular techniques (Murdock et al., 2015). When gametocytes are acquired from an avian host during a blood meal, a complex life cycle ensues, with the parasite undergoing asexual and sexual development in the blackfly over the next several days. The *Leucocytozoon* parasite is transmitted, as sporozoites, to another bird during a subsequent blood meal, where it undergoes asexual development and gametocyte production (Figs. 8 and 9). Although clinical signs of leucocytozoonosis in wild birds are often negligible, the disease can be pernicious among domesticated ducks, geese, and turkeys; hence, it is sometimes colloquially called “gnat fever,” “duck malaria,” or “turkey malaria.” The disease in domesticated birds can manifest with a variety of clinical signs, such as an enlarged spleen and liver, anemia, anorexia, and weakened immunity, sometimes leading to mortality. The devastating effects of leucocytozoonosis in North America’s poultry industry occurred largely before the 1980s when birds, such as turkeys, were raised outdoors where they were readily available to host-seeking blackflies (Adler et al., 2004). The effects of *Leucocytozoon* infections in

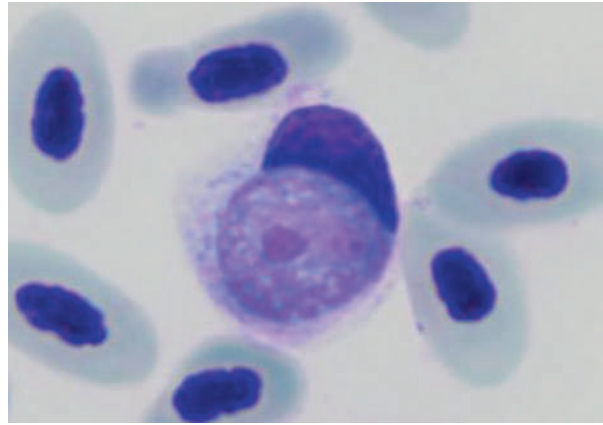


Fig. 8 Macrogametocyte of *Leucocytozoon fringillinarum* from blood of the common chaffinch, *Fringilla coelebs*. Photo by Gediminas Valkiunas.

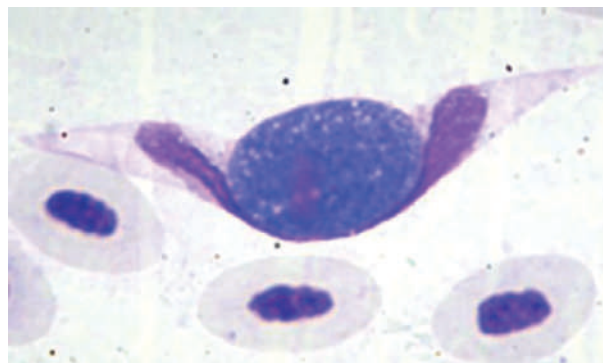


Fig. 9 Macrogametocyte of *Leucocytozoon simondi* from blood of the mallard duck, *Anas platyrhynchos*. Photo by Gediminas Valkiunas.

wild birds are difficult to separate from the direct effects of blood feeding. Both factors, however, probably have greater consequences when birds experience low food availability and other environmental stressors (Hunter et al., 1997).

Various serotypes of vesicular stomatitis virus (VSV) infect wildlife and livestock, especially cattle and horses, causing blistering epithelial lesions often followed by lameness and weight loss. Animals usually recover but major economic losses can result during outbreaks. Some states and countries restrict movement of animals from areas with cases of VSV. Multiple transmission routes include blood feeding by arthropods, such as blackflies (e.g., *Simulium vittatum*), which are epizootic vectors of the New Jersey serotype (Mead et al., 2009). The relative contribution of blackflies to outbreaks is, however, unknown.

Additional animal pathogens have been detected in blackflies, but the ability of the flies to transmit most of them in the field is unknown. These pathogens include eastern equine encephalitis virus (Anderson et al., 1961); snowshoe hare virus (Sommerman, 1977); *Francisella tularensis*, the causal agent of tularemia (Yakuba, 1963); *Zelonia australiensis*, a trypanosomatid protozoan (Barratt et al., 2017); and a trematode (Lewis and Wright, 1962). Various levels of transmission, whether biological or mechanical, have been found for the myxoma virus in domestic rabbits (Mykutowycz, 1957), Whataroa virus in mice (Austin, 1967), Venezuelan equine encephalitis virus from laboratory guinea pigs to mice (Homan et al., 1985), and *F. tularensis* in laboratory guinea pigs (Philip and Jellison, 1986). The role of simuliids as vectors in nature is considered minimal for these pathogens, with the exception of myxoma virus in rabbits. Chlamydia infections in sheep and Rift Valley fever might be simuliid-borne in South Africa (Palmer, 1995). All of these pathogen–vector relationships, and others yet to be investigated, deserve more serious attention.

Prevention and control

Defense against blackflies is an age-old effort, practiced at both personal and community levels. Yet, only in the past century did it begin developing into a science of control and prevention, with annual budgets of some programs exceeding US\$5 million annually (Adler et al., 2004).

Early realization that blackflies were concentrated in streams and rivers typically made larvae, rather than adults, the preferred targets for control. Although physical control, such as managing water levels or removing attachment substrates for the larvae, has been applied with varying levels of success, most efforts historically emphasized chemical control. Applications of kerosene, oils,

and other hydrocarbons to streams initiated the chemical era of blackfly population suppression. DDT followed in the early 1940s into the early 1970s. Subsequently, methoxychlor and temephos were popular chemicals used in larval control. However, resistance in the flies and the effects on nontarget organisms eventually spelled the reduction or elimination of chemical applications to running water.

A remarkable number of organisms—more than 190 nonbacterial species—live in larval blackflies in some form of symbiosis, mostly in parasitic relationships (McCreadie et al., 2011). The value of biological control of blackflies was recognized more than a century ago, and intense efforts were focused, especially in the 1970s, on the potential benefits of mermithid nematodes, fungi, and other natural enemies (Laird, 1981; Adler et al., 2004). The discovery of the bacterium *Bacillus thuringiensis* variety *israelensis* (*Bti*) in larval mosquitoes in 1976 initiated the modern era of simuliid control. The bacterium, specifically the endotoxins it produces during sporulation, has proved highly effective in area-wide management programs for blackflies, with little negative effect seen to date on nontarget organisms. Applications are made either by hand (Fig. 10) or by aircraft. *Bti* is now the gold standard of larval control worldwide (Gray and Fusco, 2017). The diverse microbiome of blackflies offers potential opportunities for future means of control (Tang et al., 2012).

On a more personalized level, smoke, especially from cigars and smoldering materials (smudges), has been used to ward off adult flies. All imaginable repellents have been applied to humans and their animals—greases, tars, plant oils, mud, skin softeners, and the like. Nearly all of these materials have fallen out of favor, although applications of Vaseline inside the ears of livestock, particularly horses, in small-scale operations can provide reliable protection. Various sprays, dusts, pour-ons, and ear tags, as well as sheltering, have been used to protect livestock. Birds in nest boxes can be protected by surrounding the holes with adhesives. For people, head nets and light-colored clothing fitted to exclude crawling flies can be effective, albeit uncomfortable. The most effective modern repellents for personal use typically contain N, N-diethyl-meta-toluamide (DEET) or icaridin (picaridin).

A special set of control efforts has involved the herculean battle against human onchocerciasis. The World Health Organization's Onchocerciasis Control Programme (OCP) in West Africa, followed by the African Programme for Onchocerciasis Control (APOC), operated effectively, primarily by larviciding (Fig. 11), from the early 1970s through 2015 (Davies, 1994; Coffeng et al., 2013). With the close of APOC, the World Health Organization established the Expanded Special Project for the Elimination of Neglected Tropical Diseases to coordinate technical support for five diseases, including onchocerciasis (Colebunders et al., 2018).

A new era in the fight against onchocerciasis came in 1987 with the pledge of Merck & Co. to donate ivermectin tablets (Mectizan®) as long as needed, and the efforts of other organizations (e.g., The Carter Center and the Task Force for Global Health) to mass distribute the tablets. A similar effort in Latin America, the Onchocerciasis Elimination Program for the Americas, began operations in 1993 (Blanks et al., 1998), with great success. Ivermectin is highly effective against the microfilariae, but does not kill the adult worms; doses every 6 to 12 months are, therefore, needed for the long life of the adult female worms. The latest development in the onchocerciasis battle springs from the discovery of *Wolbachia* bacteria in the filarial worms, which provides a means of killing the adult worms with antibiotics, especially doxycycline (Bouchery et al., 2013; Taylor et al., 2014). In some areas, the elimination of onchocerciasis would still leave a significant pest problem where biting rates remain high, as in the Democratic Republic of the Congo. A combination of ivermectin and doxycycline, combined with vector control, offers tremendous hope for the eventual elimination of onchocerciasis and any associated pest problems.

Continued success in the control and management of blackflies will depend on increased ability to identify target species, detect genetic changes in parasites and vectors, monitor changes in pest and vector populations brought on by habitat alteration and



Fig. 10 Application of *Bacillus thuringiensis* variety *israelensis* in a northern temperate river.



Fig. 11 Spray helicopter for larviciding, used in the World Health Organization's Onchocerciasis Control Programme in Togo, 1978. Photo by Roger Crosskey.

climate change, and maintain and develop an arsenal of environmentally safe suppression tools. Monitoring for resistance to the current tools, particularly *Bti*, ivermectin, and antibiotics aimed at *Wolbachia*, will be essential.

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Horse Flies (Diptera: Tabanidae)

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Introduction

The family Tabanidae is a well-known worldwide distributed group of hematophagous Diptera of great economic importance. Compared to other hematophagous insects, they have received only a little attention (Baldacchino et al., 2014a). Tabanids are differently named according to the taxonomic group or geographical region. Commonly they are referred to as horse flies (also written as horseflies or horse-flies) which is the name generally applied to the Tabaninae species (except the tribe Haematopini), deer flies (the term used preferentially for species of the genus *Chrysops*) or clegs (sometimes as glegs or clags; the name used for the genus *Haematopota*). On top of that, several other names are commonly used, e.g., gad-flies (from the word “gad” meaning a spike), stouts, hippo-flies, dun-flies, breeze flies (this name is used particularly in North America) or March flies (in Australia). They feed on many vertebrate hosts, including humans. Their medical and veterinary importance resides mainly in the painful bite and their role of efficient biological and mechanical vectors of various human and animal disease agents including viruses, bacteria, and parasites.

Medical and veterinary importance

A direct effect of tabanids on their hosts

Tabanids have a painful bite and persistent biting behavior, which makes them nuisance pests for humans and animals, mainly livestock, in particular large-bodied species such as horses and cattle (Fig. 1). Wildlife is also affected, mainly large mammalian species; however, *Chrysops* and other smaller species feed (occasionally) also on birds (e.g., ravens, crows, ducks) and reptiles (e.g., turtles). Their noisy flight combined with a large size produce also visual stress when they approach and land on the host. Large accumulations of flies do occur in some areas, and with their painful bite tabanids can be pests of both livestock and humans. Areas with abundant tabanid populations are negatively affected due to the impact on tourism, outdoor activities (e.g., trekking, fishing, camping, and swimming), and also farming. In fact, horse flies have directly resulted in decreases in the weight gain of cattle of 0.1–1 kg/day (Baldacchino et al., 2014a).

Except for the painful bite (Fig. 2), as tabanids are large insects, the blood loss associated with their feeding is also important and depends on the horse fly species and size: ranging from 20 to 25 μ L for many *Chrysops* species to almost 700 μ L per bite of *Tabanus atratus*. The host blood loss continues after the bite by oozing (Fig. 3) and can be aggravated by opportunistic blood-feeding flies (Fig. 4). Bite sites can be used by myiasis causing flies for ovi/larviposition. Often the bites result in local skin reactions such as nodules. High densities are also responsible for decreased milk production and weight loss mainly in cattle, due to impaired feeding. Salivary components found in tabanids correspond entirely with their feeding strategy of engorging a large blood-meal within a few minutes. They are exophilic/exophagic (outdoor blood-feeding) and telmophagous (feeding from a pool of blood caused by tissue laceration) and take blood meals during the day. The saliva of tabanids exhibits strong anticoagulant and vasodilatory activities, together with a broad repertoire of platelet aggregation inhibitors, and immunoregulatory peptides



Fig. 1 Tabanids are a nuisance to livestock, producing stress and reduced feeding like in this cow disturbed by a large number of attacking horse flies. Photo: Andrei D. Mihalca.



Fig. 2 Tabanid (*Haematopota* sp.) feeding on a horse. Photo: Andrei D. Mihalca.

(Volfová et al., 2016), it has also a toxic effect (Baldacchino et al., 2014a) and may induce allergic reactions in sensitive individuals (Bircher, 2005).

Vectorial role

Tabanids are important vectors of human and animal pathogens, acting both as biological and mechanical transmitters for a variety of pathogenic organisms. Over the past few decades, the epidemiological relevance of tabanids as mechanical vectors has been focused on the increasing incidence of some veterinary important diseases, above all equine infectious anemia, bovine besnoitiosis, and livestock trypanosomoses. Of the biologically transmitted human pathogens, the filarial nematode *Loa loa* has recently re-emerged as a disease agent of public health importance, because of its negative impact on the control of onchocerciasis and



Fig. 3 The bleeding of an attacked horse caused by the bite of the tabanids (*Hybomitra* sp.) will soon attract other insects interested in its blood. Photo: Jana Bulantová.



Fig. 4 Opportunistic flies feeding on the blood leaking from the site of the tabanid (*Haematopota* sp.) bite. Photo: Andrei D. Mihalca.

lymphatic filariasis in areas of co-endemicity. Additionally, tabanids are involved in the phoretic transmission of the human botfly *Dermatobia hominis* (Oestridae) in the Neotropical region.

Biological transmission

Tabanids are demonstrated as biological vectors for three groups of parasitic organisms: filarial nematodes, trypanosomes, and apicomplexans (Baldacchino et al., 2014a).

The filarial nematode *Loa loa*, also known as the Tropical eye worm is responsible for the disease known as loiasis, occurring mainly in rainforests of Central and West Africa (Kelly-Hope et al., 2017). The adults live in the subcutaneous connective tissue of humans, producing edematous swellings on the body known as Calabar swellings. Occasionally, the parasites can migrate under the conjunctiva of the eye (Anderson, 2000). Two species of the genus *Chrysops* are the main incriminated vectors of *L. loa* in humans: *Chrysops silacea* and *Chrysops dimidiata*. Both species are canopy dwellers of African equatorial rainforests (Kelly-Hope et al., 2017). Although the loiasis is usually not severe, high levels of microfilaremia are associated with serious symptoms and death in patients using nematocidal drugs (i.e., ivermectin or diethylcarbamazine) for other diseases such as lymphatic filariases or onchocercoses (Kelly-Hope et al., 2017). In tabanids, *Loa loa* develop from the L1 stage (microfilariae) to L3 in the fat body tissue of the body, head, and thorax. From the fat body, they migrate to the mouthpart and emerge through the proboscis during feeding. The development takes from 10 to 12 days (Anderson, 2000). Two other species, *Chrysops zahrai* and *Chrysops distinctipennis*, have lesser importance but still can act as biological vectors of human loiasis. All above mentioned *Chrysops* species plus *Chrysops centurionis* and *Chrysops langi* could be involved in the transmission of simian loiasis in several monkeys and great apes (*Cercopithecus mona*, *Cercopithecus nictitans*, *Mandrillus leucophaeus*, chimpanzee, and both species of gorillas) and various experimental attempts of interspecific transmission of *Loa* between humans and other primates using *Chrysops* tabanids were done (see Anderson, 2000; Votýpka et al., 2020).

Tabanids have been also demonstrated as biological vectors for other filarial nematodes. The larvae of *Pelecitus roemeri* (formerly *Dirofilaria roemeri*), a filarial parasite in the subcutaneous and intermuscular tissue of the knee region of several species of Australian marsupials, develop in several tabanid species belonging to the genera *Dasybasis*, *Mesomyia*, *Scaptia*, and *Tabanus* (Anderson, 2000). The intermediate hosts for *Elaeophora schneideri*, a filarial parasitic nematode of the carotid arteries and their branches in sheep and mule deer, *Odocoileus hemionus* in western North America, are also various species of the tabanid genera *Hybomitra* and *Tabanus* (Anderson, 2000).

Various tabanids act as biological vectors for the non-pathogenic kinetoplastid, *Trypanosoma theileri* species complex (Fig. 5) which includes several trypanosome species hardly distinguishable due to the lack of discriminating morphological characters (however, diseases associated with this pathogen in cattle were sporadically reported). Trypanosomes belonging to this group have been isolated from different domestic and wild bovine, ovine, and cervids in Europe, Africa, Asia, and the Americas. The principal vectors of the *T. theileri* group are considered tabanid flies (however, *T. melophagium*, also belonging to the *T. theileri* species complex, is transmitted exclusively by sheep keds). After ingestion of blood with trypomastigotes from infected hosts, the parasites multiply through a cyclopropagative stercorarian development in the posterior part of the digestive tract of various horse fly species (e.g., *Haematopota pluvialis*, *Hybomitra micans*, *Tabanus autumnalis*, and *T. bromius*—Fig. 6) (Böse et al., 1987).

Tabanid flies have been implicated as the vectors for the North American species of turtle malarial parasites, *Haemocystidium* (*Simondia*) *metchnikovi* (formerly described as *Haemamoeba metchnikovi*) belonging to the genus *Plasmodium* sensu lato (Galen et al., 2018). The infective sporozoites have been identified in the salivary glands of the tabanid *Chrysops callidus* in Michigan, USA (DeGiusti et al., 1973); hence, this tabanid species is incriminated as an intermediate host and biological vector of this hemosporean parasite in turtles. Tabanids have been assumed to be the vectors for all members of the clade, but this should be validated because their role in the transmission of South American, Middle Eastern, or other chelonophilic taxa has not been confirmed.

Mechanical transmission

The mechanical transmission does not require a biological interaction between the pathogen and the arthropod vector; the infectious disease agent does not replicate and/or develop (e.g., does not undergo physiologic changes) in/on the vector. Due to their feeding behavior with multiple bites on different hosts, tabanids are particularly important as mechanical vectors for viruses, bacteria, and parasites. Their painful bite results in the flies often being disturbed while feeding, but they are persistent and will commonly move directly to another animal to recommence feeding. Because of this, and the rather “spongy” nature of the labella,

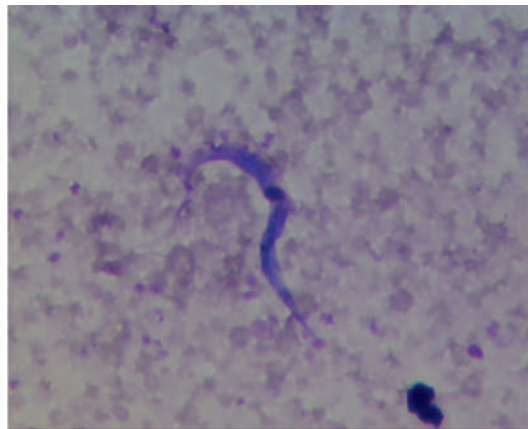


Fig. 5 *Trypanosoma theileri* s.l. in a blood smear of an asymptomatic cow sampled in Romania. Photo: Andrei D. Mihalca.

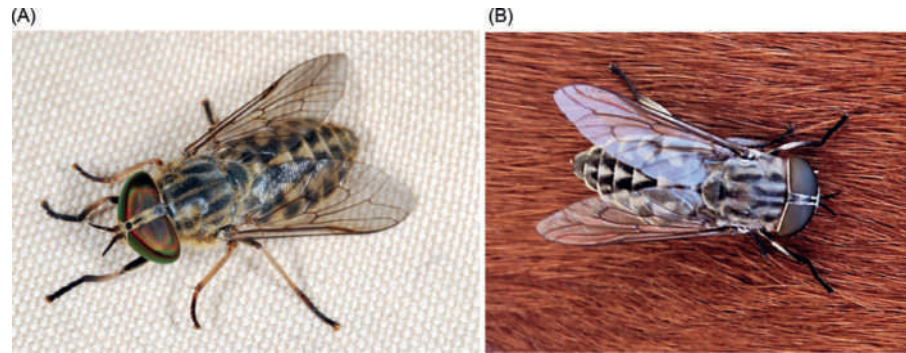


Fig. 6 *Tabanus bromius* (A) and *T. autumnalis* (B). Photo: David Modrý (A); Jana Bulantová (B).

which can hold up to a nanoliter of blood, they are excellent mechanical vectors. Their role as mechanical vectors has been extensively reviewed by Krinsky (1976) and Baldacchino et al. (2014a). One of the most important viruses transmitted by tabanids (genera *Tabanus*, *Hybomitra*, and *Chrysops*) is the Equine infectious anemia virus which is responsible for a severe disease of horses. Other viruses mechanically transmitted by tabanids in animals (mainly livestock) include the bovine leukosis virus, the bovine viral diarrhea virus, hog cholera virus, vesicular stomatitis virus, and rinderpest virus. Among bacteria, the mechanical transmission has been demonstrated for *Anaplasma marginale*, *Francisella tularensis* (this zoonotic disease is widespread throughout the Holarctic and is transmitted by a variety of agents, but in the USA the tabanid *Chrysops discalis* is a major vector), *Bacillus anthracis*, *Clostridium chauvoei*, *Pasteurella multocida*, *Listeria monocytogenes*, *Erysipelothrix rhusiopathiae*, and *Ehrlichia risticii*. Among protozoa, natural or experimental proof of transmission by tabanids has been shown for *Besnoitia besnoiti*, *Trypanosoma evansi*, *T. vivax*, *T. equiperdum*, *T. simiae*, *T. brucei* complex, and *T. congolense* (a detailed review is available in Baldacchino et al., 2014a).

Additionally, several other viruses (California encephalitis virus, western equine encephalitis virus, tick-borne encephalitis virus, influenza virus) and bacteria (*Borrelia burgdorferi* sensu lato, *Coxiella burnetii*) have been isolated from tabanids, but no proof of transmission was confirmed (Baldacchino et al., 2014a).

Phoretic transmission

Tabanids, among other dipterans, are used by the neotropical human botfly, *D. hominis*, to phoretically spread their eggs (Baldacchino et al., 2014a). Females of *D. hominis* use as a primary egg dispersal technique the oviposition directly on the body of other insects. As summarized by Catts (1982), *D. hominis* females will choose as ovicarriers insects which fulfill the following characteristics: (1) are zoophilic; (2) are diurnal; (3) have moderate size; and (4) moderate activity. The eggs are glued with a water-insoluble secretion to the abdomen of the carrier insects, with the operculum directed posteriorly. A carrier insect can transport even more than 35 eggs (Catts, 1982). Although mosquitoes seem to be the preferred transport hosts for *D. hominis* eggs, several other insect groups have been involved such as Anthomyiidae, Calliphoridae, Fanniidae, Muscidae, Sarcophagidae, Simuliidae, and Tabanidae. Among tabanids, species of genera *Chrysops* and *Tabanus* are included here (Baldacchino et al., 2014a).

Biology and ecology

Taxonomy and diversity

Tabanidae Latreille, 1802 is a family of the order Diptera (Linnaeus, 1758), suborder Brachycera (Macquart, 1834), infraorder Tabanomorpha (Hennig, 1948), and superfamily Tabanoidea (Latreille, 1802). They are a cosmopolitan (absent from some islands, e.g., Greenland, Iceland, and Hawaii), gathering almost 4400 described valid species in 137 genera worldwide (Pape et al., 2011; Pape and Thompson, 2020). This diversity eclipses any other hematophagous insect group, for instance biting midges, Ceratopogonidae (mainly genus *Culicoides*), with less than one and half thousand species, or the order Siphonaptera, the fleas, with slightly more than two thousand species, and even mosquitoes, Culicidae, with less than four thousand species. The family Tabanidae includes four subfamilies divided into tribes: Chrysopsinae (Bouvieromyiini, Chrysopsini, and Rhinomyzini), Pangoniinae (Mycteromyiini, Pangoniini, Philolichini, and Scionini), Sepsidinae, and Tabaninae (Diachlorini, Haematopini, and Tabanini). The most diverse genus is *Tabanus* (Fig. 7A) (with 1340 described species), followed by *Haematopota* (Fig. 7B) (556 species), *Chrysops* (287 species), *Hybomitra* (243 species), *Dasybasis* (176 species), *Cydistomyia* (146 species), *Philoliche* (124 species), *Scaptia* (119 species), *Esenbeckia* (111 species), *Stenotabanus* (100 species), *Fidena* (99 species), *Dichelacera* (80 species), *Atylotus* (76 species), *Catachlorops* (66 species), and *Mesomyia* (58 species) (Pape and Thompson, 2020). Most of the economically important tabanids are in the subfamily Chrysopsinae, particularly the genus *Chrysops*, and the subfamily Tabaninae (Mullens, 2002). Besides Tabanidae, also females of some genera of the families Athericidae and Rhagionidae (belonging to the infraorder Tabanomorpha) take blood from their hosts but are not involved in the pathogen transmission.

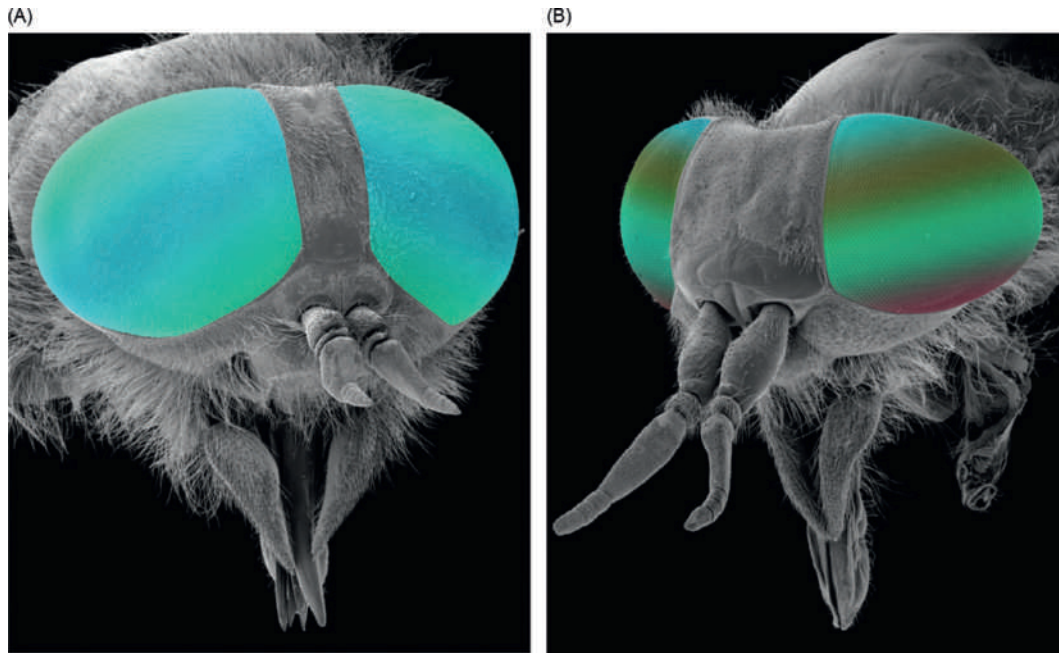


Fig. 7 Colorized SEM photo of *Tabanus* sp. (A) and *Haematopota* sp. (B) heads. Photo: Jana Bulantova.

At present, the tabanid identification is predominantly based on morphological characters; however, the generalized morphology, the rarity of male specimens, the use of variable characters like body and wing color, and in some cases the lack of reliable taxonomic characters in this family make species identification very difficult. In light of the identification problems using traditional taxonomic tools, molecular-based approaches have started to be used to solve some of these problems (Votýpka et al., 2019). The DNA barcoding region of mitochondrial cytochrome oxidase subunit I (COI) has been shown to effectively discriminate tabanid species (e.g., Cywinska et al., 2010; Banerjee et al., 2015; Changbunjong et al., 2018; Votýpka et al., 2019; see also the BOLD database: <http://www.boldsystems.org>).

Morphology

These robust and sturdy flies are strong fliers and exhibit body size ranging from 5 to 33 mm: horse flies (*Tabanus* and *Hybomitra*, just to cite key examples) are relatively large (10–33 mm) whereas deer flies (*Chrysops*) and clegs (*Haematopota*) are significantly smaller (5–13 mm). Adults are stout-bodied flies with a striking appearance, variously colored from black through brown to greens and yellows, bold stripes, and large brilliant colored eyes (Fig. 8), all providing useful taxonomic characteristics. The large, semi-lunar head bears two prominent eyes, which, in the live insects, are often patterned with iridescent reds and greens that usually fade after death. The eyes are commonly spotted in *Chrysops* spp., have zig-zag bands in *Haematopota* spp., and horizontal stripes and no patterning in *Tabanus* spp. The males are easily differentiated from female flies because eyes show striking differences in the arrangement of eyes. Females are dichoptic where eyes are separated by frons while males are holoptic where eyes are contiguous. The larger eyes of males reflect a mating strategy dominated by visual cues. The larger dorsal ommatidia may be more sensitive to UV



Fig. 8 Tabanids have large brilliant colored eyes: horizontal stripes are typical for *Tabanus* and *Hybomitra* (A); zig-zag bands for *Haematopota* (B), and *Chrysops* has spotted eyes (C). Photo: Andrei D. Mihalca (A); Jana Bulantová (B and C).

light, allowing the male to detect a fast-moving female against the sky, and the smaller ventral ommatidia may be used to resolve visual details.

Feeding

Similar to nematoceran hematophagous flies (e.g., sandflies (Phlebotominae), blackflies (Simuliidae), biting midges (the genus *Culicoides*), and mosquitoes (Culicidae)), in the family Tabanidae adult males feed on plant extracts and nectars, while females exhibit both hematophagous as well as nectarophagous feeding habit. Both sexes drink water from pools, lakes, ponds and water reservoirs in flight. In general, blood meal is necessary to carry out gonotrophic cycles in hematophagous insect females. Almost all tabanids are hematophagous, except for those who exhibit autogeny, a process where usually a single gonotrophic cycle can be completed without any blood meal (e.g., *Tabanus nigrovittatus*, *Apatolestes actives*, *Chrysops atlanticus*, *Chrysops fuliginosus*, *Chrysops hirsuticallus* or *Brennania hera*). However, many other species (e.g., *Hybomitra kaurii*, *Heptatoma pellucens*, *Haematopota pluvialis*, and *Haematopota crassicornis*) seem to be (facultatively) autogenous in the first gonotrophic cycle, probably reflecting genetic variability and the carry-over of available nutrients from the larval stage (Krčmar and Marić, 2010).

Tabanids locate their vertebrate hosts mainly by color and movement. The dark color is more attractive and dark-colored hosts, or even dark areas on black and white animals (e.g., Holstein cow), are often favored for an attack. A very interesting aspect of defense mechanism against the bite of various hematophagous arthropods, mainly tabanids, is the pattern of contrasting black and white stripes of zebras. This has been demonstrated on several occasions through experimental studies and field observations and reviewed recently by Caro et al. (2019). Moreover, this seems to be the primary role of the zebra stripes, and other possible functions such as camouflage, confusion of predators, signaling to conspecifics, and thermoregulation have not been validated. The visual repellent effect seems to work also against other biting dipterans such as tsetse flies and stable flies belonging to the genus *Stomoxys*. However, the mechanism of how these stripes prevent flies to bite zebras is not yet understood (Caro et al., 2019). Many tabanids are selective in attacking specific body regions of their hosts, regardless of color. For example, *Chrysops* spp. and *Haematopota* spp. usually feed high on the body (e.g., head and shoulders), legs are more favored by *Tabanus* spp. and *Hybomitra* spp.

Ecology

Tabanids mostly occur in warm areas with suitable moist locations for breeding, but also occupy a wide range of habitats from deserts to alpine meadows and they are found from sea level to at least 3300 m. They are largely seen in warm days with low wind speed; adult females bite during the daytime and are especially active in the sunshine. Their preferred habitat seemed to be bushy areas or grassland near aquatic bodies or wetlands. Females are often found nearby their hosts, mostly seen in and around cattle in rural areas. Adults generally take rest on tree trunks after feeding. They are almost all diurnal in the habit (but a very few species feed at dusk and during the night, particularly in the tropics) and found to breed near aquatic bodies. Although adult tabanids are strong fliers capable of considerable movement away from the breeding site and may actively travel several kilometers, they do not normally become widely dispersed.

In both temperate and tropical countries, the occurrence of adults is seasonal. In the tropics, populations often reach a peak towards the beginning of the rainy seasons; in temperate areas, the maximum is in the early summer.

Life cycle

The eggs (1–3 mm long) of tabanids are laid usually in a single compact mass (different species produce between 100 and 1000 eggs in a cluster; a few species lay several smaller batches of eggs), usually on the lower surface of leaves overhanging the water, on small branches, grasses, rocks, and stones or vegetation near water (ponds or streams), or simply on wet soil. Surfaces chosen for egg-laying are often species-specific (in general, *Haematopota* prefers relatively dry soil, *Tabanus* wet soil close to water bodies, and *Chrysops* wet mud often in semi-submerged situations). Eggs are white when laid but darken to grey, brown, or black within several hours and hatch in 1 or 2 weeks; however, in high temperature (about 30 °C) egg hatch can occur within 2 or 3 days.

Tabanid larvae are found in a wide variety of aquatic and semiaquatic habitats. Larvae pass through (four) six to nine (eleven) stadia and the time spent in the larval stage can last from a few months to one or two (for some species even three; e.g., *Tabanus atratus*, *Tabanus calens*) years. In temperate zones, most species overwinter in the larval stage and have only one generation per year (univoltine): adults usually die off at the end of the summer, with a new adult population emerging the subsequent spring. In the tropics, tabanids may produce two or more generations per year (multivoltine). Mature larvae of Tabanidae are usually 12–50 mm long, color varies from white to green, rather maggot-shaped, cylindrical, elongate, and fusiform, with a pigmented, retractile, sclerotized head capsule, and have distinct, raised, tire-like rings encircling three thoracic and eight abdominal segments with six protuberances called pseudopods (that can often be found on segments 4–10). Larvae of most species of the family Tabanidae are carnivorous (and cannibalistic) except first and second instars which are non-feeding, while the majority of *Chrysops* larvae are saprophagous, feed on detritus and decaying vegetable and animal matter. Even though most of the larvae are aquatic, semiaquatic, or edaphic and like muddy sites, they breathe in air through the very short siphon positioned on the last body segment. Mature larvae before pupation migrate to places of lower humidity.

Pupation usually occurs in drier soil near the larval habitat, with the head end oriented upward. The pupa obtecta (i.e., the pupae are capable of limited movement), which is brown and glossy, usually 10–33 mm long, is unlikely to be submerged. The pupal period varies from approximately 1 to 3 weeks and is temperature-dependent (Chvála, 1980; Chvála and Ježek, 1997).

The adult fly emerges from the pupal case via a slit located along the thorax of the case (circumscriptional names Orthorrhapha vs. Cyclorrhapha). The sex ratio is approximately 1:1, and the emergence of males precedes that of females by one to a few days.

Tabanid females usually mate before they seek a vertebrate host. Mating occurs in flight after virgin females enter male swarms. In canopy-inhabiting forest species, swarming happens above the canopy, but also along forest roads, ecotones, or above natural features (serving as markers), normally in the early morning or late afternoon. The male aggregations at the tops of hills (“hilltopping”) are common for some species. A few species are autogenous, but most require a blood meal for egg maturation. Following a blood meal, egg development takes typically 3–4 days, depending on temperature; however, time to oviposition may be several days longer.

Due to their very complex and long-time life cycle, predaceous and cannibalistic habits, and requirement of specific conditions including hibernation (for temperate species) and mating in flight (the key barrier to colonization), no tabanid species has been successfully colonized. However, several methods were described for rearing immature horse fly stages (e.g., larvae collected in mud, damp soil, humus, muddy waters, on the edges of pools and ponds, or adhere to floating vegetations) by using various substrates (including soil, bryophytes, or sand) for maintenance of horse fly species including those with a long development (see Ferreira and Rafael, 2006).

Epidemiology

In general, most tabanid species are highly seasonal, with great variations (even intraspecific) between geographical regions. There are several studies available on the seasonality of tabanids in various regions of the world, both in the northern and southern hemisphere and various ecoregions (Noireau et al., 1991; Baldacchino et al., 2014b; Krüger and Krolow, 2015; Lucas et al., 2020). In temperate regions, the peak activity is during the summer, and often short. In tropical regions, tabanids are more active during the rainy or humid seasons, but with great variation depending on the species (Baldacchino et al., 2014a). In certain areas, a seasonal succession of species has been described (De Liberato et al., 2019).

Female tabanids are diurnal insects and their activity is influenced by the photoperiod. Certain species have one peak per day (unimodal) (i.e. at noon), others have two (bimodal) (i.e. morning and afternoon). The activity during the day is influenced by climatic factors such as temperature and wind speed. They generally prefer the warmer hours of the day in temperate areas, and the cool mornings and evenings in tropical areas (Baldacchino et al., 2014a).

Prevention and control

As tabanids are a nuisance, as well as vectors of causative agents of several important diseases for animals and humans, there is an increased interest in their population control. However, the efforts have never reached the intensity of those invested in the control of other arthropod vectors such as mosquitoes. Moreover, control measures should be usually based on surveillance data, which is again far less plentiful than in the case of most other vectors. As summarized by Baldacchino et al. (2014a), the effective tabanids population reduction is challenging due to several factors related mostly to the biological features of these insects: short on-host hematophagous feeding period, use of multiple hosts for the blood meal (including wildlife), and a great variety of the biotopes use for oviposition and larval development.

Several control measures are possible, some of them only at the theoretical level, as they have rarely or never been implemented into current practices. As a curiosity, we can mention a field experiment suggested that the striped coats of zebras can prevent biting and cows painted with zebra-like striping can avoid biting fly attacks including tabanids (Kojima et al., 2019). There are very few practical measures that are effective in reducing tabanid populations. All of them have been detailed in the review by Baldacchino et al. (2014a) and will be summarized below. One of the ideal options is the use of biological control. Despite the existence of numerous pathogens, parasitoids, and predators of tabanids, there were only a few experimental approaches for this. Management of grazing seems to be an efficient method to reduce the intensity of tabanids attacks. Several studies have shown that various tabanid species are more active along the forest edges, so such areas should be avoided when grazing livestock or horses. Another suggested approach could be moving the livestock to higher altitude pastures during the peak tabanid season. Due to their obstacle avoiding flight behavior, tabanids will less likely attach hosts in shelters (even basic ones), or behind solid physical barriers.

Several chemical-based approaches are available for livestock, including pyrethroids such as permethrin, cypermethrin, deltamethrin, fenvalerate applied as pour-on, sprays, or ear tags. The reported success varies as efficacy and duration with the study, concentration, and route of application. The use of repellents is a desirable method, which should prevent tabanids from landing and biting the host. The most efficient seems to be the DEET, applied by spraying at a concentration of 75%, which protects horses for 3–4 h but induces dermatological side effects. Oxamate 20% was also efficient for 12 h when used on cattle. Plant-derived essential oils have also been used with variable efficacy (Benelli and Pavela, 2018). However, all these repellents have the disadvantage that they have a short-lasting effect (Baldacchino et al., 2014a); thus, nanoencapsulated formulations boosting their efficacy over time can be considered in further research. Placing various types of traps around livestock flocks is a control

method that is also being used. These traps (consisting of colored screens (blue, black, or red) coated with various adhesives) can be un-baited or they can use various attractants as baits such as CO₂, 1-octen-3-ol, ammonia, and phenols, or aged mammalian urine (Baldacchino et al., 2014a); however, they are no real solution for combating tabanids.

The control of tabanids which are vectors for human disease (i.e. *Chrysops*) is not currently being considered in public health programs in Africa. Some of the methods presented above for livestock have been also occasionally documented for the prevention of human bites in Africa and they included also the destruction of tabanid habitats (see Kelly-Hope et al., 2017).

Conclusions

Despite their great diversity and medical and veterinary importance, tabanids are a neglected group of hematophagous arthropods. Their study is relatively difficult also because there are no laboratory colonies available. Their control is also difficult for several reasons, and they continue to be a permanent nuisance, in many regions only seasonally.

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Horn Flies (*Haematobia* and *Haematobosca*)

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Introduction

The non-biting flies as the house fly (*Musca domestica*) and the face fly or autumn house fly (*M. autumnalis*) together with biting flies like the stable fly (*Stomoxys calcitrans*) and the horn/buffalo flies (*Haematobia irritans/exigua*) are responsible for the considerable annoyance of host animals. Wheals, crusts, and cutaneous papules and nodules have mainly been associated with the biting flies *Stomoxys* and *Haematobia* and several tabanid species. The four closely related and morphologically similar muscoid genera, *Stomoxys*, *Haematobia*, *Haematobosca*, and *Haematostoma* are widely distributed in tropical and subtropical areas with some species spread also in a temperate zone. The stable fly (*S. calcitrans*) and the horn/buffalo flies (*H. irritans/exigua*—see “[Taxonomy and diversity](#)” section) are major livestock pests that cause substantial economic losses in the cattle/buffalo industry and are vectors of many parasites and pathogens.

Horn flies are among the most economically important blood-sucking ectoparasites that affect the health and well-being of pasture livestock, particularly cattle and buffaloes. The repetitive bites of horn flies, both males and females, can lead to reduced weight gain, decreased milk production, and skin lesions. The flies cause billions of dollars of damage and loss to the cattle industry each year; however, the economic losses vary significantly among regions because environmental conditions significantly affect the growth of horn fly populations. Horn flies are vectors of several filarial nematodes such as *Stephanofilaria stilesi* in North America and *Stephanofilaria assamensis* and *Parabronema skrjabini* in Asia. They can also serve as mechanical vectors for several important pathogens of livestock (see “[Mechanical transmission](#)” section).

The adult fly (Fig. 1) is 3–5 mm long with piercing/sucking mouthparts that are painful to cattle. They are ferocious and painful biters and both sexes take blood meals multiple times a day, even more than 20 ×. Flies are in continual contact with the hosts, resting on them between feedings, and can be seen mainly on the withers, shoulders, back, and side and will move to the underbelly during the hottest parts of the day. More than 400 flies per animal will affect the health and productivity and treatment is recommended when fly counts exceed 200. Larvae breed in undisturbed dung pats, and adult populations build up during the warmer months of the year.

Medical and veterinary importance

Horn flies are particularly important as veterinary parasites, but occasionally they can also feed on humans. Their veterinary and medical importance resides in their direct effect (painful bite, blood loss, allergic reaction, decreased productions with economical loss) and their vectorial role (both biological and mechanical). All these are summarized below.

Direct effect of horn flies on their hosts

Horn flies occur mostly on the adult cow or buffalo and usually only incidentally on the calves (Moon, 2019). They usually feed along the dorsal part of the body but also the sides and ventral abdominal region. Due to their blood-feeding behaviour which involves multiple and repeated withdrawals and re-insertions of the mouth parts, the cutaneous irritation and inflammatory response



Fig. 1 *Haematobia irritans*. Photo Andrei Daniel Mihalca.

are considerable. The bites of *H. exigua* cause cutaneous hypersensitivity in cattle (Kerlin and Allingham, 1992). As for other biting dipterans, the nuisance is significant and impairs normal feeding (Wall and Shearer, 1997). Infested cattle react by licking their backs, twitching their flanks, switching their tails, and kicking at their bellies with their hind legs (Moon, 2019).

In addition to the excessive number of painful bites that occur daily, the lesions can lead to secondary infections and cosmetic defects in tanned or dyed leather (Moon, 2019). One of the significant impacts of horn flies is the economic loss on the cattle industry. The direct economic losses are though linked to decreased milk production, associated with decreased weaning weights of calves but also to decreased weight gains (Foil and Hogsette, 1994). Heavy attacks by horn flies were shown to cause a 25–50% decrease in milk production (Wall and Shearer, 1997). Studies (summarized by Foil and Hogsette, 1994) showed that in North America, a level of 200 horn flies per animal is considered to financially justify the implementation of control measures. Not treating highly infested cows can lead to a one-tenth decrease in the average daily growth rate of nursing calves. The growth rate of yearling stocker cattle and lactation rates of dairy cows may decrease by about 15%. Metabolic and behaviour responses indicate that horn flies increase the amount of energy spent by cattle when defending themselves, leaving less energy available for growth.

Vectorial role

Horn flies are biological vectors (and intermediate hosts) for various filarial nematodes of herbivores (Anderson, 2000) but also mechanical vectors for viruses, bacteria, and protozoans.

Biological transmission

Haematobia irritans and *H. titillans* are biological vectors for the filarioid nematode *Stephanofilaria stilesi* (Anderson, 2000). The role of horn flies as vectors for *S. stilesi* was suggested by Dikmans (1934), but only later (Ivashkin et al., 1963; Hibler, 1966) demonstrated their role. *Stephanofilaria stilesi* causes a circumscribed granular or crusty dermatitis mainly along with the medio-ventral line of the body of cattle (udder, belly, scrotum, and prepuce) (Foil and Hogsette, 1994; Anderson, 2000; Russell et al., 2013). Males and females of *H. irritans* feed on the cutaneous lesions and engorge blood contained the L1 of the nematodes. In the abdominal hemocoel, they undergo two molts and become infective L3 in ca. 18–21 days at room temperature (Hibler, 1966). As horn flies prefer to feed on adult cattle, stephanofilariasis is more common in these, compared to calves (Anderson, 2000). Another member of the genus *Stephanofilaria*, namely *S. assamensis* has been shown to be transmitted by its intermediate host, *H. titillans* (Anderson, 2000).

Haematobia irritans is a vector for *Habronema microstoma* (Russell et al., 2013), a gastric spirurid of horses, while *H. titillans* is a biological vector of *P. skrjabini* (Ivashkin, 1959; Anderson, 2000), a spirurid nematode inhabiting the abomasum of various species of ruminants in Africa, Asia, and some Mediterranean countries (Hasheminasab et al., 2016).

Another horn fly species, *Haematobosca stimulans*, is a biological vector for *Setaria cervi* (Osipov, 1966), a filarial parasite of the abdominal cavity and central nervous system of various cervids in Eurasia (Anderson, 2000). The infective stage in the flies develops in ca. 23 days (Anderson, 2000).

Haematobosca atripalpis is a biological vector for *Parafilaria multipapillosa* (Gnedina and Osipov, 1960), a parasite of the subcutaneous and intermuscular connective tissue of equids in the Old World and South America where it causes the disease known as “bloody sweat” or “summer bleeding” (Anderson, 2000). The larvae reach the L3 infective stage in 10–15 days at 20–36 °C (Anderson, 2000).

Mechanical transmission

Due to their frequent hematophagous feeding behaviour, horn flies, together with tabanids, are considered among the most important mechanical vectors, mainly about the diversity of transmitted pathogens (Baldacchino et al., 2018). However, from an epidemiological point of view, due to their rather sedentary behaviour (they tend to rest on the same animals between blood meals), horn flies are not as efficient as tabanids and other biting muscids in spreading diseases via a mechanical transmission (Russell et al., 2013).

Horn flies are important mechanical vectors for the summer mastitis caused by *Staphylococcus aureus* associated with other bacteria (Owens et al., 1998; Baldacchino et al., 2018). Summer mastitis is more severe during the increased activity of horn flies, and the control of flies decreases the incidence of teat lesions (Ryman et al., 2013).

Horn flies have been implicated or suggested as mechanical vectors for other bacterial pathogens such as *Salmonella enterica* (Olafson et al., 2014), *Staphylococcus saprophyticus*, *Morganella morganii*, and *Serratia marcescens* (Palavesam et al., 2012). Apart from non-transferable symbiotic *Midichloria*- and *Wolbachia*-like bacteria (Hornok et al., 2008; Zhang et al., 2009; Torres et al., 2012), various other microorganisms have been detected in *H. irritans*, but the mechanical transmission has not been demonstrated. Examples include the Nora virus and *Mycobacterium bovis* in Mexico (Torres et al., 2012) or *Mycoplasma wenyonii* in Hungary (Hornok et al., 2008, 2011).

Under experimental conditions, *H. irritans* was suggested as a mechanical vector for the bovine leukaemia virus (Buxton et al., 1985; Panei et al., 2019) and bovine viral diarrhoea virus (Chamorro et al., 2011).

The mechanical transmission by *Haematobosca squalida* is known also for the kinetoplastid *Trypanosoma brucei brucei*, the agent of Nagana in Africa (Mihok et al., 1995; Baldacchino et al., 2018). A high incidence of the bovine besnoitiosis caused by the apicomplexan parasite *Besnoitia besnoiti* was associated with the peak activity of biting flies, including *H. irritans*, but its vector role has not been demonstrated (Gollnick et al., 2015).

Haematobia flies from South America have been shown to be phoretic carriers of the eggs of the human bot fly, *Dermatobia hominis* (Pont, 1973; Leite et al., 1998).

Biology and ecology

Taxonomy and diversity

The following four biting muscid genera: *Stomoxys* (e.g., the stable fly *S. calcitrans*), *Haematobia* (e.g., the horn fly *H. irritans* and the buffalo fly *H. exigua*), *Haematobosca* (e.g., the large horn fly *H. stimulans*, the moose fly *H. alcis*, and *H. sanguinolenta*), and *Haematostoma* (*H. austeni*) are true flies (order Diptera) belonging to the family Muscidae, subfamily Muscinae, and tribus Stomoxyini (sometimes are placed in the separate subfamily Stomoxyinae or even family Stomoxyidae). (There is one more genus, *Stygeromyia*, with two species distributed in Sub-Saharan Africa and Oriental region; however, the existence and the systematic status of this genus are disputed.) Stomoxyini biting flies, comprising almost 50 species, are obligate blood-sucking ectoparasites of livestock and other warm-blooded animals, and some species are considered important pests of domestic animals with a significant economic impact in many parts of the world. The genus *Stomoxys* contains 20 described and currently accepted species, the genus *Haematobia* accommodates 9 species, the genus *Haematobosca* is comprised of 17 species, and the last genus *Haematostoma* is represented by only one species, *H. austeni* (Pape and Thompson, 2020).

In addition to the cosmopolitan species *S. calcitrans*, several other *Stomoxys* fly species, including *S. niger*, *S. sitiens*, and *S. indicus*, also readily attack domestic animals (Wall and Shearer, 1997).

The horn fly species *H. irritans* has been originally described by Linnaeus as *Conops irritans* Linnaeus, 1758; however, Lepeletier and Serville established in 1828 the genus *Haematobia* where, later, this species was accommodated. Unfortunately, the generic name *Siphona* Meigen (1803) and *Lyperosia* Rondani (1856) were also introduced (as *Siphona irritans*, *Lyperosia irritans*, or *Siphona* (*Lyperosia*) *irritans*) into literature and have been commonly used in 19th and 20th centuries.

The well-known and cosmopolitan horn fly *H. irritans* is in some geographical areas substitute by the buffalo fly *H. exigua*. The horn fly is a significant pest of cattle throughout much of the world; originally it is distributed in the Holarctic region—in Europe, North Africa, Anatolia, and central Asia, the distribution in East Asia is questionable. The species was accidentally introduced by humans and livestock to North America in the middle 1880s. It was first reported in Canada in the early 1890s; it colonized most of the continent and had reached Venezuela by the 1930s. Horn fly arrived in Brazil in the mid-1980s and has spread through much of the cattle-producing areas of North and South America, including Hawaii. The buffalo fly is an important pest in northern Australia and Southeast Asia. The species is native to southern Asia, the Orient, Indonesia, and several Pacific islands and it spread through commerce to New Guinea and Australia before 1840. Both species have occurred together through the boundary of the Palearctic and Oriental regions. The horn and buffalo flies are closely related to each other (sometimes referred to as *H. irritans sensu lato*) and the morphological differences are very minor (e.g., presence/absence of 4–6 long hairs with curled tips on the second segments of the male's hind tarsus). These two flies have often been regarded as different species or as subspecies (*H. irritans irritans* and *H. irritans exigua*). The current chemotaxonomic research (Urech et al., 2005) and studies based on molecular divergences between these two taxa indicate an intermediate status between intraspecies and interspecies genetic variability (Iwasa and Ishiguro, 2010; Low et al., 2014; Changbunjong et al., 2016; Onder et al., 2018); however, the current universally accepted agreement is to keep them as two separate species.

In addition to *H. irritans* s.l., several other species affect domestic animals, among them *Haematobia titillans* (also referred to as *Lyperosia titillans*; for the reason see above) is the most important. The species is found in the Palaearctic region, namely in southern Europe, Turkey, the Central Asian Republic, and throughout the southern part of Russia reach to India and China; in many areas is associated with *H. irritans*. Like in other Stomoxyini flies, larvae are coprophagous and develop in many kinds of ungulate dung; however, *H. titillans* is quite often associated with camels where is the most numerous biting flies. The species is, together with *H. irritans*, an intermediate host of the nematode *P. skrjabini*, the causative agent of camel parabronchitis, and the nematode *Stephanofilaria assamensis* that causes common chronic ulcerated growth ("humpsore") in the Central Asian Republics and East Asia.

The genus *Haematobosca* is relatively species-rich, with 17 currently accepted and formally described species. The best-known species *Haematobosca stimulans* (also referred to as *Haematobia stimulans*) is in some areas so-called the large horn fly or large meadow bite fly. The fly with a length from 5 up to 7 mm is native to Europe and Asia and was introduced with cattle in North America. Its activity is mainly in late summer and autumn (e.g., in Europe flight from May to October) when heavy infestations with about one hundred flies per animal can occur. The species is associated mainly with cattle and other bovids; however, flies occasionally occur also on other big animals, like on horses. Adults are found on or around cattle and on/in farm buildings. Eggs are laid in almost fresh cow-dung and there are up to four (rarely five) generations per year. Adults sit on cattle with the head upwards, which distinguishes them from *H. irritans*. While the horn flies *H. irritans* practically live on the host, *H. stimulans* leave the host and spend the night at resting places in the vegetation.

In North America, another, morphologically very similar species of the genus *Haematobosca* is associated with cervids, specifically with the moose/elk (*Alces alces*). The association of the moose fly, *H. alcis*, with its host is intimate and flies could be found in greatest numbers on those parts with the least or shortest hair (e.g., on the calcaneum, around the anus, and on the hindquarters) and even more than 500 flies per animal were recorded (Burger and Anderson, 1974). The development from egg to adult in moose excrement requires approximately 3 weeks and flies could have completed as many as five generations per year (Egan and Moon, 2013). In the temperate zone, similar to other *Haematobia* spp. and *Haematobosca* spp., only the pupal stages overwinter.

The third notable species of this genus is *H. sanguinolenta*, commonly found in livestock farms, but also zoos and national parks in tropical Asia (Changbunjong et al., 2012).

The monotypic genus *Haematostoma*, represented with the only one species, *H. austeni*, is known from Oriental regions. The species has been reported as a jungle fly attacking big mammals, but it has rarely been collected despite its wide distribution in Southeast Asia including Borneo, Malaysia, Laos, Myanmar/Burma, and Thailand.

Morphology

The adult horn/buffalo flies (*H. irritans* s.l.) live in close association with cattle or buffalo. The fly is about 3–5 mm long, thus significantly smaller than the stable fly (*S. calcitrans*) with body length 4–7 mm or the large horn fly (*H. stimulans*) 5–7 mm long. A noticeable feature of the horn/buffalo flies is that they sit close together (forming a typical swarm) and with their heads pointing downwards on the hosts (Fig. 2). They do not walk over the surface of the bovid but fly to change position.

The colour of the body is grey, with four dark stripes on the top of the thorax. The wings are broad (Fig. 3) and spread in a delta-shaped manner (held at a 45–60° angle to their bodies) at rest (Figs. 1 and 2). The eyes are large and red, widely spaced in the females and close together in the males. The mouthparts are similar to the stable fly with a heavily sclerotized labium that forms the sheath of the proboscis. The food canal is formed by the labrum-epipharynx and the hypopharynx forms the salivary canal. The labium ends in a pair of labella that bear sharp teeth used for cutting the host's skin. Species of *Haematobia* and *Haematobosca* may be readily recognized by the possession of an elongate, sclerotized proboscis, with palps that are as long as the proboscis and which are grooved internally. The palps extend in front of the head and are distinctly dilated towards their tips. On the other hand, the palps of the stable fly (*Stomoxys*) are almost invisible. In the genus *Haematobosca*, arista (a bristle arising from the third antennal segment) carries hairs on the dorsal and ventral side, contrarily in *Stomoxys* and *Haematobia* the arista carries hairs only on the dorsal side.



Fig. 2 *Haematobia irritans* resting on the back of a cow. Photo David Modrý.

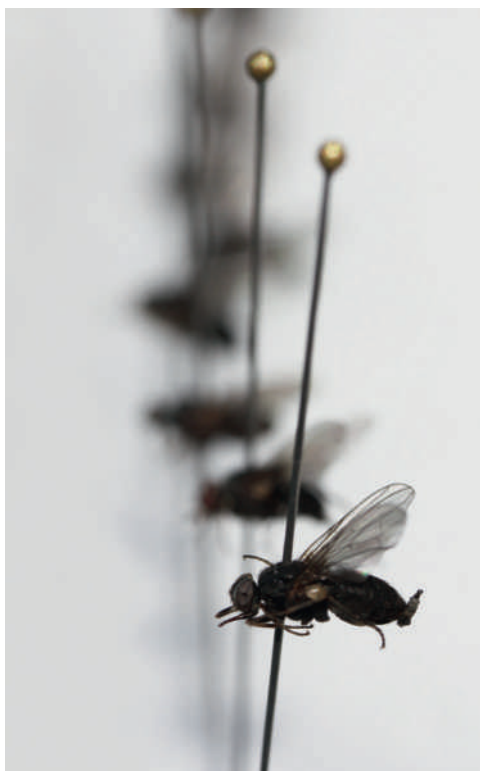


Fig. 3 *Haematobia irritans*. Photo Jana Bulantova.

Feeding

In contrast to nematoceran hematophagous flies (e.g., sand flies (Phlebotominae), blackflies (Simuliidae), biting midges (the genus *Culicoides*), and mosquitoes (Culicidae)) and horse flies (family Tabanidae), both males and females of all species of the tribus Stomoxyini (*Stomoxys*, *Haematobia*, *Haematobosca*, and *Haematostoma*) are hematophagous, similar to tsetse flies and hippoboscids flat-flies.

Although cattle and water buffalo are known to be the primary and preferred hosts for *H. irritans* and *H. exigua*, respectively, occasional feeding on other domestic animals including bison, camels, horses (Onder et al., 2018), and dogs (Kuramochi, 2000) have also been reported, sometimes in a very high density (e.g., over 500 horn flies per a horse were observed in Turkey, Onder et al., 2018). Biting on humans was also rarely observed (Marchiondo, 1987). In contrast, the stable fly is much less host-specific and its frequent attacks on people moving around typical hosts are well known.

The horn/buffalo flies stay on animals almost continuously and taking 20 or even more blood meals each day with their stiff needle-like mouthparts. They take frequent small blood meals, with each fly consuming on average 10 μ L of blood per day. Persistent blood-feeding irritates (so that's why the species name is "*irritans*") hosts and can cause significant production losses. Irritations from the bites annoy animals and occasionally, the wounds may become infected. In heavy infestations, the injection of salivary antigens stimulates the formation of circulation antibodies in highly variable concentrations. Like in other hematophagous arthropod species, the success of blood-feeding in *H. irritans* s.l. is highly dependent on its complex salivary mixture delivered at the bite site and the modulation of the host's haemostasis and immune system. It is well known that saliva from hematophagous arthropods facilitates the availability of blood as well as the transmission of etiological agents of various infectious diseases. It has been emphasized that *H. irritans* shows host specificity which relies on physiological adaptation to blood-feeding. These flies include two forms of a trypsin-like enzyme and an aminopeptidase in their midgut and Kuramochi (2000) demonstrated that aminopeptidase activity is lower in flies fed with blood from other animals than that in flies fed with cattle blood which indicates blood proteins from other hosts may not be able to digest by the horn flies. In addition to this, in the *H. irritans* saliva, a bioactive protein hematobin (HTB) has been recently identified with its ability to modulate macrophage inflammatory response (Breijo et al., 2018).

Life cycle

The horn/buffalo flies (*H. irritans* s.l.) occur worldwide, suck blood more than 20 times per day, and develop four to five generations per year. The life cycle and development of horn flies are primarily correlated with environmental temperatures and the presence of suitable hosts and manure (Lysyk, 1992). When the larvae are exposed to declining temperatures in autumn (in temperate

latitudes), pupal diapause occurs, and the adult flies emerge at the beginning of the following spring especially in temperate areas (Lysyk and Moon, 1994). Horn fly females usually only mate once, and this occurs on the host, and can oviposit on the second day after emergence. They lay eggs on fresh cattle manure only, specifically under edges of dung pat within minutes of defecation, usually up to 10 min of it hitting the ground. Horn fly maggots only develop in cattle manure and do best in the grass manure of pastured cattle; dung from other hosts (e.g., horses and sheep) remain unnoticed. Each female can lay up to 400 eggs (in batches of 20–30 eggs) so very large populations can build up over the summer. Eggs hatch within 1–2 days. The larvae are off white and have a tapered appearance on one end and rounded on the other end. After passing through three instar stages, the larvae develop to pupae (3–4 mm in length) in the manure at days 4–8, depending on environmental temperature (Lysyk, 1991; Lysyk and Moon, 1994). Pupating takes place within the dung pat or in the nearby soil and adults emerge 6–8 days later and should find a host quickly for their first blood meal. Adult horn flies spend most of their life on their host and feed multiple times a day. They leave their host only for laying eggs and/or moving to another host. The life cycle is completed in 10–18 days.

Epidemiology

Haematobia irritans is native to the Old World (Africa, Europe, and central Asia). It was introduced into North America from Europe in the middle of the 19th century and since then it has spread to most regions of the world where cattle are present. *Haematobia exigua* is native to Asia and some Pacific islands and has spread to Australia and New Guinea in the first half of the 19th century. Although the main hosts are large ruminants (cattle, bison, and water buffaloes) they can use aberrant hosts such as horses and other large mammals, and occasionally they can bite humans. However, larval development can take place only in the dung of their typical large ruminant hosts (Moon, 2019).

Several host-related intraspecific factors such as genetics and cattle breed seem to influence the infestation levels with *H. irritans* (Guglielmone et al., 2000; Calkins et al., 2019). It is unclear which factors are responsible for such differences but hair density and sebum quantities have been incriminated (Steelman et al., 1997). Horn fly resistant lines are known (McKay et al., 2019).

The seasonal variation in the activity of the horn flies is influenced by various factors such as the temperature emergence threshold (Scasta et al., 2017). In temperate areas, horn flies are present throughout the whole summer season (Fowler et al., 2015) and the overwintering is as diapausing pupae (Moon, 2019). In tropical regions, seasonality is influenced rather by the rainfall, with high peaks after the rain season (Ferraz da Costa et al., 2014). In subtropical regions of South America (e.g., Uruguay) the peak is in late summer and early autumn, and up to dozen generations per year is possible (Castro et al., 2008). The infestation levels are also dependent on the grazing system. A study from Brazil showed that *H. irritans* is more likely to occur on cattle from conventional pasture systems than from silvopastoral systems (de S Oliveira et al., 2017).

Prevention and control

Compared to other biting dipterans such as horse flies, stable flies, or mosquitoes, the control of horn flies has been considered easier, mainly to the behaviour of adults who spend most of their time (feeding or resting) on the body of their hosts. Hence, the application of insecticides on cattle is usually successful in reducing the populations (Foil and Hogsette, 1994). However, this success may be partially tempered by the development of insecticide resistance which has been reported to organochlorines, organophosphorus compounds (both banned or with very limited uses nowadays), and pyrethroids (Foil and Hogsette, 1994). Adulticides can be applied to animals through various routes such as sprays, pour-ons, wipe-ons, or various self-application devices (ear tags, dust back, or back rubbers) (Foil and Hogsette, 1994; Russell et al., 2013). Repellents are generally considered to have a limited effect on horn flies (Russell et al., 2013). In contrast, semiochemical tools to attract and trap horn flies could be considered (reviewed by Brugman et al., 2018).

Considering the biology of the reproduction of horn flies, the removal of the manure is also an effective way to limit the larval development and adult emergence. Various types of artisanal trapping systems have been tested, some of them with acceptable efficacy (Foil and Hogsette, 1994). Biological control measures such as habitat competition for larvae by dung beetles, predation by other insects (Staphylinidae and Histeridae beetles) and mites, entomopathogenic fungi (reviewed by Weeks et al., 2018), or bacteria (Baldacchino et al., 2018) have been evaluated but with various success rates (Foil and Hogsette, 1994).

The use of insecticides in a feed (benzamides, insect growth regulators, or avermectins) of cattle has been shown to be effective and limit the larval development due to elimination in the dung (Brugman et al., 2018). However, such methods can pose a significant threat to free-living arthropods and the opportunity to use this should be evaluated with care.

Several vaccine candidates (e.g., recombinant salivary gland protein, hematobin) were evaluated against *H. irritans* (Cupp et al., 2004; Breijo et al., 2017), but they have not yet been implemented as a control measure under field conditions.

In summary, control measures should be applied only when horn flies are abundant enough to justify it economically and integrated pest control measures (e.g., insecticide application and manure removal) are preferable to achieve maximum efficacy.

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Stable Fly (*Stomoxys calcitrans*, Muscidae)

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Glossary

Biological vector The pathogen infects the vector and undergoes reproduction or develops from one stage to another. Also referred to as a cyclical vector.

Dead-end host A host for a pathogen that is not part of the transmission cycle or amplification of the pathogen.

Ectoparasites Parasite living on the outside of their host.

Hematophagous Feeds upon blood.

Intermediate host A host in which development takes place but not sexual reproduction.

Mechanical vectors No development or reproduction of the pathogen takes place within the vector.

Synanthropic Domestic or associated with humans and their habitations.

Introduction

Stable flies, *Stomoxys calcitrans* L., are hematophagous synanthropic flies. Originating in Africa or southern Asia and accompanied the introductions of European livestock to the remainder of the temperate and tropical world. Although the time of their arrival to North America is not documented, their presence in Philadelphia during the signing of the Declaration of Independence, 4 July 1776 was noted by Thomas Jefferson:

The weather was oppressively warm, and the room occupied by the deputies was hard by a stable, whence the hungry flies swarmed thick and fierce, alighting on their legs and biting hard through their thin silk stockings. Treason was preferable to discomfort.

(Fuller, 1913)

Most likely, stable flies arrived in the Americas along with the 1200 men, pigs, horses, and cattle on Columbus's second voyage in 1493. It is unlikely they would have been absent from this entourage. Stable flies are persistent and painful biters making them serious pests of livestock, wildlife, and humans throughout their range. It is estimated that they reduce the productivity of United States cattle industries by over \$2 billion per year (Taylor et al., 2012b). Using 2018 cattle inventories and commodity prices, their impact on cattle productivity is estimated to be \$2.66 billion.

In addition to stable fly, other common names for *S. calcitrans* include dog fly, biting house fly, and power mower fly. The common name stable fly, is usually used in a narrow sense, referring only to *Stomoxys calcitrans*. However, occasionally it is used more broadly, referring to the other species in the genus as well.

Medical and veterinary importance

With their painful bites and blood feeding activities, stable flies reduce the productivity and comfort of livestock and companion animals, interfere with human outdoor and recreational activities, and transmit pathogens causing human and livestock diseases. As for most ectoparasites, the damage they inflict can be considered either direct, that caused directly by the parasite, or indirect, that caused by pathogens transmitted by the parasite.

Direct damage

Stable flies' painful bites disrupt feeding, reproductive and resting behaviors of livestock and induce physiological stress. Most of the economic impact of stable flies can be attributed to direct damage, the behavioral and physiological responses of livestock in response to the flies feeding activity. Stable fly bites are more painful than other biting insects (Elzinga and Broce, 1986) causing discomfort and strong defensive behaviors in the hosts. In severe infestations, host mortality may be observed (Bishopp, 1913; Cook et al., 1999; Huchzermeyer et al., 2001; Elkan et al., 2009). Both male and female stable flies require blood meals for sustenance and reproduction. Adults blood feed daily, $\approx 11 \mu\text{L}$ of blood per meal (Salem et al., 2012) and females require 3–4 blood meals to develop each batch of ≈ 150 eggs (Harris et al., 1974; Jones et al., 1999). Adults are closely associated with their hosts only while blood feeding, $\approx 2\text{--}4$ min per day (Harris et al., 1974). Each fly in an instantaneous count on a host can represent up to 180 biting flies per day or 2 ml of blood loss per day. Infestations at the economic threshold level, ≈ 15 flies per animal (Berry et al., 1983; Campbell and Berry, 1989), can result in the loss of ≈ 30 mL of blood while an infestation of 1000 or more flies (Solórzano et al., 2015) can result in the loss of >2 L of blood per day.

Cattle react to stable fly attack with behavioral and physiological responses. Fly repelling behavioral responses include tail flicking, leg stamping, head throwing, and skin twitching (Mullens et al., 2006) and fly avoidance behaviors include moving into shaded areas or enclosures, standing in water, and "bunching" (El Ashmawy et al., 2019). These behaviors consume metabolic energy and interfere with grazing. In response to stable flies, cattle may shorten grazing periods, reduce overall daily grazing time and intake (Todd, 1964). Cattle infested with stable flies tend to consume herbage faster, but for shorter periods of time, than uninfested animals (Dougherty et al., 1994). Experimental results relative to physiological responses are ambiguous. Schwinghamer et al. (1986) observed increased heart rate, respiration rate, and rectal temperature among other physiological parameters when steers were exposed to stable flies, whereas Estienne et al. (1991) observed no changes in the same parameters. Previous exposure to stable flies moderates fly induced responses in cattle (Mullens et al., 2006; Schole et al., 2011).

During the last 30 years, stable flies developing in crop residues in fields have become an increasingly serious issue in diverse regions of the world, vegetable crop residues in Western Australia (Cook et al., 2018), sugarcane residues in Brazil and Mauritius (Dominghetti et al., 2015), and pineapple residues in Costa Rica (Solórzano et al., 2015). Prior to the use of combines, when grains were threshed in the field, similar observations were made in the United States where large outbreaks of stable flies were attributed to flies developing in straw piles (Bishopp, 1913). Outbreaks resulting from flies developing in crop residues are producing infestation levels in excess of 1500–2000 stable flies per animal (Solórzano et al., 2015). Infestations of this magnitude make livestock production impossible with negative weight gains, dry dairy cows, and mortality.

Indirect damage

Relative to other blood feeding flies, stable flies are poor vectors. Other than for the horse stomach worm (*Habronema microstoma*) stable flies act as mechanical vectors; no development or reproduction of the pathogens/parasites take place in the fly. Rather, the pathogen/parasite can be transferred on contaminated mouth parts or by regurgitation of gut contents while feeding on an uninfected host. In most cases, pathogen transmission by stable flies involves interrupted feeding and resumption on a different host within a short timeframe. Since stable fly infestations can be heavy and interrupted feeding very common (Schofield and Torr, 2002), although inefficient vectors, they can still play significant roles in the transmission of pathogens and parasites. Although they have been incriminated as vectors of several pathogens/parasites, the four criteria needed to demonstrate the vectorial role an arthropod (Barnett, 1960; Table 1) have been documented for very few. Most of the pathogens/parasites that stable flies have been implicated for transmitting are not currently present in North America. Unfortunately, several are invasive and rapidly expanding their ranges. Even though they are not currently a threat in North America, we need to remain vigilant and recognize that, should they invade, we have competent vectors capable of maintaining pathogens and resulting disease.

Table 1 Barnett's criteria for incriminating a vector.

1	Demonstration of feeding on or other effective contact with the host under natural conditions
2	A biological association in time and/or space of the arthropod and host
3	Repeated demonstrations that the arthropod, under natural conditions, harbors the pathogen
4	Transmission of the pathogen under controlled conditions

Barnett HC (1960) The incrimination of arthropods as vectors of disease. *Proceeding of 11th International Congress of Entomology*, vol. 2, pp. 341–345.

Viruses

Equine infectious anemia (EIA)

Equine infectious anemia is an infectious, viral disease of horses and other equids with worldwide distribution. The virus is an RNA virus in the family Retroviridae, genus *Lentivirus*. The disease, also known as Swamp Fever, is most active during seasons and at locations where biting flies are abundant (Scott, 1922). Deer flies (*Chrysops* spp.; Foil et al., 1983), horse flies (*Tabanus* spp.; Stein et al., 1942; *Hydrometra* spp.) and stable flies (*S. calcitrans*; Stein et al., 1942; Foil et al., 1983) can transmit the disease mechanically. The relative importance of these flies as vectors depends upon season and geographical region.

Lumpy skin disease (LSD)

Lumpy skin disease is caused by a virus in the family Poxviridae, genus *Capripoxvirus* which affects cattle and water buffalo in Africa, the Middle East, and recently, expanding into southeastern Europe (EFSA, 2019). Lumpy skin disease causes considerable economic losses due to emaciation, hide damage, infertility, mastitis, loss of production, and mortality (Davies, 1991; Al-Salihi, 2014). Outbreaks of LSD in Israel are associated with high infestation levels for stable flies (Kahana-Sutin et al., 2017). Stable flies can transmit LSD virus between sheep (Kitching and Mellor, 1986) and cattle (Sohier et al., 2019; Issimov et al., 2020). Stable flies and horse flies appear to be the primary vectors of LSDV.

Bovine leukosis

Enzootic bovine leukosis is a contagious retroviral disease of cattle caused by the Bovine Leukemia Virus (BLV). BLV infection degrades lymphocyte function in cattle, diminishing immune response, milk production and longevity. Approximately 40% of dairy cattle in the United States are infected with BLV. Annual losses to dairy producers and consumers exceed \$500 million per year (Bartlett et al., 2014). Biting fly control has a significant effect on the incidence of BLV in dairy herds (Kobayashi et al., 2010; Erskine et al., 2012). Although experimental transmission of BLV by tabanid flies has been demonstrated (Ohshima et al., 1981; Hasselschwert et al., 1993), attempts to transmit the virus with stable flies have been unsuccessful (Buxton et al., 1985; Weber et al., 1988; Freitas and Romero, 1991). It is unlikely that stable flies play a significant role in the transmission of BLV.

Rift valley fever

Rift Valley Fever is an acute zoonotic disease of ruminants and humans caused by the Rift Valley Fever Virus (RVFV; Bunyaviridae, *Phlebovirus*). It causes high mortality in newborn ruminants, especially sheep and goats, and abortion in pregnant animals. Humans are dead-end hosts. Endemic to sub-Saharan Africa, RVFV spread to Egypt in 1976 causing 200,000 human cases and 600 deaths (Chevalier et al., 2010) and in 2000 to the Arabian Peninsula (Balkhy and Memish, 2003). Mosquitoes and direct contact with infected fluids are the primary routes of transmission. However, biting flies including *Stomoxys* spp. and *Glossina* spp. can transmit the virus mechanically (Hoch et al., 1985). Their role in the epidemiology of RVF is probably small in endemic areas. Should the virus invade new territories such as Europe or the Americas, *S. calcitrans* could play a larger role.

West Nile encephalitis

West Nile is an acute encephalitis caused by the West Nile Virus (WNV). WNV is in the family Flaviviridae and normally transmitted by *Culex* mosquitoes. The virus was introduced to North America in 1999 resulting in an epizootic that progressed from east to west across the United States (Kramer and Bernard, 2001). In an unusual set of circumstances, a rookery of American White Pelicans was beset by WNV and stable flies. The stable flies fed heavily upon the sick and dying pelican chicks and were suspected of transmitting the virus among chicks (Johnson et al., 2010; Doyle et al., 2011).

Acquired immunodeficiency syndrome

Acquired immunodeficiency syndrome is caused by the Human Immunodeficiency Virus (HIV). Although Brandner et al. (1992) and Eigen et al. (2002) discuss the possibilities of *Stomoxys* being involved in the transmission of HIV, no observations of experimental transmission have been published. Analysis of epidemiological evidence concludes "There was thus no evidence of insect-borne HIV" (Goddard, 2018).

Porcine reproductive and respiratory syndrome (PRRS)

Porcine reproductive and respiratory syndrome is a globally important disease of swine caused by an arterivirus. The virus causes pneumonia in growing pigs, abortion in sows and loss of vigor. PRRS is easily transmitted among hogs within a herd by contact and exchange of fluids including saliva (Cho and Dee, 2006). Airborne transmission between isolated herds is suspected (Mortensen et al., 2002) possibly implicating insect vectors. Stable flies retain infective virus for up to 24 h after feeding on infected pigs (Rochon et al., 2015). However, attempts to transmit the virus to uninfected pigs by stable fly bites were not successful (Rochon et al., 2011). Stable flies do not appear to play any role in the transmission of PRRS.

Bacteria

Enteric bacteria

Unsurprisingly, given their association with animal wastes, enteric bacteria including *Campylobacter* spp. (Szalanski et al., 2004), *Enterobacter sakazakii* (Hamilton et al., 2003; Mramba et al., 2007), and mycobacteria (Fischer et al., 2001) have been observed in or

on stable flies. Stable flies do not congregate on and around human food as do house flies so probably do not play a large role in the contamination of foodstuffs. They may contaminate the coats of livestock and companion animals while feeding thus allowing for the ingestion of enteric pathogens when the hosts groom. The role of stable flies in the dissemination of these pathogens has not been established.

Dermatophilosis

Dermatophilosis is a pustular and crusting dermatitis in sheep, goats, cattle, horses, cats, and rabbits caused by *Dermatophilus congolensis*, a facultative anaerobic, branching Actinomycetes. Although *D. congolensis* has a worldwide distribution, most cases of dermatophilosis are observed in the moist tropics (Zaria, 1993). On cattle, lesions on the lower body and legs are usually attributed to tick bites or initial damage to the epidermis by vegetation. However, lesions on the backs have been attributed to flies (Stewart, 1972). Richard and Pier (1966) demonstrated transmission of *D. congolensis* from rabbit to rabbit with stable flies. However, Philpoot and Ezech (1978) found transmission from bovine to bovine by *Stomoxys* spp. to be rare and conclude that they are probably not primary vectors because of their predilection to bite on the lower body and legs whereas the dermatophilosis lesions were on the back of the cattle.

Anthrax

Anthrax is a serious infectious disease caused by *Bacillus anthracis*. The type of illness is dependent upon the method by which the pathogen entered the body. Four types of anthrax are recognized. Cutaneous, gastrointestinal, inhalation, and injection. The most common form is cutaneous which occurs when the bacteria enter the body through a skin abrasion or insect bite. Biting flies, especially horse flies, have been associated with anthrax since the earliest recorded outbreaks in the 1800s (Hugh-Jones and Blackburn, 2009). Several studies have documented the ability of *Stomoxys calcitrans* to transmit anthrax after feeding on an infected host (Schuberg and Kuhn, 1912; Schuberg and Boing, 1914; Mitzmain, 1914; Turell and Knudson, 1987). Epidemiological and spatial evidence support the involvement of biting flies in the transmission of cutaneous anthrax (Blackburn et al., 2014). However, the relative roles of horse flies and stable flies are unresolved. Horse flies appear to be the more efficient mechanical vector. However, stable fly numbers exceed those of horse flies by several fold in many regions. As for many pathogens and diseases stable flies overwhelming numbers may overcome their low vectoral efficiency.

Protozoa

Apicomplexa

Besnoitiosis

Besnoitiosis is a chronic and debilitating, emerging/expanding disease of cattle and other ruminants caused by the Apicomplexan protozoan *Besnoitia besnoiti*. *Besnoitia besnoiti* is closely related to *Toxoplasma gondii* and has a similar life cycle. Definitive hosts for *B. besnoiti* are felids; cattle are intermediate hosts. The majority of cases of besnoitiosis are in Africa. However, the disease is spreading to the Middle East, southern Asia, southern Europe, and even South America. *Besnoitia bradyzoites* can be transmitted mechanically by the bites of blood-feeding flies (Álvarez-García et al., 2013; Duvallet and Boireau, 2015). In France, the temporal pattern of *Besnoitia* transmission coincides with that stable flies (Liénard et al., 2011) and experimental transmission from chronically infected cattle to rabbits has been demonstrated (Sharif et al., 2019). At least in some locations, stable flies appear to play a primary role in the epidemiology of besnoitiosis.

Trypanosomatids

Trypanosomes are parasitic flagellate protozoans in the class Kinetoplastea, order Trypanosomatida, family Trypanosomatidae, genus *Trypanosoma*. Several species of *Trypanosoma* cause serious and debilitating disease in humans, livestock, and companion animals. Depending upon the species of *Trypanosoma* involved and the host, Trypanosomiasis go by many names. The Trypanosomes responsible for human Trypanosomiasis, also known as African Sleeping Sickness, *Trypanosoma brucei gambiense* and *T. b. rhodesiense* undergo cyclical development in their primary vector, *Glossina* spp. (tsetse flies). Several species of *Trypanosoma* can be transmitted directly from host to host via mechanical transmission as well. Stable flies have been implicated as vectors for several of those Trypanosomes. Of special interest relative to *Stomoxys* is the phenomenon of trypanosomiasis in the absence of *Glossina* spp. This is observed in parts of Africa where *Glossina* spp. are absent as well as in parts of Latin America and Asia.

Trypanosoma evansi (= *T. brucei evansi*)

Trypanosoma evansi is widely distributed in Saharan Africa, southern Asia, and South America (Radwanska et al., 2018) and is the etiological agent of Surra, a debilitating and economically important disease of equines, bovines, and camelids (Aregawi et al., 2019). *Trypanosoma evansi* is a form of *Trypanosoma brucei* that lost functional kinetoplasts and as a result, the ability to develop cyclically in *Glossina* spp. *Trypanosoma evansi* is therefore dependent upon mechanical transmission for movement from host to host. Stable flies and horse flies (Tabanidae) have been implicated as the primary vectors of *T. evansi* (Luckins, 1988; Lun et al., 1993; Desquesnes et al., 2008). Stable flies have been associated with the transmission of *T. evansi* in time and space (Desquesnes et al., 2008; Rodríguez et al., 2014). Experimental demonstrations of transmission of *T. evansi* by *S. calcitrans* however are scant. Bouet and Roubaud (1912) demonstrated transmission in several experiments and indicated that *Stomoxys* spp. play a major role in the epidemiology of Surra. However, their experiments lacked controls and some of the rigor expected in more recent studies. Sumba

et al. (1998) demonstrated transmission with *Stomoxys niger* and *S. taeniatius*, but *S. calcitrans* was not included in their study. Desquesnes et al. (2013) discuss the biological and epidemiological properties of *Stomoxys calcitrans* that would support their involvement in the transmission of *Trypanosoma evansi*, but do not demonstrate experimental transmission. Ngeranwa and Kilalo (1994) were unable to transmit *T. evansi* between goats or camels with *S. calcitrans*. Failure of laboratory transmission studies should not be taken as strong evidence that *S. calcitrans* is not involved in the transmission of *T. evansi* because most such studies involve relatively small numbers of flies/bites. In the field, animals can be bitten by 10- to 100-thousand flies or more per day during severe outbreaks. Such numbers can overcome low odds. Additional research is needed to elaborate the role of *Stomoxys* spp. and specifically *S. calcitrans*, in the epidemiology of Surra.

Nagana

Nagana is an acute or chronic disease caused by several species of trypanosomes. *Trypanosoma congolense* causes a chronic form of the disease while *T. brucei brucei* and *T. vivax* cause an acute form. Primarily, these trypanosomes are cyclically transmitted by *Glossina* spp. However, *T. congolense* and *T. vivax* can be transmitted mechanically by Tabanids and Stomoxines. This has allowed them to expand their range beyond that of *Glossina* spp. *Trypanosoma congolense* is limited to sub-Saharan Africa while *T. vivax* has expanded its range to much of South and parts of Central America (Radwanska et al., 2018). The relative importance of Tabanids and Stomoxines in the transmission of *T. congolense* and *T. vivax* is unclear. Experimental mechanical transmission of *T. congolense* and *T. vivax* by Tabanids has been demonstrated (Ferenc et al., 1988; Raymond, 1990; Otte and Abuabara, 1991; Desquesnes and Dia, 2003, 2004). Sumba et al. (1998) demonstrated mechanical transmission of *T. congolense* by *Stomoxys niger niger* but *S. calcitrans* was not included in their study. Transmission of *T. vivax* (= *T. cazalboui*) by mixed species of *Stomoxys* was reported by Bouet and Roubaud (1912) but similar efforts by the same authors to transmit *T. congolense* (= *T. dimorphon*) with *Stomoxys* failed. Santos-Silva et al. (2004) reported that Serra-Freire and Rezende (1988) had successfully transmitted *T. vivax* mechanically with *S. calcitrans*. In conclusion, experimental data supporting the involvement of *S. calcitrans* in the transmission of *T. congolense* and *T. vivax* are thin. However, given the very high infestation levels of *S. calcitrans* observed in some locations and their propensity for interrupted feedings, their role in transmitting these pathogens cannot be ruled out even if their efficiency is very low and difficult to replicate in the laboratory.

Leishmaniasis

Leishmania spp. are the etiological agent for several diseases of humans and animals including cutaneous, visceral and mucocutaneous forms of the disease. *Leishmania* spp. are normally transmitted cyclically by phlebotomine sand flies. However, in some urban and peri-urban environments, where phlebotomine sand flies are rare or absent, it is possible that other biting flies may be responsible for mechanical transmission of *Leishmania* spp. Coelho et al. (2016) observed viable *Leishmania* spp. in field collected tabanid flies, but not in *S. calcitrans*. Berberian (1938, 1939) documented that *S. calcitrans* were able to transmit *Leishmania tropica* immediately after an interrupted meal on an open Oriental sore. These results were duplicated by Lainson and Southgate (1965) with *Leishmania mexicana*.

Nematodes

Habronemiasis

Horse stomach worms *Habronema* spp. infect the stomachs of horses. Adult worms release eggs which pass out with the feces. Eggs hatch soon after being excreted and the first instar larvae are eaten by fly larvae in the manure. The worms molt twice in the fly larvae and develop into infective L3 larvae by the time the adult fly emerges. The L3 escapes the fly host while it is feeding, usually around the nose or lips of the definitive host. L3 larvae are ingested by the host, pass to the stomach where they mature and complete the cycle (Pugh et al., 2014). Stable flies are the intermediate host for *Habronema microstoma* and house flies for *H. muscae* (Traversa et al., 2008). If the L3 nematode larva are deposited near a wound or the eyes, they are unable to develop into adults and continue to migrate in the skin causing inflammation and a condition referred to as "summer sore." This is the only situation where stable flies are considered biological or cyclical hosts of a pathogen.

Biology and ecology

Taxonomy and phylogeny

Stable flies are members of the subfamily Muscinae, tribe Stomoxyini of the Dipteran family Muscidae (Zumpt, 1973). The genus *Stomoxys* includes 18 species most of which are endemic to Africa with several species ranging to the Indian subcontinent and southeast Asia (Zumpt, 1973). *Stomoxys calcitrans* is cosmopolitan, having accompanied human mediated introductions of livestock around the world. As currently recognized, the genus *Stomoxys* is considered paraphyletic because of the segregation of *Prostomoxys saegerae* (Zumpt) (Dsouli et al., 2011).

Morphology

Adult stable flies are approximately the same size as house flies, 5–8 mm long, and generally of similar appearance. They can be differentiated from house flies by the presence of a rigid proboscis projecting forward from the head (Fig. 1). The thorax is grey with



Fig. 1 *Stomoxys calcitrans* (photo credit: Stephen Ausmus, USDA Agricultural Research Service). <https://www.ars.usda.gov/oc/images/photos/nov12/d2620-1/>.

four longitudinal dark stripes. The abdomen is grey with darker spots medially on the base of segments 3, 4, and 5 and lateral on the apex of segments 3, 4 and sometimes 5 (Zumpt, 1973). Males and females are similar with the eyes of males well separated and only slightly larger than those of females.

Stable fly eggs are whitish and ≈ 1.0 mm long and 0.3 mm wide. Eggs are slightly curved, convex on the ventral side and concave on the dorsal side. Two parallel lines of dehiscence are visible on the dorsal side (Parr, 1962; Salem, 2012).

Stable fly larvae are typical of muscoid fly maggots. Mature, third instar, larvae are 10–11 mm long and creamy white. Stable fly larvae can be differentiated those of house fly by the shape and relative position of the posterior spiracles. In house flies, the posterior spiracles are semicircular and proximate with the button located near the inner margin. In stable flies, the posterior spiracles are separated by more than the diameter of a spiracle, semi-triangular in shape, and the button is located near the center of each.

Stable fly puparia are 6–7 mm long and often slightly more reddish brown than those of house flies which are usually a darker brown. The integument of the puparia is that of the third instar larva. Therefore, larval morphological traits, especially the spiracular plates, can be used to differentiate stable fly puparia from those of house flies.

Life cycle

As all dipteran flies, stable flies are holometabolous, going through egg, larva, pupa, and adult stages during their development. Females lay up to approximately 150 eggs directly on the developmental substrate. Eggs are usually laid in small groups of several to 20 or more dispersed in small crevices and irregularities on the surface of the substrate. Under optimal conditions, 25–30 °C, eggs hatch ≈ 24 h after oviposition. Larvae are vermiform and progress through three instars, growing from ≈ 1 mm to ≈ 11 mm in 7–8 days. Mature larvae molt into coarctate puparia followed by a true pupal molt within the puparia ≈ 1 day later. Adults eclose 7–10 days after pupariation. The duration of development from egg to adult is 19–20 days. Under suboptimal conditions, temperatures between 15 °C and 20 °C, development can take 70 or more days (Parr, 1962; Lysyk, 1998; Salem et al., 2012; Florez-Cuadros et al., 2019). Stable flies are not known to have diapause or freeze tolerant stages (Greene, 1989).

Stable fly larvae develop in a broad variety of fermenting vegetative materials (Simmons and Dove 1941, 1942; Siverly and Schoof 1955; Hafez and Gamal-Eddin 1959; Sutherland 1978; Campbell and McNeal 1979; Williams et al., 1980; Hall et al., 1982; Cook et al., 1999, 2018; Solórzano et al., 2015), often contaminated with animal feces and urine. Larvae are most likely to be found in moist, slightly alkaline substrates with high levels of ammonium and coliform bacteria (Talley et al., 2009; Friesen et al., 2016). Although stable fly larvae can develop in pure, fresh bovine feces in the laboratory, they are rarely found in such in the field (Hogsette et al., 1987).

Blood feeding, digestion and ovarian development

Both male and female adult stable flies bite vertebrate hosts and feed upon blood. They will feed upon virtually any available mammal and, under unusual circumstances, birds (Johnson et al., 2010). Because their bites are quite painful, they are more likely to feed upon larger, less agile, and more docile animals such as cattle. They will rarely feed upon humans in the vicinity of livestock, but, can be severe nuisances for humans in the absence of livestock in urban or recreational areas. It takes 2–4 min for a stable fly to imbibe a full blood meal. Three fourths of feedings are interrupted, either by host defensive behaviors or by other flies, (Schofield and Torr, 2002) causing flies to initiate feeding multiple times and, often, on multiple hosts.

Males require a blood meal in order to produce seminal fluid components (Anderson, 1978). Females require multiple blood meals prior to mating (Anderson, 1966) and subsequent blood meals to develop their first batch of ≈ 150 eggs. Blood meals are usually taken at daily intervals when hosts are available (Anderson and Tempelis, 1970; Harris et al., 1974). Females can develop additional batches of eggs every 3–5 days with continued blood feeding. Both sexes bite and take blood meals throughout their adult life. In the absence of hosts, both sexes can feed on nectar for sustenance (Jones et al., 1992; Taylor and Berkebile, 2008). Although providing energy for flight and basic metabolism, nectar does not support mating or ovarian development. Adults can live up to 4–6 weeks; however, in the field, few survive more than 5–10 days.

Stable flies ingest ≈ 11 μ L of blood in a blood meal (Salem et al., 2012). Males ingest less and the amount ingested by females is dependent upon their stage of ovarian development. The first, second and third blood meals, are larger and take up to 4 days to be digested. However, females continue to feed daily even though previous blood meals are not completely digested. The final blood

meal prior to oviposition may be one-half the volume of blood relative to prior blood meals and digested within 24 h (Anderson and Tempelis, 1970). Virgin females continue to take blood meals and ovarian development proceeds to the fully gravid stage, however, they will not oviposit until mated (Venkatesh and Morrison, 1980).

Mating

Both male and female stable flies begin mating 2 days after eclosion. Prior to their first blood meal, males appear to be uninterested in attempting to mate and females are not receptive to mating. Males perch in sunny locations, often on feed bunks or fence rails, and dart out and grab passing flies, or even fly sized pebbles. Long chain methylated hydrocarbons on the cuticular surface of the female act as contact pheromones, stimulating the male to attempt to mate (Sonnet et al., 1977). If the target is not a female stable fly, the male will return to a perch, often the same one as previously occupied, to await another opportunity. If the target is a receptive female stable fly, the couple alight and remain in copula for 4–6 min (Parr, 1962). Males can inseminate up to 10 females, while females mate only once (Harris et al., 1966).

Dispersal

Stable fly dispersal and the related topic of mode of recolonization of temperate regions in the spring remain unsettled, probably because they vary relative to regions and possibly localities. Stable flies are strong fliers with the ability to disperse long distances. Flight mill studies indicate they can fly nearly 30 km per day (Bailey et al., 1973). Observations of stable flies many kilometers from shore on large bodies of water such as Lake Michigan and coastal waters attest to their flight abilities. Hogsette and Ruff (1985) recaptured several stable flies marked 160–225 km from the recapture sites on the Gulf Coast of Florida. However, the number of recaptures was small (7) and few intermediate distance traps were included in the study such that median, population level, dispersal distances could not be calculated. Studies looking at larger numbers of flies with denser trap arrays and abundant hosts observed median distances to recapture <1–1.6 km (Bailey et al., 1973; Gersabeck and Merritt, 1985; Taylor et al., 2010). In New Zealand, Todd (1964) noted that few stable flies were observed more than 1 mile (1.6 km) from larval developmental sites. Stable flies appear to move only as far from their natal site as needed to find hosts and then congregate near the hosts (Todd, 1964; Bailey et al., 1973).

Several workers have postulated that stable flies move long distances at high altitudes with weather systems (Hogsette and Ruff, 1985; Jones et al., 1999; Isard et al., 2001). This hypothesis is supported by observations of large populations of stable flies in localities lacking identified developmental habitats (Broce, 1993). However, stable flies are seldom collected on traps placed higher than 1.5–2 m above ground level (Williams and Rogers, 1976). Direct evidence, including the collection of stable flies flying at high altitudes, is needed to support high altitude migration of stable flies.

Prevention and control

Management of stable fly populations has proven to be a difficult task. The most successful programs have involved an Integrated Pest Management (IPM) approach involving cultural, mechanical and biological control methods in addition to insecticides. Insect control is most effective when the target is concentrated and immobile (Novak et al., 2010). For stable flies, the larval stage is the preferred target. Sanitation should be the primary component of stable fly management (Thomas et al., 1996). Sanitation involves the removal and proper disposal of vegetative waste and residues, especially those contaminated with animal waste, proper storage of silage and other animal feed stocks, timely removal of animal and feed wastes from pens and corrals. Unfortunately, sanitation is often not logistically feasible. The quantities of waste involved can be extremely large, for instance post-harvest pineapple residues can exceed 250 metric tons per hectare, the time frame very short, stable flies can go from egg to adult in just over 1 week, and disposal options are limited. In addition, stable fly larval developmental sites are ephemeral and often difficult to identify prior to adult emergence, especially in pasture settings (Broce et al., 2005). However, without sanitation as a component of an IPM program to limit the resources for developing flies, other methods are doomed to failure.

The most promising biological control option for stable flies is represented by the inundative release of parasitoid wasps. Pteromalid wasp in the genera *Muscidifurax* and *Spalangia* have been employed most frequently. Natural populations of native parasitoids are unlikely to reach population levels adequate to reduce stable fly populations to below the economic threshold, especially in pasture environments (Rutz, 1986). Results of parasitoid releases on stable fly populations have been mixed with the most success in defined habitats such as dairies and little success in open habitats such as pastures (Machtinger and Geden, 2018).

Mechanical approaches to stable fly management involve manipulating or modifying developmental substrates to reduce their suitability for larvae to complete development to adulthood. Covering or enclosing silage can effectively keep flies from laying eggs and adults from emerging into the environment. Likewise, covering manure piles can keep adult flies from emerging. Mechanical control has been used in Australia to bury vegetable crop residues and compress the overburden to keep adult stable flies from emerging (Cook et al., 2020). Burying pineapple residues at least 1 m deep has been used for stable fly control in Costa Rica, but the cost ≈\$4000 US per hectare is prohibitive.

Chemical control of stable fly larvae in the developmental substrates is difficult because microbial activity in the substrates rapidly breaks down most insecticides. Two classes of growth regulators, triazines and benzoylureas, can provide 8–12 weeks of

stable fly control in developmental substrates (Taylor et al., 2012a, 2014). Novaluron (a benzoylurea) is used on pineapple plants prior to chopping for field renovation in Costa Rica to control stable flies developing in the chopped plant residues.

Control of adult stable flies is difficult because the flies spend little time on the host, feed primarily on the lower legs where insecticides and repellants are easily washed off by wet grass, and the flies can move relatively long distances in search of hosts. Few products are registered for use against stable flies on food animals such as beef and dairy cattle because of the threat of residues in food products. Pyrethroids are the most commonly used class of insecticides used for stable fly control. However, pyrethroid resistance is becoming a greater and greater problem with some populations being fully resistant (Olafson et al., 2019). Long lasting stable fly repellants such as coconut fatty acids (Zhu et al., 2018) are in the developmental and formulation stages and may offer promise for reducing the impact of stable flies on livestock.

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Oestridae Causing Myiasis

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Introduction

Within the Oestroidea super-family, larvae of flies belonging to the families Oestridae, Calliphoridae and Sarcophagidae feed on dead or living animal tissues, therefore developing from first to second and third instar larvae (L1, L2 and L3). In their attitude in feeding on and infesting alive tissues, some of these larvae cause infestations known as myiasis (Colwell et al., 2006). While Calliphoridae and Sarcophagidae larvae may feed of dead or living tissues, therefore causing facultative myiasis and being also of forensic importance, the Oestridae includes flies which larvae cause only obligatory myiasis. Oestridae contains a large number of species, about 150, whose larvae parasitize a wide range of hosts, from mice to elephants. They display a high degree of host specificity and their biological life cycle may be extremely complex as a result of a longlasting adaptation to the host (Otranto, 2001). The four sub-families of medical and veterinary importance are Gasterophilinae, Oestrinae, Hypodermatinae and Cuterebrinae. Within the Gasterophilinae, the most important genus, *Gasterophilus*, consists of eight species, three of which are worldwide distributed. The sub-family Oestrinae includes nine genera and 34 species (known as nostril flies), the sub-family Cuterebrinae includes six genera and 83 species and the sub-family Hypodermatinae includes six species of the genus *Hypoderma* which are of major veterinary concern (Russell et al., 2013). In the following, we summarize the epidemiology, life cycle, the medical and veterinary importance as well as the control of the main species belonging to the family Oestridae.

Hypodermatinae

Within the sub-family Hypodermatinae and the genus *Hypoderma*, six species are of veterinary importance. All these larvae cause dermal myiasis and the most studied are *Hypoderma bovis* and *Hypoderma lineatum*, also known as cattle grubs, which have an important impact on cattle productions and a widespread distribution in the northern hemisphere (Colwell et al., 2009). Other species affect roe deer (*Hypoderma diana*), red deer (*Hypoderma actaeon*), reindeer (*Hypoderma tarandi*) and yak (*Hypoderma sinense*) and have been occasionally detected in other animal species, including humans, as subdermal and internal ocular myiasis. Another species within the Hypodermatinae subfamily is *Przhevalskiana silenus*, which causes the goat warble fly infestation, a myiasis characterized by the presence of warbles under the skin of the back and flanks of goats (Fig. 1).

Adults are typically bumble-bee-like flies with reddish yellow hairs at the posterior extremity of the abdomen and have rudimental mouthparts. The morphological identification of *Hypoderma* is mainly carried out on L3, since this is the most common developmental stage retrieved by practitioners and veterinary inspectors from live and slaughtered animals in typical warbles under the skin of the infested animals (Fig. 2). The morphological differentiation of L3 is not difficult, but can sometimes be troublesome because *Hypoderma* spp. have similar ecological, biological and morphological features. Indeed, *Hypoderma* flies are frequently present in the same environment and their larvae may sometimes share hosts (e.g. *H. bovis* and *H. lineatum* cattle, *H. diana* and *H. actaeon* red deer). For example, although host specificity is generally high, *H. bovis*, *H. lineatum* and *H. sinense* have been collected from yaks (Huang et al., 1987; Otranto et al., 2004a, 2005a). Accordingly, some *Hypoderma* species share epitopes of antigens secreted during larval migration inside host tissues (Boulard et al., 1996a).

The mature L3 is large (30 mm in length) presenting body segments with tiny spinulation (differently from Oestrinae larvae, which have dark transverse dorsal bands and transverse rows of spines on the ventral surface of each segment) and flat spiracular plates, both structures different in presentation (Otranto et al., 2003). Microscopic differences among *Hypoderma* spp. is based on

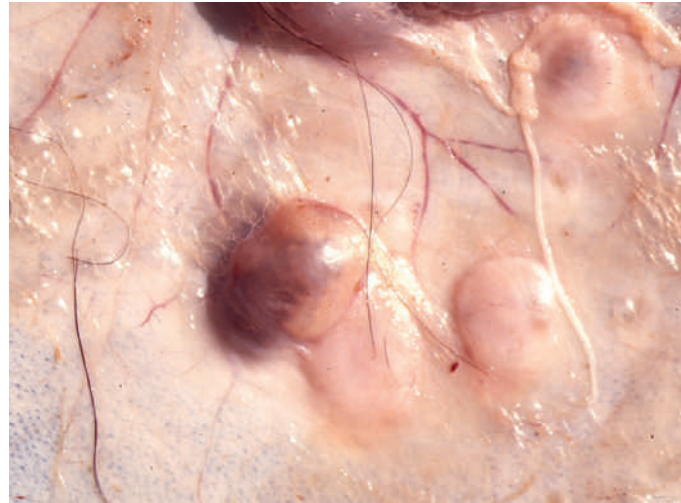


Fig. 1 Larva of *Przhevalskiana silenus* in the subcutaneous tissue of a goat. Photo: Otranto D.



Fig. 2 *Hypoderma lineatum* warbles under the skin of infested cattle during the early spring. Photo: Lia RP.

the pattern and morphology of spines on the cephalic and thoracic segments, by spine patterns on the tenth abdominal segment, and by morphology of the spiracular plates (Colwell et al., 1998; Otranto et al., 2003). The morphological differences above are also supported by the molecular characterization of the most variable region of the *cytochrome oxidase I* gene of these species, which showed to be a useful marker for the molecular identification and phylogenesis of this group of insects (Otranto et al., 2003).

As soon as they emerge, the females lay eggs (300–650) on the host's hair with adhesive material. While *H. bovis* females lay single eggs per hair on the rump and upper parts of the hind leg of cattle causing gadding, *H. lineatum* females lay 7–10 eggs in line along a single hair on the legs, which do not disturb the host. After hatching, the L1 penetrates the skin using collagenolytic enzymes secreted from the blind midgut (Boulard et al., 1996a). These enzymes are extremely immunogenic (i.e. they elicit an immunoreaction which is important for an early serological diagnosis) and enables larvae to migrate throughout the host's body for several months until they reach their final localization on the back of the host. During the winter months, migrating L1s of *H. lineatum* are found within the wall of the esophagus (Fig. 3), whereas those of *H. bovis* in the epidural fat of the spinal canal (the latter probably migrate along nerve trunks or through muscles). *Hypoderma sinense* presents a migration in the host body, similar to that of *H. lineatum* (Otranto et al., 2006a). Following this migration, L1s move to the host's back under the skin where they form nodules, also known as warbles. In this site, the L1 develops into L2 and L3 and release collagenolytic enzymes accumulated in their gut, piercing the skin and form a hole, allowing the breathing (Fig. 4). Full mature larvae drop to the ground, where they move actively, pupate and develop to adult fly in 3 to 10 weeks, depending upon environmental conditions (Fig. 5). The life cycle of larval *P. silenus* is exclusively subcutaneous without internal migration (Otranto and Puccini, 2000).



Fig. 3 First stage larvae of *Hypoderma lineatum* in the esophagus of infested cattle during their migration in the winter months. Photo: Lia RP.



Fig. 4 Mature third stage larvae of *Hypoderma lineatum* emerging from the nodule of a cattle from the respiratory hole on the skin and ready to drop in the environment to pupate. Photo: Lia RP.



Fig. 5 Mature third stage larvae of *Hypoderma lineatum* drop in the environment. Notice the dark coloration, which indicates the larva is ready to pupate. Photo: Lia RP.

Distribution and biology

Reports on clinical infestation of cattle, serum and milk serology surveillance studies, parasiticide efficacy studies and/or description of human cases published over the past years provide evidence that populations of *Hypoderma* spp. are still present in cattle in North America including Canada and the United States, as well as in Europe, e.g. in Portugal, Spain, France, Italy, Belgium, Switzerland, Germany, Poland, Slovakia and Estonia (Minář, 2003; Haine et al., 2004; Otranto et al., 2005a; Zygutiene et al., 2006; Panadero et al., 2007; Colwell, 2013; Rehbein et al., 2013). In addition, bovine hypodermosis is also reported in several countries in eastern and south eastern Europe including Belarus, Russia, Romania, Serbia, Bosnia and Herzegovina, Kosovo, Albania and Greece (Papadopoulos, 2004; Otranto et al., 2005b; Gorcea et al., 2011), and it is still abundant at high levels in resource-limited countries in northern Africa and Asia (Otranto et al., 2004a; Guan et al., 2005; Saidani et al., 2011; Dehghani et al., 2012; Yadav et al., 2013; Jaiswal et al., 2016).

Adult *H. lineatum* occur from late March to the end of May, whereas *H. bovis* from June to mid-September. Soon after eggs laying, L1s undertake migration (for *H. lineatum* from April until September) and can be found in the esophagus during the winter months with warbles appearing on the host's back from January to April. Therefore, mature L3s leave the host to pupate from March to early May (Colwell et al., 2006). The biological cycle of *H. bovis* is slightly delayed of some months also because they usually occur in northern, colder, regions. *Hypoderma sinense* infects cattle and yaks in China (Otranto et al., 2004a), where prevalence of infestation by *H. bovis* and *H. sinense* may be extremely high (up to 98–100% of animals and intensities exceeding 400 warbles per animal). According to the timing of this life cycle an early serological diagnosis of infestation (ELISA) throughout September–November (when warbles are still undetectable by palpation) and subsequent treatment strategies are advised (Otranto et al., 2016). Adults fly at temperatures above 18 °C, surviving for 3–5 days only, for mating and laying eggs (they have not a mouthpart for feeding). *Hypoderma diana* parasitize deer throughout Europe and Asia living in several different ecological zones where their hosts are distributed. The larvae reach the skin on the host's back where they develop into swellings warbles, developing in cysts containing purulent fluid. *Hypoderma tarandi* (the reindeer warble fly) infests reindeer/caribou in arctic and sub-arctic regions of northern Europe, North America and Russia. Its life cycle is similar to that of other species of this genus with the exception of female laying eggs (500–700) to the undercoat and the larvae then burrow into and under the skin during July–August without major migration in the body of the animals. During September–October, mature larvae form a warble, inducing damage to hides and leading to economic losses. As for almost all *Hypoderma* species the intensity of infection and prevalence are higher in younger animals, possibly as a consequence of the lack of acquired resistance (Lysyk et al., 1991).

Larvae of *P. silenus* penetrate the skin and develop to L2 and L3 nearby, without any migration in internal organs or in the subcutaneous tissue (Otranto and Puccini, 2000).

Medical and veterinary importance and control

Although bovine hypodermosis does not induce significant mortality and morbidity, this infestation affects the productivity and welfare of animals resulting in considerable losses to the livestock industry arising from a number of causes (Hall and Wall, 1995; Reist et al., 2002; Hassan et al., 2010). The importance of myiasis caused by *Hypoderma* spp. to livestock production and their distribution has been greatly reduced over the decades as a consequence of eradication campaigns in many countries and of the systemic use of macrocyclic lactones (Lia et al., 2019). Nonetheless, economic losses due to *Hypoderma* spp. still occur in some areas (e.g. southern Mediterranean regions, eastern Europe and China) and they are also at risk of reintroduction due to the spread in organic farms and the limited use of systemic endo-ecto-parasiticides (Colwell et al., 2009). Beyond the disturbance caused by ovipositing flies to grazing cows, reduction in weight gain and milk production is also a consequence of larval migration in the body of the animals. In addition, damage of the hide caused by holes formed by the L3s reduce the hide value in the leather industry (Hassan et al., 2010).

The presence of *Hypoderma* larvae may be detected by palpation of the warbles on the animals' backs (clinical parasitological examination) during the spring and summer months or by examination of subcutaneous tissues (Fig. 2) and internal organs of carcasses (post mortem examination). Some ELISA tests (Boulard et al., 1996a,b) have been used in many countries for the serodiagnosis of the disease. Anti-*Hypoderma* antibodies persist in infected cattle for 3–4 months after the L3s fall from their backs to the ground and antibody levels peak between November and March in Europe, making these the optimum months for testing and for achieving an early diagnosis of hypodermosis before the formation of warbles (Pruett and Barret, 1985). Testing commercial milk for antibodies has been shown to be effective for detecting the presence of hypodermosis in cattle, especially in those countries for which no data on this disease are available (Otranto et al., 2001). Cross-reactivity between *H. lineatum* antigen and anti-*P. silenus* antibodies allowed to prepare an ELISA for the detection of goat warble fly infestation demonstrating an antibody kinetics in naturally infested goats similar to that recorded in *H. lineatum*-infested animals (Otranto et al., 1999).

Human infestation by *Hypoderma* spp. occurs sporadically as ophthalmomyiasis or subdermal warbles and even skin allergies (Panadero-Fontán and Otranto, 2015). Infestation occurs in people who are closely associated with livestock or reindeer flocks as reported in case series in Norway (Kan et al., 2013). In poor socioeconomic settings of China, the infestation may occur in 0.4–7% of cattle and yak farmers.

Larvae of *Hypoderma* spp. are highly susceptible to systemically active organophosphates and macrocyclic lactones. These chemicals should be administered between September and October, but not later (mid-November and mid-March), in order to avoid anaphylactic reactions to toxins released by dead larvae, while migrating in the spinal canal or esophagus. Macrocyclic lactones, even when used at minimum dosage are highly effective as demonstrated in systemic campaigns which successfully

eradicated cattle hypodermosis in large areas of Central Europe (United Kingdom, Eire, Denmark and the Netherlands). However, the risk of reintroduction is constant. In a pilot study carried out in southern Italy on 9939 cattle bred in an area with a high prevalence of cattle hypodermosis, the use of moxidectin at 0.5% pour-on and 1% injectable formulations demonstrated an efficacy of 99.9% and 100%, respectively, showing the real opportunities of national programs for eradicating warble fly infestation (Otranto et al., 2005c). Macrocytic lactones administered with the feed may be used to eliminate oestrids from farmed deer.

Oestrinae

Larvae of the sub-family Oestrinae develop in the nasopharyngeal cavities of ungulate mammals, and occasionally infest human beings causing ophtalmomyiasis (Hall and Wall, 1994). The most important species are *Oestrus ovis* in sheep and goats, *Rhinoestrus purpureus*, in equids, *Cephalopina titillator* in camels, *Cephenemyia trompe* in reindeer, and *Tracheomyia macropi* in red kangaroo. Larvae of all these flies are firmly attached to the nasal mucosa through well-developed mouth-hooks, whereas the adults, without mouthparts, are short-lived.

While L1s of *O. ovis* (about 1 mm long) have gently curved mouth-hooks and terminal spines arranged in two groups (Fig. 6), mature L3s (about 25 mm long) have dark transverse bands dorsally and transverse rows of spines ventrally on each segment. After the development in the uterus, L1s are projected by female flies into the nose or eye of the proper host (e.g. *O. ovis* ejects about 20 larvae into the nasal cavities of sheep and goats), from where they move into the frontal and/or maxillary sinuses, developing until L3 (Fig. 7). Developed larvae, usually of a brownish color, migrate back toward the nasal cavities to be expelled by sneezing or coughing into the environment where they pupate before the next generation of adults emerge. *Rhinoestrus purpureus* larvae may localize into the pharynx of the host or even in the trachea.



Fig. 6 First stage larvae of *Oestrus ovis* (about 1 mm long) collected on the nasal mucosa soon after being projected by female fly. Note the tiny spinulation on the body segments and the two mouth-hooks which are important for the migration on the nasal mucosa. Photo: Lia RP.



Fig. 7 Mature third stage larvae of *Oestrus ovis* in the nasal cavities and sinuses. Photo: Lia RP.

Distribution and biology

Oestrus ovis has a worldwide distribution in the Palearctic ecoregion. Adults fly during summer causing disturbance in flocks, with sheep typically keeping their heads toward the ground in order to avoid them. Two fly generations usually occur per year in the northern hemisphere, with adults emerging in late spring, mate and ejecting larvae into the eyes or nose of the hosts. L1s develop rapidly and mature until L3s to leave the host in July and August. Adults may fly in autumn, larvipositing in September and October with the overwintering of L1s within the host. The biological life cycle may start again during early spring with a new generation of flies emerging from the pupae in June.

Infestation with *O. ovis* is commonly recorded in flocks where treatments are not regularly performed. Larvae may cause irritation of the mucosa, and mucopurulent discharge and impaired respiration, whereas, adult *O. ovis* causes sheep annoyance, leading to loss of grazing time (Papadopoulos et al., 2006). Late summer and/or winter treatment with macrocyclic lactones, such as ivermectin, is extremely efficacious with a clear reduction in nasal discharge and increased weight gain.

Cephenemyia spp. larvae develop in deer causing nasal discharge, sneezing, coughing and restlessness. Females eject live L1s in the nostrils of the hosts and after moving to the pharyngeal and nasal cavities they mature and migrate to the anterior pharynx before leaving the host as L3s to pupate in the environment.

Larvae of the genus *Rhinoestrus* localize in the throat region, at the base of the tongue, in the pharyngeal cavity and in the turbinates of equines. There is one generation per year and the female ejects larvae in batches at a time into the nostrils or eyes of a horse. L1s may remain in the nasal cavity for many months before moving to the sinuses to complete their development. Of the 11 species described, four are restricted to equines and seven to a wide range of wild animals (e.g. giraffe, warthog, bushpig and antelope). The most common species is *R. purpureus*, which parasitizes horses, donkeys and their crossbreeds. Both *R. purpureus* and *R. usbekistanicus* are diffuse in southern Europe with prevalence up to 21% in native horses and intensity rate as high as 65 larvae per host (Otranto et al., 2004b). L3s of the two species above are differentiated morphologically based on the shape of spiracular plates and the rows of spines on the dorsal view of the third larval segment (Otranto et al., 2005e) which is complete in *R. purpureus* (Fig. 8A) or narrowly interrupted medially in *R. usbekistanicus* (Fig. 8B).

Within the genus *Cephalopina*, *C. titillator*, the camel nasal bot fly, is extremely diffuse in dry parts of the Palearctic and Oriental ecoregions, and Australia with a prevalence of up to 90% in camel herds (Sazmand and Joachim, 2017). Due to their large size and presence of strong spinulation (Fig. 9), larvae may cause considerable irritation, breathing difficulty and lesions, which provide a suitable environment for pathogen proliferation and, eventually, pneumonia.

Medical and veterinary importance and control

For all the Oestrinae, adult fly activity and larvae depositing disturb animals, leading to a loss of grazing time with a reduction in weight gain. The parasitic rhinitis caused by the larvae is usually characterized by a mucoid nasal discharge, which may also be hemorrhagic. This could lead to histopathological changes with infiltration of inflammatory cells and squamous metaplasia and allergenic reactions provoked by molecules excreted/secreted by the larvae (Angulo-Valadez et al., 2011). Clinical signs of infestation include mild discomfort, nasal discharge, sneezing, nose rubbing or head shaking. Macrocyclic lactone endectocides are highly effective with treatments given, for *O. ovis*, twice a year (i.e. at the beginning of summer to kill newly acquired larvae, and in midwinter to kill any overwintering larvae). Infestation of Oestrinae larvae occasionally occurs in humans where they cause

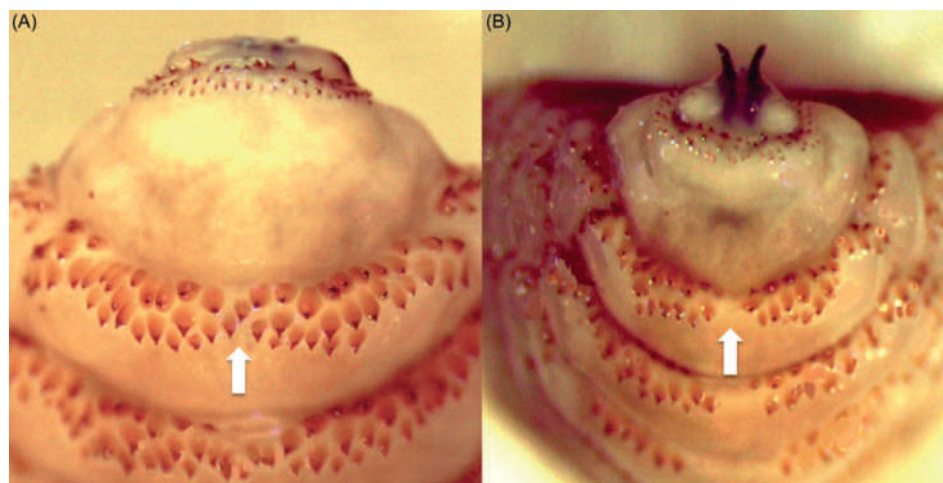


Fig. 8 Third stage larvae of *Rhinoestrus* spp. characterized by complete rows of spines on the dorsal view of the third larval segment (A) (*Rhinoestrus purpureus*) or narrowly interrupted medially (B) (*Rhinoestrus usbekistanicus*). Photo: Otranto D.

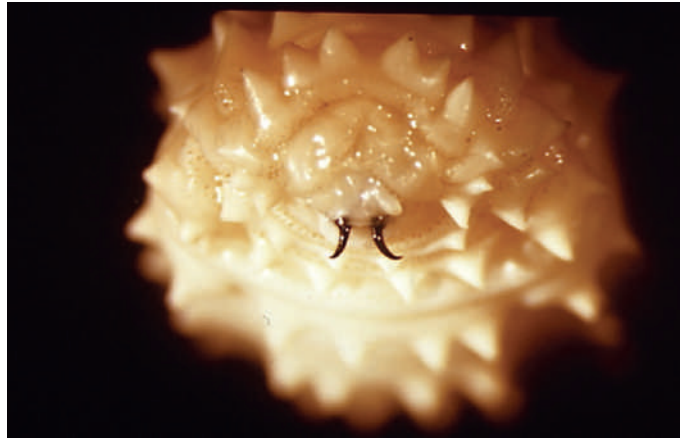


Fig. 9 Third stage larva of *Cephalopina titillator* collected in the nasal cavities of a camel. Note the strong spinulation which, cause considerable irritation, breathing difficulty and lesions in the infested animals. Photo: Otranto D.

ocular myiasis and even blindness. External infestation can be managed by physical removal of the larvae, but management of internal infestation requires expert assistance (Panadero-Fontán and Otranto, 2015).

Gasterophilinae

The subfamily Gasterophilinae includes flies whose larvae infest the gastrointestinal and pharyngeal mucosa of their hosts, causing inflammation, sloughing of the tissue and ulcerations. The genus *Gasterophilus* is the most important and it consists of eight species infesting equids, and *Gyrostigma* and *Cobboldia*, infesting rhinoceroses and elephants, respectively (Hall and Wall, 1994). Human cases with creeping dermal and oral/ocular infestations have been only occasionally reported.

Among *Gasterophilus* spp., *Gasterophilus intestinalis* is the most prevalent and widely distributed species in horses followed by *Gasterophilus nasalis*, *Gasterophilus haemorrhoidalis*, *Gasterophilus pecorum* and *Gasterophilus inermis*. The same species may also parasitize zebras. *Gasterophilus intestinalis* is the most important horse bot fly in Europe, the United States and Australia. In some countries of the Mediterranean basin, *G. intestinalis*, *G. nasalis*, *G. inermis*, *G. pecorum* and *G. haemorrhoidalis* occur in sympatry in free-grazing horses (Otranto et al., 2005d). The presence in some regions of southern Europe of an unusually large number of different species of bot flies has suggested a high degree of oestrid biodiversity in this area. The many factors that can influence parasitic species composition (e.g. host and parasite genetics, relationships with their hosts and environment and animal management) include the movement of domestic animals in association with migrating human populations in southern Europe over thousands of years (Otranto et al., 2006b).

While the adult of these insects are robust dark, large flies (10–15 mm in length). The larvae are armed with spines with robust mouth-hooks (Colwell et al., 2006). In the stomach, intestine or pharynx, mature L3s (16–20 mm) are cylindrical, reddish with two rows of spines anteriorly on most segments (*G. nasalis* has only one row). Number, shape and distribution of spines on the larvae metamers represent differential characters (Colwell et al., 2007).

Distribution and biology

Flies lay eggs (Fig. 10) on the host (i.e. *G. haemorrhoidalis* on the hairs around the lips, *G. nasalis*, *G. inermis*, and *G. intestinalis* on the intermandibular space below the jaws, on the cheeks and on the front legs, respectively) and the newly hatched L1s penetrate the tissues of the oral cavity and throat to migrate to the different tracts of the host's intestinal apparatus where they develop to L2 and L3 while attached to the mucosa. The localization of L2s and L3s varies according to *Gasterophilus* spp. For instance, *G. pecorum* resides on the soft palate and at the basis of the tongue, whereas *G. intestinalis* in the cardiac region of the stomach (Fig. 11). Moreover, *G. nasalis* locates in the pyloric region of the stomach and in the first portion of the duodenum, whereas *G. haemorrhoidalis* in the fundus of the stomach and the duodenum, and L3s in the rectal mucosa. Finally, also larvae of *G. inermis* reside in the rectal mucosa. There, the L3s stay for several months before passing into the environment with the feces and pupate. One or more generation of *Gasterophilus* spp. may occur every year in northern temperate or tropical regions, respectively.

According to their biological life cycle, infestation occurs at any time of the year with very high prevalence of infestation in horse heard living in the same area, with a high intensity of infestation (i.e. up to 738 per horse, of which 689 *G. intestinalis* in the stomach, 39 *G. nasalis* in the duodenum and 10 *G. inermis* in the rectum, in a survey conducted in southern Italy (Otranto et al., 2005d).



Fig. 10 Egg of *Gasterophilus* spp. yellow in color and attached singly to the hair of their host. Photo: Lia RP.



Fig. 11 *Gasterophilus intestinalis* third-stage larva firmly attached to the esophageal portion of the stomach. Photo: Lia RP.

Medical and veterinary importance and control

Due to their well-developed mouth-hooks, L2s and L3s of *Gasterophilus* spp. remain attached to the mucosa causing ulcers, mainly in early summer. The localization of larvae in the buccal cavity of horses may lead to stomatitis with ulceration of the tongue, which may become infected with bacteria and cause microabscesses, impairing proper chewing. Similarly, larval attachment to the stomach provokes an inflammatory reaction resulting in chronic gastritis, loss of condition and even perforation and death.

The adult fly can cause irritation and intense avoidance reactions when hovering around the host and laying eggs on the skin. Larvae of *Gasterophilus* spp. may infest humans through direct oviposition on human body hair or upon direct contact with hatching eggs on a horse. External ocular myiasis by *Gasterophilus* spp. have been reported in humans. Removal of eggs from the host's coat is not practicable, but *Gasterophilus* spp. infestations can be easily controlled by broad-spectrum macrocyclic lactone compounds, such as ivermectin, by a single treatment during the winter.



Fig. 12 *Dermatitis hominis* third-stage larva typically pyriform with stout spines on the globular anterior portion. Photo: Lia RP.

Cuterebrinae

In the Cuterebrinae subfamily there are included six genera and 83 species, with *Dermatitis hominis* as the most known because of the wide range of infected animals, including humans from southern Mexico through Argentina. Other species belong to the genus *Cuterebra* that infest wild rodents and lagomorphs (and occasionally humans) in North America (Sancho, 1988). The life cycle of *D. hominis* is peculiar in that the female glues her eggs (800–1000) to the abdomen of a hematophagous insect, which approaches a potential host. The contact with the host and the increase in the temperature, elicits egg hatching and the penetration of the larva into the skin (Hall and Wall, 1994).

Larvae penetrate individually in the skin where they develop to L2 and L3 in four to 18 weeks, before dropping from the respiratory host into the environment and pupate. While the L1 is sub-cylindrical, the L2 and L3 (Fig. 12) are typically pyriform with stout spines on the globular anterior portion and no spines on the narrower posterior part and anterior spiracles. After emerging from the pupae, adults mate, living for 1–9 days without feeding (Sancho, 1988). *Dermatitis hominis* may cause severe cutaneous myiasis in cattle as well as in humans. Lesions caused by this myiasis are usually prone to secondary bacterial infection as well as larvae causing myiasis.

Medical and veterinary importance and control

Myiasis by *D. hominis* may be characterized by severe abscesses due to secondary bacterial infections, which may cause losses to livestock production due to reduced calf growth and weight gain, decrease in milk production, and hide damage. Also, sheep and dogs may be frequently attacked. Following the penetration of L1 (also through clothing), humans may present painful, discharging, cutaneous swellings on the body which heal soon after their removal. The only way to prevent the infection by *D. hominis* is the protection of bites by carrier insects (e.g. mosquitoes). Antibiotics should be prescribed if secondary infection is present or likely. Some proteins have been characterized for the development of potential vaccines against *D. hominis* for cattle (Fernandes et al., 2012), but so far no commercial vaccine has been licensed.

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Myiasis-Causing Flies

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History and taxonomic positioning

There are several reports on the occurrence of myiasis in ancient times, e.g. “my flesh is clothed with maggots and encrusted with dust, my skin rotted and become loathsome” (Job 7:5); “the maggots will not turn into flies within you” (Papyrus Gizehn^c 18026:4 14) (Service, 1978). Infestation by maggots was considered a curse for bad men, like king Herodes (Book of Acts 12:23), also affected by high pressure, gangrene and renal insufficiency, Antioco IV Epifanius (Maccabees 2:9–12), Agrippa I and Judas Iscariotes (Montefiore, 2011). Aristotle, observing the appearance of maggots on skin, proposed the spontaneous generation theory.

Myiasis was part of a method of torture utilized by old Persians in scaphism, in which the victim was trapped between two boats, forced to eat milk and honey and exposed to flies, which produced serious anal lesions, and wasps (Lockwood, 2009).

Oestrus ovis (L.) already was referred in Biblical times as a dreaded pest of sheep, and the Greek physician Alexander Trallien referred to “sheep gad fly” in 560 CE.

Hernán Cortez, Conquistador of Mexico, branded his native prisoners on the cheek with a “G”, *Guerra* for war, sentencing many to death by infestation by screwworm attack. During second Opium War (1856–60), English prisoners died in prison after being tortured and attacked by maggots, provoking revenge by lord Elgin, which destroyed the beautiful Summer Palace, hurting Chinese people for a long time.

Myiasis (from ancient Greek *myia*, meaning fly) was proposed in 1840 by F.W. Hope for the infestation of tissues of living vertebrates by larvae of flies (Diptera), replacing scholichiasis, which referred to diseases caused by larvae of any insect.

Diptera is the fourth most diverse order of the Class Insecta, with approximately 150,000 species and 150 families described in two suborders: Nematocera and Brachycera (Brown, 2009). A little over 10% of dipteran families have at least one representative as obligatory or non-obligatory myiasis-causing. Once both types of parasitism, facultative or accidental, are nonspecific, it is impossible to quantify the exact number of species associated with this type of infestation worldwide, especially when parasitism occurs in the wild environment, where cases are rarely investigated.

In a recent review of reports of human myiasis, from 1997 to 2017, 464 cases were reported from 79 countries and caused by 41 species of flies (Bernhardt et al., 2018). A greater diversity on species in Asia, and a higher number of cases in the Americas and Caribbean were cited. Although the number of cases in Western Europe is very low, there could have been some lack of records, because the primary disease is the primary concern (Bernhardt et al., 2018) and there is some taboo (Hall et al., 2015).

The registry of parasitized vertebrates, both domestic and wild, is also numerous and includes amphibians, reptiles, birds, and mammals, including humans.

Biology of the myiasis-causing dipterans

The emergence of the habits causing myiasis and the transition to parasitism may have arisen from a broad adaptability process or by complete lack of specialization of a group to exploit the resources available in the nature (James, 1969).

According to Zumpt (1965), some species of flies that fed on corpses and feces began laying eggs on necrotic wounds or cavities of living animals due to the similarity of odors between such food resources—afterwards, some of these species have also developed the ability to invade living tissues, with the probable emergence of appropriate enzymes for this purpose. Thus, was drawn the first adaptive transition from saprophagy to parasitism.

Another proposed evolutionary route would be that larvae of some species, due to food scarcity, would have become facultative predators of other larvae. Subsequently, they were able to break the tegument of other larvae and suck their body fluids and, at a

later stage, started to pierce the skin of birds and mammals to suck blood. Finally, they would be able to go through the skin and lodge in the host's subcutaneous tissue, thus becoming an obligatory and hematophagous parasite (Zumpt, 1965).

From a parasitological point of view, i.e., taking into account the parasite-host relationship, myiasis can be classified as: (i) *obligatory*: refers to flies that feed exclusively on living tissues or other substances in the body of their hosts; in general, they exhibit parasitic specificity, since parasitism is the only resource that guarantees the perpetuation of the species involved (e.g., *Auchmeromyia* spp., *Chrysomya bezziana* (Villeneuve), *Cochliomyia homivorax* (Coquerel), *Cordylobia* spp. and *Lucilia* spp. (Calliphoridae); *Passeromyia* spp. and *Philornis* spp. (Muscidae); *Oestrus* spp., *Gasterophilus* spp., *Hypoderma* spp. and *Dermatobia hominis* Linnaeus Jr. (Oestridae); and *Wohlfahrtia* spp. and *Lepidodexia* spp. (Sarcophagidae)); (ii) *facultative*: when the larvae feed on the devitalized tissues (particularly the necrotic) of their hosts; in this case, their eating habit does not differ from that commonly observed in nature—where they are seen feeding on various sources of dead organic matter, therefore, it is a more opportunistic relationship than a parasite (among the most commonly involved fly families are Calliphoridae and Sarcophagidae); (iii) *accidental* or *pseudo-myiasis*: they can occur, but without any predictability, by unintentional ingestion of larvae or by the unusual penetration of larvae in the natural orifices of the host's body, causing disturbances of some gravity—among the most commonly involved fly families are Psychodidae (moth flies), Piophilidae (cheese skippers), Syrphidae (hover flies) and Phoridae (coffin flies).

Primary or *secondary* myiasis are also terms often used to define clinical conditions in which infestation begins without or with a pre-existing lesion, respectively. In addition, there are reports that take into account only the location where the larvae were found in the host, such as ocular, nasopharyngeal, anal, urogenital, enteric, cavitory, traumatic, furuncular, hematophagous, among others (Scholl et al., 2019).

Diptera are holometabolous, so their development, which undergoes a complete metamorphosis, includes four stages of life: egg, larva, pupa and adult. In general, myiasis-causing females lay eggs grouped in masses (Fig. 1A) (except *Oestrus ovis* and *Sarcophagidae* species that deposits larvae) on the edge of an open wound (Fig. 1B) (of whatever nature, e.g., umbilicus of young animals), in natural body openings or on/at the base of the hair (Fig. 1C) (in the last case, only *Hypoderma* spp.). It is not a rule, but infestations in natural openings are more frequent when there are injuries, poor hygiene, or in association with bacterial infections.

The larvae hatch within approximately 12–24 h and begin to feed on dead or living tissue (depending on the species of fly), reaching maturity within approximately 5–10 days (except oestrid flies, which can take 50–60 days to develop; Catts, 1982). Mature larvae leave the wound/body and burrow into loose soil to pupate. Adults emerge in 8–20 days and live on average 1–6 weeks, depending on the temperature and humidity. Females obtain protein to support egg production from wounds, excrement, or decaying animal matter, except oestrid flies that do not feed because their mouthparts are greatly reduced. Almost all females are attracted to the smell of the hosts' wound.

Although the biological cycle of myiasis-causing flies is similar, the behavior of some species to achieve a very successful parasitism can be quite peculiar. For example, *Cordylobia anthropophaga* (Blanchard) and *Auchmeromyia senegalensis* Macquart are found only in the Afrotropical region and the females of both genera laying the eggs on the soil, i.e., out of contact with the host (Zumpt, 1965). Newly hatched *Cordylobia* larvae seek a host and penetrate into their skin, causing a large abscess (= furuncle), while *Auchmeromyia* larvae remain free-living and repetitively seek blood from hosts, especially at night, causing only irritation. Females of bird parasites belongs to *Passeromyia* and *Philornis* genera lay eggs in nests and the newly hatched larvae, depending on the species, migrate to their hosts causing (furuncular) myiasis or only occasionally move to feed on blood.

Larvae of some species can also temporarily migrate to their hosts' cavities, or remain there until they reach larval maturity, causing so-called cavitory myiasis. Apparently, this behavior may increase the chance of offspring survival. Larvae of *Hypoderma bovis* (L.) and *H. lineatum* (Viller) (warble flies) migrate via the spinal column or oesophagus to skin, subsequently causing an abscess (Hall and Wall, 1995). When fully grown, larvae burrow out of the skin and drop into the ground to pupariate.

The route of *Oestrus ovis* (nasal bot flies) larvae in their host's body is not very long. Most of the time, the larvae can remain within the nasal sinus (place close to where the females originally deposit the larvae), and sometimes in the throat tissues (Fig. 1D and E). When the larvae are fully developed, they move down the nasal passage and drop into the soil to pupate. Parasitic species of *Lepidodexia*, which only parasitize anurans (Fig. 2A), have a behavior very similar to that observed for *O. ovis*.

The infestation by *Gasterophilus nasalis* (L.) or *G. intestinalis* de Geer (bot flies) starts in the mouth when the host (Li et al., 2019) licks the eggs that were deposited onto hairs by females, which are known for their persistence in flight around the hosts, causing a fear response. Larvae migrate to the stomach where they attach to the inner lining, but their bodies remain exposed to the stomach cavity. Their larvae are well adapted to such oxygen-poor environment. When ready to pupariate they release their hold on the wall of the gut, and are passed during defecation. Puparia are sometimes found in the dung.

Dermatobia hominis has a particular behavior related to the search for their hosts: females lay eggs on other insects, where they remain firmly fixed such that the insect-carrier (called phoretic) will carry them (Fig. 1F). To date, this dispersion strategy has not been observed among other myiasis-causing flies. *Musca domestica* L., *Stomoxys calcitrans* L., fanids and mosquitoes, probably due to the abundance and frequency with which they are observed living near humans and domestic animals, are among the most common *D. hominis* egg carriers (Guimarães and Papavero, 1999; Maia et al., 2018). Larvae hatch immediately in response to heat stimulation, CO₂ and probably vibratory movements of the host, after which they penetrate the subcutaneous tissue, individually, remaining at the same site until development is complete. This infestation is also referred as furuncular myiasis (Fig. 2B).

Regarding parasitic specificity, undoubtedly, oestrids (except *D. hominis*) and some species belonging to the genera *Philornis*, *Passeromyia* (Muscidae) and *Lepidodexia* (Sarcophagidae) exhibit the highest host-parasite specificity, in contrast to the low specificity observed in other families of myiasis-causing flies such as Calliphoridae and Sarcophagidae (Hall and Wall, 1995).



Fig. 1 Behavior and strategies of myiasis-causing flies to reach their hosts. (A) Blowflies females usually lay eggs, approximately 1 mm long each, grouped in masses to increase the offspring's chance of survival; (B) an example of an open wound on a cow's mammary gland: blood and injured tissue can attract flies to oviposition; (C) eggs laid at the base of the ox hair by *Hypoderma lineatum*; (D) and (E) *Oestrus ovis* larvae remain protected within the nasal sinus and sometimes in the tissues of their hosts' throat as they develop; (F) *Dermatitis hominis* eggs on *Fannia punctipennis*: the phoresy favors the wide dispersion of the "berne" fly. (B) Courtesy of Gustavo A. Faria, UNICAMP, Brazil; (C) Courtesy of Dr. Lyle J. Buss, University of Florida, USA; (D) and (E) Courtesy of Prof. Bruna F. Silva PhD, UNIPLAC, Brazil.

For example, the obligatory parasite *Wohlfahrtia magnifica* (Schiner) may deposit larvae on several healthy hosts without any predisposing conditions (Carnevali et al., 2019).

Global warming certainly is influencing distribution of myiasis-causing flies and intensity of attacks. Not only will the rise or temperatures, but also the shortening and softening of winters in the temperate regions increase the myiasis problem (Pitts and Wall, 2005).

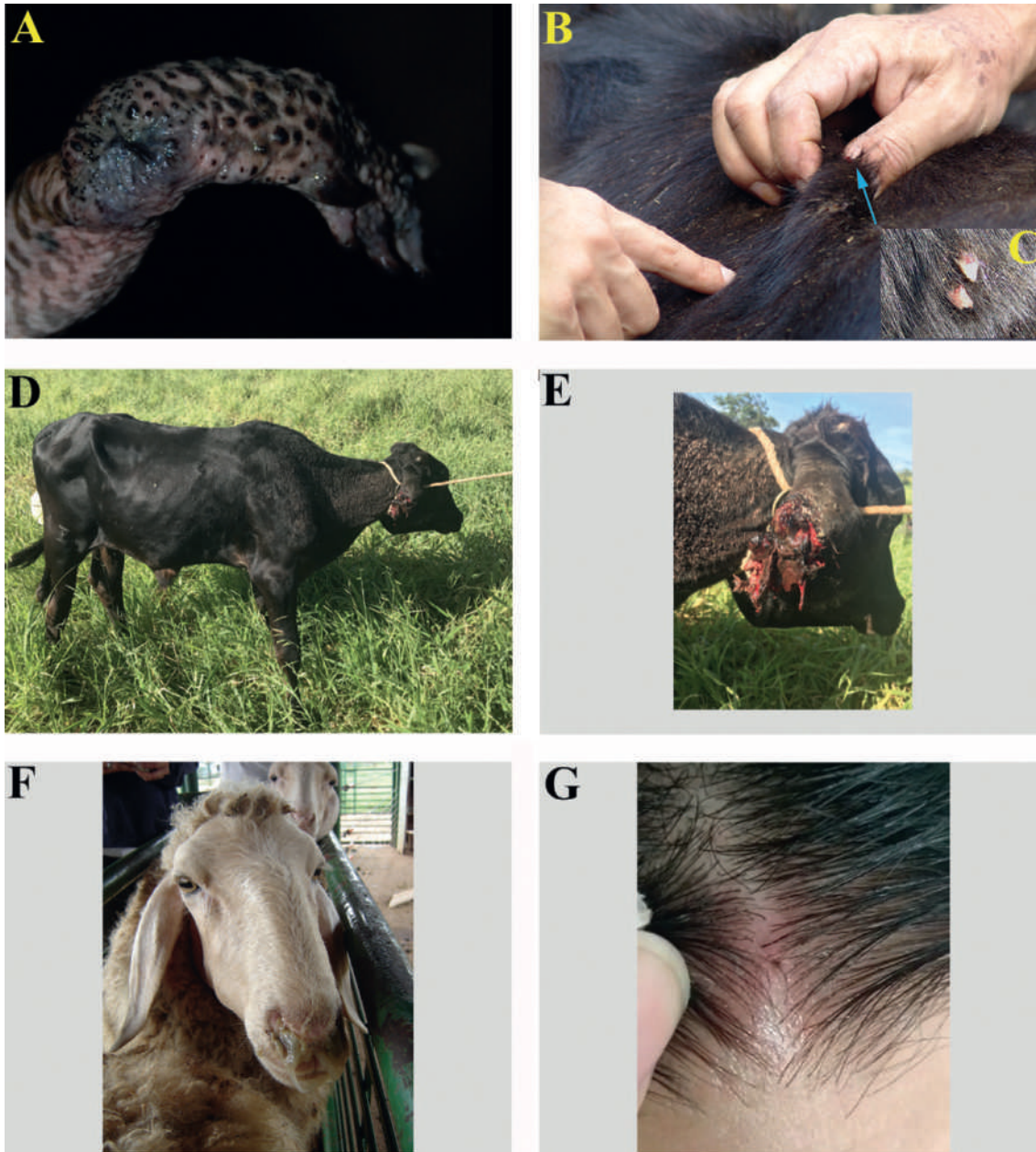


Fig. 2 Main clinical pictures and pathologies observed in the myiasis by different species. (A) furuncular or cutaneous myiasis in *Rhinella jimi* (cane toad) (Anura) by a species of *Lepidodexia* sp. (Sarcophagidae); (B) and (C) furuncular myiasis by *Dermatobia hominis* leading to perforations in the cattle leather; (D) and (E) traumatic myiasis in livestock by *Cochliomyia hominivorax*—note extensive damage to the calf's ear in (C); (F) *Oestrus ovis* causing severe rhinitis and sinusitis in goat; (G) furuncular lesion with little severity in human head by *Cordylobia anthropophaga*. (A) Courtesy of Prof. Luis F. T. R. Pereira PhD, UNICAMP, Brazil; (C) Courtesy of Gustavo A. Faria, UNICAMP, Brazil; (F) Courtesy of Prof. Bruna F. Silva PhD, UNIPLAC, Brazil.

Pathology, distribution and economic importance

There are three basic forms of infestation that may also indicate, in part, the pathological processes resulting from parasitism on their hosts: (i) traumatic (sometimes called wound myiasis); (ii) furuncular; and (iii) cavitory.

Traumatic myiasis (Fig. 2D) is very serious, and it is usual, in large infestations, to cause the death of the hosts. Although the larvae remain most of the time only in the host's skin or integument (there are those that can occupy the natural cavities of the body, but still do not go very deep), they can cause extensive damage (= trauma) due to the voracity of most species associated with this type of myiasis ("eating machines"). In this way, it is important to highlight the difference between species that cause obligatory myiasis, which feed on living tissues, and species that cause facultative myiasis, which rarely cross the borders of colonized wounds,

since they stop eating when the devitalized tissue, its only food source, ceases. Among the main species that cause traumatic myiasis are: *Chrysomya bezziana*, *Cochliomyia hominivorax*, *Lucilia cuprina* (Wiedemann), *Lucilia sericata* (Meigen) and *Wohlfahrtia magnifica*.

Chrysomya bezziana, also referred as the Old World screwworm, is widely distributed in tropical regions of the Old World, particularly in the Oriental region. It parasites a wide range of mammals; in Africa, it attacks lions, impalas, rhinos and elephants, including humans and livestock (cattle, sheep, dogs, and pigs) (Zumpt, 1965). Other species of *Chrysomya* found around the world rarely cause myiasis; when this occurs, they are restricted to facultative parasitism.

Cochliomyia hominivorax, also referred as the New World screwworm, is confined to the Nearctic and Neotropical regions. This species parasites mainly the livestock (Fig. 2E) that live close to forested areas, causing economic losses that exceed US\$ 300 million/year (Grisi et al., 2014). Neonatal calves and post-parturient cows are especially susceptible to infestation.

Before European invasion of the American continent, introducing cattle, other mammals were attacked and continued to be hosts of this fly. Killing of 80% of white-tailed deers (*Odocoileus virginianus texanus*) was reported, in bad years, in the United States, and opossums and jackrabbits used also to be affected in that country, before its eradication (see Spradberry, 1994 for a review).

Unlike other flies that lay, on average, 50 eggs at a time, adult females of *C. hominivorax* can initially lay more than 100 eggs. The odor of a wound associated with that of a primary infestation can attract more females, both from *C. hominivorax* and other species of opportunistic flies, increasing the severity of the initial condition. For example, an infestation of 3000 larvae at once can cause the host to die within a few days.

Some diseases or comorbidities can also promote circumstances that will stimulate the attractiveness of females of *C. hominivorax* for oviposition. Thyssen et al. (2012) reported a case of high infestation in an elderly patient with periodontitis and a vulnerable state of health, which culminated in the loss of part of the palate and all teeth in the upper arch. Myiasis by *C. hominivorax*, in this particular case, was used as evidence of maltreatment, given that the patient was dependent on constant care.

In addition, many cases of human infestation have been reported. *Cochliomyia hominivorax* can cause extensive lesions on several regions of the body, including the face. Jean Charles Coquerel, which described this fly in 1858, had already noticed these facial lesions in Devil Island's prisoners. Oral and maxillofacial infestations, though less frequent than in other localizations, are frequently associated to teeth extraction, mouth breathing, *cancrum oris*, among others, developing to frightening situations (e.g., Antunes et al., 2011).

The genus *Lucilia* contains a large number of species distributed worldwide (Whitworth, 2014). Most species are saprophagous and for this reason are mainly involved with cases of facultative myiasis. Both *L. sericata* and *L. cuprina* play an important role in the obligatory parasitism of woolly animal breeds. Their larvae can quickly cause extensive lesions under the skin and inflammatory processes that cause a lot of irritation; in severe or untreated cases, it can lead the host to death. *Lucilia sericata*, known as a sheep strike, causes serious economic problems for sheep farmers in Africa, Australia, Northern Europe and New Zealand (Snoep et al., 2002). It is estimated that several million dollars are spent annually in loss of animals and attempt to control flies. Since the 1930s, *Lucilia cuprina* has been the most dominant causative agent of myiasis in Australia, and also causes economic loss of some significance in New Zealand (Heath and Bishop, 2006; James, 2006).

Why is *L. cuprina* not a problem for animal health in other parts of the world? To elucidate this question, Stevens and Wall (1996) studied the genetics basis of *L. cuprina* collected in Africa, Europe, Australasia, North America and the islands of Hawaii. The results supported the existence of an intra-specific genetic variation in *L. cuprina*, possibly related to geographical isolation (Stevens and Wall, 1997b) and strong climatic influence determining which species would become predominant in different geographic regions (Stevens and Wall, 1997b). Thus, in an independent evolution of the parasitic habit (Stevens and Wall, 1997a), *L. cuprina cuprina*, distributed in Neotropical, Oriental and Southern Nearctic regions, would be responsible for facultative myiasis, while *L. cuprina dorsalis*, found in Australasian, East and Sub-Saharan Afro tropical regions, a causative agent of obligatory myiasis.

Wohlfahrtia species infest many species of livestock (e.g., cattle, horses, sheep, goat, pigs) and other animals (e.g., donkeys, camels, dogs, cats, rodents and birds). Human infestations, particularly in the nose or eyes, are relatively rare. Both *Wohlfahrtia magnifica* and *W. nuba* Wiedemann, distributed in the Palearctic and Oriental regions, are responsible for traumatic myiasis, but *W. magnifica* is one of the most important obligatory parasites (Farkas et al., 2009), since *W. nuba* is associated with facultative myiasis.

The recent surging of infestation by *W. magnifica* in sheep in central Italy is possibly associated to introduction of Merino sheep, more susceptible to infestation (Giangaspero et al., 2011). Larvae of this species can cause severe damage to hosts, if not properly treated, since they grow quickly and are quite voracious (Carnevali et al., 2019). Consequently, large economic losses may be unavoidable.

In furuncular and cavitary myiasis, the larvae are not seen outside the body of their hosts. In the first case, the larvae penetrate the healthy skin and a furuncle develops, while in the second case, the larvae reach the internal cavities of the host's body, mainly the digestive and urinary tracts. Unlike traumatic myiasis, the larvae migrate very little (through the cavities) or nothing (when they form the furuncles) and feed basically on body fluids, but not on tissues. Thus, the damage caused by parasitism is strongly associated with the number of infestations per host. There are exceptions, but in general, parasitized individuals do not die. Among the main species that cause furuncular and cavitary myiasis are: *Dermatobia hominis*, *Gasterophilus nasalis*, *G. intestinalis*, *Hypoderma bovis*, *H. lineatum*, *Oestrus ovis* and *Cordylobia anthropophaga*. All species listed here are obligatory parasites.

Dermatobia hominis, which can also be called human botfly, berne (in Brazil) or tórsalo (in Spanish), occurs in the Neotropical region. This species parasites mainly cattle, but also several other mammals, including humans. For example, large infestations cause multiple skin lesions (boils) in cattle, whose devaluation of the leather can cause serious financial losses, estimated at ca. U\$

280 million/year in Brazil (Grisi et al., 2014). Exportation of leather produces revenues of 2.48 billion dollars to Brazil, but 40–89% of it have perforations (Fig. 2C) caused by this fly (Gomes, 2002).

Gasterophilus nasalis and *G. intestinalis* can be found worldwide and live exclusively in the alimentary canal of horses. Infestations rarely cause problems. Both species in heavy infestations can cause gastric ulceration, peritonitis, gastroesophageal reflux, splenitis and pleuritis (Uzal and Diab, 2015).

Hypoderma bovis and *H. lineatum* infest cattle in the Palearctic, Nearctic and Neotropical regions; and sometimes horses or sheep, but the life cycle is not completed in the latter. Both *Hypoderma* species can cause a great amount of damage to the health of the cattle and to their hides, similar to what is observed for *D. hominis*, though these flies have been eradicated in many countries in Europe.

Oestrus ovis parasites sheep and goats worldwide, causing rhinitis and sinusitis, sometimes severe (Fig. 2F); encephalomyelitis was observed in a goat. This parasite fly rarely is found in humans. In rural areas, females can deposit larvae in the conjunctival sac of humans, particularly those who live in close contact with sheep (Angulo-Valadez et al., 2010). Infestation has also been reported in llamas and alpacas, the South American camelids (Angulo-Valadez et al., 2010).

Cordylobia anthropophaga infests humans (Fig. 2G), dogs, cats and some wild mammals. This fly is found in the Afrotropical region, though an autochthonous human case was reported in Portugal (Curtis et al., 2015). The pathology of *C. anthropophaga* infestations is generally less severe than those caused by *D. hominis*, with some swelling and infection (Hall and Wall, 1995). Zumpt (1965) reported that adult females can lay eggs on clothes hanging to dry, which explains infestations in which an individual does not observe flies flying around them.

There are other intriguing species of myiasis-causing flies and unfortunately little studied such as those of the genera *Passeromyia* (Palearctic and mostly in the Old World) and *Philornis* (in the New World), parasites of wild and captive birds; and *Lepidodexia* spp. (in Neotropical region) and *Lucilia bufonivora* (in North West Europe and Canada), both parasitizing anuran and sometimes causing relatively high mortality rates among some hosts (Weddelling and Kordges, 2008; Grzywacz et al., 2014; Arias-Robledo et al., 2018; Mello-Patiu, 2019). For example, *Philornis downsi* Dodge & Aitken, recently introduced to Galápagos Islands, has drawn worldwide attention for being responsible for the deaths of almost 100% of Darwin's finch chicks in recent years (McNew and Clayton, 2018).

Several abiotic and biotic factors can affect how a given animal species is distributed, but in the particular case of myiasis-causing flies it is important to be aware of the fact that some species can be transported from place to place together with their hosts, or accidentally, by plane and boats. For example, three *Chrysomya* species—*C. albiceps* (Wiedemann), *C. megacephala* (Fabricius) and *C. putoria* (Wiedemann)—became widely distributed in the American continent after the independence of Angola and Mozambique, with massive migration of Portuguese people, and their domestic animals, to this continent (Guimarães et al., 1978, 1979; Baumgartner and Greenberg, 1984; Wells, 1991). In the 1980s, a dog infested by *C. hominivorax* was found in France (Chermette, 1989); this same species was introduced and spread over almost 25,000 km² in Libya (Lindquist et al., 1992). The invasion of Libya by *C. hominivorax* could have been catastrophic, if it had followed Nile valley to sub-Saharan Africa (Woodford, 1992). According to an analysis by CLIMEX, potential distribution of *C. bezziana* in the American continent would be the same of *C. hominivorax*, and it could invade even the southern regions of Australia, like New South Wales (Sutherst et al., 1989).

Diagnosis

Before removing the larvae, it is recommended to identify and record the type of pathology associated with the myiasis in question, whether traumatic or furuncular. After proper cleaning of the lesions, collect the larvae, if necessary using tweezers and transfer them to a vial containing 70° GL ethanol or formalin. Although information on patients' previous travels can be useful in identifying unusual myiasis, the larvae must be sent to specialists for identification.

In general, the larvae can be identified mainly through characteristics such as body shape, size, presence and pattern of distribution of spines in the integument and conformation of respiratory stigmas (Fig. 3; Marcondes and Thyssen, 2017). Publications such as those by Zumpt (1965), Guimarães and Papavero (1999) and Szpila et al. (2014, 2015) can be very useful for identification, since they bring keys and/or illustrations of anatomical structures with diagnostic value.

For the identification of cavity myiasis, like those of *Hypoderma* spp. and *Oestrus ovis*, serological and molecular methods have been successfully utilized (Otranto et al., 2004), including ELISA for *Gasterophilus* spp. (Migueléiz et al., 2016). A molecular test developed for larvae of *Rhinoestrus* spp. infesting nasal cavities, synuses and pharynx of equids was also proposed for other myiasis-causing flies (Traversa and Otranto, 2006). Molecular differentiation has been utilized for *Hypoderma* spp. from Turkey and Pakistan (Ahmed et al., 2017).

PCR in nasal mucus produced respectively 100% of sensitivity and specificity for goats, while rhinoscopy produced 100% of sensitivity and specificity for goats, but 100% of specificity and only 81% of sensitivity for sheep for *O. ovis* infestation (Ipek and Altan, 2017). This molecular technique permits sure diagnosis without killing the host.

Recently, *D. hominis* had its Cytochrome Oxidase and ITS-2 genes characterized (Martínez-Hernández et al., 2019; Touissant-Caire et al., 2018) for diagnostic purposes. In addition, Touissant-Caire et al. (2018) found a 98% similarity between individuals from Mexico and other locations in South America. Larvae of this species can be easily identified through traditional morphological examination, but when it comes to a case involving the encounter of a non-endemic species of a region it is advisable to conduct studies such as these integrating morphological and molecular data to avoid misidentification. Grella et al. (2015) using the integrated taxonomy demonstrated that a non-endemic species of *Chrysomya* had been mistakenly registered for Brazil.

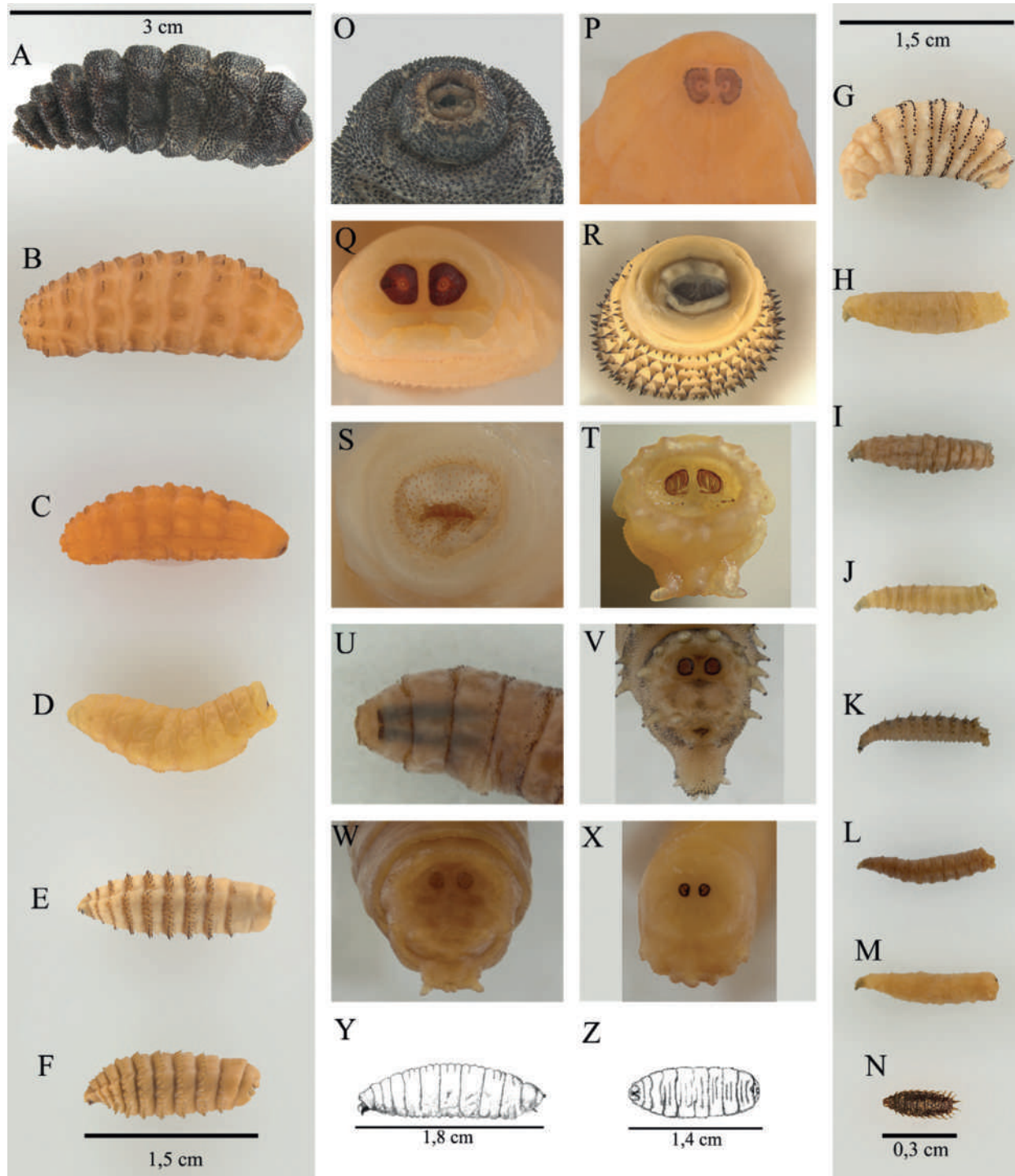


Fig. 3 Larvae of several myiasis-causing flies. From A to N, U, Y and Z, lateral or dorsal view. From O to T and from V to X, posterior view, showing respiratory spiracles with diagnostic value. In: (A) and (O) *Cuterebra* sp.; (B) *Hypoderma bovis*; (C) and (P) *Hypoderma lineatum*; (D) and (Q) *Oestrus ovis*; (E) and (R) *Gasterophilus intestinalis*; (F) *Gasterophilus nasalis*; (G) and (S) *Dermatobia hominis*; (H) and (T) *Sarcophaga* sp. (Sarcophagidae); (I) and (U) *Cochliomyia hominivorax*, showing the pigmented tracheas; (J) *Chrysomya megacephala*; (K) and (V) *Chrysomya albiceps*; (L) and (W) *Lucilia eximia*; (M) and (X) muscid fly; (N) *Fannia* sp.; (Y) *Auchmeromyia* sp.; (Z) *Cordylobia anthropophaga*. From Marcondes CB and Thyssen PJ (2017) Flies. In *Arthropod Borne Diseases*, pp. 475–502. Cham: Springer, reused with permission from Springer, 2020 (Richard Lansing). *Hypoderma* figures—http://entnemdept.ufl.edu/creatures/livestock/cattle_grub.htm.

Bernhardt et al. (2018) pointed out several problems with misidentification of myiasis-causing species and with the increase in the number of cases associated with humans in Europe. They also stressed how important the correct identification is to improve the monitoring and treatment of parasitized individuals.

The chance of misidentification of a species that is parasitizing a traveler is relatively high, when disregarding information about the regions visited. Probably, this is the case of infestation by *D. hominis* in a traveler recently visiting Africa and of a report of autochthonous cases from Saudi Arabia, in whose southwestern region *C. anthropophaga* had already been reported, and a case of infestation by *C. hominivorax* in the North of India. However, this does not mean the distribution of myiasis-causing flies cannot change, as observed by an above cited report of autochthonous infestation by *C. anthropophaga* in Portugal, diagnosed on the return of the patient to United Kingdom, and practically excluding contamination by clothes (Curtis et al., 2015).

Although furuncular myiasis is usually caused in Africa by *C. anthropophaga*, a human case of infestation by *Cordylobia rodhaini* Gedoelst (Lund's fly) in a man returning to Italy from Uganda was reported, and a review of 25 cases reported since 1902 was provided (Pezzi et al., 2015). This species usually parasitizes monkeys, rodents and small antelopes, emphasizing the need of accurate identification. Another issue to be considered is that many infestations may have been unreported.

Treatment and prevention

If a traumatic myiasis in humans is in place, all larvae must be removed from the wound or orifices, which need to be cleaned and disinfected afterwards. Careful treatment and protection of wounds in the skin or mucosal orifices are essential to prevent infestation. Rooms of clinics and hospitals where wounded or recovering after surgical procedure patients are maintained need to be protected by nets on windows and doors, or bed nets need to be utilized.

In domestic animals, some drugs have been utilized, mainly ivermectin and other avermectins, for treatment or prevention against several myiasis-causing flies. For example, 110 artificially wounded steers, castrated bull calves and newborn calves were divided in two groups: 55 of them treated by 200 µg/kg of ivermectin were totally protected from infestation by *C. hominivorax*, while from 25% to 53% of 55 control animals in similar conditions animals became infested (Anziani and Loreficce, 1993). However, a potential resistance of *D. hominis* to ivermectin and moxidectin in the usual dose (200 µg/kg) was reported in cattle, with some larvae surviving to treatment (Neves et al., 2015).

Control of myiasis in animals have also been done through the use of chemical compounds, mainly organophosphorus insecticides, macrocyclic lactones and, more recently, a foamy spray based on spinosad. For example, dicyclanil is used as insect growth regulator on castration wounds in cattle and has been quite effective in preventing infestations by *C. bezziana*.

In Italy, the efficacy of a plant-derived formulation, composed of neem oil (*Azadirachta indica* A. Juss.) and the oily extract of *Hypericum perforatum* (L.) flowers, has been investigated against *W. magnifica* with successful results. Forty-four infested domestic animals, among which sheep, bovine, goat, swine, equine, canine and rabbit, were treated and all wounds healed between 10 and 32 days, without further infestation (Camevali et al., 2019). Other researches have been conducted to test the use of essential oils and natural plant extracts to kill larvae of *Lucilia* spp. (Khater et al., 2011).

For the treatment of furuncular myiasis (e.g., by *D. hominis* or *C. anthropophaga*), one proposed method is asphyxiation of the larva for subsequent removal of the insect with the aid of a tweezer. To remove larvae of *O. ovis*, cavities in the head of host need to be washed, but this may not always be effective.

Caution is recommended when drying clothes in the environment for avoidance of *C. anthropophaga*; and it is also recommended that people sleep on beds (or hammocks) to reduce parasitism by *Auchmeromyia* sp.

Control

Inspection and treatment of all infested animals are too time-consuming. In addition, insecticides and traps have not been effective for the control of several parasite species due to short time of residence on the host and short generation time. *Cochliomyia hominivorax* was the first insect to be controlled and eradicated using sterile insect release, in a program known as Sterile Insect Technique (S.I.T.), proposed by Knipling (1955). This technique consists of releasing sterilized males on the field in numbers sufficient to overwhelm the wild population, with the idea that sterilized individuals will compete with wild normal males. Successful results have been obtained in the United States and in several Caribbean islands (Spradberry, 1994; Guimarães and Papavero, 1999). However, dogs, pigs and deers have recently been found in Florida (mainland and Florida Keys) (Hennessey et al., 2019).

Studies are currently underway to develop a synthetic odor bait to attract female *C. hominivorax*. It could be used to monitor *C. hominivorax*, besides helping to reduce the population in infested regions (Cork and Hall, 2007). For the control of *L. cuprina* and *L. sericata*, the removal of skin from the breech in certain breeds of sheep and tail-docking are the most effective non-chemical method for control, by preventing favorable ovipositing sites. However, genetic methods, by selection of breeding more resistant to sheep strike and adequate techniques of mulesing and tail-cutting, have been proposed to replace such time-consuming methods (James, 2006). Treatment of sheep for helminthic infection, to prevent diarrhea, also is important.

In England, tests conducted on sheep farms have demonstrated the effectiveness of using traps for catching adults of *L. sericata* for reducing flies in the environment, and consequently reducing the number of attacks on animals (Broughan and Wall, 2006). The

same study showed that there was no significant difference between using traps as a single strategy and using traps combined with chemical treatment of animals. This finding appears to be a promising strategy for reducing insect populations without promoting selection of resistance to the insecticide in the medium and long term.

Due to the complexity of the *D. hominis* life cycle, efficient strategies for its control are not available. Guimarães and Papavero (1999) pointed out that open formations seem to be a barrier to the dispersion of *D. hominis*, since this species is more abundantly found on the margins of primitive or secondary forests, gallery forests or eucalyptus plantations.

Vaccination of livestock has been explored both in vitro and in vivo against some species of myiasis-causing flies, but this issue is still unresolved.

Additional aspects of myiasis-causing flies

Some myths have been associated to myiasis. Carcasses of *Cuterebra*-infested squirrels and rabbits are usually discarded in United States, supposing these animals are unfit to eat, but in the Arctic and sub-Arctic cultures not only the meat of reindeer or caribou infested by *Hypoderma tarandi* is regularly eaten, and these larvae (located on animal's back) are considered a delicacy by the natives (Scholl et al., 2019). *Cephenemyia* adults were considered to fly at very high speed, supposedly to attack deer, but there are no indications of speeds higher than 28 km/h, and they attack their hosts when they are still (Scholl et al., 2019). Larvae of *Cuterebra emasculator* were supposed to consume the testis and emasculate their squirrel and chipmunk hosts, but they only prevent the seasonal descent of gonads into the scrotum, due to the presence in the scrotal sack.

The horror of harboring or viewing maggots can cause reactions severe enough to result in mental disorders and self-mutilation (Clarke, 2013). Delusional parasitosis (Ekbom syndrome) includes the so-called “matchbox sign,” in which the patient present supposed parasites, frequently incriminating maggots. However, some patients may be correct on the allegations, as an Indian patient, which had many larvae of *Musca domestica* in his large gut.

Adapted to feed and survive in contaminated environments, maggots have the capacity to kill many types of bacteria. Observation of cleaning and improvement of wounds of several origins through infestation by larvae of secondary myiasis-causers stimulated the development of laboratory colonies, and sterile larvae have been purposely applied on wounds. Mostly *Lucilia* spp., and recently *Chrysomya megacephala* (Pinheiro et al., 2015) and *Cochliomyia macellaria* (Nassu and Thyssen, 2015; Masiero et al., 2019) have been utilized, but several other species have been tested for this very efficient maggot debridement therapy (MDT). This therapy, used to heal wounds quickly with minimal scarring, has been utilized in several countries, mostly in Europe, Israel and United States, but also in Brazil, Colombia, Chile, Egypt, Australia etc. (see: www.BTERFoundation.org) (Fleischmann et al., 2004; Marcondes, 2006).

Determination of time and conditions since death is very important, mostly in cases of suspect of crime. Since this determination is difficult, infestation of corpses by insects, in particular by dipterans, can provide useful information (Thyssen et al., 2018), as part of forensic entomology.

From Spradberry (1994):

It is doubtful that the mind of man could create a more vile (sic) scene than that of worms consuming the live flesh of one's body. The imagination almost refuses—particularly in this day and age—to conjure up the horrendous pain and outright revulsion that must come to a person infested with a writhing, seething mass of worms steadily tearing and consuming his flesh.

From C. E. Scruggs (1975).

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Keds and Bat Flies (Hippoboscidae, Nycteribiidae and Streblidae)

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Introduction

The order Diptera, more than the other orders, has many families characterized by an evolutionary adaptation in the trophic behaviour: the hematophagy (Petersen et al., 2007). Representatives of these groups have modified the mouthparts in piercing-sucking appendages to feed on the blood of many vertebrate species. Thus, the mouth apparatus has evolved in different types influenced by the feeding behavioural mechanisms: species that insert their thin appendages into the skin of the host reaching the capillary net, injecting saliva and taping the blood, termed solenophages, or species that by the external teeth-like structures scratch the host skin and feed on the spilled blood termed pool feeders (Afonso et al., 2012; Mehlhorn, 2018).

Mosquitoes, which belong to the suborder Nematocera, are important vectors that carry the most severe diseases plaguing humans. A major example is represented by malaria, caused by *Plasmodium* parasites vectored by *Anopheles* spp. (Benelli and Beier, 2017), a plague yearly affecting more than 2 million of people and has produced 405,000 deaths worldwide in 2018 (WHO, 2019) of note, *Aedes* and *Culex* vectors are crucial in spreading arboviruses. For example, *Aedes aegypti* and *Aedes albopictus* act as vectors of dengue, chikungunya, and Zika in humans (Powell, 2018; Benelli et al., 2020).

In the suborder Brachycera there are important flies acting as mechanical transmitters of pathogens (e.g., bacteria) and parasites (e.g., protozoan cysts and helminth eggs), and some species play an important role also as intermediate host or biological transmitters. Furthermore, some are blood-sucking and attack both humans and animals (Iwasa, 1983; Onmaz et al., 2013). Acalyptrate flies of the family Psychodidae, Simuliidae Ceratopogonidae are important vectors of diseases such as haemosporidian parasites or bacteria of genus *Bartonella* as well as filarial nematodes (Durden and Mullen, 2019; Santiago-Alarcon et al., 2012; Afonso et al., 2012). In high Calyptrate Diptera, the family Muscidae has some important hematophagous species such as *Stomoxys calcitrans*, a worldwide distributed fly responsible of considerable economic losses in dairy farms. Flies generate direct nuisance such annoyance and blood loss in cattle, and some representatives are implicated as mechanical vectors of important viruses like West Nile Fever Virus (WNV) or the African Swine Fever Virus (ASFV) (Baldacchino et al., 2013). In the superfamily Hippoboscoidea, the family Glossinidae includes representatives responsible for the transmission of some *Trypanosoma* spp. that cause the Human African trypanosomiasis or the African animal trypanosomiasis, which cause the “sleeping disease” and the “nagana” respectively in humans and in livestock (Vreysen et al., 2013). Finally, Hippoboscidae (ked and louse flies), Streblidae and Nycteribiidae (bat flies)

are implicated in the horizontal and vertical transmission of protozoa, bacteria as well viruses and nematodes to wildlife, and often responsible of direct damage to humans causing allergic reactions by their bite (Reeves and Lloyd, 2019). The present chapter reviews current knowledge about these interesting families, describing the biology, ecology, morphology and behaviour of some species which are important from veterinary and medical point of view, and providing insights on current control tools available in the Integrated Pest/Vector Management scenario.

The superfamily Hippoboscoidea

Hippoboscoidea is a superfamily of hematophagous ectoparasites belonging to the order Diptera and includes four recognized family level-taxa: Glossinidae, Hippoboscidae, Nycteribiidae and Streblidae. All of these families were grouped under the name Puppipara because they are viviparous and deposit larvae that quickly pupate. Currently, the name Puppipara is recognized as being improper because females lay fully developed larvae instead of pupae. For this reason, it is incorrect to consider this superfamily as pupiparous but larviparous (Reeves and Lloyd, 2019).

Hippoboscidae commonly are referred to as keds or deer keds, or as feather, bird, or louse flies, while Nycteribiidae and Streblidae are known as bat, flat or spider flies (Reeves and Lloyd, 2019). The family Hippoboscidae includes several parasites of mammals and birds, while the other two families are exclusively limited to bats (Hutson, 1984).

Members of Hippoboscoidea are characterized by several peculiar morphological and biological traits. Their reproductive strategy is adenotrophic viviparity: larvae are contained singly in the mother's uterus and are nourished from structures named "milk-glands," well-described by Benoit, et al., 2015. When a third-instar larva is fully developed, it is deposited by the female and shortly thereafter pupates. Females larviposit in different substrates depending on the species. Some flies deposit larvae in nests, others on the host animal fur, but some attack larvae to roost walls or glue them to the host wool. Fully-grown larva is legless with a barrel-like soft body (Maa and Peterson, 1987), while the puparium is similar to the mature larva with more sclerotized cuticle (Fig. 1). In many hippoboscids, the emergence of a new generation occurs simultaneously in a determined range with the year, with pupae able to overwinter in diapause, depending on the larviposition period (Bequaert, 1953; Hutson, 1984).

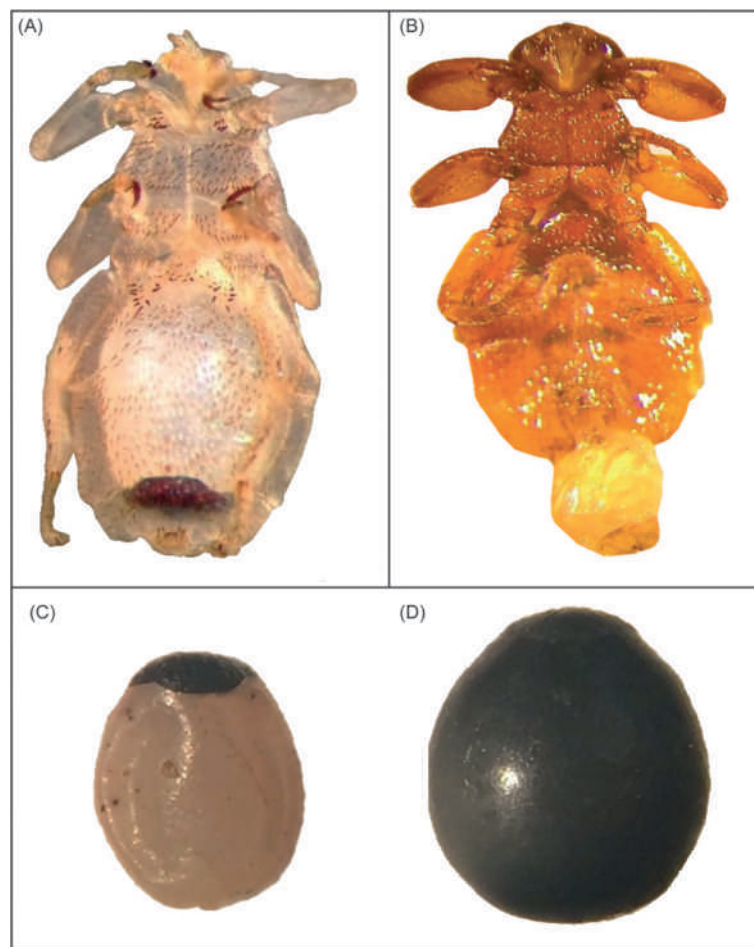


Fig. 1 *Lipoptena cervi*. Gravid female (artificially depigmented) showing a mature larva inside the uterus (A); female larvipositing (B); barrel-like larva just out of the mother's body (C); typical dark sclerotized puparium (D).

In the adult stages, all members of Hippoboscoidea are obligate and permanent blood-sucking ectoparasites, feeding on various host species. Generally, a fly is specialized in attacking a specific host or a few related groups, but occasionally can feed on other species, called “accidental hosts,” which are used as food source but on which reproduction does not occur (Maa and Peterson, 1987). Within the superfamily, species exhibit varying parasitism levels. Some species are monoxenous, meaning they are associated exclusively with a single species; others are stenoxenous, and can infest a phylogenetically related group, or oligoxenous if they parasite a limited number of hosts restricted by ecological factors only; others instead are polyxenous and are able to live on a wide range of species (Hutson, 1984; Lourenço and Palmeirim, 2008).

To live in close association with hosts, these ectoparasites have co-evolved with them adapting morphologically and physiologically (Guerin et al., 2000). For instance, parasites of birds synchronize their life cycle with host seasonality: adults die when birds migrate, and the emergence of a new generation coincides with host return (Bequaert, 1953). Similarly, aggregations of bat flies live in caves together with their gregarious hosts. When a bat exits the cave to search for food, female parasites leave the host to deposit pupae on the walls (Peterson and Wenzel, 1987). Finally, deer keds are strictly dependent on a single suitable host specimen and spend the entire life on the fur of the host (Bequaert, 1942).

Representatives of Hippoboscoidea are adapted morphologically for ectoparasitic life and display numerous structures that allow them to efficiently live together with their hosts (Petersen et al., 2007). Their body is dorsoventrally flattened with several reduced and fused regions and a sclerotized exoskeleton that is able to withstand mechanical stresses caused by host movements. Females show a strong reduction or disappearance of abdominal sternites with large intersegmental membranous areas allowing the develop of the larva in the mother's uterus (Fig. 2). Additionally, parasites have peculiar adhesion organs that permit the adherence to animals (Maa and Peterson, 1987; Petersen et al., 2018; Reeves and Lloyd, 2019). Among the three families, Nycteribiidae are modified morphologically to the extent that they no longer resemble most Diptera, while Streblidae are unusually variable in features and structures between their species (Hutson, 1984; Petersen et al., 2007). The head is prognathous in Hippoboscidae and Streblidae, while in Nycteribiidae it is protruding from the dorsal thoracic area (Maa and Peterson, 1987; Dick and Patterson, 2006). Both sexes of Hippoboscoidea are hematophagous and feed using highly adapted mouthparts consisting of a slender proboscis embraced in two concave, bristled, and sclerotized palpi. The alimentary canal is formed by the union of labrum, hypopharynx, and labium (theca sensu Snodgrass, 1943). Labella end the proboscis and bear at the tip different kinds of teeth and sensilla (Fig. 3) (Snodgrass, 1943; Peterson and Wenzel, 1987; Wenzel and Peterson, 1987; Andreani et al., 2019).

Some species are wingless in the adult stage, e.g., nycteribiids or some hippoboscids such the sheep ked, *Melophagus ovinus* (Linnaeus, 1758), while others are caducous-winged and shed wings once settled on a suitable host, as in the subfamily *Lipopteninae* of Hippoboscidae. Finally, others, instead, maintain either reduced or fully developed wings, like in Streblidae and some species of the Hippoboscidae family, such as *Hippobosca equina* (Linnaeus, 1758) or the louse fly *Pseudolynchia canariensis* (Macquart, 1840) (Hutson, 1984; Liu et al., 2019).

The lifecycles of some Hippoboscoidea have been drawn and presented below (Figs. 4–7).

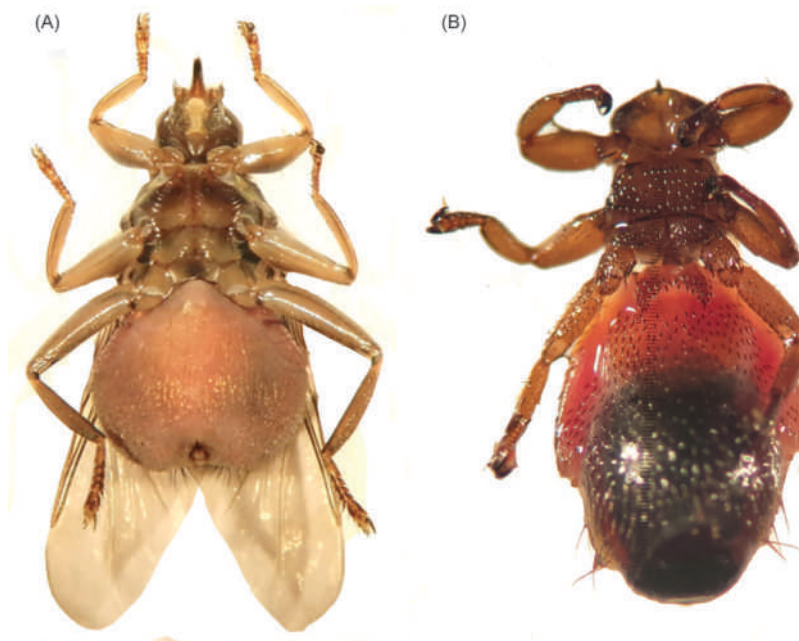


Fig. 2 Gravid females of *Pseudolynchia canariensis* (A) and *Lipoptena mazame* (B). Note the disappearance of abdominal sternites in both species allowing the development of the larva.

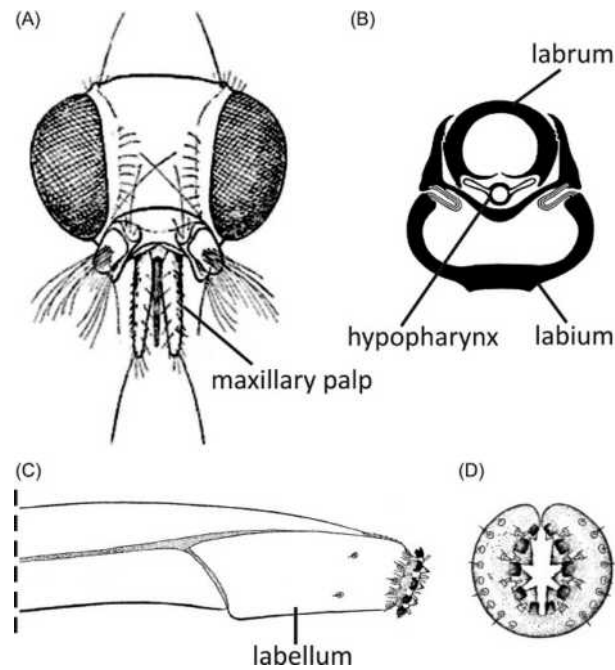


Fig. 3 Head and mouthparts of Hippoboscidae. (A) Head; (B) Cross-section of mouth appendages; (C) proboscis; (D) details of the proboscis tip. Modified from Jobling BA (1926) Comparative study of the structure of the head and mouth parts in the Hippoboscidae (Diptera Pupipara). *Parasitology* 18(3): 319-349 and Snodgrass RE (1943) The feeding apparatus of biting and disease-carrying flies: A wartime contribution to medical entomology. *Smithsonian Miscellaneous Collections* 104: 1-51.



Fig. 4 Life-cycle of *Lipoptena* spp. on red deer, *Cervus elaphus*.

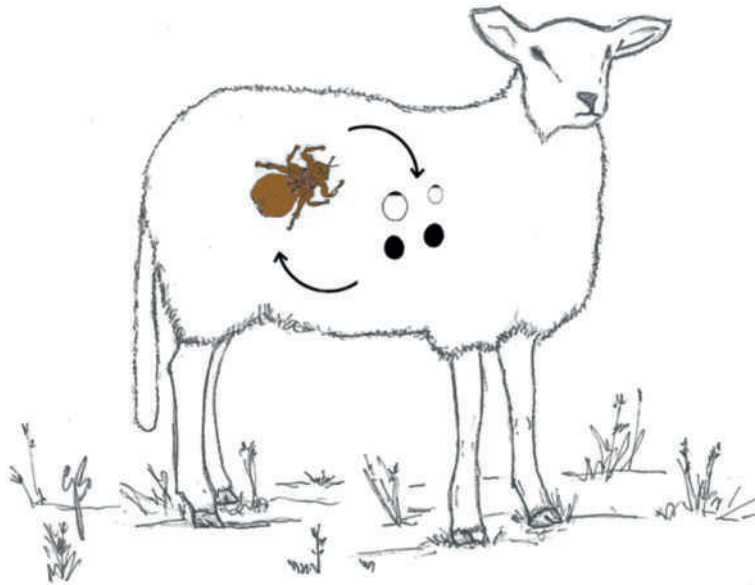


Fig. 5 Life-cycle of the sheep fly, *Melophagus ovinus*.

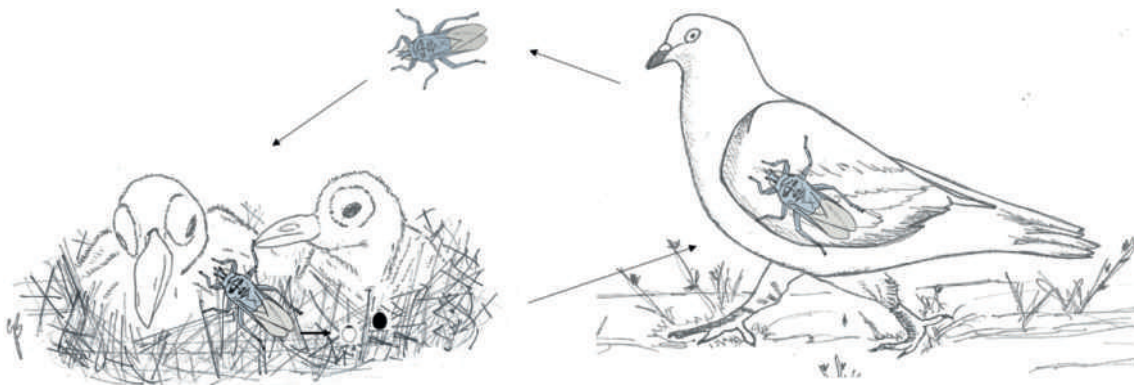


Fig. 6 Life-cycle of the pigeon louse fly, *Pseudolynchia canariensis*.

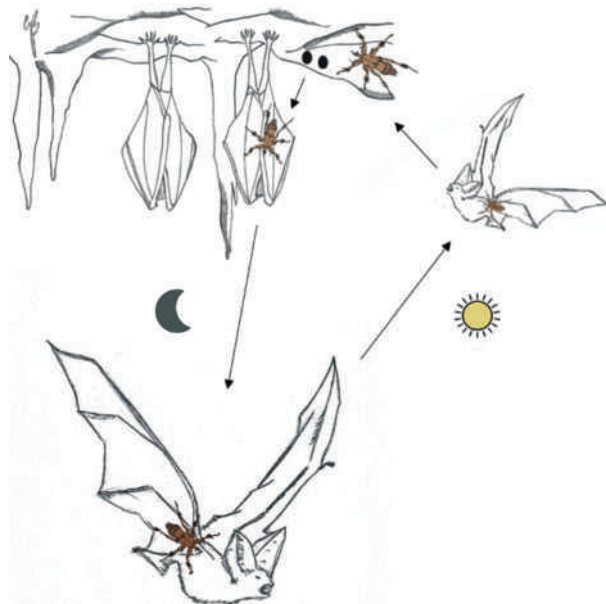


Fig. 7 Life cycle of a bat fly of the family Nycteribiidae.

The Family Hippoboscidae

Bio-taxonomy, morphological adaptations and behaviour

The family Hippoboscidae is composed by three subfamilies and includes a total of 213 described species divided in 21 genera (Dick, 2006). The subfamily Ornithomyiinae is the most numerous with 16 genera and 171 species; the subfamily Hippoboscinae, instead, consists of two genera with eight species, while three genera and 34 species form the Lipopteninae subfamily (Maa and Peterson, 1987; Dick, 2006).

Among the genera, host specificity is more marked in flies attacking mammals, while bird parasites live at the expense of a higher range of hosts (Reeves and Lloyd, 2019). Compared to the other two families belonging to the Hippoboscoidea, Hippoboscidae includes highly variable species in terms of morphology, biology, and behaviour.

The head of hippoboscids is prognathous with mouthparts consisting of two well-sclerotized palpi embracing the piercing apparatus, called also proboscis (haustellum sensu Snodgrass, 1943). It ends with two labella bearing a series of different types of sensilla and teeth-like structures which allow to scratch the skin of the host. The fly thus can feed on the blood spilled from the injury. Other interesting features strongly adapted in these flies are the legs. They are provided with an acropod (pretarsus) equipped with modified adhesion organs such as claws, pulvilli, and empodium. These structures are armed differently among species, but they allow the parasite to live together with the host during most of the life-cycle (Fig. 8).

Lipopteninae subfamily is known to infest ruminant Artiodactyl mammals, especially Cervidae and Bovidae (Bequaert, 1942; Hutson, 1984). This group is divided into three genera: *Lipoptena*, *Melophagus* and *Neolipoptena*. Except *Melophagus* genus, which is totally wingless, the others have caducous wings that show a predetermined horizontal breaking line. Flies lose these structures during the passage between the fur of the host (Haarlov, 1964). Lipopteninae species need a single suitable specimen to survive. Due to their loss of wings once on-host, it is difficult for these species to switch host. Pupae are laid on the hairs of the fur but fall to the ground as a result of the mammal movements; the emergence of a new generation occurs in a specific range of time. New adults are winged and search for a specific host, they are not able to move for long distances and remain near the emergence site (Bequaert, 1942).

Ornithomyiinae is the largest subfamily of Hippoboscidae. About 75% of hippoboscid species exclusively parasite birds and all of them belong to this subfamily (Hutson, 1984). Eighteen bird orders are infested by Hippoboscidae (Santos Murgas et al., 2014). The genera within this group are related more strictly than those of the other two subfamilies. Parasites live in close association with their victims and display different level of host specialization, some are limited to one or few species, while others affect a wider range of hosts. Pupae are deposited in bird nests and the life cycle is synchronized with those of the hosts (Bequaert, 1953). These species have peculiar morphological structures that assist them in remaining on the host during its flight.

Subfamily of Hippoboscinae is indigenous throughout the continental areas of the Old World. All species of this subfamily are ectoparasites of mammals, with the exception of *Struthibosca struthionis* (Janson, 1889) which solely parasite ostriches. Flies of this subfamily infest mainly ungulates and carnivores showing less host specificity compared to Lipopteninae subfamily. They are winged during all their adult life and are good fliers able to switch host frequently (Bequaert, 1930).

Species of Hippoboscidae family can establish phoretic associations with mites, fleas and lice (Maa, 1966; Maa and Peterson, 1987; Megat Abd Rani et al., 2011; Amaral et al., 2013). This aspect can be relevant for the public health, because of the possible transmission of zoonotic microorganisms carried by flies.

Several species of the three subfamilies have been confirmed as potential and/or vectors of various pathogenic agents from veterinary and medical point of view. Consequently, studies on the biology of these parasites are increasing thanks to the modern molecular technologies in order to understand their sanitary role as well as their economic importance.

Relevant Hippoboscidae species for animal and human health

Subfamily Lipopteninae

Lipoptena cervi (Linnaeus, 1758)

The fly is a Palearctic species, nowadays distributed worldwide (Fig. 9). Originally it has been recorded in Europe, Siberia and North China, but subsequently the species has spread into Asia, North Africa and North America, mainly as a result of both intentional and accidental introductions (Bequaert, 1942). Currently, this fly is the most widespread species in Europe (Salvetti et al., 2019). It is usually named “deer ked” as it infests several species of deer. In fact, many species belonging to the Cervidae family are suitable hosts for the parasite (Haarlov, 1964). In Europe, the fly predominantly parasites moose (*Alces alces*), red deer (*Cervus elaphus*) (Fig. 10), and to a lesser extent reindeer (*Rangifer tarandus fennicus*) roe deer (*Capreolus capreolus*) and fallow deer (*Dama dama*). Recently it adapted to few Bovidae species; in fact, some flies have been recorded on chamois (*Rupicapra rupicapra*) and mouflon (*Ovis musimon*) (Ferron, 2008; Bianchi et al., 2016). Moreover, it can occasionally parasite other species used as a food source, such as horses, cows, dogs, cats, badgers, boars, and humans (Bequaert, 1942; Hermosilla et al., 2006). *Lipoptena cervi* has become a pest for animals and humans. In the past this parasite has reached a very high density in Finland, causing such nuisance that people strongly reduced recreational and professional activities in woodland (Härkönen et al., 2009). The infestation can be surprisingly high, in fact up to 17,500 flies have been counted on a single moose, with severe consequences for the host, as skin injuries, dermatitis and alopecia (Kaunisto et al., 2008; Madslien et al., 2011). Parasitism affects also the behaviour of the hosts, increasing defensive or restless actions (scratching, grooming, shaking) with a decrease in the general animal welfare (Kynkäänniemi et al.,

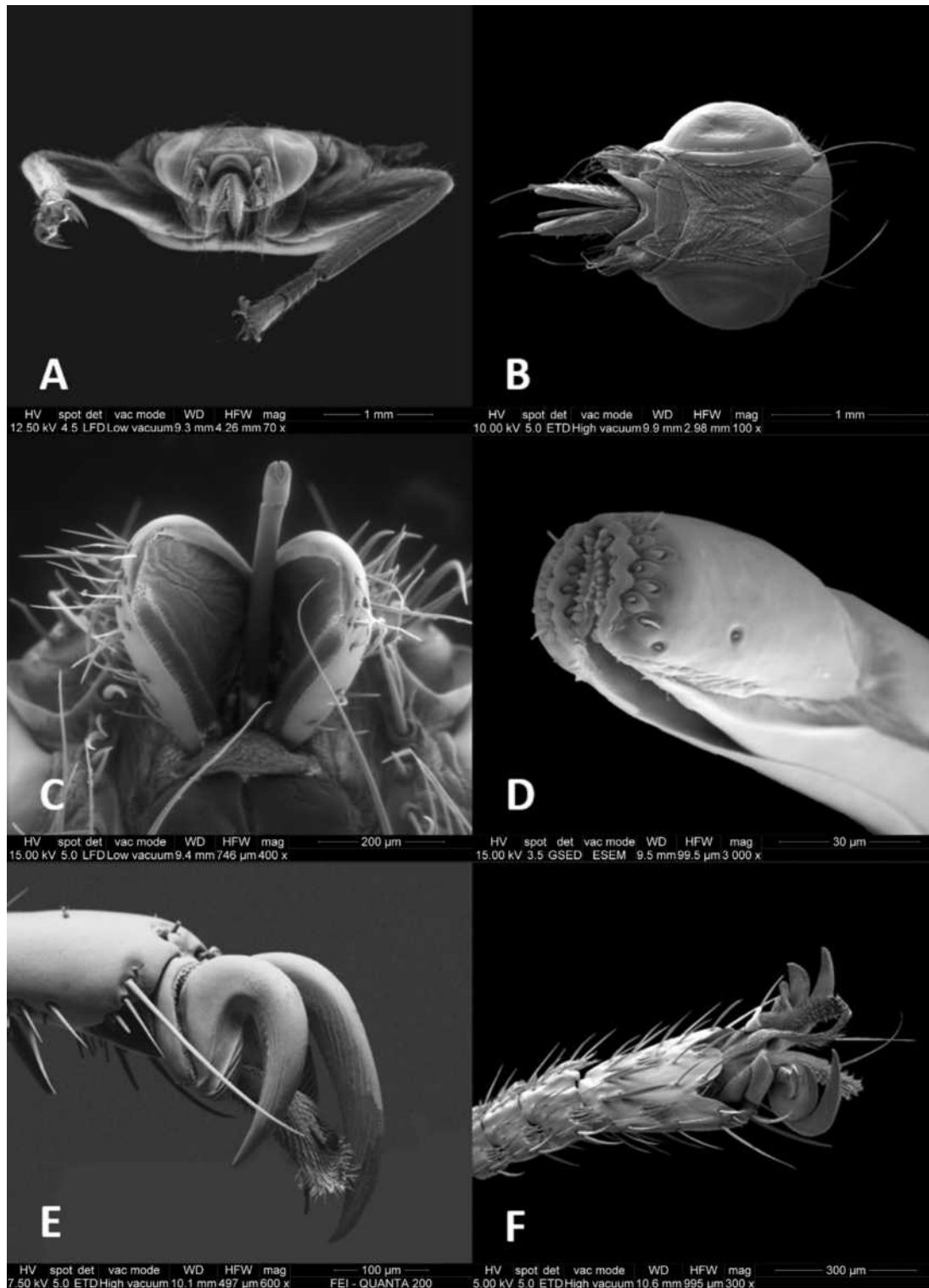


Fig. 8 *Pseudolynchia canariensis*. Frontal view of the head and anterior legs (A); dorsal view of the prognathous head (B). *Hippobosca equina*. Mouthparts with sclerotized palps embracing the proboscis (C); detail of the proboscis tip bearing teeth-like structures and sensilla (D). *Lipoptena* spp. Acropod with asymmetric claws (E). *P. canariensis*. Acropod with three differently sized claws (F).

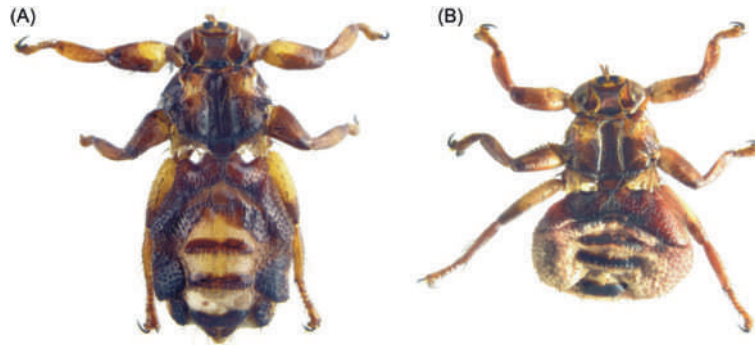


Fig. 9 *Lipoptena cervi* adults. Female (A) and male (B). Modified from: Andreani A, Sacchetti P, Belcari A (2019) Comparative morphology of the deer ked *Lipoptena fortisetosa* first recorded from Italy. *Medical and veterinary entomology* 33: 140-153.

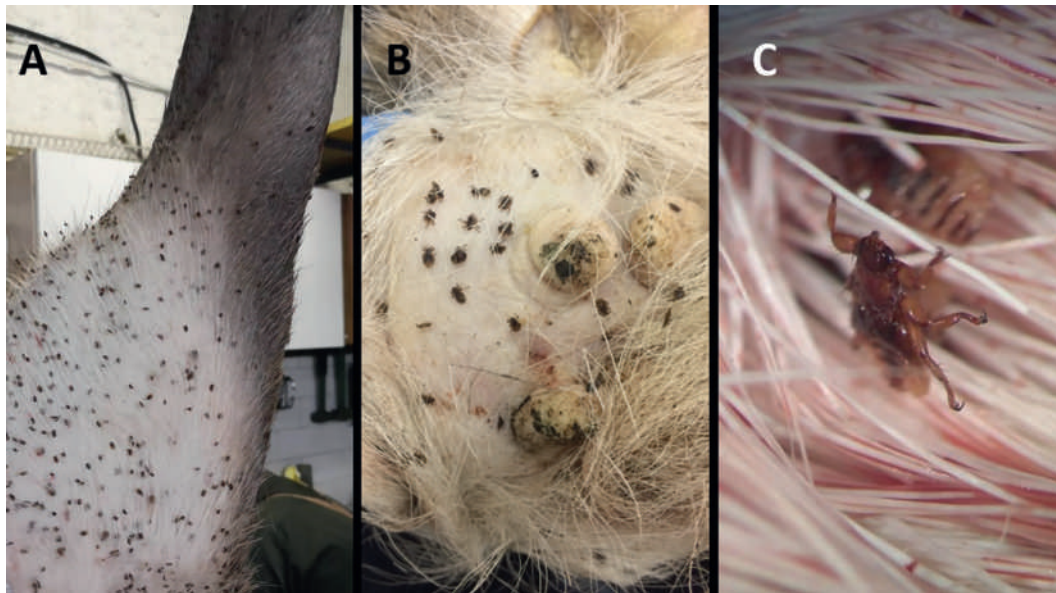


Fig. 10 Red deer thigh and breast with a high infestation of *Lipoptena cervi* and *L. fortisetosa*. (A and B); detail of an adult of *Lipoptena cervi* moving through the guard hairs of the host (C).

2014). Besides mechanical harms, recent studies have identified *L. cervi* as potential vector for several pathogens. *Borrelia burgdorferi*, etiologic agent of Lyme disease, has been detected and identified in flies, as well as *Anaplasma phagocytophylum*, responsible for human and animal granulocytic anaplasmosis; in some case the specimens were infected with both pathogens (Buss et al., 2016). It is possible that the parasite is a mechanical vector for the transmission of these agents, acquiring them from the blood of an infected deer. This hypothesis is confirmed also by lack of these pathogens in the winged flies (Víchová et al., 2011). Moreover, *L. cervi* is considered a potential carrier for *Trypanosoma* spp. (Böse and Petersen, 1991) and *Bartonella* spp. (Dehio et al., 2004; Halos et al., 2004; Regier et al., 2018), responsible of human dermatitis. Presumably, *Bartonella schoenbuchensis* is vertically transmitted from the infected females to the new generations, in fact the pathogen has been detected in larvae and pupae, winged and wingless adults, and colonize the midgut of flies (Dehio et al., 2004; de Bruin et al., 2015). In Norway *L. cervi* seems to contribute to infect moose with *Bartonella* spp. (Duodu et al., 2013). In addition, *Acinetobacter baumannii* DNA has recently been detected in one specimen of *L. cervi*, proving that the parasite can contribute to the spread of this pathogen, affecting people with compromised immune systems, in human and animals (Regier et al., 2018). Although the presence of pathogen DNA in the flies does not imply their competence as biological vector, it suggests that *L. cervi* can increase the spread of the agent to humans and animals. However, the possible mechanical transmission of the pathogen entails the risk to infect other subjects via the bite of the parasite.

***Lipoptena depressa* (Say, 1823)**

Lipoptena depressa has been divided taxonomically in two different subspecies: *L. depressa depressa* (Say, 1823) and *L. depressa pacifica* (Maa, 1969; Dick, 2006). These two subspecies are differently distributed: the first occurs in several central states of the USA, while

the other occupies the eastern part of USA and Canada (Reeves and Lloyd, 2019). *Lipoptena depressa* attacks several subspecies of the ungulate *Odocoileus hemionus* (i.e., *O. h. hemionus*, *O. h. columbianus*, *O. h. californicus*, *O. h. fuliginatus*) (Bequaert, 1953). Moreover, it is a likely parasite of *Odocoileus virginianus leucurus*, the Western white-tailed deer, and has been found on other accidental hosts, such as horses and pigeons. Although in USA the infestation areas occupied by *Lipoptena* species seem to be divided geographically, in western North America *L. depressa* and *Neolipoptena ferrisi* frequently occur together on the same host, as well as *L. cervi* and *Lipoptena fortisetosa* in Italy (Andreani et al., 2019; Skvarla and Machtinger, 2019). *Lipoptena depressa* is considered a potential vector for *Anaplasma* (Skvarla and Machtinger, 2019).

***Lipoptena fortisetosa* Maa, 1965**

The species (Fig. 11) is native to Japan but currently has been identified in few other countries in Asia and Europe (Choi et al., 2013). Its original host is the Japanese deer, named Sika deer (*Cervus nippon*) (Maa, 1965, 1967), although it has been recorded also on the other hosts, such as deer, cattle, goat, sheep, dog, passerine birds and humans (Schumann and Messner, 1993; Yamauchi et al., 2009; Sokół and Gałęcki, 2017; Kurina et al., 2019). In all the countries in which *L. fortisetosa* has been recorded, its presence is considered to be related to the Sika deer, frequently introduced and considered one of the major naturalized alien ungulates in Europe (Mogi, 1975; Sonobe, 1979; Yamauchi and Nakayama, 2006; Choi et al., 2013; Raganella Pelliccioni et al., 2013). On the contrary, Kurina et al., 2019 report that just few Sika deer recently have been counted in Estonia, suggesting that this host cannot be the mean by which *L. fortisetosa* arrived in this area. However, it is undoubted that this hippoboscid is continuously expanding its range, in fact in the last years several occurrences have been recorded in further European countries (Sokół and Gałęcki, 2017; Andreani et al., 2019; Kurina et al., 2019; Mihalca et al., 2019). *Lipoptena fortisetosa* is poorly investigated for possible implications to animal and human health, but, as other hippoboscid species, mechanical damages on the host skin have been attributed to this species, as well as anaemia and hair loss (Kurina et al., 2019). Besides, this fly is considered a potential vector for several pathogens. In fact, the presence of *Coxiella* spp., *Theileria luwenshuni* and *T. ovis* have been detected in some fly specimens, though its role in the transmission of other agents (*Babesia* spp., *Hepatozoon* spp., *Anaplasma* spp., *Ehrlichia* spp., *Rickettsia* spp., *Bartonella* spp., *Borrelia* spp.) has not been clarified (Lee et al., 2016). For this reason, further investigations are needed to verify if the parasite acts as biological vector for pathogens, as well as which effect it can produce on the host (Reeves and Lloyd, 2019).

***Lipoptena mazame* Rondani, 1878**

The species has been recorded in the south-eastern United States and several central and south America countries (Reeves and Lloyd, 2019; Skvarla and Machtinger, 2019). This parasite infests mainly Mazama deer in central and south America and white-tailed deer in the United States, but the fly may accidentally parasite pampas deer, domestic cattle, pumas and humans (Reeves et al., 2006; Graciolli et al., 2011). Recent studies have proved that *L. mazame* acts as vector for *Bartonella* spp. Every year *B. henselae* is responsible for over 20,000 human infestations in the United States; its main vectors are domestic cats, but the presence of this pathogen has been detected in *L. mazame* as well (Reeves et al., 2006). Moreover, the fly may transmit *B. schoenbuchensis*, which is well-known to cause deer ked dermatitis in humans, and it is implied also in the transmission of *Anaplasma* spp. in cattle and *Trypanosoma cervi* in cervids (Reeves et al., 2006; Trout et al., 2010).

***Melophagus ovinus* (Linnaeus, 1758)**

This parasite is an important economic species of sheep. Adults are wingless (Fig. 12), 4–7 mm long brown colored (Yevstafyeva et al., 2017). *Melophagus ovinus* is distributed in the major part of temperate and subtropical areas where sheep are bred. The life cycle of the parasite is completely carried out on the host: gravid female generates a creamy fully grown larva which is attached to the host wool by means a glue-like secretion and in few hours it molts in a dark puparium. A single female can generate up to five-six larvae and the pupal stage lasts about 19–30 days in relation to the temperature. Even if the species is wingless it can switch from one host

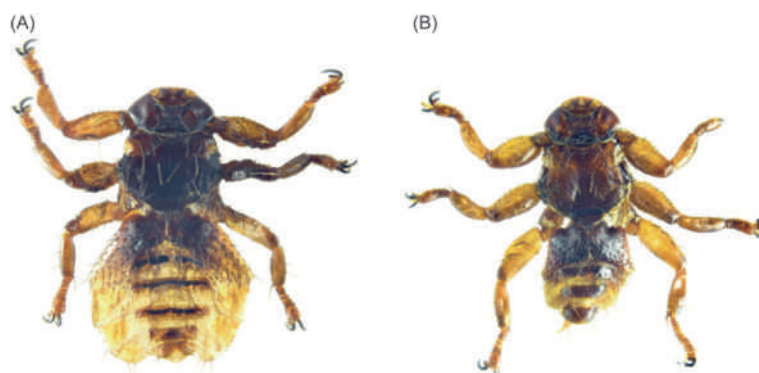


Fig. 11 *Lipoptena fortisetosa* adults. Female (A) and male (B). Modified from: Andreani A, Sacchetti P, Belcari A (2019) Comparative morphology of the deer ked *Lipoptena fortisetosa* first recorded from Italy. *Medical and Veterinary Entomology* 33: 140-153.



Fig. 12 *Melophagus ovinus* adult.

to another very easily, especially from the mother to lambs (Small, 2005). A study conducted on Wyoming unshorn lambs to investigate the distribution of keds over the bodies, showed that the most colonized area of the host was the rib for both sexes of the ked, while the next most heavily populated area for the males was the thigh. Ked populations increased in winter and spring, with an average of 400 keds/host approximately, decreased in summer and increased again in the autumn (Legg et al., 1991).

The number of infesting keds on the sheep can vary a lot: in Ukraine the mean number of the keds/host was 92.72 (Yevstafyeva et al., 2017), while in Canada the mean number was higher. In fact, the peak populations for barren ewes ranged from 61 to 659 keds and for pregnant ewes from 110 to 1348 keds; anyway a general reduction of keds in all animal categories was noticed (Nelson and Qually, 1958). Reduction of the populations seems to be due to several factors such climatic conditions, physiological status as well the intrinsic resistance of the hosts. This latter has been fully investigated in some experimental trials and it has been proved that sheep during the time develop a resistance to the insect trophic activity in terms of inflammatory response to keds. This resulted in changes to the skin that reduced the keds' ability to feed successfully. In other words, the development of resistance was at the beginning, determined by the frequency of attempts for the feeding activity as well the ability and time taken to engorge (Nelson and Kozub, 1980). Later, investigations carried out on artificially infested lambs showed elevated antibody titres within 5 months after the infestation which reached the maximum peak. Further experiments highlighted that the resistance was temporary and mainly due to an inflammatory response to the skin lesions produced by the keds activity as the direct result of the localized arteriolar vasoconstriction that makes blood unavailable to the keds (Baron and Nelson, 1985; Nelson and Kozub, 1980; Small, 2005).

Presence of keds on the host causes a skin reaction such as pruritis, aggravated by the rubbing and scratching in response to the irritation, with consequent reduction of growth rates and production. Besides, losses of the leather quality due to the nodules produced by the keds feeding activity have been observed (Legg et al., 1991; Small, 2005).

Melophagus ovinus is a species of veterinary and medical importance since it is a vector of some important diseases. A recent study conducted in China during the years 2013–17 aimed at investigating the presence of pathogens inside the sheep flies, showed as primary result, the detection of *Anaplasma ovis* DNA in pupae, confirming the potential vertical transmission. *Anaplasma ovis* is an obligate pathogen infecting sheep, goats, and some wild ruminants and its presence in animals causes the anaplasmosis which is an important disease for public and animal health producing economic losses to sheep breeding (Zhao et al., 2018). Moreover, *M. ovinus* is capable to transmit the Blue Tongue Virus (Luedke et al., 1965) as well as the Border Disease Virus, which is an important infection in sheep and goats (Liu et al., 2019). In a survey carried out in Ethiopia, on different domestic animals, it has been shown that about 86% of keds collected from sheep were infected by *Acinetobacter lowfii*. *Acinetobacter* spp. are ubiquitous bacteria implicated in different types of human infections especially in immunocompromised individuals (Kumsa et al., 2013). Halos et al., 2004 demonstrated the vertical transmission of *Bartonella* in *M. ovinus* due to the presence of *Bartonella* DNA in all sampled pupae of the sheep ked, suggesting a symbiotic association between the bacterium and the vector. Further researches allowed the identification of this bacterium as *B. melophagi* (Kumsa et al., 2013; Liu et al., 2018). Finally, some authors demonstrated the presence of *Borrelia burgdorferi* and *Rickettsia* in different sheep ked samples (Chu et al., 2011; Liu et al., 2016).

Management strategies for the sheep ked

Shearing practice can reduce about 75% of the ked population; ewes have to be shorn prior to lambing, otherwise keds can move from them and infest later their lambs. Unshorn lambs, until the following spring, could become a reservoir of infestation for the flock (Johnson, 2011).

Topical application of chemical insecticides is a spread practice and spray, or dust are usually applied in ked control programs. Best results have been obtained treating sheep after shearing; the replacement of animals should take into consideration to treat them before introducing into the flock. It is important to keep treated animals away for about 7–10 days in order to allow the insecticide to kill all the external parasites. Best results in the treatment procedures have been obtained by the application pour-on of permethrin plus piperonyl butoxide (Johnson, 2011) or diazinon and cypermethrin that showed, in addition to a remarkable effectiveness, a long-lasting preventive action (Small, 2005).

Neolipoptena ferresi Bequaert, 1935

This is the only species of *Neolipoptena* genus (Dick, 2006). It is a volant fly infesting predominantly Cervidae, to a lesser extent Bovidae, and occasionally humans (Hutson, 1984). It is well-represented in western America and occurs in the same areas of *L. depressa*. The species is considered a potential vector for *Anaplasma* spp. (Skvarla and Machtinger, 2019).

Subfamily Ornithomyinae

Crataerina pallida (Latreille, 1812)

The “swift louse fly” is a monoxenous ectoparasite, so named because commonly collected on the European swift (*Apus apus*) and, to a lesser extent, on martin (*Delichon urbicum*) (Hutson, 1984). This hippoboscids is widespread in the Palaearctic region, although its populations are decreasing due to the decrement of host species. (Oboña et al., 2019). An interesting trait of this hippoboscids is its scarce fly ability caused by reduced forewings unsuitable for this activity (Liu et al., 2019). However, it is agile inside the plumage and establishes a permanent association with a single host, adapting its life cycle with the bird seasonality. Since the swift is a migratory bird usually returning to the same places every year, *C. pallida* lays larvae directly in or around the nest and dies when birds leave the nesting site to migrate back to Africa. The new adult emergence, occurring after a dormant period until the next breeding season, coincides with host return (Bequaert, 1953; Hutson, 1984). Currently, this hippoboscids has been studied for its interesting morphological and physiological adaptations, mainly for its capability to remain adhere to the victim which is able to reach altitudes exceeding 3500 m and velocities faster than 40 km/h (Petersen et al., 2018; Liu et al., 2019). Differently to *Pseudolynchia canariensis*, *C. pallida* attacks mainly adult instead of young swifts; it has been recorded with a density of maximum 31 parasites on a single host subject (Hutson, 1984). Nevertheless, damaging effects have never been detected on hosts, although the parasite can remove up to 5% of the host's blood volume (Liu et al., 2019). Recently, this species has been proved as a potential vector of *Rickettsia bellii* and *R. monacensis*, suggesting its role in the transmission or in the spread of these pathogens (Cerutti et al., 2018).

Icosta americana (Leach, 1817)

The species belongs to the Nearctic genus *Icosta*, which is the largest of the Hippoboscidae family (Hutson, 1984). This fly is a parasite of owls, which can be infested from a single individual to more than 12 specimens. *Icosta americana* has been identified as a possible vector of West Nile Virus (Gancz et al., 2004). Positive specimens, both unengorged or with blood, have been collected from positive owls in Pennsylvania and in Canada in Ontario (Farajollahi et al., 2005). Although the competence of the vector has not been confirmed yet, it is particularly worthy of attention that some positive parasites were unengorged. It suggests that further investigations are needed to understand the role of *I. americana* as a carrier for the virus.

Pseudolynchia canariensis (Macquart, 1840)

This parasite is known as the “pigeon louse fly” because it is generally associated with tame or wild pigeons, and doves; it is the only hippoboscids attacking domestic birds (Bequaert, 1957; Maa, 1966). This species shows the highest affinity for its host, *Columba livia*, (Fig. 13) although it has been found on other birds, such as Falconiformes (Hutson, 1984; Yamauchi et al., 2011; Santos Murgas et al., 2014). The fly is present worldwide, in tropical, subtropical and temperate areas, where its host occurs. *Pseudolynchia canariensis* can attack occasionally humans, but it happens rarely, in case the parasite has lost the pigeon and encounters humans nearby. The bite is a painful annoyance for humans, but the fly seems to not transmit any diseases to them (Kern, 2014); however, *C. livia* is a reservoir for zoonotic pathogens, which means that the parasite could play a significant role in the transmission (Amaral et al., 2013). This hippoboscids is known to cause several health problems to the hosts, such as skin irritation or dermatitis. Moreover, it is a potential carrier for *Haemoproteus columbae* an avian malaria parasite highly dangerous for young birds and able to make hosts more susceptible to predation (Earle et al., 1993; Pirali-Kheirabaldi et al., 2016). It is well-known that the fly infests



Fig. 13 *Pseudolynchia canariensis* on the external plumage of a feral pigeon, *Columba livia*.

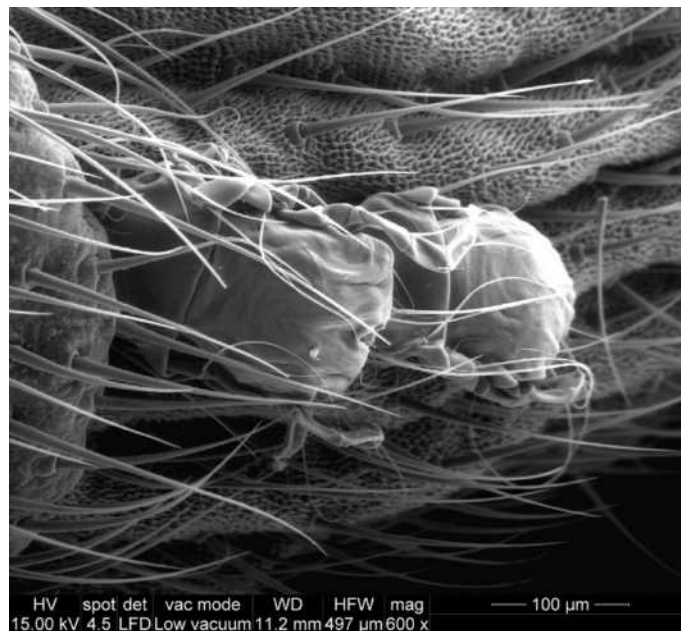


Fig. 14 Abdomen of *Pseudolynchia canariensis* with phoretic mites.

more frequently young subjects instead of adults, probably due to the immunity acquired by adults, which additionally use claws and beak as defense weapons against parasites (Amaral et al., 2013). Besides, *P. canariensis* can potentially transmit *Trypanosoma hanna* and DNA of *Bacillus burgdorferi* has been detected in this species as well (Nartshuk et al., 2018). The pigeon fly establishes phoretic association with mites (Fig. 14) (particularly genus *Myialges*), chewing lice and Mallophaga as reported by Maa (1966), Macchioni et al. (2005), Amaral et al. (2013), and Kern (2014).

Subfamily Hippoboscinae

Hippobosca equina Linnaeus, 1758

Hippobosca equina is a medium-sized hippoboscid species, spread in several temperate areas of the Palearctic and West Oriental Regions (Sóos and Hurka, 1986). The fly is an obligate parasite that feeds on several mammal host species, primarily on domestic horses. The species can reproduce also on cattle (Maa, 1969; Hutson, 1984) or on different secondary hosts such as red deer (Kadulski, 1996), camel and rabbit (Maa, 1969). Further, also birds such the grey heron (Olafsson, 1985) and northern goshawk (Kristofik and Stefan, 1980) have been recorded as occasional hosts. The species is commonly termed also “forest fly.” Wings

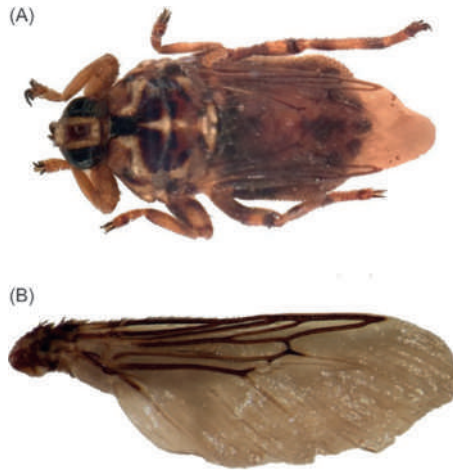


Fig. 15 *Hippobosca equina*. Adult (A) and typical wing pattern (B).

(Fig. 15) display a vein reduction typical of nearly all representatives of the family, but primary veins remain hardened allowing the adults to fly very fast and also for a long time (Turner and Mann, 2004). Gravid females larviposit a full-grown larva in suitable sites, mostly in organic litter (Roberts, 1925). Main biological information for this species have been given by Hafez et al., 1977, which report that *H. equina* was reared artificially in Egypt on guinea pigs. Main results relied on some physiological features of lab-reared adults who survived up to 40–44 days when fed on the blood of factitious hosts and only 1–2 days when starved. The female fecundity ranged from seven and nine larvae during the entire life and the duration of intrauterine life of the larvae lasts from 3 to 8 days according to different climatic conditions. A 2-year study carried out in Poland on primitive horses showed that *H. equina* was present in this area mainly with highest presence from mid-June to the end of July and the most attacked horses were working geldings, leading stallions, and 1.5-year-old colts (Sokoł and Michalski, 2015).

Hippobosca equina is a potential vector of some important animal diseases such as the haemopathogen *Anaplasma* spp. on horses in Tunisia (Selmi et al., 2019) or the Buffalo Oedematous Skin Disease (OSD) produced by *Corynebacterium pseudotuberculosis equi* which cause closed skin lesions either oedematous or nodular or open ulcerative lesion in buffaloes in Egypt (Arafa et al., 2019). In a research carried out in France in several specimens of *H. equina* the intracellular Gram-negative bacterium *Bartonella comelii* has been detected and identified (Halos et al., 2004). *Bartonella* spp. are considered to be emerging pathogens in humans and animals and are present in a wide range of wild and domestic mammals, some of which have been associated with zoonoses (Breitschwerdt and Kordick, 2000; Chang et al., 2000; Halos et al., 2004).

Some anaphylaxis cases due to the bite of *H. equina* to humans have been described. In South America, a man 54-year-old showed severe reactions developing generalized pruritus, followed shortly by generalized urticaria, palpebral and labial oedema, dyspnoea, and hypotension after a bite of *H. equina* (Vidal et al., 2007). In Italy a 48-year-old female showed after a bite a generalized pruritus and then extensive urticaria, abdominal pain, nausea, angioedema on the face, and dyspnoea (Quercia et al., 2005). Finally, in Hungary a 46-year-old female patient showed different symptoms such as hard swelling at the border of the forehead, with oedema. After, erythema and itching developed locally and all over the body, with oedema in the hands, face and lips, later accompanied by shivering, nausea and vomiting (Decastello and Farkas, 2010).

***Hippobosca longipennis* Fabricius, 1805**

Hippobosca longipennis is a common hippoboscid species spread in several countries of southern Europe, Africa and Asia, particularly China and India, associated especially to arid and semi-arid areas. This parasite is frequently called “dog fly,” because wild and domestic dogs are its principal victims, and it has been found also in mummified dogs in Egypt (Sokoł and Gałęcki, 2017). However, it has been collected from different other species, such as fox, cat, hyena, cheetah, lion mongoose or civet (Megat Abd Rani et al., 2011; Reeves and Lloyd, 2019) and occasionally from humans (Bequaert, 1942). *Hippobosca longipennis* is considered the main vector of the filarial nematode *Acanthocheilonema dracunculoides*, found in several European countries in different host species. Moreover, the involvement of this fly as vector of the filarian nematode *Acanthocheilonema* spp. has been proved in northern India (Megat Abd Rani et al., 2011; Mihalca et al., 2019). The health importance of this nematode seems to be little, with just one case in Australia: a female larva was detected in a human eye (Megat Abd Rani et al., 2011), but further investigations are needed to better know its implications. The transmission occurs through the parasite bite, since the infective larvae migrate from the fat-body cells to the mouthparts of the fly. In addition, *H. longipennis* can have a phoretic association with the mite *Cheyletiella yasguri*, zoonotic agent harmful for dogs and humans since it can cause itching, erythema and exfoliative dermatitis (Megat Abd Rani et al., 2011; Sokoł and Gałęcki, 2017).

Bat flies (Nycteribiidae and Streblidae)

Bio-taxonomy, morphological adaptations and behaviour

Bat flies are a highly specialized group that includes two families, Streblidae and Nycteribiidae, both strictly limited to bats (Dick and Patterson, 2006). A total of 520 recognized species belong to the bat flies, making this group the richest of species among the Calyptate Diptera related to mammals (Dittmar et al., 2006). Although they are spread worldwide, no species, genus or even subfamily are present in both hemispheres; in fact, streblids are well-represented in the Western Hemisphere, while nycteribiids are common in the Eastern (Dick and Patterson, 2006). Two subfamilies among the Streblidae (Nycteriboscinae, Ascodipterinae) are well-represented in the Old World, while the other three (Trichobiinae, Nycterophilinae and Streblinae) have been recorded mainly in the New World (Petersen et al., 2007). Bat fly families occur in different climate areas as well: Nycteribiidae occupy temperate regions, while Streblidae have been found in tropical and subtropical climates (Dittmar et al., 2006).

Life cycle of bat flies seems to be quite similar to that of hippoboscids, and it is likely the same among species (Peterson and Wenzel, 1987). All of them live in close association with their hosts inside caves together with gregarious bats and show a high level of host specificity although different bat species occupy the same sites (Lourenço and Palmeirim, 2008). About every 10 days, females leave the infested subject to stick on roost walls a single larva, and then actively search for a new host. Since males do not lay larvae, it could be thought that they always stay on the victim, but, actually, several male flies have been collected from cave walls, proving that they leave the host at least for brief periods (Dick and Patterson, 2006). Several factors affect host specificity in bat flies, for example climate conditions, isolation, competition or predation, and morphological or physiological adaptations (Autino et al., 2011). Bat flies are monoxenous or stenoxenous, and, although they are able to infest alternative bat species that live in the same cave, their preference in parasitizing one species have been demonstrated recently (ter Hofstede et al., 2004; Lourenço and Palmeirim, 2008; de Vasconcelos et al., 2016). Host specificity presumably is affected also by fly ability, but, despite of nycteribiids are totally wingless, they are as specific as the winged streblids (ter Hofstede et al., 2004). Different parasite species can infest the same subject, and the presence of several flies at the same time on a single host has been proved positively associated (Dick and Patterson, 2006).

Bats are the first cause of parasite death. In order to avoid the predation during host auto-grooming behaviour, flies adapted to occupy specific micro-niches, in fact, they generally are located on the pelage or under the membranous wings of bats (ter Hofstede et al., 2004). Moreover, they move very fast in all directions and have structures, e.g., claws, that allow them to adhere to the host (Dick and Patterson, 2006; Kim et al., 2012). Bat flies have a peculiar general morphology, and, like the other Hippoboscoidea, they display several adaptations that make them able to efficiently live together with the hosts (Peterson and Wenzel, 1987; Wenzel and Peterson, 1987). Nycteribiidae evolved the same structures, while, among Streblidae species, morphological adaptations are more variable. Differently from Hippoboscidae except for *Melophagus* spp., bat flies have reduced or absent compound eyes due to an evolutive response towards the dark environment in which they live (Mayberry, 2014). Despite the two families show several common characteristics in both morphological and bio-ecological traits, they have been divided in two groups because of differences in some body structures and in their geographical distribution (Table 1).

Table 1 Main differences between the families Nycteribiidae and Streblidae.

	Body	Head	Wings	Legs	Distribution	Climate
Nycteribiidae Nycteribiidae	Dorsoventrally flattened; spider-like	Backwardly folded at rest	Absent	Dorsally inserted	Eastern hemisphere	Temperate regions
						
Streblidae Streblidae	Varying from generalized convex, laterally compressed to dorsoventrally flattened; flea-like	Prognathous	Fully developed, brachypterous, stenopterous, or apterous.	Laterally inserted	Western hemisphere	Tropical and subtropical regions
						

Subfamily Ascopterinae

Genus *Ascopteron* Adensamer 1896

Members of this subfamily are distributed in tropical and subtropical areas especially in Africa, Middle East, Asia and Australia (Wenzel and Peterson, 1987). *Ascopteron* spp. represent the only exception to the ectoparasitic nature of bat flies. After mating, an eyeless female embeds herself in the tissue of the host thanks to a strongly modified mouth apparatus that has series of cheliceral blades located at the tip of the labial thecum (Hastriter et al., 2006), and becomes an endoparasite (Wenzel and Peterson, 1987; Dick and Patterson, 2006). When she settles on a host, immediately sheds wings, halteres and all legs except for coxae; moreover, thorax and mouthparts invaginate within the abdomen forming a cyst-like body. This feature is a rare example of neosomy in the adult stage; in fact, there is an additional cuticular secretion that allows the enlargement of the whole abdomen. This latter protruding from the host skin displays three pairs of respiratory spiracles close to the anal opening (Hastriter et al., 2006).

Health importance of bats and bat flies

Bats are a great diverse mammalian group, second only to rodents. They play an essential role in ecosystems and have also public health significance (Szentiványi et al., 2019). In fact, bats are known to be reservoirs of several etiological agents, such as Rabies, Marburg, Ebola, measles, mumps, parainfluenza, canine distemper and hepatitis C viruses (Han et al., 2015; Reeves et al., 2016). For many reasons these animals are likely carriers of pathogens, they are the second largest order of mammals with a high diversity of species and a long evolutionary history allowing the co-evolution with viruses. Moreover, bats are social animals that create aggregation of millions of subjects, they are able to fly long distances facilitating the spread of agents and have a relatively long-life span (Han et al., 2015). Bats are hosts for many different ectoparasites, among which Nycteribiidae and Streblidae flies are the most common. These parasites have been implicated as vectors of several pathogens, like protozoa (*Nycteria* spp.), arboviruses and trypanosomes (*Trypanosoma vespertilionis*). In addition, nycteribiids are able to transmit *Polychromophilus* spp., while filarial nematode DNA have been detected in streblids, although it is not clear if it is only present in the last blood meal (Reeves et al., 2016; Szentiványi et al., 2019). DNA of *Bartonella* spp. was detected in *Trichobius major* (Diptera: Streblidae), demonstrating that it may play a role in the transmission of the agents but not explaining the competence of the vector (Reeves et al., 2005). Bat flies more host specific display a lower diversity of *Bartonella* spp., but a general high prevalence of these pathogens, suggesting they have co-evolved with bats. Viviparity could cause vertical transmission of agents from the mother to the offspring through the “milk glands” (Szentiványi et al., 2019). Finally, parasites can affect host behaviour, inducing bats to change roost in case of a high pupal density on the walls. Moreover, a high infestation level can reduce the survival and the reproduction success of the host (Szentiványi et al., 2020).

Concluding remarks and challenges for further research

After this literature analysis and critical discussion on keds, louse and bat flies, it is clear that much work has been done in these last years especially for the detection and identification of pathogens carried by these parasites, proving in some cases that they could play an important role as vectors of etiological agents responsible of diseases in both animals and humans. Investigations have been carried out thanks to new molecular tools allowing the identification of the species as well of the different strains of pathogens. Nevertheless, we have to stress the importance of future basic studies on these flies mainly concerning their morphology, biology and behaviour that may contribute to better understand the real role of these parasitic flies, particularly in relation to both wild and domestic hosts as well to the environment. Finally, it will be particularly worthy of attention the studies dealing with the possible zoonoses transmitted to humans.

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Kissing Bugs (Triatominae)

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Glossary

Abiotic factors Physical or chemical elements of nature that characterize a given biotope or ecosystem.

Akinesia Lack, loss, or cessation of movement.

Anthropozoonotic Disease that passes from animals to humans.

Biotic factors Factors that result from activities of a living being, or any other living component of an environment, such as how the actions of one organism affect the life of another organism.

Coprophagy Feeding on feces.

DDT Diclolo-diphenyl-trichloroethane, insecticide used in various groups of insects.

Domestic cycle When infection in humans occurs through contact with intradomiciliary insects.

Ecotope Physical medium in which a biological community develops.

Endemic That habitually affects a region or country.

Enzootic Disease that affects one or more animal species in a given territory.

Exoterygote Insect that undergoes an incomplete metamorphosis, which gradually changes from the immature to adult stages; juveniles are similar in appearance to adults, but have wings that develop externally (e.g., triatomine nymphs to adults).

Fat body Tissue in insects formed by storage cells that is distributed throughout the body cavity.

Hematophagous Feeding on blood.

Hemimetabolous Term referring to insects whose metamorphosis is gradual or incomplete, and in which the juvenile is similar to the adult or imago.

Hemocoel Unlined cavity characteristic of many invertebrates in which the hemolymph is found.

Hemocytes The cells found in invertebrate hemolymph.

Hemolymph Circulatory liquid of invertebrates, analogous to blood in vertebrates.

Holometabolous Type of development in higher insects, characterized by having distinct embryo, larval, pupal, and imago stages.

Incomplete metamorphosis Development that includes only three stages: egg, nymph, and imago.

Intradomiciliary Insects vector presence inside the home.

Metacyclogenesis When metacyclic parasites are produced. In this case, when epimastigotes become metacyclic trypomastigotes.

Paratransgenesis Technique based on the use of symbiotic bacteria to express effector molecules within a target vector.

Phytophagous Feeding on plant material.

Reservoir Long-term host of a pathogen that causes an illness.

Screening Test to detect a disease.

Symbiont Organism that lives in interaction with another organism, usually a larger one.

Vector Any agent that transports and transmits a pathogen to another living organism.
Wing primordia Rudimentary state in which a developing organ is found, in this case, the wings.
Zoonanthropnotic Disease that passes from humans to wild animals.
Zoonosis Disease found in animals that can be incidentally transmitted to humans.

Introduction: What are triatomine bugs?

The triatomines are insects of the subfamily Triatominae (Hemiptera: Reduviidae), which is composed of 149 species distributed mainly on the American continent (Justi et al., 2016; Monteiro et al., 2018). Throughout the life cycles triatomines feed on the blood of vertebrates, in other words, they are hematophagous, associated mainly with the nests of birds and small mammals (Carcavallo et al., 1999; Rabinovich et al., 2011). The triatomines, also known as kissing bugs, are vectors of the six described lineages of *Trypanosoma cruzi* (Kinetoplasta: Trypanosomatidae) (Brenière et al., 2016), the etiological agent of American trypanosomiasis or Chagas Disease. American trypanosomiasis is the zoonosis limited to wild habitats, while Chagas disease refers to the illness that occurs in humans. The landscape changes that occur in natural ecotopes have been key for the invasion and adaptation of the vectors to human dwellings, and with them, the establishment of *T. cruzi* transmission in urban environments (Parker and Sethi, 2011). The World Health Organization (WHO) classified Chagas disease as one of the 10 neglected tropical diseases that pose the greatest threat to public health (Justi and Galvão, 2017; Tarleton et al., 2014). Kissing bugs acquire *T. cruzi* when they feed on a reservoir of the parasite, which is an infected mammal (Rabinovich et al., 2011). Classic transmission of *T. cruzi* is through the triatomine's feces (Beard, 2005), though there are other mechanisms.

Past, current and future distribution of triatomines

Some underlying evolutionary questions about this group are when and where it arose, whose responses, based on molecular clocks, have reached unclear conclusions (Justi and Galvão, 2017). One of the most widely accepted hypotheses is that the triatomines arose during the Oligocene, when South America was already isolated from the Antarctic and the rest of the American continent (Hwang and Weirauch, 2012; Ibarra-Cerdeña et al., 2014; Otálora-Luna et al., 2015). Whatever their origin, populations of Triatominae tend to show similar adaptive behavior. Presumably, the triatomines evolved from predators of the family Reduviidae which diversified on the continent at the same time as mammals and birds during the Jurassic (Monteiro et al., 2018). Given that the family Reduviidae contains a wide variety of predatory behaviors (Hwang and Weirauch, 2012), it is thought that the diversification of the lineages of the reduviids could be a consequence of, among other factors, changes in feeding behaviors.

Different species of triatomines are distributed from the southern United States through southern Argentina (Fig. 1) (de Fuentes-Vicente et al., 2018). However, 14 species have been reported in the Old World: eight of the genus *Triatoma* and six from *Linshcosteus* (Monteiro et al., 2018). It is likely that the triatomines from the Old World originated in the New World (Hypša et al., 2002; Justi et al., 2016). Specifically, in Asia a common ancestor is reported to have given rise to the groups of *Triatoma* with distribution in Asia/Australasia, and the *Linshcosteus* restricted to the southern Himalayas and southeastern Thar Desert (Monteiro et al., 2018; Patterson and Gaunt, 2010). Interestingly, there is only one species that is distributed both in the Old and New World, *Triatoma rubrofasciata*, but it only has epidemiological importance in the Americas (Dujardin et al., 2015). It is suggested that ships were responsible for the dispersion of *T. rubrofasciata* (Barrett, 1991). It is worth pointing out that none of the Old World triatomine species have been reported as natural vectors of *T. cruzi* (Hamilton et al., 2012). This supports the hypothesis that the protozoan *T. cruzi* originated on the American continent (Hamilton et al., 2012).

The distribution of the triatomines on the American continent is due to their tolerance to a wide range of environments. There are biotic and abiotic factors that modulate the distribution of the triatomines. Among the biotic factors, the most important are the diversity of food sources for the triatomines (Noireau et al., 2005) and the presence or absence of infection by *T. cruzi* (Villalobos et al., 2019). In the case of abiotic factors, temperature (Carmona-Castro et al., 2018) and anthropization have favored distribution (Parker and Sethi, 2011). The use of georeferencing tools have allowed the current distribution of the subfamily Triatominae to the predicted distribution with increased detail (Gurgel-Gonçalves et al., 2012; Ibarra-Cerdeña et al., 2014). While most triatomines prefer to inhabit wild environments and the cycle of transmission of *T. cruzi* occurs among the wild mammals present there (Jurberg and Galvão, 2006), there are species such as *Rhodnius prolixus*, *Triatoma infestans* and *T. dimidiata*, that have been able to colonize and adapt to human dwellings (Jurberg and Galvão, 2006).

Medical importance

A history of humankind and Chagas disease

Chagas disease is as old as the first appearances of humans in South America (Steverding, 2014). Thanks to advances in molecular techniques, it has been possible to document the oldest known infection with *T. cruzi* in a Chinchorro mummy from 9000 years ago

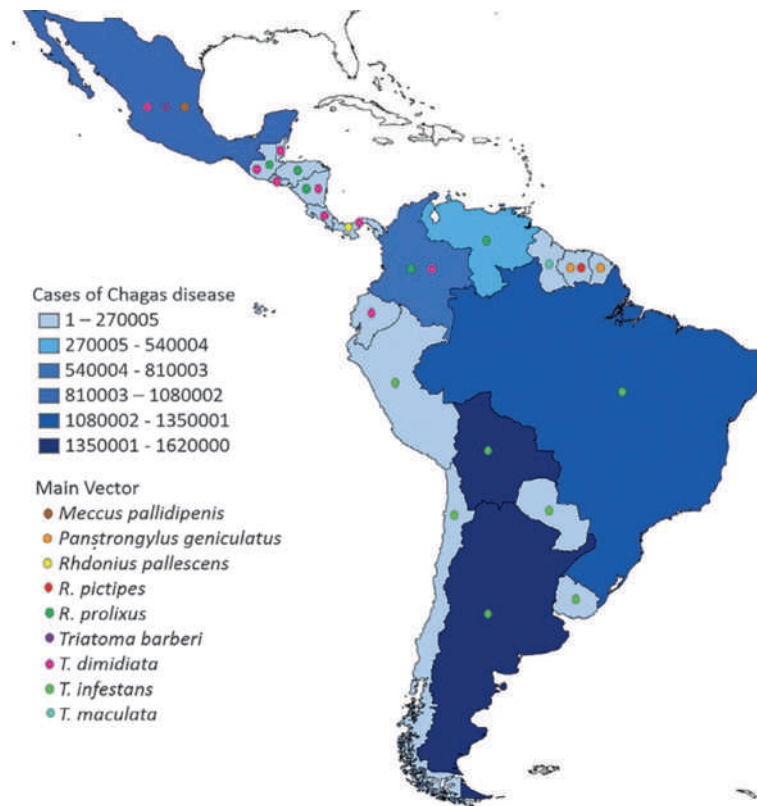


Fig. 1 Distribution of Chagas disease and the main vectors in the Americas. The nuances indicate the number of cases per country. de Fuentes-Vicente JA, Gutiérrez-Cabrera AE, Flores-Villegas AL, Lowenberger C, Benelli G, Salazar-Schettino PM, and Córdoba-Aguilar A (2018) What makes an effective Chagas disease vector? Factors underlying *Trypanosoma cruzi*-triatomine interactions. *Acta Tropica* 183: 23–31.

(Aufderheide et al., 2004). The Chinchorro culture is the name given to a group that fished along the coast of the Atacama Desert in South America between 7020 and 1500 years ago (Aufderheide et al., 2004; Vigliano et al., 2020). Based on the available paleoparasitological data, it is thought that Chagas disease originated in the Andean region. In this area, patterns of socioenvironmental occupation favored the gradual transition of *T. cruzi* from the wildlife to human homes (Ferreira et al., 2011; Steverding, 2014; Vigliano et al., 2020).

The disease was not discovered until 1909, when the Brazilian physician Carlos Chagas identified it in Minas Gerais while carrying out an anti-malaria campaign (Chagas, 1909). This discovery was a milestone in the history of medicine because it simultaneously described the causal agent, some of the clinical manifestations of the disease, and the insect vectors. Previously in the literature there were reports of kissing bugs, but their epidemiological role was unknown (Steverding, 2014). Perhaps the most famous note is the anecdote by Charles Darwin in 1835 narrating the “attack” by these bugs at night, and that Darwin let one of the bugs feed on him in order to describe its morphological changes (Darwin, 1839). This has led to suspicions that the myocardial infarction that caused Darwin’s death could have been related to the cardiomyopathy produced by Chagas disease. After Carlos Chagas’s discovery, a number of studies documented the presence of triatomine bugs over much of the American continent, and the introduction of diagnostic techniques showed that *T. cruzi* infection was widespread among humans (Gürtler et al., 2008). Toward the end of the 20th century, it was estimated that there were nearly 18 million people infected with Chagas disease worldwide, a number that has decreased thanks to programs to control triatomine bugs (Noireau et al., 2009).

Epidemiology of Chagas disease

At some point in its evolution, *T. cruzi* became adapted to life in the gastrointestinal (GI) tract of triatomines, where it replicates and is expelled along with the bug’s feces and urine. Thus, triatomines become infected with *T. cruzi* after feeding on the blood of an infected host and days later expel the parasite, infecting other hosts and completing the cycle of transmission (Beard, 2005) (Fig. 2).

The vast majority of kissing bug species live in wild ecotopes and are associated with nests and refuges of small mammals and birds (Carcavallo et al., 1999; Rabinovich et al., 2011). This is how a little over 100 species of mammals (mainly rodents, edentates, and carnivores) and some species of reptiles participate in the cycle of transmission of this parasite (San Juan et al., 2020; Teixeira et al., 2009), while birds do not become infected with *T. cruzi* because their immune system is able to eliminate it (Teixeira et al., 2011).

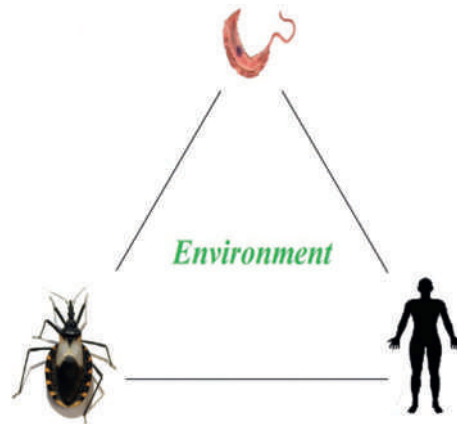


Fig. 2 Epidemiological components of Chagas disease: the *T. cruzi* parasite, the kissing bug and the human; in an environmental context.

In wild environments, the parasite circulates between vertebrate hosts and insect vectors, resulting in a wild transmission cycle that can connect with other, non-wild environments or remain independent. According to Galvão and Justi (2015), the transmission cycles can be *enzootic* (wild cycles), when only wild animals are involved; *anthropozoonotic*, when the infection is the result of contact between humans and wildlife vectors; *zoonotic–amphixenotic*, when the infection results from contact between domesticated animals and vectors from human dwellings, or the rarest mode, *zooanthroponotic*, which refers to transmission from humans to wild animals through insect vectors from human dwellings (Coura, 2013). Thus, multiple factors influence the propagation of *T. cruzi* in human populations, basically associated with the way these populations occupy and exploit their environments (Galvão and Justi, 2015; Vaz et al., 2007). When triatomines came into contact with human dwellings (da Fonseca and Robinson, 1990), humans became one of their potential food sources and, as a consequence, humans began to participate in the life cycle of *T. cruzi* as well, known as the *domestic cycle*. While other modes of transmission of the parasite have been reported, transmission by insect vectors continues to be the most important (Coura, 2015). This is because vector transmission is virtually impossible to eradicate, since it occurs in two scenarios: (1) human invasion of wild ecotopes where the transmission cycle is active, or (2) re-infestation by kissing bugs of human settlements where they were previously eliminated by vector control campaigns. As such, transmission of *T. cruzi* to humans is clearly the consequence of anthropogenic changes to the wild environment. Other mechanisms of transmission include blood transfusions (Young et al., 2007), organ transplants (Centers for Disease Control and Prevention (CDC), 2006), congenital transmission, and ingestion of food or drink contaminated with *T. cruzi* (Filigheddu et al., 2017; Santana et al., 2019).

Chagas disease has an *endemic* distribution in 21 countries in the Americas (Guhl, 2017). Currently, it is estimated that there are 6 million people who are ill and approximately 22 thousand deaths annually in Latin America alone; with Brazil, Argentina and Mexico, the most affected countries (Fig. 1). Because of a lack of screening for *T. cruzi* in donated blood and organs, Chagas disease became a public health concern in countries that are non-endemic and that do not have vectors, such as Spain, Italy, France, the United Kingdom and Switzerland (Antinori et al., 2017; Gascon et al., 2010). Thus, the WHO (2019) estimates between 6 and 7 million people infected worldwide, indicating that the cost of an infected patient is approximately US \$474 in medical expenses, with an annual cost of US\$627.46 million worldwide. The economic costs of labor losses due to disability for Chagas patients must also be added to this cost (Lee et al., 2013). This means that Chagas disease has strong economic repercussions in endemic countries.

Chagas disease involves two important stages: an acute stage characterized by the circulation of the parasites in the bloodstream, and the chronic stage in which the parasite becomes lodged in different organs such as the heart, esophagus, and colon, where they generate complications (Nunes et al., 2018). To date there is no vaccine or effective prophylaxis against Chagas disease, though there are two drugs that, if administered in time, can eliminate the infection: Benznidazol and Nifurtimox (Meymandi et al., 2018). These antiparasitic drugs, developed nearly 40 years ago, are most effective when given soon after infection, since they act much more efficiently on parasites in the bloodstream than on the invasive form in muscle. However, these medicines can have side effects such as digestive intolerance, headache and peripheral neuropathy (Kirchhoff, 2003). As such, agents like Fexinidazole are being tested clinically as an alternative and other studies seek to redesign the doses of Benznidazol to avoid these effects (Sales Junior et al., 2017).

Biology and ecology

Feeding habits

The kissing bugs' ancestors were thought to be individual predators that in turn descended from phytophagous insects (Otálora-Luna et al., 2015). One piece of evidence that supports this idea is that the triatomines' digestive enzymes include *cathepsin* like phytophagous hemipterans, rather than *trypsin*, which is used by other hematophagous insects to degrade ingested blood (Ribeiro

et al., 2014). While blood meals are their main source of food, triatomines are able to exploit other food sources, such as plant saps, blood consumed by other insects, *coprophagy*, or the *hemolymph* of other triatomines (Díaz-Albiter et al., 2016; Garrouste, 2009; Otálora-Luna et al., 2015). The variety of habitats used by the triatomines thus facilitates their relationship with their main hosts (Bar et al., 1999).

These insects tend to feed preferentially at night, while their vertebrate hosts are asleep, and during the day they remain in *akinesia*. As such, the emergence of both chemical and physical mechanisms for locating food sources were fundamental for this nocturnal behavior (Otálora-Luna et al., 2015). In the absence of light, they are able to perceive chemical signals of their hosts, including CO₂, isobutyric acid, 1-octen-3-ol ammonia, and L-lactic acid (Lazzari et al., 2013). While attraction to CO₂ is crucial for other hematophagous insects, it is apparently not as important in the process of attraction of triatomines. Curiously, after long periods of fasting there is no increase in their responsiveness to CO₂, but after a recent blood meal it acts as a repellent (Lazzari et al., 2013). The most important physical signal for triatomines is heat, and their extreme thermal sensitivity allows them to perceive thermal energy on the order of only a few $\mu\text{W}/\text{cm}^2$ from several meters away (Lazzari, 2009). In addition to its role as an orientation signal, heat is used to locate blood vessels of the host in order to feed more efficiently (Ferreira et al., 2007).

Their mouthparts are relatively small compared to other hematophagous insects, so the suction of blood must be efficient in terms of speed. For this, they have a suction pump located along the head that functions with muscles that occupy much of the head (Lehane, 2005). Once feeding begins, there is a release of the neurotransmitter serotonin into the hemolymph, which leads to the plastification of the cuticle and allows the abdominal tegument to stretch to accommodate large quantities of blood in the anterior midgut (Orchard, 2006; Sant'Anna et al., 2017). Then, the blood is digested gradually, depending on the individual's physiological requirements, allowing these bugs to endure long periods of fasting.

Individual nutrition plays a crucial role in the life cycle of the triatomines. Both sexes feed on vertebrate blood during all five nymph stages and as adults. Advantageously, triatomines are able to obtain a single feeding without suffering fitness costs (Martínez-Ibarra et al., 2018). If the amount of blood is sufficiently large, the distension of the body wall can produce a nerve stimulus that triggers the molt to the next stage. Poor nutritional condition has two developmental consequences: they take longer to molt from one stage to the next, and females produce fewer eggs (Castillo and Wolff, 2000).

If they are not disturbed during feeding, the ingestion of the complete blood meal can take between 10 and 20 min, depending on the species (Sant'Anna et al., 2017). The amount of blood ingested can be several times the bug's body weight and nymphs in the first stages consume the most blood. After a large meal, triatomines can spend several weeks without feeding, hiding in a refuge until their next feed or, in the case of adults, searching for a mate in order to breed. Triatomines in all developmental stages occupy the same general habitat and feed on the same hosts, which makes them susceptible to acquiring *T. cruzi* and remaining infected during their whole life cycle.

Life cycle

Triatomines can be observed in several different ecological scenarios. Some species are exclusive to a particular habitat or host, while others are found in different habitats (Gaunt and Miles, 2000). While habitats are diverse, all kissing bug species are limited in their tolerance to humidity (30–80%) and temperature (24–28 °C). The life cycle of these insects tends to take longer at lower temperatures (i.e. 16 °C), while temperatures above 40 °C are lethal (de Fuentes-Vicente et al., 2017; Pimentel-Melo et al., 2020).

Diverse species of vectors adapted to human dwellings are generally easy to breed in laboratory conditions, providing models for studies of their biology, physiology, genetics, and basic evolutionary tendencies (Ryckman, 1952). We know that at temperatures between 20 and 30 °C, development takes approximately 5–6 months, but this time can vary depending on the species. For example, the mean duration of the life cycle of *Rhodnius prolixus* and *Triatoma infestans* is a few months, while species like *Triatoma dimidiata*, *Panstrongylus megistus*, *Dipetalogaster maximus*, can take more than a year (Gorla and Schofield, 1989; Guarneri et al., 2000; Lima et al., 1992). These interspecific differences are probably related to different environments. For example, while *R. prolixus* lives in tropical habitats with constant environmental characteristics, this is not the case for *D. maximus*, which lives in southern Baja California (Jiménez and Palacios, 2002).

The triatomines are *exopterygotes*, which pass through different developmental stages, undergoing an *incomplete metamorphosis*. They have an egg stage, five nymph stages, and a reproductively mature adult stage (Fig. 3). Their sizes vary from 5 to 40 mm, but the most important species in the epidemiology of Chagas disease are around 20 mm (Bello-Bedoy et al., 2019; Berenger and Parola, 2017). Their eggs have an ellipsoid shape and they have an operculum. It is suggested that the geometry of the outline of the eggs could help discriminate not just among species, but also microhabitats of communities (Catalá et al., 2017). The eggs are white when laid, and by 2–3 weeks after laying are pinkish (Fig. 3) and are ready to hatch as nymph 1, which takes between 10 and 40 days after oviposition (Catalá et al., 2017). Nymphs can be differentiated from adults by their incompletely developed wings and genitalia. Between the fourth and fifth stages of development, the *wing primordia* are already visible. In the adult, the wings are completely developed, and genital differentiation is evident (Fig. 4). The internal male reproductive structures have been well characterized and are informative for taxonomic studies, particularly for distinguishing closely related species (Gonçalves et al., 2013; Pereira-Lourenço et al., 2013; Tellez-Garcia et al., 2019). On the contrary, female genitalia have been relatively little studied, mainly because it has been assumed that this characteristic is highly uniform among species and lacks taxonomic characters (Lent and Wygodzinsky, 1979; Tellez-Garcia et al., 2019).

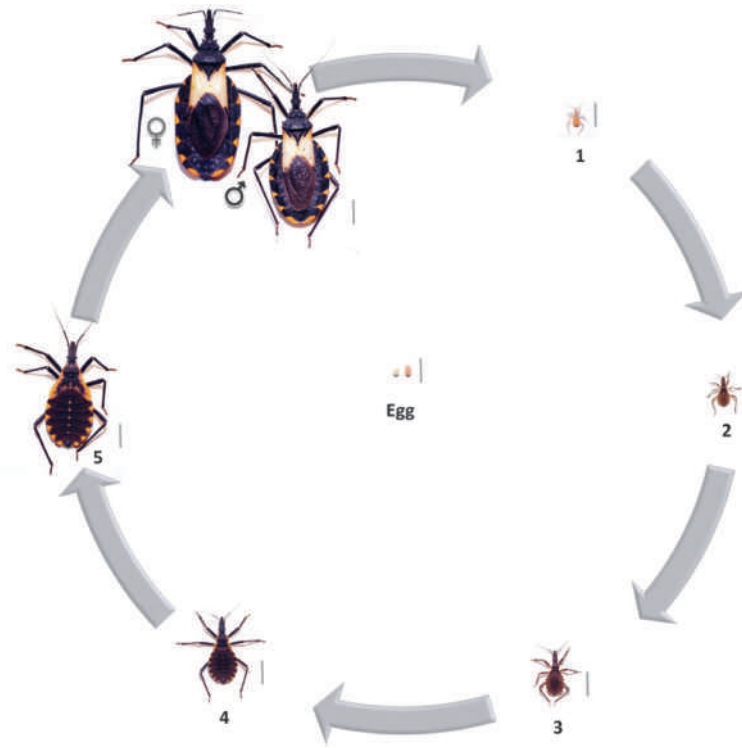


Fig. 3 Life cycle of the kissing bugs (Triatominae).



Fig. 4 Adult specimens of *Triatoma (Meccus) pallidipennis* (Triatominae). Notice the differences in the last abdominal segments that determine sex.

Reproduction

Because most studies on the reproductive behavior of triatomines have been done under laboratory conditions, little is known about their reproduction in nature. It is suggested that the beginning of mating is mediated by chemical signals (Pontes et al., 2008, 2014; Zacharias et al., 2010), since triatomines do not emit auditory signals and there is virtually no visibility at night (Manrique and Lorenzo, 2012; Pontes and Lorenzo, 2012). In some species, mating in captivity occurs at night at the beginning of *scotophase*, when movement activity reaches its maximum and females leave their refuges (Lazzari, 1992; Pontes and Lorenzo, 2012). Some species of triatomines carry out precopulatory behaviors such as dances or nuptial movements that precede copulation (Galliard, 1936; Hase, 1932) or a type of courtship like female stridulation (Jiménez and Palacios, 2002). The triatomines are apparently polygamous, and mating behavior appears to be very similar among species (Manrique and Lorenzo, 2012). According to the literature review by Tellez and collaborators (2019), there are several steps during copulation reported in several species (Fig. 5). Once a male detects a female, he approaches and adopts a vigilant posture. Then, he mounts and jumps on top of her, and in some species, turns around on top of the female. If the female is receptive, the male grabs on with his three pairs of legs while he places his

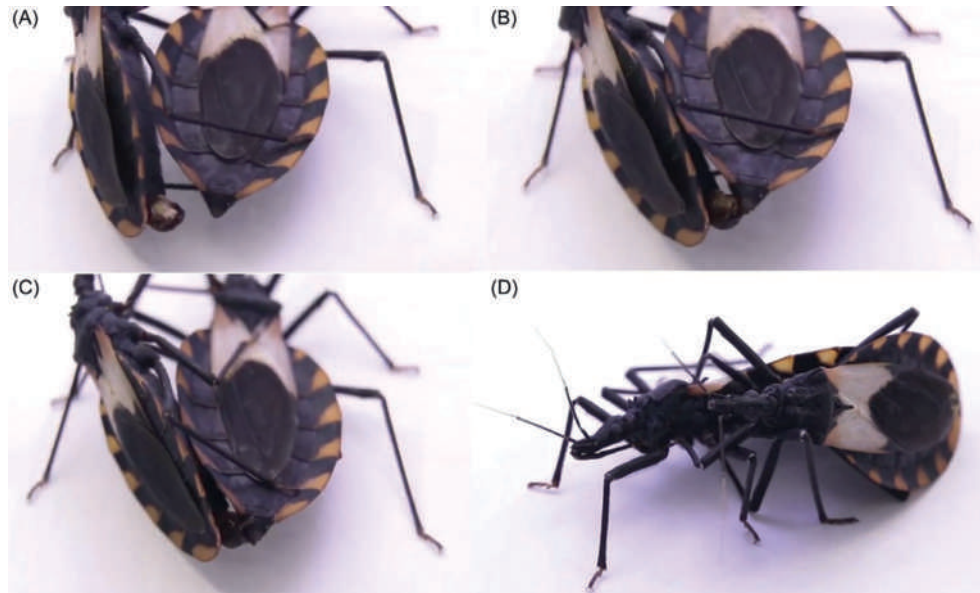


Fig. 5 Mating of kissing bugs (Triatominae): (A) the male locates the female and jumps on her, (B) the male holds onto the terminalia of the female and (C) copulation occurs; (D) when copulation is complete, the male descends.

body in ventro-lateral position. After that, the male holds onto the terminalia of the female and the copulation takes place. Finally, when the copulation is completed, the male again mounts the female then descends. Copulation duration is highly variable among species; *D. maxima*, *T. brasiliensis* and *T. infestans* are the species with the fastest copulation times, lasting about 6 min, while in *R. prolixus* copulation takes 49 min on average (Jiménez and Palacios, 2002; Manrique and Lazzari, 1995; Pontes et al., 2008; Vitta and Lorenzo, 2009). Females can obtain a single copulation or up to 27, like in *T. brasiliensis*, after which the female begins to oviposit (Brasileiro, 1982). The eggs are deposited between 10 and 30 days after a successful copulation. Eggs are not adhered to the substrate, except for some species of the tribe Rhodniini (e.g. *R. prolixus*).

Immune system of kissing bugs

Triatomines may come into contact with microorganisms through food, wounds, copulation, etc. Any immune response assumes that the host recognizes the invader as non-self. The immune response implemented by triatomines has evolved through interactions with a variety of pathogens and parasites (e.g. bacteria, viruses, fungi and protozoa), with which they have been in contact during all stages of their life cycles (Flores-Villegas et al., 2015). The immune response of these insects, indeed, of nearly all organisms, is conformed of (Fig. 6): physical barriers (i.e. the cuticle and the epithelium), cellular responses (i.e. phagocytosis, nodulation, aggregation of hemocytes and encapsulation), and humoral factors (i.e. lectins, antimicrobial peptides (AMPs), and the phenoloxidase cascade (PPO)).

Physical barriers

The cuticle and the intestinal epithelium are the main lines of defense (Moussian, 2010). Both are conformed mainly of chitin fibers embedded in a matrix of proteins, lipids, and glycoproteins (Andersen, 2010). In particular, glycoproteins cover the GI tract of triatomines, particularly in the intestinal region (Gutiérrez-Cabrera et al., 2014). These glycoproteins, that is, proteins with different sugar residues, are a main component of the perimicrovillar membrane (PMM), analogous in function to the peritrophic membrane of other insects (Gutiérrez-Cabrera et al., 2016). While the PMM functions as a physical barrier and for nutrient absorption, the diversity of glycoproteins present serves as a mechanism for interaction with the microorganisms present, including both the microbiote and pathogens, such as *T. cruzi* (Buarque et al., 2016; Gonzalez et al., 2006). It has been tested whether the PMM is essential for establishment and replication of *T. cruzi* in the vector (Alves et al., 2007; Cortez et al., 2012; Garcia and Azambuja, 2004; Gutiérrez-Cabrera et al., 2019).

Cellular response

Once the pathogen evades the first line of defense, the immune cell and humoral mechanisms are activated, which often act together in response to pathogens. Pathogen recognition begins at pattern recognition receptors (PRR) which identify pathogen-associated

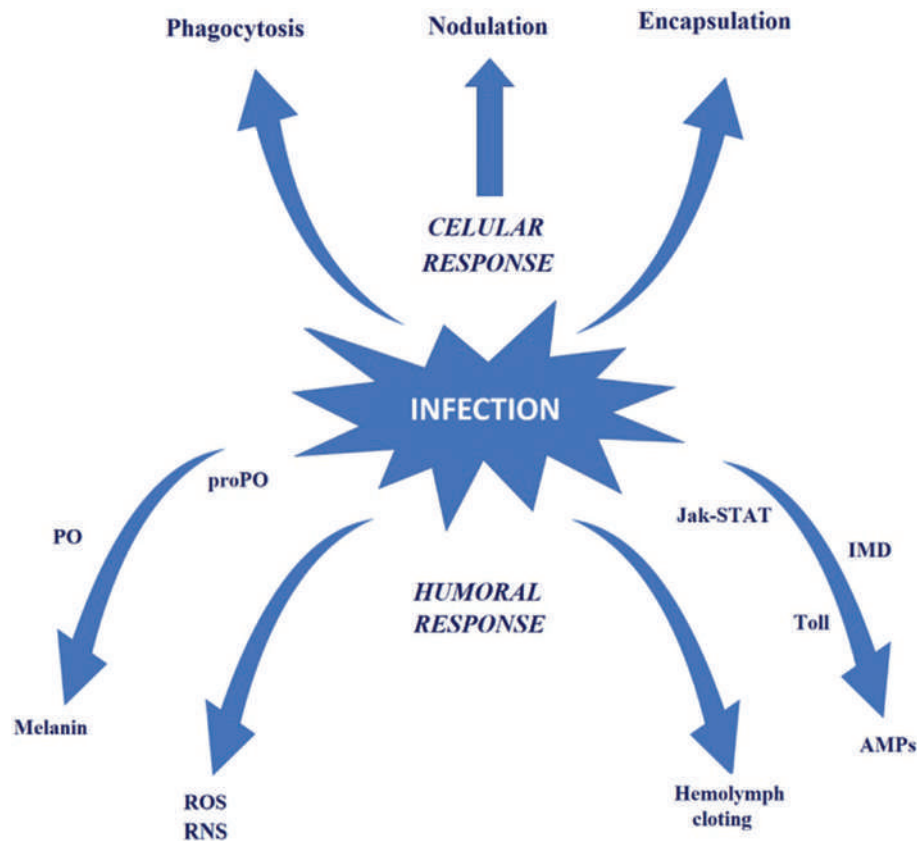


Fig. 6 Overview of the insect immune response. This is divided into cellular and humoral response.

molecular patterns (PAMPs; Gillespie et al., 1997). This mechanism allows the insect to recognize and respond to the pathogen more effectively in secondary encounters (Cisarovsky et al., 2012; Flores-Villegas et al., 2015). The cellular immune responses typically generate phagocytosis, nodulation and encapsulation (Borges et al., 2008; Hillyer, 2016). Phagocytosis is the main immune cell response in the hemocoel against small microorganisms and foreign bodies (Borges et al., 2008). Nodulation refers to hemolytic multicellular aggregates that trap large quantities of small pathogens that can then be melanized to form a nodule (Hillyer, 2016). Finally, encapsulation is the immunological process by which hemocytes bond to larger parasites, also accompanied by melanization (Hillyer, 2016). The cells involved in these processes are known as *hemocytes*, and they are also in charge of the production of humoral elements that will be mentioned below. Hemocytes in the triatomines are classified according to their structure and function (Azambuja et al., 1991a; Price and Ratcliffe, 1974), and their differentiation and the number of hemocytes produced depend on the type of pathogen, molt, blood meal and hormone action (de Oliveira and de Souza, 2003; Salcedo-Porras and Lowenberger, 2019). The most represented hemocytes in triatomines are plasmatocytes, granulocytes, cystocytes, prohemocytes, oenocytoids, giant granular cells, and adipohemocytes (Azambuja et al., 1991a). However, to date the role of each of these cell types in the innate immune response of triatomines is not known (Borges et al., 2008; de Oliveira and de Souza, 2003; Salcedo-Porras and Lowenberger, 2019).

There have been some experiments in triatomines that show how different factors modulate the cellular immune response. Such factors include production of ecdysone (Azambuja et al., 1991b; Orchard and Steel, 1980); prothoracicotropic hormone (PTTH; Orchard and Steel, 1980); abdominal distension (Ruiz et al., 2015); hemoglobin in the ingested blood (Azambuja et al., 1991b; Feder et al., 1997); eicosanoids (Garcia et al., 2004a,b); juvenile hormone (Feder et al., 1997); and platelet activation factor (PAF-Castro et al., 2009; Figueiredo et al., 2008; Machado et al., 2006).

Humoral response

Hemocytes are also involved in the production of humoral elements such as reactive oxygen species (ROS), reactive nitrogen species (RNS), prophenoloxidase (PPO) and antimicrobial peptides (AMP). The production of humoral elements is activated when the parasites are isolated for elimination or when they reach the *hemocoel* (Zumaya-Estrada et al., 2017). During the process of phagocytosis, the hemocytes release cytotoxic agents, known as a respiratory explosion, accompanied by a rapid increase in oxygen consumption (Lambeth, 2004; Rosales and Uribe-Querol, 2017). PPO in triatomines has been identified within the hemocytes (Fonseca et al., 2010; Gomes et al., 2003; Hillyer, 2016; Lu et al., 2014) and is expressed in multiple tissues (Favila-Ruiz et al., 2018;

Zumaya-Estrada et al., 2018). AMP are produced mainly in the *fat body* to be released into the hemolymph or by gastrointestinal epithelial cells to be released into the lumen of the intestine (Arrese and Soulages, 2010). In triatomines, the highly conserved AMP include defensins, lysozymes and prolixin (an atacine/diptericine type molecule), which have been described in different bug species and organs but are most common in the GI tract and fat body (Salcedo-Porras and Lowenberger, 2019). Defensins (with a molecular weight of 4 kDa) are cysteine-rich proteins, and their main role is to combat Gram-positive and Gram-negative bacteria, as well as fungi (Araújo et al., 2006; Waniek, 2009). Their mechanism of action is the formation of pores and channels that disrupt the cytoplasmic membranes of bacteria (Yi et al., 2014). Lysozymes (with a molecular weight of 15 kDa) are proteins with enzymatic activity that hydrolyze sugar residues of the peptidoglycans present in the cell walls of Gram-positive bacteria, rupturing the cell (Araújo et al., 2006; Feder et al., 1997; Flores-Villegas et al., 2015). Lysozymes can have a synergistic effect with other AMP such as cecropines and atacines (Ursic-Bedoya et al., 2008; Vieira et al., 2014) to increase antibacterial activity by degrading the bacterial cell wall (Feder et al., 1997; Kollien et al., 2003). Prolixin (with a molecular weight of 20–23 kDa) belongs to the family of glycine-rich proteins (Feder et al., 1997). Its activity is limited to a few Gram-negative bacteria (Feder et al., 1997; Ursic-Bedoya et al., 2011) in the posterior midgut, so its function may be bacterial expansion (Flores-Villegas et al., 2015; Vieira et al., 2014).

The induction of AMP expression is orchestrated by PRPs (i.e. Gram-negative binding proteins- GNBPs, and peptidoglycan recognition proteins- PGRPs), which are able to differentiate and recognize diverse microorganisms such as Gram-negative bacteria, Gram-positive bacteria, fungi and protozoa (Gottar et al., 2006; Hultmark, 2003; Lemaitre and Hoffmann, 2007). The process of microbial recognition also involves C-type lectins (CTLs; Tanji et al., 2006) and thioester-containing proteins (TEP) that bind to and mark pathogens to promote their elimination by humoral or cellular immune mechanisms (Lagueux et al., 2000). Some types of lectins are present in the hemolymph and GI tract of triatomines, where they participate in the recognition of flagellate parasites (Mello et al., 1999; Ribeiro et al., 2014).

After recognition, transduction molecules amplify this signal, resulting in the translocation of transcription NF- κ B family transcription factors (Krem and Cera, 2002) that bind to the promoters of many genes and induce AMP expression (Zumaya-Estrada et al., 2018). There are three signaling cascades involved in the expression of AMPs and with that, the elimination of invading pathogens: the immunodeficiency pathway (IMD), the Toll pathway, and the Jak-STAT pathway. In many insects, the IMD pathway is activated mainly by Gram-negative bacteria (Kleino and Silverman, 2014; Myllymäki et al., 2014), while the Toll and Jak-STAT pathways have dual function, both in embryonic development and in immunity (Lemaitre and Hoffmann, 2007), but Toll responds mainly to infection by fungi and Gram-positive bacteria (Valanne et al., 2011). The Toll and Jak-STAT pathways appear to be signaling pathways that are relatively conserved among hemipterans. On the contrary, for the IMD pathway, there is debate as to whether the pathway is incomplete or significantly reduced (Arp et al., 2016; Benoit et al., 2016; Chen et al., 2016; Saha et al., 2017; Zumaya-Estrada et al., 2018) or whether the IMD pathway is conserved in hemi- and *holometabolic* insects (Panfilio et al., 2018; Ribeiro et al., 2014; Salcedo-Porras and Lowenberger, 2019). Using molecular mechanisms (Ribeiro et al., 2014; Salcedo-Porras and Lowenberger, 2019) it was shown that the IMD pathway is actually functional in hemipterans, specifically, in *R. prolixus*, responding strongly to Gram-negative bacteria (Salcedo-Porras and Lowenberger, 2019). Despite the apparent existence of a pathway to control invasion by certain pathogens, there are experimental studies that suggest that PGRPs can activate multiple immune signaling pathways (Nishide et al., 2019). However, this is a field that needs additional research.

Triatomines and their microbiota

During the life cycle of triatomines, they are exposed to and acquire different microorganisms, especially bacteria (Balczun et al., 2008; Beard et al., 2002; Sassera et al., 2013). *Coprophyagy* is the main process by which triatomines acquire their symbiotic intestinal microorganisms (Eichler and Schaub, 2002). The acquisition of other non-symbiotic organisms can also be through coprophagy or may be associated with the environment and during the blood meal (Engel and Moran, 2013). Thus, the intestinal microbiome of the triatomines is composed of facultative and obligate symbionts, whose diversity and population dynamics (changes in growth, abundance, density, interactions, mortality and migration of each species) is little known, considering that it can vary under different physical conditions (Bolaños et al., 2016).

The microbiome of the triatomines has been described as low-complexity, with only a few dominant genera that vary depending on the species (da Mota et al., 2012; Gumiel et al., 2015), stage of development of the insect, feeding status (fasting/fed; Eichler and Schaub, 2002), food source (Dumonteil et al., 2018), intestinal region sampled (de Oliveira et al., 2018), infection by *T. cruzi* (Azambuja et al., 2005a; Díaz et al., 2016; Taracena et al., 2015) and whether the insect is wild or lab-reared (Waltmann et al., 2019). However, *symbiotic bacteria* are fundamental for the bugs' survival and development (Rodríguez-Ruano et al., 2018), participating in the biosynthesis of B-vitamins (Rio et al., 2016), homeostasis of the immune system (Mann et al., 2020; Teotônio et al., 2019), and the triatomines' ability to competently transmit *T. cruzi* (Goodrich et al., 2014; Teotônio et al., 2019).

Triatomines and *Trypanosoma cruzi*

The complete life cycle of *T. cruzi* occurs in the intestinal lumen of the insect vector and the bloodstream and nucleated cells of a vertebrate host (Azambuja et al., 2005b). There is a mobile and infectious stage known as a trypomastigote (blood trypomastigote if

bugs' immune factors linked to this are not known (Araújo et al., 2006; Ursic-Bedoya et al., 2008; Vieira et al., 2014), though bugs are known to produce AMPs such as prolixin and defensins (Moreira et al., 2014; Ursic-Bedoya et al., 2011).

The activation of the immune response imposes an energetic cost on the triatomine, which can decompensate other physiological functions like, for example, fecundity (Schwenke et al., 2016). In fact, in experimentally infected triatomines a reduction in fertility has been observed (Fellet et al., 2014), perhaps as a consequence of competition for resources between the insect and the parasite. Similarly, *T. cruzi* infection in kissing bugs delays development (Elliot et al., 2015), decreases life expectancy in adults (Cordero-Montoya et al., 2019; Schaub, 1989), decreases blood ingestion (Schaub et al., 2011) and reduces ecological niche (Villalobos et al., 2019). On the other hand, some factors, such as the vector's nutrition, food source, temperature or intestinal microbiota negatively affect the parasite (de Fuentes-Vicente et al., 2017), as well as its replication and the process of differentiation between the epimastigote forms and metacyclic trypomastigotes (metacyclogenesis) (Pimentel-Melo et al., 2020).

Curiously, *T. cruzi* also leads to physiological changes in the triatomine that may be to the parasite's advantage. For example, the levels of digestive enzymes after a meal increase (Buarque et al., 2013), which suggests that the blood consumed will be digested more quickly and the insect will need to feed again sooner, increasing the possibility of transmission of the parasite. Another similar case is that *T. cruzi* seems to promote the activation of certain immune pathways in the insect in order to decrease competition in the microhabitat of the intestine (Castro et al., 2012). All of this reveals a highly complex interaction that is the product of millions of years of coevolution between the two.

Prevention and control

By the end of the 1990s, programs to control *intradomiciliary* vectors drastically decreased the official count of the prevalence of Chagas disease in Latin American countries (Noireau et al., 2009). Vector control programs can be indirect, through housing improvement programs, or direct, using insecticides, traps, or capture of bugs inside or around the domicile (Fig. 9). The improvement of the human dwelling refers to the elimination of possible refuges for the insects to reduce the interaction between humans and triatomines. Filling in holes and cracks with cement and lime (Monroy et al., 2009) or any other material that guarantees a smooth surface (Rojas de Arias et al., 2012), has been highly effective at reducing intradomiciliary populations of some species (Monroy et al., 2009). Other interventions such as the use of screens on doors and windows are essential to prevent transient infestations (Flores-Ferrer et al., 2019; Waleckx et al., 2018). Notwithstanding, economic conditions and lack of government support in many communities have been important barriers to achieving these improvements, such that the use of lower-cost

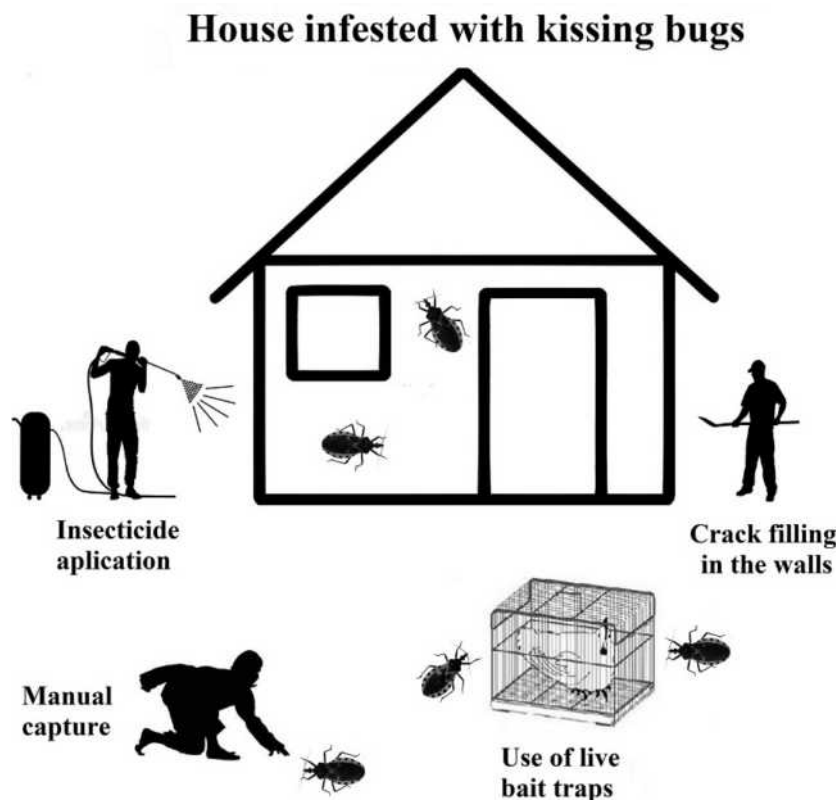


Fig. 9 Control strategies of triatomine bugs (Chagas disease vectors).

locally available materials may be a better option (Monroy et al., 2009). Given these factors, community participation through periodic checks in houses (we suggest every 15 days) seems an ideal strategy for avoiding infestation and keeping homes free of insect vectors (Abad-Franch et al., 2011).

Dusting houses with insecticide has been widely used and is the method preferred by health authorities. Since the 1980s, organochlorate and organophosphate insecticides (such as DDT) and carbamates were the most frequently used, but they were replaced with pyrethroid insecticides due to their lower cost and larger benefits at relatively low doses (Pessoa et al., 2015). While chemical control has been an ally in the reduction of intradomiciliary populations, insect resistance and re-infestation are important factors challenging its use. Resistance results in decreased mortality in the population, which has autosomal semi-dominant inheritance (Pessoa et al., 2015). It is thought that the efficiency of dusting is lower in populations where there is a flow of individuals between domestic, peridomestic, and wild ecotopes, perhaps because of gene renewal through migration (Gourbière et al., 2012) or because it leads to the recovery of the intradomiciliary population a few months after application. New alternatives are being developed to avoid resistance and re-infestation problems by kissing bugs, with encouraging results for their potential use as vector control agents. The most studied alternatives for controlling triatomines are the use of entomopathogenic fungi, paratransgenesis by modifying symbiotic bacteria, and direct capture of the insects using traps.

Entomopathogenic fungi is an example of a technique that can kill 100% of individuals (Flores-Villegas et al., 2016) and up to 92% of eggs under laboratory conditions (Flores-Villegas et al., 2019). An advantage of this biological control method is that the most widely used fungi, *Metarhizium anisopliae* and *Isaria fumosorosea* do not appear to have adverse effects on humans, unlike the toxic effects of chemical control. However, the use of this control agent must be reconsidered since there is controversy over whether a combined effect favors the survival of triatomines infected with *T. cruzi* (Flores-Villegas et al., 2019). While these alternatives are gaining ever increasing attention, more candidates for biological control need to be considered and formulations must be viable under natural conditions.

Paratransgenesis does not attempt to kill the kissing bugs, but rather to eliminate the *T. cruzi* parasite within them and thus avoid transmission to humans (Taracena et al., 2015). The use of genetic engineering to modify triatomines' symbiotic bacteria (i.e. *Rhodococcus rhodnii*) appears to substantially reduce populations of *T. cruzi* (Fieck et al., 2010). Indeed, even unmodified bacteria, such as *Serratia marcescens* have negative effects on the parasite through the pigment prodigiosin (Genes et al., 2011). As such, the bacterial microbiome living in the GI tract or other organs of the triatomines has been investigated with the objective of limiting *T. cruzi* transmission (Buarque et al., 2016). However, the use of bacteria against *T. cruzi* in triatomines may not be feasible given that bacterial taxa vary among kissing bug species and even between developmental stages in the same individual (Oliveira et al., 2018).

The use of traps has not been particularly relevant for the control of kissing bugs and their use has apparently been focused mainly on trapping live insects for research. Some models of traps use live baits or adhesive surfaces to attract or capture the insects (Angulo and Esteban, 2011; Noireau et al., 2002). Other models have replaced live bait with attraction molecules, such as CO₂ or semiochemicals (Abad-Franch et al., 2011; Cardozo et al., 2020), both related to feeding, without yet exploring molecules related to sexual behavior. Light traps have also been used with triatomines, but they have the disadvantage of mainly attracting hungry adult insects and other insects besides triatomines (Noireau and Dujardin, 2001). Thus, there is a need to find effective, highly specific, and economical traps that can be used for vector control in homes.

Finally, it is important to point out that population control of triatomines in any ecotope should be preceded by studies of species identification, behavior, ecology and genetic structure. The goal is to choose the best control strategy, and above all, avoid re-infestation by populations of the same species or by other secondary species that could invade the habitat (Gutiérrez-Cabrera et al., 2018).

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Bed Bugs and Bat Bugs (Cimicidae and Polyctenidae)

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Introduction

Bed bugs (Cimicidae) and Bat bugs (Polyctenidae) are the common names of two insect families belonging to the suborder Cimicomorpha (Hemiptera), in which both are entirely composed by hematophagous-obligated ectoparasites of homeothermic vertebrates (Henry, 2009).

The family Polyctenidae Westwood is a small group comprised of 32 species and five genera of insects that are obligate and permanent parasites living in bats (Chiroptera). There is only one genus, *Hesperoctenes* Kirkaldy, represented in the Neotropical region and the other taxa are distributed among the biogeographic regions in the Old World (Autino et al., 2009; Henry, 2009). Polyctenidae members have a narrow host-specificity among bat families but some evidence suggested they could easily colonize a new host when dispersal barriers were removed (Dick et al., 2009). The knowledge of bat bugs is poorly represented in published literature in comparison with other insect taxa that live on bats and this is also reflected by their scarce presence in museum collections, taxonomic descriptions and experimental studies (Henry, 2009; Amarga and Yap, 2017).

On the other hand, the family Cimicidae Latreille (Hemiptera) is composed of 110 species of temporary ectoparasites that feed on birds, bats, and humans (Krinsky, 2019). There are only three species of cimicids that live in close association with humans: *Cimex lectularius* Linnaeus 1758, *Cimex hemipterus* Fabricius 1803 and *Leptocimex boueti* Brumpt 1910 (Henry, 2009). Although these species are often described as human parasites, they have a wide range of hosts, which includes bats and birds as well. The geographical distribution of these species is very wide. *C. lectularius* is cosmopolitan and prefers temperate climates, whereas *C. hemipterus* is distributed in tropical and subtropical regions of the world and *L. boueti* is restricted to tropical areas of West Africa (Usinger, 1966).

Many other bed bug species can feed on humans in opportunistic host-parasite interaction. For instance, the swallow bugs *Oeciacus hirundinis* Jennyns 1839, *O. vicarius* Horvath 1912, the species that parasitize bats such as *C. adjunctus* Barber 1939, *C. pipistrelli* Jennyns 1839, *C. pilosellus* Horvath 1910, and the chicken bug *Haematosiphon inodorus* Duges 1892 are some examples of cimicids that can feed on humans as a result of the changes in their natural host or the environmental conditions (Usinger, 1966). Since multiple species of Cimicidae can inhabit the human household, accurate identification is crucial to the application of pest control practices (Usinger, 1966; Wawrocka et al., 2015; DeVries et al., 2017). It is worth mentioning that the common name of cimicid species is often formed by the name of their host (e.g., swallow or bat bugs). Herein the common name bat bug is only used to refer to Polyctenidae taxon.

Medical importance

The urban pests are those species involved in the transmission of infectious diseases to humans, habitat degradation and human wellbeing. In other words, the harmful effects of urban pests can involve not only public health but also the domestic economy and the finances of public institutions and the private sector (Harlan et al., 2008). In particular, bed bugs are a difficult urban pest to control because of their biological and behavioral characteristics. Also, their effects on health and their presence in infested environments often manifest late, which makes clinical diagnosis and detection by pest control services more complex (Potter, 2011).

In contrast, there is a lack of information about the potential risks on human health and veterinary effects of bat bugs. However, it is already known that bats could act as reservoirs as well as vectors of zoonotic agents such as *Bartonella* spp. that are commonly found in different bat species and their arthropod ectoparasites around the world (Stuckey et al., 2017). It is worth mentioning that the potential transmission of the pathogenic bacterium *Bartonella quintana*, the etiological agent of Trench fever, was evidenced in *C. lectularius* feces. Although the human body louse *P. humanus humanus* L. 1758 is the main vector of *B. quintana*, the common bed bug was able to acquire, maintain and release viable bacterium in experimental conditions (Leulmi et al., 2015). These results are

important not only to evaluate the potential vector role of bed bugs and bat bugs to transmit infectious bacteria but also for unraveling parasitism mechanisms using these hematophagous insects as a model organism (Tsai et al., 2014; Ehlers et al., 2020).

During bed bugs feeding, they inoculate saliva that contains a large load of proteins with anticoagulant and digestive function, which are potential triggers of the host-immune response (Francischetti et al., 2010). A protein involved in the transport of nitric oxide to the host tissue, nitrophorin, produces vasodilation and increased blood flow to the insect (Valenzuela and Ribeiro, 1998). However, saliva is also involved in the hypersensitivity mechanism mediated by host immunoglobulins, which causes the dermal reaction (Leverkus et al., 2006).

Cimicosis is the clinical manifestation characterized by dermatological lesions produced by bed bug bites during human sleeping hours (Rahlenbeck et al., 2016). The direct effect of cimicosis can vary from scratchy bites to maculopapular rash (Delaunay et al., 2009). However, the magnitude of the allergic reactions relies on the individual immune status. There were reported patients living in conditions of severe infestations with secondary skin infections caused by scratching that developed asthma, anaphylactic reactions, iron deficiency, and anemia (Pritchard and Hwang, 2009). Furthermore, they can lead to indirect health problems, mainly due to their action as psychosocial stressors in the form of discomfort, distress, insomnia, and anxiety in people living in places with high levels of infestation (Berenger et al., 2008; Rahlenbeck et al., 2016).

The transmission of pathogens by bed bugs remains a question that requires further investigation. So far, more than 40 species of microorganisms have been identified in their saliva, intestine, feces, and integument (Delaunay et al., 2011). For instance, resistant strains of *Staphylococcus aureus* and *Enterococcus faecium* were obtained from bed bugs collected from the residences of patients who had been hospitalized for diseases caused by these bacteria (Lowe and Romney, 2011). However, the vector capacity of bed bugs to transmit disease remains under investigation (Goddard and deShazo, 2009; Delaunay et al., 2011). The potential transmission of the hepatitis B virus and human immunodeficiency virus were studied using antigen and DNA isolation techniques from bed bug feces and hemolymph (Webb et al., 1989; Blow et al., 2001). The results revealed that the bugs were able to incorporate these viruses after feeding on infected blood, but both viruses failed to replicate their gene material as well as failed to transmit them via ovaries to their offspring.

Likewise, the role of bed bugs as a vector of the *Trypanosoma cruzi* was studied, because bed bugs and the main Triatominae vector of Chagas disease, *Triatoma infestans* Klug 1834 were found together in dwellings from the endemic zone in Argentina (Jörg, 1992). This first research showed some evidence of acquisition, replication, and transmission of *T. cruzi* in field-collected *C. lectularius*. More recently, the transmission of *T. cruzi* was assessed under in vitro conditions and raised the possibility that both *Cimex* species can act similarly as vectors of this parasite (Salazar et al., 2015; Blakely et al., 2018). One hypothesis is that the biological and behavioral similarities with the Chagas vector (species belonging to the genera *Triatoma* and *Rhodnius*), such as the excretion of feces after each blood-intake and the scratching produced by insect bites could lead to the mechanical infection in their host (Delaunay et al., 2009). However, there is not enough evidence to support the effective transmission of *T. cruzi* by bed bugs, because of experiments with mice that were bitten by infected insects did not develop the disease (Salazar et al., 2015). The potential explanation could be the lack of a sylvatic cycle (wild hosts that act as reservoirs for disease pathogens) acts as a barrier for the vector transmission by bed bugs. Likewise, insect vectors and infectious agents have a common history of co-evolution, in which their survival is guaranteed by coordinated regulation and physiological balance on both sides (Lehane, 1991).

Biology and ecology

Polyctenids are insects morphologically and ecologically adapted to develop their whole life cycle on their hosts. Their body size range between 3 and 5 mm and is dorsoventrally-flattened. They are apterous, without eyes and ocelli, have long legs and sclerotized combs that enable them to move fast through their host (Henry, 2009). The life cycle of bat bugs is noteworthy characterized by viviparity, which favors physical interaction with their host (Dick et al., 2009). The first nymphal stages occur within the female, while the three post-natal stages develop directly on the host and they can feed frequently. Because bat bug dispersal depends only by direct host-host contact, the interactions between bug and bat are assumed to be monoxenous (restricted to a single host species) (Dick et al., 2009; Amarga and Yap, 2017).

Although there are similarities between both families, the main differences are mostly related to the condition of parasitism in each group. In the case of bed bugs, the following biology description is made for the common bed bug *C. lectularius* as a candidate for the whole family. Bed bugs are wingless and oval-flattened insects, in which adults are between 3 and 5 mm in length and have a reddish-brown coloration. The nymphs are yellowish-brown and size between 0.4 and 3 mm (Fig. 1). The eggs are white and cylindrical and are attached to the substrate by a cementing substance during oviposition (Usinger, 1966).

After hatching, the nymphs pass through five stages, and each requires at least one blood intake before moving to the next stage (Davies et al., 2012). Adults ingest large volumes of blood for both their nutrition, as well as for egg production and the spermatogenesis process in females and males, respectively (Reinhardt and Siva-Jothy, 2007). Under optimal conditions, an individual can complete their life cycle in approximately 35 days and continue their adult life for more than 150 days (Polanco et al., 2011). However, the life-span time will depend on host availability, feeding frequency, lifecycle stage, and environmental conditions (Usinger, 1966).

The aggregation behavior and host searching are influenced by the circadian rhythm in bed bugs (Fig. 2). The increase in locomotion occurs in the sunset hours, which coincides with the sleep hours of human hosts (Romero et al., 2010a). Also, the emission of CO₂ by the host encourages bed bugs to leave their shelter, and the active search for the host (Wang et al., 2009). After

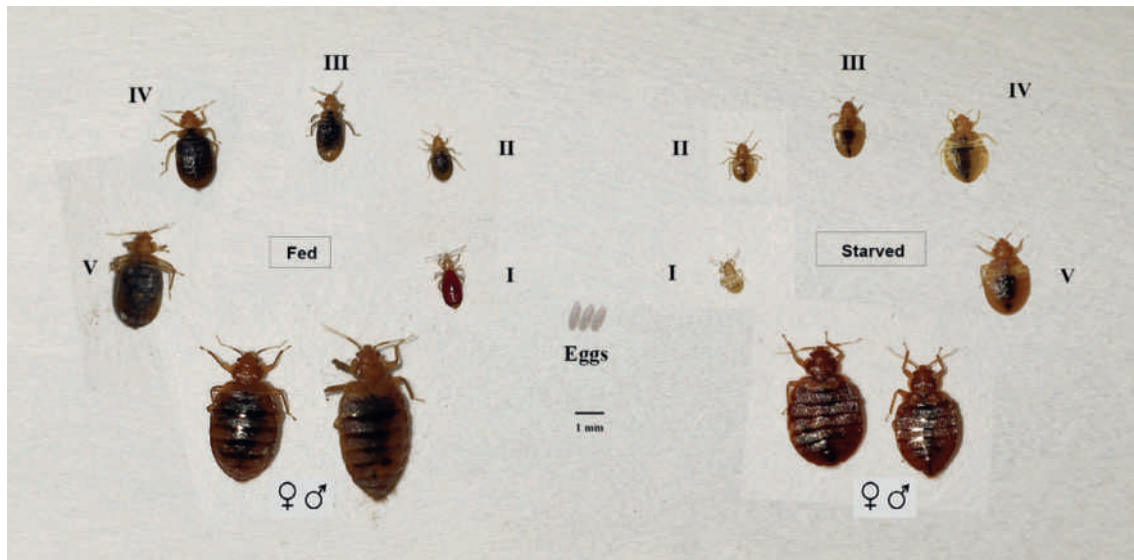


Fig. 1 The life cycle of *C. lectularius* consists of five nymphal-stages before molting to adults. Morphological differences between both sexes could be only recognized by the rounded-shape of the female abdomen, while it is more acute in males. All life-stages are hematophagous-obligated parasites. The figure show bed bugs recently fed (left) and insects starved for 10 days (right).



Fig. 2 Bed bugs live in groups composed of insects with different ages, feeding and mating status, and also they share space with their feces, exuviae, and dead insects. Bed bug aggregations stay hidden in crack or crevices in bed, walls, and furniture.

feeding, they return to the refuges where they remain hidden until again the stimuli emitted by the host are present (Reinhardt and Siva-Jothy, 2007).

Sexual reproduction in bed bugs includes a singular strategy, although it is also present in other species of insects and groups of invertebrates (Tatarnic et al., 2006; Kamimura, 2007; Hotzy and Arnqvist, 2009). Sexual behavior involves two well-defined evolutionary strategies: traumatic insemination and sexual conflict (Stutt and Siva-Jothy, 2001; Reinhardt and Siva-Jothy, 2007; Reinhardt et al., 2009a,b). Adults copulate several times after feeding, immediately and up to 72 h later (Stutt and Siva-Jothy, 2001). During copulation, the male pierces the abdomen of the female, as opposed to the genital opening, with the aedeagus (side and distal organ, usually even, of the copulatory apparatus of male) and then the sperm is released directly into the hemolymph (Usinger, 1966; Stutt and Siva-Jothy, 2001; Reinhardt and Siva-Jothy, 2007). This reproductive strategy is called “traumatic

insemination,” and the stress imposed on females can cause a reduction in their longevity and the inability to maintain the water balance (Stutt and Siva-Jothy, 2001; Morrow and Arnqvist, 2003; Benoit et al., 2012).

To counteract stress, females have developed the mesospermae, a paragenital structure whose function is to drive sperm to the ovaries (Reinhardt et al., 2003; Benoit et al., 2012). Although it is an effective strategy, it is also expensive for females due to the energy expenditure they perform to restore the damaged cuticle, the loss of hemolymph, and the increased risk of infection (Benoit et al., 2012). The genital tract of females is only functional for oviposition (Usinger, 1966).

Sexual conflict is given by the evasive behaviors that the female deploys against the male’s attempts to copulate (Siva-Jothy, 2006). To protect the insemination zone, the females approach the right side of the abdomen close to the substrate. However, the female was unable to perform these movements when it has fed, due to bloating and it is very slow movements. Therefore, insemination by one or more males is facilitated after the females feed and stay aggregated in the shelters.

Additionally, males attempt to copulate with other males or with V-nymphs. This behavior seems to be influenced by the similarity of size with females that preventing successful recognition between individuals of different ages (Stutt and Siva-Jothy, 2001). Insects, like many other organisms, perform movements in response to the perception of a specific stimulus. In bed bugs, the thigmotaxis (direct contact) and visual cues are prevalent in sexual behavior and suggest the absence of specific sexual pheromones that allow males to recognize their sexual partners. However, the presence of chemoreceptors in the aedeagus possibly plays a role in the recognition of females and their genital structures.

Epidemiology and worldwide reemergence

It is now clear that bed bugs are parasites of humans, although this interaction is the result of a much more complex evolutionary process. The suborder Cimicomorpha (Insecta: Hemiptera) shows a wide family diversity associated with a wide variety of feeding habits. However, the hematophagous habit is an uncommon character that is only present in three families and it has possibly evolved independently throughout the history of this taxon (Schuh et al., 2009; Weirauch et al., 2019). As a result of the independence of evolutionary patterns, there are differences in hematophagy between each taxon. As was previously described the Cimicidae and Polyctenidae families are both entirely composed of species of blood-sucking ectoparasites (Cannet et al., 2015). In contrast, the Triatomines are the unique tribe of the kissing bugs (Reduviidae) that parasitized hot-blooded vertebrates and include the species of vectors of Chagas disease. Besides, parasitism strategies are different depending on taxa. Triatomine bugs and bed bugs are temporary parasites of birds and mammals, while Polyctenidae species are permanent bat parasites (Usinger, 1966; Cannet et al., 2015).

The family Cimicidae is a monophyletic group within the Cimicomorpha and its origin dates back to the Cretaceous (115 million years). Recently, the hypothesis of bed bugs evolution has been discussed based on the analysis of phylogenetic, molecular and paleontological evidence (Roth et al., 2019).

The proposal developed by Roth et al. (2019) considers that bed bugs have emerged much earlier than bats (30 million years earlier according to the fossil record), and before the mass extinction event of K/T (Cretaceous-Tertiary limit) species. In other words, although the identity of the ancestral host of bed bugs is unknown, the bats were colonized later and independently by the different lineages of Cimicidae.

Also, the diversification of parasitism in bat hosts (specialist > specialist) and the transition to birds and humans (specialist > generalist) would have occurred because those hosts provided a constant source of food, which was guaranteed by their mode of social organization and the shared use of space in the caves where they live (Booth et al., 2015). Therefore, the ability of bed bugs to seize the ecological opportunity to colonize new hosts was made possible by their genetic variability and phenotypic plasticity that allowed them to change hosts. As consequence, the transition from a specialist ancestor to generalist species seems to be the hypothesis of host-preference, and it could also explain the remarkable ability of bed bugs to adapt to different types of hosts/blood when they are raised in laboratory conditions (Roth et al., 2019).

Additionally, the fossil record has established that the speciation between *C. lectularius* and *C. hemipterus* occurred 47 million years ago and the origins of both species are different. *C. lectularius* derives from a parasitic lineage of bats, while *C. hemipterus* originates from bed bugs parasitized birds. The interpretation of this evidence raises dissents with the hypothesis of co-evolution in the parasitic-host interaction of hematophagous insects (Lehane, 1991). Under this hypothesis, there is a direct relationship between the evolution of ectoparasites and mammal radiation, as has happened between the head lice *Pediculus humanus capitis* De Geer 1767 (Phthiraptera: Pediculidae) and the lineages of the genus *Homo* (Lehane, 1991; Reed et al., 2004). However, in both bed bug species, the divergence occurred before hominids radiation, and parasitism is the result of subsequent events.

Therefore, contact between humans and bed bugs is considered to have happened at the beginning of the Holocene (10,000 years ago), and the interaction between these species occurred intermittently due to the nomadic habits of the first hominins (Usinger, 1966; Potter, 2011). Then, the development of a social way of life and the establishment of communities in permanent villages would have fostered the most intimate and constant association between bed bugs and their human hosts.

The gradual expansion of civilization, urbanization, international trade, and travel allowed bed bugs to cross seas and geographical borders (Potter, 2011). Bed bugs have been exhumed from Egyptian archaeological sites dating back more than 3500 years BC (Panagiotakopulu and Buckland, 1999), arrived Italy in 77 years AD, in the 7th century in China, and in Germany and France in the 11th and 13th centuries respectively. In contrast, the presence of bed bugs in America was recorded from the European conquerors of the 18th century and in subsequent migratory waves (Usinger, 1966).

Bed bug populations persisted, albeit discontinuously and seasonally, around the world until the 1900s. From that moment on, the increase of the human population associated with improvements in well-being and comfort provided by the access to goods and services also had a favorable impact on this plague (Usinger, 1966). During the 1940s, the incorporation of heating systems into households led to an increase in bed bug populations. As a result, the first bed bug epidemics began in the major cities of developed countries (Johnson, 1941).

In the past, bed bug infestations were managed with home-made treatments and control practices aimed at improving the hygienic conditions. Thus, people living in poverty were often the most affected, because they had limited resources to overcome the infestation. However, as a result of the reduced effectiveness of the treatments applied, both the rich and the poor struggled constantly to control bed bugs (Potter, 2011).

The earliest report of bed bug control was recorded in the Greek and Roman literature around 400 years BC. The common practice was to hang the leg or skin of animals (rabbit, deer or bear) or by placing a container of water under the bed to keep the bed bugs away (Usinger, 1966). During the 18th century, pest control companies began to offer bed bug control services and included careful inspection as the key tool to obtain adequate control (Potter, 2011). Also, the use of plant extracts as insecticides and repellents was used in Europe and Asia. Thus, a systematized list of recommended plant species to be placed under the bed as bed bug repellent was published for the first time in China in 1758 (Wang and Wen, 2011).

The safest methods for control operators and residents, but less effective for insect control, were used combined with more harmful techniques that guaranteed the extermination of bed bugs. An example of this was the use of mercury chloride, popularly known as bed bug poison (Usinger, 1966). Although applying mercury chloride to cracks and crevices in beds and walls provided satisfactory short-term results, it was also the cause of poisoning and death for residents. Furthermore, re-infestation was recurrent due to the lack of residual effect of these insecticides (Potter, 2011).

During the 1940s, the massive use of dichlorodiphenyltrichloroethane (DDT) caused a significant decrease in bed bug populations, among other pest insects. The success of DDT was due to its mode of action, fumigant power, and long residual effect. Unlike other products that required direct contact with insects, DDT guaranteed the poisoning of bed bugs that remained hidden in their shelters thanks to their fumigant power or by contact when walking on previously sprayed surfaces (Usinger, 1966).

Recommendations provided by the USDA (1953) suggested the treatment of mattresses and bed frames with DDT. It should be noted that, in addition to its effectiveness, the use of DDT offered other advantages such as a variety of formulations, incorporated in paints or impregnated in wallpaper, and could be used in multiple points (e.g., beds, mattresses, and toys) without the requirement of personal protection, equipment or professional assistance. Also, DDT was easily accessible because was purchased in groceries with relatively low cost (Potter, 2011; Koganemaru and Miller, 2013).

The use of DDT and other organochlorines, as well as the subsequent development and application of new classes of insecticides (organophosphates, carbamates, and pyrethroids), collaborated in reducing bed bug populations until almost eliminating them, especially in developed countries. Infestations became sporadic and were relegated to poor areas due to a lack of adequate sanitation and a lack of economic resources to buy effective chemical products (Krueger, 2000).

The application of insecticides as part of pest control programs allowed the effective management of bed bugs and brought relief to society at the time. An exemplary case was the performance of research and community action teams within the framework of the "National and Patriotic Public Health Campaign" implemented by the Government of China during the period 1950–80. The program included bed bugs as one of the "four pests" (along with rodents, flies, and mosquitoes) that needed to be eradicated (Cao and Liu, 2000). Treatments consisted of the application of lindane (γ -cyclohexane; organochlorine), and then fenthion (O, O-dimethyl and O-4-methylthio-m-tolyl phosphorothioate; organophosphate), combined with physical control strategies and a community educational program. During the 1980s, with the incorporation of new active ingredients such as DDVP (2,2-dichlorovinyl dimethyl phosphate; organophosphate) and the pyrethroids deltamethrin [(1R, 3R) -3- (2, 2-dibromovinyl) -2 (RS) -2-dimethylcyclopropane carboxylate (RS) -cyano-3-phenoxybenzyl], cypermethrin [(1R S) -cis, trans-3- (2,2-dichlorovinyl) -2,2-dimethylcyclopropane carboxylate (RS) -cyano-3-phenoxybenzyl] and permethrin [3-phenoxybenzyl (1R S) cis, trans-3- (2, 2-dichlorovinyl)—2, 2-dimethylcyclopropane carboxylate], favorable results were achieved in the management of the pest (Wang and Wen, 2011).

It should be noted that the intensive use of insecticides can negatively affect the pest control program. This is because continuous exposure to the insecticide only eliminates susceptible individuals, and survivors will be those individuals who possess any insecticide resistance mechanism. Despite the forthcoming risk of insecticide resistance development in bed bugs, the problem did not seem relevant enough. In particular, because of bed bug infestations remained in developing countries and communities with socio-economic vulnerability (Potter, 2011).

Although prevention and control campaigns were implemented as public health and sanitization policies, the first reports of the potential development of insecticide resistance were published. The first official data on bed bug control failures were obtained at the Naval Station Pearl Harbor, (Hawaii) in 1947 (Johnson and Hill, 1948).

During the following years, DDT-resistant bed bugs became common in several countries (Chen et al., 1956; Busvine, 1958), probably associated with spraying homes with DDT as part of the eradication of malaria (Rafatjah, 1971). Due to the increase in cases of resistance to DDT, insecticides such as lindane, malathion, and diazinon were used as alternatives (Gratz, 1959). However, the health problems derived from the use of these insecticides, as well as their persistence in the environment, led most of the countries to legislate environmental toxicology and human health. Starting in the 1970s, the use of DDT and other organochlorines began to be prohibited, and restrictions were imposed on other families of insecticides (Potter, 2011).

Pyrethroid insecticides have extensively occupied the market for urban pest control insects for the past 55 years (Zaim and Guillet, 2002). The World Health Organization recommends the use of pyrethroids for the treatment of materials and environments for the control of mosquitoes of medical importance (World Health Organization, 2005). The success of pyrethroids is related to their higher toxicity compared to the original pyrethrins, and to their better residual effect and lower photolability than plant extracts (Elliott et al., 1978; Zerba, 1988). Some compounds are very effective and cause rapid overturning, that is, a state of incoordination and locomotor instability that, depending on the dose, will lead to paralysis and eventually death. Furthermore, pyrethroids can have a repellent effect on insects, or antifeedant effect (Sfara et al., 2006; Romero et al., 2009b).

Currently, bed bug populations have been restored and are spreading globally (Romero et al., 2007). Numerous investigations have reported an increased number, intensity, and distribution of bed bug infestations in Africa (Gbakima et al., 2002), Europe (Masetti and Bruschi, 2007; Kilpinen et al., 2008; Fuentes et al., 2010; Levy-Bencheton et al., 2010), United States (Krueger, 2000; Anderson and Leffler, 2008), Canada (Hwang et al., 2005), Argentina (Cáceres et al., 2019), Australia (Doggett and Russell, 2008) and Southeast Asia (Lee et al., 2008; Suwannayod et al., 2010).

The first reports of bed bugs reemergence began in the mid-1990s and the number of infestations started to increase in several countries. For instance, United Kingdom pest control companies reported an increase in bed bug infestations at least to 100% in only 3 years (Koganemaru and Miller, 2013). Similarly, Doggett and Russell (2008) surveyed over 120 pest control professionals in Australia and recorded a 4500% increase in bed bug infestations between 1999 and 2006.

Many factors are involved in the re-emergence of this pest: the increase in tourism travel and commercial exchange (Potter, 2005), the furniture recycling, the lack of surveillance actions, and the insufficient variety of insecticides available (Koganemaru and Miller, 2013). In addition to the factors mentioned, resistance to insecticides is considered one of the main causes of the worldwide resurgence of bed bugs (Doggett and Russell, 2008; Romero et al., 2007).

Insecticide resistance is a common trait in urban insect pests and with medical relevance (Scott et al., 1990; Vassena et al., 2003; Picollo et al., 2005; Roca Acevedo et al., 2009; Knox et al., 2014; Koo et al., 2014). According to the Insecticide Resistance Action Committee (IRAC), resistance to insecticides consists in the inheritable change in the susceptibility of an insect population, which manifests itself with the repeated failure of an insecticide product to produce the expected effect when applied according to the label recommendations for the insect target (IRAC, 2018).

Resistance is developed by the irrational use of an insecticide on the pest species. In this way, the artificial selection pressure exerted by the chemical treatment will produce the death of the susceptible individuals and the survival of the resistant ones, and consequently, the evolution towards populations resistant to the insecticide. In addition to selection pressure, inheritance and gene frequency are intrinsic factors that strongly influence the evolution of resistance (Brogdon and McAllister, 1998).

A major concern with this situation is the lack of insecticides with different modes of action in order to switch among active ingredients that could be applied and to allow rational insecticide resistance management (IRM). Currently, neurotoxic insecticides such as pyrethroids and neonicotinoids are the only available for urban pest control and recommended for use against bed bugs are mainly the (EPA, 2019a).

However, the loss of efficacy of pyrethroids often leads to the use of insecticides that are prohibited or with a recommendation for limited use. For each of these groups, some degree of resistance has been demonstrated in bed bugs, which could suggest not only the illegal use of insecticides but also the presence of bed bugs populations with multiple resistance, that is, they possess more of a resistance mechanism that allows surviving the effect of more than one group of these insecticides (Adelman et al., 2011; Zhu et al., 2013; Gordon et al., 2014; Benoit et al., 2016; Romero and Anderson, 2016).

Many bed bug populations possess point mutations in genes that encode voltage-dependent sodium ion channels, that is, the target site of DDT and pyrethroids. Knockdown resistance (*kdr*), as this resistance mechanism is known, usually results from a replacement or exchange of the amino acid leucine by histidine, serine, or phenylalanine in the S6 hydrophobic segment in domain II of the protein (Williamson et al., 1996; Martínez-Torres et al., 1998). These mutations cause pyrethroids to lose their effectiveness in acting on the target site. V419L and L925I mutations in the voltage-dependent sodium ion channel α subunit have been described as being responsible for pyrethroid resistance in bed bugs (Yoon et al., 2008; Zhu et al., 2010).

Furthermore, many resistant *C. lectularius* populations have been shown to have an increased ability to detoxify insecticides (Romero et al., 2009a; Mamidala et al., 2011; Zhu et al., 2012; Romero and Anderson, 2016; Cáceres et al., 2019). This mechanism may be due to gene overexpression of one or more of the main classes of detoxifying enzymes: cytochrome P450 monooxygenases (OFM), carboxylesterases (EST) and glutathione S-transferases (GST). In particular, the overexpression of several P450 genes has been related to resistance to deltamethrin in several populations of bed bugs (Zhu et al., 2013). Pyrethroid detoxification activity has also been observed by esterases at the molecular level (Adelman et al., 2011) and through the use of synergists (Cáceres et al., 2019).

Insect cuticle modification is a unique and efficient mode of insecticide resistance (Lilly et al., 2016), that can operate by the reduction in the penetration of the insecticide and its passage to the hemolymph. This may be due, in part, to the production of detoxifying enzymes in epidermal cells (Mamidala et al., 2012; Zhu et al., 2013; Benoit et al., 2016) or to the over-expression of CPR genes (cuticular proteins) in resistant bed bugs (Koganemaru et al., 2013). In turn, as evidence of the effect of reduced penetration, the susceptibility to the insecticide is greater when it is injected directly into the hemolymph, unlike application by topic (Adelman et al., 2011; Koganemaru et al., 2013).

Also, there is a known behavioral resistance mechanism in several insect species (Wang et al., 2004; Buczkowski et al., 2005; Rust and Saran, 2006; Chareonviriyaphap et al., 2013), but less studied in bed bugs. At the beginning of an insecticide poisoning, the symptoms that manifest are hyperactivity, irritability or repellency (Alzogaray and Zerba, 1997). Instead, resistant individuals can

perform behaviors that reduce exposure to the toxic compound, or that allow it to survive in an environment that is harmful to most insects (Haynes, 1988; Chareonviriyaphap et al., 2013).

In particular, it has been observed that resistant bed bugs were able to avoid pyrethroid-treated areas when a heat source, as an attractive host stimulus, was present (Romero et al., 2009b). Additionally, bed bugs can remain in shelters impregnated with insecticides, and neither aggregation behavior nor survival was affected by the residual action of the insecticide (Moore and Miller, 2009; Romero et al., 2009b). Although these types of responses are considered innate behaviors, the underlying genetic basis requires much more in-depth analysis in bed bugs.

Traditional and new strategies for bed bugs control

The infestations of *C. lectularius* and *C. hemipterus* are usually achieved through a set of strategies that include non-chemical (physical) and chemical (insecticides) methods. Nowadays, a variety of non-chemical methods are available such as aspiration, heat, passive monitoring traps (sticky traps) and exclusion, and their effectiveness will depend on the magnitude of infestation (Potter, 2011). However, visual inspection made by pest control operators is an important first step to take before the application of any control treatment. Many pest control companies are providing trained dogs, which are capable to detect (smelling bed bug odors) the bed bugs refugees in rooms or furniture. Also, the use of monitoring devices can aid in early detection and could be more effective than visual inspections. In the early stages of an infestation, mechanical removal through aspiration or the heat and steam treatments could be useful for controlling or even killing bed bugs (Koganemaru and Miller, 2013).

However, insecticides are central for any control pest service aimed to reduce bed bug infestations (Potter, 2011). Currently, insecticides with different modes of action are used to bed bugs control (Davies et al., 2012; Potter and Haynes, 2014). However, pyrethroid insecticides applied alone or in combination with neonicotinoids, are widely used by pest control professionals in the United States. Other insecticides, such as pyrroles, desiccant powders, and insect growth regulators are used but to a lesser extent than pyrethroids (Romero et al., 2010b; Goodman et al., 2012; Potter et al., 2015).

The widespread of insecticide-resistant bed bugs has renewed interest in developing more effective control methods. In particular, given that bed bugs can develop high resistance to pyrethroids and also could display multiple resistance mechanisms that making ineffective almost any known insecticide. The addition of enzymatic inhibitors, e.g., piperonyl butoxide (PBO), in pyrethroids formulations can be a good option to improve pyrethroid toxicity (Cáceres et al., 2019). However, the PBO is only effective against resistant bed bugs that possess metabolic resistance (with P450 enzymes as the main responsible for the detoxification of insecticides) (Romero et al., 2009a).

The presence of mutations at the target site can be counteracted by the selection of insecticides with different modes of action. For instance, chlorfenapyr [4-bromo-2- (4-chlorophenyl) -1-ethoxymethyl-5-trifluoromethyl-1H-pyrrole-3-carbonitrile] (Pyrroles—Group 13: decoupling of oxidative phosphorylation) is considered an alternative, although its effect is slow-acting (Romero et al., 2010b). There are a large number of insect growth regulators (IGR) but their efficacy in the control of bed bugs is poorly studied (Goodman et al., 2012; Koganemaru and Miller, 2013). For instance, there are some evidence that the continuous exposure to lufenuron [1- [2, 5-dichloro-4- (1, 1, 2, 3, 3, 3-hexafluoropropoxy) phenyl]—3- (2, 6-difluorobenzoyl) urea]—Benzoylurea, Group 15: inhibitors of chitin synthesis caused significant reduction in experimental colonies of bed bugs susceptible and resistant to pyrethroids (Garramuño, 2018). This result demonstrates the potential efficacy of this IGR in controlling bed bugs, while also being a suitable alternative for managing pyrethroid resistance.

Although neurotoxic insecticides will continue to play an important role in bed bug control, the future will also depend on the development of new insecticide formulations, safe for human health and environmentally friendly. Nowadays, formulations with organic solvents or the addition of surfactants (and cosurfactants) are needed to improve the power and residual effect of the active ingredient (Pratap and Bhowmick, 2008). Also the formulation of insecticides based on emulsions allowed to reduce the use of organic solvents, but at the same time, they require the incorporation of greater amounts of surfactants and other additives to improve their properties. All these methods have allowed more efficient use of insecticides but have not been able to solve the problem caused by their discharge into the environment, the effects on non-target organisms, or reduce production/application costs (Bhagat et al., 2013).

On the other hand, the incorporation of alternatives to insecticides should be incorporated in programs for bed bugs integrated pest management (IPM). Control strategies based on modifying the behavior of blood-sucking insects, such as repellents and attractants, are suitable approaches to overcome insecticide resistance and provide protection to people (Foster and Harris, 1997).

The use of repellents is one of the best known and most effective methods for the control and prevention of insects. As a general definition, a repellent is a chemical compound that causes insects to move away from the emitting odor source (White and Moore, 2015). N, N-diethyl-m-toluamide (DEET) is the most widely used insect repellent (Moore and Debboun, 2007) because of its great efficacy, a broad spectrum of use and low toxicity in humans (EPA, 2019b). However, most of the repellency studies of DEET and other natural and synthetic compounds have been carried out on mosquitoes (Swale et al., 2014; de Souza et al., 2019), ticks (Jaenson et al., 2005), or triatomines (Zermoglio et al., 2015; Reynoso et al., 2017). In comparison with these insect pests, the bed bug repellence was poorly studied (Kumar et al., 1995; Wang et al., 2013). For instance, the repellent effect of DEPA (N, N-diethylphenylacetamide) on *C. hemipterus* was only significant at very high concentrations, compared to the effective dose in other insect species (Kumar et al., 1995). Also, the starvation period can promote repellence by DEET in bed bugs. In contrast, this

behavior was different in those resistant bed bugs, which were more tolerant to different concentrations of DEET (Vassena et al., 2018).

Chemical communication plays an important and essential role in insect survival (Pickett et al., 1997). Semiochemicals are natural compounds used by insects as specific chemical signals that modify behavior or certain physiological processes (Wyatt, 2009). Insects use semiochemicals to host search or for mating, to avoid competition or natural enemies (Reisenman et al., 2016). These natural compounds have high specificity and are probably safer, both of which are advantages compared to insecticides and synthetic compounds used for pest control. These properties make them promising tools for the integrated management of numerous pest insect species.

A wide range of semiochemicals (volatiles and non-volatiles) are involved in numerous insect behaviors. The chemical composition of alarm and aggregation pheromones has been already identified in bed bugs (Siljander et al., 2007; Gries et al., 2015), as well as the importance of non-volatile feces compounds in aggregation behavior (Olson et al., 2017). In contrast, there are components of the insect cuticle that not only have a primary role in protecting the internal conditions and regulating dehydration but also can be a source of chemical clues that insects use for recognition in different life contexts (among social insect castes, sexes, parents and offspring). Studies done on bed bugs have not yet been able to elucidate the effects of cuticular components on behavior (Pfiester et al., 2008; Domingue et al., 2010), and the chemical composition has not been exhaustively studied either (Feldlaufer and Blomquist, 2011).

Semiochemicals have great potential for use in bed bug management, for example, bed bug semiochemicals could be incorporated in bait traps for both control treatment and monitoring (Weeks et al., 2011). Furthermore, the use of these traps after the application of insecticides would also make it possible to determine the success of the control effort, or the need to continue treating the pest (Koganemaru and Miller, 2013).

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Fleas (Siphonaptera)

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Etymology

Name: German: Floh; English: flea; origin in the old German term: fliehan = flee; French: puce; Spanish: pulga; Italian: pulce; Chinese: 跳蚤; Russian: Блошинный.

Morphology, biology and ecology

The approximately 2500 species of fleas (Tables 1–3; Figs. 1–3 and 7–9) belong to the group of the insects, which got their Latin group name (*insectus* = carved in) due to the existence of deep incisions between head and breast and between breast and abdomen. About 94% of them suck blood on mammals, the remaining ones on birds. All these fleas are characterized by the facts that they are wingless, but possess six long, strong legs, which allow jumps of up to 35 cm in length to escape enemies such as birds or to reach preys (warm blooded animals), where they may suck blood, which they need obligatorily to proceed growth and reproduction.

Their laterally depressed body is principally segmented in head, breast, and abdomen, whereby the rather tiny head and the breast are not clearly separated. The 6 ft are each equipped with a pair of claws, which allow a firm hold at the hair of their hosts (animals, humans) (Fig. 4A and B) and on the carpet (Fig. 5).

Their head capsule is rather small and dorsally equipped by a pair of segmented antennae, which might be redrawn into a deepening of the cuticular head capsule (Figs. 1, 3, 4A and B, 5), when moving in the fur of host animals or on the body of humans respectively in their clothes. At each of both lateral sides of the head one rather primitive eye (ommatidium) is located consisting of four crystal cone cells and two pairs of main and covering cells. At the ventral side of the head the mouthparts of the sucking type are present enabling the flea to penetrate with their mouthparts into the skin of their hosts and to ingest their blood, which they keep fluid by injection of saliva containing anticoagulation components.

The terminal end of the rather large abdomen is equipped with a so-called pygidial-plate, which contains numerous sense hair, which are called trichobothria and which apparently recognize air movements and odors of potential hosts, so that flea can reach them by help of their enormous jumps of up to 30 cm or become able to flee from enemies.

The individual holometabolous life cycle of fleas comprises the following stages: eggs, three larval stages, a pupa and the male and female stages. Fleas reach a life period of a maximum of about 1½ years. The copulation often takes place on a host, whereupon, for about 3–6 weeks, the female daily lays 10–25 eggs that drop from the host's surface to the ground. The cat flea for example deposits about 800–1000 eggs like this, while the human flea produces in mean only 450 eggs. After ~5 days (temperature dependent), the eyeless larva hatches from the egg, which is also called "wireworm" because of its bristled appearance. These stages mainly feed on detritus. However, they also need proteins for their development. They obtain them by ingesting portions of dead adults or by ingesting fecal drops, which contain remnants of blood being constantly excreted by the adult fleas.

After about 2–3 weeks and two molts, the larvae spin themselves a silky cocoon by help of their salivary glands. Within about 3 more days, the larva develops into a pupa and stays—depending on the microclimate—in this immobile condition for about 1–2 weeks. The hatching from the pupal cocoon is mostly triggered by an external stimulus, e.g., vibrations indicating the arrival of a host. If this stimulus is omitted—because no host visits the flea populated lair—the adult flea can stay for months in this pupal cocoon, apparently with a reduced metabolism. The arrival of the first host then provokes a sudden mass exodus of the adult fleas from all present pupae. This occurs identically in the cases of a resettlement of an old nest by birds, of a kennel by dogs or of an apartment by humans. Because 99% of a flea population is located on the ground, control measures have also to be directed against these stages. This can be done by help of sprays containing chemical compounds, which prevent the molt of the larval stages and thus stop the occurrence of new replication-competent adults. If at the same time adult insects are controlled with insecticides (today: pyrethrum or pyrethroid = original or synthesized agents from chrysanthemum), even massive flea infestations can be eliminated from dwellings and animal lair.

Table 1 Important international fleas, their hosts and their transmitted agents of diseases.

Species/geographical distribution	Length (mm)	Main hosts/humans	Transmitted pathogens	Combs at head front	Combs at pronotum
<i>Amphipsylla primaris</i> /EA, E	M 1.6 F 2.5	Rodents, humans	<i>Yersinia pestis</i> , erysipeloid	—	+
<i>Archaeopsylla erinacei</i> /E	M 4 F 3	Hedgehogs, foxes, dogs, rats, humans	Bacteria, rickettsia	+	+
<i>Ceratophyllus gallinae</i> /E	M 3.0 F 3.5	Chickens, turkeys, dogs, cats, humans	Mechanically many pathogens, Omsk hemorrhagic fever	—	+
<i>Ctenocephalides canis</i> /worldwide	M 2.5 F 3.5	Dogs, cats, humans	Larvae of cestodes (<i>Dipylidium</i> , <i>Hymenolepis</i>), <i>Yersinia pestis</i> , <i>Mycoplasma hemofelis</i> (formerly <i>Haemobartonella felis</i>)	+	+
<i>Ctenocephalides felis</i> /worldwide	M 2.5 F 3.0	Cats, dogs, humans	Larvae of cestodes (<i>Dipylidium</i> , <i>Hymenolepis</i> , <i>Dipetalonema</i>) <i>Yersinia pestis</i> , viruses, <i>Rickettsia felis</i> , <i>R. typhi</i>	+	+
<i>Ctenophthalmus breviatus</i> /E	M 2.5 F 3.0	Murine rodents, humans	<i>Yersinia pestis</i> , tularemia, pseudotuberculosis, bartonellosis	—	—
<i>Echidnophaga gallinacea</i> /worldwide	M 2.0 F 3.0	Chickens, dogs, cats, pigs, humans	Bacteria, <i>Yersinia pestis</i>	—	—
<i>Leptopsylla segnis</i> /E, EA	M 1.6 F 1.8	Murine rodents	Mechanically many pathogens, cestodes	+	+
<i>Megabothris species</i> /E, A	M 2.7 F 3.5	Murine rodents, humans	Hemorrhagic nephrosonephritis	—	—
<i>Nosopsyllus segnis</i> /E	M 1.8 F 2.0	Rats, humans	<i>Yersinia pestis</i> , erysipeloid, larvae of cestodes	—	+
<i>Pulex irritans</i> /worldwide	M 2–2.5 F 4	Humans, foxes, mice, domestic animals	<i>Yersinia pestis</i> , erysipeloid, larvae of cestodes	—	—
<i>Spilopsyllus cuniculi</i> /E	M 1.6 F 2.0	Rabbits, humans	Myxomatosis virus, <i>Francisella tularensis</i>	+	+
<i>Tunga penetrans</i> /E	M 0.7 F 5–6 (fed)	Humans, many animals	Penetrates skin, bacterial superinfections	—	—
<i>Xenopsylla cheopis</i> /warm countries	M 1.5 F 2.5	Rats, rodents, sheep, cats, humans (tropics)	<i>Yersinia (Pasteurella) pestis</i> , <i>Rickettsia typhi</i> , erysipeloid, cestodes larvae	—	—

A: Asia; E: Europe; EA: East Asia; M: male; F: female; +: present; —: absent.

Table 2 Oversight of the flea genera on the continents.

Eurasia

- *Amalaraeus*
- *Amphalius*
- *Amphipsylla*
- *Ceratophyllus*
- *Citellophilus*
- *Coptopsylla*
- *Ctenocephalides*
- *Ctenophthalmus*
- *Echidnophaga*
- *Frontopsylla*
- *Megabothris*
- *Mesopsylla*
- *Neopsylla*
- *Nosopsyllus*
- *Ophthalmopsylla*
- *Oropsylla*
- *Paradoxopsyllus*
- *Pulex*
- *Rhadinopsylla*
- *Stenoponia*
- *Stivalius*

(Continued)

Table 2 (Continued)

- <i>Xenopsylla</i>
Afrotropical region
- <i>Dinopsyllus</i>
- <i>Chiastoposyllus</i>
- <i>Ctenophthalmus</i>
- <i>Leptophylla</i>
- <i>Listropsylla</i>
- <i>Pulex</i>
- <i>Synopsyllus</i>
- <i>Synosternus</i>
- <i>Tunga</i>
- <i>Xenopsylla</i>
- <i>Xiphiopsylla</i>
North America
- <i>Anomiopsyllus</i>
- <i>Catallagia</i>
- <i>Ceratophyllus</i>
- <i>Ctenocephalides</i>
- <i>Foxella</i>
- <i>Hoplopsyllus</i>
- <i>Hystriochopsylla</i>
- <i>Malareus</i>
- <i>Meringis</i>
- <i>Opisodasys</i>
- <i>Orchopens</i>
- <i>Oropsylla</i>
- <i>Polygenis</i>
- <i>Pulex</i>
- <i>Stenistomera</i>
Central and South America
- <i>Adoratopsylla</i>
- <i>Craneopsylla</i>
- <i>Cediopsylla</i>
- <i>Delostichus</i>
- <i>Hectopsylla</i>
- <i>Hoplopsyllus</i>
- <i>Neotiphloceras</i>
- <i>Plocopsylla</i>
- <i>Plusaetis</i>
- <i>Polygenis</i>
- <i>Pulex</i>
- <i>Rhopalopsyllus</i>
- <i>Sphinctopsylla</i>
- <i>Tiamastus</i>
- <i>Tunga</i>
Australia
- <i>Ctenocephalides</i>
- <i>Pulex</i>
- <i>Stivalius</i>
- <i>Xenopsylla</i>

The most important flea species parasitizing on humans and its pets are listed in [Table 1](#). They are not only of importance because of their bloodsucking activity, but some of them serve (by contaminated mouthparts) as mechanical vectors of pathogens or as intermediate host of parasites.

More than 2000 species of fleas have been described worldwide, of which about 80 are found in Central Europe. However, only 5–10 species are really very important with respect to a potential vectorship of agents of diseases of humans and animals ([Table 1](#)). The species diagnosis of fleas is rather difficult and cannot be done just by consideration of the host, where the flea had been detected.

The last larval stage ([Figs. 1, 2, and 5](#)) of the fleas is transformed into the pupal stage wherein the adult female or male flea is developed. The whitish eggs are excreted in different series ([Fig. 5](#)). The females of *Pulex irritans* produce in total up to 450 eggs during their life, while the cat flea (*Ctenocephalides felis*) can lay up to 1000 eggs, which are excreted in daily packages of about 25. Thus, a single, fertilized female flea may start a real flea invasion in a dwelling.

Table 3 Fleas: molecular species/strain diagnosis, mt-16rDNA region differences.

	<i>Ctenocephalides felis</i>	<i>Ctenocephalides canis</i>	<i>Xenopsylla cheopis</i>	<i>Tunga penetrans</i> Human	<i>Tunga penetrans</i> Dog 1	<i>Tunga penetrans</i> Dog 2
<i>Ctenocephalides felis</i>	–	5%	18%	18%	30%	49%
<i>Ctenocephalides. canis</i>	5%	–	17%	19%	32%	61%
<i>Xenopsylla cheopis</i>	18%	19%	–	20%	33%	46%
<i>Tunga penetrans</i> Human	18%	19%	20%	–	23%	48%
<i>Tunga penetrans</i> Dog 1	30%	32%	33%	23%	–	20%
<i>Tunga penetrans</i> Dog 2	49%	61%	46%	48%	20%	–

Differences in sequences in the mt16S-rDNA-region of different flea species.

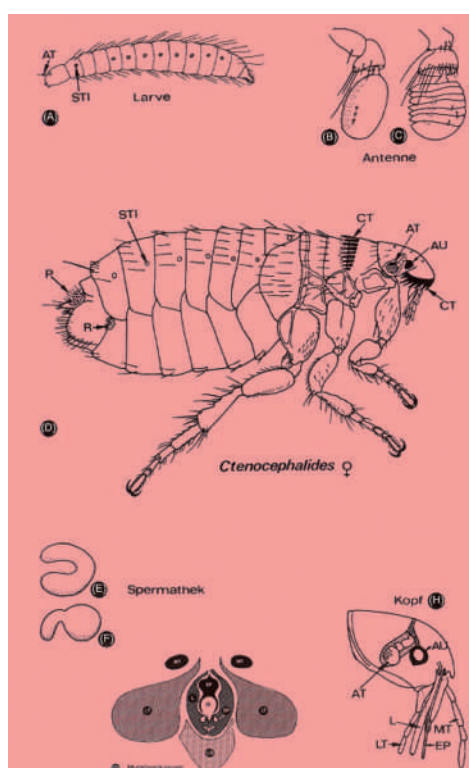


Fig. 1 Diagrammatic representation of the morphology of fleas. (A) Larva ("wireworm"). (B) Antenna of *Tunga penetrans*. (C) Antenna of *Pulex irritans*. (D) Female of *Ctenocephalides felis*. (E and F) Receptacles of *Xenopsylla cheopis* (E) and *Pulex irritans* (F). (G) Cross section through the biting apparatus (epipharynx and laciniae are injected into the skin during bite). (H) Lateral aspect of a flea head (simplified). AT: antenna; AU: eye (ocellus); B: blood, feeding channel; CT: ctenidia; EP: epipharynx (represents the food channel); L: laciniae, maxillar stinging bristles each containing a salivary ductule; LB: labium; LT: labial palpus; M: maxillar palpus; P: pygidial plate, sensillum; R: receptaculum seminis; SP: salivary ductule; STI: stigma.

The larval development takes about 14 days (at 18–29 °C and at 70–90% humidity) in the case of the so-called human sand flea *Tunga penetrans*. In the case of chicken fleas (*Ceratophyllus gallinae*), dog fleas (*Ctenocephalides canis*) and cat fleas (*C. felis*), this development is somewhat shorter. However, in the literature also periods have been reported ranging from 16 to 460 days. The larval development occurs mainly inside/on the resting sites of their hosts. Thus, to verify the existence of fleas in a dwelling it is needed to start the search for the whitish, worm-like maggots. The so-called human flea *Pulex irritans* proceeds its development in dusts close to human beds and they can only hardly be seen by naked eye, since they are very often covered by dust. These flea larvae do not suck blood since their chewing mouthparts are unable to cut the skin of humans or animals. However, on the other hand, adult fleas of both sexes have biting-sucking mouthparts (Figs. 4B, 6, and 8A), which can enter the skin of humans and animals. By their help they suck blood several times per day, whereby they inject compounds, which keep the blood fluid, but which also

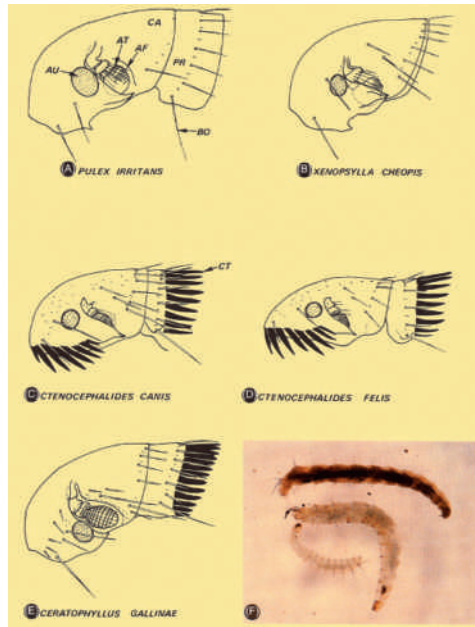


Fig. 2 (A–E) Diagrammatic representation of heads of some important fleas. The occurrence of combs (PC, GC), the arrangement of setae (SE) and the shape of the antenna (AT) are species specific. AF: groove of the antenna; AT: antenna; AU: eye, ommatidium; BO: bristle; CT: ctenidial combs. (F) Microscopical aspect of three differently aged larvae of *C. felis*.

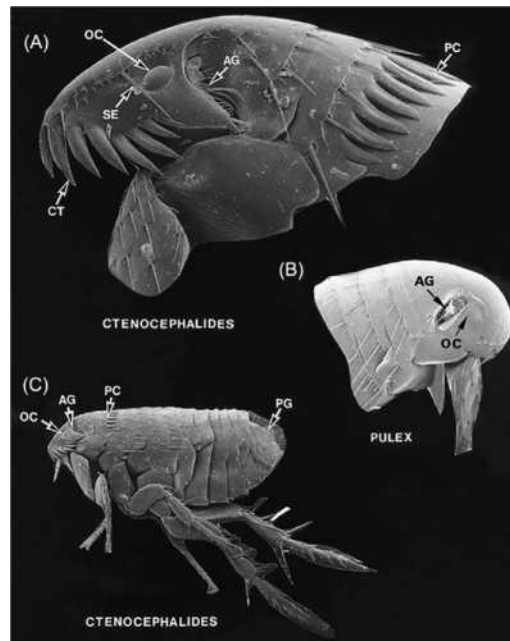


Fig. 3 Scanning electron micrographs of fleas of dogs, cats and man (A and C), *Ctenocephalides felis* (B) *Pulex irritans*. AG: antennal groove; CT: ctenidium (genal comb); OC: ocellus; PC: pronotal comb; PG P: pygidium; SE: setae.

cause allergic skin reactions and itching. Thereby they ingest in a hasty manner more amounts of blood than they can use. Thus, they excrete within their feces portions of blood, which then serve as food for their larvae. The last one of three larval stages excrete a fluid, which hardens and thus forms the pupal cover. Therein the female or male adult flea is formed within 7–10 days. The whole development from egg to an adult flea is temperature dependent, e.g., in moderate European temperatures it takes about 4–6 weeks, but it needs less time in warm regions or in very warm indoor rooms in colder regions of earth. Human fleas and related species need at least a minimum of humidity, so that the in general rather dry human dwellings are poor biotopes for their progeny.

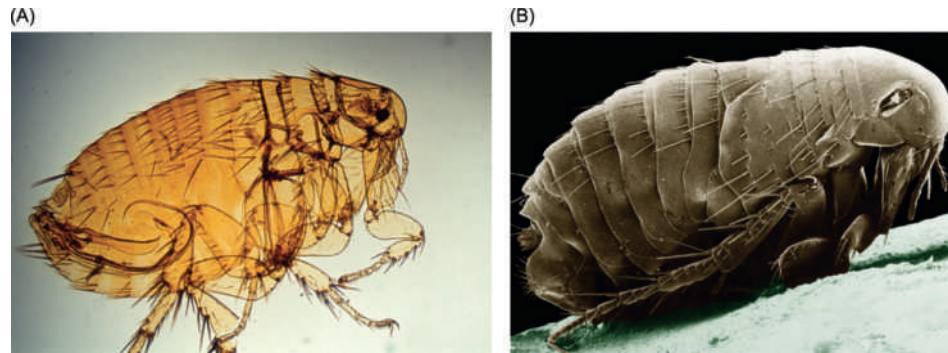


Fig. 4 (A) Light micrograph and (B) scanning electron micrograph of *Pulex irritans*.



Fig. 5 Scanning electron micrograph of stages of the cat flea on the floor. Above: adult flea; left below: egg with a hatching larva; right below: larva 2 (= "wireworm").



Fig. 6 Light micrograph of a male flea of *Ceratophyllus* sp. (main hosts: poultry).

The cat flea *C. felis* (Table. 1; Figs. 1 and 2; Vobis et al., 2003), however, is not very dependent on significant ranges of temperature and humidity and thus is now much more common in human dwellings than *P. irritans* or other flea species. This flea, which attacks besides cats also dogs and humans, represents now 80% of the fleas occurring in European human dwellings. Inside its pupal cover (cocoon), the ready to hatch adult flea may starve for long (up to 1 year), since it waits for signals like soil shaking, which announce the arrival of a potential host. This is important to know for people who move into an apartment, which was the home of dog or cat owners and stood empty for a longer period. The newcomers may become attacked by large numbers of suddenly hatching fleas or others, which had starved for long. The same behavior is described from bird fleas, which rest inside their pupa cover until another bird enters the nest or they attack when humans start cleaning bird cages, etc. The adult fleas leave dead

hosts as soon as the body cools down. This is important in the transmission of the agent of plague (the bacterium *Yersinia pestis*). If infected rats die, the fleas leave them and attack humans, whereby they transmit eventually present plague bacteria within their saliva during blood sucking. The transmission of agents of this disease into human hosts is proceeded by regurgitation of blood containing the bacteria. There are the following steps:

- (1) A flea ingests the bacteria at an infected rat or a human being.
- (2) The flea changes the host and attacks after a while or immediately another human being.
- (3) The flea starts to suck hastily human blood, which is mixed in its prestomach with the remnants of a meal of rat or human blood containing the infectious bacteria.
- (4) Then the non-stretchable anterior portion of the flea’s gut becomes overfilled and therefore the flea vomits. Thereby it regurgitates the whole blood mixture containing the bacteria into the wound of the human skin.
- (5) Then these bacteria in general start their propagation inside the human blood and thereby induce severe symptoms of disease, which may lead to death. In former times this disease was called Black Death due to the appearance of numerous black spots on the skin of dying persons.

Especially the tropical rat flea (*Xenopsylla cheopis*) (Table 1; Fig. 7) is a very important vector of the agents of plague, since it is worldwide especially high common on rats in human slums. Thus, outbreaks of plague occur even today especially in many regions of India, East Africa and even the United States (Abdullah et al., 2019; Beaucournu et al., 2012; Clark et al., 2018; Frank et al., 2013; Gilles et al., 2009; Grüntzig and Mehlhorn, 2010; Gyimesi et al., 2007; Kreppel et al., 2016; Lawrence et al., 2015, 2019; Márquez, 2015; Mead, 2018; Mehlhorn, 2012a,b; Mehlhorn, 2016a,b; Nagy et al., 2007; Oteo et al., 2014; Peus, 1938; Rzotkiewicz et al., 2015; Sanchez and Gomez, 2012; Sekeyová et al., 2012; Vobis et al., 2004; Zurita et al., 2019; Zhao et al., 2018).

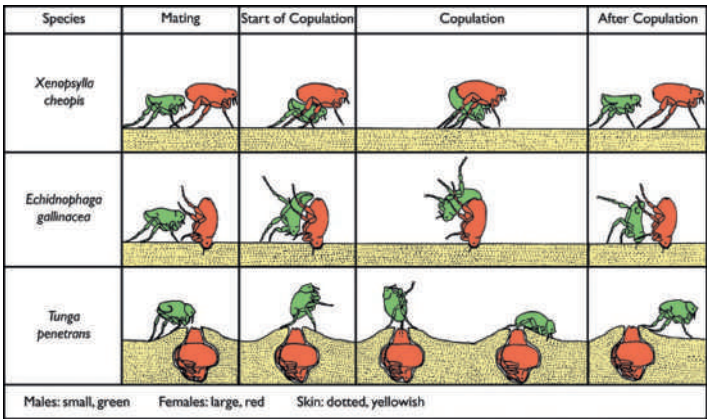


Fig. 7 Diagrammatic representation of the mating behavior of three flea types, which occur free or partly, respectively, fully attached on the skin of their hosts.

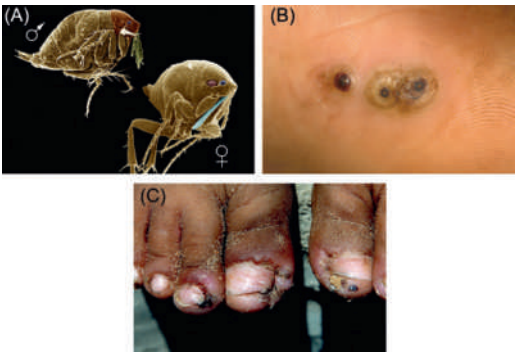


Fig. 8 (A) Scanning electron micrograph of a male and female sand flea (*Tunga penetrans*). (B) Macrophoto of human skin with three females of *Tunga penetrans*. The diameters of each of the swollen females was 7–8 mm. (C) Toes of children with penetrated sand fleas.

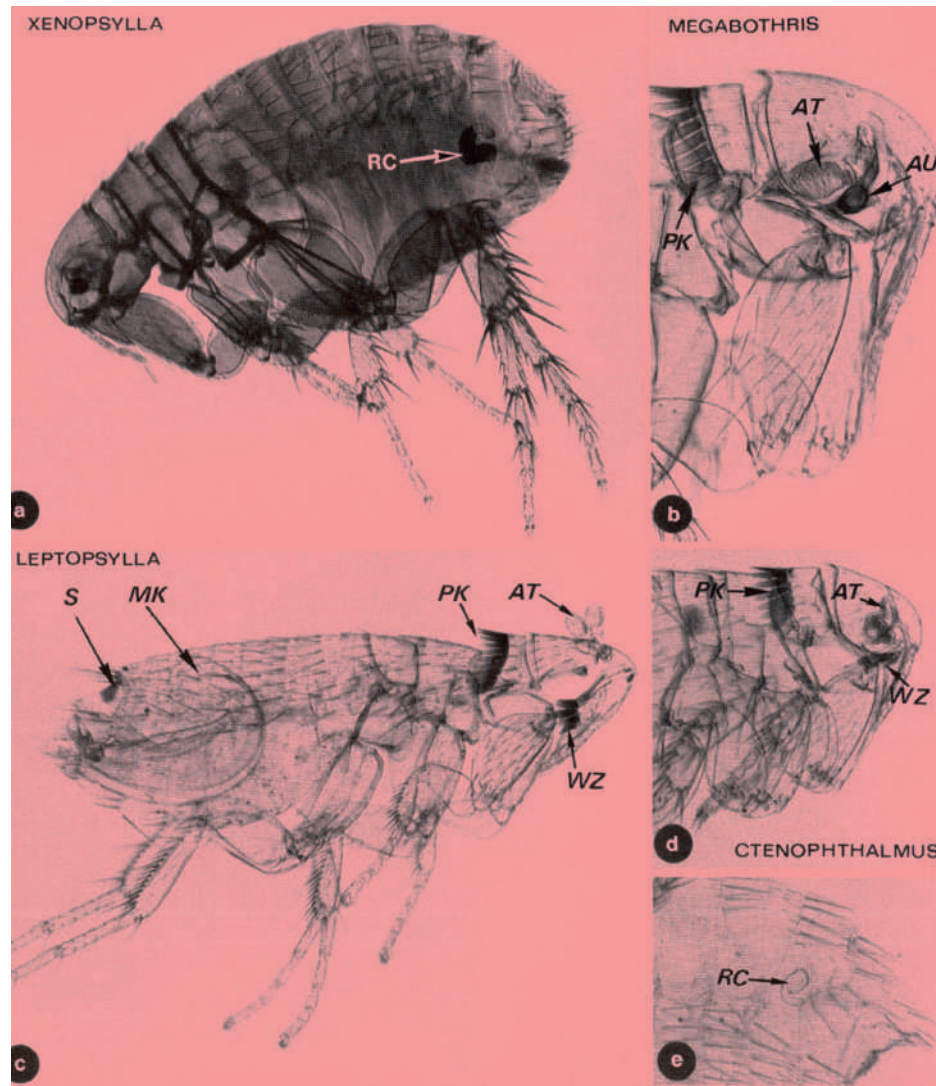


Fig. 9 Light micrographs of fleas. (A) *Xenopsylla cheopis*. (B) *Megabothris turbidus*. (C) *Leptopsylla segnis*. (D and E) *Ctenocephalimus agyrtes*. AT: antenna; AU: eye, ommatidium; MK: male copulation organs; PK: pronotal comb, ctenidium; RC: receptaculum seminis; S: sensillum, bristle; WZ: genital ctenidium.

Bite reactions

Fleas are easily disturbed during blood sucking. Then they stop it and start a new approach at a different site of the host body. Thus, there can be found real rows of bites along a human skin (Fig. 10). The injected saliva, which keeps the host's blood fluid, initiates intense itching, which is several times repeated, if a host reacts by scratching at a bite site. However, reactions on bites may range from not occurring or not noted to extremely intense reaction depending on the allergic status of a host (Fig. 11)—but in any way itching is nasty and may remain for several days.

Transmission of agents of disease

The history of mankind is full of examples, where fleas had influenced the fate of single persons but also that of whole nations, since fleas are common vectors of important agents of diseases.

Plague: This disease, which has killed millions of humans in times before the invention of antibiotics, is due to infections with the bacterium *Yersinia (Pasteurella) pestis*. This non-flagellated, gram-negative, stab-like bacterium, which was detected and described in the year 1896 in Hong Kong by co-workers of Robert Koch and Louis Pasteur: Shibasaburo Kitasato (1853–1931) and Alexandre Yersin (1863–1943) during an epidemic in Hong Kong, is transmitted by fleas during regurgitation of bacteria-containing blood.



Fig. 10 Flea bite reactions of humans. Fleas bite often in rows after having been disturbed during sucking.

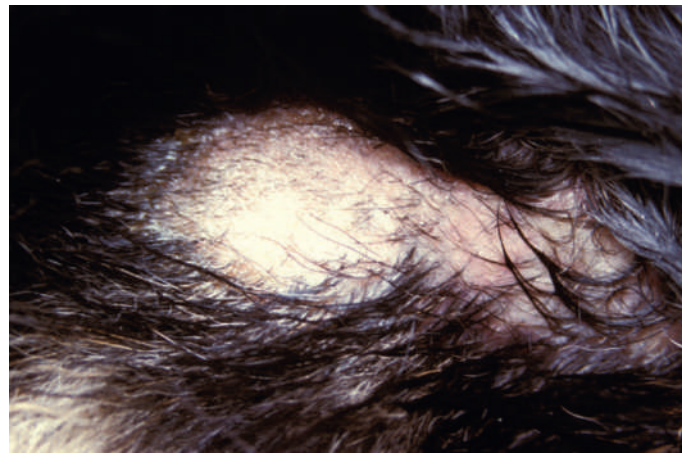


Fig. 11 Skin reaction due to flea bite allergy.

These bacteria are equipped with a capsule of proteins, which act antigenic and block phagocytosis. They reach within blood and lymph the lymph fluid nodes which during infections increase their size extremely within 3–5 days after the flea bite and appear in a hemorrhagic red color. These swollen regions occur in about 90% of all infections and were called bubones. Via blood transportation the agents of disease then may enter the lung, where they reproduce themselves enormously so that during coughing slime masses with millions of bacteria are expectorated and may infect other persons. This phase—called lung pest (plague)—leads to death without treatment. Thus, it is understandable that in pre-antibiotic times plague has extremely reduced the human populations in many countries around the world. For example, after the European so-called 30-Year Wars (1618–48) as many people had been killed that only 5 million humans survived in the region between Breslau and Paris (over more than 1200 km). Plague is still today present in India, Africa and North America and starts local outbreaks. For example, in the year 1994 there was a plague breakout in India close to a monastery (where “holy” rats had been kept) leading to thousands of human cases. This outbreak made big problems, since the urgently needed supply with antibiotic was too slow. Treatment of plague is actually done by application of antibiotics such as tetracyclines or aminoglycosides.

Murine spotted fever: The agents of this fever and related types (*Rickettsia typhi* = *R. mooseri*) are common in rats in many tropical and subtropical regions. Today treatment is done by application of tetracyclines and chloramphenicol. However, extremely important and highly needed is the intense control of the plague hosts such as rats and fleas, which live often in close surroundings of human houses.

Erysipeloid: This disease is often found in Russia and is induced by the bacterium *Erysipelothrix rhusiopathiae* which leads to severe skin damages in many animals and immune-compromised humans.

Viruses: The groups of Mehlhorn and Mencke (Germany) proved by transmission experiments that flea may transmit a broad spectrum of viruses (e.g., Caliciviruses), which are stable enough to survive for a while in the stomach of fleas (Mencke et al., 2009; Vobis et al., 2003). These agents were found as well as inside flea saliva as in flea feces, where they remained infectious for up to 60 days (!) (Beck and Prosl, 2010; Bosio et al., 2014; Cagnon et al., 2019; Contreras et al., 2018; Hajipour et al., 2014; Hornok et al., 2014; Kapoor and Elston, 2012; Klimpel et al., 2005; Kumsa et al., 2014; Mencke et al., 2003; Miarinjara et al., 2017; Miller et al.,

2019, 2020; Raking, 2003; Ranaivozanany et al., 2020; Ramasindrazana et al., 2017; Robert Koch Institute (RKI), 2020; RKI Berlin, 2020; RKI STAKOB, 2017; Schmidt-Chanasit, 2014; Simona et al., 1998).

Transmission experiments with feline leukemia virus via fleas

As described in the paper of Vobis et al. (2003), the following results were obtained in an artificial feeding system (Artificial dog from FleaData Inc., Freeville, United States), where fleas have the chance to ingest blood from small blood containers via membranes. The whole system was adjusted to keep the blood temperature at 37 °C. In the first feeding, an initial population of 110 fleas was infected with feline leukemia virus (FeLV) through feeding for 24 h with 2 mL blood from a viremic cat (Table 4). To verify the successful uptake of viruses, 10 fleas and whole feces of this initial population were tested for FeLV RNA. The viral RNA could be detected in both samples, fleas and feces. The FeLV was incorporated by the fleas via blood feeding and was excreted with the feces. The remaining 100 infected fleas were subsequently divided in two populations of 50, where population 1 was fed for 24 h and population 2 for 5 h with 300 µ uninfected blood from a healthy cat. After the defined time, 10 fleas and feces of each population were tested for FeLV. Viral RNA could be detected in fleas of both populations as well as in their feces. FeLV RNA was detectable for 5 and 24 h, respectively, after uptake by the flea and feeding with uninfected blood. To investigate whether transmission of FeLV occurred, the remaining blood from transfection 1 and 2 was examined. Viral RNA could be detected in both samples of the previously uninfected blood from a clinically healthy cat. This shows that the cat flea *C. felis* can function as a vector for feline leukemia virus RNA in this in vitro system of an artificial dog. Viral RNA was detectable in the fleas and their feces for up to 24 h. In addition, the fleas successfully transmitted FeLV RNA directly from an infected blood sample to an uninfected one.

In a third feeding study, the same fleas, their feces and blood samples were examined for FeLV. After this third feeding, viral RNA could not be detected neither in the fleas, their feces, nor in blood samples. FeLV RNA was detectable in the fleas up to 24 h after feeding. During this time, fleas also excreted viral RNA within their feces. In addition, the fleas can transmit FeLV RNA directly from one blood sample to another and functioned therefore as a vector of the virus.

In quantitation measurements (Vobis et al., 2005a,b) using the same artificial dog system as in the first series (Vobis et al., 2003), it was shown that around 70% of the ingested virus stages are able to withstand passage through the intestine with at least an intact nucleocapsid and is excreted and spread into the flea feces and thus into the surroundings. When comparing the degradation rates of the FeLV in cell culture supernatant, the nucleocapsid seems even more stable in the feces. About 55% of the initial amount of the viruses could still be detected after 15 days when keeping the fleas at room temperature. Lower temperatures led to even higher survival rates.

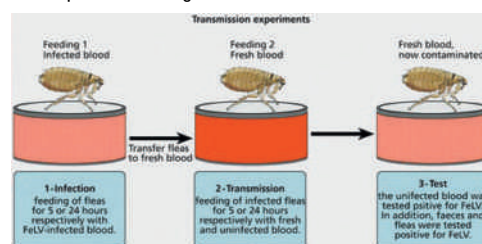
These experiments showed that the viruses were transmitted by sucking (and apparent regurgitation) and that they were definitively surviving for days within the feces thus endangering humans when reaching their dwellings.

Transmission experiments with feline calicivirus

To find out whether flea feces do not only contain the probably unchanged viruses, in vivo experiments were done using the feline calicivirus (FCV) (Mencke et al., 2009). Fleas were inoculated with the viruses in the artificial dog system containing bovine blood free of FCV antibodies but being spiked with FCV to give a final titer of 10^6 TDC₅₀/mL. The flea feces were collected daily for 10 days and incubated at room temperature. When using Crandell-Rees feline kidney (CRFK) cells, it was shown that the FCV apparently remained infectious in feces for 8 days. When exposing four specific pathogen-free (SPF) kittens oro-nasally to the virus-containing feces, all four were successfully infected (two kittens showed clinical symptoms). Four additional SPF kittens were exposed to fleas that had been fed with FCV-spiked bovine blood. One of these kittens was proven to be definitely infected in this way when testing virus isolation from pharyngeal swabs.

This series of experiments clearly showed that fleas may play an important role in the transmission of viruses under certain conditions, either during the blood meal and/or in the case that hosts get in contact with the flea feces. The only, but very important condition is that the transmitted virus is rather stable. However, there are many viruses known to fulfill these conditions, so that viruses transmitted by fleas may introduce emerging diseases under these conditions.

Table 4 Transmission experiments using the feline leukemia virus.



Prophylaxis and flea elimination

In case of temporarily keeping fleas for experiments, use first insecticides to clean the vessels (e.g., with Alugan®-Spray or pyrethrum spray, e.g., Axis®Natur, or spraying with INS 15 or CBM 8®). Repetition of the treatment should be done every 10–12 days and regular cleaning of the sleeping place (carton with a lateral opening and a heat keeping bottom consisting of wood or thick cardboard).

To avoid transmission of agents of disease by flea bites or flea feces, the pet animals should be protected by insecticidal collars and pour-on preparations containing insecticides (e.g., Advantix®, Bayer, Germany). The latter, however, might not act fully against flea larvae, which, however, may be killed by e.g., MiteStop®, Alphabiocare, Germany, an extract of neem seeds, which dries up the surface layer and thus kills the larvae (Gopinath et al., 2018; Rust, 2016, 2017).

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Blood Sucking and Chewing Lice

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Introduction

There exist two groups of lice, which attack humans: bloodsucking (Anoplura) and chewing lice (Mallophaga). The bloodsucking lice (German: *Läuse*; French: *pou*; Spanish: *piojo*; Italian: *pidocchio*) as well as the chewing lice (mallophages) belong to the animal phylum of the Arthropoda (Greek: *arthron* = segment; *pous/podos* = foot). Inside this phylum the lice are placed into the group of Insecta respectively Hexapoda, which are characterized by six legs (Greek = *hexapoda*) and by the division of the body into head, thorax and abdomen, being visibly separated from each other by more or less deep incisions. Among the insects the lice belong to the systematic order Anoplura (Greek: *anoplos* = unarmed, *ura* = tail). The lice of humans, although being much older than the recent *Homo sapiens* (Figs. 1 and 2; Aspöck and Walochnik, 2007) have coevolved with the human beings over the last 5–6 million years. Thus, they are today exclusively found on the bodies of the recent 7 billion “winners of the evolution”: *Homo sapiens*.

These exclusively human lice belong to two genera:

- *Pediculus humanus capitis* (head louse; Latin: *pediculus* = little foot; *humanus* = human; *caput* = head); measuring in length: ♀ 2.6–4 mm; ♂ 2.4–2.9 mm (Fig. 2).
- *Pediculus humanus corporis* (body louse; Latin: *corps*, *corporis* = body); measuring in length: ♀ 3–4.8 mm; ♂ 2.7–3.8 mm (Fig. 3).
- *Phthirus pubis* (pubic louse; Latin: *phthir* = louse; *pubes* = region around primary sexual organs); measuring in length: ♀, ♂ 1.3–1.6 mm (Fig. 4).

Some authors claim that the races of the genus *Pediculus* are already separate species and thus they should be named *Pediculus capitis* and *Pediculus humanus*. However, in cross-breeding experiments it has been shown that members of both races may produce a fertile progeny. However, such a “mixture” apparently does not occur on the human body, since both races prefer the different body temperatures that occur on the surface of the scalp and the rest of the body respectively.

Today lice infestations are one of the most common human infections in the world—especially in kindergarten and in school-children aged between 3 and 15 years. It is estimated that even in countries with high hygienic standards, such as the United States of America, high infestation rates exist (about 6–12 millions of kids just in the United States), that result in several million lost school

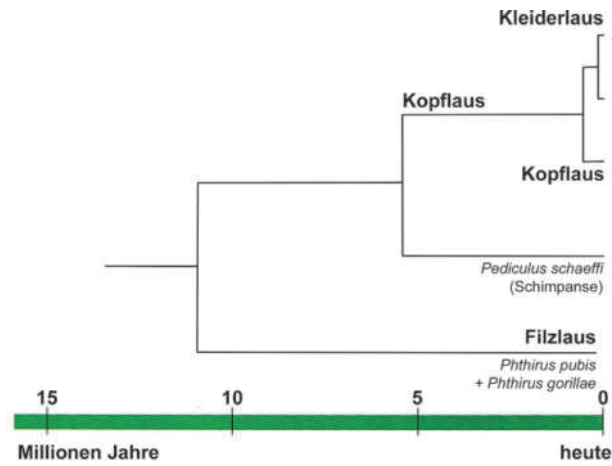


Fig. 1 History of the lice of humans (Aspöck and Walochnik 2007). Note that lice attacking humans branched off around 5–7 million years ago (Kopflaus = head louse; Kleiderlaus = body louse; Filzlaus = pubic louse).

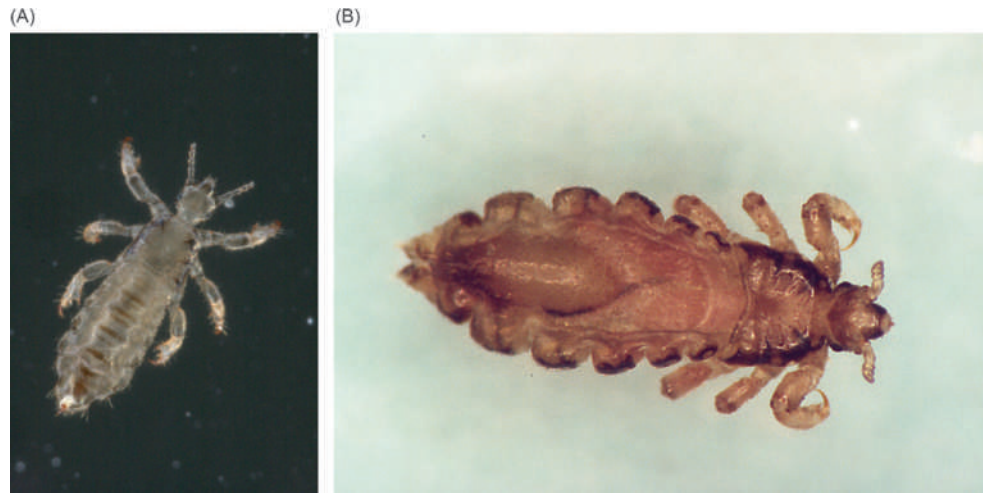


Fig. 2 (A and B) Light micrographs of the head louse (*Pediculus humanus capitis*) in dark light and overview.



Fig. 3 Scanning electron micrograph of a female of the body louse *Pediculus humanus corporis* and three eggs being glued at tissue.



Fig. 4 Light micrograph of a pubic louse (*Phthirus pubis*).

days and treatment costs of more than \$5 billion per year. Similar costs must surely exist worldwide in all countries where there is medication available for purchase. Thus it is a reasonable goal to establish significant, safe, healthy and low budget eradication methods for lice control in a world of growing resistance (Abdel-Ghaffar et al., 2010a,b, 2012; Burgess, 2004; Gur and Schneeweiss, 2009; Lebwahl and Levitt, 2007; Mehlhorn, 2016a,b,c; Mehlhorn and Mehlhorn, 2014; Meinking et al., 1986; Mumcuoglu, 1999; Amanzougaghene et al., 2016; Arnaud et al., 2016; Asala and Mbajiorgu, 1996; Bartlow et al., 2016; Boutellis et al., 2014; Boyd et al., 2017; Bressa et al., 2015; Coulaud et al., 2015; Dos Santos et al., 2011; Gustafsson et al., 2015; Gustafsson et al., 2019; Kagira et al., 2013; Leitinger and Richter, 2018; Mehlhorn, 2012; Mey, 1988; Smith et al., 2013; Valim and Cicchino, 2015; Veracz and Raoult, 2012; Wang et al., 2014).

Head lice

Morphology of head lice (*Pediculus humanus capitis*)

Among the general characteristics of head lice, the following features can be noted:

- Their body, covered by a stiff yellowish-brown appearing cuticle, is visibly divided into three regions: head, thorax and abdomen. Only in the abdomen are the different segments clearly visible, while those of the head (caput) and thorax are more or less fused (Fig. 2A and B).
- The head has a "pig-like" snout, within which the two channels of the blood sucking mouthparts are retracted.
- The head bears two rather large, somewhat protruding eyes, which are clearly pigmented.
- Furthermore, the head has two motile antennae (tentacles) bearing fine-smelling sensillae; each of these antennae consists of five segments (Fig. 2).
- The breast comprises three segments being closely melted. At the dorsal side, there appears a central depression, which is due to the fact that at its inner side strong muscles are affixed. The dorsal side of the lice does not possess wings at any stage of the development (Fig. 2).
- The thorax is equipped on each side with one spiracle = stigma (which represents the opening of the tracheal = respiratory system).
- Ventrally at the breast three pairs of feet are attached, echoing the former existence of three clearly separated thorax segments (pro-, meso- and metathorax).
- Each of the six feet consists of five typical segments (coxa, trochanter, femur, tibia and tarsus). All terminal tarsus segments are transformed into giant claws, which can clutch different single hairs. This system helps lice to avoid to become separated from their hosts, an important feature, since head lice live exclusively on humans. In exceptional cases when fallen down from a host, they need to find a new host within a short period of 5–6 h, otherwise they will die. Therefore, lice try to stay on a host.
- The hind body (abdomen) of the head lice louse is composed of 10 true segments. The first two are greatly reduced and do not bear the paired lateral openings (spiracle, stigma) of the respiration system, which are clearly visible on the lateral sides of the segments 3–8. Segments 9 and 10.
- The position of the opening of the sexual organs differs in both sexes. While the male opening can be found at the back line of the ninth segment, the female system opens one segment earlier (at the posterior end of the eighth segment).

- The copulation system of the male leads to a pointed aspect of the terminal end of the male stage, while the females are characterized by a slight terminal bifurcation.
- The intestines of all blood-sucking head lice stages start with the apical, “pig-like” snout, within which an invagination (containing the two protruding channels of the blood sucking system) has its terminal end. The mouth is adjacent to the pharynx with its numerous muscles, thus forming a strong pumping system. The esophagus transfers the engorged blood into the stomach, which forms laterally two short blinded extensions (ventricula), which are directed anteriorly. The stomach is stretched to the posterior end of the 6th abdominal segment and is followed by a small-sized intestine forming an anterior loop. At its border to the terminal portion (hind gut, colon) the tubes of the excretory system (Malpighian tubules) enter the intestine. The anus finally opens at the end of the 8th segment, while segments 9 and 10 appear as a combined block.
- The inner ventral side of the stomach is lined by a system of supporting microorganisms (bacteria) forming a so-called mycetome, which is essential during the digestion of the food.
- The male sexual organs consist of two testes (each with two lobes), from where a “ductus seminalis” transfers the sperms to two sperm bladders with glands. Finally, the ends of these bladders are united and terminate in an injector, which may become protruded and injected into the female system.
- The female sexual system consists of two ovaries each with five lobes containing the produced eggs. After fertilization, the eggs are excreted via the two oviducts, which are lined with symbiotic microorganisms being transferred to the eggs. The oviducts fuse to create a single terminal portion, which does not form a “receptaculum seminis” (a sperm storage system), but is lined by two glands (each with three lobes) producing an adhesive material that eventually covers the eggs. The females are thus able to attach them firmly to the hair close to the scalp. Due to the lack of a sperm storage pocket (receptaculum seminis), lice copulate several times during their adult life span.

Life cycle of head lice

The life of a head louse as well as of other lice (Fig. 7) starts with the copulation between a male and female louse. After fertilization, whitish eggs about 0.5–0.8 mm long become glued to the basis of hair. Then it takes 6–9 days for the six-legged larvae (some authors call them nymphs) to hatch from the egg by blasting off the covering operculum which contains several typical semi-open aeropyles that allow the entrance of oxygen. The larvae suck blood at intervals of 2–4 h and thus grow rapidly. After three moltings (over larvae 2 and 3) the adult stage is reached within 8–11 days, so that the whole generation usually only takes 17–20 days. The developmental larval stages and both adults suck blood every 2–4 h. The females live as adults for about 20–25 days and lay 2–9 eggs per day, so that a total reproduction rate of 50–150 eggs per female is reached (Reichenow, 1946). In the beginning, just after placing the eggs at the base of the hair, the eggs (nits) are brownish. Since the hair grows about 0.2 mm per day, it takes weeks before the eggs (nits) become visible as brilliant white globes at the terminal ends of the hair (Figs. 5 and 6). By this time, the nits are long since empty and thus cannot be used as a means of ascertaining the existence of a living louse population on a head.

All stages of the lice (Fig. 7) need to avoid falling off a head and have thus developed giant claws. However, females—if fertilized—have a peculiar way of transferring living and fertile females to another host (e.g. propagation of an existing louse population). Immediately after a “good” blood meal the fertilized females run to the end of the hair driven by a behavioral adaptation called positive phototaxis. If this lurking female gets cold after a waiting time of about 1–2 h, it runs back to the scalp and starts feeding again. However, in the event of hair-to-hair contact between two people, while a female louse is lurking at the end of the hair, she may enter the new host and become the mother of a new population. If the adult females drop off a head and fail in finding a new host within the next 5–7 h, most of them will die by desiccation. Gallardo et al. (2009) proved that practically all males and 97% of the females died at a room temperature of 24 °C at a rather low room humidity. This is clearly different from the



Fig. 5 Light micrograph of three lice eggs being glued at a hair.



Fig. 6 Scanning electron micrograph of an egg of the head louse being glued at a hair. Note the openings at the cover (operculum). The developing larva is inside protected from drying up by a fine membrane.

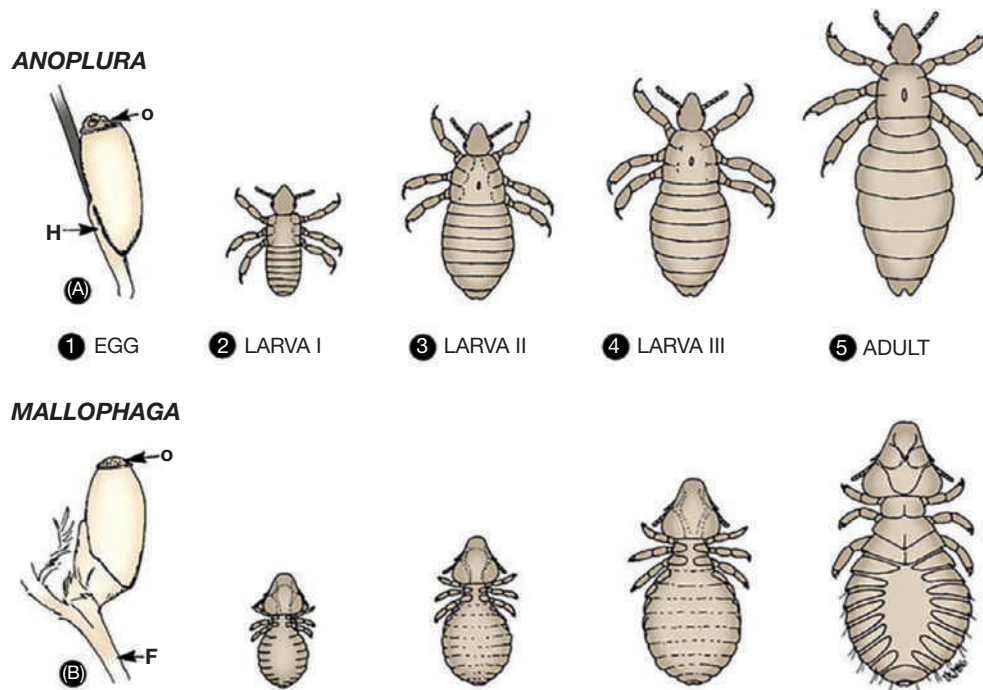


Fig. 7 Diagrammatic representation of the life cycle stages of the Anoplura and Mallophaga.

survival chances of body lice, which can survive starving periods of up to 10 days on the floor, if there are rather low temperatures in a room (0–6 °C). Heating the room significantly lowers the survival time of body lice in a room. At room temperatures of more than 20 °C, they only survive for 2–4 days (Reichenow, 1946).

Thus, there are completely different needs, when outbreaks of head or body lice occur within one room. In the case of head lice there are practically no further precautions needed due to their short survival time and the extreme rarity of them voluntarily leaving the head of a victim in the direction of the floor, etc. In the case of body lice, however, rooms have to be heated for 5 days at temperatures close to 30 °C or must be left empty for at least 10 days until the danger of being attacked by body lice from the floor or beds has passed.

Epidemiology of head lice infestations

Human head lice have co-evolved with mankind and are thus cosmopolitan parasites, found in all areas of the world and in all socio-economic classes. Contrary to popular belief, pediculosis (infestation of lice) occurs in anyone regardless of ethnic origin, economic status, family background, religion or personal habits. The only exception is that longer hair supports transmission, since this leads to higher numbers of hair-to-hair contacts with possibly infested persons. This explains why girls are generally more often infected than boys. In general, and in practically all countries, the highest prevalence rates of pediculosis are found in pre-school and elementary-school children (3–10–15 years). There are reports of prevalence rates of 5% up to 56% in different countries (Al-Shawa 2006; Bauer et al., 2009; Buczek et al., 2004; Downs et al., 1999; Falagas et al., 2008; Govere et al., 2003; Hemingway et al., 1999; Heukelbach et al., 2005; Kokturk et al., 2003; Mumcuoglu et al., 2009; Speare and Büttner 1999; Toloza et al., 2009). The increasing rates of lice infestations worldwide follow certain seasonal fluctuations (Bauer et al., 2009), which depend on climate conditions and behavioral activities. Thus, Heukelbach et al. (2005) reported that in a slum in a semi-arid region of Brazil the prevalence of pediculosis varied considerably during the year. It was 44% in the raining season (when humidity is high and temperatures are rather low), but decreased to 27% in the hot and dry season. Similar findings had been published by Castro Ddel et al. (1994), who noted that in some districts of Buenos Aires the prevalence of lice declines from 56% in the relatively cold winter to 12% in the dry hot summer. Results of Toloza et al. (2009) showed that high prevalence rates still occur in Argentina throughout the year (19–42%), while in some schools in Germany/Europe/the United States there is always an increase in the number of louse-infested kids just after school holidays (Bauer et al., 2009). Due to control methods—always dependent on the financial means of the patients—initial infection rates decrease during the year or not.

How to diagnose infestations with head lice

Infestation with lice is a hidden process, which is usually only noted weeks or even months after its start. This is because almost 1 month is required until a fertilized female louse that has switched from one head to another host, has given rise to its progeny, which needs 17–21 days to reach maturity. Furthermore, the early bites of a few lice do not introduce the typical allergic reactions (i.e. itching at the biting sites), which mostly start after 4–6 weeks. For sensitive people the first symptoms of an infestation with head lice are the intensive crawling around of the newborn louse generation, often causing restlessness in the victims. Combined with the increase in the louse population on the head, the number of biting sites increases, appearing first as fine red dots of 1 mm in diameter, and later changing into a deep blue-red color and starting to itch. In general, these dots disappear within 8 days. However, in the case of bacterial superinfections (e.g. due to scratching) scars may be formed leading to pus-producing eczema—especially in cases of mass occurrence of lice. In these patients, the itching sensation increases dramatically and the hairs often stick tightly to each other. In such heavy infestations fever and lymph node swellings often occur because of the bacterial superinfections. However, significant proof of a louse infestation can only be shown by demonstrating the motile louse stages (crawling larvae and adults) and by detecting the nits (Figs. 5 and 6) at the base of the hair. If the hair is combed using a metal comb (the best is those with 4–5 cm long, 0.2 mm narrow teeth), the lice and their dark brown feces can be seen most easily when falling off onto a white towel. The occurrence of brilliant-white nits at the tips of the hair only indicates that there had been lice, since these nits are empty. Thus, the success of an anti-lice treatment is only assured by intensive combing for at least 15 min, since lice are motile and move quickly along the hair.

Symptoms of disease during head louse infestation

As described above, louse infestation may introduce no, slight or even severe symptoms of disease depending also on the different individual allergic reactions of the patients and the degree of bacterial or fungal superinfections.

Louse infestations may therefore result in a disease called pediculosis capitis, which is characterized by the following symptoms:

- inflammation at the biting sites (red/blue dots);
- itching leading to constant pain;
- eczema and excoriations;
- exudations;
- restlessness and poor sleep;
- slight to intense fever;
- swelling of lymph nodes;
- in severe cases (under extremely poor hygienic conditions and among people with immune suppression) eczema and the fluids of the exudations may cause plica polonica, where the hair becomes glued together into a tangled mass;
- in cases of superinfection of the wounds by bacteria (e.g. MRSA or Streptococcus) severe diseases may occur (Burgess, 2004; Meinking, 1999).

If lice-infested hosts contain agents of disease in their blood, head lice may also transmit them, as it is regularly established in body lice (which may be among the vectors of the Rickettsial disease “louse spotted typhus”). However, in the case of head lice, transmissions of agents of disease are much rarer than in body lice due to the fact that head lice change their hosts only rarely. Thus, mechanical transmissions (e.g. without intense reproduction inside the intestine of the louse) can only occur in crowded

camps (e.g. of prisoners, refugees, etc.) with many infected people (Bonilla et al., 2009; Robinson et al., 2003; Sasaki et al., 2006). However, since the agents of “trench fever” and “spotted louse borne fever” (with a lethal potency) have been found inside head lice, infestations with these ectoparasites should never be under-estimated.

Public reactions to head louse infestation and most common errors

Lice infestations on human heads are as old as mankind due to the common co-evolution that started millions of years ago (Fig. 1). Therefore, all human societies—even the most developed ones in the past and present—have to handle such infestations. Thus lice are described in the oldest human writings (e.g. in the 2nd book of Moses, in Egyptian papyrus papers, in the Talmud of early Jews, in Greek papers e.g. of Aristotle, in Arabian papers of Al-Qazwini, in medieval books and also in the famous drama “Faust” of the German poet Goethe, etc.). However, the methods of considering and handling lice infestations has differed in the social history of mankind. Of course, all victims suffered from the bite reactions and primarily considered these blood suckers as “nasty companions.” But they had to bear such infestations, because they could not avoid them. In former times the obvious correlations between social standards and hygiene of a victim were not realized—there are many reports of lice infestations of kings and rich people—thus there was no discrimination against infested victims. Lice infestation of men was even positively correlated with their sexual (“womanizing”) powers and attraction.

This attitude versus the “nasty” lice changed, however, when hygiene (starting in the 18th century) became a criterion of a high social standard. Then “louse infestation” was considered synonymous with dirtiness. With the constant increase in hygiene standards—especially in the rich, money-oriented Western societies—lice infestations became a psychological problem and even led to psychological diseases followed by completely incorrect responses. Since louse infestation is still considered as a sign of dirtiness and bad hygiene, louse infections are kept secret, so that other members of a group (e.g. kindergarten, school) may become unnoticeably infested. This makes the control of lice infestation very difficult. Therefore, high costs and a lot of useless efforts are accepted, although a head louse infection is much less important and dangerous compared to common diseases in children such as measles, diphtheria, whooping cough, etc.

This completely inaccurate view of a louse infestation is based on the following main errors:

1. Wrong: Lice only infect dirty people.
Right: Anybody can get lice, if there is hair-to-hair contact.
2. Wrong: Lice can be easily removed by combing and/or by using a “normal” shampoo.
Right: Even after intense combing some lice (e.g. larvae, nits) will remain and are not killed by normal shampoos.
3. Wrong: I can kill lice in a sauna or in a hot whirlpool.
Right: The temperature along the scalp (= biting site) is always ideal for the lice to survive.
4. Wrong: If I cut away the white brilliant nits, lice infestation will be stopped.
Right: These nits are already empty and the living lice stages suck blood at the scalp.
5. Wrong: Lice only occur just after the summer holidays in September and October.
Right: Lice occur during the whole year—there are constant reinfestations among the those in a group or family.
6. Wrong: If there is a louse infestation, nightclothes and bed sheets should be washed every day.
Right: This is pointless, since lice only occasionally leave a warm head in the direction of a colder sheet. Furthermore, lice die by desiccation within a few hours.
7. Wrong: I do not have to inform those in charge in the kindergarten or the mothers of my child’s friends in case of louse infestation, because I will treat it.
Right: The whole group should be checked and treated if necessary. Otherwise reinfestations may occur immediately after a successful treatment of a child.
8. Wrong: Vinegar will kill lice inside hair.
Right: The concentration of normal = diluted vinegar will not kill lice. Higher concentrations are even dangerous for health!
9. Wrong: Any anti-lice shampoo on the market will control head lice.
Right: There are many completely useless products and in addition many dangerous ones (inflammable, harming the lungs, skin). Furthermore, lice have developed resistance to some active compounds (e.g. permethrin, allethrin, malathion, etc.).
10. Wrong: All anti-lice shampoos also kill the developing larvae inside nits.
Right: Most of the anti-lice products do not enter the nits. Thus the larvae hatch unchanged. 2–9 days after the treatment (depending on their age during treatment). Therefore you will usually have to treat children two or even three times within a period of 9–10 days.
11. Wrong: If I have treated my child against lice, he/she will be protected.
Right: The child may become re-infested the next morning after treatment, if there is contact with the lice-infested hair of another person.
12. Wrong: Within a family only the lice-infested child has to be treated.
Right: All members of the family must be treated, since one of them may have a recent and thus still hidden infestation.

Head louse control

Historical approaches and mechanical delousing

Although infestation with head lice does not constitute a serious disease, it is not merely a nuisance. In the “cultivated” societies of today infected people are often plagued by social stigma, embarrassment, low self-esteem, disgust and/or rejection. Therefore, mankind has tried since early times to develop methods to get rid of these nasty bloodsuckers. Thus, in the oldest societies people collected lice from the hair of family members and ate them. The same is done by monkeys, with members of the lower hierarchy peel the hair of dominant members. In many early societies, but even until today, complete hair removal (i.e. shaving the head and the body as did the Egyptian priests every 3 days) has been a common method of eliminating head and body lice. However, although effective, the cosmetic and social results of this rough method is less than desirable, especially for school-going teenagers. This practice might be helpful among groups of soldiers or prisoners, but even then it was not commonly used in the Middle Ages. In former times, primitive and also highly sophisticated societies used plant oils, butter, mayonnaise and/or fine crystalline sand particles to suffocate the lice stages. These practices were used until chemical remedies were developed by analytical and synthetic methods at the beginning of the 19th century. Mechanical delousing by combing wet hair (that stops motility of the lice) gets the best results, if a metal comb with 4–5 cm long teeth at a very narrow distance (0.2 mm) is used. The combing must be done for at least 20 min. Even then delousing remains incomplete, always leaving some tiny larval stages plus many of the nits. The latter are strongly attached to the hair and thus withstand combing.

Chemical remedies

Apart from fleas, lice are the insects that most frequently plague humans. Although their immediate potential as vectors of agents of diseases is rather low, lice infestations cause enormous stress within families and their elimination requires huge efforts and considerable financial expenditure. This explains why mankind and later specialized chemistry companies tried to develop specific chemicals as anti-lice medicaments or tried to use existing chemical compounds as pediculicides and/or ovicides. At the beginning of the industrial century diluted vinegar, petroleum (= light oils), kerosene or different paraffins (= saturated C_nH_{2n+2} -compounds) were used in addition to vinegar, olive oil, mayonnaise, isopropyl alcohol (~70% by volume) or even different variations of jellies, which often did not work and which had many health-threatening side effects.

Insecticidal compounds

As far as insecticidal activity is concerned, there should always be controls carried out over the course of about 22–24 h. When incubating lice just in tap water, they contract their legs, remain immotile for minutes, but recover practically in 100% of the cases (own experiments). These results were confirmed by Mougabure-Cueto and Picollo (2010), who immersed lice in distilled water for 2 or 10 min. Even then more than 98% were found living after 22 h. The immersion of lice in 70% ethanol for 2 or 10 min showed some adverse effects in 30–35% of the lice 7–8 h after incubation in either of the 2 or 10 min exposed groups. However, many of them recovered after 22 h (Mougabure-Cueto and Picollo, 2010). These experiments clearly show that stronger measures are needed in the control of lice and that efficacy control must be carried out until 22–24 h after treatment. The actual situation of the pharmacological therapies around the world is rather obscure, since the efficacy of many of the different substances, that have been used often for more than 50 years, has decreased considerably in several countries on the one hand. On the other hand, many of these products turned out to cause side effects which threaten the health of users and/or the entire environment. This resulted in health authorities of different countries or regions (e.g. Australia, the United States, European Community) making a different assessment of the balance between usefulness and risks for the population. These different official considerations and deliberations finally ended in completely different governmental regulations with respect to the use and sale of the available active compounds in the different countries. Thus, in some countries a product that is sold in some countries over the counter (OTC products, especially medications), is only available on prescription in others or even completely banned from the market (Table 1). Furthermore, the situation may change at any time during the next few years.

Therefore, the present review first considers the efficacy of the available pharmacological anti-lice products, which of course may differ depending on the situation in the various countries according to the frequency of use, or on the recommended dose or mode of application respectively.

Lindane

Lindane, which belongs to the group of gamma-benzene hexachlorides, was developed to protect plants from the relevant pests and was widely used until it was banned for agricultural application in many countries. It acts in larval and adult lice in the same way as in the plant pests by inhibition of the so-called GABA receptor, which is an inhibitory neurotransmitter. This leads to a neuronal hyperstimulation of the insect followed by paralysis, thus stopping any feeding activity (e.g. blood sucking in the case of lice). This efficacy explains why lindane acts mainly on the motile stages of lice and thus is a pediculicide rather than an ovicide acting on the stages in the nits. During recent years there have been an increasing number of reports showing that many insects have developed resistance to lindane (Meinking et al., 1986, 2001, 2002, 2004, 2007). The lindane resistance is based on mutations inside the GABA receptor, which make it less sensitive to GABA antagonists.

Due to its chemical activity lindane may act neurotoxically not only in insects but also in mammals. Therefore, in the United States it carries a black box warning sign and was recently forbidden and outlawed in the EU due to this potential health risk. Lindane was labeled (to combat head lice) just for a single use and a very short application period of only 4 min, which in practice is

Table 1 Examples of pharmacologically-acting anti-lice products (AUS = Australia; EU = European Community; FRA = France; NL = the Netherlands; SA = South Africa; UK = United Kingdom; USA = United States of America).

Trade name	Country	Active compounds	Inflammability
Infecto Pedicul medical solution	EU	Permethrin	Yes
Full Marks liquid	UK	Phenothrin	?
Goldgeist Forte medical solution	EU	Pyrethrum (pyrethrins + alcohol)	Yes
Lindane Shampoo, Kwell	USA	Lindane	?
Quellada, Gambex	SA	Lindane	?
NIX	SA	Permethrin	?
Pronto Plus, Rid, A-200, Pronto	USA	Pyrethrins plus piperonyl butoxide or acetone	?
Ovide	USA	Malathion plus isopropyl alcohol and terpineol	Yes
Priderm	NL, FRA, UK	Malathion, isopropylalcohol, terpineol	Yes
Banlice Mousse	AUS	Pyrethrins + piperonyl butoxide	?
Mosquito Louse Shampoo EU	EU	cocoamido-propylbetaine	No
Parasidose	FRA	biococcidine	?
Para speciaal Spray	NL, SA	Bioallethrine + piperonyl butoxide	Yes
Stromectol (officially for scabies)	FRA	ivermectin	?

usually much longer, since it takes time to disperse the product all over the hair. Even if lindane is used against non-resistant lice, this single short application will not eradicate the head lice and according to many researchers lindane requires at least 2, if not 3, treatments to eliminate the head louse infestation (Lebwohl and Levitt 2007). This rise in the number of treatments, however, increases the risk of health problems—on the package leaflet repeated treatments are deemed too risky because of neurotoxicity. Furthermore, a double or even triple treatment would entail enormous costs for the user (a single bottle of the lindane product was said to cost \$125; Lebwohl and Levitt, 2007).

Pyrethrins and pyrethroids

The pyrethrins are obtained from flower head extracts from the plant *Chrysanthemum* (former genus name: *Pyrethrum cinerariaefolium*) and are mainly extruded from the so-called pyrethrum component of this plant. The pyrethroids are synthetically produced pyrethrins and contain a large number of insecticides belonging to up to four generations due to their time of development. Permethrin, allethrin, phenothrin and many others have been (and some are still today) excellent insecticides with quite low toxicity to mammals due to their rather poor skin absorption and their rapid degradation to non-toxic metabolites during human metabolism. Both groups—naturally obtained ones or synthesized compounds—affect the voltage-gated sodium channels, where they introduce delayed repolarization of the neuron by blocking the closure of the sodium channel (VGSC; Clark, 2009). This leads (as in lindane) to a paralyzing effect on the muscles of lice, which impedes their blood sucking activities. While the synthesized pyrethroids (e.g. permethrin) are used as a main active ingredient in many insecticidal products, the naturally extracted pyrethrins (like the pyrethrum itself) are mostly used in combination with additional compounds, increasing the efficacy in their role as synergists. For example, piperonyl butoxide is often used in many products as a synergist, since it inhibits microsomal enzymes that would catabolize the pyrethrins inside the lice.

The main problem with the use of pyrethroids (i.e. the increasing resistance among insects) is spreading worldwide. The *kdr* in particular is rather high and based on a process that also occurs in DEET resistance. This resistance has its source in a mutation of the alpha-subunit gene of the neuronal VGSC, thus decreasing the sensitivity of the channel to pyrethroids and as a consequence, reducing the knock-down effect (i.e. killing effect). Therefore, the survivors with such a mutation will continue to reproduce, while the number of pyrethrin-sensitive lice will constantly decrease. Even the addition of piperonyl butoxide will not help to solve this problem, since it is only able to block the degradation of the now ineffective pyrethrin/pyrethroid (Kristensen 2005; Kristensen et al., 2006; Lee et al., 2000, 2003). Another way in which to produce resistance among lice to pyrethroids is because some louse populations have increased their amounts of glutathione-S-transferase and different mono-oxygenases, which are able to help in metabolizing the pyrethrins/pyrethroids. In the meantime, three louse mutations were found that lead to pyrethroid resistance. The most important of these is the amino-sequence position T 9291 (Clark, 2009).

It is therefore not surprising that reports on increasing resistance among lice to pyrethrins/ pyrethroids have become more numerous since the early 1970s, when the pyrethroids were registered as pediculicides. Broader existence of pyrethroid-resistant lice strains has been reported in France (1994, 2007), Czech Republic (1995), Israel (1995), Argentina (1998), the United Kingdom (1999), the United States (1999), Japan (2003), Australia (2003), Denmark (2005, 2006) (Barrios et al., 2010; Chosidow et al., 1994; Clark 2009; Durand et al., 2007; Gao et al., 2003; Gur and Schneeweiss 2009; Hemingway et al., 1999; Kristensen et al., 2006; Lebwohl and Levitt 2007; Mumcuoglu et al., 1995; Toloza et al., 2010; Yoon et al., 2003, 2004). The reduction of efficacy of permethrin, for example, was found in some studies to be extremely low, achieving louse-free heads in only 25% of the treated patients in Australia (Barker and Altman 2010).

Therefore, it is rather astonishing that under these conditions some US authorities and scientific authors recommend a three-time use of 1% permethrin, which nevertheless amounts to costs of around \$160 per treatment (including costs for medical care

plus loss of income due to staying at home). The market position of pyrethrin products is extremely high in the United States (Clark, 2009):

- Pyrethroids (e.g. NIX = 1% permethrin cream rinse) represents about 16% of the sales;
- Synergized pyrethrins (with piperonyl butoxide) have up to 40% of the market share (Table 1).

In order to avoid the disadvantages of the increasing resistance of lice to the pyrethrins/pyrethroids and since these compounds have low—if any—ovicidal activity, a three-fold treatment is recommended on days 0–7 and 13–15 based on the data concerning the louse development on an infested head. A two-fold treatment has often failed in the past. Meinking et al. (2004, 2006) found only 45–65% efficacy when treating with permethrin 1% on day 0 and 7.

Malathion

Malathion is inflammable and belongs to the group of organophosphates (phosphoric esters). It acquired its generic name from its smell of “bad sulfur.” Due to the generally bad effect of organophosphates on human health, malathion is not a very common ingredient of anti-lice products sold in the industrial countries. Thus, there is only one pharmaceutical preparation in the United States containing malathion (at a concentration 0.5% diluted in 78% isopropyl alcohol and 12% terpineol) at a rather exorbitant price of about \$125/per bottle. Malathion is also used in the United Kingdom, France, Australia and Denmark (Table 1).

Malathion is converted inside lice into malaoxon, which irreversibly blocks acetylcholinesterases. The subsequent increase in cholinergic activity brings about an enormous neuronal hyperexcitability that stops the feeding activity of the lice (Lebwohl and Levitt, 2007). If malathion resistance occurs (reported in the United Kingdom, France, the United States, Denmark, Australia (Burgess 1995, 2005; Downs et al., 1999, 2002; Gao et al., 2006; Hunter and Barker 2003; Izra and Brière, 1995; Kristensen et al., 2006; Yoon et al., 2004), apparently increasing levels of carboxylesterases are produced, which metabolize malathion into non-malaoxon intermediates. As a consequence, acetylcholinesterase remains at higher levels, cholinergic activity remains low and feeding of the lice remains possible. In the United States, the malathion product (Ovide) contains 78% isopropyl and 12% terpineol and so the hair is easily inflammable, if wet. The isopropyl alcohol acts synergistically by degrading louse proteins in the eggs and motile stages, while the tea tree extract (terpineol) inhibits both acetylcholinesterase and octopamine receptors and thus leads to neuronal hyperactivity and death. The whole product is fluid and thus can enter the aeropyles of the louse eggs and kill the developing lice inside the eggs. Therefore, in the United States approximately 80% of users are cured with a single application. Nevertheless, the official recommendation is a double application at an interval of 7 days. This is, however, a rather expensive “luxury” for the patient at a cost of \$125 per bottle. This treatment is recommended in the United States by the FDA for first-line use, but is only available on prescription, while in the United Kingdom it is an OTC product. Health might be endangered, if malathion is unintentionally swallowed; however, this is apparently rare due to the bad smell of malathion (Centers for Disease Control and Prevention, 2005). While in the United States over a period of 5 years (1998–2003) there were 50 cases of malathion misuse, over 300 and over 700 were recorded for pyrethroids and lindane misuse, respectively. Nevertheless—especially when looking at the reported malathion toxicity in the 1970s (Lebwohl and Levitt 2007), strict precautions are required.

Ivermectin

The chemical compound ivermectin belongs to the group of avermectins, which were originally isolated from the fungus of the *Streptomyces avermitilis* group by Professor Saitoshi Omura (Japan, Kitasato University). Ivermectin and related mectins were found to act systemically, thus it also enters tissues and may kill parasites there. Therefore, it was demonstrated that ivermectin not only kills intestinal and tissue-based round worms, but also mites such as *Sarcoptes scabiei* and *Demodex folliculorum*, which parasitize the epidermis of human and animal hosts. This effect is reached when ivermectin is orally consumed or if it is applied to the skin. Due to the efficacy of the avermectins, they were also tested as an anti-lice product, since they should be present in the blood, otherwise they would not have reached intestinal or tissue worms after topical application. Tests show that the normal “anti-worm dose” (= 0.2 mg/kg bodyweight) is sufficient to kill lice, when taken up orally (Glaziou et al., 1994; Meinking, 1999; Ribeiro et al., 2005). Thus, there are products on the market (e.g. in France), which also claim to control head lice in addition to scabies mites, if used on days 0, 7 and 13 (Table 1). Ivermectin acts on its targets (nematodes, mites, insects) by inducing an influx of chloride ions into the neurons, which causes paralysis of the parasites. Since head lice have to take up blood at least every 3–6 h, they die soon after feeding.

Trimethoprim and/or sulfamethoxazole

Head lice carry important symbiotic microorganisms inside their intestines and inside their sexual extruding channels (to be transferred to the progeny). These bacteria depend on the B-vitamin or other compounds, which might be produced by these bacteria. Therefore, several trials have been conducted using antibacterial compounds in order to control lice (Hipolito et al., 2001; Shashindran et al., 1978; Sim et al., 2003).

Suffocating chemical compounds

Today, chemical remedies that block the oxygen uptake of lice are making progress in the market for anti-lice products (Table 2), since pediculicidal-insecticidal products are posing more and more problems due to increasing resistance and/or due to their

Table 2 Examples of chemical louse-suffocating products (AUS, Australia; EU, European Community; FRA, France; UK, United Kingdom; USA, United States of America).

Trade name	Country	Active compounds	Inflammability
Jacutin Pedicul Fluid medical solution	EU	Dimethicone	No
EtoPril medical solution, XT Luis	EU	Di- plus cyclomethicones	Yes
Nyda spray	EU	Dimethicone	Yes
Nyda Plus	EU	Dimethicone	Yes
K.Louse medical solution	EU	Cyclomethicone plus 50% isopropyl myristate	Yes
Dimet 20	EU	Dimethicone plus dodecanol, isopropanol	Yes
Liberalice, Duo LP-pro medical solution	EU	Oxyphthirine	No
Pouxit	FRA	Dimethicone	?
Ulesfia	USA	Benzyl alcohol	Probably
NeutraLice Advance	AUS	Benzyl alcohol, mineral oil, triethanolamine, etc.	Probably

health-threatening side effects. Previous non-pharmacological approaches such as the use of butter, mayonnaise, paraffin/petroleum jelly, olive oil, etc., tended towards the same direction, but in general failed to eradicate lice to any considerable degree, since lice were able to survive even for 5–6 h, if the openings (spiracles, stigma) of their tracheoles (= air transporting system) became mechanically closed. This is because sufficient oxygen remains in the network of the large and finest tracheoles in the interior of the lice, which—in case of occlusion—stop their motility and thus reduce the need for metabolic activity. In addition, these former occlusion materials did not hit the eggs (nits) significantly, so the larvae continued their development until hatching.

Di- and cyclomethicones

Today di- and cyclomethicones are used in combination with a variety of solvents. These cyclo- and dimethicones are used in many cosmetic skin care products, e.g. conditioners and hair repair products, since they are known to produce fine films on the skin and/or on hair. Therefore, they are also used in anti-lice products, where they should cover the openings (stigma) of the respiratory system, which are found at the lateral sides of the thoracic and abdominal segments 3–8 (see Section [Morphology of head lice \(*Pediculus humanus capitis*\)](#)). These di- and/or cyclomethicone containing products usually need very high concentrations of up to 94% and long exposure periods of up to 12 h to kill the lice, since they apparently do not enter the tracheoles, but cover only the outer openings. These products often glue the hair together, and at the end of the long exposure period a shampoo is required to clean the hair and enable combing. Some other products add diluting substances to the di- and/or cyclomethicone containing product. For example, some products contain high percentages of alcohol, dodecanol, isopropyl myristate to make the products more fluid, so that they can enter the tiny channels (tracheoles) of the respiratory system of the lice. These additions mean that the lice are killed within about 20 min at most.

The use of these di- or cyclomethicones, however, has several serious disadvantages, which might even affect the health of users (Table 2):

- Cyclomethicones (syn. cyclopentasiloxane) are highly volatile, which makes them useful as carriers for other ingredients. However, this property also means that they are highly inflammable. Therefore, there are clear warnings (Lotioncrafter, USA) “to avoid vapor inhalation” and “keep away from sources of ignition” (Tables 2 and 3).
- Even if the product itself is not inflammable, the combination of “hair plus product” may catch fire. Several severe cases in Germany, Austria and the Netherlands have led to government warnings.
- These di- and cyclomethicones are known to produce fine films. This is—of course—not important on the skin or on hair, but if they are inhaled, the same might occur on the surface of the lung epithelial cells thus endangering children treated overnight with such a product.
- Similar effects have been described with dodecanol-diluted products. They are not toxic, if ingested, but the inhalation, for example, of 0.2 mL dodecanol killed 9 of 10 rats within 30 min.

Table 3 Examples of flashpoints of different pediculicidal compounds (= minimum temperature for which the concentration of steam is sufficient to produce a detonation in contact with a flame or flash).

Pediculicides	Flash point
Coconut oil + isopropyl alcohol	17 °C
Citronella oil + dimethicones	37 °C
Dimethicones without hair	83.2 °C
Cyclomethicones without hair	77 °C
Di- and cyclomethicones with hair (depending on the mixture)	~37 °C

- The diluting compound isopropylmyristate is used in some products to spread the active antilice compounds. Due to this efficacy inhaling should be avoided here too.
- Although the Cosmetic Ingredient Export Panel of Germany considered it unlikely that these polymers would be significantly absorbed into the skin, there are cited cases where a cream formulation with only 1% dimethicone had severe adverse effects (Nair, 2003). This shows that di- or cyclomethicones may cause harm, if they are transported by carrier substances into the skin.
- These di- and cyclomethicones, when contained in products with a low fluidity, are often not fully effective and thus there is no quick and easy use, if full activity is the aim.
- Dimethicones (syn. Polydimethylsiloxane, PDMS) belong to a group of polymeric organosilicon compounds. Dimethicones are optically clear, considered as inert and thus are used in contact lenses, lubricating oils, or in products that make hair shiny and slippery, but also as occlusives such as hair repair products. According to the Environment Canada Domestic Substance List dimethicones are “expected to be toxic or harmful” and “suspected to be an environmental toxin and be persistent or bioaccumulative.”

However, these di- and cyclomethicones have the advantage over true chemical insecticides, in that resistance may not occur, as is the case in treatment with permethrin, allethrin, malathion and/or the widely banned lindane (Mougabure-Cueto et al., 2008; Picollo et al., 2000; Vassena et al., 2003).

Benzyl alcohol

Benzyl alcohol (synonyms: hydroxytoluol, phenylcarbinol, phenylmethanol) is generally used as a solvent in the production of perfumes, paints and adhesives, etc. In nature it occurs in the flowers of jasmine (up to 6%), carnation, etc. It is reported that this compound may produce together with air explosive mixtures including benzaldehyde, which acts narcotically. The explosive limit is at 20 °C, 1.8–16.3 vol%. Its toxicity is 2–3 times higher than that of isopropanol. The local reactions seen on the skin are flush and irritations, while inhaling may induce vomiting, headache, diarrhea, and/or collapse.

The activity of benzyl alcohol on head lice was reported by Meinking et al. (2010) when testing the United States product Ulesfia, which contains 5% benzyl alcohol (Table 2). It was shown in their tests that the benzyl alcohol blocks the closing (= contraction) of the spiracles (= stigma = the external entry openings of the breathing system of the lice), so that the non-pharmacological substances of the product (mineral oils, etc.) may enter and asphyxiate the lice by covering the exchange region between cells and free oxygen. These effects are documented even after an exposure of only 10 min. Of course, the efficacy is not neurotoxic as is correctly stated by the FDA, but the potential side effects of the benzyl alcohol are considerable.

Oxyphthirine

This product, which is registered in lists 03 and 05 of the International Classification of Goods and Services for the Purposes of the Registration of Marks, is composed of a patented vector which encapsulates a combination of triglycerides, isohexadecane, sorbitan ester and water (Table 2). This galenic lotion is claimed to be able to enter the orifices of the tracheal system of the motile louse stages and should also penetrate the nits via their aeropyles thus suffocating the developmental stages inside. A clinical study by De Sousa et al. (2010) carried out in 2007 in Fortaleza (Brazil) reported a complete cure in 82 of 82 children and a 97.5% killing rate of the nits, if the treatment time was 8–24 h. However, when using shorter treatment periods, this formulation failed. Heukelbach et al. (2009) showed that after 3 min only 22–54% of the lice were killed. Abdel-Ghaffar et al. (2010b) found no effects even after an exposure of 10–20 min, which are normal convenient treating times for users. Thus, longer exposition times of at least 8 h are needed in the case of Oxyphthirine.

Minerals in nanoparticles

Advances in nanotechnology offer further possibilities in the fight against ectoparasites. Thus there is already the technology to include chemical insecticides in tiny nanoparticles, which keep the active ingredients available for a long time (e.g. at the walls of indoor stables) (Mehlhorn and Mehlhorn, 2009). This saves money due to the reduction in the required spraying action and also reduces the danger of intoxication from bodily contact. Recent trials using ZnO nanoparticles (made by mixing zinc nitrate and sodium hydroxide with soluble starch as a stabilizing agent; Kirthy et al., 2011) were promising, since even low concentrations, when brought in vitro onto the head lice, achieved a killing rate of 100% after an exposure time of only 1 h. However, further tests are needed to ascertain whether the same killing rate is achieved when lice hidden among hair are treated. Furthermore, tests are required to see whether those inhaling the tiny nanoparticles during treatment and/or exposition period get lung problems. A covering of the surface of the lung alveoles with such nanoparticles might have considerable implications.

Carbaryl

This compound is a chemical in the carbamate family. It is the third most used insecticide in the United States to control pests in domestic gardens and commercial agriculture. In the late 1960s it was also used in the control of head lice and became available in United Kingdom as a lotion (Carylterm) or as shampoo (New Suleo). Its action against lice is based on its efficacy in inhibiting cholinesterases. At first this compound was classified as “moderately hazardous.” However, when in recent years it turned out that carbaryl may be carcinogenic, it was retracted from the market, and there are no recent products available in the United Kingdom.

Plant-derived remedies

During the billions of years of evolution on earth, plants, which depend strongly on their fixed habitats, have had to develop means that would protect them against the attacks of other plants and against the feeding activities of insects, in order to survive. These supporting substances inside plants were later used by humans as precursors of medicaments and also against plant or body pests either by applying them directly, as naturally obtained extracts or by synthesizing particular ingredients, such as the pyrethrins/pyrethroids (Amer-Krim and Mehlhorn, 2006; Casida and Quishad, 1995; Food and Agricultural Association of the United Nations (FAO), 1995; Mehlhorn et al., 2005; Schmutterer, 2002; Semmler et al., 2009).

Only in the case of synthesized single substances were studies successful in defining the mode of action against mites, ticks or insects (including lice), while the activity of isolated extracts, which always contain a mixture of single, potentially active substances, was only poorly understood. The activity of extracts is also depended on the concentration used and the method of application. Thus, in the following two sections the mode of action of the selected plant extracts is either unknown, suggested and/or rather superficially understood.

Insecticidal compounds

Essential oils obtained by various methods from plants (e.g. from seeds, flowers, bark, stem, leaf, and/or roots) have been in use since mankind began undertaking cultural activities. Thus billions of humans use these oils daily for cosmetic purposes, while their use as medical or pesticidal devices is much less common, although increasing today. With respect to the control of head lice infestations, there are many products on the market (Table 4). In general, mixtures of different plant extracts are used and combined into a product in order to get a standardized formulation, which brings constant results. However, although a large variety of plant extracts are used, significant and comparable trials are rather rare. There are reports on the efficacy of mixtures containing 10% (w/v) *Melaleuca* (= tea tree) oil and lavender oil (w/v) (NeutraLice, Australia) with an efficacy of 97.56% (Barker and Altman, 2010) exceeding the 82.5% efficacy of a mixture of other essential oils (MOOV solution, Australia; Grieve et al., 2007). The efficacy of essential oils depend on the method of extraction, on the application dose, on the plant species and on the selected plant organ. This has been shown by many authors. For example, Toloza et al. (2010) reported that essential oils of four different *Eucalyptus* species needed between 47 and 112 min to kill 90% of the head lice in their test system. This is not surprising, since the different compounds in two different *Eucalyptus* species (*E. dunnii*, *E. gunnii*) varied considerably. For instance, 1.8 cineole was found in a ratio of 49.59–26.67% in identical chromatograms of both plants. Sunilson et al. (2009) showed that the mode of extraction also makes a big difference in efficacy. In the case of petroleum, they obtained using ether extracts of leaves of the Indian tree *Pongamia pinnata* lice killing rates between 50% and 100%, while methanol- or chloroform extracts revealed only low efficacy rates. The combination of essential oils in general brings better results than higher concentrations of a single oil. The in vitro and in vivo study of Prasutr et al. (2010) on a product called Biomimic lice lotion showed in vitro full (100%) ovicide activity. The pediculicide activity was 100% in vitro in three trials, but only reached 84% in a fourth. Both—eggs and motile lice—had been incubated for 30 min. When looking at the in vivo results, the activity of motile lice was around 90%. Similarly, however, varying efficacy rates were reported from oils of *Eugenia caryophyllata* and other plants (Yang et al., 2004, 2005). All these tests have to be considered under similar testing conditions.

Although there are definite proofs that essential oils may kill lice, their mode of action is unknown and generally only described as “fumigant activity” (Barker and Altman, 2010), since it was observed that there are a lot of volatile components in these oils, which are also called “etheric oils.” This effect was demonstrated to increase, if the patient wears a shower cap, so that the lice and eggs remain under complete “oil-air pressure.”

Table 4 Body characteristics of lice of humans.

Characteristics	<i>Pediculus humanus capitis</i>	<i>Pediculus humanus corporis</i>	<i>Phthirus pubis</i>
Size (length, mm)	♂~2.4–2.5 ♀~2.6	♂~2.7–3.8 ♀~3.8–4.8	♂ 1.3–1.6 ♀ 1.3–1.6
Antennae	4 short, strong segments arising from a strong shaft	4 short, strong segments arising from a strong shaft	4 short, strong segments arising from a strong shaft
Breast	Breast is smaller than hind body	Breast and hind body appear ovoid	Fused in part with short hind body, appears square-like
Shape of eyes	On both sides of the head a simple ocellus for dark-light seeing	On both sides of the head a simple ocellus for dark-light seeing	On both sides of the head a simple ocellus for dark-light seeing
Abdomen	Small, relatively deep lateral notches	Broad, not so deep notches	Appears nearly quadratic, no clear separation between breast and hind body
Sites of parasitizing	Mainly on hair of head, rarely also on hair of breast, beard, eyebrows, armpits	Mainly on body hair and on inner side of clothes, very rare in head	Mainly on pubic hair, rarely on eyebrows, hair of breast and armpits
Place of the pores of the operculum (egg cover)	On 4/5 of the whole place	On 4/5 of the whole place	Operculum is fully covered by pores

This method not only helps to increase the efficacy, but also protects the patient from the side effects of inhaling such high concentrations of those essential oils. Therefore, it is strongly recommended to check the potential side effects of certain essential oils before using them at high concentrations (Tolozan et al., 2010). This is underlined by the report of Barker and Altman (2010), who reported that 29 out of 132 patients suffered adverse reactions after a treatment with 12% tea tree oil and lavender, but only 3 or 4 suffered adverse reactions when treated either with benzyl alcohol plus mineral oil or with pyrethrins respectively.

Suffocating compounds

This section deals with extracts of neem tree seeds and grapefruit (Table 5) in combination with a patented activity of dibasic esters. Of course, it is known that components of neem and grapefruit may have insecticidal activities, if they are used in high concentrations (Schmutterer, 2002) due to their main components azadirachtin and flavonoids. The action of azadirachtin (e.g. on fleas and crop pests) was seen to proceed slowly, since this component prohibited feeding and egg deposition. Thus, this activity would be a pharmacological activity. Flavonoids have similar effects, which occur after constant and permanent application of high dosages (Adedapo et al., 2008). However, treatments for lice infestations require sudden effects (= a quick knock-down of motile stages). Therefore, a shampoo has been developed, which is based on rather low concentrations of azadirachtin (MelAza) and a combination of supporter compounds. This rather water-free but liquid shampoo (Abdel-Ghaffar and Semmler, 2007; Heukelbach et al., 2006) acts quickly and mechanically by entering the tracheal respiratory system of lice (and many other mites, ticks and insects (Abdel-Ghaffar et al., 2008; Semmler et al., 2009)). At the terminal end of tracheoles, where a fine aqueous (fluid) layer covers the membranes of the cells thus protecting them from desiccation, the electric loading of the neem-based anti-lice compound enables the closing of the terminal end of the respiratory system (Fig. 9). This was proven by transmission electron micrographs, showing three 5–10 µm sized gold particles, which had been mingled into the shampoo prior to the treatment of lice in vitro (H. Mehlhorn, unpublished data). Due to this activity the entrance of oxygen into the cell is suddenly interrupted and will be blocked until the insect dies. This process is therefore not based on pharmacological properties, but is simply a physical reaction.

A paper by Abdel-Ghaffar et al. (2010b) proved that a complete submersion of the lice into this neem-based shampoo for only 3 min is sufficient to kill 100% of the lice. This shows that the efficacy starts much quicker when using the more adhesive di- and cyclomethicones, which often only block the outer entrances (= spiracles, stigma) of the respiratory system leaving enough oxygen for a long survival period, during which the lice reduce their metabolism. Therefore the recovery rate of methicone-treated lice is high, if the treatment period is not long enough and if the products are washed off too early (by means of a normal shampoo). Nearly identical effects were seen when the neem extracts were replaced by grapefruit extracts (Abdel-Ghaffar et al., 2010a).

When using either the neem-based shampoo or the shampoo containing grapefruit, the larval stages inside the nits were also killed. However, this process took longer than 10 min. Apparently the developing stages need less oxygen, so they might survive oxygen starvation for at least 10–15 min. This was seen when cut-off nits were incubated in both shampoos and their development was followed in vitro for the next 7 days. However, in vivo treatment of nits may fail in some specimens, if these nits are not covered long enough (if at all) by the shampoo due to imperfect application of the product onto the hair. This survival of developmental stages within the nits would lead to persistence of an infestation in case of a single treatment. Therefore, a second course of treatment after 9–10 days seems reasonable. This second treatment would also extinguish new infestations with lice, which may occur if a perfectly treated child gets into hair-to-hair contact with untreated louse bearers (Al-Quraishy et al., 2015; Aspöck, 2010; Dörge et al., 2017; Iwamatsu et al., 2016; Pavela et al., 2016; Raoult and Roux, 1999; Sands et al., 2016; Sangaré et al., 2016; Semmler et al., 2017; Ulutasdemir et al., 2018; Yoon et al., 2015).

Table 5 Selected species.

Species	Host	Length	Suborder
<i>Bovicola bovis</i>	Cattle	1.5–2 mm	Ischnocera
<i>B. ovis</i>	Sheep	1.5–2 mm	Ischnocera
<i>B. caprae</i>	Goat	1.5 mm	Ischnocera
<i>Werneckiella equi equi</i>	Horse	2.0 mm	Ischnocera
<i>W. e. asini</i>	Donkey	1.8 mm	Ischnocera
<i>Trichodectes canis</i>	Dog/canids	2 mm	Ischnocera
<i>Felicola subrostratus</i>	Cats	1.35 mm	Ischnocera
<i>Anaticola anseris</i>	Goods	4 mm	Ischnocera
<i>Eomenacanthus stramineus</i>	Turkey/fowls	3.2 mm	Amblycera
<i>Menopon gallinae</i>	Chicken	1.8 mm	Amblycera
<i>Lipeurus caponis</i>	Chicken	2.3 mm	Ischnocera
<i>Columbicola columbae</i>	Pigeons	2.5 mm	Amblycera

Why is it so difficult to eradicate head lice completely?

The persistence of head lice even in societies with high hygienic standards and sufficient financial means to control them is based on many reasons and on the astonishing abilities of the lice:

- Head lice live hidden in hair and a transmission is noted only weeks to months after the infestation day.
- Due to their strong claws lice do not fall involuntarily from a host.
- Head lice deposit their eggs inside nits, which protect the developing larvae from falling off and from adverse influences (e.g. water, treatments).
- The glue used to fix the egg/nits to the hair is not water-soluble.
- Motile lice stages inside hair are very resistant to environmental influences. Even 70% alcohol does not kill them easily.
- Lice have developed resistance to many chemotherapeutical drugs, so that there are always survivors.
- Furthermore, control measurements using suffocating products fail to kill 100% of the louse population on an infested head, since often not all the hair is covered with the products.
- Even in cases of successful treatment, a re-infection may occur the next day from hair-to-hair contact with an untreated person.
- The time needed for completing the developmental cycle on a head varies considerably, so that at a given treatment period some lice stages may not come into contact with the product.
- In a population of humans there are always people who do not notify an infestation with lice and are thus a major reservoir for new infestations.
- Louse transmission is easy in today's crowded places (e.g. subways, sports events) or among playing children.
- Poor people cannot pay for anti-lice products and thus remain reservoirs. Other people—often with “green” ideology—do not use efficient products and thus also produce “happy survivors” among the nasty bloodsuckers.

Conclusions and perspectives

The present situation in the fight against head and body lice is characterized by the constant decreasing activity of chemotherapeutical remedies due to increasing resistance. This gap could be closed using chemical and/or natural products with suffocating activities. However, several of these innovative products do not fully work and thus leave reservoirs for new infections. Furthermore, many of the available anti-lice products on the global markets pose high health risks for users. These dangers are even increased if the products are wrongly used or when pharmaceutical companies avoid mentioning the dangers. It is hard to believe that on many products containing cyclo- and/or dimethicones or high percentages of inflammable substances, no warnings are given that these products are inflammable (Table 3) and that the fire can only be extinguished by covering with a towel, but not with water.

On the other hand, there are suffocating products made with neem extracts or flavonoids (see Table 4), which do not glue the hair, which are not inflammable, which do not touch the scalp and which smell rather nice. Therefore, the user should carefully read the product information leaflet before buying any anti-lice product.

Body lice (*Pediculus humanus corporis*)

1. Name: This species parasitizes on the human body surface sucking blood like their relatives, the head lice (*Pediculus humanus capitis*) and the pubic lice (*Phthirus pubis*). In some countries these body lice are also described by the Latin name *P. humanus vestimentorum* (=belonging to clothes). In Germany it is called “Kleiderlaus,” in France “Pou du corps” or “Pou blanc” and in Spain “Piojo di vestimento.”
2. Size: The adult males reach a length of 2.7–3.8 mm, while the females (Figs. 3 and 8) are 3–4.8 mm long. Their segmented antennae are considerably longer than those of males reaching a length of up to 0.4–0.5 mm (Fig. 3).
3. Body: The body of the lice is wingless and is clearly separated into three regions:
 - a. Head, which is provided with one pair of segmented antennae being composed of five segments bearing sensillae. At both lateral sides a single, rather primitive eye is located consisting of an enlarged, pigmented so-called ommatidium (Fig. 8). The mouthparts belong to the biting-sucking type. They are kept retracted in a sheath inside the mouth until the next bloodsucking.
 - b. Breast, which consists of three segments, each of which bears a pair of legs being each equipped with two claws, which may clutch ring-like together, so that a hair can be completely surrounded thus avoiding an accidental dropping down from the host's body. Each leg comprises six segments. At the proximal part closer to the thorax, they start with the coxa followed downwards by the trochanter, femur, tibia and tarsus. The lice are wingless, while in other insects the backside of two breast segments are each equipped with two wings.
 - c. Abdomen. The hind body of the body lice consists of originally 10 segments. The first two are strongly reduce and do not bear openings of the tracheal system, while starting from the third until the eight segment each has an opening (at the lateral side) of the tracheal breathing system, which is called stigma or spiracle. The terminal segments number nine and ten are (partially) fused forming one, which in case of females ends bifurcated and appears pointed in males.

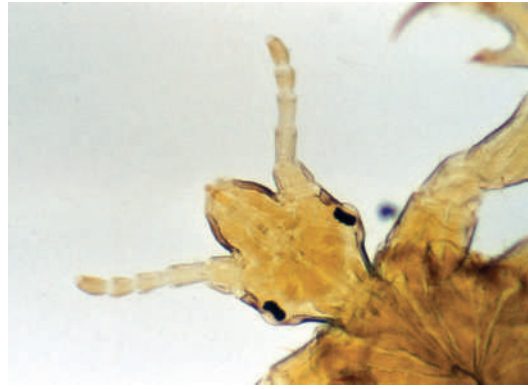


Fig. 8 Micrograph of the anterior region of a *Pediculus humanus corporis* louse showing the two antennae with 4 segments and the two lens eyes, which possess a dark bottom.

4. Development: After copulation with males the females discharge whitish eggs (Fig. 5), which become attached at the hair close to the scalp. The eggs are slender reaching a length of 0.6–0.8 mm and are covered by a spherical to ovoid lid, which is removed by the larva at the end of its development. Two-thirds of this lid contain tiny openings, which at their inner side are covered by a fine membrane, which allows apparently a limited entrance of oxygen. This arrangement of the openings is very similar in head and body lice, which in history have common sources (Fig. 1). After 5–6 days the six-legged first larva hatches from its egg by removing the cover by help of their legs. They start sucking blood along the body of their hosts for 5–7 days and then they discharge their cuticle and then they form a new one, which hardens in a few hours. After another feeding period of 5–6 days they discharge again their cuticle and reach the third larval stage. After another hatching the adult stage is reached as male or female.
5. Transmission of lice: The body lice live on humans in regions between skin and clothes, where they are anchored by help of their claws. After copulation, the females glue their eggs at the base of the body hair of humans, but also onto the inner surface of clothes and onto the surface of bed covers, from where they may reach other hosts. The lice prefer temperatures between 31 and 33 °C, which are given in the space between skin and clothes or bedcovers. These temperatures are needed to produce and distribute eggs. However, they survive also lower and higher temperatures (10–50 °C). To lay eggs the females need at least one blood meal per day. If temperature and blood meals are given, the females lay about 5–14 eggs per day (Fig. 3), which they attach at human hair or clothes. Lice are killed, when clothes are exposed to at least 50 °C upwards. Females are able to produce up to 300 eggs during their normal adult life span of about 30–40 days, which might be prolonged, if contaminated clothes are stored at low temperatures (e.g. close to 0 °C).
6. Reactions on blood sucking: While sucking blood all lice stages (larvae, adults) introduce saliva into the biting site. This saliva contains components, which keep the ingested blood fluid, so that the channels of the mouthparts are not blocked by coagulating blood. The human skin develops reactions showing 1–1.5 mm sized dots, which at first appear slightly red and bluish. These biting sites develop itching reactions the intensity of which depends on the allergic status of the person hit by such lice. Since the itching might become very intensive in cases of infections with many lice, eczema might become developed and skin may appear brownish.
7. Transmission of agents of diseases: In contrast to the effects of head lice, which in general might be very nasty, but not very dangerous, the bites of body lice can be life threatening, since they are able to transmit agents of severe diseases.
 - a. Louse-borne spotted fever: This disease is initiated by transmission of the species *Rickettsia prowazekii*, which occurs in Europe, Asia and Africa. These stages have been first described in the year 1909 by the French scientist Charles Nicolle, who received 1929 the Nobel Prize for this discovery. In the year 1907 the American Howard Tyler Ricketts (1871–1910) described the fact that the agents of the so-called Rocky Mountains spotted fever looks very similar to those of the louse-borne spotted fever. He died already 1910 in Mexico due to this fever. The German scientist Stanislaus von Prowazek (1875–1915) confirmed Ricketts' results by experiments and noted the death of hundred thousands of soldiers of both sides occurred during the First World War (1914–18). He also died as consequence of a laboratory infection with this bacterium in the year 1915. Remembering the death of both (von Prowazek and Ricketts) the agent of this louse-borne disease was named *Rickettsia prowazekii*, which today is described as a gram negative, stick-like bacterium measuring $0.8\text{--}2 \times 0.3\text{--}0.5 \mu\text{m}$. These bacteria live exclusively inside cells of their hosts. In their vectors—body lice—they live in intestinal cells and are set free within dust-like feces. When humans inhale or ingest such bacteria, they enter endothelial cells of blood vessels and start reproduction. If such endothelial cells are destroyed, they enter other endothelial cells and reproduce themselves therein again.
 - b. The clinical signs of this disease start after an incubation period of about 10–14 days with a high graded fever for about 10 days accompanied by 2–4 cm sized skin dots, giddiness, limb pain, dizziness and cough. In times before the drugs doxycycline and several tetracyclines had been developed, the lethality rate was very high reaching “normally” 10–20%, but was even considerably higher among soldiers in the trenches of the European and American wars, where very large numbers

of soldiers died in the First World War in France and in various USA-South America wars. Today drugs like doxycycline (200 mg $\times$ 1), chloramphenicols and penicillin are very helpful. For pregnant women and children amoxicillin and erythromycin had been successfully used. If the diagnosis and the start of the treatment does not start soon after an infection, the death rate is significantly increased.

8. Further transmitted agents of diseases

- a. *Rickettsia mooseri*,
- b. *Bartonella quintana*,
- c. *Borrelia recurrentis*,
- d. *Francisella tularensis*,
- e. *Salmonella* sp.
- f. *Staphylococcus* spp.

9. Mode of transmission

Body lice are rather quickly moving animals, which are able to pass larger distances in rather short times (e.g. 35 cm/min), so that in hotels, clinics, etc. they may easily reach their hosts during night time, while their presence remains undiscovered. The female glue their eggs at the hair of persons and occasionally on the surface of resting places, which allow hatching larvae to get in contact to body sites of human hosts.

10. Protection measures

- a. Check lower side of beds when sleeping in foreign beds.
- b. Check remnants of blood on blankets, which occur potentially due to hidden lice.
- c. Check blankets for dark droplets, which may have their origin in the bloody feces of lice.
- d. Check presence of tiny blackish balls, which might be excreted lice feces.
- e. Wash clothes as well as blankets in hot water (60 °C).
- f. Ask a pest exterminator to spray insecticides or molt inhibitors.
- g. If the above listed measurements are not feasible, do not enter the room for at least 10 days and keep the temperatures low therein.
- h. The occurrence of body lice in an institution should be announced to the public health institutions, otherwise punishment will follow.

Attention: The stages of *Rickettsia prowazekii* lead to a chronical infection, so that even years after survival of a spotted fever attack a relapse may occur. Thus, the control of body lice, especially in institutions with huge numbers of humans on small space is very important to avoid epidemics of louse-borne diseases.

Pubic lice (*Phthirus pubis*)

1. Name: The genus name *Phthirus* (syn. *Phthirius*) has its origin in the Greek term "phtherein," which means "to destruct," while the species name "pubis" is of Latin origin and designates the female genital region. This explains, why in French language these lice are called "pou du mont pubis" or "papillion d'amour" = butterfly of love in English. In Italian language they are named "piattola," while the English world calls them "crab louse" describing its nearly quadratic body.
2. Biology: Adult males and females measure 1.3–1.6 mm in length and nearly the same in width (Fig. 4). The claws of the six legs are equipped with claws, which help that the lice may clutch at hair and thus become fixed thereon. After a development via three larval stages the adult female starts laying eggs for up to 26 days before they die. After 5–8 days the larvae hatch from the eggs, start sucking blood and passing the larval stages within 15–17 days they become adult and start propagation mainly in the pubic region of the human body, but they occur also on the eyebrows, eyelashes, armpits and beard.
3. Bites: The bites of these lice induce less intensive itching than do other lice species and they are often only noted when they have developed huge numbers of lice descendants.
4. Transmission: During sexual intercourse. These lice are mainly transmitted from person to person.
5. Removal: Since the lice clutch firmly at hair, the quickest solution of the problem of heavy infestation is to remove the lice from the hair at heavy infested sites by help of tweezers. However, mass occurrence of these lice should be treated by help of suffocating shampoos like Licener, which kill also the stages in the eggs.
6. Effects of bites: The bites induce only low-graded itching reactions. However, due to scratching at itching sites, eczema might become developed and the skin may appear bluish (in French "taches bleues").

Pig lice (*Haematopinus suis*)

The adults of this species reach a length of 4–6 mm (Fig. 9A and B). They are specialized to live on pigs, but in rural regions they are also found on farmers. The infection occurs when farmers hold such animals on their arms in order to stay on a balance and to find out the weight of a "nervous" piglet, which would not stay alone on a balance.

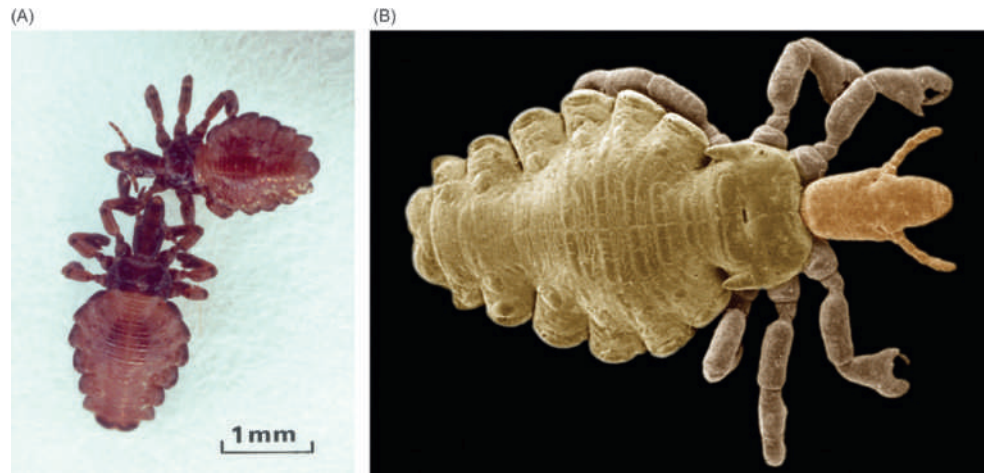


Fig. 9 Light (A) and electron microscopic (B) micrographs of the species *Haematopinus suis*, which may also reach human skin during work.

Mallophaga (Chewing lice, biting lice)

1. Name: Greek: mallos = wool; phagein = to consume, feed.
2. Geographical distribution: Worldwide in cases of dense animal farming they appear in masses and some of them attack also humans.
3. Agents of disease: The order Mallophaga (Table 5) contains two suborders: Amblycera and Ischnocera. They parasitize mainly on animals, while humans are rather seldom affected.
 - a. Ischnocera: Name: Greek: ischnos = thin; keras = horn/protrusion. Antennae: there are two antennae, which are clearly visible and possess—depending on the species—3–5 segments. Hosts: see Table 5.
 - b. Amblycera: Name: Greek: amblys = blunt; keras = horn. Antennae: They lie often in a fold of the head and thus are not or hardly visible. Hosts: see Table 5.

Besides the differences in the position of the antennae, the Amblycera possess only one claw at the feet, while feather chewing lice possess mostly two.

4. Symptoms of disease: Amblyceran species (Table 5) massive loss of blood may occur in highgraded infections. In cases with infectious ischnoceran species restlessness, strong itching and loss of hair/feathers is one of the most common symptoms.
5. Diagnosis: If animals show itching, observation of skin is required, i.e. looking for attached eggs: studying hair by help of a microscope. Detailed diagnosis is not needed, since all Mallophaga react on contact insecticides.
6. Pathway of infection: Body contact between infested and not infested specimens. These parasites may also pass onto the skin of humans, who then may suffer from the same symptoms like animals.
7. Prophylaxis: Spraying of typical insecticides onto animals or the biological agent MiteStop (produced by Alpha-Biocare GmbH, Neuss, Germany), which kills by drying the mallophages on mammals and the featherlings on birds (see Benelli et al., 2018 for a dedicated review on Mallophaga control).
8. Therapy: Macrolytic lactones have been proven to be successful against chewing and biting lice among the mallophages. In cases of infested humans normal shower shampoos are sufficient to clean the body.

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Laelapid and Dermanyssid Mites of Medical and Veterinary Interest

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Introduction

Laelapid and dermanyssid mites are two families belonging to the order Mesostigmata, one of the most morphologically and ecologically diverse and widespread order of Parasitiformes. Among this order, parasitic species are mainly grouped in one superfamily, the Dermanyssoidea (Dowling, 2006) where 11 families out of 17 comprise species parasites of vertebrate (Lindquist et al., 2009a,b). Laelapids and dermanyssids are two of these families showing a related evolutionary path and a worldwide distribution. Nevertheless, their medical and veterinary importance is very different.

Members of the laelapid family are very numerous and heterogeneous. They show a great variation of life-history strategies and associations. Several species are free-living predators in soil, others are nidicolous commensal, while others facultative or obligate ectoparasites in the nests and colonies of vertebrates and insects (e.g., Radovsky, 1985; Gettinger et al., 2005; Dowling and Oconnor, 2010; Lindquist et al., 2009b). Species that are blood-feeding parasites of commensal rodents have risen some interest. However, to date, only one species (*Laelaps echidninus* Berlese 1887) proved to have some sanitary relevance since involved in diseases transmission among rodents and suspected to be a vector of human pathogens (Mullen and Oconnor, 2019). On the other hand, considering the increasing populations of “peridomestic” rodents in urban and rural regions, the importance of the rodent associated laelapids is likely to increase as well.

A different scenario characterizes the dermanyssid family. They are a much more uniform group with all members obligate hematophagous ectoparasites of vertebrates (Lindquist et al., 2009b). Among this family, the genera *Liponyssoides* and *Dermanyssus* have a veterinary and medical interest. In particular, *Dermanyssus gallinae* (De Geer, 1778) has aroused the highest attention due to its enormous economic implications in the poultry industry, and its not negligible interest in human health. This article, after a first part focusing on aspects of systematics, evolution, and identification, discusses the complex biology and ecology of these two families, and highlights the medical and veterinary importance of the relevant species, together with the correct approach for their prevention and control.

Systematics, phylogenetic relationships and evolution

Among chelicerates, Acari (mites and ticks) are the most abundant and widespread taxon and the most diverse regarding life history patterns, behavior, external and internal morphology (Alberti and Coons, 1999; Krantz, 2009a,b). They comprise over 55,000 species described (Zhang, 2011) but 1 million species seems a very cautious estimate. The order Mesostigmata (or Gamasida) belongs to the superorder Parasitiformes (or Anactinotrichida), the smaller of the two main groups in which Acari are divided (Lindquist et al. (2009a,b)). The known fossil records for the Parasitiformes date back to the late Cretaceous Era, and ancestors of the superorder Parasitiformes probably made their terrestrial debut as predators in surface habitats of the littoral zone (Krantz, 2009a).

Mesostigmata is a monophyletic group (Li et al., 2019) comprising the largest number of species (over 11,000 species, Beaulieu et al., 2011), and it is the most ecologically diverse and geographically widespread order in Parasitiformes showing significant adaptive radiation into a broad spectrum of general niches. Fossils of mesostigmatid mites are very rare (only 15 records, including 4 named species) with the oldest record dated back to Paleogene Period (Dunlop et al., 2013, 2014).

The higher classification of Mesostigmata is still controversial. Here, we follow Lindquist et al. (2009a,b) and Beaulieu et al. (2011). Most species in the Mesostigmata are free-living predators and are beneficial, while some species have a parasitic behavior.

The ones associated with vertebrates have developed notably diverse host relationships, but nearly all are in one superfamily, the Dermanyssoidea (Dowling, 2006).

The Dermanyssoids include many species of free-living predators, which have given rise to a diverse range of families and genera that are closely associated with vertebrate and invertebrate hosts (Radovsky, 1969; Lindquist et al., 2009b). Derivative families include from commensals, phoretics, and symbionts, to facultative (e.g., Laelapidae) and even obligate blood-feeding ectoparasites (e.g., Laelapidae and Dermanyssidae) of vertebrates. Due to such wide ecological amplitude with high levels of morphological variability, it has been difficult to develop a robust phylogenetic hypothesis of dermanyssoids. However, as hypothesized by Radovsky (1969), the taxa present in the Dermanyssoidea appear to be derivative groups of a basic free-living stock represented by the subfamily Hypoaspidae (Laelapidae) while another subfamily, the Laelapinae (Laelapidae), appears to be the source of most of the other parasitic groups in the Dermanyssoidea. The same life cycle-feeding pattern present in dermanyssids and laelapids may be considered an additional indication of their evolution from a common ancestral protolaelapid type (Furman, 1966). Such evolutionary sequence, postulated for the Dermanyssoidea (Radovsky, 1969, 1985, 1994; Dowling, 2006; Krantz, 2009b; Lindquist et al., 2009b), was confirmed by molecular studies (Dowling and Oconnor, 2010).

The nesting habitat provided the basis for a close association between the mite and the nest occupant; such association may have initially been considered a kind of commensalism, i.e., mites feeding on small ectoparasites and/or on blood exuding from abrasions present on the body of the nest inhabitant. Later, specializations for strict parasitism have occurred because they give access to a more dependable and richer food supply than opportunistic feeding (Radovsky, 1994).

The adaptation to a closer parasitism is shown, for example, by the more and more specialization of the mouthparts (chela). In fact, while the primitive chelate-dentate state of some dermanyssoids (e.g., some species of *Androlaelaps* (Laelapidae)) is more efficient in grasping and shearing the prey, or other food than in piercing skin, the slender shape of the chelicerae and the presence of smaller teeth (e.g., the stylet-like chelicerae of Dermanyssidae) have driven to the hematophagous behavior of some other dermanyssoids, thus providing a more efficient host-parasite relationship. Furthermore, a reduction of the dorsal sclerotization and of the dorsal shield occurred increasing the capacity for engorgement (e.g., in Dermanyssidae, Macronyssidae and Haemogamasidae). The development of relatively long, slender legs is also associated with an increased capacity for engorgement. In fact, the body expansion sometimes is enormous and short legs would not have been able to carry it (as in the case of Dermanyssidae) (Radovsky, 1969, 1985, 1994; Krantz, 2009b; Lindquist et al., 2009b).

In the Dermanyssoidea, the intermediate gradations between the free-living and the obligatory parasitic behavior are well represented by extant species: e.g., the genera *Haemogamasus* and *Androlaelaps* (Laelapidae) include predatory species but exhibit varying degrees of dependence on a diversity of vertebrate hosts (from nidicoles to facultative, to obligatory hematophagous parasites), and the Laelapidae and Haemogamasidae show a graded transition from predatory to parasitic mites, strongly indicating that the transition began with opportunistic feeding in the vertebrate nest environment. On the other hand, these families show a lack of specific morphological modifications to parasitism. Indeed, displaying varying levels of blood-feeding and host dependence (Radovsky, 1994; Dowling, 2006).

Among the 17 families recognized in the Dermanyssoidea (Beaulieu et al., 2011), 11 families comprise vertebrate parasites (Lindquist et al., 2009b); the present chapter focuses on the families Laelapidae and Dermanyssidae, which also include ectoparasites of several terrestrial vertebrate taxa (Krantz, 2009b) (Table 1).

Table 1 Systematic classification of the families Dermanyssidae and Laelapidae according to Lindquist et al. (2009a,b) and Beaulieu et al. (2011).

Taxonomic category	Name	Notes
Superorder	<i>Parasitiformes</i> Reuter, 1909 (or Anactinotrichida)	
Order	<i>Mesostigmata</i> G. Canestrini, 1891 (or Gamasida)	Divided into three suborders
Suborder	<i>Monogynaspida</i> Camin & Gorirossi, 1955	Largest and most speciose: 12,000 described species (estimate number of species believed to exceed 94,000) Genital opening covered by a single shield
Cohort (or infraorder)	<i>Gamasina</i> Kramer, 1881	Comprises most of the described species of Mesostigmata and most families of vertebrate parasites
Subcohort (or hyporder)	<i>Dermanyssiae</i> (or Dermanyssina) Evans & Till, 1979	Female sperm access system opening by pair of small pores (solenostomes) in region of coxae II–IV. Male chelicerae with a spermatodactyl
Superfamily	<i>Dermanyssoidea</i> Kolenati, 1859	Comprises 17 families Large and diverse assemblage of free-living and parasitic taxa Many classification schemes proposed in past years for this complex group
Family	<i>Laelapidae</i> Berlese, 1892 ^a	Comprises 90 genera, more than 1300 species
Family	<i>Dermanyssidae</i> Kolenati, 1859	Comprises 2 genera, 26 species

^aAccording to Trach and Joharchi (2018), the family Laelapidae comprises 146 genera and over 1500 species.

Laelapidae—The family Laelapidae is a cosmopolitan heterogeneous group including thousands of species (Table 1) and showing the full gamut of life-history strategies and associations: they are free-living predators in soil (e.g., some *Androlaelaps* spp.), as well as nidicolous commensal, facultative or obligate ectoparasites in nests and colonies of vertebrates and insects while the possible endoparasitic association to marsupial mammals has not been fully investigated (e.g., Radovsky, 1985; Gettinger et al., 2005; Dowling and Oconnor, 2010; Lindquist et al., 2009b). Mites of the subfamily Laelapinae are the most common ectoparasites of small rodents and frequently found on the rodents' body or in their nests (e.g., Strandtmann and Wharton, 1958; Gettinger et al., 2005; Lindquist et al., 2009b; Mařán and Fenřa, 2010; Kaminskienė et al., 2017, 2020). Several species (e.g., members of the genera *Haemolaelaps*, *Echinolaelaps*, and *Laelaps*) are blood-feeding parasites of rodents and are found worldwide; only a few species can occasionally bite humans (Wall and Shearer, 2001).

Dermanyssidae—The family Dermanyssidae is a much more uniform group; they are all obligate hematophagous ectoparasites of primarily birds, and rodents, and with less extent other mammals, including humans (Lindquist et al., 2009b).

Taxonomy and identification

Acarine systematics are highly controversial and systematic problems also occur with the lower categories. Several times the same taxonomic name is used by different authors for grouping different taxa or with different meanings. An extreme example, at the highest levels, is that some authors consider the Acari a subclass, others a superorder of Arachnida (Lindquist et al., 2009a).

Even placement of a single mite in the proper suborder requires a trained scientist, slides-mounted species, a compound microscope (Dermanyssoidea are medium-sized mites, <1 mm in length), and identification keys. In this respect, keys to the Superorders, Orders, and Suborders of Acari can be found in Lindquist et al. (2009a).

Things get even more complicated with the lower levels. The taxonomy of the superfamily Dermanyssoidea has been in a perpetual state of flux since the late 19th century. Many classification schemes, often contradictory, have been proposed by different researchers, typically based more upon ecology and host associations than any character evidence. Sometimes even the taxonomic rank of the families is disputable: i.e., some acarologists considered some groups as subfamilies while others ranked them as separate families (Lindquist et al., 2009b).

Since identification keys of the Mesostigmata families (Lindquist et al., 2009b) are based on adults (normally females), it is crucial to be able to distinguish adults (males and females) and juveniles (see Life cycle, for details). Both reproductive and morphological criteria are used in the higher classification of the Mesostigmata. One major division is based on the method of insemination (podospermic or tocospermic, see "Reproduction and mating behavior", for details). Mites of the subcohort Dermanyssiae are all podospermic and characterized by the presence, in the females, of an insemination system (or sperm access system) opening by a pair of pores, by a male genital orifice in presternal position, and movable digit of male chelicera with spermatodactyl (Evans, 1992; Alberti and Coons, 1999). Among Dermanyssiae identifications of families and species is based on several diagnostic characters: ventral sclerotization of the female (e.g., sternal, genital or genito-ventral and anal shield or ventro-anal shields) (Fig. 1), chaetotaxy of the dorsum and venter that form a predictable pattern (Fig. 2A), chaetotaxy of the legs, presence of lyrifissures and pores on the dorsum, morphological diversity of the chelicerae (Fig. 2B–D), relative length of peritremes vs. coxal position.

Reliable species determinations require the preparation of slide-mounted species for microscopic examination and the assistance of an acarologist with the proper background and who has access to the appropriate taxonomic literature. In fact, there are no comprehensive publications including species identification keys and the few catalogs or reviews available do not cover the whole species comprised in the families Laelapidae and/or Dermanyssidae. Quite often, keys available in taxonomic articles refer only to species from certain geographical areas (e.g., Moss, 1968, 1978; Mařán and Fenřa, 2010; Mařán et al., 2014; Vinarski and Korralo-Vinarskaya, 2016; Kaminskienė et al., 2017). Thus, specific identification is challenging due to the lack of (detailed) descriptions, and because different dermanysoid species can share the same environment and/or host, and because the use of taxonomic identification keys—to differentiate between them—requires expertise that is no longer widely available. This is even more critical for the family Laelapidae since mites infesting small mammals have been less intensely studied. Finally, the reliability of some of these diagnostic characters seems to be doubtful or not stable enough and many intraspecific variations have been reported, both in the family Laelapidae and Dermanyssidae (e.g., Roy and Chauve, 2007; Roy et al., 2010a; Lareschi and Galliari, 2014; Silva-de la Fuente et al., 2020). Thus, the presence of cryptic¹ species and/or species complex have been hypothesized and, in some cases, confirmed by genetic studies (Gettinger and Owen, 2000; Roy et al., 2009a,b, 2010a; Roy and Buronfosse, 2011; Engelbrecht et al., 2014; Lareschi and Galliari, 2014; Silva-de la Fuente et al., 2020).

Compared to the family Laelapidae, which includes a huge amount of species, the much smaller family of Dermanyssidae includes only two genera, *Dermanyssus* and *Liponyssoides* (Evans and Till, 1962; Moss, 1967, 1968, 1978; Roy and Chauve, 2007, 2010; Lindquist et al., 2009b; Beaulieu et al., 2011). The extremely elongated second cheliceral article of females and the highly reduced digits of the chela (with stylet-like chelicera (Fig. 2C)) are diagnostic for this latter family (see Di Palma et al., 2012a for details and illustrations).

¹Cryptic species are species that do not possess any morphological divergence with their closest sister(s) so they may not be distinguished from each other based on morphology alone. They are usually the result of a recent speciation event.

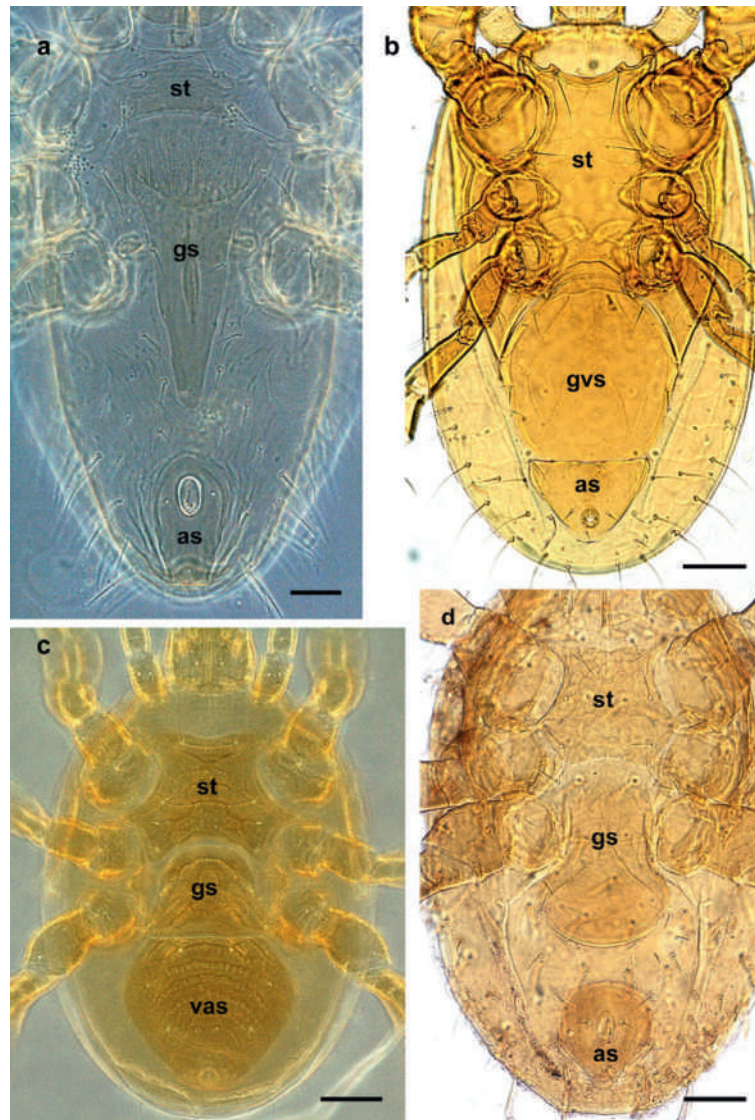


Fig. 1 Light microscopy (LM). Examples of several ventral sclerotizations in females belonging to different families of Dermanyssidae (A) Macronyssidae, (B) Pachylaelapidae, (C) Macrochelidae, (D) Laelapidae. Note: different shape and dimension of the genital (epyginal) shields (gs) and of the sternal shields (st) in all pictures, presence of a genito-ventral shield (gvs) in (B), ventro-anal (vas) shield in (C), and anal shields (as) of different shape in (A) and (D). All these characters are diagnostic for the identification of the families. Scale bar: 50 μ m.

Dermanyssus species morphologically fall into two groups: (i) those possessing a soft body adapted for sporadic and large engorgement with reduced shielding and slender legs, and (ii) those possessing a compact, more heavily sclerotized body with shorter, stouter legs. Species possessing the soft-body type are the most common and most of them are nearly indistinguishable from each other and constitute the *gallinae*-group (Roy et al., 2009a).

Biology and ecology

Reproduction and mating behavior

Among the different reproductive modes, haplodiploidy is a widespread phenomenon in mites. In haplodiploidy, males are haploid and females are diploid and it can be caused by a number of different underlying genetic systems. One of the most common is arrhenotoky: a type of parthenogenesis where haploid males are produced from unfertilized eggs, whereas diploid females result from fertilized eggs.

Arrhenotoky appears to be quite frequent among the subcohort Dermanyssidae (or Dermanyssina) in that it is the predominant form of reproduction in several families of the subcohort (e.g., Macrochelidae, Dermanyssidae, Macronyssidae) and for at least some species in the laelapid family (e.g., Furman, 1966; Mitchell, 1968; Oliver Jr, 1965, 1971; De Jong et al., 1981; Evans, 1992). In such cases, like *Dermanyssus gallinae* (Dermanyssidae), females lay haploid (unfertilized) and diploid (fertilized) eggs which

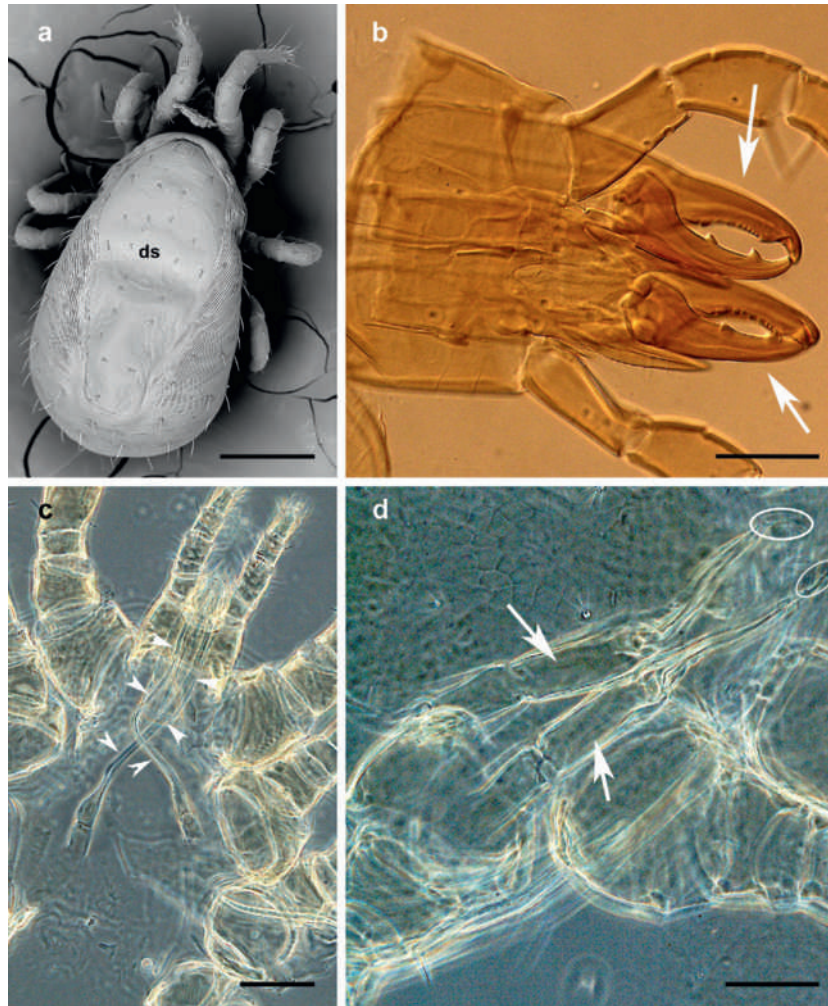


Fig. 2 (A) *Dermanyssus gallinae*, SEM. Female dorsal shield (ds) with a characteristic layout of the setae. (B) An example of chelicerae with robust chelae (arrows) in the family Laelapidae (LM). (C) *Dermanyssus gallinae*, LM. Female chelicerae modified in elongated stylets (arrowheads), this is a character diagnostic for the family. (D) *Dermanyssus gallinae*, LM. Male chelicerae stylet-like and provided with small spermatodactyls (circles) used to inseminate the females. Scale bar: 250 μm (A); 50 μm (B and D); 100 μm (C).

develop into males and females, respectively (Oliver Jr., 1966; Hutcheson and Oliver Jr., 1988). However, the Dermanyssidae contain diplo-diploid (both males and females derived from fertilized eggs) and pseudoarrhenotokous (males and females arise from fertilized eggs, but males eliminate the paternal genome) groups as well. Phylogenetic studies suggest that arrhenotoky should be derived from pseudoarrhenotoky (De Jong et al., 1981; Cruickshank and Thomas, 1999). A switch from pseudoarrhenotoky to arrhenotoky confers the advantage to a female of being able to produce offspring without mating (facultative asexuality). Indeed, females lay unfertilized eggs that produce males who can mate with their mothers. This means that, in arrhenotokous species, a new habitat patch can be colonized by a single un-inseminated female who can parthenogenetically produce males with which to mate, thus getting new females for her sons to mate with. In this way, one female can found a new colony. This is likely to be of particular importance in parasites (like Laelapidae and Dermanyssidae), in which the new habitat patch represents an uninfected host (Cruickshank and Thomas, 1999).

Regarding mating behavior, among Mesostigmata only direct sperm transfer occurs: the male introduces the sperm in the female (Alberti and Coons, 1999 and references therein). In particular, in the Dermanyssidae the males present chelicerae modified as gonopods and equipped with a sperm transfer appendage, called spermatodactyl (Fig. 2D), provided with a canal (Di Palma et al., 2006, 2009, 2013a, and references therein). Males use the spermatodactyl to inject the sperm material into a pair of female's insemination pores (sperm induction pores, solenostomes) usually located close to the base of legs II–IV. Thus, this mode of direct sperm transfer is termed “podospermy” (Alberti and Coons, 1999; Krantz, 2009c, and references therein). The insemination pores lead to a complex insemination system where sperm is stored and transferred to the ovary. This insemination system was first described by Michael (1892) and proved to have systematic relevance for families and species identification (Evans, 1992; Krantz, 2009c). Currently, two basic types of sperm access system are known in the Dermanyssidae: a laelapid and a phytoseid-type with several variations (Alberti and Coons, 1999 and references therein; Di Palma and Alberti, 2001; Di Palma et al., 2012b, 2013b,

2017); the families Laelapidae and Dermanyssidae both present insemination system of the laelapid-type (Evans, 1992; Alberti and Coons, 1999 and references therein).

In Dermanyssidae, mating occurs in the venter to venter position (more frequently) or ventro-laterally with the male laying between the legs of the female and using his second pair of legs to hold the fourth pair of legs of the female. The male produces, from his genital orifice, a sperm package (the spermatophore), then he collects the spermatophore with the help of the chelicerae and flexing the gnathosoma (Alberti and Coons, 1999; Krantz, 2009c and references therein). The sperm fluid passes from the spermatheca into the spermatodactyl canal whose tip is inserted into the female insemination pore so that sperm fluid is injected into the insemination system (Alberti and Coons, 1999; Krantz, 2009c; Di Palma et al., 2006, 2009, 2013a).

Life cycle

As in all arthropods, postembryonic development of Acari is characterized by molts that divide the life cycle into a number of instars. Ontogenetic development in the Mesostigmata includes egg, larva, protonymph, deutonymph, and adult male and female (Evans, 1992; Walter and Krantz, 2009). The adoption of a parasitic mode of life by members of the superfamily Dermanyssoidea has been accompanied by a variety of morphological and life-cycle modifications (Walter and Krantz, 2009).

Laelapidae and Dermanyssidae are normally oviparous with stages in the life cycle consisting of egg, nonfeeding larva, feeding protonymph, deutonymph, and adults (Furman, 1966; Mitchell, 1968; Maurer and Baumgärtner, 1992; Walter and Krantz, 2009). Anyway, in many laelapines the egg and even the larval stage may be suppressed, with females giving birth to larvae or even protonymphs (Radovsky, 1969, 1994; Mitchell, 1968). Usually, eggs are produced singly (Fig. 3A) and not in a cluster and not on

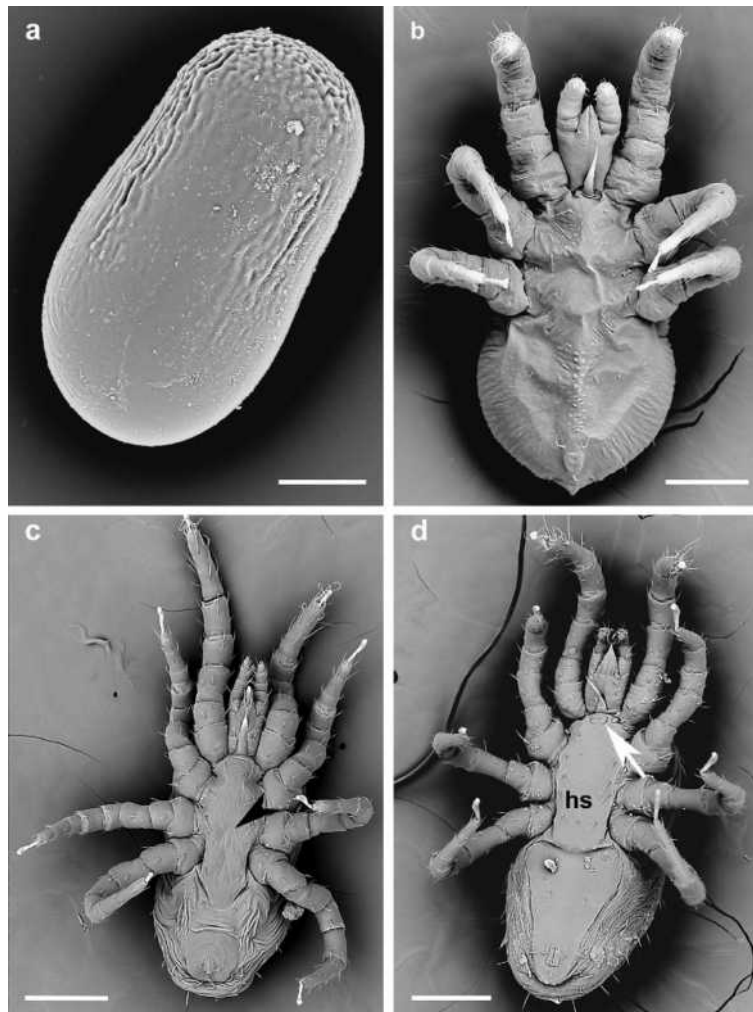


Fig. 3 *Dermanyssus gallinae*, SEM. (A) An egg deposited singly. (B) Larva with three pair of legs and no evident shields. (C) Nymph with four pair of legs and a reduced genital shield (arrowhead) without genital opening. (D) Male with an elongated holovenral shield (hs) carrying the genital opening (arrow). Scale bar: 50 μ m (A); 100 μ m (B); 150 μ m (C and D).

the host. In obligate parasites (as in *Dermanyssus* spp.), eggs are laid shortly after feeding and a blood meal is required for each gonotrophic cycle, and oviposition is sustained until all blood ingested is digested (Tucci et al., 2008).

Larvae are hexapod (Fig. 3B) lacking the fourth pair of legs, while the nymphal and adult stages are all octopod. Each stage has distinct setal and idiosomal shielding patterns. The larva typically possesses very little, if any, shielding (Fig. 3B), and reduced setation. Each successive molt increases both shielding (Fig. 3C and D) and setation until the full complement of each is reached at the adult stage (Dowling, 2006; Walter and Krantz, 2009). The sexes in the Dermanyssoidea are readily recognized by the relative shape of the shield covering the genital orifice and the shape and position of the genital opening (Figs. 3D and 4). Secondary sexual characters are represented by the spermatodactyl (Fig. 2D) on the male movable digit (see [Reproduction and mating behavior](#)) and hypertrophy of certain setae on the male legs (Evans, 1992).

The average life developmental time in several species of Laelapidae is 10 days and even less for some Dermanyssidae (Furman, 1966; Mitchell, 1968; Hutcheson and Oliver Jr., 1988; Evans, 1992; Walter and Krantz, 2009). For example, *D. gallinae* can complete the life cycle in 7 days with warm temperatures (between 25 and 37 °C) (Maurer and Baumgärtner, 1992; Chauve, 1998), but takes considerably longer (16.8 days) during colder periods (lowest 20 °C) proving that it is a poikilothermic organism whose population ecology is very much influenced by the temperature. Nevertheless, this species can develop and reproduce in a wide range of temperatures (Tucci et al., 2008) and it seems that higher temperatures (up to 35 °C) are less restrictive than lower ones.

Another important adaptation of parasitic species is their ability to survive adverse environmental conditions (Kirkwood, 1963; Nordenfors et al., 1999; Chauve, 1998): some species can survive for 9 weeks (i.e., *Liponyssoides sanguineus* Hirst) (Mullen and Oconnor, 2019) without a blood meal or as long as 55 weeks (i.e., 13 months) (i.e., *D. gallinae*) (Pavličević et al., 2007). The ability of parasitic mites to withstand starvation is of high practical importance.

Feeding habit

Parasitic genera of the dermanyssoid mites can be permanent parasites (i.e., the mite remains on the host throughout its life cycle) or nidicolous (i.e., the mite spends part of its life cycle in the nest, roost, or another dwelling but it is also found on the host). Some species have mixed feeding behavior that includes blood-feeding as well as predation and scavenging (Radovsky, 1994). Since the chelicerae of male parasitic dermanyssoids are modified for sperm-transfer (see [Reproduction and mating behavior](#)) their capacity in feeding must be greatly reduced or even lost entirely; indeed, males usually feed very occasionally (Radovsky, 1969, 1994).

For most laelapids associated with mammals, it is unknown whether they are nidicolous, facultative, or obligate parasites. Species of some genera (e.g., *Androlaelaps*, *Laelaps*) are known to feed facultatively on nesting rodents but apparently cannot penetrate with their chelicerae the unbroken skin of their host suggesting they usually feed at an abraded area made by other ectoparasites or other means (Mitchell, 1968). Perhaps blood is oozing from a small scratch on the rodent incurred during its activities, or a small blood droplet is forming where a flea has just withdrawn its mouthparts thus, mites, luckily oriented toward the blood by odor, feed to repletion (Mitchell, 1968). For other species cannibalistic and predaceous tendencies have been observed (e.g., genera *Haemolaelaps* and *Laelaps*) (Furman, 1966) while nidicolous commensal species feed on a variety of substances,

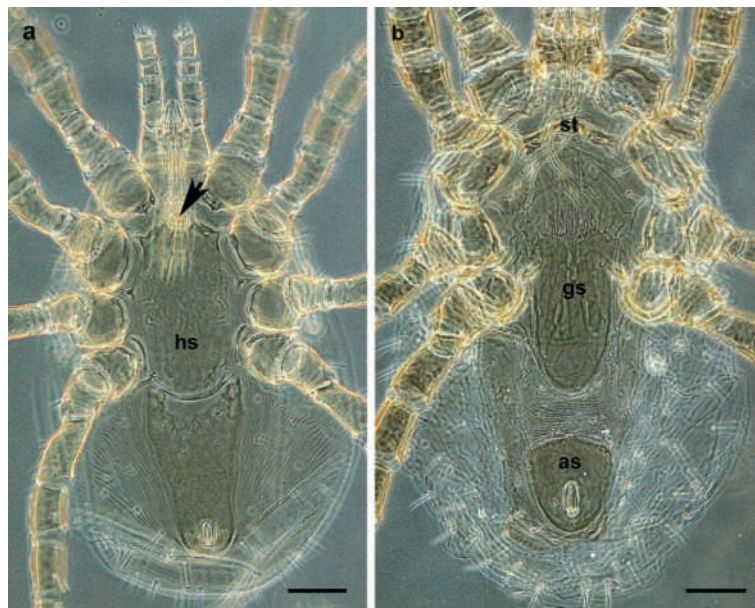


Fig. 4 *Dermanyssus gallinae*, LM showing different arrangement of the ventral shields in male and female. (A) Ventral view of a male: see the long holovenral shield (hs) with the anterior genital opening (arrow). (B) Ventral view of a female: see the small sternal (st) shield, the long genital shield (gs) and the small anal shield (as). Scale bar: 100 µm.

including small arthropods and flea feces in the nest and ectoparasites on the host itself (Krantz, 2009b). A confirmation is represented by some laelapid species previously considered parasitic and even suspected of causing dermatitis or skin irritations in humans, which have been proved to be mostly predaceous free-living or saprophagous and with loose connections to nest habitats and without any sanitary significance (Krantz, 2009b; Halliday, 2011). An example is a species very common in nests of wild birds (i.e., *Androlaelaps casalis* (Berlese, 1887)) sometimes regarded as parasitic; however, there is evidence that it is actually a predator that can feed on parasites of birds (Lesna et al., 2009). The genus *Androlaelaps* Berlese, 1903 is cosmopolitan and includes species with a variety of feeding modes (Radovsky, 1985; Martins-Hatano et al., 2011) making crucial species identification. These observations are consistent with the morphology of the chelicerae of these species which have dentate opposable digits (Fig. 2B) typical of predatory Laelapidae, not the elongate edentate digits that are commonly found in true parasites (Radovsky, 1985, 1994). Thus, the family Laelapidae shows a gradation from polyphagous to parasitic feeding with variations in the adaptation of the chelicerae from stout chelate-dentate chelicerae, more adapted for grasping and tearing the cuticle of small prey, to chelicerae modified to penetrate unbroken skin (starting with species that make crater-like holes in the skin of suckling rodents) (Radovsky, 1969).

Species of Laelapidae parasitic of vertebrates can live permanently or temporarily on their hosts (e.g., Mařán and Fenřa, 2010; Kaminskienė et al., 2017, 2020 and references therein). Among the genus *Laelaps*, the spiny rat mite (*Laelaps echidninus*) is hematophagous or tissue fluid-feeding; it is doubtful if its chelicerae are able to pierce intact skin or instead assist the mite in feeding on blood or serous exudates from abraded skin. It lives primarily in host bedding or nesting materials, moving onto the host at night to feed (Pratt, 1963; Lareschi and González-Acuña, 2010; Mullen and Oconnor, 2019).

In dermanyssoid groups, specialization of the chelicerae for skin-penetration has highly evolved in the family Dermanyssidae. The elongated second segment of the chelicerae (Fig. 2C) with highly reduced stylet-like chelae at their tips (Phillis III, 2006) function for piercing host tissue and sucking blood (Fig. 5). Thus, dermanyssids are typical blood-sucking nest parasites with a pronounced engorgement capacity and an ability to withstand starvation for months. They usually spend most of the time in the nest when not feeding; however, some species remain on the host for extended periods of time (Radovsky, 1994). Each stage (larvae excluded, since they do not feed) needs to feed before develop into the following one.

Nymphs and adults of *Liponyssoides sanguineus* (Hirst) (Dermanyssidae) are bloodsucking and feed for short periods of 1–2 h on the host while spending most of the time in the nesting materials, along the walls of infested premises, and especially in warmer areas of buildings. Nymphs generally take a single blood meal, adults move onto and off the host to take several blood meals (Fischer and Walton, 2014; Mullen and Oconnor, 2019; Ereemeeva and Muniz-Rodriguez, 2020).

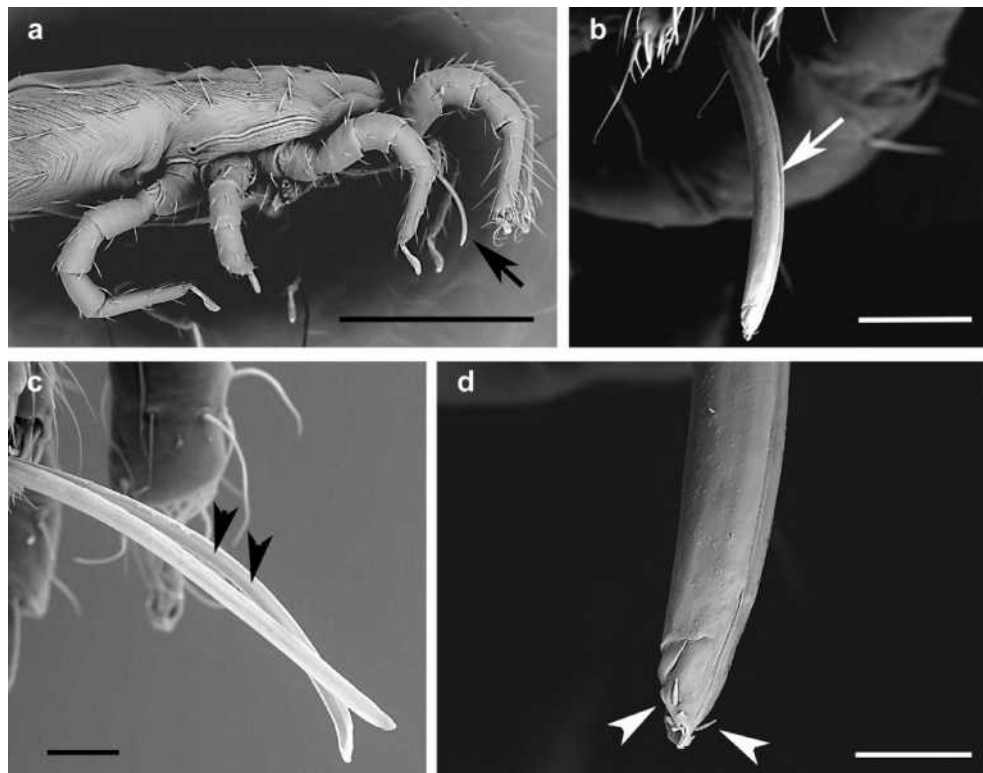


Fig. 5 *Dermanyssus gallinae*, SEM. (A) Lateral view of a female with the stylets (arrow) protracted. (B–D) Detail of the stylet-like chelicerae modified to pierce the host tissue. In (B) the stylets are coupled (see the longitudinal line (arrow)) while in (C) they are separated with the evident concave surface (arrowheads). In (D) remembrance of the chelae (arrowheads) are visible at the tip of the stylets. Scale bar: 150 μ m (A); 30 μ m (B); 25 μ m (C); 10 μ m (D).



Fig. 6 Clusters of *Dermanyssus gallinae* (arrow) hidden in the structures of laying hen cages.

Dermanyssus gallinae is blood-sucking and feeds every 2–4 days, mainly at night (although not exclusively, Nakamae et al., 1997) and normally stays on the birds approximately 30–60 min before returning to hide (Fig. 6) (Kirkwood, 1963; Chauve, 1998; Maurer et al., 1988). The mite prefers areas of the host's body with few feathers, where the skin is thin and the veins are superficial, such as the neck, back, and under the wing (Giangaspero et al., 2016).

Unlike ixodid ticks, dermanysids are characterized by multiple brief acts of blood-sucking, each accompanied by salivation. This seems supported by the dynamics of the secretory activity and in the non-simultaneous release of secretion from different cells of the salivary glands (Amosova and Stanyukovich, 2008).

Host range, host location and distribution

Mammalian ectoparasites can be monoxenous (occurring on a single species of host), oligoxenous (occurring on a few host species), and polyxenous (capable of infesting a broad array of host taxa). Polyxenous ectoparasites may be limited to host species that are closely related phylogenetically or may parasitize unrelated hosts that share ecological or behavioral similarities.

Regarding laelapid species obligate hematophagous of small mammals, it is under discussion whether there is a single polixenous species that parasites a variety of hosts, or whether there are cryptic species highly host-specific. Recent studies strongly indicate that they are primarily monoxenous (e.g., Gettinger, 1992; Martins-Hatano et al., 2002; Lareschi and Velazco, 2013) and when mites, initially assigned at the same species, infest more than one host species, an examination of the morphological variation within and among mite specimens support the hypothesis that mites infesting different hosts are reproductively isolated populations or species complex (Gettinger, 1992; Gettinger et al., 2011; Nava and Lareschi, 2012; Lareschi et al., 2013; Lareschi and Galliari, 2014). Host switching of mites among rodents has probably taken place, followed by speciation (Lareschi et al., 2013; Lareschi and Galliari, 2014; Silva-de la Fuente et al., 2020). Since some laelapids are vectors of pathogens, these processes of the new host colonization are also important from an epidemiological point of view.

The spiny rat mite, *L. echidninus*, and the smaller species, *Laelaps nuttalli* Hirst, 1915, are cosmopolitan ectoparasites. *Laelaps echidninus* is associated primarily with murid synanthropic rodents like the black rat (*Rattus rattus* Linnaeus 1758) and the Norway rat (*Rattus norvegicus* Berkenhout 1769). Moreover, it has been detected on Sigmodon, on the house mouse *Mus musculus* Linnaeus, 1758, and rarely on laboratory rodents (Pratt, 1963; Lareschi and González-Acuña, 2010; Mullen and O'Connor, 2019). *Laelaps nuttalli* is commonly found on commensal and wild *Rattus* species, while both (*L. echidninus* and *L. nuttalli*) can occasionally bite humans (Mullen and O'Connor, 2019).

Members of the family Dermanyssidae are primarily parasitic on birds, but contains a few species readily found on rodents. Rodent associated dermanysids are in the genus *Liponyssoides*, while species in the genus *Dermanyssus* are primarily parasites of birds; occasionally they have been collected from rodents and other mammals (Moss, 1967, 1968, 1978; Radovsky, 1985; Dowling, 2006; Lindquist et al., 2009b; Mullen and O'Connor, 2019).

Liponyssoides sanguineus, has been collected from many species of mammals throughout the world. The common house mouse, *Mus musculus*, is the preferred host, but this mite occurs also on peridomestic rats (*R. norvegicus* and *R. rattus*), diverse laboratory rodents, and rodents sold in pet stores. It readily attacks human beings as well (Fischer and Walton, 2014; Mullen and O'Connor, 2019; Ereemeeva and Muniz-Rodriguez, 2020).

Dermanyssus species show a rather low host-specificity with multiple host associations and a rather wide geographic distribution (Moss, 1978). For example, *Dermanyssus gallinae* is largely considered an avian-specific ectoparasite of farmed birds (e.g., chickens, turkeys, and ducks), as well as wild and synanthropic birds (e.g., pigeons, sparrows, starlings, and doves). However, it can occasionally feed on a range of mammals, such as cats (Guin, 2005; Di Palma et al., 2018), dogs (Ramsay et al., 1975; DeClerq

and Nachtegaele, 1993; Friesen et al., 2011), gerbils (Lucky et al., 2001), other rodents (Allymehr et al., 2012; Kowal et al., 2014), goat (Dorny et al., 1994), horses (Mignon and Losson, 2008), and humans (reviewed by Cafiero et al., 2019).

However, studies in the last decade have shown that most of the *Dermanyssus* species actually have more restricted host ranges than first believed (Roy et al., 2009b). In particular, *D. gallinae* might not be the only *Dermanyssus* parasitizing laying hens but that some other closely related species might often have been confused with this species (Roy and Chauve, 2007). Recent research based on morphological and molecular data (Roy et al., 2009a, 2010b; Roy and Buronfosse, 2011) has shown that *D. gallinae* is actually a species complex including at least two cryptic species. In particular, *D. gallinae* s. str. Appears to be the only pest in poultry houses, as well as on other bird species except pigeons, while *D. gallinae* special lineage L1 is restricted to pigeons only.

In general, blood-feeding ectoparasites utilize a variety of cues such as body heat, CO₂, and host-specific kairomones to locate their host. Regarding the ability of laelapid parasites to locate the host, some studies (Mitchell, 1968) highlighted the combination of responses toward carbon dioxide and blood as an important advantage for a nest-dwelling mite in actively seeking suitable hosts. Investigations on the host-searching process in dermanyssids have shown that mite-related cues (aggregation pheromones) and host-related cues (kairomones) mediate the behavior of the poultry mite (Koenraadt and Dicke, 2010) while foreleg tarsal sense organs are involved in stimuli perceiving (Soler Cruz et al., 2005). A gradual increase of CO₂ concentration, heat, and vibrations are powerful activating stimulus (Kilpinen, 2001, 2005) guiding mites toward the host thanks to the heat transmitted through the substrate or the exhaled air (as an indication that a potential host is near). After the mites have moved close to the potential host, recognition may be facilitated by skin lipids of birds, which are known to act as feeding stimulants to poultry red mites (Zeman, 1988). Anyway, CO₂ can elicit contrast behavior: mites “freeze” (i.e., they become motionless) in response to a quick increase of CO₂ when in light conditions (probably as a defensive strategy to avoid being eaten by the host) (Kilpinen, 2005). Thus, responses of mites toward CO₂ are likely to be dose and context-dependent. After the blood meal, *D. gallinae* forms aggregations of mixed developmental stages and thigmokinesis (i.e., increased locomotion in response to changes in contact with the immediate physical environment), and pheromones are thought to play a role in this (Entrekin and Oliver Jr, 1982; Koenraadt and Dicke, 2010). However, as nests are discontinuous and temporary microhabitats, an important adaptation of mite parasites involves the use of the host to disperse (Martins-Hatano et al., 2011).

Among laelapine nest mites, the female is usually the dispersal stage and most often found on the host, while males and immatures are presumed to be restricted to the nest of the host. In this way, dispersion is primarily vertical, between hosts of the same species (parents and young) occupying the same nest. The same happens with dermanyssids with females representing the dispersal stage most often collected on the host (Radovsky, 1985, 1994). Moreover, many families of Mesostigmata, including Laelapidae and Dermanyssidae, have established close phoretic relationships with other arthropods (Krantz, 2009b). Examples of phoretic behaviors on insects by *D. gallinae* have been reported twice (Flechtmann and Baggio, 1993; Pavlović et al., 2016). In this way, arthropods can contribute to mite distribution in the near vicinity, including houses and farms.

Anyway, there are parasite species whose presence is strongly related to human activities (i.e., *D. gallinae*). In this case, the most important aspect for their distribution, at least in Europe, has become trade flows (and not wild bird) that seem to be the routes that are the most often used by mites to disseminate. Mites use cages, birds, personnel that are carried by trucks during transfer as a spreading mean at high geographical scales (dozens to hundreds of kilometers) (Øines and Brännström, 2011; Roy and Buronfosse, 2011; Marangi et al., 2014).

Moreover, recent morphological studies explained the previously observed ability of some dermanyssoids (e.g., *D. gallinae*) to climb upwards on slippery surfaces as well as on a wide range of material, in the “off-host” environments (Mul et al., 2016), thanks to adaptations on the distal region of the legs (Di Palma and Mul, 2019). Such adaptations would have initially evolved in “wild-living” but would also prove beneficial for movement within poultry houses and thus explaining the possible active colonization of different facilities using the ventilation systems, wall fans, wall inlets, and air chimneys (Mul et al., 2009).

Medical and veterinary importance

The sanitary interest of Laelapidae and Dermanyssidae species stands on both their direct damage to the host (mostly related to the hematophagy) or indirect harmful effect (acting as vectors of pathogens) on their hosts. Direct damage can range from skin irritation to different degrees of blood deprivation. The indirect damage is related to their ability (thanks to the feeding behavior) to transmit a multitude of pathogens to animals and/or humans.

As to the latter effect, it should be underlined that simple detection of a vector-borne agent in an ectoparasite does not demonstrate vector competence. In fact, mites could acquire pathogens during the blood meals but might be unable to transmit them; on the other hand, not all parasitic dermanyssoid mites feed exclusively on blood but some could acquire pathogens by eating other ectoparasites such as fleas and lice. In addition, some bacterial agents (e.g., *Wolbachia* spp. and *Spiroplasma* spp.) are often endosymbionts of arthropods, and detection of these organisms might be misleading (Reeves et al., 2006; De Luna et al., 2009).

Some dermanyssoids are proven or suspected to play a role as vector and/or reservoir for pathogens of birds, reptiles, and mammals, including humans (e.g., Kocianová et al., 1993; Kocianová, 1989, 1993; Valiente Moro et al., 2005; Reeves et al., 2006; Mišková et al., 2015; Radzijejskaja et al., 2018); however, not conclusive data are available.

The medical and veterinary interest in the family Laelapidae is mainly due to the intimate association of some species with commensal rodents (see the “Biology and Ecology” section). Although some laelapid species are reported as causing occasional discomfort to humans (Mullen and Oconnor, 2019), Maaz et al. (2018) refer that such reports are very rare and mostly doubtful.

Indeed, most authors consider the spiny rat mite, *L. echidninus*, as the only significant laelapid species of sanitary interest. Although rarely, its feeding causes discernible lesions and injury to the footpads of suckling mice has been reported. However, the most important relevance of the spiny rat mite *L. echidninus* is due to the fact that it is capable of biologically transmitting disease agents among rodents (Table 2), such as the murine typhus (a worldwide rickettsial illness of commensal rats) and *Hepatozoon muris* (a blood protozoan of rats) (Mullen and Oconnor, 2019). Moreover, Junin virus, which causes Argentinian hemorrhagic fever, has been isolated from *L. echidninus* and associated rodent hosts in South America (Parodi et al., 1959) while, according to experimental studies, *L. echidninus* can transmit tularemia (a rabbit fever, caused by the zoonotic bacteria *Francisella tularensis*) (Strandtmann and Wharton, 1958). Anyway, the spiny rat mite’s role as a potential vector of human pathogens remains uncertain (Mullen and Oconnor, 2019).

Other rodent associated laelapid mites (*Haemolaelaps glasgowi* (Ewing), and *Laelaps cynognathus*), may have a role in the maintenance and transmitting Hantaan virus (the causative agent of Korean hemorrhagic fever) based on data reporting naturally infected mites and their ability to transmit the virus by bite to laboratory mice and, transovarially, to their offspring (Yu and Tesh, 2014). These findings are of particular interest since it is generally believed that the hantaviruses do not have an arthropod transmission cycle and that they are transmitted to mammals (rodents, insectivores, bats, and humans) by inhalation of aerosols of infected animal excreta or by bite. Moreover, recently, Kuo et al. (2020) and references therein highlighted the presence of spotted fever group (SFG) rickettsiae in hematophagous *Laelaps* mites, particularly in species closely associated with synanthropic rodents (i.e., *L. nuttalli* and *L. echidninus*). This calls for further investigation on the competence of these mites in transmitting rickettsiae. Finally, Alonso et al. (2020), in their survey, remark that ectoparasite-borne infectious diseases have been emerging or re-emerging throughout the world in the last years and their incidence is on the rise. Thus, considering that Norway rat is the most common vertebrate pest species in animal production systems and that it is a reservoir for several important zoonotic diseases, much attention should be paid to its parasitic fauna (e.g., Laelapidae) with potential sanitary importance and global geographical distribution. Attention should be also paid to other commensal rodents, such as house mice, *M. musculus*, or black rats, living

Table 2 Bacterial and viral pathogens and/or diseases associated with Laelapidae and Dermanyssidae species.

	<i>Laelaps echidninus</i> (Laelapidae) (see refs in the text)	<i>Lyponyssoides sanguineus</i> (Dermanyssidae) (see refs in the text)	<i>Dermanyssus gallinae</i> (Dermanyssidae) (updated from Sparagano et al., 2014)
BACTERIA	Murine typhus ^a <i>Haepatozoon muris</i> ^a Tularemia Spotted fever group (SFG) rickettsiae	<i>Rickettsia akari</i> ^{a,b} (Z) Tularemia	<i>Salmonella gallinarum</i> ^a <i>Salmonella enterica enterica</i> serovar Gallinarum ^c <i>Salmonella enterica enterica</i> serovar Enteritidis (serovar Enteritidis) ^b (Z) <i>Salmonella enteritidis</i> ^a (Z) <i>Pasteurella multocida</i> ^a (Z) <i>Chlamydia</i> spp. <i>Chlamydia psittaci</i> DNA ^d (Z) <i>Borrelia anserina</i> <i>Borrelia burgdorferi</i> DNA ^d (Z) <i>Erysipelothrix rhusiopathiae</i> <i>Listeria monocytogenes</i> <i>Coxiella burnetii</i> ^d (Z) <i>Escherichia coli</i> <i>Staphylococcus</i> spp. <i>Streptomyces</i> spp. <i>Spirochetes</i> ^a Avian leucosis Newcastle disease Fowl poxvirus ^a St. Louis encephalitis Tick-borne encephalitis Eastern equine encephalitis ^a Western equine encephalitis ^a Venezuelan equine encephalitis ^a
VIRUS	Junin virus		

^aTransmission demonstrated.

^bTransovarial and trans-stadial passage demonstrated.

^cTransmission demonstrated (Cocciole et al., 2020).

^dMites found positive during two outbreaks of human dermatitis related to sparrow and pigeon nests (Raele et al., 2018). (Z) zoonotic pathogen.

inside buildings, and wild rodents who harbor many zoonotic agents as well. In fact, the population density of these “peridomestic” rodents is even higher in urban than rural regions, due to a prolonged breeding season and better winter survival, and there are chances that they transmit diseases to domestic animals, mainly via vectors, turning them in an additional reservoir for human exposure (Zavala-Castro et al., 2009).

Regarding dermanyssid mites, the veterinary and medical concern of the genera *Liponyssoides* and *Dermanyssus* is better supported. Among the genus *Liponyssoides*, the house mouse mite *Liponyssoides sanguineus*, a cosmopolitan bloodsucking ectoparasite of synanthropic rodents, is very important as a vector of *Rickettsia akari*, the agent of the zoonotic rickettsialpox (Huebner et al., 1946; Ereemeeva and Muniz-Rodriguez, 2020; Szakacs et al., 2020 and references therein). Rickettsialpox occurs primarily in urban areas in crowded living quarters infested with the house mouse, which serves as the major reservoir. Despite being considered as a mild, self-limited zoonotic febrile illness, there is increased interest in rickettsialpox due to its clinical similarity to bioterrorism-associated diseases such as anthrax and smallpox. The transovarial and trans-stadial transmission of this rickettsia in *L. sanguineus* has also been demonstrated (see Ereemeeva and Muniz-Rodriguez, 2020). Moreover, *L. sanguineus* has been reported to transmit tularemia once (Strandtmann and Wharton, 1958) (Table 2). Among the genus *Dermanyssus* the species of greatest interest is *Dermanyssus gallinae*, also called the poultry red mite (PRM); this species deserves a more detailed discussion due to its serious economic consequences on animal welfare as well as, although marginally, to its interest on human health. As above mentioned, it is mostly considered an avian-specific ectoparasite of mostly farmed birds and among those, it is significantly related to chicken industries and a major threat to the laying hen industry, worldwide, particularly in Europe where *D. gallinae* infestation rates vary from 60% to 90% (Mul, 2017; Waap et al., 2019).

The sanitary and economic interest of *D. gallinae* is mainly related to its hematophagous behavior. As said, all legged stages, except larvae, feed on blood. While males suck blood only occasionally, one female can engorge about 200 micrograms of blood. Since the infestation level is usually high (up to 500,000 mites/head in heavily infested laying hens), hematopoiesis is insufficient to compensate for blood loss, and animals present marked anemia that is more pronounced in young subjects (Cosoroaba, 2001; Wojcik et al., 2000). Moreover, mites are responsible for amplified stress (feather-pecking, increased self-grooming, and cannibalism) (Kilpinen, 2005; Mul et al., 2009).

The consequences are a negative impact on weight gain, conversion index, and egg quality failure (due to blood spots and shell thinning) and production (up to 20% of laying reduction) (reviewed by Sparagano et al., 2014; Sparagano, 2020).

The sanitary and economic impact of *D. gallinae* in the poultry system is also related to its role as a vector of several pathogens. As shown in Table 2, several bacteria and viruses are associated with PRM; however, more robust scientific evidence of transmission has been documented only for some *Salmonella* species. Although for several pathogens the real vectorial competence of *D. gallinae* remains unconfirmed (Valiente Moro et al., 2005, 2009; Circella et al., 2011; Chu et al., 2015) its potential should not be underestimated also bearing in mind that heavy infestations are reported to suppress antibody production or reduce antibody titers to some viral vaccines by the host (Sparagano et al., 2014; Sparagano, 2020; Pugliese et al., 2019).

In non-avian hosts (e.g., dogs, cats, horse), attacks document from severe, mild, to absent skin lesions (DeClerq and Nachtegaele, 1993; Guin, 2005; Mignon and Losson, 2008; Friesen et al., 2011; Di Palma et al., 2018) and an immunological reaction in experimentally infested dogs have been described (Martins et al., 2020).

As to humans, attacks can occur in the poultry farm setting when the PRM infestation level is high and farmers, employees, or visitors are not properly protected (thus proposed as “occupational disease”), as well as in the urban context when the nests built close to residential buildings by synanthropic birds (mostly pigeons) are abandoned and hungry mites enter houses, residences, public buildings, hospitals, etc. through crevices and cracks around windows and doors, or via ventilation ducts and air-conditioning systems (Fig. 7) and attack humans (Cafiero et al., 2019; Sargison et al., 2020 for references). The symptoms of the infestation by *D. gallinae* in humans (often recurrent) range from pruritic dermatitis, erythematous rashes, small papules and blisters, urticarial plaques, and erythema—with consequent histological changes—and are mostly described on arms, hands, chest, legs (see Cafiero et al., 2019) (Fig. 7).

The importance of this mite species in public health also stems from their role as reservoirs/vectors of zoonotic pathogens (see Table 2). Though human infestations are frequently associated with adventitious feeding, the risk of transmitting arthropod-borne disease might be considered remarkable.

Finally, another important issue related to the medical and veterinary importance of these family mites is that the host switching phenomenon followed by speciation reported for both laelapid and dermanyssid species (see “Host specificity and distribution” section), which in turn could lead to the colonization of new natural and artificial biotopes by these species can have unpredictable consequences. In particular, considering that laelapid species can be associated with bats (e.g., Radovsky, 1966; Fain, 2001; Shaw, 2011) the emergence of important viral diseases associated with these mammals and possibly transmitted to humans may be a current and future worrying topic.

Prevention and control

Infestations and attacks of humans in public places or private houses by rodent and/or avian associated dermanysoid mites (e.g., *L. echidninus*, *L. nuttalli*, *L. sanguineus*, *D. gallinae*) are mainly accidental. Mites move from their hosts or from nests to human beings when the original hosts naturally leave the nest (this happens in birds when the chicks can fly) or are disturbed by treatments or restoring of the buildings. Prevention practices such as removing pigeon or other bird nests, erecting meshes to stop them from

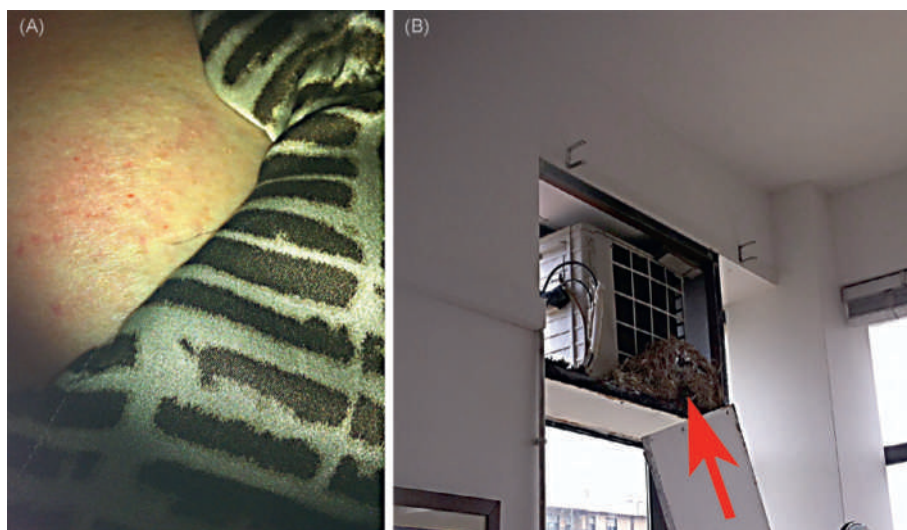


Fig. 7 *Dermanyssus gallinae*. (A) Dermatitis on a human chest caused by mites migrating from an abandoned pigeons' nest (arrow) in an air-conditioning unit in a private office (B).

nesting (Cafiero et al., 2019) and targeted control measures against commensal rodents (mainly rats) are highly recommended. Site inspection is the crucial step which allows recognitions, for examples, of the *D. gallinae* origin (abandoned birds' nests on balconies, attics, eaves, windows and holes near the building) and their possible indoor hiding places such as heated spots (under bed covers, electrical devices, laptop computers, television and radio clocks, etc.).

Failure to eliminate commensal rodents in urban contexts or to prevent mite migration during pest treatments may result in repeated infestations or new infestations by their associated mites, even at sites distant from the original foci, and regard seriously marginalized population such homeless.

In case of suspected or confirmed episodes of dermatitis it is recommended the patients should shower extensively and wash their clothes at 60 °C; infested premises should be intensively vacuum cleaned and disinfested using pyrethroids, while textiles (curtains, carpets, cushions) should be steamed clean or washed at 60 °C (Cafiero et al., 2019).

More serious is the situation related to occupational dermanysiosis by *D. gallinae* in humans which is related to the poultry farm infestation and requires improvements in control strategies (see below). To avoid infestation, poultry workers must be covered by suitable protective clothing, before entering the facilities (Cafiero et al., 2019).

The veterinary interest is mostly related to Dermanyssidae and particularly to *D. gallinae*. The overall cost of *D. gallinae* to the poultry industry—roughly estimated at € 231 million per year for the EU egg industry, with similarly significant losses elsewhere (Sparagano et al., 2014; Van Emous, 2017; Sleeckx et al., 2019)—imposes strict control strategies. However, when established in a poultry farm, the control of *D. gallinae* is very challenging due to its high reproduction rate, long survival period (even in absence of the host), feeding and resting behavior, increasing resistance to commonly used acaricides (Sparagano et al., 2014; Sparagano, 2020 and references therein).

Basic practices as cleaning and hygiene facilities between flocks can greatly reduce *D. gallinae* numbers but are often underestimated as control practices, or too costly (as steam and heat treatments), and, in the mean time, these are unable alone to eradicate infestations.

To date, worldwide, control of the key pest of the poultry industry (the PRM) is dominated by the use of synthetic acaricides. Organophosphates, pyrethrin, pyrethroids are still commonly used; only a few products are licensed in the EU for use against *D. gallinae*, and not many new molecules are marketed by pharmaceutical companies. Recently, only a fluralaner-based product belonging to the isoxazolines group has been developed and commercialized (see Sparagano, 2020). Indeed, successful treatment is more and more hampered by the ability of *D. gallinae* to develop resistance to multiple acaricides, as well as product residues (Giangaspero et al., 2016). The recent planetary scandal related to the presence of residues of fipronil (an unlicensed molecule) in eggs, represents the tip of the iceberg, i.e., the continuous needs by farmers of new molecules to combat *D. gallinae* in laying hen farms.

Consequently, farmers call for urgent alternative control strategies. In this view, natural acaricide compound formulations against *D. gallinae* are becoming increasingly more accessible, such as spinosad-based, and neem-based products. Collaterally, desiccants (i.e., inert substances such as silica dusts, able to break down the mite's waxy surface layer) are now commonly used by farmers (Sparagano et al., 2014; Sparagano, 2020 and references therein).

In the meantime, plant-derived products (PDPs) and natural enemies are beginning to provide natural solutions to control *D. gallinae*. Essential oils, herbs or plant extracts have produced some promising results especially for their primarily fumigant action able to penetrate mite refugia (see "Biology and ecology" section) and their bio-efficacies such as ovicidal, repellent, antifeeding, and biocidal activities (Sparagano et al., 2014 and references therein; Tabari et al., 2017; Lee et al., 2019). Nevertheless, variable

efficacy (due to different plant varieties, parts, geographic location, seasonality, method of oil extraction, year of harvest, storage conditions, and components used in product formulation) is widely recognized as a restraint and an impediment to their use in the field and commercial development.

Biological control approaches using PRM predators seem to reduce the mite infestation and some predator species are now commercially available (e.g., *Cheyletus eruditus*, *Androlaelaps casalis*) and can be used in combination (Sparagano et al., 2014; Sparagano, 2020). They will not eradicate PRM but have the potential to allow an acceptable low-level infestation with no harm to poultry, product, environment, and people. Future areas of development which show promises are also represented by entomopathogenic fungi (e.g., *Aspergillus* spp., *Metarhizium* spp., *Beauveria bassiana*) that can penetrate the integument of arthropods and cause a delayed pathology and, thus, become widely disseminated and, potentially, eliminate large mite populations (Sparagano et al., 2014; Pritchard et al., 2015; Sparagano, 2020; De Olivera et al., 2020 and references therein). However, further research is urgently needed concerning their practical application in poultry farms.

In conclusion, despite recent technological innovations in the poultry industry and the level of productivity achieved in recent years, *D. gallinae* is still a worrying issue worldwide. It is important to underline that an acceptable control of *D. gallinae* in poultry systems would be possible only through Integrated Pest Management (IPM) strategies; however, IPM strategies—largely limited to basic biosecurity, synthetic acaricides, sanitation, and physically acting substances—remains under-utilised in the poultry industry compared to agricultural sector, such as horticulture. There is a long way still to achieve an highly effective and sustainable control of *D. gallinae*. In the meantime, the following simple considerations should be highlighted.

The application of any control methods must be based on the correct identification of the involved pests. Once identified, the control of the red mite infestations could be achieved by strict preventive and sanitary measures. The application of hygiene regulations (cleaning and hygiene facilities, farmer and technician awareness of the problem, an interruption between the production cycles and thorough disinfection before the arrival of new birds, careful checking of new animals) on poultry farms would prevent or limit the infestation.

Regular monitoring of the burden of poultry red mite on a farm is a critical point² and would allow prompt intervention to be taken as soon as the red mite population starts to increase (Oh et al., 2020).

Anti-resistance strategies imply selection of effective products against *D. gallinae*, safe for animals, humans, and the environment, the correct dose and low application frequency, use in empty houses between flocks.

The most promising approach for controlling *D. gallinae* would be via vaccination, which offers the advantages of prolonged effectiveness, freedom from chemical residues/environmental pollution, and a reduced risk of resistance.

Although improvements in mite in vitro rearing and in vivo testing (Bartley et al., 2012; Nunn et al., 2020), application of omics strategies (Price et al., 2019; Lima-Barbero et al., 2019a,b), and delivery methods (Price et al., 2019) are helping to facilitate progress in this field,³ much has still to be done to develop a commercially viable vaccine for *D. gallinae*.

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²Traps (mimicking refuges for the fed mites), both industrially produced or simple homemade with cardboard, corrugated PVC, or pieces of knotted white cloth in cages or nearby and examined every few days or once a week (see Sparagano, 2020 for references) can be used, including, more recently, an automated mite counter (Mul et al., 2015; Mul, 2017; Mul et al., 2017).

³Recent experimental recombinant subunit vaccines have shown to induce mite mortality and importantly reduce mite oviposition (Price et al., 2019; Lima-Barbero et al., 2019a,b); and in the case of an autogenous vaccine, reduce the *D. gallinae* population in a field study (Bartley et al., 2017).

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Chigger Mites (Trombiculidae)

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Introduction

Chigger mite is a general term that can refer to larval stages of mites that belong to three main families: i.e., Trombiculidae Ewing, 1944; Leeuwenhoeikiidae Womersley, 1944; and Walchiidae Ewing, 1946 (Acariformes: Trombidiformes). Of the above, Trombiculidae is of medical and veterinary importance, given that parasitic larvae, in general, have low specificity, being able to parasitize a plethora of hosts, including humans (Mendoza-Roldan et al., 2020a). Furthermore, some species of Trombiculidae mites have been pointed out as capable vectors of pathogens, some of which are of zoonotic concern (Ewing, 1944; Fischer and Walton, 2014; Jacinavicius et al., 2018). While the adult and nymphal stages predate on the eggs and juvenile stages of other arthropods, chigger larval stages are parasitic of animals, being distributed throughout the world. The most important species are in Europe *Neotrombicula autumnalis* (harvest mites) (Fig. 1), in the Americas *Eutrombicula alfreddugesi* (chiggers mites) (Fig. 2) and in Asia and Australia *Eutrombicula sarcina* (scrub itch mites) (Diaz, 2010; Parcell et al., 2013; Santibáñez et al., 2015). The pathogenic effect of trombiculids is mainly linked to the pruritic dermatitis they cause in animals and humans.

However, some of them (e.g., *Leptotrombidium akamushi* and *L. deliense*) may be also vectors of rickettsial disease scrub typhus, caused by *Orientia tsutsugamushi* (formerly known as *Rickettsia tsutsugamushi* and *R. orientalis*) in eastern and southeastern Asia, New Guinea and northern Australia (Stekolnikov, 2013; Chakraborty and Sarma, 2017; Elliott et al., 2019; Kumler et al., 2018). Other species such as *Neoschongastia americana* are important pests of turkeys in the southern States of North America and they may also attack many other bird species (Kunz et al., 1969).

Based on the peculiar parasitism of larvae, this instar bears a robust gnathosoma with strong, blade-like anteromedial-oriented chelicerae, which are surrounded by stout, segmented palps with a typical claw-shape into the penultimate (palpal tibia) and ciliated setae on the terminal segment (palpal tarsus) (Goff et al., 1982; Kumler et al., 2018). The respiration occurs transcutaneously and there are not stigmata or tracheae. On the dorsal idiosoma, it is possible to observe the scutum, which usually bears a pair of sensilla (trichobothria), which are setaceous in many genera and inflated in other (e.g., *Schoengastia*), with four ciliated setae at each corner of the scutum and a single anteromedian seta (paired in *Apolonia*) (Fig. 3). Body surfaces carry ciliated setae, which distribution, along with the morphological characteristics abovementioned, represents important features for the taxonomic classification of trombiculids (Goff et al., 1982). The body of nymphal and adult trombiculids are covered by dense pilose setae on legs, body and palps (they are also known as velvet mites) and bear stigmata at the base of the chelicerae (Jenkins, 1947; Moniuszko et al., 2017). When larvae penetrate into the host skin, using their chelicerae, they start feeding on the host and the saliva partially digest tissues forming a feeding tube (stylostome) (Shatrov and Felska, 2017). Moreover, chigger mites in general attach to the epidermis with their chelicerae feeding of the exudates produced (Fig. 4), whereas some genera of the Leeuwenhoeikiidae family may burrow in the subcutaneous tissue mainly of amphibians (Fig. 5) (Silva-De La Fuente et al., 2016). In both cases, chigger mites feed on exudates composed by epithelial cells and lymph, and rarely some hematic cells. Larvae feed through the stylostome, which represents an artificial duct and is similar in function to the cement produced by ticks during attachment to the host (Shatrov, 2009; Bullard et al., 2016).

After feeding for several days, larvae fall to the environment and enter a quiescent phase prior to molt to the protonymph, then through other two stages to adults with a typical figure-of-eight shape body (about 1 mm long). While in tropical regions trombiculid infestations of larvae occur throughout the year, in temperate areas they perpetuate during late summer or early autumns (thus the species name of *N. autumnalis*). Therefore, hatched larvae usually climb on the grass (to a height of 60–80 mm) attacking any host (e.g., sheep, cattle, dogs, humans) they come in contact with (Guarneri et al., 2017a,b; Moniuszko et al., 2017).

The biological cycle generally includes eggs, prelarva or deutovum, followed by larvae, three nymphal instars of which deutonymphs are active and protonymphs and tritonymphs are inactive, and finally adults. Inactive prelarvae eclode from the eggs, 6 days after being laid, and active parasitic larvae emerge, feeding on the host in about three to 5 days (more than 10 days in skin-dwelling Leeuwenhoeikiidae larvae). After engorgement, larvae detach and return to the environment where they go through



Fig. 1 Larvae of *Neotrombicula autumnalis*. Lia, RP.



Fig. 2 Scanning electron microscopy of larvae of *Eutrombicula alfreddugesi*. Mendoza-Roldan, JA.



Fig. 3 Scanning electron microscopy of *Eutrombicula alfreddugesi*: dorsal view of idiosoma showing typical rectangular scutum bearing setae (sensilla arrowed). Mendoza-Roldan, JA.

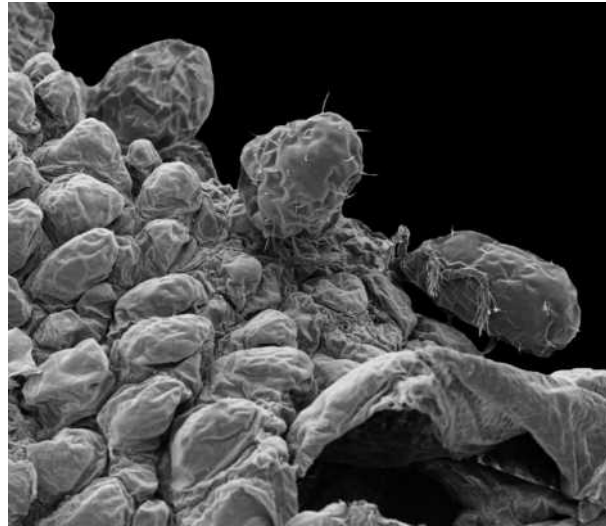


Fig. 4 Scanning electron microscopy of *Eutrombicula alfreddugesi*: larvae attached to *Kentropyx calcarata* lizard. Mendoza-Roldan, JA.

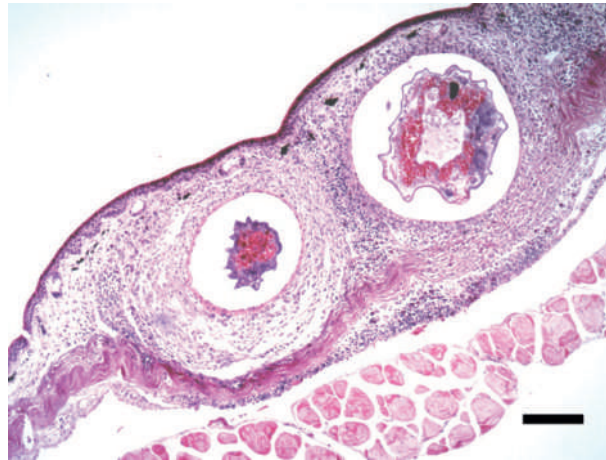


Fig. 5 Histological section of skin tissue of *Leptodactylus latrans* frog with intradermic *Hannemania hepatica* (Leeuwenhoeikiidae) larvae inside capsules. Mendoza-Roldan, JA.

different active or inactive instars (inactive protonymph or nymphochrysalis, active deutonymphs which develop four pairs of legs, and inactive tritonymphs). Finally, nymphs molt to adults, completing the cycle in two to 12 months depending on the species and quiescent stages duration. Species that occur in temperate regions can complete up to three generations, whereas tropical ones have continuous cycles all year round (Servat et al., 2018; Lv et al., 2019). Given the medical and veterinary relevance, this chapter will provide a detailed focus on Trombiculidae chigger mites, highlighting the peculiar biology, ecology and control of the species of major public health importance.

Trombiculidae Ewing, 1944

Members of the Trombiculidae family are the true chigger mites, also known as harvest mites. Larvae are red, white or yellowish in color and approximately 150–300 μm in size. Mites of the families Leeuwenhoeikiidae and Trombiculidae share morphological and life cycle similarities. The main morphological difference is the segmentation of the legs. Trombiculidae have 7-7-7 or 7-6-6 leg segmentation (Fig. 6A), and Leeuwenhoeikiidae have 6-6-6 (Fig. 6B), except for the genus *Comatacarus* Ewing, 1942, which has 7-6-6 leg segmentation (Kolebinova, 1992). For most of the described species, only the larval stage is known, and to date more than 3000 species have been described distributed in 31 genera (Furman, 1953; Brennan and Goff 1977; Jacinavicius et al., 2018).

As other Parasitengona mites, chiggers from the Trombiculidae family have low host specificity and infest the different possible hosts in a given environment (i.e., nests, urbanized areas, forested areas, etc.) (O'Callaghan et al., 1994). Specifically, in ectothermic hosts, these mites attach on soft tissues of the skin and depending of the association of the mite-hosts, some animals have developed

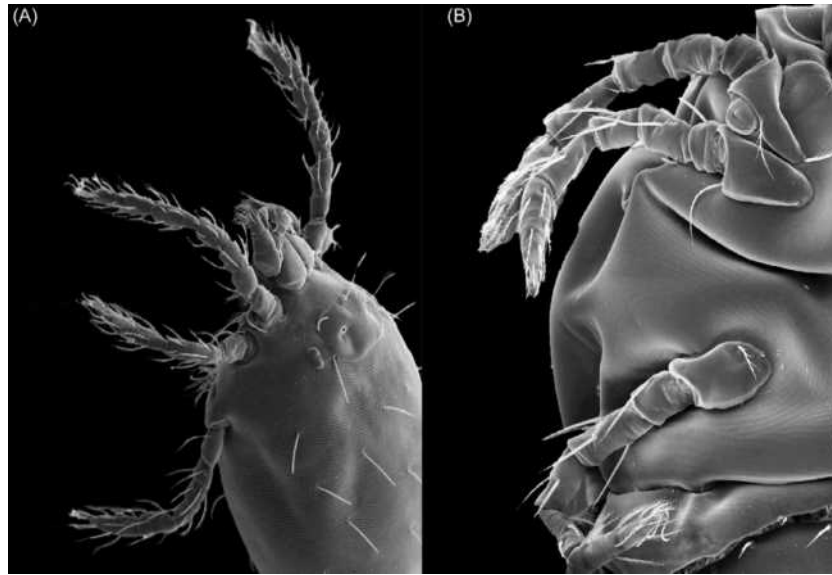


Fig. 6 Scanning electron microscopy of leg segmentation of A) Trombiculidae (7-7-7) and B) Leeuwenhoeikiidae (6-6-6). Mendoza-Roldan, JA.



Fig. 7 *Eutrombicula alfreddugesi* larvae on *Kentropyx calcarata* lizard axillar mite-pocket. Modified from Mendoza-Roldan J, Rocha Ribeiro S, Castilho-Onofrio V, Gobbi Grazziotin F, Rocha B, Ferreto-Fiorillo B, Soares Pereira J, Benelli G, Otranto D, Barros-Battesti DM (2020) Host association of mites and ticks to reptiles and amphibians in Brazil. *Acta Tropica*, 105515.

microhabitats, also called “mite pockets”, that can be from cavities to folds of the skins, located generally in the armpit, groin and neck regions (Fig. 7). Chigger mites are generally ectoparasites, though some exceptions exist, such as species of the genera *Vatacarus* and *Iguanacarus* which are endoparasitic mites of the respiratory tract of marine snakes (*Laticauda* sp.) and iguanas (*Amblyrhynchus* sp.) (Nadchatram, 2006). The distribution and diversity of chigger mites is given by the environmental conditions, region and host availability. In this sense, species of medical and veterinary importance belong to the genus *Eutrombicula* Ewing, 1938 in the Neotropics and Nearctic, *Neotrombicula* Hirst, 1925 in the Palearctic and *Leptotrombidium* Nagayo et al., 1916 in the Oriental region.

***Eutrombicula alfreddugesi* (Oudemans, 1910)**

The most common species of trombiculid mite in the Americas is *Eutrombicula alfreddugesi*, having been recorded in almost all countries from Canada to Southern Argentina and the Caribbean basin. Given the plasticity and adaptability of this species, it has been reported infesting amphibians, reptiles, birds, and mammals, including humans (García-De la Peña, 2011; Mendoza-Roldan et al., 2020b). Depending on the ecoregion (i.e., temperate, tropical), larvae can be present year round or from summer to fall in the northernmost or southernmost part of its distribution. This species has been recorded as the causative agent of trombiculosis (dermatitis produced by the bite of the mite) (Clopton and Gold, 1993). Larvae of *E. alfreddugesi* are well adapted to temperatures ranging from 18 to 38 °C, and prefer high humidity (>80%) (Jameson, 1972). Parasitic larvae have an extra-oral digestion, feeding

on the detritus produced in the epidermis on which the mite attaches. Thus, larvae have preferences for microhabitats on the host that are of difficult access such as lower abdomen, ears, armpits (mite-pocket in lizards), genitalia, base of the tail, and around the anal/cloacal region (Rocha et al., 2008).

Females of *E. alfreddugesi* lay their eggs 15 days after fertilization. Eggs develop at different times in the female body, though they hatch all at the same time. Prelarvae hatch and develop to larvae in 15 days for each stage. Parasitic larvae emerge and can infest vertebrate host for two to 48 days depending if the host is endothermic (mammals or birds) or ectothermic (amphibians and reptiles). Engorgement periods on reptiles tend to be longer and the deleterious effects on the host are minor. After larvae detach from the host, they return to the environment where they develop to protonymph (inactive stage), deutonymph (active) which can last up to 40 days. Deutonymphs molt to quiescent tritonymph and 5 to 10 days later, adults emerge and can live up to 1 year and a half (Williams, 1946; Jenkins, 1948).

Neotrombicula autumnalis (Shaw, 1790)

Neotrombicula autumnalis (i.e., harvest mites) are of veterinary and medical concern in Europe. The six-legged larvae are hairy and measure from 250 to 750 µm according to the stage of repletion (Prosl et al., 1985; Parcell et al., 2013). Mites are reddish and have a pentagonal dorsal scutum. After females *N. autumnalis* lay eggs in humid organic-enriched soil, larvae hatch from eggs in late summer or early autumn (at temperatures around 16 °C) being prevalent on grass and fallen leaves in habitats characterized by overgrown gardens and parks or forest edges and dry meadows. In the environment, larvae quest for hosts and, in warm sunny days, they are particularly active in the afternoon. Clustering together in some spots, usually hundreds or even thousands of larvae attack massively individual animals at the thin-skinned areas of the body (Poliana, 2012; Schöler et al., 2006). The infestation appears like “orange powder” (Fig. 8). In dogs and cats, the preferred sites are the paws, the face (around the eye ring, bridge of the nose, ear pinnacle), (Fig. 9) and in the inguinal region. In sheep, larvae localize on the face and ears and in cattle on the basis of the tail and the inner thigh (Fig. 10) (Caputo et al., 2018). On the skin of the host, larvae feed of fluids caused by their piercings and fall to the ground after about 3 days where they molt to octopod nymphs and then adults. Usually, the development is interrupted by the hibernation in the environment (in deep soil) with one generation of mites developing per year. Lizards and snakes are suitable hosts also with high parasitic loads throughout the body, in between the scales. For example, a prevalence of *N. autumnalis* up to 70% was recorder in southern Italy, in *Podarcis muralis*, *Podarcis siculus* and *Lacerta bilineata* lizards (Fig. 11) (Mendoza-Roldan et al., 2019). Another species of *Neotrombicula* that has been recorded as possible causative agent of trombiculosis is *Neotrombicula inopinata* (Oudemans, 1909). This species has been identified mainly in Spain and Portugal, infesting dogs, cats and humans (Stekolnikov et al., 2014; Ramilo et al., 2019; Areso Apesteguía et al., 2019).

Leptotrombidium spp. Nagayo et al., 1916

The genus *Leptotrombidium* is distributed in eastern and southeastern Asia, New Guinea and northern Australia and cause a pruritic dermatitis in humans. About 15 species in that genus (e.g., *Leptotrombidium akamushi*, *L. deliense*, *L. pallidum*, and *L. scutellare*) act as vectors of a rickettsial disease, also known as scrub typhus, caused by *O. tsutsugamushi* in humans (Stekolnikov, 2013; Tilak and Kunte, 2019). The disease is known in Asia since the 16th- century being reported in Chinese manuscripts of natural history, and it is characterized by acute febrile presentation, severe headache, rash and lymphadenopathy lasting for 2–3 weeks (Xu et al., 2017). While the severity seems to vary according to the strain of the pathogen, a mortality exceeding 30% has been recorded in untreated cases. The multiple serotypes of *O. tsutsugamushi* characterized, may cause transient (non-protective) cross-immunity in humans (Bonell et al., 2017). Therefore, prompt treatment with tetracycline antibiotics (e.g., doxycycline and chloramphenicol) may prevent



Fig. 8 Larvae of *Neotrombicula autumnalis* around the labial commissure of a sheep, with the typical “orange powder” aspect. Lia, RP.



Fig. 9 Larvae of *Neotrombicula autumnalis* around the ear pinnacle of a dog. Lia, RP.



Fig. 10 Larvae of *Neotrombicula autumnalis* around the eyelid of a cow. Lia, RP.



Fig. 11 Larvae of *Neotrombicula autumnalis* on *Podarcis siculus* lizard in association with immature stages of *Ixodes ricinus*. Modified from Mendoza-Roldan JA, Colella V, Lia RP, Nguyen VL, Barros-Battesti DM, Iatta R, Dantas-Torres F, Otranto D (2019) *Borrelia burgdorferi* (sensu lato) in ectoparasites and reptiles in southern Italy. *Parasites & vectors*, 12(1), 35.

mortality cases. The strict association between scrub typhus and mites is proven by the fact that *tsutsugamushi* is a Japanese word meaning dangerous mite (Wee et al., 2017).

Although species of *Ascoschoengastia* and other genera of chiggers such as *Blankaartia*, *Gahrlepiea*, *Eutrombicula*, *Microtrombicula* and *Odontocarus*, have been found to carry and have been implicated in the circulation of *O. tsutsugamushi* among rodents, *Leptotrombidium* spp. play the primary role in the transmission of the scrub typhus. It results in the fact that, this zoonosis is associated with enzootic foci in rodents of the subgenus *Rattus* (*Rattus*), in transitional vegetation and in suitable habitats (also known as mite islands), whereas bird- and reptile-associated species (e.g., genus *Eutrombicula* and *Schoengastia*) do not carry *O. tsutsugamushi* (Elliott et al., 2019). This may ultimately explain the reason why the distribution of scrub typhus does not necessarily overlap that of scrub itch. Scrub typhus is also associated with man-made environments that may be suitable for the perpetuation of rodents (Elliott et al., 2019).

Considering the parasitic behavior of trombiculid larvae, which feed on one individual host, *O. tsutsugamushi* is transmitted transovarially from the adult female mites to the offspring. Accordingly, *O. tsutsugamushi* has been detected in almost all organs (e.g., salivary glands, reproductive organs) and tissues (e.g., epidermal cells) of larval and adult mites (e.g., *L. pallidum*), which therefore represent not only a vector of the pathogen but also the reservoir.

Therefore, the occurrence of scrub typhus is seasonal (e.g., in the coastal areas of south-east China during summer-autumn) and outbreaks have been associated with *L. pallidum* in gardens in the vicinities of the patients' households (Nagano Prefecture, Japan) (Kelly et al., 2009). Similarly, an outbreak in a suburb of Bangkok was related to a high exposure to *O. tsutsugamushi* in *R. rattus* population (96%), to the positivity in mites (*L. delicense* and *Ascoschoengastia* spp.) collected on them and (2%) and to positivity of 30% of the human population (Low et al., 2019).

Medical importance and control

The pathogenic effect of trombiculid mites on their host depends on the parasitic load and on the stylostome length, which varies according to the species of chigger (Tudor et al., 2015). Furthermore, the stylostome morphologic features, the host reaction to the chigger mite infestation (dermic response), could affect the capacity of mites in vectoring pathogens, among others (Fleming and Chastain, 1991; Shatrov and Felska, 2017). In general, the local reaction to the larva "bite" is imperceptible, whereas when chigger mites feed on humans or accidental hosts, they tend to produce dermatitis. This reaction is called chigger dermatitis or trombiculiasis (Guarneri et al., 2017a,b). Generally, the causative agent producing skin lesions goes undetected as larvae are typically small (100–300 µm) being barely visible to the untrained eye. Depending on the host and species of mite, they can be either red, orange or light yellow and even white. Usually, large number of larvae infest the host at once, resulting in multiple lesions and discomfort. In humans, mites tend to attach in regions near to the ankles, legs, lower body and arms (Guarneri et al., 2005). Lesions are produced by the liquefaction of the tissues surrounding the site of the attachment. This generates erythema, blisters, local alopecia, and crusty dermatitis. Depending on the number of mites and host reaction, persistent pruritus, excoriation and, in severe cases, convulsions may occur and last for some days until larvae fall into the environment. The diagnosis is confirmed by the anamnesis (e.g., season or contact with trombiculiasis foci), by the clinical presentation and the microscopic observation of mites (Santibáñez et al., 2015). Though, dermatitis disappears as soon as larvae abandon the hosts, treatment similar to that used for cheyletiellosis may be considered in pet dogs and cats with fipronil and selamectin or, in dogs, even with pyrethroids (Lecru et al., 2019). In humans exposed to trombiculid larvae in the environment the primary lesion are macupapulae, hemorrhagic and itchy. In rare cases, they may localize around the eyes and cause conjunctivitis. Humans frequenting habitats endemic for the scrub typhus should prevent the mite bites through the treatment of cloths or exposed skin with topical repellents (e.g., diethyltoluamide) or pyrethroids (e.g., permethrin), particularly in the parts of the body more likely to be exposed to larval mites (e.g., lower legs, ankles and feet) (Wee et al., 2017). Control of the rodent population is effective as a long-term measure to reduce scrub typhus. However, the risk associated with trombiculid larvae seeking alternative hosts (e.g., humans) should be considered if rodents are suddenly removed from the environment (Kim et al., 2018).

Other species of *Eutrombicula* can also produce trombiculosis in humans [i.e., *Eutrombicula splendens* (Ewing)]. Larvae of *E. splendens* can infest amphibians, reptiles, birds, and mammals, including humans, though its host preference remains within reptiles (e.g., snakes and turtles). *Eutrombicula splendens* occurs in sympatry with *E. alfreddugesi*, having also similar seasonality (Jenkins, 1949).

Leeuwenhoekidae Womersley, 1944

Mites from the family Leeuwenhoekidae are encompassed in 33 genera with approximately 230 species. Of these genera, two are of noticeable veterinary importance, i.e., *Hannemania* and *Straelensia*. The genus *Hannemania* (Fig. 12) are intradermic larvae typically associated with amphibians and includes 27 valid species, of which 26 occur in America and one in Oceania. Still, the status of 11 of these species is uncertain due to succinct descriptions and most of the type material is lost (Silva-De La Fuente et al., 2016). Larvae penetrate the skin and develop inside a capsule where the mite feeds of the debris and lysates of skin tissue, remaining for weeks and even months inside the capsule (Fig. 5). This capsular process produces an inflammatory response, that can result in cysts, pustules, and limb loss due to avascular necrosis (Wohltmann et al., 2006; Alvarado-Rybak et al., 2018). On the other hand, some species of



Fig. 12 Scanning electron microscopy *Hannemania hepatica* (Leeuwenhoekidae) larvae. Mendoza-Roldan, JA.

Straelensia larvae (e.g., *Straelensia cynotis* Fain & Le Net, 2000) can parasitize dogs and Iberian ibex in Europe, attaching within the inner surface of hair follicles, and forming intradermic solid nodules (Net et al., 2002; Fain and Le Net, 2000; Cevidanes et al., 2019).

Medical importance and control

Infestations by larvae of *Straelensia* are less pruritic than those caused by trombiculid mites, nonetheless, the distinctive nodular dermatitis produces macules that can be painful. Perifollicular edema and mucinosis can be observed at the cytologic examination, also with thickening of the epithelium. Skin nodules are generally small and firm, distributed mostly on the dorsal area of the head and body. Lesions evolve from erythematous papules to firm pale nodules. In most cases, the dermal response resolves within 3 months up to a year and treatment can be achieved with systemic avermectins, whereas topical acaricides are not useful (Net et al., 2002). To avoid mite attachment, it is important to ensure animals are not housed in infested places.

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Sarcoptidae and Demodicidae Mites

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Etymology

A. Sarcoptidae: *Sarcoptes scabiei*. Greek: *sarx* = meat; *koptein* = twisted; Latin: *scaber* = rough, not clean; English: itch; scientific name of disease: scabies.

B. Demodicidae: *Demodex* species. Greek: *edemas* = body; *dex* = stretched.

Geographic distribution and epidemiology

Sarcoptes and *Demodex* species are both worldwide found in the skin of humans and many animals (as subspecies).

Biology and morphology

A. *Sarcoptes scabiei*: These so-called scabies mites (Figs. 1–3; Table 1) enter the epidermis of their hosts as soon as a female has been fertilized by a free ranging male on the skin of a host. Inside the epidermis, the females dig channels with a length of up to 1 cm (Fig. 4). Therein the females lay 1–4 eggs per day for up to 4 months. When hatched inside the epidermis channel the six-legged larvae feed on epidermis cells and become finally adults after passing two 8-legged nymph stages (Fig. 3). The development of the males and females inside the epidermis is different. It takes 12–15 days for females, but only 9–10 days in the case of males, which therefore reach the host's skin surface earlier than the females. These mites prefer special regions of the human skin (Fig. 5), within which the temperatures might be somewhat higher than in others. The adult females reach a size of $0.3\text{--}0.5 \times 0.2\text{--}0.4$ mm, while the males remain somewhat smaller (Fig. 1). The adults of this species can move 2–5 cm per minute. Thus, they can infest many regions of the host's skin before they enter the skin and dig a channel inside the stratum granulosum (Fig. 6). The females live therein for up to 60 days (Arlian et al., 1989, 2016; Castro et al., 2018; Mehlhorn, 2016a,b,c; Rider et al., 2015; Shen et al., 2018; Sary, 2010; Wei et al., 2019).

B. Demodicidae (Follicle mites): The follicle mites (*Demodex folliculorum*) and the mites of the sebaceous glands (*Demodex brevis*) measure as adults 0.3–0.4 mm in length and 0.2 mm in width. The body of both appears like a cigar (Figs. 7 and 8; Table 2). Their legs are clearly shorter than those of *Sarcoptes* specimens (Fig. 1). After copulation, the females lay several 70–90 $\times$ 20–25 μm sized eggs. From each egg hatches a six-legged larva, which molts into another larval stage or becomes directly a nymph, which molts also in time to become either the adult male or female. This development takes all in all up to 3 weeks, which is respectively rather quick and thus induces first skin lesion very soon after having reached a new host. The males stay on the host's surface. After several copulations with females they live mostly for only further 3–5 days, while the fertilized females enter the follicle region of the hair, where they depone about 20 fertilized eggs before they also die. The transmission of adult mites from host to host occurs by body-to-body contacts between humans or animal hosts, respectively.

Symptoms of disease

A. Scabies mites: Infections mainly start by occurrence of inflammation reactions in the region between the fingers, on sexual organs or along the groins, from where they may spread all over the body and thus even reach the head skin (Figs. 5 and 6). In the case of

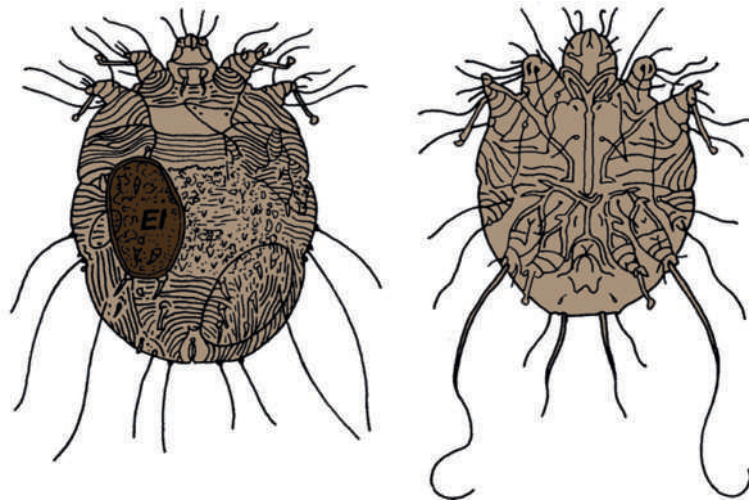


Fig. 1 Diagrammatic representation of a female (left side) and a male stage of *Sarcoptes scabiei*. In the female, an egg is shining through.



Fig. 2 Light micrograph of an adult male of *S. scabiei* showing the ventral side. Note the long hair, which help to recognize the surroundings in a skin channel. The anterior legs are equipped with sucker-like structures on a stick.

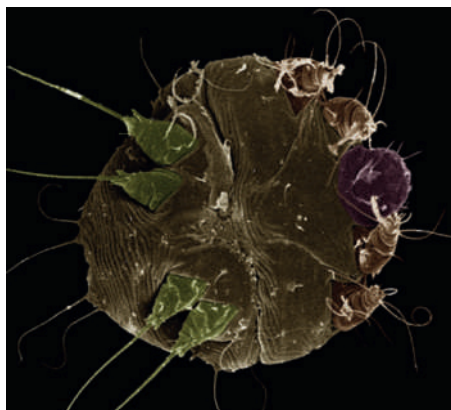


Fig. 3 Scanning electron micrograph of a female scabies mite (*Sarcoptes scabiei*), which shows clearly the eight, very short legs, which enable the mites to move inside prepared channels of the epidermis.

Table 1 *Sarcoptes* species of humans and animals.

Species	Length (mm)	Hosts/habitat	Name of disease
<i>Sarcoptes scabiei</i>	♀ 0.3–0.5 ♂ 0.2–0.3	Humans/epidermis	Scabies
<i>S. canis</i>	♀ 0.3–0.4 ♂ 0.2–0.25	Dogs/epidermis	Mange
<i>S. suis</i>	♀ 0.4–0.5 ♂ 0.25	Pigs/epidermis	Mange
<i>S. bovis</i>	♀ 0.3–0.5 ♂ 0.2–0.3	Cattle/epidermis	Mange
<i>S. cuniculi</i>	♀ 0.3–0.4 ♂ 0.2–0.24	Rabbits/epidermis	Mange
<i>S. equi</i>	♀ 0.4–0.45 ♂ 0.25	Horses/epidermis	Mange

Note that also animal species might enter humans.

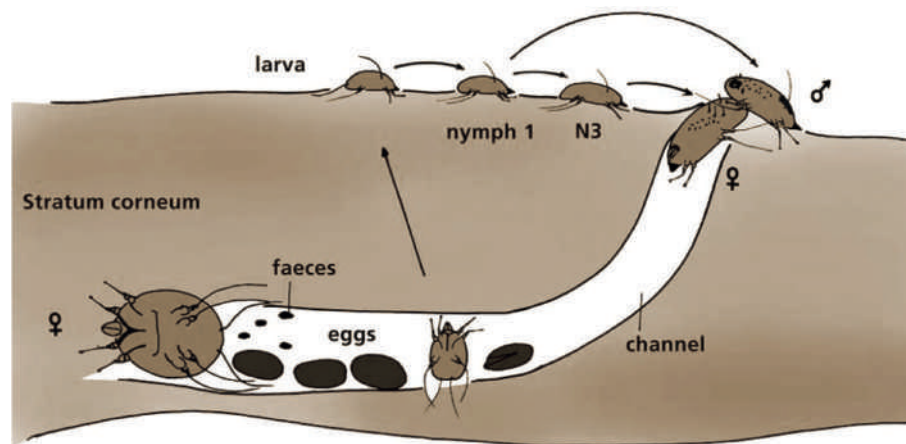


Fig. 4 Diagrammatic representation of the ongoing of the life cycle stages of *Sarcoptes scabiei* inside the epidermis of humans. As soon as adults have reached the surface of a host, they may become transmitted to other hosts during body contacts.

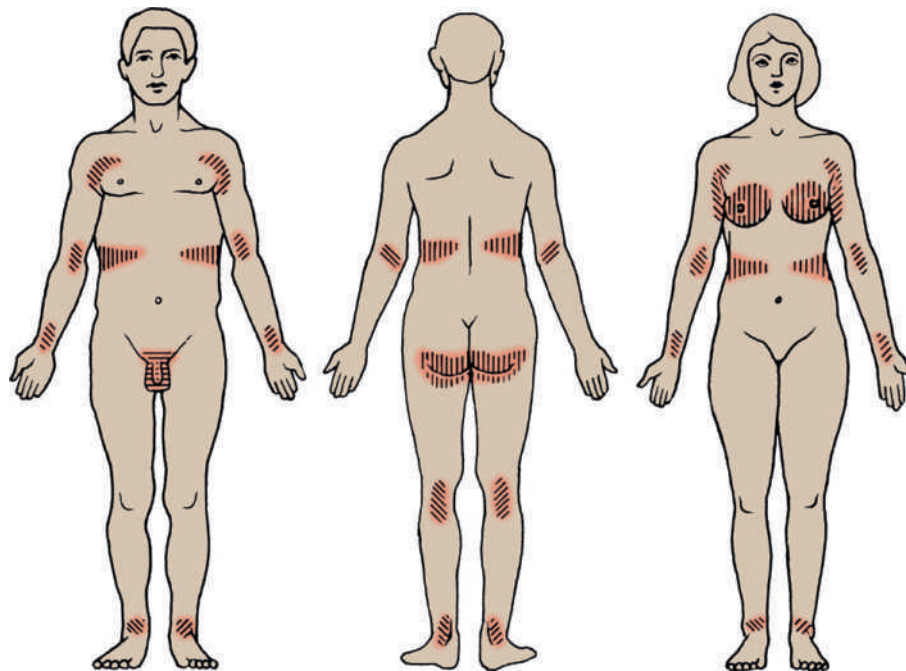


Fig. 5 Diagrammatic representation of a human skin region, on which the copulation occurs. After fertilization, the females enter the skin by digging a channel. Inside this channel they lay eggs, from which the larvae hatch and dig a channel from there to the surface of the skin. Transmission to other hosts occurs, when fertilized females enter another host during body contacts.

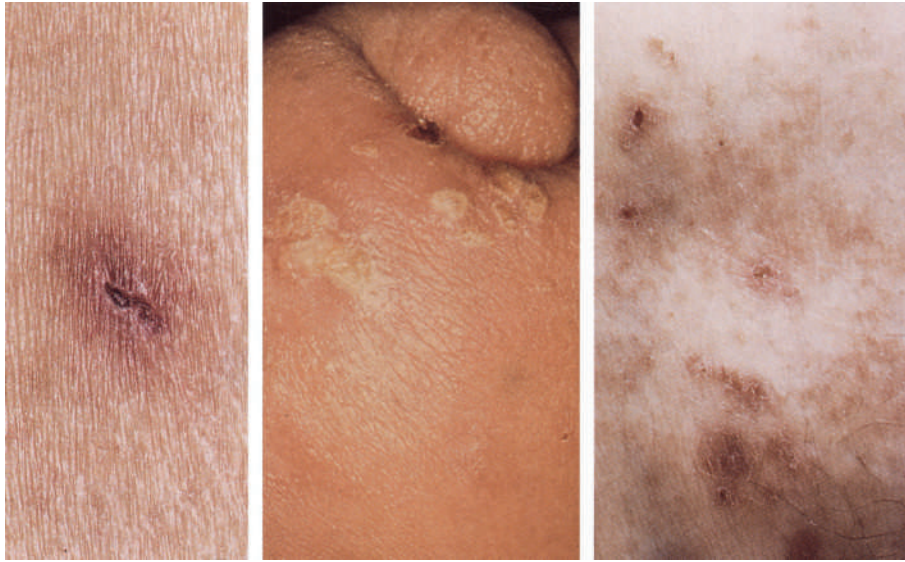


Fig. 6 Examples of three human skin regions being affected by scabies mites. Note that also sexual organs might be heavily infested.

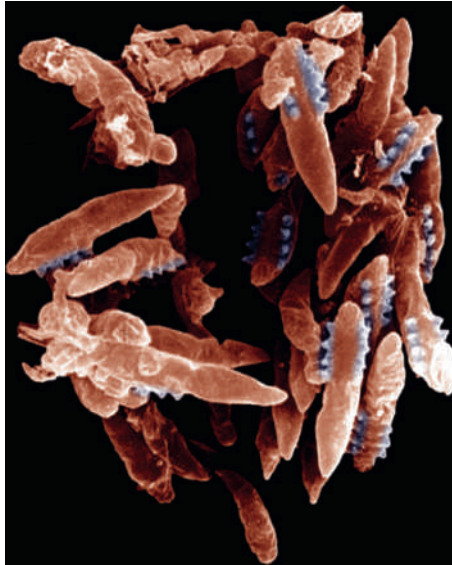


Fig. 7 Scanning electron micrograph of numerous adults of *Demodex folliculorum*. Note their cigar-like body shape and 8 short legs (in blue) inside the host's epidermis, which enable them to dig tiny tunnels inside the host's epidermis.

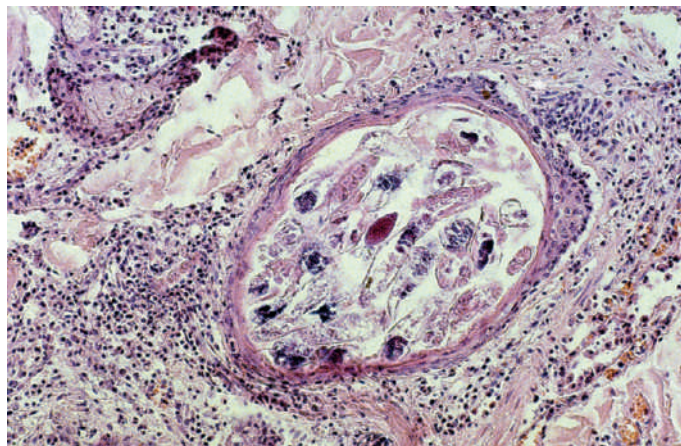


Fig. 8 Light micrograph of a section through the skin of a dog, wherein several *Demodex* mites are cut.

Table 2 Life cycle data of the *Demodex* species of humans and animals.

Species	Hosts	Body size (μm)	Egg size (μm)	Main sites of infection
<i>D. canis</i>	Dogs	0.15–0.25	70–90 $\times$ 25	Head, anterior legs, belly, breast sides
<i>D. felis</i>	Cats	0.15–0.25	70–90	Head
<i>D. suis</i>	Pigs	0.27	80 $\times$ 25	Snout, eyelids, belly, inner side of legs
<i>D. bovis</i>	Cattle	0.4	80 $\times$ 50	Head, shoulder, anterior breast
<i>D. caprae</i>	Goats	0.45	80 $\times$ 50	Head, shoulder, anterior breast
<i>D. ovis</i>	Sheep	0.45	80 $\times$ 50	Head, shoulder, anterior breast
<i>D. equi</i>	Horses	0.4	60–80 $\times$ 40	Eyelids
<i>D. folliculorum</i>	Humans	0.4	70 $\times$ 40	Many places
<i>D. brevis</i>	Humans	0.25	70 $\times$ 40	Many places

immuno-suppressed persons, the spreading of the mites on the hosts is often especially intensive leading to large regions of destroyed, suppurating remnants of the epidermis (Fig. 6). The final appearance of such large regions with typical crusts or scabs is described as Norwegian scabies, which is especially often found at persons suffering from strong immunosuppression (i.e. in cases of AIDS). Infected persons have reported, that especially in the evening and night hours, scabies is accompanied by very intense itching.

The following aspects can be differentiated along human skin after an infection by scabies mites.

- Mite channels (Fig. 4): The mostly only 1–4 mm long channels inside the skin end in a terminal slightly enlarged bladder that surrounds the fertilized female excreting the eggs. These channels appear reddish to brownish and may contain blood after intensive scratching (Fig. 6).
- Secondary exanthema: This sign appears mostly as hypersensitivity reaction on antigenic material excreted by the skin-boring females. Eczemas are also often seen during this phase of infection.
- Hyperkeratotic skin crusts (Fig. 6): This symptom is especially often found in immunosuppressed adult humans as well as in children along the whole body and occurs also in the face. If the brownish crusts reach $\frac{1}{2}$ cm in diameter, the symptom is described as Norwegian scabies (Scabies norvegica).
- Superinfection by bacteria: in heavy infections, it occurs rather often that the “channels” in the skin become superinfected by bacteria thus inducing severe infections depending on the species.

B. Demodicidae: Although the infection rates of humans may increase up to 100% during lifespan and although occasionally up to 1000 mites may be present at the same time, clinical symptoms are rarely seen in healthy persons. However, especially immuno-suppressed people or animals may show keratinization besides rosacea, areata-like alopecia, pyoderma, lupus miliaris, blepharitis, perioral dermatitis and/or micropapular lesions (Bhat et al., 2017; Chen and Plewig, 2015; Gach and Heagerty, 2000; Zhong et al., 2019).

Diagnosis

A. *Sarcoptes scabiei*: Skin inspection: Infected skin shows pruritus, papulae – often combined with exanthemas - and tiny inflamed regions. Using needles female mites can be taken out from the skin channels, wherein they stay.

Skin scrapes: Using a fine scalpel, the infested skin region is scraped, whereby portions of the inflamed skin can be obtained. If this material is placed into a 10% potash alkaline solution for 5–30 min and afterwards studied by light microscope the developmental stages of the mites and/or their eggs might be diagnosed.

B: *Demodex folliculorum*: When squeezing the skin in affected regions, which often occur in the naso-labial zone, the obtained sebaceous material often contains the cigar-shaped mites (Fig. 7) or portions of them. They are rather small and thus only will be clearly seen by help of a light microscope, when the obtained material is mixed with a drop of concentrated lactase (Kido et al., 2017).

Pathway of infection

A. *Sarcoptes scabiei*: Transmission of adult fertilized females (= telonymphs) occurs, when a new host has got body contact with the skin of infected animals or humans.

B: *Demodex folliculorum*: Transmission of adults occurs during intense body-to-body contact with unknown persons or animals.

Prophylaxis

A. *Sarcoptes scabiei*: Infections can be blocked by avoiding skin-to-skin contact with infected persons or animals and by avoidance to wear long time unwashed clothes of other persons.

B: *Demodex folliculorum*: Regular hair and body wash using skin cleaning products.

Incubation period

A. *Sarcoptes scabiei*: It takes about 10–14 days until first human skin symptoms are seen.

B: *Demodex folliculorum*: It takes in general about 2–3 weeks until (if at all) symptoms (itching) occur. However, low-graded infections remain often hidden, nevertheless mites can be transmitted to other persons.

Prepatent period

A. *Sarcoptes scabiei*: The first stage of larvae can be found about 15 days after a fertilized female has entered the skin of a host. Low-graded infestations or especially invasions of single but fertilized females need weeks until first parasites are seen.

B: *Demodex folliculorum*: Same range like in *S. scabiei*.

Patency

A. *Sarcoptes scabiei*: Females live only for 2–3 months, however, many repeated self-infections due to produced progeny keep infections ongoing and lead often to spreading all over the body.

B: *Demodex folliculorum*: Years, since repeated self-infections may occur.

Therapy

Infections of both species can be treated with the chemical compound ivermectin, which originally had been developed to control worms. There are different product names in France (Stromectol®) and in Germany (Scabioral®). Since in a family the single members might have been infected at different times and thus may show not yet clear symptoms, it makes sense to treat them all at the same time to avoid repeated “ping-pong” infections (Alipour and Goldust, 2015; Demmler et al., 1997; Fromstein et al., 2018; Jacob et al., 2019; Khalil et al., 2017; Morgan et al., 2017; Nicholls et al., 2017).

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Ixodid and Argasid Ticks

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Introduction

Ticks are the largest acarines and are obligate blood-feeding parasites of reptiles, amphibians, birds and mammals, in all or some of their developmental stages (Olivier, 1989; Keirans, 2009). They present an extensive distribution throughout all the six zoogeographical regions of the globe, being particularly abundant in the tropics (Keirans, 2009; Guglielmone et al., 2014). Nonetheless, ticks are also significant parasites of animals and humans in temperate forests, steppes, prairie habitats and even in the harsher tundra of the arctic (Keirans, 2009; Guglielmone et al., 2014).

The parasitic relationship between ticks and their hosts dates back to dinosaurs (Peñalver et al., 2017). In the course of their interminable evolutionary history, ticks evolved as primary parasites of wild animals, but some species have perfectly adapted to domestic animals as well. This is the case of brown dog ticks (*Rhipicephalus sanguineus* sensu lato), which have been infesting dogs since Ancient Egypt, at least (Huchet et al., 2013; Otranto et al., 2014b). Nonetheless, even ticks that are presently well adapted to domestic animals may still be able to parasitize wild animals. For instance, the cattle ticks *Rhipicephalus microplus* and *Rhipicephalus annulatus* are primary parasites of cattle, but can also infest wild bovids (Lohmeyer et al., 2018). Indeed, the increasing populations of wild bovids has compromised the success of the Cattle Fever Tick Eradication Program in South Texas (Lohmeyer et al., 2018). Ticks that frequently feed on both wild and domestic animals are often of medical and veterinary importance, not only for their ability to bite livestock, companion animals and humans, but also for their competence as vectors of a huge variety of pathogens to these hosts. Examples of such ticks are *Ixodes ricinus* in Europe, *Amblyomma americanum* in the United States and *Amblyomma sculptum* in South America, *Ixodes holocyclus* in Australia and *Ixodes persulcatus* in Asia.

Comparable only to mosquitoes, ticks are competent vectors of a long list of bacteria, protozoa, viruses, and helminths to animals and humans (Dantas-Torres et al., 2012a). Tick species of medical and veterinary importance are included in the families Ixodidae (hard ticks) and Argasidae (soft ticks), which will be in the spotlight in this chapter. The long evolutionary history of ixodid and argasid ticks has molded their biology and ecology, being their need to obtain a blood meal one of the main driving forces for their evolution (Mans et al., 2011). Ixodid and argasid ticks evolved in such a way that even within the same family ticks may present quite distinct biological life cycles, host specificity and habitat requirements. In the following sections, current aspects of tick origin, taxonomy and identification will be touched upon. General information on the medical and veterinary importance, biology, ecology, prevention and control of ticks are also discussed.

Origin and phylogenetic relationships

Pioneer hypotheses put the origin of ticks in the Paleozoic Era (in the Devonian, Carboniferous or Permian periods), as parasites of the ancestors of modern vertebrates such as reptiles and amphibians (e.g., Olivier, 1989; Dobson and Barker, 1999; Jeyaparakash and Hoy, 2009; Mans et al., 2011, 2016). Other authors believe that ticks originated in the Mesozoic Era, somewhere between the Triassic and Jurassic periods (e.g., Balashov, 1994; Beati and Klompen, 2019). However, fossil data indicate that ixodid and argasid ticks were already diverged in the Mesozoic Era, at least since the Cretaceous period (Poinar and Brown, 2003; Klompen and Grimaldi, 2001; Chitimia-Dobler et al., 2017; Estrada-Peña and de la Fuente, 2018). So far, no fossil evidence is available for the

Nuttalliellidae, but the fourth known tick family (Deinocrotonidae) was also described from Cretaceous amber (Peñalver et al., 2017), further indicating that nuttalliellid, deinocrotonid, ixodid and argasid ticks were already independent lineages during the Cretaceous period.

It has been postulated that ticks would have appeared in the African portion of the supercontinent Gondwana, during the Upper Carboniferous or Lower Permian, probably as parasites of diapsid or synapsid (Mans et al., 2016). This assumption is based on the restricted distribution of the species *Nuttalliella namaqua* (the only living representative of Nuttalliellidae, an ancestral lineage of ticks) as well as supported by molecular estimates (molecular clock) from the data of mitochondrial genes (Mans et al., 2016). However, an extinct species of tick, namely *Deinocroton draculi*, has recently been described based on specimens retrieved in a 99 million-year-old Cretaceous amber from Myanmar (Peñalver et al., 2017). From the morphological point of view, *Dc. draculi* [abbreviations of tick genera are according to Dantas-Torres, 2008, except for *Deinocroton* which was described afterwards] is closely related to *Nu. namaqua* and it is believed that they represent distinct evolutionary lineages that originated from a common ancestor (Peñalver et al., 2017). Unfortunately, no genetic data is presently available for *Dc. draculi*, which precludes any further consideration, which also is beyond the scope of this chapter. Nonetheless, the finding of *Dc. draculi* in Myanmar may suggest that ancestral lineages of ticks could have had a broader distribution in Gondwanan than previously thought, which calls into question the theory of the origin of ticks in South Africa (Dantas-Torres, 2018).

Phylogenetic analyzes indicate that all tick families and subfamilies are monophyletic (Mans et al., 2012). However, there are still doubts about the position of the Nuttalliellidae in relation to other extant tick families (i.e., Argasidae and Ixodidae) (Mans et al., 2016). In the past, it was believed that *Nu. namaqua* was the “missing link” in the phylogenic relationship of Argasidae and Ixodidae. Nowadays, it is accepted by some authors that the Nuttalliellidae occupies a basal position in relation to other extant tick families, representing the closest relative to tick ancestors (Mans et al., 2011). However, studies carried out with different molecular markers (mitochondrial and nuclear) have generated discrepant results in relation to the exact phylogenetic position of this family (Mans et al., 2012; Burger et al., 2014).

Taxonomy

Ticks are chelicerate arthropods (kingdom Animalia, phylum Arthropoda, subphylum Chelicerata), belonging to the class Arachnida, subclass Acari, superorder Parasitiformes and order Ixodida (=Metastigmata) (Keirans, 2009; Lindquist et al., 2009). This order is currently subdivided into four families: Argasidae, Ixodidae, Nuttalliellidae and Deinocrotonidae (extinct) (Dantas-Torres, 2018).

Out of over 50,000 named species of Acari, ~970 are tick species (updated from Dantas-Torres, 2018; Du et al., 2018; Kwak et al., 2018; Apanaskevich and Tomlinson, 2019; Apanaskevich et al., 2019; Barker, 2019; Dantas-Torres et al., 2019a; Martins et al., 2019; Sun et al., 2019; Labruna et al., 2020; Muñoz-Leal et al., 2020; Onofrio et al., 2020), eight of which have been described from fossil material. Ixodidae and Argasidae are the most speciose families with approximately 750 and 218 species, respectively. Species in the family Ixodidae are arranged in two major phyletic lines, namely Prostriata (all species in the genus *Ixodes*) and Metastriata (all other ixodid species) (Olivier, 1989). Prostriate ticks are the most ancient and primitive members of the Ixodidae (Black and Roehrdanz, 1988). Among metastriate ticks, those belonging to the genus *Haemaphysalis* are more ancient and primitive than other genera (Olivier, 1989).

The Ixodidae includes the following genera (approximate number of species in parentheses): *Africaniella* (2 spp.), *Amblyomma* (136 spp.), *Anomalohimalaya* (3 spp.), *Archaeocroton* (1 sp.), *Bothriocroton* (7 spp.), *Compluriscutula* (1 sp.), *Cornupalpatum* (1 sp.), *Cosmiomma* (1 sp.), *Dermacentor* (42 spp.), *Haemaphysalis* (173 spp.), *Hyalomma* (27 spp.), *Ixodes* (261 spp.), *Margaropus* (3 spp.), *Nosomma* (2 spp.), *Rhipicentor* (2 spp.), *Rhipicephalus* (86 spp.) and *Robertsicus* (1 sp.) (updated from Dantas-Torres, 2018). The species *Haemaphysalis cretacea*, which was described based on a nymph found in a Burmese amber from the Late Cretaceous (Chitimia-Dobler et al., 2018) is accounted for in the total number of ticks in the world, but not among the species of *Haemaphysalis*, due to uncertainties regarding the generic classification of this species.

The genus-level classification of the Argasidae has been the subject of ongoing discussion (Mans et al., 2019). However, the most frequently adopted classification includes the following genera (approximate number of species in parentheses): *Antricola* (17 spp.), *Argas* (62 spp.), *Nothoaspis* (3 spp.), *Ornithodoros* (134 spp.) and *Otobius* (2 spp.).

Identification

Ticks are large mites, whose size varies according to developmental stage and species; the largest ticks may reach over 30 mm in length (Keirans, 2009). Ixodid and argasid ticks are quite polymorphic (Figs. 1 and 2). The main morphological differences between ixodid and argasid ticks are summarized in Table 1. Although adults of some species have vestigial hypostome (e.g., *Otobius* spp., *Antricola* spp., some species of the genus *Ornithodoros* and some nidicolous males of the genus *Ixodes*), the vast majority of ticks have well-developed hypostome, covered with retrograde denticles, which are designed to anchor the tick into the host skin during the feeding phase. Ticks have no antennae, epistome, corniculi or tritosternum (Keirans, 2009). The palps are divided into four segments (articles I–IV) and can vary in morphology. In the tarsus I of all post-embryonic stages, a sensory organ called Haller's organ is present. This organ is designed to receive chemical, olfactory and microclimatic stimuli.

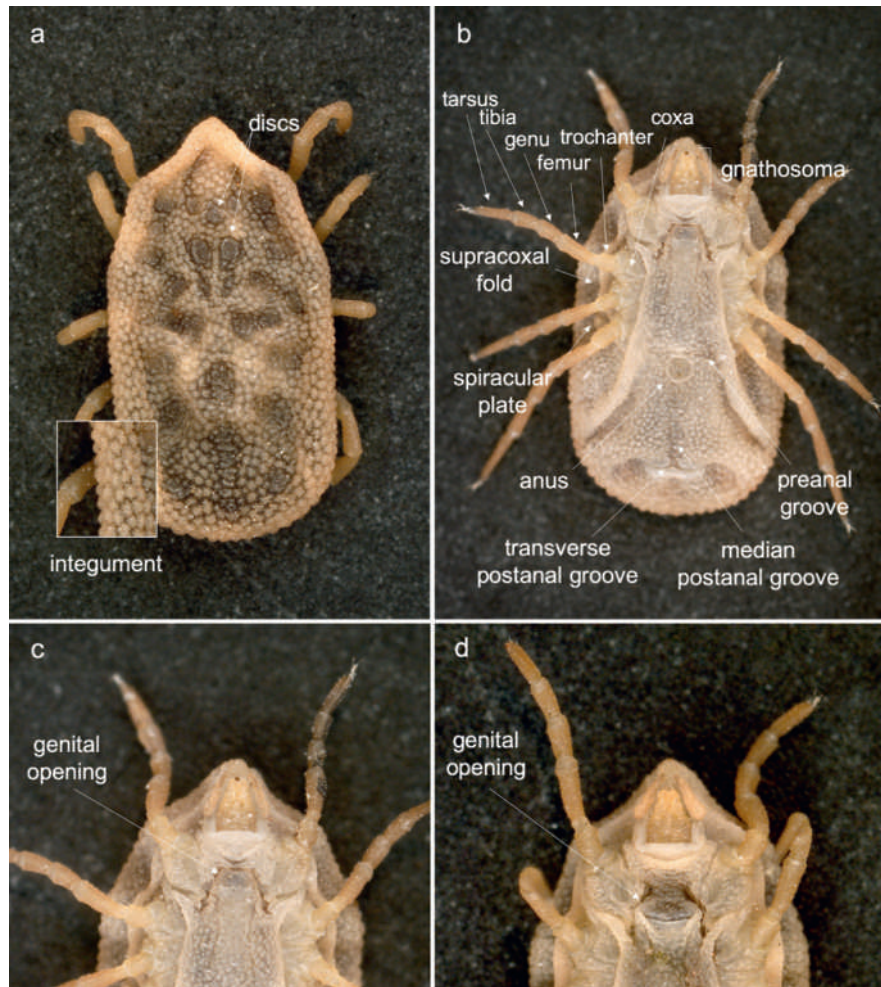


Fig. 1 Dorsal (A) and ventral view (B) of the bat tick *Ornithodoros kelleyi*. Ventral close-up views of a male (c) and a female (d) of the same species are also depicted. Main anatomical structures of taxonomic interest are indicated. Photos courtesy of CDC/William L. Nicholson and Jim Gathany, Public Health Image Library.

A single pair of eyes (ocelli) may be present or absent. When present, they are located on the lateral margins of the dorsal shield in ixodid ticks and in the supracoxal folds in argasid ticks. The larvae are hexapods and do not have spiracular plates. Nymphs and adults have four pairs of legs and have spiracular plates. Larvae and nymphs are sexually immature, having no genital opening. Larvae of most known species of argasid ticks have a dorsal plate, absent in nymphs and adults of this family; nymphs and adults of *Nothoaspis* spp. have a leathery dorsal shield, generally called pseudoscutum.

Ixodids have only one nymphal instar (=stadium). Argasid ticks have several nymphal instars; generally, three to four in most species, but may be five to eight in other species (Olivier, 1989; Apanaskevich and Olivier, 2013). Nymphal instars vary in size, but are essentially identical from the morphological point of view. In general, argasid ticks show little sexual dimorphism, whereas males and females of ixodid ticks can be easily differentiated by the dorsal scutum, which is anterior (podonotal) in females and complete (holonotal) in males. The male dorsal scutum is also called conscutum (Figs. 1 and 2).

The morphological determination of some species of ticks can be difficult. Taxonomic keys for the identification of tick genera and species can be found in reference books (e.g., Walker et al., 2000; Keirans, 2009; Durden and Beati, 2013; Estrada-Peña et al., 2017b; Nava et al., 2017) or papers (e.g., Martins et al., 2014a; Dantas-Torres et al., 2019a). A recent useful review listed 216 references which cover all tick species reported as permanent residents of the Western Palearctic, including 28 species of the genus *Ixodes*, two *Dermacentor*, seven *Haemaphysalis*, nine *Hyalomma*, eight *Rhipicephalus*, five *Argas* and about seven species of *Ornithodoros* (Estrada-Peña et al., 2017a).

Some morphological characters can be difficult to observe in fed specimens (especially engorged larvae, nymphs and females), poorly preserved (e.g., with modified ornamentation pattern of the scutum), or even damaged (e.g., without hypostome). Likewise, some species can be very similar in one or more stages of development. For example, males from *Amblyomma cajennense* and *Am. sculptum* cannot be morphologically distinguished with certainty and, therefore, molecular identification is the only way to unequivocally separate them (Nava et al., 2014; Martins et al., 2016). In fact, the molecular identification of ticks has contributed

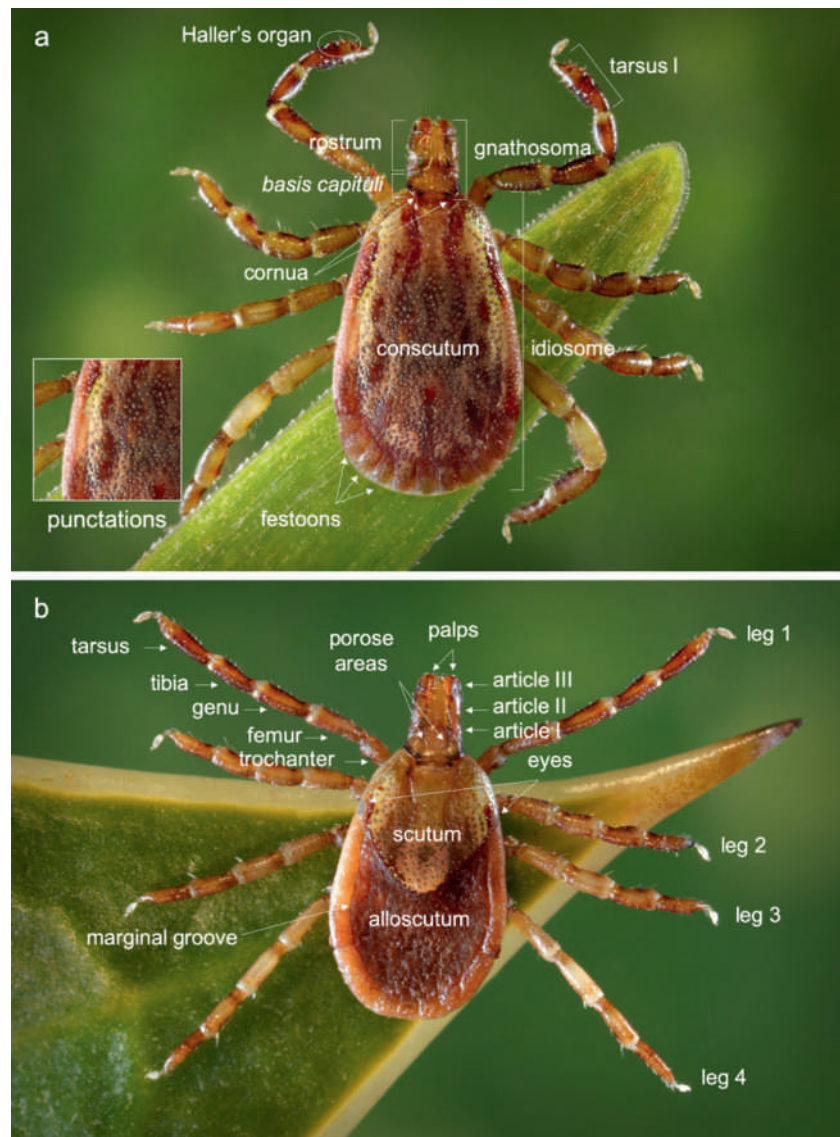


Fig. 2 Dorsal view of a male (A) and a female (B) of South American yellow dog tick, *Amblyomma aureolatum*. Main anatomical structures of taxonomic interest are indicated. Photos courtesy of CDC/Dr. Christopher Paddock and James Gathany, Public Health Image Library.

significantly to the differentiation of species that are difficult to recognize morphologically, to the discovery of new species, as well as to the revalidation of species previously placed in synonymy.

Concerning the molecular identification of ticks, ribosomal nuclear DNA genes (18S and 28 rRNA) are generally more informative at the family and subfamily levels. Mitochondrial genes (e.g., cytochrome *c* oxidase subunit 1, 12S rRNA and 16S rRNA) and transcribed internal spacers (ITS1 and ITS2) provide better resolution at the level of genus and species. Mitochondrial sequences from closely related ticks should be interpreted with caution, considering that paternal leakage and mitochondrial DNA heteroplasmy may occur (Mastrantonio et al., 2019). It is also worth mentioning data bases such as Genbank (<https://www.ncbi.nlm.nih.gov/genbank>) may contain doubtful or even incorrect information, as there is no strict control over the accuracy of the information submitted (e.g., morphological identification). It is not uncommon to find sequences erroneously attributed to a particular species. Therefore, users of these databases should always pay attention to the use of reference sequences for the species under study. These sequences can be found in taxonomic papers (e.g., Dantas-Torres et al., 2012c; Nava et al., 2014, 2018), but also in reference books (e.g., Nava et al., 2017).

Next-generation sequencing (NGS) has been suggested as a useful tool in the search for new nuclear and mitochondrial markers for the study of tick systematics (Mans et al., 2015). The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used to identify ticks and detect pathogens in these arthropods (Diarra et al., 2017). In practice, NGS and MALDI-TOF MS are restricted to a few laboratories.

Table 1 Principal morphological differences between argasid and ixodid ticks.

Structure	Argasidae	Ixodidae
Coxal glands	Present in nymphs and adults	Absent
Eyes	When present, they are located in the supracoxal folds	When present, they are located at the margins of the scutum
Festoons	Absent	Present (except in <i>Ixodes</i> spp., <i>Margaropus</i> spp. and <i>Rhipicephalus</i> (<i>Boophilus</i>) spp.)
Gnathosoma	Ventral (except in larvae, in which it is terminal)	Terminal
Integument	Wrinkled, granulated, leathery, mamillated or tuberculated (except in adults of <i>Ornithodoros marinkellei</i> , in which it is smooth with small punctations)	Smooth (scutum and ventral plates) or striate (soft parts of the integument), with punctations that vary in size, deepness, number and localization
Palps	Four subequal articles (except <i>Or. marinkellei</i> and <i>Nothoaspis</i> spp., in which the article I is broader and differs from other articles)	Four articles of various size and forms, being the article IV reduced and recessed within a cavity of article III
Porose areas	Absent	Present
Scutum	Absent in nymphs and adults (except in <i>Nothoaspis</i> spp. that have a pseudoscutum); larvae may present a dorsal shield	Present in larvae, nymphs and adults
Spiracular plates	Situated between coxae III and IV	Situated after coxae IV

Medical and veterinary importance

Ticks can cause direct damage to the host, whose degree of severity will depend on characteristics of the infesting tick species as well as on the intensity of infestation (i.e., number of ticks per infested host). When ticks insert their mouthparts (i.e., hypostome and chelicerae) into the host skin (Richter et al., 2013), they can produce a local wound, which may facilitate secondary bacterial infections or infestation of larvae causing myiasis. Due to their extreme blood-feeding capabilities (discussed in detail below, in the section *Biology and ecology*), ticks can cause blood depletion, potentially leading to anemia in massively infested animals, particularly in young animals, malnourished and in the presence of polyparasitism. In addition, tick infestations can be a nuisance, causing irritation and decline of infested animals. Components of tick saliva can also cause allergy and paralysis (also known as tick toxicosis). Last but not least, ticks are mostly important due to their ability to transmit a multitude of pathogens to animals and humans (Jongejan and Uilenberg, 2004; Dantas-Torres et al., 2012a). It is worth mentioning, however, that many microorganisms detected in ticks are non-pathogenic or are presently of unknown pathogenicity (Ogrzewalska et al., 2019).

In the tropics, ticks are important parasites of livestock. The direct damage caused to the host and the morbidity and mortality attributable to tick-borne diseases (e.g., anaplasmosis, babesiosis and theileriosis) are responsible for a considerable economic burden to the livestock industry (Jongejan and Uilenberg, 2004; Dantas-Torres et al., 2012a; Estrada-Peña et al., 2020). Companion animals, especially dogs, are also frequently exposed to ticks and tick-borne pathogens, including bacteria (e.g., *Ehrlichia* spp. and *Rickettsia* spp.) and protozoa (e.g., *Babesia* spp. and *Hepatozoon* spp.). In the same way, many ticks primarily associated to wild animals, livestock and/or companion animals may infest and eventually transmit pathogens to humans. In fact, diseases such as Lyme borreliosis (caused by *Borrelia burgdorferi* and some related genospecies), ehrlichiosis (*Ehrlichia chaffeensis*, *Ehrlichia ewingii* and *Ehrlichia muris euclairensis*), human granulocytic anaplasmosis (*Anaplasma phagocytophilum*), rickettsioses (*Rickettsia rickettsii*, *Rickettsia conorii*, *Rickettsia massiliae*, *Rickettsia parkeri*, *Rickettsia africae* and many others), and tick-borne encephalitis (tick-borne encephalitis virus, a member of the family *Flaviviridae*) are responsible for considerable morbidity and mortality in humans around the world (Dantas-Torres et al., 2012a; Parola et al., 2013; Portillo et al., 2015; Madison-Antenucci et al., 2020).

Tick-borne diseases are on the rise worldwide (Dantas-Torres et al., 2012a; Dantas-Torres, 2015; Madison-Antenucci et al., 2020). Among the main factors linked to increase risk of this group of diseases, climate change, increased outdoor activities, global traveling, urbanization, natural environment encroachment, deforestation, habitat fragmentation should be mentioned. Through different mechanisms, these factors may promote or increase the contact between ticks, wildlife, livestock, companion animals and humans, thus creating a suitable environment for tick-borne pathogen transmission to occur (Dantas-Torres, 2015).

Ticks typically become infected while feeding on an infected host, which may be infected by one or multiple pathogens (Otranto, 2018). Indeed, ticks, animals and people may be co-infected by multiple pathogens (de Caprariis et al., 2011; Latrofa et al., 2014; Otranto et al., 2014a; Wormser et al., 2019). Ticks may also become infected through co-feeding with infected ticks on the same host and this play an important role in the transmission dynamics of certain pathogens (e.g., *B. burgdorferi*) (Belli et al., 2017; States et al., 2017). In some instances, ticks may pass the infection from one developmental stage to another (i.e., transstadial transmission) or from their mother to the offspring (transovarial transmission). Besides the feeding habits in terms of feeding periodicity and host range, transstadial and transovarial transmission play a key role in determining the infection rates in ticks in nature. For instance, transovarial transmission of *R. rickettsii* is inefficient in *Am. sculptum* (the principal vector of this bacterium in South America) but highly efficient in *Amblyomma aureolatum* (a vector in the metropolitan region of São Paulo) (Labruna et al., 2011). The prevalence

of *R. rickettsii* in *Am. sculptum* in Brazil is very low (i.e., 0.05–1.28%), even in areas the Brazilian spotted fever is endemic (Costa et al., 2020). Populations of this tick species from areas where Brazilian spotted fever cases have not been reported seem to be devoid of *R. rickettsii* infection (Dantas-Torres et al., 2019c; Gerardi et al., 2019). This can be attributed to an insufficient number of competent amplifying hosts (e.g., capybaras), a low susceptibility of the tick population to *R. rickettsii*, or both. Indeed, a recent study assessing six geographically distinct populations of *Am. sculptum* showed that the frequency of *R. rickettsii* transovarial transmission in this tick may vary according to population (Gerardi et al., 2019).

A great deal of data regarding the infection of ticks with a variety of pathogens has been accumulated since the advent of molecular biology techniques. A meta-analysis of the prevalence of *B. burgdorferi* sensu lato in questing nymphs in Europe revealed that prevalence by different genospecies (e.g., *Borrelia afzelii*, *Borrelia garinii* and *Borrelia valaisiana*) range from <1% up to >90% (Estrada-Peña et al., 2018). Prevalence in ticks was associated with environmental factors, with highest prevalence occurring in areas of 6.85°–16.85°C of average annual temperature and a slow spring rise of temperature, together with a moderate spring rise of vegetation vigor (Estrada-Peña et al., 2018). Some tick-transmitted pathogens may exert a deleterious effect on their tick vectors, as it is the case of *Rickettsia conorii* and *Rh. sanguineus* s.l. (Levin et al., 2009; Parola et al., 2009). This fact may partly explain the generally low prevalence (usually <1%) of *R. conorii* and *Rh. sanguineus* s.l. in nature (Parola et al., 2009).

Tick-borne pathogen transmission primarily occurs during tick blood feeding. Exceptions are protozoa of the genus *Hepatozoon* spp., whose transmission occurs through the ingestion of ticks harboring mature oocysts or eventually by predation of infected prey (Johnson et al., 2009; Baneth, 2011). During the course of blood feeding, transmission times (i.e., time elapsed from the tick attachment to the actual transmission of the pathogen) may range from minutes to hours or days (Saraiva et al., 2014; Eisen, 2018; Otranto, 2018). Transmission times are governed by features of the pathogen itself, but may also be influenced by other factors, such as interrupted feeding. For instance, it has been demonstrated that unfed nymphs and adults of *Am. aureolatum* had to be attached to the host for >10 h to transmit *R. rickettsii* (Saraiva et al., 2014), the agent of the most severe form of spotted fever in the western hemisphere (Dantas-Torres, 2007). On the other hand, fed ticks required only 10 min to transmit the bacterium to naïve hosts (Saraiva et al., 2014). This difference was attributed to the so-called reactivation phenomenon. In practice, *R. rickettsii* remain in a nonvirulent state in unfed ticks, whereas pre-feed ticks carry the bacteria in their virulent state (i.e., reactivated). It means that the pre-feeding can revert the bacteria to a virulent state, promoting their multiplication and shortening their transmission time when blood feeding is resumed on a new or even on the same host.

All the above-mentioned aspects inherent to tick-borne pathogen transmission are pivotal for understanding the mechanisms through which hosts become infected and thereby to establish refined prevention and control strategies.

Biology and ecology

Because of their medical and veterinary importance, ticks have been a recurrent subject of biological and ecological studies. A great deal is known about various aspects of tick life cycles, including feeding, reproduction, host specificity, habitat requirements and seasonal patterns (Olivier, 1989; Vial, 2009; Apanaskevich and Olivier, 2013; Gray et al., 2013). The following sections provide an overview on general aspects of tick biology and ecology. For more details, readers are advised to consult reference books on ticks (e.g., Bowman and Nuttall, 2008; Sonenshine and Roe, 2013a,b).

Feeding

Ticks are obligatory blood feeders. Ixodid ticks feed once in all developmental stages (larva, nymph and adult), with the exception of males of some *Ixodes* spp. that have rudimentary mouthparts and do not feed (Gray et al., 2013). Argasid larvae and nymphs generally feed once, whereas adults feed several times. However, if less than a critical amount of blood has been imbibed, a nymph may feed twice before ecdysing to the next nymphal instar (Olivier, 1989). Moreover, adults of some species (e.g., *Otobius* spp. and *Antricola* spp.) that possess rudimentary mouthparts do not feed. Similarly, larvae (e.g., *Ornithodoros savignyi*) and first-instar nymphs (e.g., *Ornithodoros fonsecai*) of some argasid species are able to molt without feeding (Olivier, 1989; Santiago et al., 2019).

When ticks are on the host and find a suitable place to feed on, they use their two chelicerae to pierce the skin of the host (Richter et al., 2013). Then, a breaststroke-like motion, effected by synchronous flexure and retraction of both chelicerae, causes the barbed hypostome to enter the host's skin (Richter et al., 2013). The buccal canal of the tick is formed by the dorsal surface of the hypostome and the ventral surfaces of the chelicerae (Richter et al., 2013).

The blood feeding process of ticks may last from hours to several days, depending on species and developmental stages. While feeding, ticks inject their saliva into the bite wound, which contains several active molecules, including anti-coagulants and inhibitors of blood clotting, kinases, vasodilators, and inhibitors of host defense (Mans and Neitz, 2004). Many ticks also secrete a cement-like substance, which is produced by the salivary glands, forming a prominent feeding cone that helps tick attachment (Richter et al., 2013).

Ixodid larvae and nymphs feed for days before molting to the next developmental stage. Larvae of most species of argasid ticks also remain on the host for several days, although larvae of some species (e.g., *Argas cucumerinus*) may feed for some minutes only. In general, nymphs and adults of argasid ticks feed for short periods (usually from 30 min to a few hours) (Olivier, 1989). Ixodid females generally feed for several days before laying eggs.

Ticks have developed an extraordinary capacity of survival without a blood meal, particularly argasid ticks. Ixodid ticks can survive for more than 1 year without a blood meal, but not much longer than that (Dantas-Torres et al., 2012b). On the other hand, argasid ticks are more capable to fast and some species are well adapted to the harsh, dry, low-host-density environments. As an example, unfed larvae of *Ornithodoros lahorensis* can live for a year and unfed adults can survive for 18 years without a blood meal (Hoogstraal, 1985). This long-term survival capability allows argasid ticks to linger in inhospitable places (e.g., remote desert areas), where host availability may be limited.

Feeding rhythm is definite in most ticks. The feeding cycle is comprised of periods of sucking, salivation and resting. For instance, sucking activity of *Rh. microplus* reach a peak at night, decrease in the morning and during the day, increasing again in the evening (Olivier, 1989). In a similar manner, ixodid ticks and larvae of some argasid ticks exhibit a definite daily rhythm of detachment from the host (i.e., drop-off rhythm), which is usually coordinated with the host's activities (Olivier, 1989; Dantas-Torres, 2010). For instance, engorged larvae of *Rh. sanguineus* s.l. detach from dogs predominately during daylight hours, whereas engorged nymphs and females exhibit nocturnal drop-off rhythm (Hadani and Rechav, 1969; Jacobs et al., 2004; Paz et al., 2008). Indeed, most ticks will detach while the hosts are in their nests, burrows, or other sheltered places (Olivier, 1989). Another interesting case is the synchronism between *Haemaphysalis leporispalustris* and rabbits, with this tick species dropping from the rabbit primarily during daylight hours when the rabbits are in their nests and only rarely during nocturnal hours (George, 1971).

Hyperparasitism (i.e., the parasitism of a parasite by another parasite), seems to be not uncommon among conspecific argasid ticks (Votava et al., 1974; Moorehouse and Heath, 1975; Helmy et al., 1983; Oliver et al., 1986; Endris et al., 1991; Williamson and Schwan, 2018). It has been wondered that this may reflect an earlier predatory origin of parasitism (Olivier, 1989). This may also be a strategy of fasting ticks sharing the same niche with fed ticks that have had a meal. A recent experimental study reported that 78.6% of *Ornithodoros hermsi* males (114/145) fed on conspecific engorged ticks (Williamson and Schwan, 2018). Unfed females and nymphs also fed on other ticks but far less frequently (only 6.7% combined; 17/254). Remarkably, unfed males acquired *Borrelia hermsii* when parasitizing nymphs that had recently engorged on a bacteremic mouse, and unfed infected males transmitted spirochetes to recently engorged nymphs (Williamson and Schwan, 2018). Finally, some ticks infected via hyperparasitism subsequently transmitted *B. hermsii* to mice. The real meaning of this behavior in nature remains poorly known.

Exceptional cases of hyperparasitism have been reported in ixodid ticks (Labruna et al., 2007; Durden et al., 2018; Buczek et al., 2019) and are reputed to be more common in *Ixodes* males (Olivier, 1989). Considering the low frequency and paucity of reports on this phenomenon in ixodid ticks, one may argue that the biological meaning of hyperparasitism for ixodid ticks is probably null.

Mating

As in other arthropods, mate-finding and courtship behavior in ticks are mostly regulated by pheromones of several types, including on-host sexual aggregation pheromones secreted by female metastriate while feeding (Kiszewski et al., 2001). Copulation of ticks can occur on hosts and in the environment. Argasid ticks always copulate in the environment, except *Argas transversus*, a very unique tick that mates on the host (Hoogstraal et al., 1973). Ixodid ticks of the genus *Ixodes* (Prostriata) produce spermatids even before feeding and generally copulate in the environment, but eventually also on the host (Olivier, 1989). One exception to this rule is *Ixodes trianguliceps*, whose males copulate solely on their hosts (Randolph, 1980).

Male ticks belonging to other genera (Metastrata lineage), on the other hand, typically attain sexual maturity and mate solely on their hosts (Kiszewski et al., 2001). Rare exceptions are the species *Bothriocroton hydrosauri*, *Bothriocroton concolor* and *Amblyomma triguttatum* whose males may produce spermatids without feeding (Guglielmone and Moorhouse, 1983; Oliver and Stone, 1983). Moreover, unfed males of *Am. triguttatum* can even transfer spermatids to females off the host (Guglielmone and Moorhouse, 1983).

When a male tick attains venter-to-venter contact with a female, mating proceeds through a succession of events (spermatophore formation and transfer), which culminates in fertilization of the female. The spermatophore transfer generally occurs in less than a minute, but males and females may remain in sexual contact for several days (Kiszewski et al., 2001).

The degree of copulatory penetration varies among ixodid and argasid ticks (Kiszewski et al., 2001). While copulating, argasid males insert their chelicerae, hypostome and pils into the females' genital pore (Feldman-Muhsam and Borut, 1971). As far as ixodid ticks, metastriate males insert only the tip of their chelicerae, whereas prostriate males introduce both their chelicerae and hypostome (Kiszewski et al., 2001). Metastriate males are able to copulate several times and take blood meals between copulas (e.g., males of the lone star tick *Am. americanum* can inseminate up to 37 females; Gladney and Drummond, 1970).

Males of some tick species are extremely rare, as it is the case of the rotund tick *Amblyomma rotundatum* (Sanches et al., 2012; Luz et al., 2013). Indeed, this tick species is considered to be obligatory parthenogenetic and capable of producing diploid female offspring by thelytoky (Olivier, 1989; Sanches et al., 2012), that is, the production of diploid daughters from unfertilized eggs. While extremely rare, males of *Am. rotundatum* have been found in nature (Labruna et al., 2005; Martins et al., 2014b; Gianizella et al., 2018), which is intriguing and raises the question whether this species is also able to reproduce bisexually, as it happens with other ixodid species. Indeed, sporadic parthenogenesis in normally bisexual species of ixodid and argasid ticks may occur, but it is generally considered of little practical significance as the few larvae produced are generally weak and soon die (Olivier, 1989; Kiszewski et al., 2001). However, parthenogenetic races of normally bisexual species could possibly emerge from such occurrences, which appears to be the case in *Haemaphysalis longicornis* (Olivier, 1989).

Females of some species of argasid ticks are able to lay eggs without feeding on blood, a trait named autogeny. Autogeny may be facultative (e.g., *Argas persicus*, *Or. lahorensis*, *Ornithodoros tartakovskyi*, *Ornithodoros tholozani* and *Or. fonsecai*) or obligatory (e.g.,

Otobius spp., *Antricola* spp. and probably *Nothoaspis* spp.) (Olivier, 1989; Kiszewski et al., 2001; Santiago et al., 2019). Autogeny has not been reported for ixodid ticks.

In addition to fulfilling its basic role of egg fertilization in bisexual species, mating is very important in regulating ixodid feeding and reproductive behavior (Olivier, 1989; Kiszewski et al., 2001). In fact, most ixodid females will not complete engorgement until mated. After mating, they will enter the rapid engorgement phase and soon drop from the host. Fed females are positively geotropic and negatively phototactic. So, they usually find a secluded place for oviposition, which occurs soon thereafter, unless an ovipositional diapause occurs (Olivier, 1989).

Oviposition

The preoviposition and oviposition periods may vary according to biotic and abiotic factors, lasting from a few days to several weeks (Troughton and Levin, 2007; Dantas-Torres et al., 2011). Temperature is critical factor (Olivier, 1989).

Ixodid females present a single gonotrophic cycle. After laying her single egg batch, the female stays alive for some days and then die (Dantas-Torres et al., 2011; Apanaskevich and Olivier, 2013). Ixodid females lay in average a few thousand eggs (Troughton and Levin, 2007). However, the amount of eggs laid by each single female varies from species to species and also according to other factors such as the amount of blood ingested (Dantas-Torres et al., 2011). For example, *Haemaphysalis inermis* females usually deposit a few hundred eggs (Apanaskevich and Olivier, 2013), whereas large *Amblyomma* spp. and *Hyalomma* spp. females can produce impressive numbers of eggs. There is a record of laying 38,732 eggs by a female of *Amblyomma variegatum* (Dipeolu and Ogunji, 1980). Partially engorged, fertilized females can also oviposit, but typically lay a lower amount of eggs.

Unlike ixodid females, argasid females may have various gonotrophic cycles and lay several batches of eggs, which are intercalated by periods of feeding and blood digestion. Interestingly, argasid females use only a portion of the ingested blood for egg production, storing the remainder for subsequent gonotrophic cycles or for their sustenance during long starvation periods (Olivier, 1989). Interestingly, females of some argasid species (e.g., *Argas arboreus*, *Argas boueti*, *Argas brumpti*, *Ar. transgarepinus*, *Ornithodoros moubata* and *Ornithodoros salahi*) show a brooding behavior over their eggs during incubation, whereas others (e.g., *Antricola marginatus*, *Argas striatus* and *Argas transgarepinus*) present a maternal behavior (carrying of larvae on the idiosoma) (Olivier, 1989; Labruna et al., 2012; Pienaar et al., 2018). Brooding may last for a few days or until hatching (e.g., in *Ar. arboreus*), or even through the inactive larval stage until nymphs emerge (e.g., in *Or. moubata*) (Jobling, 1925; Hafez et al., 1972). It has been supposed that maternal behavior may be an evolutionary adaptation to ecological challenges in inhospitable habitats for larval survival (Pienaar et al., 2018). Nonetheless, both maternal and brooding behaviors are still poorly investigated.

Host specificity

Ixodid and argasid ticks feed on a wide variety of reptiles, amphibians, birds and mammals. Some ticks accept a broad range of host species (i.e., generalists), whereas others are more selective (i.e., specialists) and feed on a narrow group of host species (Hoogstraal and Aeschlimann, 1982; Olivier, 1989).

A comprehensive study based on 43,615 records for 223 species of African ticks concluded that 14 species were generalists and 39 were potential specialists (Cumming, 1998). Data available at that time were insufficient for any conclusion regarding host preference for the remainder species. On the other hand, a relatively more recent meta-analysis based on 4172 records from Neotropical hard ticks concluded that strict host specificity is uncommon (Nava and Guglielmone, 2013). An example of a medically important generalist tick in the Neotropical region is *Am. sculptum* (Martins et al., 2016). This tick species may feed on capybaras, anteaters, tapirs, foxes, maned wolves, coatis, horses, dogs, among others (Martins et al., 2016; Ramos et al., 2020). It is also a very aggressive tick that bites humans, particularly nymphs and adults (Szabó et al., 2020).

In anthropic environments, domestic animals may act as the principal hosts for certain tick populations (e.g., dogs for *Rh. sanguineus* s.l. and cattle for *Rh. microplus*). However, wild animals (e.g., rodents, marsupials, foxes, lizards, snakes, iguanas, tortoises, bats and birds) may also act as primary hosts for tick populations in anthropic environments. For instance, capybaras are the main hosts of *Am. sculptum* in anthropic areas in São Paulo, southeastern Brazil (Luz et al., 2019). In the same way, white-tailed deer (*Odocoileus virginianus*) are important hosts for *Ix. scapularis* in urban parks in New York, United States (VanAcker et al., 2019).

As for ixodid ticks, host specificity varies in argasid ticks. Some groups of argasid ticks feed exclusively or predominately on bats, birds, toads or lizards (Dantas-Torres et al., 2012c; Muñoz-Leal et al., 2016, 2018b; Sándor et al., 2019; Venzal et al., 2019). On the other hand, some argasid may be generalists and feed on bats, marsupials and humans as well (Labruna et al., 2014).

The tick-host relationship is also an important factor driving the distribution of ticks. Exotic pets, migratory birds and also livestock may contribute to the dispersion of ticks and their associated pathogens in different habitats and regions (Brianti et al., 2010; Falchi et al., 2012; Hasle, 2013; Medlock et al., 2018; Bilbija et al., 2019).

Habitat and distribution

With some exceptions, ixodid and argasid ticks spend the bulk of their lives in the environment, where they are under the selection pressure of several abiotic factors. Habitat requirements of ticks directly influence their nonparasitic stages and may also affect their chance of finding a new host (Olivier, 1989; Dantas-Torres, 2010). In fact, most argasid ticks and many *Ixodes* species have adopted a

nidicolous life cycle strategy and live in nests, caves, burrows, or in human and livestock habitations (Olivier, 1989; Vial, 2009), which may provide a more stable microclimate and also increased protection from predators. Nonetheless, cave-dwelling argasid ticks may be targeted by predators such as spiders (Bernardi et al., 2010). While relatively uncommon, ixodid ticks may also be found in caves. Some *Ixodes* ticks primarily inhabit bat caves (Hornok et al., 2014), whereas others may sporadically be found in caves (Dantas-Torres et al., 2009), probably being transported by animals living nearby or visiting those caves.

Ixodid ticks live in a variety of habitats, including in tropical and temperate forests, steppes, prairie habitats, and even in the harsher tundra of the arctic (Keirans, 2009; Guglielmone et al., 2014). Some argasid ticks are able to endure in remote inhospitable areas. They are able to subsist in arid and hot environments, thanks to the peculiar nature of oilskin and cement components of their cuticle, which makes it highly resistant and reduces water evaporation (Vial, 2009). For instance, *Or. moubata* and *Or. savignyi* tolerate air temperature up to 63 °C and 75 °C, respectively (Vial, 2009). Many ixodid ticks are also able to survive in dry and hot environments, such as deserts and xeric shrublands (Guglielmone et al., 2014).

Some ticks may be exclusively found in primary forests, whereas others may be adapted to rural and urban environments. A recent study conducted in the Atlantic forest ecoregion in Argentina concluded that forest environments are more suitable habitats than agricultural and urban environments for many native tick species (Lamattina et al., 2018). On the other hand, forest environments were considered unsuitable for exotic species that have successfully established in environments that have been modified by man (Lamattina et al., 2018). The same tick species may be found in a range of habitats, but may be more abundant in a particular type of habitat. As such, living in certain types of habitats (e.g., grasslands) may be a significant risk factor for tick infestation in some hosts (Zanzani et al., 2019).

The ability of ticks to occupy a wide range of habitats partly explains their wide geographical distribution. Indeed, some tick species may present a broad distribution in different countries or continents (e.g., *Rh. microplus*). Information on ixodid tick distribution in the world zoogeographical regions (i.e., Afrotropical, Australasian, Nearctic, Neotropical, Oriental and Palearctic regions) has been exhaustively compiled elsewhere (Guglielmone et al., 2014). Some tick genera (e.g., *Ixodes*, *Haemaphysalis*, and *Amblyomma*) are found in all six zoogeographical regions, whereas others are more restricted in distribution. For instance, *Anomalohimalaya* (three species) is exclusive to the Palearctic, whereas the *Nosomma* (two species) is exclusively Oriental (Guglielmone et al., 2014).

The distribution of ticks in the zoogeographical regions is not uniform (Guglielmone et al., 2014). Indeed, some ticks are exclusive to a particular country, or even a particular island. For instance, *Haemaphysalis kadsani* is found in the Australasian Region, but exclusively on Sulawesi Island (Hoogstraal and Wassef, 1977). A valuable dataset of tick distribution in South America containing over 4000 geo-referenced tick records has recently been made available (Estrada-Peña et al., 2019). This database also provided valuable information on the relationship between tick distribution and climate.

Life cycles

The biological cycle of ticks includes phases of egg, larva, nymph (one instar for ixodid ticks and several instars for argasid ticks) and adult (male and female). In nature, the life cycles of various tick species are regulated by seasons and the length of the cycle will depend on several factors such as photoperiodicity, temperature, moisture, and host availability (Olivier, 1989; Apanaskevich and Olivier, 2013). The number of generations per year may also vary widely according to species and geographical region. For instance, the life cycle may be greatly prolonged in harsh arid and cold climates where hosts may be scarce (Olivier, 1989).

Depending on the number of hosts generally used during their life cycle, ixodid ticks can be classified as one-host (e.g., *Rh. microplus* and *Dermacentor nitens*), two-host (e.g., *Rhipicephalus evertsi*) or three-host ticks (e.g., *Am. sculptum*, *Ix. ricinus* and *Rh. sanguineus* s.l.). In the one-host life cycle, all molts (from larva to nymph and from nymph to adult) occur on the host. In the two-host life cycle, one of the molts (from larva to nymph) occurs on the same host and another (from nymph to adult) on a second one. In the three-host life cycle, all molts occur in the environment and all stages feed on three different individual hosts. Some ticks from three hosts may eventually adopt a two-host cycle (e.g., *Am. rotundatum*), depending, for example, on the type of host (Luz et al., 2013). One-host ixodid ticks may complete several generations per year (Borges et al., 2000; Canevari et al., 2017), as may some three-host ixodid ticks in the tropics (Silveira et al., 2009). Nonetheless, it is supposed that one generation a year is likely the usual pattern for most tick species in subtropical and warmer temperate regions (Olivier, 1989).

With rare exceptions, argasid ticks feed on several hosts (multi-host life cycle) and most species have more than one nymphal instar. Molts generally occur in the environment, but a recent finding suggested that *Ornithodoros hasei* may molt from larva to nymph on bats (Muñoz-Leal et al., 2018a). Rare exceptions include *Or. lahorensis* (a two-host argasid tick) and *Otobius megnini* and *Otobius lagophilus* (one-host argasid ticks). Another extraordinary exception is the tick *Ar. transversus*, an exclusive parasite of giant Galapagos tortoises (Hoogstraal and Kohls, 1966). This diminutive tick is a unique species within the order Ixodida: it spends its entire life on its host. It is the only known species of tick whose females lay eggs on their hosts (Hoogstraal et al., 1973).

Seasonality

The seasonal rhythms of ticks in the environment and on the hosts vary widely according to species, developmental stages and geographical region. Tick seasonal patterns are influenced by photoperiodicity and host population dynamics, but also by other factors, such as temperature, precipitation and relative humidity (Olivier, 1989; Vial, 2009; Apanaskevich and Olivier, 2013). These factors will exert different effects on tick seasonal patterns in various species and developmental stages, also depending on

geographical regions (Dantas-Torres et al., 2011, 2019c; Dantas-Torres and Otranto, 2013; Gray et al., 2016). For instance, southern, central and northern European populations of *Ix. ricinus* may exhibit distinct seasonal patterns (Dantas-Torres and Otranto, 2013; Gray et al., 2016). The same seems to be true regarding different *Am. sculptum* populations in southeastern and northeastern Brazil (Dantas-Torres et al., 2019c).

Some tick species have acquired biological and ecological traits that enables a life in a specific type of environment, also during periods of low temperature and low-host density. For instance, some ticks may enter into diapause, a hormonally-controlled arrested development which usually occurs seasonally (Perret et al., 2003; Gray et al., 2016). Indeed, diapause usually occurs before environmental conditions become unfavorable for the ticks, and has a fixed latency period that must be completed before development can resume (Gray et al., 2016). It is mainly regulated by hours of sunlight, which are perceived by bilaterally placed strings of photoreceptors, which are located under the cuticle and the hypodermis of the tick (Perret et al., 2003).

Some ticks can survive underwater for certain periods (Fielden et al., 2011; Giannelli et al., 2012; Sá-Hungaro et al., 2014; Bidder et al., 2019), which may also be an advantageous strategy for ticks living in environments that experience seasonal floods or even for ticks parasitizing hosts which live close contact with the water (Luz et al., 2013, 2020; Dantas-Torres et al., 2019b; Kwak et al., 2020). For example, *Am. rotundatum* is a common parasite of reptiles and amphibians in South America, which means that they may frequently go into the water with their hosts (Luz et al., 2013; Dantas-Torres et al., 2019b). Another example is the sea snake tick *Amblyomma nitidum* that is a specialist parasite of snakes of the genus *Laticauda*, being one of the few ticks that could be regarded as semi-marine (Kwak et al., 2020). Some mammal-associated ixodid ticks may also be more adapted to humid habitats and seasonally flooding soils than others. This appears to be the case of *Amblyomma dubitatum* and *Am. sculptum* (Luz et al., 2020). These ticks parasitize several mammal hosts, but are primarily maintained by capybaras in some regions (Martins et al., 2016; Luz et al., 2020). Capybaras are semiaquatic mammals that uses water bodies to regulate their body temperature, for mating, and to escape from predators. A recent experimental study suggested that *Am. dubitatum* is more adapted to humid habitats and seasonally flooding soils than *Am. sculptum*, which in turn is more adapted to drier habitats than *Am. dubitatum* (Luz et al., 2020). This may partly explain the spatial distribution and abundance of these tick species in different landscapes and seasons.

Prevention and control

The most effective and low-cost way to prevent tick infestations is to avoid tick-infested environments (Dantas-Torres, 2007). Thus, people living in tick-free environments should avoid entering into forested areas, urban parks, grassy picnic areas, or any other environment potentially infested by ticks. For obvious reasons, this is not always a realistic recommendation. In tick-infested areas, sitting on logs, gathering wood, sitting against trees, and walking might increase the risk of tick infestation (Lane et al., 2004). Regardless the primary reason (e.g., working, hunting, walking, jogging and fishing), anyone accessing natural environments are at risk of tick infestations (Fig. 3). As commented above, the tick season vary widely according to species and geographical region. Hence, it is important to consult data from local public health authorities to avoid visiting tick-infested environment during periods of the year when larvae and nymphs are more abundant (Dantas-Torres and Otranto, 2013).

When avoidance of tick-infested environments is not feasible, people entering into risk areas should avoid places potentially infested by ticks, and whenever possible, walk in the center of trails. Wearing light colored clothing makes it easier to find crawling ticks. Likewise, wearing long-sleeve shirts and long trousers and tucking pant bottoms into tops of socks or boots, may also help preventing tick bites. While staying from several hours in risk areas, frequent self-examination and tick removal is important also to reduce the risk of tick-borne pathogen transmission (Dantas-Torres, 2007; Piesman and Eisen, 2008). Children should be checked for ticks more often, paying special attention to the head, neck and ears. Attached ticks can be removed with the aid of fine-tipped

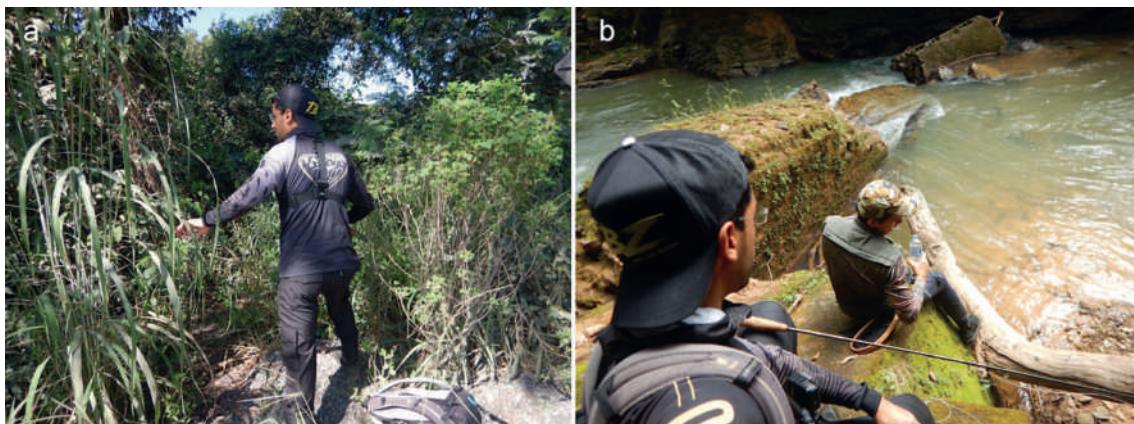


Fig. 3 Fisherman walking through a forested area (A) to access a remote stream for fly fishing (B) in center-west Brazil. In these areas, they are usually exposed to *Amblyomma* spp. ticks, especially nymphs. Photos courtesy of Brenner Ferreira, Tarpon Brazil.

tweezers, by grasping the tick with the tweezers as close as possible to the skin surface, turning it gently, and pulling upward with steady, even pressure. Whenever possible, gloves should be used and tick removal with bare hands should be avoided. Indeed, ticks should not be squeezed, crushed, or punctured, because tick saliva, body fluids, gut contents may contain infectious agents. In areas where different tick species may be found, including vector and non-vector species, it is important to keep the tick in 70% ethanol for future identification and eventually testing for pathogens.

For people visiting tick-infested habitats, commercially available repellents with efficacy against ticks can provide an effective means of personal protection against tick bites (Dantas-Torres, 2007; Benelli et al., 2019). A recent study demonstrated that low concentrations of diethyltoluamide (DEET), picaridin, 2-undecanone, citronellal and nootkatone specifically disrupt the thermal infrared radiation sense of ticks (Carr and Salgado, 2019). There also is ongoing research on novel green repellents based on essential oils (Benelli and Pavela, 2018).

Integrated tick management approaches have also been proposed to control ticks as well as infection by various pathogens in the ticks in natural environments where tick-borne diseases (e.g., Lyme disease) are endemic. For instance, combinations of white-tailed deer reduction, broadcast application of the entomopathogenic fungus *Metarhizium anisopliae* and small rodent-targeted fipronil-based bait boxes have been shown to be an effective to reduce *Ix. scapularis* and infections by various pathogens (e.g. *B. burgdorferi*, *Babesia microti* and *A. phagocytophilum*) in questing nymphs of this tick species (Williams et al., 2018; Little et al., 2020).

Companion animals frequenting tick-infested environments, including dogs and cats, are also at risk of becoming infested by ticks. Therefore, it is important to prevent companion animals from roaming freely in risk areas. If a dog visited a tick infested area, it is important to check the animal for the presence of ticks, which may be difficult to find if small in size (e.g., larvae and nymphs). In any event, controlling tick infestations on dogs is imperative for the wellbeing of dogs themselves, but also from a public health perspective, considering that ticks infesting these animals can also transmit pathogens to humans. Community-based, integrated, tick-management strategies may be effective in controlling ticks on dogs and in the environment, therefore reducing the risk incidence of tick-borne diseases such as Rocky Mountain spotted fever (Drexler et al., 2014).

The best way to keep dogs free of ticks is to apply topical products with repellent activity, such as collars and pipettes containing pyrethroid-based compounds (e.g., permethrin, deltamethrin, and flumethrin), with repel and kill ticks (Otranto et al., 2010, 2017; Dantas-Torres et al., 2013). Repellents cause irritation and toxic effect on ticks, greatly reducing tick feeding (anti-feeding effect) (Otranto et al., 2020). In turn, acaricides do not repel ticks, but kill them soon after they start feeding on a treated host. Chemical groups that possess molecules with killing effect on ticks include, for example, organophosphates (e.g., diazinon and fenthion), formamidines (amitraz), phenylpyrazoles (fipronil and pyriprole) and isoxazolines (e.g., afoxolaner, fluralaner, lotilaner and sarolaner) (Beugnet and Franc, 2012; Rohdich et al., 2014; Six et al., 2016; Cavalleri et al., 2018; Tinkruejeen et al., 2019). Ivermectin is used off-label to control *Rh. sanguineus* s.l. infestations of dogs in numerous countries, including Thailand and Brazil, but is not efficacious (Tinkruejeen et al., 2019). A wide range of formulations (e.g., solutions, shampoos, lotions, sprays, powders, spot-ons, collars and palatable tablets) are available for dogs and eventually cats. It is important to mention that cats are sensitive to several compounds that are safe for dogs. For instance, permethrin is toxic for cats (Beugnet and Franc, 2012). There are, however, specific formulation for cats with other molecules (e.g., flumethrin) that are safe for cats.

Environmental treatment with acaricides may be required in some specific situations, such as in case of high levels of environmental tick infestation in kennels, usually accompanied by high levels of tick infestations on dogs. However, due to the potential negative impacts on nature, environment treatment is not the preferred method for tick control in most of the situations. In addition, the continuous use of acaricides may lead to resistance, representing a major concern in many parts of the world (Villar et al., 2020).

Numerous approaches have been used to control ticks on livestock, including non-chemical and chemical strategies. For instance, non-chemical strategies to control ticks in cattle include the use of tick-resistant breeds of cattle or pasture rotation, which are alternative management practices that may be employed in light infestations, also to reduce the overuse of acaricides (George et al., 2004). Nonetheless, tick control on livestock still largely rely on acaricides. Many products are market to control tick infestations in livestock. These may be an emulsified concentrate, wettable powder or flowable product that can be diluted in water and applied through immersion of animals in a dipping vat, with a hand spray or spray race. Nowadays, products are also available as injectables, pour-on formulations, an intraruminal bolus, acaricide-impregnated ear tags and pheromone/acaricide-impregnated devices. The periodicity of application may vary from region to region and according to tick species (Nava et al., 2019). Nonetheless, the widespread occurrence of resistance to different acaricides has stimulated the search for additional non-chemical alternatives. In this context, anti-tick vaccines have been developed using the midgut glycoprotein Bm86 from *Rh. microplus* as antigen (Rodríguez-Mallon, 2016; Valle and Guerrero, 2018). Vaccination within integrated tick management programs has been shown to be able to reduce the number of acaricidal applications per year that are required to control some strains of *Rh. microplus* in different geographical regions (Rodríguez-Mallon, 2016; Valle and Guerrero, 2018).

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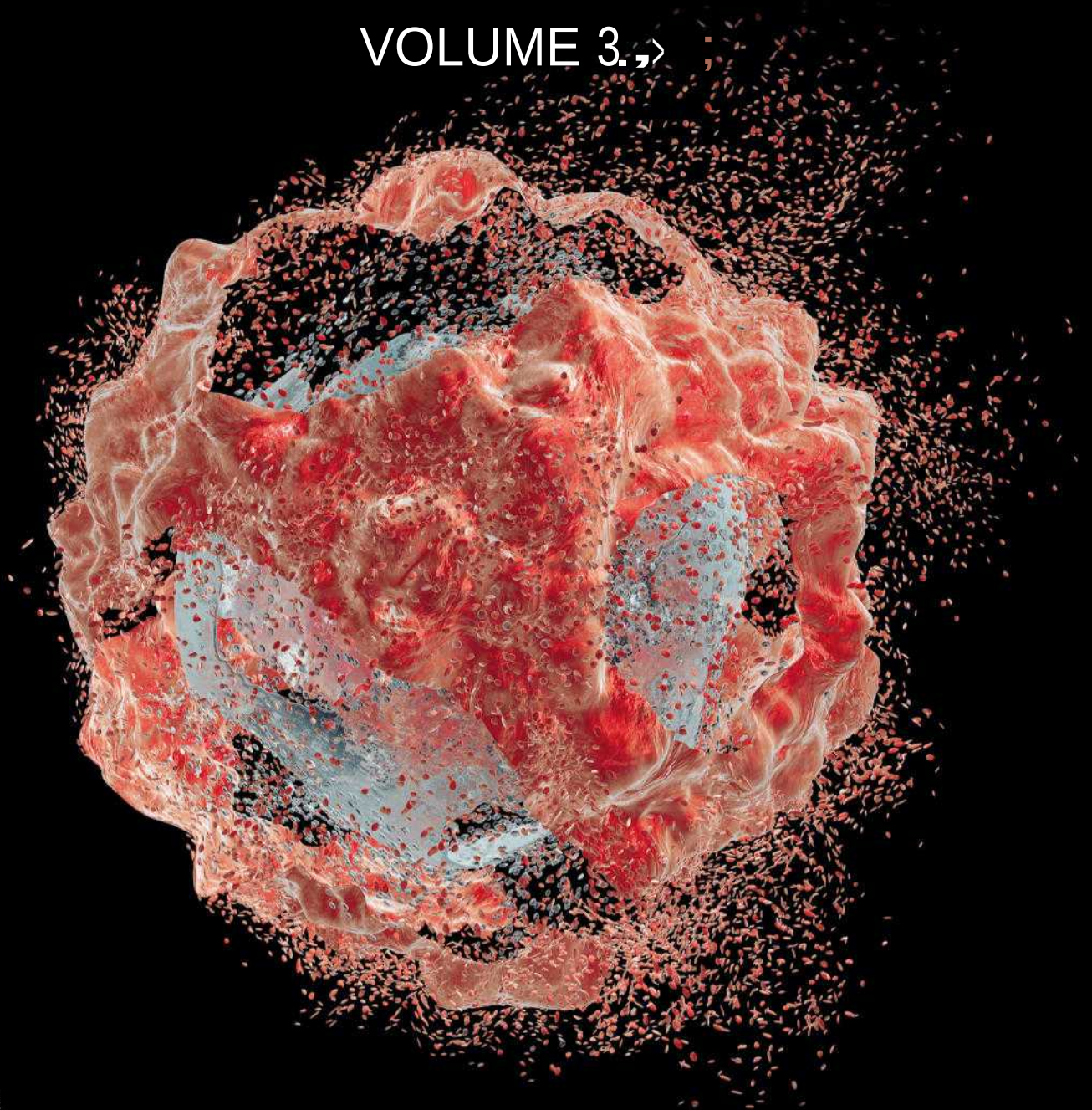


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FOREWORD

It is a great honor to be asked to introduce, with this foreword, the *Encyclopedia of Infection and Immunity*. This work will be read and enjoyed by many students, both graduate and postgraduate, including clinicians, laboratory practitioners, and scientists. Many colleagues have contributed to this important work, with over 230 original chapters assembled in sections and listed alphabetically. I offer my warm congratulations to the editors of this book, for soliciting a wide body of expertise and assembling it in a form that gives so much pleasure and information to the reader.

For those of us who work in the field of infection and immunity, the last two years have been exceptionally challenging. At the current time we are 22 months into a major pandemic caused by a novel coronavirus and its variants, and worldwide, sadly, there have been many hospitalizations and deaths. Clinicians and scientists in our field have been working flat out, caring for patients, generating or utilizing diagnostic tests, generating new knowledge in immunity to coronaviruses, or discovering, testing, and deploying new vaccines. Public Health practitioners have organized our medico-social responses to limit transmission and deal with the consequences of infection. Behavioral scientists have transformed our approach to informing the public, getting the best possible outcomes from effective communication. Governments have learned the danger of ignoring the expertise of scientists, and in many countries, the public look upon clinicians and scientists with gratitude and respect. It is a testament to the advancement of infection and immunity science that the genome of the virus was sequenced within a few weeks of COVID-19 emerging, and that within 12 months, the first trials of novel vaccines had been completed. These vaccines included some with exceptionally novel technologies, including the RNA vaccines and the viral-vectored vaccines.

Underpinning the academic agility of students and practitioners in Infection and Immunity is the universal strength of sound education. Readers of this book will be seeking the knowledge they need for a comprehensive understanding of infectious disease and immunology. We work in a fascinating discipline. Our field is unique in that pathogenesis involves at least two sets of genomes—on the one hand the microbe, and on the other, the host. The microbial genome programs its microenvironment to enable acquisition of and residence in a niche for sufficient time to replicate and further disseminate to other hosts. The human genome has evolved to tolerate and contain a wide range of symbionts and pathobionts, with highly sophisticated immune decision-making at the cellular level, but to respond to invading species with an elaborate coordinated repertoire of innate and acquired immunity. The complex conversation between many different microbes and the human host generates a wide range of disease phenotypes. This makes the job of an infectious disease physician so challenging but at the same time so fulfilling.

Our field is enriched by the many scientists and clinicians who are willing to share their experience with their peers and students. This book reflects the great variety of knowledge that has emerged over centuries of inquiry and endeavor, and I believe you will be rewarded by using it as one of your reference sources.

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PREFACE

Although infections have been one of the most important health threats since centuries ago, the mechanisms of human defense against infections have been unclear until last century. The scientific world has remarkable progresses in the field of immunology during recent decades. Indeed, the novel discovery of genes related to different pathogens has enhanced our knowledge about the immune system. Understanding different pathogens and the immune mechanisms promote diagnostic strategies and design more efficient therapeutic agents.

Encyclopedia of Infection and Immunity is a comprehensive text, which covers topics not only on basic immunology and microbiology but also on clinical immunology and infectious diseases. Section 1 focuses on the immune system, while Section 2 describes the interaction between pathogens and immunity. Bacteria, viruses, fungi, protozoan parasites and helminths, and insect and mites are explained in Sections 3–6, respectively. Section 7 discusses the clinical infectious disease challenges, whereas clinical immunology is covered in Section 8. Laboratory tests and treatment protocols are the main focuses of Sections 9 and 10, respectively.

Encyclopedia of Infection and Immunity is the result of valuable contribution of more than 400 scientists from many well-known universities/institutes worldwide. I would like to hereby acknowledge the expertise of all contributors, for generously devoting their time and considerable effort in preparing their respective chapters. I would also like to express my gratitude to Elsevier for providing us the opportunity to publish this Encyclopedia.

Finally, I hope that this comprehensive book will be of special value for researchers and clinicians who wish to extend their knowledge on infection and immunity.

Nima Rezaei, MD, PhD

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Respiratory Tract Infections: Bacteria

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Pharyngitis

Pharyngitis is a recurrent infection, both in children and adults. Most cases are caused by a virus but some of them can be of bacterial etiology. Group A β -hemolytic *Streptococcus* (GAS), known as *Streptococcus pyogenes*, is the mayor commonly identified bacterial cause of acute pharyngitis. GAS is estimated to be the cause of the United States 15–30% pediatric pharyngitis cases. Other less frequent bacterial pharyngitis agents include groups C and G β -hemolytic streptococci, *Fusobacterium necrophorum*, *Neisseria gonorrhoeae*, and *Corynebacterium diphtheriae*, among others (Arnold and Nizet, 2018).

GAS is a prominent human pathogen, that leads to more than 600 million cases of throat infections per year mainly pharyngitis, tonsillitis and on the skin, it causes non-bullous impetigo (Carapetis et al., 2005; Hurst et al., 2018). Invasive disease is an infrequent consequence of the infection, through which the organism reaches the bloodstream and can get access to deeper tissues, with high impact in terms of morbidity and mortality. GAS pharyngitis is most common in temperate climates and may peak in the winter due to the confinement of the favoring transmission through the respiratory route (Bessen et al., 2015).

The determination of different groups of GAS strains has been possible using the serology-based Lancefield classification scheme, based on the antiphagocytic M protein surface fibrils (Lancefield, 1959). The cell wall-associated M protein is attached to the peptidoglycan of the bacterial cell wall and encoded by the *emm* gene. Protective immunity to GAS is M type-specific. Recently, a sequence-based *emm* typing scheme was carried out, based on extensive nucleotide sequence differences at the 5' end of the *emm* gene. Currently, more than one hundred GAS strains types has been identified based on M and *emm* type. M protein is antiphagocytic, proinflammatory, and facilitates to mucosal adhesion. Its exerts its antiphagocytic effect by binding to complement control factors and other host proteins interfering with activation of the alternative complement pathway (Hurst et al., 2018).

The biological significance of *emm* pattern genotypes is that they are strong markers for preferred tissue sites of infection among GAS strains. The *emm* pattern A–C genotype indicates a statistically significant association with pharyngitis, while on the contrary *emm* pattern D isolates show a statistically significant association with impetigo (Bessen et al., 2015). Besides M protein, GAS strains express many virulence factors including, streptolysins, streptokinase, hyaluronidase, peptidoglycan, teichoic acid. Nucleases (DNase), but only antibodies to streptolysin O and S (ASO titers) are regularly measured in clinical practice (Hurst et al., 2018).

Clinical manifestations

GAS pharyngitis is transmitted via respiratory droplets after a short incubation period. The symptoms include fever and sore throat, erythematous pharynx, cervical adenopathy and, enlarged lymph nodes in the neck. The infection can also lead to the development of other manifestations and complications, such as Scarlet fever caused by the production of an erythrogenic toxin. It occurs when in addition to pharyngitis and tonsillitis a generalized erythematous rash occurs over the upper trunk and spreads to the extremities. Afterward, the rash begins to fade followed by fine desquamation, first on the face, and then progressing downward. The erythematous eruption produces bright red discoloration and then leads to desquamation of the tongue and later skin (Arnold and Nizet, 2018).

The different M types of GAS infection can trigger serious post infectious immune-mediated disorders, including post-streptococcal reactive arthritis, glomerulonephritis, and acute rheumatic fever. Additionally, molecular mimicry of M proteins can cause autoreactivity with heart, synovium, and brain tissue, causing damage to these tissues. Rheumatic fever is a classical autoimmune syndrome that can follow an episode of pharyngitis. It presents fever, polyarthritis, and carditis, with long-term cardiac damage and which is a notable cause of morbidity and mortality. Post-streptococcal reactive arthritis is more common than rheumatic fever and presents sooner (on average 10 versus 21 days) (Peters et al., 2017). The antigenic similarity of M protein's to the glomerular basement membrane causes glomerulonephritis. It results in the formation of an immune complex with complement activation and acute nephritis. Generally, this often happens in children within 2 weeks after skin infection, but it can occur early after the pharyngitis infection. The diagnosis is clinical, and patients usually exhibit haematuria and proteinuria (Peters et al., 2017).

Etiological diagnosis

The diagnosis of GAS pharyngitis is based on clinical signs and symptoms. However, to avoid unnecessary treatment with antibiotics in cases of viral infections, some guidelines indicate that confirmation of GAS, with either a throat culture or a rapid antigen detection test (RADT), is necessary. A throat culture is a standard for the diagnosis of GAS pharyngitis with a sensitivity of 90–95% and the disadvantage that results may take up to 48 or 72 h. Currently available RADTs have specificities of 95% with sensitivity between 70% and 90% (Ann Maes, 2011).

Treatment

There are alternative drugs for GAS infection because the pathogen is sensitive to the penicillin group. Otherwise, in patients with penicillin allergy, macrolides are frequently used for superficial infections. The optimization of the treatment in invasive disease cases is frequently a combination therapy using a penicillin group as well as a ribosome-acting agent to reduce toxin production (e.g. clindamycin, linezolid). Currently, a 10 days therapy is recommended for most syndromes of infection caused by GAS as pharyngitis, although long-acting macrolides (azithromycin) are administered for 5 days. Prophylaxis can be used in outbreak settings and is also administered to mothers and babies in the neonatal period if they are exposed to a person infected with invasive GAS (Peters et al., 2017).

Sinusitis

Acute sinusitis is an inflammation of the nasal mucosa and sinuses. Distinguishing between the common cold and sinusitis is difficult, and the distinction is often made according to symptom duration. Typical common colds are self-limited and last 7–10 days, whereas acute sinusitis has a duration of up to 4 weeks. Sinusitis can be caused by bacteria or viruses, and identification is one of the most important determinants of treatment. Bacterial causes of sinusitis include *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* (Grief, 2013).

Clinical manifestations

Symptoms of acute sinusitis are similar to the common cold, including nasal congestion with discharge, facial pain over the sinuses, decreased sense of smell, and cough. A bacterial origin is generally suggested if the patient shows purulent nasal discharge, maxillary tooth or facial pain, fever, and if symptoms worsen after initial improvement.

Etiological diagnosis

Acute bacterial sinusitis can be clinically diagnosed if purulent nasal discharge, nasal obstruction, focal facial pain, headache, and fever are present and if symptoms persist beyond 7–10 days. Nasal endoscopy and culture of secretions are good tests, but unfortunately, they are complex techniques that should be performed by a specialist. Thus, they are only used for the diagnosis of chronic sinusitis (Grief, 2013).

Treatment

General treatment for sinusitis includes maintaining adequate hydration, applying warm facial packs to help with pain relief, eliminating environmental contaminant factors, sleeping with the head of the bed elevated, and avoiding extremely cold or dry air. In some cases, the use of intranasal steroids, analgesics, and saline nasal irrigation could be prescribed.

The type of antibiotic that should be used depends on the bacteria. Amoxicillin-clavulanate is the preferred empiric antimicrobial therapy. Other antibiotics that could be used include doxycycline or combination therapy with a third-generation oral cephalosporin (cefixime or cefpodoxime) plus clindamycin. Levofloxacin could be prescribed for children older than 8 years with a history of hypersensitivity to penicillin (Grief, 2013).

Epiglottitis

Infectious epiglottitis is an acute inflammation of the epiglottis or supraglottis and can lead to airway obstruction. In almost all cases, it is caused by the bacteria *Haemophilus influenzae* type b (Hib). Nowadays, this infection has become very rare in most countries. This is due to the universal vaccination against Hib, which majorly reduces the incidence of epiglottitis. However, epiglottitis continues to remain potentially life-threatening, and the security of the airway and antibiotic treatment should be established early to ensure the life of the patient (Pham et al., 2017).

Physiopathology

Hib colonizes the pharynx of healthy people, and mainly children, through respiratory transmission from close contact. Then Hib can penetrate the mucosal barrier and invade the bloodstream, causing bacteraemia. After this Hib can cause epiglottitis, sepsis, pneumonia, meningitis, and many other infections. Epiglottitis infection leads to acute onset of inflammatory edema that significantly reduces airway aperture. Edema rapidly progresses thus involving neighboring areas.

Other species that may cause epiglottitis are other *Haemophilus* strains, some *Streptococcus* strains, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Pasteurella multocida* (Pham et al., 2017).

Clinical manifestations

In addition to edema of the epiglottis, patients exhibit stridor, dyspnoea, fever, and sore throat, among other symptoms. Acute epiglottitis may progress rapidly into life-threatening upper airway obstruction, mainly in children. In adults, symptoms tend to have a slower onset. Airway obstruction may occur by progressive cellulitis of the supraglottic area (Pham et al., 2017).

Diagnosis

Diagnosis of epiglottitis is not difficult because patients present a swollen and red epiglottis. Visualization of the posterior pharynx is the best way to confirm the diagnosis of epiglottitis. After securing airway blood cultures and epiglottic cultures, radiographs of the neck should be obtained, which can show the mass shadow resulting from the edema. Occasionally, bacterial epiglottitis could be misdiagnosed as laryngotracheobronchitis (croup). This occurs mainly in children and at the beginning of the illness; however, some symptoms and signs are common in both cases. Signs of upper airway obstruction such as drooling without coughing can help differentiate epiglottitis from croup (Pham et al., 2017).

Treatment

It is very important to secure the airways because intubation of the patient could be necessary in severe cases. Intubation is needed for less than 24 h in most cases. Most children can be extubated after 24 h of antibiotic therapy. Hospital treatment can last up to 3 days. Antibiotic treatment mainly includes cephalosporins like cefuroxime, ceftriaxone, and cefotaxime.

Some physicians administrate steroids to decrease the edema. It is important that close contacts (family, etc.) receive a prophylaxis treatment with an adequate antibiotic to avoid secondary infection (Pham et al., 2017).

Acute laryngitis

Acute laryngitis is included by many authors in the group of laryngo trachea bronchitis and it is known by the common term of croup. Croup is an inflammatory infection affecting the larynx, trachea, and bronchi.

Clinical manifestations

Croup frequently begins with a sore throat, along with fever, rhinorrhea and cough. This is followed by the appearance of characteristic symptoms such as hoarseness and non-productive cough which ends with inspiratory stridor (Grief, 2013). A radiograph of the neck can show subglottic obstruction.

Diagnosis

Croup is mainly caused by a virus but some bacterial species can also be responsible for the infection, such *Haemophilus influenzae* type B, *Mycoplasma pneumoniae*, *Corynebacterium diphtheriae*, *Moraxella catarrhalis*, and group A β -hemolytic *Streptococcus* (Caserta, 2015; Grief, 2013). The degree of severity of croup is evaluated by the Westley Croup score. This score includes the evaluation of stridor, chest wall retractions, air entry, cyanosis and level of consciousness (Bourayou et al., 2010; Johnson, 2009; Zafar et al., 2017).

Microbiologic diagnosis can be established through bacterial cultures from the pharynx or in some cases by rapid antigen detection. Radiographs of the lateral neck can be necessary to make a differential diagnosis of epiglottitis. Croup shows subglottic narrowing that is wider on expiration than on inspiration and a normal epiglottis, whereas epiglottitis shows a swollen epiglottis.

Treatment

There are various types of treatment depending on the severity of croup but in mild cases, the use of oral corticoids could be enough. However, in severe cases the use of nebulized epinephrine and dexamethasone is necessary and some cases require oxygen administration or intubation (Grief, 2013; Pham et al., 2017).

Given that almost all croup cases are caused by a virus there is no recommended empirical antibiotic therapy and so antibiotic treatment can vary depending on the bacterial agent.

Whooping cough

Whooping cough is an infection of the respiratory mucosa caused by infection with *Bordetella pertussis* or related members of the genus. The infection is highly contagious and is characterized by a paroxysmal cough.

Physiopathology

The initial step of the infection is the colonization of the ciliated epithelium of the bronchi and trachea. Then, the bacteria, produces a cytotoxin that paralyzes the cilia and leads to paroxysms of coughing as an alternative means of removing the increased mucus (Preston, 2012).

Clinical manifestations

The infection has several phases, starting with an incubation period of 1–3 weeks, which is followed by a catarrhal phase with a simple cough, rhinorrhea, increased lacrimation, conjunctival injection, and sometimes a low-grade fever.

After some days and up until a week, the catarrhal phase gives way to the paroxysmal phase, characterized by a paroxysmal cough. This cough consists of a series of uncontrollable expirations, followed by gasping inhalation that is responsible for the whooping sound. Each episode of coughing can be associated with cyanosis and vomiting. Between episodes the patient shows minor symptoms and usually does not appears to be ill. This phase can be extended for 4 weeks (Hewlett, 2012). The last phase is the convalescent phase where the patient presents a chronic cough, which can last from weeks to months (Hewlett, 2012).

Diagnosis

Owing to the characteristics of the cough attack, the diagnosis of whooping cough is usually made clinically. However, in cases of atypical or mild illness, a laboratory diagnosis could be necessary. In these cases, bacterial culture is the best choice and it has the highest specificity; bacteria can be serotyped and antibiotic susceptibility can be determined. Furthermore, *Bordetella* antigens may be detected in serum and urine in tests with specific antiserum. Further, polymerase chain reaction (PCR) is also available (Preston, 2012).

Treatment and prevention

The recommended antibiotic is a macrolide type, with the most common one being azithromycin. Potential alternatives are clarithromycin or erythromycin. Treatment reduces the severity of the illness if given before the paroxysmal stage. Moreover, it is important to eliminate the bacteria to reduce the exposure of contacts. This is a highly contagious illness so macrolide may also be given as a form of chemoprophylaxis for exposed individuals.

The best type of prevention is vaccination. A killed, whole cell vaccine and acellular pertussis vaccine, are available and are administered to infants and children in most countries (Hewlett, 2012; Preston, 2012).

Acute pneumonia

Pneumonia is a common inflammatory disease of infectious etiology which affects the pulmonary parenchyma. It can be caused by bacteria, viruses, fungi, or parasites. Pneumococcal infection is the leading etiology responsible for the incidence and mortality of lower respiratory infections among children and adults. *Streptococcus pneumoniae* are encapsulated, nonsporulating, facultative anaerobic, lancet-shaped gram-positive bacteria that reside in the human nasopharynx (Morais et al., 2019; Troeger et al., 2018). Several factors such as age, associated diseases, and the context in which the infection is acquired (community, residences, hospital), condition the frequency with which the various etiological agents cause pneumonia. Likewise, these factors influence the clinical symptomatology, radiological pattern, treatment selection, evolution, complications, and prognosis of the disease. Pneumonia is characterized by fever, variable respiratory symptomatology and the appearance of infiltrates in radiology. Therefore this entity involves clinical, radiological and evolutionary diagnosis (Torres and Rodriguez de Castro, 2020).

In the extreme ages of life, the incidence of pneumonia is higher than that in the rest of the population and it is in these patients that the consequences are more serious. Pneumonia represents a relevant problem in public health, both in asocial and economic context, which include high morbidity and mortality, high hospitalization rates, prolonged hospital stay, and high costs. In recent decades and considering the difficulty to establish the etiology of the disease in most cases, classifications have been used based on clinical characteristics and the affected population. According to the guidelines proposed by the American Thorax Association, three type of pneumonia can be established (Mandell et al., 2007; Metlay and Fine, 2003). The first type is community-acquired pneumonia (CAP), which is infection of the lung parenchyma produced by microorganisms acquired outside the hospital environment. As the reporting of CAP is not mandatory and only 20–50% of patients are hospitalized, its incidence is difficult to establish. CAP has a broad clinical presentation and causal agents. It is also a frequent cause of consultation in the emergency department, which implies that it causes significant burden on health care systems and their resources. Affected populations need to be differentiated according to age (children and adults over 65 years), comorbidities such as congestive heart failure (CHF) and chronic obstructive pulmonary disease (COPD), and disease-modifying factors. Such conditions are known to increase the risk of specific infections (*S. pneumoniae* resistant to penicillin, gram-negative bacilli, *pseudomonas*). The second type is acute in-hospital pneumonia acquired by hospital-admitted patients after 72 h or those that after nosocomial discharge exhibit symptoms within 7 days. The third type occurs in immune-suppressed patients, particularly those with AIDS and those undergoing chemotherapy or other immunosuppressive treatment. In this case, etiological agents could be different of the other two groups.

Diagnosis

In 2019, the American Thorax Society and The National Institute for Health and Care Excellence (NICE) updated the guidelines for the management of CAP, including the assessment of severity and mortality risk. Scores like pneumonia severity index (PSI), CURB65, and SMART-COP have been described to determine the severity of CAP (Metlay et al., 2019; National Institute of Health and Care Excellence (NICE), 2014; Metlay et al., 1997). The most frequently present symptoms of CAP are fever (80%), polypnea (45–70%), chest pain (30%), cough, dyspnoea, expectoration, fever, gastrointestinal symptoms, tachycardia, and pleuropulmonary findings. Given the acute nature of these symptoms, patients usually seek medical help within the first 5 days of the onset of symptoms (Metlay et al., 2019; National Institute of Health and Care Excellence (NICE), 2014).

PSI score assign 5 types of risk classes (Fine et al., 1997). Patients with less than 50 years of age and without any of the health coexisting conditions and abnormalities on physical examination showed in Table 1 are assigned to risk class I. The other risk classes are assigned according Tables 1 and 2. The risk of mortality and the treatment recommendations are shown in Table 2.

Another useful severity index is the CURB 65 score (Lim et al., 2003). This score assigns 1 point for each adverse characteristic showed in Table 3. The risk of mortality and the treatment recommendations are shown in Table 4.

Table 1 PSI score calculation.

Age		Health coexisting conditions		Health status	
Age men	Age (years)	Nursing home resident	+10	Altered mental status (disorientation)	+20
Age women	Age (years)—10	Neoplastic disease	+30	Respiratory rate >30/min	+20
		Liver disease	+20	Systolic blood pressure <90 mmHg	+20
		Congestive heart failure	+10	Temperature <35 °C or >40 °C	+15
		Cerebrovascular disease	+10	Pulse >125/min	+10
		Renal disease	+10	Arterial pH <7.35	+30
				Blood urea nitrogen >30 mg/dL (11 mmol/L)	+20
				Sodium <130 mmol/l	+20
				Glucose >250 mg/dL (14 mmol/L)	+10
				Hematocrit <30%	+10
			Partial pressure of arterial oxygen <60 mmHg	+10	
Total age score		Total health coexisting conditions		Total health status	
Total score = Total age score + Total health coexisting condition + Total health status					

Data were obtained from Fine MJ, Auble TE, Yealy DM, Hanusa BH, Weissfeld LA, Singer DE, Coley CM, Marrie TJ, and Kapoor WN (1997) A prediction rule to identify low-risk patients with community-acquired pneumonia. *New England Journal of Medicine*. <https://doi.org/10.1056/NEJM199701233360402>.

Table 2 Risk class assignment according to PSI score.

Risk class	Score	Risk of mortality %	Recommendations
I		0.1	Outpatient treatment or an abbreviated course of inpatient care
II	<70	0.6	
III	71–90	2.8	
IV	91–130	8.2	Inpatient care
V	>130	29.2	

Data were obtained from Fine MJ, Auble TE, Yealy DM, Hanusa BH, Weissfeld LA, Singer DE, Coley CM, Marrie TJ, and Kapoor WN (1997) A prediction rule to identify low-risk patients with community-acquired pneumonia. *New England Journal of Medicine*. <https://doi.org/10.1056/NEJM199701233360402>.

Table 3 CURB 65 score adverse features.

C	Confusion
U	Urea >7 mmol/L
R	Respiratory rate >30/min
B	Low blood pressure (systolic <90 mmHg or diastolic <60 mmHg)
65	Age >65

Data were obtained from Lim WS, van der Eerden MM, Laing R, Boersma WG, Karalus N, Town GI, Lewis SA, and Macfarlane JT (2003) Defining community acquired pneumonia severity on presentation to hospital: An international derivation and validation study. *Thorax* 58: 377–382.

Table 4 CURB 65 score.

Number of adverse prognostic features	Group assigned	Risk of mortality %	Recommendations
0	1	Low 1.5	Likely suitable for home treatment
1			
2	2	Intermediate 9.2	Consider hospital supervised treatment
3	3	High 22	Manage in hospital as severe pneumonia
4			
5			

Data were obtained from Lim WS, van der Eerden MM, Laing R, Boersma WG, Karalus N, Town GI, Lewis SA, and Macfarlane JT (2003) Defining community acquired pneumonia severity on presentation to hospital: An international derivation and validation study. *Thorax* 58: 377–382.

In clinical research studies, the presence of new pulmonary infiltrates in the radiological chest pair (RxTx) is used as a gold standard for the diagnosis of CAP, together with the presence of already analyzed suggestive signs and symptoms. Although the sensitivity and specificity of RxTx varies from 30% to 70%, current guidelines indicate that it has a good performance in patients with suspected pneumonia (Mandell et al., 2007; Torres and Rodriguez de Castro, 2020).

Etiology

The distribution and frequency of agents responsible for CAP are very diverse and depend on where the study is conducted and the diagnostic methodology used. Despite this, most studies have identified *S. pneumoniae* as the main cause of CAP, often followed by *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Legionella pneumophila*, *Chlamydia pneumoniae*, *Moraxella catarrhalis*, and influenza virus A. More than 90% of CAPs are produced by these agents. Respiratory viruses are the most important etiological agents during the first years of life (Cilloniz et al., 2016). *Mycoplasma pneumoniae* has the predominant etiological role in the pneumonia developed in school-age children and adolescents. *H. influenzae* type b has also been responsible for bacterial pneumonia in children. The incidence of this microorganism has decreased since the inclusion of the vaccine against it in childhood vaccination schedules (Cilloniz et al., 2016). *Staphylococcus aureus* is responsible for between 2% and 5% of cases, with particular significance among the elderly, where it can become a rare complication of the flu. The incidence of community methicillin-resistant *Staphylococcus aureus* continues to remain low but still represents a serious problem owing to its aggressive pathogenesis. More specifically, the presence of the Panton-Valentine leukocidin carrier causes serious necrotizing pneumonia even in healthy people without underlying diseases. Gram-negative bacilli are responsible for 5–10% of cases and are particularly important agents among residents of nursing homes and alcoholics (Cilloniz et al., 2016).

Pathogenesis

In absence of disease, pulmonary defense mechanisms maintain sterile infraglottic airways, except in smokers and chronic bronchitis patients, whose infraglottic airway is usually colonized by oropharyngeal flora. Through micro aspirations or aerosolized material, such as that produced by a sneeze, microorganisms enter the pulmonary parenchyma following the canalicular pathway. The presence of ciliated cylindrical epithelium and mucus-producing cells along the airway forms a defensive barrier that microorganisms must overcome to reach the pulmonary parenchyma. Other defense mechanisms are the saliva level, pH, local production of complement and secretory IgA, and macrophages that act as phagocytic cells.

Microorganisms have adhesion mechanisms such as hemagglutinins in influenza viruses and pili, exotoxins, and proteolytic enzymes in bacteria. When the pathogens overcome these anatomical, mechanical, and physiological barriers, they are then able to reach the lung tissue, and activate immune cells producing inflammatory response mediators that attract neutrophils to the site of infection. The presence of specific IgGs stimulates the activation of the classical complement pathway, fundamental in the antibacterial response. Cell-mediated immunity plays a fundamental role in intracellular infections (viruses, mycoplasmas, chlamydias, *Legionella*, and *Mycobacterium*) (Blanquer et al., 2011).

There are some factors that can interfere with defense mechanisms and must be considered to understand the pathogenesis of this infection. For example, changes in the level of wakefulness (alcoholic intoxication, central nervous system depressant drugs, stroke, etc.) may compromise epiglottic closure and produce aspiration of the content of the oropharynx. Furthermore, cigarette smoke can also disrupt mucociliary function and macrophage activity. The high viral load respiratory infections destroy the respiratory epithelium and alter the chemotaxis of neutrophils. Alcoholism, in addition to wakefulness disorders, decrease cough reflexes, which in turn predisposes the person to colonization by gram-negative bacilli, by decreasing neutrophil mobilization and secretion of IgA. Manipulations of the digestive and respiratory tract also alter the mechanisms of defense, e.g. orotracheal intubation, nasogastric tube placement, and fibroscopy. Others factors that alter local defense mechanisms are alveolar edema in CHF, hypoxemia, acidosis, malnutrition, uraemia, age (due to immaturity of the immune system in infants or old age in those over 65), alterations in the production of immunoglobulins (chronic myeloid leukemia, multiple myeloma, nephrotic syndrome, etc.), underlying respiratory system disorders (COPD, bronchiectasis, cystic fibrosis), and extrinsic compressions (adenopathies, bronchopulmonary tumors). In some cases, it is not possible to identify any of the above-mentioned predisposing factors, but it is important to keep in mind the role of virulence factors of different microorganisms in the host attack, highlighting in particular *S. pneumoniae* (Metlay et al., 2019).

Clinical manifestations

Classically, from a clinical point of view, there has been a differentiation between typical or usual bacterial and atypical pneumonia. Although currently the overall specificity of the signs and symptoms of the determined etiology have been put into doubt, it is still considered useful to maintain the mentioned subgroups for educational purposes. Typical pneumonia is usually caused by *S. pneumoniae*, *H. influenzae*, and gram-negative bacilli such as *S. aureus* (Torres and Rodriguez de Castro, 2020). The disease is characterized by its abrupt beginning, although it may be preceded by a catarrhal syndrome. Cold sores, a high fever (absent in the elderly) and an initial dry cough, which is then productive with mucopurulent, or rusty expectoration are also possible symptoms.

Such symptoms can be accompanied by side stitch pain and nasal flutter. During the clinical examination, patients present with a toxic appearance, polypnea, fever, sweatiness, and with signs of parenchymal condensation (decreased vibrations, dullness, crackling rales). Chest radiology shows homogeneous opacity with air bronchogram affecting one or several lung segments, being able to occupy an entire lobe.

Atypical pneumonia is produced by microorganisms such as *M. pneumoniae* or *C. pneumoniae*. It is characterized by an insidious onset with headache, asthenia, malaise, persistent dry cough with poor or no expectoration, fever, and thoracic pain that increases with a cough. Extra-respiratory symptoms such as nausea, vomiting, skin rash, arthromyalgia, rhinorrhea, dysphonia, and odynophagia are also frequent. Pulmonary auscultation reveals the presence of fine crackles, although this may be normal. An interstitial pattern with opacity of the reticulonodular type is observed on the radiograph. The lower lobes are the most frequently affected.

Pneumonia among the elderly is also of particular interest as it is three to five times more frequent and leads to a higher mortality (close to 30%). Indeed, it is the first cause of infectious mortality in this age group. The most frequently involved agents are *S. pneumoniae*, *H. influenzae*, *K. pneumoniae*, *S. aureus*, and *influenza virus A* and *B*. In recent years, the respiratory syncytial virus (RSV) was recognized as an agent of increasing importance in this age group. The clinical presentation of pneumonia among the elderly shows a high degree of unspecific symptoms. In some cases, unexplained tachycardia and disorders in the level of wakefulness without fever may also be present. In terms of radiology, a dimmer opacity makes the diagnosis difficult. Further, healing and radiological resolutions are slower among elderly patients, and the prognosis depends on the delay in the initiation of the treatment, the etiology and the presence of comorbidities.

Etiological diagnosis

Microbiological studies allow us to identify the causative agent of pneumonia and to determine its sensitivity and resistance to antimicrobials. Identifying the responsible microorganism allows us to provide a targeted treatment with greater therapeutic possibilities, thus reducing the antimicrobial spectrum, antibiotic resistance and costs. However, microbiological studies are not recommended for all patients with CAP. The most commonly performed tests are the cultivation of respiratory secretions, either obtained by spontaneous expectoration or through endoscopic, blood culture, serological tests and molecular biology diagnostic tests. The culture of tracheobronchial secretions can be done through non-invasive (sputum) or invasive (bronchio-alveolar lavage) methods. Laboratory processing and clinical interpretation of the results differs for each type of sample (Blanquer et al., 2011; Miller et al., 2018).

Sputum culture

Sputum culture has variable sensitivity and specificity and does not contribute significantly to the initial management of the patient. This method has limitations that condition its performance, such as the inability to obtain adequate samples in more than one-third of the patients and reduction in its sensitivity with the previous use of antibiotics. Samples must be representative of the lower respiratory tract and meet Murray's criteria: more than 25 polymorphonuclear and less than 10 epithelial cells (Blanquer et al., 2011; Miller et al., 2018).

All patients with moderate to severe CAP will be requested to be able to expectorate. In patients with severe CAP or therapeutic failure, it is recommended to extract the culture of sputum or another sample from the lower respiratory tract. Samples should arrive at the laboratory less than 2 h after extraction or stored in a refrigerator at -4°C for up to 24 h. If these measures cannot be secured, the study should not be conducted. The samples obtained by endoscopy are more representative of the parenchymal focus as they overcome the possible oropharyngeal contamination. It is a non-morbidity-free invasive method that should not be applied routinely. Thus, its performance is justified in a certain group of patients, such as immunocompromised patients, those admitted to the intensive care unit (ICU), and those with torpid evolution, among others. The universally used sample is, however, bronchio-alveolar lavage (BAL).

Blood culture

Blood cultures in general, do not modify the beginning of the empirical therapy and will be requested in cases such as severe CAP, presence of pleural effusion or cavity, leukopenia, alcoholism, chronic liver disease, anatomical or functional asplenia, or pneumococcal antigen in urine (Mandell et al., 2007; Woodhead et al., 2011). Blood culture should be preferably performed prior to the start of antimicrobial treatment. Although the objective of blood cultures is to achieve a microbiological diagnosis, they are positive in 7–16% of cases and have a high rate of false positives. *S. pneumoniae* is the most frequent microorganism found in blood cultures. Aerobic culture in blood agar with CO_2 atmosphere allows recovery of aerobic bacteria such as *S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis*, *S. aureus*, *K. pneumoniae* and *P. aeruginosa*. If Gram staining does not show a predominance of a bacterial type, but an abundant number of inflammatory cells is observed, the presence of fastidious bacteria or viral agents must be suspected. Some microbiology laboratories have designed expanded culture protocols when this situation occurs which includes viral cultures. Antimicrobial susceptibility studies are carried out on the bacterial species identified in the culture.

Urinary antigens

The urinary antigen test for *S. pneumoniae* is a simple, accessible, and well-established test in current clinical practice. It has a sensitivity of 74% and specificity of 97.2% (Lacoma et al., 2012; Sinclair et al., 2013). The test may remain positive after beginning antibiotic treatment, so it is not recommended for patients who have suffered from CAP in the month prior to the test. All patients with moderate-severe CAP will be requested to do such a test. Urinary antigen for *Legionella pneumophila* is useful to identify *Legionella pneumophila* group 1, which is responsible for 80% of legionellosis pneumonia. The test has a sensitivity of 70–90% and specificity close to 99% (Lacoma et al., 2012). The antigen is positive from day 1 of the disease, which continues for weeks. The test is recommended for the following situations: (1) very severe CAP entering the ICU, (2) CAP that does not respond to treatment with β -lactams, (3) patients with risk factors e.g. trip to endemic areas in the 10 days prior, (4) notion of use of heating or air conditioning, (5) certain occupations such as plumbing system repair, and (6) immunocompromised patients. The etiological diagnosis is validated using the PCR method. In some hospitals there is a multiple PCR team for etiological diagnosis. This test is available for patients with severe CAP who require admission to the ICU and for immune-compromised patients (Herrera et al., 2016).

In addition, there are serological tests to detect serum antibodies (IgG and IgM) against *M. pneumoniae*, *L. pneumophila*, *C. pneumoniae* and *C. psittaci*, being especially useful in the latter due its high sensitivity and specificity. In general, it is necessary to obtain two samples in the acute phase and in the convalescence phase, with an interval of 4–6 weeks, to determine seroconversion (Almirall et al., 2004; Herrera et al., 2016). These samples will be obtained from patients entering the ICU and patients with epidemiological suspicion. In addition, in cases of pleural effusion, a study of pleural fluid will be requested and a diagnostic thoracentesis will always be performed except when there are contraindications with Gram staining culture and pneumococcal antigen (Woodhead et al., 2011).

Treatment

Antimicrobial treatment (AT) in CAP is essential for its adequate resolution. Its early indication reduces complications, recovery times, morbidity and mortality (Mandell et al., 2007; Metlay et al., 2019; National Institute of Health and Care Excellence (NICE), 2014). There are currently multiple international guides, which have been a reference in the selection of treatment. One of them is the IDSA guides of the British Thoracic Society, SEPAR. However, the trend in current clinical practice is to achieve a therapeutic consensus that reflects local reality and available resources. The empirical AT selected must meet certain conditions such as to have demonstrated activity against the most frequent microorganisms that cause CAP, to have considered the patient's risk factors and comorbidities, and to have been evaluated over time, thus allowing changes related to the epidemiological evolution itself. The onset of antibiotic treatment cannot be conditioned to the isolation of the microorganism. The published series shows that the etiologic agents are usually the same, and the antibiotic sensitivity pattern must be hierarchized. This particularity makes the treatment of CAP fundamentally empirical. Therefore, there is currently no reason to defer the initiation of AT, which should be started as soon as possible. The active plan will always include an active antibiotic against *Streptococcus pneumoniae*, which is the most common etiological microorganism. The most used antibiotics are β -lactams, which are bactericides antibiotics. The risk factors that suggest a decreased sensitivity of *S. pneumoniae* are: age 65 or older, immunosuppression, having received β -lactams in the last 3 months, living in nursing home, poor socio-economic environment, and alcohol abuse. Aminopenicillins (ampicillin and amoxicillin) are equally active against sensitive and intermediately sensitive *S. pneumoniae*. For oral administration, amoxicillin is preferred as it is better absorbed. Many strains of *Staphylococcus* spp., *H. influenzae*, *K. pneumoniae*, *E. coli*, and *M. catarrhalis* (β lactamase producers) are currently resistant to aminopenicillins, so empirical use is not recommended when it is suspected that the infection is caused by these organisms. β lactamase inhibitors (clavulanic acid or sulbactam) recover aminopenicillin activity against most cited strains. Third-generation cephalosporins are the most active cephalosporins against gram-negative bacilli. Ceftriaxone and cefotaxime are the most active against gram-positive, with the exception of *Enterococcus* spp. and *Listeria monocytogenes*. Ceftazidime has antipseudomonal activity but is not very active against *S. aureus* and other microbes. Macrolides (erythromycin, clarithromycin, azithromycin), are indicated when there is suspicion of CAP for "atypical germs," because they do not reach sufficient serum concentrations. Macrolides should not be used if there is suspicion of bacteraemia, nor in patients with moderately severe or severe illness.

Tuberculosis

Tuberculosis (TB) is a re-emerging disease and also one of the most important infectious diseases worldwide. It is caused by the bacterium *Mycobacterium tuberculosis* which remains one of the most difficult pathogens to control. The annual World Health Organization (WHO) report shows that, despite advances in disease control, tuberculosis is still causing a large disease burden worldwide (Kyu et al., 2018).

In 2015, more than 10 million new or relapsed TB cases and more than a million TB-related deaths were reported. It has been estimated that a third of the global population carries a latent TB infection and that approximately 10% of these infections will reactivate and progress to infectious clinical TB in the person's lifetime, thus maintaining transmission in humans. Further, at least a third of human immunodeficiency virus (HIV)-related deaths is attributed to TB. Thus, efforts to prevent TB are advisable in countries with high rates of the disease (Kyu et al., 2018).

Physiopathology

TB transmission occurs via inhalation of droplets expelled by an individual with pulmonary or laryngeal TB coughs, sneezes, or speaks. Droplet nuclei can remain suspended in the air for hours. Moreover, only a subset of the bacteria-laden aerosol droplets of 5 µm diameter with 1–3 bacilli that are inhaled actually reach any alveolar sac. This is because larger size droplets (>5 µm) are trapped in the upper respiratory system by mucus and ciliary action. After being inhaled, bacteria are phagocytosed by alveolar macrophages. Transmission rarely occurs through direct contact with fomites. The risk of transmission is directly correlated with the closeness of contact and amount of time spent with a contagious case (Highsmith et al., 2019). In some cases, macrophages are unable to destroy the bacteria, which then grow inside the phagosome of infected macrophages. After that, a granuloma is generated in the focus of infection. In many cases, activation of the infected macrophages leads to destruction of the bacteria, which allows growth control. However, the presence of latent bacteria inside necrotic tissue can also lead to a latent infection. Some bacteria are then drained into the alveolar space, where they can reactivate. This type of latent tuberculosis infection does not generate symptoms and is not infectious. However, in 10% of cases, latent infection can turn into an active tuberculosis disease. Progress from tuberculosis infection to tuberculosis disease can occur early (primary form) or a period of time after infection (reactivation). The risk of reactivation increases with alterations of the immune system, such as immune deficiencies caused, for example, by HIV; drugs affecting the immune system and other pathologies. In primary or reactivation disease, the lungs are the most commonly affected organ. Nevertheless, the infection can sometimes spread during the primary phase of the infection and reach distant organ systems (Weinberger et al., 2019).

Clinical manifestations

The clinical manifestations of latent and active TB infection show important differences. Latent infection does not show any clinically apparent disease and the only mark of infection is a positive reaction to a skin tuberculin test for delayed hypersensitivity. Patients with latent infection are not contagious. Meanwhile, patients with active disease can show relatively nonspecific symptoms such as weight loss, anorexia, fatigue, low-grade fever, cough, and chest pain occasionally. Patients with disseminated tuberculosis can also show symptoms which result from a particular organ being affected. The most common regions affected by disseminated tuberculosis are: pleura, peritoneum, kidney, adrenal glands, bones, and central nervous system (Weinberger et al., 2019).

Diagnosis

The most commonly used diagnostic method for TB is the tuberculin skin test. This test shows whether the patient has been exposed to *M. tuberculosis*, independently of whether the disease is active, latent, or whether the patient has had a previous infection. For this test, a small amount of protein from bacteria is intradermally injected into the forearm. Individuals who have been infected with *M. tuberculosis* exhibit swelling at the site of injection after 48–72 h. The tuberculin skin test is easy to perform but has an important number of false negative and false positive results (Weinberger et al., 2019). For the diagnosis of active tuberculosis, the initial diagnostic tool is a chest radiograph. In the primary disease, the chest radiograph shows a nonspecific infiltrate and sometimes lymph node enlargement. After the primary disease heals, a radiograph can reveal small calcified lesions due to the collection of calcified granulomas.

A rigorous diagnosis of tuberculosis is done by culturing bacteria from either secretions or tissue. However, *M. tuberculosis* is a slow-growing bacterium, and approximately 6 weeks are required for sufficient growth time, identification and the establishment of antibiotic sensitivity. A more useful and faster diagnosis procedure is through staining of material obtained from the tracheobronchial tree (sputum, tracheobronchial washing or biopsy). Mycobacteria are characterized by the fact that they retain certain dyes, even after exposure to acid. The Ziehl-Neelsen, Kinyoun, or an auramine-rhodamine fluorescent stain can easily detect the presence of an acid-fast bacillus that is clinically significant in the majority of cases. Besides this, nucleic acid amplification assays (PCR) which can detect *M. tuberculosis* in respiratory specimens with good sensitivity and specificity have also been developed (Weinberger et al., 2019).

Treatment and prevention

Most cases of TB are curable with appropriate drug therapy. Patients must be treated with at least two drugs for a long period of time which is generally of 6 months. One of the most common therapy plans for TB includes isoniazid and rifampin for 6 months plus pyrazinamide for the first 2 months. Moreover, due to the presence of resistant strains, ethambutol is added at the initiation of therapy until the acquirement of the drug-resistant profile. When resistance to agents is documented by inhibitory assays, it is possible to elaborate a specific regimen and duration of therapy. Patient's whose sputum has become smear-negative on three successive samples are considered to no longer be infectious, which demonstrates a successful clinical response to therapy (Weinberger et al., 2019).

Due to the long treatment period, an important issue in TB therapy is the patient's adherence. An incomplete or intermittent therapy can lead to treatment failure and the emergence of resistant organisms. Further, due to hepatotoxicity problems which can arise as a result of the medication, patients should be monitored during therapy. Another important challenge in this field is the association between tuberculosis and HIV/AIDS. The drugs used for these diseases can interact, leading to an increase in the

probability of hepatotoxicity and other adverse reactions. In the case of patients with latent tuberculosis, treatment consists in the administration of isoniazid for 9 months, although other regimens are also used. The treatment of patients with latent tuberculosis decreases the probability of the activation of the infection (Weinberger et al., 2019).

The current vaccine for TB, *Mycobacterium bovis* bacilli Calmette-Guerin (BCG), is extensively used as part of the Expanded Program on Immunization. The vaccine has mitigated severe disseminated forms of tuberculosis in children, but shows mixed effectivity results in other age groups. Because BCG does not provide adequate protection against pulmonary disease, new vaccine candidates are being developed to control the disease. The goal of the vaccine candidates is to induce robust immune responses critical for controlling progression of *Mycobacterium tuberculosis* infection. Currently, there are 13 vaccines in clinical development, which can be divided into whole cell-derived vaccines or viral vectored subunit vaccines and adjuvanted protein subunit vaccines (Kaufmann et al., 2014). Recently, investigators reported some approaches to improve the efficacy and protection of BCG, such as development of recombinant subunit vaccines. Other options include an engineering BCG by inserting genes for *Mycobacterium tuberculosis* antigens as well as cytokines and bacterial toxin-derived adjuvants (Nieuwenhuizen and Kaufmann, 2018).

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Respiratory Tract Infections: Viruses

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Global impact of respiratory virus diseases

The reemergence of H1N1 in 2009 and the outbreak of SARS-CoV-2, a novel pandemic virus in 2019, have alerted the world to the fact that a virus can emerge and spread globally in a matter of days. Thousands of deaths and huge economic losses are the main consequences of a pandemic. The large reservoir of viruses that animals have and their potential to infect humans imply that the emergence of new respiratory pathogens is guaranteed. Furthermore, the efficient human-to-human transmission of respiratory viruses makes controlling them challenging (WHO, 2018). The respiratory viral infections are the most frequent reason for seeking medical care, which is reflected in the substantial number of hospital and emergency room visits by people from all ages groups (GBD 2017 DALYs and HALE Collaborators, 2018).

In general, respiratory viruses are transmitted directly by contact with infected respiratory droplets or by contact with contaminated fomites. The survival and infective capacity of viruses on surfaces, which can range from a few hours to days, depend on several factors such as porosity of the surfaces and the characteristics of the virus. Nonenveloped viruses are more stable in the environment than enveloped viruses and can probably cause infection through direct contact (Boone and Gerba, 2007).

Clinical outcomes

Respiratory viruses affect the upper respiratory tract and are responsible for diseases such as rhinosinusitis or the “common cold,” otitis media, sinusitis and laryngitis. Such viruses can also affect deeper structures below the larynx, causing bronchiolitis, bronchitis, acute pneumonia, exacerbation of asthma and chronic lung disorders (WHO, 2012, 2018a). Respiratory viruses increase the risk of secondary bacterial infections and also are known to have been associated in half of the pneumonia cases. They are also the main cause of cases of bronchiolitis and asthma exacerbations in infants. In adults, respiratory virus is majority implicated in asthma exacerbations, and COPD exacerbations (WHO, 2012). Although specific pathogens commonly cause characteristic clinical manifestations, there are significant similarities in the clinical symptoms associated with the different viruses causing respiratory illnesses (Table 1).

Influenza virus (IV)

Influenza viruses are the leading cause of seasonal flu epidemics that occur almost every winter and 5% to 10% of the human population. IV infection can lead to severe morbidity. It has a higher mortality rate among elderly patients, small children, and individuals with chronic respiratory disease or immune compromised condition (WHO, 2018b).

Influenza viruses belong to the Orthomyxoviridae family. They are enveloped negative-sense single-stranded segmented RNA viruses. The family comprises five genera, but only influenza A and B, which can cause seasonal and occasionally epidemic flu, are of medical importance in humans. Influenza A (IAV) are the only influenza viruses known to have pandemic potential (WHO, 2018b). There are different subtypes of IAV, based on the antigenic properties of hemagglutinin (HA) and neuraminidase (NA) that are two major viral antigens glycoproteins on the surface of the virus. In turn, there are 18 different hemagglutinin subtypes and 11 different neuraminidase subtypes (H1 through H18 and N1 through N11, respectively). Influenza A virion may display any combination of the two proteins on the surface (Centers for Disease Control and Prevention, 2017). A specific characteristic of IAV is

Table 1 Diseases and disorders commonly associated with respiratory viral pathogens.

<i>Clinical disease or disorder</i>	<i>Main causes agents</i>	<i>Other agents</i>	<i>References</i>
Rhinosinusitis or “Common cold”	RV, PIV, CoV	IV, RSV, ADV, EV, hMPV	Nokso-koivisto et al. (2015)
Flu	IV	PIV, CoV	Uyeki (2017) and Wen et al. (2019)
Pneumonia	RSV, IV	hMPV, ADV, RV, PIV, CoV	Barrera-Badillo et al. (2020), Lee et al. (2019), Self et al. (2016), Uyeki (2017) and Wen et al. (2019)
Bronchitis	PIV, IV, RV	EV, CoV, ADV	Heimdal et al. (2019) and Wen et al. (2019)
Bronchiolitis	RSV	PV, EV, IV, RV, hMPV, ADV	Barrera-Badillo et al. (2020), Uyeki (2017) and Wen et al. (2019)
Otitis media	RSV, PIV	IV, RV, hMPV	Nokso-koivisto et al. (2015) and Uyeki (2017)
Pharyngitis	ADV, PIV	RV, CoV, EV, IV, RSV	Heimdal et al. (2019)
Laryngitis	PIV	ADV, CoV, hMPV, IV	Nokso-koivisto et al. (2015)

RV, Rhinovirus; IV, influenza virus (includes A and B); PIV, Parainfluenza virus; CoV, Coronavirus; RSV, Respiratory syncytial virus; EV, Enterovirus; ADV, Adenovirus; hMPV, human metapneumovirus.

that they spread in domestic animals, pigs, horses, poultry, and wild migratory birds. These animals are a source of antigenically diverse HA and NA genes that can be exchanged between viral strains by reassortment after co-infection of the same host, causing the generation of human pandemic influenza virus strains with HA and/or NA derived from animal strains. Pandemic influenza arises every 10–50 years and is the result of the emergence of a new IAV virus strain that is antigenically very different from circulating strains already known. Indeed, the lack of preexisting immunity in humans is often correlated with the severity of the infection and high mortality (Krammer et al., 2018). In 2009, the WHO declared an influenza pandemic caused by a new H1N1 strain (pdm09H1N1). The outbreak of the pandemic started in Mexico and the infected subjects presented severe pneumonitis which leads to a high case-mortality rate. Afterward, the disease rapidly disseminated to 72 other countries leading to hundreds of thousands of infected subjects and numerous hundred deceased (Centers for Disease Control and Prevention, 2009).

Clinical manifestations

Transmission of influenza in humans occurs through direct contact with infected subjects, or inhalation of small respiratory droplets from infected subjects (WHO, 2018b). NA plays a role in facilitating viral entry into epithelial cells lying underneath the mucus in the nasopharynx by cleaving the sialic acids from the mucus glycoproteins to free the mucus trapped-virus. HA attachment to the receptor allows cellular entry of the virus (Taubenberger and Morens, 2008). Signs and symptoms of uncomplicated influenza frequently overlap with those of other respiratory viral infections. Initial clinical signs include chills, fever, myalgia, fatigue and headache. The onset of these symptoms can be sudden, especially a high fever. Symptoms such as dry cough, sore or dry throat (possibly with hoarseness) and nasal obstruction can help in the differentiation from other respiratory viral infection. Elderly people and immune suppressed individuals may not develop a fever. Presentation in children can include diarrhea and in adults vomiting may also occur. Flu symptoms tend to last for 2 weeks, and not all flu patients display all of the clinical manifestations. People at greater risk of severe disease or complications include pregnant women, children under 59 months, elderly people, individuals with immunosuppressive conditions or with chronic medical conditions, such as allergic asthma and chronic lung disease. Complications include secondary pneumonia caused by bacteria such as *Staphylococcus aureus*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* (Krammer et al., 2018; Uyeki, 2017; van der Sluijs et al., 2010; WHO, 2018b).

Antivirals and vaccines

The treatment of flu includes blockers of polymeric M2-mediated ion-channel activity and neuraminidase inhibitors (NAIs), both drugs are approved by the FDA, but the first one is globally no longer recommended for clinical use due to the widespread resistance among the circulating IAV. NAIs inhibit the release of virus progeny from infected cells and thus reduce viral spread. NAIs include Oseltamivir (commercially named Tamiflu), Zanamivir (Relenza), and Laninamivir octanoate (Inavir). All patients with serious or progressive clinical illness associated with suggested or confirmed influenza virus infection should be treated with antiviral drugs at the earliest (Krammer et al., 2018; Takashita et al., 2020; Uyeki, 2017; WHO, 2018b).

The most effective way to prevent IV infection is vaccination. The WHO recommends annual vaccination against influenza for pregnant women, children aged between 6 months to 5 years, elderly people, and individuals with chronic medical conditions and healthcare workers. Vaccination may be less effective in preventing illness among elderly or individuals with chronic conditions, but it reduces severity of disease as well as the incidence of complications and deaths. The composition of seasonal influenza inactivated vaccines may be trivalent, that is consisting of the three most representative types of virus in circulation (two subtypes of influenza A viruses and one influenza B virus) or quadrivalent, to provide wider protection against influenza B virus infections. Virus strains included in vaccines need to be changed annually due to constantly antigenic drift of the circulating viruses. The WHO Global Influenza Surveillance and Response System continuously monitor the influenza viruses circulating in humans and updates the viruses that match both antigenically and genetically with the causative viruses of the particular flu season (northern and southern hemisphere) for influenza vaccine inclusion (WHO, 2018b).

Coronavirus (CoV)

Until the outbreak of the severe acute respiratory syndrome (SARS) in 2002 (Ksiazek et al., 2003), corona viruses were known as an ordinary cause of the common cold and were not considered to be highly pathogenic in humans. SARS alerted the WHO of the emergence of new corona viruses, which was later confirmed by the Middle East respiratory syndrome (MERS) outbreak in 2012 (WHO, 2019) and the recent coronavirus disease (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 was first recognized in Wuhan, China, in December 2019 and rapidly spread worldwide. Globally, at the date of this publication, 120.383.919 COVID-19 cases had been confirmed, including 2.664.386 deaths reported to WHO (WHO, 2021a).

Coronaviridae is a large family of enveloped positive-sense single-stranded RNA viruses, classified into different genera as alpha, beta, gamma, and delta virus. Their tendency for recombination and the inherently high mutation rates allow these RNA viruses to adapt to multiple species, and crossover from animals to humans, which leads to outbreaks. Alpha and Beta coronaviruses only infect mammals, causing respiratory disorders in humans and gastroenteritis in animals. Gamma and Delta coronaviruses infect birds, but can also infect mammals (Woo et al., 2012). All seven human coronaviruses are known to have animal origins.

The novel coronavirus SARS-CoV-2, is a beta coronavirus that is structurally and functionally related to two bat-derived SARS-like coronaviruses, and to the epidemic SARS-CoV (79% similarity) and MERS-CoV (50% similarity) (Lu et al., 2020). Coronaviruses entry into the host cells is mediated by the external glycoprotein spike S. The protein consists of two functional subunits responsible for binding to the host cell receptor and fusion of the viral and cellular membranes. In SARS-CoV-2, angiotensin-converting enzyme 2 (ACE2) has been recently confirmed as the main virus receptor (Hoffmann et al., 2020). ACE2 populates the apical membrane of the respiratory epithelial cells and is also present in the nose and mouth. Like SARS-CoV, SARS-CoV-2 spike recognizes and binds to the ACE2 receptor, primed by the transmembrane protease serine type 2 (TMPRSS2), although affinities remain to be compared (Hoffmann et al., 2020; Walls et al., 2020).

Clinical manifestations and outcomes of SARS-CoV-2

SARS-CoV-2 is transmitted efficiently by human-to-human via droplets or direct contact. It is stable in aerosols for hours and on surfaces up to days (van Doremalen et al., 2020). It has a basal reproductive number of 2.68 that could go up to 4, which is higher than that for SARS-CoV and MERS-CoV. SARS-CoV-2 infection has been estimated to have a mean incubation period of 6.4 days (Wu et al., 2020). The clinical spectrum of SARS-CoV-2 infection can range from no symptoms or mild respiratory symptoms to severe acute respiratory disease and death. People infected with SARS-CoV-2 transmit the virus without recognizing, or prior to recognizing symptoms. This poses a great difficulty for infection control, because these unspecific symptoms are difficult to identify and quarantine. The risk for infection is high for individuals of all ages; however, most COVID-19 patients are middle-aged adults and elderly people. The most frequently found symptoms are fever and cough, but patients can also present with other symptoms such as myalgia, headache, dyspnea, fatigue, chest pain, and nasal congestion. Most patients present with fever together with other symptoms, rather than only fever. Some patients also experience diarrhea, nausea, or vomiting (Wu et al., 2020; Yang et al., 2020). Other manifestations in mild patients are olfactory and gustatory disorders, which appear in patients with no nasal symptoms or before these symptoms appear, these were prevalent symptoms reported outside China (Lechien et al., 2020). The population at the highest risk of developing a severe disease is the elderly and people with certain comorbidities. Severe COVID-19 cases were more likely to have comorbidities than non-severe cases. The most common underlying diseases found in all patients were cardiovascular disease, hypertension, diabetes, chronic respiratory disease, cancer, renal disease, and obesity (Chen et al., 2020; Wang et al., 2020b; Yang et al., 2020). Radiological findings of SARS-CoV-2 pneumonia are variable, with focal unilateral to diffuse bilateral lung involvement with ground-glass opacities being the most typical finding on chest computed tomography (Wang et al., 2020b). The progression of the high-risk disease requires hospitalization, oxygen support, and admission to an intensive care unit (ICU). Moreover, common complications include shock, acute respiratory distress syndrome, arrhythmia, acute cardiac injury, acute kidney injury, secondary infection, and death (Huang et al., 2020; Wang et al., 2020b; Yang et al., 2020).

Clinical management

There is no specific treatment with confirmed efficacy for COVID-19 and clinical management is based on supportive care, such as in other respiratory diseases. Considerations for patients with other severe acute respiratory illness include the early use of empirical antimicrobials and routine corticosteroids but these should be avoided unless they are indicated for asthma exacerbation or chronic obstruction pulmonary disease (WHO, 2021a). Given the lack of a specific antiviral therapy against SARS-CoV-2, several drugs with potential antiviral activities are under active investigation, although the clinical effectiveness of these drugs for the treatment of patients with COVID-19 has not yet been confirmed. Initial reports suggest that the protease inhibitors lopinavir and ritonavir, used to treat infection with HIV, could be potential candidates. However, no benefit was observed with lopinavir-ritonavir treatment in hospitalized adult patients with severe COVID-19. Despite this, treatment combination including these two antivirals and type I interferon seems to be a more promising option (Cao et al., 2020). Another candidate is Remdesivir, an adenosine analogue that has demonstrated activity against RNA viruses in several families, including *Coronaviridae* (such as SARS-CoV and MERS-CoV) (Sheahan et al., 2017) showing recently to be effective in preventing virus replication in in vitro assays (Wang et al., 2020). There are several clinical trials ongoing, some results already available showed that Remdesivir compared to placebo, shortened the recovery

time in adults hospitalized with COVID-19 (Beigel et al., 2020). Hydroxychloroquine is a widely used anti-malarial and autoimmune disease drug, reported to block virus infection interfering with the glycosylation of ACE2 cellular receptors (Wang et al., 2020). Although it has been tested in clinical studies, they have not obtained conclusive results and its use is not even recommended for the treatment of COVID-19 (US NIH, 2020). Inhibition or modulation of the receptor ACE2 or TMPRSS2 is one of the proposed host-based strategies for the treatment of SARS-CoV-2. The soluble recombinant human ACE2 could inhibit the attachment of the virus to the cells (Monteil et al., 2020). Furthermore, a clinically proven TMPRSS2 inhibitor has also been proposed as a potential treatment option (Hoffmann et al., 2020). Other therapeutic targets involve actors of the innate immune system acting by enhancing or attenuating the inflammatory response. Multiple clinical trials on the use of the above-mentioned and other candidates for treatment of COVID-19 are currently underway. Research efforts are also focused on the development of a vaccine, which represents a more long-term strategy for future outbreaks of SARS-CoV-2 (Clinicaltrials.gov, n.d.).

Effective SARS-CoV-2 vaccines are required to control the COVID-19 pandemic. Research efforts were focused around the world to develop and launch effective vaccines against SARS-CoV-2 through advanced molecular biology and biotechnology approaches. Several vaccine platforms are in development, including inactivated viruses, live attenuated viruses, virus-like particles, DNA, RNA, protein subunits, and viral vector vaccines (replicating and non-replicating). To date, the WHO overview summary has included 264 candidate vaccines against COVID-19, of which 82 are under clinical evaluation (WHO, 2021b). Currently there are 3 vaccines that have passed phase IV trials and have been licensed for massive use worldwide: BNT162b2 (Pfizer / BioNTech), mRNA-1273 (Moderna) and AZD1222 (University of Oxford and AstraZeneca) (WHO, 2021b). Vaccination started mainly with these, but in addition at least seven different vaccines have been administered around the world. Vulnerable populations, the elderly and healthcare workers, are the highest priority for vaccination in all countries. Globally a total of 363.691.238 vaccine doses have been administered (WHO, 2021a).

Respiratory syncytial virus (RSV)

RSV is a leading global cause of morbidity and mortality. It is the most common viral pathogen identified in infants and young children with pneumonia or bronchiolitis and the major single cause of hospitalization during infancy. It is also the second most-likely single death-causing pathogen, especially among infants in the first 6 months of life (Nair et al., 2010; Shi et al., 2017). RSV also has been increasingly recognized as a cause of illness in adults, mainly in the elderly and high-risk adults with heart failure or COPD. It has a disease burden comparable to that of the influenza virus in the elderly (Falsey et al., 2005).

RSV is an enveloped non-segmented negative-sense single-stranded RNA virus that belongs to the *Pneumoviridae* family. The envelope of the virus has two glycosylated surface proteins essential for virus infectivity and pathogenesis. The attachment glycoprotein G is responsible for the attachment of the virus to respiratory epithelial cells, while the fusion protein F mediates virion and host cell fusion, thus creating large multinucleated characteristic syncytia by fusing with neighboring cells. Moreover, the F and G proteins stimulate the neutralizing antibody immune response by the host (Collins and Graham, 2008; Vandini et al., 2017). Based on antigenic differences in their glycoproteins G and F, there are two major RSV groups, RSV-A and RSV-B. The differences between the two groups are an extensive antigenic and nucleotide sequence on the G protein. Both groups generally coexist and multiple genotypes within the groups can also co-circulate during an epidemic season. The predominance of one genotype over the others leads to alternating infection incidences each the year and that can be the reason for the ability of RSV to infect previously exposed individuals and circumvent preexisting immune responses (Vandini et al., 2017; Liu et al., 2016; Sullender, 2000).

Clinical manifestations

In children, RSV causes respiratory tract infection from mild upper respiratory tract symptoms to severe lower airway involvement that needs hospital admission and mechanical ventilation. The highest RSV infection occurs in infants that have an increased possibility of lower airway involvement causing bronchiolitis and pneumonia. Host factors like premature delivery, malnutrition, or congenital heart diseases increase the risk of developing an acute disease (Nair et al., 2010). RSV is a highly contagious virus; transmission occurs by inoculation of the nose or eyes, but rarely the mouth, with contaminated secretions or by virus-containing respiratory droplets of an infectious patient (Hall, 2001; Meissner, 2016). The virus can also survive for prolonged periods of time on the skin, clothes, and other objects, thus facilitating its spread (Hall, 2001). The incubation period of RSV is of 2–8 days and after inoculation in the nasopharyngeal epithelium it rapidly spreads to the lower respiratory tract where it infects ciliated epithelial cells. The virus rather infects the bronchioles and type 1 pneumocytes in the alveoli, but can also infect non-ciliated epithelium and intraepithelial dendritic cells (Collins and Graham, 2008; Meissner, 2016). It has been demonstrated that RSV can infect progenitor basal cells and induce morphogenetic changes in the epithelium, resulting in an increased number of goblet cells and reduced number of ciliated cells (Persson et al., 2014). The virus binds to cellular receptors of the target cell using G glycoproteins and the viral attachment begins a conformational change in F protein, promoting fusion of the virus envelope with the host cell membrane to insert its nucleocapsid and begin its intracellular replication (Meissner, 2016). Among the initial clinical manifestations appear upper respiratory tract symptoms like coryza, congestion and rhinorrhea. Also, fever is a predominant symptom in infants. A consequence of the nasal congestion and various symptoms causes of malaise can be poor feeding and subsequent dehydration. Increasing cough is frequently the first sign of lower respiratory tract involvement, followed by the appearance of clinical signs of

bronchiolitis like tachypnea, dyspnea, and the acute phase of the infections is often followed by episodes of increased airway resistance, air trapping, and wheezing. Furthermore, symptoms of lower airway infection are also common in infants with mild disease (Collins and Graham, 2008; Meissner, 2016; Smyth and Openshaw, 2006). Along with the health burden of RSV infection, there is a high possibility of long-term consequences on respiratory function caused by RSV infection, with the development of persistent airway disease (Meissner, 2016; Smyth and Openshaw, 2006). Moreover, adults have less distinctive clinical findings compared to children and the cause is often unclear. Among clinical manifestations are sore throat, fever and malaise as well as lower respiratory tract symptoms such as cough. There is an increased morbidity and mortality in aged patients or patients with underlying immune or cardiorespiratory disease (Falsey et al., 2005; Hall, 2001).

Clinical management

The majority of RSV cases are treated with nonspecific medical intervention. Therapy for acute RSV bronchiolitis in infants is supportive care and includes maintenance of nutrition, hydration and an oxygen saturation of 90%. Antibiotics, bronchodilators or epinephrine are not recommended. Ribavirin is a widely used broad-spectrum antiviral agent. Despite this, it has shown no benefits for RSV bronchiolitis (Smyth and Openshaw, 2006). Developing RSV vaccines has been a challenge, and the difficulty of inducing protective immunity is highlighted by repeat infections throughout life. Currently, there are numerous RSV vaccines in various stages of clinical trials. In the late 1960s a formalin-inactivated RSV vaccine was administered to infants resulting in enhanced RSV disease but with a high rate of hospitalization and two deaths. It was immunogenic but induced poor antibody affinity maturation and antibody avidity for virus epitopes (Kim et al., 1969). The reason for the failure of this vaccine is unclear, but is thought to be, in part, due to formalin-mediated disruption of the neutralizing epitopes (Murphy and Walsh, 1988). It has also been argued that the lack of affinity maturation leads to insufficient TLR activation in B cells (Delgado et al., 2009). An antiviral therapy licensed for RSV prophylaxis, and only available for high-risk infants, is a specific monoclonal antibody called Palivizumab. It is recommended in the first year of life for infants born before 29 weeks, with hemodynamic significant heart disease or chronic lung disease. Palivizumab is not recommended for otherwise healthy infants born at or after 29 weeks, and more information is needed before its use in RSV-primed older children (Simoes et al., 2018). Throughout history, several therapeutic interventions for RSV have failed and still remain a source of scientific interest. RSV molecular epidemiological surveillance of seasonal circulation of different RSV genotypes is essential for identify new viral strains, predict their clinical relevance, and select candidate strains for vaccine and therapeutic development.

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Respiratory Tract Infections: Fungi

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Introduction

A common route of fungi infection is via the respiratory tract. The most common fungus diseases of the respiratory system are shown in Table 1. Environmental exposure to spores of pathogenic fungi during occupational or recreational tasks frequently leads to subclinical disease infections or to more severe life-threatening diseases (Queiroz-Telles et al., 2017). Most pulmonary fungal infections only imply temporal colonization of the respiratory tract but sometimes the infection can represent serious infection, leading to different management and prognosis. Generally, in the lung's alveoli, fungus is phagocyted by macrophages and other cells involved in the primary immune response. Some fungi have developed a way to disable the macrophage's neutralization and have acquired the ability to grow and multiply inside macrophages. In these cases, cells from the adaptive immune system can control the infection. In healthy individuals the fungus is contained in most cases, but when cellular immunity is impaired, as for instance, in AIDS patients, infection with an endemic fungus cannot be controlled and could generate a systemic disease.

Aspergillosis

Aspergillus species are a ubiquitous fungus which widely present in the environment. *Aspergillus fumigatus* is the most frequent cause of human infections. Other pathogenic species of *Aspergillus* are *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus nidulans*.

Table 1 Common fungus diseases.

Disease	Etiological agent
Aspergillosis	<i>Aspergillus fumigatus</i> (most common), <i>Aspergillus flavus</i> , <i>Aspergillus niger</i> , <i>Aspergillus terreus</i> and <i>Aspergillus nidulans</i> .
Histoplasmosis	<i>Histoplasma capsulatum</i>
Coccidioidomycosis	<i>Coccidioides immitis</i> and <i>Coccidioides posadasii</i>
Blastomycosis	<i>Blastomyces dermatitidis</i>
Paracoccidioidomycosis	<i>Paracoccidioides brasiliensis</i>
Cryptococcosis	<i>Cryptococcus neoformans</i> and <i>Cryptococcus gattii</i>
Pneumocystis pneumonia	<i>Pneumocystis jirovecii</i>

The inhalation of the spores (conidia) is a common phenomenon while tissue invasion is infrequent and occurs mainly in patients with some degree of immunosuppression. The mortality of invasive forms depends on the clinical form and the host's immune background but is usually higher than 50%.

Under normal immune function, alveolar macrophages are capable of eliminating the spores inhaled every day. Despite this, the spores that escape macrophage phagocytosis can germinate and become hyphae, in which case they are eliminated by cellular components of the immune system as polymorphonuclear neutrophils. From a clinical point of view, *Aspergillus* species can lead to different types of pathogenesis, from aspergillosis bronchopulmonary allergic (ABPA) and chronic pulmonary forms to invasive and disseminated forms (Fortún et al., 2012; García-Navarro, 2010).

Clinical manifestations

The clinical manifestations depend on the type of infection. ABPA occurs most frequently in patients with chronic asthma or cystic fibrosis and is characterized by episodic airway obstruction and fever. In the case of aspergilloma these cases are characterized by cough, dyspnoea, weight loss, fatigue, chest pain, or fever. Chest radiographs show a ball in a cavity because the fungus matted hyphae and debris are deposited in a preformed cavity from previous pulmonary tuberculosis, histoplasmosis, or fibrocystic sarcoidosis.

Chronic aspergillosis occurs in patients with chronic lung disease or immunosuppressed, commonly due to the use of corticosteroids for a long period of time. Clinical manifestations including cough and dyspnoea could be indistinguishable from the primary chronic respiratory disease. Invasive pulmonary aspergillosis (IPA) occurs in immunocompromised hosts and is characterized by fever, focal pulmonary infiltrates, cough, pleuritic pain, and sometimes hemoptysis (Walsh and Stevens, 2012).

Diagnosis

Due to its nonspecific symptoms which can be confused with chronic pulmonary disease ABPA is an under diagnosed disease. Diagnosis is based on clinical and radiologic aspects and can vary in cases of patients who have other pulmonary diseases such as cystic fibrosis. Diagnosis is based on a positive cutaneous reaction to *A. fumigatus* antigen, immunoglobulin E (IgE) concentration in blood above 1000 IU/mL, presence of *Aspergillus*-specific IgE, peripheral blood eosinophilia, and pulmonary infiltrates.

Aspergillus-specific IgE is the most sensitive screening test for ABPA. If patients with uncontrolled asthma show a positive *Aspergillus*-specific IgE but a total IgE level below 1000 IU/mL and do not meet the additional characteristics, they could be classified as severe asthma with fungal sensitization. The diagnosis of IPA is difficult. Computed tomography could reveal features that can be confused with another type of infection. Microbiologic culture or direct microscopy from bronchoalveolar lavage or biopsy is important to differentiate aspergillosis from other infections. Detection of galactomannan (polysaccharide of *Aspergillus* cell wall) by immunoassay could be also useful (El-Baba et al., 2020; Walsh and Stevens, 2012).

Treatment

There are different treatment alternatives for allergic forms that include glucocorticoids and antifungals. For systemic ABPA the preferred treatment is with glucocorticoids such as prednisolone. The intention of the therapy is to decrease the hypersensitivity response.

Antifungals (itraconazole or voriconazole) could be beneficial in some patients, including those showing a poor response to glucocorticoid monotherapy.

The treatment for invasive forms differs from that for allergic forms because in these cases most patients have immunodeficiencies. Treatment consists of antifungal therapy, a reversal of immune suppression when possible, and in some cases, surgical resection of lesions. The recommended antifungal is voriconazole. In cases where patients have contraindications or a history of hypersensitivity to voriconazole, liposomal amphotericin B is indicated. Second-line therapy may include posaconazole, isavuconazole, or itraconazole.

In the case of immunosuppressed patients, it is important to decrease or suppress corticosteroid treatment and it may also be necessary to establish treatments to accelerate the recovery from neutropenia as the administration of granulocyte colony-stimulating factor or granulocyte-macrophage colony-stimulating factor.

Surgical intervention could be necessary in some patients with several forms of invasive aspergillosis, for example those with massive or recurrent hemoptysis or with pulmonary lesions contiguous to important vessels or the pericardium. Furthermore, surgical intervention may be useful in localized disease refractory to antifungal treatment (El-Baba et al., 2020; Walsh and Stevens, 2012).

Histoplasmosis

Histoplasmosis is one of the most common infections by fungi. It is caused by a dimorphic fungus, *Histoplasma capsulatum*. The subspecies *capsulatum* generally causes subclinical infection but high levels of exposure can lead to an asymptomatic pulmonary infection that in immune-compromised patients can lead to a severe infection.

The typical habitat of *H. capsulatum* is soil with high nitrogen content coming from the excrement of birds or bats. Moreover, other risk areas are sites such as chicken coops, bat-infested caves or other places with bird excrement (Fraser et al., 2006; Navarro et al., 2013). Another subspecies of *H. capsulatum*, *duboisii*, is common in Africa and can have different disease manifestations with cutaneous and skeletal affections (Navarro et al., 2013).

H. capsulatum grows as mold below 35 °C while at 37 °C it appears as yeast. The infectious particles are the microconidia spores produced by the mold. After inhalation, spores germinate within bronchioles and alveoli where they grow as yeast-like forms. The alveolar macrophages proceed to control the infection, resulting in localized inflammation. However, in some cases, *H. capsulatum* can survive and reactivate within macrophages. In these cases, complementary immune mechanisms such as specific T-lymphocyte activation, interleukin-12 and interferon γ stimulate activation of macrophages killing the fungus. In immune-compromised patient fungal infection can lead to a disseminated infection and it can be found poorly formed granulomas and a large fungal burden (Christenson and Kleiman, 2018; Navarro et al., 2013).

Clinical manifestations

Clinical manifestations are varied and depend on the inoculum level and health status of the patient. Immunodeficiencies or chronic lung diseases can lead to more severe diseases. Acute pulmonary histoplasmosis infection is generally an asymptomatic or self-limited illness. In the case of showing symptoms, these appear some weeks after exposure and are characterized by fever, fatigue, non-productive cough, and myalgias. Chest radiography shows lobar or multilobe nodular infiltrate.

In the case of patients that were exposed to a high level of spores or in immune-suppressed patients the infection could be severe including high fever, dyspnoea, cough, pericarditis, cyanosis, hemoptysis, and could lead in respiratory failure and disseminated disease.

In contrast, chronic cavitary pulmonary histoplasmosis is a progressive and often fatal respiratory infection of older patients who have chronic obstructive pulmonary disease (COPD). Patient's symptoms include fever, fatigue, anorexia, productive cough, and hemoptysis. Chest radiography shows infiltrates in upper lobe and extensive fibrosis in the lower lobes. Acute disseminated histoplasmosis occurs mostly in immune-suppressed patients. Patients show fever, anorexia, hypotension, dyspnoea, and diffuse lesions in skin, disseminated intravascular coagulation, adult respiratory distress syndrome and acute respiratory failure. This syndrome resembles a sepsis of any bacterial or viral cause.

Finally, chronic progressive disseminated histoplasmosis is another severe form of histoplasmosis. The illness is characterized by fever, ulcerations in the buccal mucosa or nasal vestibule, anorexia, and fatigue. Involvement of most of the organ systems has been detected due to disseminated infection including bowel obstruction or perforation, cerebral granulomas, endocarditis with aortic and mitral valve vegetations, adrenal insufficiency (Kauffman, 2012a; Navarro et al., 2013).

Etiological diagnosis

It is a challenge to obtain a differential diagnosis of symptomatic pulmonary histoplasmosis from tuberculosis and other fungi infections like blastomycosis and coccidioidomycosis when clinical and radiographic findings are similar.

The culture of the *H. capsulatum* is a reliable diagnostic test but unfortunately it involves a long incubation time of 6 weeks. Alternatively, serology diagnosis by complement fixation assays and immunodiffusion tests are available. Another helpful option is an immunoassay that detects *H. capsulatum* polysaccharide antigen in blood and urine. This assay can achieve a sensitivity from 75% to 90% in patients who have acute pulmonary histoplasmosis and disseminated histoplasmosis, but it is not suitable for patients who have chronic cavitary histoplasmosis and mediastinitis histoplasmosis. Moreover, the test can have some cross-reactivity with other fungi infections.

Treatment

The treatment depends on the type of infection and patient health status.

Generally, for mild to moderate infections itraconazole (azoles antimycotic group drug) is the drug of choice, while lipid formulations of amphotericin B are used as initial therapy for severe infections. Acute pulmonary histoplasmosis is usually treated with amphotericin B in severe forms and alternatively with itraconazole in milder infections. Itraconazole is also recommended for chronic cavitary pulmonary histoplasmosis. Immunocompromised patients with severe pulmonary histoplasmosis disease are treated with amphotericin B for 1–2 weeks followed by itraconazole (Kauffman, 2012a; Navarro et al., 2013).

Coccidioidomycosis

Coccidioidomycosis is a pulmonary and systemic disease caused by fungi of the genus *Coccidioides*. Two species are pathogenic, *C. immitis* and *C. posadasii*. Both species caused a similar disease. *Coccidioides* is endemic in semi-arid and arid zones in the Americas exclusively. Endemic zones include the southwestern United States, northern Mexico, and other countries of Central and South America (Guatemala, Honduras, Nicaragua, Colombia, Venezuela, Brazil, Argentina, Bolivia, and Paraguay). Endemic areas are characterized by arid or semi-arid climates, low altitude, alkaline soil, limited rainfall, and mild winters. Mycelia grow in soil during periods of rain, while in dry months arthroconidia develops and aerosolizes, causing the infection. In the United States only, coccidioidomycosis affects 150,000 persons every year.

Patients are infected via inhalation of arthroconidium, leading to fungal proliferation. The first steps of the immune response include the participation of macrophages and neutrophils forming granulomas. Fungi may resist effective phagocytosis in which case infection is controlled by the participation of other cells of the immune system like dendritic cells, and B and T lymphocytes. Disseminated disease is rare and occurs in patients with immunodeficiencies (Anstead et al., 2011; Galgiani, 2012).

Clinical manifestations

Coccidioidomycosis is asymptomatic in more than 60% of patients. These individuals can only be detected by a positive skin test.

Other patients can show the “Valley Fever” (called by San Joaquin Valley of California, an endemic area of coccidioidomycosis) that includes fever, erythema nodosum and multiforme and migratory arthralgias. Most symptomatic patients manifest pulmonary infection symptoms as fever, dry cough, and chest pain. In most patients, acute pulmonary coccidioidomycosis resolves without treatment in a month. However, in some patients, the disease progresses into chronic pulmonary infiltrates and persists for months or years. Aggressive pulmonary coccidioidomycosis occurs more often during pregnancy and in immunosuppressed patients. These patients may also suffer from dissemination outside the lungs. The most common locations are the skin, joints, bones, and meninges. Lesions can range from subacute to chronic but in some patients they may be fatal with detectable fungemia (Anstead et al., 2011; Anstead and Graybill, 2013; Galgiani, 2012).

Etiological diagnosis

Due to the nonspecific symptoms, a history of residence or travel to endemic areas is important information for a suspected diagnosis. Search in tissues, sputum or exudates can be achieved through direct microscopy (spherules with endospores or mycelia) or culture. Because of the high possibility of transmission to lab personnel, culture must be made according to proper biosafety practices. Serological tests are also a useful tool for diagnosing and monitoring the course of infection. The skin test is performed with either coccidioidin (mycelia phase) or spherulin (spherule phase). In the benign forms of coccidioidomycosis, the skin test is positive in almost all of the patients some weeks after the start of infection. However, patients with disseminated disease, have often a negative reaction to the skin test (Anstead et al., 2011; Fraser et al., 2006).

Treatment

Most patients are asymptomatic and most cases of primary pulmonary infection resolve without treatment. However, in detected cases, observation is required for some years until the resolution of the infection to prevent complications.

Antifungal therapy is necessary for patients with severe disease, disseminated disease, or high risk of dissemination. The therapy can include amphotericin B or azole group antifungal (itraconazole, fluconazole, voriconazole) or a combination of both groups (first weeks with amphotericin B and then azole antifungal). Treatment should be prolonged for at least 1 year (Anstead et al., 2011; Galgiani, 2012).

Blastomycosis

Blastomycosis is a pulmonary and sometimes systemic disease caused by *Blastomyces dermatitidis*. Endemic areas include south central and north central regions of the United States and Great lake areas of United States and Canada. Specific zones are those with soil near river basins or lakes. Occasional cases are also reported in Africa, Europe, Asia, and Latin America. Outbreaks are infrequent and are generally related to outdoor activities along waterways. *B. dermatitidis* can infect dogs, cats, and other mammals. Transmission from animals to humans is not possible except, exceptionally, through a dog bite. Infected animals can act as

sentinels to detect sources of outbreaks and to help in the diagnoses. Infection occurs by inhalation of conidia that, upon arrival to the alveoli, transform into yeast and induce an inflammatory response. Alveolar macrophages and neutrophils can inhibit the transition into yeasts. *B. dermatitidis* yeast is relatively resistant and some of them can survive in macrophages. Most infections are asymptomatic and resolve without treatment. Some patients may develop severe pneumonia while others can present with chronic pneumonia.

Infrequently, the infection can disseminate to other sites such as bones and prostate.

Unlike other fungi, blastomycosis has not been reported as an important infection in patients with AIDS. Nevertheless, patients with immunosuppressive conditions have a marked susceptibility (Anstead et al., 2011; Vyas and Bradsher, 2013).

Clinical manifestations

The incubation period of blastomycosis can range from several weeks up to 4 months. Most patients are asymptomatic. Symptomatic patients with acute pneumonia show fever, malaise, and non-productive cough. Skin lesions in the form of painless papules, plaques, nodules, or ulcers along with pneumonia symptoms are suggestive of blastomycosis and other fungus pneumonia. Chest radiography shows pulmonary lobar or multilobar irregular infiltrates, or nodular infiltrates. Patients with chronic pneumonia can show fever, night sweats, fatigue, cough, hemoptysis, and dyspnoea. Chest radiography can show cavitary, nodular or fibrotic lesions. Disseminated disease is characterized by osteomyelitis and skin lesions (Gauthier and Klein, 2018; Kauffman, 2012b).

Etiological diagnosis

Diagnoses can be made by direct examination or culture. A direct examination can be made based on sputum or bronchial washings usually treated with potassium hydroxide.

Culture of *B. dermatitidis* from sputum or bronchial washing gives a definitive diagnosis but results can take up to 4 weeks. Serological tests have low sensitivity and specificity values and have extensive cross-reactivity with other fungal diseases such as histoplasmosis. PCR and antigen test are in development and could be available in the near future (Anstead et al., 2011; Gauthier and Klein, 2018).

Treatment

For severe cases or in immunocompromised patients, amphotericin B or lipid formulation of amphotericin B is recommended for initial therapy. Following improvement of the patient, treatment switches to oral itraconazole. This treatment is prolonged by 6–12 months. In cases of less severe disease, itraconazole is used as initial therapy for 6–12 months (Anstead et al., 2011; Vyas and Bradsher, 2013).

Paracoccidioidomycosis

Paracoccidioidomycosis or South American blastomycosis is an acute or chronic mycosis endemic in humid areas of Central and South America. It is caused by *Paracoccidioides brasiliensis*, a thermally dimorphic fungus. The disease presents in two general forms. The acute form occurs in children and immunocompromised patients and is characterized by dissemination disease. The chronic form affects adults and is mainly characterized by progressive pulmonary lesions.

The fungus can be found in soil and infection occurs when the individual inhales the conidia, resulting in a primary pulmonary lymph node complex. As in other fungus infections, *Paracoccidioides* entry into alveolar macrophages and conidia convert into yeast form. The infection remains localized in the lung but in some cases, systemic dissemination can occur. The infection is predominantly present in males (the male-to-female ratio is 14:1). The reason why adult females are resistant to the disease is that estradiol, inhibits the transformation of the mold into yeast (Kauffman, 2012c; Negroni et al., 2011).

Clinical manifestations

Primary infections are generally asymptomatic, and most individuals will remain free of disease. A positive skin test is the unique evidence of previous infection. Acute or juvenile forms affect pre-puberty children that visited or lived in an endemic area. After a short incubation period, they present with fever, asthenia, anorexia, subcutaneous abscesses, lymphadenopathy, liver and spleen enlargement. Lungs or mucous membrane affection are infrequently. The chronic form usually occurs in adult males that lived in the endemic area and slowly progress over the years. Patients present with asthenia, loss of weight, dyspnoea, and productive cough. Some patients only show pulmonary infection characterized by fever, cough, and dyspnoea. Most patients show multifocal presentation affecting the skin, mucous membranes, lungs, lymph nodes, adrenal glands, abdominal organs, and infrequently, the central nervous system. Typical lesions include oropharyngeal ulcers, lips, and gingival mucosa edema, laryngeal dysphonia, dyspnoea, dysphagia, skin lesions at the face and neck, and hypertrophic cervical lymph nodes. In Brazil, paracoccidioidomycosis is associated with AIDS patients, in which an acute disseminated juvenile type infection is observed (Negroni, 2013; Queiroz-Telles et al., 2017).

Etiological diagnosis

The accurate diagnosis of paracoccidioidomycosis is made through culture, but direct examination could also help to obtain a rapid presumptive result. Direct examination of the samples of bodily fluids and sputum is carried out by treating them with potassium hydroxide and calcofluor fluorescent stain to visualize the fungus. Although culture is the definitive assay, it has the disadvantage that it takes up to 4 weeks to grow. Besides this, immunological assays such as immunodiffusion, ELISA and complement fixation are available to complement the diagnosis (Kauffman, 2012c).

Treatment

Paracoccidioides brasiliensis is sensitive to most antimycotics drugs. The most recommended therapy is treatment with itraconazole or ketoconazole for 6–12 months. Amphotericin B is usually reserved for severe disease. Other treatments include trimethoprim with sulfamethoxazole and sulfadiazine. HIV infected patients are initially treated with amphotericin B followed by trimethoprim-sulfamethoxazole long life suppressive therapy.

Cryptococcosis

Cryptococcosis is a severe infection that mainly affects immunocompromised patients.

The disease is caused by two species of encapsulated yeasts: *Cryptococcus neoformans* and *Cryptococcus gattii*. *C. neoformans* has a worldwide distribution and is an important opportunistic infection in AIDS patients. In contrast, *C. Gattii* is associated with tropical or subtropical regions and usually infects the immune competent host.

Cryptococcus capsular polysaccharide and melanin production are the most important virulence factors. Primary infection occurs via inhalation of yeast into the lung where it is found by macrophages and neutrophils. Moreover, *Cryptococcus* capsules help prevent phagocytosis, so the capsular opsonization is important for appropriate elimination of the yeast. T-cell- mediated immunity also has an important activity against infection by limiting the replication of the yeast. In immunocompetent patients, the infection generally goes asymptomatic and remains localized in the lungs. However, in immune-suppressed patients, yeast can disseminate to other organs, and preferably to neural systems, thus causing meningoencephalitis (Dromer and Lortholary, 2013; Kauffman, 2012d; Thompson and Patterson, 2018).

Clinical manifestations

Many patients with cryptococcal primary pneumonia are asymptomatic, including some immunocompromised patients. In contrast, symptomatic patients can present with fever, chest pain, dyspnoea, and dry cough. AIDS and other immunocompromised patients have a high risk of dissemination that can result in meningoencephalitis and infection of other organs. In this case, patients can suffer from severe headaches, nausea, lethargy, and nuchal rigidity among others signs (Dromer and Lortholary, 2013; Kauffman, 2012d; Thompson and Patterson, 2018).

Etiological diagnosis

The diagnosis could be made through the culture of yeast from sputum, blood, or cerebrospinal fluid, in the case of disseminated disease. *Cryptococcus* suspicious samples are incubated in conventional fungal media for 2 weeks but, in most cases, the organism grows in a few days. Identification can be made based on the capsular staining. Another useful test is a latex agglutination assay that detects cryptococcal polysaccharide antigen. This assay has high sensitivity and specificity in the blood and cerebrospinal fluid of patients with disseminated disease. Sensitivity is reduced in patients with non-disseminated disease. In addition, other commercial immunoassays are also available for detection of cryptococcal antigen, with similar efficacy to the latex assay (Kauffman, 2012d; Thompson and Patterson, 2018).

Treatment

The treatment of pulmonary non-disseminated cryptococcosis depends on the severity of the infection. Most patients have a mild infection and are usually treated with oral fluconazole for 6–12 months. In the case of severe infection or with cryptococcal meningitis, treatment includes an induction phase with amphotericin B and flucytosine for 2 weeks. Then, a consolidation phase with fluconazole for 8–10 weeks. In immunocompromised patients, maintenance treatment with fluconazole could be necessary for a long time. The treatment protocol for *C. gattii* is the same as for *C. neoformans*, albeit some authors prefer voriconazole instead fluconazole for the consolidation phase (Diaz, 2020; Dromer and Lortholary, 2013; Kauffman, 2012d; Thompson and Patterson, 2018).

Pneumocystis jirovecii

Pneumocystis jirovecii, formally called *P. carinii*, is a fungus that causes *Pneumocystis* pneumonia (PCP) in immunocompromised patients. *Pneumocystis jirovecii* has been listed for a long time as a protozoan but currently, based on large molecular genetics studies, is classified as ascomycete fungus. However, information on its life cycle and the route of infection remains unknown.

Serological studies show that pneumocystis has a worldwide distribution and anti-pneumocystis antibodies are found in most people at an early age. Pneumocystis seems to be transmitted by respiratory route between human hosts and there is no evidence of transmission from animals, water, soil, or fomites. Despite the fact that serological studies indicate that pneumocystis infection is widespread, immunocompetent hosts do not show any symptoms or signs of significant disease (Kovacs, 2012).

Clinical manifestations

Patients with AIDS or medically immunosuppressed patients are the primary target of pneumocystis pneumonia. Patients present with a fever, non-productive cough, and dyspnoea. Pulmonary symptoms could be accompanied by other systemic symptoms like fatigue and weight loss (Kazanjian, 2013; Kovacs, 2012).

Etiological diagnosis

Because pneumocystis cannot be cultured definitive diagnosis requires direct examination of the suitable sample. The preferred sample is sputum. Alternative samples can be obtained by bronchoalveolar lavage (BAL) or biopsy. Direct examination could be made by Giemsa or fluorescein-labelled antibody sample staining. Moreover, molecular PCR assays have been extensively evaluated but currently have a limited availability (Gigliotti and Wright, 2018; Kazanjian, 2013; Kovacs, 2012; Miller and Doffman, 2014).

Treatment

The most common treatment for PCP is trimethoprim-sulfamethoxazole (co-trimoxazole) for 2 to 3 weeks. In the case of patients with intolerance or toxicity to co-trimoxazole, an alternative treatment could be carried out using other combinations of drugs. Alternative effective treatments can be dapsone with trimethoprim, clindamycin with primaquine, or atovaquone alone. In patients with glucose 6-phosphate dehydrogenase deficiency, dapsone, or primaquine should be avoided because of an increased risk of hemolysis. In addition, adjunctive therapy with glucocorticoids could be useful and should be evaluated for patients at risk of respiratory failure (Gigliotti and Wright, 2018; Kovacs, 2012; Miller and Doffman, 2014).

Other fungus infections

Other species of fungus can cause opportunistic infections in specific situations or under special conditions, such as in immunosuppressed patients or pulmonary invasive procedures. Pulmonary infection by *Candida albicans* is very infrequent but some cases have been reported in immunosuppressed patients. Other types of fungus occasionally reported as etiological agents in atypical pneumonias are *Rhizopus*, *Geotrichum*, *Sporothrix*, and *Actinomyces* (Fraser et al., 2006).

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Respiratory Tract Infections: Parasites

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Respiratory infections caused by helminths

Helminths are designated as both parasitic and free-living worms. In humans, parasites determine the appearance of zoonoses related to socio-environmental living conditions. They constitute a global public health problem due to the high incidence and endemicity as well as the possibility of generating epidemic outbreaks. There are three mayor types of helminth causing respiratory infections (Table 1) (Cox, 2009; Pérez Arellano and Carranza, 2003; Shah et al., 2016).

Cestodes

Hydatid disease

This disease is a zoonosis of worldwide distribution (OPS, 2011). The most important species that affect humans are *E. granulosus* and *E. multilocularis*. They cause cystic echinococcosis (CE) and alveolar echinococcosis (AE). The disease has a distribution pattern that has remained unchanged for decades and continues to be endemic in many countries in South America, Africa, and Asia, with hundreds of cases being diagnosed annually. The regular deworming of dogs introduced in most countries has decreased human infestation, but the disease continues to be endemic. Hydatid disease integrates the list of diseases that WHO focuses on their elimination and/or control by 2050. New plans for the eradication and control of hydatid disease are continuously implemented, including vaccination and diagnosis in dogs (de Salud, 2015; Wen et al., 2019). *Echinococcus* spp. was traditionally defined as causing CE. However, at least 10 genotypes or strains have been currently recognized by DNA sequencing. The genus *Echinococcus* has the following species: *E. granulosus sensu stricto*, *E. canadensis*, *E. orthepi*, *E. felidis*, *E. equinus*, *E. multilocularis*, *E. oligathra*, *E. vogeli*, *E. shiquicus*. Moreover, without appropriate management, both CE and AE can be chronic diseases, leading to death. The definitive hosts are mainly canines that become infected by ingesting viscera with fertile hydatid cysts containing protoscolices larvae. In the dog's intestine, the larvae transform into adult worms that release eggs and mature proglottids that are eliminated through feces. This also leads to the contamination of the environment in which they are found. The eggs are ingested by an intermediate host (sheep, goats, swine, cattle) forming hydatid cysts mainly liver and lungs (Craig et al., 2007; McManus et al., 2012).

Human infection is produced by accidental ingestion of eggs, mainly in rural areas, where contact with the definitive infected host is recurrent. The ingested eggs hatch in the intestinal lumen, giving rise to oncospheres that, through enterohepatic circulation, reach the first filter, made up of the liver, where 70–90% of the cysts are located. Lung disease, which occurs in 15–20% of cases, can be caused by the arrival of the larvae directly to the lung via the blood or lymphatic route, where its maturation leads to the formation of the pulmonary hydatid cyst. Sometimes, pleuropulmonary invasion occurs by the extension of a hepatic cyst that in its growth can reach the pleural cavity.

The slow growth of the cysts can cause the pulmonary hydatid cyst to remains asymptomatic for years and become a radiological finding during general studies. On radiological images, the pulmonary hydatid cyst appears as a solid, rounded mass, which can sometimes present with the so-called meniscus sign or even as a complete hydro aerial image with the endomembrane floating

Table 1 Three mayor types of helminth causing respiratory infections.

Cestodes (taenia)	<i>Echinococcus granulosus</i>	<i>E. multilocularis</i>				
Trematodes (flatworms)	<i>Schistosoma mansoni</i>	<i>S. haematobium</i>	<i>S. japonicum</i>	<i>S. intercalatum</i> , <i>Paragonimus westermani</i>	<i>P. africanus</i>	<i>P. caliensis</i>
Nematodes (cylindrical worms)	<i>Ascaris lumbricoides</i> , <i>Toxocara canis</i>	<i>T. cati</i> , <i>Necator americanus</i>	<i>N. brasiliensis</i> , <i>Ancylostoma duodenale</i>	<i>A. caninum</i> , <i>Strongyloides stercoralis</i> , <i>Trichinella spiralis</i> , <i>Wuchereria bancrofti</i>	<i>Brugia malayi</i> , <i>Dirofilaria immitis</i>	

inside the cystic fluid. Nonspecific symptoms (cough, dyspnoea) may occur when the cyst reaches a diameter close to 10 cm. Symptomatic patients should be immediately investigated by imaging and serological studies. Rupture of the cyst to the pleural cavity (hydropneumothorax or pneumothorax) or even to the airway (vomiting) may occur, accompanied by symptoms such as fever, cough, and mild to fatal anaphylactic reactions.

Serology is a useful diagnostic test intended to detect antibodies in the serum of patients after surgery or drug treatment. Antigen B and Antigen 5 are widely used, particularly in developing countries, although there are doubts about their effectiveness for clinical detection and screening (Craig et al., 2007; McManus et al., 2012). The sensitivity of serology for the detection of serum antibodies is higher than for the detection of free circulating *E. granulosus* antigens. Current serology for human CE basically studies the earliest immunoglobulin IgG response against native or recombinant B antigen using the enzyme immunoassay (EIA) or western blotting. The definitive diagnosis of echinococcosis is carried out through ultrasonography, tomography, and magnetic resonance imaging (Menghi et al., 2017). The treatment for cure of the disease is surgery but it no averts a recurrence. Taking into account the slow growth, location, and possibility of calcification of the cyst, observation and waiting may be also an option. However, this decision is not supported by systematic studies that define its indication and limitations.

AE is a rare disease, caused by *E. multilocularis*. It is endemic in China, Russia, and in some countries in Europe (Holland, France, Germany). Increases in the fox population have determined the expansion of the parasite into habitually non-endemic areas. The final hosts are foxes, coyotes, and, less frequently, dogs. Humans are an intermediate host and they are accidentally infected via contaminated food or contact with animals. The cysts of AE settle predominantly in the liver, leading to the slow growth of multiple irregular cysts poorly delimited from the rest of the parenchyma. In its growth, it spreads to neighboring organs, and at a distance, by the lymphatic or hematogenous route, it can reach the brain, lungs, etc. Moreover, without treatment, death occurs in 100% of cases. The treatment of choice is surgical but is sometimes supplemented with deworming drugs (WHO, 2020).

Trematodes

Schistosomiasis

Schistosomiasis constitutes a public health problem in tropical and subtropical countries since it frequently affects young populations (OPS, 2011; Pérez-Arellano et al., 2006; Pérez Arellano and Carranza, 2003; WHO, 2002). In Africa, Central and South America the most frequent species are *S. mansoni* and *S. haematobium*, while *S. japonicum* predominates in Southeast Asia, China, and the Philippines. Immigrants and travelers are responsible for imported cases in countries where the disease does not exist.

All *Schistosoma* species have an intermediate cycle in freshwater snails, with humans being the definitive host. The form of contagion is by transcutaneous penetration of the cercariae, the intermediate form of the *Schistosoma*. Inside the body, the larvae develop into adult schistosomes, which live in the blood vessels, where the females lay their eggs. Some of those eggs leave the body with feces or urine and continue the parasite's life cycle, while others. Others become trapped in body tissues, where they cause an immune reaction and progressive organ damage. Two forms of schistosomiasis are recognized: intestinal and urogenital, which are caused by the five main species of *Schistosoma*. However, pulmonary manifestations may occur with early symptoms, during larvae transit through the pulmonary capillaries, or in late stages due to embolization of the pulmonary arteries by *Schistosoma* eggs. In the first stage, respiratory symptoms may appear with dyspnoea, dry cough, and wheezing, accompanied or not by fever. The most significant findings in blood tests are leukocytosis with eosinophilia. Nonspecific and diffuse infiltrates are common in radiological studies of the chest.

In advanced forms of the disease, embolization can cause arthritis of the pulmonary arteries, leading to pulmonary hypertension. Dyspnoea is the most characteristic symptom and on radiology, military-type micronodular infiltrates can be found.

For the definitive diagnosis, it is necessary to find the parasite's eggs in urine or stool samples (Ciddio et al., 2017).

Paragonimiasis

Paragonimus westermani is responsible for most infections, and the definitive host (humans) acquire it by ingesting raw crustaceans infected by larval forms of the parasite (metacercariae). *Paragonimus westermani* has an indirect cycle, with intermediate hosts first in freshwater snails and then in crustaceans (Kuzucu, 2006; OPS, 2011; Pérez-Arellano et al., 2006). Larvae arrive at the lung via migration from the intestine, more particularly through the peritoneal cavity. Once they reach the respiratory system, they perforate the diaphragm and colonize the pleural cavity and then the lungs. Once in the lungs, a fibrous layer forms around the larvae, which then continue their maturation, followed by the laying of eggs in the lung at 6–8 weeks (Pérez Arellano and Carranza, 2003).

In the first phase, infected patients may suffer from abdominal and/or pleuritic pain, which is generally bilateral. During pulmonary migration, irritative cough, chest pain, and blood expectoration appear. In the next phase blood expectoration typically becomes chocolate, and recurrent haemoptysis appears (Gómez-Seco et al., 2011).

Leukocytosis with eosinophilia is the most common laboratory finding in the early stages. Eggs of *Paragonimus* can be found in the expectorated material. Another way to establish the diagnosis in the late phase is through BAL or serological tests (ELISA is available).

Nematodes

Ascariasis and hookworm infections

Ascaris lumbricoides is one of the most prevalent parasites in humans (OPS, 2011; WHO, 2005), with a particular incidence in childhood. The infection is acquired by ingesting eggs present in other humans, food, water, or soil (Pérez-Arellano et al., 2006). Eggs reach the small intestine, releasing larvae that cross the intestinal wall and then reach the lungs via the hematic or lymphatic system. Once in the interior of the alveoli, larvae migrate upwards via the tracheobronchial tree until it reaches the larynx after which they are swallowed. In the digestive tract, larvae complete their maturation to adult worms, producing eggs that will be eliminated with feces. Respiratory symptoms develop in the lung phase of the parasite, between 9 and 12 days after the ingestion of the eggs (Coello Kuon Yeng and Rey Guevara, 2019; Dall'Orso et al., 2014). Infections are characterized by transient eosinophilic pulmonary infiltrates, called Löffler's syndrome. *A. lumbricoides* is the main cause of this syndrome worldwide. Symptoms such as irritative cough, retrosternal pain, and mild hyperthermia are frequent findings. Dyspnoea, and hemoptoic expectoration can occur in more severe cases. Approximately 50% of patients present crackling rales and wheezing, and urticaria may appear in the first 4–5 days after respiratory expression of the disease. Hepatomegaly may appear in some patients. The above-mentioned acute symptoms are usually self-limiting and generally remit in 5–10 days (Dall'Orso et al., 2014). Radiological findings consist of small rounded or oval infiltrates in both lung fields. These infiltrates are migratory, and generally disappear after several weeks. Worms produce irritation of the intestinal mucosa by mechanical action. The formation of tangles can lead to intestinal obstruction. At the respiratory system level, larval migrations induce TH2-type responses with increased IgE, IgG, and eosinophils; a decrease in TH1 lymphocytes favors susceptibility to bacterial infections. The increase in IgE favors infections by reducing the action of polymorphonuclear cells (PMN). In the liver and lung, larvae and their antigens determine a granulomatous reaction with central necrosis that facilitates the entrapment of bacteria and the formation of abscesses.

The definitive diagnosis of ascaris pneumonitis depends on the isolation of their larvae in respiratory secretions or gastric aspirate.

Ancylostoma duodenale and *Necator americanus* (OPS, 2011; Pérez-Arellano et al., 2006; Pérez Arellano and Carranza, 2003) are common causes of disease throughout the world. Its larvae enter via the skin or orally. Larvae migrate through lungs causing a hypersensitivity response, and symptoms similar to those caused by *Ascaris*.

Strongyloidiasis

Strongyloides stercoralis infection in humans occurs via penetration of larvae through the skin due to walking or working barefoot in places contaminated by human feces. Larvae enter the body and are carried via the blood into the lung. From the alveoli, they ascend to the airways, including the upper ones, where they are swallowed (Mascarini-Serra, 2011; OPS, 2011; Pérez Arellano and Carranza, 2003).

In the digestive tract, larvae turn into adult worms. The eggs produced by adult worms are eliminated through feces or they hatch into larvae that restart the cycle through the intestinal lumen. The latter is responsible for recurrent infections (self-infection) (Pérez Arellano and Carranza, 2003).

Transpulmonary larval migration can produce dry cough, pharyngeal irritation, dyspnoea, wheezing, and haemoptysis. Some patients with chronic strongyloidiasis experience recurrent episodes of fever and pneumonitis, asthma attacks (which worsen, paradoxically, with the use of corticosteroids) or dyspnoea due to restrictive disease pulmonary. The definitive diagnosis of pulmonary strongyloidiasis is based on the finding of larvae in sputum or BAL.

Trichinosis

This parasitosis is acquired by ingesting raw or semi-cooked meat, which in most cases is pig meat infected with *Trichinella spiralis* larvae (OPS, 2011; Pérez-Arellano et al., 2006). Trichinosis has an intestinal phase, followed by a muscular phase with encystment of larvae that remain viable for prolonged periods of time.

Pulmonary involvement occurs first by the passage of larvae through the pulmonary circulation and then by myositis of the main and accessory respiratory muscles (Valle-Velazco González, 2005).

In the first stage, symptoms are nonspecific (irritative cough) and radiology bibasal infiltrates that spontaneously resolve can be found. In the stage of myositis, deterioration of respiratory function, chest pain, and dysphonia may occur. Calcified intramuscular cysts are visible on a chest radiograph. A definitive diagnosis is based on the presence of larvae in a muscle biopsy.

Visceral larva migrans or toxocariasis

Visceral larva migrans or toxocariasis is a prevalent disease in childhood, especially in areas with high infestation of dogs and cats by *Toxocara canis* and *T. cati*. Humans are not a definitive host for this parasite and human infection occurs due to contact with infected

animals or by ingestion of soil contaminated by canine or feline feces (Mascarini-Serra, 2011; OPS, 2011; Pérez-Arellano et al., 2006). In the lung, larvae cause the formation of eosinophilic granuloma. Chronic cough and bronchospasm are common symptoms, as well as Löffler's syndrome and asthma attacks and/or wheezing. Radiology shows poorly defined consolidations and laboratory shows are characterized by leukocytosis with eosinophilia, increased IgE, and a positive ELISA. Rheumatoid factor and hypergammaglobulinemia may also be found.

T. canis does not complete its life cycle in the human and does stool examinations are negative. Hence, a definitive diagnosis is established by identifying larvae in biopsy samples.

Tropical pulmonary eosinophilia (filariasis)

The causal agents, *Wuchereria bancrofti* and *Brugia malayi*, are inoculated into humans (the exclusive host) by the bite of mosquitoes (*Culex quinquefasciatus*, *Aedes*, and *Mansonia*) that inoculate larvae. These larvae spread lymphatically and are mostly trapped in the lungs (OPS, 2011; Pérez-Arellano et al., 2006; Valle-Velazco González, 2005). At the respiratory level, affected patients usually develop a dry, nocturnal cough, and episodes of bronchospasm. Concomitantly, dyspnoea and weight loss appear.

Typical laboratory findings include leukocytosis with eosinophilia, a rise in IgE, and the presence of eosinophilic alveolitis in BAL. Antifilaria antibody test is positive. In chest radiography, there may be inhomogeneous opacity with a nodular reticulum pattern (up to 5 mm in diameter) and there may be a restrictive effect in the functional respiratory study (World Health Organization, 2014).

Dirofilariasis (heart worm)

Dirofilaria immitis is transmitted from dog to human through the bite of mosquitoes of different species and genera belonging to different parts of the world. Examples of such mosquitoes include *Culicidae*, *Anopheles*, and *Aedes* (OPS, 2011; Pérez-Arellano et al., 2006; Valle-Velazco González, 2005). The infection is not transmitted from person-to-mosquito-to-person nor from person-to-person because humans are "dead-end" hosts (Sileli et al., 2016). In the lung, infection can induce vasculitis of the pulmonary arteries if the worm lodges in its immature form in the right ventricle. The patient may be asymptomatic or present nonspecific symptoms: cough, fever, chest pain, and dyspnoea. The development of pulmonary nodules is considered to be due to an inflammatory reaction against the deadly parasite. On radiology, it appears as a solitary nodule on the periphery of the lung. It does not require specific treatment but should be considered as a differential diagnosis in the study of pulmonary nodules.

Respiratory diseases caused by protozoa

Amoebiasis

Entamoeba histolytica is a worldwide protozoan that is transmitted to man through contaminated food and water. Two stages are recognized in its life cycle: the invasive vegetative form amoeboid (trophozoite) and the cystic resistance and infective form (OPS, 2011; Valle-Velazco González, 2005). Amoebiasis has worldwide distribution and transmission occurs via ingestion of mature cysts present in contaminated food and water.

The main organ affected by *Entamoeba histolytica* is the intestine, causing amoebic dysentery. However, it also has extra-intestinal locations that affect the liver and lung (Cox, 2009). In this case, the infection can reach the thorax through blood from the primary intestinal lesion, or a liver abscess that, when it extends to the subdiaphragmatic space, reaches the pleural cavity or lung tissue. In the first case, it causes empyema and in the second a lung abscess. Other less frequent forms are hepatobronchial or biliopulmonary fistula. These conditions are always very serious, life-threatening situations that require urgent treatment. Clinical findings are supplemented through laboratory investigation of amoebic antigens by ELISA and PCR.

Malaria

Malaria is a life-threatening disease caused by parasites transmitted to humans by the bite of infected female mosquitoes of the genus *Anopheles*. It is a preventable and curable disease (Jiménez et al., 2005; OPS, 2011; WHO, 2019).

According to WHO data, more than 200 million people were infected with malaria during 2018. The disease has a high incidence in Africa, mainly affecting children under 5 years of age. The species causing malaria are *Plasmodium vivax*, *P. malariae*, *P. ovale*, *P. falciparum*, and *P. knowlesi*.

The biological cycle of malaria

Plasmodium needs two hosts to complete its cycle. During the hematophagy process, the female *Anopheles* mosquito inoculates the sporozoites into the human. Sporozoites infect liver cells and multiply by schizogony, leading to the formation of merozoites. After multiplication in the liver (exoerythrocytic schizogony), the parasite invades the red blood cells and reproduces by schizogony (erythrocytic schizogony). Merozoites invade red blood cells. Annular trophozoites become schizonts that will give rise to new merozoites. Some parasites are sexually differentiated (gametocytes). When the parasite is in the blood, the clinical manifestations of the disease appear. Male (microgametocyte) and female (macrogametocyte) gametocytes are ingested by an *Anopheles* mosquito during hematophagy. The multiplication of the parasite in the mosquito is known as the sporogonic cycle. Microgametes fertilize

macrogametes and develop zygotes. The zygote becomes a mobile ookinete, invades the wall of the intestine, and becomes an oocyst. The oocyst develops sporozoites inside, which once released ascend to the salivary glands. Inoculation of sporozoites into a new host perpetuates the malaria cycle.

In general, lung involvement is part of a general systemic affection, with high parasitemia, multiple organ failure, and severe acute respiratory distress. The diagnosis of malaria is based on the finding of plasmodium in a thick drop of blood. Species differentiation however requires a fine drop of blood.

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Urinary Tract Infections: Virus

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Introduction

Thousands of human viruses challenge human life daily by affecting virtually any anatomic site or function. The urinary tract is no exception. Urologic practice copes frequently with bacterial urinary tract infections (UTIs), but most Urologists are not familiar with urinary infections of viral origin. These are usually seen in immunocompromised patients, especially in solid organ and stem cell transplantation recipients.

While it is assumed that no bacteria should be found in the urine of healthy people, especially in men, some viruses can be found in healthy, asymptomatic, and immunocompetent patients. For example, the polyomaviruses JC virus (JCV) and BK virus (BKV) infect the majority of humans asymptotically, but are an increasing cause of morbidity and mortality in immunocompromised patients. The prevalence of BKV and JCV infection ranges from 65% to 90% and from 20% to 60% in the adult population, respectively (Knowles, 2006). Despite this difference in prevalence, JCV is more commonly found in the urine of healthy adults, making BKV excretion a more sensitive indicator of immune status. Therefore, the presence of an identifiable viral organism with inflammatory symptoms is required to define a viral infection.

Viral infections can cause clinical syndromes of the lower (bladder) or upper urinary tract (kidneys, ureters). The most common presentation is hemorrhagic cystitis. The clinical frame and the natural history of UTIs depend on anatomic abnormalities and more importantly on the immunologic status of the host. Whereas mortality among healthy patients with UTIs is extremely low, viral UTIs with high viral load can result in multiorgan failure and high risk of mortality in immune-compromised patients. The kidneys can also be the target of several glomerular syndromes associated with chronic (HBV, HCV, HIV) or acute/subacute (Dengue, Hantavirus, etc.) viral infections through a direct or immune-mediated injury. As a final topic, the involvement of the urinary tract by a SARS-CoV-2 infection is investigated. The aim of this chapter on viral UTIs is to present an overview and to provide a practical approach supporting clinical decisions.

Epidemiology

Recent diagnostic improvements led to identify viruses in many more clinical settings as compared to the past. As such, it was found that viral infections are a common cause of hemorrhagic cystitis. Hemorrhagic cystitis is the most common presenting symptom of viral UTI. Hemorrhagic cystitis is a well-recognized complication of hematopoietic stem cell transplantation (HSCT) with an incidence of 15–25%. It comprises a wide range of symptoms varying between microscopic hematuria and severe hemorrhage.

In a large series of 407 adult patients undergoing allogeneic HSCT, hemorrhagic cystitis was viral-induced in 87.5% (Dosin et al., 2017). Similarly, in a prospective study of more than 102 children undergoing bone marrow transplantation, a viral cause was identified in all children with hemorrhagic cystitis (Gorczyńska et al., 2005).

Although the prevalence is lower than in bone marrow transplantation recipients, renal transplantation is another setting for viral UTIs. Human polyomavirus, Adenovirus, and Citomegalovirus, account for the vast majority of viral infections.

Polyomavirus

Polyomaviruses are small, non-enveloped viruses, which are widespread in nature and highly adapted to grow in the species and the tissue they infect. BK and JC viruses are human polyomaviruses infecting most people throughout the world, as shown by the presence of antibodies against viral proteins in about 80% of the human population (Pinto et al., 2014).

BK virus (BKV)

Polyomavirus primary infection occurs during childhood in 60–100% of general population, with approximately 97% of the adult population having antibodies against it (Demeter, 2000). BK Virus (BKV) has a significant homology to JCV, a neurotropic virus causing progressive leukoencephalopathy. It can be acquired through a respiratory or oral route. While the primary infection is usually asymptomatic, the virus seeds the urinary system and the brain, often remaining in a latent form (Shah, 2000). BKV has a urotheliotropic nature and can be identified in the epithelium of the collecting ducts and of the transitional tissue. Extensive experience over the past 3 decades has shown the prevalence and clinical importance of BKV reactivation in patients with bone marrow transplantation (BMT) and solid organ transplantation. The first evidence of BKV in BMT was reported in 1981, with the detection of BKV in the urine of 13 patients, 1 to 6 weeks after transplantation. The typical cytopathologic changes of BKV are often found in a healthy person, and it is believed to represent transient shedding of virions in urine, most likely secondary to stress or decreased immune response. The patient's cytopathologic changes resolve within 3 months, and there seems to be no clinically significant sequelae of BKV-positive cytology in immunocompetent people. Different disease processes have been linked to BKV infection, but the two most important diseases are polyomavirus-associated nephropathy (PyVAN) affecting kidney transplant patients and polyomavirus-associated hemorrhagic cystitis (PyVHC) affecting allogeneic hematopoietic stem-cell transplantation (HSCT) patients. Other pathological processes that have been associated with BKV are ureteric stenosis, encephalitis, meningoencephalitis, pneumonia, vasculopathy and bladder cancer. The following sections are dedicated to BKV-related syndromes affecting the urinary tract.

a) *Polyomavirus-associated hemorrhagic cystitis (PyVHC)*

Hemorrhagic cystitis (HC) is a frequent complication after HSCT. According to the time of occurrence after HSCT, HC is defined as early- and late-onset. Early-onset HC occurs typically during or within 48 h after the end of conditioning regimen, and it is the result of a direct toxic effect of drug metabolites and radiotherapy on the bladder mucosa. Late-onset HC usually starts around the time of the period of neutrophil engraftment (weeks 2–4) or in second-third month after HSCT (Hirsch and Pergam, 2016a, b). The concurrent presence of prohemorrhagic abnormalities of coagulation, severe thrombocytopenia and mucosal inflammation are predisposing factors for any type of HC.

The pathogenesis of BKV is not well understood as similarly high urine BKV loads are found in kidney transplant patients, most of whom do not develop cystitis or gross hematuria (Funk et al., 2008; Leboeuf et al., 2017). Rather, a sequence of events has been suggested, starting with subclinical urothelial damage by the conditioning regimen, high-level BKV replication leading to viral denudation of the pre-damaged regeneration-impaired urothelial lining and urinary leakage in the submucosa followed by hemorrhagic exacerbation with abundant inflammatory cell infiltrates following allogeneic stem cell engraftment (Bedi et al., 1995; Li et al., 2013). Indeed, conditioning involving cyclophosphamide, busulfan and total body irradiation has been implicated, and the pronounced toxic, inflammatory properties of cyclophosphamide and its metabolite acrolein are supported by clinical and experimental studies (Romih et al., 2001).

Adenovirus, JCV, citomegalovirus (CMV) and other infectious (bacteria, parasite) and non-infectious etiologies (bleeding disorders with or without low platelet count, primary or metastatic neoplasia, vesical catheter or ureteric stenting) may also cause hemorrhagic cystitis, and must be excluded for appropriate diagnosis and management (Hirsch and Pergam, 2016a, b). The severity of hematuria is commonly described as microscopic (grade 1); macroscopic (grade 2); macroscopic with clots (grade 3); or macroscopic with clots and postrenal failure secondary to urinary tract obstruction (grade 4).

b) *Polyomavirus-associated nephropathy (PyVAN)*

The first reports describing disease resembling polyomavirus-associated nephropathy (PyVAN) in kidney transplant patients, came as early as 1978 (Coleman et al., 1978). However, it was not until the late 1990s that PyVAN emerged and that BKV was found to be its etiologic agent (Binet et al., 1999). At that time, new and more potent immunosuppressive regimens were being taken into use, capable of decreasing the rejection rates at the expense of increased risk of opportunistic infections like BKV. PyVAN affects between 1 and 10% of kidney transplant patients during the first 2 years post-transplantation. PyVAN rarely affects patients other than kidney transplant recipients, and so far only 26 cases of biopsy confirmed PyVAN, in native kidneys of other immunocompromised patients, have been reported (Sharma et al., 2013). This clearly suggests that there must be risk factors associated with the allograft and not only with the suppressed immune status. Prognostication is complicated by the many putative risk factors, including HLA-mismatch, donor gender (female) and age (high), recipient of male gender, type and dose of immunosuppressive medication and transplantation events such as ureteric stents, steroid exposure and acute rejection (Brennan et al., 2013). The pathogenesis of PyVAN is characterized by high-level BKV replication in the renal-tubular epithelial cells of the transplanted kidney leading to cytopathogenic loss and thereby denudation of the epithelial monolayer in the allograft tubulus. As a consequence, the virus leaks into the tissue and bloodstream and inflammatory cells infiltrate the interstitium leading to tubular atrophy and interstitial fibrosis. This reduces the graft function and increases the risk of graft loss. Of note, the urothelial cells may also play an important role in PyVAN. Mathematical modeling of BKV replication in kidney transplant patients with PyVAN suggests that viral replication starts in the renal tubular epithelial cells but is then carried to the urothelial cell compartment where more than 90% of urine BKV loads are generated (Funk et al., 2008). This is supported by histopathologic data revealing extensively infected urothelial cells in the bladder of patients with PyVAN. In agreement with this, primary human urothelial cells from bladder were found to be very permissive to BKV infection.

c) Ureteric stenosis

BKV was first identified in the urine of a renal transplantation recipient with the initials B.K. by Dr. Sylvia Gardner in 1971: the kidney transplant patient from whom BKV was initially isolated, was suffering from ureteric stenosis (Gardner et al., 1971). Since then, there have been several reports on BKV-associated ureteric stenosis, usually in kidney transplant patients, both pediatric (Rajpoot et al., 2007) and adult (Coleman et al., 1978) but also in allogeneic HSCT patients (Hwang et al., 2013). The pathogenesis of ureteric stenosis is still not completely resolved. Coleman suggested in 1978 that high-dose steroids, given to kidney transplant patients post-transplantation, permitted reactivation of BKV (Coleman et al., 1978). The ureteric epithelium which was damaged by ischemia or inflammation supported infection and was replaced with granulation tissue. In a study of kidney transplanted cynomolgus monkeys receiving immunosuppression, a reactivation of a new polyomavirus called cynomolgus polyomavirus occurred in 12–57 monkeys (Van Gorder et al., 1999). The virus was detected in the urothelium of graft ureters in association with inflammation and in the smooth muscle cells of the ureteric wall showing signs of apoptosis. A significant incidence of late onset stenosis was seen. Apparently, ureteric stenosis is now less frequently reported, possibly due to better surgical techniques and a decline in the use of ureteral stents (Hirsch, 2005).

JC virus (JCV)

BKV and JCV have been shown to establish latency in several tissues including the urinary system. In renal transplant patients and more generally in those suffering from immune system impairments, human polyomaviruses can on rare occasions cause tubulo-interstitial nephritis. Nevertheless, data regarding the ability of JCV to induce ureteral and bladder damage are very scarce (Di Maida et al., 2021). The triggering mechanisms for JC virus reactivation are not well defined yet. It has recently been suggested that there is a higher risk of JC virus reactivation in immunocompromised patients and those treated with immunomodulatory drugs for chronic inflammatory disorders, hematological malignancies, or following organ transplantation (Kartau et al., 2019). However, drug specific causality is difficult to assess, since most patients receive multiple immunomodulatory medications concomitantly or sequentially, or present with several immunocompromising factors related to their underlying disease. At the level of the urinary tract, the association between patient immune status and JCV pathogenicity appears even more ambiguous, due to the limited available evidence. Moreover, low levels of JC viruria can also occur in approximately 13–20% of healthy individuals (Boukhoum et al., 2016), making the interpretation of data even more difficult.

The central nervous system and kidney are considered as the reservoirs of JC virus infection, in which the virus can persist in latency after primary infection. The possible role of JC virus in urinary tract involvement has only recently been recognized. Based on laboratory findings and PCR studies, several authors have long considered BK virus as the only polyomavirus eventually affecting the kidneys and urinary tract. Indeed, although genomic sequences of both JCV and BKV DNA have been detected by PCR in renal biopsies of transplanted patients, combined immunohistochemical and molecular biology studies suggested that renal impairment was secondary to the replication of BK virus, whereas JC virus was usually present as a co-infecting agent in a latent, non-replicative phase (Boldorini et al., 2001). As such, JC virus has long been considered unable to elicit pathological effects.

Nickeleit et al. (2000) proposed that JC viral particles produced after a viral lytic cycle in the renal pelvis or ureter may enter the capillary vessels and infect the tubular cells of the kidney or, alternatively, follow an ascending route of infection from transitional cells to kidney tubular cells. Conversely, Boldorini et al. (2003) first reported morphological evidence of lytic JC virus infection in four renal specimens from AIDS patients, supporting the hypothesis that, in immunocompromised patients, active JC viral replication can also occur in the kidney and urinary tract. JC virus-related nephropathy is now recognized as a rare cause of nephropathy in transplant recipients (Wiegley et al., 2021). Ureteral and bladder involvement is more rarely reported in different types of immunosuppressed patients (Cavallo et al., 2007).

Diagnosis of polyomavirus

As the initial presentation of PyVAN is insidious, it is strongly recommended to screen kidney transplant patients regularly for early diagnosis (Hirsch, 2005). Screening should be performed at least every 3 months the first 2 years after transplantation and then annually until the fifth year of post-transplantation. Screening is usually accomplished by quantitative PCR of urine and/or plasma for detection of high-level BKV viruria $\geq 7 \log_{10}$ copies/mL or viremia $\geq 4 \log_{10}$ copies/mL (Randhawa et al., 2004). Alternatively, cytological examination of urine in search of decoy cells (Mackenzie et al., 1978) or electron microscopy in search of viral aggregates can be performed (Singh et al., 2009). Plasma PCR has a higher positive predictive value than urine PCR as episodic viruria is quite frequent in this patient group, while viremia is less common and usually precedes PyVAN. Usually the diagnosis of PyVAN requires a histological demonstration of BKV replication (Purighalla et al., 1995). However, due to the focal nature of PyVAN, a negative biopsy result cannot rule it out (Hirsch and Pergam, 2016a, b; Brennan et al., 2013). To distinguish PyVHC from the more frequent early-onset hemorrhagic cystitis occurring prior to the engraftment due to urotoxic conditioning or total body irradiation, the triad of cystitis, hematuria (grade II or more) and high-level BKV replication with urine loads of $\geq 7 \log_{10}$ GEq/mL, must be present.

Treatment of polyomavirus

No specific anti-viral therapy has been developed to treat PyVAN. Thus, early treatment before the development of irreversible histopathological damage are important to ensure a favorable prognosis. The AST (American Society of Transplantation) guideline recommends starting interventions at the high-level BK viremia stage, which is indicative of probable or presumptive PyVAN (Hirsch et al., 2019). Since no BKV-specific antiviral therapy has been developed, reducing the level of maintenance immunosuppression is the most common and effective treatment for BK viremia or PYVAN. Currently, the AST guideline recommends two strategies (Hirsch et al., 2019): (1) first reduce the dose of the calcineurin inhibitor by 25–50% in one or two steps, and then reduce by 50% and ultimately discontinue the antimetabolites; (2) first reduce the antimetabolites by 50%, and then reduce the calcineurin inhibitors by 25–50% and discontinue the antimetabolites. At this time, the “calcineurin inhibitor first” and “antimetabolite first” approaches (as the first step) are considered largely equivalent (Hirsch et al., 2019; Bischof et al., 2019; Hardinger et al., 2010).

In addition, several medical treatments have been tried, including intravenous immunoglobulin (Piburn and Al-Akash, 2020), cidofovir (Kuten et al., 2014), and fluoroquinolone (Gabardi et al., 2010). Cidofovir (Vistide®, Gilead, Foster City, CA, USA), is an intravenously administered nucleoside analog of deoxycytidine monophosphate that is licensed by the U.S. Food & Drug Administration for the treatment of cytomegalovirus (CMV) retinitis in AIDS patients. In 2000, a patient with PyVHC and simultaneous CMV infection was successfully treated with cidofovir and shortly after it was reported to be useful in the treatment of one patient with PyVAN (Bjorang et al., 2002). The drug is taken up via the organic anion transporters (OAT1), which are mainly expressed on the basolateral side of renal tubular epithelial cells. Cidofovir is nephrotoxic and has less potent anti-BKV activity than the other therapies. Intravenous immunoglobulin through osmotic injury causes vacuolation in proximal tubular epithelial cells, in turn leading to acute kidney injury (Levy and Pusey, 2000). Fluoroquinolones are synthetic broadspectrum antimicrobial agents targeting the bacterial enzymes topoisomerase II and IV (158) and are also suggested to interfere with the helicase activity of BKV LTag (Ali et al., 2007). Critical aspects of Fluoroquinolone in this setting are related to their gastrointestinal and central nervous system side effects, given the lack of significant effects on BKV replication and hemorrhagic cystitis severity, and the selection of antibiotic resistance (Walker, 1999). None of these treatments have sufficient clinical data supporting their use as standard practices. PyVAN sometimes accompanies acute rejection; these cases are difficult to treat. Due to the lack of effective anti-viral drugs, the main therapeutic strategy is to reduce immunosuppression; at the same time, clinicians must pay attention to the risk of allograft rejection.

Therapy for PyVHC is purely supportive, involving symptom relief by analgesia, hyperhydration to increase diuresis and continuous bladder irrigation to prevent clot formation and urinary tract obstruction (Hirsch et al., 2019). Moreover, lost platelets and erythrocytes are substituted. Other treatments of PyVHC aim at repair and regeneration of the urothelial mucosa through hyperbaric oxygen therapy or by topical application of fibrin glue. The main drawback of hyperbaric oxygen is its limited availability on a larger scale, the requirements for dedicated hyperbaric room facilities, the risk of ear barotrauma/pressure intolerance and claustrophobia linked to the procedure (Hosokawa et al., 2014). Finally, topical applications to the damaged bladder mucosa to achieve hemostasis through cystoscopy have been reported in single-center retrospective series of 35 patients. The complete response rate was 83% (Tirindelli et al., 2014). Similarly, several compounds to reduce bleeding have been used in case studies and small series, e.g. FXIII concentrate (Demesmay et al., 2002), intravesical sodium hyaluronate (Cipe et al., 2009), estrogens (Ordemann et al., 2000) or choreito extract granules (Kawashima et al., 2015). The same adjuvant treatments have been tried for PyVHC as for PyVAN again without any documented benefit.

As the understanding regarding the pathogenesis of PyVHC is evolving, new potential therapeutic targets are expected to emerge. Recently, Schneidewind et al. reported the successful use of an innovative three-dimensional (3D) organotypic cell culture model for studying BKV life cycle. Such culture methods will highlight the interactions between BKV and human urothelial cells which might unveil the unknown nuances associated with BKV pathogenesis. Furthermore, they might facilitate the in vitro testing of antiviral drugs against new potential therapeutic targets (Schneidewind et al., 2020a, b).

In a recent experimental study, a novel 3D cell culture approach was used to study BKV pathogenesis and potential new therapeutic targets. The results favored the involvement of the STAT3 pathway in the pathogenesis of BKV infection, and IL-11 was recognized as the potential therapeutic target. The utilization of antibody against IL-11 showed promising in vitro activity against

BKV. Although tocilizumab (an anti-IL-6 antibody) did not yield significant results in this study, its role in the treatment of BKV infection will need detailed further exploration, as IL-6 is an important component of the STAT3 pathway (Schneidewind et al., 2020a, b). Likewise, it may be expected that further experimental studies focussed on the STAT3 pathway in BKV infection might unveil more potential therapeutic targets.

Synopsis and current recommendations on BKV management

Polyomavirus BK persistently infects the majority of people at an early age, usually without causing disease. Pathological consequences appear mainly in immunodeficient individuals, an expanding patient group due to transplantations, use of immunosuppressive therapy and the increasing average age of the population. However, the management of PyVAN and PyVHC differs.

Safe and effective antiviral treatment of BKV diseases is still lacking and for patients with PyVAN, the only treatment strategy with documented effect is reduction of immunosuppressive therapy. On the other side, PyVHC remains a challenging complication after allogeneic HSCT with average rates of 13%. Its diagnosis requires the clinical and laboratory triad of cystitis, gross hematuria and high urine BKV loads $7 \log_{10}$ copies/mL, but a significant role of other etiologies must be excluded. Plasma BKV loads 1000 copies/mL has a role for the management and follow-up of triad positive allogeneic HSCT recipients. Screening of asymptomatic HSCT patients at risk remains an area of investigation and is presently not recommended, as pre-emptive therapy is not established (DII). Specific antiviral prophylaxis is not available and fluoroquinolones are not recommended. PyVHC treatment is based on the best supportive therapy, such as hyperhydration, bladder irrigation, platelet transfusions as needed to reduce bleeding, and pain treatment, particularly when using myeloablative conditioning based on cyclophosphamide, busulfan and total body irradiation. The risk/benefit ratio of reduction of immunosuppression is not clear in PyVHC and must be balanced with the risk of worsening or triggering an acute graft-versus-host disease. Antiviral treatment with intravenous cidofovir is controversial due to the absence of randomized controlled studies. Until the availability of safe and effective antivirals, the use of cidofovir may be an option although there is uncertainty of efficacy, the best dose schedule and the need to balance any benefit against its renal side effects. Non-specific measures aimed at speeding the healing process of the damaged urothelial lining such as hyperbaric oxygen therapy or urologic fibrin glue application have been successful in a limited number of uncontrolled studies. No recommendation is possible for several other treatments such as the administration of intravesical sodium hyaluronate, intravenous FXIII concentrate, leflunomide, estrogens, mesenchymal cells and cellular immune therapy because these treatments have only been used sporadically in a very limited number of patients or are still experimental. These evidence-based recommendations are applicable for both pediatric and adult patients. In conclusion, despite much progress in understanding the pathogenesis, epidemiology and risk factors of PyVHC, this complication still represents a disabling unmet clinical need with limited prophylactic and therapeutic options. To overcome this deficiency will require novel antiviral treatment approaches supported by proper clinical trials.

Our institutional BKV protocol in transplanted patients

BKV PCR-based search is performed every 3 months in the first year post-transplantation, every 6 months in the second year, and annually thereafter.

In case a billion copies/mL is found in the urine, BKV is searched in the blood. The threshold for an active treatment is a BKV plasma level of $5 \log_{10}$ copies/mL. The search for SV40 on renal tissue is also performed in order to quantify the renal injury.

Our strategy in case of clinically significant BKV infection is based on three progressive steps:

- 1) reduction or suspension of antimetabolite therapy (mycophenolate)
- 2) switch calcineurin-inhibitor therapy from tacrolimus to everolimus
- 3) antiviral therapy with levoflunamide, in case the above steps were ineffective

Adenovirus

Adenoviruses (ADV) are double-strand DNA viruses with at least 51 serologic subtypes. They are known to cause upper respiratory, gastrointestinal, and conjunctival infections in healthy people and children; however, their pathogenicity is altered by the immunologic status of the host, and in immunocompromised patients, ADV can affect many other systems (Ison, 2006). Human ADV are classified into 7 species (A–G) and 67 types (1–67). Types 1–51 were identified by serotyping, whereas types 52–67 were identified by genomic sequencing and bioinformatic analysis. As the assay for ADV detection covers only a proportion of the currently known virus types, the occurrence of ADV infection of the urinary tract may have been underestimated. In addition, ADV has been identified as the causative agent of various diseases, including acute upper respiratory inflammation, pneumonia, pharynx conjunctivitis, gastroenteritis, hepatitis, and myocarditis (Echavarria, 2008). Normally, ADV causes asymptomatic infection of lymphoepithelial tissues, but in the immunocompromised patient, they can reactivate the latent infection or cause de novo infection. The adenoviral infections are more common in stem cell transplantation and solid organ transplantations. ADV can be detected in 10% of urine samples after transplantation, and over 12 months of follow-up, adenoviral UTIs occurred in 9% of patients (Runde et al., 2001). ADV are ubiquitous infectious agents that cause significant morbidity and even loss of life, particularly in select groups of at-risk individuals. Troubling outbreaks of ADV infection occur far too frequently, and these can also affect otherwise healthy individuals not normally at risk for serious infection.

Diagnosis

Adenoviral cystitis can present as gross or microscopic hematuria in up to 20% of patients (Allen and Alexander, 2005). History of solid organ or bone marrow transplantation and use of immunosuppressants aids in diagnosis because ADV cystitis occurs almost exclusively in immunocompromised patients. Most cases of ADV-related hemorrhagic cystitis occur within 12 months of transplantation (Hofland et al., 2004). The adenoviral infection can often coexist with Aspergillosis and CMV in immunocompromised patients, and broad cultures should be obtained. A urine bacteriological culture, urine cytology, and the presence of urine ADV and BKV DNA were examined in those with a diagnosis of urinary tract infection based on symptoms of macrohematuria and dysuria (i.e., urinary frequency, urgency, and micturition pain). A definitive diagnosis of ADV infection of the urinary tract should be confirmed by positive results for the presence of ADV DNA in the urine. In addition, blood culture and the presence of ADV DNA in the blood were examined in patients with fever. ADV DNA in the urine and blood is detected using a qualitative polymerase chain reaction assay (Lion, 2014) for ADV types 1–6, 8, 19, and 37; types 7 and 11 were also included because of hemorrhagic cystitis. BKV DNA in the urine is also detected using a qualitative polymerase chain reaction assay. Furthermore, it is necessary to perform a cystoscopy, computed tomography (CT), and/or magnetic resonance imaging (MRI) of the abdomen and pelvis in cases of prolonged hematuria, and renal graft biopsy in cases of renal graft dysfunction.

Treatment

Unfortunately, until recently there was no single antiviral drug that would be potent and devoid of drug toxicity. For treatment of ADV infection of the urinary tract after renal transplantation, reduction in immunosuppression is necessary, similar to other viral infections (Asim et al., 2003). Both B and T lymphocytes play an important role in recovery from ADV infection. Therefore, if clinical improvement cannot be achieved by the reduction or discontinuation of mycophenolate only, dosages of calcineurin inhibitors, such as tacrolimus or cyclosporine, should be reduced. Moreover, an antiviral effect can be expected following intravenous immunoglobulin administration, particularly in cases of hypogammaglobulinemia. Antiviral therapies include CDV, ganciclovir, ribavirin, and brincidofovir. The primary anti-viral agent for the treatment of ADV infections is CDV. Cidofovir can display a dose-limiting nephrotoxicity, and the incidence is relatively low (6.9%). Intravesical administration of cidofovir achieved favorable outcomes in case of non-response to intravenous cidofovir or renal failure.

The findings also suggested that this method could be an alternative to intravenous administration of cidofovir to reduce nephrotoxicity. Owing to less nephrotoxicity and high potency against all clinically significant ADV subtypes, brincidofovir is an attractive antiviral therapy for the management of ADV disease (Painter et al., 2012). However, given the small sample size, more studies are expected to provide important evidence for the efficacy of brincidofovir (Lion et al., 2010). An appreciable benefit among patients with various manifestations of ADV disease was found to be associated with ribavirin, although the small number of cases in the studies limits these conclusions. Based on current data, there is no recommendation regarding the use of ganciclovir for ADV systemic infection but there is possible benefit of ganciclovir against urinary tract infection. Previous study observed that hemorrhagic cystitis and nephritis probably can generally be treated only with reduction in immunosuppression without specific antiviral therapy (Florescu et al., 2013).

The realization that we need more effective and safer antiviral drugs for HAdV infection seems to have passed a critical threshold in recent years, and the surge of studies in the field of anti-ADV development is encouraging. The past decade has seen exponential growth in the field of ADV antivirals. However, there is still ground to be covered before an effective therapy is clinically available. Progress in this field is intimately related to the strength and power of the tools available. Specifically, drug screening platforms and model organism systems for preclinical testing are critical tools to advance research on ADV antivirals (Mackenzie et al., 2021). In conclusion, although urinary tract infection is a common complication after renal transplantation, a differential diagnosis of ADV infection should be considered. ADV infection can be cured, although nephritis or viremia might be developed. Hence, the possibility of exacerbation should always be considered. After a definitive diagnosis of ADV infection of the urinary tract, adequate follow-up observation should be conducted, and diligent and aggressive therapeutic intervention is required before the condition worsens.

Cytomegalovirus

Cytomegalovirus (CMV) infection is common, and more than 60% of adults are seropositive. CMV belongs to a large group of herpes viruses with limited pathologic significance in immunocompetent patients. CMV reactivation or new infection is common in transplantation patients, and it can cause significant mortality and morbidity; hence CMV prophylaxis is commonly employed in patients after transplantation. CMV is a relatively rare cause of lower UTIs; however, circumferential evidence supports the association between CMV and hemorrhagic cystitis (Spach et al., 1993). Although rare, CMV cystitis can also occur in immunocompetent patients (Basquiera et al., 2003). Hemorrhagic cystitis has been clearly associated with reactivation of CMV (Childs et al., 1998). CMV is also believed to cause ureteritis and ureteral stenosis (Mueller et al., 1995).

Cytomegalovirus (CMV) is the most common viral infection following kidney transplantation. Without prophylaxis, CMV disease occurred in 45% of recipients with high-risk CMV serostatus (donor CMV seropositive and recipient CMV seronegative) (Lowance et al., 1999). CMV infection has been associated with reduced renal allograft function and survival (Kliem et al., 2008; Helanterä et al., 2006). However, invasive CMV disease involving the renal allograft is apparently uncommon. This may be

attributed to the rarity of detecting CMV inclusion in renal allograft biopsies (<1%) despite the presence of concurrent renal dysfunction and CMV disease. The spectrum of renal allograft pathology caused by CMV can demonstrate tubulointerstitial changes or glomerular involvement. Tubulointerstitial nephritis caused by CMV is the more common manifestation in renal allograft. CMV glomerulopathy, which can occur with or without concomitant tubulointerstitial disease, has been reported (Rane et al., 2012). A case of CMV-induced necrotizing and crescentic glomerulonephritis in renal transplant patient has been described (Detwiler et al., 1998).

Diagnosis

CMV can be detected by seroconversion in a previously negative host and increase in IgM and IgG antibodies titers (Kalil et al., 2005), but a high prevalence of latent CMV infection makes serologic diagnosis difficult, and detection of CMV antigen (pp65), RNA, or DNA is commonly used. The pp65 antigenemia and real-time PCR have been compared with each other, and real-time PCR seems to correlate better with the clinical picture (Mengoli et al., 2004).

Treatment

Letermovir has been shown in a placebo-controlled randomized trial to decrease the risk for clinically significant CMV infection (need for preemptive antiviral therapy and/or CMV disease) and also decrease all cause mortality in CMV (+) patients. Most patients receive prophylaxis with ganciclovir after solid organ transplantation; however, in active diseases, other drugs, such as foscarnet, 60 mg/kg IV twice daily for 14 days, can be used because they are active against CMV (Bielorai et al., 2001). Ganciclovir can reduce the risk for CMV disease but is associated with significant toxicity. High-dose acyclovir/valaciclovir can reduce the risk for CMV replication. The data regarding prophylactic Ig is conflicting and its use is currently not recommended.

Ganciclovir (valganciclovir) and foscarnet are the most used drugs for CMV disease. The addition of high-dose Ig for treatment of CMV pneumonia has been commonly used, but the data supporting this combination is limited. There is no data supporting the addition of Ig to antiviral treatment for other types of CMV disease. Cidofovir is also a possibility for second- or third-line antiviral therapy (Ljungman et al., 2019), whose duration has to be decided on a case-by-case basis, but normally longer therapy is needed compared to preemptive therapy (6–8 weeks). Cidofovir is active against both BKV and CMV and has been used if both viruses are detected. For this reason, cidofovir may become a drug of choice in patients who present with hemorrhagic cystitis after solid organ or bone marrow transplantation. New antiviral drugs are in development but have not been proven efficacious on this indication. Leflunomide and artesunate have been tested, but data supporting their use is very limited.

Several groups have tried to prevent or treat CMV infection and disease following allo-HSCT by the transfer of CMV-specific T-cells. The transfer of these cells was shown to reconstitute virus-specific T-cell immunity. When given therapeutically to patients with chemotherapy-refractory CMV infection, a drop in the viral load after an increase in the number of CMV-specific T-cells could be documented. In patients with chemotherapy-refractory CMV infection postHSCT, adoptive T-cell therapy is a valid therapeutic option (Ljungman et al., 2019).

Human papilloma virus

Recently, human papillomavirus (HPV) has been suggested as a causative agent for squamous cell carcinoma (SCC) of the urinary bladder (Kim et al., 2014). Whether HPV also plays a significant role as etiological factor in other urinary cancers is less clear.

HPV is a double-stranded circular DNA virus of approximately 8000 base pairs, containing eight genes; E1, E2, E4, E5, E6, E7, L1, and L2 (Narisawa-Saito and Kiyono, 2007). The L1 gene encodes the principal capsid protein. Today, approximately 120 different genotypes are known. To be classified as a genotype the sequence of the L1 region has to be >10% different from the L1 region in any other HPV genome. The most common types, among women with HPV DNA positive cervical uterine cancer, are in descending order of frequency, HPV 16, 18, 45, 31, 33, 52, 58, and 35. Furthermore, HPV can be classified into low risk (LR), probable high risk (pHR), and high risk (HR) HPV types according to their prevalence in benign and malignant lesions where causality has been established (Munoz et al., 2003). The tumor suppressor p16INK4a (p16) is well known to be associated with HR HPV infection in cervical uterine cancer and oropharyngeal cancer, which is why it has been suggested as a surrogate marker for HPV infection in bladder cancer.

HPV is a common sexually transmitted infection among women and men, with a life-time risk of infection of approximately 80% for sexually active women. The virus is linked to almost 10% of cancers worldwide, especially a subset of squamous cell carcinoma of the cervix uteri, vulva, penis, anus, and oropharynx. It is therefore anticipated that the virus has affinity for squamous cells. In as much as 90% of cases, the HPV infection is cleared spontaneously by an effective cell-mediated immune response. HPV evades the innate immune system, delaying activation of the adaptive immune system which is the agent responsible for removal of the HPV infection. The remaining 10% with a persistent infection thus have increased hazard of oncogenic progression in case of an HR HPV infection (Stanley, 2010).

A geographical variety in HPV prevalence exists. The HPV prevalence has been presented to be highest in Asia. Compared with this, a significantly lower prevalence was demonstrated in Europe. Genetic difference, differences in environmental risk factors (including sexual behavior) and other unknown sources may explain such geographical variety. Comparing studies from different regions of the world should be taken into consideration such geographical variety (Li et al., 2011).

Studies investigating the simultaneous presence of HPV DNA and p16 overexpression have shown as high as 94% incidence in bladder cancer and 8.8% in other urinary with squamous cell differentiation. In the latter study on 35 cases with a HPV prevalence of 17.1%, a high p16 overexpression of 45.7% was demonstrated (Kim et al., 2014). A meta-analysis including 37 studies with 2246 cases of bladder cancer focused on the topic of an association between p16 and prognosis or clinicopathology in bladder cancer cases. The meta-analysis showed a downregulation of p16 in bladder cancer (Gan et al., 2016). Consequently, the knowledge on the association of BC, HPV, and p16 is even more uncertain than the association between HPV and bladder cancer.

Association between HPV presence and positive p16 staining seems to be equally unclear and, therefore, negative p16 staining cannot rule out HPV infection in bladder cancer tissue. Although HPV infection has been found in bladder cancer studies, it is important to keep in mind that such an association is not equivalent to causation. Thus, clear etiological association between HPV and bladder cancer development cannot be established at this point.

Viral-induced glomerulonephritis

Viruses are capable of inducing a wide spectrum of glomerular disorders with variable immune response and different histologic forms of glomerular injury. Although emerging data emphasizes the role of occult viral disease in the development of glomerulonephritis (GN), the criteria to define a viral illness should include: the demonstration of active viremia primarily through serologic PCR testing, the identification of viral proteins/nucleic acid residues within renal tissue and/or within immune complexes deposited in the glomerular basement membrane, and the regression of the glomerular lesion concomitant with the host immune clearance of viremia or eradication of viremia through antiviral therapy. The present classification is based on the duration of active viremia, chronic or acute/subacute.

Glomerular syndromes associated with long-term chronic infections

HCV

Similar to HBV, HCV-associated glomerular disease is primarily a consequence of viral antigen – immune complex formation with glomerular basement membrane deposition. The histologic aspect include type 1 membranoproliferative glomerulonephritis (MPGN) as a consequence of type 2 mixed cryoglobulinemia, polyarteritis nodosa from immune complex deposition, mesangial proliferative and focal proliferative GN and IgA nephropathy. It is important to note that most renal diseases in HCV patients with liver disease are a consequence of acute tubular necrosis, hepatorenal syndrome, prerenal azotemia, chronic kidney disease, and not GN. Therefore, it is essential to be aware of the characteristics of acute GN in HCV patients, differentiating them from the more common diagnoses causing acute kidney insufficiency in this population (Kupin, 2017a, b). Oral direct-acting antivirals (DAAs) against HCV replaced Interferon and became the mainstay of treatment, providing long-term successful eradication of HCV and elimination of cryoglobulinemia. A variety of drugs in this class have been developed that are used in combination over a 12-week period without Interferon and result in sustained viral remissions of 95% (Sise). It has also been proved that persistent HCV infection was independently associated with chronic kidney disease, and that antiviral treatment with IFN plus ribavirin can improve renal function and reverse chronic kidney disease (Zhang et al., 2019).

HIV

The HIV pandemic involves 37 million active carriers in the world, significantly less than the 240 million carriers of HBV and the 170 million carriers of HCV. The differential diagnosis of glomerular disease in this setting will be influenced by the treatment status in relation to combined Antiretroviral Therapy (cART-naïve versus actively on cART), and by their coinfection status with HBV (5–10%) or with HCV (25–30%). As with HCV, the prerequisite need for ongoing active viremia is essential for the development of HIV-related glomerular syndromes: HIV-associated nephropathy (HIVAN) and HIV immune complex disease of the kidney (HIVICK) (Sise et al., 2016).

HIVAN embraces glomerular injury arising from direct viral infection, particularly the visceral and parietal epithelial cells, without the presence of immune complexes. The lifetime risk of HIVAN in an untreated HIV-infected black patient is 2–10%, rising to 50% in homozygous for the APOL1 variants (Kruzel-Davila et al., 2016). It should be considered in any cART-naïve HIV patient of black race ancestry with any degree of abnormal albuminuria or proteinuria. New emerging therapy for HIVAN includes renin-angiotensin-aldosterone system inhibition, interruption of the mTOR pathway, and retinoic acid derivatives (Kumar et al., 2010).

Unlike HIVAN, HIVICK includes a constellation of histopathologic diseases with a different presentation and prognosis (Booth et al., 2016). It derives from the deposition of immune complexes in the glomerulus and it is a complication of active HIV viremia in a cART-naïve patient or a patient with cART resistance. Like HIVAN, HIVICK glomerular lesions from coinfecting HIV patients with HBV or HCV are not considered HIVICK and are classified separately as secondary lesions due to their individual viruses. Because 50% of the lesions are postinfectious, these would not be expected to improve with cART, whereas the remaining immune complex glomerular diseases should theoretically show benefit depending on their degree of chronicity. However, the heterogeneity of HIVICK and the limited number of studies makes it difficult to make a recommendation on the benefit of cART (Foy et al., 2013).

HBV

Overall, renal disease may occur in 3–5% of patients with chronic HBV infection (Gupta and Quigg, 2015). Immune-complex GN (MPGN and IgA nephropathy) or immune complex–related vasculitis (polyarteritis nodosa) are the histologic manifestations of HBV-associated renal disease. Of note, many patients with chronic HBV may also harbor coexistent HIV (5–10%) and HCV (10–30%). Checking for these viruses in all HBV carriers is essential (Basnayake and Easterbrook, 2016). Antiviral agents and corticosteroids are mainstay of treatment. The Kidney Disease Improving Global Outcomes recommends the use of IFN or oral antiviral agents (tenofovir, adefovir) or nucleoside (lamivudine, entecavir, telbivudine) reverse transcription inhibitors (KDIGO, 2012). Corticosteroids may be given for a period of <6 months without a significant effect on liver disease, HBV viremia, or patient morbidity or mortality as long as concomitant antiviral therapy is used (Zheng et al., 2012). Universal HBV vaccination reduced those childhood cases of HBV membranoproliferative related to horizontal transmission of the virus (Liao et al., 2011).

Glomerular syndromes associated with acute/subacute viral infections

This focus is on individual types of glomerular lesions seen with a variety of acute and subacute viral infections including cytomegalovirus (CMV), parvovirus, Epstein–Barr virus (EBV), hantavirus, and dengue. An accurate differential diagnosis in case of GN should rule out acute or chronic viral infections. Familiarity with the main renal syndromes associated with viral diseases is essential in order to begin accurate testing and therapeutic interventions (Kupin, 2017a, b).

Although any acute viral illness can lead to an immune complex–proliferative glomerulonephritis (GN), a select group of viral syndromes can induce glomerular injury: dengue, hantavirus, and non-HIV viruses associated with collapsing FSGS (cFSGS) (parvovirus, EBV, and cytomegalovirus).

Dengue

Dengue is a worldwide infection with estimated 100 million new per year. It is classified into dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. GN in dengue results not only from immune complex deposition but also from direct viral entry into renal tissue (Lizarraga and Nayer, 2014). Treatment remains supportive in all categories of dengue.

Hantavirus

Hantaviruses are RNA viruses of the *Bunyaviridae* family which are associated with two major syndromes: hanta pulmonary syndrome and hemorrhagic fever with renal syndrome, characterized by diffuse endothelial cell injury as a consequence of direct viral infection. Similar to dengue, no specific therapy is available for hantavirus infection but preventive strategies with widespread vaccination are undergoing clinical trials (Kruger et al., 2015).

Collapsing focal segmental glomerulosclerosis

Viral-induced Collapsing Focal Segmental Glomerulosclerosis (cFSGS) has long been associated with parvovirus B19, CMV, and EBV. These viruses can cause acute immune complex GN especially with vertical transmission and membranous glomerulopathy has been the most common renal histology reported in this setting. In addition, CMV causes a distinct tubulointerstitial nephritis in transplant patients and may play a role in the development of transplant glomerulopathy (Beneck et al., 1986).

Covid-19 and urinary tract involvement

The current pandemic has seen a new character arising from the multitude of human viral pathogens. SARS-CoV-2 has been shown to be a global player, potentially involving any anatomic site or function. Although the abundance of the virus genetic material in both urine (ca. 10^2 – 10^5 gc/mL) and feces (ca. 10^2 – 10^7 gc/mL) is much lower than in nasopharyngeal fluids (ca. 10^5 – 10^{11} gc/mL) (Jones), it is believed that urinary symptoms are caused by a direct action of SARS-CoV-2. Urinary frequency has been identified as an additional symptom of SARS-CoV-2 infection independent of acute renal injury or urinary tract infection in a small series of hospitalized patients (Mumm et al., 2020). It has been also suggested that the urinary tract may become the target of life-threatening involvement by SARS-CoV-2, especially in patients with pre-existing urologic conditions (Luciani et al., 2020). This assumption is based on the finding that the bladder urothelium, as well as the kidney, harbors cells expressing ACE2, the receptor for the viral spike protein (Zou et al., 2020; Sise et al., 2020). In the face of a possible prolonged or recurring waves of the pandemic, clinicians should be aware that a nontypical course of hematuria or other urinary symptoms might be related to a COVID-19 infection. Although the likelihood of infection due to contact with sewage-contaminated water is low, it has also been speculated that a risk of transmission might occur in clinical and care home settings where secondary handling of people and urine/fecal matter occurs (Jones et al., 2020).

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Urinary Tract Infections: Fungi (*Candida* spp.)

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Glossary

Balanitis Infection of the penis head.

Candiduria Defined as the detection of ≥ 1000 CFU/mL of yeast from at least 2 urine cultures; urinary symptoms can be present (symptomatic) or absent (asymptomatic).

Community-associated UTI (CAUTI) UTI that is acquired outside hospital setting or at least the patient is not admitted in the hospital for more than 48 h.

Complicated UTI UTIs that are complicated by several risk factors that can result to treatment failure, recurrence of infection, significant morbidity and even mortality.

Cystitis Bladder inflammation, usually as a result of infection.

Dysuria Pain or discomfort when urinating.

Healthcare-associated UTI (HAUTI) UTIs acquired from the hospitals (nosocomial).

Hematogenous Distributed or spread via the bloodstream.

Lower UTI UTI localized to the lower urinary tract (bladder, prostate and urethra).

Non-*albicans* *Candida* species (NACS) Clinically-relevant *Candida* spp. that is not *Candida albicans*.

Perianal Area around the anus.

Prostatitis Infection of the prostate that may present symptoms such as pain in the groin, painful and difficulty urinating.

Pyelonephritis Inflammation of the kidney arising as a result of infection often accompanied by fever.

Uncomplicated UTI UTIs where there are no relevant functional or anatomical abnormalities presented and no relevant concomitant diseases promoting the UTI. The risk of uncomplicated UTI developing serious complication is low.

Upper UTI UTI localized to the upper urinary tract (kidney).

Urethritis Inflammation and irritation of the urethra, usually due to infection.

Urinary Tract Infection (UTI) Infection of the urinary tract, which can be either upper or lower UTI, uncomplicated or complicated.

Urogenital Refers to both urinary and genital organs.

Vulvovaginal candidiasis (VVC) *Candida* infection localized to the vulvovaginal area of the female reproductive organ.

Introduction

Urinary tract infections (UTIs) are among the most common microbial infections in humans with an annual worldwide prevalence of approximately 150 million people affected and an estimated lifetime incidence of 50–60% among women (Medina and Castillo-Pino, 2019). Worldwide, there were roughly 8.3 million office visits and more than 1 million hospitalizations to treat UTIs with an overall associated annual cost >\$1 billion. The prevalence of UTIs increases with age though they remain a significant cause of morbidity in different human population groups ranging from neonates/infants, older men, and women of all ages. In a European prevalence survey from 2016 to 2017, UTIs accounted for 18.9% of healthcare associated infections (HAI) in acute care hospitals (ACH) and 32% in long term care facilities (LTCF) (Suetens et al., 2018). Although most infections were of bacterial etiology, fungal-related UTIs represented a significant proportion of the total UTI cases (Medina and Castillo-Pino, 2019).

Among the fungal species frequently isolated, *Candida* spp. remain the most important. *Candida* spp. represent the fourth leading cause of hospital-acquired infections in the US and account for about 8–10% of all nosocomial blood stream infections (Wisplinghoff et al., 2004; reviewed in Pfaller and Diekema, 2007). The overall mortality of systemic *Candida* spp. infections can be as high as 40% (reviewed in Kullberg and Arendrup, 2015). The increasing trend of fungal infection has been attributed to the widespread use of broad-spectrum antibiotics and immunosuppressive drugs like corticosteroids, the increase in the number of HIV and AIDS patients, the application of immunosuppressing medical procedures such as chemotherapy, and the alarming epidemics of obesity and diabetes (reviewed in Pfaller and Diekema, 2007; Kullberg and Arendrup, 2015; Bongomin et al., 2017). Although the majority of the reports regarding fungal UTIs are focused on *C. albicans*, the most important representative species of the *Candida* group, the contribution of other *Candida* species collectively known as Non-*albicans Candida* Species (NACS), which include *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and *C. auris* to UTI pathogenesis is not well understood despite the increasing incidence of NACS-related genitourinary tract infections (Dorko et al., 2002; Powell et al., 2016; Mekanjuola et al., 2018; Biagi et al., 2019; Jabeen et al., 2020). Compared to *C. albicans* and *C. glabrata*, literature describing the fundamental biology of NACS is limited and currently an area of active investigation (Butler et al., 2009; Lackey et al., 2013; Deorukhkar et al., 2014; Feistel et al., 2019; Du et al., 2020).

Candida spp. exist as natural commensals and routinely colonize mucosal surfaces, the external genitalia, and urethral meatus even of healthy premenopausal women (Sobel, 2011). *Candida* spp. have been found in 1% of “clean” voided urine specimens in a healthy population, 5% of all urine cultures in the hospital setting, and 10% of urine isolates in a tertiary care facilities (Nayman Alpat et al., 2011; Rivett et al., 1986). Recently, *C. auris*, first identified in Japan in 2009 from an external ear canal discharge from a female patient, has received global recognition as an emerging and serious global health threat, especially in nosocomial settings, due to their highly invasive nature and multidrug resistance properties (Biagi et al., 2019; Du et al., 2020; Lockhart et al., 2017; Iguchi et al., 2019). It should be noted that other fungal species such as *Cryptococcus neoformans* (Poloni et al., 2013), *Aspergillus* spp. (Flechner and McAninch, 1981; Rao, 2015; Shirwany et al., 1998; Hadaya et al., 1998; Zhou et al., 2017), *Rhizopus* spp. (Barnes et al., 2017), *Mucor* spp. (Perez de la Espejo et al., 2004; Mathew et al., 2019), *Trichosporon asahii* (Sood et al., 2006; Kumar et al., 2011; Sun et al., 2012; Khan et al., 2015; Iken et al., 2015; Urs et al., 2018) and *Blastomyces* spp. (Vahidi et al., 2018) have been found in very rare instances of UTIs.

UTIs can be categorized according to the region of the urinary tract affected; they can either be upper or lower urinary tract infections, which can be presented with or without symptoms. Upper UTIs involve the kidney (pyelonephritis), whereas lower UTIs involve the bladder (cystitis), urethra (urethritis), and prostate (prostatitis) in men (Najar et al., 2009) (Fig. 1). In actual clinical practice, differentiating between these two regions is often difficult as infection often spreads from one area to the other. In most cases, the term UTI is used to describe pyelonephritis and cystitis, although urethritis and prostatitis are infections that also involve the urinary tract. UTIs can occur as a result of ascending or through hematogenous spread. In ascending UTIs, the infection originates in the lower urinary tract; for example, the vulvar region (urethral region included) in women, which may be periodically colonized by *Candida* spp. originating from the rectum as a result of migration across the perianal area (Nayman Alpat et al., 2011; Tomczak et al., 2014). Usually, ascending UTI occur in patients with in-dwelling catheters where *Candida* can form biofilms, facilitating their persistence for extended times. These organisms can ascend to the bladder or in rare cases, to the kidney, causing infection in all of these regions. Of grave concern is once *Candida* spp. have infected the kidneys, the infection can spread to neighboring tissues or to distant regions via the bloodstream (candidemia). Most observational studies have found that candiduria (presence of yeast cells in urine) in asymptomatic patients does not commonly lead to candidemia (1.3–8%), and in cases where candiduria progressed to candidemia, a urinary tract obstruction was found, the consequence of a urinary tract procedure (Kauffman et al., 2000; Bougnoux et al., 2008; Ang et al., 1993). *C. albicans* prostatitis occurs infrequently in patients with diabetes. In hematogenous routes, renal (kidney) infection can occur as a result of dissemination from the blood, likely during invasive candidiasis. Other complications of *Candida* infection include the appearance of fungus balls in the renal pelvis, ureter, or bladder that can lead to obstruction in these areas. Papillary necrosis and intra-renal and perinephric abscesses may form resulting in reduced kidney function.

Less common, the sources of the infection have been traced to sexual activity, originating from an infected asymptomatic partner carrying *Candida*. In males, the penile foreskin can harbor *Candida*, which can be a source of infection during intercourse (Lisboa et al., 2010a,b; Wray et al., 2020). Although they share some general overlapping symptoms and affect the urogenital tract and may predispose the infected individuals to UTI development when left untreated, it is important to note that the common yeast infection affecting the genitals of women (vulvovaginal candidiasis; VVC) and men (candida balanitis; CB) are distinct types of infection.

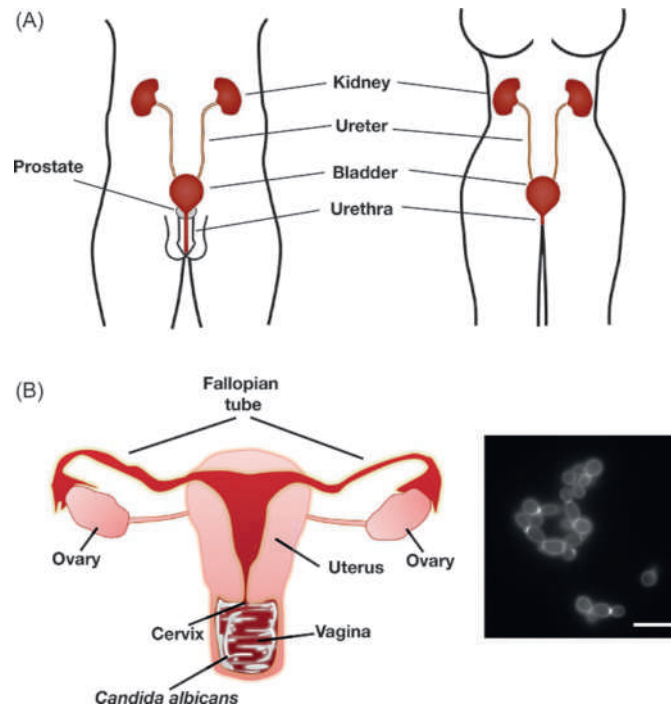


Fig. 1 (A) Male (left) and female (right) urinary tracts. (B) *Candida albicans* infection in the vaginal cavity of women causing vulvovaginal candidiasis (VVC). (Right) *C. albicans* yeast cells stained with the fluorescent cell wall stain, calcofluor white. Scale bar = 10 μ .

Candida spp. as a major etiological agent of UTI in humans

Candida spp. belong to the phylum *Ascomycota*, subphylum *Saccharomycotina*, which includes a great variety of human pathogenic fungi. Most pathogenic yeasts that include *C. albicans* and *C. tropicalis* belong to the CTG clade where they decode CUG (CTG) codons as serine instead of leucine. The second main group of pathogenic yeasts such as *C. glabrata* belong to the Nakaseomyces clade, one of the so-called post-WGD (post whole genome duplication) lineages where the genomes of member species have undergone a complete duplication and is largely represented by the non-pathogenic brewer's yeast, *Saccharomyces cerevisiae* (Gabaldon et al., 2016). The non-canonical codon usage of *C. albicans*, as of all CTG-clade species, represents one of the major challenges that has hampered genetic studies of these *Candida* species. As already mentioned, of the *Candida* spp., *C. albicans* is the most frequent etiology of fungal infection in humans. *C. albicans* are integral part of our natural microbiome and can be found inhabiting the skin and mucosal surfaces, and is detectable in the majority of healthy individuals. On standard yeast-peptone-dextrose (YPD) or Sabouraud Dextrose (SD) nutrient plate, *C. albicans* (and other *Candida* spp.) form white- to cream-colored colonies (Fig. 2A, left). Differential medium, such as CHROM-Pal agar, can be used for the presumptive identification of *Candida* spp. from clinical samples (Fig. 2A, right) (Sahand et al., 2009).

C. albicans can exist in different cellular morphologies inside the human body, and hence it is known as a pleomorphic organism (Fig. 2B). Important are the three major morphologies: yeast, pseudohyphae, and true hyphae, where the latter two are classically known as filamentous morphologies (Sudbery, 2011; Berman, 2006). Morphological transitions, will be discussed in more detail later in this review. Yeast-like cells can exist in various morphotypes. In addition to the well-known white phenotype, yeast cells can become opaque, which are elongated cells characterized by "pimple"-containing structures on their cell surface. More recently, the GUT and Grey yeast morphotypes have been identified and have been implicated in both commensalism and virulence (Noble et al., 2017). In addition, in certain conditions like starvation, *C. albicans* can form a large distinct cellular structure at the terminal cells of mycelia known as the chlamydo-spore, the function of which is not fully understood (Noble et al., 2017; Gow, 2013).

C. albicans was among the first eukaryotic pathogens with a completely sequenced genome. Other *Candida* spp. that have important clinical significance such as *C. glabrata* and *C. tropicalis* have also been sequenced. *C. albicans* is a diploid (2N) yeast with a haploid genome size of around 16 MB distributed to 8 chromosomes (Chromosomes R and 1–7) (van het Hoog et al., 2007; Jones et al., 2004). The genome is highly polymorphic and exhibits considerable natural heterozygosity. *C. albicans* is a naturally occurring diploid organism that predominantly reproduces asexually by budding. Although long considered to be an obligate diploid, *C. albicans* can exist and tolerate discrete ploidy states, ranging from the haploid state to tetraploid; each state associated with distinct cellular properties (Noble et al., 2017; Ene and Bennett, 2014) (Fig. 2C). Like the closely related brewer's yeast, *Saccharomyces cerevisiae*, *C. albicans* possess a and α mating type alleles at a conserved mating type locus (MTL). Unlike *S. cerevisiae*, *C. albicans*

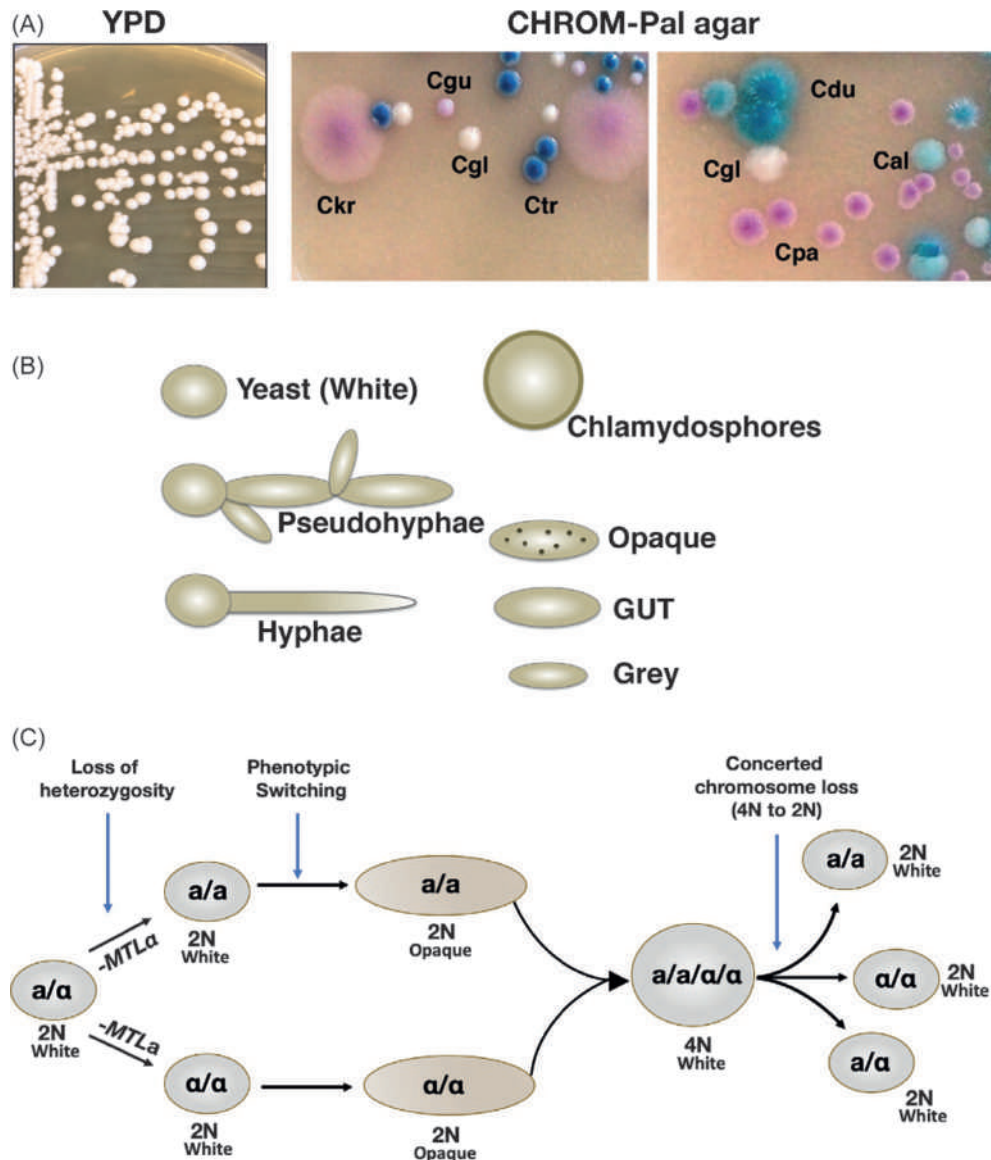


Fig. 2 (A) (Left) *Candida albicans* (SC5314 laboratory strain) colonies grown on YPD agar for 48 h at 30 °C. (Right) Colony color and morphologies of different *Candida* spp. growing on CH-Pal agar medium. Cal, *C. albicans*; Cdu, *C. dubliniensis*; Cgl, *C. glabrata*; Cgu, *C. guilliermondii*; Ckr, *C. krusei*; Cpa, *C. parapsilosis*; Ctr, *C. tropicalis*. (B) Pleomorphism in *C. albicans*. *C. albicans* can assume different morphologies ranging from the classical yeast (white) form to the filamentous pseudohyphal and hyphal forms. The yeast-like form can exist in distinct morphotypes such as the opaque, GUT, and grey phenotypes. In addition, *C. albicans* can form large spherical suspensor structures at the end of hyphal tips known as chlamydospores. Most of these forms (e.g., yeast and hypha) have been implicated in virulence whereas the functions of the other forms are relatively less understood and are actively investigated. (C) Parasexual cycle of *C. albicans*. Parasexual cycle in *C. albicans* involves the Mating Type-Like Locus (*MTL*) that is similar to *S. cerevisiae* *MAT* locus. The *MTL* locus is naturally heterozygous ($MTLa/MTL\alpha$) (Ene and Bennett, 2014; Bennett, 2015). However, under certain conditions such as growth on Sorbose, one of the *MTL* alleles is eliminated due to the loss of one chromosome 5, resulting in an aneuploid diploid cell that possesses only *MTLa* or *MTL\alpha* allele (Noble and Johnson, 2007). An epigenetic change can facilitate the phenotypic switching from the white (a/a or α/α) to the opaque phenotype (a/a or α/α). Opaque cells are mating-competent and can mate forming a tetraploid ($a/a/\alpha/\alpha$), which subsequently revert to the diploid state, and sometimes to aneuploid progeny, through concerted chromosome loss (Noble and Johnson, 2007). (A) Image adapted from Sahand IH, et al. (2009) Evaluation of CHROM-pal medium for the isolation and direct identification of *Candida dubliniensis* in primary cultures from the oral cavity. *Journal of Medical Microbiology* 58(Pt 11): 1437–1442.

appears unable to undergo meiosis (Ene and Bennett, 2014; Bennett, 2015). However, under conditions of stress, either chromosome loss or rare gene conversion events at the *MTL* result in a or α aneuploid, or a/a or α/α diploid strains, respectively, that can mate in a parasexual manner. This involves the formation of unstable tetraploid (4N) progeny that then revert to the diploid state via concerted chromosome loss (Noble and Johnson, 2007).

Candida albicans pathogenicity mechanisms

As a human commensal, *C. albicans* can asymptotically colonize virtually all anatomical sites in the body, including the skin, the oral and oropharyngeal cavities, the gastrointestinal and urogenital tracts, each serving as discrete niches (Kullberg and Arendrup, 2015; Moyes and Naglik, 2011). Relative to other sites, the vagina represents an entirely distinct microenvironment in terms of nutritional composition, competing microbiota, local environmental stress, and presence and rigor of innate immune defenses (Brown et al., 2014). Clearly, *C. albicans* needs to properly adapt to this environment to ensure successful colonization, and later on establish infection (Brown et al., 2014). The capacity of *C. albicans* to successfully infect a niche is attributed to a large set of virulence factors that include the expression of adhesins facilitating adhesion to host cells, biofilm formation, secretion of hydrolytic enzymes and cytolytic peptides (i.e., Candidalysin), morphological switching between the yeast and filamentous forms, and phenotypic switching (Fig. 3). Recently, other fitness attributes, including tolerance to host-imposed stress, efficient nutrient acquisition systems, and the presence of robust and flexible metabolic machinery, are now considered contributing factors to *C. albicans* pathogenicity (Brown et al., 2014; Mayer et al., 2013; Malavia et al., 2017). Interestingly, some of these attributes are also linked to the pathogenicity of other clinically-relevant *Candida* spp. such as the filamentous growth (*C. tropicalis* and *C. dubliniensis*), adhesion to epithelial surfaces (*C. glabrata*), phenotypic switching (*C. tropicalis*), tolerance to host-imposed stress (*C. glabrata*), and metabolic flexibility (*C. glabrata*).

Adhesion and invasion

Adherence to host tissues is often considered as an essential early step in the colonization of different niches. A critical step during colonization of the urinary tract is the ability of *C. albicans* to adhere to epithelial cells in the vagina, urethra, bladder, or even kidney (Lentz, 2009). Adherence to host cells is primarily attributed to cell surface glycoproteins encoded by the *ALS* (agglutinin-like sequence) and *HWP1* genes acting as multi-functional adhesive molecules (Hoyer, 2001). The *C. albicans* *ALS* gene family consists of eight genes, *ALS1*–*ALS7* and *ALS9* (Hoyer, 2001), one of them (i.e., *ALS3*) is known to encode for a glycoprotein that is also a downstream effector of hyphal growth that facilitates adhesion and flocculation of *C. albicans*. Another well-characterized adhesin in *C. albicans* is Hwp1, a hyphae-specific cell surface protein, initially synthesized as a glycosyl-phosphatidylinositol (GPI) anchored protein that eventually becomes covalently cross-linked to $\beta(1,3)$ -glucan via $\beta(1,6)$ -glucan in the cell wall (Staab et al., 2004). Hwp1 mediates tight adherence to epithelial cells by a mechanism that requires the participation of a specific host cell surface protein. The N-terminal domain of Hwp1 serves as a substrate for mammalian transglutaminases that crosslink this protein to the protein present in the host cell surface (Staab et al., 2004). Null mutations in *HWP1* (*hwp1* Δ/Δ) impair the capacity of *C. albicans* to adhere to oral epithelial cells and display reduced virulence in a murine systemic infection model (Staab et al., 1999). In *C. glabrata*, the process of tissue adherence is associated with glycoproteins encoded by the *EPA* (epithelial adhesion) genes.

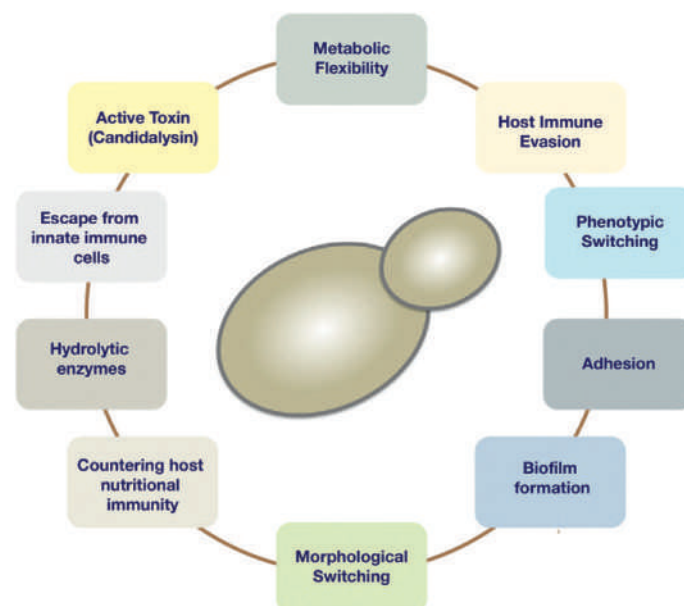


Fig. 3 Virulence factors of *Candida albicans*. *C. albicans* can show a plethora of virulence factors that enables it to colonize and later on establish infection in a certain host niche. Virulence factors that are linked to *C. albicans* pathogenicity include the capacity to undergo morphological (yeast to hypha) switching, adhere and invade host cells, secrete hydrolytic enzymes to degrade host tissues, secrete a cytolytic toxin (candidalysin), form complex biofilms, assimilate trace nutrients (e.g., trace metals) that are actively withheld by the host (known as nutritional immunity), escape or persist in the phagosome of innate immune cells such as macrophages, phenotypic switching which enables mating, and the presence of a robust and flexible metabolic machinery.

Morphological switching

In response to a large repertoire of environmental cues, *C. albicans* cells can reversibly switch between yeast and filamentous (hyphae and pseudohyphae) morphologies (Fig. 4A). Well-studied inducers of morphogenesis reflecting the diversity of microenvironments within the host include serum, CO₂, certain nutrients (e.g., glucose, amino acids, *N*-acetylglucosamine), temperature, and nitrogen starvation (Cottier and Muhlschlegel, 2009). Yeast forms are oval cells that asymmetrically divide into mother and daughter cells by axial or bipolar budding. Pseudohyphae and hyphae are two distinct morphologies that can easily be confused with each other, as pseudohyphae can sometimes superficially resemble true hyphae; both filamentous forms exhibit similar long elongated structures. However, these two forms are quite distinct. Hyphae, for example, initiate as germ tubes at a bud site, and assume polarized growth with elongated cells with completely parallel sides that lack constrictions at the sites of septation, whereas pseudohyphae are much wider and become clearly constricted at every septal junction (Fig. 4A; Sudbery, 2011). The yeast form is well-tolerated by the host and are maintained at low numbers on the vaginal epithelial surface by several mechanisms that also inhibit the transition to the filamentous form. In the laboratory, when this capacity to switch is genetically disabled in *C. albicans*, for example by inactivating transcription factors Efg1 and Cph1 that are important for transducing hyphal-inducing signals to target genes, *C. albicans* fail to mount infections as assayed in a number of in vitro and in vivo infection models. Cumulated results consistently support the concept that morphogenesis is an essential virulence attribute (Sudbery, 2011; Lo et al., 1997; Sudbery et al., 2004; Lorenz et al., 2004). Other *Candida* spp., such as *C. tropicalis* and *C. dubliniensis* are known to form true hyphae though they are considered less robust than *C. albicans*. *C. glabrata* does not form hyphae or pseudohyphae, even under conditions that trigger robust hyphal growth in *C. albicans* (Fig. 4B).

As will be discussed later in this review, the higher incidence of *Candida* UTI in women is linked to the vagina being an important reservoir for *C. albicans*. The vaginal environment is dynamic (Fig. 5). The vaginal epithelium together with associated physiological secretions (vaginal fluid/cervical mucus) and resident commensal bacterial microflora contribute to maintaining an acidic environment that represses filamentation of *C. albicans* (Boskey et al., 1999; Gorodeski et al., 2005). However, the vaginal epithelium (matrix) represents an inherently conducive environment for morphological switching of fungal cells. Particularly, the physical contact to epithelial surfaces, the constant temperature of 37 °C and elevated levels of CO₂ are established factors that favor filamentous growth (Biswas et al., 2007). In sexually mature women the vaginal epithelium undergoes transient changes that can induce filamentation. The pH of cervical mucus is usually acidic (pH of 4–4.5; non-inducing), however during menses it increases to pH 6.8 (inducing). The introduction of seminal fluid, which is alkaline (pH of 7–8.5), can transiently raise the pH within the vagina (Masters and Johnson, 1966). Serum present during menstruation and the deposition of seminal fluid during coitus introduces high concentrations of known morphogenic factors, including amino acids and proteins, and in the case of seminal fluid, *N*-acetylglucosamine (GlcNAc) (Alvarez et al., 2015). Morphological transition to hyphae enables *C. albicans* to strongly adhere to, then penetrate the mucosal layer and become intertwined with the vaginal epithelium resulting to hyperemia (increase in organ

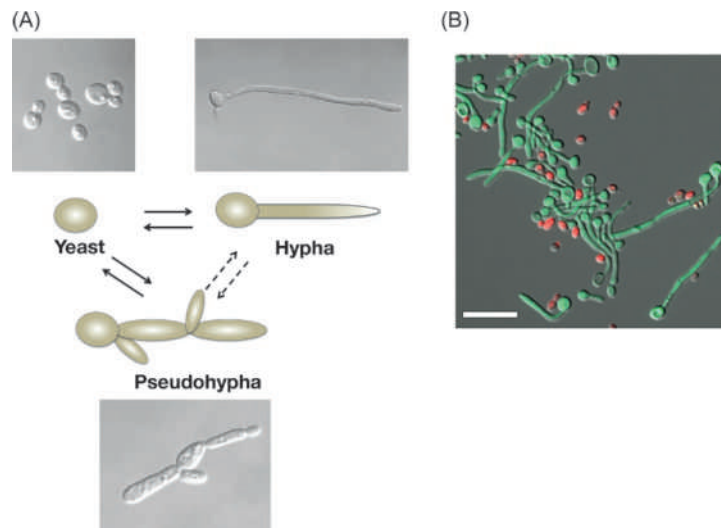


Fig. 4 (A) *C. albicans* can exist in three main distinct morphological forms: the yeast, pseudohyphae, and true hyphae. Similar to diploid strains of the baker's yeast *Saccharomyces cerevisiae*, *C. albicans* can grow as ovoid yeast like-cells that can undergo morphological switching forming pseudohyphal cells, which have an elongated form, initiated by asymmetrical budding. *C. albicans* can also form true hyphae, which are similar to hyphae observed in other filamentous fungi that grow by continuous polarized growth and involves significant changes in cell cycle processes. Each of these forms can interconvert to one another, with the yeast-to-hyphal transition being the most well-characterized; (B) *C. albicans* constitutively expressing green fluorescent protein under the control of a strong *ADH1* gene promoter and *C. glabrata* constitutively expressing red fluorescent protein under the control of the *TDH3* gene promoter were grown together in cell culture medium (Dulbecco's Modified Eagle Medium, DMEM) supplemented with 10% fetal bovine serum for 2 h at 37 °C and 5% CO₂. Under these conditions, *C. albicans* switched to filamentous growth whereas *C. glabrata* remain in the yeast form (Silao and Ljungdahl, unpublished data). Scale bar = 10 µm.

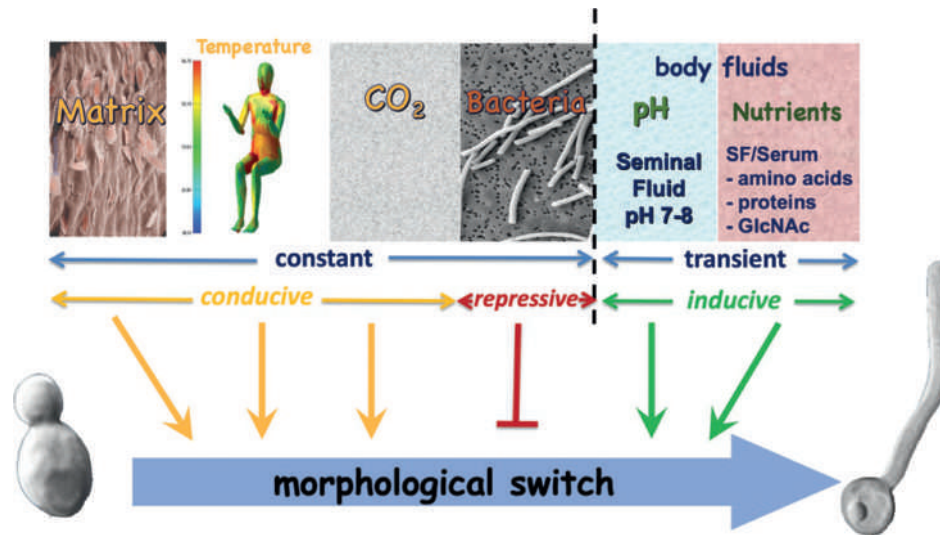


Fig. 5 The vulvovaginal environment is dynamic. With respect to the development of VVC, it is important to differentiate between conductive, repressive and inducing environmental signals. There are relatively constant environmental factors, including the vaginal epithelium (matrix), temperature (37 °C), CO₂ levels, and bacterial microflora. In addition to the monthly hormonal cycle, there are transient fluctuations due to the introduction of body fluids, including serum and seminal fluid (SF). The matrix, 37 °C and CO₂ levels are inherently conductive, whereas the bacterial microflora contributes to repress morphological switching. Serum and SF induce morphological switching due to their elevated pH and to the high content of amino acids and protein in serum and semen, and GlcNAc in semen.

blood flow). When filamentous cells detach together with the recruited inflammatory cells from the invaded epithelial layers, debris from lysed cells and vaginal fluid come off as the characteristic cottage cheese-like vaginal discharge, which is one of the clinical signs and symptoms typical of VVC (Lobo et al., 2017; Cassone, 2015).

Biofilm formation

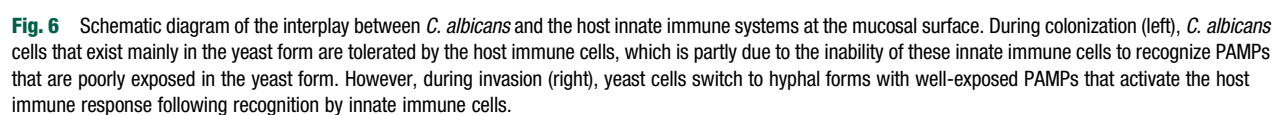
A consequence of *Candida* spp. adherence to surfaces is that they readily contribute to the formation of biofilms. Biofilms are often comprised of communities of cells embedded on a secreted extracellular matrix associated with mucosal surfaces or non-biological surfaces, such as those of implanted medical devices. *Candida* spp. biofilms are of major medical concern as cells growing in biofilms remain shielded from antifungal treatments and commonly gain resistance. As cells sluff off or detach from biofilms, cells with enhanced virulence properties can more readily disseminate and colonize other tissues and organs of the body, resulting in difficult-to-treat systemic infections. *C. albicans* is the most common fungus isolated from medical devices (Kojic and Darouiche, 2004), and catheters are, after diabetes, a major predisposing factor for fungal UTIs (Gharanfoli et al., 2019).

C. albicans biofilms contain a mix of both round, yeast-like cells and filamentous cells, all embedded in an extracellular matrix. In vitro and in vivo studies have shown that *Candida* biofilms develop in a concerted way with four clear stages initiating with adherence to a surface and ending with the dispersion of planktonic cells. The progression through these stages is dependent on a highly complex interplay of more than 50 transcriptional regulators (Lohse et al., 2018). The adhesion between hyphal cells is a very important feature of biofilm maturation, as it is the secretion of the extracellular matrix, composed mostly of glycoproteins. This, together with cellular components from the host, gives stability to the biofilm and generates protection against the immune system and antifungal drugs characteristic of *Candida* biofilms (Lohse et al., 2018).

All mucosal surfaces of the human body have a complex microbiome with several species of bacteria living alongside *C. albicans*. Interactions between *Candida* and bacteria are often mediated by small quorum sensing molecules and secreted virulence factors that exert synergistic and/or antagonistic effects (reviewed in Nogueira et al., 2019) and that influence the ability of *Candida* cells to participate in the formation of biofilms. As an example, *Candida* cells are able to suppress the growth of *E. coli* cells, in what may constitute a protection against bacterial UTI infections (Hall and Noverr, 2017). In the female reproductive tract, *Lactobacillus* spp. reduce the adhesion of *Candida* cells to the epithelium and their formation of hyphae (Sobel, 2007). On the other hand, the presence of seminal fluid in the vaginal tract transiently induces *C. albicans* filamentation (Alvarez et al., 2015; Rolo et al., 2020). The presence of *Candida* in the female reproductive tract also increases in situations of bacterial dysbiosis (van de Wijgert et al., 2014). *Candida* biofilms protect their cells from recognition and damage by the human immune system. For instance, neutrophils fail to activate, macrophages are pierced by *Candida* hyphal cells, while the complement and antimicrobial peptides are blocked (reviewed in Lohse et al., 2018). On top of that, *Candida* cells within the biofilms are also resistant to most antifungal drugs, in part due to the upregulation of efflux pumps (Ramage et al., 2002), but also due to the presence of β -1,3 glucan polysaccharides in the extracellular matrix that act as both a binder and a barrier against drug penetration (Nett et al., 2007). Hence, to fight against *Candida* biofilms, novel substances are needed that can disrupt the architecture of biofilms with the intention to promote access of antifungal drugs.

Another group of important hydrolases are phospholipases that play an important role in host cell membrane damage and invasion. Currently, there are four phospholipases identified in *C. albicans*: phospholipases A, B, C, and D. Increased production of phospholipases is often linked to virulence in animal models. Phospholipases are found concentrated at the hyphal tips and do not appear to increase adherence of *C. albicans* to host cells but rather increase the penetration of *C. albicans* by damaging the lipid components of the membrane, permitting invasion to other sites (Ghannoum, 2000). Recently, a novel secretory cytolytic peptide, candidalysin, was discovered from *C. albicans* that induces damage to epithelial membranes causing activation of the epithelial immune response and killing of macrophages via NLRP3 inflammasome activation (Moyes et al., 2016; Naglik et al., 2019; Kasper et al., 2018). The secreted peptide fragment is derived from the Kex2-dependent proteolytic processing of the hyphal specific protein Ece1. Only the third peptide fragment of Ece1 (Ece1-III) is shown to induce epithelial damage.

The capacity of our immune system to discriminate between colonization and invasion is paramount to controlling disease progression. The interaction of *C. albicans* and host immune cells during colonization and invasion has been extensively reviewed (Gow, 2013; Gow et al., 2012; Erwig and Gow, 2016; da Silva Dantas et al., 2016; Netea et al., 2008, 2015; Netea and Marodi, 2010; Cheng et al., 2012; Lionakis and Netea, 2013; Camilli et al., 2020). As an overview, mucosal surfaces such as those in the female reproductive tract are often colonized by *C. albicans* that exist mainly in the yeast form. However, this does not induce epithelial cell damage eliciting an immune response from epithelial cells or from macrophages (MC) and dendritic cells (DC). In the yeast form



pathogen-associated molecular patterns (PAMPs) are hidden or less exposed, which limits recognition by innate immune cells responsible for inflammasome activation. When yeast cells switch to hyphae, *C. albicans* becomes invasive and induces damage on epithelial cells. The switch exposes hidden PAMPs, which activate an immune response via inflammasome activation in MC and DC. This process results to the release of the pro-inflammatory cytokine, IL-1 β , which then stimulates the release of IL-17 and IL-22 from mature T_H17 cells. IL-17 and IL-22 cytokines are both crucial to mucosal antifungal response; IL-17 activates neutrophils whereas IL-22 stimulates the release of defensins from epithelial cells.

Commensal microbial communities such as those of *Lactobacillus* spp. (yellow cells, Fig. 6), antagonize *C. albicans* colonization, either through competition for sites of adhesion on epithelial cells or through secretion of surfactants that decrease fungal binding. As can be deduced from these observations, the vaginal microflora is an important factor that influences the incidence of UTIs in women. Alterations in the vaginal microflora disrupting the normal balance of the commensal vaginal organisms, often leading to reduced levels of *Lactobacillus* spp., are known to be associated with enhanced fungal growth and the development of UTIs (Czaja et al., 2009; Beerepoot and Geerlings, 2016) and recurrent UTIs (Kirjavainen et al., 2009). This is supported by Hooton et al., who reported that only 50% of premenopausal women with recurrent UTI have H₂O₂-producing lactobacilli in their vagina (Hooton et al., 2005).

Clinical management of Candida UTI

Risk factors

Most common risk factors of candiduria include diabetes mellitus, prolonged hospitalization, ICU admission, use of broad-spectrum antibiotics, instrumentation used to examine the urinary tract, and indwelling urinary tract devices. Other risk factors identified in some reports include: extremes of age, female sex, bladder dysfunction, urinary stasis, nephrolithiasis, renal transplantation, concomitant bacteriuria, congenital abnormalities of the urinary tract, structural abnormalities of the urinary tract, and presence of obstructions (bladder stones) (reviewed in Achkar and Fries, 2010). Fungal-related UTIs are more often diagnosed in women than in men due to anatomical structure differences such as a shorter urethra and the close proximity of the vagina and anus (Tomczak et al., 2014; Behzadi et al., 2010). Approximately 27% of women have recurrent infections. The vagina is the key anatomical site in the pathogenesis of *Candida* UTI in women as it serves as a main reservoir for the infecting organism. In addition, different epidemiological studies have identified additional risk factors for the development of UTI in women such as active sexual activity, delayed post-coital voiding, use of diaphragm and spermicide, and even pregnancy. The act of sexual intercourse can physically displace the microbiota into the introitus and further into the urethra. Delayed post-coital voiding allows stasis and may cause the organism to proliferate. The use of diaphragm and spermicide disrupts the vaginal microbiota by killing the *Lactobacillus* spp. which balances vaginal pH. Antimicrobial use alters the vaginal flora and could be a risk factor for UTI (Lentz, 2009; Lobo et al., 2017).

Diagnosis

The definition of candiduria is often unclear. It may be defined as the presence of ≥ 1000 CFU/mL of *Candida* spp. in cultures (Jacobs et al., 2018) or microscopic visualization of *Candida* spp. in urine sediment samples (Poloni and Rotta, 2020). Since *Candida* spp. are integral components of the human microbiota, the presence of fungal cells in cultures of urine samples does not necessarily indicate candiduria. Yeast colonization, specifically the presence of *Candida* cells in the urinary tract, does not necessarily require treatment because *Candida* spp. is very often cultured in the urine (Tomczak et al., 2014; Lentz, 2009; Fisher et al., 2011a). In the study by Tomczak in Poland, as many as 71% positive urine cultures were from women and the most common isolates were *C. albicans* and *C. glabrata* (Tomczak et al., 2014). This may have been due to vaginal candidiasis and urine contamination. Thus, further investigation should be done requiring the urine test to be repeated in newly obtained, clean-voided urine specimens to rule out the possibility of contamination prior to initiating antifungal therapy in these cases (Tomczak et al., 2014). In cases when obtaining clean-voided urine samples is not possible due to medical conditions of the patient, clean specimens can be obtained using catheter. In direct microscopic examination, *Candida* spp. can be easily identified via visualization of yeast and/or pseudo(hyphal) morphologies without the need to utilize special staining procedures (Fig. 7A). However, other very rare fungal species causing UTI such as *Cryptococcus neoformans* also exist in yeast form but this can be differentiated with *Candida* spp. via the presence of an encapsulating structure made up of polysaccharides around the yeast cell that can be readily observed using special stains such as India (or China) ink or mucicarmine and in some cases, even in unstained fresh urine samples (Fig. 7B) (Poloni et al., 2013; Poloni and Rotta, 2020).

Antifungal drugs

Commercially available antimycotic drugs used for treating patients include fluconazole, amphotericin B, caspofungin and/or flucytosine that can be topically, orally, or intravenously administered. Fluconazole belongs to the azole family, the largest group of antimycotic drugs that contains imidazole- and triazole-containing compounds (Fig. 8) (Mast et al., 2013). Azoles specifically inhibit the activity of the cytochrome P450 enzyme, lanosterol 14- β -demethylase (i.e., Erg11 or CYP51A1), required for ergosterol biosynthesis, which compromises the integrity of fungal cell membrane. Fluconazole is often the drug of choice for *Candida* UTI due

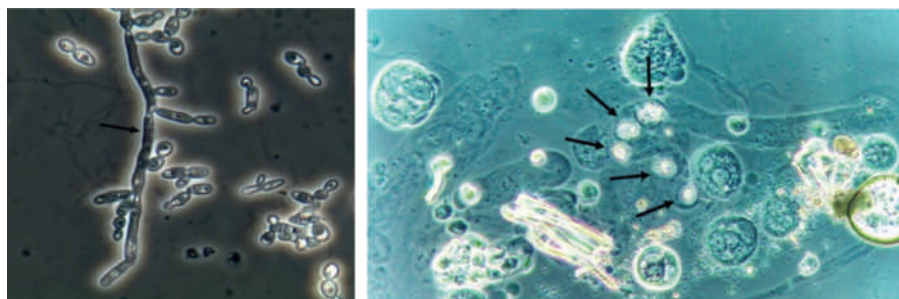


Fig. 7 (A) *C. albicans* identified in unstained urine sediments. (B) *Cryptococcus* spp. identified in a urinary cast. Images were adopted from Poloni JAT and Rotta LN (2020) Urine sediment findings and the immune response to pathologies in fungal urinary tract infections caused by *Candida* spp. *Journal of Fungi* 6(4): 245.

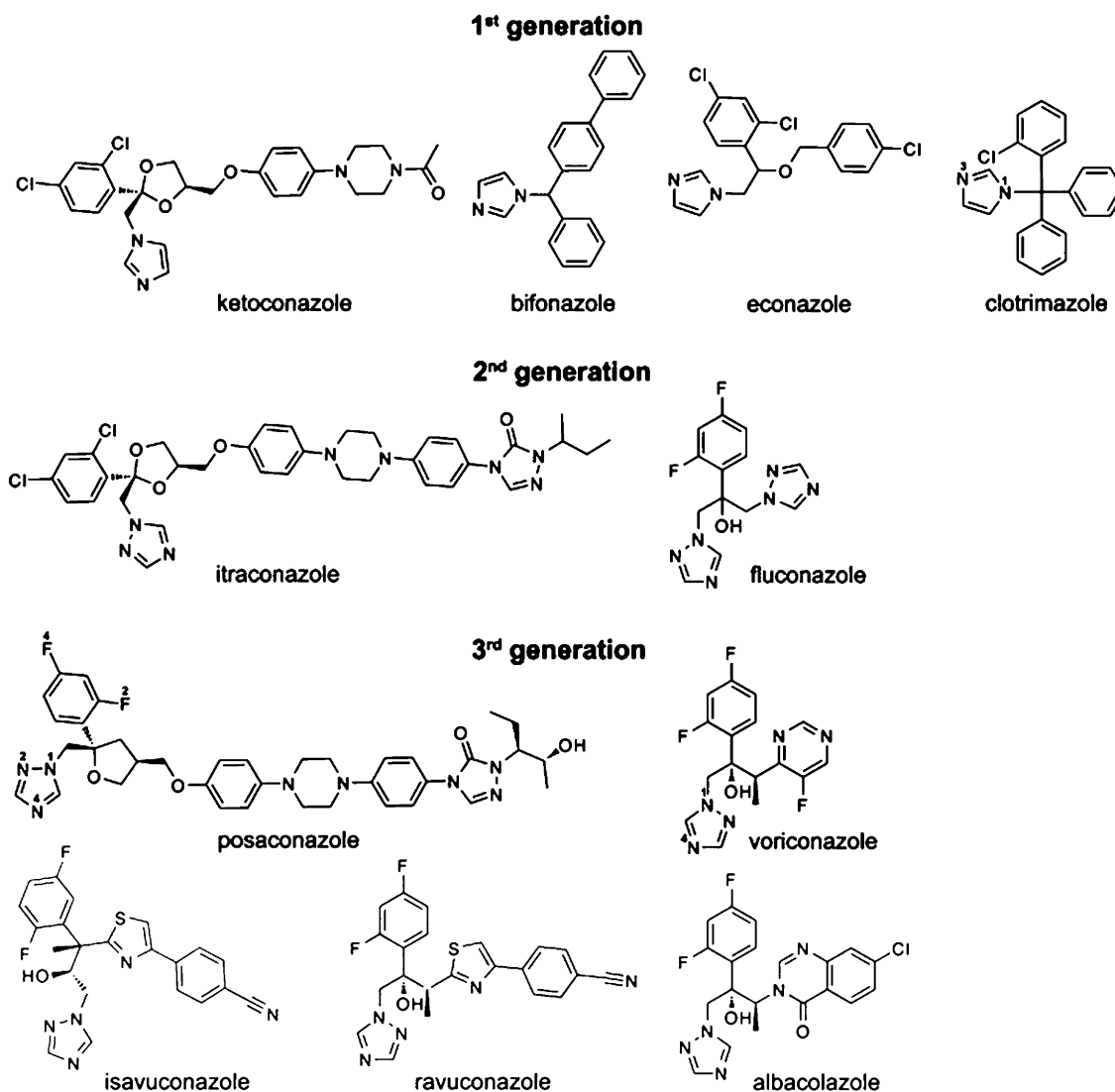


Fig. 8 Molecular structures of three generations of marketed (black) and experimental (gray) azole compounds developed to treat human mycoses. Azoles contain a five-membered heterocyclic ring containing a nitrogen atom and at least one other non-carbon atom, which can be nitrogen, sulfur, or oxygen. The numbering of heteroatom in clotrimazole, posaconazole, and voriconazole were indicated. Image was reprinted with permission Mast N, et al. (2013) Antifungal azoles: Structural insights into undesired tight binding to cholesterol-metabolizing CYP46A1. *Molecular Pharmacology* 84(1): 86–94.

to its safety profile and the ability to achieve a high urinary tract concentration. Other azole antifungals have minimal excretion of active compounds in the urine and are not useful for *Candida* UTI (Felton et al., 2014). Amphotericin B, another antifungal drug, is a polyene fungicidal agent that directly binds to cell membrane ergosterol forming transmembrane pores that facilitate the leakage of monovalent ions thereby disturbing cell membrane electrochemical potential. Amphotericin B (deoxycholate; AmB-d) is active

against most *Candida* species and are often used when fluconazole-resistant isolates are recovered from patients. Though effective, AmB-d is known for its renal toxicity. It has a lipid formulation (AmB-lip) designed to decrease renal toxicity; however, unlike its deoxycholate form (AmB-d) that can be excreted in high concentration in urine, AmB-lip does not achieve adequate levels in urine and kidney (Felton et al., 2014). When fluconazole-resistant *Candida* spp. are isolated from candiduria patients, the administration of AmB-d (with or without flucytosine) is necessary for all high-risk patients with or without symptom presentation. In pregnant women, Amphotericin B remains to be the drug of choice for the treatment of systemic fungal infections. Caspofungin belongs to echinocandin drugs, which is a new class of antifungal drugs that target fungal cell wall biosynthesis through inhibition of the enzyme β -1,3-glucan synthase. Other members of this class are micafungin and anidulafungin that can be also used as alternative antifungal therapy. Echinocandins, in general, have low concentrations (i.e., <2% of the dose) of active drug in urine and they are generally ineffective in treating *Candida* UTI (Kane and Muzevich, 2016; Sobel et al., 2007). However, there are case reports of successful treatment if the *Candida* infection is localized in the kidney and the infection is due to hematogenous spreading (Felton et al., 2014). Flucytosine (5-FC) is a synthetic nucleoside analog that exhibits antimycotic property through intracellular formation of 5-fluorouracil (5-FU), disrupting normal pyrimidine metabolism. It has good antifungal activity against many *Candida* species with the exception of *C. krusei*. 5-FC has been shown to have synergistic effect with azoles and amphotericin B, and are often used in tandem to treat severe infection cases. Interestingly, 5-FC excretion in urine is high. About 97% of flucytosine is excreted in the urine without structural changes. Known side effects of this antifungal drug include bone marrow suppression, GI disturbance, rash, and hepatotoxicity (Felton et al., 2014).

Treatment

Between 16.5% and 40% of candiduria diagnosed in patients being treated in ICU are caused by *Candida albicans* (>50%), followed by *C. glabrata* and *C. tropicalis* (Achkar and Fries, 2010; Alvarez-Lerma et al., 2003; Zarei-Mahmoudabadi et al., 2012). Diagnosis of candiduria due to UTI is often challenging as *Candida* spp. can also inhabit the urinary tract as a natural commensal. Consequently, this represents a therapeutic challenge confronting physicians that can be encountered ranging from asymptomatic ambulatory patients to severely ill hospitalized patients in an ICU setting. However, once candiduria is confirmed by repeat positive urine cultures and microscopy, the therapeutic options for patients vary and are dependent upon several factors such as the identity of the fungal species, the clinical status of the patient (symptomatic or asymptomatic), the severity and localization of the infection, and other intrinsic and extrinsic factors affecting the efficacy of specific antifungal drugs. New updated IDSA guidelines divide between asymptomatic and symptomatic candiduria (Pappas et al., 2016). Candiduria presented without symptoms in patients with no risk factors rarely requires therapy. A comprehensive review of patient's medical history, accompanied by additional physical examination and laboratory studies are required to obtain a full diagnostic appreciation including predisposing factors. When candiduria is linked to colonization of in-dwelling catheters, these catheters are removed to improve the condition. However, in high risk patients with symptoms, the administration of antifungal drugs is necessary to prevent the onset of invasive candidiasis, a potentially lethal complication of candiduria. If no explanation for candiduria is found, a follow-up examination of the urine is generally all that is necessary because candiduria can be expected to resolve within weeks to months without therapeutic intervention in the vast majority of individuals.

Asymptomatic candiduria

In a randomized, double-blind study (Fluconazole (200 mg) vs. placebo for 14 days) involving 316 asymptomatic candiduric hospitalized patients, candiduria recurred in 40% of the catheterized subjects and 30% of those without a bladder catheter. None of the patients in the study developed candidemia (Sobel et al., 2000). The recommendation is to eliminate predisposing factors such as discontinuation or removal of Foley catheter. Treatment with antifungal agents is not recommended unless the patient is at high risk for dissemination. Patients at risk for dissemination include neutropenic patients, very low birth weight infants (i.e., <1500 g), and patients undergoing urologic manipulation. Prophylactic use of oral Fluconazole (400 mg/day) or Amphotericin B deoxycholate (0.3–0.6 mg/kg/day) several days before and after the urologic procedure is recommended (Pappas et al., 2016). Patients with asymptomatic candiduria in ICU setting requires evaluation for the possibility of disseminated candidiasis. In this setting, 46–80% of patients with candidemia have accompanying candiduria. Removing the catheter in ICU patients resolves 20–40% and reinsertion of a new catheter can resolve in 20% of candiduria (Fisher et al., 2011b). In a large prospective study, only 7 (1.3%) of 530 patients who had candiduria and were followed for 12 weeks developed candidemia (Guler et al., 2006). In a retrospective study, 11 (10.5%) of 105 patients with candiduria developed candidemia (Lundstrom and Sobel, 2001). In a treatment trial that specifically enrolled asymptomatic or minimally symptomatic patients with candiduria, none of the 316 patients developed candidemia (Binelli et al., 2006). In the presence of an obstruction, candidemia can follow candiduria (Malani and Kauffman, 2007; Jacobs, 1996). A 1-year prospective observational study in ICUs included 262 patients with nosocomial candidemia and/or candiduria. The mean incidences of candidemia and candiduria were 6.7 and 27.4 per 1000 admissions, respectively. About 8% of candiduric patients developed candidemia with the same species (Bougnoux et al., 2008). Predisposing factors for the development of candidemia in ICU setting include neutropenia and for non-neutropenic host: prolonged broad-spectrum antibiotics, intravascular catheterization, abdominal & thoracic surgery/procedure, parenteral nutrition, and severe burn.

Discontinuing antibiotics that are no longer necessary and treating other predisposing conditions simultaneously should also be done in patients in the ICU. Antifungal treatment of candiduria in an inpatient should be reserved for those patients who have solid clinical evidence of infection of the kidney or collecting system or disseminated candidiasis. For asymptomatic candiduria, predisposed patients with strong evidence for disseminated candidiasis or unstable/neutropenic patients, the following antifungal treatments may be given: Fluconazole (400–800 mg IV/day), Caspofungin (70 mg IV loading then 50 mg/day), Anidulafungin (200 mg IV loading then 100 mg/day), Voriconazole (6 mg/kg IV q12 then 4 mg/kg q12), or Amphotericin B (0.5–0.7 mg/kg/day with or without flucytosine). For non-neutropenic patients, empiric antifungal therapy should be considered in critically ill patients with risk factors for invasive candidiasis and no other known cause of fever and should be based on clinical assessment of risk factors, surrogate markers for invasive candidiasis, and/or culture data from non-sterile sites (strong recommendation; moderate-quality evidence). Empiric antifungal therapy should be started as soon as possible in patients who have the above risk factors and who have clinical signs of septic shock (strong recommendation; moderate-quality evidence) (Pappas et al., 2016).

Symptomatic candiduria

Symptomatic candiduria may be from an ascending infection from the lower urinary tract to the upper urinary tract or via hematogenous spread to the kidneys. Patients may have cystitis and it presents as dysuria, frequency, suprapubic discomfort. They may have pyelonephritis (fever and flank pain) and rarely oliguria and stranguria due to the presence of a fungal ball. Rarely among males, they may have prostatitis, epididymitis, and orchitis. The treatment of symptomatic candiduria depends on the antifungal susceptibility of the organism and adequate antifungal concentration in the urine. About 80% of the administered dose of azole drugs remain intact in the urine. It is active against most *C. albicans* isolates.

For patients with symptomatic *Candida* cystitis, it is highly recommended (if feasible) to remove any indwelling bladder catheter, while for patients with symptomatic ascending *Candida* pyelonephritis, urinary tract obstruction (if present) must be first eliminated, or, when nephrostomy tubes or stents are in place, must be considered for removal or replacement (Pappas et al., 2016). When fluconazole-susceptible organisms are isolated from patients with symptomatic *Candida* cystitis, an oral dose of fluconazole at 200 mg (3 mg/kg) per day for 2 weeks is recommended; for patients with symptomatic ascending *Candida* pyelonephritis, an oral daily dose of 200–400 mg (3–6 mg/kg) for 2 weeks is recommended. However, when fluconazole-resistant *C. glabrata* isolates are recovered, patients with cystitis may be treated with monotherapy of either Amphotericin B deoxycholate (AmB-d) at 0.3–0.6 mg/kg per day for 1–7 days or oral flucytosine (5-FC) at 25 mg/kg 4 times per day for 7–10 days; for patients with pyelonephritis, AmB-d can be administered together with 5-FC (25 mg/kg 4 times per day) for 1–7 days. When *C. krusei* is isolated from patients with symptomatic cystitis or pyelonephritis, a daily dose of AmB-d at 0.3–0.6 mg/kg per day for 1–7 days is recommended (Pappas et al., 2016). In more severe cases where *Candida* urinary tract infection is associated with fungus balls, surgical removal is strongly recommended in adults accompanied by appropriate antifungal treatment as described for symptomatic cystitis or pyelonephritis (Pappas et al., 2016). If feasible, AmB-d can also be administered by irrigation through nephrostomy tube at 25–50 mg in 200–500 ml of sterile water (Pappas et al., 2016). The use of AmB-d bladder irrigation (50 mg/L in sterile water daily for 5 days for cases of cystitis due to fluconazole-resistant species) has not been studied in the pregnant population (Pappas et al., 2016).

Vulvovaginal candidiasis (VVC)

All common *Candida* species are capable of causing UTI and this infection and may start from an undiagnosed or untreated VVC. A gynecological assessment may be warranted to exclude the possibility of vaginal mycosis. VVC can be categorized as uncomplicated or complicated depending on clinical presentations, etiology, host response, and treatment required. Repeat urine collection for microbiological examination should be performed. A single test result with candiduria should not be a basis for therapy. If on gynecological examination, there is evidence of vaginal candidiasis, the woman should be given ideally topical, locally-acting antifungal treatment. This treatment is safe for both pregnant and non-pregnant populations (Table 1) (Tomczak et al., 2014; Malani and Kauffman, 2007). Another urine culture should be performed after treatment and if *Candida* spp. remains present, this indicates a case of urinary tract mycosis. However, a panel of microbiological tests (e.g., CRP, leukocyte count, general urine test, etc.) needs to be done and correlated with the clinical picture because the diagnosis of UTI does not rely only on the urine culture results. Due to the limited available antifungal drugs, it is always necessary to determine which part of the urinary tract is infected so that the appropriate therapy, with the appropriate drug penetration may be given (Tomczak et al., 2014).

Another problem is the non-*albicans* *Candida* UTI. These non-*albicans* fungi colonize the vulvovaginal area and are known to be resistant to fluconazole. Treatment of this complicated VVC with azoles other than fluconazole is important, in order to control the possible source of the fungi causing the UTI. Topical application of azole drugs for a limited duration of time can effectively treat uncomplicated VVC. Short-course topical formulations (i.e., single dose and regimens of 1–3 days) effectively treat uncomplicated VVC. Table 2 lists the treatment options for non-*albicans* VVC (Behzadi et al., 2010; Verma-Gaur et al., 2015; Sobel and Chaim, 1997; Sobel et al., 2003; Saxon Lead Author et al., 2020). The treatment regimen becomes more complicated when the woman is pregnant. The use of topical azoles for the treatment of superficial or intravaginal fungal infections is safe and efficacious. However, data suggest that there is a dose-dependent increased risk of teratogenicity associated with use of systemic azoles. These agents are capable of penetrating the placental barrier and entering the fetal cord blood, therefore adverse effects of these agents on the fetus are a valid concern (Moudgal and Sobel, 2003; Pilmsin et al., 2015). Systemic azoles are generally classified as pregnancy Category C or D. Voriconazole and Flucytosine are both Category D, while azoles and Echinocandins are Category C, with serious risk for fetal

Table 1 CDC recommended intravaginal regimen for uncomplicated VVC.

Drug	Prescription status	Dose per day	Duration (Days)
Clotrimazole (1% cream)	OTC	5 g	7–14
Clotrimazole (2% cream)	OTC	5 g	3
Miconazole (2% cream)	OTC	5 g	7
Miconazole (4% cream)	OTC	5 g	3
Miconazole (200 mg, suppository)	OTC	1 suppository	3
Miconazole (1200 mg, suppository)	OTC	1 suppository	1
Tioconazole (6.5% ointment)	OTC	5 g	1
Butoconazole (2% cream)	Prescription	5 g	1
Terconazole (0.4% cream)	Prescription	5 g	7
Terconazole (0.8% cream)	Prescription	5 g	3
Terconazole (80 mg, suppository)	Prescription	1 suppository	1

Table 2 Treatment options for complicated Non-*albicans* VVC.

Drug	Prescription status	Dose per day	Duration (Days)
<i>C. glabrata</i>			
Boric acid (600 mg, suppository)	Prescription	5 g	7–14
Nystatin (100,000 units tablet)	Prescription	5 g	14
Flucytosine cream (17% cream) or Amphotericin B cream (3%)	Prescription	5 g	14
<i>C. krusei</i>			
Clotrimazole (1% cream)	OTC	5 g	7–14
Clotrimazole (2% cream)	OTC	5 g	3
Miconazole (2% cream)	OTC	5 g	7
Miconazole (4% cream)	OTC	5 g	3
Miconazole (200 mg, suppository)	OTC	1 suppository	3
Miconazole (1200 mg, suppository)	OTC	1 suppository	1
Boric acid (600 mg, suppository)	OTC	1 suppository	14
Itraconazole (100 mg, capsule)	Prescription	200 mg (2 ×)	7–14
Ketoconazole (200 mg, tablet)	Prescription	200 mg (2 ×)	7–14

malformations. There is insufficient data regarding the use of caspofungin in pregnancy (Moudgal and Sobel, 2003; Pilimis et al., 2015). Fluconazole has been found to be embryo-toxic and teratogenic in rodents and rabbits. Fluconazole dose of >300 mg is considered teratogenic and remains contraindicated during pregnancy. However, a single low dose (total dose of <300 mg) does not increase the risk of congenital disorders. This low dose fluconazole may be considered as systemic antifungal treatment in the absence of topical alternative, beyond first trimester, especially in cases of VVC (Moudgal and Sobel, 2003; Pilimis et al., 2015).

Concluding remarks

As discussed in this review, fungal-related urinary tract infections that predominantly are due to *Candida* spp. are common although their medical and socioeconomic impact is likely underestimated. The development of UTI as a result of *Candida* spp. switching from a benign commensal to a pathogenic agent is a complex process and is influenced by different factors such as the health and immune status of the host, expression of virulence factors, interaction with competing microbiota, and the presence of robust nutrient acquisition and metabolic machinery. The treatment of fungal UTI is challenging and highly variable depending upon several factors such as variation in clinical manifestations of the disease among different risk groups, limited antifungal drugs, emergence of fluconazole-resistant non-*albicans* *Candida* species (NACS), and thus must be used with utmost consideration. Although *C. albicans* constitutes most of the reported cases of fungal UTI, it is likely that UTI cases due to NACS or even other fungal species is under-reported highlighting the need to develop more reliable diagnostic procedures. Clearly, there are several important aspects of fungal-related UTI that need to be resolved in order to get a clearer overall picture of its impact in the society.

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Urinary Tract infections: Urinary Schistosomiasis

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Abbreviation

ANA	Antinuclear Antibodies
CRP	C-Reactive Protein
CYP	Cytochrome
DEET	N,N-diethyl-m-toluamide
HIV	Human Immuno Deficiency Syndrome
HSS	Hepatosplenic Schistosomiasis
IL	Interleukin
INF γ	Interferon Gamma
MDA	Mass Drug Administration
NTD	Neglected Tropical Disease
PBMCs	Peripheral-Blood Mononuclear Cells
PSAC	Preschool age children
PZQ	Praziquantel
RTD	Rapid Diagnostic Tools
SAA	Serum Amyloid A
SLE	Systemic Lupus Erythematosus
SNP	Single Nucleotide Polymorphisms
SS	Sjögren's Syndrome
SWAP	Soluble Worm Antigen Preparation
Th	T Helper
TNF- α	Tumor Necrosis Factor Alpha
WASH	Water Sanitation and Hygiene
WHO	World Health Organization

Urinary tract infections

Urinary tract infections (UTIs) are the inflammatory disorders of the urinary tract caused by the abnormal growth of pathogens causing short-term morbidity in terms of fever, dysuria, and lower abdominal pain (LAP) and may result in permanent scarring of the kidney (Odoki et al., 2019). These symptoms may occur as a result of delayed and inadequate treatment (Gökçe et al., 2017). UTIs are commonly classified by the site of infection (the bladder [cystitis], kidney [pyelonephritis], or urine [bacteriuria]) and can be asymptomatic or symptomatic, characterized by a wide spectrum of symptoms ranging from mild irritative, bacteremia, sepsis, or even death (Nicolle, 2002).

UTIs are caused by a range of pathogens the most common ones include *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus saprophyticus*. UTIs are one of the most common infections in childhood (Leung et al., 2019). Antibiotic treatment is the mainstay treatment of UTI and is effective in reducing the morbidity caused by UTI (Yüksel et al., 2006). However, there is a growing concern over antibiotic resistance in many parts of the world (Gökçe et al., 2017; Yüksel et al., 2006).

In this chapter we will be focusing on urinary schistosomiasis which affects mostly the urinary bladder, the lower ends of the ureters, and the seminal vesicles. Urinary schistosomiasis is one of the neglected tropical diseases and therefore the need to look at it apart from the common UTIs.

Schistosomiasis

Human schistosomiasis or bilharzia is a parasitic infection that is caused by trematode flukes of the genus *Schistosoma*. Approximately 250 million people are infected with *Schistosoma* species worldwide (Colley et al., 2015). Urogenital schistosomiasis caused by *Schistosoma haematobium* is endemic in 53 countries in the Middle East and most of the African continent and results in 150,000 deaths annually in sub-Saharan Africa (Hammad et al., 2018). Schistosomiasis is a neglected tropical disease (NTD) which represents a group of 17 major debilitating diseases. These diseases thrive among the poor, increase poverty by reducing, marring, stigmatizing, isolating victims and destroying their ability to learn, work, and contribute to their families and the nation at large (Karunamoorthi et al., 2017).

Schistosomiasis is particularly common in poor communities without access to safe drinking water and adequate sanitation (Weerakoon et al., 2015); as a result people at the highest risk of infection are persons involved with agricultural, domestic and recreational activities, which expose them to contaminated water. Schistosomiasis infection prevalence and intensity curves show peaks in children aged 6–15 years and decline gradually with age, hence, the current global strategy emphasizes preventive chemotherapy to reduce morbidity, which primarily targets school-age children (Coulibaly et al., 2013). Children are mainly at risk of *Schistosoma* infection (Fig. 1) as they engage in multiple high-risk behaviors for acquiring schistosomiasis because of poor knowledge on disease transmission, lack of understanding on the severity of disease-associated consequences, and lack of alternatives for water-related activities of daily living and recreational play (Person et al., 2016). There are 2 types of schistosomiasis affecting humans, intestinal and urogenital schistosomiasis caused by five main species of blood fluke which are *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma mekongi*, *Schistosoma intercalatum* and *Schistosoma haematobium*. The most common forms are *Schistosoma haematobium*, *Schistosoma mansoni*, and *Schistosoma japonicum*.

Urogenital schistosomiasis

S. haematobium which is most prevalent in Africa and the Middle East causes urogenital schistosomiasis (Darren and Weerakoon, 2013). Deposit of human waste in water containing intermediate hosts is the single most important epidemiological factor in transmission of schistosomiasis and the availability of a suitable snail host. The transmission of schistosomiasis is associated with water development projects including dams for irrigation and fish-farming, as snails infected with *Schistosoma* breed in them and human water contact also takes place in such water bodies. Urogenital schistosomiasis being a water-based disease is spread through contact with contaminated water in which snails harbor and shed the infective cercariae are present (Nwele et al., 2017).

Typical symptoms are blood, protein and leukocytes in urine, nutritional deficiencies, lesion of the bladder, kidney failure, and child growth retardation (Afiukwa et al., 2019). In areas of overall high levels of infection, urogenital schistosomiasis is known to peak in the 6–20 age groups with areas of lower levels of infection more prevalent in older age groups which is attributed to the development of acquired immunity (Mitchell et al., 2011).

Transmission of *Schistosoma* spp.

Transmission of the *S. haematobium* or *S. mansoni* parasite starts when infected people with urinary schistosomiasis contaminate freshwater sources with urine that contains the eggs of *S. haematobium* which hatch in the water to form miracidia. Miracidia infect the species-specific intermediate hosts, freshwater snails of the genus *Bulinus* (*S. haematobium*, *S. intercalatum* and *S. guineensis*), *Biomphalaria* (*S. mansoni*), *Oncomelania* (*S. japonicum*) and *Neotricula* (*S. mekongi*). The miracidia multiply asexually in the intermediate hosts. Although most *Schistosoma* species have humans as the only definitive host, *S. japonicum* may infect a wide range of domestic and wild animals, including cattle, dogs, pigs, water buffaloes and rodents. The cercariae are produced in the snail and invade the human via percutaneous penetration (Bourke et al., 2013). After penetration, cercariae migrate from the skin into the portal vein, where they reach adult stage, mate and migrate to the venous plexus of the bladder and genitals and start producing eggs (European Centre for Disease Prevention and Control, 2015).



Fig. 1 School going children at risk of Schistosomiasis. Many communities rely on nearby rivers or dams for water for carrying out household chores such as laundry in (A). These water sources are likely to be contaminated by the *Schistosoma* parasites. The water bodies also provide recreational activities like swimming in (B).

The adult female and male worms reside in permanent pairs in the perivesical for *S. haematobium* or portal veins and mesenteric blood vessels for other *Schistosoma* species (Knopp et al., 2013). Eggs produced by the female *S. haematobium* or *S. mansoni* worms reach the bladder or intestine respectively, from where they can be excreted with urine or feces. The laying of eggs causes damage to the walls of the bladder and genital tissues in the form of lesions in blood vessels and tissues leading to the common symptom of blood in urine known as hematuria (Appleby et al., 2012; Ekpo et al., 2010). A later stage of clinical pathology called hepatosplenic schistosomiasis (HSS), is frequently seen in endemic areas (Gasim et al., 2015). Most clinical patterns of the infection depend on the interaction of various factors including strain of parasite, host genetic background, host immunity and nutritional status, and co-infections.

Prevention and control of schistosomiasis

The main method of preventing *Schistosoma* infection is avoiding contact with fresh water infested with *Schistosome* parasites including avoiding swimming, wading, or any other aquatic activities in *Schistosoma*-contaminated sources, which exposes the skin to possible penetration by the cercariae (Inobaya et al., 2014). Topical lipid formulations of N, N-diethyl-m-toluamide (commonly known as DEET) are highly effective in killing schistosome cercariae on the skin (Ramaswamy et al., 2003).

The lifecycle of schistosome can be interrupted by chemotherapy, intermediate host control, and the complementary roles of water sanitation and hygiene (WASH) (Braun et al., 2018). Zimbabwe launched a mass drug administration (MDA) program against schistosomiasis and soil-transmitted helminths in WHO | Zimbabwe (2012). The MDA program is very effective and is the main method used for killing the adult worm using PZQ, however PZQ does not prevent reinfection (Braun et al., 2018). Control of the intermediate host in Zimbabwe was done using methods including mollusciciding with niclosamide which was implemented in the Kariba dam and Hippo Valley estate. Other methods of controlling the intermediate host include plant-based molluscicides, also controlling snails using biological agents such as ducks, fish or competitive snails (Chimbari, 2012).

Data from the 2012 National Population Census in Zimbabwe show that more than 40% of the family households do not have any type of toilet facility with the majority of the population being the rural population which practices open defecation (UNICEF Zimbabwe, 2019). The WASH programs that are being implemented in Zimbabwe may reduce the prevalence of schistosomiasis if programs are integrated. WASH reduces transmission by containing schistosome eggs and reducing human water contact. People with safe water and adequate sanitation have been shown to be less likely to have *Schistosoma* infection (Grimes et al., 2015).

Diagnosis of schistosomiasis

Schistosomiasis can be diagnosed by direct or indirect methods. All of which have limitations. Direct parasitological methods of schistosomiasis involve the detection of parasite eggs in fecal or urine samples or in the tissues or detection of schistosome-derived antigens in the circulation and excreta. Indirect immunological tests are used to detect the specific antibodies induced against the different stages of the parasite in blood (Lu et al., 2012). Microscopic examination of eggs in urine is currently considered gold standard of detecting *Schistosoma*. *S. haematobium* eggs are released in urine and are detected by microscopy in a sample of urine concentrated by sedimentation, centrifugation, or by filtration forced through a nitrocellulose filter. A urine sample is preferably collected between 10 am and 2 pm to coincide with the maximum excretion of eggs. Reported blood in the urine and micro-hematuria on reagent strips indicates potential infection in those living in endemic areas (Gray et al., 2011). Other methods include microscopic examination of tissue biopsies, serological and reagent strip diagnosis of circulating parasite proteins detectable in blood and urine and, more recently, detection of parasite DNA in urine or vaginal lavage samples (Mutapi, 2011). Methods such as detection of hematuria (blood in the urine) and proteinuria (protein in the urine) which results from the passage of parasite eggs through the bladder wall. Ultrasound scans of the urinary tract are also used to detect morbidity but are less practical in the majority of field settings owing to the requirement of specialized equipment and trained personnel. Microscopic methods of diagnosing schistosomiasis are cheap, time-consuming and have poor sensitivity, hence, sensitive serological tests have the potential to increase diagnostic yield, especially in those with light infection who excrete few eggs (Turner et al., 2004). The specificity of currently available serological tests for the diagnosis of schistosomiasis is generally reduced due to cross-reactivity, making it impossible to differentiate between schistosomiasis and infections with some other helminths (Hinz et al., 2017). The sensitivity of antibody-detection methods was previously shown to decline in areas of low infection. Despite the decline in sensitivity, antibody detection methods remain one of the best available tools for diagnosis in areas of low infection (Nausch et al., 2014).

Praziquantel (PZQ)

Urinary schistosomiasis, as for other types of schistosomiasis, can be treated with praziquantel (Kay et al., 2015). Praziquantel (PZQ) is a pyrazinoisoquinoline derivative (Vale, 2017). PZQ was first used in the 1970's to treat veterinary cestode and trematode infections (Andrews et al., 1983). PZQ has been utilized to treat an assortment of human trematode diseases and is presently the medication of choice for all types of schistosomiasis. PZQ cannot be used for chemoprophylaxis because of its short half-life (1–1.5 h) and cannot kill migrating larvae that are three to 21 days old (McManus et al., 2009). With over 30 years in use the precise mechanism of action of PZQ is not known (Aragon et al., 2009). However, the effects of the drug on schistosomes have been reported where it has been shown that PZQ may interrupt inorganic ion transport. When male *S. mansoni* interacted with PZQ in vitro, the parasite instantly experienced an intense muscular paralysis that was accompanied by a rapid influx of calcium ions, a slower influx of sodium ions and potassium ions (Pax et al., 1978). PZQ disrupts the worm tegument which leads to the exposure of antigens on the parasite surface this has been linked to the disruption of calcium ion homeostasis. It has been proposed that the mechanism of action of PZQ involves alteration of the schistosome membrane fluidity, reduction of schistosome glutathione concentration enabling the host immune system to be more effective, binding to schistosomal actin leading to tegument disruption as well as inhibition of nucleoside uptake.

PZQ is widely metabolized by cytochrome P450 (CYP) enzymes where the CYP3A are most likely to be the major enzymes responsible for PZQ metabolism. Rifampicin was shown to significantly decrease plasma concentrations of single and multiple oral doses of PZQ to levels lower than that of the minimum therapeutic concentration. An example is in Thailand where rifampicin is used for the treatment of tuberculosis and PZQ for the treatment of liver flukes (Ridititid et al., 2002). In this way, the use of both rifampicin and PZQ must be avoided in medical practice to maximize the therapeutic efficacy of PZQ. Joint administration of PZQ and grapefruit juice could lead to a further improvement in the effectiveness of PZQ therapy by enhancing the bioavailability of PZQ (Castro et al., 2002).

Resistance to praziquantel

PZQ replaced all other anti-schistosomal drugs and is the only drug that is currently used for the treatment of schistosomiasis for the past 30 years and the emergence of drug resistance could be a major concern (Wang et al., 2012). The repeated use of PZQ to reduce worm burden makes the evolution of resistant worms a possibility (Melman et al., 2009). Worm reproduction in the mammalian host is sexual and the generation time is relatively long, resistance is likely to take many years to become an important clinical and public health issue (Gray et al., 2011).

Surveys conducted on Egyptian villagers where PZQ has been heavily used show that *S. mansoni* has gained resistance to PZQ. *S. mansoni* has been shown to have gained resistance to PZQ among the Kenyan and Senegalese (Melman et al., 2009; Southgate, 1997). The global burden of schistosomiasis may have increased over the last three decades despite of the regular MDA with PZQ suggesting that MDA with PZQ alone will not be adequate for the global elimination of schistosomiasis (Merrifield et al., 2016). Research should continue on developing alternative drugs (Gray et al., 2011). Currently there is no vaccine for schistosomiasis hence, a need to increase efforts in understanding the immunological responses when one is infected and treated with PZQ.

In the preschool age children (PSAC) there is lack of information as to prescribing PZQ by pharmaceutical companies on toxicity, method of administration, adverse effects and pharmacokinetics which may result in clinical disease that is not managed (Kimani et al., 2018). The oral bioavailability of drugs might vary in PSAC and adult populations due to differences in gastric pH and emptying time, intestinal transit time, immaturity of secretion, and the activity of bile and pancreatic fluid. Further, the drug distribution in children and adults differs as well as the immaturity of enzyme systems (cytochrome P450), glomerular filtration, renal tubular secretion, and tubular reabsorption in children account for a different excretion of drugs in the pediatric population compared with adults (Coulibaly et al., 2017). Praziquantel in adults is mainly used at 40 mg/kg body weight it may be beneficial to carry out a survey using a 20 mg/kg, 40 mg/kg and 60 mg/kg concentration of PZQ to obtain the most effective dosage to be used for the preschool-age children.

Immune response to schistosomiasis

During acute schistosomiasis, before the appearance of eggs in the urine or stool, there is a dominant T-helper 1 (Th1) response characterized by the production of large quantities of Tumor necrosis factor alpha (TNF- α), interleukin 1 and 6 (IL-1 and IL-6) by peripheral-blood mononuclear cells (PBMCs). As the disease progresses the developing egg-antigen induces a T-helper 2 (Th2) response which downregulates the production and effector functions of pro-inflammatory mediators (Pearce and MacDonald, 2002). Raising an immune response has harmful effects that include damage to host's immune system. The evolution of a beneficial immune protection has had to develop alongside potentially lethal damage that immune responses can cause, namely immunopathology (Zinkernagel and Hengartner, 2001).

T helper 1 response

T helper type 1 (Th1) responses have been shown to provide protective immunity against schistosome infection (Butterworth, 1968). Although Th1 immune responses are important in providing protective immunity against schistosome infection, a rapidly induced and excessive Th1 response may also cause damage to tissues of the infected host during parasite killing (Pearce and MacDonald, 2002; Xu et al., 2010).

Interferon gamma (IFN γ) and TNF- α are regarded as markers of the Th1 and pro-inflammatory response. Other cytokines involved are IL-1 and IL-6 which are part of the acute phase of infection (Badr et al., 2015). The pro-inflammatory response is needed to hinder the multiplication of the parasite and favor its clearance in human and animal models. While important for parasite clearance a powerful Th1 and pro-inflammatory response could also be detrimental for the host if uncontrolled, leading to tissue damage and severe disease (Ateba-Ngoa et al., 2015).

T helper 2 response

T helper type 2 (Th2) responses provide a double role in schistosomiasis, to mediate granuloma formation and to regulate inflammation (Riner et al., 2013). Chronic helminthiasis is usually characterized by a marked Th2 response as well as by the induction of a regulatory network that could consequently impair the host immune response to other antigens (Ateba-Ngoa et al., 2015). The Th2 response is characterized by low levels of IFN- γ , and high production of IL-4, IL-5, IL-13, and IL-10 (Badr et al., 2015). IL-10 is a key anti-inflammatory, anti-immune, and anti-fibrotic cytokine secreted by Th2 cells, and it is responsible for the regulation of the inflammatory response and modulation of hepatic fibrogenesis (Silva et al., 2014).

Hygiene hypothesis

The hygiene hypothesis states that increased exposure to microbes early in life may prevent allergic diseases and the theory was first proposed by Strachan, who observed an inverse relationship between hay fever and the number of older siblings when following more than 17,000 British children born in 1958 (Okada et al., 2010). The hygiene hypothesis proposes that the stimulation of the immune system by microbes protects from the development of inflammatory diseases; therefore a reduced exposure to infectious agents may explain the rise in allergic and autoimmune diseases in industrialized countries (Yazdanbakhsh and Matricardi, 2004).

Parasitic infections are a major aspect of the hygiene hypothesis, as allergies and autoimmune diseases are less prevalent in countries with higher burdens of helminths and other parasitic organisms. Helminths establish chronic infections indicating successful down modulation of the host immune system resulting in lower immunopathology, reduce host susceptibility to, and severity of allergic and autoimmune diseases (Wilson and Maizels, 2004). Studies in mice have established that regulatory immune responses induced by helminth can suppress Th2 and Th1 or Th17 responses that mediate allergy and autoimmunity, respectively. The studies in mice have provided an explanation for the hygiene hypothesis' and have led to the exploitation of helminths or their immunomodulatory products in the development of new immunosuppressive therapies for inflammatory diseases in humans (Finlay et al., 2014). Helminth products generate regulatory responses capable of inhibiting not only anti-parasite immune responses but also allergic sensitization (Rujeni et al., 2012). Helminth infections and allergic diseases are both associated with Th2 cytokines and high levels of immunoglobulin E (IgE) and eosinophilia (De Oliveira et al., 2014).

Antinuclear antibodies (ANA) are a spectrum of auto antibodies targeted to various nuclear and cytoplasmic components of the cells and are useful serological markers for different autoimmune disease, like Systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), scleroderma, polymyositis, or mixed connective tissue disease (Vlagea et al., 2018). We measured ANA and their relation to schistosomiasis (Chimponda et al., 2019). We found levels of the antinuclear antibodies to be significantly higher in schistosome-uninfected children than infected children. Furthermore, *S. haematobium* infection intensity inversely associated with antinuclear antibody levels supporting the hygiene hypothesis.

Pediatric schistosomiasis

Probably from as early as 1898 it has been reported that children 5 years and below were infected with schistosomiasis (Bustinduy et al., 2017). Investigators have reported prevalence rates of pediatric schistosomiasis infection ranging from 18% to 63% in Mali, Niger, Sudan, Uganda and Zimbabwe (WHO, 2010). Children under 5 years of age are often exposed daily to infected water very early in life, although infection is not apparent, infection generates inflammation that predisposes to organ fibrosis, which can endure for many years (Colley et al., 2015).

Water infested with the cercariae may be used in bathing the children, putting the children at risk of infection to *S. haematobium* despite not having been in contact with the river. The justification for the neglect in treatment of young children is that, the group was considered a non-infected to lightly infected population and therefore thought to be at low risk for schistosomiasis-associated morbidity; as well as lack of a child-friendly formulation for oral treatment that would decrease the risk of choking (Bustinduy et al., 2017). Other problems which resulted in the exclusion of this group include operational problems associated with accessing preschool children and lack of information on the safety of the drug of selection for schistosome management, PZQ, in children aged 5 years and below (Mutapi et al., 2011). The first expert meeting on the inclusion of preschool-age children in schistosomiasis control efforts was convened at the WHO in 2010. Strong evidence emerged that very young preschool-age children do indeed harbor egg-patent infection (Bustinduy et al., 2017). These children are both at risk of infection and a potential reservoir for the parasite in communities successfully targeted by mass anti-helminthic drug treatment (Mutapi et al., 2011). Early care for and attention to children 5 years old and below may provide an opportunity to treat schistosomiasis-associated anemia, reduce occult blood loss and reduce inflammatory responses (Mduluzza et al., 2017). Fig. 2 is a picture showing PSAC at risk of *S. haematobium* infection.

When dealing with schistosomiasis in pediatrics we have to look at the wholistic picture. Fig. 3 shows some issues that come with pediatric schistosomiasis.

Co-infections with schistosomiasis

Schistosomiasis exists in communities that are infected with other pathogens. Schistosomiasis and malaria exhibit almost similar distribution patterns, making coinfection a predominant occurrence in malaria endemic countries where there is very high poverty (Ojo et al., 2019). Many studies have been carried out revealing the co-infection of schistosomiasis with other diseases on adults but there is still a gap on the effects of co-infections of *S. haematobium* with other pathogens in PSAC (Mduluzza-Jokonya et al., 2020). In a study multiple parasite infections were prevalent in school and PSAC of Magu district in Tanzania and these coinfections result in poor growth development, reduced learning and school achievements as well as increased susceptibility to other infections (Kinung'hi et al., 2014).

Children in Senegal with a light *S. haematobium* infection (1–9 eggs/mL of urine) presented significantly lower *P. falciparum* parasite densities than children not infected by *S. haematobium* showing that schistosomiasis is protective to malaria (Lemaitre et al., 2014). Schistosomiasis induces a polarized Th2-enriched environment which modulates the human immune response, possibly affecting the incidence and severity of concomitant falciparum malaria (Lyke et al., 2006). Anemia has been proposed as a marker for morbidity reduction in schistosomiasis control programs because it is easy to quantify and is affected by presence and intensity of schistosome infection, however, the contribution of schistosomiasis to anemia is concealed by anemia due to malaria therefore should not be used to determine the impact of a *Schistosome* program in populations of malaria and schistosomiasis coinfections (Valice et al., 2018).

Schistosomiasis appears to promote an increased susceptibility to HIV, a more rapid disease progression, and transmission of HIV to others by stimulating the Th2-type cytokine production that leads to eosinophilia in contrast with the Th1-type response in

(A)



(B)



Fig. 2 PSAC are at risk of schistosomiasis. Pediatrics at a water source suspected to be contaminated with snails infested with *S. haematobium* parasites in (A). Mothers or care givers often bathe their young children in the dams or rivers as shown in (B).

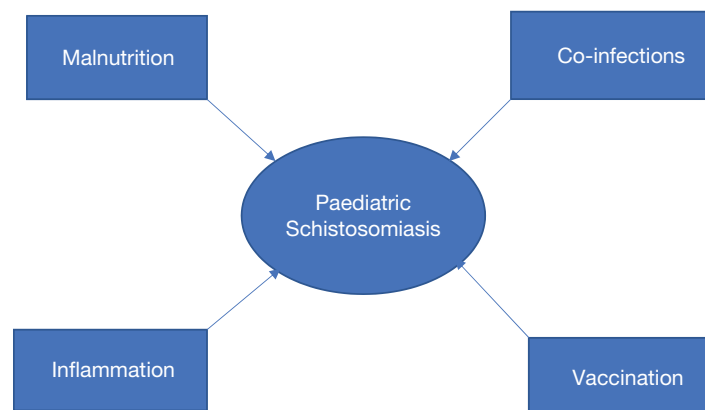


Fig. 3 Paediatric schistosomiasis and its complications. PSAC living in areas where schistosomiasis is endemic also suffer from malnutrition and are subject to other infections such as malaria and HIV. This age group has underlying inflammatory conditions and also receive vaccinations, whose efficacy may be affected by the infection.

lymphocytes, which is reduced leading to a less efficient control of HIV replication in the host (Knopp et al., 2013). HIV infection comes before schistosomiasis in infants and young children, whereas in adults schistosomiasis precedes HIV (Bustinduy et al., 2014). Children with light intensity of *Schistosoma* infection may have compromised immune systems from HIV infection (or other immune deficiencies), which impairs efficient egg excretion and, for this reason alone, HIV infection should be considered as a possible contributing factor on the rate of egg excretion (Bustinduy et al., 2014). This has a direct bearing on diagnosing schistosomiasis. In that case the urine filtration method which is used mostly to diagnose *S. haematobium* infection in these low resource areas because it is cheap may have to be combined with other techniques such as serology in order to identify these children with light infections.

Schistosomiasis and malnutrition

Malnutrition refers to deficiencies, excesses, or imbalances in a person's intake of energy and/or nutrients and applies to three groups of conditions such as undernutrition, micronutrient and overweight (WHO, 2020a,b). It was estimated worldwide that one in every three preschool children is malnourished (Wong and Nair, 2014). According to WHO 47 million children under 5 years of age are wasted, 14.3 million are severely wasted and 144 million are stunted, while 38.3 million are overweight or obese (WHO, 2020a,b). Malnutrition among children is a marker of development and represents the socio- economic status of populations (Payandeh et al., 2013).

Parasitic infections cause anorexia and poor absorption of nutrients and promote the deviation of nutrients to the organism's defense mechanisms and contribute to the onset or exacerbation of weight and height deficits (Mekonnen et al., 2014). Blood losses from hematuria and fecal occult blood from schistosomiasis affects iron balance and subsequently nutrition (Adeniran et al., 2017). *S. haematobium* causes losses of a milligram or more of iron a day and the amount of iron lost by children infected with *S. haematobium* can be similar to daily menstrual losses of iron averaged out over a month (Hall, 2007).

Iron deficiency impairs the immunity, increases the susceptibility of infected individuals to reinfection with schistosomiasis and other tropical diseases (Mwanakasale et al., 2009).

Factors affecting growth and development of preschool children are well-documented (Mekonnen et al., 2014). However, since the PSAC are now being considered for treatment for schistosomiasis we need to revisit the issue of malnutrition. In a population receiving multi-micronutrient supplementation along with broad spectrum anti-parasitic anthelmintic treatment saw reduced infestation with *Schistosoma* spp. (Morales-Suarez-Varela et al., 2019). The gap that may need to be filled is that instead of treating the PSAC with PZQ only it may be necessary to supplement the treatment with micronutrients. The benefits of micronutrient supplementation with PZQ treatment have been shown in other studies. Mwanakasale et al. (2009) showed that providing iron supplements once a week decreased reinfection of *S. haematobium* after 6 months in school age children. Supplementing with micronutrients was shown to reduce the side effects of PZQ treatment in swiss female albino mice.

Childhood vaccinations and schistosomiasis

PSAC receive vaccinations from diseases such as tuberculosis, measles, rubella, diphtheria, tetanus and whooping cough. There is clinical evidence to suggest that chronic prenatal parasitic infection as well as parasitic infections in the first few years of life can highly alter infant immune responses to standard childhood vaccinations (Labeaud et al., 2009). Helminth-induced immunomodulation reduces host responses, allowing parasite survival and minimizing tissue damage and this immunomodulation may also alter responses to unrelated organisms and vaccines (Elliott et al., 2007).

Studies on the effect of schistosomiasis on response to vaccines need to be undertaken for the PSAC. Twayongyere et al. (2019) found that *S. mansoni* infection among pre-school children is associated with a reduced antibody response to catch-up measles immunization, and that PZQ treatment improves the response. *S. mansoni* infection was shown to reduce the protective efficacy of BCG vaccination against *M. tuberculosis* (Elias et al., 2005). Nono et al. (2018) found lower immune responses to measles vaccine in *S. mansoni* infected participants than uninfected participants which was not observed in *S. haematobium* infected participants. According to (Hartmann et al., 2019) helminth infection reduces the quantity and quality of antibody responses to vaccination against seasonal influenza. This implies that considerations have to be made when designing vaccines to be utilized by individuals living in areas where helminths are endemic.

Inflammation in schistosomiasis

Inflammation is the body's way of signaling the immune system to heal and repair damaged tissue and defend itself against foreign invaders, such as viruses and bacteria. Inflammation can be a fundamental factor in the development of a disease (Malenica et al., 2017). In chronic *S. haematobium* infections, eggs lodged in the urinary tract elicit immune responses that result in inflammatory cell infiltration, granuloma formation, urinary tract fibrosis and renal dysfunction (Njaanake et al., 2014). Antigens produced by viable ova in tissues stimulate a host driven granulomatous inflammation that disrupts the epithelial barrier of the bladder, which permits ova to move from the venule into the lumen of the bladder. The inflammatory process results in varying degrees or collateral damage to tissues, and the extent of this damage is accompanied by the intensity of the granulomatous response and its anatomic location. Inflammation at the site where the ureter enters the bladder may obstruct the ureter, leading to a dilation of the ureter, calyces of the kidney, and eventual kidney failure (Wamachi et al., 2004). In schistosomiasis, excessive inflammation and cytokine production are associated with severe immunopathology and mortality.

Inflammatory markers

Measurement of inflammatory markers is used to detect acute inflammation that might indicate specific diseases or to give a marker of treatment response (Watson et al., 2012). Biomarkers of inflammation if identified early may allow for better prognosis (Chisango et al., 2018). Inflammatory markers are good diagnostic markers especially for diseases which do not show any clinical symptoms and help to diagnose and possibly predict disease outcome (Kiszevska et al., 2015). Identification of additional biomarkers for detection of schistosomiasis related morbidity would be an invaluable tool for schistosome control programs, especially in schistosome infections where such markers may facilitate timely treatment to prevent severe morbidity (Vennervald et al., 2000). *Schistosoma* infection is known to induce inflammatory reactions in the host leading to changes in serum concentrations of immunoglobulins as well as acute phase proteins (Salawu and Arinola, 2010). IL-6 is the major regulator of the acute phase response in hepatocytes and promotes the systemic effects of the acute phase response by stimulating the growth and differentiation of many cell types (Bresnahan and Tanumihardjo, 2014).

There are different types of inflammatory markers such as cytokines, reactive oxygen species and acute phase proteins such as serum amyloid A (SAA) and C-Reactive Protein (CRP) (Brenner et al., 2015). Acute phase proteins are plasma proteins whose concentrations increase by at least 25% during inflammation states (Gabay and Kushner, 1999). The acute phase response accompanies both acute and chronic inflammatory states. Conditions that normally lead to substantial changes in the plasma concentrations of acute-phase proteins include infection, trauma, surgery, burns, tissue infarction, various immunologically mediated and crystal-induced inflammatory conditions, and advanced cancer (Gabay and Kushner, 1999). Identification of additional biomarkers for detection of schistosomiasis related morbidity would be an invaluable tool for schistosome control programs, especially in schistosome infections where such markers may facilitate timely treatment to prevent severe morbidity (Vennervald et al., 2000). In a study on inflammatory markers in *S. haematobium* infection, we measured levels of Resistin, P-selectin and CRP in PSAC living in a *Schistosoma* endemic area. We found P-selectin to be significantly associated with *S. haematobium* infection and that infected participants had significantly higher levels of this marker than uninfected children and hence a potential biomarker of *S. haematobium* infection. Participants exposed to cercarial antigens also had higher levels of P-selectin, Resistin and C-reactive protein showing an underlying inflammatory condition (Chimponda et al., 2019).

NTDs including urogenital schistosomiasis in the era of COVID-19

The world's attention and focus is on COVID-19 and many diseases have been left out including the already neglected tropical diseases (Molyneux et al., 2020). COVID-19 has brought with it many problems including access to chemotherapy for urinary schistosomiasis as many countries were on strict lock downs. The WHO has designed a frame work for implementing MDA programs for NTDs. The proposed process relies on two steps: a risk-benefit assessment, to decide if the planned NTD activity should proceed, and an examination of a list of precautionary measures, to decide how the planned activity should be implemented (WHO, 2020a,b). MDA programs have thus been put on hold as deliberations on how to best carry out these programs are being discussed. The milestones that have been reached especially in the context of pediatric schistosomiasis have been derailed. According to (Toor et al., 2020), schistosomiasis, STH and trachoma found in high transmission areas are bound to have a fast resurgence impacting the gains made of the MDA programs up to 2020 and are bound to affect the 2030 goals. COVID-19 has also impacted on the manufacturing of rapid diagnostic tools (RDT) for many neglected tropical diseases with many RDTs being made for COVID-19 diagnosis (de Souza et al., 2020). The lessons learnt from the manufacturing of RDTs for COVID-19 must now be implemented for schistosomiasis.

Other concerns are that co-infection of pre-existing infection with any parasitic infection with SARS-CoV-2 may lead to changes in susceptibility and/or severity of COVID-19 (Gutman et al., 2020). (Ssebambulidde et al., 2020) have given a possible hypothesis for the comparatively low COVID-19 cases/deaths in parasite-endemic areas such as Africa is the immunomodulation induced by parasites.

Concluding remarks

Schistosomiasis will continue to be with the poor and causing devastating effects especially to the young still growing up. Mass treatment may be the only interim control measure, to reduce development of severe morbidity and pathology. Mass treatment through MDAs is not sustainable especially in communities where the governments are already riling under poverty and failing to provide basic health services to its populations. Integrated control strategies need to be devised that target several of the prevalent neglected tropical diseases in the tropical regions. With the spread of SARS-Cov2, the poor communities may have exacerbating conditions due to an already inflammatory status. However, more work is required to understand the contribution of the NTD prevalences and incoming of SARS-Cov2. The most urgent requirement is to integrate NTDs control with WASH program as most of the NTDs are related to poor wash conditions. However, health education need to be intensified and prevent the continued deposition of waste and unhygienic conditions in the communities, and intensify the supply of medicines to cut the *Schistosoma* life cycle.

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Bacterial Gastrointestinal Infections

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Gastrointestinal bacterial infections

Gastrointestinal (GI) infections, including diarrheal diseases and infections of the gastric pathogen *Helicobacter pylori*, are very common in regions of the world with poor water sanitation and cramped living conditions. Outbreaks of, e.g., cholera or bacterial dysentery, might occur in refugee camps and after flooding or earthquakes when access to clean potable water and sanitation is scarce; however, these diseases are also endemic in several regions and are able to cause pandemics. Food poisoning and gastroenteric problems such as inflammatory bowel disease (IBD), inflammatory bowel syndrome (IBS), and nosocomial antibiotics-induced *Clostridioides difficile* infections (CDI) are common globally and are associated with bacterial dysbiosis in the GI tract and severe health problems. Hence, gastrointestinal infections include a large number of bacterial species that cause a variety of symptoms. In addition, although not addressed in this article, it is important to remember that viruses and parasites are also major causative agents of gastroenteric infections worldwide.

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Food poisoning and foodborne gastroenteritis

Foodborne bacterial infections are common globally and can cause a range of diseases, from diarrhea to cancer. Foodborne infections are often spread by contaminated food or drink and can cause regional outbreaks or epidemics, affecting larger areas and populations. Several of the most common foodborne bacterial pathogens can cause both epidemics and pandemics. Diarrheal diseases, particularly affecting people living in low-income regions, are often endemic but may cause seasonal epidemic peaks; this will be described separately below.

According to World Health Organization (WHO), enterohemorrhagic *E. coli* (EHEC), *Salmonella enterica* serovar Typhimurium, and *Campylobacter* spp., are the most common causes of bacterial foodborne gastroenteritis globally and cause similar symptoms including bloody dysenteric stools and inflamed epithelium, abdominal pain, and fever. Other foodborne diseases are common also in high-income regions and include acute food poisoning or intoxication caused by bacterial toxins that remain in food after preparation. Such enterotoxins are produced by species such as *Staphylococcus aureus*, *Clostridium perfringens*, and *Bacillus cereus*. They may cause acute nausea and vomiting as well as diarrhea within less than an hour after ingestion. Symptoms of foodborne infections range from mild problems to potentially life-threatening conditions caused by cholera, listeriosis, or botulism.

Campylobacter

Campylobacteriosis is the most common gastroenteritis globally, and it is caused by *Campylobacter*. The genus *Campylobacter* encloses Gram-negative and microaerophilic bacterial species, including *Campylobacter jejuni* and *Campylobacter coli*. They may be present in raw milk, water, and raw poultry and are considered as zoonotic bacteria that can spread by improper handling of meat after slaughter. The onset of bloody diarrhea, abdominal pain, and fever typically occur after 2–5 days. Most infections are mild and not life-threatening, but young children and immunocompromised persons may get severe symptoms. *Campylobacter* adhere by means of an adhesin encoded by the *cadF* gene. Following adhesion, some species are invasive and may also express a cytolethal distending toxin and hemolysin that damage the epithelial cells. Most infections result in self-limiting diarrhea that only requires oral rehydration.

Campylobacteriosis has been linked to Guillain-Barré syndrome, where the immune system attacks the nerves causing muscle weakness and paralysis. This autoimmune disorder is believed to be caused by molecular mimicry where *Campylobacter* has *N*-acetyl neuroamine acid (sialic acid) epitopes on its surface that induce autoimmune response due to its similarity to neural gangliosides. *Campylobacter* infections have also been associated with the onset of inflammatory bowel disease (IBD), discussed below.

Salmonella enterica

The genus *Salmonella* belongs to the family Enterobacteriaceae and consists of only two species, *Salmonella enterica*, and *Salmonella bongori*. Salmonellosis in humans is caused by *S. enterica*, which has a large number of so-called serotypes or serovars and are grouped into typhoid infection caused by *S. enterica* serovar Typhi, and non-typhoid *Salmonella* (NTS) that cause gastroenteritis, respectively. The most common causes of NTS are *S. enterica* serovar Typhimurium (*S. Typhimurium*) and *S. enterica* serovar Enteritidis (*S. Enteritidis*). *Salmonella*, as well as *Campylobacter*, are frequently isolated foodborne pathogens and usually contracted from water or food contaminated with human feces. The severity of the *Salmonella* infection in humans depends on the serotype of the infective strain, and the most common clinical manifestations are enteric fever, gastroenteritis, bacteremia, and chronic carrier stage. Most of the *Salmonella* strains are capable of invading, replicate, and survive in human host cells. *Salmonella* induces phagocytosis by injecting effectors through a Type 3 Secretion System (T3SS) inside the cytoplasm of epithelial cells to trigger actin cytoskeleton rearrangements. The genes responsible for *Salmonella* invasion are found in the *Salmonella* pathogenicity islands (SPIs) located in the bacterial chromosome.

The incubation time for gastroenteric NTS infections ranges from 6 to 12 h. The infections are often self-limiting and last for a few days. A slow increase of fever (from 37°C to 41.5°C) in a couple of weeks is characteristic of the typhoid infection. This infection is associated with high morbidity and mortality in the developing world. Complications of the typhoid infection include pancreatitis, hepatitis to hemorrhages, and they have been reported in 15% of infected patients. Patients shedding bacteria in the stool for a period longer than a year are considered chronic carriers. Patients with *S. Typhi* infections are more likely to become chronic carriers than patients with NTS infection. Currently, two types of vaccines (inactive parental and oral live attenuated) are approved against the enteric fever, but no licensed vaccines are available for NTS infections (Eng et al., 2015).

Enterohemorrhagic *Escherichia coli*

Enterohemorrhagic *E. coli* (EHEC) is a subset of the *E. coli* pathotype STEC (Shiga toxigenic *E. coli*) that produce Shiga-like toxins, also known as verotoxins, due to their activity against cultured Vero cells. Like *Campylobacter* and *Salmonella*, EHEC is spread by raw or undercooked meat or contaminated vegetables and fruit. Symptoms of EHEC infection include diarrhea and abdominal pain, and sometimes fever. Cattle are considered as the main reservoirs. Once EHEC invades the human gut, it forms attaching and effacing (A/E) lesions on the mucosal epithelium (pedestal-like structures), allowing intimate contact of the bacteria with the cell followed by secretion of Shiga toxins. A total of 41 genes are involved in the formation of A/E lesions and are encoded within a pathogenicity island called locus for enterocyte effacement (LEE). The T3SS is part of the LEE and resembles a needle used to inject effector proteins into the host cells. The Shiga-like toxins Stx1 and Stx2 inhibit protein synthesis and can cause cell death, sloughing of the mucosa, inflammation, intestinal vasculitis, and, ultimately, bloody diarrhea. Most EHEC diarrheal patients recover with fluid replacement, and no further treatment is necessary (Woodward et al., 2019). In humans, EHEC may progress to a serious

life-threatening condition called HUS (Hemolytic uremic syndrome), which causes acute renal failure and anemia. Although the most studied and dangerous serotype is EHEC O157:H7, a pandemic clone capable of causing severe disease, other non-O157 EHEC strains also represent a significant public health concern.

Staphylococcus aureus

S. aureus causes a large variety of clinical infections, including bacteremia, infective endocarditis, skin infections, and soft tissue infections. It may also be present as part of the normal microbiota in the nose and skin. Studies on medical students at the Karolinska Institutet performed over nearly 10 years showed that *S. aureus* is present in the nose of one-third of the population at a given time without symptoms (Sjöling, unpublished data). *S. aureus* can also cause gastrointestinal problems, typically by food poisoning. *S. aureus* is able to grow in a range of temperatures from 7° to 48.5 °C and at a high concentration of sodium chloride (up to 15%), characteristics that favor this microorganism's ability to contaminate and grow in food during preparation and processing. During *S. aureus* growth, an assortment of enterotoxins can be produced, such as Staphylococcal enterotoxins (SEs). SEs are part of the pyrogenic toxin superantigen family, which is highly stable and heat-resistant to conditions such as freezing and drying as well as to the proteolytic activity of enzymes found in the gastrointestinal tract. The foodborne disease caused by *S. aureus* has a quick onset that takes approximately 3–5 h after ingesting contaminated food. The severity and incubation time depend on the concentration of toxin ingested. The symptoms are often immediate, including nausea, vomiting, abdominal pain, and diarrhea.

The overexposure of methicillin and β -lactam antibiotics to treat *S. aureus* infections caused the rise of methicillin-resistant *S. aureus* (MRSA) strains. MRSA strains are resistant to several antibiotics and are a serious global healthcare problem. Recently, a new strain of *S. aureus* (livestock-associated methicillin-resistant *S. aureus*, LA-MRSA) mainly found in livestock has emerged and now is considered an emerging pathogen and cause of infections in humans. In particular, LA-MRSA strains belonging to the multilocus sequence type 398 (ST398) were the most common MRSA type isolated in retail meat, and they have been found to be responsible for more than 20% of cases of MRSA. ST398 LA-MRSA have shown to have an increased capability to colonize and cause infection in human (Kadariya et al., 2014).

Public awareness and educational programs are public health interventions to prevent *S. aureus* outbreaks. Safeguarding the cold chain during food handling and preparation is crucial for preventing staphylococcal foodborne disease.

Bacillus cereus

B. cereus is a pore-forming Gram-positive microorganism well known for causing food poisoning, but also increasingly recognized as an etiological pathogen of wounds and systemic infections. Due to its capability to produce resistant endospores, *B. cereus* strains have wide dissemination in the environment, including food products, and they persist under adverse conditions, including stress-induced by pH, temperature, or nutrient deprivation. When the spores are exposed to favorable conditions (i.e., amino acids) or temperatures (>25 °C), their germination is triggered, leading to toxin production. *B. cereus* produces three different enterotoxins and one emetic toxin. The emetic toxin named cereulide is produced by cells growing in the food and results in emesis (vomiting) after ingestion, whereas the enterotoxins, which are elaborated when vegetative bacterial cells grow in the small intestine, cause diarrhea, cramps, and nausea. The three pore-forming enterotoxins Nhe (nonhemolytic enterotoxin), Hbl (hemolytic enterotoxin), and CytK (cytotoxin K), are responsible for provoking diarrhea; however, other putative virulence factors might also contribute to the diarrheal syndrome. The genes encoding the enterotoxins are located in the chromosome and broadly spread in the *B. cereus* group. The genes encoding the biosynthetic machinery for the cereulide toxin are the cereulide synthetase (*ces*) gene cluster, which is present in a 270-kb megaplasmid and limited to closely related strains. For the emetic-type illness, the onset of symptoms occurs rapidly, ranging from 30 min to 6 h after eating contaminated food. In the diarrheal type, the symptoms start after 6–15 h, typically after a meal where food was left at room temperature for several hours (Ehling-Schulz et al., 2019).

Clostridium perfringens

Clostridia are obligate anaerobic bacteria that reside in the small and large intestine and well as in soil and water. The genus includes several medically interesting species, including *Clostridium tetani* that cause tetanus, and *Clostridium botulinum* that secrete the most potent nerve toxin on earth, the botulinum toxin, commonly known as botox. Although *C. botulinum*, or its toxin, can be ingested through food and cause paralysis, this is a rare condition and will not be described in this article. The species *C. perfringens* is, however, a common cause of gastroenteritis. *C. perfringens* can give rise to serious wound infection gas gangrene characterized by large necrotic wounds and degradation of tissue. However, *C. perfringens* might also be part of the normal microbiota, and approximately 20% of humans carry the species without symptoms. But due to its ability to survive in anoxic environments and to secrete enterotoxins during sporulation, *C. perfringens* is a cause of food poisoning in food that is prepared in large batches and either transported and/or chilled and then reheated before serving. Although symptoms can be mild, elderly persons may develop severe and life-threatening diarrhea. The stress of heating and chilling may cause contaminating *C. perfringens* to sporulate and release heat stable toxins. In some cases, vegetative bacteria die during reheating leaving the toxin itself to be the only agent causing diarrhea. Prevention of anaerobic bacterial growth is taught to personnel handling food in order to abrogate outbreaks of food poisoning at hospices and schools.

Listeria monocytogenes

Infection caused by *Listeria monocytogenes* or listeriosis is a foodborne disease that affects elderly people, newborns, and immunocompromised individuals more severely. The risk is also high to pregnant women as it can lead to stillbirth or spontaneous

abortion. Typically, listeriosis causes diarrheal disease, but in risk group individuals, the pathogen can disseminate systemically, causing life-threatening sepsis or central nervous system infection. In the intestinal tract, *L. monocytogenes* can induce phagocytosis through the expression of surface proteins (internalins) encoded by two non-redundant operons, and the pore-forming toxin Listeriolysin O (LLO). LLO also contributes to the bacterial escape from the phagocytic vacuole, thus permitting *L. monocytogenes* intracellular replication. As a non-motile Gram-positive, *L. monocytogenes* induces actin biosynthesis by the host cell to propel itself through the host cell cytoplasm. Actin nucleation at one pole of the bacterium and subsequent assembly of actin filaments enables the movement of the bacterium within the cell and, eventually, its escape and invasion of a neighboring host cell.

Listeriosis is relatively uncommon, yet it has a death rate of 20–30%. Listeriosis outbreaks are associated with the consumption of food contaminated with the pathogen, typically in food-processing environments. The largest outbreak to date occurred in South Africa between June 2017 and April 2018, with 937 cases and 193 deaths (Thomas et al., 2020).

Diarrheal diseases in low-and-middle-income countries

Diarrheal diseases are a leading cause of illness and death among children under 5 years of age. Although vaccine strategies and access to clean water have improved the situation, diarrheal diseases are still estimated to cause 1.4 million deaths every year. The WHO estimates show that diarrheal diseases also rank among the top 10 causes of deaths in low income and lower-middle-income countries.

In two extensive multicenter studies, the Global Enteric Multicenter Study (GEMS) (Kotloff et al., 2013), and the Malnutrition and Enteric Disease Study (MAL-ED) (Platts-Mills et al., 2018). *Shigella* spp. together with ST toxin positive enterotoxigenic *E. coli* (ETEC) and *Campylobacter* spp. were identified as the most common bacterial causes of diarrhea in children in Africa, Asia, and Latin America. These pathogens are particularly common in young children under 2 years of age with symptoms of diarrhea and are hence serious threats to child health. Additional studies using the large material collected in these two multicenter studies, as well as follow-up studies, showed that *Shigella* and ST positive ETEC were even more common than previously thought. They were also, together with, e.g., enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC), linked to death cases in children under 2 years with both severe and mild-to-moderate diarrhea.

Diarrheagenic *Escherichia coli*

Pathogenic *E. coli* encompass several well-described pathogens; two subspecies are extraintestinal, i.e., uropathogenic *E. coli* (UPEC) that cause urinary tract infections and sepsis, and neonatal meningitis causing *E. coli* (NMEC) that can cause infections of the brain as well as sepsis. These subspecies are important pathogens and implicated in the global spread of antibiotic resistance genes but are not the topic of this article, although they might be part of the gut microbiota. Intestinal pathogenic *E. coli* include the common diarrheal pathogens enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC). This group is common in low-and-middle-income countries and frequent causes of diarrhea. In addition, EHEC and EIEC (enteroinvasive *E. coli*) may cause bloody diarrhea but these pathotypes seem to be less common in low-income settings.

Enterotoxigenic *Escherichia coli*

ETEC is regarded as the most common cause of tourist's diarrhea. It is contracted by the ingestion of contaminated food and drink and give rise to a range of mild symptoms of diarrhea to profuse watery diarrhea that resembles severe cholera. Vomiting and fever are sometimes associated with ETEC diarrhea. ETEC colonizes the distal ileum and adheres to the epithelium by means of colonization factors (CFs) termed CFA/I and CS1-CS8, CS12-14, CS17-23, CS26-31. Each isolate typically expresses 1-3 CFs and one or two toxins. The toxins give rise to watery diarrhea and secretion of Na⁺ and Cl⁻ characteristic of ETEC diarrhea and cholera. The A-B toxin heat-labile toxin (LT) is homologous to the cholera toxin (CT) and binds GM1 on the epithelial surface. It activates adenylate cyclase and increases cAMP in the cell, causing deregulation and opening of the ion channel cystic fibrosis transmembrane regulator (CFTR). The other toxin type of ETEC is the heat-stable toxin with the two variants STh and STp commonly found in human isolates. ST toxins are small peptides that increase intracellular cGMP and also open the CFTR. While large studies repeatedly linked ST positive ETEC with diarrheal cases, ETEC strains expressing only-LT are found both in asymptomatic and symptomatic carriers.

Enteropathogenic *Escherichia coli*

EPEC is another major pathogen infecting children and a frequent cause of persistent diarrhea. EPEC can be divided into two groups: typical EPEC (tEPEC) or atypical EPEC (aEPEC) strains. Typical EPEC strains express virulence genes *eae* (involved in an AE phenotype similar to EHEC) and *bfp* (bundle forming pili) but lack Shiga toxin genes found in STEC. Atypical EPEC are *eae* positive and *bfp* and *stx* negative. EPEC attach to the small and large intestine through Bfp, promoting microcolony formation. As EHEC, EPEC injects virulence factors encoded by the LEE region and the EAF plasmid into the host cell via the T3SS and form AE lesions. During adherence, tEPEC strains are able to display a localized adherence pattern, while aEPEC strains have more diverse patterns (diffuse, aggregative, or localized). The major reservoir of tEAEC are humans, but aEPEC can be found in both animals and humans. The GEMS reported that tEPEC is not a leading pathogen associated with moderate to severe diarrhea in children; however, EPEC remains an important etiological agent in some countries of the developing world and is the most frequent pathogen detected in mixed infections. Other studies have shown significant proportions of EPEC, specially aEPEC, in asymptomatic cases. tEPEC cause secretory diarrhea with significant water discharges and mucus. EPEC infection can also trigger severe malabsorption of nutrients

that can result in nutritional aggravation and persistence of diarrhea. Some aEPEC strains have the serotype O55:H7 and share virulence and genetic features with EHEC O157:H7. Atypical EPEC might be precursors for *stx*-positive isolates. For example, one study showed that the aEPEC O103:H25 infected by the *stx* bacteriophage became STEC with increased virulence (Sekse et al., 2008).

Enteraggregative *Escherichia coli*

EAEC is a heterogeneous emerging pathogen that causes acute and persistent diarrhea (more than 14 days) among infants. EAEC is also an important pathogen associated with travelers' diarrhea and outbreaks in industrialized countries. EAEC has a distinct ability to produce a "stacked-brick" adherence pattern on epithelial cell lines. To cause infection, EAEC first employs several adhesins (aggregative adhesion fimbria, AAF/I to AAF/IV) to attach to enterocytes and form a biofilm. EAEC also secrete enterotoxins (i.e., EAST1S, SHET1, and pic) and cytotoxins (pet) that cause secretion of electrolytes and inflammation of the mucosa. EAEC is able to disperse along the intestinal mucosa through the secretion of an anti-aggregation protein called dispersin, which helps to establish new infection sites. A large number of EAEC virulence factors are regulated by the transcriptional factor AggR—that controls the expression of 44 plasmid and chromosome-borne virulence genes.

EAEC infection can be characterized by the presence of watery diarrhea and sporadically mucoid diarrhea. Children that have reported EAEC infection suffered from growth impairment regardless of the presence of diarrhea. Most of the studies in the developing world have associated EAEC with diarrhea, but others have also found EAEC in an equal number from cases and control individuals. Acquired immunity as a consequence of frequent exposure to this pathogen where poor sanitation is prevalent, as well as the circulation of strains with low pathogenicity, can be the explanation of asymptomatic carriers of EAEC. The broad diversity in virulence genes makes the establishment of international surveillance and therapeutic approaches challenging (Jensen et al., 2014).

Vibrio cholerae and other *Vibrio* spp.

Vibrio cholerae is a Gram-negative, single-flagellated, and highly motile bacterium native to estuarine and marine environments worldwide. The species includes harmless strains as well as strains that have been responsible for epidemic and pandemic cholera disease. Virulent strains contain the CTX ϕ bacteriophage that encodes the cholera toxin.

Environmental persistence of *V. cholerae* is facilitated by bacterial attachment and biofilm formation to abiotic and biotic surfaces, including chitinous organisms that provide chitin as a carbon source facilitated by the bacterium's chitinase activity. *V. cholerae* has a complex lifestyle that includes viable but non-culturable (VBNC) cells, predominantly found within the bacterioplankton, and culturable cells, predominantly found as aggregates or as part of biofilms. Environmental conditions such as temperature and nutrient availability induce the switch from VBNC to culturable (Lutz et al., 2013).

V. cholerae survives the action of bile salts and host antimicrobial peptides through the secretion of outer membrane vesicles. During infection, it secretes the cholera toxin (CT), composed by an A subunit and five B subunits. Toxin-coregulated pili contribute to the bacterial attachment to intestinal epithelial cells. The CT B subunit binds to GM1 gangliosides on the surface of intestinal epithelial cells, inducing endocytosis of the toxin and the release of the A subunit, leading to increased adenylate cyclase activity and the secretion of water and electrolytes to the intestinal lumen that can cause profuse, watery diarrhea. Two serogroups, O1 and O139, have been responsible for seven pandemics between 1817 and 1971. Most infections are asymptomatic or mild. The Centers for Disease Control (CDC) estimates that only 5–10% of the carriers develop severe disease. Estimations calculate 1.3–4.0 million cases per year, causing 21,000–143,000 deaths, primarily in South Asia, Africa, and areas of Latin America. Treatment includes rehydration therapy, antibiotic administration, and in pediatric cases, zinc supplementation. In 2016, the FDA approved an oral vaccine against cholera.

Non-cholera *Vibrio* spp. like *Vibrio vulnificus* or *Vibrio parahaemolyticus* are causative agents of vibriosis, a less severe diarrheal disease than cholera associated with the consumption of contaminated raw seafood. Vibriosis is not restricted to the developing world and is responsible for over 80,000 cases of infections every year only in the US, according to the CDC.

Shigella spp.

Dysentery or shigellosis caused by the invasion of the intestinal epithelium by *Shigella* spp. in the terminal ileum, colon, and rectum, manifests as bloody diarrhea and was described for the first time over 100 years ago. It only affects primates, including humans. Pathogenic *Shigella* are classified into four major serological groups: Group A (*Shigella dysenteriae*), Group B (*Shigella flexneri*), Group C (*Shigella boydii*), and Group D (*Shigella sonnei*). Disease caused by *S. sonnei*, which is prevalent in the developed world, tends to be mild. The most severe manifestation of the disease is caused by *S. flexneri*. A hallmark of shigellosis is the low infective dose needed, which can be as low as 10 microorganisms for some virulent strains (Kothary and Babu, 2001).

Shigella infection requires a conserved T3SS encoded on a large plasmid common to all *Shigella* spp. Upon transcytosis to the epithelial sub-mucosa through M cells, *Shigella* spp. are phagocytosed by macrophages, but a swift induction of apoptosis enables bacterial survival and spread. Destruction of the epithelial lining occurs upon recruitment and infiltration of polymorphonuclear neutrophil leukocytes, originating bloody diarrhea typical of the disease. Some strains produce the Shiga toxin, which inhibits protein biosynthesis by targeting the 28S rRNA of the 60S ribosomal subunit. *Shigella* infections are endemic to temperate and tropical regions and are estimated 80–165 million annually, causing 600,000 deaths.

Yersinia enterocolitica

Yersinia enterocolitica is the causative agent of yersiniosis, causing enteritis, including fever and sometimes bloody diarrhea, especially in children. Domestic pigs constitute the main reservoir of *Y. enterocolitica*. Thus, consumption of infected undercooked pork is usually the cause of infection, although outbreaks associated with consumption of vegetables or contaminated water have also been reported. Pathogenic *Yersinia* spp. harbor a virulence plasmid named pYV that encodes a T3SS called the Ysc injectisome, as well as proteins secreted by Ysc, collectively termed “Yops” (*Yersinia* outer proteins). Expression of the genes encoding the Ysc-Yop system depends on the AraC-family transcriptional activator VirF, whose expression is controlled by temperature, and contributes to bacterial survival to host defenses by arresting phagocytosis (Shoaib et al., 2019). *Y. enterocolitica* is unable to chelate iron and relies on siderophores produced by other bacteria to acquire this element. Variants of pYV harbor a high pathogenicity island encoding the siderophore yersiniabactin that contributes to bacterial virulence (Pelludat et al., 2003). Indeed, iron supplementation is associated with higher severity of the disease. *Y. enterocolitica* also harbors a chromosomally encoded heat-stable enterotoxin similar to that of ETEC, although its contribution to infection is thought to be minor. The CDC estimates more than 100,000 annual cases of yersiniosis only in the US every year.

Gastric infections

In the stomach, ingested food is blended with highly acidic gastric juice. The luminal content of the stomach is hence acidic and typically ranges from pH 1–5. This environment was considered to be too hostile for bacterial colonization until the discovery of *Helicobacter pylori* in 1983. This finding by Barry J. Marshall and J. Robin Warren was later rewarded with the Nobel prize in Physiology or Medicine in 2005.

Helicobacter pylori

H. pylori is a Gram-negative, microaerophilic bacterium that thrives in the gastric epithelium and lumen, where oxygen levels are around 5%. *H. pylori* is not an acidophilic bacterium but uses the action of secreted urease to neutralize the surrounding acidic environment. *H. pylori* adheres to the gastric epithelium by means of the adhesion factors BabA and SabA and secretes the major toxins CagA and VacA. CagA induces cell rearrangements and alters signaling cascades, while VacA induces apoptosis and formation of vacuoles in the target cell.

H. pylori infection is common, and it is estimated that half of the world’s population is infected. Infection is more common in low socioeconomic populations where up to 90% can be infected. Although infection always causes inflammation of the gastric epithelium, the majority of the infections do not give symptoms. However, 10–15% of cases develop gastric or duodenal ulcers and 1–2% gastric cancer.

H. pylori entirely dominates the stomach microbiota in persons with ongoing *H. pylori* infection. Sequence analyzes often record more than 90% of the total content to be *Helicobacter*. However, other species might be found in the healthy stomach; the absolute numbers of bacteria are much lower, and many species are probably transient bacteria that do not reside permanently in the stomach mucosa. Lactobacilli and oral species such as *Streptococcus*, *Veillonella*, and *Treponema* are often found in the stomach both in the presence and absence of *H. pylori*.

Emerging pathogens

Improvements in bacterial identification methods, boosted by next-generation sequencing technologies and microbiome studies, have expanded the spectrum of species associated with human gastrointestinal disease. One of the most renowned cases is that of *Fusobacterium nucleatum*, a commensal bacterium and strict anaerobe that colonizes the oral cavity that is rarely isolated from extra-oral samples in healthy individuals. There is, however, a growing body of evidence that shows an association between *F. nucleatum* abundance and gastrointestinal diseases like inflammatory bowel disease (IBD), colorectal cancer (CC), or appendicitis (Allen-Vercor et al., 2011). There is currently no clear evidence of whether *F. nucleatum* is an opportunistic colonizer, perhaps facilitated by disease-induced dysbiosis upon translocation from the oral cavity, or an active driver of disease.

Waterborne bacteria are frequent causative agents of gastrointestinal disease, normally after exposure to water or consumption of raw fish or seafood. In recent years, occasional reports *Shewanella algae* gastrointestinal infection have been published. Microbiome studies have detected an increased abundance of *Shewanella* spp. in colorectal cancer mucosa (Flemer et al., 2017; Kinross et al., 2017). The role of *Shewanella* spp., if any, in gastrointestinal disease remains unclear. Other waterborne bacteria reported causing human GI infection include members of the genera *Aeromonas* and *Plesiomonas* (Vila et al., 2003).

Development of Crohn’s disease (CD) has been linked to exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP), an intracellular bacterium that is transmitted to humans primarily through milk consumption or upon contact with the pathogen in the environment (McNees et al., 2015). As with other potential emerging pathogens, further research is needed to prove it as a causative agent of CD.

Bacterial dysbiosis

Bacterial dysbiosis is a disturbed composition of bacterial species residing at a specific location. There is an increasing recognition that a highly diverse consortium of bacterial species in the GI tract is beneficial for health. Hence, when the balance is disturbed by an overgrowth of individual species or shifts towards specific members of the gut microbiota with certain metabolic properties, disease symptoms might occur.

Inflammatory diseases of the GI tract

Inflammatory bowel disease

IBD is characterized by chronic inflammation of the GI tract with alternating clinical relapse phases and remissions. IBD is divided into ulcerative colitis (UC) and Crohn's disease (CD). IBD is believed to be an overreaction of the immune system towards the resident microbiota, and although genetic predisposition, environmental factors, and composition of the microbiota seem to play a role, the factors behind the onset of UC and CD are not fully known. The mucosa of IBD patients is inflamed, and high levels of pro-inflammatory cytokines such as TNF α and IL-6 are secreted by activated immune cells. Hence, e.g., anti-TNF therapy has proven to help a subset of patients. Recent studies indicate that analyzes of the composition of the microbiota might be able to predict if anti-TNF treatment is successful or not in IBD patients.

Inflammatory bowel symptoms

IBS is a common condition characterized by disturbed bowel functions. The symptoms are diffuse and include abdominal pain, bloating, and dysfunctional bowel habits, including diarrhea and constipation. It has been estimated that 6–10% of the general population suffer from IBS. Several studies have indicated that a change in diet might improve IBS. The FODMAP regime that include a diet low in fermentable oligosaccharides, di- and monosaccharides, and polyols has been successful for some IBS patients. Several studies suggest that the resident microbiota composition can play a role in whether the patient improves by the diet or not.

Acute GI infections have been linked to later development of persistent symptoms such as induction of IBD and IBS. A systematic review identified *Salmonella*, *Campylobacter*, and *Clostridioides difficile* to be associated with increased risk of incident IBD (Axelrad et al., 2020). Increasing evidence have associated the *E. coli* pathobiont (neither commensal nor a true pathogen) adherent-invasive *E. coli* (AIEC) with Crohn's disease (CD). AIEC has also been found in healthy volunteers, and no virulence marker has been described yet in comparison to the other well-characterized pathotypes of *E. coli*. However, it can cause perturbations in the normal intestinal environment and due to the presence of some genetic determinants such as adhesins and invasins, allow the bacteria to invade the intestine, expand and exacerbate inflammation (Shaler et al., 2019).

Clostridioides difficile

The normal microbiota often contains low numbers of *Clostridioides difficile* (formerly *Clostridium difficile*). *C. difficile* is associated with a common type of bacterial dysbiosis caused by the use of broad-spectrum antibiotics that kill several species and allow expansion of resistant *C. difficile*. The resulting so-called antibiotic-induced diarrhea is a common nosocomial infection and can be life-threatening if it develops to pseudomembranous colitis. The overgrowth of *C. difficile* cause severe inflammation of the large bowel due to the action of two toxins A and B (TcdA and TcdB). As in many other pathogens, there are increasing concerns over the occurrence of hypervirulent clones. In *C. difficile* the ribotype 027 emerged in 2003 and has since then spread globally. It is responsible for outbreaks globally and produces more toxins, have increased adherence to the epithelium and is resistant towards fluoroquinolones and erythromycin. Other emerging clones are ribotype 078 and 106, where the latter has increased in recent years.

Since the cause of CDI often is antibiotic treatment, it is imperative to stop administration of the first antibiotics. Thereafter treatment with vancomycin or metronidazole, effective towards anaerobes like *C. difficile*, is often the next therapy. Some CDIs are cured by this second administration of antibiotics, but recurrent diarrhea occurs in a subset of patients. Fecal microbiota transplants (FMT) is an effective therapy for CDI. Although the exact mechanisms that confer mitigation of the CDI diarrheal symptoms are unknown, it is believed that the transferred FMT contains a healthy gut microbiota that establishes itself in the recipient and restores normal gut functions.

There are some indications that the metabolites of the FMT are responsible for the cure of treated patients. The primary bile taurocholic acid (TCA) promote spore germination of *C. difficile*, whereas secondary bile acids like deoxycholic acid inhibit vegetative growth and toxin activity. In humans, primary bile acids secreted by the gall bladder into the duodenum are converted by large bowel resident microbiota species expressing bile salt hydrolases and bile converting enzymes (i.e., 7- α -dehydroxylase). In support of this CDI, patients have been found to have low levels of secondary bile acids and higher levels of TCA, which would support active growth and toxin production in CDI patients. Other gut factors of importance are short-chain fatty acids (SCFA). Short-chain fatty acids, particularly acetate, propionate, and butyrate, are produced by resident microbiota, and lower levels of SCFA are found in CDI patients. It is, however, unclear if this is a consequence of antibiotic treatment. Valerate, a five-carbon SCFA, seems to inhibit the growth of *C. difficile*, which supports the role of SCFA.

Colonization mechanisms in the intestinal tract

The GI tract is normally colonized by bacterial species of the phyla Firmicutes and Bacteroidetes, while Actinobacteria, Verrucomicrobia, and Proteobacteria are present at lower levels. These phyla cover over 1000 species that normally live in the GI tract and constitute the normal microbiota. The presence of this vast number of different species provides so-called colonization resistance (CR) to invading pathogens by competing for nutrients and space. CR is often very efficient, but some pathogenic species elaborate intriguing mechanisms to overcome this. The ability of pathogenic species to outcompete the normal microbiota is a new research field with interesting findings.

Several pathogens utilize specific host factors present in the GI tract to induce virulence genes encoding adhesion factors and toxins that facilitate inflammation and disease progression. In addition, certain pathogenic members of, e.g., Proteobacteria, have the ability to outcompete the resident microbiota, and reports have shown that, e.g., *V. cholerae*, *S. Typhimurium*, and ETEC can constitute over 50% of all bacteria in the gut during acute diarrheal sequelae. There is evidence that this overgrowth is due to an inherent ability to cause inflammation of the epithelium, followed by the specific use of competing nutrients or alternative metabolic pathways in the inflamed gut. Pathogenic bacteria are able to adapt to and survive the gastrointestinal tract and cause symptoms by adherence to the epithelium. While some bacteria like *Salmonella enterica* and *Shigella* spp. induce massive inflammation and/or invasion others like *V. cholerae*, and ETEC are believed to cause only mild inflammation and instead induce diarrheal symptoms by cytotoxic toxins that deregulate electrolyte and water secretion of epithelial cells. Regardless, the onset of both mild and severe inflammation might release new factors into the gastrointestinal tract, and pathogens that can use these for metabolism have an advantage over resident microbiota that cannot metabolize the specific factors.

Interactions with host factors that facilitate colonization and virulence

Mucin degradation

The surface of the GI epithelium is covered by a layer of mucus (mucin), whose composition, structure, and thickness varies along the GI tract. This protective layer prevents epithelial cells from damage caused by harsh substances, and at the same time, acts as a barrier against pathogens. A major component of mucin is O-glycans, which constitute a carbon source for the associated microbiota, thus offering a complex niche whose microbial composition varies longitudinally and transversally in the GI (Tailford et al., 2015). The inner layer of mucin, closer to the epithelial lining, is reported to be sterile (Hansson and Johansson, 2010).

The ability to degrade mucin is a fitness advantage for several gut pathogens, not only for its utilization as a carbon source but also as a way to gain access to host cells. Mucinase activity has been demonstrated for *Shigella flexneri*, *Helicobacter pylori*, pathogenic *E. coli*, and *V. cholerae*, among other pathogens (Carlson-Banning and Sperandio, 2016). The diverse O-glycan composition of mucin also provides a primary attachment point for GI pathogens. *S. enterica*, for example, while unable to degrade complex glycans, attaches to intestinal glycans and uses monosaccharides released upon mucin degradation by the commensal consortium (Josenhans et al., 2020). Expression of mucin-binding adhesins and lectins is thus included in the 'toolbox' of an array of pathogens, including *S. enterica*, *C. jejuni*, EHEC, *V. cholerae*, and *L. monocytogenes*.

Bile salt tolerance

Pathogenic bacteria that aim to colonize the human gut have to encounter the antimicrobial detergent-like activity of bile acids, which are secreted upon digestion. In addition to the physiological roles of bile, enteric pathogens have evolved to tolerate and use bile acids as signal molecules for the activation of virulence. For example, bile acids activate the expression of virulence genes of ETEC involved in adherence but inhibit toxin production (Joffe et al., 2019). In *Shigella flexneri*, bile induces over 50 genes, many of them involved in colonization and T3SS effectors. Bile acids also activate several pathogenic features of *V. cholerae*, including biofilm formation, T3SS, T6SS, and virulence regulation. *Salmonella* has shown to be more tolerant to bile acids, which is achieved by pumping out the bile acids and modifying the lipopolysaccharides of the outer membrane for increased fitness in a bile-rich environment. Among Gram-positive pathogens, bile acids can be crucial for *C. difficile* infection by interacting with the germinant receptors within the spores' inner membrane that trigger spore germination (Sorg and Sonenshein, 2008). Overall, these and more other examples highlight that bacterial pathogens can respond to their environmental surroundings to effectively regulate the virulence to successfully cause infection (Sistrunk et al., 2016).

Utilization of local or host-derived oxygen and anaerobic electron acceptors

A signature of gut inflammation is an unbalanced proportion of strict anaerobes with respect to Proteobacteria, including Enterobacteriaceae, a phenomenon that is at least in part driven by oxidation of formate and respiration of oxygen in vivo as shown in a murine model of colitis (Hughes et al., 2017). Inflammation induces oxygenation after increased blood flow, and aerobic metabolism is thus a key factor in the establishment and overgrowth of facultative anaerobic pathogens, including, e.g., *V. cholerae* (Van Alst and DiRita, 2020). Oxygen levels in the small intestine are higher in intestinal crypts than in the lumen and the tip of villi, where the microenvironment is nearly anoxic. Hence, the observed requirement of oxygen for colonization and/or virulence of pathogenic facultative anaerobic bacteria infecting the small intestine, including ETEC and *V. cholerae*, might indicate that intestinal crypts are the main site of infection for these pathogens.

While aerobic metabolism is the most energetically favorable, the intestinal tract provides niches that are rich in other substances that can fuel bacterial anaerobic metabolism. During *Salmonella* infection, inflammation of intestinal tissues generates reactive oxygen species that react with local thiosulfate to form tetrathionate, a compound that *Salmonella* can use as an electron acceptor for anaerobic respiration and whose utilization gives a growth advantage to the pathogen (Winter et al., 2010). In vivo oxidation of trimethylamine generates trimethylamine N-oxide (TMAO), a compound that also forms part of the repertoire of alternative electron acceptors used by *Salmonella* or *E. coli*. Nitrate, a major alternative electron acceptor, used by many facultative anaerobes, has been shown to be generated in situ in the gut during inflammation and used by commensal *E. coli* to colonize the intestines in a mouse model (Winter et al., 2013). The genome of *E. coli* encodes two respiratory and one dissimilatory nitrate reductases, which are conserved across pathotypes; therefore, it is reasonable to assume that such a capacity could also be used by pathogenic *E. coli* strains to thrive during infection. Taken together, this exemplifies how a versatile facultative metabolism, together with swift adaptation of cellular programs to changing tissue environments, contribute to pathogen spread and disease progression.

Summary

In summary, bacterial gastroenteric diseases and/or symptoms are caused by interactions between a diverse set of pathogenic bacteria and the human host. The ability of these bacteria to cause disease in humans seems to be dependent on the ability to sense host factors and interact with, and utilize, the resident microbiota as well as the gut epithelium. It is evident that we still need to understand much more how pathogens use environmental factors and other species to be able to colonize the hostile environment that constitutes the gastrointestinal tract in order to be able to prevent or fight gastrointestinal diseases more efficiently. This is important since gastrointestinal diseases still are significant causes of global morbidity and mortality.

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Gastrointestinal Tract Infections: Viruses

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Introduction

Diarrheal diseases are a leading cause of morbidity worldwide, particularly among young children (Global Burden of Disease (GBD) 2019 Diseases and Injuries Collaborators, 2020). An estimated 4.5 billion episodes of diarrhea and >1.5 million diarrhea-related deaths occur annually, with the highest burden in the youngest and oldest age groups (Global Burden of Disease (GBD) Collaborators, 2017a,b; Global Burden of Disease (GBD) 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017). Viral etiologies of diarrheal illnesses are common: it has been estimated that 3 of the top 10 causes of pediatric diarrheal deaths are due to illnesses caused by viruses (Global Burden of Disease (GBD) Collaborators, 2017b).

Major viral etiologies of diarrheal disease include rotavirus, norovirus, sapovirus, astrovirus, and enteric adenoviruses (Fig. 1) (Global Burden of Disease (GBD) Collaborators, 2017b). Among these pathogens, rotavirus group A (RVA) is associated with the greatest morbidity and mortality burden, concentrated among young children <5 years of age (Global Burden of Disease (GBD) Collaborators, 2018). Although RVA burden has been declining due to increasing implementation of national vaccination programs, RVA remains a leading cause of severe pediatric diarrheal illness and deaths, especially in lower-resource countries (Global Burden of Disease (GBD) Collaborators, 2018). Adenovirus has been estimated to cause the next highest numbers of diarrheal episodes and deaths, also disproportionately affecting children <5 years (Global Burden of Disease (GBD) Collaborators, 2018; Lee et al., 2020). Although norovirus commonly causes illness across the age spectrum (Burke et al., 2021; de Wit et al., 2001; Harris et al., 2017; Kirk et al., 2015; O'Brien et al., 2016), it is an increasingly prevalent etiology of pediatric diarrhea in settings with high coverage of RVA vaccine (Hassan et al., 2019; Koo et al., 2013; Lartey et al., 2020; McAtee et al., 2016; Payne et al., 2013). Astrovirus and sapovirus, while not as prevalent as RVA, norovirus, or adenovirus, also contribute to the burden of viral gastroenteritis, especially among young children (Burke et al., 2021; Mattison et al., 2021; Vielot et al., 2021). This chapter will discuss molecular, immunological, and epidemiological aspects of each of these five enteric viruses, including methods of diagnosis, prevention, and control.

Clinical presentation and management

Viral gastroenteritis commonly causes sudden-onset diarrhea, vomiting, abdominal pain, and fever. Although illnesses are usually self-limiting, symptoms can be severe, leading in some cases to dangerous dehydration; severe outcomes, such as hospitalization and death, are more common in lower-resource settings, as well as among young children and older adults (Global Burden of Disease (GBD) Collaborators, 2017a). There are no specific treatments for viral gastroenteritis: clinical management focuses on

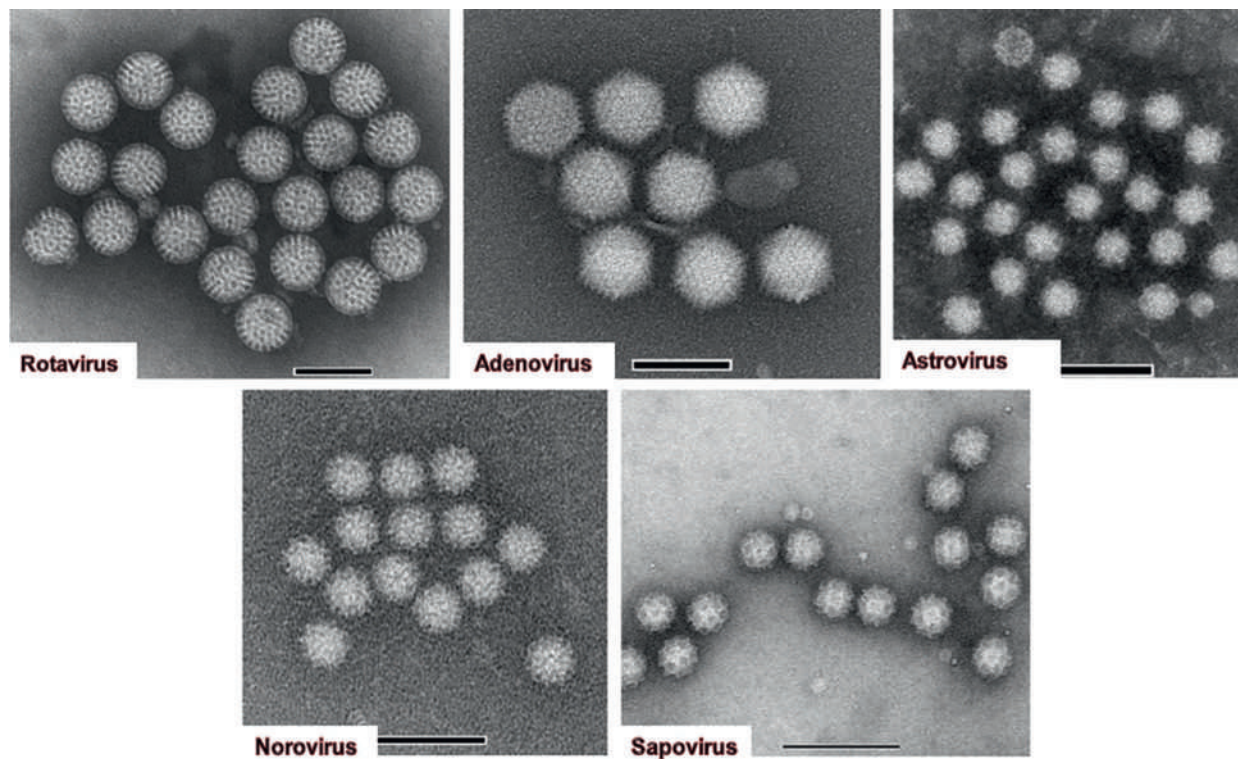


Fig. 1 Electron micrographs of negatively strained gastroenteritis viruses. Bar = 100 nm. Courtesy: Charles Humphrey, CDC.

prevention of or treatment for dehydration or through oral or, in severe cases, intravenous rehydration (Banyai et al., 2018; Hartman et al., 2019). Antiemetics and probiotics are sometimes used as adjunct therapies, and may help reduce duration of symptoms, although more evidence is needed to recommend universal use (Collinson et al., 2020; Hartman et al., 2019). Antimotility drugs are not recommended for treatment of acute gastroenteritis (AGE) in young children (Banyai et al., 2018; Hartman et al., 2019).

Enteric viruses are transmitted through the fecal-oral route, making sanitation and hygiene key elements of prevention and control. Handwashing with soap and water is especially important for norovirus, as alcohol-based hand cleaners have been shown to be less effective against norovirus (Liu et al., 2010; Tuladhar et al., 2015). Additionally, many enteric viruses, particularly norovirus, can be environmentally persistent (Espinosa et al., 2008; Kotwal and Cannon, 2014); thus, surface cleaning with effective disinfectants is an important part of halting onward transmission. Because the infectious dose for some enteric viruses is quite low, and prolonged fecal shedding can be common, it is recommended that sick individuals exclude themselves from food preparation and some types of work (e.g., work in a healthcare setting) for at least 48 h following symptom resolution (Banyai et al., 2018). Among the five viruses discussed in this chapter, only RVA has licensed vaccines available, although several norovirus candidate vaccines are in development (Mattison et al., 2018; Burke et al., 2019; Cates et al., 2020).

Rotavirus

History

Rotaviruses were first described in diarrheic mice (Adams and Kraft, 1963), monkeys (Malherbe and Harwin, 1963) and cattle (Mebus et al., 1969) in the 1960s. The first human-associated RVA was discovered in 1973 by electron microscopy (EM) in duodenal biopsy samples from children with severe diarrhea and in diarrheic fecal samples from children (Bishop et al., 1973; Flewett et al., 1973). Rotavirus is derived from the Latin word *rota* (meaning wheel), for its wheel-like appearance (Estes and Greenberg, 2013; Flewett et al., 1974).

Morphology

RVAs are nonenveloped, icosahedral-shaped viruses as observed by negative-stain EM (Fig. 1). Three types of RVA particles are observed under EM: (1) triple-layered particles (TLP) composed of a 3-capsid structure: an inner core, an intermediate capsid, and an outer capsid with short radiating spikes; (2) double-layered particles lacking an outer capsid; and (3) single-layered particles lacking outer and intermediate capsids (Crawford et al., 2017; Estes and Kapikian, 2007). TLP are ~100 nm in diameter and are considered infectious (McClain et al., 2010).

Structure

RVAs possess a genome of 11 double-stranded RNA (dsRNA) segments. Six segments encode genes for structural proteins (VP1, VP2, VP3, VP4, VP6, and VP7), and five encode non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5/NSP6) (Estes and Kapikian, 2007; McClain et al., 2010; Estes and Greenberg, 2013). Each segment encodes a single protein except for segment 11, which can encode for two non-structural proteins, NSP5 and NSP6, in some strains. The 11 dsRNA segments are surrounded by the VP2 protein, which forms the inner capsid, and the VP6 protein that forms the intermediate capsid. The outer capsid is comprised of the VP7 protein, which forms the shell around the virion (Aoki et al., 2009), and the VP4 protein, which forms the protease-activated spikes that protrude outward from the capsid for attachment (Dormitzer et al., 2002) (Fig. 2A).

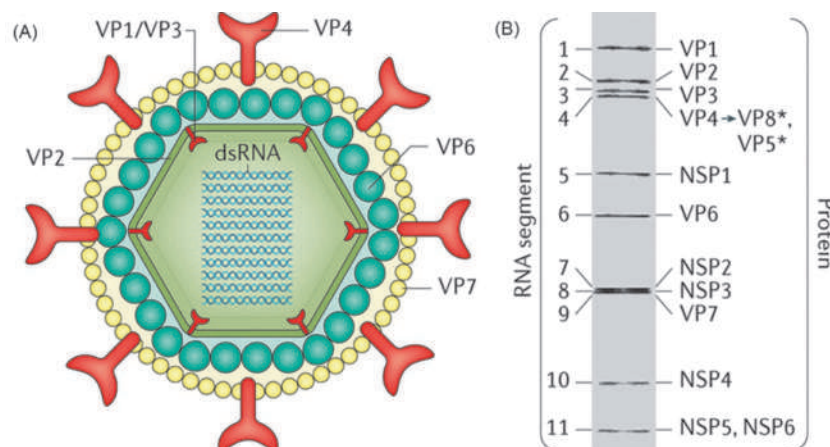


Fig. 2 Rotavirus group A (RVA) structure. (A) Cross-sectional schematic of the triple-layered particles RVA. (B) Electrophoretic migration profile of the 11 segments of RVA dsRNA and the encoded proteins for the simian RVA SA11 strain. Used with permission from Crawford SE, Ramani S, Tate JE, Parashar UD, Svensson L, Hagbom M, Franco MA, Greenberg HB, O’Ryan M, Kang G, Desselberger U and Estes MK (2017) Rotavirus infection. *Nature Reviews. Disease Primers* 3: 17083.

Nomenclature

Rotaviruses belong to the family *Reoviridae* with at least 10 distinct groups (A–J) based on the antigenic properties of VP6 gene (Matthijnssens et al., 2012). RVA causes >90% of human infections, although rotavirus group B (RVB) and rotavirus group C (RVC) are also associated with AGE. RVA can be classified into 2 major genome patterns: long or short RNA profiles or electropherotypes [E-type], based on differences in the relative migration pattern of genome segments 10 and 11 on polyacrylamide gels (Fig. 2B). Two outer capsid proteins are used traditionally to classify RVA: VP7 determines the G (glycoprotein) serotypes/genotypes, and VP4 determines the P (protease-cleaved) serotype/genotype. Since 2008, a classification scheme based on nucleotide sequence identity cutoff percentages has been used to assign a genotype to each of the 11 RVA genes: Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx for VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 (Matthijnssens et al., 2008). To date, RVAs of human and animals have been classified into at least 36 G, 51 P, 26 I, 22 R, 20C, 20 M, 31 A, 22 N, 22 T, 27 E, and 22 H genotypes (Table 1) (Banyai et al., 2018). Currently, >80 G and P genotype combinations of RVA are known to infect humans, but six combinations (G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8]) account for about 90% of severe infections (Banyai et al., 2018).

Replication and specific aspects of infection

RVA enters the body through the mouth and infects the mature enterocytes on the tips of the absorptive intestinal villi of the small intestine (Ramig, 2004). The incubation period of RVA in young children is 1–3 days (Bishop, 1996) and symptoms typically last 3–7 days, although in more severe cases symptoms can last up to 14 days (Bernstein, 2009). RVA infection is usually localized to the intestinal tract; however, antigenemia and viremia have been reported in children with RVA diarrhea (Blutt et al., 2003; Moon et al., 2012).

RVA entry into host cells is a multistep process. Both outer-capsid proteins VP7 and VP4 play important roles in virus entry and infection (Crawford et al., 2017). After infection, the VP4 protein is cleaved by intestinal trypsin into two polypeptides: VP8*, which forms the head of the protein, and VP5*, which forms the stalk and base of the protein (Arias et al., 1996). The VP8* domain of the RVA VP4 protein mediates initial interaction with the host cell surface, followed by the interactions of RVA proteins VP5* and VP7 with multiple co-receptors (Arias et al., 1996; Lopez and Arias, 2004; Desselberger, 2014). RVA attachment to host cells is also mediated by binding partners on the host cell surface, including sialoglycans and human histo-blood group antigens (HBGAs), carbohydrates found on the surface of gut epithelial cells (Arias et al., 2015). Following entry, RVA travel to different endosomal compartments and lose their outer layer before entering the cytosolic space (Arias et al., 2015). After cellular uptake, RVA replication and assembly occur in cytoplasmic viroplasms, and newly produced RVA are released from the cells through cell lysis or vesicular transport (Fig. 3) (Crawford et al., 2017).

Table 1 Rotavirus group A (RVA) genes and genotypes.

Gene segment	Encoded protein	Size in bp	Location in virus particles	Protein	Protein function	Number of genotypes	% Identity cutoff
1	VP1	3302	Core	RNA-dependent RNA polymerase	Complex with VP3	22R	83
2	VP2	2687	Core	Inner capsid core protein	Required for replicase activity of VP1	19C	84
3	VP3	2592	Core	Methyltransferase	Complex with VP1	20M	81
4	VP4	2362	Outer Capsid	Protease-sensitive outer capsid protein, P-type neutralization antigen	Attachment protein, protease-enhanced infectivity, virulence, fusion with cell membrane	51P	80
5	NSP1	1581	Nonstructural	Interferon antagonist	RNA binding, E3 ligase	31A	79
6	VP6	1356	Inner Capsid	Intermediate capsid protein	Intracellular neutralization, required for transcription	26I	85
7	NSP3	1074	Nonstructural	Translation enhancer	Inhibits host protein translation	22 T	85
8	NSP2	1059	Nonstructural	NTPase	Inhibits host protein translation	22 N	85
9	VP7	1062	Outer Capsid	Glycoprotein, G-type neutralization antigen	Glycoprotein calcium-dependent trimer	36G	80
10	NSP4	751	Nonstructural	Enterotoxin	Plays role in morphogenesis of TLPs, interacts with viroplasms and modulates intracellular calcium and RNA replication	27E	85
11	NSP5	666	Nonstructural	Phosphoprotein	Essential for viroplasm formation, RNA binding, interaction with VP2 and NSP6	22H	91
	NSP6		Nonstructural		Interaction with NSP5, localization in viroplasms		

Modified from Desselberger U (2014) Rotaviruses. *Virus Research* 190: 75–96.

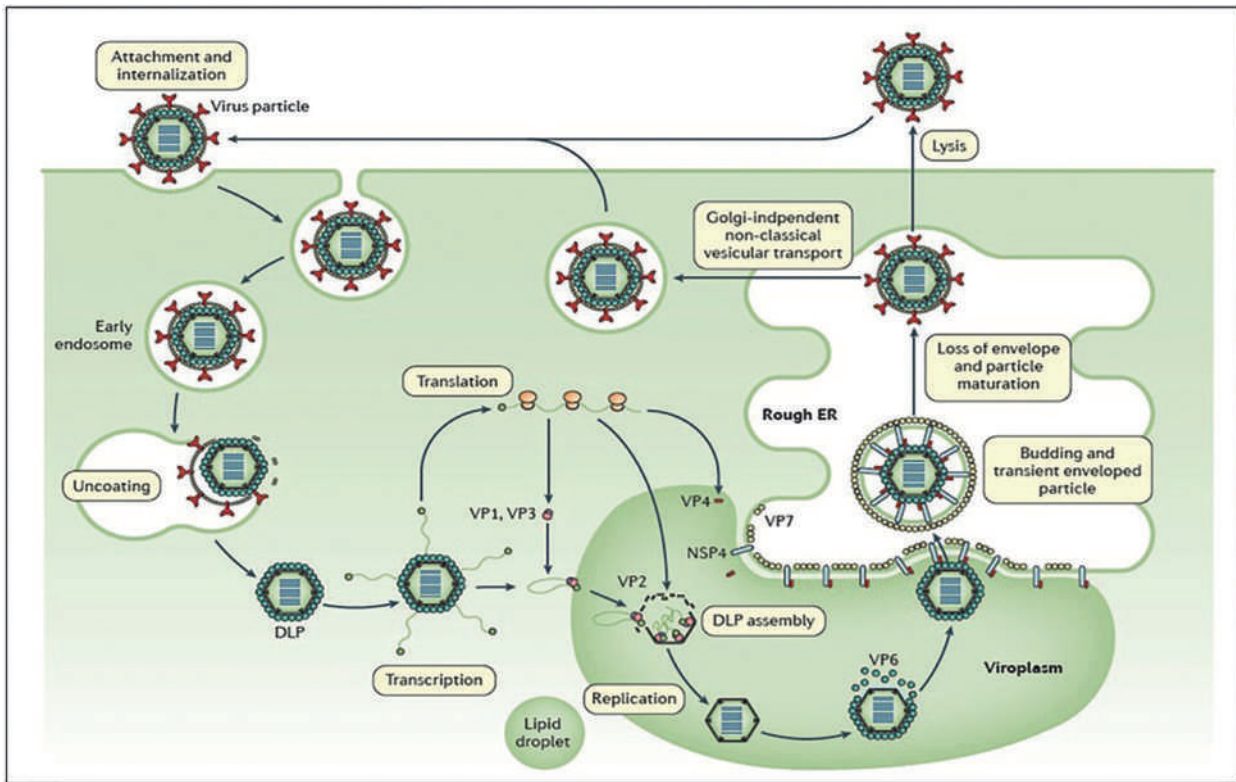


Fig. 3 The rotavirus group A (RVA) replication cycle. (A) RVAs attach to different glycan receptors on the host cell surface through interaction with the viral protein 8* (VP8*) domain of VP4. (B) The virus is internalized into cells by endocytic pathways. The low calcium levels inside the endosome trigger the removal of the outer capsid layer, which releases the transcriptionally active double-layered particle (DLP) into the cytoplasm. (C) Viral mRNA is used as a template for RNA synthesis during genome replication; the RNA is then packaged into new DLPs within viroplasm. (D) Triple-layered particle assembly involves the binding of newly formed DLPs to non-structural protein 4 (NSP4), which serves as an intracellular receptor, followed by the budding of DLPs into the endoplasmic reticulum (ER). (E) In the ER, transient enveloped particles can be observed and the outer capsid proteins VP4 and VP7 are added onto the DLPs. The envelope is then lost, the virus particles mature, and progeny virions are released from cells through cell lysis or by a Golgi-independent non-classical vesicular transport mechanism in polarized epithelial cells. Used with permission from Crawford SE, Ramani S, Tate JE, Parashar UD, Svensson L, Hagbom M, Franco MA, Greenberg HB, O’Ryan M, Kang G, Desselberger U and Estes MK (2017) Rotavirus infection. *Nature Reviews. Disease Primers* 3: 17083.

Immunity

Natural infection with RVA reduces the frequency of subsequent RVA episodes, and specifically protects against subsequent RVA AGE. Acquired immunity is important to prevent both recurrent disease and, to a lesser extent, reinfection following primary infection (Velazquez et al., 1996). The first level of immunity to human RVA is an innate susceptibility or resistance to infection based partially on an individual’s HBGAs phenotypes expressed on the gut surface epithelium. The ABO blood group, fucosyltransferase 2 (FUT2), and fucosyltransferase 3 (FUT3) genes define the ABO blood group antigens, secretor/nonsecretor, and Lewis antigen phenotypes, respectively, of HBGAs, which function as binding ligands and initial receptors required for RVA binding to host cells (Banyai et al., 2018) (Loureiro Tonini et al., 2020). Binding of the VP4 surface protein to the HBGAs occurs in a genotype-dependent manner, suggesting that HBGAs are putative receptors for RVA binding (Huang et al., 2012). The distribution of HBGAs types in different human populations is thought to affect the prevalence of common RVA genotypes across the globe, as well as susceptibility to some strains of animal origin (Nordgren et al., 2014).

RVA immunity is multifactorial, achieved through the combined action of secretory antibodies and humoral and cell-mediated immunity (Franco et al., 2006). Following RVA infection, the innate immune system (mediated by macrophages, interferons, and cytokines) is rapidly triggered, which provides a potent antiviral state by suppressing the RVA replication in the host cell (Angel et al., 2012).

Diagnostic testing

RVA AGE cannot be diagnosed without laboratory tests. Diagnosis of RVA infection relies on direct viral detection from fecal specimens (Banyai et al., 2018). Several immunological and molecular techniques have been developed to detect, genotype, and characterize RVA strains in stool samples (Table 2).

Table 2 Comparison of different types of assays for rotavirus group A (RVA) detection, genotyping, and strain characterization.

Assay method	Assay name	Cost	Time	Throughput	Sensitivity	Specificity	Advantages	Disadvantages	References
Detection	Electron microscopy	High	Days	Low	Low	High	High specificity	Subjective interpretation of results	Flewett et al. (1974)
	Cell culture isolation	Low	Days	Low	Medium	Low	Low cost	Low throughput, prone to contamination	Ward et al. (1984) and Arnold et al. (2009)
	Poly acrylamide gel electrophoresis (PAGE)	Low	Days	Low	Low	High	RV detection and electropherotype determination	Low throughput, labor intensive	Herring et al. (1982) and Matsui et al. (1990)
	Enzyme immuno assay (EIA)	Low	Hours	High	Medium	High	High throughput	Variable sensitivity and specificity of different EIA kits	Dennehy et al. (1988) and Gautam et al. (2013)
	Latex agglutination	Low	Hours	High	Low	Low	Least complex	Low sensitivity and specificity	Cevenini et al. (1983)
	Immunochromatographic tests	Low	Hours	High	Low	Low	Low cost and high throughput	Low sensitivity and specificity	Lee et al. (2007)
	Reverse transcription-polymerase chain reaction (RT-PCR)	Low	Hours	High	High	Medium	High throughput and high sensitivity	Cross reactivity of primers	Wilde et al. (1991)
	Quantitative reverse transcription-polymerase chain reaction (qRT-PCR-RVA)	Low	Hours	High	High	High	High throughput and high sensitivity	Cross reactivity of primers and probes	Gutierrez-Aguirre et al. (2008), Kottaridi et al. (2012), Kang et al. (2004), Katz et al. (2017), and Mijatovic-Rustempasic et al. (2013)
	Multipathogen diagnostic assays	Low	Hours	High	High	High	High throughput and high sensitivity	Cross reactivity of primers and probes	Gray and Coupland (2014), Navidad et al. (2013) and Reddington et al. (2014)
Serotyping/Genotyping	Serotyping	Medium	Days	Low	Low	Low	Low cost	Low sensitivity and limited Mabs	Gomara et al. (2000)
	RT-PCR	Low	Hours	High	High	Medium	High throughput and high sensitivity	Cross reactivity of primers	Esona et al. (2015)
	qRT-PCR	Low	Hours	High	High	High	High throughput and high sensitivity	Cross reactivity of primers and probes	Gautam et al. (2016), Kottaridi et al. (2012), Liu et al. (2015), and Podkolzin et al. (2009)
Sequencing	Sanger sequencing	High	Days	High	Medium	Medium	High throughput and sequence availability	High cost and medium sensitivity/specificity	Das et al. (1994), Gentsch et al. (1992), and Iturriza-Gomara et al. (2001)
	Next generation sequencing using MiSeq	High	Days	High	Medium	Medium	High throughput and sequence availability	High cost and medium sensitivity/specificity	Katz et al. (2019), and Sarkar et al. (2020)
	Next generation sequencing using Minlon	High	Days	High	Medium	Medium	High throughput and sequence availability	High cost and medium sensitivity/specificity	Kim et al. (2020)

RVA detection assays

RVA detection assays have included EM, cell culture isolation, polyacrylamide gel electrophoresis (PAGE) of viral segments, enzyme immunoassays (EIAs), latex agglutination tests, immunochromatographic tests, RT-PCR and real time or quantitative RT-PCR (qRT-PCR), but only Ag-detection EIAs and singleplex/multiplex PCR assays are used widely today. Several multipathogen diagnostic kits are commercially available to rapidly detect enteric pathogens in a high throughput format, including the Luminex Gastrointestinal Pathogen Panel (xTAG GPP) and Film Array Gastrointestinal Panel (Table 2) (Esona and Gautam, 2015).

RVA serotyping and genotyping assays

RVAs are traditionally classified based on the serologic/genetic characteristics of the aforementioned outer capsid proteins VP7 (G) and VP4 (P) that elicit neutralizing antibodies in the host. During RVA surveillance studies, both G and P serotypes/genotypes are monitored because they reassort independently from one another in vivo. RVA strains can be characterized for VP7 and VP4 by molecular-based assays, including RT-PCR and qRT-PCR, which have increased sensitivity, higher throughput, and faster turnaround time (Table 2) (Esona and Gautam, 2015).

RVA genotyping by sequencing assays

Sequence analysis is the definitive method for confirmation of RVA genotypes. Since the 1990s, the Sanger dye-termination sequencing method has been the most widely used method of automated DNA sequencing of VP7 and VP4 RVA genes. Currently, the genetic characterization of all 11 RVA genes (Esona and Gautam, 2015) is typically accomplished using next generation sequencing platforms such as the Illumina MiSeq (Illumina, San Diego, CA) (Katz et al., 2019; Sarkar et al., 2020) or the Nanopore MinIon (Oxford Nanopore Technology, United Kingdom). Whole genome level characterization enables identification of reassortant strains beyond VP7 and VP4 genotyping. (Kim et al., 2020) (Table 2).

Epidemiology

RVAs are a major cause of AGE in infants and children <5 years of age in both high- and lower-income countries; however, RVAs can cause illness in adults and has been estimated to cause >200,000 deaths globally per year across all ages (Banyai et al., 2018; Global Burden of Disease (GBD) Collaborators, 2018; Tate et al., 2016). Although infection and severe disease from RVA are prevalent worldwide, >90% of annual RVA deaths globally occur in developing countries, where ready access to rehydration therapy is less available (Banyai et al., 2018; Tate et al., 2016). Infections in the first 3 months of life are generally milder, probably because of protection from maternal antibodies. Children aged 4–23 months are at greatest risk for severe RVA disease, with frequent infections due to lack of immunity and more frequent exposure (Velazquez et al., 1996). Immunity progressively increases with each subsequent infection, and thus symptoms are milder upon reinfection (Greenberg and Estes, 2009).

RVA infection can remain asymptomatic or lead to AGE with mild to severe diarrhea, vomiting, and fever, resulting in dehydration, electrolyte imbalance, abdominal cramps, and death in some cases (Bernstein, 2009). RVA are shed in high concentrations in the stools of infected children and are transmitted primarily by the fecal-oral route, both through close person-to-person contact and through fomites (Butz et al., 1993). Infected children start shedding RVA in their stools before onset of symptoms and may excrete more than 10^{10} – 10^{11} RVA particles per gram of feces. RVA is highly contagious and requires <100 virus particles to cause infection in a susceptible person (American Academy of Pediatrics, 2003). RVA symptoms subside within days in immunocompetent children but can last for several months in immunocompromised children (Saulsbury et al., 1980). RVA infections in adults are generally asymptomatic or cause milder symptoms, but symptomatic infections, including moderate to severe illness, have been observed in older adults (Burke et al., 2018, 2021; Cardemil et al., 2012; Pacilli et al., 2015).

RVA AGE shows prominent winter seasonality in many higher-resource countries, with infection prevalence substantially decreasing during summer months. In many developing countries, however, RVA disease is less seasonal and may occur year-round; seasonal peaks can occur during the dry season in tropical climates (Chao et al., 2019; Levy et al., 2009; Patel et al., 2013).

Vaccines and other methods of control

Prior to RVA vaccine introduction, RVA infections caused about 40% of AGE hospitalizations in children <5 years of age worldwide. In 2009, The World Health Organization (WHO) recommended inclusion of RVA vaccines in national immunization programs globally, as an optimal strategy to decrease the burden associated with severe and fatal RVA diarrhea worldwide (World Health Organization, 2009; Global Burden of Disease (GBD) Collaborators, 2018; Patel et al., 2011). This led to substantial reductions in AGE-related hospitalizations, emergency department visits, and mortality in countries that introduced the vaccine (Ai et al., 2020; Aliabadi et al., 2019; Burnett et al., 2017; Cortese et al., 2009). Among children <5 years of age, the median percentage reduction in AGE hospitalizations was 38% overall and 41%, 30%, and 46% in countries with low, medium, and high child mortality, respectively. Hospitalizations and emergency department visits due to RVA AGE were reduced by a median of 67% overall and 71%, 59%, and 60% in countries with low, medium, and high child mortality, respectively (Burnett et al., 2017).

Four live attenuated, oral RVA vaccines—RotaTeq[®] (Merck and Co., West Point, PA), Rotarix[®] (GlaxoSmithKline Biologicals, Rixensart, Belgium), ROTAVAC[®] (Bharat Biotech, Hyderabad, India) and Rotasiil[®] (Serum Institute of India, Pune, India)—have been WHO prequalified and introduced into the national vaccination program in more than 100 countries (Fig. 4) (International Vaccine Access Center (IVAC), 2021; Banyai et al., 2018).

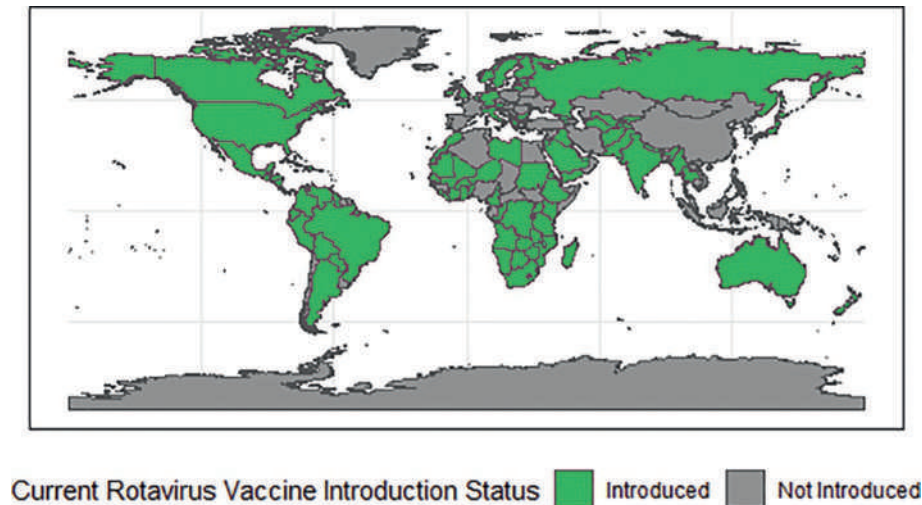


Fig. 4 Countries that have introduced Rotavirus group A (RVA) vaccine into immunization schedules (regional or national), 2021. Data from International Vaccine Access Center (IVAC) (2021) Johns Hopkins Bloomberg School of Public Health. Available online: www.view-hub.org.

RotaTeq®: In 2006, RotaTeq® was licensed by the FDA and recommended by the Advisory Committee on Immunization Practices (ACIP) for routine vaccination of U.S. infants; in 2008, the vaccine was prequalified by WHO. RotaTeq® is a pentavalent (G1, G2, G3, G4, P[8]) human-bovine reassortant vaccine administered orally to infants as 3 doses, 4 weeks apart, beginning as early as 6 weeks of age (Cortese et al., 2009; Dennehy, 2008). In clinical trials and observational studies in high-income settings such as the United States and Finland, RotaTeq® was highly efficacious, reducing incidence of medical office visits by 86%, emergency department (ED) visits by 94%, and RVA-associated AGE hospitalizations by 96% (Vesikari et al., 2006). RotaTeq® vaccine effectiveness is lower (45%), however, in low-income countries with high child mortality (Burnett et al., 2018).

Rotarix®: In 2008, ACIP recommended Rotarix® vaccine for routine vaccination of U.S. infants; in 2009, the vaccine was prequalified by WHO. Rotarix® contains a live, attenuated monovalent G1P[8] human RVA strain and is recommended for oral administration to infants in 2 doses, at least 4 weeks apart, beginning at 6 weeks of age (Cortese et al., 2009). In a randomized, double-blind, placebo-controlled study conducted in 6 European countries, Rotarix® was highly immunogenic and efficacious. Its efficacy through 2 RVA seasons against severe AGE was 90% and in reducing hospitalizations was 96% (Vesikari et al., 2004, 2007). Rotarix® vaccine effectiveness is lower (57–75%) in medium and low-income countries with high child mortality (Burnett et al., 2018).

ROTAVAC®: In 2018, ROTAVAC® vaccine received WHO prequalification. This live attenuated vaccine is derived from a naturally occurring human reassortant strain 116E (G9P[11]) isolated from an Indian child. The ROTAVAC® vaccine is administered orally in 3-dose series 4 weeks apart, beginning at 6 weeks of age up to 8 months of age. In a clinical trial in India, the efficacy of ROTAVAC® for severe non-vaccine RVA AGE was 56.4% in the first year of life and 49% in the second year (Bhandari et al., 2014).

Rotasiil®: In 2018, Rotasiil® vaccine received WHO prequalification. Rotasiil® is a live attenuated bovine-human reassortant RVA vaccine [BRV-PV] containing RVA serotypes G1, G2, G3, G4, and G9. It is administered as a 3-dose regimen at 6, 10, and 14 weeks of age. Rotasiil® is the world's first thermostable RVA vaccine, able to be transported at ambient temperature and stored at <25 °C. In a clinical trial of Indian infants, the vaccine efficacy of Rotasiil® against the very severe RVA cases (Vesikari score ≥ 16) was 60.5% at the time of the primary analysis and 54.7% for the complete follow-up period; results from a trial in Niger were similar (Isanaka et al., 2017; Kulkarni et al., 2017).

Nationally licensed RVA vaccines

Two additional live, attenuated orally administered RVA vaccines—Lanzhou Lamb (Lanzhou Institute of Biological Products) and Rotavin-M1 (POLYVAC Center for Research and Production of Vaccines Vietnam)—have obtained national licensure (Table 3).

Lanzhou Lamb (LLR): The Lanzhou Lamb vaccine is a live, attenuated oral vaccine licensed in China in 2000. It contains a G10P[12] lamb RVA strain isolated in 1985. This vaccine is administered to children aged 2–36 months, one dose between 6 and 12 months of age followed by yearly boosters. LLR vaccine effectiveness estimates have ranged from 35% to 53% among children <3 years of age (Fu et al., 2012; Zhen et al., 2015).

Rotavin-M1™: Rotavin-M1™ vaccine is a live, attenuated oral vaccine that was licensed for use in Vietnam in 2012. This vaccine contains a G1P[8] human RVA strain (KH0118-2003) isolated from a child in Vietnam (Dang et al., 2012). Rotavin-M1™ is administered orally in a 2-dose schedule, with the first dose at age 6 weeks or later and the second dose 60 days after the first. Rotavin-M1™ vaccine is safe and immunogenic, with an RVA IgA seroconversion rate of 73% in a trial of Vietnamese infants (Skansberg et al., 2021).

Table 3 Key characteristics of currently WHO prequalified and nationally licensed rotavirus group A (RVA) vaccines.

Name	Manufacturer	Composition	Doses	Formulation/Storage and Shelf life	Licensure	Study sites for phase III Trials	Efficacy	Safety
RotaTeq® (RV5)	Merckand Co.	Live attenuated bovine-human reassortant strains, G1, G2, G3, G4, P1[8]	3	Liquid/2–8 °C for 24 months	WHO pre-qualified in 2008	Bangladesh, Belgium, Costa Rica, Finland, Germany, Ghana, Guatemala, Italy, Kenya, Mali, Mexico, United States, Sweden, Taiwan, Vietnam	51–64% in developing countries and 94–100% in developed countries	WHO concluded that the benefits of rotavirus vaccination exceeded the risk of intussusception
Rotarix® (RV1)	GSK Biologics	Live human-attenuated rotavirus strain, G1P [8]	2	Liquid/2–8 °C for 36 months	WHO pre-qualified in 2009	Argentina, Brazil, Chile, Colombia, the Dominican Republic, Finland, Honduras, Malawi Mexico, Nicaragua, Panama, Peru, Venezuela, South Africa	49–72% in developing countries and 72–97% in developed countries	WHO concluded that the benefits of rotavirus vaccination exceeded the risk of intussusception
ROTAVAC® (RV1)	Bharat Biotech, India and PATH, United States	Live attenuated neonatal rotavirus strain, G9P[11] (aka 116E)	3	Liquid/2–8 °C for 6 months Frozen/–20 °C for 5 years	WHO pre-qualified in 2018	India	56% in India	Intussusception risk in vaccinated group was not higher than in placebo group. No increased risk seen in several observational studies
ROTASIL® (RV5)	Serum Institute of India, India and PATH, United States	Bovine-human reassortant rotavirus vaccine, G1, G2, G3, G4, G9	3	Lyophilized/<40 °C for 18 months Lyophilized/<25 °C for 30 months	WHO pre-qualified in 2018	India and Niger	66% in Niger and 39% in India	Intussusception risk in vaccinated group was not higher than in placebo group
Lanzhou Lamb Rotavirus (LLR) (RV1)	Lanzhou Institute of Biological Products, China	Live attenuated lamb rotavirus strain, G10P [12]	5	Liquid/–20 °C for 30 months	In China	China	Not applicable	Not known
Rotavin-M1™ (RV1)	POLYVAC, Vietnam	Live attenuated human rotavirus strain, G1P [8]	2	Liquid/2–8 °C for 2 months Frozen/–20 °C for 24 months	In Vietnam	Vietnam	Not applicable	Not known

Burke RM, Tate JE, Kirkwood CD, Steele AD and Parashar UD (2019) Current and new rotavirus vaccines. *Current Opinion in Infectious Diseases* 32(5): 435–444.

RVA vaccines under development

Several RVA vaccine candidates are under clinical development utilizing different strains, formulations, and routes of administration to overcome challenges associated with live, attenuated oral vaccines (Burke et al., 2019). The candidate vaccine RV3-BB (PT BioFarma, Bandung, Indonesia) is an oral vaccine based on a naturally attenuated neonatal strain of G3P[6] RVA and has the ability to replicate in the newborn gut even in the presence of maternal antibodies (Bines et al., 2018).

Non-replicating parenterally delivered RVA vaccines are considered an alternative to the current live oral vaccines. The advantages of these vaccines are improved vaccine safety with respect to intussusception, improved efficacy due to lack of competition from maternal antibodies, and lower manufacturing costs (Kirkwood et al., 2019). Some non-replicating RVA vaccine candidates are inactivated RVA vaccine (Resch et al., 2018; Wang et al., 2010), expressed proteins (based on VP6 inner capsid) (Lappalainen et al., 2015), virus-like particles (Bertolotti-Ciarlet et al., 2003), and VP8* expressed proteins (Groome et al., 2017).

Norovirus

History

Norwalk virus, the prototype strain of genus *Norovirus* within the family *Caliciviridae*, was identified in 1972 by immune EM (IEM) of fecal specimens obtained from human volunteers who had ingested stool filtrates from a gastroenteritis outbreak at a school in Norwalk, Ohio that had occurred in 1968 (Kapikian, 2000; Kapikian et al., 1972). Several other norovirus reference strains, such as Hawaii virus and Snow mountain virus, were subsequently discovered in 1977 and 1982, respectively (Dolin et al., 1982; Thornhill et al., 1977). Noroviruses, named after the prototype Norwalk virus, are now recognized as a major cause of epidemic and sporadic acute gastroenteritis among humans of all ages.

Morphology, structure, and nomenclature

Noroviruses consist of a single-stranded RNA in a nonenveloped icosahedral capsid. They are small round viruses 27–35 nm in diameter (Fig. 1). Two major domains can be found in the capsid: the shell (S) and the protruding arms (P). The S domain represents the inner part of the capsid that surrounds the genome, while the P domain forms the archlike capsomeres that protrude from the virion. Norovirus virions contain 180 copies, or 90 dimers, of capsid protein, forming a shell from which 90 archlike dimers protrude. Norovirus has $T = 3$ icosahedral symmetry. Unlike other caliciviruses, norovirus virions do not display a typical cup-like morphology (Glass et al., 2009; Robilotti et al., 2015).

The norovirus genome is composed of a linear, positive-sense RNA that is approximately 7.6 kb in length and covalently linked to the viral protein genome (VPg) at the 5' end and polyadenylated at the 3' end (Jiang et al., 1993; Thorne and Goodfellow, 2014). There are three open reading frames (ORFs), designated ORF1, ORF2, and ORF3, encoding eight viral proteins (Fig. 5). ORF1 encodes a polyprotein that is proteolytically processed into the 6 nonstructural proteins, including the norovirus protease and RNA-dependent RNA polymerase (Thorne and Goodfellow, 2014). ORF2 encodes a viral capsid protein (VP1) with a significant role in virus replication, and ORF3 codes for a small basic protein (VP2) which interacts with genomic RNA during virion formation (Robilotti et al., 2015; Thorne and Goodfellow, 2014).

Norovirus is one of the eleven genera in the family *Caliciviridae* (Vinje et al., 2019). Based upon phylogenetic analysis and $2 \times$ standard deviation of VP1, they are currently classified into 10 genogroups (GI–GX), and the genogroups can be further subdivided into 48 genetic clusters or genotypes (Chhabra et al., 2019). Viruses belonging to GI and GII are most frequently associated with human infections. Of the 35 genotypes that have been detected in humans, GII.4 viruses are the predominant circulating genotype causing outbreaks worldwide (Bull et al., 2006; Glass et al., 2009; Robilotti et al., 2015). The rapid evolution of GII.4 variants has

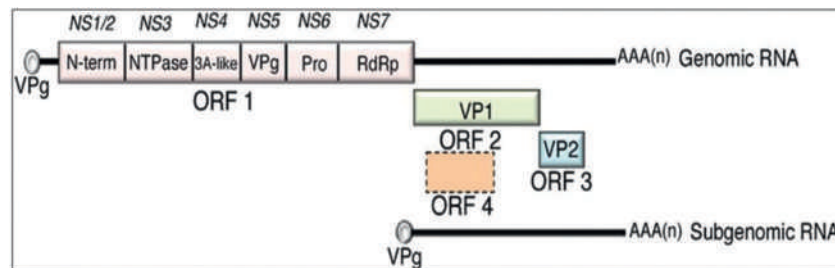


Fig. 5 The human norovirus genome. The genome is comprised of a linear, positive-sense RNA, 7.3–8.3 kb in length, covalently linked to the viral protein genome (VPg) at the 5' end and polyadenylated at the 3' end. There are three open reading frames (ORFs), designated ORF-1, ORF-2, and ORF-3, encoding 8 viral proteins. ORF-1 encodes the 6 nonstructural (NS) proteins that are proteolytically processed by the virally encoded cysteine proteinase (Pro). ORF-2 and ORF-3 encode the structural components of the virion, major capsid protein 1 (VP1) and minor capsid protein 2 (VP2), respectively. VP1-Major capsid protein. VP2-Minor capsid protein. p48 (NS1/NS2)-N-term (amino terminal protein). NTPase (NS3)-Nucleoside triphosphatase. P22 (NS4)-3A-like protein. VPg (NS5)-Genome-linked viral protein. Pro (NS6)-Proteinase. Pol (NS7)-RNA-dependent RNA polymerase. From Amir Yunus M (2021) Molecular Mechanisms for Norovirus Genome Replication, in *Norovirus*. IntechOpen, doi:10.5772/intechopen.96032.

led to the emergence of novel strains associated with global epidemics, as new norovirus strains are capable of infecting newly susceptible populations lacking protective immunity (van Beek et al., 2018).

Replication and pathogenesis

As in all other positive-strand RNA viruses, norovirus replication begins with the attachment of the VP1 protein of the viral particles through a specific receptor on the membrane of the host cell. In the case of norovirus, the specific cellular receptor is currently unknown. However, several binding ligands have been described, including HBGA and bile acids (Campillay-Veliz et al., 2020; Lin et al., 2014, 2020; Marionneau et al., 2002; Tan et al., 2003), which have been linked to susceptibility to norovirus in a genotypic-specific manner (Huang et al., 2003; Lindesmith et al., 2003). Individuals with two non-functional copies of *FUT2* are termed “non-secretors,” as they do not produce the H-type 1 antigen in saliva or epithelial cells. Non-secretors have been found to be resistant to norovirus infections, particularly those caused by GII viruses (Reyes et al., 2021; Thorven et al., 2005).

Following viral entry, virions are disassembled and viral RNA is released into the cytoplasm. The exact mechanism by which these two processes take place is not yet known for noroviruses. Once the viral genome is released, the VPg protein, which is covalently linked at the 5′ end of the viral genome, starts the translation process (Daughenbaugh et al., 2003) and generates three protein precursors—p48/NTPase, p22/VPg, and Pro/Pol—that are needed to carry out the replication process (Campillay-Veliz et al., 2020). A viral polyprotein is produced from the translation of the viral genome, and VP1 and VP2 proteins are generated from the sub-genomes (Cao et al., 2007). The structural proteins then assemble in complete virions which are released through a mechanism that remains to be elucidated (Fig. 6) (Campillay-Veliz et al., 2020; Hutson et al., 2002).

Noroviruses infect predominantly by the oral route, and most infections result in mild and self-limiting illness. Symptom onset, in particular vomiting, is frequently abrupt, and usually occurs within 12–48 h after exposure (Robilotti et al., 2015). Human volunteer studies have shown that noroviruses have a low infectious dose (median infectious dose <20 virus particles) and that infected persons excrete viruses in large amounts (10^8 to 10^{10} copies of RNA per gram of feces). Norovirus shedding can continue for >2 weeks after the symptomatic phase of the disease, as well as in cases of asymptomatic infection (Okhuysen et al., 1995; Parashar et al., 1998). There is little evidence that noroviruses cause chronic infections; however, a study performed in immunocompromised children and adolescents reported norovirus shedding for at least 8 months (Levett et al., 1996).

The virus replicates in intestinal epithelial cells (Karandikar et al., 2016), observed in adult volunteers infected with Norwalk virus (Green, 2013). The mechanisms responsible for diarrhea are not known, but the lesions caused by blunting of the intestinal villi may cause disruption of ion transport (Green, 2013). The development and refinement of human intestinal enteroids for cultivating human noroviruses has led to substantial advances in the understanding of viral replication and host-pathogen interactions (Chan et al., 2021; Costantini et al., 2018; Estes et al., 2019; Ettayebi et al., 2016, 2021; Karandikar et al., 2016; Karst and Tibbetts, 2016). These enteroids are now being used to study the cellular processes and signaling pathways involved in restricting replication of human noroviruses.

Clinical manifestation

Individuals infected with norovirus typically present with symptoms consistent with acute gastroenteritis, which include one or more of the following: nausea, vomiting, abdominal pain and cramps, diarrhea, myalgias, headache, and chills. Norovirus presents as a vomiting-only disease more frequently than other enteric viruses, particularly among young children (Burke et al., 2021; Vanderkooi et al., 2019). Symptoms may develop with or without early signs and typically persist for 1–3 days (Atmar et al., 2018; Banyai et al., 2018; Sell and Dolan, 2018). Treatment is aimed toward prevention of dehydration and includes either oral rehydration or intravenous administration of fluids. Moderately to severely dehydrated infected individuals may show signs such as tachycardia, orthostatic hypotension, decreased skin turgor, and dry mucous membranes. In severe cases, infection can lead to death due to dehydration. Rarely, infected individuals may present with significant neurologic symptoms such as a seizure or encephalopathy (Green, 2013; Randazzo et al., 2018; Robilotti et al., 2015).

Diagnostic testing

Diagnostic testing available for norovirus includes real-time reverse transcriptase-polymerase chain reaction (RT-qPCR, multi-gastrointestinal-pathogen diagnostic platforms (such as Luminex xTAG GPP, Biofire FilmArray GI and Verigene Enteric Pathogens Test), and commercial EIAs (Chhabra et al., 2017; Green, 2013; Vinje, 2015). RT-qPCR is presently regarded as the gold standard for norovirus detection, being both sensitive and specific as well as able to provide results within hours (Morillo and Timenetsky Mdo, 2011; Vinje, 2015). However, this method requires skilled technicians and specialized equipment and facilities. Although less sensitive than RT-qPCR, ELISA and lateral flow assays (such as IDEIA Norovirus EIA, SRSV (II)-AD, and RIDASCREEN) allow for rapid detection of viral proteins in fecal specimens (Green, 2013), and their ease of use, cost effectiveness, ability to detect a range of genotypes, and ability to provide results within hours make them useful for confirming norovirus as the etiology in outbreak settings. Norovirus can also be genotyped by sequence analysis of an RT-PCR product encompassing the 3′-end of the polymerase gene (region B) and 5′-end of the capsid gene (region C) in a single reaction for either genogroup I or genogroup II noroviruses (Chhabra et al., 2021). Sequences can then be typed using online typing tools such as <https://www.rivm.nl/mpf/norovirus/typingtool> or <https://norovirus.ng.philab.cdc.gov/> (Tatusov et al., 2021). Both typing tools use the exact same reference sequences (Chhabra et al., 2019).

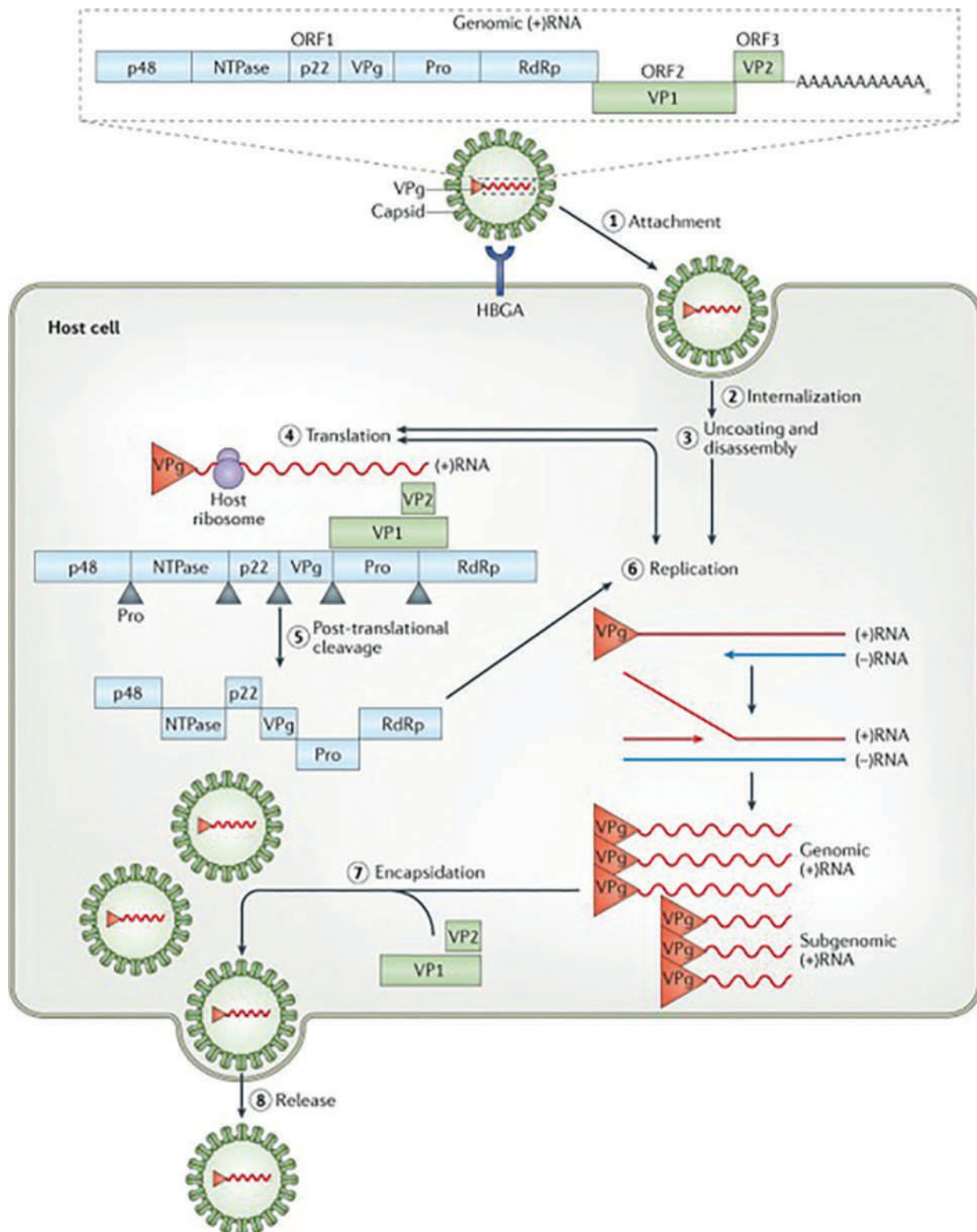


Fig. 6 The composition and replication cycle of human noroviruses. The viral capsid protein attaches to the cell surface through interactions between VP1 and host histo-blood group antigens (HBGAs) (step 1), and is subsequently internalized, uncoated and disassembled (steps 2, 3). The (+)RNA is then transcribed and translated in the cytoplasm of the host cell. Translation is mediated by host translation factors that are recruited by the non-structural virus protein VPg, which covalently binds to the 5' end of the genome (step 4). The polyprotein that is encoded by ORF1 is post-translationally cleaved (step 5) by the virus-encoded protease, Pro (also known as NS6 or 3C-like), into individual proteins: p48 (also known as NS1/2 or N-term), NTPase (also known as NS3 or 2C-like), p22 (also known as NS4 or 3A-like), VPg, Pro and RNA-dependent RNA polymerase (RdRp). During genome replication, (+)RNA is transcribed into negative-sense RNAs (–)RNAs, which are used as templates for the synthesis of new genomic and subgenomic (+)RNAs, respectively (step 6). Subgenomic (+)RNAs contain only ORF2 and ORF3 and are used for the production of VP1 and VP2. During encapsidation (step 7), genomic- and possibly subgenomic- (+)RNAs are packaged into new virions, which are subsequently released from the infected host cell (step 8), although the mechanism by which release occurs remains largely unknown. Used with permission from De Graaf M, Van Beek J, and Koopmans MPG (2016) Human norovirus transmission and evolution in a changing world. *Nature Reviews Microbiology* 14(7): 421–433.

Epidemiology, transmission, prevention, and control

Noroviruses are a leading cause of AGE worldwide, with estimated annual episodes ranging as high as 684 million and estimated annual deaths as high as 200,000 (Kirk et al., 2015). Norovirus burden is particularly high among older adults and young children (Burke and Hall, 2019). Noroviruses are one of the leading causes of severe gastroenteritis in children <5 years of age globally; as wider use of RVA vaccines has decreased RVA burden, prevalence of norovirus has increased among young children (Hassan et al., 2019; McAtee et al., 2016; Payne et al., 2013). Noroviruses are distributed globally with GII.4 viruses being more common than GI viruses (Vega et al., 2014). Norovirus cause both outbreaks and sporadic acute gastroenteritis. Outbreaks occur regularly in crowded or congregate living settings such as childcare centers, hospitals, nursing homes, restaurants, and cruise ships (Wikswa et al., 2021). Norovirus is extremely contagious. Though aerosol spread of norovirus is possible, transmission occurs mostly via fecal-oral and vomit-oral pathways by four general routes: direct person-to-person, foodborne, waterborne, or through environmental fomites. Since humans are the only known reservoir of human norovirus, all transmission is ultimately person-to-person (Gotz et al., 2001; Lopman et al., 2016). Though several norovirus vaccine candidates are in development, there are presently no licensed vaccines or antiviral therapies for norovirus gastroenteritis (Cates et al., 2020). Prevention and control strategies for norovirus are limited to standard infection control guidelines, such as hand hygiene, cleaning and disinfection of diarrhea or vomitus, and work exclusions where appropriate (Lopman et al., 2016).

Enteric adenoviruses

History

Adenoviruses were first isolated and characterized from human lymph nodes in 1953 (Rowe et al., 1953). The first adenovirus identified was isolated from adenoidal tissue of children and named “adeno” after the Greek word for gland (Rowe et al., 1953). Adenoviruses are ubiquitous, have been isolated from nearly every class of vertebrates, and are species-specific (Berk, 2013; Strauss and Strauss, 2002). Today, adenoviruses cause various human diseases, including acute gastroenteritis, respiratory tract infections, conjunctivitis, and in rare cases acute hemorrhagic cystitis, meningitis, and pneumonia (Berk, 2013; Ghebremedhin, 2014). This section will focus on adenoviruses that cause acute gastroenteritis.

Morphology, structure, and nomenclature

Adenoviruses are non-enveloped icosahedral particles with virions of an estimated 70–100 nm in diameter. The virions exhibit a characteristic icosahedral capsid layer of 252 capsomers of which 240 have a six-fold symmetry (hexons) and form facets, and 12 have a five-fold symmetry (pentons) and form vertices. Fiber-like projections protruding from the pentameric penton base proteins at the vertices of the icosahedron are responsible for host receptor binding. Additional proteins (pIIIa, pVI, pVIII, and pIX) are located on the capsid. The capsid surrounds a DNA-containing core that consists of a single copy of DNA with proteins such as the terminal protein (TP), polypeptide (pV), pVII, and pX (Fig. 7) (Berk, 2013; Davison et al., 2003; Ginsberg et al., 1966; Goncalves and de Vries, 2006; Russell, 2000; Seiradake and Cusack, 2005).

The adenovirus genome consists of double-stranded (ds) DNA ranging about 26–45 kbp in length. Structurally, the genome is linear and non-segmented and encodes for more than 30 structural and non-structural proteins. The genome has a terminal 55 kDa protein that is linked to the dsDNA 5'-ends, where it is used as a primer during replication (Berk, 2013; Chaberny et al., 2003;

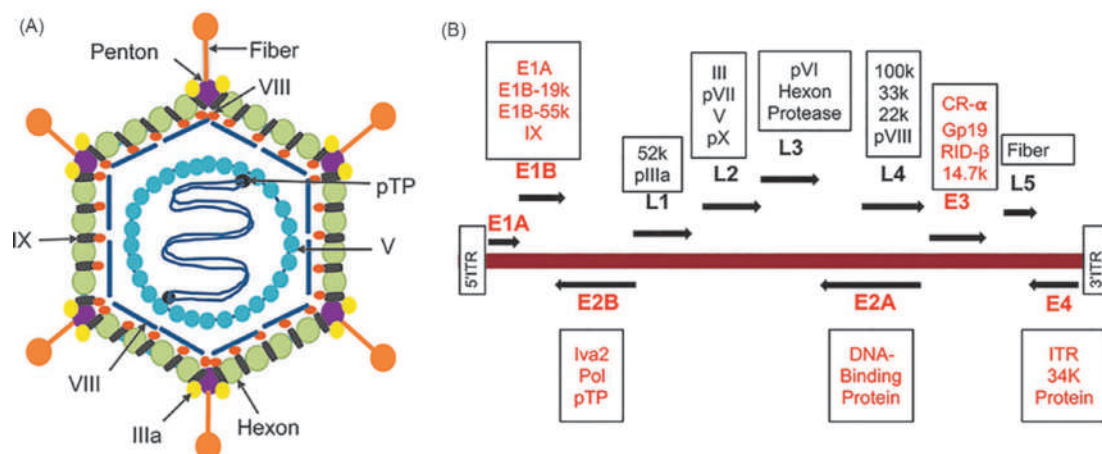


Fig. 7 Structure and genome of adenovirus. (A) Depiction of adenovirus structure and different proteins. (B) Adenovirus genome organization showing various early (E) and late (L) transcripts and proteins encoded by each transcript. Red indicates early and black indicates late. From Singh S, Kumar R, and Agrawal B (2019) *Adenoviral Vector-Based Vaccines and Gene Therapies: Current Status and Future Prospects*, IntechOpen.

Friefeld et al., 1984). In addition, the genome encodes for several transcription units: early units (E1A, E1B, E2, E3, and E4), intermediate units, and a major late transcription unit (Fig. 2) (Berk, 2013; Ruuskanen et al., 2002).

Classification and nomenclature

Adenoviruses are classified into five genera (*Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*, and *Ichtadenovirus*) based on the host from which they were first isolated (Berk, 2013). Of those, only members of *Mastadenovirus* cause human infections (International Committee on Taxonomy of Viruses, 2018. <https://talk.ictvonline.org/taxonomy/>). Currently, human adenovirus (HAdV) is classified into nine subgroups (A to I): within each subgroup, 90 genotypes have been identified (International Committee on Taxonomy of Viruses, 2018. <https://talk.ictvonline.org/taxonomy/>; Human Adenovirus Working Group, 2018. Available from: <http://hadvwg.gmu.edu/>; Ghebremedhin, 2014). Although other adenovirus types have been associated with gastrointestinal infections, only viruses belonging to subgroup F (genotypes 40 and 41) have been associated with acute gastroenteritis and are responsible for an estimated 5–20% of acute gastroenteritis in young children (Banerjee et al., 2017; Kim et al., 2017; Qiu et al., 2018; Uhnoo et al., 1984).

Replication and pathogenesis

Adenoviruses replicate in the nucleus (Fig. 8). First, the virion attaches to the host cell by binding its glycoprotein fiber structure to an adenovirus-specific receptor followed by interaction of the penton base with cellular integrin, which promotes receptor-mediated internalization (Leen and Rooney, 2005). Next, the virus is internalized into the clathrin-coated endosome, which due to its high pH helps uncoat the virus and transport viral DNA through microtubules into the nucleus. At this point, early transcripts undergo translation to produce about 20 different early proteins that induce the host cell to enter into the S-phase of the cell cycle and to create conditions favorable for viral replication (Shenk, 2001). DNA replication takes place in the nucleus as the 5' end of the viral DNA strand acts as a primer for initiation of viral DNA synthesis (Goncalves and de Vries, 2006; Shenk, 2001). During late transcription/translation and viral morphogenesis and release, a large single primary transcript is synthesized from viral DNA that is spliced into 18 fragments; each fragment acts as mRNA and is transported to the cytoplasm, where translation occurs and viral structural proteins are synthesized. Viral DNA then gets packaged into preformed capsid-forming mature virus particles that are stable, infectious, and resistant to nuclease enzyme of a host cell. Finally, the mature virus is released from the host cell by budding (Berk, 2013; Lenaerts et al., 2008).

Adenoviruses can infect and replicate in epithelial cells of the gastrointestinal tract and various viral factors contribute to pathogenesis. One unique feature of adenovirus is that its penton protein has a toxic effect on cells leading to atrophy of the villi and

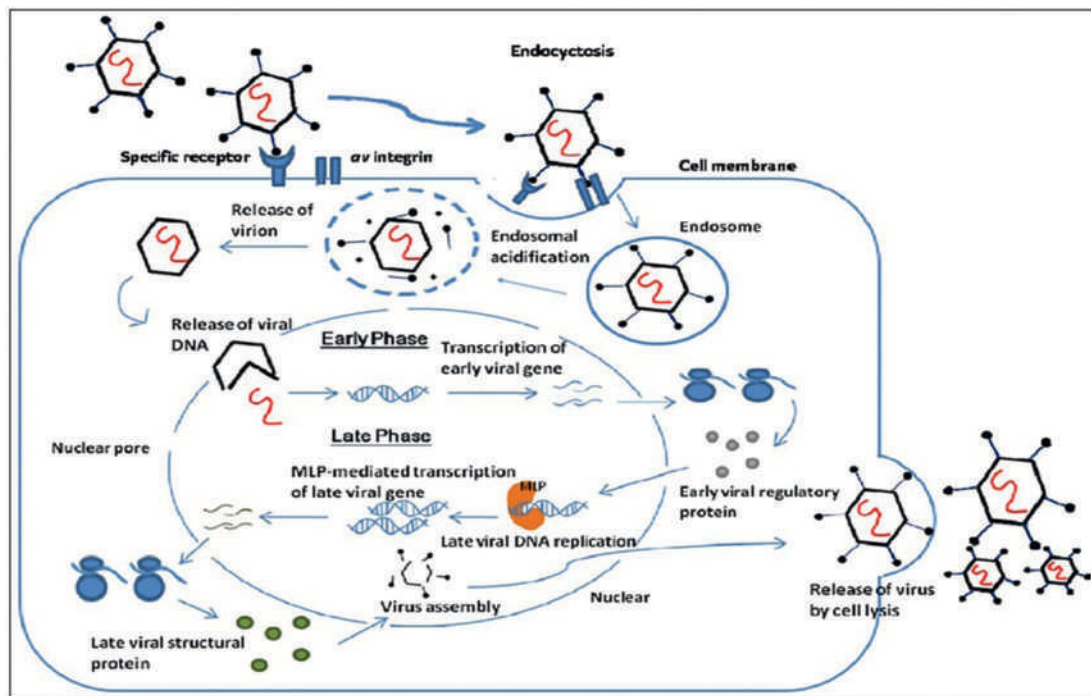


Fig. 8 A schematic illustration of the adenovirus infection and replication cycle. Used with permission from Waye MMY and Sing CW (2010) Anti-viral drugs for human adenoviruses. *Pharmaceuticals* 3(10): 3343–3354.

compensatory hyperplasia in the crypts with subsequent malabsorption and loss of fluids (Berk, 2013; Fox et al., 1977; Wadell et al., 1999). Adenovirus infection results in an immune response that probably renders long-lasting protection against re-infection with the same virus type (Berk, 2013).

Clinical manifestation

The symptoms caused by adenovirus types 40 and 41 are typically mild and self-limiting, and infections occur throughout the year, mostly in young children. Patients normally present clinical symptoms such as acute watery diarrhea and vomiting (Hierholzer, 1992; Scott et al., 2016). The incubation period of adenoviruses is 8–10 days, and diarrhea can last 3–11 days. Illness can be severe in immunodeficient patients and, although rarely, infection can lead to chronic diarrhea (Berk, 2013; Bresee et al., 1999).

Diagnostic testing

Laboratory diagnosis of adenovirus infection requires (Martin and Kudesia, 1990) isolation of virus in cell culture (Berk, 2013), demonstration of antigen-infected cells by immunofluorescence (IF) (Tsutsumi et al., 1999), detection of virus antigen by EIA (Fodha et al., 2006), demonstration of viral nucleic acid by molecular-based assays such as PCR (Reither et al., 2007), or demonstration of IgM antibody or a four-fold rise in antibody titer in blood specimens (Berk, 2013). Adenoviruses can also be detected using multipathogen assays such as Luminex Gastrointestinal Pathogen Panel (Navidad et al., 2013), TaqMan Array Card (Liu et al., 2013), and Film Array Gastrointestinal Panel (Reddington et al., 2014).

Epidemiology, transmission, prevention, and control

Adenoviruses are widespread in nature and infect mammals and birds. They cause a variety of symptoms and are an important cause of acute gastroenteritis in children <5 years of age, responsible for an estimated 15% of all childhood diarrhea (Clark and McKendrick, 2004; Hassan et al., 2019; Koopmans, 2005; Lee et al., 2020). The transmission of enteric adenoviruses occurs via the fecal-oral route, and they can be transmitted from person to person (direct contact) or via environmental routes (Bresee et al., 1999). Adenoviruses have been linked with outbreaks in communities such as schools, day-care centers and military camps, and the virus is able to contaminate environmental sources including food and water (Filho et al., 2007; Fong and Lipp, 2005; Huh et al., 2009; Lazic et al., 2015; Mattison et al., 2021).

There are currently no publicly available vaccines or antivirals against adenovirus infections. Whole-virus vaccines are not used because of the potential risk of oncogenesis. Other vaccines, including recombinant vaccines, are under development (Doerfler, 1996).

Astrovirus

History

In 1975, Appleton and Higgins used EM to detect viral particles of 28–30 nm in size in stools of children suffering from vomiting and diarrhea (Appleton and Higgins, 1975). Later that year, Madeley and his colleague reported occurrence of the same viral particles in feces of infants hospitalized with gastroenteritis and in outbreaks of gastroenteritis in newborn nurseries (Madeley and Cosgrove, 1975). They used the term astrovirus to describe these viruses.

Morphology, structure, and nomenclature

The name astrovirus is derived from the Greek word “astron,” which means star. Visualized by EM, astrovirus virions have a well-defined five- or six-pointed, star-like appearance with a white center and triangular surface hollows (Mendez and Arias, 2013). They are small icosahedral nonenveloped particles with a smooth margin; the distinctive star shape is visible on the surface of approximately 10% of astrovirus virions (Mendez and Arias, 2013). The astrovirus genome is a positive-sense single-stranded polyadenylated RNA molecule of 6.8–7.9 kb in length. It contains three ORFs, named from the 5′ end to the 3′ end, ORF1a, ORF1b, and ORF2. The genome consists of the 5′ and 3′ untranslated regions of 11–85 bases and 80–85 bases, respectively, and a poly-A tail at the 3′ end. ORF1a and ORF1b encode for the nonstructural proteins involved in RNA transcription and replication, while ORF2 encodes for the capsid precursor proteins, which are expressed from a sub-genomic RNA (Fig. 9) (Bosch et al., 2014; Firth and Atkins, 2010; Mendez and Arias, 2013; Monroe et al., 1993; Walter and Mitchell, 2003; Wilhelmi et al., 2003; Willcocks and Carter, 1993).

Classification and nomenclature

Astroviruses have been found in the feces of a wide variety of mammalian species (Bosch et al., 2014; Castro et al., 2013; Hoshino et al., 1981; Martella et al., 2011; Mendez and Arias, 2013), as well as avian species (Chu et al., 2012; Koci et al., 2000; Mendez and Arias, 2013). The family *Astroviridae* is divided into two genera, based on the hosts of origin: *Mamastrovirus* (MAstV) and *Avastrovirus*

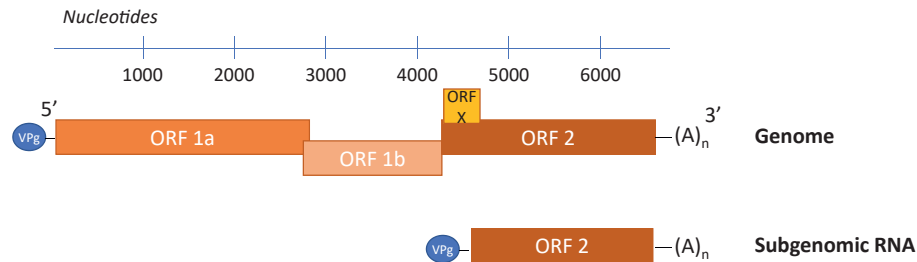


Fig. 9 Genome organization human astrovirus. Genomic and subgenomic RNA organization, with open reading frames (ORFs) ORF1a, ORF1b, ORF2, and putative ORFX represented as boxes. Adapted from Bosch A, Pinto RM and Guix S (2014) Human astroviruses. *Clinical Microbiology Reviews* 27(4): 1048–74.

(AAstV), infecting mammalian (human, bovine, feline, mink, ovine, and porcine) and avian species (chicken, duck, and turkey), respectively. Within the genus MAstV there are two genogroups, GI (consisting of 10 genotype species) and GII (nine genotype species); both encompass viruses of human and animal origin (Zhu et al., 2009). Human astroviruses (HAstVs) are classified into eight distinct genotypes based on the nucleotide sequence of ORF2 (Noel et al., 1995) which correlate well with astrovirus serotypes, as defined by clear differences in neutralization. Genotypes 1 and 2 are most frequently detected worldwide (Mendez and Arias, 2013).

In the late 2000s, two divergent HAstV genotypes were discovered which are referred to as a group of non-classical astroviruses. They include the HAstV-MLB (Melbourne) clade containing at least three strains (MLB1, MLB2, MLB3) and the HAstV-VA/HMO (Virginia/Human-Mink-Ovine-like) clade containing at least five strains (VA1, VA2, VA3, VA4, VA5). (Mendez and Arias, 2013). The prevalence of these non-classical viruses varies greatly according to geographic location, and their association with clinical disease remains unclear.

Replication and pathogenesis

Viral replication is cytoplasmic, and entry into the host cell is achieved by attachment to host receptors that mediate endocytosis. Several investigators have suggested that astrovirus RNA replication is associated with membranes derived from the endoplasmic reticulum at the perinuclear region, and ultrastructural analysis has revealed the presence of viral aggregates close to the nuclear periphery surrounded by a high number of double-membrane vacuoles. Although the exact composition of these replication complexes is not known, it is likely that all nonstructural proteins are required and that the temporary regulation of protein processing of the replicase complex may play a role in the regulation of the minus- and plus-strand RNA synthesis processes. Of interest, variability within the protein encoded at the C-terminal region of ORF1a has been shown to affect the RNA replication pattern, and a functional effect of phosphorylation of the protein has been suggested. Although some nonstructural proteins have been detected by IF within the nucleus of infected cells (Fig. 10) (Bosch et al., 2014; Mendez and Arias, 2013; Speroni et al., 2009; Willcocks et al., 1999), involvement of the nuclear phase in the replication cycle of astrovirus is not fully understood.

Although a few spike crystal structures of the astrovirus spike domain have been studied and defined, little is known about their pathogenesis and how the capsid structures relate to the mechanism of pathogenesis (Dong et al., 2011). Common mechanisms involved in astrovirus pathogenesis include destruction, distention, and thinning of the intestines as well as fluid accumulation due to inhibition of usual absorption mechanisms, loss of secretory functions, and reduction in epithelial permeability in the intestines (Bosch et al., 2014; Mendez and Arias, 2013).

Clinical manifestation

The main clinical symptom of HAstV infection is self-limiting acute gastroenteritis, which on rare occasions has been shown to cause systemic infection in immunocompromised individuals (Wunderli et al., 2011). Symptoms typically last 2–3 days. Some non-classical astroviruses have also been associated with central nervous system infections mostly in immunocompromised patients (Espul et al., 2004; Sato et al., 2016). Viruses can be excreted in feces for up to 14 days, although shedding can be prolonged in immunodeficient patients. Asymptomatic excretion of astroviruses can occur in up to 20% of young children (Caballero et al., 2003; Clark and McKendrick, 2004; Espul et al., 2004).

Diagnostic testing

Laboratory assays to detect astroviruses include EIAs and RT-qPCR (Beuret, 2004; Glass et al., 1996; Kurtz and Lee, 1987; Mendez and Arias, 2013; Risco et al., 1995). The classical astroviruses (types 1–8) can also be detected using multipathogens assays such as TaqMan Array Card (Liu et al., 2013) and Film Array Gastrointestinal Panel (Reddington et al., 2014).

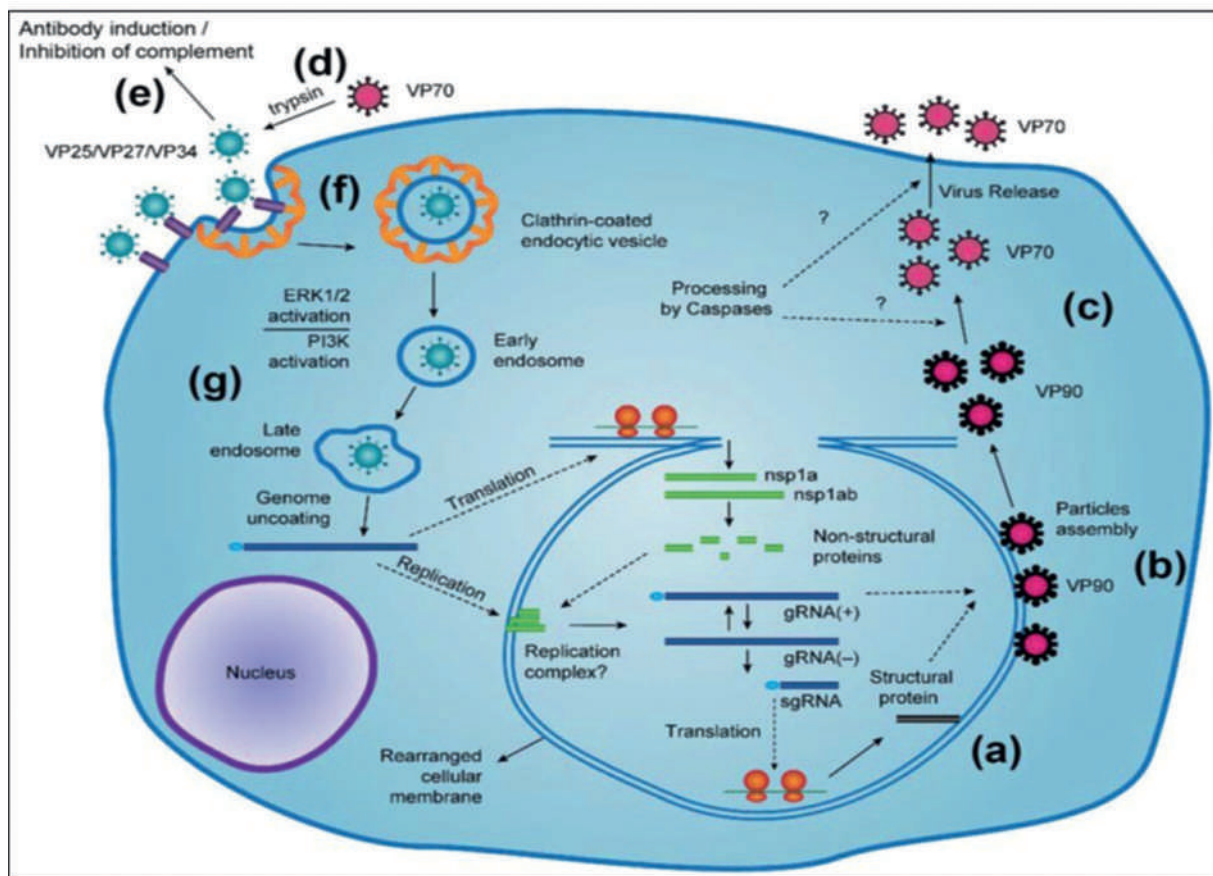


Fig. 10 Replication cycle of human astroviruses. (A) Synthesis of the 90 kDa human astroviruses (HAsV) capsid protein (VP90) from viral subgenomic RNA (sgRNA); (B) assembly of VP90 proteins (180 copies) with viral genomic RNA into HAsV particles; (C) caspase-mediated cleavage of VP90 C-termini to form VP70 and subsequent release of immature HAsV particles; (D) extracellular protease cleavage of immature HAsV particles to form mature, infectious HAsV particles. In cell culture, trypsin is used to produce mature, infectious HAsV particles; (E) extracellular HAsV particles induce host antibody production and inhibit host complement activation; (F) attachment and clathrin-dependent endocytosis of HAsV particles; (G) Uncoating of the virus genome in the late endosome. Extracellular signal-regulated kinase (ERK1/2) and phosphoinositide 3-kinase (PI3K) are activated during HAsV binding or entry into the cell. From Arias C and Dubois R (2017) The astrovirus capsid: A review. *Viruses* 9(1): 15.

Epidemiology, transmission, prevention, and control

Astroviruses are distributed worldwide, typically infect the young of mammalian and avian species, and account for an estimated 10% of all sporadic human diarrhea cases (Mendez and Arias, 2013). On rare occasions, they cause outbreaks in adults, but more often cause single cases of mild gastroenteritis in children. Astroviruses are often detected together with other gastrointestinal viruses, especially rotaviruses (Glass et al., 1996; Monroe et al., 2001). Astroviruses are spread predominantly through the fecal-oral route but can also be transmitted through contaminated food and water. Astroviruses are endemic throughout the world, with a winter seasonality in temperate climates and a higher prevalence during the rainy season in tropical regions. Asymptomatic infections occur in neonates and young children, especially in nurseries, childcare centers, and hospitals (Bresee et al., 1999; Caul, 1996a,b). Currently, no astrovirus vaccine is available. After infection, immunity against astrovirus lasts for several years, and by adulthood, most people contract very few symptomatic infections. Astrovirus infection can be prevented by frequent hand washing, disinfection of contaminated surfaces, prompt washing of soiled clothes, and avoidance of consumption of contaminated food or water. In addition, disinfection of contaminated fomites is important to stop transmission (Bosch et al., 2014).

Sapovirus

History

Sapovirus was first detected in human diarrheic stool samples in 1976 in the United Kingdom using EM (Madeley and Cosgrove, 1976). A year later, the virus was detected in an outbreak of gastroenteritis in an orphanage in Sapporo, Japan (Chiba et al., 1979). The virus was soon recognized as a novel etiology of diarrhea infection in both humans and animals (Nakata et al., 2000; Oka et al., 2015; Saif et al., 1980).

Morphology, structure, and nomenclature

Sapoviruses are small round viruses with a diameter of approximately 30–40 nm. They are morphologically distinct from other enteric viruses because of their characteristic star-of-David morphology and depression on the surface, formed by 32 cups or “calices,” visible under EM (Madeley, 1979; Oka et al., 2015; Roingear, 2008; Saif et al., 1980). Sapoviruses are non-enveloped, linear, positive-sense, single-stranded RNA viruses. The genome is approximately 7.1–7.7 kb in size with a poly(A) tail at the 3′-end and a genome-linked viral protein (VPg) covalently attached at the 5′-end. The virus has a $T = 3$ icosahedral symmetry, and the capsid consists of 180 capsid proteins (Oka et al., 2015). The genome is organized into two ORFs. ORF-1 encodes the nonstructural proteins (NS1–NS5 and NS6–NS7), as well as the major capsid protein, VP1, while ORF-2 encodes the minor capsid protein, VP2 (Miyazaki et al., 2015; Oka et al., 2015; Okada et al., 2006). Sapovirus VP1 capsid protein can be separated into several domains: the N-terminal variable region (NVR), N-terminal region (N), central variable region (CVR), and C-terminal region (C) (Fig. 11) (Oka et al., 2015).

Sapoviruses belong to the genus *Sapovirus* in the family *Caliciviridae* (Mayo, 2002). They have been detected not only in humans, but also in pigs, minks, dogs, sea lions, bats, chimpanzees, and rats (Oka et al., 2016). Sapoviruses are genetically highly diverse and classified into 19 genogroups (G) based on complete VP1 protein sequences (Yinda et al., 2017). Strains that infect humans belong to four genogroups (GI, GII, GIV, GV) (Barer, 2011; Oka et al., 2015; Scheuer et al., 2013). These viruses have distinctive conserved amino acid sequences in NS3 (GAPGIGKT), NS5 (KGKTK and DDEYDE), NS6 (GDCG), NS7 (WKGL, KDEL, DYSKWDST, GLPSG, and YGDD), and VP1 (PPG and GWS) (Oka et al., 2015).

Replication and pathogenesis

Due to the lack of a cell culture model, the replication cycle of sapoviruses has not been determined, yet; however, it is thought that, like other members of the family *Caliciviridae*, replication occurs in the cytoplasm (Oka et al., 2015).

Clinical manifestation

The main clinical symptoms of sapovirus infection include vomiting, diarrhea, nausea, abdominal cramps, chills, headache, myalgia, and malaise, all of which are indistinguishable from noroviruses. Although hospitalization has been reported, most sapovirus symptoms are self-limiting and patients usually recover within 1–4 days (Oka et al., 2015).

Diagnostic testing

Laboratory diagnosis of sapovirus relies on detection of viral RNA by real-time RT-PCR assay (Nagai et al., 2020; Oka et al., 2006, 2015; Saif et al., 1980). Sapoviruses can also be detected using of the commercially available multi-enteric pathogen assays such as the TaqMan Array Card (Liu et al., 2013) and Film Array Gastrointestinal Panel (Reddington et al., 2014).

Epidemiology, transmission, prevention, and control

Sapovirus infections are increasingly detected because of the increased use of the multi-enteric pathogen panels and are known to cause disease outbreaks in people of all age groups, including the elderly; persons in crowded or congregate living settings (such as

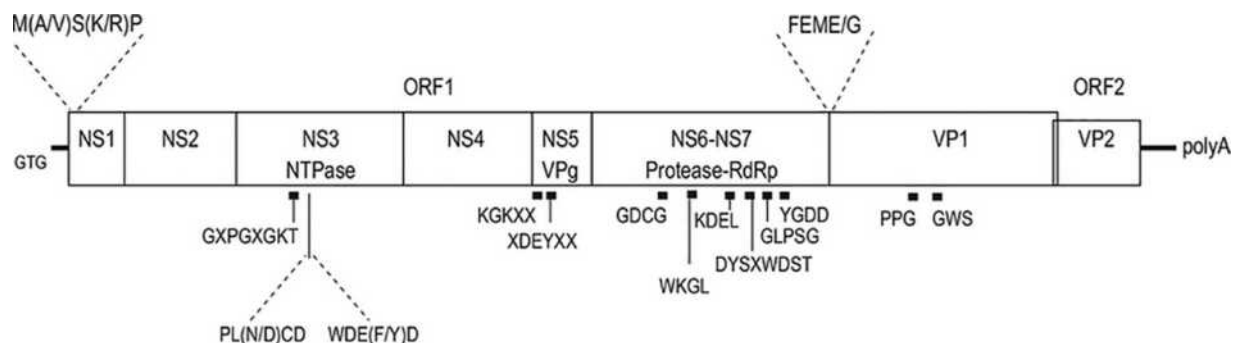


Fig. 11 A schematic diagram of the sapovirus genomic organization. This diagram shows two open reading frames, ORF1 and ORF2, as well as predicted viral nonstructural proteins and capsid proteins. Nonstructural proteins NS1, NS2, NS3 [NTPase], NS4, NS5 [VPg], NS6–NS7 [protease–RNA-dependent RNA polymerase (RdRp)], and the major capsid protein (VP1) are encoded by ORF1. ORF2 encodes VP2, the minor capsid protein. The following amino acid or nucleotide motifs are also shown and conserved among known sapoviruses: GXPGXGKT, PL (N/D) CD, and WDE (F/Y) D of NS3, KGKXX and XDEYXX of NS5, GDCG of NS6, WKGL, KDEL, DYSXWDST, GLPSG, and YGDD of NS7, PPG and GWS of VP1, and the first three nucleotides (GTG) at the 5′-end. The first five amino acids of NS1 (M [A/V] S [K/R] P) and around the NS7–VP1 cleavage site (FEME/G, where the slash indicates the putative cleavage site by viral protease NS6) are shown and are conserved among GI–GV, GVIII, and GXIII sapoviruses. From Oka T, Lu Z, Phan T, Delwart EL, Saif LJ, and Wang Q (2016) Genetic characterization and classification of human and animal Sapoviruses. *PLoS One* 11(5): e0156373.

daycares or assisted living facilities) may be especially vulnerable to outbreaks (Burke et al., 2021; Hansman et al., 2006, 2007; Lee et al., 2012; Oka et al., 2015; Page et al., 2016; Platts-Mills et al., 2018; Sanchez et al., 2018; Vielot et al., 2021). Although sapovirus gastroenteritis is generally mild, severe cases have been reported (Robinson et al., 2002; Vielot et al., 2021). The viruses can be transmitted from person to person through including contaminated materials/surfaces, or through contaminated food and drinking water (Oka et al., 2015). Like norovirus, sapovirus is probably transmitted efficiently via aerosol during vomiting. Currently, there are no vaccines or antiviral drugs available to prevent or treat sapovirus infections.

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Fungal Infections of the Gastrointestinal Tract

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Introduction

Both yeasts and filamentous fungi may infect the gastrointestinal (GI) tract, and are a particular risk for immunocompromised patients such as organ transplant recipients, patients receiving chemotherapy or immunomodulator therapy, and individuals with AIDS and other immune compromising conditions (Enoch et al., 2017; Pagano et al., 2017; Gavalda et al., 2014; Lionakis, 2019). As the number of patients with solid organ and bone marrow transplants and on immunosuppressive therapy has increased over the past few decades, so have invasive fungal infections (Antinori et al., 2016), and the endemic mycoses are one of the most significant infectious causes of morbidity and mortality in this population (Malcolm and Chin-Hong, 2013; Ellis, 2001; Fleming et al., 2002). Other risk factors for invasive fungal infections include repetitive abdominal surgeries, widespread use of antimicrobial agents, intrusive vascular lines, diabetes, total parenteral nutrition, neonatal prematurity, and advancing age (Enoch et al., 2017; Pagano et al., 2017; Gavalda et al., 2014; Lionakis, 2019). Although gastrointestinal fungal infections occur most commonly in immunocompromised patients, virtually all of the fungi discussed in this chapter can cause infections in immunocompetent persons as well. Knowledge of the patient's geographic and/or travel history may be very helpful in evaluating these cases.

Signs and symptoms of gastrointestinal fungal infections are often similar, regardless of the type of fungus. Esophageal fungal infections typically present with odynophagia and dysphagia, whereas gastric and intestinal infections may present with diarrhea, vomiting, melena, frank GI bleeding, abdominal pain, and fever. It is important to note that although fungal infections of the GI tract are often a part of a disseminated disease process, gastrointestinal symptoms and signs may be the presenting manifestations. Although *Candida* and *Aspergillus* species have historically represented the majority of fungi diagnosed in immunocompromised patients (Parize et al., 2012; Schwesinger et al., 2005), other fungi are emerging as more commonly encountered pathogens, such as *Fusarium* and the phaeohyphomycoses (Fleming et al., 2002; Arcobello and Revankar, 2020).

General principles of diagnosis

Despite advances in laboratory technology, particularly in the areas of serologic and molecular testing, the recognition, diagnosis, and classification of fungal infections in the immunocompromised patient population remains challenging. Tissue biopsy remains one of the most important tools available in the diagnosis of fungal infections, as fungal cultures may require days to weeks for adequate growth and analysis, and molecular assays may also have longer turnaround times. In addition, cultures are often not obtained as often as pathologists might wish or expect. The surgical pathologist can play an important role in both detecting the organism and identifying it, based on characteristic morphologic features (Guarner and Brandt, 2011; Procop and Pritt, 2018). Organisms may be identifiable on routine hematoxylin and eosin (H&E) stained sections, particularly in heavy infections, but Gomori methenamine silver (GMS) and periodic acid Schiff (PAS) stains remain invaluable diagnostic aids as they highlight most fungal elements and allow for enhanced microscopic identification. It is often possible to at least presumptively identify fungi based on morphologic features, particularly in cases of infection by *Candida* spp., the Mucorales, and the endemic dimorphic fungi (Lamps, 2012) (Tables 1 and 2). Similarly, *Aspergillus* spp. and similar-appearing fungi (e.g., *Fusarium* and other hyaline fungi with regularly-septate hyphae) can usually be differentiated from the Mucorales based on morphologic features; however, there is significant overlap in morphologic features among *Aspergillus* spp. and the *Aspergillus*-like fungi, and other techniques are required for definite identification (Guarner and Brandt, 2011; Lamps, 2012). It is also important to note that fungi exposed to antifungal therapy or ambient air may produce bizarre and unusual forms, and these may complicate the morphologic identification. Fungal infection should be strongly considered in any immunocompromised patient with ischemic lesions of the gastrointestinal tract, particularly ischemic lesions of the stomach; because of its rich blood supply, the stomach is not usually affected by ischemia due to atherosclerosis.

Identification of the specific genus is necessary because antifungal therapy may differ based on the involved organism, and therefore culture or molecular assays such as nucleic acid amplification testing (NAAT) and broad-range fungal sequencing may be required (Guarner and Brandt, 2011; Lamps, 2012). Culture provides the ability to perform antifungal susceptibility testing, results of which can vary significantly for some species (e.g., *Candida*), and which have growing importance in treatment of mold infections as well (Procop and Pritt, 2018).

Most diagnostic assays are based on detecting fungal cell wall elements and/or their antibodies in host tissue and body fluids. Nucleic acid amplification tests (NAATs) such as PCR are highly sensitive and specific, but cannot differentiate fungal colonization from true invasion. Thus, the results of NAATs must be correlated with the histopathologic findings whenever possible. Immunohistochemical (IHC) and in situ hybridization (ISH) tests may aid in this regard as they are performed directly on the FFPE tissue and can be viewed in the context of the histopathologic features. However, these tests suffer from cross-reactivity between fungal species and are not widely available. This is a promising area of active clinical research.

Other non-invasive fungal diagnostics that can complement a histologic diagnosis include antibody- and antigen-based detection tests. Urine antigen testing is particularly useful for endemic fungi such as *H. capsulatum*, and the combination of antigen testing with antibody testing through enzyme immunoassay (EIA), immunodiffusion and/or complement fixation increases the overall diagnostic yield. As with IHC and ISH, fungal antigen and antibody tests exhibit significant cross-reactivity among different fungal species, and thus need to be interpreted in the context of other test results.

Table 1 Morphologic features of filamentous fungi involving the gastrointestinal tract.

Organism (Disease)	Primary Geographic Distribution	Morphologic Features	Host Reaction	Major Differential Diagnoses
<i>Aspergillus</i> species (Aspergilliosis)	Worldwide	Hyphae- Septate Uniform width Branching-Regular Acute angles Conidial head formation in cavitary lesions	Ischemic necrosis with angioinvasion Acute inflammation Occasionally granulomatous	Mucorales, <i>Fusarium</i> spp., <i>Scedosporium</i> species complex (include fungus formerly known as <i>Pseudallescheria boydii</i>)
<i>Candida albicans</i> ; <i>Candida</i> spp. other than <i>C. glabrata</i> (Candidiasis); See also Table 2.	Worldwide	Mixture of budding yeast and pseudohyphae; occasional septate hyphae	Usually suppurative, with variable necrosis and ulceration Occasionally granulomatous	Occasionally <i>Aspergillus</i> , Also dematiaceous fungi (particularly if marked pleomorphism)
Mucormycores (Mucormycosis; older term = zygomycosis)	Worldwide, associated with diabetics more than any other mycosis	Thin-walled, pauciseptate, ribbon-like hyphae with thin walls and haphazard branching	Occasional angioinvasion Similar to <i>Aspergillus</i> spp.	<i>Aspergillus</i> spp., <i>Basidiobolus ranarum</i>
<i>Basidiobolus ranarum</i> (Basidiobolomycosis)	Saudi Arabia, Africa, Parts of Asia; Arizona	Similar to the Mucorales; fewer organisms, "cellophane ball" appearance	Eosinophilia, necrosis, granulomas, Splendore-Hoeppli reaction to organisms	Mucorales
<i>Fusarium</i> spp. (Fusariosis)	Worldwide	Similar to <i>Aspergillus</i> spp.; hyphae may be constricted at sites of origin	Similar to <i>Aspergillus</i> spp.	<i>Fusarium</i> spp., <i>Scedosporium</i> species complex

Table 2 Characteristics of yeasts involving the gastrointestinal tract.

Organism (Disease)	Primary Geographic Distribution	Histology
<i>Histoplasma capsulatum</i> (Histoplasmosis)	Ohio River Valley/Lower Mississippi River (North America); East Southern Cone (South America)	Small, round to oval yeast (2–4 μ m) with narrow based budding; "halo" effect
<i>Histoplasma duboisii</i> (African histoplasmosis)	Central Africa	Oval thick-walled yeast, 8–15 μ m, with narrow-based budding
<i>Talaromyces</i> (formerly <i>Penicillium</i>) <i>marneffei</i>	Southeast or Far East Asia	Small, round to oval yeast (2–5 μ m), occasional elongated forms; no budding, division by septation planes
<i>Cryptococcus neoformans/C. gattii</i>	Worldwide distribution	Small, round to oval yeast (2–20 μ m) with narrow based budding; pleomorphic in size; "soap bubble" gross appearance
<i>Candida albicans</i> ; <i>Candida</i> spp. other than <i>C. glabrata</i> (Candidiasis); See also Table 1.	Worldwide distribution	Mixture of budding yeast (5–8 μ m) and pseudohyphae; occasional septate hyphae
<i>Candida glabrata</i>	Worldwide distribution	Budding yeast without hyphae; may be extracellular
<i>Pneumocystis jirovecii</i> (pneumocystosis)	Worldwide distribution	4–6 μ m spherules, typically extracellular, with cup or crescent shapes when collapsed, Paired comma-shaped internal structure
Microsporidia (Microsporidiosis)	Worldwide distribution	0.7–4 μ m spores; size varies by genus

Finally, there are two commonly-used serum-based detection methods that detect components of the fungal cell wall that can aid in the diagnosis of invasive fungal infection. The galactomannan assay detects a component of the *Aspergillus* cell wall that is released during growth, and thus is helpful in diagnosing invasive aspergilliosis (Pfeiffer et al., 2006). The beta (1,3) D glucan assay is another test that detects fungal antigen that is a component of the cell wall of most fungi (Karageorgopoulos et al., 2011). Thus, this test is positive in many disseminated fungal infections, with important exceptions including the Mucorales, *Cryptococcus* spp. and *Blastomyces* spp.

When fungal infection is in the differential diagnosis prior to obtaining tissue, a separate specimen should be submitted for fungal stain and culture. Consideration should also be given to setting a piece of fresh tissue aside for targeted or broad range

molecular tests. The clinical microbiology laboratory generally employs primary fluorescence stains such as calcofluor white to perform a fungal smear, which allow for rapid and relatively sensitive screening of the specimen while awaiting the more sensitive culture results. If a fungal infection is an unexpected finding and fresh tissue is not available, PCR can be performed on FFPE tissue. These assays are either species-specific for common pathogens such as *H. capsulatum* and *Pneumocystis jirovecii*, or consist of broad-range assays that use primers that anneal to conserved DNA regions and sequencing to identify the genus and species. Unfortunately, the analytical sensitivity of these assays is limited by the degradation of nucleic acids by formalin, and thus having stored fresh tissue for molecular analysis is desirable. Molecular tests are often expensive, have relatively long turnaround times (several days), and are only available at specialized reference laboratories.

A detailed discussion of the major mycoses affecting the gastrointestinal tract is presented in the following sections. Other fungal infections that occasionally involve the gastrointestinal tract, but are not discussed in detail here, the phaeohyphomycoses (dematiaceous, or naturally pigmented, fungi), *Coccidioides immitis*/*C. posadasii*, *Blastomyces dermatitidis*/*B. gilchristii*, and *Paracoccidioides brasiliensis* (South American blastomycosis), which can mimic chronic idiopathic inflammatory bowel disease both clinically and radiographically (Arcobello and Revankar, 2020; Weatherhead et al., 2015; Martinez et al., 1979; McKenzie and Khakoo, 1985).

Aspergillus species (aspergillosis)

Aspergillus is a genus of ubiquitous non-pigmented (hyaline) environmental fungi. Infection of the gastrointestinal tract occurs almost exclusively in immunocompromised patients, particularly those with prolonged neutropenia, and thus bone marrow transplant recipients are at particular risk for invasive aspergillosis (Pagano et al., 2017; Gavalda et al., 2014; Malcolm and Chin-Hong, 2013; Ellis, 2001; Prescott et al., 1992). Aspergillosis in immunocompromised hosts can be a medical emergency leading to rapidly life-threatening disease and requiring urgent treatment. Disease in immunocompetent hosts is uncommon.

Aspergillus fumigatus and *A. flavus* are the most common pathogens, but several other species including *A. niger*, *A. terreus*, *A. versicolor*, and *A. nidans* may also cause human disease (Centers for Disease Control and Prevention, 2018). Infection is acquired almost exclusively through inhalation of microscopic airborne spores, leading to primary infection of the respiratory tract, with the potential for subsequent hematogenous dissemination to other organs in immunocompromised hosts (Prescott et al., 1992; Eggimann et al., 2006). Extrapulmonary involvement has been reported in 25–60% of cases of invasive aspergillosis, and is seen almost entirely in the context of primary pulmonary infection (Eggimann et al., 2006). Primary intestinal aspergillosis is rarely, presumably secondary to ingestion of infectious spores (Prescott et al., 1992; Eggimann et al., 2006).

Clinical features

The bowel (particularly colon) is the most frequent site of gastrointestinal involvement, followed by the esophagus and stomach. Multiple sites of concomitant involvement are common. Aspergillosis is much less frequently seen in the esophagus compared to candidiasis. Most patients have clinical or radiologic features of concomitant pulmonary disease (Lamps, 2012; Centers for Disease Control and Prevention, 2018; Eggimann et al., 2006). Common intestinal symptoms are fever, vomiting, abdominal pain, diarrhea, and GI bleeding (Lamps, 2012). First line therapy of invasive disease is voriconazole (Centers for Disease Control and Prevention, 2018).

Gross findings

The characteristic histologic lesion of aspergillosis is a nodular infarction, which may be transmural, consisting of a zone of ischemic necrosis centered on blood vessels containing fungi. These nodular infarctions may produce mucosal “target lesions” with a central necrotic zone surrounded by a ring of hemorrhage (Fig. 1). The surrounding mucosa is commonly erythematous, edematous, and ulcerated (Lamps, 2012). Occasionally an inflammatory mass (aspergilloma) may be seen (Ibrahim, 2000).

Microscopic findings

Aspergillus is highly angioinvasive, a feature that results in many of the characteristic morphologic findings. As noted above, transmural infarction of the bowel wall is common, and fungal hyphae often extend outward from the infarct in parallel or radial arrays, with associated ischemic necrosis (Fig. 2) (Guarner and Brandt, 2011; Lamps, 2012). Depending on the extent of immune compromise, the associated host response may range from minimal inflammation to marked neutrophilic infiltrates (Lamps, 2012) (Fig. 3). Granulomatous inflammation may be present as well. The typical hyphae of *Aspergillus* are narrow (3–12 µm diameter), uniform, and regularly septate, classically with parallel walls and acute angle, dichotomous branching (Fig. 4). Occasional atypical enlarged/bulbous forms are commonly seen in tissue.

Differential diagnosis

Several hyaline molds, such as *Fusarium*, *Paecilomyces*, and *Scedosporium* species, have nearly identical histologic appearance to that of *Aspergillus*, so that definite identification cannot be made from tissue morphology alone (Guarner and Brandt, 2011; Procop and

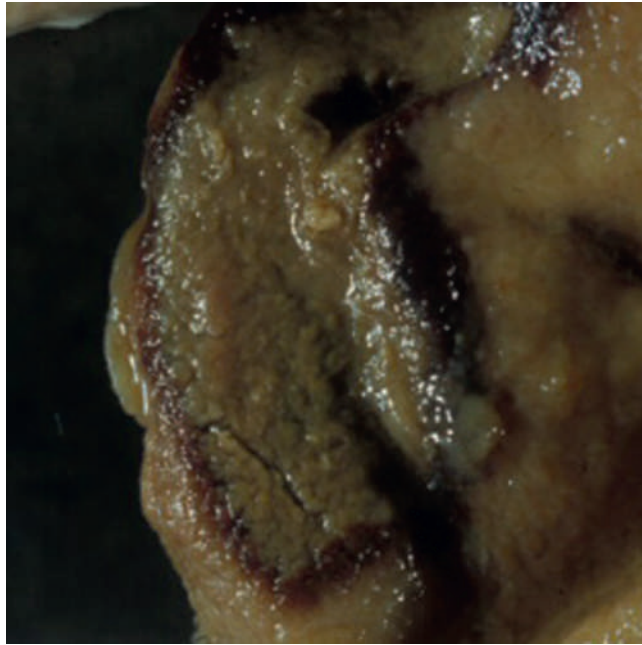


Fig. 1 This autopsy specimen shows a stomach with the characteristic “target lesion” of *Aspergillus* infection. The nodular infarction consists of a central necrotic zone surrounded by a ring of dark brown hemorrhage.

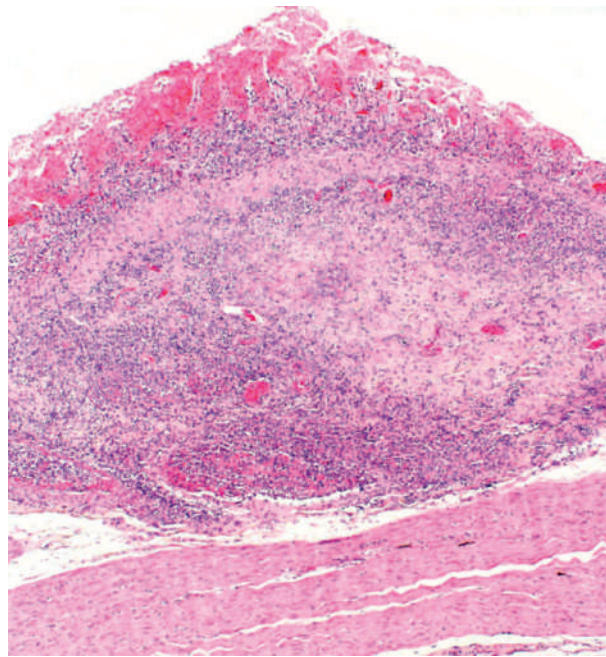


Fig. 2 A nodular infarction with overlying ischemia in the stomach of a bone marrow transplant patient.

Pritt, 2018). The only exception to this is when *Aspergillus* spp. fruiting heads are seen in tissue; this is a rare occurrence that is typically seen when the infection is contiguous with an airspace. Given the limitations of morphology, suggested phrasing in the pathology report is, “Narrow, regularly-septate hyphae with acute angle branching. Differential diagnosis includes *Aspergillus* and similar sp. Culture or molecular identification is required for definite diagnosis.” The tissue reaction of *Aspergillus* spp. and the Mucorales can certainly mimic each other, but in contrast to *Aspergillus*, the Mucorales have broad, ribbon-like, pauciseptate hyphae with irregular walls, which branch randomly at various angles.

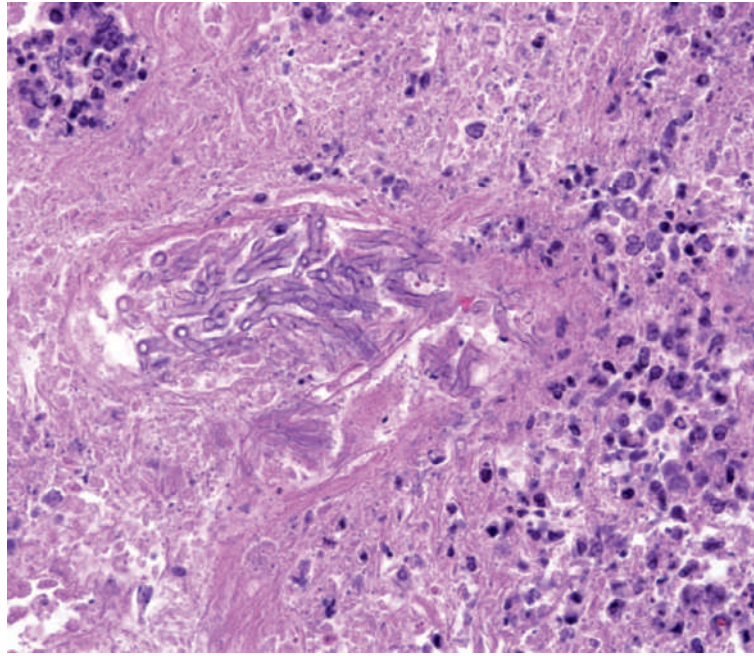


Fig. 3 Fungi occlude a small vessel in the wall of the bowel, with associated neutrophilic inflammation and necrosis.

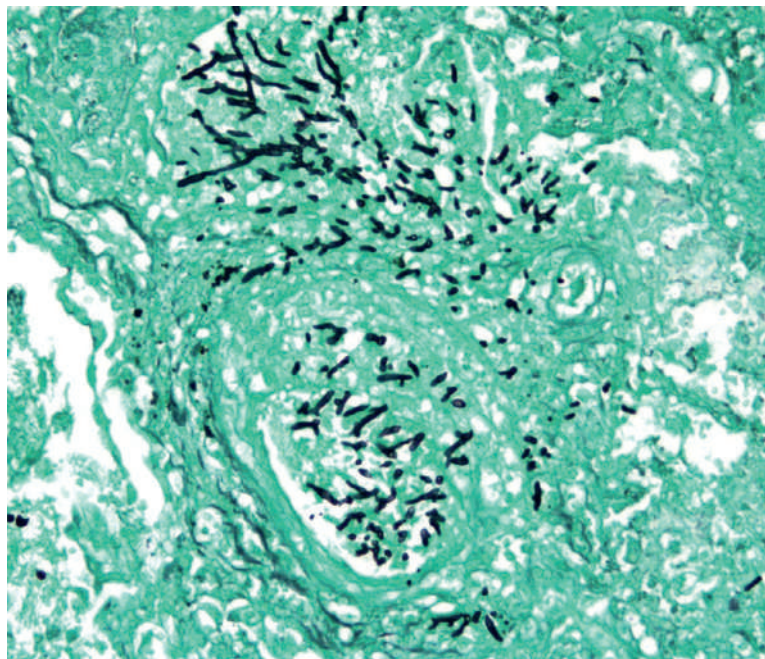


Fig. 4 Typical hyphae of *Aspergillus* spp. are narrow and regularly septate, with parallel walls and acute angle, dichotomous branching. These fungi are seen within a small vessel in the bowel wall (GMS stain).

Mucorales (mucormycosis)

The Mucorales is an order within the Zygomycetes comprising multiple genera that cause human mucormycosis (formerly zygomycosis) (Guarner and Brandt, 2011; Centers for Disease Control and Prevention, 2018). Like *Aspergillus*, the mucorales are ubiquitous environmental fungi that rarely cause disease in immunocompetent hosts, but can cause serious, life-threatening disease in immunocompromised hosts (Centers for Disease Control and Prevention, 2018). *Rhizopus* spp. are the most common cause of

mucormycosis, but several other genera including *Mucor*, *Rhizomucor*, and *Lictheimia* also cause disease (Guarner and Brandt, 2011). Although *Aspergillus* species have long been recognized as the most commonly encountered filamentous fungi in the immunocompromised patient population, for reasons that remain poorly understood, the incidence of mucormycosis is increasing, particularly in patients with diabetes, hematologic malignancies, and bone marrow transplants (Dictar et al., 2000). Mucormycosis is associated with poorly-controlled diabetes and other causes of metabolic acidosis, deferoxamine therapy, organ or bone marrow transplant, neutropenia, skin and soft tissue breakdown, intravenous drug use, neonatal prematurity, and malnourishment. Interestingly, HIV/AIDS does not appear to be a risk factor for this infection. The mortality rate is quite high (over 40% in general, and even higher in patients with hematologic malignancies and those status post bone marrow transplant). Gastrointestinal mucormycosis is an uncommon form of infection seen primarily in the setting of severe immune compromise and concomitant pulmonary infection (Centers for Disease Control and Prevention, 2018; Hosseini and Lee, 1998; Kahn, 1963; Thomson et al., 1991).

Clinical features

Gastrointestinal mucormycosis (Hosseini and Lee, 1998; Kahn, 1963; Thomson et al., 1991) is relatively uncommon, but often fatal. Patients with gastrointestinal involvement commonly present with nausea, vomiting, abdominal pain, and occasionally, gastrointestinal bleeding (Centers for Disease Control and Prevention, 2018). In neonates, the presentation may resemble that of necrotizing enterocolitis. Treatment is usually with lipid formulations of amphotericin B (Centers for Disease Control and Prevention, 2018). The Mucorales do not respond to voriconazole, an agent commonly used for prophylaxis and treatment of aspergillosis, which underscores the importance of proper fungal identification.

Gross findings

Any level of the GI tract can be involved, but gastric and colonic involvement are most common. The gross findings are similar to those of aspergillosis, with ulceration and infarction being common findings. Ulcers may be large with irregular, rolled edges, mimicking malignancy (Lamps, 2012). The Mucorales may also superinfect previously ulcerated tissues.

Microscopic findings

As with aspergillosis, nodular infarction with angioinvasion is a characteristic microscopic finding of mucormycosis (Lamps, 2012). The inflammatory response is variable, but neutrophils are usually prominent. The hyphae are usually visible with H&E, particularly in areas of necrosis (Fig. 5). The hyphae differ from those of *Aspergillus* spp., as they are pauciseptate, with irregular diameters ranging from 5 to 20 μ m (Guarner and Brandt, 2011). The appearance of optically clear centers is often seen on cross-section (Fig. 6). The hyphal walls are very thin and easily collapse during histologic processing, giving the appearance of “crumpled cellophane” (Guarner and Brandt, 2011; Lamps, 2012). Branching occurs at various angles including acute and right angles (Procop and Pritt, 2018; Lamps, 2012).

Differential diagnosis

The Mucorales can often be differentiated morphologically from the uniformly narrow, regularly septate molds such as *Aspergillus* by their broad, irregular, crumpled, pauciseptate hyphae (Guarner and Brandt, 2011).

A rare entity that must also be considered in the differential diagnosis is gastrointestinal infection with *Basidiobolus ranarum*, a fungus in the order Entomophthorales (see below).

Basidiobolus ranarum (basidiobolomycosis)

B. ranarum is a zygomycete of the order Entomophthorales, which are closely related to the Mucorales, both being members of the Zygomycetes; however, they have very different pathogenic potentials. Basidiobolomycosis is a chronic infection seen primarily in immunocompetent patients. Risk factors include pediatric age group, peptic ulcer disease, diabetes, pica, ranitidine use, and living in an endemic area. Of note, unlike many of the other filamentous fungi, typical causes of immunocompromise such as neutropenia, HIV/AIDS, and organ transplantation are not risk factors for this infection. Infection by this world-wide soil saprophyte was originally described in Africa, Saudi Arabia, and Southeast Asia, but has recently gained attention in the United States, primarily in Arizona (Centers for Disease Control and Prevention (CDC), 1999; Yousef et al., 1999; Lyon et al., 2001).

Clinical features

Unlike the skin and subcutaneous infection in which this organism was originally described, *B. ranarum* has typically affected the gastrointestinal tract in the United States. Abdominal pain is the most common presenting symptom (Centers for Disease Control and Prevention (CDC), 1999; Yousef et al., 1999; Lyon et al., 2001).

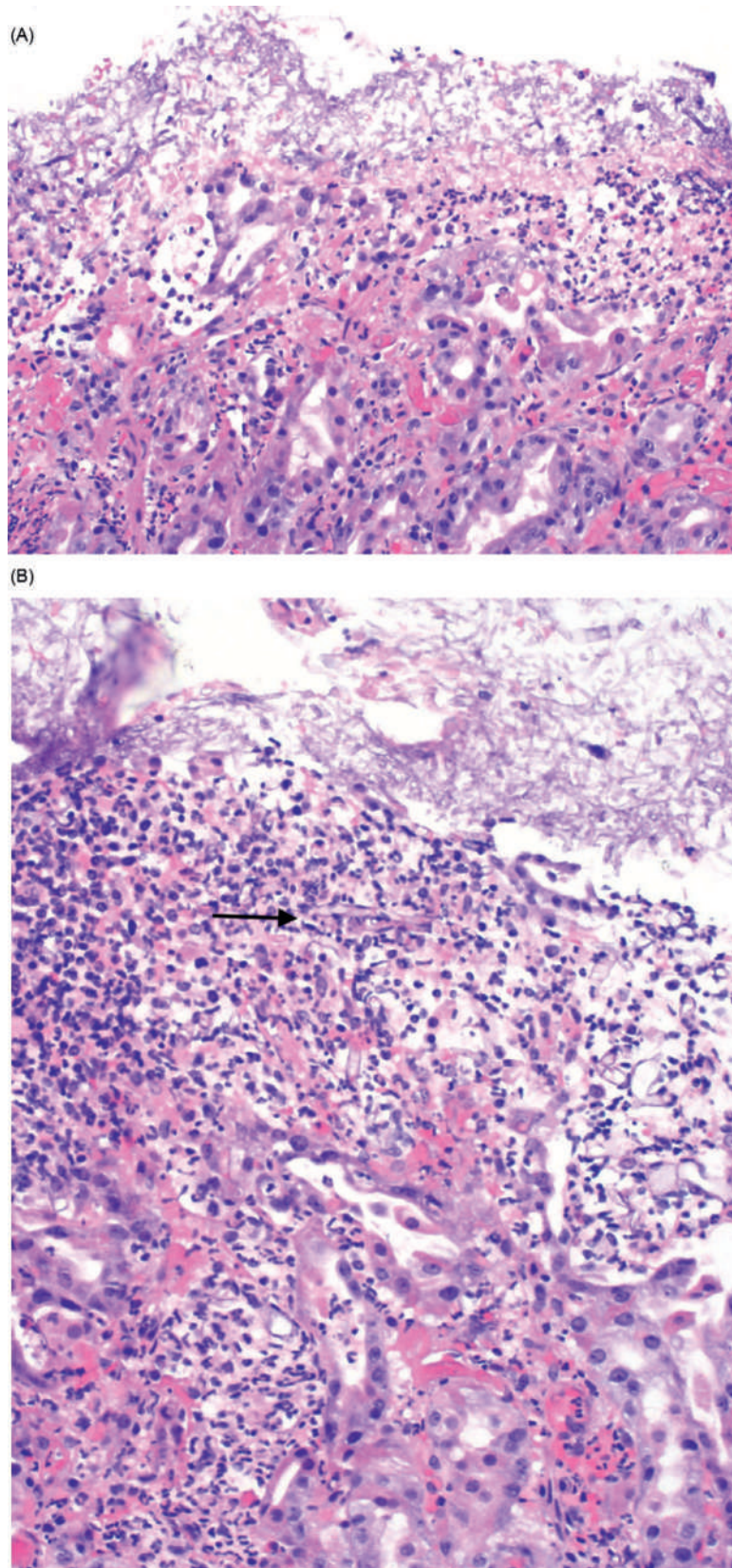


Fig. 5 This gastric biopsy with invasive Mucorales infection shows ischemic-appearing mucosa with associated neutrophils and an exudate (top of image) that contains fungi (A). Invasive organisms are also seen in the mucosa (B, arrow).

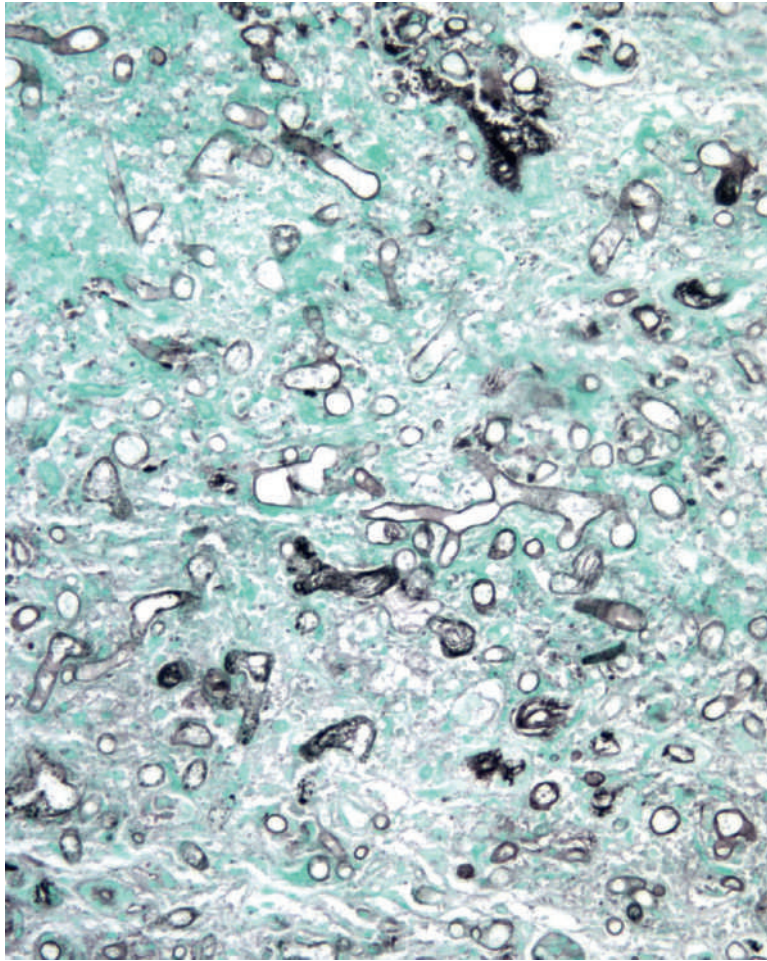


Fig. 6 The hyphae of the Mucorales are broad and ribbon-like, with optically clear centers on cross section. They are pauciseptate with variable diameters, and branch at random angles.

Gross findings

The colon and rectum are most frequently involved, followed by the small bowel and stomach. Gastrointestinal infections may mimic malignancy or chronic idiopathic inflammatory bowel disease clinically, and many patients have presented with pericolic masses that are worrisome for colon cancer (Nemenqani et al., 2009).

Microscopic findings

The hyphae are very similar to those of the mucorales, but the tissue reaction is quite different, featuring intense granulomatous inflammation with prominent eosinophils, neutrophils, necrosis, and fibrosis (Guarner and Brandt, 2011) (Fig. 7). The hyphae are commonly associated with peripheral bright pink Splendore-Hoeppli reaction (Fig. 8).

Differential diagnosis

Although the fungi themselves are morphologically similar to those that cause mucormycosis, there are typically fewer of them in a given tissue section. In addition, *B. ranarum* may have more pronounced “cellophane-ball” or crumpled appearance (Fig. 9). *Basidiobolus* is not angioinvasive, in contrast to the Mucorales, and mucormycosis does not have prominent eosinophilia or Splendore-Hoeppli reaction to organisms.

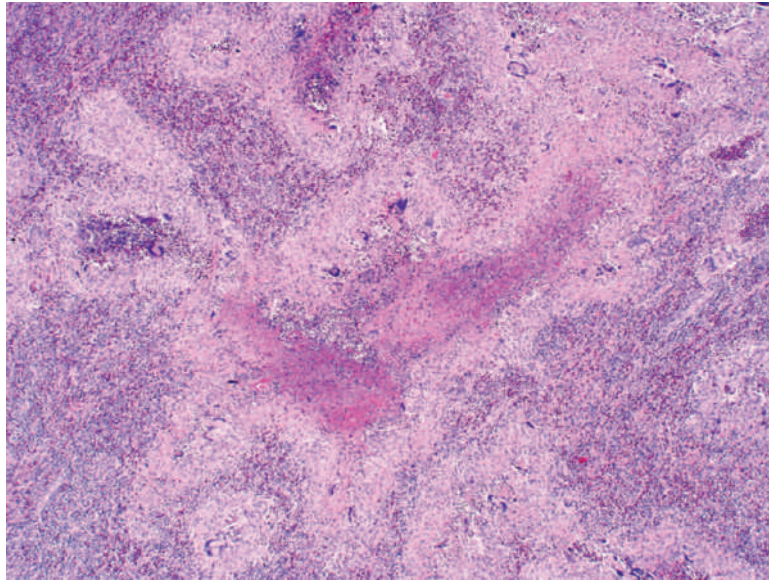


Fig. 7 This section of a colonic wall mass shows necrosis and granulomatous inflammation with giant cells and striking associated eosinophilia.

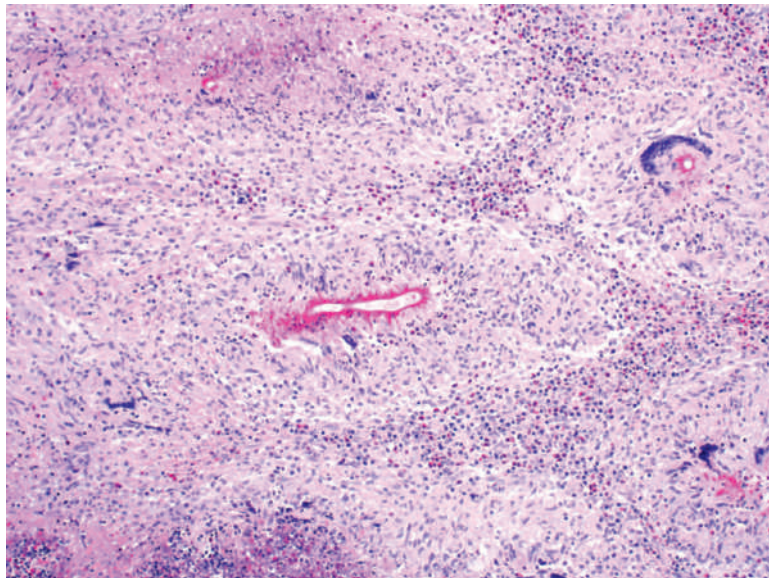


Fig. 8 The fungi have associated bright pink Splendore-Hoeppli protein deposits on their walls, which highlight them within the granulomas. Note the numerous eosinophils.

***Candida* species**

The gastrointestinal tract is a major portal for disseminated candidiasis, as *Candida* often superinfects ulcers that develop from other causes. *C. albicans* is most commonly isolated of the *Candida* species, but *C. tropicalis* and *C. (Torulopsis) glabrata* may produce similar clinical and pathologic manifestations. Other non-*albicans* species (such as *C. krusei* and *C. parapsilosis*) are also emerging as important causes of invasive fungal infection. Common risk factors for invasive candidiasis include immunosuppression, chemotherapy, corticosteroids, and major abdominal surgery (Enoch et al., 2017; Pagano et al., 2017; Gavalda et al., 2014; Lionakis, 2019; Antinori et al., 2016).

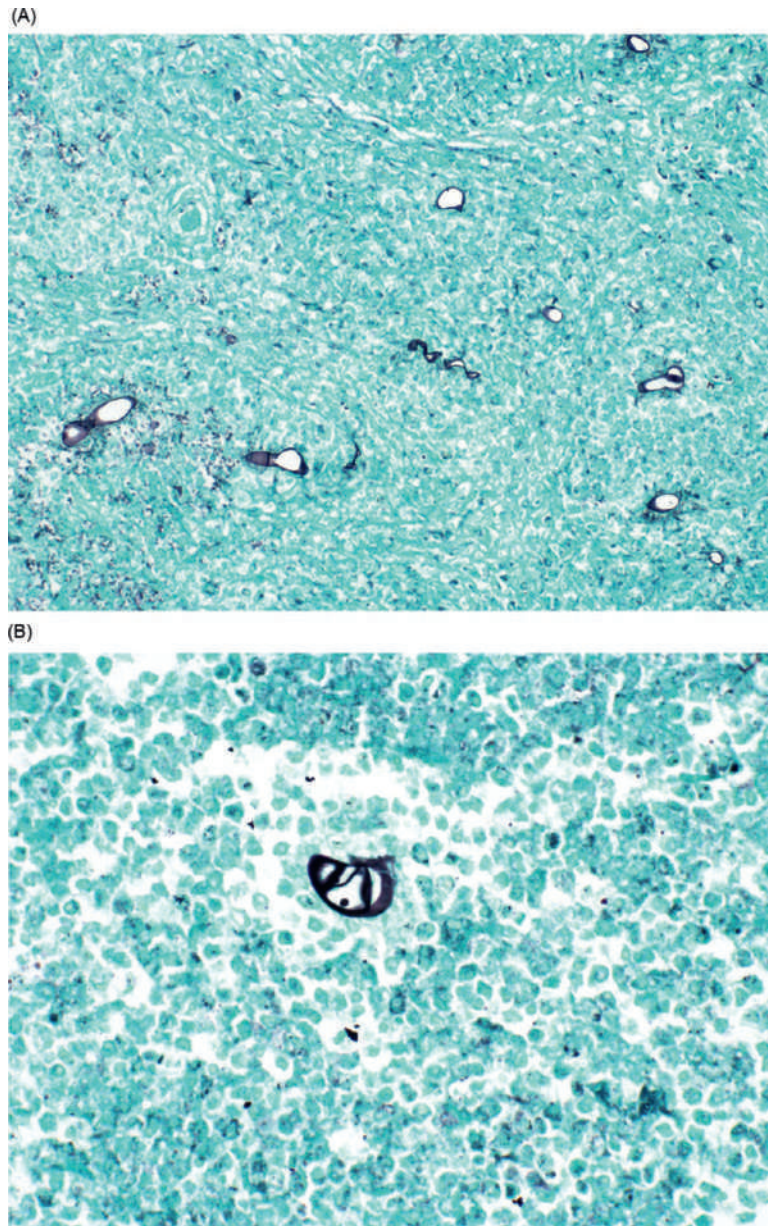


Fig. 9 *B. ranarum* has a similar morphologic appearance as the Mucorales, but there are typically fewer organisms present in the tissue, and they are commonly surrounded by the Splendore-Hoeppli protein. Note the thin walls with a crumpled appearance, and rare septa (A, GMS stain). Some fungi have a “cellophane ball” morphology (B, GMS stain).

Clinical features

Candida is the most common infection of the esophagus, but may infect any level of the GI tract (Prescott et al., 1992; Kondo and Terada, 2017; Hoversten et al., 2018; Hissong et al., 2020).

Gross findings

Esophageal infection typically features white plaques that easily scrape away to reveal ulcerated mucosa underneath (Fig. 10). The gross features of candidiasis in the stomach and intestines are variable and include ulceration, pseudomembrane formation, and inflammatory masses (Prescott et al., 1992) (Fig. 11). The ulcers are often multiple, irregular, and hemorrhagic, and may be confluent in advanced cases. Deep linear ulcers also have been described. If vascular invasion is prominent, the bowel may appear infarcted. Involvement may be diffuse or segmental.

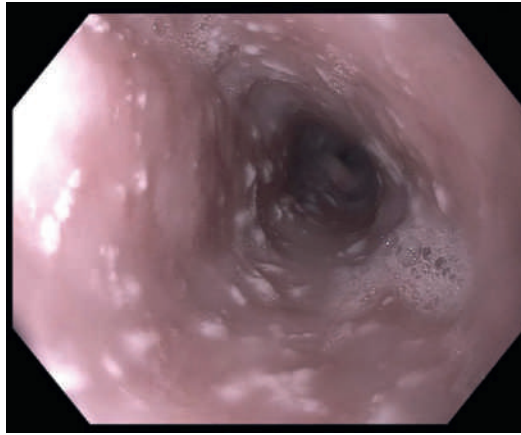


Fig. 10 This endoscopic photograph from an AIDS patient shows white plaques throughout the esophagus. Courtesy Dr. Rhonda Yantiss.

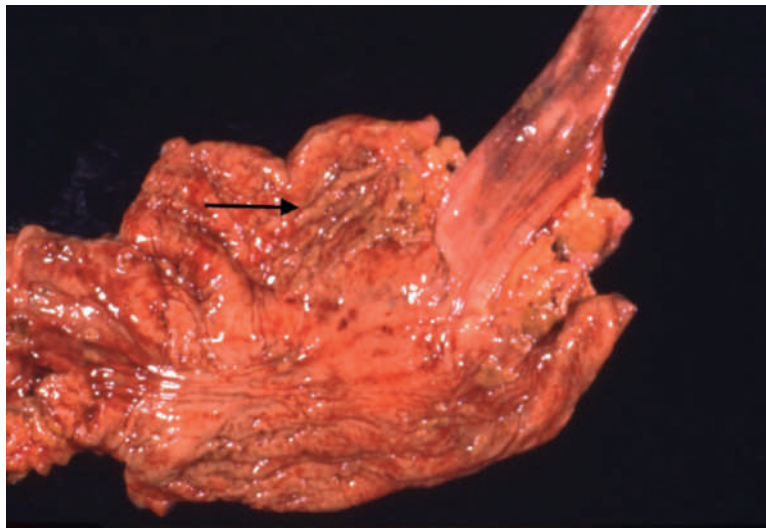


Fig. 11 This gastric specimen with invasive *Candida* infection, from an autopsy, shows variably sized ulcerations with yellow plaques (arrow).

Microscopic findings

The associated inflammatory response ranges from minimal (especially in immunocompromised patients) to marked with prominent neutrophilic infiltrates, abscess formation, erosion/ulceration, and necrosis (Fig. 12). When pseudomembranes are present, they are composed of a mixture of yeast, necrotic debris, and fibrin. Granulomas are occasionally present. Numerous intra-epithelial neutrophils in the squamous mucosa of the esophagus is a common finding in esophageal candidiasis, and should provoke the use of fungal stains if organisms are not appreciated on H&E. Fungi may invade any level of the gut wall, and invasion of mucosal and submucosal blood vessels is sometimes a prominent feature (Lamps, 2012; Prescott et al., 1992).

C. albicans, *C. tropicalis* and species other than *C. glabrata* produce a mixture of budding yeast forms intermingled with pseudohyphae and occasional true hyphae (Fig. 13). *C. glabrata* features tiny budding yeast forms similar to *H. capsulatum*, but does not produce hyphae or pseudohyphae (Fig. 14).

Differential diagnosis

Grossly, intestinal candidiasis may mimic other inflammatory process including chronic idiopathic inflammatory bowel disease, pseudomembranous colitis, and ischemic colitis. As above, fungal stains should be strongly considered in any immunocompromised patient with ischemic lesions of the gastrointestinal tract.

It is important to morphologically differentiate between invasive candidiasis and superficial colonization, as *Candida* is capable of colonizing benign ulcers and mucosal surfaces without invasion. In the esophagus, care should be taken to avoid confusing oral contamination by *Candida* for true invasive *Candida* esophagitis. In invasive candidiasis, fungi should be present within the squamous epithelium, often (but not always) with associated acute inflammation (Fig. 15) (Hissonig et al., 2020).

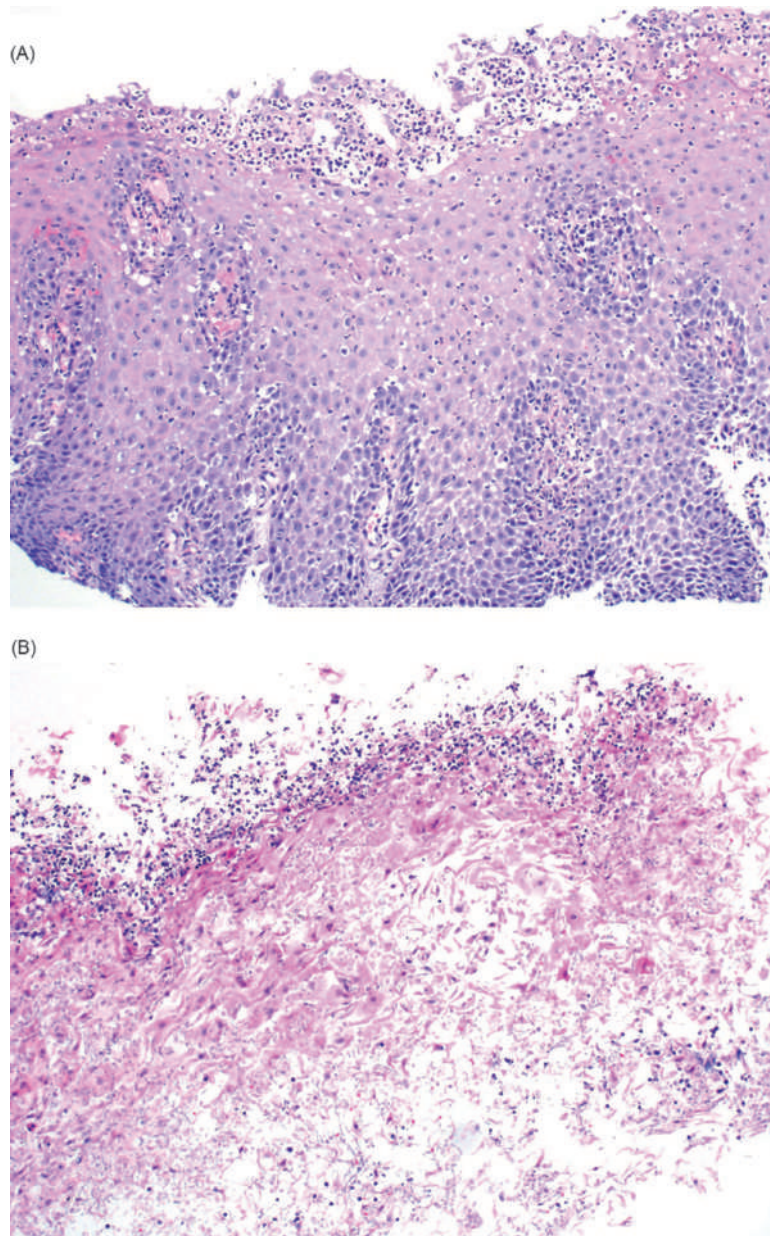


Fig. 12 Esophageal candidiasis typically features neutrophilic inflammation with acantholysis or sloughing of the superficial squamous mucosa (A). This esophageal biopsy contains an exudate consisting of yeast, sloughed epithelial cells, and inflammation (B).

***Cryptococcus* species (cryptococcosis)**

Cryptococcus is a ubiquitous environmental fungus that is a rare but important cause of gastrointestinal infection (Centers for Disease Control and Prevention, 2018). The two most common species are *C. neoformans* and *C. gattii*. *C. neoformans* is found throughout the world and causes disease primarily in immunocompromised hosts; it is a saprophytic organism, commonly associated with pigeons. *C. gattii* is found in the tropics, subtropics, and Pacific Northwest, and associated with eucalyptus trees; it may infect both immunocompromised and immunocompetent hosts (Centers for Disease Control and Prevention, 2018). Infection is acquired through inhalation of fungal spores, resulting in a primary respiratory infection in susceptible individuals (Centers for Disease Control and Prevention, 2018). Both *C. neoformans* and *C. gattii* are capable of disseminating from the lungs to other organs (Centers for Disease Control and Prevention, 2018), and have a predilection for the central nervous system.

Gastrointestinal involvement occurs almost exclusively in immunocompromised hosts and is due to dissemination from a primary respiratory source; however, rare cases of GI involvement in immunocompetent hosts, and of primary intestinal cryptococcosis, and have been reported (Melato and Gorji, 1998; Washington et al., 1991; Harris et al., 2011).

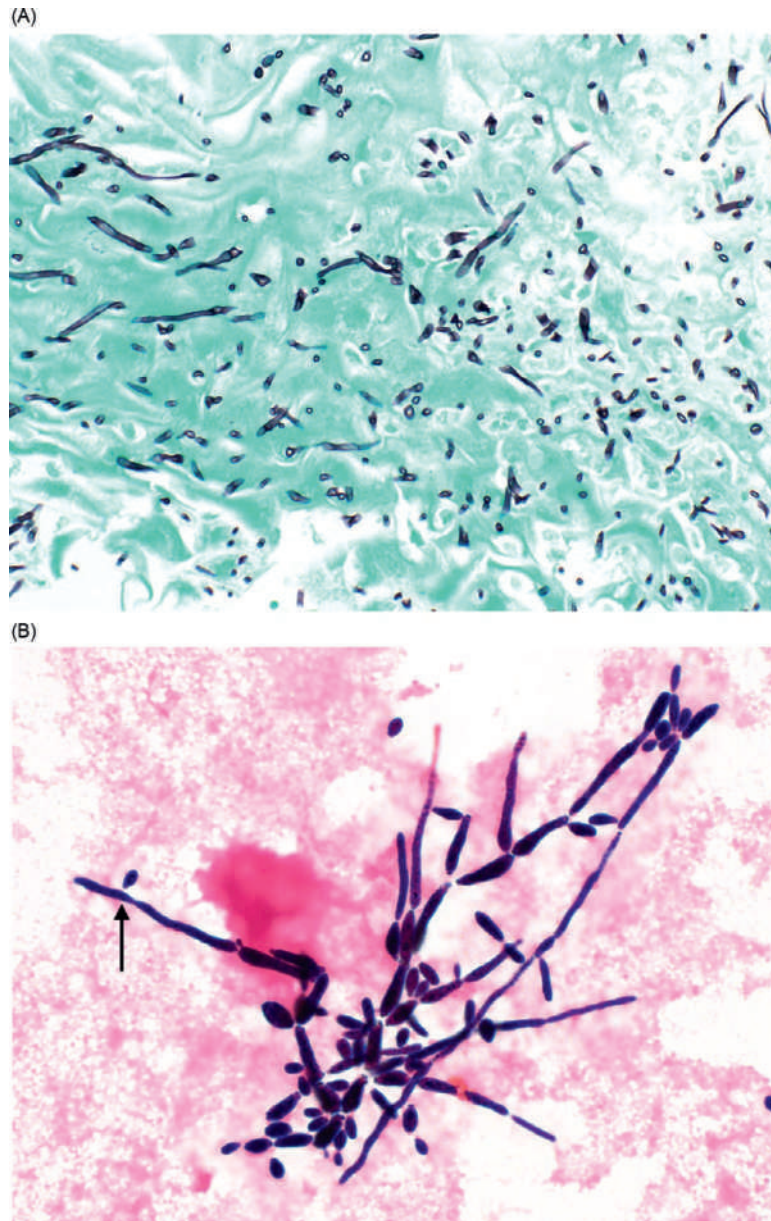


Fig. 13 The morphology of *Candida albicans* is a mixture of budding yeast and pseudohyphae, with occasional true hyphae as seen in this image (A, GMS stain). Fig. (B) (Gram stain) shows pseudohyphae, from which yeasts bud directly (arrow).

Clinical features

Most patients have clinical or radiographic evidence of pulmonary and central nervous system involvement (Lamps, 2012; Washington et al., 1991). Symptoms of intestinal involvement include abdominal pain and distension. Treatment varies with the severity of disease, and severe extra-pulmonary disease is treated with liposomal amphotericin B plus flucytosine (Perfect et al., 2010).

Gross findings

Any part of the gastrointestinal tract may be involved, but the colon is the most frequently involved site, followed by the esophagus. Endoscopic lesions include nodules and ulcers, sometimes associated with thick white exudates (Lamps, 2012; Washington et al., 1991). However, the mucosa is normal in many cases, even when deeply invasive organisms are present, as the organisms are often

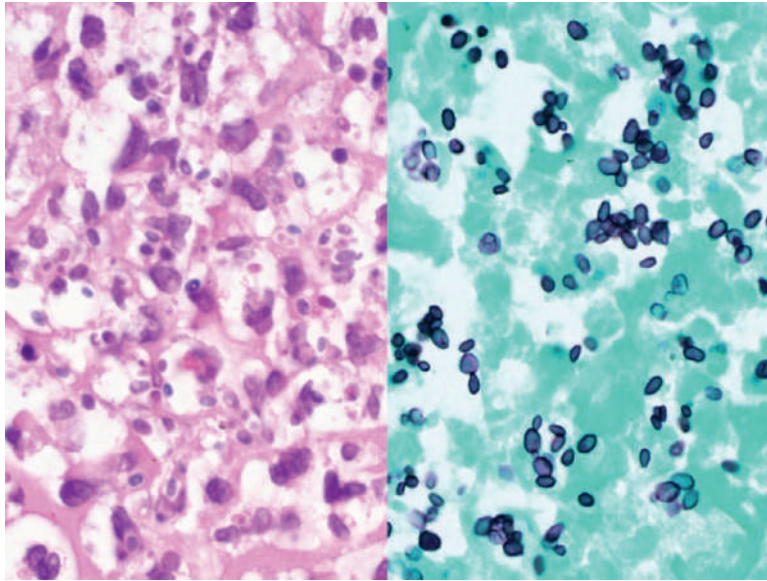


Fig. 14 Unlike the other *Candida* species, *Candida glabrata* is only present as small, oval-shaped, yeasts with narrow-based budding, and thus can be difficult to differentiate from *Histoplasma capsulatum*. Unlike *H. capsulatum*, *C. glabrata* is usually visible on H&E (left side of image), is commonly extracellular, and may have an associated suppurative host response. Thus it is important to carefully examine the H&E-stained slide as well as the GMS (right side of image) to differentiate these 2 morphologically-similar fungi.

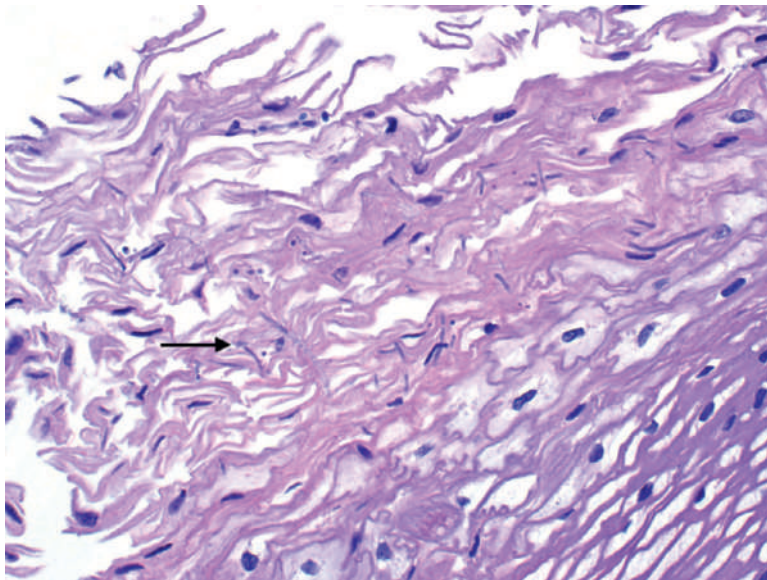


Fig. 15 Some patients have invasive candidiasis (arrow), but no associated inflammation.

located within lymphatics. Inflammatory polypoid masses have been described rarely. In heavy infections with abundant mucopolysaccharide capsular material, small gelatinous pseudocysts may form which are visible grossly and radiologically as “soap bubble” lesions, particularly in the brain.

Microscopic findings

Histologically, the inflammatory reaction is variable and depends on the immune status of the host, ranging from a suppurative, necrotizing inflammatory reaction (Fig. 16) to virtually no reaction in profoundly immunocompromised hosts (Guarner and Brandt, 2011; Procop and Pritt, 2018; Lamps, 2012; Washington et al., 1991) (Fig. 17). Both superficial and deep involvement may

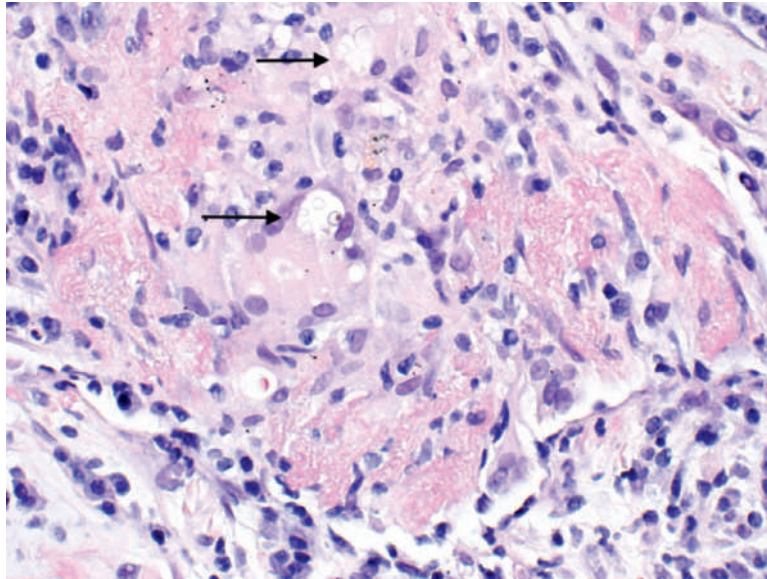


Fig. 16 *Cryptococcus neoformans* is seen within a giant cell in a granuloma (arrows). Note the non-staining "soap bubble" appearance associated with the organism, caused by the capsule.

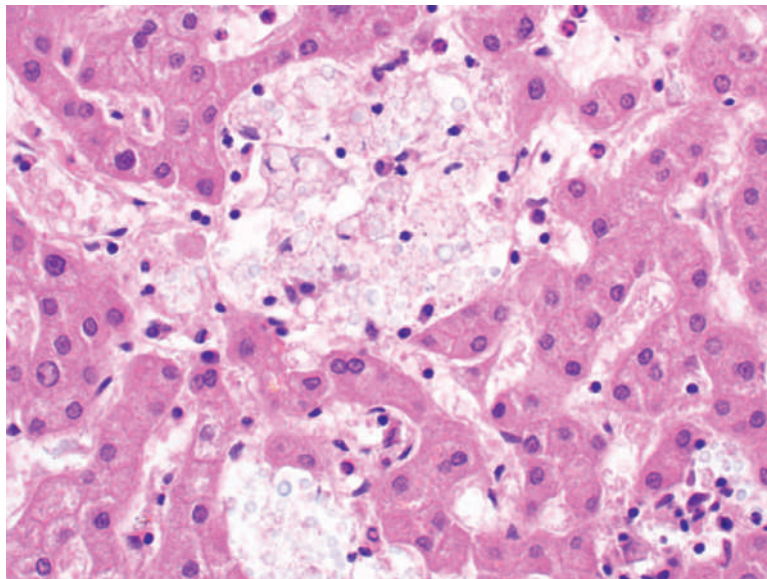


Fig. 17 This liver biopsy from an AIDS patient contains innumerable *Cryptococcus* sp. within macrophages, but virtually no inflammatory reaction.

occur, and lymphatic involvement is common. In patients with some degree of native immunity, a granulomatous response may be seen with occasional formation of a mass lesion (cryptococcoma).

The two species are morphologically identical. The diagnostic feature is the presence of variably-sized (2–20 μm), round, intra- and extra-cellular yeasts within the mucosa and/or submucosa (Guarner and Brandt, 2011; Procop and Pritt, 2018; Lamps, 2012; Washington et al., 1991). Variation in size is a typical feature of cryptococcosis (Fig. 18). The yeasts typically have a non-staining halo corresponding to the presence of a mucopolysaccharide capsule, which may also be described as having a "soap bubble" appearance (Fig. 16). Yeasts divide by narrow-based budding. Rare pseudohyphae may also be seen (Procop and Pritt, 2018). The yeast capsule often stains deeply with mucin stains such as mucicarmine (Fig. 19) and Alcian blue, although capsule-deficient strains may be mucicarmine negative (Ro et al., 1987). The cell wall may also be highlighted using a Fontana-Masson stain (Fig. 20), although this varies widely by the stain formulation being used, and may stain other similar-appearing yeasts such as dematiaceous fungi (Guarner and Brandt, 2011; Procop and Pritt, 2018; Ro et al., 1987).

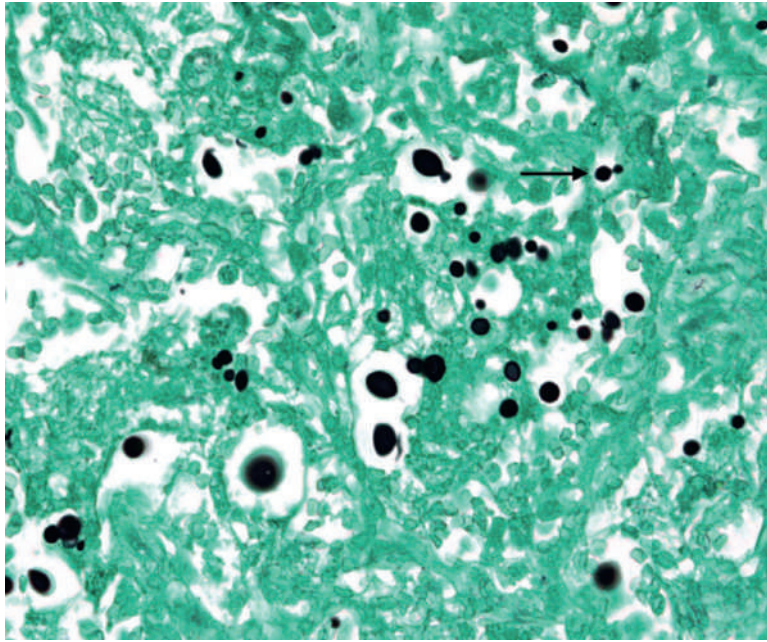


Fig. 18 GMS stain of *C. neoformans* highlights numerous yeasts with variation in size, and rare narrow-based buds (arrow).

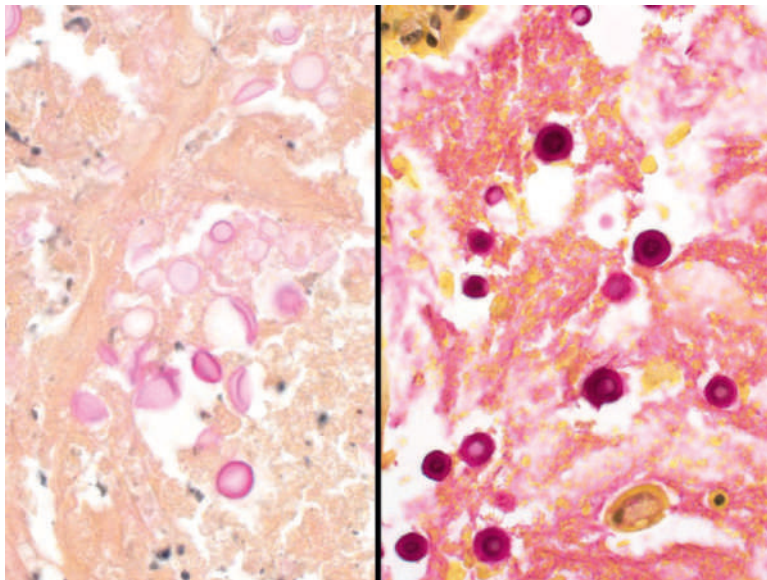


Fig. 19 *Cryptococcus* spp. often, but not always stain with mucicarmine (left). Some strains are capsule deficient, and may be partially or entirely mucicarmine negative. In other cases, abundant mucin production forms mucicarmine-positive pools with deeply-staining yeasts within.

Differential diagnosis

The yeasts of *Cryptococcus* spp. can usually be differentiated from other yeasts by their variable size, capsule, and narrow-based budding (see Table 2). Other yeasts that infect humans do not have a true capsule, and thus its presence is diagnostic for *Cryptococcus*. It is important to note that the cell wall of *Blastomyces dermatitidis*, which only rarely infects the GI tract, may be faintly mucicarmine positive and should not be interpreted as evidence of a capsule. Features useful for differentiating *B. dermatitidis* from *Cryptococcus* spp. are the uniformly large size of the former (8–15 μm diameter) and thick refractile cell wall on H&E. *Histoplasma capsulatum* may also be in the differential diagnosis, particularly when only small forms are seen, but is more oval rather than round and lacks a capsule (see below). In addition, *H. capsulatum* does not stain with mucicarmine. In cases of capsular-deficient cryptococcosis, the cell wall will often still stain with Fontana-Masson (Ro et al., 1987).

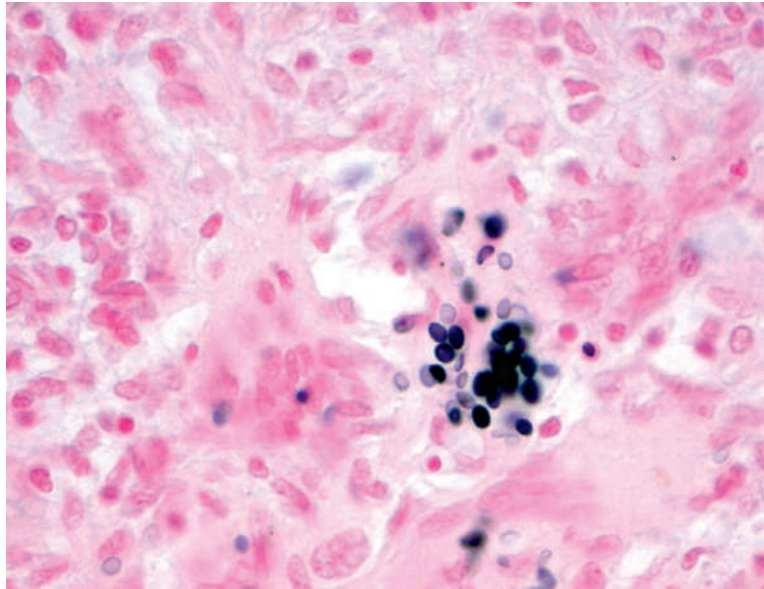


Fig. 20 Cryptococcal cell walls will also stain with Fontana-Masson, including capsule-deficient strains.

***Histoplasma capsulatum* (histoplasmosis)**

Histoplasma capsulatum is a saprophytic fungus with a near-worldwide distribution (Centers for Disease Control and Prevention, 2018; Goodwin and Des Prez, 1978; Goodwin et al., 1980, 1981). *H. capsulatum* var. *capsulatum* is found in close association with river valleys in all continents except Antarctica, while *H. capsulatum* var. *duboisii* (a.k.a., *H. duboisii*) is found in Africa (Centers for Disease Control and Prevention, 2018). This section will focus on *H. capsulatum* var. *capsulatum*, which is the most common cause of intestinal histoplasmosis. *H. capsulatum* var. *capsulatum* is endemic to the central United States, especially within the Ohio, Missouri, and Mississippi river valleys; other endemic areas in the Western hemisphere include Mexico, Guatemala, Peru, and Venezuela. Histoplasmosis has been reported in many nonendemic areas as well.

H. capsulatum is a thermally-dimorphic fungus, growing as a mold in the environment (25 °C), predominantly in soil enriched with bird or bat droppings, and as a yeast in humans (36 °C). Infection is acquired primarily through inhalation of microscopic airborne microconidia, resulting in a primary pulmonary infection with the potential for dissemination to other organs. It has a marked affinity for dissemination through the mononuclear phagocyte system, and gastrointestinal tract involvement is seen in 70–90% of cases with disseminated infection (Lamps, 2012; Cappell et al., 1988; Yang et al., 2013). Most patients are profoundly immunocompromised, but intestinal histoplasmosis can rarely occur in immunocompetent patients, as well as in patients such as young children and the elderly, who may not have fully functional immune systems (Lamps, 2012; Cappell et al., 1988; Yang et al., 2013). Isolated gastrointestinal histoplasmosis has been reported rarely. It is the most common endemic mycosis in patients with AIDS, and has also been described in association with infliximab therapy, an anti-tumor necrosis factor alpha drug used in the treatment of Crohn's disease and rheumatoid arthritis.

Clinical features

Most patients will have clinical or radiographic evidence of pulmonary and disseminated disease (Cappell et al., 1988; Lamps et al., 2000), but patients may initially present with signs and symptoms of gastrointestinal illness, and do not always have concomitant pulmonary involvement. Notable presenting gastrointestinal symptoms include diarrhea, gastrointestinal bleeding, abdominal pain, dysphagia, nausea, vomiting, weight loss, and signs of small bowel obstruction (Lamps et al., 2000). The majority of patients are febrile, and many have had symptoms for weeks to months. Gastrointestinal bleeding and anorectal disease are more common in patients with AIDS and other immunocompromising conditions.

Gross findings

The ileum is the most common site of involvement, followed by colon, stomach, and esophagus; any portion of the gastrointestinal tract may be involved. Associated lymphadenopathy is common. The esophagus may also be impinged upon by mediastinal lymphadenopathy secondary to histoplasmosis, or by *Histoplasma*-associated mediastinal fibrosis. Ulcers are the most common gross lesion; they are often multiple, with annular raised borders, associated hemorrhage, and necrosis at the base. Nodules (often centered on lymphoid aggregates) and obstructive masses or strictures are also common. Often, a combination of lesions is present (Lamps et al., 2000).

Microscopic findings

Histologic findings include diffuse lymphohistiocytic infiltrates and nodules (Fig. 21A), usually involving the mucosa and submucosa, with overlying ulceration. In the ileum, these lesions are often associated with Peyer patches (Cappell et al., 1988; Lamps et al., 2000). In addition to histiocytes and lymphocytes, there may be numerous associated eosinophils, neutrophils, and plasma cells. Nonspecific ulceration and inflammation with numerous organisms present in the bowel wall may be seen as well. Discrete granulomas and giant cells are present in only a minority of cases (Fig. 21B). In contrast to gastrointestinal lesions, abdominal lymph nodes often show necrotizing granulomas. In immunocompromised patients or very young children, endoscopic findings may be normal, and large numbers of organisms may be seen with virtually no tissue reaction histologically.

H. capsulatum yeasts are small (2–5 µm) and ovoid with single small buds at the more pointed pole (Fig. 22), typically within histiocytes. Multiple organisms are often clustered within a macrophage. The yeasts may be extremely difficult to see on H&E, but can occasionally appear as small intracellular objects with a narrow surrounding clear zone or halo (Fig. 23). The halo (pseudo-capsule) results from the poorly-stained cell wall rather than the presence of a true capsule as seen with *Cryptococcus* spp. (Procop and Pritt, 2018). The yeasts exhibit narrow-based budding, which is best seen with GMS. Unlike other yeasts, *H. capsulatum* does not always stain well with PAS.

Differential diagnosis

The overall histologic picture of intestinal histoplasmosis is similar to that of other diffuse histiocytic processes such as Whipple's disease, malakoplakia and *Mycobacterium avium* complex infection. The presence of small intracellular yeasts allows these processes to be excluded, and narrows the differential to other yeast infections. The morphology of *H. capsulatum* is similar to that of *Cryptococcus* spp. (see above), but with uniformly small rather than variably-sized forms. Unlike *Cryptococcus* spp., *H. capsulatum* also lacks a capsule. Other small yeasts that may enter the differential include *Talaromyces* (formerly *Penicillium*) *marneffei* (usually patients from Southeast Asia) and *Candida glabrata*. *T. marneffei* can be differentiated by its lack of budding. *P. carinii* can also be differentiated by its lack of buds, as well as its internal structure, the fact that it is usually extracellular, and the commonly-seen "foamy cast" tissue reaction. *C. glabrata* is more difficult to differentiate, but causes a suppurative rather than histiocytic inflammatory reaction and is more easily visible on H&E (Fig. 14). The GMS appearance of *C. glabrata* is identical to that of *H. capsulatum*, so that culture or NAATs may be required to differentiate them (Procop and Pritt, 2018). Blastomyces is much larger and has broad-based buds and *Leishmania* spp., have a characteristic kinetoplast and are GMS negative. Erythrocytes and naked cell nuclei may mimic *Histoplasma* in sections overstained with GMS; comparison with H&E slides often helps to resolve this.

Clinically, the differential diagnosis for the inflammatory lesions of histoplasmosis includes idiopathic inflammatory bowel disease, sarcoidosis, and other infections. Obstructive masses may mimic neoplasia as well. GMS and/or PAS should easily identify organisms.

Several other laboratory methods are available for diagnosing histoplasmosis (Azar and Hage, 2017). Fungal cultures are the gold standard when positive, but are not useful for rapid diagnosis. Histoplasmin skin testing and serologic tests may be useful in immunocompetent adults, but are unreliable in immunocompromised patients, young children, elderly patients, and those with disseminated disease. Urine antigen detection may be helpful.

Talaromyces (formerly *Penicillium*) *marneffei*

T. marneffei is a dimorphic fungus that is endemic in Thailand, China, Hong Kong, Vietnam, and Indonesia. Patients with this infection who are encountered in Western countries have almost always either traveled to these areas or lived there (Ko et al., 1999; Borradori et al., 1994; Cooper and McGinnis, 1997). This infection is emerging as one of the most common opportunistic infections in Asian patients with AIDS (Borradori et al., 1994; Bulterys et al., 2013). Patients with who are not HIV positive usually have other immunocompromising conditions, such as hematologic malignancies, autoimmune diseases, malnutrition, or other debilitating conditions. It has rarely been described in immunocompetent persons. Dissemination can occur in a matter of a few weeks and be quickly fatal, especially in immunocompromised patients. Months of antifungal therapy may be required for effective therapy.

Clinical features

T. marneffei most commonly involves the lungs and liver, followed by the GI tract. Common presenting symptoms include fever, anemia, weight loss and skin lesions; diarrhea is common in patients with GI involvement (Ko et al., 1999). The mortality rate is very high, in large part due to delay in diagnosis and treatment.

Gross findings

Any level of the GI tract may be involved. Endoscopic findings include ulcers, erosions, and inflammatory masses (Ko et al., 1999).

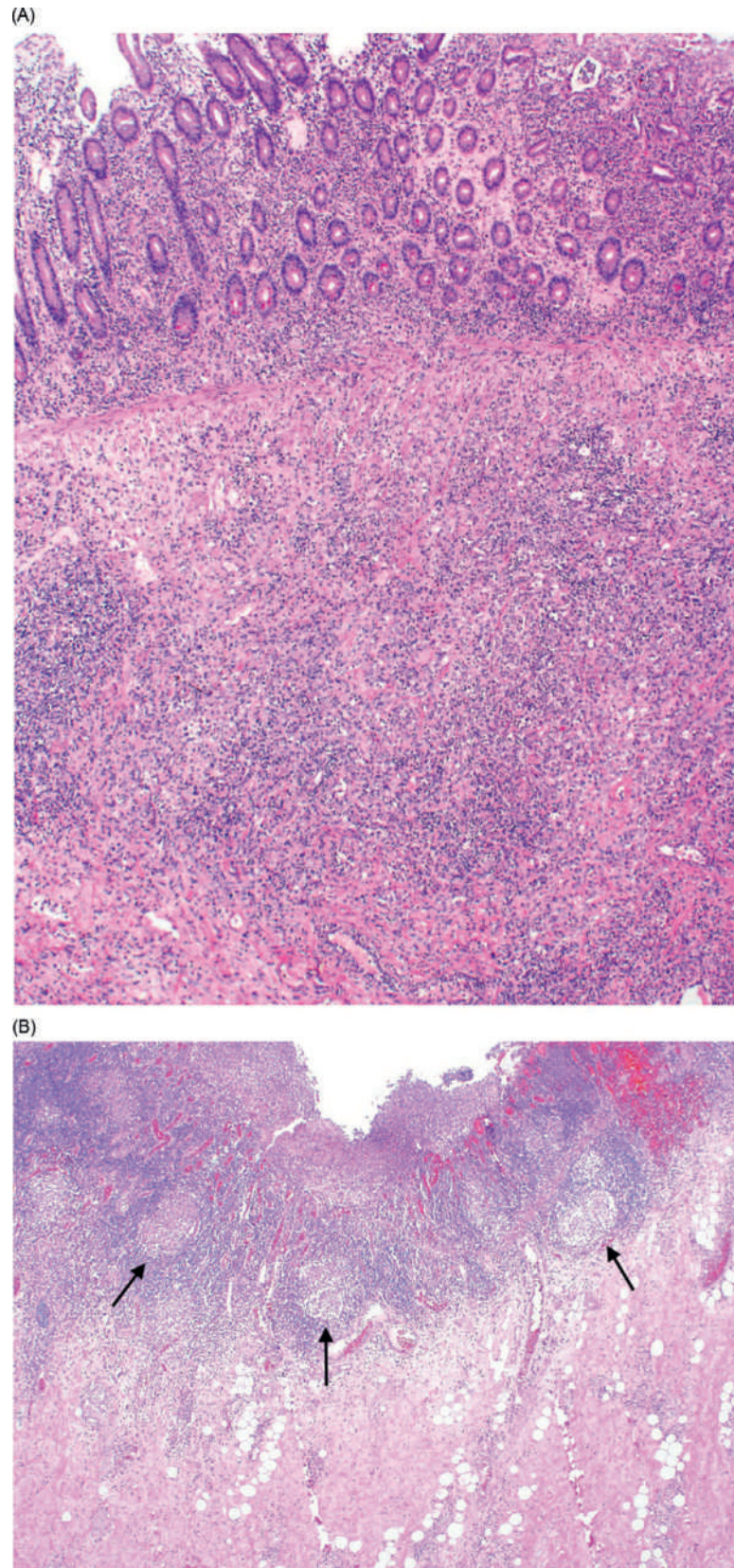


Fig. 21 A nodular lymphohistiocytic infiltrate involves the mucosa and submucosa of the small bowel in a case of histoplasmosis (A). This patient on long term Humira developed a large ileal ulcer due to histoplasmosis. The arrows point to the germinal centers of lymphoid follicles comprising the Peyer's patches of the ileum. (B) Courtesy Dr. Joel Greenson.

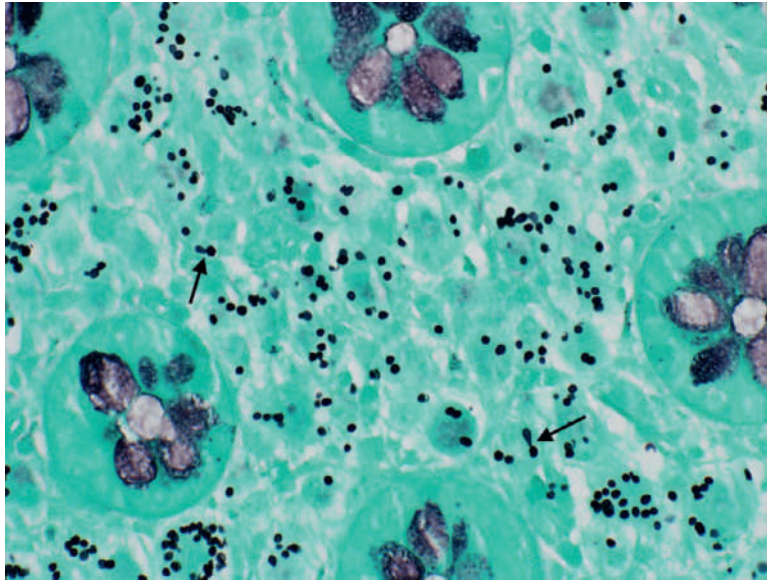


Fig. 22 The yeasts of *Histoplasma capsulatum* are uniformly small, oval, and divide by narrow-based budding (GMS, arrows).

Microscopic findings

As with the other dimorphic fungi, *T. marneffei* grows as a yeast in humans and as a mold in the environment. The yeasts of *T. marneffei* infect the mononuclear phagocyte system, and multiply within histiocytes that enlarge to accommodate increasing numbers of organisms (Fig. 24). The inflammatory response may be granulomatous, suppurative, or mixed. As the lesions expand, necrosis predominates, and the macrophages may lyse and release free organisms (Cooper and McGinnis, 1997).

The organisms themselves are spherical to ovoid, and fill and distend the involved macrophages (Fig. 25). The fungi are typically small (2–5 μm) and are about the same size as *H. capsulatum*; however, occasional elongated and/or curved forms may be present, and these may measure up to 20 μm . Of note, *T. marneffei* does not bud, as they divide at their central septum by fission. Elongated forms with the central septum are sometimes referred to as “pill capsule” forms.

Differential diagnosis

The major organism in the morphological differential diagnosis is *H. capsulatum*, as both are typically intracellular and are of similar size (Cappell et al., 1988; Yang et al., 2013). As noted above, *H. capsulatum* yeasts divide by narrow-based budding at one pole, whereas *T. marneffei* do not bud. On H&E staining, *H. capsulatum* also often has a surrounding small “halo.” In addition, the areas in which these two yeasts are endemic are very distinct. *Cryptococcus* spp. (measuring 2–20 μm) are larger than the yeasts of *T. marneffei*, show much more variation in size, and also shows frequent narrow-based budding (Fig. 18). *Pneumocystis jirovecii* are also larger than *T. marneffei* (4–6 μm) and are round, but have cup or crescent shapes when collapsed. They lack the transverse septum of *T. marneffei*, and do not pack and distend macrophages. Many contain characteristic single or paired comma-shaped internal structures. *Candida glabrata* features tiny budding yeast forms of similar size to *T. marneffei*, but is extracellular and has frequent narrow-based budding (Fig. 14). *C. glabrata* also lacks the transverse septum of *T. marneffei*. The galactomannan assay for detection of *Aspergillus* infection will cross-react with *T. marneffei* (Dieterich et al., 1992), which may have utility as the organisms appear quite different in tissue.

Pneumocystis jirovecii (formerly *carinii*)

P. jirovecii has a life cycle that more closely resembles a protozoan, but over time there has been convincing molecular evidence indicating that it has greater homology with fungi. *P. carinii* pneumonia is a major cause of morbidity in the AIDS population, and extrapulmonary (including gastrointestinal) involvement is not uncommon. In addition to patients with AIDS, *P. jirovecii* infection has been occasionally reported in the context of organ transplant, hematologic malignancy, other immunodeficiency states, and steroid therapy. *P. jirovecii* infection has also associated with infliximab therapy, an immunosuppressive treatment for Crohn’s disease and rheumatoid arthritis (Dieterich et al., 1992; Kaur and Mahl, 2007). Risk factors for extrapulmonary infection include severe underlying immunodeficiency, overwhelming pulmonary infection, and aerosolized pentamidine prophylaxis for *Pneumocystis jirovecii* pneumonia (a.k.a. PJP).

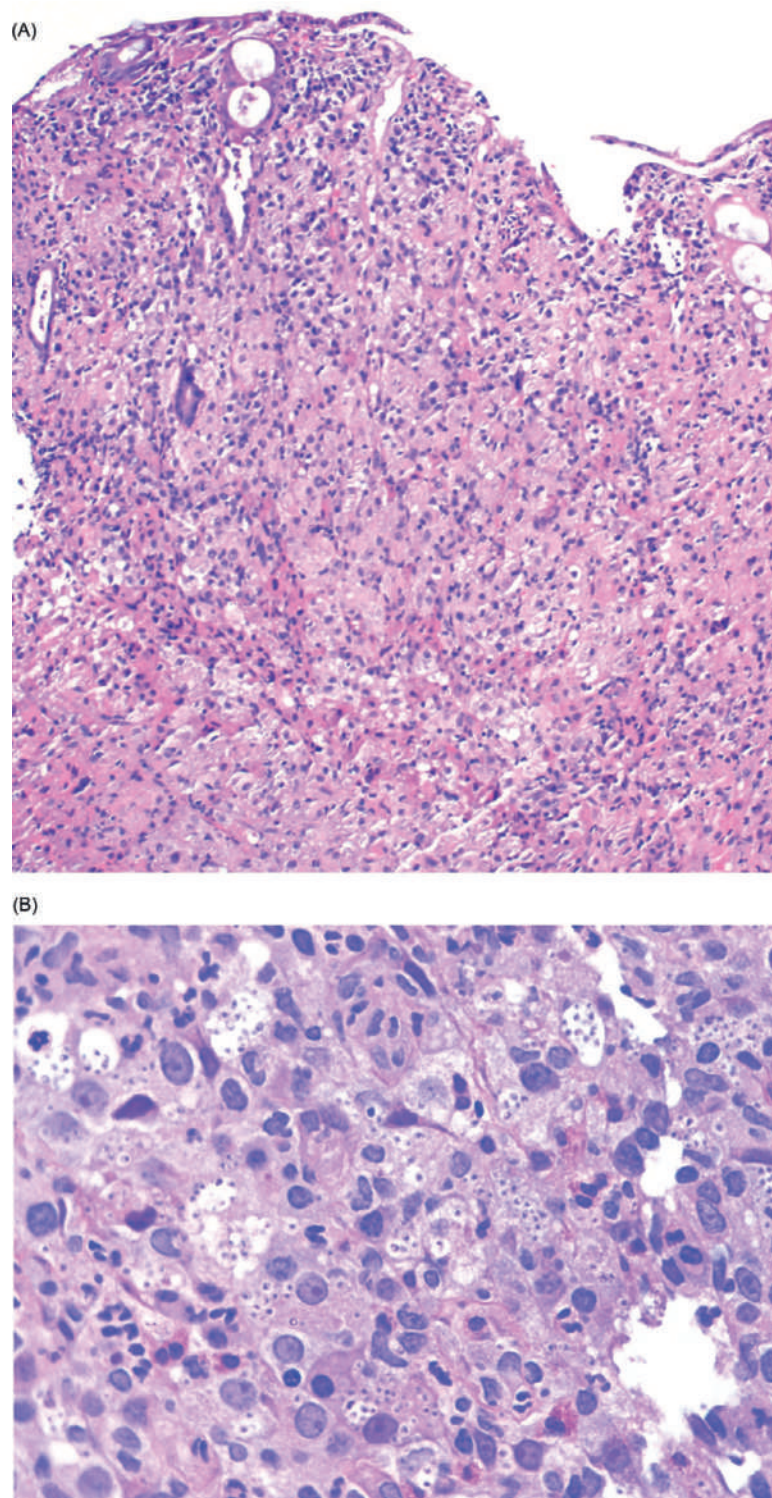


Fig. 23 This colon biopsy contains a histiocytic infiltrate with numerous intracellular yeasts of *Histoplasma capsulatum* (A). The yeasts appear as small intracellular objects with a surrounding halo, a result of the poorly-stained cell wall (B). (A) Courtesy Dr. Maria Westerhoff. (B) Courtesy Dr. Martha Yearsley.

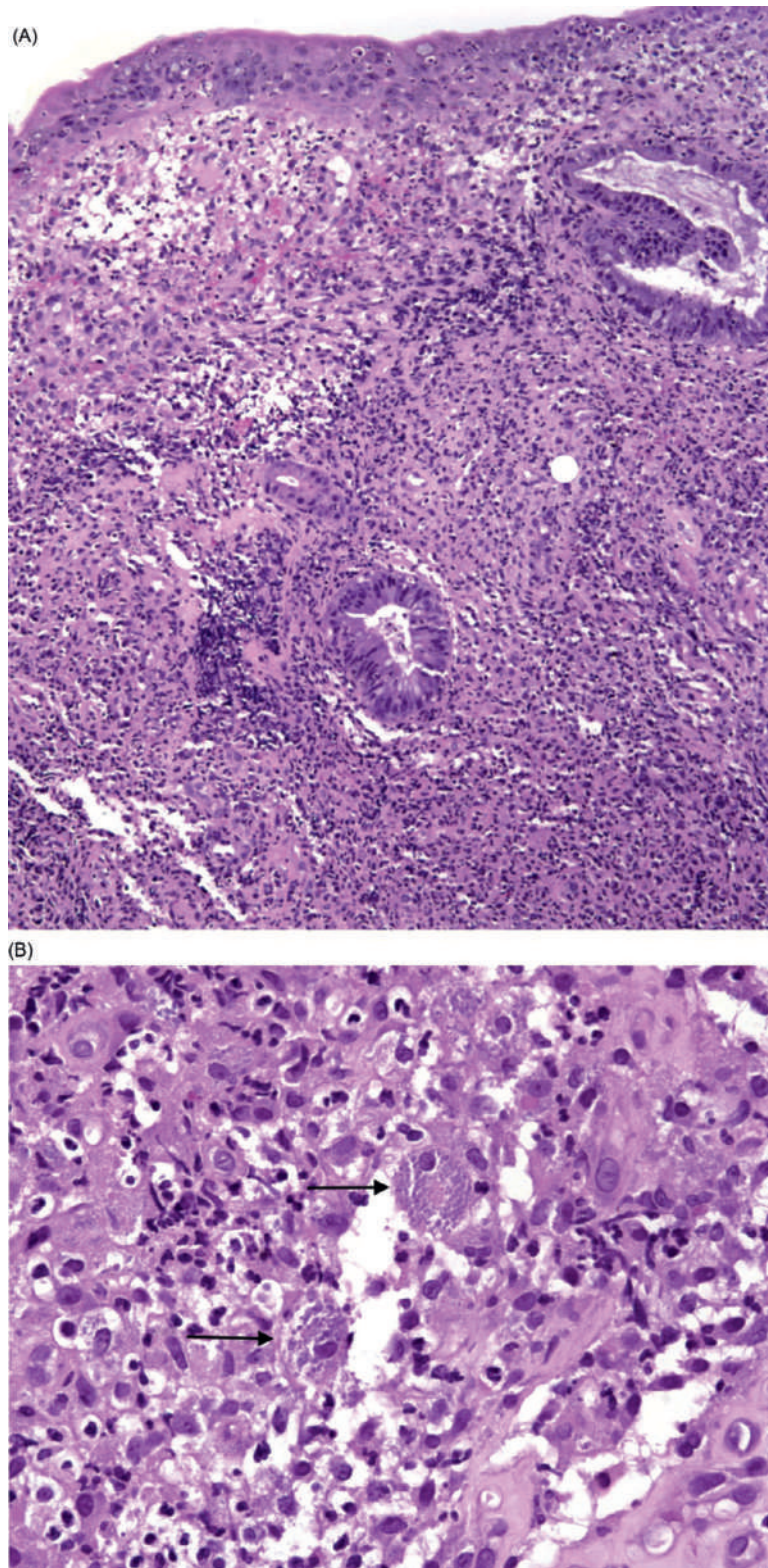


Fig. 24 This colon biopsy from a patient who had lived in Vietnam shows extensive mucosal destruction with acute, chronic, and loosely granulomatous inflammation (A). At high power, macrophages packed with small organisms can be seen (B, arrows).

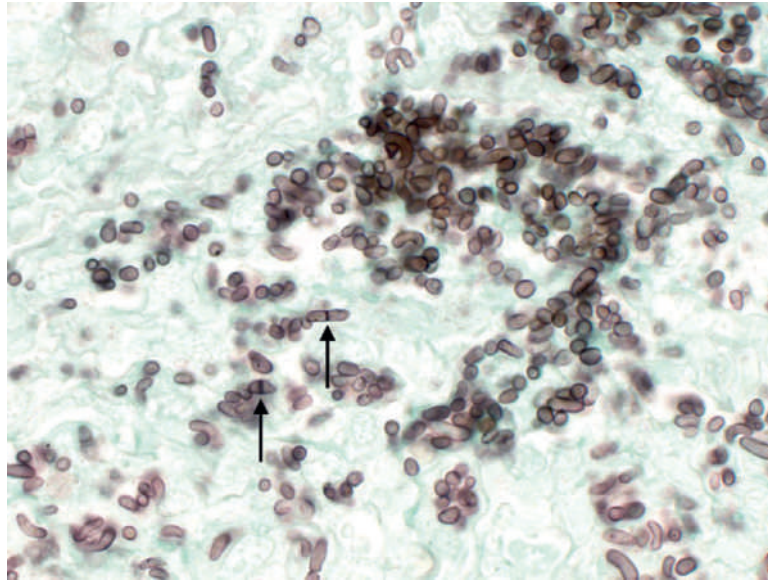


Fig. 25 *T. marneffei* are small, ovoid, and do not bud (A, GMS stain). Instead, they divide by forming transverse septations (arrows, GMS).

Clinical features

Patients with gastrointestinal involvement typically present with fever and diarrhea. Most, but not all, cases of gastrointestinal pneumocystis also have pulmonary involvement (Dieterich et al., 1992; Bellomo et al., 1992).

Gross findings

Macroscopically, *P. jirovecii* infection produces a nonspecific, often erosive, esophagogastritis or enterocolitis, occasionally with small polypoid nodules. Any portion of the gastrointestinal tract may be involved, including mesenteric lymph nodes (Dieterich et al., 1992).

Microscopic findings

As in the lung, a wide variety of inflammatory responses may occur in the gastrointestinal tract, including prominent macrophage infiltrates, necrosis, and occasionally granulomas (Cooper and McGinnis, 1997). The classic granular, foamy eosinophilic casts seen in pulmonary *Pneumocystis* infection can also be found in the GI tract, either in mucosal vessels or in the lamina propria and submucosa (Fig. 26). Because granulomatous reactions are rare with *Pneumocystis*, concurrent pathogens should be excluded when granulomas are seen. The organisms are 4–6 μm spherules, typically extracellular, that have cup or crescent shapes when collapsed (Fig. 27). Many contain characteristic paired comma-shaped internal structures.

Differential diagnosis

P. jirovecii can usually be correctly classified in tissue sections based on features and the characteristic foamy casts. As noted above, the major organisms in the differential diagnosis are *H. capsulatum*, *Cryptococcus* spp., and *T. marneffei*. *P. jirovecii* does not bud, unlike *H. capsulatum* and *Cryptococcus* spp., and is generally larger and lacks the transverse septum of *T. marneffei*. The beta (1.3) D glucan assay is also helpful in this situation, as it is positive in *P. jirovecii* infection but the morphologic features are significantly different from those of the filamentous fungi.

Microsporidiosis

Microsporidiosis is an infection caused by multiple species of a highly specialized fungus that for years was believed to be a parasite (coccidian) (Han and Weiss, 2017). Microsporidia have a worldwide distribution, and are present in many environmental sources. They have many animal hosts as well. Many are capable of infecting multiple organ systems, but *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, and *Encephalitozoon hellem* are most commonly seen in the GI tract. This infection is particularly important when considering the differential diagnosis of diarrhea in patients with AIDS (Curry and Smith, 1998; Franzen et al., 1999;

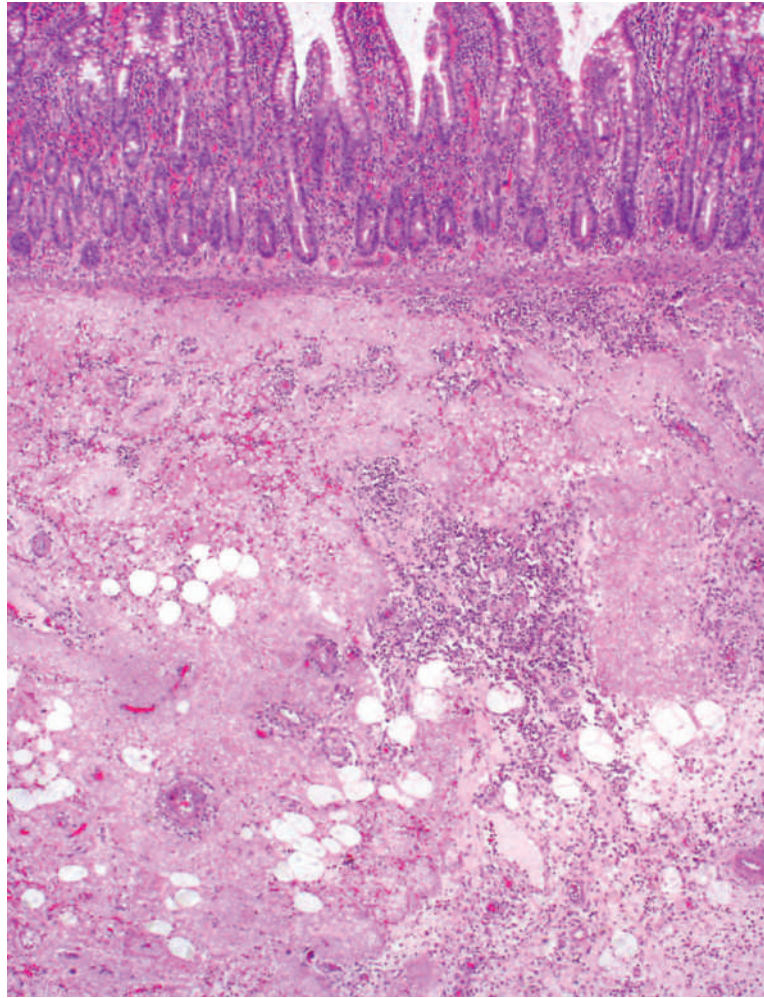


Fig. 26 This case of *Pneumocystis jirovecii* enteritis shows the characteristic foamy casts filling the submucosa.

Goodgame, 1996). Transmission is normally via the fecal-oral route, either directly or through contaminated food and water. These fungi are also a cause of self-limited traveler's diarrhea. Albendazole is the mainstay of treatment, although *E. bienensei* and some other species are resistant to this drug, posing a significant challenge for profoundly immunocompromised individuals with persistent disease. In some cases, immune restoration is the only treatment option.

Clinical features

Chronic nonbloody, nonmucoid diarrhea is the most common presentation, variably accompanied by fever, weight loss, abdominal pain, and malaise. Stool does not usually contain red blood cells or leukocytes (Curry and Smith, 1998; Franzen et al., 1999; Goodgame, 1996; Field and Milner Jr., 2015; Orenstein et al., 1994). In immunocompetent persons, infection is usually self-limited, but immunocompromised patients are at risk for chronic, severe diarrhea, with malabsorption, dehydration, and eventual death. Disseminated infection may occur, especially with *E. intestinalis* infection. Endoscopic findings are usually absent or mild.

Gross findings

Any level of the gastrointestinal tract may be affected, including the biliary tree, but the small bowel is the most common site of infection.

Microscopic findings

Microsporidia are notoriously difficult to detect in routine H&E stained sections due to their incredibly small size (Field and Milner, 2015). Characteristic findings include mild villous blunting, a patchy lamina propria lymphoplasmacytic infiltrate, variably

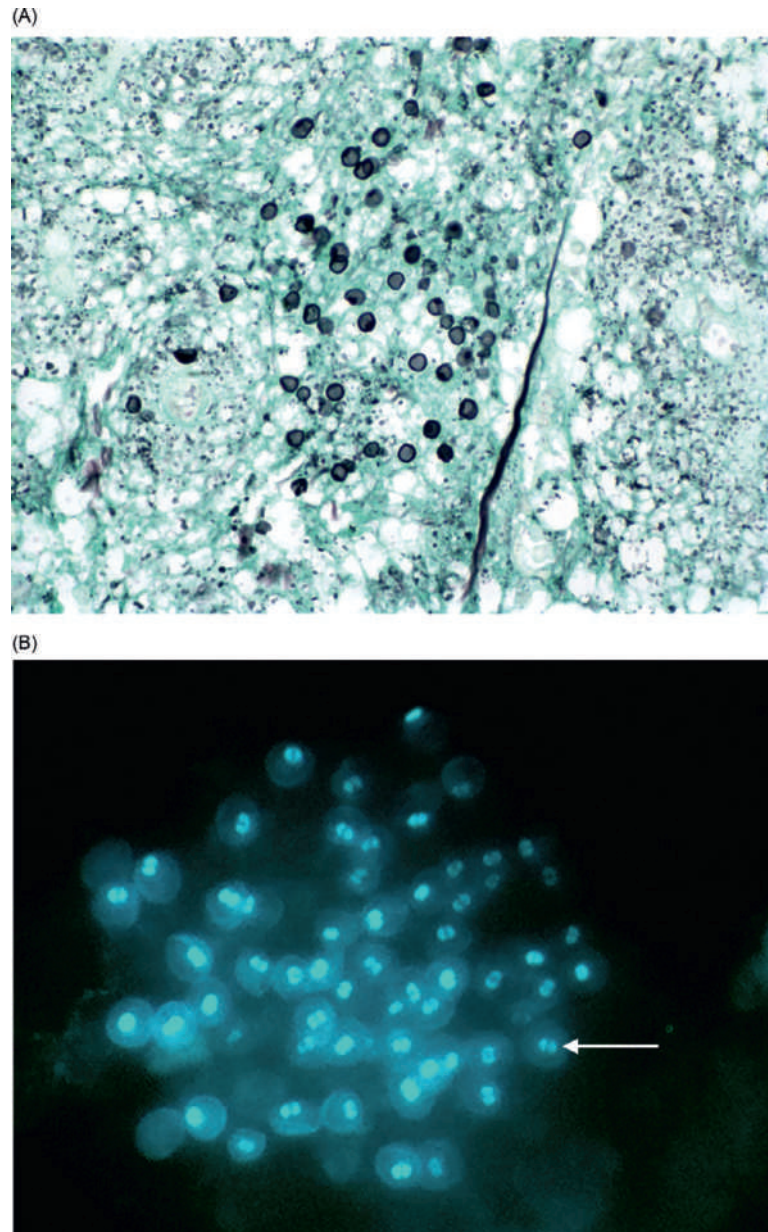


Fig. 27 *Pneumocystis jirovecii* organisms are extracellular and are seen on GMS as spheres with a cup or crescent shapes when collapsed. Occasional dot or comma-like internal structures can be seen (A, GMS). Fig. (B) shows a calcofluor white fungal smear, which clearly shows the internal comma-like structures (arrows).

increased surface intraepithelial lymphocytes, vacuolization of the surface epithelium, and surface epithelial cell disarray (Fig. 28). There may be minimal to no tissue reaction, however.

The organisms are present as small spores within host cells. While the size of the microsporidia that infect humans ranges from 0.7 to 4 μm , the spores of *E. bienersi* and the *Encephalitozoon* species are only 0.7–1.4 μm in greatest dimension. In intestinal microsporidiosis, the spores are located within the supranuclear cytoplasm of epithelial cells or occasionally lamina propria macrophages (most common with *E. intestinalis*). Associated vacuoles may cup or flatten the enterocyte nucleus.

A modified trichrome stain can aid greatly in the diagnosis (Fig. 29), and the organisms also stain with Warthin-Starry, Giemsa, Gram, Ziehl-Neelsen, and GMS stains, although staining with the latter two is often focal (Field and Milner, 2015; Lamps et al., 1998). PAS stain may show a positive granule at one end of each spore. Occasionally, microsporidial spores polarize within biopsy specimens, since the chitin-rich internal polar filament of the organism is birefringent under polarized light. However, this method is unreliable, due to unpredictability of spore birefringence, and variability depending on the type of microscope and light source used (Lamps et al., 1998).

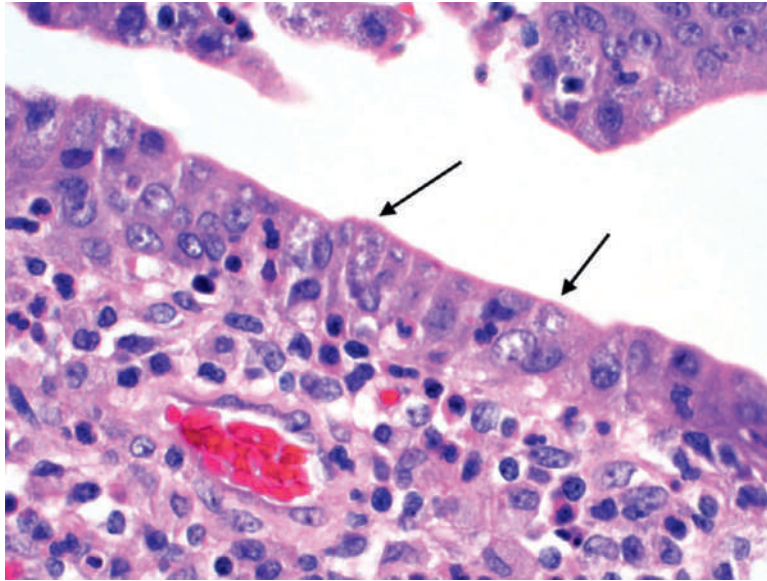


Fig. 28 This small bowel biopsy with microsporidia yeasts shows a subtle supranuclear vacuolization (arrows) with surface epithelial cell disarray and scattered intraepithelial lymphocytes.

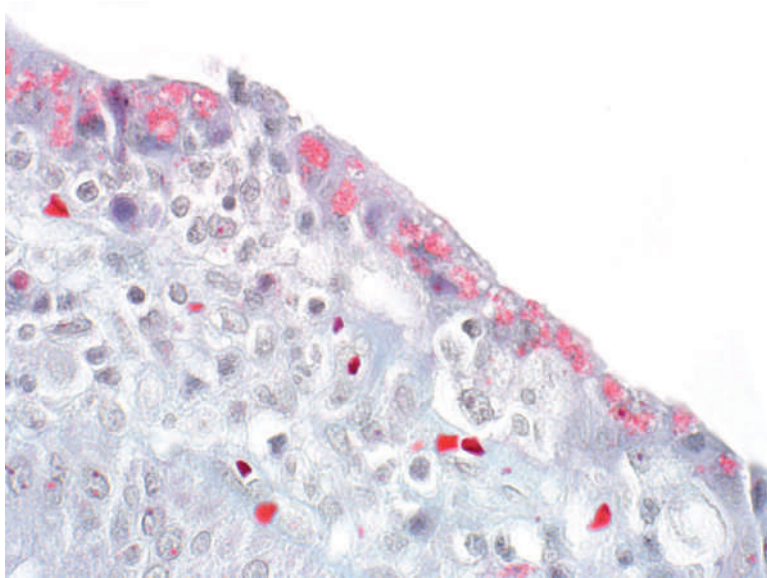


Fig. 29 A modified trichrome stain highlights the spores in microsporidial spores infection.

Differential diagnosis

The differential diagnosis includes other small yeasts, as well as *Leishmania* spp., intracellular bacteria, and phagocytosed material. Although often difficult to appreciate on H&E stain, the microsporidia are the only fungi in the differential diagnosis that are typically present within surface epithelial cells. *Leishmania* spp. are typically present within macrophages in the GI tract, and are Giemsa positive, GMS negative, and have a kinetoplast. *Histoplasma capsulatum* yeasts are also present within macrophages, produce buds, and are uniformly GMS positive; they have a "halo" around the organism on H&E stain. Similarly, *C. glabrata* has buds and is uniformly GMS positive, and is typically not present within surface epithelial cells. *T. marneffe* has a transverse septum, and is usually also present within macrophages. Transmission electron microscopy is still considered the gold standard for confirming diagnosis, although it is being replaced with sequence analysis. The availability of both is severely limited. There are commercially available immunofluorescence assays available for some species, and PCR is available at some laboratories. Stool O&P studies may be helpful if special stains (chromotrope 2R modified trichrome) are used, but diagnosis may be challenging due to the resemblance to small yeasts or bacteria.

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Parasites of the Gastrointestinal Tract

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Glossary

Commensal A parasite that benefits from its host but does not damage its host.

Cyst The environmentally hardy stage in the protozoan life cycle responsible for transmission.

Definitive host A host in which sexual maturity and reproduction of the parasite takes place.

Dead-end host A host in which a parasite cannot develop to adulthood; the term is usually applied a host that is not part of the natural life cycle.

Embryonation The process of in the development of a helminth egg that results in the formation of a larva that is infectious to the next host. Eggs may be shed in the feces of the definitive host unembryonated (no differentiation of the developing larva), partially embryonated, or fully embryonated (containing an infectious larva).

Intermediate host A host in which larval development or asexual reproduction takes place.

Oocyst The environmentally hardy state in the life cycle of apicomplexan parasites; in *Cryptosporidium* and the coccidians, it is the stage responsible for transmission.

Operculum A small lid-like opening on the eggs of trematodes (exclusive of *Schistosoma* spp.) and diphyllbothriid cestodes.

Paratenic host A host in which harbors the larva or immature stage of a parasite, but further development of the parasite does not take place.

Trophozoite The stage of a protozoan responsible for feeding and replication by binary fission.

Vector An arthropod or other animal that mechanically transfers a pathogen to a host, usually while feeding.

Zoonotic Refers to a parasite that does not routinely infect humans as part of its natural life cycle.

Introduction

Gastrointestinal (GI) parasites encompass a broad array of parasites, including protozoa, helminths, and rarely, arthropods. Each of these groups of parasites can be divided into further categories: the protozoa by their type of motility (e.g., flagellates, ciliates, amebae), and the helminths by their overall morphology (roundworms, flatworms, and the acanthocephalans). Some of these parasites are commensal organisms (i.e., parasite that do not damage their host), whereas others are potential pathogens. This chapter will cover important parasites that involve all regions of the GI tract, from mouth to anus, including the liver and biliary tree (See Tables 1–5).

Parasites were the earliest type of GI pathogens reported in human writings, owing to the ability of some to be seen with the unaided eye and their association with clearly-defined clinical syndromes (Cox, 2002). The Egyptian Ebers papyrus (circa 3000 to 400 BCE) described parasitic infections which are now clearly recognized as ascariasis, echinococcosis, enterobiasis, and schistosomiasis. Descriptions of these infections later appeared in writings by physicians in Greece, China, India, Rome and the Arab Empire. Today, parasites remain an important cause of human morbidity and mortality and have a significant economic impact in many parts of the developing world. Several GI parasitic diseases are categorized by the World Health Organization (WHO) as Neglected Parasitic Diseases (NTDs) due to the devastating effect they have on individuals living in poverty (WHO, 2020a). These diseases are estimated to affect more than one billion people worldwide, and result in substantial losses in productivity, income, and human lives. Among the 20 NTDs, seven categories involve the GI tract: foodborne trematodiasis, soil-transmitted helminthiasis, schistosomiasis, Chagas disease, echinococcosis, leishmaniasis, and taeniasis/cysticercosis. Chagas disease is also recognized by the United States Centers for Disease Control and Prevention (CDC) as a Neglected Parasitic Infection (NPI), targeted as a priority for public health action (CDC, 2020b). This group of five parasitic infections has been selected based on the number of individuals impacted in the United States, the severity of the associated illnesses, and the ability to prevent and treat infections. Cysticercosis is another relevant NPI that will be considered in this chapter as it is linked to the GI infection, taeniasis, caused by infection with the cestode *Taenia solium*. In addition to the NTDs and NPIs, there are a large number of parasitic infections that are recognized by the WHO, CDC, and other public health organizations as important causes of diarrheal illnesses. In 2010, the WHO estimated that infections with the enteric protozoan parasites, *Entamoeba histolytica*, *Giardia duodenalis*, and *Cryptosporidium*, were responsible for more than 3.5 million illnesses, and 60,000 deaths worldwide (Kirk et al., 2015).

Infections with potentially-pathogenic GI parasites are often asymptomatic, particularly at low levels of infection. However, infections may be associated with a range of symptoms due to the mechanical or chemical damage caused by the parasite and the associated host immune response. While some parasites cause only localized disease, others may cause extraintestinal

Table 1 Parasites of the oral cavity.

Disease [agent(s)]	Clinical Manifestations	Diagnosis [secondary, alternative methods]
Protozoan Infections		
<i>Entamoeba gingivalis</i>	None; often associated with periodontitis	Microscopy
<i>Trichomonas tenax</i>	None; often associated with periodontitis, rarely pleural empyema	Microscopy
Mucocutaneous Leishmaniasis [<i>Leishmania braziliensis</i> , <i>L. panamensis</i> , <i>L. amazonensis</i> , <i>L. guyanensis</i>]	Initially epistaxis, rhinorrhea, nasal stuffiness, lymphadenopathy; progresses to chronic expanding ulceration of the lips, nostril or palate. Previous scar from healed cutaneous disease commonly present.	Microscopy, culture, nucleic acid amplification tests
Chagas Disease Sialorrhea [<i>Trypanosoma cruzi</i>]	Drooling	Antibody detection; microscopy is rarely useful (amastigotes not usually seen)
Nematode Infections		
Gongylosomiasis [<i>Gongylonema pulchrum</i>]	Oral sensations, nausea, vomiting	Microscopy (gross examination of adult worms; histopathology)
Anatrichosomiasis [<i>Anatrichosoma</i> spp.]	Painful blisters on the oral mucosa, lips, tongue, hard and soft palate	Microscopy (histopathology)
Trichinellosis (trichinosis) [<i>Trichinella</i> spp.] ^a	Diarrhea, abdominal pain, vomiting, eosinophilia, myalgia, muscle weakness, facial edema, hoarse voice	Antibody detection [histopathology]
Arthropod Infections		
Oral Myiasis [<i>Cochliomyia hominivorax</i> , <i>Wohlfahrtia magnifica</i> , <i>Musca domestica</i> , <i>Calliphora vicina</i> , <i>Chrysomya bezziana</i> , <i>Oestrus ovis</i> , <i>Hypoderma bovis</i> , <i>H. tarandi</i> , <i>Gasterophilus intestinalis</i>]	Pain, swelling, live maggots observed in oral mucosa	Gross morphology of extracted fly larvae

^aTrichinellosis will be covered in this chapter under Parasites of the Small Intestine.

Table 2 Parasites of the esophagus and stomach.

Disease [agent(s)]	Clinical Manifestations	Diagnosis [secondary, alternative methods]
Protozoan Diseases Chagasic Achalasia/Chagasic Megaesophagus [<i>Trypanosoma cruzi</i>]	Dysphasia, regurgitation, weight loss, malnutrition	Antibody detection; microscopy is rarely useful (amastigotes not usually seen)
Nematode Infections Anisakiasis [<i>Anisakis</i> spp., <i>Pseudoterranova</i> spp., <i>Contracaecum</i> spp.]	Acute onset of severe epigastric pain; live larva may be retrieved from the mouth, esophagus or stomach; allergic symptoms	Microscopy (gross morphologic identification of intact worms expelled live or extracted by endoscopy; histopathology) [Antibody detection]

manifestations during the usual course of their life cycle or as a result of ectopic spread within their host. Examples of extraintestinal manifestation include a pruritic cutaneous lesion at the site of initial host skin penetration (hookworm and *Strongyloides stercoralis*), bacterial sepsis (*S. stercoralis*, *Trichuris trichiura*), and abscess-like lesions in various organs (*Entamoeba histolytica*).

Parasite taxonomy

Parasite taxonomy has evolved substantially over the past few decades. Parasites were traditionally placed into two Kingdoms: the Protozoa (single-celled eukaryotic organisms) and Animalia (helminths and arthropods). Parasites were further categorized into phylum, class, order and family groupings based on physiologic, ecologic and subjective morphologic criteria. While this hierarchical system is still used in some settings, parasitologists have increasingly recognized the inability of the traditional system to reflect true phylogenetic parasite relatedness. In contrast, modern classification schemes incorporate molecular analyses such as whole genome sequencing to place parasites into clades based upon genetic relatedness (Adl and Mathison, 2019; Adl et al., 2019). In the widely-recognized system by the International Society of Protistologists, human parasites are placed within five *supergroups*: the Amoebozoa, Archaeplastida, Excavata, SAR, and Opisthokonta. Many familiar groupings have been retained in this schema to facilitate correlation with the traditional taxonomic system. At this time, the modern classification system is used primarily for the protozoan parasites. It remains common for helminth and arthropod parasites to be described using the traditional taxonomy scheme.

Diagnosis of gastrointestinal parasites

A variety of methods are used in the diagnosis of gastrointestinal parasitic infections. Traditional morphologic examination of gross and microscopic preparations remains the gold standard method for detection and identification many parasites, including the intestinal helminths and commensal protozoa. Light microscopic examination of unstained and stained preparations can also be used for detection of common intestinal protozoa, although stool immunoassays and/or nucleic acid amplification tests (NAATs) generally offer greater sensitivity and specificity, and there are commercial options available for common pathogenic parasites (Garcia et al., 2018). Antibody detection has the greatest utility for detecting evidence of parasites that are not readily seen within easily-acquired specimens such as stool and sputum. Situations in which serology is particularly useful are for supporting the clinicoradiologic diagnosis of amebic liver abscess and hepatic echinococcosis, and diagnosing schistosomiasis in the absence of identifiable eggs in the stool. Finally, culture methods are used for identification and study of select parasites in research settings. These methods are not commonly used for routine diagnosis in the clinical laboratory, and are generally limited to the specialty reference/public health laboratory or research setting. In the United States, several parasite culture methods are performed at the CDC's Division of Parasitic Diseases and Malaria. For specific procedures, recipes, and guidance on Quality Assurance, the reader is directed to the CDC's DPDx website (<https://www.cdc.gov/dpdx/diagnosticprocedures/index.html>) as well as several excellent references (Ash and Orihel, 1987, 2007; Carroll et al., 2019; Couturier et al., 2015; Garcia, 2016; Garcia et al., 2018). All specimens must be handled with great care since they may pose an infection risk to humans.

Diagnostic methods for specific parasites will be discussed in detail below under the specific diseases they cause.

Parasites of the oral cavity

A small number of parasites can involve the oral cavity (Table 1). While some like *Trichomonas tenax* are considered commensal organisms, not thought to cause disease, others such as *Leishmania* can cause destructive and potentially life-threatening damage.

Table 3 Parasites of the small intestine.

Disease [agent (s)]	Clinical Manifestations	Diagnosis [secondary, alternative methods]
Protozoan Diseases		
Giardiasis [<i>Giardia duodenalis</i>]	Commonly asymptomatic; explosive foul-smelling watery/fatty/mucous diarrhea, belching, flatulence	Microscopy (O&P examination), antigen detection, NAATs
Cryptosporidiosis [<i>Cryptosporidium</i> spp.]	May be asymptomatic; profuse watery, prolonged diarrhea, nausea/vomiting, abdominal pain, low-grade fever. Worse in immunocompromised individuals.	Microscopy (modified acid-fast, safranin stains), antigen detection, NAATs
Cyclosporiasis [<i>Cyclospora cayetanensis</i>]	May be asymptomatic; profuse watery, prolonged diarrhea, nausea/vomiting, abdominal pain, low-grade fever. Worse in immunocompromised individuals.	Microscopy (modified acid-fast, safranin stains; UV autofluorescence), NAATs
Cystoisosporiasis [<i>Cystoisospora belli</i>]	May be asymptomatic; profuse watery, prolonged diarrhea, nausea/vomiting, abdominal pain, low-grade fever. Worse in immunocompromised individuals. Occasionally peripheral eosinophilia.	Microscopy (O&P examination, modified acid-fast, safranin, UV autofluorescence, histopathology)
Intestinal Sarcocystosis [<i>Sarcocystis hominis</i> , <i>S. suis</i>]	May be asymptomatic; profuse watery, prolonged diarrhea, nausea/vomiting, abdominal pain, low-grade fever. Worse in immunocompromised individuals. Occasionally peripheral eosinophilia.	Microscopy (O&P examination, modified acid-fast, UV autofluorescence)
Chagas Disease [<i>Trypanosoma cruzi</i>]	Chronic diarrhea	Antibody detection; microscopy is rarely useful (amastigotes not usually seen)
Nematode Infections		
Ascariasis [<i>Ascaris lumbricoides</i>]	May be asymptomatic; manifestations are often proportional to the worm burden: Loeffler's syndrome, abdominal discomfort, anorexia, diarrhea, small bowel obstruction, ectopic migration with appendicitis, pancreatitis	Microscopy (wet mounts of stool, gross morphology of adults, histopathology)
Intestinal Capillariasis [<i>Capillaria philippinensis</i>]	Abdominal pain, chronic watery diarrhea, vomiting, anorexia, weight loss, malabsorption; may be fatal overtime due to ongoing autoinfection.	Microscopy (wet mounts of stool, histopathology)
Intestinal Hookworm Infection [<i>Necator americanus</i> , <i>Ancylostoma duodenale</i> , <i>A. ceylanicum</i>]	May be asymptomatic; manifestations are often proportional to the worm burden: rash at initial skin penetration, Loeffler's syndrome, abdominal pain, diarrhea, anemia (may be severe), hypoalbuminemia, malnutrition	Microscopy (wet mounts of stool, gross morphology of adults)
Trichostrongyliasis [<i>Trichostrongylus</i> spp.]	Usually asymptomatic; nausea, anorexia, diarrhea, pulmonary symptoms, eosinophilia	Microscopy (wet mounts of stool, Kato-Katz)
Strongyloidiasis [<i>Strongyloides stercoralis</i> , <i>S. fuelborni</i>]	Commonly asymptomatic; rash at initial skin penetration, Loeffler's syndrome, hyperinfection in immunocompromised patients may cause bloody diarrhea, recurrent bacterial sepsis and meningitis, pulmonary hemorrhage, respiratory collapse, death	Microscopy (wet mounts of stool, Kato-Katz, Baermann technique, string test, histopathology), Culture, Antibody Detection
Trichinellosis (trichinosis) [<i>Trichinella</i> spp.]	Intestinal and muscular/systemic phases; intestinal phase with self-limited diarrhea and abdominal pain. Muscular/systemic phase with peripheral eosinophilia, facial edema, vasculitis, intravascular thrombosis, conjunctivitis, muscle pain, elevated muscle enzymes.	Antibody Detection [Microscopy (pepsin-digestion, histopathology), NAATs]
Human Abdominal Angiostrongyliasis [<i>Angiostrongylus costaricensis</i>]	Eosinophilic enteritis, fever, anorexia, vomiting, and RUQ pain	Morphology (histopathology)
Anisakiasis [<i>Anisakis</i> spp., <i>Pseudoterranova</i> spp., <i>Contracaecum</i> spp.]	Abdominal pain	Microscopy (gross morphologic identification of intact worms expelled live or extracted by endoscopy; histopathology) [immunodiagnosis]
Trematode Infections		
Fasciolopsiasis [<i>Fasciolopsis buski</i>]	May be asymptomatic; manifestations are often proportional to the worm burden: epigastric pain, nausea, diarrhea, malabsorption, protein-losing enteropathy, peripheral eosinophilia, anemia.	Microscopy (wet mounts of stool, gross morphology of adult)
Heterophyiasis, Metagonimiasis [<i>Heterophyes heterophyes</i> , <i>Metagonimus yokogawai</i> , many others]	May be asymptomatic; manifestations are often proportional to the worm burden: abdominal pain, diarrhea, peripheral eosinophilia	Microscopy (wet mounts of stool)
Nanophyetiasis [<i>Nanophyetis salmincola</i>]	Abdominal pain, nausea, vomiting, weight-loss, increased number of bowel movements, fatigue, eosinophilia	Microscopy (wet mounts of stool)
Echinostomiasis [<i>Echinostoma</i> spp., several others]	epigastric and abdominal pain, malaise, weight loss, anorexia, nausea, vomiting, and diarrhea	Microscopy (wet mounts of stool)

<i>Neodiplostomum</i> infection [<i>Neodiplostomum seoulense</i>]	Abdominal pain, fever	Microscopy (wet mounts of stool)
Intestinal Schistosomiasis ^a [<i>Schistosoma japonicum</i> , <i>S. mekongi</i>]	Abdominal pain, anorexia, bloody diarrhea	Microscopy (wet mounts of stool; histopathology); Antibody Detection
Cestode Infections		
Diphyllobothriasis (fish tapeworm disease) [<i>Dibothriocephalus</i> spp., <i>Diphyllobothrium</i> spp., <i>Adenocephalus</i> spp.]	Commonly asymptomatic; abdominal pain, diarrhea, constipation, fatigue, vitamin B ₁₂ deficiency, passing chains of proglottids.	Microscopy (wet mounts of stool, gross morphology of adults)
Intestinal Taeniasis [<i>Taenia saginata</i> , <i>T. solium</i> , <i>T. sui hominis</i>]	Commonly asymptomatic; abdominal pain, diarrhea, constipation, fatigue, passing individual or chains of proglottids.	Microscopy (wet mounts of stool, gross morphology of adults) [coproantigen testing]
Hymenolepiasis [<i>Hymenolepis nana</i> , <i>H. diminuta</i>]	Commonly asymptomatic; poor growth and development in children	Microscopy (wet mounts of stool, gross morphology of adults)
Dipylidiasis (dog tapeworm disease) [<i>Dipylidium caninum</i>]	Commonly asymptomatic; diarrhea, restlessness, abdominal discomfort, anal pain and itching	Microscopy (gross morphology of adults, wet mounts of stool)
Miscellaneous Cestode Infections [<i>Raillietina</i> spp., <i>Inermicapsifer</i> <i>madagascariensis</i> , <i>Bertiella stuederi</i> , <i>B. mucronotata</i> , <i>Mesocestoides</i> spp.]	Commonly symptomatic; generalized gastrointestinal symptoms	Microscopy (gross morphology of adults, eggs liberated from proglottids)
Acanthocephalan Infections		
Acanthocephaliasis [<i>Macracanthorhynchus hirudinaceus</i> , <i>M. ingens</i> , <i>Moniliformis moniliformis</i>]	May be asymptomatic, abdominal pain, rarely intestinal perforation.	Microscopy (gross morphology of adults and eggs liberated therefrom)

Abbreviations: NAAT – nucleic acid amplification test.

^aIn this chapter, intestinal schistosomiasis is covered under Parasites of the Appendix, Large Intestine, and Anus.

Table 4 Parasites of the appendix, large intestine, and anus.

Disease [agent(s)]	Clinical Manifestations	Diagnosis [secondary, alternative methods]
Protozoan Infections		
Amebiasis [<i>Entamoeba histolytica</i>]	Commonly asymptomatic; watery to bloody diarrhea, dysentery, fulminant necrotizing colitis, ameboma, disseminated disease (e.g., liver involvement), death	Microscopy (O&P examinations of stool, histopathology), antigen detection, antibody detection, NAATs
<i>Dientamoeba fragilis</i> Infection [<i>Dientamoeba fragilis</i>]	Pathogenesis is controversial; associated with abdominal pain, diarrhea, constipation, peripheral eosinophilia	Microscopy (O&P examinations of stool), NAATs
Balantidiasis [<i>Balantidium coli</i>]	May be asymptomatic; abdominal pain, watery to bloody diarrhea, dysentery, fulminant necrotizing colitis, disseminated disease, death	Microscopy (O&P examinations of stool, histopathology)
Blastocystosis [<i>Blastocystis</i> spp.]	Pathogenesis is controversial; associated with abdominal pain, diarrhea, increased flatulence, anorexia	Microscopy (O&P examinations of stool), NAATs
Miscellaneous nonpathogenic protozoa [<i>Entamoeba hartmanni</i> , <i>E. coli</i> , <i>E. polecki</i> , <i>E. chattoni</i> , <i>Endolimax nana</i> , <i>Iodamoeba buetschlii</i> , <i>Chilomastix mesnili</i> , <i>Pentatrichomonas hominis</i> , <i>Enteromonas hominis</i> , <i>Retortamonas intestinalis</i>]	None; organisms are nonpathogenic	Microscopy (O&P examinations of stool)
Chagasic Megacolon [<i>Trypanosoma cruzi</i>]	Chronic constipation	Antibody detection; microscopy is rarely useful (amastigotes not usually seen)
Nematode Infections		
Enterobiasis (Pinworm Infection) [<i>Enterobius vermicularis</i>]	May be asymptomatic; nocturnal pruritus ani, insomnia, ectopic disease (e.g. appendicitis, vulvovaginitis)	Microscopy (Observation of eggs on cellulose tape preps; gross morphology of adult worms; histopathology) [wet mounts of stool]
Trichuriasis (Whipworm Infection) [<i>Trichuris trichiura</i>]	May be asymptomatic; manifestations are often proportional to the worm burden: abdominal pain, diarrhea, anemia (may be severe), malnutrition, rectal prolapse	Microscopy (wet mounts of stool; gross morphology of adult worms; histopathology)
Oesophagostomiasis [<i>Oesophagostomum</i> spp.]	Acute abdomen, low-grade fever, anorexia, vomiting, diarrhea, purulent peritonitis	Microscopy (wet mounts of stool)
Trematode Infections		
Intestinal Schistosomiasis [<i>Schistosoma mansoni</i> , <i>S. intercalatum</i> , <i>S. guineensis</i> , <i>S. japonicum</i> , <i>S. mekongi</i>]	May be asymptomatic in early disease; cercarial dermatitis, Katayama fever, abdominal pain, anorexia, blood diarrhea.	Microscopy (wet mounts of stool; histopathology), Antibody Detection
<i>Gastrodiscoides</i> Infection [<i>Gastrodiscoides hominis</i>]	Diarrhea with mucus	Microscopy (wet mounts of stool; gross morphology of adults)

Entamoeba gingivalis

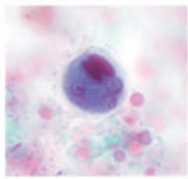
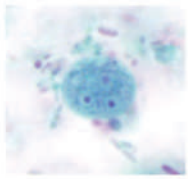
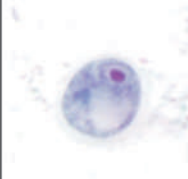
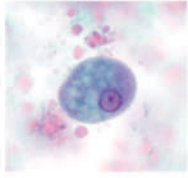
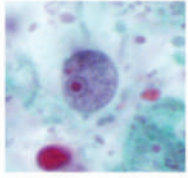
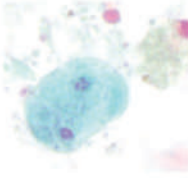
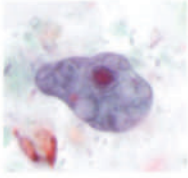
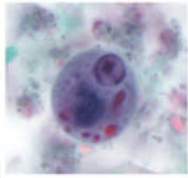
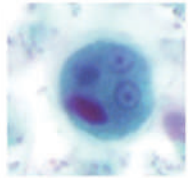
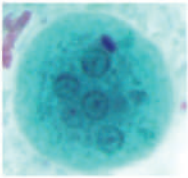
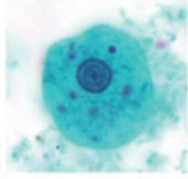
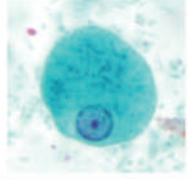
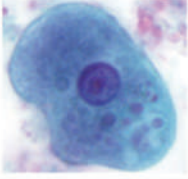
Entamoeba gingivalis is generally considered a commensal in the human oral cavity. While it has been associated with certain clinical conditions, such as periodontitis (Bonner et al., 2018) and osteomyelitis of the mandible (Bhaijee and Bell, 2011), its presence under such circumstances is not entirely understood, and it is generally considered incidental and the amebae are probably feeding on bacteria associated with these conditions (Dubar et al., 2020). HIV-positive patients may show a higher prevalence of *E. gingivalis* due to the accelerated development of chronic periodontitis in these patients (Cembranelli et al., 2013), but its role as a pathogen is still unclear. Ectopic colonization of the female urogenital tract has been associated with contaminated intrauterine devices (IUDs) (Bradbury et al., 2019).

Diagnosis of *E. gingivalis* infection is made by microscopic examination of tooth and gum scrapings stained with routine microbiologic stains (e.g. Wheatley's trichrome stain), or rarely, tissue biopsies stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and Masson's trichrome (Bhaijee and Bell, 2011). *Entamoeba gingivalis* exists only in the trophozoite stage; unlike the other human-infecting *Entamoeba* spp., there is no cyst stage (Vráblík et al., 1991). The trophozoites have the classic morphologic features of all amebic trophozoites, including a round-to-oval-to irregular shape with pseudopod projections. There is a single nucleus with the features of typical "Entamoeba-type" nuclei including a relatively-small karyosome and peripheral chromatin (Fig. 1). Subtle variations of this appearance are used to differentiate the various *Entamoeba* spp.: the trophozoites of *E. gingivalis* are 8.0-20.0 µm in greatest dimension and have a nucleus with a centrally-located karyosome and equally-distributed peripheral chromatin. The cytoplasm is finely granular and may contain ingested erythrocytes, leukocytes, epithelial cells, and bacteria.

Table 5 Parasites of the liver and biliary tree.

<i>Disease [agent(s)]</i>	<i>Clinical Manifestations</i>	<i>Diagnosis [secondary, alternative methods]</i>
Protozoan Infections		
Hepatic Amebiasis [<i>Entamoeba histolytica</i>]	Right upper quadrant pain, fever, complications: rupture into peritoneal cavity, obstructive jaundice, bacterial superinfection.	Imaging, Antibody Detection, NAATs [Microscopy (permanent stained smears; histopathology)]
Visceral Leishmaniasis (kala-azar) [<i>Leishmania donovani</i> , <i>L. infantum</i>]	May be asymptomatic in latent stage; active disease fatal if untreated: persistent fever, hepatosplenomegaly, pancytopenia, weight loss, wasting, skin hyperpigmentation	Antibody Detection [Microscopy (Giemsa-stained smears; histopathology); NAATs; culture]
Nematode Infections		
Hepatic Capillariasis [<i>Capillaria hepatica</i>]	Persistent fever, peripheral eosinophilia, abdominal pain, elevated hepatic enzymes, watery diarrhea, asthma-like symptoms	Microscopy (histopathology)
Visceral Larval Migrants (toxocariasis, baylisascariasis) [<i>Toxocara canis</i> , <i>T. cati</i> , <i>Baylisascaris procyonis</i>]	May be asymptomatic; fever, hepatosplenomegaly, asthma-like symptoms, high peripheral eosinophilia	Antibody Detection [Microscopy (histopathology)]
Trematode Infections		
Hepatic Schistosomiasis ^a [<i>Schistosoma mansoni</i> , <i>S. japonicum</i> , <i>S. mekongi</i>]	Portal hypertension, leading to ascites, esophageal varices, severe anemia, delayed growth in children.	Microscopy (histopathology), Antibody Detection
Fascioliasis [<i>Fasciola hepatica</i> , <i>F. gigantica</i>]	Commonly asymptomatic; fever, myalgia, arthralgia, peripheral eosinophilia, abdominal pain, cholelithiasis, cholangitis, pancreatitis, biliary cirrhosis	Microscopy (detection of eggs in wet mounts of stool), Antibody Detection [histopathology, gross morphology of adults]
Opisthorchiasis, Clonorchiasis, Metorchiasis [<i>Opisthorchis viverrini</i> , <i>O. felinus</i> , <i>Clonorchis sinensis</i> , <i>Metorchis conjunctus</i>]	Commonly asymptomatic; cholangitis, cholelithiasis, hepatomegaly, obstructive jaundice, cholangiocarcinoma.	Microscopy (detection of eggs in wet mounts of stool) [antibody detection, NAATs]
Dicrocoeliasis [<i>Dicrocoelium dendriticum</i> , <i>D. hospes</i>]	Peripheral eosinophilia, abdominal distention, RUQ pain, diarrhea, constipation, anemia, and weight loss, cholecystitis, liver abscesses	Microscopy (detection of eggs in wet mounts of stool)
Cestode Infections		
Cystic Echinococcosis [<i>Echinococcus granulosus</i> , <i>E. canadensis</i> , <i>E. equinus</i> , <i>E. ortleppi</i> , <i>E. oligarthra</i> , <i>E. vogeli</i>]	May be asymptomatic for many years; abdominal discomfort, decreased appetite, enlarging abdominal mass over many years; cyst leakage can cause life-threatening allergic reactions	Imaging Analysis, Antibody Detection [Microscopy (histopathology; wet mount examination of aspirates)]
Alveolar Echinococcosis [<i>Echinococcus multilocularis</i>]	Fatal if not treated; abdominal pain and damage to neighboring organs due to infiltrating cysts.	Imaging Analysis, Antibody Detection, Microscopy (histopathology)

^aIn this chapter, schistosomiasis is covered in detail under Parasites of the Appendix, Large Intestine, and Anus.

Species	<i>Entamoeba hartmanni</i>	<i>Endolimax nana</i>	<i>Dientamoeba fragilis</i>	<i>Iodamoeba buetschlii</i>
Cyst			Existence of cyst form is not universally accepted	
Trophozoite				
Species	<i>Entamoeba polecki</i>	<i>Entamoeba histolytica/E. dispar</i>	<i>Entamoeba coli</i>	
Cyst				
Trophozoite				

Scale 20 μ m

Fig. 1 Common intestinal amebae (Wheatley's Trichrome stain, 1000 $\times$ original magnification). The amebae are differentiated from one another by the morphologic features of the trophozoite and cyst forms, such as the overall size, number of nuclei, nuclear chromatin pattern, and characteristics of the nuclear karyosome and cytoplasm. The flagellate *Dientamoeba fragilis* is also included in this chart as it has an ameboid appearance and would enter the differential diagnosis of amebae found in human stool specimens. From Pritt B (2014) *Parasitology Benchtop Reference Guide*. Northfield, IL: College of American Pathologists.

Trichomonas tenax

Trichomonas tenax, like *E. gingivalis*, is considered a commensal in the human oral cavity. Similarly, *T. tenax* has been associated with periodontal disease, but also like *E. gingivalis*, it is generally believed its presence under such conditions is associated with increased bacterial growth (Dubar et al., 2020). There is no specific pathologic response associated with *T. tenax* in the oral cavity, however its presence could be an indicator of periodontal disease caused by other etiologies (Marty et al., 2017). *Trichomonas tenax* has been associated with extra-oral disease, such as pleural empyema, but it is generally believed its presence in such sites is associated with bacteria present and *T. tenax* itself is not an agent of disease (Bellanger et al., 2008).

Diagnosis of *T. tenax* is made primarily by microscopy, either wet mount examinations or permanent smears stained with Giemsa or Wheatley's Trichrome (Marty et al., 2017). Trophozoites are 12–30 μ m long and ellipsoidal or ovoid in shape. Each has a single large nucleus, a prominent axostyle, and four anteriorly-directed flagella. Morphologically, it is very similar to *T. vaginalis* observed in urogenital specimens (Marty et al., 2017). There is no cyst stage.

Leishmania spp. (mucocutaneous leishmaniasis)

Biology and epidemiology

Leishmaniasis is a serious and often debilitating parasitic disease that is classified as a Neglected Tropical Disease by the WHO (WHO, 2020a). Leishmaniasis is caused by flagellated protozoa in the genus *Leishmania*; approximately 19 species of *Leishmania* have been implicated in human disease. There are three broad clinical categories of leishmaniasis: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL; also referred to as mucosal leishmaniasis), and visceral leishmaniasis (VL). MCL is caused by three species in the subgenus *Viannia*, including *L. (V.) guyanensis*, *L. (V.) panamensis*, *L. (V.) braziliensis*, as well as *L. amazonensis* which is in the subgenus *Leishmania* (David and Craft, 2009; Torres-Guerrero et al., 2017). All four species occur in tropical Central and South America. *Leishmania amazonensis* occurs in Brazil, Venezuela, and Bolivia. *Leishmania braziliensis* occurs in the Western Amazon Basin of Brazil, Venezuela, Bolivia, and Peru, and in Guatemala. *Leishmania guyanensis* is found in northern South America (French Guiana, Brazil, Suriname, and Bolivia). *Leishmania panamensis* occurs in Central and South America (Steverding, 2017).

Leishmaniasis is a vector-borne disease transmitted by sand flies in the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World). Infection is initiated when infected sand flies introduce the motile (flagellated) promastigote stage of *Leishmania* intradermally when taking a blood meal. The promastigotes are taken up by primarily by macrophages, but also neutrophils and dendritic cells. Within macrophages, the parasites employ a variety of mechanisms to evade the microbicidal activities of the phagolysosome and convert it into a parasitophorous vacuole in which it can survive and multiply (Arango Duque and Descoteaux, 2015). Promastigotes transform into amastigotes, the small non-motile form of the parasite, and divide by binary fission. The infected cells migrate away from the bite site and carry the parasite to other organs, where they continue to replicate and infect other macrophages. Infected macrophages in the skin may be picked up by a new sand fly taking a blood meal, thus continuing the parasite lifecycle. Within the gut of the sand fly host, the amastigotes transform into promastigotes and migrate to the salivary glands, where they can infect another host during subsequent sand fly blood feeding.

Pathogenesis, clinical presentation, and treatment

Mucocutaneous leishmaniasis is a disseminated form of cutaneous infection resulting in metastasis from the original cutaneous lesion to the mucosa of the oro- and naso-pharynx (CDC, 2020a; Burza et al., 2018; Palumbo, 2010). Up to 90% of patients with MCL have a scar from previous CL. Mucosal involvement generally occurs within several years to decades of the initial cutaneous infection, and often begins with non-specific nasal symptoms such as epistaxis (nasal bleeding), rhinorrhea (runny nose), and nasal stuffiness. Lymphadenopathy is also common, and generally precedes ulceration (David and Craft, 2009). Systemic symptoms such as fever and hepatomegaly may also be present. Ulcerative lesions generally appear first on the lips or nostrils and expand to involve the palate and nasal septum (Burza et al., 2018; Palumbo, 2010). Over time, expansive tissue damage resulting from infiltrating infected macrophages and the associated host T-cell related cytotoxicity can result in life-threatening complications, including complete destruction of cartilaginous and soft tissue structures, airway obstruction and secondary infection (Fig. 2). Factors that determine the extent of the clinical manifestations of MCL include host cell-mediated immunity and virulence of the parasite (David and Craft, 2009).

Given the potential for severe, destructive disease, MCL should be treated aggressively using intravenous or intramuscular antimonial therapies (e.g., sodium stibogluconate), intravenous amphotericin, or oral miltefosine (CDC, 2020a; Palumbo, 2010; Medical Letter, 2013).

Diagnosis

MCL should be considered in all patients with an appropriate exposure history (i.e., sand fly bites in South or Central America) and ulcerative lesions of the oral/nasal mucosa, particularly when there is a history of prior cutaneous leishmaniasis (David and Craft,



Fig. 2 Moderately-advanced mucocutaneous leishmaniasis in a patient living in Paraguay. There is involvement of both nasal vestibules and erosion of his nasal septum. Courtesy of the U.S. Centers for Disease Control and Prevention Public Health Image Library.

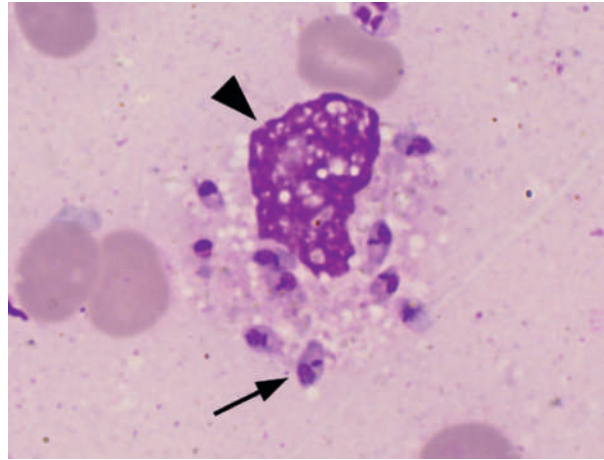


Fig. 3 *Leishmania* sp. amastigotes within the cytoplasm of a macrophage (Giemsa, 1000 ×). Each amastigote (arrow) has an eccentric nucleus and rod- to dot-shaped kinetoplast. The macrophage nucleus is denoted by an arrow head.

2009). Definitive diagnosis is by microscopy examination for intracellular and free amastigotes within tissue biopsies or aspirates. The amastigotes are small (2–4 μm), round to oval, and have an eccentric nucleus and rod-shaped kinetoplast (Fig. 3). The preferred microscopy preparation is Giemsa-stained impression preparations of sampled tissue. Histopathologic preparations can also be examined for the presence of amastigotes within the cytoplasm of macrophages, although the amastigotes are harder to identify due to the shrinkage that occurs during formalin fixation and tissue process. It is important to note the culture and/or NAAT offer greater sensitivity than microscopy and should be considered in cases where microscopy is non-diagnostic. The CDC provides diagnostic testing services for patients in the United States, including telediagnosis, culture, and NAAT (David and Craft, 2009).

Nematode infections

Gongylonema pulchrum (gongylonemiasis)

Gongylonema pulchrum is a parasite of livestock and other ruminants that has a nearly worldwide distribution. The parasite cycles between the vertebrate definitive host and an insect intermediate host, and human infection is acquired from the incidental (or intentional) ingestion of insects harboring infectious larvae (Libertin et al., 2017).

The most common clinical presentation of *Gongylonema* infection is the sensation of something moving in the oral mucosa and tongue (Allen and Esquela-Kerscher, 2013; Libertin et al., 2017). Nausea and vomiting are also common symptoms. Grossly, the oral mucosa shows an irregular “zigzag”-shaped blister-like lesion (Libertin et al., 2017). Removal of the worm is considered curative, although albendazole may be considered in patients with a heavier worm burden or prolonged duration of infection (Libertin et al., 2017).

Human infection of *G. pulchrum* is diagnosed by the identification of adult worms in the oral and esophageal mucosa, either by histopathology or extraction and examination of intact gross specimens. Rarely, eggs may be detected in wet mounts of stool. Adult female worms measure up to 14.0 cm while adult male worms measure up to 6.2 cm. Adults of both sexes have characteristic raised plaques (bosses) surrounding the anterior end of the worm (Naem et al., 2000). Eggs of *G. pulchrum* 50–70 μm long by 25–37 μm wide, have a thick shell, and are embryonated when shed in feces. The polar ends of the eggs often have a hyaline clearing (Ash and Orihel, 2007).

Anatrichosoma spp. (anatrichosomiasis)

Anatrichosoma is an enigmatic genus of nematodes related to the familiar human parasites *Trichuris trichiura* and *Trichinella spiralis*. Human infection is thought to be rare, with only three cases of human oral cavity involvement reported in the literature. All three cases were in patients living in the midwestern United States with recent travel to Mexico, and all were diagnosed by histopathologic examination of oral biopsies. *Anatrichosoma* species have not been morphologically described by histopathology, and therefore, the specific etiologic agents in these cases are not known. Based on the epidemiology, however, *A. buccalis*, a parasite of the oral cavity of opossums in the Americas, is the presumptive agent (Eberhard et al., 2014). The life cycle of *Anatrichosoma* species is not completely understood, but the history of the two most recent patients, a mother and daughter, participating in “snail facials” while on vacation in Mexico suggests the possible role of mollusks as an intermediate host (Eberhard et al., 2014). A second daughter participated in all of the same activities in Mexico with the exception of the snail facial and did not have any manifestations of infection.

Clinically, patients have presented with painful blisters on the tongue, lips, hard and soft palate, and oral mucosa. Grossly, raised track-like lesions and ulcerated lesions were also present at these sites (Eberhard et al., 2014, 2010). The patients' symptoms resolved following mebendazole or albendazole administration (Eberhard et al., 2014).

Diagnosis has been made by identifying adult worms in biopsy specimens of the oral mucosa and associated tissue. Worms in cross section will display typical trichuroid features such as bacillary bands and stichocytes (Eberhard et al., 2010). This morphologic appearance, in conjunction with the location, allows for the diagnosis of anatrchosomiasis.

Arthropod infections

Oral myiasis

Biology and epidemiology

Myiasis is colonization of living or dead animal tissue with fly larvae. Clinically, myiasis can be classified as obligatory (flies that have adapted to a parasitic lifestyle and require healthy host tissue), facultative (colonization of pre-existing wounds, often with dead or necrotic tissue), or accidental/incidental (incidental colonization of mucus membranes or biofilms on invasive medical devices, such as catheters) (Mathison and Pritt, 2014). The most common sites of infection in humans are the skin (furuncular myiasis), pre-existing, and often necrotic, wounds (facultative myiasis, wound myiasis), eyes (ophthalmomyiasis), and the ears, nose, and throat (ENT, cavitary myiasis) (Francesconi and Lupi, 2012). Oral myiasis has been reported worldwide, but is not common and is often seen in people with poor oral hygiene. Factors associated with oral myiasis include those that result in breaks in the oral mucosa (e.g. gingival disease, trauma, recent tooth extractions, malignancy) and those that result in prolonged mouth opening (e.g. mouth breathing while sleeping, alcoholism, dementia, intellectual disability, incapacitation). Infants who breastfeed from mothers with wound myiasis of the breast may also be at increased risk of oral myiasis (Francesconi and Lupi, 2012; Novo-Neto et al., 2015; Shikha et al., 2015).

Because oral myiasis usually involves infestation of pre-existing wounds and other unsanitary conditions, it is usually caused by species that normally cause facultative myiasis. One exception is the primary screwworm fly, *Cochliomyia hominivorax*, which will initially colonize a pre-existing wound but will continue to damage and consume healthy tissue. Other species implicated in oral myiasis include *Lucilia sericata*, *Wohlfahrtia magnifica*, *Musca domestica*, *Chrysomya bezziana*, *Oestrus ovis*, *Hypoderma bovis*, *H. tarandi*, *Gasterophilus intestinalis*, and *Calliphora vicina* (Francesconi and Lupi, 2012). Infection occurs when gravid female flies directly lay eggs (oviposition) or larvae (larviposition) on wounds and tissues.

Pathogenesis, clinical presentation, and treatment

Live maggots invade diseased, and occasionally healthy, tissue, causing pain and swelling in the affected area. Live maggots may be felt and seen within the tissue.

Treatment for oral myiasis is removal of larvae (Fig. 4) followed by cleaning of the wounds. Antibiotics may be prescribed to treat or prevent secondary bacterial infections (Shikha et al., 2015). Ivermectin has been shown to be effective in killing larvae by blocking the nerve impulses on nerve endings through the release of gamma amino butyric acid, which leads to paralysis and subsequent death of the larvae (Shikha et al., 2015).

Diagnosis

Diagnosis of oral myiasis is made by the gross examination of extracted fly larvae. Because removal of the larvae is curative, a definitive identification is not necessary. However, myiasis-causing fly larvae are often sent for identification for educational



Fig. 4 *Lucilia* sp. third instar larva. *Lucilia* sp. cause facultative wound myiasis and have been reported to cause oral myiasis. The dark, sclerotized, hook-like mandibles can be seen at the upper left of the image. This is the anterior portion of the larva, and the end that embeds into tissue while feeding. The posterior end (right) remains in contact with the environment to allow for respiration through two spiracles. Characteristics of the spiracular plate (inset) are used in the identification of the larva. From Mathison BA and Pritt B (2015) Arthropod Benchtop Reference Guide. Northfield, IL: College of American Pathologists.

purposes, scientific curiosity, and epidemiologic data. Identification of fly larvae to the genus or species level can be challenging, and best left to experienced parasitologists or entomologists. Helpful morphologic features include the arrangement of cuticular spines, shape of mandibles, and form of anterior and posterior spiracles and spiracular plates (Mathison and Pritt, 2015, 2014).

Parasites of the esophagus and stomach

As with the oral cavity, there are only a small number parasites that involve the esophagus and stomach (Table 2). While parasitic involvement of these regions is not usually life-threatening, infections can result in extensive morbidity and loss of productivity.

Protozoan infections

Trypanosoma cruzi (chagasic achalasia/chagasic megaesophagus)

Biology and epidemiology

Chagas disease, also known as American trypanosomiasis, is caused by the hemoflagellate *Trypanosoma cruzi*, which is endemic the New World, from the southern US to South America. In the natural life cycle, the parasite is transmitted to a mammalian host via the feces of an infected triatomine (kissing) bug (Fig. 5A). Efficient vectors of *T. cruzi* defecate while taking a blood meal from a human host. When the person scratches the bite site in response to an inflammatory response to the bug's bite, metacyclic trypomastigotes in the feces of the bug are rubbed into the wound. These metacyclic trypomastigotes invade surrounding tissues and transform into amastigotes that divide by binary fission. After about 4 days, the amastigotes transform into trypomastigotes and the host cells rupture, releasing these trypomastigotes into circulation (Fig. 5B). The trypomastigotes travel via the bloodstream to other tissues and initiate new replicative cycles. Without treatment, the infection can last for the lifetime of the patient (Bern et al., 2011). Humans can also be infected with *T. cruzi* by blood transfusion, organ donation, congenital transmission, and the ingestion of unpasteurized fruit and sugar cane juice contaminated with bugs and their feces (Bern et al., 2011).

Although Chagas disease is endemic only to the New World, international travel has made it a global concern (Connors et al., 2016). It is estimated that 6–7 million people are infected worldwide, with over 300,000 infected patients in the United States (CDC, 2020b; WHO, 2020a). The vast majority of these infections are believed to be in immigrants from endemic areas, although rare cases of locally-acquired infection have been reported in the southern United States (Bennett et al., 2018; Kuehn, 2016).

Chagas disease can be divided into acute and chronic forms of disease. Patients are thought to remain infected indefinitely, and approximately 20–30% of patients will go on to develop manifestations of chronic disease. The most common organ affected is the heart, with the GI tract being the second most common (CDC, 2019). The focus of this section is on the gastrointestinal manifestations associated with chronic Chagas disease.

Pathogenesis, clinical presentation, and treatment

Trypanosoma cruzi infection results in damage to the enteric nervous system, leading to various gastrointestinal motor disorders (e.g., altered peristalsis, failure of smooth muscle to relax) and “mega” syndromes (megaesophagus, megastomach, megaduodenum, megajejunum, megagallbladder, and megacolon) (Matsuda et al., 2009). Megacolon is the most commonly associated GI manifestation, and is associated primarily with chronic constipation. The esophagus is the second most common organ to be involved in GI Chagas disease, and results in achalasia (failure of the lower esophageal sphincter to relax), and resultant chagasic megaesophagus. The inability to pass food from the esophagus into the stomach leads to dysphagia, malnutrition, and severe weight loss (Matsuda et al., 2009).

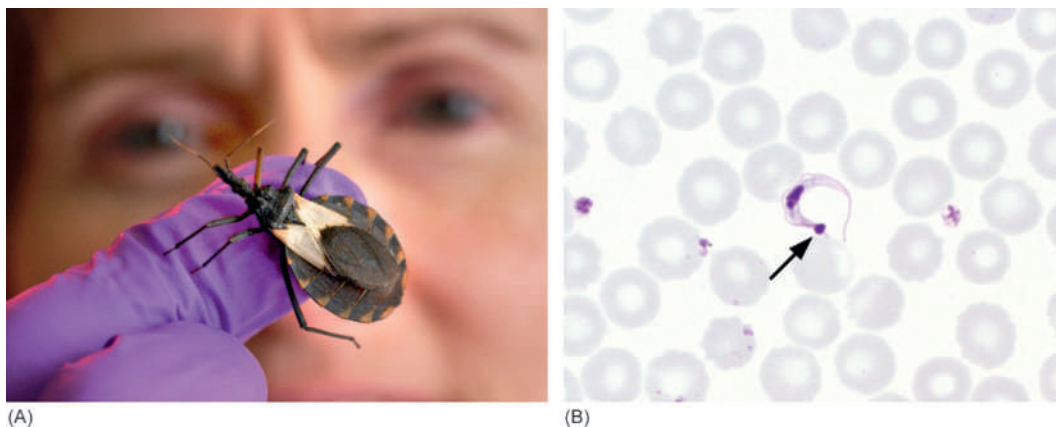


Fig. 5 (A) A triatomine bug, *Triatoma pallidipennis*, capable of transmitting *Trypanosoma cruzi*, the causative agent of Chagas disease. Note the bug's large size and prominent ornamentation. (B) Trypomastigote stage of *Trypanosoma cruzi* in a Giemsa-stained peripheral blood smear (1000 $\times$). Note the large kinetoplast at the posterior end of the organism (arrow). (A) Courtesy of the U.S. Centers for Disease Control and Prevention Public Health Image Library.

Treatment varies by the stage of disease, age of the patient, and presence of cardiomyopathy. Antiparasitic treatment with benznidazole or nifurtimox is strongly recommended for all children with chronic infection, as well as adults ≤ 50 years with chronic infection and no evidence of advanced cardiomyopathy. In older patients, and those with cardiomyopathy, the decision to give antiparasitic therapy must be considered on a case-by-case basis, considering the patient's clinical status, preference for treatment, and overall health (CDC, 2019). In cases of achalasia, surgical or medical interventions may also be indicated to relax the lower esophageal sphincter and improve esophageal emptying (Matsuda et al., 2009).

Diagnosis

The primary means of diagnosing chronic Chagas disease is by antibody detection. Unfortunately, immunodiagnosis of Chagas disease is challenging due to modest sensitivity and specificity provided by any single assay (Afonso et al., 2012). In some parts of the world, including the US, it is common to run two assays concurrently, or to screen with an assay that has high sensitivity, such as an enzyme-linked immunosorbent assay (EIA), and then confirm with an assay with greater specificity, such as immunoblot (Whitman et al., 2019). The CDC (Atlanta, Georgia, USA) uses the Chagatest-ELISA (Weiner lab Group, Rosario, Argentina) alongside an immunoblot using trypomastigote excreted-secretory antigens (TESA). An immunofluorescence assay (IFA) can be used as a tie-breaker when results are discordant (<https://www.cdc.gov/dpdx/trypanosomiasisamerican/index.html>). Antibody detection methods are also used for screening blood donors.

Tissue biopsy for microscopy and/or NAAT may be useful for detecting parasites in cardiac biopsies, but has limited utility in diagnosing GI Chagas disease. Amastigotes are rarely seen within GI tissues, except in profoundly immunocompromised patients (Meyers et al., 2011).

Unlike cardiac disease, amastigotes are not usually seen in biopsies of the GI tract in patients with Chagas disease.

Nematode infections

Anisakis spp., *Pseudoterranova spp.*, and *Contracaecum spp.* (anisakiasis)

Biology and epidemiology

Anisakiasis is a zoonotic disease caused by nematodes in the genera *Anisakis*, *Pseudoterranova*, and *Contracaecum*. The natural definitive hosts for these anisakid nematodes are fish-eating marine mammals and birds, where the larvae develop into adults in the gastric mucosa. Fully-embryonated eggs (eggs containing an infectious third-instar, or L3, larva) are shed in the feces of the definitive host and hatch in marine and brackish water. The L3 larvae then infect a crustacean intermediate host, which is in turn eaten by a fish or squid that serves as a paratenic host. The worms become encapsulated in the flesh of the fish or squid and the definitive host becomes infected after eating a paratenic host (Ortega and Sterling, 2017; Xiao et al., 2015). Humans also become infected after eating raw or undercooked paratenic hosts. However, humans are a dead-end host for anisakid nematodes as the worms cannot develop to sexual maturity. Human infection is usually confined to the gastrointestinal tract, but ectopic anisakiasis can occur when worms penetrate the gastric or intestinal mucosa and enter the peritoneal cavity. Ectopic disease has been reported to involve the mesentery, omentum, mesocolic lymph nodes, spleen, pleural cavity, and parametrium (Ramanan et al., 2013).

Human anisakiasis is most common in parts of the world where raw or undercooked fish is eaten as part of the cultural norm, most notably in Japan, coastal western Europe and South America, and in recent decades, North America (Audicana and Kennedy, 2008; Ramanan et al., 2013), although the disease can occur almost anywhere due to the global seafood trade. Commonly implicated dishes include sushi, sashimi, and ceviche. Anisakid nematodes generally have low host specificity for the piscine and molluscan hosts, and some of the most common commercial marine fish and squid may serve as paratenic hosts, including salmon, cod, snapper, herring, tuna, grouper, and Japanese flying squid (Fig. 6) (Ortega and Sterling, 2017). Therefore, a variety of fish and

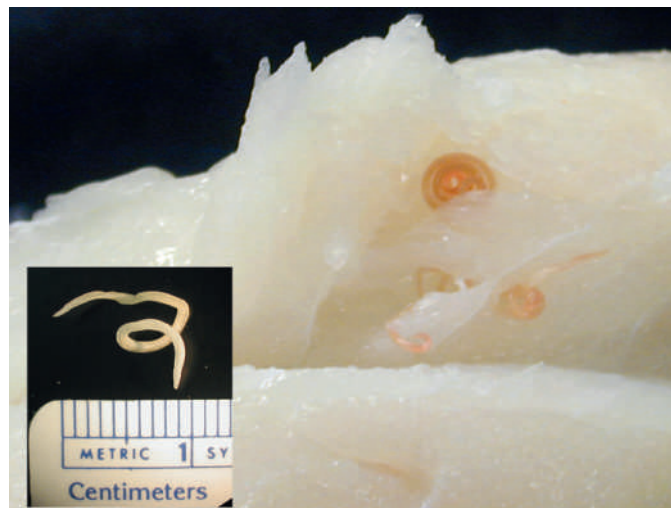


Fig. 6 Coiled anisakid (*Pseudoterranova* sp.) larvae in a fresh cod fillet. The inset shows a the size of the anisakid larva following removal from the fillet. From Mathison BA and Pritt BS (2018) Chapter 5: Parasitic diseases. In: Atlas of Fundamental Infectious Diseases Histopathology: A Guide for Daily Practice. Pritt BS (ed.). Northfield, IL: College of American Pathologists.

squid may pose a risk of anisakiasis if not fully cooked before ingestion. The U.S. Food and Drug Administration (FDA) recommends freezing fish at -20°C for 7 days or -35°C for 15 h to kill parasites prior to serving raw (FDA, 2020).

Pathogenesis, clinical presentation, and treatment

Clinical manifestations of anisakiasis are due to the presence of the live worm and/or the host immune response to larval antigens (Ramanan et al., 2013). The infection may come to the patient's attention when the larva is expelled through the mouth or nose. Of greater concern is when the larva burrows into the gastric or intestinal mucosa, which results in acute onset of epigastric or abdominal pain, respectively. Nausea, vomiting, and diarrhea are also common in this setting. Acute gastric symptoms typically occur within a few hours of ingesting the larva in undercooked seafood, whereas acute intestinal anisakiasis presents approximately 5–7 days after ingestion (Ramanan et al., 2013). If the larva is not immediately retrieved, chronic gastric or intestinal symptoms may occur as a result of the inflammatory reaction to the dying worm. If the larva penetrates the wall of the stomach or intestine, then peritonitis may occur. Additional symptoms may also be associated with the presence of the larva in the ectopic location. Removal of the embedded larva via endoscopy or surgery is curative.

Allergic reactions may be observed in susceptible individuals following exposure to anisakid allergens from either live or dead worms. Manifestations range from mild urticaria to life-threatening anaphylactic shock (Audicana et al., 2002; Audicana and Kennedy, 2008). Patients with severe allergies are advised to permanently avoid ingesting seafood products that may contain anisakid larvae. Treatment may include antihistamines and epinephrine.

Diagnosis

Anisakiasis is diagnosed based on the clinical presentation and exposure history, followed by confirmatory laboratory testing. When worms are expelled live from the nose or mouth, diagnosis is made by the morphologic identification of the intact L3 larvae. Grossly, L3 anisakid larvae resemble small specimens of *Ascaris lumbricoides*, measuring 10–50 mm long and possessing three fleshy anterior 'lips' (Fig. 11). Anisakids can be differentiated from *A. lumbricoides* from the presence of a boring tooth near the buccal opening or a terminal mucron, a small spine-like process which can be found in all species of *Pseudoterranova*, but only in some species of *Anisakis* and never in *Contracaecum* (Meyers et al., 2000). Worms embedded in the gastric or intestinal mucosa can be removed via endoscopy and identified grossly, or identified in biopsy specimens processed by histopathology. Anisakids can be identified to the genus level by examining structures of their alimentary canal and lateral chords, however a genus-level identification is not needed for patient management, as removal is curative (Meyers et al., 2000, Ortega and Sterling, 2017).

Diagnosis of ectopic anisakiasis is usually based on the incidental finding of dead, sometimes calcified, worms in biopsy specimens that were collected based on the suspicion of other etiologies, such as tumors (Mathison and Pritt, 2018a). The worms can usually be identified by the characteristics of the musculature, lateral chords, and the absence of sexual structures (Mathison and Pritt, 2018a). Degrading worms may be difficult to distinguish from ectopic *Ascaris lumbricoides*, the latter of which may possess sexual structures and eggs, which would not be present in anisakid larvae (Mathison and Pritt, 2018a).

Diagnosis of allergic reactions to anisakid nematodes is initially performed by a positive skin prick test in conjunction with compatible clinical manifestations and a recent history of eating fish or shellfish. The skin prick test should be confirmed by specific IgE antibodies using an additional assay (e.g. radioimmunoassay) in conjunction with a lack of antibodies to the host fish proteins (Audicana et al., 2002).

Serologic assays that detect parasite specific IgE are commercially-available at select reference and public health laboratories. There are no widely available molecular assays for routine clinical diagnosis of anisakiasis, and such tests are only likely to be offered by specialized reference laboratories.

Parasites of the small intestine

A large number of protozoa and helminths are capable of infecting the small intestine, and they are associated with a significant amount of human morbidity (Table 3). While some infections are rarely fatal (e.g., cryptosporidiosis, trichostrongylosis, cestode infections), others are important causes of global mortality (e.g., strongyloides hyperinfection, ascariasis with small bowel obstruction, hookworm infection with severe anemia, intestinal capillariasis).

Protozoan infections

Giardia duodenalis (giardiasis)

Biology and epidemiology

Giardia duodenalis (syn. *G. intestinalis*, *G. lamblia*) is a cosmopolitan flagellated protozoan parasite, with higher prevalence rates reported in regions with poor sanitation. There are eight genetic assemblages of *G. duodenalis*, two of which (A and B) are parasites of both humans and animals and have been implicated in both person-to-person transmission and zoonotic infections. The remaining six assemblages (C–H) exhibit some degree of host specificity for non-human animals and do not cause disease in humans (Ryan and Caccio, 2013). The WHO estimates that *G. duodenalis* caused 183,842,615 illnesses in 2010, but fortunately, no associated deaths were noted (Kirk et al., 2015). In resource-rich regions with adequate sanitation, infection is commonly associated with ingestion of untreated surface water (e.g., by hikers, campers) and in institutional settings (e.g., daycare, institutions for intellectually disabled individuals). Water- and food-borne outbreaks have also been reported (Adam et al., 2016).



Fig. 7 Scanning electron microscopic images of a single *Giardia duodenalis* trophozoite (left) and numerous *G. duodenalis* trophozoites attached to the duodenal mucosa (right). Courtesy of the U.S. Centers for Disease Control and Prevention Public Health Image Library.

Giardia duodenalis has a simple one-host life cycle. Infection occurs from the ingestion of mature cysts in fecally-contaminated water, or, less-commonly, food and fomites. The parasite excysts in the duodenum to produce two trophozoites. Trophozoites replicate by binary fission in the small intestine and feed on the surface of the intestinal mucosa (Figs. 7 and 8). After exposure to biliary fluid in the jejunum, some trophozoites form cysts and are excreted in the feces along with trophozoites. While both cysts and trophozoites are shed in feces, only cysts are infectious for a new host (Adam, 2001). Giardiasis is also regarded as a sexually-transmitted infection (STI) and parasites can pass between sexual partners via the anal-oral route (Escobedo et al., 2014).

Pathogenesis, clinical presentation, and treatment

Many patients with giardiasis are asymptomatic, particularly in endemic settings. When present, common symptoms include profuse, foul-smelling, watery diarrhea, flatulence, belching, bloating, vomiting, and weight loss. Less commonly, low grade fever and urticaria may also be present. Symptoms usually occur following an incubation period of 1–2 weeks, and usually last for 1–4 weeks. Many patients experience full resolution of symptoms. However, chronic giardiasis may occur, and has been reported in up to 50% of patients. Patients with chronic giardiasis often experience loose stools, malabsorption, flatulence, weight loss, and cramping. These symptoms may wax and wane over a period of several months. Some patients may also develop lactose intolerance.

Species and stage	<i>Retortamonas intestinalis</i>	<i>Enteromonas hominis</i>	<i>Chilomastix mesnili</i>	<i>Pentatrichomonas hominis</i>	<i>Giardia duodenalis</i>
Cyst				No known cyst form	
Troph					

Scale 10 μ m

Fig. 8 Intestinal flagellates, showing both cyst and trophozoite forms (Wheatley’s Trichrome stain, 1000 $\times$). From Pritt B (2014) *Parasitology Benchtop Reference Guide*. Northfield, IL: College of American Pathologists. Images of *Retortamonas intestinalis* and *Enteromonas hominis* courtesy of the Centers for Disease Control and Prevention DPDx.

Giardia does not invade into the intestinal wall, but adheres to the mucosal surface using its ventral sucking disc. In this way, it is thought to lead to the structural and functional abnormalities of the intestinal lining that result in the symptoms of giardiasis. Treatment is with metronidazole, tinidazole, or nitazoxanide. Rehydration may also be necessary.

(Lalle and Hanevik, 2018) Diagnosis

Diagnosis of giardiasis has historically been performed by O&P examination of stool and duodenal aspirates. Today there are numerous commercial antigen detection assays and NAATs for the diagnosis of giardiasis with improved sensitivity and susceptibility over microscopy.

Microscopy

Both cysts and trophozoites of *G. duodenalis* can be detected in O&P examinations of stool and duodenal aspirates. Because organisms are shed sporadically, multiple stools may need to be examined to ensure an accurate diagnosis. In fresh, unpreserved stool, living trophozoites may be detected in a saline wet mount preparation. *Giardia duodenalis* moves in a liquid substrate with a fluttering-like motion referred to as “falling leaf” motility. In preserved stool, both cysts and trophozoites may be detected in both wet mount concentrations and permanent-stained smears. Trophozoites are pyriform (pear-shaped) and measure 10–20 µm long (Fig. 7). They possess two large nuclei, a large sucking disc occupying the ventral surface of the anterior portion of the body, and prominent curved median bodies. Trophozoites possess eight flagella, although these are often not readily visible in routine permanent-stained slides (Fig. 8). Cysts are ovoid in shape and measure 8–19 µm in length. Mature cysts have four nuclei, axostyles, and curved median bodies (Fig. 8). The cytoplasm may be pulled away from the cyst wall, creating a halo-like effect (Ash and Orihel, 2007).

Occasionally, giardiasis is diagnosed by histopathologic examination of duodenal biopsies, particularly in chronic cases in which giardiasis is not suspected. Classic features include flattened duodenal villi and associated trophozoites (Mathison and Pritt, 2018a).

Antigen detection

There are many commercial assays available for the detection of *G. duodenalis* in stool and duodenal aspirates, some of which can be used for the simultaneous detection of *Cryptosporidium* and *Entamoeba histolytica*. Some assays require fresh, unpreserved stool while others can be performed using stool in fixatives. Also, some assays may not detect both cysts and trophozoites (Cama and Mathison, 2015; García et al., 2018). Available formats include lateral flow immunochromatography, enzyme immune assay, and direct fluorescence antigen detection, the latter of which is considered the gold standard method.

NAATs

There are several commercial NAATs available for the detection of *G. duodenalis* and other intestinal parasites. Some labs also employ on LDTs, often only targeting specific organisms for confirmation after morphological analysis. There are several multiplex NAATs for the detection of *G. duodenalis*, as well as other intestinal protozoa, three of which are FDA-approved for use in the US: (1) BioFire® FilmArray® Gastrointestinal GI Panel (BioMérieux, Marcy-l'Étoile, France), (2) BD MAX™ Enteric Parasite Panel (Becton Dickinson, Franklin Lakes, NJ), and (3) xTag® Gastrointestinal Pathogen Panel (Luminex, Austin, TX). In addition, there are several assays with CE approval but are not FDA-approved for use in the US.

Cryptosporidium spp. (cryptosporidiosis)

Biology and epidemiology

Cryptosporidiosis is caused by apicomplexan parasites in the genus *Cryptosporidium*. Historically, *Cryptosporidium* was classified among the coccidians, such as *Cyclospora cayetanensis* and *Toxoplasma gondii*. However, molecular and morphologic analyses place *Cryptosporidium* among the gregarines within Apicomplexa (Ryan et al., 2016). In the clinical realm, however, *Cryptosporidium* is still often referred to as a coccidian for familiarity and ease of communication, and because it is diagnosed by the same staining methods used for the “true” coccidians. At least 16 species of *Cryptosporidium* have been implicated in human infection, although the vast majority of cases are caused by two species: *C. hominis*, an anthropophilic species that only infects humans, and *C. parvum*, a parasite of livestock and other ruminants that causes zoonotic disease in humans. Both species are ubiquitous worldwide and infection is usually associated with contaminated water, although foodborne and direct person-to-person infection is possible (Cama and Mathison, 2015). *Cryptosporidium* infection can be a common cause of diarrheal disease in resource-limited parts of the world (Korpe et al., 2018). The WHO estimates that *Cryptosporidium* spp. caused 64,003,709 illnesses in 2010, with 27,553 deaths (Kirk et al., 2015). In resource-rich societies with adequate sanitation, infection is most commonly associated with exposure to recreational waters (natural sources of freshwater, public swimming pools, water parks, splash pads), the consumption of undercooked or raw food or unpasteurized milk and fruit juices, animal contact at petting zoos and fairs, and in day care centers (Cama and Mathison, 2015; Conrad et al., 2017; Turabelidze et al., 2007). Cryptosporidiosis can be classified as an STI, especially in MSM (Danila et al., 2014). *Cryptosporidium* has a complex life cycle involving both sexual and asexual reproduction in a single host. Infection is initiated by the ingestion of fully-sporulated oocysts in fecal-contaminated water, food, and fomites. Oocysts excyst in the upper small intestine and release sporozoites that infect the intestinal epithelial cells. The parasites form a parasitophorous vacuole between the host cell membrane and cytoplasm and derives nutrients from the host cell by means of a feeder organelle.

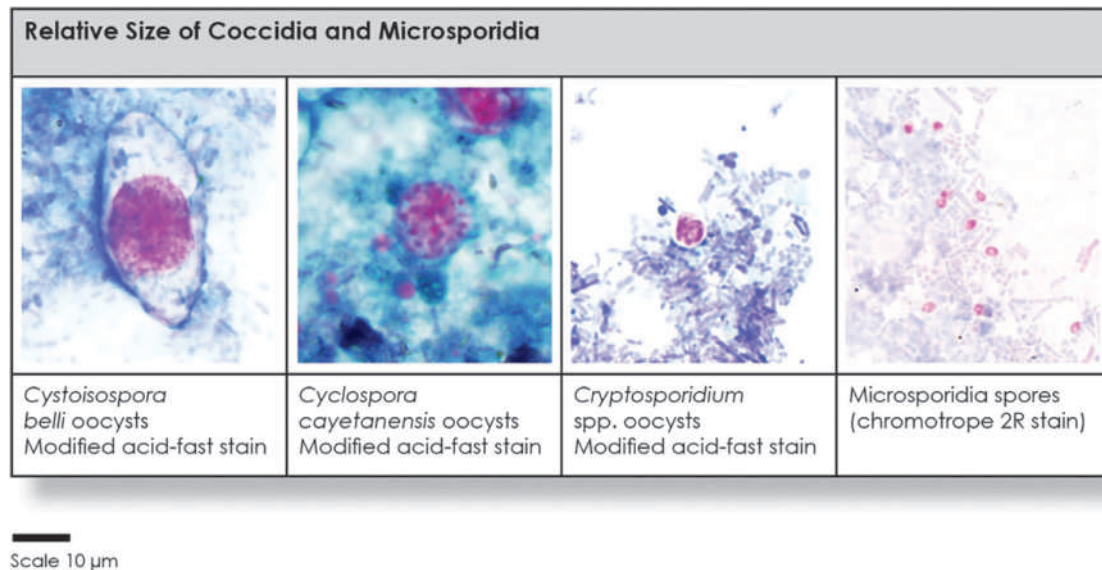


Fig. 9 Relative size of *Cryptosporidium* spp. and coccidia oocysts as seen by modified acid fast stain (1000 $\times$). Microsporidia spores are also shown for size comparison (chromotrope 2R stain). While once thought to be parasites, microsporidia are now known to be highly-specialized fungi. From Pritt B (2014) *Parasitology Benchtop Reference Guide*. Northfield, IL: College of American Pathologists.

Within the parasitophorous vacuole, sporozoites undergo cycles of asexual reproduction (merogony). Mature meronts rupture from the host cell and take one of two pathways. Type I meronts infect surrounding host cells and perpetuate the asexual cycle, while Type II meronts give rise to microgametes and macrogametes that initiate the sexual cycle (gametogony). Fertilization results in the formation of zygotes. Zygotes differentiate into oocysts that contain four naked (lacking a sporocyst wall) sporozoites. There are two kinds of oocysts produced: thin-walled oocysts rupture in the lumen of the intestinal tract and infect neighboring cells (autoinfection); thick-walled oocysts are shed via the feces into the environment to infect a new host (Fig. 9). Oocysts of *Cryptosporidium* are shed fully-sporulated and are infectious immediately out of the host (Cama and Mathison, 2015).

Pathogenesis, clinical presentation, and treatment

Cryptosporidiosis may have a range of manifestations, from asymptomatic infection, to mild diarrhea, to persistent, life-threatening disease (Bouzid et al., 2013). When present, symptoms are thought to be due to the parasites' interference with normal small intestinal secretory and absorptive functions, either through direct injury by the parasite or the resultant host immune response (Bouzid et al., 2013).

The extent and outcome of infection are largely dependent on the host's immune status and parasite-specific virulence factors. Immunocompetent individuals generally present with profuse watery, and occasionally prolonged, diarrhea, as well as nausea, vomiting, abdominal pain, and/or low-grade fever. Non-specific systemic symptoms including myalgia, headache, malaise and anorexia may also be present. Infection will generally resolve in 2–3 weeks, and illness may be shortened with the antiparasitic agent, nitazoxanide (Medical Letter, 2013). Infection may be more severe in immunocompromised hosts, particularly patients with HIV/AIDS and CD 4 T lymphocyte cell counts of less with 50 cells/mm³ (Bouzid et al., 2013). These patients may experience prolonged diarrhea, dehydration, anorexia and wasting, as well as biliary tract disease, pancreatitis, and rarely, involvement of the respiratory tract. Unfortunately, there is currently no effective antiparasitic therapy for cryptosporidiosis in profoundly immunocompromised patients (Medical Letter, 2013). Immune reconstitution (e.g., stopping immunosuppressive therapies, administering antiretrovirals for patients with AIDs) should be considered for severe, unremitting cases.

Diagnosis

Diagnosis of cryptosporidiosis can be performed by microscopy, antigen detection, and NAATs.

Microscopy

Cryptosporidium is not easily detected by traditional O&P examination as the oocysts do not stain well with Wheatley's trichrome or iron hematoxylin stains. The visualization of oocysts in stool specimens is enhanced by the use of special stains, such as Kinyoun's modified acid-fast (MAF) (Fig. 9), Ziehl-Neelsen acid-fast, aurimine O, and modified safranin (Cama and Mathison, 2015). Oocysts of *Cryptosporidium* are 4–6 μ m in diameter; they possess a thick outer wall and contain four sporozoites which may be visible in stained smears (Ash and Orihel, 2007). The different species of *Cryptosporidium* cannot be separated by morphology, but a species-level identification is not needed for clinical management. Unlike the true coccidians *Cyclospora*, *Cystoisospora*, and *Sarcocystis*, oocysts of *Cryptosporidium* do not autofluorescence under UV light.

Occasionally, cryptosporidiosis is diagnosed by histopathologic examination of duodenal biopsies, particularly in chronic cases in which infection is not suspected. The parasites can be seen within the small intestinal brush border as small (3–5 µm), spherical, blue-purple objects. Microvillous atrophy and mild inflammation may also be noted (Mathison and Pritt, 2018a).

Antigen detection

As with giardiasis, there are many commercial antigen detection assays available for the detection of *Cryptosporidium*, with an increased sensitivity over morphologic analysis. Some of the assays allow for concurrent diagnosis of giardiasis and amebiasis. There are several different methods, including DFA, EIA, and lateral flow assays (LFA), and for the most part the sensitivity and specificity is similar among the various platforms. For most assays, fresh, frozen, and formalin-preserved stool is acceptable for testing (Garcia et al., 2018).

NAATs

For routine clinical diagnosis and screening, many commercial multiplex assays for *G. duodenalis* will also detect *Cryptosporidium* spp., and with increased sensitivity and specificity over both morphologic analysis and antigen detection.

NAATs and sequence analysis can also be used for species-level identification. While this extent of identification is not important for clinical patient management, it can be very useful for outbreak investigations and in better understanding the molecular epidemiology of cryptosporidiosis. Genotyping has become a useful tool in identifying species and strains of *Cryptosporidium*. In the United States, for example, the CDC has developed *CryptoNet* (<https://www.cdc.gov/parasites/crypto/cryptonet.html>), a multi-disciplinary, molecular-based surveillance system to identify clusters and outbreaks in the US. Many European countries have their own local surveillance systems (Morris et al., 2019). Historically, genotyping was performed by using conventional PCR in combination with either restriction fragment length polymorphism (RFLP) or Sanger sequencing analysis (Xiao and Feng, 2017). Multilocus genotyping has shown some potential in outbreak investigations, but for it to be successful, would require a standardized typing scheme across multiple agencies and disciplines. While whole genome sequencing has had success for bacterial diseases, it is probably a while before it could be used for *Cryptosporidium* (Morris et al., 2019).

Cyclospora cayetanensis (cyclosporiasis)

Biology and epidemiology

Cyclosporiasis is caused by the intestinal coccidian *Cyclospora cayetanensis*. While other species of *Cyclospora* have been identified from non-human primates (Eberhard et al., 1999), *C. cayetanensis* is the only species to have been recorded from humans, and humans are believed to be the only host for *C. cayetanensis* (Cama and Mathison, 2015). Cyclosporiasis is primarily a disease of the tropics and subtropics and areas of endemicity include Central and South America, parts of the Middle East (Egypt and Turkey), the Indian subcontinent (including Nepal), southeast Asia, and Indonesia (Almeria et al., 2019). In the United States, cyclosporiasis is a seasonal illness and most infectious occurring from May to August; vehicles of transmission are primarily fresh produced imported from Latin America (Casillas et al., 2019).

Cyclospora cayetanensis has a life cycle similar to *Cryptosporidium*, with some important caveats. Infection occurs from the ingestion of fully-sporulated oocysts, usually on fresh produce contaminated with infected water. In the small intestine, oocysts release two sporozoites, each containing two infectious sporozoites. Sporozoites infect intestinal epithelial cells and form a parasitophorous vacuole in the cytoplasm of the host cell. As with *Cryptosporidium*, the parasite undergoes cycles of asexual and sexual reproduction, eventually leading to the production of oocysts (Fig. 9). Unlike with cryptosporidiosis, oocysts of *Cyclospora* are shed into the environment unsporulated. Factors accounting for sporulation and infectivity are not well understood, but are believed to include temperature, humidity, and possibly sunlight exposure. In the environment, sporulation takes on average 1–2 weeks (Cama and Mathison, 2015). Given that the oocysts must mature in the environment before they become infectious, direct person-to-person spread is not seen with cyclosporiasis. Instead, infection is only acquired via ingestion of oocysts from the environment in fecally-contaminated food or water. Also, there are no animal reservoirs. Therefore, infection can be prevented by providing adequate sanitation facilities and keeping human waste out of soil and water where crops are grown for human consumption.

Pathogenesis, clinical presentation, and treatment

Cyclospora cayetanensis infection results in acute and chronic inflammation, reactive vascular changes in the intestinal mucosa, and partial atrophy of the intestinal villi, leading to disruption of the intestinal barrier and malabsorption. Resultant symptoms commonly include watery diarrhea, abdominal pain, nausea, anorexia, weight loss and low-grade fever being commonly-reported symptoms (Ortega and Sanchez, 2010). Occasionally, the diarrhea may contain mucus or blood. Most patient living in non-endemic settings experience symptoms, while asymptomatic cases are more frequent in endemic settings. Patients at risk for severe disease include the very young, very old, and patients with AIDS. If untreated, these patients may experience life-threatening dehydration and wasting (Ortega and Sanchez, 2010). Biliary disease has also been reported, primarily in patients with AIDS. Treatment is recommended in all patients to shorten the duration of symptoms, which can otherwise last for weeks to months. The antiparasitic treatment of choice is trimethoprim-sulfamethoxazole (Ortega and Sanchez, 2010, Medical Letter, 2013).

Diagnosis

The diagnosis of cyclosporiasis is made primarily by microscopy. Molecular diagnosis is improving, but there are currently no antigen-detection assays for routine clinical diagnosis.

Microscopy

As with *Cryptosporidium*, oocysts of *C. cayetanensis* do not take up traditional stool permanent stains and are not as easily detected by traditional O&P examination. The preferred staining methods for *C. cayetanensis* are Kinyoun's modified acid-fast and modified safranin, which both stain the oocysts bright pink-red (Fig. 9). In specimens stained with MAF, the parasite exhibits irregular staining patterns and many oocysts may fail to take up the stain ("ghost forms"). The hot safranin method may provide more uniform staining (Cama and Mathison, 2015; Visvesvara et al., 1997). Oocysts of *C. cayetanensis* are consistently larger than those of *Cryptosporidium*, measuring 8.0–10.0 µm in diameter. The oocysts do not possess sporozoites in freshly-collected stool specimens. The oocyst is refractile and exhibits a "ground-glass" appearance (this feature is best observed in ghost forms); the outer wall may be folded or collapsed (Cama and Mathison, 2015; Ash and Orihel, 2007).

An interesting biological phenomenon of *C. cayetanensis* is the ability of its oocysts to autofluoresce when wet mounts are examined under UV illumination. The oocyst wall will fluoresce blue when examined with an excitation filter of 330–365 nm, and exhibit a less intense fluorescence when examined with an excitation filter of 450–490 nm (Cama and Mathison, 2015). Suspicious objects observed in the wet mount of an O&P examination can be easily confirmed by examining the specimen under UV illumination with an appropriate filter. However, iodine used in O&P exams is inhibitory to the autofluorescence, so if iodine is used routinely, a fresh wet mount without iodine should be prepared.

Occasionally, cyclosporiasis is diagnosed by histopathologic examination of small intestinal biopsies, particularly in chronic cases in which infection is not suspected. Various forms parasites can be seen within parasitophorous vacuoles in epithelial cells (Mathison and Pritt, 2018a).

NAATs

Currently, there only two multiplex assays for the clinical diagnosis of cyclosporiasis, the BioFire™ FilmArray™ GI Panel, which has both FDA and CE approval, and the Seegene Allplex Gastrointestinal Panel, which only has CE approval. These multiplex panels detect *C. cayetanensis* as well as other common bacterial, viral and parasitic causes of diarrhea.

Unlike with *Cryptosporidium*, the use of genotyping for outbreak investigations of cyclosporiasis is still in its infancy. Several genetic markers have been studied as potential tool for cluster and outbreak investigations, including single-nucleotide polymorphisms and small insertions and deletions spanning the apicoplast genome (Cinar et al., 2016) and the mitochondrial junction region (Nascimento et al., 2019). Most recently, sequencing analysis of the complete mitochondrial genome shows potential for linking cases during outbreaks (Cinar et al., 2020).

Cystoisospora belli (cystoisosporiasis)

Biology and epidemiology

Cystoisosporiasis is a coccidian infection caused by *Cystoisospora belli*. Historically placed in the genus *Isospora*, morphologic and molecular data support the exclusion of *C. belli* (and all historical *Isospora* parasites of mammalian hosts) from *Isospora*, which, as currently delineated, are parasites of birds (Barta et al., 2005). *Cystoisospora belli* has a worldwide distribution, but most human cases are seen in the tropics and subtropics of Central and South America, the Caribbean, Africa, and Southeast Asia. Most cases seen in the US and Europe are in travelers that have visited, or immigrants from, endemic areas, especially those that are immunocompromised (Dubey and Almeria, 2019).

The life cycle of *C. belli* is very similar to that of *C. cayetanensis*. Infection occurs from the ingestion of fully-sporulated oocysts, usually in contaminated food, water, and fomites. In the small intestine, oocysts release two sporocysts, each containing four infectious sporozoites. Sporozoites infect intestinal epithelial cells and form a parasitophorous vacuole in the cytoplasm of the host cell. As with *C. cayetanensis*, the parasite undergoes cycles of asexual and sexual reproduction, eventually leading to the production of oocysts which are shed unsporulated or partially sporulated into the environment via the feces (Fig. 9), (Cama and Mathison, 2015).

Pathogenesis, clinical presentation, and treatment

Like cryptosporidiosis and cyclosporiasis, cystoisosporiasis is associated with disruption of the intestinal barrier and malabsorption (Lindsay and Weiss, 2011). Patients generally presents with profuse watery diarrhea, abdominal pain, nausea, vomiting, and low grade fever (Dubey and Almeria, 2019). Steatorrhea and acalculous cholecystitis may also occur. Infection is generally self-limited, but may be severe, prolonged and life-threatening in immunocompromised patients such as those with AIDS. Treatment is with trimethoprim-sulfamethoxazole (Medical Letter, 2013).

Diagnosis

Cystoisosporiasis is diagnosed primarily by microscopy, either by traditional stool examinations, or less commonly, histopathology. Because of the large size of the oocysts, *C. belli* is more easily recognized in traditional O&P examinations. However, oocysts are shed in much smaller numbers than *Cryptosporidium* and *C. cayetanensis*, so multiple wet mounts may need to be examined. If permanent stains are used in diagnosis, Kinyoun's MAF and modified safranin are preferred over Wheatley's trichrome. The oocyst wall and developing sporoblast(s) will autofluoresce when examined under UV illumination with a proper excitation filter (see under *Cyclospora cayetanensis*, above) (Cama and Mathison, 2015). When observed in wet mounts, oocysts of *C. belli* are 20–33 µm long by 10–19 µm wide, ellipsoidal in shape, and possess one or two immature sporoblasts (Fig. 9). Oocysts in patients who have been treated may appear devoid of sporocysts (Ash and Orihel, 2007).

Occasionally, cystoisosporiasis is diagnosed by histopathologic examination of small intestinal biopsies, particularly in chronic cases in which infection is not suspected. Several asexual and sexual developmental stages of *C. belli* can be observed in biopsy specimens of the intestinal epithelium. Parasitophorous vacuoles tend to be located more basal in the cytoplasm of the epithelial cell, as compared to *C. cayetanensis* which tends to be located closer to the apical margin (Mathison and Pritt, 2018a). In AIDS patients, *Cystoisospora belli* can be observed outside the intestinal epithelium, such as in mesenteric lymph nodes. Although *C. belli* can infect the gallbladder mucosa, several recent reports of gall bladder involvement with *C. belli* in immunocompetent patients are now believed to represent misidentification of non-parasitic epithelial inclusions (Dubey and Almeria, 2019; Swanson et al., 2018).

Of note, *C. belli* infection may elicit a peripheral eosinophilia, which is not of feature of most other intestinal protozoal infections. The other protozoan parasites associated with peripheral eosinophilia are *Sarcocystis* spp. (intestinal and muscular forms of disease) and *Dientamoeba fragilis* (see below).

Sarcocystis hominis, S. suihominis (intestinal sarcocystosis)

There are two clinical diseases associated with coccidians in the genus *Sarcocystis*: intestinal sarcocystosis, which is caused by *S. hominis* and *S. suihominis*, and muscular sarcocystosis, which is caused by zoonotic species of *Sarcocystis*. The focus of this chapter will be intestinal sarcocystosis.

Biology and epidemiology

Sarcocystis spp. have a complex two-host life cycle. Humans are the natural definitive host for *S. hominis* and *S. suihominis* and infection occurs nearly worldwide (Fayer et al., 2015). Humans become infected after the ingestion of undercooked beef (*S. hominis*) or pork (*S. suihominis*) harboring infectious cysts (sarcocysts). The sarcocysts rupture in the small intestine and release bradyzoites, which infect intestinal epithelial cells. The bradyzoites differentiate into microgametes and macrogametes, which initiate sexual reproduction eventually resulting in the formation of oocysts. Oocysts sporulate in the lumen of the intestine; mature oocysts are thin-walled and possess two sporocysts, each with four sporozoites. Both intact oocysts and individual sporocysts liberated from ruptured oocysts are shed into the environment in the feces. Cattle and pigs become infected after eating sporocysts and oocysts while grazing on plants contaminated with human feces. Sporozoites liberated in the intestines of the intermediate host infect the endothelial cells of blood vessels. The parasites undergo two generations of schizogony. Merozoites from the second-generation schizonts invade muscle tissue and form sarcocysts containing many infectious bradyzoites (Fayer et al., 2015).

Pathogenesis, clinical presentation, and treatment

Like infection with the other coccidia, intestinal sarcocystosis results in disruption of the intestinal barrier and malabsorption. Many infected patients are asymptomatic or have only mild symptoms (Fayer, 2004; Fayer et al., 2015). When present, symptoms commonly include present with watery diarrhea, nausea, vomiting and abdominal pain. Occasionally a peripheral eosinophilia may be observed. Symptoms generally appear between 2 days to 2 week following ingestion of tissue sarcocysts in undercooked pork or beef (Fayer, 2004). Symptoms are self-limited in most cases and resolve within 1–2 days; however, infected individuals may shed oocysts and sporocysts in their stool for a year or more. There is no antiparasitic agent for intestinal sarcocystosis with proven efficacy (Fayer, 2004).

Diagnosis

The diagnosis of intestinal sarcocystosis is made by microscopy, primarily examinations of wet mounts of stool. Individual sporocysts will autofluoresce when examined under UV illumination, but the oocyst wall will not (Fayer et al., 2015). Sporocysts can also be visualized in MAF-stained stool specimens (Abou El-Naga and Gaafar, 2014). Oocysts measure 15–19 µm long by 15–20 µm wide; individual sporocysts are ovoid and measure 15–19 µm long by 8–10 µm wide (Ash and Orihel, 2007).

Nematode infections

Ascaris lumbricoides (ascariasis)

Biology and epidemiology

Ascariasis is caused by *Ascaris lumbricoides*, the largest nematode inhabiting the human intestinal tract. *Ascaris lumbricoides* has a nearly worldwide distribution and pigs are a major animal reservoir, especially in industrialized societies. Ascariasis is one of the soil-transmitted helminthiases, and is thus categorized as an NTD by the WHO. Like the other soil-transmitted helminthiases, the prevalence of infection is highest in poor regions of the world without proper sanitation and access to clean water (Kirk et al., 2015; Jourdan et al., 2018; Else et al., 2020). It is estimated there are 760 million human cases worldwide (Betson et al., 2014), with an associated 923,000 years lost due to disability and 511,000 years of life lost in 2016 (WHO, 2020b).

Human infections associated with pigs are often attributed to *A. suum*. There has been an ongoing debate for decades on the relationships between *A. lumbricoides* and *A. suum* and the validity of the latter as a distinct species. In general, morphological, molecular, and ecological data suggests that *A. suum* is synonymous with *A. lumbricoides* (Leles et al., 2012; Liu et al., 2012). While there are genetic differences in worm populations around the world, and that humans and pigs may harbor different genetic populations in endemic areas, it is still unclear whether those differences represent two or more species with a common ancestor, or there were multiple human colonization events from pigs (or vice versa) over time in different geographic areas, or there is a single species with geographic variations, the latter of which becomes more problematic given the global migration of humans and the pigs they bring with them (Betson et al., 2014). From a clinical standpoint, ascariasis is managed the same regardless of the species implicated in human disease.

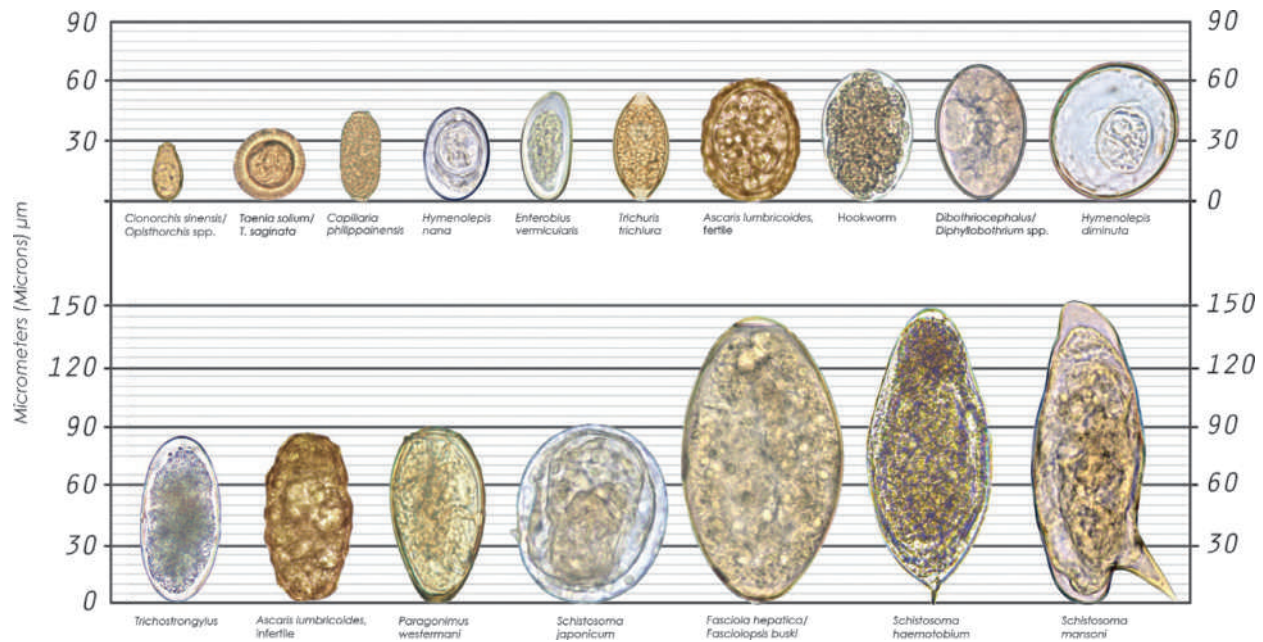


Fig. 10 Intestinal helminth eggs arranged according to increasing size (unstained). From Pritt B (2014) *Parasitology Benchtop Reference Guide*. Northfield, IL: College of American Pathologists.

Ascaris lumbricoides has a complex life cycle involving a single host. Adults reside in the small intestine of the human host. Female worms may shed fertilized (if mated) or unfertilized eggs into the environment via the feces (Fig. 10). As with other soil-transmitted helminthiases, the eggs are not immediately infectious, and must develop further in the soil. Therefore, there is no person-to-person spread of this parasite. Unfertilized eggs are a dead-end stage, but fertilized eggs will embryonate over 2–4 weeks depending on environmental conditions such as temperature and humidity. Fully embryonated eggs contain an infectious L3 larva. Humans become infected after ingesting fully-embryonated eggs on fomites, food, and water contaminated with soil containing the eggs. The eggs hatch in the upper small intestine. The L3 larvae penetrate the intestinal epithelium and are carried via the bloodstream to the lungs. After about 3 weeks, the L3 larvae penetrate the alveolar walls and ascend the bronchial tree to the epiglottis, where they are coughed up and swallowed. Once back in the small intestine, they molt twice and become adult worms. Adults live for about 1–2 years (Mathison and Pritt, 2018b). Occasionally, adult female worms will migrate to ectopic sites, such as the liver, gall bladder, pancreas, abdominal cavity, kidneys, umbilicus, and pleural cavity. This life-cycle can be interrupted by incorporating good sanitation practices, including proper disposal and treatment of human and pig feces.

Pathogenesis, clinical presentation, and treatment

Like all of the soil-transmitted helminths, the clinical presentation of ascariasis varies by the stage and extent of infection (Jourdan et al., 2018). Light infections are commonly asymptomatic, while heavy infections are associated with significant morbidity, particularly in young children. Death from infection is rare. During the lung migration stage, the larval worms elicit a type-1 hypersensitivity reaction which may produce transient pulmonary symptoms referred to as Loeffler's syndrome. This syndrome generally occurs 9–14 days after and is characterized by dry cough, urticaria, wheezing, and dyspnea. Peripheral eosinophilia (>500 cells per microliter) is common (Akuthota and Weller, 2012). Chest radiograph characteristically show patchy areas of consolidation that may migrate on repeat imaging, and pleural effusion might be noted (Jourdan et al., 2018). Hemoptysis and respiratory distress may occur with heavy infections (Akuthota and Weller, 2012).

The intestinal stage of infection is usually asymptomatic. When present, patients commonly experience abdominal discomfort, anorexia, and altered bowel patterns including diarrhea (Jourdan et al., 2018). Potentially life-threatening complications are due to the large size of the worm; heavy infections, particularly in young children, may present with small bowel obstruction, whereas ectopic migration of one or more adult worms into the biliary tree may result in acute pancreatitis, acute cholecystitis, and hepatic abscess (Jourdan et al., 2018).

Treatment is primarily with the benzimidazole drugs, albendazole or mebendazole (Jourdan et al., 2018). Ivermectin is an alternative therapy. In endemic regions, it common to administer an a benzimidazole drug on a periodic basis to susceptible individuals (e.g. annual school-based deworming programs) to prevent significant morbidity from infection.

Diagnosis

Diagnosis of ascariasis is done by microscopy, including examinations of wet mount of stool to detect eggs, gross morphology of expelled adult worms, and rarely, histopathology for ectopic colonization of adult worms. Larvae can rarely be seen in sputum during the lung migratory stage through sputum microscopy.

Four morphotypes of eggs may be observed in wet mounts of stool, and technologists need to be familiar with all four to ensure a reliable diagnosis. Fertile eggs are 55–75 μm long by 35–50 μm wide and have a thick, multi-layered shell with a bumpy, albuminous layer (mamillated) (Fig. 10). The embryo is at a single-cell stage of development in freshly-processed stool specimens, but will embryonate over time, even in stool some fixatives, such as 10% formalin. Occasionally, the albuminous layer is lacking and the outer shell is smooth (decorticated). Infertile eggs are 85–85 μm long by 43–47 μm wide with internal contents consisting of disorganized granules. As with fertile eggs, infertile eggs may be mamillated or decorticated. Infertile, decorticated eggs can easily be confused for the eggs of other parasites, such as *Fasciola hepatica* and *Fasciolopsis buski*, or plant cells (Ash and Orihel, 2007).

Adult worms expelled in stool are large, smooth, and cream-colored (Fig. 11). Adult females are 20–35 cm long, while males are smaller at 15–31 cm long. Males also tend to have a curved tail. Adults of both sexes have three, fleshy anterior 'lips' (Ash and Orihel, 2007). Adult *A. lumbricoides* needs to be distinguished from L3 larvae of anisakid nematodes, which are generally less than 5 cm long.

Adult worms in ectopic sites may be discovered via radiologic imaging or surgical exploration in acutely symptomatic patients. Alternatively, they may be discovered incidentally in biopsy specimens collected under the suspicion of other etiologies. In the latter situation, the worms are usually dead and calcified or in varying stages of degradation. Sometimes only eggs remain in granulomas. The worms can usually be recognized by the structure of the cuticle, musculature, and intestinal cells; however degrading worms can be very difficult to distinguish from ectopic anisakids. The presence of reproductive structures or eggs would rule-out anisakiasis, as anisakid nematodes do not develop to sexual maturity in the human host (Mathison and Pritt, 2018a). L3 larvae in lung biopsy specimens have several features in common with other ascarid nematodes, including large excretory canals and prominent lateral alae. L3 larvae of *A. lumbricoides* need to be distinguished from visceral larval migrans caused by *Toxocara* spp. (Mathison and Pritt, 2018a).

Capillaria philippinensis (intestinal capillariasis)

Biology and epidemiology

Intestinal capillariasis is caused by the trichuroid nematode *Capillaria philippinensis*. The species was first described from a human patient in the Philippines, but endemic human infection occurs throughout much of Southeast Asia, including China, Korea, Thailand, Laos, Malaysia, and Japan. The disease is established in Egypt as well, and has been identified in a patient from Colombia (Eiras et al., 2018). Additional cases have been reported from the United Kingdom, UAE, Iran, Italy, and Spain (Attia et al., 2012). It is uncertain whether the disease is more widely distributed than historically believed, or if it has been introduced to new areas by human activity or migrating birds.

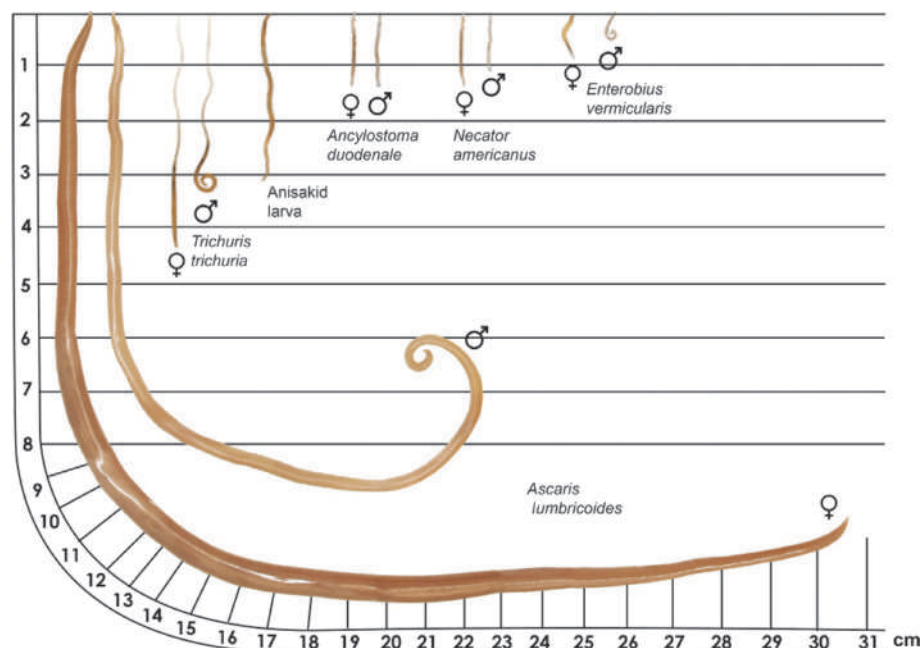


Fig. 11 Gross morphology of common intestinal helminths arranged according to size (unstained). From Pritt B (2014) *Parasitology Benchtop Reference Guide*. Northfield, IL: College of American Pathologists.

Capillaria philippinensis has a complex two-host life cycle that includes direct and indirect cycles. In nature, the disease is believed to cycle between fish and piscivorous (fish-eating) birds. Humans become infected after eating raw or undercooked fish harboring infectious *C. philippinensis* larvae. The larvae mature to adults in the small intestine. After mating, first-generation females release larvae which infect the host's intestinal epithelium and mature into second-generation adults. After mating, most of these second-generation females release eggs, although some will continue to release larvae, the latter of which results in autoinfection. The autoinfection process results in long-standing infection of the host, which eventually progresses to hyperinfection. Eggs are shed into the environment unembryonated or partially embryonated (Fig. 10). Eggs embryonate in 5–10 days under normal environmental conditions. Fully-embryonated eggs are ingested by fish and hatch in the fish's intestinal tract. The liberated larvae colonize the tissue of the fish and remain there until eaten by a suitable definitive host (Cross, 1992).

Pathogenesis, clinical presentation, and treatment

Intestinal capillariasis is a serious, progressive infection, which may lead to death if untreated due to ongoing destruction and disruption of the intestinal mucosa (Cross, 1992). Patients most commonly present with chronic watery diarrhea, abdominal pain, vomiting, anorexia, malaise, and weight loss. Symptoms progress over time due to ongoing autoinfection. Eventual hyperinfection results in severe weight loss, malabsorption, protein-losing enteropathy, and electrolyte imbalances (Cross, 1992; Limsrivilai et al., 2014). Treatment is albendazole or mebendazole (Medical Letter, 2013).

Diagnosis

Intestinal capillariasis is diagnosed by microscopy, including wet mount examinations of stool and histopathology of intestinal biopsy specimens.

Eggs of *C. philippinensis* can be detected in wet mounts of stool, and in patients with heavy worm burdens, larvae and occasionally adult worms can also be detected. Eggs are 36–45 µm long by 21 µm wide and have a thick striated shell and inconspicuous polar protuberances on each end (Fig. 10). The eggs are often slightly indented medially giving them a peanut-shaped appearance (Ash and Orihel, 2007). Larvae are 130–300 µm in length and possess stichocytes along the esophagus (Cross, 1992).

Biopsy specimens of the small intestine may reveal adults, larvae, and eggs. Adults and larvae have typical trichuroid morphologic features, such as stichocytes and bacillary bands, and need to be distinguished from *Trichuris trichiura* and *Trichinella* spp. Adults of *T. trichiura* are larger than those of *C. philippinensis* and have a marked difference in the diameter of anterior and posterior regions of the body. Adults of *Trichinella* are smaller than those of *C. philippinensis* and do not produce eggs in tissue or stool. Because of a similar autoinfective cycle, *C. philippinensis* may resemble *Strongyloides stercoralis*, the latter of which lacks features common to trichuroid nematodes, such as stichocytes and bacillary bands (Mathison and Pritt, 2018a).

***Necator americanus* and *Ancylostoma* species (intestinal hookworm infection)**

Biology and epidemiology

Most cases of intestinal hookworm infection are caused by one of three species: *Necator americanus*, *Ancylostoma duodenale*, and *A. ceylanicum*. Classified as a Neglected Tropical Disease (NTD) along with other soil-transmitted helminths, it is estimated there are over 439 million hookworm infections worldwide (Hotez et al., 2014). The WHO estimates that hookworm infections were responsible for 1,682,000 disability-adjusted life years in 2016. *Necator americanus* and *A. duodenale* have a nearly worldwide distribution in the tropics and subtropics (WHO, 2020a). Major areas of endemicity for *N. americanus* include southern and southwestern China, southern India, Southeast Asia, sub-Saharan Africa, and Latin America. Coastal areas of these regions appear to have increased transmission. Major areas of endemicity for *A. duodenale* include northern regions of China and India, Egypt, northern Australia, and Latin America (Brooker et al., 2004). Hookworm infection has been largely eradicated from the United States, but sporadic cases do still occur, especially in impoverished areas of the South. Mixed infections of *N. americanus* and *A. duodenale* are not uncommon where the two species overlap (Brooker et al., 2004). *Ancylostoma ceylanicum*, a common parasite of dogs and cats in Asia which historically been regarded as an uncommon zoonotic parasite in humans, is now believed to be one of the leading causes of human hookworm infection in Southeast Asia (Traub, 2013). *Necator gorillae* causes uncommon zoonotic infection in western Africa (Kalousova et al., 2016).

Hookworms have a complex one-host life cycle. Adults reside in the small intestine. Mated females lay unembryonated or partially-embryonated eggs into the environment in the feces. Under optimal conditions in soil, eggs embryonate within 24–48 h and hatch, releasing first-instar (L1) larvae. The L1 larvae molt twice and become infectious L3 larvae in the soil. Humans are infected when L3 larvae penetrate intact skin when walking barefoot or in sandals over contaminated soil; *A. duodenale* can also cause infection from ingestion of L3 larvae on food or fomites contaminated with soil. The larvae are carried via the bloodstream to the right side of the heart and then to pulmonary vessels. The larvae rupture pulmonary capillaries and enter the lung parenchyma, ascend the bronchial tree to the epiglottis, and then are coughed up and swallowed. Once in the small intestine, they molt twice to become adults. The adult worms attach to the intestinal mucosa using specialized mouthparts and feed on the blood of the host (Figs. 12 and 13). It takes approximately 6–8 weeks from the time L3 larvae penetrate the skin to sexual maturity of the adults (Hotez et al., 2004).



Fig. 12 Mouthparts of adult hookworms used to attach to the small intestine and feed from blood vessels within the mucosa. *Ancylostoma* species have “teeth” (left), while *Necator americanus* has cutting plates (right). From Mathison BA and Pritt BS (2018) Chapter 5: Parasitic diseases. In: Atlas of Fundamental Infectious Diseases Histopathology: A Guide for Daily Practice. Pritt BS (ed.). Northfield, IL: College of American Pathologists.



Fig. 13 Two adult hookworms embedded in the intestinal mucosa. Courtesy of the U.S. Centers for Disease Control and Prevention Public Health Image Library.

Pathogenesis, clinical presentation, and treatment

Hookworm infections are commonly asymptomatic (Jourdan et al., 2018). When present, symptoms vary by the stage of infection and worm burden. Patients may initially present with a pruritic rash (i.e., “ground itch”) at the site of skin penetration by the infective larvae. Later, Loeffler’s syndrome may rarely occur when larvae migrate through the lungs. Finally, the intestinal stage of infection may be accompanied with abdominal pain, diarrhea, blood in the stool (microscopic, or rarely macroscopic), and anemia. Patients with a heavy worm burden may experience severe anemia due to ongoing blood loss from the feeding adult worms. Young children and pregnant women are particularly at risk for poor outcomes due to anemia, hypoalbuminemia, and malnutrition (Jourdan et al., 2018). Treatment is albendazole or mebendazole (Medical Letter, 2013, Jourdan et al., 2018). In endemic regions, it is common to administer a benzimidazole drug on a periodic basis to susceptible individuals (e.g. annual school-based deworming programs) to prevent significant morbidity from infection. Patients should also be educated on preventative measures such as adequate sanitation and regular use of footwear (Jourdan et al., 2018).

Diagnosis

Intestinal hookworm infection is diagnosed primarily by the detection of eggs, and less-commonly L1 larvae, in wet mount examinations of stool. Adults may be removed by endoscopy and identified grossly by the structures of their mouthparts. Routine molecular diagnosis for intestinal helminths is still in its infancy; however, regional laboratories have developed LDTs for the detection of hookworm.

Female hookworms have a relatively high fecundity, and finding eggs in a positive stool specimen does not usually present with any problems if the specimen is processed properly. Eggs of *N. americanus* are 60–75 μm long by 36–40 μm wide, have a thin, colorless shell, and are in an early cleavage stage in freshly-processed feces. The eggs of *Ancylostoma* spp. are slightly smaller, at

55–65 μm long by 36–40 μm wide. Due to these inappreciable differences in size, it is essentially impossible to morphologically separate the eggs of *N. americanus* and *Ancylostoma* spp. in stool specimens. When hookworm eggs are detected in wet mounts of stool, it is common practice to report them generically as “hookworm eggs” and not at the genus or species level (Ash and Orihel, 2007). Identification to the genus or species level is not needed for clinical management of patients. Hookworm eggs are also very similar to the eggs of *Trichostrongylus* spp. (Fig. 10; see below).

If there is a delay in processing stool specimens, the eggs may embryonate and hatch and L1 larvae may be present in O&P exams. The L1 larvae of hookworm need to be distinguished from L1 larvae of *Strongyloides stercoralis*, which are also detected in stool. The L1 larvae of hookworm are 250–350 μm long, have a long buccal canal, and an inconspicuous genital primordium. In contrast, the L1 larvae of *S. stercoralis* 180–380 μm long and possess a short buccal canal and a prominent genital primordium (Ash and Orihel, 2007).

Adult males of *N. americanus* measure 5–9 mm long; females are slightly larger at 9–11 mm long (Fig. 11). Adults of both sexes have a buccal capsule (mouth) containing prominent cutting plates. Adults of *Ancylostoma* spp. are similar in size; males measure 8–11 mm long and females measure 10–13 mm long. Instead of cutting plates in the buccal capsule, adults of *Ancylostoma* have two sets of teeth (Fig. 12) (Ash and Orihel, 2007). Adults of *A. duodenale* and *A. ceylanicum* can be separated by the form of the teeth. In *A. ceylanicum*, the outer (lower) teeth are much larger than the inner (upper) teeth, whereas the two pairs of teeth in *A. duodenale* are similar in size and shape (Yoshikawa et al., 2018).

Trichostrongylus species (trichostrongylosis)

Trichostrongylosis is a zoonotic helminth infection caused by nematodes in the genus *Trichostrongylus*, including *T. orientalis*, *T. columbiformis*, and *T. axei*. Normally a parasite of herbivorous mammals, humans can become infected after the ingestion of infectious L3 larvae on contaminated produce. Human infection is not common, but sporadic cases have reported from many countries, including Laos, Thailand, China, South Korea, Iran, Australia, and the United States (Sato et al., 2011).

The clinical picture of trichostrongylosis is not well described. Most patients are asymptomatic, but some may present with diarrhea, abdominal pain, anorexia, pulmonary symptoms, and eosinophilia. Cutaneous manifestations are rare (Ghanbarzadeh et al., 2019). Treatment options for trichostrongylosis include albendazole, mebendazole, and pyrantel pamoate (Medical Letter, 2013).

Trichostrongylosis is diagnosed by the detection of eggs in wet mount examinations of stool (Sato et al., 2011). Eggs of *Trichostrongylus* are morphologically very similar to hookworm eggs and the disease may be under-reported in endemic areas. Eggs of *Trichostrongylus* spp. tend to be slightly larger, measuring 75–95 μm long by 40–50 μm wide (Fig. 10). The eggs taper noticeably to one end and the embryo is usually in an advanced stage of cleavage when shed in feces (Ash and Orihel, 2007).

Strongyloides stercoralis (strongyloidiasis)

Biology and epidemiology

Most cases of strongyloidiasis are caused by *Strongyloides stercoralis*, a nematode with a nearly cosmopolitan distribution. As the molecular epidemiology of the disease becomes better understood, it is apparent that some human infections in Africa, Southeast Asia, New Guinea, and Australia are attributed to *S. fuelleborni*, a parasite of non-human primates in Africa and Asia (Barratt et al., 2019). Classified as an NTD, the global burden of *S. stercoralis* is difficult to calculate due to poor or nonexistent reporting in some areas and the low sensitivity of traditional diagnostic assays, such as stool exam. It is believed there are anywhere from 3 to 100 million people infected worldwide, although these numbers are not believed to be close to the true prevalence of the disease globally. Major areas of soil-borne transmission are Latin America, sub-Saharan Africa, and Southeast Asia. In developed countries or areas where fecal contamination of soil is rare, major risk factors include HIV infection, HTLV-1 infection, alcoholism, organ transplantation, and patients undergoing corticosteroid therapy (Schar et al., 2013).

Strongyloides stercoralis has a complex life cycle including both parasitic and free-living stages. Adult females (only) reside in the epithelium of the small intestine. They reproduce parthenogenetically (without fertilization by a male) and shed embryonated eggs containing an L1 (rhabditiform) larva into the lumen of the intestine. The eggs hatch in the small intestine and the L1 larvae take one of three pathways, two environmental and one internal. L1 larvae shed in the feces take one of two pathways in soil. They either molt twice and become infectious L3 (filariform) larvae that infect a new host, or they mature all the way to adults. In the latter, both free-living adult males and females occur. They mate and the females produce embryonated eggs containing L1 larvae. When the eggs hatch, the larvae molt twice to become infectious L3 larvae. There is only one generation produced in the environment; perpetuation of the free-living cycle requires continued contamination of the environment with human feces. Regardless of the pathway to the production of L3 larvae, human infection occurs when L3 larvae penetrate intact skin when people walk barefoot or in sandals on contaminated soil. Historically, it was believed that the larvae were carried via the bloodstream to the heart and then the lungs, before being coughed up and settling in the small intestine, much like hookworm. It is now believed that migration to the small intestine is random through tissues in the body. Once in the small intestine, the L3 larvae molt twice to become parthenogenetic females. The third pathway the L1 larvae can take is autoinfection. The L1 larvae become L3 larvae while still in the intestinal tract. These L3 larvae penetrate the intestinal epithelium and mature to adult females, which continue the cycle. Autoinfection can lead to chronic disease lasting decades, with the infection largely being kept in check by the host's immune system. If a patient becomes immunocompromised, (e.g. immunosuppression prior to organ transplantation, corticosteroid use), the L3 larvae can disseminate in large numbers to other parts of the body, including the kidneys, skin, and lungs, leading to life-threatening hyperinfection (Page et al., 2018).

Pathogenesis, clinical presentation, and treatment

Infection is usually asymptomatic in immunocompetent, otherwise healthy individuals (Jourdan et al., 2018). L3 larval migration through various organs may result in localized symptoms; a Loeffler's like syndrome may occur with the initial pulmonary migration, whereas a serpiginous urticarial rash called larva currens may be seen during chronic infection. A skin rash ("ground itch") may also be noted at the initial site of skin penetration, like what is seen with hookworm. Immunocompromised individuals are at risk of hyperinfection syndrome, where the host immune response is no longer able to keep the autoinfection cycle under control. In this case, the large migration of L3 larvae from the intestinal tract to the lungs and other organs can result in recurrent sepsis and/or meningitis with bowel bacterial flora, mucosal and pulmonary bleeding, and respiratory collapse (Jourdan et al., 2018). Patients are risk for hyperinfection syndrome include those receiving chemotherapy for hematologic malignancies, patients with human T-cell lymphotropic virus type 1 (HTLV-1) infection, and patients with hypogammaglobinemia (Jourdan et al., 2018). Unlike the other soil-transmitted nematodes, the first line anti-helminthic drug is ivermectin (Medical Letter, 2013).

Diagnosis

The diagnosis of strongyloidiasis is challenging. Historically, strongyloidiasis was diagnosed by the detection of L1 larvae in wet mounts of stool. Unfortunately, this method has low sensitivity. Detection of *S. stercoralis* in stool can be enhanced by methods such as the Baermann technique, or preferably, the stool agar culture (De Kaminsky, 1993; Salazar et al., 1995). Disseminated disease and hyperinfection can be diagnosed by the finding of L3 larvae in respiratory specimens and other body fluids or by the histopathologic examination of biopsy specimens. Adult females, larvae, and eggs may be detected in biopsy specimens of the small intestine. Diagnosis of chronic strongyloidiasis and the screening of patients prior to organ transplantation or immunosuppression for malignancy is performed by antibody detection, although available assays have varying degrees of sensitivity and specificity. NAATs are not widely used for routine clinical diagnosis, but large reference labs and labs in endemic areas may employ them as LTDs.

Microscopy

Although insensitive, the "gold standard" for the diagnosis of strongyloidiasis is the detection of L1 larvae in stool by the O&P examination. Due to the low sensitivity of morphologic analysis, upwards of seven serial stool specimens should be examined (Siddiqui and Berk, 2001). Detection in stool can be enhanced by the Baermann technique. For this method, stool is placed in a coarse mesh screen and placed suspended in a funnel that is filled with warm water. The bottom of the funnel is connected to a clamped tube. L1 larvae in the stool develop to L3 larvae and enter the water, which is drained into the tube, centrifuged, and examined by microscopy. Laboratorians performing this technique must be careful, as live L3 larvae are infectious if they come in contact with skin (Requena-Mendez et al., 2013). The string test (see under *Giardia duodenalis*, above) can also enhance diagnosis.

L1 larvae in stool are 180–380 μm long by 14–20 μm wide. They possess a short buccal canal and a prominent genital primordium (Fig. 14). L3 larvae observed in stool exams (especially if there was a delay in processing), retrieved from a Baermann funnel, or in extraintestinal specimens such as bronchial washings and BALs are longer, measuring upwards of 600 μm in length. They have a notched tail and there is a 1:1 ratio of esophagus to intestine. Unlike with L1 larvae, the form of the buccal canal and genital primordium in the L3 larvae are not diagnostically important (Ash and Orihel, 2007).

Adult females in biopsy specimens of the small intestine are small, measuring 30–40 μm in diameter. They can usually be recognized by their prominent alimentary canal and paired uterine tubes that usually contain eggs in various stages of development. L3 larvae in cross section will reveal tiny, paired lateral alae (Mathison and Pritt, 2018a).



Fig. 14 *Strongyloides stercoralis* L1 (rhabditiform) larva in stool (wet mount, 400 $\times$).

Stool culture

Probably the most sensitive test for diagnosing strongyloidiasis is the agar plate culture (APC) method. For this method, fresh stool is placed in a petri dish containing agar culture medium, such as blood agar. After L1 larvae in the stool become L3 larvae, they migrate over the surface area of the agar plate, carrying bacteria with them. Bacterial growth in the tracks becomes visible to the unaided eye (Fig. 15). Strongyloidiasis should be strongly suspected when these tracks are seen, however the specimen should be confirmed by microscopy, as hookworm L3 larvae will make visible tracks as well. While more sensitive, the APC method has its disadvantages, most notably being time consuming and presenting a safety risk to laboratorians performing the assay (Requena-Mendez et al., 2013).

Antibody detection

Anybody detection is used primarily for diagnosing chronic strongyloidiasis and for screening organ transplant recipients prior to immunosuppressive therapy. Several different serologic methods have been developed for the diagnosis of strongyloidiasis. Unfortunately, few have a sensitivity greater than 80% on average, and that can decrease in immunocompromised patients. Also, several of these assays cross-react with other nematode infections. In patients where the predictive positive value is high, EIA assays are more sensitive than IFA. One commercial assay, the IVD *Strongyloides* Serum Antibody Detection Microwell ELISA kit (IVD Research, Inc., Carlsbad, California) has a sensitivity of 73–100% and a specificity of 29–93%, but may exhibit lower sensitivity in immunocompromised patients. It also exhibits high cross reactivity to other nematode infections. The *Strongyloides* ELISA produced by Bordier Infinity Products (Crissier, Switzerland) was developed using antigens to *S. ratti* and exhibits a sensitivity of 88% and a specificity of 94% in patients without other helminth infections. The specificity drops to 77% in patients with other helminth infections, however (Requena-Mendez et al., 2013). Western blots show promise of high sensitivity, but also struggle with cross reactivity in patients with other helminth infections and are not widely available outside of specialized reference labs (Requena-Mendez et al., 2013). A luciferase immunoprecipitation system (LIPS) using recombinant *Strongyloides* antigen shows high sensitivity (97%) and specificity (100%) with no cross reaction with filarial infections. Unfortunately, the assay is not available outside of specialized reference laboratories (Bisoffi et al., 2014; Requena-Mendez et al., 2013).

Antibody detection can be an important part of the algorithm when screening pre-transplant recipients for strongyloidiasis. Normally potential transplant recipients are screened by EIA. However, given the lower sensitivity of this assay, it is recommended that pre-transplant patients with a negative EIA result but high epidemiological risk for strongyloidiasis or unexplained eosinophilia still get treated prior to immunosuppressive therapy (Mobley et al., 2017; Roxby et al., 2009).

NAATs

Like with many helminth infections, molecular diagnosis of *S. stercoralis* is still not routinely available outside of specialized reference labs. Currently, it is generally not believed that PCR is adequate for screening for *S. stercoralis* as it does not provide sensitivity superior to traditional methods, such as the Baermann technique (Buonfrate et al., 2018). Loop-mediated isothermal amplification (LAMP) assays has been developed which show promise, but are currently not superior to conventional or real-time PCR assays (Watts et al., 2019).

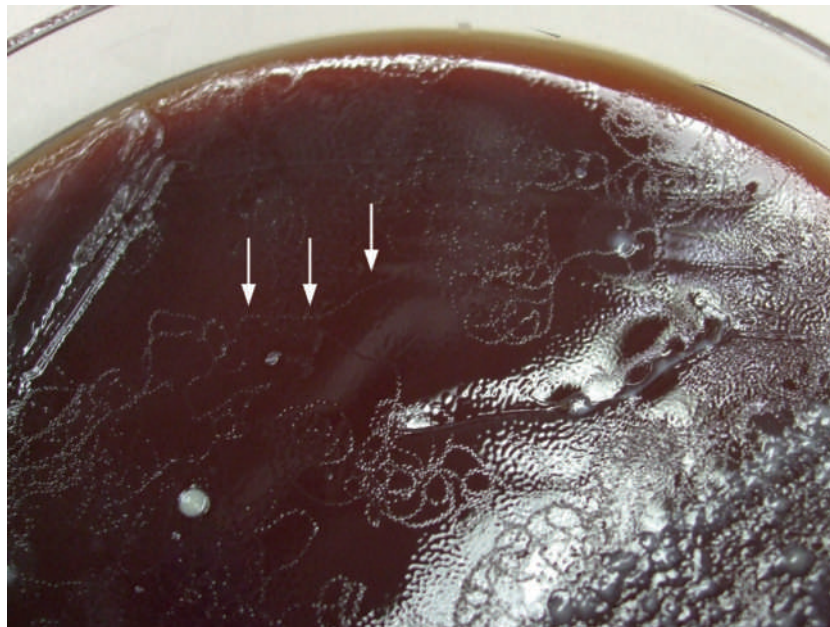


Fig. 15 Bacterial growth along serpiginous *Strongyloides stercoralis* tracks (arrows) on sheep blood agar. This was an incidental finding on a stool bacterial culture.

Trichinella* species (trichinellosis, trichinosis)*Biology and epidemiology**

Trichinellosis (trichinosis) is a zoonotic disease caused by trichuroid nematodes in the genus *Trichinella*. Depending on the taxonomy applied, up to eight species and genotypes have been implicated in human infection. These species, along with their geographic distribution and common sources of human infection, include *T. spiralis* (cosmopolitan; domestic and wild pigs, horses), *T. nativa* (Arctic regions of North America and Eurasia; bears, walrus), *T. britovi* (North America; wild and domestic pigs, wild carnivores, horses), *T. murrelli* (North America; bears, horses), *T. nelsoni* (sub-Saharan Africa; bush pigs and warthogs), *T. pseudospiralis* (cosmopolitan; domestic and wild pigs), *T. papuae* (Papua New Guinea, Thailand; wild pigs), and *Trichinella* genotype T6 (North America; bear, mountain lion) (Gottstein et al., 2009).

Human infection is usually described as originating from a domestic cycle or sylvatic cycle. In the domestic cycle, *Trichinella* cycles between domestic animals, such as pigs and horses, and anthropophilic rodents. Due to improvements in animal husbandry practices in developed countries, the domestic cycle is significantly less common, and is usually seen in home-raised livestock or small community farms rather than large commercial meat producers. In the sylvatic cycle, the worms cycle between wild carnivorous and omnivorous animals, and human infection usually occurs from the ingestion of wild game, such as wild pigs and bears (Mathison and Pritt, 2018b).

Trichinella species have a complex life cycle in which a host serves as a definitive host, intermediate host, and possibly a dead-end host. Infection occurs when L1 larvae are ingested in the undercooked muscle of an infected animal. The larvae are released in the stomach of the host and travel to the small intestine where they penetrate the intestinal epithelium. The L1 larvae molt four times within 48 h to become sexually-mature male and female worms. Mated females release L1 larvae into the lymphatics and the larvae migrate to striated muscle. They penetrate the muscle cells and develop to an infectious form contained within a “nurse cell,” the host muscle cell programmed by the parasite to sustain its existence for a period of time. Of the species that have been implicated in human disease, all except *T. pseudospiralis* and *T. papuae* are encapsulated in the nurse cell. The larvae remain dormant in the nurse cells until the muscle of the host is consumed by a new host. If the host is not eaten, the larvae will eventually calcify and die. Continuous cycles of carnivory are needed to keep the life cycle going. Humans are typically dead-end hosts for *Trichinella* spp. (Gottstein et al., 2009).

Pathogenesis, clinical presentation, and treatment

There are two phases of human infection: the intestinal phase and invasive phase (Gottstein et al., 2009). Some scientists break the invasive phase into 2 parts: the parenteral (blood stream) phase and the muscle encystment phase. Any stage may be asymptomatic, but patients are more likely to present with symptoms when more than a few hundred larvae are released by the adult worms in the intestinal tract. Thus, the initial burden of ingested larvae and the percentage that develop into adult worms are important drivers of clinical manifestations. The infecting *Trichinella* species may also impact extent and duration of observed symptomatology (Gottstein et al., 2009).

The intestinal (enteral) phase is caused the presence of the adult worms and the newly-released larvae in the intestinal mucosa, and is characterized by self-limited abdominal pain and diarrhea appearing approximately 2 days after ingestion. The invasive (parenteral and encystment) phase begins approximately 1 week after infection when the larvae enter the blood stream. Their presence invokes a host inflammatory response characterized by eosinophilia and a Th2-oriented cytokine profile (Gottstein et al., 2009), including production of IgE. Mast cells, monocytes and lymphocytes are also recruited. The resultant release of serotonin, histamine, prostaglandins, and bradykinin causes fever, edema, vasculitis and intravascular thrombosis – hallmarks of acute-stage systemic disease. Characteristic findings include conjunctivitis, periorbital edema and subungual (“splinter”) hemorrhages (Fig. 16). As the larvae penetrate skeletal muscle, muscle pain and elevated serum muscle enzymes (creatine phosphokinase and

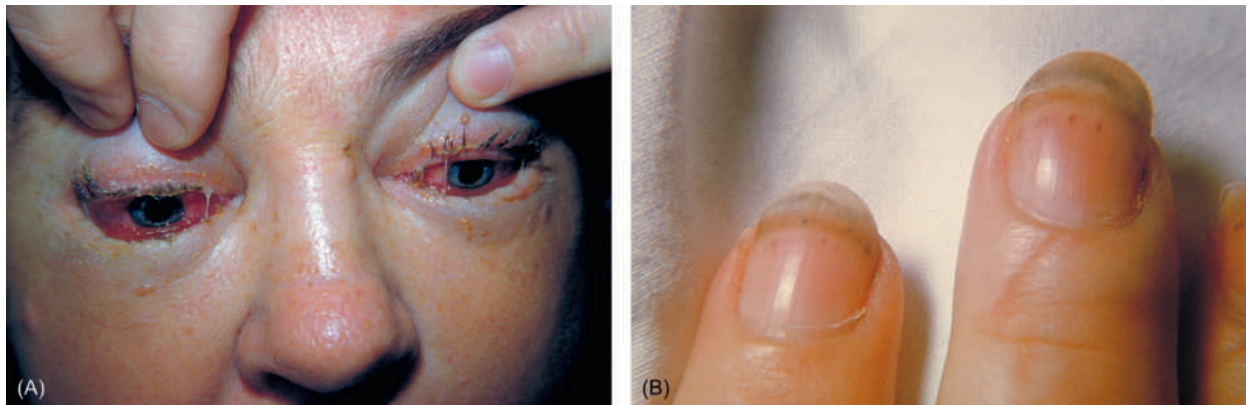


Fig. 16 Characteristic features of acute disseminated trichinosis: periorbital swelling and conjunctivitis (left) and splinter hemorrhages of the nail beds (right). These clinical manifestations are due to the host's immune response to the migrating *Trichinella* sp. larvae. Courtesy of the U.S. Centers for Disease Control and Prevention Public Health Image Library.

lactate dehydrogenase) are observed. These symptoms generally last for 1–3 weeks and may be associated with life-threatening myocarditis, encephalitis, and thromboembolic disease. After 3 weeks, patients enter the encystment (chronic) stage of systemic trichinellosis, which may be characterized by encephalitis and cortical infarcts, as well as secondary bacterial pneumonia and sepsis. Of note, patients may experience persistent numbness, decreased muscle strength, conjunctivitis, and excessive sweating for months to years after infection (Gottstein et al., 2009). Treatment is with albendazole or mebendazole, in conjunction with glucocorticosteroids to decrease the damage caused by the host immune response. Additional treatment may be required for pain management and correction of fluid and electrolyte imbalances (Gottstein et al., 2009; Medical Letter, 2013).

Diagnosis

The diagnosis of trichinellosis is made by considering the patient's clinical presentation in combination with a thorough epidemiological investigation, and confirmed by laboratory testing. Antibody detection is the gold standard for laboratory diagnosis of trichinellosis. Larvae can also be detected in partially-digested muscle specimens or by histopathologic examination, although biopsies are rarely collected when trichinellosis is suspected. Molecular assays are not widely used for primary diagnosis, but may be used for epidemiologic investigations in determining the source of infection.

Microscopy

L1 larvae of *Trichinella* may be detected in muscle biopsies, either by pepsin-digestion methods or histopathology. The pepsin-digestion method involves placing a small sample of muscle in a solution of 0.5% pepsin and 0.7% hydrochloric acid and incubating at 37 °C in a closed petri dish for a minimum of 4 h. After incubation, the partially-digested muscle can be pressed between two glass microscope slides and examined under low magnification for coiled and, for most species, encapsulated larvae (Ash and Orihel, 1987). Larvae in muscle biopsies processed for histopathology are coiled and, with the exception of *T. pseudospiralis* and *T. papuae*, contained within a hyaline cyst. Larvae of *Trichinella* possess typical trichuroid features such as stichocytes and bacillary bands (Mathison and Pritt, 2018a). The presence of a coiled, encapsulated nematode in muscle is pathognomonic for trichinellosis (Fig. 17).

Antibody detection

The primary serologic assay for the clinical diagnosis of trichinellosis is EIA targeting IgG antibodies, which are usually present 12–60 days post infection. Depending on the assay, IgE, IgA, and IgM may be detected, but do not provide diagnostic relevance for routine clinical diagnosis. IFA is also available for diagnosing trichinellosis, but it is more time consuming and expensive than EIA (Gottstein et al., 2009).

NAATs

Molecular diagnosis of trichinellosis is primarily performed on meat samples in order to get a species-level identification for epidemiological purposes. Typically, PCR is used targeting the ITS1 and ITS2 subregions and the expansion segment V region of the rRNA repeat (Gottstein et al., 2009). Recently, a multiplex qPCR assay was developed for the detection and identification of encapsulated North American *Trichinella* species in both human clinical specimens and meat samples (Almeida et al., 2018). Species and genotype data are collected and archived by the International Trichinella Reference Centre (<http://www.iss.it/site/Trichinella/index.asp>).

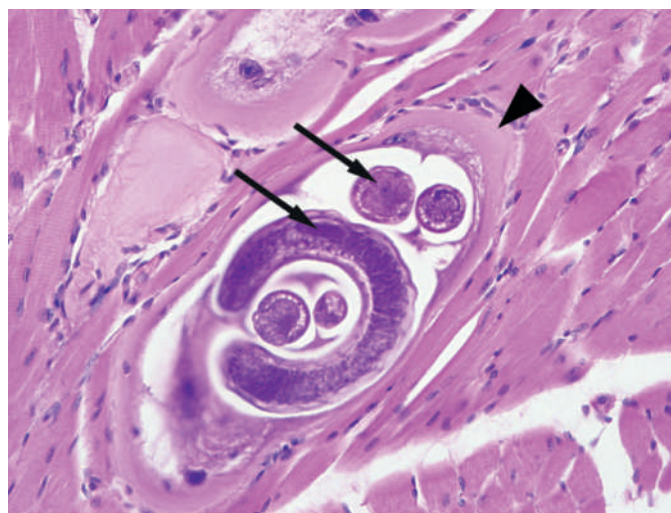


Fig. 17 *Trichinella* sp. larva within a host muscle (“nurse”) cell (hematoxylin and eosin stained, 400 ×). The coiled larva is seen in both longitudinal and cross sections in this histologic preparation. Note the stichosome (arrows) and thick outer capsule (arrow head). From Mathison BA and Pritt BS (2018) Chapter 5: Parasitic diseases. In: *Atlas of Fundamental Infectious Diseases Histopathology: A Guide for Daily Practice*. Pritt BS (ed.). Northfield, IL: College of American Pathologists.

Angiostrongylus costaricensis (human abdominal angiostrongyliasis)

Human abdominal angiostrongyliasis (HAA) is caused by *Angiostrongylus costaricensis*, a zoonotic nematode that occurs throughout much of Latin America, from southern Texas in the US to South America. Most human cases have been reported Costa Rica, with additional cases from Honduras, Venezuela, Brazil, Panama, and several Caribbean islands (Romero-Alegria et al., 2014). *Angiostrongylus costaricensis* has a complex two-host life cycle. The natural definitive hosts are rodents, primarily members of the family Cricetidae (New World rats and mice), Heteromyidae (kangaroo rats), and Muridae (Old World rats and mice) (Dard et al., 2018). Adults reside in the ileocecal mesenteric arteries of the definitive host. Eggs hatch in the ileum and L1 larvae are shed into the environment via the feces. L1 larvae are ingested by an appropriate intermediate host, which are terrestrial mollusks, including snails, slugs, and semi-slugs. In the mollusk host, the parasite molts twice to become in infectious L3 larva. A new rodent host becomes infected after eating infected mollusks, and the L3 larvae molt twice to become adults in the mesenteric vessels. Humans also become infected after the ingestion of raw or undercooked mollusks or parts thereof on contaminated produce. The parasite behaves similarly in the human host as it does the rodent host (Ortega and Sterling, 2017).

The hallmark clinical presentation of HAA is eosinophilic enteritis. Patients may present with fever, anorexia, vomiting, and pain in the right iliac fossa, right flank, or upper right quadrant, and symptoms may mimic appendicitis. Pathologically, there are three principle findings. First is a heavy infiltration of eosinophils in the intestinal wall, especially in the intestinal mucosa. Next is a granulomatous reaction surrounding enteric vessels consisting primarily of giant cells, epithelioid cells, and eosinophils. Third is eosinophilic vasculitis of the arteries, veins, capillaries, and lymphatics, often with accompanying epithelial ulceration (Ortega and Sterling, 2017; Mathison and Pritt, 2018a). There is no proven anthelmintic treatment for HAA.

The diagnosis of HAA is made by the finding of worms and their eggs in biopsy specimens processed for histopathology. Adult worms are usually localized in blood vessels while embryonated and unembryonated eggs are usually found in associated tissues. Adults are characterized by coelomyarian/polymyarian musculature, large, nucleated intestinal cells, and conspicuous dome-shaped lateral chords. Gravid females have paired uterine tubes that usually contain eggs in various stages of maturation. Eggs are usually thin-shelled and measure 68–74 µm long by 46–48 µm wide (Mathison and Pritt, 2018a).

Trematode infections

Several trematodes (i.e., flukes) can infect the human gastrointestinal tract, including those that infect the intestine, biliary tree, and mesenteric blood vessels. All but the schistosomes (see below) are acquired through ingestion of raw or undercooked food containing the infective form of the parasite (metacercariae) for humans. These food-borne trematodiasis are classified as NTD by the WHO, and are estimated to cause 2 million life years lost to death and disability each year (WHO, 2020a). As zoonotic infections, they also have a significant economic impact due to losses in aquaculture and livestock.

The food-borne trematodes have a complex life cycle that includes two non-human intermediate hosts, the first of which is always a freshwater snail, and a definitive host (e.g., humans and other animals). Transmission to humans is largely dependent on culinary practices and access to fresh water, and thus infections are often limited to specific geographic regions of endemic countries. The extent of symptomatology varies with the worm burden, and many light infections are asymptomatic. The anti-helminthic drug of choice is praziquantel for all trematodiasis except for *Fasciola hepatica* infection (fascioliasis).

Fasciolopsis buski (fasciolopsiasis)

Biology and epidemiology

Frequently known as the “giant intestinal fluke,” *Fasciolopsis buski* is a large intestinal trematode endemic to freshwater habitats in Southeast Asia. Natural reservoirs for human infection are primarily pigs, but also non-human primates, dogs, and rabbits (Ma et al., 2017). Hot spots of human infection include China, Nepal, Cambodia, India, Laos, Myanmar, Taiwan, Thailand, and Vietnam (Sah et al., 2019).

Like all trematodes, *F. buski* has a complex life cycle involving multiple hosts. Adult flukes reside in the small intestine of the definitive host (Fig. 18). Unembryonated eggs are shed into the environment via the feces. Eggs in freshwater develop over a period of 3–7 weeks, resulting in the formation of a first stage larva (miracidium) within each egg. The eggs hatch and the miracidia infect an appropriate snail host, which for *F. buski* are primarily members of the genera *Segmentina* and *Hippeutis*. Within the snail, the parasite undergoes several cycles of asexual reproduction, resulting in the formation of cercariae. The cercariae leave the snail host and encyst on freshwater vegetation as metacercariae. The definitive host, including humans, becomes infected after eating uncooked vegetation harboring metacercariae or from drinking water contaminated with liberated metacercariae (Sah et al., 2019).

Pathogenesis, clinical presentation, and treatment

As with all of the intestinal flukes, the clinical presentation varies by the extent of infection. The adult flukes attach to the intestinal wall with their oral and ventral suckers, causing localized hemorrhage, inflammation and ulceration. The flukes may also interfere with absorption, and their large size can cause transient small bowel obstruction. Infection is frequently asymptomatic in patients with a low worm burden (Maguire, 2020), whereas heavy infections (e.g., >100 worms) are more likely to be clinically significant. Common symptoms include epigastric pain, nausea, and diarrhea (Sah et al., 2019). Edema of the extremities and face may be seen in heavy infections due to protein loss from malabsorption, protein-losing enteropathy and/or a hypersensitivity reaction to worm metabolites (Maguire, 2020). Eosinophilia and anemia are most pronounced in heavy infections, but may be seen in light infections as well. Treatment is with praziquantel (Medical Letter, 2013).

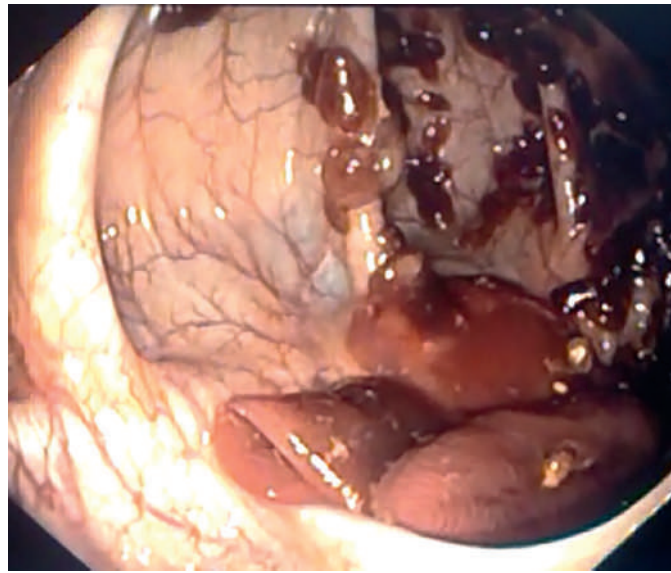


Fig. 18 *Fasciolopsis buski* adult flukes seen in the large intestine during colonoscopy. The smaller flukes are *Gastrodiscoides hominis*. Courtesy of Dr. Sandeep T.

Diagnosis

Fasciolopsiasis is diagnosed by the finding of eggs in wet mounts of stool (Fig. 10) or the gross examination of adult flukes removed by endoscopy (Figs. 18 and 19). In heavy infections, flukes may also inhabit the large intestine.

Eggs of *F. buski* are 130–150 μm long by 63–90 μm wide, have a small, inconspicuous operculum, and are unembryonated when shed in stool (Fig. 10). The eggs are morphologically nearly indistinguishable from those of *Fasciola hepatica* and it is common for laboratories to slash call the two species when reporting results (e.g. *Fasciola hepatica*/*Fasciolopsis buski* eggs). The eggs of *F. buski* are also very similar to other large intestinal flukes, such as *Gastrodiscoides hominis* and *Echinostoma* spp. A definitive identification can be challenging in endemic areas where multiple species coexist.

F. buski is the largest of the intestinal flukes. Adults removed by endoscopy are large, flat flukes, measuring 20–75 mm long by 20 mm wide (Figs. 18 and 19). The adults are similar to those of *F. hepatica*, but the location in the host can be helpful in separating them, as *F. buski* lives in the lumen of the small intestine while *F. hepatica* lives in the bile ducts of the liver. Also, the anterior end of *F. buski* is rounded when the specimen is relaxed, compared with that of *F. hepatica* which has a prominent cephalic cone.

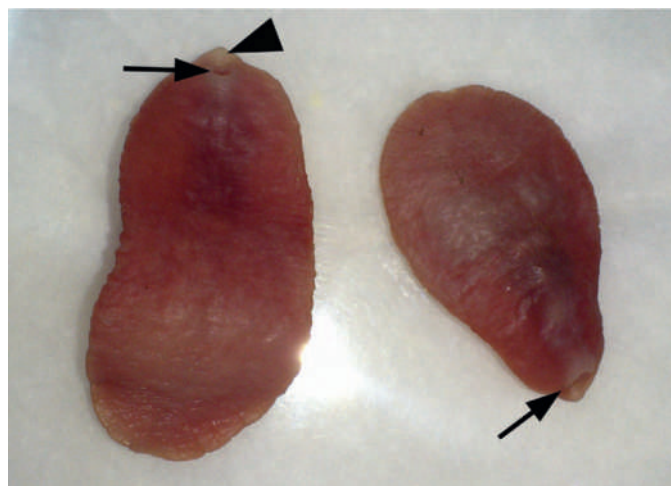


Fig. 19 Two *Fasciolopsis buski* flukes removed from the case shown in Fig. 18. The ventral aspect of the flukes is shown, revealing the acetabulum (ventral sucker; arrows). The mouth and oral sucker are directly anterior to the ventral sucker (arrow head). Courtesy of Dr. Sandeep T.

Heterophyes heterophyes, Metagonimus yokogawai, and other small intestinal trematodes (heterophyiasis, metagonimiasis)

Biology and epidemiology

Nearly 30 species of small trematodes have been reported causing zoonotic intestinal human infections in Asia and other parts of the world. Genera implicated in human disease include *Heterophyes*, *Metagonimus*, *Haplorchis*, *Pygidiopsis*, *Heterophyopsis*, *Stellantchasmus*, *Centrocestus*, *Stictodora*, *Acanthotrema*, *Apophallus*, *Procerovum*, *Ascocotyle*, and *Cryptocotyle* (Chai and Jung, 2017). The two species that are most familiar in the clinical realm are *Heterophyes heterophyes* and *Metagonimus yokogawai*. Human infection with *H. heterophyes* occurs in Egypt, Sudan, Palestine, Turkey, India, Japan, and Korea, while human infection with *M. yokogawai* occurs in China, Japan, Korea, Taiwan, Russia, and India (Chai and Jung, 2017).

Regardless of the species implicated in human disease, all of these small intestinal trematodes have a similar multi-host life cycle. Adults reside in the lumen of the small intestine of the definitive host, which in nature are fish-eating mammals and birds. Fully-embryonated eggs are shed into freshwater via the feces. The eggs are ingested by an appropriate snail host and the liberated miracidia penetrate the snail's alimentary canal. The parasite undergoes several cycles of asexual reproduction resulting in the formation of cercariae. The cercariae leave the snail host and infect a fish. In the flesh of the fish, the cercariae encyst as metacercariae, and the definitive host becomes infected after eating raw or undercooked infected fish (Chai and Jung, 2017).

Pathogenesis, clinical presentation, and treatment

As with fasciolopsiasis, the clinical manifestations are dependent on the number of flukes present (Maguire, 2020). The flukes attach to the small intestinal wall, resulting hemorrhage, inflammation and ulceration. Light infections may be asymptomatic, although eosinophilia may be noted. Moderate to heavy infections may result in abdominal pain and diarrhea. The drug of choice is praziquantel (Medical Letter, 2013).

Diagnosis

Regardless of the species, all of these small intestinal trematodes are diagnosed by the detection of eggs in wet mounts of stool. Eggs are often shed in low numbers, and multiple stool specimens may need to be collected and examined for a reliable diagnosis. The eggs of all the species are very similar in size and shape, and in endemic areas where multiple species coexist, a definitive identification may not be possible.

On average, the eggs are 20–30 µm long by 15–17 µm wide and are fully embryonated when shed in feces. The operculum is usually obvious, but the eggs do not have prominent opercular “shoulders” as are seen with some trematodes. Also, the abopercular end does not possess a knob. Because of the morphologic similarity of the eggs of the various species, it is common for laboratories to slash-call names (e.g., *Heterophyes heterophyes*/*Metagonimus yokogawai* eggs). The eggs of small intestinal flukes are also very similar to the eggs of small opisthorchid liver flukes in the genera *Clonorchis* and *Opisthorchis*, although separating the eggs of heterophyid and opisthorchiid trematodes can usually be achieved by the presence of prominent opercular shoulders and an abopercular knob in the latter (Ash and Orihel, 2007).

Miscellaneous intestinal trematodes

Nanophyetus salmincola (nanophyetiasis)

Nanophyetus salmincola is an intestinal fluke of fish-eating mammals and birds in the Pacific Northwest of North America. Humans become infected after the ingestion of raw or undercooked salmonid fish containing infective metacercariae. The clinical picture of nanophyetiasis is not well-described, but patients have presented with generalized gastrointestinal symptoms, such as abdominal pain, diarrhea, nausea, vomiting, increased number of bowel movements, as well as weight-loss, fatigue, and increased eosinophilia (Mathison and Pritt, 2018b). Praziquantel is the drug of choice (Fritzsche et al., 1989). *Nanophyetus salmincola* carries rickettsiae in the genus *Neorickettsia* that can cause a fatal disease in dogs called “salmon poisoning”; humans do not appear to be affected by these bacteria (Greiman et al., 2016).

Nanophyetiasis is diagnosed by the detection of eggs of *N. salmincola* in wet mounts of stool. The eggs are brown, broadly oval, and measure 64–97 µm long by 34–55 µm wide. The operculum is inconspicuous and the abopercular end is noticeably thickened. Eggs of *N. salmincola* need to be distinguished from those of *Paragonimus* spp. and diphylobothriid cestodes (Ash and Orihel, 2007).

Echinostoma spp. and related (echinostomiasis)

Echinostomiasis is a zoonotic disease caused by intestinal trematodes in the family Echinostomatidae (echinostomes). Most cases of human infection have been attributed to members of the genus *Echinostoma*, but several other genera have been implicated in human disease, including *Acanthoparyphium*, *Artyfechinostomum*, *Echinochasmus*, *Euparyphium*, *Himasthla*, *Hypoderaeum*, and *Isthmiophora*. Human cases have been reported nearly worldwide, but endemic foci of human infection are China, India, Indonesia, Korea, Malaysia, the Philippines, and Thailand (Toledo and Esteban, 2016).

Like all trematodes, echinostomes have a complex multi-host life cycle. The natural definitive hosts are aquatic and semi-aquatic mammals and birds. The second intermediate hosts vary greatly depending on the species, and animals implicated in human infection include snails, clams, mussels, freshwater crustaceans, frogs and tadpoles, snakes, and freshwater fish. Water contaminated with metacercariae has also been suggested as a source of human disease (Toledo and Esteban, 2016).

Clinically, echinostomes may cause more severe disease than other intestinal trematodes due to physical damage to the intestinal epithelium caused by the presence of spines surrounding the oral sucker on adult worms. Symptoms include epigastric and abdominal pain, malaise, weight loss, anorexia, nausea, vomiting, and diarrhea. Pathologically, the intestinal epithelium shows mucosal ulceration and erosion, bleeding, and chronic gastritis. Echinostomes have been implicated in adenocarcinoma (Toledo and Esteban, 2016). Praziquantel is the drug of choice (Toledo and Esteban, 2016).

Diagnosis of echinostomiasis is made by the finding of eggs in wet mounts of stool. Eggs are broadly-oval, unembryonated when shed in stool, and have an inconspicuous operculum. There is a broad size range depending on the species, measuring 77–130 μm long by 52–80 μm wide. Because of this broad range, there is considerable overlap with other trematodes, including *Fasciola*, *Gastrophilus*, *Gastrodiscoides*, *Neodiplostomum*, and *Paragonimus*, and a definitive diagnosis may be challenging (Toledo and Esteban, 2016; Ash and Orihel, 2007).

Neodiplostomum seoulense (Neodiplostomum infection)

Neodiplostomum seoulense is a zoonotic intestinal trematode endemic to China and Korea. The natural definitive hosts are rats and the second intermediate hosts are amphibians. Human infection has been attributed to the ingestion of undercooked snakes which serve as paratenic hosts. The clinical picture is not well understood, but patients have presented with acute abdominal pain and fever (Chai and Jung, 2020). Diagnosis is made by the detection of eggs in wet mounts of stool. The eggs are morphologically very similar to those of the echinostomes, measuring 80–102 μm long by 51–63 μm wide, are unembryonated, and possess an inconspicuous operculum (Ash and Orihel, 2007).

Cestode infections

Dibothriocephalus spp., Diphyllbothrium spp., and Adenocephalus spp. (diphyllbothriasis, fish tapeworm disease)

Biology and epidemiology

Diphyllbothriasis is a zoonotic cestode infection. Historically, human infection was attributed to members of the genus *Diphyllbothrium*, until recent morphologic and molecular studies resulted of the splitting of *Diphyllbothrium* into multiple genera. The species implicated in human disease, and their current taxonomic status and broad geographic distributions, are *Adenocephalus pacificus* (southern South America, southern Asia), *Dibothriocephalus latus* (Holarctic), *D. nihonkaiensis* (Asian Pacific, eastern Russia, Pacific Northwest), *D. dendriticus* (Holarctic), *D. dalliae* (Alaska and Siberia), *D. ursi* (North America), *Diphyllbothrium stemmacephalum* (circumpolar), and *D. balaenopterae* (circumpolar). Two additional species implicated in human disease, "*Diphyllbothrium*" *cordatum* and "*D.*" *lanceolatum*, both circumpolar in distribution, are tentatively placed in *Diphyllbothrium* but will probably undergo generic transfer in the future (Kuchta et al., 2015; Waeschenbach et al., 2017).

Like all tapeworms, the diphyllbothriid cestodes have a complex, multi-host life cycle. Adults reside in the small intestine of the definitive host, which in nature are fish-eating mammals and birds. The adults are attached to the intestinal wall by a specialized scolex, while the body (strobila) elongates from the mitotically-active neck region, forming individual units called proglottids. Gravid proglottids, and eggs liberated therefrom, are shed into water (freshwater or marine, depending on the species) via the feces. The eggs are shed unembryonated and after 18–20 days under optimum conditions, release the first instar larva, the coracidium. The coracidium infects a microscopic crustacean and develops into a proceroid larva. The crustacean is eaten by a small fish and develops into a plerocercoid larva (sparganum). A larger, predaceous fish eats the smaller fish and becomes a paratenic host for the plerocercoid larva. The definitive host, including humans, becomes infected after eating undercooked or raw fish harboring plerocercoid larvae. The plerocercoid larva is liberated in the upper small intestine, attaches to the intestinal mucosa via the scolex, and matures to adulthood. Eggs appear in the stool of the definitive host approximately 5–6 weeks post infection (Scholz et al., 2019).

The three species that account for the majority of human infections are *D. latus*, *D. nihonkaiensis*, and *A. pacificus*. Common fish sources of human infection for *D. latus* in North America are pikeperch and walleye and in Europe are perch, pike, and burbot. Common fish sources of human infection for *D. nihonkaiensis* in the North Pacific are salmonid fishes, primarily cherry, pink, chum, and sockeye salmon (Scholz et al., 2009). The epidemiology of *A. pacificus* is less understood. In coastal Peru, infection occurs after the consumption of dishes containing raw seafood, such as ceviche, tiradito, and chingurito, but the host fish is not always known. Plerocercoid larvae of *A. pacificus* have been identified in Eastern Pacific bonito, Atlantic Spanish mackerel, lorna drum, and Peruvian banded croaker, among several other phylogenetically unrelated fish (Kuchta et al., 2015).

Pathogenesis, clinical presentation, and treatment

An estimated 80% of patients with diphyllbothriasis are asymptomatic (Scholz et al., 2009). In these cases, infection may only come to the patient's attention when a large chain of proglottids is passed through the anus. When present, symptoms commonly include abdominal pain, diarrhea, constipation, and fatigue. The latter may be due to a unique feature of some parasites, in which 80% of the host's vitamin B₁₂ intake is absorbed, resulting in megaloblastic anemia (Scholz et al., 2009). Treatment is with praziquantel, with niclosamide as an alternative therapy (Medical Letter, 2013). Vitamin B₁₂ replacement may also be indicated.

Diagnosis

The diagnosis of diphyllbothriasis is made by the detection of eggs in wet mounts of stool (Fig. 10) or the gross examination of adult tapeworms and their proglottids expelled in stool of passed through the anus (Fig. 20). The cestodes causing diphyllbothriasis are the longest to infect humans, reaching lengths of more than 15 m! Chains of proglottids will occasionally break free from the strobila (body) and recovered by the patient.

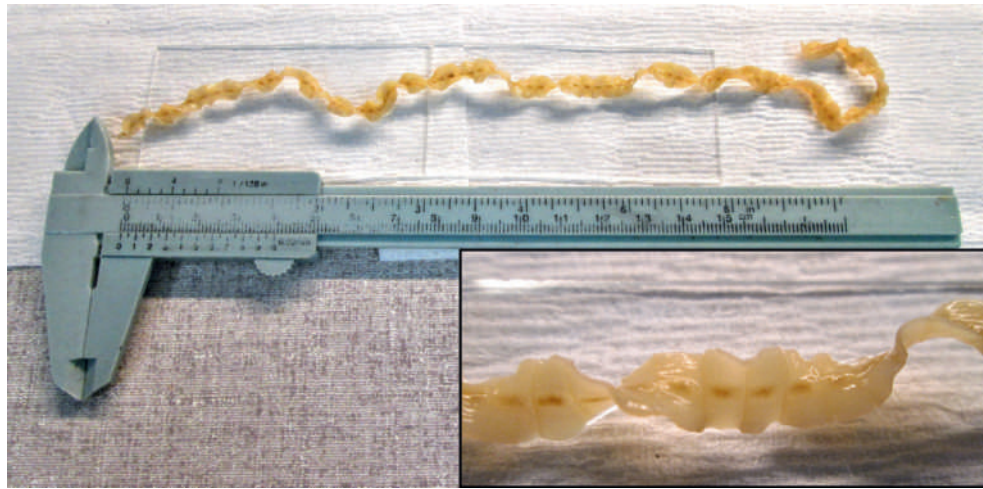


Fig. 20 Chain of *Diphyllobothrium/Dibothriocephalus* proglottids passed by an otherwise asymptomatic individual. The individual proglottids are wider than they are long, and the dark central uterus is easily appreciated. Courtesy of Phong Duong.

Eggs of all diphylobothriid cestodes are similar in size and shape, and a genus or species-level identification cannot be made by egg morphology. However, a definitive identification is not needed for clinical patient management. The eggs measure 58–75 μm long by 40–50 μm wide, have a moderately thick shell, and are unembryonated when shed in feces (Fig. 10). Unlike other intestinal cestodes of humans, the eggs are operculated, as seen in the eggs of most trematodes. For the most part, the eggs of diphylobothriid cestodes do not overlap in size with those of trematodes that routinely infect humans. Diphylobothriid eggs usually possess a visible abopercular knob, which is also not seen on the eggs of most human trematodes except for those of *Opisthorchis*, *Clonorchis*, and other small liver flukes (Ash and Orihel, 2007).

Proglottids shed in stool are usually in short to long chains (Fig. 20). The mature proglottids are usually broader than long, measuring on average 3.0 mm long by 11.0 mm wide. They possess a characteristic centrally-located rosette uterus, which in a gross specimen may appear as a dark spot in the center of an otherwise pale proglottid. The genital pore is on the mid-ventral surface and the testes are laterally located. The scolex is rarely seen in clinical specimens, as it is small and remains attached to the small intestinal mucosa unless the patient receives antihelminthic therapy. It has dorsal and ventral longitudinal grooves (bothria) used for attachment to the intestinal mucosa of the definitive host (Ash and Orihel, 2007). A genus or species-level identification is possible by analyzing the scolex or the sexual structures of the proglottids, but most clinical laboratories lack the expertise and reference materials to make such an identification.

Taenia saginata, Taenia solium, Taenia sui hominis (intestinal taeniasis)

Biology and epidemiology

Intestinal taeniasis is caused by cestodes in the genus *Taenia*. Three species cause human disease: *T. saginata* (the “beef tapeworm”), *T. solium* (the “pork tapeworm”), and *T. sui hominis* (syn. *T. asiatica*, the “Asian tapeworm”). Taeniasis is classified as an NTD by the WHO (WHO, 2020a). *Taenia saginata* occurs nearly worldwide where raw or undercooked beef is eaten, with a higher prevalence in resource-poor areas of Central Asia, Middle East, and sub-Saharan Africa. *Taenia solium* also has a very wide distribution wherever pigs are raised, but has a higher prevalence in resource-poor areas of Latin America, Africa, India, and Southeast Asia. Neither *T. saginata* nor *T. solium* have a high natural prevalence in North America and Europe where better sanitation and animal husbandry practices occur (Ortega and Sterling, 2017). *Taenia sui hominis*, which historically has been classified as a subspecies of *T. saginata*, is endemic to Southeast Asia with foci of human infection in China, Nepal, Thailand, Sumatra, Indonesia, Taiwan, Korea, and Japan (Ale et al., 2014).

All three species have a complex, two-host life cycle. The definitive hosts are humans, and adult tapeworms reside in the lumen of the small intestine where they are attached to the intestinal wall via a specialized scolex. Proglottids, or eggs liberated therefrom, are shed into the environment in the feces. The eggs are fully-embryonated when passed in feces and contain an infectious first-stage larva called an oncosphere (Fig. 10). Eggs contaminate the environment and are eaten by an appropriate intermediate host (cattle for *T. saginata*; pigs for *T. solium* and *T. sui hominis*) when grazing or foraging. The eggs hatch in the small intestine of the intermediate host, and the oncosphere penetrates the intestinal wall, enters circulation, and is carried to musculature. In the muscle of the intermediate host, the oncosphere develops into the second-stage larva called a cysticercus. After 60–90 days, it reaches its maximum size of approximately 1.0 cm and consists of a protoscolex contained within a thin-walled bladder. A human becomes infected after eating raw or undercooked beef or pork harboring cysticerci. In the small intestine, the liberated cysticercus evaginates and the protoscolex penetrates the intestinal mucosa. The protoscolex matures into the scolex and proglottids start to develop, forming the strobila made up of individual proglottids. Maturation to a sexually-mature adult cestode takes approximately

10–12 weeks. While the adult tapeworms are hermaphroditic and can produce eggs by themselves, the preferred method of reproduction is cross-fertilization between two individual worms in the same host. After approximately 2 months post infection, gravid proglottids break off the strobila and are shed into the environment (Ortega and Sterling, 2017).

Taenia solium is unique among the *Taenia* species as it can cause larval infection (cysticercosis) in the human host after the consumption of eggs in food or fomites contaminated with human feces. Essentially, the human assumes the role of the intermediate host and harbors the cysticerci larvae in musculature and other organs. This is a more dangerous form of infection than taeniasis, as the cysticerci have a predilection for the CNS, resulting in an often debilitating or fatal disease known as neurocysticercosis (Ortega and Sterling, 2017).

Pathogenesis, clinical presentation, and treatment

Patients with intestinal taeniasis are usually asymptomatic (Okello and Thomas, 2017). In this setting, infection may only come to medical attention when the patient passes proglottids through the anus. Freshly-passed proglottids are motile, are may be seen on the surface of stool or found in the undergarments. When present, symptoms commonly include abdominal pain and weight loss. Rarely, proglottids may obstruct the small bowel or biliary tree. Treatment is with praziquantel, with niclosamide as an alternative therapy (Medical Letter, 2013).

Diagnosis

Intestinal taeniasis is diagnosed by the detection of eggs in wet mounts of stool, gross examination of adult tapeworms and proglottids, and coproantigen testing. Molecular testing is only available in specialized reference labs, usually in endemic areas.

Microscopy

The eggs of all three *Taenia* species are morphologically indistinguishable from one another. They are spherical, measure 31–43 μm in diameter, and have a thick, striated shell (Fig. 10). They eggs are fully embryonated and contain an oncosphere with six refractile hooklets. Identification to the species level requires examination of mature proglottids and/or the scolex.

Adults of *Taenia* are large worms, measuring 2–8 m in length (Fig. 21). The scolex of *T. solium* has four suckers and an armed rostellum with two rows of hooklets (usually 26 in total, 13 large and 13 small); the scolex of *T. saginata* also has four suckers but lacks the armed rostellum (snout-like protrusion with a circular row of hooklets), (Fig. 22). Mature proglottids of both species are longer than wide and possess a prominent lateral genital pore. Grossly, the proglottids of *T. saginata* and *T. solium* are morphologically identical, and the number of primary uterine branches need to be counted to identify the species. Proglottids may be pressed between two slides and backlighted in order to count the branches. This may be facilitated with clearing in lactophenol. If needed, the proglottids can be injected with India ink to enhance visualization of the uterine branches after clearing with lactophenol. Proglottids can also be cleared, mounted and stained with carmine to visualize the uterine branches. *Taenia saginata* has 12–30 primary uterine branches coming off one side of the central uterine stem, while *T. solium* has 7–13 primary uterine branches. The primary uterine branches of *T. saginata* tend to be thin, while those of *T. solium* tend to be thicker. *Taenia asiaticus* has morphologic features similar to both species, with proglottids similar to *T. saginata*, but an armed rostellum similar to *T. solium* (Fig. 23), (Ash and Orihel, 2007). In areas where *T. sui hominis* occurs with other species, the morphologic criteria need to be scrutinized in detail in combination with an epidemiologic investigation, since *T. sui hominis* appears nearly-identical to *T. saginata*.

Until a confident identification is made, it is imperative that laboratorians use proper PPE when handling unknown cestode proglottids, due to the infectious nature of *T. solium* eggs.



Fig. 21 *Taenia saginata* adult worm measuring 4 m in length. Note the small size of the scolex, and the mature proglottis (left end) that are longer than they are wide. Courtesy of the U.S. Centers for Disease Control and Prevention Public Health Image Library.



Fig. 22 Scolex (head) and neck region of a *Taenia solium* adult. The scolex contains 4 suckers (arrow points to 1 of the 4) and a prominent snout (rostellum) with a row of hooklets. The suckers and hooklets are used for attachment to the small intestinal mucosa. The scolex of *Taenia saginata* also has 4 suckers but lacks the rostellum with hooklets. Courtesy of the U.S. Centers for Disease Control and Prevention Public Health Image Library.

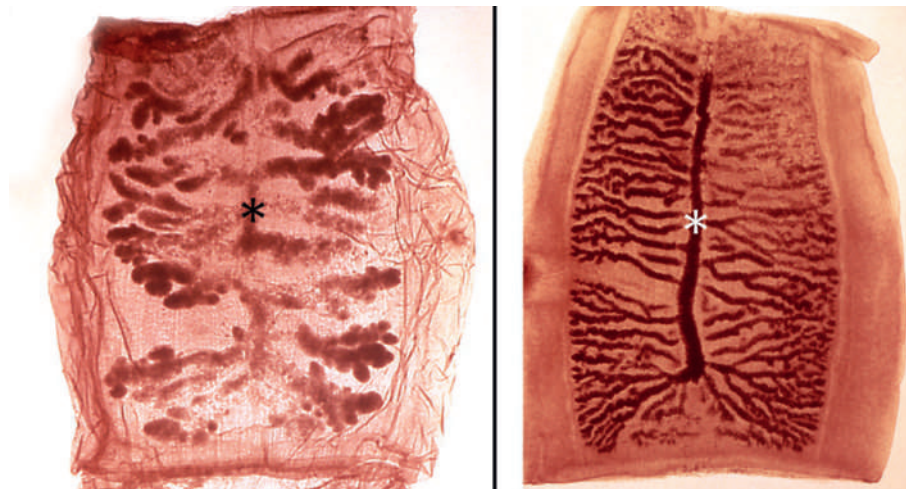


Fig. 23 Carmine-stained proglottids of *Taenia solium* (left) and *T. saginata* (right). The two species can be differentiated by counting the primary uterine branches that originate from one side of the uterine stem (marked by an “*”): *T. saginata* has 12–30 primary uterine branches, while *T. solium* has 7–13. Courtesy of the U.S. Centers for Disease Control and Prevention Public Health Image Library.

Coproantigen testing

Coproantigen assays allow for *Taenia*-specific diagnosis targeting parasite metabolic byproducts in stool. Most platforms are an antigen-capture EIA using antibodies targeting somatic or excretory-secretory antigens. The specificity and sensitivity vary based on the platform and quality of reagents, but are generally high for a genus-level identification. Target antigens disappear from stool post-treatment, so coproantigen testing can be used to monitor treatment success (Mwape and Gabriël, 2014). Coproantigen assays are usually only available in large reference laboratories or laboratories in endemic areas.

Most coproantigen assays are only specific at the genus level, even though they are primarily used to detect *T. solium* in endemic areas. Researchers in Peru have developed an assay that uses polyclonal antibodies against whole-worm extract and excretory-secretory antigens from *T. solium* in a hybrid sandwich-EIA. This assay is reported to demonstrate 95% sensitivity and 100% specificity to *T. solium* with no apparent cross-reactivity to *T. saginata* or *T. suihominis* (Guezala et al., 2009).

Hymenolepis nana, *Hymenolepis diminuta* (hymenolepiasis)

Biology and epidemiology

Hymenolepiasis is a zoonotic cestode infection caused by rodent parasites of the genus *Hymenolepis*. Two species implicated in human disease are the “dwarf tapeworm” *H. nana* and the “rat tapeworm” *H. diminuta*. Both species have a nearly worldwide distribution, but are more commonly seen in children in resource-poor regions of the world (Thompson, 2015; Tiwari et al., 2014). *Hymenolepis nana* is more commonly reported due to its ability to directly infect a new host in the absence of an intermediate host. In developed countries, *H. diminuta* is very rare, and usually only seen in rural or degraded areas (Marangi et al., 2003).

In the natural life cycle, both *H. nana* and *H. diminuta* are intestinal parasites of rodents, and adults reside in the small intestine of the rodent host. Gravid proglottids typically disintegrate while in the lumen of the intestine and only eggs are usually shed in feces. Eggs are eaten by an insect intermediate host, commonly beetles, and the oncosphere develops into a cysticercoid larva. Rodents and humans become infected after eating insects harboring cysticercoid larvae. Children are at particular risk, due to their habit of putting objects, such as insects, in their mouths. *Hymenolepis nana* is unique in that it can bypass the intermediate host, and humans can also become infected by ingestion of eggs on contaminated fomites. In this direct cycle, the eggs hatch in the lumen of the small intestine to release an oncosphere, and the oncosphere invades an intestinal villus. After approximately 4 days, it develops into a cysticercoid larva. The cysticercoid larva ruptures, releasing it from the villus, and then attaches to the intestinal mucosa and develops in to an adult. Adult worms attach between intestinal villi of the mucosa using hooklets and an armed rostellum, much like the scolex of *Taenia solium*. The pre-patent period takes about 4 weeks (Thompson, 2015).

Pathogenesis, clinical presentation, and treatment

Hymenolepis spp. cause ongoing damage to the host during the cysticercoid stage of autoinfection (*H. nana*), as well as attachment of the adult worms to the intestinal mucosa (*H. nana* and *H. diminuta*) (Thompson, 2015). Infected patients are commonly thought to be asymptomatic, although *Hymenolepis* infection may result in poor growth and development in children, particularly in cases of heavy infection or in the presence of other parasitic infections. The anti-helminthic drug of choice is praziquantel, with niclosamide as an alternative therapy (Medical Letter, 2013).

Diagnosis

Hymenolepiasis is diagnosed by the finding of eggs in wet mounts of stool (Fig. 10). Because the proglottids usually disintegrate while in the lumen of the small intestine, they are rarely seen in clinical specimens. However, adult tapeworms are sometimes removed by endoscopy and can be identified by gross morphology (Fig. 24).

Eggs of *H. nana* are ovoid, measuring 30–47 µm in diameter and have a thin, hyaline shell. The oncosphere has six hooklets and is surrounded by a membrane with bipolar thickenings. Four to eight filaments arise from these polar thickenings and extend into the space between the oncosphere and the outer shell. Eggs of *H. diminuta* are larger, measuring 60–85 µm in diameter, and have a thicker outer shell. The membrane surrounding the oncosphere lacks polar filaments (Ash and Orihel, 2007).

Adults removed by endoscopy are relatively small compared to *Taenia* spp. and the diphylobothriid cestodes. *Hymenolepis nana* is 2.5–4.0 cm long and has a scolex with four suckers and an armed rostellum with a ring of 20–30 hooklets. *Hymenolepis diminuta* is larger, measuring 20–60 cm long, and has an unarmed scolex. In both species, gravid proglottids are wider than long and are usually obviously craspedote (overhanging, like roof shingles) (Ash and Orihel, 2007).

Dipylidium caninum (dipylidiasis, dog tapeworm disease)

Biology and life cycle

Dipylidiasis, also commonly referred to as dog tapeworm disease or double-pored dog tapeworm disease, is caused by the zoonotic cestode, *Dipylidium caninum*. The disease occurs worldwide, but the sparsity of case reports and the asymptomatic nature of most infections probably belies its true prevalence. Most published reports have come out of the United States, Europe, the Philippines, China, Japan, and Latin America (Cabello et al., 2011). Most case reports have been in diaper-aged children, probably due to astute observations of the parents in combination with diapers being an efficient collection apparatus.

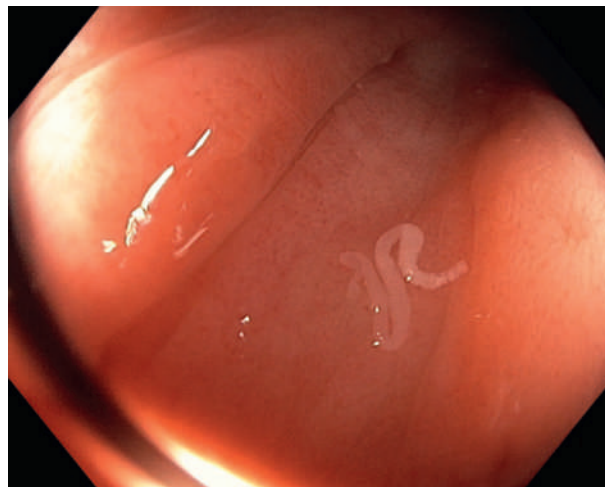


Fig. 24 *Hymenolepis* sp. adult seen in the terminal ileum during colonoscopy. Note that the body is comprised of short proglottids. Courtesy of Dr. Steffen Muehldorfer.

The natural definitive hosts for *D. caninum* are primarily dogs and cats. Adults live in the small intestine of the definitive host, attached to the intestinal mucosa by the scolex, and gravid proglottids are shed into the environment in the feces. Proglottids, and eggs derived therefrom, are eaten by an insect intermediate host, predominately flea larvae and parasitic lice. Common intermediate hosts are cat and dog fleas (*Ctenocephalides* spp.), human fleas (*Pulex irritans*), and dog chewing lice (*Trichodectes canis*). In the arthropod host, the oncosphere develops into a cysticercoid larvae. Fleas are holometabolous insects, and infected flea larvae harbor the cysticercoid larvae through their metamorphosis to adulthood. Cats and dogs become infected after ingesting infected adult fleas and lice when they groom themselves (Cabello et al., 2011). Humans also become infected from the incidental ingestion of infected insects. Children are of particular risk due to their more intimate associations with pets (e.g., kissing, cuddling pets) and tendency to put things in their mouths.

Pathogenesis, clinical presentation, and treatment

Most infected patients are thought to be asymptomatic, although non-specific symptoms such as diarrhea, restlessness and abdominal discomfort may be noted (Cabello et al., 2011). Older children may complain of anal pain and itching. Treatment is primarily with praziquantel (Medical Letter, 2013). Niclosamide is an alternative anti-helminthic drug.

Diagnosis

The diagnosis of dipylidiasis is primary made by the gross morphology of proglottids shed in stool (Fig. 25). The proglottids are harder than other intestinal cestodes, so eggs and characteristic egg packets are not commonly seen in wet mounts of stool.

Adult tapeworms are 10–70 cm long, and proglottids seen in clinical specimens are usually single or in small chains. Gravid proglottids are longer than wide and have two genital pores, one on each lateral margin. Gravid proglottids can be upwards of 23 mm long, but as humans are atypical hosts, proglottids seen in clinical specimens are usually smaller (e.g., a few mm long), and have been described as resembling pumpkin seeds or rice grains (Fig. 25). Proglottids are compartmentalized and contain egg packets. The scolex is not usually recovered in clinical specimens, but in conical and has a retractile armed rostellum (Ash and Orihel, 2007). Proglottids submitted to a laboratory that resemble *D. caninum* should be dissected to look for characteristic eggs packets (Fig. 25) and to rule out the less common, but morphologically similar, members of the genera *Raillietina* and *Inermicapsifer* (Davis et al., 2019).

Egg packets observed in wet mounts of stool or liberated from proglottids contain 8–15 eggs on average. The eggs are thin-shelled and measure 25–40 µm in diameter. The oncosphere contains 6 hooklets (Ash and Orihel, 2007).

Miscellaneous cestode infections

Raillietina spp. (raillietiniasis)

Raillietina is a genus of cestodes that normally parasitize birds, rodents, lagomorphs, and non-human primates. Human infection is rare, but has been reported from Central and South America, Cuba, Iran, Japan, Southeast Asia, the Philippines, French Polynesia, Australia, and the United States (Hawaii). Human infection is believed to be acquired from the incidental ingestion of infected insects and other invertebrates that serve as the natural intermediate hosts (Davis et al., 2019; Sapp and Bradbury, 2020). Most cases have been reported from children, probably for the same risk factors and collection methods described for *D. caninum*.

The clinical picture of raillietiniasis is not well described. Most cases have been asymptomatic, but pediatric patients have presented with generalized intestinal symptoms such as nausea, vomiting, diarrhea, headaches, weight-loss, and retarded growth. Non-intestinal symptoms have been reported in children in Latin America, such as personality changes, convulsions, tachycardia, arrhythmia, and lipothymia. However, given the presence of other endemic parasitic diseases in this region, it is unclear whether those symptoms were caused by *Raillietina* (Acha et al., 1980). There is no defined treatment regimen for raillietiniasis, but praziquantel has been used with success (Davis et al., 2019).

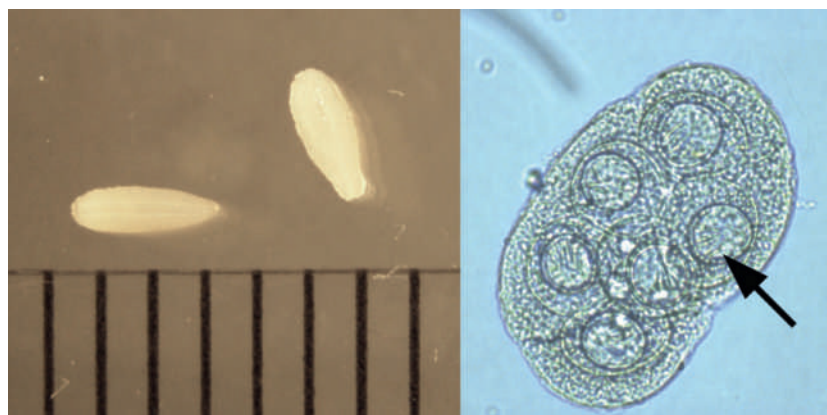


Fig. 25 *Dipylidium caninum* proglottids (left; markings represent millimeters) and characteristic egg packet (right). Note the presence of a 6-hooked within each egg (arrow).

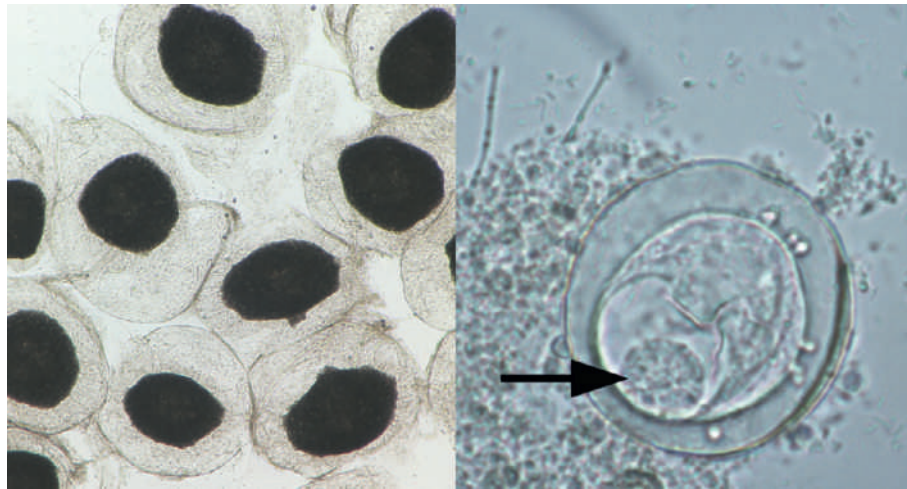


Fig. 26 (A) Egg pouches of *Raillietina* sp., liberated from a gravid proglottid. (B) Characteristic egg of *Bertiella* sp., demonstrating inner and outer shells. A hooked oncosphere (arrow) is housed within the inner shell. (A) Courtesy of Dr. Richard E. Davis.

Diagnosis of raillietiniasis is made by the gross morphology of adult worms and proglottids, and the morphology of eggs liberated from the proglottids. Grossly, the proglottids are very similar to those of *D. caninum*, and should be dissected to examine the eggs. The eggs are 25–35 μm in diameter and contain a six-hooked oncosphere. The eggs are contained within pouches that are roughly 250–300 μm in diameter on average (Fig. 26A) (Davis et al., 2019; Ash and Orihel, 2007). The proglottids, egg pouches, and eggs are morphologically indistinguishable from those of *Inermicapsifer madagascariensis*. The two genera can be separated by the morphology of the scolex; the scolex of *Raillietina* is armed with hooklets, while the scolex of *I. madagascariensis* is unarmed (Davis et al., 2019).

***Inermicapsifer madagascariensis* (*Inermicapsifer* infection)**

Inermicapsifer madagascariensis (syn. *I. cubensis*, *I. arvicanthidis*) is an enigmatic cestode parasite of rodents. The life cycle is not completely understood, but it is presumed the intermediate hosts are arthropods and that human infection occurs after the incidental ingestion of arthropods. Human infection has been reported in sub-Saharan Africa, Madagascar, Comoro Islands, Venezuela, and Cuba (Sapp and Bradbury, 2020; Goldsmid and Muir, 1972). Nearly all human cases have been in predominately asymptomatic young children, or children with generalized gastrointestinal symptoms (Goldsmid and Muir, 1972). Treatment for *Inermicapsifer* infections is praziquantel or niclosamide (Sapp and Bradbury, 2020).

The diagnosis of *I. madagascariensis* is made by the gross examination of adult worms and their proglottids passed in stool (Goldsmid and Muir, 1972). The proglottids, egg pouches, and eggs are morphologically indistinguishable from those of *Raillietina* (above). To separate the genera morphologically in regions where both occur, it is necessary to examine the scolex; the scolex of *Raillietina* is armed with hooklets, while the scolex of *I. madagascariensis* is unarmed (Davis et al., 2019).

***Bertiella* species (*bertiellosis*)**

Bertiellosis is a zoonotic cestode infection caused by members of the genus *Bertiella*. Most cases from humans have been attributed to *B. stuederi* in Africa, India, Southeast Asia, and South America and *B. mucronotata* in South America and Cuba, although a species-level identification can be challenging. The natural hosts are non-human primates, rodents, and Australian marsupials, and most of the published human cases are in children that have had some contact with monkeys (Lopes et al., 2015; Sharma et al., 2018). The intermediate hosts are oribatid mites, and human infection is believed to be acquired from the incidental ingestion of infected mites. Patients have presented with common gastrointestinal problems, including diarrhea, recurrent and chronic abdominal pain, anorexia, weight loss, vomiting, and constipation (Sharma et al., 2018). Praziquantel is the drug of choice (Sharma et al., 2018).

Diagnosis of bertiellosis is made by the morphologic analysis of proglottids shed in stool and eggs liberated from proglottids or observed in wet mounts of stool. Gravid proglottids of *Bertiella* spp. are broader than long and often craspedote. Eggs of *B. stuederi* are on average 45–50 μm in diameter; those of *B. mucronotata* are smaller at 38–41 μm in diameter. The eggs of both species have an external membrane, a middle envelope composed of mucin, and an inner chitinous shell in the form of a “pyriform apparatus” that contains the oncosphere which can have 6–12 hooklets in pairs (Fig. 26B) (Meyers et al., 2000).

***Mesocestoides* spp. (*mesocestoidiasis*)**

Mesocestoidiasis is an enigmatic zoonotic disease caused by cestodes in the genus *Mesocestoides*. Human infection is rare, and most cases have been reported from the United States, as well as Greenland, Japan, Korea, China, and Africa (Meyers et al., 2000; Fuentes

et al., 2003). The life cycle of *Mesocostoides* is not completely described, but it is believed to have a three-host cycle. The natural definitive hosts are carnivorous birds and mammals, especially cats and dogs. The first intermediate host is believed to be an arthropod or other invertebrate, but nothing has been successfully described, either in nature or under laboratory conditions. Eggs experimentally fed to natural intermediate hosts has not resulted in infection, supporting the three-host life cycle hypothesis. The second intermediate and paratenic hosts are small mammals, birds, and reptiles. The intermediate host harbors a larval stage called a tetrathyridium that is the infectious stage for the definitive host. Sometimes, tetrathyridia or acephalic adult worms will cause a proliferative disorder in the definitive host (Padgett and Boyce, 2004; Mathison and Pritt, 2018b).

The source of human infection is not always known, but is believed to be from the consumption of undercooked meat or other animal products harboring infectious tetrathyridia. A cluster of cases from Japan was linked to the consumption of raw snake and turtle organs and blood, but most of the US cases do not have a confirmed source of infection. Most patients are asymptomatic, but have presented with anorexia, abdominal colic, anemia, and irritability (Meyers et al., 2000). Praziquantel and niclosamide have been used to treat mesocostoidiasis (Meyers et al., 2000).

All human cases have been diagnosed by the morphologic analysis of proglottids shed in stool. Gravid proglottids and contain a large characteristic parauterine organ that contains the eggs (Meyers et al., 2000). The eggs are small, measuring 19–24 µm in length, and have a thin membranous wall surrounding the six-hooked oncosphere (Ash and Orihel, 2007).

Acanthocephalan infections

Macracanthorhynchus species, Moniliformis moniliformis (acanthocephaliasis)

Biology and epidemiology

Acanthocephaliasis is a rare to uncommon zoonotic infection caused by the acanthocephalans, commonly referred to as “thorny-headed worms”. Acanthocephalans are an enigmatic group of animals. While grossly they are somewhat similar to nematodes, they are more closely related to rotifers, or possibly nested within the phylum Rotifera proper (Garcia-Varela et al., 2000). Most cases of intestinal human infection have been attributed to *Macracanthorhynchus hirudinaceus*, *M. ingens*, and *Moniliformis moniliformis*. *Macracanthorhynchus hirudinaceus* and *M. moniliformis* are widespread and sporadic human cases have reported throughout much of the world, while to date cases of *M. ingens* have only been recorded from the eastern United States (Mathison et al., 2016).

Acanthocephalans have a complex two-host life cycle with a vertebrate definitive host and an arthropod intermediate host. The natural definitive hosts for *M. hirudinaceus* and *M. ingens* are pigs and raccoons, respectively, while the definitive hosts for *M. moniliformis* are rats, although a variety of wild and domestic animals may serve as definitive hosts for any of the worms. Adult acanthocephalans reside in the small intestine of their definitive host, embedded in the intestinal mucosa by means of an armed proboscis (Fig. 27). Like cestodes, acanthocephalans absorb nutrients through their tegument. Gravid females shed eggs into the environment via the feces. The eggs are fully embryonated and contain an infectious first-stage larva called an acanthor. Eggs are ingested by an intermediate host, which in nature are typically beetles, millipedes, and cockroaches for *M. hirudinaceus*, *M. ingens*, and *M. moniliformis*, respectively. In the hemocoel of the intermediate host, the acanthor molts and becomes the second-stage larva known as an acanthella. After 6–12 weeks, the acanthella develops into a cystacanth, which is the infectious stage for the definitive host. Most human cases are reported in young children, who are more likely to put objects, such as insects, in their mouths (Mathison et al., 2016).

Pathogenesis, clinical presentation, and treatment

The clinical presentation in humans may range from asymptomatic or mild infection, to severe painful disease (Mathison et al., 2016). The thorny proboscis may cause hemorrhage, ulceration and abdominal pain when inserted into the intestinal wall, and rarely, intestinal perforation may occur. Other symptoms include nausea, vomiting, hematochezia, anorexia, weight loss, diarrhea and constipation. Local and peripheral eosinophilia are common. Treatment is not well described. Patients have been successfully treated with mebendazole and pyrantel pamoate. Surgery and anti-bacterial drugs may be necessary in cases of intestinal perforation (Mathison et al., 2016).

Diagnosis

Acanthocephaliasis is diagnosed by the gross morphology of adult worms shed in stool (Fig. 27), and eggs derived therefrom. Eggs of acanthocephalans are rarely reported from wet mounts of stool.

Adult acanthocephalans are large pseudocoelomate animals. Adult females of *Macracanthorhynchus* spp. are 12–32 cm long while the males are smaller at 7–8 cm long. They are white to pale pink in colors and have wrinkles and depressions which may give the false impression of segmentation. *Moniliformis moniliformis* is slightly smaller and more slender, measuring 10–27 cm for females and 4–10 cm for males (Fig. 27). Adults of *M. moniliformis* have more pronounced pseudosegmentation and often appear beaded. Adults of all acanthocephalans have an armed proboscis, which in clinical specimens may be retracted and must be dissected to appreciate (Ash and Orihel, 2007; Meyers et al., 2000).

Eggs of *Macracanthorhynchus* species are morphologically indistinguishable from one another and measure 89–100 µm long by 50 µm wide. They have a thick, dark brown shell that has a rough texture. Eggs of *M. moniliformis* are slightly larger, measuring 90–125 µm long by 65 µm wide and have a thick, smooth, clear shell (Fig. 27) (Ash and Orihel, 2007).

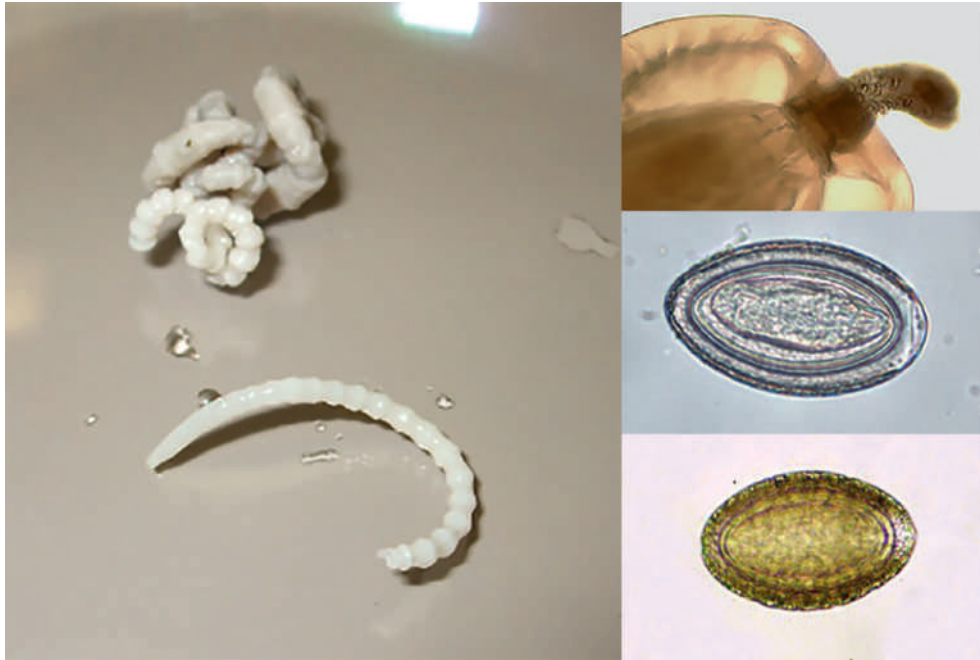


Fig. 27 Eggs and adult acanthocephalans. *Moniliformis moniliformis* adults (left) have pseudosegmentation, sometimes with a beaded appearance. They have a small armed proboscis (upper right) which is often retracted in adult worms submitted for examination. The eggs of *M. moniliformis* have a thick, smooth clear shell (right, middle panel), whereas those of *Macracanthorhynchus* have a thick, dark brown shell that has a rough texture (lower right). Both eggs contain a larva (acanthor) with rostellar hooks. The *Macracanthorhynchus* egg is courtesy of Phong Duong.

Parasites of the appendix and large intestine, and anus

Like the small intestine, there are a large number of protozoa and helminths that can infect the large intestine (Table 4). Some such as *Entamoeba histolytica*, *Balantidioides coli*, and *Schistosoma* spp. are responsible for a significant amount of human morbidity and mortality in endemic regions of the world.

Protozoan infections

Entamoeba histolytica (intestinal amebiasis)

Biology and epidemiology

Clinical amebiasis is caused by *Entamoeba histolytica*, one of 11 species of amebae, and one of nine species of the genus *Entamoeba*, that inhabit the human GI tract (Hooshyar et al., 2015). *Entamoeba histolytica* belongs to a complex of four morphologically-identical species, the others being *E. dispar*, *E. bangladeshi*, and *E. moshkovskii*. Other nonpathogenic intestinal amebae will be discussed elsewhere in the chapter. Historically, *E. histolytica* was considered the only pathogenic *Entamoeba* in the human host, however, the pathogenic role of *E. bangladeshi* and *E. moshkovskii* is not completely understood (Ali, 2015).

Entamoeba histolytica has a worldwide distribution, with clinical disease being most prevalent in resource-poor regions of Latin America, Africa, and Asia. Most patients in the US and Europe with clinical amebiasis are returning travelers, particularly from South America, the Middle East, and Southeast Asia. In developed nations, the prevalence in non-travelers is highest in MSM, immunocompromised patients, and institutionalized patients (Shirley et al., 2018). Overall, the global burden is difficult to define due to a lack of specificity in many of the diagnostic assays. The WHO estimates that there were 103,943,952 cases in 2010, with 27,553 deaths (Kirk et al., 2015). *Entamoeba dispar* also has a worldwide distribution and non-human primates may serve as reservoir hosts (Hooshyar et al., 2015). The epidemiology of *E. moshkovskii* and *E. bangladeshi* is less understood. Cases of *E. moshkovskii* have been sporadically reported from the US, Latin America, the Middle East, Africa, Australia, and Southeast Asia, with higher prevalence in Australia, India, Bangladesh, Tanzania, Pakistan, and Iran (Ali, 2015; Lopez et al., 2015). *Entamoeba bangladeshi* is endemic to Southeast Asia and sub-Saharan Africa (Ngoben et al., 2017).

Members of the *E. histolytica*-complex have a simple one-host life cycle. Trophozoites multiply by binary fission in the lumen of the large intestine. Both cysts and trophozoites are shed into the environment via the feces (Fig. 1). Infection is initiated by the ingestion of mature cysts in fecal-contaminated food, water, and fomites or via anal-oral sex. The amebae excyst in the small intestine and migrate to the lumen of the large intestine. Although trophozoites usually reside in the colonic lumen and feed on bacterial flora, *Entamoeba histolytica* has the ability to adhere to colonic mucins via the Gal/GalNAc lectin and then degrade the

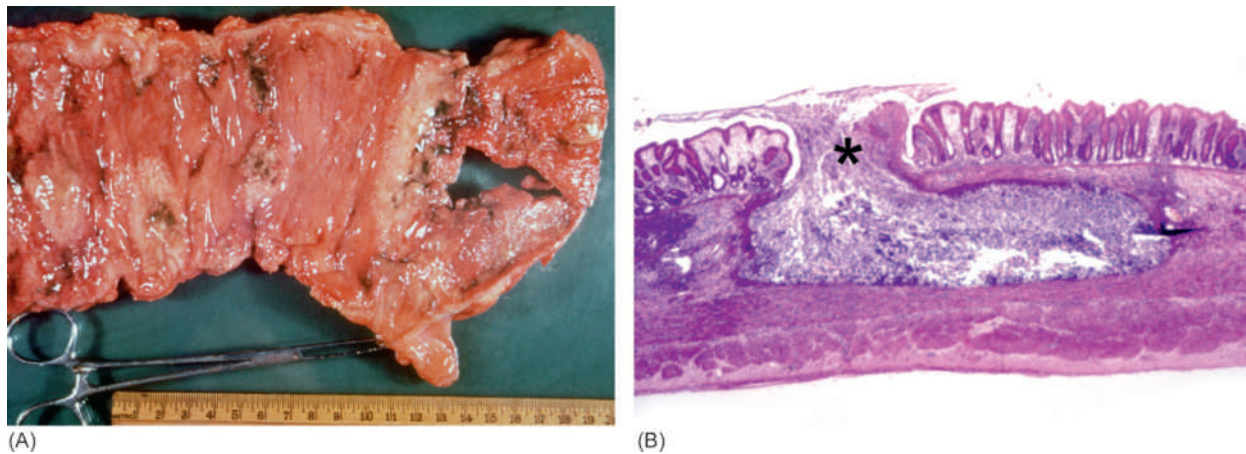


Fig. 28 Intestinal amebiasis with ulceration. Image A depicts a section of resected colon which has been opened to reveal the mucosa. Several ulcers and a region of perforation are clearly visible. By histopathologic examination, the ulcers classically have a “flask-shape” (B). This occurs as trophozoites invade through the intestinal mucosa and then invade laterally into the underlying submucosa, producing an ulcer apex (marked by an “*”) that is narrower than the ulcer base. Images courtesy of the U.S. Centers for Disease Prevention and Control Public Health Image Library.

protective colonic mucous barrier using glycosidases and other enzymes (Kantor et al., 2018). Trophozoites may then penetrate the colonic epithelium, causing erosions, ulcerations, mass-like lesions (ameboma), and rarely, perforation (Fig. 28A). Trophozoites may also enter the circulation and infect other sites. The most common site of extraintestinal infection is the liver, followed by the brain and lungs (Shirley et al., 2018). Hepatic amebiasis will be discussed elsewhere in the chapter.

Pathogenesis, clinical presentation, and treatment

The majority (~90%) of cases of *E. histolytica* infection are asymptomatic (Kantor et al., 2018). When present, intestinal manifestations may range from mild watery diarrhea, to bloody diarrhea, to severe dysentery and even fulminant necrotizing colitis due to invasion of the trophozoites into the intestinal wall. Fever and abdominal pain are common with invasive disease. Complications include toxic megacolon and perforation, as well as formation of an ameboma which may cause partial obstruction or mimic malignancy. Risk factors for severe disease include extremes of age, immunocompromised state, alcoholism, malnutrition, and pregnancy (Kantor et al., 2018). Treatment of infection is warranted even in cases of asymptomatic disease, to prevent the spread of infection to other individuals. The treatment regimen varies by the type of disease. Noninvasive colonic colonization is treated only with a luminal agent such as paromomycin, iodoquinol, or diloxanide furoate to eradicate the cyst form of the organism (Medical Letter, 2013). For cases of invasive and/or disseminated disease, metronidazole or tinidazole is added to the regimen.

Diagnosis

The diagnosis of intestinal amebiasis can be accomplished by O&P examinations of stool, histopathology, antigen detection, antibody detection, and molecular diagnosis. In specialized reference labs, methods such as culture or isoenzyme analysis may be available.

Microscopy

The traditional diagnosis of amebiasis is the detection of *E. histolytica* cysts and trophozoites in O&P examinations of stool. Unfortunately, in most cases, the morphologic features are inadequate for reliably separating *E. histolytica* from non-pathogenic members of the complex. Trophozoites of *E. histolytica* measure 10–60 μm in size, with pathogenic strains typically greater than 20 μm (Fig. 1). They possess a single nucleus with evenly-distributed peripheral chromatin and a discrete, usually centrally-located, karyosome. The cytoplasm is finely granular and may include bacteria, yeast, or erythrocytes. The presence of ingested erythrocytes is considered pathognomonic for *E. histolytica*, and is the only morphologic feature that allows for a presumptive species-level identification by stool microscopy. Pseudopodia may also be present. The cysts of *E. histolytica* are 10–20 μm in diameter (Fig. 1). Immature cysts typically have a large single nucleus, a large glycogen vacuole, and irregularly-shaped peripheral chromatoid bodies. Mature cysts have four small nuclei and the chromatoid bodies are broad and bluntly-rounded. Members of the *E. histolytica*-complex cannot be separated by cyst morphology (Ash and Orihel, 2007).

Trophozoites can also be detected in intestinal biopsy specimens processed for histopathology. *E. histolytica* classically forms “flask-shaped” ulcers in the colonic mucosa, in which the apex of the ulcer in the epithelium is narrower than the base in the submucosa (Fig. 28B). The trophozoites are morphologically similar to those observed in stool, although with a higher prevalence of ingested erythrocytes. *Entamoeba* observed in intestinal biopsy specimens or extraintestinal sites can be reported as *E. histolytica* at the species level as it is the only species that can invade tissue and become systemic. Cysts are not observed in extraintestinal specimens (Mathison and Pritt, 2018a).

Antigen detection

There are several commercially-available antigen detection assays for the diagnosis of intestinal amebiasis. There are several platforms available, including EIA, DFA, and lateral flow immunochromatography, and a few will simultaneously detect other intestinal protozoa such as *Giardia duodenalis* and *Cryptosporidium* spp. (Garcia et al., 2018). In general, these assays have good sensitivity and specificity, but they require fresh or frozen unpreserved stool and some were developed using trophozoite-specific antigens and may have false negative results in stool in which only cysts were shed (Garcia et al., 2018; Ali, 2015). Also, some assays are unable to distinguish between *E. histolytica* and *E. dispar* (Ali, 2015).

Antibody detection

Antibody detection is primarily used for diagnosis of extraintestinal infection of *E. histolytica* and is not sensitive for diagnosing non-invasive intestinal illness. Most commercially-available assays are EIA. These tests have a high sensitivity (greater than 90%) for extraintestinal infections, but only about 70% for invasive intestinal infection. Also, because IgG antibodies to *E. histolytica* persist over time, a positive result cannot distinguish between current and past infections, and patients who are from, or spent substantial time in, highly endemic areas may be positive due to past exposure (Ali, 2015).

NAATs

NAATs are available in both single analyte and multiplex formats. While the former are not widely used outside of specialized reference and research centers, there are now several commercially-available, easy-to-use multiplex assays that detect *E. histolytica* that have FDA or CE approval, and these assays are increasingly used in the clinical microbiology laboratory in developed countries. Multiplex assays may have slightly lower sensitivity than monoplex NAATs, but still have a higher sensitivity than morphologic analysis or antigen detection, and have become a test of choice for rapidly evaluating patients with infective diarrhea in some settings. Unfortunately, the relatively high cost of these multiplex molecular panels tends to be cost-prohibitive outside of large reference labs or health care systems that have consolidated testing. It is also important to note that some of these multiplex assays may not be able to distinguish *E. dispar* from *E. histolytica* when the former is in higher concentrations (Ali, 2015; Garcia et al., 2018). Regional reference laboratories may have specialized NAATs for the diagnosis or differentiation of *Entamoeba* species. The CDC in conjunction with researchers in Brazil developed a proof-of-concept Luminex[®]-based assay that can detect *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. coli*, and *E. hartmanni* (Santos et al., 2013). The CDC also offers a confirmatory real-time PCR to separate *E. histolytica* from *E. dispar* in stool specimens positive by morphologic analysis (Qvarnstrom et al., 2005). LAMP-mediated assays show promise in increasing simplicity and lowering the cost of molecular diagnosis (Ali, 2015).

Dientamoeba fragilis (Dientamoeba infection)

Biology and epidemiology

Dientamoeba fragilis is an enigmatic protozoan. While morphologically similar to amebae, it is actually a flagellate and is more closely related to *Trichomonas vaginalis* and *Pentatrichomonas hominis* than it is to other protozoa that parasitize humans. Unlike other trichomonads, the flagella of *D. fragilis* are internalized and the organism moves via pseudopodia like amebae. *Dientamoeba fragilis* occurs worldwide, but the prevalence varies by region depending on the diagnostic method. It is unusual in that its prevalence is higher in developed countries, probably due to improved diagnostic techniques. There are currently two recognized genotypes, genotype 1 and genotype 2, the former of which is more common in clinical specimens (Stark et al., 2016).

There has been controversial debate in recent years as to the nature of the life cycle of *D. fragilis* and how it is transmitted to humans. For decades, it was believed that *D. fragilis* lacked a cyst stage, which is typical for trichomonads, and that infection occurred from the ingestion of trophozoites or was carried passively by other parasites, such as in *Enterobius vermicularis* (pinworm) eggs. The pinworm theory has for the most part been abandoned, but in recent years the description of precyst and cyst stages in mouse models and detection of such cysts in human clinical specimens has been proposed (Munasinghe et al., 2013; Stark et al., 2014). It should be noted that these claims are still not universally accepted among protozoologists and eukaryotic taxonomists. The role of animals in the transmission of *D. fragilis* is also not well understood. While generally considered a human parasite, *D. fragilis* has been reported from non-human primates and domestic pigs (Stark et al., 2016). Regardless of the mode of transmission, *D. fragilis* resides in the lumen of the intestine where it multiplies by binary fission and feeds on bacteria, yeast, and other organic material in the fecal stream. There is no evidence to date on whether *D. fragilis* can invade the intestinal epithelium and the pathogenic status of the organism remains unresolved.

Pathogenesis, clinical presentation, and treatment

The role of *D. fragilis* as a pathogen remains controversial, despite the large body of evidence from clinical studies showing its association with symptomatology and resolution of symptoms following treatment (Stark et al., 2016). Although it is clear that many infected patients are asymptomatic, the clinical manifestations range from acute and chronic abdominal pain, diarrhea, and constipation. Eosinophilia has been reported in up to 50% of infected patients (Stark et al., 2016). There is no well-accepted treatment regimen for *D. fragilis* infection. Commonly used antiparasitic drugs include paromomycin, iodoquinol, metronidazole, tinidazole, and tetracycline (Stark et al., 2016; Medical Letter, 2013).

Diagnosis

Infection is diagnosed primarily by the detection of trophozoites in permanently-stained fecal smears. If they exist, cyst forms are likely to be rare and difficult to detect. Culture and PCR may be available in large or specialized reference laboratories. There are currently a few commercially-available multiplex NAATs that include *D. fragilis* as a target; they all have CE approval, but to date none are FDA-approved or cleared for in vitro diagnostic use in the United States.

Trophozoites of *D. fragilis* measure 5–15 µm and possess one or two nuclei, with binucleate forms predominating (Fig. 1). The nuclei contain a mass of karyosomal material that is often in a cluster of 4–8 granules; there is no peripheral chromatin. The cytoplasm is finely to heavily granular and often contains bacteria and yeast. Motility is non-progressive and a thin, nearly transparent pseudopod may be evident extending from the cytoplasm (Ash and Orihel, 2007).

Balantioides coli (balantiosis)

Biology and epidemiology

Balantiosis (also known as balantidiasis) is caused by *Balantioides coli*, the largest parasitic protozoan, and only ciliated protozoan, of humans. This species has a complicated taxonomic history. It was originally described in 1857 in the genus *Paramecium* from two patients presenting with dysentery. It was transferred to *Balantidium* in 1863. In 1931, the genus *Balantioides* was described to accommodate the parasite, but unfortunately that name apparently went unnoticed for decades and *Neobalantidium* was described in 2013 to accommodate *B. coli*. In 2014, attention was brought to the original description of *Balantioides*, and in 2018 *Balantioides* was restored as the valid genus to accommodate *B. coli* (Mathison and Pritt, 2020). To complicate matters, *B. coli* is sometimes incorrectly placed in the genus *Balantidioides*, which is a genus of endosymbiotic ciliates.

Balantidioides coli is uncommonly reported, but has a worldwide distribution. Many mammal species can serve as hosts for *B. coli*, but pigs are a common reservoir for human infection, and human cases are more common in resource-poor areas that engage in pig farming. Areas of higher prevalence include Latin America (especially the Altiplano region of Bolivia), Philippines, New Guinea, and the Middle East (Schuster and Ramirez-Avila, 2008).

Balantioides coli has a simple, one-host life cycle. Infection occurs from the ingestion of cysts in fecal-contaminated food, water, and fomites. The parasite excysts in the small and large intestine and multiplies by binary fission in the lumen of the large intestine. Both cysts and trophozoites are shed in stool, but only cysts are infectious for a new host. Like *Entamoeba histolytica*, *Balantioides coli* can invade the colonic mucosa, and may rarely cause ectopic infection. Regardless, most cases of *B. coli* reported from respiratory specimens are believed to be misidentifications of ciliocytophthoria, a condition whereby motile ciliated epithelial cells and ciliary tufts detach and are observed in fresh bronchial washings and BALs (Schuster and Ramirez-Avila, 2008).

Pathogenesis, clinical presentation, and treatment

The pathogenicity of *Balantioides coli* is not well understood. Trophozoites may be able to invade the intestinal mucosa through the use of proteolytic enzymes such as hyaluronidases (Schuster and Ramirez-Avila, 2008). Many infected individuals are asymptomatic, or experience only mild abdominal pain and watery diarrhea. Extensive intestinal invasion can result in fulminating disease with bloody diarrhea, dysentery, tenesmus, and weight loss. Fatalities have been caused by intestinal hemorrhage and perforation (Schuster and Ramirez-Avila, 2008). Risk factors for severe disease include malnourishment, immune compromise, achlorhydria, alcoholism, and concurrent chronic diseases (Schuster and Ramirez-Avila, 2008). Treatment is with tetracycline, metronidazole, or iodoquinol (Schuster and Ramirez-Avila, 2008; Medical Letter, 2013).

Diagnosis

The diagnosis of balantiosis is made primarily by the finding of cysts and trophozoites in wet mounts of stool, less commonly in permanent-stained stool specimens (Fig. 29). Trophozoites measure 50–200 µm long by 40–70 µm wide. Living trophozoites in fresh wet mounts move by a rotary, boring motion with the cilia maintaining a constant, synchronized motion. Trophozoites taper to the anterior end where a prominent cystostome is located. There are two nuclei, a large kidney-shaped macronucleus and a smaller micronucleus, the latter of which can be difficult to discern. The cytoplasm may contain ingested debris and food vacuoles. Cysts are spherical and measure 50–70 µm in diameter. Cilia may be visible under the thick cell wall. Both the macronucleus and micronucleus are present. Because ingested objects are not expelled during encystment, food and vacuoles may be seen in the cysts as well (Ash and Orihel, 2007).

Trophozoites of *B. coli* can be observed in colonic biopsy specimens, usually in relation to flask-shaped ulcers like those described for intestinal amebiasis. The trophozoites in biopsy specimens share the same morphologic features as those seen in stool specimens (Mathison and Pritt, 2018a).

Blastocystis spp. (blastocystosis)

Biology and epidemiology

Blastocystosis is caused by enigmatic protozoan parasites in the genus *Blastocystis*. Historically, the genus has been allied with yeasts, amebae, and coccidians, but is now believed to be a stramenopile in the Stramenopiles-Alveolates-Rhizaria (Sar) super-group (Adl et al., 2019). Traditionally, species have been described based on the host (e.g., *B. hominis* from humans, *B. ratti* from rats) and historically all isolates of humans were referred to as *B. hominis*. However, molecular analyses appear to demonstrate that there is no population unique to humans and all human isolates appear to have animal reservoirs. Nine (possibly ten) primary subtypes have been found in mammals and birds, with subtypes 1 and 3 making up most of the cases of human blastocystosis (Stensvold et al., 2007).

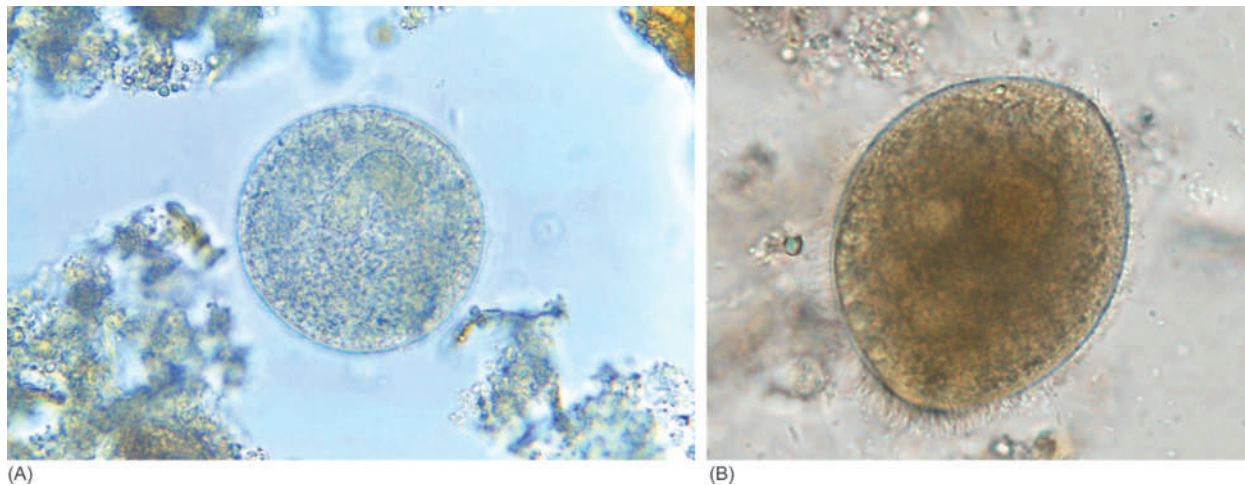


Fig. 29 *Balantioides coli* cyst (A) and trophozoite (B), (unstained wet preparation, 400 $\times$). Note the large ‘kidney bean-shaped’ macronucleus (best seen here in the cyst) and the circumferential cilia on the trophozoite. Images courtesy of the U.S. Centers for Disease Control and Prevention.

The life cycle of *Blastocystis* is not completely understood, and several models have been proposed. In a recent proposal, vacuolar forms (which includes both the “vacuolar” and “cyst-like” forms in historical nomenclature) reproduce by binary fission in the lumen of the large intestine. These vacuolar forms can become cysts, and both vacuolar forms and cysts are shed into the environment via the feces, however it is believed only the true cyst forms are infectious for a new host. After ingestion of cysts in fecal-contaminated food, water, and fomites, the parasite excysts and becomes a granular form, which is a precursor to the vacuolar form. It has also been suggested that in the lumen of the host’s large intestine, the parasite can become an amoeboid form which plays a role in pathogenesis. This belief is still not generally accepted, however, as these amoeboid forms might simply be aberrant vacuolar forms (Tan, 2008).

Pathogenesis, clinical presentation, and treatment

The potential pathogenicity of *Blastocystis* is unclear. Infection has been associated with a variety of symptoms, among which abdominal pain and diarrhea are most common (Tan, 2008). Other symptoms include anorexia, abdominal bloating, increased flatulence, nausea, urticaria, and the irritable bowel syndrome. Risk factors for symptomatology may include immune compromise and parasite genotype, although this warrants further study (Tan, 2008). There is no evidence that *Blastocystis* can invade tissue, but presumed colonization of invasive and extraintestinal lesions due to other pathogens has been described (Tan, 2008). There is no widely-accepted treatment regimen, but metronidazole, trimethoprim sulfamethoxazole and nitazoxanide have been used with varying degrees of success (Sekar and Shanthi, 2013).

Diagnosis

The diagnosis of blastocystosis is primarily made by the finding of organisms in O&P examinations of stool. Molecular diagnosis may be available at large reference laboratories, and there are a couple commercially-available multiplex assays that include *Blastocystis* as a target. These assays currently are CE marked, but not FDA approved/cleared.

The most common stage of *Blastocystis* observed in stool specimens is the vacuolar form, which includes the traditional descriptions of both vacuolar and cyst-like forms (Fig. 30). The vacuolar form is highly variable in size, measuring 4.0–63.0 μm in diameter in human stool specimens. A large central vacuole takes up approximately 90% of the body, with the nuclei and other organelles located within a thin rim between the vacuole and outer cell wall. Traditionally, the “cyst-like” forms tend to be smaller and have a larger rim containing the nuclei and organelles, whereas the traditional vacuolar forms are larger and have a very thin rim around the central vacuole. Dividing forms may be seen in permanent stained smears (Tan, 2008). In specimens stained with Wheatley’s trichrome, the central vacuole typically stains blue-green while the nuclei and other organelles stain red-purple (Ash and Orihel, 2007). The cyst, which is the most recently-described stage, is rarely reported from stool, probably due to its small size (2.0–5.0 μm) and unfamiliarity to diagnostic technologists (Tan, 2008). The cysts are ovoid and contain 1–4 nuclei. Due to the historic unfamiliarity of this stage, it is possible it has been misidentified for years as cysts of *Endolimax nana* or *Enteromonas hominis*. Because the taxonomy of *Blastocystis* is in a state of flux, it is becoming more common for diagnostic laboratories to report *Blastocystis* at the genus-level only rather than *B. hominis*. As with leukocytes and erythrocytes, it is common practice to report the relative quantity of *Blastocystis* forms (e.g., rare, few, moderate, many), as the quantity may be positively associated with symptoms (Garcia et al., 2018).

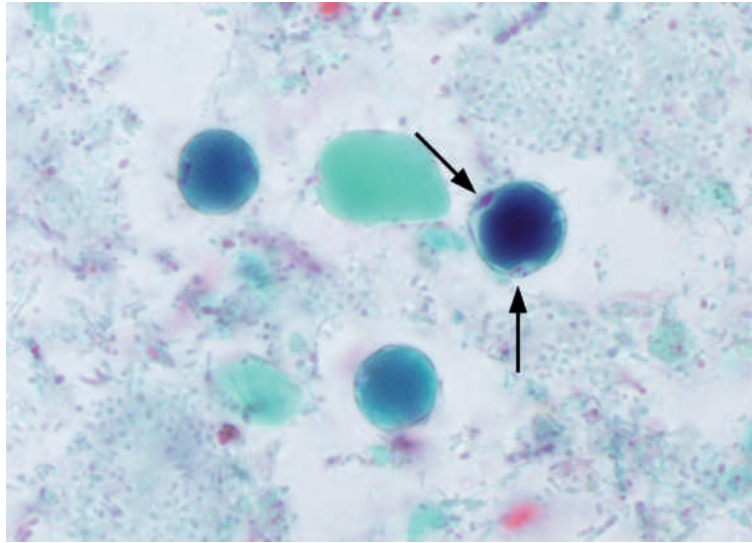


Fig. 30 Vacuolar form of three *Blastocystis* sp. parasites (Wheatley's trichrome stain, 1000 $\times$). Note the large central vacuole and peripheral nuclei (arrows).

Non-pathogenic intestinal protozoa

The following protozoa inhabit the human intestinal tract as commensals but do not cause disease. It is important for laboratorians and diagnosticians to learn to recognize them and distinguish them from potentially pathogenic species (Figs. 1 and 8). It is also helpful to report nonpathogenic protozoa, especially in symptomatic patients, as it could be an indicator of exposure to fecal-contaminated food or water sources, even if the protozoan is not the cause of disease. It would be helpful such reports indicated which species were not pathogenic or otherwise provided guidance for treating physicians to prevent undue burden and costs of unnecessary treatment (Lee et al., 2000).

Entamoeba hartmanni

Entamoeba hartmanni is a small nonpathogenic ameba with a worldwide distribution. It has many morphologic characteristics in common with *E. histolytica*, and historically has been referred to as a "small race" of *E. histolytica*. In cases where members of the *E. histolytica*-complex are on the small end their size range, separation from *E. hartmanni* can be challenging. Trophozoites measure 8–12 μm but are usually consistently less than 10 μm . They have single nucleus with even peripheral chromatin and a discrete, usually centrally-located karyosome. Mature cysts are 5–10 μm , have four nuclei, and spherical to elongate chromatoid bodies with blunt ends (Ash and Orihel, 2007).

Entamoeba coli

Entamoeba coli has a worldwide distribution and is one of the most commonly reported *Entamoeba* species in clinical specimens. Non-human primates may serve as reservoir hosts. Trophozoites of *E. coli* measure 15–50 μm and have a single nucleus with irregular peripheral chromatin and a usually eccentric karyosome. The cytoplasm is often described as "dirty" and may contain vacuoles and ingested food particles, including bacteria, yeast, and even other intestinal protozoa. Cysts are 10–35 μm in diameter. Immature cysts typically have a large glycogen vacuole and two nuclei. Mature cysts have eight nuclei, with hypernucleate forms having 16 or more nuclei. The presence of an *Entamoeba* cyst in human stool with more than four nuclei is pathognomonic for *E. coli* (Ash and Orihel, 2007).

Entamoeba polecki and *E. chattoni*

Entamoeba polecki and *E. chattoni* are enigmatic zoonotic *Entamoeba* species. The epidemiology of these two species is not well described. *Entamoeba polecki* is primarily a parasite of pigs and monkeys and has spots of endemicity in Venezuela, Guinea, Iran, and Southeast Asia, where the prevalence in Papua New Guinea can reach 30% (Hooshyar et al., 2015; Verweij et al., 2001). *Entamoeba chattoni* is normally a parasite of non-human primates and is endemic to Africa (Hooshyar et al., 2015). Both species morphologically identical and in areas where both species occur may be difficult to separate. Cysts are 9–24 μm in diameter; the single nucleus (rarely binucleate forms may be seen) is large in relation to the cytoplasm and has variable peripheral chromatin and a karyosome that can be minute to large or even obsolete. Cysts often contain a large inclusion mass that usually stains grey to green with Wheatley's trichrome. Chromatid bodies are numerous and highly variable in size and shape. Trophozoites measure 10–25 μm and contains a single large nucleus with characteristics similar to those of the cysts. The trophozoites can be difficult to separate from other *Entamoeba* spp. (Ash and Orihel, 2007).

Endolimax nana and Iodamoeba buetschlii

Endolimax nana and *Iodamoeba buetschlii* are common and widespread nonpathogenic amebae. The cysts of *E. nana* are 5–10 µm and have four small nuclei with a large karyosome and no peripheral chromatin. There are no chromatoid bodies present. Cysts of *I. buetschlii* are 5–20 µm and have a large single nucleus with a large karyosome and no peripheral chromatin. As with *E. nana*, chromatoid bodies are not present. A unique characteristic of the cysts of *I. buetschlii* is the presence of a large glycogen mass that appears red-brown in wet mounts of stool stained with iodine. In permanent-stained smears, this glycogen mass does not take up stain, but is evident as a colorless, well-defined mass. The trophozoites of both species are nearly identical and possess a single nucleus with a large karyosome and no peripheral chromatin. The published size ranges are slightly different; trophozoites of *E. nana* are 6–12 µm while those of *I. buetschlii* are slightly larger at 8–20 µm. When cysts are not present, the two species can be difficult to separate. Luckily neither is pathogenic (Ash and Orihel, 2007; Poulsen and Stensvold, 2016).

Chilomastix mesnili

Chilomastix mesnili is a nonpathogenic flagellate of the human large and small intestine that occurs worldwide. Like most nonpathogenic intestinal protozoa, it has a simple one-host life cycle whereby infection occurs from the ingestion of cysts and trophozoites multiply by binary fission in the lumen of the intestine. Trophozoites are usually pyriform and measure 6–24 µm long. They have a large, single, anteriorly-located nucleus that contains a small karyosome and granular, fragmented peripheral chromatin. In permanent-stained specimens, the cytostome and fibrils may be evident. *Chilomastix* has three anterior flagella which are not usually visualized on stained smears. Trophozoites may not stain well and often round-up in improperly-preserved specimens, which could cause issues in microscopic detection. Cysts are uninucleate, lemon-shaped, and measure 6–10 µm in length. Fibrils are usually prominent and give the appearance of a “safety pin” alongside the cytostome (Ash and Orihel, 2007).

Pentatrichomonas hominis

Pentatrichomonas (formerly *Trichomonas*) *hominis* is a cosmopolitan nonpathogenic flagellate of the human large intestine. Its prevalence is unknown and the species is probably underreported. Other mammals may serve as reservoir hosts, including non-human primates, dogs, cats, foxes, rabbits, cattle, sheep, goats, and deer (Li et al., 2018, 2016, 2017). Like other trichomonads, there is no known cyst stage and infection is believed to occur from the ingestion of trophozoites on fecal-contaminated fomites. Trophozoites are pyriform and measure 6–20 µm long. The nucleus is elongated and in the anterior portion of the organism. They possess five flagella: four anterior and a fifth that runs along the outer margin of an undulating membrane and extends beyond the posterior end of the organism. Flagella are usually visible in stained smears. Trophozoites also possess a prominent axostyle that extends beyond the posterior end of the organism (Ash and Orihel, 2007).

Enteromonas hominis and Retortamonas intestinalis

Enteromonas hominis and *Retortamonas intestinalis* are two species of widespread but uncommonly reported nonpathogenic intestinal flagellated protozoa. Their scarcity in reports may be due to their small size, unfamiliarity to laboratorians, or misidentification of other small protozoa such as *E. nana* and *C. mesnili* due to some morphologic similarities. Like *C. mesnili*, they have a simple life cycle whereby infection occurs from the ingestion of cysts and trophozoites multiply by binary fission in the lumen of the intestinal tract. Trophozoites of *E. hominis* are rounded to oval and measure 6–8 µm long. They have an anterior, single nucleus with a large karyosome and four flagella, three anterior and one posterior. The flagella can be visualized in stained smears. Cysts of *E. hominis* are ellipsoidal and measure 4–8 µm long. They may possess 1, 2, or 4 nuclei; when two or four nuclei are present, they usually have a bipolar arrangement. Trophozoites of *R. intestinalis* are pyriform to rounded and measure 4–10 µm long and have a single, anterior nucleus with a small karyosome. There are two flagella present, one anterior and one posterior. The cysts of *R. intestinalis* are ovoid and measure 4–7 µm long. Mature cysts have a single nucleus with a compact karyosome and prominent fibril (Ash and Orihel, 2007).

Nematode infections

Enterobius vermicularis (enterobiasis, pinworm infection)

Biology and epidemiology

Enterobiasis, commonly referred to as pinworm infection, is a cosmopolitan disease caused by *Enterobius vermicularis*. It is one of the most common helminth infections worldwide; it is estimated there are 40 million people infected at any one time in the United States alone. The disease is seen most commonly in patients under the age of 18 years old and in institutional settings, day care centers, and other crowded environments, and usually occurs in families (Rawla and Sharma, 2020). There is a 2:1 ratio of males to females infected. Prevalence varies from country to country based on availability of testing procedures and reporting regulations. The true global burden is not known.

Enterobius vermicularis has a simple one-host life cycle and humans are the only known host. Adults live in the lumen of the large intestine. At night, gravid females migrate to the anal opening and deposit partially-embryonated eggs in the perianal folds. Eggs embryonate quickly and within approximately 4 h are already infectious for the next host. This is in marked contrast to most other intestinal nematodes, which must embryonate in the environment over a period of days to weeks. Enterobiasis occurs from the ingestion of fully-embryonated eggs on fomites, including hands, clothing, bedding, furniture, and personal care products.

Enterobiasis can be complicated to control after an environment becomes contaminated. Eggs hatch in the small intestine and migrate to the large intestine where they become adults. Ectopic colonization of the appendix is not uncommon (Rawla and Sharma, 2020). On rare occasions in female patients, after oviposition, the female worms will accidentally enter the urogenital tract and cause ectopic infection of the vulva, vagina, uterus, fallopian tubes, and ovaries (Choudhury et al., 2017).

Pathogenesis, clinical presentation, and treatment

An estimated 1/3 of infected individuals are asymptomatic (Rawla and Sharma, 2020). The most common symptom is nighttime anal itching (“nocturnal pruritis ani”) due to the presence of the adult female and eggs in the perianal area. Heavy infections may cause insomnia, daytime sleepiness and irritability. Appendicitis, vulvovaginitis, salpingo-oophoritis, and pelvic inflammatory disease may be caused by ectopic migration of adult worms. Drugs of choice include albendazole, mebendazole, or pyrantel pamoate (Medical Letter, 2013).

Diagnosis

Enterobiasis is diagnosed by the detection of eggs (and occasionally adult females) on cellulose tape preparations and specialized collection devices, and rarely, by histopathology. Unlike other intestinal nematodes, eggs of *E. vermicularis* are only rarely reported from concentrated wet mounts of stool.

The traditional method of diagnosis is the “cellulose tape test” whereby non-frosted cellulose tape is applied to the perianal area of suspect patients in the early morning hours before showering and defecation and examined microscopically for the presence of eggs. Nowadays there are commercial collection kits available, including the SWUBE™ (Becton Dickinson, Franklin, New Jersey) and the Pinworm Collector (Scientific Device Laboratory, Des Plaines, Illinois) (Carroll et al., 2019). Eggs of *E. vermicularis* are 50–60 µm long by 20–30 µm wide, are flattened on one side, and have a thick hyaline shell (Fig. 10). Eggs are partially-embryonated when observed on paddles, cellulose tape, or in wet mounts of stool (Ash and Orihel, 2007).

Adult females of *E. vermicularis* are commonly submitted to diagnostic laboratories when observed grossly in stool specimens, around the perianal folds during clinical evaluation, in colonic washings, and by colonoscopy (Fig. 11). Adult males are rarely seen due to their small size; they are small enough that they may be observed in concentrated wet mounts of stool. Adult females are 8.0–13.0 mm long and have cephalic inflations and a characteristic pointed tail (Fig. 31). Males measure 2.5 mm long and also have cephalic inflations, but have a bluntly-rounded tail (Ash and Orihel, 2007).

Adult females of *E. vermicularis* observed in appendix and intestinal biopsy specimens have distinct morphology that usually does not cause diagnostic problems. They have large platymyarian muscle cells, a strongly-muscled esophagus, and characteristic lateral alae on the cuticle that are formed from ridges that run along the external body of the worm. The uterine tubes of gravid females are usually loaded with characteristic eggs (Mathison and Pitt, 2018a).



Fig. 31 Adult female *Enterobius vermicularis*. Note the characteristic pointed tail, from which the term “pinworm” comes. The uterus if full of eggs (inset). From Mathison BA and Pitt BS (2018) Chapter 5: Parasitic Diseases. In: *Atlas of Fundamental Infectious Diseases Histopathology: A Guide for Daily Practice*. Pitt BS (ed.). Pathologists: Northfield, IL: College of American.

***Trichuris trichiura* (trichuriasis, whipworm infection)**

Biology and epidemiology

Trichuriasis, commonly referred to as whipworm infection, is caused by *Trichuris trichiura*, a nematode with a nearly worldwide distribution. The zoonotic potential of *T. trichiura* is not well understood. Historically, *Trichuris* species were classified based on host data, but as more genotyping data becomes available, it is unclear whether there are multiple species infecting humans or whether other animals, such as nonhuman primates and pigs, can harbor *T. trichiura* and serve as a reservoir for human infection (Betson et al., 2015). Classified as a “soil-transmitted helminth” (STH) along with *Ascaris lumbricoides* and hookworms, *T. trichiura* is believed to infect around 2.8 million people worldwide, especially in resource-poor countries in the tropics and subtropics (Flueckiger et al., 2019). Human infection is primarily a disease of poverty where there is inadequate waste disposal and limited access to clean water (Else et al., 2020). According to the WHO, *T. trichiura* was responsible for 337,000 years lost to disability worldwide in 2016 (WHO, 2020b). Prevalence of infection is greatest in children ages 5–15 years, and declines in older individuals.

Trichuris trichiura has a simple one-host life cycle. Adults reside in the large intestine and gravid females shed unembryonated eggs into the environment in the feces. After 15–30 days under optimal conditions in soil, eggs embryonate and contain an infections L1 larva. Infection occurs when these embryonated eggs are ingested in food, water, and fomites contained with soil. In the small intestine, the eggs hatch and the larvae migrate to the large intestine (especially the colon and caecum) where they burrow into the intestinal mucosa and molt to become adults. Mated females begin to release eggs 60–70 days post infection (Xie et al., 2018).

Pathogenesis, clinical presentation, and treatment

The pathology and clinical manifestations associated with trichuriasis are primarily due to the localized inflammatory response and blood loss from the adult worms embedded in the colonic mucosa (Else et al., 2020). The extent of tissue damage and symptomatology is strongly linked with the worm burden. Most infected individuals have a low worm burden (<15) and have mild symptoms. In contrast, heavy infection (≥ 800 worms) is associated with clinically-significant colitis and iron deficiency anemia, and patients are likely to have diarrhea (which may be bloody), malnutrition, and abdominal pain (Else et al., 2020). Young children may present with growth retardation and failure to thrive. The most severe manifestation of infection is massive infantile trichuriasis, characterized by chronic mucoid diarrhea, rectal bleeding, iron deficiency anemia, and rectal prolapse (due to mucosal edema and resultant tenesmus) (Else et al., 2020). Treatment is with albendazole, mebendazole, or ivermectin (Medical Letter, 2013).

Diagnosis

Trichuriasis is diagnosed by the detection of eggs in wet mounts of stool and adult worms removed during colonoscopy.

Eggs of *T. trichiura* measure 50–55 μm long by 22–24 μm wide and are barrel-shaped with bipolar hyaline plugs (Fig. 10). They have a thick brown shell and are unembryonated when shed in feces. On rare occasions, eggs of the canine whipworm *T. vulpis* are observed in human stool. The presence of such eggs may reflect spurious passage and not always represent true infection. Eggs of *T. vulpis* are larger, measuring 72–90 μm long by 32–40 μm wide, and are often embryonated in freshly-processed stool specimens (Ash and Orihel, 2007).

Adult worms of *T. trichiura* measure 30–50 mm in length (Fig. 11). They have a long, slender anterior end and a short, thicker posterior end, giving the appearance of a whip, hence the common name. The poster end of the male is often coiled. Adults of *T. trichiura* processed for histopathology will demonstrate traditional characteristics of trichuroid nematodes such as stichocytes around the esophagus and bacillary banding, the former only in the whip-like anterior end (Ash and Orihel, 2007).

***Oesophagostomum* spp. (oesophagostomiasis)**

Oesophagostomiasis is caused by zoonotic nematodes in the genus *Oesophagostomum*. Most human infections have been attributed to *O. bifurcum* in sub-Saharan Africa, but also *O. stephanostomum* in Africa and *O. aculeatum* in Southeast Asia. Infection is initiated by ingestion of L3 larvae on plant material or fomites. The most common clinical presentation is acute abdomen mimicking appendicitis, followed by low-grade fever, anorexia, vomiting, diarrhea, and LRQ pain. Rarely worms will penetrate the intestinal mucosa, causing purulent peritonitis. Diagnosis is made by the finding of eggs in stool. Unfortunately the eggs are very similar to those of hookworm, and separating the two can be difficult in areas of coendemicity (Ghai et al., 2014; Polderman and Blotkamp, 1995).

Trematode infections

***Schistosoma* species (intestinal schistosomiasis, bilharzia)**

Biology and epidemiology

Schistosomiasis, one of the leading causes of morbidity and mortality among parasitic diseases, is caused by “blood flukes” in the genus *Schistosoma*. Six species are usually implicated in human disease: *S. mansoni*, *S. haematobium*, *S. japonicum*, *S. mekongi*, *S. intercalatum*, and *S. guineensis*. *Schistosoma mansoni* is widely distributed in sub-Saharan Africa, as well as in parts of the Middle East, South America (Brazil, Suriname, Venezuela), and the West Indies. *Schistosoma haematobium* is also widely distributed in sub-Saharan Africa and parts of the Middle East, and has been established in Corsica, France where it has hybridized with the

zoonotic *S. bovis*. *Schistosoma intercalatum* is endemic to the Democratic Republic of the Congo and *S. guineensis*, which was historically referred to as the “Guinea strain” of *S. intercalatum*, is distributed in west-central Africa. *Schistosoma japonicum* is distributed in Southeast Asia (primarily in China and the Philippines), while *S. mekongi* is endemic to the Mekong River Valley of Laos and Vietnam (Colley et al., 2014; Gautret et al., 2015; Weerakoon et al., 2015).

Schistosoma species have a complex two-host life cycle involving a vertebrate definitive host and a mollusk intermediate host. Paired adults live *in copula* in the blood vessels of the definitive host. Most human species reside in the mesenteric vessels of the large intestine and rectum except for *S. haematobium*, which resides in the venous plexus of the urinary bladder. *Schistosoma japonicum* and *S. mekongi* can also be found in the superior mesenteric veins draining the small intestine. Ectopic deposition of adults can occur in other sites, such as the liver and lungs. After mating, gravid females leave the male and deposit eggs in the capillaries. Eggs make their way to the lumen of the intestine and are shed into freshwater via the feces. Freshly-shed eggs contain a fully-developed miracidium which hatch in water and infect an appropriate freshwater snail host. In the snail, the parasite undergoes several cycles of asexual reproduction eventually leading to the formation of cercariae. The cercariae leave the snail and swim freely in the water until they come in contact with the skin of a person who is swimming, bathing, or washing laundry in the water. The cercariae penetrate the skin, shed their tails, and become schistosomulae, which are carried via the bloodstream to the lungs or liver where they mature to adults; females will not mature to adulthood unless paired with a male. Paired worms enter the bloodstream and are carried to the mesenteric blood vessels for mating and oviposition (Colley et al., 2014).

Schistosomiasis is considered a neglected tropical disease by the WHO, and there are now global campaigns to prevent and treat this disease in endemic regions. Preventative measures consist of periodic large-scale administration of praziquantel to at-risk individuals (preventative chemotherapy), in addition to access to safe water, snail control, hygiene education, and improved sanitation (WHO, 2020a). While preventative chemotherapy alone will not prevent re-infection in endemic regions, it has been shown to reduce overall disease morbidity and mortality when delivered periodically to at-risk individuals. The WHO estimates that 290.8 million individuals required preventative treatment for this infection in 2018 (WHO, 2020a).

Pathogenesis, clinical presentation, and treatment

Clinical manifestations vary by the stage of infection. Initially, individuals may have a self-limited hypersensitivity response at the site of skin penetration (cercarial dermatitis) in response to dead and dying cercariae (McManus et al., 2018). This is followed by the acute stage of disease in weeks to months after exposure, during which cercariae that successfully mature into schistosomula migrate to the liver, mature, and begin egg deposition. Antigens released from the parasite during this stage trigger a systemic hypersensitivity reactions and immune complex formation. This stage is also known as Katayama syndrome. Clinical manifestations include fever, headache, myalgia, abdominal pain, non-productive cough, eosinophilia, and transient pulmonary infiltrates on radiologic imaging (McManus et al., 2018). Most individuals recover spontaneously after a period of 2–10 weeks. During established active and chronic infection, symptoms are due to the ongoing release of eggs. The eggs secrete glycoproteins which facilitate their passage from the blood vessel through the wall of the colon and into the intestinal lumen. In GI schistosomiasis, the eggs are also swept away by the portal blood supply to the liver, where they induce the formation of eosinophilic granulomas (see Section “Hepato-splenic schistosomiasis”). The tissue granulomas are eventually replaced with fibrous tissue (McManus et al., 2018). Of note, the adult worms are largely able to avoid detection by the host immune response by changing their surface antigens and binding host antigens to their outer tegument.

Intestinal schistosomiasis is characterized by transmural inflammation associated with eggs that are trapped or passing through. Mucosal granulomatous inflammation results in microulcerations, hemorrhage, and pseudopolypoidosis (McManus et al., 2018). This stage is marked by abdominal pain, anorexia, and bloody diarrhea. Treatment is with praziquantel (Medical Letter, 2013).

Diagnosis

Intestinal schistosomiasis is diagnosed by microscopy, including the detection of eggs in wet mounts of stool and histopathologic examination of biopsy specimens, or antibody detection. Antibody detection is especially useful in cases with light infections where egg shedding is below the detection limits of microscopy.

Microscopy

Eggs of *Schistosoma* species are large and conspicuous, but are often shed in small numbers and may not be detected when only one wet mount is examined per stool specimen. In endemic areas, the Kato Katz method and/or microscopic examination of rectal snips is used to enhance diagnosis. The miracidium hatching test can also enhance diagnosis but is rarely used outside of institutional research settings (Weerakoon et al., 2015).

Eggs of all species of *Schistosoma* are large (>50 µm), nonoperculated, possess a lateral or terminal spine, and contain a fully-developed miracidium when shed in feces (Fig. 10). Eggs of *S. mansoni* are 114–175 µm long by 45–70 µm wide and have a conspicuous lateral spine suggestive of a shark fin. Eggs of *S. intercalatum* and *S. guineensis* are 140–240 µm long by 50–85 µm wide, elongate with an equatorial bulge, and have a prominent terminal spine. Eggs of *S. japonicum* are subspherical and measure 70–100 µm long by 55–65 µm wide. They possess a small, inconspicuous lateral spine that is not readily detectable. Eggs of *S. mekongi* are similar to those of *S. japonicum* but slightly smaller, measuring 51–78 µm long by 39–66 µm wide. Eggs of *S. haematobium* are not shed in stool, but may be observed in fecal specimens if contaminated with urine. They are similar to the eggs of *S. intercalatum* and *S. guineensis* and measure 112–170 µm long by 40–70 µm wide but lack the equatorial bulge (Ash and Orihel, 2007).

Eggs, and on rare occasions adults, can be detected in biopsy specimens processed for histopathology. The size and shape of the eggs is often altered due to the angle of cuts, and the spines are often not visible. The presence of large eggs with a mature miracidium in a biopsy specimen is typically pathognomonic for schistosomiasis (Mathison and Pritt, 2018a).

Antibody detection

Antibody detection can be particularly useful in patients with light infections, who have an appropriate travel history and whose stool examinations are negative. Many assays have been developed, including circumoval precipitin test (COPT), indirect hemagglutination (IHA), LFA, IFA, and EIA, and the assays vary in sensitivity and specificity based on the antigen preparation used (crude or purified, adult, egg, or cercarial). Many of these assays have been developed using antigens from *S. mansoni*, which can decrease sensitivity and specificity for other species. For species-specific diagnosis, the most reliable assay is immunoblot (Weerakoon et al., 2015).

There are potential issues to consider regarding antibody detection of schistosomiasis. First, anti-schistosomal antibodies remain for many years post-infection, so a positive antibody test cannot distinguish between past and present infections. Secondly, assays made using crude antigen have a higher rate of cross reactivity to other trematode infections and some nematode infections. In areas of high endemicity, tests should have high specificity to avoid false-positives. However, in areas of nonendemicity, such as travelers returning from endemic areas to the US or Europe, a higher sensitivity assay is preferred for screening, and confirmed with an assay with higher specificity, such as immunoblot (Kinkel et al., 2012; Weerakoon et al., 2015). The CDC in the USA screens with FAST-ELISA using *S. mansoni* adult microsomal antigen (MAMA) and then confirms with immunoblot for the species *S. mansoni*, *S. haematobium*, and *S. japonicum* based on the patient's travel history and clinical presentation. The assays have not been assessed for *S. mekongi*, *S. intercalatum*, and *S. guineensis*, and so sensitivity and specificity might be lower for these three species (<https://www.cdc.gov/dpdx/schistosomiasis/index.html>). While reliable commercially-available assays are available, confirmatory assays such as immunoblot may only be available in endemic areas or large, specialized reference laboratories.

Gastrodiscoides hominis (*Gastrodiscoides* infection)

Gastrodiscoides hominis is a zoonotic trematode infection endemic to Southeast Asia. Hot spots of human infection include India, Pakistan, Myanmar, Vietnam, Philippines, Thailand, and China (Chai et al., 2009). The natural definitive hosts are pigs, although non-human primates, mouse deer, and rodents may serve as reservoir hosts. Adults reside in the lumen of the cecum and colon of the definitive host. Unembryonated eggs are shed into water via the feces. After embryonation, newly-hatched miracidia infect freshwater snails in the genus *Helicorbis*. After cycles of asexual reproduction in the snail host, cercariae leave the snail and encyst as metacercariae on aquatic vegetation, tadpoles, frogs, and crayfish. The definitive host, including humans, becomes infected after eating contaminated plants and animals (Mas-Coma et al., 2006; Chai et al., 2009).

Most infections of *G. hominis* are asymptomatic, and the most common clinical presentation is diarrhea with mucus. Pathologically, there is surface desquamation of the epithelium, and the submucosa and lamina propria are infiltrated with eosinophils, lymphocytes, and plasma cells. Blood vessels are often hyperemic and show thickening of the tunica intima infiltrated with eosinophils and lymphocytes (Mas-Coma et al., 2006). Praziquantel is the drug of choice for *G. hominis* (Mas-Coma et al., 2006).

Gastrodiscoides infection is diagnosed by the detection of eggs in wet mounts of stool or the gross examination of adult flukes removed by endoscopy. Eggs of *G. hominis* are 130–160 µm long by 62–75 µm wide, have an inconspicuous operculum, and are unembryonated in fresh stool specimens. The eggs of *G. hominis* can be very difficult to differentiate from those of *Fasciola* species, *F. buski*, and some echinostomes. Adult flukes 5–14 mm long and are pyriform in shape, with a small, conical anterior end and a large, bulbous posterior portion (Ash and Orihel, 2007).

Parasites of the liver and biliary tree

The parasites that involve the liver and biliary tree encompass both protozoa and helminths (Table 5). Consequences of infection may be life-threatening, such as amebic liver abscess (*Entamoeba histolytica*), hepatic fibrosis (several *Schistosoma* spp.), and cholangiocarcinoma (*Clonorchis sinensis* and *Opisthorchis* spp.).

Protozoan infections

Entamoeba histolytica (hepatic amebiasis)

Hepatic amebiasis is caused by liver infection of the *Entamoeba histolytica*, the only intestinal ameba that causes extraintestinal infection (see Parasites of the Appendix, Large Intestine, and Colon for information on the biology and epidemiology of *E. histolytica* and related amebae). As mentioned previously, the liver is the most common site of extraintestinal *E. histolytica* infection. Other sites include the lungs and brain. Men are 10 times more likely than women to have hepatic amebiasis, although the reasons for this disparity are not fully understood (Wuerz et al., 2012). Trophozoites reach the liver through the portal blood supply and generate a localized inflammatory response resulting in hepatocyte necrosis. The resultant lesion is traditionally referred to as an abscess, although this is a misnomer as neutrophils are not commonly present.

Clinically, patients with hepatic amebiasis present with acute to subacute onset of right upper quadrant pain and fever. Concurrent intestinal symptoms are present in less than 50% of cases. Complications of hepatic amebiasis include rupture into the peritoneal cavity, obstructive jaundice, and bacterial superinfection (Wuerz et al., 2012). Treatment is primarily with metronidazole or tinidazole. An intraluminal agent to clear residual colonic *E. histolytica* cysts (e.g., iodoquinol, paromomycin) is also indicated to prevent recurrent of disease and spread to others. Drainage of the lesion is not generally indicated unless there is a high risk for rupture (e.g., lesion >10 cm in diameter, and/or location in the left lobe of the liver). Diagnosis of hepatic amebiasis is by imaging analysis followed by confirmatory laboratory testing such as antibody detection and molecular analysis. Trophozoites may be detected in permanent-stained smears of liver abscess fluid or in biopsy specimens, but the sensitivity is low and differentiating trophozoites from host WBCs. The nature of the lesional material in hepatic amebiasis is described as resembling “anchovy paste” (Fig. 32).

Ultrasonography and computed topography (CT) scans usually reveal a single abscess measuring on average 4–12 cm; amebic abscesses are more commonly seen in the right lobe of the liver. While amebic abscesses are usually single, multiple abscesses may also be seen (Wuerz et al., 2012).

Antibody detection is the preferred laboratory method for supporting the diagnosis of hepatic amebiasis, especially when corresponding stool examinations are negative (which occurs in 25–50% of cases) (Wuerz et al., 2012). Commercial EIAs tend to have a high sensitivity (greater than 90%) for hepatic and other extraintestinal infections, but cannot reliably differentiate between current and past infection, and so may not a good positive predictor in patients who are from, or spent considerable time in, endemic areas (Ali, 2015).

NAATs can be performed on liver abscess fluid and biopsy specimens. Because *E. histolytica* is the only member of its genus to cause extraintestinal infections, monoplex assays specific for *E. histolytica* can be used over the generally less-sensitive multiplex assays (Ali, 2015; Qvarnstrom et al., 2005). Commercially-available multiplex assays designed for stool specimen should not be used for liver abscess fluid unless thoroughly validated for that specimen type.

Leishmania species (visceral leishmaniasis, kala-azar)

Biology and epidemiology

VL is caused primarily by two species of *Leishmania* in the *donovani*-complex: *L. donovani* and *L. infantum*. *Leishmania donovani* causes anthroponotic infection and endemic areas include India, Nepal, Bangladesh, Sri Lanka, China, Sudan, Ethiopia, and Kenya, with potential hot spots in Bhutan, Chad, Yemen, and Uganda. *Leishmania infantum* causes zoonotic infection with dogs and wild canids as the primary reservoir host and endemic areas of human infection include Mediterranean Europe, North Africa, Southwest Asia, China, and parts of Latin America. *Leishmania archibaldi* (Africa) and *L. chagasi* (Latin America) are believed to be conspecific with *L. donovani* and *L. infantum*, respectively (Ready, 2014). A third species, *L. martiniquensis*, has been implicated as a cause of VL on the Caribbean island of Martinique and in Thailand. *Leishmania siamensis* has also been implicated as a cause of VL in Thailand, but that species is now considered to be conspecific with *L. martiniquensis* (Jariyapan et al., 2018; Pothirat et al., 2014). Leishmaniasis is classified by the WHO as a NTD, and is estimated to cause disease in 50,000–90,000 individuals each year (WHO, 2020a). VL is also recognized as an important opportunistic infection associated with human immunodeficiency virus (HIV). HIV increases the risk of developing active VL, whereas VL accelerates HIV replication; thus coinfection has important clinical and prognostic implications (WHO, 2020c).

The life cycle of *Leishmania* species was covered earlier (Parasites of the Oral Cavity, above).

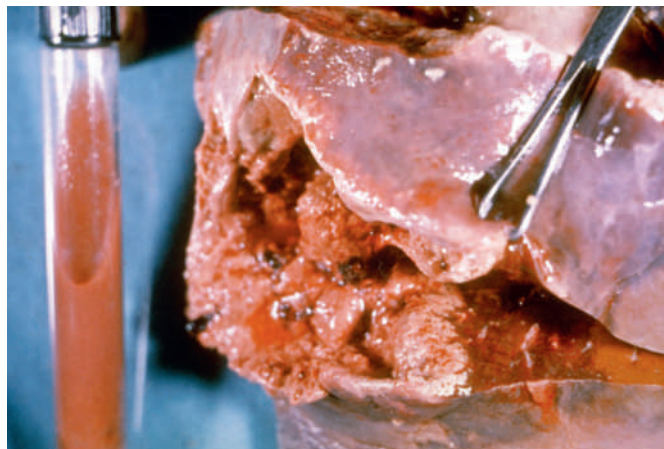


Fig. 32 Hepatic amebiasis, showing an incised liver lesion (right) and fluid resembling “anchovy paste” (left) that had been extracted from the lesion. Image courtesy of the U.S. Centers for Disease Prevention and Control Public Health Image Library.

Pathogenesis, clinical presentation, and treatment

Visceral leishmaniasis is the most severe form of infection, and active disease nearly always fatal within 2 years if untreated (Burza et al., 2018). The clinical manifestations are due to the infiltration of macrophages carrying amastigotes into the spleen, liver and bone marrow, and include persistent fever, hepatosplenomegaly, pancytopenia, weight loss, and wasting. Non-protective hypergammaglobulinemia is common. Skin hyperpigmentation is seen in the Indian subcontinent resulting in the name, *kala-azar*, meaning black fever. Death is usually a consequence of bone marrow involvement with severe anemia, and leukopenia resulting in opportunistic infections including secondary bacterial infections (Burza et al., 2018). Treatment for VL requires systemic therapy, often with parenteral antimonial drugs (e.g., sodium stibogluconate) or liposomal amphotericin B (Burza et al., 2018; Medical Letter, 2013). Oral miltefosine and parenteral paromomycin may also be used, alone, or in combination with other agents to reduce the likelihood of parasite resistance forming. Patients with HIV co-infection require longer treatment and higher doses, and are at increased risk of relapse (Burza et al., 2018).

Diagnosis

VL is traditionally diagnosed by antibody detection, but can also be diagnosed by microscopy (presence of amastigotes in tissue aspirates and biopsy specimens), culture, isoenzyme analysis, and NAATs. In some larger reference laboratories, NAATs have replaced isoenzyme analysis. It is recommended that more than one method be used to enhance the likelihood of a positive result (Aronson et al., 2016).

Antibody detection

Several serologic assays have been developed for the diagnosis of VL and their sensitivity and specificity varies based on assay type, antigens used, and host factors. Assays include direct agglutination test (DAT), gel diffusion, complement fixation, IHA, IFA, EIA, and LFA (Lockwood and Sundar, 2006; Sundar and Rai, 2002). IgG to *Leishmania* persists for years, so antibody detection should not be used to assess response to treatment and cannot necessarily differentiate present and past infections. The potential for false-negative results can be high in immunocompromised patients and cross reactivity to *Trypanosoma cruzi* can also occur (Aronson et al., 2016).

Dipstick (LFA) assays targeting the rK39 antigen of *L. infantum* shows high specificity and sensitivity. When compared with the CDC's IFA, the Kalazar Detect™ Rapid Test (InBios, Seattle, Washington) shows 98% agreement with 90% sensitivity and 100% specificity (Welch et al., 2008). This assay is FDA-cleared for in vitro diagnostic use in the US.

Microscopy

Tissue aspirates and biopsy specimens can be collected for microscopic analysis, as well as for culture as a prelude to molecular analysis. When VL is suspected, bone marrow is the specimen of choice, but liver, lymph nodes, and whole blood (buffy coat) are also adequate specimens (Aronson et al., 2016).

Aspirates can be stained with Giemsa and examined for the presence of amastigotes. Amastigotes of all *Leishmania* species are morphologically similar to one another, and technically are morphologically indistinguishable from those of *T. cruzi*. Amastigotes are ovoid, measuring 1–5 µm long by 1–2 µm wide and possess a large nucleus and a small rod-like kinetoplast (Fig. 3); (Ash and Orihel, 2007).

Amastigotes can be more challenging to detect in biopsy specimens processed for histopathology due to the angle of the cuts and possible shrinkage during processing. The nucleus is often visible, but the kinetoplast may be more difficult to discern. Promastigotes are not naturally found in clinical specimens but may be observed if there is a substantial delay in processing (Mathison and Pritt, 2018a).

NAATs

In recent years, NAATs have started to replace the traditional diagnostic method of isoenzyme analysis, which is labor intensive and time consuming as it is performed on positive culture isolates which can take a week or more to become detectable (De Almeida et al., 2011). NAATs can also be more useful in immunocompromised patients who are less-likely to have detectable IgG and thus have negative serology results (Sundar and Singh, 2018). NAATs for the detection of VL are likely only to be available in large reference labs in nonendemic areas or reference and academic institutions in endemic areas. Unlike for CL or MCL, where there are many more species implicated in human disease, many PCR assays for VL allow for species-level identification. In the US, the CDC uses a PCR targeting rRNA internal transcriber spacer 2 (ITS2) subunit followed by sequencing analysis for detection and specification of *Leishmania* spp. (De Almeida et al., 2011). LAMP assays also show promise for sensitive, specific, and rapid molecular diagnosis of VL (Sundar and Singh, 2018).

Nematode infections

Capillaria hepatica (hepatic capillariasis)

Biology and epidemiology

Hepatic capillariasis is a relatively rare zoonotic disease caused by the trichuroid nematode *Capillaria hepatica*. The parasite occurs nearly worldwide, with sporadic human cases reported from several countries (Li et al., 2010).

Capillaria hepatica has an unusual one-host life cycle. The natural hosts are rodents, but many mammals, including humans, can serve as adequate hosts. Parasite maturation, mating, and oviposition all take place in the liver parenchyma of the host. Mated females lay eggs that are sequestered in the liver and are not shed into the environment by the host. In order for the eggs to embryonate, they must be released into the environment and come in contact with moist soil, usually by one of two means: (1) death and decomposition of the host, or (2) spurious passage into the environment after the host is eaten by a predator. Once in the soil, the eggs embryonate slowly over a period of about 1.5–6.0 months under optimal conditions and contain an infectious L1 larva. Embryonated eggs are ingested by a new host on food or fomites contaminated with soil; children with pica are at an increased risk of infection. Eggs hatch in the cecum, penetrate the intestinal mucosa, and travel to the portal system where they settle in the liver parenchyma. Once in the liver, L1 larvae molt four times to become adults. Gravid females lay eggs over a period of about 30 days, after which they die (Aghdam et al., 2015; Rocha et al., 2015).

Pathogenesis, clinical presentation, and treatment

The presence of *Capillaria hepatica* developing larvae, adults, and eggs invoke a necrotizing, eosinophilic granulomatous inflammatory response in the liver parenchyma (Li et al., 2010). The worms will eventually die calcify, and the lesions resolve by septal fibrosis. A 2019 report of 16 patients with hepatic capillariasis revealed that eosinophilia, leukocytosis, and sustained fever was the most common findings of infection, being found in 100%, 93.75%, and 56.25% of cases, respectively (Wang et al., 2019). Other signs, symptoms and laboratory findings included elevated hepatic enzymes (60%), abdominal pain (37.5%), pulmonary manifestations (37.5%; e.g., cough and wheezing), and watery diarrhea (25%). Patients have been successfully treated using albendazole or pyrantel tartrate and corticosteroids (Li et al., 2010; Wang et al., 2019; Medical Letter, 2013).

Diagnosis

Hepatic capillariasis is diagnosed by the finding of eggs, and sometimes adult worms, in liver biopsy specimens processed for histopathology. Because of the unique life cycle of the parasite, eggs detected in wet mounts of stool represent spurious passage (i.e., following ingestion of infected liver), and follow-up specimens should be collected and examined to rule-out true infection.

Eggs of *C. hepatica* measure 48–66 µm long by 28–36 µm wide, possess a thick, striated, two-layered shell and bipolar plugs, similar to those of *C. philippinensis* (Fig. 10), but with thicker shell and more ovoid shape. Eggs in biopsy specimens are unembryonated. Adults observed in biopsy specimens will have typical trichuroid features in cross-section, including bacillary bands, stichocytes, and a single reproductive tube (Meyers et al., 2000).

Toxocara canis, *T. cati* (visceral larval migrans, toxocariasis)

Biology and epidemiology

Visceral larval migrans (VLM) is a zoonotic disease caused by ascarid nematodes that in their natural life cycle parasitize carnivorous mammals. VLM and ocular larval migrans (OLM) are predominately caused by *Toxocara canis* and *T. cati* (toxocariasis), cosmopolitan parasites of dogs and cats (Despommier, 2003). VLM can also be caused by the raccoon parasite *Baylisascaris procyonis* (baylisascariasis), however that species has a predilection for the CNS where it causes neural larval migrans (NLM) (Gavin et al., 2005).

In their natural life cycle, adults of *T. canis* and *T. cati* live in the small intestine of dogs and cats, respectively (although both species can infect either host). Unembryonated eggs are shed into the environment via the feces, and in 1–4 weeks under optimal conditions in soil, they mature to contain an infectious L3 larva. New dog and cat hosts become infected after the ingestion of fully-embryonated in contaminated soil. In the small intestine, the eggs hatch and the L3 larvae penetrate the gut wall, migrate through the lungs to the bronchial tree. After a short time in the lungs, the L3 larvae are coughed up and swallowed and mature to adulthood in the small intestine. Dogs and cats can also become infected by eating paratenic hosts harboring eggs, such as earthworms, or harboring L3 larvae, such as rodents and birds. Usually younger dogs and cats are more likely to acquire patent infections. When older animals become infected, the larvae usually become arrested in tissues. In pregnant dogs and cats, these arrested larvae can become reactivated during gestation and can be transferred to their fetuses transplacentally. In cats, *T. cati* can also be transferred to nursing kittens via transmammary route (Despommier, 2003). Thus kittens and puppies may be born infected, or acquire infection during nursing.

Humans become infected after the incidental ingestion of embryonated eggs in food and fomites contaminated with soil. Young children are most likely to become infected, given their close exposure to soil. In particular, children with pica are at higher risk. Egg shedding is highest in puppies and kittens and households with the greatest risk of environmental contamination are those that have adopted feral animals or animals that have had prolonged exposure to outdoor environments. Once a yard or other environment is contaminated with eggs control can be difficult, and under optimal conditions, eggs can remain viable in soil for months or years (Despommier, 2003).

Pathogenesis, clinical presentation, and treatment

The presence of dead and dying larvae within host tissues elicits an immediate- and delayed-type hypersensitivity response, resulting in the formation of necrotizing, eosinophilic granulomas (Despommier, 2003). The extent of host damage and clinical presentation is largely dependent on the burden of infection and the tissues that have been invaded. Eye involvement can lead to impaired sight, and even blindness in some cases. Some cases had been previously misdiagnosed as retinoblastoma, resulting in extreme treatment measures such as enucleation. Fortunately, this misdiagnosis is uncommon today (Despommier, 2003). Visceral larva migrans

typically presents with fever, hepatosplenomegaly, asthma-like respiratory symptoms, and high levels of eosinophilia (e.g., approaching 70%). Hypergammaglobulinemia is also commonly present (Despommier, 2003). Albendazole is the preferred anti-helminthic drug for treating toxocariasis, and may be administered in conjunction with corticosteroids to decrease harmful inflammation and suppress allergic manifestations (Despommier, 2003; Medical Letter, 2013).

Diagnosis

VLM is diagnosed primarily by clinical presentation and confirmed by antibody detection. L3 larvae may be observed in liver biopsy specimens, however biopsies are rarely collected when VLM is suspected.

Antibody detection

Antibody detection for VLM is primarily performed by EIA. These assays can usually detect both *T. canis* and *T. cati*, but cannot distinguish between the two. *Toxocara* serology will also commonly detect antibodies to *B. procyonis*. Arrested larvae in human tissues usually survive for months to years, continuously releasing antigens that keep the immune response stimulated. As such, antibodies may persist for years. After antihelminthic treatment, IgG levels have been shown to decline but may remain detectable for 4–5 years (Wilkins, 2014).

Historically, EIAs were developed using antigens extracted from embryonated eggs or L3 larvae cultured in vitro. The development of EIAs using *Toxocara* excretory-secretory (TES) antigens showed improved sensitivity and specificity. Assays may detect either IgG or IgE, but it is debatable which offers better specificity (Wilkins, 2014).

Microscopy

L3 larvae of *Toxocara* spp. in liver biopsy specimens are 290–350 μm long and 15–21 μm in diameter (Fig. 33). They possess typical features of ascarid larvae such as prominent excretory canals, few muscle cells per quadrant, and prominent lateral alae. As they remain in their larval state, reproductive structures are not present. Larvae of *B. procyonis* share similar features but are slightly larger, at upwards of 1.4 mm long and 62 μm in diameter (Mathison and Pritt, 2018a). While larvae are only rarely seen in biopsies, evidence of the host immune response (e.g., eosinophilic granulomas and abscesses) is commonly appreciated.

Trematode infections

Schistosoma species (hepatosplenic schistosomiasis)

Biology and epidemiology

Hepatosplenic schistosomiasis is caused by the same species that cause intestinal schistosomiasis. The biology and epidemiology of these trematodes are discussed previously (Parasites of the Appendix, Large Intestine, and Anus Above).

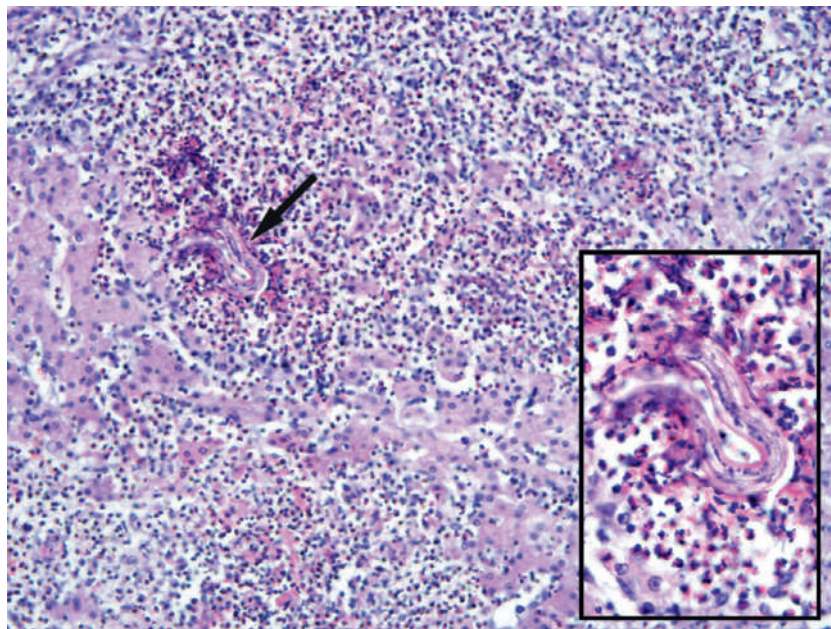


Fig. 33 Longitudinal section of a *Toxocara* sp. larva (arrow, inset) in a liver biopsy, associated with eosinophilic abscesses (H&E, 200 $\times$; inset 1000 $\times$). From Mathison BA and Pritt BS (2018) Chapter 5: Parasitic Diseases. In: *Atlas of Fundamental Infectious Diseases Histopathology: A Guide for Daily Practice*. Pritt BS (ed.). Pathologists: Northfield, IL: College of American.

Pathogenesis, clinical presentation, and treatment

As mentioned above, clinical manifestations vary by the stage of infection. This section will only address hepatic manifestations that occur during established active and chronic infection, when eggs are swept to the liver via the portal blood supply and induce the formation of eosinophilic granulomas in the pre-sinusoidal periportal tissues. This initially results in substantial enlargement of the liver and spleen (McManus et al., 2018) (Fig. 34). The tissue granulomas are eventually replaced with fibrous tissue and the size of the liver shrinks. Collagen is deposited around branches of the portal vein, leading to a distinct entity called Symmer's pipestem (or clay pipe) fibrosis, named due to the appearance of the thickened portal tracts in gross specimens, which are thought to resemble a clay pipe stem in cross-section. Of note, there is usually no hepatic parenchymal damage in hepatic schistosomiasis, and cirrhosis does not occur. Fibrosis is irreversible, and may lead to occlusion of small branches of the portal vein, with resultant portal hypertension and associated sequelae (e.g., esophageal varices, ascites). Since there is no damage to the hepatic parenchyma, liver enzymes are within normal ranges (McManus et al., 2018). Treatment is with praziquantel. Unfortunately, sequelae associated with fibrosis may be irreversible.

Diagnosis

See the section above on intestinal schistosomiasis. Eggs may be seen in liver biopsies in association with a granulomatous or fibrous host cell response (Fig. 35).

Fasciola hepatica and *F. gigantica* (fascioliasis)

Biology and epidemiology

Fascioliasis is a zoonotic trematode infection caused by *Fasciola hepatica* and *F. gigantica*. *Fasciola hepatica* has a worldwide distribution where cattle, sheep, and other ruminants are farmed. Hot spots of endemicity for human disease include the Bolivian Altiplano, Ecuador, Peru, Cuba, Portugal, Spain, Turkey, the Nile Delta, Iran, and Vietnam (Tolan, 2011). The WHO estimates 2.4 million people are infected in more than 70 countries (https://www.who.int/foodborne_trematode_infections/fascioliasis/en/). *Fasciola gigantica* is more geographically restricted, being endemic to tropical Africa, the Middle East, and Southeast Asia, in areas where *F. hepatica* also occurs (Tolan, 2011).

Fasciola spp. have a complex two-host life cycle. Adults reside in the biliary ducts of the mammalian definitive host. Natural hosts for *Fasciola* spp. are primarily herbivorous ruminants, such as sheep, cattle, goats, deer, and camels. Unembryonated eggs are discharged into the biliary ducts and are passed into the environment in feces. In freshwater, eggs embryonate over a period of approximately 2 weeks. Embryonated eggs hatch, releasing miracidia that infect an appropriate snail intermediate host. Snail hosts for *Fasciola* are members of the family Lymnaeidae, including *Galba*, *Lymnaea*, and *Pseudosuccinea*. In the snail host, the parasite undergoes several asexual cycles resulting in the formation of cercariae. Cercariae leave the snail host and encyst as metacercariae on aquatic vegetation such as watercress. The definitive host becomes infected after ingesting contaminated aquatic plants or drinking water contaminated with liberated metacercariae. Metacercariae excyst in the duodenum, penetrate the intestinal wall, and migrate to the biliary ducts where they mature to adults. Eggs are usually shed in stool 8–2 weeks post infection (Tolan, 2011).



Fig. 34 Young Puerto Rican boy with a visibly-distended abdomen due to hepatosplenomegaly caused by schistosomiasis. Image courtesy of the U.S. Centers for Disease Control and Prevention Public Health Image Library.

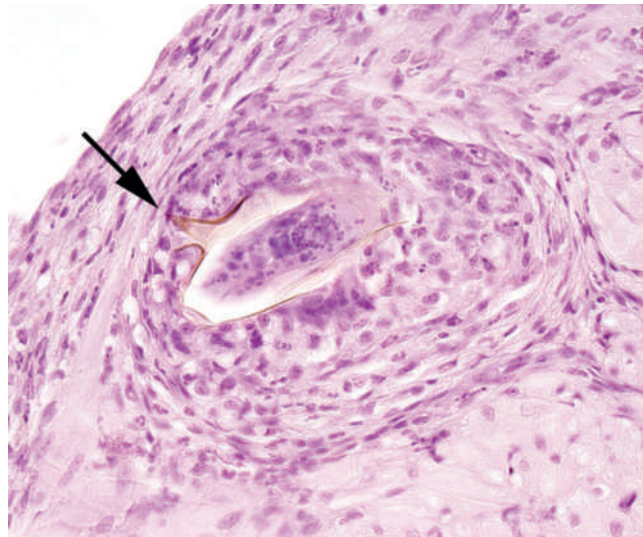


Fig. 35 *Schistosoma mansoni* eggs within a granuloma (H&E, 400 $\times$). Note the presence of a characteristic lateral spine (arrow) which is defining for this species, as well as the nuclei of the miracidium within the egg. It is not always possible to identify spines on eggs in tissue, and care must be taken not to overinterpret egg shell irregularities that occur during tissue processing as true spines.

Pathogenesis, clinical presentation, and treatment

The pathology and clinical manifestations of fascioliasis are due to the migration of the maturing fluke through Glisson's capsule of the liver and the hepatic parenchyma, and the eventual presence of the adults within the biliary tree (Saba et al., 2004; Tolan, 2011). More than 50% of infections are thought to be asymptomatic. When present, manifestations of the liver migration stage commonly include fever, myalgias, arthralgias, eosinophilia and leukocytosis. Urticaria, asthma-like respiratory symptoms, weight loss and headache may also be seen (Tolan, 2011). Later in infection, the presence of the adults in the biliary tract may result in cholelithiasis, ascending cholangitis, pancreatitis, cholelithiasis, biliary cirrhosis, and hepatic fibrosis. Unlike infection with *Clonorchis* and *Opisthorchis*, cholangiocarcinoma is not seen with fascioliasis (Tolan, 2011). Triclabendazole is the treatment of choice. Of note, the drug of choice for other trematode infections, praziquantel, is ineffective in treating fascioliasis (Medical Letter, 2013).

Diagnosis

Fascioliasis is diagnosed primarily by the detection of eggs in wet mounts of stool or by antibody detection. Because eggs are usually be detected in stool until about 8–12 weeks post infection, antibody detection may be used for diagnosing early infection. Occasionally adults may be observed via endoscopic retrograde cholangio-pancreatography (ERCP) or adults and eggs may be observed in biopsy specimens processed for histopathology.

Microscopy

Fascioliasis may be diagnosed microscopically by the detection of eggs in wet mounts of stool, less commonly by the finding of adult flukes via ERCP or biopsy specimens stained for histopathology. Eggs of *F. hepatica* measure 130–150 μm long by 63–90 μm wide (Fig. 10); those of *F. gigantica* are larger at 160–190 μm long by 70–90 μm wide. Eggs of both species have a thick shell, a small, inconspicuous operculum, and are unembryonated when shed in stool. The eggs of *F. hepatica* are morphologically indistinguishable from those of *Fasciolopsis buski*, and can be difficult to separate from other flukes such as *Gastrodiscoides hominis* and the echinostomes. It is therefore common for laboratories to report the finding of characteristic eggs as *Fasciola* spp./*Fasciolopsis buski* eggs (Ash and Orihel, 2007).

It is important to remember that the presence of *Fasciola* eggs in stool might represent spurious passage after the ingestion of infected beef or sheep liver, rather than true infection. Follow-up stool exams on positive patients should be ordered to rule-out spurious passage (Ash and Orihel, 2007). Patients should be instructed not to ingest liver during this follow-up period.

Grossly, adult flukes are large, measuring up to 30 mm long by 13 mm wide. They are flat, leaf-like in shape, and the anterior end is distinctly cone-shaped (Ash and Orihel, 2007). Adults observed in biopsy specimens will have inherent features of trematodes. The tegument will typically have flattened, scale-like spines. Eggs observed in biopsy specimens are large and unembryonated. The only fluke eggs in a liver biopsy specimen what would near *Fasciola* in size are those of *Schistosoma*, which should contain a mature miracidium (Mathison and Pritt, 2018a).

Antibody detection

Antibody detection allows for diagnosis of fascioliasis that can be particularly helpful for diagnosing early or acute infection. Antibodies can be detected as early as 2 weeks post infection, nearly 2–3 months earlier than when eggs are typically observed in stool. Early diagnosis and treatment of fascioliasis can prevent chronic severe liver damage. While sensitivity and specificity of most assays are high, some cross-reactivity with other helminth infections does occur (Sarkari and Khabisi, 2017).

The most common platform for immunodiagnosis of fascioliasis is EIA. There are some commercially-available assays, including the DRG Fasciola IgG ELISA (DRG International, Springfield, New Jersey) which has FDA and CE approval. Assays are usually developed using excretory-secretory antigens. The most-studied antigens used in development of assays include the 27 kDa and cathepsin L1 (CL1) antigens. The saposin-like protein 2 (SLP-2) also demonstrated high sensitivity and specificity. Most assays are developed using antigens from *F. hepatica* and may express varying degrees of specificity for *F. gigantica*. Assays that can detect both cannot differentiate between the two species, but species-level identification is not needed for clinical management (Sarkari and Khabisi, 2017). Researchers at the CDC developed immunoblot and Luminex-based assays using the glutathione S-transferase recombinant antigen GST-FhSAP2 for the immunodiagnosis of fascioliasis. Both assays exhibited relatively high sensitivity and specificity, but there was some cross-reactivity with other helminth infections. The immunoblot assay showed some cross-reactivity to hookworm and toxocariasis with total IgG and hookworm with IgG4. The Luminex assay some cross-reactivity to schistosomiasis and toxocariasis with total IgG and some cross-reactivity to hookworm with IgG4 (Shin et al., 2016).

Clonorchis sinensis, Opisthorchis viverrini, O. felinus (clonorchiasis, opisthorchiasis)

Biology and epidemiology

Clonorchis sinensis, *Opisthorchis viverrini*, and *O. felinus* are often referred to as the ‘small liver flukes.’ *Clonorchis sinensis* (the ‘Chinese liver fluke’) is endemic to East Asia, with hot spots of human infection in Korea, China, Taiwan, and Vietnam (Qian et al., 2012). *Opisthorchis viverrini* is endemic to Southeast Asia, with hot spots of human infection in Thailand, Laos, Vietnam, and Cambodia (Kaewpitoon et al., 2008). *Opisthorchis felinus* is endemic to Eastern Europe, Central Asia, and Russia (Pakharukova and Mordvinov, 2016). In Canada and the northern USA, can humans rarely become infected with another small hepatic trematode, *Metorchis conjunctus* (Behr et al., 1998; Maclean et al., 1996).

All opisthorchid trematodes have a similar three-host life cycle. Adults reside in the biliary ducts of the definitive hosts. Embryonated eggs containing a fully-developed miracidium are discharged from the biliary ducts and passed in stool. In fresh water, the eggs are ingested by a suitable freshwater snail host. In the snail host, the miracidium undergoes cycles of asexual reproduction resulting in the formation of cercariae. The cercariae leave the snail host and infect freshwater fish. Over 100 species of fish have been documented as suitable hosts. In the flesh of the fish, the cercariae encyst as metacercariae. The definitive host, including humans, becomes infected after eating raw or undercooked fish harboring metacercariae. The metacercariae excyst in the duodenum and ascend the biliary tree via the ampulla of Vater. Maturation to adults takes approximately 1 month (Kaewpitoon et al., 2008).

Pathogenesis, clinical presentation, and treatment

Most of the pathology associated with *Clonorchis sinensis* and *Opisthorchis* spp. infection is due to the presence of the adult flukes in the bile ducts (Kaewpitoon et al., 2008). The extent of symptoms depends on the parasite burden, duration of infection, and host factors. Many infections are asymptomatic. When present, common manifestations include cholangitis, cholecystitis, cholelithiasis, hepatomegaly, and obstructive jaundice. There is also a strong association of long-standing infection with cholangiocarcinoma. Pathologic findings include bile duct dilation, periductal fibrosis and inflammation, marked biliary epithelial hyperplasia, and biliary metaplasia (Choi et al., 2004). The etiology of cholangiocarcinoma is thought to be multifactorial, and include mechanical damage caused by the flukes within the bile ducts (e.g., from their suckers and metabolic by-products), parasite secretions, and perhaps most importantly, epithelial damage caused by the host immune response (Choi et al., 2004; Kaewpitoon et al., 2008). The patient’s genetic makeup and exposure to environmental carcinogens (e.g. N-nitroso compounds in food) may also contribute to progression to cholangiocarcinoma (Kaewpitoon et al., 2008).

Signs and symptoms include malaise, fever, abdominal discomfort, distension, anorexia, weight loss, and diarrhea. Testing commonly reveals a peripheral eosinophilia and hyperbilirubinemia. Late stage infection may be associated with hepatosplenomegaly, portal hypertension and ascites (Choi et al., 2004). As with most other trematode infections, the treatment of choice is praziquantel (Medical Letter, 2013).

Diagnosis

The diagnosis of opisthorchiasis and clonorchiasis is primarily made by the finding of eggs in concentrated wet mounts of stool, although antibody detection and NAATs may be available in endemic areas (Kaewpitoon et al., 2008).

Eggs of the opisthorchid trematodes are all very similar in size and shape. Eggs of *C. sinensis* are 27–35 µm long by 12–19 µm wide, have prominent opercular shoulders, an abopercular knob, and contain a miracidium with passed in feces (Fig. 10). Eggs of *Opisthorchis* spp. are smaller at 19–29 µm long by 12–17 µm wide. Despite the smaller size, there is sufficient overlap that a genus-level identification may be challenging. It is not uncommon for labs to issue a combined report (e.g., *Clonorchis sinensis*/*Opisthorchis* spp. eggs) (Ash and Orihel, 2007). Eggs of *M. conjunctus* are 28.5 µm long by 15.6 µm wide on average (Maclean et al., 1996).

Dicrocoelium dendriticum, D. hospes (dicrocoeliasis)

Dicrocoeliasis is a rare zoonotic disease caused by liver flukes in the genus *Dicrocoelium*. Most true human infections are caused by *D. dendriticum*, which occurs in North America, Asia, Europe, northern Africa, and the Middle East. *Dicrocoelium hospes* causes rare human disease in West Africa (Cengiz et al., 2010; Odei, 1966). Like other trematodes, *Dicrocoelium* spp. have a complex multi-host life cycle. Definitive hosts are typically ruminants, including cattle, sheep, and goats. The first intermediate hosts are terrestrial snails. The second intermediate hosts and source of infection for the definitive hosts are ants (Cengiz et al., 2010).

Infection is often asymptomatic. When present, patients with dicrocoeliasis may present with abdominal distention, RUQ pain, diarrhea, constipation, and weight loss. Peripheral eosinophilia and anemia are common laboratory findings. Heavy infections may result in cholecystitis and liver abscesses (Cengiz et al., 2010). Successful treatment has been reported using praziquantel or triclabendazole (Cengiz et al., 2010; Schweiger and Kuhn, 2008).

Diagnosis of dicrocoeliasis is made by the finding of eggs in concentrated wet mounts of stool. Humans can spuriously pass *Dicrocoelium* eggs after ingesting infected animal livers, so multiple specimens should be collected and examined to rule-out or confirm true infection (Cengiz et al., 2010). Eggs of *D. dendriticum* measure 38–45 µm long by 22–30 µm wide, have a thick shell, an inconspicuous operculum, lack an abopercular knob, and contain a fully-developed miracidium when shed in feces (Ash and Orihel, 2007).

Cestode infections

Echinococcus granulosus-complex (cystic echinococcosis)

Biology and epidemiology

Cystic echinococcosis (CE) is a zoonotic cestode infections caused by members of the genus *Echinococcus*, primarily by species in the *granulosus*-complex. There is currently not a taxonomic standard for the number of valid species in the complex. Some systems recognize one species, *E. granulosus*, with numerous genotypes or strains, while others recognize multiple species. A recent study on the molecular epidemiology of members of the *granulosus*-complex recognizes three species that have been implicated in human disease: *E. granulosus* (genotypes G1-G3, the sheep-buffalo strains), *E. canadensis* (genotypes G6-G8, G10, pig, camel, and cervid strains), and *E. ortleppi* (genotype G5, cattle strain). Most of these species have wide distributions in North America, Europe, Asia, and the Middle East. In South America, two additional species cause neotropical cystic (a.k.a. polycystic) echinococcosis, *E. oligarthra* and *E. vogeli* (Romig et al., 2015). The WHO has classified echinococcosis as an NTD and estimates that more than 1 million people are infected by *Echinococcus* species at any one time (WHO, 2020a).

All species have a similar life cycle that cycles between a carnivore definitive host and an herbivore intermediate host. Most species use dogs and wild canids as definitive hosts (*E. oligarthra* uses wild felids as a definitive host). Adult cestodes reside in the small intestine of the definitive host. Eggs are shed via the feces and contaminate the surrounding environment. The natural intermediate host becomes infected after the ingestion of infectious eggs while grazing. The eggs hatch in the intestine of the intermediate host and the liberated oncosphere is carried via the bloodstream to other organs where it develops into a hydatid cyst (metacestode). Within the primary cyst, daughter cysts and protoscoleces develop (Fig. 36). Each protoscolex has the potential of becoming an adult tapeworm when eaten by a suitable definitive host. A new definitive host becomes infected after eating infected intermediate hosts. Humans serve as an incidental, dead-end intermediate host after the ingestion of tapeworm eggs in food and fomites contaminated with canine feces (Eckert and Deplazes, 2004). In humans, hydatid cysts can localize in many organs, with the liver being the most common site, followed by the lung (Eckert and Deplazes, 2004).



Fig. 36 Echinococcal cyst that has been incised to reveal multiple collapsed daughter cysts within. Courtesy of the U.S. Centers for Disease Control and Prevention Public Health Image Library.

Pathogenesis, clinical presentation, and treatment

The pathogenesis and clinical manifestations vary based on the size of the lesion and location in the host. Cystic echinococcosis begins as a microscopic lesion and slowly grows over time, so that many years may elapse between infection and clinical presentation (Wen et al., 2019). Lesions become clinically relevant when impinge upon neighboring structures. Patients with liver cysts commonly present with abdominal discomfort and decreased appetite. Jaundice may occur with compression of the biliary tree. Cyst leakage or frank rupture can result in life-threatening allergic reactions, ranging from urticaria, fever and peripheral eosinophilia to anaphylactic shock (Wen et al., 2019). Leakage into the surrounding tissue can also result in local dissemination of the parasite. Treatment varies with the type and size of the cyst (see Imaging below) (Wen et al., 2019; Medical Letter, 2013). Of note, it is no longer considered ‘taboo’ to insert a needle in CE lesions, so long as proper precautions for preventing anaphylaxis and dissemination are in place. For cysts that are amenable to drainage via percutaneous aspiration, the preferred treatment is now the PAIR technique (puncture, aspiration, injection and reaspiration), or the modified catheterization technique (MoCAT). These methods use percutaneous drainage in conjunction with injection of a protoscolicidal agent to deflate the cyst and kill the inner reproductively-active germinal layer without the need for surgery. The MoCAT represents an improvement upon the PAIR technique in that parasitic membranes are aspirated in addition to the cyst contents. The antihelminthic drug, albendazole, is administered in conjunction with PAIR and MoCAT therapy. For more complex cysts, surgical removal is required, in addition to albendazole.

Diagnosis

The diagnosis of CE is usually initially performed by imaging analysis followed up with serologic testing (antibody detection) for confirmation. Echinococcosis can also be diagnosed microscopically by the finding of cysts in biopsy specimens or liberated protoscoleces in aspirate specimens.

Imaging

Hydatid cysts are classified into four categories based on their radiologic findings. Type I cysts are simple cysts with no visible internal structures; they are believed to represent early and active cysts. On CT, Type I cysts appear as well-defined water-attenuated lesions. Magnetic resonance imaging (MRI) shows hypointensity on T1-weighted images and hyperintense lesions on T2-weighted images. Type II cysts are cysts that have daughter cysts within the primary cyst. On imaging analysis, Type II cysts usually appear with a rosette or honeycomb pattern caused by the daughter cysts. Type III cysts are calcified cysts. On CT, Type III cysts appear as round hyperattenuating areas while on MRI they appear as hypointense areas. Type IV cysts are classified as ‘complicated cysts’ due to an abnormal size and profound mass effect on neighboring organs or because they have ruptured. In the case of ruptured cysts, fissures can be seen in the cyst wall on both CT and MRI (Marrone et al., 2012; Polat and Atamanalp, 2009).

Antibody Detection

Many assays have been developed for antibody detection of *Echinococcus* spp., including EIA, IFA, immunochromatographic card tests (ICTs), immunoelectrophoresis, and immunoblot. The sensitivity and specificity of these assays varies from platform to platform. Generally, assays such as EIA and IFA are good for screening, but assays with higher specificity, such as immunoblot, are better for confirmation (Zhang et al., 2012). Patients with single, well walled-off cysts might not have a measurable antibody response, so a negative result does not rule-out disease. Likewise, false positives can occur in patients with other helminth infections, tumors, or autoimmune disorders (Chen et al., 2014). In particular, a high degree of cross-reactivity has been noted with cysticercosis.

Microscopy

In liver biopsy specimens, a hydatid cyst appears as a well-defined cyst surrounded by a thick fibrous wall of host origin. The outer layer is thick, acellular, and elastic. It often appears to be made of several smaller layers (‘laminated layer’). The inner cyst layer is nucleated and serves as a germinal base for daughter cells which contain multiple protoscoleces. An individual protoscolex are invaginated and contain hooklets and many calcareous corpuscles which are a hallmark of cestode anatomy (Fig. 37).

The presence of multiple protoscoleces rules-out cysticercosis caused by *Taenia solium*, which possesses only one protoscolex per cyst. In cases of coenurosis (a zoonotic disease caused by canine parasites in the genus *Taenia*), multiple protoscoleces can also occur. However, in cases of coenurosis, there are no daughter cysts within the primary cyst (Mathison and Pritt, 2018a). Wet mount aspirates of cysts may show liberated protoscoleces and freed hooklets and calcareous corpuscles from disintegrated protoscoleces (‘hydatid sand’) (Ash and Orihel, 2007).

Echinococcus multilocularis (alveolar echinococcosis)

Biology and epidemiology

Alveolar echinococcosis (AE) is a zoonotic cestode infection caused by *Echinococcus multilocularis*. The disease occurs in the Northern Hemisphere, including Canada and northern USA, Europe, Russia, and Central Asia. The life cycle is similar to members of the *E. granulosus*-complex (above), with foxes serving as natural definitive hosts and rodents serving as intermediate hosts. When humans become infected with *E. multilocularis* after the ingestion of eggs in food or fomites contaminated with canid feces; the most common site of infection is the liver. However, the destructive nature of the alveolar cyst can lead to infection of neighboring organs and the CNS (Wen et al., 2019).

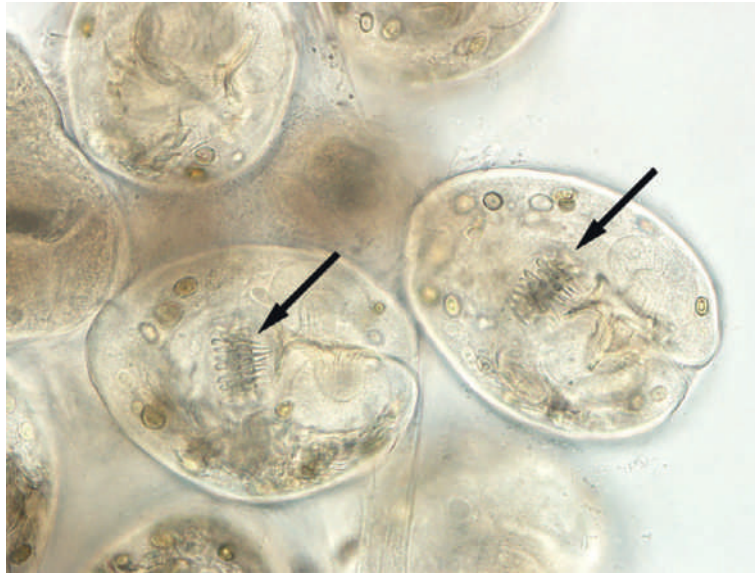


Fig. 37 Protoscolexes aspirated from a case of cystic echinococcosis. Note the refractile hooklets (arrows) within each protoscolex (400 ×). From Mathison BA and Pritt BS (2018) Chapter 5: Parasitic Diseases. In: *Atlas of Fundamental Infectious Diseases Histopathology: A Guide for Daily Practice*. Pritt BS (ed.). Pathologists: Northfield, IL: College of American.

Pathogenesis, clinical presentation, and treatment

Unlike the single cystic lesion of cystic echinococcosis, AE results in a proliferation of infiltrating cysts that invade neighboring structures and organs (Fig. 38) (Wen et al., 2019). The disease is usually fatal if not treated. The preferred treatment, when possible, is complete resection of the lesions, in conjunction with prolonged albendazole therapy. Life-long suppression with albendazole may be required when complete surgical resection is not possible (Wen et al., 2019; Medical Letter, 2013).

Diagnosis

The diagnosis of alveolar echinococcosis is made initially by imaging analysis followed by serology or microscopy (histopathology) for confirmatory diagnosis.

Imaging analysis

On ultrasonography, cysts of *E. multilocularis* appear as mass lesions with a mixed heterogeneous echogenic pattern with irregular contours, cystic necrotic areas, and calcified foci. On CT, cysts appear tumor-like masses with irregular borders, variable internal structures, and random calcified foci. MRI can be helpful for extra-abdominal cysts, but is not as sensitive for detecting calcifications

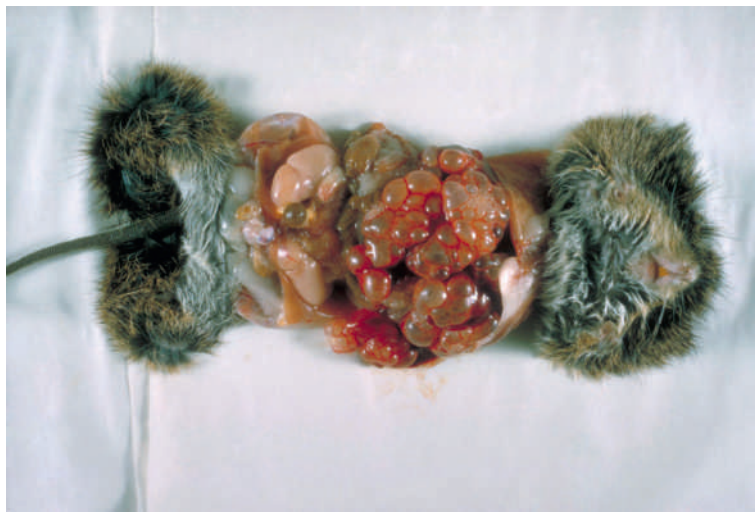


Fig. 38 Alveolar echinococcosis in a cotton rat (*Sigmodon* sp.) in which the liver and other abdominal organs have been infiltrated by cysts of varying sizes. Courtesy of the U.S. Centers for Disease Control and Prevention.

seen with CT. The typical finding on MRI is a mass lesion with infiltrative characteristics having irregular borders. There is usually hypointensity or isointensity on T1-weighted images and hypotensity, isotensity, or hypertensity on T2-weighted images (Bulakçı et al., 2016).

Antibody detection

Antibody detection for the diagnosis of AE is usually made by EIA targeting excretory-secretory antigens derived from intact cysts. The Em2^{plus} EIA (Bordier, Crissier, Switzerland) had been used in Europe extensively for the diagnosis of AE with sensitivity and specificity approaching 90% (Wen et al., 2019).

Microscopy

On histopath examination, cysts of *E. multilocularis* grow in an invasive fashion as cysts arise indefinitely from the germinal layer. Laminated and germinal layers are convoluted and folded on themselves, scattered throughout the host tissue. Protoscoleces are absent, as are calcareous corpuscles, usually (Mathison and Pritt, 2018a).

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Bacterial Eye Infections

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Infections of the eyelids

The eyelid shields the anterior surface of the eye from the environment and injury; it is a physical barrier and contains a row of eyelashes that protect the eye from dust particles, foreign bodies and perspiration. The eyelids have a vascular supply, lymphoid tissue and lymphatic drainage as well as innervation to a central muscle. An imbalance of these local and systemic defenses can cause bacterial infections of the eyelid.

Bacterial blepharitis

Anterior bacterial blepharitis is typically caused by staphylococcal infection and affects the base of the eyelashes producing inflammation of the lid margin. Other causative organisms include *Propionibacterium acnes* and *Corynebacterium* species (Jackson, 2008). The disease can occur in all ages, ethnicities, and gender. However, it is more common in adults older than 50 years of age (Pflugfelder et al., 2014). Ocular symptoms include itching, burning, gritty, red eyes, dry or watery eyes, blurred vision, foreign body sensation, and crusting of the eyelids. Symptoms are often worse in the morning (Fig. 1). Patients typically exhibit eyelash loss and/or misdirection, a feature that is rarely seen with other types of blepharitis. Diagnosis is based on patients' signs and symptoms, and bacterial cultures can be performed to confirm bacterial infection.

Treatment consists of a combination of lid hygiene, warm compresses (10 mins, two to four times a day) and topical antibiotics. Topical ointments such as erythromycin or bacitracin applied to the eyelid margin have been shown to provide symptomatic relief and are effective in eradicating bacteria from the eyelid margin (Lindsley et al., 2014). With the increase of antibiotic resistance to *Staphylococcus*, Fusidic acid is a good alternative, as it covers *Staphylococcus epidermidis*, *Staphylococcus aureus* and methicillin-resistant *S. aureus*. Aminoglycosides (e.g., gentamicin or tobramycin) can be used but do not have the same broad coverage against gram-positive bacteria and are more likely to cause toxicity with prolonged use (Jackson, 2008). Fluoroquinolones (e.g., ciprofloxacin and levofloxacin ophthalmic solutions) have shown effectivity in managing bacterial blepharitis, but their long-term use has been discouraged due to the increase in antibiotic resistance (Yactayo-Miranda et al., 2009; Jackson, 2008). Topical vancomycin and azithromycin have also been used to treat blepharitis (Fleischer et al., 1986; Haque et al., 2010). Intermittent topical antibiotic treatment can be repeated using different medications to prevent the development of antibiotic resistant (Preferred Practice Pattern, 2018). Short-term topical corticosteroids (less than 2 weeks) may be beneficial in controlling inflammation in severe cases. The lowest effective topical steroid dose should be employed, and a tapering course administered (Preferred Practice Pattern, 2018).



Fig. 1 Clinical presentation of blepharitis.

Antibiotic-steroid combinations are also available in either ointment or eye drop form and may be suitable for some patients (Jackson, 2008). To prevent blepharitis, good eyelid hygiene and elimination of triggers that exacerbate symptoms such as thorough removal of eye makeup should be performed (Fig. 1).

Preseptal cellulitis

Preseptal cellulitis is an infection of the soft tissues localized anterior to the orbital septum. The condition most commonly affects children and primarily caused by trauma or sinusitis (Hauser and Fogarasi, 2010). The most common causative organisms for preseptal cellulitis are *Staphylococcus*, *Streptococcus* and *Haemophilus*. The infection is usually unilateral and presents with edema and erythema of the peri-orbital skin. Patients may experience eye pain, redness and a tender eyelid whilst maintaining full extraocular movements, without proptosis, and no changes to vision. This is because the extent of the infection is superficial; it does not extend posteriorly into the orbit. Preseptal cellulitis rarely leads to serious complications, however, the infection may extend posteriorly via facial and ophthalmic veins causing orbital cellulitis (Chaudhry et al., 2012). In extreme cases, it can extend further to cause subperiosteal abscess, orbital abscess, intracranial infection or cavernous sinus thrombosis (Baring and Hilmi, 2011).

Diagnosis is based on patient history, clinical features and radiologic findings. Patients with preseptal cellulitis typically will have a history of sinusitis or upper respiratory infection, trauma, or infection or insect bites in a nearby area. Preseptal and orbital cellulitis have similar presentations making the differential diagnosis difficult. It is important to note the patient's temperature (i.e., fever) and ophthalmic complaints as these may indicate orbital cellulitis. In orbital cellulitis there is typically eyelid edema and erythema, diminished visual acuity, proptosis, a relative afferent pupillary defect, reduced color saturation, chemotic conjunctiva and reduced extraocular movements (Basic and Clinical Science Course, 2007). Preseptal cellulitis tends to present with eyelid edema and erythema, but with normal visual acuity, pupil reaction to light, color saturation, conjunctiva and ocular movements. A computerized tomography (CT) scan of the orbits and sinuses can be used to differentiate between the two conditions, as well as depicting the extent of the infection. A CT scan of preseptal cellulitis will show eyelid swelling, no proptosis, no fat stranding of orbital contents, and no involvement of the extraocular muscles (Carlisle and Digiovanni, 2015; Hauser and Fogarasi, 2010).

The mainstay of treatment is broad-spectrum systemic antibiotics with coverage against gram-positive and gram-negative bacteria. Most patients with preseptal cellulitis are managed with oral antibiotics, such as fluoroquinolones or azithromycin. Intravenous antibiotics such as third-generation cephalosporins or ampicillin are recommended for children less than 1 year of age, immunosuppressed patients and those with severe systemic infections (Hauser and Fogarasi, 2010; Ferguson and McNab, 1999). Initial antibiotic therapy should be altered if there is no response to treatment or laboratory cultures indicate otherwise.

Hordeolum

An acute infection of the sebaceous glands of Zeis, usually caused by *S. aureus* is known as a hordeolum (Lindsley et al., 2017). Hordeolum can form on the external eyelid and are commonly referred to as a "stye" and are common in both children and adults. Predisposing risk factors include blepharitis, meibomian gland dysfunction, ocular rosacea, seborrheic dermatitis and diabetes mellitus (McAlinden et al., 2016). It typically presents in the form of focal abscess manifesting as a painful, swollen, edematous and erythematous inflammation of the eyelid (Carlisle and Digiovanni, 2015). It can also occur on the inner eyelid and be mistaken for a chalazion. Diagnosis is based on clinical features. The disorder is usually self-limiting, lasting 1–2 weeks and often resolves on its own via spontaneous rupture. Warm compresses and massage therapy (10 mins four times a day) can assist by softening the granulomatous tissue and facilitating drainage. Persistent or complicated lesions may require antibiotic therapy. Incision and drainage may be required for persistent abscess (McAlinden et al., 2016).

Impetigo

A highly contagious infection of the superficial layers of the epidermis, commonly caused by gram-positive bacteria. It is prevalent in children ages 2–5 years old but can occur at any age (Cole and Gazewood, 2007). Impetigo of the eyelid is usually associated with infections of the face, caused by skin disorders such as eczema, insect bites, poison ivy, and cuts or scrape due to minor trauma, by *S. aureus* or *Streptococcus pyogenes* (May et al., 2019). Diagnosis is typically based on clinical features. The infection starts as a tiny blister, which eventually bursts, leaving multiple lesions. Bacterial cultures can be performed to confirm diagnosis. Impetigo often resolves spontaneously, however, topical or oral antibiotic treatments decrease the duration of the infection and spread of lesions (Smith et al., 2018). Antibiotics prescribed should cover both *S. aureus* and *S. pyogenes*.

Erysipelas

A bacterial skin infection mainly caused by Group A *Streptococcus*. Individuals with obesity, lymphedema, leg ulcers, smokers, diabetes mellitus, and dermatitis are predisposed to erysipelas (Raff and Kroshinsky, 2016). Patients typically experience high fever, chills, fatigue, headaches and vomiting within 48 h of initial infection. Eyelid symptoms include erythematous, swollen, warm, hardened, and enlarged painful skin lesions. Diagnosis is based on clinical features and the patients' medical history. Initial treatment should be with *Streptococcus* susceptible antibiotics. Monotherapy oral penicillin is sufficient for most cases and should be given for 5–10 days (Stevens et al., 2014). For patients' allergic to penicillin, first-generation cephalosporin or macrolides such as erythromycin or azithromycin can be prescribed (Dalal et al., 2017).

Cornea and conjunctiva

The cornea is the clear outer layer of the eye that allows light to enter and become focused through the lens onto the retina. The conjunctiva is the thin membrane that covers the front surface of the eye and the inner surface of the eyelids. The main purpose of this outer layer is to protect structures inside the eye, as it is the interface between the eye and the environment. This part of the eye is the body's most valuable defense against infection (Biber, 2013).

Conjunctivitis

Conjunctivitis is a common condition that causes dilation of the conjunctival blood vessels, resulting in inflammation. It presents with a red eye and is highly contagious. Diagnosis of conjunctivitis includes assessment of visual acuity (which should be minimally affected) and corneal, including with fluorescein staining to exclude corneal ulceration or infection (Watson et al., 2018). Fig. 3 is an algorithmic approach to diagnosing and treating conjunctivitis, based on signs and symptoms.

Acute bacterial conjunctivitis

Acute bacterial conjunctivitis is more common in children. The most common bacteria are *Haemophilus influenzae*, *Streptococcus pneumoniae* and *S. aureus* (Sheikh and Hurwitz, 2006). Direct transmission of pathogens onto the conjunctiva leads to infectious conjunctivitis. The onset of the infection is sudden, and patients usually complain of irritation, foreign-body sensation, redness and sticky lids in the morning. Discharge is purulent or mucopurulent and symptoms are usually unilateral but may be bilateral (Fig. 2) (Watson et al., 2018).

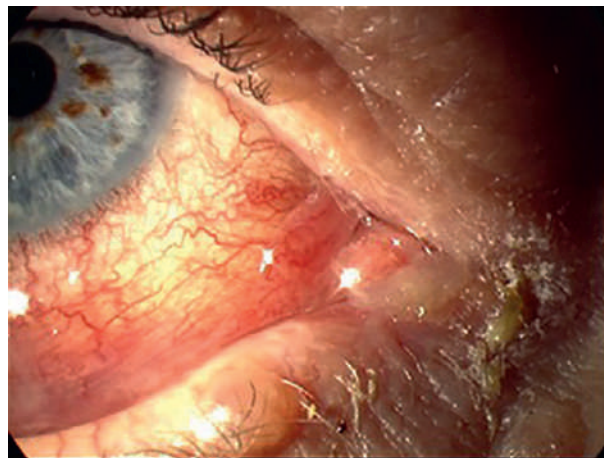


Fig. 2 Conjunctivitis—discharge at medial canthus.

Diagnosis can be made through clinical examination alone. However, conjunctival swabs can be obtained where copious purulent discharge, along with clinical suspicion, make the diagnosis of gonococcal or chlamydia infection more likely (Beal and Giordano, 2016; Azari and Barney, 2013) or in the event of recurrent conjunctivitis.

In general, most cases resolve within 7 days without treatment. In patients who present with purulent discharge or with a compromised ocular surface (e.g., dry eyes), it is reasonable to prescribe a topical antibiotic. Studies have found that topical antibiotics confer modest benefits in improving symptom resolution (Varu et al., 2019). Treatment may reduce transmissibility with broad-spectrum topical antibiotics recommended for 5–7 days. The initial treatment recommended by Therapeutic Guidelines is chloramphenicol 0.5% or framycetin 0.5%, 1 drop into the affected eye, four times a day for up to 7 days (Table 2) (Guidelines, 2014) (Fig. 2).

Gonococcal conjunctivitis

Conjunctivitis caused by *Neisseria gonorrhoeae* is uncommon but should be considered in sexually active young adults and neonates. The occurrence of Gonococcal conjunctivitis in neonates is most likely due to maternal transmission during birth. In older age groups, it is associated with sexually transmitted infections (STIs). If left untreated it can lead to meningitis and/or blindness (Belga et al., 2019).

Patients may experience erythema of the eyelids, discharge and conjunctival hyperemia. In non-neonatal populations, Gonococcal conjunctivitis should be considered in sexually active individuals that present with conjunctivitis with or without genital symptoms. Conjunctival swabs for gram staining and culture for *Neisseria* species should be obtained to confirm the diagnosis. Gonococcal conjunctivitis requires systemic treatment as topical antibiotic therapy alone is not adequate. Recommended antibiotics include a single dose of intramuscular Ceftriaxone and oral Azithromycin or oral doxycycline (twice a day for 7 days) (Table 2). Patients should irrigate the infected eye with saline and add therapy to cover chlamydia (Varu et al., 2019) (Fig. 3).

Chlamydia conjunctivitis

Chlamydia the most common STI worldwide is caused by the bacteria *Chlamydia trachomatis*. Chlamydia conjunctivitis typically occurs in neonates (2.1.5), and adults with exposure to STIs (WHO, 2016).

Most cases of chlamydial conjunctivitis are unilateral and in adults, there is concurrent genital infection. Patients' usually experience conjunctival hyperemia, mucopurulent discharge and lymphoid follicle formation. Diagnosis is usually based on clinical findings. However, polymerase chain reaction (PCR) conjunctival swabs can be performed to confirm *C. trachomatis*. The treatment for chlamydial conjunctivitis consists of systemic treatment with a single dose of oral azithromycin or oral doxycycline (twice a day for 7 days) (Varu et al., 2019). There is no evidence that concomitant topical therapy improves outcomes.

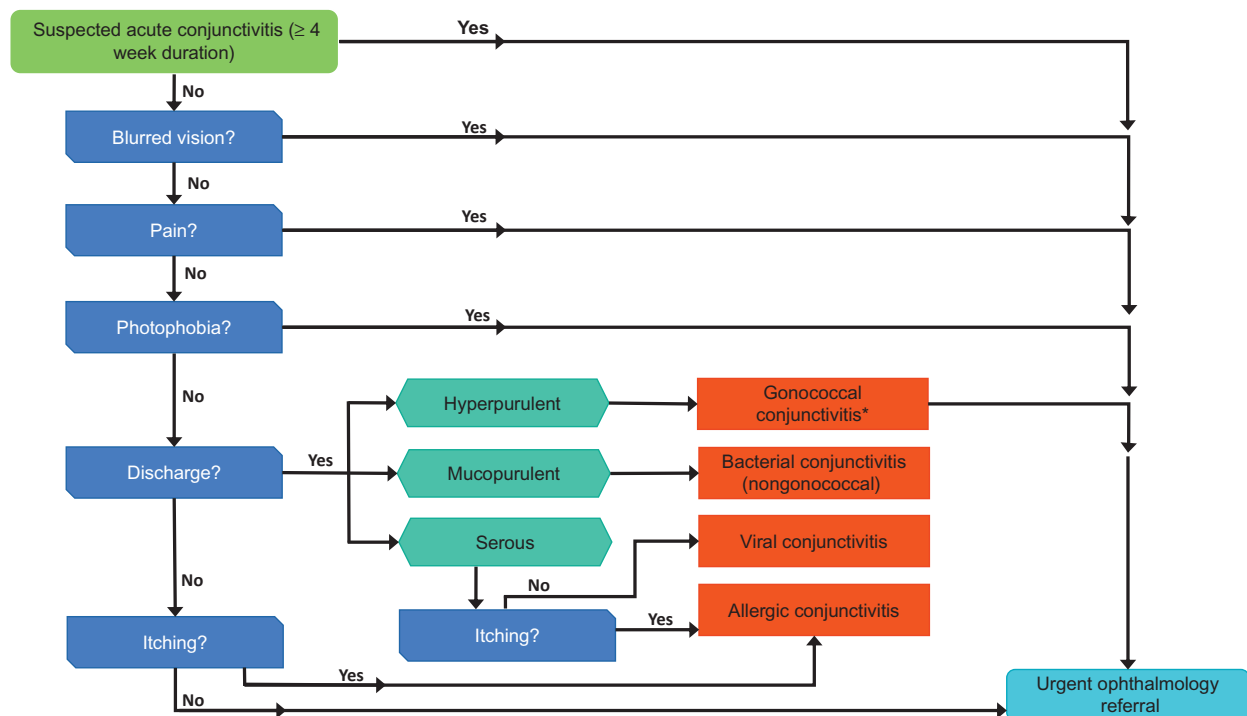


Fig. 3 Suggested procedure for clinical approach to suspected acute conjunctivitis.

Trachoma

Trachoma is a form of chronic conjunctivitis caused by repeat infection with *C. trachomatis*. It is the leading cause of preventable blindness, globally (Resnikoff et al., 2004). It is generally seen in communities with poor hygiene and inadequate sanitation. Transmission of the disease occurs through direct personal contact with an infected person, contaminated fomites, and flies that have come in contact with the eyes or nose of an infected person (Wright et al., 2007).

C. trachomatis is part of the chlamydia genus, which is differentiated into 18 serovars (serologically variant strains). The serovars correlate with multiple medical conditions, with serovars A, B, Ba, and C correlated with trachoma which only occurs in humans (Morre et al., 2000). Patients typically experience conjunctival cicatrization which leads to entropion and trichiasis, this can then disrupt the anatomic integrity of the corneal epithelium predisposing to ulceration (Whitcher et al., 2001). Without treatment, recurrent infection can lead to scarring of the eyelids, corneal ulceration, corneal scarring and loss of vision.

Diagnosis of trachoma is made through identification of typical clinical features including conjunctival hyperemia and/or edema, mucopurulent discharge, lymphoid follicle formation and/or subepithelial keratitis with corneal neovascularization (Ubani, 2013). The presence of limbal or conjunctival follicles suggest the infection is active; however, edema may mask the presence of follicles (Tabbara, 2014).

The World Health Organization recommends the “SAFE” strategy when managing trachoma. “S” surgery for trichiasis, “A” Antibiotics for *C. trachomatis* infection, “F” Facial cleanliness, “E” Environmental changes to improve sanitation and increase access to clean water (WHO, 2020). The recommended drug of choice is azithromycin (Table 2). Doxycycline is an alternative, but azithromycin is preferred as it is a single-dose therapy. In patients with entropion and trichiasis, surgery is necessary to prevent secondary ulceration and/or infections of the ocular surface (West, 2012).

Neonatal conjunctivitis

Ophthalmia neonatorum also known as neonatal conjunctivitis presents during the first month of postnatal life (Tabbara, 2014). Neonatal conjunctivitis is classified according to its cause as either transmitted bacteria, non-sexually transmitted bacteria, viral, and/or chemical. Less than 1% of cases are caused by *N. gonorrhoeae*, however, the incidence increases to 48% in babies born to mothers infected with *N. gonorrhea* (Laga et al., 1989). Non-sexually transmitted bacterial conjunctivitis account for 30–50% of cases, and is usually caused by *S. aureus*, *Streptococcus* species, *Pseudomonas aeruginosa* and gram-negative bacteria (Infectious Diseases and Immunization Committee of the Canadian Paediatric Society, 2002).

Patients develop erythema of the eyelids, discharge and conjunctival hyperemia. Subconjunctival lymphoid tissues are absent in neonates, and therefore, conjunctival follicles are not seen in this age group, however, edema of the palpebral and bulbar conjunctiva may be noted. Gonococcal conjunctivitis is more severe than chlamydial conjunctivitis, as gonococci have the ability to penetrate intact corneal epithelium, leading to corneal edema and ulceration, which can then lead to corneal perforation and endophthalmitis if left untreated (Table 2) (Mallika et al., 2008).

Diagnosis of neonatal conjunctivitis is based on clinical examination and bacterial cultures. Conjunctival swabs and scrapings are essential in neonatal conjunctivitis, especially if the infection is caused by *N. gonorrhoeae*, as it is a medical emergency. *C. trachomatis* and *N. gonorrhoeae* are usually transmitted at the time of delivery. Treatment for neonatal conjunctivitis is shown in Table 1.

Table 1 Treatment for neonatal conjunctivitis.

Type of infection	Onset of infection after birth	Treatment
<i>Neisseria gonorrhoeae</i>	2–5 days	Hospitalization, isolation (>24 h after therapy is initiated). Topical irrigation with normal saline to remove mucopurulent discharge. Ceftriaxone in a single dose (25–50 mg/kg IM or IV, up to a maximum of 125 mg). If there is a systemic disease, treatment is required for 7–14 days depending on the nature of the invasive infection, then topical 0.5% erythromycin ointment every 2–4 h. Note: Gonococcal conjunctivitis should also be treated for chlamydia. Mothers and sexual partners should be treated as well.
<i>Staphylococcus aureus</i> , <i>Streptococcus</i> species or other gram-positive	2–5 days	Topical 0.5% erythromycin ointment four times a day for 2 weeks
<i>Chlamydia trachomatis</i>	5–14 days	Oral erythromycin syrup, 30–50 mg/kg/day and 0.5% Erythromycin ointment four times a day for 2 weeks
<i>Pseudomonas aeruginosa</i> or other gram-negative	5–18 days	Topical 0.3% gentamicin or 0.3% tobramycin four times a day for 2 weeks

Bacterial keratitis

Bacterial keratitis is the most common cause of microbial keratitis. It is an ophthalmic emergency requiring immediate attention as it can progress rapidly. It is a significant cause of corneal blindness (Allan and Dart, 1995; Cabrera-Aguas et al., 2019; Watson et al., 2019), and one of the most common causes of visual impairment in working age adults (Whitcher et al., 2001). Predisposing risk factors include contact lenses, trauma, ocular surface disease, history of a corneal transplant and topical steroid use (Green et al., 2008). The type of causative organism varies according to the climate and geographical region and patient's risk factors (Cabrera-Aguas et al., 2019; Asbell et al., 2008).

The most common causative organisms include *S. aureus*, *Coagulase-negative staphylococci* (CoNS), *S. pneumoniae*, *P. aeruginosa* and *H. influenzae* (Cabrera-Aguas et al., 2019; Asbell et al., 2008). *P. aeruginosa* has been reported to be the most common causative organism implicated in bacterial keratitis among contact lens wearers (Willcox, 2007; Khoo et al., 2020). Patients with bacterial keratitis usually experience pain, photophobia, irritation, purulent discharge and reduced vision. They will also present with redness, discharge, corneal ulcer, corneal infiltrates and/or a hypopyon (Fig. 4).

Diagnostic laboratory investigation such as corneal scrapes should be performed to identify the causative organism and antibiotic susceptibility and resistance. Corneal ulcers should be scraped for staining and culturing. Samples can be collected at the area of corneal infiltration. Superficial corneal samples are inoculated onto 2 glass slides for microscopy and culture media (which includes 2 blood agars, chocolate agar, Sabouraud's agar slope and cooked meat medium) (Ngo et al., 2019). Other media such as brain heart infusion broth, thioglycolate broth, non-nutrient agar may be used (Leck, 2009; Preferred Practice Pattern, 2013). A corneal biopsy can also be performed in progressive cases where a negative result has been obtained from corneal scrapes or the organism identified does not match the clinical picture. A lamellar corneal biopsy can be taken using a dermal trephine or freehand dissection, the specimen is divided into two halves for histopathological and microbiological analysis (Robaei et al., 2018).

The severity of bacterial keratitis can be divided into mild, moderate and severe; this is typically done for clinical trials (Constantinou et al., 2007; Herretes et al., 2014). Mild corneal ulcers are those less than 2 mm in size with the depth of the ulcer less than 20% or 100 µm corneal thickness. Superficial infiltrates near the ulcer may also be seen. Moderate corneal ulcers range between 2 and 5 mm in size, depth of 20–50% (100–275 µm) of the cornea, with dense infiltrates extending to the mid stroma. Severe ulcers are 5 mm or larger, with a depth of more than 50% (>275 µm) and dense infiltrates reaching the deep layers of the corneal stroma (Tabbara et al., 2014). Increasing severity is generally associated with poorer outcomes (Khoo et al., 2020; Green et al., 2007).

The treatment has to be empiric as laboratory results can take over 48 h, and the infection can progress rapidly without treatment. Broad-spectrum topical antibiotics are the mainstay of treatment and should be used until culture results are available (Table 2). For mild to moderate ulcers, 1–2 drops of fluoroquinolone (e.g., ciprofloxacin 0.3% or ofloxacin 0.3%) every hour for 48 h, tapered to every 4 h until healed is recommended. For severe keratitis, fortified antibiotics (e.g., cephalothin 5% plus gentamicin 0.9%) 1–2 drops hourly for 48 h, then reduce frequency according to treatment response (Alam and Bastakoti, 2015). Treatment should be modified based on the results of culture and susceptibility testing (Preferred Practice Pattern, 2013; Guidelines, 2014) (Table 2).

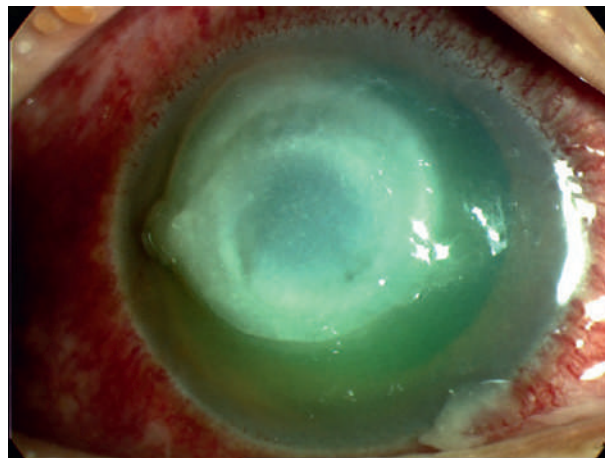


Fig. 4 Bacterial keratitis. The central cornea has an epithelial defect and conjunctival inflammation.

Table 2 Bacterial infections of the cornea and conjunctiva.

Disease	Pathogen	Signs and symptoms	Transmission	Treatment
Acute bacterial conjunctivitis	<i>H. influenzae</i> , <i>S. pneumoniae</i> , <i>S. aureus</i>	Inflammation of conjunctiva with purulent crusting, discharge	Exposure to secretions from infected individual	Broad-spectrum topical antibiotics
Gonococcal conjunctivitis	<i>N. gonorrhoeae</i>	Inflammation of conjunctiva with hyperpurulent discharge, eyelid erythema	Direct sexual contact with infective secretions	Systemic Ceftriaxone, Azithromycin or Doxycycline
Trachoma	<i>C. trachomatis</i>	Chronic conjunctivitis, trichiasis, corneal scarring; may lead to blindness	Exposure to infected individual, contaminated fomites, or through flies	Systemic Azithromycin
Neonatal conjunctivitis	<i>C. trachomatis</i> , <i>N. gonorrhoeae</i>	Inflammation of conjunctiva, purulent discharge, eyelid erythema, corneal ulceration/perforation; may lead to blindness	Exposure to pathogens in the birth canal of infected mother with chlamydia or gonorrhea	Systemic Erythromycin for <i>C. trachomatis</i> Topical Erythromycin for <i>N. gonorrhoeae</i>
Bacterial keratitis	<i>CoNS</i> , <i>S. aureus</i> , <i>S. pneumoniae</i> , <i>P. aeruginosa</i> , <i>H. influenzae</i>	Blurred vision, redness and irritation to eye, photophobia, corneal ulceration, progressive corneal scarring; may lead to blindness	Exposure to pathogens (e.g., contact lens wear, trauma)	Broad-spectrum topical antibiotics (preferably fluoroquinolones)

Uveitis

Uveitis is not a single disease; it is a generic term that encompasses more than 30 different entities; some infective, some either autoimmune or auto-inflammatory and many where the cause is unclear. Some uveitides are ocular manifestations of systemic disease while others are disorders restricted to the eye. The term is used to describe inflammation of the uveal tract of the eye (iris, ciliary body, and the choroid). Uveitis can be classified as anterior, intermediate, posterior, or pan-uveitis based on the primary anatomical location of the inflammation in the eye (Table 3) (Jabs et al., 2005).

It can affect people of all ages, with the incidence varying significantly due to geographic location and age of patient (Acharya et al., 2013; Hwang et al., 2012; Gritz and Wong, 2004). Anterior uveitis is the most prevalent form of uveitis, accounting for approximately 50% of cases (Wakefield et al., 2011). Symptoms can range from conjunctival injection, photophobia and pain to complete loss of vision. Bacterial causes of uveitis include: tuberculosis (*Mycobacterium tuberculosis*), syphilis (*Treponema pallidum*), Lyme disease (*Borrelia burgdorferi*), and Whipple's disease (*Tropheryma whipplei*).

Diagnosis of uveitis is based on the patients' medical history, clinical features and laboratory testing. Symptoms and signs vary depending on the location of inflammation (Table 3). Identifying the likely cause of uveitis critically depends taking a careful thorough history, a review of systems and complete examination of both eyes. Multi-modal ocular imaging such as digital fundus photography, fundus fluorescein angiography (FFA), fundus autofluorescence (FAF), optical coherence tomography (OCT) and ultrasonography are often performed to characterize different forms of posterior and pan-uveitis. Laboratory testing can be performed to diagnose a specific underlying infectious disease based on clinical suspicion such as: QuantiFERON gold testing to determine exposure to tuberculosis, and Fluorescent treponemal antibody absorption (FTA-ABS) and rapid plasma regain (RPR) to confirm a diagnosis of syphilis (Table 3).

Treatment for infectious uveitis remains a therapeutic challenge for ophthalmologists. The mode of treatment varies greatly based on the infectious etiology. Management typically involves systemic antibiotic therapy with or without the use of corticosteroids. This will be discussed in more detail.

Table 3 Anatomical classification of uveitis and common key signs and symptoms.

Type of uveitis	Primary site of inflammation	Signs and symptoms
Anterior	Anterior chamber	Symptoms: redness, painful eye Signs: anterior chamber cell and flare, posterior synechiae, keratic precipitates
Intermediate	Vitreous cavity and pars plana	Symptoms: worsening floaters, decreased vision Signs: vitreous cell and haze, pars plana snowbank, cystoid macular edema
Posterior	Retina and choroid	Symptoms: worsening vision, visual field changes Signs: chorioretinal lesions, retinal whitening, vascular sheathing
Panuveitis	Anterior chamber, vitreous cavity, pars plana, retina and choroid	Symptoms: red, painful eye, severely decreased visual acuity, floaters Signs: anterior chamber cell and flare, vitreous cell and haze, chorioretinal lesions, retina whitening

Intraocular tuberculosis

Tuberculosis (TB) is an infection caused by *Mycobacterium tuberculosis*. The disease is thought to affect the lungs in 80% of patients, with the remaining 20% being affected in areas such as the gastrointestinal system, lymphoreticular system, central nervous system, musculoskeletal system, skin, liver and eyes (Shakarchi, 2015). Adults in their productive years and people infected with HIV are at risk of TB. However, the disease can affect all age groups. Ocular TB is an important cause of uveitis, the prevalence varies from 0.5–11% depending on the region (Henderly et al., 1987; Al-Shakarchi, 2014; Mercanti et al., 2001). A recent multinational study identified immigrants as a high-risk group to develop intraocular TB (Agrawal et al., 2018).

Intraocular TB is a complex disease as it is a great mimicker of various uveitis entities. This is partly due to the location of the infection, host response and the virulence of the organism (Gupta et al., 2007). Clinical presentations of intraocular TB are anterior uveitis, intermediate uveitis, posterior uveitis and pan-uveitis, retinitis, retinal vasculitis, neuroretinitis, optic neuritis, endogenous endophthalmitis, panophthalmitis, and scleritis.

Anterior uveitis can present as unilateral or bilateral chronic granulomatous disease that manifests as granulomatous keratic precipitates and is sometimes associated with iris nodules or granulomas (Tabbara, 2005; Gupta et al., 2007). The presence of anterior chamber inflammation associated with small grayish-yellow or reddish nodules in the iris or small translucent nodules at the pupillary margin (Albert and Raven, 2016). During relapse, mutton fat keratic precipitates may develop, along with extensive posterior synechiae, cataract and vitritis (Gupta et al., 2007).

In intermediate uveitis, patients present with a low-grade, chronic vitritis, vitreous snowballs and snow banks and peripheral vascular sheathing (Ahmed and Biswas, 2016).

In posterior and pan-uveitis, the ocular changes can be divided into choroidal tubercles, choroidal tuberculoma, subretinal abscess and serpiginous-like choroiditis.

- Choroidal tubercle is the most common manifestation of TB posterior uveitis. It has been suggested that this occurs as a result of hematogenous spread. They appear as small grayish white to yellow nodules and may be unilateral or bilateral (Ahmed and Biswas, 2016). Occasionally, a tubercle may continue to grow into a solitary mass called tuberculoma (Gupta et al., 2007).
- Choroidal tuberculoma is less common. They can be located anywhere in the choroid (e.g., macula, posterior pole, equator, or in juxtapupillary location). These large solitary mass may mimic a tumor. Hemorrhages and retinal folds may be seen on the tuberculoma surface and in later stages, the overlying retina may detach (Ahmed and Biswas, 2016).
- Subretinal abscess may be present in patients with disseminated TB with little overlying vitreous inflammation. Multiplication of the bacilli in the caseous material of the granulomas can lead to liquefaction necrosis and abscess formation. They are usually yellow in color indicating liquefaction necrosis (Gupta et al., 2007).
- Serpiginous choroiditis is a rare, chronic, recurrent inflammation that primarily involves the choroid and choriocapillaris. The lesions typically appear as gray-white to yellow with ill-defined edges, beginning in a peripapillary location and spreading centrifugally with multiple recurrences. Atypical patterns of serpiginous choroidopathy are now recognized as caused by TB. Such patients may have unilateral involvement or not involve the optic disc and peri-papillary retina. The choroiditis regresses following anti-tubercular drug therapy (Ahmed and Biswas, 2016).

Tuberculosis isolated in the retina is rare and involvement may be mostly secondary to choroiditis. Tubercular retinal vasculitis typically presents as peripheral occlusive retinal vasculitis with inflamed retinal vessels and features of distal retinal ischemia (Fig. 5) (Gunasekeran et al., 2019). Tubercular retinal vasculitis presenting features are often similar to Eales disease. Eales disease is a

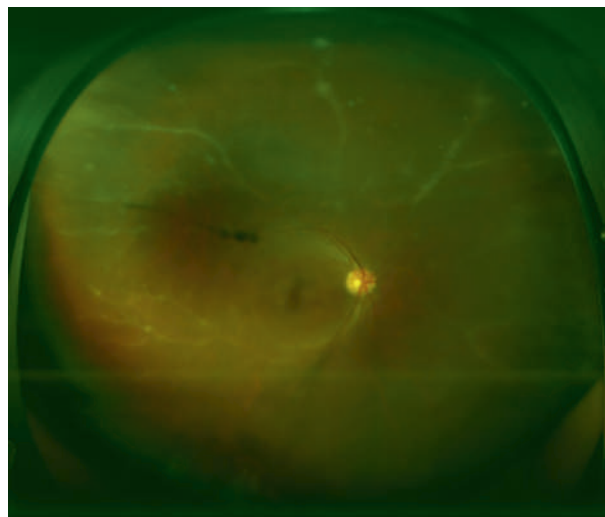


Fig. 5 Tuberculosis occlusive retinal vasculitis. Optos fundus image showing widespread severe occlusive retinal vasculitis.

poorly understood form of retinal vasculitis, that is typically described as an idiopathic obliterative vasculopathy involving peripheral retina of young adults (Bodaghi and Lehoang, 2000). Recently, this has been relabeled as either tubercular retinal vasculitis or idiopathic retinal vasculitis, depending on whether it is accompanied by evidence of TB (Agrawal et al., 2017a; Gunasekaran et al., 2019; Biswas et al., 2013).

Optic neuropathy develops either from direct infection induced by *M. tuberculosis* or from a hypersensitivity to the infectious agent. The involvement of the nerve may manifest as an optic nerve tubercle, papillitis, papilledema, optic neuritis, retrobulbar neuritis, or neuro-retinitis (Gupta et al., 2007; Ray and Gragoudas, 2001). Neuro-retinitis may develop from the spread of the mycobacteria to the juxtapupillary retina from the choroiditis (Ray and Gragoudas, 2001).

Endogenous endophthalmitis has an acute onset and progresses rapidly with the destruction of the intraocular tissue (Fig. 6). Severe inflammation causes hypopyon, purulent material in the anterior chamber and involvement of the cornea (Gupta et al., 2007). These clinical presentations sometimes hinder the diagnosis of intraocular TB. In the posterior segment, intense inflammation of the choroid causes the formation of large subretinal abscesses. If left untreated, it can eventually burst into the vitreous cavity presenting itself as endophthalmitis or panophthalmitis. Furthermore, in panophthalmitis, the sclera may sometimes be involved resulting in globe perforation (Raina et al., 2000).

The difficulty in managing TB uveitis is contributed by its ability to affect any tissue in the eye (Agrawal et al., 2017a; Gupta et al., 2007; Sharma et al., 2017). Furthermore, the lack of uniformity in diagnostic criteria makes the diagnosis and management of TB uveitis difficult. The Collaborative Ocular Tuberculosis Study (COTS)-1 group developed the following diagnostic criteria to aid the diagnosis of TB uveitis. A diagnosis of TB is established if a patient satisfied both criteria 1 and 2 and at least one of criteria 3 and 4 (Agrawal et al., 2017b).

1. Clinical signs suggestive of TB uveitis, including the following:
 - i) Anterior uveitis (granulomatous or non-granulomatous), iris nodules and ciliary body granuloma.
 - ii) Intermediate uveitis (granulomatous or non-granulomatous with exudates in the pars plana, with or without inflammatory cells in the vitreous).
 - iii) Posterior and pan-uveitis, choroidal tubercle, choroidal granuloma, subretinal abscess, and serpiginous-like choroiditis.
 - iv) Retinitis, retinal vasculitis, neuroretinitis, optic neuritis, endogenous endophthalmitis, panophthalmitis, and scleritis.
2. Exclusion of other uveitic entities, where relevant, based on clinical manifestations of disease and regional epidemiologic findings.
3. Corroborative investigations
 - i) Positive Mantoux test result.
 - ii) Interferon γ release assay, such as QuantiFERON TB Gold.
 - iii) Evidence of healed or active TB on chest radiography.
4. Investigations that document the mycobacteria or its genome:
 - i) Demonstration of acid-fast bacilli by microscopy or culture of *M. tuberculosis* from ocular fluid.
 - ii) Positive PCR from ocular fluid for IS 6110 or other conserved sequences in mycobacterial genome.
 - iii) Evidence of confirmed active extrapulmonary TB (by microscopic examination or culture of a tissue sample from the affected tissue).



Fig. 6 Endogenous Endophthalmitis. Ill-defined white area of focal choroiditis that has broken through into the retina.

The treatment of intraocular TB is complex, as inappropriate management can result in life-threatening consequences. Ocular TB is generally treated under the same guidelines as active pulmonary and extrapulmonary TB. Anti-tuberculosis treatment (ATT) for intraocular TB consists of a combination of isoniazid, rifampicin, ethambutol and pyrazinamide initially for 2 months. Followed by a choice of two of the ATT over the following 4–7 months (American Thoracic Society; CDC; Infectious Diseases Society of America, 2003; World Health Organization, 2010). Low-dose systemic adjunctive corticosteroid therapy may be used for 4–6 weeks with multidrug ATT to limit the damage to the ocular tissues. However, corticosteroid alone without concomitant ATT should be avoided as it can cause panophthalmitis or cause a flare-up of systemic tuberculosis by activating a latent infection.

Syphilis

Syphilis is a sexually transmitted infectious disease caused by *Treponema pallidum*. The most common ocular manifestation of syphilis is uveitis. Risk factors include: men who have sex with men, unprotected sexual intercourse and co-infection with HIV (Aldave et al., 2001). Syphilis is commonly called the great mimicker and it can present as any form of inflammatory eye disease. Excluding syphilis infection serologically is the only investigation routinely performed in all patients presenting with uveitis. Syphilis can cause any form of uveitis: granulomatous or non-granulomatous anterior uveitis, intermediate uveitis, posterior or pan-uveitis (Fig. 7) (Barile and Flynn, 1997).

Diagnosis of the infection is based on serologic testing. Most laboratories have switched to a reverse screening algorithm and perform Treponemal specific tests to screen for exposure to syphilis as these antibodies are positive for life following infection. These include enzyme immunoassay, FTA-ABS, *T. pallidum* particle agglutination, *T. pallidum* hemagglutination, and chemiluminescence immunoassay. Non-treponemal tests are used to determine active untreated infection. Their titer will fall following treatment (Davis, 2014; Kiss et al., 2005).

Management for syphilitic uveitis consist of 10–14 days of intravenous antibiotics of penicillin G at 3–4 million units every 4 h (Lin, 2015). Adjunctive therapy with topical and oral steroids can be used to decrease the inflammatory response generated by active infection and to prevent a Jarisch-Herxheimer reaction associated with treating the infection (Kiss et al., 2005; Barile and Flynn, 1997).

Lyme disease

Lyme disease is a tick-borne infection caused by *Borrelia burgdorferi* and is a common tick-borne infection worldwide, that is endemic in Europe and North America (Piesman and Gern, 2004). The disease is commonly reported in Caucasians but can infect all races (Fix et al., 2000).

The clinical manifestation of the disease has been divided into three stages: early, disseminated, and late. The early localized disease is distinguished by erythema migrans at the site of the tick bite that occurs 7–10 days after the bite, with fever and lymphadenopathy. The disseminated stage develops 3–12 weeks after the initial infection and may manifest with erythema chronica migrans, lymphocytoma, neurologic (e.g., dizziness, headache) and cardiac manifestations (e.g., chest pain, palpitations and dyspnea). Late-stage Lyme disease may occur months or years after the initial infection. Typical features include neurological and rheumatological involvements and skin disorders.

Ocular inflammation typically occurs in the disseminated and late stages of the disease; however, it is uncommon. During the disseminated stage it may manifest as anterior, intermediate, posterior or pan-uveitis. Like syphilis it can mimic many forms of uveitis. In the late stage, keratitis is the most common ocular manifestation, episcleritis is less common (Amer et al., 2009; Mikkila et al., 2000).

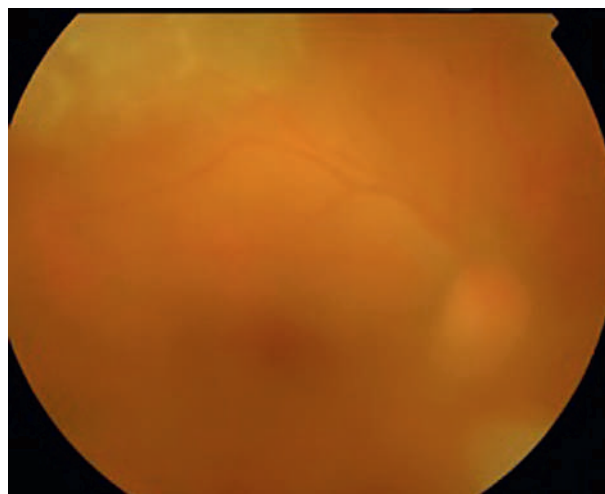


Fig. 7 Syphilitic retinitis. Severe vitreous inflammation resulting in hazy view of the retina. Swollen inflamed optic disc and large area of peripheral retinitis.

The diagnosis of Lyme disease is based on clinical assessment and importantly travel to or residence in an endemic region. There may or may not be a history of tick bite. Serological testing such as ELISA for IgM and IgG, followed by a confirmatory Western blot test are needed to confirm the diagnosis. The specificity and sensitivity of testing varies widely and care should be taken as a false-positive serological test may lead to incorrect diagnosis (Johnson et al., 1996).

The treatment for Lyme disease is based on a combination of antibiotics and corticosteroids. The recommended antibiotics include doxycycline, amoxicillin, cefuroxime or ceftriaxone (Wormser, 2006). Topical and systemic corticosteroids are used to treat the associated inflammation (Winward et al., 1989).

Posterior segment

The posterior segment of the eye is made up of the vitreous humor, retina, choroid and optic nerve. Bacterial infection of the posterior segment, with manifestations such as optic neuropathy, retinitis and choroiditis most commonly occur in conjunction with uveitis for example with tuberculosis and syphilis, however, cat-scratch disease, Lyme disease and Whipple's disease.

Cat scratch disease

Cat scratch disease (CSD) is a worldwide, self-limited, systemic illness caused by the gram-negative bacillus, *Bartonella henselae* (Ksiaz et al., 2019). Cats are the main carriers of the infection, and the cat flea is an important vector for the transmission of the organism among cats (Pennisi et al., 2013). The disease is transmitted to humans by scratches, licks, and bites of domestic cats (Ksiaz et al., 2019). It typically causes a mild illness in immunocompetent humans.

The clinical systemic manifestation of CSD includes fever, malaise, anorexia, and lymphadenopathy at the site of inoculation. The onset of symptoms occurs approximately 2–3 weeks after inoculation. Typical ocular features are adnexal and anterior segment involvement, particular Parinaud oculo-glandular syndrome, which is common in CSD. The infection may also cause unilateral or bilateral neuro-retinitis and optic papillitis classically associated with a macular star. There is often mild anterior chamber and vitreous inflammation (Mabra et al., 2018). The onset of visual symptoms occur approximately 4 weeks following inoculation and is usually unilateral. Patient experience decreased in visual acuity ranging from 6/6 to light perception (Solley et al., 1999). Neuro-retinitis usually has a self-limited course, and most patients recover within several weeks to months (Ksiaz et al., 2019; Anderson and Neuman, 1997). The macular star resolves within 8–12 weeks but can be present for up to 1 year.

Diagnosis of CSD is made through patient history, clinical features and laboratory results. The typical history of prodromal symptoms, lymphadenopathy, and cat exposure strengthens the diagnosis, specifically when presenting in younger adults or children. There are various laboratory and histological techniques can be used to diagnose CSD. A lymph node or conjunctival biopsy can be performed, and the tissue stained with Warthin-Starry staining. However, the most common diagnostic technique is serological testing, either with ELISA or indirect fluorescence assay (IFA). The sensitivities and specificities differ with both techniques. The preferred test is the IFA test as it has a higher specificity of 95% (Ksiaz et al., 2019). PCR can also be performed on tissue or blood. PCR can differentiate between different *Bartonella* species (Relman et al., 1990).

For immunocompetent patients, the disease has a self-limited course, and for patients with mild to moderate case, treatment may not be necessary. However, treatment is given to patients with severe ocular or systemic complications. Antibiotics such as azithromycin, doxycycline, ciprofloxacin, trimethoprim-sulfamethoxazole, or rifampicin may be used (Prutsky et al., 2013). Preventative measures such as washing and disinfecting any wounds immediately after a cat scratch or bite, and avoiding stray cats is recommended to prevent CSD (Nelson et al., 2016; Gupta et al., 2004).

Whipple's disease

Whipple's disease is a rare but chronic, multisystem disease caused by the gram-positive bacillus, *Tropheryma whippiei*. It occurs more frequently in middle-aged white men, with a male to female ratio of 4:1 and the mean onset of symptoms at 55 years of age (Dolmans et al., 2017). Involvement of the eye is rare in patients with Whipple's disease but may occur in isolation or with gastrointestinal, neurologic or other systemic manifestations. A number of ocular manifestations in Whipple's disease have been reported such as uveitis, retinitis, choroiditis, retinal vasculitis, retinal hemorrhages and cystoid macular edema (Jelliti et al., 2014; Chan et al., 2001).

Ocular diagnosis of Whipple's disease remains a challenge, especially in the absence of gastrointestinal symptoms. Diagnosis is usually via biopsy of the duodenal mucosa but can also be confirmed via PCR analysis of cerebrospinal or vitreous fluids and/or peripheral blood (Relman et al., 1992). Positive results are determined either through positive PCR detection of *T. whippiei*, PAS staining showing foamy macrophages in biopsy specimen or immunohistochemical staining with *T. whippiei* antibodies (Chan et al., 2001; Jelliti et al., 2014).

Treatment of ocular Whipple's disease consists of oral antibiotics (trimethoprim-sulfamethoxazole (TMP-SMX) and rifampin) for at least 1 year to prevent relapse (Keinath et al., 1985). Third-generation cephalosporins are effective for *T. whippiei* resistant to TMP-SMX (Touitou et al., 2012).

Endophthalmitis

Infectious endophthalmitis is an inflammatory condition of the intraocular cavity (i.e., the aqueous or vitreous humor) caused by microorganisms (Callegan et al., 2002). The most common causative organisms are CoNS, *S. aureus*, *Streptococci* species, and *Bacillus* species (Durand, 2013; Okada et al., 1994). It is an ocular emergency requiring immediate attention to prevent permanent loss of vision. Endophthalmitis is uncommon and its incidence is dependent on the cause.

There are two types of endophthalmitis; exogenous and endogenous. Exogenous endophthalmitis is the most common cause of endophthalmitis and arises from infecting organisms gaining entry into the eye by direct inoculation, such as from cataract surgery, intravitreal injections (for conditions such as age-related macular degeneration and diabetic retinopathy), penetrating ocular trauma, corneal infection or via a filtering bleb (for glaucoma) (Durand, 2017). Endogenous endophthalmitis occurs when infectious organisms are hematogenously disseminated to the eye from a distant source of infection, such as the brain, heart, liver, lungs and urinary tract (Callegan et al., 2002; Scott et al., 1996). Risk factors include: recent dental surgery, intravenous cannula and IV lines, sepsis, surgery, immunocompromised, malignancy and IV drug use (Relhan et al., 2018).

Endophthalmitis usually presents 3–5 days after the procedure or injury with acute onset of pain, floaters, photophobia, blurred vision and light perception visual acuity. Endophthalmitis from lower virulence organisms may not present for weeks after infection. Clinical features usually include: redness, hazy media, anterior chamber cells and hypopyon (Fig. 8) (Durand, 2017; Taban et al., 2005). The most common causative organisms are CoNS, *S. aureus*, streptococci species (Ng et al., 2005; Durand, 2017). Bacteria such as CoNS typically colonize the conjunctiva and can be introduced to the inner ocular cavity during injury, surgery or trauma.

The diagnosis is based on patient history, clinical features and laboratory results. The onset of symptoms is a good indication of whether it has a bacteria or fungal etiology. Bacterial endophthalmitis typically presents acutely in a shorter timeframe, often within days of a penetrating event compared to fungal endophthalmitis which has a more sub-acute presentation, worsening over days to weeks (Durand, 2017). Exogenous endophthalmitis is commonly unilateral and there is a history of a recent penetrating event (Kernt and Kampik, 2010). Endogenous endophthalmitis is also most often unilateral, but in a third of cases can be bilateral (Chee and Jap, 2001; Sadiq et al., 2015).

Microbiological specimens of the vitreous should be performed to identify the causative organism, the vitreous can be obtained either by vitreous tap or pars plana vitrectomy (PPV). According to the Endophthalmitis Vitrectomy Study (EVS), the decision between a vitreous tap and vitrectomy for patients should be determined based on the presenting visual acuity (Anon, 1995). For patients with light perception vision or diabetes, a PPV should be performed as it increased the likelihood of achieving a better visual outcome. For patients with visual acuity better than light perception, there was little difference with either method (Anon, 1995). Additional tests can be performed to confirm diagnosis such as fundoscopic examination can reveal intraocular inflammation. Ultrasound evaluation can be performed to identify vitritis, or chorioretinal infiltrates (Sadiq et al., 2015).

Urgent management with intravitreal, broad spectrum antibiotics is critical to preserve vision (Guidelines, 2014). Broad-spectrum intravenous, topical and potentially intravitreal antibiotics should be administered after microbial sampling. Treatment should be modified based on the results of culture and susceptibility testing (Sadiq et al., 2015). Antibiotics recommended for empirical intravitreal therapy include vancomycin, ceftazidime, amikacin and 4th generation fluoroquinolones such as moxifloxacin (Guidelines, 2014; Barry et al., 2007). Often antibiotic treatment is combined with PPV as besides providing a diagnostic sample, it reduces the infective and inflammatory load (Anon, 1995).

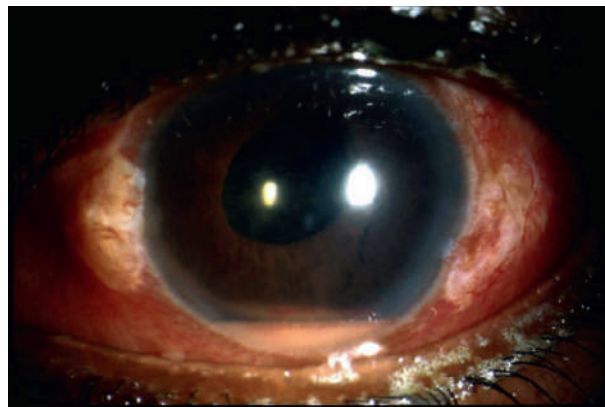


Fig. 8 Endophthalmitis. Photo shows a hypopyon inside the front of the eye.

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Viral Ocular Infections

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Herpes simplex virus (HSV), varicella-zoster virus (VZV) and cytomegalovirus (CMV) belong to the Herpesviridae family, a large group of enveloped, double-stranded DNA viruses that commonly affect humans (Liesegang, 2001; Koganti et al., 2019; Tsatsos et al., 2016).

Herpes simplex virus has two forms: HSV-1, associated with ocular and perioral pathology and HSV-2 with anogenital infections (Farooq and Shukla, 2012; Guess et al., 2007; Tsatsos et al., 2016). Ocular HSV infections represent a significant burden of disease worldwide (White and Chodosh, 2014). An estimated 500,000 people have ocular HSV in the United States alone, and treatment of new and recurrent cases costs the country US\$ 17.7 million annually (Farooq and Shukla, 2012). Extrapolation of the incidence of new and recurrent cases to 2011 estimated the United States population yields an estimate of 64,499 new and recurrent cases per year in the United States (White and Chodosh, 2014).

Varicella-zoster virus along with HSV-1 and HSV-2 belong to the subfamily alphaherpesvirinae. These viruses have the widest host range and short replicative cycle compared to the subfamilies beta and gammaherpesvirinae (Gershon et al., 2015; Koganti et al., 2019). Primary infection with VZV manifests as varicella (chickenpox). After the episode of varicella, the virus travels in a retrograde manner to the dorsal root and cranial nerve sensory ganglia becoming latent. As cellular immunity declines with age or in immunocompromised patients, VZV reactivates causing herpes zoster (HZ or shingles; Gershon et al., 2015; Bowling, 2016; Li, 2018; Liesegang, 2008). Following HZ patients may be left with ongoing pain in the area affected, this condition is known as post-herpetic neuralgia (PHN). Epidemiological data has been reported from developed countries with long life expectancies. The population incidence of zoster is 3–4 per 1000 person-years of observation (Weitzman et al., 2013; Yawn et al., 2007; Li, 2018). The incidence of HZ and PHN increases significantly with age (Weitzman et al., 2013; Yawn et al., 2007). In an Israeli study, 12% and 3% of patients older than 65 years developed PHN or ophthalmic complications, respectively (Weitzman et al., 2013). Similarly, in a study in Olmsted County, MN, USA, PHN occurred in one-fifth of adult patients and in one-third of patients aged 79 or older (Yawn et al., 2007). By the age of 85, more than 50% of population presents at least one episode of HZ (Gershon et al., 2015).

The herpesvirus human cytomegalovirus (HCMV) belongs to the subfamily betaherpesvirinae (Koganti et al., 2019). Primary infection usually occurs in healthy individuals who may not exhibit symptoms and leads to lifelong latency of the virus in the individual. However, a serious burden of the disease results in latently infected individuals with a re-activation of the virus, particularly in those who are immunocompromised (Harthan et al., 2016; Dupont and Reeves, 2016).

Blepharoconjunctivitis

Herpes simplex virus

Blepharoconjunctivitis caused by HSV can be a manifestation of a primary infection or result from reactivation of the virus (Guess et al., 2007; Bowling, 2016). It presents after an incubation period of between 1 and 28 days, with self-limited skin lesions and a

follicular conjunctival inflammatory response which may lead to keratitis (Guess et al., 2007; Valerio and Lin, 2019). Prodromal facial and lid tingling lasts 24 h followed by the eruption of skin lesions over 48 h (Bowling, 2016). The lesions consist of grouped vesicular or vesiculopustular eruptions on the eyelids and periocular area (Guess et al., 2007; Valerio and Lin, 2019). The lesions are limited to a dermatome but not present in a sharply delineated unilateral pattern like in HZO. Other symptoms include discharge and lid swelling (Bowling, 2016). Misdiagnosis is frequent as this condition is rare. Differential diagnosis is follicular conjunctivitis such as chlamydia and meibomian gland dysfunction (Valerio and Lin, 2019).

In some patients, this condition will settle without treatment in over a week. Topical antiviral agents such as acyclovir and trifluridine are the first line therapy. Oral antiviral may be also used (Valerio and Lin, 2019; Bowling, 2016).

Herpes zoster ophthalmicus

Herpes zoster ophthalmicus (HZO) refers to herpes zoster affecting the dermatome supplied by the ophthalmic division of the fifth cranial (trigeminal) nerve (Bowling, 2016; Li, 2018; Liesegang, 2008). HZO presents more frequently in the sixth or seventh decade of life, accounting for 10–20% of HZ cases (Bowling, 2016; Tran et al., 2016; Liesegang, 2008; Vadoothker and Jeng, 2018).

Ocular involvement is common through different mechanisms: direct viral invasion causing conjunctivitis and epithelial keratitis; secondary inflammation and occlusive vasculitis causing episcleritis, scleritis, keratitis, uveitis, optic neuritis and cranial nerve palsies; and reactivation causing necrosis and inflammation in the affected sensory ganglia with cornea anesthesia and neurotrophic keratopathy (Bowling, 2016). The Hutchinson sign refers to the presence of skin lesions in the tip, side and root of nose. These areas are supplied by the external nasal nerve which is a branch of the nasociliary nerve. This sign correlates strongly to ocular involvement (Bowling, 2016; Li, 2018; Liesegang, 2008; Wehrhahn and Dwyer, 2012; Chan and Chee, 2019).

A prodromal phase of 3–5 days with tiredness, fever, malaise and headache precedes the onset of the rash. Symptoms in the involved dermatome include itching, tingling or burning sensation to a severe lancinating constant or intermittent pain, poor vision and photophobia (Vadoothker and Jeng, 2018; Bowling, 2016; Li, 2018). Older individuals with early severe pain and a large affected area are most at risk of presenting PHN (Bowling, 2016; Li, 2018). The typical cycle of skin lesions in HZO are described in Fig. 1 (Bowling, 2016). Some patients can develop “*zoster sine herpette*” which manifest without any skin rash (Li, 2018; Vadoothker and Jeng, 2018; Liesegang, 2008). Ocular symptoms tend to appear 1–4 weeks post rash onset (mean 1.82 weeks) (Vadoothker and Jeng, 2018).

In immunosuppressed patients, large hemorrhagic lesions are more common (Bowling, 2016). Long-term manifestations can present in one third of patients with HZO, increasing to 70% in patients over the age of 80 years despite the current availability of antivirals (Liesegang, 2008).

Complications associated with HZO not only affect the skin and the anterior segment but may also involve the retina, optic nerve and central nervous system (Liesegang, 2008). Periorbital edema and ptosis may occur and as the inflammation from the vesicles subsides, residual ptosis, lid scarring, deep scalp pitting, entropion, ectropion, loss of normal pigmentation and lid necrosis may follow. Conjunctivitis may also present with hyperemia, petechial hemorrhages, a papillary reaction and infrequently pseudomembranes (Liesegang, 2008). The development of any complication does not relate with the severity of the rash (Liesegang, 2008).

If the clinical diagnosis is unclear, mostly in immunodeficiency, vesicular fluid can be tested using PCR, immunomicroscopy or culture. PCR may be positive in 40% of cases as the viremia lasts for some days (Bowling, 2016).

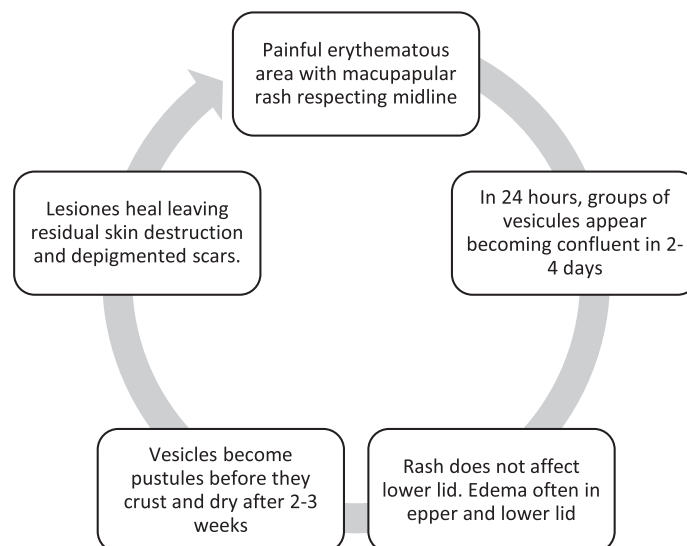


Fig. 1 Typical cycle of skin lesions in herpes zoster ophthalmicus.

Oral antiviral must be preferably commenced within 72 h of the rash onset. The antivirals reduce the severity, duration of the episode and the risk of PHN. Antiviral therapies include the following (Bowling, 2016; Li, 2018; Wehrhahn and Dwyer, 2012; Jain et al., 2019; Dworkin et al., 2007; Therapeutic-Guidelines-Ltd, 2019):

- Aciclovir 800 mg, five times daily for 7 days.
- Valaciclovir 1 g, three times daily for 7 days.
- Famciclovir 500 mg, three times daily for 7 days.

Intravenous acyclovir 10 mg/kg, three times daily is only indicated in severe disease, especially for encephalitis, and for moderate to severe immunocompromised patients.

Systemic steroids are controversial but are usually prescribed for moderate to severe disease with neurological complications. A typical regimen is prednisolone 60 mg daily for 4 days, followed by 40 mg for 4 days, then 20 mg for 4 days.

Conjunctivitis

Adenovirus, a non-enveloped double-stranded DNA virus, is the causal organism of 90% of viral conjunctivitis cases (Bowling, 2016; Watson et al., 2018; Azari and Barney, 2013). It presents more commonly in adults and is self-limiting (Watson et al., 2018; Bowling, 2016). Incubation and communicability periods are approximately 5–12 days and 10–14 days, respectively (Azari and Barney, 2013).

This is a highly contagious infection with a risk of transmission between 10% and 50%. The virus can survive on dry surfaces for weeks and the viral shedding may occur for many days before the symptom's onset. Transmission occurs by direct contact with respiratory or ocular secretions via contaminated fingers, medical instruments, swimming pool water or personal items (Azari and Barney, 2013; Bowling, 2016).

Clinical presentation includes:

- Non-specific acute follicular conjunctivitis which is the most common clinical form of adenoviral conjunctivitis. Symptoms include unilateral watering, redness, itching and mild photophobia. The contralateral eye is commonly affected 1–2 days later but typically less severely. Moreover, respiratory symptoms such as sore throat or rhinorrhoea can occur. Signs include eye lid edema, pre-auricular lymphadenopathy in up to 50% of cases, prominent conjunctival hyperemia and follicles and papillae in the superior tarsal conjunctiva (Bowling, 2016; Azari and Barney, 2013) (Fig. 2).
- Pharyngoconjunctival fever is caused by adenovirus serovars 3,4 and 7. It spreads within families with upper respiratory tract infections. Keratitis may be seen in a third of the cases and rarely severe. Clinical features include sore throat which is characteristically severe with a sudden onset of high fever, bilateral conjunctivitis and periauricular lymphadenopathy (Bowling, 2016; Azari and Barney, 2013).
- Epidemic keratoconjunctivitis is caused by adenovirus serovars 8, 19 and 37 and is the severe form of adenoviral infection. Clinical features include watery discharge, hyperemia, chemosis and ipsilateral lymphadenopathy (Azari and Barney, 2013). Most cases (80%) present with a severe keratitis and photophobia (Bowling, 2016).

Diagnostic tests are usually not needed; however, they should be considered for non-resolving cases or for unclear diagnosis. PCR is sensitive and specific for viral DNA. Viral culture is the gold standard but is costly and slow. Sensitivity is variable but specificity is 100%. A “point-of-care” immunochromatography test takes 10 min to detect adenoviral antigen in tears with an excellent sensitivity and specificity (Bowling, 2016).

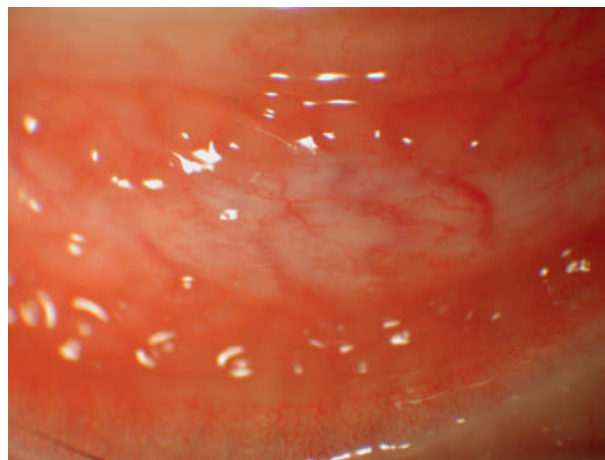


Fig. 2 This photograph illustrates follicles in adenoviral conjunctivitis.

Adenoviral conjunctivitis resolves within 2–3 weeks spontaneously. There are no specific medications for this condition. To decrease transmission risk is important to maintain a strict hand hygiene, rigorous instrument disinfection, and avoid eye rubbing and towel sharing (Azari and Barney, 2013). For symptomatic relief, lubricants four times daily and cold (or warm) compresses can be beneficial. Preservative-free preparations bring an advantage as they come in single-dose units reducing transmission risk. Topical antihistamines and vasoconstrictors may improve symptoms, especially itching. Other measures include discontinuation of contact lens wear until resolution of symptoms (Azari and Barney, 2013; Bowling, 2016; Watson et al., 2018).

Topical steroids such as prednisolone 0.5% four times daily may be needed for severe membranous or pseudomembranous adenoviral conjunctivitis. They only control the inflammation but do not shorten healing time. Topical steroids should be used with caution as they may enhance viral replication, extend the period that the patient is infectious and increase intraocular pressure if the treatment is prolonged. Removal of symptomatic pseudomembranes or membranes may be needed (Bowling, 2016).

Available antiviral medications are not beneficial and topical antibiotics do not prevent secondary bacterial infections. Their use may complicate the course of this infection by causing toxicity or allergy and/or delaying the diagnosis of other possible causes. Moreover, the indiscriminate use of topical antibiotics may increase the antimicrobial resistance of the most common causal organisms. If the condition does not improve between 7 and 10 days or the conjunctivitis is severe, patients should be referred to an ophthalmologist (Azari and Barney, 2013; Watson et al., 2018).

Keratitis

Herpes simplex virus

Herpes simplex virus keratitis (HSK) is an important cause of unilateral blindness in the developed countries due to stromal opacification (Cabrera-Aguas et al., 2018; Guess et al., 2007; Watson et al., 2018). About 500,000 people are infected with ocular herpes simplex virus in the United States alone (Azher et al., 2017; Farooq and Shukla, 2012). One fifth of people with ocular HSV infection develop stromal HSK with the attendant risk of blindness (Guess et al., 2007). Worldwide the incidence of HSK in 2012 was estimated conservatively at about 1.5 million, with 40,000 new cases of severe monocular visual impairment or blindness each year (Farooq and Shukla, 2012).

The appropriate therapy for each type of HSK depends on the correct diagnosis made by clinical observation on slit-lamp examination. A simple classification system was introduced in the “Herpes Simplex Virus Keratitis: a treatment guideline” by the American Academy of Ophthalmology (AAO) in 2014. This classification includes epithelial HSK, stromal HSK, and endothelial HSK and depends on which layer of the cornea is involved (White and Chodosh, 2014). It may also be considered as either primary or recurrent depending on whether it is the patient’s first episode (Guess et al., 2007). A history of labial cold sores or prior HSK can be the first clue to the diagnosis (Watson et al., 2018).

The severity and frequency of recurrent HSK episodes is determined by the susceptibility of the host to the virus and the local susceptibility of the host target tissue. The general susceptibility of the host to the HSV depends on their immune status. Any inherited or acquired condition that suppresses the immune system and age increase the risk of frequent ocular HSV recurrences or severe disease, mostly HSK (White and Chodosh, 2014). For example in organ transplant recipients, the immune system is compromised due to corticosteroids and immune suppressive medications (White and Chodosh, 2014). In terms of age, diagnosing and treating children with ocular HSV disease is challenging. Children tend to present with severe ocular HSV inflammatory disease, more recurrences, and complications compared to adults (White and Chodosh, 2014; Schwartz and Holland, 2000). The inflammatory response of the stromal HSK is more aggressive leading to stromal scarring, corneal opacification, and irregular astigmatism. If the vision is reduced by these changes children, typically before the age of 8 years, are likely to develop amblyopia (White and Chodosh, 2014). Bilateral ocular HSV disease occurs more frequently in children with rates of 3.4–26% and a recurrence rate within the first year of 45–50%, when compared to adults (1.3–12% and 18%, respectively). Bilateral presentation usually occurs as a manifestation of primary infection and causes more complications (White and Chodosh, 2014).

Cornea susceptibility may increase under certain conditions, including with the administration of medications, trauma, and local inflammation (White and Chodosh, 2014). Medications such as prostaglandin agonists (such as latanoprost) used to manage elevated intraocular pressure and corticosteroids may increase the risk of recurrent ocular HSV disease (White and Chodosh, 2014). Any type of ocular surgery on an eye with previous ocular HSV disease increases the risk of recurrence of the disease (Valerio and Lin, 2019). The trauma of the surgery with the local immunosuppression of the perioperative corticosteroids may be contributors. Antiviral prophylaxis is therefore recommended in the immediate perioperative period especially while the patient is on corticosteroids (White and Chodosh, 2014).

A diagnosis of HSK is mainly made clinically after assessing the patient’s history, symptoms and signs. Investigations are performed for atypical or complicated cases or when the diagnosis is uncertain and in all cases of suspected neonatal HSV infection. Viral culture and antigen or DNA detection can be useful in primary epithelial infection but not in stromal HSK as most adults are latently infected with HSV (antibody-positive) (White and Chodosh, 2014; Guess et al., 2007). Polymerase chain reaction test can detect viral DNA and quantify the number of viral copies differentiating viral shedding from replication. Variable reports of the sensitivity of PCR testing exist in different studies 70% (El-Aal et al., 2006), 98% (Kowalski et al., 2006; Kowalski) and 100% (Subhan et al., 2004). PCR is more likely to diagnose patients with typical lesions or patients who have not used antiviral

medications than the Direct Fluorescent Antibody (DFA) test. Advantages of PCR include its high sensitivity and fast results. Disadvantages include the need for a skilled technician, special equipment and appropriate parameters for ocular specimens in a clinical setting. In addition, the interpretation of this test is limited by the inability to differentiate HSV disease level from asymptomatic HSV shedding in the tear film (White and Chodosh, 2014).

The clinical features of HSK, shown in Table 1, can be identified on slit lamp examination (White and Chodosh, 2014; Azher et al., 2017; Rowe et al., 2013; Guess et al., 2007; Cabrera-Aguas et al., 2018) (Figs. 3 and 4). Commonly, the treatment for epithelial HSK is acyclovir ointment five times daily for 14 days (Watson et al., 2018). However, in some cases oral antivirals may be needed (White and Chodosh, 2014). Management of stromal HSK, endothelial HSK and keratouveitis involves referral to an ophthalmologist for oral antivirals (Acyclovir or Valacyclovir), topical steroids and close follow up (Watson et al., 2018; Fung et al., 2020).

The AAO released a treatment guideline in 2014 for epithelial HSK, stromal HSK with/without ulceration, endothelial HSK and prophylaxis (White and Chodosh, 2014). This guideline recommended for epithelial HSK, ganciclovir as the first line topical therapy and includes alternatives such as oral acyclovir, famciclovir and trifluridine. Nevertheless, ganciclovir and trifluridine are not easily accessible in Australia as there are in the United States. A study from Sydney, Australia, reported a diverse prescribing patterns for the treatment and prevention of HSK which were not in alignment with the recommendations from clinical trials (Cabrera-Aguas et al., 2018). Therefore, an evidence-based treatment guideline was developed, implemented and evaluated to standardize the initial treatment for HSK (Table 1) (Cabrera-Aguas et al., 2020; White and Chodosh, 2014; Valerio and Lin, 2019). This guideline was endorsed by the Royal Australian and New Zealand College of Ophthalmologists (RANZCO) in April 2020.

Recurrent episodes of HSK can lead to damage to the cornea nerves causing neurotrophic keratopathy. Patients present with decreased corneal sensation, blink reflex and tear production due to the damage to the sensory fibers innervating the cornea. Corneal sensation is reduced during HSV infection through unclear mechanisms but probably involving the immune system and events within the sensory ganglion. The effects of reduce corneal sensitivity ranges in severity from a irregular epithelial surface to an

Table 1 Herpes simplex keratitis treatment guideline (Cabrera-Aguas et al., 2020; Watson et al., 2018).

Indication	Signs	Antiviral therapy	Topical corticosteroid therapy
Epithelial HSK	Dendritic ulcer Geographic ulcer	Topical acyclovir 3% five times daily for 1–2 weeks. Valacyclovir 500 mg twice daily for 7 days ^a	
SHSK-U	Stromal haze/opacity without ulceration Lipid keratopathy Stromal edema Scarring Sectoral or diffuse neovascularization Corneal thinning Immune ring	Valacyclovir 500 mg once daily during topical steroid use	Prednefrin Forte 4–6 times daily tapered over >10 weeks
SHSK+U	Stromal haze/opacity with ulceration Lipid keratopathy Stromal edema Scarring Sectoral or diffuse neovascularization Corneal thinning Immune ring	Valacyclovir 1 g three times daily for 7–10 days ^b	Prednefrin Forte twice daily tapered slowly as disease comes under control
Endothelial HSK	Central focal circular area stromal edema Keratic precipitates	Valacyclovir 500 mg once to 1 g three times daily for 7–10 days ^b (There is a lack of clinical evidence to guide dosage in this situation)	Prednefrin Forte 4–6 times daily tapered over >10 weeks
Keratouveitis ^c	Stromal edema Keratic precipitates Stromal keratitis Anterior chamber cells	Valacyclovir 1 g three times daily for 7–10 days ^b	Prednefrin Forte 4–6 times daily tapered over >10 weeks
Prophylaxis ^d		Acyclovir 400 mg twice daily Valacyclovir 500 mg once daily	

Key: HSK, herpes simplex keratitis; SHSK-U, stromal herpes simplex keratitis without ulceration; SHSK+U, stromal herpes simplex keratitis with ulceration.

^aIndications: immunocompromised patients; non-compliance, inability to use or tolerate, or ocular toxicity from topical acyclovir.

^bReduce valacyclovir to prophylactic dose after 7–10 days and maintain for as long as frequent topical steroids are in use.

^cRefer patient to cornea/uveitis clinic, respectively depending on degree of corneal or uveal involvement.

^dIndications: multiple recurrences of any type of HSK, especially stromal HSK; and patients with history of ocular HSV following any ocular surgery including penetrating keratoplasty or during immunosuppressive treatment.

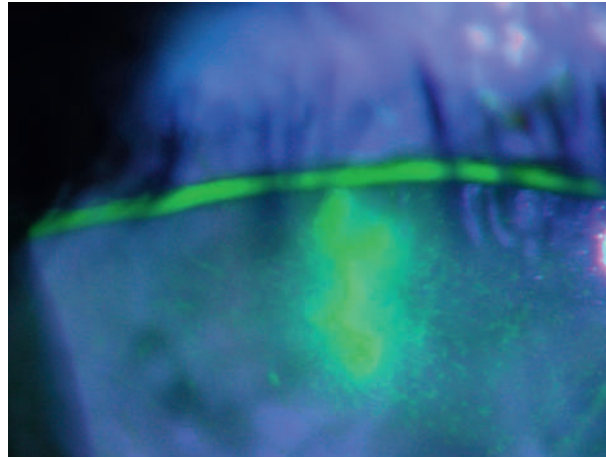


Fig. 3 This photograph illustrates an epithelial dendrite from herpes simplex keratitis seen with fluorescein staining and a cobalt blue light.

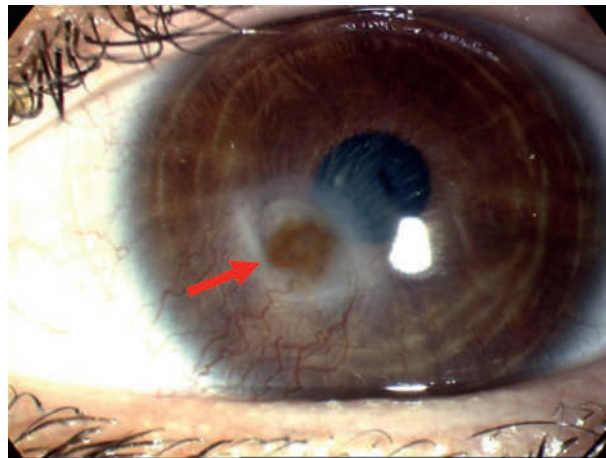


Fig. 4 This photograph illustrates a stromal herpes simplex keratitis with ulceration (arrow).

oval-shaped neurotrophic ulcer with a heaped-up border (Valerio and Lin, 2019; Tsatsos et al., 2016). After the infection is resolved, the cornea reinnervates with a different organization of its fibers, peptidergic content or function. If the breakdown of the epithelium is not appropriately treated as soon it is diagnosed, it may lead to corneal scarring, thinning, vascularization, perforation, or secondary corneal infection resulting in vision impairment or blindness (Tsatsos et al., 2016).

Treatments to promote epithelial growth include ocular lubricants, tarsorrhaphy, botulinum toxin-induced ptosis, growth factors and autologous plasma for early to moderate cases; and collagenase inhibitors, tissue adhesives, conjunctival flap, amniotic membrane use and penetrating or lamellar keratoplasty for more severe and complicated cases (Tsatsos et al., 2016; Valerio and Lin, 2019).

Varicella zoster virus

Herpes zoster keratitis (HZK) generally presents within 1 month of the onset of HZO involving any layer of the cornea. Epithelial and stromal HZK have been identified as risk factors for vision loss after acute phase due to the development of long-term scarring or haze. In a study from Minnesota, 6% of patients developed long-term vision loss (Li, 2018).

Epithelial HZK presents in up to 50% of patients with HZO with ocular involvement. Punctuate epithelial lesions appear 2 days after the vesicular eruption being a transient finding or coalescing into pseudodendrites around day 6 (Liesegang, 2008; Li, 2018; Aggarwal et al., 2014). Pseudodendrites are small and fine lesions, with a similar branching pattern as HSV dendrites. They are formed by swollen and heaped up epithelial cells located in the periphery of the cornea. Pseudodendrites differ from HSV dendrites as they lack the terminal bulb appearance and the central ulceration of HSV dendrites. Pseudodendrites may resolve spontaneously or progress to stromal HZK in half of the cases (Li, 2018).

Stromal HZK presents in 6–16% of patients with ocular involvement of HZO. It usually develops at day 10 of the onset of HZO after the manifestation of the epithelial lesions. Complications includes long-term nummular corneal scarring, lipid keratopathy

and dellen (Li, 2018; Liesegang, 2008). Keratouveitis/endotheliitis occurs in 1–7% of patients within a week of onset of HZO. Clinical features include localized corneal edema, cell and flare and a complement-mediated immune Wessely ring (Liesegang, 2008; Li, 2018). The degree of anterior chamber involvement varies being severe with associated hypopyon or hyphema from the vasculitis. Elevated intraocular pressure may be also present. This type of keratitis may lead to long term endothelial cell loss and corneal decompensation.

A disciform stromal keratitis may represent an HZV active infection or a zoster immune reaction. It resolves within months and may become chronic with edema and lipid keratopathy. Corneal mucous plaques or delayed pseudodendrites occur months or year later typically in a quiescent eye. Pseudodendrites vary in size, migrate and move around the cornea (Liesegang, 2008). Interstitial keratitis results from long-term HZV inflammation with corneal vascularization and lipid keratopathy, scarring and possible perforation. Nerve damage leads to neurotrophic keratopathy diminishing corneal sensation with the loss of corneal epithelial integrity and tear dysfunction (Liesegang, 2008).

Neurotrophic keratopathy may suddenly present months after HZO with diffuse epitheliopathy and chronic surface dysfunction. Subsequent calcareous plaque formation may develop. Corneal edema can be temporary but usually represents the chronic end stage of corneal endothelial destruction by the virus or the related inflammation (Liesegang, 2008).

Episcleritis or scleritis may appear in an early stage of the infection and become persistent or appear later. The scleritis may involve the limbus presenting as limbal vasculitis and sclerokeratitis resulting in a patchy scleral atrophy (Liesegang, 2008).

Oral antiviral agents must be commenced within 72 h of onset of HZO. Acyclovir, valacyclovir and famciclovir are comparable in efficacy and in the prevention of ocular complications (Li, 2018; Colin et al., 2000; Beutner et al., 1995; Tyring et al., 2000; Jain et al., 2019). Despite treatment recommendations for patients presenting with HZO, there is little consensus on the management of acute complications such as keratitis (Aggarwal et al., 2014). Diverse antiviral agents alone or in combination with topical steroids have been found to be effective in some studies for pseudodendritic keratitis despite current or recent oral antiviral therapy (Hu et al., 2010; Mortemousque, 2006; Magone et al., 2005). For example, topical ganciclovir 0.15% gel was reported to be useful in these cases (Aggarwal et al., 2014; Li, 2018). The use of corticosteroids has been controversial. Corticosteroids control the inflammation in stromal, keratouveitis/endotheliitis cases, however tapering off the drops may be challenging. In some cases, patients may need long-term topical steroids, however they need to be warned about side effects such as glaucoma and cataracts (Li, 2018).

Complications from HZO are associated with chronic and/or recurrent active HZV infections. There is evidence that suppressive oral antiviral therapy is effective in HSV disease, and as HSV share similarities with HZV; it may be reasonable to consider the use of suppressive oral antiviral therapy for HZV infections. Despite the lack of evidence, clinicians prescribe suppressive antiviral therapies in different dosages and frequencies (Lo et al., 2019). Consequently, the Zoster Eye Disease Study (ZEDS) is currently investigating whether suppressive antiviral therapy (valacyclovir 1 g daily for 1 year) reduces chronic and recurrent HZV disease in the United States with over 200 investigators at 60 participating centers (Lo et al., 2019; Jain et al., 2019; Clinicaltrials.gov, 2020).

Uveitis

Uveitis refers to the inflammation of the middle, pigmented, vascular structure of the eye. It may affect one or multiple structures in the uveal tract. Anatomically, uveitis is divided in anterior, intermediate, posterior uveitis and pan-uveitis. Anterior uveitis affects the anterior chamber, intermediate uveitis, the vitreous, posterior uveitis, the retina or choroid; and pan-uveitis, the anterior chamber, vitreous, and retina or choroid (Jabs et al., 2005).

Anterior uveitis

Anterior uveitis (AU) is the most common form of uveitis worldwide (Tsirouki et al., 2018). Anterior uveitis can be acute (sudden onset with a duration of less than 3 months), recurrent (episodes separated by a quiet period lasting over 3 months), or chronic (episodes lasting over 3 months). Acute AU typically presents with unilateral eye pain, redness, blurred vision, photophobia and tearing. Chronic AU may be asymptomatic or present with mild blurred vision. Concomitant scleritis and posterior uveitis need to be excluded. Elevated intraocular pressure or iris atrophy may also suggest a diagnosis of viral AU. The most common viruses associated with AU include HSV, VZV and CMV (Chan and Chee, 2019).

Cytomegalovirus

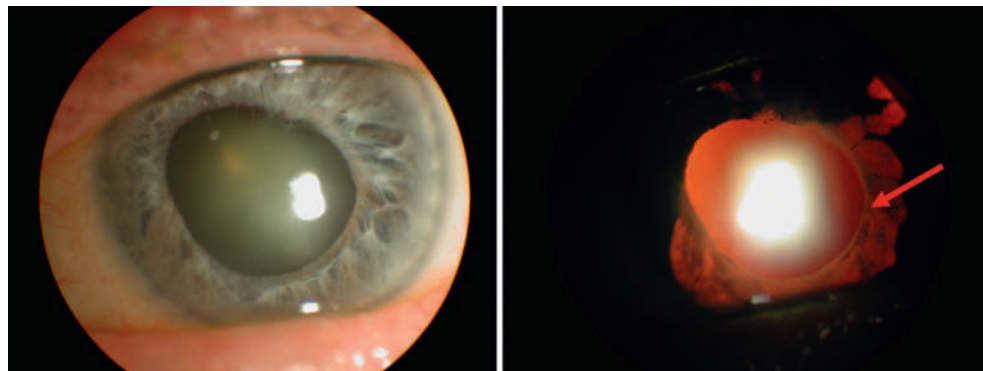
Over 85% of Asian populations are seropositive for CMV compared to 51.5–54.4% in the West (Chee and Jap, 2010; Lin, 2015). CMV AU presents mainly in males in their fourth to fifth decade of life, mostly affecting the anterior segment with a variety of clinical features (Lin, 2015; Chan and Chee, 2019; Pleyer and Chee, 2015; Touhami et al., 2018).

CMV AU may follow two courses: a Posner-Schlossman Syndrome (PPSS) pattern of recurrent acute attacks or chronic anterior uveitis, the Fuchs uveitis syndrome (FUS) like pattern. In acute AU, patients typically present with recurrent, acute evanescent episodes of mild unilateral AU and a small number of granulomatous keratic precipitates (KPs) (typically 1–5 KPs) and markedly elevated IOP (up to 50 mmHg) causing mild ocular discomfort, mild blurred vision and haloes (Chan and Chee, 2019; Bowling, 2016; Lin, 2015). Clinical features include low-grade anterior chamber inflammation with no or minimal flare, diffuse iris stromal atrophy, a round pupil with no posterior synechiae and a normal posterior segment (Chan and Chee, 2019; Pleyer and Chee, 2015). Common clinical features are summarized in Table 2 (Fig. 5).

Table 2 Clinical features of anterior uveitis caused by herpes simplex virus, varicella-zoster virus and cytomegalovirus (Chan and Chee, 2019; Pleyer and Chee, 2015).

<i>CMV acute—PPSS like pattern</i>	<i>CMV Chronic-FUS like pattern</i>	<i>HSV</i>	<i>VZV</i>
• Male patients • 20–50 years • Mild conjunctival injection • Very high IOP • Endotheliitis • Single or few coin-shaped KPs • Linear KPs • Minimal AC activity • Diffuse iris atrophy (43%)	• Male patients • Asian population • 40–60 years • Mild conjunctival injection • Very high IOP • Fine, white, stellate diffusely distributed KPs • Mild AC activity • Diffuse iris atrophy (60%) • Cataract (>80%)	• <50 years • No gender predilection • Moderate to severe conjunctival injection • IOP with acute spikes • Vesicles in border eyelids • Dendrites or stromal keratitis • Small-medium size KPs • Moderate AC activity • Sectoral iris atrophy • Posterior synechiae • Vitritis (43%)	• >60 years • No gender predilection • Moderate to severe conjunctival injection • IOP with acute spikes • Vesicular rash dermatome CN V1 • Pseudodendrites or nummular keratitis • Small-medium size KPs • Severe AC activity • Sectoral iris atrophy • Posterior synechiae • Vitritis (83%)

Key: *CMV*, cytomegalovirus; *FUS*, Fuchs uveitis syndrome; *HSV*, herpes simplex virus; *IOP*, intraocular pressure; *KPs*, keratic precipitates; *PPSS*, Posner-Schlossman syndrome; *VZV*, varicella zoster virus.

**Fig. 5** These photographs illustrate extensive iris atrophy (arrow) in varicella zoster virus uveitis on a slit-lamp (left) and a retro-illuminated image (right).

In chronic AU, patients present with a chronic unilateral low-grade inflammatory disorder with raised intraocular pressure, resembling Fuchs uveitis syndrome (FUS) (Touhami et al., 2018). It is less aggressive than the other uveitides with majority of patients asymptomatic for a long period of time. It mostly presents in males in the third or fourth decade of life from Asian populations (Japan, Taiwan and Singapore). It presents with minimal ocular discomfort, blurred vision and variably elevated IOP up to 50 mmHg. Clinical features include mild-to-moderate anterior chamber activity, no ciliary injection, fine stellate uniformly white distributed KPs over the entire endothelium. Other signs include late posterior subcapsular cataract in 81% patients and diffuse iris stromal atrophy and fragile vasculature (Chan and Chee, 2019; Bowling, 2016; Pleyer and Chee, 2015). Common clinical features are summarized in Table 2. In European patients, there are fewer and small pigmented KPs located inferiorly, minimal anterior chamber inflammation with posterior subcapsular cataract developing late in the disease in 75% of eyes (Chan and Chee, 2019).

Corneal endotheliitis presents with KPs, stromal edema and Descemet folds, isolated or associated with AU. These findings may be localized or manifest similarly to bullous keratopathy with severe corneal edema and elevated intraocular pressure. Immune ring formation can also be associated with CMV endotheliitis (Chan and Chee, 2019).

Herpes simplex virus

Herpes simplex virus type 1 mainly causes this condition affecting both genders in their fourth and fifth decade of life. It occurs due to reactivation of the primary disease (Chan and Chee, 2019; Pleyer and Chee, 2015). HSV/VZV accounted for 8% of uveitis cases in the Pacific Ocular Inflammation Study (Lin, 2015; Acharya et al., 2013). The patient may have a past history of fever or blisters (Chan and Chee, 2019). The infection may manifest in the periocular skin, in the cornea or only in the uvea (Pleyer and Chee, 2015).

Clinical features include: diffuse small to medium-size, stellate or granulomatous KPs, elevated intraocular pressure, sector iris atrophy, moderate anterior chamber activity with cells and flare (Valerio and Lin, 2019; Bowling, 2016; Chan and Chee, 2019; Lin, 2015). Posterior synechiae develop in a third of cases and anterior vitritis in nearly half of involved eyes. IOP is high due to

trabeculitis (Chan and Chee, 2019). Sectorial iridoplegia and localized flattening of the pupil border in the affected area with poor reaction to light may be present. After the episode resolves, the pigment epithelium is destroyed resulting in patchy or sectorial iris atrophy with transillumination defects (Chan and Chee, 2019). Common clinical features are summarized in Table 2.

Corneal scars and hypoesthesia may be present as result of previous epithelial or stromal keratitis (Chan and Chee, 2019; Lin, 2015). HSV uveitis commonly occurs in eyes with active or prior keratitis and the uveitis correlates with the severity of keratitis. Chronic stromal or endothelial disease are associated to more severe uveitis (Chan and Chee, 2019).

Varicella zoster virus

Varicella zoster virus is latent in neural sensory ganglia after primary infection and reactivates when immunity wanes usually in older patients (immunosenescence) or in an immunocompromised patient (Chan and Chee, 2019; Pleyer and Chee, 2015). VZV anterior uveitis may present months after the rash associated with HZO resolves or rarely without skin involvement (Zoster sine herpette; Chan and Chee, 2019). In patients over age 60, unilateral AU in association with sectoral iris atrophy with transillumination defects, and no evidence of prior or concurrent epithelial or stromal keratitis is a common presentation (Pleyer and Chee, 2015). Common clinical features are summarized in Table 2; Fig. 6.

Diagnostic tests

Aqueous humor analysis is a useful and common method to determine viral etiology and severity of the disease as the diagnosis may not be possible based only on the clinical features. However, several taps maybe needed as false negatives are frequent due to prior or current antiviral therapy (Chan and Chee, 2019; Valerio and Lin, 2019; Lin, 2015; Pleyer and Chee, 2015). An anterior chamber tap is performed under aseptic technique and aqueous humor is sent for PCR analysis.

Polymerase chain reaction (PCR) can detect minimal amounts of viral DNA and enable rapid confirmation of the diagnosis (Chan and Chee, 2019; Lin, 2015). The aqueous tap should be done during the IOP spike, prior to initiating therapy especially when suspecting CMV (Lin, 2015). Negative PCR does not exclude viral etiology. It may be due to low intraocular viral load, limited volume of aqueous humor sample, a short-lived phase of DNA release that may have been missed during the time of aqueous sampling due to delay in presentation or testing. A positive result may only be obtained after repeated aqueous taps. PCR may give a false positive by detecting CMV DNA from latent virus in leucocytes present in the anterior chamber during inflammation (Chan and Chee, 2019).

PCR tends to be positive at the outset and/or during early reactivation, but viral DNA levels may fall below the detection limit in later stages of the disease. HSV-VZV commonly present during the acute phase when the viral load is higher and are frequently detected by PCR (Chan and Chee, 2019).

Treatment

The mainstay of treatment is a combination of topical and/or oral antivirals, topical corticosteroids, topical nonsteroidal anti-inflammatory drugs (NSAIDs) and IOP-lowering agents if needed (Pleyer and Chee, 2015; Fung et al., 2020).

For CMV, ganciclovir topical gel 0.15%, five times daily is the preferred antiviral therapy due to its efficacy, affordability and safety (Pleyer and Chee, 2015; Chee and Jap, 2010). Topical steroids must be given if there is severe inflammation (prednisolone acetate 1% twice daily). They have less systemic side effects compared to periocular or systemic steroids agents (Fung et al., 2020). This may be tapered and replaced by NSAIDS ketorolac tromethamine 0.4%, four times daily (Pleyer and Chee, 2015). Recurrences tend to present even after successful 3-month treatments. Some patients are unable to come off the therapy and may require topical ganciclovir, and low dose of topical steroids or NSAIDS with or without antiglaucoma medications indefinitely.

For HSV and HZV uveitis, antiviral agents including oral acyclovir, valacyclovir or famciclovir are used along with topical steroids for example prednisolone acetate 1% four times daily, and a topical cycloplegic, atropine 1% twice daily, if anterior chamber inflammation exceeds of 1+ cell. This is to reduce pain and prevent posterior synechiae (Pleyer and Chee, 2015) (See Table 3). Topical corticosteroids must be avoided if epithelial HSK is present. Furthermore, IOP must be treated as needed (Lin, 2015;

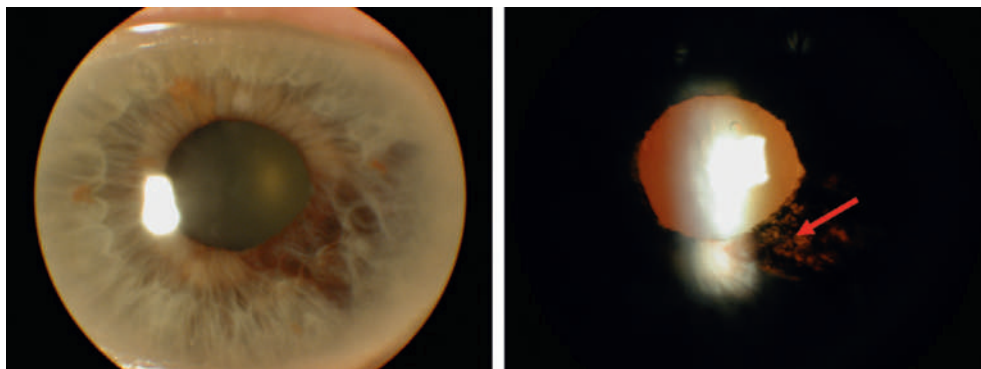


Fig. 6 These photographs illustrate a localized iris atrophy (arrow) in herpes simplex uveitis on a slit-lamp examination (left) and a retro-illuminated image (right).

Table 3 Treatment of anterior and posterior uveitis (Chee and Jap, 2010; Aizman et al., 2007; Lee et al., 2017; Wu et al., 2019; Baltinas et al., 2018; Valerio and Lin, 2019; Schoenberger et al., 2017; Stryjewski et al., 2018; Pleyer and Chee, 2015; Scott et al., 2002; Tseng et al., 2015).

Indication	Dosage
AAU	HSV/VZV <i>Therapeutic:</i> • ACV 400–800 mg, 5 times daily for 4–6 weeks • VLC 500 mg⁻¹ g, TDS for 6 weeks • FCV 250–500 mg TDS <i>Prophylaxis:</i> • ACV 400 mg BD-TDS • VLC 500 mg⁻¹ g BD • FCV 250–500 mg BD CMV Topical gel GCV: 0.15%, apply five times daily for 3 months
ARN	• IV ACV 10–15 mg/kg TDS for 5–14 days FOLLOWED BY ACV 400–800 mg, five times daily for 6–12 weeks • VLC 2 g TDS for 6 weeks • Intravitreal injection Foscarnet 2.4 mg/0.1 mL
PORN	• IV ACV 10–15 mg/kg TDS PLUS • IV foscarnet 60 mg/kg three times or 90 mg/kg twice daily for 2 weeks • IV GCV 5 mg/kg twice daily PLUS • IV foscarnet, 60 mg/kg three times or 90 mg/kg twice daily for 2 weeks, FOLLOWED BY oral valganciclovir, 900 mg once daily PLUS • IV foscarnet 90–120 mg/kg/day until healing is achieved • IV GCV 5 mg/kg twice daily PLUS • IV ACV 10–15 mg/kg TDS • Intravitreal GCV 2 mg/0.05 mL PLUS • IV ACV 10–15 mg/kg TDS OR • IV foscarnet 60 mg/kg three times or 90 mg/kg twice daily • Intravitreal GCV 2 mg/0.05 mL PLUS • Intravitreal foscarnet 1.2 mg/0.05 mL, three times weekly for 2 weeks, FOLLOWED BY maintenance injections once or twice weekly until retinitis is stabilized
CMV retinitis	• IV GCV 5 mg/kg, TDS weekly as induction dose for up to 3 weeks FOLLOWED BY 5 mg/kg as a maintenance dose • Valganciclovir 900 mg BD for 3 weeks as induction therapy; then 450 mgs BD as maintenance dose NB. Dose adjusted depending on renal function

Key: AU, anterior uveitis; ARN, anterior retinal necrosis; BD, twice daily; CMV, cytomegalovirus; FCV, famciclovir; GCV, ganciclovir; HSV, herpes simplex virus; IV, intravenous; PORN, progressive outer retinal necrosis; TDS, three times daily; VZV, varicella zoster virus.

Bowling, 2016). When treating VZV uveitis, systemic antivirals should be commenced within 72 h of rash onset (Pleyer and Chee, 2015). Acute trabeculitis responds very well to topical corticosteroids even without antihypertensive medication. However, inadequate antiviral therapy can lead to recurrent episodes and risk of glaucoma development (Valerio and Lin, 2019) (Table 3).

Posterior uveitis

Posterior uveitis refers to the inflammation of the posterior uveal tract formed by the retina and the choroid (Lee et al., 2017). Studies from United States (Merrill et al., 1997; Rodriguez et al., 1996; McCannel et al., 1996; Henderly et al., 1987); Europe (Pivetti-Pezzi et al., 1996; Smit et al., 1993); Australia and New Zealand (Zagora et al., 2017; Wakefield et al., 1986; Hart et al., 2019; Wong et al., 2017); and Asia (Al Dhibi et al., 2017; Al-Shakarchi, 2014; Rahman et al., 2018; Amin et al., 2019; Chen et al., 2017; Abaño et al., 2017; Pathanapitoon et al., 2008) have reported the prevalence of posterior uveitis from 5% to 46%. Most cases are idiopathic or inflammatory in origin (Tsirouki et al., 2018), however the proportion of infectious cases can fluctuate from about 40% reported in studies from Australia (Zagora et al., 2017) and New Zealand (Wong et al., 2017) to about 80% reported in a study from Iran (Kianersi et al., 2015; Lee et al., 2017).

The latent-reactivation cycle of HSV, VZV, CMV depends on the interaction with the host immune system and external factors. Depressed cell-mediated immunity can lead to reactivation of latent virus causing the disease. HSV and VZV retinitis occur both in immunocompetent and immunocompromised patients; whilst CMV retinitis only occurs in immunocompromised patients. Progressive outer retinal necrosis (PORN), a rare hyperacute form of VZV retinitis is only seen in immunocompromised patients.

Symptoms of posterior uveitis include: decreased visual acuity, visual field defects, floaters, photopsia, photophobia and occasionally pain. Signs include: conjunctival injection, variable anterior uveitis, and keratic precipitates, raised IOP, vitreous cellular infiltrate, vitreous haze and a wide range of retinal and choroidal changes. Important clinical features include: macula edema, optic disk swelling focal or diffuse retinal and choroidal lesions, and retinal vasculitis (Lee et al., 2017).

Diagnosis of posterior uveitis is based on clinical assessment by a detailed history, thorough ocular examination of each eye and a careful review of systems. Multi-modal imaging such as wide field retinal imaging, digital fundus photography, fundus autofluorescence, fundus fluorescein angiography, indocyanine green angiography and optical coherence tomography can be used for diagnosis and monitoring response to treatment. The tetraplex PCR analysis for HSV, VZV and CMV can confirm the causing organism in 59–100% of cases with a PCR sensitivity of 80.9–84% and a specificity of 97.4–100% (Lee et al., 2017).

Treatment of infectious posterior uveitis includes: appropriate systemic or intravitreal anti-microbials which are often combined with oral corticosteroid therapy. Serious complications include cataracts, band keratopathy, uveitic glaucoma, cystoid macular edema and retinal detachment leading to blindness (Lee et al., 2017).

Acute retinal necrosis

Acute retinal necrosis (ARN) syndrome is a sudden onset, vision threatening, rapidly progressive retinal infection characterized by circumferentially spreading areas of retinal necrosis, retinal vasculitis, optic neuropathy and intraocular inflammation. ARN is rare with an annual incidence of 0.5–0.63 new cases per million population according to British studies (Cochrane et al., 2012; Muthiah et al., 2007; Schoenberger et al., 2017). It is caused by VZV, HSV-1 or HSV-2 and occurs in both immunocompetent and immunosuppressed patients (Pleyer and Chee, 2015; Valerio and Lin, 2019; Aizman et al., 2007; Lee et al., 2017; Chong et al., 2009). In many parts of the world solid organ transplantation is now the commonest cause of immunocompromised rather than HIV infection. CMV is the commonest cause of retinitis in patients with acquired immunodeficiency syndrome (Dabil et al., 2001). Clinically it can sometimes be difficult to differentiate CMV infection from other herpetic retinitis in immunocompromised patients. Whenever possible it is best to confirm the diagnosis by PCR testing.

VZV and HSV-1 is more likely to be found in middle aged and old patients, and HSV-2 in children and young adults (Lin, 2015; Pleyer and Chee, 2015; Cochrane et al., 2012). VZV infections have poor prognosis needing more aggressive antiviral treatments. This may be explained by viral features and/or age-related changes. Retinitis occurs from reactivation of latent virus; however, the exact pathogenesis remains unclear (Pleyer and Chee, 2015).

Early identification of the causative virus is important to guide treatment (Dabil et al., 2001). ARN presents with a rapidly progressive pan-uveitis with unilateral vision loss, photophobia, floaters and pain with up to one third of cases progressing to bilateral disease (Lin, 2015). The infection spreads rapidly over hours to several days. The diagnosis is typically made by clinical examination and confirmed by PCR from vitreous or aqueous. Standard diagnostic criteria published by the American Uveitis Society include (1) >1 foci of retinal necrosis with discrete borders in the peripheral retina, (2) rapid circumferential progression of necrosis in the absence of antiviral therapy, (3) evidence of occlusive vasculitis and arteriolar involvement, and (4) prominent inflammation in the vitreous and anterior chambers (Valerio and Lin, 2019; Pleyer and Chee, 2015; Lee et al., 2017; Aizman et al., 2007; Lin, 2015). The risk of a second eye involvement may be reduced by systemic antiviral therapy. Second eye involvement most frequently develops within 6 weeks of the initial infection but may long delayed. The progression of retinitis is usually controlled within 2–4 weeks. Once the acute episode is controlled, severe retinal atrophy develops with well-demarcated diaphanous lesion borders predisposing to retinal breaks. Vitreous organization may lead to proliferative vitreoretinopathy which further increases the likelihood of retinal breaks and retinal detachment which occurs in up to 70% of ARN affected eyes. Other complications include: vitreous haze and membranes, macular edema, optic atrophy, epiretinal membrane formation and viral relapse following antiviral cessation (Pleyer and Chee, 2015) Fig. 7.

Generally, the diagnosis of ARN is made by clinical examination. Laboratory tests can assist in the diagnosis in less typical cases. Polymerase chain reaction test is the standard method to identify viral DNA from small aqueous or vitreous samples (Schoenberger et al., 2017). Several studies have reported PCR testing of aqueous and vitreous samples. Only one study found a significant difference when comparing aqueous and vitreous samples (93% positive for vitreous samples and 46% for aqueous samples). Other studies found no significant differences between both type of samples. There is insufficient evidence to recommend one type of sample over the other (Schoenberger et al., 2017; Wu et al., 2019).

Antiviral treatment must be commenced as soon as clinical diagnosis is made (Lee et al., 2017; Schoenberger et al., 2017). There are no randomized clinical trials to establish standard therapies. However, the standard therapy remains as intravenous acyclovir 10–15 mg/kg, three times daily at least for 7 days; or 1500 mg/m² daily for 5–10 days, followed by oral acyclovir 800 mg, five times daily for 6–12 weeks (Aizman et al., 2007; Pleyer and Chee, 2015; Valerio and Lin, 2019; Lee et al., 2017). Side effects of the intravenous treatment include nausea, vomiting, diarrhoea, headache, photosensitivity, rash, myelosuppression, reversible rise in the serum creatinine levels, urinary calculi, elevation in liver function tests, and central nervous system toxicity such as lethargy, delirium and seizures (Wu et al., 2019; Aizman et al., 2007). Studies have shown that valacyclovir 2 g, four times daily can be used instead of the intravenous acyclovir (Valerio and Lin, 2019; Baltinas et al., 2018). Valacyclovir is a well-tolerated medication with few side effects. For initial non-responders or patients with retinitis lesions threatening the optic nerve or the macula, adjunctive intravitreal foscarnet therapy can be used (Aizman et al., 2007; Pleyer and Chee, 2015; Valerio and Lin, 2019; Stryjewski et al., 2018; Wu et al., 2019). See Table 3 for antiviral dosages.

Retinal detachment occurs in up to 75% of patients (Lee et al., 2017; Lin, 2015). Baltinas et al. found that patients treated with oral valacyclovir (66%) or IV acyclovir (62%) had similar risk of developing retinal detachment usually approximately 60 days after presentation (Baltinas et al., 2018; Wu et al., 2019). Prophylactic barrier laser should be done within 6 weeks of diagnosis to allow adequate chorioretinal adhesion before vitreous detachment. Baltinas et al. reported that the prophylactic barrier was given to a third of patients within a week of the diagnosis. This procedure did not reduce the risk of retinal detachment regardless the treatment given to the patients (Baltinas et al., 2018). Other complications include: cataract development, epiretinal membrane formation, recurrent AAU and cystoid macular edema, hypotony/phthisis and reactivation of the infection either in the same eye or in the fellow eye (Baltinas et al., 2018).

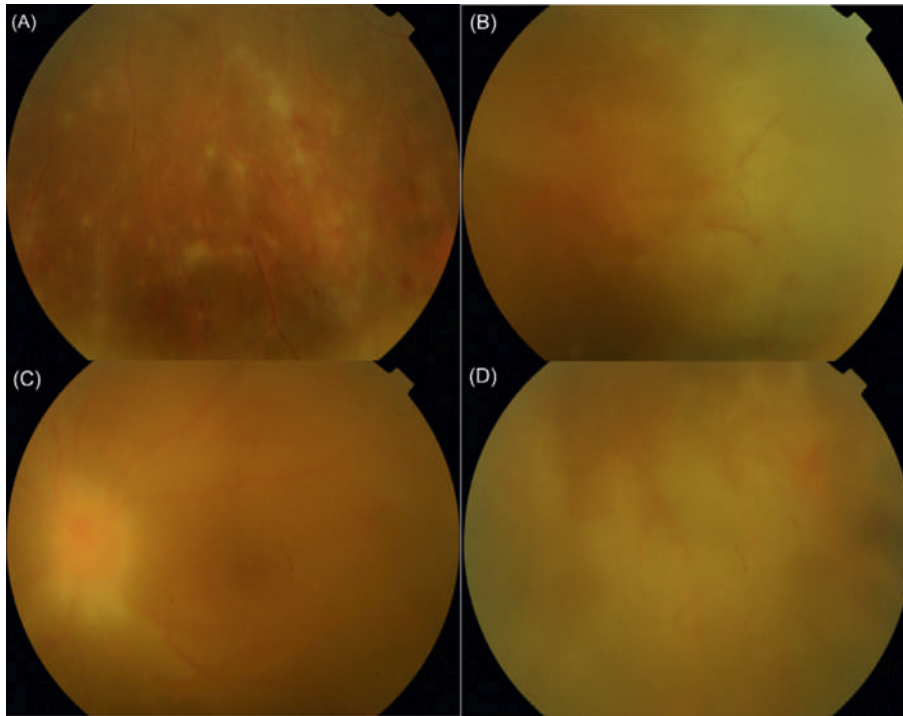


Fig. 7 (A) This photograph illustrates an early acute retinal necrosis on the left eye with multiple small foci of peripheral retinitis. (B–D) These photographs illustrate 24 h later: a peripheral retinitis much worse, swollen disk from optic neuropathy and hazy media from severe inflammation.

Oral prednisone 40–80 mg daily can be added to the treatment in case of severe inflammation for example in severe vitritis, serous retinal detachment, or vasculitis (Wu et al., 2019; Lee et al., 2017). Topical corticosteroids may decrease anterior vitritis and cystoid macular edema (Wu et al., 2019).

Progressive outer retinal necrosis

Progressive outer retinal necrosis is a very rapidly progressive bilateral devastating form of ARN only seen in very immunocompromised patients, for example in AIDS patients with low CD4⁺ T lymphocyte counts (<50 cells/ μ L) or in solid organ transplant patients on very intense immunosuppressive therapy (Lee et al., 2017; Wu et al., 2019). VZV is the causative organism of PORN (Lee et al., 2017; Wu et al., 2019). This condition presents with acute rapidly progressive blurred vision, visual field loss, without pain or photophobia. There is extensive multifocal necrotizing retinitis at the posterior pole that rapidly spreads to the retinal periphery. The typical macular lesion is a white, necrotic, parafoveal opacification with a central cherry red spot (Lee et al., 2017; Wu et al., 2019) Fig. 8.

Diagnosis of PORN is based on a diagnostic criteria proposed by Engstrom et al. and PCR analysis of intraocular fluids for VZV (Lee et al., 2017; Wu et al., 2019). The diagnostic criteria include (1) multifocal, deep retinal opacification without granular borders, (2) lesions may be confluent, (3) peripheral lesions with or without macular lesions, (4) very rapid progression, (5) inconsistent direction of disease spread, (6) no vascular inflammation, (7) none or minimal intraocular inflammation (Engstrom Jr. et al., 1994; Wu et al., 2019).

Aggressive systemic and intravitreal antiviral therapy must be commenced early, as PORN progresses rapidly, to prevent progression of retinitis, induce remission, prevent the infection in the contralateral eye and preserve useful vision (Lee et al., 2017). Systemic antiviral agents include intravenous foscarnet 24 mg/mL three times daily, intravenous ganciclovir 5 mg/kg/24 h, and intravenous acyclovir (Lee et al., 2017). A combination of antiviral agents leads to better visual outcomes. The following combination are recommended: intravenous acyclovir plus foscarnet, intravenous ganciclovir plus intravenous foscarnet, intravenous ganciclovir and intravenous acyclovir, intravitreal ganciclovir plus intravenous acyclovir, or intravitreal ganciclovir plus intravitreal foscarnet (Lee et al., 2017; Wu et al., 2019; Scott et al., 2002). When patients respond to treatment oral valacyclovir 1 g, three times daily, valganciclovir or famciclovir is given for maintenance. Oral prednisolone 40 mg/day may be given to reduce intraocular inflammation, for example vitritis (Lee et al., 2017).

Panretinal photocoagulation can be considered but is not useful in preventing retinal detachment. Retinal detachment may be treated with vitrectomy with silicon oil tamponade (Lee et al., 2017). Two-thirds of patients with PORN progress to no light perception. Generally, PORN has poorer visual outcomes than ARN due to its poor respond to antiviral therapy. Complications of PORN include retinal detachment in 70% cases, optic disk edema or atrophy, vitreous hemorrhage, macular edema and infection in the fellow eye (Lee et al., 2017).

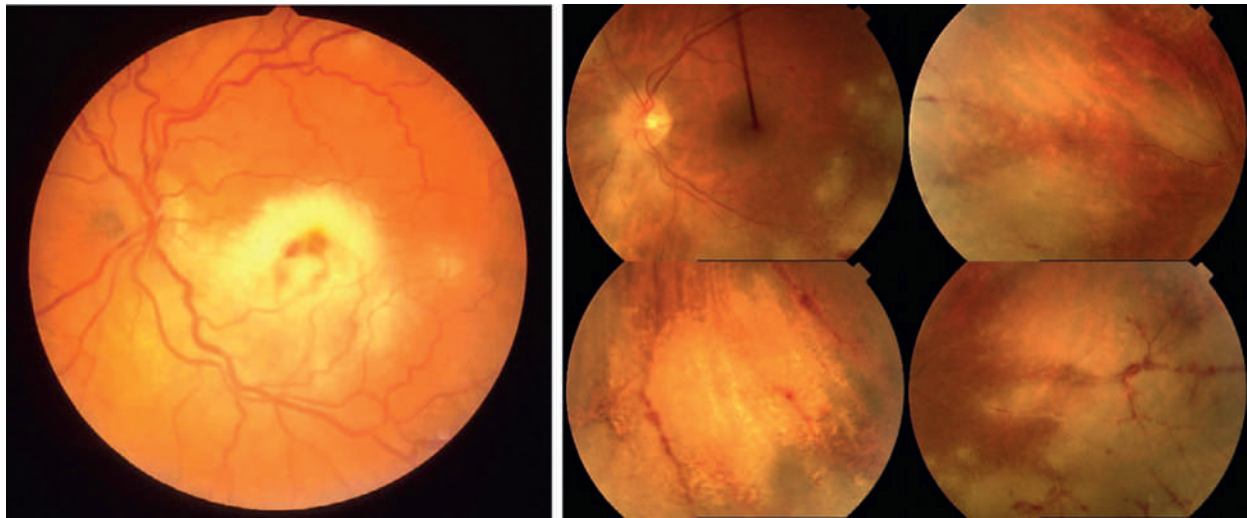


Fig. 8 These photographs illustrate progressive outer retinal necrosis (PORN) with widespread retinal lesions, posterior pole involved and typical macular retinitis.

Cytomegalovirus retinitis

CMV retinitis is an opportunistic infection in severe immunocompromised patients (Lee et al., 2017; Wu et al., 2019). During the last century, CMV retinitis occurred mainly in severe immunocompromised AIDS patients with CD4⁺ counts below 100 cells/ μ L. Currently, susceptible patients include those on immunosuppression after solid-organ transplantation, malignancy, hematopoietic stem cell transplantation or those receiving immunosuppressive medication for other reasons (Wu et al., 2019; Lee et al., 2017). Up to 85% of AIDS patients with CD4⁺ counts less than 50 cells/ μ L present CMV retinitis. CMV retinitis can rarely occur in seemingly immunocompetent patients. Such patients are mild to moderately immunocompromised from advanced age, diabetes mellitus, corticosteroid or noncytotoxic immunosuppressive medication use. CMV retinitis can present at any age, with an unilateral presentation progressing to the contralateral eye in 20% of cases within 6 months (Lee et al., 2017).

CMV retinitis is asymptomatic in up to 50% of patients. Symptoms include: blurred vision, floaters, visual field loss and ocular discomfort (Lee et al., 2017). Clinical features include: mild anterior segment inflammation keratic precipitates, anterior chamber cells, and minimal vitritis (Lee et al., 2017; Wu et al., 2019). The characteristic fundus clinical feature is a single focus of full thickness yellow-white necrotizing granular retinitis in the peripheral retina with a perivascular distribution which expands centrifugally (Lee et al., 2017). In other patients with CMV retinitis there is more overlying vitritis and the involved retina has a characteristic yellowish white color and there is associated retinal hemorrhages and vasculitis. The appearance is often described as “pizza retinitis” (lesion appearance similar to mozzarella cheese and tomato sauce) There can be extensive vascular occlusion and severe intraocular inflammation widespread necrotizing retinitis (Wu et al., 2019) Fig. 9.

The diagnosis of CMV retinitis is made by indirect ophthalmoscopy with documentation on digital fundal photography and supporting clinical history, laboratory and positive CMV PCR in intraocular fluid samples (Lee et al., 2017; Wu et al., 2019).

The treatment of CMV retinitis consists of reversing the immunodeficiency and addressing the direct damage to the retina (Wu et al., 2019; Lee et al., 2017). In HIV/AIDS patients, combination anti-retroviral therapy must be initiated or optimized if the CD4⁺ count remains low despite antiretroviral therapy (Lee et al., 2017). CMV retinitis is often a manifestation of systemic CMV infection. Peripheral blood branched chain CMV PCR at diagnosis and during follow up to determine the systemic viral load is useful to document the response to treatment. It is important to exclude sub-clinical infection in other organ systems such as the gastrointestinal tract, liver and lungs (Wu et al., 2019). In HIV patients, an increase in CD4⁺ T-cells over 200 cells/ μ L for more than 3–6 months is considered an acceptable recovery with reduced risk of CMV reactivation. In non-CD4⁺ specific immunosuppression or ongoing systemic immunosuppression to control autoimmune conditions, ophthalmologists need to work closely with other physicians to determine appropriate management of the systemic condition and the retinitis (Wu et al., 2019).

Antiviral agents including ganciclovir, valganciclovir and foscarnet are used to treat CMV infection (Lee et al., 2017). First-line therapy is intravenous ganciclovir or its oral prodrug valganciclovir which block viral thymidine kinases (See antiviral dosages in Table 3). In cases with resistance (UL97 mutations) or inability to tolerate ganciclovir, intravenous foscarnet which inhibits CMV DNZA polymerase is recommended. Intravitreal foscarnet and ganciclovir can also be used when clinically indicated for posterior pole involvement or very extensive retinitis. A typical intravenous regimen may be three times weekly as induction, reducing to twice weekly until retinitis is controlled and immune function is improved. Systemic antivirals are used to protect the contralateral eye. Complications include extension of retinitis, involvement of contralateral eye, epiretinal membrane formation, retinal neovascularization, posterior subcapsular cataracts, phthisis, optic atrophy and rhegmatogenous retinal detachment in up to 30% of cases (Lee et al., 2017).

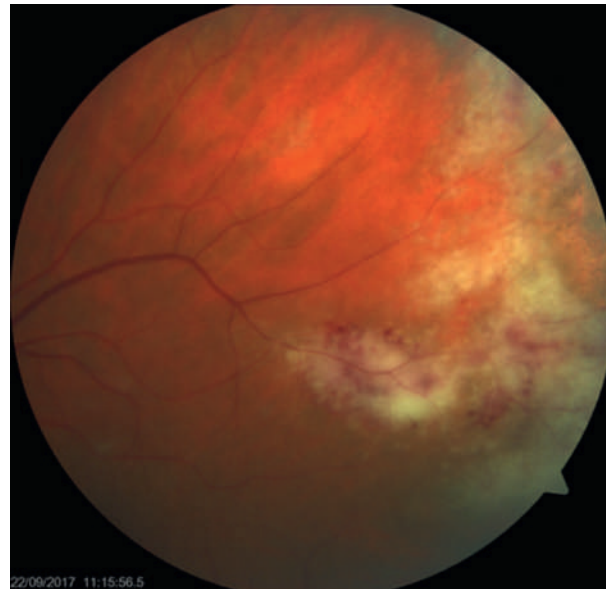


Fig. 9 This photograph illustrates a typical CMV retinitis involving the retinal periphery.

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Fungal Ocular Infections

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A fungal infection of the eye is a significant cause of vision impairment and blindness (Thomas, 2003; Spadea and Giannico, 2019). It affects mainly the cornea but other parts of eye such as the orbit, lids, conjunctiva, sclera and the lacrimal system and internal structures can also be involved (Thomas, 2003; Spadea and Giannico, 2019; Kalkanci and Ozdek, 2011). Fungi infrequently cause infection in intact ocular tissues. Minimal disruption of the corneal epithelium can predispose to fungal keratitis. Numerous fungal species have been associated with ocular infections. Thomas et al. in their review grouped fungal species into hyaline filamentous fungi, dematiaceous fungi, yeasts and zygomycetes, thermally dimorphic fungi and other with uncertain classification (*Pythium insidiosum*, *Rhinosporidium seeberi*, and *Pneumocystis carinii*) (Thomas, 2003).

Hyaline filamentous fungi include *Fusarium*, *Aspergillus*, *Scedosporium*, *Paecilomyces* and *Acremonium* species. *Fusarium* species are a saprobic fungus causing infections in plants and immunocompromised patients. *Aspergillus* species thrive in substrates like corn, decaying vegetation and soil and are commonly found in hospital air (Thomas, 2003). *Scedosporium* species has been found in soil, sewage, polluted water and manure from farm animals. This fungus has been associated with severe ocular infection following trauma by vegetative material, contact with polluted water and immunosuppression. *Paecilomyces* species are found in soil and decaying vegetation. They may also contaminate sterile solutions and culture media. *Acremonium* species is also found in soil, decaying vegetation and the air (Thomas, 2003).

Dematiaceous fungi include species such as *Bipolaris*, *Curvularia*, *Exophiala*, *Exserohilum* and *Lasiodiplodia theobromae*. The dark pigmentation of their hyphae is the common denominator among them. They are a common cause of keratitis after *Fusarium* and *Aspergillus*. *Lasiodiplodia theobromae* is prevalent in tropical regions in corn, yams, citrus and banana plants (Thomas, 2003).

Most yeasts associated with keratitis are from the *Candida* species, especially *Candida albicans*. This occurs in patients with a history of immunosuppression, diabetes mellitus, ocular disease (lid abnormalities or dry eye) or use of topical corticosteroids (Thomas, 2003; Qiao et al., 2020; Klotz et al., 2000). *Cryptococcus* species is associated with immunocompromised patients (Thomas, 2003). Zygomycetes include *Rhizopus* spp., *Mucor* spp. *Rhizomucor* spp. and *Absidia* spp. They can cause rhino-orbitocerebral zygomycosis and keratitis (Thomas, 2003; Klotz et al., 2000; Mukherjee et al., 2016).

Thermally dimorphic fungi include *Paracoccidioides brasiliensis*, *Coccidioides immitis*, *Blastomyces dermatitidis*, and *Histoplasma capsulatum*. These fungi are found in soil and decaying vegetation in endemic sites in United States, Central and South America. They cause severe and chronic disease affecting skin, lungs and lymphoid organs. Ocular manifestations occur after reactivation of the disease and may manifest as eyelid lesions, orbital disease, keratitis and endophthalmitis (Thomas, 2003; Slowik et al., 2015).

Orbital infections

Infections of the orbit usually occur as secondary infections from nearby structures including paranasal sinuses, skin and the nasopharyngeal cavity (Klotz et al., 2000). The inflammatory conditions affecting the eyelids and the orbit are classified into preseptal (periorbital) and postseptal (orbital) (Kalkanci and Ozdek, 2011).

Orbital cellulitis is mainly caused by bacteria. Fungal and viral infections rarely occur. Fungal orbital cellulitis is seen in patients with uncontrolled diabetes mellitus or other immunodeficiencies. Orbital cellulitis is always invasive. Causal organisms include Zygomycetes, *Aspergillus* and less common *Blastomyces*, *Sporothrix* spp. and *Bipolaria* spp. (Kalkanci and Ozdek, 2011; Mukherjee et al., 2016). Early diagnosis is paramount for a timely treatment.

Mucormycosis

The zygomycete, *Rhizopus oryzae* is the major culprit of the following conditions: Rhino-orbital-cerebral zygomycosis (ROCZ), rhino-orbital-cerebral mucormycosis (ROCM), zygomycosis, phycomycosis and orhyphomycosis (Klotz et al., 2000; Mukherjee et al., 2016; Thurtell et al., 2013). Other zygomycetes include *Mucor ramosissimus*, *Rhizomucor pusillus*, *Absidia corymbifera* and *Apophysomyces elegans*. A zygomycetes is a filamentous fungus found in soil, decaying fruit, vegetables and animal feces. ROCZ mainly occurs in immunocompromised patients. Risk factors include patients with uncontrolled diabetes with ketoacidosis, neutropenia, patients with iron overload, intravenous drug abuse, chemotherapy, hematologic malignancy, bone marrow transplant, solid organ transplant and use of corticosteroids and immunosuppressant medications (Mukherjee et al., 2016; Thurtell et al., 2013).

Clinical features include fever, sinusitis, nasal discharge, epistaxis, orbital and periorbital edema and pain, nasal mucosal ulceration, crusting and necrosis (Mukherjee et al., 2016; Yohai et al., 1994). Ocular features include: decreased vision, proptosis, complete external ophthalmoplegia, ptosis, chemosis, internal ophthalmoplegia and corneal anesthesia (Johnson, 2000; Mukherjee et al., 2016) (Fig. 1). The orbital apex syndrome (OAS) encompasses three elements: visual loss due to optic neuropathy, ophthalmoplegia due to third, fourth and sixth cranial neuropathy, and ocular-orbital pain and/or anesthesia due to involvement of the ophthalmic division of the trigeminal nerve (Thurtell et al., 2013). Sudden blindness can occur due to central retinal artery occlusion, thrombosis of the posterior ciliary arteries, infarction of the intraorbital part of optic chiasm (Mukherjee et al., 2016; Downie et al., 1993). ROCZ can also manifest as a painless OAS without orbital cellulitis. The infection can spread to the brain via the cribriform plate and the orbital apex. Invasion of the cavernous sinus and cavernous part of the carotid artery can lead to carotid occlusion, cerebral infarction, mycotic intracranial aneurysm/hemorrhage, meningitis, mycotic abscess and death (Mukherjee et al., 2016; Bae et al., 2012; Thurtell et al., 2013).

The sequence of signs and symptom onset is important in the differential diagnosis of this condition. Generally, OAS in invasive fungal sinusitis presents with constant eye pain, followed by visual loss and ophthalmoplegia. If visual loss and ophthalmoplegia appear simultaneously, it is evident that the patient had OAS and fungal infection should be considered as a cause. If there is only eye pain, followed by visual loss, the differential diagnosis is of an acute painful optic neuropathy. Moreover, if there is eye pain followed by ophthalmoplegia, fungal infection is not one of the top causes; therefore, the diagnosis and adequate treatment are delayed if fungal infection is in fact causal (Thurtell et al., 2013). See a list of the differential diagnosis for painful OAS in Table 1. Patients with eye or facial pain are sometimes empirically treated with steroids for presumed orbital inflammatory disease or giant cell arteritis. As steroids may contribute to a devastating outcome in a patient with invasive fungal infection, a fungal infection should be suspected in a diabetic or immunocompromised patient with a painful eye even without ophthalmoplegia or visual loss (Thurtell et al., 2013).

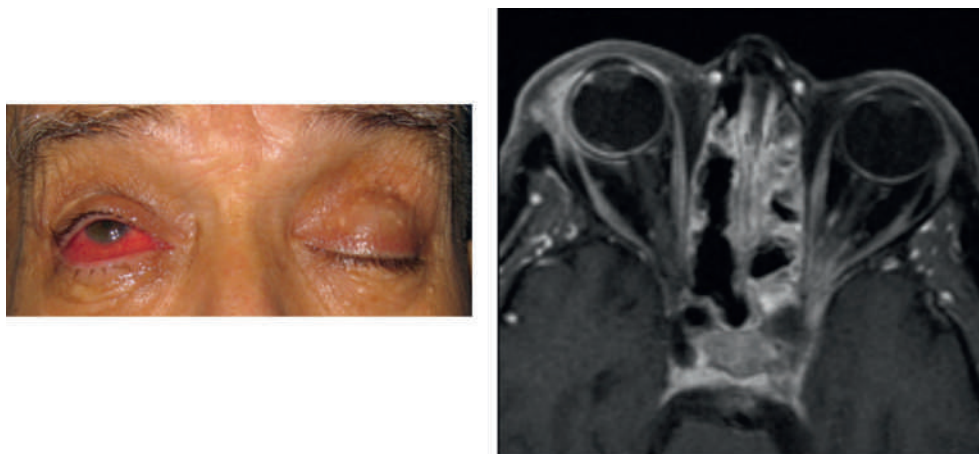


Fig. 1 Orbital Apex Syndrome: severe chemosis (left image) and magnetic resonance imaging (right).

Table 1 Causes of orbital apex syndrome.

Type of condition	Name of condition
Inflammatory	– Sarcoidosis – Vasculitis: Churg-Strauss syndrome, Wegener's vasculitis – Giant cell arteritis – Thyroid Eye Disease – Idiopathic inflammation – Idiopathic orbital inflammation – Tolosa-Hunt syndrome – Systemic lupus erythematosus – Orbital inflammatory pseudotumor
Infectious	– Fungi: <i>Aspergillus</i> spp., <i>Rhizopus oryzae</i> – Bacteria: <i>Mycobacterium tuberculosis</i>, <i>Streptococcus</i> spp., <i>Staphylococcus</i> spp., <i>Actinomyces</i> spp., Gram-negative bacilli, anaerobes – Herpes zoster ophthalmicus
Vascular	– Carotid cavernous fistula – Carotid cavernous aneurysm – Cavernous sinus thrombosis – Sickle cell anemia
Neoplastic	– Head and neck tumors: nasopharyngeal carcinoma, adenoid cystic carcinoma, squamous cell carcinoma – Neural tumors: neurofibroma, meningioma, ciliary neurinoma, schwannoma – Metastatic lesions: lung, breast, renal cell, malignant melanoma – Hematologic: Burkitt lymphoma, non-Hodgkin lymphoma, leukemia – Perineural invasion of cutaneous malignancy
Other	– Mucocoele – Trauma: Penetrating and Nonpenetrating injury, orbital apex fracture, retained foreign body – Iatrogenic: sinonasal surgery, orbital/facial surgery

Adapted from Thurtell MJ, Chiu AL, Goolid LA et al. (2013) Neuro-ophthalmology of invasive fungal sinusitis: 14 consecutive patients and a review of the literature. *Clinical & Experimental Ophthalmology* 41(6): 567–576; Yeh S and Foroozan R (2004) Orbital apex syndrome. *Current Opinion in Ophthalmology* 15(6): 490–498.

Nasal and oral mucosa must be thoroughly examined, with samples and biopsies sent for microbiology examination and culture. Calcofluor white, Gomori's methenamine silver (GMS), hematoxylin and eosin (H and E), and periodic acid–Schiff stains are used for a prompt identification of the fungus. Sabouraud's agar without cycloheximide is used for culture. Polymerase chain reaction (PCR) test for fungal DNA can also be used for identifying the pathogen in the sample. The PCR sensitivity and specificity for zygomycetes is 100% and 92%, respectively. PCR's advantages include a fast result - within 2–4 h, compared to histopathology examination which can take up to 5 days (Hata et al., 2008).

Treatment for ROCZ must be commenced immediately without waiting for tissue confirmation of the diagnosis as there is at least 50% mortality. Consultation with an infectious disease specialist is mandatory. Management of predisposing factors, early surgical debridement, sinus drainage and antifungal agents are required. Amphotericin B is the preferred medication. The dosage is slowly increase from 0.7 to 1 mg/kg intravenous to a cumulative dose of 2–4 g over weeks to months after giving an initial test of 1 mg. Common side effects include fever, headache, myalgia, anorexia, malaise, anemia, hypokalemia, vomiting and nephrotoxicity (Mukherjee et al., 2016). Ketoconazole, clotrimazole, fluconazole, voriconazole, posaconazole and caspofungin are also alternatives to amphotericin B (Mukherjee et al., 2016). Amphotericin B in combination with flucytosine, fluconazole, itraconazole, or micafungin has been also used. Posaconazole alone was reported to be effective for *Rhizopus oryzae* (Kok et al., 2007).

Extensive surgical excision and debridement of necrotic orbital tissues and sinuses are important to decrease the fungal load. Debridement of the tissue until bleeding occurs is a good indicator of the extent of surgical resection needed. Repeated procedures may be needed. Exenteration may be life-saving and should be performed when there is an inflamed orbit with a blind eye or advanced infection of the orbit (Kalkanci and Ozdek, 2011; Mukherjee et al., 2016; Thurtell et al., 2013).

Aspergillosis

Aspergillosis is a rare infection of the orbit which affects both immunocompetent and immunosuppressed patients. *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger* are the main causal organisms (Klotz et al., 2000). The main route of infection is from contiguous spread from sinuses. *Aspergillus fumigatus* commonly affects immunocompromised patients while *Aspergillus flavus* to immunocompetent patients (Mukherjee et al., 2016).

Predisposing factors include neutropenia (count $<1000/\text{mm}^3$), hematologic malignancy, use of corticosteroids and other immunosuppressive medications, solid organ transplantation, diabetes mellitus, prosthetic devices, trauma, environmental exposure, endemic area (Sudan), and advanced age (Mukherjee et al., 2016; Boes et al., 1994; Brown et al., 1994). Other factors include patients with burns and with occlusive dressings due to the warm and humid environment they create and loss of tissue integrity (Mukherjee et al., 2016; Warwar and Bullock, 1998).

Non-invasive aspergillosis occurs in immunocompetent patients who may harbor fungal balls or present with allergic sinusitis with the signs and symptoms of allergic rhinitis, chronic sinusitis and nasal obstruction. Orbital involvement is unusual. Invasive aspergillosis occurs in immunocompromised patients and severe neutropenia. Clinical features vary leading to misdiagnosis. Initial diagnoses include bacterial orbital cellulitis, idiopathic orbital inflammatory disease, temporal arteritis and sino-orbital malignancies. Fungal etiology must be ruled out before initiating corticosteroid therapy in a patient with painful proptosis. In immunocompromised patients, it manifests with acute proptosis with severe pain and visual loss with minimal orbital inflammation. In patients with AIDS, it presents with headache, periorbital pain, gradual proptosis and limitation of ocular movement without loss of vision over a period of weeks to months (Mukherjee et al., 2016).

Magnetic resonance imaging (MRI) provides better images of the posterior orbit, optic nerve and cavernous sinus and detects enhancement of optic nerve and cerebral dura adjacent to involved paranasal sinuses. In addition, MRI with contrast and fat suppression shows soft tissue abnormalities at the orbital apex and skull base by the time the OAS is complete and sometimes before that. These soft tissues changes show a markedly decreased signal of T2-weighted imaging. Orbital and perimaxillary fat stranding can be an early sign that the inflammatory process has spread outside the paranasal sinuses. Loss of mucosal contrast enhancement on MRI is a specific sign for invasive fungal syndrome indicating mucosal infarction (Thurtell et al., 2013) (Fig. 1). A normal brain computerized tomography (CT) cannot exclude an invasive fungal syndrome as asymmetric sinus mucosal thickening is common. Bony erosion can be seen in a paranasal sinus or orbit CT; however, it could be a sign in either an invasive fungal syndrome or in non-invasive fungal syndrome with paranasal sinus mucocoele. Evident sinusitis on CT, particularly with bony erosion, requires a nasendoscopy urgently. The causes are mostly bacterial or allergic in origin (Thurtell et al., 2013).

If there is evidence of partial OAS with extra-sinus disease on CT or on MRI, an urgent consultation with otorhinolaryngology for a biopsy of the paranasal sinuses must be sought, even if the tissues look clinically normal (Thurtell et al., 2013). Multiple biopsies must be taken from different sinuses, as fungal invasion can be patchy and in case the initial biopsy is negative. If the sinus biopsy is negative, an orbital apex biopsy must be done. This biopsy should be directed toward areas of imaging change for example contrast enhancement or fat stranding using an anterior approach (medial orbital apex biopsy via medial lid crease incision and lateral orbital apex biopsy via lateral canthotomy) or via the sinuses if the imaging change is medial (Thurtell et al., 2013). Samples should be sent for direct microscopic examination under KOH and calcofluor white; and the culture should be done in Sabouraud dextrose agar (Mukherjee et al., 2016; Klotz et al., 2000).

Fungal culture and histopathological examination of the samples is essential for diagnosis and therapy. There are no standardized guidelines; however, intravenous amphotericin B or voriconazole is preferred, followed by oral azoles. Oral itraconazole/voriconazole can be commenced after the completion of intravenous amphotericin B or as alternatives when the patient does not tolerate amphotericin B. In fact, some reports suggest that voriconazole may contribute to better outcomes at an intravenous dose of 6 mg/kg twice daily from day 1, followed by 4 mg/kg for 1 week and then oral voriconazole 200 mg twice daily for 12 weeks (Herbrecht et al., 2002; Mukherjee et al., 2016; Thurtell et al., 2013).

There is no consensus regarding surgical approach. Some authors recommend exenteration in all patients with retrobulbar or apical involvement; while others recommend less aggressive options. Debridement helps in reducing the fungal load and the penetration of the medication; but not all patients need this procedure. In some cases, prolonged systemic therapy has cured the infection without recurrence (Mukherjee et al., 2016). In immunosuppressed patients, the first step is to reverse immunosuppression and the prognosis is poor if this cannot be done. Exenteration is usually needed in severe cases (Thurtell et al., 2013). Local irrigation of amphotericin B with indwelling catheters is recommended to improve survival (Mukherjee et al., 2016).

Blepharitis

Bacteria are the common culprit of blepharitis; however, fungi infrequently can infect the superficial and deeper layers of the eyelid. *Candida* species infection presents in patients treated with broad-spectrum antibiotics or immunosuppressive medications. It manifests differently depending on the fungal species (Słowik et al., 2015). For example, *Cryptococcus* species cause ulcerative lesions while productive lesions are seen in blastomycosis (Słowik et al., 2015; Klotz et al., 2000). A nodule with a central ulcer coexisting with conjunctivitis have been reported in half of cases of paracoccidioidomycosis. This infection mainly affects agricultural male workers over 30 years of age in endemic areas. These lesions required antifungal therapy and surgical removal of the fibrotic lesions (Słowik et al., 2015).

Tear ducts

Fungal infection of the tear ducts is unusual and often accompanied by conjunctivitis. A major predisposing factor is the use of topical antibiotics or corticosteroids. Poor response to antibiotics or corticosteroids may indicate a fungal cause. About 5% of cases of lacrimal sac infection are caused by fungus. The most common fungi include *Aspergillus*, *Candida* and *Rhizopus* species. This infection is generally chronic with epiphora as a major symptom accompanied by conjunctivitis and edema of the medial canthus. Purulent discharge can appear from the lacrimal puncta of the lower eyelid when pressure is applied (Słowik et al., 2015).

Treatment includes topical natamycin, rinsing of the tear ducts and lacrimal sac with amphotericin or nystatin. If there is no response to the initial therapy, an incision of the lacrimal ducts can be performed. Silicone tube are then used to reconstruct the lacrimal ducts. Sometimes surgical procedures such as removal of the lacrimal sac or anastomosis of the lacrimal sac with the mucosa of the nasal cavity are required (Słowik et al., 2015).

Conjunctivitis

Fungal conjunctivitis is uncommon outside India. It is endemic in countries such as India, Sri Lanka and the Balkans caused by *Rhinosporidium* species. Closed reservoirs of water are a major risk factor. Fungal conjunctivitis is typically secondary to infection in the cornea, lacrimal sac and tear.

Causal organisms include *Candida*, *Aspergillus*, *Sporotrichum*, *Blastomyces*, *Coccidioides* and *Malassezia* species and dermatophytes (Słowik et al., 2015; Spadea and Giannico, 2019). The infection manifests with acute inflammation of the conjunctiva with a mucopurulent discharge (Spadea and Giannico, 2019). The lesions produce a grayish deposit on the conjunctiva with further formation of eyelid-eyeball adhesions from *Candida* infection or papular changes of a white-yellow color for *Sporotrichum* species. *Rhinosporidium* species cause pink or red inflammatory changes in the palpebral conjunctiva. Fungal conjunctivitis can cause severe complications needing intensive antifungal medications, even surgical debridement for example for *Rhinosporidium* infection (Słowik et al., 2015).

Keratitis

Fungal keratitis is a sight-threatening condition and one of the leading causes of blindness in Asia (Kalkanci and Ozdek, 2011; Watson et al., 2020; Bhartiya et al., 2007). It represents between 6% and 53% of all cases of infectious keratitis depending on the country (Klotz et al., 2000; Thomas, 2003; Kumar et al., 2010; Maharana et al., 2016; Bhartiya et al., 2007). Predisposing factors, causal organisms and outcomes vary depending on the geographic location, weather, local environment, occupation, availability of antimicrobials and gross national income (Watson et al., 2020; Kredics et al., 2015; Ali Shah et al., 2017; Garg et al., 2016). Filamentous fungi are more prevalent in developing countries with tropical climates and yeasts in developed countries with temperate climates (Qiao et al., 2020; Kumar et al., 2010). A study in India reported that 90% of fungal keratitis cases were caused by trauma while only 11–44% of fungal keratitis in USA were trauma related. In general, most the fungal keratitis cases are caused by filamentous saprophytic fungi (Klotz et al., 2000).

Predisposing factors associated with fungal keratitis include trauma with organic or vegetable matter or objects contaminated with soil, contact lens wear, history of systemic conditions, ocular surface disease and topical corticosteroid use (Kalkanci and Ozdek, 2011; Klotz et al., 2000; Kumar et al., 2010; Maharana et al., 2016; Qiao et al., 2020). Corneal trauma with vegetative matter either introduces the fungus directly into a corneal epithelial defect or the defect may become infected during the trauma. It has been associated more with agricultural or outdoor occupations (Klotz et al., 2000; Thomas, 2003).

Contact lens wear is mainly associated with *Pseudomonas aeruginosa*-related bacterial keratitis; but *Candida albicans* can also adhere to contact lenses. These organisms secrete exopolymers almost impenetrable to antibiotics. In addition, contact lens wear causes relative hypoxia of the corneal epithelium leading to changes in the cell surface glycoproteins. Microscopic defects are introduced by the contact lens wear enhancing organism adherence to the otherwise non-adherent epithelium. Fungi and bacteria adherent to contact lenses come from poor contact lens handling including during cleaning and storage of the lenses (Klotz et al., 2000).

Filamentary fungi are more associated with corneal trauma; while yeasts are related to patients with abnormal or compromised cornea, or systemic immunodeficiency for example in dry eye disease, chronic ulceration, erythema multiforme and Human Immunodeficiency Virus (Thomas, 2003; Klotz et al., 2000; Qiao et al., 2020; Galarreta et al., 2007; Ong et al., 2016). The most common filamentary fungi include *Fusarium* species, *Aspergillus* species and *Curvularia* species and the most common yeasts, *Candida albicans* and *Candida parapsilosis* (Qiao et al., 2020; Thomas, 2003; Watson et al., 2020; Maharana et al., 2016; Garg et al., 2016).

Clinical presentation

Clinical findings include coarse granular infiltration of the corneal epithelium and anterior stroma. The corneal defect appears 24–36 h after the trauma with minimal or absent host cellular infiltration. The infiltrate is often surrounded by a ring which may represent the junction of fungal hyphae and host antibodies (Klotz et al., 2000). The most common findings of culture-positive fungal keratitis include a gray or dirty-white surface, anterior chamber cellular reaction, irregular feathery margins, elevated borders, dry rough texture, satellite lesions, Descemet's folds, hypopyon, ring infiltrate, endothelial plaque, and keratic precipitates (Thomas, 2003; Maharana et al., 2016).

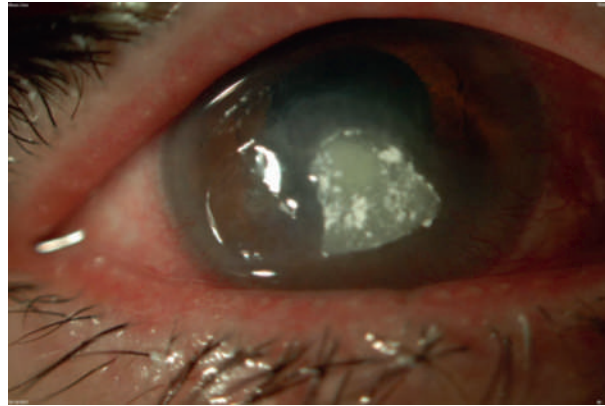


Fig. 2 A case of *Scedosporium prolificans* (filamentous fungal) keratitis.

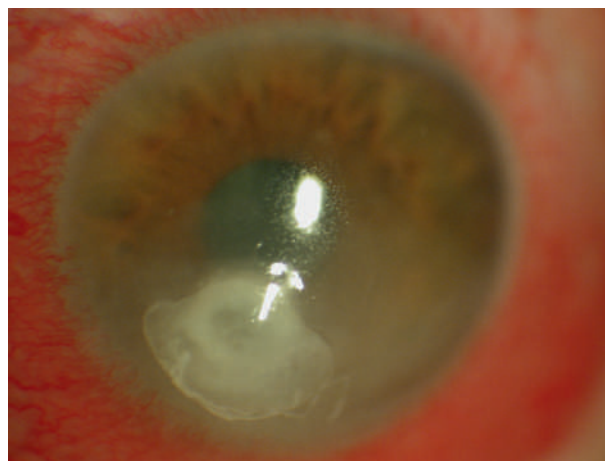


Fig. 3 A case of candida keratitis in a patient on high dose oral prednisone.

In summary, common clinical features of filamentous fungi include dry elevated slough, stromal infiltrate with hyphate margins, satellite lesions and thick endothelial exudate (Fig. 2); and for yeast fungi, stromal keratitis similar to bacterial keratitis, overlying epithelial defect, discrete infiltrate, slow progression, and inferocentrally location (Thomas et al., 2012) (Fig. 3).

Diagnostic methods

Smear and culture of corneal scrapings are the preferred diagnostic methods for diagnosis of fungal keratitis (Klotz et al., 2000; Chidambaram et al., 2017; Kumar et al., 2010). A prompt suggestive diagnosis of fungal keratitis can be made if fungal hyphae or yeast cells are seen in a stained smear by direct microscopic examination (Thomas et al., 2012). About 65–75% of Gram and Giemsa stains are positive to fungal hyphae in cases of fungal keratitis (Labbé et al., 2009). Corneal scrapings should be inoculated onto plates of culture media such as blood agar and Sabourauds. Following inoculation, fungal culture media should be maintained at 22–25 degrees in a cooling incubator if available. A diagnosis of fungal keratitis can be confirmed following isolation of a fungus which takes a minimum of 48–72 h. Cultures have limited sensitivity, even lower than 25%, as it can take days or weeks to grow any organism (Kumar et al., 2010). Sometimes corneal biopsies may be needed to isolate the causing organism as filamentous fungus grow slowly in the culture (Robaei et al., 2018). Therefore, a diagnosis of fungal keratitis may be delayed after aggressive bacterial and viral keratitis failed treatments leading to poor outcomes (Klotz et al., 2000; Kalkanci and Ozdek, 2011; Brasnu et al., 2007; Thomas et al., 2012).

In vivo confocal microscopy (IVCM) is a non-invasive imaging modality to assist in diagnosing fungal keratitis. It provides high contrast in vivo images of the cornea, from the epithelium to endothelium, nerves and cells for an immediate visualization of filamentous fungi (Chidambaram et al., 2017; Brasnu et al., 2007). A third type of IVCM, the laser scanning confocal microscope Heidelberg Retinal Tomograph (HRT3) uses 670 nm red wavelength and high-resolution images, close to 1 μ m improving the sensitivity of this technique allowing the identification of yeasts, which could not be reported previously with first generation confocal microscopes (Brasnu et al., 2007; Kumar et al., 2010; Maharana et al., 2016). Sensitivity of this imaging technique for

fungal keratitis has been reported at between 80% and 94%, and specificity between 78% and 91.1% (Maharana et al., 2016; Kumar et al., 2010; Thomas et al., 2012; Kanavi et al., 2007; Labbé et al., 2009). Advantages of this imaging technique include being non-invasive, early identification of the organism, monitoring and guidance of treatment and determination of depth of infection. Limitations include the need for an experienced operator, patient collaboration, presence of motion artifacts, dense corneal infiltrates or scarring which can affect proper tissue penetration and visualization (Maharana et al., 2016; Kumar et al., 2010).

The polymerase chain reaction (PCR) test involves the enzymatic amplification of a specific sequence of DNA (Thomas, 2003). Several investigators have reported the sensitivity of the PCR ranging from 75% to 100%, and specificity from 50% to 100% for the diagnosis of fungal keratitis in relation to culture (Maharana et al., 2016; Gaudio et al., 2002; Menassa et al., 2010; Ferrer and Alió, 2011; Ghosh et al., 2007; Stone and Tan, 2014; Kim et al., 2008). It has the highest positive detection rate compared with cases with culture or smear negative results. Its advantages include that only a small sample is needed for diagnosis and the provision of prompt results - within 4–8 h, compared with cultures for which results are available between 2 and 7 days. Major disadvantages are its cost and limited availability. In some countries, it has been validated for corneal scrape samples. Its use as the only diagnostic modality in fungal keratitis is not advisable. Nevertheless, it can be used to guide early antifungal therapy while awaiting the other diagnostic test results (Maharana et al., 2016).

Management

Fungal keratitis is managed with medical therapy or surgical interventions. Medical therapy includes antifungal agents, cycloplegics to relieve anterior uveitis, and antibiotics for secondary bacterial infection (Thomas, 2003). The prognosis of fungal keratitis is generally poor due to the nonavailability of antifungal agents with good ocular penetration (Sharma et al., 2019) and the difficulty in diagnosing and choosing the adequate initial therapy (Watson et al., 2020). In fact, there is a lack of consensus for an initial therapy among specialists worldwide leading to diverse antifungal therapies (Sharma et al., 2019).

The selection of antifungal agents may depend on discussions with infectious diseases specialists, availability of antifungals or clinician's preference of medications in alignment with a Cochrane Review (FlorCruz and Evans, 2015). Topical natamycin 5% is the preferred empiric agent as it is commercially available and has been associated with better outcomes in filamentary keratitis (Watson et al., 2020; Prajna et al., 2013; Bhartiya et al., 2007; Thomas, 2003). Topical voriconazole and amphotericin B 0.15% are alternatives. Oral agents such as voriconazole, ketoconazole, itraconazole or oral fluconazole may be added (Watson et al., 2020; Thomas, 2003; Bhartiya et al., 2007); although MUTT trial 2 concluded that oral therapy was not useful (Prajna et al., 2016). Severe cases may need intracameral or intrastromal antifungals (Sharma et al., 2019).

Sharma et al. proposed and evaluated the Topical, Systemic, and Targeted Therapy (TST) protocol for the management of fungal keratitis in India, illustrated in Fig. 4 (Sharma et al., 2019). This algorithm had been used for 4 years by the time of the study.

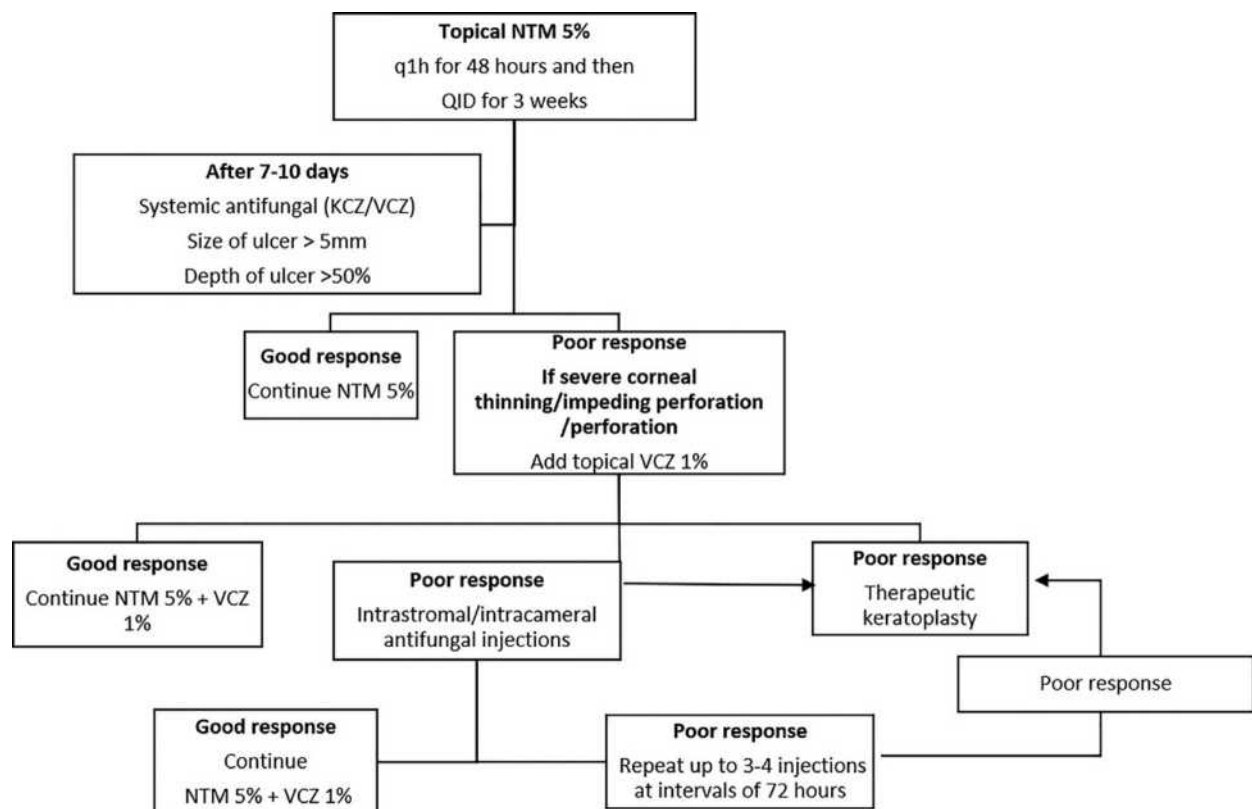


Fig. 4 Topical, systemic and targeted therapy protocol for fungal keratitis treatment. Key: KCZ = ketoconazole, NTM = natamycin, QID = every 4 h, q1h: every hour, VCZ = voriconazole. Adapted from Sharma N, Sahay P, Maharana PK, et al. (2019) Management algorithm for fungal keratitis: The TST (Topical, Systemic, and Targeted Therapy) protocol. *Cornea* 38(2) 141–145.

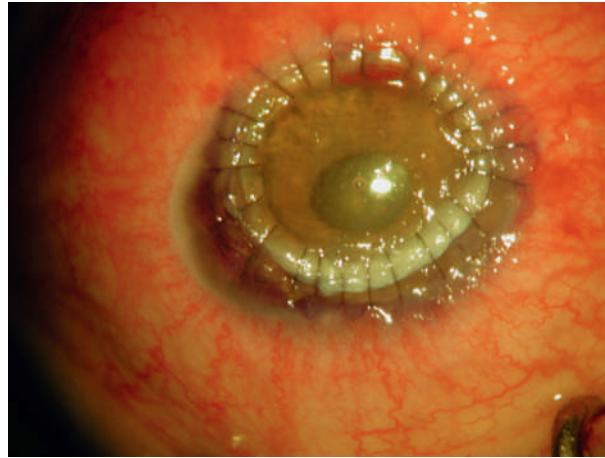


Fig. 5 A large corneal grafting being performed for progressive candida keratitis that had failed medical therapy.

All cases were commenced with topical natamycin 5%, every hour for first 48 h, then every 2 h during waking hours until the epithelial defect healed and then every 4 h for 3 weeks; plus, a cycloplegic, homatropine 2%, four times daily. In case of poor response, topical voriconazole 1% was added every hour for first 48 h and then every 2 h until healed; or penetrating keratoplasty (PK) performed if there was severe corneal thinning or perforation. Systemic therapy with voriconazole or ketoconazole was recommended if the ulcer measured over 5 mm or its depth was over 50%. In case of poor response to natamycin 5% plus topical voriconazole 1%, intracameral or intrastromal antifungals were given, or a PK performed (Fig. 5). If there is a poor response after the injected antifungals, they were repeated up to 3–4 times every 72 h. If the infection worsened, a PK was performed (Sharma et al., 2019). This protocol had a success rate of 79.8% similar to other clinical trials with rates ranging from 66.7% to 73.7%. There was a low proportion of corneal perforation (6.7%) and need for PK (20.2%) (Sharma et al., 2019; Parchand et al., 2012).

Uveitis

Presumed ocular histoplasmosis syndrome

Histoplasma capsulatum infection occurs when the yeast form of this fungus is inhaled producing the pulmonary infection. This infection affects immunocompetent patients with 2000 new cases annually leading in some cases to vision impairment and blindness (Klotz et al., 2000). This infection is endemic in the Ohio and Mississippi river valleys in USA (Bowling, 2016).

More than half of patients (60%) present a bilateral disease. This syndrome is commonly asymptomatic unless macular choroidal neovascularisation (CNV) appears. The classic triad includes multiple white atrophic chorioretinal 'histo' spots about 200 µm in diameter, peripapillary atrophy without vitritis. Choroidal neovascularisation is a late manifestation present in less than 5% of patients. It is associated with a pre-existing macular histo spot. Associated subretinal fluid and hemorrhage lead to a decrease in vision. Acute chorioretinitis is mostly asymptomatic. Discrete oval-round whitish lesions <400 µm in diameter may have developed into classic punched-out histo spots have been described. Some lesions burn out and other can reactivate (Bowling, 2016; Klotz et al., 2000).

Active endophthalmitis in patients with spread of histoplasmosis secondary to AIDS or immunosuppression is associated with abundant budding yeast cells in the choroidal tissue and endothelium. Sometimes yeast cells are found in the anterior chamber structures such as iris, ciliary muscle and canal of Schlemm (Klotz et al., 2000).

This syndrome is associated with HLA-B7 and DR w2. Serological testing would be helpful but is only positive when systemic mycosis is present. Fluorescein angiography (FA) and optical coherence tomography (OCT) when CNV is suspected (Bowling, 2016).

Spontaneous regression of CNV may occur but without treatment 60% of eye with CNV would have a final visual acuity of less than 6/60. Treatment includes intravitreal anti-vascular endothelial growth factor (VEGF) injection for CNV (Bowling, 2016).

Endophthalmitis

Endogenous endophthalmitis refers to the infection of the interior of eye resulting from hematogenous spread of the initial infection from a distant site. Exogenous endophthalmitis occurs when the pathogen enters the eye through an ocular trauma or surgery (Kalkanci and Ozdek, 2011; Klotz et al., 2000). Most cases of endophthalmitis are exogenous. The incidence of endophthalmitis ranges from 0.03% to 1.3% after cataract surgery to up to 40% following open globe trauma. Fungal endophthalmitis accounts for 8.6–18.6% of culture-positive cases (Spadea and Giannico, 2019). Endogenous endophthalmitis mainly occurs in

immunocompromised patients (Klotz et al., 2000). Previous studies have reported that yeasts, *Candida* species and *Cryptococcus neoformans*, are the most common causal organisms, followed by molds, *Aspergillus* and *Fusarium* species (Sridhar et al., 2013; Essman et al., 1997; Binder et al., 2003).

Endogenous endophthalmitis

Candida species

Candida albicans is the most common causal organism of fungal endophthalmitis (Spadea and Giannico, 2019; Durand, 2017). It has been associated with immunocompromised patients treated for sepsis with broad spectrum antibiotics or parenteral nutrition, post-organ transplantation (Spadea and Giannico, 2019), neutropenia, intravenous drug use, a septic focus associated with a catheter, chronic lung disease or diabetes mellitus (Bowling, 2016; Klotz et al., 2000). A third of patients with systemic candidiasis develop endophthalmitis (Bowling, 2016; Lin, 2015). The initial manifestation is most commonly a unifocal or pauci-focal choroiditis, seen clinically as fluffy white choroidal lesions usually in the posterior pole (Fig. 6). The patient is asymptomatic at this stage. Symptoms will appear when the infection progresses to involve the retina and vitreous (Durand, 2017). Peripheral fundus lesions are uncommon and may cause little disturbance, in contrast central lesions or severe vitritis will manifest earlier (Bowling, 2016). Occasionally patients will present with a unifocal retinitis rather than choroidal lesions (Fig. 6). As ocular candidiasis may be asymptomatic until late in the infection and patients with candidemia are severely ill, an emergent fundus examination needs to be performed on any patient with candidemia (Durand, 2017). Vitritis presents with a creamy white, well-circumscribed cottonlike lesion involving the retina and choroid and extending into the vitreous cavity. Vitreous involvement is often a series of white fluffy lesions, the so called “string of pearls” sign (Fig. 6) (Bowling, 2016; Kalkanci and Ozdek, 2011; Lin, 2015).

Aspergillus species

Aspergillus species are the second most common cause of fungal endophthalmitis. They have been associated with immunocompromised patients, for example malignancy, diabetes mellitus, use of immunosuppressive medications, solid-organ transplantation, HIV, neutropenia, use of oral corticosteroids, central venous catheters, and intravenous drug use (Kalkanci and Ozdek, 2011; Spadea and Giannico, 2019). Clinical manifestations include eye pain, burning, foreign body sensation, tearing, hyperemia and blurred vision. On the slit lamp examination, thickened eyelid margins, conjunctival injection, grayish-white corneal stromal infiltration with fluffy margin located centrally, corneal ulcer with hypopyon, corneal satellite lesions, flare and anterior chamber cells can be found (Spadea and Giannico, 2019) (Fig. 7).

When the infection reaches the posterior segment, vitreous haze, vitritis or vitreous abscess may cause severe proliferative vitreoretinopathy, subretinal exudates and retinal detachment. Extensive fluffy white preretinal lesions with creamy white deep retinal and choroidal lesions and intraretinal hemorrhages, large wedge-shaped quadrants of pigmented chorioretinal atrophy and scarring occur as the infection is controlled (Spadea and Giannico, 2019) (Fig. 8).

Exogenous endophthalmitis

The source of this type of infection is the ocular surface or the environment. Ocular surface includes post-surgery, post-injection, keratitis-related, bleb-related or device-related), environment means post-trauma. Cataract surgery and corneal transplantation are the surgical procedures most associated with this infection. It may have a period of latency of weeks to months before it manifests. Even the infection is confined to the anterior chamber. The most common fungi associated with post-surgical procedures include

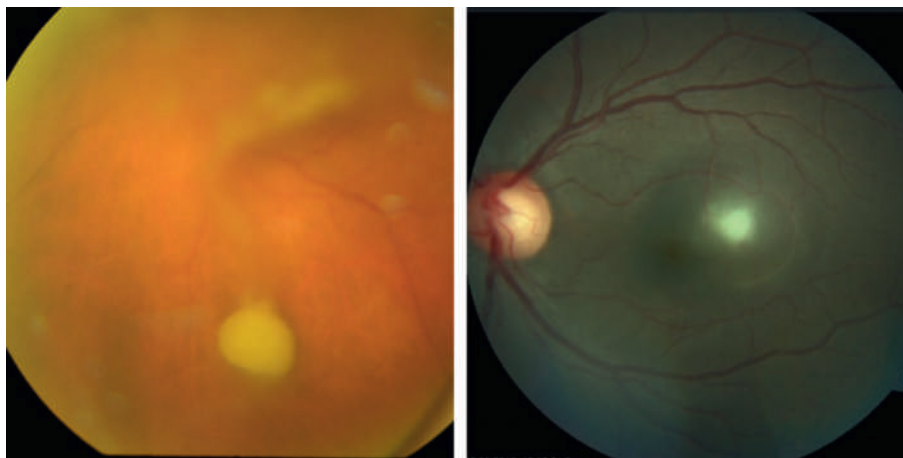


Fig. 6 Retina & vitreous “string of pearls” (left image), single retinal focus (right image).

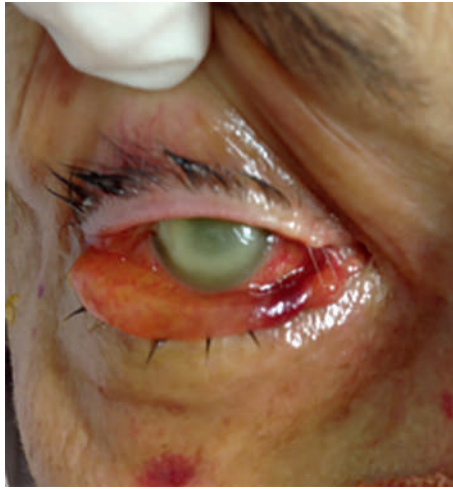


Fig. 7 Clinical examination of a patient with *Aspergillus* endophthalmitis.

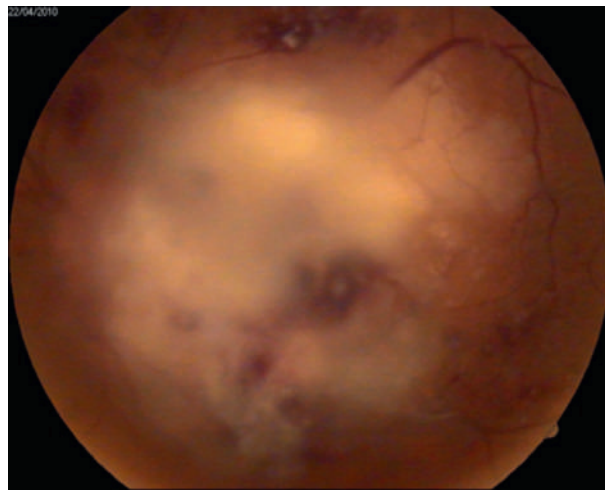


Fig. 8 Extensive fluffy white preretinal lesions with creamy white deep retinal and choroidal lesions and intraretinal hemorrhages in *Aspergillus* endophthalmitis.

yeast, especially *Candida glabrata* and *Candida formata*; while *Fusarium* species were found in post-trauma and post-keratitis cases and *Paecilomyces lilacinus* in post-surgery or contaminated sterilization solutions. Zygomycosis in the surrounding soft tissue and cryptococcal neuroretinitis may also lead to exogenous endophthalmitis (Klotz et al., 2000).

Diagnostic tests

Culture of the vitreous and/or aqueous and blood cultures assist with the diagnosis of endophthalmitis (Durand, 2017; Lin, 2015). Vitreous culture shows a high proportion of positive results (70–80%); however, molecular techniques may be used to identify the pathogen for negative cultures (Durand, 2017; Spadea and Giannico, 2019). Small samples of aqueous (0.1 ml) or vitreous (0.2–0.3 ml) may be taken for culture by needle aspiration. Some vitreous samples are ‘dry taps’ due to difficulty in aspirating the gel. In this case, some of the vitreous maybe removed via a vitrectomy in the operation room. Culture positivity rate of vitrectomy specimens is 90%, 50–70% for vitreous aspirates and 40% for aqueous aspirates (Durand, 2017).

Fluorescein angiography shows hyperfluorescence of the preretinal granuloma and the surrounding deep retinal lesions, and staining of the chorioretinal scars, focal vasculitis and leakage from the involved vessels (Spadea and Giannico, 2019).

Ultrasound examination is mandatory when fundus is not accessible for examination. Ultrasounds show dense opacities in the vitreous chamber for vitritis, thickening of the retinochoroidal layer due to subretinal exudative lesions, pre- and intraretinal hemorrhages, preretinal layering of exudates that lead to epiretinal membranes and retinal detachment (Spadea and Giannico, 2019).

Spectral domain optical coherence tomography (SD-OCT) has become a useful diagnostic method for retinal and choroidal disease. This method allows the visualization of infected structures and the detection of changes in response to treatment. Some

studies have reported different tomographic features for *Candida* species (Cho et al., 2011; Lavine and Mititelu, 2015; Adam and Rahimy, 2015) and for *Aspergillus* species (Adam and Sigler, 2014; Invernizzi et al., 2018). Invernizzi et al. identified two major patterns of chorioretinal changes, chorioretinal and intraretinal, on the OCT of their patients in a case series. Chorioretinal pattern consists of choriocapillaris alterations overlaid by retinal pigmented epithelium (RPE) disruption, full thickness retinal lesions extending from the subretinal region up to the preretinal space. A saw-tooth-shaped hyperreflective change was seen in the subretinal space adjacent to the penetrating lesion in the patients with full thickness involvement. The chorioretinal pattern illustrates the spread pathway of fungi from the choriocapillaris growing centripetally in the eyeball toward the vitreous chamber, for example in cases of *Candida* (Invernizzi et al., 2018).

The intraretinal pattern involves only the retina and the preretinal space. Invernizzi et al. reported that one third of patients in their case series showed an intraretinal pattern sparing retinal structures beyond the inner nuclear layer, the subretinal space and the choriocapillaris. In these cases, the organisms probably had entered the eye through the proper retinal vasculature contained into the inner nuclear layer, spread into the retina, the preretinal space and the vitreous chamber (Invernizzi et al., 2018). The *Candida* preference for the vitreous chamber rather than spreading within the retina or in the subretinal space is the reason for a better outcome of patients with endogenous *Candida* endophthalmitis compared to other patients with endophthalmitis caused by other fungi (Invernizzi et al., 2018).

Treatment

Patients should be co-managed by uveitis specialists, medical team and infectious disease specialists. If there is evidence of vitreous involvement or the internal limiting membrane (ILM) breached, vitreo-retina team should also be involved.

Early vitrectomy is required for lesions that have breached the ILM with vitreous seeding. Vitrectomy provides significant culture specimen, reduces fungal and antigen load, facilitates therapeutic agent penetration and clears the ocular media (Bowling, 2016; Lin, 2015). Intravitreal voriconazole may be given beforehand for example in patients too unwell for a vitrectomy or if the vitrectomy is delayed in cases of fungal lesions close to the fovea. Tap-and-inject could be considered in selected cases.

Voriconazole has good penetration when used systemically and is safer than amphotericin B as an intravitreal treatment. Intravitreal dose of voriconazole up (0.05 mg in 0.1 mL) is considered safe for the retina (Spadea and Giannico, 2019). If the diagnosis is still unclear, antibiotics may be also injected. Further intravitreal injection should be given in theaters as patients find them very distressing.

Blood tests including full blood count (FBC), electrolytes, urea, creatinine (EUC), liver function tests (LFT), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), Treponema serology, HIV, three sets of blood tests should be ordered. Calcium and magnesium should be also ordered if the patient was prescribed systemic voriconazole and/or moxifloxacin due to possible prolongation of QT interval. HIV, hepatitis V virus (HCV), HBsAg, HBcAb tests should be ordered to patients who inject drugs and be immunized against hepatitis B virus (HBV). If a non-*Candida* fungal endophthalmitis is suspected, a serum cryptococcal antigen should be tested for. Other tests to request include urine culture, cardiac investigations such as electrocardiogram to assess conduction abnormalities and transoesophageal echocardiogram to check for colonization of valves, MRI brain, CT chest, abdomen and pelvis, in some cases).

Topical therapy includes a steroid every 2 h and atropine. Systemic treatment includes fluconazole or voriconazole as they are preferred due to the toxicity of amphotericin B (Durand, 2017). The usual starting dose for oral voriconazole is 400 mg, twice daily for the first two doses followed by 200 mg, twice daily thereafter. Monitoring levels of voriconazole is needed once the patient is discharged. If no fungus is found elsewhere in the body, oral voriconazole is given for 6 weeks.

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Parasitic Eye Infections

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Introduction

A parasite is an organism that lives on or lives in a host organism, obtaining nourishment and protection. The geographic location is an important determining factor in the development of parasitic infections. The prevalence depends primarily on the geographical distribution of the parasite, socioeconomic environment and immune status of the patient (Padhi et al., 2017; Malla and Goyal, 2016). These infections may originate from a large number of sources, with ingestion of contaminated water and food (i.e., freshwater fish, undercooked or raw beef, or pork) being the most common route. Other sources include blood-sucking insects, houseflies, and household pets.

Parasitic infections of the eye may be a manifestation of generalized systemic disease or a localized phenomenon. In most ocular parasitic cases the cause is by aberrant migration of the parasite in host tissues and sometimes due to direct inoculation of the eye (Padhi et al., 2017). Several parasites that usually strike other organs may inhabit the eye because it is a unique structure with an immunologically weak microenvironment. Once infection occurs it is usually treatable but causes significant ocular morbidity and mortality due to late diagnosis or misdiagnosis, often from unfamiliarity with the disease.

Parasitic infections in humans are more predominant in areas where environmental factors and poor sanitary conditions favor transmission from the animal host. In recent years, the increase in immigration and widespread international travel have facilitated the spread of certain parasitic infections from endemic to nonendemic areas. Knowledge of the type of parasites, dietary habits, sanitary measures, food and water conditions, and their prevalence in certain geographical regions may aid in diagnosing unusual symptoms in patients.

Interaction between the host and the parasite determines whether the parasite will die or spread throughout the body, and defines the location, type and extent of infection. The route of infection to humans varies with the parasitic species. There are three main classes of parasitic organisms, these include protozoans, helminths and ectoparasites. This article will review parasitic infections involving the eye.

Protozoans

Protozoa are one-cell organisms that can be free-living or parasitic in nature (Table 1). They can multiply in humans, which contributes to their survival and also permits serious infections to develop from just a single organism.

Toxoplasmosis

Toxoplasmosis is caused by an intracellular protozoan parasite known as *Toxoplasma gondii*. Approximately, one-third of the human population has been infected with the parasite (Hill and Dubey, 2002). Cats play an important role in the spread of toxoplasmosis, they become infected by eating contaminated rodents, birds or other small animals. The parasite is then passed in the cat's stool in the form of an oocyst. Infected cats can then contaminate the soil or water in the environment. These oocysts can survive in the soil for long periods (Dubey et al., 1970, 1998). Infection of human then occurs either by consumption of oocysts present in raw or

Table 1 Summary of protozoans parasitic infections with ocular involvement.

<i>Disease (causative agent)</i>	<i>Mode of transmission</i>	<i>Geographical distribution</i>	<i>Ocular pathology</i>	<i>Treatment</i>
Toxoplasmosis (<i>Toxoplasma gondii</i>)	Ingestion of raw or uncooked meat, exposure to infected cat feces, transplacental	Worldwide	Posterior uveitis, retinochoroiditis	Pyrimethamine, sulfadiazine, clindamycin, corticosteroid
Chagas disease (<i>Trypanosoma cruzi</i>)	Bite by triatomine bug, ingestion of contaminated food, blood transfusions or organ transplant, transplacental	Regions of the Americas, Europe, Africa, Eastern Mediterranean and Western Pacific	Skin lesions or palpebral edema with possible facial swelling	Benznidazole and nifurtimox
Malaria (<i>Plasmodium</i> spp.)	Bite by infected female anopheles mosquito	Africa, Central and South America, Oceania and Asia	Retinal whitening, vessel discoloration, retinal hemorrhage, optic disk edema	Artemisinin-based combination therapy
Leishmaniasis (<i>Leishmania</i> spp.)	Bite by infected female phlebotomine sandfly	Region of the Americas, Eastern Mediterranean region, Southern Europe, South-East Asia	Nodular lesions in the eyelids, trichiasis, nodular conjunctivitis, interstitial and ulcerative keratitis, dacryocystitis, episcleritis	Intralesional or systemic stibogluconate
Acanthamoebiasis (<i>Acanthamoeba</i> spp.)	Contamination of contact lenses, corneal trauma	Worldwide	Keratitis	PHMB, chlorhexidine, penetrating keratoplasty
Microsporidiosis (<i>Microsporidia</i> spp.)	Poor sanitary conditions, close contact with domestic animal hosts, topical steroid use, immunocompromised individuals and contact lenses	Worldwide	Keratoconjunctivitis, epithelial keratopathy, anterior uveitis, stromal keratitis	Albendazole, fumagillin, fluoroquinolones and voriconazole, penetrating keratoplasty

Key: *spp.*, species, *PHMB*, polyhexamethylene biguanide.

uncooked meat, exposure to infected cat feces or mother to child transmission during pregnancy (Hill and Dubey, 2002). Within days of exposure, *T. gondii* multiplies and differentiates within cells that line the human digestive tract. From there the parasite can penetrate the intestinal mucosa and reach distant organs such as the brain, eyes, liver, spleen, lymph nodes, heart, skeletal muscles and the placenta by blood and lymphatic stream (Harker et al., 2015). Dendritic cells and macrophages guide the parasite through the blood-brain barrier allowing infection of the brain and eye (Harker et al., 2015; Feustel et al., 2012).

Children and young adults (25–45 years) are affected by Toxoplasmosis which is often recurrent (Ng and McCluskey, 2002). Ocular toxoplasmosis usually manifests in immunocompromised *T. gondii* infected individuals and in neonates who acquire infection trans-placentally. Congenital ocular toxoplasmosis usually involves both eyes, whereas acquired ocular toxoplasmosis is typically unilateral (Holland, 2004). *T. gondii* is the most common cause of posterior uveitis in adults and children (Butler et al., 2013; Holland, 2003) followed by retinochoroiditis (Dupont et al., 2012). Patients with ocular toxoplasmosis typically experience floaters, reduced vision, photophobia, redness and eye pain. Hallmark clinical signs are a vitreous cellular infiltrate associated with a white retinal lesion that is typically adjacent to a pigmented chorioretinal scar (Ng and McCluskey, 2002) (Fig. 1).

Diagnosis is based on clinical features and can be supported by a serology test or polymerase chain reaction (PCR). Enzyme-linked immunosorbent assay (ELISA) can be used for the estimation of immunoglobulin G (IgG) or M (IgM) antibodies. However, serological tests have a limited role in establishing the diagnosis of ocular toxoplasmosis as they are not conclusive. Patients with positive toxoplasmosis always indicate positive for *Toxoplasma*-specific IgG, but so do infected individuals who present with no signs of ocular involvement. Hence, the use of serology testing for ocular toxoplasmosis is of low diagnostic value (Garweg et al., 2011) PCR, on the other hand, can be performed on either aqueous humor or vitreous and is both sensitive and specific (Bou et al., 1999).

The need for therapy, type of drug and duration of therapy must be tailored to the patient. An episode of ocular infection with mild inflammation and no involvement of the optic disc or macula region of the retina is self-limiting in immunocompetent patients (Ng and McCluskey, 2002). However, in immunocompromised patients, anti-parasitic medication may be required and treatment is usually required for 6–12 weeks (Padhi et al., 2017). The ideal treatment is combination drug therapy as it results in rapid resolution, whilst minimizing inflammatory damage and antibiotic resistance. The most commonly used treatment combinations are pyrimethamine, sulfadiazine and corticosteroids, or clindamycin and corticosteroid (Ng and McCluskey, 2002).

Ocular toxoplasmosis can be prevented by washing of fruits and vegetables, avoidance of cross-contamination in the kitchen with raw meat, and adequate cooking of meat to destroy any harbored cysts (Padhi et al., 2017). Pregnant women should also avoid cat litter pans as they can transmit the infection transplacentally to their unborn fetus and contraceptive precautions should be administered to women of childbearing age for 6 months following primary toxoplasmosis infection as reactivation of *T. gondii* can occur during pregnancy (Ng and McCluskey, 2002).

Chagas disease

Chagas disease is caused by *Trypanosoma cruzi*, a protozoan hemoflagellate. It was once confined to continental rural areas of the Region of the Americas (specifically Latin America). However, with increased population mobility in the last decades, most people infected live in urban settings and the disease has been increasingly detected in the United States of America, Canada, Europe, Africa, Eastern Mediterranean and Western Pacific countries (World Health Organization, 2020a). It is usually transmitted to humans via a bite from an infected blood-sucking triatomine bug. During its feed, the triatomine bug urinates or defecates while ingesting human blood, thereby excreting infective trypanosomes (Rassi and De Rezende, 2012). The parasite enters the body through mucous membranes, breaks in the skin, eyes, or mouth. Transmission can also occur by consumption of contaminated food with *T. cruzi*, blood transfusions from infected donors, passage from an infected mother to her newborn during pregnancy or childbirth, or via the transplantation of organs from infected donors (Coura and Viñas, 2010).

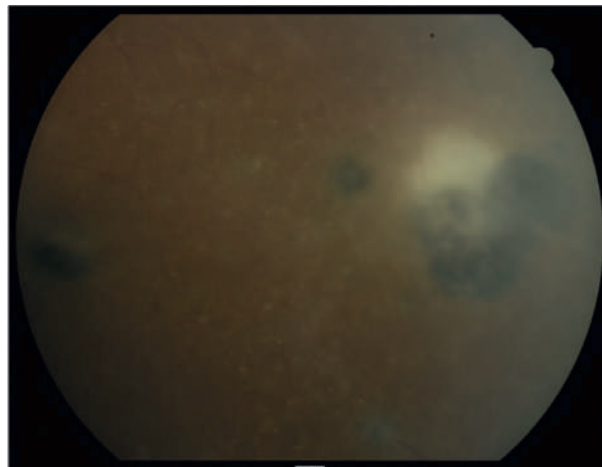


Fig. 1 Fundus photograph of a patient with Toxoplasmosis. Note the presence of retinitis adjacent to an old chorioretinal scar and vitritis. Image courtesy of Professor Peter McCluskey from The University of Sydney, Save Sight Institute.

There are two stages of Chagas disease, an initial acute and a chronic phase. The initial acute phase lasts for around 2 months after infection. During this phase, patients are asymptomatic or, in less than 50% of cases, individuals bitten by the triatomine bug show visible signs of skin lesions or a purplish swelling of an eyelid (World Health Organization, 2020a). Once the parasite has entered the body, the trypomastigotes circulate throughout the body with a preference for invading muscle cells, neural tissue and the reticuloendothelial system. If the initial bite is near the orbit, the patient may experience palpebral and periorbital edema. The edema is often painless and frequently accompanied by symptoms of fever, malaise and anorexia (Padhi et al., 2017). During the chronic phase, the parasites are hidden mainly in the heart and digestive muscles and can cause cardiac arrhythmias or progressive heart failure (Moncayo and Ortiz Yanine, 2006).

The diagnosis of Chagas disease is made by the detection of trypomastigotes in the blood as during the acute phase there is a large number of trypanosomes in the blood and they can be readily detected through light microscopy of blood smears (Nimir et al., 2012). In chronic Chagas disease, serological tests such as indirect fluorescent antibody (IFA) or ELISA are often performed to detect antibodies to *T. cruzi*. Although these tests are sensitive, they may yield false-positive results in patients with certain diseases, such as leishmaniasis (Santos et al., 2016).

In the acute phase, treatment with anti-trypanosome therapy (i.e., benznidazole and nifurtimox) is often effective if given early enough (World Health Organization, 2020a). The efficacy of the treatment decreases as the duration of the infection lengthens and side effects occur more frequently with old age (Padhi et al., 2017; Nimir et al., 2012).

Malaria

Malaria is a mosquito-borne infectious disease caused by parasitic protozoa of the genus *Plasmodium*. Nearly half the world's population is at risk of contracting the infection. It is endemic in Africa, Central and South America, Oceania and Asia (World Health Organization, 2019b). The natural life cycle of malaria involves cyclical infection of humans and the female anopheles mosquito. In humans, the parasite grows and multiplies first in the liver cells and then the red blood cells. The parasite then destroys the red blood cells releasing daughter parasites (i.e., merozoites), that continue the cycle by invading other red blood cells. The bite of a female anopheles mosquito, infected with the active form of the parasite (i.e., sporozoite), starts another human infection (Ashley et al., 2018).

Four species of *Plasmodium* are associated with malaria (i.e., *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*). *P. falciparum* is associated with cerebral malaria; most of the retinal changes occur in severe cerebral malaria. Signs of malaria retinopathy include retinal whitening, vessel discoloration, retinal hemorrhage, and optic disc edema (Harding et al., 2006; Biswas et al., 1996). Retinal whitening may be seen in the macula, though some patients may only develop peripheral retinal whitening. Retinal vessels may appear orange or white, particularly in the periphery in discrete sections of the vessels or in a branching pattern (Beare et al., 2006). Intra-retinal hemorrhages are often seen with a white center and resemble Roth spots. Patients with asymptomatic to mild infections present with no too few hemorrhages, while those with severe malaria may present with numerous hemorrhages (>120 in each eye) (White et al., 2009). Optic disc edema accompanies retinal features of cerebral malaria in several cases and its presence is typically a sign of poor prognosis (Padhi et al., 2017).

Studies have shown that in cerebral malaria, retinopathy is associated with subsequent death and an increased severity of retinal signs has been related to increased risk of fatal outcome (Beare et al., 2004). It should be noted, that whilst these retinal signs indicate disease severity they do not alter the management of the disease (Nimir et al., 2012). Additionally, while retinal findings may be seen in non-cerebral malaria, they are quite rare and less severe (Lewallen et al., 2008; Beare et al., 2006, 2011).

The gold standard for diagnosing malaria is transmission electron microscope (TEM), however, the procedure is expensive, time-consuming and not feasible for routine diagnosis. Therefore, light microscopy examination of the parasite in blood smears is typically performed (Nimir et al., 2012). Although not widely available, PCR is slightly more sensitive than microscopy. However, PCR results are often not available quickly enough to be of value in establishing the diagnosis of malaria infection. Though, the technique is useful for confirming the infecting *Plasmodium* species of the malarial parasite after the diagnosis has been established; this can aid with management. Serology tests such as IFA and ELISA detect antibodies against malaria parasites, although, they do not detect current infection but rather measure past exposure. Indirect ophthalmoscopy and fluorescein angiography can also aid the diagnosis of malaria when there is retinal involvement.

The treatment of malaria is complex and dependent on the parasite species, geography and drug resistance patterns. Patients with the infection should be provided systemic antimalarial therapy, guided by local anti-malarial sensitivity and resistance patterns based on where the infection occurred. Artemisinin-based combination therapy, such as oral artemether/lumefantrine, is the most rapidly active treatment and in many situations the drug of choice. Malaria is a preventable and treatable disease, chemoprophylaxis can be administered to individuals travelling to affected areas as the drug suppresses the blood stage of the infection (World Health Organization, 2020c).

Leishmaniasis

The infection is caused by *Leishmania* species which are transmitted by the bite of infected female phlebotomine sandflies. The disease is found in parts of the region of the Americas, Eastern Mediterranean region, Southern Europe and South-East Asia (World Health Organization, 2020b). After inoculation by a sandfly, promastigotes are phagocytized by host macrophages. Within these cells, they transform into amastigotes where they replicate and may spread either systemically or cutaneously (Bates, 2007). The disease can be divided into three categories based on the organs involved: cutaneous, mucocutaneous and visceral (World Health Organization, 2020b).

Ocular involvement, commonly seen in cutaneous and mucocutaneous leishmaniasis, includes nodular lesions in the eyelids, trichiasis, nodular conjunctivitis, interstitial and ulcerative keratitis, dacryocystitis, and episcleritis (Padhi et al., 2017). Visceral leishmaniasis also known as “kala-azar,” represents the systemic forms of the disease. Ocular manifestations are typically uncommon, however patients may present with keratitis, uveitis and/or blepharoconjunctivitis (Nandy et al., 1991; Khalil et al., 2011; Pradhan et al., 2018).

Diagnosis can be confirmed by blood smears or biopsies of the lesion. Amastigotes are usually demonstrated fairly easily in ocular cases of cutaneous and mucocutaneous leishmaniasis. Other tests available include the *Leishmania* skin test, direct fluorescent antibody test, indirect hemagglutination test, ELISA, and PCR.

The treatment for leishmaniasis is dependent on the clinical syndrome, infecting *Leishmania* species, geography, organism susceptibility to treatment and immune status of the host. The most common treatment for leishmaniasis is intralosomal or systemic stibogluconate (i.e., sodium stibogluconate) (Padhi et al., 2017). Treatment of ocular leishmaniasis is more challenging. Systemic antimoniate (e.g., glucantime) and amphotericin has shown to be effective in treating ocular leishmaniasis (Reithinger et al., 2007; Nikandish et al., 2016).

Acanthamoebiasis

The genus *Acanthamoeba* contains at least 24 free-living amoebic protozoa species that are ubiquitous in nature. They are present worldwide and exist in both soil and nearly all water sources. Their life cycle exists in two forms: trophozoites (i.e., the infective form) and cysts. The trophozoite form can replicate asexually by binary fission (Alkharashi et al., 2015), while cysts remain dormant and can withstand adverse environmental conditions for years (Sriram et al., 2008).

Human ocular involvement with *Acanthamoeba* presents in the form of keratitis. *Acanthamoeba* keratitis (AK) is a rare, sight-threatening infection. Various *Acanthamoeba* species can cause chronic and progressive keratitis (i.e., corneal infection). The main predisposing risk factor is contact lens wear and corneal trauma (Carnt et al., 2018; Bharathi et al., 2007; Watson and Dart, 2012; Bartimote et al., 2019).

Patients typically present with a unilateral or paracentral epithelial defect, often with a ring-shaped peripheral infiltrate. In the early stages, patients may also present with eyelid ptosis, conjunctival hyperemia, and pseudodendrites. In the later stages, patients may present with deep stromal infiltrates, corneal perforation, satellite lesions, scleritis, and anterior uveitis with hypopyon. Clinical symptoms of the infection include severe pain, decreased vision, foreign body sensation, photophobia, tearing and discharge (Dart et al., 2009; Höllhumer et al., 2020) (Figs. 2 and 3).

A provisional diagnosis of AK can be made from the patient history, clinical features and confocal microscopy. *Acanthamoeba* cysts appear as hyperreflective, spherical and well-defined double-wall structures on confocal microscopy, while trophozoites are difficult to discriminate from leukocytes and keratocyte nuclei (Villani et al., 2014). Diagnosis should be confirmed via corneal scrape to identify the *Acanthamoeba* species. If clinical suspicion exists, the epithelial defect should be scraped using a sterile blade or needle under topical anesthesia (Ngo et al., 2020). The culture specimen should then be inoculated onto *Escherichia coli* plated over non-nutrient agar. As the clinical picture is often non-specific, cultures should be taken for bacterial, fungal and viral infection as well. Although culture on *E. coli* agar plates is the gold standard, polymerase chain reaction (PCR) testing can be performed as it is well established and has higher sensitivity; *E. coli* plates may also not be available in all centers (Ikeda et al., 2012; Lorenzo-Morales et al., 2015). The sample may also be placed on nutrient agar for transfer to *E. coli* plates in the laboratory (Dart et al., 2009). In the case of deep corneal involvement, a corneal biopsy maybe needed for diagnosis (Robaei et al., 2018) (Fig. 4).

Early diagnosis and aggressive medical therapy have improved the management of this often complicated infection. A combination of various antiacanthamoebal agents are utilized; there is no single drug that can eliminate both cystic and trophozoite forms. Due to the existence of cyst form which is highly resistant to therapy, a combination of agents is generally used. Topical



Fig. 2 Chronic *Acanthamoeba* keratitis. The cornea has stromal opacity, vascularization and an area of thinning.

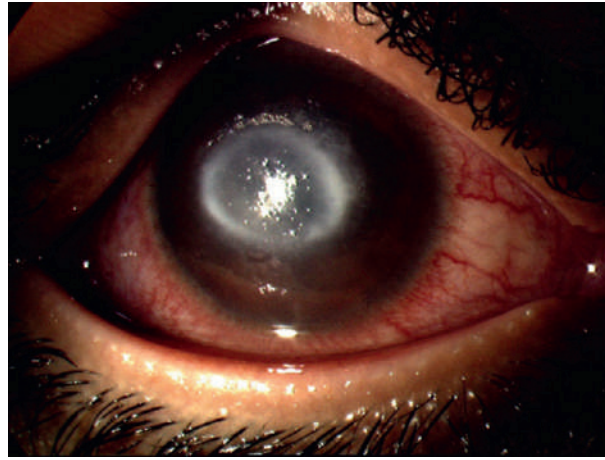


Fig. 3 Ring infiltrate in acanthamoeba.

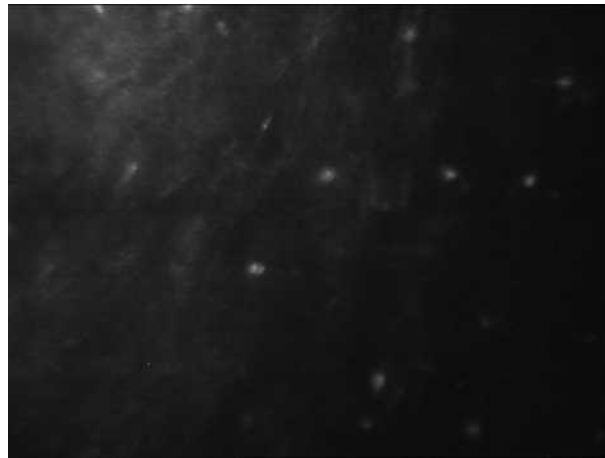


Fig. 4 Confocal microscopy of acanthamoeba.

agents effective against acanthamoeba trophozoites and cysts are polyhexamethylene biguanide (PHMB) and chlorhexidine (Lorenzo-Morales et al., 2013, 2015). Both PHMB and chlorhexidine are effective against trophozoites with variable efficacy against cysts. Chlorhexidine is often used in combination with propamidine or hexamidine and has shown good results if the treatment is commenced early in the course of infection (Roberts and Henriquez, 2010). However, propamidine and hexamidine are not available in all countries. Topical anti-acanthamoeba drugs should be administered every hour after corneal scrape procedures for the first several days with the frequency then reduced depending on clinical response. Treatment is recommended for 6–12 months with close observation to prevent recurrent infection. Therapeutic penetrating keratoplasty is reserved as a measure of last resort in cases of impending corneal perforation. Robaei et al. suggests delaying corneal transplantations where possible, until the eye is no longer inflamed and after completion of antiacanthamoebal treatment (Robaei et al., 2015).

Should be considered when the infection spreads to the paracentral corneal stroma, as performing this procedure on a more localized infection may allow for the total removal of the organism (Cohen et al., 1987). Topical steroids may be needed to control inflammation but only after antiacanthamoebal therapy has been commenced (Carnt et al., 2016).

Acanthamoeba keratitis can be difficult to treat; it can lead to complications including scleritis and treatment toxicity. To prevent Acanthamoeba keratitis, clinicians should instruct patients on proper cleaning of contact lenses and remind patients to avoid wearing contact lenses while swimming or showering (Carnt and Stapleton, 2016).

Microsporidiosis

Microsporidiosis is an intestinal infection caused by *Microsporidia* species. The infection occurs worldwide and predominantly in patients with AIDS and causes chronic diarrhea, disseminated infection, and corneal disease. The source of *microsporidia* infection is still uncertain, but the genotype that infects humans have been identified in domestic, farm and wild animals (Mathis et al., 2005). Predisposing factors include poor sanitary conditions, close contact with domestic animal hosts (such as cats and birds), prolonged topical steroid use, immunocompromised, and contact lens use (Theng et al., 2001; Didier and Weiss, 2006). Once the parasite is

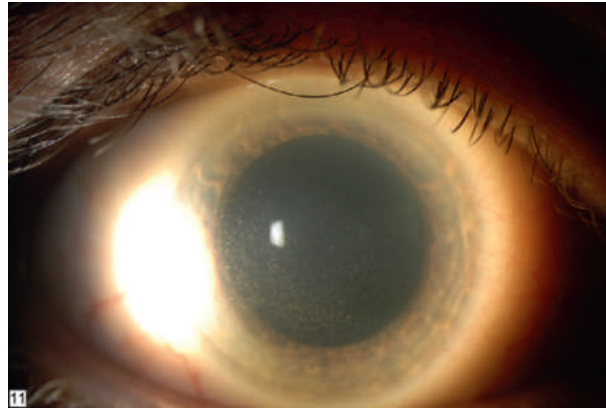


Fig. 5 Microsporidia keratitis caused by exposure to water well. Note the speckled fine opacities in the cornea. Image courtesy of Assistant Professor Tyler Hall from the University of Alabama Birmingham.

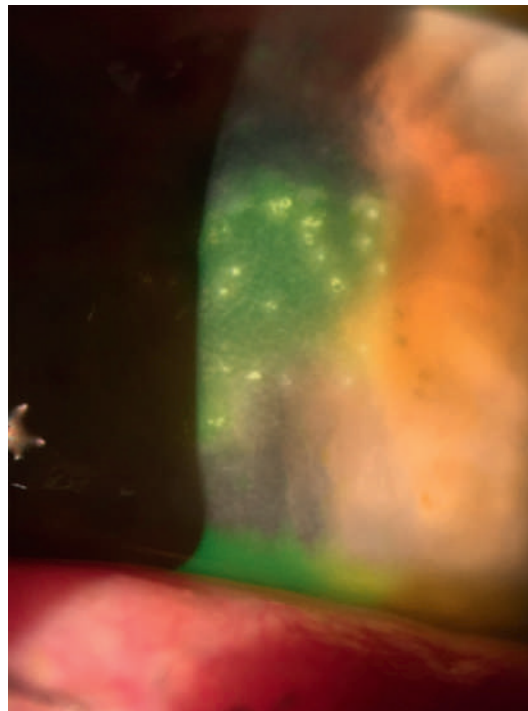


Fig. 6 Microsporidia keratitis. The images shows atypical punctate epithelial lesions. Image courtesy of Dr. Joshua Teichman from the University of Toronto.

inside the host, they inoculate the host cell with an infective sporoplasm. Intracellularly, the sporoplasm divides and multiplies, producing sporoblasts that mature into spores. The spores are then disseminated throughout the body or pass into the environment via respiratory aerosols, stool or urine. An inflammatory response occurs when the spores are released from the host cells (Didier and Weiss, 2006) (Figs. 5 and 6).

Clinical signs and symptoms vary depending on the parasite species and the immune status of the host. In immunocompetent patients, *microsporidia* can cause asymptomatic infection or self-limited watery diarrhea. In patients with AIDS, chronic diarrhea and malabsorption occur. Superficial keratoconjunctivitis can occur in both immunocompetent and immunocompromised patients. Both immunocompetent and immunocompromised patients typically experience redness, photophobia, pain, lacrimation and blurred vision, and present with signs of diffuse, raised, punctate, round epithelial lesions in the cornea, mimicking adenoviral keratoconjunctivitis (Loh et al., 2009; Das et al., 2012; Joseph et al., 2006). In immunocompromised patients, the presence of epithelial keratopathy, anterior or mid stromal infiltration, anterior uveitis and corneal necrosis and perforation have also been seen. Stromal keratitis has also been reported mostly in immunocompetent individuals (Joseph et al., 2006).

The gold standard for the identification of *microsporidia* is via TEM. In routine clinical practice, TEM is not feasible; corneal scrape or biopsy should be performed to identify the species of *microsporidia*. IFA and PCR can also be used to identify *microsporidia* species (Didier and Weiss, 2006).

The recommended treatment for microsporidiosis is albendazole as it is effective in controlling diarrhea. Stromal keratitis is generally refractory to medical therapy, and usually requires debridement of superficial corneal epithelium or penetrating keratoplasty. However, a case report has shown oral albendazole to be effective in treating stromal keratitis (Sangit et al., 2011). Microsporidial keratoconjunctivitis may be self-limited (Das et al., 2010) or require topical antibiotics such as fumagillin, fluoroquinolones and voriconazole (Loh et al., 2009).

Helminths

Helminths are large, free-living, multicellular worms that are generally visible to the naked eye in their adult stage (Table 2). In their adult form, helminths cannot multiply in humans. There are three main groups of helminths: flatworms (platyhelminthes), thorny-headed worms (acanthocephalins) and roundworms (nematodes).

Onchocerciasis

Onchocerciasis, also known as river blindness, is a disease caused by the parasitic worm *Onchocerca volvulus*. The infection is transmitted by bites from infected black Simulium fly, which breeds in rapidly flowing waters. It is an endemic in many areas of sub-Saharan Africa (World Health Organization, 2019c). Humans are the only host of *O. volvulus*, the larvae are transmitted through the bite of female black flies and develop into mature adult worms that form subcutaneous nodules. The adult female releases millions of microfilariae that can migrate throughout the body, particularly to the skin and eyes (Hoerauf et al., 2003).

Ocular manifestations of onchocerciasis are caused by the presence of dead parasites within the eye. The microfilariae infiltrate the eye through the bulbar conjunctiva at the limbus and invade the cornea, aqueous humor, iris, retina and choroid. Patients may present with punctate keratitis, sclerosing keratitis, anterior uveitis, iridocyclitis, glaucoma, chorioretinitis, corneal opacity and optic atrophy (Padhi et al., 2017; Hoerauf et al., 2003).

The diagnosis of onchocerciasis is via a combination of clinical signs and symptoms and a histopathological examination (i.e., skin, subcutaneous nodules, or sclerocorneal biopsy) (Udall, 2007). Microfilariae may be visible in the cornea and anterior chamber of the eye during slit-lamp examination. PCR can also detect their DNA and is more sensitive than standard techniques (i.e., skin snips or biopsies) but is typically only used in research settings (Boatin et al., 2002).

The recommended treatment is ivermectin; it reduces the production of microfilariae in the eye. Ivermectin does not kill the adult female worms, instead, in cumulative doses, it decreases their fertility. A single dose of 150 µg/kg, repeated for 6–12 months interval is recommended (World Health Organization, 2019c). Doxycycline has also been used to decrease microfilaria load and corticosteroids to reduce inflammation.

Loiasis

Loiasis, also known as the African eye worm, is caused by the *Loa loa* worm. It only occurs in Africa, primarily in rainforest areas of Central and West Africa (Barnett, 2007). Individuals living in the rainforest in Central and West Africa and travelers visiting those areas are at high risk of infection (Centers for Disease Control and Prevention, 2015). Transmission of the disease is caused by bites from tabanid flies (also known as deer fly or horse fly). Immigrants from or travelers to an endemic area are at risk of contracting the infection (Barnett, 2007).

Table 2 Summary of helminths parasitic infections with ocular involvement.

Disease (causative agent)	Mode of transmission	Geographical distribution	Ocular pathology	Treatment
Onchocerciasis (<i>Onchocerca volvulus</i>)	Bites by female black flies	Sub-Saharan Africa	Punctate keratitis, sclerosing keratitis, anterior uveitis, iridocyclitis, glaucoma, chorioretinitis, corneal opacity and optic atrophy	Ivermectin, doxycycline, corticosteroids
Loiasis (<i>Loa loa</i>)	Bites by tabanid flies	Central and West Africa	Worm under bulbar conjunctiva, chemosis, pruritus, keratitis, iritis, retinal hemorrhages	Removal of worm, DEC
Toxocariasis (<i>Toxocara canis</i> , <i>Toxocara cati</i>)	Ingestion of contaminated food or water of stool from infected animal, ingestion of undercooked infected transfer hots (e.g., rabbits)	Worldwide	Posterior pole granuloma, peripheral granuloma, nematode endophthalmitis	Albendazole, corticosteroids
Cysticercosis (<i>Cysticercus cellulosae</i>)	Ingestion of water or food contaminated with eggs or tapeworms, ingestion of raw or undercooked infected pork	Developing countries of Africa, Asia, Latin America	Orbital or intraocular cyst	Albendazole, corticosteroids, surgical removal of cyst

Key: spp., species; DEC, diethylcarbamazine.

Transmission occurs when humans are bitten by the fly which passes microfilariae to the human, where they develop into mature adult worms (Centers for Disease Control and Prevention, 2015). The adult worms migrate through cutaneous and deep connective tissue, producing microfilariae. The presence of microfilaria or adult worms can cause ocular diseases. A migrating worm in the periorbital area may cause intense itching, swelling and a moving foreign body sensation, while a dead worm can incite inflammation, leading to conjunctival congestion, chemosis, pruritus, keratitis, iritis, retinal hemorrhages and even blindness (Padhi et al., 2017; Klion et al., 1991; Bowler et al., 2011).

Loiasis is usually diagnosed by the detection of circulating microfilariae. In cases of conjunctival involvement, removal of an adult worm confirms the diagnosis. Serology can be performed to detect microfilariae in the blood with samples taken between 10 am and 2 pm when microfilariae levels are the highest (Centers for Disease Control and Prevention, 2015). Treatment for the infection requires manual removal of adult worms present in the conjunctiva and the use of oral diethylcarbamazine (DEC). Clinicians should monitor patients with severe infection closely, as patients prescribed DEC are at risk of potentially fatal encephalopathy, due to the release of antigens from dying microfilariae (Padhi et al., 2017).

Toxocariasis

Toxocariasis is a zoonotic disease caused by the larva of *Toxocara canis* (dogs) or *T. cati* (cats). It is distributed worldwide and is the most prevalent helminthic infection in tropical, developing countries (Mattos et al., 2016). Animals of canine and feline species act as definite host, humans are the accidental host. Geophagia and exposure to and/or ownership of puppies and kittens have been identified as significant risk factors (Nathwani et al., 1992). Humans may accidentally ingest eggs in food or water contaminated by stool from infected animals or from ingesting of undercooked infected transfer hosts (e.g., rabbits).

In the definite animal host (i.e., dogs and cats), the larvae mature into adult forms and liberate eggs in their feces. In humans, the parasite faces a dead end, that is the larvae doesn't mature into the adult form, though can remain alive in the human host for many months. In the human host, the larvae hatched in the intestines, penetrate the bowel walls and migrate through to the liver, lungs, eyes and other tissues (Despommier, 2003). Patients usually present with systemic symptoms of fever, cough, hepatomegaly and rash (Padhi et al., 2017). In certain cases, ocular toxocariasis can occur. This is caused by a single larva entering the ocular circulation and typically affects older children. It is usually unilateral with little to no evidence of systemic manifestations. The main ocular findings include posterior pole granuloma, peripheral granuloma, and nematode endophthalmitis (Ahn et al., 2014a) (Fig. 7).

Diagnosis of ocular toxocariasis is presumptive and typically based on patient history, clinical observation and serologic findings. The standard tests for diagnosing toxocariasis in humans is the detection of serum IgG using ELISA based on the *Toxocara* larval antigen (Ahn et al., 2014a). A biopsy to obtain the infected tissue can be performed but is risky and difficult in eyes with ocular toxocariasis, and thus rarely performed.

The infection is usually self-limiting in asymptomatic patients and those with mild symptoms. For patients with moderate to severe infection, systemic albendazole is the recommended drug of choice (Ahn et al., 2014a; Padhi et al., 2017). For ocular toxocariasis, currently, no optimal treatment regime has been established. However, systemic and periocular steroids are usually administered to control inflammation (Ahn et al., 2014b). For structural complications in the retina (e.g., retinal detachment, epiretinal membrane, and persistent vitreous opacity) vitreoretinal surgery may be performed (Ahn et al., 2014a).

Cysticercosis

Cysticercosis is a systemic illness caused by *Cysticercus cellulosae* larvae of the adult tapeworm *Taenia solium* and *Taenia saginata* (Dhiman et al., 2017). It can affect the skin, skeletal muscles, heart, eye and central nervous system. It is a common cause of ocular



Fig. 7 Toxocariasis. The fundus photograph shows a granuloma in the macula region with connective tissue bands extending into the vitreous from the lesion. Image courtesy of Professor Peter McCluskey from The University of Sydney, Save Sight Institute.

inflammation in developing countries of Africa, Asia and Latin America (World Health Organization, 2019a). Cysticercosis is caused by ingestion of water or food contaminated with tapeworms or eggs, or ingestion of raw or undercooked infected pork. In ocular cysticercosis, the egg matures into larvae and penetrates the intestinal mucosa and spreads hematogenously into the eye (Carpio et al., 2013). Ocular cysticercosis typically affects the younger age group, occurring most commonly between the ages of 10 and 20 years (Pushker et al., 2004; Dhiman et al., 2017).

The cysticercus larvae can affect almost any part of the eye such as the anterior orbit, subconjunctival space, posterior orbit and eyelid. Depending on the location of the cyst, patients may present with the following: decreased visual acuity, pupillary blocking leading to glaucoma, conjunctiva or scleral redness, proptosis, lid swelling, exudative or rhegmatogenous retinal detachment or optic disc edema (Dhiman et al., 2017).

Diagnosis is based on clinical features, serological and radiological examination. Depending on the location of the cysts, the use of B-scan ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI) are most helpful in providing a definitive diagnosis. Serologic tests such as ELISA can identify antibody against excretory-secretory antigens (i.e., IgG) but have a low sensitivity in diagnosing cysticercosis (Dhiman et al., 2017; Diwan et al., 1982).

The treatment for ocular cysticercosis is oral albendazole (Sihota and Honavar, 1994) and may lead to increased inflammation around the lesions requiring the use of corticosteroids. Surgery may also be required for isolated ocular lesions to prevent vision loss.

Ectoparasites

These are parasites that live on the external surface of the host and depend on blood meals from a human host for their survival, for example, fleas and lice (Table 3).

Ophthalmomyiasis

Ophthalmomyiasis is the result of an invasion of dead or living vertebrate animal tissue by fly larvae into the eye. Genera commonly associated with human myiasis include *Dermatobia*, *Gasterophilus*, *Oestra*, *Cordylobia*, *Chrysomya*, *Wohlfahrtia*, *Cochliomyia*, and *Hypoderma* (Francesconi and Lupi, 2012). It is common in rural farming regions. Myiasis usually follows a seasonal pattern, in accordance with the insect's life cycle, i.e., most cases occur during spring and summer in temperate climates (e.g., Europe, Northern America and Northern Africa) (Padhi et al., 2017).

Many flies do not lay their eggs on humans but on other insects, animals or objects that may come into contact with skin. Female flies retain their eggs within their body until they hatch. Once they hatch the larvae can burrow into the skin and develop and grow into mature larvae. Although some flies are attracted to open wounds some species infest unbroken skin (Francesconi and Lupi, 2012). When they occur in the eye, larval infections are usually restricted to the conjunctiva and cornea. There are three categories of ophthalmomyiasis: ophthalmomyiasis externa, ophthalmomyiasis interna and orbital myiasis.

Symptoms vary based on the biology of the larvae, the location in the eye, and route of penetration. Ophthalmomyiasis externa refers to the superficial infection of ocular tissue, i.e., conjunctival involvement. Patients typically experience itching, pain, conjunctival hyperemia, lid edema, lacrimation and foreign body sensation (Anane and Hssine, 2010). These signs may mimic conjunctivitis. This type of ocular myiasis is typically mild, self-limited and benign.

Ophthalmomyiasis interna is an infection involving the anterior or posterior segment of the eye. The fly larva may be seen in the anterior segment, vitreous and/or subretinal space (Jakobs et al., 1997). This type of infection is not common and clinically appears as anterior uveitis, sometimes accompanied by posterior segment inflammation. Patients may present with iritis, retinal detachment or subretinal tracks. Symptoms usually include red eye, vision loss, floaters, pain and scotomas (Francesconi and Lupi, 2012).

Table 3 Summary of ectoparasites parasitic infections with ocular involvement.

Disease (causative agent)	Mode of transmission	Geographical distribution	Ocular pathology	Treatment
Myiasis (<i>Dermatobia</i> , <i>Gasterophilus</i> , <i>Oestra</i> , <i>Cordylobia</i> , <i>Chrysomya</i> , <i>Wohlfahrtia</i> , <i>Cochliomyia</i> , and <i>Hypoderma</i>)	Insects, animals or objects that may come into contact with skin	Europe, Northern America and Northern Africa in rural farming regions	Ophthalmomyiasis externa: itching, pain, conjunctival hyperemia, lid edema, FBS ophthalmomyiasis interna: iritis, retinal detachment, subretinal tracks orbital myiasis: proptosis, congestion, chemosis and discharge	Removal of worms from accessible location, corticosteroids, exenteration
Lice (<i>Pediculus pubis</i>)	Intimate "skin to skin" contact or transmitted by fomites	Worldwide	Small erythematous papules, crusting of the eyelid margin	Manual removal with forceps of visible lice and eggs, petrolatum, physostigmine ointment, oral ivermectin

Key: FBS, foreign body sensation.

Orbital myiasis is a severe infestation characterized by the intraocular invasion of maggots from the eyelid myiasis. Eyelid tumors are the most common predisposing factor for this type of ophthalmomyiasis (Çaça et al., 2006; Cavuşoglu et al., 2009; Jain et al., 2007). Patients typically present with proptosis, congestion, chemosis and discharge (Padhi et al., 2017).

Diagnosis is made via patient history and physical examination. Establishing a history of a patient's recent close contact with farm animals (specifically sheep or goats) in a rural area may aid diagnosis (Nimir et al., 2012). Additionally, a slit lamp exam may reveal small translucent larvae up to 5 mm in length on the conjunctiva. Because the larvae are photophobic, it is prudent to examine the eye and discharge under moderate slit lamp illumination and high magnification (Francesconi and Lupi, 2012). Ocular myiasis should be considered in cases of unilateral foreign body sensation.

The mainstay of treatment is the removal of the larvae, often at the slit lamp, with topical analgesics and antibiotics. Removal of the larvae may be difficult due to their strong attachment to tissue. Corticosteroids may be used to treat inflammation. Symptoms tend to resolve immediately after removal of the larvae. In severe cases, exenteration may be required to prevent the intracranial extension of tissue destruction (Francesconi and Lupi, 2012).

Lice

Lice are wingless, blood-sucking insects that infest the head (*Pediculus humanus* var. *capitis*), body (*P. humanus* var. *corporis*) or pubis (*P. pubis*). Involvement of the eyelashes and eyelids is caused by *P. pubis* (Rundle and Hughes, 1993). The infestation occurs worldwide and is associated with poor hygiene, overcrowding and is more common in the sexually active age group of 15–45 years (Kiran et al., 2012). Transmission occurs from close contact, often intimate “skin to skin” contact, or is transmitted by fomites (e.g., towels, bedding, clothing) (Rundle and Hughes, 1993).

Depending on the species, eggs or nits are laid and glued to body hairs or clothing fibers. This results in nymphs emerging to feed on the host, leading to symptoms of pruritus. Small erythematous papules with evidence of excoriation and crusting of the eyelid margin may also be seen (Karabela et al., 2015).

Diagnosis can be confirmed via slit lamp examination, as the lice anchors to the eyelashes or eyebrows. Manual removal with forceps of visible lice and eggs is the standard treatment but can be tedious and unsuccessful due to the firm adherence of eggs to the eyelash. Application of petrolatum, physostigmine ointment, and/or oral ivermectin can increase the effectiveness of treatment (Karabela et al., 2015).

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Otitis Media and Ear Infections: Bacteria

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Introduction

The human ear is exposed to the external environment and the internal environment of the upper airway (Fig. 1). Thus it is susceptible to both exogenous and endogenous acquired bacterial infections. Those infections that occur within the ear canal are otitis externa while those that occur in the space behind the tympanic membrane are otitis media (OM).

OM is among the most common human infections. OM can occur at any age, but OM is most commonly seen in the first 3 years of life. OM is the most frequent cause for sick-baby visits to the pediatrician, and the reason for most antibiotic prescriptions (Gonzales et al., 2001).

OM is an inflammatory disease of the middle ear of which there are various forms. This includes acute otitis media (AOM) (Fig. 2), otitis media with effusion (OME), and chronic supportive otitis media (CSOM). Each of these forms of OM are sometimes described as distinct diseases. There is overlap among the different forms, and it is sometimes not easy to distinguish between the progressions of the disease. However, AOM is described as acute suppurative OM with pus in the middle ear and rapid onset of fever and irritability or ear pain in young children. OME presents as middle ear fluid without the signs of bacterial infection. OME can occur in children after the AOM subsides. CSOM involves a perforation in the tympanic membrane and a persistent drainage for weeks from the middle ear. Additionally, recurrent AOM involves multiple episodes of AOM over a short period of time.

In contrast to OM, infections that result in inflammation of the ear canal are defined as otitis externa. Fig. 3 is from an animal model showing inflammation as a result of bacterial infection. Acute otitis externa (AOE) is commonly referred to as swimmers ear. Cerumen, or ear wax, is an important component in the defense of the external ear canal. Too much or too little cerumen can predispose the external ear canal to infection.

Pathology, epidemiology, and pathogenesis of bacterial OM

The bacteria that cause OM colonize the nasopharynx. They can then transition to the middle ear via the Eustachian tube. The Eustachian tube functions to maintain air pressure within the middle ear and clear mucus from the middle ear to the nasopharynx. To accomplish this, the tube opens and closes periodically. This can provide a pathway for bacteria to reach the middle ear. Additionally, in young children the angle of the Eustachian tube between the middle ear and the nasopharynx is relatively flat becoming more acute as the child develops. This lack of angle increases access to the middle ear from the nasopharynx.

There are a number of factors that can contribute to the development of OM. Probably younger age is the chief risk factor. Attendance at daycare can also predispose young children to the development of OM. Daycares provide a stressful environment of close contact and sharing of objects. This can increase the rate of colonization in the nasopharynx by those bacteria that cause OM. Household smoking can impact the overall health of young children and increase the occurrence of OM. OM is more frequently seen in children who are formula fed from birth. In contrast breast feeding can be protective.

As much as 70% of AOM is caused by bacteria. This is often preceded by viral infections of the upper respiratory tract. The viral infection can alter the responses in the nasopharynx leading to an over production of proinflammatory cytokines. Additionally, there may be cellular damage during the viral infection. These and other factors can increase the potential for a bacterial infection of the middle ear.

The leading bacterial causes of OM include *Streptococcus pneumoniae* (pneumococcus), nontypeable *Haemophilus influenza* (NTHi), and *Moraxella catarrhalis*. Infections with these bacteria can occur singularly or in combination. Prior to the introduction of the pneumococcal conjugate vaccine (PCV), the pneumococcus was considered the leading cause of OM, and the capsule polysaccharide was considered the major virulence factor for the organism. This formed the basis for the development of the PCV.

Most cases of OM are treated empirically, and the etiological agent is not determined. While the PCV has had a dramatic effect on the pneumococcal invasive disease, the incidence of OM has not decreased. This is due in part to the other otopathogens, NTHi and *M. catarrhalis*. However, a consequence of the PCV has been the selective pressure on the pneumococcus. This has resulted in serotype replacement by pneumococcal serotypes that are not covered by the vaccine. Additionally, there has been the emergence of nonencapsulated *S. pneumoniae* (NESp) as a cause of OM (Bradshaw and McDaniel, 2019). It is not clear if the NESp lineage

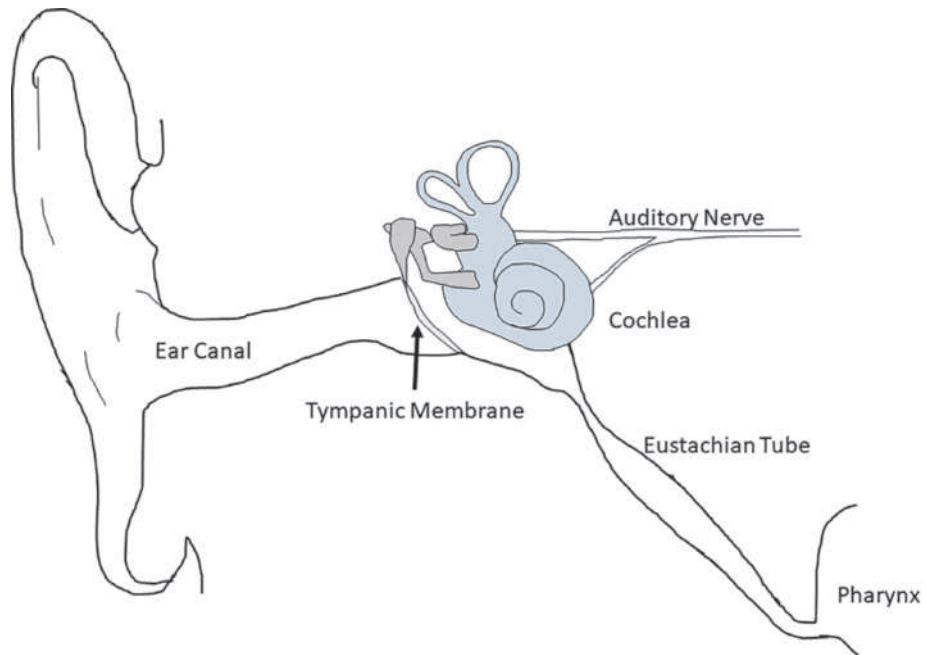


Fig. 1 A topographical representation of the human ear.

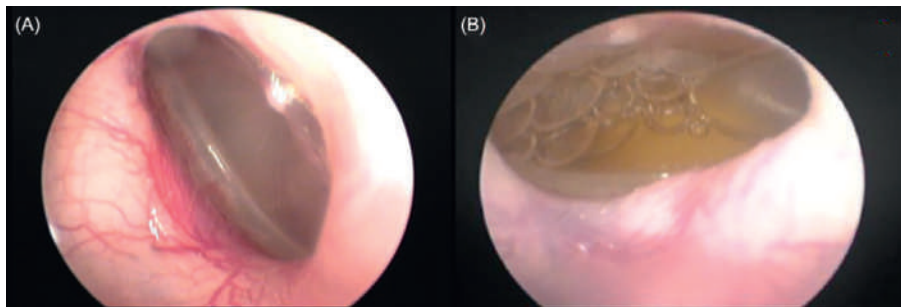


Fig. 2 Acute otitis media. A view through an otoscope of (A) a normal ear and (B) an infected ear with fluid and bubbles behind the tympanic membrane. Note that these images are from an animal model of infection.

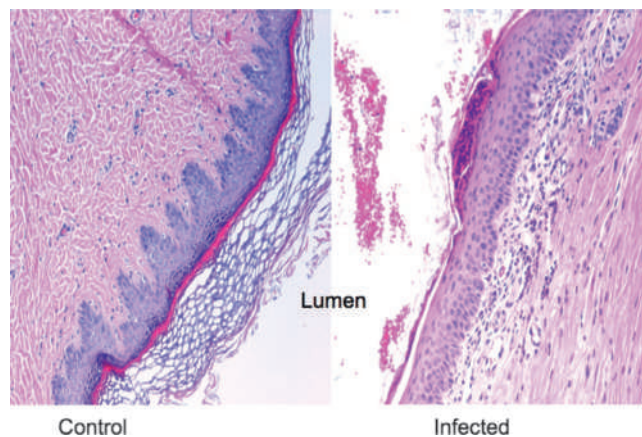


Fig. 3 Otitis externa. Guinea pigs were infected in the OEC with *Pseudomonas aeruginosa*. The ear canals were harvested, sectioned, and stained with hematoxylin and eosin. The control is from a sham infected animal. It shows intact squamous epithelial cells with underlying tissue. The section from the infected animal shows desquamation of the epithelium, inflammation, and infiltration of neutrophils.

represents variants that arose because of the selective pressure of the PCV. Alternatively, NESp may have been in circulation and are now being detected through screening for disease isolates following the introduction of the vaccine. It is clearly known that NESp do play a role in human disease.

NESp have been primarily associated with upper respiratory diseases such as OM, sinusitis, adenoiditis, and pneumonia (Keller et al., 2016b). These NESp naturally lack a polysaccharide capsule but are able to compensate, survive within the host, and cause infection. NESp form biofilms and are efficient in taking up DNA from other strains. NESp can be resistant to multiple antibiotics. Thus, NESp may serve as a resistance reservoir for encapsulated strains and possibly other bacterial strains.

The mechanism that allow NESp to cause disease in the absence of pneumococcal capsular polysaccharide is not fully understood. At least three virulence factors have been identified that are encoded in place of the genes required for capsule production. These are PspK, AliC, and AliD. PspK has been demonstrated to increase adherence to epithelial cells and is required for full virulence in OM in the chinchilla model. AliC and AliD are permeases that transport small peptides. The peptides act as environmental signals that cause a change in the bacterial surface depending on its location within the host. These changes have been shown to lead to increased complement resistance when AliC and AliD are expressed. This has implications for OM and other NESp infections.

Encapsulated pneumococci have additional factors that allow them to cause OM. Pneumolysin, a pore forming toxin, and pneumococcal surface proteins are involved in pneumococcal virulence in the chinchilla model of OM (Keller et al., 2016a). Additionally, DNA methylation can alter pneumococcal gene expression and plays a role in virulence.

NTHi has increased as a causative agent of OM. There are a number of surface exposed virulence factors that are being studied. These include adhesins that bind to lectins within the airway. Also, surface proteins have been shown to bind to human extracellular matrix which can contribute to persistence in the middle ear.

Like most airway pathogens, NTHi exist in biofilms during disease. DNA plays a role in the formation of biofilm by NTHi. These structures are important both in colonization and OM. The formation of biofilm during infection reduces the chances of clearance of the NTHi. Also, the regulation of gene expression within the host environment contributes to the ability of NTHi to cause disease. For example, the lack of iron in the middle ear leads to increased expression of iron acquisition genes by NTHi.

M. catarrhalis is a cause of OM in children. Like NTHi, adhesins are important in the attachment of *M. catarrhalis* to human epithelial cells and extracellular matrix. *M. catarrhalis* has a family of trimeric autotransporter adhesins (TAAs) that act as multi-functional virulence factors. Proteins among this family are thought to play a role in blocking complement activation which is the first line of defense against many pathogens.

Biofilm formation by *M. catarrhalis* is also important for persistence in the middle ear. Extracellular DNA is an important part of the scaffolding of the biofilm. Regulation of a nuclease that can degrade extracellular DNA could allow for aggregation of *M. catarrhalis* early in infection and then release of the bacteria later in infection which could lead to spread.

Co-infection of the middle ear with more than one bacterial species at a time can contribute to disease progression. *M. catarrhalis* produces a beta-lactamase that can inactivate penicillin. Thus an OM co-infection of *M. catarrhalis* and *S. pneumoniae* could result in protection of the pneumococcus from penicillin therapy.

Complications

AOM often resolves spontaneously without any complications. In some cases there can be perforation of the tympanic membrane. Perforations can be acute or chronic. This can progress to CSOM. Also, recurrent episodes of OM can lead to scarring of the tympanic membrane which results in hearing loss. In rare cases there can be intracranial complications such as meningitis. Morbidity can be significant despite the use of systemic antibiotics for treatment of OM. In fact, the systemic antibiotics can cause problems by altering the normal bacterial flora outside of the targeted area.

Otitis externa

Acute otitis externa (AOE), commonly known as swimmer's ear, can occur in up to 10% of the population. It presents as a generalized inflammation of the external auditory canal (EAC) and is almost exclusively a bacterial infection. Infection can occur in any age group but is more frequently seen in 2–18 year olds with a peak incidence in 5–14 year olds. Also, cases have been linked to the use of aural irrigation instruments contaminated with bacteria. When infections do occur, bacteria cause >90% of AOE with *Pseudomonas aeruginosa* being the most common causative isolate. AOE results in a substantial personal burden due to increased medication use and an inability to carry out daily activities.

The EAC is a tube running from the outer ear to the tympanic membrane and is covered by a thin layer of skin. Cerumen is thought to be an important component in the defense of the EAC against infection. Cerumen is acidic and contains lysozyme which may impede bacterial growth to help protect the EAC. It is likely that changes in the EAC environment can predispose the EAC to infection.

P. aeruginosa is an opportunistic pathogen that causes a range of infections, is present in many different environments, and is often part of the skin flora of humans. It is likely that the external ear canal is frequently exposed to *P. aeruginosa*. However, the mechanisms by which *P. aeruginosa* can establish an infection in the ear canal and persist are largely unknown. The pathogenesis of

P. aeruginosa is complex. There is variability in *P. aeruginosa* isolates compared to those that cause AOE. This suggests that there may be specific pseudomonas virulence factors that are required for infection of the EAC. Identification of these virulence factors, and their role in AOE could result in better interventions and possible prevention of infection.

There have been a number of studies that have identified risk factors associated with AOE. Probably the most significant risk factor is swimming in fresh water. Previous episodes of AOE can be a risk factor as well as the regular use of devices that block the ear canal. This includes hearing aids, ear plugs, and earphones. These devices can cause trauma and irritation to the skin in the ear canal predisposing to infection. Hot humid weather can be a contributing factor. Also, excessive cleaning of the ear canal can remove ear wax which can potentially lead to infection. Comorbidities such as diabetes and malnutrition can predispose to infection of the EAC.

The overall impact of AOE is not known, but it has been estimated that millions of prescriptions are processed in the United States each year as a result of this infection. This does not take into account physician visits, loss of productive time, and personal discomfort. Often, AOE is thought to be a self-limiting and self-resolving disease. Current clinical guidelines recommend topical treatment for AOE without complications. However, many patients with uncomplicated AOE receive systemic antibiotics. Clearly there is a need to better understand this medically important infectious disease.

Complications resulting from AOE are usually uncommon. Some individuals will have repeated episodes of AOE or persistent inflammation of the EAC for more than 3 months. This condition is known as chronic otitis externa. Other complications may include abscesses in the EAC which are painful pus-filled growths persisting after the infection. Stenosis, a narrowing of the ear canal, can result from a build-up of thick dry skin. This is more likely to occur as a result of chronic otitis externa. Cellulitis may occur as a result of AOE. The bacteria may penetrate deeper layers of the EAC skin as a result of damage during AOE. This would require more extensive treatment to resolve. Malignant otitis externa is a very rare but serious complication of AOE that occurs mainly in immunocompromised adults. The infection spreads to the bone surrounding the EAC.

Summary

Bacterial ear infections can occur in anyone at any age. OM is an infection of the middle ear. This infection occurs primarily in young children and remains the main reason for sick baby visits to the pediatrician. This is true even with available vaccines. The economic burden is not just that of the doctor visit, but also, there is the lost work time for parents that have to tend to the child. AOE has different concerns because it tends to occur in people of a productive age and can impact their ability to work. The range of infections involving the ear can be from very mild to severe with significant complications. There needs to be a continued focus on understanding host-pathogen interactions in the diseases of the ear.

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Ear Infection and Otitis Media Infections: Virus

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Acute middle ear infection, acute otitis media (AOM) is one of the most common infectious diseases in children; about two thirds of children have at least one AOM episode by the age of 2 years (Nokso-Koivisto et al., 2015; Schilder et al., 2016). In adults, acute otitis media occurs rarely, but the role of viruses in the pathogenesis of acute middle ear infection in adults do not differ from children. The role of viruses in the pathogenesis of middle ear infection with effusion is uncertain and studies very limited. Thus, this chapter will focus on the role of respiratory viruses in acute middle ear infections.

AOM is considered to be a bacterial complication of viral upper respiratory tract infection (URI). However, viruses alone can cause AOM. The high incidence of AOM results from the high burden of viral URIs. About 35% of URI episodes are complicated by AOM, occurring mainly within the first week of URI onset.

The association between respiratory viruses and AOM

Infections caused by respiratory viruses are the most common diseases in children around the world. Viral upper respiratory infection is usually mild and self-limiting, but sometimes complicated by bacterial infections such as AOM. Multiple respiratory viruses are of hundreds of different serotypes which can cause the common cold or respiratory infection leading to AOM.

The association of viral infections and development of AOM has been shown in several different studies (Chonmaitree et al., 2015, 2016; Nokso-Koivisto et al., 2015). An epidemiologic association between the outbreaks of viral infections and a simultaneous increase in AOM incidence has been demonstrated e.g., in a 14-year longitudinal study in which concurrent or previous respiratory infection was associated with one-fourth of AOM episodes (Henderson et al., 1982).

Viral respiratory infections have been shown to alter middle ear pressure, which in turn can cause accumulation of mucus and pathogenic agents in the middle ear. Studies of experimental and natural viral infections in children and adults have shown that up to two thirds of patients develop negative middle ear pressure during upper respiratory infection and may develop middle ear effusion negative for bacteria.

Although AOM is often bacterial or viral-bacterial co-infection, there is evidence that respiratory virus alone can cause AOM. Experimental studies in adults and in chinchillas have shown that induced virus infection can lead to the development of AOM. It has been reported that children infected with respiratory syncytial virus (RSV) have an increased risk of AOM even without colonized bacteria (Ruohola et al., 2013). In addition, respiratory viruses have been detected in majority of nasopharyngeal specimens and up to 70% middle ear fluid samples in children with AOM, and viruses or viral nucleic acids have been detected from middle ear fluid of children with AOM even in absence of bacteria. Table 1 presents selected studies of viruses detected from middle ear fluids at the time of AOM including the very first study from the year 1955 (Yoshie (1955)). The table also demonstrate the development of viral detection methods from culture to antigen detection and polymerase chain reaction (PCR), and the difficulties in detecting viruses. The wide variety in the proportion of virus positivity in middle ear fluids is mainly due to difficulties in isolating viruses and different sensitivities of the tests, although seasonal variation of the viral infections may have a role. In addition, as all respiratory viruses are not detectable with a single test, combining different detection methods (culture, antigen detection, PCR, serology) has been necessary to cover most of the important causative viruses. For example in a study (Ruohola et al., 2006) using multiple detection methods, the proportion of virus positive middle ear fluids was the highest. Lately PCR has been the main detection method, as it is usually more sensitive than viral culture or antigen detection methods and has been developed for many respiratory viruses.

Evidence of an association between viruses and AOM has also been based on virus-specific intervention studies. AOMs occurring during influenza outbreaks can be prevented by immunizing children with influenza virus vaccine or by giving specific antiviral treatment at the time of influenza virus infection (Block et al., 2011; Heikkinen et al., 2013; Heinonen et al., 2010). In addition, high doses of RSV-enriched immunoglobulin have been reported to prevent development of AOM in high-risk children (Simoes et al., 1996).

Table 1 Selected data from studies of viruses detected from middle ear fluid (MEF) in children with acute otitis media (AOM).

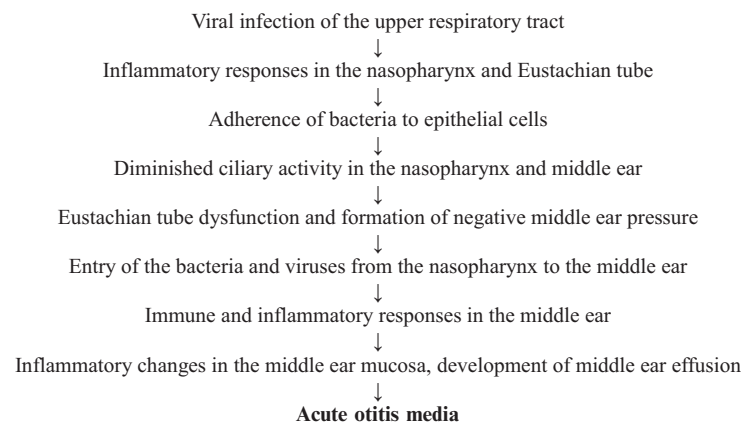
Study	No. of children with AOM	No. of MEF	Virus detection method ^a	Proportion of virus-positive MEF (%)
Yoshie (1955)	10	10	Culture	40
<i>Ag detection included</i>				
Klein et al. (1982)	53	53	Ag	25
Heikkinen et al. (1999)	456	815	Culture, Ag	17
<i>PCR included</i>				
Pitkäranta et al. (1998)	92	92	PCR	48
Chonmaitree and Henrickson (2000)	40	65	Culture, PCR	74
Nokso-Koivisto et al. (2004) ^b	940	3210	Ag, PCR	38
Ruohola et al. (2006)	79	79	Culture, Ag, PCR	70
van Dongen et al. (2015)	230	230	PCR	21
Yatsyshina et al. (2016)	179	216	PCR	15

^aAg, antigen detection; PCR, polymerase chain reaction.^bFollow-up study with multiple visits of the same study children.

Pathogenesis of virus-induced AOM

AOM usually occurs concurrently or just after upper respiratory tract infection. More than 90% of children with AOM have previous or concurrent respiratory infection. The three most common bacterial otopathogens: *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae*, and *Moraxella catarrhalis* can colonize the infant's nasopharynx from early age. However, usually these pathogens do not infect the respiratory tract or cause symptoms until viral infection, which causes nasopharyngitis and changes of the nasopharyngeal milieu. The coexistence of bacterial otopathogens and the interactions between bacteria and respiratory viruses effect on the respiratory infection course and eventually on AOM outcome. To current knowledge, viral infection is needed to start this cascade of development of AOM.

The role of viruses in the pathogenesis of AOM has been studied in experimental animal models, in vitro studies, studies of adult volunteers infected with respiratory viruses, and studies of children with URI (Nokso-Koivisto et al., 2015; Schilder et al., 2016; Thornton et al., 2020). At start respiratory viruses cause inflammation of the nasopharynx and the Eustachian tube, generating host immune and inflammatory responses, including the generation of cytokines, chemokines and inflammatory mediators. Viral infection also increases nasopharyngeal colonization and adherence of bacteria to epithelial cells, and diminish the normal ciliary clearance of the nasopharyngeal and tympanomastoid mucosal cells. This leads to Eustachian tube dysfunction and negative pressure in the middle ear and entrance of colonized bacteria and respiratory viruses from the nasopharynx into the middle ear cavity. Thus the middle ear mucosa is infected, leading to middle ear fluid accumulation and development of signs and symptoms of AOM (Fig. 1).

**Fig. 1** Pathogenesis of virus induced acute otitis media.

Viruses as AOM pathogens

The most common respiratory viruses in humans are adenoviruses, human bocaviruses, coronaviruses, enteroviruses, influenza viruses, human metapneumoviruses, respiratory syncytial virus (RSV) and rhinoviruses. Any respiratory virus that can cause upper respiratory infection can initiate development of AOM as described earlier. However, it has been suggested that certain viruses are more likely to cause AOM than others, to be more “ototropic.” Among the common respiratory viruses, RSV has long been identified as AOM-associated virus. Because of the high prevalence of rhinovirus-URI in general, even without an increased rate of AOM development after URI, rhinoviruses are also considered common AOM-associated viruses. Respiratory virus testing of more than 96,000 specimens from children over 8 years showed that detection of RSV, human metapneumovirus and influenza was temporally associated with increased diagnoses of AOM (Stockmann et al., 2013).

With the widespread use of molecular diagnostics, respiratory viruses have been detected in up to 50% of asymptomatic young children. However, it has been shown that AOM development following URI requires an inflammation severe enough to initiate the expression of URI symptoms. It has been shown that AOM does not occur following asymptomatic viral infections, but symptomatic URI can lead to AOM in 27% of children in the first year of life (Chonmaitree et al., 2015).

Role of specific virus types in AOM

RSVs are some of the most important respiratory viruses to cause infections in children. RSV infections are present in all age groups, but they predominate in children and especially in infants. Up to 70% of infants have been reported to be infected with RSV during the first year of life, and the rest are infected by the age of 2 years. RSVs are the leading cause of bronchiolitis and acute wheezing in young children. Typically, RSV epidemics occur during the winter months, sometimes starting in the late fall and continuing until early spring. It has been suggested that RSV infection often leads to AOM, and AOM can develop in almost two thirds of the children with RSV infection.

Human metapneumovirus is a virus related to RSV and the clinical symptoms caused by metapneumovirus are similar to those due to RSV. This virus is an important cause of lower respiratory infections and wheezing in children, but it does not seem to be prone to cause AOM. Metapneumovirus infections occur mainly between winter and early spring.

Human rhinoviruses are important in AOM development since rhinoviruses are the predominant cause of the common cold all over the world and in all age groups. Many follow-up studies have shown that rhinoviruses are the most commonly detected viruses at the time of URI and often associated with AOM in children. Rhinovirus infections typically occur in the early fall and in the spring.

Enteroviruses, which belong to the same virus family Picornaviridae as rhinoviruses, are very common worldwide, and most primary infections occur in childhood. Often enteroviruses have been thought to cause mild diseases with characteristic signs (e.g., hand, foot, and mouth disease and herpangina) or more severe diseases such as meningitis. However, it has been shown that enteroviruses are also a common cause of URI and AOM in children.

There are three types of influenza viruses in humans, types A, B and C. However, most data about influenza viruses are for types A and B because, due to detection difficulties, influenza C viruses have not been included in the studies. Influenza type A and B cause infections that can range from asymptomatic infections and common colds to serious illnesses with systemic complications, such as pneumonia. Influenza A and B infections typically occur in a seasonal pattern and in winter epidemics, which have variable intensities. Especially influenza vaccine and treatment studies have shown that prevention of influenza infections will reduce the incidence of AOM and influenza viruses are important cause of AOM.

Human bocavirus has been associated with respiratory infections and has been suggested to be ototropic (Nokso-Koivisto et al., 2015). The possible prolonged presence of bocaviruses in the nasopharynx have made the causality of virus detections to the disease difficult to interpret. However, population-based studies with control groups have shown associations between bocavirus detection (high viral load) or seropositivity and symptoms of URI and the diagnosis of AOM.

It seems that human coronavirus infections are not very common cause of respiratory infections in young children, whereas later in life, clinical and subclinical infections occur more often. Pandemic coronavirus COVID-19 does not seem to be prone to cause AOM.

Viral-bacterial interactions in AOM

The most important risk factors for AOM development are the colonized bacterial otopathogens in the nasopharynx, the presence of respiratory viruses and their complex interactions (Marom et al., 2012). Respiratory viruses pave the way for otopathogens. In children with URI, those who are colonized with otopathogens are more likely to have concomitant AOM than those who are not. AOM risk seems to differ by the specific viruses and bacteria involved, e.g., detection of *M. catarrhalis* together with RSV or rhinovirus from the nasopharynx increases the risk of AOM (Chonmaitree et al., 2016). Studies have also aimed to determine the level of bacterial and viral loads that are sufficient enough to cause AOM, but the results so far have been conflicting.

The presence of respiratory viruses in the nasopharynx is not enough for the development of AOM; viral replication and the symptoms caused by the viral infection are also necessary. In animal studies e.g., infection with adenovirus increased the proportion of *S. pneumoniae* AOM compared to infections with bacteria alone. However, infection with nonreplicating adenovirus did not

increase the incidence of bacterial AOM (Murrah et al., 2015). Nasopharyngeal microbiota studies have also shown that the more URIs the children have in the first months of life, the more otopathogens there are in the nasopharynx. In addition, the presence of otopathogens is associated with symptomatic viral infection (Chonmaitree et al., 2017).

Viruses in AOM -Clinical implications

The knowledge on the importance of viruses in AOM has clinical implications, specifically on the treatment and prevention. The overall goal would be prevention of viral URI and to reduce or prevent nasopharyngeal colonization of bacterial otopathogens. In addition to reducing environmental risk factors for URI and AOM, the use of bacterial and viral vaccines would make a significant impact.

Prevention of viral URI is complicated because of the variety of numerous respiratory virus types that are associated with URI. So far, the only effective and widely used respiratory virus vaccine in influenza vaccine. It has been shown that trivalent, inactivated influenza vaccines and live-attenuated influenza vaccines effectively prevent influenza and influenza-associated AOMs (Block et al., 2011; Heikkinen et al., 2013). Surprisingly, intranasal live-attenuated influenza vaccines can also decrease the overall rate of AOM during influenza season despite the cause of AOM (Heikkinen et al., 2013). In addition, there has been studies on prevention of AOM by early treatment of influenza in children; treatment with oral oseltamivir significantly reduced new AOM in young children with laboratory-confirmed influenza infection (Heinonen et al., 2010).

Unfortunately, no other vaccines for common respiratory viruses are available. Any future progress towards vaccination against common respiratory viruses in addition to bacteria would have an impact on reducing the burden of AOM (Alderson et al., 2020).

Respiratory viruses in otitis media with effusion

It has been suggested that respiratory viruses, along with bacteria, also have a role in otitis media with effusion (OME) by being a latent infections in the middle ear or by presenting a pathogen reservoir in the nasopharynx and adenoid. Respiratory viruses have been detected from the adenoid tissue and middle ear secretion or middle ear washes of children with OME. However, respiratory viruses have been detected in similar proportions of the children without OME. The role of the viruses in the pathogenesis is uncertain and studies limited. Respiratory viruses do not seem to have major role in OME.

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Ear Infections: Fungi

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Otomycosis in history

Andral and Gavarret (1843) were the first to describe a case of otomycosis in 1843; a year later, Mayer reported a disease of the external auditory canal caused by a fungus, documenting the presence of a “mycotic parasite in the push of a draining ear” (Mayer, 1844). Highet wrote: “*Otomycosis, or the growth of fungus in the external auditory meatus, is apparently a rare disease in temperate climates if one may judge from the scanty literature on the subject. During eight years’ work in Singapore and Bangkok, however, my experience has been that it is quite a common—if not the most common—disease affecting the external auditory meatus which one meets with in the tropics*” (Highet, 1900).

Regarding the etiology, Hall stated that the first fungus to be isolated from ears was identified as an *Aspergillus* by Prof. Schwartz of Halle (Hall, 1877). *Aspergillus fumigatus* was cited in a paper of Whale (1915) and *Mucor mucedor* is described in McKenzie (1926). One of the first etiological classifications of otomycosis appeared with Wolf, who created a list of fungi responsible for this pathology: Aspergillaceae (*Aspergillus*, *Penicillium* and *Scopulariopsis*), Mucoraceae (*Lichtheimia*, *Mucor*, *Rhizopus* and *Syncephalas-trum*), Yeast-like fungi, Dermatophytes, Actinomycetaceae and miscellaneous (Wolf, 1947). Haley distinguished from kinds of fungi isolated from normal ears in contrast with those isolated from infected ears (Haley, 1950).

From the 1950s until the 1990s, attention has been drawn to the increasing problem of otomycosis and its prevalence (Blank and Stuart, 1955; Holt and Morgan Jr, 1958; Gregson and La Touche, 1961; Sood et al., 1967; Youssef and Abdou, 1967a,b; Yamashita and Yamashita, 1972; Yassin et al., 1978; Mugliston and O’Donoghue, 1985; Chow and Ho, 1986), pointing out especially cases of aspergillosis and dermatophytosis of the external auditory meatus (Stuart and Blank, 1955; English, 1957; Ismail, 1962; Yassin et al., 1964; Watanabe, 1986; Stern and Lucente, 1988), despite some authors denying the existence of this pathological entity—“*Infections of the ear with pathogenic fungi have something in common with the snakes of Ireland; there are none*”—(Kingery, 1965).

The first experiments were conducted to explain the increased incidence of otomycosis during the warm seasons, finding that there are some factors that equally contribute together in the etiology: the pH value, the incubation temperature, the humidity and the alkalinity of water supply (Ismail, 1962; Yassin et al., 1964). Furthermore, some authors redefined the term otomycosis to include fungal infection of the external ear, middle ear and open mastoid cavity (Yassin et al., 1964; Paulose et al., 1989).

Otomycosis of the external ear

In terms of superficial infections, otomycosis represents about the 10–30% of the all the affections related to the external auditory meatus (EAM) (Pontes et al., 2009; Alarid-Coronel et al., 2018).

Risk factors

The anatomy of the EAM simulates a culture medium with skin that guarantees ideal conditions for fungal growth, and a narrow auditory canal facilitates the development of otomycosis, especially if is associated with eczema and dermatitis (Prasad et al., 2014). Furthermore, warm and humid climate, the use of earphones/earplugs, swimming in polluted waters, self-injury (Yassine and Haiet, 2018), metabolic disorders and/or immunodeficiencies (Patel et al., 1999; Viswanatha and Naseeruddin, 2011; Viswanatha et al., 2012),

malnutrition, abuse of topical eardrops (Schrader and Isaacson, 2003; Jackman et al., 2005) represent well known risk factors (Enweani and Igumbor, 1997; Tasić-Otašević et al., 2020), even if fungal organisms (especially *Candida* spp.) may be isolated from cases of otitis externa without risk factors for fungal infections (Enoz et al., 2009).

Geography

Prevalence of otomycosis in tropical and humid environments, particularly during rainy months, has been described by many authors (Stern and Lucente, 1988; Paulose et al., 1989; Pontes et al., 2009; Prasad et al., 2014; Gughani et al., 1989; Ozcan et al., 2003a,b; Kumar, 2005; Fasunla et al., 2007; Aneja et al., 2010).

Regarding otomycosis' epidemiology, this pathology is widespread worldwide (Yehia et al., 1990; Zaror et al., 1991; Pradhan et al., 2003; Dorko et al., 2004; Jia et al., 2012; Adoga and Iduh, 2014; Ismail et al., 2017; Li and He, 2019), and many studies were conducted in Middle East countries, especially Iran (Barati et al., 2011; Saki et al., 2013; Nemati et al., 2014; Kiakojuri et al., 2015; Gharaghani et al., 2015; Kazemi et al., 2015; Abastabar et al., 2019; Aboutalebian et al., 2019; Sabz et al., 2019; Kamali Sarwestani et al., 2019).

Sex, occupation and age

The gender prevalence is heterogeneous: some authors report that otomycosis was seen in women more than in men (Yehia et al., 1990; Fasunla et al., 2007; Pontes et al., 2009; Aneja et al., 2010; Barati et al., 2011; Jia et al., 2012; Kiakojuri et al., 2015; Nemati et al., 2014; Fayemiwo et al., 2010), others say the opposite (Mugliston and O'Donoghue, 1985; Pradhan et al., 2003; Yehia et al., 1990; Prasad et al., 2014; Garcia-Martos et al., 1993; Vennewald and Klemm, 2010; Garcia-Agudo et al., 2011; Degerli et al., 2012; Agarwal and Devi, 2017).

The highest incidence of otomycosis was found inside rural communities (Prasad et al., 2014; Agarwal and Devi, 2017; Abdelazeem et al., 2015) and in people of lower socioeconomic status (Barati et al., 2011; Aneja et al., 2010). The mean age was estimated between 21 and 30 years old by Prasad et al. (2014), and between 15 and 35 years old for Agarwal and Devi (2017), but Jackman et al. (2005) reported that "the number of paediatric patients diagnosed with otomycosis has increased over the past decade" (Westby et al., 2020).

Microbiology

Among the latest studies focused on causative fungal pathogens of external otitis, we find respectively those conducted by Abastabar et al. (2019), Aboutalebian et al. (2019), Rath et al. (2019), Sabz et al. (2019) and Tasić-Otašević et al. (2020).

The most frequent fungi associated with otomycosis are *Aspergillus* spp. and *Candida* spp. (Abdelazeem et al., 2015; Agarwal and Devi, 2017; Jadhav et al., 2003; Ozcan et al., 2003a,b; Hueso Gutiérrez et al., 2005; Ali et al., 2018; Hagiwara et al., 2019; Tasić-Otašević et al., 2020). Regarding *Aspergillus* spp., many updated molecular studies found black *Aspergillus* species other than *A. niger* as the most frequent causative agents (Szigeti et al., 2012; Kamali Sarwestani et al., 2018), that is in contrast with the previous known microbiological spectrum of otomycosis. In this sense, molecular studies should be mandatory to provide specific results related to the microbial epidemiology of fungal infections of the ear. According to Aboutalebian et al. (2019), if sequence analysis had not been adapted, basing on morphology, all isolated *A. tubingensis*, *A. niger*, *A. awamori*, *A. foetidus*, etc. would be identified as *A. niger*, making this organism the most frequent genera. In fact recently Sabz et al. (2019) demonstrated that, in their analysis, *A. tubingensis* was the dominant causative agent of otomycosis.

Some researches prove non-albicans *Candida* species domination, at first place *C. parapsilosis* followed by *C. guilliermondii*, *C. glabrata*, *C. tropicalis* (Kurnatowski and Filipiak, 2001; Garcia-Agudo et al., 2011; Aboutalebian et al., 2019); the majority of *Candida* strains isolated are protease positive (Arsović et al., 2009). Moreover, *C. auris* has been proven to be an emerging otomycotic pathogen (Abastabar et al., 2019; Satoh et al., 2009; Pekard-Amenitsch et al., 2018).

Combined infections (*Aspergillus* + *Candida*) must also to be taken into consideration, even if they are demonstrated in only the 5.5% of patients (Tasić-Otašević et al., 2020). In this group, recurrences and chronic entities may be avoided choosing the right drugs' combination, effective against both yeasts and molds (Jia et al., 2012).

Regarding less common pathogens, we find *Exophiala dermatitidis* (Kerkmann et al., 1999), *Trichoderma longibrachiatum* (Hennequin et al., 2000), *Trichophyton rubrum* (Buzina et al., 2004), *Aspergillus sclerotiorum* (Harima et al., 2004), *Malassezia* spp. (Latha et al., 2010) *Chaetomium brasiliense* (Hubka et al., 2011), *Pseudallescheria apiosperma* (Neji et al., 2013), *Scedosporium apiospermum* (Huguenin et al., 2015; Salamat et al., 2015), *Schizophyllum commune* (Matos et al., 2016; Maeda et al., 2019), *Scedosporium prolificans* (Wen et al. 2016), *Saksenaea vasiformis* (Trabelsi et al., 2020).

Signs and symptoms

Even if fungi were often isolated in patients without clinical signs of fungal infection (Saunders et al., 2011), symptoms of otomycosis include otalgia, itching, hearing loss and tinnitus (Ho et al., 2006; Anwar and Gohar, 2014), while signs of fungal infection could be otorrhoea and discharge. Aspergillosis is characterized by the presence of large keratinic scales with a sort of wet tissuepaper aspect. Typically the manifestation of *A. niger* includes black tufts and spots (Fig. 1), while *A. fumigatus* looks greenish.

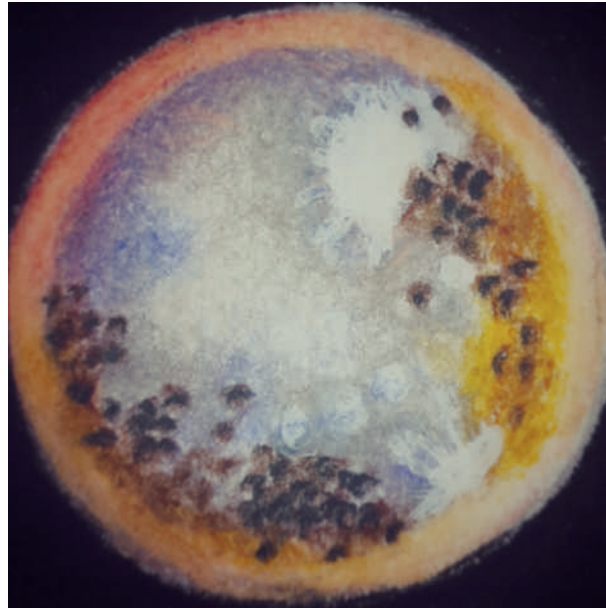


Fig. 1 Otoscopic view of external otomycosis (*A. niger*).

Candida usually provokes a significant edema of the EAM with skin maceration and white creamy secretions; much more rarely otomycosis could occur with aural mass (Patel et al., 1999; Hoshino and Matsumoto, 2006). Ear fungal infections can also lead to tympanic membrane perforations (Pradhan et al., 2003; Jia et al., 2012; Hurst, 2001; Abou-Halawa et al., 2012; Song et al., 2014) and sometimes the presentation is bilateral (Mishra et al., 2004; Kirschner et al., 2017).

We can divide fungal infections in:

- Primary otomycosis (in the EAM of an immunocompetent patient, no tympanic membrane perforation, no middle ear pathology)
 - a) without external otitis
 - b) with external otitis, previously absent (that is, due to otomycosis and not vice versa)
- Secondary otomycosis (pre-existing otitis media or externa, history of trauma and/or otosurgery, history of and/or fungal infection of other anatomical districts)
 - a) without immunocompromise
 - b) with immunocompromise (Prasad et al., 2014).

Diagnosis

Apart from patients' history and direct otoscopy, conventional diagnostic methods include microscopic examination of the samples and also immunofluorescence microscopy (Gurr et al., 1997), but the gold standard is represented by detection and identification of the source of fungal infection through the culture of ear secretions on specific growth media (Tasić-Otašević et al., 2020). In well-equipped Units, antimycogram is done to study sensitivity of strains to drugs in vitro. However, recent researches are focused on molecular diagnosis (Hagiwara et al., 2019) and matrix-assisted laser desorption ionization time of flight mass spectroscopy (Rath et al., 2019). Radiologic studies as computed tomography and resonance magnetic imaging are more useful in case of suspected fungal malignant external otitis and fungal middle ear otitis (see below).

Fungal malignant external otitis

The first detailed reports of fungal malignant external otitis (FMEO) are described in 1985 by Petrak et al. (1985), Bickley et al. (1988) and Cunningham et al. (1988)—although, a report of mixed *Serratia* and *Aspergillus* infection has been briefly mentioned in a series from Fisher et al. (1981). Invasive external otitis generally occurs in patients with underlying pathologies (diabetes, immunodeficiencies, hematologic illnesses, etc.): this applies both for bacterial (especially *Pseudomonas aeruginosa*) and fungal forms (Petrak et al., 1985; Bickley et al., 1988; Cunningham Phillips et al., 1990; Reiss et al., 1991); among other things, these two forms can be added together (Bovo et al., 2012).

FMEO is characterized by an aggressive infection that develops from the EAM and spreads along the soft tissues (Noroy et al., 2020) and bones of the skull base (Chandler, 1968). Symptoms are non-specific and include ear pain, otorrhea, hearing loss and

possible facial nerve palsy (Lilic et al., 2012; Walton and Coulson, 2014). From a demographic point of view, FMEO affects in primis elderly diabetic patients (Tarazi et al., 2012), the 75% of which present intense otalgia, especially during night (Bovo et al., 2012) while purulent otorrhea ranges from 50% and 80% of the cases (Bovo et al., 2012). The histologic examination typically shows granulation tissue with non-specific signs of inflammation (Bovo et al., 2012).

Computed tomography and magnetic resonance imaging can document swelling of the EAM, involvement of the mastoid, bones erosion, tissue infiltration and/or complications as skull base osteomyelitis and abscesses (Pichon et al., 2020), on the basis of the stage of the pathology (from a limited infection of the EAM and the nearby soft tissue, to a non-complicated osteitis or mild cranial nerves neuropathies, to infiltration of intracranial structures and neck spaces). Technetium Tc 99m, methylene diphosphonate scintigraphy (bone scan) result positive in almost 100% of MEO, allowing early diagnosis of osteomyelitis. Gallium citrate Ga 67 accumulates in soft tissues' inflammation and bone infections and can be beneficial in the diagnosis recurrent disease (Vennwald and Klemm, 2010).

The most common causative pathogens are *Aspergillus* spp.: after *A. fumigatus* (van Tol and van Rijswijk, 2009; Clark et al., 2011), *A. flavus* is the second mycotic cause (Pichon et al., 2020; Hedayati et al., 2007); less frequent are *A. niger* (Bellini et al., 2003; Zriba et al., 2014) and *A. wentii* (Halsey et al., 2011), followed by *Candida* spp. (Bae et al., 2007; Mani et al., 2008; Hamzany et al., 2011; Elayoubi et al., 2016). Much more rarely we find *Malassezia sympodialis* (Chai et al., 2000), *Scedosporium apiospermum* (Yao and Messner, 2001; McLaren and Potter, 2016; Doss and Doss, 2018), mucormycosis (Tuzcu et al., 2006) and *Scopulariopsis brevicaulis* (de Miguel-Martinez et al., 2018).

The diagnosis of FMEO is often delayed:

- generally, anti-pseudomonal treatment is started even if the bacterial samples don't show growth, so frequently it takes a long time to change management strategy;
- culturing fungi from tissue-collected specimens yields a very low rate of positive results;
- there isn't clear clinical, laboratory or radiological finding that allows to differentiate fungal from bacterial infection, except for tissue sampling for direct smear or polymerase chain reaction (Gruber et al., 2015), and specific fungal cultures. (Abu Eta et al., 2018).

Thus, culture of biopsy samples and histologic examination are mandatory in cases of persistent ear pain that resists to apparently appropriate antimicrobial therapy (Pichon et al., 2020; Gordon and Giddings, 1994).

The consequence of FMEO can be dramatic, with described dissemination from the ear to other organs (Harley et al., 1995), skull base osteomyelitis (Shelton et al., 2002) and jugular thrombosis (Moniot et al., 2019).

About the therapy, it will be discussed in more detail later: generally, the treatment can include conservative solutions (Finer et al., 2002; Ling and Sader, 2008; Marchionni et al., 2016), sometimes in association with more aggressive approaches (Marzo and Leonetti, 2003).

It was demonstrated that the efficacy of FMEO therapy depends on the assessment of cranial nerve state, the causative pathogen and imaging findings: absence of facial nerve palsy, *Aspergillus* spp. and absence of radiological signs correlate with better prognosis (Mion et al., 2015).

Fungal otitis media

Smyth evidenced that "Towards the end of 1956, fungi were repeatedly isolated from a high proportion of chronically infected postoperative mastoid cavities", pointing out the relevance of fungal infections of the middle ear (Smyth, 2006).

Those at greatest risk are agricultural field workers (Wadhvani and Srivastava, 1984) and immunocompromised patients: in this last group, *Pneumocystis carinii* is particularly common as causative pathogen (Gherman et al., 1988; Smith et al., 1988; Sandler et al., 1990; Park et al., 1992; Menger and v d Berg, 2003). Other fungi responsible for middle ear infections are *Aspergillus* spp. (Talwar et al., 1988; Hall and Farrior, 1993; Chen et al., 1999; Tiwari et al., 1995; Ibekwe et al., 1997; Yates et al., 1997; Juyal et al., 2014) that can affect predisposed subjects as in case of autoimmune disease (Amonoo-Kuofi et al., 2005), but also immunocompetent patients (Ohki et al., 2001; Youssef et al., 2014); *Candida* spp. alone (Kurnatowski and Filipiak, 2001; Martin et al., 2005; Choi et al., 2017) or in association with *Aspergillus* spp. (Punia et al., 2019). More rarely we find *Scedosporium apiospermum* (Bhally et al., 2004; Baumgartner et al., 2007; Koay et al., 2015), *Blastomyces dermatitidis* (Istorico et al., 1992; Nguyen et al., 2013), *Apophysomyces elegans* (Goyal et al., 2007), entomophthorales (Arya et al., 2011) and *Paracoccidioides brasiliensis* (Lucinda and Polanski, 2017).

Two cases of fungal allergic otomastoiditis have been described (Chen and Chiang, 2013; Salamah and Al-Shamani, 2019), showing the existence of allergic reactions to fungi in the middle ear, as well as the presence of fungal hyphae have been demonstrated in fluids collected from eosinophilic otitis media (Murakami et al., 2012); furthermore, fungal colonizations of cholesteatoma are documented (Singh et al., 2018).

Age ranges from childhood to elderly subjects; signs and symptoms don't differ from those of classic middle otitis, as well as imaging findings—for instance, presence of soft tissue density in the mastoid antrum, mastoid air cells and/or in the middle ear, with or without destruction of tegmen tympani—(Punia et al. 2019).

The complications of fungal middle otitis can be very serious: from otomastoiditis and tympanic membrane perforations (Koltsidopoulos and Skoulakis, 2020) to skull base osteomyelitis (Menachof and Jackler, 1990; Stodulski et al., 2006; Marechal et al., 2018), petrous apicitis (Rushton et al., 2004; Eloy et al., 2007; Bhatt et al., 2013) and meningitis (Morgand et al., 2015; Okada et al., 2015). Specific resistance allele found in some strains of fungi can explain the aggressiveness in certain infective forms (Mushi et al., 2016; Zhang et al., 2020).

The diagnosis is usually performed using samples of discharge from middle ear (directly in cases of tympanic membrane perforation or intra-operatively during mastoidectomy), then the mycological examination can be conducted by microscopy, fungal cultures and polymerase chain reaction (Kim et al., 2002; Vennewald et al., 2003).

The histologic examination of surgical specimens can highlight:

- fungal hyphae in the stratum corneum of EAM's epithelium, in the middle ear's cavity or between the lamelle of cholesteatomas;
- chronic hyperplastic/polypoid inflammation of the middle ear's mucosa;
- growth of mycelium in blood vessels and bones (Vennewald and Klemm, 2010).

Fungi in the inner ear

The literature documents only a case of fungal invasion of the inner ear: Haruna et al. (1994) describe the presence of *Mucor* species within internal auditory nerve found during the autoptical examination of a patient died for acute myelogenous leukemia.

Treatment of otomycosis

In 1948, Bryant wrote a "Do nots" list from the observation of 4600 cases of otomycosis:

1. Do not neglect to cleanse the ear thoroughly.
2. Do not neglect to ascertain the presence of acute external otitis by otoscopy and by manipulation of the auricle and pressure on the tragus.
3. Do not treat the otomycosis until the superimposed acute external otitis has been treated and cured.
4. Do not treat otomycosis without first removing all exudate and debris.
5. Do not hesitate to use aqueous solutions for irrigating.
6. Do not neglect to dry the canal thoroughly.
7. Do not use colored preparations in the treatment of the ear at any stage.
8. Do not discontinue the prescription used for otomycosis until 6 weeks have elapsed following cessation of all symptoms and signs.
9. Do not presume that every ear canal containing moist debris indicates mycosis, for all is not mold that litters (Bryant, 1948).

In consideration of the fact that the first studies showed *Aspergillus* spp. as the main causative pathogens, Damato reported the great efficacy of topical Tolnaftate against *A. niger* (Damato, 1973), while Schönebeck and Zakrisson demonstrated the value of topical 5-fluorocytosine (Schönebeck and Zakrisson, 1974)—result confirmed few years later by Lopez and Evens (1980). Cresylate revealed non-specific antimicrobial activity, while nystatin and mostly clotrimazole showed specific antifungal power (Lawrence et al., 1978; Maher et al., 1982; Bassiouny et al., 1986; Stern et al., 1988). Clotrimazole drops, miconazole cream and fluconazole drops showed same therapeutic efficacy (Navaneethan and Yaadhava Krishnan, 2015), furthermore topical use of clotrimazole alone (Abou-Halawa et al., 2012; Dundar and İylen, 2019) or in association with ceftizoxime powder (Mahdavi Omran et al., 2018) appears safe also for otomycosis with tympanic membrane perforations and seems to be more powerful than tolnaftate as a therapy (Jimenez-Garcia et al., 2020) and in the prevention of infections' recurrences (Kiakojuri et al., 2019)—that are estimated around the 7.3%—(Kiakojuri et al., 2018).

About the effect of Aqueous Merthiolate, someone believed that this solution has not specificity against fungi (Lawrence et al., 1978; Stern et al., 1988), others reported a sure effect against otomycosis (Lopez and Evens, 1980; Schneider, 1981).

Some researches are focused on more natural remedies (alone or with a synergistic effect with antifungal drugs) as fixed oils treated with herbal seeds (Jain et al., 1993), garlic extract (Pai and Platt, 1995), volatile vapours of perfumes as musk and jasmine (Jain and Agrawal, 2002) and other organic volatile substances (Jain and Agrawal, 1994), honey and starch (Boukraâ and Bouchehrane, 2007), *Aloe Vera* leaf extract (Saniasiya et al., 2017), thyme oil and clove extracts (Ghaly et al., 2020).

Applications of mercurochrome are suggested in case of external otomycosis (Chander et al., 1996; Mgbor and Gugnani, 2001), while boric acid seems to be less effective compared to other solutions as ciclopiroxolamine and clotrimazole (del Palacio et al., 2002; Romsaithong et al., 2016).

Povidone iodine applications appear valid also against recalcitrant forms of external otomycosis (Philip et al., 2013; Swain et al., 2018; Mofatteh et al., 2018).

Hyperbaric oxygen is useful especially in cases of FMEO, probably for the microangiopathy typical of diabetic patients with the consequent local hypoxia and ischemia: necrotizing inflammation of soft tissues and resistance to osteomyelitis treatment are generally accepted indications for hyperbaric oxygen therapy (Narozny et al., 2006), and many studies proved its efficacy against FMEO due to *Aspergillus* spp. (Bellini et al., 2003; Yao and Messner, 2001; Shelton et al., 2002; Finer et al., 2002).

Regarding in vitro investigations, terbinafine seems to be valid against otomycosis (Rotoli et al., 2001; Karaarslan et al., 2004; Zarei Mahmoudabadi et al., 2015; Yang and Young, 2019) and the last researches confirm these data also with clinical trials (Xu et al., 2020). Isolated studies were conducted on luliconazole (Moslem and Mahmoudabadi, 2020) and clioquinol (Cimolai, 2020).

Munguia and Daniel propose a detailed review of the main ototopical antifungal drugs, finding that the azoles class such as clotrimazole, fluconazole, ketoconazole and miconazole are more effective, followed by nystatin and tolnaftate (Munguia and Daniel, 2008); fluconazole exhibited broad spectrum effect *Aspergillus* spp. and *Candida* spp. (Ahmed et al., 2018).

Many studies were performed on voriconazole: Ayçiçek et al. proved that caspofungin and voriconazole in vitro are effective as amphotericin B and itraconazole (Ayçiçek et al., 2009), even if Kaya et al. found that some *A. fumigatus* and some *A. niger* result resistant against itraconazole (Kaya and Kiraz, 2007), furthermore voriconazole has been shown more potent in vitro than itraconazole (Yenişehirli et al., 2009). In vivo, local application of a 1% sterile solution of voriconazole resulted successful in the treatment of nystatin-resistant *A. niger* otomycosis (Chappe et al., 2018). Oral voriconazole is extensively used also in FMEO (with surgical debridement where required), as demonstrated by many reviews of the literature (Mion et al., 2015; Parize et al., 2009; Ho et al., 2014): the administered doses depend on the stage and the gravity of the infection.

In summary, superficial otomycosis and chronic colonization should be treated with cleansing and debridement under otomicroscopic vision associated with ototopical antifungal drugs (solutions, creams, drops, oil, gel, etc.). Given the possibility of tympanic membrane perforations, low risk of ototoxicity is a fundamental property of the chosen topical treatment, waiting for application of paper patches or waiting to perform cartilage myringoplasty (both are suitable solutions in front of perforations with otomycosis) (Görür et al., 2019; Lou, 2020).

Systematic therapy should be reserved for severe cases, as fungal middle otitis with mastoiditis or other complications, and for FMEO with important inflammation of the soft tissues and the bones; in this last situation, glycemic control is mandatory. Nowadays, early antimycotics as ketoconazole and amphotericin B have been replaced with fluconazole and voriconazole, orally and intravenously administrable. The triazoles should be preferred for mastoiditis and otogenic cerebral mycosis given their low molecular weight (Vennewald and Klemm, 2010). Voriconazole has very good penetration of bone and synovial fluid, that is essential in case of fungal mastoiditis or otogenic meningitis (Vennewald and Klemm, 2010).

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Parasites of the Ear

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Glossary

Infestation Arthropods living in or on a person as a parasite.

Maggot Fly larva.

Myiasis Parasitic infestation of live animals by dipterous fly larvae.

The human ear is a fascinating organ of hearing and equilibrium, which is only rarely involved in parasitic infections. Most parasitic infections occur in the outer portion of the ear, as this is the area that communicates with the outside world. The short external auditory canal may be entered by a variety of arthropods, and while many are not capable of surviving in this environment, some such as parasitic fly larvae and mites may cause patent infestations. There have also been rare reports of infection of the outer ear canal with free-living nematodes in the genus *Rhabditis* (Teschner et al., 2014). The auditory canal ends with the tympanic membrane (ear drum) which separates the outer and middle portions of the ear and presents a thin, delicate barrier against inward parasite invasion. The middle ear is a narrow, air-filled cavity in the temporal bone which contains three tiny bones involved in sound conduction from the tympanic membrane to the inner ear. Parasites may enter the middle ear through a ruptured tympanic membrane, through the Eustachian tube, or by direct invasion from neighboring structures. As the middle ear is connected to the nasopharynx through the Eustachian tube, migrating worms such as *Ascaris lumbricoides* (Fagan and Prescott, 1993; Jain and Pahuja, 1988; Maqbool, 1975), and possibly *Enterobius vermicularis*, may rarely pass into the middle ear from the gastrointestinal tract and cause acute otitis media and associated physical damage. *Ascaris lumbricoides* and *E. vermicularis* are covered in greater detail elsewhere, and therefore will not be covered in this chapter. The innermost portion of the ear is referred to as the inner ear, and houses the vestibular system (involved in balance) and cochlea (the organ of hearing). Worms such as *Lagochilascaris minor*, and rarely, *Gnathostoma* spp. (Cutchavaree et al., 1985), may invade any part of the outer, middle and inner ear as it migrates throughout the head and neck.

This chapter will cover the most common parasitic infections and infestations of the ear: aural myiasis (otomyiasis), otoacariasis, and lagochilascariasis. The biology of each parasite will be described, along with the clinical manifestations, diagnosis and treatment of the associated infection or infestation.

Aural myiasis (otomyiasis)

Biology and epidemiology

Aural myiasis, also known as otomyiasis, is the infestation or colonization of the ear by dipterous fly larvae. There are different ways to classify myiasis, with the most common being based on anatomic and ecological features (Francesconi and Lupi, 2012; Mathison and Pritt, 2014, 2015). When considering its anatomic classification, aural myiasis is characterized as a form of cavitary myiasis, in which a cavity such as the ear canal, nose, eye or brain is infested by larvae (Francesconi and Lupi, 2012). Since aural myiasis commonly involves a pre-existing wound or diseased tissue, it may also be characterized as a form of wound myiasis, particularly when the wound involves the outer earlobe (auricular or pinna). The ecological classification of myiasis considers the nature of the

infesting larvae, and whether they are obligatory, facultative or accidental parasites. Obligatory parasites require a human host to complete their lifecycle and readily invade healthy tissue. In contrast, facultative parasites are free-living, and usually feed on dead and decaying matter in the environment. They may become parasites when they colonize pre-existing wounds and feed on decaying tissue (Mathison and Pritt, 2014). Occasionally facultative parasites will attack healthy tissue as well. Accidental or incidental myiasis, sometimes referred to as pseudomyiasis, occurs when free-living larvae incidentally colonization mucus membranes or biofilms on invasive medical devices, such as catheters. They do not feed on host tissue, but may cause a pathological host reaction from their presence.

Cases of aural myiasis have been reported from around the world, with most cases being found in tropical climates. Both obligatory and facultative parasites have been implicated, with *Cochliomyia hominivorax* (New World screwworm fly), *Chrysomya bezziana* (Old World screwworm fly), *Chrysomya megacephala* (latrine fly), *Wohlfahrtia magnifica* (spotted flesh fly), *Sarcophaga* spp. (Oriental flesh fly) being the important agents (Francesconi and Lupi, 2012) (Fig. 1 and Table 1).

Dipterous flies have four life cycle stages: egg, larva, pupa and adult (Mullen and Durden, 2019). Aural myiasis begins when eggs or larvae are deposited near the aural cavity or in pre-existing wounds involving the ear and neighboring structures (Francesconi and Lupi, 2012). Eggs hatch to release first instar larvae. If left undisturbed, the larvae will feed on living and/or dead tissue and molt into second, and eventually third, instar stages. In nature, the third stage larva will leave the host to form a pupa, from which the adult fly eventually emerges. The entire lifecycle spans a period of several weeks and may vary based on the temperature and humidity.

Clinical presentation, pathology, and treatment

The clinical presentation of aural myiasis varies with the location and extent of the infestation. As larvae feed on decaying and/or healthy tissue, patients may experience ear pain, bleeding, malodorous discharge, restlessness, foreign body sensation, tinnitus, vertigo, and impaired hearing. Larvae are observed within the ear, or crawling out of the ear canal (Francesconi and Lupi, 2012; Hernando et al., 2017). Diffuse erythema and edema of the external auditory canal, and perforation of the tympanic membrane may also be noted on otological examination (Francesconi and Lupi, 2012). Most cases of aural myiasis have been reported in young children (including neonates) (Casanova-Roman et al., 2010; Yuca et al., 2005; Al Jabr, 2015; Tligui et al., 2007), patients with preexisting aural disease, and debilitated individuals (e.g., intoxicated, physically or mentally disabled) (Francesconi and Lupi, 2012; Werminghaus et al., 2008; Magliulo et al., 2000; Jiang et al., 2020). Poor hygiene and socioeconomic status have been identified as risk factors (Hernando et al., 2017). Chronic otorrhea may also be a risk factor, particularly in otherwise healthy individuals (Arora et al., 2009; Yuca et al., 2005). The infestation is nearly always unilateral, although rare cases of bilateral disease have been reported (Bayindir et al., 2010; Yuca et al., 2005).

If untreated, the infestation may lead to destruction of the middle ear, deafness, invasion of the mastoid cavity, penetration of the central nervous system, and rarely, death (Francesconi and Lupi, 2012; Arora et al., 2009). Infection with larvae causing obligatory myiasis are of particular concern (e.g., *C. hominivorax*, *C. bezziana*), given their ability to actively invade healthy tissue and cause wide-spread damage. Radiologic imaging such as computed tomography is helpful for assessing the extent of invasion. Treatment is by removal of the larvae by ear irrigation, suction, and otoscopic and/or surgical extraction. Cases involving the middle ear or mastoid may require extensive debridement and multiple procedures to ensure removal of all larvae (Francesconi and Lupi, 2012; Uzun et al., 2004). Antibacterial agents are also commonly administered to prevent bacterial superinfection.

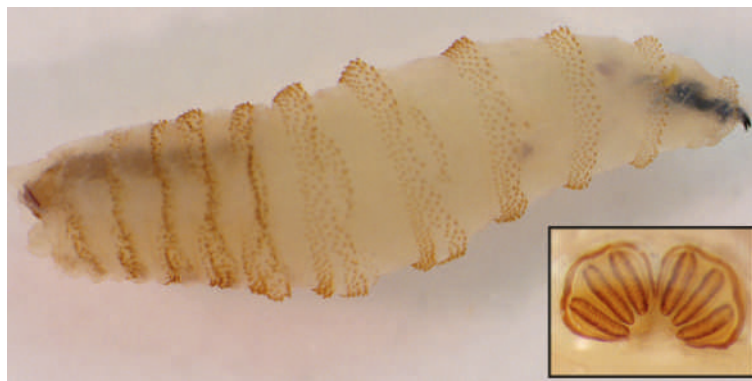


Fig. 1 Third instar larva of *Cochliomyia hominivorax*, an important cause of aural myiasis in Latin America. The overall body shape, arrangement of the cuticular spines, and features of the posterior spiracles are used for identifying the dipterous fly larvae. The third instar larva of *C. hominivorax* has an elongate, screw-shaped body with cuticular spines on all body segments, and the spiracular plate (inset) has three relatively-straight slits that point toward the opening of the peritreme (plate that surrounds the spiracles). From Mathison BA and Pritt BS (2015) Arthropod Benchtop Reference Guide. Northfield, IL: College of American Pathologists, used with permission.

Table 1 Flies implicated in aural myiasis with select references (the references are not necessarily exhaustive for a given species).

Genus and species	Geographic location	Reference(s)
Facultative myiasis		
<i>Calliphora</i> spp.	India	Bapat (2000) and Jain et al. (2008)
<i>Chrysomya megacephala</i>	Malaysia	Lee and Yong (1991)
<i>Lucilia sericata</i>	Poland, Italy, South Korea	Kaczmarczyk et al. (2011), Berlot and Calderan (2017), and Cho et al. (1999a)
<i>Lucilia</i> sp.	Turkey	Cetinkaya et al. (2008)
<i>Musca domestica</i>	Turkey	Demirci et al. (2006)
<i>Fannia canicularis</i>	Morocco	Arsalane et al. (2001)
<i>Sarcophaga crassipalpis</i>	Australia	Morris (1987)
<i>Sarcophaga haemorrhoidalis</i>	Israel	Braverman et al. (1994)
<i>Sarcophaga dux</i>	Thailand	Chaiwong et al. (2014)
Sarcophagidae, gen. sp.	Indonesia, Malaysia, Italy, Germany	Hernando et al. (2017), Ahmad et al. (2009), Magliulo et al. (2000), and Werminghaus et al. (2008)
Obligatory myiasis		
<i>Chrysomya bezziana</i>	Malaysia, Algeria, Saudi Arabia, Spain	Rohela et al. (2006), Abed-Benamara et al. (1997), Al-Abidi et al. (2003), and González Poggioli and Vázquez Barro (2009)
<i>Cochliomyia hominivorax</i>	Belize, Colombia, Ecuador, Argentina, Paraguay	Mendivil and El Shammas (1979), Cardozo and Espinal (2018), Calvopina et al. (2020), Menghi et al. (2010), Neira et al. (2002), and Visciarelli et al. (2007)
<i>Wohlfahrtia magnifica</i>	Saudi Arabia, Turkey; Egypt, Morocco, Spain, Italy	Al Jabr (2015), Ata and Güzelkara (2017), Karaman et al. (2009), el Kadery and el-Begermy (1989), Tligui et al. (2007), Casanova-Roman et al. (2010), Atmaca et al. (2009), Uzun et al. (2004), Bayindir et al. (2010), and Magliulo et al. (2000)

Diagnosis

Diagnosis of aural myiasis is made by finding fly larvae within the ear. As removal is curative, the specific identification of the infesting fly may not be necessary, except when investigating the route in which the infestation was acquired (Francesconi and Lupi, 2012). Fly larvae are identified based on the geographic distribution and several morphologic features such as the overall body size and shape, arrangement of the cuticular spines, and form of the posterior spiracles (Mathison and Pritt, 2014, 2015).

Otoacariasis

Biology and epidemiology

Otoacariasis is infestation of the ear canal with mites and ticks. Several species of ticks and mites have been implicated in otoacariasis, and while uncommon, the condition can occur anywhere in the world (Table 2).

The most common agents associated with otoacariasis are hard ticks (family Ixodidae), including members of the genera *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, and *Rhipicephalus*. Ticks in these genera are typically zoonotic on humans and their presence in the ear canal is usually incidental and atypical.

One species of soft tick (family Argasidae) that is adapted to feed in the ear canals of its mammalian hosts, *Otobius megnini*, causes rare zoonotic infections in humans. *Otobius megnini* has a one-host life cycle. Natural hosts are primarily ungulates, such as cattle, goat, sheep, deer, and horses, but other mammals can be infected. Native to western North America, *O. megnini* now occurs nearly worldwide where livestock is raised. Only larvae and nymphs feed on the vertebrate host, and stay attached for prolonged periods of time; adults live off the host and do not feed. The number of nymphal stages in the life cycle is unresolved and various studies have proposed one, two, or three nymphal instars (Rajakaruna and Diyes, 2019). *Otodectes cynotis*, the common ear mite of pets (especially cats) can also rarely cause infections in humans, usually among people with poor hygiene practices or who have prolonged exposure to infected natural hosts (Van de Heyning and Thienpont, 1977).

A number of other mites, that either parasitize non-human animals or are free-living in the environment, have been implicated in human otoacariasis, including members of the genera *Dermanyssus*, *Cosmoglyphus*, *Sancassania*, *Rhizoglyphus*, *Chorotoglyphus*, and even household dust mites in the genus *Dermatophagoides*. Most of these are self-limiting following proper control measures.

Clinical presentation, pathology, and treatment

When symptoms are present, the most common complaint of otoacariasis is acute ear pain. Other symptoms include dizziness, tinnitus, bleeding, hearing loss, facial paresis, otitis externa and otitis media. Toxins in the tick's saliva may induce facial or respiratory paralysis or facial palsy (Cakabay et al., 2016). Patients may report hearing unusual sounds, such as crackling in the ear (Grady et al., 2011). Physical trauma can result in rupturing of the tympanic membrane. Symptoms usually wane after successful removal of the tick. However, it is possible in some cases that physical trauma and pain may be caused more by haphazard removal of the tick rather than the host reaction to the tick.

Table 2 Mites and ticks implicated in otocariasis with select references (the references are not necessarily exhaustive for a given species).

Species	Geographic location	Reference(s)
Family: Ixodidae (hard ticks)		
<i>Amblyomma integrum</i>	Sri Lanka	Dilrukshi et al. (2004)
<i>Amblyomma testudinarium</i>	Japan	Nakao et al. (2017)
<i>Dermacentor astrosignatus</i>	Malaysia	Mariana et al. (2008)
<i>Dermacentor auratus</i>	Sri Lanka	Ariyaratne et al. (2011)
<i>Dermacentor compactus</i>	Malaysia	Mariana et al. (2008)
<i>Dermacentor steini</i>	Malaysia	Mariana et al. (2008)
<i>Dermacentor variabilis</i>	United States	Grady et al. (2011) and Kasle and Waldman (2019)
<i>Haemaphysalis longicornis</i>	South Korea, Japan, New Zealand	Choi et al. (2018), Hornibrook and Pollard (2011), and Ohi et al. (1992)
<i>Haemaphysalis</i> spp.	Malaysia	Mariana et al. (2008)
<i>Hyalomma brevipunctata</i>	Sri Lanka	Dilrukshi et al. (2004)
<i>Hyalomma marginatum</i>	Sri Lanka	Dilrukshi et al. (2004)
<i>Hyalomma</i> spp.	Turkey	Dalgic et al. (2010) and Gokdogan et al. (2016)
<i>Ixodes ovatus</i>	Japan	Iwasaki et al. (2007)
<i>Ixodes persulcatus</i>	Japan	Kogoashiwa et al. (2009)
<i>Rhipicephalus haemaphysaloides</i>	Sri Lanka	Dilrukshi et al. (2004)
<i>Rhipicephalus sanguineus</i>	Sri Lanka, Jerusalem	Dilrukshi et al. (2004) and Uspensky (2009)
Family: Argasidae (soft ticks)		
<i>Otobius megnini</i>	Turkey, South Africa, Zimbabwe, Chile, United Kingdom, Sri Lanka, India, Mexico, Argentina, Peru, United States	Avci (2019), Naudé et al. (2001), Burchard et al. (1984), Mazlumoglu (2018), Simpson (1901), Ariyaratne et al. (2016), Broom (1920), Chellappa (1973), Christophers (1906), Dios and Knopoff (1931), Eads and Campos (1984), Escomel (1929), Hoelscher (1985), Merten and Durden (2000), and Norval (1986)
Family: Dermanyssidae (bird, rodent mites)		
<i>Dermanyssus gallinae</i>	United Kingdom	Rossiter (1997)
Family: Acaridae (mold, grain mites)		
<i>Cosmoglyphus</i> sp.	India	Pal et al. (2018)
<i>Rhizoglyphus</i> sp.	Iran	Kiakojouri et al. (2018)
<i>Sancassania berlesii</i>	South Korea, United Kingdom	Paleri and Ruckley (2001) and Cho et al. (1999b)
Family: Chorotoglyphidae (chorotoglyphid mites)		
<i>Chorotoglyphus arcuatus</i>	Lebanon	Abi-Akl et al. (2017)
Family: Psoroptidae (psoroptid mites)		
<i>Otodectes cynotis</i>	Belgium	Van de Heyning and Thienpont (1977)
Family: Pyroglyphidae (dust mites)		
<i>Dermatophagoides farinae</i>	Taiwan	Liao and Chang (2012)
Family: Histiostomatidae (histiostomatid mites)		
Histiomatidae, gen. sp.	Saudi Arabia	Al-Arfaj et al. (2007)

In areas that are endemic for tickborne pathogens, ticks in the ear may vector disease-causing agents, including those responsible for Lyme disease, Rocky Mountain spotted fever, ehrlichiosis, anaplasmosis, Crimean-Congo hemorrhagic fever, and babesiosis.

Treatment for otoacariasis is the successful removal of the offending agents, especially with ticks that are larger and generally visible to the naked eye. For mites, topical solutions can be used to kill and flush out the mites. A 2% solution of hydrogen peroxide was used to successfully treat a case of otoacariasis caused by mites in the genus *Rhyzoglyphus* (Kiakojouri et al., 2018). Ear drops containing crothamiton were used to treat an unidentified histiostomatid mite (Al-Arfaj et al., 2007). One percent permethrin was used to treat a case of otoacariasis caused by a mite in the genus *Cosmoglyphus* (Pal et al., 2018). A case of otoacariasis caused by the common dust mite *Dermatophagoides pteronyssinus* was successfully treated using eardrops containing triamcinolone, nystatin, neomycin, and gramicidin (Liao and Chang, 2012). These topical solutions are often complimented with oral antibiotics, also to prevent secondary bacterial infections in the lesions caused by the ticks as well as to preemptively treat bacterial pathogens that may have been transmitted by the arthropod.

Diagnosis

Diagnosis of otoacariasis is by the morphologic identification of the ticks and mites removed from the ear. Removal is generally curative, and a definitive identification may not be necessary, except when determining the risk for tickborne diseases endemic to where the tick was acquired (Cakabay et al., 2016).

Lagochilascariasis (*Lagochilascaris minor*)

Biology and epidemiology

Lagochilascariasis is caused by the rare zoonotic nematode *Lagochilascaris minor*. The parasite occurs throughout Central and South America. Natural hosts are presumed to be wild felines. Prior to 2016, *L. minor* was only documented from humans until a wild mountain lion was found parasitized in Mexico (Falcon-Ordaz et al., 2016). Human cases have been documented in Mexico, Costa Rica, Suriname, Trinidad and Tobago, Bolivia, Colombia, Venezuela, Ecuador, Paraguay, Peru, and Brazil; over 70% of the cases in the literature come from Brazil (Campos et al., 2017).

The natural life cycle is still unknown; however, experimental models using cats and mice suggest a two-host life cycle whereby the feline definitive host becomes infected after consuming infected intermediate hosts. The sources of human infection are not known, but it is believed humans become infected after eating undercooked meat, especially that of rodents such as cavies, agoutis, and Guinea pigs (Campos et al., 2017).

Clinical presentation, pathology, and treatment

The most common clinical presentation of lagochilascariasis is the presence of a subcutaneous abscess on the neck, middle ear, tonsils, mastoid, and sinuses. Other sites of infection include lungs, eyes, and central nervous system (Campos et al., 2017). Patients may present with the sensation of moving in the throat and the discharge of young adult and mature worms from the nose, throat, and ears (Orihel and Ash, 1995). The presence of multiple parasite stages (eggs, larvae, adults) in some sites suggests the possibility of autoinfection in the human host (Campos et al., 2017; Orihel and Ash, 1995).

Aural involvement of *L. minor* usually presents as an ulcer in the middle ear with discharge. Perforation of the tympanum can occur and young adult or mature worms are often expelled from the ear canal. Aural infection can often be secondary to other areas of the head and neck and may present as Gradenigo's syndrome (Douma et al., 2016).

Several drugs have been used in cases of lagochilascariasis. Treatment often multiple anthelmintic agents and is initiated with thiabendazole, followed by either diethylcarbamazine (DEC) or mebendazole, and then lastly with levamisole. In some cases surgical removal may be warranted (Campos et al., 2017). Left untreated, infection can last years.

Diagnosis

Lagochilascariasis is usually diagnosed by the morphological identification of expelled worms or histopathologic examination of biopsy specimens (Fig. 2).

Adult female worms measure approximately 2.0 cm in length; males are slightly smaller at 1.7 cm. Adults of both sexes possess three fleshy anterior "lips" that are characteristic of ascarid nematodes. Conspicuous lateral alae run along the cuticle, which itself is finely striated (Orihel and Ash, 1995).

On histopathologic examination, *L. minor* usually does not present with diagnostic difficulty, taking into consideration epidemiology, anatomic site, and parasite morphology. Adults of *L. minor* possess many of the characteristics inherent to other ascarid nematodes, including coelomyarian/polymyarian musculature and tall, slender uninucleate intestinal cells. Typically, *L. minor* occurs in anatomical sites not parasitized by other ascarid nematodes, with the exception of ectopic anisakiasis. The prominent lateral alae are a key diagnostic feature, especially when seen in conjunction with features of an adult worm, such as ovaries or testes; other ascarid nematodes that present with alae are larval, including L3 larvae of *Ascaris lumbricoides* (lung), *Baylisascaris procyonis* (liver, CNS), and *Toxocara* spp. (liver, lung, CNS) (Orihel and Ash, 1995).



Fig. 2 Cross section of *Lagochilascaris* in a biopsy specimen stained with hematoxylin-and-eosin (H&E), showing prominent lateral alae, coelomyarian/polymyarian musculature, and tall intestinal cells. This figure represents *L. spreuti*, a parasite of the opossum, but the species shares the same morphologic features as *L. minor* in humans. Image courtesy of Dr. Sarah Sapp, Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention.

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Brain Infections, Encephalitis, and Meningitis: Bacteria

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Introduction

Infectious diseases such as brain infection, encephalitis, and meningitis are less common in developing countries, but its outcome always potentially fatal (Sarrazin et al., 2012). Increased understanding of risk factors, clinical features, diagnostic tools, and advancement in microbiology considerably decreased the number of people suffering from these infectious diseases. However, infectious diseases are tenacious and continue to be a significant health concern in low- and middle-income countries (Fonkwo, 2008).

Encephalitis is an acute inflammation of brain parenchyma with relatively severe neurological outcomes (Ellul and Solomon, 2018). Sometimes, acute meningitis can be clinically indistinguishable from acute encephalitis. Worldwide incidence of encephalitis ranges from 1.5 to 7/100,000 inhabitants/year with a fatality of 7% (Boucher et al., 2017). Although *Herpes simplex virus* (HSV) infection is the most common cause of encephalitis (Quist-Paulsen et al., 2013) the bacterial strains such as *Streptococcus pneumoniae*, *S. pyogenes*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Bacillus anthracis*, *Chlamydia trachomatis*, *Leptospira* sp., *Borrelia burgdorferi*, and *Salmonella* sp. also causes encephalitis (Granerod et al., 2010). *L. monocytogenes* may cause meningoencephalitis, a syndrome where features of both meningitis and encephalitis are present. The survivors' from encephalitis often presents residual physical or neuropsychological deficits (Ellul and Solomon, 2018).

Meningitis is an infection caused by pathogens that leads to inflammation of the brain and spinal cord coverings called meninges. In the United States, infection of viral, bacterial, fungi and parasites, and unspecified reasons accounts for the meningitis cases. The higher incidence is still prevalent in the meningitis belt of sub-Saharan Africa, with incidence rates varying between 10 and 40 per 100,000 inhabitants. In contrast, in the United States and Europe, the incidence rate varied between 0.7 and 7.1 per 100,000, which accounts for all causative pathogens (Castelblanco et al., 2014; Figueiredo et al., 2018). Recently published systematic review reports the global frequency of meningitis causing microorganisms in all ages reveals that in America, *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*; in Europe *N. meningitidis*, *S. pneumoniae*, *Staphylococcus aureus*, *S. agalactiae*, *H. influenzae*, and *L. monocytogenes*; in Africa *S. pneumoniae*, *N. meningitidis*, *H. influenzae*, *E. coli*, *S. aureus*, *L. monocytogenes*, and *S. agalactiae* (Oordt-Speets et al., 2018). The bacterial meningitis is either community-acquired or nosocomial (hospital-acquired). It often results from the invasion of the bacteria into the meninges from blood, or it may reach from local infection. The most common bacterial strains that lead to infection vary by age (Table 1). *S. pneumoniae* was the most common cause of bacterial

Table 1 Invasion of CNS pathogen and clinical diseases.

Bacterial pathogen	Age	Sites of entry and colonization	Bacterial adherence and invasion	Entry to CNS	Clinical diseases
<i>Streptococcus pneumoniae</i>	1–3 months 3 months to 3 years 3–10 years	Nasopharynx and lung	Cell wall-anchored proteins, cytolysin, capsule	Invasion across the blood-brain barrier (BBB) and blood cerebrospinal fluid barrier (BCSFB)	Neonatal, pediatric and adult meningitis, brain abscess, and encephalitis (Liu et al., 2010; Pick, 2016)
<i>Streptococcus agalactiae</i>	0–3 months 3 months to 3 years	Hematogenous spread from mother to infant, nasopharynx, intestinal tract	Cell wall-anchored proteins, hemolysin, capsule, LTA, pili	Invasion across the BBB and B-CSFB	Neonatal meningitis and brain abscess (Pick, 2016; Stoner et al., 2015; Dando et al., 2014)
<i>Neisseria meningitidis</i>	1–3 months 10–19 years	Nasopharynx	Capsule, type IV pili, outer membrane proteins (Opa, Opc, FBA, ACP, MspA)	Invasion across the BCSF	Pediatric, infant and adult meningitis (Pick, 2016; Chauhan et al., 2008)
<i>Listeria monocytogenes</i>	0–3 months 3 months to 3 years	Intestinal tract and blood-borne	Internalin, InlI	Invasion across the BBB	Neonatal and adult meningitis, encephalitis and brain abscess (Pick, 2016; Mosa et al., 2009; Popowska et al., 2017)
<i>Haemophilus influenzae</i>	Below 15 year	Nasopharynx	Capsule, Hap, HMW1, HMW2, Hia/Hsf, and hemagglutinating pili	Remains unclear	Meningitis and brain abscess (Galdiero et al., 2001; Kim, 2008; Doran et al., 2016; Rodriguez et al., 2003)
<i>Escherichia coli</i>	1–3 months	Hematogenous spread from mother to infant, nasopharynx, intestinal tract	OmpA, K1 capsule, CNF1, fimbriae, lbeA	Invasion across the BBB	Meningitis, brain abscess, and choroiditis (Pick, 2016; Krishnan et al., 2012)
<i>Mycobacterium tuberculosis</i>	2–4 years	Nasopharynx and blood-borne	ESAT-6, Mpt64 and CFp2	Invasion across the BBB	Meningitis, CNS tuberculosis (Rock et al., 2005; Mezocho et al., 2017; Ramsugit and Pillay, 2016)
<i>Klebsiella pneumoniae</i>	3 months to 50 years	Nasopharynx	Outer membrane protein KpOmpa	Invasion across the BBB	Meningitis (Lee et al., n.d.; Li et al., 2014; Pichavant et al., 2003)
<i>Pseudomonas aeruginosa</i>	3 months to 50 years	Nasopharynx and lung	Outer membrane protein EstA, OprD, OprG and PA3923.	Invasion across the BBB	Meningitis (Gallagher et al., 2017; Bodey et al., 1983; Paulsson et al., 2019)

meningitis (58.0%), *S. agalactiae* (18.1%), *N. meningitidis* (13.9%), *H. influenzae* (6.7%), and *L. monocytogenes* (3.4%). The other strains, such as *E. coli*, *Klebsiella* sp., *Enterobacter* sp., and *Pseudomonas aeruginosa* accounts for less common causes of infection. The bacterial strains, such as *S. pneumoniae*, *S. aureus*, *S. epidermidis*, and some Gram-negative bacilli, account for the hospital-acquired meningitis infection (Chacon-Cruz et al., 2019; Linder and Malani, 2019). Recent studies have put forth evidence that the prevalence of bacterial meningitis has dramatically diminished with the utilization of conjugate vaccines, antimicrobial therapy, and adjuvant treatment in developed countries. However, survivors from meningitis often suffer from neurological sequelae, including hearing loss, focal neurological deficits, and cognitive impairment (Lucas et al., 2016).

Both bacterial meningitis and encephalitis are acute infections which need medical attention promptly to avoid mortality and poor neurological outcome. Several extensive studies in children with bacterial meningitis and encephalitis have been done, but there are no large-scale epidemiologic studies evaluating all causes of meningitis and encephalitis in the pediatric population in the United States. In this chapter, we aimed to summarize the mechanism by which the bacterial pathogen invades the immune-privileged central nervous system (CNS), the response of the immune system toward the bacterial colonization, and long-term neurological complications after this dreadful infection.

Colonization of bacteria to cause encephalitis and meningitis

The route of infection in bacterial invasion generally has the cascade of events involving colonization (nasopharynx, respiratory tract, middle ear, maternal vaginal and intestine), bloodstream dissemination, blood-brain barrier (BBB) and blood-cerebrospinal fluid (BCSF) barriers crossing, and finally entry into the subarachnoid space, survival and subsequent infection (de Steenhuijsen Piters et al., 2015; Dando et al., 2014). In general, the nasal cavity easily exposed to inhaled microbes, allergens, and particulate material. The epithelium lining the nasal nares and the cavity harbor normal bacterial flora, and CNS pathogens, such as *S. aureus*, *S. pneumoniae*, *N. meningitidis*, and *H. influenzae*, may asymptotically colonize in the nasopharynx (Oikawa et al., 2014; Bogaert et al., 2011; Rasmussen et al., 2000). Several events that occur following infection of human nasopharyngeal is associated with mucus independent of capsular polysaccharide, fimbriae, or immunoglobulin A subclass 1 (IgA1) protease production; (i) cytotoxicity characterized by the breakdown of epithelial-cell tight junctions, called ciliostasis; (ii) selective attachment to nonciliated epithelial cells; (iii) multiplication and formation of microcolonies on the epithelial surface; (iv) invasion of the epithelium by intracellular or intercellular routes; and (v) passage of organisms to the submucosa (Tunkel and Scheld, 1993). The details of bacterial pathogen and their route of entry, respective bacterial adhesion protein involved, and their route of entry to CNS is detailed in Table 1.

Maternal vaginal colonization

The first step involves the successful bacterial colonization of the mucosal surface in adherence to luminal epithelial cells and/or surface host proteins. Vaginal pH facilitates the increased bacterial adherence to vaginal epithelial cells and extracellular matrix (ECM) proteins, which has been observed *in vitro* as pH shifts from acidic to neutral (Park et al., 2012). Few surface-expressed determinants such as serine-rich repeat (Srr) proteins, Srr-1 and Srr-2, alpha-like proteins, the pilus protein PilA of the GBS pilus island (PI)-2a, bacterial surface adhesins (Bsa) B, BspA, and immunogenic bacterial adhesin (BibA) have been shown to contribute to vaginal and cervical epithelial cell adherence protein (Santi et al., 2007). Other than these surface-expressed determinants, some bacterial constituents, including β -hemolysin/cytolysin toxin and carotenoid pigment, and MntH, an H^+ -dependent manganese transporter elicits adherence or vaginal persistence to host (Patras and Nizet, 2018).

Nasopharyngeal colonization

The transmission of the pathogen occurs between individuals by coughing and sneezing. The pili identified is most commonly responsible for the initial attachment to the host. The first pilus is an oligomeric appendage encoded by the *rlrA* operon, and the second pilus is encoded by pilus islet 2. To reach and colonize in nasopharynx, the pathogen degrades mucus by exoglycosidases such as neuraminidase A, β -galactosidase, β -N-acetylglucosaminidase, and neuraminidase B, decreasing mucus viscosity and preventing mucus entrapment (Burnaugh et al., 2008). Then they interact with additional adhesins such as phosphorylcholine, which binds to the platelet-activating factor receptor (PAF-R), CbpA (Rosenow et al., 1997). They facilitate transcytosis through the mucosal epithelial cell layer from the apical membrane to the basal membrane (Prager et al., 2017). Binding and crossing the epithelial cell layers allows access to the submucosal layer, leading to invasive illness.

Bacteraemia

The host defense opposes the bacterial entry in the blood include complement-mediated opsonization and phagocytosis. For survival, the pathogen increases its capsule size, reduces complement deposition on its surface, and reduces subsequent interaction with phagocytes (Kadioglu et al., 2008). This is achieved by inhibiting complement-mediated clearance by recruiting factor H that inhibits the complement cascade (Li et al., 2007). Some other defense is recognition of the bacteria by antigen-presenting cells

through the binding of pattern recognition receptor (PRR). These events result in the release of cytokine further induce the recruitment of neutrophils and lymphocytes and simultaneously cause cerebrovascular damage (Prager et al., 2017).

S. pneumoniae is internalized by human and rat BMECs via receptor-mediated endocytosis. This uptake does not involve the formation of cellular membrane protrusion (Ring et al., 1998). Polysaccharide capsule may also be an essential virulence factor for invasive disease caused by *S. pneumoniae*. *S. pneumoniae* induces the translocation of β -arrestin from the cytosol to the plasma membrane, then colocalizes with the platelet-activating factor (PAF) receptor. It stimulates mitogen-activated protein kinases (MAPK) signaling required for pneumococcal uptake. Vacuoles containing *S. pneumoniae* colocalized with endosomal markers; however, increased expression of β -arrestin promotes the transcytosis of viable bacteria. The interactions between PAF receptor and β -arrestin contribute to the transcellular penetration of HBMECs by *S. pneumoniae* (Radin et al., 2005).

N. meningitidis makes initial attachment and colonization in nasopharyngeal mucous membranes in humans. Studies have confirmed that pili are essential mediators of attachment of meningococci to human cells. The receptors for meningococcal pili are found to be more in nasopharyngeal mucous surfaces (Salit and Morton, 1981; Stephens and McGee, 1981). An experimental model of human columnar nasopharyngeal tissue in organ culture demonstrated the interaction of encapsulated, piliated *N. meningitidis* with the mucosal surface. Electron microscopic results showed that meningococci attached selectively to nonciliated columnar cells of the nasopharynx (Stephens et al., 1983). Type IV pili mediate the attachment of *N. meningitidis* to HBMECs. Although the initial attachment is inhibited by cerebral microcirculation once attached, *N. meningitidis* proliferates and able to resist its thorough, strong bacterium-bacterium and bacterium-host interactions (Mairey et al., 2006). Identified CD46 dependent and independent attachment to host cells (Kirchner and Meyer, 2005). Furthermore, studies with human bronchial epithelial cells show that the meningococcal type IV pilus binds to the PAF receptor, and that synergy between a pilin-linked glycan and phosphorylcholine decorating moieties is required for pili to efficiently engage the receptor (Jen et al., 2013).

L. monocytogenes utilize the nasopharyngeal route to enter the CNS. Then the invasion occurs using the Trojan horse mechanism or by transcellular penetration of HBMECs. In human umbilical vein endothelial cells (HUVECs) and HBMECs, it was demonstrated that *L. monocytogenes* could invade endothelial cells directly or via cell-to-cell spread from adherent and infected mononuclear phagocytes in the neonatal period (Drevets et al., 1995). In HUVECs, *L. monocytogenes* attachment induces membrane ruffling that leads to internalization (Parida et al., 1998). In HBMECs, *L. monocytogenes* invasion preceded by intimate interactions between the bacterium and microvilli on the cell surface (Greiffenberg et al., 2000). The invasion of *L. monocytogenes* into HBMECs requires actin microfilaments; however, the activation of phosphatidylinositol 3-kinase has not involved (Greiffenberg et al., 1998).

E. coli proteins contribute to the adhesion to and invasion of HBMECs; the virulence factors promote the destruction of tight junctions between BMECs. In addition to triggering the internalization of *E. coli* K1 into HBMECs, the binding of outer membrane protein A (OmpA) to its gp96 homolog receptor stimulates inducible nitric oxide synthase and nitric oxide production, which enhances the generation of cyclic GMP (Krishnan et al., 2012; Mittal and Prasadarao, 2010). This increase in cyclic GMP activates protein kinase C (Sukumaran and Prasadarao, 2003).

Central nervous system bacterial invasions cause brain infection, encephalitis, and meningitis

The BBB is a dynamic and complex system that separates the brain from the blood. It prevents neurotoxic plasma components, blood cells, and pathogens from entering the CNS (Sweeney et al., 2019). The BBB composed of endothelial cells, and it is regulated by pericytes, microglia, astrocytes, and neurons. BBB's specialized endothelial cells contain in their membranes, tight junctions by occludin, claudins, and junctional adhesion molecules and water channels that have altogether guard the transport of polar substances and infectious organisms (Cereijido et al., 1998). The traversal of the BBB by the pathogen is a prerequisite for all types of brain infections. The pathogen successfully crosses the BBB by transcellular, paracellular, and infected phagocytes (Trojan-horse) mechanism to infect the host. Microbial traversal through barrier cells occurs (i) With no trace of microorganism and no evidence of tight-junction disruption called transcellular traversal; (ii) With or without tight-junction disruption called paracellular traversal; and (iii) Using transmigration within infected phagocytes called the Trojan-horse mechanism (Kim, 2006, 2008).

The bacterial pathogen such as *E. coli*, *S. agalactiae*, *S. pneumoniae*, and *N. meningitidis* utilize transcellular traversal to penetrate the BBB. The bacterial pathogen, such as *L. monocytogenes* and *M. tuberculosis*, penetrate the BBB through both transcellular and Trojan-horse mechanism.

Pathogen ligand-receptor interaction

S. pneumoniae crosses the BBB partly through interactions between cell-wall phosphorylcholine and the PAF-receptor. It was demonstrated by the PAF-receptor antagonist, which partially inhibited the pneumococcal invasion of HBMECs (Ring et al., 1998). In PAF-receptor knockout mice, it delayed the translocation of *S. pneumoniae* from the lung to blood and blood to cerebrospinal fluid (CSF) (Cundell et al., 1995).

N. meningitidis interacts with endothelial and epithelial cells, and this interaction involves type IV pili, PilC, *N. meningitidis* adhesin A (NadA), and the Opa and Opc (outer membrane) proteins. The unencapsulated *N. meningitidis* invade the HBMECs by Opc binding to fibronectin, which anchors the bacteria to the integrin $\alpha 5 \beta 1$ receptor on the surface of HBMEC (Unkmeir et al., 2002). However, it is unclear, the encapsulated *N. meningitidis* and the in vivo relevance of Opc-fibronectin-mediated binding. *N. meningitidis* type IV pili bind to CD46 on HBMECs, and their LPS has been shown to contribute to a high level of bacteremia and penetration into the CNS (Plant et al., 2006).

L. monocytogenes invasion into CNS is mediated by internalin B (Greiffenberg et al., 1998). The HBMEC receptors for internalin B, includes gC1q-R (globular head of the component C1q-receptor) and Met tyrosine kinase but their contributions to *L. monocytogenes* invasion of HBMECs is little known (Braun et al., 2000). The internalin B does not compete for the same interaction site on Met as the natural ligand hepatocyte growth factor (Braun et al., 2000). The CNS penetration attributed to the transmigration of *L. monocytogenes*-infected monocytes and myeloid cells across the BBB (Drevets et al., 2004). However, further studies are needed to determine the major route of *L. monocytogenes* penetration into the CNS.

The neonatal meningitis pathogens *S. agalactiae* accommodates a number of proteins that allow binding and invasion of HBMECs. *S. agalactiae* binds to HBMECs through laminin-binding protein (Lmb), the fibrinogen-binding protein (FbsA), pili, and invasion-associated gene A (IagA) occurs through LTA anchoring (Tenenbaum et al., 2007; Doran et al., 2005; Maisey et al., 2007). Still, it is not clear that the above proteins are unique to *S. agalactiae* CSF isolates and its role in contributing to penetration into the CNS (Kim, 2008).

E. coli binding to HBMECs is contributed by the determinants, Type 1 fimbriae and OmpA (Teng et al., 2005; Khan et al., 2003). It has also been shown that the amino-terminal region and surface-exposed loops of OmpA contribute to binding to HBMECs (Shin et al., 2005) and that OmpA interacts with HBMECs through N-acetylglucosamine residues in HBMEC glycoproteins, including gp96 (Prasadarao et al., 2003; Khan et al., 2003). A recent study used an *E. coli* DNA microarray to compare an OmpA mutant with its parental *E. coli* K1 strain and revealed that the OmpA mutant expressed significantly fewer film cluster genes (Teng et al., 2006). Other *E. coli* determinants that contribute to invasion of HBMECs include recombinant Ibe proteins (Huang et al., 2001). It was supported as the polyclonal antibody raised against IbeA inhibited *E. coli* K1 invasion of HBMECs (Prasadarao et al., 1999). Besides, CNF1 contributes to *E. coli* K1 invasion of HBMECs in vitro and traversal of the BBB in vivo that depends on RhoA activation (Khan et al., 2002).

M. tuberculosis penetrates the BBB through a transcellular traversal or Trojan-horse mechanism. A study showed that strains of *M. tuberculosis* invade and traverse HBMECs; the invasion was significantly increased for the *M. tuberculosis* strains (H37RV and CDC 1551) than for the non-pathogenic strains (Jain et al., 2006). Using DNA microarray analysis 33 upregulated genes and 147 downregulated genes in *M. tuberculosis* that was associated with HBMECs compared with *M. tuberculosis* that was not associated with HBMECs. Mutants of five *M. tuberculosis* genes were attenuated for their ability to invade and/or survive in the brain (Be et al., 2008).

H. influenzae penetrates between epithelial cells via an unknown mechanism. Interaction with PAF receptor demonstrated in *H. influenzae* serotype b (Weiser et al., 1998), but the role of the PAF receptor in *H. influenzae* penetration into the CNS is not clear. Intracisternal inoculation *S. pneumoniae* diminished the pleocytosis by PAF-receptor antagonist but had no effect on the pleocytosis induced by *H. influenzae* (Cabellos et al., 1992). Zyxin is a vital protein protecting the tight junction of the BBB against cell transmigration across the BBB. Notably, tumor necrosis factor (TNF) α mediated decrease in zyxin significantly increased the migration of the *H. influenzae* (Miyazaki et al., 2014). In an experimental model using macaques, the earliest histopathologic lesions were observed on choroid plexus after *H. influenzae* infection (Smith, 1987). *H. influenzae* isolated from CSF after intranasal inoculation was associated with intravascular inflammatory infiltration and mild choroid plexitis (Daum et al., 1978). As explained above, the pathogenic bacteria utilize different transport mechanisms to penetrate the BBB to reach the brain (Fig. 1).

Recognition of bacterial infection by innate immune sensors

The general function of innate and adaptive immunity allows the host to detect pathogen entry and to initiate an inflammatory response to clear the invaders, thereby prevents host homeostasis. However, during the fight against the invaders, the CNS with lower regenerative profile, damage to host tissues is more prominent (van der Flier et al., 2003).

When the pathogen reaches the subarachnoid space, the bacteria multiply and establishes its colony, which further releases the cell wall fragments, lipopolysaccharide (LPS), lipoteichoic acid (LTA), teichoic acid, pneumolysin, and peptidoglycan (Mook-Kanamori et al., 2011). All these compounds are immunogenic and elicit an inflammatory response to the host. The concept of pattern recognition receptors (PRRs), a sensor for the innate immune system, is essential to understand the innate immune response. This non-clonal receptor can recognize structures common to many pathogenic organisms, known as pathogen-associated molecular patterns (PAMPs) (Janeway, 2001). Recognition of PAMPs occurs through toll-like receptors (TLRs), RLRs (retinoic acid-inducible gene-1-like receptor), cytosolic DNA sensors, and Nod-like receptors (NLRs). However, RIG-I-like receptors (RLRs) are a family of cytosolic PRR that is essential for detecting viral RNA (Kawai and Akira, 2009; Hanke and Kielian, 2011).

Toll-like receptors (TLRs)

Studies have identified a family of membrane-bound TLRs (TLR1-TLR10) 10 in humans and (TLR1-TLR9, TLR11, and TLR13) 12 in mice (Kawasaki and Kawai, 2014). TLRs expressed in dendritic cells (DCs), macrophages, fibroblasts, and epithelial cells. TLRs generally serve as PRRs to recognize a wide range of PAMPs, including lipids, lipoproteins, proteins, glycans, and nucleic acids, either on the cell surface or on the lumen of intracellular vesicles such as endosomes (Kawai and Akira, 2009). The cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10; the intracellular TLRs include TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 (Kawai and Akira, 2010). TLR2 recognizes triacyl lipopeptides from bacteria and mycobacteria, diacyl lipopeptides from mycoplasma, peptidoglycan, and LTA from Gram-positive bacteria, porin from *Neisseria*, lipoarabinomannan from mycobacteria (Hoebe et al., 2005). TLR4 activates pneumolysin, and TLR9 activates pneumococcal DNA containing CpG motifs within

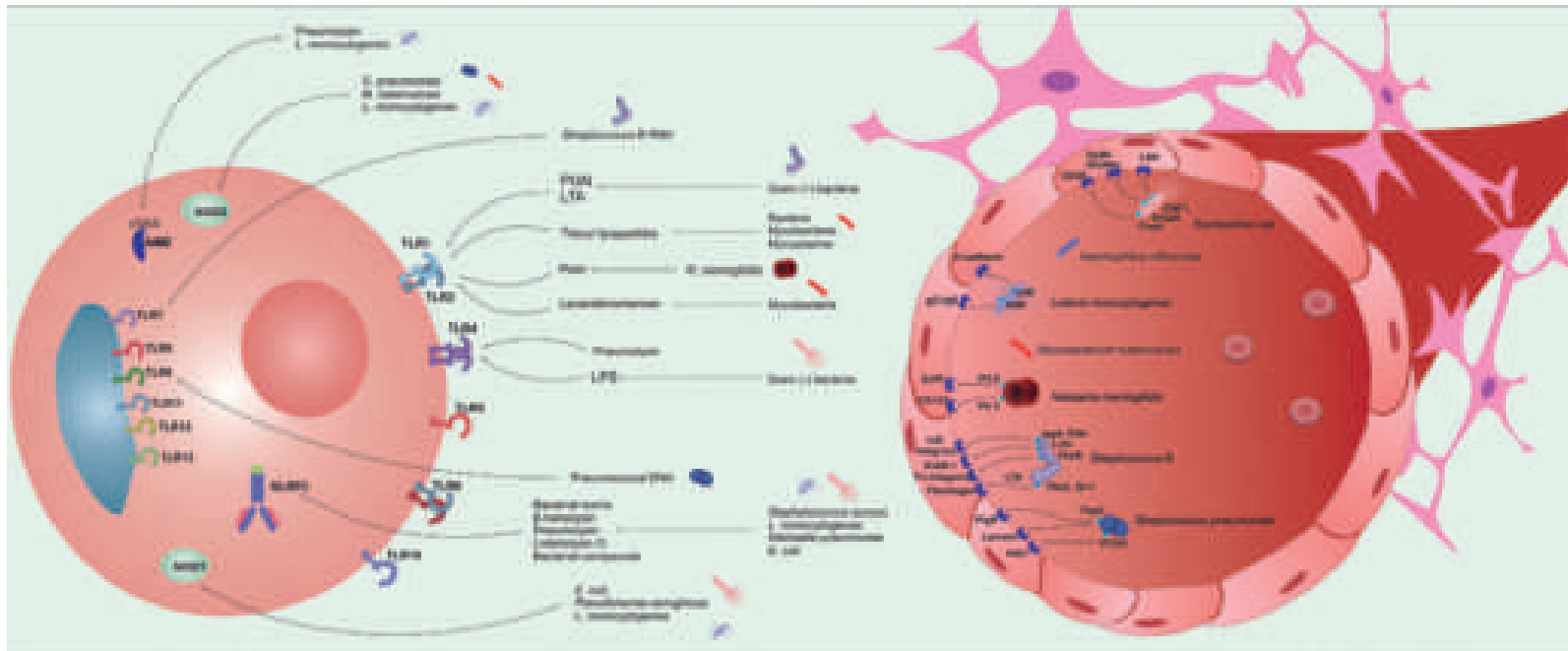


Fig. 1 Mechanisms of bacterial recognition and BBB disruption that facilitates bacterial-host interactions upon bacterial infection. NOD1 and NOD2 cytosolic receptors specifically recognize peptidoglycans present within the bacterial cell wall *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *P. aeruginosa*. *Staphylococcus aureus*, *E. coli*, *L. monocytogenes*, and *Klebsiella activates* NLRP3 through bacterial toxins. Lipopolysaccharide from Gram-negative bacteria recognized through TLR4 transmembrane protein. Invasion of the pathogen to the central nervous system achieved by specific protein present on the blood-brain barrier or blood-cerebrospinal fluid barrier.

endosomes. TLR4 activated by LPS, a principal constituent of the outer membrane of Gram-negative bacteria. It enables the adaptor protein (AP), myeloid differentiation primary response gene 88 (MyD88)-dependent pathway, and the TIR-containing adapter inducing interferon (IFN) β (TRIF)-dependent pathway (Akira et al., 2006; Hanke and Kielian, 2011). TLR5 recognizes *Listeria* sp. through its flagellin (Hayashi et al., 2001).

MyD88-dependent pathway

The MyD88 acts as a canonical adaptor for inflammatory signaling pathways downstream of members of the TLR and interleukin (IL)1 receptor families (Gay and Keith, 1991). It links the IL1 receptor (IL1R) or TLR family members to IL1R-associated kinase (IRAK) family kinases (Deguine and Barton, 2014). TLR engagement induces the formation of the Myddosome, which is based on MyD88 and also contains IRAK1 and IRAK4. IRAK1 activation also induces TNF-receptor (TNFR)-associated factor (TRAF) 6 activation following K63-linked polyubiquitination on TRAF6 itself and transforming growth factor- β -activated kinase (TAK)1. TAK1 activation leads to the activation of I κ B kinase (IKK) complex nuclear factor kappa B (NF κ B) and MAPK. Thus, IRAK family kinases activation leads to a variety of functional outputs, including the activation of NF κ B, MAPKs (Chen, 2012; Ajibade et al., 2013). MAPK activation further activates AP1 transcription factor activation. During bacterial infection, TRAF6 translocated to mitochondria, where it interacts with evolutionarily conserved signaling intermediate in Toll pathway (ECSIT) and results in increased mitochondrial and cellular reactive oxygen species (ROS) generation. Interestingly, studies demonstrated that deficiency of MyD88, adapter protein in children, increases their susceptibility to invasive pneumococcal meningitis (von Bernuth et al., 2008). In a preclinical model, MyD88-deficient mice have demonstrated a lack of responsiveness to LPS, the ligand for TLR4. Though MyD88 deficiency protected the mice from endotoxic shock, MyD88-deficient cells retained some residual signaling when stimulated with LPS, suggesting MyD88-independent signaling mechanisms downstream of TLR4 (Kawai et al., 1999). TLR7 is predominantly expressed in plasmacytoid DCs and recognizes RNA from streptococcus B bacteria (Kawasaki and Kawai, 2014). The details on signaling pathways triggered by Toll-like receptor (TLR) and Nod-like receptor (NLR) are given in Fig. 2.

TRIF-dependent pathway

TRIF-related adapter molecule (TRAM)—TRIF (TLR4 signaling) induce inflammatory responses via the recruitment of TRAF6, a member of the TRAF family of proteins. TRAF6 recruits the receptor-interacting protein (RIP)-1, which in turn interacts and activates the TAK1 complex, leading to activation of NF κ B, MAPKs, and induction of inflammatory cytokines. On the other hand, TRAF3 recruits the IKK-related kinases TANK-binding kinase (TBK)1 and IKKi (epsilon; IKK ϵ) along with NF κ B essential modulator (NEMO) for IRF3 phosphorylation. Subsequently, IRF3 forms a dimer and translocates into the nucleus from the cytoplasm, where it induces the expression of type I IFN genes (Akira et al., 2006; Kawai and Akira, 2010).

A well-recognized function of Peli family members—E3 ubiquitin ligases is participation in the signal transduction mediated by TLRs and IL1 receptors (Jin et al., 2012). Pellino-1-deficient mice display impaired TRIF-dependent NF κ B activation and cytokine production (Chang et al., 2009). TBK1/IKKi phosphorylates Pellino-1 that facilitates the ubiquitination of RIP-1; further, recruitment of RIP-1 is essential for TRIF-dependent NF κ B activation. Furthermore, Pellino-1 regulates IRF3 activation by binding to deformed epidermal autoregulatory factor (DEAF)-1, a transcription factor that facilitates the binding of IRF3 to the IFN- β promoter. Recently, IRF3 activation was demonstrated to be regulated by an inositol lipid, phosphatidylinositol 5-phosphate (PtdIns5P). PtdIns5P binds to both IRF3 and TBK1, and thus facilitates complex formation between TBK1 and IRF3. The accessibility of TBK1 to IRF3 mediated by PtdIns5P causes IRF3 phosphorylation (Fig. 2; Kawasaki et al., 2013).

Nod-like receptors (NLRs)

In the cytosol, the NLR (NOD-like receptor) family detects the presence of PAMPs and endogenous molecules. NLRs consist of three domains characterized by an N-terminal protein interaction domain, a central nucleotide-binding domain, and a C-terminal leucine-rich repeat (LRR). Members of the NLR family categorized into at least five subfamilies distinguished by their N-terminal structures (Meylan et al., 2006; Fritz et al., 2006; Kanneganti et al., 2007). The ligands of nucleotide-binding oligomerization domain-containing protein (NOD)1 and NOD2 categorized as NLRC1 and NLRC2, respectively, and are well-characterized members of the NLR family that recognize distinct structural motifs derived from peptidoglycan. NOD1 recognize pathogenic bacteria such as *E. coli*, *Shigella flexneri*, *P. aeruginosa*, and *Chlamydia* species intracellularly. NOD2 participates in sensing *Streptococcus pneumoniae* and *M. tuberculosis* (Kanneganti et al., 2007). *L. monocytogenes* activates both NOD1 and NOD2. Signaling via NOD1 and NOD2 results in the induction of inflammatory cytokines and other anti-microbial genes, thereby contributes to host defense mechanisms (Kobayashi et al., 2005). *H. influenzae* commonly found in the upper respiratory tract classified encapsulated or nonencapsulated form. Encapsulated serotype B is the most virulent one of the leading causes of meningitis young children. NOD1 is implicated in the intracellular recognition of this enteroinvasive pathogenic bacterial *H. influenzae* (Kanneganti et al., 2007).

Cytosolic DNA sensors

Cytosolic DNA sensors can also detect bacterial pathogens. For example, cyclic GMP-AMP (cGAMP) synthase (cGAS) recognizes a variety of intracellular bacteria, including *L. monocytogenes* (Hansen et al., 2014) in macrophages and activates absent in melanoma (AIM) 2 inflammasomes (Tan et al., n.d.). This recognition also occurs in the cytoplasm of many cell types, including immune and non-immune cells, and it triggers a type I IFN response via TBK1 that phosphorylates the transcription factor IRF3 (Stetson and Medzhitov, 2006).

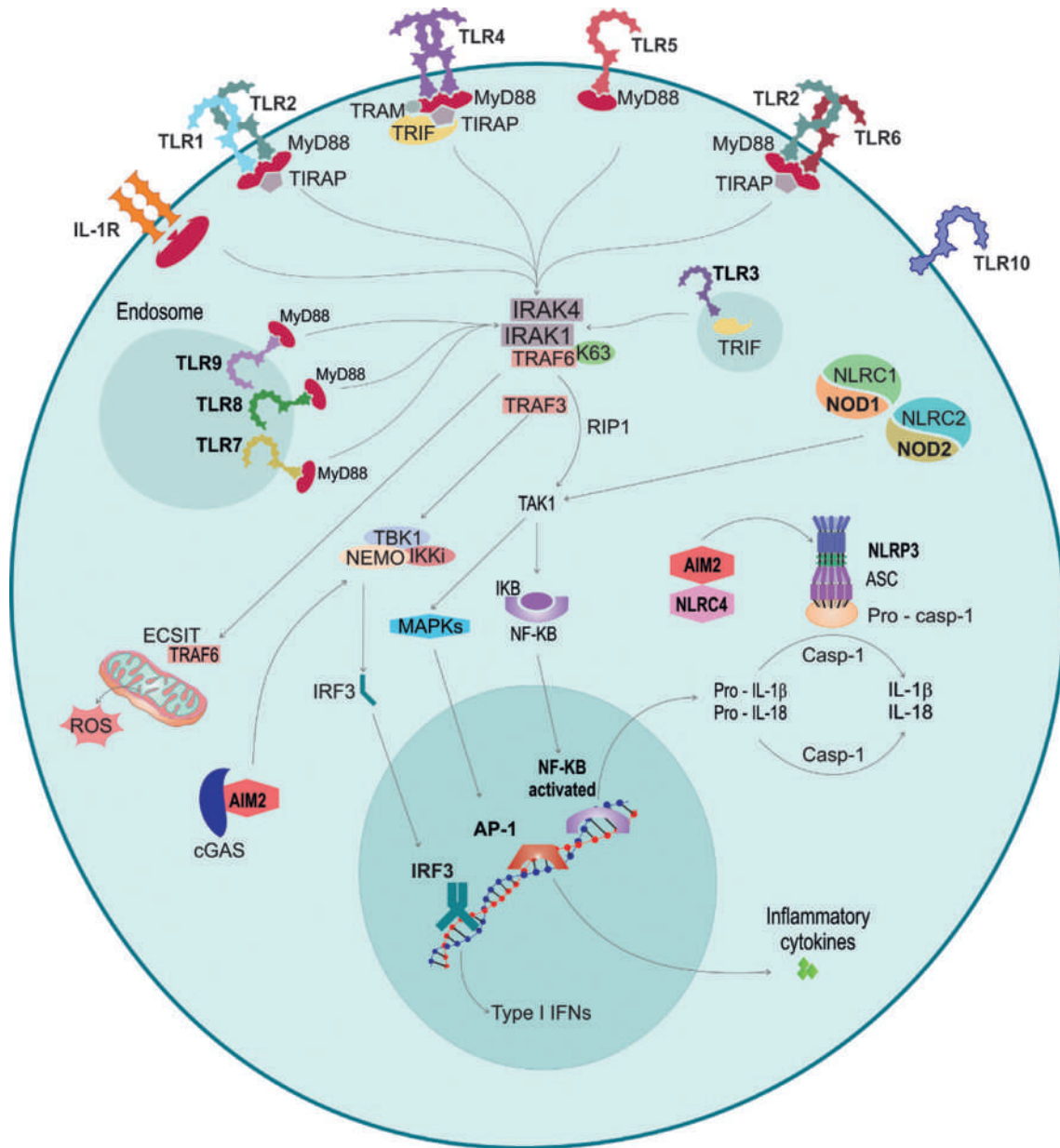


Fig. 2 Signaling pathways triggered by Toll-like receptor (TLR) and Nod-like receptor (NLR). The TLR complex recruits a TIR domain-containing adaptor complex, including TRAM—MyD88 and TRIF adaptors. MyD88 pathway: MyD88 recruits IRAK family members and TRAF6 to activate TAK1. The TAK1 complex activates the IKK complex composed of IKK α , IKK β , and NEMO (IKK γ), which catalyze the phosphorylation of I κ B proteins. Phosphorylated I κ B proteins are degraded, allowing NF- κ B to translocate to the nucleus. TAK1 simultaneously activates the MAPK pathway. The activation of NF- κ B and MAPK results in the induction of inflammatory cytokine genes. TRIF pathway: TRAM-TRIF recruits TRAF6 for activation of TAK1 as well as TRAF3 for activation of TBK1-IkKi that phosphorylates and activates IRF3. Whereas NF- κ B and MAPK regulate expression of inflammatory cytokine genes in both pathways, IRF3 regulates expression of type I IFN in the TRIF-dependent pathway only. NOD-like receptor: The ligands NOD1 is NLRC1, and NOD2 is NLRC2; they recognize distinct structural motifs derived from peptidoglycan.

NLRP-3 inflammasome

The NLRs respond to many PAMPs from pathogens and lead to the release of the IL1 family of inflammatory cytokines, including IL1 β , IL18, and IL33, through the formation of the NLRP-3-inflammasome, which involves caspase-1 apoptotic pathway (Meylan et al., 2006; Yu and Finlay, 2008; Fritz et al., 2006). Upon activation, NLRP3 oligomerizes with apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase 1. Procaspase-1 is converted to activated caspase-1, which subsequently matures and secretes the pro-inflammatory cytokines IL1 β and IL18 in their mature form caspase-1 mediates the processing of the pro-form of these cytokines into mature forms, which results in the secretion of bioactive cytokines. The stimuli such as bacterial toxins, β -hemolysin, pneumolysin, listeriolysin O, and bacterial compounds activate the inflammasome. *Pneumococcus* pneumolysin

exotoxin activates NLRP3 and AIM2 100 and β -hemolysin. *S. agalactiae* invasion activates NLRP3. NLRP3 also has been associated with the responses to several microorganisms, such as *S. aureus*, *L. monocytogenes*, *K. pneumoniae*, and *E. coli* (Davis et al., 2011). Infection by *Listeria* sp. sensed by AIM2 and NLRC4, which activates NLRP3 and leads to strong caspase1 activation and pro-inflammatory response (Wu et al., 2010).

Central nervous system immune response to bacterial infection

Microglia

Microglia are one of the major resident mononuclear phagocytes that participate in innate immune responses in CNS. Microglia act as a baseline immune sentinel that defense against invading pathogens into the CNS before peripheral leukocyte infiltration (Norris and Kipnis, 2019). When bacterial pathogen crosses the BBB, microglia responds either directly to the bacteria or the cell wall antigens. In response to cell wall components, microglia produces inflammatory mediators such as IL6, TNF α , IL12, keratinocyte-derived chemokine (CXCL1/KC), CCL2/MCP1, CCL3/MIP1 β , CXCL2/MIP2, and CCL5/RANTES, as well as soluble TNF receptor II, TNF α antagonist (Rock et al., 2004; Hanisch, 2002). The inflammatory mediators' production involved activation of the extracellular signal-regulated protein kinases ERK1 and ERK2 via a MAPK intracellular signaling pathway (Hanisch, 2002). To elicit antibacterial defense, this microglial-derived cytokine profile is effective at leukocyte recruitment into the CNS. Along with the complement pathway, microbial activation leads to post-infection cognitive impairment by the loss of hippocampal synapses (Vasek et al., 2016). In dogs with meningoencephalitis, the marked levels of Th1, Th2, and Th17 related cytokine, CXCR3, CCR2 chemokine receptor mRNA, microglia/macrophage levels were reported (Park et al., 2013). We recently demonstrated the in vivo microglial activation after *S. pneumoniae* induced meningitis in the rat model using [^{11}C]PBR28-PET imaging (Giridharan et al., 2020). Besides, several studies have reported the microglial activation after *S. pneumoniae* infection (Djukic et al., 2006). In the animal model, neuro-invasive infection by *L. monocytogenes* demonstrated activation of microglia (Zhang et al., 2018). A study by Hoogland et al., reported that systemic challenge with live *E. coli* induces microglial activation 3 days after inoculation, at the same time no production of pro-inflammatory cytokines in the brain was found. In CNS tuberculosis patients monocyte, *M. tuberculosis* upregulates microglial matrix metalloproteinase 1 and 3 expressions and secretion via NF κ B and activator protein 1 dependent monocyte networks (Green et al., 2010). Challenge of infant rats' brains with *H. influenzae* type b intraperitoneally resulted in the time-dependent expression of MIP2, MIP1 α , MCP1, and RANTES, the expression of RANTES was localized predominantly to resident microglia (Diab et al., 1999).

Astrocytes

The astrocytes are macroglial resident cells of the CNS, play critical roles in and elimination of neurotoxins, and invading bacterial pathogen by the production of trophic factors. Astrocytes orchestrate with microglial cells to regulate the recruitment and activity of infiltrating hematogenous cells through their expression of cytokines, proteases, protease inhibitors, adhesion molecules, and extracellular matrix components (Mucke and Eddleston, 1993). In addition to its role in the immune system, the functions of astrocytes include the maintenance of BBB. Glial fibrillary acidic protein (GFAP) is an astrocyte-specific protein belonging to the intermediate filament protein at grade III, whose increased level is strongly correlated with brain development. The level of GFAP has been shown to exhibit elevations in cases of bacterial meningitis, highlighting the leakage of the BBB.

Pneumolysin a major *S. pneumoniae* neurotoxin as a factor in remodeling the structure of the host brain tissue by astrocyte reshaping and interstitial fluid retention, thereby increases the penetration of toxic macromolecules and bacteria into the tissue and worsens the disease course (Hupp et al., 2012). In murine astrocyte cultures, *S. pneumoniae* upregulates the macrophage receptor with collagenous structure (MARCO), that mediates the production of IL1 β in astrocytes (Braun et al., 2011). Astrocytes recognize *L. monocytogenes* infection uses TLR2 and NOD1 PRR system during neonatal and adult meningitis (Mosa et al., 2009). Intracerebral injection of *E. coli* K1 E44 strain into mice induced expression of the astrocyte differentiation marker glial fibrillary acidic protein and the inflammatory mediators' cyclooxygenase 2 and nitric oxide synthase 2 (Wu et al., 2009). Challenge of infant rats' brains with *H. influenzae* type b intraperitoneally resulted in the time-dependent expression of MIP2, MIP1 α , MCP1, and RANTES, the expression of RANTES was localized predominantly to resident microglia (Diab et al., 1999).

Future studies dissecting the potential roles of inflammatory mediators and their receptors in the context of CNS infectious diseases may provide insights into the complex regulatory network inflammatory responses.

Glymphatic system

The CNS was believed to be devoid of a lymphatic system. The identification of the *glymphatic* pathway in rodent brain changed this scenario (Baciyinski et al., 2017). This pathway subserves, the flow of CSF to the perivascular spaces, penetrating arteries of the brain, mixes with interstitial fluid (ISF) and solutes in the parenchyma and then exits along with the perivascular spaces of draining veins which are facilitated by aquaporin 4 (AQP4) water channels (Rasmussen et al., 2018). It gained importance when it was identified as a pivotal contributor to the clearance of interstitial solutes from the brain, including amyloid β , a major misfolded protein in Alzheimer's disease. Hence recently, attention raised to increase the knowledge of glymphatic dysfunction, which might provide a new perspective for understanding the pathophysiology of brain infection.

Cytokines

Release of the inflammatory mediator cytokine is a one of a significant event that occurs after bacterial invasion. As underactivation of cytokine results in an inadequate immune response, whereas overactivation can be extremely destructive. Proinflammatory cytokines such as TNF α , IL1, IL6, and IL8 and the anti-inflammatory cytokines IL10 and TGF β are involved in the pathophysiology of the bacterial pathogen. The cerebrospinal fluid neonates with bacterial meningitis presented with high levels of TNF α , IL1 β , and IL6 (Krebs et al., 2005; Fida et al., 2006). In children with bacterial meningitis, exhibited an increase in TNF α , IL6, and IL8 concentrations in the CSF (Prasad et al., 2014). The results from meta-analysis reported that the TNF α and IL1 β levels were identified as useful markers for the prediction of bacterial meningitis (Panato et al., 2014). IL6 is produced in response to infections and tissue injuries. It contributes to the host immune defense through the stimulation of acute-phase proteins, fever, leukocytosis, and the activation of the complement cascade (Tanaka et al., 2014). The concentration of IL6 in patients with acute bacterial meningitis CSF (Vazquez et al., 2012; Takahashi et al., 2014), and the serum were significantly elevated (Hamed et al., 2012). Elevated levels of IL6 in the CSF of pediatric patients with bacterial meningitis observed with no association of bacteremia, neurologic complications, or sequelae (Rusconi et al., 1991). In experimental meningitis, IL6 deficient mice presented an increase in the inflammatory response in the CSF; however, there was also a reduction of vascular permeability, intracranial pressure, and BBB disruption during the bacterial meningitis infection (Paul et al., 2003). In experimental models, IL6 has shown a dual role as both a pro-inflammatory and anti-inflammatory cytokine (Paul et al., 2003; Cohen and Cohen, 1996).

The cytokines play the significant role as an inflammatory mediator after encephalitis infection. The CSF concentration of IL6, IFN γ , IL10, and sTNFR1 was significantly higher in encephalitis patients (Ichiyama et al., 2008). IL5 is expressed intrathecally in encephalitis act as a mediator and marker (Grygorczuk et al., 2016, 2018a,b). In a mice model of encephalitis resistant C57BL/6 mice protected against encephalitis independently of signaling via TNFR1 or TNFR2 (Lundberg et al., 2007). The IL12 is chemotactic and activates natural killer (NK) cells, and induces their binding to endothelial cells CD4 T cells preferentially differentiate toward Th1 cells in the presence of IL12. Besides, these cell types, neurons have IL12 receptors exogenous IL12 treatment results in host recovery from encephalitis. IL12 administration to mice results in rapid clearance of the pathogen, increased survival, and enhanced recovery from infection (Ireland et al., 1999). Understanding the levels of cytokines is a crucial intervention is one of the possible strategies. The intervention of inflammatory response at the cytokine level can be achieved by different avenues (i) inhibition of transcription of the cytokine gene; (ii) blockage of the cytokine itself; (iii) by cleavage to activate the procytokine; and iv. antagonism of the cytokine receptor and its downstream signaling pathways (van der Flier et al., 2003).

Chemokines

Chemokines are the other inflammatory mediators released after bacterial entry. They are produced at sites of inflammation at the luminal side of endothelial cells or on the surface of the heparan sulfates (Middleton et al., 1997). The CSF of patients with bacterial meningitis contains detectable levels of the C-X-C chemokines (IL8) and C-C chemokines (monocyte chemotactic protein 1 [MCP1], macrophage inflammatory proteins MIP1 α and MIP1 β) (Spanaus et al., 1997). In experimental models of meningitis, neutralization of C-X-C had a sustained effect, and neutralization of MCP1 reduced macrophage infiltration, whereas systemic neutralization of MIP1 α or MIP2 with antibody temporarily reduced neutrophil influx into the CSF. However, intrathecal neutralization of IL8 did not affect immune cell recruitment (Diab et al., 1999; Ostergaard et al., 2000). In encephalitis, CSF-CXCL1 concentrations correlated with neutrophil count was higher in patients with encephalitis (Grygorczuk et al., 2016, 2018a,b). The CSF concentration of CCL5 was increased in encephalitis, the highest in the most severe presentation (Grygorczuk et al., 2006, 2016, 2018a,b).

Complement system

Infection by bacterial pathogen strongly activates the complement system of plasma proteins (Jack et al., 1998; Jarvis and Griffiss, 1989). The major pathways by which complement may be activated after bacterial colonization is through the antibody-mediated classical pathway (C1q, C1r, C1s, C4 and C2); direct binding of complement components to the pathogen surface called alternative pathway (Factor B and Factor D); and the mannose (or mannan) binding lectin (MBL) pathway (Lewis and Ram, 2014). MBL, as the name suggests, can recognize and bind to mannose on pathogen surfaces. Notably, individuals who are deficient in complement proteins have an increased risk of meningococcal disease (Walport, 2001). In particular, the interaction of molecules of the complement system with the bacterial pathogen has proven important in disease pathogenesis as it establishes a significant contribution to the development of newer vaccine formulations.

Clinical consequences and post-mortem findings of brain infection, encephalitis, and meningitis

Brain infection, such as meningitis and encephalitis, is highly fatal without treatment. Even with treatment, fatality is 5–10% within two days of symptom development, and more than 10% of the survivors are left with severe sequelae (Meningococcal meningitis fact sheet #141, 2018). The most common neurological disabilities in children surviving meningitis infection are sensorineural hearing impairment, seizures, academic and behavioral difficulties, and a decrease in intelligence (Saez-Llorens and McCracken, 2003) while in adults hearing loss and cognitive impairment are the most common neurological complications (van de Beek et al., 2006).

Growing evidence also confirms the increased risk of psychiatric and behavioral problems such as post-traumatic stress disorder, attention deficit hyperactivity disorder, separation anxiety, depression as well as schizophrenia and psychotic symptoms in the life of children surviving meningitis (Abraham et al., 2005; Khandaker et al., 2015; Olbrich et al., 2018). Khandaker et al. demonstrated that children with a childhood meningitis history showed poor performance on all neurocognitive and academic measures, showed increased depression, anxiety symptoms, psychological and behavioral issues, and showed a twofold increase in the risk of psychotic experiences in the long-term compared to healthy controls (Khandaker et al., 2015). Hence, it is very crucial to acknowledge and try to minimize these severe sequelae in survivors.

The most common complications during meningitis infection, mainly caused by pneumococcus, were cerebrovascular diseases (van de Beek et al., 2006; Weisfelt et al., 2006b). Around 30% of the patients suffered from an arterial stroke, and around 9% suffered from cerebral venous thrombosis, while intracranial hemorrhages were seen in approximately 9% of patients (Kastenbauer and Pfister, 2003; Weisfelt et al., 1996, 2006a). Inflammatory infiltrates seen in cerebral arteries and veins in various post-mortem studies (Buchan and Alvord, 1969; Cairns and Russell, 1946; Dodge and Swartz, 1965), which along with the segmental arterial narrowing seen on angiography, leads to the hypothesis that vasculitis could be the underlying pathophysiological mechanism of infarction seen in bacterial meningitis patients. Delayed cerebral thrombosis (DCT), which is a rare but devastating complication seen in 2–4% cases of pneumococcal meningitis, causes multiple cerebral infarcts (Lucas et al., 2013; Schut et al., 2009). An autopsy study comparing the histopathology of brain samples from pneumococcal meningitis patients with DCT and without DCT with non-meningitis controls reported that several factors could cause DCT, such as inflammation of vessels, thromboembolism of large arteries and infectious intracranial aneurysms (Engelen-Lee et al., 2018). Pneumococcal capsule components were also reported to be present in the meninges for a long-duration after meningitis and might be responsible for the increase in inflammation after the initial immunosuppression by dexamethasone wears off ultimately leading to DCT and cerebral infarcts (Engelen-Lee et al., 2018). Another post-mortem study demonstrated that fibrin thrombi and brain infarcts were frequently present in the brain samples of pneumococcal meningitis patients. At the same time, inflammatory vessel wall infiltrates were absent, suggesting that diffuse cerebral intravascular coagulation can be another etiologic factor causing ischemic stroke in pneumococcal meningitis (Vergouwen et al., 2010).

Apart from infarction, other mechanisms of neuronal damage have also been reported by several post-mortem studies. Apoptotic neuronal damage in the dentate gyrus of hippocampal formation has been reported in autopsy samples from bacterial meningitis patients, which might explain the learning deficits frequently seen in the survivors of bacterial meningitis (Nau et al., 1999). Though the exact mechanism of apoptosis of neurons in bacterial meningitis is not known, free radicals and excitatory amino acids may be responsible for it. A substantial contribution of axonal pathology to neuronal damage in bacterial meningitis was also demonstrated in another post-mortem study. Extensive axonal injury caused predominantly by ischemia was reported to be present in the white matter of human and animal post-mortem brain samples (Nau et al., 2004).

Similarly, the selective synaptic loss was also seen in the frontal neocortex in autopsy samples from pneumococcal meningitis patients. In this study, pneumolysin is hypothesized to play a synaptotoxic and dendritotoxic role in pneumococcal meningitis by releasing glutamate from astrocytes and ultimately causing excitotoxic synaptic damage (Wippel et al., 2013). In conclusion, various mechanisms causing neuronal damage should be further explored to find new therapeutic targets for decreasing the frequency of debilitating neuropsychiatric sequelae in survivors of meningitis.

Conclusion

The recognition of immune privilege to CNS is attributed to its various tissue properties such as the presence of physical barriers, the absence of traditional lymphatics, and the lack of major histocompatibility complex class II expressing antigen-presenting cells. Classically, immune privilege in the CNS is a double-edged sword. Although it makes the neuroprotective mechanism, in the context of CNS infections, and immune privilege not considered beneficial to the host. In this chapter, we summarized the bacterial invasion, the central immune response to the invaded bacteria, and its long-term neurological sequelae after meningitis and encephalitis. It is crucial to understand the interactions between the complex immune network, inflammatory profile, and bacterial virulence factors as it may aid in developing potential therapeutic strategies for these infectious diseases.

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Brain and Central Nervous System Infections: Viruses

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Introduction

Several viruses exhibit tropism for the central nervous system and/or peripheral nervous system. The majority are RNA viruses (Tables 1 and 2). Viral infection in the nervous system can result in a multitude of clinical presentations, including acute or chronic meningitis, encephalitis, myelitis, polyradiculitis, and peripheral neuropathy.

Viruses can directly affect the nervous system, such as in meningitis and encephalitis, or indirectly (parainfectious or post-infectious) from inflammatory or immunologic reactions, such as in Guillain-Barré syndrome (GBS) or acute disseminated encephalomyelitis (ADEM) (Barah et al., 2014; Helgeson et al., 2018; de Almeida et al., 2007; Steiner et al., 2005).

Clinical aspects

All segments of the nervous system, central or peripheral, can be involved in viral infections. Neurological complications of RNA and DNA virus are summarized in Tables 1 and 2. Concerning the central nervous system, the main clinical presentations are meningitis and encephalitis.

Meningitis is the most common infectious CNS syndrome, defined as an inflammation of the meninges. Viruses can cause acute or chronic meningitis. The clinical symptoms of acute meningitis are fever, malaise, and vomiting, and in some cases, petechial rashes. Signs of meningeal irritation include neck stiffness, Kernig's sign (an inflection of the knee when the limb is placed at a certain degree of relative inflection to the trunk), and Brudzinski's sign (an involuntary inflection of the limb following a head inflection). These signs are poorly sensitive in adults; both Kernig and Brudzinski signs have a sensitivity of only 5%, while the sensitivity of nuchal rigidity is 30%. The nonspecific nature of the symptoms and clinical signs means that we often overtreat and require additional exams to confirm a diagnosis (Thomas et al., 2002). Signs of meningeal irritation are rare among younger children. Instead, small children can present other signs, such as an inability to feed, vomiting, drowsiness, convulsions, and a bulging fontanel.

Encephalitis includes clinical signs of brain parenchyma, involving fever, chronic headache, and conscience alteration, which can be followed by focal neurological signals or seizures of recent onset. Meningoencephalitis occurs when meningitis is followed by involvement of the brain parenchyma (de Almeida et al., 2007).

The spinal cord can be affected by virus infection in any of its segments. The clinical presentation of acute viral myelitis can be divided into poliomyelitis-like gray matter syndrome, or partial or complete white matter syndrome. First, pure motor deficit occurs, which consists of weakness and one or more flaccid limbs without sensory deficit or urinary bladder. Then, prominent sensory disturbances, urinary retention, and weakness with either hypo- or hyperreflexia with myelitis occur, due to the involvement of the white matter. This involvement usually affects only part of the transverse expanse of the spinal cord and manifests as asymmetric motor and sensory symptoms. In acute transverse myelitis, both halves of the spinal cord are affected (Kincaid and Lipton, 2006).

Table 1 Neurological complications of RNA viruses.

Agent		Meningitis	Encephalitis	Myelitis	ADEM	GBS	Cognitive impairment
Non-polio enteroviruses	Echovirus	+++	+	+			
	Coxsackievirus	+++	+	+			
	EV-A71	+	+	++			
Parechovirus		+	+			+	
Arboviruses	Dengue		+	+		+	
	Zika virus	+		+		++	+
	Yellow fever		+				
	Chikungunya virus	+	+	+		+	
	West Nile virus	+	++	++			
	St Louis encephalitis virus	+	++	++			
	Powassan		++				
	Japanese encephalitis virus (JEV)		++	+		+	
Lymphocytic choriomeningitis virus (LCMV)		++	+	++			
Mumps virus		++	+	+	+		
Human immunodeficiency virus (HIV)		++	+	+		+	+
HTLV 1/2 ^a				+			
Rabies virus ^b			+++	+	+		
Measles virus			+	+	+++		
Rubella virus				+	+		
Enterovirus poliovirus ^c		+	++	+++	+		
Influenza				+	+		
Parainfluenza		+	+		+		
Rotavirus		+					
Filovirus	Ebola	+	+				+
	Marburg		+				
	SARS-CoV	+	+				
	MERS-CoV	+	+		+		
	SARS-CoV-2	+	+	+	+	+	?
Picornavirus							
HAV		+	+	+	+	+	
Flavivirus			+	+	+	+	+
Calicivirus			+	+		+	

The number of crosses "+" indicates the frequency of the neurological syndromes, one cross less frequent, three crosses more frequent.

ADEM, acute disseminated encephalomyelitis; GBS, Guillain-Barré syndrome; HAV, hepatitis A virus; HCV, hepatitis C virus; HEV, hepatitis E virus.

Monticelli et al., 2018; Thompson and Thakur, 2017; Billioux et al., 2016.

^aHuman T-lymphotropic virus type 1 (HTLV-1) and type 2 (HTLV-2) cause *tropical spastic paraparesis*.

^bRabies virus is a significant cause of encephalitis worldwide, with nearly 100% mortality. It is enzootic in all continents except Australia and Antarctica, as well as certain island states or nations (Great Britain, Ireland, Iceland, Japan, New Zealand, Hawaii, the Bahamas, and Bermuda).

^cPoliovirus was eradicated from the Western hemisphere, but is endemic in Nigeria, Afghanistan, and India.

Table 2 Neurological complications of DNA viruses.

Agent		Meningitis	Encephalitis	Myelitis	ADEM	GBS	Cognitive impairment
Herpes viruses	Herpes simplex virus (HSV)-1	+	++	+		+	+
	HSV-2	++	+	++		+	
	Varicella-zoster virus (VZV)	+	++	++	+		
	Cytomegalovirus (CMV)	+	++	++		+	
	Epstein-Barr virus (EBV)	+	+	+	+	+	
	Human herpesvirus (HHV)-6	+	++	+			
	HHV-7	+		+			
	HHV-8		+				
	Herpes B virus		+++				
Adenovirus		+	++				
Parvovirus B-19		+	++	+		+	
Hepadnavirus				+	+	+	+
HBV				+	+	+	+

The number of crosses "+" indicates the frequency of the neurological syndromes, one cross less frequent, three crosses more frequent.

ADEM, acute disseminated encephalomyelitis; GBS, Guillain-Barré syndrome; HBV, hepatitis B virus.

Monticelli et al., 2018; Thompson and Thakur, 2017; Barah et al., 2014.

Cognitive impairment is present in several CNS virus infections, mainly in chronic infections, such as HIV or HCV, or as sequelae of acute encephalitis, as HSV-1 encephalitis (Tables 1 and 2).

Acute meningitis

Viral meningitis is a global disease that can be either sporadic or epidemic. The main cause of acute viral meningitis are non-polio enteroviruses, with around 80–95% of the cases, especially during the summer months. The remaining cases are caused by several other virus (Tables 1 and 2) (de Almeida et al., 2007; Vidal et al., 2011, 2019). Despite its low mortality rates, viral meningitis can be associated with a high morbidity. Coxsackie virus sub-group B is responsible for 60% of cases of meningitis among children under 3 years of age. Enterovirus 70 and 71 show strong neurotropism, which is associated with meningoencephalitis, polio-like paralytic syndromes, GBS, or meningitis (Teoh et al., 2015).

The viruses of the herpes family are collectively responsible for 4% of meningitis cases. Meningitis is more frequently caused by HSV-2, while HSV-1, and EBV are associated with recurrent lymphocytic meningitis (de Almeida et al., 2007). Parotid disease can be associated with meningitis in 10–20% of cases. It is more frequent during winter months, and it occurs in males in a 3/1 ratio (Rhie et al., 2016).

Encephalitis

Herpes simplex, mainly HSV-1, is the most common cause of sporadic encephalitis, while arboviruses being the most common overall, mainly during epidemic. Human herpes virus 6 is an emerging cause of encephalitis in immunocompetent and immunodeficient patients.

Neuroimaging findings, such as edema and necrosis in one of the temporal lobes, are strongly indicative of herpetic encephalitis.

Arboviruses

Arbovirus (arthropod-borne virus) causes emergent and re-emergent viruses. Outbreaks of these viruses have become more frequent due to increases in climatic change, deforestation, and urbanization (Carod-Artal et al., 2013; Tournabize et al., 2009). Several arboviruses are restricted to specific geographical areas, and can thus be considered traveler diseases (Thompson and Thakur, 2017; Petersen et al., 2016). Arboviruses encompass viruses of distinct genera or families, which are transmitted by arthropod vectors. They comprise a group of over 100 RNA neurotropic and neurovirulent viruses, which mainly cause encephalitis but also meningitis. Some examples include Flavivirus: dengue, yellow fever virus, Saint Louis encephalitis virus, Powassan virus, Japanese encephalitis virus (JEV), West Nile virus (WNV), Murray Valley Encephalitis virus, Tickborne Encephalitis virus, Louping III virus, Kyasanur Forest Disease virus, Rocio virus, Ilheus virus, and Zika virus. Alphavirus: Chikungunya virus, equine encephalitis virus (Eastern (EEE), Western (WEE), Venezuelan (VEE)), Semliki Forest virus, Me Tri virus, Mayaro; Bunyavirus: California (La Crosse), Oropouche, Phlebovirus: Rift Valley Fever virus, Toscana virus; Reoviridae: Colorado tick fever; Rhabdoviridae: Chandipura virus (Weaver and Reisen, 2010; Solomon et al., 2000; Solomon, 2004; Figueiredo, 2007; Fragoso et al., 2016; Liu and Chambers, 2001; Qureshi et al., 2012; Simon et al., 2016; Wang et al., 2020; Muñoz et al., 2016; Soares et al., 2020).

Hepatitis viruses

Hepatitis viruses encompass a range of diverse and unrelated groups of viruses from different families, with the common characteristics of hepatotropism, hepatovirulence, and hepatotoxicity. The hepatitis viruses of neurological interest primarily comprise RNA viruses, hepatitis A, C, and E viruses (HAV, HCV, and HEV, respectively), and a DNA virus, hepatitis B virus (HBV) (Sellner and Steiner, 2014). Viral hepatitis can be accompanied by various extrahepatic manifestations in both acute and chronic infections.

HCV and HBV are the most frequent hepatitis viruses with nervous system involvement (Yimam et al., 2013), although it has also been described with HAV and HEV, albeit with a lower frequency (Ferro et al., 2016).

Hepatitis viruses can affect the entire nervous system, including the brain, spinal cord, motor neurons, peripheral nerves, and muscles. The most common neurological manifestations of HCV and HBV are peripheral neuropathy.

Hepatitis C virus

The extrahepatic manifestations of HCV are independent of the severity of the underlying chronic liver disease and hepatic encephalopathy. Neurological complications of HCV occur mainly in chronic infections, but can also occur in acute cases. Nervous system impairment occurs in more than 50% of chronically infected HCV individuals (Iriana et al., 2017). HCV mainly affects

neurons responsible for motor function, memory, and concentration (Monaco et al., 2015). Although the incidence of HCV infection is lower than that of HBV, chronicity occurs in up to 85% of cases (Sellner and Steiner, 2014); this could explain the higher frequency of neurological complications.

Nervous system complications in patients with HCV include peripheral neuropathy, which is the most common neurological complication among subjects with chronic HCV infection (Adinolfi et al., 2015). HCV infection is known to cause motor, sensory, and sensorimotor mononeuropathy, mononeuropathy multiplex, or polyneuropathy (Adinolfi et al., 2015). Symptomatic peripheral neuropathy was observed in 9% of cases (Cacoub et al., 2000). In contrast to the brain, there is currently no evidence for HCV replication in peripheral nerves (Adinolfi et al., 2015). Peripheral neuropathy associated with HCV is usually associated with cryoglobulinemia, which is mainly characterized by axonal damage. It was postulated that nerve damage is secondary to epineural vessel changes caused by occlusion or vasculitis induced by longstanding cryoglobulin precipitation with complement fixation and rheumatoid factor (RF) deposition. Vasculitis or vascular occlusion causes fascicular ischemia, leading to axonal degeneration (Nemni et al., 2003). GBS associated with HCV infection is rare. The mechanism of GBS is based on the autoimmune-induced demyelination of peripheral nerves, which often occurs after an infectious episode. Chronic inflammatory demyelinating polyneuropathy associated with HCV has also been described (Ferro et al., 2016).

A significant proportion of patients with chronic HCV experience cognitive impairment and HCV neurocognitive disorders in the absence of advanced liver disease, which may interfere with their daily living activities and quality of life (de Almeida et al., 2018). The rates of cognitive impairment vary widely across studies (0–82%), reflecting differences in co-morbidities and experimental methods (Huckans et al., 2009). Several studies have investigated domain-specific cognitive impairments in HCV-positive patients and found deficits in attention, concentration, working memory, executive function, and psychomotor speed (Perry et al., 2008; de Almeida et al., 2018). Cognitive impairment was not found to be correlated with HCV RNA in the peripheral blood or cerebrospinal fluid (CSF) or the presence of cirrhosis (Clifford et al., 2015; de Almeida et al., 2018), and was not dependent on the HCV subtype (Monaco et al., 2015; de Almeida et al., 2018).

Human immunodeficiency virus

Neurological complications of human immunodeficiency virus (HIV) infection are summarized on Table 3.

Acute meningitis is present in 5–10% of HIV-infected patients, usually before seroconversion, as well as during or after the observation of mononucleosis-like signs and symptoms. Its clinical evolution is self-limited. It can be asymptomatic or accompanied by focal neurological signs, such as peripheral facial palsy. The CSF has a biochemical and cellular pattern compatible with that of acute viral meningitis (Heaton et al., 2010). Anti-HIV enzyme-linked immunosorbent assays (ELISA) often yield negative results with blood and CSF samples. Patients at risk of HIV infection are advised to have a second serological diagnosis test for HIV after 4–6 weeks. However, no specific biomarker in the CSF or blood helps diagnose acute meningitis caused by HIV. The p24 antigen test for the early diagnosis of HIV using blood samples is neither sensitive nor standardized for CSF analysis.

Chronic meningitis is present in 40% of infected individuals at any stage of HIV infection, while 59% are asymptomatic. Because it is an exclusion diagnosis, other causes of chronic meningitis must be ruled out (de Almeida et al., 2008). CSF presents with pleocytosis (5–50 cells/mm³) and increased total protein level (50–100 mg/dL), while glucose, the CSF blood glucose ratio, and lactic acid are in the normal range (Fishman, 1992; Zink and Clements, 2000; de Almeida et al., 2011a).

HIV associated neurocognitive disorder (HAND) is the main neurological complication directly associated with HIV infection, and is a clinical diagnosis (Heaton et al., 2010; Antinori et al., 2007; de Almeida et al., 2013). CSF analysis is important for ruling out opportunistic infections and co-infections. Several biomarkers in CSF with the potential to diagnose HAND have been studied. However, they are not clinically applicable (Brew and Letendre, 2008; de Almeida et al., 2005; Cinque et al., 2007; Gisslen et al., 2007; Hagberg et al., 2010) and are only used in research. With the aging of the HIV population, other biomarkers have become important and have been investigated (Brew et al., 2005; Gisslén et al., 2009; Krut et al., 2013; Krut et al., 2014). The same can be

Table 3 Neurological complications of HIV infection.

<i>Directly related to HIV infection</i>	<i>IRIS</i>	<i>Opportunistic infections</i>	<i>Co-infections</i>
Acute meningitis	opportunistic infections	<i>Cryptococcus</i> sp.	Tuberculosis
Chronic meningitis	Inflammatory disease	Toxoplasmosis	Syphilis
HAND	HAND	PML (JC virus)	Community-acquired bacterial meningitis
Peripheral neuropathy		Primary lymphoma	Cysticercosis
Vacuolar myelopathy			Hepatitis C virus
Parkinsonism			Chagas disease
			Malaria
			Schistosomiasis
			NPCM

HAND, HIV-associated neurocognitive disorder; *IRIS*, immune reconstitution inflammatory syndrome (after starting antiretroviral); *NPCM*, neuroparacoccidioidomycosis; *PML*, progressive multifocal leukoencephalopathy.

applied to polyneuropathies and myelopathy associated with HIV. HIV vacuolar myelopathy occurs in 10% of patients with AIDS; it is an exclusion diagnosis. Other frequent causes of myelopathy, such as neurosyphilis, HTLV, CMV, and specific endemic etiologies, must be ruled out.

Neurons do not have a CD4 receptor, the main receptor involved in HIV cell invasion. Consequently, HIV does not infect neurons. Neuronal injury in HIV infection results from an indirect mechanism due to the complex interaction of anions, inflammatory and neuronal injury proteins, and constitutional HIV proteins. These proteins have been investigated as biomarkers in the CSF or serum for CNS HIV infection. It is possible that pyroptosis plays an important role in neuronal lesion and death. Pyroptosis markers in the CSF of patients infected with HIV have not yet been studied. The mechanism of cell death, namely, apoptosis, which can be described as the death of cells with a productive infection caused by HIV, does not depend on the inflammatory process. Rather, it is associated with the activation of caspase-3. Recently, a mechanism of cell death in HIV-infected cells, called pyroptosis, was described. The term pyroptosis refers to proinflammatory programmed cell death, a form of cell death that lies between apoptosis and necrosis. Pyroptosis is dependent on caspase-1, which in turn depends on inflammatory processes. Caspase-1 is not involved in apoptotic cell death, and caspase-1-deficient cells respond normally to most apoptotic signals. The HIV-1-induced death of CD4 T lymphocytes is mediated by pyroptosis. The two mechanisms of programmed cell death, apoptosis and pyroptosis, are not exclusive (Doitsh et al., 2014a; Doitsh et al., 2014b; Monroe et al., 2014).

IRIS-HIV is characterized by the occurrence of opportunistic infections or non-infectious inflammatory disease within weeks of initiating or changing ARV treatment, despite improvements in immunological status. Approximately 15–25% of patients who receive ARV treatment develop IRIS HIV within the first few months of therapy. The nervous system is a frequent target. Biomarkers in the CSF that diagnose IRIS have yet to be identified, and the diagnosis is based on clinical criteria (Lipman and Breen, 2006; Venkataramana et al., 2006).

The CNS acts as an important reservoir for HIV (Karris and Smith, 2011; Zárate et al., 2007; Haggerty and Stevenson, 1991). Several constitutional characteristics specific to the CNS support the view that the CNS is an immunologically privileged site. HIV can infect two types of cells in the CNS: cells derived from monocytes (microglia and macrophages) and astrocytes. Specific CNS immunological characteristics, the blood-brain barrier (BBB), rapid mutation and recombination of HIV, and poor ARV penetration in the CNS contribute to the compartmentalization of HIV in the CNS. Some organs, such as the CNS, genital tract, and gastrointestinal lymphoid tissue, are viral compartments and reservoirs that allow HIV to persist despite ARV therapy, which eliminates the virus from the peripheral blood. Compartments are defined as anatomical regions that restrict the genetic flow of HIV, thereby enabling viral evolution and divergence from the virus circulating in the peripheral blood. On the other hand, reservoirs are cells or anatomical sites where HIV or HIV-infected cells survive because the viral kinetics are slower than those in the peripheral blood. Compartments and reservoirs protect HIV from specific immune responses, ARV therapy, and biochemical changes, thereby providing an environment for the pathogen to freely interact with its host (Karris and Smith, 2011; North et al., 2010).

In treatments with a combination of ARV drugs, the main objective is to suppress HIV replication in all cells and tissues (Dore et al., 1999). The effective treatment of HAND most likely entails the complete suppression of HIV replication in the CNS. The incomplete suppression of the virus in the CNS, caused by factors such as lack of ARV penetration in the CNS, can promote viral mutations and resistance to ARV drugs, both of which allow the virus to redistribute to non-CNS tissues. In this context, the CNS is considered a possible reservoir or sanctuary for HIV (Abbott, 1987).

HIV exhibits high genetic and antigenic variability. Mutation and recombination are the main mechanisms underlying the genetic diversity of HIV and the evolution of the HIV-1 pandemic (Thomson and Nájera, 2005; Nájera et al., 2002). The high genetic diversity of HIV can be attributed to the lack of a control mechanism for reverse transcriptase activity and the consequent high error rate (0.2–2 mutations per genome per cycle), in association with a high replication rate, accompanied by rapid viral turnover (Korber et al., 2001; Domingo, 1998).

The failure of some ARV drugs to penetrate the CNS contributes to persistent neurocognitive deficits and allows for slow replication in the CNS. Improvements in neurocognitive performance after 12 weeks of HAART is greater in those who receive ARV drugs with better CNS penetration (Ariën et al., 2007). Despite the limited CNS penetration of most ARV drugs, HAART is partially effective in suppressing HIV replication in the CNS. Although some HAART-based treatments are better than others with regard to CNS penetration (Langford et al., 2006), ARV drugs must be selected to prevent viral activity in the CNS, limit neuron dysfunction, and prevent or treat HIV-infected patients with HAND. In addition, these strategies may help prevent the development of ARV resistance (Letendre et al., 2010).

Despite the effective suppression of viremia with ARV therapy, HIV can still replicate in the CNS, with the development of resistant strains in the CNS in patients with acute and sub-acute neurological manifestations (Zárate et al., 2007; Haggerty and Stevenson, 1991; Harrington et al., 2009). Discordance between the HIV viral loads in the plasma and CSF is defined by an HIV RNA viral load in the CSF that is ≥ 1 log higher than that in the plasma. CNS escape is defined as any detectable value of CSF HIV RNA while plasma HIV RNA is undetectable (Canestri et al., 2010).

Coronaviruses

Coronaviruses (CoVs) are enveloped viruses with a positive-sense single-stranded RNA genome that belong to the Coronaviridae family and the Nidovirales order. CoVs are classified into four genera: alpha, beta, gamma, and delta. Alpha, beta, and delta

coronaviruses infect mammals, whereas delta and gamma coronaviruses infect avian species (Li, 2016). However, CoV has the ability to jump between species, leading to the emergence of severe respiratory syndromes in humans, such as severe acute respiratory syndrome (SARS) caused by SARS-CoV-1, Middle East respiratory syndrome (MERS) caused by MERS-CoV, and COVID-19 caused by SARS-CoV-2 (Li, 2016). The neurotropic, neuro-invasive, and neurovirulent capabilities of CoV have been described in animals and humans, leading to encephalitis, meningitis, or encephalomyelitis. However, the capacity of CoV to infect the CNS in humans is not well characterized (Bohmwald et al., 2018). Both SARS-CoV and MERS-CoV have been proven to be neurotropic and neurovirulent (Tsai et al., 2005; Saad et al., 2014); however, nervous system complications are more frequent with SARS-CoV2 (Alshebri et al., 2020; Almqvist et al., 2020). Encephalitis and meningitis have been reported in SARS-CoV, MERS-CoV, and SARS-CoV2 (Alshebri et al., 2020).

The SARS-CoV-2 virus was first described in Wuhan, China in December 2019. In a few months, it spread to nearly every country in the world, with large economic, social, and health impacts, and a great number of deaths (Thompson, 2020). In addition to systemic and respiratory symptoms, 36.4% of patients with SARS-CoV-2 infection develop neurological symptoms (Mao et al., 2020).

Neurological parainfectious complications of SARS-CoV-2 infection include anosmia, myalgia, meningitis, encephalitis, stroke, and metabolic encephalopathy. Viral encephalitis caused by SARS-CoV-2 was described in Beijing, China, with the presence of the virus confirmed in CSF (Xiang et al., 2020; Favas et al., 2020). Post-infectious complications include ADEM, acute necrotizing hemorrhagic encephalopathy, transverse myelitis, GBS, and sensory neuropathy (Atakla et al., 2020). The post-infectious complications of SARS-CoV-2 are of inflammatory and immunological origin. In some cases, encephalopathy has been reported to present symptoms or manifestations of SARS-CoV-2 infection (Poyiadji et al., 2020; Alshebri et al., 2020).

The interval of 5–10 days between the onset of viral illness and the first symptoms of GBS is similar to that seen with that which occurs during or after other infections (Wijdicks and Klein, 2017; Kim et al., 2017; Sharma et al., 2019; Toscano et al., 2020).

Cognitive impairment is being considered as a manifestation of SARS-CoV-2 infection, with severely affected COVID-19 cases experiencing high levels of proinflammatory cytokines and acute respiratory dysfunction. All these factors have been suggested to cause cognitive decline. Pathogenetically, this may result from the direct negative effects of the immune reaction, the acceleration or aggravation of pre-existing cognitive deficits, or the de novo induction of neurodegenerative disease (Heneka et al., 2020; Fernandez-Gonzalo et al., 2020).

Ischemic stroke was described in SARS-CoV, MERS-CoV, and SARS-CoV2. The mechanism likely involves endothelial dysfunction, immune-mediated injury leading to cytokine storm and coagulation abnormalities, and hypoxia-induced injury (Alshebri et al., 2020). The majority of reported stroke cases in the literature were related to SARS-CoV2 infection, most of which had an elevated D-dimer level (Alshebri et al., 2020).

Brain autopsies in COVID-19 showed chronic neurological disease and acute abnormalities; low levels of viral SARS-CoV-2 RNA were present; however, the cellular source remains unknown. Acute hypoxic injury, hemorrhage, and minimal inflammation were frequently observed (Mukerji and Solomon, 2021).

John Cunningham virus (JCV)

JCV is a polyomavirus, a DNA virus. Seroepidemiological studies have indicated that asymptomatic primary infection with JCV occurs in childhood before 5 years of age, and that, by adult life, roughly 85% of the population is seropositive. Following primary infection, JCV becomes latent in sites including the kidney, bone marrow, and tonsils. By contrast, it is unclear whether CNS latency occurs. JCV reactivation in immunocompetent hosts usually takes the form of asymptomatic viruria (Tan and Koralnik, 2010).

Progressive multifocal leukoencephalopathy (PML), a subacute demyelinating disease of the CNS, is a result of the infection of oligodendrocytes by JCV (Koralnik, 2006). In the context of impaired cell-mediated immunity, including immunosuppressive drug treatment, lymphoproliferative disorders, chronic infectious or inflammatory diseases, such as tuberculosis and sarcoidosis, AIDS, and treatment with immunomodulatory biologicals, JCV can reactivate to produce PML (Clifford et al., 2010; Major, 2010; Tan and Koralnik, 2010).

Laboratory investigation

The diagnostic investigation of virus infection is performed by nucleic acid amplification tests (NAAT) in cerebrospinal fluid (CSF). The diagnostic characteristics of NAAT vary between viruses. Multiplex assays include several viruses, which cause nervous system infections. Despite its high sensitivity, polymerase chain reaction (PCR) can still provide false-negative results. Pre-analytical care is crucial to obtain the best results. CSF samples must be collected on DNase and RNase tubes to avoid destroying viral genetic material, and transported on ice to the laboratory. This is particularly important for RNA viruses, which are less stable. The presence of hemoglobin in body fluid samples has been considered an important inhibitory factor for PCR. Other factors include the quality of samples submitted to the lab, small recovery rates of genetic material from the sample during processing, low specificity of primers, optimization of reagents, failure to use nuclease-free materials, or the presence of a series of PCR inhibitory factors in the samples. Various body fluids, such as saliva, urine, serum, stool, amniotic fluid, and cerebrospinal fluid (CSF), contain intrinsic agents that can inhibit PCR (Davies et al., 2005; Ochert et al., 1994; Spreer, 2015; de Almeida et al., 2016). Other clinical samples, such as feces, urine, and blood, can be analyzed in association with the CSF; however, a positive reaction does not confirm CNS infection.

Currently, the study of viral agents is performed using molecular biology methods, where real-time polymerase chain reaction (qPCR) is the most suitable method. The positivity of molecular biology methods depends on a series of factors, such as sample quality; thus, samples must always be transported on ice and collected in suitable tubes. The time between the onset of symptoms and sample collection is also important (Davies et al., 2005).

The polymerase chain reaction (PCR) in the CSF, which amplifies the HSV DNA, is the method of choice for HSV diagnosis (Al-Shekhlee et al., 2006; Anderson et al., 1993). The PCR results were positive 24–48 h after the beginning of neurological symptoms and remained positive for 2–5 days after the beginning of treatment with antivirals. The sensitivity of the PCR reaction depends on a series of factors. The sensitivity of PCR for HSV is 94%, with a specificity of 98%, a positive predictive value of 95%, and a negative predictive value of 98% (Tyler, 2004; Lakeman et al., 1995).

There is a relationship between the detection of the virus by PCR in the CSF and the onset of neurological symptoms. The highest positivity of the PCR for enterovirus occurs between the 3rd and 14th day (Davies et al., 2005).

HIV RNA in the CSF

HIV particles present in the CSF can have different origins: they can drain from perivascular spaces or infected cells of the meninges, or they can originate in the plasma and pass through the choroid plexus during CSF production, particularly in the case of damage to or inflammation of the choroid plexus (Zink and Clements, 2000). In clinical practice, determining the HIV viral load in the CSF is important for monitoring the therapeutic effects of ARV treatment, identifying patients with CNS escape (compartmentalization), and for determining the differential diagnosis in psychiatric disorders (Tyler and McArthur, 2002).

The correlation between HAND and HIV viral load in the CSF is dependent on the degree of immunosuppression. In cases with a CD4 count of >200 cells/mm³ the HIV viral load in the CSF correlates positively with that in the peripheral blood, but not with HAND. After the initiation of ART, the HIV viral load in the CSF and blood decreases. In patients with a CD4 count <200 cells/mm³, the HIV viral load in CSF correlates positively with cognitive symptoms, but not with the viral load in the peripheral blood. The initiation of ARV treatment leads to a decrease in the CSF viral load, although the decrease is slower than the decrease in the blood (Ellis et al., 1997, 2002; Langford et al., 2006).

Opportunistic infections and co-infections increase the HIV viral load in the peripheral blood. Some opportunistic infections or co-infections in the CNS can increase the viral load in the CSF, as noted in patients with neurosyphilis (de Almeida et al., 2010).

SARS-CoV-2 identification in the CSF

Concerning SARS-CoV-2 identification in CSF, the majority of cases have been undetectable (Espíndola et al., 2020; Lewis et al., 2021), most likely due to a low CSF viral load. The molecular biology tests available are not validated for CSF. A negative RT-qPCR for SARS-CoV-2 in the CSF does not rule out CNS SARS-CoV-2 infection. The diagnosis of SARS-CoV-2 in the CNS is based on cases with neurological symptoms, which fulfill the World Health Organization (WHO) clinical diagnostic criteria for SARS-CoV-2 infection. Cases positive for SARS-CoV-2 by RT-qPCR on nasopharyngeal samples and probable cases negative for SARS-CoV-2 by RT-qPCR on nasopharyngeal samples both presented the clinical and radiological criteria for SARS-CoV-2 infection (WHO, 2020).

Herpes encephalitis

CSF is altered in 97% of cases of herpes encephalitis. However, there are no pathognomonic alterations. There is an increase in CSF white blood cells (WBC) of 5–500 cells/mm³, with a predominance of lymphocytes, moderate CSF total protein increase, and a normal or slightly reduced glucose (Steiner et al., 2005). The presence of red blood cells in the absence of traumatic lumbar puncture occurs in 40% of cases, while xanthochromic CSF occurs in 11% of cases. These two CSF characteristics help to distinguish the diagnosis from other types of encephalitis (Fishman, 1992). Increased IgG levels occurs after the second week of illness. Specific levels of CSF anti-HSV IgG are elevated, corresponding to increased levels in the serum, which can remain high for 3 months to 3 years after acute illness (Kapur et al., 1994). Most patients with HSV have previously developed serum antibodies; therefore, serological tests do not have a diagnostic value. A fourfold increase in serum antibodies does not represent sensitivity or specificity. Intrathecal anti-HSV antibody synthesis occurs, with an increase of four times. Although the relationship between anti-HSV antibodies in the CSF serum has diagnostic value, this increase occurs slowly and is used for diagnostic confirmation retrospectively (Whitley et al., 1982). The detection of specific antibody anti-HSV can be calculated using the relation (antibodies index (AI)) between the CSF serum quotients for the specific antibodies (Q spec) and the IgG quotient (Q IgG), $AI = Q \text{ spec}/Q \text{ IgG}$. Values greater than 1.5 indicate that specific antibodies are synthesized locally (Reiber and Lange, 1991).

New methods

Current data suggest that over one-third of cases of encephalitis remain unidentified, even in the best-equipped medical centers (Granerod and Crowcroft, 2007; Glaser et al., 2006; Venkatesan et al., 2013). Other methods have been developed, such as syndromic panel-based testing (Tansarli and Chapin, 2020) and next-generation sequencing (NGS) methods (Brown et al., 2018).

Diagnosis of lymphocytic meningitis

CSF examination is essential to establish the diagnosis of meningitis and to identify the etiological agent. It is not possible to perform a differential diagnosis between acute viral or bacterial meningitis only on a clinical basis. The CSF characteristics of viral meningitis include an increase in white blood cells (>5 cells/mm³) in the CSF, normal levels of glucose, and normal or increased values of total protein. The CSF lactate levels were within the normal range (<3.5 mmol/L) (Fishman, 1992). Sometimes, the differential is difficult to calculate due to overlapping of CSF characteristics. The predominance of polymorphonuclear (PMN) cells (50%) can occur within the first 6 h of the onset of viral meningitis. After this time, there is a change in the characteristics of the CSF to the typical pattern of viral meningitis. A predominance of lymphocytes can be observed in cases of acute bacterial meningitis in young children, neonates, older patients, immunosuppressed individuals with atypical bacteria, and partially treated cases (Fishman, 1992).

The best test to differentiate bacterial from viral meningitis is the CSF lactate test (Sakushima et al., 2011). CSF lactate concentrations greater than 3.5 mmol/L are characteristic of acute bacterial meningitis. The increase in lactate levels is closely related to low glucose levels (de Almeida et al., 2009). Unlike glucose, the lactate concentration in the CSF is independent of that of the serum. Therefore, there is no need to collect matched serum samples. The CSF lactate levels are also useful for the diagnosis of post-surgical acute bacterial meningitis, when there is no specific increase in cells or proteins (de Almeida et al., 2020).

Other biochemical biomarkers quantified in the CSF that can assist in the differential diagnosis of acute meningitis are shown in Fig. 1 (de Almeida et al., 2011b; Gnutzmann et al., 2016; Kleine et al., 2003).

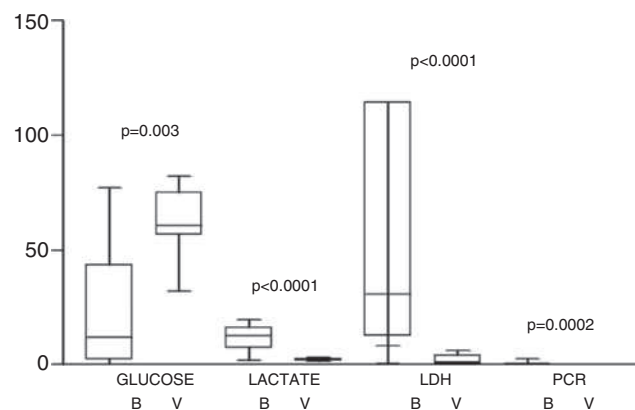


Fig. 1 Cerebrospinal fluid biomarkers for differential diagnosis of acute bacterial (B) and viral (V) acute meningitis, glucose (mg/dL), lactate (mmol/L), lactate dehydrogenase (LDH, U/L) and C-reactive protein (PCR, mg/dL) (de Almeida et al., 2011b).

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Brain Infections, Encephalitis, and Meningitis: Fungus

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Introduction

Fungal central nervous system (CNS) infection is a cause of mortality and neurological morbidity globally. The highest incidence occurs in low-income countries, followed by middle-income and high-income countries (Robertson et al., 2018; Kumar et al., 2020). Global estimates demonstrate that more than 1.5 million people die due to fungal infection, and more than 1 billion people are affected by a fungal infection (Bongomin et al., 2017). In the Leading International Fungal Education (LIFE) platform, the incidence of acquired immunodeficiency syndrome (AIDS)-related cryptococcal meningitis estimated 278,250 cases each year. The global number of disseminated histoplasmosis cases in AIDS worldwide is estimated to be 100,000 annually. Over 30 million can be contaminated with *Aspergillus* sp. each year because of corticosteroid or other therapies, and over 300,000 patients develop infection aggressively annually. Also, candidemia causes ~350,000 cases worldwide every year (Bongomin et al., 2017; Infection, 2020; George et al., 2020).

The fungal brain infection occurs as a consequence of the host immune system impairment, and other health conditions, including the use of medications, chemotherapy, corticosteroid therapies, broad-spectrum antimicrobial, surgical procedures, infection, AIDS, stroke, organ transplantation, end stage renal disease (ESRD) on hemodialysis (HD), neutropenia, and pre-term babies (Bongomin et al., 2017; McCarthy et al., 2014). The respiratory tract is the main route of fungal colonization and infection, followed by hematogenous dissemination and subsequent reaching the CNS. Besides, there are other direct inoculation routes in the CNS, including surgery, trauma, and intravenous drug use. The fungus can also colonize structures underlying the CNS, including sinuses, mastoids, and orbits (Guida, 1988; McCarthy et al., 2014; George et al., 2020).

The most common yeasts that cause brain infection are *Cryptococcus* sp., *Candida* sp., and *Trichosporon* spp. (Guida, 1988). The moniliaceous molds common pathogens are *Aspergillus* sp., *Fusarium* sp., and *Scedosporium* sp. (McCarthy et al., 2014). The Mucoromycetes CNS infections are uncommon, but the most common include the *Mucor* spp. and *Rhizopus* spp. The dimorphic fungi include the *Blastomyces dermatitidis*, *Coccidioides* spp., and *Histoplasma capsulatum*, and dematiaceous fungi *Cladophialophora bantiana* and *Exophiala dermatitidis* (McCarthy et al., 2014; Guida, 1988). In this article, we focus on fungal brain infection. We also explain the mechanisms of fungal invasion, the CNS immune response to the fungal invasion, and its long-term clinical consequences.

Routes of colonization, mechanisms of pathogenesis of fungi to reach the brain

Fungal infections of the CNS follow colonization and dissemination routes, and the respiratory tract is the main route of entry. After the fungi spread via hematogenously, cross the blood-brain barrier (BBB) and subsequently reach the CNS. Also, colonization and infection can occur in structures close to the CNS, including sinuses, mastoids, and orbits. The direct colonization in the CNS occurs after surgery, trauma, and intravenous drug use (McCarthy et al., 2014; Guida, 1988).

The yeasts: *Candida* sp., *Cryptococcus* sp., and *Trichosporon* spp.

Candida genus is a group of Ascomycetes yeast-like fungi that comprises over 30 different species that cause candidiasis, and most of them are rarely isolated from patients. *C. albicans* utilizes different mechanisms of pathogenicity to invade the host cells. *C. albicans* transits between morphological forms of yeast and hyphae. Presents adhesins and invasins on the cell surface, form biofilms, secretes hydrolytic enzymes, adapts to fluctuations of pH, acquires essential nutrients from the host, and protects itself against reactive oxygen species (ROS). The *Candida* sp. can induce cell endocytosis through specialized proteins invasins agglutinin-like sequence 3 (Als3) and Ssa1 on the cell surface that binds to E-cadherin and N-cadherin endothelial cells. Also, the secreted aspartic proteases (Saps) contribute to its active penetration (Mayer et al., 2013). *C. albicans* Als3 invasin binds to the gp96 heat shock protein on the brain endothelial cells' surface and crosses the BBB (Liu et al., 2011).

C. neoformans and *C. gattii* are agents of cryptococcosis disease. However, researchers recognized genetic diversity and the informal nomenclature of *C. neoformans* species complex and *C. gattii* species complex. The species complex demonstrates that two or more cryptococcal species are present in one species name (Hagen et al., 2017; Kwon-Chung et al., 2017). *Cryptococcus* sp. can be inhaled and spread into the host's lungs and disseminate to the bloodstream. *Cryptococcus* sp. crosses the BBB directly by transcytosis through the endothelial cells or using a Trojan-horse strategy that involves transport in phagocytic cells (Kronstad et al., 2011). The capsule is composed of glucuronoxylomannan (GXM) and galactoxylomannan (GalXM) polysaccharides, and these polysaccharides have immunomodulatory properties, and mutants without capsules are avirulent. Cryptococcal cells are resistant to ROS and nitrosative stress (NS) through neutralizing enzymes and protective proteins and metabolites, including superoxide dismutase, flavohemoglobin denitrosylase, glutathione peroxidase, glutathione reductase, thiol peroxidases, thioredoxins, and trehalose (Kronstad et al., 2011). The *Cryptococcus* sp. also uses the lipid signaling pathway diacylglycerol to activates the transcription factor 2 (Atf2) to upregulate expression of the antiphagocytic protein anti-phagocytic protein 1 (App1) (Rhome and Del Poeta, 2010). In summary, the mechanism of pathogenesis includes thermotolerance, resistance to ROS and NS, gluconeogenesis, acetyl-CoA, and melanin synthesis, capsule, and resistance of phagocytosis (Rhome and Del Poeta, 2010; Kronstad et al., 2011).

Trichosporon asahii, *T. asteroides*, and *T. mucoides* are responsible for the trichosporonosis cases in patients undergoing chemotherapy, patients who developed severe and prolonged neutropenia, and those exposed to invasive medical procedures (Colombo et al., 2011; Duarte-Oliveira et al., 2017). The fungus presents galactosaminogalactan (GAG), a virulence factor required to produce biofilms. GAG inhibits neutrophil infiltration and promotes fungal colonization. Also, GAG is an immunosuppressive polysaccharide and facilitates fungal survival (Fontaine et al., 2011; Lee et al., 2016). The *Trichosporon* sp. produces many enzymes, including lipases that hydrolysis triglycerides, phospholipases that hydrolyze phospholipids present in cell membranes, proteases that catalyze proteolysis, and DNases (Bentubo and Gompertz, 2014; Duarte-Oliveira et al., 2017). The fungus is disseminated hematologically and could cross the BBB by a transcellular or paracellular mechanism and colonize the brain.

The moniliaceous molds: *Aspergillus* sp., *Fusarium* sp., and *Scedosporium* sp.

Patients that present a risk factor for aspergillosis include systemic or paraspinal glucocorticoid treatments, neutropenia, mastoidectomy, tuberculosis, asthma, and cancer (Antinori et al., 2013; Ruhnke et al., 2007). After inhalation occurs, the contiguous dissemination spreads to the CNS from the orbits, periorbital regions, middle ear, or paranasal sinuses. The fungus also spreads through the bloodstream into the CNS (Dagenais and Keller, 2009). *Aspergillus* spp. produces aflatoxins and gliotoxins; these mycotoxins inhibit phagocytosis and reduce conidial opsonization. The gliotoxin inhibits assembly of the human cell respiratory burst NADPH oxidase (Yoshida et al., 2000), suppresses the human cellular immune response mediating cell apoptosis (Stanzani et al., 2005), induces a collapse of the actin cytoskeleton around the nucleus leading to cell shrinkage (Coméra et al., 2007). The gliotoxin penetrates and impairs the BBB decreasing the transendothelial electrical resistance (TEER) and increasing permeability, facilitating the fungus invasion into the CNS (Patel et al., 2018; Miceli, 2019).

Fusariosis, an invasive fungal infection caused by *Fusarium* spp., is listed as the second most common opportunistic mold infection after aspergillosis. The *Fusarium* sp. routes of invasion include lungs, sinuses, vascular catheters, and paronychia in neutropenic patients (Antinori et al., 2013). *Fusarium* sp. produces mycotoxins, including deoxynivalenol, 3-acetyldeoxynivalenol, and moniliformin. Deoxynivalenol reduces cell viability, increases BBB permeability, and reduces the expression of claudins facilitating brain infection (Behrens et al., 2015).

CNS infections secondary to *Pseudallescheria boydii* or its anamorph *Scedosporium apiospermum* occur after the aspiration of a large inoculum of the fungi into the lungs (e.g., near drowning in fresh water), which reaches the bloodstream and spreads into the brain. Other routes of infection include orbital infection, neurosurgical procedures or ventriculoperitoneal shunting, and sphenoidal sinusitis (Kowacs et al., 2004). The CNS infection occurs in immunocompetent patients by direct inoculation through neurosurgery, shunts, cerebrospinal fluid (CSF) drainage devices, etc. (Miceli, 2019). The fungus presents a glycoconjugate, peptidorhamnomannan, on the conidia and hyphal cell. The peptidorhamnomannan is associated with fungal adhesion and endocytosis by immune cells facilitating its colonization, virulence, and dissemination in the host (Ramirez-Garcia et al., 2018).

The Mucoromycetes: *Mucor* spp. and *Rhizopus* spp.

Mucoromycetes are the most aggressive mold brain infection, and the risk factor for the development of mucormycosis includes uncontrolled diabetes mellitus, iron-overload conditions, neutropenia or patients receiving glucocorticoid therapy (Ramírez-García et al., 2018; Ibrahim et al., 2012). Patients with elevated unbound iron in plasma are susceptible to mucormycosis. Mucoromycetes has the capacity to acquire iron from the host and interact and colonize the endothelial cells that line blood vessels (Ibrahim et al., 2012). Hemodialysis patients treated with the iron chelator deferoxamine are also susceptible to an invasive and deadly form of mucormycosis (Boelaert et al., 1991; Ibrahim et al., 2012). *Rhizopus* sp. secretes rhizoferrin, and this siderophore supplies iron to *Rhizopus* sp. through an energy-dependent process mediated by receptor. The iron plays a central role in mucormycosis infections, which causes extensive angioinvasion resulting in vessel thrombosis and subsequent tissue necrosis (Spellberg et al., 2005; Ibrahim et al., 2012). The route of invasion of mold infection is rhino-orbital-cerebral mucormycosis or pulmonary infection following hematogenous dissemination and CNS infection (McCarthy et al., 2014).

The dimorphic fungi: *Blastomyces dermatitidis*, *Coccidioides* spp., and *Histoplasma capsulatum*

Blastomyces spp. is a dimorphic fungal that presents the filamentous mold characteristics in the environment and present as a yeast in the human tissues. The conidia and fragments of the fungi can be inhaled, and inside of the lung, in the host the fungus convert fragments into pathogenic yeasts. *Blastomyces* sp. and other dimorphic fungi, including *H. capsulatum*, and *Coccidioides* spp. can survive inside the macrophages spreading the disease to the whole human body (McBride et al., 2017). Cerebral blastomycosis is a rare fungal disease triggered mainly by inhalation of conidia (Stavrakis et al., 2015). However, immunosuppressed patients are at risk for severe and disseminated blastomycosis disease (Ryan et al., 2019).

Coccidioidomycosis is a fungal infectious disease caused by the *Coccidioides* spp. The patients with T-helper2 lymphocytes dysfunction or immune deficiency have been found to be a risk factor of the disease. After inhalation and bloodstream dissemination, the spherules or endospores migrate to the meninges or into brain tissue triggering the CNS coccidioidomycosis. Histoplasmosis affects immunosuppressed patients, AIDS patients, transplant recipients, corticosteroid and tumor necrosis factor (TNF) antagonist therapies, and patients with ventriculoperitoneal shunts (Veeravagu et al., 2013; Góralaska et al., 2018). Histoplasmosis begins through spore inhalation, followed by a lung infection. In the sequence, the yeasts are phagocytized and spread inside of the immune cells in the host organism and into the CNS (Hariri et al., 2015).

The dematiaceous fungi: *Cladophialophora bantiana* and *Exophiala dermatitidis*

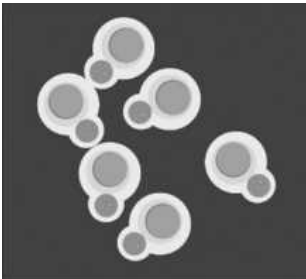
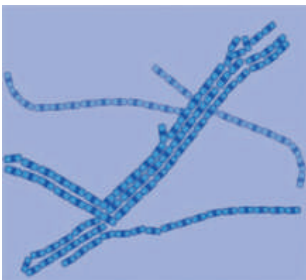
Dematiaceous fungi present a common route of transmission by inhalation or inoculation from surgery or trauma, with following hematogenous or contiguous spread into the CNS (Góralaska et al., 2018). The most important mechanism of pathogenicity of the phaeohyphomycosis is the presence of melanin in their cell walls that protect the fungi by the ROS and NS produced by the phagocytic cells (Gopalakrishnan et al., 2017). *Cladophialophora bantiana* is a melanized mycelial fungus mold that is a rare cause of human disease. The infection may start from the sinuses or via the bloodstream causing primary infection in the lung and disseminating to the CNS (Chakrabarti et al., 2016). *Cladophialophora* sp. presents a tropism to the CNS triggering phaeohyphomycosis in immunocompetent patients (Revankar et al., 2004). The neurotropic melanized *Exophiala dermatitidis* presents various properties. The fungus is extremophilic, oligotrophicity, assimilating volatile toluene and other aromatic or phenolic hydrocarbons as sole carbon sources. The CNS is a source of many monoaromatic catecholamine neurotransmitters, and it could be an explanation for the *Exophiala* sp. neurotropism (Lavrin et al., 2020). For more details, see Table 1.

Central nervous system immune response to fungal infection

The CNS is protected against toxins, drugs, and pathogens through the BBB and the intrinsic immune system of the brain (Koutsouras et al., 2017). Fungal infections in the CNS have become more common in the past two decades. Also, the fungal invasion in the CNS depends on the host's immune status, the ability of the fungi to avoid the immune system, and the virulence factors that facilitate the colonization and spread of the fungus. Fungi can cross the BBB through different mechanisms. The transcellular penetration involving either pinocytosis or receptor-mediated mechanisms to cross the endothelial cells; the *C. neoformans* and *C. albicans* use a transcellular mechanism to cross the BBB (Liu et al., 2011; Kronstad et al., 2011; Colombo and Rodrigues, 2015). Paracellularly, the pathogen crosses through intercellular junctions; for example, *C. neoformans*, *Aspergillus* sp., and *Fusarium* sp. In the Trojan-horse mechanism, the pathogen crosses the BBB internalized within an immune cell; for example, *C. neoformans* (Kronstad et al., 2011). For more details, see Fig. 1.

Glial cells, consisting of microglia, astrocytes, and oligodendrocytes, have several functions, including cerebral homeostasis, myelin production, BBB stability, and support and protection for neurons (Nimmerjahn et al., 2005). When the invading pathogen reaches the CNS, it provokes immediate and focal activation of CNS-resident cells. Microglia and astrocytes, together with

Table 1 Characteristics of fungal infections of the central nervous system.

	Taxonomy	Pathogenicity mechanisms	Risk factors of the host	Clinical consequences	References
Yeasts <i>Candida albicans</i>	<i>Candida</i> sp. Phylum: Ascomycota	Adhesins, invasins, form biofilms, hydrolytic enzymes, adapts to fluctuations of pH, acquires essential nutrients from the host, protection ROS and Saps	Genetic factors, prolonged antibiotic therapy, neutropenia, steroid therapy, transplantation, chronic granulomatous disease, CARD9 deficiency, neurosurgery, contaminated devices, prematurity in infants, and immunocompetence	Meningoencephalitis, meningitis, subpial ischemic lesions, local necrotic lesions, and brain abscess	Mayer et al. (2013), Góralska et al. (2018), Barton et al. (2014), and Neves et al. (2014)
					
<i>Cryptococcus neoformans</i>	<i>Cryptococcus</i> sp. Phylum: Basidiomycota	GXM and GalXM polysaccharides, SOD, flavohemoglobin denitrosylase, GPx, GR, thiol peroxidases, thioredoxins, trehalose, thermotolerance, gluconeogenesis, acetyl-CoA, melanin synthesis, and resistance of phagocytosis	Contamination by bird feces, immunocompetence	Meningoencephalitis, meningitis, subpial ischemic lesions, and chronic lymphocytic fungal meningitis	Rhome and Del Poeta (2010), Kronstad et al. (2011), and Góralska et al. (2018)
					
<i>Trichosporon asahii</i>	<i>Trichosporon</i> spp. Phylum: Basidiomycota	GAG, lipases, phospholipase, protease, DNAase, and form biofilm	Chemotherapy, severe depletion of neutrophils, and immunocompetence	Meningitis, brain abscess, and hydrocephalus	Colombo et al. (2011), Duarte-Oliveira et al. (2017), and Góralska et al. (2018)
					

Moniliaceous molds
Aspergillus fumigatus



Aspergillus sp.
Phylum:
Ascomycota

Aflatoxins, gliotoxins, nitrogen absorption,
protease, and elastase

Deferoxamine therapy, systemic or
paraspinal glucocorticoid treatments,
neutropenia, mastoidectomy,
tuberculosis, asthma, and cancer

Stroke, brain
abscess, skull-base
syndromes, and
meningitis

Góralaska et al. (2018), Black and
Baden (2007), Antinori et al.
(2013), Ruhnke et al. (2007), and
Yoshida et al. (2000)

Fusarium verticillioides



Fusarium sp.
Phylum:
Ascomycota

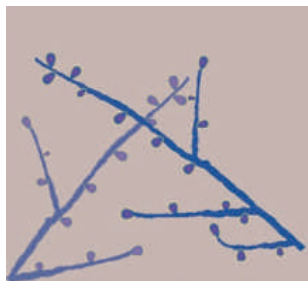
Deoxynivalenol, 3-acetyldeoxynivalenol,
moniliformin, trichothecenes, and
fumonisins

Prolonged neutropenia, hematological
malignancies, and transplant recipients

Hemorrhagic
infarction, brain
abscess, and
meningitis

Behrens et al. (2015), Koutsouras
et al. (2017), Garcia et al. (2015),
and Peterson et al. (2014)

Scedosporium apiospermum



Scedosporium sp.
Phylum:
Ascomycota

Peptidorhamnomannan, thermotolerance,
adaptation to low oxygen environment,
melanin, SOD, protease, phosphatase,
proteolytic and amyolytic enzymes

Immunocompetence, neurosurgery, and
sphenoidal sinusitis

Cerebral abscess and
meningitis

Ramirez-Garcia et al. (2018), Nesky
et al. (2000), and Lackner and
Guarro (2013)

Mucoromycetes
Mucor indicus



Mucor spp.
Phylum:
Zygomycota

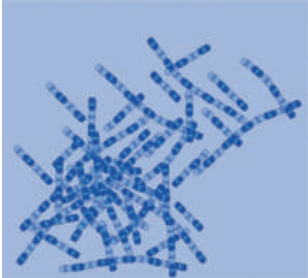
Iron uptake system

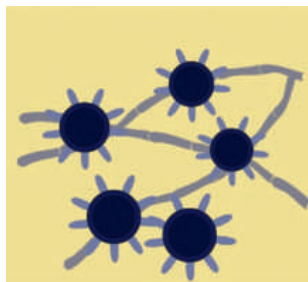
Diabetic ketoacidosis, necrotic burns, renal
failure, intravenous drug use, diabetes
mellitus, iron-overload conditions,
neutropenia, and glucocorticoid therapy

Meningoencephalitis,
stroke, brain
abscess, and
rhino-cerebral
infection

Góralaska et al. (2018), Bongomin
et al. (2017), McCarthy et al.
(2014), and Ibrahim et al. (2012)

Table 1 (Continued)

	Taxonomy	Pathogenicity mechanisms	Risk factors of the host	Clinical consequences	References
<i>Rhizopus arrhizus</i> 	<i>Rhizopus</i> spp. Phylum: Zygomycota	Rhizoferrin, mycotoxin rhizoxin, iron uptake system, lytic enzymes, ketone reductase system, growth in the acidic and glucose-rich environment	Diabetes mellitus, iron-overload conditions, neutropenia, and glucocorticoid therapy	Stroke, rhino-orbital-cerebral mucormycosis	McCarthy et al. (2014) and Ibrahim et al. (2012)
<i>Dimorphic fungi</i> <i>Blastomyces dermatitidis</i> 	<i>Blastomyces</i> sp. Phylum: Ascomycota	Thermally dimorphic, during in vivo infection, upregulates the BAD1; secrete DPPIVA, cysteine synthase, CDG, SSU1, PRA1, ZRT1, ZRT2, NIC1, DRK1, and RYP1-4 genes.	Immunocompetence	Meningitis, subpial ischemic lesions, brain abscess, epidural abscess, and mass lesions	Panackal and Williamson (2015), and McBride et al. (2019)
<i>Coccidioides immitis</i> 	<i>Coccidioides</i> spp. Phylum: Ascomycota	SOWgp, arginase I, coccidioidal urease, ammonia, and enzymatically active urease	Immunocompetence, T-helper 2 lymphocytes dysfunction, diabetes mellitus, and chronic steroid treatment	Meningoencephalitis, granulomatous meningitis, subpial ischemic lesions, and brain abscess	Panackal and Williamson (2015), Góralaska et al. (2018), and Hung et al. (2007)

Histoplasma capsulatum

Histoplasma capsulatum
Phylum:
Ascomycota

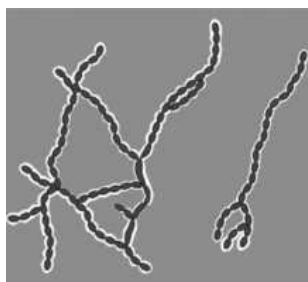
Capsule, CBP1, α -glucan, adhesins, CatA and CatB catalase protein, resistant to ROS and RNS, Eng1 and Exg8 β -Glucanases

Contamination by bird feces, immunocompetence, transplant recipients, corticosteroid and tumor necrosis factor antagonists, and patients with ventriculoperitoneal shunts

Meningitis, subpial ischemic lesions, and brain abscess

Veeravagu et al. (2013), Panackal and Williamson (2015), Hung et al. (2007), Edwards and Rappleye (2011), Mittal et al. (2019), Missall et al. (2004), and Garfoot et al. (2017)

Dematiaceous fungi
Cladophialophora bantiana



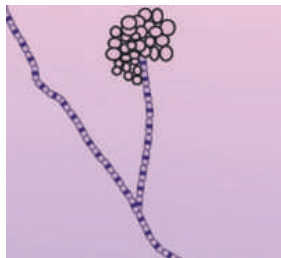
Cladophialophora sp.
Phylum:
Ascomycota

Melanin, extremophilic oligotrophicity, hydrolyzes peptides, urease, lipase, phospholipase, and DNase

Immunocompetence, transplant recipients, hematologic malignancy, tumors, and neutropenia

Brain abscess, granulomatous inflammation, meningitis, myelitis

Revankar et al. (2004) and Souza et al. (2008)

Exophiala dermatitidis

Exophiala sp.
Phylum:
Ascomycota

Melanin, extremophilic, oligotrophicity, hydrolyzes peptides, urease, lipase, phospholipase, and DNase

Immunocompetence, transplant recipients, hematologic malignancy, tumors, and neutropenia

Brain abscess, granulomatous inflammation, meningitis, myelitis, or encephalitis

Lavrin et al. (2020), Revankar et al. (2004), and Souza et al. (2008)

Abbreviations: BAD1, blastomyces adhesin-1; Cat, catalase gene; CDG, cysteine dioxygenase; DPPIVA, dipeptidyl-peptidase IVA; DRK1, dimorphism-regulating kinase; Eng1, endo-1,4-beta-glucanase; Exg8, *exo*- β 1,3-glucanase; GAG, galactosaminogalactan; GalXM, galactoxylomannan; GPx, glutathione peroxidase; GR, glutathione reductase; GXM, glucuronoxylomannan; NIC1, nickel transporter; PRA1, exogenous zinc uptake; RNS, reactive nitrogen species; ROS, reactive oxygen species; RYP1-4, required for yeast phase 1-4; Saps, secreted aspartic proteases; SOD, superoxide dismutase; SOWgp, spherule outer wall glycoprotein; SSU1, sulfite efflux pump; ZRT1, high-affinity zinc transporter; ZRT2, low-affinity zinc transporter.

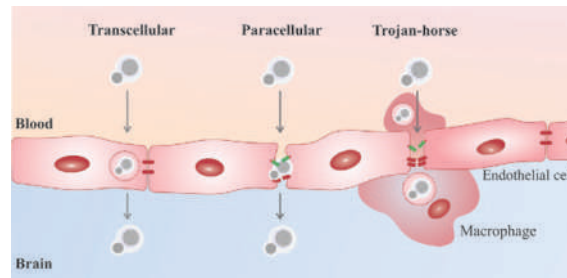


Fig. 1 Types of mechanisms involved in the BBB fungal crossing. Pathogens can cross the blood-brain barrier transcellularly, paracellularly, or using the Trojan-horse strategy. In the transcellular mechanism, fungi cross the BBB between endothelial cells, such as *C. neoformans*, *C. albicans*, and *Trichosporon* sp. In the paracellular mechanisms, fungi, such as *Cryptococcus* sp., *Aspergillus* sp., *Fusarium* sp., and *Trichosporon* sp. they cross through intercellular junctions. The Trojan-horse mechanism fungi, such as *Cryptococcus* sp., reach the CNS and are transported in phagocytic cells.

peripheral immune cells, orchestrate the brain homeostasis and restoration (Dokalis and Prinz, 2019). Microglial cells recognize fungal compounds known as pathogen-associated molecular patterns (PAMPs). These PAMPs bind to pattern recognition receptors (PRRs) and non-PRRs, which are essential constituents of the immune system. The detection of PAMPs by immune receptors triggers a cascade of signaling pathways that activate various transcription factors and promote pro-inflammatory molecules production to eliminate invading fungi (Koutsouras et al., 2017).

Microglia, as a resident macrophage of the CNS, expresses Toll-like receptors (TLRs) 1 to 9 and C-type lectin receptors (CLRs), in which microglia is active after interaction with opsonized or non-opsonized fungal antigens. TLR-2 recognizes fungal β -glucans present in *Candida* spp., *C. neoformans*, *A. fumigatus*, and *H. capsulatum* (Camilli et al., 2018). The heterodimers TLR2/TLR1 and TLR2/TLR6 recognize GXM of the *C. neoformans*, and phospholipomannan (PLM) of the *C. albicans*. TLR4 recognizes O-linked mannans of *C. albicans*, GXM of *C. neoformans*, and *A. fumigatus* conidia (Koutsouras et al., 2017; Bourgeois and Kuchler, 2012). The endosomal TLR3, TLR7, and TLR9 recognize RNA or DNA of many fungi, including *C. albicans*, *C. neoformans*, and *A. fumigatus* (Bourgeois and Kuchler, 2012; Góralaska et al., 2018). The TLR1/TLR2, TLR6/TLR2, and TLR4 signal through the central pathway recruiting the myeloid differentiation factor (MyD)88-dependent adaptor protein that acts via nuclear factor- κ B (NF- κ B) to induce pro-inflammatory cytokines production (Broad et al., 2007). The cytokines production interferon (INF)- γ , TNF- α , interleukin (IL)-1 β , IL-6, and IL-12, and the chemokines play essential roles in preventing infection by inhibiting fungal growth and dissemination in the CNS (Broad et al., 2007; Góralaska et al., 2018). Microglia phagocytizes the *C. neoformans* and upregulates the expression of inducible nitric oxide synthase (iNOS) to eliminate the invasion pathogen (Drummond, 2017).

CLRs are an important class of receptors responsible to eliminate fungus infection. The most important CLRs are dectin-1, complement receptor 3 (CR3), mincle, and mannose receptor-1. Microglia expresses dectin-1, CR3, mincle, and mannose receptor-1 (McKimmie et al., 2006). Dectin-1 recognizes β -1,3-glucan of the *A. fumigatus*, *C. neoformans*, *H. capsulatum*, *Coccidioides* sp., *C. albicans*, *F. solani*, *E. rostratum*, *C. cladosporioides*, and *T. asahii* (Goyal et al., 2018). Dectin-1, together with TLRs, presents a synergism activating the NF- κ B to produces pro-inflammatory cytokines and exacerbating the ROS production to eliminate the invasion (Kasper et al., 1988; Goyal et al., 2018). CR3 on the surface of microglia recognizes mannose and β -glucans on the surface of *A. fumigatus* and *C. albicans* (Mogensen, 2009), increasing the phagocytosis and production of the ROS (Siew and Chern, 2018). Mincle recognizes *A. fumigatus*, and *C. albicans*, this receptor activates second messengers protein and the transcription factor NF- κ B to produces pro-inflammatory cytokines via the Syk/CARD9 pathway (Marakalala and Ndlovu, 2017; Richardson and Williams, 2014).

Brain infections, encephalitis, and meningitis: Fungus

Fungal infections of the CNS have a lower incidence when compared to viral infection or bacterial infection. However, fungal infections are severe and have a high mortality rate. The prevalence of the fungal neuro-infections disease is related to immunosuppressed and immunocompetent individuals, mainly hospitalized people and patients undergoing neurosurgery (Bongomin et al., 2017). In states where the patient has reduced immunity, BBB permeability increases, facilitating fungal penetration into the brain by paracellular, transcellular, and Trojan-horse mechanisms, Fig. 1. Fungi reach the brain parenchyma and proliferate, causing inflammation and cerebral infection. Since fungal pathogenic factors need to overcome the significant barriers surrounding the brain, invasions are mainly associated with the host's immunocompromised state (Sharma et al., 2012; Góralaska et al., 2018). The clinical syndromes of neuroinvasion caused by fungi are meningitis, meningoencephalitis, brain abscess, rhino-cerebral infection, and skull-base syndromes. Among the fungus that causes meningitis, we can list *Cryptococcus* sp., *Coccidioides* sp., *Exserohilum* sp., *Candida* sp., and *Histoplasma* sp. The incidence of meningoencephalitis is associated with *Cryptococcus* sp., *Coccidioides* sp., and *Candida* sp. In the brain, the abscess is listed *Aspergillus* sp., *Candida* sp., *Mucoromycetes* sp., *Blastomyces* sp., *Coccidioides* sp., and *Histoplasma* sp. The rhino-cerebral infection agents are *Mucor* sp., *Rhizopus* sp., *Absidia* sp., *Rhizomucor* sp., and *Syncephalastrum* sp. The skull-base syndromes are associated with *Aspergillus* sp. After a stroke or infarction, the main fungus systemically disseminated and

can reach the CNS are *Mucoromycetes* sp., *Aspergillus* sp., and disseminated mucoromycetes *Candida* sp., *Cryptococcus* sp., *Aspergillus* sp., *Coccidioides* sp., (Góralaska et al., 2018). For more details, see Table 1.

Conclusion

Fungal infections in the CNS remain a serious global health problem and pose a threat to the lives of patients. Besides, the clinical picture of manifestations of CNS infections caused by fungi are often unspecific and pose a challenge for diagnosis and treatment. In this article, we present the course and role of the immune response in cerebral fungal infection. A better understanding of the immunological pathways that are activated in an attempt to eliminate these pathogens, particularly in a cerebral context, is crucial and can provide insights for a faster diagnosis and, consequently, a better prognosis for patients affected by these severe infections. At the same time, it can assist the development of effective antifungal therapy to decrease mortality.

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Fungal Meningitis

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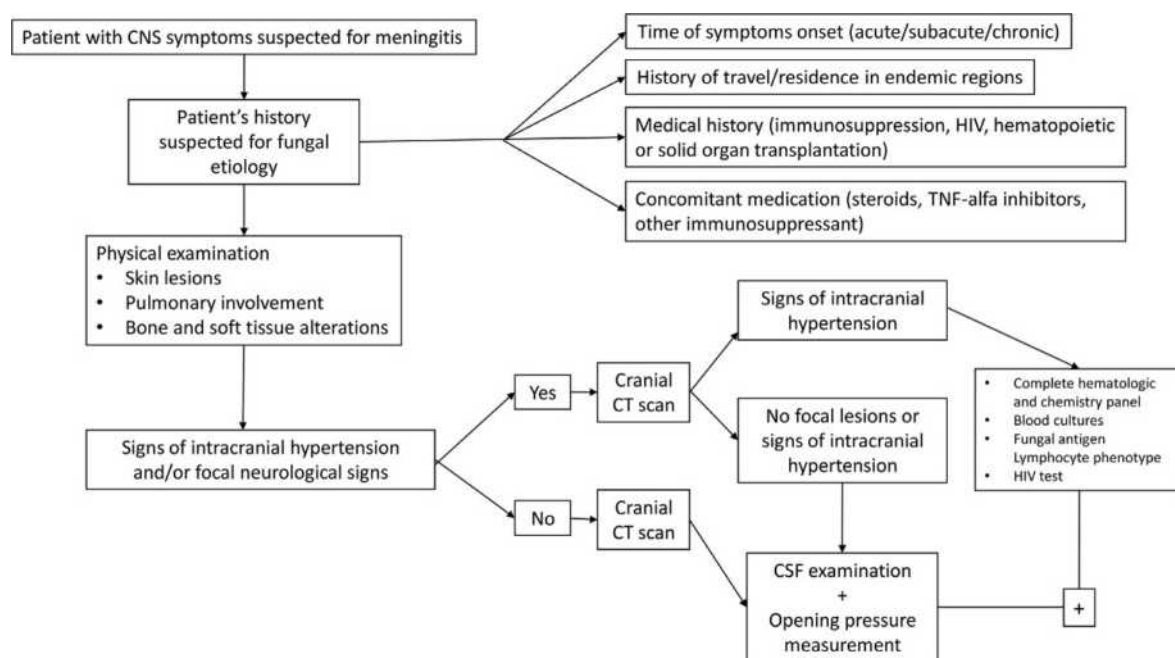
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Introduction

Fungal meningitis is one of the possible clinical manifestations of invasive fungal diseases (IFDs) (Donnelly et al., 2020). Typically, IFDs are diagnosed in subjects with underlining medical conditions leading to alteration of the immune system caused by some diseases (i.e. human immunodeficiency virus (HIV)) or by the use of different medications leading to iatrogenic immune suppression (i.e. steroids, anti-rheumatic drugs, etc.) (Antinori and Giacomelli, 2020). Nevertheless, especially in recent years a rise in IFDs have been observed also in hosts without underling medical condition leading to immune system dysregulation (Vallabhaneni et al., 2016). Central nervous system involvement by fungi can manifest with focal intracerebral abscess(es), spinal cord lesions or more frequently with meningitis. Fungi can reach the central nervous system with different modalities: (1) hematogenous spread; (2) direct focal extension from anatomical regions such as paranasal sinuses, ear or orbits; (3) traumatic introduction. Fungal meningitis is a serious syndrome with an important burden of morbidity and mortality generally presenting as subacute or chronic meningitis (Table 1) (Pagliano et al., 2020). The incidence of fungal meningitis in England and Wales according to an 8-year observational study was 0.09/100,000 inhabitants (Okike et al., 2014). The authors reported only data regarding *Candida albicans* and non-*C. albicans* making impossible to infer regarding the incidence of other more frequent causes of fungal meningitis (i.e. *Cryptococcus* spp.). Moreover, it is likely that an underestimation of the real fungal meningitis burden occurred in this study (Antinori et al., 2014). In another study conducted in United States between 2000 and 2012 among fungal meningitis the most common causative organism was *Cryptococcus* spp. (70.1% of cases), followed by *Coccidioides* spp. (16.4%), *Histoplasma capsulatum* (6.0%) and *Candida* spp. (7.6%) (Charalambous et al., 2018). When considering the incidence estimates it should be considered that fungal meningitis is often underdiagnosed due to a low grade of suspect for fungal infection when meningitis is diagnosed outside selected populations (i.e. patients with underline immune system disorders) and standard cultures performed on cerebrospinal fluid (CSF) showed a low sensitivity for fungi (Barenfanger et al., 2004). Because infrequent, fungal meningitis requires a high suspicion index and an appropriate diagnostic workup to prompt diagnose and properly treat (Fig. 1). The epidemiology of the patient is important and an accurate anamnesis is fundamental to assess the potential exposure to contaminated environment, geographical areas with potential endemic fungi or to invasive procedures with the potential

Table 1 Fungal species responsible of meningitis.

Fungal species	Type of meningitis	Risk factors	Frequency
<i>Aspergillus</i> spp.	Acute meningitis	Neurosurgery, neutropenia	+
<i>Blastomyces dermatitidis</i>	Chronic meningitis	Travel/residence in endemic regions	-/+
<i>Candida</i> spp.	Subacute meningitis	Neurosurgery, premature newborn, CARD 9 deficiency	+
<i>Coccidioides immitis</i>	Subacute meningitis	Travel/residency in endemic regions, Hispanic, African and Asian background	+
<i>C. posadasii</i>	Chronic meningitis	Travel/residency in endemic regions, Hispanic, African and Asian background	+
<i>Cryptococcus neoformans</i>	Subacute meningitis	HIV infection	+++
	Chronic meningoencephalitis	Solid organ transplantation, immunosuppressive therapy	
<i>C. gattii</i>	Chronic meningoencephalitis	Travel/residency in endemic regions	++
<i>Paracoccidioides brasiliensis</i>	Chronic meningitis	Travel/residency in endemic regions	+
<i>Exserohilum rostratum</i>	Chronic meningitis	Contaminated lots of methylprednisolone	+
<i>Histoplasma capsulatum</i>	Chronic meningitis	Travel/residency in endemic regions	+
<i>Scedosporium/Pseudallescheria</i>	Chronic meningitis	Diabetes, immunosuppression	-/+
<i>Sporothrix schenckii</i>	Chronic meningitis	Immunocompetent	-/+
	Acute meningitis	HIV infection	
<i>Talaromyces marneffe</i>	Disseminated infection	Travel/residency in endemic regions, HIV infection	-/+

**Fig. 1** Diagnostic workup of patients with suspected fungal meningitis. CT, computed tomography; HIV, human immunodeficiency virus; TNF, tumor necrosis factor; CSF, cerebrospinal fluid.

inoculation of fungi in sterile sites. The implementation of additive laboratory tests other than cultures on CSF such as (1 → 3)-β-D-glucan, although non fungal specific, could increase the diagnostic accuracy (Davis et al., 2020).

In this narrative review we will focus on the most common causative agents of fungal meningitis starting from their epidemiology to pathogenesis, clinical manifestations, diagnosis and treatment.

Cryptococcosis

Microbiology and epidemiology

Cryptococcosis, first identified in 1894 by Otto Busse and Abraham Buschke, emerged as a major life-threatening meningoencephalitis with the onset of AIDS pandemic at the beginning of the 1980s (Williamson et al., 2017).

The initial attribution of a single fungal species named *Cryptococcus neoformans* has been challenged by phylogenetic and molecular studies that in 2002 led to classify *C. neoformans* var. *gattii* as a distinct species, *Cryptococcus gattii* (Kwon-Chung et al., 2002).

More recently, studies based on a multilocus sequence typing (MLST) and other molecular typing techniques showed a more complex taxonomic issue with the proposal of several separate species: *C. neoformans* (formerly *C. neoformans* var. *grubii*), *C. deneoformans* (formerly *C. neoformans* var. *neoformans*), *C. gattii* (VG1/AFLP4), *C. deuterogattii* (VGII/AFLP6), *C. bacillosporius* (VGIII/AFLP5), *C. tetragattii* (VGIV/AFLP7), *C. decagattii* (VGIV and VGIIIc/AFLP10) (Hagen et al., 2015, 2017; Kwon-Chung et al., 2017). To further complicate this taxonomic classification at least four diploid/aneuploidy hybrids have been recognized: AD (*C. neoformans* × *C. deneoformans*), DB (*C. deneoformans* × *C. gattii*), AB (*C. neoformans* × *C. gattii*) and AB (*C. neoformans* × *C. deuterogattii*) (Kwon-Chung et al., 2017).

Although cryptococcosis generally affects immunocompromised patients, with HIV/AIDS as the most common underlying condition, *C. gattii* is considered a true pathogen able to infect healthy immunocompetent individuals (Bielska and May, 2016; Franco-Paredes et al., 2015; Kronstad et al., 2011; Acheson et al., 2018).

It has been estimated that globally, cryptococcal meningitis was responsible for 15% of AIDS-related deaths and the most significant burden of the disease is in the Sub-Saharan Africa accounting for 73% of the estimated cryptococcal meningitis cases in 2014 (162,500 cases [95% CI 113,600–193,900]). Moreover, the annual global deaths from cryptococcal meningitis were estimated at 181,100 (95% CI 119,400–234,300), with 135,900 (75%; [95% CI 93,900–163,900]) deaths in sub-Saharan Africa (Rajasingham et al., 2017).

As far as *C. gattii*, this fungus has been considered to be endemic only in tropical and subtropical areas but, following the outbreak started in 1999 on Vancouver Island (British Columbia, Canada), it has been observed a more widespread geographic distribution including also temperate climate regions of Northern United States, Canada and Northern Europe (Herkert et al., 2017; MacDougall et al., 2007).

C. neoformans spores are particular abundant in bird's droppings as well in leaves and decaying woods whereas, *C. gattii* environmental niche is represented by different trees (especially Australian eucalyptus in tropical and subtropical regions and *Pseudotsuga menziesii* in temperate regions).

As previously mentioned HIV infection is the highest driven of cryptococcal meningitis but other risk factors (Table 2) such as solid organ transplantation (SOT) recipients and several heterogeneous HIV-negative/non-transplant (NHNT) conditions as well as some drug treatments have been identified to be as risk factors for cryptococcal meningitis (George et al., 2017; Marr et al., 2020; Bratton et al., 2012; Wald-dicker and Blodget, 2017; Singh et al., 2007; Brizendine et al., 2013; Williamson et al., 2017).

Two large retrospective studies conducted in the United States reported an overall incidence of cryptococcosis among SOT recipients of 0.2–0.37% (George et al., 2017; Pappas et al., 2010). In the setting of SOT, cryptococcosis is the third most common fungal infection, with a life time risk of 1–2%, presenting with meningitis in 44% to 60% of patients (George et al., 2017; Singh et al., 2007; Brizendine et al., 2013; Davis et al., 2009; Henao-Martínez and Beckham, 2015). Cryptococcosis is infrequent in the immediate post-transplant period being diagnosed after a median time from transplant of 20 to 26 months (Bratton et al., 2012; Pappas et al., 2010). An early onset of disease (occurring 30 days after transplantation) is observed in 10% of patients and it is sometime expression of donor-derived cryptococcal infection (George et al., 2017; Sun et al., 2010; Baddley et al., 2011, 2019). Lung transplantation recipients had the highest risk of cryptococcosis (Hazard ratio 2.1) compared with other organ transplant recipients (George et al., 2017). In NHNT cryptococcal meningitis is diagnosed in 34–43% of those with cryptococcal infection and for individuals taking immunosuppressive agents the median duration of drug exposure was 7 months (Bratton et al., 2012; George et al., 2018).

Pathogenesis

Cryptococcal infection occurs with the inhalation of both desiccated yeast cells (3 µm of size) and spores (1–2 µm of size) which are able to deposit deep in the alveoli of the lung (Kronstad et al., 2011). The next step following the pulmonary infection is the ability of *Cryptococcus* spp. to disseminate and cross the blood-brain barrier either by transcytosis and migration of phagocytic cells

Table 2 Predisposing conditions associated with non-HIV cryptococcal meningitis.

Predisposing conditions associated with non-HIV cryptococcal meningitis

- Solid organ transplant recipients
- Sarcoidosis
- Autoimmune diseases
- Chronic steroid treatment
- Idiopathic CD4+ lymphopenia
- Liver cirrhosis
- Autoantibodies to IFN-gamma
- Chronic granulomatous diseases
- Job syndrome (autosomal dominant hyper-IgE syndrome)
- Hyper IgM syndrome (X-linked CD40 L deficiency)
- Treatment with Bruton's tyrosine kinase inhibitors (ibrutinib)

containing the fungus thus causing a meningoencephalitis. The *Cryptococcus* spp. pathogenic mechanisms rely on the ability to replicate at 37 °C of temperature and on the ability to form an extracellular capsule. In particular, the polysaccharide capsule provides a physical barrier able to interfere with the macrophage phagocytosis and avoiding the clearance by the immune system (Bose et al., 2003). Moreover, phospholipase-B, laccases (LAC 1 and LAC 2) which catalyze melanin synthesis, and urease have been identified as potential additional virulence factors implicated in the dissemination of *Cryptococcus* spp. from the lung through the lymphatic system and blood to the central nervous system. In particular, both *C. neoformans* and *C. gattii* express phospholipase-B (Plb) enzyme B1 (PLB1) and the phosphatidylinositol (PI)-preferring phospholipase-C (Plc) which are involved in the fungal pathogenic mechanism (Djordjevic, 2010). Regarding the function of *Cryptococcus* spp. extracellular-bound urease (URE1), they show a role in the survival and multiplication of yeasts within the macrophages with potential implication in yeast crossing of epithelial and endothelial barriers (Feder et al., 2015). In addition, the emergence of an unusual lineage of *C. gattii* infecting otherwise healthy individuals first in Vancouver Island and then spread to the Canadian mainland and the Pacific Northwest shed new light on the evolution of pathogenic mechanisms. In this outbreak isolates comprise two distinct genotypes, one hypervirulent with an identical genotype that is unique to the Pacific Northwest and the other significantly less virulent and sharing an identical genotype of isolates from an Australian recombining population. These observations suggest that cryptic same-sex reproduction can enable expansion of a human pathogen to a new geographical niche and contribute to the ongoing production of infectious spores (Fraser et al., 2005). Another pathogenic factor related to hypervirulent strains (i.e. VGIIb clonal group) is the ability to proliferate within host macrophages where reactive oxygen species stimulate tubular mitochondrial morphology in a subset of cells, which appears to be a protective mechanism from autophagic degradation (Farrer et al., 2016). The genome plasticity of *Cryptococcus* spp. which relies in the modification of the number of chromosomes have been linked to virulence and the development of azole resistance (Farrer et al., 2013). The ability of the fungus to increase the number of azoles target (ERG11) or transporters (AFR1) lead to the development of azole-resistant strains (Kwon-Chung and Chang, 2012).

Clinical features

Cryptococcal meningitis presents usually with a subacute course over a period of days to weeks with a combination of several signs and symptoms including mild to moderate fever, headache, vomiting, cranial neuropathies, altered mentation, lethargy, memory loss, and signs of meningeal irritation (Table 3) (Maziarz and Perfect, 2016; Dromer et al., 2007; Hevey et al., 2019; Brizendine et al., 2013; George et al., 2018; Bratton et al., 2012; Antinori et al., 2001). However, the presentation is heterogeneous and in HIV infected subjects the onset could also be abrupt in the absence of signs of meningeal irritation but characterized by severe headache with high opening pressure at CSF examination (Fig. 2A–C). Due to the characteristics of the disseminated disease the patients with cryptococcal meningitis could present with a variable involvement of other sites such as lung, skin, eye, prostate, abdominal and bones (Antinori et al., 2020). The co-occurrence of meningeal involvement and focal cerebral lesions “criptococcoma” and/or obstructive hydrocephalus, frequently associated with *C. gattii* infection, suggest a poor prognosis and the patient could present with

Table 3 Clinical and mycological characteristics of cryptococcal meningitis in HIV positive patients, solid organ transplant recipients and HIV negative/non transplant.

Clinical and mycological parameters	HIV positive ^a %	SOT ^b %	NHNT ^c %
Meningitis or meningoencephalitis	69–88	42–60	34.4–50
Fever	44–70	40–49	28–49
Headache	57–73	43–50	40–44
Altered mental status	21–40	29–30	25–38
Cranial nerve palsy	3–11	0–4	3–8
Weight loss	24	13–20	17–29
Visual changes	10–22	2–6	5–12
Cough	14–20	24–37	23–34
Vomiting	21–38	44	13
Positive CSF culture	87–88.6	85	68
Positive serum CrAg	87–97.3	51–86	35–86
Positive CSF CrAg	97.2	32	38
Positive India ink	87.8	50–51	35–51
Positive blood culture	44–46.4	28–44	24–28
CSF serum glucose ration <0.6	89	89	85
Ninety-day mortality	14.6–19	17–20.7	13.7–27
One-year mortality	19.6–26	24–26	27.8–35

HIV, human immunodeficiency virus; SOT, solid organ transplant; NHNT, non HIV/non transplant; CSF, cerebrospinal fluid; Ag, antigen.

^aCompiled by using: Dromer et al. (2007); Hevey et al. (2019); Brizendine et al. (2013); George et al. (2018); Bratton et al. (2012); Antinori et al. (2001).

^bCompiled by using: Hevey et al. (2019); Brizendine et al. (2013); George et al. (2018); Bratton et al. (2012).

^cCompiled by using: Hevey et al. (2019); Brizendine et al. (2013); George et al. (2018).

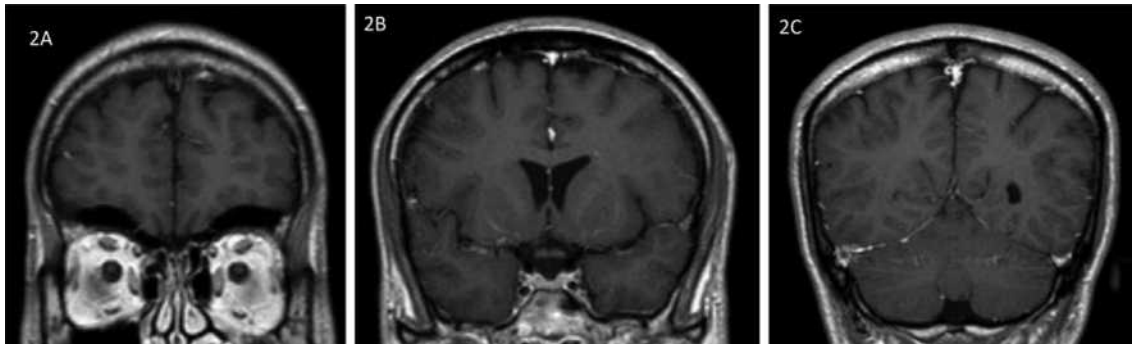


Fig. 2 (A–C) Magnetic resonance findings in an AIDS presenter patient with disseminated cryptococcal disease with meningitis. The magnetic resonance imaging showed no meningeal contrast enhancement although the cerebrospinal fluid revealed a positive cryptococcal antigen, a positive India ink examination and the cerebrospinal culture turned positive. The cerebrospinal fluid examination revealed normal cell count and proteins level with only a reduced cerebrospinal on plasmatic glucose ratio.

signs of focal lesions (Fig. 3A–D) (Phillips et al., 2015). HIV negative patients present less frequently with meningitis and fever is observed in less than 50% of cases, a factor that can delay the diagnosis especially in SOT recipients with one quarter of patients diagnosed more than 1 month after symptoms onset (Marr et al., 2020). A delayed diagnosis of cryptococcal meningitis has been associated with long-term neurological deficits (i.e. hearing and visual loss, ataxia, seizures) (Aye et al., 2016). CSF examination in cryptococcal meningitis include a raised opening pressure (more frequently in HIV-positive patients), a mild elevated white cell count with lymphocyte predominance, low CSF glucose and elevated CSF protein (Kashef Hamadani et al., 2018; Dromer et al., 2007; Antinori et al., 2001; Tseng et al., 2013). However, in HIV-associated cryptococcal meningitis the CSF white cell count is generally lower and can be normal in comparison with HIV negative individuals (Jarvis et al., 2014; Antinori et al., 2001; Dromer et al., 2007). Moreover, the rate of positive CSF cultures, positive CSF cryptococcal antigen (CrAg) and positive India ink are higher among HIV positive patients. Significantly higher CrAg titer either in serum and CSF are observed in HIV infected subjects than NHNT subjects and transplant recipients probably as expression of more severe immunodeficiency and fungal burden (Hevey et al., 2019; Dromer et al., 2007; Tseng et al., 2013). An important complication of cryptococcal meningitis identified both in HIV positive patients and SOT recipients is the immune restoration inflammatory syndrome (IRIS), the presentation of which mimics worsening or relapsing cryptococcal diseases (paradoxical IRIS) or the “de novo” diagnosis of cryptococcal meningitis in HIV-positive subjects with subclinical cryptococcal disease once the antiretroviral therapy is started (unmasking IRIS) (Lortholary et al., 2005; Bicanic et al., 2009; Antinori et al., 2009; Singh, 2012; Haddow et al., 2010). In a study conducted in United States which included the three groups of patients with cryptococcosis, the prevalence of IRIS was 3% although higher prevalence has been reported either in AIDS patients (8–19%) and SOT recipients (5–11%) (Bratton et al., 2012; Lortholary et al., 2005; Bicanic et al., 2009; Antinori et al., 2009; Singh, 2012).

Diagnosis

The initial diagnostic workup relies on a high index of clinical suspicion derived from an appropriate medical history. The gold standard is the isolation of *Cryptococcus* spp. from a sterile site (i.e. CSF, blood). In HIV positive patients with cryptococcal meningitis CSF and blood cultures are positive in up to 90% and 70% of patients, respectively (Antinori, 2013). Moreover, the CSF negativity on antifungal treatment has prognostic implication and the quantitative culture on CSF correlate with survival (Bicanic et al., 2007). The diagnosis could be reached also with antigenic assays on cerebrospinal fluid and blood combined with the direct examination of the CSF by India ink stain. The sensitivity reported for India ink depends mainly on the fungal burden and consequently is higher, up to 80%, in people living with HIV who developed AIDS when compared to non-AIDS patients (below 60%) (Antinori, 2013). The diagnostic capacity significantly improved with the introduction of methods who searched for the cryptococcal polysaccharide capsular antigen (CrAg), which is shed during infection. Latex agglutination and enzyme immunoassay performed on serum and CSF samples showed an overall sensitivities and specificities of 93% to 100% and 93% to 98%, respectively (Wu and Koo, 1983; Tanner et al., 1994). More recently, a lateral flow assay was approved for use in serum and CSF, with sensitivity and specificity of greater than 98% in both specimen types (including whole blood from finger stick samples) and sensitivity of 85% in urine (Jarvis et al., 2011; Huang et al., 2015). The histopathology could be helpful in selected cases (i.e. bone or soft tissue involvement) or when an initial diagnosis due to poor suspicion index is not reached. In the end molecular methods are mainly used for research purposes and are to date not yet implemented in clinical practice.

Treatment

The therapeutic strategy for cryptococcal meningitis relies on appropriate antifungal treatment and in patients with AIDS with the choice of an appropriate timing of antiretroviral start to minimize the risk of IRIS (van der Horst et al., 1997). The treatment of

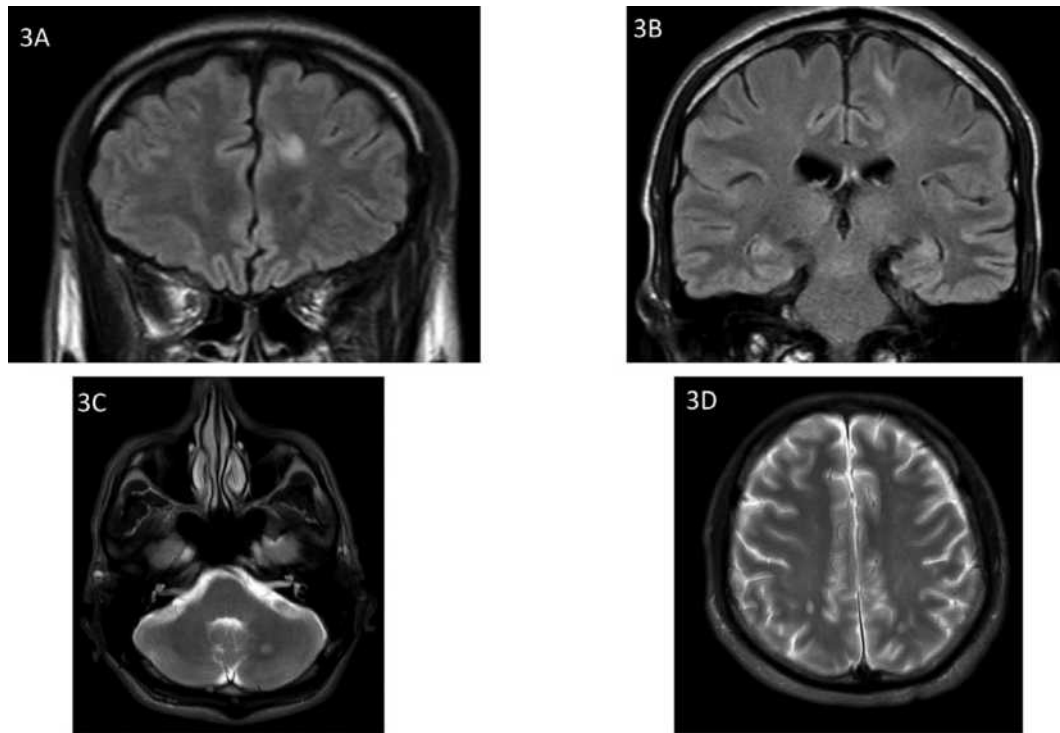


Fig. 3 (A–D) Magnetic resonance findings in an AIDS presenter patients presenting with worsening headache, multiple cutaneous lesion and pneumonia. The MR showed multiple contrast enhanced lesions compatible with opportunistic infection of the brain parenchyma. Blood cultures turned positive for *Cryptococcus neoformans* var. *neoformans*.

Table 4 Treatment option for cryptococcal meningitis.

Phase	First drug options	Alternative options	Administration route	Duration
Induction	Liposomal amphotericin B 3–4 mg/kg or amphotericin B deoxycholate 0.7–1 mg/kg/day and 5-flucytosine 100 mg/kg/day	Liposomal amphotericin B 3–4 mg/kg or amphotericin B deoxycholate 0.7–1 mg/kg/daily and fluconazole 800–1200 mg/daily	Iv + Os	HIV +: 2 weeks SOT: ≥2 weeks All other patients ^a : 4–6 weeks
Consolidation	Fluconazole 400 mg/daily	Itraconazole 400 mg/daily	Os	At least 8 weeks
Maintenance	Fluconazole 200 mg/daily		Os	At least 12 months ^b

Iv, intravenous; Os, oral subadministration; HIV, human immunodeficiency virus; SOT, solid organ transplantation.

^aIncluding patients with *Cryptococcus gattii* infection.

^bIn HIV-positive patients consider discontinuation of maintenance if CD4 cells count is >100 cell/μL and HIV-RNA is suppressed for more than 3–6 months.

cryptococcal meningitis consists on three phases: induction, consolidation, and maintenance therapy (Table 4). The induction phase aims to achieve rapid fungicidal activity to obtain CSF sterility using the combination of amphotericin B deoxycholate (amB) (0.7 mg/kg/day) and 5-flucytosine (5-FC) (100 mg/kg/day) for 2 weeks. The combination of amB and 5-FC is more rapidly fungicidal than amB alone and is associated with a trend toward a higher proportion of patients achieving a sterile CSF at 2 weeks and a reduced relapse rate (van der Horst et al., 1997; Perfect et al., 2010). The consolidation phase is the subsequent 8-week treatment period with either fluconazole (400 mg/day) or itraconazole (400 mg/day) as an alternative for patients with fluconazole intolerance. The maintenance phase is crucial in people living with HIV to avoid relapses. Oral fluconazole at a dosage of 200 mg/day has been shown to be superior to placebo (Bozzette et al., 1991) and both weekly intravenous amB (Powderly et al., 1992) and oral itraconazole (Saag et al., 1999) and should be continued for at least 1 year. After that the discontinuation of maintenance therapy could be considered in those with an HIV-RNA below 50 cp/mL for at least 3 months and a CD4 cell count above 100 cell/μL. It has been demonstrated that the substitution of amB deoxycholate with liposomal amB does not reduce the

probability of therapeutic success and is associated with better tolerability in particular by reducing the probability of kidney toxicity. Consequently, where available, the liposomal formulations of amB are now the preferred first line options (Hamill et al., 2010; Migone et al., 2018).

Regarding the correct choice of antiretroviral start in people living with HIV with cryptococcal meningitis international guidelines (Panel on Guidelines for the Prevention and Treatment of Opportunistic Infections in Adults and Adolescents with HIV (2020)) suggest an optimal administration time between 2 and 10 weeks after the start of antifungal therapy with the precise starting dates based on individual conditions and local experience and preferably after 5 weeks. The latter recommendation is based on a randomized clinical trial showing an increased probability of survival in those delaying the antiretroviral initiation after 5 weeks (Boulware et al., 2014).

The treatment in patients with SOT and receiving immunosuppressant treatment follow the same scheme of that of people living with HIV. The management of the immunosuppressant should take into account the risk of IRIS also in these patients (Singh et al., 2005). The treatment of non-HIV and without underline classical risk factors is complicated by the usual late presentation of these patients.

The management of intracranial pressure in patients with cryptococcal meningitis is crucial because a CSF opening pressure of more than 250 mmH₂O is associated with an increased mortality and over a quarter of HIV positive patients presents with a very high intracranial pressure (Rolfes et al., 2014; Jarvis et al., 2014; Graybill et al., 2000). Therefore, when raised intracranial pressure is observed several international treatment guidelines recommended to perform repeated therapeutic lumbar punctures ad adjunctive management (Chang and Perfect, 2016). Surgical management of intracranial pressure with either external lumbar drainage or ventriculoperitoneal shunting has been reported in case series but specific rules for their use are still lacking (Woodworth et al., 2005; Bach et al., 1997; Park et al., 1999; Cherian et al., 2016; Manosuthi et al., 2008).

Outcomes

Despite aggressive treatment a significant proportion of patients with cryptococcal meningitis persists with poor outcomes and neurological disabilities. In a study from United States, the overall mortality rate was 28.8% with a 90-day mortality rate higher for NHNT subjects (20.7%) in comparison with HIV infected (14.6%) and SOT recipients (13.7%) ($P < .001$) (George et al., 2018). Moreover, the overall mortality was also higher among NHNT subjects (33.2%) and SOT recipients (37.2%) than in HIV positive patients (25%). Possible explanation for the worst outcome among HIV negative included advanced age, underlying comorbid disease and delayed diagnosis (George et al., 2018; Brizendine et al., 2013). In a retrospective cohort study, patients with end-stage liver disease had a significantly higher mortality than HIV positive and HIV negative patients (80% vs 13.6% vs 22.7%, respectively; $P < .001$) (Spec et al., 2015). A systematic literature review showed that in HIV associated cryptococcal meningitis the main independent risk factor for long term mortality were an abnormal neurological status, high CSF fungal burden, low CD4+ cell count and older age at diagnosis (Pasquier et al., 2018). Among HIV-negative patients with cryptococcal meningitis, the main independent risk factors for 1-year mortality were delayed diagnosis, altered neurological status, high CSF CrAg and non-amB based induction therapy (Pasquier et al., 2018).

Candidiasis

Epidemiology and clinical manifestations

Meningitis caused by *Candida* spp. is an uncommon clinical entity usually related to the presence of an iatrogenic predisposing condition such as a neurosurgical procedure with the placement of a shunt or an intracranial device (Nguyen and Yu, 1995). In a 12-year study from Brazil, *Candida* spp. meningitis represented only 1.1% of death attributed to fungal meningitis and 0.05% of all deaths due to meningitis (Alves Soares et al., 2019). Moreover, in this setting the previous exposure to broad spectrum antibiotics is a quite common event (Nguyen and Yu, 1995). Among the infections of shunts and intracranial devices *Candida* spp. infections represent only a minority with a so far higher prevalence of bacterial infections. In a recent case series of 24 patients with *Candida* spp. involving the central nervous system the attributed overall mortality was 42%: 53% in case of disseminated infection and 14% in case of localized infection. In the same series the most frequent causative agent was *C. albicans* (70%) followed by *C. tropicalis* and *C. parapsilosis* (Chaussade et al., 2020). CSF analysis in case of *Candida* spp. meningitis shows high cellularity with either neutrophil or lymphocyte predominance (Casado et al., 1997; Sánchez-Portocarrero et al., 1994; Goldani and Santos, 2010); hypoglycorrhachia is observed in 50% of cases and CSF culture is also positive in half of patients (Chaussade et al., 2020). Fever and intracranial hypertension are observed in 86% of patients. In another case series of 11 central nervous system candidiasis following neurosurgery *C. albicans* accounted for 73% of episodes. All the infections were associated with foreign intracranial material (external ventricular drains in 82%). In the same study a mortality up to 30% was reported (O'Brien et al., 2011).

Candidiasis of the central nervous system is a rare clinical entity which can be observed also in neonate and premature infants with predisposing conditions (shunt/intracranial devices/broad spectrum antibiotics) and children affected by hematologic malignancy and primary immune deficiencies following haematogenous spread with a consequent central nervous system seeding (Walsh et al., 2019). Haematogenous disseminated *Candida* meningoencephalitis is a life-threatening infection with the potential of serious neurologic morbidity if not recognized and treated early. In a case series of 12 children with cancer *C. tropicalis* accounted for 11 out of 12 central nervous system infections (McCullers et al., 2000).

Pathogenesis

The pathogenic mechanism underlying the *Candida* spp. penetration into the subarachnoid space is the direct inoculation of the fungus through contaminated instruments or the disruption of the natural blood-brain-barriers combined with the presence of device which creates a milieu for yeast attachment and proliferation. Haematogenous seeding is another important mechanism through which the yeast disseminates in central nervous system. Moreover, *Candida* spp. are able to produce a biofilm which protect the fungus from the immune-system protective mechanism such as phagocytosis and oxidative stress (Cavalheiro and Teixeira, 2018).

Diagnosis

The detection of $(1 \rightarrow 3)$ - β -D-glucan on CSF in *Candida* spp. meningitis is an early diagnostic indicator which could also be helpful for the subsequent therapeutic management (Salvatore et al., 2016; Lyons et al., 2015). The definite diagnosis is reached when *Candida* spp. is isolated from a sterile site such as blood and CSF but CSF culture is relative insensitive. The resistance pattern of the fungus is crucial to guide the treatment. The diagnostic workup of candidiasis of the central nervous system requires the inclusion of diagnostic procedures to exclude the presence of other septic sites (i.e. endocarditis) or localized region (i.e. brain abscess, eye and abdominal localization).

Treatment

The treatment of central nervous system is based on liposomal amB, 5 mg/kg daily, with or without oral 5-FC, 25 mg/kg four times daily. Fluconazole 400–800 mg (6–12 mg/kg) daily is recommended after response to the initial treatment as step-down therapy after the patient has responded to initial treatment and should be continued until symptoms have been resolved such as the radiological abnormalities. A prompt evaluation of the infected central nervous system devices should be performed with the aim to remove the infected focus. If not possible, amB deoxycholate could be administered through the device into the ventricle at a dosage ranging from 0.01 to 0.5 mg in 2 mL 5% dextrose in water (Pappas et al., 2016). The preferred regimen for neonates is amB deoxycholate, 1 mg/kg intravenous daily with liposomal amB, 5 mg/kg daily, as an alternative. The step-down treatment is recommended with fluconazole, 12 mg/kg daily, if the clinical isolate is susceptible (Pappas et al., 2016).

Aspergillosis

Epidemiology and clinical presentation

Aspergillus spp. meningitis is an extremely rare clinical entity which can occur both in immunocompetent and immunocompromised hosts with a case fatality rate up to 72% (Antinori et al., 2013). The most frequent causative agent is *A. fumigatus* followed by *A. flavus* and *A. terreus*. The most common clinical presentation is acute meningitis, followed by meningoencephalitis. Meningeal signs are uncommon and when present are indicative of subarachnoid hemorrhage (McCarthy et al., 2014; Ma et al., 2020). Headache is frequent in 56.5% of patients with *Aspergillus* spp. meningitis associated with visual loss (39.1%), diplopia (35.8%) and hemiplegia (13%) (Ma et al., 2020). Chronic meningitis could also be observed especially in immunocompetent hosts with a subacute onset with mild symptoms. The evolution of *Aspergillus* spp. meningitis could include spinal arachnoiditis, ventriculitis and the formation of a granuloma or an abscess (McCarthy et al., 2014).

Pathogenesis

Central nervous system aspergillosis is usually associated with classical risk factors such as neutropenia and high dose steroids. Nevertheless, in the cases observed in immunocompetent hosts a direct route through the subarachnoid space was usually found (i.e. direct seeding from contiguous sites such as orbital or sinus tract infection, neurosurgical procedures, spinal anesthesia) (Antinori et al., 2013). Moreover, also the haematogenous dissemination is possible in intravenous drug users or in those with infective endocarditis (Antinori et al., 2013).

Diagnosis

The culture performed on CSF shows low sensitivity and often requires multiple attempts to turn positive. The diagnosis is quite often reached after autopsy in misdiagnosed cases. The probability of a positive culture is higher in immunocompetent hosts (36.9%) when compared to those with immune system impairment (18.2%) (Antinori et al., 2013). The CSF examination usually reveals pleocytosis with a predominance of granulocytes and hypoglycorrhachia. The galactomannan antigen could be helpful during the diagnostic workup if turned positive on serum whereas, due to the absence of standardized cut-off, the results on CSF are difficult to be interpreted (Verweij et al., 1999; Viscoli et al., 2002). Moreover, the diagnostic accuracy of $(1 \rightarrow 3)$ - β -D-glucan on cerebrospinal fluid is still unclear with only anecdotal reports (Davis et al., 2020). The PCR performed on CSF in subject with hematologic disorders showed a good sensitivity (100%) and specificity (93%) but it is not widely implemented (Hummel et al., 2006; Reinwald et al., 2013).

Treatment

The first line regimen is based on voriconazole starting with 6 mg/kg iv every 12 h the first day followed by 4 mg/kg iv every 12 h (Schwartz et al., 2005) with a recommended therapeutic trough concentration between 2 and 5.5 mg/L. Liposomal amB at the dosage of 3–5 mg/kg daily should be considered as an alternative option (Ullmann et al., 2018). The treatment duration should be tailored according to treatment response and clinical improvement. In case of focal lesions or signs of intracranial hypertension a neurosurgical evaluation is warranted.

Endemic mycoses

Certain fungi which are endemic in some areas because of high concentration in the environment are able to cause IFDs with meningitis (Kauffman, 2019; Ashraf et al., 2020). Among these, *Coccidioides* spp., *Paracoccidioides* spp., *Histoplasma* spp., *Talaromyces* spp., *Sporothrix* spp. and *Blastomyces* spp. are able to cause IFDs both in immunocompetent and in immunocompromised hosts (Challa, 2020). A schematic representation of the recommended antifungal treatment for meningitis caused by endemic mycoses is reported in Table 5.

Talaromycosis

Talaromycosis is caused by the dimorphic fungus *Talaromyces* (previously *Penicillium*) *marneffe* which is endemic in China and South East Asia. The fungus is widely distributed in soil and it could be found in several species of bamboo rats (Nelson et al., 2011). The burden of *T. marneffe* infections significantly increased with the HIV epidemic (Duong, 1996). Nevertheless, the risk is also increased in those receiving immunosuppressive agents in particular TNF- α inhibitors (Chan et al., 2015; Antinori et al., 2020). Talaromycosis could seldom be observed also in immunocompetent hosts.

The fungus is acquired through inhalation and after the entrance in the lung it is disseminated by the reticuloendothelial system. The vast majority of infections run asymptomatic in those with an intact T cellular immunity. The occurrence of meningitis in subjects with disseminated *T. marneffe* infection is a very uncommon event (Le et al., 2010). The diagnosis is made by the isolation of the pathogen from sterile sites (blood and CSF) or by the culture or histopathologic examination of skin samples. The usual CSF findings are mild cellularity, slight protein increase and a normal or reduced glucose level. The treatment of talaromycosis is based on liposomal amB 5 mg/kg daily for 4–6 weeks followed by a step-down therapy with oral itraconazole 200 mg twice daily or higher to achieve trough serum concentrations above 2 μ g/mL.

Table 5 Treatment regimens for meningitis caused by endemic mycoses.

Meningitis caused by endemic mycoses	Treatment induction	Step-down	Notes
Talaromycosis	Liposomal amphotericin B 5 mg/kg daily for 4–6 weeks	Itraconazole 200 mg per os every 12 h until CD > 100 cell/ μ L for at least 6 months	– Trough concentration of itraconazole >2 μ g/mL
Blastomycosis	Liposomal amphotericin B 5 mg/kg daily for 4–6 weeks	Itraconazole 200 mg per os every 12 h for 12 months or voriconazole 200–400 mg every 12 h	– Alternative regimen for induction voriconazole – Lifelong treatment in immunosuppressed patients should be considered
Paracoccidioidomycosis	Liposomal amphotericin B 5 mg/kg daily for 4–6 weeks	Itraconazole 200 mg per os every 12 h for 12 months	– Monitor trough concentration of azoles during step-down treatment – Alternative step-down therapy with voriconazole or trimethoprim/sulfamethoxazole
Coccidioidomycosis	Fluconazole 400–1200 mg per os daily	Chronic azole treatment	– Life long suppressive treatment with trimethoprim/sulfamethoxazole should be considered – Alternative regimens based on itraconazole, voriconazole, isavuconazole and liposomal amphotericin B – Refractory cases could be treated with intrathecal amphotericin B
Sporotrichosis	Liposomal amphotericin B 5 mg/kg daily for 4–6 weeks	Itraconazole 200 mg per os every 12 h for 12 months	– Lifelong suppressive therapy is recommended – Lifelong treatment in immunosuppressed patients should be considered
Histoplasmosis	Liposomal amphotericin B 5 mg/kg daily for 4–6 weeks	Itraconazole 200 mg per os every 12 h for 12 months	– Lifelong treatment in immunosuppressed patients should be considered

Coccidioidomycosis

Coccidioidomycosis is an endemic mycosis caused by the soil-dwelling fungi *Coccidioides immitis* and *C. posadasii* which are only present in the western hemisphere (Johnson and Einstein, 2006). Although the clinical presentation of the disease is the same between the two species, *C. immitis* is more common in California, and *C. posadasii* is more common in Texas, Central and Latin America (McCotter et al., 2019). The number of reported coccidioidomycosis cases has increased in recent years probably due to environmental changes in endemic area combined with an increasing number of immunocompromised hosts (Brown et al., 2013).

The vast majority of *Coccidioides* spp. infections run asymptomatic. Nevertheless, influenza like illness is common such as symptoms deriving from a mild primary lung involvement. A small percentage of subjects develop the disseminate form of the disease. Lymphohematogenous spread to the leptomeninges almost always occurs within weeks to months following the initial untreated lower respiratory infection and frequently involves areas of the basilar, sylvian, and interhemispheric cisterns (Wrobel et al., 1992). The clinical presentation is usually subacute or chronic. *Coccidioides* spp. and the course of the disease is usually insidious because headache may not be severe, the main hallmark being its persistence over weeks or months. In a case series of 71 patients from a referral center from Arizona headache was present in 77% of cases whereas neck rigidity was found in only 23% (Drake and Adam, 2009). As the disease progress undiagnosed, the patients develop confusion, emotional lability, disorientation, diplopia and grit disturbances. At physical examination nuchal rigidity is uncommonly observed but subjects show slowed speech and cognitive disturbances (Bouza et al., 1981; Drake and Adam, 2009; Kelly, 1980). Nearly 40% of patients with coccidioid meningitis will present or develop hydrocephalus which is the most common complication of this meningitis and a major contributor of mortality (Blair, 2009; Galgiani et al., 2016). Vasculitic infarction are observed in 10% of patients with stroke (aphasia, hemianopsia, hemiparesis) as clinical picture of presentation (Williams et al., 1992). Lumbar pain, neurological bladder, paraplegia and quadriplegia are the expression of arachnoiditis (Mathisen et al., 2010).

Neuroimaging studies (preferably gadolinium enhanced magnetic resonance scan) are an essential part of the work-up of suspected coccidioid meningitis (Wrobel et al., 1992; Arsura et al., 2005). Leptomeningeal enhancement of the basilar cistern is observed in 91% of cases and hydrocephalus in 78% (Lammering et al., 2013). The high frequency of spinal involvement (37% nerve root clumping, 16% intramedullary and spinal cord hyperintensity on T2 signal, 16% with intradural masses) suggests the need to make both intracranial and spinal imaging for patients with coccidioid meningitis (Crete et al., 2018).

Because untreated meningitis is nearly always fatal, early diagnosis and initiation of therapy is important to prevent death and many of the complications that ongoing meningeal inflammation produces (Vincent et al., 1993).

Coccidioid meningitis diagnosis requires laboratory analysis of the CSF, a must in patients with a diagnosis of coccidioidomycosis with persistent headache. The CSF findings of coccidioid meningitis include cellular pleocytosis (between 20 and 200 cell/ μ L). CSF examination usually shows a lymphocytic predominance but CSF eosinophilia is considered suggestive (Jackson et al., 2019; Ragland et al., 1993) and hypoglycorrhachia is frequent and severe (Drake and Adam, 2009). The definite diagnosis could be reached by the culture and isolation of *Coccidioides* spp. from CSF but it is positive in less than one third of cases. Several non-culture techniques can be employed to diagnose coccidioid meningitis (Binnicker et al., 2011). A classical assay is the complement fixation (CF) coccidioid antibody test based on a complement-fixing methods or immunodiffusion (ID). This test detects IgG antibody and is positive in over 95% of patients with coccidioid meningitis (Pappagianis and Zimmer, 1990; Pappagianis, 2001). In the largest study so far conducted, the CF with ID had a sensitivity of 78%, 100% specificity and positive predictive value and 74% negative predictive value (Stevens et al., 2020). Interestingly, a lateral flow assay (one strip and two strip) showed the highest sensitivity (92–95%) and specificity (100%) with a negative predictive value of 86–91%. Beta-D-glucan (with a cut of 31 pg/mL) had a good sensitivity (96%) but a lower specificity (82%) and a negative predictive value (90%) (Stevens et al., 2020).

The best available treatment for coccidioid meningitis is fluconazole at the recommended dose of 400 mg daily although some experts would prefer an initial dose of 800 mg daily (Tucker et al., 1990; Jackson et al., 2019). A maintenance therapy (fluconazole 400 mg) should be continued life-long to avoid the high rate of relapse (80%) observed in patients discontinuing it (Dewsnup et al., 1996). All the other azole (itraconazole 400–800 mg daily, voriconazole 4 mg/kg every 12 h, posaconazole oral suspension 400 mg every 12 h, isavuconazole) can be used as an alternative therapy for coccidioid meningitis (Johnson et al., 2018).

Paracoccidioides

Paracoccidioidomycosis is caused by two species *Paracoccidioides brasiliensis* and *P. lutzii* (Hahn et al., 2019). The epidemiology of paracoccidioidomycosis is restricted to Latin America with most of cases reported in Brazil (*P. lutzii* near the Mato Grosso region), Argentina, Venezuela, Ecuador, and Colombia (Martinez, 2017). Such as for other endemic mycoses with a direct relation to soil exposure the disease is more often reported in men without apparent underlying medical conditions. Nevertheless, in recent years the disease is increasingly observed in people living with HIV (Morejón et al., 2009) such as in SOT (de Almeida Jr et al., 2018). Due to the latency of the fungus, the infection could be acquired during the childhood and then reactivate in case of immune suppression. The central nervous system involvement is uncommon in paracoccidioidomycosis (<15% of patients) and more often observed in the chronic form of the disease with space-occupying lesions. Meningitis is a very infrequent manifestation of this endemic mycosis and is a consequence of a disseminated form of the disease both in acute or chronic cases (Elias Jr et al., 2005).

The diagnosis could be reached by the isolation of the fungus from CSF or by a positive culture or histopathology on tissue obtained by a brain biopsy (Elias Jr et al., 2005). The detection of a circulating antigens (gp43 and gp70) of *P. brasiliensis* in serum and CSF has been suggested as a diagnostic method (da Silva et al., 2005). Nevertheless, the diagnosis is often presumptive when *Paracoccidioides* spp. is isolated from other sites and symptoms of central nervous system involvement are present.

The treatment is based on liposomal amB (5 mg/kg daily for 4–6 weeks) followed by itraconazole for at least 12 months. In some circumstances, i.e. immunosuppressed patients, a suppressive lifelong treatment with trimethoprim/sulfamethoxazole should be considered (Shikanai-Yasuda et al., 2017).

Histoplasmosis

Histoplasmosis is a disease caused by the fungus *Histoplasma capsulatum*. *H. capsulatum* var. *capsulatum* is widely distributed whereas *H. capsulatum* var. *duboisii* is limited to sub-Saharan Africa. The disease is highly endemic in some regions of North America, Central America, and South America but it is also reported in certain countries of Asia such as China, India and Thailand (Antinori, 2014; Bahr et al., 2015). Histoplasmosis more often involves subjects with underlying medical conditions and in particular people living with HIV causing approximately 10–15% of AIDS related deaths in the Americas (Pan American Health Organization and World Health Organization, 2020). In particular, in such patients the clinical characteristic of disseminated histoplasmosis makes the differential diagnosis with tuberculosis extremely difficult.

Central nervous system involvement occurs in 5–10% of disseminated histoplasmosis cases (Wheat et al., 2018) and the most frequent clinical feature is represented by chronic meningitis with or without focal brain, or spinal cord lesions, stroke syndromes, encephalitis, and hydrocephalus (Wheat et al., 2007). It is worth mentioning that approximately one third of subjects affected by central nervous system histoplasmosis have no underlying medical conditions leading to immunosuppression (Wheat et al., 2018). Although histoplasmosis involving the central nervous system was first described in 1934, it was not until 1990 that a clinical series reported the clinical course of patients with central nervous system histoplasmosis (Wheat et al., 1990). In this case series meningismus was described in only 8% of patients whereas in a more recent study by Wheat et al. neck rigidity was observed in 13% of patients (Wheat et al., 1990, 2018).

The diagnosis of *Histoplasma* spp. meningitis is delayed as observed in case of chronic meningitis with only 26% of patients presenting with symptoms for 2 weeks or less (Wheat et al., 1990, 2018). CSF findings show a lymphocytic pleocytosis in 50% of cases but a predominance of neutrophils occurs in 11% of cases; low glucose level (<40 mg/dL) are reported in 53% of patients.

Histoplasma spp. can be isolated from fungal culture of CSF in approximately 25% of cases. Moreover, the slow growing nature of this fungus often causes a treatment delay (Wheat et al., 2018). The most sensitive method for diagnosis of central nervous system histoplasmosis is based on the detection of antibody and antigen in the CSF. The combination of both the assays lead to an accuracy of 98% in detecting central nervous system histoplasmosis cases (Bloch et al., 2018). As expected, the sensitivity of antigen detection in central nervous system histoplasmosis was better among immunocompromised patients (81%) in comparison with those not immunocompromised (31%) and in patients with more severe disease (93% vs 55%) (Wheat et al., 2018). According to a recent study the sensitivity of *Histoplasma* antigen detection in CSF was 78% with a specificity of 93% (Bloch et al., 2018).

The treatment of *H. capsulatum* meningitis requires a prompt antifungal treatment with liposomal amB followed by itraconazole for at least 12 months. As for other endemic mycoses a suppressive lifelong treatment with azoles should be considered (Wheat et al., 2007).

Blastomycosis

Blastomycosis is an infection caused by several species of a dimorphic fungus named *Blastomyces*. The fungus able to cause human diseases are *B. dermatitidis*, *B. gilchristii* and *B. helicus* (Schwartz et al., 2019). The fungus lives in the environment, particularly in moist soil and in decomposing matter in the United States and Canada, in particular around the Ohio and Mississippi River valleys and the Great Lakes. The cases are more often sporadic and it could be possible to observe outbreaks when an epidemiological link between subjects is present, i.e. work activities involving soil disruption in endemic area. Probably due to working exposure to contaminated environment blastomycosis is more often observed in men.

Blastomyces spp. disseminate through the lung and the central nervous system involvement is less common when compared to other endemic mycoses. The clinical manifestation of central nervous system involvement comprehends both space occupying-lesions and/or meningitis, meningoencephalitis (Gonyea, 1978; Bariola et al., 2011; Bush et al., 2013). The central nervous system involvement is more frequent in immunocompromised patients and it is estimated that occur in 1–10% of patients with blastomycosis (Pappas et al., 1992; Chapman, 2005) but it could seldom be observed in hosts without underlying medical conditions (Bush et al., 2013). The clinical manifestations of meningitis caused by *Blastomyces* spp. range from a chronic syndrome with headache, mental status alteration and signs of raised intracranial pressure to a disseminated disease characterized by multiple organ involvement more often observed in immunocompromised individuals (Kauffman, 2019).

The definite diagnosis is obtained by the isolation of the fungus from a sterile site such as blood or CSF. Due to the slow growing nature of *Blastomyces* spp. the diagnosis is often reached by the histological examination of tissues in disseminated diseases with the help of silver or periodic acid Schiff (PAS) colorations. Moreover, a presumptive diagnosis could be inferred by the positivity of *B. dermatitidis* antigen on serum or urine (Bariola et al., 2011). The specific antigen could also be searched in the cerebrospinal fluid (Bariola et al., 2010). The galactomannan antigen could be helpful but it is worth mentioning the cross-reactivity with another endemic fungus, *H. capsulatum*. Liposomal amB is the drug of choice for the initial phase of treatment followed by a prolonged treatment with azoles for at least 12 months. In those subjects with underlying immune systems disorders a life-long antifungal treatment should be considered (Chapman et al., 2008).

Sporotrichosis

Sporotrichosis is a ubiquitous mycosis caused by a complex of six phylogenetically different dimorphic fungi, including *Sporothrix schenckii* sensu stricto, *S. albicans*, *S. brasiliensis* (in Brazil), *S. mexicana* (in Mexico), *S. globosa* (in United Kingdom, Spain, Italy, China, Japan, United States, and India) (Marimon et al., 2006). The fungus usually penetrates through the skin but seldom could be acquired by inhalation. *S. brasiliensis* is able to be transmitted by cats and family clusters such as zoonotic outbreaks have been described so far (Brandolt et al., 2018).

The central nervous system involvement is rare and classically described as a subacute or chronic meningitis secondary to a disseminate disease. Most of cases shows an underlying medical condition leading to immunosuppression such as HIV infection or use of immune-suppressive agents (Queiroz-Telles et al., 2019). The clinical presentation is often with a slow onset of headache, confusion and symptoms of intracranial pressure increase (Kauffman et al., 2007).

The definite diagnosis is made with isolation of *Sporothrix* spp. on sterile sites such as CSF, blood or from biopsies. The fungal burden is higher in immunocompromised hosts and consequently in these patients the fungal culture in CSF more often turned positive when compared to that without disseminate disease (Moreira et al., 2015). The biochemical characteristics of CSF are comparable to that of other chronic meningitis with increased cellularity (mononuclear), increase in proteins and reduced glucose level.

The treatment of *Sporothrix* spp. meningitis is the same of the disseminate disease and is based on an induction with liposomal amB for 4–6 weeks followed by a step-down treatment with itraconazole for at least 12 months. The prosecution of a lifelong azole suppressive treatment should be considered in case of persistent immune suppression (Kauffman et al., 2007).

Dematiaceous mold (*Exserohilum rostratum*)

Dematiaceous fungi are rare cause of fungal meningitis which usually occur in immunocompetent hosts (McCarthy et al., 2014). An invasive form which can involve the central nervous system is known as phaeo-hyphomycosis. In a literature review of 101 cases approximately 50% showed an underlying medical condition leading to immunosuppression and meningitis was the clinical presentation in 9% of cases (Revankar et al., 2004).

Among dematiaceous fungi, *Exserohilum rostratum* emerged as a cause of fungal meningitis in 2012 when the Centers for Disease Control and Prevention reported a large outbreak of infections in patients who received injections, primarily epidural, of methylprednisolone acetate (Pettit et al., 2012). The investigations performed highlighted how the infection derived from the contamination of the product produced from a single company during the manufacturing production (Centers for Disease Control and Prevention (CDC), 2012). The outbreak comprehended 753 patients accounting from 64 (8%) death related to the infection by *E. rostratum*. Moreover, almost all patients with spinal or paraspinal involvement showed persistent disability at 12 months of follow-up (Malani et al., 2020). After the injection the first manifestation was fungal meningitis with some patients developing stroke and subsequently a wide range of other syndromes caused by the IFD. The symptoms reported in those who developed meningitis were headache in 91% of cases, fever in 38%, and decreased cognitive function in 28% of case which was classified as persistent at 12 months as mild-moderate in 11% of patients. Patients who developed meningitis with or without stroke were those at higher risk of death with an eightfold higher risk of death when compared to the spinal/paraspinal (hazard ratio, 7.62; 95% CI, 3.17–18.37) (Malani et al., 2020).

The initial antifungal treatments used was voriconazole at a dosage of 6 mg/kg the first day every 12 h followed by 4 mg/kg daily every 12 h, in case of refractory disease in can be used in combination with liposomal amB 5 mg/kg daily. Moreover, a prompt neurosurgical assessment is warranted with appropriate surgical debridement in case of spinal or paraspinal involvement (McCarthy et al., 2014). The treatment duration is prolonged (usually of 4 (2–9) months) and the relapse of the infection is not infrequent after a median of 3 (1–14) months from treatment interruption (Malani et al., 2020).

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Placenta and Fetus Infections: Fungi

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Candidiasis

Introduction

Candida species is the most common causative agent of intra-uterine infections involving the placenta, umbilical cord, and fetus. Over 100 case reports and short case series have been published since the first case of chorioamnionitis caused by *Candida albicans* that resulted in preterm birth and neonatal death in 1958 (Benirschke and Raphael, 1958). As the prevalence of this disease is very rare in the general population, the largest study series comprised only 32 cases (Qureshi et al., 1998). Therefore, clinical manifestation is not well understood. According to the largest literature review, intra-uterine *Candida* infection may result in stillbirth, preterm delivery, and neonatal death (Maki et al., 2017).

This topic will discuss clinical manifestations, diagnosis, and treatment of intra-uterine *Candida* infection.

Epidemiology

Candida species rarely causes intra-uterine infections that result in chorioamnionitis, funisitis, and congenital candidiasis despite the high incidence of vulvovaginal candidiasis during pregnancy (13–40%) (Kiss et al., 2004; Roberts et al., 2011; Barton et al., 2017). The published literature includes a few case reports and case series. As there is no report that assessed the prevalence of intra-uterine *Candida* infection in the general population, the actual prevalence is unclear. In the largest case series, four among approximately 10,000 placentae from 70,000 deliveries had *Candida* infection (Qureshi et al., 1998). At regional referral centers, the prevalence of *Candida* chorioamnionitis is reported to be 0.5% in the south central region of Ontario, Canada (Whyte et al., 1982), and 0.3% in the southern region of Japan (Maki et al., 2017). A review of intra-amniotic infection published in 1992 showed that the prevalence of *Candida* species isolated from the amniotic fluid obtained by transabdominal amniocentesis was 6.5% (5/773) in cases with preterm labor with intact membranes and 2.2% (4/625) in cases with preterm prelabor rupture of membranes (pPROM) (Chaim et al., 1992). Another study showed that the isolation rate of *Candida* species from the amniotic fluid was 5% and 1.2% using polymerase chain reaction (PCR) method and 3.2% and 0.6% using culture-based methods in patients with pPROM and preterm labor with intact membranes, respectively (DiGiulio et al., 2008).

Transmission

As observed with bacterial intra-uterine infection, ascending *Candida* infection from the vagina and cervix to the uterine cavity is probably the most common pathway. Therefore, the rupture of membranes is a risk factor of intra-uterine *Candida* infection. The incidence of *Candida* species isolation from the amniotic fluid of patients with pPROM is higher than that from the amniotic fluid of patients with intact membranes (DiGiulio et al., 2008). A previous study reported that 25% cases were associated with pPROM

(Maki et al., 2017). Interestingly, an in vitro study using human fetal membranes showed that *C. albicans*, but not *C. glabrata*, *C. pseudotropicalis*, *C. guilliermondii*, and *C. tropicalis*, penetrates the fetal membrane, accompanied by the degeneration of the structure of the membrane epithelium (Gürkan et al., 1994). Why *Candida* species rarely causes intra-uterine infection is less understood, although its colonization in the vagina is very common (Kiss et al., 2004; Roberts et al., 2011; Barton et al., 2017). A previous review failed to show the association between vaginal *Candida* colonization and intra-uterine *Candida* infection, owing to the lack of information on vaginal *Candida* colonization in most cases (Maki et al., 2017). A possible case of the inoculation of the maternal skin flora into the uterine cavity by transabdominal amniocentesis has also been reported (Rode et al., 2000).

Microbiology

A case control study involving 18 cases with *Candida* chorioamnionitis reported *C. albicans* in 15 cases, and the remaining three cases were one each of *C. parapsilosis*, *C. tropicalis*, and *C. stellatoidea* (Whyte et al., 1982). *C. albicans* is a predominant species accounting for 71.3% cases (72/101), followed by *C. glabrata* (21.7% [21/101]) (Maki et al., 2017). Recently, there has been an increase in case reports of *C. glabrata* infection associated with in vitro fertilization (IVF). There are a few reports of cases with other *Candida* species infection, including *C. tropicalis* ($n = 3$) (Nichols et al., 1995; Barth et al., 2002; Canpolat et al., 2011), *C. lusitanae* ($n = 2$) (DiLorenzo et al., 1997; Huang et al., 2012), *C. parapsilosis* ($n = 2$) (Waguespack-LaBiche et al., 1999; Krallis et al., 2006), *C. famata* ($n = 1$) (Maki et al., 2017), and *C. kefyr* ($n = 1$) (Pineda et al., 2012). Coinfection with *C. albicans* and *C. parapsilosis* has also been reported (Waguespack-LaBiche et al., 1999; Krallis et al., 2006), including a case of twin pregnancy where blood cultures were positive for *C. albicans* in one of the two twin infants and *C. parapsilosis* in another twin (Krallis et al., 2006).

Risk factors

Several studies suggest foreign bodies in the uterus to be associated with intra-uterine *Candida* infection. Intra-uterine device (IUD) is a well-known risk factor. A case series revealed 3 of 32 cases of *Candida* funisitis to carry IUD (Qureshi et al., 1998). Further, a case control study showed that 8 of 18 cases with *Candida* chorioamnionitis had IUD (Whyte et al., 1982). In addition, a review on fetal *Candida* infection found 17 of 54 cases to be associated with IUD and that the presence of IUD was associated with systemic (heart, brain, liver, gastrointestinal, and lung) fetal infection (Roqué et al., 1999). In a review of 123 cases, 21% *Candida* chorioamnionitis cases had IUD (Maki et al., 2017). Statistical studies strengthened the association between IUD and intra-uterine *Candida* infection. A retrospective cohort study involving 196 cases with IUD during pregnancy concluded the presence of IUD as a risk factor for late miscarriage, preterm delivery, vaginal bleeding, histological chorioamnionitis, clinical chorioamnionitis, and placental abruption. Furthermore, the prevalence of intra-amniotic infection caused by *Candida* species was higher in pregnant women with IUD than in those without IUD (31.1% vs 6.3%, $P < 0.001$) (Kim et al., 2010). Another retrospective study showed that pregnancies with IUD were associated with higher prevalence of *Candida* invasion of the amniotic cavity than those without IUD (28% vs 3%, $P < 0.01$) (DiGiulio et al., 2010). The mechanism of transmission of *Candida* species to the pregnant uterine cavity with IUD is not known, but some theories are proposed. The IUD string can serve as a route of fungal transmission from the vagina to the uterine cavity (Delprado et al., 1982). Contamination with *Candida* species may occur at the time of IUD insertion (Schweid and Hopkins, 1968). An in vitro study showed that *C. albicans* isolated from vaginal discharge of patients with vulvovaginal candidiasis strongly adhered to all parts of IUD and produced biofilm (Chassot et al., 2008).

Cervical cerclage is also reported as a risk factor for intra-uterine *Candida* infection. *Candida* infections associated with cervical cerclage were reported in 2 of 32 cases (Qureshi et al., 1998) and 5 of 18 cases (Whyte et al., 1982) in previous studies. Another study reported that 8 of 123 cases (6.5%) had cervical cerclage (Maki et al., 2017). However, there is no statistical evidence supporting this information.

In recent years, there has been an increase in case reports on intra-uterine *C. glabrata* infection following IVF. A literature review of 20 cases of intra-uterine *C. glabrata* infection showed that 13 of them were associated with IVF (Ganer Herman et al., 2015). Cases of intra-uterine infection caused by other *Candida* species, including *C. albicans*, *C. parapsilosis*, *C. kefyr*, and *C. famata*, have also been reported. In a review on 123 cases, 20% were related to IVF (Maki et al., 2017). Any steps in the process of IVF may contaminate the fertilized embryo or introduce *Candida* species into the uterine cavity.

Clinical manifestations

Maternal symptoms are unspecific. Preterm labor with an intact membrane is the most common symptom (42%), and pPROM and cervical dilatation without uterine contraction are also frequent (25% and 9%, respectively). Fever was reported in only 13% cases (Maki et al., 2017).

Diagnosis

Isolation of *Candida* species from the amniotic fluid or pathological findings of the presence of fungal cells with inflammation in the placenta, fetal membrane, umbilical cord, and/or fetus is the gold standard for diagnosis of intra-uterine *Candida* infection. A limitation of pathological findings is that these are available only after delivery. Therefore, amniotic fluid test through transabdominal amniocentesis is imperative for antenatal diagnosis. According to a previous study, antenatal treatment was attempted in

13 cases. All these cases were diagnosed using amniotic fluid culture obtained by transabdominal amniocentesis (Maki et al., 2017). Transvaginal sampling of amniotic fluid should be avoided, owing to the risk of vaginal colonization with *Candida* species.

Standard culture method is commonly used in clinical practice, whereas PCR is used in the research field. A retrospective study showed the combination of PCR and culture method achieved higher detection rate of *Candida* species than culture method alone (5% vs 3.2%) (DiGiulio et al., 2010). Beta-glucan, a constituent of the cell wall of fungi, test in the amniotic fluid can be useful for rapid diagnosis (Pacora et al., 2019).

Pathological findings in the placenta, umbilical cord, and fetus

Chorioamnionitis and funisitis are common like bacterial intra-uterine infection. Macroscopically, yellow-white nodules on the umbilical cord are typically observed in intra-uterine *C. albicans* infections (Fig. 1). These nodules are formed by subamniotic abscesses with fungal cells (Qureshi et al., 1998). Grocott and periodic acid-Schiff staining can highlight yeasts with pseudohyphae in the fetal membrane and umbilical cord (Fig. 2). The nodules may not be clearly visible in *C. glabrata* infection (Ibara et al., 2004; Matsuzawa et al., 2005). Although the typical yellow-white nodules may be absent, the infiltration of yeast-like organisms without pseudohyphae consistent with the morphology of *C. glabrata* can be observed in the fetal membrane upon Grocott and periodic acid-Schiff staining in cases with *C. glabrata* infection (Ibara et al., 2004; Özer et al., 2013; Sfameni et al., 1997; Jackel and Lai, 2013; Freydiere et al., 2005).

There are several reports on fetal autopsy of stillbirth cases. Skin rash is commonly observed on gross examination. Microscopic examination has revealed fungal cells on the epithelial surface and hair follicles with inflammatory cells (Buchanan et al., 1979; Donders et al., 1997; Spaun and Klünder, 1986). Fungal invasion into the respiratory tract and lungs along with inflammation is the most frequent feature, suggesting that the direct aspiration of *Candida* species in the amniotic fluid is the main cause of fetal infection (Nichols et al., 1995; Huang et al., 2012; Schweid and Hopkins, 1968; Sfameni et al., 1997; Buchanan et al., 1979; Meizoso et al., 2008; Chaim et al., 1993; Bittencourt et al., 1984; Ho and Aterman, 1970; Lee and Lo, 2011). On the other hand, *Candida* species can be ingested into the gastrointestinal tract. Fungi are often seen in the stomach and intestine, but the bowel wall is usually free of fungus and inflammation (Huang et al., 2012; Ibara et al., 2004; Meizoso et al., 2008; Bittencourt et al., 1984; Ho and Aterman, 1970; Lee and Lo, 2011; Rivasi et al., 1998). In cases with severe infection, dissemination of fungi is observed in multiple organs, including the kidney, liver, spleen, and brain (Engelhart et al., 1998; Levin et al., 1978).

Treatment

Prompt delivery and treatment with an antifungal agent individually for the mother and the infant is preferable because no optimal antenatal treatment for intra-uterine *Candida* infection is yet established, although the immaturity of the infant is often crucial. Various antenatal treatment regimens have been tested. Amphotericin B offers some advantages for antenatal treatment, as it crosses the placenta, does not exert teratogenic effects or fetotoxicity (Briggs and Freeman, 2017), and is generally effective against *C. glabrata*, the second most prevalent species of intra-uterine *Candida* infection. Amphotericin B is recommended as the first-line treatment against invasive candidiasis in pregnant women and neonates (Pappas et al., 2016). A case of successful intravenous amphotericin B treatment was reported that resulted in pregnancy extension by 4 weeks and delivery of twin infants with no signs of chorioamnionitis or funisitis (Tan et al., 2015). However, there are some cases of stillbirth after intravenous amphotericin B treatment (Jackel and Lai, 2013; Garcia-Flores et al., 2016; Akhanoba et al., 2014). Transcervical intra-amniotic administration of amphotericin B for a case with pPROM caused by intra-uterine *C. albicans* infection has been reported. Intra-uterine *C. albicans* infection was confirmed by amniotic fluid culture obtained by transabdominal amniocentesis. Amphotericin B 2 mg in 1000 mL of normal saline was infused into the uterine cavity via the transcervical catheter at first time, and the dose was subsequently increased to 10 mg. After 5 days of daily infusion, culture of the amniotic fluid turned negative for *C. albicans* and spontaneous labor occurred on day 7 of treatment (Shalev et al., 1994).



Fig. 1 Yellow-white nodules on the umbilical cord.

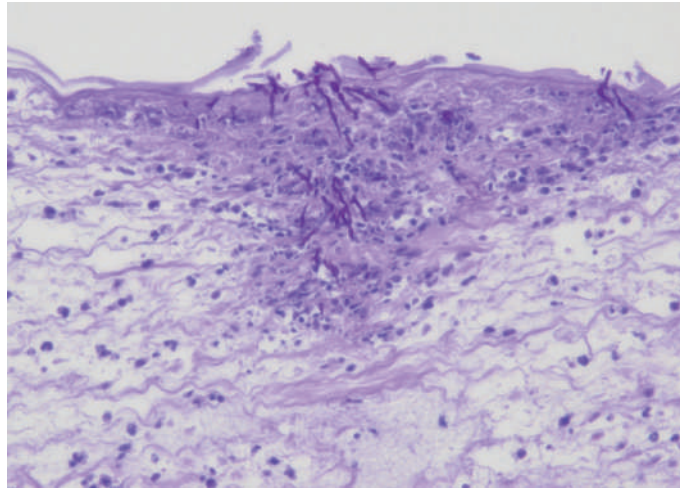


Fig. 2 Subamniotic abscesses with fungal cells on the umbilical cord (PAS stain).

Fluconazole is the most popular agent in case reports (Ganer Herman et al., 2015; Arai et al., 2002; Crawford et al., 2006; Poliquin et al., 2015; Bean et al., 2013), although whether it crosses the placenta is unknown (Briggs and Freeman, 2017). Further, it exerts less activity against *C. glabrata*. Weekly intra-amniotic administration of 40–100 mg fluconazole combined with oral administration succeeded in pregnancy extension for 6–8 weeks and delivery of infants without congenital candidiasis (Bean et al., 2013). Given the potential risk of teratogenic effects on the fetus, careful patient selection is necessary (Briggs and Freeman, 2017; Zhu et al., 2020). The efficacy and safety of micafungin is unclear. The characteristics of micafungin, such as high molecular weight, low lipid solubility, and very high protein-binding ability, suggest the limitation of its transfer to the fetus (Briggs and Freeman, 2017). Intravenous micafungin administration was attempted in one case that failed to eradicate *C. glabrata* from the amniotic fluid but extended pregnancy for 10 days and led to live infant delivery (Alfei et al., 2014).

Prognosis

In a case series of 32 cases, 24 had premature delivery and 7 cases were delivered within 26 weeks. All fetuses and neonates died perinatally. There was no perinatal death among the remaining 25 cases born after 26 weeks, although 4 of these 25 cases had congenital candidiasis (Qureshi et al., 1998). In a literature review of 123 cases, 27% of all singleton cases were delivered before 22 weeks and 60% were delivered between 22 and 36 weeks. Almost 30% singletons born after 22 weeks died. As the description of the clinical features is heterogeneous among the reports, whether the cause of death is congenital candidiasis is unclear. The authors concluded that immaturity and severe fetal inflammation may lead to high mortality rate (Maki et al., 2017).

Less common fungal infections

Placental and fetal infections with fungi other than *Candida* species are extremely rare. Given the rarity of such infections, clinical information is limited. Several cases of neonatal fungal infections have been reported. These cases may include vertical transmission in utero, aspiration or swallowing of infected vaginal secretions during delivery, and acquisition after delivery. As this chapter focuses on placental and fetal infections, we discuss only cases with placental infection, vertical transmission in utero confirmed by fetal autopsy, and early neonatal infection accompanied with placental lesions.

Pneumocystis infection

Pregnant women are at increased risk of colonization with *Pneumocystis jirovecii*. The positive rate of *P. jirovecii* DNA in the nasal swabs of healthy pregnant women was higher than that from samples of nonpregnant controls (15% vs 0%) (Vargas et al., 2003). *P. jirovecii* pneumonia was the most common cause of death in pregnant women with human immunodeficiency virus (HIV) infection in the United States in 1980s (Koonin et al., 1989). A literature review of *P. jirovecii* pneumonia in HIV-infected pregnant women found 22 cases and reported a very low fetal/neonatal survival rate (10/22, 54.5%). There is only one report describing fetal infection with *P. jirovecii* where an HIV-infected pregnant woman was diagnosed with *P. jirovecii* pneumonia at 24 weeks of gestation. Despite the treatment, the pregnancy resulted in stillbirth and the patient died after delivery. Autopsy of the fetus revealed *P. jirovecii* in the interalveolar macrophages in the fetal lung without any signs of interstitial pneumonitis. *P. jirovecii* was also found in placental sections (Mortier et al., 1995), although there is a controversial opinion (Hughes, 1995). There are a few cases of congenital pneumocystic pneumonia reported before the acquired immunodeficiency syndrome (AIDS) epidemic

(Pavlica, 1962; Bazaz et al., 1970). A study detected *P. jirovecii* DNA from the placenta and lungs of aborted fetuses delivered from immunocompetent women (Montes-Cano et al., 2009). These findings suggest the possibility of maternal-fetal transmission of *P. jirovecii*.

Blastomycosis

Blastomycosis is caused by *Blastomyces dermatitidis*, a dimorphic environmental fungus. Whether it is transmitted from the mother to the fetus is yet unknown. In a literature review of 23 cases of blastomycosis during pregnancy, two neonates died from blastomycosis (Baker et al., 2017). Symptoms were presented at 18 days and 3 weeks of age. Both neonates were not examined at birth and were asymptomatic until then. The placenta was not evaluated (Maxson et al., 1992; Watts et al., 1983). Therefore, the route and timing of infection are unclear (Baker et al., 2017). There is one case of placental blastomycosis confirmed by pathology that demonstrated dimorphic blastomycosis spores on both maternal and fetal sides. Culture of placenta was positive for *B. dermatitidis*, but all cultures of the fetus were negative (MacDonald and Alguire, 1990).

Cryptococcosis

Cryptococcosis, which is caused by *Cryptococcus neoformans*, is an opportunistic infection in immunocompromised patients. There are four case reports of placental cryptococcosis diagnosed by placental pathology demonstrating mucin-positive yeasts in the intervillous space (Kida et al., 1989; Molnar-Nadasdy et al., 1994; Darko et al., 2009; Patel et al., 2012) and chorionic villi (Darko et al., 2009; Patel et al., 2012). There is another case of numerous cryptococcus accompanied with a granulomatous response found in aborted specimen from a HIV-positive mother (Rahimi et al., 2009). These reports suggest that *Cryptococcus* can invade the placenta. Among the four cases of placental cryptococcosis, one neonate had congenital cryptococcosis (Patel et al., 2012). The mother was HIV positive and her blood and cerebrospinal fluid cultures were positive for *C. neoformans* at 25 weeks of gestation. Intravenous amphotericin B treatment improved her symptoms. The neonate was born by caesarean section at 28 weeks of gestation because the mother developed preeclampsia. The neonate had positive serum cryptococcal antigen, but the cerebrospinal fluid analysis was normal. The neonate was treated with amphotericin B followed by fluconazole, which resolved antigenemia.

Coccidioidomycosis

Coccidioidomycosis is caused by *Coccidioides immitis* or *C. posadasii*. Pregnancy is a risk factor for coccidioidomycosis. In particular, severe primary and disseminated coccidioidomycosis may develop in third trimester and after delivery (Peterson et al., 1993; Crum and Ballon-Landa, 2006; Bercovitch et al., 2011). There is no evidence of confirmed maternal-fetal transmission of *Coccidioides* species in utero. However, several reports have shown the placental involvement. A study reported over 15 cases with placental lesions of coccidioidomycosis showed that *Coccidioides* spherules containing endospores localized by necrosis and deposits of fibrin with acute and/or chronic granulomatous inflammation in the placenta (Labuschagne et al., 2016). The fetuses/neonates were not infected with *Coccidioides* species even in these cases. Fibrin deposition is related to the pathogenesis of coccidioidomycosis. It may help to inhibit vascular access and prevent transmission to the fetus (Labuschagne et al., 2016). There are several reports of neonatal coccidioidomycosis. These cases may be infected by aspiration of vaginal secretions at the time of delivery (Bercovitch et al., 2011).

Histoplasmosis

Histoplasmosis is caused by a dimorphic fungus, *Histoplasma capsulatum*. The first case of placental and congenital histoplasmosis was reported in 2004. The report comprised three cases of histoplasmosis during pregnancy. The mother developed acute respiratory distress after cholecystectomy at 25 weeks of gestation. The bronchoalveolar lavage and blood culture were positive for *H. capsulatum*. The neonate was delivered by emergency cesarean section. *H. capsulatum* was detected in the blood, cerebrospinal fluid, and peritoneal fluid culture of the neonate. The neonate was discharged after 103 days of amphotericin B treatment. Placental histology showed clusters of yeasts in the villi and trophoblast. Morphological features in Grocott-Gomori methenamine-silver staining and Periodic acid-Schiff staining were consistent with those of *H. capsulatum*. The other two cases had no evidence of placental histoplasmosis by pathological examination. One fetus died from maternal ketoacidosis, while the other had no symptoms and abnormal examinations (Whitt et al., 2004). Another congenital histoplasmosis has been reported. The neonate was born at 36 weeks of gestation from the mother with disseminated histoplasmosis. The mother had severe inflammatory bowel disease and received anti-tumor necrosis factor (TNF) monoclonal antibody every 6 weeks by 32 weeks of gestation. Histoplasma antigen was detected in the umbilical cord, although the neonate was asymptomatic. Histoplasma antigen was detectable in the urine and serum at 3 weeks of age. The patient was treated with amphotericin B and itraconazole. Interestingly, histological findings of the placenta were normal, and no fungal forms were detected by Grocott-Gomori methenamine-silver staining (Carlucci et al., 2016). Histoplasmosis was also reported in another infant that developed fever and rash at 4 weeks of age and was diagnosed with disseminated histoplasmosis. HIV test was positive for both the infant and the mother. Further, the mother showed elevated urine histoplasma antigen level. The authors suspected vertical transmission (Carlucci et al., 2016). However, there is no evidence that the infant had histoplasmosis at birth, and there is no description of placental pathology.

Zygomycosis

Zygomycosis is caused by fungi of the class *Zygomycetes* consisting of the orders *Mucorales* and *Entomophthorales*. *Rhizopus*, *Mucor*, and *Lichtheimia* are the most common fungi of this group (Petrlikos et al., 2012). There are two reported cases of placental zygomycosis. In the first case, the neonate delivered vaginally had respiratory failure but no signs of infection and could recover. The mother had habit of smoking cocaine and marijuana. Placental pathology showed broad, ribbonlike, and aseptate fungal hyphae consistent with morphology of *Zygomycetes* in the chorionic plate and the umbilical vessels without inflammation. Culture was not performed (Kambham et al., 1998). Another case involved a stillborn case at term. The mother had no comorbidity. Numeral fungal hyphae consistent with the morphology of *Zygomycetes* were noted in the villi, membranes, and umbilical cord without inflammatory cells. The dead fetus had no signs of infection upon microscopic examination (Marcorelles et al., 2005). As both cases showed no inflammation in the placenta or signs of infection in the fetus or neonate, the clinical importance of placental zygomycetes is unclear.

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Oral and Dental Infections: Bacteria

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Oral health

The attention to oral health dates back to thousands of years ago, with written evidence of a dentistry job recovered from a 4600-year-old Egyptian tomb and the use of basic dental practices dating back to the Ancient Greeks (Blackburn, 1977). Throughout the centuries, both the field of dentistry and the importance of oral health experienced a constant growth, reaching new heights with the discovery of the oral microbiome and its effects on multiple diseases and body niches. A clear example of the importance of oral health can be given by its impact on human health and the ensuing economic burden. Despite the difficulties, research efforts from the past two and a half decades have yielded some of the first comprehensive studies based on Big Data science regarding the impact of oral diseases in global health, as depicted in the first global burden of disease (GBD) epidemiological reports (Marcenes et al., 2013; Kassebaum et al., 2015). These reports showed that at least 3.5 billion people are affected worldwide by some of the most common oral diseases (untreated dental caries, severe periodontitis, and edentulism) (Kassebaum et al., 2017). According to the newest GBD report from 2019, the morbidity and mortality of oral disorders (including lip and/or oral cancer) accounted for a global median loss of 28,641,313.63 years of healthy life due to early death and disability, known as Disability Adjusted Life Years (DALYs) (IHME GBD, 2019). This means that oral disorders represent 1.13% of the total global DALYs for 2019, a 1.82-fold increase compared to data from 1990. This last number is worthy of notice since it depicts a result of what is regarded as the “Global Epidemiological Transition” (The Lancet, 2016). This concept aims to describe how the health problems transition in economically developed states and result in the prevalence of non-communicable and chronic diseases, such as oral disorders, in comparison to communicable diseases (which have decreased their impact on morbidity and mortality from 31.7% in 1990 to 15.59% in 2019, a 2.03-fold decrease) (IHME GBD, 2019). Moreover, it is estimated that the economic burden to treat dental diseases (including periodontal disease) is approximately USD\$298 billion per year, which is equivalent to an average of 4.6% of the global health expenditure per year (Listl et al., 2015).

Oral microbiome

The term “microbiome” describes the collection of commensal, symbiotic, and pathogenic microorganisms inhabiting our bodies, with an untold and underestimated potential for human health and diseases (Lederberg and McCray, 2001). As there are almost as many microbiome communities as there are body niches suited to their growth, there are several different types of human microbiomes, with potentially more to discover (Scotti et al., 2017). One of the most important microbiomes is the oral one, and the discovery of some of its members coincides with the discovery of bacteria by Antony van Leeuwenhoek in 1676, with a self-constructed microscope (Yamashita and Takeshita, 2017). The human oral microbiome comprises all the microorganisms that are found on or in the human oral cavity and its contiguous extensions, stopping at the distal esophagus (Dewhirst et al., 2010). The

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oral cavity is the gatekeeper of the human body and it is the second largest microbial habitat in humans, colonized by one of the most diverse communities of microbes, including viruses, bacteria, fungi, archaea, and protozoa (Huttenhower et al., 2012; Li et al., 2012). The mouth is, in fact, uniquely suited to microorganisms, being a warm and moist environment perfect for the growth of different kinds of microbes (Van'T Hof et al., 2014) and providing non-shedding surfaces that can help microbial colonization, development of thick biofilms, and microbial persistence (Marsh and Devine, 2011). However, as humans evolved countermeasures against oral bacterial colonization, the mouth can also be hostile to microbial life, resulting in only a subset of microbes entering the oral cavity being able to survive and colonize this niche (Marsh and Devine, 2011) on few sites such as dental surfaces, cheek, hard palate, tongue, and saliva. The microorganisms differ significantly on these different oral habitats, which show distinct microbial profiles (Papaioannou et al., 2009). Interestingly, in healthy individuals, the dental samples from different surfaces display the highest diversity (Zaura et al., 2009). According to the Human Oral Microbiome Database (HOMD), a total of 619 taxa has been identified in the human oral cavity, only 65,6% of which have been cultured (Aas et al., 2005; Dewhirst et al., 2010). However, most of the oral microbiome is still under investigation, with some bacterial species not being officially named, or even not cultivated (Wilson, 2005), as of yet resulting in a compendium of only 700 bacterial species (Deo and Deshmukh, 2019).

The oral microbiome forms an ecosystem, which is responsible for maintaining oral homeostasis, protecting the oral cavity health and preventing disease development (Luftig, 2009; Welch et al., 2016; Deo and Deshmukh, 2019). However, when the ecosystem is imbalanced or ecological shifts occur in the microbiome, pathogens can seize the opportunity to cause infection and diseases (Avila et al., 2009; Deo and Deshmukh, 2019). These changes in the microbiome composition, collectively known as dysbiosis, can lead to oral diseases such as oral cancer (Zhao et al., 2017), and systemic diseases such as cardiovascular diseases (Beck and Offenbacher, 2005), metabolic diseases, and autoimmune disorders (Cho and Blaser, 2012; Muszer et al., 2015; Levy et al., 2017). The definition of dysbiosis as an imbalance in the microbiome profile and a change from its healthy, eubiotic, stage props the question of the existence of a common healthy microbiome. Although oral microbiomes in different people show some diversity, the major portion of the oral microbial community was found to be shared across unrelated healthy individuals (Zaura et al., 2009). This supports the concept of a core microbiome common to all individuals, albeit affected by age, unique lifestyle, genotypic differences, host genetics, and diet habits (Zarco et al., 2012; Deo and Deshmukh, 2019). At the time of birth, the human oral cavity represents an essentially microbe-free niche that becomes colonized within hours by maternal bacteria (Dominguez-Bello et al., 2010). With the host's growth, the oral microbiome changes and the number and diversity of periodontal pathogens in it increases, showing a pattern of colonization in children similar to that previously found in adults, resulting in higher susceptibility to oral diseases and a gradual maturation of the oral microbiota (Papaioannou et al., 2009; Lira et al., 2018). The juvenile population, instead, was observed to present a significant increase in bacterial species compared to adults (Lira et al., 2018).

The different European, African, Asian, and American populations present different oral microbial compositions. For instance, the saliva microbiomes of Alaskans, Germans, and Africans displayed a significant degree of diversity between and within individuals (Li et al., 2014a). Furthermore, two Caucasian and African American populations from the United States, which shared common food, nutritional and lifestyle heritage demonstrated significant microbial differences in the oral cavity, showing that host genetics is also very important for oral microbiome composition (Mason et al., 2013). Nonetheless, focusing on the evolution and changing ecology of the human oral microbiome, the core microbiome of primarily biofilm structural taxa has been maintained throughout African hominid evolution, but strain-level differences are also indicated within the core taxa, according to reconstructions of oral metagenomes from up to 100 thousand year ago (Yates et al., 2021).

Main oral diseases

A dysbiotic oral microbiome, or a specific pathogenic member of the oral microflora, can lead to the development of oral diseases, which can vary greatly from oral infections to cancer. With a few exceptions, in fact, most oral ailments are microbial in nature, or have oral bacteria playing a role in their etiopathogenesis. This, coupled with the knowledge of the staggering economic burden caused by oral diseases, shows the importance of studies trying to uncover the molecular mechanisms and the microbial actors underlying the etiology of these diseases. The most important oral disorders, whose pathogenesis involves oral bacteria are tooth decay, oral cancer, periodontal diseases, and noma.

Tooth decay

Tooth decay, also known as dental caries, is one of the most common chronic diseases and the major cause for tooth loss worldwide. Caries is the deterioration of dental hard structures through acidic by-products of bacterial carbohydrate fermentation in a process called demineralization (Selwitz et al., 2007). It affects the majority of adults and 60–90% of school children, varying with age, geographic location, food habits, sex, and socio-economic status. Risk factors for the development of tooth decay include an insufficient fluoride exposure, high amounts of cariogenic bacteria, recession of the gingiva, as well as genetic and immunological factors and previous caries experience. The symptoms of tooth decay include toothache and sensitivity, especially when eating or drinking something cold, hot, or sweet, as well as a dark-brown or white staining of the tooth surface, visible pits or holes in teeth and bad breath, while complications may include tooth loss, discoloration, Ludwig angina and Cavernous sinus thrombosis, which may even be lethal (Yadav and Prakash, 2016). The most common diagnostic method is the visual examination of the teeth and may include the use of a dental probe (Selwitz et al., 2007). Caries is initiated by changes in the bacterial composition of the dental biofilm (plaque). With the thickening of the biofilm and the increased production of exopolysaccharides, the saliva is unable to

buffer the decrease in pH caused by the bacteria and cannot therefore enhance the re-mineralization of the tooth structure, leading to disease progression (Baker and Edlund, 2019). Disease evolution is slow in most patients since it can be halted at any moment for prolonged periods of time. Several bacterial genera are involved in tooth decay, most of which are facultative or strict anaerobes. The main species associated with dental caries are *Streptococcus mutans* and other mutans streptococci, lactobacilli, *Bifidobacterium* spp., and *Actinomyces* spp. (Selwitz et al., 2007). Cariogenic biofilms change their bacterial composition over time, depending on disease progression and depth of the lesions (Yadav and Prakash, 2016; Lamont et al., 2018). The interplay between acidogenic bacteria and species able to tolerate such a decrease in pH is key for the establishment of caries and its progression. In particular, *S. mutans* has been found to play a major role in this transition. The bacterium, in fact, is able to decrease surrounding pH, resulting in toxicity for certain microorganisms and a shift in the types of species growing on the tooth. Therefore, *S. mutans* can be seen as a keystone species involved in the caries biofilm and matrix formation (Lamont et al., 2018). Mutans streptococci and other cariogenic bacteria may be acquired via vertical and horizontal transmission routes, for example from a mother to her child. Under healthy conditions and with a diet low in fermentable carbohydrates certain bacterial commensals, especially Mitis streptococci, are able to outcompete cariogenic species and keep homeostasis. Apart from bacteria, *Candida albicans* has also been found to initiate severe caries *in vivo* when associated with cariogenic bacteria, especially *Streptococcus mutans* (Selwitz et al., 2007; Lamont et al., 2018).

The progression of tooth decay is influenced by factors such as lifestyle, including (dietary) fluoride exposure, oral hygiene, the consumption of fermentable carbohydrates, as well as salivary composition and flow (Yadav and Prakash, 2016; Baker and Edlund, 2019). Caries can cause cavitation in the crown or the root of the tooth (coronal and root caries). It may affect the enamel (the outer layer of the crown), the cementum (the outer layer of the root), or the dentin (lying below cementum and enamel) and may lead to dentin exposure (Selwitz et al., 2007).

Management of caries in most countries mainly involves restorative treatment, regardless of its limitations and tendency to create the need for further treatment. Restorations usually have a short durability and caries may form around the reconstructed areas. Relapse occurs in 40% of all cases after the first year (Alazmah, 2017). Preventive care is therefore increasingly being applied due to its high effectiveness, especially in Scandinavian countries (Mjör and Toffenetti, 2000; Selwitz et al., 2007). The prevention of tooth decay involves lifestyle and nutritional changes such as the improvement of oral hygiene and health education programs, as well as the removal of biofilm (plaque) and the application of fluoride, and the use of sealants for tooth protection (Bader et al., 2001; Selwitz et al., 2007). According to a 2015 Cochrane study, one of the most effective ways to prevent caries has been found to be the self-application of fluoride toothpaste (Marinho et al., 2015).

Early childhood caries is a serious and destructive form of tooth decay affecting the primary teeth of young children worldwide, with a higher incidence in families with poor economic conditions (Çolak et al., 2013). It may start at the eruption of primary teeth and occurs up to the age of 5. With its high incidence amongst childhood chronic conditions, early childhood caries highly influences the child's well-being by causing bacteremia, inflammation, tooth loss, and pain if left untreated. It may also lead to a decrease in confidence of the child, as well as speech disorders and growth alterations (Alazmah, 2017). Affected individuals have a much greater probability of developing caries in adulthood (Çolak et al., 2013). Early childhood caries initiates as white spots close to the gingival margin, usually on anterior teeth and maxillary molars, and may destroy the crown if it remains untreated. In severe cases, maxillary teeth may be completely destroyed and the disease might spread to the lower teeth (Selwitz et al., 2007). An important risk factor apart from socioeconomic status and dietary sugar intake are feeding practices such as the improper use of baby bottles. The prolonged exposure to lactose due to a longer bedtime use of bottles, in fact, represents a major risk factor for early childhood caries. Tooth extraction is the primary way of treating early childhood caries, which may however result in further orthodontic problems, especially upon removal of molars (Çolak et al., 2013).

Oral cancer

The term oral cancer refers to several malignant oral tumors, 90% of which are oral squamous cell carcinoma (OSCC), the most common oral carcinoma, which develops from the mucosal lining of the oral cavity including the anterior part of the tongue (Bagan et al., 2010). Approximately, 450,000 individuals are diagnosed with oral cancer worldwide every year, specifically with a 1.7% incidence in men and a 0.71% incidence in women. Notably, the five-year survival rate is only 50% (Marsh et al., 2011), mainly due to the poor prognosis in the early stages. Furthermore, studies have shown individuals at high risk for developing recurrent oral cancer after survival of the first oral carcinoma (Alfano and Horowitz, 2001). The epidemiological studies in the past have established certain risk factors for cancer, including diet, tobacco use, age, genetic factors, inflammation, and viral infections (Perera et al., 2016). OSCC is of multifactor origin, where several factors act either individually or in combination to lead to pathogenesis. The chief risk factors of OSCC are smoking (with or without tobacco) and alcohol consumption, which accounts for a risk of 75% for the population to develop oral cancer (Markopoulos, 2012). Additionally, viral (human papillomavirus) infections are another major risk factor for OSCC, leading to increased incidence of cancer in the younger population and among female non-smokers (Bleyer, 2009). Surprisingly, these successful studies led to believe that there was no room left for any other causative agent, such as bacteria, to be instrumental in causing cancer development. However, studies in the 1990s established *Helicobacter pylori* to be the first bacterial species to be associated with gastric cancer and, since then, supporting research has linked microbial communities in the oral cavity with various types of cancers. Two oral bacteria, *P. gingivalis* and *F. nucleatum*, have also been linked to pancreatic cancer and colorectal cancer (CRC), respectively, but their connection with OSCC has been investigated more in depth. In particular, these bacteria might cause dysbiosis and dysregulated immune responses resulting in periodontal diseases and periodontitis, but they can also play a role in the pathogenesis of OSCC (Fitzsimonds et al., 2020).

Oral microbes can cause oral oncogenesis via several mechanisms, including inhibition of apoptosis, induced cell growth, host cell invasion, provoked inflammatory responses and production of carcinogens. Dysregulated inflammation is a key factor for modulating a tumor microenvironment. Both oral pathogens *P. gingivalis* and *F. nucleatum* survive intracellularly within epithelial cells and their persistence causes systemic spread of the disease and disrupts the immune functions (Han and Wang, 2013). A study, in fact, showed *F. nucleatum* recruiting tumor-infiltrating immune cells for the progression of CRC in an Apc^{Min/+} murine model (Kostic et al., 2013). In contrast, *P. gingivalis* can elicit both pro- and anti-inflammatory responses, thereby disrupting immune functions in the oral cavity (Lamont and Jenkinson, 1998; Takeuchi et al., 2013). However, both *P. gingivalis* and *F. nucleatum* impinge upon several aspects of epithelial cell signaling that have relevance to cancer progression.

Apoptosis inhibition

Studies have shown *P. gingivalis* to inhibit apoptosis in primary gingival epithelial cells (GECs) (Nakhjiri et al., 2001). This periodontal organism stimulates the JAK1/STAT3 and PI3K/Akt signaling pathway, which regulates pathways associated with mitochondrial apoptosis (Yilmaz et al., 2004; Mao et al., 2007). In the mitochondria, reduced release of cytochrome c is induced due to decreased activity of BAD (BCL-2-associated death promoter, proapoptotic) causing an elevated expression of BCL2 (antiapoptotic) (Yao et al., 2010). This affects the downstream genes where caspase-9 and -3 are blocked (Mao et al., 2007; Yao et al., 2010). Additionally, *P. gingivalis* induces microRNA-203 in GECs, which results in the activation of STAT3 (signal transducer and activator of transcription 3), thus further leading to inhibition of apoptosis (Moffatt and Lamont, 2011). *P. gingivalis* is also known to secrete a nucleoside diphosphate kinase (NDK), which also inhibits ATP-dependent apoptosis in gingival epithelial cells (Yilmaz et al., 2008). A study in a murine model showed that co-infection with *P. gingivalis* and *F. nucleatum* leads to oral cancer development via induction of the IL6/STAT3 pathway (Gallimidi et al., 2015).

Cell proliferation induction

P. gingivalis accelerates the growth of gingival epithelial cells through the S and G2 phases of the cell cycle. This is caused by activation of cyclins (A, D, E) and phosphorylation of CDKs (cyclin-dependent kinases) and further reduces expression of p53 (Kuboniwa et al., 2008; Pan et al., 2014). Adhesins are chief factors in inducing cell proliferation, as a mutant of *P. gingivalis* lacking the fimbrial gene *fimA* does not modulate CDK activity (Kuboniwa et al., 2008). Its LPS also plays a crucial role in dysregulating p53 (Whitmore and Lamont, 2014). Additionally, *P. gingivalis* also activates β -catenin, leading to a proliferation in GECs (Zhou et al., 2015). On the other hand, *F. nucleatum* infection results in the induction of kinases involved in cell growth, cell survival, and DNA repair signaling pathways (Uitto et al., 2005). *F. nucleatum* also activates the β -catenin pathway via the FadA adhesion protein, which binds to E-cadherin in colon cancer cells, thus leading to downstream activation of oncogenes, the Wnt pathway and proinflammatory responses, and subsequent cell proliferation (Rubinstein et al., 2013).

Cell invasion

Both periodontal pathogens *P. gingivalis* and *F. nucleatum* invade OSCC cells. During infection in OSCC, *P. gingivalis* increases the production of pro-matrix metalloproteinase-9 (pro-MMP-9) by activating signaling pathways (ERK1/2-ETS1, p38/HSP27, PAR/NF- κ B), and subsequently the active MMP-9 is produced after the proenzyme is cleaved by gingipains (Inaba et al., 2014, 2015). MMP-9 causes degradation of the basal membrane and extracellular matrix, which further enhances cellular invasion thereby causing the dissemination of cancer cells into blood and lymph for metastasis at systemic sites (Inaba et al., 2014). This causes *P. gingivalis* to stimulate tumor invasion in OSCC. Additionally, continuous exposure to the pathogen also induces the expression of MMP-1 and MMP-10 in OSCC, causing enhanced invasion (Ha et al., 2015). Likewise, *F. nucleatum* infection in the host epithelium upregulates the expression of MMP-13 via the activated p38 MAPK, thereby promoting cell migration (Uitto et al., 2005).

Inflammatory responses modulation

Inflammation induced by either infections or environmental stress is instrumental in different stages of cancer. The initiation of oncogenesis is mediated by ROS, RNS, and cytokines produced by the host cells thereby triggering genome instability and gene mutations that lead to proliferation, invasion, and metastasis (Perera et al., 2016). Proinflammatory cytokines cause the activation of STAT3 and NF- κ B transcription factors, which subsequently lead to a tumor-induced inflammatory environment (Grivennikov et al., 2010; Landskron et al., 2014). Chronic inflammation is the major pathway by which periodontal pathogens cause oral cancer. For instance, *F. nucleatum* induces high inflammatory levels in CRCs indicating a positive correlation to further cancer progression (Kostic et al., 2013). Moreover, *P. gingivalis* enhances the expression of B7-H1 and B7-DC receptors in OSCC, thereby contributing to inflammation (Groeger et al., 2011). B7-H1 expression, in turn, causes T-regulatory cell development, which further reduces the functioning of effector T-cells, contributing to immune disruption by oral oncogenesis. The induction of cytokines, such as IL-1, IL-8, and TNF- α due to *P. gingivalis* infection are key to oral carcinogenesis (Andrian et al., 2004). A similar inflammatory phenotype is also induced by other oral pathogens like *Streptococcus anginosus* and *Streptococcus mitis* (Yumoto et al., 2001; Narikiyo et al., 2004).

Carcinogen production

Acetaldehyde is categorized as a group I carcinogen since it causes point mutations, epithelial proliferation, and DNA adducts (Seitz et al., 1994). Certain oral bacteria like *Streptococcus salivarius*, *Streptococcus intermedius*, *S. mitis*, and oral *Candida* spp. harbor alcohol dehydrogenases, which lead to the production of acetaldehyde, causing mutations in the host (Kurkivuori et al., 2007).

Treponema denticola is also associated with OSCC, esophageal squamous cell carcinoma, as well as colorectal cancers (Landskron et al., 2014). *T. denticola*, in fact, secretes dentilisin (Fenno, 2012), which is primarily known for its proteolytic characteristics and is associated with early stages of cancer, enhanced tumor invasion, and tumor size (Listyarifah et al., 2018). Dentilisin also causes degradation of cytokines such as IL-8 and TNF α and cleaves inactive forms of MMP8 and MMP9 into their active forms, creating a proteolytic micro-environment for better cell invasion (Jo et al., 2014; Nieminen et al., 2018).

Periodontal disease

Evolution is driven by natural selection, where variable genetics and variable environments interact together, determining immunological phenotypes. Infections historically have been the major determinants of selection, favoring variability of the immune system. The major histocompatibility region (MHC) specifically, is one of the most variable genetic regions in many species (Parham, 2018) with the genes coding for T and B cell receptors (Han et al., 2014) providing variants for immune defense. It follows that different combinations of genes, environmental factors, and the exposure to pathogens may be active in different subsets of diseases. An example of multifactorial chronic inflammatory illness is periodontal disease (PD), which affects 10–15% of the human population. This disease usually shows few symptoms over many years, which are often not perceived or correctly classified by the patient. Therefore, awareness and recognition of an unhealthy periodontal condition are essential to prevent the disease's onset. A healthy periodontium is described as a state free from inflammation, and it is characterized by shallow pockets and the absence of gingival bleeding (Mariotti and Hefti, 2015).

Periodontitis can be classified as either aggressive or chronic periodontitis. The first type is related to young individuals presenting recent periodontal breakdown and unknown time lapse between the biological start and the clinical evidence of the disease. Chronic periodontitis affects adults which, in 85% of the cases, show no progression even after treatment (Lindhe et al., 1983).

A healthy periodontal ecosystem is established by the relationship between the virulence factors of periodontal pathogens and the tightly regulated immune responses of the host. Innate and adaptive cellular responses, together with the host's indigenous oral microbes, play in fact an important role in the prevention of pathogen invasions (Hajishengallis, 2015). To counteract the host immune defenses, periodontopathic bacteria (Hajishengallis, 2015) have evolved a range of mechanisms to circumvent the host immune reactions (Contreras et al., 2014). At the start of periodontitis, bacterial antigens attach to Toll-like pattern recognition receptors on the host immune cells. These receptors activate signaling transduction pathways which, in turn, trigger immune and inflammatory responses to eliminate invading pathogens. Next, the activated lymphocytes differentiate into subtypes. A prolonged inflammatory host response, however, might also introduce counter effects such as T- or B-cell dysregulation, DNA damage, cellular senescence, and oxidative stress, which then can potentially cause immunodeficiency and opportunistic infections. To control the latter, host-destructive immune reactions, regulatory T-cells, and noncoding RNA regulation serve to restrain or abrogate excessive inflammation, autoimmunity, and immunopathology (Kebschull and Papapanou, 2015).

Traditional periodontal therapy was originally focused on eliminating gingival bleeding and controlling patients' plaque. However, this goal was hard to achieve in practice, and modern treatments are more focused on reducing all the undesirable clinical symptoms. This goal can be achieved by treating the periodontal disease as an opportunistic infection and reducing the predisposing factors with a continuous assessment and supportive periodontal care. As periodontitis is a multifactorial disease, innovative approaches for its treatment consider an individual's genes, environment, and lifestyles. Thus, patients can be classified into diverse groups and customized clinical treatments can be established (Barros et al., 2016). One current sophisticated diagnostic test for periodontal disease is based on a proteomic or genomic assessment of body fluid such as saliva and gingival crevicular fluid (Taylor and Preshaw, 2016). Additionally, due to its complex multifactorial status, periodontitis' clinical forms and outcomes are a result of the individual's host inflammatory response. Accordingly, when diagnosing, treating, and managing periodontal diseases, the individual's inflammatory response must be considered (Giannobile et al., 2013). Today, personalized periodontics exploits modern technologies to allow the assessment of individual risks based on the analysis of biological responses, and other patient factors (Razzouk and Termechi, 2013).

Among the environmental factors influencing periodontal disease, tobacco, alcohol, medications, diabetes, obesity, malnutrition, and psychological stress are to be considered in personalized approaches for disease management (Sabbah et al., 2018). Tobacco has shown adverse side effects on periodontitis, which are associated with damage of periodontal ligament cells, an acceleration of the alveolar bone loss, and negative remodeling of alveolar bone during implantation and orthodontic treatment (Zhang et al., 2019b). Another recognized factor with an adverse effect on the periodontium is the use of drugs for both therapeutic and recreational purposes. The negative effects of drug (ab)use on the periodontium are well-documented and include drug-induced gingival overgrowth, increased gingival bleeding, and an altered inflammatory response (Bharti and Bansal, 2013).

Interestingly, many other systemic conditions are associated with poor periodontal health, and among them diabetes has been studied in detail (Borgnakke, 2016). The importance of prediabetes and poorly controlled diabetes on the periodontium has been explored, and periodontal treatment may assist in the management of prediabetes and poorly controlled diabetes and vice versa in the context of personalized periodontal care. Obesity has also a strong correlation with an increased risk for periodontitis. Specifically, studies analyzing the association between obesity and periodontal diseases indicate that obese individuals are more likely to have periodontal diseases, which can be more severe than in non-obese people. Diet and nutrition therefore have a strong impact on periodontal health and disease (Dommisch et al., 2018). In fact, certain micronutrients have an influence on biological functions, such as innate and adaptive immunity. Hence, in the context of personalized periodontics, an assessment of micronutrients intake is essential for the patients.

Another factor which has a strong impact on periodontal disease is stress (Sabbah et al., 2018). Frequent exposure to stressors leads to systemic effects and results in what is termed allostatic load. Therefore, the link of allostatic load and periodontal diseases is an important variable to consider in the field of personalized periodontics.

Aside from environmental factors, however, individual genetics has a considerable role in the manifestation of the disease (Michalowicz et al., 1991). PD forms or stages are also correlated to individual genetic susceptibility (Lauritano et al., 2015) and, therefore, the evaluation of genetic predisposition in the host-bacterial interaction may represent a crucial element to be considered for the disease's early detection, pathogenesis, and progression. Interestingly, transcriptome analyses on tissue samples have elucidated common pathways and molecular regulatory networks specific for distinct stages of PD (Sawle et al., 2016), and these insights may provide a new perspective on understanding PD pathogenesis as a response to plaque bacteria. Similarly to other common polygenic diseases, such as diabetes, coronary disease, or hypertension, the susceptibility to PD can be explained by the hypothesis that in the population there are some common disease-associated genetic variants or single nucleotide polymorphisms (Gibson, 2012). Genome-wide association analysis, therefore, represents an efficient means to identify the traits associated with the illness, reflecting different PD phenotypes at a genomic level (Zhang et al., 2016). In this regard, previous studies have confirmed the upregulation or downregulation of certain genes involved in inflammation or bone resorption (Kim et al., 2016). Specifically, two genetic variants have been implicated in the colonization of bacteria in biofilm (Nibali et al., 2014). The first variant is characterized by a single nucleotide polymorphism which may alter the function of genes in bacterial sensing and recognition such as receptor genes, or genes encoding binding. The second variant is represented by variations in genes that may lead to an excessive inflammatory environment in the subgingival niche, thus triggering the growth of certain biofilm bacteria. Notably, tissue breakdown leads to an excessive inflammatory response, which then feeds pathogenic bacteria orchestrating the biofilm formation (Moutsopoulos et al., 2014). Not by chance, immune responses and their pathways were found to be the most frequently enriched ones in the case of biofilm formation. For example, B-cell development, leukocyte extravasation signaling, phosphoinositide 3-kinase signaling in B lymphocytes, B-cell receptor signaling, and granulocyte adhesion and diapedesis were the most upregulated in periodontitis' gingival tissues once assessed with RNA sequencing (Kim et al., 2016). Moreover, an anticipatory upregulation or downregulation of some genes involved in inflammation or bone resorption has been confirmed (Langrish et al., 2005). For example, p38, c-Jun N-terminal kinase, and mitogen-activated protein kinase were identified as significantly upregulated in diseased tissues (Langrish et al., 2005). Also, an upregulation for chemokines, growth factors, tumor necrosis factor signaling, and cell adhesion molecules, was observed to be positively associated with the disease (Langrish et al., 2005).

Transcriptome studies also allowed scientists to identify novel molecules involved in epithelial function and barriers which appeared to be downregulated in the gingival tissues of patients affected by PD. As an example, the desmocollin 1 gene, expressed in epithelial cells and required for cell adhesion, was the gene most significantly downregulated. In addition, the expression of most keratin family genes, involved in the epithelial barrier, was significantly suppressed in diseased gingival tissue (Kim et al., 2016). Furthermore, transcriptome sequencing of gingival biopsies from chronic periodontitis patients has facilitated the identification of novel gene expression and splicing patterns. Specifically, downregulation of genes involved in the gingival or mucosal epithelial barrier may enable the easier penetration of pathogens (or their metabolites) into the connective tissue, thus leading to inflammation in the subepithelial region. It has also been noticed that molecules involved in the recruitment of neutrophils at the mucosal surface play a key role in the onset of inflammatory disease such as psoriasis, lupus erythematosus, and ulcerative colitis (Konig et al., 2015). Interleukin-17, primarily secreted by T helper 17 lymphocytes and involved in signaling in epithelial cells, for example, resulted in being upregulated in gingival tissues of periodontitis patients (Kim et al., 2016). This molecule plays a role against pathogen invasion by recruiting neutrophils but, if excessively secreted, leads to negative effects which may be related to inflammatory diseases.

Phenotypes, however, are not only determined by genetic sequences, but also by variable heritable factors which can influence gene expression with epigenetics being the emergent field of periodontology considering these heritable variations (Barros et al., 2018). In periodontitis, factors such as microbes became relevant in the evaluation of gene expression patterns. Recent modern technologies allowed analysis of the complex interactions between diseases and traits of epigenetic biomarkers, implying that they may serve to develop novel diagnostic and therapeutic agents in periodontal disease (Barros et al., 2018). Altogether, personalized periodontics offers an interesting move toward a new medical model of disease management, recognizing the individual as the principal component of complex diseases. An analysis of lifestyle, behavior, genetic factors, and how these interplay in periodontal diseases will probably become an integral part of everyday clinical practice. However, a considerable amount of work still needs to be undertaken to refine our diagnosis and treatment.

Bacteria use pathogen-associated molecular patterns and pattern recognition receptors to elicit inflammatory responses in different host cell populations (Poltorak et al., 1998). The bacterial lipopolysaccharide, for example, is a potent inflammation inducer by activating in the host the Toll-like receptor-4, a recognition receptor, which is found on the cell surfaces of macrophages or dendritic cells (Hawn et al., 2003). Other pathogen-associated molecular components, such as the bacterial cell wall or the flagellin protein of motile bacteria can be recognized by Toll-like receptor-2 and Toll-like receptor-5 on the host cell surfaces respectively and trigger inflammatory responses (L  e et al., 1986). However, as aforementioned, although bacteria in the plaque biofilm are responsible for initiating the inflammation, intrinsic host and environmental factors are then determinants in modulating the magnitude, duration, and extent of the periodontal inflammatory response (Sawle et al., 2016). The current periodontal microbiology theory proposes that infectious oral diseases are the result of a dysbiotic biofilm in the host, and 16S ribosomal RNA gene-sequencing technologies allowed the evaluation of the phylogenetic relation among oral bacteria. According to these studies, the notion emerged that bacterial diversity in subjects with PD is more consistent than in subjects with periodontal health (Maeda and Takeda, 2017), where health-associated species are not lost or replaced but are suppressed, and that PD is associated with shifts in the composition of species of the oral microbiota.

The future of periodontitis research is connected to the progress in biological sciences. Today's molecular technologies, such as real-time PCR, have already significantly enhanced our insights in the negative impact of pathogenic microbes on periodontal health (Slots, 2015). In particular, real-time PCR can be used to analyze the gene expression levels of inflammatory molecules such as cytokines. Other more advanced technologies, such as next-generation sequencing and metagenomics, have the potential for rapid identification and classification of pathogenic communities of periodontal viruses, bacteria, fungi, and parasites. Metagenomics can also be applied to classify the periodontal microbial communities and to run longitudinal studies on patients and control groups (Wang et al., 2013a). The understanding that shifts in the composition of the periodontal microbiome can cause disease-related perturbations in the periodontal ecosystem should allow the development of innovative methods for periodontal disease prevention. Thus, the identification of disease-related markers in oral biofluids may lead to the development of tools for the early detection of disease, treatment planning, the prediction of treatment response, therapeutic solutions, and personalized therapies (Jaedicke et al., 2016). This may be augmented by computerized algorithms. In the long term, novel diagnostic and therapeutic methodologies may even permit patients to self-detect and arrest periodontitis (Kornman et al., 2017).

Complexes in periodontitis

Periodontal bacteria in subgingival plaque were originally divided into different complexes, known as the Socransky complexes (Socransky et al., 1998). This classification reflects in part the succession of colonization by microbial species that ultimately form the dental biofilm that causes periodontitis. In fact, early bacterial colonizers are classified in the green complex (including *Eikenella corrodens*, *Campylobacter concisus*, and *A. actinomycetemcomitans* serotype "a"), the yellow complex (including *Streptococcus* species such as *S. mitis*, *S. oralis*, and *S. gordonii*) and the purple complex (including *Veillonella parvula* and *Actinomyces odontolyticus*) (Socransky et al., 1998; Sakamoto et al., 2005; Hajishengallis and Lamont, 2012). These bacteria are able to adhere to the pellicle on the tooth surface and form a basis for other bacteria to colonize the gingival sulcus. The Socransky complexes are also defined by other criteria including their association with the severity of the disease. In particular, the complexes that encompass periodontal pathogens are the orange and red ones. It is important to note that even though defined microbial complexes are a convenient concept that has been commonly adopted worldwide, this view has been challenged by newer studies on the microbiome in periodontitis (Hajishengallis and Lamont, 2012; Caselli et al., 2020). In fact, the increased use of advanced molecular-based technologies for the detection of bacteria, instead of the use of culture-based approaches only, has significantly advanced our understanding of the complexity of the periodontal microbiome in health and disease (Hajishengallis and Lamont, 2012; Bankur et al., 2014; Pérez-Chaparro et al., 2014; Ye et al., 2020). For instance, the distinction between pathogens and commensals is not as clear as previously thought, as it is also dependent on the interactions of bacteria with the host. This would explain the fact that red complex bacteria can be present, although in very low numbers, in healthy individuals (Griffen et al., 1998; Bankur et al., 2014; Carrouel et al., 2016). Moreover, a higher diversity and heterogeneity of the oral microbiome has been recognized recently. Among the newly identified members of the oral microbiome, there are new bacterial taxa that also correlate strongly with periodontitis, including Gram-positive bacteria which, contrary to Gram-negative bacteria, were thought to be less involved in periodontitis (Ye et al., 2020). Newly identified periodontitis-associated bacteria include species of the genera of *Bacteroidetes*, *Prevotella*, *Peptostreptococcaceae*, *Streptococcus*, *Pseudomonas*, and *Selenomonas* (Socransky et al., 1998; Pérez-Chaparro et al., 2014). Thus, it is likely that the species included in each complex might need to be redefined and subjected to possible alterations based on other variables, such as specific polymicrobial interactions and the host-microbiome crosstalk happening in the oral cavity (Yamada et al., 2005; Suzuki et al., 2013). Bacteria of the established periodontal complexes will therefore be grouped and discussed as such, mainly for the sake of convenience, with a clear focus on the more virulent bacteria belonging to the red, orange, and the so-called AA complex.

Red complex

The red complex is considered the climax bacterial community and includes *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, the most important pathogens involved in adult periodontitis (Socransky et al., 1998; Mohanty et al., 2019). These bacteria produce specialized virulence factors that mediate the efficient destruction of periodontal tissues and alveolar bone resorption. *T. denticola* is a small oral spirochete, which is strictly anaerobic and mainly located within the surface layers of the subgingival plaque (Dashper et al., 2011; Mohanty et al., 2019). Important *T. denticola* virulence factors include proteinases, peptidases, cytotoxic factors, flagella, and chemotaxis factors. Notably, its motility and chemotaxis are key for host colonization, its deep penetration into periodontal pockets, and invasion of host cells. Indeed, it has been shown that this bacterium is able to adhere and invade several host cells, including epithelial cells and gingival fibroblasts. Conversely, *T. forsythia* is a Gram-negative anaerobe which requires demanding growth conditions in the lab setting (Malinowski et al., 2019). Recent studies have shown that obese individuals are more likely to develop periodontal diseases due to colonization by this bacterium. Amongst *T. forsythia* virulence factors, cysteine-like proteases are involved in the host tissue damage and dysregulation of the host immune system. Other virulence factors include the protease carilysin and a serpin protein that interferes with several immune mechanisms protecting the bacterium (Malinowski et al., 2019). Interestingly, several studies using periodontitis animal models revealed cooperative interactions between these periodontopathogens, such as coaggregation and modulation of virulence factors (Yamada et al., 2005; Mohanty et al., 2019).

Among the members of the red complex, however, the most studied is probably *Porphyromonas gingivalis*, a Gram negative, non-motile, anaerobic bacterium. Originally a model for the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, *P. gingivalis*' importance grew concomitantly with the discoveries of its connection to periodontitis and to a plethora of non-oral diseases, such as

rheumatoid arthritis, atherosclerosis, diabetes, and, more recently, Alzheimer's disease. Additionally, *P. gingivalis* has also been associated with oral and pancreatic cancer, respiratory diseases, and nonalcoholic fatty liver disease. Periodontitis' connection with rheumatoid arthritis has been suspected since ancient times, but *P. gingivalis*' role in it, and the underlying molecular mechanisms, had only been recently discovered. *P. gingivalis*' peptidylarginine deiminase (PPAD) or citrullinating enzyme, in fact, appears to drive one of the molecular lynchpins of the association between the two diseases. Citrullination of certain human proteins by this bacterial enzyme is believed to elicit production of anti-citrullinated protein antibodies in the host (Mangat et al., 2010; de Smit et al., 2011; Farquharson et al., 2012; Quirke et al., 2014), potentially leading to rheumatoid arthritis development. Atherosclerosis has also been linked to periodontitis, with this oral disease being a potential risk factor (Humphrey et al., 2008) and its treatment being capable of reducing atherosclerosis biomarkers (Teeuw et al., 2014). This may be due to *P. gingivalis* specifically, as the pathogen was found in 82.61% of clinical samples (Xie et al., 2020), showing several pejorative effects in atherosclerosis pathogenesis (Li et al., 2002; Xie et al., 2020). Conversely, *P. gingivalis*' role in the etiology of diabetes revolves around its role in the induction of insulin resistance in the host. This is achieved through an increase in systemic inflammation, due to several mechanisms, including gut microbiota modulation (Arimatsu et al., 2014), and release of pro-inflammatory mediators (Pradhan et al., 2001; Le Sage et al., 2017; Seyama et al., 2020). Similarly, the connection between *P. gingivalis* and Alzheimer's disease chiefly lies in the pro-inflammatory capabilities of this bacterium. *P. gingivalis*, in fact, seems to be responsible, in mice, for neuroinflammation via cathepsin B expression (Wu et al., 2017), the activation of the TLR4/NF- κ B signaling pathway through its lipopolysaccharide (LPS) (Zhang et al., 2018) and, less directly, by causing the release of host inflammatory molecules such as TNF- α , IL-1, IL-6, and IL-8 (Amano et al., 2004). These inflammatory mediators could also increase blood-brain barrier permeability, further boosting *P. gingivalis*' capacity to cause neuroinflammation (Al-Obaidi and Desa, 2018), and cognitive damage. Additionally, this pathogen seems capable of hindering the host immune response, thereby furthering the inflammation environment that is paramount to Alzheimer's disease pathogenesis. Gingipains, the specialized cysteine proteinases of *P. gingivalis*, also appear to drive neuroinflammation through the proteolytic activation of protease-activated receptor 2 (Liu et al., 2017) and could potentially cause neuronal damage (Dominy et al., 2019).

P. gingivalis has also been associated with several cancer types, albeit with different levels of confidence, possibly due to the different depth of the research in each of these fields. There appears to be an inverse relation between *P. gingivalis* and the survival rate of esophageal cancer patients (Zhang et al., 2019a) and a direct relation between *P. gingivalis* antibody and disease severity (Gao et al., 2018). As a potential mechanism, *P. gingivalis* appears to stimulate tumoral cells and metastasis via the NF- κ B pathway (Meng et al., 2019) or miR-194/GRHL3/PTEN/Akt axis activation (Liang et al., 2020). The association of this periodontal pathogen with pancreatic cancer, instead, has been proposed after studies showed that high levels of serum antibodies against it were linked with a 2-fold increase in disease risk (Michaud et al., 2013) and *P. gingivalis* and *A. actinomycetemcomitans* are related to subsequent increased risk (Fan et al., 2018). Possible underlying mechanisms include expression of TLR pathway-associated genes (Shaik-Dasthagirisahab et al., 2015) and p53 activation (Inaba et al., 2018). Similarly to pancreatic cancer, *P. gingivalis* is associated with a higher risk of oral squamous cell carcinoma, with its presence in tumoral cells reducing patient survival (Wen et al., 2020). Several studies have delved into the potential mechanisms underlying this connection, as described in the "oral cancer" section of this chapter.

Respiratory diseases, such as pneumonia and chronic obstructive pulmonary disease, have also been connected to *P. gingivalis*. In the case of pneumonia, however, this bacterium might contribute to the disease development via interactions with other respiratory pathogens, such as the H1N1 virus (Chen et al., 2018) and *Pseudomonas aeruginosa* (Li et al., 2014b). Similarly, it is believed that the role played by *P. gingivalis* in the etiopathogenesis of chronic obstructive pulmonary disease also involves other pathogens, as their colonization of the airways appears to be facilitated by *P. gingivalis*-associated proteases (Vadiraj et al., 2013).

Lastly, the connection of periodontitis with nonalcoholic fatty liver disease prompted the analysis of an eventual connection between this disease and *P. gingivalis*, showing a higher presence of bacterial DNA in patients, as opposed to controls, (Yoneda et al., 2012) and disease worsening in several *in vivo* studies (Furusho et al., 2013; Nakahara et al., 2018; Sasaki et al., 2018; Nagasaki et al., 2020). Potential mechanisms explaining this connection, aside from the aforementioned ones inducing insulin resistance, include gut microbiome modulation and cytokine cascade induction (Alakhali et al., 2018).

The multifaceted damaging potential of *P. gingivalis* is certainly partly due to its vast array of virulence factors. Among them, the most infamous and best studied are LPS, gingipains, PPAD and fimbriae. LPS is a bacterial endotoxin comprising the conserved region lipid A, core oligosaccharide, and the variable O-specific polysaccharide (Schromm et al., 2000). *P. gingivalis* possesses two forms of LPS, the O-antigen polysaccharide O-LPS, commonly found among Gram-negative bacteria, and the anionic polysaccharide A-LPS. Notably, the latter anchors several virulence factors secreted by the type IX secretion system, such as gingipains and PPAD, to the outer membrane, thus becoming paramount to such proteins' expression of their virulence potential (Gabarrini, Heida, et al., 2018c). The LPS of *P. gingivalis*, however, is also a pro-inflammatory molecule, potentially contributing to neuroinflammation in Alzheimer's disease (Zhang et al., 2018), and to the pathogenesis of atherosclerosis, diabetes (Bhat et al., 2014; Le Sage et al., 2017), and nonalcoholic fatty liver disease (Furusho et al., 2013). Gingipains, on the other hand, are cysteine proteinases located on the outer membrane via A-LPS modification, in outer membrane vesicles (OMVs) and secreted in the outer milieu. They are either arginine-dependent (RgpA and RgpB) or lysine-dependent (Kgp) and are responsible for 85% of the proteolysis occurring outside the cell (De Diego et al., 2014). Gingipains have a potential role in the development of diabetes (Ilievski et al., 2017; Seyama et al., 2020) and Alzheimer's disease (Liu et al., 2017) in mice and rheumatoid arthritis (Wegner et al., 2010; Laugisch et al., 2016). PPAD is one of the most studied proteins of *P. gingivalis* in the context of the association between this bacterium and rheumatoid arthritis, as it is believed to play a major role in the etiology of this disease. This enzyme, which can

convert arginine into citrulline residues in a process termed citrullination, appears to be able to target specific human proteins, thus eliciting the rise, especially in genetically predisposed individuals, of autoantibodies involved in the pathogenesis of rheumatoid arthritis (Gabarrini et al., 2015). Notably, PPAD is the only peptidylarginine deiminase present in a human pathogen, one of the only three present in prokaryotes (Gabarrini, Chlebowicz, et al., 2018a), and it has no phylogenetic relation with mammalian PAD enzymes (Goulas et al., 2015; Gabarrini, Chlebowicz, et al., 2018a). Lastly, fimbriae are employed by *P. gingivalis* in adhesion and invasion processes, biofilm formation, and mobility (Enersen et al., 2013), and appear to be involved in every interaction this pathogen has with its host and other bacteria (Hamada et al., 1998). A class of extracellular polymers, fimbriae are proteinaceous structures stemming from the outer membrane and extending toward the extracellular milieu. *P. gingivalis* possesses two fimbriae: the long fimbriae, composed of FimA subunits, and the short fimbriae, consisting of Mfa1 subunits (Amano, 2010). While structurally different, both fimbriae contribute to periodontitis pathogenesis (Amano et al., 2010).

It is worth noting that most virulence factors of *P. gingivalis* are present in OMVs, nanostructures originated from blebbings of the outer membrane (Gabarrini, Medina, et al., 2018b). The vast majority of their cargo is composed of outer and periplasmic proteins (Ellis and Kuehn, 2010), but they appear enriched in virulence factors, especially gingipains (Veith et al., 2014). The encasing of virulence factors and highly efficient proteases in its OMVs, provides protection to this bacterium from its own highly proteolytic environment. Considering the other functions of *P. gingivalis* OMVs, such as biofilm formation and protein secretion, as they were shown to possess invasive capabilities (O'Brien-Simpson et al., 2009; Xie, 2015; Gui et al., 2016) and were capable of entering host epithelial cells and degrade key receptor proteins via gingipains (Furuta et al., 2009a, b; Zhu et al., 2013), they should be considered an additional and efficient mechanism of delivery of virulence factors, contributing to the already high damaging potential of this oral pathogen.

Orange complex

Orange complex bacteria include many periodontal pathogens such as *Fusobacterium nucleatum*, *Campylobacter rectus*, *Campylobacter gracilis*, *Eubacterium nodatum*, *Prevotella intermedia*, *Prevotella nigrescens*, and *Parvimonas micra*. In the case of *F. nucleatum*, it is known that this bacterium acts as a bridging species linking the early colonizers with late colonizers, which are often highly pathogenic and are classified in the red complex (Diaz et al., 2002; Suzuki et al., 2013). A characteristic that makes *F. nucleatum* a key organism in the formation and maturation of oral biofilms is the adhesin-rich outer membrane, which allows adhesion to other microorganisms and host cells (Thurnheer et al., 2019). Interestingly, the metabolism of the orange complex bacteria creates appropriate living conditions for the red complex bacteria which are strict anaerobes (Diaz et al., 2002; Bourgeois et al., 2019). For instance, *C. rectus* produces protoheme, which enhances the growth of the red complex bacterium *P. gingivalis* (Aruni et al., 2015). Moreover, orange complex bacteria are responsible for the increase in pocket depth and are the most abundant bacteria of the interdental biofilm.

Arguably the most notorious member of the orange complex, *Fusobacterium nucleatum* is a strictly anaerobic Gram-negative bacterium which is one of the most abundant species in the oral cavity (Socransky et al., 1998; Yang et al., 2014). *F. nucleatum* plays an important role in development and progression of oral diseases, such as periodontitis and gingivitis (Saygun et al., 2011), and it is also one of the most prevalent species found in extra-oral infections (Han, 2015). *F. nucleatum*, in fact, plays a role in diseases such as infective endocarditis (Shammas et al., 1993), pneumonia (Nagaoka et al., 2017), neonatal sepsis (Wang et al., 2013b), colorectal cancer (Brennan and Garrett, 2019), and Lemierre's syndrome (Brook, 2013).

F. nucleatum subspecies are very heterogeneous, especially regarding their virulence properties, thus the potential for different diseases varies among subspecies of *F. nucleatum* (Conrads et al., 2002; Henne et al., 2018). Originally, *F. nucleatum* was divided into five subspecies: *vincentii*, *animalis*, *nucleatum*, *polymorphum* and *fusiforme*. According to genetic analyses, the *fusiforme* subspecies was absorbed into the *vincentii* clade (Kim et al., 2010).

F. nucleatum subsp. *animalis* is the most dominant subspecies associated with human colorectal cancers (Ye et al., 2017), while *F. nucleatum* subsp. *polymorphum* is the most prevalent subspecies in the human oral cavity (Henne et al., 2018). *F. nucleatum* was initially found in overabundance in colorectal cancer tissue using metagenomics in two studies (Castellarin et al., 2012; Kostic et al., 2012). Subsequently, more and more reports indicated that *F. nucleatum* played a key role in the acceleration of development and progression of colorectal cancer (CRC) (Flynn et al., 2016; Yamamura et al., 2016; Casasanta et al., 2020).

Although the microbial translocation route of *F. nucleatum* is not clear, it is well accepted that the bacterium may originate from the oral cavity and that it results abundantly in CRC (Brennan and Garrett, 2019; Komiya et al., 2019). One report showed that more than 40% of CRC patients exhibited identical strains of *F. nucleatum* in their CRC and saliva specimens, suggesting the oral-gastrointestinal route as one possible route for translocation (Komiya et al., 2019). Another study showed that an *Apc*^{Min/+} murine infection model, which was fed with *F. nucleatum*, developed enhanced intestinal tumors, thus strengthening this hypothesis (Kostic et al., 2013). However, recently, a study reported that the intravenous route of oral fusobacteria to CRC is more efficient compared to the gastrointestinal route (Abed et al., 2020). A previous study also demonstrated that *F. nucleatum* localized to colorectal tumors via transient physiologic bacteremia (Wilson et al., 2008), while yet another study suggested that *F. nucleatum* can translocate from the mouth to CRC tissues via multiple routes (McCoy et al., 2013).

A better understanding of the mechanisms underlying the role played by *F. nucleatum* in CRC carcinogenesis could develop novel therapeutic strategies for CRC patients. One group demonstrated that *F. nucleatum* can stimulate the proliferation of CRC cells. In the first study, in fact, they showed that FadA, the adhesin and virulence factor of this bacterium, can bind E-cadherin, activate β -catenin signaling, and increase the expression of Wnt genes and inflammatory genes, stimulating the proliferation of CRC cells (Rubinstein et al., 2013). In a later study, the authors showed that ANXA1, either a tumor suppressor or promoter, was activated by *F. nucleatum*, thus selectively stimulating the growth of CRC cells (Rubinstein et al., 2019). Additionally, this bacterium can generate

a proinflammatory microenvironment through recruitment of tumor-infiltrating immune cells, thus promoting tumor cell proliferation (Kostic et al., 2013). Yet, another important mechanism is based on bacterial enrichment. Metagenomic analyses, in fact, demonstrated that *F. nucleatum* localized to and was enriched in human CRC cells and recruited multiple other bacterial species playing a role in the development and progression of CRC (Mitsuhashi et al., 2015). This was possibly due to the binding of *F. nucleatum*'s Fap2 lectin to the Gal-GalNAc expressed by tumor cells (Abed et al., 2016) and to the binding of fusobacterial adhesins, such as RadD (Guo et al., 2017), Aid1 (Kaplan et al., 2014), FomA (Liu et al., 2010), and CmpA (Lima et al., 2017) to other bacterial species such as *P. gingivalis* and *Streptococcus* spp. (Abed et al., 2016). The third mechanism underlying the involvement of *F. nucleatum* in CRC carcinogenesis is based on antitumoral immune evasion. *F. nucleatum*-bound tumors, in fact, are protected from NK-mediated killing and immune cell attack via an interaction between Fap2 and the immune cells' inhibitory receptor TIGIT (Gur et al., 2015). Fap2 was shown to be also involved in *F. nucleatum*-mediated hemagglutination with the hemagglutination potency of Fap2 directly correlated with TIGIT inhibition (Gur et al., 2015). The role of this bacterium in CRC chemoresistance is a prime target for investigation since a deeper understanding of it could easily optimize current treatment. A study reported that autophagy-related pathways are enriched and activated in CRC patients with a high amount of *F. nucleatum*, and that *F. nucleatum* promotes CRC chemoresistance via selectively targeting specific miRNAs and autophagy elements. The ULK1 and ATG7 pathways, in fact, were reported to be activated by *F. nucleatum*, which also caused a selective target loss of miR-18a* and miR-4802, thus this activation of autophagy pathways results in a modulation of CRC chemoresistance *in vitro* and *in vivo* (Yu et al., 2017).

Orange-associated complex and green complex

The bacteria associated with the orange complex are also considered "moderate risk" pathogens of the oral cavity. These bacteria consist of two Firmicutes species and three Proteobacteria, namely *Eubacterium nodatum* (Clostridiales) and *Streptococcus constellatus* (Lactobacillales), and *Campylobacter rectus*, *Campylobacter showae*, and *Campylobacter gracilis* (Campylobacteriales), respectively (Socransky et al., 1998). The green complex, on the other hand, is composed of "early colonizers" of the oral cavity. This complex includes one species of Proteobacteria named *Eikenella corrodens* (Neisseriales) and three Bacteroidetes from the genus *Capnocytophaga* (Flavobacteriales), which include *Capnocytophaga gingivalis*, *Capnocytophaga ochracea*, and *Capnocytophaga sputigena* (Socransky et al., 1998).

AA-complex

The AA-complex comprises the bacterium *Aggregatibacter actinomycetemcomitans*. *A. actinomycetemcomitans* is a Gram-negative, facultative anaerobic, capnophilic bacterium with non-motile and non-sporulating qualities (Henderson et al., 2010; Gholizadeh et al., 2017). *A. actinomycetemcomitans* is associated with both oral and non-oral infections. Localized aggressive periodontitis (LAP) in adults and juveniles is an oral infection associated with this bacterium (Slots et al., 1980), which results in loss of connective tissue and of alveolar bone (Krueger and Brown, 2020). Therefore, the presence of *A. actinomycetemcomitans* can act as an indicator for the initiation of LAP (Slots et al., 1980; Fine et al., 2007). Soft tissue abscesses, osteomyelitis, and endocarditis are the non-oral infections linked with *A. actinomycetemcomitans*, of which the latter one is the most common (Van Winkelhoff and Slots, 1999).

The known isolates of this bacterium can be divided into seven serotypes (a–g). The differences between these serotypes relate to the structural differences in lipopolysaccharide O-antigens, also called specific polysaccharide antigens (SPAs) (Perry et al., 1996; Fujise et al., 2008; Johansson et al., 2017). Other structural differences are evident in the division between rough and smooth phenotype. Fresh isolates of the bacterium display the rough phenotype, which results in transparent colonies and is characterized by fimbriae on the surface and a star-shaped colony structure. In the smooth phenotype, the fimbriae and star-shaped colony structure have disappeared, and the colonies are opaque. This conversion from rough to smooth occurs spontaneously after long-term passages (Wang et al., 2005; Pei et al., 2013).

Zambon et al. showed that strains of *A. actinomycetemcomitans* isolated from patients with periodontal disease produced significant amounts of the leukotoxin LtxA, while isolated strains from healthy patients produced sensibly less LtxA (Zambon et al., 1983). LtxA is a 113 kDa protein and one of the main virulence factors of *A. actinomycetemcomitans*. The *ltx* operon contains four genes (*ltxC*, *ltxA*, *ltxB*, and *ltxD*), with an upstream promoter (Johansson, 2011). The secretion of LtxA is facilitated by the type I secretion system but, to transport LtxA to the outer membrane, a periplasmic channel (LtxD) and an outer membrane pore (LtxC) are required. LtxB is an ATPase that transports LtxA throughout the whole process (Johansson, 2011). An increased concentration of LtxA was shown to decrease the proliferation of human microvascular endothelial cells in a tetrazolium-based assay (Dietmann et al., 2013). LtxA was further shown to induce apoptosis in human microvascular endothelial cells and increased expression of ICAM-1 and VCAM-1, leading to pro-inflammatory effects in human brain endothelial cells (Dietmann et al., 2013).

Another important virulence factor of this bacterium is the genotoxic cytolethal distending toxin (CDT) (Gholizadeh et al., 2017), encoded by the *cdtABC* operon. CDT is capable of disrupting the cell cycle in fibroblasts during the G1 and G2/M phase through the p53-dependent pathways. Gingival fibroblasts incubated with the wild-type strain of *A. actinomycetemcomitans* showed an increased proportion of cells in the G2/M phase, while a *cdt* mutant showed the same distribution of cells in the G1 and G2/M phases as the untreated control group (Mayer et al., 1999; Belibasakis et al., 2004). CdtB is the functional subunit of CDT, which is able to induce cell cycle arrest due to its type I deoxyribonucleases activity that leads to DNA double-strand breaks (Lara-Tejero and Galan, 2000). Confocal microscopy showed that CdtB on its own does not bind or enter HeLa cells, but CdtA and CdtC do bind the cell wall. CdtB and CdtC together were shown to localize in the cytoplasm of HeLa cells, so CdtC appears to enter the cell along with CdtB (Akifusa et al., 2005). Jurkat cells were exposed to different *cdt* mutants (*cdtA*, *cdtB*, *cdtC*, *cdtAB*, *cdtBC*, and *cdtABC*), and all

these mutant *A. actinomycetemcomitans* bacteria were shown to result in a lower percentage of cells in the G2 phase compared to the wild-type strain. Thus, a mutation in one or more of the three *cdt* genes results in a decrease in toxin activity (Shenker et al., 2004).

Another important virulence factor of *A. actinomycetemcomitans* is its LPS, which stimulates bone resorption (Raja et al., 2014; Belibasakis et al., 2019). LPS has, in fact, multiple virulence-related properties. For example, LPS causes the widening of the spaces between the cells in primary tissue cultures (Belibasakis et al., 2019). Fibroblasts of gingival tissues naturally ingest, and digest collagen, but this process was enhanced by LPS. This can result in an imbalance in the regeneration of gingival tissue. LPS also enhanced the production of cytokines including IL-8, IL-6, and IL-15, which results in a cascade of immune responses (Belibasakis et al., 2019). The patterns of LPS are different in rough and smooth strains of *A. actinomycetemcomitans*. As measured with the classical Limulus lysate assay, the endotoxin activity was higher in the rough isolates than in the smooth strains, suggesting that the rough phenotype has a higher potential for LPS-related pathogenicity (Fine et al., 1999).

There are several additional virulence factors in *A. actinomycetemcomitans*, including the morphogenesis protein C (MorC), which has an influence on the fimbrial selection and microcolony formation that is essential for membrane morphology and leukotoxin secretion (Smith et al., 2016). The tight adherence (Tad) protein is required for production of fimbriae and, accordingly, this protein is important for colonization and virulence of *A. actinomycetemcomitans* (Kachlany et al., 2000; Oscarsson et al., 2019). Further research, however, needs to be done on the virulence factors of *A. actinomycetemcomitans* and their concerted actions, as this will be key to develop novel treatment strategies for all the diseases in which this pathogen is implicated.

Noma

Noma, or cancrum oris, is an opportunistic gingival infection mainly affecting poor and undernourished children aged 2–16 years in low-income countries (Falkler et al., 1999a, b; Enwonwu et al., 2000). Some communities in Sub-Saharan Africa reach an estimated frequency of 1 to 7 cases in every 1000 children, even though actual numbers are thought to be significantly higher, since most cases remain unreported. Without treatment, the mortality rate lies between 70% and 90% (Barnes et al., 1997). Noma causes orofacial necrotizing lesions and severe deterioration of multiple soft and hard tissues, leaving survivors with surgery-requiring disfigurements that may reach up to the cheeks, upper and lower lips, and the nose (Coupe et al., 2013). The infection causes irreversible deterioration of the maxilla, muscles, and mucosal tissues (Lazarus and Hudson, 1997). Key risk factors for Noma include malnutrition, especially vitamin A deficiency, poverty, poor (oral) hygiene and infectious diseases (Enwonwu et al., 2000). These factors, in fact, result in impaired host resistance, which again promotes secondary infections and the etiopathogenesis of this disease. This, in a way, makes poverty the main causative agent for the appearance of the disease.

Noma was for a long time thought to start as a necrotizing ulcerative gingivitis, which is an inflammation of the interdental papillae. However, this theory has been challenged and the cause for the development of Noma remains unclear. Accordingly, no specific infectious agents have been identified to cause the development of Noma from necrotizing ulcerative gingivitis (Enwonwu et al., 2006). Even with the high prevalence of ulcerative gingivitis presented in undernourished Sub-Saharan African children, in fact, only few of them develop Noma (Enwonwu et al., 2006). The disease onset of Noma usually implies the presence of an accompanying bacterial, viral, or parasitic infection (Barnes et al., 1997). Microbiologically, the infection appears polymicrobial in nature, involving bacteria such as the spirochete *Treponema vincentii*, in association with an anaerobe, usually a *Fusobacterium*, and *Prevotella intermedia* (Falkler et al., 1999a, 1999b; Feller et al., 2019). Additionally, HIV, herpesviruses, and measles have been frequently observed as co-infections or reported shortly prior to the diagnosis with Noma in the medical history of affected individuals (Feller et al., 2019). Descriptions of the initial stages of the infection remain inconsistent since patients usually seek medical help once the infection is already well-established. Early symptoms include foul-smelling halitosis, purulent oral discharge, dark coloration of the affected region, and a rotten taste (Baratti-Mayer et al., 2003). When left untreated, Noma usually progresses to the labiogingival fold and subsequently invades the mucosal tissues of the lips and cheek (Coupe et al., 2013). As soon as the infection has reached the soft tissues surrounding the gingiva, further progression causing cheek perforation happens within days and therapy is mostly ineffective (Enwonwu et al., 2006). In those cases, death is typically caused by starvation, as well as sepsis or pneumonia which are common complications of the infection (Baratti-Mayer et al., 2003). Severe disease evolution can be prevented in case of early detection in the gingival stage. The treatment of Noma includes broad spectrum antibiotics, disinfection of the affected area, nutritional recovery, and treatment of the predisposing infection (Feller et al., 2014).

Noma prevention in form of educational campaigns, improvement of hygiene, proper nutrition, vaccination and/or treatment of possible accompanying and co-infections such as measles are main strategies to control Noma outbreaks. However, healthcare workers and individuals in affected communities are still largely under-informed about Noma, which makes educational work a priority to avoid fatal disease development (Enwonwu et al., 2006). Simultaneously, further research to find the cause for Noma development and an effective therapeutic approach for late-stage Noma infections remain urgently needed.

Conclusion

In conclusion, the plethora of bacteria living in the oral cavity and forming the oral microbiome plays an important role in the preservation of our oral and general health. Dysbiotic changes in the oral microbiome, or even specific microbial profiles, can result in a multitude of highly damaging oral diseases, but they may also lead to complications or disorders in other body sites. Specific oral pathogens, especially, are capable of bringing about several far-reaching consequences. For example, *Porphyromonas gingivalis* is

either a causative agent, risk factor, or believed to play a role in the etiopathogenesis of several diseases, oral or systemic, such as periodontitis, cardiac and respiratory diseases, diabetes, Alzheimer's disease, nonalcoholic fatty liver disease, oral and pancreatic cancer, and rheumatoid arthritis. Similarly, *Fusobacterium nucleatum* has been connected to several different cancer types, such as oral, colorectal, and pancreatic cancers. However, with new analytical technologies and the ever-increasing number of new studies focused on oral diseases, oral pathogens, and the molecular mechanisms connecting the two, it will be easier to unlock the full damaging potential of the oral microbiome, thus relieving the global economic burden of oral diseases and improving, or saving, the life of millions of patients worldwide.

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Oral and Dental Infections: Virus

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Introduction

The oral cavity includes a number of different habitats, such as teeth, tongue, cheeks and gingiva, which are colonized by bacteria. The Human Oral Microbiome Database currently includes some 775 microbial species (Escapa et al., 2018). Viruses are difficult to detect with traditional methods such as in vitro cultivation; however, the advent of the tools of molecular biology, particularly various PCR-based methods, has changed the situation. We can now readily detect not only bacteria that do not grow in culture, but also viruses. Viruses are surprisingly common in the oral cavity; consequently, it is important to know what role they play, if any, in oral disease. The topic has been reviewed elsewhere (Clarkson et al., 2017; Grinde and Olsen, 2010), below follows an updated overview.

It is well known that most of the bacteria associated with your body do not cause any harm. Viruses have a more dubious reputation. The main reason why they in the eyes of clinicians are more closely associated with diseases is presumably the fact that they have been less visible. While it is easy to find numerous species of bacteria in samples from a healthy mouth, even after the advent of PCR, people rarely look for viruses except as suspects when diagnosing a particular clinical condition. If a virus is detected, it is typically assumed to contribute to that condition. We now know that some viruses are highly prevalent in the human body. Viruses of the family *Anelloviridae* are present in more than 90% of adults (Moen et al., 2002; Huang et al., 2001); they have been suggested to play a role in a number of diseases, but so far no obvious role in pathogenicity has been elucidated. The same can be said about the closely related *Circoviridae* (Sauvage et al., 2018; Rosario et al., 2017). Polyomaviruses are present, at least in some points in life, in a majority of the population, but are only rarely associated with any symptoms (Jiang et al., 2009). Moreover, even viruses that are known to be capable of serious complications can be highly prevalent in people without any overt symptoms; examples include the herpesvirus family and enteroviruses. Many of these viruses presumably rely on an oral or fecal-oral route of transmission, thus a presence in the oral cavity would not be unexpected.

The ubiquity of the above-mentioned viruses implies that their detection in samples taken from diseased oral tissue is not sufficient to implicate the virus in pathological changes. The focus of this review is on viruses that may be directly responsible for clinical symptoms – as opposed to those that simply use an oral route of transmission without manifesting themselves in the mouth. Some viruses affect oral health indirectly. HIV is one example as the immunodeficiency caused by the virus, typically manifest itself by oral infection of other agents such as *Candida*.

Clinical aspects

The process of evolution has shaped viruses according to the same principles as other organisms, that is, they are tuned to survival and procreation. As with any obligate parasite, a winning strategy requires not just efficient replication, but also a means of transmission between hosts. Consequently, it is not an optimal strategy to kill the host; in fact, it is generally preferable to reside in a physically active, implying healthy, host. Unfortunately, evolution rarely forms optimal organisms.

One feature that distinguishes viruses is their extreme form of what in biology is referred to as ‘r-selection.’ The term is used for organisms that produce as much progeny as possible without bothering about quality assurance or putting resources into each one of them. This strategy allows the virus to have a more sloppy machinery for RNA or DNA replication than any cellular organisms; viruses can afford to make thousands of worthless copies of themselves for each competent viral particle. The point is a nightmare for clinicians, in that viruses relatively easily develop resistance to antiviral medication, in that they escape vaccines or the immunological memory of previous infections, and in that their variability makes the DNA/RNA based diagnostic tools less certain.

Although viruses as a rule of thumb are best served by having a healthy host, they still cause serious disease. Three factors are of particular importance for the clinical outcome of a viral infection:

Table 1 Common clinical symptoms caused by viruses – according to transmission strategy.

<i>Strategy</i>	<i>Symptom/disease</i>	<i>Relevant viruses</i>
'Hit-and-run' viruses	Coughing Diarrhea Bleeding	Influenza, rhino- and coronaviruses Noro- and rotaviruses Ebola- and hantaviruses
Chronic viruses	Blisters, soars, ulcers Behavioral change, e.g. aggression	Herpes- and enteroviruses Rabies virus
Clinical symptoms with no obvious viral strategic cause	Cancer Immunodeficiency Fever Gross immunological overreaction and secondary infections	Papilloma- and polyomaviruses HIV and coronaviruses Many viruses Pathogenic influenza and SARS coronaviruses

- 1) Pathology depends on how advanced the relationship between virus and host is. Viruses that have a long evolutionary history with a particular species generally evolve mechanisms to avoid unnecessary damage to their host; while viruses that recently jumped from one species to another, have not had the chance to do so. Consequently, zoonotic viruses pose a particular threat to human health.
- 2) Symptoms also depends on whether the virus is designed to remain within the host for a long time, preferably throughout the life span; or if it is a 'hit-and-run' virus. In the latter case, there is obviously less selection as to restraining the virus from causing harm.
- 3) The final factor is whether the virus requires particular behavior or symptoms of the host in order to be transmitted to another individual. Coughing, vomiting and diarrhea are perhaps the most obvious and well-known examples. In [Table 1](#), the various strategies and clinical symptoms are outlined schematically.

Viruses can infect any type of cell in the human body, with the exception of erythrocytes; still the oral cavity has a particular significance. For one, the mouth offers a perfect entrance to a new host. We breathe, drink and eat every day; thus all that is required for the virus is a strategy that allows transmission either through air or through water/food. The former is typically cared for by the infected host when he coughs and thus sends out aerosols containing viral particles (but simple breathing can be sufficient); the latter is reflected in transmission by the fecal-oral route; that is, the virus infects the guts and is thus released to the environment through the feces. The central location of the mouth reflects that several viruses can be diagnosed by the use of oral/throat samples (e.g., swabs) even though they are unlikely to contribute to oral pathogenicity ([Corstjens et al., 2016](#)). Some viruses will occasionally manifest themselves in the oral cavity, although they primarily cause disease elsewhere. The measles virus, for example, can trigger small, white spots in the mouth, while its typical manifestations are skin rash and systemic symptoms.

Respiratory viruses

Respiratory infections, including the common cold, are mostly due to viruses. The more common culprits belong to rhinoviruses, coronavirus, adenoviruses, and influenza. These viruses primarily replicate in the upper respiratory tract; more rarely, they are active in the oral cavity or find their way down to the lungs. Their typical strategy of aerosol transmission does not require replication in the mouth, in fact in order to induce a cough, it is more appropriate to cause irritation of the throat.

This statement is true for SARS-CoV-2, the virus responsible for covid-19, as well. Although the main receptor for this virus (ACE2) is expressed in oral mucosa, particularly on the tongue (as well as in a variety of other tissues), the virus is apparently not very active in the oral cavity ([Xu et al., 2020](#)). There are, however, some case reports that associate SARS-CoV-2 with vesiculobullous lesions (painful ulcers or blisters) in the oral mucosa, as well as pain in the tongue ([Carreras-Presas et al., 2020](#)). Although the causative nature of the association is not definite, it would neither be unexpected.

Herpesviruses

In the case of some viruses, replication in the mouth may be preferable. Humans are not the only species that have developed a delight in kissing, but probably no other species are prone to engage in the exchange of sputum to the extent that we humans are. Some viruses have apparently adapted to this behavior in their approach to the question of transmission. In order to assure continuous viral presence in sputum, it is preferable to replicate in cells lining the oral cavity and to release viral particles to the saliva. Herpesviruses (and possibly papillomaviruses) are among the viruses that presumably use this strategy. Replication in oral tissue places the oral cavity more at risk for clinical symptoms.

Table 2 Human herpesviruses (HHV) and their associated diseases.

Type	Primary target cell	Oral affection	Other pathology
Herpes simplex virus-1	Mucoepithelial	Herpes ulcers ("cold sores")	Genital ulcers
Herpes simplex virus-2	Mucoepithelial	Herpes ulcers ("cold sores")	Genital ulcers
Varicella Zoster virus	Mucoepithelial	Possible oral manifestations of chicken pox and herpes zoster	Chicken pox, herpes zoster (shingles)
Epstein-Barr virus	B-cells and epithelial cells	Hairy leucoplakia, oral lymphomas, periodontitis?	Mononucleosis, lymphoma
Cytomegalovirus	Monocytes, lymphocytes and epithelial cells	Oral ulceration, periodontitis?	Mononucleosis
Human herpesvirus-6	T-cells and possibly others	No known oral manifestation	Roseola in infants
Human herpesvirus-7	T-cells and possibly others	No known oral manifestation	Roseola in infants
Human herpesvirus-8	Probably lymphocytes and epithelial cells	No known oral manifestation	Kaposi's sarcoma (in AIDS patients)

Herpesviruses have a double-stranded DNA genome and are among the largest and most complex human viruses. There are eight members of the human herpesvirus (HHV) family (Table 2). The more common, as to oral health problems, are the two herpes simplex viruses (HSV-1 and -2), which cause recurrent herpetiform ulcerations referred to as cold sores. These ulcers typically occur on the lips, but the viruses can also cause similar lesions in the mucosa, such as in the case of gingivostomatitis. The mucosal affection is normally associated with a primary herpes infection in children, and is accompanied by bodily symptoms such as fever and malaise. Both HSV-1 and -2 are involved in the above oral manifestations, although the latter is traditionally associated with the genitals.

Epstein-Barr virus (EBV) and cytomegaloviruses (CMV) are present in the vast majority of adults, but in most cases presumably without causing any overt disease. Both can, however, cause mononucleosis; EBV being responsible for most of the cases. Mononucleosis is also known as 'kissing disease,' suggesting that the virus spreads by direct mouth-to-mouth contact. The condition is associated with teenagers; the name reflects that this is the time when people start kissing. The cause of the disease, however, is most likely a lack of contact with these viruses early in life. EBV and CMV are adapted to be reasonably ubiquitous and not to cause overt symptoms in youngsters. The sanitary conditions of modern societies are likely the main culprit as to the prevalence of mononucleosis.

EBV's potential for affecting the mouth is further underlined by oral hairy leucoplakia. The disease is characterized by white patches, typically on the side of the tongue, with a hairy appearance. The condition is restricted to immunosuppressed patients.

Varicella-Zoster virus (VZV) is associated with chicken pox, when active in a primary infection, and with herpes zoster if reactivated later in life. The vesicular rash formed occurs primarily in the skin, but may also affect the mucosa. The three remaining human herpesviruses (HHV-6, -7 and -8) rarely cause serious disease, but the former two are responsible for a particular type of skin rash (roseola) with associated fever in infants.

The herpesviruses typically form chronic infections. The virus remains with its host until death do them apart; much of the time in latency, but with occasional bursts of activity. As these viruses are contact transmitted, either by means of virus production in the skin accompanied by rashes and blister or viral presence in sputum, it is not surprising to find viral activity in the oral cavity. In addition to the traditional clinical picture referred to above, EBV and CMV have been associated with periodontitis (Grinde and Olsen, 2009). Several laboratories have demonstrated that these viruses are found significantly more frequently in samples taken from affected 'pockets' around the teeth compared to healthy pockets (Slots, 2004; Sunde et al., 2008a), however, this association does not necessarily mean that they are involved in the pathology. The number of viral genomes observed is generally low, which has led some to doubt any clinical significance (Aggarwal et al., 2017). One person with recurrent periodontal disease, who had a particularly high EBV-load subgingivally, was successfully treated with antiviral treatment (Sunde et al., 2008b), suggesting that EBV does play a role at least in some patients.

In general, it makes sense to be skeptical as to pathogenicity when the viral load is limited. Both EBV and CMV are found occasionally in any mouth, as long as a relevant sample of sufficient size is analyzed by a sensitive technique. EBV, in particular, tends to be cyclically active in the body and thus periodically present in sputum. Associations between viruses and the severity of the condition, or between viruses and the presence of particular bacterial species, may be explained by confounding factors; for example, a more active inflammation would be expected to correspond to the presence of certain bacteria, and cause more pain, but also to contain more lymphoid cells and/or more body fluids. These two factors could explain a viral association, as both EBV and CMV replicate in leukocytes and are shredded to the extracellular fluid.

Antiviral treatment seems to be the best way to indicate the actual role of EBV or CMV in periodontitis. Moreover, if successful, it may save the patients from considerable pain and agony. Quantitative antiviral tests, such as real-time PCR, are relevant in the management of these patients, maybe particularly in chronic and aggressive cases for which other therapy fails. If the tests find appreciable amounts of virus, antiviral treatment should be considered as an adjunct to conventional periodontal therapy. It should be noted that the latter form of therapy has also been reported to reduce the viral load (Grenier et al., 2009).

Enteroviruses

The enteroviruses belong to the family *Picornaviridae*. Enteroviruses have a single-stranded RNA genome and are classified into five species with all together more than a hundred subtypes. Although the majority of human enterovirus infections are asymptomatic (Witso et al., 2006), they can cause upper respiratory illness, febrile rash, aseptic meningitis, pleurodynia, encephalitis, acute flaccid paralysis and neonatal sepsis-like disease (Romero and Modlin, 2015).

As to oral affection, the enteroviruses are primarily associated with hand, foot and mouth disease. This is a febrile illness with tender papulovesicular lesions of both the hand, feet and oral mucosa (Wong et al., 2010). It occurs mostly in children, but can also affect adults. The association with enteroviruses primarily concerns the type A viruses (including Coxsackie virus A16 and enterovirus-71), but other enteroviruses may also be involved. Herpangina is a related condition where the clinical manifestation is primarily in the oral cavity in the form of ulceration and blisters. Again, the condition is rare and restricted to children.

Papillomaviruses

Human papillomavirus (HPV) is a DNA virus that can chronically infect either skin or mucosal epithelium. Parts of the viral genome are occasionally integrated in the DNA of the host cells, and some of the genes are assumed to have a malignant potential, as reviewed elsewhere (Berman and Schiller, 2017). The role of HPV in cervical cancer is well accepted and has led to the widespread use of papillomavirus vaccines for young women. Related carcinomas occur in the mouth cavity, as well as in the oropharyngeal area.

HPVs are considered responsible for various forms of papillary lesions in the oral cavity, including squamous papillomas, condylomas, and multifocal epithelial hyperplasia (Betz, 2019). Although these viruses appear to be involved in many cases of malign oropharyngeal cancers, there is a distinct cohort of cancer patients that are HPV negative (Evans et al., 2013). In this cohort, the disease is typically associated with other risk factors such as tobacco and alcohol. The observed prevalence of oral cancers is considerably lower than what is reported for cervical cancers; still the case favoring a role of these viruses is reasonably strong. Based on the viral role in cervical carcinomas, the viruses are classified as having either high (primarily subtype 16 and 18) or low (including subtype 6 and 11) oncogenic potential.

In a recent systematic review, it was concluded that approximately 7.7% of healthy subjects harbor HPV in the oral region (Tam et al., 2018). The prevalence is typically reported to be higher in biopsies from oral lesions such as leucoplakia or cancers. In the former case, the association with oncogenic HPVs is less obvious; while in the case of malignant cancers, most laboratories find a definite overrepresentation of the more malignant HPVs (Giuliano et al., 2008).

HPV vaccines have been generally available since 2006, and appear to have caused a major reduction of cervical cancer (de Sanjose et al., 2019). There is also a reduction in penile cancers and warts, presumably due to the indirect effect of men being less exposed to these viruses when having unprotected sex due to their drastically reduced prevalence in women. There is a general reduction in oral HPV infections, but the expected reduction of oral cancers is not yet verified.

The main argument against vaccinating both sexes is the calculated cost/benefit ratio. Relevant forms of cancer have a considerably lower prevalence in males compared to females. Moreover, as the virus forms chronic infections, vaccination of individuals who already contain the potentially malign subtypes is considered less useful.

Interactions between viruses and other microbes

Herpesviruses are well known for their capacity to manipulate the immune system; for example by producing cytokine mimics designed to modulate the host's immune defense. Although the obvious purpose of manipulation is to boost viral replication, it is easy to envision that a down-regulation of immunological surveillance may also benefit other agents present, such as bacteria. There are data suggesting that viral activity in periodontal tissues may impact on the local immune response in a way that benefits opportunistic bacteria and thus leads to aggravated symptoms (Chen et al., 2020). For example, the bacterial profiles in samples obtained from periodontic sites depend on the presence of virus. It should be noted that the microbial activity can stimulate viral replication, as has been shown in the case of EBV and malaria (Chène et al., 2007).

Bacteriophages

It has been estimated that the mouth is typically home to some six billion bacteria, and possibly 35 times as many viruses (Edlund et al., 2015). The majority of the latter are presumably not human viruses, but bacteriophages – viruses that replicate in bacteria. The reason for this imbalance is that bacteria vastly outnumber human cells associated with the oral cavity. The fact that the bacteriophages only indirectly have a human host, does not make them irrelevant as to oral health. They do modulate the microbial ecosystems they inhabit; for example by transferring genes such as those responsible for antibiotic resistance. As such, they can either act to the benefit of a patient suffering from oral bacterial infections, or increase the severity of the symptoms. The point is relevant in connection with periodontitis (Edlund et al., 2015).

Diagnostics

Viral diagnostics have become more relevant in clinical dentistry. This is partly because of an increased awareness that viruses are possible etiological agents, and partly because the methods of viral detection have become considerably cheaper and easier. The preferred methods are based on variants of real-time PCR, which not only offer a test for the presence of virus, but also yield quantitative data. The latter point is particularly relevant as several viruses can be present in healthy oral mucosa. However, a high viral load in a sample taken from affected tissues does suggest a pathogenic association with the underlying condition.

A major limitation as to this association is that some of the viruses that are chronically present in the body replicate in leukocytes (e.g. EBV and CMV). Clinical samples are typically taken from inflamed tissues, such as periodontal pockets or ulcerations; consequently, one might expect a presence of these viruses due to the accumulation of leukocytes. The point has been demonstrated in the case of CMV and periodontitis (Sabeti et al., 2009). Again, a clinical role is suspected if the titer is particularly high, and even more so if the condition improves upon antiviral treatment.

A main limitation of PCR-based methods is that they only detect the viruses (or nucleic acid sequences) they are designed to detect. Several novel human viruses have appeared during the last decade, and most likely the human body is the host to a range of viruses that are yet to be described. Moreover, the cost of the methods restricts analyses to relatively few viral types; thus the total spectrum of potentially relevant viruses is rarely tested. Two strategies compensate for this limitation: microarrays and pyrosequencing. In microarrays, probes detecting different viruses (or other agents) can be applied to a slide and the sample DNA or RNA hybridized onto the slide, thus offering a possible detection of all known viruses. In pyrosequencing, the complete nucleic acids present in the sample are sequenced to look for recognizable viral sequences by searching relevant databases. Both these methods have the same two limitations; they are less sensitive than PCR, and they are considerably more expensive. Consequently, the techniques are not suitable for routine diagnostics, but are relevant in scientific inquiries seeking to outline viral involvement in various conditions. One such case is the common aphthous ulcers, also known as canker sores. Although viruses have been implicated by their presence in clinical samples (Lin et al., 2005), it seems unlikely that the true viral culprit, if any, has yet been found.

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Oral and Dental Infections: Fungi

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Introduction

Fungi are a large and complex group of eukaryotes organisms. Among these, *Candida* species should be highlighted, which produces most of the fungal infections that affect the oral cavity. *Candida* is a ubiquitous polymorphic fungus that is commonly found in the gastrointestinal tract and other mucous membranes, as well as, on the skin. The genus *Candida* comprises more than 200 species and most of them are dimorphic yeasts (Bhattacharya et al., 2020; Bandara and Samaranayake, 2019; Lewis and Williams, 2017; Telles et al., 2017; Hellstein and Marek, 2019). Of the different *Candida* species (Table 1), *C. albicans* is the most prevalent in the oral cavity and is isolated in 90–95% of cases. Apart from *C. albicans*, there are other isolated species with much less frequency such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. pseudotropicalis*, *C. dubliniensis*, *C. lusitaniae*, *C. krusei*, *C. guilliermondii*, *C. stellatoidea*, *C. inconspicua*, *C. norvegensis*, *C. rugosa*, and *C. auris* (Lombardi and Ouanounou, 2020; Bhattacharya et al., 2020; Hellstein and Marek, 2019; Hoare et al., 2017; Lewis and Williams, 2017; Telles et al., 2017). In some cases, several *Candida* species can be associated, being *C. albicans* and *C. glabrata* the most frequent association, followed by *C. albicans* and *C. tropicalis* (Swidergall and Filler, 2017).

Besides the species of the genus *Candida*, there are other types that lead to fungal oral infections less frequently, especially in immunosuppressed patients. They are *Geotrichum*, *Aspergillus*, *Histoplasma*, *Cryptococcus*, *Blastomyces*, *Coccidioides*, *Saccharomyces*, *Penicillium* and *Geotrichum*, among others (Bandara and Samaranayake, 2019; Lewis and Williams, 2017).

Fungal infections of the oral cavity are common, especially oral candidiasis (OC). In most cases, they are superficial infections but in some cases are invasive and can progress to disseminated infections, increasing morbidity and mortality (Bhattacharya et al., 2020; Telles et al., 2017). The prevalence of OC varies according to age and the population studied. It should be noted that *Candida* carriage is present in the oral cavity of 20–85% of healthy patients. Therefore, the presence of *Candida* is not a good predictor of the disease (Bandara and Samaranayake, 2019; Bugshan et al., 2017; Hellstein and Marek, 2019; Lewis and Williams, 2017). In fact, according to certain studies that do not use cultures to identify the oral fungal microbiome but molecular detection methods, *Candida* species are present in all the patients studied (Peters et al., 2017).

Pathogenesis

C. albicans is a commensal fungus that is commonly found in the oral cavity in balance with the rest of the oral microbiome and host's immune system. In certain cases, when this balance is altered *Candida* yeast can change from commensal to pathogen, causing superficial infections and in some cases, especially in immunosuppressed patients, it can produce systemic infections (Hube and Naglik, 2001). The change from commensal to pathogen is complex and it is usually due to changes in the local environment and

Table 1 Fungal species associated to oral lesions.

Fungal species	
<i>Candida</i>	<i>C. albicans</i> <i>C. glabrata</i> <i>C. parapsilosis</i> <i>C. tropicalis</i> <i>C. pseudotropicalis</i> <i>C. dubliniensis</i> <i>C. lusitaniae</i> <i>C. krusei</i> <i>C. guilliermondii</i> <i>C. stellatoidea</i> <i>C. inconspicua</i> <i>C. norvegensis</i> <i>C. rugosa</i> <i>C. auris</i>
<i>Geotrichum</i>	<i>Geotrichum candidum</i>
<i>Aspergillus</i>	<i>Aspergillus fumigatus</i> <i>Aspergillus flavus</i>
<i>Histoplasma</i>	<i>Histoplasma capsulatum</i>
<i>Cryptococcus</i>	<i>Cryptococcus neoformans</i> <i>Cryptococcus gattii</i>
<i>Blastomyces</i>	<i>Blastomyces dermatitidis</i>
<i>Coccidioides</i>	<i>Coccidioides immitis</i> <i>Coccidioides posadasii</i>
<i>Geotrichum</i>	<i>Geotrichum candidum</i>

systemic state of the host. This results in an increase in the growth of *C. albicans* strains and in the expression of its virulence factors (Quindos et al., 2019). Likewise, the oral microbiome also has an influence, when the number of bacteria decreases the number of *Candida* species increases (Lewis and Williams, 2017). The most relevant points of OC pathogenesis will now be reviewed.

Ability to form biofilms

C. albicans has the ability to form biofilms on different surfaces such as oral mucosa, teeth, dentures and orthodontic appliances (Quindos et al., 2019). The formation of biofilms is a dynamic process, which begins with the adherence of *C. albicans* strains which must proliferate, form hyphae, and accumulate extracellular matrix, as will be seen below.

Two types of *C. albicans* cells are involved in biofilm formation: the blastospores or small yeast-form cells, and the long tubular hyphal cells (Wibawa, 2016). The biofilm matrix of *C. albicans* is composed of polysaccharides β -1,3-glucan, β -1,6-glucan, and mannans that form the mannan-glucan complex (MGCx) (Vila et al., 2020). When the biofilm is established, the expression of *Candida* virulence factors increases, and the susceptibility to host phagocytosis and antimicrobials decreases. Evidence shows that the formation of *C. albicans* biofilm is associated with resistance to antifungal therapy. Biofilm protects and promotes metabolism cooperation, and improves resistance to physical and chemical stress (Wibawa, 2016). In addition, the presence of bacterial components in the oral microbiome also play an important role in the appearance and exacerbation of lesions (Vila et al., 2020).

Adherence to oral mucosa and other surfaces

Adherence to oral mucosal epithelial cells, dentures, and orthodontic appliances is the first step in the development of *C. albicans* infection. By adhering, it prevents be damaged by salivary antimicrobial agents, be removed by saliva and allows to repopulate the epithelial tissue when it is replaced (Lewis and Williams, 2017). *C. albicans* adhesion is carried out through electrostatic interactions with the epithelial surface by cell-wall receptors. Besides, there are some virulence factors called adhesins that collaborate binding *C. albicans* to epithelial cells and polymers of dentures (Wibawa, 2016). Some of the most studied *C. albicans* adhesins are the agglutinin-like sequence (ALS) glycoproteins such as Als3p and hyphal wall protein Hwp1 (Vila et al., 2020).

Invasion and cell damage

C. albicans invades the oral epithelium by two different mechanisms. The first is endocytosis. To do so, it changes its morphology and phenotype which facilitates penetration into the epithelium (Quindos et al., 2019). *C. albicans* has the ability to grow both in the form of yeast and mold. This ability to change from a yeast form to a hypha form is called dimorphism. Dimorphism is a fundamental characteristic of *C. albicans* that favors its virulence. Passage from yeast to filamentous form collaborates in the different stages of the infection (Wibawa, 2016). Besides, *C. albicans* has another feature called phenotypic switching. It has been observed how certain *C. albicans* isolates, which are homozygous at the mating type locus (*MTL*, *a/a* or α/α), can change between two different cell morphologies: white and opaque. These cell morphologies are different in their mating ability and expression of genes related to adhesion and metabolism (Wibawa, 2016).

The changes of phenotype and morphology are due to different environmental stimuli. A relevant property of hyphal cells is thigmotropism or their ability to grow after contact with a surface, which favors endocytosis and invasion of intercellular junctions (Vila et al., 2020). The hyphae of *C. albicans* express the adhesins Als3p and invasin Ssa1 (member of the HSP70 family of heat shock proteins), which bind to E-cadherin and a heterodimer composed of the epidermal growth factor receptor (EGFR) and HER2 on the surface of the epithelium (Sun et al., 2010; Swidergall and Filler, 2017). These processes cause clathrin-dependent endocytosis machinery that induces epithelial cells to produce pseudopods that engulf the fungus and drag it into the interior. There are signaling pathways that are necessary for endocytosis to occur, including platelet-derived growth factor BB (PDGF BB) and neural factor protein pathway 9 (NEDD9). The activation of these signaling pathways requires the formation of *C. albicans* hyphae and the expression of Als3 adhesin. The second mechanism is active penetration. The hyphae of *C. albicans* elongate to penetrate epithelial cells. Both processes, endocytosis and active penetration, can occur at the same time (Swidergall and Filler, 2017).

C. albicans can also penetrate epithelial cells by a paracellular route. It does this by secreting extracellular hydrolytic enzymes into the local environment (Swidergall and Filler, 2017; Lewis and Williams, 2017; Vila et al., 2020). These hydrolytic enzymes facilitate the adherence to oral epithelium, the rupture of intercellular junctions and the invasion of oral mucosa evading host defenses (Wibawa, 2016). These enzymes include:

- *Secreted aspartyl proteinases (SAPs)*. One of the most studied groups of *C. albicans* hydrolytic enzymes are SAPs that degrade E-cadherin and other proteins that hold epithelial cells together (Wibawa, 2016; Swidergall and Filler, 2017; Lewis and Williams, 2017; Vila et al., 2020). This group consists of 10 proteins from Sap1 to Sap10. SAPs increase adherence, headline damage and evasion of host defense (Hube and Naglik, 2001). *C. albicans* can also stimulate calpain in epithelial cells, which is a cysteine protease that degrades E-cadherin (Swidergall and Filler, 2017). Although the function of SAPs is not entirely clear, it seems that these proteins provide the nutrition for the yeast cells, allowing *C. albicans* to continue to penetrate and invade the tissues.
- *Phospholipases (PLs)*. PLs hydrolyze certain phospholipids in the cell membranes, thus destabilizing them. Therefore, these enzymes collaborate in the tissue invasion and in the acquisition of nutrients (Wibawa, 2016; Lewis and Williams, 2017; Vila et al., 2020).

- **Hemolysins.** Another virulence factor of the genus *Candida* is the production of hemolysins. These enzymes allow the fungus to obtain iron from the host tissues that it uses for growth, metabolism and invasion. For this purpose, *C. albicans* is able to bind to erythrocytes and subsequently obtain iron from them (Wibawa, 2016).

Evasion to the host immune responses

The patient with OC tries to defend himself by producing an innate and adaptive immune response. The innate response is the first line of defense, it is non-specific and broad. It includes complement system and cells such as neutrophils and macrophages. However, the adaptive immune response is specific because it recognizes *Candida* antigens (Wibawa, 2016). Immunosuppression can encourage the invasion of *Candida* species and damage. There are many immunosuppressed patients, such as intensive care hospital patients, patients with neutropenia, HIV/AIDS patients and patients receiving immunosuppressive drugs in which the immune response against *C. albicans* may not work properly (Hube and Naglik, 2001). However, in immunocompetent patients the cells secrete antimicrobial peptides that destroy *C. albicans* and release pro-inflammatory cytokines that cause neutrophils to migrate to the area, eliminating *C. albicans* (Swidergall and Filler, 2017).

Even so, *C. albicans* has a number of virulence factors that help it evade host defenses. For example, SAPs proteinases are able to destroy immune system molecules such as antimicrobial peptides and antibodies. Another factor that favors the evasion of host defenses is candidalysin that is a cytolytic peptide toxin. Candidalysin is considered a true virulence factor of *C. albicans* and is important in both mucosal and invasive infections (Vila et al., 2020; Swidergall and Filler, 2017; Naglik et al., 2019).

Host defense peptides (HDP) are the first line of defense against fungal infections. Epithelial cells release multiple types of HDP such as β -defensins and cathelicidins that destroy or slow down the growth of *C. albicans*. HDPs act by different mechanisms that target the fungal cell membrane and the mitochondria. *C. albicans* is able to evade the effects of HDPs by releasing Msb2, a mucin-like membrane protein, which inactivates HDPs and secretes SAPs, which break down HDPs (Saraswat et al., 2016). It also releases FLU1 efflux pump which reduces intracellular HDPs levels (Swidergall and Filler, 2017).

The continuing exposure to HDPs creates a stressful response in *C. albicans*. Activation of the high-osmolarity-glycerol (HOG) pathway allows *C. albicans* to resist histatin 5 and human β -defensins 2 and 3. The RNA-binding protein SSD1 acts in part through the transcription factor Bcr1 to mediate resistance to human β -defensin 2 (Gank et al., 2008). The activation of the mitogen-activated protein kinase Cek1 (MAPK) improves the surface exposure of β -1,3-glucans, raising the uptake of histatin 5 and increasing the sensitivity to HDPs (Swidergall and Filler, 2017).

Predisposing factors to OC

There are different risk factors (Table 2) that have been associated with the presence of OC. Among them, are systemic factors such as age (elderly and infants populations), primary and secondary immunosuppression, the use of systemic corticosteroids, diabetes mellitus, active malignancies, radiotherapy, chemotherapy, anemia, the use of antibiotics, Cushing's syndrome, malnutrition, and the use of drugs that reduce salivary flow (Bandara and Samaranayake, 2019; Bugshan et al., 2017; Hellstein and Marek, 2019; Hoare et al., 2017; Lombardi and Ouanounou, 2020; Swidergall and Filler, 2017; Williams and Lewis, 2011). On the other hand, there are a number of local factors that have classically been associated with OC such as the use of dentures, poor denture and oral hygiene, hyposalivation, the use of topical corticosteroids and steroid inhalers, high carbohydrate diet and smoking (Bandara and Samaranayake, 2019; Bugshan et al., 2017; Lombardi and Ouanounou, 2020). The most important risk factors associated with the development of OC will be reviewed below.

Table 2 Risk factors of oral candidiasis.

Systemic factors	Local factors
Age (elderly/infants)	Use of dentures
Immunosuppression	Poor oral hygiene
	Poor denture hygiene
Use of systemic corticosteroids	Use of topical corticosteroids
Diabetes Mellitus	Use of steroid inhalers
Malignancies	High carbohydrate diet
Head & Neck radiotherapy	Smoking
Chemotherapy	Hyposalivation
Use of antibiotics	
Malnutrition	
Drugs-induced dry mouth	

Age

Age is a risk factor for OC. It has been observed that OC often appears at extreme ages in life, such as infants and elderly (Vila et al., 2020). OC often occurs in older patients, because this group of patients suffers from a greater number of comorbidities, such as diabetes, which increases the risk of OC. In addition, the elderly take a lot of drugs which produce hyposalivation, increasing the risk of OC. In addition, they sometimes wear dentures to replace lost teeth and do not have proper oral hygiene (Bianchi et al., 2016). In children, the prevalence of OC varies between 4% and 15%. *C. albicans* colonizes the newborn through the birth canal or the environment (Vainionpää et al., 2019; Telles et al., 2017).

Immunosuppression

C. albicans can change from commensal to pathogenic altering oral biofilm when immunosuppression is present. These alterations may in turn increase the resistance of *Candida* species to antifungal treatment favoring systemic infections (Hellstein and Marek, 2019). There are different diseases and treatments that can cause immunosuppression. Among them are HIV/AIDS, the use of immunosuppressants, chemotherapy, use of immunomodulators, graft-versus-host disease and agranulocytosis (Vila et al., 2020).

Before the introduction of antiretroviral therapy, OC was very common in HIV/AIDS patients. In fact, the presence of oropharyngeal candidiasis is considered a predictor of HIV disease progression (Telles et al., 2017). Nowadays, OC is the most frequent oral lesion in HIV patients. In addition, it has been described how these patients are more likely to suffer from candidiasis caused by other *Candida* species such as *C. dubliniensis*. HIV patients also have fewer antimicrobial protective factors such as histatin 5. In addition, HIV patients suffer highly drug resistant, mainly to fluconazole (Vila et al., 2020; Ramirez-Amador et al., 2020).

In renal transplant patients, OC has been described as the most frequent oral lesion. It has also been observed that in the immediate post-transplant period the risk of OC is higher, probably due to the higher dose of immunosuppressive treatment in this period (Lopez-Pintor et al., 2010, 2013). There are currently other patients receiving immunosuppressive treatments for rheumatological diseases or chronic inflammatory diseases. In these patients, OC can also appear. There are studies carried out in patients with antineutrophil cytoplasmic antibody-associated vasculitis who received treatment with cyclophosphamide, rituximab or other immunosuppressants, in addition to corticoids, which showed how OC was a predictor of subsequent severe infections, due to a decrease in the immune response (Yamaguchi et al., 2019).

Head & neck radiotherapy and chemotherapy

It has been observed that patients who are treated with head & neck radiotherapy often suffer from OC. According to certain studies, the presence of OC is more frequent in those patients with a low leukocyte count, those who suffer from oral mucositis and who have a lower salivary flow (Nishii et al., 2020). Similarly, patients who were treated in the past with head & neck radiotherapy suffer from chronic hyposalivation and therefore from OC. In these patients, it has been observed that not only does the colonization of *C. albicans* increase, but also that of other species such as *C. glabrata* and *C. tropicalis* (Tarapan et al., 2019).

The incidence of OC varies between 20% and 40% in patients treated with chemotherapy, depending on the treatment, dose and duration. Patients who receive this treatment due to hematological malignancies suffer a higher incidence of OC (Diaz et al., 2019).

Diabetes

There are studies that have shown how patients with diabetes mellitus are more frequently colonized by *C. albicans*, so they frequently suffer from OC. There are studies that showed an association between poorer glycemic control and higher *Candida* load (Zomorodian et al., 2016). Diabetic patients are also more prone to OC if they wear dentures (Gonzalez-Serrano et al., 2016). It has also been observed how these patients have a higher prevalence of *C. dubliniensis*, and how strains isolated from patients with diabetes are more resistant to antifungal treatments (Zomorodian et al., 2016).

Use of antibiotics, systemic steroids, and other treatments

The use of broad-spectrum antibiotics is one of the factors most associated with the appearance of OC. The decrease of the bacterial flora causes alterations in the levels of *Candida*, favoring its proliferation (Vila et al., 2020; Farah et al., 2010; Shenoy and Gottlieb, 2019).

In the same way, systemic steroids also encourage the development of OC. This is due to their immunosuppressive effect and to adverse effects such as hyperglycemia (Shenoy and Gottlieb, 2019).

The use of IL-17 blockers also promotes the development of candidiasis. Examples of these drugs are brodalumab, ixekizumab and secukinumab. These drugs are used in rheumatological and dermatological conditions such as ankylosing spondylitis and psoriasis (Shenoy and Gottlieb, 2019).

Nutritional disorders

There are a number of nutritional alterations that have been linked to the appearance of OC. A high-carbohydrate diet and hematic deficiencies should be highlighted. Also, deficiencies of iron, magnesium, zinc, folic acid, selenium and vitamins B12, B6, A and C have been linked to OC (Vila et al., 2020).

Use of steroid inhalers

Steroid inhalers used to treat asthma, such as fluticasone, increase the risk of OC (Shenoy and Gottlieb, 2019). This is due to local decrease in cellular immunity and phagocytosis (Telles et al., 2017).

Reduced salivary flow

The decrease in salivary flow favors the appearance of OC. Saliva is one of the first lines of defense against *Candida* and contains different agents with antifungal effect such as defensins, lysozymes, IgA, mucins, calprotectin, histatin 5, lactoferrin and lactoperoxidase, among others (Hoare et al., 2017; Telles et al., 2017). In addition, a correct salivary flow prevents *Candida* overgrowth due to its washing effect (Hoare et al., 2017). Therefore, when the saliva decreases, the washing effect and the antifungal components are reduced. The incidence of salivary alterations increases not only in elderly patients (mostly due to the use of drugs) but also in other patients such as those receiving chemotherapy, radiotherapy, and HIV patients (Vila et al., 2020).

Patients with Sjögren's syndrome are at increased risk for OC. In fact, there are studies that showed how OC is the most frequent lesion in patients with primary Sjögren's syndrome (Billings et al., 2017; Serrano et al., 2020a). In these patients, it has been shown how the colony forming units of *C. albicans* increase when the salivary flow decreases (Serrano et al., 2020b).

Use of dentures and other oral appliances

As already shown above, *C. albicans* is able to adhere to the surface of dentures and to form biofilms in them. The prolonged use of dentures, in addition to poor hygiene and continued trauma favors the emergence of OC. Dentures are in themselves a favorable environment for the growth of *Candida*: it avoids the presence of saliva, acid pH and low amount of oxygen which promotes SAPs. In immunosuppressed patients who are denture wearers, the appearance of denture stomatitis is more frequent. The material of the denture is also important, acrylic dentures favor the appearance of candidiasis more than metallic ones. Orthodontic appliances also favor their emergence (Vila et al., 2020). In addition, angular cheilitis is more frequent in patients with dentures, due to the loss of vertical dimension in patients wearing dentures for many years. In these cases, the corners of the mouth are moist, which favors *Candida* and *Staphylococcus* infections (Telles et al., 2017).

Use of topical steroids

Treatment with topical steroids, frequently used in patients with chronic inflammatory and autoimmune oral diseases, has also been associated with the appearance of OC. These treatments generate local immunosuppression and alter the oral environment. These alterations usually revert when treatment ceases. In cases where treatment with topical steroids will be of long duration, prophylactic antifungal treatment is recommended (Vila et al., 2020; Tejani et al., 2016; Marable et al., 2016).

Smoking

Patients who smoke have higher levels of *Candida*, which increases the risk of OC. This is mainly due to a reduction of salivary flow from tobacco. This leads to a lower pH, reduces IgA and depresses neutrophil function, which promotes the appearance of OC (Vila et al., 2020). There is a lack of information regarding e-cigarettes and the appearance of OC. However, in vitro studies have shown how these devices induce the genes of virulence factors SAP2, SAP3 and SAP9 (Vila et al., 2020; Sultan et al., 2018).

Clinical presentations of oral candidiasis

It is unknown why different forms of OC are produced by the same fungal species. Nor is it known why a subject can present one clinical form of candidiasis and not another. What is understood is that *C. albicans*, which is the most frequently detected agent, is a genotypically and phenotypically heterogeneous species what could explain these variations (Lewis and Williams, 2017). OC can be divided into three categories: pseudomembranous, erythematous and hyperplastic forms. In addition, there are a number of multifactorial lesions associated with *Candida* such as denture stomatitis, angular cheilitis and linear gingival erythema. Below, the clinical forms of oral candidiasis will be reviewed (Table 3).

Table 3 Clinical presentations of OC.

<i>Clinical presentations of OC</i>	
Acute erythematous candidiasis	• Located in the dorsum of the tongue which present as depapillated. • Can be asymptomatic, sometimes associated with sensitivity to certain foods. • Related to treatment with antibiotics. • Other risk factors: steroid therapy, immunosuppression.
Pseudomembranous candidiasis	• Creamy white plaques that can be removed by gently scraping, showing an underlying erythematous mucosa. • Can be asymptomatic or cause tenderness, burning sensation and dysphagia. • Risk factors: immunosuppression, neonates, elderly and patients under steroid treatment.
Chronic hyperplastic candidiasis	• Uncommon form of OC. • White lesions, often asymptomatic, on the dorsum or lateral of the tongue, commissural angles, or the anterior buccal mucosa. • Risk factors: Middle-aged to older males and smokers. • Clinical forms: homogeneous or heterogeneous.
Angular cheilitis	• Secondary form of OC. • Red lesions ulcerated or fissured at the commissural angles of the mouth. • Risk factors: decrease in vertical dimension, constant humidity, a deficiency in iron and B12 vitamin. • Sometimes associated to other species: <i>Staphylococcus aureus</i> or streptococcus.
Median rhomboid glossitis	• Secondary form of OC. • Symmetrical shaped lesion in the dorsum of the tongue, usually asymptomatic. • Risk factors: smokers or use of steroid inhalers.
Denture stomatitis	• Asymptomatic, sometimes causes pain or burning sensation. • Under dentures, more frequently on the hard palatal, showing a red mucosa beneath the fitting surface. • Risk factors: poor oral hygiene, not removing the denture or when they are deteriorated or not well adapted dentures.
Chronic mucocutaneous candidiasis	• Secondary form of candidiasis. • Superficial infections of the skin, eyes, mouth, nails, and other mucous membranes. • It can appear as angular cheilitis or hyperplastic candidiasis. • Risk factors: impaired immune function, endocrinopathies, autoimmune diseases.

Erythematous candidiasis

This form of OC usually appears after a treatment with broad spectrum antibiotics, due to the reduction in the levels of the bacteria forming the oral microflora. Some other predisposing factors as steroid therapy specially inhale steroids, and immunosuppressants may also contribute to local immunosuppression and therefore to the overgrowth of *Candida* (Lewis and Williams, 2017; Williams and Lewis, 2011). Although it is frequently asymptomatic, it can appear as a painful bright erythematous lesion, usually located in the dorsum of the tongue (Fig. 1). The tongue may present as depapillated and sometimes is associated with sensitivity to certain foods (Lewis and Williams, 2017; Bandara and Samaranayake, 2019; Hellstein and Marek, 2019). Usually, once the antibiotic intake is completed, this type of lesions will resolve without any need of additional treatment. When chronic forms of erythematous candidiasis appear, mirror images may appear, with lesions on the dorsum of the tongue and the hard palate (Lewis and Williams, 2017).



Fig. 1 Candidiasis erythematous in a patient who was treated with amoxicillin for a week for a dental infection.

Pseudomembranous candidiasis

Pseudomembranous candidiasis (also known as oral thrush) is characterized by the appearance of creamy white plaques in the oral mucosa (Fig. 2) that can be easily removed by gently scraping them, showing an underlying normal or erythematous mucosa (Williams and Lewis, 2011; Lewis and Williams, 2017; Lombardi and Ouanounou, 2020). This clinical form frequently appears in immunosuppressed patients, neonates, elderly and patients under steroid treatment (specially inhaled corticosteroids), and it could be acute or chronic. It can present at different parts of the oral cavity as the palate, oropharynx, dorsal tongue, or the buccal mucosa. It could be either asymptomatic or it may course with tenderness, burning sensation or dysphagia (Lombardi and Ouanounou, 2020). In case of using inhaled corticosteroids, patients should be instructed to rinse their mouth with water after using the inhaler to prevent this condition (Lewis and Williams, 2017). The lesions contain fungal hyphae, bacteria, desquamated epithelial cells, keratin, fibrin and inflammatory cells (Lombardi and Ouanounou, 2020).

Chronic hyperplastic candidiasis

Chronic hyperplastic candidiasis (CHC) is a less common type of candidiasis. It presents as a white lesion, often asymptomatic, which cannot be removed by gently scraping. It appears most frequently at the commissural angles, the anterior buccal mucosa, and on the dorsum or lateral of the tongue. It usually affects middle-aged to older males and smokers. There are two clinical forms: homogeneous CHC (Fig. 3) when having smooth and white lesions, or heterogeneous CHC (Fig. 4) when appear erythematous areas accompanied with the white lesions (Lewis and Williams, 2017; Hellstein and Marek, 2019; Lombardi and Ouanounou, 2020). It has been proposed that this type of OC could develop epithelial dysplasia (especially in heterogeneous CHC or speckled lesions) and may progress to oral cancer. Therefore, it has been recommended to treat this type of lesions with antifungals prior taking a biopsy (Lewis and Williams, 2017; Lombardi and Ouanounou, 2020). If the antifungal therapy is not enough for resolving the lesion, a biopsy must be taken and a histopathological study should be carried out in these cases (Hellstein and Marek, 2019; Lombardi and Ouanounou, 2020).



Fig. 2 Pseudomembranous candidiasis in a liver transplant patient being treated with immunosuppressants.

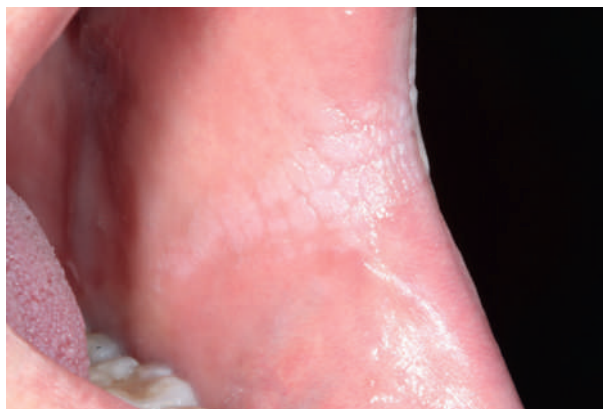


Fig. 3 Chronic hyperplastic candidiasis in a smoking patient.

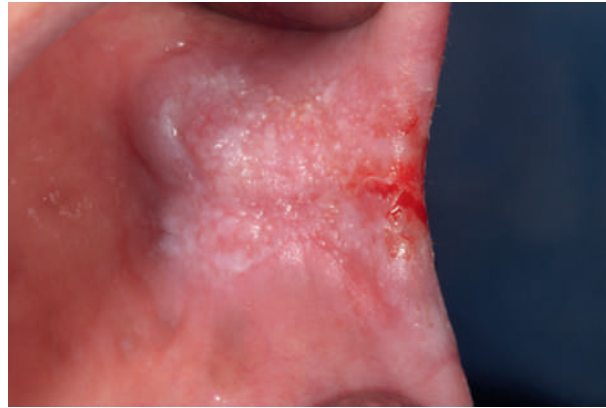


Fig. 4 Chronic hyperplastic candidiasis of speckled appearance in a patient who smoked 20 cigarettes a day.

Angular cheilitis

Angular cheilitis is considered a secondary form of OC. It appears as red lesions, ulcerated or fissured, at the commissural angles of the mouth which typically causes soreness and pain (Fig. 5). Several predisposing factors are related. Among them are decrease in vertical dimension which result in keeping constantly humid this area, and iron and B12 vitamin deficiencies. Angular cheilitis can present as an isolated lesion, or with concomitant intraoral candidiasis, and sometimes other species as *Staphylococcus* or *Streptococcus* may be also present (Lewis and Williams, 2017; Bandara and Samaranayake, 2019; Hellstein and Marek, 2019; Lombardi and Ouanounou, 2020; Vila et al., 2020).

Median rhomboid glossitis

Median rhomboid glossitis is also considered a secondary form of OC. It presents as a symmetrical shaped lesion in the middle of the dorsum of the tongue anterior to the circumvallate papilla (Fig. 6). Lesions are atrophic and erythematous, due to the loss of filiform papilla. It usually courses with no symptoms and appears in patients who smoke or use steroid inhalers (Lewis and Williams, 2017; Hellstein and Marek, 2019; Lombardi and Ouanounou, 2020).

Denture stomatitis

Denture stomatitis or chronic erythematous/atrophic candidiasis is the most common type of OC. It is usually asymptomatic but sometimes it may cause pain or burning symptoms (Lewis and Williams, 2017; Lombardi and Ouanounou, 2020). It develops under acrylic or metallic dentures, more frequently on the hard palatal than in the alveolar ridge, showing a reddening of the mucosa beneath the fitting surface (Fig. 7). It can be associated with the use of orthodontic appliances. In the literature, three groups of denture stomatitis have been described (Newton's classification). Type I is characterized by small petechial and inflammation, pin-point erythema of the palatal mucosa. Type II presents diffuse erythema in the oral mucosa in contact with the denture, partially or completely. Finally, type III is defined as papillar or granular hyperplasia of the oral mucosa in the middle of the palate or at the alveolar ridges (Lombardi and Ouanounou, 2020; Vila et al., 2020).



Fig. 5 Angular cheilitis in a patient wearing a denture with diminished vertical dimension.



Fig. 6 Median rhomboid glossitis in an asthmatic patient being treated with steroid inhalers.



Fig. 7 Denture stomatitis in a patient wearing an acrylic denture.

Several risk factors are related to this condition, as poor oral hygiene, not removing the denture, deterioration or lack of adaptation. When this happens, *Candida* can colonize the denture (Lombardi and Ouanounou, 2020; Lewis and Williams, 2017). Therefore, it is of great importance to advise on correct oral and denture hygiene. Dentures should be cleaned with a brush/toothbrush and several commercial products for its sanitation, as specific lozenges, hypochlorite if there are no metal components, or chlorhexidine if there are, can be used. It is also important to insist to remove them while sleeping (Lewis and Williams, 2017; Hellstein and Marek, 2019).

Chronic mucocutaneous candidiasis

Chronic mucocutaneous candidiasis is a form of candidiasis which appears in those patients with an impaired immune function. There are cases described in the literature in patients suffering endocrinopathies (Addison disease, hypothyroidism, hypoparathyroidism, and diabetes mellitus), autoimmune diseases and immunosuppressed for different reasons. Certain types of mucocutaneous candidiasis have been described as having a recessive autonomic genetic origin. They are also associated with an altered function or interleukin 17 deficiency (Lewis and Williams, 2017; Lombardi and Ouanounou, 2020; Telles et al., 2017).

It presents with a variety of superficial infections of different parts of the body, as the skin, eyes, mouth, nails, and other mucous membranes. When it affects the oral cavity, it can appear as angular cheilitis or hyperplastic candidiasis (Lewis and Williams, 2017; Lombardi and Ouanounou, 2020; Telles et al., 2017). Mucocutaneous candidiasis has a poor response to antifungal treatment, and this lack of response is related to the level of immunodeficiency (Lombardi and Ouanounou, 2020).

Linear gingival erythema

Linear gingival erythema is a *Candida*-associated lesion that has been described in HIV patients. It is characterized by linear erythematous lesions affecting the gingiva of the anterior teeth. It may not be accompanied by symptoms but in other patients it may be associated with discomfort and bleeding. It is believed to be due to subgingival colonization of *Candida* species in patients with an inadequate activity of polymorphic nuclear neutrophils. This lesion has been described as a predictor of

immunosuppression in these patients (Goncalves et al., 2013). Its possible association with *C. albicans* has always been controversial and currently due to the use of retroviral therapy its incidence has decreased considerably (Ryder et al., 2020).

Other non-Candida-associated oral fungal lesions

Other fungal infections (Table 4), much less frequent than OC, which can occur with oral lesions, are described below. These infections usually appear in immunosuppressed patients.

Aspergillosis

Aspergillosis is the second most prevalent fungal infection. In cases of maxillary sinus infection by *Aspergillus* the most common species isolated is *Aspergillus fumigatus* (Bandara and Samaranayake, 2019; Maschmeyer and Ruhnke, 2004; Bugshan et al., 2017). *Aspergillus flavus* is related to invasive infection (Bugshan et al., 2017). This type of infection often occurs in hospitals and in places where there may be a lack of hygiene. It can also appear in immunosuppressed patients (Bandara and Samaranayake, 2019; Maschmeyer and Ruhnke, 2004). In the oral cavity, it has been described how *Aspergillus* can enter through the marginal gingiva or the gingival sulcus. It has been associated with painful yellow or white gingival ulcers. It can also lead to grey or purple swellings of the soft tissues. The most common oral locations of these infections are the palate or posterior tongue (Bandara and Samaranayake, 2019; Maschmeyer and Ruhnke, 2004). It can be confused with allergic-type sinusitis when it affects the maxillary sinus giving rise to an aspergilloma or mass formed by the hyphae (Bugshan et al., 2017). *Aspergillus* hyphae can invade the oral mucosa and lead to systemic infections (Bandara and Samaranayake, 2019; Maschmeyer and Ruhnke, 2004).

Histoplasmosis

Histoplasmosis is caused by *Histoplasma capsulatum*, which is found in warm, humid environments, and in places where there are bird and bat excreta, as well as where soils are high in nitrogen (Bandara and Samaranayake, 2019; Hernández et al., 2004). It is the most common systemic fungal infection in the United States. Transmission of the disease often occurs through airborne spores from the mycelial form (Bugshan et al., 2017; Hernández et al., 2004). It usually affects more men than women. It often occurs in immunosuppressed patients (Bandara and Samaranayake, 2019; Hernández et al., 2004). Histoplasmosis is usually accompanied by pulmonary alterations with chest pain, cough, dyspnea and joint pain that usually last between 1 and 2 weeks. When the form is limited it is called primary acute pulmonary form. These alterations can become chronic in older people and smokers. On rare occasions, disseminated histoplasmosis can occur when the microorganism spreads through the bloodstream, especially in immunosuppressed such as AIDS patients, infants and elderly patients (Bugshan et al., 2017; Hernández et al., 2004). It rarely gives oral lesions, the most frequent oral lesion being the ulcerative lesion, but it can also be associated with papular, nodular or granulomatous lesions. Lesions have been described on the tongue, buccal mucosa, palate, gingiva and lips (Bandara and Samaranayake, 2019; Hernández et al., 2004). When ulcerous lesions appear, a differential diagnosis must be made with oral squamous cell carcinoma (Bugshan et al., 2017; Telles et al., 2017).

Cryptococcosis

Cryptococcosis is an infection caused by *Cryptococcus neoformans* in immunosuppressed patients and by *Cryptococcus gattii* in immunocompetent patients. *C. neoformans* is found in the soil and feces of birds such as canaries, parrots and pigeons. *C. gattii* is endemic to Australia and is found in the koala bear. *Cryptococcus* is the most common cause of meningitis in southern Africa. Like other fungal infections it appears in immunocompromised patients such as HIV patients, patients on immunosuppressive therapy and cancer patients. It can also appear in smokers. Oral lesions associated with cryptococcosis can present as purple nodules of granulation tissue, swelling and ulcers. They can appear on the palate, gingiva and in the tooth socket after extraction. Sometimes the ulcerated lesions associated with Cryptococcosis can be indurated (Bandara and Samaranayake, 2019; Telles et al., 2017).

Coccidioidomycosis

Coccidioidomycosis is caused by *Coccidioides immitis*. It is called valley fever, as it appears in the soil of the southwest part of the United States, Mexico and Central and South America. It affects people over the age of 60. As in other cases, its incidence is higher in immunosuppressed patients such as AIDS and HIV patients, transplant patients, patients treated with immunosuppressors, diabetic patients and pregnant women. Oral lesions are rare; if they appear, they are presented as ulcerated granulomatous nodules and non-specific ulcers (Bandara and Samaranayake, 2019; Telles et al., 2017).

Table 4 Characteristics of fungal infections other than candidiasis.

Fungal Infection	Causative agents and epidemiology	Risk factors	Oral lesions	Diagnosis	Treatment
<i>Aspergillosis</i>	- 2nd most prevalent - Most common species isolated <i>Aspergillus fumigatus</i> (sinus infection) and <i>Aspergillus flavus</i> (invasive infection)	- Immunosuppression - Hospitalization - Lack of hygiene - Dental extraction	- Gingival ulcers - Swellings of the oral mucosa - Fungal sinusitis (mass or aspergilloma in the maxillary sinus) - Can lead to systemic infections	- Culture - Serology (antigen galactomannan) - PCR - Biopsy - Limulus assay	- Antifungal treatment: amphotericin B, voriconazole, posaconazole, itraconazole, and caspofungin. - Debridement of aspergilloma in maxillary sinus
<i>Histoplasmosis</i>	- Caused by <i>Histoplasma capsulatum</i> - Men are more affected - Most common systemic fungal infection in United States	- Immunosuppression - Warm and humid environments - Bird and bat excreta - Soils high in nitrogen	- Rare - Ulcerative lesion - Papular, nodular or granulomatous are also described	- Clinical - Biopsy - Culture - Serology (immunodiffusion, histoplasmin skin test, complement fixation test) - PCR (Polymerase chain reaction)	- Improve immunocompetence - Antifungal therapy: amphotericin B (drug of choice), itraconazole and ketoconazole
<i>Cryptococcosis</i>	- <i>Cryptococcus neoformans</i> present in the soil and feces of birds. It affects immunosuppressed patients - <i>Cryptococcus gattii</i> endemic to Australia. It affects immunocompetent patients	- Immunocompromised patients - Smokers	- Purple nodules of granulation tissue, swelling and ulcers (sometimes indurated) - Affect palate, gums and tooth socket after extractions	- Isolation of the pathogen in blood, sputum or bronchoalveolar samples in pulmonary and disseminated infections - Biopsy - Serology (circulating antigens on serum)	- Systemic antifungals: amphotericin B, flucytosine, fluconazole
<i>Coccidioidomycosis</i>	- <i>Coccidioides immitis</i> - <i>Coccidioides posadasii</i> - Southwest of the United States, Mexico and Central and South America	- >60 years - Immunosuppressed patients - Diabetic patients - Pregnant women	- Ulcerated granulomatous nodules - Non-specific ulcers	- Clinical presentation - Biopsy - Serology - Coccidioidin skin test	- Systemic antifungals: fluconazole, amphotericin B, ketoconazole and itraconazole
<i>Blastomycosis</i>	- <i>Blastomyces dermatitidis</i> - North, Central, and South America	- Immunocompromised patients - Forest areas	- Ulcerous lesions - Granulomatous or verrucous lesions	- Clinical - Biopsy - Serology - Coccidioidin skin test	- Systemic antifungals: fluconazole, amphotericin, ketoconazole and itraconazole
<i>Mucormycosis</i>	- <i>Rhizopus arrhizus</i> - Spread by inhaling spores or through wounds	- Immunocompromised patients (diabetes patients)	- Swelling of maxillary ridge or the palate due to maxillary sinus affection	- Biopsy - Culture	- Surgical debridement - Systemic antifungals: amphotericin B
<i>Geotrichosis</i>	- <i>Geotrichum candidum</i> - Present in soil, fruits, plants, and vegetables	- Immunocompromised patients (HIV/AIDS, diabetes mellitus, malignancies, used of steroids or long-term antibiotic treatment, advanced age)	- <i>Candida albicans</i> like lesions	- Culture - Microscopic examination	- Topical antifungals: nystatin and gentian violet - Systemic: voriconazole and amphotericin B

Blastomycosis

Blastomycosis is caused by *Blastomyces dermatitidis*. It appears in North, Central, and South America. In addition to immunocompromised patients, it can appear in forest areas (farmers and hunters). In the oral cavity it can lead to ulcerous lesions and granulomatous or verrucous lesions (Bandara and Samaranayake, 2019; Telles et al., 2017).

Mucormycosis

Mucormycosis is mainly caused by *Rhizopus arrhizus*. But it can also be produced by *Rhizopus microsporus*, *Rhizopus stolonifer*, *Mucor circinelloides*, *Mucor velutinosus*, among other species. It is usually spread by inhaling spores or through wounds. Like candidiasis, it is an opportunistic infection and is more common in immunocompromised patients. Blood vessels involvement may lead to embolism, resulting in necrosis of the affected tissue. The sinus, lungs and brain structures can also be affected. If the maxillary sinus is affected, it can lead to intraoral swelling on the maxillary ridge or the palate, which if not treated correctly can cause necrosis (Bugshan et al., 2017; Telles et al., 2017).

Geotrichosis

Geotrichosis is another opportunistic infection caused primarily by *Geotrichum candidum*. It produces similar lesions to OC and therefore it is often misdiagnosed. This fungus is found in plants, vegetables, fruits and soil. Normally, it is not pathogenic but in people with immunodeficiencies it can lead to invasive diseases (Telles et al., 2017).

Diagnosis of oral fungal lesions

Anamnesis

Diagnosis of OC is usually made with a complete and guided medical history and the clinical examination findings. It is important to take into account that several risk factors have been related to the presence of *Candida* infections, such as poor oral hygiene, impaired salivary gland function, denture use and associated conditions as diabetes, xerostomia, tobacco use, immunosuppression (HIV-infection, leukemia, solid organ transplantation) or medication as antibiotics, corticosteroids, psychotropics or immunosuppressives.

Clinical diagnosis

As previously mentioned, there are multiple clinical forms of OC, either as acute or chronic lesions, which may appear in more than one location at the same time. The dentist should look for any clinical signs and symptoms that patients suffering from OC may experience. The range of symptoms varies from being in some cases asymptomatic to experiencing superficial signs (as itching, burning or soreness). In more severe cases, fever or tachycardia could be present (Vila et al., 2020; Telles et al., 2017). A differential diagnosis with other white lesions (chemical burn, frictional keratosis, leukoplakia or dysplasia) should be carried out for chronic hyperplastic candidiasis. For diagnosing pseudomembranous candidiasis, the white lesions are wiped off by gentle scraping them, showing an underlying normal or red mucosa. When observing erythematous candidiasis, other possible erythematous lesions should be ruled out such as erythroplakia, iron or vitamin B12 deficiency and thermal burns (Bandara and Samaranayake, 2019; Bugshan et al., 2017). In most cases the clinical aspect together with the symptomatology and associated risk factors are sufficient to make a presumptive diagnosis. The diagnosis can also be made by the response to antifungal therapy (Telles et al., 2017).

Culture

Different methods for collecting *Candida* from the oral cavity can be used, including swab, oral rinse and imprint. One of the most widely used is the plain microbial swab that can be applied directly to the lesion and sent for culture. Samples are inoculated onto Sabouraud dextrose agar and incubated at 37 °C for 24–48 h and the results are expressed as *Candida* colony forming units per ml (CFU/mL). There are also chromogenic media, which allow to differentiate the growth of the different species of *Candida* according to the color and appearance of the colonies (Lewis and Williams, 2017). These techniques are frequently enough for the identification of *Candida* from those cases of erythematous and pseudomembranous candidiasis. Nevertheless, it should be noted that since *Candida* is part of the commensal microbiota of healthy population, its simple identification does not mean infection (Lombardi and Ouanounou, 2020). This techniques have a low sensitivity and in addition, it can lead to false-positive results (Bhattacharya et al., 2020).

Cytology

Exfoliative cytology is another technique that could be used for the identification of *Candida*, especially in those cases of pseudomembranous candidiasis. A periodic acid-Schiff staining is used for detecting fungal pseudohyphae. This technique is

non-invasive, cost effective and simple. However, again, the identification of *Candida* does not prove tissue invasion (Lombardi and Ouanounou, 2020; Hellstein and Marek, 2019; Bugshan et al., 2017). This technique provides information about the prevalence of fungal organisms (Hellstein and Marek, 2019). There are cases where a microscopic study with ethylene blue staining or with 10% potassium hydroxide can also be performed. These stains show the yeasts, hyphae and pseudohyphae, but do not show the invasion of the tissues (Lombardi and Ouanounou, 2020).

Hematological test and sialometry

A hematological test should be checked, especially values of full blood count, ferritin, vitamin B12 and folate. In diabetic patients, blood glucose and glycated hemoglobin (HbA1c) need to be measured (Lombardi and Ouanounou, 2020; Lewis and Williams, 2017; Telles et al., 2017). The performance of a sialometry will help to check if the patient has a reduced salivary flow, which as we have shown is associated with the appearance of OC.

Biopsy

In most cases of *Candida* infection, a biopsy is not needed. Nevertheless, when hyperplastic candidiasis is present and has not respond to antifungal therapy, a tissue biopsy is indicated, for confirming the invasion of the epithelium by *Candida*. In these circumstances, an acid-Schiff (PAS) technique is regularly used (Bandara and Samaranayake, 2019; Lewis and Williams, 2017). In the tissue, it can be observed hyperkeratosis of the epithelium with elongation of rete ridges and chronic inflammation in the connective tissue (Bugshan et al., 2017). This test also serves to rule out the possible presence of dysplasia or malignancy (Telles et al., 2017).

The biopsy is also indicated in cases of ulcerous and granulomatous lesions produced by histoplasmosis and other fungi. In these cases, granulomatous inflammation is observed accompanied by plasma cells, multinucleated giant cells, macrophages and lymphocytes (Bugshan et al., 2017).

Other diagnostic tools

Molecular methods can be used to identify different species of *Candida* with species-specific polymerase chain reaction (PCR). This technique is very useful in refractory lesions and research studies (Lombardi and Ouanounou, 2020; Lewis and Williams, 2017; Hernández et al., 2004). Although these tests are frequently used for viral and bacterial infections, in the case of fungal infections their use is still limited (Sanguinetti et al., 2019).

Today, despite scientific advances, fungal infections are classically diagnosed through morphological analysis using cultures. However, there are other non-culture-based tests such as immunodiffusion (ID), enzyme immunoassays (EIA) and the complement fixation test. For example, aspergillosis can be diagnosed by the detection of serum antigens such as galactomannan and β -1,3 D-glucan. Furthermore, for the diagnosis of *Cryptococcus* infection one of the most reliable serological tests is the detection of *Cryptococcus neoformans* antigens. However, today the most promising tests are the molecular tools such PCR, sequencing and mass spectrometry. These tests are mainly used in invasive infections or in those cases where the diagnosis is difficult (Sodre et al., 2020).

Treatment

Identification and removal of risk factors

Prior to prescribing an antifungal treatment, it is of great importance to recognize and remove any predisposing factor. As it has been previously said, a thorough medical history should be performed. If anemia or vitamin deficits are present, they need to be solved. In the same line, patients should be advised of the importance of having a correct glycemic control. If smoking habit is present, patients should be encouraged to cease the habit. In addition, whenever possible, the use of antibiotics, topical or systemic corticoids and immunosuppressants should be avoided. In patients who use steroid inhalers, it is advisable to rinse their mouths with water after their use (Lombardi and Ouanounou, 2020; Lewis and Williams, 2017; Williams and Lewis, 2011).

Likewise, oral hygiene and proper denture cleansing need to be recommended. It is necessary to give a series of indications such as cleaning the dentures daily after meals, removing the dentures at night and cleaning when necessary with ultrasound the biofilm. In addition, it is necessary to improve retention, stability, fit and occlusion of dentures (Lombardi and Ouanounou, 2020; Telles et al., 2017).

Antifungal treatment

There are four types of antifungal agents (Lewis and Williams, 2017; Lombardi and Ouanounou, 2020; Telles et al., 2017):

- Polyene antifungal agents, which disrupt the cell membranes of the fungus. This group includes nystatin and amphotericin B. These agents bind to the ergosterol in the fungal cell membrane, increasing permeability and leakage of intracellular contents (K^+ , Na^+ , Ca^{2+}) producing cell necrosis. They were the first antifungal agents. They are mainly used topically. Amphotericin B can be used by intravenous route but it produces toxicity and numerous side effects.

- Azole antifungals, which inhibit the synthesis of ergosterol by interference with lanosterol demethylase. They are fungistatic and include miconazole, fluconazole and itraconazole. Miconazole is used topically, but the rest of azole antifungals are used systemically through oral administration. They interfere with warfarin and statins. However, they do not interact with dabigatran. Nowadays, resistance to azole antifungals is frequent.
- Echinocandin antifungals, which inhibit the enzyme β -1,3 D-glucan synthetase, which inhibits the synthesis of the cell wall of the fungus. Among them are anidulafungin, micafungin and caspofungin.
- Pyrimidines antifungals, which disrupt RNA, DNA and protein synthesis of fungus, such as flucytosine.

Below, the most frequently used local and systemic antifungals for the treatment of OC will be reviewed (Table 5).

Local treatment

Topical treatment is the treatment of choice for most cases of OC. Local antifungals have little side effects since their absorption is limited. Therefore, they do not interact with other systemic drugs. There are several topical formulations as tablets, oral suspension, or gels, among others. Nystatin and miconazole should be the first line treatment for OC, due to their efficacy, cost, and little adverse effects. Nystatin is frequently prescribed as oral suspension in doses of 100,000 IU/mL, 4–6 mL four times daily rinses for 2–3 min, or pastilles (100,000 IU, four times a day), both formulations for 7–14 days (Bandara and Samaranayake, 2019; Quindos et al., 2019). Patient should be instructed to not eat or drink for 30 min afterwards. It is important to prolong the treatment at least 48 h after oral symptoms are resolved, to prevent from OC recurrence (Telles et al., 2017).

For angular cheilitis or denture stomatitis nystatin may be prescribed as cream (100,000 IU/g) three times daily for 10–14 days (Lombardi and Ouanounou, 2020). Topical miconazole (gel or cream) is also effective against OC, especially in those cases of denture stomatitis. Miconazole gel 2% can be applied to the fitting surface of the denture, two to three times a day, for 2 or 3 weeks. It could also be applied as a lacquer to dentures, once a week to dentures for 3 weeks (Williams and Lewis, 2011; Bandara and Samaranayake, 2019; Telles et al., 2017). Miconazole cream (2%) is as well a good therapeutical option for treating angular cheilitis, applied directly in the lesions two times a day for 2–3 weeks (Williams and Lewis, 2011; Lombardi and Ouanounou, 2020). It is important to consider that miconazole, even topically, may interact with warfarin and should also be avoided in pregnancy and patients with porphyria (Lombardi and Ouanounou, 2020; Quindos et al., 2019). Other agents as ketoconazole, clotrimazole, itraconazole or fluconazole could also be used topically (Lombardi and Ouanounou, 2020). Another topical antifungal is the Gentian violet solution. It is used in doses of 1.5 mL twice a day. This antifungal can cause oral ulcers, skin irritation and staining (Lombardi and Ouanounou, 2020).

Systemic treatment

In those cases where there is a widespread, recurrent, or refractory OC, systemic antifungals such as fluconazole or itraconazole should be used (Bandara and Samaranayake, 2019). Since ketoconazole causes hepatotoxicity, several drug interactions and potential teratogenicity, its clinical use has been very limited, and it should not be the first choice for treating OC (Lewis and Williams, 2017; Lombardi and Ouanounou, 2020). In the same line, as said before, it should be reminded when prescribing these

Table 5 Topical and systemic antifungal therapy most used in oral candidiasis.

Drugs (Class)	Dose
Nystatin (Polyene)	• Oral suspension, 100,000 IU/mL, 4–6 mL, four times daily, during 2–3 min for 7–14 days. • Pastilles (100,000 IU, four times daily) for 7–14 days.
Amphotericin B (Polyene)	• Cream (100,000 U/g), three times daily for 10–14 days. • Oral suspension (100 mg/mL), 100–200 mg for 14 days. • Lozenge (10 mg), for 14 days. • Cream, for 14 days.
Miconazole (Imidazole)	• Gel (2%), two to four times a day for 2–3 weeks. • Lacquer, once a week to dentures for 3 weeks. • Cream (2%), two times daily for 2–3 weeks.
Ketoconazole (Imidazole)	• Cream (2%), two to three times daily for 14–28 days. • Oral tablets, 200–400 mg, four times daily for 14 days.
Clotrimazole (Imidazole)	• Troches (10 mg), five times daily for 14 days. • Solution (1%), 5 mL, four times daily for 14 days. • Cream (1%), two to three times daily for 21–28 days.
Fluconazole (Triazole)	• Oral capsules, initial dose of 200 mg followed by 100 mg daily for 1–2 weeks.
Itraconazole (Triazole)	• Oral capsules, initial dose of 200 mg, three times daily for 3 days, followed by 100 mg for 11 days for refractory candidiasis. • Oral capsules, initial dose of 100 mg, four times daily for 14 days for initial candidiasis. • Oral suspension, 100–200 mg, two times daily for 7–14 days.
Voriconazole (Triazole)	• Oral, for refractory OC, at a dose of 200 mg two times daily for 28 days.
Posaconazole (Triazole)	• Oral suspension, for refractory candidiasis, 400 mg two times daily for 3 days, followed by 400 mg 4 times daily for 25–28 days. • For initial cases, 100 mg two times on the first day, followed by 100 mg four times daily for the next 13 days.

drugs that azole antifungal agents (including itraconazole and fluconazole) interact with warfarin (Lewis and Williams, 2017). Fluconazole is the most widely used, and it is frequently dispensed as oral capsules, in an initial dose of 200 mg followed by 100 mg a day for 1–2 weeks. Itraconazole should be chosen for treating refractory candidiasis. Other systemic antifungal drugs are voriconazole, isavuconazole and posaconazole. These new triazoles along with echinocandins are promising alternatives therapeutic options (Lombardi and Ouanounou, 2020; Quindos et al., 2019). Although the efficacy of certain antifungals such as nystatin, amphotericin B, clotrimazole, miconazole, ketoconazole, itraconazole has been demonstrated, there are studies that have shown how fluconazole is superior to other antifungals. As with antibiotics, there is also resistance among *Candida* species, which can also be a public health problem (Fang et al., 2020).

Table 4 summarizes systemic treatments for other fungal infections that may affect the oral cavity less frequently.

Adjunctive agents: Sialogogues, chlorhexidine, probiotics

There are other adjunctive therapeutic options that may contribute to *Candida* eradication. In those patients with reduced salivary flow and residual glandular capacity, the use of sialogogues may be assessed. This treatment aims to increase salivary flow and, therefore, the fungicidal power of saliva. Systemic sialogogues as pilocarpine have several side effects, and should be avoided in patients with glaucoma, hypertensive patients or those with uncontrolled asthma (Lombardi and Ouanounou, 2020).

Oral rinses with chlorhexidine gluconate (0.12%) for 1 min 2 times a day for 2 weeks can be used as a preventive measure in recurrent OC (Lombardi and Ouanounou, 2020). Other mouthwashes with antifungal activity as triclosan, or essential oil formulations (usually containing natural plant extracts as eucalyptol, thymol or bioflavonoids) may also be used. In cases of denture stomatitis, if the denture has metal components chlorhexidine 0.2% should be used for its cleanse (Bandara and Samaranayake, 2019).

Since there is an increasing resistance to the antifungal therapies, alternative treatments, as probiotics have emerged (Ribeiro et al., 2020; Shenoy and Gottlieb, 2019). Its usefulness in the treatment of OC is due to enhance immune response and mucosal barrier and reduce pathogens. In addition, they adhere to epithelial cells and mucus and can therefore reduce adhesion to the surfaces of fungal microorganisms. They also produce inhibition of *Candida* producing certain metabolites or competing for adhesion sites preventing *Candida* colonization and growth. Different studies have proven their positive effects as a treatment for OC, decreasing the levels of yeast and increasing IgA (Hoare et al., 2017; Ribeiro et al., 2020; Shenoy and Gottlieb, 2019). Different species of the genus *Lactobacillus* and *Bifidobacterium* may be a good option especially in elderly patients, or as a adjuvant treatment to antifungal therapies (Ribeiro et al., 2020; Shenoy and Gottlieb, 2019). There are studies that have used *B. longum*, *B. bifidum*, *L. rhamnosus*, *L. reuteri*, *L. casei*, *L. acidophilus*, *L. bulgaricus*, *S. thermophilus* or *Propionibacterium freudenreichii* to reduce *Candida* species levels in the oral cavity and dentures, and the oral pain produced by OC (Hoare et al., 2017; Shenoy and Gottlieb, 2019). However, probiotics have not shown superiority to the usual treatments for OC such as nystatin. Therefore, scientific evidence today shows their usefulness more in prevention than in the treatment of OC (Shenoy and Gottlieb, 2019).

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Oral and Dental Infections: Parasites

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Intro

The mouth is an organ involved in several activities, mainly respiration, phonation, alimentation, and interpersonal contacts. The anatomic bases of these functions are supported by different tissues providing structural and mechanical specificities. The surface of these tissues and their interfaces offer distinct barriers between the oral cavity and the internal milieu, as well as varied microenvironments possibly hosting microbiota. These interactions are not static but rather depend on the influence of flows in the mouth corresponding to the function of this complex organ: air for breathing and talking; liquids, solids, nutrients, and other molecules while feeding; microorganisms during interpersonal contacts or vectorial contamination by food or hands, among others. It is thus important to consider that the mouth can be contaminated by some substances or organisms, while it will not be properly infected by the latter. For instance, in taxonomic studies using 18s RNA gene sequencing to detect parasites or fungi in different oral sites, results can be filtered to exclude species of organisms coming from the food, like vegetables and animals. This obvious example questions us about the relevance of repeated detection, even if in high abundance: do we measure frequent habits of seeding the mouth with some microorganisms or do we evaluate their capacity to colonize this environment?

To define a true infection, the durability of the interaction between the host and the microorganism must be evaluated in the absence of any recontamination. Oral hygiene is a Sisyphean task that leads to the—often daily—disorganization of microbial communities. The main goal of this strategy in the dental environment is to hinder the maturation of biofilms to dental plaque and, later, to tartar. Supragingival accumulation of bacteria can initiate the formation of cavities. Subgingival calculi and plaque cause inflammation (gingivitis) and can lead to irreversible bone loss (periodontitis). In these biofilms are detected in vast majority bacteria, but it is now widely accepted that other infectious agents are present in the oral cavity, in particular eukaryotes and viruses, and that they may affect other tissues, like the mucosa. Fungal infections are not uncommon and oral candidiasis is a pathologic manifestation of the colonization of the mouth mucosae by *Candida albicans*. Other fungal genera are also frequently detected in the human periodontal sulcus, like *Malassezia*, *Aspergillus*, *Penicillium*, and *Rhodotorula*, while their role in periodontitis is still to be deciphered (reviewed in Karkowska-Kuleta et al., 2021).

Parasites—motile eukaryotic microorganisms—are also detected in the mouth: those for which this entry route is a dead-end (for instance, *Plasmodium falciparum* and *Amoeba proteus*), those that travel through it when contaminating the human host via an oral route (like *Entamoeba histolytica*, *Taenia solium*, and *Toxoplasma gondii*), and those that will settle a durable infection within the human host (*Leishmania* spp., *Trichomonas tenax*, and *E. gingivalis*). This chapter will focus on the latter group.

Amoebae in periodontitis

The parasites

Discovery

Amoebae are eukaryotic unicellular organisms with a characteristic motility. They adhere to the substrate, emit one or several pseudopods and drag the rest of the cell body onwards. Pseudopods are cytoplasm projections delimited by the plasma membrane, budding in the direction of the movement. This type of mobility has been described since the middle of the 18th century and the mere name of amoeba reflects the ability to move, observed in free-living amoebae, i.e., amoebae that do not need a host to survive and divide (Rösel von Rosenhof, 1755). It is noteworthy that the then-recent improvement of microscopes allowed to observe the deformations of these “big” eukaryotic cells, which probably increased the perception of their active movements as compared to flagellated protozoans or bacteria.

Amoebae were detected in association with humans as early as the middle of the 19th century: in the dental plaque (Fig. 1), with no mention of their possible involvement in pathogenic processes (Gros, 1849):

«Au milieu des productions du tartre des dents, on voit des vibrios, une sorte de végétation qui est quelque fois très régulière; mais on n'avait pas encore mentionné les vésicules que nous avons représentées Pl. VI. B. Ces vésicules ont un mouvement si lent et si obscur qu'il faut en être averti pour remarquer qu'elles prennent toutes les formes, par une extension et contraction amoebéenne, laissant toujours voir à l'intérieur des globules qui semblent se déplacer un peu, et être l'analog de ce que nous connaissons chez de certains infusoires soi-disant polygastriques. Leur origine, leur rôle et leur fin sont ignorés. Elles se trouvent surtout à la face interne des dents. Est-ce encore une génération spontanée?»

In the middle of the production of tartar of the teeth, vibrios can be observed, a sort of vegetation that is sometimes very regular; but were never mentioned before the vesicles we represented Pl. VI. C. These vesicles have such a slow and obscure motion that one must be aware of it to note that they can take any forms, by an amoeban extension and contraction. Inside of them, some globules are always visible—they seem to move a bit—and seem to be the analog to what is known in some infusoria, so-called polygastric. Their origin, their role, and their aim are ignored. They are found in particular on the internal face of the teeth. Is this again a spontaneous generation? (translation from the author).

In the era preceding Pasteur's and Koch's discoveries—though contemporary of John Snow's striving research on cholera and thus of the beginnings of modern epidemiology—these amoebae from clinical samples were not assumed or suspected to be pathogenic. Furthermore, periodontitis was such a common condition that its cause was not especially sought: it was rather considered part of the normal aging process.

The end of the 19th century was marked by the emergence and maturation of the concept of infectious disease, and an infectious etiology for all ailments was investigated. In this quest, *E. histolytica* was found to cause dysentery (Lösch, 1875). This close-related organism to *E. gingivalis* drew attention to the possible link between the oral parasite and periodontitis (Kartulis, 1893):

Der Substanzverlust entspricht darnach einem echten Knochengeschwür. Die Amöben liegen gewöhnlich am Grunde des Geschwüres, und auch dies erinnert an Bilder von dysenterischen Geschwüren. [...] Darüber, ob Amöben schliesslich Entzündung, beziehungsweise Verschwärung oder Abscessbildung bedingen können, möchte ich auf die Schlussfolgerungen verweisen, welche in meinen Arbeiten über die Aetiologie der Dysenterie, beziehungsweise der Leberabscesse zu finden sind.

The loss of substance corresponds to a real bone ulcer. The amoebae are usually at the base of the ulcer, and this too is reminiscent of images of dysenteric ulcers. [...] Regarding whether amoeba can ultimately cause inflammation, either by exacerbating or producing abscesses, I would like to refer to the conclusions that can be found in my work on the etiology of dysentery and liver abscesses. (translation from the author).



Fig. 1 *Amoeba gingivalis*, first description of *Entamoeba gingivalis*. Figure from Table VI C in Gros G (1849) Fragments d'helminthologie et de physiologie microscopique. *Bulletin de la Société Impériale des Naturalistes de Moscou* 22 : 549–573.

Beside this injunction to consider the association between *E. gingivalis* and periodontitis, the lack of rigorous epidemiological studies impeded to formerly conclude and thus to validate the first Koch's postulate, leaving the field with a blurry message about the etiology of the disease (Barrett, 1914):

It is well known with what persistent difficulties the dental profession has labored in connection with the condition usually spoken of as pyorrhea alveolaris; nor is there occasion here to go into details as to the various views which have been presented as to its etiology and the methods by which it is to be handled in treatment. Pyorrhea alveolaris has always been regarded as a form of bacterial infection in which local irritative conditions—such as the presence of tartar or the downward growth of the enamel organ in form of Hertwig's sheath—are thought to play at least a predisposing part, if no more, and in which systemic faults have very commonly by variously been looked upon as etiological factors by different writers at different times. [...].

From this standpoint and from a general interest in the fact that the studies of the protozoan parasitology of the mouth—a location appreciably suited to the protozoa from the viewpoint of its conditions of moisture, temperature, provision of appropriate food material and openness to the chances of infestation—have been relatively infrequent and unsystematized, the present study was undertaken. It seemed entirely probable that not only forms of rhizopods, such as the amoebae described by Kartulis and by Prowazek and others, in a comparatively limited number of cases, but possibly also flagellates and ciliates might be accommodated in this part of the body, and that those at least which are not mere surface habitants, but actually penetrate into the tissues, might in some of the inflammatory conditions of the mouth, particularly in the chronic ulcerative forms, in analogy with the chronic ulcerations due to dysenteric amoebae, be of pathogenic importance.

In this study, the 46 samples from periodontal pockets contained amoebae, as detected by microscopy (Barrett, 1914). Soon after this, a study detected *Entamoeba buccalis* in 199 of the 200 evaluated samples (Bass and Johns, 1914). The only sample in which amoebae were not detected came from a patient who recently used ipecac, whose root is used for emetine extraction. Ipecac root and emetine have been used to cure amoebic dysentery and liver abscess (Rogers, 1912), and have been shown to be effective against both inflammation and amoebae in the context of periodontitis (Barrett, 1914; Bass and Johns, 1914). However, this was not a formal demonstration for causation, and the specificity of the drug was soon questioned (Howitt, 1925). Reports about emetine cardiotoxicity changed the perception of the risk-benefit ratio (Chopra and Ghosh, 1922; Dale, 1915) and led to give up its use against amoebae.

This scientific odyssey about *E. gingivalis* involvement in periodontitis was not stopped by this failed attempt to indirectly demonstrate its etiologic role. Back to the first Koch's postulate, the association of the parasite with the disease was legitimately questioned: these amoebae are not rarely detected in "healthy" samples {14/46 = 30.4%; (Chiavaro, 1914)}. This was proposed to be due to common and recurrent contamination sources from the environment, also making the cure of deep pockets more fastidious (Bass and Johns, 1914):

As long as the lesion is not healed up it naturally offers favorable soil for reinfection. The disease is almost universal, and, therefore, chances of getting infection from others must exist almost daily.

To confirm the second and third Koch's postulates, microorganism culture and an adequate animal model are needed. However, *E. gingivalis* culture, though feasible (Howitt, 1925), is uneasy and polyxenic. Even if experimental periodontitis was developed in canine models, it was impossible to exclude the bacterial microbiota as the cause or a necessary adjuvant for the pathophysiology (Kofoid et al., 1929).

The stammering field of infectiology had then exhausted its resources to study the possible causative link between periodontitis and the presence of *E. gingivalis* or other microorganisms. The most convincing model for periodontitis was then non-infectious, and microorganisms were mainly thought to be opportunistic or saprophytic (Belding and Belding, 1933; Bruske, 1928). This status quo was maintained until new methods allowed a deeper characterization of the microbiota in parallel to the rise of new concepts about disease causation (Hill, 1965).

Cellular characteristics

Amoebae from the genus *Entamoeba* possess a cystic form in their life cycle allowing them to survive environmental stresses like high oxygen pressure from the air and desiccation and thus to be transmitted from host to host. Though early reports described the presumed cysts of *E. gingivalis* (Chiavaro, 1914; Craig, 1916; Smith and Barrett, 1915), it is now believed to be the only cyst-deprived species of the genus *Entamoeba*, existing only as trophozoites, its vegetative form. Several hypotheses can be formulated. First, transmission may be more direct than thought and oxidative stress may not be sufficient to kill the vegetative form of the parasite, the trophozoite. Second, interaction with bacteria could lead to higher resistance to oxidative stress, like for its close relative *E. histolytica* (Varet et al., 2018). Finally, cysts may not be produced in all individuals and in all the niches where trophozoites are found, meaning that they might not be found in some dental plaque samples in humans, where they are sought.

The genus *Entamoeba* comprises amoebae that emit a unique lobose (blunt) pseudopodia. The cells constantly deform, allowing them to displace and to feed. They are variable in size: in 1915, a review mentioned that, according to the sources, parasites could measure from 6 to 40 µm (Smith and Barrett, 1915). The intracellular composition of the parasite has also been described very early (reviewed in Howitt, 1925), mentioning its large spherical nucleus and intracytoplasmic food vacuoles. These food vacuoles are formed by the phagocytosis of human cells or microbes.

Genetic diversity

Before the emergence of genetic methods, morphological and physiological criteria of microorganisms were used to identify species. The amoebae of the oral sphere face different environments, from crevicular fluid or saliva to solid tartar deposits, from health-related microbiota to periodontitis-linked microenvironments, and finally are observed in different conditions for microscopy. It is thus expected to have strong variations in shape, composition, motility, and size, which led to the propose the existence of several species: *Amoeba gingivalis* (Gros, 1849), *Amiba buccalis* (Steinberg, 1862), *Amoeba dentalis* (Grassi, 1879), *Entamoeba buccalis* (Prowazek, 1904), and *Entamoeba kartulisi* (Kartulis, 1893). These were soon reunited as the same species by Smith and Barrett (1915), and confirmed in a close to consensual manner (Craig, 1916).

Later, phylogenetic relationships in the *Entamoeba* genus have been studied by riboprinting and revealed two ribodemes for the *E. gingivalis* species, confirming some genetic differences exist (Clark and Diamond, 1997). These isolates were however very closely related, and more similar between them than with any other isolate from other *Entamoeba* species. Further analyzes showed that single nucleotide polymorphisms exist in the 18S rRNA gene for different *E. gingivalis* isolates and allowed, together with low-stringency single specific primer PCR (LSSP-PCR), the identification of 4 different strains in addition to the reference strain (ATCC30927) (Cembranelli et al., 2013). Interestingly, these five strains belong to the same *E. gingivalis* subtype (ST1), as opposed to the newly characterized “kamaktli variant,” or *E. gingivalis* ST2 (Garcia et al., 2018b). It is noteworthy that the sequencing of the rRNA:18S-ITS1-5.8S-ITS2 shows a similarity between the subtypes that is lower than this observed between the species *E. dispar* and *E. histolytica*. Furthermore, a differential link between the subtypes and the periodontal health status has been proposed but is still to be investigated (Garcia et al., 2018a; Zaffino et al., 2019).

It is noteworthy that the full *E. gingivalis* genome is still not available.

Entamoeba gingivalis and periodontal diseases

Epidemiology

Periodontitis is characterized by irreversible bone loss, leading to clinical attachment loss and to the formation of periodontal pockets. The subsequent retraction of gingival tissues will result in a visible marginal recession, which testifies for an older bone loss. Beside the irreversible loss caused by periodontitis, the inflammation and gum swelling in gingivitis is defined as reversible, without bone loss and deep pockets. Following this model, it was early questioned if the transition from gingivitis to periodontitis was due to a change in the microbiota and, more precisely, to the colonization by a pathogen, as opposed to a model in which gingivitis would be an initial, lower-grade periodontitis with undetectable irreversible damage, though possibly existent.

The development of genetics-based diagnostic methods, which are less impacted by the user's subjectivity and technical skills, allowed the reassessment of the link between *E. gingivalis* and periodontitis. By PCR, the prevalence of the amoeba in periodontal pockets is 76.9% (95%-CI: 71.5%–81.7%), while it is 29.6% (95%-CI: 24.4%–35.3%) in healthy sulci, giving a risk ratio of 3.96 (95%-CI: 1.57–9.98) by meta-analysis (Martin-Garcia et al., 2021). The ST1 was subtype more prevalent (70.6%, 95%-CI: 65.0%–76.2%) than ST2 (43.9%, 95%-CI: 35.5%–52.4%) in periodontal pockets, though their risk ratios are equal: 3.30 (95%-CI: 1.27–8.55) for ST1 and 3.30 (95%-CI: 0.42–26.03) for ST2. In parallel, the subtypes might exhibit a different prevalence in gingivitis (Garcia et al., 2018a).

Pathophysiology

Besides the correlation between parasite presence and disease, the possible virulence of *E. gingivalis* was based on its ability to phagocytose human cells and bacteria in plaque smears (Child, 1926). Most of the virulence traits of *E. gingivalis* were suspected due to the vicinity of the morphologically indistinguishable pathogenic species *E. histolytica*. Recent studies have shown that *E. gingivalis* can provoke inflammation and tissue destruction in vitro (Bao et al., 2020, 2021). The mechanisms leading to these effects could be conserved with *E. histolytica* and are still to be investigated.

Conclusive remarks: Therapeutic management

Diagnosis of periodontal disease is based on the clinical detection of irreversible damage. As *E. gingivalis* is strongly correlated with periodontal diseases, it can be hypothesized that it is a good indicator of ongoing pathogenic processes. Thus, clinical and microbiological diagnosis may not be equivalent, and the detection of amoebae may allow the early detection of periodontally compromised sites.

Prophylactic strategies against the parasites are difficult to implement since both its life cycle and its role in the pathophysiology of the disease are not defined. The consensually assumed absence of a cystic stage reminds us of the possible inadequacy of the conclusions extrapolated from *E. histolytica*. However, treatment using emetine led to the clearing of parasites in humans (Barrett, 1914; Bass and Johns, 1914), while metronidazole is currently used with this aim by some dentists (Bonner et al., 2013) and was shown effective against *E. gingivalis* (Eloufir et al., 2014). As metronidazole is often used to target anaerobic bacteria, it may already take part into clearing *E. gingivalis* from periodontal pockets.

Trichomonads in periodontitis

The parasites

Discovery

Trichomonads are anaerobic flagellated protists. The members of the genus *Trichomonas* have a typical pear shape with a broad front end and a finer tail, as described for the species *Trichomonas tenax* in 1773 by Otto Friedrich Müller, who named it “*Cercaria tenax*” (Müller, 1773):

Membranous *Cercaria*, well-defined and somewhat more solid anterior part, rear-end three times as short. Substantially bigger than *Monas lens*. Transparent, inverted egg-shaped membrane with a neat and strong front; it ends as a sharp at its posterior extremity or in a very short tail. No internal apparatus. Moves in all the directions with quick turns [...] In infusion of tooth dirt within 4 days. (Translation from Latin by the author).

Donné proposed in 1836 to give the name “*Trico-monas vaginale*” to the microbes found in the purulent vaginal discharge who are close to “*Monas*” because of their “trunk,” and to “*Tricodes*” because of their cilia (Donné, 1836) (Fig. 2), later respelled “*Trichomonas vaginalis*.” For decades, several species of *Trichomonas* were suspected to live in the mouth, as reported later (Dobell, 1939). The consolidation of the *Trichomonas* genus in man, with three species, each one unique in its niche as we know now (*T. tenax* in the mouth, *T. vaginalis* in the urogenital tract, *T. hominis* in the intestine) was fixed by Wenrich (1947).

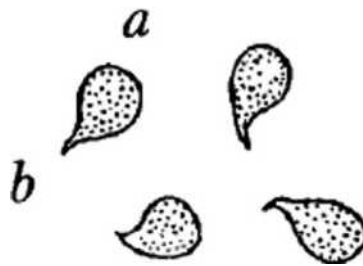


Fig. 2 *Cercaria tenax* (Müller, 1786), with a “stronger front” (A) and a “small tail” (B). Reproduction by Dobell C (1939) The common flagellate of the human mouth, *Trichomonas tenax* (O.F.M.): Its discovery and its nomenclature. *Parasitology* 31(1) : 138–146.

Cellular characteristics

The life cycle of the parasite *T. tenax* is still undeciphered. Beside the pear-shaped trophozoite form commonly found in culture, a rounded, an amoeboid, and a pseudocyst form were described when interacting with cells (Ribeiro et al., 2015). Interestingly, a pseudocyst form also exists in the close-related *T. vaginalis* species, which is devoid of a typical cyst wall, undergoes membrane deformation, and causes strong pathogenic effects (Afzan and Suresh, 2012; Dias-Lopes et al., 2017). An additional cyst-like structure has been produced in vitro and detected in clinical samples (Berl et al., 2019). They possess a thick electron-dense layer consistent with a cyst wall and are more resistance to chlorination than trophozoites. Cyst-like structures could play a role in the non-sexual transmission of the parasite (Pereira-Neves and Benchimol, 2008). The existence of such structures in *T. tenax* is still questioned.

As the parasite *T. vaginalis* is more extensively studied, it is tempting to speculate about the biology of *T. tenax* based on the data obtained with the close-related species from the feminine sexual tract. However, the environments in which they evolve exhibit differences and axenic trophozoite cultures are available since 1962 (Diamond, 1962). Thus, caution must be taken when extrapolating from the parasite from the feminine sexual tract and fundamental research using *T. tenax* should be promoted.

The parasite *T. tenax* revealed it has a fibronectin-like surface molecule probably involved in adherence to microbes and to host cells (Ribaux et al., 1983). It also harbors proteases (Bozner and Demes, 1991a,b; El Sibaei et al., 2012; Yamamoto et al., 2000) and hemolysins (Nagao et al., 2000), which may be involved in target lysis. Virulence of *T. tenax* is now undoubted and the parasite is cytotoxic towards several types of cells (Ribeiro et al., 2015). However, the mechanisms involved in the interaction with host cells still remain to be elucidated.

Genetic characteristics

Only one species of trichomonads is suspected to colonize the human mouth since the reunification of the different candidate species in 1939 (Dobell, 1939). The first full genome sequence of *T. tenax* has been recently released (accession number PRJEB22701) and is still to be assembled and annotated. The same research team already published the link between *T. tenax* genetic background and the severity of periodontitis in the same geographical area (Benabdelkader et al., 2019).

Trichomonas tenax and periodontal diseases

Epidemiology

The parasite *T. tenax* has been associated to the dental plaque since its identification. However, the extent of its association with periodontitis is still discussed. By PCR, the prevalence of trichomonads in periodontal pockets is 38.6% (95%-CI: 27.2%–50.0%), while it is 1.6% (95%-CI: 0.0%–3.3%) in healthy sulci, giving a risk ratio of 21.82 (95%-CI: 6.71–70.96) by meta-analysis

(Martin-Garcia et al., 2021). This is consistent with the results obtained in another meta-analysis pooling all diagnostic methods, including culture and microscopy, which revealed a prevalence of *T. tenax* of 27% (95%-CI: 10%–48%) during periodontitis (Eslahi et al., 2021).

The results vary consistently between the studies and may be explained by the differences in the definition of health and disease. Indeed, tooth mobility, accumulation of plaque, and clinical attachment loss were correlated to the presence of *T. tenax* (Bisson et al., 2018). This suggests that *T. tenax* may be associated to specific stages or grades of the disease, or even to other periodontal disease types. This underlines the need to better study the link between clinical parameters and the parasites that are not independent and may be of biological relevance.

Pathophysiology

Though *T. tenax* is cytotoxic to cells in culture and *T. vaginalis* is the etiologic agent of one type of vaginosis, the precise role of *T. tenax* in certain types of gum inflammation and periodontal diseases is still to be formally established. Several hypotheses have been presented and *T. tenax* may exacerbate gingivitis, as discussed elsewhere (Marty et al., 2017). By integrating recent data, we can hypothesize that *T. tenax* may be a superinfecting microorganism over an already present plaque-induced gingivitis, resulting in a rapid and direct cytotoxicity towards host cells. This parasite may be the etiologic agent of necrotizing gingivitis. However, formal evidence is missing, and this hypothesis should be studied by idoneous scientific explorations before further conclusions.

Conclusive remarks: Therapeutic management

Infection by *T. tenax* does not appear to be correlated to initial stages of periodontal diseases, but rather seems to be associated with some types of advanced and fast-evolving periodontal diseases. Microbiological diagnosis may help the identification of cases with a risk for rapid progression of damage. The link of immunodepression with necrotizing gingivitis, through neutropenia (Feller et al., 2012), highlights the interest to study the possibility of different etiologies and aggravating factors for these diseases with similar clinical presentations.

Trichomonads are anaerobic and vaginal trichomoniasis is efficiently treated with nitroimidazoles, which may be suitable for the elimination of *T. tenax* in the periodontal sulci. Interestingly, scaling and root planing consistently decrease parasite detection in diseased sites (Dubar et al., 2020). This result is consistent with the association of the parasite with dental plaque (Bisson et al., 2018) and recalls the importance of prophylactic or therapeutic strategies involving hygiene and non-surgical periodontal therapy in the prevention of periodontal diseases.

Trypanosomatids in oral mucosal lesions

The parasites

Discovery

Trypanosomatid parasites were described for the first time in 1841 (Valentin, 1841), while the name of genus “Trypanosoma” was used for the first time in 1843 (Gruby, 1843). The description of flagellated protozoans in cutaneous lesions (“Sart sore”) was made by Borovsky in 1898 (Hoare, 1938) but the name of the genus was later given by Ross (1903).

The oral mucocutaneous form of leishmaniasis was originally proposed to be either due to metastatic dissemination of parasites (“espundia”), or by local extension of cutaneous lesions on the face (“uta”) (Escomel, 1911) (Fig. 3). Though they may differ in

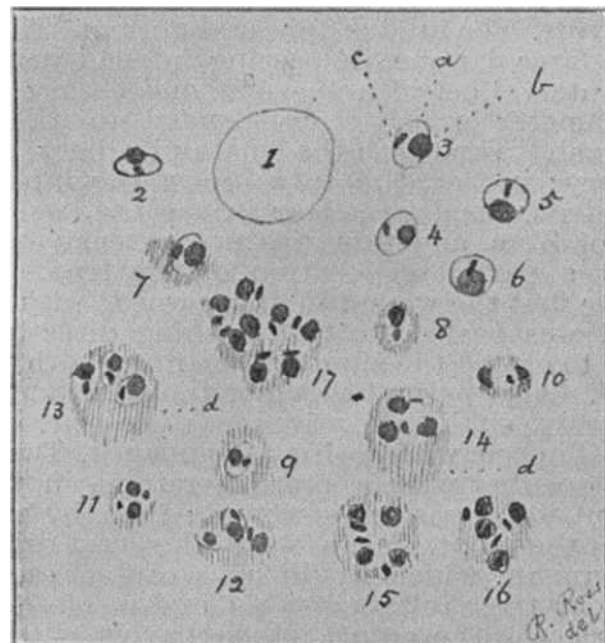


Fig. 3 “*Leishmania donovani*, Laveran: 1, red corpuscle; 2–6, free forms; 7–17, embedded forms; (A) contour line; (B) macronucleus; (C) micronucleus; (D) matrix.” From Ross R (1903) Further notes on Leishman’s bodies. *British Medical Journal* 2(2239): 1401.

clinical presentations and outcomes, a great number of the species of the genus *Leishmania* are now known to cause mucocutaneous leishmaniasis (MCL).

Cellular characteristics

Parasites from the genus *Leishmania* have two main hosts. The mammalian hosts support an intracellular mitotic replication of amastigote forms, which are rounded and short-flagellated (originally detected as unflagellated by microscopy, hence the name). These forms can infect phagocytic and non-phagocytic cells, in which they replicate until their release. Infected macrophages can be ingested during the blood meal of the insect host, the phlebotomine sandfly. When exiting the cells, the parasite differentiates into extracellular elongated promastigote forms, which possess a long flagellum and are endowed with motility. These forms replicate by mitosis within the digestive tract of the insect, to which they adhere before they are injected into a new mammalian host. They are actively internalized by professional phagocytes, in particular monocytes, in which they differentiate into amastigotes.

Genetic characteristics

The *Leishmania* genus is complex, with an increasing number of identified species, of which several can provoke pathologies in humans. Common classification of the species is done according to the geographic area where they are identified (Paleotropics or Old World, as compared with Neotropics or New World), to the site where they divide in the phlebotomine vector (parasites from the subgenus *Viannia* divide in the hindgut, promastigotes from the subgenus *Leishmania* develop in the midgut), or to the type of disease they yield (tegumentary or visceral presentations). MCL is generally attributed to *L. tropica* in the Old World; *L. braziliensis*, *L. panamensis*, and *L. guyanensis* in the New World. However, increasing numbers of studies report that other species can cause MCL.

Furthermore, the picture is more complex because the great genomic diversity of the *Leishmania* genus is enhanced by sexual genetic exchanges (Patino et al., 2020). Indeed, parasites can mate in the phlebotomine after meiosis-like events, and hybrids can be transmitted to mammals (Akopyants et al., 2009). Thus, the sandfly can be considered a definitive host, while the mammals are intermediate hosts.

Interestingly, in *Trypanosoma cruzi*, another parasite from the Trypanosomatidae family, genetic characteristics were not the only determinant for the clinical presentation of the disease in mammals, evidencing the role of host-related and infection-linked parameters (Santi-Rocca et al., 2017). Infection by *Leishmania* species might also be influenced by these parameters, causing the variety of presentation within the same taxonomic entity.

Leishmania spp. and oral mucosal leishmaniasis

Epidemiology

The parasite needs two host pools to spread: a mammal one—which can be human—and an insect one. Consequently, the areas in which human leishmaniasis is present are limited by the geographic distribution of its insect host, from the *Lutzomyia* genus in the New World and the *Phlebotomus* genus in the Old World. Strategies preventing contact between humans and insects can be efficient to control the transmission of the parasite. Thus, socio-economic parameters also impact the incidence of the disease. The WHO warns that more than 90% of the cases of MCL occur in Brazil, Bolivia, Ethiopia, and Peru (<https://www.who.int/es/news-room/fact-sheets/detail/leishmaniasis>).

The global burden of oral leishmaniasis is difficult to evaluate since the most frequent site for MCL is the nose and often indistinctly classified with cutaneous leishmaniasis. Furthermore, the declaration of the disease is not mandatory in all endemic countries, introducing a strong bias.

Pathophysiology

The possible and frequent evolution from long-lasting cutaneous forms of leishmaniasis and MCL has been identified long ago (Pessoa, 1941). Indeed, parasites can migrate from cutaneous lesions, probably carried by lymphoid cells or directly by blood, triggering a new lesion in the oral-nasal environment from weeks to years after the scarring of the initial ulcer (Handler et al., 2015).

The damage in MCL seems to be fueled by an excessive Th1 response, as compared to cutaneous lesions. However, inflammation is needed to control infection, as evidenced by the exacerbated parasite replication caused by the use of immunomodulators (TNF inhibitors), which can lead to death (Fromm et al., 2015). In parallel, ablation of Tregs results in exacerbation of the cutaneous disease provoked by *L. panamensis* infection, while adoptive transfer of naïve Tregs hindered the pathophysiology, with reduced cutaneous damages, parasite load, and cytokine production (IL-10, IL-13, IL-17, and IFN- γ) (reviewed in Conceicao-Silva et al., 2018).

It is noteworthy that data missing from the description of the pathophysiology of human MCL are completed by results for MCL in animal models or extrapolated from other types of lesions provoked by infection with parasites from the *Leishmania* genus. There is a lack of homogeneity in the species used for these description and caution should be taken when assuming mechanisms are conserved between hosts, parasite species, and clinical presentations.

Therapeutic management

The prevention of MCL resides mainly in the protection from primary infections, may they be visceral or cutaneous. The first strategy is to prevent contamination by avoiding contact with the parasite-transmitting insect vector (Stockdale and Newton, 2013). This can be done using insecticide-impregnated nets. Then, the canine reservoir of the parasite can be controlled.

The molecular identification of *Leishmania* parasites by PCR is the most sensitive method for MCL diagnosis (Cantanhede et al., 2021). However, more affordable techniques like immunohistochemistry can be an acceptable option in some endemic regions (Sanchez-Romero et al., 2020). Serological tests are of limited use due to the cross-reactivity of antibodies directed towards other trypanosomatids, and with the possible persistence of antibodies developed during visceral or cutaneous forms of the disease (discussed in Abadias-Granado et al., 2021).

It is noteworthy that, though several species can provoke MCL, differential diagnosis and treatment are not routinely made. The therapeutic management relies on the identification of parasites from the *Leishmania* genus, which is sufficient to justify the choice of a systemic therapy involving either liposomal amphotericin B (intravenous) or pentavalent antimonials (intravenous or intramuscular). These treatments are nephrotoxic and cardiotoxic, respectively. Other strategies can be considered, like the use of azoles, miltefosine, and pentamidine (reviewed in Abadias-Granado et al., 2021).

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Sexually Transmitted Diseases*

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Introduction

Global estimates indicate there are approximately 374 million new curable sexually transmitted infections (STI) each year and, in many regions of the world, STI incidence is on the rise. STI comprise a diverse category of pathogens including viruses, bacteria, fungi, and protozoa, that spread primarily through sexual contact (Fig. 1). Specifically, there are currently more than 30 recognized bacterial, viral and parasitic pathogens that may be transmitted and acquired through sexual contact (World Health Organization [WHO], 2021a), and which manifest in a number of clinical symptoms (McGough, 2008). The most prevalent STI include human immunodeficiency virus, herpes simplex virus, human papillomavirus, and hepatitis B virus, syphilis, gonorrhea, chlamydia, and trichomoniasis. The spread and control of such STI is determined by a complex interplay of biological, behavioral and social factors which are influenced by political, economic, demographic, and cultural conditions (Garnett, 2008).

STI constitute a considerable disease burden and persist as a major source of morbidity and mortality around the world. Globally, the incidence and prevalence of STI varies significantly. Health inequity remains widespread, with some global regions and communities bearing a disproportionate burden of STI and sequelae. Ultimately, STI continue to have a significant impact on sexual and reproductive health, thus the epidemiological study and prevention of STI are critical to advancing global public health.

STI and epidemiology

Epidemiology is the study of the patterns, causes and risk factors of disease (Garnett, 2008). Epidemiological evidence is used to address and prevent health problems, such as sexually transmitted infections. STI epidemiology examines the patterns, causes, risk, and outcomes of acquisition at both the individual and population levels. The epidemiology of STI has shifted dramatically over the past century as a result of social, behavioral, political, economic, and demographic forces, as well as advances in diagnostic testing and procedures (Lowndes and Hughes, 2010; Aral and Holmes, 2008). The ultimate determinants of transmission include sexual behaviors and changes in sexual practices, social network formations, and the uptake of effective prevention messaging, all of which contribute to shifting STI epidemiology.

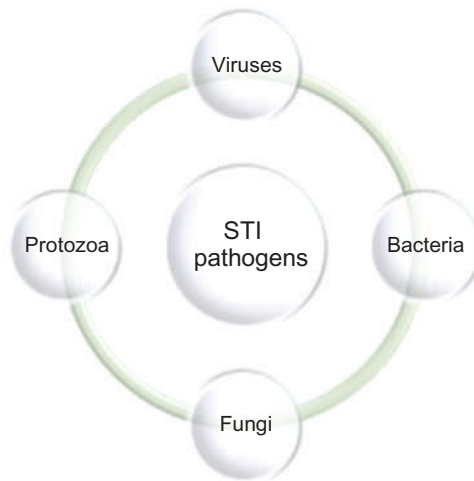


Fig. 1 STI comprise a diverse category of pathogens including viruses, bacteria, fungi, and protozoa, which manifest themselves in a number of clinical symptoms.

Importance of STI study

STI are among the most common and potentially devastating group of infections in the world. Historically, major investment from government and medical actors has been limited, however, in recent years there have been some new efforts directed at the health sector response to STI epidemics. Most notably, the *World Health Organization Global health sector strategy on Sexually Transmitted Infections, 2016–2021*, which aims to “contribute to a radical decline in new sexually transmitted infections and in deaths related to such infections (including still births and cervical cancer), while improving individual health, men’s and women’s sexual health, and the well-being of all people” (WHO, 2016, p. 2). Such initiatives are critical and must be paralleled by the ongoing study of STI, the importance of which cannot be understated, for reasons including:

1. Advances in biomedical knowledge and technology have led to new and improved diagnostic options. New diagnostic approaches have facilitated epidemic investigations that have identified various modes of transmission, prevention strategies, and the consequences of sexually transmitted infections and their sequelae.
2. New risk groups have been identified and the number of individuals affected by STI, including HIV, has increased. STI are unequally distributed; social and structural determinants of STI, including stigma and discrimination, put certain people and communities at greater risk for infection. Disadvantaged and marginalized persons, people who inject drugs, prisoners, sex workers, and men who have sex with men are disproportionately impacted by STI and sequelae.
3. STI affect hundreds of millions of people globally each year, presenting high rates of morbidity and mortality. STI and sequelae are tied to serious health complications including cancers, male and female infertility, and poor birth outcomes.
4. Left untreated, STI can have serious implications for reproductive, maternal and child health and well-being. Specifically, STI are the main preventable cause of infertility in women (WHO, 2011) and may lead to certain types of genital cancers. In addition, STI may be transmitted through mother-to-child transmission and cause adverse birth outcomes (e.g. stillbirths, prematurity, neonatal conjunctivitis).
5. Global health inequity remains a major issue. Access to treatment and prevention measures is limited in many areas of the world and continues to challenge practitioner, government, and nongovernment organization efforts to curb STI rates.
6. Sexual behaviors, which are inextricably tied to STI, are constantly evolving which means researchers, public health professionals, and clinicians are presented with new and shifting challenges.

Key factors affecting public health

The global burden of STI is high. According to estimates, there are more than 1 million new daily cases of curable STI in persons aged 15–49 years (this number includes chlamydia, gonorrhoea, trichomoniasis, and syphilis; it does not include HIV and other STI), making STI among the most common communicable diseases around the world. STI continue to adversely affect the health and well-being of individuals and communities (Rowley et al., 2019; WHO, 2019a), and remain epidemic in most of the world.

The complexities of STI transmission present major challenges to traditional public health interventions and control efforts (Fenton and Lowndes, 2004). Moreover, prevention programs that aim to curb transmission, exist in highly diverse, complex, and dynamic social, cultural and health-service settings. There are also major differences in the availability of resources, as well as in the range and extent of both prevention and intervention services among different global regions and populations. These differences may be influenced by a number of factors such as (but not limited to): the level of various STI and health conditions in

communities—communities that bear a significant burden of infection may experience a strain on often already limited resources; the level of preventive health services available; access to diagnostic tools; and the amount of financial resources put into STI prevention or management services. While in many countries access to clinics is readily available, in resource-limited regions and countries with developing public health systems, clinics and health care centers may be sparse or nonexistent altogether.

Populations at risk

There are significant differences in STI prevalence and incidence between populations. The incidence, prevalence, and population distribution of STI are largely determined by a complex interplay of dynamic environmental, demographic, economic, political, social, cultural and behavioral forces, as well as the response of health systems to emergent STI morbidity patterns (Aral and Holmes, 2008). The social and structural determinants of transmission often operate in tandem (Lowndes and Hughes, 2010), influencing risk and making certain global communities especially vulnerable to infection. Consequently, some persons and population group are disproportionately affected by STI.

1. **Adolescents:** Adolescents and young adults are particularly vulnerable to STI. Research suggests that there is a global, national, and local epidemic of STI among individuals aged 15–24 years (Reed and Huppert, 2011; Shannon and Klausner, 2018). In all societies, STI tend to concentrate in certain populations, with highest rates among sexually active adolescent females followed by adolescent and young adult men. This pattern is particularly pronounced in the West where effective prevention and control efforts result in concentrated STI morbidity.
2. **Women:** Studies indicate a gender disparity – women under 25 have higher rates of STI than any other population (Reed and Huppert, 2011). In addition, STI adversely affect the health of women and can have serious implications for reproductive, maternal, and newborn health (WHO, 2011). Women in resource-limited countries are disproportionately impacted and experience the adverse effects of STI most acutely.
3. **People experiencing poverty:** Poverty is well understood as a pathway to STI, with studies drawing a causal link between socioeconomic vulnerability and risk of infection. STI are unevenly distributed and disproportionately affect resource limited global regions. STI diagnosis and treatment are often unavailable, inaccessible, delayed, or inadequate (Bertozzi and Opuni, 2008).
4. **Marginalized persons and populations:** Men who have sex with men, transgender women, injection drugs users, prisoners, and sex workers, also experience a greater burden of disease, in part due to a lack of targeted testing and treatment, as well as poor access to care due to stigma and discrimination. Consequently these risk groups bear a greater burden of infection and disease. Moreover, there are often intersections among these groups, making certain persons and communities particularly vulnerable to STI acquisition and sequelae.

Trends in STI

Viral infections

Incurable STI are characterized by persistent viral infections that are often lifelong in duration (Mabey, 2010). Though viral STI are long lasting, they are characterized by lower transmission probabilities. Over time, and especially over the past two decades, viral infections have been increasing in prevalence in many regions of the world. At the same time, diagnosis, management and control of viral STI have changed as the introduction of new diagnostic technologies has increased recognition of viral infections.

The four most common viral STI are:

1. human immunodeficiency virus,
2. herpes simplex virus,
3. human papillomavirus,
4. hepatitis B virus.

Human immunodeficiency virus

The epidemiology of human immunodeficiency virus (HIV) has shifted since the beginning of the HIV epidemic nearly 40 years ago. HIV is transmitted through various means including exposure to infected blood or blood products, mother-to-child-transmission, and sexually—the predominant mode of transmission. There has been major progress in the prevention, diagnosis and treatment of HIV. A cure, however, remains elusive. As such, while the world has seen a decline in new infections and mortality, HIV persists as a considerable global public health issue. See Epidemiology of HIV in this edition.

Herpes simplex virus

Infection with herpes simplex virus (HSV) is common among all sexually active populations. Two types of herpes simplex virus (HSV) have been identified: HSV-1 and HSV-2. The majority of cases (85–90%) of recurrent HSV are caused by HSV-2 (Da Ros and Da Silva Schmitt, 2008; Centers for Disease Control and Prevention [CDC], 2021a). Genital herpes, caused by HSV-2, is chronic and lifelong with recurrent episodes of painful genital ulceration. Infections take an average of 3 weeks to resolve and then enter a latent

state from which recurrences of symptomatic lesions occur with decreasing frequency over time. These recurrences create a long infectious period that can expose subsequent sexual partners to infection. While HSV-1 is primarily an oral infection, it is suggested to be emerging as a rising cause of genital herpes in some settings (Looker et al., 2020).

Genital ulceration is generally more frequent, more severe, and longer lasting in immunocompromised individuals. A potential “epidemiological synergy” has been noted between HSV-2 and HIV, with HSV-2 linked to a threefold increase in sexual transmission and acquisition of HIV (Harfouche et al., 2021; Looker et al., 2020). Risk increases due to ulcers caused by HSV-2 infection which provide a direct physical route of entry for HIV, and HIV-prone activated T-cells and dendritic cells, resulting from the immune system’s response, are present in significant numbers at the site of HSV-2 infection.

HSV is one of the most common genital ulcerative STI in the world (Aral and Holmes, 2008; Hawkes, 2008). The WHO (2019a,b) estimates that globally nearly 500 million people are living with HSV, with prevalence of HSV-2 infection highest in Africa and followed by the Americas. Greatest HSV disease burden is experienced by populations in sub Saharan Africa, where HSV-2 incidence remains high and significantly higher than other parts of the world (Harfouche et al., 2021). Incidence of HSV-2 is greatest in women and particularly women living in the WHO African Region.

Seroepidemiological studies provide useful insight into the cumulative incidence and prevalence of HSV infection as they confirm the strong association between HSV seroprevalence and increasing age, number of partners during the previous year, higher seroprevalence in females compared with males, as well as the increasing proportion of first episodes of genital herpes being caused by HSV-1 instead of HSV-2 (Aral and Holmes, 2008). There is currently no cure for herpes, however antiviral medications may reduce the severity and frequency of symptoms. Available prevention modalities, such as condoms and antiviral therapy, do not sufficiently control infection spread; therefore the integration of prevention and control measures is needed. Consequently, the WHO and global partners have identified the development of an HSV vaccine and topical microbicides as a critical global priority.

Human papillomavirus

Human papillomaviruses (HPV) belong to the family *Papillomaviridae* and present among all sexually active groups. *Condylomata acuminata*, or genital warts, are caused by HPV and are spread through skin-to-skin contact (Ayadpoor and Hall, 2011). Genital warts are the clinical manifestation of infection with certain types of HPV (specifically types 6 and 11) (Lowndes and Hughes, 2010). There are more than 100 subtypes of HPV (Ayadpoor and Hall, 2011), with as many as 40 transmitted through genital-to-genital contact.

HPV is the most common STI in the world, with most sexually active persons acquiring HPV at some point in their lives. Roughly 1 million new cases of HPV are diagnosed each year and prevalence is between 24 and 40 million. Women bear a disproportionate burden of infection; globally, nearly 300 million women live with HPV (WHO, 2019a). HPV is responsible for a substantial global burden of cervical and other cancers (Chesson et al., 2017), and types 16 and 18 are particularly carcinogenic to humans, accounting for at least 70% of cervical cancer cases (Tota et al., 2011; WHO, 2022).

Although HPV is currently incurable, it is preventable. A first major step in prevention was seen in June 2006 when a quadrivalent HPV vaccine was licensed by the Food and Drug Administration for use in the US (Bryer, 2010). There are now 3 vaccines that have been prequalified and, as of late 2020, HPV vaccines were made available as part of routine immunization programmes in 111 countries (WHO, 2021a). The vaccines all provide protection against types 16 and 18—the serotypes most commonly associated with cervical cancers—and two also protect against types 6 and 11, which cause genital warts (WHO, 2020b). Vaccination is indicated for females aged 9–26 and received ideally before women become sexually active. The US guidelines recommend routine vaccination for females 11–12 years and “catch up” vaccination for young women 13–26 who have not been vaccinated previously (Ryan et al., 2008; Bryer, 2010).

While the WHO indicates, “HPV vaccination could prevent the deaths of millions of women over the next decade in low- and middle-income countries, where most cases of cervical cancer occur, if high (>80%) vaccination coverage of young women (ages 11–15) [is] achieved,” the vaccine remains inaccessible to many women in resource-limited settings, in large part due to the expensive cost. Implementation is therefore a key challenge, with significant obstacles to vaccine integration in national immunization programs (see *Program for Appropriate Technology in Health (PATH)*, 2011). Moreover, although the vaccine targets the serotypes that are most often associated with cervical cancer, the serotypes most often responsible for genital warts are not covered. Treatment costs associated with HPV and its related disease are exponential; in 2010, in the US alone, treatment costs were estimated to be in excess of \$4 billion per year (Bryer, 2010).

Hepatitis B virus

Hepatitis B infection is prevalent in much of the world, although overall incidence is declining. There are many routes of hepatitis B virus (HBV) transmission including, most commonly perinatal transmission to newborn infants, exposure to infected blood products, the reuse of unsterilized needles, syringes, or medical equipment, the use of contaminated needles and paraphernalia associated with injection drug use, and sexual exposure (Ayadpoor and Hall, 2011).

HBV is 50–100 times more infectious than HIV (WHO, 2012). Two billion people worldwide have acquired HBV and, in 2019, an estimated 296 million people were living chronic HBV (WHO, 2021b). Certain global regions bear a disproportionate burden of infection, with HBV endemic in several parts of the world including parts of Asia and many regions of Africa (Shepard et al., 2006).

The WHO (2021b) estimates that 116 million people in the WHO Western Pacific Region and 81 million in the WHO African Region have a chronic HBV infection. In contrast, in western Europe and North America less than 1% of the population is infected (WHO, 2012).

Most (90–95%) adults are able to fight the virus while the infection is in its early (acute) stage, however, between 5 and 10% experience a progression of the disease, at which point it becomes chronic. The likelihood of HBV becoming chronic depends on an individual's age at infection as chronic infection is likely to occur in younger populations (Workowski and Berman, 2010). Acute hepatitis presents few long-term consequences, however chronic cases may lead to cirrhosis and hepatocellular carcinoma (liver cancer); approximately 15–25% of people with chronic HBV die of liver disease. It is estimated that approximately 600 000 people die each year due to the consequences of HBV (WHO, 2012). Moreover, HBV-HIV coinfection is common. According to the WHO (2021b), roughly 1% of persons with HBV infection are also living with HIV and the global prevalence of HBV infection in persons with an HIV infection is more than 7%.

There is not a specific treatment course for acute HBV, though chronic HBV infection may be treated with antivirals. Given high HBV-HIV coinfection, as of 2015, the WHO has recommended treatment for anyone diagnosed with HIV infection, regardless of disease stage.

HBV transmission can be prevented through the use of condoms and education of the disease, particularly to partners regarding the potential of transmission through body fluids (Ayadpoor and Hall, 2011). There is also a safe and effective vaccine that provides 98–100% protection, with protection lasting at least 20 years. The HBV vaccines series is recommended as part of routine vaccination and the WHO recommends that all infants are vaccinated as soon as possible after birth. However, adoption of this has been slow and is limited in resource-poor countries.

Bacterial infections

Most treatable STI are caused by bacteria and protozoa (Mabey, 2010). This group of STI is characterized by a relatively short lifespan and a high probability of transmission (Garnett, 2008). The prevalence data and the estimated duration of infection suggest that there were more than 376 million new cases of bacterial STI in individuals 15–49 years of age worldwide in 2016 (Rowley et al., 2019). The introduction of new diagnostic technologies has improved sensitivity in identification of bacterial STI. The use of urine and vaginal swabs has expanded the coverage of screening services and has led to the availability of true population-based estimates of the prevalence of STI (Aral and Holmes, 2008).

Despite a period of decline in some parts of the world, more recently the incidence of bacterial STI has increased significantly. Rates of some bacterial STI remain high or have seen an uptick in certain subpopulations (e.g., gay, bisexual or other men who have sex with men; Grant et al., 2020); reinfections and co-infections are also common (Rowley et al., 2019). An individual is susceptible to infection or reinfection due to a lack of acquired immunity.

The major bacterial STI include:

1. syphilis,
2. gonorrhea,
3. chlamydia.

Syphilis

Treponema pallidum, the spirochete that causes venereal syphilis, has a highly variable clinical course (Ayadpoor and Hall, 2011). It is a genital ulcerative disease that may cause significant complications if left untreated (Da Ros and Da Silva Schmitt, 2008). It is spread through direct contact with a syphilitic lesion or rash during vaginal, anal, or oral sex as the bacteria can enter the body through the penis, anus, vagina, mouth, or through broken skin. A pregnant woman living with syphilis can also pass the disease to her infant through vertical transmission in utero or during birth. Like other bacterial STI, syphilis facilitates the transmission of HIV.

While prevalence has declined in some regions, syphilis continues to cause morbidity and mortality around the globe. Estimates suggest that, annually worldwide, there are approximately 6 million new cases of syphilis in persons 15–49 years of age (Kojima and Klausner, 2018). Despite efforts to reduce syphilis incidence, some high-income countries (e.g., regions in Western Europe, the United States, United Kingdom and Canada) have recently seen concerning rise in rates of infection and outbreaks in specific populations. However, low- and middle-income countries continue to bear the greatest burden of disease, where rates of syphilis infection remain at endemic levels among general populations (Kojima and Klausner, 2018).

Outbreaks of syphilis disproportionately impact specific populations globally, including men who have sex with men, transgender women, sex workers, in part due to a lack of targeted testing and treatment, as well as poor access to care due to stigma and discrimination (Kojima and Klausner, 2018). Untreated syphilis has a number of severe health outcomes, including increasing risk of HIV infection through direct skin-to-skin contact with syphilis sores. Pregnant women and newborn children also bear a disproportionate burden of adverse health outcomes; untreated infection in pregnancy can result in mother-to-child transmission and accounts for approximately 200,000 preventable stillbirths and newborn deaths each year (Lawn et al., 2016).

Rapid, point-of-care testing for syphilis are available; rapid testing can be done without electricity or laboratory equipment and can be performed on blood samples obtained by a single finger-stick (Mabey, 2010). The management of syphilis outbreaks focuses on active partner notification, enhanced surveillance of infectious syphilis and the investigation of sexual networks facilitating disease transmission. Interventions center on enhancing both public and health care worker awareness, venue-based screening, rapid testing within- and outside treatment centers, targeted sexual health promotion and syphilis screening of HIV positive individuals. Single-dose benzathine penicillin given intramuscularly remains the mainstay of treatment for primary syphilis and is a highly important medication for resource-limited settings.

Gonorrhea

Gonorrhea, caused by *Neisseria gonorrhoeae* bacterium infection, grows easily in the reproductive tract including the cervix, uterus, and fallopian tubes in women and in the urethra in women and men. Gonorrhea can also grow in the mouth, throat, eyes, and anus. There is little evidence suggesting protective immunity and, more recently, antimicrobial resistance to antibiotics has increased rapidly (WHO, 2019a,b). Consequently, a person can be infected with gonorrhea multiple times throughout their lifespan. There is significant concern about the threat of increasing global incidence of drug-resistant gonorrhea (e.g., Eisinger et al., 2020), as it ultimately presents a challenge to public health efforts aimed at reducing STI impact on a global scale (WHO, 2019a,b).

Gonorrhea is the second most commonly reported notifiable STI and recent global incidence is estimated at more than 82 million infections annually among adolescents and adults aged 15–49 years (WHO, 2021b). Women under 25 years of age are at highest risk of infection. The relatively large increase in the number of cases in young women since the mid-1990s suggest that heterosexually acquired infections have increased significantly, yet, infections acquired through sex between men continue to account for a disproportionate number of infections. An increase in gonorrhea among men who have sex with men and bisexual men has also been reported in some world regions, such as the US.

Education on transmission and prevention, condom use, partner notification, STI screening, and limiting the number of sexual partners are key prevention strategies. However, the emergence of antimicrobial resistant gonorrhea has created additional obstacles to the control of infection. In many parts of the world, quinolone antibiotics are no longer effective and more expensive cephalosporin antibiotics are required to successfully treat infections. The development of a vaccine against the infection is also essential. The combination of persistently high gonorrhea morbidity in some populations and the threat of drug-resistant gonorrhea reinforce the need to better understand the epidemiology of the disease.

Chlamydia

Chlamydia is a common STD caused by *Chlamydia trachomatis* (*C. trachomatis*). While infection is generally asymptomatic (symptoms are usually mild or absent altogether), chlamydial morbidity can lead to serious health complications and irreversible damage. Chlamydial infections appear to be rising—in 2005, there were 101 million cases of chlamydial infection worldwide (Mabey, 2010), just over ten years later, in 2016, the WHO reported an estimated 127 million cases (WHO, 2019b). It is the most commonly reported bacterial STI in high income countries; for example, in 2016, the U.S. had nearly 1.6 million known cases of chlamydia (CDC, n.d.).

More cases of STI are caused by *C. trachomatis* than by any other bacterial pathogen, making such infections an enormous public health problem throughout the world. Infection is persistent in groups with good access to treatment due to a typically longer disease course (Mabey, 2010). Women bear a disproportionate burden of chlamydial incidence and it is most common among women aged 15–24 years. Women in resource-poor countries experience the impact of infection most acutely, as the serious health risks and adverse outcomes associated with chlamydia are magnified.

There has been an increase in global diagnoses of chlamydia due to changes in sexual behaviors (specifically among youths) and improvements in diagnostic testing. Cure rates are currently 97–98% (Ayadpoor and Hall, 2011), with appropriate antimicrobial therapy. Untreated infection, however, can lead to serious health complications including infertility, pelvic inflammatory disease (PID) and neonatal complications.

The range of chlamydia control strategies and activities is broad. In high income settings, there tends to be a focus on screening for asymptomatic cases. For instance, the US CDC recommends chlamydia screening for all sexually active women under 26 years of age (Da Ros and Da Silva Schmitt, 2008). Where screening has been introduced, the prevalence of infection and rates of PID are decreasing. It has been suggested that the screening of sexually active young men should also be considered in clinical settings with a high prevalence of chlamydia, such as adolescent clinics and detention facilities (e.g., prisons and jails) (Da Ros and Da Silva Schmitt, 2008), though the WHO *Global health sector strategy on STI, 2016–2021* reports that “best strategies to control and measure chlamydia infections are still to be defined” (p. 17).

The treatment of chlamydia consists of single-dose Azithromycin, or when not available, a 7 day course of Doxycycline. Treatment should also be given as presumptive therapy for individuals presenting with confirmed or presumptive gonorrhea.

General care and management of STI

Reporting

The underreporting of STI that are deemed to be “reportable” to public health departments is of concern. Few countries outside Western Europe and North America have accurate reporting systems for STI, thus, in most of the world, the true incidence of STI is

generally unknown (Mabey, 2010). The UK has a unique network of treatment centers that are dedicated entirely to the management of STI (genitourinary medicine or GUM clinics), from which statistical reporting constitute the basis of national STI surveillance programs (Fenton and Lowndes, 2004). In the US, it is estimated that reported cases of STI represent only 50–80% of reportable STD, reflecting both limited screening and low-disease reporting. By contrast, many EU states rely on a combination of sentinel reporting, mandatory disease notification or laboratory reporting to monitor trends (Fenton and Lowndes, 2004). Epidemiological knowledge is based on the results of ad hoc prevalence surveys of convenience populations, of concern such data may be unrepresentative of the wider population (Mabey, 2010). Over time, the epidemiology of STI in sub-Saharan Africa has been improved through the introduction of large population-based prevalence surveys (Mabey, 2010). The results of these surveys have confirmed the high prevalence of STI such as syphilis in the region.

STI diagnosis

Adequate screening for STI should be done on a routine basis in every part of the world. Many STI are asymptomatic and thus can be difficult to control. The purpose of screening is to ensure that persons who experience infection will be quickly diagnosed and appropriately treated to avoid serious health consequences and reduce the spread of STI.

Diagnostic testing

Since the 1990s, a number of new initiatives have promoted diagnostic test development for STI control. Over the years, progress in science and technology and funding from major international donors have led to the development of “rapid” STI tests (Ryan et al., 2008). The introduction of new diagnostic technologies has greatly increased the recognition of viral STI, enhanced the sensitivity of detecting bacterial STI, and expanded the repertoire of usable specimens (Aral and Holmes, 2008). New, simple, rapid, cheap point-of-care serological tests for syphilis, for example, which can be performed on whole blood obtained from a fingerprick are now available and hold much potential for improving STI control. The use of vaginal swabs and urine has also significantly expanded the screening service coverage and has led to the availability of true population-based estimates of the prevalence of STI (Aral and Holmes, 2008). The STI Diagnostics Initiative located in the UNICEF/UN Development Program/WB/WHO Special Programme for Research and Training in Tropical Disease is one of the most notable of diagnostic initiatives (Ryan et al., 2008). The *WHO Global health sector strategy on STI, 2016–2021*, also reflects a growing recognition of the importance of integrating rapid point-of-care tests into STI control strategies. Diagnostic testing is crucial as, in diagnosing and treating patients with STI, effective prevention efforts may be advanced and consequently reduce the spread of STI around the world. However, diagnostic testing is not readily available to all global regions.

Syndromic diagnosis

The biggest challenge in STI management is ensuring that adequate-quality, rapid tests are accessible to resource-limited settings that need them most. Laboratory tests provide the most specific evidence of infections, however, such tests are not readily available at all clinical sites (Ryan et al., 2008), as they are often unavailable or simply too expensive (even when available, test results may not be available soon enough to guide appropriate treatment) (WHO, 2011). As such, low- and middle- income countries are especially dependent on “syndromic management.” The basis of this approach is the identification of a syndrome through consistent and easily recognizable signs and symptoms associated with a limited number of well-defined etiologies (Ryan et al., 2008; WHO, 2019a,b). This approach uses clinical algorithms and flowcharts to guide diagnosis and treatment (WHO, 2011, 2019b). In essence, treatment are determined by the likely etiology, as opposed to etiologic diagnosis.

Syndromic management is relatively simple and cost effective, thereby making it a more feasible option for regions that lack adequate laboratory infrastructure. However, syndromic diagnosis also has several limitations. This approach cannot detect asymptomatic infections, can lead to overdiagnosis and an overuse of treatment (due to the conflation of symptoms with those of another condition), and drug resistance. Moreover, a syndrome-based approach may impede accurate surveillance data. As an example, while syndromic management of genital ulcers and genital discharge in men has been shown to be relatively straightforward and cost effective, the same cannot be said of syndromic management of genital ulcers and genital discharge in women as symptoms are poor predictors of the presence of an STI. Inexpensive, simple, dipstick-type tests (i.e., point-of-care tests) for gonorrhea and chlamydia infection would significantly aid in diagnosis, but the availability of such testing remains limited.

Behavioral prevention

STI prevention programs have relied heavily on biomedical models, however, behavioral interventions must continue to be at the center of STI prevention and control efforts. Behavioral interventions aim to curb STI transmission by changing risk behaviors to safer sex strategies. Interventions target various behaviors and attempt to modify individual behaviors, partner behaviors, and community behaviors. Interventions target social norms and influence social networking, resources and opportunities. Some such interventions include educational programs, treatment programs or face-to-face counseling programs for high risk populations.

Behavioral approaches may have the capacity to more broadly prevent STI (as opposed to more singular STI prevention typical of biomedical interventions). By incorporating the risk behaviors of a particular population or subpopulation and considering local

(i.e., contextual) factors, more appropriate interventions may be created. Moreover, the implementation of integrated behavioral prevention interventions may result in decreased transmission of STI. However, behavioral change is complex (WHO, 2021a). Some researchers have pointed to a “limited public health impact of behavioral interventions” (Aral, 2011, p. ii32) as the efficacy of this approach rests on the knowledge, attitudes and beliefs of individuals, nevertheless counseling and education have been shown to be particularly useful or effective in some settings.

Contact tracing

Contact tracing, or “partner notification,” is an essential prevention strategy used by public health practitioners to help limit the spread of STI. When an individual is diagnosed with a traceable STI, any individual with whom they have been in sexual contact must be notified. In some settings, upon diagnosis an individual is required to contact sexual partners directly; in others, public health officials attempt past partner notification of potential exposure. Although contact tracing is widely promoted, the contribution of this intervention in reducing the transmission of STI continues to be debated. There are obvious limitations to this approach including having accurate information about the potential contacts, finding the contacts when identified, and creating risky situations for the individuals providing the information to public health officials.

Treatment challenges

Despite many decades of modern public health interventions, new biomedical tools, good antimicrobial options, and a comprehensive understanding of STI transmission, the severe health and social consequences of STI persist. Human sexual behaviors create a level of complexity that has challenged public health policy and practice. In addition, issues related to resource access, service delivery, technology dissemination, and acceptability of the interventions pose considerable challenges. The development of effective vaccines and better drugs should continue to be a priority while available STI prevention tools are scaled up. Moreover, ensuring these are available and accessible to persons and communities across all global regions remains imperative.

Conclusion

Opportunities for sexual health gain and the control of STI

In recent years there have been renewed efforts to combat STI. In 2016, the WHO released the *Global health sector strategy on Sexual transmitted infections, 2016-2021* to “accelerate and focus comprehensive prevention efforts through scaling up evidence-based combined behavioural, biomedical and structural approaches; facilitate people’s access to information on their sexually transmitted infection status; improve access to treatment and comprehensive long-term care when needed; and challenge pervasive stigmatization and discrimination” (p. 2). Some nations have also indicated a political commitment to sexual health (for instance the Government of Canada 2019 Action Plan *Accelerating Our Response—Government of Canada Five-Year Action Plan on Sexually Transmitted and Blood-Borne Infections*; Public Health Agency of Canada (PHAC), 2019). However there continues to be a pressing need for government commitment, strategic leadership and action globally and across nations.

Public health intervention is also critical to sexual health gain and control of STI. “Care management” provides a useful way forward for both prevention and management of STI and includes the management of both asymptomatic and symptomatic individuals (Ryan et al., 2008). The major steps in effective care management include:

- risk assessment and clinical evaluation;
- identification of individuals with STD who are unlikely to seek diagnostic and treatment services;
- appropriate diagnosis and treatment of infected persons;
- strategies to avoid new infection and reinfections, such as prevention education, counseling, and partner notification;
- effective evaluation, treatment and counseling of sex partners of STI-infected persons;
- pre-exposure vaccination of at-risk persons of vaccine-preventable STI (Ryan et al., 2008).

Given the evolving epidemiology of STI, it is clear that substantial health gains can be obtained from such an approach. Further, greater investment in efficient and effective treatment, diagnosis, prevention, and surveillance is also urgently required. Recent reviews of effectiveness of prevention should be used to not only guide, but prioritize investment in STI (Lowndes and Hughes, 2010). Stigma and discrimination also pose key obstacles for effective STI prevention, care and treatment; efforts to address stigma and discrimination, including racism, sexism, and gender-based violence, are urgently needed (Jackson and Tremblay, 2019).

Moreover, there is a need for prolonged investment in STI prevention, microbiology, and surveillance research (Fenton and Lowndes, 2004). Public health gains are also likely to be achieved through interdisciplinary and multi-sectoral research and collaborations that focus on key areas and issues of concern. Given sufficient resources and commitment, it is possible to control STI and sequelae through provision of accessible, acceptable, and affordable clinical services, combined with partner notification and screening programs in high risk groups. Control of STI could significantly reduce the incidence of sexually transmitted infections worldwide and have a major impact on the overall health and well-being of hundreds of millions of people each year.

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Relevant websites

World Health Organization

<http://www.who.int/en/>—World Health Organization.

<https://www.who.int/news-room/fact-sheets/detail/hiv-aids>—HIV/AIDS

<https://www.who.int/reproductivehealth/publications/rts/chlamydia-treatment-guidelines/en/>—WHO guidelines for the treatment of Chlamydia trachomatis.

Centers for Disease Control and Prevention

<http://www.cdc.gov/>—Centers for Disease Control and Prevention.

<https://www.cdc.gov/std/statistics/default.htm>—STD Data and Statistics.

<https://www.who.int/news-room/fact-sheets/detail/cervical-cancer>—Cervical Cancer.

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Bacterial Skin Infections

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Introduction

Skin and soft tissue infections (SSTIs) have a pivotal role among bacterial infections, as they cause about 10% of hospital admission in developed countries, as epidemiologic investigations in the United States report (Esposito and Noviello, 2016). Many factors contribute to the increase of SSTIs in terms of frequency and severity, including the aging of the general population, the growing number of critically ill or immunocompromised patients, and the emergence of multidrug-resistant (MDR) pathogens. A study comparing cases of SSTI observed in the US in the year 2000 and in the year 2012 highlighted an increase of the total number of ambulatory and Emergency Department visits for SSTI >30%, which was mainly associated with a higher burden of SSTI in older adults. This increase of SSTI incidence was evident in all health-care settings and reported a considerable increase in terms of the costs associated to the disease (Tun et al., 2018).

SSTIs presentation can range from mild to severe life-threatening manifestations, which can affect all age groups regardless they are immunocompromised or having chronic diseases. Underlying conditions can affect SSTI severity and prognosis and several factors can influence their etiology, and clinical presentation. Immunologic status, previous antimicrobial treatments, geographic localization, recent history of trauma or surgery, animal exposure, and bites should be considered in establishing the diagnostic and therapeutic strategies for a patient reporting an SSTI (Sartelli et al., 2018a).

All these considerations on SSTI suggest that every case needs an accurate evaluation and clinical observation to avoid both the severe, life-threatening complications of under-treatment and the problems related to over-diagnosis of SSTIs as those occurring in patients suffering stasis dermatitis or honeybee stings with venom reactions. A large multicenter trial of patients presenting to an emergency department, demonstrated that a presumptive diagnosis of cellulitis, which is the most common skin infection, accounting for 650,000 hospitalizations in the United States annually, was not confirmed in 30% of the patients after dermatological consultation. In this study, venous stasis dermatitis, erythema migrans, contact dermatitis, eczema, and erythema nodosum were the most common mimics of cellulitis (Raff and Kroshinsky, 2016).

Blood and skin cultures from patients with SSTI have low yields, making the etiologic investigations of relatively low value outside selected population reporting extensive and chronic infected lesions. As a general concept, microbiologic investigations of SSTIs are positive in 30% of the cases only and *Staphylococcus aureus* and β -hemolytic streptococci (BHS) are the commonest etiologic agents. The majority of SSTIs reports a monomicrobial etiology, and a polymicrobial etiology can be demonstrated in about 20% of the cases (Esposito et al., 2019a).

SSTI: Classifications and definitions

Intact skin is a physical barrier, which provides an effective protection from the external injuries and maintains a microbiologic environment that makes difficult the growth of pathogenic organisms. SSTIs can occur after microorganisms invade otherwise

healthy skin. In some cases, an underlying disease or trauma favors the infection due to the loss of physiological barrier function. The damage is due to the destruction of affected tissues that causes an inflammatory response, which is clinically characterized by pain, local warmth, and erythema. In particular settings, such as in patients living with diabetes mellitus or neuropathies, the degree of skin damage can be high and healing of lesions can be delayed, making SSTI management more complicated. In these settings, recurrent episodes or the evidence of non-healing lesions warrant further investigations to assess vascular and lymphatic compromise, suggesting the need for a multidisciplinary approach (Labreche et al., 2013).

SSTIs represent a heterogeneous array of disorders, and several classifications have been proposed. Every attempt of classification organizes SSTIs based on the characteristics of a specific variable, such as anatomic localization, causative agent, extension of the skin lesion, progression rate, clinical presentation, and severity. Each of these classifications has its own usefulness, but some of them have poor value in driving toward the most appropriate management of the condition. In the attempt to satisfy the need of a practical, useful, and accurate evaluation, the Infectious Diseases Society of America (IDSA) classification adopts three different distinctions: (i) skin extension: uncomplicated typically superficial infections (uSSTIs) and complicated infections (cSSTIs), usually with deep involvement; (ii) rate of progression: acute and chronic wound infections; and (iii) tissue necrosis: necrotizing and non-necrotizing infections. However, IDSA classification does not fulfill the need to standardize the evaluations to be adopted to SSTI patients involved in pivotal trials evaluating the efficacy of antibiotic treatments. For this purpose, the US Food and Drug Administration (FDA) introduced a new definition of acute bacterial skin and skin-structure infection (ABSSSI) that includes cellulitis/erysipelas, wound infections, and major cutaneous abscesses. Thus, an ABSSSI is currently defined as a bacterial infection of the skin with a lesion size area of ≥ 75 cm² (lesion size measured by the area of redness, edema, or induration) (Esposito et al., 2017a).

Table 1 provides a practical classification of SSTIs based on the severity of local and systemic findings.

Microbiologic characteristics of SSTI

Microbiologic characteristics of SSTI are strongly influenced by the site of infection. For example, a study evaluating patients with facial infections reported group A β -hemolytic streptococci (GABHS) in about 75% of the cases and highlighted staphylococci as the second most frequent causative agent (Rath et al., 2018). Instead, if we look at lower extremity infections, these are more frequently caused by non-GABHS such as *Streptococcus agalactiae*. *S. aureus* and BHS are the main etiologic agents of uncomplicated SSTIs; BHS is the primary cause of erysipelas (Jones et al., 2003).

Staphylococci and streptococci sustain most cases with skin abscesses, and the bacteria that constitute the normal skin flora boost their virulence. When we look at the patients affected by cellulitis, we find that GABHS or *S. aureus* are frequently involved. *S. aureus* is considered the most common pathogen, with an increasing prevalence of MRSA strains in emergency department and ambulatory outpatient visits. Considering the data deriving from the analysis of particular groups of patients, those with chronic venous stasis or with saphenous vein harvest for coronary artery bypass surgery, showing a significant body weight and reporting a surgical procedure during 30 days prior to medical consultation, can have a Gram positive microorganism cultured from the site of infection. *S. aureus* is frequently isolated during the immediate post-operative period and BHS is cultured during the following period. Similarly, recurrent episodes of cellulitis in patients with lymphedema are frequently sustained by BHS. Staphylococci and BHS are also the most common pathogens in bacterial infections among drug users, which can be complicated by hematogenous dissemination. Gram negative and anaerobic bacteria are more common in association with surgical site infections (SSIs) of the abdominal wall or with infections of the soft tissue in the anal and perineal region, due to the specific characteristic of the bacterial flora in these parts of the body (Esposito et al., 2019b).

SSIs and SSTIs can have particular epidemiologic aspects in those with cytotoxic therapy-induced granulocytopenia. In these cases, extended-spectrum β -lactamase (ESBL)-producing Enterobacteriaceae and carbapenem-resistant *Pseudomonas aeruginosa* constitute about 10% of the etiologic agents reported (Jääskeläinen et al., 2017). Other settings that deserve particular attention include patients with hematologic malignancies or living with a transplanted organ. In these cases, we can observe an *Ecthyma gangrenosum*, which is caused by the cutaneous hematologic seeding of hospital-acquired microorganisms such as *P. aeruginosa*, *Stenotrophomonas maltophilia*, and other bacteria and fungi otherwise infrequently involved in SSTIs. Patients reporting neutrophil defects, cellular immunodeficiency including HIV, transplant, immunosuppressive medications, and diabetes can develop cellulitis due to unusual organisms, such as Gram negative bacilli, anaerobes, and other opportunistic pathogens such as *Mycobacteria* and

Table 1 SSTI classification according to the severity of local and systemic signs.

Class	Patient criteria	Site of care
1	Afebrile and healthy, other than cellulitis	Send home on oral therapy
2	Febrile and ill appearing, but no unstable comorbidities	OPAT with possibility to switch to oral therapy
3	Toxic appearance, or at least on unstable comorbidity, or limb-threatening infection	OPAT with possibility of hospital admission
4	Sepsis syndrome or life-threatening infection (e.g., necrotizing fasciitis)	Hospital admission

OPAT, Outpatient parenteral antibiotic therapy; SSTI, skin and soft tissue infection.

fungi. Subcutaneous injection of illegal drugs can cause cellulitis from uncommon bacterial species, such as *Enterobacteriaceae*, *Enterococcus faecalis*, *Bacteroides* spp., and *Clostridium* spp. Other uncommon causes of cellulitis are *Neisseria meningitidis*, *Mycobacterium avium*, *Pasteurella multocida* (which is associated with animal bites), *Aeromonas hydrophila* (after contact with freshwater), *Chromobacterium violaceum*, and *Vibrio vulnificus* (after contact with seawater) (Barbier and Timsit, 2000).

When we analyze the spectrum of hospital-acquired bacteria, focusing on patients requiring intensive care, we find that *Acinetobacter baumannii* is the main emerging MDR pathogen causing soft tissue infections, including cellulitis, after the use of invasive devices. Current data indicate that methicillin-resistant *S. aureus*, *A. baumannii* and *Pseudomonas aeruginosa* can be important pathogen in patients with burn wounds, due to their relevant profile of resistance, which makes the treatment difficult and favors an increase in terms of sepsis related mortality (Joy et al., 2020).

Necrotizing fasciitis is frequently sustained by *S. aureus* and GABHS, alone or in combination, in selected areas also *Vibrio vulnificus* can be identified and reports the highest mortality rate (Tsai et al., 2021).

Moreover, Gram positive cocci, especially *S. aureus*, are the main pathogens causing diabetic foot infections, but Gram-negative pathogens can be reported in those with chronic infection receiving multiple antibiotic treatments. Infections in patients suffering foot ischemia or gangrene can be caused by obligate anaerobic pathogens (Watkins and David, 2021).

Polymicrobial infections account for about 10% of cases of SSTI, and both Gram-positive and Gram-negative organisms can be the causative agent. According to general considerations, polymicrobial infections provide a complex environment in which a variety of interactions occurs between local flora and causal pathogens, which finally share virulence determinants and trigger the synergistic release of cytokines, boosting the severity of the infection. Most of the polymicrobial SSTIs can be included in the following: diabetes-related foot infections (DFIs), pressure ulcer infections, and burn infections (Esposito et al., 2019a).

Microbiologic diagnosis

Patients with SSTI receive different diagnostic workup with respect to underlying condition and infection severity (Esposito et al., 2011).

Blood cultures should be considered as part of the diagnostic work-up in all cases with severe infection requiring hospitalization, but they have a very low yield in patients affected with an uncomplicated SSTI. A study on 132 patients admitted because of an SSTI highlighted that only a minority of cases (10.6%), satisfied IDSA SSTI criteria for obtaining blood cultures: six animal bites, five patients with severe cellular immune deficiency and three immersion injuries in non-sterile water, but blood cultures were attempted in about 50% of the cases with positive results in 6% only. Moreover, only 2 patients had a positive culture that was consistent with the clinical findings, instead in 2 cases the bacteria retrieved were contaminant (Sturkie et al., 2021).

Although swabs from infected sites are the samples cultured with the highest frequency among patients with SSTI, it is important to emphasize that superficial swabs of open ulcers and drainage from fistulous material usually do not correctly identify the microbiologic etiology. Cultures and microscopic examination of cutaneous aspirates, pus, biopsies should be considered in complex infection, septic and immunosuppressed patients, water- or soil-related injuries, and animal bites. In patients with deep tissue infections, the results of superficial techniques do not reflect the etiologic pathogen because of the presence of commensal microorganisms on the wound surface, which explains the low specificity of the diagnostic investigation. Quantitative cultures can be proposed to distinguish commensal microbiota from clinically significant bacterial growth, but the usefulness of this technique has been questioned by prospective studies (Esposito et al., 2017b).

Yields of cultures attempted from materials obtained after needle aspirations of the inflamed skin are positive in an extremely variable number of cases (<5–40%, on the basis of the different studies) (Esposito et al., 2016a). Cultures of punch biopsy specimens yield an organism in 20–30% of cases. If an abscess is present, puncture and aspiration of fluid should be performed. SSTIs without any evidence of liquid collection should be investigated by a small biopsy of skin or soft tissue, after superficial disinfection and excision of necrotic tissue. Chronic wounds can be investigated by pus or fluid culture; alternative samples to biopsy include aspiration of saline that has just been infused into the wound depth (Esposito et al., 2016a).

Molecular technologies can be considered an effective, time-saving alternative to overcome the delay in the results associated with traditional bacterial culture. Polymerase chain reaction (PCR)-based techniques are equally sensitive as cultures, but they can be useful for the diagnosis of SSTIs sustained by *S. aureus*, potentially providing crucial information to the choice of appropriate antibiotic regimen, such as the rapid detection of Panton-Valentine leucocidin-encoding genes from pus samples or to the identification of cryptic resistances. A recent study investigating the impact of a SSTI management based on a Gram stain plus a specific PCR assay highlighted that such approach resulted in several advantages including a reduction of the treatment period, relative costs, incidence *Clostridium difficile* colitis, and finally mortality (Bouza et al., 2020; Saeed et al., 2018).

Imaging studies

Imaging studies ameliorate the accuracy of SSTI diagnosis. Plain radiography has a relevant role in detecting the presence of gas in the soft tissues, which is suggestive of a necrotizing infection, or in diagnosing an osteomyelitis. However, an X-ray examination has poor value in patients with cellulitis, unless a history of trauma or chronic wound are present, as assessed by a study investigating over 150 patients (Stranix et al., 2015). Computed tomography (CT) scans can give useful information on the extent of the

infectious process and can be crucial in guiding fluid aspiration and in revealing the presence of foreign objects or even small fluid–air collections in the soft tissues (Mower et al., 2019).

Magnetic resonance imaging (MRI) has the advantage of a better definition of soft tissues and can be particularly helpful in differentiating cellulitis from pus and abscess formation. Moreover, MRI provides better accuracy than CT in detecting necrosis, inflammatory edema, and muscular fascia involvement, but its use is limited because of cost. Moreover, the value of MRI can be high in diagnosing patients with severe cellulitis and osteomyelitis, distinguishing cellulitis, phlegmon, adjacent soft tissue ulcers, and cortical bone destruction (Klein et al., 2020).

Ultrasonography (US) can be a sensitive technique for SSTI diagnosis, reporting important advantages over other imaging studies, including MRI due to it is easy to perform, cost-effective, and can be used in guiding infected purulent secretion drainage (Alsaawi et al., 2017). In fact, ultrasonography provides useful information to differentiate cellulitis from abscess, therefore limiting more expensive imaging studies and unnecessary harmful procedures like incision and drainage. Moreover, ultrasonography is an easy and rapid technique without side effects that can be performed in many cases where MRI cannot be performed. A study on 62 patients in a rural tertiary care academic medical setting highlighted that an US examination as part of the preliminary assessment of patients with SSTI changed the diagnostic decision in 16% of the cases, increasing the number of patients treated by drainage or needle aspiration. A systematic review identified 5 studies investigating the value of US examination. Sensitivity of US examination ranged between 75 and 90%, and specificity between 55 and 83%. Use of US examination as diagnostic tool led to appropriate changes in the therapeutic approach in 16 to 39% of patients (Knaysi et al., 2021).

Radionuclide scanning studies generally lack specificity in the acute situation, but the use of hybrid techniques (such as single-photon emission tomography [SPECT]/CT and positron emission tomography [PET]/MRI) can increase the specificity of nuclear medicine imaging techniques, eventually revealing the compromise of surrounding structures. The role of these techniques in supporting SSTI diagnosis needs further investigations (Esposito et al., 2017a).

Indications for hospital admission

SSTIs are among the most common reasons for hospitalization of adults in the United States. Hospitalization costs of SSTIs can range from \$6000 to \$13,000 per patient (average cost of \$8000 per patient), with multi-day room and board expenses comprising 50% of total costs (Lodise et al., 2015).

Several authors have attempted to identify the items to be considered for assessing the need for hospital admission of patients with SSTI and several aspects have to be evaluated to establish the circumstances that warrant hospital admission for patients with SSTI. Medical history of previously untreated or relapsing skin lesions, presence of fever or relevant co-morbidities (e.g., vascular insufficiency, neuropathy, diabetes, and immunosuppression), the site of the lesion itself (the hand or head has the potential for more significant damage), aspects of laboratory investigations (e.g., elevated white blood cell count and lactate levels), and the degree of the body surface (>9%) involvement must be considered to recommend hospital admission (Estrada et al., 2020). A multicenter, prospective study on adult patients with SSTI seeking Emergency Department cares with symptoms onset within 7 days highlighted that about 15% of the cases were hospitalized with the highest frequency among those needing parenteral antibiotics for SSTI treatment (about 40% with SSTI as the only reason for admission), those requiring surgery and those reporting an underlying disease (Talan et al., 2015). Overall, most patients with uncomplicated SSTIs can be managed as outpatients. Complicated infections often require hospital admission, especially if muscle or fascial involvement is suspected, the process is rapidly progressing toward relevant lesions or septic shock, signs of toxemia are developing, the diagnosis or prognosis is in doubt, exploratory surgery is contemplated, or the patient cannot adequately comply with outpatient treatment.

Treatment of SSTI

The following aspects have to be considered in establishing the treatment of an SSTIs

- Anatomic localization
- Etiologic agent
- Skin extension (localized or widespread infection)
- Rate of progression (acute or chronic disease)
- Clinical presentation (primary or secondary infection)
- Severity (presence of co-morbidities)

In general, superficial infections are not complicated or severe, and an oral antibiotic treatment or even topical treatment can be sufficient for the resolution. The extension of the lesion on the skin, the rate of progression, and finally the necrotizing or non-necrotizing characteristics of the infection are the main aspects to be considered in treating deep infection. All these considerations are extremely important for the choice of the antibiotic regimen, particularly when necrotizing lesions are present (Sartelli et al., 2018b).

As a general concept, SSTIs can need hospitalization for the treatment of complication and sometimes of the septic status. Several authors have attempted to identify predictors for admission, as their correct evaluation would play a central role in choosing appropriately the best site of care. One of the best attempts comes from Eron et al. (2003), who took into consideration the general clinical conditions of the patient affected by an SSTI, considering four different classes, with each class corresponding to different needs regarding the site of care (Tables 2 and 3). This kind of predictor can drive clinical choice, identifying the patients that need an outpatient parenteral antibiotic therapy (OPAT) approach instead of hospitalization (Eron et al., 2003; Palit et al., 2021).

OPAT is a treatment model widely utilized in the United States and several European countries and refers to antibiotic administration given in settings other than a traditional hospital stay, including the patient's home, the general practitioner's office, the hospital ambulatory unit, and public or private infusion centers. This treatment model reports the relevant advantage of reducing hospitalization, giving the same therapeutic opportunities. SSTIs are a common indication for OPAT treatment. Several controlled trials comparing patients receiving intravenous antibiotic treatment of cellulitis at home with those receiving hospital treatment not only showed no difference in outcome and greater patient satisfaction with home treatment, but also significant cost savings accrued from real or potential reductions in hospital stay. Antibiotics used for OPAT treatment of SSTI must be effective

Table 2 Treatment of non-necrotizing infections.

Diagnosis	Etiology	Antibiotic Choice	Note
Impetigo	<i>Staphylococcus aureus</i> , BHS	Local mupirocin or chlortetracycline	In case of suspected or proven MRSA: doxycycline, clindamycin
Furuncles and Carbuncles	<i>Staphylococcus aureus</i>	As impetigo	As impetigo
Animal bites	Gram-negative (<i>Capnocytophaga Canimorsus</i> and <i>Pasteurella multocida</i>)	Oral amoxicillin ampicillin/sulbactam	In case of severe wounds: parenteral piperacillin/tazobactam or carbapenems
Cutaneous ^a abscesses	<i>Staphylococcus aureus</i>	Incision and drainage Amoxicillin/clavulanate	In case of suspected and proven MRSA: TMP-SMX clindamycin, Tetracyclines, linezolid, fusidic acid As alternatives: first- and second-generation cephalosporins
Erysipelas ^a	<i>Streptococcus pyogenes</i>	Amoxicillin	In case of <i>Vibrio vulnificus</i> : cefotaxime Plus doxycycline
Cellulitis ^a	<i>Staphylococcus aureus</i> , <i>Streptococcus pyogenes</i>	Amoxicillin, clavulanat As alternatives: Fluoroquinolones, macrolides, and lincosamides	In case of <i>Aeromonas hydrophila</i> : Ceftazidime plus gentamicin In case of MRSA: glycopeptides and newer antimicrobial options (linezolid, daptomycin, telavancin, and tigecycline)
Surgical site ^a infections	<i>Staphylococcus aureus</i>	Amoxicillin/clavulanate Ampicillin/sulbactam	In case of MRSA: glycopeptides and newer antimicrobial options (linezolid, daptomycin, telavancin, and tigecycline)

Whenever the lesion size is $\geq 75\text{cm}^2$ it is classified as ABSSSI.

BHS, β -hemolytic streptococci; MRSA methicillin-resistant *S. aureus*; TMP-SMX.

^aDalbavacin and tedizolid have been approved for their empirical treatment.

Table 3 Empiric antimicrobial therapy for chronic SSTIs.

Infection	Route of administration	Antibiotic/dose	Alternative/dosage
Mild	Oral	Levofloxacin 500 mg od Amoxicillin/clavulanic acid 100 mg BID/TID	Cotrimoxazole 800/160 mg BID/TID
Moderate	Intravenous (possible switch to oral)	Ampicillin/sulbactam 1000 mg QID Ertapenem 1000 mg od Tigecycline 50 mg (with loading dose)	Piperacillin/Tazobactam 4,5 g TID Linezolid 600 mg BID Daptomycin 300/500 mg od
Severe	Intravenous	Clindamycin 600 mg TID plus Ciprofloxacin Imipenem 500 mg Meropenem 1000 mg TID Clindamycin 600 mg TID plus Amikacin 1 g od plus Ampicillin mg ² g TID	Clindamycin 600 mg TID plus Ceftazidime 2 g TID Vancomycin 1 g BID Linezolid 600 mg BID Linezolid 600 mg BID plus Aztreonam 2 g TID plus Metronidazole 500 mg TID

BID = twice a day; TID = three times a day; QID = four times a day.

against common pathogens such as *S. aureus* and BHS and have a convenient once- or twice-daily dosing schedule. Ceftriaxone is most frequently used, with teicoplanin, vancomycin, and daptomycin reserved for SSTIs with suspected or proven methicillin-resistant *S. aureus* (MRSA) etiology. A study evaluating patients suffering with cellulitis who were receiving antibiotics following an OPAT program demonstrated that hospitalization occurred in 7% only of the patients and mean duration of the treatment was days. Analysis of a multivariate model established that age, peak C-reactive protein (CRP) level and number of medical reviews during OPAT therapy were found to be associated with longer duration of IV therapy. Eighty-nine percent of the patients were discharged on oral therapy. Ceftriaxone was the antibiotic administered with the highest frequency (Palit et al., 2021; Esposito et al., 2016b).

Acute non-necrotizing infections: Therapy

Impetigo

Impetigo, which is most often due to *S. aureus*, can be usually treated topically with mupirocin or chlortetracycline twice daily for 5 days. Oral therapy can be a 7-day regimen with an antistaphylococcal penicillin or cephalexin. When MRSA is suspected or confirmed, doxycycline, minocycline, clindamycin, or sulfamethoxazole trimethoprim (SMX-TMP) report similar efficacy in terms of favorable outcome. It remains unclear if oral antibiotics are superior to topical antibiotics, and the therapeutic choice remains an individual decision mostly based on the number of lesions and their location. Based on a systematic review comparing different guidelines for impetigo treatment, a systemic antibiotic therapy is always recommended, and 20 guidelines (39%) do not mention an antiseptic treatment (Hall et al., 2021).

Furuncles and carbuncles

Furuncles' treatment choice is based on lesions dimension as small furuncles can be treated with warm compresses to promote drainage, whereas larger furuncles and carbuncles and all abscesses require incision and drainage. Empiric antibiotic therapy with an anti-staphylococcal (and an anti-MRSA activity in areas of high prevalence) antibiotic can be considered as part of these lesions' treatment. A systematic review on different treatment strategies for furuncles and carbuncles failed in retrieving randomized controlled trials assessing the efficacy and safety of topical antibiotics versus antiseptics, topical versus systemic antibiotics, or phototherapy versus sham light for treating bacterial folliculitis or boils. All the studies tailored to compare different antibiotic treatments did not find a significant difference in terms of efficacy and safety evaluating different antibiotics investigated (Lin et al., 2021).

Recurrence of furuncles can be prevented by using soap containing chlorhexidine gluconate with isopropyl alcohol and a 1- to 2-month course of low-dose oral clindamycin (150 mg daily).

Animal and human bites

Antibiotic administration for those reporting an animal or human bite depends on the severity of lesion, its location, and type. Pre-emptive antibiotic therapy is generally recommended for those wounds at risk of infection. Such therapy must be done with oral amoxicillin/clavulanate or ampicillin/sulbactam after accurate cleaning and copious irrigation. As a general concept, antibiotic treatment should consider both aerobic and anaerobic coverage looking at the bacteria that colonize the skin and the oral environment. In the case of severe wounds, especially in patients with co-morbidities (immunodepression, diabetes, end-stage liver disease) or patients with clinical signs of systemic infections (sepsis), parenteral therapy with piperacillin/tazobactam or carbapenem is recommended due to the risk of an infection sustained by MDR bacteria such as *Pseudomonas* (Greene and Fritz, 2021).

Cutaneous abscesses

Prior to an antibiotic treatment, incision and drainage of pus and debris of cutaneous abscesses is strongly recommended as primary management to reduce the bacterial burden and to allow microbiologic investigations. Needle aspiration - even ultrasonographically guided - should not be performed due to its low rate of success (Kamath et al., 2018).

Concomitant administration of antibiotics does not modify the patient's outcome for localized and small abscesses, but in case of major abscesses, the administration of antibiotics effective against *S. aureus*, which is the main pathogen responsible, is recommended, especially in the presence of clinical symptoms of sepsis, such as temperature $>38^{\circ}\text{C}$, tachypnea, tachycardia, or white blood cell count above $12,000/\text{mmc}$. A placebo-controlled study evaluating the efficacy of antibiotic treatment for small size abscess ($<5\text{ cm}$) demonstrated that in these cases antibiotic administration can be effective if *S. aureus* is cultured (Daum et al., 2017). If an MRSA is suspected, as in geographic areas with a high prevalence of community-associated MRSA (CA-MRSA), or the case of MRSA isolation by cultures, treatment should consider an oral agent, such as TMP-SMX, clindamycin, tetracyclines, oxazolidinones, and fusidic acid. All these antibiotics can be administered in the outpatient setting either after a course of intravenous antibiotic or as first-line therapy (Giacobbe et al., 2021).

Erysipelas

Erysipelas is always caused by *Streptococcus pyogenes*, which is the pathogen in most of the cases responsible for this infection. As *S. pyogenes* has remained sensitive to penicillin over time, this class of antibiotics remains the first choice in establishing a therapeutic plan. Although coverage against *S. aureus* has been proposed, the recommendation for this coverage is weak and based on low-quality investigations. The route of administration - oral or intravenous will be defined according to the severity of the infection. Amoxicillin is the best choice, but also first- and second-generation cephalosporins can be an easy to administer alternative (Karakonstantis, 2020).

Cellulitis

Cellulitis can be a serious infection requiring appropriate antibiotic treatment, such as amoxicillin/clavulanate (oral or intravenous, depending on the disease severity), which is active against the most frequent responsible microorganisms (*S. aureus* and *S. pyogenes*). The therapy must be initiated promptly, monitoring the clinical evolution, and keeping in mind that other pathogens can be present. Fluoroquinolones and lincosamides can be an appropriate alternative even though the resistance to clindamycin is increasing and side effects of fluoroquinolones should be considered. High rates of macrolide/azalide resistance among BHS present in some areas of the world makes use of these drugs a concern, unless susceptibility results are available. If additional bacterial species are likely to be involved after unusual exposures, such as abrasion or laceration occurring after saltwater exposure, where *V. vulnificus* might be the pathogen, treatment with cefotaxime plus doxycycline is effective (Raff and Kroshinsky, 2016).

Similarly, in the setting of cellulitis after an abrasion or laceration occurring with freshwater exposure, where *A. hydrophila* could be suspected, treatment with ciprofloxacin (along with an antimicrobial targeted to the common pathogens) is indicated. When severe lesions are present, a combination of ceftazidime plus gentamicin can be suggested to ensure the best coverage against Gram negative bacteria. If MRSA is suspected (both hospital-acquired and CA-MRSA), glycopeptides, TMP-SMX, and newer antimicrobial options, including linezolid, daptomycin, telavancin (only available in the United States), and tigecycline, are suitable agents. Although several studies investigate the effectiveness of different antibiotic treatments, data regarding route and duration of treatment cannot be considered conclusive, because data beyond 30 days of follow-up available are not sufficient and data regarding side effects of prolonged treatments are not always reported (Walsh et al., 2016; Cross et al., 2020).

Surgical site infections

Similarly, to cutaneous abscesses, treatment of SSIs is based on incision and drainage, which remain the most important aspects of therapy.

Adjunctive systemic antimicrobial therapy should be administered, especially when a significant systemic response is present, as in the cases presenting with fever or signs of sepsis. Antibiotic treatment recommendations are based on the site of operation, considering that *S. aureus* remains the most frequent etiologic agent. Thus, an anti-staphylococcal agent should be preferred, such as amoxicillin/clavulanate or ampicillin/sulbactam, with the route of administration depending on the general conditions of the patient. Whenever the prevalence of methicillin resistance is high, an anti-MRSA is mandatory, with glycopeptides being the gold standard of therapy, although several newer antibiotics have demonstrated similar efficacy in terms of success rate (Norman et al., 2016; NICE, 2015).

ABSSSI

Recently, as noted earlier, the US FDA introduced the new definition of ABSSSI to define cSSTI more closely for the purpose of registration trials.

The new drugs for ABSSSI have important activity also against MRSA, especially dalbavancin and tedizolid. Consequently, these new drugs can be used for prompt empirical treatment of ABSSSI.

Tedizolid, which has long half-life, can be administered once a day only, giving an advantage in terms of compliance to therapy. Dalbavancin was approved by the FDA and EMA with a two-dose regimen of 1000 mg followed 1 week later by 500 mg administered intravenously over 30 min. One of the strengths of dalbavancin is the prolonged half-life which leads to the possibility of a single 1500 mg intravenous (IV) administration, finally shortening the hospitalization period and avoiding the problems related to low compliance to treatment (Hsu et al., 2021; Lan et al., 2019).

Chronic non-necrotizing infections: Therapy

DFIs, pressure ulcer infections, burn infections, and infected chronic ulcers are distinct clinical entities that have the common characteristic in that they can become chronic with a high degree of colonization from the bacteria present in the surrounding environment. These microbiologic characteristics are relevant as bacteria involved can acquire resistance, which is often against multiple antibiotics and can limit the choices for both empiric and subsequent microbiologically driven therapy (Lemaire et al., 2016).

Initial therapy must be empirical in many cases, such as those occurring after burns. A careful evaluation based on the severity of the clinical presentation and on the history of previous antibiotic treatment guides the empirical treatment, which must be changed looking at the microbiologic investigations (Table 3). The spectrum of antibiotic therapy should be broad, considering the possible polymicrobial etiology and should include coverage for *S. aureus*, considering an anti-MRSA agent in those areas with high prevalence of methicillin resistance. Moreover, coverage against Gram negative bacilli is mandatory in all cases with moderate to severe infection and in those failing narrower-spectrum treatment. After culture from tissue biopsy are available, the antibiotic spectrum can be narrowed according to the results. The quality of the specimen should be considered when deciding which isolates need to be covered (Hill et al., 2021; Sakabe and Del Fiol, 2016).

In most cases, treatment of the most likely pathogens, such as *S. aureus*, streptococci, and any *Enterobacteriaceae* provides effective coverage. When cultures are obtained, therapy should be tailored with respect to microbiologic findings. When a fungal infection can be suspected based on histopathology, cultures must be attempted to identify those fungi whose coverage is not provided after amphotericin B administration (Ertuğrul et al., 2020).

Among patients with a recent ineffective treatment, an infection due to MDR microorganisms should be suspected. In these cases, alternative therapies should be administered: vancomycin, linezolid, or daptomycin for MRSA infections; colistin or tigecycline for MDR Gram-negative microorganisms. Antibiotic treatment can be administered in patients with pressure ulcers, but only based on the presence of local or systemic clinical signs of sepsis and after an intensive local curettage (Goei et al., 2020).

Treatment of burn injuries must consider early excision followed by grafting to reduce infection risk and length of hospital stay restoring a barrier against bacteria present in the hospital environment, which frequently report a high degree of resistance to antibiotics; these surgical procedures are an essential part of a burn patient's management. Topical antibiotics are currently employed; their utility can differ with respect to preparation, as demonstrated for silver-sulfadiazine. Antibiotic prophylaxis appears to have no significant value except in those with more severe injuries (Wang et al., 2018).

Chronic trophic ulcers need surgical treatment of vascular complications and control of underlying predisposing factors such as hyperglycemia to ameliorate the outcome. Once infection is diagnosed, chronic ulcers must be treated aggressively. Treatment includes debridement, surgical drainage of abscesses, debridement of infected bone, and tissue culture-guided antimicrobial therapy. Although a lot of topical therapies/antimicrobials and dressings have been proposed for the treatment of chronic ulcers, very few have prospective data to support their effectiveness in promoting chronic ulcer repair (Ertuğrul et al., 2020).

Necrotizing infections: Therapy

All necrotizing SSTIs (myositis, myonecrosis, necrotizing fasciitis, and Fournier gangrene) require immediate surgical debridement because prompt surgery ensures a better likelihood of survival. Surgical debridement must be continued until tissue necrosis ceases and the growth of fresh viable tissue is observed. In addition, an early surgical treatment minimizes tissue loss, reducing the risk for amputation of the infected extremity. Surgical intervention may be required to ultimately confirm the diagnosis and the severity of illness, and lack of evolution control requires immediate surgery before a diagnosis is confirmed by diagnostic investigations. Empiric antibiotics should be started immediately as well (Table 4). Initial antimicrobial therapy should be broad-based to cover aerobic Gram positive and Gram negative organisms and anaerobes, with the highest tolerable doses of the antibiotics considering the patient's weight and liver and renal status. Empiric antibiotic therapy can be employed until wound culture isolates are identified. Acceptable monotherapy regimens include carbapenems, piperacillin/tazobactam, or tigecycline (Jung and Eckmann, 2019).

However, an optimal choice in the management of necrotizing fasciitis can also be the association of ampicillin/sulbactam plus clindamycin and ciprofloxacin or gentamicin. In cases of suspected MRSA infection, TMP-SMX, vancomycin, or daptomycin can be used; alternative drugs include the newer compounds linezolid, tedizolid, dalbavancin, and ceftaroline, although large scale data on these pathogens are lacking. The association of ceftazidime/avibactam or similar agents with an anti-anaerobic agent

Table 4 Empiric antibiotic treatment of necrotizing infection.

Necrotizing infection	Antibiotic choice
Synergistic (aerobic and anaerobic pathogens)	Imipenem, Meropenem, Piperacillin/Tazobactam As alternative: ampicillin/sulbactam plus clindamycin and ciprofloxacin or gentamicin ^a
Penicillin allergy (skin rash only)	Cefepime + metronidazole
Penicillin allergy (anaphylaxis)	Ciprofloxacin + metronidazole
If MRSA is suspected	Vancomycin or daptomycin
If CA-MRSA is suspected	Clindamycin, TPM-SMX
New anti-staphylococcal (including MRSA) alternatives	Linezolid, Tedizolid, Dalbavancin, Ceftaroline
Difficult to-treat gram-negative bacteria	Ceftazidime/Avibactam

^aConsider the patient's renal function CA-MRSA, TPM-SMX.

(metronidazole or clindamycin) can be a useful option in the case of difficult-to-treat Gram-negative bacteria such as an ESBL-producing strain and/or MDR *Pseudomonas* (Urbina et al., 2021).

Hyperbaric oxygen has been reported to reduce associated tissue loss and mortality and is considered an essential part of these patients' management in many cases. Anyway, well-controlled, randomized clinical trials demonstrating a statistically significant benefit of hyperbaric oxygen are difficult to be proposed, and consequently, its use as an adjunctive therapy for necrotizing fasciitis remains controversial. Thus, the mainstay of treatment remains surgical debridement, and this should never be delayed while arrangements for hyperbaric oxygen treatments are being made (Peters et al., 2020).

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Superficial Fungal Infection

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Superficial fungal infection (SFI) is one of the most frequent forms of infection worldwide (Havlickova et al., 2008; Hay et al., 2014; de Hoog et al., 2017) with prevalence of more than 20%.

The dominant causative agents of this infection are dermatophytes, the group of fungi which invade the keratinized tissue of mammals (Hay et al., 2014; Otašević et al., 2018). In addition to dermatophytes, SFI are often caused by *Malassezia* yeasts (Barac et al., 2015) and *Candida* spp. (Otašević et al., 2016). Moreover, yeast of *Cryptococcus* spp. (Noguchi et al., 2019), *Trichosporon* spp., could cause this form of mycoses primarily in immunocompromised patients. Recently, there has been an increase in published data on the pathogenic potential of non-dermatophytic molds as *Aspergillus* spp., mucorales, the hyalohyphomycetes and dematiaceous fungi in SFI, not only in immunodeficient patients, but also in immunocompetent subjects (Brandt and Warnock, 2003; Kuruvilla and Dias, 2012).

Dermatophytes

Etiology

Dermatophytosis is one of the most prevalent skin diseases due to dermatophytes infection. Classification, or in other words differentiation and identification, of genus and species of this group of fungi were initially based on their morphological characteristics (Sabouraud, 1910; Gräser et al., 1999). However, no standardization, possibility of determination of species only when fresh isolates are examined followed by need of reference strains are the problems when conventional methods are used in process of identification of strains.

The first attempt in effort to overcome these deficiencies was introduction of assays for determination of physiological parameters of dermatophytes. Unfortunately, this approach has limitations too (Weitzman et al., 1983).

Recently, the molecular methods were established and it can be said that it revolutionized dermatophyte taxonomy (Gräser et al., 2000; Makimura et al., 1999; Rezaei-Matehkolaei et al., 2014; Sharma et al., 2006). In one of the newest proposed taxonomy, de Hoog et al. (2017) state, “the number of genera in dermatophytes group has increased (*Trichophyton*, *Epidermophyton*, *Nannizzia*, *Microsporium*, *Lophophyton*, *Arthroderma*, *Ctenomyces*, *Guaromyces* and *Paraphyton*), but species that are relevant to routine diagnostics now belong to smaller groups, which enhances their identification”.

However, in medical mycology the most important are genus *Trichophyton*, *Epidermophyton*, *Microsporum*. Very important differences between mentioned species are their natural habitat. Regarding natural habitat, dermatophyte species are classified in three ecological groups: anthropophilic, zoophilic, or geophilic, where some species are still unclassified due to insufficient data (Ajello, 1962; Weitzman and Summerbell, 1995; Georg, 1960).

Anthropophilic species naturally colonize humans, but rarely animals (de Hoog et al., 2017); in this group, human to human (direct contact or indirectly through contaminated objects) transmission is present; usually cause chronic, mild, noninflammatory infections; the changes on the skin are usually hyperemic with desquamation, sharply demarcated from the healthy tissue, on the hairy part of the head there are small beaches with superficial desquamation, similar to dandruff, while the hair is thinned; minor or major epidemics may occur in collectives. The dominant species all over the world are *Trichophyton violaceum* (*T. violaceum*), *T. tonsurans*, *T. soudanense*, *Microsporum (M.) audouinii*, *Epidermophyton (E.) floccosum* (Otašević et al., 2019).

Zoophilic species parasitize animals, but can infect humans, transmission to humans usually occurs through symptomatic or asymptomatic animals-reservoirs; Mostly species *M. canis*, *T. mentagrophytes*, *T. verucosum* are causative agents; cause strong inflammatory processes, can cause chronic infection that usually takes a long time to treat, sometimes up to a year, not frequently, this infection is accompanied by fever and regional lymphadenitis. After healing, they can cause scarring and permanent alopecia. There is no interhuman transmission except in extremely rare cases (Chmel, 1980; Mayr, 1989).

Geophilic species live in the soil and sporadically lead to human infection; in addition to the soil as a reservoir, they are often found on the fur of animals; in the case of infections, geophilic just like zoophilic species cause acute, inflammatory mycoses in humans, while most infections are quickly resolved (Feuerman et al., 1975; Otašević et al., 2011, 2018).

Clinical manifestations

Based on the literature data, virulence factors of dermatophytes include: (i) adhesion factors that allow fungal spores to bind to the most superficial layer of the skin; (ii) a sulfite pump that allows fungi to cleave disulfide bonds in keratinized tissue, which enables the decomposition, and lyses of proteins; (iii) keratinase production and other proteinases that hydrolyze keratin; (iv) immunomodulatory structures in the composition of the cell wall that suppress the cellular immune response (Weitzman and Summerbell, 1995).

Superficial dermatophytosis is characterized by a slow but constant development of the infection. The changes are sharply demarcated from the healthy skin, accompanied by dandruff, without or with weak inflammation if SFI is caused by anthropophilic species. The most common zoophilic species can cause deep infections that affect the dermis, with a pronounced inflammatory response thus they are manifested by painful pustules, abscesses and lymphadenopathy (Weitzman and Summerbell, 1995; Nenoff et al., 2014).

Symptoms and signs i.e., clinical findings in patients with dermatophytosis depend on the location of the infection, the causative species as well as the immune status of the host.

Tinea pedis

The most common causative agents of *t. pedis* are *T. rubrum*, *T. interdigitale*, *E. floccosum*.

Clinical manifestation: The clinical picture is not pathogen-specific, it can be acute but frequently turns into a chronic form. Intertriginous form localized in the interdigital space is usually associated with onychomycosis (Bardazzi et al., 2013; Piccardo-Geisinger et al., 2014). This form is characterized by maceration, dandruff, itching, vesicle formation and later with burning sensations and pain. Secondary bacterial infections often occur. In addition, the hyperkeratotic type of *tinea pedis*, Moccasin-type *tinea* caused by *T. rubrum* and the third variant the inflammatory vesiculobullous or dyshidrotic type are also described (Romano et al., 2006; Boboschko et al., 2005; Canavan and Elewski, 2015).

Risk factors: *T. pedis* and onychomycosis are becoming more common as a result of changes in lifestyle, including increased urbanization, the use of communal bathing facilities, and occlusive footwear. The increasing incidence of diabetes and HIV infection are also important contributory factors. Certain occupations (miners, soldiers) and recreational activities (marathon runners) predispose *t. pedis* (van der Snoek et al., 2013).

Tinea manuum

The most common causative agents of *T. manuum* are *T. rubrum*, *T. interdigitale*, *E. floccosum*.

Clinical significance: occurs more rarely than *t. pedis*; infection is the most often localized on the palms; it is manifested as a hyperkeratotic form, but can be developed to more severe forms with the appearance of vesicles, maceration, ragads. In addition to this form, intertriginous form occurs very often due to *E. floccosum* infection (Weitzman and Summerbell, 1995; Nenoff et al., 2014).

Risk factors: *T. manuum* occur in people whose profession predispose infection such as bakers, cooks, bartenders. In both *t. pedis* and *t. manuum*, poor hygiene has significant effect on the occurrence of infection (Zhan et al., 2013).

Rarely, *tinea manuum* occurs in bullous form- Bullous *tinea* (Aste et al., 2005). The most common causative agents are *T. rubrum*, zoophilic *Trichophyton* species, as well as *Arthroderma benhamiae*. Clinical examination reveals erythematous-squamous plaques with blister formation. Diagnosis includes detection of fungal hypha forms in PAS stained tissue sections. However, in the beginning of disease, conventional mycological examination can provide confirmation of infection.

Tinea corporis

The most common causative agents: *T. rubrum*, *T. interdigitale*; zoophilic dermatophytes *M. canis*, *T. verrucosum* in children and young adolescents; *T. violaceum*, *M. audouinii* in Africa *T. concentricum* in Southeast Asia Africa and South America (Weitzman and Summerbell, 1995; Nenoff et al., 2014; Otašević et al., 2019; Nenoff and Haustein, 1997).

Clinical significance: Changes are called Herpes tonsurans or ringworm. They occur on the skin, and spread slowly but steadily, most often in circular manner. Lesions are clearly demarcated from healthy skin by an erythematous rim with different types of skin efflorescence, such as papules, vesicles, pustules and crusts, appearing on the affected area, depending on the degree of inflammation. In the center of the change, lighter fields with less pronounced desquamation can be seen. The change is often accompanied by itching of varying intensity. Atypical lesions are seen in immunocompromised patients. Zoophilic species can cause intense inflammation with severe folliculitis.

One of the forms is *t. inguinalis* characterized by macerated and scaly lesions. It is so-called “eczema marginatum” and *T. rubrum*, *T. interdigitale* and *E. floccosum* are dominant causative agents (Bonifaz and Vázquez-González, 2011; Seebacher et al., 2008).

T. glutealis frequently misdiagnosed as anal dermatitis, is characterized by inflammatory follicular papulopustules with typical erythematous-squamous plaques with prominent margins (Ely et al., 2014).

Risk factors: The most common infections in prepubertal children are *T. corporis* and *T. capitis* (Ely et al., 2014). Additionally, diabetes mellitus, long-term treatment with antibiotics or immunosuppressives, compromised peripheral circulation, wearing inadequate synthetic clothing, hyperhidrosis, elevated body mass index, poor personal hygiene and in case of infection by zoophilic species keeping pets or engaging in livestock farming (Weitzman and Summerbell, 1995; Nenoff et al., 2014; Otašević et al., 2018).

Tinea barbae and capitis

The most common causative agents of *T. barbae* and *T. capitis* are zoophilic dermatophytes *M. canis*, *T. mentagrophytes*, *T. verrucosum* (Havlickova et al., 2008; Rodríguez-Cerdeira et al., 2021; Kovitwanichkanont and Chong, 2019).

Clinical manifestation: it is an infection commonly involving the scalp and the beard area, and may be superficial or a severe inflammatory form (Kovitwanichkanont and Chong, 2019). Mild form is usually subclinical and appears as slight erythema and a few patchy areas of scalp with dull gray hair stumps. Contrary, highly inflammatory reaction manifests with folliculitis, kerion formation, and, sometimes accompanied by fever, malaise, and regional lymphadenopathy. Both the skin surface and hairs are involved which can lead to extensive areas of scarring and alopecia. Such infections on the scalp are called *Kerion celsi*, and on the men's beard *Sycosis barbae* (Rodríguez-Cerdeira et al., 2021; Weitzman and Summerbell, 1995; Nenoff et al., 2014; Otašević et al., 2018).

Favus or tinea favosa is a severe and chronic inflammatory dermatophyte infection, due to *Trichophyton schoenleinii* infection. This form of tinea capitis has become a history, except in some areas in Eurasia and Africa. Clinically, favus is characterized by scutula (yellow saucer-shaped crusts composed of epithelial debris and fungal mycelia formation) around hair follicles. Disease is manifested by severe scarring alopecia, and besides scalp, it may affect the glabrous skin and nails (Rippon, 1985; Kwon-Chung and Bennett, 1992; Daadaa and Ben Tanfous, 2020).

Tinea incognito/tinea atypical, unrecognized tinea, by definition is a dermatophyte infection that has lost its typical clinical appearance. It frequently occurs due to: (i) uncritical use of topical corticosteroids or calcineurin inhibitors; (ii) improper treatment; (iii) self-medication; (iv) the lack of differential diagnostic consideration of SFI (Seitz et al., 2013; Zisova et al., 2013).

It should be point out that there has been a worldwide increase in *T. incognito* incidence. Clinically, pruritic erythematous and erosive lesions, often described as eczema-like and psoriasis-like are seen in these patients and often occurs on the trunk and face. *T. rubrum* was found to be the most common causative agent (Kim et al., 2013), but *M. canis* was also described as the pathogen (Amano et al., 2013). The general opinion is that if in patients with *t. pedis* or onychomycosis, eczema-like dermatoses develop on the trunk or face, *T. incognito* occurrence should always be considered.

Trichophyton rubrum syndrome represents a chronic and generalized dermatophytosis with at least four body sites are affected: feet (plantar), hands (palmar), nails, as well as one other site. Depending on the used criteria, syndrome is diagnosed by mycological analyses-detection of fungal elements using mycological examination of material from four sites or cultivation of *T. rubrum* from material from three sites, where in both used criteria inguinal region explicitly has to be excluded (Piñeiro et al., 2010).

Majocchi granuloma (granuloma trichophyticum) is a chronic (Matthes et al., 2001) superficial dermatophytosis in which comes to the appearance of granulomatous follicle-associated inflammatory reaction as a complication of fungal infiltration along hair follicles. The skin lesions are pruritic, erythematous-squamous plaques with follicular papules and pustules and this form of dermatophytosis affects immunosuppressed patients (Steiner et al., 2012). In the most cases of Majocchi granuloma, *T. rubrum* is the causal pathogen, but *T. tonsurans* is also described (Kurian and Haber, 2011).

Dermatophytids or mycids, so-called ID reaction is a consequence of induced, type IV hypersensitivity reactions to dermatophyte components, very frequently as a result of antimycotic treatment (Ilkit et al., 2012). The localization of the lesions is far from the primary dermatophytes infection; they can be as popular or as nodular and multiform eruption. Fungi cannot be isolated from these changes and dermatophytids are remediated when primary fungal infection is cured (Romano et al., 2006; Cheng et al., 2011).

Mycological analyses-diagnostics of superficial fungal infection caused by dermatophytes

Specimen collection

For mycological analyses adequate specimen collection, transport and storage is of crucial importance. The most important principles during specimen collection are:

- The use of aseptic technique
- The material should be sampled appropriately -from the appropriate place (for all infection the material is sampled from the periphery of the change, except in the case of Pityriasis versicolor infection when material for mycological analyses is sampled from the central part).
- Material has to be sampled before starting empirical therapy
- Extra care should be taken if sharp scalpel blade or scissors are used to collect samples
- The quantity of sampled material must be sufficient for all analyses
- The material must be accompanied by clinical findings
- Sacrifices and hair/hairs must be transported in dry sterile containers or envelopes (to prevent bacteria and/or mold overgrowth) which are put in sealed plastic bags
- In case of skin and hair samples, direct inoculation in media for cultivation is recommended immediately after sampling. However, if that is not possible, fast transport, in a period of 2 h, is considered optimal for successful mycological analyses
- The hairbrush technique is favored over scalp scraping in *T. capitis* when causative agents are anthropophilic dermatophytes. On the contrary, epilation of hairs and simple swabs are preferred in purulent infections (Nasir et al., 2014; Nenoff et al., 2014).

Microscopic examination

Despite the efforts for introduction of rapid, non-culture-based assays, the microscopic examination (ME), as user-friendly and rapid method, is irreplaceable for detection of fungi in patient's material. Wet mount with chlorolactophenol or KOH is still convenient technique for screening and direct microscopy examination provides rapid detection of fungal blastoconidia-yeast and pseudohyphal-hyphal forms in patient's material (Figs. 1 and 2) (Brooks et al., 2013; Otašević et al., 2011, 2018). Additionally, specific staining with chemicals or fluorescent dyes can improve visualization and detection of fungi (Arsić-Arsenijević et al., 2012). Calcofluor white binds to the chemical structures (cellulose and chitin) of the cell wall so using the fluorescent microscopy fungal elements could be easier detected.

Culture method—The propagating method

Cultivation and isolation of the causative agent is superior, yet time-consuming procedure that enables antigen-detection, genetic and biochemical identification of etiological agents. In addition, it also allows antimicrobial susceptibility testing.

Dermatophytes can be cultivated on selective media that contain the higher concentration of carbohydrates and antibacterial dugs which prevent bacterial growth and cycloheximide (Actidion®) to suppress mold growth. The optimal temperature for cultivation is 28 °C, and incubation last for three (or four) weeks. Since the time of incubation is so long, growth of fungi is visually checking twice weekly (Nenoff et al., 2014; Arsić-Arsenijević et al., 2012; Otašević et al., 2011). Depending on laboratory equipment, even some selective media, such as dermatophytes test media (DTM), can be used. This particular agar reveals

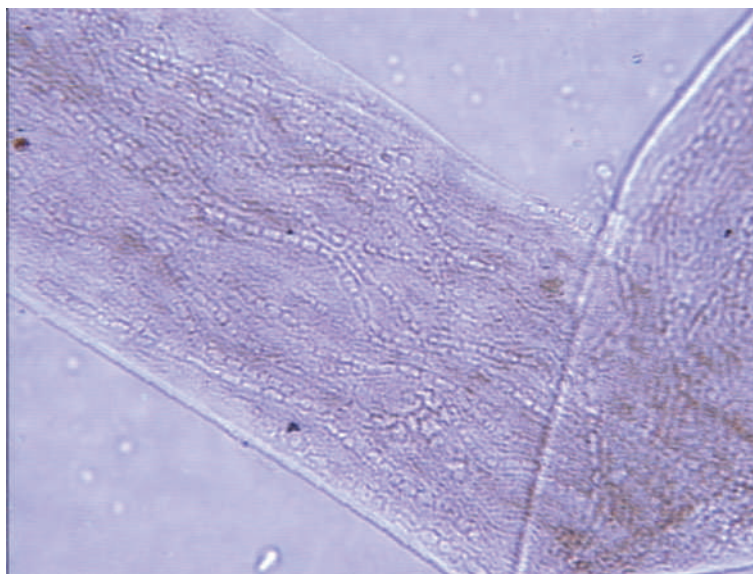


Fig. 1 Chlorine lactophenol wet microscopic preparation showed spores in the hair.

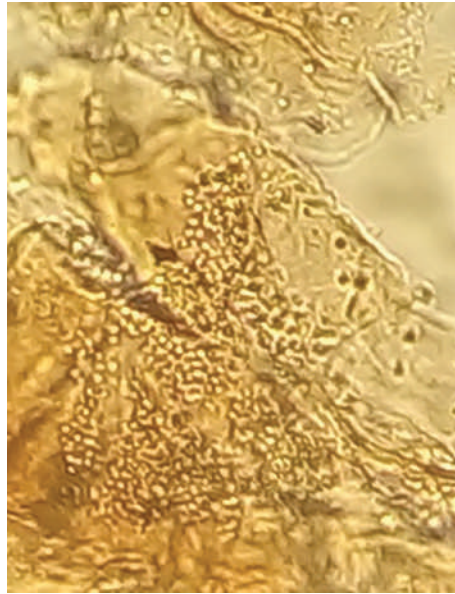


Fig. 2 Chlorine lactophenol wet microscopic preparation showed numerous spores in the scarification of the skin.



Fig. 3 Whitish-yellow fluffy colony of species *Microsporum canis* on Sabouraud dextrose agar.

dermatophytes growth by color changes from yellow to red due to production of alkaline metabolites. However, in this way the pathogens natural pigment, which is important for identification of species, may be masked. Thus, simultaneous cultivation on both Sabouraud agar and DTM remains mandatory (Figs. 3 and 4).

The differentiation of dermatophytes is based on macroscopic and microscopic morphological characteristics. Macroscopic characteristics include appearance, structure and pigmentation of vegetative and reproductive mycelium (visualization of upper and bottom side of colonies), and growth rate. Microscopic morphological characteristics as sporulation type—formation of macro and microconidia, growth rate schedule and appearance of hyphae are crucial in differentiation of dermatophytes (Fig. 5) (Otašević et al., 2011).

In addition to fungal morphology analysis, physiological tests such as in vitro hair perforation test, hydrolysis of urea, subcultivation on special nutrient media and temperature tolerance test can also be useful in differentiation of dermatophytes species (Otašević et al., 2011).



Fig. 4 Whitish fluffy colony of species *Microsporum canis* on Dermatophytes test medium.



Fig. 5 Characteristic numerous macroconidia, spindle shape with taper to knoblike ends of species *Microsporum canis*.

Immunodiagnostic test—Immunochromatographic kits

Since conventional mycological analyses require time and expert knowledge, a lot of effort is put into the design of rapid immunochromatographic tests (Hayette and Sacheli, 2015). One IC strip uses *Trichophyton* antibodies coated with colloidal gold particles to capture antigens of seven different dermatophyte species. When compared with ME, IC shows sensitivity and specificity of up to 80% for detection of dermatophytes in skin and nail samples (Higashi et al., 2012; Noriki and Ishida, 2016; Tsunemi et al., 2014). Although these tests are convenient and rapid method for screening of dermatophytosis, for mycological diagnosis ME and culture are required.

Molecular methods

Over the last 40 years, a large number of molecular techniques have been developed to identify dermatophytes to the level of a genus or species (Otašević et al., 2018). Molecular techniques using different specific primers are capable of accurate discrimination between zoophilic, geophilic and anthropophilic species. There are even studies which imply that these tests may change the whole perspective on taxonomy, ecology and epidemiology of dermatophytes (Cafarchia et al., 2009, 2013; Tamura et al., 2007). Moreover, due to the possibility of identification they caused a “revolutionary change” in the diagnosis of SFI caused by dermatophytes.

Recently published results (Motamedi et al., 2017) showed that real-time PCR with specific pan-dermatophyte primers for detection of the most common species in clinical samples, identify more infected patients than conventional methods. The use of “in-house” or rare commercial molecular tests enabled rapid detection directly in the patient’s sample in just 48 h (Kondori et al., 2010). In addition, developed single-tube qPCR with ITS1 sequences has a design that allows the detection of 11 species of dermatophytes belonging to the genera *Trichophyton*, *Microsporum* and *Epidermophyton* directly in a patient sample (Bergmans et al., 2010; Dhib et al., 2014). Multiplex PCR for the detection of *T. rubrum* and *T. mentagrophytes* in nail samples with chitin synthetazome 1 and ITS primers, proved to be a molecular test with excellent diagnostic performance (sensitivity 95% and specificity 100%). Also, there are new data on the possibility of using the PCR-ELISA method for the fast and accurate detection of anthropophilic species (*T. violaceum*, *M. audouinii*, *T. soudanense* and *T. rubrum*) in the material of patients with tinea capitis, primarily in endemic areas of Africa. Such testing allows rapid diagnosis, application of effective and appropriate therapy, determination of a possible source of infection and application of preventive measures in order to prevent the spread of the causative agent and infection (Wiegand et al., 2017).

Despite the claims of some authors that molecular techniques cannot be a substitute but only a support to conventional diagnostics due to lower sensitivity and lower positive predictive value of 66.5%, development, standardization and commercialization of molecular tests are of great importance for diagnosis of SFI.

Treatment of superficial fungal infection caused by dermatophytes

The first antimycotic drug that was introduced in clinical practice during the middle of the 20th century is griseofulvin. Afterwards, in the 1980s, ketoconazole was started to be used, while eventually, a decade later, fluconazole, itraconazole and terbinafine were developed. The increased resistance of dermatophytes to antifungal drugs, observed in the last few years, raised the interest of researchers. Therefore, many studies have been conducted in order to single out the most efficient antimycotics and to update the therapeutic guidelines for fungal infections (Bishnoi et al., 2018; Khurana et al., 2018).

The effectiveness of systemic antifungals depends on various factors, such as: fungal characteristics (host adaptability, virulence, variation in drug susceptibility, biofilm formation); host factors (immunocompetence); drugs features (mechanism of action, MFC/MIC ratio, absorption, levels achieved in skin, keratin adherence) and finally clinical presentation of SFI.

It appears that resistance established in vitro correlates with therapeutic failure. On the other hand, good in vitro susceptibility does not guarantee favorable clinical outcome (Espinel-Ingroff, 2000). Accordingly, the term recalcitrance is now preferred over the term resistance since it better define in vivo antimicrobial activity by referring to both in vitro resistance and all the other potential contributors to treatment failure (Jones, 1990; Sardana and Khurana, 2018). Dermatophytosis may be treated with azole derivatives, allylamines, griseofulvin, polyenes and others. The azole-derived agents act by inhibiting the lanosterol 14 α -demethylase and that way interfere with synthesis of ergosterol. They represent the biggest group of antifungals which includes both systemic (Fluconazole, Ketoconazole, Itraconazole, Voriconazole, Posaconazole, Isavuconazole) and local (Clotrimazole, Miconazole, Econazole, Luliconazole, Lanoconazole, Efinaconazole, Sertaconazole, Oxiconazole, Eberconazole, Fenticonazole, Bifonazole) agents. Allylamines inhibit squalene epoxidase and also encompass systemic (Terbinafine) and topical (Terbinafine, Butenafine, Naftifine) drug forms. Heterocyclic benzofuran—Griseofulvin was one of the first antimycotic and it inhibits the microtubule aggregation. Polyenes (Nystatin, Natamycin, Amphotericin B) affect cytoplasmic membrane permeability by formation of pores and they can be applied locally. Echinocandins (Anidulafungin, Caspofungin, Micafungin) which inhibit glucan synthesis, have only demonstrated good in vitro effect, however there are still no results from clinical trials.

Apart from mentioned groups of antifungals, following drugs can be used for local application: Morpholine Amorolfine (Inhibition of C-14 reductase and C8 isomerase); Thiocarbamate Tolnaftate (Inhibition of squalene epoxidase); Hydroxypyridones-Ciclopirox (Inhibition of metal dependent enzymes—catalase, peroxidase); Tavabarole (cytoplasmic leucyl tRNA synthetase—inhibition of protein synthesis and termination of cell growth) (Khurana et al., 2018).

Systemic antimycotics being used for dermatophytoses treatment are azoles ITR, FLU, KTZ and allylamine TRB. Over the last three decades, ITR showed favorable results in treatment of dermatophytoses. This drug achieves sufficient concentration in SC, good accumulation, with concentration being higher than in plasma (Cauwenbergh et al., 1988); obtained concentration is maintained for weeks after the therapy; there are no public data of dermatophytes resistance to ITR, except occasional reports of *T. interdigitale* higher MIC (Singh et al., 2018). The common dose is 100 mg for 15 days or 200 mg for 7 days, while the therapy course may be prolonged if the infection is not cured (Khurana et al., 2018; Sardana and Gupta, 2017; Rajagopalan et al., 2018).

Another azole used in treatment of dermatophytoses is FLU, which rapidly accumulates in SC exhibiting high concentration. However due to low binding avidity, concentration is not long lasting (Wildfeuer et al., 1994). Fluconazole is considered effective in treatment of tinea capitis (Gupta and Cooper, 2008). It is prescribed in a dose of 150 mg weekly, given for 2–6 weeks (Stary and Sarnow, 1998; Suchil et al., 1992; Sardana and Khurana, 2018). Unfortunately, it was noted that this weekly regimen may not lead

to clinical improvement (Balci and Cetin, 2008), while several studies established high in vitro MICs (Ghannoum et al., 2006; Gupta et al., 2005; Khurana et al., 2018; Rudramurthy et al., 2018). A study from India reported that almost 50% of isolates were resistant to FLU (Singh et al., 2018) where it is not considered a first-line drug for dermatophytoses anymore.

Ketoconazole has good clinical efficacy, high keratin adherence and steady levels in the SC even 10 days after the end of treatment (Jones, 1990). Additionally there are no reports of resistance while reports of high in vitro MICs are rare (Singh et al., 2018). Nevertheless, due to hepatic toxicity, it is used with caution and restrictions while in some countries its use is completely forbidden (Gupta and Lyons, 2015). The drug is still used locally for superficial mycoses (Gupta and Lyons, 2015) whereas it is sporadically employed in a dose of 200–400 mg/day as a “last resort” option for severe recalcitrant dermatophytoses, with mandatory liver function monitoring.

Allylamine TRB has good penetration to SC mainly because of secretion into sebum and maintains high concentration even after treatment is finished (Faergemann et al., 1991, 1993). It is well tolerated and has minimal drug interactions, hence it is drug of choice for dermatophytoses. Recommended dose is 250 mg, 2–3 weeks, which can be prolonged if needed.

Griseofulvin is the first systemic antifungal used for treatment of dermatophytoses. Regarding its no special binding forces existing to hold GRI in SC (Epstein et al., 1972), GRI require prolonged treatment of several weeks to months. Studies showing resistance of dermatophytes isolates to GRI (Yenişehirli et al., 2013; Mistik et al., 2006) limited the use of GRI to tinea capitis (Gupta and Cooper, 2008).

Topical antifungals that are used for dermatophytosis treatment are shown in Table 1 (Evans et al., 1993; Givens et al., 1995; Goldstein et al., 2000; Noble et al., 1998; Shear et al., 1998).

Epidemiology of dermatophytes infection

The prevalence of certain types of dermatophytes which are causative agents of SFI and the localization of these infections differs depending on the geographical, socio-economic, and ecological characteristics of the region and people's habits and customs in certain areas.

In Asia, *T. rubrum* and *T. mentagrophytes* are the most commonly isolated causative agents of tinea pedis and tinea unguium. The most common causes of tinea capitis are zoophile dermatophytes, species *M. canis* and *T. verrucosum* (Rodríguez-Cerdeira et al., 2021). Also, as a very dominant species causing tinea capitis and corporis, the fungus *T. violaceum* has been proven on this continent (Celik et al., 2003; Lari et al., 2005). Similarly, based on the available data, in Australia, the same species as in Asia are most common causes of tinea pedis and tinea unguium, but (Merlin et al., 1999) in this area of the world, *T. violaceum* as a cause of tinea

Table 1 Local therapy of dermatophyte infections.

Agent	Drug formulation and dosing frequency
<i>Allylamine</i>	
Naftifine	1% Cream once daily
	1% Gel once to twice daily
Terbinafine	1% Cream solution once to twice daily
<i>Benzylamine</i>	
Butenafine	1% Cream once to twice daily
<i>Imidazole</i>	
Clotrimazole	1% Cream, solution, twice daily
Econazole	1% Cream once daily
Ketoconazole	1% Cream once daily
	1% Shampoo twice weekly
Miconazole	2% Cream, spray, lotion, powder—twice daily
Oxiconazole	1% Cream or lotion once to twice daily
Sulconazole	1% Cream or lotion once to twice daily
<i>Other</i>	
Ciclopirox	1% Cream or lotion twice daily
Tolnaftate	1% Cream or solution or powder twice daily

Note. Local therapy is used in *Tinea corporis*, *Tinea pedis* (uncomplicated), *Tinea cruris*, *Tinea manuum* and *Tinea faciei*. From Givens TG, Murray MM and Baker RC (1995) Comparison of 1% and 2.5% selenium sulfide in the treatment of tinea capitis. *Archives of Pediatrics and Adolescent Medicine* **149**: 808–811; Evans EG, Dodman B, Williamson DM, Brown GJ and Bowen RG (1993) Comparison of terbinafine and clotrimazole in treating tinea pedis. *BMJ* **307**: 645–647; Shear NH, Einarson TR, Arikian SR, Doyle JJ and vanAssche D (1998) Pharmacoeconomic analysis of topical treatments for tinea infections. *International Journal of Dermatology* **37**: 64–71; Noble SL, Forbes RC and Stamm PL (1998) Diagnosis and management of common tinea infections. *American Family Physician* **58**: 163–174; Goldstein AO, Smith KM, Ives TJ and Goldstein B (2000) Mycotic infections. Effective management of conditions involving the skin, hair, and nails. *Geriatrics* **55**: 40–52; Tasić S and Pešić S (2006) *Fungal Infections: Diagnosis and Treatment Possibility*. University of Niš, Medical Faculty: Niš (in Serbian).

capitis has been proven mainly in immigrants from Africa. Additionally, unlike in Asia the predominant causative agents of tinea capitis in this territory are anthropophilic species (*T. soudanense* and *M. audouinii*) (Rodríguez-Cerdeira et al., 2021). In America, the most common dermatophytes are *T. rubrum*, *T. tonsurans*, and *T. mentagrophytes* (Brilhante et al., 2000; Welsh et al., 2006). A dramatic increase in the incidence of *T. tonsurans* infections has been registered, especially among athletes who practice martial arts, which is why the term “Tinea corporis gladiatorum” was introduced. In the United States, *T. rubrum* is the cause of tinea manuum/pedis in 70% of patients (Foster et al., 2004). *T. tonsurans* has been proven in as many as 95% of patients with tinea capitis, after long-term dominance of the anthropophilic species *M. audouinii* (Kemna and Elewski, 1996). In Canada, in a period of only 10 years (1985–96), an incredible increase in the prevalence of tinea capitis also caused by *T. tonsurans* was recorded (Gupta and Summerbell, 1998). In the previous period, the most common causes of superficial mycoses were *T. verrucosum* and *Microsporum* spp. In South America, the zoophilic species *M. canis* and *T. mentagrophytes* and the geophilic species *M. gypseum* are the most common causes of tinea capitis in children (Rodríguez-Cerdeira et al., 2021). *T. rubrum* is the predominant cause of tinea corporis in these areas, and *T. tonsurans* is most common in tinea capitis in adults (Foster et al., 2004). In the central part of the American continent, the species of dermatophytes with the highest prevalence are *T. rubrum*, *T. mentagrophytes*, *M. canis*, *T. tonsurans* and *E. floccosum* (Welsh et al., 2006). Fungal infections are very often diagnosed in Africa. The incidence of tinea in 2005 in Sub-Saharan Africa was around 78 million cases (Hay et al., 2006). The species *M. audouinii*, *T. violaceum*, *T. soudanense*, and *T. gourvilii* are the most common causes of tinea capitis. *T. mentagrophytes* and *T. rubrum* are the most common causative agents of tinea corporis, especially tinea cruris or tinea axillaris in women (Enweani et al., 1996; Ellabib et al., 2002; Abu-Elteen and Malek, 1999).

The most common causes of superficial mycoses in European countries are *T. rubrum*, *M. canis*, *T. mentagrophytes*, and *T. verrucosum*. The dominant causative agents of tinea capitis are *M. canis*, *T. mentagrophytes*, *T. tonsurans*, and *T. violaceum* (Rodríguez-Cerdeira et al., 2021). *T. rubrum* is the most common cause of SFI in urban centers of France, Germany, Switzerland, Finland, and Russia (Foulet et al., 2007; Seebacher et al., 2008; Lehenkari and Silvennoinen-Kassinen, 1995; Monod et al., 2002; Khaldin et al., 2005). In the United Kingdom the predominant species that causes tinea capitis is *T. tonsurans*. After the long-term dominance of *T. verrucosum* in Belgium and the Netherlands, *M. canis* has been recorded as a causative agent of superficial mycoses in recent years in 40% of cases (Korstanje and Staats, 1994). In the countries of Eastern Europe, the Mediterranean, as well as in the countries of the former Yugoslav republics, zoophilic species still dominate, among which *M. canis* is also the most dominant (Nowicki, 1996; Panasiti et al., 2007; Lange et al., 2004; Dolenc-Voljc, 2005; Prohic, 2008; Otašević et al., 2019).

Malassezia spp.

Additionally to dermatophytes group of fungi, *Malassezia* species, lipophilic yeasts, recognized as skin commensals could be the causative agents of skin infection with very high prevalence. These species are capable of producing an extensive array of bioactive indolic compounds especially in pathogenic condition (Gupta et al., 2003) and can cause. Pityriasis versicolor (PV), pityriasis/ *Malassezia* folliculitis (PF) and seborrhoeic dermatitis (SD). Apart from ultraviolet radiation, recent data showed that the presence of different *Malassezia* species, different yeast density and enzyme production, could be important for *Malassezia* role in pathogenesis of melanoma (Vekic et al., 2016). The core mechanism by which *Malassezia* yeasts could probably mediate the carcinogenesis acts through the production of locally acting potent indolic AhR ligands (Gaitanis et al., 2011).

Additionally, there is the evidence that *Malassezia* spp. contribute to the development of atopic dermatitis by T-cell cross-reactivity due to similarity between fungal and human antigens (Nowicka and Nawrot, 2019). However, for explanation of the pathogenetic mechanisms and link between fungi and immune defense further research is required.

In *Malassezia* genus at least 14 different species were differentiated as: *M. furfur*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta*, *M. slooffiae*, *M. dermatis*, *M. japonica*, *M. nana*, *M. yamatoensis*, *M. equina*, *M. caprae*, *M. cuniculi* and *M. pachydermatis* (Naldi and Reborá, 2009).

Clinical manifestation

Pityriasis versicolor is SFI caused by *Malassezia* yeast that transform from commensal to pathogen form. Invasion of yeast to stratum corneum influences pathological changes in skin areas. Infection is characterized by round or irregularly shaped macular change with squamation which may be accompanied by lesions, wrinkles and widespread skin may be affected. Lesions are hypopigmented in dark skin or hyperpigmented in white skin individuals. Mild itching may accompany the visible changes (Faergemann, 1993, 2000).

Seborrhoeic dermatitis is eczema where beside genetic predisposition *Malassezia* species may be causative agents. SD is characterized by poorly defined erythematous, flaking, and greasy-looking patches, following by itching in some cases (Schwartz et al., 2013).

Malassezia/pityriasis folliculitis is an inflammatory condition of the sebaceous glands. In spite some evidence of inflammatory reaction which is followed by focal rupture of the follicular epithelium, pathological mechanism is still unclear (Faergemann, 2000; Gupta et al., 2004). A rash of uniform erythematous papules or pustules are often seen in patients (Abdel-Razek et al., 1995).

Diagnosis

The diagnosis is made on the basis of the clinical findings and microscopic examination of skin scraping samples can be taken from the surface of skin (area of 4 cm²) by scraping or using adhesive tape on the skin for 1920s (Pechere et al., 1999). Detection of active reproduction of yeast with forms like meatballs and spaghetti pattern during microscopic examination goes in favor of infection. In Wood's light, that can be used, a bright yellow color may be emitted from the lesions and thus visualize the extent of the affected skin areas. It has to be highlighted that negative Wood's light examination does not exclude PV as not all *Malassezia* species fluorescence (Hald et al., 2015).

For the isolation, selective media as modified Dixon agar (mDIX), Leeming-Notman agar (LNA), potato dextrose agar with olive oil (Oil-PDA) for the isolation of lipophilic species, and standard mycological media Sabouraud dextrose agar (SDA) for the isolation of *M. pachydermatis* can be used. Inoculated media incubated for 10 days at 32 °C under aerobic conditions and growth on culture has to be observed daily (Guillot and Gueho, 1995; Leeming et al., 1989).

A preliminary differentiation of the lipophilic *Malassezia* species can done by detection of colonies on selective media, Dixon and LNA, followed by absence of growth on SDA. Identification of isolated strains included microscopic examination (cell characteristics) and testing of physiological and biochemical characteristics (catalase production and Tween assimilation) (Leeming et al., 1989; Barac et al., 2015). Recently, modified CHROMagar Candida with ox bile, glycerol monostearate, glycerol and Tween 60 as an essential ingredients for *Malassezia* growth, make possible the simultaneous isolation, species differentiation and identification of *Malassezia* and *Candida* species (Kaneko et al., 2005).

Treatment of SFI caused by *Malassezia* spp.

Pityriasis versicolor is usually successfully treated with topical agents that has antimycotic or keratolytic effect. Systemic treatment, alone or in combination with topical antimycotic, is occasionally used in case of serious infection or when local treatment was ineffective (Montero-Gei et al., 1999; Faergemann, 2000; Dehghan et al., 2010; Partap et al., 2004).

It should be kept in mind that sometimes for complete resolution of skin changes several months are needed, especially when hypopigmentation is present. Subsequent mycological analysis is required for confirmation of healing (Drake et al., 1996; Fredriksson and Faergemann, 1983; Hull and Johnson, 2004; Faergemann and Fredriksson, 1980).

Considering high recurrence rate of this infection, different maintenance therapy modalities are proposed. Application of selenium disulfide every 3 months for 2 years is appropriate since it reduces chance of relapse from 82% to 20%. Likewise, the use of ketoconazole 2% shampoo once daily for at least 3 days at the beginning of summer session may also be useful (Piérard-Franchimont et al., 2002; Stratigos et al., 1988).

In case of Seborrheic dermatitis in the majority of patients, local therapy is sufficient. Systemic treatment approach is undertaken when large area of skin is affected or when there is resistance to topical treatment. Most commonly antifungals and corticosteroids are used in combination. The maintenance therapy is frequently required (Peter and Richarz-Barthauer, 1995; Faergemann, 1986; Taieb et al., 1990; Hald et al., 2015).

Data regarding treatment of *Malassezia* folliculitis are limited since studies are done on small number of subjects. It is considered that systemic therapy is more effective than local, although the best results are achieved when these both are combined. Here also, maintenance therapy is preferable in order to avoid relapse. Local antimycotics can be combined with keratolytics (Levy et al., 2007; Abdel-Razek et al., 1995; Jacinto-Jamora et al., 1991; Yu et al., 1998).

Candida spp.

Superficial fungal infection caused by *Candida* spp. can be divided into three distinct entities, intertrigo, paronychia and onychomycosis. *Candida*-intertrigo, as its name implies, occurs on intertriginous areas of skin, namely between fingers or in axillary, pubic and submammary regions (Tasić and Pešić, 2006). The main predisposing factors for this condition are moisture, heat, friction, and maceration. It is especially common among young children living in poor sanitary conditions and elderly people (Yibin et al., 2018; Lane, 1995; Varade and Burkemper, 2013). Typical skin lesions are erythematous plaque with erosions and satellite pustules that have tendency to spread on healthy skin. Chronic forms of infection are part of clinical picture of chronic mucocutaneous candidosis, but may also be seen in other immunodeficiencies (Jautová et al., 2001).

Superficial candidosis (SC) is routinely established using conventional mycological methods specifically ME (cytology) and fungal cultivation. Microscopy is a method that may quickly provide initial diagnosis. Although fast and simple method that enables rapid detection of fungi (blastospores, pseudohyphal or hyphal forms) in the patients' material, microscopy, in the most cases, cannot be used for determination of genus or species (Otašević et al., 2018). In mycological laboratories selective media, Sabouraud dextrose or maltose agar with gentamicin and chloramphenicol, are used for isolation of *Candida* spp. optimal cultivation time of yeast is 48–72 h at 37 °C in aerobic atmosphere. Following their growth on the selective media, yeast are identified through biochemical characteristics. Two tests used for determination of phenotypic characteristic of *C. albicans* species and its differentiation from other species are germination test and chlamydoconidia production test (Savić et al., 2020). Additionally, differentiation of yeast is made using assimilation test. Interpretation of assimilation or other biochemical tests is based on opaqueness or change of indicator color which is done by mycologist or in more advanced laboratories by automatized systems

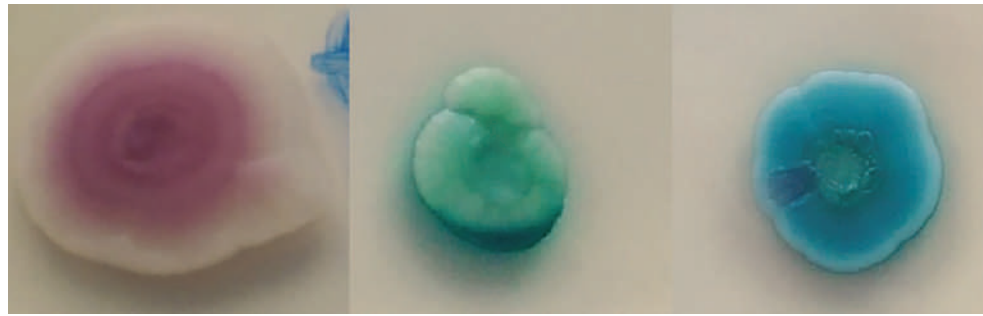


Fig. 6 Differentiation of three *Candida* species on the basis of colony color during primoisolation: *C. krusei*—pink colony with rough appearance *C. albicans*—green colony, *C. tropicalis*—blue colony.

(VITEC- automatized system) (Arsić-Arsenijević et al., 2012; Otašević et al., 2011). Recently, chromatogenic selective medias allow differentiation of three *Candida* species on the basis of colony color during primoisolation (*C. albicans*—green colonies, *C. tropicalis*—blue colonies, *C. krusei*—pink with rough appearance; Fig. 6). The importance of these media is heightened by the fact that it gives opportunity to diagnose infection caused by different *Candida* species, which is impossible on other medias (Arsić-Arsenijević et al., 2012; Otašević et al., 2011).

Cryptococcus spp.

The yeast of *Cryptococcus* genus may cause skin infection which can be primary or secondary. According to newest classification primary infection is defined as localized infection in the absence of systemic dissemination, cryptococcal fungemia or antigenemia (Noguchi et al., 2019). This form is rare and is usually seen in immunocompromised patients, although it may also develop in immunocompetent hosts thus should be included in differential diagnosis of skin infection (Christianson et al., 2003).

On the other hand, secondary infection is developed as part of disseminated cryptococcosis (Srivastava et al., 2015).

Skin lesions vary from small papules, vesicles, purpura, or acne like lesions while later they may ulcerate and thus cause abscess, erythematous nodules or cellulitis formation (Savić et al., 2020). The lesions are treated with systemic antimycotics.

Patients with cutaneous cryptococcosis should be closely monitored since they are at increased risk of developing disseminated form or CNS cryptococcosis. On the contrary, secondary cryptococcal skin infection in AIDS patients implies dissemination of *Cryptococcus* spp. (French et al., 2002; Jarvis et al., 2009; McKenney et al., 2015).

Nondermatophytic molds

Nondermatophytic molds rarely cause skin infections. However these fungi are widespread, persisting in immediate human surroundings, on soil and other living organisms (i.e., plants or animals), therefore skin infection are possible. Primary infection usually develops on skin damaged by laceration, burns or some medical procedures like cannula insertion, needle puncture and use of adhesive tapes (Douglas et al., 2016; Spesso et al., 2018; Obradovic-Tomasev et al., 2014). Additional risk factors are contaminated tattoos, as well as cosmetic treatment for rejuvenation such as soft tissue fillers (Shin et al., 2017). Wide presence of molds even on walls and floors of buildings contribute to infection especially when construction works are done in hospital or in the surroundings.

Cutaneous nondermatophytic mold infection may also be the result of fungal dissemination which suggest poor prognosis of underlying disease. Besides, in immunocompromised patients, skin infection caused by these fungi may be the source of pathogen dissemination causing fungemia and invasive mold infection (Durand-Joly et al., 2003; Gallais et al., 2017).

The great number of fungal genera are found in the nature. One proposed classification divide infections into two broad groups, those caused by *Aspergillus* species and those caused by non-*Aspergillus* molds. Non-*Aspergillus* SFI can be caused by *Mucorales* fungi, hyalohyphomycetes (bright pigmented fungi) and phaeohyphomycetes (dark pigmented fungi or dematiaceae fungi) (Douglas et al., 2016; Revankar and Sutton, 2010).

Aspergillus SFI: Primary cutaneous aspergillosis is typically seen after the trauma is coupled with contamination by the fungi of *Aspergillus* spp. The lesions are maculopapular yet inflammatory nodules, abscess or necrosis can develop (Frias-De-León et al., 2018; Shin et al., 2017; Gallais et al., 2017).

Mucorales fungi usually produce skin infection in intravenous drug addicts or in those tattooed with the contaminated ink. Additionally, infection also occur following extensive burns (Kang et al., 2014; Spesso et al., 2018; Xu et al., 2018; Devauchelle et al., 2019).

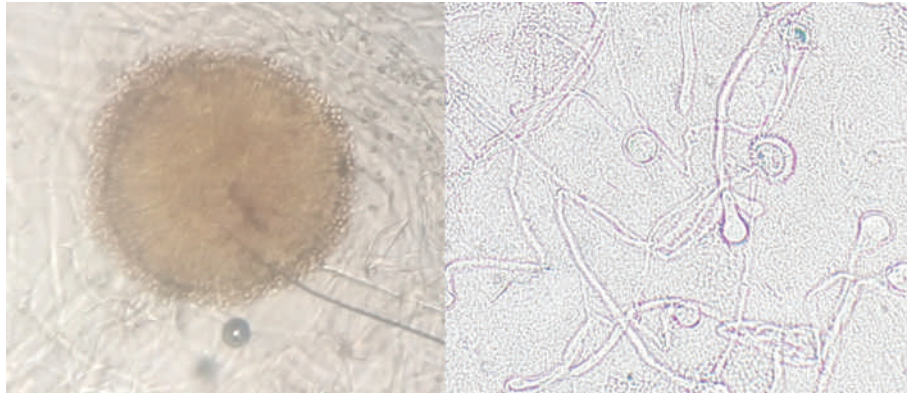


Fig. 7 Characteristic microscopic morphological characteristic of *Aspergillus* spp.: Septate hyphae, hyaline conidiophores with phialides borne directly on the vesicles.

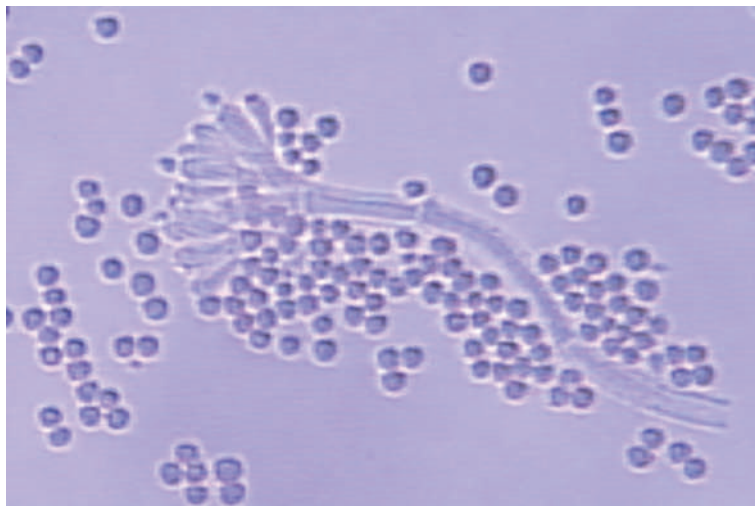


Fig. 8 Microscopic morphological characteristics of *Penicillium* spp.: Conidiophores showing two-stage branching and forming flask shape phialides in groups.

Hyalohyphomycosis are SFI commonly caused by species of genus *Fusarium*, *Scopulariopsis*, *Acremonium*, *Trichoderma* and by species *Penicillium marneffei* (Figs. 7–9). For the last 10 years there has been a significant rise in rate of these infections mainly in patient with neutropenia due to hematological malignancy. If neutropenia is not managed it contributes to failure of antifungal treatment. Multiple red or gray macules along with pustules and subcutaneous nodules with central ulceration are characteristic for *Fusarium* infection (Hay, 2007; Durand-Joly et al., 2003; Bodey et al., 2002).

Acremonium genus comprise of saprophytic molds which often cause problems in laboratory work since they contaminate medium. Skin infection is manifested by pustules and eventually may lead to erythema and edema of large skin area with ulcerations and pus drainage (Sharma et al., 2013; Hilmioğlu et al., 2015).

Scopulariopsis spp., more precisely species *Scopulariopsis brevicaulis*, can induce chronic granulomatous skin condition in both immunodeficient and immunocompetent persons (Wu et al., 2009; Gavril et al., 2016).

In the genus *Penicillium*, species *P. marneffei* is commonly labeled as causative agent of fungal infections, including infections of the skin. This species is, in some parts of the world, the most common cause of fungal infection in AIDS patients (Ungpakorn, 2000).

Over the last few years cutaneous infections presenting as necrotizing ulcer due to *Trichoderma* species have been reported in immunodeficient patients (Román-Soto et al., 2019).

In addition to mentioned non-*Aspergillus* molds, skin infection can be caused by dematiacea fungi. In the literature, reports of the possible pathogens include *Alternaria* spp. (Williams et al., 2008; Gürçan et al., 2009). *Bipolaris* spp. (Ge et al., 2017; Bilu et al., 2004; Dubois et al., 2008) *Cladophialophora bantiana*, *Curvularia* spp. (Moody et al., 2012; Berg et al., 1995), *Exophiala* species (Lucas-Truyols et al., 2019; Espanhol et al., 2019), *Scedosporium prolificans*, *Scytalidium dimidiatum* (Brandt and Warnock, 2003; Xavier et al., 2010). Dematiacea infections occur in immunocompetent subjects and are more common in tropical regions (Neoh et al., 2007; Miyagawa et al., 2017). The changes appear as erythematous or squamous lesions accompanied by rosacea-like popular lesions.

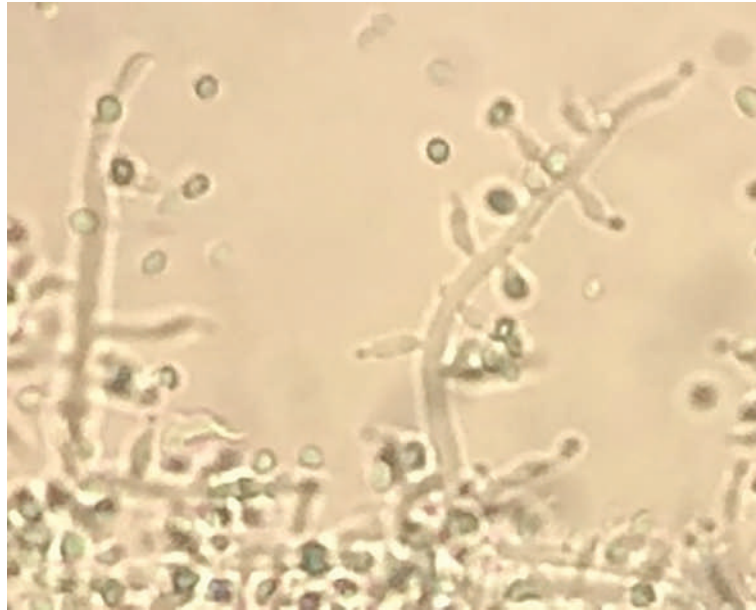


Fig. 9 Microscopic morphological characteristics of *Trichoderma* spp.: Hyphomycete with repeatedly branched conidiophores bearing clusters of flask-shaped phialides.



Fig. 10 Microscopic morphological characteristics of *Fusarium* spp.: characteristic fusiform to sickle shaped macroconidia.

Diagnosis of SFI caused by *Aspergillus* and non-*Aspergillus* molds is made by standard mycological analysis (MI and cultivation) (Figs. 7–12). Furthermore, histopathological examination is considered as the golden standard and is used for confirmation of pathogenic process induced by fungi. Of crucial importance for correct diagnosis is to avoid possible mold colonization, contamination of skin or sampled material. The growth of non-dermatophyte molds should always be investigated further and not automatically regarded as contamination. The best practice is to combine clinical findings and the results of microscopic examination and cultivation which should be successively repeated (Demitsu et al., 2020). Only by such multidisciplinary approach and inclusion of clinicians/dermatologists it is possible to establish the right diagnosis.



Fig. 11 Microscopic morphological characteristics of *Alternaria* spp.: Obclavate, ovoid or ellipsoidal, pale brown multicelled macroconidia with short conical breaks.



Fig. 12 Microscopic morphological characteristics of *Bipolaris* spp.: Development of pale brown, fusiforms to ellipsoidal, pseudoseptate poroconidia.

Trichosporon* spp.; *Piedraia hortae*; *Hortae werneckii

Among members of genus *Trichosporon*, species *Trichosporon* (*T.*) *asahii* or *T. beigeli* as it was previously known, is of the biggest clinical significance (Pulvirenti et al., 2006). It cause the development of whitish nodules adhere to the hair shaft that are referred to as white piedra. On the other hand, black piedra, caused by fungus *Piedraia hortae*, is characterized by black nodules, small in size and firmly attached to the hair (Schwartz, 2004).

The non-contagious, superficial fungal infection named tinea nigra is caused by species *Hortae werneckii* (*Exophiala werneckii*). It is usually seen following an injury on palms and soles as black or brown patchy lesions without scaling (María Consuelo Giordano et al., 2018).

After clinical suspicion, diagnosis is made by detection of fungal elements in the material using microscopic examination. Fungal culture can also be performed with identification of species based on either biochemical (*Trichosporon* spp.) or morphological characteristics (*Piedraia hortae*) (Savić et al., 2020).

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Human Primary Immunodeficiencies

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Glossary

Antibody Serum proteins, immunoglobulins, that recognize (bind) a specific antigen.

Antigen Molecules that are recognized by antibody molecules.

Autosomal inheritance Inheritance of genes on all chromosomes except the X and Y chromosomes.

Cytokines Proteins made by cells; they bind to specific receptors on other cells and affect their behavior.

Immunoglobulin replacement therapy Is the infusion of Immunoglobulin G obtained from healthy individuals into patients with immunodeficiency either intravenously (directly into the blood stream) or subcutaneously (under the skin).

Opportunistic organism Microorganism that cause disease only in immunodeficient patients such as in AIDS.

Signal transduction Process by which events on the cell surface are translated into changes in the nucleus of a cell; the process usually involves activation of a series of molecules inside the cell, one after the other, until activated molecules move into the nucleus and either turn on or turn off expression from one or more genes.

Stem cell transplantation Also known as bone marrow transplantation is a procedure where bone marrow derived stem cells from a healthy individual are transplanted (infused) into an immunodeficient patient to correct the immunodeficiency.

Thymus An organ located in the chest, just behind the breastbone, where T lymphocytes develop from precursor cells that leave the bone marrow and enter the thymus.

Human primary immunodeficiencies

The main function of the immune system is to protect against invading microorganisms (bacteria, viruses, fungi, and parasites) that cause disease (pathogenic microorganisms or pathogens). In addition, it must destroy any of the body's own cells that develop into cancer. Primary Immune Deficiency Diseases are inborn errors of immunity (IEI) resulting from genetic defects that affect the development or function of one or more components of the immune system, leading to an increased susceptibility to infection and, in some cases, to cancer, autoimmune, inflammatory or allergic disease. These immunodeficiency diseases demonstrate the

importance of each of the altered components of the immune system in immunity. This chapter is organized according to the classification of the International Union of Immunological Societies (IUIS) (Tangye et al., 2020), however, with the large number of genetic defects causing primary immunodeficiency disease, this chapter provides only an introduction to the various types of immune defects with more detailed information found in specific chapters.

Immunity to invading pathogens

The first line of defense against pathogens involves mechanical barriers such as the skin and mucous membranes of the respiratory and gastrointestinal tracts (Janeway and Medzhitov, 2002). Once a pathogen has broken through these barriers, a variety of soluble proteins and phagocytic cells attack the invading pathogen and destroy it. Phagocytic cells are white blood cells (WBC) that can ingest pathogens and play an important role in the early response to an infection. Phagocytes must be able to reach the site of infection and must be able to ingest and kill the pathogen. The initial immune response to an invading pathogen is called the innate immune response and involves the recognition of the pathogen as a danger signal by proteins found on the surface and inside the cell called pattern recognition receptors. The receptors recognize molecules common to all members of a class of pathogen (pathogen-associated molecular patterns, PAMPs) but cannot differentiate between the different pathogens within the specific class. This early response triggers an inflammatory reaction that is necessary for the activation of the adaptive immune system consisting of antibodies secreted by B lymphocytes as well as T lymphocyte responses that can kill infected cells directly, help B lymphocytes make antibody and help phagocytic cells kill intracellular pathogens.

Some pathogens have a cell wall structure that prevents their ingestion by phagocytes. Such pathogens are cleared by antibodies, which recognize specific molecules on the surface of the pathogen (antigen). Antibody molecules recognize the three-dimensional structure of antigen and have at least three basic mechanisms of action: (1) they can directly neutralize toxins, (2) they can kill pathogens by binding to and activating proteins of the complement system, and (3) they can facilitate the uptake of pathogens by phagocytic cells through surface receptors for immunoglobulin (Murphy and Weaver, 2017). A fourth mechanism of action has been discovered and involves high affinity receptor to immunoglobulin (TRIM21) found inside cells (McEwan et al., 2013). Antibodies bound to DNA or RNA nonenveloped viruses or intracellular bacteria bind to this receptor with high affinity and trigger inflammatory and antiviral processes inside the cell. Immunoglobulin molecules have two identical variable regions, antigen-binding sites and a constant region that determines the isotype and function of the immunoglobulin molecule (Murphy and Weaver (2017). There are five major isotypes of immunoglobulin molecules (IgM, IgD, IgG, IgA, and IgE), each with characteristic functions. The main function of antibodies is to clear the extracellular space of pathogens and their toxins (Murphy and Weaver, 2017) as well as some intracellular viruses and bacteria (McEwan et al., 2013). IgM is secreted as a pentamer of five molecules attached to each other. Because of their large size, IgM molecules remain within the blood vessels. IgG antibodies are secreted as single molecules and therefore are found inside and outside of blood vessels, cross the placenta to protect the newborn, and bind to the intracellular immunoglobulin receptor (TRIM21) to clear nonenveloped viruses and some intracellular bacteria. IgA antibodies are secreted as dimers at mucous membranes and in breast milk; they help prevent pathogens from entering through the mucous membranes. IgE antibodies play a role in fighting parasitic diseases, but also cause allergic diseases. When B lymphocytes are first activated by an antigen, they synthesize and secrete IgM antibodies. Immunoglobulin isotype switching, from IgM/IgD to IgG, IgA, or IgE, is the mechanism by which a B lymphocyte can synthesize an antibody with the same antigen specificity (same variable region) but with different function (different constant region, isotype). Isotype switching in human B lymphocytes requires a contact-dependent signal from T lymphocytes (Stavnezer et al., 2008).

Some pathogens, such as viruses, invade cells directly and take over the cell's machinery. While nonenveloped viruses can be cleared by antibody inside the cell, other pathogens including enveloped viruses, remain inside the cell and out of the reach of antibody molecules. T lymphocytes play a key role in clearing such intracellular pathogens by recognizing infected cells and killing them (Murphy and Weaver, 2017). T lymphocytes recognize antigens by antigen receptors on their surface called T cell receptor (TCR). TCRs have a variable region similar to that of immunoglobulin molecules, but are different in that they do not recognize the whole antigen in its three-dimensional structure, they recognize a fragment of the antigen (peptide) in association with (presented by) antigen-presenting molecules on the surface of infected cells. Antigen-presenting molecules are called major histocompatibility complex (MHC) molecules because they determine whether an individual tolerates a tissue graft from another individual. There are two types of MHC molecules: MHC class I and class II molecules. MHC class I molecules are present on all cells, present peptide fragments from proteins that are produced by a virus-infected or cancer cell and present the peptide antigen to the CD8 subpopulation of T lymphocytes. Therefore, the main function of CD8 T lymphocytes is to destroy virus-infected or cancer cells. MHC class II molecules are found on the surface of professional antigen-presenting cells, B lymphocytes, monocytes, and dendritic cells; can be induced on the surface of other cells; present peptide fragments of proteins from pathogens that are taken up and degraded by antigen-presenting cells; and present antigen to the CD4 subpopulation of T lymphocytes. The main functions of CD4 T lymphocytes, therefore, are to help B lymphocytes make antibodies against the invading pathogen and to help monocytes kill ingested pathogens.

IEI are inherited genetic defects affecting the development or function of one or more components of the immune system resulting in an increased susceptibility to infection, immune dysregulation with autoimmunity and/or inflammation, allergic diseases or cancer. There are over 400 different gene defects causing IEI and many more to be discovered.

Defects of adaptive immunity

Adaptive immunity is composed of a humoral component, antibodies secreted by B cells, and a cellular component, activated T cells, which help B cells make antibodies, help phagocytes kill intracellular pathogens, and directly kill virally-infected and cancer cells (cytotoxic T cells) (Murphy and Weaver, 2017). The adaptive immune system is characterized by specificity that is determined by antigen receptors on B cells (Immunoglobulin molecules) and T cells (T cell receptors, TCR); variability, that is determined by the random rearrangement of the genes that encode for the variable region of the antigen receptors; memory, that allows rapid responses at subsequent exposure to a pathogen, and tolerance; that helps prevent inflammatory, autoimmune and allergic diseases.

Immunodeficiencies affecting cellular and humoral immunity

Severe combined immunodeficiency disease

Severe combined immunodeficiency disease (SCID): results from the absence or lack of function of T lymphocytes with or without B lymphocytes and/or Natural Killer (NK) lymphocytes (Notarangelo, 2010; van der Burg and Gennery, 2011). Patients with SCID lack T cell immunity and, in most cases, antibody-mediated immunity. In cases where B lymphocytes are present, the B lymphocytes may be dysfunctional or they may lack T lymphocyte help. The result is a severe immunodeficiency that presents early in life with recurrent bacterial, viral, fungal, and opportunistic infections, chronic diarrhea, and failure to thrive in some cases. Patients are prone to develop disease by attenuated live-viral vaccines, especially BCG given at birth and therefore should not receive any of the live attenuated vaccines. Patients are also susceptible to a fatal reaction from a blood transfusion known as Transfusion graft vs. host (T-GVH) disease (Park et al., 1974). This results from the presence of lymphocytes in the transfused blood which recognize the patient's tissues as foreign and initiate a rejection reaction that is usually fatal if not treated. The reaction starts as a rash but can extend to involve the liver, lung, and/or intestinal tract. Normally, the immune system of the recipient of a blood transfusion will destroy any lymphocyte or other WBC in the donor blood. However, in SCID, the patient has no functioning immune system to destroy the foreign lymphocytes and is thus a target for T-GVH disease. It is therefore very important to irradiate all blood products given to SCID patients, which inhibits the capacity of lymphocytes to mount a GVH reaction. In addition, patients with SCID may have maternal lymphocytes especially T cells (maternal T cell engraftment) that cross the placenta and may result in sufficient numbers of T cells that may delay the diagnosis of SCID but can also result in maternal T cell induced GVH disease, which is rarely fatal. Treatment of SCID requires stem cell transplantation (Dvorak et al., 2013), although some conditions such as adenosine deaminase deficiency (ADA) may be treated with enzyme replacement (Serana et al., 2010) and some SCID patients have been treated successfully by gene therapy such as X-linked SCID and ADA deficiency (Booth et al., 2011; Fischer et al., 2011). However, some of the X-SCID patients treated with gene therapy developed leukemia, which has prompted a revision of the viral vectors used in gene therapy trials.

The severity of the immune deficiency in SCID and the availability of treatment, stem cell transplantation, that has a higher success rate if performed early (Pai et al., 2014), as well as gene therapy or enzyme replacement, has led to the development of newborn screening for SCID (Buckley, 2012; Chase et al., 2011; Chan and Puck, 2005), which has been implemented in several states. The screening method makes use of DNA circles that are formed in new T lymphocytes (naïve T cells) when the TCR genes are rearranged (*T cell Receptor Excision Circles*, TRECs) and can be amplified by quantitative polymerase chain reaction to identify newborns with low or absent T lymphocytes. The identified newborns are then screened for SCID, DiGeorge syndrome [See below] and other T lymphocyte deficiencies (Kwan et al., 2014).

T-B+ SCID

X-linked SCID results from a defect in the gene that encodes the common gamma chain, a molecule that is part of many cytokine receptors including the receptors for interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15, and IL-21 (Tangye et al., 2020). IL-7 is necessary for T cell development and IL-15 for NK cell development and therefore, X-SCID is characterized by the absence of T cells and NK cells but the presence of B cells that do not function properly. Similarly, defects in the signaling pathway of these receptors such as JAK-3 kinase results in a similar phenotype but affects boys and girls equally. There are many other gene defects that affect T cell development only (IL-7R alpha chain, members of the CD3 complex and CD45 among others) but nevertheless result in SCID because of the central role of T cells in adaptive immunity (Tangye et al., 2020).

T-B- SCID

Deficiency in enzymes such as adenosine deaminase and purine nucleotide phosphorylase result in the accumulation of metabolites that are toxic to all lymphocytes and usually present with very low or absent numbers of T cells, B cells and NK cells (Table 1). In contrast, defects in the enzymes that mediate recombination of the variable regions of antigen receptors (immunoglobulin in B cells and TCR in T cells) affect the development of T cells and B cells only with normal or elevated numbers of NK cells (Tangye et al., 2020).

A group of T-, B- and NK+ SCID are associated with radiation sensitivity with microcephaly such as in ligase IV deficiency and without microcephaly such as in Artemis deficiency (Tangye et al., 2020).

Table 1 Phenotype and genetic causes of some forms of SCID.

Phenotype	Type of defect	Examples of gene defects
T- B- NK-	Global defect in lymphocytes +/- other hematopoietic cells	Adenosine deaminase Purine nucleotide phosphorylase Reticular dysgenesis (AK2 deficiency) Recombinase activation gene (RAG)-1 RAG-2
T- B- NK+	Defect in antigen receptor recombination	Artemis DNA-PKcs
T- B+ NK-	Defect in cytokine receptor common gamma chain signaling pathway	Common gamma chain (X-SCID) Janus kinase 3 (JAK3)
T- B+ NK+	T cell specific defect	IL-7R α chain CD45 deficiency CD3 $\delta/\epsilon/\zeta$ chain defect
CD8 deficiency	CD8 T cell specific defect	CD8 α chain ZAP-70
CD4 deficiency	CD4 T cell specific defect	MHC class I deficiency MHC class II deficiency

Combined immunodeficiency

X-linked immunodeficiency with normal or elevated IgM (X-linked hyper IgM, X-HIGM): is a rare genetic disorder characterized by markedly decreased or absent serum levels of IgG and IgE immunoglobulins with normal or elevated levels of IgM (Qamar and Fuleihan, 2013). The syndrome was first described in 1961 (Rosen et al., 1961; Burtin, 1961) and since then over 200 cases have been reported worldwide (Levy et al., 1997; Winkelstein et al., 2003; Lee et al., 2005). Affected males are susceptible to recurrent bacterial infections, opportunistic infections with *Pneumocystis jiroveci* and with *Cryptosporidium*, that may be complicated with sclerosing cholangitis and cancer (Hayward et al., 1997). X-HIGM can be differentiated from XLA by the presence of normal numbers of mature B lymphocytes and by the ability to make specific antibodies in response to immunization. However, these antibodies are restricted to the IgM isotype, with a failure to switch to the production of other isotypes (IgG, IgA, and IgE). Some patients are able to synthesize low or normal levels of IgA. In addition, there is a lack of a memory response, which is characterized by a rapid production of larger quantities of antibodies upon re-exposure to a pathogen or vaccine. The lymph nodes, while present, have an abnormal architecture (Facchetti et al., 1995). The underlying defect in X-HIGM appears to be an inability to undergo immunoglobulin isotype switching from IgM/IgD secretion to the production of other immunoglobulin (Ig) isotypes, IgG, IgA, or IgE. In 1993, five independent groups identified the genetic defects in X-HIGM to be in the gene encoding for a protein that is expressed on the surface of activated T lymphocytes and provides the signal B lymphocytes require for isotype switching by binding to CD40 on the surface of B lymphocytes (Allen et al., 1993; Aruffo et al., 1993; DiSanto et al., 1993; Fuleihan et al., 1993; Korthauer et al., 1993). This molecule was therefore named CD40 ligand (CD154). Numerous mutations that disrupt the ability of CD40 ligand to bind to CD40 have been found in X-HIGM. There are autosomal recessive forms of hyper IgM that affect the signaling pathway of CD40 including mutations in CD40 itself (Ferrari et al., 2001), which has a clinical picture similar to X-HIGM.

The defect in X-HIGM is not limited to the lack of T lymphocyte help to B lymphocytes because CD40 has been found on the surface of many other cell types. CD40 ligand on T lymphocytes also provides help to monocytes via CD40 to kill intracellular pathogens, which may be important in clearing infections by opportunistic organisms. Furthermore, interaction of CD40 and its ligand has been found to play many other important roles in the immune response to infection and in the development of immune-mediated diseases (Elgueta et al., 2009).

Treatment of X-HIGM consists of immunoglobulin replacement therapy, PJP prophylaxis, and general precautions to prevent *Cryptosporidium* infection but patients remain susceptible to severe complications including cancer. Stem cell transplantation has been used with success to treat patients with X-HIGM (Ferrua et al., 2019; de la Morena et al., 2017) as well as CD40 deficiency (Mazzolari et al., 2007).

Other combined immunodeficiency diseases include a group of immunodeficiency diseases that are associated with autoimmunity, recurrent infection and hypogammaglobulinemia such as in ICOS & ICOSL deficiency, the latter may also be associated with neutropenia (Tangye et al., 2020).

Autoimmunity and/or immune dysregulation with normal immunoglobulin are caused by a group of genetic defects such as CD8 deficiency in which patients have absent CD8, others are associated with low CD8 such as in ZAP70 (LOF/GOF) and MHC class I deficiency (Tangye et al., 2020). In contrast MHC class II, deficiency, characterized by low CD4 T cell numbers, reduced MHC II expression on lymphocyte, normal or low immunoglobulin, can present in infancy with infections & failure to thrive mimicking SCID, called the bare lymphocyte syndrome (Shrestha et al., 2012). MHC molecules are important for T cell development in the thymus. CD4 T cells develop if they can receive a signal from MHC class II molecules and CD8 T cells from MHC class I molecules (Murphy and Weaver, 2017). In their absence, there is a marked deficiency of the respective T cell subset, but unlike mice, the deficiency is not complete. However, the T cells that do develop do not function properly because there are no molecules to present the peptide antigens necessary for antigen recognition and T cell activation.

Dedicator of cytokinesis 8 (DOCK8) is critical for cytoskeletal organization, and deficiency of DOCK8 impairs dendritic cell transmigration, T cell survival, and NK cell cytotoxicity (McGhee and Chatila, 2010). Deficiency of DOCK 8 is an autosomal recessive disease, characterized by recurrent sino-pulmonary infections, cutaneous viral infections especially Herpes skin infections (Zhang et al., 2009; Randall et al., 2009; Engelhardt et al., 2009). Patients with the disease may have severe eczema, multiple food allergies, elevated IgE and eosinophils. DOCK2 deficiency has recently being reported as a cause of combined immunodeficiency.

Combined immunodeficiencies with associated or syndromic features

Thymic defects with additional congenital anomalies

The DiGeorge anomaly: is a genetic disorder that results in a developmental anomaly affecting several tissues, including the thymus, parathyroid glands, heart, and some structures of the face (McDonald-McGinn and Sullivan, 2011). Patients usually have a congenital defect of the heart, low calcium levels in the blood secondary to low or absent parathyroid hormone, and low numbers of T lymphocytes because of a small or absent thymus. Congenital heart disease is the major life-threatening defect in this disease. Low calcium results in tetany (cramp-like involuntary spasms). The immunodeficiency varies from mild to severe and is related to the number and function of mature T lymphocytes. If patients survive the neonatal period, they become susceptible to recurrent infections, including fungal and opportunistic infections, diarrhea, and failure to thrive as well as autoimmune disease. Patients, especially with milder immunodeficiency, tend to develop improved immune function with age. The anomaly is associated with a deletion in chromosome 22 at 22q11.2 or a mutation in the TBX1 gene located in this region (Tangye et al., 2020). In rare cases, there is complete absence of thymic tissue and T cells, (Complete DiGeorge), which has been treated successfully by thymic transplantation (Markert et al., 2009).

Immunodeficiency with congenital thrombocytopenia

Wiskott-Aldrich syndrome (WAS) is characterized by the triad of eczema, thrombocytopenia with small platelets and a combined immunodeficiency resulting in recurrent sino-pulmonary infections as well as a susceptibility to autoimmunity and/or cancer (Massaad et al., 2013). WAS results from defects in an X-chromosome gene encoding for a protein that is a regulator of the cytoskeleton and termed WAS protein (WASP) (Derry et al., 1994). There is a large variability in the clinical phenotype ranging from thrombocytopenia alone (X-linked thrombocytopenia, XLP) to the full spectrum of the disease. Defects in the gene encoding a WASP-interacting protein (WIP) also results in an immunodeficiency with congenital thrombocytopenia (Lanzi et al., 2012). WAS can be treated by stem cell transplantation (Burroughs et al., 2020) and possibly by gene therapy, which is under investigation currently (Ferrua et al., 2020).

Hyper IgE syndromes

Autosomal dominant mutations in the signaling molecule STAT3 results in the classic Hyper IgE syndrome also known as Job's or Buckley syndrome (Bergerson and Freeman, 2019). Patients have severe eczema, elevated serum IgE levels and are susceptible to Staph infections (cold abscesses) as well as invasive fungal infections, likely due to the failure of differentiation of TH17 T cells. The disease is also associated with non-immunological features include connective tissue, skeletal and vascular abnormalities. Autosomal dominant mutations in the genes encoding for ERBIN and CARD11 as well as autosomal recessive defects in the genes for DOCK8 (see combined immunodeficiency above), IL-6 receptor, IL-6 signal transducer (IL6ST) and phosphoglucosyltransferase 3 (PGM3) also cause an hyper IgE syndrome with susceptibility to infection and variable expression of non-immunologic manifestations (Tangye et al., 2020).

Predominantly antibody deficiencies

Agammaglobulinemia: the prototype antibody deficiency: X-linked agammaglobulinemia (XLA) was the first immunodeficiency to be described. In 1952, Ogden C. Bruton described a young boy with recurrent bacterial infections who had no immunoglobulins in his serum (Bruton, 1952). He also demonstrated that administration of immunoglobulins (plasma) derived from normal individuals resulted in a reduction of the frequency of the patient's bacterial infections. Plasma cells, which secrete immunoglobulins, were found to be lacking in patients with XLA (Good, 1954), and it was later shown that the cellular defect in XLA was the absence of mature B lymphocytes (Geha et al., 1974). These studies on XLA patients discovered that antibodies (immunoglobulins) were secreted by plasma cells, which were derived from B lymphocytes and highlight the role of investigating IEL in our knowledge and understanding of the development and function of the immune system in health and in disease. Affected males develop recurrent bacterial and viral infections early in infancy or childhood; usually after 4–6 months of age, when the protective maternal antibodies that crossed the placenta during the third trimester of pregnancy have decreased (Minegishi et al., 1999). The main finding on physical examination is the absence of tonsils and lymph nodes. Levels of all immunoglobulin isotypes in the serum are markedly decreased if not absent, and, despite repeated immunization, the patients cannot synthesize specific antibodies. Few if any mature B lymphocytes are found in the blood. Although in vitro studies of T lymphocyte number and function are normal, patients

with XLA are prone to develop chronic infections with enteroviruses. Indeed, these patients can contract vaccine-associated paralytic poliomyelitis, for which reason attenuated live-viral vaccines (especially poliomyelitis) should be avoided in patients with XLA. The use of immunoglobulin replacement therapy intravenously or subcutaneously has allowed patients to lead practically normal lives with a marked reduction in the number and severity of infections (Garcia-Lloret et al., 2008).

Immunoglobulins are synthesized by B lymphocytes, which develop in the bone marrow from stem cells that also give rise to other cells found in the blood. B lymphocyte precursors undergo several stages of development before they become mature B lymphocytes, characterized by the presence of surface immunoglobulin molecules capable of recognizing foreign pathogens [See B lymphocytes, Development of]. The defect in XLA affects the development of B lymphocytes such that fewer and fewer B lymphocytes present at each successive stage of development. The gene responsible for XLA was identified in 1993 by two independent groups (Tsukada et al., 1993; Vetrie et al., 1993). Its protein product was found to be a tyrosine kinase, which has been named Btk (for Bruton's tyrosine kinase). Autosomal recessive forms of agammaglobulinemia result from other gene defects that also affect the development of B cells and result in a similar immunodeficiency affecting males and females equally (Conley et al., 2005). Because of the absence of mature B lymphocytes, patients with agammaglobulinemia cannot synthesize immunoglobulin molecules and thus lack the protective role of antibodies. The absence of antibodies in agammaglobulinemia results in a susceptibility to bacterial and viral infections, some of which can be debilitating or fatal and demonstrate the importance of antibodies in immunity (Fig. 1).

Common Variable Immunodeficiency (CVID): is the most common symptomatic primary immunodeficiency disease (Resnick and Cunningham-Rundles, 2012). CVID has its highest incidence in the second and third decades of life. Diagnostic criteria include a low IgG level, low IgA and/or IgM levels and defective specific antibody synthesis in an adult or child, older than 2 years of age at the time of diagnosis, with recurrent infections and other causes of primary immunodeficiency ruled out. CVID is associated with the development of autoimmune disease particularly affecting cells of the blood such as platelets and red blood cells, and the development of granulomatous disease especially in the lung and bronchiectasis. Some CVID patients have low immunoglobulin levels and autoimmune disease without a history of recurrent infections. Several gene defects affecting B lymphocyte function and survival have been identified, the genetic causes of CVID in most patients have yet to be discovered. Treatment of CVID includes antibody replacement with intravenous or subcutaneous immunoglobulin as well as treatment of any complications that are present at the time of diagnosis or that develop over time.

Hyper IgM syndrome with predominantly antibody deficiency: Defects in the genes that encode for the enzymes important for immunoglobulin isotype switching, activation-induced cytidine deaminase (AID) (Revy et al., 2000) and uracil N-glycosylase (UNG) (Imai et al., 2003), which lead to a HIGM clinical picture that is characterized by an antibody deficiency and lymph node enlargement without a susceptibility to opportunistic infections or cancer because these enzymes are only found in B cells and do not affect T cell function. AID and UNG also mediated somatic hypermutation, a mechanism by which B lymphocytes mutate the variable region to produce antibodies with higher affinity, which is also defective in HIGM patients (Fig. 2).

Selective IgA deficiency: is the most common primary immunodeficiency affecting as many as 1:600 individuals but most patients have few infections or complications (Wang and Hammarstrom, 2012). The prevalence of IgA deficiency, defined as IgA level < 10 mg/dl, varies among different ethnic groups. Recurrent infections, bronchiectasis and autoimmune diseases occur in some patients and may be related to association with IgG subclass deficiency, poor specific antibody responses or low class-switched memory B cells. Autoimmune diseases include autoimmune thyroid diseases, type 1 diabetes mellitus, systemic lupus erythematosus, juvenile arthritis and celiac disease. The genetic cause of IgA deficiency remains elusive but appears to be associated with genes within the Major Histocompatibility Complex (MHC) area on chromosome 6 as well as non-MHC genes. Treatment with IgG replacement therapy is usually not indicated unless there is a demonstrated specific antibody deficiency associated with severe or recurrent infections that are not preventable by antibiotic prophylaxis. Exposure of patients with complete absence of IgA to blood products including IgG replacement products, which contain variable amounts of IgA, may elicit an anti-IgA antibody response that

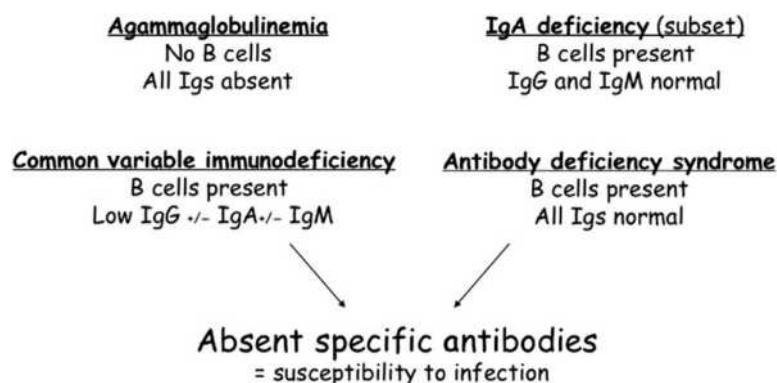


Fig. 1 Antibody deficiency results in a susceptibility to infection, which may be severe in agammaglobulinemia and CVID or more limited susceptibility in IgA deficiency and specific antibody deficiency.

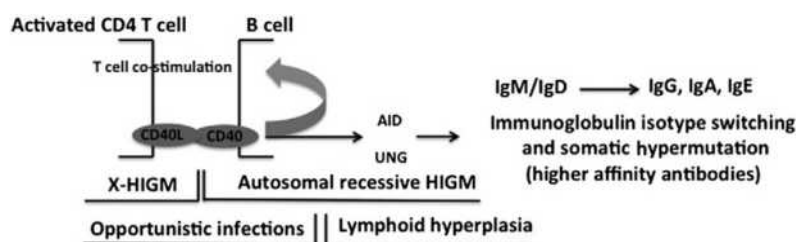


Fig. 2 Schematic representation of interaction between an activated CD4 T lymphocyte and a B lymphocyte. CD40 ligand (CD40L) binds to CD40 and activates immunoglobulin isotype switching from IgM to IgG, IgA or IgE as well as somatic hypermutation mediated by AID and UNG. In addition, CD40 stimulates expression of molecules that provide co-stimulation to the CD4 T cell. Mutations in CD40 ligand cause X-HIGM, whereas mutations in CD40, AID or UNG result in autosomal recessive HIGM. Susceptibility to opportunistic infections occurs with proximal defects (CD40 ligand and CD40) whereas the distal defects (AID and UNG) are associated with lymphoid hyperplasia.

can result in an allergic reaction upon subsequent transfusions/infusion (Rachid and Bonilla, 2012). While this has been documented, it is not very common and should not delay the lifesaving administration of blood products especially if it is the patient's first infusion. The patient can be tested subsequently for anti-IgA antibodies prior to receiving further blood products.

Specific antibody deficiency: is an immunodeficiency defined as an inability to synthesize antibody to specific antigens usually the polysaccharide antigens of encapsulated bacteria with normal serum levels of immunoglobulins and normal numbers of B cells (Leiva et al., 2013). Patients are susceptible to recurrent infections and may be treated with prophylactic antibiotics or IgG replacement if they have severe infections or if they fail antibiotic prophylaxis. Specific antibody deficiency can be associated with other conditions including IgG subclass deficiency, IgA deficiency, Wiskott-Aldrich syndrome or DiGeorge syndrome [See below] among others. The underlying cause of this immunodeficiency has not been determined yet.

Diseases of immune dysregulation

The association of autoimmunity with several immunodeficiency diseases such as selective IgA deficiency, CVID and DiGeorge Syndrome hinted to the presence of immune dysregulation as a result of a defect of immunity or the co-inheritance of autoimmune disease susceptibility with the immune deficiency (Cunningham-Rundles, 2011). However, the discovery of the genetic cause of Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) highlighted the role of regulatory T cells in immune homeostasis and opened up the field of immune dysregulation as a defect in immunity even if not associated with susceptibility to infection (Bennett et al., 2001; Chatila et al., 2000). IPEX results from defects in the gene encoding for FoxP3, a transcription factor necessary for the generation of thymic regulatory T cells. The disease is associated with both autoimmune as well as allergic disease, namely food allergy, emphasizing the role of regulatory T cells in controlling or preventing autoimmunity as well as food allergy and likely other allergic diseases. Other defects in regulatory T cell function such as in IL-10 or its receptor, CD25 deficiency, CTLA-4 haploinsufficiency or autosomal recessive LRBA deficiency lead to immune dysregulation including autoimmunity, enteropathy and inflammatory bowel disease (Tangye et al., 2020).

Predisposition to autoimmunity is probably best exemplified by APECED resulting from a defect in the AIRE gene that is responsible for expression of self-proteins, particularly proteins expressed in endocrine organs, in the thymus, where negative selection occurs (Tangye et al., 2020). AIRE also has a role in regulatory T cell development. Autoimmunity to IL-17 and IL-22 appears to underlie the association of chronic mucocutaneous candidiasis in APECED (Puel et al., 2010; Kisand et al., 2010).

Autoimmunity with lymphoproliferation results from defects in the FAS apoptotic pathway leading to Autoimmune Lymphoproliferative Syndrome (ALPS) with defects in Fas, Fas ligand and downstream Caspases 8 and 10 (Tangye et al., 2020). This condition was first reported by Canale and Smith (Canale and Smith, 1967) and later found to be the same disease as ALPS (Rieux-Laucat et al., 1995; Fisher et al., 1995), when Fas mutations were demonstrated in patients described by Canale and Smith (Drappa et al., 1996).

Other disease of immune dysregulation result from defects in NK cell and cytolytic T cell function such as defects in perforin and UNC13D that lead to familial hemophagocytic lymphohistiocytosis (FHL) or LYST, Rab27a, and AP3B1 and AP3D1 that lead to FHL with hypopigmentation, Chediak-Higashi syndrome, Griscelli syndrome type 2, and Hermansky-Pudlak syndrome types 2 and 10, respectively (Tangye et al., 2020).

Susceptibility to EBV infection and lymphoproliferation also results from defects in NK cell function as well as NKT cell development (Nichols et al., 2005) characterized by X-linked lymphoproliferative disease (XLP) resulting from defects in the SH2D1A gene (Sayos et al., 1998; Nichols et al., 1998). Defects in X-linked inhibitor of apoptosis (XIAP) also results in a susceptibility to EBV and lymphoproliferation (Rigaud et al., 2006) as well as recurrent HLH (Marsh et al., 2010) and inflammatory bowel disease (Kelsen et al., 2015).

Defects of innate immunity

The innate immune system is important for the initial recognition of a foreign pathogen, mounting an immediate response that often clears the invading pathogen, and if not, initiates an inflammatory response that controls the infection while activating the adaptive immune system (antibodies made by B cells and activated T cells that can kill virally infected cells) to clear the infection (Medzhitov and Janeway, 1997). Defects in any of the components of the innate immune system can lead to frequent or severe infections (Rosenzweig and Holland, 2011). In some cases, while there is an initial serious and life-threatening infection, recurrent infections do not develop because the adaptive immune system with immunological memory is able to control subsequent infections (Alcais et al., 2010).

Congenital defects of phagocyte number or function

The phagocytic system plays a very important role in killing pathogens early in an infection. Phagocytes include neutrophils, eosinophils, and basophils. Neutrophils are the most abundant of the phagocytic cells [See Neutrophils]. Their main function is to ingest (phagocytose) and kill pathogens. Defects in the number or function of neutrophils result in a susceptibility to recurrent bacterial and fungal infections (Rosenzweig and Holland, 2011). Patients suffer from soft tissue inflammation and abscesses. They have an enhanced susceptibility to infection by some bacteria (*Staphylococcus aureus*, *Pseudomonas*, and *Serratia*) and by some fungi (*Aspergillus* and *Candida*). Defects in neutrophil numbers are mostly secondary; however, congenital neutropenia does occur, leaving affected individuals susceptible to recurrent infections. Defects in neutrophil function result from a variety of genetic defects:

Leukocyte Adhesion Deficiency: results from defects in molecules found on the surface of neutrophils (integrins), which allow neutrophils to bind (adhere) to the cells lining the blood vessels (vascular endothelium). Adherence to vascular endothelium is the first step in the process of neutrophil migration to the site of infection (Rosenzweig and Holland, 2011). As a result of this deficiency, neutrophils remain in the blood vessels where they are found in higher numbers than normal. Leukocyte adhesion-deficient neutrophils cannot reach the sites of infection, resulting in the absence of pus. One of the earliest signs of a leukocyte adhesion deficiency is delayed separation of the umbilical cord beyond 2 weeks especially if it is associated with an infection around the umbilical cord (Omphalitis).

Chronic Granulomatous Disease (CGD): results from an inability to kill ingested bacteria. Killing of bacteria by neutrophils involves a burst of respiratory activity to generate superoxide (Rosenzweig and Holland, 2011). Defects in any of the enzymes involved in the respiratory burst can lead to a defect in intracellular killing by neutrophils. Five enzyme defects have been described to date and are inherited as autosomal recessive disorders (p22-phox, p40-phox, p47-phox, and p67-phox), except for the most common gene defect in CGD (gp91-phox), which is located on the X chromosome. The diagnosis of CGD is made by a test known as the nitroblue tetrazolium dye (NBT) test, where a soluble yellow dye becomes an insoluble blue dye in activated neutrophils, and more recently, by a flow cytometry-based dihydrorhodamine 123 (DHR) assay, which is a more sensitive test converting DHR into a brightly fluorescing rhodamine. Female carriers of the X-linked form of this disease will demonstrate NBT dye reduction in only a subset (average 50%) of their neutrophils, the subset carrying the normal gene, or will show two peaks of fluorescence in the DHR assay (a normal and an abnormal peak). Although no specific therapy exists, patients have been found to benefit from the regular administration of interferon- γ (IFN- γ) (1991), as well as from the administration of prophylactic antibiotics and antifungals (Kang et al., 2011). Stem cell transplantation offers a potential cure (Cole et al., 2013), is possible when the patient has a suitable related or unrelated donor and is recommended in patients with absent or very low oxidative burst (Kuhns et al., 2010).

Other Phagocytic Deficiencies: result from defects in other enzymes such as severe deficiency of (G6PD) glucose-6-phosphate dehydrogenase (Tangye et al., 2020) or deficiency in myeloperoxidase (Pahwa et al., 2021).

Defects in intrinsic and innate immunity

Mendelian susceptibility to mycobacterial disease (MSMD) results from defects in genes involved in the production of interferon gamma (IFN- γ) or in the IFN- γ signaling pathway including IL-12 and its receptor, IFN- γ receptors, STAT1, Macrophage gp91phox, IRF8, Tyk2, ROR γ t, and JAK1 (Tangye et al., 2020; Rosain et al., 2019). Defects that affect IFN- γ production can benefit from treatment with IFN- γ , however, defects of the IFN- γ receptor and its downstream signaling pathway are resistant to addition of IFN- γ and may require stem cell transplantation.

TLR signaling pathway deficiency with bacterial susceptibility: Toll-like receptors (TLR) are pattern-recognition receptors found on the surface of cells or inside cells and recognize classes of pathogens including gram positive bacteria, gram negative bacteria, fungi, DNA or RNA viruses (Janeway and Medzhitov, 2002). Most TLR signal via a similar pathway to activate an inflammatory reaction that helps in the control of the invading pathogen and in the activation of the adaptive immune system to clear the infection. Genetic defects in several of the TLR or their signaling pathway have been associated with severe or recurrent infections, each TLR pathway defect is associated with a narrow window of susceptibility (Picard et al., 2011). Defects in TLR or their respective signaling pathways have been associated with invasive Staphylococcal and Streptococcal infections with minimal fever and inflammation (IRAK-4, MyD88 defects) or herpes virus encephalitis (TLR3, UNC93B and TRAF-3) among others (Tangye et al., 2020).

Autoinflammatory disorders

Type 1 interferonopathies result from gene defects that affect the regulation of type I interferons resulting in inflammatory conditions resembling rheumatologic diseases such as systemic lupus erythematosus and vasculitis (Rodero and Crow, 2016). Affected genes include *Trex1*, *RNASEH2B*, *C* and *A*, *SAMHD1* and other genes causing Aicardi-Goutieres syndrome, *TMEM173* causing STING-associated vasculopathy, infantile-onset (SAVI), *ADA2* and others.

Defects affecting the inflammasome lead to periodic fever syndromes exemplified by Familial Mediterranean Fever caused by defects in the *MEFV* gene, Hyper IgD syndrome caused by defects in the *MVK* gene, Muckle-Wells syndrome, Familial cold autoinflammatory syndrome and neonatal onset multisystem inflammatory disease (NOMID) caused by defects in the *NLRP3* gene, *PLCγ2*-associated antibody deficiency and immune dysregulations (PLAID) caused by mutations in the *PLCG2* gene and other familial cold autoinflammatory syndromes (Tangye et al., 2020).

Non-inflammasome-related conditions include a variety of clinical phenotypes including recurrent fever, arthritis, myositis, Panniculitis, and chronic recurrent multifocal osteomyelitis (Tangye et al., 2020). Diseases include TNF receptor-associated periodic syndrome (TRAPS) caused by defects in the *TNFRSF1A* gene, Majeed syndrome caused by defects in the *LPIN2* gene, deficiency of the interleukin 1 receptor antagonist (DIRA) caused by defects in the *IL1RN* gene and others.

Deficiency of the complement system

Deficiency of proteins: The complement system involves a large number of serum proteins (at least 30) that participate in the killing of pathogens (Rosenzweig and Holland, 2011). Nine of these proteins are numbered C1-C9. Activation of the complement system results in the generation of a complex formed by the proteins C5-C9, which can destroy bacteria. Three mechanisms activate the complement system: the classical pathway via antibodies which utilizes the early components, C1, C2 and C4 proteins, the alternative pathway which activates C3 spontaneously and the lectin pathway, which is activated by mannose binding lectin and ficolins that bind to carbohydrate molecules on pathogens. All three pathways lead to activation of C3 and then the formation of the membrane attack complex C5-C9. Therefore, complement proteins can directly kill some bacteria or can help antibodies to kill bacteria. Deficiency of any of the early components leads to a decreased efficiency of antibody function and, therefore, to an enhanced susceptibility to infections. In addition, the early complement proteins appear to play a role in the clearing of antibody/antigen complexes. Therefore, patients deficient in one of the early complement proteins are also susceptible to diseases generated by immune complexes. Deficiency of any of the late components, C3, C5-C8 proteins, results in an enhanced susceptibility to *Neisseria meningitidis* infections. Deficiency of C9 does not result in as severe an immunodeficiency because the C5-C8 complex has some antibacterial activity.

Bone marrow failure syndromes

Inherited diseases that lead to bone marrow failure can cause immunodeficiency by affecting the development of immune cells including neutrophils, monocytes, and various lymphocyte subsets. Examples include gene defects causing Fanconi anemia and Dyskeratosis congenita (Tangye et al., 2020).

Phenocopies of inborn errors of immunity

Acquired immune deficiency or dysregulation resembling inherited diseases in children can result in adults from somatic mutations in gene leading to autoimmune lymphoproliferative syndromes, cryopyrinopathies and hypereosinophilia (Tangye et al., 2020). In addition, the development of autoimmunity directed against cytokines and their receptors can result in disease mimicking the inherited childhood diseases (Browne and Holland, 2010) including chronic mucocutaneous candidiasis from autoantibodies to IL-17 and its receptor, susceptibility to mycobacteria with autoantibodies to IFN- γ , recurrent skin infections with autoantibodies to IL-6, susceptibility to severe viral infections including SARS-CoV2 with autoantibodies to type I interferons (Bastard et al., 2020).

Concluding remarks

There are many other primary immunodeficiency diseases and diseases of immune dysregulation that affect the innate and/or adaptive immune system with susceptibility to severe infections, including opportunistic infections, cancer, autoimmune, inflammatory or allergic disease (Tangye et al., 2020). This article describes only a few of the known genetic immune deficiency diseases within each category of immune deficiency or dysregulation that illustrate how a single gene defect can have such a profound effect on the immune system. Studying patients with these diseases can lead to important findings on how the immune system works and allows for the development of novel therapeutic approaches for immunodeficiency diseases, cancer, allergic diseases, autoimmune diseases, inflammatory diseases including autoinflammatory defects in the innate immune system. The immune system is at the center of health and disease and primary immunodeficiency diseases are experiments of nature that teach us about the immune system and how its different components work to fight infection or cause disease.

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Combined T and B Lymphocyte Deficiencies

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Introduction

The key cells of the adaptive immune response are T-lymphocytes and B-lymphocytes. Defects in T-lymphocytes alone or both T- and B-lymphocytes give rise to combined immunodeficiency, as B-lymphocyte function requires T-lymphocyte interaction. Combined T- and B-lymphocyte deficiencies are the most common immunodeficiencies after antibody deficiencies and in the 2019 International Union of Immunological Societies classification, are defined in [Tables 1 and 2](#) of the latest publication ([Tangye et al., 2020](#)).

Severe combined immunodeficiencies

Severe combined immunodeficiencies (SCID) are the most profound combined immunodeficiencies and have been described as a pediatric emergency. They comprise a group of inherited disorders whose hallmark is deficient T-lymphocyte differentiation, due to genetic defects causing disruption of lymphocyte development ([Fischer et al., 2015](#); [Tangye et al., 2020](#)). Contingent on the genetic

Table 1 Genetic causes of severe combined immunodeficiency.

<i>Disorder</i>	<i>Gene</i>	<i>Phenotype</i>	<i>Inheritance</i>
Cytokine Signaling	<i>IL2RG</i>	T ⁻ B ⁺	XL
γC deficiency			
JAK3 deficiency	<i>JAK3</i>	T ⁻ B ⁺	AR
IL7Rα deficiency	<i>IL7Rα</i>	T ⁻ B ⁺	AR
Nucleotide biosynthesis	<i>ADA</i>	T ⁻ B ⁻	AR
Salvage pathway defects			
ADA deficiency			
Defects affecting signaling through the T lymphocyte antigen receptor			
CD45	<i>PTPRC</i>	T ^{-/low} B ⁺	AR
CD3δ	<i>CD3D</i>	T ^{-/low} B ⁺	AR
CD3ε	<i>CD3E</i>	T ^{-/low} B ⁺	AR
CD3ζ	<i>CD3Z</i>	T ^{-/low} B ⁺	AR
LAT deficiency	<i>LAT</i>	T ^{low/+} B ^{low/+}	AR
VDJ recombination defects			
RAG deficiency	<i>RAG1</i>	T ⁻ B ⁻	AR
DCLRE1C (Artemis) deficiency	<i>RAG2</i>	T ⁻ B ⁻	AR
DNA-PKcs deficiency	<i>DCLRE1C</i>	T ⁻ B ⁻	AR
	<i>PRKDC</i>	T ⁻ B ⁻	AR
Cernunnos-XLF deficiency	<i>NHEJ1</i>	T ⁻ B ⁻	AR
DNA Ligase 4 deficiency	<i>LIG4</i>	T ⁻ B ⁻	AR
Mitochondrial defect	<i>AK2</i>	T ⁻ B ^{+/-}	AR
Reticular dysgenesis	<i>RMRP</i>		
Cartilage hair hypoplasia			
Other	<i>CORO1A</i>	T ⁻ B ⁺	AR
Coronin-1A deficiency			
Thymic defects	22q11 deletion	T ⁻ B ⁺	AD
DiGeorge syndrome	<i>CHD7</i>	T ⁻ B ⁺	AD
CHARGE syndrome			
T-lymphocyte immunodeficiency with thymic aplasia	<i>FOXP1</i>	T ⁻ B ⁺	AR

XL, X-linked; AR, autosomal recessive; AD, autosomal dominant.

Table 2 Causes of Erythroderma in the Infant associated with Immunodeficiency.

<i>Disease</i>	<i>Cause</i>
Omenn syndrome	'Leaky' form of severe combined immunodeficiency
Materno-fetal graft versus host disease	Maternal T lymphocyte engraftment in patient with severe combined immunodeficiency
Transfusion-related graft versus host disease	Blood transfusion donor T lymphocyte engraftment in patient with severe combined immunodeficiency
Comel-Netherton Syndrome	Mutation in <i>SPINK5</i>

defect, B-lymphocytes may be affected: they may be absent, or intrinsically non-functioning. However, if B-lymphocytes are present and intrinsically functional, the absence of CD4⁺ T-lymphocytes in SCID prevents B-lymphocyte activation and generation of normal antibody responses producing a combined cellular and humoral immune deficit. Natural killer (NK) cells may be present or absent. Almost 20 different genetic causes of SCID are now described (Table 1), but clinical presentation is similar regardless of genotype.

Clinical presentation

First clinical presentation of an infant is within the first few months of life. Without curative treatment, most patients do not survive beyond the first 12–18 months. Infection is the most common manifestation—persistent respiratory tract infection is frequently encountered, with a failure to clear viruses accompanied by persistent bronchiolitic-like signs including tachypnea, nasal flaring, subcostal and intercostal recession, with widespread crepitations and rales, and cyanosis. Persistent viral-associated diarrhea with accompanying growth failure is also common, and vaccine-strain viruses (e.g., rotavirus) may be detected if the infant has been vaccinated. Infants with SCID are often initially well and growing normally, but fall away from the growth centile when infection occurs, because of malabsorption secondary to intestinal villous atrophy, which in severe cases causes malnutrition (van der Burg and Gennery, 2011). Progressive respiratory symptoms accompanied by an oxygen requirement and radiological evidence of



Fig. 1 Chest radiograph from an infant with severe combined immunodeficiency showing hyperinflated lungs with mid-line pleural borders of the upper lobes visible in the absence of a thymic shadow, and interstitial pneumonitis secondary to *Pneumocystis jirovecii* pneumonia and parainfluenzae type 3 infection. Courtesy of the Bubble Foundation, UK.

interstitial pneumonitis and hyperinflation, suggests *Pneumocystis jirovecii* infection (Fig. 1) (Berrington et al., 2000). There may be oral or perineal candidiasis and other superficial infections. Frequently, patients are infected with multiple organisms. Immunization with the live attenuated Bacillus Calmette-Guérin (BCG) vaccine can result in disseminated BCG infection, affecting bones, marrow, liver, brain and respiratory tract (Marciano et al., 2014).

Whilst most genetic forms of SCID present with similar clinical features, there are some gene defects that warrant specific mention.

Adenosine deaminase deficiency

Adenosine deaminase (ADA)-deficient SCID, caused by loss of function mutations in the *ADA* gene, is a systemic metabolic defect. Adenosine deaminase is an indispensable purine salvage pathway enzyme, in which deficiency causes one of the more common autosomal recessive SCID syndromes, accounting for approximately 10–15% of cases in outbred populations (Gaspar, 2010). Adenosine deaminase is essential for the irreversible deamination of adenosine to inosine and 2′deoxyadenosine to 2′deoxyinosine and absent or impaired function of ADA causes intracellular and extracellular accumulation of these toxic substrates, and deoxyadenosine triphosphate (dATP). ADA-deficient SCID is typified by severe pan-lymphocytopenia affecting T- and B-lymphocytes and NK cells. Whilst the infectious complications encountered by patients are the same as for other genetic causes of SCID, the systemic distribution of the enzyme is manifest in non-immunological features, including neurodevelopmental deficits (Rogers et al., 2001) sensorineural deafness (Albuquerque and Gaspar, 2004) and skeletal abnormalities, particularly of the scapulae and ribs (Fig. 2) (Sauer et al., 2009; Manson et al., 2013).



Fig. 2 Chest radiograph from an infant with adenosine deaminase-deficient severe combined immunodeficiency showing abnormal squaring of the scapulae and cupping deformities at the ends of the ribs. Courtesy of the Bubble Foundation, UK.

A particular feature is the presence of early-onset severe respiratory symptoms caused by direct toxicity of adenosine metabolites on lung tissue, confirmed in experimental models: *Ada*^(-/-) mice demonstrate severe pulmonary inflammation, with accompanying accumulation of activated macrophages and eosinophils. The resultant inflammation initiates airway remodeling, which is reversible if treated early with polyethylene-glycosylated ADA enzyme replacement therapy (Blackburn and Kellems, 2005). Prolonged exposure of lung tissue to high adenosine concentrations due to inadequate treatment with low dose enzyme replacement therapy causes pulmonary fibrosis, although the changes can be reversed by decreasing pulmonary adenosine levels (Chunn et al., 2005). ADA-deficient patients exhibit similar non-infectious pulmonary manifestations including pneumonitis and pulmonary alveolar proteinosis, which are described more frequently in this group than in other genetic forms of SCID (Booth et al., 2012). Pulmonary alveolar proteinosis in patients with ADA-deficient SCID resolves upon commencing treatment with polyethylene-glycosylated ADA (Grunebaum et al., 2012). However, co-existence of adenosine-induced pulmonary toxicity and respiratory infection is common, and chest radiography does not differentiate the pathogenesis—therefore, patients with ADA-deficient SCID who exhibit pulmonary symptoms should receive both appropriate anti-microbial treatment and polyethylene-glycosylated ADA enzyme replacement therapy.

Reticular dysgenesis

Patients with loss-of-function mutations in *AK2* that encodes adenylate kinase 2 have a particular form of SCID characterized by agranulocytosis in addition to pan-lymphocytopenia with absence of T- and B-lymphocytes and NK cells. Classically, patients are born pre-term, or are small for gestational age. There is associated sensorineural deafness. Whilst the majority of infants with SCID present in the first few months of life, the agranulocytosis associated with *AK2* deficiency confers a severe phenotype and patients develop severe symptoms in the first few days or weeks of life. Circulating lymphocytes and granulocytes are generally absent, and the agranulocytosis does not respond to G-CSF. Other hematological indices may be low, including hemoglobin or thrombocytes. An examination of bone marrow may show hypoplasia or hyperplasia. The most common presentation is invasive bacterial infection, usually bacterial sepsis, with respiratory features. Omphalitis is frequently co-existent. Implicated bacteria include *S. aureus*, streptococcal commensal species, *K. pneumonia*, and *E. coli*. Candida sepsis is also described (Hoenig et al., 2017).

Omenn syndrome and atypical SCID

Omenn syndrome is a form of SCID with a characteristic clinical and immunological phenotype. Patients present with an extensive, thickened dermatosis, which is often scaling with erythematous exfoliation, and protein-losing erythroderma, which presents a 'leathery' consistency (Fig. 3). Alopecia, often including eyebrows and eyelashes, develops as the rash evolves. The rash may be present at birth or shortly afterwards, or evolve over the first few weeks of life. There is an associated lymphadenopathy, particularly of the axillary and inguinal nodes. Hepatosplenomegaly is frequently present (Omenn, 1965). The immunophenotype is



Fig. 3 Infant with Omenn syndrome, demonstrating thickened, scaling erythematous exfoliation with alopecia, including eyebrows and eyelashes. Courtesy of the Bubble Foundation, UK.

characteristic, and quite different to classic SCID. A T-lymphocytosis with a highly activated phenotype is found, dominated by a restricted oligoclonal expansion of a few autologous TCRV β families with absence of other families (Harville et al., 1997; de Saint-Basile et al., 1991). Markers of thymopoiesis, such as CD4⁺ CD45RA⁺ CD31⁺ T-lymphocytes, or T-lymphocytes bearing T-lymphocyte Receptor Excision Circles (TRECs), which are episomal DNA formed during T-lymphocyte receptor re-arrangement, representative of a recent thymic emigrant and absent in SCID, are absent. B-lymphocytes are usually absent, and NK cells are generally present in normal numbers. T-lymphocytes fail to proliferate in response to stimulation with phytohemagglutinin. Although serum immunoglobulins IgM, IgA and IgG are absent, with absence of vaccine antigen responses, the serum IgE is usually raised, with an associated eosinophilia.

An important distinction between Omenn syndrome and classical SCID is the presence of intense auto-inflammation. Inflammatory pneumonitis, enteritis, hepatitis may be present (Altmann et al., 2018), usually with coexisting infection with conventional or opportunistic pathogens. The clinico-immunophenotype is similar to that found in patients who have classical SCID with materno-fetal engraftment with graft-versus host disease (Wahn et al., 1991), or classical SCID and blood transfusion-associated graft-versus host disease (Sebnem Kilic et al., 2010), and can be distinguished by determining the origin of T-lymphocytes by molecular techniques (Table 2).

Infants with a clinical diagnosis of classical SCID and mutations in the genes listed in Table 1 demonstrate absent circulating T-lymphocytes. However, those with 'atypical SCID' have normal or elevated T-lymphocyte numbers, and harbor hypomorphic mutations in SCID-causing genes, which permit residual T-lymphocyte development, and most commonly present with severe recurrent pneumonia, rarely due to opportunistic micro-organisms such as *Pneumocystis jirovecii* (Felgentreff et al., 2011).

Other presentations of SCID

Infection is the most common presentation in patients with SCID, particularly of the respiratory or gastrointestinal tract, but other rare presentations are described (Table 3). Very rarely, polymorphous lymphoproliferative lesions, resembling B-lymphocyte lineage lymphomas, have been described, sometimes associated with Epstein-Barr viral infection (Slatter et al., 2011).

Hemophagocytic lymphohistiocytosis, is a life-threatening hyperinflammatory syndrome, more usually linked to defects in genes of the cytotoxic granule-mediated cell death pathway. It has been described in patients with SCID, sometimes in association with engraftment of maternal T-lymphocytes (Bode et al., 2015).

Management

Accurate identification of pathogens is crucial for effective anti-microbial treatment. Nasopharyngeal secretions may yield viruses causing infection of the lower respiratory tract, such as respiratory syncytial virus, but isolates from these samples may indicate upper respiratory tract colonization or infection only (e.g. rhinovirus), and so broncho-alveolar lavage is recommended in these patients—non-bronchoscopic examination is adequate for most patients, but directed fiber-optic bronchoscopy, should be employed in patients with isolated lesions identified on chest imaging (Slatter et al., 2007). Lung biopsy should be considered in symptomatic patients if no pathogen is identified by other means (Hughes, 1991). More than one pathogen may co-exist in these patients (Berrington et al., 2000). Appropriate microbial therapy should be directed at the specific pathogen. Viral infection is difficult to treat and without restoration of T-lymphocyte function, viral clearance will not be achieved (Crooks et al., 2000), although some control may be gained with antiviral agents. In severe cases, donor or third party adenovirus- or cytomegalovirus-specific cytotoxic T-lymphocytes may be administered (Keller et al., 2016) but an intense inflammatory pneumonitis may result and so co-administration of corticosteroids is recommended. Palivizumab, an anti-respiratory syncytial virus monoclonal antibody prevents respiratory syncytial virus infection in high-risk infants, and should be administered to infants with SCID who present during the winter months. The role of palivizumab in treating infants with established lower respiratory tract respiratory syncytial virus infection is less clear—it appears safe to give (Boeckh et al., 2001), but efficacy has yet to be demonstrated (Chávez-Bueno et al., 2007). All infants with SCID should receive immunoglobulin replacement therapy until B-lymphocyte function is restored.

Respiratory impairment confers a significant adverse outcome following hematopoietic stem cell transplantation (Gennery et al., 2010), especially in infants with active infection at time of transplantation (Pai et al., 2014) and the physical condition of infants should be optimized prior to the transplant procedure. Early diagnosis of SCID before infection has occurred gives the best outcomes (Brown et al., 2011), which is why newborn screening for SCID is being introduced in increasing numbers of countries (Kwan et al., 2014).

Table 3 Presentations of severe combined immunodeficiency.

Common Presentations	Rare Presentations
Respiratory infection	Bacterial septicemia
Gastrointestinal infection	Disseminated BCG infection
Candidiasis	Materno-fetal graft-versus-host disease
<i>Pneumocystis jirovecii</i> pneumonitis	Hemophagocytosis
Failure to thrive	Autoimmune cytopenias
	Lymphoid malignancy

Combined immunodeficiencies

Background

Patients with combined immunodeficiency (CID) often present with recurrent respiratory and gastrointestinal tract infections, caused by a wide range of pathogens. Clinical presentation shares features with those of patients with atypical SCID due to residual T-lymphocyte function, although patients with CID usually have a less severe presentation with a later age of onset (>1 year of age). Because of impaired T-lymphocyte function, viral infections can be particularly severe, but depending on the specific immunodeficiency, many different infectious agents are described. Autoimmune manifestations are also common, and in some diseases, lymphoproliferation and lymphoid malignancy is well described. The latest International Union of Immunology Societies classification lists 39 different genes associated with non-syndromic CID (Tangye et al., 2020).

Immunoglobulin class switch recombination deficiencies affecting CD40-CD40L (CD40LG deficiency, CD40 deficiency)

Different genetic defects are described that interrupt the capacity of B-lymphocytes to switch from producing IgM immunoglobulin, to immunoglobulin isotypes with alternative constant regions (IgA, IgE, IgG). Defects are either extrinsic to the B-lymphocyte signaling pathway, or impact the intrinsic B-lymphocyte switch pathway (Table 4). Accurate identification of the defect enables the management approach to be appropriately targeted, according to the specific gene involved (Durandy et al., 2013). CD40 ligand (CD40L) deficiency [X-linked hyper-IgM syndrome type 1], is the most common immunoglobulin isotype class switch defect. It is a rare X-linked primary immunodeficiency caused by mutations in *CD40LG*, on chromosome Xq26.3-Xq27.1, and encodes the transmembrane CD40L glycoprotein (CD154), which is expressed on the surface of activated CD4 T-lymphocytes. Mutations in *CD40LG* alter co-stimulatory T-lymphocyte function, impairing B-lymphocyte isotype switching and dendritic cell signaling, and resulting in increased susceptibility to intracellular and bacterial pathogens. Patients commonly present in infancy or early childhood with recurrent upper and lower respiratory tract infections, including *Pneumocystis jirovecii* interstitial pneumonia (Levy et al., 1997). Cryptosporidium infection is a common cause of acute or chronic diarrhea, and is not cleared. Chronic inflammation may lead to severe biliary tract disease, including sclerosing cholangitis, cirrhosis, and rarely cholangiocarcinoma, hepatocellular carcinoma, and adenocarcinoma (Hayward et al., 1997). Infrequently, infection of the central nervous system, particularly enteroviral meningoencephalitis, or JC virus progressive multifocal leukoencephalopathy, cause progressive neurodegeneration (Cunningham et al., 1999; Aschermann et al., 2007).

Although long-term survival with conservative therapy of immunoglobulin replacement and cryptosporidial prophylaxis has been historically poor, with 20–50% survival to the third decade (Levy et al., 1997; Toniati et al., 2008), recent data demonstrate an improved median survival from diagnosis of 25 years in 109 patients reported with CD40L deficiency (de la Morena et al., 2016). Hematopoietic stem cell transplantation is curative. A multi-center study of 130 patients reported 80% overall survival after transplantation. Better outcomes were associated with transplantation before 10 years of age, or transplantation early after diagnosis. Use of myeloablative conditioning regimens conferred higher overall and disease-free survival, whilst reduced intensity and non-myeloablative conditioning was associated with poor donor cell engraftment (Ferrua et al., 2019). Transplant survivors experienced significantly better well-being, as measured by using Karnofsky/Lansky scales, than those not transplanted (de la Morena et al., 2016).

Mutations in the *CD40* gene, the receptor for CD40L, mimic the phenotype of CD40L deficiency, although inheritance is autosomal recessive, and it is much less common. Expression of CD40 on monocytes and B-lymphocytes is absent or where present, the CD40 protein function is absent or impaired, whereas expression of CD40L is normal. Clinical features overlap those of CD40L deficiency and include bacterial sino-pulmonary infection due to antibody deficiency, as well as infection with opportunistic micro-organisms such as *Pneumocystis jirovecii*. Severe bronchiectasis can develop (Ferrari et al., 2001; Lougaris et al., 2005). Hepatic

Table 4 Immunodeficiencies associated with raised IgM.

Disease	Genetic Defect	Affected cell type	Inheritance
CD40 Ligand deficiency	<i>CD40LG</i>	CD4 ⁺ T-lymphocytes	XL
CD40 deficiency	<i>CD40</i>	B-lymphocytes, monocytes, dendritic cells	AR
Ataxia Telangiectasia	<i>ATM</i>	B-lymphocytes	AR
DNA ligase IV deficiency	<i>LIG4</i>	B-lymphocytes	AR
Cernunnos-XLF deficiency	<i>NHEJ1</i>	B-lymphocytes	AR
Nijmegen Breakage syndrome	<i>NBN</i>	B-lymphocytes	AR
NF-KAPPA-B Essential Modulator (NEMO) deficiency	<i>IKBKG</i>	Polymorphonuclear cells, monocytes, dendritic cells, lymphocytes	XL
Activation-induced cytidine deaminase deficiency	<i>AICDA</i>	B-lymphocytes	AR
Activation-induced cytidine deaminase deficiency, C-terminus variant	<i>AICDA</i>	B-lymphocytes	AD
uracil-DNA glycosylase deficiency	<i>UNG</i>	B-lymphocytes	AR
IL21R Deficiency	<i>IL21R</i>	T-lymphocytes, B-lymphocytes, NK cells	AR

disease associated with cryptosporidial infection is reported (Kutukculer et al., 2003). Treatment is the same as for patients with CD40L deficiency—immunoglobulin replacement and antibiotic prophylaxis to prevent *Pneumocystis jirovecii* and *Cryptosporidium* infection. Hematopoietic stem cell transplantation is curative (Al-Saud et al., 2019a).

ICOS and ICOSL deficiency

The inducible T cell co-stimulator (ICOS) belongs to the CD28/CTLA4 family of proteins, and is expressed on activated CD4 T-lymphocytes; signaling through ICOS is induced by interaction with ICOS ligand which is highly expressed on B-lymphocytes, and dendritic cells and weakly present on T-lymphocytes. Signaling through ICOS has wide-ranging effects on the adaptive immune response and is important for augmenting T-lymphocyte proliferation, cell surface molecule expression and cytokine secretion, as well as T-B-lymphocyte co-activation, CD40-CD40L mediated Ig class switch recombination, and development of Th1 and Th2 immune responses. ICOS signaling also has important roles in regulating generation of Th17 cells, and differentiation of FoxP3 regulatory T-lymphocytes. The first descriptions of patients with ICOS deficiency were from cohorts of patients with common variable immunodeficiency, with clinical features including recurrent bacterial infections of the respiratory and gastrointestinal tracts but no other CVID features such as splenomegaly, autoimmunity or granulomas. (Grimbacher et al., 2003; Salzer et al., 2004). ICOS deficiency is listed as a combined immunodeficiency in the IUIS classification and the phenotype expanded, although only 22 patients have been described to date (Takahashi et al., 2009; Robertson et al., 2015; Schepp et al., 2017; Abolhassani et al., 2020). Frequent clinical features include respiratory infections, severe viral infections and enteropathy, but less common presentations are described with autoimmunity, lymphoproliferation and malignancy. Only one patient has been described with ICOSL deficiency (Roussel et al., 2018). The patient had respiratory infections, widespread human papillomavirus warts and oro-labial HSV infections. Hypogammaglobulinemia is described in all patients to date, most with vaccine antigen specific antibody deficiency. Pan-lymphocytopenia is described but not invariable. All have had reduced class-switched memory B-lymphocytes and reduced follicular CD4⁺ T-lymphocytes. Treatment is with antimicrobial prophylaxis (including *Pneumocystis* prophylaxis) and immunoglobulin replacement. Hematopoietic stem cell transplantation has been successfully offered in some patients.

CD3 γ chain deficiency

The CD3 molecule is an essential part of the CD3/T-lymphocyte receptor complex, and is critical for T-lymphocyte maturation. The CD3 molecule itself is composed of a single δ chain and γ chain and two ϵ chains. Defects in CD3 δ and CD3 ϵ confer a SCID phenotype. CD3 γ chain-deficient mice demonstrate a similar phenotype to that of humans with CD3 δ - or CD3 ϵ -deficiency. Although there are few patients described with CD3 γ chain deficiency, the phenotype is less severe and broader, as the defect is permissive for T-lymphocyte development (Arnaiz-Villena et al., 1992; Ozgür et al., 2008; Tokgoz et al., 2013; Gokturk et al., 2014; Rowe et al., 2018; Lee et al., 2019). The age of patients ranges from early childhood to adulthood. Bacterial and viral infections, particularly of the respiratory tract are most commonly described, and atopic dermatitis is frequent. Additionally, autoimmunity is frequently encountered, particularly autoimmune thyroiditis, inflammatory bowel disease and autoimmune hemolytic anemia. Immunologically, lymphocyte numbers are generally normal, although CD3 expression is impaired. Proliferation to mitogens may be diminished. The regulatory T-lymphocyte proportion, repertoire diversity and function is also restricted in some patients, possibly accounting for the autoimmune features. Treatment with antimicrobial prophylaxis and immunosuppression has been reported—hematopoietic stem cell transplantation has been performed in a few patients.

CD4 deficiency

The CD4 molecule, a member of the immunoglobulin superfamily, is a co-receptor of the T-lymphocyte receptor and binds to distinct regions of the MHC class II molecule, defining T-helper lymphocytes. Two patients, both adults, are published to date with different homozygous mutations in *CD4*, resulting in absent or reduced protein function (Fernandes et al., 2019; Lisco et al., 2021). Both patients had severe, longstanding papillomatous warts, whilst the patient with absent CD4 protein also experienced recurrent respiratory tract infections and severe pneumonia. Immunophenotyping revealed normal CD3⁺ and CD8⁺ lymphocyte numbers but complete absence of CD4⁺ T-lymphocytes. Immunoglobulin levels were normal, although the most severely affected patient had impaired antibody responses to some vaccine antigens (Lisco et al., 2021). Interestingly, this patient had a relative expansion of CD4[−]CD8[−]TCR $\alpha\beta$ ⁺ (double negative) lymphocytes which retained phenotypic and functional characteristics of normal CD4⁺ T-lymphocytes, which included a division of naïve and memory subsets, expression of regulatory T-cell markers FOXP3 and CD25, and expression of CD40, that paralleled that of normal CD4⁺ T lymphocytes, suggesting that double negative T-lymphocytes may be able to accomplish certain specific CD4⁺ T-lymphocyte functions. Patients were treated conservatively, but in severe cases, hematopoietic stem cell transplantation would be expected to cure the disease.

CD8 α chain deficiency

The CD8 T-lymphocyte co-receptor, expressed on the cell surface as $\alpha\alpha$ chain homodimers or $\alpha\beta$ chain heterodimers, is essential for intra-thymic positive thymocyte selection, without which CD8⁺ T-lymphocytes are not released into the periphery. The expression of CD8 β on the cell surface requires expression of CD8 α ; failure to express CD8 α results in degradation of CD8 β in the

endoplasmic reticulum. Few patients with CD8 α chain deficiency are described (de la Calle-Martin et al., 2001; Mancebo et al., 2008; Dumontet et al., 2015) and younger family members sharing the same genotype and immunotype were asymptomatic. Patients present with recurrent viral and bacterial sino-pulmonary infections from early childhood, which evolve to severe bronchiectasis in later life. Immunological evaluation demonstrated normal lymphocyte numbers, with normal CD4 $^{+}$, CD19 $^{+}$ values and normal NK cells, but completely absent CD8 $^{+}$ T-lymphocytes. A high percentage of CD4-CD8-TCR $\alpha\beta^{+}$ T-lymphocytes was identified. Proliferation to mitogens was normal. Immunoglobulin levels were normal, with positive antibody responses to vaccine antigen. Optimal treatment has yet to be determined:- antibiotic prophylaxis and immunoglobulin replacement has been reported.

TCR α subunit constant chain deficiency

Most T-lymphocytes express a T-lymphocyte receptor heterodimer with extracellular and transmembrane domains consisting of α and β peptides. These associate with transmembrane and intracellular subunits of the CD3 complex. During intra-thymic development, the antigen-capture (variable) region of the T-lymphocyte receptor is constructed following random recombination of specific coding segments in the V (variable), D (diversity), and J (joining) regions of the T-lymphocyte receptor loci, which then joins with the constant subunit of the T-lymphocyte receptor α and β chains. Two patients from unrelated families are reported, who demonstrated a mutation at the base following a termination codon, initiating abnormal splicing leading to a leaky termination signal. Clinically, recurrent upper and lower sino-pulmonary tract infection led to chronic lung damage. Candidiasis, diarrhea, and growth failure were apparent. One patient experienced chronic varicella, EBV and human herpesvirus 6 infection. Hepatosplenomegaly and lymphadenopathy were present. Autoimmunity with vitiligo, alopecia and hemolytic anemia was described.

The immunotype was similar between patients. CD3 $^{+}$ T-lymphocytes expressed the $\gamma\delta$ T-lymphocyte receptor. An abnormal population of CD3 lo cells expressed the $\alpha\beta$ T-lymphocyte receptor at extremely low levels. Proliferation of lymphocytes in response to phytohemagglutinin was variably impaired. Class-switched memory B-lymphocytes were present, and immunoglobulin levels were normal (although IgE was elevated), with normal vaccine responses. Autoantibodies were also documented.

Infections responded to conventional antimicrobial treatment and did not recur upon institution of antibacterial and antifungal prophylaxis. Both patients were cured by hematopoietic stem cell transplantation (Morgan et al., 2011).

IL21 and IL21R deficiency

Interleukin 21 (IL-21) is principally produced by CD4 $^{+}$ T-lymphocytes, encompassing T follicular helper lymphocytes, as well as T_H17 T-lymphocytes. Upon ligation to the interleukin-21 receptor (IL21R), the associated constituent, the common γ chain transmit signals through the JAK-signal transducer and activator of transcription (STAT) pathways to regulate of CD4 $^{+}$ T-lymphocyte, T_H17 and Treg differentiation, CD8 $^{+}$ T-lymphocyte proliferation, B-lymphocyte cell differentiation, and NK cell cytotoxicity. IL-21 production by T follicular helper lymphocytes and increased IL-21R expression on germinal center B-lymphocyte provokes brisk B-lymphocyte proliferation, and directs immunoglobulin isotype switching and plasma cell differentiation as well as memory B-lymphocyte formation.

To date, only one consanguineous family is described with IL21 deficiency (Salzer et al., 2014). Clinically, the proband, 11 years old at the time of the report, presented with inflammatory bowel disease and failure to thrive at 2 months of age. Recurrent, severe upper and lower respiratory infections were notable through childhood and may have contributed to finger clubbing. Immunological investigations revealed raised IgE, but low IgG. T-lymphocyte numbers were normal, although there was an increase in TCR $\gamma\delta$ -bearing T-lymphocytes. B-lymphocyte numbers were low, with virtual absence of class-switched memory B-lymphocytes. A homozygous mutation in IL21 was found in a conserved residue, predicted to reduce protein stability.

Loss-of-function mutations in *IL21R* have been identified in 13 patients (Ives et al., 2013; Kotlarz et al., 2013, 2014; Erman et al., 2015; Stepensky et al., 2015; Cagdas et al., 2021). Clinical symptoms are evident in childhood, with respiratory infection and gastro-intestinal disease most common symptoms. Chronic upper and lower respiratory infection is frequently described, leading in some cases to bronchiectasis. *Pneumocystis jirovecii* infection is reported in a third of patients. Progressive cholangitis secondary to cryptosporidial infection is described in almost half of patients, leading to cirrhosis and end stage liver disease. Immunological findings are variable. T- and B-lymphocyte numbers were variably low, in the normal range or high. Class switched memory B-lymphocyte numbers were reduced and class switch induction was impaired. Immunoglobulin levels were variably low or normal, except IgM and IgE which were often raised.

Immunoglobulin replacement was offered as treatment, but the severity of the immune defect, and risk of serious lung and liver disease suggest that hematopoietic stem cell transplant should be offered early in the course of disease, particularly as mortality was seen in older patients with infection and end-organ damage.

OX40 deficiency

T-lymphocyte co-stimulatory receptor, OX40, is a member of the tumor necrosis factor superfamily and enhances T-lymphocyte receptor-induced responses. It is expressed on many activated T-lymphocytes, including CD8 $^{+}$ T-lymphocytes, and CD4 $^{+}$ T-lymphocyte subtypes:- T_H1, T_H2 and T_H17 and CD4 $^{+}$ Foxp3 $^{+}$ Tregs. Only one patient has been described to date with loss of function mutations in *OX40* (Sahin et al., 2010; Byun et al., 2013), a female from a consanguineous union who presented in

childhood with leishmaniasis, and at the age of 14 years with HHV8-associated Kaposi sarcoma. Immunological investigation demonstrated a normal number of T- and B-lymphocytes in blood. However, the naive CD4⁺ T-lymphocyte population was expanded with lower proportions of non-naive subsets, particularly for the effector memory and Treg populations. Similarly, the memory B-lymphocyte compartment was diminished. Given the high level of OX40 expressed in Kaposi sarcoma, OX40 deficiency may be selectively susceptible to this complication of HHV8 infection.

MHC class I deficiency

MHC class I molecules are transmembrane glycoprotein heterodimers constructed from α chains, the genes for which are encoded on the short arm of chromosome 6, and a β_2 microglobulin protein. MHC class I molecules present peptides derived from intracellular protein, to CD8 T-lymphocyte receptors and induce the cytotoxic T-lymphocyte response. They are important for clearing intra-cellular pathogens. Additionally, the expression of MHC I molecules on the thymic epithelial cell surface, enables positive selection of CD8⁺ T-lymphocytes during intra-thymic T-lymphocyte development. MHC I deficiency is caused by defects in the genes encoding one of the proteins essential for transporting the peptide to the endoplasmic reticulum and loading it onto the MHC I molecule, namely transporter associated with antigen processing (TAP) 1 and TAP2, and tapasin proteins. Rarely, defects in β_2 microglobulin lead to the same phenotype (Ardeniz et al., 2015; Yabe et al., 2002). Without antigen loading, the MHC I molecule is not presented at the cell surface.

Patients usually present usually during childhood with recurrent upper respiratory tract disease causing sinusitis, otitis media, nasal disease and pharyngitis. Recurrent lower respiratory tract bacterial infections cause chronic inflammatory lung disease and bronchiectasis. Sterile necrotizing granulomatous skin lesions resembling Wegeners granulomatosis are described in some patients, and may cause midface deformities. Immunological findings include diminished CD8⁺ TCR $\alpha\beta$ lymphocyte numbers. MHC I expression is severely diminished or absent. Natural killer cell numbers may be increased. Treatment is supportive with aggressive use of prophylactic antibiotics—immunoglobulin replacement may be necessary. Treatment of the granulomata can be extremely challenging and include immunosuppression with local topical steroid (Gadola et al., 2000). Hematopoietic stem cell transplant has rarely been attempted (Gao et al., 2016).

MHC class II deficiency

MHC class II molecules are transmembrane glycoprotein heterodimers constructed from α and β chains, the genes for which are on the short arm of chromosome 6. MHC class II molecules offer exogenous peptides to CD4⁺ T-lymphocyte receptors to commence the normal adaptive response. The expression of MHC II molecules on thymic epithelial cells enables positive selection of CD4⁺ T-lymphocytes during thymic development. MHC II deficiency is caused by mutations in one of four regulatory genes (*CIITA*, *REFX5*, *REFXANK*, *REFXAP*) that code for transacting regulatory factors, which are required for MHC II molecule expression on the cell surface. Mutations in any of these genes lead to a similar clinical picture.

Patients usually present at an older age than patients with classical severe combined immunodeficiency. Severe, persistent respiratory or gastrointestinal tract infections are characteristic of the disease, with infections caused by bacterial, viral, fungal or protozoal pathogens including *Pneumocystis jirovecii*. Severe sino-pulmonary infections are common. Growth failure secondary to viral-induced enteropathy is frequently encountered. Cryptosporidial liver disease is well described. Enteroviral infections are frequent and may cause meningoencephalitis. Autoimmunity, particularly autoimmune cytopenia, is a distinct feature in some patients (Ouederni et al., 2011; Al-Herz et al., 2013; Ben-Mustapha et al., 2013).

The immunophenotype includes CD4⁺ lymphocytopenia, with diminished immunoglobulin levels and impaired vaccine antibody responses. T-lymphocyte proliferative responses to phytohaemagglutinin may be normal. Most patients will not be detected by newborn screening for severe combined immunodeficiency as some TRECs are present, although occasional patients will be picked up (Marcus et al., 2018).

The only curative treatment is hematopoietic stem cell transplantation. Historically, results have been less good for this disease compared with other immunodeficiencies (Gennery et al., 2010), in part because of increased mortality from viral infection or graft versus host disease (Renella et al., 2006). Recent results are more encouraging with a variety of donor and stem cell sources (Elfeky et al., 2018; Lum et al., 2020).

ZAP-70 deficiency

The zeta-chain associated protein kinase 70 kDa (ZAP-70) is important for effective T- and B-lymphocyte receptor signaling and critical for T-lymphocyte differentiation and function (Kaur et al., 2014). Mutations in *ZAP70* cause an autosomal recessive combined immunodeficiency characterized by CD8⁺ T-lymphocytopenia. The majority of patients present in the first year of life with severe infections including *Pneumocystis jirovecii* infection, and failure to thrive (Elder et al., 1994; Turul et al., 2009). However, patients with hypomorphic mutations may present later (Picard et al., 2009), when autoimmunity may predominate (Chan et al., 2016; Sharifinejad et al., 2020). Hematological cytopenias are most common, autoimmune enteropathy and autoimmune dermatoses are described. Lymphoproliferation may be seen at an older age, and lymphoma is reported which is not invariably associated with EBV infection.

Typically, patients demonstrate normal or elevated peripheral lymphocyte numbers, associated with normal or increased CD4⁺ T-lymphocytes. Conversely, CD8⁺ T-lymphocytes are low or absent, but may increase over time suggesting development of limited thymic output, although TREC levels are very low (Roifman et al., 2010; Suresh et al., 2020). Despite normal CD4⁺ T-lymphocyte numbers, cells fail to proliferate in response to T-lymphocyte mitogen stimulation. However, by-passing stimulation of the T-lymphocyte receptor with PMA and ionomycin does induce proliferation (Elder et al., 1994). B-lymphocyte numbers are normal, although most patients are hypogammaglobulinemic.

Management of patients with ZAP-70 kinase deficiency is as for other patients with a severe combined immunodeficiency—antimicrobial prophylaxis and immunoglobulin replacement. Hematopoietic stem cell transplantation is curative (Cuvelier et al., 2016).

IKAROS deficiency

Ikaros family zinc finger 1 (*IKZF1*) encodes a transcription factor, which regulates gene transcription during hematopoietic cell development in the bone marrow, by recruiting target DNA to proteins that modify chromatin.

Heterozygous mutations in *IKZF1* have been associated with a variety of immune defects, autoimmunity and malignancy. Pancytopenia with anemia, thrombocytopenia and neutropenia portending B-lymphocytopenia and diminished NK cells is described (Goldman et al., 2012). A common variable immunodeficiency-type clinical picture with progressive B-lymphocytopenia and associated hypogammaglobulinaemia is also recognized, which is inherited in an autosomal dominant fashion with incomplete penetrance (Kuehn et al., 2016; Bogaert et al., 2018). Combined immunodeficiency with increased CD8⁺ T-lymphocyte counts but reduced regulatory T-lymphocyte numbers, a skewed T-lymphocyte receptor repertoire, with increased T-lymphocyte receptor $\alpha\beta^+$ CD4⁺ CD8⁺ double-negative T-lymphocyte counts, and impaired proliferation to mitogens are reported (Hoshino et al., 2016). Haploinsufficiency defects are associated with a common variable immunodeficiency phenotype, whereas combined immunodeficiency is associated with dominant negative mutations. Mutations leading to a dimerization defect are associated with predominantly hematological manifestations including cytopenia, but also malignancy manifesting as T-ALL and Burkitt lymphoma (Kuehn et al., 2021).

The clinical presentation is inconsistent, but severe bacterial infection, with recurrent sino-pulmonary infection described is reported, as well as infection with *Pneumocystis jirovecii*. Autoimmune hematological cytopenias, vasculitis and systemic lupus erythematosus are recognized (Kuehn et al., 2016). Treatment is directed at replacing immunoglobulin, and appropriate management of autoimmune symptoms, or of malignancy. The place of hematopoietic stem cell transplantation in selected patients is yet to be determined, although it has been successfully performed (Hoshino et al., 2016).

DOCK8 deficiency

Although previously classified as autosomal recessive Hyper IgE syndrome (Zhang et al., 2009; Engelhardt et al., 2009), patients with bi-allelic mutations in dedicator of cytokinesis 8 (*DOCK8*) are now considered to have a combined immunodeficiency, reflected in the new ISUS classification (Engelhardt et al., 2015; Aydin et al., 2015; Tangye et al., 2020). *DOCK8* is highly expressed highly in immune cells and failure of *DOCK8* function impairs T-lymphocyte proliferation, production of antiviral T_H1 cytokines, decreased production of T_H17 cytokines, and increased production of T_H2 cytokines, which is associated with allergic responses (Su et al., 2011). The immune synapse formation is impaired, which disrupts antiviral NK cell cytotoxicity.

Patients can present from infancy with severe bacterial, viral and fungal infections. Typical clinical features include eczema, recurrent sinopulmonary infections, recurrent or severe viral skin infections (particularly Herpes simplex, severe molluscum contagiosum and severe human papilloma virus infections), and malignancy (Aydin et al., 2015) often leading over time to end organ damage. Bronchiectasis may develop from recurrent pneumonias. Severe food allergies are common in patients with *DOCK8* deficiency and maybe associated with anaphylaxis. Common bacterial pathogens include *Staphylococcus aureus* and *Streptococcus pneumoniae*. *Pneumocystis jirovecii* can cause interstitial pneumonitis. Mucocutaneous candidiasis is frequently reported. Vasculitis is well described, including cerebral vasculitis. Malignancies are common, and include lymphoma, associated with chronic EBV infection, and squamous cell carcinoma associated with chronic HPV infection. Severe eczema is one of the most common complications. Asthma is also common.

Immunological features include a highly elevated IgE and eosinophilia. IgG and IgA levels can be normal or high, IgM levels are often progressively reduced. Vaccine antibody responses may be subnormal and isohemagglutinins diminished or absent. T-lymphocyte subsets are diminished, and B-lymphocyte counts reduced with low or absent class switched memory B-lymphocytes. NK cell counts can be reduced (Engelhardt et al., 2015; Aydin et al., 2015). Some patients with *DOCK8* deficiency may be detected at birth by newborn screening for severe combined immunodeficiency, due to low or absent TRECs (Dasouki et al., 2011).

Aggressive treatment of infection is key, and antimicrobial prophylaxis is required. Immunoglobulin replacement is recommended. However, even with these conservative measures, the long term prognosis is poor with survival less than 40% by 30 years of age, and event-free survival (life-threatening infection, central nervous system involvement, malignancy or death) only 4% by 30 years of age (Aydin et al., 2015). Hematopoietic stem cell transplantation is therefore considered the treatment of choice for these patients, with 84% survival in a large multi-center cohort (Aydin et al., 2019). Although most manifestations respond well to transplantation, resolution of food allergy is variable (Happel et al., 2016; Aydin et al., 2019).

DOCK2 deficiency

Like DOCK8, Dedicator of cytokinesis 2 (DOCK2) is primarily expressed in hematopoietic cells and has an important role in the reorganization of the actin cytoskeleton. Few patients have been described to date, with loss of function mutations in *DOCK2*. The predominant clinical phenotype appears to be combined immunodeficiency with viral susceptibility, particularly to varicella, and mycobacterial infection. One kindred presented with 'leaky' SCID phenotype, and a further patient had a raised IgM. The immunophenotype shows T-lymphocytopenia and impaired in vitro T-lymphocyte activation in response to phytohemagglutinin. Naïve T-lymphocytes were reduced, with diminished TREC numbers on dried blood spots taken at birth. Immunoglobulins are generally normal, with impaired vaccine responses. Hematopoietic stem cell transplantation appears to be curative (Dobbs et al., 2015; Alizadeh et al., 2018; Moens et al., 2019).

Polymerase (DNA-Directed), DELTA 1, catalytic subunit (POLD1) and DELTA 2, regulatory subunit (POLD2) deficiency

DNA polymerase delta (POLD) is indispensable for leading and lagging DNA strand synthesis, and in mammals is a heterotetramer composed of four subunits: POLD1 is a catalytic subunit with polymerase and exonuclease functions and is critical in several synthetic and DNA repair processes. POLD2, with POLD3 and POLD4 regulate the activity of the complex by stabilizing the structure, establishing protein-protein interactions, and regulating the cell cycle. Heterozygous mutations in the POLD1 exonuclease domain are reported in patients with inherited colorectal cancers. A heterozygous deletion in the catalytic domain, that disrupts DNA polymerase activity but preserves exonuclease activity, is described in patients with a developmental disorder of mandibular hypoplasia, sensorineural hearing loss, progeria-like features, and lipodystrophy with insulin resistance. More recently, patients with a combined immunodeficiency have been described, who have T-lymphocytopenia with a CD8 oligoclonal repertoire restriction. The defect appears to confer impaired T-lymphocyte development and survival, consistent with the role that POLD plays in DNA repair. One patient with a combined immunodeficiency phenotype has been described with mutations in POLD2. The optimum treatment pathway for these patients has yet to be determined (Conde et al., 2019; Cui et al., 2020; Nichols-Vinueza et al., 2021).

RHOH deficiency

The ras homolog family member H is an essential negative regulator of hematopoietic cell growth and survival. To date, only two adult patients have been described with bi-allelic loss-of-function mutations in *RHOH*. The defining clinical phenotype was characterized by severe and persistent Epidermodysplasia verruciformis from early childhood—one patient experienced less severe molluscum (Crequer et al., 2012a). Burkitt lymphoma developed in one patient. The immunological phenotype demonstrated normal T-lymphocyte counts with slightly reduced CD4⁺ T-lymphocytes and high CD8⁺ T-lymphocytes. Recent thymic emigrants were severely diminished. There was restricted TCR usage with clonal expansion of certain Vβ families. Lymphocyte proliferation was impaired after stimulation through the T-lymphocyte receptor. Immunoglobulin levels and vaccine antibody responses were normal. Treatment for these patients was symptomatic—however, the T-lymphocytopenia was corrected in mice by hematopoietic stem cell transplantation, suggesting that this treatment modality may have utility in human patients.

Serine-threonine protein kinase 4 deficiency (Mammalian sterile 20-like protein deficiency)

The serine/threonine kinase 4 gene (*STK4*) encodes Mammalian sterile 20-like protein (MST1), which has a central role in regulating lymphocyte homing, cell survival and proliferation. Bi-allelic loss-of-function mutations have been described in a few individuals (Nehme et al., 2012; Abdollahpour et al., 2012; Crequer et al., 2012b; Dang et al., 2016; Sherkat et al., 2017; Schipp et al., 2018; Moran et al., 2019; Al-Saud et al., 2019b; Ashrafi et al., 2020; Radwan et al., 2020; Jørgensen et al., 2021). Clinical features are varied, but severe lower respiratory infection with bacterial infection is common. Skin manifestations are frequent including extensive molluscum contagiosum, eczema, recurrent skin abscesses, and significant human papillomavirus infection. In some patients, mucocutaneous candida infection was a feature. Autoimmune cytopaenias have been documented in a few patients. Lymphoma, commonly but not invariably associated with EBV, has been described in a number of individuals.

A variable but consistent lymphocytopenia is observed, and in some patients, CD4⁺ lymphocytopenia was remarkable. Low numbers of naïve T-lymphocytes are present. B-lymphocyte numbers are generally normal, and immunoglobulin levels were normal or high. Autoantibodies were present in some patients. Intermittent neutropenia has been a significant feature in several patients.

Treatment with antimicrobial prophylaxis and immunoglobulin replacement was effective for some patients, but given the severity of the combined immunodeficiency, hematopoietic stem cell transplantation seems a reasonable approach and has been successful for a few patients.

LCK deficiency

The lymphocyte-specific LCK protein, which is encoded by *LCK*, is a protein-tyrosine kinase of the SRC oncogene family. It is important for transduction through the T-lymphocyte receptor. The p56(LCK) protein is plasma membrane-bound and cooperates

with intracellular domains of CD4⁺ and CD8⁺ co-receptors. There are only a few reports of mutations in *LCK* in patients. A young female who had homozygous mutations in *LCK* is reported. From infancy, she had failure to thrive secondary to chronic diarrhea. She presented with recurrent respiratory tract infections, and experienced pneumonia complicated by a pneumatocele. In early childhood, she had multiple nodular skin lesions diagnosed as neutrophilic panniculitis with interphalangeal joint inflammation accompanied by daily fevers (Hauck et al., 2012). Immunologically, there was a profound CD4⁺ T-lymphocytopenia with a diminished proportion of TCRαβ⁺ T-lymphocytes and increased TCRγδ⁺ T-lymphocytes. B-lymphocyte and NK cell counts were normal or slightly low. A further patient with a common variable immunodeficiency phenotype is reported with CD4⁺ T-lymphocytopenia and an aberrantly spliced *LCK* transcript lacking exon 7 of *LCK* (Sawabe et al., 2001). The same defect was found in an infant with severe combined immunodeficiency, who presented with oral candidiasis and rotavirus-associated diarrhea and poor weight gain (Goldman et al., 1998). *Enterobacter cloacae* was isolated from the blood, and cytomegalovirus from the urine and intestinal biopsies. Hematopoietic stem cell transplantation was successful in this patient, but although the first patient underwent hematopoietic stem cell transplantation, she succumbed to complications (Bader-Meunier et al., 2018).

Interleukin-2- inducible T-lymphocyte kinase deficiency

Interleukin-2-inducible T-lymphocyte kinase (ITK), encoded by *ITK*, is involved in T-lymphocyte receptor activation, signaling and proliferation. Upon T-lymphocyte receptor activation, ITK moves to the cell membrane, activates PLCγ1, causing intracellular calcium release and activation of nuclear factor κB (NF-κB), mTOR, and MAPK/ERK intracellular pathways.

Patients with mutations in *ITK* characteristically present with EBV-associated lymphoproliferation and are highly susceptible to developing EBV-related lymphoma—nine of sixteen (56%) in a review of ITK-deficient patients developed lymphoma (Fig. 4), (Ghosh et al., 2018). Other features included EBV-driven hemophagocytic lymphohistiocytosis, and CMV or varicella infection. Epidermodysplasia Verruciformis has also been described (Youssefian et al., 2019). Autoimmune features were documented in three patients, and autoimmune lymphoproliferative syndrome is described (Wallace et al., 2021). Pulmonary interstitial nodules have been described in many patients (Huck et al., 2009; Stepensky et al., 2011; Linka et al., 2012; Mansouri et al., 2012; Ghosh et al., 2014; Serwas et al., 2014; Alme et al., 2015; Bienemann et al., 2015; Cipe et al., 2015; Cagdas et al., 2017). CD4⁺ T-lymphocytopenia is present in most patients, although CD3⁺ and CD8⁺ lymphocyte values were normal. Natural Killer T-cells, when measured, were reduced. Progressive hypogammaglobulinemia was documented in some patients. Hematopoietic stem cell transplantation seems curative, and should be offered to these patients once malignancy has been controlled—pre-emptive transplant is likely to prevent malignancy (Ghosh et al., 2018).

CARD11/BCL10/MALT1 (CBM) complex deficiencies

NF-κB is a critical component of lymphocyte activation, survival, proliferation and inflammatory cytokine production. Upstream physiological regulation of NF-κB activation is co-ordinated by a protein complex composed from caspase activation and recruitment domain 11 (CARD11), B-cell CLL/lymphoma 10 (BCL10) and mucosa associated lymphoid tissue lymphoma translocation protein 1 (MALT1), the CBM complex. Loss-of-function and gain-of-function mutations in the *CARD11*, *BCL10*



Fig. 4 Chest radiograph of a 4 year old child with ITK deficiency demonstrating extensive bilateral pulmonary nodules of variable sizes. Biopsy of the nodules demonstrated an EBV-driven lymphoproliferative disorder. Courtesy of the Bubble Foundation, UK.

and *MALT1* genes encoding these protein components are reported in a few patients (Snow et al., 2012; Greil et al., 2013; Jabara et al., 2013; Stepensky et al., 2013; McKinnon et al., 2014; Torres et al., 2014; Turvey et al., 2014; Pérez de Diego et al., 2015; Frizinsky et al., 2019; Van Den Rym et al., 2020).

Autoimmune features are prominent in these diseases, particularly severe inflammatory bowel disease and enteropathy in *MALT1* and *BCL10* deficiency, but severe eczematous dermatitis is also reported (Wiegmann et al., 2020). Periodontal disease including aphthous ulcers, cheilitis, and gingivitis is described. Infection is also a prominent feature—particularly sino-pulmonary infections due to bacterial, viral and fungal pathogens—infection with *Pneumocystis jirovecii* is reported in patients with *CARD11* mutations. Atopy is described in a few patients (Fig. 5).

The immunotype shows wide variation. The lymphocyte numbers are generally normal, although B-lymphocytopenia has been described in a patient with *MALT1* deficiency. Regulatory T-lymphocytes and T_H17 lymphocytes are generally reduced in *CARD11*-deficient patients, but normal in *MALT1*-deficient patients. The patients with *BCL10* deficiency showed a profound hypogammaglobulinemia, and progressive hypogammaglobulinaemia is found in *CARD11*-deficiency, with absent antibody responses to vaccine antigens. In *MALT1*-deficient patients, immunoglobulin levels are normal, and antibody response to vaccine antigen is variable. In *CARD11* dominant negative mutations, B-lymphocytosis is reported with diminished class switch memory B-lymphocytes (Dadi et al., 2018; Desjardins et al., 2018).

Optimum treatment is yet to be determined—antimicrobial prophylaxis and immunoglobulin replacement may be of some value, but there are a few reports of successful hematopoietic stem cell transplantation for patients with *CARD11* or *MALT1* defects (Punwani et al., 2015; Rozmus et al., 2016; Charbit-Henrion et al., 2017).

IKBKB deficiency

Activation of cells of the immune system requires complex signaling to tightly regulate transcription of genes required for activation, proliferation, differentiation, survival and signaling. The key regulators are the nuclear NF- κ B transcription factors, the canonical pathway for which operates through signaling via immune receptors, including T- and B-lymphocyte receptors, tumor necrosis factor α (TNF- α) and interleukin-1 β receptors and toll-like receptors. In the non-activated state, NF- κ B molecules are maintained in latent form in the cytoplasm by molecules of the I κ B family. Upon signaling, the complex comprising IKK1, IKK2, and NEMO (which are encoded by *IKBKA*, *IKBKB*, and *IKBKG*, respectively), phosphorylates I κ B α , enabling it to undergo partial degradation in the proteasome, leading to liberation of the NF- κ B molecules RelA, c-Rel, and p50 which translocate to the nucleus as transcription factors. Several individuals have been described with bi-allelic loss of function mutations in *IKBKB* (Pannicke et al., 2013; Nielsen et al., 2014; Burns et al., 2014; Cuvelier et al., 2019; Alsum et al., 2020; Qin et al., 2021). Clinical features include delayed separation of the umbilical cord stump, as well as recurrent infections with a range of bacterial, viral and fungal micro-organisms. Many patients had localized or disseminated infection with BCG, or other atypical mycobacteria. In a few patients, the presence of conical teeth was noted. The immunotype demonstrates normal T- and B-lymphocyte numbers, with naïve cells present, normal T-lymphocyte receptor repertoire and proliferations. Class switched memory B-lymphocytes were low in some patients. IgM was variably low, normal or high—other immunoglobulin isotypes were low. Hematopoietic stem cell transplantation has been curative, and death in early childhood seems common without curative treatment.

Four patients from two families have been described with heterozygous gain of function mutations in *IKBKB* (Cardinez et al., 2018). Recurrent sinopulmonary infection was reported in all patients, with one developing bronchiectasis. Subcutaneous abscesses and mucocutaneous candidiasis were also reported. Ectodermal dysplasia was reported in one. The immunological findings included variable lymphopenia, but absent class switched memory B-lymphocytes and variable hypogammaglobulinemia. Treatment was symptomatic.

RelA deficiency

RelA mediates activation of NF- κ B through the canonical pathway, and is activated through stimulation of various cell membrane receptors including tumor necrosis factor receptor, IL-1 receptor, Toll-like receptors, and the T- and B-lymphocyte receptor. To date, five patients from two families have been described with heterozygous loss of functions in *RELA* causing haploinsufficiency. In the first family, four individuals had mucocutaneous ulceration, and two had bowel disease (Badran et al., 2017). In the second, the clinical features were of autoimmune lymphoproliferative syndrome, with tri-lineage cytopenias, and episodes of aseptic meningitis (Comrie et al., 2018). The immunotype described from two patients was limited but largely unremarkable, although the patient with autoimmune lymphoproliferative syndrome had increased activated CD4⁺ T-lymphocytes and decreased naïve T-lymphocytes.

c-Rel deficiency

c-Rel is a member of the NF- κ B family of transcription factors and, with RelA, mediates activation through the canonical pathway and binds to gene promoters encoding for cytokines including IL-2, IFN γ , IL-12, IL-21, and IL-23. One patient is described to date with a homozygous loss of function mutation in *REL*. Clinically, the patient presented in early childhood, with chronic infectious diarrhea associated with multiple pathogens, including *Salmonella enterica*, cytomegalovirus, and *Cryptosporidium* leading to sclerosing cholangitis. *Mycobacterium tuberculosis* osteomyelitis subsequently developed, despite prior vaccination with BCG. Immunologically, there were increased numbers of CD4⁺ and CD8⁺ T-lymphocytes and decreased memory T-lymphocytes and

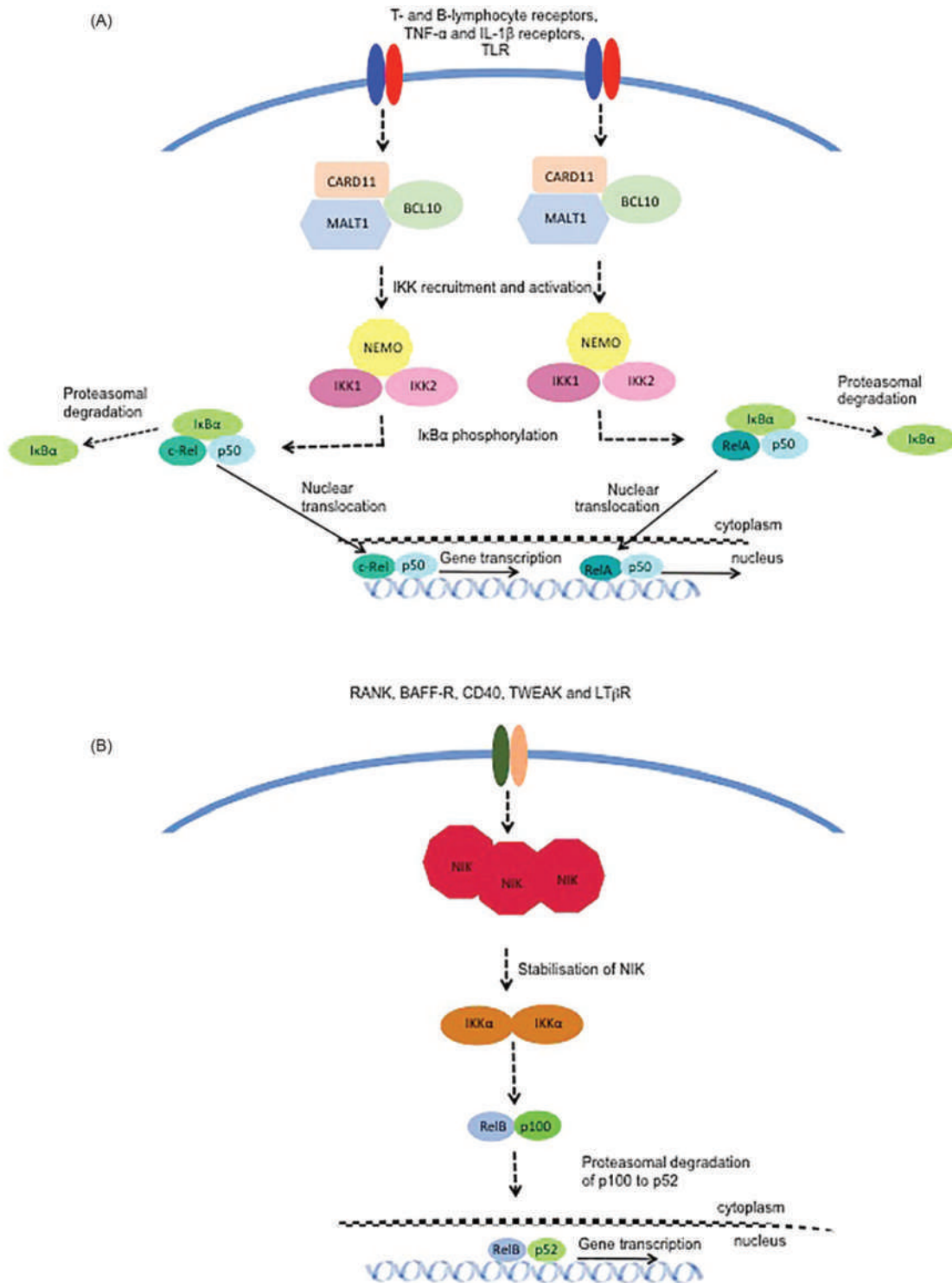


Fig. 5 (A) NF- κ B canonical signaling pathway, demonstrating the role of CARD11, MALT1, BCL10, IKK2, c-Rel and RelA. (B) NF- κ B non-canonical signaling pathway, demonstrating the role of NIK and RelB.

associated reduced mitogen-induced T-lymphocyte proliferation. There was also B lymphocytopenia, hypogammaglobulinemia and non-protective antibody titers to the tetanus and diphtheria vaccines. Treatment was with intravenous immunoglobulin replacement and prophylactic antibiotics and the patient was evaluated for hematopoietic stem cell transplantation (Beaussant-Cohen et al., 2019).

NIK deficiency

NF- κ B inducing kinase (NIK), encoded by mitogen-activated protein kinase 14 (*MAP3K14*), is integral to the non-canonical NF- κ B signaling pathway to assimilate signals from tumor necrosis factor alpha receptor family members. A number of signaling molecules activate this pathway, including receptor activator of NF- κ B ligand (RANKL), B-cell activating factor (*BAFF*), CD40L, TNF-like weak inducer of apoptosis (TWEAK) and lymphotoxin beta (LT β). Binding of the ligand to the appropriate receptor enables phosphorylation of I κ B kinase- α (IKK α) and leads to activation of downstream signaling proteins and formation of heterodimeric RelB-p52 complexes, which enter the nucleus to activate target genes. This pathway is important for activation of dendritic cells, for B-lymphocyte maturation and lymphoid organogenesis. Only seven patients have been described to date with three homozygous loss of function mutations in *MAP3K14* (Willmann et al., 2014; Schlechter et al., 2017; Ben Farhat et al., 2019). All presented in childhood with symptoms of combined immunodeficiency, including recurrent respiratory infections leading to bronchiectasis, gastrointestinal infections, and in one case, post-vaccination disseminated BCG infection. Lymphoid tissue is scarce or absent. The characteristic immunophenotype is hypogammaglobulinaemia. T-lymphocyte numbers are reported as variably low, normal or high, although class switched memory B-lymphocytes are low. Two patients received hematopoietic stem cell transplantation—in one full immune reconstitution was documented (Ben Farhat et al., 2019).

RelB deficiency

NF- κ B activation through the non-canonical pathway is mediated by RelB, which is downstream of NIK, and is activated by the same molecules, namely RANKL, *BAFF*, CD40L, TWEAK and LT β , although simultaneous activation of the canonical pathway is observed. Three patients from an extended family of Irish descent have been described to date with homozygous stop-gain mutations in *RELB* (Merico et al., 2015). All presented in early infancy with recurrent sinopulmonary infections, including multiple episodes of lobar pneumonia—other features included ecthyma gangrenosum in one patient and arthritis in one patient. The immunotype demonstrates expanded circulating T-lymphocytes, but diminished naïve T-lymphocytes, determined by TREC quantification. Thymic biopsy from one patient showed a hypoplasia and dysplasia with disorganized cortico-medullary architecture and absent Hassall's corpuscles. T-lymphocyte proliferations to mitogens were diminished. Although all patients had increased B-lymphocyte numbers and cells responded to the T-lymphocyte independent mitogen, *Staphylococcus aureus* Cowan, which implies that there was no fundamental block in B-lymphocyte proliferation, immunoglobulin levels were variable, T-lymphocyte dependent antibody responses to vaccine antigens were low to absent, and activated and class switched memory B-lymphocyte numbers were diminished (Sharfe et al., 2015). B-lymphocytes appeared to exhibit maturational arrest, and had reduced BAFF receptor expression. Two patients underwent hematopoietic stem cell transplantation—both immune reconstituted although thymic output remained low, and one developed a recrudescence of arthritis 18 months after transplant (Ovadia et al., 2017).

Moesin deficiency

Moesin is one of three members of the ezrin-radixin-moesin (ERM) protein family, with ezrin and radixin, that connect cortical actin filament C-terminal domains to the cell plasma membrane. Moesin is expressed strongly in the hematopoietic system where it maintains cell rigidity, predominately in circulating lymphocytes. Chemotaxis and T-lymphocyte receptor and B-lymphocyte receptor signaling stimulate prompt ERM protein dephosphorylation enabling relaxation of the cytoskeleton and cell polarization. Moesin is important for the immunologic synapse formation by microclustering of the antigen receptor. Nine male patients have been described with heterozygous loss of function mutations in *MSN* on the X chromosome. Clinically, patients present in childhood, with recurrent bacterial and viral infections, particularly recurrent respiratory infections, and severe *Varicella* and *Molluscum contagiosum* infection. Autoimmune manifestations included cytopenias, alopecia and thrombotic thrombocytopenic purpura (Lagresle-Peyrou et al., 2017; Bradshaw et al., 2018). The immunotypes demonstrated pan-lymphocytopenia with reduced naïve T-lymphocytes, detectable on newborn bloodspot screening (Delmonte et al., 2017). There was pan-hypogammaglobulinemia with diminished or absent antibody responses to vaccine antigens. Patients received immunoglobulin replacement, and one successfully underwent hematopoietic stem cell transplantation. At the time of report, all patients were alive aged 4–69 years.

TFRC deficiency

The cell surface receptor, transferrin receptor 1, is required for receptor-mediated endocytosis of iron, which is critical for lymphocyte development and proliferation. Bi-allelic loss of function mutations in *TFRC* have been described in 23 individuals from the Middle East (Jabara et al., 2016; Aljohani et al., 2020; Whangbo et al., 2021). Characteristic clinical features from infancy and early childhood included recurrent respiratory infection, gastrointestinal infection with failure to thrive, and less commonly sepsis and viral meningitis, autoimmunity and hemophagocytic lymphohistiocytosis in one patient. There was a significant family history of childhood death. T-lymphocytes were of normal number, as were naïve T-lymphocytes, but B-lymphocytes were low with diminished or absent class switched memory B-lymphocytes. There was pan-hypogammaglobulinemia. Additionally, intermittent thrombocytopenia and neutropenia was noted, and mild anemia. Dysmyelopoiesis was described in a few patients. Thirteen patients underwent successful and curative hematopoietic stem cell transplantation.

FCHO1 deficiency

The internalization of molecules and proteins across cellular membranes is dependent on clathrin-mediated endocytosis. The *F-BAR domain only protein 1* (FCHO1) is key in the early events of clathrin-mediated endocytosis formation. Fifteen patients are described with bi-allelic loss of function mutations in *FCHO1* (Calzoni et al., 2019; Lyszkiewicz et al., 2020). Clinical presentation was characterized by severe bacterial, viral or fungal infections, Respiratory infection was predominant. Four patients developed B-cell lymphoma. Immunological investigation revealed CD4⁺ and CD8⁺ T-lymphocytopenia. B-lymphocyte numbers were variably low to normal, but patients had hypogammaglobulinemia. Hematopoietic stem cell transplantation is curative.

Conclusion

The exploration of immune function networks from the discovery of previously undescribed disease-causing gene mutations helps predict other uncharacterized diseases and broaden our understanding of the immune connectome. Of diseases above that have been identified and described for the longest periods of time, and for which there are large patient cohorts, the functional defect, natural history and current optimal treatment pathways are well established. For the more newly described diseases, much more information is required before we can confidently recommend the best investigative and treatment approach—for many of the diseases above, only a few patients are described, it is not apparent if these represent the dominant clinical and immunological phenotype, or rather are extremely severe examples of a more common disease manifestation. Furthermore, we should be aware of the impact of the internal and external environment on disease manifestation. For the newly described diseases, patients may come from specific ethnic and geographical backgrounds, and often from single kindred's, and so the disease manifestation may not represent that of patients with the same disease in other parts of the world.

The advent of next-generation sequencing has transformed our ability to interrogate emerging phenotypes, coupled with our realization that the manifestations of immunodeficiencies are much broader than simply infection susceptibility, and include predisposition to some lymphomas, as well as autoimmunity. Despite the growing list of new combined immunodeficiencies, there are many more molecules involved in lymphocyte development, differentiation and activation, and it is certain that the current list will continue to grow.

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Combined Immunodeficiencies With Syndromic Features

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Combined immune deficiencies (CID) with syndromic features are a subgroup of primary immune deficiency (PID) disorders, also known as inborn errors of immunity (IEI), caused by single gene defects. Affected patients have in common an increased susceptibility to bacterial, viral and opportunistic infections, and, in addition, may suffer from growth retardation, connective tissue pathology, ectodermal dysplasia, cardiac malformation, intestinal atresia, autoimmunity, radiosensitivity, chromosomal instability or an increased incidence of malignancies (Tangye et al., 2020). Of note, the symptoms suggesting immune deficiency may have delayed onset and develop gradually over time. To address the extent of the immune defect, a basic assessment of the patient's adaptive and innate immune systems is important for implementing proper preventive therapy. A detailed patient and family history may reveal signs and symptoms of possible immunodeficiency and syndromic features suggesting a genetic origin with autosomal recessive (AR) or autosomal dominant (AD) inheritance (Table 1). A carefully executed examination to detect syndromic features in a patient with a history of chronic infections is the preferred approach to recognize and eventually molecularly

Table 1 Syndromic features, genetic and immunologic defects

Disease and combined immunodeficiencies with syndromic features	Genetic defect	OMIM	Inheritance	Immunologic defects		
				T	B	Immunoglobulins specific antibodies
1. Congenital thrombocytopenia						
(a) Wiskott-Aldrich Syndrome Bloody diarrhea, eczema, bacterial, viral infections, autoimmunity, malignancies, small platelets	WAS	300392	XL	Lymphopenia, decreased proliferation to anti-CD3	Normal numbers	IgA and IgE elevated, depressed antibody responses to some antigens
(b) X-linked thrombocytopenia Milder form of WAS	WAS	313900	XL	Decreased proliferation to anti-CD3	Normal	IgA, IgE elevated
(c) WIP deficiency WAS phenotype, WAS protein is absent, but mRNA is present	WIPF1	602357	AR	Lymphopenia Poor response to anti-CD3	Normal	IgA, IgE elevated
(d) ARP2/3 deficiency WAS phenotype, vasculitis, SCID-like	ARPC1B	604223	AR	Normal numbers	Normal numbers	IgA, IgE elevated
2. DNA repair defects						
(a) Ataxia Telangiectasia (A-T) Sino-pulmonary infections. Progressive cerebellar ataxia, ocular and skin telangiectasia; chromosomal instability, radiosensitivity, malignancies; increased alpha fetoprotein	ATM	607585	AR	Lymphopenia, may have low TRECS at NBS, low mitogen responses	Variably reduced	Low except for normal or elevated IgM, abnormal antibody responses by some patients
(b) Nijmegen breakage syndrome (NBS) respiratory infections. Microcephaly, dysmorphic features; chromosomal instability, radiosensitivity, malignancies, normal alpha fetoprotein	NBS1	602667	AR	Progressive lymphopenia, low TRECs at NBS	Variably reduced	Low, except for normal or elevated IgM, antibody responses may be decreased
(c) Bloom syndrome (BS) Mild bacterial infections; short stature, dysmorphic facies, sun sensitive erythema; chromosomal instability, lymphoma/leukemia; normal alpha fetoprotein	BLM	604610	AR	Normal	Normal	Hypogammaglobulinemia (in some)
(d) Immunodeficiency, centromeric instability, facial anomalies (ICF syndrome) Recurrent bacterial, viral, opportunistic infections; formation of multi-radial chromosomal figures; malignancies; hypomethylation			AR	Normal, or low numbers and function	Normal or decreased	Hypogammaglobulinemia, variable antibody deficiency
• Type 1	DNMT3B	602900				
• Type 2	ZBTB24	614064				
• Type 3	CDCA7	609937				
• Type 4	HELLS	603946				
(e) PMS2 deficiency Recurrent infections; increased risk for malignancies	PMS2	600259	AR	Normal	Decreased	Hypogammaglobulinemia, elevated IgM
(f) Radiation sensitivity, immunodeficiency, dysmorphic features, learning difficulties (RIDDLE Syndrome) Short stature, microcephaly; occasionally ataxia telangiectasia and increased alpha fetoprotein	RNF168	612688	AR	Normal	Normal	Hypogammaglobulinemia
(g) DNA ligase 1 deficiency Growth retardation; sun and radiation sensitivity	LIG1	126391	AR	Decreased, mitogen responses low	Normal	Hypogammaglobulinemia, Abnormal antibody responses

3. CID associated with thymic defects							
(a) DiGeorge syndrome (22q11.2 deletion syndrome) Abnormal facies; hypoparathyroidism; cardiac malformation	<i>Large deletion 22q11.2</i>	188400	AD	Normal or decreased, low TRECs at NBS	Normal	May have abnormal antibody responses	
(b) TBX1 deficiency DiGeorge phenotype	<i>TBX1</i>	602054	AD	Normal or decreased, low TRECs at NBS	Normal	May have abnormal antibody responses	
(c) CHARGE syndrome Coloboma, heart defect, choanal atresia, growth retardation, genital and ear abnormalities,	<i>CHD7</i>	608892	AD	Decreased, low TRECs, ±SCID-like	Normal	May have hypogammaglobulinemia	
(d) Winged helix nude FOXN1 deficiency Bacterial and viral infections; congenital alopecia, nail dystrophy; abnormal thymic epithelium	<i>SEMA3E</i>	608166	AD				
(e) FOXN1 haploinsufficiency Mild viral infections; often nail dystrophy; symptoms improve with age	<i>FOXN1</i>	601705	AR	Decreased, low TRECs at NBS; no CD45RA	Normal	Hypogammaglobulinemia, Abnormal antibody responses	
4. CID associated with immuno-osseous dysplasia							
(a) Cartilage hair hypoplasia (CHH) Bacterial, viral fungal infections; growth failure, hair hypoplasia; neuronal dysplasia (colon), bone marrow failure; autoimmunity; malignancies	<i>FOXN1</i>	618806	AD	Low at birth, low TRECs at NBS	Normal	Normal	
(b) Schimke immuno-osseous dysplasia Bacterial, viral, fungal infections; short stature, spondyloepiphyseal dysplasia; nephropathy;	<i>RMRP</i>	157660	AR	Decreased or normal; decreased TRECs at NBS	Normal	Immunoglobulin levels normal or reduced, specific antibodies may be normal or decreased	
(c) Other osseous dysplasia syndromes	<i>SMARCAL1</i>	606622	AR	Low CD45RA, low mitogen responses	Normal	Reduced antibody responses	
• MYSM1 deficiency	<i>MYSM1</i>	612176	AR	Decreased, low CD45RA	Block in early B cell development	Hypogammaglobulinemia, reduced antibody response	
• MOPD1 deficiency	<i>RNU4ATAC</i>	601428	AR	decreased NK cell function	Low memory B cells	Hypogammaglobulinemia, reduced antibody response	
• EXTL3 deficiency	<i>EXTL3</i>	617425	AR	decreased	Normal	Low or normal Ig's	
5. Hyper IgE syndromes (HIES)							
(a) STAT3 (LOF) deficiency (Job syndrome) bacterial, fungal infections, mucocutaneous candidiasis, boils; pulmonary abscesses, pneumatoceles; neonatal onset eczema, coarse facial features, hyperextensible joints, scoliosis, pathologic fractures, retained primary teeth;	<i>STAT3</i>	147060	AD Dom. Neg.	Th17 lymphopenia	Reduced memory and switched B Cells	High IgE, reduced antibody responses	
(b) DOCK8 deficiency Bacterial respiratory infections, cutaneous viral infections; squamous cell carcinoma; eczema, allergies (food), asthma; CNS manifestations (aneurisms, vasculitis)	<i>DOCK8</i>	243700	AR	T lymphopenia Low (±) TRECs at NBS, low responses to mitogens	Reduced numbers of memory B cells	High IgE, reduced antibody responses	
(c) ZNF341 deficiency Clinical phenotype of AD STAT3 deficiency due to disrupted STAT3 transcription	<i>ZNF341</i>	618282	AR	Th17 lymphocytes Decreased	Reduced memory and switched B cells	High IgE, decreased antibody responses	
(d) IL-6 signaling defects Clinical phenotype of AD STAT3 deficiency		147880					
• IL-6 receptor deficiency	<i>IL6R</i>	618523	AR	normal	Low switched B	High IgE, low antibody	
• IL-6 signal transducer (GP130) deficiency	<i>IL6ST</i>	617638	AR	Th17 cells low	Low switched memory B cells	High IgE, decreased antibody responses	

(Continued)

Table 1 (Continued)

Disease and combined immunodeficiencies with syndromic features	Genetic defect	OMIM	Inheritance	Immunologic defects		
				T	B	Immunoglobulins specific antibodies
(e) PGM3 deficiency Bacterial, viral, fungal infections; allergies; fascial dysmorphism, skeletal abnormalities; neurologic manifestations	PGM3	172100	AR	Decreased (±)	Decreased total and memory B cells	IgE often elevated
(f) CARD11 deficiency Bacterial, viral infections of skin; early onset asthma, food allergies, eczema; some with autoimmunity and cytopenia; variable penetrance	CARD11	617638	AD (LOF)	Low mitogen and anti-CD3 responses,	Normal	High IgE, reduced antibody responses
(g) Comel-Netherton syndrome Bacterial infections; congenital ichthyosis, bamboo hair; atopic diathesis	SPINK5	605010	AR	Normal	Low switched memory B cells	High IgE, decreased antibody responses
6. Defects of vitamin B12 and folate metabolism						
(a) Transcobalamin 2 deficiency Bacterial, viral infections, PJP; megaloblastic anemia; pancytopenia, hyper segmented neutrophils	TCN2	613441	AR	Decreased	Decreased	Hypogammaglobulinemia, reduced antibody response
(b) PCFT deficiency causing folate malabsorption Bacterial, viral infections, PJP; megaloblastic anemia	SLC46A1	229050	AR	Decreased	Decreased	Hypogammaglobulinemia
(c) MTHFD1 (a folate-dehydrogenase) deficiency Bacterial, viral infections, PJP; megaloblastic anemia, neutropenia	MTHFD1	172460	AR	Decreased	Decreased	Decreased antibody responses
7. Anhidrotic Ectodermal Dysplasia with Immunodeficiency (EDA-ID)						
(a) IKBKG (NEMO) deficiency Variable phenotype, including bacterial, atypical mycobacterial, viral, fungal infections; colitis; variable defects of skin, hair, teeth (conical)	IKBKG	300248	XL	Normal or decreased, impaired function	Reduced memory and switched B cells	Often decreased, some with elevated IgM, abnormal antibody responses
(b) EDA-ID due to heterozygous GOF mutation in IKBA Bacterial, viral, fungal infections; colitis; variable defects of skin, hair, teeth (conical)	NFKBIA	164008	AD GOF	Normal, impaired function	Reduced memory and switched B cells	Decreased with elevated IgM, abnormal antibody responses
(c) EDA-ID due to GOF mutation in IKBKB Bacterial, viral, fungal infections; variable ectodermal defects; can be SCID-like	IKBKB	618204	AD GOF	Decreased number and function	Normal numbers Reduced function	Hypogammaglobulinemia
8. Calcium channel (CRAC) defects						
(a) ORAI1 deficiency Bacterial, viral, fungal infections; EDA; autoimmunity; congenital myopathy	ORAI1	160277	AR	Impaired T cell activation, NK function reduced	Normal	Abnormal antibody responses
(b) STIM1 deficiency Phenotype similar as ORAI1 deficiency	STIM1	605921	AR	Impaired T/NK cell function	Normal	Abnormal antibody responses
9. Purine nucleoside phosphorylase (PNP) deficiency Bacterial, opportunistic infections; autoimmunity; neurologic defects	PNP	164050	AR	Decreased, reduced function	Normal or low	Hypogammaglobulinemia
10. Hepatic veno-occlusive disease with immunodeficiency (VODI) Opportunistic infections; thrombocytopenia; hepatosplenomegaly	SP110	604457	AR	Normal, decreased memory T cells	Decreased memory B cells	Hypogammaglobulinemia
11. Other CID disorders with syndromic features						
(a) Immunodeficiency with multiple intestinal atresia, Bacterial, fungal, viral infections; intrauterine polyhydramnios; may be SCID-like	TTC7A	609332	AR	Low or absent, low TRECs at NBS	Normal or low	Hypogammaglobulinemia

(b) Tricho-Hepato-Enteric Syndrome (THES) Respiratory infections; facial dysmorphism, growth retardation; wooly hair; diarrhea; liver disease	<i>TTC37</i>	222470	AR	Impaired IFN γ production	Decreased switched memory B cells	Hypogammaglobulinemia abnormal antibody responses
	<i>SCIV2L</i>	614602	AR			
(c) Neurologic deficiencies associated with CID Recurrent infections; developmental delay, absent corpus callosum; cutaneous manifestations				low TRECs at NBS, low CD4 SCID-like	Normal Abnormal function	Normal Hypogammaglobulinemia
• BCL11B deficiency	<i>BCL11B</i>	617237	AD			
• EPG5 deficiency (Vici syndrome)	<i>EPG5</i>	615068	AR			
(d) Defects of the Linear Ubiquitination chain Assembly Complex (LUBAC) Bacterial infections; autoinflammation; amylopectinosis						
• HOIL1 deficiency	<i>RBCK1</i>	610924	AR	Normal	low memory B	Hypogammaglobulinemia
• HOIP deficiency	<i>RNF31</i>	612487	AR	Normal	cells	abnormal antibody responses
(e) STAT5B deficiency Bacterial, viral infections, interstitial pneumonitis; short stature due to growth hormone insensitivity; dysmorphic features	<i>STAT5B</i>	245590	AR	Decreased, reduced Treg function	normal	Hypogammaglobulinemia elevated IgE
(f) Kabuki Syndrome Respiratory infections, typical facial features (long palpebral fissures); skeletal abnormalities, high arched palate, short stature; congenital heart defects,	<i>STAT5B</i>	604260	AD Dom. Neg.	Normal	normal	Elevated IgE
• Type 1	<i>KMT2D</i>	602113	AD	Normal	Normal	Low IgA and occasionally low IgG
• Type 2	<i>KDM6A</i>	300128	XL	Normal	Normal	

define these unique syndromes. To address the extent of the adaptive immune deficiency, the number and function of B and T cells have to be evaluated including quantitation of serum immunoglobulins (IgG, IgA, IgM, IgE), lymphocyte subset analysis, and assessment of in vitro lymphocyte proliferation to mitogens, anti-CD3 and specific antigens such as candida and tetanus toxoid (Table 1). Dependent on the extent of the immune deficiency, appropriate therapies can be initiated, including prophylactic antibiotics or antivirals, IgG replacement, biologics, hematopoietic stem cell transplantation (HSCT) or gene therapy (GT).

Wiskott-Aldrich syndrome and other congenital Thrombocytopenias

As originally described by Wiskott (Wiskott, 1937), classic Wiskott-Aldrich Syndrome (WAS) is characterized by the triad of recurrent bacterial and viral infections, eczema and hemorrhagic diathesis secondary to congenital micro-thrombocytopenia. The syndrome was subsequently expanded to include increased risk for autoimmunity and hematologic malignancies (Albert et al., 2011). The discovery of the WAS gene (Derry et al., 1994) allowed the identification of variable clinical phenotypes in addition to “classic” WAS, namely X-linked thrombocytopenia (XLT), a mild form of WAS with close to normal life expectancy but dismal event-free survival (Albert et al., 2010a), and X-linked-neutropenia (XLN) due to mutation in the Cdc42 binding domain of the WAS gene (Beel et al., 2009). WAS, XLT and XLN are X-linked disorders caused by mutations in the WAS gene that encodes the WAS-protein (WASP) which is expressed only in hematopoietic stem cell derived lineages.

WASP is stabilized by WASP-interacting protein (WIP). Mutations in the *WIPF1* gene result in unstable (absent) WASP and a clinical phenocopy of classic WAS while the sequence of the WAS gene and mRNA expression are normal (Lanzi et al., 2012; Schwinger et al., 2018). Activated cytoplasmic WASP links signaling pathways to actin cytoskeleton reorganization by inducing actin polymerization. This process is mediated by actin-related protein (ARP) 2/3, coded for by *ARPC1B*. Biallelic mutations in the *ARPC1B* gene result in a WAS-like clinical phenotype characterized by congenital micro-thrombocytopenia, eczema, vasculitis, inflammatory bowel disease (IBD) and recurrent infections. Most patients with *ARPC1B* deficiency develop eosinophilia, elevated serum IgA and IgE and autoantibodies similar to WAS patients. (Kahr et al., 2017). Other single gene defects characterized by actinopathy and congenital thrombocytopenia include AR mutations in the *WDR1* (Pfajfer et al., 2018) and *RASGRP1* genes (Salzer et al., 2016), and several other genes recently reviewed (Etzioni and Ochs, 2020). Each of these actinopathy/thrombocytopenia entities has to be molecularly confirmed by sequencing the causative genes.

Treatments for patients with CID associated with thrombocytopenia and actinopathy include conventional measures such as prophylactic antibiotics and antiviral agents, IgG replacement for patients with demonstrated antibody deficiency, and trauma prevention measures. Platelet transfusions are reserved for major bleeding episodes (acute CNS hemorrhage, gastrointestinal bleeding or during major surgery). Thrombopoietin receptor agonists have been used with some success in WAS/XLT patients while waiting for HSCT (Khoreva et al., 2021; Gerrits et al., 2015). Elective splenectomy for WAS/XLT patient will result in increased platelet number, but is not recommended because of an increased risk to develop septicemia (Lum et al., 1980). HSCT is a readily available curative treatment for WAS/XLT patients with excellent overall survival (Burroughs et al., 2020). Gene therapy is an alternative, potentially curative, therapy under investigation (Ferrua et al., 2019).

DNA repair defects

Ataxia-telangiectasia

Ataxia-telangiectasia (A-T) is an autosomal recessive multisystem disorder characterized by progressive neurological impairment, oculocutaneous telangiectasia, variable CID resulting in recurrent sinopulmonary infections, cancer susceptibility and sensitivity to ionizing irradiation, making A-T a prominent example of DNA repair defects (Rothblum-Oviatt et al., 2016). The worldwide incidence of A-T is estimated to be 1:40,000 to 100,000 live births, more frequent in countries with founder effect mutations, such as Poland and Costa Rica. A-T is caused by biallelic mutations in the *ATM* gene which encodes a protein (ATM) that is involved in the cellular responses to DNA double strand breaks (DSB), genomic stability, checkpoint control and cell cycle regulation. Cerebellar ataxia is generally the presenting symptom and becomes apparent at age 1–2 years when an affected child begins to walk and the gait remains ataxic with truncal movements. The ataxia is progressive affecting the extremities, speech, swallowing, and eventually requiring the use of a wheelchair. Telangiectasias develop typically during childhood between 3 and 6 years of age. Predisposition to sinopulmonary infections occurs in up to 80% of A-T patients. Recurrent episodes of pneumonia may progress to bronchiectasis, pulmonary fibrosis, eventually leading to cor pulmonale and premature death. The recurrent infections are the result of combined immunodeficiency which is characterized by hypogammaglobulinemia, except for elevated IgM levels due to defective class switch recombination (CSR). In vitro lymphocyte proliferation to mitogens and antigens is generally impaired. The characteristic chromosomal instability and frequent translocations involving chromosomes 12 and 14 have been associated with the high incidence of leukemia and lymphoma (Hecht et al., 1973).

The diagnosis of A-T is based on the characteristic clinical presentation and laboratory findings that include elevated serum α -fetoprotein levels beyond early infancy, decreased IgA, IgG and often elevated IgM, abnormal antibody production, lymphopenia, radiosensitivity, chromosomal instability and chromosomal translocation. Lymphopenia may be present at birth, resulting in low T cell receptor excision circles (TREC) at newborn screening (NBS). The diagnosis is confirmed by mutation analysis of the *ATM* gene, which allows the differentiations of A-T from the A-T like disorder (ATLD) caused by mutations in the *MRE11* gene, which encodes a

double-stranded repair nuclease, and from other DNA repair defects (none of which has elevated α -fetoprotein) (Yel et al., 2014). Therapy is symptomatic including antibiotic prophylaxis and IgG replacement. Irradiation, either naturally or by diagnostic X-ray, should be avoided.

Nijmegen breakage syndrome

The clinical hallmarks of Nijmegen Breakage Syndrome (NBS) patients are severe microcephalic growth retardation, typical facial appearance, CID, radiosensitivity and increased risk for malignancies. NBS is an AR multisystem disorder with similarities to A-T (Chrzanowska et al., 2012). The microcephaly, due to the stunted growth of the brain, is present in most cases at birth. Lack of sexual maturation is a frequently seen consequence, especially in girls. Susceptibility to infections varies greatly but respiratory infections are reported by most patients and may result in bronchiectasis or chronic lung disease (Wegner et al., 2014). The immune deficiency, if present, affects both humoral and cellular immunity. Some NBS patients have normal serum immunoglobulin levels, others may present with hypogammaglobulinemia, elevated IgM due to defective CSR, and decreased antibody production. In vitro responses of lymphocytes to mitogens and/or specific antigens are variably defective. In contrast to A-T, α -fetoprotein is normal in NBS patients. Most but not all NBS patients have features of chromosomal instability, such as chromosomal rearrangements involving chromosomes 7 and 14, possibly linked to the predisposition to malignancies, which are mostly of lymphoid origin (B and T cell lymphoma). NBS is caused by biallelic mutations in the *NBS1* gene, making a molecular diagnosis possible. The gene product, nibrin, forms the complex MRE11-RAD50-Nibrin which detects DSB of DNA and activates ATM. Treatment is symptomatic and includes IgG replacement if antibody deficiency is present. HSCT has been successfully performed in selected cases (Albert et al., 2010b).

Bloom syndrome

The most consistent physical features of Bloom Syndrome is the small but well-proportioned stature, dysmorphic facial features, a sun-sensitive erythematous “butterfly” rash, infertility, susceptibility to bacterial infections and early development of lymphoid malignancies and cancer (colon, skin, breast) (Cunniff et al., 2017). The immunodeficiency in Bloom Syndrome patients is mild, consisting of hypogammaglobulinemia, but generally normal antibody responses to immunizations and normal lymphocyte proliferation to mitogens and specific antigens. The chromosomes of phytohemagglutinin (PHA) stimulated lymphocytes of Bloom Syndrome patients develop large numbers of characteristic and diagnostic sister chromatid exchanges (SCE). Bloom Syndrome is caused by biallelic mutations in the *BLM* gene which encodes BLM, a DNA helicase involved in DNA replication and repair. Mutations in the *BLM* gene are rare, except in the Ashkenazi Jewish populations where as many as 1:110 individuals is a carrier. Treatment is symptomatic, antibiotics for acute infections, IgG replacement if antibody deficient, and sun protection. Avoiding irradiation, and close observation for malignancies is recommended (Wegner et al., 2014).

Immunodeficiency, centromeric instability, facial anomalies (ICF) syndrome

This rare AR syndrome is often associated with consanguinity, and characterized by mild facial anomalies that include hypertelorism, epicanthic folds, broad flat nasal bridge, low set ears, macroglossia, immunodeficiency and chromosomal instability (Hagleitner et al., 2008). Infections are more frequent and severe than in other DNA repair defects. Recurrent episodes of pneumonia are frequently caused by gram positive and gram negative bacteria, but may include opportunistic infections such as *Pneumocystis jirovecii* (PJ), CMV, JC virus and candida. Hypogammaglobulinemia, abnormal antibody responses and T cell function have been reported. Centromeric instability involving chromosomes 1, 9 and 16 are characteristic findings in cultured lymphocytes and include gaps, breaks, deletions and multiradial figures. The common pathogenic mechanism of ICF appears to be hypomethylation caused by defective DNA methyl transferase activity. To date, 4 genes causing ICF have been identified, *DNMT3B* (ICF1 present in 60% of ICF patients), *ZBTB24* (ICF2, 25%), *CDCA7* (ICF3, 5%) and *HELLS* (ICF4, 5%) (Thompson et al., 2018; Unoki et al., 2019).

Other CID syndromes with DNA repair defects

Genetic disorders included in this group of rare syndromes characterized by genomic instability have in common defects in the DNA repair machinery that utilizes cell cycle checkpoints to stop cell cycle progression in the presence of DNA damage. Since DNA repair mechanisms are also responsible for DNA rearrangements required for the generation of highly diverse T and B cell antigen receptors, defects in this process result in abnormal adaptive immunity associated with characteristic syndromic features. Increased radiosensitivity and susceptibility to cancer, recurrent bacterial and viral infections, facial dysmorphism and short stature are common features (van Gent et al., 2001).

Disorders assigned to this category have AR inheritance and include:

- PMS2 is involved in the mismatch repair machinery and plays a role in CSR-induced DSB generation. As a result of biallelic mutations in the *PMS2* gene, affected patients present with hypogammaglobulinemia except for elevated IgM, and are at high risk for malignancies (Peron et al., 2008).

- Biallelic mutations in the *RNF168* gene causes a syndrome characterized by Radiosensitivity, Immunodeficiency, Dysmorphic features, Learning Difficulties (RIDDLE). *RNF168* participates in the repair mechanism of DNA DSB (Stewart et al., 2009). Affected patients present with short stature, mild ataxia, and occasionally develop microcephaly, telangiectasia and increased serum levels of α -fetoprotein. The immune deficiency is mild and limited to hypogammaglobulinemia, rarely resulting in chronic lung disease. A-T has to be considered in the differential diagnosis (Devgan et al., 2011).
- MCM4 deficiency is associated with short stature, adrenal insufficiency, recurrent infections including viral infections (EBV, herpes simplex and varicella—zoster virus), and an increased incidence of B cell lymphoma. MCM4 functions as a DNA helicase that is required for DNA replication and cell proliferation. The prominent immune defect involves NK cells that are low in number and function (Hughes et al., 2012).
- POLE1 and POLE2 deficiencies, caused by biallelic mutations in polymerase ϵ subunit 1 and 2, are characterized by facial dysmorphism, short stature due to intra-uterine growth retardation, telangiectasia, recurrent bacterial and viral infections, hypogammaglobulinemia, lymphopenia, low B cell numbers, and decreased in vitro lymphocyte proliferation (Logan et al., 2018). Mutations in POLE1 cause FILS syndrome (Fascial dysmorphism, Immunodeficiency, Livedo, Short stature) often presenting at birth with discolored skin of cheeks and upper and lower extremities known as livedo (Pachlopnik Schmid et al., 2012). POLE2 deficiency causes more extensive T cell defects that may result in absent TRECs at NBS, disseminated BCG, and autoimmune diseases (type 1 diabetes and hypothyroidism).
- DNA Ligase 1 deficiency, caused by biallelic mutations within *LIG1*, presents with growth retardation/short stature and sun sensitivity. This rare syndrome is the consequence of defective repair of DNA DSB via homologous recombination, resulting in lymphopenia, decreased T cell proliferation, hypogammaglobulinemia and macrocytic red blood cells. The severity of the symptoms can vary considerably. Patients with severe T cell deficiency may require HSCT (Maffucci et al., 2018).
- ERCC6L2 (Hebo) deficiency is associated with facial dysmorphism, microcephaly and may progress to bone marrow failure. Lymphopenia affecting both B and T cells is a common finding. ERCC6L2 is a helicase involved in chromatin unwinding, transcription regulation and DNA recombination, translocation and repair (Zhang et al., 2016).
- Patients with GINS1 deficiency present with intrauterine growth retardation, mild facial dysmorphism, recurrent bacterial and viral infections, lymphopenia (affecting both T and B cells), low NK cell number and neutropenia that responds to G-CSF treatment. Hypogammaglobulinemia except for normal or elevated IgA is a common feature (Cottineau et al., 2017).

CID associated with thymic defects

DiGeorge syndrome (22q11.2 del; *TBX1* deficiency)

The thymus and parathyroid glands originate from the 3rd and 4th pharyngeal arches which interact with the cardiac neural crest during the 4th week of embryonic development. Disruption of this process results in CID associated with AD thymic defects known as DiGeorge/velocardiofacial syndrome (chromosome 22q11.2 deletion syndrome, or heterozygous *TBX1* deficiency), characterized by thymic aplasia, hypoparathyroidism, conotruncal cardiac malformation and mild to moderate immunodeficiency, defined as predominant T cell defect with secondary abnormalities in B cell function (Sullivan, 2019). The spectrum of CID is broad, ranging from near normal T cell numbers and function to a partial DiGeorge anomaly with susceptibility to bacterial and viral infections to complete DiGeorge syndrome with SCID/Omenn like features requiring a thymic transplant (Deshpande et al., 2021). The *TBX1* gene, located within the 22q11.2 deletion, encodes a transcription factor, that, if mutated on a single allele, is responsible for a syndrome with many features of DiGeorge anomaly. If associated with severe lymphopenia, the DiGeorge syndrome may present with low numbers of TRECs at NBS. The decreased serum concentration of Ca^{++} and phosphorus may cause tetany. If severely lymphopenic and if T cell function is absent, life vaccines are contraindicated.

CHARGE syndrome, a multiorgan disorder with AD inheritance

CHARGE syndrome is caused by heterozygous mutations in the *CHD7* or *SEMA3E* genes, presenting with growth retardation, coloboma, heart defects, atresia of the choanae, genital hypoplasia and CNS malformation (van Ravenswaaij-Arts et al., 1993). Treatment is symptomatic; experimental thymic transplants have shown promising results (Legendre et al., 2017).

Winged helix nude *FOXP1* deficiency

Biallelic mutations in the transcription factor *FOXP1* result in severe respiratory viral and bacterial infections, congenital alopecia, nail dystrophy and erythroderma. *FOXP1* acts as master regulator of thymic epithelial development and its absence is responsible for athymia and CD4 lymphopenia, absence of CD45RA lymphocytes and low TRECs at NBS, resulting in combined immune deficiency. In addition, *FOXP1* plays a crucial role in the differentiation of keratinocytes and hair follicles. HSCT and thymic transplantation have resulted in immune reconstitutions in a subset of patients (Gallo et al., 2017). Heterozygous pathogenic mutations were found in a cohort of infants with very low TRECs at NBS associated with severe T cell lymphopenia which persisted through infancy. Nail dystrophy and mild viral infection were noted in half of these infants. With age, the lymphopenia and symptoms resolve, except for nail dystrophy that persists in some adults with *FOXP1* haploinsufficiency (Bosticardo et al., 2019; Auricchio et al., 2005).

CID associated with immuno-osseous dysplasia

This group of syndromic CID patients presents with spondyloepiphyseal dysplasia associated with intra/extrauterine growth retardation, dysmorphic facial features and variable immune defects resulting in recurrent bacterial and viral infections.

Cartilage-hair hypoplasia

Cartilage-hair hypoplasia due to biallelic mutations in the *RMRP* gene is characterized by short-limbed growth failure due to metaphyseal dysplasia, hair hypoplasia (thin and sparse hair growth), neuronal dysplasia of the colon resembling Hirschsprung anomaly, impaired spermatogenesis and increased risk of malignancies. The *RMRP* gene encodes the untranslated RNA component of the mitochondrial RNA-processing endoribonuclease which contributes to cell cycle regulation and telomere maintenance. The extend of immune deficiency varies and may present as congenital lymphopenia (low TRECs at NBS) and impaired lymphocyte proliferation to mitogens, hypogammaglobulinemia and antibody deficiency. Approximately half of reported cases present with increased susceptibility to bacterial infections, such as URI, pneumonia, sepsis, and meningitis, but may also suffer from viral (CMV, EBV, parvovirus, varicella-zoster virus, HSV) and fungal (aspergillus) infections. Chronic lung disease, including bronchiectasis, fibrosis, emphysema and asthma are common complications (Vakkilainen et al., 2020). Treatment depends on the extend of immune deficiency and frequency of infections. Anti-microbial therapy, IgG replacement, attention to early onset GI—disease and observation for malignancies are part of symptomatic treatment. HSCT has been reported to successfully cure the immune defect but with only 62% long term survival (Bordon et al., 2010).

Schimke immuno-osseous dysplasia

Schimke immuno-osseous dysplasia, due to biallelic mutations in *SMARCA1*, is a rare multisystem disorder characterized by growth retardation, spondyloepiphyseal dysplasia, nephropathy often progressing to renal failure, and frequent bacterial, viral and fungal infections (Bertulli et al., 2020). *SMARCA1* encodes a chromatin remodeling helicase that is considered a genome maintenance protein. Most patients have low numbers of recent thymic emigrants, decreased number and function of T cells, absence of IL-7 R α expression and reduced humoral immune responses. Treatment is symptomatic. Renal transplantation has been successfully performed but the outcome of HSCT has been dismal (Baradaran-Heravi et al., 2013).

Other immuno-osseous dysplasia syndromes that have in common short stature, skeletal anomalies and recurrent infections

- MYSM1 deficiency is a rare syndrome, observed in consanguineous Middle East families, presenting with progressive bone marrow failure, myelodysplasia, gingival hyperplasia, cataracts, and developmental aberrations. Recurrent respiratory tract infections, associated with lymphopenia, reduced number of naive T cells and hypogammaglobulinemia, require antibiotic prophylaxis unless curative HSCT can be performed. MYSM1 is a transcriptional regulator mediating histone deubiquitination (Bahrami et al., 2017).
- Roifman syndrome, also known as MOPD1 deficiency, is caused by biallelic mutations in the *RNU4ATAC* gene, and is characterized by extreme intra-uterine growth retardation, retinal dystrophy, facial dysmorphism and microcephaly. Decreased NK cell function, low memory B cell numbers, hypogammaglobulinemia and antibody deficiency are responsible for the frequent bacterial infections that are typical for this syndrome (Merico et al., 2015).
- Immuno-skeletal dysplasia with neurodevelopmental abnormalities is caused by biallelic mutations in the *EXTL3* gene and typically presents with hypogammaglobulinemia, recurrent bacterial infections, lymphadenopathy, retinal dystrophy and intrauterine growth retardation (Volpi et al., 2017).

Hyper IgE syndromes

Hyper IgE syndromes (HIES) are a group of single gene defects characterized by eczema, recurrent infections, combined immuno-deficiency and elevated serum IgE (Al-Shaikhly and Ochs, 2019).

STAT3 LOF mutations with dominant negative effect, also known as Job syndrome

Neonatal onset eczema is present in 57% of affected patients and recurrent cutaneous staphylococcal infections in the form of “cold” abscess formation develop in 74%. Recurrent episodes of pneumonia caused predominately by staph aureus (72%) and *Aspergillus fumigatus* (27%) often result in pneumatoceles. Deep organ abscesses and mucocutaneous candidiasis are the direct result of defective innate and adaptive immunity. Non-immunologic manifestations include characteristic facial features, retained primary teeth (41%), hyper-extensible joints (8.5%), scoliosis (34%), recurrent pathological fractures (39%) and vascular anomalies (aneurisms) (Gernez et al., 2018). Based on these clinical findings, combined with elevated IgE and eosinophilia, the NIH-HIES scoring system was designed to accurately diagnose the syndrome (Grimbacher et al., 1999). In most cases, the IgE

levels start to rise shortly after birth, reaching 2000–100,000 IU/mL, often decreasing, or even normalizing during adulthood. IgE concentration does not correlate with disease severity. IgG, A, M are normal, but antibody responses are defective and B cell maturation is impaired as exemplified by decreased CD27⁺ switched memory B cells (Meyer-Bahlburg et al., 2012). Heterozygous loss of function (LOF) mutations in the DNA binding domain, SH2 domain and transactivation domain, resulting in a non-functional STAT3 protein that is no longer capable of forming dimers or penetrating the nuclear membrane and act as a transcription factor, thus generating a dominant negative effect with only 25% of normal STAT3 function (Renner et al., 2008). Treatments include prophylactic anti-staphylococcal and anti-fungal agents, meticulous skin care and IgG replacement if antibody deficiency is present. The aim is to prevent pneumatocele formation and the permanent loss of lung function. HSCT, performed on an experimental basis, has given conflicting results (Goussetis et al., 2010).

DOCK8 deficiency

DOCK8 Deficiency typically results in atopic diathesis. Affected patients present with eczema, allergies (85% have food allergies) and often suffer from asthma. Due to pronounced combined immune deficiency, DOCK8 deficient patients develop recurrent bacterial and fungal sinopulmonary infections, and are highly susceptible to cutaneous viral infections, most frequently herpes simplex virus (HSV), human papilloma virus (HPV) and molluscum contagiosum. Persistent HPV infection may be responsible for the high prevalence of vulvar and head/neck squamous cell carcinoma. Neurologic manifestations include CNS-infections, vasculitis, aneurysms, and brain infarcts. (Aydin et al., 2015). Besides elevated IgE and eosinophilia, the finding of T cell lymphopenia, reduced naïve CD8⁺ and increased exhausted CD8⁺ T cells, low numbers of NK and regulatory T (Treg) cells with decreased function, depressed mitogen responses, reduced memory B cells and decreased antibody responses indicates a SCID phenotype. DOCK8 deficiency is caused by biallelic deletions spanning multiple exons of the *DOCK8* gene or less frequently by point mutations, leading to premature stop codons and resulting in lack of protein expression, allowing rapid diagnosis by flow analysis. DOCK8 acts upstream of WASP and regulates cytoskeletal rearrangement required for cell-migration and synapse formation (Randall et al., 2009). Since DOCK8 is expressed uniquely in hematopoietic stem cell derived cellular elements, early HSCT is highly successful and curative (Aydin et al., 2015).

Phosphoglucomutase 3 (PGM3) deficiency

PGM3-deficient patients share characteristic features of STAT3 and DOCK8 deficiency including facial dysmorphism and skeletal abnormalities, allergies, and recurrent bacterial, viral, and fungal cutaneous infections. As has been observed in other glycosylation disorders, neurologic manifestations (developmental delay, ataxia) distinguish PGM3-deficient patients from other HIES (Zhang et al., 2014). Lymphopenia affecting both T and B cells (but with normal or elevated immunoglobulin levels), reduced mitogen responses and neutropenia are responsible for recurrent infections. Since PGM3 is involved in protein glycosylation, loss of function mutations causes a broad spectrum of clinical manifestations. Treatment is symptomatic. Oral *N*-acetyl glucosamine supplementation has been implemented in a few cases, with some positive results. HSCT was performed successfully in two patients (Stray-Pedersen et al., 2014).

CARD11 deficiency

Genetic evaluation of a group of patients with severe atopy (early onset asthma, eczema, food allergies) and recurrent bacterial and viral infections revealed heterozygous missense mutations with dominant negative effect in caspase recruitment domain family member (CARD)11, a scaffold protein that plays a critical role in T and B cell signaling via NF- κ B and mTor activation. Affected patients have normal immunoglobulin levels, except for elevated IgE. In vitro lymphocyte proliferation to mitogens and anti-CD3 is decreased and antibody responses are depressed, indicating combined immune deficiency (Dadi et al., 2018). A recent analysis of 48 patients from 27 unrelated families expanded the clinical phenotype to include IPEX-like symptoms of autoimmunity and cytopenias, and immunologic defects resembling those seen in STAT3 loss of function, DOCK8 deficiency and CVID (Dorjbal et al., 2019).

Disrupted STAT3 transcription by biallelic mutations in the *ZNF341* gene

ZNF341 is a transcription factor that binds to a specific DNA motif within the STAT3 promotor, controlling transcription, translation, and production of the STAT3 protein. As a result of ZNF341 deficiency, only a small amount of STAT3 is generated in response to STAT3-activating cytokines, resulting in a clinical phenocopy resembling AD-HIES. All affected patients reported to-date have been the offspring of consanguineous marriages and presented with recurrent infections, reduced memory B cells, decreased antibody production, high serum IgE and IgG levels, and decreased numbers of Th17 cells (Frey-Jakobs et al., 2018; Beziat et al., 2018). Treatment is symptomatic and includes antimicrobial compounds, as recommended for AD-HIES patients.

Defects in IL-6 signaling mimicking AD-HIES

IL-6, a proinflammatory cytokine, interacts with a multi-subunit receptor complex by directly binding to its receptor (IL-6R) resulting in the ligation of the signal-transducer subunit (IL6ST) also known as glycoprotein 130 (GP130) transducer chain, leading to JAK and STAT-phosphorylation, the dimerization of pSTAT3 and its nuclear import resulting in transcription of genes involved in cell growth and differentiation.

Loss of the IL-6R due to homozygous mutations was reported in two unrelated patients, presenting with atopic dermatitis, skin abscesses (described as lacking the formation of erythema), bacterial infections, hypogammaglobulinemia, very high IgE levels, eosinophilia and markedly elevated serum IL-6 (Spencer et al., 2019).

Loss of function mutations in the *IL6ST* gene, encoding GP 130, result in a clinical phenotype resembling AD STAT 3 deficiency. Two unrelated patients were identified to have homozygous missense mutations each involving a different codon (Shahin et al., 2019); both were offspring of consanguineous marriages. A cohort of 12 patients from 8 unrelated kindreds had an AD-HIES phenotype due to seven different truncating heterozygous *IL6ST* mutations with dominant negative effect (Beziat et al., 2020). STAT3 phosphorylation in response to stimulation with GP130-dependent cytokines was impaired in patient lymphocytes and monocytes.

HIES imitators

In addition to the “classic” HIES with a characteristic clinical phenotype and interconnected molecular mechanisms, there is a group of patients with distinct clinical symptoms and laboratory findings that resemble in part the classic HIES phenotype (Al-Shaikhly and Ochs, 2019).

- Patients with atypical complete DiGeorge syndrome due to 22q11.2 deletion may develop an Omenn-like phenotype with recurrent respiratory infections, erythematous rash, elevated IgE and eosinophilia, restricted T cell repertoire and lymphadenopathy, in addition to hypocalcemia and congenital heart disease (Vu et al., 2013).
- Omenn syndrome is a distinct autoinflammatory disorder associated with genetically diverse leaky SCID-causing gene mutations. Affected infants develop viral and fungal infections, chronic diarrhea, severe erythroderma, enlarged lymphoid organs, increased IgE levels and eosinophilia. The process is triggered by oligoclonal T cells with autoreactive activity. Treatment is symptomatic, HSCT is curative (Villa et al., 2008).
- WAS is an X-linked disorder with variable clinical phenotypes that correlate with type and location of mutations in the *WAS* gene (Ochs and Thrasher, 2006) (see Section “Wiskott-Aldrich syndrome and other congenital Thrombocytopenias” of this review). Patients with “classic WAS” present with recurrent bacterial, viral, and fungal infections, microthrombocytopenia and bleeding tendency, eczema, autoimmune symptoms, and increased risk of malignancies. The CID of WAS is characterized by abnormal function of B, T, NK and T regs cells; serum IgA and IgE and the number of eosinophils are elevated.
- Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX), is a rare PID caused by hemizygous mutations in the *FOXP3* gene which encodes the transcription factor FOXP3 responsible for the generation of regulatory T (Treg) cells. IPEX is a member of the increasing family of Primary Immune Regulatory Disorders (PIRD), but, unlike most HIES, susceptibility to infection is not a prominent complication. Affected males present early in life with autoimmune endocrinopathies (type 1 diabetes, hypothyroidism) and cytopenias, enteropathy and eczema. Eosinophilia and elevated IgA and IgE are common findings. Most IPEX patients have reduced or lack completely CD4+CD25+FOXP3+Treg cells (Torgerson and Ochs, 2007). While Rapamycin and other immunosuppressive agents effectively turn down the overactive immune system, the only curative treatment is HSCT (Barzaghi et al., 2018).
- Comel-Netherton syndrome, an AR disorder caused by mutations in *SPINK5* encoding LEKTI, is characterized by increased bacterial infections, congenital ichthyosis, bamboo hair, atopic diathesis, elevated IgE and eosinophilia. Based on the finding of reduced switched memory B cells and abnormal antibody responses, IVIG therapy has been initiated in a cohort of Comel-Netherton patients with marked improvement of symptoms (Renner et al., 2009).
- Heterozygous loss of function mutations with dominant negative effect in *CARD14* were associated with severe atopic dermatitis, skin infections, asthma, and food allergies in four members of three non-related families, resulting in decreased NFκB signaling. IgE levels were persistently very high (Peled et al., 2019).
- Severe atopic dermatitis is the most common cause of elevated IgE and needs to be differentiated from HIES caused by single gene defects. The immune system is generally intact in non-specific atopic disease and the bacterial and viral infections are the consequence of a defective skin barrier and rarely result in deep abscesses, organ infections or immune dysregulation (Wollenberg et al., 2003).

Defects of vitamin B12 and folate metabolism

Vitamin B12 and folate (vitamin B9) play critical roles in cellular metabolism. Deficiencies of these vitamins affect rapidly proliferating cell populations such as hematopoietic stem cell derived lineages that provide adaptive immunity and generate

phagocytes. Three single gene defects affecting cobalamin or folate uptake and transport into cells have been identified in patients with clinical symptoms of immune deficiency. (Watkins and Rosenblatt, 2020).

Transcobalamin 2 deficiency

Transcobalamin 2 deficiency, caused by biallelic mutations in *TCN2*, interrupts the delivery of B12 to bone marrow cells, causing megaloblastic anemia, pancytopenia including low numbers/decreased function of T and B cells, and neutropenia with hyper-segmented granulocytes. Recurrent infections, such as candidiasis and Pneumocystis Jirovecii Pneumonia (PJP), improve when affected patients are treated parenterally with high dose Vitamin B12 (Unal et al., 2019).

Biallelic mutations in SLC46A1

Biallelic mutations in *SLC46A1* encoding the proton-coupled folate transporter (PCFT) result in hereditary folate malabsorption. In addition to megaloblastic anemia, patients develop variable B and T cell deficiencies and recurrent infections including oral candidiasis, PJP, hypogammaglobulinemia (Borzutzky et al., 2009).

Methylene-tetrahydrofolate dehydrogenase 1 (MTHFD1) deficiency

Methylene-tetrahydrofolate dehydrogenase 1 (MTHFD1) deficiency is associated with low thymic output, decreased B cell numbers and abnormal antibody responses, neutropenia, megaloblastic anemia and PJP. Treatment with oral folic acid corrects the hematologic and immunologic abnormalities (Ramakrishnan et al., 2016).

Anhidrotic ectodermal dysplasia

Anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) is caused by molecular defects in the NF-KB pathway. Typical findings are sparse hair, conical teeth and absent sweat glands associated with various degrees of combined immunodeficiency. In unstimulated normal cells the NF-KB complex is sequestered in the cytoplasm by the inhibitory protein, IKBA (I κ B α) which is encoded by *NFKBIA*. Activation of the upstream I κ B kinase (the complex IKK α /IKK β /IKK γ) induces the proteolytic degradation of IKBA and enables the NF-KB complex (p50, p65/c-Rel) to translocate into the nucleus where it activates the transcription of cytokine associated genes (Heller et al., 2020).

Mutations in the *IKBKG* gene

Mutations in the *IKBKG* gene also known as NF κ B essential modulator (NEMO) lead to the X-linked form of EDA-ID. The clinical phenotype and extend of the immune deficiency vary substantially and depend on the severity of the NEMO mutation (Heller et al., 2020). Most patients with NEMO mutations suffer from bacterial infections, and some, in addition, from infections with atypical mycobacteria, viruses, and parasites. T and NK cell function can be normal or depressed, T cell activation is often impaired, and the number of memory and isotype switched B cells may be decreased. Hypogammaglobulinemia and decreased antibody responses are observed in half the NEMO deficient patients. Carrier females with non-functional heterozygous mutations may develop incontinentia pigmenti.

Heterozygous gain of function (GOF) mutations in NFKBIA

Heterozygous gain of function (GOF) mutations in *NFKBIA* impairs the degradation of its product, IKBA and results in AD EDA-ID. Most patients have a severe clinical phenotype and guarded prognosis. (Schimke et al., 2013).

Heterozygous GOF mutation in the *IKBKB* gene

Heterozygous GOF mutation in the *IKBKB* gene that encodes IKK β was found in two unrelated families with ectodermal dysplasia, decreased T cell number and function, impaired T cell receptor (TCR) activation, hypogammaglobulinemia and recurrent bacterial and candida infections (Cardinez et al., 2018). A different clinical phenotype, resembling SCID, was observed in members of four families of Northern Cree ancestry with a history of early onset of severe bacterial, viral and fungal infections. Affected members carried a homozygous duplication in the *IKBKB* gene resulting in frameshift and premature termination, and loss of expression of IKK β (Pannicke et al., 2013). A similar SCID phenotype was described in a female infant of consanguineous parents of Turkish descent with a homozygous nonsense mutation in *IKBKB* and lack of IKK β expression (Nielsen et al., 2014).

Treatment of patients with anhidrotic ectodermal dysplasia requires a multidisciplinary approach to address the multiple and variably severe symptoms of recurrent infections, ectodermal defects, inflammation, and immune dysregulation. HSCT can be curative, but with less than optimal outcome (Pannicke et al., 2013).

Calcium channel defects

Calcium signals play a key role in the activation and effector function of lymphocytes. Calcium channel defects are autosomal recessive disorders of CA^{2+} influx from the extracellular space into lymphocytes through the calcium release-activated calcium (CRAC) channels. Mutations in genes coding for proteins that form (*ORAI1*) or control (*STIM1*) CRAC channels result in CID associated with ectodermal dysplasia, autoimmunity and congenital myopathy (Feske et al., 2006; Fuchs et al., 2012).

Mutations in the *ORAI1* or *STIM1* genes typically cause severe viral, bacterial and fungal infections due to defective TCR mediated activation resulting in impaired NFAT signaling. The numbers of T, B and NK cells are normal but in vitro lymphocyte proliferation is reduced or absent, and NK cell cytotoxicity is severely impaired. While symptomatic treatment with antimicrobials and IVIG is partially effective, HSCT to correct the immune deficiency should be considered, although HSCT does not correct myopathy nor the ectodermal dysplasia.

Purine nucleoside phosphorylase (PNP) deficiency

PNP, similar to adenosine deaminase, plays an important role in purine metabolism. Biallelic mutations in the *PNP* gene result in the intracellular accumulation of the toxic metabolite deoxyguanosine triphosphate, which inhibits DNA synthesis causing impaired lymphocyte proliferation and CID. Because PNP is an ubiquitous enzyme required by dividing cells, PNP deficient patients also suffer from non-immunological abnormalities, in particular neurologic defects such as cerebral palsy, ataxia and developmental delay (Yeates et al., 2017). Recurrent bacterial and opportunistic infections, associated with T cell lymphopenia, occur usually within the first months of life, but may be delayed to early childhood. In some cases, neurologic symptoms precede the susceptibility to infections. Autoimmunity is common and includes non-infectious arthritis, pericarditis, thyroiditis, vasculitis and cytopenias. In vitro lymphocyte proliferation in response to mitogens and antigens is reduced or absent. The defect in purine metabolism leads to reduced serum levels of uric acid, elevated deoxyguanosine in plasma and increased concentrations of d-GTP in erythrocytes. HSCT is curative and indicated early, before neurologic deficits occur (Yeates et al., 2017).

Hepatic veno-occlusive disease with immunodeficiency (VODI)

VODI is a rare syndrome with AR inheritance that presents in infancy with hepatic vein occlusion, hepatomegaly and hepatic failure, associated with hypogammaglobulinemia and clinical evidence of T cell dysfunction (Cliffe et al., 2012). Accordingly, affected infants suffer from recurrent bacterial and opportunistic (PJP, Candida, CMV) infections. While normal in number, T and B cells are mostly immature, switched memory B cells are absent and antibody responses are decreased. VODI is caused by biallelic mutations in the transcriptional regulator gene *SP110*. Treatment is symptomatic with antimicrobials, PJP prophylaxis, IgG replacement; HSCT and liver transplantation are of limited success.

Other CID disorders with syndromic features

This group of rare disorders includes monogenic defects that involve proteins with functions limited to specific organ systems.

Immunodeficiency with multiple intestinal atresia due to biallelic mutations in the *TTC7A* gene

This syndrome is often complicated by intrauterine polyhydramnios and intestinal growth restriction resulting in short bowel syndrome (Notarangelo, 2014). The combination of malabsorption, recurrent bacterial, fungal, and viral infections requires a multidisciplinary approach to manage affected patients.

Tricho-Hepato-Enteric syndrome (THES) caused by biallelic mutations in *TTC37* or *SKIVL2*

THES is characterized by facial dysmorphism, intrauterine growth retardation, wooly hair, chronic diarrhea, liver disease and skin abnormalities (Monies et al., 2015). Most patients have recurrent respiratory infections and antibody deficiency.

Neurologic deficiencies associated with CID

These syndromes include AD BCL11B deficiency with severe T cell abnormalities that may resemble SCID with low TRECs at newborn screening (Punwani et al., 2016) and biallelic mutations in *EPG5* (VICI syndrome) associated with early onset recurrent infections (Abidi et al., 2020). Both syndromes present with CNS abnormalities (absence of corpus callosum), developmental delay and cutaneous manifestations.

HOIL1 and HOIP deficiency

These deficiencies are due to biallelic mutations in *RBCK1* (HOIL1) and *RNF31* (HOIP), respectively, and are associated with hypogammaglobulinemia, lymphopenia, bacterial infections, autoinflammation and amylopectinosis. HOIL1 and HOIP are components of the linear ubiquitination chain assembly complex (LUBAC), a constituent of the NF- κ B cascade involved in the IKK complex activation (Boisson et al., 2015).

STAT5B deficiency

STAT5B deficiency is characterized by short stature due to growth hormone insensitivity, dysmorphic features, chronic pulmonary disease presenting as interstitial pneumonitis, and recurrent bacterial and viral infections. Together with other STATs, STAT5B is an important component of the IL-2 signaling pathway, which is of critical importance for lymphoid development. STAT5B plays a specific role in the transcription of the insulin-like growth factor-1 (*IGF-1*) gene. In most STAT5B deficient patients, the inheritance is AR and the protein is absent (Vidarsdottir et al., 2006), but AD inheritance with mutations resulting in a dominant negative effect has been reported (Klammt et al., 2018).

Kabuki syndrome

This syndrome is characterized by typical facial features (long palpebral fissures with eversion of the lateral lower eyelid, arched and broad eyebrows), moderate intellectual disability, growth deficiency and other developmental anomalies (Adam et al., 2019). Recurrent respiratory infections are reported by the majority of affected individuals and autoimmune complications, such as ITP and hemolytic anemia, occur in half of the patients. The inheritance is AD involving the gene *KMT2D*, often reflecting a de novo mutation, or X-linked affecting the *KDM6A* gene.

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Predominantly Antibody Deficiencies

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Severe reduction in all serum immunoglobulin isotypes with profoundly decreased or absent B cells, agammaglobulinemia

(BTK deficiency, μ heavy chain deficiency, $\lambda 5$ deficiency, Ig α deficiency, Ig β deficiency, BLNK deficiency, p85 deficiency, E47 transcription factor deficiency, LRRC8 deficiency, SLC39A7 (ZIP7) deficiency, p110 δ deficiency, TOP2B deficiency).

Pathogenesis

Absence of peripheral B cells with severe reduction in all serum immunoglobulin levels is the hallmark of agammaglobulinemia, a rare form of PID; X-linked, autosomal recessive and autosomal dominant forms of the disease have been described. Affected patients present with early onset of recurrent bacterial infections, typically within the first 12 months of life. The incidence of the disease varies from 1:100000 to 1:200000 depending on ethnicity and specific genetic defect involved.

The most frequent form of this group of PADs is X-linked agammaglobulinemia (XLA). XLA or BTK deficiency represents the prototype for PID and was the first immunological disorder for which the genetic cause was discovered. The first patient was described by colonel Bruton (1952), but was only in the early 1990s that two different groups described the underlying genetic defect as mutations in Bruton's tyrosine kinase (BTK, OMIM 300,300), a member of the Tec family of kinases (Tsukada et al., 1993; Vetrie et al., 1993). Early B cell development takes place in the bone marrow and is characterized by specific maturational steps starting from pro-B to pre-B to immature and then mature B cells that exit the bone marrow and enter the periphery (Conley, 2002). Pre-B cells express the pre-BCR receptor complex that requires BTK for the initiation of the downstream signaling cascade, necessary for further maturation. Mutations in BTK result in a developmental arrest of B cell development in the bone marrow at the pro-B to pre-B stage. This early block in B cell development results in less than 1% of B cells in the periphery of these patients. Immunoglobulin levels are very low for all classes and there is virtually no humoral response to recall antigens. The almost total lack of circulating B cells results in reduced size of lymph nodes and tonsils, tissues normally highly populated by B cells. On the other hand, both number and function of T cells are conserved, with the former being slightly increased.

BTK maps on the X-chromosome and mutations can be both familial and de novo ones; in the first case, mothers of affected individuals are healthy carriers.

Agammaglobulinemia with autosomal recessive transmission (autosomal recessive agammaglobulinemia; ARA) is even more rare than XLA. ARA affects both males and females and is characterized by severe reduction of all serum immunoglobulin classes and absence of peripheral B cells, in the absence of BTK mutations. The underlying genetic defect has been identified only in a limited number of patients. As stated earlier, early B cell development in the bone marrow depends on the sequential expression of specific gene products that promote B cell differentiation from the pro-B to pre-B, then to immature B and to mature B cell that enters the periphery (Conley, 2002). Pre-B cells express the pre-BCR, a receptor complex formed by the μ (mu) heavy chain, Ig α , Ig β , VpreB and λ 5. The pre-BCR complex is essential for the initiation of downstream signaling essential for B cell differentiation, which in turn depends on kinases such as BTK (Martensson et al., 2007). In fact, mutations in the μ heavy chain (IGHM, OMIM*147020), Ig α (CD79A, OMIM*112205), Ig β (CD79B, OMIM*147245), λ 5 (IGLL1, OMIM*146770), B-cell linker protein (BLNK, OMIM*604515), p85 α (PIK3R1, OMIM*171833) genes have been identified in a limited number of patients affected with ARA (Yel et al., 1996; Minegishi et al., 1998, 1999; Wang et al., 2002; Dobbs et al., 2007; Ferrari et al., 2007a,b; Conley et al., 2012).

Clinical manifestations

Clinical symptoms in affected patients initiate typically between the ages of 6–12 months, when the maternal IgGs are catabolized. Nonetheless, a minority of patients may remain asymptomatic for the first year of life. Recurrent bacterial respiratory and/or gastrointestinal infections are the hallmark of this disorder.

XLA and clinical manifestations

XLA patients suffer from recurrent otitis media, sinusitis, bronchitis, pneumonia, skin and gastrointestinal infections (Plebani et al., 2002; Aghamohammadi et al., 2006a,b; Winkelstein et al., 2006; Lougaris et al., 2020) mainly sustained by encapsulated pyogenic bacteria, namely *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* and others. *Pseudomonas* species has been reported to be the most frequently isolated pathogen in septicemia, followed by *H. influenzae*, *S. pneumoniae* and *S. aureus*. Septic arthritis in these patients is mainly caused by *H. influenzae* and *S. pneumoniae* before intravenous immunoglobulin (IVIG) therapy, whereas a viral cause is considered responsible upon initiation of IVIG therapy. Bacterial meningitis can also complicate the history of these patients, especially before Ig replacement treatment is initiated (Plebani et al., 2002; Winkelstein et al., 2006; Aghamohammadi et al., 2006a,b; Lougaris et al., 2020).

Typically, XLA patients suffer from recurrent infections of the upper and lower respiratory tract. Chronic sinusitis is present in more than 65% of patients. Recurrent bronchitis and/or pneumonia continue to occur even when regular IVIG therapy has been established, leading to the development of bronchiectasis in almost 47% of patients after 40 years of follow-up, in the presence of chronic sinusitis in the majority of cases (84%) (Lougaris et al., 2020).

Besides bacterial infections, viral infections may complicate the natural history of XLA. Affected patients are particularly susceptible to enteroviruses, namely poliovirus, echovirus and coxsackie virus. Vaccine associated poliomyelitis after live-attenuated oral vaccine (Sabin) has been reported and is complicated by a high mortality rate. Enteroviral meningoencephalitis in XLA patients tends to manifest slowly over the years, although fulminating infection with fever, headache and seizures has also been reported (Bearden et al., 2016). Unfortunately, isolation of the viral pathogen is very difficult, and the lack of an isolate cannot exclude the infection in XLA patients. High dose IVIG treatment has been shown to be efficient in controlling the infection and limiting the CNS (central nervous system) involvement, however, the limited number of patients studied does not allow statistical conclusions. Chronic enteroviral infection eventually results in cerebral edema, diffuse inflammation and progressive cerebral atrophy (Misbah et al., 1992; Bearden et al., 2016).

Arthritis has been reported in almost 20% of XLA patients (Plebani et al., 2002; Winkelstein et al., 2006; Aghamohammadi et al., 2006a,b; Lougaris et al., 2020). Clinical findings are indistinguishable from classical Rheumatoid Arthritis (RA), including motion limitation, effusion, pain and destructive pannus formation. Although no isolates are found in the majority of cases, these manifestations tend to respond to IVIG therapy, suggesting a potential infectious cause. Antibiotics are usually associated with the IVIG treatment. In many reported cases, an enteroviral or mycoplasma infection was associated with the rheumatic manifestations.

Neutropenia has also been reported in XLA in a percentage that is variable from 10–25% (Aghamohammadi et al., 2006a,b; Winkelstein et al., 2006), although the clear involvement of BTK in neutrophil development has not yet been elucidated.

ARA and clinical manifestations

Clinical symptoms in patients with μ heavy chain deficiency are similar to those observed in XLA, although frequently in a more severe presentation and at an earlier age (Yel et al., 1996; Minegishi et al., 1998, 1999; Wang et al., 2002; Dobbs et al., 2007; Ferrari et al., 2007a,b; Conley et al., 2012). In fact, age at diagnosis also appears younger for this disorder when compared to XLA. Chronic enteroviral encephalitis, recurrent bronchitis, pneumonia, *Pseudomonas aeruginosa* sepsis, otitis media and other symptoms characterize the onset of the disease. The clinical history ameliorates after immunoglobulin replacement therapy is initiated at a regular basis. Neutropenia has also been reported in almost a third of patients with this disorder.

The clinical history of the first patient affected with λ 5 deficiency started at the age of 2 months with recurrent otitis media; he was found to be hypogammaglobulinemic with absence of peripheral B cells at the age of 5 years, when he was hospitalized for *Haemophilus meningitis*. Bone marrow studies showed a specific block at the pro-B to pre-B cell stage of differentiation (Minegishi et al., 1998).

The first female patient affected with Ig α deficiency presented with chronic diarrhea and failure to thrive within the first month of life (Wang et al., 2002). At 1 year of age, she was hospitalized for bronchitis and neutropenia. Immunological work-up showed severely reduced levels of all immunoglobulin classes and absence of peripheral B cells. Bone marrow analysis evidenced a specific block at the transition from pro-B to pre-B cell no lymph nodes were detectable during clinical examination. The second patient was a male with a history of respiratory infections, diarrhea and a dermatomyositis-like picture who died of a pulmonary infection.

The patient with the hypomorphic mutation in Ig β presented with recurrent lower respiratory tract infections at the age of 5 months (Dobbs et al., 2007). After initiation of IVIG therapy at the age of 15 months, her clinical symptoms improved significantly. The patient carrying a non-sense mutation in the Ig β gene was first admitted at the age of 8 months for pneumonia and Salmonella-caused enteritis (Ferrari et al., 2007a); his immunological workup revealed a complete absence of peripheral B cells (CD19 <1%) and pan-hypogammaglobulinemia. When IVIG therapy was initiated, the patient's clinical history was complicated by recurrent bronchitis, sinusitis, otitis media and bacterial conjunctivitis.

The first reported patient with BLNK deficiency (Minegishi et al., 1999), suffered from recurrent otitis since the age of 8 months and reported two episodes of pneumonia before the age of 6 months. The first immunological workup revealed undetectable serum IgG, IgA and IgM levels in the absence of peripheral B cells. Once on regular IVIG therapy and during an 18-year period of follow-up, his clinical history was complicated by chronic otitis and sinusitis, hepatitis C secondary to contaminated immunoglobulin preparation, and protein-losing enteropathy.

The single female patient affected with p85a deficiency due to a premature stop codon suffered from colitis without particular infectious history. Peripheral B cells were absent with an early block in bone marrow B cell development (Conley et al., 2012).

The condition of agammaglobulinemia with absent B-cells may be observed in other conditions beside the ones described so far. E47/TCF3 deficiency was found in four patients with agammaglobulinemia and reduced peripheral B cells that expressed CD19 but lacked BCR expression on the cell surface. The leucine-rich repeat-containing 8 (LRRC8), another gene implicated in the pathogenesis of agammaglobulinemia, was identified to be mutated in a female patient with agammaglobulinemia and dysmorphic features (Sawada et al., 2003). Recently, the condition of agammaglobulinemia was associated with biallelic mutations in SLC39A7 encoding ZIP7, a Zn transporter, in six patients (Anzilotti et al., 2019). All patients presented with early onset infections, agammaglobulinemia and absence of circulating B cells (CD19 B cells: <1%). Two of them, due to co-morbidities, underwent HSCT with positive outcome. A single patient with autosomal recessive deficiency of p110delta and agammaglobulinemia complicated with autoimmune thrombocytopenia and enterocolitis was recently described (Swan et al., 2019). Patients with autosomal dominant mutations in TOP2B and agammaglobulinemia have also been reported (Broderick et al., 2019).

Diagnosis

The typical laboratory findings of agammaglobulinemia consist of low to undetectable immunoglobulin serum levels and the almost complete absence of peripheral B cells (<2% of lymphocytes), reflecting the early block in B cell development. However, rare cases of patients with peripheral B cells and/or near normal Ig levels have been reported. In such cases, antibody responses to vaccines were abnormal and this may be used for further characterization. T cell numbers and function are typically normal, although the former may be slightly elevated. Once the clinical suspicion is supported by laboratory findings, molecular analysis of the BTK gene should be performed in male patients in order to identify the mutation. If the molecular analysis is not available, evaluation of the expression of BTK protein by means of flow cytometry in monocytes, is an alternative valuable and a rapid diagnostic tool. If the protein is not expressed a definite diagnosis of XLA is made, and genetic analysis can be done subsequently in order to define the causative mutation. Cytofluorimetric analysis of BTK expression can also be used for carrier detection. Once the mutation is defined, carrier diagnosis and prenatal diagnosis can also be performed. If mutation analysis for BTK is negative or if the patient is female, genetic analysis for the above-mentioned genes should be undertaken.

Treatment and prognosis

Immunoglobulin replacement treatment (IgRT), intravenous or subcutaneous is the first line treatment for all patients affected with agammaglobulinemia of any type. Every acute bacterial infectious episode should be treated with antibiotics. In some cases, antibiotic prophylaxis may be necessary, depending on the type and number of infectious episodes. The most recent longitudinal study of 168 XLA patients showed that, independently of IgRT, affected patients show reduced overall survival at 40 years of age which is further reduced in the presence of bronchiectasis (Lougari et al., 2020). These data suggest that current treatment strategies are not sufficient to prevent these complications and to guarantee a normal survival for affected patients.

Adverse reactions to immunoglobulin administration should be monitored during therapy. The most common reactions include nausea, vomiting, chills, low-grade fever, myalgia and fatigue. Adverse effects may occur within 30 min from infusion and usually last for several hours. Slowing the rate of infusion or interrupting the infusion for a few minutes greatly helps in preventing symptoms. These reactions may be minimized by pre-medication with anti-inflammatory drugs including corticosteroids.

Severe reduction in at least 2 serum immunoglobulin isotypes with normal or low number of B cells, CVID phenotype

(ICAROS deficiency, TACI deficiency, BAFFR deficiency, CD19 deficiency, CD20 deficiency, D81 deficiency, CD21 deficiency, PRKCD deficiency, p110 δ deficiency, p85 α deficiency, TWEAK deficiency, LRBA deficiency, NFKB2 deficiency, NFKB1 deficiency, CTLA-4 deficiency, PTEN deficiency, TRNT1 deficiency, IRF2BP2 deficiency, ATP6AP1 deficiency, ARHGEF1 deficiency, SH3KBP1 deficiency, SEC61A1 deficiency, RAC2 deficiency, MOGS deficiency).

Pathogenesis

Hypogammaglobulinemia with normal or low number of B-cells represents a group of PADs of which common variable immunodeficiency (CVID) is the prototype. CVID is a heterogeneous group of disorders, characterized by hypogammaglobulinemia, defective specific antibody production, increased susceptibility to recurrent and chronic infections (Aghamohammadi et al., 2005; Cunningham-Rundles and Bodian, 1999) and an increased incidence of autoimmune disorders and cancers (Cunningham-Rundles et al., 1987; Knight and Cunningham-Rundles, 2006).

CVID has an estimated prevalence ranging from 1:10,000 to 1:50,000 (Darragh, 1982; Fasth, 1982; Hammarstrom et al., 2000) with equal distribution between males and females. It may present at any age but the peak of presentation occurs in early adult life (Luzi et al., 1983; Notarangelo et al., 2004) with an average period of 5–6 years between the onset of symptoms and diagnosis (Aghamohammadi et al., 2005; Cunningham-Rundles and Bodian, 1999).

Diagnosis of CVID is based on a decrease of immunoglobulins of at least two isotypes (serum IgG, IgA, and/or IgM) by two or more standard deviations from the normal mean for age and genetic exclusion of other antibody deficiencies associated with well-defined single gene defects.

Although most CVID cases are sporadic, it has been estimated that 10–20% are familial, mostly with autosomal dominant inheritance (Ashman et al., 1992; Hammarstrom et al., 2000; Nijenhuis et al., 2001). In multiple-case families, CVID is often present in one parent, accompanied by IgA deficiency (IgAD) in the descendants (Vorechovsky et al., 1999) and it has been estimated that about 15% of the patients with CVID have a first degree relative with either IgAD or CVID (Burrows and Cooper, 1997; Vorechovsky et al., 1995). Attempts to identify the genes responsible for CVID have resulted in finding several monogenic defects.

The first genetic defect leading to CVID was identified in inherited homozygous deletions in the *ICOS* gene (*ICOS*, OMIM*604558) in 4 adult-onset CVID patients (Grimbacher et al., 2003). T cells from these individuals were normal with regard to subset distribution, activation, cytokine production and proliferation. In contrast, naive, switched and memory B cells were reduced. The phenotype of human *ICOS* deficiency, which differs in key aspects from that of the *Icos*^{-/-} mice, suggests a critical involvement of *ICOS* in T cell help for late B cell differentiation, class-switching and memory B cell generation. Following the description of mutations in TACI and BAFFR, that are associated or causative of CVID (Salzer et al., 2004; Salzer et al., 2009; Warnatz et al., 2009; Pieper et al., 2014; Losi et al., 2005; Lougaris et al., 2016). CD19 deficiency (OMIM#107265) was identified in four patients (van Zelm et al., 2006). Three patients carried the homozygous deletion 1384del(GA), while one harboured the homozygous insertion 972ins(A), both causing a premature stop codon leading to lack of CD19 expression by B cells. CD27⁺ memory B cells were decreased in affected patients. BCR cross-linking failed to induce a vigorous B cell activation and recall response to vaccinations was poor (van Zelm et al., 2006). Another patient with CD19 deficiency was identified after the initial description, carrying a compound heterozygous mutation (Kanegane et al., 2007). Mutation analysis of *CD19* in this patient revealed a mutation in the splice acceptor site of intron 5 (IVS5-1G >T) of the maternal allele, resulting in skipping of exon 6, and thus a truncated protein product. The paternal allele was disrupted by a gross deletion encompassing at least the *ATP2A1*, *CD19* and *NFATC2IP* genes (Kanegane et al., 2007).

After the description of CD19 deficiency, CD20 deficiency was identified due to a homozygous splice site mutation in the *CD20* gene in a single patient with hypogammaglobulinemia. The patient showed defective antibody formation upon T-independent antigen stimulation, similarly to what has been observed in *cd20* knockout mice (Kuijpers et al., 2010).

Lack of CD19 expression by B cells was described in a patient with hypogammaglobulinemia and severe nephropathy (van Zelm et al., 2010). However, the lack of CD19 was not due to CD19 deficiency but to CD81 deficiency, a member of the tetraspanin family that interacts with CD19 and CD21 on the B cell surface. Genetic analysis showed a homozygous c.561 + 1G >A mutation in the *CD81* gene leading to a complete lack of CD81 expression. Memory B cells were reduced in this patient as well. As observed in CD19 deficiency, BCR cross-linking failed to activate properly B cells; in contrast, T cell activation was conserved (van Zelm et al., 2010).

Immunological evaluation of an adult patient with recurrent infections and hypogammaglobulinemia demonstrated lack of CD21 expression by B cells (Thiel et al., 2012). Sequence analysis revealed a compound heterozygous deleterious mutation in the *CR2* gene (encoding CD21) (c.1225 + 1G >C/W766X). Class-switched memory B cells were reduced. While vaccination responses to protein antigens were normal, the response to pneumococcal polysaccharide vaccination was impaired (Thiel et al., 2012).

The genetic defects described so far as associated or causative of CVID are related to receptors expressed on the cell surface. Genetic defects affecting cytoplasmic proteins were subsequently reported in patients with clinical and immunological phenotypes compatible with CVID. Salzer et al. (2013) reported a single patient from a consanguineous family, with progressive B cell lymphopenia, hypogammaglobulinemia and severe autoimmunity, who was affected with PRKCD deficiency due to a homozygous

(c.1352 + 1G >A) splice site mutation leading to absent protein expression. PRKCD deficiency due to a R614W homozygous mutation was reported in a single patient with chronic, low-grade Epstein-Barr virus infection (Kuehn et al., 2013). The patient had chronic lymphadenopathy, splenomegaly, autoantibodies, hypergammaglobulinemia and natural killer cell dysfunction. A homozygous mutation in PRKCD (p.G510S) was identified in three patients affected with systemic lupus erythematosus, who also showed B cell defective apoptosis and hyperproliferation (Belot et al., 2013).

Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110 δ were identified in patients with a CVID-like phenotype (APDS-1; Activated phosphoinositide 3-kinase δ syndrome) (Angulo et al., 2013; Lucas et al., 2014a,b). Affected patients presented with variable features, including lymphopenia, hypogammaglobulinemia, variable B and T cell maturational defects and, of note, T cell senescence. A comprehensive study of a large cohort of affected patients revealed an increased prevalence of B-cell lymphomas in patients affected with APDS-1 (Kracker et al., 2014).

Besides the identification of dominant mutations in the p110 δ catalytic subunit of PI3K, monoallelic mutations in PIK3R1, the gene encoding for the regulatory subunit p85 α of PI3K, were also identified (Deau et al., 2014; Lucas et al., 2014a,b; Lougaris et al., 2015a,b), causing APDS-2. Affected patients present with recurrent respiratory infections, gut involvement, enlarged lymph nodes and tonsils, normal to elevated IgM serum levels, low IgG and IgA, and variable lymphopenia. NK cell evaluation in patients with APDS-1 and APDS-2 revealed important functional NK cell defects (Lougaris et al., 2016; Ruiz-Garcia et al., 2018).

Considering that in APDS, both type 1 and 2, the genetic defect leads to hyperactivation of the PI3K pathway, and thus of mTOR and AKT, targeted inhibition of this cascade, for example with rapamycin, an mTOR inhibitor, or specific PI3K inhibitors, may become an effective strategy for the treatment of affected patients (Angulo et al., 2013; Lucas et al., 2014a,b; Deau et al., 2014).

TWEAK deficiency was identified in a single family with three affected members (father, daughter and son). All patients showed variable antibody deficiency, recurrent infections and defective vaccine responses (Wang et al., 2013). They carried a rare mutation in the sixth exon of TWEAK, p.R145C, within the conserved TNF-homology domain of the full-length protein. Functional in vitro experiments showed that the mutant protein caused inhibition of BAFF-dependent B-cell survival and proliferation, suggesting a causative pathogenetic role of this mutant for the CVID phenotype in this family (Wang et al., 2013).

LRBA (lipopolysaccharide responsive beige-like anchor protein) deficiency was described in patients with CVID and/or autoimmune disorders (Lopez-Herrera et al., 2012; Burns et al., 2012). Lopez-Herrera et al. identified five CVID patients harboring homozygous mutations (I2657S, R1683X, E59X and a large deletion that includes exons 1 and 2) in the gene encoding for LRBA causing loss of protein expression. All patients had early onset hypogammaglobulinemia and severe autoimmune manifestations. Immunological evaluation revealed disturbed B cell development, abnormal in vitro B cell activation, immunoglobulin secretion and proliferation, and defective B cell autophagy. LRBA deficiency due to a large homozygous deletion including exons 1 to 30 was recently reported in a single patient with autoimmunity but without hypogammaglobulinemia, suggesting that LRBA defects may present with variable immunological phenotypes (Burns et al., 2012). Considering the complicated clinical course of LRBA deficiency, based on long-term follow-up of numerous affected patients (Gamez-Diaz et al., 2016), HSCT has been implemented in a small number of cases, with variable results (Gamez-Diaz et al., 2016; Backthiar et al., 2017). Since LRBA deficiency leads to lack of CTLA-4 expression by T regulatory cells, Abatacept has become a promising therapeutic option (Lo et al., 2015; Alroqi et al., 2017).

Alterations in components of the NF- κ B pathway were recently associated with the pathogenesis of CVID. Germline mutations in NFKB2, a component of the non-canonical NF- κ B pathway, were described in a small number of patients affected with early onset hypogammaglobulinemia, recurrent infections, autoimmune features and adrenal insufficiency (Chen et al., 2013). These NFKB2 mutations cause altered processing of p100 and affect p52 nuclear translocation. Upon the initial description of NFKB2 deficiency, additional CVID patients with NFKB2 mutations have been described (Lindsley et al., 2014; Lee et al., 2014; Lougaris et al., 2015a,b). Of interest, evaluation of NK cells from affected patients revealed defective cytotoxic activity, a rather unusual feature for CVID (Lougaris et al., 2015a,b).

Monoallelic mutations in NFKB1, a component of the canonical NF- κ B pathway, were recently identified in adult-onset CVID patients that presented with hypogammaglobulinemia, recurrent infections and variable autoimmune features (Fliegauf et al., 2015). The identified mutations altered the normal processing of p105 and the nuclear localization of p50 (Fliegauf et al., 2015). Evaluation of B cell maturation showed both early and late B cell developmental alterations in NFKB1 mutated patients (Lougaris et al., 2016a). Following the identification of this genetic defect, additional cases have been described, broadening the clinical and immunological phenotype (Schippe et al., 2016; Boztug et al., 2016). Of interest, as observed in NFKB2 deficiency, NK cell evaluation in affected patients revealed functional and developmental abnormalities (Lougaris et al., 2016b).

Monoallelic mutations in Cytotoxic T lymphocyte antigen 4 (CTLA-4) were identified as responsible for a complex CVID-like condition (Schubert et al., 2014; Kuehn et al., 2014). CTLA-4 is expressed in regulatory T cells upon activation and exhibits an inhibitory function on T cell biology. Affected patients present a complex syndrome of immune dysregulation and may present with hypogammaglobulinemia, lymphopenia, autoimmune cytopenia, lymphoproliferation and granuloma formation, resembling LRBA deficiency (Lopez-Herrera et al., 2012; Gamez-Diaz et al., 2016). Of note, affected patients seem to present an increased risk of gastric cancer, which has been experimentally confirmed in animal models (Miska et al., 2018). Considering the available experience with the biology of CTLA-4, targeted treatments with CTLA-4-Ig may become a promising tool in the treatment of patients affected with CTLA-4 or LRBA deficiency, since LRBA regulates CTLA-4 expression (Lo et al., 2015; Alroqi et al., 2017).

An immunological phenotype compatible with CVID may be observed in a number of additional genetic defects.

Autosomal-dominant germline mutations in *PTEN* have been reported in patients with hypogammaglobulinemia associated with reduced CD27⁺ memory B cells, and normal naive, memory, and effector CD4 and CD8 T cells (Driessen et al., 2016). Biallelic

mutations in *TRNT1* lead to defective mitochondrial translation and cause a phenotype referred to as congenital sideroblastic anemia with immune deficiency, fevers and developmental delay (SIFD). Patients typically have hypogammaglobulinemia with poor maturation of B cells (Wiseman et al., 2013). Heterozygous germline *IKZF1* mutations have been identified in patients with a B cell immunodeficiency mimicking CVID (IKAROS deficiency). These mutations demonstrated incomplete penetrance leading to haploinsufficiency (Kuehn et al., 2016). Autosomal dominant mutations in the *IRF2BP* gene have been reported in a small number of patients with a CVID-like phenotype, combining hypogammaglobulinemia with colitis (Keller et al., 2016). ATP6AP1 deficiency has been observed in a complex form of CVID associated with liver damage and neurological involvement (Jansen et al., 2016). Compound heterozygous mutations in *ARHGEF1*, resulting in the loss of ARHGEF1 protein expression were recently identified in 2 antibody-deficient siblings presenting with recurrent severe respiratory tract infections and bronchiectasis (Bouafia et al., 2019). Patients harboring a germline deletion within the *SH3KBP1/CIN85* gene on the X chromosome show primary antibody deficiency with B lymphocyte intrinsic defects in distinct effector pathways of the B cell antigen receptor, most notably NF- κ B activation and up-regulation of CD86 expression on the cell surface (Keller et al., 2018). Patients with hypogammaglobulinemia and heterozygous mutations in the *SEC61A1* gene show defective plasma cell differentiation (Schubert et al., 2018). While monoallelic dominant negative mutations in *RAC2* result in neutrophil defects, monoallelic germline activating mutations result in hypogammaglobulinemia with lymphocyte defects compatible with a combined immunodeficiency. To date only two siblings born to consanguineous parents presented with a CVID-like phenotype due to homozygous deficiency of *RAC2* (Lougaris et al., 2020). Mutations in the gene encoding MOGS (also known as glucosidase 1) cause severe hypogammaglobulinemia with susceptibility to viral infections, often associated with neurological involvement (Sadat et al., 2014).

Clinical manifestations

The main clinical symptoms associated with CVID patients are recurrent infections, autoimmune manifestations, and an increased incidence of lymphoma and other selected cancers. The age at onset of symptoms is variable, ranging from childhood to late adult life, with some evidence of a bimodal distribution, demonstrating two peaks between 1 and 5 years and also 18 and 25 years (Aghamohammadi et al., 2005; Cunningham-Rundles and Bodian, 1999). In contrast to patients with XLA, patients with CVID have normal sized or enlarged tonsils and lymph nodes and approximately 25% of patients develop splenomegaly (Holm et al., 2005).

Almost all patients with CVID have a history of acute, chronic, or recurrent infections, particularly pneumonia, sinusitis and otitis (Aghamohammadi et al., 2005; Cunningham-Rundles and Bodian, 1999; Hermaszewski and Webster, 1993). Approximately 89% of patients with CVID have had at least one episode of chronic sinusitis and 70% have had recurrent otitis media before establishing the diagnosis (Aghamohammadi et al., 2007; Martinez Garcia et al., 2001). Between 75% and 84% of CVID patients have experienced at least one episode of pneumonia before diagnosis, and many have suffered multiple episodes (Busse et al., 2002; Pourpak et al., 2006; Sweinberg et al., 1991). Bronchiectasis, an irreversible lung complication, has been reported in a variable percentage (37.5–73%) of CVID patients (Kainulainen et al., 1999; Park et al., 2005; Thickett et al., 2002). It has been documented that subgroup of CVID patients who have low numbers of IgM memory B cells and reduced levels of anti-pneumococcal polysaccharide IgM antibodies are at an increased risk of developing recurrent bacterial pneumonia and bronchiectasis. Measurement of these parameters may be useful for the attending physicians and may result in more aggressive and targeted treatment strategies. Asthma represents an obstructive lung complication, which has been observed in 9–15% of patients with CVID (Mullighan et al., 1997). The etiology of asthma in patients with CVID is unknown.

Non-caseating, granulomatous infiltrations have been reported in less than 25% of patients with CVID (Fasano et al., 1996; Mechanic et al., 1997). These lesions are not clearly distinguishable from sarcoidosis. Non-caseating granulomas are occasionally also found in lymphoid tissues or the liver (Mechanic et al., 1997).

Lymphoid interstitial pneumonitis (LIP) may develop in the lower airways of patients with CVID (Buckley, 2004; Davies et al., 2000). LIP can be suspected based on findings on high resolution CT (HRCT) scan. Presence of granulomatous lung disease and/or LIP are associated with a worse prognosis and higher rate of lymphoproliferative disease (Bates et al., 2004; Koss et al., 1987). There is a high prevalence of inflammatory and infectious gastrointestinal disorders in patients with CVID (Washington et al., 1996). Mild, watery diarrhea is common and occurs periodically in about 20% of patients, with 10% having a more severe enteropathy resulting in malabsorption and weight loss (Teahon et al., 1994). Gastrointestinal pathology in these patients includes nodular lymphoid hyperplasia, inflammatory bowel disease (ulcerative colitis, ulcerative proctitis, or Crohn's disease), sprue-like illness with flat villi, chronic giardiasis and nonspecific malabsorption. Defects in cellular immunity, rather than antibody deficiency alone, appear to predispose patients to such symptoms (Washington et al., 1996). *Helicobacter pylori* is an important pathogen in CVID resulting in chronic active gastritis involving both antrum and corpus.

Approximately 10% of CVID patients have significant liver dysfunction, due to hepatitis B and C virus infection, primary biliary cirrhosis, and granulomatous disease.

Approximately one fourth of patients with CVID have, at the time of diagnosis or later, developed one or more autoimmune conditions such as autoimmune hemolytic anemia, thrombocytopenia, rheumatoid arthritis, or pernicious anemia (Aukrust et al., 1996; Cunningham-Rundles et al., 1991; Hermaszewski and Webster, 1993).

While there is an increased susceptibility to autoimmunity, the mechanism underlying this complication is unknown. Most CVID patients with idiopathic thrombocytopenia purpura (ITP) or autoimmune hemolytic anemia have been successfully treated with infusions of high doses of intravenous immunoglobulin (IVIG), coupled with a short course of corticosteroids. However, due to a higher incidence of medical complications associated with the use of immunosuppressive drugs in patients with CVID, this type of therapy should be used with caution (Cunningham-Rundles et al., 1991).

Individuals with CVID are susceptible to malignancy, particularly lymphoma. The incidence of malignancy is increased (11–13%) in CVID during the fifth and sixth decades of life (Hermaszewski and Webster, 1993). The majority of these malignancies involve the gastrointestinal tract and lymphoid tissues (Cunningham-Rundles et al., 1991; Filipovich et al., 1994; Kinlen et al., 1985; Kokron et al., 2004; Mellemkjaer et al., 2002; Sander et al., 1992). An association between Non-Hodgkin Lymphoma (NHL) and congenital immunodeficiency is well established, and most of the NHL cases associated with immunodeficiency appear in patients with T cell defects. In a survey of malignancy in CVID patients, a 438-fold increased likelihood of developing NHL was reported for females compared to the age-adjusted expected incidence in normal controls (Cunningham-Rundles et al., 1987). A 1.8 to 5-fold increase in all types of cancers has been described in CVID patients, with a 47-fold increase for stomach cancer and a 30-fold increase for lymphoma. Benign manifestations of lymphoproliferation, including nodular lymphoid hyperplasia, splenomegaly and generalized lymphadenopathy, have also been reported (Kinlen et al., 1985; Kokron et al., 2004; Pulvirenti et al., 2018).

Mucosa-associated lymphoid tissue (MALT) lymphoma, which represents a subset of low-grade B-cell non-Hodgkin lymphomas, is a rare complication in CVID patients (Aghamohammadi et al., 2006a,b; Reichenberger et al., 2001).

Diagnosis

The most important laboratory criterion for establishing the diagnosis of CVID is a low serum IgG concentration, ranging from profoundly reduced (<100 mg/dl) to just 2 SDs below the normal values for age (International Union of Immunological Societies, 1999; Conley et al., 1999). Most patients have low levels of IgA, and approximately half show reduced IgM levels. Documenting impaired production of specific antibodies (isohemagglutinins and/or poor responses to one or more vaccines) is part of the diagnostic work-up for CVID.

Flow cytometry is important in evaluating numbers of peripheral B cells in patients with profound hypogammaglobulinemia. Numbers of B cells in the peripheral blood may be normal or reduced and approximately 13% of patients will have a B-cell count of less than 3% in peripheral blood (Cunningham-Rundles and Bodian, 1999). Approximately half of the patients with CVID have reduced T-cell numbers and diminished lymphocyte proliferative responses to mitogens and antigens.

As there is no single diagnostic immunological or genetic test for CVID, it is important that patients are investigated to exclude other well-defined causes of hypogammaglobulinemia.

Treatment and prognosis

The mainstay of treatment for CVID is immunoglobulin replacement therapy (IgRT) (Buckley and Schiff, 1991; Ochs et al., 1986; Orange et al., 2006). Intravenous (IVIG) (Orange et al., 2006) or subcutaneous (SCIG) (Fasth and Nystrom, 2007; Ochs et al., 2006) IgRT can be used on a regular basis to maintain a trough level of at least 400–500 mg/dl. A dose of 400–600 mg/kg every 3–4 weeks is usually required. It has been shown that doses of 600 mg/kg every 4 weeks achieved serum IgG trough levels of greater than 500 mg/dl (Roifman et al., 1987). In patients with structural lung damage, a trough level of 700–800 mg/dl is required. Higher doses of immunoglobulin may be necessary for patients with severe chronic sino-pulmonary infections and to prevent bronchiectasis (Quartier et al., 1999).

Adverse reactions to immunoglobulin administration should be monitored during therapy. The most common reactions include nausea, vomiting, chills, low-grade fever, myalgias and fatigue. Adverse effects may occur within 30 min from starting the infusion and usually last for several hours. Slowing the rate of infusion or interrupting the infusion for a few minutes greatly helps in preventing symptoms. These reactions may be minimized by pre-medication with anti-inflammatory drugs including corticosteroids.

In addition to IVIG or SCIG, other forms of supportive care, such as the use of prophylactic antibiotics and lung physiotherapy, are important as bacterial infections may continue, albeit at a reduced rate, even with appropriate immunoglobulin replacement (Busse et al., 2002; Cunningham-Rundles and Bodian, 1999).

Treatment options may become more “tailored” in some cases, as described above for APDS-1 and -2 (rapamycin, p110 inhibitors) or for CTLA-4 and LRBA deficiency (Abatacept).

The prognosis for patients with CVID depends on the frequency of infections, extent of structural lung damage and concomitant presence of autoimmune disease. Other major factors in determining the prognosis are the extent of end-organ damage and the success of prophylaxis against infections. Patients and their families should be educated about early signs of infection in order not to delay treatment.

Severe reduction in serum IgG and IgA with normal/elevated IgM and normal numbers of B cells

(autosomal hyper IgM) (AID deficiency, UNG deficiency, INO80 deficiency, MSH6 deficiency, Other CSR selective deficiency).

Pathogenesis

These types of Hyper IgM (HIGM) syndromes are a consequence of defect in the class switch recombination (CSR) machinery (Durandy et al., 1997) and are currently termed Ig-CSR deficiency. Defect in these syndromes is selectively an intrinsic and pure B-cell defect, caused by mutations in Activation-Induced Cytidine Deaminase (*AICDA* or AID, OMIM*605257), Uracyl-DNA

Glycosylase (*UNG*, OMIM*191525) and others undefined genes (Durandy et al., 2004; Imai et al., 2003a; Imai et al., 2003b; Quartier et al., 2004). These disorders are clinically characterized by low or absent IgG, IgA and IgE levels, normal or elevated IgM, recurrent and chronic bacterial (not opportunistic) infections, lymphoid hyperplasia and autoimmune disorders (Durandy et al., 1997). In contrast to HIGM due to defects in CD40-mediated signaling (CD40 ligand and CD40), they have a better prognosis (Korthauer et al., 1993; Nonoyama et al., 1993).

The maturation of the antibody repertoire produces several antibody isotypes with high affinity for antigen, which is an important and basic component of humoral immunity. The maturation of the antibody repertoires takes place in primary (fetal liver and bone marrow) and secondary (lymph nodes, tonsils, and spleen) lymphoid organs. In primary lymphoid organs B-cell undergo the DNA recombination event. At this stage, the process of V(D)J and VJ recombination, which is antigen and T cell independent, results in the expression of surface immunoglobulins (IgM and IgD) on B cells.

Once surface IgM positive B cells are generated in the bone marrow, they migrate to the secondary lymphoid organs. Such as spleen, lymph nodes, where they may encounter antigens that bind to their receptor (Manis et al., 2002). In secondary lymphoid organs, mature B lymphocytes can be stimulated to undergo second DNA modifications. During this specific activation process, two distinct antigen independent events can take place in B cells including class switch recombination (CSR) and somatic hypermutation (SHM) (Chaudhuri and Alt, 2004; Dudley et al., 2005).

Occurrence of CSR and SHM requires three successive steps including transcription of target DNA sequences, DNA cleavage and DNA repair. As result of this transcriptional step, RNA/DNA hybrids are formed on the template DNA strand, leaving the non-template DNA strand accessible to cleavage (Chaudhuri et al., 2003; Petersen et al., 2001; Ramiro et al., 2003; Yu et al., 2003). During cleavage of DNA, different components are necessary for the generation of double stranded DNA breaks (DSBs). *AID* (Catalan et al., 2003; Petersen et al., 2001) and *UNG* (Di Noia and Neuberger, 2002; Rada et al., 2002) are B-cell components required to generate DSBs. Deamination of deoxy-cytidine to deoxy-uracil and then removal of uracil on single-stranded DNA by *AID* and *UNG* respectively are an important step for the base-excision repair pathway leading to the generation of DSBs.

Defect in function of the AID protein, caused by recessive mutations in the *AID* gene, results in Ig-CSR deficiency type 1 (Durandy et al., 2006). In AID deficiency, defect in Ig-CSR leads to absence or very low levels of IgG, IgA, and IgE associated with normal or elevated serum IgM levels (Durandy et al., 2006). In affected individuals, antibody responses are restricted to the IgM isotype, without change in affinity after repeating immunization (Durandy et al., 2005; Notarangelo et al., 2006, 2007). B cells are unable to undergo CSR when activated by CD40 agonists plus cytokines.

Mutations in the gene encoding *UNG* results in profound impairment in CSR (Ig-CSR deficiency type 2) (Imai et al., 2003a,b; Lee et al., 2005). B cells are unable to undergo CSR when activated by CD40 agonists plus cytokines. *UNG* deficiency is inherited as autosomal recessive.

More than half of the HIGM patients due to intrinsic B-cell defects are not secondary to either AID or *UNG* deficiency and the molecular basis remains unknown (Durandy et al., 2004; Imai et al., 2003a,b). Similar to AID or *UNG* deficiency, B cells from these patients are not able to undergo CSR after activation by CD40 agonists and cytokines. The CSR defect of these patients is milder than patients with AID deficiency because of residual serum IgG levels. However, their clinical phenotype is similar to AID deficiency.

Recently, nonsynonymous homozygous mutations in *INO80* have been shown to interfere with the CSR process in two siblings (Kracker et al., 2014). Mutations in *MSH6*, typically associated with colorectal cancer, have also been seen in CSR defects in a limited number of patients (Gardès et al., 2012).

Clinical manifestations

AID deficiency manifestations

Patients with AID deficiency are susceptible to severe and recurrent infections in respiratory and gastrointestinal tracts (Quartier et al., 2004). Recurrent infections lead to chronic sinusitis and bronchiectasis. Gastrointestinal infection with giardia can persist and cause malabsorption in these patients. Infections of central nervous system caused by *Haemophilus influenzae* and Herpes simplex virus (HSV) were reported in studied patients (Quartier et al., 2004). In contrast to patients with X-linked HIGM and CD40 deficiency, patients with AID deficiency are not susceptible opportunistic infection such as *Pneumocystis jirovecii* or *Cryptosporidium*. Pneumonia is in accordance with a pure B-cell deficiency. Patients are much less likely to develop neutropenia.

The hallmark of AID deficiency is enlargement of the lymphoid organs. It has been reported that 69% patients with AID deficiency develop lymphoid hyperplasia (Minegishi et al., 2000; Quartier et al., 2004; Revy et al., 2000; Zhu et al., 2003). One possible explanation for the intense lymphoid proliferation observed is the possibility that in the absence of functional AID, antigens continuously induce the proliferation of B cells, assuming that no successful antibody maturation has occurred (Fagarasan et al., 2001). It has been shown that in 75% of the cases, lymphoid hyperplasia decreases under IVIG therapy (Quartier et al., 2004).

Autoimmunity and related inflammatory disorders are the third most common complication in patients with AID deficiency, occurring in 21%, including autoimmune hepatitis, hemolytic anemia, thrombocytopenia, Crohn's disease, uveitis, chronic active hepatitis and Type 1 diabetes mellitus (Lee et al., 2005). Patients have autoantibodies of the IgM isotype (Quartier et al., 2004).

In contrast to HIGM due to defects in CD40-ligand and CD40, patients with AID mutation are generally older at age of onset, have a less severe disease and are generally recognized to have immunodeficiency later in life (Minegishi et al., 2000; Revy et al., 2000).

UNG deficiency manifestations

To date few cases of UNG deficiency have been reported in human subjects (Kavli et al., 2005). The clinical manifestations of UNG deficiency are similar to patients with AID deficiency. Patients with UNG deficiency present with a history of recurrent sinopulmonary infections and lymphoid hyperplasia and have very low serum IgG and IgA levels, increased serum IgM levels and lack of IgG antibody responses to vaccine antigens due to a profound defect of CSR. Patients have normal numbers of B- and T-cell subsets. Clinically UNG deficiency is indistinguishable from AID deficiency.

Diagnosis

The most important laboratory criterion for establishing the diagnosis of Hyper IgM is a low serum IgG, IgA, and IgE concentration and normal or elevated serum IgM levels. Antibody responses in these patients are restricted to the IgM isotype (Notarangelo et al., 2006, 2007). In contrast to patients with X-linked hyper-IgM and CD40 deficiency who have minimal lymphoid tissue, most patients with AID deficiency have prominent lymphadenopathy and tonsillar hypertrophy. Definitive diagnosis is made by mutation analysis and detection of *AID* or *UNG* mutations (Quartier et al., 2004).

Treatment and prognosis

The mainstay of treatment for HIGM is IgRT that effectively reduces the incidence and severity of infectious complications in this group of patients.

IVIG can be used on a regular basis to maintain a trough level of 400–500 mg/dl. It has been shown that IVIG also decrease lymphoid hyperplasia (Quartier et al., 2004). Every acute infectious episode should be treated with antibiotics. As a general rule, adverse reactions to immunoglobulin administration should be monitored during therapy.

Isotype, light chain, or functional deficiencies with generally normal numbers of B cells

(Ig heavy chain mutations and deletions, κ light chain deficiency, Isolated IgG subclass deficiency, IgA with IgG subclass deficiency).

Pathogenesis

Human IgG is subdivided into four subclasses, IgG1, IgG2, IgG3 and IgG4, each encoded by a separate constant (C) region gene and endowed with unique biological and functional properties. IgG1 makes up most of the total IgG (66%), followed by IgG2 (24%), IgG3 (7%) and IgG4 (3%). IgG1 and IgG3 appear early in ontogeny (Maguire et al., 2002; Schur et al., 1970), are efficient activators of the classical complement pathway (Bruggemann et al., 1987; Dangel et al., 1988) and are directed mainly against protein antigens. IgG2 contains the preponderance of antibodies to polysaccharide antigens of encapsulated bacteria. IgG2 subclass serum levels do not reach adult levels until 5–10 years of age. Other differences include a shorter half-life for IgG3, and less placental passage for IgG2.

IgG subclass deficiency was first described in Schur et al. (1970). It is defined as a deficiency of one or more IgG subclasses, (less than 2 SD below the mean for age) in the presence of normal total IgG levels (Lemmon and Knutsen, 2006; Maguire et al., 2002). In most patients, the IgM level is normal. In some patients, abnormal IgG subclasses are associated with a low level of IgA (French et al., 1995). The clinical significance of IgG subclass deficiency in patients with recurrent infections is unclear (Buckley, 2002; Maguire et al., 2002) since almost 2.3% of the general population have an IgG subclass deficiency of one or more IgG subclasses. Therefore, a low level of one or more IgG subclasses alone is generally not considered sufficient for a diagnosis of immunodeficiency.

The basic pathogenesis causing IgG subclass deficiencies is unknown. In a few cases the lack of expression of Ig isotypes has been shown to be due to homozygous deletions of the corresponding C region genes (Bottaro et al., 1990; Migone et al., 1984; Olsson et al., 1993; Pan and Hammarstrom, 2000; Smith et al., 1989). Most IgG subclass deficiencies are due to dysregulation of the expression of the γ genes.

The most common type of IgG subclass deficiency is IgG4 deficiency (40%), followed by IgG2 deficiency (28%), IgG3 deficiency (17%) and IgG1 deficiency (14%). Isolated IgG1 deficiency is rare, since a deficiency of this subclass usually result in a decreased level of total IgG.

Immunoglobulin heavy chain deletion is an autosomal recessive disease, caused by chromosomal deletion of a cluster of genes, the IgG heavy chain locus (*IGHC*, OMIM*147100), at 14q32. One or more IgG and/or IgA subclasses as well as IgE may be absent, but some affected cases may be asymptomatic (Plebani et al., 1993; Brusco et al., 1999; Geha et al., 2007).

κ light chain deficiency is an autosomal recessive disease caused by mutations in immunoglobulin kappa constant region (*IGKC*, OMIM*147200) gene, located on chromosome 2p11. Although this disease could be seen in association with some other conditions, it could also be asymptomatic. The pathogenesis of the disease is failure to express kappa chains, but the reason is unknown (Bernier et al., 1972; Liu et al., 2005; Stavnezer-Nordgren et al., 1985; Zegers et al., 1976).

Clinical manifestations

The most frequent complication observed in patients with low levels of 1 or more IgG subclasses is recurrent respiratory infections including otitis, sinusitis and bronchitis (Plebani et al., 1985; Shackelford et al., 1986; Shield et al., 1992; Umetsu et al., 1985, 1986) mainly caused by encapsulated bacteria. Serious systemic infections (e.g., sepsis, pneumonia, meningitis, cellulitis) are less common. Some patients also exhibit frequent viral illnesses. Environmental allergy is also frequently encountered in patients with IgG subclass deficiency (Lacombe et al., 1997) and many patients are atopic, and asthmatic bronchitis often accompanies the respiratory infections.

IgG2 deficiency is the most common subclass disorder associated with recurrent infection and may be accompanied by IgA and or IgG4 deficiencies. Many of these patients have impaired polysaccharide responsiveness. IgG4 deficiency, the most common form of IgG subclass deficiency, is usually not of clinical significance. However recurrent pneumonia and bronchiectasis have been described.

Diagnosis

In patients with history of recurrent respiratory infections and normal IgG levels, IgG subclass should be considered. IgG subclass levels must be compared with age-matched controls (Plebani et al., 1989). In some cases, the total IgG level may be low; in such a circumstance it should be determined whether a diagnosis of CVID might be more appropriate, especially if there is impaired specific antibody production.

Insufficient polysaccharide responses are observed commonly among young patients with IgG2 subclass deficiency (Umetsu et al., 1985, 1986). A clinically significant IgG subclass deficiency must be established by measuring the antibody response to a vaccine antigen, particularly pneumococcal polysaccharide vaccine. Impaired antibody production may not be seen among adult patients with IgG3 subclass deficiency (Barlan et al., 1993). In individuals with recurrent infections and low levels of one or more IgG subclasses, a demonstrable impairment in antibody response to vaccination is considered the most important determinant of disease (Buckley, 2002). Susceptibility to infection may wane over time, although immunologic abnormalities may persist (Herrod, 1997).

Tests for cellular immunity, complement activity and phagocytic function should be taken into consideration based on the clinical history of the patients under investigation. Chest X-ray, sinus imaging, and pulmonary function studies should be considered.

Treatment and prognosis

Asymptomatic patients with IgG subclass deficiency and normal antibody responses to polysaccharide antigens need no therapy. Many patients do well with prompt medical management and early use of antibiotics in the course of a respiratory infectious episode.

Some patients with recurrent infections or chronic respiratory infections need to be treated with prophylactic antibiotics, particularly during the winter months. Patients with severe symptoms and persistent radiographic abnormalities despite prolonged use of prophylactic antibiotics may benefit from IVIG therapy at the usual therapeutic doses. The presence of a subclass deficiency alone is not an indication for IVIG.

Some children may recover from a subclass deficiency spontaneously, particularly if there is not a complete absence of the subclass. In contrast, a subgroup of symptomatic patients can progress to common variable immunodeficiency. Therefore, a repeat immunoglobulin determination at yearly intervals is indicated in patients with IgG subclass deficiency.

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Congenital Defects of Phagocytes

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Abbreviations

AD	autosomal dominant
AR	autosomal recessive
CGD	chronic granulomatous disease
CyN	cyclic neutropenia
ER	endoplasmic reticulum
G-CSF	granulocyte-colony stimulating factor
GFI1	growth factor independent-1
H ₂ O ₂	hydrogen peroxide
HAX1	HCLS1-associated protein X-1
HSCT	hematopoietic stem cell transplantation
ICAMs	intracellular adhesion molecules
LAD	leukocyte adhesion deficiency
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
NE	neutrophil elastase
ROS	reactive oxygen species
SCN	severe congenital neutropenia
SLeX (CD15a)	sialyl Lewis X
WASP	Wiskott-Aldrich syndrome protein
XLN	X-linked neutropenia

Introduction

More than 100 years ago, George Bernard Shaw wrote in Act 1 of his famous play, *The Doctor's Dilemma*, "There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes" (Martinez and Gordon, 2015). Indeed, neutrophils play an essential role in the innate immune response of the human body. The role of neutrophils in phagocytosis, was observed in 1884 by Metchnikov, who coined the name phagocytes for this group of blood cells (reviewed by Dale et al., 2008).

Neutrophils are the first mediators of the rapid innate host defense against various infectious agents, which occurs before the humoral and cellular adaptive immune responses are activated (Mantovani et al., 2011). Neutrophils are produced in the bone marrow from committed hematopoietic progenitor cells—common myeloid progenitors—which in turn are transformed to granulocyte-monocyte progenitors that mature into neutrophils (reviewed by Cowland and Borregaard, 2016). This process is regulated by many different transcriptional factors (Manz and Boettcher, 2014), and involves progression through the promyelocyte, myelocyte, metamyelocyte, band cell, and finally polymorphonuclear segmented cell stages (Klein, 2011). Fully differentiated neutrophils are released into the circulation in response to infectious stimuli. The regulation of both granulopoiesis and neutrophil release from the bone marrow is governed by granulocyte-colony stimulating factor (G-CSF) (Chatta et al., 1994). Millions of cells are released every day into the circulation and their life span is very short. They remain 4–6 h in the circulation and can survive up to 2 days in other body tissues (Dale et al., 2008). The four major categories of neutrophil defects affect production, blood vessel emigration, tissue migration, and bacterial killing (Fig. 1).

This review focusses only on the primary genetic disorders in each category.

Severe congenital neutropenia

Severe congenital neutropenia (SCN) is a group of inherited disorders in which there is a defect in granulocyte maturation in the bone marrow. Generally, neutropenia is defined as less than 1500 granulocyte/ μ L; but severe neutropenia, which is associated with serious infections, is observed with less than 500 cells/ μ L. Infections start early in life and usually involve the lungs, skin, and mucosal surfaces. Periodontitis and gingivitis are common features later in life (Zeidler et al., 2000). A large variety of gram-positive and gram-negative bacteria, as well as fungal infection, can cause deep-seated and severe infections (Klein, 2020). Without appropriate treatment, more than 80% of SCN patients may die from infections in early childhood (Gilman et al., 1970).

Kostmann (1950) was the first to recognize SCN as a genetic condition, describing children with an autosomal recessive trait. Subsequently, cases with autosomal dominant inheritance were discovered. Today mutations in more than 25 distinct genes have been associated with SCN (Klein, 2020; Skokowa et al., 2017). Nevertheless, in more than 20% of the cases a genetic mutation has not been found. The prevalence of SCN appears to range from 0.77 to 6 per one million, depending on ethnicity (Klein, 2020).

The diagnosis of SCN relies on clinical, hematological, and genetic findings. In most cases, infants under 1 year of age will present with a serious infection and the blood count will show severe neutropenia.

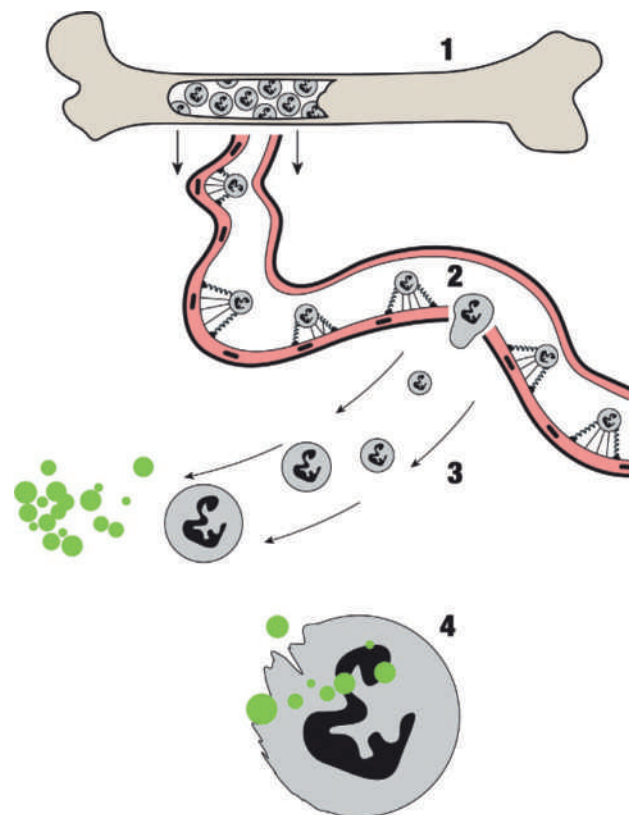


Fig. 1 Position of neutrophil defects: (1) severe congenital neutropenia; (2) leukocyte adhesion defects; (3) motility defects; (4) chronic granulomatous disease.

It should be noted that the most common cause of infant neutropenia is viral infection, in which case the neutropenia resolves spontaneously after several months (Dale, 2017). Only those cases with severe infections and persistent neutropenia should be further evaluated with bone marrow aspiration. In classic SCN, only a few normal neutrophil granulocytes are seen, indicating maturation arrest (Donadieu et al., 2017). Genetic testing is gaining an increasing role in its diagnosis. Since mutations in *ELANE* account for approximately 50% of the cases (Skokowa et al., 2017), one should begin by looking at the *ELANE* gene. If no mutation is found, a panel of genes reported to be associated with SCN can be requested. Finally, whole exome sequencing provides an opportunity to look for additional genes that may be involved in the disease.

In cases of severe infection, intravenous broad-spectrum antibiotics should be started. While in the past the only definite therapy was hematopoietic stem cell transplantation (HSCT), today every SCN case should be initially treated with G-CSF (Dale et al., 2017). Most patients respond well to 3–5 µg/Kg G-CSF, with an increase of the neutrophils to above 1500 µL and a marked decrease in episodes of sepsis (Zeidler and Welte, 2007). It seems that by increasing G-CSFR mediated signaling, G-CSF therapy induces compensatory mechanisms of granulopoiesis in SCN patients. In those few cases, where increasing the dose to 50 µg/Kg has no effect on granulopoiesis, HSCT is indicated (Rosenberg et al., 2006). Aside from failure to respond to G-CSF, another indication for HSCT is development of leukemia. The pathophysiology of leukemogenesis involves mainly somatic mutations in the *CSF3R* gene and high therapeutic doses of G-CSF (Germeshausen et al., 2007). The outcome of transplantation without malignancy is very good, with a 90% survival rate; however if leukemia is present, survival is only about 50% (Connelly et al., 2012). Recently, the use of ex vivo CRISPR/Cas9 mediated *ELANE* knockout in patient hematopoietic stem cells followed by autologous transplantation was suggested as a future alternative therapy for SCN (Nasri et al., 2020).

Specific defects in severe congenital neutropenia

Several excellent reviews have been published recently, which discuss all presently known defective genes associated with SCN (Klein, 2020; Skokowa et al., 2017); therefore this section only concentrates on the more common genetic defects.

ELANE mutations

In 1999, using a genome wide screen for genetic linkage, Horwitz et al. (1999) studied 13 families with cyclic neutropenia (CyN). All patients were found to have autosomal dominant (AD) heterozygous mutations in the *ELANE* gene, which is located at the end of the short arm of chromosome 9. A year later the same group reported that more than 50% of SCN cases harbor AD mutations in the *ELANE* gene (Dale et al., 2000). To date, more than 100 unique mutations in the *ELANE* gene have been detected which are randomly distributed across all exons (Makaryan et al., 2015). Generally, no genotype-phenotype correlation exists and the same mutation can cause SCN in one patient and CyN in another (Makaryan et al., 2015).

ELANE encodes for neutrophil elastase (NE), which belong to a class of cytotoxic serine proteases and is involved in several intracellular processes. It is expressed exclusively in mature myelomonocytic cells and their precursors (Takahashi et al., 1988). Since NE is a protease, it was reasonable to think that mutations would affect its enzymatic properties. But, in fact, protease activity is not defective since the mutant elastase has no effect on the normal NE (Horwitz et al., 2007). Several theories have been proposed to explain the granulocyte maturation arrest seen in *ELANE* mutations. The prevailing theory is as follows: since the mutant NE cannot be properly folded, it accumulates intracellularly, induces reticulum endoplasmic stress, and activates the unfolded protein response (Nanua et al., 2011). This response has evolved to protect cells from the damaging effect of improperly folded protein (Zhang and Kaufman, 2008). Therefore, the unfolded protein response initiates cell apoptosis to overcome potential damage to the integrity and maintenance of the stem cell pool. Indeed, increased apoptosis has been observed in NE mutant cells (Kollner et al., 2006; Grenda et al., 2007). While this theory can explain the pathophysiology of neutropenia, it is not clear how the same mutation can lead to SCN in one patient, while another one suffers from CyN. Recently, it was suggested that some hematopoietic stem cell progenitor cells escape the unfolded protein response and can proliferate in response to G-CSF, up to a certain threshold, at which point, the stress response again affects the majority of cells. This can lead to the cyclic balance between the proliferative effects of G-CSF on the one hand, and the stress-induced apoptosis on the other hand, eventually causing CyN (Mir et al., 2020).

HAX1 deficiency

Kostmann originally described a syndrome that was named after him, in which patients had autosomal recessive inheritance (Kostmann, 1950), and thus could not have had *ELANE* mutations. In 2007, Klein et al. (2007) described a mutation in the HCLS1-associated protein X-1 (HAX1) in DNA samples from the original Kostmann cases, as well as in other SCN patients with autosomal recessive inheritance. Thus, today Kostmann Syndrome is confined only to patients with biallelic HAX1 mutations (Klein, 2020). Although relatively rare, HAX1 mutations are more frequent in Europe (11% of SCN) than in the United States due to higher consanguinity in several European countries (Skokowa et al., 2017). HAX1 is located mainly in the mitochondria but can also be found in other parts of the cell. Its exact function remains unclear, but several studies have shown an antiapoptotic effect (Klein et al., 2007). HAX1 has also been reported to be involved in calcium homeostasis, cell motility and other cell functions. There are two HAX1 splice isoforms, A and B. Mutations affecting both isoforms lead to SCN with neurological symptoms. Mutations occurring only in isoform A cause SCN with no neurological manifestations, indicating a genotype-phenotype association

(Germeshausen et al., 2008). Since HAX1 regulates the inner mitochondrial membrane potential, it has been proposed that HAX1 mutation cause apoptosis due to mitochondrial disruption and thus may contribute to the pathogenesis of neutropenia (Klein et al., 2007). However, the exact mechanism remains elusive. Most cases of HAX1 deficiency respond very well to G-CSF therapy; however, in rare cases, especially if leukemia occurs, HSCT is considered treatment of choice. Hematopoietic stem cell gene transfer or genetic correction of induced pluripotent stem cells may in the future offer an alternative approach for a cure (Morishima et al., 2014).

Jagunal homolog 1 (*JAGN1*) deficiency

Using homozygosity mapping and exome sequencing, several cases of SCN were found to have AR mutations in the *JAGN1* gene located on chromosome 3 (Boztug et al., 2014). Clinically, these cases are very similar to patients with *ELANE* or HAX1 mutations. In some *JAGN1*-deficient patients, bone and teeth defects associated with short stature occur (Skokowa et al., 2017). No genotype-phenotype correlation was observed in patients with *JAGN1* mutations. *JAGN1* is located in the endoplasmic reticulum (ER) and plays a role in protein transport from the ER to the Golgi apparatus (Klein, 2016). The neutrophils of *JAGN1*-deficient patients have ultrastructural defects, mainly in the ER, a reduced number of granules and increased apoptosis, and defective N-glycosylation of multiple proteins (Boztug et al., 2014). Since the G-CSF receptor is also poorly glycosylated, the clinical response to G-CSF in *JAGN1* deficient patients is not as favorable compared to cases of *ELANE* or HAX1 deficiency (Klein, 2016). It is not clear why these patients suffer only from neutropenia, as *JAGN1* is ubiquitously expressed. *JAGN1* also plays a role in antifungal defense and it was recently found that myeloperoxidase (MPO), an important player in antifungal defense, is markedly reduced in patient cells (Khandagale et al., 2018). Interestingly, GM-CSF but not G-CSF, restores MPO expression in the cells of these patients, suggesting a novel therapeutic option for this condition.

Growth factor independent-1 deficiency

Since mice lacking growth factor independent-1 (GFI1) have been found to be neutropenic, a genetic analysis was performed in SCN patients without an *ELANE* or HAX1 mutation. Several cases of AD GFI1 mutations were identified (Person et al., 2003). GFI1 is a zinc finger transcriptional repressor that controls hematopoietic stem cell self-renewal and differentiation. It has been implicated in the transcriptional control of various genes important for granulopoiesis, including *ELANE* (Horman et al., 2009). GFI1 has also emerged as a critical factor in granulopoiesis due to its multiple regulatory functions. Several distinct heterozygous mutations have been identified, which generate dominant negative GFI1 variants and thus interfere with transcriptional networks, leading to maturation arrest in the myeloid lineage (Zarebski et al., 2008). Aside from severe neutropenia, affected patients have marked monocytosis. It was proposed that wild type GFI1 represses *ELANE* protein expression, thus linking mutations in the two genes to a common phenotype (Person et al., 2003).

X-linked neutropenia due to *WAS* gene mutation

While all known SCN gene mutations are classified as loss of function mutations and are transmitted as autosomal recessive or dominant traits, several patients with SCN have been found to harbor mutations in the Wiskott-Aldrich syndrome (*WAS*) gene, which is located on the X-chromosome (Devriendt et al., 2001). Most mutations in this gene cause the classical Wiskott-Aldrich syndrome or X-linked thrombocytopenia and are loss of function mutations. However, in X-linked neutropenia (XLN) the mutations lead to gain of function (GOF) resulting in a constitutively active WAS protein (WASP) expression. These GOF mutations are located in the region encoding the conserved GTPase binding domain, which allows auto-inhibition of WASP (Kim et al., 2000); therefore, mutations in this domain will lead to a continuously activated WAS protein (Devriendt et al., 2001), decreased stability of the auto-inhibited structure of WASP and thereby stimulating actin polymerization. The exact role of the GOF WAS mutations in causing maturation arrest of neutrophils is not clear. It has been suggested that the constitutive activation of WASP causing increased F-actin polymerization might account for the neutropenia (Devriendt et al., 2001). Using a mouse model, it was observed that WASP activating mutations also interfere with lymphocyte cell survival and alter cytoskeleton responses (Westerberg et al., 2010). Interestingly, the small number of neutrophils that exist in the blood are hyperactive, which may explain the milder clinical symptoms of XLN patients compared to other forms of SCN (Keszei et al., 2018).

Leukocyte adhesion deficiency (LAD)

Once the neutrophils are mature, have exited the bone marrow, and entered the blood stream, they must adhere to the vessel endothelium in order to transmigrate and enter the tissue inflammation site. This emigration is highly dynamic, involving the multi-step leukocyte adhesion cascade, which is mediated by several families of leukocyte and endothelial adhesion molecules (Etzioni and Alon, 2014). There are four distinct phases in the adhesion cascade which are critical to this cascade: rolling, integrin activation, firm adhesion, and transmigration.

Under normal conditions, leukocytes speed through the blood vessels with the normal shear flow and do not adhere to the endothelium. When inflammation occurs, the resulting chemokines and cytokines activate the endothelial cells on the blood vessels to express molecules that slow the velocity of leukocytes which begin to roll along the venular wall. This rolling phase is mainly mediated by selectins, which are expressed only after endothelial cell activation, interacting with their ligands that are constitutively present on leukocytes. The three selectins E, L, and P, bind to their ligands, which share a core of sialylated and fucosylated lactosamine tetrasaccharides, known as Lewis X (SLeX, CD15a) (Zarbock et al., 2011). Following the rolling phase, integrins mediate firm adhesion of the leukocyte to the endothelial wall. However, a prior activation process of these molecules is mandatory (Sun et al., 2019). Upon leukocyte activation by different mediators, mainly chemokines generated from the inflamed area, the integrins undergo transformational changes from a low to high affinity state (Shattil et al., 2010). The full integrin activation process leads to the next phase, referred to as “the sticking” or “the firm arrest step,” which occurs through the binding of the activated integrins to their ligands. The main ligands on the vascular endothelium are members of the immunoglobulin superfamily: the intracellular adhesion molecules (ICAMs). The final step of the extravasation process is the trans-endothelial movement of the leukocytes by diapedesis from the blood vessel to the surrounding tissue (Schmidt et al., 2013).

The importance of this orchestrated adhesion cascade was shown when specific genetic defects in the various phases of the process were discovered, leading to severe clinical symptoms. To date, defects in the first three steps of the adhesion cascade have been identified, and they all share two main manifestations: A very high leukocyte count that can exceed 100,000/mL, and a tendency for severe bacterial infections with defective wound healing (Etzioni and Alon, 2014).

LAD-1

LAD-1 was the first and the most frequent LAD syndrome to be recognized. Described originally almost 40 years ago, to date hundreds of patients worldwide have been diagnosed (Novara et al., 2018). As an autosomal recessive disease, an increase incidence has been found in countries with high consanguinity. The primary genetic defect, mutations in the gene encoding the beta subunit of integrin (CD18) (Van de Vijver et al., 2012), leads to a decrease or absence of CD18 expression on the leukocyte surface, resulting in a markedly defective firm adhesion on the vascular endothelium. While patients’ leukocytes can roll normally along the endothelium, they fail to stop and therefore cannot exit the blood vessels to reach sites of inflammation (Fischer et al., 1988). LD-1 is characterized by delayed separation of the umbilical cord, recurrent severe soft tissue infections, impaired wound healing, and marked leukocytosis (van de Vijver et al., 2013). The clinical severity and magnitude of the functional deficit of LAD-1 are directly related to the degree of CD18 expression deficiency. Patients with less than 1% expression suffer from a severe form of LAD-1 and will usually not survive beyond infancy. Those with 2–20% expression have the moderate form, and with antibiotic therapy can achieve adulthood (Hanna and Etzioni, 2012).

Initial clinical symptoms are severe omphalitis together with delayed separation of the umbilical cord. Later in life, infections—mainly of the skin and mucosal surfaces—are predominant; for those who survive infancy, severe chronic periodontitis is very common. However, it seems that the periodontitis in LAD-1 is not a pure infection; it also displays an increased potential to stimulate inflammatory responses and to particularly trigger the induction of interleukin IL-17 and IL-23-mediated immune responses in the gingival area. Indeed, ustekinumab, a human monoclonal antibody that blocks IL-23 and IL-12, has been found to be effective in reducing the oral inflammation in LAD-1 (Moutsopoulos et al., 2017). Aside from infections, a high percentage of patients with LAD-1 have also been found to suffer from autoimmune complications (De Rose et al., 2018).

In most cases, the diagnosis of LAD-1 can be established by the clinical symptoms of delayed separation of the umbilical cord, omphalitis, defective wound healing and marked leukocytosis. Flow cytometry analysis is helpful to confirm the diagnosis, and genetic testing can also be used for early prenatal diagnosis (Etzioni, 2007).

For all LAD-1 cases, prophylactic antibiotics should be started as soon as possible. In addition, for the severe form, HSCT should be performed as it has a very good survival rate (Qasim et al., 2009). While some success has been reported for gene therapy in animal models of LAD-1, it has not yet been reported in LAD-1 patients (Bauer Jr. and Hickstein, 2000).

LAD-2

LAD-2 is a very rare condition, with less than 10 cases reported worldwide. It is an autosomal recessive disorder that is characterized by marked leukocytosis, increased rate of infection, short stature, mental retardation, facial dysmorphism, and the rare Bombay blood group (Yakubenja and Wild, 2006). LAD-2 has a general defect in fucose metabolism due to mutations in *SLC35C1*, a gene that encodes the Golgi-localized GDP-fucose transporter (Lubke et al., 2001). These mutations lead to defective synthesis of various fucosylated proteins, including the selectin ligand, sialyl Lewis X (SLeX), thus causing a rolling defect in the adhesion cascade. The general defect in fucosylation also leads to the non-immunologic phenotypes of the disease, such as Bombay blood group and mental retardation (Hanna and Etzioni, 2012). In general, LAD-2 associated infections are milder than those observed in LAD-1 patients. A significant decrease in infection frequency is noticed in older children, although severe periodontitis remains their major infectious issue (Etzioni et al., 1998).

Aside from antibiotic therapy for infections, in some cases fucose supplementation can improve immunological findings (Marquardt et al., 1999). Since the mental retardation and short stature are not due to the leukocyte adhesion defect, HSCT is not recommended in LAD-2.

LAD-3

LAD-3, which has also been referred as LAD-1 variant, is a general defect in all integrins, as opposed to LAD-1 in which only the beta2-integrin is defective (Alon and Etzioni, 2003). Thus, aside from the classical LAD-1 symptoms, these patients also suffer from severe bleeding diathesis due to the activation defect in beta3-integrin (Etzioni, 2014). The condition is inherited as autosomal recessive trait, with several dozens of patients reported. All LAD-3 patients have mutations in *FERMT3*, a gene that encodes for Kindlin3 which is essential for integrin activation (Sun et al., 2019). Although integrin expression is normal on the leukocyte surface of most patients, integrin activation fails to occur after chemokine stimulation, and hence no firm adhesion of leukocyte or thrombocyte aggregation occurs (Harris et al., 2013).

Kindlin3 is exclusively expressed on hematopoietic cells and transfection of patient's lymphocytes with wild type kindlin3 restored integrin activation (Svensson et al., 2009). Due to the bleeding diathesis, patients require repeated blood transfusions. Because Kindlin3-mediated signaling by integrins is required for osteoclast-induced bone resorption, LAD-3 patients suffer from an osteopetrosis-like state (Schmidt et al., 2011). Currently, HSCT is the only curative therapy that can alleviate both the leukocyte and the platelet defects (Wolach et al., 2019).

Leukocyte migration defects

Once the leukocytes have transmigrated from the blood vessel toward the site of inflammation, they migrate toward the pathogens in a gradient dependent manner, a process known as chemotaxis. The main chemoattractant molecules that emerge from the site of a bacterial infection are fMLP and the cytokines produced by the host, mainly IL-18 (Amulic et al., 2012). The main factor ensuring leukocyte movement is the actin cytoskeleton. Actin exists in two forms: as monomers that can readily diffuse into the cytoplasm, called granular or G-actin, and as an actin filament designated F-actin, which supports the formation of the lamellipodia which are crucial for cell migration (Gressin et al., 2015). The regulation of cytoskeleton dynamics is complex and depends on a variety of actin binding proteins. Some lead to the formation of F-actin, while others are involved in severing it from G-actin. AIP1/WDR1, which is expressed in most leukocytes, enhances cofilin activity, thereby severing F-actin filaments. In order to assure an ameboid movement, the actin filaments must quickly disassemble. Several proteins were found to be involved in this process, one of which is cofilin. Actin-interacting protein 1 (Aip1), also called WD repeat containing protein 1 (WDR1), interacts with cofilin to regulate actin dynamics (Kanellos and Frame, 2016). Another important molecule is Megakaryoblastic Leukemia 1 (MKL1), which forms a complex with G-actin in the cytoplasm, and is essential for F-actin formation (Filippi, 2015).

In recent years several genetic defects caused by mutations in actin regulatory proteins were described and are referred to collectively as actinopathies.

This section focuses only on actinopathies with the main clinical symptom being a neutrophil motility defect. The reader is referred to some recent comprehensive reviews on all other disorders with a motility defect as part of the syndrome (Janssen and Geha, 2019; Burns et al., 2017).

Lazy leukocyte syndrome associated with WDR1 mutation

In 1971 Miller described two unrelated children with recurrent infections, severe stomatitis, otitis, gingivitis, and fever, but with a normal bone marrow examination. They named this new condition, "lazy leukocyte syndrome" (LLS), due to the findings of poor neutrophil chemotaxis with severely impaired random migration (Miller et al., 1971). Very few patients have been reported over the years, although it has been found that autosomal recessive mutations of actin interacting protein 1 (Aip1)—which is encoded by WDR1—were in some cases the cause of an LLS-like phenotype (Kuhns et al., 2016).

Originally it was thought that LLS was a classical immune deficiency condition of neutrophil dysfunction; however, it is now clear that in some cases autoimmunity and autoinflammation are dominant features. Although no genotype-phenotype correlation was found, a more pronounced defect has been observed only in patients with near absent WDR1 expression. WDR1, which is expressed in most leukocytes, enhances cofilin activity, which is required for severing F-actin filaments. Indeed, in LLS patients, high levels of F-actin were found, which led to defective leukocyte migration (Pfajfer et al., 2018).

LLS is not just an innate immune deficiency due to neutrophil dysfunction, but also an adaptive immune deficiency since T- and most B-cells of WDR1 deficient patients have abnormal function.

Aside from the immunological symptoms, children with LLS have facial dimorphism, severe stomatitis with oral stenosis and impaired wound healing, and in many cases intellectual disability. Without HSCT early in life, WDR1 deficient patients do not survive infancy (Pfajfer et al., 2018).

β -Actin deficiency

The β -actin gene (*ACTB*) encodes an abundant cytoskeleton housekeeping protein involved in many cellular functions, including cell motility. Only very few cases of β -actin deficiency associated with immunodeficiency have been reported. Recurrent infections, furunculosis, and septicemia were noted and associated with multisystem manifestations (Nunoi et al., 1999). The defect impairs actin polymerization resulting in abnormal neutrophil locomotion without affecting actin synthesis (Laurence, 1974). More recently it has been shown that heterozygous loss of function *ACTB* mutations cause a distinct pleiotropic malformation syndrome

with intellectual disability (Cuvertino et al., 2017). A review of 30 patients with AD ACTB mutations suggested that there could be an important mutation-specific variability in the phenotype, which does not always lead to immunodeficiency but may cause other organ defects (Sandestig et al., 2019).

Megakaryoblastic leukemia 1 deficiency

Megakaryoblastic Leukemia 1 (*MKL1*) gene, located on chromosome 22, encodes a transcription factor involved in the regulation of actin transcription as well as actin cytoskeleton-related genes. Actin polymerization releases MKL1 from the complex it forms with G-actin and allows its translocation to the nucleus where it initiates transcription of actin and actin related genes. MKL1 silencing in a myeloid cell line resulted in reduction of G-actin stores and abnormal expression of several actin-regulatory genes. Only three cases of homozygous MKL1 deficiency have been reported, all suffering from severe bacterial infections (Record et al., 2015; Sprenkeler et al., 2020). Two of the patients died of pneumonia due to progressive pseudomonas infection, while the younger patient underwent HSCT shortly after birth. A pronounced reduction in motility and chemotactic response was observed with a reduced F-actin level in neutrophils, but not in myofibroblasts (Sprenkeler et al., 2020), the latter probably due to compensatory mechanisms by MKL2 which is not expressed in neutrophils.

Despite the low number of reported patients to date, it seems that MKL1 is a non-redundant regulator of cytoskeleton associated functions involving mainly neutrophils.

ARPC1B mutation

Recently several patients with combined immune deficiency were found to have bi-allelic mutations in ARPC1B, which is part of the ARP2/3 complex. The ARP2/3 complex is involved in actin branching essential for cell migration. Loss of ARP2/3 leads to decreased lamellipodia formation, defective chemotaxis, and impaired cell migration. Mutations in ARPC1B also result in defective cytoskeletal rearrangement and altered immunological synapse formation, leading to severe loss of T-cell function (Brigida et al., 2018). Furthermore, reduced cytotoxic CD8 T-cell numbers and impaired function were observed. This is due to defects in lamellipodia formation, actin reorganization and T-cell receptor maintenance (Randzavola et al., 2019). ARPC1B-deficient platelets are micro-thrombocytes similar to those seen in Wiskott-Aldrich syndrome, are low in numbers and show aberrant spreading consistent with loss of Arp2/3 function (Kahr et al., 2017). Clinically, the disease has an early onset within the first month of life, manifesting skin rash, infections, and bleeding episodes, mainly in the gastrointestinal tract. Aside from the infections and bleeding, immune dysregulation manifestations are common and include eczema with leukocytoclastic vasculitis, arthritis, and inflammatory bowel disease (Volpi et al., 2019). Most patients require prophylactic antibiotic-treatment, and several have undergone successful HSCT, which appears to be the treatment of choice for ARPC1B deficiency.

Bacterial killing defect

After arriving at the site of infection, neutrophils must ingest and kill the pathogen. During phagocytosis of bacteria, neutrophils are activated, and a respiratory burst occurs. This oxygen consumption is essential for killing the bacteria, and is associated with the activation of the NADPH oxidase complex subunits, producing several bactericidal factors crucial for killing bacteria. The NADPH oxidase complex is comprised of five subunits. Two are membrane bound (gp91phox and p22phox) and form a heterodimer named cytochrome *b558*. Upon activation by contact with surrounding pathogens, the remaining three cytoplasmatic subunits (p47phox, p67phox and p40phox) form a heterodimer that trans-locates to cytochrome *b558* on the membrane (Anjani et al., 2020). Once activated (known as a respiratory burst), the NADPH oxidase transfers electrons to molecular oxygen and generates superoxide anion, which is dismutated to hydrogen peroxide (H₂O₂). This leads to production of other reactive oxygen species (ROS) with high microbicidal activity. Normally, the electron transfer from NADPH to molecular oxygen creates a negative charge within the phagolysosome, initiating potassium influx, which is essential for the activation of the primary granule proteins, neutrophil elastase and cathepsin G. Decrease of these two proteins is associated with defective neutrophil killing (Reeves et al., 2002).

While no defects in phagocytosis have been reported to date, a genetic defect in the killing process is not uncommon and frequently identified.

Chronic granulomatous disease

Chronic granulomatous disease (CGD) is the most common neutrophil defects, affecting 1 in 200,000 live births (Winkelstein et al., 2000). It was one of the first immunodeficiencies described and the second in which the genetic defect was found (the first being adenosine deaminase deficiency). It was initially described as “fatal granulomatous disease of childhood,” but with advances in therapeutic options and subsequently by diagnosing it in adults suffering from the disease, it is now designated as CGD. The disease can be transmitted in two forms: X-linked (~76% of cases) or autosomal recessive (AR) (24% of cases) (Winkelstein et al., 2000). The autosomal form is more prevalent in regions of the world where consanguinity is common (Wolach et al., 2017). The hallmark of CGD is the inability of the neutrophils to kill bacteria intracellularly, although phagocytosis is completely normal. Clinically, CGD is characterized not only by recurrent severe bacterial and fungal infections with tissue granuloma formation but also by an

increased incidence of inflammatory bowel disease and other autoimmune disorders including lupus, rheumatoid arthritis and Crohn's disease (Dinauer, 2019).

Mutations in all five subunits (gp91phox, p22phox, p47phox, p67phox, p40phox) of the NADPH oxidase complex were reported as causes of CGD. The *CYBB* gene, which encodes for gp91phox is located on the X-chromosome and is the most common mutation of the five subunits. The genes for the other subunits are located on different autosomes the most common being mutations in the *NCF1* gene which encodes p47phox (Roos et al., 2010). In addition to these five subunits, homozygous loss of function mutation in *CYBC1*, which is required for assembly and expression of cytochrome b558, has also been reported to cause CGD (Arnadottir et al., 2018).

Diagnosis can be made by measuring residual NADPH oxidase enzyme activity. Superoxide production can be measured by the classical Nitroblue Tetrazolium (NBT) reduction test. The generation of H₂O₂ can also be assessed using dihydrorhodamine-1,2,3 (DHR) in a flow cytometric assay (Anjani et al., 2020). Mutation analysis should be performed to identify the precise genetic defect needed for genetic counseling and prenatal diagnosis.

For many years the pathogenesis of CGD was attributed to defective ROS production, thereby impairing the killing of microorganisms, leading to severe bacterial and fungal infections. Other theories were subsequently proposed. A defective production in neutrophil elastase and cathepsin G was found to be a major factor in the pathogenesis of CGD. Indeed, these two proteins were found to be markedly decreased in cells of CGD patients, leading to a defect in intracellular killing of staphylococcus and candida (Reeves et al., 2002).

Most patients with CGD present with infections early in life, although in some cases the infections appear in adolescence or even adulthood (van den Berg et al., 2009). Patients with X-linked CGD tend to present with earlier onset and more severe disease than patients with AR-CGD (Marciano et al., 2015). CGD patients have an increased incidence of infections with catalase positive organisms such as staphylococcus, Burkholderia, and aspergillus. Common sites of infection are the lungs, lymph nodes, skin, and liver (Feld et al., 2008). Liver abscesses are usually multi-locular and need aggressive therapy (Yu et al., 2020), but surgical intervention may not be required since treatment with corticosteroids is often successful (Straughan et al., 2018). CGD patients have increased risk for infection with *Mycobacterium tuberculosis*, mainly in endemic areas, and BCG vaccination can lead to severe local and systemic disease.

Aside from severe infections, patients suffer frequently from inflammatory complications, mainly granuloma formation, colitis and increased frequency of autoimmunity. Granulomas in the gastrointestinal tract and the urinary tract can obstruct the lumen (Marciano et al., 2004). There are several theories why patients with CGD develop hyper-inflammatory conditions. It has been suggested that the lack of ROS leads to enhanced inflammation. Another mechanism points to the decreased degranulation of bacterial material causing persistent cellular activation. More recently, it was shown that CGD neutrophils produce higher amount of leukotriene B₄ (LTB₄) than normal neutrophils. LTB₄ plays an important role in lung inflammation (Song et al., 2020).

Because CGD is a chronic disorder with frequent serious complications, CGD children are reported to have more difficulties in socialization and learning, as well as emotional problems which may persist even after being treated successfully with HSCT (Pulvirenti et al., 2020).

Symptoms of infection, which can initially be quite vague, should be treated aggressively for a prolonged period of time. Microbiological diagnosis is crucial and every effort (including surgical procedures) should be made to identify the pathogen to ensure appropriate antibiotic or anti-fungal therapy.

Prophylactic antibiotics such as, trimethoprim/sulfamethoxazole, together with itraconazole have dramatically reduced the number of severe infections and are the cornerstone for successful treatment (Slack and Thomsen, 2018). The use of interferon γ is somewhat controversial: It is part of conventional prophylactic therapy in the USA, but it is not generally used in Europe (Leiding and Holland, 2020). For severe CGD cases, granulocyte transfusions have been tried, although the effect has only been anecdotally reported, without double-blind studies (Marciano et al., 2017). In view of the heterogeneity of the presentation and with many hyper-inflammatory symptoms, several new biological agents have been evaluated, but no beneficial effects have been observed so far (Dinauer, 2019).

Currently, HSCT is the only curative treatment for CGD. Excellent results have been achieved using reduced-intensity conditioning regimens, with 80–90% survival. In a recent study on 712 children and adults with CGD who underwent HSCT, the overall 3-year survival rate was 85.7%, with a low incidence of graft failure (Chiesa et al., 2020). As in many other hematopoietic genetic disorders, the possibility of gene therapy had been the subject of clinical research for two decades. While results have been poor in the past, use of the new lentiviral vector has had promising results: six out of seven patients demonstrated stable vector copy numbers and the persistence of 16–46% oxidase positive neutrophils 1 year after gene therapy. No new infections were seen and prophylactic antibiotics were discontinued (Kohn et al., 2020). Gene therapy may indeed be an excellent alternative approach for the treatment of CGD.

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Above the Regular Tide: Primary Immune Regulatory Disorders (PIRD) Diagnosis and Treatment Considerations

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Introduction

Primary immune deficiency disorders (PIDD) are classically described as diseases that cause susceptibility to common and unusual infections. PIRD are monogenic causes of PIDD dominated by autoimmunity, auto- or hyper-inflammation, lymphoproliferation, malignancy, and severe atopy (Fig. 1) (Chan and Torgerson, 2020; Chan et al., 2020). The genetic variants that cause PIRD encode proteins that play essential roles in regulating immune tolerance, the inflammatory response, and malignancy eradication (Bousfiha et al., 2020). The International Union of Immunological Societies classification schema has identified over 129 PIRD within the 430 genetically described IELs (Bousfiha et al., 2020; Tangye et al., 2020).

The clinical diversity observed within PIRD stems from the genetic defects of diverse immune regulatory pathways. Multiple gene variants have been identified that contribute to PIRD pathogenesis. Tregopathies arise from defects in T regulatory cell (Treg) development or activity. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome and related disorders are part of this category (Cepika et al., 2018). Other Tregopathies display similar clinical features and/or pathophysiology of IPEX and have thus been coined IPEX-like syndromes (Chan and Torgerson, 2020).

Non-malignant lymphoproliferation as has been described in Autoimmune Lymphoproliferative Syndrome (ALPS) encompass another subcategory of PIRD characterized by nonmalignant lymphoproliferation (López-Nevado et al., 2021). Clinical manifestations of ALPS include lymphadenopathy, splenomegaly, and autoimmunity secondary to abnormal FAS-mediated apoptosis (Oliveira et al., 2010). There are a group of diseases that present similarly to ALPS but show abnormal lymphocytic infiltration of non-lymphoid organs, which earns them the title of ALPS-like disorders. Another category of PIRD includes congenital atopic hypersensitivity with amplified atopic features. Monogenic inflammatory bowel diseases (IBD) are also included among primary immune dysregulation of the intestine that may present with other rheumatologic conditions that result from a breakdown in

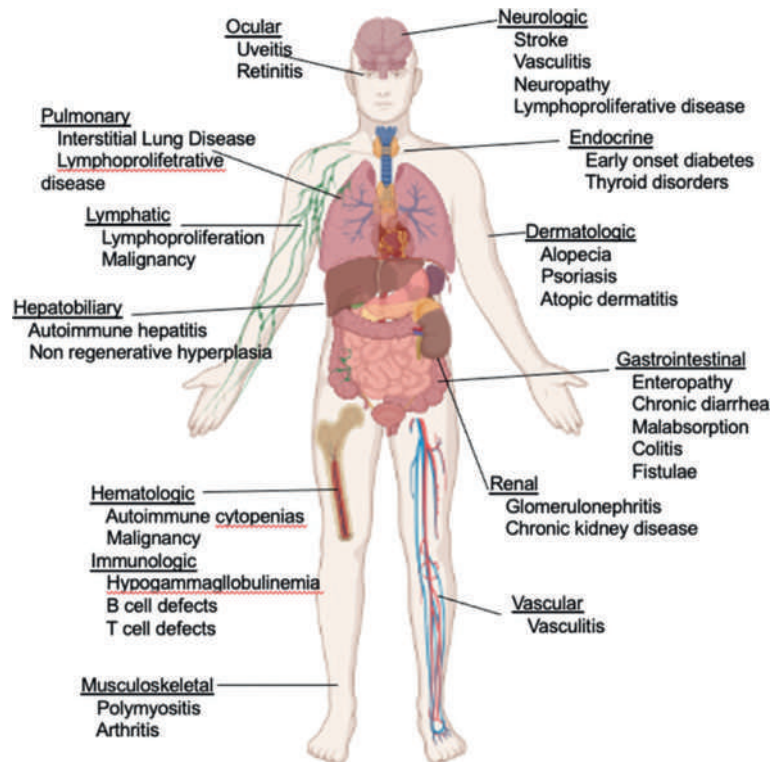


Fig. 1 Symptoms of PIRD.

self-tolerance (Kelsen et al., 2020). A mixed combination of the above phenotype can also occur in PIRD when a gene defect overlaps multiple immune regulatory pathways (Chan and Torgerson, 2020).

The pathophysiology of hyperinflammation and autoimmunity in PIRD is not well understood. Overactive innate and adaptive inflammatory responses with aberrant inflammatory cytokine release including exaggerated interleukin (IL)-1 signaling, Type I Interferon (IFN) signaling, and constitutive Nuclear factor (NF)- κ B activation are commonly implicated (de Jesus et al., 2015).

The increasing understanding of PIRD pathophysiology has promoted the development of innovative precision-based therapeutic approaches by targeting specific immunological pathways. Further, the management of PIRD is often led by a multi-disciplinary team, balancing precise immune modulation and potential increased risk of infection (Chan and Torgerson, 2020). Targeted therapy has been deemed beneficial in controlling PIRD related symptoms but its role in prevention of disease manifestations is not understood. Definitive therapy with HSCT has also been effective in several PIRDs. Mechanism-based therapy in PIRD can be used as a bridge for definitive therapy with hematopoietic stem cell transplantation (HSCT) (Arnold et al., 2021).

Tregopathies

Immunodysregulation-polyendocrinopathy-enteropathy-X-linked (IPEX) syndrome

IPEX results from a germline X-linked recessive mutation in *FOXP3* which encodes a transcriptional regulator protein crucial for Tregs development (d'Hennezel et al., 2012). IPEX generally presents in early infancy with enteropathy, endocrinopathies, and other severe autoimmune phenomena (Verbsky and Chatila, 2013; Gambineri et al., 2018).

Tregs modulate the immune response by engaging with antigen presenting cells via Cytotoxic T cell Antigen (CTLA)-4, or by secretion of IL-10 and transforming growth factor (TGF)- β . The defective peripheral tolerance in IPEX leads to uncontrolled CD4+ effector T cell proliferation and lymphocytic tissue infiltration (Gambineri et al., 2018). The disorder's most prominent and common feature is enteropathy, characterized by severe watery diarrhea and malabsorption that causes failure to thrive. Under microscopy, the bowel often shows villous blunting and lymphocytic infiltration of the damaged bowel lining. Dermatologic manifestations include rashes resembling eczema and alopecia which can vary in severity. The cutaneous biopsy often shows spongiotic psoriasiform features. Type I diabetes mellitus is the most common IPEX endocrinopathy. Hematologic manifestations include autoimmune hemolytic anemia and autoimmune thrombocytopenia. Additionally, patients may present autoimmune neurological dysfunction, lymphoproliferation, and increased susceptibility to infections (d'Hennezel et al., 2012; Bennett et al., 2001; Torgerson and Ochs, 2007).

Immunophenotyping often shows diminished FOXP3+ Tregs, elevated immunoglobulin (Ig) E, and eosinophilia. Additionally, IgM and IgG can be normal to slightly low due to enteric protein loss (d'Hennezel et al., 2012). Sanger sequencing or next

generation sequencing are often used for the detection of *FOXP3* mutations (Jamee et al., 2020). Various *FOXP3* mutations have been identified in most exons, introns, upstream, and downstream (poly A) of the coding region of *FOXP3* (Bennett et al., 2000).

Affected males often die within the first or second year of life, if IPEX is left untreated (Gambineri et al., 2018). Often, treatment plans are directed at patient stabilization with total parenteral nutrition, correction of glucose abnormalities secondary to endocrine deficits, and treatment of infections prior to considering immunomodulation (Park et al., 2020). Immunosuppression is the mainstay of therapy. Corticosteroids in combination with calcineurin inhibitors such as tacrolimus and cyclosporine, are often employed in the management of IPEX (Gambineri et al., 2018; Jamee et al., 2020). Steroids alone have shown minimal effectiveness (Gambineri et al., 2018). Sirolimus has also been effectively used, particularly in patients that do not tolerate calcineurin inhibitors (Yong et al., 2008).

The use of monoclonal antibody in IPEX is often in conjunction with other immunosuppressive agents. Tumor necrosis factor (TNF)- α inhibitors, anti-CD20, and abatacept have commonly been used for treatment with mixed success. Immunomodulation in IPEX, while beneficial, has been linked to increased disease recurrence, negatively impacting long-term disease-free survival (Barzaghi et al., 2018).

HSCT remains the only definitive approach in restoring normal numbers of competent Tregs and clinical improvement even with low-level mixed donor chimerism (Barzaghi et al., 2018; Lucas et al., 2007). T cell depletion using alemtuzumab or anti-thymocyte globulin have been employed to stabilize patients prior to HSCT. In general, reduced-intensity conditioning has leads to less morbidity and mortality but increased mixed chimerism (d'Hennezel et al., 2012; Lucas et al., 2007).

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) haploinsufficiency

CTLA-4 is an inhibitory regulator of T cell activation expressed by activated T cells and constitutively expressed by Tregs. CTLA-4 competes with CD28 in binding with CD80/86 on the surface of high affinity antigen presenting cells. While CTLA-4 binding provides an inhibitory response, CD28-CD80/86 binding activates T cells by co-stimulation (Farmer and Uzel, 2021). Thus, a defect in expression of CTLA-4 to increased CD28 co-stimulation, a lack of inhibition of T cell activation and ultimately the activation of self-reactive T cells against various tissues (Verma et al., 2017).

CTLA-4 haploinsufficiency presents with increased susceptibility to infections and varying forms of autoimmunity, frequently displaying incomplete penetrance and variable expressivity among members of the same family (Kuehn et al., 2014; Schwab et al., 2018). Autoimmune cytopenias, splenomegaly, and lymphocytic infiltration of non-lymphoid organs are features of CTLA-4 haploinsufficiency. Respiratory manifestations of the disease include pulmonary fibrosis, bronchiectasis, and infiltrative or granulomatous lymphocytic interstitial lung disease. Autoimmune enteropathy, autoimmune hepatitis, adrenal insufficiency, type I diabetes, arthritis, psoriasis, uveitis, and vitiligo are common CTLA-4 haploinsufficiency complications. Notably, malignancy risk for Hodgkin's lymphoma and gastric cancer is higher among this population (Verma et al., 2017; Schwab et al., 2018; Chellapandian et al., 2020).

The immunophenotype of CTLA-4 haploinsufficiency patients commonly shows hypogammaglobulinemia, decreased vaccine specific antibody titers, decreased CD19+ B cells, low switched-memory B cells, and expansion of autoreactive CD21^{low} B cells (Schwab et al., 2018; Ise et al., 2010). The T cell compartment characteristically shows increased polyclonal T cell activation, normal FOXP3+ Tregs numbers, and skewing toward a memory T cell phenotype (Verma et al., 2017; Kuehn et al., 2014).

Treatment requires attention to both the immunodeficiency and immunodysregulatory features. Immunoglobulin replacement therapy with or without prophylactic antibiotics should be considered to prevent severe infections (Verma et al., 2017). Historically, corticosteroids have been utilized to treat autoimmune enteropathy, cytopenias, infiltrative lung disease, and inflammation of the central nervous system (Schubert et al., 2014). Rapamycin has shown success in managing autoimmune cytopenias, nonmalignant lymphoproliferation affecting primarily the spleen and lymph nodes, and increased immunoglobulin consumption (Schwab et al., 2018). Other corticosteroid-sparing treatments that have been tried in the management of the multiple autoimmune complications in CTLA-4 deficiency include mycophenolate mofetil, cyclosporin, anti-thymocyte globulin, rituximab, and anti-tumor necrosis factor (TNF), all of which have shown some positive response (Verma et al., 2017). Abatacept and belatacept are CTLA-4 immunoglobulin fusion proteins (CTLA-4-Fc-IgG1) that act as soluble forms of CTLA-4. In more recent years, these mechanism-based therapies have shown promising results in a targeted approach to treating CTLA-4 functional loss (Chellapandian et al., 2020; Lee et al., 2016).

HSCT has been successful in treating severe immunodysregulatory symptoms or treatment refractory symptoms (Chellapandian et al., 2020).

Lipopolysaccharide-response beige-like anchor (LRBA) deficiency

LRBA deficiency is a autosomal recessive PIRD caused by a bi-allelic mutations in the *LRBA* (Habibi et al., 2019). LRBA is a cytoplasmatic protein that plays an essential role in regulating the traffic of intracellular vesicles, therefore preventing CTLA-4 protein lysosomal degradation, and allowing CTLA-4 re-circulation to the Tregs surface membrane (Lo et al., 2015).

The clinical spectrum of LRBA deficiency, resembles CTLA4 haploinsufficiency (Lo et al., 2015; Serwas et al., 2015). LRBA deficiency patients, most commonly present with antibody deficiency, and immunodysregulatory features such as autoimmune cytopenias, lymphocytic interstitial pneumonia, early onset type I diabetes, autoimmune hepatitis, alopecia, uveitis, and polyarthritis (Habibi et al., 2019; Kardelen et al., 2021; Lopez-Herrera et al., 2012; Alroqi et al., 2018; Gamez-Diaz et al., 2016). Malignancies such as gastric adenocarcinoma have been reported in this population (Chellapandian et al., 2020).

Neurologic disease such as central nervous system (CNS) demyelinating disease, myasthenia gravis, and brain granulomas have been reported (Gamez-Diaz et al., 2016). Immunophenotyping in LRBA deficiency, often reveals hypogammaglobulinemia, reduced switched memory B cells and plasmablasts, and variable numbers of CD4+ and/or CD8+ T cells (Habibi et al., 2019). Circulating T follicular helper (cTFH) are often increased and correlate with disease activity (Chellapandian et al., 2020).

Given the shared mechanism of disease between LRBA deficiency and CTLA-4 haploinsufficiency, both resulting in diminished CTLA-4 protein expression, abatacept has been used to treat LRBA deficiency manifestations with success as well. Abatacept has proven to be beneficial in the management of autoimmune cytopenias, interstitial lung disease, nonmalignant lymphoproliferation and autoimmune enteropathy; even though in selected cases the addition of sirolimus was necessary to achieve remission of enteropathy (Lo et al., 2015; Kiykim et al., 2019; Leiding et al., 2018). Clinical improvement with the use of abatacept correlates with reduction in cTFH, reinforcing the role of cTFH quantities as a biomarker of disease activity (Kiykim et al., 2019). HSCT has been curative for LRBA deficient patients with severe autoimmune complications (Kardelen et al., 2021; Tesch et al., 2020).

Signal transducer and activator of transcription 1 (STAT1) gain of function (GOF)

STAT1 GOF is a monogenic disorder due to heterozygous mutation in *STAT1* that confer GOF. STAT1 is a 1 of a 7-member family of transcription factors that includes essential for transmitting various cytokine signals from the cellular membrane to the nucleus to change gene expression. STAT1 in particular increased IFN- γ responsive genes.

Some of the prominent clinical features observed in STAT1 GOF include early-onset chronic mucocutaneous candidiasis, susceptibility to dimorphic fungal infections, bacterial and viral susceptibility, and immune dysregulation. Early onset type 1 diabetes, thyroid disease, autoimmune cytopenias, interstitial lung disease, and enteropathy are the most frequent immunodysregulatory features (Zhang et al., 2021a). Interestingly, patients with STAT1 GOF are also at risk of cerebral aneurysms. Cerebral aneurysms and cancers have also been noted in 6% of these patients (Toubiana et al., 2016). The pathophysiology of autoimmunity in STAT1 GOF is thought to be secondary to aberrant lymphocyte activation and signaling, abnormal Th1 and follicular helper T cell responses, impaired function of natural killer cells, and reduction in Th17 quantities (Chaimowitz et al., 2020).

Antimicrobial prophylaxis, including antifungal and antibiotics, are often first line treatment to address the increased infection susceptibility while immunoglobulin replacement therapy is often used in patients with frequent respiratory tract infections (Toubiana et al., 2016). Janus kinase (Jak) inhibitors are an emerging category of targeted therapy for patients with STAT1 GOF (Chan and Torgerson, 2020; Chellapandian et al., 2020; Chaimowitz et al., 2020; Forbes et al., 2018). HSCT for STAT1 GOF has been pursued in the setting of life-threatening complications such as severe infections, hemophagocytic lymphohistiocytosis (HLH), or refractory autoimmunity. The HSCT experience is limited ($n = 15$), with a poor overall survival (40%) and elevated rate (50%) of secondary graft failure (Leiding et al., 2018).

Signal transducer and activator of transcription 3 (STAT3) GOF

STAT3 is a transcription factor critical for various cellular processes. Heterozygous, somatic *STAT3* mutations have been described in malignancies such as large granular lymphocytic leukemia. In more recent years, germline, heterozygous GOF mutations in *STAT3* have been identified in patients presenting with early onset multi-organ autoimmunity, nonmalignant lymphoproliferation, and growth failure (Vogel et al., 2015).

On average, STAT3 GOF patients debut with symptoms at the age of 4 years (Fabre et al., 2019). Common autoimmune manifestations include autoimmune cytopenias, lymphadenopathy, splenomegaly, type I diabetes, thyroid disease, lymphocytic interstitial pneumonia, and enteropathy among others (Vogel et al., 2015). Immune phenotyping varies significantly among STAT3 GOF patients; decreased class-switched memory B cells, decreased natural killer cell numbers, reduced plasmacytoid dendritic cells, and increased double negative T cells are some of the features seen in STAT3 GOF. Additionally, some patients display low circulating FOXP3+ Tregs (Vogel et al., 2015).

Immunosuppression is the mainstay of treatment in STAT3 GOF. Tocilizumab, an IL-6 receptor blocker has been shown to be beneficial in the management of polyarthritis, scleroderma, autoimmune hepatitis, interstitial lung disease, enteropathy, and nonmalignant lymphoproliferation. Additionally Jak inhibitors such as tofacitinib, have been successful at treating life threatening disease complications (Forbes et al., 2018; Milner et al., 2015). HSCT has been performed in a very limited number of patients with one patient surviving (Leiding and Forbes, 2019).

STAT5B deficiency

STAT5b is one of two STAT5 proteins which are necessary for transducing signals to the IL-2 receptor (Verbsky and Chatila, 2011). STAT5b deficiency is an autosomal recessive disease first described in an Argentinian girl with failure to thrive, prominent forehead, saddle nose, growth failure, and delayed puberty, secondary to growth hormone deficiency (Verbsky and Chatila, 2013). Severe postnatal growth failure is primarily due to growth hormone insensitivity and severe insulin-like growth factor (IGF-1) deficiency. What distinguishes STAT5b deficiency patients from those with growth hormone insensitivity syndrome due to growth hormone receptor mutations is the presence of chronic pulmonary disease and dysregulated T cell function (Hwa et al., 2011). Other manifestations of STAT5b deficiency include growth hormone resistant failure to thrive, recurrent bacterial and viral pneumonia,

lymphoid interstitial pneumonitis, autoimmune cytopenia, eczema, and idiopathic arthritis (Verbsky and Chatila, 2013; Bezrodnik et al., 2015). Immunophenotyping often shows decreased circulating FOXP3+ Tregs, T memory phenotype skewing, presence of autoantibodies, and elevated levels of IgE (Foley et al., 2021). Patients can usually live through adulthood with supportive care; however, overall management is guided by specific end-organ pathology with antimicrobial prophylaxis and, in selected cases, solid organ transplant (e.g., lung) (Hwa et al., 2011; Kanai et al., 2012). HSCT has shown promising results in murine studies (Bezrodnik et al., 2015).

IL2R α chain or CD25 deficiency

CD25 deficiency is a rare PIRD initially described in 1997 by Sharfe et al., as a combined immunodeficiency (Roifman, 2000). CD25 deficiency results from autosomal recessive mutations in *IL2RA*. As of 2019, there were only 9 reported cases from 5 unrelated families (Vignoli et al., 2019).

CD25 (IL2R α chain) represents the alpha chain of the high affinity IL-2 receptor, a structural complex consisting of one alpha, one beta (CD122), and one gamma (CD132) subunit. This IL-2R α BY complex is constitutively expressed on Tregs and plays an essential immunoregulatory role by competing with effector T cells for IL-2 during an immune response (Verbsky and Chatila, 2011; Vignoli et al., 2019). In addition, signaling through the high affinity IL-2 receptor that includes CD25, promotes Treg transcription of *FOXP3* via the IL2/IL2R α -STAT5b transduction pathway and Treg induced IL-10 production (Zorn et al., 2006; Caudy et al., 2007). CD25 deficiency manifests similarly to IPEX with autoimmune manifestations and infection susceptibility (Vignoli et al., 2019; Caudy et al., 2007). CD25 deficiency immune-mediated features include bullous pemphigoid, autoimmune thyroiditis, and alopecia universalis (Verbsky and Chatila, 2013).

IL-15 and IL-2 use the IL-2 receptor β and γ chains for signal transduction, only differing in the alpha subunits for ligand specificity. In vitro data suggest that deficient CD25 CD4+ T cell can restore their proliferation capability when exposed to high IL-5 or IL-2, however further studies are needed in humans to assess for efficacy and safety of these agents in this study population (Caudy et al., 2007). HSCT was recently pursued in an infant with successful immune reconstitution after a second matched unrelated donor transplant with RIC (Vignoli et al., 2019).

Hyperinflammatory disorders predisposing to hemophagocytic lymphohistiocytosis (HLH)

HLH is a rare life threatening hyperinflammatory syndrome due to uncontrolled activation of cytotoxic lymphocytes and macrophages in conjunction with increased cytokine release (Chellapandian et al., 2013). Organ dysfunction in HLH (e.g., bone marrow, liver, and brain) is often mediated by activated lymphocytes and macrophages infiltration (Chan and Torgerson, 2020; Janka and Lehmborg, 2014).

The HLH 2004 diagnostic criteria constitute the most commonly used tool in both pediatric and adult populations. A patient must meet at least 5 of the 8 diagnostic criteria: fever, splenomegaly, ≥ 2 cell lineage cytopenia, hyperferritinemia, increased soluble IL2 receptor, decreased natural killer cell cytotoxicity, hemophagocytosis (in bone marrow or spleen or lymph nodes), and hypertriglyceridemia or hypofibrinogenemia (Table 1) (Lehmborg et al., 2015; Henter et al., 2007).

Primary HLH is caused by defects in genes involved in 3 immune pathways: cytolytic defects (*LYST*, *PRF1*, *RAB27A*, and *UNC13D*); signaling defects (*MAGT1* and *SH2D1A*); and inflammasome defects (*NLRP4* and *XIAP*) (Chan and Torgerson, 2020).

Table 1 Diagnostic criteria for hemophagocytic lymphohistiocytosis.

1. A molecular diagnosis consistent with HLH is made
2. Diagnostic Criteria for HLH are fulfilled (5 of 8 criteria below)
 1. Fever
 2. Splenomegaly
3. Cytopenias (affecting 2 of the 3 lineages in the peripheral blood):
 - a. Hemoglobin <90 g/L (in infants <4 weeks of age, hemoglobin <100 g/L)
 - b. Platelets $<100 \times 10^9$ /L
 - c. Neutrophils $<1.0 \times 10^9$ /L
4. Hypertriglyceridemia and/or hypofibrinogenemia
 - a. Fasting triglycerides ≥ 3.0 mmol/L (i.e., ≥ 265 mg/dL)
 - b. Fibrinogen ≤ 1.5 g/L
5. Hemophagocytosis in bone marrow or spleen or lymph nodes
6. Low or absent NK cell activity (according to local laboratory reference)
7. Ferritin ≥ 500 mg/L
8. Soluble IL-2 receptor (i.e., soluble CD25) ≥ 2400 U/mL

Adapted from Henter JL, Horne A, Aricó M et al. (2007) HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatric Blood & Cancer* 48(2):124–131.

Secondary HLH can occur secondary to severe infections, malignancies, or rheumatological conditions (e.g., systemic onset juvenile rheumatoid arthritis [sJIA]) (La Rosee et al., 2019). Once HLH criteria are met, a diagnosis can be made by assessing protein quantities encoded by HLH relevant genes. Targeted genetic testing can also aid in establishing a diagnosis. Additionally, several clinical and laboratory considerations may help distinguish some of these disorders (Canna and Marsh, 2020). For example, in a male patient with EBV-induced lymphoproliferation, X-linked lymphoproliferative syndrome(s) should be considered (Chandrakasan et al., 2019; Sepulveda and de Saint, 2017).

To effectively treat HLH, induction of remission and hyperinflammation control first must be achieved. Elimination of the eliciting trigger, and finally definitive therapy of the underlying primary disorder should follow (Arnold et al., 2021). Chemotherapeutic and potent immunosuppressive drugs such as etoposide and dexamethasone derived from HLH-94 and HLH-2004 protocols, have effectively been used to induce HLH remission (Arnold et al., 2006; Bergsten et al., 2017). A chemotherapy sparing single center ($N=38$) study of HLH patients who underwent induction of remission with steroids, anti-thymocyte globulin (ATG), and cyclosporine showed a remission rate of 70%, but increased relapse rate prior HSCT (Mahlaoui et al., 2007). Alemtuzumab (anti-CD52 humanized monoclonal antibody) co-administered with methylprednisolone and cyclosporine had a rate of remission of 64% and may constitute an acceptable alternative modality for etoposide refractory/intolerant HLH patients (Marsh et al., 2013). In a recent open label phase II-III clinical trial enrolling refractory, recurrent, progressive or intolerant to conventional HLH therapy primary HLH pediatric patients, a human anti-IFN- γ antibody: emapalumab, in combination with dexamethasone, showed significant efficacy (64.7%) in controlling HLH hyperinflammation, and an overall survival to HSCT of 79% with concomitant decrease in disease activity biomarkers such as IFN- γ and CXCL9 (Locatelli et al., 2020). Several trials are underway, assessing ruxolitinib efficacy and tolerability for treatment of secondary HLH and refractory HLH (Das et al., 2016; Albeituni et al., 2019). After remission is achieved definitive treatment with HSCT should be pursued in patients with primary HLH (Chandrakasan et al., 2019).

Autoinflammatory disorders

Autoinflammatory disorders represent a group of diseases characterized by an exaggerated and hyperinflammatory response to innate danger signals, infection, and other exogenous and endogenous factors. Inflammasomes are a key component of the innate immune system and are responsible for regulating the inflammatory response to danger signals. Through the rapid production and signaling of cytokines such as IL-1, IL-18, and IL-33, the innate immune system begins the cascade of killing and elimination of the point of danger.

TNF receptor associated periodic fever syndrome (TRAPS)

First described in 1982, TRAPS is one of the first identified autoinflammatory disorders (Menon and Efthimiou, 2018). Recessive and dominant mutations in tumor necrosis factor receptor (*TNFR1*) have been reported as has variability in penetrance. Those with high penetrance mutations present with early disease onset, whereas low penetrance mutations can manifest as an adult-onset disease with less severe features (Chan and Torgerson, 2020; Menon and Efthimiou, 2018). Patients most often present with periodic episodes of fever, pericarditis, cervical lymphadenopathy, conjunctivitis, abdominal pain, and serositis. Due to excessive chronic inflammation, a long-term complication observed in TRAPS is systemic amyloidosis with SSA fibril deposition in organs (Menon and Efthimiou, 2018).

Systemic steroids and non-steroidal anti-inflammatory drugs are often first line therapy. Targeted IL-1 inhibitor therapy with IL-1 inhibitors such as anakinra or canakinumab have been effective as well. TNF- α inhibitors are not preferred monotherapy due to high side effect profile and discontinuation rate and loss of efficacy (Menon and Efthimiou, 2018; Bettiol et al., 2019; Brogan et al., 2019).

Cryopyrin-associated periodic syndromes (CAPS) and deficiency of IL-1 receptor antagonist (DIRA)

Mutations in *NLRP3* were first described as the cause of familial cold autoinflammatory syndrome (FCAS) and Muckle-Wells Syndrome (Hoffman et al., 2001) but have now been coined as members of a larger group of disorders, cryopyrin-associated periodic syndromes (CAPS) (Martinon et al., 2002). More recently, mutations in *NLRP3* have been described as the cause of neonatal-onset multi-system inflammatory disease (NOMID) (Goldbach-Mansky et al., 2006). *NLRP3* encodes for cryopyrin, GOF autosomal dominant and de novo mutations cause *NLRP3* activation and upregulation of cleavage of inactive pro IL-1 β and pro-IL-19 into their active forms (Martinon et al., 2002). There are distinct features of FCAS, MWS, and NOMID but in general, patients present with early onset periodic fevers and arthralgias. NOMID is the most severe form of CAPS with manifestations of central nervous system involvement. DIRA is caused by biallelic loss of function mutation in *IL1RN* leading to reduced expression of an antagonist of IL-1 signaling. Patients present in the first few weeks of life with continuous fever, pustular rash, arthralgias, and periosteal lesions (Aksentijevich et al., 2009; Feldmann et al., 2002).

Three IL-1 blocking drugs are approved by the FDA for treatment of CAPS and/or DIRA (Goldbach-Mansky et al., 2006; Hoffman et al., 2004, 2008). Anakinra is a short acting recombinant IL-1 receptor antagonist that blocks IL-1 α and IL-1 β binding the IL-1 receptor; rilonacept is a fusion protein made of IL-1R1 and IL-1RAcP complexed to the Fc portion of IgG1 acting as a soluble decoy

receptor preventing interaction of IL-1 and its receptor; canakinumab is also an IL-1 fusion protein that targets IL-1 β . Canakinumab has proven to show more persistent improvement in clinical and laboratory parameters in patients with CAPS. This is likely secondary to a longer half-life (28–30 days), less frequent need for administration (every 8 weeks), and minor injection-site reactions. However, the risk of infection is higher compared to the other therapeutic choices (Brydges et al., 2013).

Familial Mediterranean fever (FMF)

FMF is the most common autosomal recessive disorder among all autoinflammatory diseases, most prevalent among people with an eastern Mediterranean or Middle eastern background. Autosomal recessive mutations in *MEFV* cause FMF (Nature Genetics, 1997). *MEFV* encodes for pyrin which is important for innate system regulated inflammasome activation. Clinical manifestations include recurrent attacks of fever, polyserositis, elevated inflammatory markers, abdominal pain, and arthralgias. Inflammatory markers are also elevated during attacks (Egeli and Ugurlu, 2020). Secondary amyloidosis and end stage renal disease are the most severe complications of FMF. For nearly 50 years, colchicine has been the mainstay of treatment. It has proven efficacy in preventing or improving inflammatory attacks and in preventing secondary amyloidosis (Zemer et al., 1974). For patients poorly responsive to colchicine, IL-1 inhibitors have been effective in reducing inflammatory attacks (Atas et al., 2021).

NLR containing CARD domain-containing protein 4 (NLRC4) gain of function

GOF mutations in *NLRC4* cause early onset autoinflammation that presents as macrophage activation syndrome or enterocolitis (Canna et al., 2014; Romberg et al., 2014). *NLRC4* activation leads to caspase-1 and caspase-8 activation and consequent overproduction of pro-inflammatory cytokines IL-18 and IL-1 β . Laboratory findings include elevated inflammatory markers and pancytopenia. Treatment with IL-1 inhibitory agents has mixed success. Recently, targeted therapy with IL-18 binding protein (tadekinig -alfa) has been successful in improving disease manifestations (Canna et al., 2017). A phase 3 clinical trial (NCT03113760) is active using IL-18 binding protein for the treatment of IL-18 driven autoinflammation including *NLRC4*.

Pyogenic, arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome

PAPA is a devastating autosomal dominant disease first described in 1975 (Jacobs and Goetzl, 1975). Dominant mutations in proline-serine-threonine phosphatase-interacting protein 1 lead to hyperphosphorylation and increased binding of pyrin (Yeon et al., 2000). As a consequence, elevations in TNF- α and IL-1 β are observed (Waite et al., 2009). Clinical manifestations include early onset painful flares of recurrent sterile arthritis with a prominent neutrophilic infiltrate, skin ulceration, pyoderma gangrenosum, and cystic acne. Symptoms persist into adulthood and lead to joint destruction. IL-1 blocking agents have led to limited improvement but TNF- α inhibitors have shown promise in treating disease manifestations (Stichweh et al., 2005).

Blau syndrome

Blau Syndrome is a hyperinflammatory disease caused by mutations in nucleotide oligomerization domain protein 2 (*NOD2*). *NOD2* an intracellular bacterial pattern recognition receptor that activates receptor interacting protein2, mitogen-activated protein kinase, NFkB, to turn on antimicrobial genes. Clinical manifestations are characterized by early-onset hereditary granulomatosis with a triad of polyarthritis, rash, and uveitis (Blau, 1985). Treatment with TNF- α inhibitors have shown variable success (Nagakura et al., 2017).

Deficiency of adenosine deaminase 2 (DADA2)

DADA2 is an autosomal recessive disease secondary to LOF mutations in *ADA2* (formerly called *CECR1*) (Zhou et al., 2014). ADA proteins regulate purine metabolism by catalyzing adenosine and deoxyadenosine. ADA2 is highly expressed in monocytes and macrophages and DADA2 leads to M1 polarization of macrophages promoting inflammation and tissue damage. Clinical manifestations include: vasculopathy of small medium sized arteries, acute and chronic strokes, polyneuropathy, and vasculitis. Strokes can begin as early as infancy (Zhou et al., 2014). In a subset of patients, severe anemia may be the only symptom resembling Diamond-Blackfan anemia (Hashem et al., 2017a). Corticosteroids have been the mainstay of treatment, but TNF- α inhibitors have been shown to be effective as well (Zhang et al., 2021b). HSCT has also provided definitive therapy (Hashem et al., 2017b).

Interferonopathies

Overactivation of type I interferon response leads to type I interferon driven autoinflammatory diseases. Hyperactivation of type I interferons causes a variety of organ specific manifestations including fever with elevated inflammatory markers.

Stimulator of interferon genes (STING) -associated vasculopathy with onset in infancy (SAVI)

STING via retinoic acid-inducible gene I aids cyclic guanosine monophosphate-adenosine monophosphate synthase to recognize DNA sequences and initiate an inflammatory response. STING then signals through NFkB or interferon regulator factor 3 to stimulate the immune response (Burdette and Vance, 2013; Ishikawa and Barber, 2008). GOF mutations in *TMEM173*, which encodes for STING, cause STING associated vasculopathy (Liu et al., 2014) and familial chilblain lupus (Konig et al., 2017). Patients present with early onset rash, elevated acute phase reactants, and fever. The rash is blistering and telangiectatic and worsens with cold temperature exposure. Digital necrosis can result from the small vessel vasculopathy (Liu et al., 2014; Konig et al., 2017). Treatment with JAK-STAT inhibitors, coined jakinibs, has shown reduced disease burden (Kim et al., 2016; Sanchez et al., 2018).

Proteasome associated autoinflammatory syndromes (PRAAS)

Proteasome-autoinflammatory syndromes are caused by defects in immune proteasomes such as *PSMA3*, *PSMB8*, *PSMB9*, and *POMP* (Brehm et al., 2015; Poli et al., 2018). Mutations in proteasome subunits lead to accumulation of ubiquitinated proteins, endoplasmic reticulum stress, and an exacerbated interferon response (Manthiram et al., 2017). Clinical symptoms include chronic autoinflammation, fever, neutrophilic dermatosis, and lipodystrophy. *POMP* is a chaperone for proteasome assembly. Autosomal dominant mutations in *POMP* cause a form of PRAAS termed *POMP*-related autoinflammation and immune dysregulation (PRAID). Markedly elevated type I interferon responses occur in PRAAS. Treatment with JAK-STAT inhibitors has been effective at controlling disease related manifestations (Boyadzhiev et al., 2019). Curative treatment with HSCT has also been effective in one patient with PRAID (Martinez et al., 2021).

ALPS And ALPS-like diseases

Autoimmune lymphoproliferative syndrome (ALPS)

ALPS is a disorder of abnormal lymphocyte apoptosis associated with polyclonal populations of double negative T cells that are CD3+, express αβ antigen receptors, and are CD4- and CD8-. Multiple defects have been described among genes involved in extrinsic and intrinsic apoptosis pathways. Most patients with ALPS have variants in *FAS* or *FASL* disrupting programmed T cell death. Clinical manifestations include chronic persistent and recurrent benign lymphadenopathy, splenomegaly, hepatomegaly, and autoimmune cytopenias. Malignancies are more common in ALPS, specifically Hodgkin and non-Hodgkin lymphoma. Diagnosis relies on fulfilling a set of criteria (Table 2) (Oliveira et al., 2010; Teachey and Lambert, 2013). Immune suppression with agents such as MMF and sirolimus have proven effective (Miano et al., 2015; Rao et al., 2005).

ALPS-like diseases

Approximately 20% of ALPS patients do not have an identifiable monogenic defect. ALPS-like diseases are those primary immunodeficiency diseases that mimic an ALPS phenotype but are due to defects outside of the Fas-FasL pathway (López-Nevado et al., 2021). Patients may have similar disease manifestations but have other symptoms not consistent with ALPS, have no identified genetic variant in ALPS associated genes, and may not fulfill other diagnostic criteria for ALPS. *CTLA4* haploinsufficiency, *LRBA* deficiency, and *STAT3* GOF are classic examples of ALPS-like diseases described earlier in this chapter.

Table 2 Diagnostic criteria for autoimmune lymphoproliferative syndrome.

Required
1. Chronic non malignant lymphoproliferation (>6 months lymphadenopathy and/or splenomegaly)
2. Elevated peripheral blood αβ + CD4-CD8- T cells
Accessory
Primary
1. Defective in vitro Fas-mediated apoptosis (in 2 separate assays)
2. Somatic or germline mutation in ALPS causative gene (<i>FAS</i> , <i>FASL</i> , <i>FADD</i> , <i>CASP10</i> , <i>CASP8</i>)
Secondary
1. Elevated biomarkers (Any of following)
a. Plasma soluble FASL >200 pg/mL
b. Plasma IL-10 > 20 pg/mL
c. Plasma or serum vitamin B ₁₂ > 1500 ng/L
d. Plasma IL-18 > 500 pg/mL
2. Immunohistochemical findings consistent with ALPS as determined by experienced histopathologist
3. Autoimmune cytopenias and polyclonal hypergammaglobulinemia
4. Family history of ALPS or nonmalignant lymphoproliferation

Definitive Diagnosis: Required plus 1 primary accessory criterion.
Probable Diagnosis: Required plus 1 secondary accessory criterion.
Modified from Teachey DT and Lambert MP (2013) New advances in the diagnosis and treatment of autoimmune lymphoproliferative syndrome. *Current Opinion in Pediatrics* 24:1–8, Table 2, p. 4.

Activated PI3K delta syndrome

GOF mutations in the genes encoding p110 δ (*PIK3CD*) catalytic subunit and p85 (*PI3KR1*) regulatory subunit of phosphoinositide 3-kinase (PI3K) cause a combined immunodeficiency (Coulter and Cant, 2018; Elkaim et al., 2016; Lucas et al., 2014). PI3K is a lipid kinase that phosphorylates phosphatidylinositol-4,5 biphosphate generating phosphatidylinositol 3,4,5-triphosphate. Non-malignant lymphoproliferation that manifests as lymphadenopathy, hepatosplenomegaly, nodular mucosal lymphoid hyperplasia is hallmark of the disease. Additionally, patients are predisposed to bacterial sinopulmonary infections and invasive viral infections particularly with EBV, CMV, HSV, and VZV. The immunophenotype includes B and T cell dysfunction. Immunoglobulin levels and vaccine responses are variable, serum IgM is often elevated, and reduction in naïve T cells with increased quantities of senescent T cells are observed. Immunosuppression is the mainstay of treatment; both rituximab and sirolimus have shown clinical benefit (Maccari et al., 2018). Leniolisib, a PI3K δ inhibitor is under investigation for treatment of APDS. Early results are promising showing reduction or resolution in lymphoproliferation, normalization of circulating naïve and transitional B cell quantities, decreases in IgM, and normalization of senescent T cell quantities (Rao et al., 2017). HSCT has also been successful in the treatment of several APDS patients (Nademi et al., 2017).

Very early onset inflammatory bowel disease (VEO-IBD)

Inflammatory bowel disease that presents in children < 6 years of age is considered very-early onset. Inborn errors of immunity are detected in 15–20% of patients with VEO-IBD. The VEO-IBD is a presenting or associated symptom of the primary immunodeficiency disease. Patients with infantile onset (<2 years age), perianal disease, susceptibility to infections, and abnormal family history are more likely to be affected by a monogenic disease. More than 50 genetic variants have now been described as a cause of VEO-IBD. Whole exome sequencing and targeted sequencing panes are key components of the diagnostic approach to patients with VEO-IBD (Kelsen et al., 2020).

IL-10/IL-10R deficiency

Defects in the IL-10/IL-10 receptor signaling pathway were one of the first recognized monogenic cause of VEO-IBD. IL-10 is an anti-inflammatory cytokine that maintains homeostasis through prevention of an excessive pro-inflammatory immune response. Biallelic LOF mutations in *IL10*, *IL10RA*, *IL10RB* cause severe intestinal inflammation, particularly enterocolitis, folliculitis, and perianal disease in the infantile period (Moore et al., 2001; Shim et al., 2013). HSCT has been lifesaving as treatment in these patients (Engelhardt et al., 2013).

Severe atopic disease

Primary atopic disorders are a group of monogenic inborn errors of immunity in which atopic atopy is significant symptom of disease. Atopic clinical manifestations include atopic dermatitis, allergic rhinitis, food allergy, drug allergy, and other allergic disorders. Co-morbidities such as infection susceptibility and variable immune system abnormalities occur as well. Defects in impaired T cell signaling and cytoskeletal remodeling, cytokine signaling, T cell repertoire restriction, tolerance failure, glycosylation, skin barrier disruption, and mast cell degranulation all have allergic disease as a major feature (Milner, 2020).

Conclusions

Primary Immune Regulatory Disorders are a new and evolving group of inborn errors of immunity in which immune mediated autoimmunity, hyperinflammation, and lymphoproliferation are key features. Nearly every organ system can be affected in PIRD diseases, which can delay diagnosis. Targeted therapies have become increasingly more available as the mechanism of various PIRDs is realized. Recognition of the key clinical features of PIRD are critical to establishing a diagnosis and providing targeted therapies to treat disease related manifestations.

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Defects in Innate Immunity: Receptors and Signaling Components

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Introduction

The human immune system, designed to provide defense against pathogens, is comprised of innate and adaptive immunity responses. The innate immune system presents fast responses that precede the adaptive responses. It is composed of a diverse range of components that need no additional training to confront a pathogen and function by means of preformed receptors. On the contrary, adaptive immunity presents slow responses by a limited variety of cells, mainly lymphocytes, which need additional training to develop unique receptors and become capable of instigating more efficient responses.

For detecting invading pathogens, the innate immune system relies upon diverse components, key members of which are cytoplasmic and membrane-bound pattern recognition receptors (PRRs), including [Toll-like receptors \(TLRs\)](#), [C-type lectin receptors \(CLRs\)](#), [NOD-like receptors \(NLRs\)](#), and [RIG-I-like receptors \(RLRs\)](#). These receptors bind to specific proteins of various microorganisms and [pathogen-associated molecular patterns \(PAMPs\)](#). The minor variety of innate immunity receptors is compensated by targeting shared microbial components and common infection danger signals ([Wong et al., 2013](#)). Some other cellular and humoral components of innate immunity include macrophages, natural killer (NK) cells, granulocytes, dendritic cells, cytokines, chemokines, defensins, and the complement system.

Genetic defects in the innate immune system cause primary immunodeficiencies (PIDs), also known as inborn errors of immunity (IEIs), resulting in increased susceptibility to serious infections by miscellaneous pathogens, such as mycobacteria, viruses, and fungi. These pathogens cause a severe form of infection in patients with PIDs while are non-pathogenic or cause mild infection in normal people. In the last three decades, there have been considerable advances in genetic and sequencing technologies, which dramatically increased our understanding of innate immunity components and receptors. Consequently, the breakthrough in the discovery of mutations enabled us to develop our knowledge of PID phenotypes affecting the innate immune system. Some of the identified mutations cause general defects in the function of innate immunity, whereas others cause more specific defects in

particular pathways of the innate immunity. Thus, a golden opportunity has been created for the clinical field to provide tentative diagnosis and proper treatment for patients. However, as the field of innate PIDs is tremendously varied, some PIDs and their pathogenesis may have not yet been identified, indicating the inevitability of further investigations.

In this chapter four groups of defects associated with innate immunity receptors and signaling will be discussed:

1. Defects in the PRRs (namely TLR, CLR, and NLR) signaling;
2. Defects causing Mendelian susceptibility to mycobacterial diseases;
3. Defects causing WHIM (Warts, Hypogammaglobulinemia, Infections and Myelokathexis) syndrome;
4. Defects causing Epidermodysplasia verruciformis.

Defects of the pattern recognition receptors

The innate immune system takes the pivotal first steps in creating a defense barrier against invading pathogens. Its ability in restraining infections early in the disease relies strongly on the deployment of germline-encoded pattern recognition receptors (PRRs), expressed mainly on immune cells. It initiates host defense against infection through the recognition of common microbial structures, termed pathogen-associated molecular patterns (PAMPs), by PRRs. To date, five main families of PRRs categorized into two groups have been described. The first group contains membrane-bound PRRs namely Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), while the second group contains intracellular cytoplasmic PRRs that are Nod-like receptors (NLRs), AIM2-like receptors (ALRs), and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). PAMPs are common conserved structures between different pathogen classes and can be nucleic acids, cell wall components, or metabolic products. PRRs can also recognize damage-associated molecular patterns (DAMPs), which are host signals associated with cell damage or death in both intracellular and extracellular spaces. Recognition of PAMPs by intracellular or membrane PRRs induces infection signals resulting in the activation of some transduction pathways that contribute to host defense in two ways. Firstly, they give rise to the synthesis of proinflammatory factors including cytokines and chemokines, and secondly, it builds a bridge between the innate and adaptive immune system and triggers the activation of adaptive immunity.

Given the fact that PRRs play a crucial role in pathogen recognition, genetic defects and polymorphisms in the genes encoding PRRs may lead to a considerable increase in susceptibility to infections, either broad or restricted and permanent or transient (Table 1). PRR-associated PIDs usually decrease in inverse proportion to patients' age, have broadly variable severity, and are quite specific for certain families of pathogens. To date, PIDs have been described in three major families of PRRs with a substantial focus on the TLR family. Enhanced practical understanding of the pathophysiology of infections have provided novel therapeutic targets.

TLRs

The discovery of the TLR family dates back to the mid-1990s when a crucial defense mechanism in flies against fungal infections by *Drosophila* Toll proteins was detected (Lemaitre et al., 1996). This led to the introduction of similar protein receptors in the human immune system called TLRs. Consequently, being important type I transmembrane proteins of the innate immunity, the TLR family in humans was extensively studied. TLRs are expressed by a wide variety of cells, including macrophages, dendritic cells, fibroblasts, endothelial and epithelial cells, and B and T lymphocytes. They recognize both DAMPs and PAMPs, resulting in induction of early response against bacteria, viruses, fungi, and parasites as well as triggering the first steps of adaptive immunity. Human innate immunity has 10 TLRs divided into intracellular and extracellular categories. The former category consists of TLR3, TLR7, TLR8, and TLR9 and the latter includes TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10. As a consequence of TLRs binding to pathogenic ligands,

Table 1 Common infectious agents in human innate immunity defects.

Innate immunity defect	Examples of possible invading pathogens
IRAK/MYD88 deficiency	<i>Streptococcus pneumoniae</i> , <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i>
TLR3 deficiency	HSV-1
TLR5 deficiency	<i>Legionella pneumophila</i>
TLR4 deficiency	Gram-negative bacteria
TLR2 deficiency	<i>Mycobacterium leprae</i> , <i>Mycobacterium tuberculosis</i>
Anhidrotic ectodermal dysplasia	<i>S. pneumoniae</i> , <i>Haemophilus influenzae</i> , <i>S. aureus</i> , <i>Mycobacteria</i> , HSV, CMV, HPV, <i>Pneumocystis jirovecii</i> , parasites
Dectin-1/CARD9 deficiency	<i>Candida</i> , invasive aspergillosis, <i>Phialophora verrucosa</i> , <i>Corynespora cassiicola</i>
MBL deficiency	<i>Neisseria meningitidis</i> , vulvovaginal candidiasis
NLRs deficiency	<i>S. aureus</i> , <i>Salmonella</i> , mucocutaneous candidiasis, <i>Giardia intestinalis</i>
MSMD	<i>Mycobacterium bovis</i> , <i>Mycobacterium avium</i> , <i>M. tuberculosis</i> , <i>Salmonella</i> , HSV-1, CMV, HHV-8
WHIM syndrome	bacterial infections, HPV, EBV
Epidermodysplasia verruciformis	HPV

HSV-1, Herpes simplex virus-1; CMV, Cytomegalovirus; HPV, Human papillomavirus; HHV-8, Human herpes virus 8; WHIM syndrome, warts, hypogammaglobulinemia, infections, and myelokathexis syndrome; MSMD, Mendelian susceptibility to mycobacterial diseases.

some signals are sent to the cell nucleus that provoke the secretion of cytokines, type I interferon (IFN), and antimicrobial peptides. This pathway also induces a cascade of adaptive immune responses that have a prolonged protective effect in the process of combating the invading pathogen.

It has been demonstrated that each TLR has a particular microbial ligand and becomes activated by specific PAMPs. TLR4 recognizes and binds to lipopolysaccharide (LPS) component of Gram-negative bacteria, heat-shock protein 60 and 70, the fusion protein of the respiratory syncytial virus, and fungal mannan (Bulut et al., 2002; Tada et al., 2002). TLR2, in concert with TLR1 or TLR6, detects peptidoglycan, lipopeptide, di- or triacylated lipoprotein of Gram-positive bacteria and regulates anti-streptococcal and anti-staphylococcal responses (Takeuchi et al., 1999, 2002). TLR3 recognizes and binds to double-stranded RNA (dsRNA), produced from many viruses during replication while TLR7/8, and TLR9 sense single-stranded RNA and bacterial and viral unmethylated CpG DNA motifs, respectively (Alexopoulou et al., 2001; Diebold et al., 2004; Heil et al., 2004; Hemmi et al., 2000). The agonist for TLR5 is flagellin protein (Gewirtz et al., 2001). TLR10 ligand has not yet been identified. However, it has been proposed that it plays a role in inflammatory regulation during viral infections (Lee et al., 2014; Oosting et al., 2014). Polymorphisms in each TLR family affect susceptibility to pathogens containing that specific ligand. The interesting characteristic of this intricate system is that as an invading pathogen contains different PAMPs, it can be recognized by different TLRs and also several structurally unrelated ligands can be detected by described TLRs. A prime example of this is the capacity of TLR4 in recognizing different ligands (Akira et al., 2006).

Several PIDs have been identified that impair TLR signaling either directly or indirectly. These immunodeficiencies affect signal transmission from TLRs to the cell nucleus and impede cytokine secretion. As the TLR signaling pathways are key elements in defending the human body against infections, the mentioned PIDs increase susceptibility to infections. The identification of patients with monogenic immunodeficiencies, including Myeloid differentiation primary response 88 (MYD88) and interleukin(IL)-1 receptor-associated kinase 4 (IRAK-4) deficiency, UNC-93B deficiency, TLR3/2/4/5 deficiency, and hypohidrotic ectodermal dysplasia with immunodeficiency, shed light on our understanding of these infections.

IRAK-4 and MYD88 deficiency

Pathogenesis and etiology

PIDs that impair TLR signaling occur due to mutations in MYD88, IRAK-4, NF- κ B essential modulator (NEMO), inhibitor of NF- κ B kinase subunit- γ (IKBK γ), inhibitor of NF- κ B kinase subunit- β (IKBK β), and NF- κ B inhibitor- α (NFKBIA). The induction of intracellular signals to instigate the release of anti-inflammatory cytokines, including Tumor necrosis factor (TNF) α , IL-1 β , IL-6, and IL-12, depends greatly on a number of adaptor molecules. MYD88 and IRAK-4 are two examples acting downstream from TLRs, the inborn errors of which evade the proper function of the TLR signaling pathway. The reason lies in the dependency of all human TLRs, with the exception of TLR3, on MYD88 and IRAK-4 for inducing intracellular signals.

Toll/IL-1 receptor (TIR) acts as an intracellular signaling domain with an important role in both TLR signaling and IL-1 receptor (IL-1R) and IL-18R. MYD88 is a TIR-containing adaptor molecule involved in TLR signaling. It leads to the activation of nuclear factor kappa B (NF- κ B) and secretion of the aforementioned cytokines via activation of a cascade of protein kinases, among which a serine-threonine kinase named IRAK-4 is of great importance (Parvaneh et al., 2008).

As MYD88 and IRAK-4 deficiencies are autosomal recessive conditions, patients may have homozygous or asymptomatic compound heterozygous mutations in IRAK-4 or MYD88. Of note is that mutations leading to IRAK-4 deficiency truncate the kinase domain and are predominantly Q293X mutations (Ku et al., 2005b).

Clinical perspective

As the function of a great number of TLRs and signaling pathways depend on IRAK-4 and MYD88, their mutations seem to cause a broad and considerable immunologic loss. However, the spectrum of infecting pathogens to which patients are vulnerable is surprisingly narrow.

MYD88 and IRAK-4 deficiency predispose patients to recurrent invasive (e.g., septicemia, meningitis, arthritis, osteomyelitis, abscesses) and non-invasive (e.g., skin and upper respiratory tract infections) pyogenic bacterial infections. Pathogens predominantly include *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. However, infection with other species, including salmonella, shigella sonnei, neisseria meningitides, were also reported, though not common in these patients (Medvedev et al., 2003; Chapel et al., 2005).

Despite being unable to show considerable fever and rise of systemic inflammatory markers, including C-reactive protein (CRP) plasma level, pus formation can be observed in these patients (Picard et al., 2010). Positive blood or cerebrospinal culture may be beneficial for the diagnosis. In IRAK-4 and MYD88 deficient patients, the amount of IL-6 and IL-8 released in response to IL-1 and TLR activation is small (Picard et al., 2011). The antibody response to polysaccharides can be impaired (Ku et al., 2007b; Von Bernuth et al., 2008). Separation of the umbilical cord is also delayed in these patients (Picard et al., 2010). No sign of developmental abnormalities, including ectodermal dysplasia, osteoporosis, and lymphoedema in their growth process has been observed (Bustamante et al., 2008).

The intriguing point is that although IRAK-4 and MYD88 immunodeficiency are life-threatening in the first years of life, their clinical features tend to improve with age. It has been proposed that the reason for the decrease in their frequency and severity is the efficient adaptive immune response that comes into effect to compensate for the mentioned defensive defects against infections (Puel et al., 2004). It has been demonstrated that IRAK-4-deficient patients suffer from an impaired pneumococcal-specific immunoglobulin (Ig)G response that does not always match with the occurrence of invasive bacterial infections (Picard et al.,

2010; Ku et al., 2007a; Borgers et al., 2010). Also, it has been indicated that these patients have reduced circulating IgM⁺IgD⁺CD27⁺ B cells that produce rapid T-independent IgM against bacteria, leading to increased susceptibility to infections (Weller et al., 2012). It is presumed that T-independent IgG responses mature as age increases so that they compensate impaired T-independent IgM responses and infection susceptibility diminishes in adolescence (Douglas et al., 1983).

IRAK-4-deficient patients have a narrow susceptibility to pyogenic bacterial infections, while they seem to have normal resistance against many other pathogens, including common viruses, fungi, parasites, and mycobacteria (Ku et al., 2005b) which suggests that these invading pathogens are suppressed by other signaling pathways. As an example, viral pathogens are controlled through TLR3-dependent immunity. This narrow spectrum of susceptibility also indicates the non-redundancy of the TLR system in combating pathogens (Turvey and Hawn, 2006).

Being life-threatening conditions and causing death in half of the identified patients (Picard and Casanova, 2005), IRAK-4 and MYD88 deficiencies require appropriate administration of prophylactic and therapeutic measures. It is imperative that patients receive conjugated and nonconjugated vaccines to be immunized against encapsulated bacteria, including *S. pneumoniae*, *Klebsiella*, *Haemophilus influenza*, *Neisseria meningitidis*. Moreover, prophylactic antibiotic therapy with cotrimoxazole and penicillin V throughout life is necessary for these patients (Ku et al., 2005b). Intravenous and subcutaneous immunoglobulin replacement therapy until at least the age of 10 may be of use in some patients to resist severe bacterial infections, especially those with impaired antibody response to polysaccharides (Parvaneh et al., 2008; Picard et al., 2010). Despite receiving relevant prophylaxis, it is of the utmost importance to instantly start empirical parenteral antibiotic therapy when a bacterial infection is suspected and further implement specific antibiotic treatment to prevent invasive infection and death (Picard et al., 2011).

TLR3 deficiency

Pathogenesis and etiology

A number of PIDs affecting TLR3 signaling, an MYD88-independent pathway, have been identified. They include genetic mutations of TLR3, UNC93B, Toll/IL-1R domain-containing adaptor inducing interferon- β (TRIF), TNF receptor-associated factor3 (TRAF3), and TANK-binding kinase 1 (TBK1). Viral nucleic acids can be detected by intracellular TLRs and the RIG-I helicase family. TLR3 plays an important role in our defense against viruses by recognizing double-stranded RNA.

UNC-93B was first known for its function as a regulatory subunit of a potassium channel in *Caenorhabditis elegans* (Greenwald and Horvitz, 1980). It carries out a task in human TLR signaling, including TLR3 signaling pathway (Casrouge et al., 2006; Tabeta et al., 2006) that can strongly induce IFN- α/β and - γ secretion.

Autosomal recessive UNC-93B deficiency impairs fibroblastic cell responses to TLR3 agonists of viruses. TLR3 Heterozygous mutations have been documented as a contributing factor to Herpes simplex encephalitis (HSE) in children (Zhang et al., 2007). Also, homozygous mutations of UNC-93B were found as a potent factor that makes patients highly susceptible to isolated herpes simplexvirus-1 (HSV-1) encephalitis (Casrouge et al., 2006). In this respect, studies on pluripotent stem cell-derived neurons and oligodendrocytes derived from TLR3 or UNC93B deficient patients have discovered the decreased capacity of type I IFN production and heightened vulnerability to this infection through increased viral replication and cell death (Lafaille et al., 2012). Exposure of cells to recombinant IFN β was shown to be effective in ameliorating the destructive effects of the virus (Zhang et al., 2007). Based on this evidence, we can assume that TLR3/TRIF/UNC-93B-dependent signaling pathway and interferon type I responses are crucial for defense against HSV-1.

Clinical perspective

As explained above, mutations of TLR3/UNC-93B/TRIF/TRAF3, all central in the protective mechanism against viruses, heighten the susceptibility to HSV-1 encephalitis. It is also possible to observe incomplete clinical penetrance, which means having a mutated gene while being asymptomatic (Sancho-Shimizu et al., 2011). We should note the intriguing point that the spectrum of infections observed in these children is remarkably restricted as they are affected exclusively by HSV-1 and exhibit normal resistance to other pathogens. This surprising fact implies the redundancy of the TLR3-dependent signaling pathway in human beings except for protection against HSV-1 in the central nervous system (CNS).

Infection primarily happens in early childhood though there is a possibility of its recurrence even later in life (Bousfiha et al., 2010). Clinicians should be aware that recurrent HSE infection could be a sign of TLR3/UNC-93B deficiencies; even so, conventional immunological workup shows normal results. On the contrary, L-selectin measurement following the addition of diverse TLR agonists is practical in diagnosing TLR signaling defects (Parvaneh et al., 2008). Treatment with IFN α combined with acyclovir is the proposed therapeutic strategy for managing these patients (Casrouge et al., 2006; Zhang et al., 2007).

TLR5 deficiency

Pathogenesis and etiology

Genetic polymorphism of TLR5 that recognizes flagellin of flagellated bacteria is reported to be associated with total loss of receptors and increased susceptibility to Legionnaire's disease caused by *Legionella pneumophila*. An example of these polymorphisms happens in the TLR5 ligand-binding domain (Hawn et al., 2003). However, as TLR5 polymorphisms have a considerable frequency in human population and have subtle immunodeficiency presentations, this defect is more considered as a disease risk factor rather than a classical PID, suggesting its dispensable role in host defense (Wlasiuk et al., 2009).

Clinical perspective

Individuals with TLR5 polymorphism are highly prone to infections with *L. pneumophila* and recurrent cystitis, while it is unlikely for them to become at risk of typhoid fever infection by *Salmonella Typhi* (Hawn et al., 2003, 2009; Dunstan et al., 2005). They do not present severe manifestations of the disease. Moreover, TLR5 polymorphism has been shown to exert protective effects against systemic lupus erythematosus and Crohn's disease, which is highly prevalent in carriers of NOD2 mutations (Gewirtz et al., 2006).

TLR4 deficiency

Pathogenesis and etiology

TLR4 contributes to innate immunity function by binding to its ligands, LPS and endotoxin. The most important identified TLR4-related genetic defects are Asp299Gly and Thr399Ile single nucleotide polymorphisms (SNPs), leading to an altered amino acid sequence of the extracellular domain and impaired recognition of inhaled LPS (Arbour et al., 2000).

Clinical perspective

Asp299Gly and Thr399Ile SNPs affect patients' susceptibility to Gram-negative bacteria. These conditions may also be associated with sepsis (Turvey and Hawn, 2006). The intriguing point is that these patients have shown resistance against Legionnaire's disease. This can be explained by the unusual LPS structure of this flagellated Gram-negative bacterium which is recognized by TLR2 rather than TLR4 (De Tiege et al., 2008). Also, Asp299Gly SNP was demonstrated to have no impact on susceptibility to meningococcal disease (De Tiege et al., 2008).

TLR2 deficiency

Pathogenesis and etiology

TLR2 detects Gram-positive microorganisms and mycobacteria by recognizing their lipopeptides. TLR2 SNPs, Arg753Gln and Arg677Trp, lead to defective TLR2 signaling pathways and make patients more susceptible to some infections.

Clinical perspective

Arg753Gln was demonstrated to increase patients' susceptibility to tuberculosis. However, it is interesting that this SNP evades the development of late-stage Lyme disease (Hayashi et al., 2001; Hawn et al., 2003). Arg677Trp SNP increases susceptibility toward lepromatous leprosy and tuberculosis (Hawn et al., 2005; Gewirtz et al., 2006).

TLR defects with ectodermal dysplasia

Pathogenesis and etiology

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is a protein complex of the Rel family and a transcription factor with crucial roles in TLR-dependent inflammatory responses, cell adhesion, and cell survival (Temmerman et al., 2012). Under normal conditions, a family of inhibitors called I κ Bs (I κ B α , I κ B β , and I κ B ϵ) retain NF- κ B inactive and in the cytoplasm. Following cell stimulation via several pathways such as TLRs, IL-1R, and TNF-R, I κ B kinase (IKK) complex phosphorylate I κ Bs and lets NF- κ B move into the nucleus. IKK complex is composed of IKK α and IKK β catalytic subunits and IKK γ regulatory subunit, which is also called NF- κ B essential modulator (NEMO). NF- κ B translocation to the nucleus promotes transcription of genes that shape the host immunity system (Israel, 2000; Karin and Ben-Neriah, 2000). NEMO and I κ B α mutations bring about a rare primary immunodeficiency condition named Anhidrotic ectodermal dysplasia with immune deficiency (EDA-ID) characterized by a broad phenotype of defects in immunity, ectodermal structures and bones. EDA is not observed in other signaling defects and was the first TLR-signaling defect observed in human patients.

X-linked anhidrotic ectodermal dysplasia with immunodeficiency (XL-EDA-ID) happens due to hypomorphic mutations in the NEMO gene. It is a rare condition with a predominant incidence in male subjects; however, it can rarely happen in females as well. The first cases of XL-EDA-ID were discovered in 2000 and 2001 (Döffinger et al., 2001; Zonana et al., 2000). On the other hand, in 2003, autosomal dominant ectodermal dysplasia with immunodeficiency (AD-EDA-ID) that happens due to hypermorphic heterozygous mutation of I κ B α was discovered. This defect impedes I κ B α phosphorylation and causes NF- κ B retention in the cytoplasm, and impairs NF- κ B activation (Courtois et al., 2003).

Clinical perspective

Clinical manifestations of EDA-ID are broad and can be discussed in three subcategories:

Developmental standpoint: The wide range of developmental manifestations in EDA-ID patients mainly involve ectodermal-derived structures. Symptoms include sparse hair, thin skin, abnormal nails, Hypohidrosis, hypodontia or anodontia, heat intolerance, and in more severe cases, osteoporosis and lymphedema. However, it is possible to identify patients without EDA phenotype or with just a few signs of EDA like conical incisors and hypodontia (Ku et al., 2005a). It is also likely that patients only display the infectious signs without presenting EDA phenotype (Niehues et al., 2004; Orange et al., 2004b).

Infectious standpoint: EDA-ID patients are vulnerable to infections that affect both peripheral tissues and deep-seated tissues throughout the blood stream. The former include skin, respiratory tract, digestive tract, and their draining lymph nodes, while the latter include spleen, liver, bones, and joints. These patients present with meningitis and septicemia during infancy and may show recurrent infections with different groups of pathogens as discussed below.

The first group of pathogens that affects these subjects encompasses encapsulated invasive pyogenic bacteria, including *S. pneumoniae*, *Haemophilus influenzae*, and *S. aureus*. They lead to meningitis, sepsis, arthritis, osteomyelitis, abscesses, recurrent pneumonia with bronchiectasis, gut infection, and ENT infections.

Another group comprises mycobacteria that does not typically cause serious infection in normal individuals. Examples are *Mycobacterium kansasii*, *Mycobacterium avium*, and *Mycobacterium bovis*, causing cellulitis, osteomyelitis, lymphadenitis, pneumonia, and disseminated infections in patients (Döffinger et al., 2001; Hanson et al., 2008). In spite of the fact that these mycobacteria are weakly pathogenic, ADA-ID patients are highly susceptible to them.

Other less common pathogens include parasites, viruses, and fungi. Typical viruses that infect patients encompass herpes simplex virus (HSV), cytomegalovirus (CMV), adenovirus, Molluscum contagiosum, and human papillomavirus (HPV). They can cause herpes simplex virus encephalitis, severe adenoviral gastroenteritis, or severe cytomegalovirus infection (Döffinger et al., 2001; Uzel, 2005). *Pneumocystis jirovecii* pneumonitis is an example of opportunistic fungal infection (Orange et al., 2004a). Patients are highly susceptible to other opportunistic infections, including pulmonary pneumocystosis and chronic mucocutaneous candidiasis.

Autoimmune standpoint: Autoimmune manifestations in patients involve inflammatory bowel disease (IBD)-like colitis, hemolytic anemia, and arthritis (Niehues et al., 2004; Ørstavik et al., 2006; Uzel, 2005).

NEMO- and $\text{I}\kappa\text{B}\alpha$ -deficient patients usually have a delayed inflammatory response to severe infections. Also, many patients display a persistent abnormal antibody response to polysaccharides, giving rise to invasive pneumococcal diseases (Döffinger et al., 2001; Orange et al., 2004a; Picard et al., 2003). Nevertheless, a few patients have shown acceptable antibody responses to carbohydrates of conjugated vaccines (Ku et al., 2005). IgG hypogammaglobulinemia has been observed in a large number of patients (Courtois and Smahi, 2006). Moreover, some patients may present with hyper-IgM syndrome due to impaired CD40 signaling, which further contributes to *Pneumocystis* pneumonitis (Kenwrick et al., 2001; Seyama et al., 1999; Agou et al., 2004). In some patients, NK cell abnormalities accounting for higher susceptibility to herpes viruses have been detected (Hoffmann et al., 2003). NEMO-deficient patients may also demonstrate a lack of $\text{TNF}\alpha$ -mediated IL-10 production in whole-blood assays (Hanson et al., 2008; Puel et al., 2004). Impaired T-cell proliferation and function may be observed in some but not all patients.

A wide variety has been observed in the prognosis of these patients ranging from death in childhood to staying relatively well until older age after the application of proper prophylactic and therapeutic measures.

These patients should be immunized and vaccinated against encapsulated bacteria, including *S. pneumoniae*, *H. influenzae*, and *N. meningitidis*. However, Bacille-Calmette-Guérin (BCG) vaccination is contraindicated for them. Intravenous and subcutaneous immunoglobulin replacement therapy is applicable for patients presenting with impaired function of B-cell immunity. Prophylactic antibiotic therapy with cotrimoxazole and penicillin V in the absence of allergy may be of use, though it is controversial. For treatment of mycobacterial infection in NEMO- and $\text{I}\kappa\text{B}\alpha$ -deficient patients, intensive four-drug regimens should be applied for at least 12 months (Parvaneh et al., 2008). It has been suggested to implement empirical parenteral antibiotic treatment against *S. pneumoniae*, *S. aureus*, *P. aeruginosa*, and *H. influenzae* prior to specific antibiotic adaptation in case of suspected infection. As these patients are susceptible to life-threatening infections, hematopoietic stem cell transplantation (HSCT) may be considered to ameliorate the immunodeficiency state (Dupuis-Girod et al., 2002; Orange et al., 2004a).

CLRs

C-type lectin receptors (CLRs), a large family of PRRs and expressed by a number of innate immune cells, especially myeloid cells, are central in signaling pathways that induce essential cytokines for T cell polarization. They can also activate NF- κB directly or indirectly via TLRs. CLRs initiate antifungal immune responses by detecting fungal carbohydrates and are capable of further regulating adaptive immune responses.

Dectin-1 and CARD9 deficiency

Pathogenesis and etiology

Dectin-1 is a CLR found on monocytes and macrophages that recognizes *Mycobacterium tuberculosis* and fungal cell wall through binding to β -1,3-glucan polymer present in the fungal cell wall. Dectin-1 bound with its ligand transmits signals to CARD9, a key regulator of inflammatory responses and consequently, antifungal immunological responses become activated.

Several individuals have been identified with dectin-1 and recessive CARD9 mutations that impair immunological pathways involving IL-17. Impairment of Th-17 dependent IL-17 production has been reported in these patients (Glocker et al., 2009). A mutation in the dectin-1-encoding gene has been identified that generates a misfolded dectin-1 molecule which is not expressed on the cell membrane. Thus, impairments in the ability of mononuclear phagocytes to bind to their ligand have been observed, which lead to mucocutaneous fungal infections (Netea et al., 2012). It is notable that these mutations are not associated with a severe PID phenotype and cause milder symptoms than classical chronic mucocutaneous candidiasis (Puel et al., 2010; Lilic et al., 2003).

The absence of severe invasive candidiasis in these patients can be explained by an observation in mononuclear phagocytes and neutrophils. It demonstrates that monocytes and macrophages of homozygous patients show an impaired cytokine release which ultimately leads to mucocutaneous fungal infections. Conversely, neutrophils display normal phagocytosis function and provide protection. This fact suggests that dectin-1 is inessential for the function of neutrophils (Ferwerda et al., 2009).

CARD9 mutations bring about more severe phenotypes compared to dectin-1 deficiency. This can be traced back to CARD9 downstream location as an adaptor molecule that receives signals from several CLRs, only one of which is dectin-1. Thus, its deficiency causes more invasive fungal infections (Savides and Shaker, 2010).

Clinical perspective

Life-threatening fungal infections are the key clinical presentation of dectin-1 and CARD9 deficiency. Leading to defective β -glucan recognition and decreased cytokine release by monocytes and macrophages, dectin-1 Y238X polymorphism increases the odds of being infected with *Candida* and invasive aspergillosis (Plantinga et al., 2009; Cunha et al., 2010; Chai et al., 2011; Sainz et al., 2012). Also, CARD9 mutation makes patients susceptible to persistent and invasive fungal infections. These predominantly involve skin, nails, scalp, mucosal tissues, occasional contiguous dissemination to subcutaneous tissue, lymph nodes, bones, and also systemic fungal diseases, a common presentation of which is meningoencephalitis and CNS involvement (Lanternier et al., 2013; Wang et al., 2014; Drummond et al., 2015; Gavino et al., 2014). Mucocutaneous candidiasis (CMC) is a term referring to Persistent and recurrent mucosal infections, which usually happen earlier than serious presentations affecting the brain (Drummond et al., 2015; Grumach et al., 2015; Lanternier et al., 2015). Pathogens include *Trichophyton* species, *Phialophora verrucosa*, and the plant pathogen *Corynespora cassiicola* (Lanternier et al., 2013; Wang et al., 2014; Yan et al., 2016).

Since acquired predisposing factors for invasive fungal infections are certain and include HSCT, immunosuppressive therapies, chemotherapy, human immunodeficiency virus (HIV) infection, Supportive Oligonucleotide Technique (SOT), and hematological malignancies, gene sequencing should be done to screen CARD9 mutations in individuals with unexplained invasive fungal disease (Limper et al., 2017; Pappas et al., 2010).

Understanding how the impairment of the CARD9 signaling pathway leads to reduced cytokine release and increased likelihood of fungal infections has enabled us to explore the field of therapeutics extensively and implement tailored treatment for the patients. Suggested treatments for fungal infections encompass life-long treatment with systemic and topical azole agents for CMC, oral azole therapy for subcutaneous infection, intravenous treatment followed by oral fluconazole consolidation therapy for Invasive *Candida* infections, topical agents for Superficial dermatophytosis, and systemic antifungal agents for Extensive/deep dermatophytosis, Onychomycosis and scalp lesions (Firinu et al., 2011; Pappas et al., 2016; Pires et al., 2014; Gupta and Cooper, 2008). In case of severe deep dermatophytosis surgery is indicated for controlling the disease progression (Pires et al., 2014; Gupta and Cooper, 2008). Local excision for extensive skin lesions, and complete removal of brain abscesses by surgery are also suggested (Oberlin et al., 2017). Immune-based Adjuvant therapies for fungal meningoencephalitis were reported effective in inducing a lasting clinical remission (Celmeli et al., 2016). Vaccination against mycobacterium tuberculosis is of use in boosting immunity in these patients (Ostrop et al., 2015).

However, reoccurrence of fungal disease after stopping treatment in CARD9 deficient patients is considerably reported. This fact elucidates the need for longer courses of treatment or life-long treatment (Corvilain et al., 2018). Allogeneic stem cell transplantation leading to leukocyte-mediated CARD9 immunity reconstitution was reported successful in inducing complete clinical remission (Queiroz-Telles et al., 2019). Moreover, granulocyte-macrophage colony-stimulating factor (GM-CSF) that recruits neutrophils and curbs CNS candidiasis brought about favorable results in CARD9-deficient patients (Gavino et al., 2014).

MBL deficiency

Pathogenesis and etiology

Mannose-binding lectin (MBL) is a soluble CLR, the deficiency of which stems from changes in the MBL2 gene. As MBL has a specific role in recognizing microorganisms, decreased MBL level predispose patients to an increased susceptibility to infections. However, MBL polymorphism is quite common in the general population and thus, accounts for an infection risk factor rather than a critical PID (Sprong and van Deuren, 2008).

Clinical perspective

MBL-deficient individuals are more susceptible to fungal, viral, and bacterial infections, including *Neisseria meningitidis*. They are also likely to present with recurrent vulvovaginal candidiasis, though it is not common in the general population (Liu et al., 2006; Donders et al., 2008; Giraldo et al., 2007; Babula et al., 2003). Therapeutic strategies for patients with relapsing infections primarily include antibiotic therapy and vaccination.

NLRs

NOD-like receptors (NLRs) are a group of intracellular PRRs. NOD1 and NOD2 constitute a group of NLRs whose function is recognizing bacterial peptidoglycans leading to nuclear translocation of NF- κ B and expression of inflammatory cytokines. NLRPs are another subgroup of NLRs that are involved in the formation of the inflammasome and activation of caspase-1 protease. This activation leads to proteolytic processing of the proinflammatory cytokines, including IL-1 β and IL-18. To date, genetic mutations and polymorphisms in several members of the NLRP family and NOD2 receptor have been described. Defective NLRPs usually cause autoinflammatory diseases stemming from the dysregulated release of IL-1 cytokines.

NOD2 deficiency**Pathogenesis and etiology**

Defects of the NOD2 gene, including a mutation in the Leucine-rich repeat (LRR) domain, have been reported in association with Crohn's disease and Blau syndrome (Hugot et al., 2001; Ogura et al., 2001; Miceli-Richard et al., 2001).

Clinical perspective

A loss-of-function genetic mutation has been reported to cause a PID of gut mucosal immunity in NOD2-deficient patients. It leads to impaired cytokine response, neutrophil function, and consequently weakened immune response against invading pathogens presenting with a granulomatous reaction. Thus, in these patients, Crohn's disease, an inflammatory bowel disease (IBD), happens due to impaired mucosal host defense (Kobayashi et al., 2005; Kullberg et al., 2008). Patients also display reduced protection against bacterial infection. On the other hand, a gain of function mutation in the NOD2 encoding gene accompanied by increased activity of transcription factor NF- κ B causes Blau syndrome. Patients display an uncontrolled granulomatous inflammation (Miceli-Richard et al., 2001).

Vitamin D treatment for upregulating NOD2 may be beneficial in enhancing immunity against invading bacteria, including *S. aureus* and *Salmonella typhimurium* (Subramanian et al., 2017; Liu et al., 2017).

NLRP3 deficiency**Pathogenesis and etiology**

NLRP3, expressed in polymorphic mononuclear cells and monocytes, is an inflammasome required for the activation of cytokine-releasing pathways. Specific gain of function mutations in the NLRP3 genes lead to a cryopyrin-associated periodic syndrome (CAPS), with the sub-syndromes of familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and chronic infantile neurological cutaneous and articular syndrome (CINCA/NOMID) (Hull et al., 2003; Aksentijevich et al., 2002; Hoffman et al., 2001; Aganna et al., 2002). The range of symptoms varies from mild to severe, and patients may present with overlapping symptoms of the three aforementioned phenomena (Van Der Made et al., 2020).

Clinical perspective

All these phenotypes cause systemic inflammation with fever, raised CRP level, and neutropenia. Also, localized neutrophilic inflammation in skin, joints, muscles, conjunctiva, and cerebrospinal fluid has been detected. FCAS phenotype is associated with fever, systemic symptoms, non-pruritic urticaria-like rash, conjunctivitis, and arthralgia, all of which happen after exposure to cold (Hoffman et al., 2001). In MWS-affected patients, the same symptoms can be observed, though without specific relation to cold. Sensorineural hearing loss and amyloidosis have a higher incidence in these patients. Conversely, conjunctivitis is less probable in them (Aganna et al., 2002). Manifestations of NOMID patients include fever, urticaria, deforming arthropathy, epiphyseal overgrowth of the long bones, and chronic meningitis, uveitis, and sensorineural hearing loss (Aksentijevich et al., 2002).

Therapeutic strategies include rilonacept and interleukin-1 trap, canakinumab, an antibody that neutralizes IL-1 β , and anakinra, an antagonist of IL-1 α receptor, all of which have shown favorable results (De Koning et al., 2015; Jesus and Goldbach-Mansky, 2014).

NLRP12 deficiency**Pathogenesis and etiology**

NLRP12 is another inflammasome whose gain of function mutations also cause FCAS, though with milder presentations. Autoinflammatory disorders, Crohn's disease, and heightened susceptibility to infections are consequences of NLRP12 mutations (Kostik et al., 2018).

Clinical perspective

Patients have been reported with negatively regulated TNF- α induced NF- κ B signaling (Allen et al., 2012). They were treated with anakinra. However, recurrence happened with an increased TNF- α level (Jeru et al., 2011). Glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDs), IL-1 blockade therapy, and anti-TNF- α therapy for Crohn's disease are other therapeutic choices that result in better clinical response (Başaran et al., 2018; Kostik et al., 2018).

NLRP1 deficiency**Pathogenesis and etiology**

NLRP1 mutations cause a wide range of symptoms. The reason lies in the fact that NLRP1 is expressed by a large number of cells, including myeloid cells, T-cells, and Langerhans cells. Thus, the dysregulated function of NLRP1-induced pathways causes multiple symptoms, including various autoimmune disorders. Mutations can be found between the NACHT and LRR domains and in the function-to-find (FIIND) domain (Grandemange et al., 2017).

Clinical perspective

Autoimmune phenotypes comprise autoimmune hemolytic anemia, diabetes type I, Addison's disease, autoimmune thyroiditis, celiac disease, Systemic lupus erythematosus (SLE), and Rheumatoid arthritis (RA) (Jin et al., 2007). Moreover, an autoinflammatory syndrome named NAIAD syndrome has been described in these patients that present with systemic inflammation, diffuse skin

dyskeratosis, arthritis, vitamin A deficiency, high transitional B-cell levels, neutrophilia, decreased number of T-cells, and dramatically increased serum IL-18 and caspase-1 (Grandemange et al., 2017). Precancerous manifestations including multiple self-healing palmoplantar carcinoma (MSPC), familial keratosis lichenoides chronica (FKLC), and inherited corneal intraepithelial dyskeratosis were also detected (Alehashemi and Goldbach-Mansky, 2020; Drutman et al., 2019). The reason for the higher susceptibility of the skin to these manifestations is the high level of NLRP1 expression in keratinocytes (Zhong et al., 2016). Reported infections in these patients include mucocutaneous candidiasis and *Giardia intestinalis* (Grandemange et al., 2017). IL-1 blockade for treating systemic features and retinoic acid and vitamin A for the treatment of skin manifestations are the therapeutic strategies (Grandemange et al., 2017).

NLR4 deficiency

Pathogenesis and etiology

NLR4-related gain of function mutations are characterized by autoinflammation and infantile enterocolitis (AIFEC) (Canna et al., 2014; Romberg et al., 2014), NOMID-like symptoms, and macrophage activation syndrome (MAS)-like symptoms with cytotoxic T-cell dysfunction, IFN γ -related hemophagocytosis, and myeloid cell activation (Romberg et al., 2017).

Clinical perspective

Patients may display increased IL-18 serum levels and IL-18/CXCL9 ratio (Romberg et al., 2017). A study has reported clinical improvement in an infant patient with MAS and enterocolitis after treatment with recombinant human IL-18 binding protein. The patient had been resistant to IL-1 blockade, corticosteroids, and anti-TNF- α treatment (Canna et al., 2017). Anakinra may be useful in the treatment of NOMID-like symptoms; however, it was not shown effective enough in alleviating AIFEC flares (Romberg et al., 2017).

Mendelian susceptibility to mycobacterial diseases-causing defects

Pathogenesis and etiology

The IFN γ -mediated immunity is an integral part of host defense in controlling mycobacterial infections. Recognition of mycobacterial microorganisms by PRRs leads to the secretion of cytokines including ISG15, IL-12, and IL-23 that further bind to their receptors on T-helper and NK cells. IL-12R consists of IL-12R β 1 and IL-12R β 2 chains (Rosenzweig and Holland, 2005). Their cooperation with Tyk2 and Jak2 kinases and promotion of signal transducer and activator of transcription-4 (STAT4) ultimately lead to IFN- γ induction (Watford et al., 2003). Moreover, this pathway produces IL-17 and TNF- α that contribute to the initiation of adaptive immune responses by promoting differentiation of naïve T-helper cells toward an antigen-specific T-helper type 1 (Th1) (Mahdaviyani et al., 2020). Coordination between mononuclear phagocytes of the innate immunity and T cells of the adaptive immunity plays a crucial role in defense against mycobacteria. Additionally, when IFN- γ binds to its receptor, IFN- γ R, on macrophages, it induces IFN- γ production via association with Jak1 and Jak2 and STAT1 (Casanova and Abel, 2002). This IFN- γ enhances the ability of macrophages to phagocytose and confine mycobacteria through some mechanisms, including granuloma formation (Parvaneh et al., 2008).

Immune responses against invading mycobacteria heavily depend on the function of IL-12 and IFN- γ . The identification of IL-12R β 2 and IL-23R deficiencies and their association with infections have shed more light on the importance of IL-12- and IL-23-dependent IFN- γ immunity for resistance against mycobacteria (Bustamante, 2020). Mendelian susceptibility to mycobacterial diseases (MSMD) is a condition caused by inherited defects in IFN- γ immunity that predispose patients to infections with weakly virulent mycobacteria. They include environmental mycobacteria and substrains of the BCG vaccine.

A number of germline mutations, either autosomal or X-linked, have been identified in MSMD patients. These genes include IFNGR1, IFNGR2, STAT1, IL12B, IL12RB1, IL12RB2, SPPL2A, IL23R, ISG15, NEMO, and CYBB. SPPL2A, TYK2, JAK1, and ROR γ /ROR γ T at the molecular level and IFN- γ -producing Th1 and $\gamma\delta$ T cells at the cellular level are the newest discovered genetic defects contributing to MSMD symptoms (Rosain et al., 2019). TYK2 defects share some characteristics with IFN- γ signaling defects, though they are not categorized as classical MSMD genetic defects (Parvaneh et al., 2008). IFNGR1 mutations that can be recessive or dominant were the first described genetic defects in association with MSMD (Jouanguy et al., 1996). Genetic defects of IL-12R β 1 and IFN γ R1 are the most common etiologies of MSMD (Mahdaviyani et al., 2020). IL12B defect is known as the single cytokine gene defect leading to PID (Parvaneh et al., 2008). STAT1 deficiency can be heterozygous or homozygous with more severe outcomes.

Next-generation sequencing is a useful technique for genetic diagnosis, especially in patients whose diagnosis has not yet been defined.

Clinical perspective

As mentioned before, MSMD patients are susceptible to weakly virulent non-tuberculous mycobacteria, including *M. bovis* and *M. avium*. *M. avium* may be present at infection sites like teeth without causing much harm (Parvaneh et al., 2008). They are also susceptible to the more virulent but less frequent *M. tuberculosis* (Ozbek et al., 2005). Mycobacterial infections, beginning in early childhood, present with a wide range of manifestations that can be localized or disseminated and may reoccur (Cottle, 2011; Bustamante et al., 2014). In addition to mycobacteria, Patients have presented with systemic non-typhoidal or typhoidal

Salmonella serotypes. Other infections comprise parasites such as leishmaniasis and toxoplasmosis, intramacrophagic bacteria such as listeriosis and klebsiellosis, fungi such as histoplasmosis, CMC, and viruses, such as CMV and human herpesvirus 8 (HHV-8) (Mahdavian et al., 2020; Camcioglu et al., 2004; Ouederni et al., 2014). TYK2-deficient patients may display disseminated BCG, recurrent HSV-1, and molluscum contagiosum skin infections (Jouanguy et al., 2007).

IFN- γ therapy combined with antibiotic therapy constitutes the main therapeutic strategy in MSMD patients. Recessive complete forms of IFN- γ deficiency have a poor prognosis, and their responses to subcutaneous administration of IFN- γ are not favorable (Dorman et al., 2004). However, IFN- α therapy has shown more positive outcomes (Ward et al., 2007). Also, HSCT may be helpful though graft rejection is probable (Chantraine et al., 2006). On the other hand, recessive partial forms of the disease respond well to antimycobacterial prophylaxis, subcutaneous administration of IFN- γ , and tailored antibiotic therapy in case of an ongoing infection (Haverkamp et al., 2006). Likewise, IL12B/IL12RB1- deficient patients demonstrate a favorable response to antibiotics and IFN- γ (Parvaneh et al., 2008).

WHIM syndrome-causing defects

Pathogenesis and etiology

WHIM syndrome is the first identified chemokine receptor-dependent PID and consists of Warts, Hypogammaglobulinemia, Infections, and Myelokathexis. Myelokathexis is a condition caused by the accumulation of mature neutrophils in the bone marrow that leads to neutropenia. WHIM-causing genetic defects are traced back to mutations of G protein-coupled chemokine receptor CXCR4 (Hernandez et al., 2003). CXCR4, expressed in the immune system and the CNS, is central for homing of cells in the bone marrow. A gain of function and usually autosomal dominant mutation in this gene causes pathological holding of cells within the bone marrow.

Clinical perspective

The most important clinical manifestation of germline gain-of-function CXCR4 mutation is HPV-induced uncontrolled lesions and warts. Also, benign bacterial infections with a good remission after antibiotic therapy may occur in these patients. However, patients with considerable neutropenia rarely suffer from invasive bacterial infections (Gulino, 2003; Diaz, 2005). Opportunistic infections are not common in CD4⁺ T lymphopenia patients, except HPV (Heusinkveld et al., 2019). Epstein-Barr virus (EBV)—induced lymphoma affecting the skin and gut, and infrequent complex congenital heart defects were reported in these patients (Imashuku et al., 2002; Chae et al., 2001; Gulino, 2003).

Myelokathexis is almost always observed in these patients. Mild thrombocytopenia, Hypogammaglobulinemia, reduced number of CD27⁺ B cells, neutropenia, monocytopenia, and lymphopenia have also been described (Heusinkveld et al., 2019; Gulino et al., 2004).

Measures for the management of WHIM syndrome patients include laser ablation of warts, close monitoring of genital warts, antibiotic therapy for recurrent infections, HSCT with favorable results in complete clinical cure of the patients, and granulocyte colony-stimulating factor (G-CSF) and GM-CSF for increasing neutrophil count (Parvaneh et al., 2008). Inactivated vaccines are not contraindicated, while live vaccinations should be administered after assessing potential risks according to the patient's condition (Heusinkveld et al., 2019).

Epidermodysplasia verruciformis-causing defects

Pathogenesis and etiology

Epidermodysplasia verruciformis (EV) is an inherited rare genodermatosis that heightens the susceptibility of patients toward HPV infection and cutaneous malignancies, especially cutaneous squamous cell cancer (SCC). EV is a heterogeneous autosomal recessive disorder with mutations in EVER1 and EVER2 genes, also called transmembrane channel-like (TMC) 6 and TMC8. It was the first identified disease to link viral infections to cancers in humans (Burger and Itin, 2014). A classification has been proposed that categorizes types of EV in three classic genetic, nonclassic genetic, and acquired EV classes (Huang et al., 2018).

Clinical perspective

Clinical manifestations of EV begin to appear in early childhood and infancy. Patients usually display verrucae planae-like lesions on the extremities or pityriasis versicolor-like lesions on the trunk with mild acanthosis and hyperkeratosis (Burger and Itin, 2014). They are at increased risk of developing precancerous skin lesions in sun/UV-exposed parts of the body during the second or third decade of life that may transform to skin cancers, usually after the second decade of life (Lutzner, 1978; De Oliveira et al., 2003). In spite of the fact that these cancers progress slowly, the risk of their destructive extension and metastasis exists (Lutzner et al., 1984). Mucous membranes are not commonly involved in developing these lesions. Being susceptible and often infected with HPVs, especially HPV types 5 and 8, patients demonstrate increased serum antibody reactivity against β -HPVs (Michael et al., 2010).

The treatment consensus of EV patients is mainly comprised of symptomatic therapies and sun avoidance advice immediately after the disease is diagnosed. The first line measure toward precancerous lesions is cryotherapy. Surgical removal is beneficial in the case of localized aggressive SCC (Gubinelli et al., 2003). Finally, topical treatment with imiquimod, retinoids, 5-interferon, fluorouracil, and tacalcitol is recommended for EV treatment (Burger and Itin, 2014).

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Monogenic Systemic Autoinflammatory Diseases

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Abbreviations

GoF	Gain of function
GWAS	Genome wide association studies
HSCT	Hematopoietic Stem Cell Transplantation
IFN	Interferon
LoF	Loss of function
NF- κ B	Nuclear factor κ light-chain enhancer of activated B-cells
PBMC	Peripheral blood mononuclear cells
SNP	Single nucleotide polymorphism

Introduction

The innate immunity is the most evolutionarily conserved arm of the immune system and the first line of defense against invading pathogens. Innate immune responses are not specific to any antigen and are carried by cells such as macrophages, NK cells, neutrophils, mast cells, dendritic cells, and innate-like lymphocytes. It is only in the past two decades that monogenic diseases of aberrant activation of the innate immunity has been recognized, and they are referred to as Systemic Autoinflammatory Disorders (SAIDs) (Manthiram et al., 2017; Aksentijevich et al., 2020). Originally, the concept of *autoinflammation* was introduced to denote that the inflammatory phenotype in these patients is predominantly driven by myeloid cells. However, as innate and adaptive immunity are closely intersected, cells of the adaptive immune system may eventually become dysregulated by overactivated myeloid cells. Subsequently, the boundaries of what defines a systemic autoinflammatory condition have expanded to include features of immunodeficiency, autoimmunity, and atopy (Tangye et al., 2020). In particular, several of these diseases manifest with a prominent B-cell deficiency. SAIDs have been linked to germline and somatic pathogenic variants in over 40 genes (Aksentijevich and Schnappauf, 2021). They are caused by defects in the cytosolic sensors, cytokine receptors, proteasome-mediated protein degradation, protein misfolding, lipid metabolism, and ubiquitylation. The molecular classification has become challenging as many mutated proteins converge to upregulate common inflammatory pathways, NF- κ B, IL-1, and interferons (Savic et al., 2020). This chapter will attempt to categorize diseases based on the function of mutated protein and discuss associated pathogenic variants, clinical presentation, and therapeutic management of patients with SAIDs (Tables 1 and 2).

Inflammasome-mediated autoinflammatory diseases

Inflammasomes are cytosolic sensors that have emerged as important regulators of innate immune responses (de Vasconcelos and Lamkanfi, 2020). They can sense various exogenous and endogenous ligands known as pathogen and danger-associated molecular patterns (PAMPS) and DAMPS (e.g., cytosolic nucleic acids, crystalline particles). *Inflammasomes* function in the form of multi-protein complexes that upon its activation cause caspase-1 mediated cleavage of the IL-1 family of proinflammatory cytokines. Besides, activated caspase-1 also cleaves gasdermin D, whose N-terminal cleavage product forms pores in the cell membrane, causing pyroptosis, a type of inflammatory cell death. Most inflammasomes are nucleated by proteins that belong to the family of nucleotide-binding leucine-rich repeat-containing receptors (NLR). Via its N-terminal pyrin domain (PYD) or a CARD domain, these proteins bind to the adaptor protein, apoptosis-associated speck-like protein with a caspase recruitment domain (ASC), which subsequently cause activation of caspase-1. The pyrin inflammasome is the only functionally characterized inflammasome nucleated by the structurally different protein, although it comprises an N-terminal PYD. Pyrin is a unique intracellular sensor because it does not recognize and bind pathogen-derived antigens directly. Rather it is activated in response to pathogen-associated virulence factors, generated through bacterial toxin-induced inactivation of host GTPase RhoA proteins.

Erroneous activation of the inflammasomes can lead to diseases that are known as *inflammasomopathies* (Alehashemi and Goldbach-Mansky, 2020). Nearly all of these conditions are caused by heterozygous gain-of-function mutations in the centrally located NACHT domain of these proteins, which lead to a variable degree of activation of the respective inflammasome. Patients with the most severe phenotypes carry a *de novo* pathogenic variant that results in constitutive inflammasome activation. A milder disease-associated mutation may affect the function of the autoinhibitory domain or protein stability. Other environmental factors such as cold temperature, heat, physical or emotional stress can facilitate inflammasome activation. Variability in clinical presentations can be explained by cell or tissue expression of the mutated protein. Most inflammasomes are highly expressed in hematopoietic cells, particularly myeloid cells. Patients who carry pathogenic variants in the pyrin, NLRP3, and NLRP12 inflammasomes present with a constellation of features that include chronic or recurrent fever, skin rash, arthralgia/arthritis, and in severe cases, CNS inflammation. As the NLRP1 inflammasome is highly expressed in epithelial cells, the linked disease phenotype manifest with hyperplastic dyskeratosis and susceptibility to skin cancers. The NLRC4 inflammasome is highly expressed in intestinal epithelial cells, where it restricts replication of Gram-negative bacteria, and the associated phenotype includes gastrointestinal inflammation (Duncan and Canna, 2018). The common feature of *inflammasomopathies* is an excessive release of IL-1 β , and that they are highly responsive to therapies with IL-1 inhibitors (De Benedetti et al., 2018).

Table 1 Monogenic systemic autoinflammatory disorders.

<i>Disease acronym</i>	<i>Disease name</i>	<i>Gene/Genes</i>	<i>Mode of inheritance</i>	<i>Gain of function/Loss of function</i>	<i>OMIM number</i>
AGS (Type 1–7)	Aicardi-Goutières syndrome	<i>ADAR, DNASE2, IFIH1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, TREX1</i>	AD/AR	GoF/LoF	615010, 615846, 610333, 610181, 610329, 612952, 225750
AIADK/FKLC/MSPC/JRRP	Familial Keratosis Lichenoides Chronica/Multiple Self-Healing Palmoplantar Carcinoma	<i>NLRP1</i>	AD/AR	GoF	617388, 615225, 618803
AIFEC/NLRC4-MAS/FCAS4	Autoinflammation with Infantile Enterocolitis/NLRC4 Macrophage Activation Syndrome/Familial Cold Autoinflammatory Syndrome 4	<i>NLRC4</i>	De novo/AD	GoF	616115, 616050
AILJK	Autoimmune Interstitial Lung, Joint, Kidney Disease	<i>COPA</i>	AD	Dominant negative	616414
APLAID/PLAID/FCAS3	Autoinflammation and PLCG2-associated Antibody Deficiency and Immune Dysregulation/Familial Cold Autoinflammatory Syndrome 3	<i>PLCG2</i>	AD	GoF	614878, 614468
BS/EOS	Blau syndrome/Early-onset Sarcoidosis	<i>NOD2</i>	AD/Somatic	GoF	186580
CAPS/FCAS1/MWS/NOMID/CINCA	Cryopyrin-associated Periodic Syndromes	<i>NLRP3</i>	De novo/AD/Somatic	GoF	120100, 191900, 607115
CAMPS/PSORS2	CARD14-associated Psoriasis	<i>CARD14</i>	AD	GoF	173200, 602723
Cherubism	SH3BP2 Deficiency	<i>SH3BP2</i>	AD	GoF/Dominant negative	118400
CRIA	Cleavage-resistant RIPK1-induced Autoinflammatory	<i>RIPK1</i>	AD	GoF	618852
DADA2	Deficiency of Adenosine Deaminase 2	<i>ADA2</i>	AR	LoF	615688
DIRA	Deficiency of IL-1-Receptor Antagonist	<i>IL1RN</i>	AR	LoF	612852
DITRA/Psoriasis 14	Deficiency of IL-36-Receptor Antagonist	<i>IL36RN</i>	AR	LoF	614204
EOIBD/IL-10 Deficiency/IBD28	Early-onset Inflammatory Bowel Disease	<i>IL10, IL10RA, IL10RB</i>	AR	LoF	613148
FCAS2	NLRP12-associated Autoinflammatory	<i>NLRP12</i>	AD	LoF	611762
FMF	Familial Mediterranean Fever	<i>MEFV</i>	AR	GoF	249100
PAAD/PAAND	Pyrin Associated Dominant Diseases/Pyrin Associated Autoinflammation with Neutrophilic Dermatitis	<i>MEFV</i>	AD	GoF	134610
HA20	Haploinsufficiency of A20	<i>TNFAIP3</i>	AR	LoF	616744
MKD/HIDS/MA	Mevalonate Kinase Deficiency/Hyperimmunoglobulinemia D Syndrome/Mevalonic Aciduria	<i>MVK</i>	AR	LoF	260920, 610377
HOIL1 deficiency	LUBAC HOIL1 deficiency	<i>RBCK1 (HOIL1)</i>	AR	LoF	615895
HOIP deficiency	LUBAC HOIP Deficiency	<i>RNF31 (HOIP)</i>	AR	LoF	NA
Immunodeficiency 38	ISG15 Deficiency	<i>ISG 15</i>	AR	LoF	147571
Immunodeficiency 57	RIPK1 Deficiency	<i>RIPK1</i>	AR	LoF	618108
LACC1-associated diseases	LACC1-associated Systemic JIA and Early-onset IBD	<i>LACC1</i>	AR	LoF	618795
LPIN2 deficiency	Majeed Syndrome	<i>LPIN2</i>	AR	LoF	609628
NOCARH	Neonatal Onset of Cytopenia, Autoinflammation, Rash, Hemophagocytosis	<i>CDC42</i>	De Novo	LoF	NA
Otulipenia/ORAS	OTULIN-associated Autoinflammatory Syndrome	<i>OTULIN</i>	AR	LoF	617099
PAPA	Pyogenic Arthritis, Pyoderma Gangrenosum and Acne Syndrome	<i>PSTPIP1</i>	AD	Not known	604416

Table 1 (Continued)

<i>Disease acronym</i>	<i>Disease name</i>	<i>Gene/Genes</i>	<i>Mode of inheritance</i>	<i>Gain of function/Loss of function</i>	<i>OMIM number</i>
PFITS	Periodic Fever, Immunodeficiency, Thrombocytopenia Syndrome	<i>WDR1</i>	AR	LoF	150550
PRAAS/CANDLE	Proteasome-associated Autoinflammatory Syndromes/Chronic Atypical Neutrophilic Dermatitis with Lipodystrophy and Elevated Temperature	<i>PSMB8, PSMB9, PSMB10, PSMA3, PSMB4, PSMG2, POMP</i>	AR/De Novo/Digenic	LoF	256040, 617591, 613386
Pseudo-TORCH syndrome 2	USP 18 Deficiency	<i>USP18</i>	AR	LoF	617397
ROSAH	Retinal dystrophy, Optic nerve edema, Splenomegaly, Anhidrosis, Migraine Headache	<i>ALPK1</i>	AD	GoF	614979
SAVI	STING-associated Vasculopathy with Onset in Infancy	<i>TMEM173</i>	AD	GoF	615934
SIFD	Sideroblastic Anemia, Immunodeficiency, Fevers, Developmental Delay	<i>TRNT1</i>	AR	LoF	616084
TRAPS	TNFR1-associated Periodic Syndrome	<i>TNFRSF1A</i>	AD	Not known	142680
VEXAS	Vacuoles, E1, X-linked, Autoinflammatory, Somatic syndrome	<i>UBA1</i>	Somatic	LoF	301054
Immunodeficiency 71	ARPC1B Deficiency	<i>ARPC1B</i>	AR	LoF	617718
None	Coagulation factor XII/FCAS	<i>FXII</i>	AD	GoF	NA
None	RELA haploinsufficiency	<i>RELA</i>	AD	LoF	618287
None	TRAP1-associated Disease	<i>TRAP1</i>	AD	LoF	NA

GoF, Gain-of-function; LoF, loss-of-function; NA, not applicable.

Online Mendelian Inheritance in Man (OMIM): <https://www.ncbi.nlm.nih.gov/omim>.

Table 2 Novel therapeutic approaches for systemic autoinflammatory diseases (SAIDs).

<i>Drug</i>	<i>Molecular structure</i>	<i>Molecular target</i>	<i>Indication</i>
IL-1 inhibitors	Recombinant human IL-1R antagonist (anakinra)	IL-1R1	NLRP3-associated diseases/CAPS TRAPS, FMF/PAAND MKD/HIDS PAPA, DIRA CDC42 deficiency/NOCARH LPIN2 deficiency/Majeed Sy
	Human anti IL-1 β mAb (canakinumab)	IL-1 β	NLRP2-associated disease NLRP4-assoc diseases/MAS/FCAS4 FMF, MKD/HIDS Hz/Hc/PAMI LPIN2 deficiency/Majeed Sy
IL-6 inhibitor	Humanized anti IL-6 mAb	IL-6R	LACC1 deficiency/JIA CRIA HA20
IL-12/23 inhibitor TNF inhibitors	Human anti IL-12/23 mAb (ustekinumab)	IL-12R β 1	DITRA, CAPS
	Fusion protein (etanercept)	TNF	DITRA
	Chimeric mAb (infliximab)		PAAND
	Humanized mAb (adalimumab)		BS/EOS HA20, ORAS LACC1 deficiency DADA2 Hz/Hc/PAMI SH3BP2 deficiency/Cherubism
IFN γ inhibitor	Human mAb (emapalumab)	IFN- γ	NALRC4-associated diseases CDC42 deficiency/NOCARH
IL-18 inhibitor	Recombinant IL-18 binding protein	IL-18	NLRP4-associated disease/MAS WDR1 deficiency/PFITS
Jak inhibitors	Small molecule inhibitors:	JAK/STAT	AGS, SAVI
	JAK 1&2-Ruxolitinib JAK1&3-Baricitinib, Tofacitinib		PRAAS/CANDLE USP18, ISG15 APLAID, HA20

NLRP1-associated autoinflammatory diseases (MSPC/FKLC/NAIAD)

NLRP1 pathogenic variants can be inherited in a dominant or recessive manner, depending on their position in the affected protein domain. They are linked to a spectrum of phenotypes from systemic inflammation and arthritis to non-inflammatory skin disease. Skin manifestations include hyperplastic dyskeratosis, upper respiratory tract papillomatosis and susceptibility to malignant squamous cell carcinoma (SCC) and multiple self-healing palmoplantar carcinoma (MSPC) (Zhong et al., 2016; Drutman et al., 2019). Mutant primary keratinocytes have increased production of proinflammatory cytokines IL-1 and IL-18. Common low-penetrance variants (SNPs) in *NLRP1* have been associated with susceptibility to vitiligo and other autoimmune and autoinflammatory diseases, however, their functional consequences need to be investigated (Jin et al., 2007).

NLRP3-associated autoinflammatory diseases (FCAS/MWS/NOMID)

Heterozygous GoF variants in *NLRP3* have been linked to a continuum of inflammatory phenotypes denoted as cryopyrin-associated periodic syndrome (CAPS) from the milder phenotype named familial cold autoinflammatory syndrome (FCAS), to Muckle-Wells syndrome (MWS), and most severe neonatal-onset multisystem inflammatory disease (NOMID; also known as CINCA) (Booshehri and Hoffman, 2019). While features of fevers, urticaria-like rash, and joint involvement are shared among all three disease entities, patients with MWS/NOMID can develop CNS inflammation and bony overgrowth. The most common CNS manifestations are sensorineural hearing loss, vision loss, and aseptic meningitis that can result in cognitive impairment. Systemic inflammation in NOMID/CINCA is chronic and almost persistent, whereas in patients with FCAS flares are triggered by cold temperature. Systemic A amyloidosis (SAA) has been reported in patients with a severe phenotype.

CAPS-associated variants can be inherited as germline or somatic mutations. Identification of a de novo variant in *NLRP3* is consistent with the diagnosis of NOMID/CINCA. Somatic (post-embryonic) variants originating in the myeloid lineage have been identified in about 30% of patients with classical CAPS or with late-onset CAPS (Louvrier et al., 2020). Testing for somatic variants may require the use of next-generation sequencing-based diagnostic methods. Low-penetrance variants, Val198Met (Val200Met), Lys488Arg (Lys490Arg), and Gln703Lys (Gln705Lys) have been linked to a childhood autoinflammatory disease PFAPA (periodic fever, aphthous stomatitis, pharyngitis, adenitis), adult-onset disease Schnitzler syndrome, and other inflammatory phenotypes.

The clinical significance of these variants remains unclear. Activating mutations in NLRP3 cause caspase-1 mediated excessive production of IL-1 β , and CAPS prognosis has been dramatically changed with the use of IL-1 inhibitors.

NLRP12-associated (FCAS/MWS/NOMID) diseases (FCAS2)

NLRP12 is highly expressed in hematopoietic cells, however, it is unclear whether it forms a functional inflammasome. NLRP12 may function as an inhibitory protein by suppressing inflammatory responses in activated myeloid cells. Heterozygous LoF mutations in *NLRP12* are reported in several families with dominantly inherited cold-induced fever, localized or generalized urticaria-like rash, myalgia, and headaches (Borghini et al., 2011). The onset of symptoms is typically in childhood and avoiding exposure to cold temperatures prevents the disease flare. Given that NLRP12 is an anti-inflammatory protein, haploinsufficiency is the proposed mechanism of the disease that is yet to be characterized. Patient monocytes spontaneously secrete IL-1 β cytokine however, the efficacy of IL-1 inhibitors has been limited.

NLR4-associated autoinflammatory diseases (MAS/FCAS-4)

Heterozygous pathogenic missense variants in *NLR4* are gain-of-function, and they have different effects on the protein function, which ultimately influence disease severity. Severe de novo inherited disease-associated mutations spontaneously activate the protein and can cause life-threatening enterocolitis or a potentially lethal macrophage activation syndrome (MAS) (Canna et al., 2014; Romberg et al., 2014). The gastrointestinal inflammation can be very severe, particularly in infancy, however, some patients can survive the initial GI insult to develop a chronic milder GI disease. It is thought that the gut microbiota plays an important role in the expressivity of GI inflammation. Milder pathogenic variants that reduce the threshold for protein activation have been linked to a dominantly inherited phenotype with FCAS-like features. Primary patient myeloid cells spontaneously secrete IL-1 β and IL-18 cytokines. Patients with milder disease respond well to anti-IL-1 therapy, while patients with severe phenotype require aggressive treatment. New biological therapies with recombinant IL-18 binding protein (IL-18 BP), or blockade of interferon γ have shown promising results (Canna et al., 2017).

Pyrin-associated autoinflammatory diseases

Familial Mediterranean fever (FMF)

Pathogenic variants in pyrin, encoded by *MEFV*, have been associated with the most common autoinflammatory disease, familial Mediterranean fever (FMF), which has a high prevalence in eastern Mediterranean populations including, Arabs, Armenians, Iranians, Jews, and Turks. FMF is characterized by recurrent episodes of short-lasting fever accompanied by severe abdominal and/or chest pain, monoarthritis, and erysipelas-like erythematous rash. SAA amyloidosis is the most severe complication of the disease and was a major cause of mortality before colchicine therapy. FMF is caused by recessively inherited biallelic pathogenic missense variants that reside almost exclusively in the C-terminal the B30.2/SPRY domain, encoded by exon 10. Despite its recessive inheritance, FMF variants act as gain-of-function alleles by reducing a threshold for the activation of the pyrin inflammasome (Schnappauf et al., 2019).

Pathogenic mutations in other domains of pyrin have been associated with dominantly inherited severe inflammatory phenotypes. These pathogenic variants activate the pyrin inflammasome either by preventing pyrin inhibition or by enhancing its oligomerization. Heterozygous pathogenic variants in exon 2 are linked to a distinct disease, named pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND), manifesting with severe skin inflammation, polyarthralgia, and longer-lasting episodes of fever. Monocytes from PAAND patients produce higher levels of interleukin IL-1 β and IL-18 compared to cells from FMF patients. Dominantly inherited variants in exons 5–8 have been linked to severe colchicine-resistant fever syndrome manifesting with longer episodes of fever, serositis, severe polyarticular migratory arthritis, and renal SAA amyloidosis. The clinical significance of low-penetrance variants, Glu148Gln and Pro369Ser/Arg408Gln is still debated, although these variants may act as susceptibility alleles to inflammation (Gattorno et al., 2009; Berkun et al., 2011; Taniuchi et al., 2013; Batu et al., 2016). Most FMF patients have an excellent response to treatment with colchicine, while patients with more severe phenotype and/or amyloidosis require therapy with IL-1 inhibitors (Sag et al., 2020).

Diseases of cytoskeletal dysregulation/inflammatory actinopathies

The actin cytoskeleton is a dynamic network of actin and actin-binding proteins that are indispensable for many cellular functions, including, cell division, cell morphology, cell motility, intracellular trafficking, and endocytosis. Proteins that regulate actin polymers' assembly and organization, have been implicated in the pathogenesis of primary immunodeficiencies and autoinflammatory diseases, which corroborate the importance of actin cytoskeleton in modulating immune responses (Tangye et al., 2019).

The actin related protein complex (ARP) 2/3 is essential for the assembly and branching of actin polymers. Wiskott-Aldrich syndrome protein (WASP) is one of many regulatory proteins that promotes activation of the Arp2/3 complex. Besides, the WASP function is regulated by several proteins including, WIP (WASP-interacting protein), PSTPIP1 (proline serine threonine

phosphatase-interacting protein 1), and CDC42 (cell division cycle 42). Loss of WASP is associated with a severe X-linked disease (WAS) that manifests with immunodeficiency, thrombocytopenia, and autoimmunity. PSTPIP1 is thought to negatively regulate WASP by permitting PTP-PEST-mediated dephosphorylation of WASP. CDC42 is a member of the Rho GTPase family of proteins that induces activation of WASP to enable the interaction with the ARP2/3 complex. The Nck-associated protein 1-like (NCKAP1L/HEM1) is required for the RAC-mediated stabilization and activation of the WASP-family verprolin-homologous protein (WAVE2) complex. WAVE2's function is similar to WASP in allowing the ARP2/3 complex to initiate actin polymerization.

WD-repeat protein 1 (WDR1), also known as actin-depolymerizing factor (ADF/AIP1), is critical for the reorganization of the actin cytoskeleton by inducing disassembly of actin filaments. Abnormalities in the actin cytoskeleton organization affect the differentiation and function of innate and adaptive immune cells.

Pyogenic arthritis pyoderma gangrenosum and acne (PAPA) syndrome (PAPA)

Heterozygous pathogenic missense variants in *PSTPIP1* are associated with a severe type of neutrophilic dermatosis named pyogenic arthritis pyoderma gangrenosum and acne (PAPA) syndrome. PAPA usually present in childhood with sterile arthritis, while skin inflammation often starts in puberty and can be triggered by minor injuries. The most common PAPA-associated mutations, Ala230Thr and Glu250Gln, act by reducing the affinity for PTP-PEST and increasing the affinity of PSTPIP1 to pyrin. This finding suggests that inflammation in PAPA patients is partially due to an increased pyrin inflammasome activity and heightened IL-1 β production. Patients with severe mutations, Glu250Lys and Glu257Lys, have a more severe phenotype with abnormalities in the bone marrow and hematological manifestations (Belelli et al., 2017). This phenotype is named hyperzincemia and hypercalprotecinemia (Hz/Hc) or PSTPIP1-associated myeloid-related proteinemia inflammatory syndrome (PAMI) (Holzinger et al., 2015). PAPA patients with joint and mild skin inflammation respond well to treatment with IL-1 inhibitors, while patients with pyoderma gangrenosum require a higher dose of biologics (Omenetti et al., 2016; Mistry et al., 2018). HSCT has been successful in several PAMI patients resistant to medical therapies (Laberko et al., 2021).

Periodic fever, immunodeficiency, and thrombocytopenia syndrome (PFITS)

PFITS is a recessively inherited disease caused by LoF mutations in *WDR1*, which cause an impairment in the actin depolymerization. PFITS patients present with recurrent invasive opportunistic infections, periodic fevers, severe stomatitis, thrombocytopenia, impaired wound healing, pyoderma gangrenosum, and acne. Growth failure, dysmorphic face, and neurological impairment are seen in patients who have nearly absent protein function. WDR1-deficient myeloid cells produce exceedingly high levels of IL-18 cytokine, while interestingly the IL-1 β cytokine production is not affected. Treatment modalities for PFITS include immunoglobulin replacement, antibiotic prophylaxis, and IL-18 blockade (Kuhns et al., 2016; Standing et al., 2017; Pfajfer et al., 2018; Seppanen, 2018).

ARPC1B deficiency

Biallelic LoF mutations in the ARPC1B subunit of the ARP2/3 complex have been identified in patients who present with an early-onset immunodeficiency and low platelet count, similar to the Wiskott-Aldrich syndrome (Kahr et al., 2017; Brigida et al., 2018; Volpi et al., 2019; Randzavola et al., 2019). Other disease features include failure to thrive, small vessel vasculitis, early onset eczema, and inflammatory bowel disease. The platelets are small and deficient in dense granules and lamellipodia. The immune phenotype is characterized by eosinophilia, auto-antibody production, elevated IgE and IgA levels, and T-cell lymphopenia. Clinically, patients are predisposed to severe bacterial and viral infections. Treatment is symptomatic.

CDC42 deficiency

Dominantly inherited LoF mutations in the ubiquitously expressed isoform of cell division cycle 42 (CDC42) cause a neurodevelopmental phenotype, while inactivating mutations in the transcript isoform highly expressed in hematopoietic cells has been identified in patients with a severe potentially fatal hematological disease named NOCARH (neonatal onset of pancytopenia, autoinflammation, rash, and hemophagocytosis) (Lam et al., 2019; Gernez et al., 2019). Mutated protein displays an aberrant subcellular localization and rearrangement in actin cytoskeleton that affects all hematopoietic cells' function. BM mononuclear cells spontaneously release IL-1 β and IL-18 cytokines, while high levels of CXCL9 and IFN- γ are found during HLH episodes. Treatment with a monoclonal antibody targeting IFN- γ was successful in one patient, while some patients responded to IL-1 therapy.

NCKAP1L/HEM1 deficiency

Pathogenic biallelic LOF variants in HEM1 disrupt activation of the WAVE complex and lead to a complex immune dysregulation with features of immunodeficiency, lymphoproliferation, atopy, and severe inflammation (Cook et al., 2020; Castro et al., 2020). Deficiency of WAVE activity results in defective F-actin polymerization, besides, mutant HEM1 protein is shown to abrogate mTORC2-mediated activation of the AKT signaling pathway. Treatment is symptomatic.

Diseases of dysregulated ubiquitination

Ubiquitylation is a type of post-translational reversible protein modification that is critical for regulating all biological processes, including immune responses. Ubiquitylation involves the attachment of highly conserved 76-aa ubiquitin (Ub) molecules to target proteins in the form of a monomer or polymers (Ub chains). The type of linkage connecting the monomers in Ub chains ultimately determines the function of the modified protein. The attachment of Lys63 and Linear (Met1) Ub chains stabilize and upregulate protein activity, while the attachment of Lys48 Ub chains tags proteins for proteasomal degradation. The ubiquitylation process is counter regulated by a class of enzymes known as deubiquitinases (DUBs), and they have cell and target-specific functions.

The attachment of Met1 Ub chains to various immune molecules by the LUBAC (Linear Ubiquitin Chain Assembly Complex) is important for the assembly of receptor signaling complexes, for instance, TNFR1, TLRs, and IL-1R. LUBAC function is counteracted by OTULIN, a highly conserved deubiquitinase, with a specific activity to hydrolase Met1-linked Ub chains from target proteins (Shimizu et al., 2015). TNFAIP3, also referred to as A20, is another ubiquitin-editing enzyme that removes K-63 Ub polymers and facilitates the addition of K48 Ub to various target molecules. Thus, both OTULIN and A20 operate as negative regulators of the NF- κ B pathway. Defect in DUB activity affects other inflammatory pathways as well. In the past few years, several autoinflammatory diseases have been explained by dysregulation in the ubiquitin signaling pathway (Aksentjevich and Zhou, 2017; Beck et al., 2020).

Linear Ubiquitin Chain Assembly Complex (LUBAC) deficiencies

LUBAC consists of the catalytic subunit HOIP (HOIL1 interacting protein; *RNF31*) and two accessory proteins: HOIL-1 (Heme-oxidized IRP2 Ubiquitin ligase 1; *RBCK1*) and SHARPIN (SHANK-interacting protein like 1; *SIPL1*). Defect in one subunit of LUBAC destabilizes the expression of the whole LUBAC complex. LUBAC depletion leads to attenuation of NF- κ B and other inflammatory pathways. LUBAC activity is also important for proper B and T cell development, and maintenance of adaptive immune responses.

HOIL-1 and HOIP deficiencies are recessively inherited diseases caused by biallelic LoF mutations in respective proteins (Boisson et al., 2012, 2015; Oda et al., 2019). LUBAC-deficient patients have compromised immune responses and suffer from recurrent invasive bacterial infections. In addition to immunodeficiency, they present with systemic inflammation manifesting with fevers and GI inflammation. LUBAC-deficient patients are predisposed to develop muscular weakness and cardiomyopathy that is potentially life-threatening due to amylopectin deposition. Milder pathogenic mutations may cause an early-onset progressive myopathy/cardiomyopathy without overt immunodeficiency (Nilsson et al., 2013). Treatment is symptomatic.

Haploinsufficiency of A20 (HA20)

Heterozygous germline LoF variants in *TNFAIP3* cause a childhood-onset disease with a constellation of features from autoinflammation to autoimmunity (Zhou et al., 2016a). Typical signs include early-onset fevers, mucocutaneous ulcers, gastrointestinal and eye inflammation, arthralgia, lymphadenopathy, and a wide range of autoimmune findings (Aeschlimann et al., 2018; Berteau et al., 2018; Lawless et al., 2018; Kadowaki et al., 2018). A defect in HA20 function results in increased activity in the canonical NF- κ B pathway. During flares, high levels of many proinflammatory cytokines and chemokines are found in blood samples. Treatment with cytokine inhibitors especially TNF inhibitors and JAK inhibitors are efficacious in controlling systemic inflammation (Schwartz et al., 2020). HSC transplantation has been successful in one patient (Duncan et al., 2018). Common variants in the *TNFAIP3* gene locus, SNPs, have been linked with susceptibility to many autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, Crohn's disease, systemic sclerosis, psoriasis. Somatic variants in *TNFAIP3* are found in patients with Hodgkin's lymphoma and B cell lymphomas.

Otulin deficiency/otulipenia/ORAS

Recessively inherited LoF mutations in the *OTULIN* gene have been associated with the early-onset severe inflammatory disease, named otulipenia, also known as otulin-related autoinflammatory syndrome (ORAS) (Zhou et al., 2016b; Damgaard et al., 2016). This disease is rare and reported mainly in patients from founder populations. Clinical picture comprised failure to thrive, recurrent fevers, joint swelling, GI inflammation, an erythematous rash with skin nodules, lipodystrophy. A defect in DUB activity results in excessive linear ubiquitination and constitutive activation of key molecules in the canonical NF- κ B pathway. The inflammatory signature is stronger than in many other autoinflammatory disorders and characterized by spontaneous production of many proinflammatory cytokines and chemokines. Therapy with TNF inhibitors is very effective in suppressing systemic inflammation and reducing disease morbidity and mortality. HSC transplantation has been effective in one patient (Damgaard et al., 2019).

VEXAS (vacuoles, E1, X-linked, autoinflammatory, somatic) syndrome

UBA1 is a X-linked gene that encodes the major E1 enzyme required to initiate ubiquitylation in all cellular proteins. Pathogenic somatic missense mutations at the methionine 41 amino acid residue are found to affect the protein translation of the canonical cytoplasmic isoform and to give rise to a novel, catalytically impaired protein isoform (Beck et al., 2020). Patients present with an

often-fatal syndrome in late adulthood, manifesting with fevers, vasculitis, dysplastic bone marrow, cytopenias, venous thromboembolism, chondritis, vasculitis, neutrophilic cutaneous and pulmonary inflammation, and characteristic vacuoles in common myeloid and erythroid progenitor cells. The somatic variants originate in early marrow progenitor cells, however, in peripheral blood they are found exclusively in myeloid cells at an allele frequency often higher than 50%. Mutant lymphocytes either fail to proliferate or are eliminated from peripheral blood. Mutant monocytes display decreased ubiquitylation and upregulation in cellular stress responses and highly activated inflammatory signature. Patients are refractory to treatment with multiple disease-modifying anti-rheumatologic drugs (DMARDs) and cytokine inhibitors, while glucocorticoids in high doses can ameliorate severe inflammatory symptoms.

USP18 deficiency

Ubiquitin-specific peptidase 18 (USP18) is a type I interferon response gene that functions as a negative feedback regulator. Defect in type I IFN negative regulation leads to persistently high IFN-1 activity. Patients with biallelic LoF pathogenic variants in USP18 present at birth with a pseudo-TORCH-like phenotype, which resembles sequelae of congenital infections. Clinical signs include systemic inflammation, polymicrogyria, brain calcifications, seizures, liver dysfunction, severe thrombocytopenia, petechiae, necrotizing skin lesions, and respiratory failure (Meuwissen et al., 2016; Martin-Fernandez et al., 2020). The disease is lethal if left untreated. JAK inhibitor ruxolitinib was used successfully in one patient to induce sustained suppression of the disease activity (Alsohime et al., 2020).

ISG15 deficiency

ISG15 is a ubiquitin-like molecule with two functions; free intracellular ISG15 is crucial for USP18-mediated downregulation of type I IFN-signaling, while free extracellular ISG15 is crucial in IFN- γ -dependent antimycobacterial immunity (Hermann and Bogunovic, 2017). ISG15 expression is induced by type I interferons to stabilize the USP18 protein, thus the loss of ISG15 results in lower levels of USP18 and less efficient inhibition of type I IFN signaling. Patients with ISG15 deficiency have a relatively mild inflammatory phenotype with enhanced peripheral blood interferon gene expression signature, however, they may be predisposed to develop brain calcifications and neurological abnormalities (Bogunovic et al., 2012). Some patients present with a clinical disease only in response to BCG vaccination as the result of insufficient ISG15-dependent interferon- γ production.

Diseases mediated by activated NF- κ B pathway

Multitude of cellular processes converge at the point of NF- κ B, transcription factor (TF), activation. NF- κ B is a protein complex made up of five different members that combine to create homo- and hetero-dimers and exert wide range of biological effects. Activation of NF- κ B is critical for differentiation of immune cells, propagation of inflammatory response, and maintenance of cell viability. There is a number of monogenic disorders linked to dysregulation in activity of NF- κ B pathway, and the associated phenotypes are often mixed, having both features of autoinflammation and immunodeficiency (Schnappauf and Aksentijevich, 2020).

RIPK1-associated diseases

Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) has a critical role in a range of cellular functions, including the TNF signaling pathway. RIPK1 interacts with a variety of adaptors, ubiquitin ligases and kinases, to regulate not only pro-inflammatory effects but also cell survival.

Biallelic LoF variants in *RIPK1* have been described in patients who present with a combination of features comprising immunodeficiency, arthritis, and gastrointestinal inflammations (Cuchet-Lourenco et al., 2018; Li et al., 2019). RIPK1-deficient cells display compromised NF- κ B activity, decreased production of proinflammatory cytokines, and are hypersensitive to cell death by necroptosis. The inflammatory phenotype might be explained by the NLRP3 inflammasome activation and increased IL-1 β production. Successful HSCT has been reported; however, the intrinsic intestinal phenotype may not be rescued.

In contrast, patients with heterozygous activating pathogenic variants that affect the residue critical for the cleavage and inactivation of RIPK1 by caspase-8, the Asp (D) 342, present with systemic inflammation and without immunodeficiency (Tao et al., 2020; Lalaoui et al., 2020). This condition was named Cleavage-resistant RIPK1-induced Autoinflammatory (CRIA) syndrome. The main features of disease are high fevers, splenomegaly, lymphadenopathy, oral ulceration. Patients respond well to targeted therapy with IL-6 inhibitors.

Blau syndrome/NOD2-associated granulomatous disease

NOD2 is an intracellular pattern recognition receptor which is able to recognize specific PAMPs, such as muramyl dipeptide (MDP), and to consequently activate NF- κ B signaling pathway.

Heterozygous GoF germline or somatic mutations in the central NACHT domain cause a severe autoinflammatory disorder termed Blau Syndrome (BS) (Miceli-Richard et al., 2001; de Inocencio et al., 2015; Mensa-Vilaro et al., 2016). BS is characterized with a triad of early-onset inflammatory granulomatous polyarthritis, dermatitis and panuveitis (Rose et al., 2015). The arthritis is fairly severe leading to contractures and camptodactyly. Up to 42% of patients with BS report moderate to severe visual impairment (Sarens et al., 2018), which involves both the anterior (iridocyclitis) and posterior (retinal vasculitis, vitritis, and choroiditis) chambers of the eye. Other organs are affected at variable degrees of inflammation. The treatment of BS remains difficult. Anti-TNF treatments combined with methotrexate are largely effective for joint and visceral complications (Milman et al., 2006). The treatment of eye inflammation is more challenging since the response to anti-TNF is not universal. Other cytokine inhibitors, IL-1 or IL-6 blockade, might also be effective.

RELA haploinsufficiency

RelA (p65), encoded by *RELA* is one of the five members of NF- κ B family, which typically forms a heterodimer with NF- κ B1 (p50). Pathogenic variants in this gene are dominantly inherited and act by the mechanism of haploinsufficiency (Badran et al., 2017). Despite impaired NF- κ B activation in response to TNF, the affected patients presented with a childhood-onset inflammatory disease manifesting with fevers, mouth ulceration, vomiting, acute ileitis, and elevated inflammatory markers. These symptoms are explained by increased susceptibility of mutant cells to TNF-induced apoptosis. A second case of ReLA haploinsufficiency was reported to have a very different phenotype consistent with autoimmune lymphoproliferative syndrome, cytopenia, recurrent aseptic meningitis, and splenomegaly. Interestingly, investigations of the patient's fibroblasts showed a propensity to apoptosis, thus it has been postulated that the ReLA haploinsufficiency results from compromised stromal and epithelial cell recovery following a mucosal injury and TNF release from the injured mucosa. The positive effects of anti-TNF therapy in this case (infliximab) supports this explanation (Comrie et al., 2018).

CARD14-associated psoriasis (CAMPS)

Caspase recruitment domain-containing protein 14 (CARD14), has a specific role in controlling activation within the skin. Here, CARD14 participates in dectin-1 and IL-17 receptors initiated signaling pathways to regulate local expression of NF- κ B-inducible cytokines and chemokines.

Heterozygous high impact GoF mutations in the *CARD14* gene that result in enhanced NF- κ B activation and upregulation of psoriasis-associated genes have been associated with a spectrum of skin inflammatory phenotypes of variable disease expressivity and penetrance. These include psoriasis vulgaris (PV), familial pityriasis rubra pilaris (PRP), and pustular psoriasis (Jordan et al., 2012b; Fuchs-Telem et al., 2012; Berki et al., 2015). Constitutional symptoms may also be present. CAMPS explains only a minority of patients with familial psoriasis. Besides, rare low-penetrance variants are reported in sporadic patients with late-onset psoriasis. Targeted sequencing of *CARD14* in seven psoriasis cohorts with over 6000 cases and 4000 controls, identified many rare missense variants at a significantly higher frequency in cases compared to controls (Jordan et al., 2012a). These variants have a variable effect on the NF- κ B activation, and it is thought that other genetic modifiers and environmental factors further influence the disease onset and expressivity. Treatment with ustekinumab, a monoclonal antibody directed against the shared p40 subunit of interleukin (IL)-12 and IL-23, has been beneficial to some patients (Eytan et al., 2014).

ROSAH

ALPK1 which stands for (alpha-kinase 1)-TIFA (TRAF-interacting protein with forkhead-associated domain) protein has recently been identified as a cytosolic innate immune receptor for bacterial ADP- β -D-manno-heptose (ADP-hep) (Zhou et al., 2018). In this role ALPK1 is able to directly bind bacterial origin ADP-hep and subsequently activate NF- κ B.

A heterozygous missense variant in *ALPK1*, Thr237Met, was identified in five unrelated families to cause retinal dystrophy, optic nerve edema, splenomegaly, anhidrosis, and migraine headache (Williams et al., 2019). Based on these most consistent clinical features the condition was named ROSAH syndrome. Although fever was not initially described, additional studies identified the same variant in patients with the PFAPA syndrome (Sangiorgi et al., 2019; Zhong et al., 2020). Collectively these findings suggest that rare variants in *ALPK1* might be contributing towards an inflammatory phenotype. It is thought that the Thr237Met variant acts as GoF by stimulating the ALPK1 kinase domain and causing activation of downstream targets in the NF- κ B signaling pathway. Elevated TNF levels were detected in both cases, and patients with ocular disease were treated with anti-TNF, however the treatment was not fully effective.

Diseases of cytokine dysregulation

The members of the IL1 family of cytokines are pivotal for promoting inflammation and as such have been implicated in the pathogenesis of many autoinflammatory diseases (Dinarello, 2018). Since they are potent mediators of inflammation, release of proinflammatory cytokines IL-1, IL-18 and IL-36 must be under tight control by anti-inflammatory members of the same family. IL-1 β is predominantly expressed in peripheral blood and bone marrow, while IL-1 α and IL-36 are highly expressed in skin.

Mechanisms that prevent active IL-1 β cytokine from triggering the signal-transducing IL-1 type I receptor include the naturally occurring IL-1 receptor antagonist (IL-1Ra) and a soluble decoy IL-1 receptor type II. Autoinflammatory diseases may arise either from gain-of-function mutations in proteins that promote IL-1 signaling or from a defect in function of inhibitory proteins. IL-1 blockade is efficacious therapy for this group of diseases (Jesus and Goldbach-Mansky, 2014).

Deficiency of IL-1 receptor antagonist (DIRA)

Patients with DIRA carry biallelic LoF mutations in the *IL1RN* gene that encodes the IL-1R antagonist protein (IL-1Ra) (Aksentijevich et al., 2009; Reddy et al., 2009). DIRA is more common in patients from founder populations. The deficiency of IL-1Ra protein leads to unrestrained IL-1 β and IL-1 α activity. Onset of disease is post-natal or in infancy and comprises severe pustular dermatitis, multifocal sterile painful osteomyelitis, and characteristic overgrowth of epiphyseal bones. Osteolytic bone lesions are typically present in long bones, vertebrae and ribs. Biopsies of inflamed skin demonstrate infiltration with MPO+ neutrophils, mostly in epidermis. The disease course is severe and accompanied by persistently high levels of acute phase reactants. DIRA patients have rapid and dramatic improvement in the skin condition upon treatment with a recombinant IL-1 receptor antagonist, anakinra.

Deficiency of IL-36 receptor antagonist (DITRA)

IL-36 subfamily of cytokines and IL-36 receptor antagonist (IL-36Ra) are highly expressed in keratinocytes. IL-36Ra prevents IL-36 cytokine mediated signaling in a similar way as IL-1Ra blocks IL-1 cytokines. Deficiency of IL-36Ra is linked to a disease manifesting with recurrent episodes of high fever, generalized pustular eruptions and erythematous scaly plaques, nail dystrophy, impetigo herpetiformis, and acral pustular lesions (Marrakchi et al., 2011; Hussain et al., 2015). Disease flares are often triggered by infections and pregnancy. Skin biopsies show dense neutrophilic, macrophage and lymphocytic infiltrates. DITRA is a recessively inherited disease, like DIRA, and most patients are identified in founder populations. Although IL-36 and IL-1 are closely related proteins, IL-1 inhibitors are not very effective in treatment of DITRA patients. Some patients experienced good response to treatment with IL12/23 targeting drugs (Bonekamp et al., 2018).

IL-10 deficiency-associated inflammatory bowel disease (EO-IBD)

IL-10 is a critical cytokine for downregulation of pro-inflammatory signals. Binding of IL-10 to its cell surface receptor induces expression of anti-inflammatory genes and decreases expression of pro-inflammatory cytokines (Engelhardt and Grimbacher, 2014). Recessively inherited LoF mutations in two genes encoding components of the IL-10 receptor (*IL10RA* and *IL10RB*) or in the gene encoding IL-10 (*IL10*) lead to early-onset inflammatory bowel disease (Glocker et al., 2009, 2010). Patients present with fevers, oral ulcers, recurrent folliculitis, severe perianal fistula, colonic abscesses, and bloody diarrhea. EO-IBD is a treatment refractory disease, and patients often require surgical interventions. HSCT is the only curative therapy (Engelhardt et al., 2013).

Diseases mediated by endoplasmic-reticulum (ER) stress

The ER stress is typically triggered by the accumulation of misfolded proteins within the ER. The resulting endoplasmic-reticulum-associated protein degradation (ERAD) and unfolded protein response (UPR) are evolutionally conserved pathways activated in the event of ER stress to maintain cell homeostasis. Through ERAD, misfolded proteins are targeted for degradation by the proteasome, whilst UPR has more diverse effects including, a reduction in protein synthesis, and ultimately activation of apoptosis in the case ER stress failed to be resolved. ER stress has other biological effects including, increased responsiveness to TLR signaling, activation of the NF- κ B pathway, and activation/phosphorylation of IRF3, a transcription factor inducing type I IFN gene expression. This section describes several autoinflammatory diseases where the interplay between ER stress, proteasome function and mROS is important for the disease pathogenesis.

Tumor necrosis factor receptor-associated periodic syndrome (TRAPS)

Heterozygous missense mutations in the *TNFRSF1A* gene, which codes for TNF receptor 1 (TNFR1), affect the structure and folding of the extracellular domain of the receptor (McDermott et al., 1999). The immunopathogenesis of TRAPS is complex with several biological pathways being implicated. A defect in TNFR1 leads to reduced levels of soluble TNFR1 that blocks binding of TNF to the cell surface. There is also evidence for ligand-independent activation of mutant TNFR1, enhanced ER stress as results of TNFR1 misfolding, increased generation of mitochondrial oxygen species, and activation of NLRP3 inflammasome (Simon et al., 2010; Bulua et al., 2011). Typical symptoms associated with TRAPS include recurrent fevers lasting for weeks, serositis, myalgia, centrifugally spreading erythematous rash, and periorbital oedema (Cudrici et al., 2020). Patients who have infrequent flares and no evidence of ongoing systemic inflammatory response can be adequately treated with on-demand therapy such as short courses of NSAIDs and/or corticosteroids. The mainstay of treatment for patients with more severe disease is with IL-1 inhibitors anakinra or canakinumab, both of which achieve rapid remission (Gattorno et al., 2008; De Benedetti et al., 2018).

Sideroblastic anemia, immunodeficiency, periodic fevers, developmental delay (SIFD)

The primary function of tRNA nucleotidyl transferase 1 (TRNT1), encoded by *TRNT1*, is catalyzing the addition of cytosine-cytosine-adenosine (CCA) trinucleotides to 3' end of all precursor tRNAs. The resulting aminoacylation enables mature tRNAs to fully participate in protein biosynthesis. In humans, TRNT1 has a non-redundant function and complete deficiency is embryonically lethal.

Patients with SIFD carry biallelic LoF variants in the *TRNT1* gene (Chakraborty et al., 2014). The principal clinical features include sideroblastic anemia, immunodeficiency, periodic fevers, neurological complications such as ataxia, seizures, sensorineural hearing loss, and developmental delay. The condition tends to present in early life, and in many cases results in multi-organ involvement and premature death (Slade et al., 2020). However, milder cases have been recognized in two families with non-syndromic retinitis pigmentosa and no other overt neurological features or cardiomyopathy (DeLuca et al., 2016). The multi-system manifestations in SIFD are not surprising considering the ubiquitous expression of TRNT1 and its critical function related to protein translation. The neurological and hematological features are most likely related to the impaired mitochondrial function; however, B cell lymphopenia/pan-hypogammaglobulinemia and systemic inflammation are more difficult to link directly to the defective TRNT1. It is thought that increased ER stress, resulting from the accumulation of damaged proteins, likely contributes to selective apoptosis in lymphocytes. The management of SIFD remains difficult and is mostly supportive with red blood cell transfusion used to correct anemia, immunoglobulin replacement in patients with immunodeficiency, and anti-TNF therapy in patients with inflammatory features (Giannelou et al., 2018). To date there is one reported case where HSCT resulted in an effective cure of the autoinflammatory phenotype (Wiseman et al., 2013).

TRAP1-associated disease

Tumor necrosis factor receptor associated protein 1 (TRAP1) belongs to the heat shock protein (HSP) 90 family. TRAP1 has a role in processing and folding of nascent and proteins damaged from cellular stress (Chien et al., 2011). TRAP1 also functions to limit mitochondrial damage and production of mROS, which can otherwise drive inflammatory innate-immune pathways.

Bi-allelic hypomorphic mutations in *TRAP1* contribute to a severe autoinflammatory disorder described in a consanguineous Pakistani family (Standing et al., 2020). The clinical phenotype of these children was complex and presented at 2 weeks of age with severe oral ulceration, recurrent fevers, failure to thrive, and persistently elevated inflammatory markers. Another unrelated case was identified with a long-standing history of the anakinra-responsive disease characterized by recurrent fevers, arthralgia, and cervical lymphadenopathy. Patients' monocytes showed increased production of mROS and a higher expression of spliced X-box protein 1 (sXBP1), which is consistent with ER stress. Lastly, LPS stimulation of PBMCs showed increased production of both IL-1 β and in particular IL-18, associated with caspase-1 activation. The disease is resistant to therapies and potentially lethal. Allogeneic HSCT was successful in a single patient.

Proteasome-associated autoinflammatory syndromes (PRAAS)

One of the main functions of the ubiquitin-proteasome system (UPS) is to catalyze the degradation of short-lived intracellular proteins. The 26S proteasome complex consists of a 20S proteolytic core and two 19S regulatory elements, and recognizes K-48 ubiquitinated proteins destined for degradation. Immunoproteasomes, which is typically found in immune cells, have standard β type subunits replaced by three inducible β -type subunits β 1i, β 2i, and β 5i.

Biallelic LoF mutations in *PSMB8*, encoding the β 5i catalytic subunit, or in other proteasomes the 20S subunits (*PSMB4*, *PSMB9*, *PSMB10*) and the proteasome chaperon (*PSMG2*) are associated with a severe autoinflammatory disorder named PRAAS or chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) (Agarwal et al., 2010; Arima et al., 2011; Kitamura et al., 2011; Liu et al., 2012; de Jesus et al., 2019; Sarabay et al., 2020). The genetics of PRAAS is complex and include monogenic recessive inheritance, and digenic inheritance pattern due to monoallelic mutations in two different proteasome genes (i.e., *PSMA3/PSMB8*, *PSMB4/PSMB9*, and *PSMB4/PSMB8*) (Brehm et al., 2015). Most patients present in the neonatal period or infancy. The characteristic clinical picture comprises fevers, an annular acral rash with raised edges, progressive lipodystrophy affecting the face, upper extremities, trunk, myositis, periodic fevers, and periorbital/perioral oedema. Although this disease has a chronic course, many innocuous stimuli such as viral infections, ambient cold air, and physical/psychological stress can trigger disease flares. The reduced function of the immunoproteasome is linked to upregulation in ER stress and UPR (Ebstein et al., 2019). The inflammatory signature is predominantly mediated by type I interferons, which led to the beneficial use of JAK inhibitors in this disorder (Sanchez et al., 2018).

Coatomer subunit α (COPA) syndrome

COPA syndrome is caused by dominantly inherited mutations in the *COPA* gene, which encodes the α -subunit of the coatomer complex I (COPI) (Watkin et al., 2015). COPI has a role in retrograde trafficking of cargo proteins from Golgi to the endoplasmic reticulum and within cis-Golgi compartments (Beck et al., 2009). All pathogenic mutations affect the highly conserved WD40 domain of COPA, resulting in defective protein trafficking, ER stress, and activation of the UPR and NF- κ B signaling. One such transport affected is with the STING protein (Lepelletier et al., 2020). Predictably, the clinical phenotype of affected patients is

complex (Fremond and Nathan, 2020). The COPA syndrome tends to present in childhood or early adulthood, however, the disease penetrance is incomplete and asymptomatic cases have been described. The main clinical features include interstitial lung disease, occasionally accompanied by pulmonary hemorrhage, immune-mediated kidney disease, and inflammatory arthritis. Common immunological features include the presence of autoantibodies, increased Th17 levels, and upregulation of interferon-stimulated genes (ISGs) (Volpi et al., 2018). The important role of type I IFNs in the disease pathogenesis has been further supported by two recent case reports which describe a positive response to JAK1/2 inhibition (Krutzke et al., 2019; Fremond et al., 2020).

Diseases caused by dysregulation in nucleic acid metabolism and sensing

The presence of foreign or self-nucleic acids (DNA, ssRNA or dsRNA) in the cytoplasm creates a danger signal in the cell. The mammalian cells have evolved a complex machinery to rapidly recognize and respond to the accumulation of intracellular nucleic acids. In the case of microbial invasion, one important outcome from the recognition of foreign nucleic acids is the generation of type I interferons (IFN). Although, the type I IFN is critical for an adequate immune response to infection, these powerful cytokines can cause significant host damage if left unchecked. There are several monogenic disorders, collectively termed type I interferonopathies, linked to dysregulated type I IFN signaling. These diseases result either from GoF variants in PRRs that recognize cytoplasmic nucleic acids, or from failure to remove largely self-nucleic acids (Uggenti et al., 2019).

Aicardi-Goutières syndrome (AGS)

AGS is the prototypic type I interferonopathy characterized by early-onset neurological disease mimicking an in utero viral infection. Following advancements in genetic sequencing, seven subtypes of AGS have now been recognized, each associated with a specific gene. The disease-associated genes are involved with the processing of endogenous nucleic acids (TREX1, RNASEH2 subunits, SAMHD1, ADAR1) or detection and sensing of exogenous nucleic acids (*IFIH1*) (Crow and Manel, 2015). The LoF mutations in nuclease activity that result in the accumulation of nucleic acid molecules, or GoF mutations in the sensing gene *IFIH1* lead to an excessive type I IFN signaling.

AGS is a heterogenous disorder with a wide range of clinical features. There is a phenotypical variation between different causative genes, and even among patients carrying mutations in the same gene (Rice et al., 2020). One-fifth of all cases has neurological involvement with spasticity, dystonia, seizures, microcephaly, and basal ganglia calcification. Seemingly spontaneous fevers, as well as chilblains, are also associated with AGS as is a lupus-like syndrome. Low-titer autoantibodies are commonly found in the sera of AGS patients (Crow et al., 2015). The role of type I IFN in the AGS pathogenesis is further supported by reports of successful use of treatment strategies that downregulate the IFN pathway (Briand et al., 2019).

STING-associated vasculopathy with onset in infancy (SAVI)

STING, which stands for Stimulator of Interferon Genes, is a protein involved in coupling sensing of exogenous DNA to the type I IFN response. STING is typically activated by binding of cyclic GMP-AMP (cGAMP), produced by cGAMP synthase (cGAS) after cGAS binds to DNA of microbial or host origin. Subsequently, after transmigration from the ER to the Golgi, STING causes activation of interferon regulatory factor 3 (IRF3), resulting in the expression of interferon-stimulated genes (ISGs) and the production of type I IFN (Li and Chen, 2018; Ablasser and Chen, 2019).

SAVI is caused by heterozygous GoF mutations in the *TMEM173* gene, which encodes STING, resulting in constitutive activation of the protein (Liu et al., 2014). Patients tend to present with severe interstitial lung disease, severe vascular inflammation and skin ulceration, and acral necrosis. It is assumed that these clinical features develop as a result of high levels of interferon activity, however, in animal models neither type I IFN or IRF3 are necessary to recapitulate some disease features, and functioning T cells were required for the development of lung inflammation (Luksch et al., 2019). Nevertheless, similarly to AGS, therapeutic strategies that specifically block type I INF signaling (JAK inhibitors) have been used to ameliorate symptoms in some patients with SAVI (Sanchez et al., 2018).

Diseases of dysregulated lipid metabolism

Mevalonate kinase deficiency (MKD)

Mevalonate kinase (MVK) is a ubiquitously expressed enzyme with a key role in the biosynthesis of cholesterol and isoprenoids. Patients with mevalonate kinase deficiency can present with variable phenotypes including, mevalonic aciduria (MA), hyper IgD syndrome (HIDS), and disseminated superficial actinic porokeratosis (DSAP) (Drenth et al., 1999; Houten et al., 1999; Zhang et al., 2012). Patients with nearly absent enzymatic activity manifest severe developmental disabilities and fevers, while biallelic hypomorphic variants cause HIDS (van der Burgh et al., 2013).

Patients with HIDS typically develop symptoms in infancy manifesting with episodes of high fever, abdominal/joint pain, diarrhea, erythematous rash, swollen lymph nodes, and the disease onset is often triggered by immunization. The most common

HIDS associated variants are Ile268Thr and Val377Ile, accounting for more than 50% of patients across multiple populations. DSAP has been reported in patients of Asian ancestry and is caused by a monoallelic germ-line pathogenic variant and a second somatic pathogenic variant present in keratinocytes (Kubo et al., 2019). Defect in MVK activity leads to a lack of isoprenoids that play a role in post-translational protein modifications by prenylation of proteins that regulate the activity of pyrin and NLRP3 inflammasomes (Akula et al., 2016; Park et al., 2016). Mutant MKD cells have excessive production of IL-1 β . The inflammatory phenotype in patients with HIDS is ameliorated with anti-IL-1 therapy, while patients with MA may require HSCT (De Benedetti et al., 2018; Neven et al., 2007; Arkwright et al., 2007).

LPIN2 deficiency

LPIN2 is an enzyme with phosphatidate phosphatase activity that plays a role in downregulation of proinflammatory signaling induced by saturated fatty acids. LPIN2 catalyzes the conversion of phosphatidic acid to diacylglycerol (DAG), which works as a secondary lipid messenger by activating protein kinases and RAS signaling pathways, among others (Donkor et al., 2007).

LPIN2 loss of function leads to Majeed Syndrome, a rare, recessively inherited disorder characterized by the triad of early-onset chronic recurrent multifocal osteomyelitis (CRMO), dyserythropoietic anemia, and neutrophilic skin lesions (Ferguson et al., 2005; Cox and Ferguson, 2018). All reported cases of Majeed syndrome are identified in consanguineous families of Middle Eastern or Indian ancestry. The affected patients carry homozygous mostly truncated mutations that lead to reduced protein expression. LPIN2 has been shown to regulate the activity of the NLRP3 inflammasome. IL-1 blockade results in a dramatic improvement in clinical and laboratory parameters of inflammation in Majeed syndrome, corroborating that a loss of LPIN2 activity leads to upregulation in the IL1 signaling pathway (Herlin et al., 2013).

PLC γ 2-associated diseases

Phospholipase C2 (PLC γ 2) enzyme is activated by B cell and Fc receptors to catalyzes the formation of secondary messengers, inositol triphosphate (IP $_3$) and DAG from phosphatidylinositol 4,5-bisphosphate (PIP2). Binding of released IP $_3$ to IP $_3$ receptors results in intracellular calcium release and activation of various signaling pathways including, MAP kinases (MAPK) (Gorjestani et al., 2011).

Heterozygous pathogenic mutations in *PLCG2* lead to two distinct phenotypes: PLC γ 2-associated antibody deficiency and immune dysregulation (PLAID) and autoinflammation, antibody deficiency, and immune dysregulation (APLAID). In both conditions, B cell immunodeficiency is the cause of recurrent bacterial respiratory and GI infections. PLAID patients present with early-onset symptoms of cold-induced urticarial or blistering rash, variable degrees of immunodeficiency and autoimmunity, and potentially tissue destructive skin granulomas (Ombrello et al., 2012; Milner, 2015). This phenotype is caused by in-frame genomic deletions spanning the cSH2 autoinhibitory domain of PLC γ 2, which results in a release of the protein active site. The mutant protein exhibits a reduction in PLC2-mediated activity at physiologic temperature and enhanced signaling at sub-physiologic temperatures. Mast cells are spontaneously activated when exposed to lower temperature.

APLAID is a rare severe disease manifesting early in life with fever, blistering or erythematous skin lesions, progressive interstitial lung disease, enterocolitis, ocular inflammation, and arthralgia (Zhou et al., 2012; Moran-Villasenor et al., 2019; Neves et al., 2018; Martín-Nalda et al., 2020). APLAID patients carry GoF missense mutations in the same cSH2 autoinhibitory domain of PLC γ 2, which cause increased release of intracellular Ca $^{2+}$ and upregulation of the NLRP3 inflammasome (Chae et al., 2015). PLAID and APLAID patients with immunodeficiency require immunoglobulin replacement therapy, while patients with severe inflammatory phenotype are treated with corticosteroids and cytokine inhibitors.

LACC1 deficiency

LACC1 encodes for the protein FAMIN (Fatty Acid Metabolism and Immunity Nexus), which promotes fatty acid oxidation and lipogenesis to ensure energy sources available for proper macrophage function (Cader et al., 2016).

Common missense variant, Ile254Val variant (rs3764147) in *LACC1* have been linked to leprosy and Crohn's disease (CD) by GWAS (Liu et al., 2015; Assadi et al., 2016; Wang et al., 2018). The novel pathogenic variant Cys284Arg is identified in children with recessively inherited early-onset CD and in children with systemic juvenile idiopathic arthritis (SoJIA) (Patel et al., 2014; Wakil et al., 2015). Patients diagnosed with SoJIA have the characteristic quotidian fevers, evanescent rash, and symmetrical polyarthritides like children with a typical polygenic SoJIA. Juvenile idiopathic arthritis has emerged as another *LACC1*-associated phenotype. All reported patients have been identified in consanguineous families from Saudi Arabia and other Middle Eastern countries (Karacan et al., 2018). Functional confirmation experiments of *LACC1* pathogenic variants demonstrated a decrease in protein expression, indicating that the disease is caused by loss of function of FAMIN. Effective treatment modalities include TNF blockade with adalimumab or IL-6 inhibition with tocilizumab (Kallinich et al., 2016).

Other monogenic autoinflammatory diseases

SH3BP2 deficiency/cherubism

The SH3-binding protein (SH3BP2) is an adaptor protein expressed in most cell types with a role in regulation of various signal transduction pathways. Dominantly inherited or de novo missense mutations in *SH3BP2* lead to activation of various inflammatory pathways and stimulate excessive osteoclastogenesis (Ueki et al., 2001). Activated tissue macrophages and osteoclasts give rise to disproportionate bone resorption. Cherubism occurs in children and is characterized by painless bone cyst-like lesions and excessive bone degradation of the upper and lower jaws. These lesions are then replaced with fibrous tissue masses, which cause a prominent swelling and deformities in the lower portion of the face. In some patients, the condition is associated with problems with teething, speech, and swallowing. The limitation of bone lesions to jaws is likely explained by the requirement for rapid bone remodeling during childhood in connection with secondary dentition. Enlargement of the jaw usually stabilizes during puberty, and some patients fully recover without the need for any intervention (Papadaki et al., 2012).

Coagulation factor XII (FXII)-associated autoinflammatory disease

Coagulation factor XII (FXII) is a plasma protein with thromboinflammatory properties and drives production of the inflammatory peptide bradykinin. Pathogenic variants in the *F12* gene, which encodes FXII, have been associated with potentially life-threatening hereditary angioedema (HAE). A specific missense mutation, Trp268Arg, which results in protein activation has been identified in a four-generation family with FCAS-like phenotype manifesting with cold-inducible generalized urticarial rash, fever, chill, headache, arthralgia, and fatigue (Scheffel et al., 2020). This variant causes changes in protein conformation and exposes the activation loop, which is otherwise buried in wild type protein. The mutant protein activates the kallikrein-kinin pathway and induces increases in IL-1 β secretion. Biopsies of lesional skin upon cold exposure revealed moderate dermal oedema, and perivascular infiltrates composed of macrophages. Neutrophils are identified as the source of FXII in the skin of patients. All patients reported complete unresponsiveness to oral antihistamines. Treatment with the bradykinin B2 receptor antagonist, icatibant, resolves symptoms of oedema and cold-induced inflammation.

Deficiency of adenosine deaminase type 2 (DADA2)

Adenosine deaminase (ADA) plays a role in purine metabolism by catalyzing the deamination of adenosine (Ado) and 2'-deoxyadenosine (dAdo) into inosine and deoxyinosine, respectively. ADA2 is a secreted protein produced by activated myeloid cells.

Deficiency of adenosine deaminase type 2 (DADA2) is a recessively inherited disease associated with hypomorphic or LoF mutations that result in low or absent ADA2 activity (Zhou et al., 2014; Navon Elkan et al., 2014). Diagnosis is established either by genetic testing or by measuring plasma or serum ADA2 activity. DADA2 is a type of medium-size vessel vasculitis with histologic findings consistent with polyarteritis nodosa (PAN) (Meyts and Aksentjevich, 2018). Livedoid rash is the most common skin manifestation; subcutaneous nodules, Raynaud's, warts, ulcerations, and distal vascular occlusion are other skin features. Neurological impairments are the consequence of lacunar ischemic infarcts in the deep-brain nuclei, midbrain, and/or brainstem, in addition, hemorrhagic strokes have been observed in a subset of patients. Hematological manifestations, including aplastic anemia, pure red cell aplasia, lymphopenia, neutropenia, and thrombocytopenia, have been reported in patients with absent ADA2 activity. A subset of patients was diagnosed with CVID or autoimmune manifestations resembling systemic lupus. Hypogammaglobulinemia, in particular low IgM levels, in children presenting with fevers indicates DADA2 in the differential diagnosis. Deficiency of ADA2 causes an imbalance in the differentiation of macrophages with a paucity of anti-inflammatory M2 macrophages and activation of neutrophils (Carmona-Rivera et al., 2019). Elevated levels of the proinflammatory cytokines TNF and IL-1 have been identified in skin biopsies and blood samples of DADA2 patients. Primary treatment of DADA2 relies on anti-TNF agents in patients with an inflammatory phenotype, while patients with bone marrow failure may require HSCT (Hashem et al., 2017, 2021; Ombrello et al., 2019).

Conclusion

The family of autoinflammatory disorders has grown rapidly to incorporate many novel biological mechanisms involved in the inflammatory processes. Various attempts have been made to classify SAIDs. However, irrespective of the approach used, be it according to the genetic cause, predominant clinical features, the principal biological pathway, or response to treatment, there have always been exceptions. For example, FMF and PAAND are both caused by the GoF mutations in *MEFV*, but the clinical features and response to therapy of these two disorders vary considerably. Similarly, HA20, which is classified as a disorder of dysregulated ubiquitination, might also have interferonopathy-like features, since it seems that type I IFN signature predicts a positive response to JAKi in some patients.

Nevertheless, some way of classifying these disorders is necessary, if nothing else, to provide a framework to deal with new discoveries in the field. However, an alternative approach to patients who present with clinical features suggestive of SAID, might be

to perform, rapid, unbiased, genotyping, combined with detailed cytokine profiling and immunological characterization, to fully appreciate all factors involved in the disease pathogenesis. Such an approach, which has recently been described, might help to inform treatment choices even in the cases where the genetic basis of the disease is unknown (De Jesus et al., 2020) and bring forward the concept of personalized medicine closer to reality. For selective patients with unusually severe phenotypes of systemic inflammation and/or features of combined immunodeficiency, severe immune dysregulation or bone marrow failure, as well as those unresponsive to conventional and/or biologic therapies, allogeneic HSCT is a valid treatment possibility (Greco et al., 2019; Chan et al., 2020; Abinun and Slatter, 2021). Cautious patient selection is mandatory, as well as performing HSCT in experienced transplant centers with multidisciplinary team involvement.

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Complement Deficiencies

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Introduction

The knowledge about inborn errors of immunity (IEI), also named as primary immunodeficiencies has increased very fast in the last years. The access to genetic defects improved our comprehension about the variability of phenotypes related to molecular findings (Tangye et al., 2020). Complement deficiencies are usually hereditary. The most common type is autosomal recessive form, including C1, C2, C3, C4, C5, C6, C7, C8, C9, and Mannan-binding lectin deficiencies (Schröder-Braunstein and Kirschfink, 2019). A X-linked recessive pattern has been described for properdin deficiency. Probably more often, complement deficiencies are acquired through complement overconsumption, protein synthesis dysfunction, protein loss disorders, autoimmunity, and high catabolic states. Findings such as complement defects underlying atypical hemolytic uremic syndrome (aHUS) or in the lectin pathway are subject to diverse interpretations (Hirt-Minkowski et al., 2010; Michalski et al., 2015).

The actual prevalence of complement deficiencies is still unknown. Most estimations have been based on selected populations such as tertiary hospitals, or single-center registries. National registries show a wide variability in the frequencies of these defects, comprising between 1% and up to 30% of all primary immunodeficiencies (Abolhassani et al., 2018, 2020; Mukhina et al., 2020). According to the European Society for Immunodeficiencies (ESID) registry, deficiencies of complement proteins were responsible for approximately 5% of all primary immunodeficiencies (PID) between 2004 and 2020 (<http://esid.org/Working-Parties/Registry/ESID-Database-Statistics>; <https://cci-reporting.uniklinik-freiburg.de/#/>).

There are several aspects influencing the diagnosis of complement defects. The awareness about clinical symptoms is still restricted (Grumach and Kirschfink, 2014). Nowadays, the best example is the hereditary angioedema (HAE) with a high investment in the diagnosis, reflected by a more frequent identification of patients. Some casuistries of complement deficiencies showed half of the patients being diagnosed with hereditary angioedema (Mukhina et al., 2020; Genel et al., 2018). However, the estimated prevalence for HAE with C1 inhibitor deficiency is 1:50,000 and it is less common than MBL, C2, C5-C9 deficiencies (Mukhina et al., 2020). The analysis of complement-deficient patients included in ESID registry ($n = 77$) excluding HAE patients (Turley et al., 2015), revealed that 43% had defects in the classical pathway (CP), 31% in the alternative pathway (AP), and 26% defects of terminal complement components. C2 deficiency was the most common of the observed deficiencies (29% of the total) (Turley et al., 2015). Partial deficiencies, such as of C4, may be of relevance in certain clinical situations (Grumach et al., 2006).

Complement plays an essential role in both innate and adaptive immune responses. In cooperation with other immune and physiological systems, complement mediates the clearance of immune complexes, cellular debris and apoptotic cells, contributes to normal tissue and organ development, and promotes tissue repair after injury (Hajishengallis et al., 2017). A dysfunction in this system in terms of upregulation, downregulation, or dysregulation can cause a wide range of effects, including disturbance of normal host defense and altered inflammatory response (Botto et al., 2009).

Clinical history is the most important initial diagnostic tool for ruling out a complement deficiency. Severe or multiple infections, caused by capsulated bacteria as *Streptococcus*, *Haemophilus* and *Neisseriae* or autoimmune diseases in particular with childhood onset are the main clinical manifestations associated with complement deficiency (Brodzski et al., 2020). In a recent

study with more than 200 patients, infections corresponded to approximately 70% of the clinical picture, mostly caused by *Meningococcus* or *Pneumococcus*. Autoimmune disorders occurred in 14% of the cases. In addition, kidney disease (aHUS) or C3 Glomerulopathy, C3G) was also reported (El Sissy et al., 2019). The milder symptomatology related to some complement defects in comparison with other Inborn Errors of Immunity could justify the reduced identification of affected patients; nevertheless, the most relevant point to be emphasized is the difficulty to access specific assays and manipulate the samples for complement diagnosis. The thermolability and the involvement of complement proteins as acute phase reactants often leads to misdiagnosis. In addition, there are only few laboratories developing laboratorial and molecular diagnosis for primary complement deficiencies (Prohászka et al., 2018; Grumach and Kirschfink, 2014; Costa-Carvalho et al., 2017).

Complement system

The complement system forms an elaborate network of fluid-phase, cell surface-associated, and intracellular proteins, which either function as pattern-recognition molecules, proteases/convertases, regulators, or signaling receptors, collectively mediating immune surveillance and tissue homeostasis (Ricklin et al., 2010).

There are three major complement activation pathways (Fig. 1): the classical pathway (CP) initiated via antigen-antibody complexes, the alternative pathway (AP) through any permissive surface and the lectin pathway (LP) by the interaction of pattern-recognition mannose-binding lectins (MBL) or ficolins with carbohydrate ligands on the surface of the pathogens.

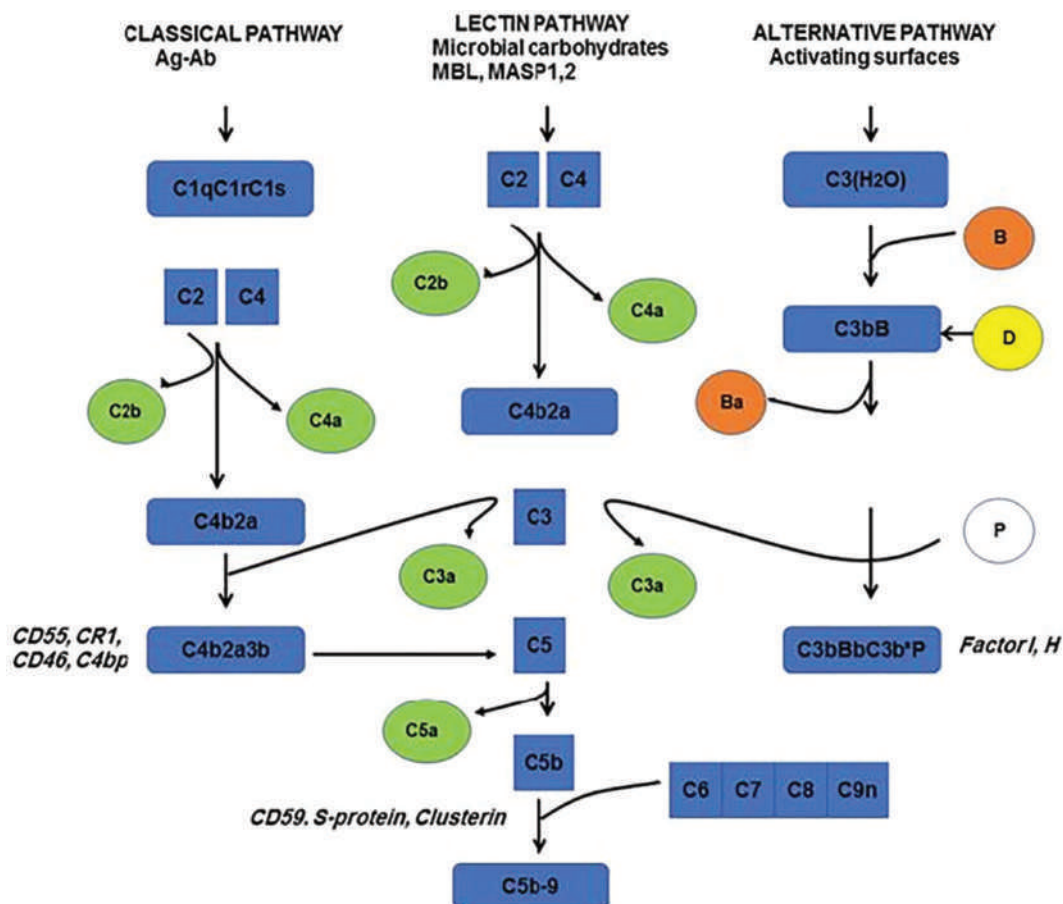


Fig. 1 Complement activation pathways and assembly of the terminal pathway. The classical pathway is initiated by the binding of the C1 complex to antibody already bound to antigen, leading to the formation of the C4b2a enzyme complex (C3 convertase). The lectin pathway is activated by the binding of either MBL or ficolin and MASP 1, 2 and 3, respectively, to an array of mannose groups on the surface of bacterial cells and the generation of C3 convertase of the classical pathway. The alternative pathway is initiated by hydrolyzed C3 and factor B and the subsequent formation of the alternative pathway C3-convertase, C3bBb. Generation of the C3-convertase allows the formation of the C5 convertase, which initiates the formation of the C5b-9 terminal complement complex. The complement system is regulated at several levels: CD55, CR1, CD46, C4bp, and factors I and H regulate the activity of the C3-convertase and C5-convertase, and CD59 blocks the final assembly of MAC by preventing the binding of C9. S protein/vitronectin binds to C5b-7 and leads to the formation of a cytolytically inactive sC5b-9 complex.

The convergence point of complement activation pathways is the formation of the C3 convertase which cleaves the C3 molecules into C3a (one of two major anaphylatoxin molecules) and C3b, which is a potent opsonin. C3b and its degradation products iC3b and C3d bind to intact target cells or cell debris and act as ligands for complement receptors CR1 (CD35), CR3 (CD11b + CD18), and CR2 (CD21), respectively, expressed by myelomonocytic cells, lymphocytes and follicular dendritic cells. The binding of C3b or its metabolites to the correspondent cell receptors is crucial for cell to cell interaction of the innate and adaptive immune responses and for removing apoptotic and necrotic cells (Ricklin et al., 2010). Consecutive activation and binding of C3 leads to the generation of the C5 convertase complex which cleaves C5 into C5a and C5b. The anaphylatoxin C5a recruits neutrophils to the areas of inflammation and exerts tissue damage. The membrane attack complex (MAC or C5b-9) is formed by generating a complex between C5b, C6, C7, C8 and multiple C9 which inserts into the cell membrane of target cells. MAC formation disrupts the phospholipid bilayer of the cell membrane, which causes massive calcium influx, loss of mitochondrial membrane potential and cell lysis (Hajishengallis et al., 2017). Collectively, the main three consequences of complement activation are (1) tagging target cells by C3b-metabolites for phagocytosis, (2) recruitment of inflammatory cells by C3a and C5a, and (3) MAC-mediated cell lysis (Fig. 1).

Complement deficiencies

Deficiencies of the classical pathway

Patients deficient in the initial components of the classical pathway are prone to autoimmune connective tissue diseases, such as SLE, and other autoimmune diseases, e.g., dermatomyositis, Henoch-Schönlein purpura, juvenile rheumatoid arthritis and glomerulonephritis (Macedo and Isaac, 2016). Out of all cases of SLE only a small fraction is associated with complement deficiency, indicating that SLE is a highly multifactorial disease (Degn et al., 2011). Genetic factors influencing the predisposition to SLE was verified evaluating monozygotic and dizygotic twins (Deapen et al., 1992), however environmental factors are also involved (Macedo and Isaac, 2016). Deficiency of the early components of the complement system classical pathway (CP), mainly C1q (90–93%), C1r/C1s (50–57%), C4 (75%), and C2 (10%) represent clearly a genetic susceptibility to SLE.

C4 deficiency is also extremely rare. This could be due to variations in C4 gene copy number with two to seven copies found in the diploid genome. Multiple mutations have to occur for deficiency to result (Degn et al., 2011). Individuals with heterozygous C2 or C4 deficiency often remain asymptomatic (Macedo and Isaac, 2016).

Deficiency of C2 is, on the other hand, generally associated with a slight increase in the prevalence of autoimmune disease. Furthermore, about half of the patients identified with C2 deficiency had a history of invasive infection with encapsulated bacteria. Family members lacking C2 but not showing clinical symptoms were often encountered (Degn et al., 2011).

Although the occurrence of autoimmunity and autoantibodies have been highlighted, patients with CP defects also develop an increased susceptibility to infections. It is estimated that ~50% of patients develop severe bacterial infections including meningitis, pneumonia, arthritis, or septicemia. These infections are caused by encapsulated bacteria, most commonly *Streptococcus pneumoniae*. Infections and vascular diseases represent the leading causes of death in these patients (Brodzski et al., 2020).

Deficiencies of the alternative pathway

Defects in proteins of the classical and terminal pathways are more frequent than those of components of the alternative pathway (properdin, factor D, and factor B) which account for only 2.5% of all complement deficiencies (Audemard-Verger et al., 2016). Properdin deficiency is the only complement protein inherited in a X-linked pattern and is often associated with fulminant meningococcal disease (López-Lera et al., 2019). It manifests with either complete absence of the molecule (type I), partial deficiency (type II) or a normal level of dysfunctional protein (type III). Properdin-deficient individuals are susceptible to meningococcal disease, which is frequently complicated by sepsis and most commonly occurs in adolescence (Brodzski et al., 2020). The risk of meningococcal infection in healthy individuals is usually greatest in children aged less than 2 years, when protective antibodies against meningococcal serotypes have not developed. In patients with properdin deficiency, the median age at the time of meningococcal infection is much higher at approximately 14 years of age (Overturf, 2003). Factor D deficiency was reported in less than 10 cases and it is also considered as a predisposing factor for meningococcal infection. Factor B deficiency has been described only twice and both patients presented recurrent bacterial infections caused by encapsulated bacteria. It is important to mention that the investigation of these proteins is scarce (Slade et al., 2013; Gauthier et al., 2020). Factor B variants were identified only rarely as the genetic cause in cases of aHUS (Brodzski et al., 2020).

C3 deficiency

C3 plays a central role in all three activation pathways and it is not surprising that its deficiency has been associated with severe recurrent pyogenic bacterial infections and immune complex-mediated diseases (Reis et al., 2006). The infections develop early in life and have a tendency to recur (da Silva et al., 2016). They are mainly caused by gram-negative bacteria, such as *Neisseria meningitidis*, *Enterobacter aerogenes*, *Haemophilus influenzae* and *Escherichia coli*. In addition, besides *Streptococcus pneumoniae*, infections caused by other gram-positive organisms, including *Streptococcus pyogenes* and *Staphylococcus aureus*, have also been observed in C3-deficient patients. The infections affect the upper and lower respiratory tract including pneumonia, episodes of sinusitis,

tonsillitis and otitis. Severe infectious complications also occur, e.g., pneumonia, meningitis, osteomyelitis, or bacteremia meningitis. Patients may also experience membranous glomerulonephritis, while symptoms consistent with SLE are less frequent. Rare C3 gain of function (GoF) mutations may lead to aHUS (Brodzski et al., 2020). One common and several rare variants in C3 have been associated with an increased risk of age-related macular degeneration (AMD) (Schramm et al., 2014).

Lectin pathway

Lectin pathway impairment due to insufficient production of any of these components is common and may be associated with several phenotypes as increased risk of infection, especially in young children and otherwise immunocompromised individuals. An association to autoimmunity has also been reported (Heitzeneder et al., 2012). In addition, there are studies showing the influence of lectin pathway on the severity of diseases (Jensenius et al., 2003; Brodzski et al., 2020). As mentioned before, polymorphisms in complement genes are linked to, for example, atypical hemolytic uremia and age-dependent macular degeneration. Polymorphisms in the collectin and ficolin genes cause variable degrees of insufficiency and no measurable ficolin 3 was associated with recurrent infections diseases in a 32-year-old man (Hummelshoj et al., 2005; Munthe-Fog et al., 2009).

The clinical impact of low MBL levels has attracted considerable interest in 'MBL deficiency'. MBL deficiency is still being used to classify some state of immunodeficiency, however, the definition of what constitutes MBL deficiency is unclear (Heitzeneder et al., 2012). MBL deficiency was identified in 5% of European descent, considering plasma levels below 100 ng/ml. A higher frequency was described for Sub-Saharan Africans (Degen et al., 2011). MBL insufficiency is, in combination with other factors, associated with more severe forms of sepsis and fatal outcomes, irrespective of the causal microorganisms (Brodzski et al., 2020).

MBL is a pattern recognition molecule involved in opsonization as well as in complement activation. Therefore, it seems evident that low MBL levels could be disadvantageous in circumstances in which the integrity of innate immunity is essential for protection against infections, e.g., in infancy. On the other hand, low MBL levels may be advantageous if strong complement activation is linked to extensive tissue damage. This notion implies that the impact of a given MBL status is context-dependent (Heitzeneder et al., 2012).

MBL deficiency was related to upper respiratory infections in children and it was reported a higher risk for severe meningococcal or pneumococcal infections at this age (Grumach and Sullivan, 2019). A controversial role of MBL deficiency has been described in severe infections, septicemia, or affecting the severity in certain diseases. Also, patients with HIV infection, under chemotherapy and after stem cell transplantation appear to be more susceptible to severe infections if MBL deficiency is associated (Heitzeneder et al., 2012). On the other side, the MBL deficiency could be beneficial in certain infections caused by *Leishmania* or *Mycobacteria* (de Messias-Reason et al., 2007). MBL levels could also influence the susceptibility and severity of autoimmune disease, reported for inflammatory bowel disease and systemic lupus erythematosus (Vignesh et al., 2017). Thus, the MBL status appears as a modifier, either aggravating the severity or improving the course of a disease according to its polymorphism.

Mannose-associated serine protease 1 (MASP1), the most abundant protease of the lectin pathway, has a central role in pathway activation via MASP2. 3MC syndrome is characterized by facial dysmorphism and other developmental defects such as cleft lip and palate, postnatal growth deficiency, cognitive impairment, and hearing loss. It is caused by homozygous mutations in the MASP1 gene (3MC syndrome 1) or members of the collectin subfamily COLEC10 or COLEC11 (3MC syndrome 2). Excess or unusual infections and autoimmunity have not yet been described in this syndrome (Brodzski et al., 2020).

A case of MASP-2 deficiency was originally described in a patient with persistent inflammatory disease, episodes of severe pneumococcal pneumonia and progressive lung fibrosis (Stengaard-Pedersen et al., 2003). Additional studies revealed that only a low clinical penetrance of MASP-2 deficiency is seen (Beltrame et al., 2015).

Ficolin-3 deficient cases manifest variable clinical symptoms, particularly infections, autoimmune disorders and neurological complications. Only a few homozygous cases have been described and different clinical symptoms including enterocolitis, respiratory and skin infections, brain abscess, refractory seizures and nephrotic syndrome have been observed (Babaha et al., 2020).

Deficiency of terminal components

Defects of terminal complement components (C5 through C9) are almost exclusively associated with an increased risk for recurrent invasive meningococcal disease and disseminated gonococcal infection (Figueroa and Densen, 1991). C9 deficiency, which is extremely rare in Caucasians, was detected in about 0.1% of Japanese blood donors, however without clinical consequences (Figueroa and Densen, 1991; Grumach and Kirschfink, 2014). Although meningococcal infection may occur at all ages, there is a peak incidence in children aged <2 years, which can be explained by the absence of specific protective antibodies (Grumach and Kirschfink, 2014). The average age of onset of symptoms in patients with terminal pathway deficiencies is 17 years. In children above 5 years of age with meningococcal meningitis there is a higher probability for a complement deficiency. Uncommon serogroups, W-135, X, Y, or Z, are more frequently related to a terminal complement component deficiency (Grumach and Kirschfink, 2014). Recurrence of diseases is another characteristic feature; almost 50% of individuals with deficiencies in components of the terminal pathway will experience at least one additional episode (Lewis and Ram, 2020). Access to specific immunization may change this profile (Ram et al., 2010). Persons with inherited terminal component deficiency experience a lower mortality per meningococcal episode compared to complement-sufficient patients (1.5% versus 19% mortality per 100 episodes). Mortality and severity of meningococcal disease is proportional to the degree of complement activation and endotoxin levels in plasma (Lewis and Ram, 2020).

Deficiencies of complement regulation

The powerful activation cascades of complement system require tight regulation to maintain appropriate homeostasis and to avoid excessive damage to self. An impaired function of these regulatory proteins is often deleterious (Liszewski and Atkinson, 2015). A broad clinical phenotype is associated with the impairment of this control system. A number of genetic alterations are associated with increased risk of developing inflammatory renal (aHUS, C3G) and ophthalmic disorders (AMD). The spectrum of clinical presentations also includes angioedema (C1 inhibitor [C1-INH] deficiency), protein-losing enteropathy (CD55 deficiency), and paroxysmal nocturnal hemoglobinuria (PNH) (CD55 + CD59 deficiency) (Noris and Remuzzi, 2009; Liszewski and Atkinson, 2015; Brodzski et al., 2020). It is important to note that, in contrast to complete deficiencies of complement regulatory proteins that result in consumption of multiple components in a pathway, haploinsufficiency can cause excessive local inflammation occurring at sites of tissue injury or debris accumulation, potentially initiated by endothelial cell damage. For example, haploinsufficiency of FH predisposes to aHUS and AMD, while homozygous FH deficiency results in alternative pathway activation, cleavage and consumption of C3 and FB, leading to increased susceptibility to pyogenic infections. Heterozygous deficiency of FI is also associated with both aHUS and AMD. PNH results from mutations in the glycosylphosphatidylinositol anchor affecting the two membrane-bound inhibitors of the complement system, decay-accelerating factor (DAF, CD55) and membrane inhibitor of reactive lysis (MIRL, CD59) (Degen et al., 2011). The complement system attacks the erythrocytes lacking these control proteins, causing PNH. Guillain-Barré-like neurological symptoms with hemolysis are the hallmark of rare cases of isolated CD59 deficiency.

Hereditary angioedema (HAE) caused by deficiency of C1-INH is inherited in an autosomal dominant trait. Besides controlling complement system activation, of C1 inhibitor regulates the fibrinolytic, coagulation and contact systems. Lack of inhibition results in excessive bradykinin generation, which in turn increases vascular permeability, leading to angioedema. The onset of the disease is early in life, causing subcutaneous and submucosa edema. The symptoms are edema affecting the face, periphery, genitals, abdomen and larynx. The upper airway obstruction can result in asphyxia if not treated. An acquired form of C1 inhibitor deficiency mostly occurs above the 4th decade of life and is associated with lymphoproliferative disorders and the presence of autoantibodies to C1 inhibitor. Another type of HAE was identified in patients with normal C1-INH levels. Mutations in genes coding for factor XII (FXII-HAE), plasminogen (PLG-HAE) and, in few families, angiopoietin-1, kininogen-1 or myoferlin have been found in this newly-defined group of primary angioedema patients. In a significant proportion of HAE patients with normal C1 inhibitor, mutations have not been detected yet (Bork et al., 2020).

Factor H and Factor I are regulators of the AP causing secondary C3 deficiency. Recurrent infections are expected in the deficiency of any of these factors (Leitão et al., 1997; Falcão et al., 2008). Approximately 50% of patients with aHUS have genetic mutations of FH, FI, C3, FB and/or MCP, and deletion of complement FH-related proteins 1 and 3 (CFHR1/CFHR3) (Nester et al., 2015). Mutations in FH, MCP and FI have also been reported in C3 glomerulopathy (formerly known as membranoproliferative glomerulonephritis (MPGN)), as well as those presenting with pre-eclampsia and hemolysis, elevated liver enzyme levels, and low platelet levels (HELLP) syndrome. Partial FI deficiency has also been previously associated with clinical manifestations including recurrent tonsillitis, urinary infections, otitis, pyelonephritis, severe meningitis and sepsis (Grumach et al., 2006).

Dysregulation of the AP appears to also play a significant role in the pathogenesis of age-related macular degeneration (AMD), which is the most common cause of vision loss in developed countries. Polymorphisms in genes encoding for FH, C3 and FI, have been associated with AMD (Brodzski et al., 2020).

Laboratory diagnosis of complement deficiencies

The work-up of complement deficiencies requires the combined laboratory analysis of multiple aspects of the complement system such as the functionality and the activation state of the different pathways, the concentration and function of single component and regulators, the presence of auto-antibodies as well as the molecular analysis of complement related genes (for reviews see: Prohaszka et al., 2018; Ekdahl et al., 2018; Schröder-Braunstein and Kirschfink, 2019; Gavrilaki and Brodzki, 2020).

When complement deficiency is considered as a differential diagnosis, in a first step, a set of tests assessing the functionality of the classical and the alternative pathway (CH50 and AH50) as well as the activation state of the complement system should be performed ("complement status"). If indicated clinically, the integrity of the lectin pathway should also be investigated. The combined results of these assays allow to determine whether a loss of pathway functionality is due to a primary (inherited) defect of complement factors or mostly likely caused by consumption of complement factors following strong complement activation (secondary defect). Furthermore, they allow to distinguish between defects affecting pathway-specific components (C1/ C2/C4 vs. FB /FB/Properdin vs. MBL/MASP1/2) and impairment the common terminal pathway (C3-C9).

In case the results of these assays suggest a primary complement defect, the analysis of individual complement components (including regulators) is indicated. The latter includes the determination of the concentration of single factors as well as functional test such as resubstitution and titration assays. Furthermore, flow cytometric analysis is employed to examine expression levels of complement regulators, such as CD55 or CD59 on cell membranes. Finally, genetic analysis will provide insight into gene variants underlying suspected inherited complement defects (Liszewski et al., 2017).

Importantly, the laboratory analysis of complement deficiencies also involves the analysis of inhibitory or activating autoantibodies targeting individual complement components including regulators (e.g. anti-C1q, anti-FH, C3 nephritic factor). By interfering with the function of the respective components, these autoantibodies represent clinically relevant causes of secondary complement deficiencies (Ekdahl et al., 2018).

Preanalytics

Conclusive complement analysis critically depends on correct sampling and subsequent preanalytical handling of the samples. Whereas serum is required for functional analysis of the complement pathways and suitable for measuring the concentration of complement components as well as autoantibodies, the quantification of activation products needs to be performed using EDTA plasma. By chelating divalent cations such as Ca^{2+} and Mg^{2+} , EDTA at concentrations of 10 mM or higher inhibits convertase activity and thereby prevents complement activation as occurring rapidly *ex vivo*. Importantly, as another measure to prevent *ex vivo* complement activation, serum and EDTA plasma have to be separated from whole blood samples as rapidly as possible. Subsequently, they need to be subject to immediate analysis (e.g. within 4 h if stored on ice or at 4 °C) or to be frozen at -80 °C until being assayed or shipped to specialized laboratories on dry ice (Mollnes et al., 1988).

For more details, the reader is referred to the detailed description of a comprehensive complement analysis in this encyclopedia.

Treatment of complement deficiencies

General considerations

Treatment of complement deficiencies includes antibiotic prophylaxis, immunization covering encapsulated bacteria, pneumococcal, *Haemophilus* and meningococcal [types A, C, W, Y, and B] vaccines, and prompt workup for sepsis if the clinical symptoms suggest bacterial infection (Ochs and Petroni, 2018). Autoimmune diseases are treated symptomatically, using the same immunosuppressive agents and anti-inflammatory medications as those used in the general population. Management of hereditary angioedema has been revolutionized by C1-esterase inhibitor concentrate, and by kallikrein inhibitor or bradykinin receptor antagonist preparations (Betschel et al., 2020).

Vaccination

There is no contra indication to use routine vaccination for complement deficient patients. Considering the susceptibility to encapsulated bacteria, it is important to indicate vaccines against pneumococcus, *Haemophilus influenzae*, and *Neisseria meningitidis*. Conjugated vaccines can be applied early in life and they represent the best choice (Ram et al., 2010). Children less than 2 years do not respond to nonconjugated vaccine. A two-dose of meningococcal conjugate vaccine has been recommended for patients with complement deficiencies and asplenia and it is important to check vaccine response in order to offer protection against bacterial infections (Brady et al., 2011). House contacts should also receive the same vaccines in order to reduce the possibility of transmission. For patients with HAE who may require blood products as part of their therapy, hepatitis B vaccination is recommended (Betschel et al., 2020).

Antibiotics

The use of antibiotic prophylaxis in complement deficiencies is aimed at protecting against infection by encapsulated organisms and it is best reserved for patients exhibiting recurrent infections despite appropriate vaccination (Bonilla et al., 2005; Brodzski et al., 2020). Monthly benzathine penicillin was described to be protective against *Neisseria* infection in 40 patients with homozygous C6 deficiency (Potter et al., 1990). This study was performed before general application of meningococcus vaccines. Other protocols evaluated the use of amoxicillin prophylaxis in children with recurrent acute otitis media where reduction of the infections was more evident in the first 6 months of treatment in comparison with placebo (Roark and Berman, 1997). In contrast, antimicrobial prophylaxis wasn't advised by others (Teele et al., 2000).

Specific therapies

Specific complement targeted therapies has developed enormously in this century. aHUS and PNH is now properly controlled with Eculizumab, a humanized monoclonal antibody that binds to C5, which prevents its cleavage C5a and C5b, thereby inhibiting the generation of the terminal complement complex C5b-9 (Mache et al., 2009). Hemolysis may still occur in some patients with PNH despite treatment with eculizumab, most probably due to unrestricted extravascular hemolysis. More recently, Ravulizumab, a long-acting, second-generation C5 inhibitor, was approved, to be administered intravenously every 8 weeks, with good comparable results to Eculizumab (Kulasekararaj et al., 2019). More complement inhibitors are now in phase 2 and phase 3 clinical studies potentially allowing a better disease-tailored complement intervention (Mastellos et al., 2019).

The treatment of Hereditary Angioedema progressed in the last years with the inclusion of new drugs for prophylaxis. Besides the use of intravenous plasma derived C1 inhibitor in several countries, the recombinant C1 inhibitor and the formulation for subcutaneous use of C1 inhibitor were available. More recently, the therapy focusing kallikrein has been developed with the launching of Lanadelumab (subcutaneous use) and Berotralstat (oral use). Regarding the attacks of angioedema, Icatibant, an inhibitor of bradykinin receptor, and Ecallantide, a kallikrein inhibitor, in addition to the intravenous C1 inhibitor, have been available decreasing the risk of death due to asphyxia (Cicardi et al., 2014; Betschel et al., 2020). Several drugs acting in the contact system have been under clinical trials (phases I and II) in patients with Hereditary Angioedema. In addition, the expansion of the indications for HAE with normal C1 inhibitor and children will be accessible soon (Fijen et al., 2021).

Conclusions

Complement deficiencies mainly manifest by recurrent infections caused by encapsulated bacteria, autoimmunity, recurrent angioedema without wheals and inflammatory disorders involving kidney and eyes. Family history of consanguinity directs to an autosomal recessive inheritance, described for most of the components excepts for Properdin (X-linked) and C1 inhibitor (autosomal dominant) deficiencies. Due to the limited access to laboratorial diagnosis, we can suppose that complement deficiencies are underdiagnosed. The progress on therapies for complement deficiencies and its manipulation have opened new perspectives for the patients. A better approach to the patients will certainly establish the need for adequate laboratorial diagnosis.

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Phenocopies of Inborn Errors of Immunity

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Introduction

The study of immune deficiencies is mainly focused on inborn errors of immunity. Yet, it is not that uncommon for a patient to have a presentation resembling that of a well-known primary immunodeficiency (PID) yet presenting at an older age or without any demonstrable genetic etiology. Two groups of phenocopies can lead to diseases with a clinical picture compatible with a heritable immunodeficiency (Table 1). Both somatic mutations and disease related to autoantibodies affecting the same pathway implicated in the inborn error can cause disease that resembles a heritable immunodeficiency. It is critical to recognize these diagnostic possibilities. Only a small number of scenarios have been identified, yet it is likely that they occur much more often. The recognition of patients with disease caused by a somatic mutation in a gene known to be critical for immune function is limited by technology and analytic approaches unless that pathologic cells are isolated and identification of patients with autoantibodies is limited by the lack of testing. Therefore, the diagnosis suggested by a strong clinical suspicion requires partnering with a laboratory experienced in the field.

Diseases associated with autoantibodies

AutoAb to IL-17A, IL-17F and/or IL-22/chronic mucocutaneous candidiasis

Chronic mucocutaneous candidiasis (CMC) is characterized by recurrent or persistent symptomatic mucocutaneous fungal infections affecting the nails, skin, and oral and genital mucosae. The lesions are usually caused by the commensal *Candida albicans*. Patients with severe combined immunodeficiency (SCID) and combined immunodeficiencies (CIDs), are also prone to CMC. However, in these PIDs associated with severe T cell defect, patients are also susceptible to many other infections. By contrast, in patients with autosomal dominant hyper IgE syndrome (AD-HIES) bearing dominant negative mutations of the *STAT3* gene, and in patients with autosomal recessive autoimmune polyendocrinopathy syndrome type I (APECED or AR APS-I) due to *AIRE* mutations, CMC is the principal or only infectious disease frequently observed (Puel et al., 2012). CMC has also been reported in one family with *CARD9* deficiency (Glocker et al., 2009). Patients with syndromic CMC have very small proportions of IL-17- and IL-22-secreting T cells. In a series of 33 APECED patients, anti-IL17 autoantibodies were identified in all of them, 29 of whom had CMC. They confirmed function of the anti-IL-17A antibody by inhibiting IL-6 production from IL-17 responsive fibroblasts (Puel et al., 2010). In another report on 162 APECED patients, anti-IL-17 A, IL-17F, and IL-22 autoantibodies were found in up to 90% of cases, also strongly associated with CMC (Kisand et al., 2010). High levels of neutralizing autoantibodies against IL-17A, IL-17F and/or IL-22 were documented in patients with APS-I presenting with CMC as their only infectious disease (Puel et al., 2012). These autoantibodies may precede onset of other features of APS-I (Wolff et al., 2013).

Some patients present with “isolated” CMC or CMC disease (CMCD), a clinical entity that usually begins early in infancy and affects otherwise healthy individuals. The molecular study of these patients led to the identification of the first three genetic aetiologies of CMCD: AR IL-17RA and AD IL-17F deficiencies and AD *STAT1* gain-of-function, impairing IL-17-producing T cell development. These data provided formal proof that IL-17 cytokines are crucial for mucocutaneous protection against *C. albicans*. However, no genetic etiology has yet been identified for a number of patients with CMCD. In addition, autoantibodies against IL-22 have been detected in patients with an APS-1 phenotype in the absence of a genetic diagnosis (Eriksson et al., 2018).

Table 1 Phenocopies of inborn errors of immunity.

	<i>Genetic defect/ presumed pathogenesis</i>	<i>Circulating T cells</i>	<i>Circulating B cells</i>	<i>Serum Ig</i>	<i>Associated features/similar PID</i>
<i>Diseases associated with autoantibodies</i>					
Chronic mucocutaneous candidiasis	AutoAb to IL-17 and/or IL-22	Normal	Normal	Normal	Chronic mucocutaneous candidiasis/CMC
Adult-onset immunodeficiency with susceptibility to mycobacteria	AutoAb to IFN γ	Decreased naive T cells	Normal	Normal	Mycobacterial, fungal, <i>Salmonella</i> , VZV infections/MSMD, or CID
Recurrent skin infection	AutoAb to IL-6	Normal	Normal	Normal	Staphylococcal infections/STAT3 deficiency
Pulmonary alveolar proteinosis	AutoAb to GM-CSF	Normal	Normal	Normal	Pulmonary alveolar proteinosis, cryptococcal meningitis, disseminated nocardiosis/CSF2RA deficiency
Acquired angioedema	AutoAb to C1 inhibitor	Normal	Normal	Normal	Angioedema/C1 INH deficiency (hereditary angioedema)
Thymoma with hypogammaglobulinemia (Good syndrome)	AutoAb to various cytokines	Increased CD8 + T cells	No B cells	Decreased	Invasive bacterial, viral or opportunistic infections, autoimmunity, PRCA, lichen planus, cytopenia, colitis, chronic diarrhea
<i>Diseases associated with somatic mutations</i>					
Autoimmune lymphoproliferative syndrome (ALPS–SFAS)	Somatic mutation in <i>TNFRSF6</i>	Increased CD4 – CD8 – double negative (DN) alpha/beta T cells	Normal, but increased number of CD5 + B cells	Normal or increased	Splenomegaly, lymphadenopathy, autoimmune cytopenias, Defective lymphocyte apoptosis
RAS-associated autoimmune leukoproliferative disease (RALD)	Somatic mutation in <i>KRAS</i> (GOF)	Normal	B cell lymphocytosis	Normal or increased	Splenomegaly, lymphadenopathy, autoimmune cytopenias, granulocytosis, monocytosis
RAS-associated autoimmune leukoproliferative disease (RALD)	Somatic mutation in <i>NRAS</i> (GOF)	Increased CD4–CD8–double negative (DN) alpha/beta T cells	Lymphocytosis	Normal or increased	Splenomegaly, lymphadenopathy, autoantibodies
RAS-associated autoimmune leukoproliferative disease (RALD)	Somatic mutation in <i>NRAS</i> (GOF)	Increased CD4 – CD8 – double negative (DN) alpha/beta T cells	Lymphocytosis	Normal or increased	Splenomegaly, lymphadenopathy, autoantibodies
Cryopyrinopathy, (Muckle-Wells/CINCA/NOMID-like syndrome)	Somatic mutation in <i>NLRP3</i>	Normal	Normal	Normal	Urticaria-like rash, arthropathy, neurological signs
Large Granular Lymphocyte leukemia	Somatic mutation in <i>STAT3</i> (GOF)	T or NK lymphocytosis with an activated phenotype	Normal	Normal	Neutropenia, anemia, splenomegaly
Hypereosinophilic syndrome	Somatic mutation in <i>STAT5B</i> (GOF)	Normal	Normal	Normal	Eosinophilia, atopic dermatitis, urticarial rash, diarrhea

AutoAb to IFN γ /susceptibility to mycobacteria

Mendelian susceptibility to mycobacterial disease (MSMD) is a rare inherited condition defined by selective susceptibility to weakly virulent mycobacteria, including Bacille Calmette-Guérin (BCG) vaccine substrains and various environmental mycobacteria, in otherwise healthy patients without overt immunological abnormalities. Patients with MSMD may also suffer from tuberculosis, caused by *Mycobacterium tuberculosis* or from invasive infections with intracellular-macrophage microorganisms, such as *Salmonella*. The genetic dissection of MSMD in these patients has revealed this condition to be caused by inborn errors of interferon (IFN)- γ immunity (Rosain et al., 2019). These findings confirm that IFN- γ is a critical macrophage-activating factor and most patients with MSMD have molecular defects in the IFN γ /IL-12 axis. Susceptibility to tuberculosis and nontuberculous mycobacterial infection as well as listeriosis, salmonellosis, histoplasmosis, coccidioidomycosis, melioidosis, and penicilliosis has been demonstrated in patients with IFN- γ R deficiency (Dorman and Holland, 2000). Mutations in 14 different genes, most of them impacting the same pathway, (*IL12B*, *IL12RB1*, *ISG15*, *TYK2*, *IRF8*, *SPPL2A*, *CYBB*, *IFNGR1*, *IFNGR2*, *STAT1*, *NEMO*, *RORC*, *JAK1*, *GATA2*) have been shown to cause MSMD (Rosain et al., 2019).

The first cases of immunodeficiency caused by autoantibodies to IFN- γ were described in 2004 (Döffinger et al., 2004; Höfllich et al., 2004). These reports have shown autoantibodies against IFN- γ associated with severe nontuberculous mycobacterial infections, mainly in female patients of East Asian descent. In a large series from Thailand and Taiwan, 81% of the 52 patients with disseminated nontuberculous mycobacterial infection had high titers of anti-IFN- γ autoantibodies (Browne et al., 2012). The prevalence was even higher (96%) in the 45 patients with other opportunistic infections (e.g., *Cryptococcus neoformans*, *H. capsulatum*, *P. marneffei*, disseminated salmonellosis or severe varicella-zoster virus infection). Overall, 88% of the patients with adult-onset immunodeficiency with opportunistic infections had anti-IFN- γ autoantibodies. These autoantibodies against IFN- γ in patients with disseminated nontuberculous mycobacterial infection were shown to be associated with two HLA class II subtypes; HLA-DRB1*15/16 and HLA-DQB1*05 (Pithukpakorn et al., 2015; Angkasekwinai et al., 2019).

AutoAb to IL-6/recurrent and severe bacterial skin infection

Increased susceptibility to staphylococcal diseases is observed in several PIDs, including chronic granulomatous disease, autosomal dominant hyper-IgE syndrome, anhidrotic ectodermal dysplasia with immunodeficiency and diseases associated with TLR signaling pathway deficiencies such as *IRAK-4*, *MyD88*, *IRAK-1* and *TIRAP* deficiencies. Infectious episodes are typically associated with mild or delayed signs of inflammation, such as fever and increase in C-reactive protein (CRP) levels as a consequence of an impaired production of proinflammatory cytokines such as IL-6, a cytokine that regulates the acute phase response in the liver, inducing production of C-reactive protein and other inflammatory markers.

Recurrent or severe skin infections involving *Staphylococcus aureus*, *Escherichia coli* or *Streptococcus intermedius*, have been described in a child and two adults in the absence of detectable serum CRP. The patients' serum contained high titers of neutralizing autoantibodies against IL-6, accounting for the lack of CRP response and, possibly, for the development of recurrent or severe infection (Puel et al., 2008; Nanki et al., 2013).

AutoAb to various cytokines/thymoma and good syndrome

Good syndrome is a rare sporadic condition with a late onset in which thymoma is associated with hypogammaglobulinemia. It is characterized by increased susceptibility to bacterial, viral and fungal infections, as well as autoimmunity. Most patients have no circulating B cells and defects in T cell-mediated immunity are frequently observed (Malphettes et al., 2015). Pathogenic autoantibodies are common in thymoma, most often in the form of myasthenia gravis caused by anti-acetylcholine receptor antibodies. Also described are a wide range of anti-cytokine autoantibodies, most commonly including IL-12 p35 and p40 subunits and type I interferons, but also other neutralizing anti-cytokine autoantibodies including those to IL-1 α , IL-17A, IL-22 (Burbelo et al., 2010). Lack of *AIRE* expression has been shown in thymoma tissue suggesting that beyond APECED, *AIRE* may play a role in other autoimmune conditions and contribute to the development of anti-cytokine autoantibodies, which are striking features of both APECED and thymoma (Offerhaus et al., 2007; Scarpino et al., 2007). It has been shown that in patients with thymoma, all patients with opportunistic infection had multiple anticytokine autoantibodies with blocking activity in vitro, suggesting that anticytokine autoantibodies may be important in the pathogenesis of adult-onset immunodeficiency associated with thymoma. The main cytokine targets for these autoantibodies were: IFN- α , IFN- β , IL-1 α , IL-12 and IL-17A (Burbelo et al., 2010).

AutoAb to GM-CSF/pulmonary alveolar proteinosis

Primary pulmonary alveolar proteinosis (PAP) is a rare syndrome characterized by accumulation of surfactant in the lungs that is mediated by disruption of granulocyte/macrophage colony-stimulating factor (GM-CSF) and alveolar macrophage dysfunction (Carey and Trapnell, 2010). Mutations or deletions in the GM-CSF Receptor alpha chain (*CSF2RA*) have been described in hereditary forms of the disease, reducing GM-CSF binding, receptor signaling, and GM-CSF functions in myeloid cells (Martinez-Moczygemba et al., 2008; Suzuki et al., 2008).

Autoimmune PAP accounts for approximately 90% of the PAP cases and is characterized by high levels of neutralizing GM-CSF autoantibodies (Inoue et al., 2008). This rare disease occurs in all ethnic groups and is approximately twice as common in males, presumably due to the association with smoking. Individuals usually present in the third to fourth decades of life with progressive dyspnea of insidious onset, diffuse bilateral lung infiltrates. Pulmonary alveoli are filled with a granular, eosinophilic, periodic acid-Schiff (PAS)-positive material. Secondary infections can occur and account for 18% of the reported mortality in PAP, presumably due to myeloid cell defects. The predisposition to infection is systemic, rather than confined to the lungs. Although organisms common in community-acquired infections have been reported in PAP (*Streptococcus*, *Haemophilus*, *Staphylococcus*, *Pseudomonas*), more often reports have identified unusual or opportunistic pathogens (*Mycobacteria*, *Aspergillus*, *Nocardia*, *Cryptococcus*) (Rosen et al., 2013).

AutoAb to C1 inhibitor/acquired angioedema

Hereditary angioedema (HAE) is a rare autosomal dominant disease due to C1 esterase inhibitor deficiency (C1-INH) (Zuraw and Christiansen, 2016). The disease is characterized by subcutaneous and submucosal edema in the absence of urticaria due to the accumulation of bradykinin (Agostoni et al., 2004). Acquired angioedema (AAE) is a very rare condition with a very similar clinical

phenotype (Cicardi and Zanichelli, 2010). Similar to HAE patients, patients with AAE have angioedema without urticaria. Angioedema recurs at unpredictable intervals, lasting from 2 to 5 days and presenting with disfiguring, nonpitting, non-pruritic edema of the skin (face, limbs, genitals), severe abdominal pain, life-threatening edema of the upper respiratory tract and edema of the oral mucosa and of the tongue. The only significant clinical difference between HAE and AAE is the age of onset of symptoms: before the second decade of life for more than 90% of patients with HAE, after the fourth decade for those with AAE. Angioedema of the gastrointestinal mucosa causing abdominal pain is less frequently reported in patients with AAE (Bouillet-Claveyrolas et al., 2003). Anti-C1-INH autoantibodies are responsible for functionally inactivation and/or increase catabolism of the protein. A component detected in some AAE patients corresponds to the anti-C1-INH autoantibodies and can be associated with low-grade lymphoma. Based on these findings, AAE is considered as a condition associated with different forms of abnormal B cell proliferation ranging from autoreactivity to malignant lymphoma (Cicardi et al., 2003). Whether the different degrees of lymphoproliferation found in AAE patients are evolutionary stages of the same process starting from expansion of anti-C1-INH autoreactive clone(s) has not yet been clarified. Although lymphoproliferative diseases represent the main group encountered in AAE and a direct pathogenic relationship between the two conditions cannot be questioned, SLE, different neoplasia and infections have also been described in association with AAE.

Diseases associated with somatic mutations

Somatic mutation in *TNFRSF6*/autoimmune lymphoproliferative syndrome (ALPS-sFAS)

Autoimmune lymphoproliferative syndrome (ALPS) is a genetic disorder characterized by early-onset, chronic, nonmalignant lymphoproliferation, autoimmune manifestations, and susceptibility to lymphoma. In ALPS, lymphocyte homeostasis is disrupted by defects in the apoptosis process mediated by Fas, a cell-surface receptor (CD95) from the TNF receptor superfamily. An excess of circulating TCR $\alpha\beta^+$, CD4 $^-$ CD8 $^-$ double-negative (DN) T lymphocytes and elevated plasma levels of Fas ligand (FasL), vitamin B $_{12}$, and IL-10 are diagnostic hallmarks of the disease. The majority of ALPS patients carry heterozygous germline mutations in the *TNFRSF6* gene coding for Fas (ALPS-FAS) (Neven et al., 2011). The penetrance of the disease in carriers ranges between 50 and 75%. The variable clinical penetrance of *TNFRSF6* mutations and the differing phenotype found in patients from the same family indicate that additional genetic or environmental factors determine the clinical manifestations of ALPS. Indeed, the additional presence of a somatic event impairing the second *TNFRSF6* allele (i.e., somatic mutation or duplication of the mutant allele with loss of the wild-type allele) have been described in patients with heterozygous germline mutation. Interestingly, patients with germline mutations and somatic changes in the second *TNFRSF6* allele were significantly older at disease onset than other ALPS-FAS patients, suggesting that the second “hit” may be acquired later in life.

In addition, somatic *TNFRSF6* mutations have been described in patients with an ALPS phenotype but no germline mutation in *TNFRSF6* (Holzelova et al., 2004). These dominant heterozygous Fas mutations are detected in the DN T cells. It represents the second most common genetic etiology of ALPS (ALPS-sFAS) as diagnosed in 12 out of a series of 31 patients with an ALPS phenotype but no germline mutation of the *TNFRSF6* gene (Dowdell et al., 2010). Similar to classic ALPS patients, the somatic ALPS patients had increased DNT cell numbers and elevated levels of serum vitamin B $_{12}$, interleukin-10, and sFAS-L. ALPS patients with somatic variants showed variable lymph node pathology. Most biopsies exhibit characteristic features of ALPS-associated lymphadenopathy, including expansion of DNTs, and in some patients, increased S-100-positive cells with emperipolesis resembling Rosai-Dorfman disease. These data support testing for somatic *TNFRSF6* mutations in DN T cells from ALPS patients with no detectable germline mutation and a similar clinical and laboratory phenotype to that of ALPS.

Somatic mutation in *KRAS* or *NRAS*/RAS-associated autoimmune leukoproliferative syndrome (RALD)

Ras-associated autoimmune leukoproliferative disorder (RALD) is a nonmalignant clinical syndrome initially identified in a subset of putative autoimmune lymphoproliferative syndrome (ALPS) patients. Similar to patients with ALPS, RALD patients present with lymphadenopathy, massive splenomegaly, increased circulating B cells, hypergammaglobulinemia, and autoimmunity (Takagi et al., 2011). In contrast to ALPS, biomarkers such as TCR $\alpha\beta^+$ CD4 $^-$ /CD8 $^-$ double negative T cells and serum vitamin B $_{12}$ levels are not always increased, and germline or somatic mutations in *FAS*, *FASL*, or *CASP10* are absent in RALD. Persistent absolute or relative monocytosis is a cardinal feature of RALD. Bone marrow and peripheral blood smear findings overlap with those of juvenile myelomonocytic leukemia (JMML) in children or chronic myelomonocytic leukemia (CMML) in older patients (Calvo et al., 2015).

Activating somatic mutations that cause amino acid substitutions that affect *KRAS* or *NRAS* were identified in myeloid and lymphoid lineages (Oliveira et al., 2007; Niemela et al., 2011). JMML is an aggressive malignant hematopoietic neoplasm of childhood with myelodysplastic and myeloproliferative features. Patients present with splenomegaly, fever, thrombocytopenia, monocytosis, and excess myelomonocytic cells that infiltrate skin and vital organs (Murakami et al., 2018; Niemeyer and Flotho, 2019). JMML accounts for 20–30% of myelodysplastic/myeloproliferative disorders in the pediatric population. The prognosis for JMML is poor with median survival of 1 year for untreated patients. Hematopoietic stem cell transplantation has become the standard of care for JMML. CMML has a variable course in adults and often requires chemotherapy. Distinguishing these conditions from RALD is critical.

Somatic mutation in NLRP3/cryopyrinopathy

The cryopyrin-associated periodic syndromes (CAPS) are a group of autoinflammatory disorders including familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and Neonatal-Onset Multisystem Inflammatory Disease (NOMID) also known as Chronic Infantile Neurological Cutaneous and Articular (CINCA). These dominantly inherited diseases are caused by heterozygous missense gain-of-function mutations in the *NLRP3/CIAS1* gene encoding NLRP3, also known as cryopyrin, leading to the activation of the inflammasome and overproduction of IL-1 β (Booshehri and Hoffman, 2019).

Somatic *NLRP3* mutations account for up to 70% of NOMID/CINCA patients who test negative for a germline mutation. Patients with somatic mutations present with symptoms comparable to patients with germline mutations, with slightly older age of disease onset and possibly milder CNS disease. The frequency of the mutant allele was reported to be similar in various cell types including myeloid cells, T cells and B cells and epithelial cells (Aróstegui et al., 2010; Tanaka et al., 2011; Rowczenio et al., 2017). A small number of monocytes carrying the *NLRP3* mutation are sufficient to evoke systemic inflammation. Two patients with the variant-type of Schnitzler syndrome have been reported with a myeloid lineage-restricted somatic mosaicism of *NLRP3* mutations (Rowczenio et al., 2018).

Somatic mutation in STAT3 or STAT5B (GOF)/large granular lymphocytosis

Large granular lymphocyte (LGL) leukemia belongs to the rare chronic mature lymphoproliferative disorders of the T/natural killer (NK) lineage (Lamy et al., 2017). T-LGL leukemia and chronic NK-cell lymphocytosis share the same clinical and biological presentation, as well as treatment options. Pathogenesis of the disease is dominated by a clonal expansion of LGL resistant to activation-induced cell death due to constitutive survival signaling. LGL leukemia principally affects elderly people with a median age of 60 years. Very rare pediatric cases have been reported. About one-third of patients are asymptomatic at the time of diagnosis. Initial presentation is mainly related to neutropenia and includes recurrent oral aphthous ulcerations and fever secondary to bacterial infections. These infections typically involve skin, oropharynx, and perirectal areas, but severe sepsis may occur. However, some patients may have profound and persistent neutropenia without any infections over a very long period of time. Splenomegaly is frequent and lymphadenopathy is rare. Most patients present with mild or moderate lymphocytosis with LGL usually ranging from 1 to $6 \times 10^9/L$. Severe neutropenia ($<0.5 \times 10^9/L$) is observed in 16–48% of cases. Anemia is frequent and sometimes secondary to pure red cell aplasia and moderate thrombocytopenia is less frequently observed (Ishida et al., 2014). The first diagnosis is based on identification of increased numbers of circulating LGL, easily identified on blood smears by their specific morphology; however, they are not cytologically distinguishable from normal reactive cytotoxic lymphocytes. T-LGL leukemias show a constitutive mature post-thymic phenotype. In the vast majority of cases, T-LGL leukemia shows a CD3⁺, TCR $\alpha\beta$ ⁺, CD4[−], CD5^{dim}, CD8⁺, CD16⁺, CD27[−], CD28[−], CD45RO[−], CD45RA⁺, and CD57⁺ phenotype, which represents a constitutively activated T cell phenotype. NK-LGL leukemia and NK-LGL lymphocytosis are characterized by the following phenotype: CD2⁺/sCD3[−]/CD3e⁺/TCR $\alpha\beta$ [−]/CD4[−]/CD8⁺/CD16⁺/CD56⁺. The transcriptome of LGL leukemia patients is that of *STAT3*-regulated genes suggesting that constitutive activation of *STAT3* plays a fundamental pathogenic role in LGL leukemia. Constitutive activation of *STAT3* in LGL leukemia patients was first discovered in 2001 (Epling-Burnette et al., 2001). In 2012, common somatic gain-of-function *STAT3* mutations were demonstrated in 28–75% of T-LGL leukemia and 30–48% of NK-LGL lymphocytosis. These differences may be due to the sequencing technique and the selection of patients (Jerez et al., 2012; Koskela et al., 2012). Detection of identical *STAT3* mutations in both T- and NK-subtypes suggests a unifying pathogenesis for these similar disorders. It has been suggested that a particular *STAT3* mutation, Y640F, predicted response to initial therapy with methotrexate. The first evidence of *STAT5B* mutations in human disease was discovered in LGL leukemia, but this mutation is not frequent (2%). The N642H mutation was associated with more aggressive disease and an unusual CD3⁺CD56⁺ phenotype.

Somatic mutations in STAT5B (GOF)/hypereosinophilic syndrome

Somatic, gain-of-function mutations in *STAT5* have been described in leukemias and lymphomas and, more recently the *STAT5B* N642H mutation has been reported in patients with hypereosinophilic syndrome (Cross et al., 2019). The syndrome associates eosinophilia, atopic dermatitis, urticarial rash and diarrhea. Additional clinical manifestations includes infections such as pneumonia and a measles-like illness after receiving the measles, mumps, and rubella vaccine in one girl (Ma et al., 2017). In most cases the syndrome was associated with a diagnosis of myelodysplastic/myeloproliferative neoplasia.

Conclusions

The conditions described here most likely represent just the beginning of conditions that will ultimately be identified as clinical comparable to inborn errors of immunity but driven by either somatic mutations or autoantibodies.

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Immunology of Hematopoietic Stem Cell Transplantation

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Abbreviations

ALL	Acute lymphocytic leukemia
APC	Antigen presenting cell
ATG	Anti-thymoglobuline
B reg	Regulatory B lymphocyte
BAFF	B-cell Activating Factor of the TNF family
BM	Bone marrow
CLA	Cutaneous lymphocyte-associated antigen
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DAMPs	Damage-associated molecular pattern molecules
DC	Dendritic cell
DLI	Donor lymphocyte infusion
DNA	Deoxyribonucleic acid

EBV	Epstein-Barr virus
ECP	Extracorporeal photopheresis
FLT3	Fms-like tyrosine kinase 3
GI	Gastro intestinal
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
HHV6	Human herpes Virus 6
HLA	Human leucocyte antigen
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes simplex virus
IDO	Indoleamine 2,3-dioxygenase
IFN γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
Jak	Janus kinases
KIR	Killer-cell immunoglobulin-like receptor
LC	Langerhans cell
LN	Lymph node
LPS	Lipopolysaccharide
Mad-CAM	Mucosal Addressin Cell Adhesion Molecule
MDSC	Myeloid-derived suppressor cell
mHa	Minor histocompatibility antigens
MHC-	Major histocompatibility complex
MMF	Mycophenolate mofetil
MSC	Mesenchymal stem cells
mTor	Mammalian Target of rapamycin
NFAT	Nuclear factor of activated T cells
NF-kB	Nuclear factor-kB
NK	Natural killer
NLR	NOD-like receptors
NO	Azote monoxide
NPM1	Nucleophosmin
PAMPs	Pathogen-Associated Molecular Patterns
PBSC	Peripheral blood stem cells
SNPs	Single nucleotide polymorphisms
T reg	Regulatory T lymphocyte
TAA	Tumor associated antigen
TGF- β	Tumor growth factor
TLRs	Toll-like receptors
TNF α	Tumor necrosis factor
VZV	Varicella-zoster virus
WT1	Wilms tumor 1

Introduction

The principle of allogeneic hematopoietic stem cell transplantation (allo-HSCT) was first explored in the mid of the 1950s to overcome the aplasia induced by lethal radiation accidents. Experiments in mice rapidly showed that transplanting lethally irradiated mice with allogeneic hematopoietic stem cells (HSC) not only allowed engraftment of hematopoietic cells but was also associated, when leukemic cells were injected with the stem cell graft, with the development of an anti-leukemic effect called “graft versus leukemic effect” (GVL effect). However, allografted mice developed an immune mediated «secondary disease» called “graft-versus-host disease” (GVHD) (Granot and Storb, 2020).

In humans, the first case of leukemia cured by an allogeneic hematopoietic stem cell transplantation (allo-HSCT) performed between syngeneic brothers was reported by Donnall Thomas in 1957. Then this procedure had been expanded with the discovery of the HLA system at the end of the 1950s and the better knowledge of how to use it in the context of allo-HSCT in the aim to decrease the risk of GVHD. Because of the GVL effect, allogeneic HSCT is being used as curative treatment of leukemias since the beginning of the 1970s (Granot and Storb, 2020).

Nowadays, allo-HSCT is still the only potential curative treatment for some hematopoietic malignant and non-malignant diseases. Because the success of this therapy is still compromised by the development of the life-threatening GVHD, various immunosuppressive and immunomodulatory therapies are associated to prevent GVHD. However, cumulating these therapies with

long-lasting immunosuppressive effects may increase the incidence of malignancy recurrence and infections. Therefore, cellular strategies allowing more transient modulation of alloreactivity with regulatory cells are gaining interest in the field.

Post-transplant immune reconstitution

Immune reconstitution after allogeneic HSCT is a slow process requiring the development of a new immune system from the donor cells. If myeloid lineage reconstitutes in the first weeks following transplantation, the recovery of lymphoid cell subtypes, particularly of T and B cells, is affected by the use of immunocompromising drugs, affecting both recipient and donor immunity. These latter include the chemotherapy and/or total body irradiation of the conditioning regimen as well as *in vivo* T cell depleting agents such as anti-thymocyte globulin (ATG) or post-transplantation cyclophosphamide, that are required to allow HSC engraftment and prevent lethal GVHD. The deepness and length of these secondary humoral and cellular deficiencies are influenced by several factors such as the age of recipient, the source of HSC, the level of HLA disparity between donor and recipient, the intensity of conditioning regimen, the use and type of T cell depletion but also the occurrence of GVHD and the duration of immunosuppressive treatments (Ogonek et al., 2016).

With respect to lymphocyte deficiency, while the NK cell population reconstitution occurs within the first weeks after HSCT, T lymphocytes remain quantitatively and qualitatively deficient during the first 6–12 months post-transplant. The lymphocyte quantitative defect mainly concerns naïve and CD4⁺ T cells with a prolonged inversion of the CD4/CD8 ratio. Indeed, CD8⁺ T cells recover within the first 3–6 months. In addition, during the first year post-HSCT, T cell expansion is usually oligoclonal and the T cell repertoire diversity is poor and particularly affected in the case of the development of acute GVHD.

Recipient antigen presenting cells (APC) disappear during the first 6 months post-transplant while donor APCs have distinct recovery patterns. While monocytes reconstitution occurs in the first weeks post-transplant, dendritic cells (DCs) recover between 6 and 12 months after HSCT. A slow B cell reconstitution associated with severe and prolonged hypogammaglobulinemia is frequently observed during the first 6–12 months after transplantation (Fig. 1) (Ogonek et al., 2016).

Despite granulocyte recovery, the lymphocyte immune deficiency is responsible for a susceptibility towards infectious agents mainly viruses (HSV, CMV, VZV, EBV, HHV6, BK, Polyomavirus, Enterovirus, Respiratory viruses, Adenovirus), fungi (*Candida*, *Aspergillus*, *Pneumocystis jiroveci*, *Cryptococcus*, *cryptosporidium*), some encapsulated bacteria (*Streptococcus pneumoniae*, *Haemophilus*, mycobacterias) and parasites (*Toxoplasma gondii*) (Fig. 2A). The risk of infection or reactivation is increased in case of occurrence of GVHD requiring prolonged immunosuppressive therapies and is dependent on the level of CD4 T cell recovery (Fig. 2B) (Ogonek et al., 2016).

The graft versus leukemia (GVL) effect

Proof of concept

The demonstration of the role of donor T cells in the generation of the life threatening GVHD led to perform T cell depleted allogeneic HSCT. If this approach could effectively reduce the risk of acute GVHD, the strategy was also associated with higher incidence of disease relapse. The anti-leukemic effect of allogeneic HSCT was also suggested by higher leukemia response rates observed in patients receiving an allogeneic HSCT rather than a syngeneic HSCT. In addition, the possibility of achieving disease control after administration of donor lymphocyte infusions (DLI) in patients relapsing with chronic myeloid leukemia following allogeneic HSCT confirmed the role of donor T cells in the induction of the GVL effect (Dickinson et al., 2017).

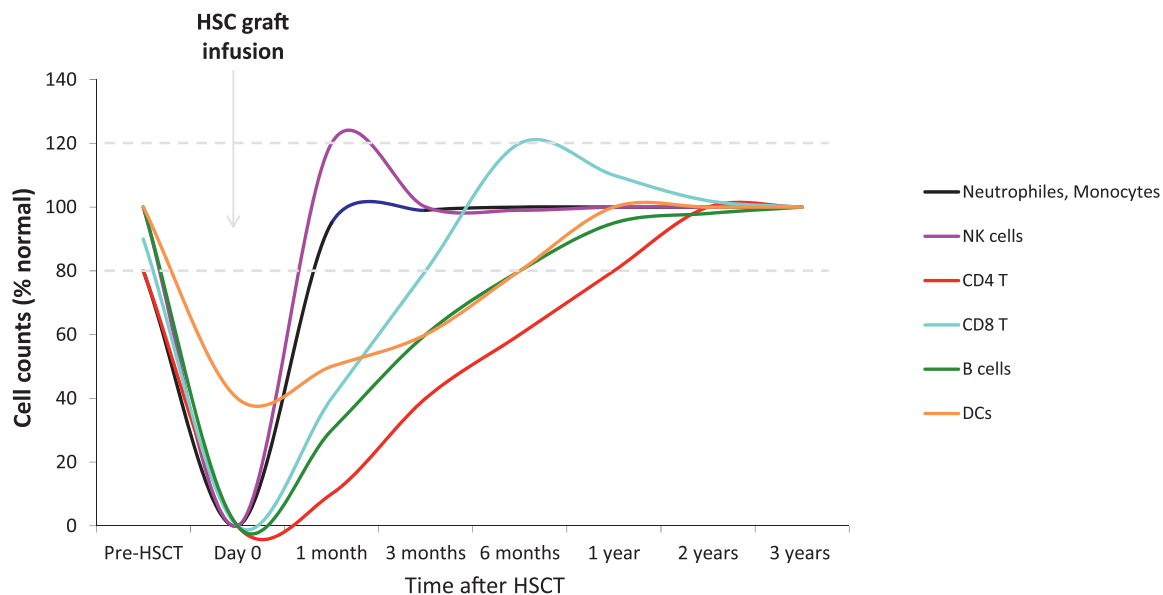
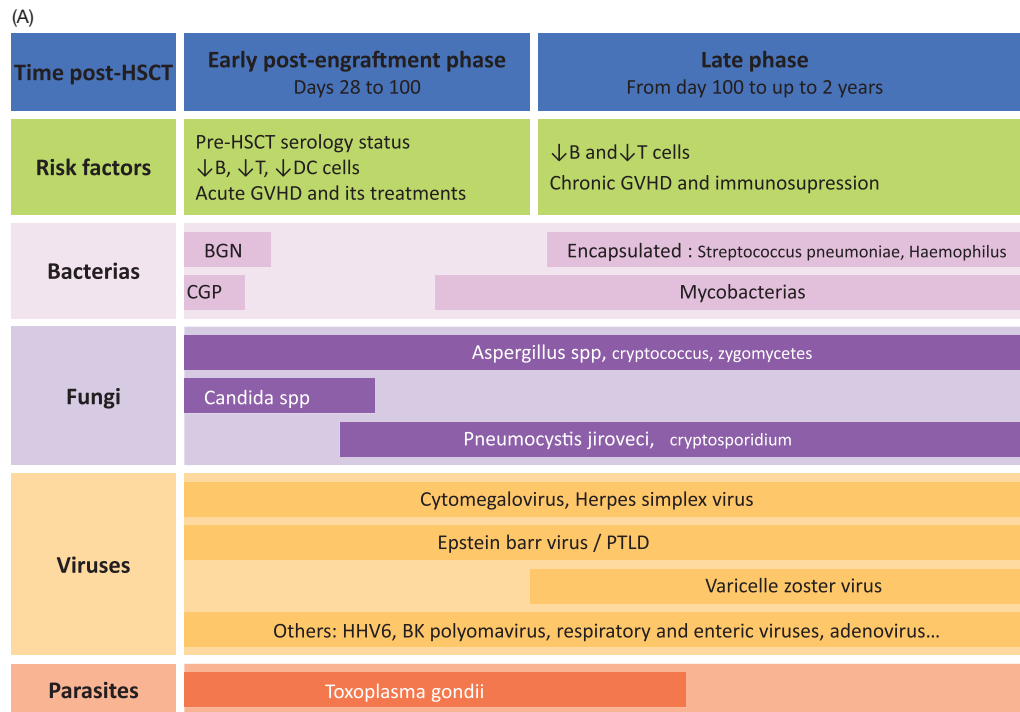


Fig. 1 Immune cell recovery after allogeneic HSCT.



DC: dendritic cells, BGN: Bacille Gram negative, CGP: Cocci gram positive, PTLN: post-transplant lymphoproliferative disease

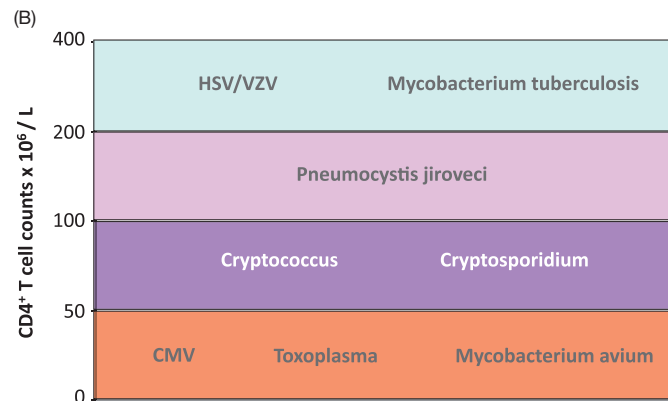


Fig. 2 (A) Potential infections after allogeneic HSCT. (B) Risk of infection/reactivation according to CD4 T cell counts after allogeneic HSCT.

Initially demonstrated in leukemias, the GVL effect has also been shown in other hematological malignancies. Therefore, pre-emptive DLI (performed in case of detectable minimal residual disease (MRD) and/or of mixed chimerism status) have been well studied to improve patient outcomes. This approach induces a high rate of conversion from mixed chimerism to complete chimerism and allows durable remissions with a risk of developing GVHD (Dickinson et al., 2017).

Immunological mechanisms

Several subsets of immune cells and immunological mechanisms are involved in the graft versus leukemia/tumor effect (Dickinson et al., 2017).

Antigen presenting cells

A large pool of potential APCs can be considered including normal subsets of DCs, leukemia-derived DCs, B cells, macrophages and leukemic cells themselves. It has been demonstrated in mice that host APCs can present allo-antigens and tumor associated antigens and thus are able to trigger naïve CD4⁺ and CD8⁺ T cell primed and licensed to kill (Fig. 3). Moreover, donor-derived APCs are able to take over the GVL effect by inducing long term anti-tumor responses, particularly if the tumor burden is low.

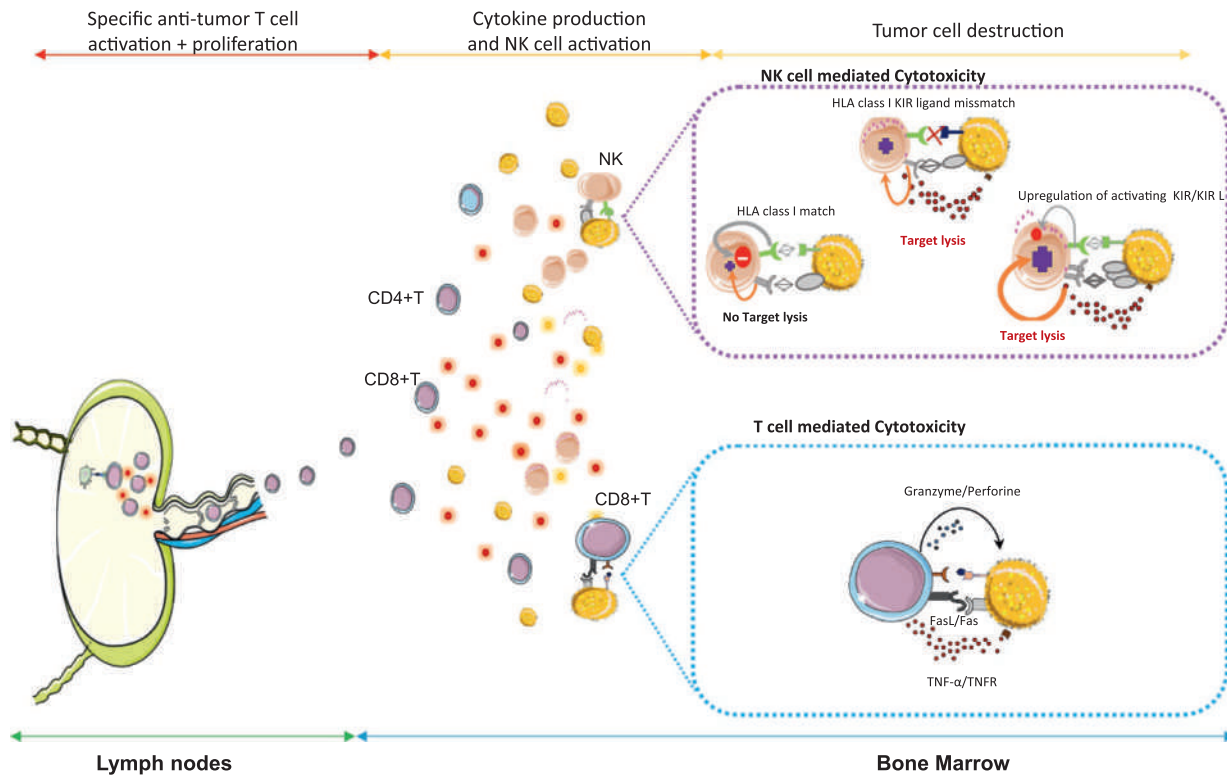


Fig. 3 Mechanisms of graft versus Leukemia effect.

T cells

Both donor CD4⁺ and CD8⁺ T cells are required for the GVL response. This observation led to investigate which allo-antigens could be involved in the immunotherapeutic effect of allogeneic HSCT. Gene polymorphisms between donor and recipient induce differences in sequences of homologous peptide derived proteins that are called minor histocompatibility antigens (mHa). These class I or class II MHC-restricted epitopes can be recognized by donor CD8⁺ or CD4⁺ specific T cells and can eradicate leukemic cells after experimental adoptive transfer. Currently, three classes of allo-antigen are described:

- First, tissue restricted mHas like HA-1, HA-2, HB-1, BCL2A1 or LRH-1. These antigens are only expressed on hematopoietic cells and should not induce GVHD. Specific CTLs for these antigens have been found in patients after allo-HSCT or DLI in correlation with responses against leukemic cells. Moreover, HB-1 and BCL2A alloantigen specific CTLs can induce in vitro the lysis of leukemic cells.
- Second, ubiquitously expressed mHas. These antigens, such as HA-8 and UGT2B17 can be involved in the GVHD and/or GVL effects depending on their tissue expression. LRH-1 represents an interesting target due to its preferential expression on CD34⁺ leukemic cells, which might explain the low incidence of GVHD in patients with LRH-1 specific CTLs.
- Third, tumor associated antigens (TAAs). These antigens can be proteins aberrantly expressed on tumor cells such as WT1, Proteinase 3 or Survivin. Despite their expression in normal tissues, TAAs overexpression could allow T cells to discriminate between normal and tumor cells, and bypass natural tolerance to self-antigens, which explains that TAA specific CTL generation correlates with a better outcome. Some trials of WT1-based vaccination have reported vaccine-induced immunological and clinical responses. Other tumor antigens are malignant-cell-restricted proteins generated either from chromosome translocations or gene mutations, for instance NPM1 or internal tandem duplication of FLT3. Such tumor specific mutated proteins can also be recognized by the immune system. These allo-antigens are interesting targets because they can induce specific CTLs in vitro with cytolytic activity against leukemic but not normal CD34⁺ cells in patients.

Different cytotoxic mechanisms leading target cell killing by T cells have been recognized: CD8⁺ cytotoxic T-cells (CTL) mainly use perforin/granzyme degranulation following TCR-mHa/MHC Class I binding. CD4⁺ T cells, besides their helper function in producing IL-2 (interleukin 2) and IFN- γ (Interferon gamma), can also act as CTLs by the release of perforin. Other cytotoxic pathways have been reported such as Fas/FasL or membrane-anchored or soluble TNF α via TNF-receptors but their implication in the induction of the GVL effect in humans remains to be demonstrated (Fig. 3).

NK cells

Natural Killer (NK) cells are characterized by their capacity to kill target cells such as tumor cells depending on their expression of killer-cell immunoglobulin-like receptors (KIRs), which recognize activating or inhibitory ligands expressed on target cells.

The signal induced by the recognition of inhibitory KIRs (for instance CD94/NKG2A) by inhibitory ligands, mainly HLA class I molecules, leads to the inhibition of the cytotoxicity of the NK cell. After specific ligand recognition, the signaling through activating KIRs (for instance CD94/NKG2C and NKG2D recognize the stress induced MIC-A and MIC-B molecules on tumor or infected cells) induce the lysis of the target cell. The expression of activating receptors and ligands can be upregulated in the context of inflammation or stress such as in viral infections or in tumors. NK cells will kill a target cell either in the absence of inhibitory signals (HLA class I missing) or in the presence of a relative higher expression of activating ligands (inflammation/stress) (Fig. 3).

The implication of NK cells in the GVL effect was first suggested in the context of haplo-identical HSCT in the presence of HLA class I mismatch. The main HLA epitopes involved in cytotoxicity are HLA-C1, C2 and Bw4. In this setting, the GVL is driven by donor NK-cell expressing KIRs not inhibited by the recipient HLA class I molecules (recipient missing the inhibitory KIR ligands). In this context, KIR mismatched NK cells might also kill recipient APCs and hematopoietic stem cells and thus reduce the risk of GVHD and facilitate engraftment (Ruggeri et al., 2002).

Because KIR and HLA genes are localized in distinct chromosomes (chromosome 19q13.4 and 6p21, respectively) and thus segregate independently, an HLA matched donor-recipient pair can be KIR-mismatched. More recent studies exploring NK alloreactivity on transplant outcomes has then been performed in matched unrelated HSCT (MUD). However, in this setting, the role of the KIR-ligand mismatches on the risk of disease relapse is still controversial.

Immune evasion

Accumulating evidence recently suggest that disease relapse after allo-HSCT is mediated by tumor cell escape to the alloreactive immune response (Zeiser and Vago, 2019). Because donor cell exhaustion has been observed in relapsing patients, immune checkpoint blockade has been shown to be able to improve the antitumor effect of allo-HSCT with a risk of inducing severe acute GVHD. Recent studies have demonstrated that relapse post-HSCT is associated with dysregulation of adaptive and innate immunity, including down-regulation of major histocompatibility complex (MHC) class II molecules inducing a permissive environment to promote T cell immune evasion in myeloid and lymphoid malignancies. Higher proportion of early-differentiated Memory Stem (T_{SCM}) and Central Memory T cells expressing multiple inhibitory receptors on their cell surface (PD1, Tim3, LAG3...) with impaired functions have been observed in patients relapsing after allo-HSCT. Reduced cytotoxic capacities of anti-leukemic effector T cells have been correlated with metabolic alterations such as decrease in glycolytic and oxidative metabolisms associated with impaired effector cytokine production (such as IFN- γ and TNF- α) (Zeiser and Vago, 2019).

Graft versus host disease

Two distinct clinical presentations of graft versus host disease are described (Ferrara et al., 2009). Acute graft versus host-disease usually occurs in the 3–6 first months post-transplantation and is characterized by acute organ toxicity mainly involving the skin, the gastro-intestinal tract and the liver. Chronic GVHD classically develops after 3 months post-transplant and presents features of auto-immune diseases with inflammatory and fibrotic features. The chronic form of the disease can affect multiple organs most frequently the skin and subcutaneous connective tissues, lacrimal and salivary glands, oral mucosa, lungs, gastrointestinal tract, liver and joints.

Immunobiology of acute GVHD

The “3 phases”

The immunopathophysiology of GVHD has been essentially explored in mouse models in which it has been classically described in 3 phases (Fig. 4) (Zeiser and Blazar, 2018).

In the first phase, the chemotherapy and radiation used for transplant conditioning induce tissue damages, particularly in the gut, associated with epithelial cell destruction and endothelial inflammation leading to the release of inflammatory signals (cytokines such as TNF- α , damaged tissue release DAMPs, microbial flora release PAMPs including LPS and CpG). Such inflammatory signals induce the maturation and activation of recipient APCs, characterized by the up regulation of surface co-stimulatory molecules, the production of cytokines and the capacity to migrate to secondary lymphoid organs.

The second phase takes place in secondary lymphoid organs where fully mature recipient APCs present a broad spectrum of antigens (major or minor histocompatibility antigens) to donor T lymphocytes. Activated APCs and T lymphocytes produce pro-inflammatory cytokines that include IFN- γ , TNF- α , IL-2, IL-12, IL-18, IL-21 and IL-17. This “cytokine storm” induces the activation of a large spectrum of other immune cells (NK, monocytes, macrophages). Alloreactive T cells can acquire the expression of specific tissue homing molecules and migrate towards inflamed GVHD target tissues (skin, GI, liver).

In the third phase, inflamed GVHD target tissues attract polynuclear cells, monocytes, mast cells, macrophages, and activated donor T cells that release several inflammatory cytokines enhancing the immune response. Host epithelial and endothelial cells can be attacked and destroyed by cytotoxic molecules released by activated macrophages and T cells, such as TNF- α , IFN- γ , IL-1 and azote monoxide (NO) and by alloreactive cytotoxic T cells expressing or secreting death molecules (Fas-L, Perforin).

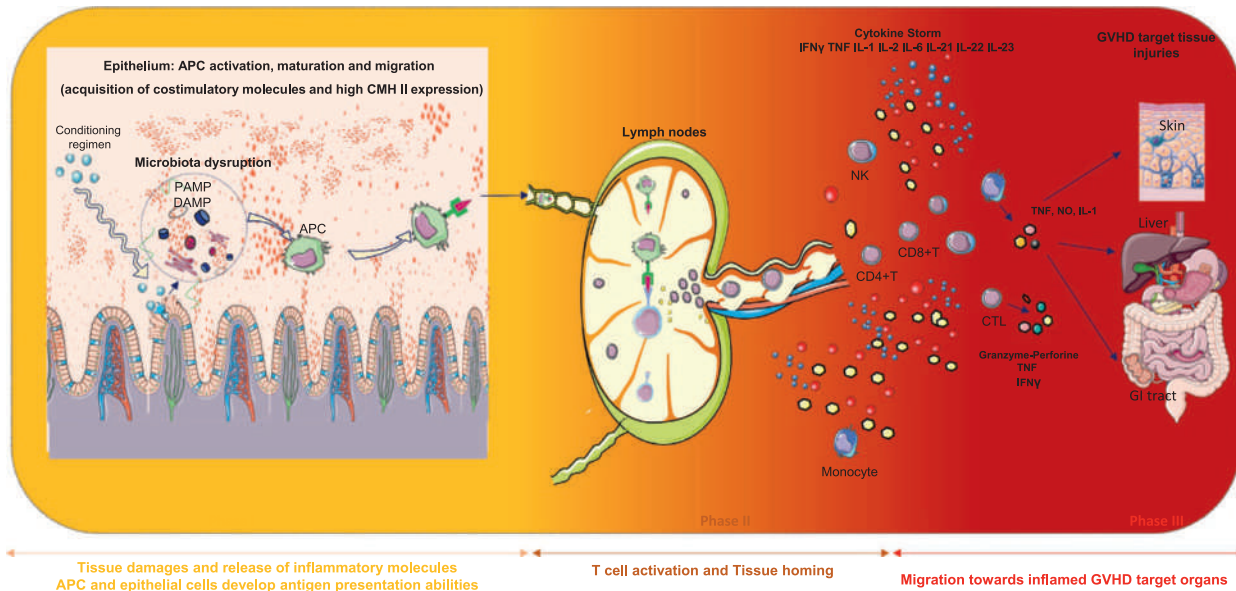


Fig. 4 Pathophysiology of acute GVHD.

Role of conditioning, danger signals and microbiota

The conditioning regimen performed to allow the engraftment of donor allogeneic stem cells is usually based on chemotherapy or radio-chemotherapy, both of which induce tissue damages and the release of “danger signals.” This phenomenon is classically attributed to the release of inflammatory cytokines, tissue and microbial release proteins (DAMPs and PAMPs such as CpG and LPS) from injured, stressed, or dying cells and bacteria, in particular in the gastrointestinal tract. Those bacterial-derived and cellular danger components signal via toll-like receptors (TLRs) or NOD-like receptors (NLR) expressed on APCs. Gut microbiota play an important role in the regulation of GVHD (Fredricks, 2019). Microbiota diversity can be strongly impaired after allo-HSCT secondary to conditioning regimen and the frequent use of broad-spectrum antibiotics. A disequilibrium with loss of butyrate producing bacteria and other beneficial commensals associated with a relative increase in pathogenic bacterial such as *Enterococcus*, *Klebsiella*, *Escherichia*, *Staphylococcus* and *Streptococcus* has been described in allografted mice and patients. Colonization, before allo-HSCT, by multi-drug resistant bacteria and *Clostridium difficile* have been correlated with a higher risk of developing acute GVHD. Enterococcal expansion has been described in patients developing acute GI GVHD. Reduction of blautia, a clostridial specie producing butyrate, has been associated with increased mortality due to GVHD. This microbiota disequilibrium induces the activation of APCs through TLRs increasing T cell stimulation and innate cell recruitment but also reduces GI epithelium integrity (loss of protection by butyrate, reduction of protective mucus . . .) and enhances the destruction of Paneth cells explaining the sera increase in antimicrobial peptides (AMPs), such as Reg3 α , that play a crucial role in the regulation of the equilibrium between activated and regulatory T cells in the mucosa (Fredricks, 2019).

Role and origin of antigen presenting cells

There are multiple types of APCs in diverse anatomic locations and with various recirculatory patterns that could be implicated in activating allogeneic T cells.

Professional APCs include dendritic cells, macrophages and B lymphocytes and have the capacity of antigen uptake, processing, and so-called direct presentation of peptides on major histocompatibility complex (CMH) class II to CD4 $^{+}$ T lymphocytes. Among those, DCs are not only the most efficient in direct presentation but also have the specific ability to cross present soluble antigens on CMH class I to CD8 $^{+}$ T lymphocytes.

DCs represent a heterogeneous population, schematically composed of two main distinct subtypes: conventional and plasmacytoid DC.

Conventional lymphoid DCs reside in lymphoid organs such as lymph nodes, spleen and thymus and lack migratory properties. By contrast, some conventional migratory dendritic cells are found in peripheral tissues such as skin, lung, intestinal tract, liver, and kidney. They are characterized by their unique ability to acquire antigens and subsequently migrate to the draining lymph nodes, where they interact with T cells. Among those, within the skin, Langerhans cells (LCs) and dermal DCs have a particular slow turnover and prolonged half-life.

Plasmacytoid DCs (pDCs) comprise a distinct DC subset found both in lymphoid and non-lymphoid organs and are characterized by rapid production of type I interferon in response to viral infections and the ability to differentiate into mature DC with high T cell stimulatory activity.

Several studies have been performed to determine which subsets of APCs are implicated in the activation of allogeneic T cells. In terms of subsets of APCs, circulating DCs and pDCs rapidly acquire a full donor chimerism, suggesting that circulating recipient's APCs are rapidly replaced by donor's ones. By contrast, a consistent pool of recipient LC and dermal DC can survive within weeks after allogeneic HSCT, even after myeloablative conditioning regimen, in humans and animal models. These different DC subsets have been reported from murine models to be the main players, rather than B cells and macrophages, in the induction of GVHD. A role of pDCs has been suggested in humans since patients with skin or intestinal acute GVHD have an increased infiltration of pDCs in those GVHD target organs.

In terms of origin of APCs, there is strong evidence in murine models for a crucial role of host's APCs in donor T cell allogeneic activation and effector differentiation, essentially via the direct presentation of recipient antigens to donor T cells. Other experimental data have also demonstrated a minor role for donor APCs cross presenting host antigens in initiating acute GVHD. However, these could drive further tissue injuries and exacerbate GVHD or pave the way for the installation of chronic GVHD (cGVHD).

Besides professional APC, there is growing evidence that non-hematopoietic cells can acquire a significant role in antigen presentation and the capacity to trigger alloreactivity, especially in an inflammatory context such as in the allogeneic HSC transplant setting. Among candidates, MHC II⁺ myofibroblasts and epithelial cells within the gastrointestinal tract, stellate cells within liver, especially intra-hepatic Ito cells and endothelial cells seem to be involved even if the extent of their implications in GVHD remains to be fully characterized.

To increase the complexity of the process, in some cytokine environment, some tolerogenic DCs can emerge. Those can present antigens without co-stimulatory molecules or deliver inhibitory signals driving immune tolerance. Indeed, they can produce T-cell inhibitory cytokines such as IL-10 and TGF- β , induce T cell anergy and/or generate regulatory T cells. Although induced regulatory/tolerogenic DCs can control GVHD in mice, their physiological role in the context of human allogeneic HSCT is unknown (Stenger et al., 2012).

Role of T cell subsets

Both the CD4⁺ and CD8⁺ T cells are involved in the pathobiology of acute GVHD. The CD4⁺ counterpart has a predominantly helper function but can also become cytotoxic, while the CD8⁺ counterpart is mainly cytotoxic.

In murine experimental models, naive CD3⁺CD44⁻ or CD62L⁺ and CCR7⁺ T cells exacerbate acute GVHD lethality while memory CD3⁺CD44⁺ or CD62L⁻CCR7⁻ protect mice from GVHD. In humans, a relative higher proportion of CCR7⁺ naive CD3⁺T cells in the stem cell graft has been associated with an increased risk of acute GVHD.

Depending on the cytokine context, murine and humans CD4⁺ T cells can differentiate into Th1, Th2, Th-9, Th17, Th22 or T regulatory cells. In experimental murine models of GVHD, naive alloreactive CD4⁺ T cells predominantly differentiate into Th1 cells, but a portion of them also differentiate into Th2 or Th17 cells. Th1 differentiation leads to tissue damage in the gut and liver, while Th2 and Th17 differentiation induces tissue damage in lung (mainly Th2) and skin (predominantly Th17). Down regulation of Th1 differentiation induces an increase in Th2 and Th17 responses responsible for an exacerbation of lung and skin GVHD while sparing the gut and vice versa. In addition, IL-22 producing T cells have been shown to protect mice from gut GVHD. In humans, the respective role of CD4⁺ Th differentiation remains poorly explored. Th1 and Th17 signatures have been observed in acute GVHD, while increased Th2 T cells in the stem cell graft is associated with a reduced risk of aGVHD.

Role of homing molecules and tissue specific receptors

Grafted T cells first transit through secondary lymphoid organs where they encounter APC before taking a direction to a specific target organ. In that context, lymph node homing markers (CCR7 and CD62L) are of major importance. Murine models of ASCT confirmed that CCR7^{-/-} T cells were not competent to induce GvHD and blockage of CD62L could reduce GvHD. In humans, adoptive transfer of CD62L⁺ T cells in MHC-mismatched recipients could reduce the risk of GvHD.

CCR5, a chemokine involved in migration to both lymph nodes and target organs including gastro-intestinal tract, has been extensively studied in murine models of ASCT. CCR5 polymorphisms have been shown to influence the risk of GvHD in patients.

Many selectins, integrins and chemokine receptors have been described in the migration of T cells towards peripheral tissues. Migration of donor T cells to gut-associated lymphoid tissues is dependent on the integrin α 4 β 7 and its ligand Mucosal Addressin Cell Adhesion Molecule (Mad-CAM). The integrin CD103 (the ligand of epithelial cell-specific E-Cadherin) has been implicated in the migration and cytolytic function of gut-infiltrating CD8⁺ lymphocytes during GvHD. Migration of lymphocytes to skin is directed by CLA, a ligand of E-selectin which is constitutively expressed on human dermal microvasculature and further increased by inflammation. The molecular effectors of lymphocyte migration to the liver in acute GVHD is more obscure although some evidence suggest the implication of integrin α 4 β 1 (VLA4), CCR5, and CD44.

Role of immunoregulatory cells

Allogeneic activation of donor T cells can be controlled by several immunosuppressive cell subsets (Fig. 5).

Regulatory T lymphocytes

A subset of CD4⁺T cells deriving from the thymus, named the natural T CD4⁺CD25^{high}FoxP3⁺ regulatory lymphocytes (T regs), can inhibit the proliferation of alloreactive T cells in vitro and can separate GVH and GVT in mouse models of allogeneic HSCT. T regs inhibit the interaction between APCs and T cells by different mechanisms: (i) by contact dependent-means mainly via their expression of CTLA4, which inhibits the interactions APC/T and induces indoleamine 2,3-dioxygenase (IDO), an inhibitor of T

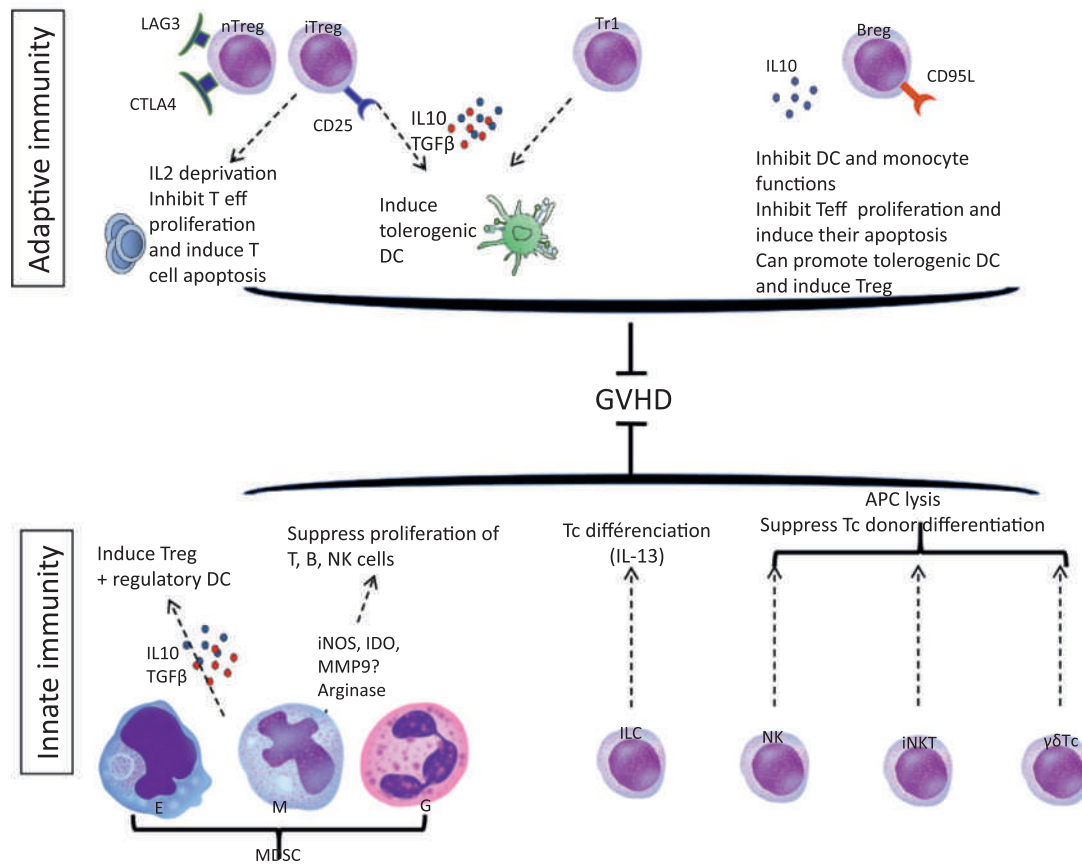


Fig. 5 Immunoregulatory cells implicated in the regulation of GVHD.

cell activation and proliferation, (ii) by competitive uptake of IL-2 and (iii) by the production of immunosuppressive cytokines such as IL-10 and TGF-β.

In humans, natural T regs are defined as CD4⁺CD25⁺FoxP3⁺CD127^{low} T cells. Inducible T regulatory type 1 (Tr1) cells are characterized by the co-expression of CD49b and lymphocyte-activation gene 3 (LAG-3) and the ability to secrete IL-10, TGF-β, and granzyme B, in the absence of IL-4 and IL-17. The chief mechanisms by which Tr1 cells control immune responses are secretion of IL-10 and TGF-β and killing of myeloid cells via Granzyme B. The possibility to generate and expand Tr1 cells in vitro in an Ag-specific manner has led to their clinical use as cell therapy in patients.

A subgroup of CD4⁺CD8⁺CD3⁺TCRαβ⁺ T cells can inhibit alloreactive T cell functions in mice and humans by trogocytosis of APCs' alloantigens, reduction of the expression of co-stimulation molecules CD80/CD86 on DCs and induction of CD8 T cell apoptosis by Fas/FasL. They can control experimental GVHD in mice. Their role in human GVHD is unknown (Hefazi et al., 2021).

γδT cells

They represent a subgroup of T cells expressing a γδ-TCR specialized in the recognition of heat shock proteins from tumor cells and some non-peptidic ligands. They are present in epithelial tissues and implicated in the innate anti-infectious and tumor immunity by their production of high amounts of IFN-γ and TNF. They also express some KIR, which enable them to kill target cells.

Some experimental data suggest a beneficial role of γδ T cells on the separation of GVH/GVL. An improved post-transplant reconstitution of γδ T cells in patients has been associated to a significant reduced risk of relapse without increasing the risk of GVHD but the mechanisms involved in their regulatory functions are unknown.

Invariant NKT lymphocytes (iNKT)

iNKT cells represent another immunoregulatory T cell population characterized by the expression of a TCR molecule composed of an invariant Vα chain (mVα14/hVα24Jα18) associated to a limited repertoire of Vβ chains. These cells recognize glycolipids presented by the CD1d molecule. They can produce, upon stimulation either by their TCR signal or in response to inflammatory cytokines (IL-12, IL-18, IL-33), important amounts of cytokines (Th-1, Th-2 or Th-17) and drive the immune response towards

tolerance or the elimination of pathogens or the removal of tumor cells. iNKT cells can also separate GVH et GVT in mouse models by the induction of Th-2 immune responses and the expansion of T regs (Kohrt et al., 2010).

An enhanced early post-transplantation recovery of total iNKT cells has been associated with reduced risk of acute GVHD while sparing the GVL effect (Rubio et al., 2012). A higher content of the stem cell graft in CD4⁺ iNKT cells has been reported to be associated with a reduced risk of acute GVHD (Chaidos et al., 2012). Altogether, these data argue for a regulatory role of iNKT cells in the control of GVHD. In a preclinical model of xeno-GVHD, CD4⁺ iNKT cells have been shown to be able to regulate GVHD by killing recipient APCs and therefore reducing alloreactive T cell activation (Rubio et al., 2012). In T cell depleted haplo-identical HSCT in pediatric ALL, the reconstitution of iNKT cells was inversely correlated with the risk of relapse suggesting a role for iNKT in the GVL effect.

Mesenchymal stem cells (MSC)

MSCs are a heterogeneous population of multipotent, self-renewing progenitors, that can be differentiated in adipocytes, chondrocytes, and osteocytes. They may be isolated from many adult and fetal tissues, particularly bone marrow (BM-MSCs), adipose tissue, dental pulp, amniotic fluid, embryonic placenta, umbilical cord, and other tissues. MSCs from various origins can suppress the activation and functions of most of the immune cells involved in the pathophysiology of GVHD (T cells, B cells, NK cells, monocytes, DCs). They display low immunogenicity in vitro, reason why they are studied as allogeneic adoptive cell therapy.

MSCs can inhibit T cells by cell contact interaction involving several molecules expressed on MSCs (particularly programmed death ligand 1 and 2, HLA-G, Galectin-1), and by the production of immunosuppressive mediators and metabolic changes (particularly tryptophan depletion and tryptophan metabolites via indoleamine 2,3 deoxygenase (IDO) activation, Transforming Growth Factor TGF- β , Hepatocyte Growth Factor, Prostaglandin E2, heme-oxygenase-1, leukemia inhibitory factor, and adenosine).

In addition, MSCs can modulate T cell functions by skewing Th-1 T-cell responses towards Th-2 T-cell responses, but also by the induction of CD4⁺CD25⁺Foxp3⁺ T cells (T regs) and the inhibition of naive CD4⁺ T differentiation into Th-17 cells. Moreover, MSCs impair the differentiation and maturation of monocytes and DCs and induce the generation of regulatory DCs such as plasmacytoid DCs. In vivo, allogeneic MSCs probably do not live long and could be killed by cytotoxic CD8⁺ T cells, and quickly phagocytosed. Their phagocytosis could lead to a key mechanism of immune modulation in vivo, by the development of anti-inflammatory monocytes which produce IDO. In patients, BM-MSCs have shown promising results to treat severe acute GVHD in children and adults. Other sources of MSCs (placenta and umbilical cord notably) are on clinical development to prevent or treat GVHD (Zhao et al., 2019).

Myeloid-derived suppressor cells (MDSCs)

In the late 1970s, a suppressive cell population, called "natural suppressor cells", was first identified in murine and rat bone marrow, spleen, and lymphatic tissues. These cells displayed an ability to suppress T-cell responses in vivo and in vitro without regard to the typical restrictions imposed by the major histocompatibility complex (MHC). Nowadays, MDSCs represent a heterogeneous group of cells. Based on the expression of CD49d, two distinct subpopulations have been described in mice: the first was the monocytic CD49d⁺ subpopulation, which also displayed CD11b⁺ Ly6C⁺ Ly6G^{low} CD115⁺ and the second was the granulocytic CD49d⁺ counterpart, which displayed CD11b⁺ Ly6C⁺ Ly6G^{high} CD115⁺. In humans, three distinct MDSC subsets have been described, based on monocytic, granulocytic, and early-stage characteristics. They commonly do not express markers of mature myeloid and lymphoid cells (lineage negative). Thus, monocytic human MDSCs (M-MDSCs) are defined as: CD11b⁺CD33⁺ HLA-DR^{low/-} and CD14⁺; granulocytic MDSCs (G-MDSCs) are defined as: CD11b⁺ CD33⁺ HLA-DR^{low/-} CD15⁺ but CD14⁻; and early-stage MDSCs (called e- or P-MDSCs) are defined as: CD33⁺ CD11b^{low} HLA-DR^{low/-} CD14⁻ CD15⁻. MDSCs are mainly defined by their capacity to inhibit the proliferation of allogeneic T cells. To date, these immunosuppressive properties have been attributed to five main mechanisms demonstrated in vitro and in vivo, including: NO production; arginase 1-mediated L arginine depletion; indoleamine 2,3-dioxygenase (IDO)-mediated tryptophan (an essential amino acid) conversion; and T regulatory lymphocyte (Treg) induction and reactive oxygen species (ROS) production. MDSCs can prevent GVHD while preserving the GVH effect in mouse models. Their role and potential impact on the GVL effect in humans is still debated (D'Aveni et al., 2020).

Regulatory B lymphocytes (B regs)

B cells derived from precursor cells that mature in the bone marrow. Briefly, immature B cells acquire B-cell receptors (BCRs) on their surfaces and undergo negative selection to delete or edit self-reactive B cells. Nonreactive immature B cells proceed through the circulation to the spleen and become transitional B cells, retaining high levels of immunoglobulin M (IgM) on their surfaces. In the spleen B-cell follicle, transitional B cells change into mature B cells and translate to the peripheral blood. B cells that have not encountered antigens are called naive B cells. In blood circulation, mature B cells receive stimulation from exogenous antigens and migrate towards lymphoid follicles as a result of germinal centre (GC) formation. In the GC, B cells interact with antigens presented by follicular dendritic cells, and B cells with low affinity move towards apoptosis, while high-affinity B cells proceed to plasma cells or memory B cells. This process is called positive selection. A subset of human and mouse B lymphocytes producing IL-10, named B-regs, has been reported to downregulate innate and adaptive immunity, inflammation, and autoimmunity. Since the first description, multiple subsets of B-regs, often with overlapping surface markers but diverse functions, have been reported. Among them, we can cite:

- CD19⁺CD24^{high}CD38^{high} B cells play immunosuppressive roles by retaining Tregs as well as limiting T-helper type 1 (Th1) and Th17 differentiation.
- CD24^{high}CD27⁺ and CD27^{high}CD38^{high} plasmablast B-cell compartments play roles in inhibiting monocyte activation and cytokine production from CD4⁺ T cells.
- CD73⁻CD25⁺CD71⁺ human B regulatory could produce IL10.

In mouse models, B-reg reduction are observed in sclerodermic chronic GVHD and decreased numbers of lymphoid progenitors and immature B cells in bone marrow are observed in the bronchiolitis obliterans syndrome (BOS). In patients with chronic GVHD, significant differences in their B-cell subsets are described. Circulating pre-GC B cells (IgD⁺CD38^{high}CD27⁺) and post-GC “plasmablast-like” cells (IgD^{low}CD38^{high}CD27⁺) increase along with elevated BAFF in a BAFF-dependent way and can produce antibodies without the help of BCR or secondary signal stimulation (Li et al., 2019).

Immunology of chronic GVHD

The pathophysiology of chronic GVHD (cGVHD) has been also described in 3 phases (Fig. 6) (Chaidos et al., 2012). During the first phase, the conditioning regimen induces the destruction of epithelial thymic cells. Thymic damage leads to both defective negative selection of auto- and allo-reactive T cells that can support a reduction in the number or function of regulatory cells such as Tregs and a pathogenic B-cell development. In addition, thymic damages and reduction in regulatory cells are aggravated by the emergence of alloreactive T cells in case of acute GVHD occurrence. In the second phase, B cells become potent antigen-presenting cells, mature, and gain the functional capacity to process and present minor histocompatibility antigens for HLA-matched donor-recipient pairs to donor CD4 T cells. The induction of CD4⁺ T cell polarized towards TH2/TH17 subsets triggers B-cell maturation and production of B-cell survival cytokines (IL-21). During the third phase, T and B cells clonally expanded and resistant to apoptosis, traffic to chronic GVHD target organ sites where IL-13, IL-22, and IL-33 are released. They create a chronic inflammatory environment that can recruit macrophages which produce platelet-derived growth factor alpha (PDGF- α) and transforming growth factor beta (TGF- β) leading to fibrosis. The analysis of peripheral blood in patients suffering from chronic GVHD has shown an increase of (i) a factor implicated in the activation and survival of B cells (B cell activating factor of the tumor necrosis family, BAFF), (ii) high plasma BAFF levels and high BAFF/B-cell levels result in increased numbers of circulating pre-germinal center (GC) B cells and post-GC plasmablast-like cells, capable of producing donor-derived alloantibodies without antigen restimulation (iii) and a delay in reconstitution of naïve B cells (Zeiser and Blazar, 2017).

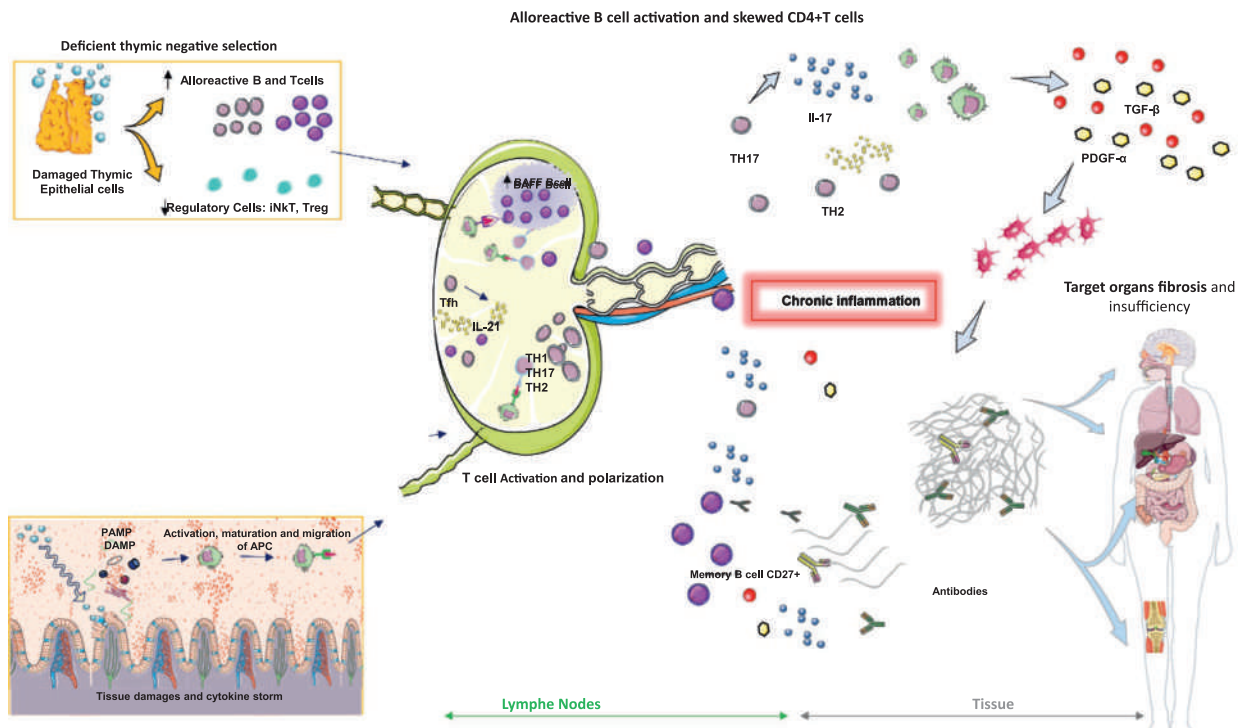


Fig. 6 Pathophysiology of chronic GVHD.

Therapeutic immune modulation

In clinical practice, the prevention and treatment of GVHD is based on the use of pharmacological agents mainly interacting with the activation of T cells by APCs (Penack et al., 2020). Such approaches have reduced the risk of developing acute and chronic GVHD and have allowed the development of mismatched allogeneic HSCT such as with partially matched cord blood units or haplo-identical HSC.

Conventional immunosuppressive agents

Immunosuppressive agents can interfere with at least one of the three signals implicated in the activation and proliferation of T cells following their interaction with APCs (Fig. 7). The first signal (signal 1) is mediated by the TCR stimulation which induces a cascade of intracellular protein phosphorylation leading to the activation of the serine threonine phosphatase calcineurin. This latter allows the dephosphorylation and activation of the transcriptional factor NFAT (nuclear factor of activated T cells). The NF- κ B pathway is also activated following TCR activation leading to the translocation to the nucleus of a transcription component. The latter induces the transcription of cytokines (predominantly IL-2), cytokine receptors (CD25 or IL-2R) and co-stimulation molecules (CD40L). The second signal (signal 2) is mediated by co-stimulation molecules expressed on APCs (CD80/86 and CD40) and their ligands expressed on activated T cells (CD28 and CD40L, respectively). The third signal (signal 3) is the consequence of the interactions between cytokines (IL-2, IL-15, IL-7) and their cognate receptors expressed on T cells. The stimulation of CD25 activates some janus kinases (Jak3/Stat5) and the mTor protein (mammalian Target of rapamycin), both of which regulate the progression from the G1 to the S phase of the cell cycle leading to T cell proliferation, which also requires functional de novo DNA synthesis.

Commonly used immunosuppressive drugs to prevent and treat GVHD are: inhibitors of calcineurin (cyclosporin and tacrolimus), inhibitors of the cycle cell or DNA synthesis (MMF, Methotrexate), inhibitors of mTOR (sirolimus or everolimus), steroids (inhibiting APC/T cell interaction and cytokine production by T cells as well as T cell homing to inflamed tissues), anti-JAK (ruxolitinib, itacitinib) and ibrutinib that inhibits the Bruton's tyrosine kinase.

In addition to pharmacological agents targeting T cell activation, there are monoclonal antibodies and immunosuppressive strategies. They include T and B cell depleting antibodies including anti-thymocyte globulin (ATG), anti-CD52 (mabcampath), anti-CD20 (rituximab), anti-IL2 receptor or anti-CD25, and extracorporeal photopheresis (ECP), an ex vivo therapy of leukapheresis collected lymphocytes by a psoralen sensitizer exposed to ultraviolet light and subsequently re-infused to the patient. This procedure induces apoptotic T cells, tolerogenic DCs and increased T regs.

In addition, cyclophosphamide, an alkylating agent, that triggers the apoptosis of activated T cells while sparing Tregs has more recently introduced into the different strategies of GVHD prevention.

Indeed, all patients receiving allo-HSCT will receive a combination of immunosuppressive drugs to prevent acute and chronic GVHD. A combination of two pharmacological inhibitors is usually used in ex vivo non-T cell depleted allo-HSCT: a calcineurin inhibitor targeting signal 1 (cyclosporin A or tacrolimus) in association with an inhibitor of signal 3 (either the anti-metabolite methotrexate or the inhibitor of purines mycophenolate mofetyl (MMF) or the mTor inhibitor rapamycin/Sirolimus).

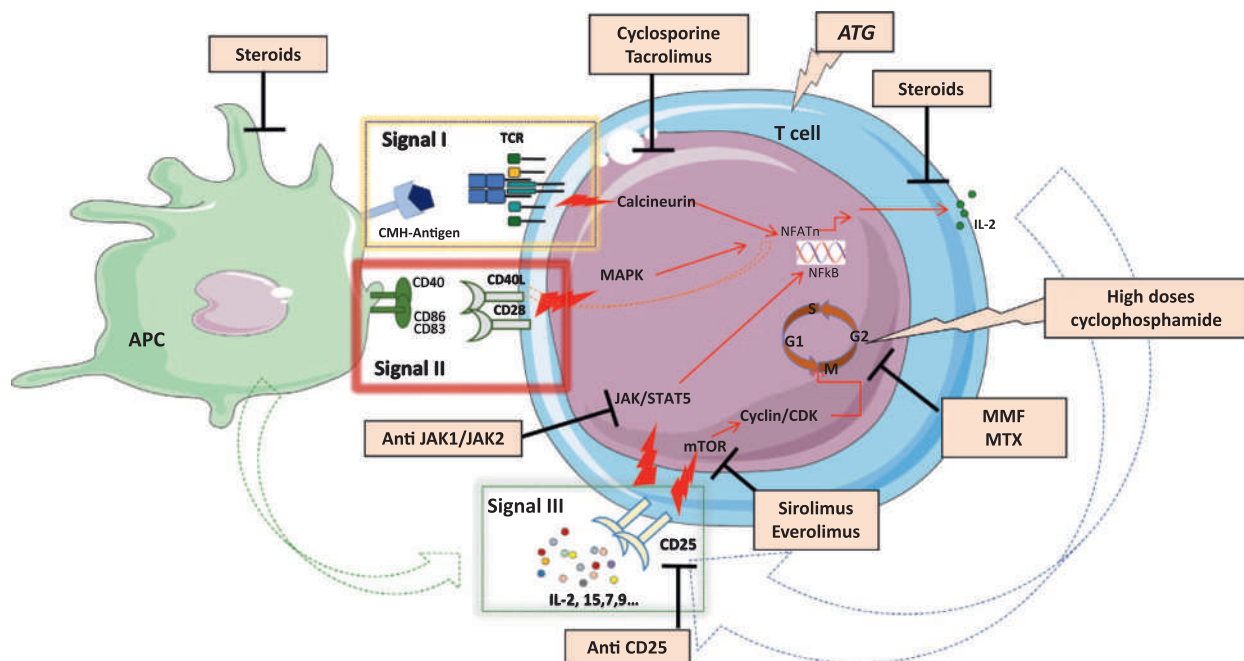


Fig. 7 Mechanisms of action of usual pharmacological immunosuppressive drugs.

In addition, administration of ATG before HSCT has been shown to prevent severe acute and chronic GVHD and is recommended in matched or mismatched unrelated or related donors. In haploidentical allo-HSCT, different GVHD prevention are used but the most developed is the administration of high doses of cyclophosphamide in a narrow window after transplantation (days 3 and 4 or 5) instead or in combination with ATG (Penack et al., 2020).

The first line treatment of acute or chronic GVHD is based on the use of steroids. Currently, ruxolitinib (JAK2 inhibitor) is the only approved drug for the treatment of steroid refractory acute and chronic GVHD (Zeiser et al., 2020). In third line, there is no consensus of treatment. None of the immunosuppressive drugs previously cited have significantly improved the poor prognosis of steroid resistant acute GVHD (OS <40% at 1 year) or steroid resistant severe chronic GVHD (30–50% mortality at 5 years) (Toubai and Magenau, 2020).

Novel immunomodulatory approaches

The challenge of allogeneic HSCT is the development of novel immunomodulatory agents that can specifically target alloreactivity without impairing graft versus tumor beneficial effect. Maintenance therapies after allo-HSCT that specifically target the hematological malignancy to prevent their relapses can also prevent GVHD.

Tyrosine kinase inhibitors

- (i) Imatinib: Imatinib, the first tyrosine-kinase inhibitor developed to treat chronic myelogenous leukemia, is a potent dual inhibitor of both transforming growth factor- β and platelet-derived growth factor receptor (PDGF-R) pathways, 2 cytokines involved in the fibrogenic and inflammatory processes of chronic GVHD with systemic sclerosis.
- (ii) Ibrutinib: Bruton's tyrosine kinase (BTK) activation is critical for B-cell survival, proliferation, and migration. Ibrutinib was designed as a selective and irreversible inhibitor of the BTK protein and inhibits B-cell tumor growth such as chronic lymphocytic leukemia and B-cell lymphomas. In addition to inhibiting BTK, ibrutinib is an irreversible inhibitor of interleukin-2 inducible kinase (ITK) and is FDA-approved for the treatment of chronic GVHD.
- (iii) Itacitinib: A JAK1 inhibitor that reduces inflammatory cytokines and T cell proliferation. In a phase II trial, itacitinib seemed promising for the prevention of acute GVHD in addition to conventional GVHD prevention. A phase III in first line treatment of acute GVHD failed however to demonstrate an interest over steroids alone.

Belumosudil

An oral rho-associated coiled-coil-containing protein kinase-2 (ROCK2) inhibitor has been shown to reduce the Th-17 alloreactive response, increase T regs and control fibrotic pathways. Belumosudil treatment in a phase II randomized trial resulted in high response rates in steroid refractory chronic GVHD, improvement of quality of life and high overall survival rates. Belumosudil has been recently approved by the FDA for the treatment of chronic GVHD after failure of at least two prior lines of systemic therapy.

5-Azacytidine

The demethylating agent 5-azacytidine (AZA) has proven its efficacy in the treatment of myelodysplastic syndrome and acute myeloid leukemia. In mice, AZA improves both survival and GVHD severity. In humans, AZA significantly decreases human T-cell proliferation as well as IFN- γ and TNF- α serum levels, reduces the expression of Granzyme B and Perforin by cytotoxic T cells and might increase T reg proliferation therefore modulating alloreactivity (Fransolet et al., 2016).

Anti-BCL2

Venetoclax is a selective inhibitor of the anti-apoptosis factor B-cell lymphoma 2 (BCL2), which has been revealed to abundantly express in leukemia stem cells. Moreover, longitudinal analysis of T-cell transcriptomes in patients before the appearance of GVHD symptoms revealed the upregulation of anti-apoptotic regulator BCL2 at GVHD initiation. BCL2 RNA is elevated in multiple organs affected by GVHD and expression correlated with transplant-related mortality and steroid-refractory GVHD. Inhibition of BCL2 might induce apoptosis of alloreactive T cells and become a new targeted therapy in the treatment of steroid refractory GVHD.

Cellular immunotherapy

Besides pharmaceutical agents, the infusion of immunoregulatory cells appears an attractive option: they can control immune homeostasis, reduce detrimental T-cell responses to foreign antigens, facilitate tissue repair with believed short-lasting immune effects avoiding increase of relapse and infections (Voermans and Hazenberg, 2020).

Adaptive lymphoid cells: Regulatory T cells

In mice, it has been clearly demonstrated that freshly isolated donor T regs or ex vivo expanded T regs added to donor CD3⁺ T cells early after allo-HSCT reduced GVHD while maintaining the GVT effect. The ability of natural CD4⁺ CD25⁺ FoxP3⁺ CD127^{low} T cells to control GVHD while maintaining the GVT effect in humans is debated. The "first-in-man" infusion of in vitro expanded natural T regs were published in 2009 for the treatment of one acute and one chronic GVHD. Freshly isolated donor-derived T regs have been injected in prophylaxis of GVHD in some small series of mismatched cord-blood or haplo-identical HSCT and several clinical trials are still recruiting with diverse sources of T regs or purified Tr1 cells. There is however a concern about their possible inhibition of the GVL effect. Indeed, Treg depletion in donor lymphocytes injections (DLI) has been associated with enhanced anti-leukemic and

anti-lymphoma responses in patients relapsing after transplantation. A way of research to modulate GVHD without impairing the GVT effect by increasing natural Tregs in vivo could be obtained with $\alpha 1$ -Antitrypsin, a naturally abundant serine protease inhibitor, a new treatment testing in GVHD treatment, downmodulating inflammation and increasing ratios of regulatory (Tregs) to effector T cells (Teffs).

Innate lymphoid cells

Lymphoid members of the innate immune system include natural killer (NK) cells, innate lymphoid cells (ILCs), iNKT cells and $\gamma\delta$ T cells.

ILC type 2 (ILC2) and iNKT cells have been infused in preclinical models and shown to reduce GVHD. Improved iNKT reconstitution or increased iNKT cells in HSC grafts have been associated with reduced GVHD and relapse. Trials in humans with expanded iNKT cells are expected.

Donor NK cells also have been tested in preclinical models and in the clinic. Transfer of donor-activated murine NK cells reduced acute GVHD as they can eliminate host antigen-presenting cells (APCs), limiting donor T-cell expansion. In humans, however, a pilot study of patients given IL-15/4-1BB ligand-activated NK cells from HLA-matched sibling or HLA-matched unrelated donors showed that five of nine of the patients (55%) experienced aGVHD including three severe forms. Thus, NK cell therapies are mainly currently explored to treat or prevent relapse after allo-HSCT in high risk patients.

Higher donor $\gamma\delta$ T cell content in HSC grafts has been associated with increased incidence of acute GVHD. However, enhanced post-transplant $\gamma\delta$ T cell has been correlated to improved event-free survival and OS, particularly in T cell depleted haploidentical HSCT. Different strategies to enhance $\gamma\delta$ T cell recovery are used in the context of haploidentical HSCT in order to avoid GVHD while sparing the GVL effect.

Myeloid cells: MDSCs

Generating MDSCs for clinical applications might be difficult, because MDSCs represent a rare subpopulation of myeloid cells. To date, few researchers have proposed to expand MDSCs in vitro for cellular therapy. MDSCs can be expanded from cord blood unit or donor apheresis after G-CSF mobilization or steady state PBMC with a cocktail of cytokine including G-CSF $\pm$ GM-CSF $\pm$ SCF $\pm$ FLT3 $\pm$ IL-6. Few preclinical proofs of concept are available (D'Aveni et al., 2020).

Non hematopoietic cells MSCs

In humans, co-transplantation of MSCs with allogeneic HSC has been shown to prevent the development of acute or chronic GVHD. They also have been used for the treatment of high-risk and/or steroid refractory acute GVHD. Since the first successful treatment of acute GVHD by intravenous infusion of bone marrow-derived haploidentical MSC reported in a 9-year-old patient with severe resistant grade IV acute GVHD, similar results have been observed with MSCs from diverse sources (adult tissues including adipose tissue and extra-embryonic tissues such as placenta/umbilical cord and Wharton's Jelly). If no serious acute infusion-related toxicity and no serious treatment-related adverse events have been reported, controversial results on the potential of MSC to increase infection and relapse incidences have been reported. It is demonstrated that their immunosuppressive properties may vary with the donor of MSCs. To erase this high inter-individual variability some authors pool MSCs from eight voluntary donors and some teams license WJ-MSCs with pro-inflammatory cytokines, for instance IFN- γ , which improves their function to prevent GVHD (Pochon et al., 2022). We lack clinical imaging systems to determine how MSCs patrol after intravenous infusion and how long they do persist in vivo. Preclinical models demonstrated that 2 h after intravenous injection, MSC are in the lungs followed by an accumulation in the liver and spleen from 24 to 96 h following injection with a hepatobiliary and renal clearance and a probably very quick death after lung trapping, due to monocyte phagocytosis. That is why repeated infusions are now usually scheduled in clinical trial, with no data on the risk of HLA alloimmunization (Zhao et al., 2019).

Conclusion

The better understanding of the immune system regulation has provided better insight in the physiopathology of alloSCT. New therapeutic approaches for the prevention of graft versus host diseases has already led to cross the HLA barrier and expand donor availability for nearly all patients. In addition, the use of these new combined pharmaceutical and cellular approaches will allow a better control of GVHD without impairing the graft versus tumor effect, thus making allo-HSCT a safer and more efficient procedure to cure hematological and immunological disorders.

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Asthma Mechanisms

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Introduction

Asthma is an impactful and common chronic pulmonary disease, affecting both children and adults, and one which disproportionately impacts ethnic minorities and people of low socioeconomic status (Akinbami et al., 2016). The most prevalent chronic respiratory disease globally, asthma is debilitating and potentially deadly, associated with hundreds of thousands of deaths annually and a significant burden in healthcare-associated costs and disability-adjusted life years (GBD Chronic Respiratory Disease Collaborators, 2017). The disease of asthma is inextricably tied up in, and potentiated by, the social determinants of health. Therefore, understanding the epidemiology of this disease is an important aspect in its management. Simply put, one cannot effectively manage an individual patient's asthma without engaging, on some level, with that patient's social, economic, and physical environment.

Asthma, as a clinical entity, defies easy definition. The complex nature of asthma pathophysiology and its myriad clinical manifestations makes concision difficult. One broadly encompassing definition of asthma is as *a chronic inflammatory disorder of the lungs causing generally reversible small airway obstruction*. The Global Initiative for Asthma (GINA) expands on this definition further:

Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation.

(GINA, 2020)

The pathophysiology of asthma is not fully understood, but appears to be mediated by a complex interaction of pulmonary genetics, immunology, prenatal development and perinatal course; and is modulated further by environmental exposures, socioeconomic stressors, and adversity (Williams et al., 2009; Flanagan et al., 2018).

Epidemiology

The prevalence of asthma has increased globally since the mid-20th century, alongside an increasing burden of allergic disease, such as eczema and allergic rhinitis (Dharmage et al., 2019). Recent data indicates that this epidemic of asthma has perhaps plateaued in wealthier countries over the past decade; however, improvements in global asthma morbidity and mortality has stalled despite increased awareness of asthma and an ever-expanding armamentarium of asthma therapies (Akinbami et al., 2016; Ebmeier et al., 2017). Despite the higher prevalence of asthma in wealthier nations, low-middle income countries suffer a disproportionate mortality from the disease (Dharmage et al., 2019). Mirroring global inequities, historical segregation of communities in the United States of America (US) has contributed to severe and pervasive socioeconomic and health inequities which persist to this day (Williams et al., 2009). Chronic stressors, including exposure to violence, may confer increased susceptibility to environmental potentiators of asthma, such as poor air quality, through dysregulation of the autonomic tone of the airways (Wright et al., 2005). Recent US Centers for Disease Control and Prevention (CDC) National Health Interview Survey (NHIS) data from 2016 indicates that 1 in 12 children 0–17 years of age in the US has asthma, and suggests a slight decrease in asthma prevalence and the incidence

of asthma exacerbations since 2001 (Zahran et al., 2018). Despite this, asthma remains quite common and burdensome for US children, with an overall prevalence of around 8%, which is increased to about 10% for children at or below the federal poverty level (Zahran et al., 2018).

For many, asthma begins in early childhood (GINA, 2020). In children 5 years of age and younger, viral respiratory illnesses are a predominant cause of asthma exacerbation and wheeze; and with age aeroallergen sensitization becomes an increasingly important driver of asthma symptoms. Asthma with comorbid allergic disease, such as eczema, may confer a worse pulmonary function trajectory over time (Dharmage et al., 2019). Boys have a higher incidence of asthma than girls in early life; however, young women experience a proportionally higher burden of disease in adolescence (Dharmage et al., 2019). This shift persists into adulthood, where healthcare utilization and mortality is highest (Dharmage et al., 2019). For those patients with true adult-onset asthma, as much as 20% of disease is proximally attributable to chronic occupational exposure to inhaled irritants (Dharmage et al., 2019).

Manifesting as reversible bronchoconstriction of the small airways, acute asthma exacerbations (characterized generally by wheeze, shortness of breath, and increased work of breathing) are managed with bronchodilators and other medications, often inhaled, which modulate the muscular tone of the airways, easing the transit of air and ameliorating the obstructive defect for a time. Longitudinally, those patients with persistent asthma often require the use of daily immune-modulating medications such as inhaled corticosteroids (ICS) and leukotriene receptor antagonists. A subset of patients, perhaps as high as 8% by some estimates, experience severe asthma, as defined by symptoms which require high-dose ICS and a second, adjunctive controller to maintain symptom control, or asthma which is refractory to this maximal outpatient therapy (Dharmage et al., 2019). In those whose asthma is mediated by allergy and is particularly refractory to management, a burgeoning class of asthma biologic medications has been important.

Poorly-controlled and severe asthma may negatively impact childhood lung development, and adults with significant asthma may be at higher risk for the development of fixed airway obstruction and chronic obstructive pulmonary disease (COPD) over time (Dharmage et al., 2019; McGeachie et al., 2016). Importantly, the impact on childhood development is not only manifested in pulmonary disease and exercise limitation, as children with high disease burden are also at risk for behavioral and academic adversity, as well as anxiety and depression attendant to chronic disease (Schneider, 2020; Halterman et al., 2006). Similarly, adults may experience job instability and other economic hardship as a result of persistent asthma symptoms and lost productivity. Strikingly, employment instability itself is associated with incidence of asthma in adults (Loerbroks et al., 2014). Uncontrolled asthma may potentiate the health consequences of comorbid diseases, for example sickle cell disease and obstructive sleep apnea, and indeed in many cases these relationships may be bidirectional (Serrano-Pariente et al., 2017; Samarasinghe and Rosch, 2019). Smoking clearly contributes to the pathogenesis of asthma and COPD in some patients, as does the aforementioned occupational exposures (GINA, 2020).

Genetic risk factors

The overall prevalence of asthma has been increasing in the United States and throughout the developing world in recent decades (Moorman et al., 2012; Eder et al., 2006). The rise in asthma prevalence is driven by the complex interaction of genetic factors and multiple environmental risk factors. Early twin studies showed that genetic factors contribute to the development of asthma. Twin studies assess the heritable risk of a disease by comparing the rate of occurrence (concordance rate) among genetically identical monozygotic twins to genetically similar dizygotic twins. Concordance rates are higher among monozygotic twins, but range from 50% to 60%, suggesting the contribution of environmental factors to the development of asthma (Koppelman et al., 1999). In addition, the predictive value of a positive family history on the development of childhood asthma only ranges from 11% to 37% (Burke et al., 2003). These findings illustrate that the inheritance pattern is more complex than the Mendelian pattern seen in monogenic disorders.

Modern advances in the field of molecular genetics offer new strategies for finding susceptibility genes associated with asthma pathogenesis. Genome-wide linkage studies and candidate-gene association studies have largely been replaced by genome-wide association studies (GWAS) over the last decade. GWASs provide an affordable and unbiased approach to identify genetic factors underlying complex diseases in large populations. GWASs scan the genome of patients with and without asthma for genetic variations called single nucleotide polymorphisms (SNPs). A polymorphism that occurs more frequently among patients with asthma suggests the genetic variant is involved in the pathogenesis of asthma. However, the GWAS approach requires a high threshold for statistical significance to limit the large number of false positive signals (Visscher et al., 2017). The GABRIEL Consortium in Europe and EVE Consortium in the United States are two of the most important GWASs on asthma. Both studies identified multiple asthma-susceptibility genes with functional roles in cellular inflammation and airway remodeling.

The GABRIEL Consortium analyzed 582,892 SNPs using data from 10,365 participants with asthma and 16,110 healthy controls (Moffatt et al., 2010). The EVE Consortium analyzed a more ethnically diverse group of participants with asthma in the United States (Torgerson et al., 2011). The strongest association with childhood-onset asthma was seen in the *ORMDL3/GSDMB* locus on chromosome 17q21. The role of *ORMDL3* in asthma development is unclear. Recent mouse studies suggest *ORMDL3* expression regulates sphingolipid synthesis. Sphingolipids, such as ceramide, are implicated in the inflammatory response and airway hyperreactivity underlying asthma (James et al., 2019). Both studies identified *IL-33* and *IL1RL1* as asthma-susceptibility genes. Interleukin-33 (IL-33) is a cytokine released in response to airway epithelium damage. The receptor for IL-33 is interleukin 1 receptor-like 1 (IL1RL1 or ST2) which is located on T helper 2 (Th2) cells and group 2 innate lymphoid cells (ILC2). IL-33 activation

of Th2 and ILC2 cells promotes the production of type 2 inflammatory cytokines responsible for allergic asthma (Chan et al., 2019). The EVE Consortium also identified thymic stromal lymphopoietin (TSLP) as a susceptibility locus. TSLP released by airway epithelial cells contributes to allergic asthma by activating ILC2 cells and promoting Th2 cell polarization (Cianferoni and Spergel, 2014). TSLP is the target of emerging biologic therapies because expression is increased in the airway epithelium of patients with asthma (Corren et al., 2017). Interleukin-13 (IL-13) is an example of a type 2 inflammatory cytokine produced by both Th2 and ILC2 cells. IL-13 contributes to asthma pathogenesis by promoting B cell class switching to produce immunoglobulin E (IgE) and mucus secretion from airway epithelial cells. IL-13 is located on chromosome 5q31, a region that contains multiple candidate genes linked to asthma development. The role of IL-13 is highlighted by the improvement in asthma control observed with the use of biologic medications that block IL-4R α and subsequent signaling through the IL-4 and IL-13 receptors.

GWAS and positional cloning also identified susceptibility genes involved in tissue repair and remodeling, including *ADAM33* and *SMAD3* (Van Eerdewegh et al., 2002). *ADAM33* is a metalloprotease present in airway smooth muscle and fibroblasts. The distribution of *ADAM33* and fact that its expression is increased in patients with asthma suggests a role in airway remodeling (Li et al., 2019). *SMAD3* is a key transcription factor for TGF β signaling. TGF β is implicated in cellular inflammation and airway remodeling by promoting differentiation of T helper 17 (Th17) cells and regulating fibrosis, respectively. Recent epigenetic studies show that methylation of *SMAD3* is associated with an increased risk of childhood asthma in infants born to mothers with asthma (DeVries et al., 2017). This is an example of how environmental risk factors can induce changes to DNA, such as acetylation and methylation. Epigenetic modifications can alter asthma susceptibility gene expression and may explain the complex interaction of genetic factors and environmental risk factors (Potaczek et al., 2017). Examples of early life risk factors include exposure to environmental allergens, lifestyle choices, and exposure to disease states and medications, among others. For example, in utero exposure to maternal smoking leads to DNA methylation and maternal smoking is associated with an increased risk of asthma development (Breton et al., 2014; Tinuoye et al., 2013).

The generalizability of the genetic findings for asthma is limited by the lack of ethnic diversity in genetic studies. The majority of GWASs on asthma have been performed on people of European descent even though asthma disproportionately affects ethnic minorities in the US (Burchard et al., 2015; Akinbami et al., 2016). The inclusion of a more ethnically diverse population in the EVE Consortium showed that certain asthma-susceptibility loci may be ancestry specific. The association of *ORMDL3/GSDMB*, *IL-33*, *IL1RL1*, and *TSLP* with asthma was replicated across different ethnic groups; however, *PYHIN1* was identified as a novel susceptibility locus in subjects with African ancestry (Torgerson et al., 2011). Several studies have identified inconsistent association signals between SNPs and asthma susceptibility among different ethnic groups. For example, SNPs in *ORMDL3* that are associated with asthma in European subjects were not detected in African American subjects (Sleiman et al., 2008). The genetic heterogeneity of asthma is best illustrated by the Consortium on Asthma among African-ancestry Populations in the Americas (CAAPA), a GWAS of 7009 participants with asthma and 7645 healthy controls of African ancestry. The CAAPA study replicated multiple loci previously reported in predominantly European populations (e.g. chromosome 17q21) and identified two novel susceptibility loci in 8p23 and 8q24 among subjects with African ancestry. Genetic heterogeneity not only exists between ethnic groups but may also exist among individuals with similar ancestry. For example, the magnitude of association between SNPs in chromosome 17q and asthma risk among African populations was directly proportional to the degree of European ancestry (Daya et al., 2019).

Environmental risk factors

The risk of developing asthma likely depends on the influence of multiple environmental risk factors rather than one modifiable risk factor (Burbank et al., 2017). It has been proposed that environmental risk factors increase the likelihood of asthma in genetically susceptible individuals by altering the development of the respiratory tract and the immune system (Martino and Prescott, 2013). Identification of the most impactful risk factors of asthma contributes to the development of primary prevention strategies and deepens our understanding of asthma pathogenesis (Abreo et al., 2018). Primary prevention strategies aim to prevent the development of asthma in susceptible individuals (Brunwasser and Hartert, 2019). For example, the Canadian Asthma Primary Prevention Study (CAPPS) showed that multiple early life interventions (e.g. dust mite control, pet avoidance, tobacco smoke avoidance, breastfeeding) could reduce asthma prevalence by 7 years of age (Chan-Yeung et al., 2005). The remainder of this section will review the impact of several early life influences on childhood asthma development.

Microbial exposure

The human microbiome is a collection of microorganisms located in the skin, respiratory tract, and gastrointestinal tract (Lynch and Pedersen, 2016). Commensal bacteria have a symbiotic relationship with humans and contribute to the development of the immune system. Germ-free mouse models lacking a gut microbiome have significant defects in immune system maturation and function that contribute to allergic disease (Cahenzli et al., 2013). Environmental factors such as mode of delivery, maternal and infant antibiotic use, and dietary changes can induce dysbiosis, or an alteration in the composition of commensal bacteria (Feehley et al., 2012). The “hygiene hypothesis” illustrates the clinical importance of dysbiosis, and postulates that early life contact to a microbe rich environment confers allergen tolerance and protection from the development of asthma (Arrieta et al., 2015). Early observational studies after German reunification showed that the prevalence of asthma was higher among children in well-developed West Germany than more polluted East Germany (von Mutius et al., 1994). It was subsequently shown that

decreased diversity of the gut microbiome in early infancy was associated with an increased risk of asthma in school-aged children (Abrahamsson et al., 2014). The gut microbiome of children with allergic disease contains a greater abundance of *Bacteroidaceae*, *Clostridiaceae*, and *Enterobacteriaceae* with a lower abundance of *Bifidobacteriaceae* and *Lactobacillaceae* (Zimmermann et al., 2019). Early life changes to the respiratory microbiome are also suspected to contribute to asthma susceptibility. Bacterial colonization of the neonatal upper respiratory tract with *Streptococcus pneumoniae*, *Haemophilus influenzae* and/or *Moraxella catarrhalis* is associated with an increased risk of childhood asthma (Bisgaard et al., 2007). The abundance of *Streptococcus* and *Haemophilus influenzae* is increased in the nasopharynx of young children infected with respiratory syncytial virus (RSV), a known risk factor for childhood asthma. The altered nasopharyngeal microbiome is associated with a pro-inflammatory response and more severe clinical outcomes, suggesting a link between dysbiosis and the host immune response (de Steenhuijsen Pijters et al., 2016).

Mode of delivery and antibiotic exposure are two environmental risk factors that can alter the microbiome. The cesarean birth rate increased by 27% between 1965 and 2015, and multiple studies show that cesarean section is associated with an increased risk of developing asthma (Taffel et al., 1987; American College of Obstetricians and Gynecologists and Society for Maternal Fetal Medicine, 2014; Huang et al., 2015). The infant microbiome is shaped by the mode of delivery. Infants delivered by vaginal delivery are colonized by vaginal flora (*Lactobacillus*, *Bacteroides*), while infants delivered by cesarean section are colonized with skin flora (*Staphylococcus*, *Corynebacterium*, *Propionibacterium*) (Dominguez-Bello et al., 2010). Recent studies suggest that the microbiome of infants born via cesarean section can be partially restored by transfer of maternal vaginal flora (Dominguez-Bello et al., 2016); however, there are no randomized controlled trials to assess this approach. A majority of pregnant women and infants in the first year of life receive at least once course of antibiotics. Prenatal and infant antibiotic use are both associated with a dose-dependent increase in the risk of childhood asthma (Turi et al., 2020; Donovan et al., 2020). It is postulated that inappropriate antibiotic use may reduce the diversity of the infant microbiome beginning in utero (Yassour et al., 2016).

The Urban Environment and Childhood Asthma (URECA) study showed that early childhood exposure to house dust samples enriched with diverse bacteria and perennial allergens (e.g. mouse, cockroach, cat) conferred protection from asthma. The findings suggest that human interaction with diverse microbial products augments the host microbiome and induces immune tolerance to environmental allergens (Lynch et al., 2014). The lower prevalence of asthma in farming environments lends further support to the importance of microbial exposure. However, the prevalence of asthma differs among the Amish and Hutterite farming communities. Although both communities have similar genetic ancestry, the Hutterites practice industrialized farming while the Amish practice traditional farming. For example, Amish children grow up on single-family dairy farms with a dependence on horses for daily living. The prevalence of asthma was four times lower among Amish children than Hutterite children. A key difference was higher microbial diversity and endotoxin levels in Amish homes. Mice exposed to Amish house-dust extracts were protected from airway hyperresponsiveness, suggesting that diverse microbial products within the home confer protection from asthma. The protective effect of Amish house-dust is postulated to involve stimulation of the innate inflammatory response (Stein et al., 2016).

Acute respiratory infections

Acute respiratory infections (ARI) during infancy are most commonly caused by respiratory syncytial virus (RSV) and human rhinovirus (HRV). HRV often leads to a mild upper respiratory infection while RSV is the primary cause of infant lower respiratory tract infection (LRTI) (Tregoning and Schwarze, 2010). Both viruses induce a pro-inflammatory response; however, RSV causes more significant cytopathic damage to the airway epithelium (Vandini et al., 2019). Numerous studies have shown that early life RSV and HRV lower respiratory tract infections are associated with early childhood wheezing (Regnier and Huels, 2013; Liu et al., 2017). The Tucson Children's Respiratory Study (TCRS) followed 1246 newborns and showed that RSV LRTI in the first 3 years of life is associated with an increased risk for wheezing until 11 years of age (Stein et al., 1999). Two prospective studies supported a potential causal role by showing that the administration of RSV immunoprophylaxis to high-risk infants was associated with a decreased risk of recurrent wheezing (Simoes et al., 2010; Blanken et al., 2013). The Children in the US Childhood Origins of Asthma (COAST) study followed 259 newborns, and showed that HRV LRTI in the first 3 years of life was associated with an increased risk of subsequent asthma at 6 years of age (Jackson et al., 2008). The mechanistic relationship between seasonal HRV infections and viral-induced asthma was illustrated by the Preventative Omalizumab or Step-up Therapy for Severe Fall Exacerbations (PROSE) study. The PROSE study found that omalizumab reduced the duration of seasonal HRV infections and reduced the rate of asthma exacerbations in the fall by enhancing IFN- α responses to HRV (Esquivel et al., 2017). It remains unclear whether prevention of respiratory illness can significantly reduce the risk of developing asthma. There are multiple candidate RSV vaccines in ongoing clinical trials to determine if active immunization of infants and/or pregnant women can prevent RSV infection (Mazur et al., 2015). The development of immunization strategies against HRV is limited by lack of cross-protective immunity between numerous HRV serotypes (McLean, 2014).

Allergen exposure

Sensitization to perennial aeroallergens in early childhood is associated with an increased risk of developing asthma (Illi et al., 2006). Perennial allergens include house dust mites, furry animals, mice, cockroach, and fungi. House dust mites are found in mattresses, carpet, and upholstered furniture in humid environments. As many as 50% of children with asthma exhibit sensitization to house dust mite (Ulrik and Backer, 1999). Exposure to the dominant house dust mite allergen Der p 1 in early childhood has been shown to be an important risk factor for childhood asthma (Sporik et al., 1990). Mechanistically, house dust mite allergens

have been shown to activate toll-like receptor 4 on airway epithelial cells to promote a type 2 inflammatory response. House dust mite control as part of two multifaceted intervention trials was associated with a decreased prevalence of asthma (Chan-Yeung et al., 2005; Arshad et al., 2007). Fungi can be found inside and outside the home. *Cladosporium*, *Aspergillus*, and *Penicillium* are indoor fungi that are commonly found in the homes of patients with asthma (Sharpe et al., 2015). Indoor dampness contributes to the growth of certain indoor fungi, and a systemic review found that both indoor dampness and indoor mold are predictors of asthma development (Quansah et al., 2012). Cockroaches and mice are commonly found in inner-city and urban settings. Children exposed to progressively higher concentrations of cockroach allergen Bla g 1 in the home were at a higher risk for developing asthma (Litonjua et al., 2001). Allergen exposure is not limited to the home, and primary prevention strategies must consider all potential areas of exposure. The School Inner-City Asthma Study followed 284 school-aged students attending inner-city schools and showed that asthma morbidity is associated with exposure to high mouse allergen levels in schools (Sheehan et al., 2017). The evidence of early life exposure to furry pets on asthma development is contradictory. Several initial studies suggested a protective effect of early dog exposure (Remes et al., 2001; O'Connor et al., 2018). However, a pooled analysis of over 22,000 children showed no effect of early pet ownership on the risk of asthma in school-aged children (Lodrup Carlsen et al., 2012). It is postulated that pet ownership, like a farm, creates a protective microbe rich environment. Additional randomized controlled trials are needed to establish causality of these risk factors and the effectiveness of primary prevention strategies (Van Mason and Portnoy, 2020).

Asthma phenotypes and endotypes

Asthma is best viewed as a heterogeneous group of disorders leading to a chronic inflammatory pulmonary disease. As new therapies become available, each of which targeting a specific point in the immunologic milieu, it becomes increasingly relevant to distinguish specific forms of asthma at the point of care. Asthma phenotypes refer to the spectrum of clinical presentations, while the different underlying mechanistic pathways are termed endotypes. Currently, endotypes are broadly categorized as type 2 (T2)-high (eosinophilic) or T2-low (non-eosinophilic) (Kuruvilla et al., 2019). Understanding of the cell types and cytokines involved in the pathology of asthma, as well as conceptualizing the manner in which the endotype contributes to the phenotype, is of increasing clinical importance. T2-high phenotypes include early-onset allergic asthma, late-onset eosinophilic asthma, and aspirin-exacerbated respiratory disease (AERD) (Kuruvilla et al., 2019). The T2-low classification is associated with phenotypes which include obesity-related, smoking-related, and advanced age-related asthma (Kuruvilla et al., 2019). The categorization of asthma will continue to evolve as scientific knowledge expands.

Immunology of asthma

Based on inflammatory cell counts in induced sputum, asthma can be broadly classified into four cellular subtypes: eosinophilic, neutrophilic, mixed granulocytic, and paucigranulocytic (Tliba and Panettieri, 2019). Eosinophilic asthma describes patients with elevated sputum eosinophils of greater than 3% (Tliba and Panettieri, 2019). Eosinophilic asthma can be either non-allergic or allergic based on the presence or absence of allergic sensitization (Lambrecht and Hammad, 2015). Neutrophilic asthma describes patients with sputum neutrophilia greater than 64% without sputum eosinophilia (Svenningsen and Nair, 2017). Mixed granulocytic asthma describes patients having both elevated neutrophils and eosinophils (Tliba and Panettieri, 2019). Paucigranulocytic asthma characterizes patients without elevated neutrophils (<64%) or eosinophils (<3%) (Svenningsen and Nair, 2017). Each form may present a unique set of challenges in the clinical realm, including unique responses to treatment. For the purposes of describing the immunologic mechanisms of asthma, we will focus primarily on eosinophilic asthma and neutrophilic asthma, where the greatest depth of immunologic understanding currently exists.

Non-allergic eosinophilic asthma

The discovery of innate lymphoid cells (ILCs), previously termed “nuocytes,” “natural helper cells” or “Innate Helper Type 2 cells (Ih2s)” has allowed much deeper insight into asthma pathogenesis (Monticelli et al., 2012). ILCs are non-antigen dependent lymphoid cells. Type 2 innate lymphoid cells (ILC2s) are significant mediators of eosinophilic inflammation (Lambrecht and Hammad, 2015). ILC2 cells are abundant in the lung, among other tissues, and appear to play an integral role in mediating eosinophilic inflammation even in non-atopic patients, owing to their lack of antigen-specific receptors (Mjosberg and Eidsmo, 2014). Recognition of the importance of ILC2s in the pathophysiology of asthma led to the change in terminology from “Th2-high asthma,” which implies that T helper type 2 (Th2) cells are the predominant driver, to “type-2 (T2) asthma” (Kuruvilla et al., 2019). ILCs respond to a group of cytokines termed “alarmins” which are released by the epithelial barrier in response to external insults. These cytokines include thymic stromal lymphopoietin (TSLP), IL-33, and IL-25 (Roan et al., 2019). A summary of prominent cytokines is provided in Table 1.

TSLP is a member of the IL-2 family of cytokines. Epithelial cells are a significant source of TSLP, with the lungs, skin, and gastrointestinal tract being the primary locations of production (Roan et al., 2019). Although some TSLP is produced under homeostatic conditions, expression and release is increased when epithelial cells are stimulated by infection, mechanical injury, inflammatory cytokines, and proteases (Roan et al., 2019). Other cell types, including dendritic cells (DCs), basophils, and mast

Table 1 Prominent cytokines and their respective roles in asthma.

<i>Cytokine</i>	<i>Primary source</i>	<i>Role in asthma</i>
Type 2 Cytokines		
IL-4	Th2 cells T _H cells +/- ILC2s	Principal cytokine which stimulates B cell class switching to IgE. Induces Th2 differentiation. Eotaxin synthesis by airway epithelium. Airway remodeling.
IL-5	Th2 cells ILC2s	Primary cytokine for eosinophil development, activation, and migration. Proliferation of B cells.
IL-13	Th2 cells ILC2s	B cell class switching to IgE. Goblet cell hyperplasia and mucus production. Upregulates iNOS. Eotaxin synthesis by airway epithelium. Airway remodeling: promotes collagen synthesis from fibroblasts and macrophages. Promotes tissue inflammation.
Alarmins		
IL-25	Epithelial cells Th2 cells	Induces Th2 inflammation. Promotes eosinophil survival. Activates ILC2s.
IL-33	Epithelial cells	Enhances Th2 activation. Activates ILC2s and promotes ILC2 egress from bone marrow. Promotes airway remodeling.
TSLP	Epithelial cells	Primes DCs to induce Th2 differentiation. Activates ILC2s.

The primary cell sources of each cytokine are listed. Cytokines may be derived from other cell sources not listed in this table.

cells, can express TSLP (Roan et al., 2019). High levels of TSLP expression have been linked to multiple atopic diseases, including asthma (Ziegler, 2010). TSLP can directly promote Th2 differentiation of naïve T cells, and increased production of IL-5 and IL-13 by Th2 cells (Rochman et al., 2018). In addition, TSLP signaling during the primary CD4 T cell response can program toward greater effector cytokine production in Th2 memory cells (Rochman et al., 2018). Basophils and ILCs have also emerged as important effector cell populations downstream of TSLP (Roan et al., 2019). The TSLP/ILC axis appears to play an important role in steroid-resistant asthma, at least in part by controlling the phosphorylation of STAT5 (Kabata et al., 2013). Evidence suggests that blockade of TSLP or inhibition of STAT5 restores corticosteroid sensitivity. Thus, the TSLP-STAT5 pathway has been a point of investigation in the treatment of corticosteroid-resistant asthma (Kabata et al., 2013).

IL-33 promotes collagen synthesis and airway remodeling, and has been associated with increased reticular basement membrane thickness on endobronchial biopsies from children with steroid resistant asthma (Sagiani et al., 2013). When evaluating children with steroid resistant asthma via endobronchial biopsy, Sagiani et al. documented similar numbers of IL-13+ cells in the airway of patients and controls. However, IL-33+ cell numbers were significantly increased in the submucosa of asthmatic patients (Sagiani et al., 2013). Following steroid treatment, IL-33 levels were maintained, while IL-13 levels were reduced, in the airway of house dust mite-exposed mice (Sagiani et al., 2013). Even in the absence of CD4+ T cells, asthma may develop through innate mechanisms involving IL-33, natural helper cells, and natural killer T (NKT) cells (Kim et al., 2012; Barlow et al., 2012).

IL-25 is a member of the IL-17 cytokine family (Hong et al., 2020). Unlike other members of the IL-17 family which lead to Th1 inflammation, IL-25 induces Th2 inflammation and production of IL-4, IL-5, and IL-13 (Hong et al., 2020). IL-25 uses both innate (ILC2) and adaptive (Th2 cell) pathways (Hong et al., 2020). A wide variety of cells can secrete IL-25, including but not limited to airway epithelial cells, Th2 cells, eosinophils, basophils, and alveolar macrophages (Hong et al., 2020). IL-25 leads to the polarization of memory Th2 cells (Hong et al., 2020). IL-25-treated eosinophils demonstrate enhanced viability, suggesting that IL-25 delays apoptosis and sustains eosinophilia in the airway (Cheung et al., 2006). ICAM-1 expression on the cell surface of eosinophils, which is essential for recruitment and transendothelial migration of the cells, is also increased by IL-25 (Cheung et al., 2006).

Allergic eosinophilic asthma

Asthma has traditionally been viewed as a purely atopic/eosinophilic disorder mediated by Th2 cells with overproduction of associated cytokines IL-4, IL-5, and IL-13. IgE-mediated sensitization to airborne allergens is the main driver of the pathology in this phenotype. Sensitized Th2 cells produce IL-4, IL-5 and IL-13 upon contact with their antigen (allergen) presented by antigen presenting cells such as DCs (Jonckheere et al., 2019). Allergens directly trigger alarmin release from epithelial cells in a non-IgE mediated manner. Therefore, ILC2 cells also play an active role in producing type 2 cytokines in patients with allergic eosinophilic asthma (Jonckheere et al., 2019). ILC2s are rapidly increased and activated in the asthmatic airway in response to allergen and are potent producers of IL-5 and IL-13 in response to allergen challenge (Chen et al., 2017; Kuruvilla et al., 2019).

There is significant overlap in the roles of IL-4 and IL-13, due in part to the fact that the IL-4 receptor alpha unit is shared by both the IL-4 receptor and the IL-13 receptor. Among the type 2 cytokines, IL-4 is the primary driver of B cell class switching to IgE (Jonckheere et al., 2019). IL-4 is also a major inducer of differentiation of Th2 cells, leading to production of their associated cytokines (Moran and Pavord, 2020). Both IL-4 and IL-13 lead to activation of eotaxin synthesis by airway epithelium, leading to increased airway eosinophilia (Moran and Pavord, 2020). Both cytokines play a role in airway remodeling. IL-4 is known to upregulate fibronectin and collagen synthesis (Moran and Pavord, 2020). IL-13 induces smooth muscle cell proliferation, collagen deposition, and fibroblast transformation and is a major player in remodeling (Moran and Pavord, 2020). IL-13 also enhances goblet cell hyperplasia and mucus production (Moran and Pavord, 2020). IL-13 upregulates inducible nitric oxide synthase (iNOS) in airway epithelial cells (Chibana et al., 2008). This allows for increased production of exhaled nitric oxide (FeNO) which can be used as a biomarker in asthma management (Chibana et al., 2008). The role of IL-13 in lung remodeling is also discussed in the Structural Changes section.

IL-5 is a major factor in eosinophilic inflammation. It is responsible for eosinophil development, activation, and migration. IL-5 promotes eosinophil differentiation from hematopoietic progenitor cells in both the bone marrow and the bronchial mucosa (Pelaia et al., 2019).

Neutrophilic asthma

Clinically, neutrophilic asthma is associated with increased clinical severity of asthma, airway remodeling with irreversible obstruction, and poor response to glucocorticoid therapy (Chang et al., 2017). Neutrophilic asthma is dominated by a mixed Th1 and Th17 cytokine milieu (Lambrecht and Hammad, 2015; Chang et al., 2017). IL-17 contributes to remodeling by promoting fibroblast proliferation, counteracting the anti-inflammatory role of Treg cells, and causing direct contraction of bronchial smooth muscle (Lambrecht and Hammad, 2015). Th17 cells and IL-17 + ILCs (commonly termed ILC3s) demonstrate steroid resistance (Nagakumar et al., 2019). In fact, in vitro assays demonstrate that IL-17 + ILCs are induced by budesonide (Nagakumar et al., 2019). ILC2s and ILC3s have also been implicated in obesity-induced asthma, a specific neutrophilic subtype which is known to be associated with corticosteroid resistance (Everaere et al., 2016).

Structural changes

Airway tissue remodeling resulting from chronic inflammation is characteristic of asthma, including a thickened subepithelial layer often accompanied by fibrosis, smooth muscle hyperplasia, enhanced vascularity, and increased collagen deposition (Banno et al., 2020). Hence, severe asthmatics show abnormal computed tomography (CT) findings including thicker airway walls with narrower lumen and potentially focal bronchial stenoses (Kim et al., 2018). Subepithelial fibrosis is predominantly mediated by submucosal fibroblasts that differentiate into myofibroblasts under the influence of TGF- β (Hough et al., 2020). Increased vascularity also contributes to thickening of the airway walls. New blood vessels generated during asthma-induced angiogenesis may have increased permeability, leading to airway edema (Banno et al., 2020). Tissue remodeling is an inherent part of asthma pathophysiology, as it is directly mediated by the same cytokines that drive the immune response. IL-4, IL-5, and IL-13 have all been shown to drive goblet cell hyperplasia and airway collagen deposition in mice (Banno et al., 2020). IL-13 is perhaps one of the biggest players in tissue remodeling. Mouse models have demonstrated that IL-9's effects on airway remodeling is dependent on IL-13. IL-9 is produced by a T-helper subset termed Th9 lymphocytes (Seumois et al., 2020) as well as ILC2s (Verma et al., 2018). In murine asthma models, IL-9 promotes bronchial hyperresponsiveness, mucus cell metaplasia, is a potent stimulator of mast cells, and plays a significant role in airway remodeling (Lambrecht et al., 2019; Kearley et al., 2011). In addition, IL-13 promotes TGF- β , the powerful mediator of airway fibrosis (Banno et al., 2020). A significant role of TGF- β is that it promotes metalloproteinase-9 (MMP-9) production (Hough et al., 2020). MMP-9, also known as gelatinase-B, alters the extracellular matrix and drives structural changes in the asthmatic airway (Hough et al., 2020). STAT-6, which is downstream of the signaling cascade induced by IL-13, mediates mucus production and promotes subepithelial fibrosis (Banno et al., 2020). Production of periostin in bronchial epithelial cells is upregulated by IL-13 and IL-4 (Izuhara et al., 2016). Periostin is a matricellular protein with participates in modulating the expression of several products such as collagen, chemokines, and TGF- β and plays a role in the extracellular matrix and airway remodeling (Izuhara et al., 2016). While Th2 driven chronic inflammation is a major driver, it does not account for the entire process of airway remodeling in asthma. Characteristics of airway remodeling have been documented in individuals with both allergic and non-allergic asthma (Banno et al., 2020).

Conclusion

Asthma is a complex disease driven by the interaction of genetic factors and early life environmental risk factors. The main features of asthma include airway inflammation leading to bronchial hyperresponsiveness and variable airflow limitation. The prevalence of asthma has increased over time, and disproportionately impacts ethnic minorities and people of low socioeconomic status. There are numerous early life influences associated with the development of asthma, including microbial exposure, acute respiratory infections, and allergen exposure. Multiple asthma phenotypes and endotypes have been identified based on different pathologic

mechanisms, including non-allergic eosinophilic asthma, allergic eosinophilic asthma, and neutrophilic asthma. Airway inflammation is driven by various cytokines, chemokines, and inflammatory cells, and associated with structural changes of the epithelium, basement membrane, and smooth muscle. Knowledge of asthma pathogenesis contributes to the understanding of the diagnostic and therapeutic modalities discussed in the next chapter.

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Asthma Diagnostics, Testing and Treatment

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Introduction

Asthma is a common, chronic respiratory disease that affects both children and adults worldwide. Initially thought to be one condition, and treated as such, molecular advances have proven it to be a heterogeneous disease with different clinical manifestations and underlying mechanisms. Current treatment options are much more diverse and targeted than previously. This chapter focuses on the diagnosis of asthma, classification into different phenotypes and endotypes, and discussion of the vast array of treatment considerations. These range from traditional medications to more novel, targeted, biologic therapies as well as non-pharmacologic interventions such as environmental modification.

Diagnosis

Diagnosis of asthma includes a history of characteristic symptoms and demonstration of variable expiratory airflow limitation. Ideally the diagnosis should be made at the initial presentation of symptoms, prior to treatment initiation, which could alter the clinical picture.

History

Vital components of the asthma history include symptoms, their pattern, and precipitating/exacerbating factors. Symptoms suggestive of asthma include wheezing, chest tightness, shortness of breath, or cough. These symptoms usually occur variably over time, sometimes with a seasonal component, and can fluctuate in intensity. Many patients experience nocturnal cough or awakening. Specific triggers include environmental allergens (pollen, dust mites, mold, cockroach, animal dander and secretions), irritants such as cigarette smoke, as well as cold air, exercise, laughter, and viral infections. Prolonged cough after viral infections can also be a clue to the diagnosis of asthma (Reddel et al., 2019).

Spirometry

Spirometry is a physiological test that measures the maximal volume of air that an individual can inspire and expire with maximal effort (Graham et al., 2019). This maneuver measures expiratory airflow limitation or obstruction and is a vital part of the diagnosis of asthma. If the history is suggestive of asthma, patients should undergo spirometry with bronchodilator challenge to assess for reversibility. A reduced FEV₁ with low FEV₁/FVC ratio is indicative of airflow obstruction and bronchodilator reversibility confirms the diagnosis of asthma.

Bronchodilator reversibility refers to an increase in FEV₁ % predicted of more than 12% and >200 mL in adults (in children, an increase from baseline of >12% predicted) 10–15 min after administration of a short-acting β_2 agonist (SABA). Importantly, reversibility is more likely to be present when no SABA has been taken for ≥ 4 h and no long-acting beta agonist taken for ≥ 15 h.

Bronchoprovocation testing

In patients with a history suggestive of asthma but with normal spirometry or absent bronchodilator reversibility, several bronchoprovocation tests can be used to document airway hyperresponsiveness.

Direct challenge tests are highly sensitive and thus are most useful in excluding a diagnosis of asthma. Methacholine is an example of a direct airway bronchoconstrictor and the methacholine challenge test (MCT) is the most widely used direct challenge. During the MCT, a decrease in FEV₁ by 20% with a provocative concentration (PC₂₀) of methacholine <4 mg/dL confirms airway hyperresponsiveness (Coates et al., 2017).

Indirect bronchial stimuli include exercise, hypertonic saline, mannitol, and eucapnic hyperventilation. The most commonly used among these is the exercise test. During an exercise challenge test, a patient must reach a HR >180 for the last 3 min of an 8 min test. A decrease in FEV₁ of >10% predicted value and >200 mL in adults, or >12% predicted in children constitutes a positive exercise test (Reddel et al., 2019).

Phenotypes/endotypes

Historically asthma has been considered to be one disease and a common treatment approach used for nearly all patients. However, over time, the entity of asthma has been increasingly recognized as a heterogeneous condition with a variety of clinical presentations. The term phenotype has been used to describe clinically observable features of a disease without respect to underlying pathophysiology. Such phenotypes include atopic/allergic asthma, late-onset asthma, aspirin-exacerbated respiratory disease (AERD), non-atopic asthma, obesity-associated asthma and asthma of the elderly (Kuruvilla et al., 2019).

More recently, molecular advances have enabled physicians to pair a molecular basis, or an endotype, with a corresponding phenotype. Asthma endotypes describe distinct underlying pathophysiologic mechanisms at a cellular and molecular level thereby facilitating precision medicine. With the advent of biologic medications, endotyping is becoming increasingly important in order to provide the most targeted treatment for patients.

Phenotypes

Phenotypes have traditionally been divided into atopic and non-atopic asthma though other clinical phenotypes have been described based on disease severity, triggers, age at onset, inflammatory patterns and airflow obstruction.

A more modern approach to phenotyping involves the use of cluster analyses in an effort to describe phenotypes in an unbiased manner. Cluster analyses group together asthmatics using algorithms that integrate multiple components. Examples of these cluster analyses include the Severe Asthma Research Program (SARP study) (Moore et al., 2010), sponsored by the NHLBI, Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes (U-BIOPRED), a European consortium of asthmatics, and Airways Disease Endotyping for Personalized Therapeutics (ADEPT). The consensus among these three consortiums is that two main clusters have emerged—T2 high and T2 low (or non-T2). Of note, the terms Th2 high and Th2 low are also referenced in the asthma literature; however, as innate lymphoid cells have been recognized as part of the inflammatory cascade in asthma, the broader terms T2 high and T2 low have emerged.

Another important finding from these cluster analyses is that eosinophils were present in patients both with and without atopic disease suggesting that patients can have eosinophilic, or T2 inflammation, in the absence of atopy. Some T2 high patients are steroid responsive and others are steroid resistant, further reinforcing the need for precision medicine with biologics.

Endotypes

In 2008, the term endotype developed as a means of describing a molecular basis, or mechanism, underlying a specific phenotype. As targeted therapeutics have become increasingly available, establishment of biomarkers is even more important to characterize a patient's asthma as specifically as possible (Table 1).

Table 1 Endotypes and phenotypes of asthma in adults and children.

Endotype	Phenotype	Clinical Characteristics	Molecular Mechanism	Biomarkers	Natural History
T2 High	Atopic (Adult)	Well-defined, early onset, steroid sensitive	Allergic sensitization	Blood/sputum eosinophils, allergen specific IgE, FeNO, total IgE	Identifiable and treatable, preserved lung function
	Atopic (Pediatric)	Uncontrolled symptoms, atopy, frequent exacerbations, steroid sensitive	Allergic sensitization	Blood eosinophils, allergen specific IgE, FeNO, total IgE	Early-onset severe asthma, impaired lung function
	Late-onset	+/- concomitant CRSwNP, steroid refractory	<i>S. aureus</i> enterotoxin	Blood/sputum eosinophil count, FeNO	Severe from onset, frequent exacerbations
	AERD	Adult-onset	Dysregulated arachidonic acid metabolism	Blood/sputum eosinophil count, urinary LTE4	Severe from onset, frequent exacerbations
Non-T2	Non-atopic (Adult)	Paucigranulocytic or neutrophilic	NLRP3/IL-1B, altered miRNA expression, TH17	Induced sputum neutrophils, MMP-9 in BAL	Variable course and lung function
	Non-atopic (Pediatric)	Neutrophilic	IL-17	Serum IL-17, sputum neutrophils, TWEAK ^a	Better lung function than atopic asthma
	Smokers	Older adults	Oxidative stress, mixed Th2 high/Th2 low	Induced sputum neutrophil count	Frequent exacerbations lower lung function
	Obesity-related	Female sex	Oxidative stress, neutrophils, ↑ innate activation	Serum IL-6	Severe symptoms, preserved lung function
	Elderly	>50 to >65 years at onset	Immunosenescence, Th1, Th17 inflammation	Induced sputum neutrophils	Steroid-resistant

Compiled from Kuruvilla et al., 2019, Licari et al., 2018, and Zhu et al., 2018.

^aHuman Tumor Necrosis Factor –like weak inducer of apoptosis: novel biomarker under investigation.

Biomarkers

Historically, bronchoscopy with bronchoalveolar lavage and bronchial brushings have been considered the gold standard for diagnosis of airway inflammation and remodeling, but these procedures are too invasive for routine use. Hence alternative, non-invasive biomarkers are attractive tools.

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of an intervention. In the field of asthma, biomarkers are useful in categorizing patients as either T2 high or T2 low which ultimately establishes candidacy for specific therapies. Biomarkers for T2 high asthma include sputum eosinophils, peripheral eosinophils, total serum IgE, periostin, allergen specific IgE, FeNo, and to a lesser extent, dipeptidyl peptidase 4 (DPP-4), and LTE4. Unfortunately, biomarkers for T2 low asthma are lacking, and this endotype is characterized primarily by either neutrophilic or paucigranulocytic sputum samples.

Peripheral eosinophils

Peripheral eosinophil count is a non-invasive and easy to obtain biomarker for asthma. In both pediatric and adult cohorts, absolute eosinophil counts (AECs) have correlated with the risk of acute exacerbations (Mogensen et al., 2018). In the INFANT study of children ages 12–59 months, the presence of aeroallergen sensitization and an absolute eosinophil count >300 identified children at high risk for exacerbation and predicted good response to daily ICS (Fitzpatrick et al., 2016). In adults as well as children who require a step-up in management to include biologic therapy, peripheral eosinophil count can help guide targeted therapy. One limitation of this biomarker is the inconsistent correlation between sputum and peripheral eosinophilia over time.

Sputum eosinophils

Sputum eosinophils are typically considered the gold standard for diagnosing T2 high asthma in the office and levels >2% are considered positive (Miranda et al., 2004). A recent study found an association between sputum eosinophil counts and asthma control. The authors noted that in patients with uncontrolled asthma who are on ICS therapy, residual sputum eosinophilia suggests opportunity for improvement in asthma control with ICS (Demarche et al., 2017). An unfortunate limitation of sputum eosinophilia is that quality sputum samples are difficult to obtain in regular practice, particularly in children. When obtained in children, however, they correlate directly with airway hyperreactivity and inversely with FEV₁ (Licari et al., 2018).

FeNO

FeNO, or fractional exhaled nitric oxide, is a safe and non-invasive measurement of eosinophilic airway inflammation. This test is a simple, quantitative means of assessing airway inflammation directly, as compared to spirometry which measures inflammation indirectly. Previously, this biomarker was used mostly in research settings, but in the era of biologics, measurement in clinical practice is becoming increasingly important. Values <25 ppb in adults and <20 ppb in children suggest that eosinophilic inflammation and steroid responsiveness are less likely; values >50 ppb in adults, and >35 ppb in children, suggest that eosinophilic inflammation and steroid responsiveness are more likely (Dweik et al., 2011).

A 2018 review of NHANES data from 1419 subjects, ages 6–79 years from 2007 to 2012 found that simultaneous elevation in both FeNO and blood eosinophils correlated with reduced lung function (FEV_1 <80%) and wheezing symptoms in asthmatics (Mogensen et al., 2018).

Total and allergen specific IgE

Total IgE level and allergen specific IgE levels are biomarkers of atopic status. As allergic asthma is the most common phenotype, assessment of allergic sensitization is a critical component in the evaluation of asthmatic patients. In the SARP study group with early-onset asthma 80% of patients were atopic, and among those with late onset eosinophilic asthma, 50% of patients were atopic (Moore et al., 2010). In infants with viral-induced wheeze, elevated total IgE levels predict later asthma development (Lemanske Jr, 2002) and high levels of allergen specific IgE correlate with asthma severity in children (Szeffler et al., 2012).

Periostin

Periostin is an extracellular matrix protein secreted by airway epithelial cells and lung fibroblasts after induction by IL-4 and IL-13 (Takayama et al., 2006). It localizes to the subepithelial region of the airway and plays a role in airway remodeling, subepithelial fibrosis, eosinophil recruitment, and regulation of mucous production from goblet cells. In adults, it may be a useful biomarker due to its upregulation by IL-4 and IL-13. Its role in the pediatric population is unclear though, as children have inherently higher levels compared to adults, presumably due to rapid bone growth. It may also be elevated in other atopic conditions such as atopic dermatitis and allergic rhinitis which is another potential limitation (Licari et al., 2018).

Urinary LTE4

LTE4 is a byproduct of the cystinyl leukotriene pathway that can be detected in urine samples and serves as biomarker of upregulation of this pathway. It has been observed to be elevated in allergen-induced asthma and in AERD and is helpful in diagnosing aspirin sensitive respiratory disease (Divekar et al., 2016).

Dipeptidyl peptidase 4 (DPP-4)

DPP-4 is a glycoprotein present in cell membranes and as a soluble blood protein. A recent study noted that DPP-4 expression is enhanced in bronchial epithelial cells of asthmatics, particularly in steroid naïve patients. Expression was also upregulated in the presence of IL-13 (Shiobara et al., 2016). Further studies of DPP-4 suggest that it may be another helpful biomarker for asthma (Emson et al., 2018).

After diagnosis of asthma with history and spirometry, and subsequent assessment of phenotype and endotype using the appropriate biomarkers, clinicians can develop a targeted treatment plan.

Treatment and management

Optimal treatment or management of asthma is directed towards reducing impairment from disease symptoms, abating the risks from use of asthma medications and minimizing long term disease sequelae. This requires multifaceted targeting of asthma triggers, avoiding exacerbations, and preventing deterioration. When targeting asthma triggers, comorbid conditions exacerbating asthma as well as environmental factors must be considered. Concurrent active management of common comorbid conditions such as gastroesophageal reflux disease, obstructive sleep apnea, obesity, and vocal cord dysfunction should be done (Boulet, 2014). Avoidance or mitigation of environmental irritants such as cigarette smoke, strong odors, and air pollution. Particulate matter (PM) in different sizes from coarse (2.5–10 μm), fine (0.1–2.5 μm) and ultrafine (<0.1 μm) can deposit in the bronchi, bronchioles and cellular structures directly, respectively (Wu et al., 2018).

Avoidance of allergic triggers for the patient such as pet dander, mold, or other aeroallergens, is also an important aspect of targeting triggers. Specific subcutaneous and sublingual aeroallergen immunotherapy (AIT) is a disease modifying process that amends the immunological milieu towards tolerance to aeroallergen, inducing allergen specific regulatory T cells, regulatory B cells, and innate lymphoid cells (ILCs), which lead to increased IL-10, and TGF- β . Clinically, this has been shown to prevent the development of asthma and improve control. Several studies have shown the disease modifying effects of AIT on allergic asthma, including several studies which show an improvement in symptom scores and medication use (Alvaro-Lozano et al., 2020).

Table 2 Stepwise approach to asthma controller medication management categorized by age (bold-preferred).

Age	Mild intermittent or Step 1 (and rescue)	Mild Persistent ^a or Step 2/3	Moderate Persistent ^a or Step 3/4 ^d	Severe Persistent ^a or Step 4/5 ^d	Alternative Add on ^b
Children 0–4 years	SABA as needed	Low dose ICS ^c	Low dose ICS + LTRA ^c	Medium dose ICS ^c - Medium dose ICS + LABA ^c or - Medium dose ICS + LTRA ^c	Cromolyn LTRA
Children 5–11 years	SABA as needed	Low dose ICS ^c	Medium dose ICS ^c - Medium dose ICS + LTRA ^c or - Medium dose ICS + LABA ^c	High dose ICS ^c - High dose ICS + LABA ^c or - High dose ICS + LTRA ^c	Cromolyn LTRA Nedocromil Theophylline Biologic
Children ≥ 12 years, and Adults	ICS + LABA as needed - SABA as needed	Low dose ICS ^e	Low dose ICS + LABA ^e - Medium dose – ICS ^e or - Medium dose ICS + LTRA ^e or - Medium dose ICS + LABA ^e	Medium/High dose ICS + LABA ^e - High dose ICS ^e or - High dose ICS + LTRA ^e	Cromolyn LTRA Nedocromil Theophylline Tiotropium Biologic

SABA, short acting beta agonist; LABA, long acting beta agonist; ICS, Inhaled Corticosteroid; LTRA, Leukotriene Receptor Antagonist.

^aDaily medications.

^bThese medications are used as add on therapy to a maintenance regimen treatment for asthma; If needed, oral corticosteroids can be used but can be associated with significant side effects if used long term.

^cRescue: SABA.

^dReferral to asthma specialist

^eRescue: ICS + LABA.

Optimal management of asthma also requires routine monitoring of asthma symptoms, and tailoring the short- and long-term treatment regimen in response. The process starts with a baseline assessment of asthma severity, history of control/exacerbations, current control, triggers, exposures to triggers, and personal goals for the particular patient. Then, after implementation of a treatment plan, re-assessment must continue, followed by adjustment of treatment plan for achievement of personal goals. This monitoring and modification process should continue long term. Traditionally, the medications used to treat asthma are separated into immediate response medications (*rescue*), long-term or preventative medications (*controller*) and *add on therapies*. Add-on treatments are often used for severe asthma or if controller medications have not fully achieved the patient's personal goals.

The management of asthma with medications is categorized based on the age of the patient, with categories of infant and children age 0–4 years, children 5–12, and adolescents and adults. A stepwise approach in escalating the number and type of medications as needed after assessing response, is recommended. Table 2 provides a brief overview of these recommendations, and the individual medications are discussed below. Although many guidelines have gone away from rigid classifications of asthma as mild, moderate and severe, these are used as loose methods of classification at baseline, followed by reassessment after initial implementation of a treatment plan.

Immediate response medications (*Rescue*)

β_2 Agonists

β_2 Agonists bind to the β_2 adrenergic receptor on airway smooth muscle, leading to activation of adenylate cyclase and a signaling cascade that results in relaxation of airway smooth muscles. Early β_2 agonists included albuterol, terbutaline, and fenoterol, each with a short duration of action (Cazzola et al., 2013). Short acting β_2 agonists (SABA), or bronchodilators, were the immediate response medications of choice for acute asthma symptoms such as shortness of breath or wheezing, and are typically used to relieve bronchospasm within minutes of use, peaking at 1 h after use, with a duration of action of 4–6 h (FDA, 2008). Numerous initial studies have established their efficacy and routine clinical practice support their use (Horak et al., 2016). Many different formulations exist. However, recent Global Initiative for Asthma (GINA) guidelines prefer the use of long acting bronchodilators (LABA) in combination with an inhaled corticosteroid (ICS) as the immediate response medication of choice for the relief of acute bronchospasm in adolescents and adults with asthma, and the use of SABA was considered the alternative option. For children under the age of 11 years, SABA are still the first line option for relief of acute symptoms (GINA Guidelines, 2020). Minimizing the use of SABA or rescue ICS + LABA is an important goal of asthma management.

Anticholinergics

Ipratropium bromide, a short acting muscarinic antagonists (SAMA), was introduced in the market in the 1970s, for use in the relief of obstructive airway disease (Chervinsky, 1977). Since its introduction, several studies have examined the efficacy of SAMA such as ipratropium bromide in comparison to SABA such as salbutamol. Most of the studies found that SABA had greater bronchodilation

than SAMA with noted difference in forced expiratory volume in the first second (FEV1) up to 6 h post administration to patients with asthma (Novelli et al., 2012). Thus, SAMA are usually not used alone for the treatment of acute symptoms, but have shown efficacy when used in combination with SABA in the acute setting (Durrani and Busse, 2014).

Magnesium sulfate

Magnesium Sulfate (MgSO_4) which is a tocolytic/antiarrhythmic, has been shown to have bronchodilator and mild anti-inflammatory effects in the severe exacerbations. It is for parenteral use and when given in the acute setting for unresponsive asthma, was shown to decrease rates of hospital admission in several studies. However, this remains a controversial area (Camargo Jr and Rowe, 2014).

Systemic corticosteroids

Many evidence based guidelines support the use of oral or intravenous systemic corticosteroids in the acute treatment of uncontrolled asthma. Systemic steroids have a potent anti-inflammatory effect and are a principle treatment of choice for a severe exacerbation that is not responsive to intensive treatment with short acting bronchodilators. The onset of action is thought to occur within an hour to 6 h after administration. The usual dosing is weight dependent in children and 40–60 mg per day in adults; higher doses are sometimes chosen for severe exacerbations (Castillo et al., 2017). However, long term use has a high incidence of adverse effects, including endocrine, immunologic, cardiovascular, gastrointestinal, musculoskeletal, dermatologic and hematologic effects.

Anti-inflammatory medications (Maintenance)

Asthma controller medications are used to decrease airway inflammation, obstruction, prevent exacerbations, and long term to minimize the decline in lung function. Long term goals should be to achieve normal activity including achievement of patient specific personal goals, while minimizing side effects from medications. This requires continual assessment of asthma control, medication effects, patient communication, and personalized management of controller medications.

Inhaled corticosteroids (ICS)

Inhaled corticosteroids are the first line treatment of persistent asthma, regardless of age or severity of asthma (Barnes, 1998). They are the most effective long-term treatment option for asthma and many studies have shown their safety and efficacy in asthma (Ye et al., 2017). Corticosteroids bind to the glucocorticoid receptor complex in the cytoplasm, which attaches to the glucocorticoid response element that promotes the production of anti-inflammatory proteins and inhibits pro-inflammatory proteins. Inhaled corticosteroids have been shown to not only decrease airway inflammation, but also to decrease bronchial hyper responsiveness by several fold, decrease asthma exacerbation frequency and severity, increase lung function, improve quality of life and be beneficial in decreasing hospitalizations as well as asthma mortality (Fanta, 2009).

This class of medications also block the late phase allergen induced reactions but do not change the underlying severity of the disease or its progression. The use of these medications also should be weighed against the small risks of local or systemic adverse effects such as oral candidiasis, or growth effects in children. A step-up approach is recommended to limit exposure to the lowest amounts to achieve therapeutic control of symptoms. This can clinically require consideration of many factors that include the potency of a specific ICS, patient age, type of delivery device used, insurance coverage/cost, prior response to the medication, systemic absorption rate, and taste, to name a few (Jackson et al., 2014). There are many ICS products on the market and they all target the inflammatory cascade. Overall, they have much fewer adverse effects in comparison to oral corticosteroids, although due to expected long term use of these medications, risk, benefits and alternatives must always be considered in making treatment decisions. However, given the long standing favorable safety profile, clear evidence of efficacy, manageable costs, and wide availability, ICS class of medications are the mainstay of the long term maintenance management of asthma in asthma guidelines.

Long acting bronchodilators

Similar to SABA, long acting beta agonists (LABA) improve airway airflow obstruction by bronchodilatory effects but last for at least 12 h in comparison. They only recommended for use in combination with an ICS for long term preventative use in children with asthma but may also be used for both maintenance control of asthma when ICS alone has failed, or for rescue at times of uncontrolled symptoms, in adolescents and adults (GINA Guidelines, 2020). The most common medications of this class that are on the market are Salmeterol and Formoterol.

Long acting anticholinergics

Long acting anticholinergic medications (LAMA) such as tiotropium bromide were approved for the treatment of chronic obstructive pulmonary disease (COPD) in 2004. Since that time, this class of medication shown to improve symptoms and lung function in asthma when added to ICS for patients with poorly controlled asthma (Peters et al., 2010). Tiotropium has also been approved for add on therapy of poorly controlled severe asthma in children (Radovanovic et al., 2017).

Xanthine derivatives

Methylxanthines in general and theophylline in particular, were used much more commonly in the past due to their bronchodilator activity. However, their routine use has become much more limited due to the risks for systemic toxicity and a narrow therapeutic window of efficacy. Monitoring of serum theophylline levels would be required. Its use is not recommended in children but may be considered for add on therapy in adults and adolescents with poor response to bronchodilators and other first line asthma medications (GINA Guidelines, 2020).

Immunological modulation using biologic therapies

Asthma phenotypes and endotype have helped characterize observable, and laboratory features of the disease for a more targeted approach to management and treatment of the disease. As such, the presence or absence of T_H2 type inflammation including T_H2 cytokines, IL-4, 5 and 13 as well as allergen specific IgE, or peripheral eosinophilia is known to identify subsets of asthma patients who have higher response rates to biologic therapies. Treatment with the commercially available monoclonal antibodies is typically reserved for patients with severe asthma and with a phenotype or biomarker profile suggestive of higher likelihood of response. The risks, benefits and alternatives of treatment with biologics must always be considered.

Köhler and Milstein first developed the method for immortalization of B cells for the purpose of antibody production in 1970. Primary animal lymphocytes were fused onto immortalized lymphoblastoid or myeloma cells, and the fused cells were isolated using hybridoma technology. However, initial efforts were limited by human anti-mouse antibodies (HAMA), and soon led to the “humanization” of the monoclonal antibodies by substituting a human constant region. However, the large-scale production of monoclonal antibodies (mAb) for human therapy soon evolved to include recombinant DNA technology expressed in a mammalian host cell such as in cultured Chinese Hamster Ovary (CHO) cells or murine myeloma non-secreting cells (NSO) (Kunert and Reinhart, 2016).

Currently, there are five FDA approved monoclonal antibodies for the treatment of asthma. These are Omalizumab, Mepolizumab, Reslizumab, Dupilumab, and Benralizumab. These products are typically used in older children and adults, with persistent asthma, poorly controlled on inhaled corticosteroids, and may be considered in situations where the use of conventional asthma treatments is contraindicated. Table 3 lists the details of each of these treatments, including the indications for use, mechanism of action, dates of approval, source, and age of approved use. Fig. 1 shows the target sites for each of the currently approved mAb.

Each of these products targets the inflammatory cascade at different points, and the success of treatment is dependent on choosing the right product for the patient. Recent studies have compared the response rate of biologics in correlation with exacerbation rates prior to commencement of the biologic and on baseline serum eosinophilia. The clinical trial results have been most favorable in improving rates of asthma exacerbation for dupilumab, mepolizumab, and reslizumab. Clinical trials for benralizumab and dupilumab have shown a steroid sparing effect, and an improvement in lung function. (Doroudchi et al., 2020) Therefore, depending on the goal for a patient, their biomarker and phenotype, the appropriate biologic treatment can be initiated.

Table 3 Currently available biologic treatments for asthma.

Medication	Brand Name	Mechanism	Source	Dosage	Age approved for	Required	Comorbid conditions treated	Year of US approval
Omalizumab	Xolair	Anti IgE receptor antibody	Humanized (mouse), CHO	Subcutaneous injection every 2–4 weeks	≥ 6 years	IgE in range, +IgE (skin or serum) to perennial aeroallergen, failed ICS	Chronic Urticaria	2003
Mepolizumab	Nucala	Anti-IL-5 antibody	Humanized (mouse), CHO	Subcutaneous injection, every 4 weeks	≥ 6 years	Eosinophilic Asthma	Eosinophilic granulomatosis with polyangiitis (EGPA)	2015
Reslizumab	Cinqair	Anti-IL-5 antibody	Humanized (rat), NSO	Intravenous infusion, every 4 weeks	≥ 18 years	Severe, eosinophilic asthma		2016
Dupilumab	Dupixent	Anti-IL-4 receptor alpha chain antibody	Human IgG4 antibody, CHO	Subcutaneous every 2–4 weeks	≥ 12 years	Moderate to severe eosinophilic asthma, or oral corticosteroid dependent asthma	Atopic Dermatitis, Chronic Rhinosinusitis with nasal polyposis	2017
Benralizumab	Fasenra	Anti-IL-5 receptor antibody	Humanized (mouse), CHO	Subcutaneous injection every 4 weeks for first 3 doses, then every 8 weeks	≥ 12 years	Severe, eosinophilic asthma		2017

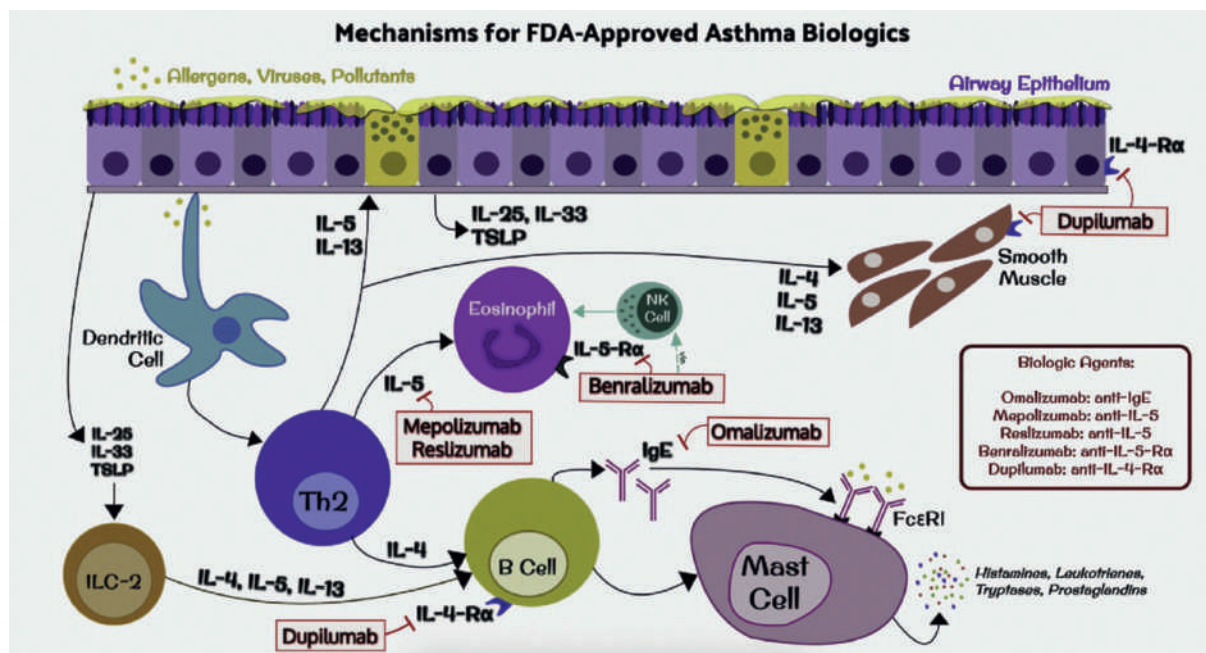


Fig. 1 Mechanisms for currently approved asthma biologics. From Doroudchi A Pathria M and Modena BD (2020) Asthma biologics: Comparing trial designs, patient cohorts and study results. *Annals of Allergy Asthma and Immunology* 124: 44–56.

Conclusion

To summarize, asthma is a multifactorial chronic disease with a wide spectrum of severity, which requires a personalized approach to diagnosis, classification, and management. Clinicians must not only accurately diagnose asthma from a large differential of asthma mimickers, but they must also consider the phenotype/endotype of asthma in each patient, and follow a stepwise approach to management. Concurrently, it is important to address not only the pharmacological treatment, but also the social and environmental triggers of asthma, keeping in mind, the individual patient goals that are specific to patient age and preference. In addition, the side effect profile from pharmacotherapy, cost of treatment, and response to therapy should routinely be reassessed to tailor or adjust the management. New treatment options continue to be developed and the clinician's toolbox must be periodically updated to adapt to evidence based guidelines.

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Food Allergies

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Glossary

Allergen A specific protein capable on initiating the formation of allergen-specific IgE antibodies in susceptible individuals.

Anaphylaxis An allergic response involving multiple organ systems that is often severe.

Basophil Blood cell that contains mediators of allergic reactions.

Celiac disease Malabsorption syndrome apparently caused by cell-mediated immune response to proteins from wheat, rye, barley, and related grains.

Food allergy Illness occurring in some individuals upon ingestion of certain foods that elicit an abnormal immune response in that individual to specific proteins (allergens) from certain foods.

IgE A class of immunoglobulins (antibodies) involved in allergic reactions.

Mast cell Type of cell present in many tissues that contain mediators of allergic reaction.

Introduction

“Quod ali cibus est allis faut acre venenum.” One man’s food is another man’s poison. Although not the original intent, this very old quote from Lucretius, a Roman philosopher, defines food allergy. Some individuals in the population experience adverse reactions upon ingestion of foods that are safe and nutritious for the vast majority. These individualistic adverse reactions to foods occur through a variety of mechanisms including food allergies, metabolic food disorders also known as food intolerances, and food sensitivities. The most serious of these illnesses are food allergies. Food allergies affect only a small fraction of the overall population. Food allergies involve an abnormal immunological response to a particular food or food component, usually a naturally occurring protein component of the food. The most common form of food allergy occurs through an antibody-mediated mechanism involving immunoglobulin E or IgE. These allergies are known as immediate hypersensitivity reactions because the symptoms ensue within minutes to a few hours after ingestion of the offending food. However, cell-mediated

food allergies can also occur. These allergies are known as delayed hypersensitivity reactions because the symptoms occur 24–72 h after ingestion of the food. While celiac disease, also known as gluten-sensitive enteropathy, is not often identified as a form of food allergy, it is a cell-mediated intestinal inflammatory response.

IgE-mediated food allergy

From a public health perspective, IgE-mediated food allergies are arguably the most important class of food allergy. While the number of affected individuals is modest, the reactions of some affected individuals can be quite severe, even life-threatening, especially if a significant quantity of the offending food is inadvertently ingested. Furthermore, the degree of tolerance for the offending food is small making the implementation of safe and effective avoidance diets more difficult.

Mechanism

The mechanism of IgE-mediated food allergy occurs through two phases: sensitization followed by elicitation (Anvari et al., 2019). Many foods contain one or more proteins that can trigger the formation of IgE antibody development among susceptible individuals, a process known as sensitization. While all humans have IgE antibodies, the majority will not produce IgE antibodies specific to a particular allergen. Even in susceptible individuals, IgE antibodies are typically formed to relatively few proteins. In many cases, exposure to a potentially allergenic food will often result in no immune response, known as oral tolerance. IgE-mediated food allergy can be viewed as a breakdown in oral tolerance (Chinthrajah et al., 2016). Some food proteins are more likely to elicit IgE antibody formation than others. Many factors including the susceptibility of the individual, the immunogenic nature of the food and its constituent proteins, the age of exposure, the route of exposure, and the dose, duration, and frequency of exposure are likely to influence the formation of allergen-specific IgE antibodies. Sensitization can occur from exposure to a food allergen through the gastrointestinal tract or the skin when their barrier functions are compromised (Sicherer and Sampson, 2018). In young infants, exposure to a food by the oral route seems to induce tolerance, while exposure to small amounts of the food through abraded skin in infants with eczema may induce sensitization (du Toit et al., 2018).

The IgE antibodies produced during the sensitization phase are highly specific and will recognize only a specific portion of the food protein that they are directed against. Occasionally, IgE antibodies produced against one particular protein in a specific food will confer sensitivity to another food either because the food is closely related or because it shares a common segment with the allergenic protein.

Allergen-specific IgE antibodies, once produced, attach themselves to the membrane surfaces of two types of specialized effector cells: mast cells that are found in many different tissues and basophils that are found in the blood. In this process, the mast cells and basophils become activated and ready to respond to subsequent exposure to that specific food allergen (the elicitation phase). Adverse reactions will then occur on any subsequent exposure to the specific allergenic protein or some closely related protein. Sensitization to a particular food allergen does not invariably mean that an adverse reaction will occur upon subsequent exposure to that food. It is possible to be sensitized but not reactive to a particular allergen. When reactions occur in the elicitation phase, the food allergen cross-links to IgE antibody molecules on the surface of the mast cell or basophil. This interaction between the allergen and the allergen-specific IgE elicits the release of a host of mediators of allergic disease which are either stored or formed by the mast cells and basophils. Several dozen different mediators have been identified including histamine, prostaglandins, and leukotrienes (Anvari et al., 2019; Valenta et al., 2015). These mediators are responsible for the symptoms associated with allergies through interactions with receptors in various target organs in the body. The interaction of a small amount of allergen with the allergen-specific IgE antibodies results in the immediate release of comparatively large quantities of the various mediators into the bloodstream and tissues. Thus, exposure to extremely small amounts of allergens can elicit symptoms.

While ingestion is the most common route of exposure to an allergenic food, symptoms can be provoked by exposure through the respiratory tract or the skin. Occupational food allergies can occur in food industry workers who are exposed by inhalation or skin contact (Fukutomi, 2019; Jeebhay et al., 2019). However, consumers may also show reactions when respiratory or cutaneous exposures occur. Often symptoms from such exposures are localized as occupational asthma or contact dermatitis or urticaria (hives).

IgE-mediated reactions can also occur from environmental exposures to many other sources of allergens including pollens, mold spores, animal danders, bee venom, and pharmaceuticals. In these cases, the source of the allergen is often environmental and not food-related. Exposure is through the respiratory route typically for pollens, mold spores, and animal danders and by injection for bee venoms.

Symptoms

IgE-mediated food allergies are designated as immediate hypersensitivity reactions because of the short onset time between the ingestion of the offending food and the onset of symptoms. Since the mediators released from the mast cells and basophils can interact with receptors in a number of different tissues in the body, a rather wide variety of symptoms can be associated with IgE-mediated food allergies as shown in Table 1 (Taylor and Baumert, 2020). Most food-allergic individuals experience only a few of the many possible symptoms.

Table 1 Symptoms of IgE-mediated food allergies.

<i>Gastrointestinal symptoms</i>	<i>Cutaneous symptoms</i>
Nausea	Urticaria
Vomiting	Dermatitis or eczema
Diarrhea	Angioedema
Abdominal cramping	Pruritis
<i>Respiratory symptoms</i>	<i>Other symptoms</i>
Rhinitis	Anaphylactic shock
Asthma	Laryngeal edema

Most of the symptoms of IgE-mediated food allergies are not particularly definitive which can make diagnosis rather difficult. For example, the gastrointestinal manifestations of food allergies can also be associated with many other foodborne illnesses and a variety of other diseases as well. Additionally, there are millions of asthmatics, but only a small percentage are allergic to foods.

Anaphylactic shock is, by far, the most serious manifestation of food allergies. Anaphylactic shock involves gastrointestinal, cutaneous, and respiratory symptoms in combination with a dramatic fall in blood pressure and cardiovascular complications. Death can ensue within minutes of the onset of anaphylactic shock. Fortunately, relatively few individuals with food allergies are susceptible to such severe reactions after the ingestion of the offending food.

The severity of an allergic reaction will be dependent to some extent on the amount of the offending food that is ingested. Severe reactions are more likely to occur when an allergic individual inadvertently ingests a large amount of the offending food, especially if that individual happens to be exquisitely sensitive. However, exposure to even trace quantities can elicit noticeable reactions due to the large release of mediators.

Perhaps the most common and possibly the mildest form of IgE-mediated food allergy is the so-called oral allergy syndrome (OAS) associated with the ingestion of various fresh fruits and vegetables (Muluk and Cingi, 2018). OAS symptoms are confined to the oropharyngeal area including pruritis, urticaria, and angioedema. In OAS, sensitization occurs to allergens present in pollens such as birch and ragweed. With OAS, sensitization to the pollen allergens increases the likelihood of sensitization to specific foods. Although fresh fruits and vegetables contain comparatively low quantities of proteins, sufficient exposure occurs to elicit symptoms in the oropharyngeal region. The allergens involved in OAS are quite susceptible to digestive proteases in the gastrointestinal tract, thus systemic reactions to these foods are infrequent. These fruit and vegetable allergens are also heat-labile. Individuals with OAS can often safely consume the heat-processed versions of these foods. In some cases, a more stable allergen called lipid transfer protein has been implicated in allergic reactions to certain fruits and vegetables and these reactions are more likely to be systemic and severe (Asero et al., 2018).

Exercise-induced food allergies are a subset of the immediate hypersensitivity reactions to foods (Foong et al., 2019). In these cases, exercise must be done coincident with ingestion of the food for symptoms to occur. Exercise-induced food allergies have been associated with several foods including shellfish, wheat, and celery. The symptoms of exercise-induced food allergies are individualistic, variable, and similar in nature to those involved in other food allergies. Exercise-induced allergies can also exist without any role for food intake. The mechanism of this illness is not well understood, although the involvement of IgE antibodies is apparent.

Sources

The most common allergenic foods are peanuts, tree nuts (almonds, walnuts, pecans, cashews, etc.), soybeans, cows' milk, eggs, fish, crustacean shellfish (shrimp, crab, lobster, etc.), and wheat (Bjorksten et al., 2008). On a worldwide basis, these eight foods or food groups are thought to be responsible for 90% of all food allergies. Allergies to crustacean shellfish and peanuts are the most common food allergies in the US. Cows' milk allergy is the most common food allergy among infants due to the widespread consumption of milk during the first months of life. Eggs are another commonly allergenic food among infants and young children because of the frequent use of eggs as a weaning food. In the US, peanut allergy is also relatively common among young children because of the popularity of peanut butter. However, in some other countries, peanut butter is less popular and peanut allergy is less prevalent especially in childhood. Thus, the prevalence of any specific type of food allergy is related to some extent to cultural food preferences. For example, in some Asian countries, buckwheat is a common allergenic food because of the popularity of soba noodles leading to frequent exposure to buckwheat (Shek and Lee, 2006). By contrast, buckwheat allergy is not common in the US probably because buckwheat is not frequently eaten in the US. Different regions of the world recognize different foods as commonly allergenic or priority allergens for the purposes of labeling regulations. Milk, eggs, fish, crustacean shellfish, peanut, soybean, tree nuts, and cereal grain sources of gluten (including wheat) are recognized by most countries. Molluscan shellfish (clams, oysters, squid, snails, etc.), mustard, and lupine are considered as priority allergenic foods in Canada and the European Union. Sesame seed is identified as a commonly allergenic food in Canada, the European Union, and Australia/New Zealand. The European Union also recognizes celery as commonly allergenic.

Any food that contains protein has the potential to elicit an allergic reaction in someone. The most common allergenic foods tend to be foods with high protein content that are frequently consumed. The exceptions are beef, pork, chicken, and turkey which are rarely allergenic despite their frequent consumption and high protein content.

Food allergens

The vast majority of the allergens in foods are naturally occurring proteins (Sathe et al., 2016). But foods contain millions of proteins and most do not provoke a sensitizing response. Thus, only a very small percentage of all known food proteins are known to be allergens. Even with the commonly allergenic foods, only a few of the many proteins in those foods are identified as allergens. Allergenicity does not appear to be an inherent property of proteins although all proteins are certainly capable of provoking immune responses under selected circumstances of exposure. Some allergenic foods, such as peanuts, eggs, milk, shrimp, and soybean contain multiple allergens, while other foods, such as Brazil nut, appear to contain only a single major allergen (Sathe et al., 2016). Major allergens are generally defined as proteins for which 50% or more of the allergic patients studied have specific IgE. Food allergens tend to fall into certain functional classes such as some of the pathogenesis-related proteins, certain types of seed storage proteins, or EF hand proteins (Lorenz et al., 2015; Radauer et al., 2008). For example, the 2S albumins, storage proteins rich in sulfur-containing amino acids, are major allergens in peanuts, Brazil nuts, sesame seeds, walnuts, sunflower seeds, and mustard. Similarly pan allergens exist across many species of fish (parvalbumin) and crustacean shellfish (tropomyosin). However, both fish and crustacean shellfish also possess other protein allergens (Sathe et al., 2016).

Prevalence

The overall prevalence of IgE-mediated food allergies is not precisely known and appears to be increasing in many countries for reasons that are also not entirely clear. The overall prevalence of food allergies among infants and children in the United States and Australia falls in the range of 5–10% (Gupta et al., 2011; Peters et al., 2017). Although many studies indicate that the prevalence of food allergies is higher among children than adults, recent evidence in the United States indicates that the prevalence of food allergies among adults is approximately 10% (Gupta et al., 2019). In a comprehensive study involving allergy centers in 8 different countries across Europe, the self-reported prevalence of food allergy ranged from 2% to 37% of survey respondents, while, on follow-up, the probable prevalence of food allergy ranged from a low of 0.3% in Greece to a high of 5.6% in Switzerland (Lyons et al., 2019). The bases for these differences are not entirely clear.

The prevalence of allergies to specific foods is also not precisely known. Based upon a telephone survey, which is probably less accurate than diagnostic evaluation, shrimp allergy appears to be the most common food allergy in the United States at 1.9% (Sicherer et al., 2004; Gupta et al., 2019). In 7 of 8 EU countries, the prevalence of shrimp allergy ranged from 0.35–0.82% with no shrimp allergy observed in Greece. Peanut and tree nut allergies were believed to affect about 0.75% and 0.6% of the overall population in the United States based on a survey published in Sicherer et al. (2010), while the prevalence leapt to 1.8% for peanut and 1.2% for tree nuts based on a survey published in Gupta et al. (2019). Peanuts are less commonly consumed in some European countries. The prevalence of peanut allergy was very low in Iceland (0%) and Greece (0.02%) but was higher in other countries including 0.45% in Spain and 0.37% in Switzerland (Lyons et al., 2019). The prevalence of milk allergy among young infants is more consistent among countries ranging from 0.5–2.2% among studies done in the United States, Israel, Denmark, and in a European birth cohort (Flom and Sicherer, 2019; Lam et al., 2008). While milk allergy has historically been considered as relatively uncommon in adults (Flom and Sicherer, 2019), several recent studies have indicated a high prevalence of 2.0–2.4% among United States adults (Gupta et al., 2019; McGowan and Keet, 2013). In the study of Gupta et al. (2019), 22% of adults who reported milk allergy also indicated an adult onset of this allergy. Two-thirds of adults with cows' milk allergy reported experiencing severe symptoms (Lam et al., 2008). Sorting out the true prevalence of food allergies in countries around the world will require considerable effort that will likely be made more difficult by the increasing prevalence of food allergies in many locations. Certainly the factors associated with the low prevalence of food allergies in Greece would be worthy of future investigation.

An increase in the prevalence of IgE-mediated food allergies is most apparent in developed countries, although variability is noted between developed countries such as Switzerland and Greece. The prevalence appears lower in less developed countries but is, as yet, poorly understood making firm comparisons difficult. Multiple factors may be involved in the increased prevalence. Certainly improvements in diagnosis mean that fewer cases are overlooked. The increased general cleanliness of the home environment is another factor that led to the promulgation of the hygiene hypothesis (Julge et al., 2001). The prevalence of food allergies is higher in urban environments than rural areas perhaps because the immune system is busy counteracting bacteria that are more prevalent on farms (Botha et al., 2018). The hygiene hypothesis has more recently evolved into a recognition that the intestinal microbiota play an important role in the balance between tolerance and allergy to foods (Shu et al., 2019). The microbiota is influenced by another factor, the increase in infants born by Caesarean sections, which is associated with an increase in food allergies (Koplin et al., 2008). After Caesarean birth, the infant's intestinal tract is devoid of beneficial maternal microbiota. Weaning practices and epigenetic factors (see Section "Prevention") may be additional important factors in prevalence.

Persistence

Many infants and young children outgrow their IgE-mediated food allergies through the eventual development of oral tolerance. Allergies to some foods, such as cows' milk and eggs are more frequently outgrown than allergies to other foods such as peanuts (Savage et al., 2016). The time period for development of tolerance ranges from a few months to several years from the time of the onset of the food allergy. As many as 80% of food-allergic children are able to tolerate the offending food by the age of 3–4 years (Peters et al., 2017; Savage et al., 2016). While peanut allergy tends to be very persistent, perhaps 20% of peanut-allergic children do

ultimately become tolerant. While milk allergy is frequently outgrown, some individuals do fail to achieve tolerance and become milk-allergic adults. However, food allergies can have their initial onset in adulthood in some cases (Gupta et al., 2019). Studies on milk- and egg-allergic children indicate that some may first become tolerant of certain processed forms of milk and egg e.g., baked foods, though being still sensitive to less heated forms of milk and eggs such as pasteurized milk and scrambled egg, although more rigorous proof of this hypothesis is needed (Lambert et al., 2017).

Prevention

The prevention of allergic sensitization in infants would counteract the development of IgE-mediated food allergies. Since most infants have a low risk of development of food allergies, the implementation of effective prevention strategies requires the early identification of those high-risk infants most likely to develop IgE-mediated food allergies. High-risk infants include those infants born to parents with histories of allergic disease of any type (pollens, mold spores, animal danders, bee venoms, food, etc.). Several strategies have been advanced for the prevention of allergic sensitization in high-risk infants. For many years, a pragmatic approach of excluding the infant from exposure to commonly allergenic foods. This strategy was coupled with breast-feeding for an extended period, the possible use of hypoallergenic infant formulae, and the exclusion of commonly allergenic foods from the diet of the nursing mother and possibly during pregnancy (Zeiger, 2003). While this approach seemed logical, it was not based upon clinical evidence of its efficacy (Sicherer, 2002). Furthermore, the prevalence of food allergies continuously increased while this strategy was being widely practiced. Exclusive breast-feeding may only delay and not prevent the development of IgE-mediated food allergies. High-risk infants may be sensitized through cutaneous exposure to food allergens on abraded, eczematous skin (du Toit et al., 2018) so avoidance of exposure to the allergen by the oral route alone is likely not sufficient. Maternal avoidance diets during pregnancy appear unwarranted and such exposure is more likely to assist in tolerance induction in the infant (Fujimura et al., 2019). Maternal avoidance during exclusive breast-feeding may decrease the risk of allergic sensitization in the infant but this could be due to the possibility of environmental exposure to the allergen via the skin (Fujimura et al., 2019). In nursing mothers, allergenic food proteins consumed in her diet may get absorbed and passed into breast milk (Schocker et al., 2016). This route of exposure could possibly sensitize the infant. Overall, the avoidance strategy seems to pale in comparison to the benefits of early introduction of allergenic foods.

Recent evidence demonstrated that the early introduction of peanut into the diets of high-risk infants (those having eczema and egg allergy at 4-months) would dramatically lower the likelihood for development of peanut allergy (du Toit et al., 2015). A similar strategy has been applied to other allergenic foods with mixed success (Fisher et al., 2018). Further research is needed to refine the factors involved in success for this strategy such as the age of the infant, the specific foods involved, and the degree of processing of the food. Widespread recommendations have now been made to feed peanuts to infants at an early age as a preventive measure. Breast-feeding is still advocated but with supplementation with peanuts or perhaps other allergenic foods. Commercially, several weaning foods are now available to allow the early introduction of peanuts and other commonly allergenic foods to young infants.

Nutrition could play a role in prevention of food allergies as well. Factors such as vitamin D, omega-3 fatty acids, folate, and antioxidants have been proposed to have effects on the development of food allergies (Sicherer and Sampson, 2018). Specific genetic loci have been identified that are associated with peanut allergy (Hong et al., 2015). In milk-allergic individuals, altered DNA methylation was found in several loci associated with T cells that are critically involved in development of IgE-mediated food allergy (Hong et al., 2016).

The use of probiotic bacteria and prebiotic infant formula may also help to lessen the likelihood of allergic sensitization (Berni Canani et al., 2019; Jeurink et al., 2013). Many of the studies with administration of probiotic bacteria have been conducted in animal models and their protective effects require further elucidation. Infant formula containing prebiotic oligosaccharides that promote the growth of beneficial microbiota in the intestinal tract have offered promising results (Jeurink et al., 2013). Commercial formulae are on the market.

Hypoallergenic infant formulae will also prevent the development of food allergies in high-risk infants in the early months of life in situations where exclusive breast-feeding is not possible. However, these formulae, especially extensively hydrolyzed and/or elemental formulae, are more often used to prevent reactions after sensitization has already occurred. The use of partial whey hydrolysate formula has been advocated for prevention (Vandenplas et al., 2019). Partial hydrolysates are more palatable to the infant.

Diagnosis

The diagnosis of food allergies is typically approached in a stepwise fashion (Boyce et al., 2010; Sicherer and Sampson, 2018). The diagnosis of food allergies by an allergist is often critical because parental diagnosis and self-diagnosis are often incorrect leading to identification of the wrong, incorrect foods or the identification of too many foods as allergens. The diagnosis begins with the physician taking a careful history of the patient's adverse reactions, taking note of the foods eaten immediately before the onset of symptoms, the amount of various foods consumed, the type, severity, and consistency of symptoms, and the time intervals between eating and the onset of symptoms. Sometimes, histories are needed from several episodes to reach a probable diagnosis. Oral challenge tests with the suspected food(s) may be used to establish with certainty the role of a specific food in the reaction. The double-blind, placebo-controlled food challenge (DBPCFC) is the most definitive clinical approach to document the cause-and-effect relationship with a particular food. History alone can be sufficient to make the diagnosis in some situations if the

cause-and-effect relationship is particularly compelling. A positive challenge test indicates that the food is associated with the adverse reaction but cannot be used to determine if IgE antibodies are involved. The diagnosis of an IgE-mediated mechanism can be made with either the skin prick test or a blood test for the presence of food-specific IgE antibodies.

Treatment

The specific avoidance diet is the primary means of treatment for IgE-mediated food allergies (Taylor et al., 1999; Grimshaw, 2006). For example, if allergic to peanuts, do not eat peanuts. Some individuals are allergic to more than one food which complicates the avoidance diet. With IgE-mediated food allergies, low amounts of the offending food can provoke reactions. Thus, the construction of a safe and effective avoidance diet can be quite difficult. Ingredients derived from commonly allergenic foods must also be avoided in many cases such as casein or whey from milk or semolina from wheat. The sources of ingredients derived from commonly allergenic sources must be labeled in many countries and food-allergic consumers are advised to avoid them even in situations where the risks may be quite small. In the United States, source labeling is not necessary for refined oils from allergenic sources such as peanut oil or soybean oil because these ingredients do not contain enough of the allergenic protein to provoke reactions. Less refined oils may contain sufficient allergenic protein to pose a risk to allergic consumers. Careful reading and complete understanding of food labels is critical to the implementation of safe and effective avoidance diets. Of course, the manufacturers of packaged foods have the responsibility to assure that the label statements on packages are accurate. Occasionally, errors are made by food processors that result in the presence of undeclared residues to allergenic foods in a packaged food. The contamination of food with another from the use of shared food processing equipment is one of the most common errors occurring in food manufacturing. However, restaurant and other food service meals can present an even bigger challenge for food-allergic individuals. Residues of allergenic foods can arise from the use of shared food preparation equipment (utensils, cooking surfaces, pots and pans, etc.). Additionally, the accurate identification of all of the ingredients in food service and restaurant meals can sometimes be quite difficult and such foods are not labeled in most countries. As a result, many inadvertent exposures occur among consumers who are attempting to avoid their offending food(s).

Cross-reactions are another perplexing issue for food-allergic consumers as they attempt to develop effective avoidance diets (Kazatsky and Wood, 2016). Cross-reactions can occur but do not inevitably occur between closely related foods. For example, many individuals are allergic to peanuts, but most of these individuals are not allergic to other legumes such as soybeans, lupine, peas, and green beans (Chan et al., 2019). Many peanut-allergic individuals are sensitized to other legumes but most can ingest these other legumes with no ill effects. Lupine, a legume which is unfamiliar to many consumers cross-reacts with peanuts in some allergic individuals (Chan et al., 2019). As a result, peanut-allergic individuals are often advised to avoid lupine even though most will not react. Cross-reactions frequently occur among the various crustacea (shrimp, crab, lobster and crayfish) and among species of fish (Pedrosa et al., 2016; Kobayashi et al., 2016). Cows' milk and goats' milk invariably cross-react as do eggs from various avian species (Kazatsky and Wood, 2016). Cross-reactions can also occur between foods and other environmental allergens. The most common examples are the cross-reactions that occur between some fresh fruits and vegetables and certain pollens allergies in some individuals (Werfel et al., 2015) and the cross-reactions that occurs in a few individuals with allergies to natural rubber latex with several foods including banana and chestnut (Raulf-Heimsoth et al., 2007).

Pharmacological approaches can be used to treat the symptoms of allergic reactions. In particular, epinephrine (also known as adrenalin) is prescribed to individuals who experience life-threatening food allergies (Greenhawt et al., 2019). The early administration of epinephrine after inadvertent exposure to the offending food can be life-saving for such patients. Antihistamines and other medications can be used to treat less-serious symptoms of food allergies (Boyce et al., 2010; Kobernick et al., 2015).

Immunotherapy (IT) has emerged as a promising therapy for the control of food allergies (Burks et al., 2018). With IT, small doses of the allergenic food are administered to the food-allergic patient over time causing the patient to become progressively more tolerant to the offending food. In most cases, IT is not a curative treatment because most patients do not achieve sustained unresponsiveness. Instead, patients must continue to regularly consume a maintenance dose of the allergenic food to achieve some level of non-responsiveness to the food. The up-dosing protocol must be approached carefully to avoid eliciting adverse reactions. In the end, the patient may not be able to consume large quantities of the food but will be protected from experiencing reactions to small doses encountered from contamination of one food with another during harvesting, processing or preparation. The development of additional treatments for food allergies are being pursued (Burks et al., 2018).

Minimal eliciting dose (Threshold)

In some individuals with IgE-mediated food allergies, reactions can be triggered by very low doses of the offending food. The low threshold for the offending food complicates effective avoidance as great care must be taken. The low eliciting dose results from the interaction of a rather small amount of allergen with IgE antibodies on the surfaces of mast cells and basophils which stimulates the release of large quantities of biologically active mediators. Accordingly, exposure to a small dose of the allergen can elicit a clinically significant reaction.

With avoidance diets, the complete avoidance of the offending food must be emphasized to all allergic individuals. However, clinical research has documented that minimal eliciting or threshold doses do exist below which allergic individuals will not experience adverse reactions. Low-dose DBPCFC can be used to determine the threshold dose for an individual patient to a specific food. For peanut, a wide range of individual threshold doses from 0.4 mg up to 10 g of whole peanut has been observed (Taylor and

Baumert, 2010). Individual threshold doses could be collected and used by public health authorities to establish population thresholds (Madsen et al., 2009). Dose-distribution modeling has been advocated as a scientifically based, transparent approach to the estimation of safe doses for allergic individuals using the data acquired from existing challenge trials (Gendel et al., 2008). Individual threshold data have been collected using consistent criteria and modeled to predict safe population thresholds for many of the most common allergenic foods (Remington et al., 2020; Westerhout et al., 2019). These population thresholds have been demonstrated to be safe for peanut-allergic individuals (Hourihane et al., 2017). Population thresholds could be used to guide food-allergic consumers in the construction of safe, effective avoidance diets and labeling and other control strategies used by the food industry.

Occurrence of allergic reactions

Since no cure exists for IgE-mediated food allergies, the prevention of reactions focuses on avoidance diets. However, food-allergic consumers will occasionally experience reactions by inadvertent consumption of the offending food. Records from emergency rooms indicate that tens of thousands seek emergency treatment for food allergy reactions each year (Clark et al., 2011). In Canada, peanut-allergic individuals studied over a 10-year period had a 12.4% annual incidence rate of accidental exposures to peanut (Cherkaoui et al., 2015). Many of these Canadians did not seek medical attention for their reaction even if it involved modest to severe symptoms (Cherkaoui et al., 2015) so the rate of presentation in emergency rooms likely underestimates the incidence of allergic reactions. In the Netherlands, 73 of 157 adult patients experienced allergic reactions to a variety of food products during a 1-year period (Blom et al., 2018). The factors leading up to these visits are poorly understood. Certainly, a small fraction of emergency room visits occur among individuals who are experiencing an allergic reaction to food for the first time. But most of these reactions likely occur among individuals who are aware of their food allergy and who should be avoiding the offending food. Individual who had received counseling from an allergist and received an epinephrine prescription were less likely to show up in emergency rooms for treatment of a food allergy reaction (Clark et al., 2014). Evidence also suggests that some individuals have repeated emergency room visits for food allergy reactions (Clark et al., 2014).

Food allergy reactions can occur in any location where food is sold or eaten (Taylor and Baumert, 2010). Homes and restaurants are the most frequent locales for such reactions. Obviously, the food-allergic individual or their caregiver could make an error that leads to inadvertent ingestion of the offending food. Failure to read or understand food labels can be a contributing factor. Avoidance diets require constant vigilance and errors are likely to happen. The frequency of such errors is unknown.

However, food manufacturers or restaurants may also contribute to food allergy reactions. Prevention of errors by food manufacturers or restaurants has become an important public health focus.

A variety of food manufacturing errors can occur including placing the incorrect product in the package, the failure to adequately clean shared equipment, the use of re-work (a common practice in certain segments of the industry involving the incorporation of left-over or misformulated quantities of a food product into subsequent batches of related products with identical or related formulations), and the inadvertent addition of an ingredient that is not supposed to be in the formulation. However, with packaged foods, the majority of food ingredients are declared on the ingredient statement on the label. Additionally, many food processors have begun to add precautionary allergen labeling (PAL) to food products including such terms as 'made on shared equipment with peanuts' or 'may contain peanuts'. This sort of labeling alerts allergic consumers to the potential presence of residues on allergenic foods in a product. However, the recent proliferation of PAL has led some food-allergic consumers to begin to ignore these statements (Allen and Taylor, 2018). In some countries, products with PAL statements frequently do not contain detectable allergen residues (Allen and Taylor, 2018) while consumers in other countries frequently experience allergic reactions from consumption of products with PAL (Blom et al., 2018).

Restaurants and other foodservice facilities constitute another big challenge for food-allergic consumers (Taylor and Baumert, 2010). In most countries, foods in restaurants are not labeled although allergen labeling is required in European countries. Wait staff sometimes fail to provide correct information on menu components. Many adverse reactions have occurred in such settings. Additionally errors can occur in food preparation including the use of shared cooking/serving equipment or cooking surfaces or cooking media and mistakes or substitutions in adherence to recipes.

Regulations

Public health agencies such as the US Food & Drug Administration regulate the labeling of packaged foods (Taylor and Baumert, 2015). As noted, the ingredient statements on packaged foods identify the vast majority of the allergenic foods or the ingredients sourced from such foods. Regulations differ around the world but the clear labeling of commonly allergenic foods is a key component of regulations in most countries.

The enforcement of existing regulations is another task for the public health agencies. In the United States, the wider recognition of food allergies has extended to FDA and other regulatory agencies. One of the fastest growing areas of recalls of packaged foods in the United States is the undeclared presence of allergenic foods. Improved methods for the detection of residues of allergenic foods have been pivotal to the investigation of allergic incidents and the enforcement of labeling regulations.

Detection of allergenic food residues

Reliable methods exist for the detection of the presence of undeclared or unwanted allergen residues in foods and food ingredients (Prado et al., 2016). The food industry uses the detection tools to assure that their manufacturing practices are adequate to eliminate the presence of unwanted allergen residues in other foods. When residues cannot be effectively and consistently eliminated, then PAL can be used. The regulatory agencies use the detection methods for enforcement of labeling provisions on packaged foods and to investigate alleged reactions that might be attributable to undeclared allergen in packaged foods. Commercial enzyme-linked immunosorbent assays or ELISAs with detection limits in the low ppm (mg kg^{-1}) range are available for the detection of residues of peanuts, soybeans, egg, cows' milk, almonds, gluten (wheat, rye, barley), walnuts, cashews, pistachios, pecans, hazelnuts, Brazil nuts, macadamia nuts, crustacean shellfish, mustard, lupine, and buckwheat. Polymerase chain reactions assays are also available for the detection of DNA from allergenic food sources. Mass spectrometry methods are being developed also for the specific detection of selected proteins from allergenic food sources.

Effects of food processing on allergenicity

Food processing operations have limited effects on the allergenicity of most of the commonly allergenic foods since the allergenic food proteins tend to be remarkably stable to food processing conditions (Sathe and Sharma, 2009; Cabanillas and Novak, 2019). Food-allergic individuals react to processed foods so allergens must be stable to food processing with some exceptions. For example, some of the allergens in fruits and vegetables are heat-sensitive. Additionally, many food allergens tend to be resistant to proteolysis allowing these proteins to survive digestive processes and arrive in the intestinal tract in immunologically active form (Lorenz et al., 2015). Thus food allergens may survive, in whole or in part, the acid and enzymatic hydrolysis methods used to prepare protein hydrolysates often used as food ingredients. Hypoallergenic infant formula can be manufactured with extensive hydrolysis of milk proteins but is one of the few examples where processing has been used commercially with the intent of drastically reducing or eliminating allergenicity (Host and Halken, 2014). The removal of almost all protein residues in edible oil refining serves as another example.

Food-allergic individuals may also vary in their susceptibility to processed forms of commonly allergenic foods. With milk and egg, some children develop tolerance to baked products containing milk and egg proteins (Lambert et al., 2017). These individuals seem to be reactive to allergens in milk and egg that are denatured by the heat used in the baking process. But other milk- and egg-allergic children do not share this experience and remain reactive to both baked and unbaked forms of milk and egg.

Celiac disease

Celiac disease is also known as celiac sprue, non-tropical sprue, or gluten-sensitive enteropathy. Celiac disease occurs in sensitive individuals upon the consumption of wheat, rye, barley, and related grains as illustrated in Table 2. This illness is a good example of delayed hypersensitivity reactions associated with food.

Mechanism

Celiac disease is associated with a cell-mediated, localized inflammatory reaction in the intestinal tract (Schuppan et al., 2009). The inflammatory response results in the so-called 'flat lesion' in the small intestine. The absorptive epithelium lining the small intestine becomes damaged by the inflammatory process resulting in a decreased number of epithelial cells that are critical to digestion and the absorption of dietary nutrients. Thus, the absorptive function of the small intestine is compromised leading to the development of a malabsorption syndrome. The inflammatory response in the gut is characterized by villous atrophy along with crypt hyperplasia, lymphoid infiltration of the epithelium, edema of the lamina propria, and the impaired epithelial absorptive function. Increased fluid secretion and enhanced permeability of the epithelium are also noted. A defect in mucosal processing of gliadin

Table 2 Cereal sources of gluten.

Wheat
Rye
Barley
Spelt
Kamut™
Triticale
Durum wheat or semolina
Club wheat
Emmer
Einkorn
Farro

(one of the gluten protein components from wheat) in celiac patients appears to provoke the generation of toxic peptides that contribute to abnormal immunologic response and the subsequent inflammatory reaction.

Symptoms and sequelae

Celiac disease results in a severe malabsorption syndrome characterized by diarrhea, bloating, weight loss, anemia, bone pain, chronic fatigue, weakness, various nutritional deficiencies, muscle cramps, and, in children, failure to thrive. While the risk of severe reactions is low, untreated celiac disease is associated with considerable discomfort in many celiac sufferers. Individuals suffering from celiac disease for long periods are also at increased risk to development of T cell lymphomas. Celiac patients are also more likely than others to have various other diseases especially diseases of the autoimmune nature including dermatitis herpetiformis, thyroid diseases, Addison's disease, pernicious anemia, autoimmune thrombocytopenia, sarcoidosis, insulin-dependent diabetes mellitus, IgA nephropathy, and Down's syndrome (Diamanti et al., 2016).

Causative factor

The prolamin protein fractions of wheat and related grains are implicated in the causation of celiac disease (Cabanillas, 2020). The prolamin fraction of wheat is known as gluten, so celiac disease is sometimes referred to as gluten-sensitive enteropathy. In wheat, gliadin, the alcohol-soluble fraction of gluten, is involved in the elicitation of celiac disease. Glutenin, the alcohol-insoluble fraction is also likely involved however. The prolamins are the major storage proteins in these grains, so all of these grains contain ample gluten and are considered hazardous for celiac sufferers.

Prevalence and persistence

The prevalence of celiac disease on a global basis is uncertain. In the United States, the prevalence is considered to be approximately 1% of the population (Green et al., 2015). The diagnosis of celiac disease can be rather difficult. Celiac disease appears to be latent or subclinical in some individuals with symptoms only appearing occasionally. The clinical presentation of celiac disease is quite variable with anemia and osteoporosis as two of the more frequent manifestations in adults in the United States (Green et al., 2015). Serological and endoscopic histological approaches are often needed to confirm the diagnosis of celiac disease.

Management and minimal eliciting dose

Celiac disease is treated by the implementation of a gluten avoidance diet (Green et al., 2015). Affected individuals should avoid all sources of wheat, rye, barley or related grains including a wide variety of common food ingredients derived from these grain. The need to avoid ingredients that do not contain detectable protein from the implicated grains is somewhat debatable, but widely practiced. Most of these individuals also avoid oats, but that is likely wise considering the frequent contamination of oats with wheat from shared farms, harvesting equipment, and storage facilities. Gluten-free foods are available with increasing frequency in the marketplace. The definition of gluten-free in most countries is below 20 ppm gluten.

The minimal eliciting dose for wheat, rye, barley and related grains among celiac sufferers is unknown. Several studies have concluded that levels of 10 mg of gluten per day will be tolerated by most patients with celiac disease (Catassi et al., 2007; Akobeng and Thomas, 2008).

Conclusion

While food allergies affect a small percentage of the population, reactions can range in severity from mild to life-threatening. Food allergies are caused primarily by naturally occurring proteins in foods. Food allergies can be triggered by rather small amounts of the offending foods.

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Celiac Disease[☆]

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Introduction

Celiac disease is an immune-mediated enteropathy that occurs in genetically susceptible persons. The disorder is characterized by intolerance to gluten-containing proteins found in different but related grains, such as wheat, rye, and barley. Another cereal grain, oats, may be contaminated by other gluten-containing grains. The disorder typically presents with diarrhea, nutrient malabsorption, and weight loss due to a mucosal inflammatory process that extends for a variable extent from the most proximal duodenum into the distal jejunum and ileum. It is possible that the severity of the individual inflammatory response to the offending proteins, the timing of its appearance, and its extent and localization within the small intestine are genetically programmed. In recent years, the disorder has been increasingly recognized in the absence of significant gastrointestinal symptoms, with a recorded prevalence rate (based on serological studies) of up to 2% in many countries. Not only is celiac disease common, but there is an increased appreciation that celiac disease is a phenotypically heterogeneous disorder. Increased recognition of celiac disease may reflect a true increase in incidence per se, but other factors likely play a more significant role. Increased diagnostic awareness among clinicians is evident coupled with widespread use of serological screening tests. Finally, in the eye of the public, perhaps more than ever before, the disorder has emerged from obscurity into the popular spotlight (Gujral et al., 2012).

Clinical features

Often, the disorder presents with diarrhea, multiple nutrient deficiencies, and weight loss. In children, failure to thrive with short stature and delayed puberty may occur. In recent years, however, it has become increasingly appreciated that a wide array of clinical presentations may develop, often without overt intestinal features. Anemia solely due to iron or folate deficiency may occur (Freeman, 2015a). In addition, isolated calcium deficiency has been noted along with hypoalbuminemia and peripheral edema, osteomalacia, or osteoporosis. Occasionally, unexplained chronic liver disease may develop, and several neurological disorders, such as dementia (Freeman, 2008), along with reproductive changes have been described (Freeman, 2015c). These or other extraintestinal disorders may actually dominate the clinical presentation, particularly endocrine disorders (Freeman, 2016a,b) such as autoimmune thyroid disease, insulin-dependent diabetes and other unusual disorders like, acetylcholine receptor antibody positive myasthenia gravis, and systemic lupus erythematosus.

Because celiac disease has been traditionally defined as a gluten-sensitive disorder, a definitive diagnosis ultimately depends on demonstration of a response to a gluten-free diet (Freeman, 2008). A biopsy of the small-intestinal mucosa is traditionally done to demonstrate the typical histopathologic features of untreated celiac disease. Serological antibody markers are usually positive, including antibodies to tissue transglutaminase and endomysium. After treatment with a gluten-free diet, resolution of the diarrhea and weight gain usually occur along with normalization of serological studies. Normalization of abnormal small-intestinal biopsy changes follows, even months to years later on a gluten-free diet (Freeman, 2017a).

[☆]Change History: October 2021. Hugh J Freeman updated the text and references to this entire chapter.

Age, sex, race, and global distribution

Although celiac disease is often detected in childhood, the disorder is most frequently diagnosed in adults, particularly between ages 25 and 45 years. Some studies have suggested that the clinical onset is bimodal, with the initial peak developing at 8–12 months of age, and the second during the third to fourth decades of life. In recent years, the disease has also been frequently recognized for the first time in the elderly (Freeman, 2015a), even in octogenarians! In adult age groups, there is a preponderance of females, although, in the elderly, the ratio of females to males appears to be more equivalent. In part, this may reflect the greater likelihood that females, as young adults, often seek medical care more frequently and at an earlier age than males. In addition, the necessary genetic markers, the HLA haplotypes DQ2 and DQ8, are more frequent in female compared to male celiacs (i.e., 94% vs 85%). In the few DQ2- and DQ8-negative celiac disease patients, a male excess has been noted. Factors that appear to lead to precipitation of celiac disease in the genetically predisposed have not been well defined, but environmental factors, including viral infections (e.g., adenovirus and rotavirus) and an altered immune state, associated with surgical procedures (i.e., post-gastrectomy, post-colectomy), pregnancy, or the puerperium, have been noted.

In the past, celiac disease was well described in Caucasians, especially from European centers, particularly the United Kingdom, Scandinavia, and Italy. Some believed that the west of Ireland, with about one individual with celiac disease in 300 persons, was the most frequent geographic site, and early published studies suggested a decreasing gradient in prevalence from the northwest to southeast of Europe. In part, this impression reflected limitations in data on celiac disease in most countries. For example, little information was available for most countries in North and South America, although the disease was suspected to be common in some countries there; it was attributed to emigration from Europe, particularly to Canada and Argentina. Interestingly, in the United States, celiac disease was considered to be a relatively rare disorder only a few decades ago. The disease has also been rarely reported from Asia, particularly China and the Indian subcontinent, or from North Africa. In recent years, however, a much wider global occurrence of celiac disease has become better appreciated.

The highest reported rates to date have come from centers in Finland. Other European countries and countries with immigration from Europe, especially the Americas and Australia, also all have high rates. Celiac disease has now also been more frequently recorded in several different Asian countries, including India and Sri Lanka, as well as China, the Middle East, and North Africa. Finally, Asian immigrant populations and their descendants in the United Kingdom and Canada, along with First Nations populations in Canada, have been recorded with celiac disease.

Although up to 2% of the general populations of some countries may be affected, the risk is much greater in first-degree relatives of affected sibling pairs (17%), monozygotic twins (75%), and HLA-identical siblings (40%). Indeed, the single most important risk factor for celiac disease is a first degree relative with already-defined celiac disease, especially a sibling.

History

Celiac disease is believed to have initially appeared in the Neolithic period with the evolution of humans from the hunter-gatherer phase to an agrarian or farming phase, with the appearance of modern crop cultivation methods in different regions to provide food and a survival advantage. Indeed, even now, varieties of wheat around the world have now been genetically traced to the Eikhorn form of wild wheat from the Fertile Crescent area, especially in eastern Turkey.

In 1856, Francis Adams delivered a lecture to the Sydenham Society that represented a translation of a Greek manuscript on the 'Coeliac Affection' from the second century by Aretaeus, a physician residing in Cappadocia (in eastern Turkey). The word 'celiac' appears to have arisen from a Greek word, 'koiliakos,' meaning 'abdominal.' Aretaeus believed that the cause of the diarrhea in adults with this disorder, usually females, was a 'lack of heat' needed to digest food.

In 1887, Samuel Gee provided a more modern description of the disorder in children during a lecture at the Hospital for Sick Children, located on Great Ormond Street in London. Gee believed that the disease may be curable, specifically with diet, and noted the exacerbations of symptoms in children with lactose-containing products. In 1908, Herter, a physician from the United States, further noted in detail 'intestinal infantilism,' emphasizing the deleterious effects of the disorder on growth in affected children. Both pediatricians were ultimately recognized for their descriptive accounts of childhood celiac disease with the appearance of the eponym 'Gee-Herter disease.' In 1924, Haas, another pediatrician from the United States, promoted a banana diet for the disorder, and this persisted as a popular form of celiac disease treatment for decades.

The next significant step in the historical emergence of this disease came during World War II. A Dutch pediatrician, Dicke, noticed that children with celiac disease seemed to clinically improve with fewer deaths during periods of famine and scarcity of wheat flour, in particular the winter of 1944 (the so-called *hongerwinter*). As wheat became increasingly available again, the high death rate documented in these children recurred. Later, Dicke and his colleagues developed a laboratory method for measurement of fat in fecal material and demonstrated increased fecal fat excretion with ingestion of wheat flour and reduced excretion when wheat flour was removed from the diet. In 1952, Anderson and her colleagues from the United Kingdom suggested that gluten was the actual component in wheat that caused worsening of the disease.

Up to this time, most information on the pathology of the small intestine in celiac disease had been accumulated using autopsy material. This was not satisfactory because of the rapid autolytic mucosal changes that appear during the immediate postmortem period. In 1954, Paulley provided the first histopathological description of atrophied villi using surgical specimens. Almost

immediately, an explosion in instrumentation for exploration of the luminal intestinal tract occurred. As a result, mucosal biopsies were collected with small-intestinal capsules and with multipurpose and hydraulic instruments, and, concomitantly, the emergence of endoscopic imaging permitted sampling under direct vision. Precise clinical and histopathological descriptions of the disease were developed based on its gluten-sensitive nature as well as recognition that the biopsy changes were not specific for celiac disease. In addition, more reproducible and more specific serological assays for population screening were developed. Extraintestinal disorders, such as dermatitis herpetiformis, were explored, and an improved appreciation for clinical complications of celiac disease emerged, including lymphoma, carcinoma, and collagenous colitis. In recent years, a much improved understanding of the complex immunopathogenesis of celiac disease has been detailed, with efforts in several centers to develop alternative therapies.

Diagnosis of celiac disease

Two criteria, based on the concept that celiac disease is a gluten-sensitive enteropathy, have emerged for a definitive diagnosis of classical celiac disease. The diagnosis is based on two sequential criteria: first, a small-bowel biopsy that shows the histopathological features of untreated disease; and, second, documentation of a response to a gluten-free diet. The biopsy features of celiac disease are listed in detail in Table 1 (Figs. 1–5).

After a biopsy has documented changes of untreated celiac disease, a gluten-free diet is instituted. An unequivocal clinical and/or histopathological response is usually necessary to establish a definitive diagnosis. Most often, the diarrhea should resolve and weight gain should occur. In some with few or no symptoms, it may be necessary to repeat biopsies from similar sampling sites to demonstrate an improved biopsy appearance. In recent years, some have estimated that over 50% of those with celiac disease may now actually be classified as overweight or obese. Even in these patients, it was possible to demonstrate weight gain in response to a gluten-free diet.

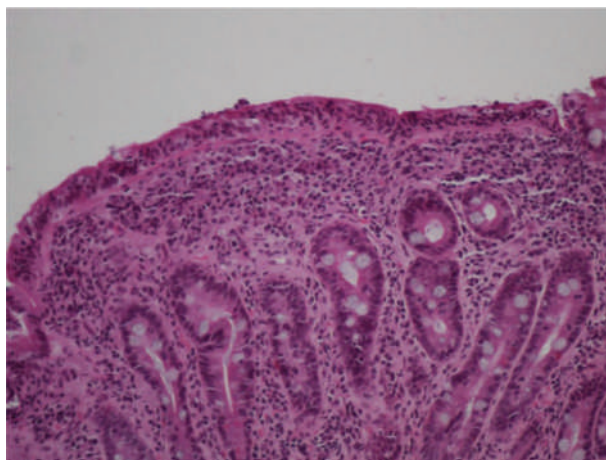


Fig. 1 Small-bowel mucosal biopsy showing severe ‘flat’ changes characteristic of celiac disease, or so-called total villous atrophy (see text). Villi are rudimentary, crypts are elongated with an increased mitotic index, and increased numbers of mucosal lymphocytes and plasma cells are evident. Hematoxylin and eosin (H & E), magnification $\times 200$.

Table 1 Biopsy features of celiac disease (gluten-sensitive enteropathy and celiac sprue; see Figs. 1–5).

Severe ‘flat’ lesion

Absent or rudimentary villi, crypt epithelial hyperplasia with an increased epithelial cell mitotic index, increased inflammatory cell content of the lamina propria region in the mucosa, intraepithelial lymphocytosis, cuboidal (rather than columnar) epithelial cells; CD3 and CD8-positive lymphocytes predominate in the mucosa, particularly in the intraepithelial lymphocyte compartment.

Another term is ‘crypt hyperplastic villous atrophy.’ Similar to a ‘Marsh 3’ lesion.

Moderate lesion

Similar changes, but not as pronounced, with only limited but definite alterations in the architecture of the villous.

‘Variably severe’ architectural change. Similar to a ‘Marsh 2’ lesion

Mild lesion

Villi are unaltered or minimally changed in size with a loss of epithelial cell polarity and marked increase in intraepithelial lymphocytes

Marginal increase in lamina propria region cellularity. Similar to a ‘Marsh 1’ lesion.

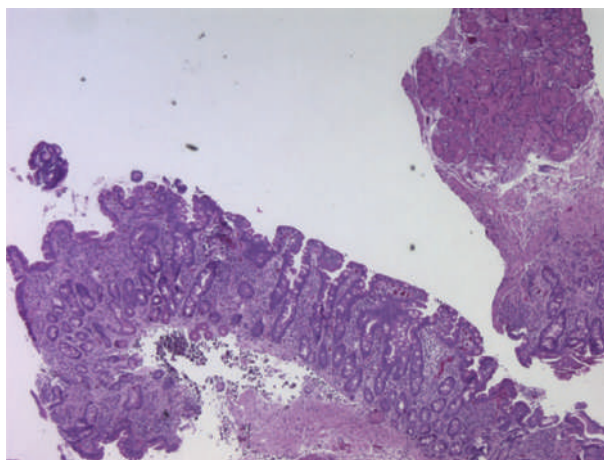


Fig. 2 Endoscopic biopsies showing moderately severe changes of celiac disease, or so-called partial villous atrophy (see text). One fragment also shows Brunner's glands typical of biopsies taken from the duodenum. H & E, magnification $\times 40$.

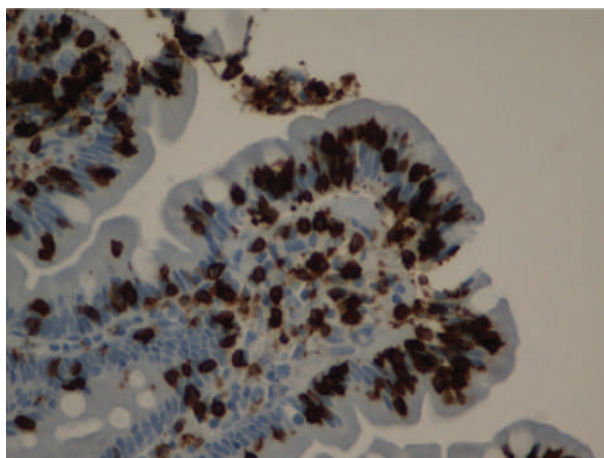


Fig. 3 CD-3-positive intraepithelial and lamina propria lymphocytes in celiac disease mucosa (see text). Immunohistochemical stain, magnification $\times 400$.

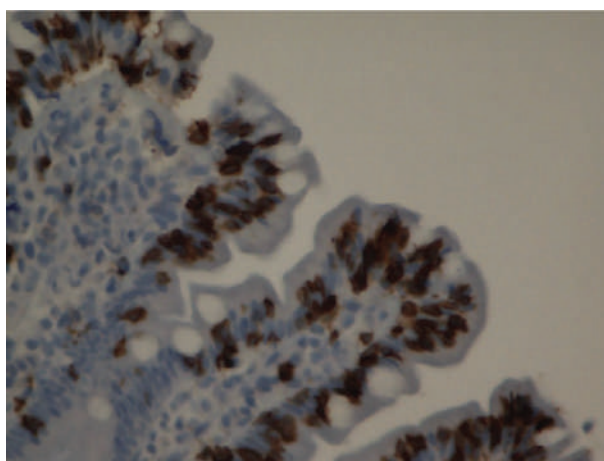


Fig. 4 CD-8-positive intraepithelial and lamina propria lymphocytes in celiac disease mucosa (see text). Immunohistochemical stain, magnification $\times 400$.

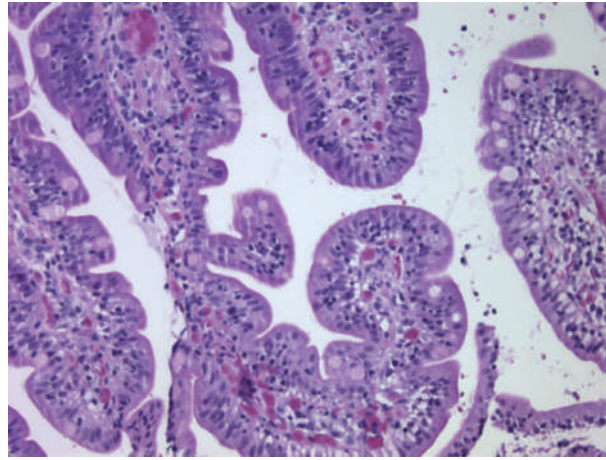


Fig. 5 Endoscopic mucosal biopsy showing mild changes in villous architecture with increased intraepithelial lymphocytes. About 10% of patients diagnosed with celiac disease may have only limited changes (see text). H & E, magnification $\times 200$.

Serological testing has become very useful as a screening measure for celiac disease, particularly newer IgA and IgG antibody methods (i.e., anti-tissue transglutaminase). However, these cannot be relied on solely to establish a diagnosis. In genetically at-risk children, for example, studies have shown the spontaneous disappearance of these antibodies in a normal diet. With immunoglobulin deficiency syndromes, false negative serological testing may also occur (e.g., IgA deficiency). If there is a clinical suspicion of celiac disease, a biopsy should be done to confirm the disorder. This includes serologically positive patients since false positive tests have occasionally been recorded in adults with normal biopsies.

The biopsy changes in classical celiac disease are prominent in the most proximal small intestine, and less so in the more distal small intestine. These changes may also be classified based on the degree of altered architecture or a combination of pathological features. Some degree of intraobserver and interobserver disagreement is common in biopsy interpretation and in the interpretation of the degree of biopsy changes. However, this error factor appears to be more significant with more cumbersome classification schemata, even among expert pathologists with a special interest in the interpretation of intestinal biopsy material.

In classical celiac disease, the architectural changes usually appear as a 'severe lesion' (or 'flat lesion') with rudimentary villi, increased mucosal and intraepithelial inflammatory cells, and hyperplasia of crypt epithelial cells. These changes may revert to normal with a strict gluten-free diet. Sometimes, this improvement may require prolonged periods of time to occur, even months to years, especially in older adults. In some, however, less severe or more variable degrees of inflammatory change are noted. In a 'mild lesion,' only limited alterations in villus architecture may occur, with loss of polarity of epithelial cells and intraepithelial lymphocytosis alone. These changes most often occur in asymptomatic serologically positive patients and family members, particularly first-degree relatives with few or no symptoms. These may also be observed with the closely linked disorder, dermatitis herpetiformis. With a 'moderate lesion,' definitive changes in the structure of the villi are also evident.

Changes in celiac disease also develop along the length of the small intestine. The extent of these inflammatory changes appears to correlate with the severity of the clinical presentation. In classical celiac disease with diarrhea and weight loss, severe biopsy changes may extend well into the jejunum, while only limited changes are present distally in the ileum. Some clinicians have hypothesized that this 'proximal-to-distal' gradient in the severity of histopathological changes is reflective of higher concentrations of dietary gluten (or the offending peptides) in the proximal small intestine. Other 'indirect' effects may result elsewhere in the gastrointestinal tract, including the gastric and colonic mucosa, due to the immune-mediated effects of recirculating memory T cells. Even though the ileum may be virtually normal, prior classical studies using infused peptide solutions through long intestinal tubes demonstrated that the ileum is very sensitive to infused gluten peptides. With more extensive involvement of the small bowel, diarrhea and malabsorption of numerous macro- and micronutrients may occur. In contrast, if the changes are limited to the most proximal small bowel, such as the duodenum, diarrhea and weight loss may not be present. In some, isolated iron deficiency may develop, due in part to localization of most significant pathological effects of celiac disease in the duodenum, the main site of absorption of iron (as well as other nutrients) (Freeman, 2015b).

Administration of a gluten-free diet usually results in resolution of diarrhea and weight gain. Normalization of serological studies may also occur, even though mucosal histopathological changes may still be present. Biopsies also improve, initially in more distal sites of small-bowel involvement and eventually in the proximal small bowel. Prolonged gluten restriction may be needed, however, to define histological improvement, particularly in biopsies from the most proximal small intestine (Freeman, 2017a).

Subclinical disease

This category of celiac disease occurs in at least two distinct forms: occult disease and latent disease. Although genetically susceptible, most patients appear for the first time in their adult years with the disease. It is conceivable that some had full-blown disease from childhood and simply were not recognized, but more likely, some other environmental factor precipitated the disease. For example, the disorder may occur after an infection, usually a viral form of gastroenteritis (e.g., adenovirus and rotavirus). Here, it has been hypothesized that the mechanism for mucosal injury is due to a form of molecular mimicry that plays a role after an initial viral infection in a genetically predisposed individual. A viral peptide sequence might lead to activation of the immune system by consumption of gluten-containing grains having a similar peptide sequence.

In some with subclinical disease that is clinically occult, mucosal involvement may be limited to the most proximal small intestine, and isolated deficiencies of some nutrients (e.g., iron, calcium, and folic acid) may occur without diarrhea or weight loss. Malabsorption of other nutrients is absent because there is more than enough small intestine present. Others with occult intestinal disease may have prominent extraintestinal clinical features, for example skin disorders, dermatitis herpetiformis, or other autoimmune endocrine disorders, such as autoimmune thyroid disease, pituitary disease, or insulin-dependent diabetes (Freeman, 2016a). Some patients may initially present with a malignancy such as a lymphoma or small-bowel carcinoma, and biopsy evidence of celiac disease is detected only later (Freeman, 2009). Other intestinal disorders may be associated with celiac disease, including microscopic forms of colitis or gastritis, including collagenous colitis or collagenous gastritis. In all of these disorders, a predominance of intraepithelial lymphocytes is a key pathological element (Freeman, 2005).

Latent celiac disease is also a subclinical form of celiac disease, first documented in patients with dermatitis herpetiformis or intestinal lymphoma. Although most with dermatitis herpetiformis have a gluten-sensitive small-bowel disorder fulfilling the diagnostic criteria for a diagnosis of celiac disease, some appear to have limited or minimal small-intestinal biopsy changes. In others, the biopsies are normal. In these patients, typical changes of variable severity were induced with a high-gluten diet, demonstrating that the mucosa was gluten sensitive. In both disorders, these gluten-induced changes were then improved with a gluten-free diet.

Other causes of sprue-like biopsy changes

Other disorders may cause severe or variably severe changes in the small-intestinal biopsy and lead to diarrhea and weight loss. However, only celiac disease responds to a strict gluten-free diet (Tables 2 and 3). Some studies, particularly in children, have suggested that oats may be safely consumed by some with documented celiac disease, at least in small amounts, but oats may also be contaminated before consumption by other grains (e.g., during processing). In addition, a specific oats-induced enteropathy has been documented with histopathological changes that mimic celiac disease. Given this concern, many adults with celiac disease elect to also restrict oats from their diet.

Several other disorders may have a similar clinical and pathological appearance to celiac disease. For example, different infectious agents may produce a spectrum of histological changes, including severe and extensive changes typical of celiac disease. In adults, these changes may also be variable in severity and patchy in distribution. Usually, these are temporary, appear to respond to antibiotics, and develop more often in adults than children. Some may be due to an undetected viral agent or bacterial agent that has spontaneously resolved even without antimicrobial agents. With giardiasis, due to the protozoan agent *Giardia lamblia*, for example, a wide spectrum of pathological alteration may be present. Up to 15% may have changes that are so abnormal that celiac disease may be erroneously diagnosed, until, of course, the typical organisms are recognized in fecal material, small-intestinal fluid

Table 2 Classification of sprue syndromes.

<i>Disorder</i>	<i>Treatment</i>
Celiac disease ^a (classical, occult, and latent)	Gluten-free diet
Oats-induced villous atrophy	Oats restriction
Refractory sprue (refractory celiac disease)	Not known
Collagenous sprue (collagenous enteritis and enterocolitis)	Not known
Mesenteric lymph node cavitation syndrome	Not known
Other protein-induced mucosal injury (e.g., soy and milk)	Delete protein
Unclassified sprue (sprue-like intestinal disease)	Not known

Each of these may cause severe ('flat') or variably severe biopsy lesions. Refractory celiac disease requires evidence of an initial gluten-free diet response. In unclassified sprue or sprue-like intestinal disease, no response to a gluten-free diet could be defined.

^aCeliac disease has also been termed 'celiac sprue' or 'gluten-sensitive enteropathy.'

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Table 3 Causes of sprue-like biopsy changes.

Disorder	Treatment
<i>Infectious disorders</i>	
Infections (e.g., parasite, protozoan, and mycobacteria)	Treat specific agent
Tropical sprue	Antibiotics and folic acid
Stasis syndrome (contaminated bowel syndrome)	Antibiotics
Whipple's disease (due to <i>Tropheryma whipplei</i>)	Antibiotics
<i>Deficiency syndromes</i>	
Nutrients (zinc, vitamin B12, and folic acid)	Replace specific agent
Malnutrition (including kwashiorkor)	Adequate diet protein
Immune deficiency syndromes (transplant, HIV, and congenital)	No specific treatment
<i>Others</i>	
Intestinal lymphangiectasia	Not known
Crohn's disease (with duodenal involvement)	No cause known
Graft-versus-host disease	Treat graft rejection
Immunoproliferative disease (lymphoma)	Often chemotherapy
Macroglobulinemia	Often chemotherapy
Zollinger–Ellison syndrome (with high luminal acid)	Antisecretory therapy
Drug-induced small-bowel injury (see Table 4)	Remove drug

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aspirates, or even the biopsy per se. Other protozoan agents that can cause severe or variably severe changes in the small-intestinal mucosal architecture include *Isospora belli*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, and microsporidians, including *Enterocytozoon bieneusi*. In the acquired immune deficiency states that follow either transplantation or retroviral infection (e.g., HIV), a severe enteropathy may occur. The precise cause of the biopsy changes may be difficult to determine in this setting because of the propensity for other concomitant infectious agents. Sometimes, multiple agents may be present. In HIV infection causing AIDS, it has been speculated that the retroviral agent may be directly responsible, or, alternatively, that an immunologic dysfunction indirectly results. These changes may be associated with superimposed malnutrition or other infectious agent(s) to cause the small-intestinal pathological changes. *Mycobacterium avium-intracellulare* infection frequently occurs in AIDS and forms a distinctive pathological lesion with severely altered architecture. Foamy macrophages, located mainly in the lamina propria region, positively stain for acid-fast organisms. These also develop a positive staining reaction with the periodic acid–Schiff reagent, reminiscent of the staining pattern in Whipple's disease. However, with the latter disorder, now recognized to be caused by Whipple's bacillus (i.e., *Tropheryma whipplei*), acid-fast stains are negative. Parasites may also cause severe but patchy or variable changes, and some may be morphologically identified in the same biopsy specimen or fecal material (e.g., *Strongyloides stercoralis*, hookworm, and *Schistosoma* or *Capillaria* species). Some viral agents (e.g., cytomegalovirus) may be seen in the small intestine, often in patients with a compromised immune system. Bacterial overgrowth, or a contaminated small-bowel syndrome, may also be superimposed on celiac disease, and some have suggested that an empirical course of a broad-spectrum antibiotic may be sufficient to resolve changes.

Other disorders may cause diarrhea with or without malabsorption and may cause severe changes in small-intestinal biopsy architecture. Distinctive pathognomonic changes may be detected with lymphangiectasia, macroglobulinemia, abetalipoproteinemia, amyloidosis, lipid storage disorders, and radiation injury. In addition, some medications may cause sprue-like small-intestinal mucosal changes, but respond completely to removal of the offending agent (Table 4). One medication that is no longer available

Table 4 Drug-induced sprue-like small-bowel disease^a.

Drug class	Example
Nonsteroidal anti-inflammatory drugs (NSAIDs)	Sulindac
Immunosuppressives	Azathioprine
Antimicrobials	Neomycin
Chemotherapeutic agents	Busulfan
Vinca alkaloids ^b	Vincristine
Antimetabolites	Methotrexate
Angiotensin II receptor antagonists	Olmesartan

^aDrugs listed are agents that may cause a small bowel lesion that may mimic untreated celiac disease.

Removal of the offending agents results in resolution of small intestinal changes (Freeman, 2014).

^bVincristine also induces a small-intestinal enteropathy with some features of celiac disease; however, it may be distinguished by its stathmokinetic effects, causing prominent metaphase arrest from mitotic blockade.

for clinical use, triparanol, was previously used to create changes similar to those of celiac disease for research purposes. The precise mechanism for these drug-induced changes is not known.

Olmesartan enteropathy has not only been shown to histologically mimic both celiac disease (Freeman, 2016b), but also the closely associated small bowel mucosal disorder, collagenous sprue (Freeman, 2020).

Crohn’s disease may cause duodenal mucosal granulomas, but severe architectural changes without granulomas, indistinguishable from untreated celiac disease, may also occur in the proximal small intestine. In Crohn’s disease, these changes do not improve with a gluten-free diet.

Another more recently recorded entity is an unusual and relatively distinctive diffuse proximal small-intestinal enteritis that occurs rarely in patients following colectomy for extensive ulcerative colitis or Crohn’s colitis (Freeman, 2019). In these, a gluten-free diet has no effect, and, occasionally, powerful immune suppression with high-dose corticosteroids or a calcineurin inhibitor is needed.

Refractory celiac disease

In celiac disease, diarrhea or malabsorption may recur. Although the mucosa may initially respond to a gluten-free diet, severe or moderately severe histopathological changes may recur. A broad array of possible causes should be considered (Table 5). Most commonly, gluten is being consumed. This may be due to an intentional failure of compliance, or it may be completely unintentional and unrecognized. Gluten is found in many ingested sources, including pill capsules and communion wafers. There may also be a difference in tolerance to gluten among patients, with a minimum threshold in some patients.

In some with celiac disease, an unrecognized infectious cause may be superimposed. Alternatively, celiac disease may predispose to a nutrient deficiency that may cause pathological changes per se. Folic acid deficiency or zinc deficiency may cause independent histopathological changes that may be difficult, in themselves, to confidentially differentiate from celiac disease alone. In patients with long-standing celiac disease, pancreatic exocrine insufficiency may be superimposed, especially if there is concomitant malnutrition. In others, the original diagnosis may have been incorrect. Documentation of a response to a gluten-free diet is critical, since the biopsy alone is not diagnostic. In Crohn’s disease with involvement of the duodenum, for example, pathological changes fail to respond to a gluten-free diet (Freeman, 2008).

Occasional cases of celiac disease appear to be complicated by other disorders, such as collagenous sprue (Freeman, 2005). This is a rare entity and may be detected without a definite background of celiac disease. Usually, there are features of severe pan malabsorption of multiple nutrients. Biopsies reveal a pathologically distinctive subepithelial band of collagen that may be demonstrable with histochemical staining reactions (e.g., trichrome) and electron microscopy showing the typical 640A bandwidths of collagen fibers. Long-term parenteral nutrition support may be needed. Free perforation of the small intestine or lymphoma has been recorded in collagenous sprue. Another descriptive entity has been recorded that complicates celiac disease with recurrent pathological changes of variable severity, splenic hypofunction, and cavitation of mesenteric lymph nodes. In this disorder, lymphoma has been recorded.

Lymphoma, and very rarely other malignancies (e.g., small-bowel adenocarcinoma), may complicate celiac disease. Both B-cell and T-cell types have been described in celiac disease. In tertiary care centers, an overall rate of 8–10% has been recorded, but, in recent years, the lymphoma risk appears to be falling, in part possibly because serological screening studies have increased the detection rate for celiac disease in those with limited symptoms. Age at the time of the initial diagnosis of celiac disease may be a critical factor. If celiac disease is initially diagnosed late in life, lymphoma may occur more often. Duration of the disease prior to initiation of a gluten-free diet may be important. Most often, lymphoma develops in the jejunum, but localization in the ileum can occur. Colonic and gastric lymphomas have also been described. Usually, lymphomas produce an ulcerating or stenosing and obstructing tumor. Rarely, lymphoma may be diffuse and multifocal with only mucosal localization. If erosions or ulceration are present in the small intestine, particularly the duodenum, lymphoma may be exceedingly difficult to diagnose; Crohn’s ulceration or so-called ulcerative jejunoileitis may be recorded. In some of these, only later, frankly neoplastic lymphoma cells may be detected that were previously difficult to appreciate in an inflamed small intestine. Free perforation of the small intestine may be due to a

Table 5 Causes of refractory celiac diseasea.

Limited gluten-free diet compliance ^a
Unrecognized source of dietary gluten
Superimposed cause (e.g., folic acid or zinc deficiency)
Second cause for symptoms (e.g., infectious diarrhea)
Wrong initial diagnosis (e.g., Crohn’s disease involving the duodenum)
Associated disease (e.g., collagenous sprue or collagenous colitis)
Complicating disease (e.g., lymphoma, specifically T-cell enteropathy)

^aFailure to respond initially to a gluten-free diet suggests ‘sprue-like’ intestinal disease or unclassified sprue, not refractory celiac disease.

lymphoma, and it should be suspected if celiac disease is known to be present. Diagnosis of lymphoma may be challenging. In some, full-thickness biopsies may be needed, or evaluation using flow cytometry, special immunohistochemical studies, or polymerase chain reaction.

Sprue-like intestinal disease or unclassified sprue

This refers to a severe ('flat') or variably severe (moderate to mild) mucosal lesion that has not responded to a gluten-free diet. Although some with this entity have been labeled as having 'refractory celiac disease,' a response to a gluten-free diet has not been documented. Usually, repeated biopsies have been abnormal despite a gluten-free diet. Most continue to have symptoms and fail to show any clinical response to a gluten-free diet. In some, dietary compliance may be difficult to prove and histological improvement may be difficult to document, especially with duodenal biopsies alone. This may represent a heterogeneous group of small-bowel disorders, a 'wastebasket group' with no definitive cause. It has been speculated that some may have a 'resistant form' of celiac disease, while others may have a 'difficult-to-diagnose' lymphoma. In some, an abnormal subset of intraepithelial lymphocytes has been described, suggesting an important prognostic marker for later lymphoma development.

Genetics

Celiac disease is a heterogeneous disorder, reflected not only by its differing clinical presentations but also pathologically with different degrees of architectural change and variable extents of inflammatory change along the length of the small intestine. Some serologically positive patients may have architecturally normal small-intestinal mucosa, although minimal changes with only a slight increase in the number of intraepithelial lymphocytes may be evident. Recent studies have reported that immunohistochemical evidence of immune activation alone may be present. Traditionally, a gluten-free diet has not been recommended if only an architecturally normal small intestine is present, but increasingly sophisticated immunochemical or other laboratory methods may eventually result in an expanded definition of the limits of 'normal' requiring more intense clinical monitoring.

It has been estimated that approximately 40% of the heritability of celiac disease can be explained on the basis of the human leukocyte antigen heterodimers, HLA-DQ2 and HLA-DQ8. These are believed to be necessary to develop celiac disease, but they are not, in themselves, sufficient for phenotypic expression of the disease. Other non-HLA genes may also be involved. One may relate to genetic variants on chromosome 19, in the myosin IXB gene (i.e., MYO9B), and may potentially predict the responsiveness of celiac disease to a gluten-free diet. Other genetic studies have identified a total of nine non-HLA loci that contribute to celiac disease risk (Gujral et al., 2012).

Celiac disease-associated HLA-DQ molecules serve to bind and present gluten peptides to antigen-specific T cells in the intestinal mucosa. These induce T-cell proliferation and cytokine secretion. Siblings with shared HLA haplotypes have a greater likelihood of concordance with celiac disease than those without shared haplotypes. It has also been hypothesized that specific genetic markers might have value in predicting the clinical course of disease. For example, two copies of DQB1*02 have been associated with a greater disease risk, but they were not predictive of an earlier age of onset or the severity of the disease. Finally, in twin studies, with one twin being biopsy defined, the cumulative concordance in the second twin was almost 80% for symptomatic or 'silent' forms of celiac disease within 5 years.

Pathogenesis

Gliadins, from an alcohol-soluble fraction of gluten, are storage proteins in wheat, barley, and rye (along with other gluten-containing grains). All gliadins have a high glutamine and proline content. Glutenins are insoluble in alcohol and differ in their biochemical structure from gliadins. It has been hypothesized that the initial immune reaction in celiac disease patients is focused on several of these gliadin peptides, while the long-standing inflammatory response may be defined by gliadin peptides that have been deamidated or cross-linked by tissue transglutaminase. These latter peptides are also more tightly linked to HLA-DQ2 and HLA-DQ8 (Gujral et al., 2012).

The tissue transglutaminase-catalyzed changes in gliadin are not restricted to single gliadin types. For example, prolamines in barley and rye are known as hordelin and secalin, respectively, and these may induce a messenger RNA-mediated interferon-gamma response in the small-intestinal mucosa of celiacs. An alpha-2-gliadin-33-mer appears to pass through the enterocyte brush border membrane by a dose-dependent translocation mechanism, and this is further enhanced by interferon-gamma, possibly mediated by a delayed epidermal growth factor endocytosis. In active celiac disease, this immune-mediated response is very complex and involves many different mediators that are still being studied. For example, a marked accumulation of specific Th-1 immune cells occurs that produces interferon-gamma. A number of other transcription factors and other mediators (e.g., interleukin-21) may be increased in celiac disease. Interleukin-21 is a key regulator, being present in activated CD4 + T cells as well as in natural killer T cells, and these cells, in turn, regulate the production of cytokines by different T-cell subsets.

With genetic susceptibility, gliadin may interact with enterocyte tight junctions and cause their disassembly. Gliadin peptides may bind to the chemokine receptor, CXCR3. This induces zonulin release from the tight junction region and causes a

resultant increase in intestinal permeability. Permeability increases have also been demonstrated prior to the onset of clinically apparent celiac disease, and even with a gluten-free diet, this altered tight junction permeability may not completely return to normal (Gujral et al., 2012).

Another important central or superimposed component to the pathogenesis of celiac disease may involve the luminal intestinal microflora. It has been hypothesized that these normally present resident intestinal microbes may play a critical role in the expression of the disease. Alternatively, it has been suggested that some infectious agents may actually have contributed to a Swedish childhood epidemic of celiac disease. These infectious agents, however, may not be restricted to the luminal microbiome, but instead the luminal virome. In a recent report, reovirus, a normally innocuous viral agent, was observed to trigger inflammatory responses to dietary antigens and development of celiac disease (Bouziat et al., 2017).

Treatment

At present, the treatment of celiac disease remains a lifelong gluten-free diet. However, the definition of a gluten-free diet may differ from one nation to another. The World Health Organization has proposed that gluten-free foods should not contain more than 200 ppm of gluten. Each individual celiac, however, may also have a unique genetically determined threshold or tolerance to dietary gluten. It is believed that a daily intake of less than 10 mg will not cause significant histological changes in the small intestine of celiac disease patients. A continuing difficulty in management relates to monitoring of compliance with a gluten-free diet. For most who adhere to a gluten-free diet, intestinal symptoms resolve, serological parameters normalize, but biopsies, especially if obtained only from the most proximal small intestine, may take prolonged periods to improve, sometimes months or years (Freeman, 2017a, b). It appears that normalization of the biopsy changes also requires longer periods in elderly celiacs, especially if the disease was initially diagnosed late in life.

Most celiacs who carefully follow their gluten-free diets respond well to the diet alone; however, there may be a role in some individuals for added supplementation with micronutrients. For example, a recent double-blind placebo controlled clinical trial demonstrated that supplementation with vitamin B provided significant improvement in general well-being. In others, iron, folic acid, zinc, and calcium supplements may be considered.

Gluten-free products are costly and difficult to readily obtain in some countries. As such, a number of alternatives to the gluten-free diet are being investigated. To date, none have been recommended as a sole treatment strategy. Some in clinical trials, or considered in a discovery phase, have included orally administered enzymes (e.g., endopeptidases), antagonists to S100B protein, interleukin-15 blockers, elemental diets, and transamidation of wheat flour. Lactobacilli, added to sourdough for fermentation, may also serve to hydrolyze the proline- and glutamine-rich gluten peptide (Gujral et al., 2012).

Oral prolyl-endopeptidases may digest ingested gluten and, theoretically, might represent a new approach to treatment for celiac disease. These proteases may be derived from different bacterial sources and function by reducing the quantity of immunostimulatory gliadin peptides present in the small-intestinal lumen. Other approaches include the modification of gluten proteins to eliminate their capacity to stimulate interferon-gamma release from CD4 + T cells or, alternatively, impair gluten-reactive T cells through an immunotherapeutic method, such as vaccination. Polymeric binders examined using in vitro model systems and in transgenic mice have been shown to reduce the deleterious effects of gliadin on the intestinal epithelial cells.

Another focus has been to alter the action of antigen presenting cells, particularly a unique subset of dendritic cells responsible for local activation of gluten-reactive T cells in celiac disease. Enteric glial cells have been shown to release neurotropic factors and appear to be activated by inflammatory insults. A protein released from these cells in the duodenum, S100B protein, is reduced on a gluten-free diet and may be a useful product that can be used to interfere with the usual immune response.

Finally, another investigative area has focused on intestinal permeability changes that may be increased in celiac disease associated with altered tight junction proteins, specifically zonulin. AT-1001 is an inhibitor of paracellular permeability and reduced increased permeability in celiac disease challenged with gluten. AT-1001 was also shown to diminish the anticipated rise in interferon-gamma levels with gluten feeding and so may have significant theoretical potential for treatment of celiac disease.

Conclusion

Celiac disease is an immune-mediated small-intestinal mucosal disorder associated with intolerance to gluten peptides in genetically predisposed individuals. Although the disorder classically causes diarrhea, malabsorption, and weight loss, it may also be associated with other autoimmune disorders and suspected with serological screening. The disease affects 0.5–2% of populations in some nations examined to date, but the most significant risk factor is established celiac disease in an immediate family member, particularly a sibling. Definition of the disease includes an intestinal biopsy before treatment with a gluten-free diet along with documentation of a response to a gluten-free diet. Serological testing may provide added evidence that celiac disease is present and may potentially be useful for monitoring gluten-free diet compliance. The disorder has a complex pathogenesis that may ultimately lead to alternative forms of treatment.

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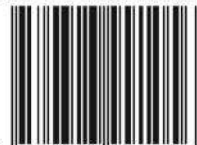
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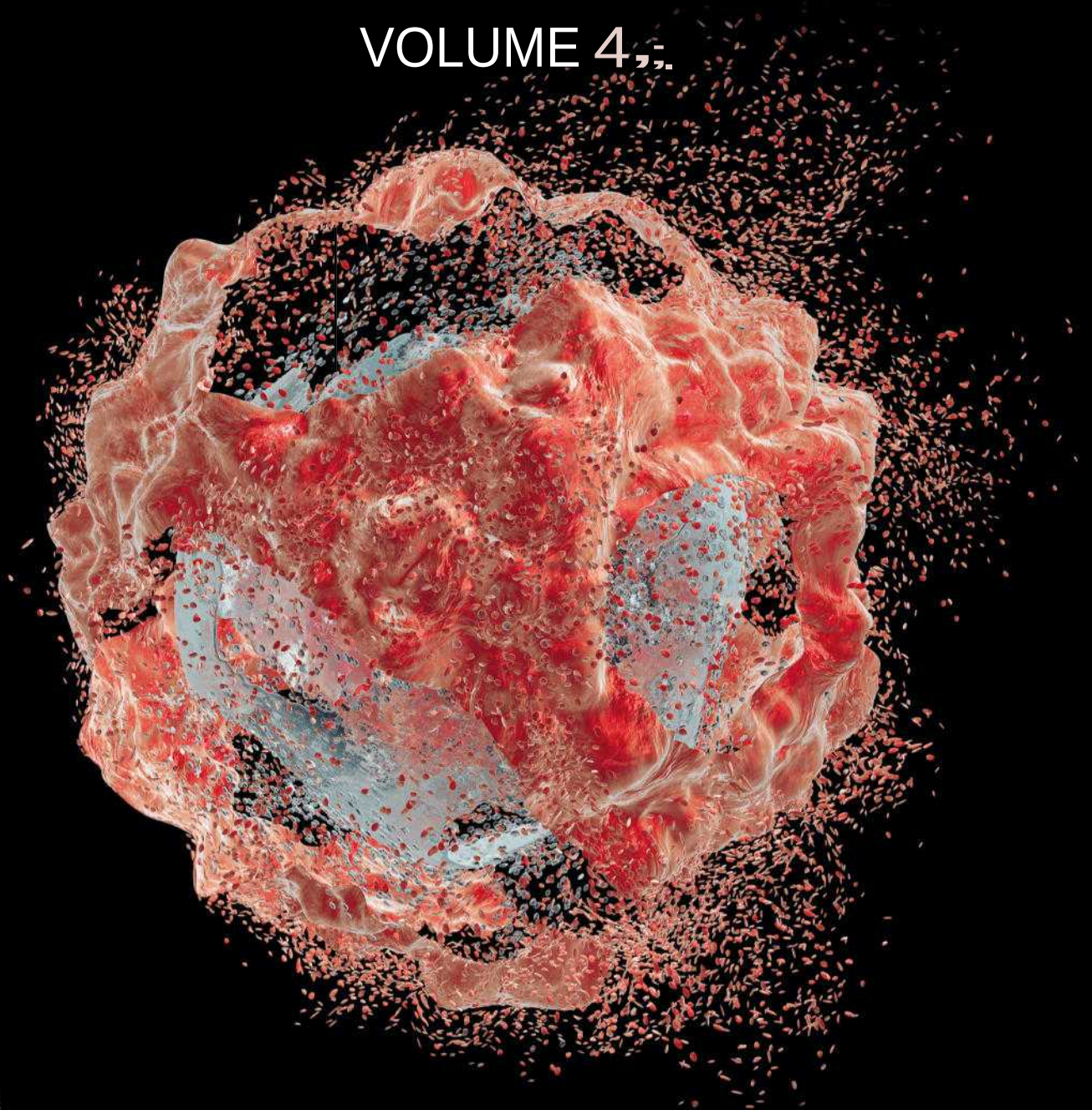


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FOREWORD

It is a great honor to be asked to introduce, with this foreword, the *Encyclopedia of Infection and Immunity*. This work will be read and enjoyed by many students, both graduate and postgraduate, including clinicians, laboratory practitioners, and scientists. Many colleagues have contributed to this important work, with over 230 original chapters assembled in sections and listed alphabetically. I offer my warm congratulations to the editors of this book, for soliciting a wide body of expertise and assembling it in a form that gives so much pleasure and information to the reader.

For those of us who work in the field of infection and immunity, the last two years have been exceptionally challenging. At the current time we are 22 months into a major pandemic caused by a novel coronavirus and its variants, and worldwide, sadly, there have been many hospitalizations and deaths. Clinicians and scientists in our field have been working flat out, caring for patients, generating or utilizing diagnostic tests, generating new knowledge in immunity to coronaviruses, or discovering, testing, and deploying new vaccines. Public Health practitioners have organized our medico-social responses to limit transmission and deal with the consequences of infection. Behavioral scientists have transformed our approach to informing the public, getting the best possible outcomes from effective communication. Governments have learned the danger of ignoring the expertise of scientists, and in many countries, the public look upon clinicians and scientists with gratitude and respect. It is a testament to the advancement of infection and immunity science that the genome of the virus was sequenced within a few weeks of COVID-19 emerging, and that within 12 months, the first trials of novel vaccines had been completed. These vaccines included some with exceptionally novel technologies, including the RNA vaccines and the viral-vectored vaccines.

Underpinning the academic agility of students and practitioners in Infection and Immunity is the universal strength of sound education. Readers of this book will be seeking the knowledge they need for a comprehensive understanding of infectious disease and immunology. We work in a fascinating discipline. Our field is unique in that pathogenesis involves at least two sets of genomes—on the one hand the microbe, and on the other, the host. The microbial genome programs its microenvironment to enable acquisition of and residence in a niche for sufficient time to replicate and further disseminate to other hosts. The human genome has evolved to tolerate and contain a wide range of symbionts and pathobionts, with highly sophisticated immune decision-making at the cellular level, but to respond to invading species with an elaborate coordinated repertoire of innate and acquired immunity. The complex conversation between many different microbes and the human host generates a wide range of disease phenotypes. This makes the job of an infectious disease physician so challenging but at the same time so fulfilling.

Our field is enriched by the many scientists and clinicians who are willing to share their experience with their peers and students. This book reflects the great variety of knowledge that has emerged over centuries of inquiry and endeavor, and I believe you will be rewarded by using it as one of your reference sources.

Robert C. Read

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PREFACE

Although infections have been one of the most important health threats since centuries ago, the mechanisms of human defense against infections have been unclear until last century. The scientific world has remarkable progresses in the field of immunology during recent decades. Indeed, the novel discovery of genes related to different pathogens has enhanced our knowledge about the immune system. Understanding different pathogens and the immune mechanisms promote diagnostic strategies and design more efficient therapeutic agents.

Encyclopedia of Infection and Immunity is a comprehensive text, which covers topics not only on basic immunology and microbiology but also on clinical immunology and infectious diseases. Section 1 focuses on the immune system, while Section 2 describes the interaction between pathogens and immunity. Bacteria, viruses, fungi, protozoan parasites and helminths, and insect and mites are explained in Sections 3–6, respectively. Section 7 discusses the clinical infectious disease challenges, whereas clinical immunology is covered in Section 8. Laboratory tests and treatment protocols are the main focuses of Sections 9 and 10, respectively.

Encyclopedia of Infection and Immunity is the result of valuable contribution of more than 400 scientists from many well-known universities/institutes worldwide. I would like to hereby acknowledge the expertise of all contributors, for generously devoting their time and considerable effort in preparing their respective chapters. I would also like to express my gratitude to Elsevier for providing us the opportunity to publish this Encyclopedia.

Finally, I hope that this comprehensive book will be of special value for researchers and clinicians who wish to extend their knowledge on infection and immunity.

Nima Rezaei, MD, PhD

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Introduction on Laboratory Tests for Diagnosis of Infectious Diseases and Immunological Disorders

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Introduction

Laboratory tests are extremely valuable in validating a diagnosis, predicting disease severity, and also in following up on the disease activity in patients who are suspected of having infectious diseases or immunological disorders. Diagnostic reliability and health benefits could be considered as two important factors when testing for such diseases. Meanwhile, timely diagnostic assessment and implementation of accurate tests are highly valuable in disease management (Caliendo, 2013). Microbiological methods, including culture and staining, have previously become the gold standard for detecting infectious diseases. These techniques can directly detect microorganisms such as bacterial, viral, parasitic, or fungal agents in fluids or tissues of the patient. Although these tests are reliable, characteristics such as time-consuming testing hamper quick diagnosis (Laupland and Valiquette, 2013). Currently, utilizing indirect laboratory techniques to evaluate infectious diseases is of utmost importance for detecting specific antibodies or pathogen antigens in blood or body fluids. Several laboratory methods have been designed for detecting either pathogens or specific antibodies in the suspected subjects. Pathogen isolation and serological methods are the conventional diagnostic tests that are widely applied to recognize the cause of a disease (Amar, 2014). Furthermore, molecular diagnostics became an important tool in healthcare in the early 1990s, when specific monoclonal antibody technology was developed. Molecular tests have higher sensitivity

and specificity compared to traditional methods. These methods can be helpful in the surveillance and control of infectious diseases, but more precise and sensitive diagnostic methods are still required (Lu et al., 2020). In this article, we will review the most important laboratory tests that are widely used to diagnose infectious diseases or various immunological disorders, including autoimmune and immunodeficiency disorders.

Diagnostic laboratory tests: Focusing on infectious diseases

Direct detection of microorganisms: Culture, staining, microscopy

Culture is usually the gold standard for identifying organisms, but results can take days or weeks. Furthermore, because culture cannot be performed for all infections, alternative tests are required. Following culture and identifying the organism, evaluation of the pathogen's susceptibility to antimicrobial drugs can also be performed. This can be helpful in implementing the appropriate antimicrobial therapy. Molecular methods can also be used to find particular resistance genes.

In order to identify a pathogen and microscopic detection, different staining methods can be used. Gram staining (using crystal violet dye) is frequently used in microbiology laboratories to classify bacteria into gram-positive or gram-negative and to characterize bacteria morphology (e.g., cocci, bacilli) (Srinivasan et al., 2012). Another staining method known as acid-fast staining (or Ziehl-Neelsen staining) is used to identify mycobacterium species. Carbol fuchsin is used in acid-fast staining that can dissolve the lipid components of the Mycobacteria cell wall. Using heat allows further penetration and therefore dye entrance into the cytoplasm. As a result, all cells appear red. Acid fast cells remain red following the decolorization step, while non-acid fast organisms become colorless. So, the colorless cells appear blue following staining with methylene blue in the next step. Therefore, the red and blue colors represent acid-fast and non-acid fast bacteria, respectively (Lamanna, 1946).

Serological methods: Assay based on antigen-antibody reaction

"Max von Gruber" was the first to use serologic methods to diagnose infectious agents (Von Gruber and Durham, 1896). He used sera from infected animals and humans to agglutinate typhus and cholera bacteria. The invention of the complement fixation procedure by "Bordet and Gengou" in 1901 is also regarded as another milestone in detecting infectious agents through serologic methods (Silverstein, 2009). Serologic tests are simple and inexpensive methods that provide immediate results. Serologic tests for infectious diseases can provide information about the disease even when there is no virus or its components in the sample. In addition, these tests can also confirm the presence of infection in asymptotically infectious diseases (Vainionpää et al., 2015).

In general, serologic tests are based on the specific interaction between antigen and antibody. Antigens can be a part of a pathogen like bacteria and viruses or a non-pathogenic agent such as red blood cells (RBC), hormones, tissue proteins, etc. Binding of antigen to an antibody is a specific reaction that can be influenced by several factors such as the pH of the environment (optimum pH is approximately 7.2), ionic strength, concentration ratio of antigens and antibodies, environment's temperature, and incubation time (Vainionpää et al., 2015). Optimal reaction between antigen and antibody and forming antigen-antibody complexes can occur in the appropriate condition. Importantly, the higher or lower concentration of either antibody or antigen causes false negative results. Higher concentrations of antibodies, for example, in a variety of tests such as Wright and Widal (serologic tests for the detection of Brucellosis and typhoid, respectively), can result in a phenomenon known as the "Prozone effect," in which the ability of antibodies to form immune complexes is impaired. Similarly, the excess concentration of antigens in tests such as CRP-latex (for detection of C-reactive protein in the serum) can result in the "Postzone effect" causing inappropriate formation of antigen-antibody complexes (Jin and Zehnder, 2016).

Serological diagnosis is generally based on the production of specific antibodies in the sera or other body fluids. IgM is the first class of antibody produced and peaks at 7–10 days following the disease occurrence, and then begins to decrease. The IgG antibody is then produced and persists for a long time after infection, facilitating the detection of infection. Therefore, an increase in IgG antibody titer over a period of about 4 weeks can usually be a sign of infection. For this reason, taking two samples from the patient at intervals of 1–4 weeks will usually be valuable in diagnosing the disease through serological tests (Vainionpää et al., 2015). Although these tests are very useful, they also have disadvantages or limitations. In certain infections, the antibody response is insufficient, or the antigens used in the test have a limited specificity, making the findings difficult to interpret. The serological response in immunocompromised patients is often too weak. In neonatal infections, the presence of maternal antibodies may make it difficult to demonstrate a response in the infant. Therefore, other diagnostic procedures should be performed in these contexts.

There are various types of serological tests that can detect antibodies or antigens in a sample. These tests can be categorized based on the following properties:

- 1) Antigen nature: including agglutination tests, precipitation tests, flocculation tests.
- 2) The function of antibodies: including neutralization test, complement fixation, hemolysis-based tests.
- 3) Labeling techniques: including ELISA, IFA, RIA, CLIA.
- 4) Blotting test: including western blotting.

In the following part we will review these tests with more details.

Agglutination tests

Agglutination occurs when an insoluble or particle antigen interacts with an antibody. A positive reaction can be detected macroscopically in a short time. However, the antigen-antibody complex may be seen with the naked eye if the complex size is large. Both IgG or IgM could be involved in the agglutination reaction. So, this test is not appropriate for distinguishing between these two classes of antibodies (Smits et al., 2000). Agglutination reactions can be divided into three categories based on the nature of antigenic determinants in antigen molecules (Oss, 2000).

- 1) Direct (active) agglutination: in this reaction, the antigenic determinants are the intrinsic component of an insoluble (or particle) antigen (Fig. 1A). For example, in bacterial agglutination assays such as the Wright and Widal tests, killed *Brucella* and *Salmonella* bacteria used to diagnose Brucellosis and Typhoid (enteric fever), respectively. In fact, in this test, dead or inactivated bacteria suspensions are used as antigens. In this test, a dilution series of the patient's serum is incubated with a defined antigen suspension. This could be done in tubes, microscope slides, or microtiter plates. A positive reaction occurs when antibodies are present in the serum. Other examples include the hemagglutination assay for identifying blood groups and the Paul Bunnell reaction for diagnosing infectious mononucleosis (Kim et al., 2015; Park et al., 2012; Tilton et al., 1988).
- 2) Indirect (passive) agglutination: in this type of agglutination, the antigen is soluble (not a particle) that binds to insoluble particles such as polystyrene latex, gelatin particles, or erythrocytes (Fig. 1B). Examples include the RA-latex test for detecting rheumatoid factor in the serum. Antibody (IgG) against rheumatoid factor is bound to a latex particle to detect rheumatoid factor in the patient's serum. Rheumatoid factor is mostly a pentameric IgM, which is found in the serum of patients with rheumatoid arthritis. The principle of the CRP-latex test is also based on indirect agglutination in which anti-CRP antibodies are attached to the latex particles. Therefore, the presence of C-reactive protein (CRP) in the patient's serum leads to a positive test (Chandra et al., 2014; Plotz and Singer, 1956).

Another example is the pregnancy test through the detection of human chorionic gonadotropin (hCG) in the urine (Fig. 1C). This test is based on agglutination inhibition, in which two forms of antigen, soluble and insoluble, compete for a specific antibody. hCG, a hormone produced during pregnancy, is a "soluble" protein and can be detected in serum or urine for pregnancy diagnosis. In this test, suspected urine is firstly mixed with specific anti-hCG antibodies. Subsequently, the latex particles coated with hCG (hCG-latex) are added to this mixture. If hCG is absent in the urine, the specific antibodies against the hormone remain free, and later, when hCG-latex particles are added, these antibodies form a complex with hCG-latex and therefore agglutination can occur. Therefore, if agglutination is observed, the test is negative. On the other hand, if hCG is present in the urine, it first binds to specific antibodies but their complex is not visible and cannot be detected because of the solubility of hCG hormone. The hCG-latex particles that are added to this complex in the next step remain free due to the lack of specific antibodies against them and therefore agglutination does not occur (Santomauro and Sciarra, 1967).

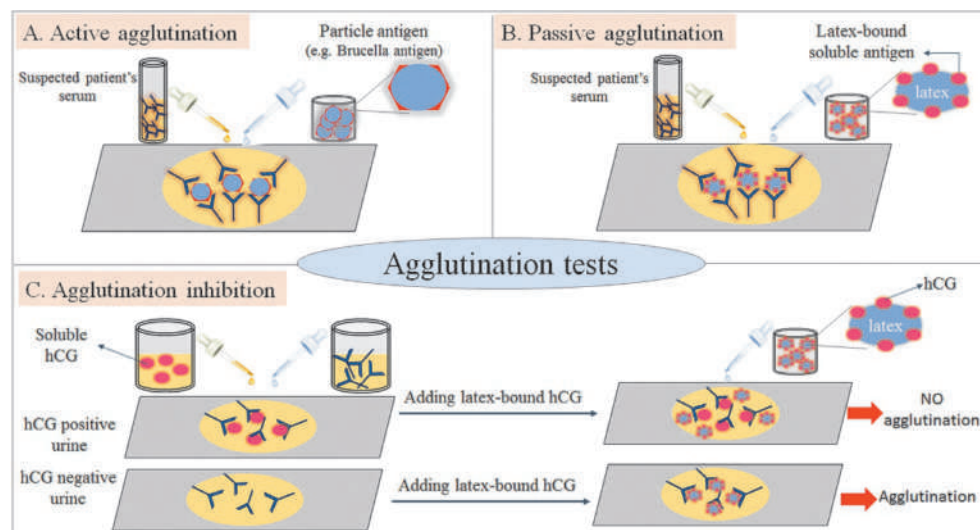


Fig. 1 Three types of agglutination tests. (A) Direct agglutination: interaction between particle (insoluble) antigen and antibodies results in the formation of large agglutinated particles which can be observed with the naked eye or by a microscope; (B) In the indirect agglutination method, soluble antigens are bound to an insoluble particle such as latex to make the reaction visible; (C) In the agglutination inhibition test, such as detection of hCG in the urine (in a pregnancy test), soluble and insoluble antigens are competing for binding to antibodies. In a pregnant person, soluble hCG in the urine binds to the anti-hCG antibodies in the first step. But, this reaction cannot be seen because of the solubility of the formed immune complexes. hCG coated latex is added in the next step. Since antibodies are bound to urine's hCG in the first step, therefore, no free antibodies remain to react with hCG-coated latex particles. As a result, the outcome of agglutination is negative. In contrast, the lack of hCG in the urine of a non-pregnant person causes the antibodies to remain free in the first step. Therefore, by adding latex particles, the agglutination occurs. hCG, human chorionic gonadotropin.

Precipitation tests

Precipitation refers to serological reactions in which the antigen is soluble. These tests can be performed on a slide, in a tube, or on agar or gel for the detection of antigen or antibody. The immunodiffusion in agar gel is the most frequent test based on precipitation. Two common types of immunoprecipitation assay include single radial immunodiffusion (SRID or Mancini method), and double immunodiffusion (DID or Ouchterlony method). In DID, antigens and antibodies are placed separately in two wells punched on the agar gel. After incubation in an appropriate condition (e.g., at 25 °C for at least 24–48 h in a humidity chamber), both antigens and antibodies are diffused in the agar and may react once they interact. In SRID, one component, for example an antigen, is placed in a pre-punched well in the agar containing antibody (antibodies are dissolved in the agar before it is placed on the slide). Therefore, the precipitation lines are formed if the antigen-antibody complex is made. An amido black staining may be used to make the precipitates more visible. These tests are rapid and simple, but they are relatively insensitive and non-quantitative. However, they can be used for the detection of either antigens (e.g., viral, bacterial) or antibodies in infectious diseases (Oss, 2000).

Flocculation tests

Flocculation refers to a reaction between a colloidal antigen and an antibody resulting in the formation of flaky and coarse immune complexes. Cardioliipin antigen is an example of a colloidal antigen that is used in the VDRL test to diagnose syphilis. Cardioliipin is prepared from beef heart extraction and is used to detect syphilitic reagin in the patient's serum. In VDRL, a suspension containing cardioliipin, lecithin, and cholesterol is added to the patient's serum. If the antibody is present in the serum, it interacts with antigenic solution and the formed immune complexes are then observed simply by a microscope. VDRL can be performed on serum or CSF samples (Vogelsang and Haaland, 1951).

Neutralization tests

Neutralization tests are based on the binding of antibodies to the pathogen or to toxins, resulting in the inhibition of their biological properties. This method can be applied to detect pathogens (with defined immunosera) or their toxins or to identify antibodies (with defined pathogens). An example the anti-streptolysin O (ASO) test for detecting antibody against streptolysin O (SO) in the patient's serum produced following streptococcal infections. The ASO test can be used for diagnosing post-streptococcal sequelae such as rheumatic fever or acute glomerulonephritis. In this test, different dilutions of the serum are incubated with a defined amount of SO enzyme (commercially ready). If the anti-SO antibodies are present in the patient's serum, they bind to the SO and neutralize it. RBC is used as an indicator to determine if the enzyme is neutralized. SO has the ability to lyse the RBCs and the neutralized enzyme would not be able to lyse RBCs (Gerber et al., 1990).

Complement fixation test (CFT)

Using the complement fixation test, the antibody titer and the amount of serum complement can be measured. Complement fixation testing is a very sensitive method based on the reaction between three components: antigen, antibody and complement. This test is performed by the macro method in tubes and by the micro method microtiter plate.

In this test, animal sensitive RBCs, usually sheep's RBCs (sRBCs) bounded to an antibody against sRBC (known as hemolysin), are used as a detector. In this procedure, the own complement of the sample should be inactivated at first. Then, known antigen and complement are added to the suspected serum. If the antigen-antibody complexes are formed, the complement system binds to them and consumed. Sensitive RBCs are then added to the mixture in the next step. If the serum is positive for antibodies, the complement is consumed in the first step because of the formation of the antigen-antibody complexes. Therefore, hemolysis-mediated complement will not occur after adding erythrocytes. As a result, if hemolysis happens, the test is likely to be negative. The titer is described as the reciprocal of the sample's highest dilution that resulted in a 50% lysis in the indicator system. In general, IgM, IgG1, and IgG3 are the most important antibodies that could be detected by CFT (Vainionpää et al., 2015).

The CFT test is a very sensitive method by which at least 0.01 µg of antibody per mL of serum can be detected. The sensitivity of this method is more than agglutination and perspiration, but less than radioimmunoassay, immunofluorescence and ELISA. This test is a common method used to serologically diagnose mycoplasma pneumoniae, fungal infections such as coccidioides, histoplasma and most viruses. However, antibodies against a variety of pathogens, including the measles virus, the mumps virus, listeria, enteroviruses, Yersinia, and the rubella virus, are no longer detectable using CFT (Amanfu et al., 2000; Stern, 1965; Shepherd et al., 1986).

This test is very complicated and tedious. Important factors are involved in the CFT, including RBC count and sensitivity, the nature and amount of hemolysin to sensitize RBCs, the hemolysis activity of complement, the ionic strength and pH of the reaction environment, and incubation time.

Hemolysis-in-gel test

In this test, antigen is attached to the surface of erythrocytes that are incorporated into the gel. The patient's serum is then added to the wells punched in the gel. Subsequently, the complement is added. If the antigen-antibody complex is formed in the well, the erythrocytes will undergo lysis. This would provide a hemolytic area around the serum sample well. This test is used to detect measles antibodies in a patient's serum. The zonal diameter increases with increasing antibody concentration. The erythrocytes remain intact if no specific antibodies are present in the serum. Both IgG and IgM antibodies can be detected in a hemolysis-in-gel test. This test could be applied for screening of infectious diseases or immunity following vaccination (Wesslén, 1978).

Enzyme-linked immunosorbent assay (ELISA)

ELISA refers to immunoassay tests using enzymes. The test is based on a colorimetric assay and is usually performed on a microtiter plate. In this procedure, enzyme-conjugated antibodies are used and the enzyme can generate measurable photometric signal following adding chromogenic substrate. Different types of enzymes are commonly used in ELISA, including alkaline phosphatase (ALP), horseradish peroxidase (HRP), and β -galactosidase. ELISA has the same sensitivity as radio immune assay (RIA) but is safer and less expensive (Aydin, 2015).

A variety of ELISA tests have been established providing the quantitative or qualitative detection of antigens or antibodies, including direct, indirect, sandwich, and competitive ELISA methods (Fig. 2).

Direct ELISA is a simple method that was first developed in 1971. This method is well suited for determining the amount of antigens or antibodies. In this regard, antigens or antibodies are coated on the surface of a microtiter plate. The photometric signal can be detected following adding enzyme-conjugated antibodies and then appropriate substrate. The generated signal is directly proportional to the amount of antigen or antibody (Fig. 2A) (Engvall, 2010).

Indirect ELISA is most commonly applied in serological testing for infectious diseases or immunological disorders. In this method, specific antigens are coated in the microtiter plates for detecting specific antibodies in the patient's serum which is added in the next step (Fig. 2B). Antigen-specific antibodies (named as primary antibodies) in the serum can bind to the coated antigens if the antibodies are present in the sample. Following washing the wells, enzyme-conjugated antibodies (specific to the primary antibodies) are added. Again, after the washing step, the appropriate chromogenic substrate is added. The photometric signal resulting from this enzymatic reaction can be measured with a spectrophotometer. Indirect ELISA is frequently used to detect pathogen-specific antibodies or antibodies against certain antigens in immune disorders. The example includes identifying the

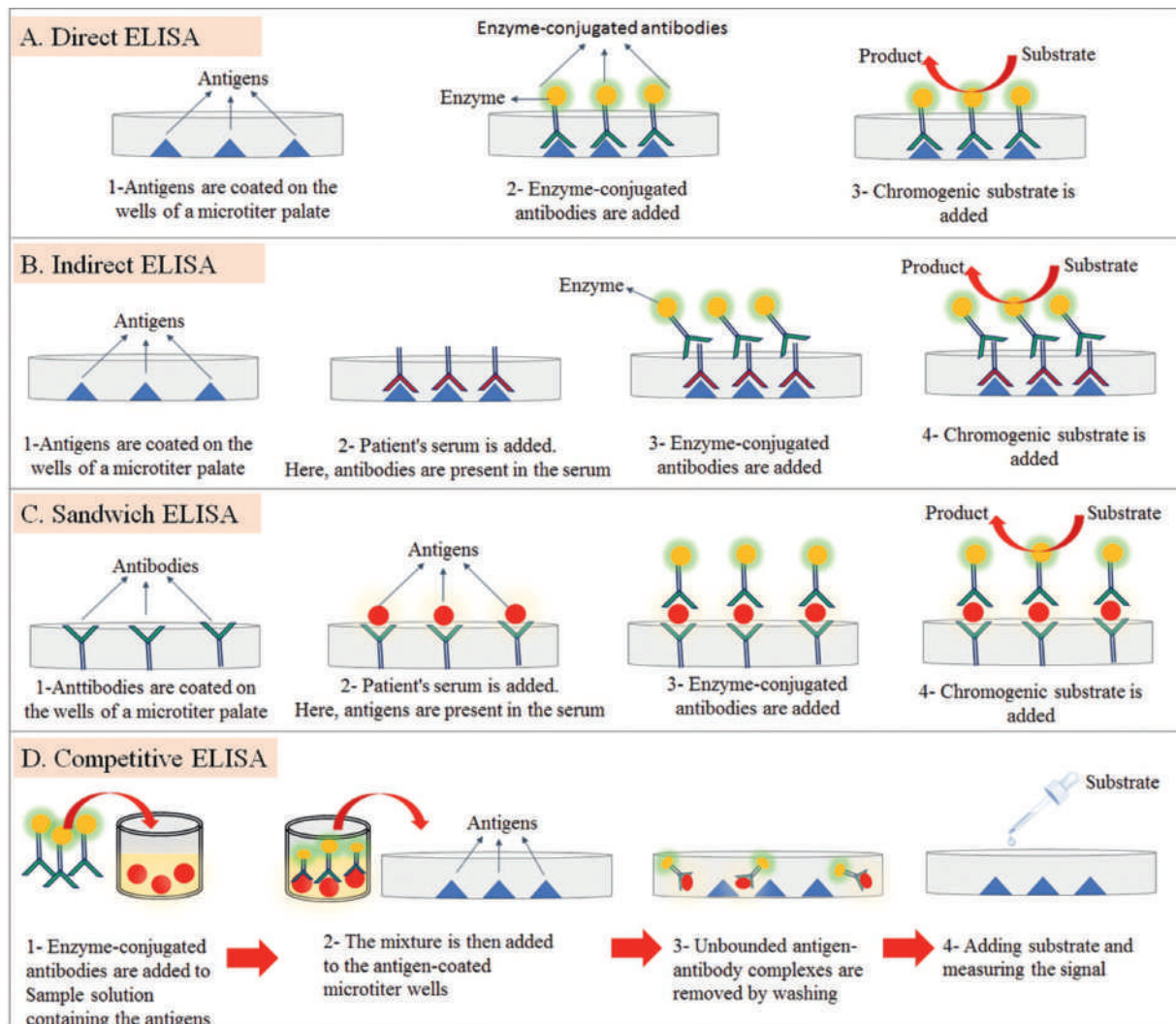


Fig. 2 Schematic diagram depicting four types of ELISA. (A) Direct ELISA; (B) Indirect ELISA; (C) Sandwich ELISA; (D) Competitive ELISA.

presence of HIV-specific antibodies in the suspected serum. For this reason, suspected serum is added to the wells of a microtiter plate coated with recombinant structural proteins of HIV (Lindström and Wager, 1978; Aydin, 2015).

Another type of ELISA technique, known as sandwich ELISA, is designed for the detection of antigens in the sample. In this method, antigen-specific antibodies (primary antibodies) are coated on a microtiter plate (Fig. 2C). By adding the unknown sample in the next step, the antigen in the sample binds to primary antibodies (if the sample is positive). Enzyme-conjugated antibodies are then added in the next step. These antibodies are specific to the primary antibodies. By adding a substrate, the photometric signal can be measured with a spectrophotometer (Kato et al., 1977).

Another procedure to assess the level of antigens or antibodies in the sample is through competitive ELISA, in which the antibodies or antigens in the patient's sample compete with a defined, enzyme-conjugated antibody or antigen over a binding site. In this method, the enzyme-conjugated antibodies are first added to the sample solution containing the antigen (Fig. 2D). This antibody-antigen mixture is then added to the antigen-coated microtiter wells. The high presence of antigen in the sample causes less free antibody to remain to bind to the coated antigens of the well. As a result, the analyte concentration has an inverse relation to the measurement signal (Yorde et al., 1976).

Another technique is designed to increase the sensitivity of the ELISA method. Enzyme-linked fluorescent assay (ELFA) is developed in which a fluorescence signal (e.g., 4-Methylumbelliferyl phosphate) is detected instead of a photometric color signal. The ELFA procedure time is quicker than ELISA and it can detect up to 10–18 mol/assay compared to ELISA that can detect only up to 10–16 mol/assay (Yolken and Stopa, 1979).

Immunofluorescence assay (IFA)

The Immunofluorescence assay can detect either antigen or pathogen-specific antibodies (Bayer et al., 2001). In this procedure, antibodies are labeled with a fluorochrome dye such as fluorescein isothiocyanate (FITC), rhodamine, and phycoerythrin. Therefore, immune complexes containing these fluorescence-labeled antibodies can be detected by colored light emitted (after being excited by light of the appropriate wavelength). The emitted light can be seen with a fluorescent microscope equipped with ultraviolet light and suitable fluorochrome filter systems. Each fluorochrome absorbs a wavelength of light and emits light at a higher wavelength. Fluorescein absorbs blue light (490 nm) and emits a fluorescent yellow-green (517 nm). Rhodamine absorbs yellow-green light (515 nm) and emits a red fluorescent dye (546 nm). Rhodamine can be used in two-color immunofluorescence tests because it emits fluorescence at a higher wavelength than fluorescein. For example, by binding fluorescein to one antibody and rhodamine to another antibody, two membrane antigens can be observed on one cell simultaneously. Phycoerythrin absorbs slightly blue-green/yellowish light (565 nm) and emits slightly orange-yellow light (573 nm) (Mullins, 2010).

Using fluorescent antibodies, the antigen or antibody of interest can be identified by a direct or indirect immunofluorescence method, respectively. Direct immunofluorescence (DIF) is a one-step histological staining procedure in which tissue antigens (fixed in a solid phase, mostly slides) can be recognized directly by adding fluorochrome-labeled antibodies. The signal is observed if the antigen-antibody complex is formed (Vainionpää et al., 2015; Aoki et al., 2010). Indirect immunofluorescence (IIF) is a two-step serological technique in which a labeled antibody is used to detect a specific antibody against an antigen in the serum. For this reason, suspected serum is added to a glass slide with antigen-coated fields, followed by adding specific labeled-antibodies. If the serum is positive for antibodies, the formed complex can be detected by a fluorescent microscope (Aoki et al., 2010; Huang et al., 2012). Pathogens such as *Toxoplasma gondii* can be detected by this method (Bernal-Guadarrama et al., 2014). This procedure is also commonly used for detecting pathogen-specific antibodies in autoimmune diseases such as systematic lupus erythematosus (SLE) (Ghosh et al., 2007). The example includes identifying the antinuclear antibodies (ANA) in the serum. ANA may be produced against different nuclear antigens in the serum of patients with a variety of rheumatic and nonrheumatic disorders. Another example is the fluorescent treponemal antibody absorption (FTA-ABS) test (Hunter, 1975). Treponema-specific antigens are coated on the slide, then the unknown serum is added. After washing and adding the labeled antibody, the result can be seen by a microscope. This test is usually performed if the initial screening tests (e.g., rapid plasma regain (RPR), venereal disease research laboratory (VDRL)) for syphilis are positive, so that this test can help confirm whether the results of screening tests are accurate. In general, the IIF has more sensitivity compared with DIF. In another technique based on IF staining, known as double IF, the presence of two different antigens on a cell can be identified.

Radio immunoassay (RIA)

RIA is an immunoassay using radiolabeled antibodies or antigens. Iodine isotopes such as ^{125}I , ^{131}I or tritium can be used for labeling. This method has high sensitivity but needs specialized equipment (e.g., gamma counter) for detecting radioactive signals. Furthermore, it requires special handling and disposal of radioactive waste. In this method, the unlabeled and labeled antigens compete for interaction with antibody molecules to form an antigen-antibody complex. The formed complex is then separated and precipitated with anti-immunoglobulin. The radioactivity of the precipitate indicates the bound antigens that is inversely correlated to the unlabeled antigen concentration (Goldsmith, 1975).

Chemiluminescence immunoassay (CLIA)

The basis of the CLIA method is similar to that of ELISA, except that CLIA substrates can generate light emission in the presence of an enzyme, providing a more sensitive process compared to ELISA. Substrates such as isoluminol or acridinium ester produce the luminescence signal in the presence of hydrogen peroxide and enzyme. The electro-chemiluminescence immunoassay (ECLIA),

another type of CLIA, uses electrical current for oxidizing substrate. CLIA and ECLIA methods have higher sensitivity compared to ELISA and ELFA and have a shorter analysis time (Liu et al., 2018).

Immunoblotting method

Immunoblotting or western blotting (WB) is a technique recognizing specific antibodies or antigens (e.g., pathogen antigens) in the sample. The presence of a certain protein and its molecular weight can be identified by WB. The method is based on a combination of gel electrophoresis and formation of the antigen-antibody complex (Ni et al., 2017; Magi and Liberatori, 2005). The procedure involves four important steps, as follows:

- 1) Protein separation by SDS-PAGE: the protein mixture is separated by SDS-PAGE based on the molecular weight of proteins.
- 2) Protein transfer from the gel to a nitrocellulose membrane by electrophoresis.
- 3) Incubation of the nitrocellulose membrane with serum sample: antigen-specific antibodies in the serum (primary antibodies) bind to the antigens on the nitrocellulose membrane.
- 4) Adding labeled secondary antibodies (specific to the primary antibodies) and detecting signals using either a chromogen or an X-ray film.

The antigen-antibody complexes can generate a visible band on the carrier membrane as a result of a dye reaction. By using known antigens, blot methods reveal a high specificity for detecting antibodies (e.g., HIV antibodies). However, WB has lower sensitivity compared to ELISA, ELFA and CLIA methods depending on the pathogen specificity. Therefore, it can be used as a confirmatory test (e.g., in borreliosis diagnosis) or as an additional test (e.g., toxoplasmosis, CMV, EBV diagnosis).

Nucleic acid-based identification methods

The principle of nucleic acid-based methods is the detection of specific DNA or RNA sequences of the microorganism. Nucleic acid-based approaches provide precise and sensitive identification and diagnosis of pathogens causing infectious diseases, as well as knowledge of the disease's epidemiology. These tests are rapid and have high specificity and sensitivity and can be used for the detection of all types of microbes. The recent development of the multiplex assay provides the detection of several different antigens in one analytical procedure. However, these tests are less sensitive or less specific than the single-target tests. In the following section, the most important nucleic acid-based methods are reviewed (Mothershed and Whitney, 2006; Mianzhi and Shah, 2017; Yu et al., 2012).

Polymerase chain reaction (PCR)-based amplification

PCR is an in vitro enzymatic reaction that enables large amounts of a particular DNA fragment to be generated. When compared to serologic tests, this method is more accurate and faster (Garibyan and Avashia, 2013; Ashford et al., 1995). Serologic tests are limited because of the window period required for production of sufficient levels of antibodies, so false-negative results can occur during this time. PCR can be applied even in the early phase of infection to detect the presence of a pathogen. For example, tuberculosis bacteria multiply slowly, making diagnosis via culture or serological methods time-consuming. In this condition, the PCR technique allows rapid amplification of the pathogen genes, leading to increased detection sensitivity and decreased detection time. Another example that highlights the importance of PCR in clinical diagnosis is Lyme disease, caused by a tick-borne spirochete. Using culture for detecting these bacteria is approximately impossible since they are too small to be seen by a microscope. Other examples are detecting Influenza virus, blood screening for human immunodeficiency virus (HIV), hepatitis B, hepatitis C and syphilis. Three important steps of PCR are as following (Fig. 3):

- 1) Denaturation: the use of high temperature at this step causes the double-stranded DNA to become single-stranded.
- 2) Annealing: as the temperature is reduced, the primers recognize and bind to the target DNA.
- 3) Extension: at this step, the complementary strand is made by a DNA polymerase enzyme at the appropriate temperature.

These three steps are generally repeated for 30–40 cycles. Importantly, the specificity of the designed primer to the target sequence determines the specificity of the PCR. After the reaction is over, the amplified DNA can be visualized using gel electrophoresis. Although computer software can estimate the amount of amplified DNA from the intensity of the band on the gel, PCR is not suitable for quantitative studies. For this purpose, quantitative PCR (qPCR) or real-time PCR (RT-PCR) has been developed. The PCR reaction can be monitored in RT-PCR through the emission of fluorescence during the reaction. These signals are recorded by the RT-PCR machine and are therefore traceable at the same time as the reaction is done. When compared to PCR, RT-PCR has a much higher degree of accuracy (Freeman et al., 1999).

Isothermal amplification

Isothermal nucleic acid amplification is a simple process that uses a constant temperature for the amplification of a nucleic acid target sequence instead of thermal cyclers required for PCR (Wong et al., 2018). Several isothermal amplification methods have been developed based on the mechanism of the reaction. Nucleic acid sequence-based amplification (NASBA) and Loop-mediated isothermal amplification (LAMP) are the most widely used isothermal methods. NASBA is an isothermal and continuous amplification based on RNA transcription, providing accurate results with high sensitivity (Compton, 1991; Morré et al., 1996).

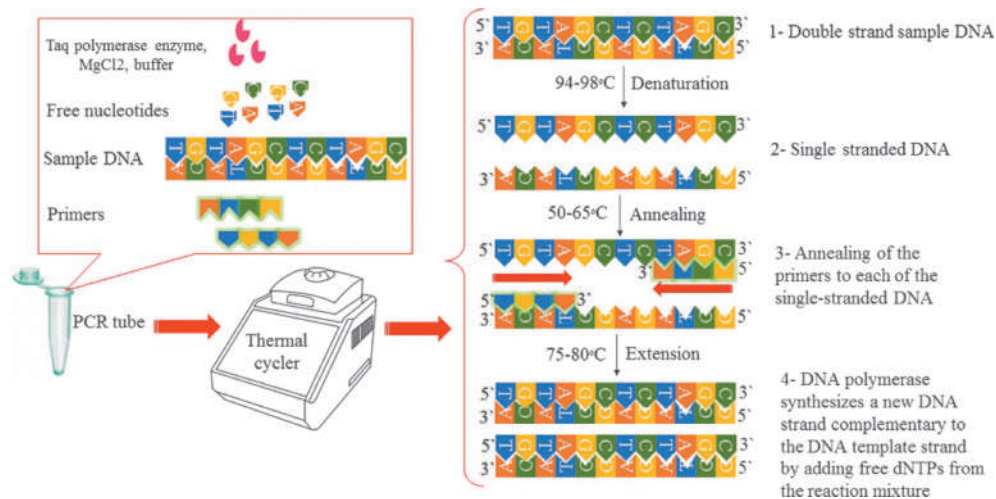


Fig. 3 Schematic diagram of the polymerase chain reaction (PCR). These three steps are generally repeated for 30–40 cycles. Here, only 1 cycle is depicted.

It can be easily done in a water bath without the need for a thermocycler. The reaction takes place in the presence of a mixture of reverse transcriptase, RNA polymerase, ribonuclease-H, and two specially designed DNA oligonucleotide primers. A 5' extension of the forward primer includes the promoter sequence for bacteriophage T7 DNA-dependent RNA polymerase. A 5' extension of the reverse primer includes a sequence for binding a probe tagged with ruthenium-based electrochemiluminescent (ECL) that can be detected by a chemiluminescence detector (Lau et al., 2006). Under ideal conditions, the yield of amplification is nearly 10^{12} -fold, compared to 10^9 -fold amplification by PCR (Boom et al., 1990).

NASBA has been used extensively in the identification of a variety of viruses, including HIV-1, avian Influenza virus, Newcastle disease virus, Simian immunodeficiency virus, and Respiratory syndrome virus. This method can also be used in the detection of cytokines, bacteria (e.g., *Salmonella enterica*), fungi, and parasites (Romano et al., 1996, 2000; Collins et al., 2003; Lau et al., 2006).

LAMP is another isothermal amplification method conducted in the presence of a DNA polymerase (with a function for strand displacement) as well as two sets of primers (four different primers) (Zhao et al., 2015). Since amplification only occurs when all six sequences of a target DNA are accurately identified by the primers, the LAMP assay has a high specificity. However, the difficulties in developing usable primers restrict the practical application of LAMP. The amplification process continues in a cyclical manner. The end product is stem-loop DNA with multiple inverted copies of the target sequence. Positive LAMP reaction products can be identified using a variety of detection methods, such as incorporation of SYBR Green I stain or using polyethylenimine (PEI). This technique has been used in the detection of several viruses (e.g., Noroviruses, SARS-CoV, H5N1, avian Influenza subtypes, Influenza subtype 1, Influenza subtype 3, and Influenza B virus) and bacteria (e.g., *Salmonella*, *Escherichia coli* and *Mycobacterium tuberculosis*) (Hara-Kudo et al., 2005; Hill et al., 2008; Iturriza-Gómara et al., 2008; Poon et al., 2004; Imai et al., 2006; Geojith et al., 2011).

Microarray/chip-based technologies

The development of microarray/chip-based technology enables the analysis of samples with a large number of sequences in a single test (Raghavachari, 2013; Marzancola et al., 2016). Pathogen screening involves immobilizing the expressed sequences of various pathogens on a filter to determine which pathogens are present in the suspected samples. Therefore, a single procedure allows the detection of multiple pathogens. The basic principle of microarrays is the hybridization between two strands of DNA. In this procedure, a high number of specific DNA sequences known as probes or oligos are immobilized on a solid surface that are used to hybridize a nucleic acid of interest in the sample. The sample's nucleic acid is purified and labeled, typically with a fluorescent tag. The labeled samples are then added to the pinholes of the microarray. After hybridization has occurred, all nonspecific binding is washed off. Finally, the microarray is scanned by a machine through a laser to excite the dye and measures the emission levels with a detector.

Microarray-based methods have been used widely in screening clinical samples for viruses. This technique is also applied in single nucleotide polymorphisms (SNPs) analysis and in cancer diagnosis, such as detecting P53 mutations, and distal metastasis of lymph-node-negative primary breast cancer (Ahrendt et al., 1999; Wang et al., 2002, 2005; Chiu et al., 2006; Schick et al., 2011).

Non-nucleic acid-based identification methods

It is essential to identify an organism after it has been isolated by culture. The importance of identification is in the management of infectious diseases, such as choosing a better drug for treatment. Rather than genetic recognition, non-nucleic acid-based identification approaches depend on phenotypic characteristics of organisms, including functional or morphologic. There are several biochemical tests available, each of which is limited to species of a certain type (e.g., aerobic or anaerobic bacteria). However, two techniques, including chromatographic methods and mass spectrometry, are widely used for identifying pathogens.

High-performance liquid chromatography (HPLC) or gas chromatography can be used to isolate and identify microbial components or products. Typically, recognition is accomplished by comparing an organism's fatty acids to a database. In general, aerobic and anaerobic microbes, mycobacteria, and fungi can all be identified using chromatographic techniques. The accuracy of the test is determined by the circumstances under which the specimen was cultured and the accuracy of the database, which could be unreliable or inadequate (Larsson and Mårdh, 1977; Lima et al., 2018; Emonet et al., 2010).

Pathogens have distinct proteins, and the relative concentration and mass of each protein may often be used to classify a microorganism. Different proteins with different masses in a sample can be detected using mass spectrometry. A type of mass spectrometry known as matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) is currently being applied to recognize mycobacteria, yeasts, molds, and viruses (Patel, 2015).

Diagnostic laboratory tests: Focusing on immunological disorders

Autoimmune disorders

Autoimmunity is defined by a lack of immune homeostasis, which causes the organism to develop an abnormal reaction to its own tissue. Laboratory tests can be helpful in disease management and determining disease severity. The development of self-reactive T cells, autoantibodies, and inflammation are common features of autoimmune diseases. Diagnosing autoimmune disorders is complex and several laboratory tests are usually needed. These laboratory diagnosis tests include specific and non-specific tests such as complete blood count (CBC), evaluation of acute phase reactants and inflammatory markers, serological tests, cytokine analysis, and immunologic studies (Castro and Gourley, 2010). Laboratory diagnostic tests for evaluating autoimmune disorders are summarized in Table 1.

Inflammation-related markers

Inflammatory markers are serum proteins that are produced by the liver in response to inflammation. Pro-inflammatory cytokines such as IL-1, IL-6, and TNF-alpha produced by immune cells in the inflammatory condition play a significant role in stimulating the production of acute phase proteins such as C-reactive protein (CRP), fibrinogen, and haptoglobin. Inflammatory markers cannot be used to diagnose inflammation, although they can reveal abnormalities in a variety of conditions such as infections, autoimmune diseases, and malignancies (Gruys et al., 2005).

Erythrocyte sedimentation rate (ESR) is a test applied for the evaluation of the RBC's quantity. ESR is performed by analyzing the RBC sedimentation rate in a tube at a defined time. While ESR is a non-specific test and cannot be considered as a diagnostic test, it can be useful in controlling disease severity and therapeutic response, and can reflect the presence of inflammatory or infectious stress (Lahita and Weinstein, 2007).

CRP is an acute phase protein which can be induced under the influence of pro-inflammatory cytokines. This protein has an important activity in inducing phagocytosis through pathogen opsonization as well as complement activation. Since serum CRP concentrations shift faster than ESR, CRP could be a better indicator of current inflammation. On the other hand, in contrast to ESR, CRP is a stable serum protein that is not influenced by other serum components. CRP can be measured quantitatively and qualitatively (e.g., by CRP-latex described in the serological methods). The level of inflammation is proportional to the level of CRP. Levels of less than 0.2 mg/dL are regarded as normal, whereas levels greater than 1.0 mg/dL are indicative of inflammation and/or infection (Gruys et al., 2005; Lahita and Weinstein, 2007).

Ferritin is another serum protein that its increased levels may suggest acute or chronic sepsis, inflammation, or cancer. Ferritin is frequently increased in diseases such as systemic-onset juvenile idiopathic arthritis and lymphohistiocytosis. Other inflammatory markers include Ceruloplasmin, Fibrinogen, and Haptoglobin that can be increased during inflammation and autoimmunity disorders (Breda et al., 2010; Koulaouzidis et al., 2009).

Autoantibodies

The existence of autoantibodies in a patient does not imply a diagnosis of an autoimmune disorder. The signs and symptoms of the patient should also be carefully considered. Currently, ELISA (described in serological methods) is frequently used to detect autoantibodies in a suspected patient (Eggert et al., 2010).

"Rheumatoid factor" (RF) is an autoantibody produced against the FC region of IgG. RF is used to evaluate patients with rheumatoid arthritis (RA) with a sensitivity and specificity of about 70%. The RF can be any class of antibodies. However, most methods identify the IgM rheumatoid factor. It should be considered that approximately 15% of rheumatoid arthritis patients are negative for RF. On the other hand, RF may be positive in some healthy individuals. Although RF is considered as a non-specific test, patients with positive RF may experience progressive arthritis, extraarticular manifestations, and secondary Sjögren's syndrome. RF can be detected in other autoimmune diseases such as SLE, Sjögren's syndrome, and cryoglobulinemia. Identifying another autoantibody known as anti-citrullinated protein (anti-CCP) is also valuable in diagnosing RA patients (with about 95% specificity). Anti-CCP can be positive in psoriatic arthritis, Sjögren's syndrome, juvenile idiopathic arthritis (JIA), and inflammatory myopathies (Lahita and Weinstein, 2007; Klippel et al., 2008).

"Anti-nuclear antibody" (ANA) is another autoantibody which is mostly used in the diagnosis of SLE. However, ANA can be found in other diseases, including RA, scleroderma, Sjögren's syndrome, mixed connective tissue disease, and polymyositis/dermatomyositis. The immunofluorescence method is frequently used for detecting ANA in patients' serum. ELISA technique is

Table 1 Laboratory diagnostic tests for evaluating autoimmune diseases.

<i>Autoimmune disorder</i>	<i>Laboratory diagnostic tests</i>
Rheumatoid arthritis (RA)	• CRP and ESR: non-specific, initial screening, indication of inflammation • RF: not specific, present in about 80% of patients with RA, associated with poor prognosis • Anti-CCP: more sensitive and specific compared to RF, present in about 80% of patients with RA • ANA: about 60% specificity and 45% sensitivity in patients with RA • HLA-Typing: HLA-DRB1 variations
Systemic lupus erythematosus (SLE)	• CBC: anemia, low WBC or platelet count maybe seen • ANA: positive in about 100% of the patients with SLE, higher sensitivity (about >95%) • Anti-dsDNA, higher specificity (about 95%), usually associate with active disease • Anti-Extractable Nuclear Antigens: Anti-sm (high specificity), anti-RNP, Anti-SSA (anti-Ro), Anti-SSB (anti-La) • Urinalysis in renal involvement: hematuria, proteinuria, present WBC • Complement assay: decreased level of C3 and C4, decreased CH50 • Kidney biopsy
Goodpasture's syndrome	• Urinalysis: indicates proteinuria, dysmorphic RBCs, WBCs, and RBC cellular and granular casts • Anti-GBM • Kidney biopsy
Sarcoidosis	• The levels of serum ACE: elevated levels may be seen, but this test is non-specific • The levels of Vitamin D: increased levels of 1, 25(OH)2D3 vitamin D are commonly seen • The levels of SAA: increased levels of SAA are commonly seen
Churg-Strauss syndrome	• CBC: indicates a peripheral eosinophilia • ESR and CRP: elevated levels are seen • ANA and a p-ANCA against MPO • A biopsy of the involved organ: for evaluation of vasculitis and granuloma formation
Wegener's granulomatosis (WG)	• ESR and CRP: elevated levels are seen • C-ANCA-PR3 • HLA-Typing: WG can be associated with HLA-DR4, DR13 • Urinalysis: proteinuria, hematuria, cellular casts • The levels of serum albumin: hypoalbuminemia
Myositis	• CBC: May show leukocytosis or thrombocytosis • Muscle biopsy • MSA: Anti-tRNA synthetase antibodies (anti-JO-1 antibodies), Anti-SRP, Anti-PM/ScI, Anti-Mi-2 • HLA-Typing: myositis can be associated with HLA-DRB1 *0301 and HLA-DQA1 *0501 • The levels of CK, AST, ALT and LDH
Autoimmune hemolytic anemia (AIHA)	• Peripheral blood smear: polychromasia, spherocytosis, fragmented and nucleated erythrocytes Reticulocytosis, erythrophagocytosis by monocytes in severe cases • The levels of haptoglobin: decreased levels are seen • The levels of LDH: increased levels are seen • DAT: evaluation of immunoglobulin and/or complement proteins bound to the patient's erythrocytes
Antiphospholipid syndrome	• CBC: thrombocytopenia • aPL
Idiopathic Thrombocytopenia	• Thrombocytopenia • human platelet antigen—1a antibodies

CRP, C-reactive protein; ESR, Erythrocyte sedimentation rate; RF, Rheumatoid factor; CCP, Cyclic citrullinated peptide; ANA, Anti-nuclear antibody; HLA, Human leukocyte antigen; CBC, Cell blood count; WBC, White blood cell; SLE, Systemic lupus erythematosus; dsDNA, Double stranded DNA; Anti-sm, Anti-smith; anti-RN, Anti-ribonucleoprotein; Anti-SSA, Anti-Sjögren's-syndrome type A; Anti-SSB, Anti-Sjögren's syndrome type B; CH50, Hemolytic complement; Anti-GBM, Anti-glomerular basement membrane antibodies; ACE, Angiotensin converting enzyme; SAA, Serum amyloid A; ANCA, Antineutrophil cytoplasmic antibodies; p-ANCA, Perinuclear-ANCA; c-ANCA, Cytoplasmic-ANCA; MPO, Myeloperoxidase; PR3, Proteinase 3; WG, Wegener's granulomatosis; MSA, Myositis-specific antibodies; Anti-PM/ScI, Antibody to nucleolar granular component; Anti-SRP, Antibody to signal recognition protein; CK, Creatinine kinase; AST, Aspartate aminotransferase; ALT, Alanine transaminase; LDH, Lactate dehydrogenase; DAT, Direct anti-globulin test; aPL, Antiphospholipid antibody.

also used with high sensitivity but low specificity. ANA is also found in nonrheumatic disorders such as autoimmune hepatitis, Hashimoto's thyroiditis, primary pulmonary hypertension, Graves' disease, and primary autoimmune cholangitis (Sur et al., 2018; Grygiel-Górniak et al., 2018). Furthermore, ANAs are also associated with several infections, such as *Mycobacterium tuberculosis*, *Treponema pallidum*, *Orientia tsutsugamushi*, *Escherichia coli*, *Bartonella henselae*, and human immunodeficiency virus (Im et al., 2020).

Other antibodies that are important and highly specific in diagnosing SLE are "anti-double stranded DNA" (anti-dsDNA). Although anti-dsDNA was previously detected by radioimmunoassay, IFA and ELISA are more commonly used currently. Crithidia luciliae, a flagellated protozoa with a dsDNA-containing small organelle called a kinetoplast, is used as a target antigen by the IFA. Semiquantitative detection of antibodies against dsDNA is accomplished by detecting IgG attached to the kinetoplast. In ELISA, the dsDNA is coated on a microtiter plate and the bound IgG is detected after adding the suspected serum (Lahita and Weinstein, 2007; Mummert et al., 2018; Conrad et al., 2009).

“Antineutrophil cytoplasmic antibodies” (ANCA) are produced against the cytoplasmic granules of neutrophils. The immunofluorescent patterns can be useful in diagnosing various ANCA associated vasculitis syndromes. Cytoplasmic pattern (cANCA) is frequently found in Wegener’s Granulomatosis (WG), microscopic polyangiitis (MPA) and Churg-Strauss syndrome. Perinuclear pattern (pANCA) can be seen in MPA as well as in RA, inflammatory bowel disease (IBD), JIA, and SLE. The vasculitis type and disease severity can also be identified by detection of proteinase 3 (PR3) and myeloperoxidase (MPO). For example, MPO-cANCA is particularly associated with WG. MPO-pANCA is positive in MPA and less often for Churg-Strauss. It should be considered that ANCA can also be positive in some conditions, such as during thyroid medication (e.g., PTU) and other immune disorders. Tissue biopsy can be helpful for confirming patients suspected of vasculitis (Schönermarck et al., 2001; Csernok and Gross, 1995).

“Anti-extractable nuclear antigen” (anti-ENA) are a group of antibodies against extractable antigens, including SSA (Ro) and SSB (La) nuclear antigens, Smith (Sm) antigen, and ribonuclear protein (RNP) or U1RNP. Anti-SSA and anti-SSB antibodies are usually seen together in Sjögren’s syndrome patients and some patients with SLE.

Anti-Sm is particularly specific for SLE; However, it is only present in 25% of SLE patients. Antibodies to U1 ribonucleoproteins have been detected in several autoimmune diseases such as SLE, systemic sclerosis, and in patients with mixed connective tissue disease (Agarwal et al., 2009).

Autoantibodies known as anti-JO-1, anti-Mi-2, anti-SRP, and anti-PM/Scl have been detected in the autoimmune inflammatory myopathies (IIM) with high specificity. Anti-JO-1 antibodies are produced against histidyl-tRNA synthetase and are particularly seen in anti-synthetase syndrome, an inflammatory myopathy. Anti-Mi2 antibodies can target a nuclear complex involved in transcription. These autoantibodies are specific for diagnosing dermatomyositis. Anti-signal recognition particles (SRP) are antibodies against a cytoplasmic RNA protein involving 7S RNA and 6 other proteins. Anti-SRP has been detected in the serum of polymyositis patients. These antibodies are considered as serological markers for identifying necrotizing myopathy. Anti-PM/Scl is a type of antinuclear antibody associated with idiopathic inflammatory myositis-systemic sclerosis overlap syndromes (also called scleromyositis or sclerodermatomyositis). These types of autoantibodies have also been found in patients with polymyositis, dermatomyositis and systemic sclerosis without features of overlap syndromes (Satoh et al., 2017).

Autoantibodies named as antiphospholipid autoantibodies (aPL) or lupus anticoagulant (LAC) or anti-cardiolipin (aCL) are produced against phospholipids as well as phospholipid-binding proteins. These antibodies have been found in patients with anti-phospholipid antibody syndrome (APS) as well as SLE patients. aPL antibodies may also be detected in healthy individuals (McIntyre et al., 2003; Coín et al., 2015).

Complement activity

The complement system is an enzymatic cascade consisting of several proteins in the serum. Complement activation can be considered as an important pathway in defending against pathogens. Meanwhile, the levels of complement components in the serum can be used to identify disease activity. The methods for measuring complement components include nephelometry and ELISA for detecting components such as C3, C4 and factor B. The hemolytic activity of complement can be assayed using CH50 and AH50 which analyze the functional activity of the classic and alternative pathways, respectively. In diseases in which the immune complex is formed in tissues, such as glomerulonephritis in patients with SLE, the serum levels of complement’s proteins decrease. In contrast, elevated levels of C3 and C4 in the acute phase of the disease can suggest an inflammatory disorder. A decreased level of complement components has been found in nonrheumatic disorders such as post-streptococcal glomerulonephritis or bacterial endocarditis (Conigliaro et al., 2019; Boackle, 2003).

Cytokines

Cytokines are glycoproteins produced by several immune and non-immune cells, playing an important role in immune function and the development of inflammation. Flow cytometry and ELISA are laboratory techniques used to evaluate cytokine levels. For this reason, sandwich ELISA is frequently used by commercial kits. Although evaluation of the gene expression levels of cytokines can be applied by molecular tests, particularly Real-Time PCR, this process is usually performed in research laboratories.

Assessing pro-inflammatory cytokines, especially IL-1, IL-6, and TNF-alpha can be helpful in detecting an inflammatory disorder. Since the level of pro-inflammatory cytokines is increased in autoimmune disorders, targeting these molecules is used as an effective therapeutic approach. For example, targeting TNF-alpha through monoclonal antibodies (mAbs) can significantly reduce disease severity in patients with RA. Using mAbs, including anti-TNF, anti-IL-1 and anti-IL-6 therapies, is currently being used in several immune disorders (O’Shea et al., 2002; Moudgil and Choubey, 2011).

Human leukocyte antigen (HLA) typing

The association between HLA or Major Histocompatibility Complex (MHC) and immune disease susceptibility has been described in many studies. HLA-A, -B and -C are the classic types of MHC class I molecules, and HLA-DR, HLA-DQ, and HLA-DP are MHC class II molecules. It has been found that many rheumatic disorders are associated with HLA class I and II. For example, HLA-B27 is highly associated (about 90–95%) with susceptibility to ankylosing spondylitis. HLA-DR1 and HLA-DR4 are associated with an increased risk of polyarticular juvenile idiopathic arthritis (JIA). In Caucasian populations, HLA-DR3 and HLA-DR2 are associated with lupus, while HLA-DRB1 is associated with an increased risk of rheumatoid arthritis (Troshina et al., 2020).

HLA-typing is also considered an important test before organ transplantation (especially bone marrow transplantation) to identify which people can provide the safest tissue transplants. In this regard, the best potential donors have HLAs that closely match the recipient’s HLA profiles. Several methods can be used to detect HLA types, including serological- and molecular-based methods (Wiebe and Nickerson, 2020).

Serological typing (traditional method) is frequently used in many laboratories for identifying HLA-A and HLA-B with low resolution. This method is performed using a complement-dependent cytotoxicity (CDC) test or micro lymphocytotoxicity assay. In this procedure, a panel of sera containing HLA-specific alloantibodies is added to a well of a microtiter Terasaki plate consisting of the patient's lymphocytes. Rabbit serum containing complement is added after a short incubation time. The lymphocytes that have been recognized by a specific alloantibody undergo lysis, allowing the fluorochrome ethidium bromide to enter. The lysed cells can simply be identified by microscopy (Saito et al., 2014).

Molecular testing provides HLA-typing with more resolution using the PCR technique. These tests including sequence-specific primer (SSP) and sequence-specific oligonucleotide (SSO) are very useful and both of them can apply either for generic (low resolution) and allelic (high resolution) typing. SSP provides identification of HLA-A, -B, -C, -DRB1, -DRB3, -DRB4, -DRB5, and -DQB1 alleles. SSP typing includes using primers that are complementary to specific HLA allele sequences (e.g., HLA*02:03) or a set of identical alleles (e.g., all A*02 alleles). The presence of the product is then detected by agarose gel electrophoresis. This method is performed rapidly and is ideally appropriate for small numbers of samples (Madden and Chabot-Richards, 2019; Kulcsarova et al., 2000).

Sequence-specific oligonucleotide (SSO) was first described for HLA class II and then for HLA class I typing. In this method, locus-specific amplification of the genomic DNA segment is initially performed by PCR. After that, the amplified DNA is blotted onto a solid support, normally a nylon membrane, and hybridized with a series of sequence-specific oligonucleotide probes (SSOP) (direct hybridization). The probes are bound to the fluorochromes, allowing their detection by chemiluminescence. In indirect hybridization, SSO probes are attached to a solid phase and then hybridized with a labeled PCR product. The SSO method is very suitable for the typing of large sample numbers. However, a method known as reverse-SSO has been developed for testing low sample numbers. In this method, a pre-made SSOs bound membrane is used. Sample DNA is specifically amplified using specific primers for certain HLA locus. Amplified DNA is labeled (e.g., with biotin) and then added to the SSOs bound membrane. Finally, the hybridization result is detected using a colorimetric or chemiluminescent detection system. Recently, a reverse-SSO system is developed based on Luminex technology. All methods, including SSP, SSO and reverse-SSO, are all appropriate for application in research and also in routine tissue typing laboratories (Madden and Chabot-Richards, 2019; Kulcsarova et al., 2000).

Another technology known as Sequence-based typing (SBT) is a comprehensive molecular-based method with high resolution. This technique is based on the sequencing of amplified DNA. Both alleles for each HLA locus are amplified and then sequenced. Although PCR-SSO is able to detect single-base variations in DNA sequences within two alleles, it is less likely to detect new undefined alleles unless the variation occurs at the particular site detected by the probe or the restriction enzyme. Furthermore, this method is more expensive compared to other methods (Voorter et al., 2014).

Immunodeficiency disorders

Immunodeficiency disorders are referred to a group of diseases in which the immune system is compromised because of defects in innate immune system (e.g., complement pathway, phagocytosis) or adaptive immune system (e.g., cellular and humoral immunity). Primary immunodeficiencies are determined genetically and they are usually revealed during infancy or childhood. Secondary immunodeficiencies can result from other disorders or conditions such as diabetes, radiation therapy, chemotherapy, bone marrow ablation before transplantation, etc. There are a variety of diagnostic laboratory tests for diagnosing defects in the immune system (Rezaei et al., 2009; Raje and Dinakar, 2015). Laboratory diagnostic tests for evaluating primary immunodeficiencies are summarized in Table 2. We will review these tests in the following sections.

Initial screening

In general, recurrent infections are a common symptom of immunodeficiency. The patient can experience up to 10 respiratory infections/year. Therefore, a careful history of the patient to assess infectious diseases and other complications is crucial to diagnosis of an immunodeficiency disorder. In this regard, manifestations such as failure to thrive (FTT), upper and lower respiratory tract infections (e.g., pneumonia, sinusitis, bronchitis), and chronic diarrhea can be considered as frequent manifestations importantly in a patient with primary immunodeficiency. Therefore, it is essential to evaluate clinical manifestations along with laboratory diagnostic assays. Initial laboratory tests for screening such patients should include complete blood counts (CBC) and differentials (platelet volume, absolute lymphocyte count, neutrophil and eosinophil counts), immunoglobulin levels (IgG, IgM, IgA), lymphocyte subpopulations, vaccine titers, and complement assessment (e.g., CH50, AH50).

CBC results can detect abnormalities in one or more cell types, which can be a clue to detect a specific disorder. These abnormalities include anemia, neutropenia, thrombocytopenia, and lymphopenia. For example, since T cells account for about 70% of circulating lymphocytes, lymphopenia could suggest a T-cell associated deficiency. A patient with persistent leukocytosis, especially between infections, may suspect a leukocyte adhesion deficiency. Wiskott-Aldrich syndrome can be initially screened by thrombocytopenia. On the other hand, decreased levels of immunoglobulins can suggest antibody deficiency. However, these assessments are often inadequate to make a diagnosis. Therefore, several complementary tests should be performed when the screening tests and clinical manifestations are in favor of an immunodeficiency disorder. These tests are usually performed to assess immune cell function (Locke et al., 2014; Eghbali et al., 2015).

Humoral immunity deficiency

When a humoral immunity-related immunodeficiency is suspected, quantitative immunoglobulin analysis (IgG, IgA, IgM, and IgE) is the most important early screening. Furthermore, the evaluation of specific antibody titers is important. In this regard, serum titers to common vaccinations such as diphtheria, tetanus, and pneumococcus can be used. ELISA is frequently used to detect antibody titers in the serum.

Table 2 Laboratory diagnostic tests for evaluating primary immunodeficiencies.

<i>Laboratory diagnostic tests</i>	
Initial screening	• CBC and differential (platelet volume, absolute lymphocyte count, neutrophil and eosinophil counts) • Immunoglobulin levels (IgG, IgM, IgA) • Lymphocyte subpopulations • Vaccine titers (e.g., diphtheria, tetanus, and pneumococcus) • Complement assessment (e.g., CH50, AH50)
Humoral immunity deficiency	• CBC and differential • Quantitative immunoglobulin analyzes (IgG, IgA, IgM, and IgE) • Specific antibody titers • B cell immunophenotyping (e.g., IgM, IgD, IgG, CD19, CD20, CD27, and CD38) • Genetic mutational analysis (BTK, TACI, BAFFR, ATM, CD79a, CD79b, BLNK, LRRC8, AID, UNG) • KREC
Cellular immunity deficiency	• CBC and differential • T cell immunophenotyping (e.g., CD3, CD4, CD8) • T cell cytotoxicity assay • CD45RA/RO status • DTH tests • Stimulation with mitogens • TREC • Genetic mutational analysis
Combined immunodeficiency	• CBC and differential • Immunophenotyping: T, B, NK cells • Genetic mutational analysis RAG1, RAG2, IL-2 gamma chain, JAK3, CD3, Artemis, Cernunnos, Ligase 4, IL-7 receptor alpha, ADA, PNP, AK2, ZAP70, CD45, DNA-PK, NHEJ1, CD40, CD40L
Innate immunity deficiency	• CBC and differential • Absolute neutrophil count, and morphological analysis of neutrophils • CD11, CD18, CD15c levels: evaluating for LAD • Integrin function • DHR assay • NBT assay • G6PD analysis • NK cytotoxicity and NK markers • Complement assay • Genetic assessment: ELA2, GFI-1, HAX1, GCSFR, MPO

CRP, C-reactive protein; *Ig*, Immunoglobulin; *CH50*, Hemolytic complement (classic pathway); *AH50*, Hemolytic complement (alternative pathway); *BTK*, Bruton's tyrosine kinase; *TACI*, Transmembrane activator and CAML interactor; *BAFFR*, B-cell activating factor receptor; *ATM*, Ataxia telangiectasia mutated; *BLNK*, B-cell linker protein; *LRRC8*, leucine-rich repeat-containing 8; *AID*, Adenosine deaminase; *UNG*, Uracil N-Glycosylase; *KREC*, Kappa-deleting recombination excision circle; *DTH*, Delayed-type hypersensitivity; *TREC*, T-cell replication excision circle; *RAG*, Recombination-activating gene; *JAK3*, Janus kinases; *ADA*, Adenosine deaminase; *PNP*, purine nucleoside phosphorylase; *AK2*, Adenylate Kinase 2; *ZAP70*, Zeta-chain-associated protein kinase 70; *NHEJ1*, non-homologous end joining-1; *CD40L*, CD40-Ligand; *LAD*, Leukocyte adhesion deficiency; *DHR*, Dihydrorhodamine; *NBT*, Nitro blue tetrazolium; *G6PD*, Glucose-6-phosphate dehydrogenase; *NK*, Natural killer cell; *ELA2*, Elastase 2; *GFI-1*, Growth Factor Independent 1; *HAX1*, HCLS1 Associated Protein X-1; *GCSFR*, Granulocyte colony-stimulating factor receptor; *MPO*, Myeloperoxidase.

There are several advanced methods which should be performed on a patient suspected of humoral immune deficiency. Analysis of the expression of B cell markers can be used to assess the B cell phenotype as well as the maturation state of B cells. Important markers used for B cell immunophenotyping include IgM, IgD, IgG, CD19, CD20, CD27, and CD38. IgM and IgD are expressed on mature B cells, but CD27 and CD38 are absent on these cells. CD27 can represent active/memory B cells and CD38 is a marker for antibody-secreting cells and plasma cells. For example, in a patient with common variable immunodeficiency (CVID) decreased levels of memory B cells (CD27⁺, IgM⁻, IgD⁻) are found. Or in patients with X-Linked Agammaglobulinemia (XLA or Bruton), a low or absent circulating B cell population is seen (Aghamohammadi et al., 2005; Moin et al., 2004).

Genetic mutational analysis is another important assessment to detect certain immunodeficiency. This approach can be performed by genome sequencing accompanied with flow cytometry for analyzing the levels of associated protein. For example, a genetic defect in the Bruton's Tyrosine Kinase (BTK) gene is linked to XLA, while a defect in the transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI) and BAFFR genes is linked to CVID. Or alterations of the ataxia-telangiectasia mutated (ATM) gene are associated with Ataxia-Telangiectasia. By using flow cytometry, intracellular expression of BTK, TACI, BAFFR, and ATM can be identified (Moin et al., 2004; Mohammadi et al., 2009; Zaki-Dizaji et al., 2017; Losi et al., 2005).

Kappa-deleting recombination excision circle (KREC) analysis is another approach to identifying a patient with a humoral immunodeficiency disorder. KRECs are episomal DNA fragments produced during the development of B lymphocytes, importantly when the kappa light-chain genes are being rearranged. PCR can be used to detect KRECs in newborn blood spots, providing a screen for B cell-related deficiencies like XLA and ataxia-telangiectasia (Serana et al., 2013).

Cellular immunity deficiency

In a patient who is suspected of having cellular immunodeficiency, a CBC with differential can be performed to distinguish infants with low/absent absolute lymphocyte counts. Subsequently, flow cytometry and in vitro functional tests can be applied to evaluate the quantity and function of T lymphocytes. Flow cytometry can distinguish between CD3, CD4, CD8, natural killer (NK), and B cell numbers. Immunophenotyping is also important for the diagnosis of severe combined immunodeficiency (SCID). CD3, CD4, and CD8 are T lymphocyte markers that are frequently decreased in defects associated with cellular immunity such as DiGeorge Syndrome. CD45RA is a marker identifying naïve T cells in infants, while CD45RO is considered as a memory T cell marker for identifying maternal T cells. In Omenn syndrome, a variant of SCID, increased frequencies of CD45RO+ T cells are found (Locke et al., 2014; Shahbazi et al., 2019).

Another method for assessing T cell function is the cutaneous delayed-type hypersensitivity (DTH) tests. In these tests, the cellular mediated memory response is detected by intradermal injection of different antigens, including purified protein derivative (PPD), *Candida albicans*, and mumps. The result is assessed 48–72 h later by measuring cutaneous induration. It should be noted that DTH is not recommended for children less than 12 months of age.

The Lymphocyte mitogen assay is another important test for assessing T cell function. In this test, T-cell mitogens are used to stimulate the lymphocytes. The quantification is performed by adding radiolabeled thymidine that is able to be incorporated into the DNA of proliferating cells. Anti-CD3 antibodies, Phytohemagglutinin (PHA), concanavalin (ConA), pokeweed (PWM), and *Escherichia coli* lipopolysaccharide (LPS) are commonly used as mitogens. In this regard, anti-CD3 antibodies, PHA, and ConA are T cell mitogens; LPS is a B cell mitogen; PWM can induce both T cells and B cell responses (Raszka et al., 1996; Ahmed and Blose, 1983).

T-cell replication excision circle (TREC) was recently developed for earlier screening of newborns with severe T cell lymphopenia. Assessing patients using TREC has helped to improve early diagnosis and reduce morbidity and mortality. TREC is referred to the circular, non-replicating pieces of DNA produced during the rearrangement of T cell receptor and they can be considered as a marker for naïve T cells (Somech, 2011).

Genetic investigation of certain genes is considered as another approach to detect cellular immunity defects as well as combined immunodeficiency in which both cellular and humoral immune systems are affected. The genetic assessment can be performed along with the analysis of protein level in the patient's sample.

Quantitative real-time PCR provides the detection of mutations with high sensitivity and specificity. For example, a mutation in the WAS protein (WASp) is associated with WAS. The detection of WASp using Flow cytometry can rapidly screen for this disorder. However, there is a newer method to detect gene alterations known as fluorescent in situ hybridization (FISH). Deletions at 22q11 that occur in DiGeorge Syndrome can be detected by this method (Stewart et al., 2007; Stachon et al., 2007).

Various phenotypes of SCID have been identified by their causative genetic deficiencies, including T-/B+/NK-, T-/B+/NK+, T-/B-/NK+, T-/B-/NK-. Genetic analysis and flow cytometry can detect defected genes. Common gamma chain (CD132) or Janus Kinase 3 (Jak3) deficiency can lead to X-linked SCID (T-/B+/NK- phenotype), the most frequent type of SCID. Deficiency in IL-7 receptor alpha, CD3, CD45, ZAP70, and Coronin-1A are related to T-/B+/NK+ Phenotype of SCID. The T-/B-/NK+ phenotype is associated with gene defects in recombination activating genes-1 (RAG-1) or RAG-2, Artemis, Ligase4 (LIG4), DNA-dependent protein kinase (DNA-PK or PRKDC), and Cernunnos (NHEJ1). In addition, adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP), and adenylate kinase 2 (AK2) deficiencies are associated with the T-/B-/NK- phenotype (Fallah et al., 2018; Locke et al., 2014).

Innate immunity deficiency

There are several disorders related to deficiencies in the innate immune system. Neutrophil-associated disorders are usually characterized by recurrent skin and respiratory tract infections. Other manifestations include delayed umbilical cord separation, poor wound healing, recurrent oral stomatitis, and omphalitis. CBC, absolute neutrophil count, and morphological analysis of neutrophils are considered as initial screening tests (Duchamp et al., 2016; Wong et al., 2013).

Leukocyte Adhesion Deficiency (LAD) is characterized by recurrent bacterial infections, delayed umbilical cord separation, or omphalitis. Three different types of LAD are defined by different gene mutations in molecules involved in leukocyte adhesion. The decreased level of expression of CD11 or CD18 using flow cytometry is considered as a diagnostic test for LAD type 1 (LAD-1). Abnormal expression of CD15c (sialyl lewis X) on neutrophils by flow cytometry is suggestive of LAD-2. In another type of LAD, LAD-3, the function of adhesion molecules is impaired. Therefore, analyzing the function of integrins in leukocytes or platelets is helpful in diagnosing LAD-3 (Etzioni, 2007; Parvaneh et al., 2010).

Chronic granulomatous disease (CGD) is another deficiency related to a defect in the innate immune system components. CGD is characterized by recurrent bacterial and fungal infections due to reduced or absent oxidative burst. CGD can be diagnosed through flow cytometric assessment of the neutrophil oxidative burst by the dihydrorhodamine (DHR) assay. This test is performed by incubation of neutrophils with DHR dye. After that, neutrophils are stimulated by adding phorbol myristate acetate (PMA). PMA induces oxidative burst in neutrophils, resulting in DHR oxidization and fluorescence production. This test is usually abnormal in a patient with CGD. Nitro blue tetrazolium (NBT) is another colorimetric assay previously defined for CGD evaluation. In general, DHR is more sensitive than NBT (Movahedi et al., 2004; Arnold and Heimall, 2017).

Two other disorders also have similar manifestations to CGD, including myeloperoxidase deficiency and glucose-6-phosphate dehydrogenase deficiency (G6PD). Although the DHR assay can be a testing option for diagnosing an MPO-deficient patient,

histochemical staining of MPO is considered as the definitive diagnosis of this disorder. G6PD is also detected by DHR, but the assessment of G6PD activity is also required in patients with recurrent infections or chronic anemia (Frank, 2005; Froissart et al., 2011).

Defects in NK cell numbers and their function result in recurrent infections and the clinical phenotype of hemophagocytic lymphohistiocytosis (HLH), respectively. For screening of suspected patients, immunophenotyping should be performed to verify the existence or absence of NK cells. CD16, CD56, and CD57 markers are usually used for immunophenotyping of NK cells using flow cytometry. On the other hand, evaluation of NK cell function can be performed by a cytotoxicity assay. This test can be done by incubating the peripheral blood mononuclear cells (PBMCs) isolated from suspected patients with labeled target cells. Subsequently, analyzing the markers associated with cell death or apoptosis, such as annexin V by flow cytometry, should be performed. In addition, assessment of CD107a by flow cytometry can be done to investigate the existence of intracellular cytotoxic proteins such as perforin and granzyme. CD107a is a marker involved in cell degranulation. Importantly, the defective expression of CD107a is considered as a diagnostic marker in patients with Griscelli syndrome, Chediak-Higashi, and familial HLH. Cytotoxicity assay can also be done for evaluation of the cytotoxic function of cytotoxic T lymphocytes (CTLs), the cells involved in cellular immunity (Orange, 2013).

Another defect in the innate immune system, complement system deficiency, can be evaluated by analyzing the level or function of the complement system using CH50 and AH50 methods. The hemolytic activity of the classic pathway is measured by CH50 assay, while the function of the alternative pathway is measured by AH50. Therefore, the deficiency in the components of the classic pathway, including C1, C2, and C4 deficiency, leads to a decreased level of CH50 but a normal AH50. On the other hand, a low level of AH50 (with normal CH50) can suggest a defect in the alternative pathway's components such as factor B, factor D, or properdin (Kirschfink and Mollnes, 2003; Frank, 2000; Pettigrew et al., 2009).

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Laboratory Identification of Bacterial Infections

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Identification of bacteria—general considerations

Rationale and justification

The goal for clinical microbiology laboratories is to provide a rapid and accurate identification of all agents including bacteria involved in infectious disease processes. While laboratories try to achieve 100% accuracy, many factors impact how successful a laboratory is in approaching this theoretical goal.

There are a number of reasons why an accurate species identification of medically important bacteria is essential and some of these are listed in Table 1. By far the single most important reason is to provide a physician with critical information regarding the cause of an infection (diagnosis and prognosis) and treatment modalities (intervention). Other important reasons for definitive species identifications include epidemiological considerations involving tracking and monitoring of infectious outbreaks in communities or healthcare settings or identifying vehicles associated with illnesses resulting from the consumption of contaminated consumable products. Outbreaks, often linked to specific pathogens and subtypes, can take on national or global importance when they evolve from a local stage. Definitive bacterial identifications can also lead to the discovery of new infectious agents or novel disease syndromes associated with classic pathogens with new virulence-associated characteristics. Finally, and not to be overlooked, is the need for a definitive identification when publishing peer-reviewed articles in scientific journals. Failure to correctly identify bacteria to species in such situations can lead to a medical literature replete with incomplete or inaccurate descriptions of phenotypic or genetic properties, species—disease associations, syndromes, and appropriate treatment regimens (Janda, 2002). Illustrative examples of the importance of definitive species identifications in certain situations are shown in Table 2.

The evolution in testing methodologies

Beginning in the late 1960s and continuing to this day technologic developments have ushered in a new era in regards to test menu options in the laboratory identification of bacteria. Fig. 1 shows the evolutionary process with some of the notable accomplishments in the development of these testing technologies between 1960 and 2020. Early seminal achievements included the miniaturization and automation of standardized test panels and combination panels with both identification and antimicrobial susceptibility results (Janda and Abbott, 2014). Such tests as the API 20E (bioMérieux Inc., Durham NC), still in use today, were developed during the early 1970s and consisted of a panel of 21 tests including an oxidase reaction. By the mid-1980s to the early

Table 1 Reasons for the Accurate Species Identification of Bacteria.

Relative priority	Category	Comment	CLIA Regulated
1	Diagnosis or prognosis	Including recurrent infections	Yes
1	Therapeutic intervention	Species-specific chemotherapy	Yes
2	Epidemiology	Disease investigations including outbreaks, epidemics and pandemics	No
2	Infection Control	Environmental surveillance for vehicles of infection	No
3	Detection of new infectious agents	New species descriptions isolated from clinical samples	Yes
3	New syndromes	In recognized or novel taxa	No
4	Research	Species-associated pathogenicity	No
5	Publications	Defining diseases, syndromes, outbreaks, or treatments associated with specific species or groups	No

CLIA, Clinical Laboratory Improvement Amendments.

Table 2 Examples of the importance of definitive bacterial identifications.

Category	Agent	Distinguish from	Disease Associations	Comments	References
New Infectious Agents	<i>Acinetobacter seifertii</i> <i>Acinetobacter variabilis</i>	Other <i>Acinetobacter</i> species	Sepsis, Wounds, Respiratory	Large groups of strains	Nemec et al. (2015)
Treatment Epidemiology	<i>Aeromonas</i>	<i>Vibrio</i> spp.	Sepsis, Wound infections	Inducible β -lactamases; carbapenemases; environmental source	Sinclair et al. (2016)
Prognosis	<i>Burkholderia cenocepacia</i>	<i>Burkholderia multivorans</i>	Cystic Fibrosis	Poorer prognosis associated with <i>B. cenocepacia</i>	Drevinek and Mahenthiralingam (2010)
Species-Associated Pathogenicity; Treatment	<i>Clostridioides difficile</i>	Same as <i>Clostridium difficile</i>	Gastrointestinal disease	Hypervirulent strain (NAP1/B1/027); poor clinical response to metronidazole	Lo Vecchio and Zacur (2012)
Diagnosis Prognosis Epidemiology	<i>Clostridium septicum</i> <i>Elizabethkingia anophelis</i>	Other clostridial species <i>Elizabethkingia meningosepticum</i>	Aortitis Gas Gangrene Sepsis	Linked to colorectal carcinoma Multiple Midwest outbreaks; new pathogenic species	Jessamy et al. (2016) Janda and Lopez (2017)
New Syndrome Species-Associated Pathogenicity	<i>Klebsiella pneumoniae</i>	No change	Pyogenic liver abscess Pneumonia Endophthalmitis	Hypervirulent (muroid K1/K2 strains)	Jun (2018)
Diagnosis	<i>Pseudomonas aeruginosa</i>	Fluorescent pseudomonads	Sepsis	Septicemia vs. pseudo-bacteremia or catheter-associated	Scales et al. (2014)
Treatment	<i>Staphylococcus pseudintermedius</i>	<i>Staphylococcus aureus</i> and <i>S. intermedius</i> group	Sepsis Chronic rhinosinusitis SSTI	Methicillin resistance	Somayaji et al. (2016) and Canver et al. (2019)
Diagnosis Prognosis	<i>Streptococcus bovis</i>	<i>S. equinus</i> , <i>S. gallolyticus</i>	Endocarditis Sepsis	Linked to colonic carcinoma	Riyaz et al. (2016)
Diagnosis Prognosis Epidemiology	<i>Vibrio cholerae</i>	<i>Vibrio mimicus</i>	Gastroenteritis	Enteritis vs. cholera; 01/0139 vs. non-01	Thompson et al. (2008)

SSTI, skin and soft tissue infections.

1990s standalone molecular platforms were introduced involving the genetic sequencing of housekeeping genes such as 16S (Janda and Abbott, 2007). Today, there are literally dozens of commercially available tests that can identify single species or targeted groups of bacteria.

Obviously the more state-of-the-art analytical instrumentation employing molecular strategies offers a more precise and definitive identification of bacteria than does conventional testing platforms developed years ago. In the case of bacterial identifications, the accuracy of such systems ranges from spot tests $\rightarrow$ commercial panels $\rightarrow$ PCR $\rightarrow$ Matrix-Assisted Laser

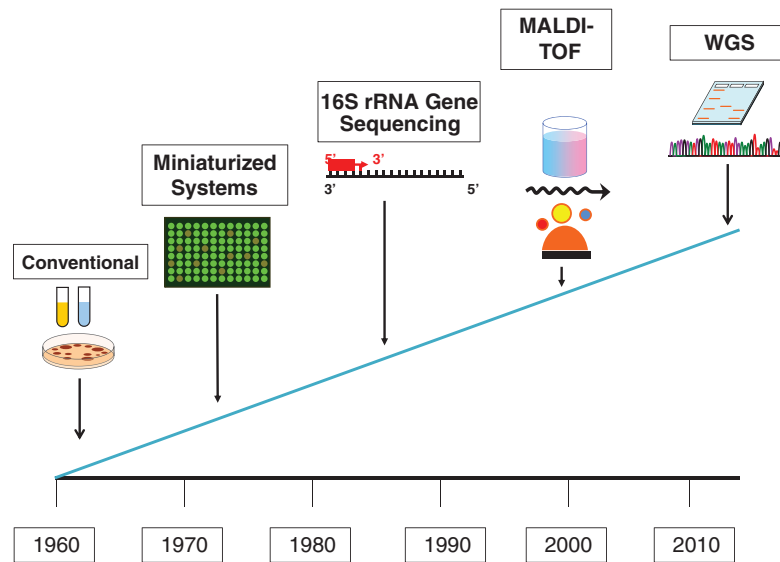


Fig. 1 Technologic Developments in the Identification of Bacteria (1960–2010).

Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF) → 16S gene sequencing → Whole Genome Sequencing (WGS). However, the choice of identification systems used in each laboratory can vary significantly because of a number of factors.

General principles of testing strategies

The “gold standard” for laboratory testing is diagnostic accuracy which theoretically implies 100% sensitivity and specificity. Other principles that need to be considered in evaluating the usefulness of any diagnostic test for infectious diseases include performance, operational characteristics, and price versus clinical impact (Banoo et al., 2006; Cantón et al., 2017). In the broader sense, laboratory tests are only one of three components needed to accurately diagnose a patient’s illness. These components are analytical validity (lab testing), clinical validity, and clinical utility (Wolk, 2020).

The choice in testing methodologies used in clinical laboratories to identify bacteria must be counterbalanced by the analytical validity of each assay against a number of competing factors including size and staffing of the microbiology laboratory and type of medical or public health facility. Sample testing volumes can vary dramatically from ~10,000 to more than half a million/year. These values do not even include significant variations in numbers or types of tests offered. These driving forces will dictate to a great extent options available for the type of test formats than can be used in particular situations. A second competing factor is cost. The cost of tests useful in identifying bacteria can range from a few cents for phenotypic assays to >\$100 for a single multiplex PCR cassette. Costs are even much higher for WGS. While a test itself can cost pennies to perform (MALDI-TOF), the initial capital investment can be expensive as the cost of instrumentation can exceed \$200,000. As the accuracy of a definitive test for bacterial identification increases with the use of molecular platforms so do the costs.

Regulations

In the United States (USA) the identification of bacteria in the clinical laboratory in most instances is judged to be a high-complexity test. Identification of bacteria leading to a disease-specific condition, patient prognosis, or treatment falls under the Clinical Laboratory Improvement Amendments (CLIA) of 2003 (Anderson et al., 2009). CLIA is administered by Centers for Medicare and Medicaid (Baltimore MD). CLIA regulates the identification of bacteria as it relates to patient care and treatment but does not regulate epidemiologic investigation or environmental surveillance (Table 1). CLIA certifies diagnostic laboratories in the US by monitoring testing practices, quality control, proficiency testing, and test validation among other things.

The second category of testing regulations for bacterial identification involves medical devices. Devices used for the diagnosis of infectious diseases are reviewed by the Division of Microbiology Devices, Center for Devices and Radiological Health, Food and Drug Administration (FDA) (Shawar and Weissfeld, 2011). Commercial-based tests are rigorously tested and evaluated by the FDA prior to formal approval. The category of test complexity (waived, moderate, high) is determined by the evaluation of seven factors which include knowledge needed, training and experience, reagents and materials preparation, characteristics of operational steps, calibration, quality control, and proficiency testing materials, test system troubleshooting and equipment maintenance, and interpretation and judgment required.

A key area not considered before the advent of many molecular testing platforms are standalone tests, also known as “home brews” which are not FDA approved. Such technologies were previously classified as in vitro diagnostics by the FDA whose use and development were operated within a single clinical laboratory facility (Genzen, 2019). One example of this type of technology

would be genome sequencing which is now classified by the FDA as Laboratory-Developed Tests or LDT when used in diagnostic settings. A long-term resolution regarding oversight of LDTs is uncertain (Genzen, 2019). Currently to use such a test or to modify another test (e.g., enhance database) means that such an assay automatically defaults to high-complexity testing. Extensive validations to meet CLIA requirements is required. Two examples of the validation of Illumina platforms, one designed to meet CLIA requirements, have been recently published (Kozyreva et al., 2017; Arnold et al., 2018).

Bacterial identification and modern molecular taxonomy

The final identification of a bacterium associated with human infections is intimately linked to the field of bacterial taxonomy. The appropriate use of bacterial taxonomy is how we communicate worldwide regarding classic or emergent illnesses or disease syndromes, species-associated clinical manifestations, appropriate medical intervention including antimicrobial therapy, and epidemiologic investigations ranging from outbreaks to global pandemics (Janda, 2021). The simple assignment of the appropriate scientific name (genus and species) to a pathogenic bacterium immediately also allows for a historical review of medical and scientific literature regarding disease symptoms, treatment, prognosis, and infection control-related aspects. No matter what system is used to provide a final bacterial identification, the key to its usefulness and function is the correct placement of a scientific name on the infectious agent in question.

Innovative molecular methods have impacted the field of modern taxonomy such that the number of proposed taxa has exploded over the past 15 years with over one thousand new species often described on an annual basis (Janda, 2017). FDA approved commercial identification systems have internally constructed databases for bacteria tested and identified on each system which allow for genus and species recognition. However, in the case of non-FDA approved molecular systems the construction of databases may be proprietary in nature and of unknown phylogenetic range and depth while other platforms producing sequence identifications are linked to public or private databases such as GenBank. The end result is that both FDA and non-FDA approved systems used to provide bacterial identifications have a difficult time keeping up with a rapidly evolving prokaryotic taxonomy.

Identifications need to be up-to-date and conform to present rules and regulations concerning bacterial nomenclature and taxonomy. The List of Prokaryotic names with Standing in Nomenclature (LPSN) is the most comprehensive source for all bacterial names. It has recently been acquired by Leibniz Institute DSMZ—German collection of Microorganisms and Cell Cultures GmbH (Parte et al., 2020). For clinical microbiology, two journals (*Diagnostic Microbiology and Infectious Diseases*, *Journal of Clinical Microbiology*) provide periodic updates on new taxa and classification changes associated with bacteria isolated from clinical samples (Janda, 2017; Munson and Carroll, 2021).

Laboratory decisions regarding test selection and menus

Emerging trends

The modern era for diagnostic microbiology has witnessed a number of increasingly important trends concerning the identification of bacteria in the clinical laboratory. These emerging trends can be summarized as follows: (1) A gradual transition from conventional testing formats to molecular-based platforms as depicted in Fig. 1 (Laupland and Valiquette, 2013). Such technologies include targeted- or multiplex PCR, loop-mediated isothermal amplification, 16S gene sequencing, mass spectrometry and WGS (Wolk and Dunne, 2011; Mitsuma et al., 2013; Laupland and Valiquette, 2013; Li et al., 2017). Driving forces behind implementation of these newer technologies in the diagnostic laboratory are a more rapid and definitive identification of bacteria; (2) Automation in clinical microbiology is slowly evolving from inoculation systems to partial or completely automated systems for bacteriology (Croxatto et al., 2016; Leo et al., 2020). European labs have been innovators in the emergence of this technology but it is now being implemented in some US laboratories as well; (3) An increasing trend towards higher sample volume requests most likely due to molecular assays, increasingly limited laboratory budgets, and a greater percentage of lab expenditure versus overall medical costs. In the United States, estimated healthcare figures for 2018 totaled \$4.4 trillion dollars which is more than 20% of gross domestic product (Ricaldi et al., 2014). A much earlier figure from 2002 found laboratory expenses at \$2.5 billion with an annual growth rate of 10% (Ricaldi et al., 2014). Precipitously high lab costs have led to financial constraints by limiting laboratory budgets and management initiatives for “lean microbiology” (Samuel and Novak-Weekly, 2014); (4) Concurrent to the expansion in molecular testing methods has been potential increases in federal or national regulatory oversight of in vitro devices commonly referred to as LDTs (Dunne Jr, 2011; Genzen et al., 2017; Genzen, 2019). Such regulations could impact how and if LDTs can be used in a clinical environment (Bank et al., 2020).

Laboratory logistics

With the increasing demand on laboratories for more accurate and rapid testing versus increasing test requests, dwindling budgets, and limited peripheral resources the laboratory director and staff are faced with a series of complex and competing issues. These issues dictate to a large extent what methods for identifying bacteria can be introduced into the clinical laboratory. In the United States, federal testing requirements under CLIA are intertwined with state or university-required licensure or certifications such as those provided by the American Society of Clinical Pathology to provide high complexity testing. These personnel requirements are

offset by fewer hospitals or universities offering laboratory-based programs for certification which translates into fewer certified personnel entering the workforce (Dunne, 2011; Croxatto et al., 2016). Other notable factors that dictate the choice of testing algorithms include test reimbursement rates, laboratory space, staffing and hours of operation, types of clinical and anatomic samples received, and patient population served (Samuel and Novak-Weekly, 2014).

Clinical factors

A number of clinical factors impact the selection of test menus for the identification of bacteria. These factors are outside the realm and control of clinical laboratories. The introduction of rapid molecular diagnostic tests to quickly identify bacteria causing bloodstream infections (BSIs) allows for improved patient care by changing from empirical therapy to species-directed chemotherapeutic intervention (Wu et al., 2020). Use of such technology also leads to more accurate BSI diagnosis, patient care, reducing timelines for effective therapy, and cost-effectiveness (Sakarikou et al., 2018; Wu et al., 2020; Briggs et al., 2021). Such innovative developments or methods drive testing volumes upward with associated costs impacting the laboratories that provide such resources.

Laboratory tests, which include the identification of pathogenic bacteria in the clinical microbiology laboratory, are the main data source that supports or influences physician decisions on patient care (Bindraban et al., 2018; Ekblom and Peterson, 2018). The oft-quoted but poorly substantiated figure of 70% refers to the percentage of clinical decisions made in medical practice based upon test results (Samuel and Novak-Weekly, 2014; Bindraban et al., 2018; Hicks et al., 2021). With the advent of rapid molecular tests this has led to a staggering increase in test requests which impacts the microbiology laboratory. Data suggests, however, that at least 20% of test requests are either not needed or not appropriate for a particular clinical picture (Bindraban et al., 2018; Cadamuro et al., 2018). These contributing factors again raise the financial costs incurred by the diagnostic laboratory. Reducing test utilization is a key component of helping to maximize the diversity and number of tests a microbiology lab can offer by reducing both the type of test selected and test volume (Ricaldi et al., 2014).

Bacterial identification: Test categories and strategies

There is presently a large litany of tests, technologies, and devices that have been developed over the past 40-odd years useful in providing a definitive identification for clinically significant bacteria (Wolk and Dunne, 2011; Laupland and Valiquette, 2013; Tshikhudo et al., 2013; Li et al., 2017; Fairfax et al., 2018). Many systems are no longer in general use as they become outdated by newer technology, cost factors, or clinical applications. Bacterial identification strategies have been broken down into four broad categories (Fig. 2). These include rapid tests, phenotypic or biochemical analyses, PCR-based assays, and molecular platforms which include mass spectrometry. Each of these categories has witnessed technologic developments of newer and more innovative approaches to the identification of pathogens over time (Table 3). PCR tests are separated out as many of these assays have been commercially developed and FDA-approved in contrast to most other molecular platforms. This section will cover those tests that presently have found the greatest widespread application in the field of bacterial identification worldwide.

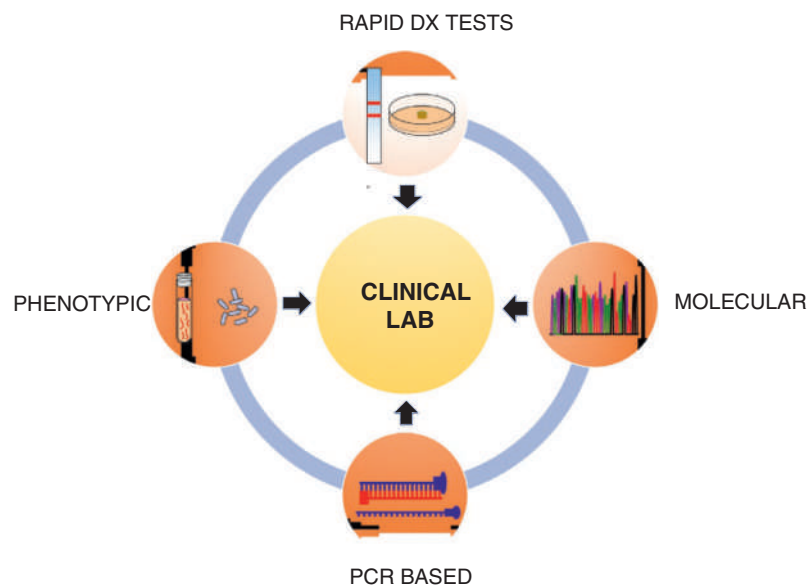


Fig. 2 Groups of Tests Currently Used in Bacterial Identification in Laboratories.

Table 3 Identification of bacteria by standard and advanced methods.

Type of test		Phenotypic	Rapid diagnostic tests	PCR	Molecular
Clinical applications	Standard methods	• Conventional (TSI, LDC, etc.) • Biochemical test strips (API 20E)	• Spot assays (indole, MUG, PYR, etc.) • Gram stain • Hemolysis (BAP)	• Monoplex PCR	• Sanger sequencing
	Advanced applications	• Combo ID/AST plates (MicroScan, VITEK, Phoenix) • Accelerate PhenoTest • Carbon Source Utilization • FAME • Latex agglutination assays • ELISA	• Lateral flow immunoassays • Dipsticks	• Multiplex PCR • Syndromic panels (GI, RTI)	• MALDI-TOF • 16S rDNA gene sequencing • pyrosequencing • WGS

AST, antimicrobial susceptibility testing; BAP, blood agar plate; ID, identification; ELISA, enzyme-linked immunosorbent assay; FAME, fatty acid methyl ester; GI, gastrointestinal; LDC, lysine decarboxylase; MALDI-TOF, Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry; MUG, 4-methylumbelliferyl- β -D-glucuronide; PYR, pyrrolidonyl aminopeptidase; PCR, polymerase chain reaction; RTI, respiratory tract infection; TSI, triple sugar iron; WGS, whole genome sequencing.

Rapid assays

There are many clinical and epidemiologic settings where a rapid test may be needed or warranted. Most rapid tests are either a general biochemical test used to screen or identify a certain bacterial species, a species-specific rapid immunologic or molecular test (<2 h) in clinical situations, or a field test where a traditional laboratory workup is not practical. Almost all of these tests are species-specific excluding syndromic panels that are discussed elsewhere.

Although out of vogue, standalone or “spot” biochemical tests can be used with a fairly high degree of accuracy to identify a limited but significant set of commonly encountered bacteria. The advantages of such tests are that they are extremely cost-effective, can generate a final identification in just a few minutes, and have an overall accuracy of at least 95%. Caveats associated with such testing require a pure culture, a microbiologist knowledgeable regarding colonial morphology and test reactions, and the understanding that a misidentification using such a scheme is unlikely to impact patient care (York, 1999). Successful multi-laboratory validations of individual tests, such as the spot indole, L-pyrrolidonyl- β -naphthylamide (PYR), to correctly identify *E. coli* show an error rate of 0.3% (York et al., 2000). Collective guidelines for use of abbreviated or rapid testing schemes have subsequently been published (Baron, 2001; CLSI, 2008).

Another standard test useful in the rapid detection of bacteria is the lateral flow immunoassay or LFIA (Andryukov, 2020). LFIA tests are relatively inexpensive, easy to read and can yield a final identification quickly. Bouzid et al. (2021) have recently reviewed the use of LFIA in the emergency department. Among bacterial species that can be rapidly identified correctly using LFIA are Group A streptococci, *Streptococcus pneumoniae*, *Legionella pneumophila*, and *Treponema pallidum*. A disadvantage of this system is a lack of a viable culture.

Rapid tests are also useful for many orphan diseases or for important public health pathogens that require an immediate diagnosis. Such is the case of *Vibrio cholerae*, the causative agent of epidemic or pandemic cholera. Many cases of cholera occur in remote locations of Africa or Asia where it is extremely difficult to get a specimen transported promptly to a clinical laboratory. Such delays can impact the health of an infected person and can impact the spread to others through contaminated food or water. Immunochromatographic tests have been developed to rapidly detect cholera on-site allowing for immediate public health intervention. One such test, the VC[®] Rapid Dipstick (Span Diagnostics, India) can detect *V. cholerae* in stools directly (or after a 4–6 h incubation in alkaline peptone water enrichment) within 15–30 min (Harris et al., 2009; Ontweka et al., 2016). Enrichment cultures showed a sensitivity of 100% (Ontweka et al., 2016). The test detects both *V. cholerae* O1 and O139 serotypes.

Phenotypic assays

Despite being the oldest of the current testing venues for bacterial identifications in the clinical laboratory, phenotypic methods remain the mainstay for most laboratories (Laupland and Valiquette, 2013; Li et al., 2017; Chakraborty and Doijad, 2018). Biochemical tests are by far the most commonly employed method by which most laboratories render final identifications. These collective systems share a number of distinct advantages in common including they are low-cost, easy to perform, and are highly accurate in the identification of established genera and species. In contrast, such systems have a number of inherent disadvantages including (1) delayed reporting times of 1–3 days for final identifications, (2) a pure culture is required such that identifications cannot be directly made from anatomical specimens such as blood, (3) test results that can theoretically be influenced by growth medium or environmental conditions, and (4) commercially-associated databases which cannot keep up with an expanding list of newly described taxa (Wolk and Dunne, 2011; Janda, 2017; Tshikhudo et al., 2013; Li et al., 2017).

Manual microidentification systems

The “Grandfather” of phenotypic identification systems is the API 20E strip (bioMérieux, Durham NC) and its progeny such as the API NH and Rapid ID 32 A. First introduced in the 1970s it miniaturized standard tests into microcapsules. In the case of API 20E, the strip consists of 20 tests (plus a manual oxidase test) that generates a 7-digit code based upon 21 reactions after an 18 h incubation. Inputting this code into the APIWEB™ database yields a final identification. This strip has now been used for over 40 years in clinical laboratories. Another such system is the BBL Crystal Enteric/Nonfermenter system (Becton, Dickinson and Company, Sparks MD). The Crystal E/NF panel contains 29 dehydrated test substrates plus a control. Like API it produces a 10-digit code leading to an identification. While both systems provide good to excellent identifications for common bacteria, they suffer from several disadvantages in addition to those noted above. Foremost among these is a bacterial identification without an accompanying antimicrobial susceptibility profile. Secondly, as in the case of the API 20E strip the test configuration has not changed in 40 years. Since this test invention preceded many of the bacterial species described since 1980 the battery of tests on the strip may not be ideal for more recently described taxa.

Automated microidentification combo panels

Offspring of manual identification systems were commercially developed during the late 1970s and early 1980s. These “Expert Systems” rely on the use of artificial intelligence with clinical applications useful for diagnostic microbiology (Winstanley and Courvalin, 2011). Among the major new features of these systems were (1) the development of a combination (combo) panels providing both an identification (ID) and antimicrobial susceptibility (AST) profile concurrently for each bacterium tested, (2) fully automated systems including reading of test results and test interpretation, thereby eliminating manual reader bias and errors, (3) expanded panels with biochemical tests to more accurately identify newly described infectious agents, (4) enhanced commercial databases for both ID and AST, and (5) periodic updated versions of software and peripherals (Winstanley and Courvalin, 2011; Li et al., 2017). Commercially automated systems commonly used on a global basis included the BD Phoenix (Hunt Valley MD), bioMérieux VITEK 2 (Durham NC), and DxM MicroScan Walkaway (Beckman Coulter Inc., Carlsbad CA). These automated systems have been the primary “workhorses” in diagnostic labs for years. However, they still lack as rapid a turnaround time (ID and AST) as physicians would like for patient care.

Bloodstream (BSIs) are one of the most serious and life-threatening complications associated with hospitalization. BSIs represent the second largest cause of healthcare-associated mortality in USA hospitals (Briggs et al., 2021). To decrease the incidence of negative outcomes of BSIs and associated costs of medical management requires more rapid system of bacterial ID and AST to detect cases of bacteremia and to revise medical management and empirical therapy as quickly as possible (Sakarikou et al., 2018; Özenci and Rossolini, 2019).

Recently, a more rapid bacterial identification and susceptibility testing system that can be used directly on positive blood cultures was FDA-approved in 2017. The Accelerate PhenoTest™ system (Accelerate Diagnostics Inc., Tucson AZ) relies on fluorescent *in situ* hybridization or FISH with morphokinetic cellular analysis to generate a final bacterial ID and AST result in 2 and 7 h respectively. When compared to standard methods, the PhenoTest™ system exhibited an identification specificity of between 91% and 99.5% for organisms in their database and a sensitivity of 95.6% (Marschal et al., 2017; Charnot-Katsikas et al., 2018; Ullberg and Özenci, 2020). Overall categorical agreement for AST was 95.5–96.4% with very major errors reported at 1% (Marschal et al., 2017; Charnot-Katsikas et al., 2018). The Accelerate system also reduced the time to ID by 23–27 h and AST results by >40 h (Marschal et al., 2017; Charnot-Katsikas et al., 2018). The system presently has difficulty with streptococcal species and coagulase-negative staphylococci as well as some less frequently encountered gram-negative bacteria (Marschal et al., 2017; Ullberg and Özenci, 2020).

PCR

The invention of PCR technology by Kary Mullis, for which he won the Nobel Prize in 1993, shepherded in the modern molecular era in the diagnostic microbiology laboratory (Fairfax et al., 2018). PCR technology allows for the amplification of DNA (genes) through a series of thermocycling reactions coupled to a heat-stable DNA polymerase (Laupland and Valiquette, 2013). Amplified products (targets) can then be detected utilizing a number of different techniques including reverse transcriptase-PCR, transcription-mediated amplification, and nucleic acid sequence-based amplification (Li et al., 2017). Targeted DNA can be present as a pure culture, as an unknown pathogen embedded in soft tissue, or as part of complex microbial flora of the gastrointestinal or respiratory tracts as examples.

The advantages of PCR are many. PCR assays are rapid, precise, can have a broad range of targets (Laupland and Valiquette, 2013; AlMasoud et al., 2020). When utilized in a real-time PCR format the product can be detected and quantified over time. This can be incredibly useful when trying to detect which bacteria may be causing clinical disease in a complex microbial environment such as the respiratory tree. However, like all techniques it has several disadvantages. These disadvantages include knowing the sequence of a specific target, the inability to distinguish live and dead cells (DNA target), time-consuming, possible contamination issues, and requiring a certain amount of expertise (AlMasoud et al., 2020).

There are several basic configurations for PCR assays. The oldest and the forerunner of more modern-day applications is the monoplex reaction where the target is a single bacterial agent. Numerous commercial PCR tests have been developed in this configuration for bacterial diseases like *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae*. Monoplex PCR reactions provide quicker definitive test results when compared to standard culture allowing for faster medical and public health intervention practices to be instituted. An offshoot on the monoplex reaction is the LDT. The advantage of an LDT is lower cost and the ability to detect orphan diseases which are not commercial practical for manufacture. LDTs are not FDA-approved and require validation to be used as an in vitro diagnostic device for human infections. Recently, multiplex PCR reactions, which are currently in vogue in clinical labs involve the use of multiple primers to detect multiple targets in different organisms. They offer several advantages that monoplex reactions cannot provide.

Syndromic PCR panels

The view of bacteria-associated infections has radically changed over the years from a one disease = one pathogen concept (diphtheria, tetanus) to a model of syndromic illnesses (gastroenteritis, bloodborne) which are caused by many different taxa. Over the past 10 years, commercial companies have developed and introduced multiple panels based upon multiplex PCR technology that can detect the many bacterial agents associated with a variety of syndromic diseases (Patel, 2016; Ramanan et al., 2018; Couturier and Dien Bard, 2019; Dien Bard and McElvania, 2020). The recognized leader in this field is BioFire (Salt Lake City, UT) which has received federal clearance for a number of diagnostic panels which can detect microorganisms including bacteria associated with syndromes such as gastroenteritis, septicemia, upper and lower respiratory tract infections, and meningitis/encephalitis.

While many different types of syndromic panels have been developed, the oldest of these panels involve the GI tract which in theory can serve as standalone-tests replacing conventional methodologies (Patel, 2016). A number of GI panels that are commercially available are listed in Table 4. All of these automated panels are based upon multiplex PCR technology but differ in their approaches, number and types of bacterial targets detected, whether the panels also detect viral and parasitic pathogens, and turnaround times. All systems essentially detect the four most common bacterial enteropathogens, namely *Campylobacter*, *Salmonella*, *Shigella* and STEC (Shiga toxin-producing *E. coli*). These are so-called “culture-independent” diagnostic tests (CIDT) as a final molecular identification of pathogens occurs without traditional culture. Thus, some of the major advantages of this technology (automation, ease-of-use, rapid identification) are partially counterbalanced by the fact that viable strains are not isolated, and therefore antimicrobial susceptibility and epidemiologic profiles cannot be generated (Janda, 2014).

There is considerable controversy regarding GI panels whether their use should be as a first-line testing option or of a more restricted nature (Schreckenberger and McAdam, 2015). On the positive side of first-line testing is the elimination of excessive technical time, maintenance and quality control of enteric media, and a rapid definitive diagnosis. On the negative side is test expense (>\$100/test), the limited number of enteric pathogens that require antimicrobial intervention, and the fact that many recognized enteropathogens are not detected by any of the commercially available panels. Table 5 lists some of the pluses and minuses of GI panels as they presently exist. Clinical situations where GI panels are useful include (1) individuals with severe dehydration, bloody diarrhea, colitis, or dysentery, (2) persons with subacute (14–28 days) or chronic diarrhea (>28 days) where the detection of enteroaggregative *E. coli* or *Plesiomonas* is entertained (3) for infectious agents that, if detected, require treatment

Table 4 Commercial syndromic panels to detect bacterial gastroenteritis.

Panel	Manufacturer	Targets		Time to Detection (hours)	FDA Approved
		Total ^a	Bacterial		
BioCode [®] Gastrointestinal Pathogen Panel	Applied BioCode (Santa Fe Springs, CA)	16	10	4	Yes
BD MAX [™] Enteric Bacterial Panel	BD Diagnostics (Sparks, MD)	4 (7) ^b	4 (7) ^b	3–3.5	Yes
EntericBio [®] Gastro Panel 2	Serosep (Limerick, Ireland)	6	4	3	No
FTD Bacterial Gastroenteritis	Siemens Healthcare (Erlangen, Germany)	7	7	6	No
FilmArray GI Panel	BioFire bioMérieux (Salt Lake City, UT)	22	13	1	Yes
ProGastro	Hologic Prodesse (San Diego, CA)	4	4	4	Yes
Seeplex [®] Diarrhea-B1 ACE Detection	Seegene (Seoul, South Korea)	5	5	9–10	No
Verigene [®] Enteric Pathogens	Luminex Corp. (Austin, TX)	8	6	2	Yes
XTAG GPP	Luminex Corp. (Austin, TX)	14	8	5	Yes

^aIncludes viruses and parasites.

^bThe extended bacterial panel (xEBP).

Table 5 Advantages and disadvantages associated with gastrointestinal syndromic molecular panels.

PRO	CON
• Rapid final identification (hours) • Automated; ease of use • Standalone technology • Eliminates or reduces enteric media and technical time • Can detect multiple enteric pathogens simultaneously • Use may reduce the number of endoscopies and related procedures	• Expense (cost per test) • No viable culture for antimicrobial or epidemiologic profile • Potential false-positives • Targets of questionable clinical significance (e.g., <i>Clostridioides difficile</i>) • Many accepted enteropathogens not covered (<i>Aeromonas</i>, specific <i>Vibrio</i> species) • Multiplex GI testing useful only for specific subpopulations

(e.g., shigellosis), and (4) for epidemiologic purposes such as outbreaks (DuPont, 2009). Some of the clinical questions one needs to consider in the use of molecular tests are stressed in a recent commentary by Tarr et al. (2019).

From the laboratory perspective, a number of issues remain unresolved. One surprising finding has been the number of positive stools that contain 2 or more enteropathogens. In a study of traveller's diarrhea 75.8% of FilmArray GIP-positive samples contained multiple pathogens (Kutsuna et al., 2021). Other studies using the Luminex GPP found 56.2% of pediatric patients positive for enteropathogens while 24.2% of controls contained enteropathogens as well (Tilmanne et al., 2019). A question to be asked is what does this mean and how do you interpret such test results as a significant number of false-positives have been reported (Dien Bard and McElvania, 2020)? Contamination has also been a problem leading to false-positive test results on the FilmArray system for *Vibrio cholerae* O1 and *Yersinia enterocolitica*. How common this situation is, is not known but has led to some panel revisions by the manufacturer. Finally, one large study found that an enteropathogen could only be recovered (reflex culture) from 57% of CIDT-positive samples (Imdad et al., 2018). Such results if repeated in other investigations could have a severely negative impact on epidemiology and public health.

MALDI-TOF

One of the remarkable achievements in modern diagnostic microbiology has been the introduction of MALDI-TOF into the mainstream of clinical microbiology to rapidly identify pathogenic bacteria (Fairfax et al., 2018; Welker et al., 2019; Tsuchida et al., 2020). Unlike other molecular techniques, MALDI-TOF relies on proteomics and the characterization of bacterial proteins at the molecular level. The technique, developed and refined for diagnostic use over a quarter of a century (1975–2000), involves a laser that emits soft ionization which then transfers energy to a matrix containing bacteria eventually leading to protein ions for analysis (Wolk and Clark, 2018).

MALDI-TOF requires only a small sample of a pure bacterial colony spotted onto a metal plate allowed to dry before being overlaid with a crystalline matrix typically composed of either α -cyano-4-hydroxycinnamic acid or 4-chloro- α -cyanocinnamic acid (Patel, 2016; Wolk and Clark, 2018). Bacterial molecules (proteins) are subsequently desorbed by short laser pulses in a vacuum tube producing ionized proteins with a mass ranging from 20 to 100 kDa. The ionized proteins are then accelerated through a positively charge electrostatic field into an analyzer where a taxon's molecular protein fingerprint composed of individual mass spectra is compared to reference spectra in a database (Patel, 2016). In theory each microorganism produces a unique protein signature identifiable as a spectral fingerprint resolvable at the genus and species level.

MALDI-TOF has a wide range of potential medical applications including the diagnosis of both infectious and non-infectious conditions. Currently the primary role MALDI-TOF plays in a majority of clinical laboratories is in providing a final identification of clinically significant bacteria. Two MALDI-TOF systems have FDA approval in the United States. These are the VITEK[®] MS (bioMérieux, Marcy-l'Etoile, France) and the MALDI Biotyper[®] (Bruker Daltonics, Billerica, MA). These are the two leading mass spectrometry systems in use today. For laboratories and hospitals, they offer a number of distinct advantages including a definitive identification in <5 min (Patel, 2016; Fairfax et al., 2018), considerable material and labor saving, the potential to streamline early empirical therapy based upon a rapid diagnosis (Florio et al., 2018), and reduced length of stays for hospitalization with associated costs (Psaroulaki and Chochlakis, 2018). The two MALDI-TOF systems, while similar, differ in several aspects. The Biotyper[®] produces identification scores between 0 and 3 (Florio et al., 2018). A score of ≥ 2.0 indicates a high confidence in genus and species identification while a score of ≥ 1.7 to <2.0 suggests confidence to the genus level only. Scores below 1.7 are interpreted as "no identification." With the VITEK[®] MS a score is generated based upon a probability percentage. Percentages are color-coded with a green square >90% identity, a yellow triangle for 85–89.9% identity, and red circle for <85%. Identifications with a green square are considered reliable while those with a yellow triangle are considered acceptable. These systems also differ in their respective databases and construction of diagnostic algorithms (Patel, 2016; Tsuchida et al., 2020). Several other mass spectrometer systems are available for the identification of bacteria that are not FDA approved. These include Autof MS1000 (Autobio Diagnostics Co. Ltd, Zhengzhou, China) and ASTA MicroIDSys (ASTA, Suwon, Korea).

As of 2019, there were more than 3,000 published articles on "MALDI-TOF and microbiology" listed on PubMed which are beyond the scope of this section for discussion (Angeletti and Ciccuzzi, 2019). Both FDA-approved MALDI-TOF systems provide a high percentage of correct identifications for many common bacteria and some more difficult-to-identify species (Levesque et al.,

2015). While the cost-per-test is remarkably small, the initial cost to purchase a system is sizable running in excess of \$200,000 USD. This figure does not include such items as maintenance contracts and associated reagents. The cost of such technology actually precludes many smaller to moderate-sized laboratories from adopting this technology (Welker et al., 2019). Hopefully, the cost will decline in the future. Other factors to consider in whether to purchase a MALDI-TOF system or not include the number and types of bacteria that require a molecular identification and the patient population served.

Despite its common use in laboratory settings, MALDI-TOF is still in the developmental stage as a diagnostic tool. How good a final identification is depends upon how well each MALDI-TOF system's database is curated and how extensive and diverse is the collection of strains used to represent a single taxon (genomospecies). Additionally, a number of reports have repeatedly documented that one or both FDA-approved systems have issues identifying species or phenotypically similar groups or complexes. These taxa include mycobacteria, *Streptococcus pneumoniae* and the *Streptococcus oralis/mitis* group, *Listeria* spp., *Enterobacter* spp., some strains of *Stenotrophomonas maltophilia* and members of the *Burkholderia* and *Acinetobacter baumannii* complexes (Florio et al., 2018; Oviaño and Rodríguez-Sánchez, 2020). Of particular importance is the resolution of *Shigella* species from one another and their separation from *Escherichia coli* since this entire group constitutes a single genetic species although their separation has important medical as well as public health implications.

At the time of writing, the VITEK® MS system included approximately 400 bacterial species in their database. Some organisms that this system could not previously identify (*Brucella*, *Elizabethkingia anophelis*) have been recently added and are FDA-approved. Many other taxa are currently not available, particularly those that are less frequently encountered in the clinical environment. This requires development of an in-house custom library through supplementation of the existing database with additional spectral fingerprints from different taxa. An interesting letter by Pandey et al. (2019) detailed how issues occurred in regards to bacterial identifications after introduction of the MALDI Biotyper® into their laboratory as the primary method of identification for ~90% of cultures. The laboratory found a number of serious errors including where *Neisseria meningitidis* was misidentified as *N. gonorrhoeae*. Other issues noted were the separation of *Salmonella* and *Citrobacter* species and species-level identifications for *Klebsiella variicola* and *Haemophilus* spp. The authors now use conventional tests in many of these scenarios. Collectively, this data strongly suggests that MALDI-TOF is not at present a “standalone” instrumentation that can be used unequivocally to identify all bacteria in the laboratory. Rather MALDI-TOF can be a useful adjunct to a bacterial identification scheme encompassing more than one technique.

16S rDNA gene sequencing

A standard technique often seen in moderate- to larger-sized diagnostic laboratories since the mid- to late-1990s is 16S rDNA gene sequencing for the definitive identification of bacteria. This technique has a number of advantages over phenotypic methods. Besides being based upon nucleic acid sequences instead of the phenotypic expression of genes, the method is highly accurate, quantifiable, and objective and can be used to identify rare bacteria with atypical biochemical profiles, slow-growing organisms, and the identification of novel species (Petti, 2007; Woo et al., 2008). Furthermore, broad-range 16S rDNA PCR has the capability to directly identify bacteria to genus and species from clinical samples without culture (Akram et al., 2017). This can be medically important in culture-negative cases and to target antimicrobial therapy directly against a particular microbe. Finally, 16S gene sequence analysis has often been used as an arbitrator in final bacterial identifications when two or more other phenotypic or molecular methods yield conflicting results (Pandey et al., 2019). In many clinical laboratories it is now considered to be the “gold standard” in routine bacterial identifications (Laupland and Valiquette, 2013).

The 16S housekeeping gene encodes for a portion of the 30S ribosome (Petti, 2007). The full sequence is approximately 1500 bp in length (Janda and Abbott, 2007). Due to the fact that all bacteria possess at least 1–15 rRNA operons with most species (>80%) possessing more than one, it provides an excellent target for gene amplification and sequencing (Espejo and Plaza, 2018). The 16S gene is composed of both conserved and hypervariable regions, the latter designated V1–V9 (Tshikhudo et al., 2013). The variable regions provide for sequence diversity in regards to taxonomic identification. An advantage of the 16S gene is that because of its overall functional importance to all bacteria it is highly conserved on an evolutionary scale although “drift” in some species has been detected.

Standard analysis of the 16S rDNA gene employs Sanger sequencing (chain terminating dideoxynucleotides) although 16S rDNA next-generation sequencing is also quite common (Rizal et al., 2020). The sequential process includes DNA extraction, PCR amplification with universal primers of the 16S gene, purification, and then subsequent 16S sequencing (Laupland and Valiquette, 2013). Sequencing and final bacterial identifications can be achieved using a commercial product with included database or by importing sequence results into public or private databases such as GenBank or the Ribosomal Database Project. The most common commercial system used is the MicroSEQ™ microbial identification system (Thermo Fisher, Waltham, MA). MicroSEQ™ databases range in size from 1,400 to over 7,000 entries. The MicroSEQ™ system is sold as a “Research Use Only” application and thus requires validation for diagnostic use in the United States.

Final bacterial identifications based upon 16S rDNA sequencing are determined by % similarity or % divergence of the test strain when compared to reference sequences such as the type strain. While no single value is universally recognized for a 16S species match, a threshold value above 98.7% is generally accepted as a minimum requirement for species identification (Rossi-Tamisier et al., 2015; Janda, 2018). In most instance values above 99% sequence similarities are used clinically for species identification. Genera can be assigned when a sequence similarity score of $\geq 97\%$ is obtained. Various other algorithms have been proposed for assigning genus and species names to medically important bacteria (Park et al., 2015). Most bacteria can be correctly identified to

species from partial (500 bp, V1–V3) rather than full (1500 bp) 16S sequences (Hong and Farrance, 2015). However, no single hypervariable region can distinguish all medically important bacterial species (Chakravorty et al., 2007). In some instances, species can only be identified by sequencing the entire 1500 bp gene (Hong and Farrance, 2015). For laboratories that only occasionally need more definitive identifications via 16S, a variety of companies offer commercial services for around \$50 USD an isolate.

As in the case of MALDI-TOF, 16S rDNA gene sequencing has a number of limitations. 16S platforms are not easily amenable to lower- to middle-income countries or smaller to moderate-size clinical labs in the United States when costs are compared to routine culture and biochemical identification (Rizal et al., 2020). Due to the fact that ribosomal genes are highly conserved because of their essential cellular function, their ability to taxonomically distinguish between two closely related species can be limited (Janda and Abbott, 2007; Rossi-Tamisier et al., 2015). Common species or groups containing many members difficult to resolve by 16S include the family *Enterobacteriaceae*, the *Streptococcus mitis* group, some *Campylobacter* species, mycobacteria, and complex members belonging to the genus *Burkholderia* as examples (Srinivasan et al., 2015; Hong and Farrance, 2015; Rossi-Tamisier et al., 2015; Rizal et al., 2020; Church et al., 2020). Improved species identification for some groups such as the *Enterobacteriaceae* can be achieved using *dnaJ* sequencing (McLean et al., 2019; Rizal et al., 2020). Similarity values produced from 16S rDNA sequencing are also subject to misinterpretation. The highest 16S similarity match to a test strain does not necessarily imply genetic identity. DNA-DNA hybridization data on select species does not always correlate with 16S values and when multiple 16S matches or scores deviate from each other by <0.5%, a final identification is always tentative unless supported by other methods (Janda and Abbott, 2007; Park et al., 2015). For a comprehensive and exhaustive overview of the role 16S plays in the routine identification of pathogenic bacteria the reader is encouraged to consult the authoritative review of Church et al. (2020). The review covers various aspects of 16S rDNA gene sequencing including sequence analysis, troubleshooting technical problems, and the identification of many major groups of bacteria causing infectious diseases (Church et al., 2020).

Next-generation sequencing (NGS)

Until the early 2000s, Sanger capillary sequencing, also known as dye- or chain-terminator or first-generation sequencing, was the backbone platform for the sequencing of bacterial genomes in academic and clinical settings (Tshikhudo et al., 2013; Besser et al., 2018). Sanger capillary sequencing began to be replaced post-2005 by NGS. NGS is primarily defined as a high-throughput method where millions of DNA fragments can be sequenced in parallel simultaneously and independently (Mitchell and Simmer, 2019). The earliest of these NGS technologies to have a diagnostic application in the microbiology laboratory was pyrosequencing with the introduction of the Roche 454 Life Sciences platform (Glenn, 2011; Besser et al., 2018). Pyrosequencing is often called second-generation sequencing since the platform requires amplification of the template molecules prior to sequencing (Glenn, 2011). This method of sequencing-by-synthesis involves the detection of released pyrophosphate (PPi) during DNA synthesis (Harrington et al., 2013). As opposed to the Sanger method where longer (500–1000 bp) DNA sequences are produced, pyrosequencing works with shorter reads of 50–400 bp (Besser et al., 2018). Pyrosequencing was found to be especially useful in the identification of certain groups of bacteria including mycobacteria. Unfortunately, this technology went out of vogue and the 454 Life Sciences platform was discontinued by Roche in 2015 (Rizal et al., 2020).

Third-generation sequencing involves platforms that directly sequence individual DNA molecules (Glenn, 2011). NGS involves two separate steps in this overall method, namely a “wet” process involving the actual preparation and sequencing of a bacterial strain and a “dry” process of bioinformatics analysis (Rosen et al., 2018). Excluding first-generation sequencing, NGS relies on one of three different basic technologies including pyrosequencing, reversible terminator chemistry, and sequence-by-ligation (Tshikhudo et al., 2013). Detection methods also vary. The premier system at present in this category is the Illumina platform (Illumina Inc., San Diego, CA) in which sequencing occurs by synthesis of the complementary strand with fluorescent-based detection of reversibly blocked terminator nucleotides; this platform has similarities to the Sanger approach (Tshikhudo et al., 2013; Besser et al., 2018). Illumina Inc. produces a series of benchtop sequencers (NextSeq 550, iSeq100) with different applications and capabilities and which vary in cost, throughput size, run times, maximum reads per run, and maximum read length. Other popular systems include the IonTorrent (Thermo Fisher, Waltham, MA) and PacBio RS II platforms (Pacific Biosciences) (Menlo Park, CA). The IonTorrent detection system involves solid state pH meters measuring H⁺ release during DNA polymerization. The MiniON system (Nanopore, Oxford, United Kingdom) is a long-read sequencing platform that offers low-cost sequencing and portable real-time devices for both DNA and RNA analysis. All of these systems differ in certain processes but follow a general workflow schema of DNA extraction → library preparation → template preparation → and automated sequencing bp (Besser et al., 2018).

NGS offers three main advantages to microbiologists in the identification and sequencing of bacteria over previous technologies which are high-speed, high throughput, and greater discriminatory power (Fairfax et al., 2018; Rosen et al., 2018). There are three currently useful applications of NGS which include WGS, targeted NGS, and metagenomic analysis (Mitchell and Simmer, 2019). WGS is the application that fits into the potential workflow of diagnostic laboratories. Presently, the primary use of NGS/WGS has been in the area of molecular epidemiology and the ability to track and identify outbreaks of infection associated with varying genotypes (Tagini and Greub, 2017; Fairfax et al., 2018; Besser et al., 2018; Mitchell and Simmer, 2019; Boers et al., 2019). Other current uses include detection of virulence characteristics and novel resistance determinants, fingerprinting, and the potential detection and identification of taxa in vivo or those that are difficult to grow in vitro. It has also been shown that full genome sequences with average nucleotide identity values of ≥95% correlate with species identity by other standard methods including 70% relatedness by DDH and >99% 16S sequence similarity. However, whether or not WGS could ever be used in a routine diagnostic application is questionable.

WGS is not without issues. The cost of any of these platforms can be sizeable with minimum costs ranging for a single whole genome sequence from \$120–\$250 USD or more. WGS is also set up for batch testing, not on a case-by-case basis such as needed in hospital settings (Rosen et al., 2018). Long reads with newer third-generation platforms are prone to error rates of 11–15% so there are tradeoffs between use of longer and shorter reads (Besser et al., 2018). Unlike many other laboratory tests, WGS requires dedicated staff for both the “wet” and “dry” portions as results are not simply “cookbook” in nature but require professional interpretation. Run times can also vary from 19 to 65 h depending upon the platform used (Rosen et al., 2018). Finally, WGS is not FDA approved which means extensive validation studies are needed (Kozyreva et al., 2017; Arnold et al., 2018). How proficiency testing, quality assurance and quality control practices are intertwined with testing is also problematic (Rosen et al., 2018). Considerable improvements are still need to be made for WGS to logistically fit into the clinical laboratory workflow.

Bacterial identification—ongoing issues

No system is perfect

One of the cardinal tenets of bacterial identifications is that no system is foolproof and errors occur. The literature is replete with case reports and comparative studies where either a non-molecular or molecular identification system has misidentified an important pathogen (Poonawala et al., 2018; Pandey et al., 2019; Mesa-Radiilla et al., 2019; Tarini et al., 2019; Sukhum et al., 2021). Some of the common groups often misidentified by multiple systems include *Neisseria gonorrhoeae*—*Neisseria meningitidis*, *Brucella*, *Acinetobacter baumannii* complex, *Burkholderia pseudomallei*, and the *Streptococcus mitis* group. In many instances such misidentification has enormous medical or public health consequences.

Whatever system is chosen by any laboratory as the primary method for bacterial identification it needs to come with the realization that it will at least occasionally yield incorrect identification results. Labs need to be aware of the major taxa each system has problems providing correct identifications for. As a result, each laboratory needs to have a backup system that is substantially different in methodology to provide a correct identification when needed. Difficult-to-identify bacteria can also be submitted to commercial or reference centers for final identifications by more sophisticated methodologies.

Databases

A less well appreciated problems facing labs is the composition of databases attached to commercial products. It is well known that the degree public or private databases are curated varies considerably (Janda and Abbott, 2007). This is particularly true for 16S data as many databases contain unidentified or poorly annotated sequences (Boers et al., 2019). A United Kingdom study found at least 5% of the 16S entries in the Ribosomal Database Project to contain substantial errors with at least 14% of these being unidentified sequencing errors (Ashelford et al., 2005). Another study found errors in the identification of *Pseudomonas* genomes at the rank of species (Tran et al., 2017). A 2020 investigation of *P. fluorescens* sequence data deposited in GenBank determined that all 28 strains were incorrectly identified to species when deposited (Morimoto et al., 2020). These same authors found similar results when *P. putida* strains were analyzed. Collective findings suggest that whatever system is used to identify bacteria the ability to generate a correct final identification is dependent upon how well the databases used is curated and updated. Sequence comparisons should always be to type strains or reference strains where DNA-DNA relatedness values are known.

An expanding bacterial taxonomy

There are now over 20,000 validly published bacterial names in the scientific literature and these numbers continue to rise (Pallen et al., 2021). While many of these newly proposed species are of environmental origin, a sizeable number continue to be recovered from clinical samples whether as infectious agents or colonizers of mucosal surfaces. The rapid expansion in species presents several problems. First, commercial products (FDA-approved or not approved) will have a great deal of difficulty keeping pace with taxonomic proposals and potentially incorporating some of these species into databases. This becomes even more difficult when the cumulative number of known strains per taxon is <25. How well these species are curated is another issue. Finally, these changes present numerous issues regarding whether or not modern bacterial taxonomy is relevant to clinical microbiologists (Janda, 2018).

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Laboratory Identification of Fungal Infections

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Introduction

Within clinical mycology we face important challenges, because the fungal species that cause diseases are increasingly numerous and diverse. Diagnosing these diseases is a complex task that requires a series of classical tests, which despite continuous changes and advances in new methodologies, continue to be very useful in the routine microbiology laboratory. To make a good diagnosis, the coordinated action of the physician and the laboratory professional is essential, and the latter must have experience and knowledge to confirm or exclude a fungal etiology.

Conventional microbiological diagnosis of fungal diseases

This diagnosis is based mainly on the direct microscopic study of clinical samples and the fungi isolation by culture in artificial media. The first allows the observation of the fungus morphological structures in the sample, together with other elements that show the presence or absence of an inflammatory response. The second allows the identification of the fungus. Both steps are essential to establish the etiology of the fungal disease and depend on the collection of a clinical sample in optimal conditions, followed by its adequate handling, conservation and transport to the microbiology laboratory.

Direct microscopic study of clinical samples

Once the clinical sample has been received in the microbiology laboratory, it is processed for direct microscopic study and later for culture. The main objective of direct microscopic study is to quickly and efficiently detect the presence of fungi in clinical samples (Arango Arteaga and Castañeda Del Gordo, 2003; Canteros et al., 2017; Clinical and Laboratory Standards Institute (CLSI), 2012; Sutton, 2015).

The choice of the microscopic preparation depends on the type of sample and the suspected fungus; therefore, the diagnostic impression provided by the doctor in the examination request is very important and will initially guide the choice of the preparation. Direct microscopic examination can be performed by means of wet mounts, and microbiological and histopathological stains. Most of the fungi can be observed in the microbiology laboratory with the first two preparations, since they are for routine use, while histopathological stains are not for routine use and are performed in the histopathology laboratory (Arango Arteaga and Castañeda Del Gordo, 2003; Canteros et al., 2017; Clinical and Laboratory Standards Institute (CLSI), 2012). Table 1 shows the advantages and disadvantages of direct microscopic study of clinical samples for mycological diagnosis.

Table 1 Advantages and disadvantages of direct microscopic study of clinical samples for mycological diagnosis.

Advantages	Disadvantages
It is the fastest, most useful and most cost-effective method for the detection of fungal elements, especially for the diagnosis of Invasive Fungal Disease (IFD). Most fungi can be seen with routine preparations and stains used in the microbiology laboratory.	It depends on the experience of the laboratory professional, who has to be well trained in the observation of fungal elements. Its diagnostic sensitivity is low (10^3 – 10^5 fungal elements per mL) and depends on the sample, the patient, and the type of microscopic preparation chosen, in addition to the experience of the laboratory professional.
In many cases, it may be the only evidence of fungal infection when the culture is negative or the clinical sample sent to the laboratory is scarce.	A negative result never rules out the presence of a fungal infection
It is a fundamental method to guide antifungal treatment while the growth of the fungus develops in the culture.	Elements such as cell debris, fat droplets, myelin, leukocytes, fungal mosaic, pollen, tissue fibers, and bubbles can cause confusion and be identified as fungal elements.
Its negativity helps in the interpretation of contaminating fungi isolates in cultures.	It is recommended to carry out various types of preparations (wet mounts and staining) to increase the sensitivity of fungal detection.
Guides the selection of culture media to promote the recovery of the fungus.	It lacks specificity. In most cases the fungus cannot be identified.
As part of the conventional diagnosis, it is the only method that allows diagnosing mycoses produced by non-cultivable fungi such as <i>Pneumocystis jirovecii</i> .	
Can detect the presence of non-viable fungal structures in clinical samples from patients receiving antifungal treatment.	

Based on data from: Arango Arteaga M and Castañeda Del Gordo E (2003) *Micosis humanas. Procedimientos diagnósticos. Exámenes directos*. Colombia: Corporación para Investigaciones Biológicas (CIB)—Instituto Nacional de Salud (INS). pp. 3–20.; Canteros C, Cantón E, Córdoba S, Rivas P and Melhem M (2017) Diagnóstico convencional de la enfermedad fúngica invasora. In: Rivas P, Pemán J, Córdoba S and Melhem M (eds.). *Aproximación Clínico-Diagnóstica de la Enfermedad Fúngica Invasora*. 1st edn, pp. 83–116. España: Fundación Micellium.; Cuenca-Estrella M (2012) Diagnóstico de laboratorio de la enfermedad fúngica invasora. *Enfermedades Infecciosas y Microbiología Clínica* **30**: 257–264.; Pemán J, Martín-Mazuelos E and Rubio Calvo MC (eds.) (2007) *Guía práctica. Identificación y diagnóstico en micología clínica*. 2nd edn. Bilbao: Revista Iberoamericana de Micología, pp 3–1–3–22.

Wet mounts

Wet mounts, also known as direct examinations, are one of the main tools used in the routine microbiology laboratory, since they are capable of providing a presumptive diagnosis and allow early therapeutic behavior if fungal elements are observed, such as for example, septate or aseptate hyphae compatible with opportunistic fungi, or characteristic structures of dimorphic fungi (Canteros et al., 2017; Cuenca-Estrella, 2012).

Some samples can be observed directly at low or medium magnification with physiological saline solution or with a Lugol's solution, which provides some coloration to pale fungal structures, but in general, other more effective mounting media are used that favor their observation (Arenas, 2014; Bonifaz, 2012).

Potassium hydroxide (KOH)

It is the most widely used universal mounting medium in the routine microbiology laboratory for wet mounts of clinical samples. It is used primarily to examine opaque and viscous samples such as sputum, exudates, and abscess material, as well as for skin flakes, nails, and hairs. All these samples have in common that they need to be clarified, digested and/or softened before being viewed under the microscope to favor the observation of the fungus morphology and pigmentation. KOH digests the protein and purulent material, and also dissolves the intercellular cement that holds the cells together, which allows to observe the fungal structures that are present in the keratinized tissue (Arango Arteaga and Castañeda Del Gordo, 2003; Arenas, 2014; Bonifaz, 2012; Canteros et al., 2017; Lindsley et al., 2015; Quindós, 2015; Sutton, 2015).

KOH can be prepared at concentrations between 10% and 30% with dyes such as methylene blue, Evans blue and Quink® blue-black ink or Super Quink® permanent blue (both available from Parker), which improve the visualization of fungal structures. The use of the trademark of the two dyes mentioned above is recommended, since inks of other colors or brands are degraded by KOH (Arango Arteaga and Castañeda Del Gordo, 2003; Lindsley et al., 2015). For viscous and opaque samples, as well as those containing a high amount of keratin such as skin flakes, nails, hairs and even corneal scrapings, it is recommended to use a 10% KOH solution. This concentration can be increased to 20% or 30% in the case of samples that also have high fibrin content, such as meliceric crusts and material from warty lesions; they are also often used to digest portions of very thick nails (Arango Arteaga and Castañeda Del Gordo, 2003; Arenas, 2014; Bonifaz, 2012).

The procedure consists of placing one or two drops of KOH (10–30%) with or without additional dyes on a clean microscope slide, adding a drop of the sample in the case of liquid samples, and small fragments in the case of solid samples, add a drop of 10% glycerin, mix gently and cover with a coverslip. The slide is then gently heated to the lighter flame several times, without boiling, to accelerate the action of the KOH on the sample. Subsequently, the preparation is left to rest for 20 min in a humid chamber and is examined under a microscope at low and medium magnification (200× and 400×, respectively). The addition of 10% glycerin and the maintenance of the preparation in a humid chamber allows its observation to be repeated up to 72 h (Arango Arteaga and Castañeda Del Gordo, 2003; Bonifaz, 2012; Canteros et al., 2017; Lindsley et al., 2015).

It is important to consider that the clinical sample, when reacting with KOH, usually generates artifacts that can be confused with fungal structures. On the other hand, KOH, especially at concentrations of 20% and 30%, is more corrosive and over time digests the sample, deteriorating it, which makes it difficult to observe. Heating the slide to a flame accelerates the degradation of keratin, fibrin, mucin and fats saponification, but it also generates the formation of needle shape potassium crystals, which makes the sample difficult to observe; hence the importance of adding 10% glycerin, which minimizes this effect by avoiding evaporation and dehydration (Arango Arteaga and Castañeda Del Gordo, 2003; Bonifaz, 2012; Canteros et al., 2017). It is important to note that KOH is very corrosive, so it is recommended to protect hands from its contact, as well as the microscope stage (Arango Arteaga and Castañeda Del Gordo, 2003).

It is a very efficient technique for the detection of budding blastoconidia, with or without pseudomycelia or true mycelium, septate hyaline (Fig. 1) and dematiaceous mycelial fungi, aseptate mycelial fungi and characteristic structures of endemic dimorphic fungi such as those of the *Paracoccidioides* species complex, *Coccidioides* species complex, and *Blastomyces dermatitidis*, with the exception of *Histoplasma capsulatum* var. *capsulatum* and *Penicillium marneffeii*, whose structures are very small (2–4 µm) and cannot be observed with this mount or with low and medium magnification, since they require microbiological staining and observation at high magnification (1000×) (Arango Arteaga and Castañeda Del Gordo, 2003; Bonifaz, 2012; Canteros et al., 2017).

The experience of the laboratory personnel is essential; both for the preparation of clinical samples and for their subsequent observation and interpretation, since the use of the fresh KOH examination does not ensure that it can effectively differentiate colonization from infection, except in endemic mycosis caused by dimorphic fungi (with the exceptions mentioned above), where the observation of compatible fungal structures is sufficient to initiate a targeted antifungal treatment (Bonifaz, 2012; Canteros et al., 2017).

Other substances similar in action to KOH, which are used for direct examinations are dimethylsulfoxide (DMS) at various concentrations (10–50%), sodium hydroxide (NaOH) at 10–20%, lactic acid at 10–50% and Black Chlorazol E (Arenas, 2014; Bonifaz, 2012; Lindsley et al., 2015).

Black Chlorazol E

It is a commercial dye that uses Chlorazol E (Sigma®) and 10% DMS plus 7% KOH as lighteners, which improves the observation of fungal structures, because it binds especially to chitin, and stains them light green or dark-green, making it a useful reagent for unexperienced or training professionals. The slides should be viewed the same day, since the dye dehydrates over time, and precipitates and degrades with light (Arango Arteaga and Castañeda Del Gordo, 2003; Bonifaz, 2012).

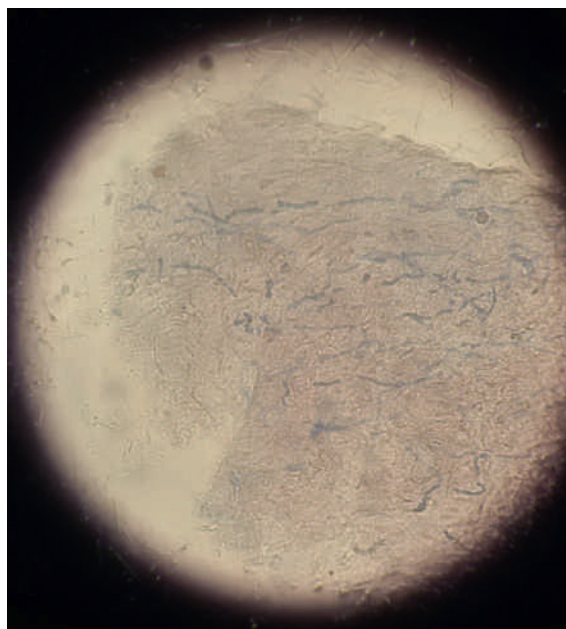


Fig. 1 Septate hyphae in toe nail sample. KOH 10% + Super Quink® permanent blue (400 ×). Photo by María Mercedes Panizo.

Chinese ink and nigrosine

They are colloidal suspensions of carbon particles that are used to specifically detect in clinical samples the presence of the *Cryptococcus neoformans* and *C. gattii* species complexes, by observing their polysaccharide capsule, which is visualized as a clear and sharp halo that surrounds a blastoconidia with or without buds, on a black background, since the capsule prevents the penetration of the suspension. They are not stains; they are used as contrast methods. The clinical sample par excellence is cerebrospinal fluid (CSF), however, the use of this suspension is also useful in other sample where the presence of this fungus is suspected: sputum, bronchoalveolar lavage (BAL), urine, biopsies, secretions, purulent material and other body fluids (Arango Arteaga and Castañeda Del Gordo, 2003; Canteros et al., 2017; Lindsley et al., 2015; Sutton, 2015).

They have a high sensitivity for the detection of these encapsulated yeasts, since their observation guides the start of antifungal treatment, but it is essential that the laboratory personnel has experience in their observation, since the presence in the sample of red blood cells and leukocytes, as well as artifacts that can be generated during sample process such as air bubbles, talc particles and fat droplets, can cause confusion, leading to the report of a false positive, especially when the yeasts capsule are small (Arango Arteaga and Castañeda Del Gordo, 2003; Canteros et al., 2017; Lindsley et al., 2015).

The procedure consists of placing a drop of the India ink suspension (Pelikan® recommended) or water-soluble nigrosin for microscopy (Merck®) on a clean microscope slide, adding a drop or a small portion of the clinical sample, mixing gently and covering with a coverslip sheet. The amount of suspension to be used affects the sensitivity of the preparation and increases the risk of artifacts, so it must be ensured that it is not excessive or insufficient. The visualization is performed at low (200 ×) and medium magnification (400 ×) immediately after making the preparation, since it dehydrates quickly, making it difficult to observe (Arango Arteaga and Castañeda Del Gordo, 2003; Canteros et al., 2017; Lindsley et al., 2015).

Microbiological stains

These stains are very useful in the routine microbiology laboratory, since they can provide a presumptive diagnosis if yeast or mycelial structures are observed in the sample; provide a definitive diagnosis if very characteristic structures of some fungi are observed; allows to diagnose non-culture fungi when other tools or resources are not available, and the host cellular response generated by the fungus can also be observed (Arango Arteaga and Castañeda Del Gordo, 2003; Ayats et al., 2011; Canteros et al., 2017).

To perform these stains, a small portion of the clinical sample is placed on a clean microscope slide and spread thinly, or an apposition smear is made in the case of biopsies. Then it is left to dry and the chosen stain is carried out. The procedures are in widespread use in microbiology laboratories. To increase the sensitivity and specificity of the diagnosis, it is recommended to make several preparations with various stains.

Gram stain

It is the most used stain in the microbiology laboratory, characterized by being quick and easy to perform. Although yeasts and mycelial fungi are usually Gram positive, some fungi do not take the dyes well with this stain, such as *Histoplasma capsulatum* var. *capsulatum*, *Blastomyces dermatitidis* and both *Paracoccidioides* and *Coccidioides* species complexes. This stain has low sensitivity and specificity, but it will always be useful in the hands of a well-trained and experienced laboratory personnel (Fig. 2) (Arango Arteaga and Castañeda Del Gordo, 2003; Canteros et al., 2017; Pontón and Del Palacio, 2007; Quindós, 2015; Sutton, 2015).

It is advisable to make a first observation of the preparation at medium magnification ($400\times$) in order to locate the presence of fungal structures, and a second one at higher magnification ($1000\times$) to observe particular characteristics. Some hyaline mycelial fungi such as *Aspergillus* spp., *Fusarium* spp. and the *Scedosporium* species complex have similar morphological aspects and stain irregularly, therefore the diagnosis of an infection caused by any of these fungi is presumptive and must be confirmed by culture. In addition, another important aspect related to low specificity, is that it does not allow to differentiate colonization from infection (Arango Arteaga and Castañeda Del Gordo, 2003; Canteros et al., 2017; Pontón and Del Palacio, 2007).

Giemsa and wright stains

They are the stains of preferential use in the microbiology laboratory for mycological diagnosis. Most fungi turn blue-violet or purple (Fig. 3) (Larone, 2011; Lindsley et al., 2015; Sutton, 2015). The Giemsa stain and its modification the Diff-Quick, as well as the Wright stain are effective in the diagnosis of folliculitis caused by the *Malassezia* species complex, in the detection of the intracellular yeast phase of *Histoplasma capsulatum* var. *capsulatum*, from yeasts of the *Sporothrix* species complex, and from trophozoites (ascospores) of *Pneumocystis jirovecii*. Like the Gram stain, they have low sensitivity, and the specificity is subject to the experience of the laboratory personnel (Arango Arteaga and Castañeda Del Gordo, 2003; Canteros et al., 2017; Lindsley et al., 2015).

These stains detect the presence of blastoconidia with and without buds, pseudomycelium or true mycelium, septate and aseptate mycelium, but like the Gram stain, they are not highly recommended for detecting fungal structures compatible with the *Coccidioides* species complex and *Blastomyces dermatitidis*, because they are not stained properly (Canteros et al., 2017).

Giemsa stain also allows differential diagnosis with intracellular parasites such as *Leishmania* spp., but, once again, the experience of the personnel in charge of the diagnosis is vital to differentiate the kinetoplast from the characteristic amastigote forms of this parasite from the intracellular yeasts of *Histoplasma capsulatum* var. *capsulatum*, and even intracytoplasmic granulations of phagocytic cells, which can cause confusion and lead to the report of a false positive (Canteros et al., 2017).

Ziehl-Neelsen staining

This stain is useful to visualize the presence of actinomycetes that are partially acid-fast, such as *Nocardia* spp. (pink), involved in cases of nocardiosis or actinomycetoma (Fig. 4) to differentiate them from other aerobic actinomycetes (Larone, 2011). It is also useful for cases of nontuberculous mycobacteriosis and to rule out or verify co-infection by *Mycobacterium tuberculosis*, particularly in respiratory samples. The Kinyoun stain can be used, which is a modification of the Ziehl-Neelsen (Arango Arteaga and Castañeda Del Gordo, 2003; Bonifaz, 2012; Larone, 2011).

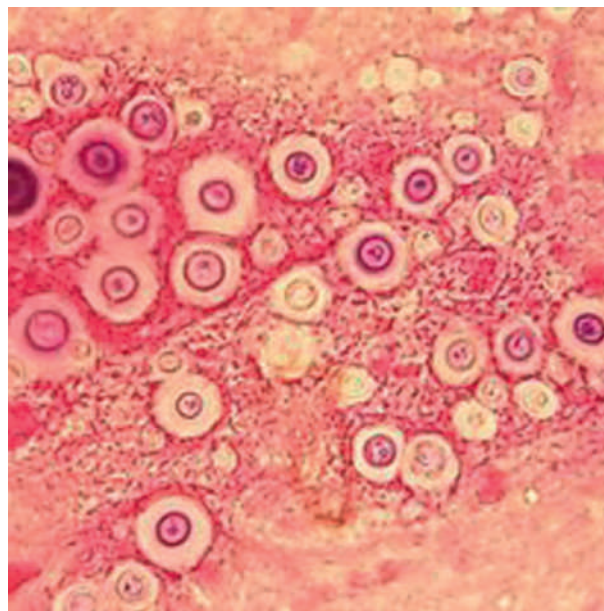


Fig. 2 Gram stain of sputum sample with *Cryptococcus neoformans* ($1000\times$). Photo by Xiomara Moreno.

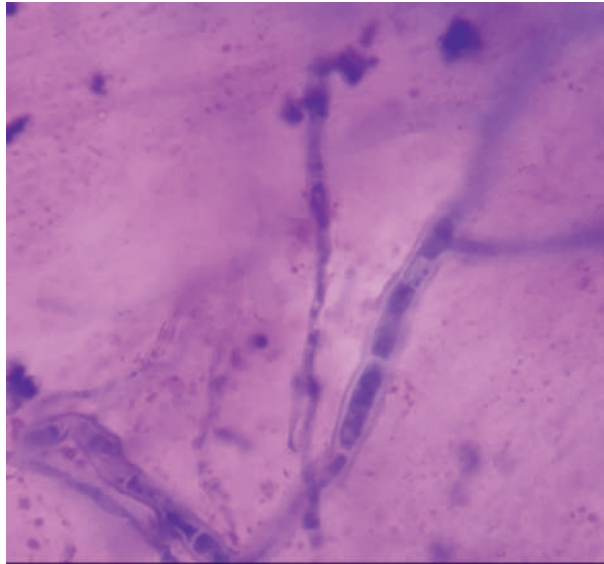


Fig. 3 Giemsa stain of sputum with hyphae of *Aspergillus* spp. (1000×). Photo by Xiomara Moreno.

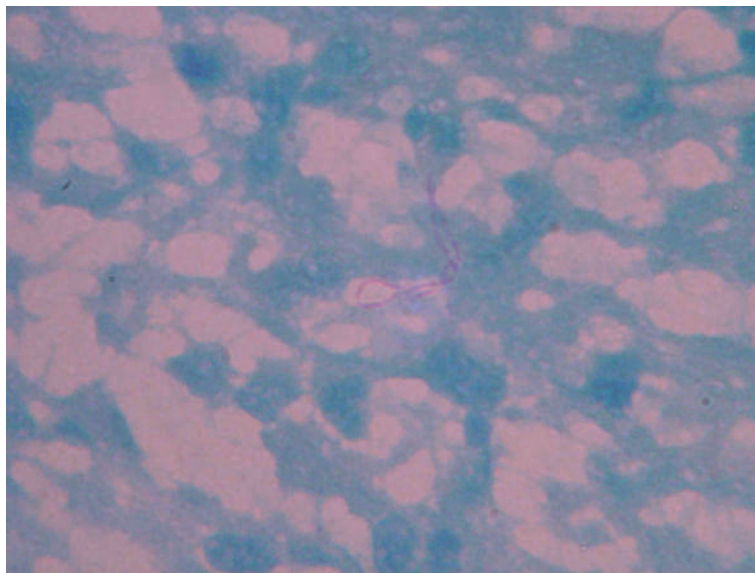


Fig. 4 Ziehl-Neelsen of *Nocardia* spp. from an actinomycetoma (1000×). Photo by María Mercedes Panizo.

Toluidine blue O stain

It is a very useful stain for the detection of *Pneumocystis jirovecii* cysts (ascus) in respiratory samples such as lung biopsies and BAL. The glacial sulfuric and acetic acids present in the reagent remove all the viscous material from the samples, facilitating the visualization of the cysts, which are colored reddish blue or purple, with a slightly blue contrast background. The reagent does not color the trophozoites (ascospores) and has the disadvantage that it must be prepared and used in an extraction chamber and with great caution, due to the acids that compose it (Arango Arteaga and Castañeda Del Gordo, 2003; Lindsley et al., 2015).

Calcofluor white

Calcofluor white is a bleaching agent, widely used in the textile and papermaking industries, that naturally binds to cellulose and chitin. On coming into contact with clinical samples, it clarifies cell debris and coloring fungal structures bright green, with to the use of a fluorescence microscope. It is a very sensitive and simple stain, capable of demonstrating the presence of hyphae, pseudohyphae and yeasts in clinical samples, and particularly useful for the detection of *Pneumocystis jirovecii* structures, which is a non-culturable fungus, but does not allow the visualization of endospores of the *Coccidioides* species complex (Arenas, 2014; Bonifaz, 2012; Lindsley et al., 2015; Sutton, 2015; Zaitz et al., 2010).

The reagent is composed by the union of two solutions: (1) 10 g of KOH in 90 mL of distilled water plus 10 mL of glycerol; (2) 0.1 g of calcofluor in 100 mL of distilled water. This final solution can in turn be diluted 1:10 with 0.05% Evans blue aqueous solution, which will act as a contrast dye. Unfortunately, the disadvantage is that the dye is expensive and that not all routine microbiology laboratories have a fluorescence microscope with 390–420 nm wavelength filters to make observations (Arenas, 2014; Bonifaz, 2012; Zaitz et al., 2010).

Table 2 presents the techniques to perform direct microscopic study according to the clinical samples and Table 3 presents a summary of characteristic features of fungal structures observed in direct examinations of clinical samples.

Histopathological stains

These stains are generally available in specialized laboratories in histopathological diagnosis, so they are not routine stains in the microbiology laboratory. They allow confirming the presence of an invasive mycosis both *ante-mortem* and *post-mortem*.

The tissue obtained by biopsy or cytology is fixed with 10% formalin, it is subsequently included in paraffin, and cuts are made with a microtome, which depending on the tissue will be 3–8 μm thick, which are placed on a clean microscope slide to carry out the stains. The material obtained must be representative of the infectious process in order to provide adequate diagnostic performance. Another important aspect is that the laboratory personnel must be familiar and well trained in the recognition of fungal morphology in tissue and the cellular response that it induces (Bennett, 2015; Canteros et al., 2017; Guarner and Brandt, 2001; Mayayo-Artal, 2004; Quindós, 2015).

The sensitivity and specificity of these stains is given by the possibility of recognizing characteristic fungal morphological structures that can diagnose an invasive mycosis, as well as observing the tissue response of the host, which groups together a wide variety of reactions conditioned by the immune response. Furthermore, fungal structures do not always exhibit their most specific morphological characteristics in the tissue, necessary for their identification, and their observation is generally possible when their presence is abundant, which frequently occurs in the most advanced stages of the disease, when tissue damage it is very important and practically irreversible (Guarner and Brandt, 2001; Mayayo-Artal, 2004; Quindós, 2015). An experienced professional will first perform an observation of the slide at low and medium magnification ($200\times$ and $400\times$), to observe the tissue response, and later will review it using higher magnification ($1000\times$), to search for fungal structures.

Table 2 Techniques for direct microscopic study according to the clinical sample.

	Wet mounts				Stains			
	Direct sample ^a	KOH ^b	Black Chlorazol E	Chinese ink Nigrosin	Gram	Giemsa Wright	Ziehl-Neelsen	Calcofluor White
<i>Sterile samples</i>								
Bone marrow					X	X		X
Blood ^c				X	X	X		
CSF	X			X	X	X	X	
Other body fluids	X			X	X	X	X	X
Biopsies	X	X	X	X	X	X	X	X
<i>Non-sterile samples</i>								
Skin flakes		X	X					X
Nails		X	X					X
Hairs		X	X					X
Exudates and abscesses	X	X	X	X	X	X	X	X
Sputum ^d	X	X	X	X	X	X	X	X
BAL ^d	X	X	X	X	X	X	X	X
Urine	X			X	X	X		X
Secretions	X	X	X	X	X	X	X	X
Ocular and otic secretions	X	X	X		X			X
Purulent material	X	X	X	X	X	X	X	X
Granules	X	X	X		X		X	X

^aA portion of the sample between slide and coverslip.

^bCan be used alone or with dyes.

^cOnly in samples from immunocompromised patients.

^dAlso use Toluidine Blue O together with Giemsa and Wright when *Pneumocystis jirovecii* is suspected.

Based on data from: Arango Arteaga M and Castañeda Del Gordo E (2003) *Micosis humanas. Procedimientos diagnósticos. Exámenes directos*. Colombia: Corporación para Investigaciones Biológicas (CIB)—Instituto Nacional de Salud (INS). pp. 171.; Canteros C, Cantón E, Córdoba S, Rivas P and Melhem M (2017) Diagnóstico convencional de la enfermedad fúngica invasora. In: Rivas P, Pemán J, Córdoba S and Melhem M (eds.). *Aproximación clínico-diagnóstica de la enfermedad fúngica invasora*. 1st edn, pp. 86. España: Fundación Micellium.

Table 3 Summary of characteristic features of fungal structures observed in direct examinations of clinical samples.

Morphology found in the samples	Fungus	Size (μm)	Characteristic features	Laboratory report
Blastoconidia (yeasts with and without budding)	<i>Histoplasma capsulatum</i> var. <i>capsulatum</i>	2–4	Small yeasts, oval to rounded, solitary, with one or more buddings. Often intracellular, located inside macrophages and monocytes, difficult to detect if are scarce. They remain in clusters when are extracellular. They are only observed in stains and with $1000\times$ magnification. When staining, the retraction of the cell membrane simulates the appearance of a capsule, a phenomenon that is not observed with the Gomori-Grocott stain	Intracellular blastoconidia suggestive of <i>Histoplasma capsulatum</i> var. <i>capsulatum</i> With the Gomori-Grocott stain: Small blastoconidia with shape and size compatible with <i>Histoplasma capsulatum</i> var. <i>capsulatum</i>
	<i>Sporothrix schenckii</i> complex	2–6	Small, oval to rounded or cigar-shaped yeasts, with single or multiple budding. They are not common in clinical samples of subcutaneous lesions; they are more frequent in systemic forms. Sometimes it is possible to observe the asteroid body and the Splendore-Hoeppli phenomenon	Blastoconidia suggestive of the <i>Sporothrix schenckii</i> complex. The asteroid body is reported if it is observed
	<i>Cryptococcus</i> species complex	2–20	Oval to rounded yeasts of variable size, with thin dark walls, solitary, with single or multiple budding with a narrow base. It has intracytoplasmic inclusions. The capsule also usually varies in size, thick-walled and refractive, which is sometimes not evident.	Encapsulated blastoconidia suggestive of the <i>Cryptococcus</i> spp. complex.
	<i>Blastomyces dermatitidis</i>	8–15	Oval to rounded yeasts, usually large, thin-walled and birefringent, with single budding attached to the stem cell by a broad base.	Single budding suggestive of <i>Blastomyces dermatitidis</i>
	<i>Paracoccidioides brasiliensis</i> complex	2–60	Oval to rounded yeasts of multiple sizes (anisometric), with intracytoplasmic inclusions. They may be solitary with multiple narrow-based rudder-like buddings or have single budding or short chains of varying size. They have a thick and refractive wall.	Multiple budding blastoconidia suggestive of <i>Paracoccidioides brasiliensis</i> complex
	<i>Lacazia loboi</i>	7–12	Oval to rounded yeasts, characterized by their uniformity in size, very thick-walled and with intracytoplasmic inclusions. They can be found isolated or with multiple budding, and the presence of long yeast chains linked together by an apparent cytoplasmic bridge is usual.	Blastoconidia suggestive of <i>Lacazia loboi</i>
Blastoconidia (yeasts that divide by fission)	<i>Penicillium marneffei</i>	3–4	Oval, intracellular yeasts, located within macrophages and monocytes. They can elongate when they are at the extracellular level. They have a central septum that is formed by fission. They do not give rise to budding. They are only observed in stains and with $1000\times$ magnification.	Intracellular blastoconidia suggestive of <i>Penicillium marneffei</i>
Blastoconidia, pseudohyphae and/or true hyphae	<i>Candida</i> spp.	3–6 (yeast) 5–10 (pseudohyphae)	Yeasts oval to rounded, solitary, with single or multiple buds, often in chains, thin-walled. In pseudohyphae, the blastoconidia remain attached by a depression at the attachment site and are narrow-ended. True hyphae are continuous and septate	Blastoconidia suggestive of <i>Candida</i> spp. Blastoconidia and pseudohyphae suggestive of <i>Candida</i> spp.
Blastoconidia and hyphae	<i>Malassezia</i> spp. complex.	3–8 (yeasts) 2.5–4 (hyphae)	Oval to rounded yeasts, single budding, grouped in compact clusters, thick-walled and refractive. Hyphae are short, thick, and curved, and not always present	Blastoconidia suggestive of the <i>Malassezia</i> species complex. Blastoconidia and hyphae suggestive of the <i>Malassezia</i> species complex.
Aseptate (coenocytic) hyphae	Mucorales	3–25 (average 12)	Large, broad, ribbon-like hyphae often fractured or twisted due to the absence of septa, although they may be occasional. The branches are at right angles (90 degrees)	Hyaline, broad, aseptate (coenocytic) hyphae, suggestive of mucorales
Septate hyaline hyphae	Dermatophytes	3–10	In skin and nails: septate hyaline hyphae of variable diameter and thickness. Intercalary or terminal chains of arthroconidia and chlamydoconidia associated with hyphae, solitary or in clusters, may occur. In hairs: arthroconidia on the periphery of the hair that form a sheath that covers it, indicating ectothrix infection. Arthroconidia formed by fragmentation of hyphae within the hair indicate endothrix infection. Long hyphae forming channels within the hair indicate a favic infection.	Skin and nails: thin, septate, branched and arthrospored hyaline hyphae Hair: Endothrix-like invasion and ectothrix-like invasion.
	<i>Aspergillus</i> spp.	3–12	Hyaline septate hyphae, thick-walled, with dichotomous branching at an angle of 45 degrees, with a tendency to grow in a radial pattern.	Dichotomous septate hyaline hyphae at 45 degrees angle suggestive of <i>Aspergillus</i> spp.
	<i>Scedosporium</i> / <i>Pseudallescheria</i> complex	3–12	It is impossible to differentiate hyphae from those of <i>Aspergillus</i> spp. and other hyaline fungi	Hyaline septate hyphae
	<i>Fusarium</i> spp.	3–12	Hyphae are similar to those of <i>Aspergillus</i> spp., <i>Scedosporium</i> / <i>Pseudallescheria</i> complex and other hyaline mycelial fungi. Both chlamydoconidia and irregular hyphae are common.	Hyaline septate hyphae with chlamydoconidias suggestive of <i>Fusarium</i> spp.

(Continued)

Table 3 (Continued)

<i>Morphology found in the samples</i>	<i>Fungus</i>	<i>Size (μm)</i>	<i>Characteristic features</i>	<i>Laboratory report</i>
Hyaline septate hyphae and arthroconidia (Hyalohyphomycosis)	<i>Geotrichum</i> spp. <i>Trichosporon</i> spp.	2–8 2–8	Rectangular hyphae and arthroconidia with rounded ends Rectangular hyphae and arthroconidia with rounded ends, with solitary and single budding blastoconidia plus pseudohyphae	Hyphae, arthroconidia, blastoconidia and pseudohyphae suggestive of <i>Trichosporon</i> spp.
Dematiaceous septate hyphae (Pheohifomycosis)	<i>Bipolaris</i> spp. <i>Curvularia</i> spp. <i>Exserohilum</i> spp. <i>Exophiala</i> spp. <i>Phialophora</i> spp. <i>Wangiella dermatitidis</i> <i>Xilohypha bantiana</i>	2–6	Polymorphic dematiaceous hyphae. Budding blastoconidia with isolated septa and conidia in chains	
Tinea nigra	<i>Phaeoannelomyces werneckii</i>	1.5–5	Usually large number of branched, thick-walled hyphae and oval blastoconidia, with a single bud and some with a septum. Well defined annelids.	
Spherules	<i>Coccidioides</i> species complex.	10–200 (spherules) 2–5 (endospores)	Spherules of variable size and stages of development, thick-walled and oval to rounded, some with endospores and others empty.	
Eumycetoma granules	<i>Curvularia</i> spp. <i>Exophiala</i> spp. <i>Leptosphaeria</i> spp. <i>Madurella</i> spp. <i>Pyrenochaeta romeroi</i> <i>Acremonium</i> spp. <i>Aspergillus</i> spp. <i>Fusarium</i> spp. <i>Pseudallescheria boydii</i>	65–1000 or greater	Intertwined septate hyphae forming a mass or sclerot of variable shape. The hyphae can be hyaline or dematiaceous, sometimes associated by cementum	Granules (sclerots) suggestive of eumycetoma
Actinomycetoma granules	<i>Nocardia</i> spp.	25–500	Slender, irregular, branched bacilli approximately 1 μm in diameter, intertwining to form soft masses of variable color and shape	Granules suggestive of actinomycetes
Sclerotic cells	<i>Fonsecae pedrosoi</i> <i>ladophialophora carrionii</i> <i>Phialophora verrucosa</i> <i>Rhinocladiella aquaspersa</i>	4–12	Rounded, dematiaceous, thick-walled cells that divide into several planes and form clusters. On some occasions, separate thick-walled dematiaceous hyphae may be found	Sclerotic cells
Black Piedra nodule	<i>Piedraia hortae</i>	Variable	Dematiaceous, multilobed ascocarpus, containing asci with transparent banana-shaped ascospores	<i>Piedraia hortae</i> (Black Piedra) nodule on hair
White Piedra nodule	<i>Trichosporon</i> spp.	Variable	Compact mass formed by hyaline hyphae that break into rectangular arthroconidia. Blastoconidia are difficult to differentiate from arthroconidia	Nodules of <i>Trichosporon</i> spp. (White Piedra) in the hair
Cysts (asci) and trophozoites (ascospores)	<i>Pneumocystis jirovecii</i>	5–12 (cysts—asci) 1–5 (trophozoites—ascospores)	The morphology varies depending on the stain. Gomori-Grocott, toluidine blue O and calcofluor: useful for identifying cysts (asci) as they color their wall. They are rounded or cup-shaped. Trophozoites do not color Giemsa and Wright: useful for identifying trophozoites (ascospores), which are small, with blue cytoplasm and purple nuclei. The cysts do not stain.	Depending on the stain: Trophozoites (ascospores)/cystic forms (asci) suggestive of <i>Pneumocystis jirovecii</i>

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From the didactic point of view, the groups of tissue reactions induced by pathogenic fungi are presented below, based on the model described by Baker (1947) and Zaitz et al. (2010).

- a) Group I: chronic inflammatory process, with the presence of suppuration, macrophages, giant cells, caseous necrosis and fibrosis. This group includes the majority of deep and subcutaneous mycoses: North American Blastomycosis, Paracoccidioidomycosis, Coccidioidomycosis, Sporotrichosis and Lacaziosis or Jorge Lobo's disease.
- b) Group II: predominance of suppuration, combined with an inflammatory infiltrate at the expense of macrophages, giant cells and fibrosis, without caseous necrosis. This group is represented by mycetomas (actinomycotic and eumycotic) and chromomycosis.
- c) Group III: suppuration generally absent, without macrophages, giant cells, necrosis or fibrosis. This group includes Histoplasmosis, whose etiological agent is intracellular, and Cryptococcosis, which occur in immunocompromised individuals.
- d) Group IV: acute inflammation and necrosis. This group includes fungal diseases with tissue necrosis and an acute exudative inflammatory response, in which fungal structures are found. The fungi that cause these diseases have a similar morphological presentation in the tissue; therefore, they must be confirmed by biopsy culture. The diseases are Aspergillosis, Mucormycosis, Hyalohyphomycosis and Pheohifomycosis, the latter two caused by a wide variety of different genera.
- e) Group V: absence of inflammatory reaction. This group includes superficial candidiasis and superficial mycoses, where the fungus is confined to the corneum stratum of the skin or to keratinized structures, respectively.

Hematoxylin-Eosin (H&E) stain

It is a very versatile and useful stain for the histopathological diagnosis of fungal diseases, particularly invasive diseases and eumycotic mycetomas, since it allows to show both the presence of the fungus (hyaline or dematiaceous), as well as the tissue response of the host against it. Fungal structures such as blastoconidia and pseudohyphae of *Candida* spp., as well as the hyphae of *Aspergillus* spp. and of the mucorales are well stained, while other fungi are partially stained or not stained. However, in the latter case, it is possible to appreciate the contour of the non-colored elements and thus show the presence of the fungus. Therefore, the professional's experience is essential (Arango Arteaga and Castañeda Del Gordo, 2003; Bennett, 2015).

Due to the fact that some fungi have similar morphologies in the tissues, the differentiation and interpretation of the stain is usually difficult, generating confusion and possible reports of erroneous results (Canteros et al., 2017). The hyphae of hyaline mycelial fungi are usually colored purple (eosinophils) and those of dematiaceous fungi retain their pigment and can be seen as brown. Yeasts tend to be colored blue or purple (basophilic), although some may not take the stain efficiently. In all cases, careful and expert observation is necessary in order to establish a presumptive diagnosis (Arango Arteaga and Castañeda Del Gordo, 2003; Larone, 2011).

Gomori-Grocott methenamine silver stain

It is one of the most used stains to confirm the presence of fungal structures in tissues, being the stain of choice for the diagnosis of invasive fungal diseases (IFD) (Arango Arteaga and Castañeda Del Gordo, 2003; Bennett, 2015; Bonifaz, 2012; Canteros et al., 2017; Lindsley et al., 2015). The fungal structures are well outlined in brown to black color, due to the deposit of reduced silver in the places where the aldehydes are located, a product of the oxidation of the hydroxyl groups of the fungal cell wall polysaccharides in the presence of the chromic acid. The contrast dye is Malachite green, which provides a green background that highlights the dark color of the fungal structures (Arango Arteaga and Castañeda Del Gordo, 2003).

This stain is considered of choice for the diagnosis of Pneumocystosis in BAL samples, being also very useful for diagnosing Paracoccidioidomycosis and Mucormycosis, however, it has limitations in the case of intracellular fungi, since it is not possible to observe the tissue reaction of the host, and it tends to color the fungi densely, making it difficult to observe structural details (Bonifaz, 2012; Larone, 2011).

Periodic acid schiff stain (PAS)

Like the Gomori-Grocott stain, it is considered a routine stain for the identification of fungal structures in tissues, since many of the fungal structures have basophilic and eosinophilic characteristics that allow their visualization clearly and sharply, much better than with H&E stain (Bonifaz, 2012; Lindsley et al., 2015). The stain is based on the property of the fungal wall neutral mucopolysaccharides to oxidize in the presence of periodic acid to form aldehydes, which fix the fuchsin of the Schiff's reagent and color from dark pink to magenta with variable intensities (Arango Arteaga and Castañeda Del Gordo, 2003; Lindsley et al., 2015).

With this stain, intracellular fungal structures can be observed, such as yeasts in the case of Histoplasmosis and spherules with endospores in cases of Coccidioidomycosis. It is also useful in cases of actinomycotic mycetoma and botryomycosis (Bonifaz, 2012) and to demonstrate the refractive double-contour wall of *Blastomyces dermatitidis*, which is not visible with the Gomori-Grocott stain (Larone, 2011).

Mayer's Mucicarmine stain

This stain is ideal for the identification of the mucopolysaccharide capsule of *Cryptococcus neoformans* and *C. gattii* species complexes, since the process of tissue fixation and paraffin removal shrinks the capsule around the cryptococcal cell, providing an intense red to pink border, but the wall itself is not stained. This also happens in clinical samples such as CSF and other biological fluids (Arango Arteaga and Castañeda Del Gordo, 2003; Bennett, 2015; Bonifaz, 2012). It also colors the cell walls of *Blastomyces dermatitidis* and *Rhinosporidium seeberi*, the latter a parasite frequently studied in medical mycology (Larone, 2011).

Fontana-Masson stain

This stain is very useful for the presumptive diagnosis of mycoses caused by dematiaceous fungi, such as Pheohifomycosis and black grain eumycetomas, and the *Cryptococcus neoformans* and *C. gattii* species complexes, since it highlights the melanin they contain in their walls (Bennett, 2015; Bonifaz, 2012; Larone, 2011; Lindsley et al., 2015). Melanin has polyphenolic bodies that, when combined with the silver salts in the reagent, are reduced to their metallic state, allowing it to be observed in black (Arango Arteaga and Castañeda Del Gordo, 2003).

Cultures

The collection and transport to the microbiology laboratory of a clinical sample in optimal conditions, the pretreatment of the sample if necessary, as well as the choice of culture media, temperature and incubation time are the factors that influence the recovery of pathogenic fungi. Currently, there is a wide variety of culture media for primary isolation and it is important to note that no single culture medium is sufficient for the recovery and growth of all medically important fungi; therefore, it is necessary to use a suitable battery. On the other hand, their choice in the laboratory basically depends on the preferences and experience of the professional in charge of the diagnosis (Lindsley et al., 2015; McGowan, 2015; Sutton, 2015; Tille, 2017).

In order to select a suitable battery of culture media, the following factors need to be considered:

- a) The choice of culture media depends on the sample type to be processed (Clinical and Laboratory Standards Institute (CLSI), 2012; Ellis et al., 2007). In the case of non-sterile samples, which may contain associated bacterial flora, it is necessary to choose a culture media battery that includes media with antibiotics that inhibit bacterial growth but allow the fungus growth, and another medium with or without antibiotics that also contains cycloheximide, to inhibit contaminating fungi or saprophytes. The battery for sterile samples should include one or two non-antibiotic media plus one medium with antibiotics (Clinical and Laboratory Standards Institute (CLSI), 2012; Ellis et al., 2007; Lindsley et al., 2015; McGowan, 2015; Sutton, 2015; Tille, 2017).
- b) Sabouraud Dextrose Agar (SDA) without antibiotics is the traditional medium for the primary isolation of fungi (Scognamiglio et al., 2010). Chloramphenicol alone or with other antibiotics such as gentamicin is included in some primary culture media to inhibit bacterial growth. However, if the clinical suspicion is *Nocardia* spp. and other aerobic actinomycetes, only SDA should be used (Clinical and Laboratory Standards Institute (CLSI), 2012; Lindsley et al., 2015; Scognamiglio et al., 2010; Sutton, 2015; Tille, 2017).
- c) Endemic dimorphic fungi grow best in enriched culture media such as heart-brain with antibiotics and enriched with 5–10% lamb blood. The latter has the disadvantage that inhibits sporulation, therefore, these fungi must be subcultured immediately to enriched blood-free media to promote conidia formation and perform molecular tests (Clinical and Laboratory Standards Institute (CLSI), 2012; Lindsley et al., 2015; Sutton, 2015; Tille, 2017).
- d) When it is required to detect the growth of a specific pathogen, the culture media battery can vary. In the case of *Candida* spp. a chromogenic medium can be used, which is both selective and differential (Murray et al., 2005). In the case of dermatophytes, Mycosel™ can be included. For *Malassezia* spp. SDA must be supplemented with sterile olive oil, adding 0.5 mL on the surface of the plate after seeding the sample; Dixon and Leeming media can also be used if available in the laboratory (Clinical and Laboratory Standards Institute (CLSI), 2012; Lindsley et al., 2015).
- e) The use of culture media dispensed in plates or tubes is frequently a matter of discussion, since it is necessary to consider the biosafety measures related to the seeding of clinical samples and the subsequent growth of the fungi, especially if it is endemic. The plates provide more surface area for the samples seeding and better contact with the air, which favors the fungus growth, the recovery of the colonies and the preparation of wet mounts, however, the culture medium tends to dehydrate and get contaminated faster. In these cases, it is recommended to seal the plates individually or place them in a bag with permeable gas, which avoids environmental contamination and increases safety when working with cultures, an activity that should always be carried out using a biological safety cabinet. The use of agar slants in tubes reduces contamination and increases safety, but decreases the growth surface for the fungus and makes it difficult to access the colonies due to the narrow mouth of the tube (Clinical and Laboratory Standards Institute (CLSI), 2012; Lindsley et al., 2015; Sutton, 2015; Tille, 2017). A low-cost and accessible alternative for laboratories is the use of Parafilm® laboratory film to seal the plates individually. The use of the culture media is ultimately at the discretion of the laboratory personnel.
- f) The culture media already seeded must be incubated aerobically at room temperature or in an incubator between 25 °C and 30 °C. The incubation time depends on the presumptive clinical diagnosis and the culture type requested. For example, for *Candida* spp. incubation does not exceed 72 h; cultures for dermatophytes are incubated for 7–15 days, while cultures for endemic fungi are routinely incubated for up to 4 weeks (Clinical and Laboratory Standards Institute (CLSI), 2012; Lindsley et al., 2015; Sutton, 2015; Tille, 2017). For the other pathogenic fungi, an incubation time of up to 3 weeks is sufficient (Labarca et al., 1998; Sutton, 2015). Cultures should be checked every 2 to 3 days for the first 2 weeks and then once a week, and should be incubated for 21–30 days before being reported as negative (Lindsley et al., 2015; Sutton, 2015; Tille, 2017).

Considerations for yeast identification

The identification of yeasts by conventional methods can be carried out according to three criteria: morphological, biochemical-physiological and immunological.

Morphological criteria

Most of the morphological methods are fast and allow the identification of some yeast until genus and species in a time not exceeding 48 h. The most used are the visualization of the macroscopic characteristics in solid culture media, germ tube formation, chlamydoconidia production, the Dalmau method that allows the study of microscopic morphology in Corn Meal Agar (CMA), and the chromogenic culture media (Campbell et al., 2013; Freydière et al., 2001; Howell et al., 2015; Larone, 2011; Linares Sicilia and Solis Cuesta, 2007; Pincus et al., 2007; Zaitz et al., 2010).

- a) *Macroscopic characteristics in solid culture media*: the appearance of the colonies is observed (mucous or shiny, opaque, butyric, smooth, rough, raised or flat, velvety), size and color (Fig. 5).
- b) *Germ tube production*: consists of placing a small portion of a colony obtained from a 24-h culture in 0.5 mL of serum and incubating at 37 °C for 2 h. This test is positive for *Candida albicans*/*Candida dubliniensis*, which produce a thin continuous tube, without constriction at the site of origin. *Candida tropicalis* can also produce a germ tube after incubation in serum for 2 h at 37 °C, but the difference is that the germ tube presents a constriction at the exit point of the yeast and the diameter is greater; the tube is not continuous or thin like that produced by *Candida albicans*/*Candida dubliniensis*.
- c) *Chlamydoconidia production*: to obtain this resistance structure characteristic of *Candida albicans* and *Candida dubliniensis*, it is necessary to use the bile agar medium, placing a small portion of a yeast colony obtained from a 24-h culture in it, and incubating at 28 °C for 24–48 h (Fig. 6).
- d) *Dalmau method*: consists of streaking a portion of the colony in the middle of CMA and placing a coverslide on top of it. Subsequently, incubated at 28 °C for 24–48 h and then the characteristic morphology for each species is observed under the microscope. The characteristic morphology of the five *Candida* spp. most frequently isolated in clinical samples is described below (Fig. 7).
 - Microscopic morphology of *Candida albicans*/*Candida dubliniensis* in CMA: they produce branched pseudohyphae with blastoconidia of regular size, round and budding, with smooth walls, arranged in clusters at the points of constriction of the pseudohypha; the characteristic chlamydoconidia of these species may or may not be present. Chlamydoconidia are round, smooth and thick-walled, with cytoplasmic inclusions; they are generally located in a terminal position in the pseudohypha, although they can also appear interspersed (Fig. 7A).
 - Microscopic morphology of the *Candida parapsilosis* species complex in CMA: they produce branched pseudohyphae with small oval or elongated blastoconidia, with smooth walls, arranged in small clusters along the pseudohypha. The blastoconidia may have buds and the pseudohypha, in most cases, has a curved appearance on one side (Fig. 7B).
 - Microscopic morphology of *Candida tropicalis* in CMA: produces branching pseudohyphae with single round or oval blastoconidia of regular size or in small groups with little budding, smooth walls, widely distributed throughout the pseudohypha. It rarely produces chlamydoconidia and if they occur they are very scarce (Fig. 7C).
 - Microscopic morphology of *Candida glabrata* in CMA: it does not produce pseudohyphae or pseudomycelium. Small, oval blastoconidia with uni or bipolar budding are observed; they are smooth and thin walled (Fig. 7D).

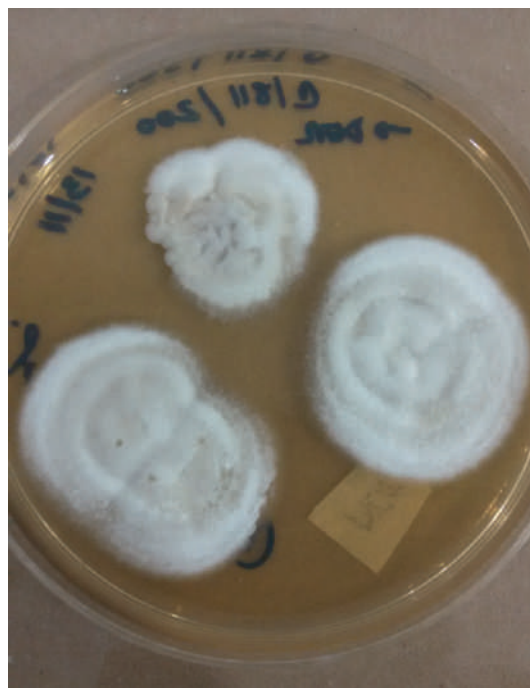


Fig. 5 *Epidermophyton floccosum* on PDA. Photo by María Mercedes Panizo.



Fig. 6 Chlamydoconidia of *Candida albicans* (1000 ×). Photo by María Mercedes Panizo.

- Microscopic morphology of *Candida krusei* in CMA: produces long, slightly thick or robust branched pseudohyphae with elongated or cylindrical blastoconidia of medium size and smooth walls, cluster at regular intervals, arranged as crossed trunks (Fig. 7E).
- e) *Chromogenic culture media*: is based on the detection of enzymatic activities by yeasts through the specific hydrolysis of a chromogenic substrate in the presence of an indicator.
 - With the commercial culture media CHROMagar™ *Candida* (CHROMagar Company, France) and CHROMagar™ (Becton-Dickinson, France), three species of *Candida* can be presumptively identified with high reliability by colony color. *Candida tropicalis* produces smooth, creamy colonies with an intense blue color or dark blue-green colonies, both with a metallic hue; *Candida krusei* produces pale pink, downy or slightly velvety, dull or dry colonies with a flat surface; *Candida albicans* produces emerald green, shiny, smooth, and creamy colonies. The colonies identification of these *Candida* species, as well as the colonies of other colors obtained on these media, must be confirmed by conventional methods (Fig. 8).
 - It is important to remember that *Candida dubliniensis* behaves phenotypically and biochemically the same as *Candida albicans*, and therefore also produces green colonies, making one species indistinguishable from the other, although some authors point out that *Candida dubliniensis* produces dark green colonies and it does not grow at 42 °C. To differentiate them it is necessary to use molecular techniques, not always available in the routine microbiology laboratory.
 - The use of this medium is recommended to obtain primary isolation from clinical samples coming from critically ill patients with clinical suspicion of candidiasis, and thus offer preliminary guidance in confirming the diagnostic impression. This makes it possible to establish an adequate and effective antifungal therapy in the shortest possible time. Another important advantage of chromogenic medium is the detection of two or more *Candida* species that may be present in clinical samples and cannot be detected in common culture media.
 - This culture medium has been widely commercialized, with differences in the detection of the enzymatic activities of *Candida* spp., among which are chromID™ *Candida* (bioMérieux, France), CandiSelect™ 4 (BioRad, United States) and Colorex *Candida*® (Biomedics).

Biochemical-Physiological criteria

Physiological methods can be classified into rapid and conventional tests. Rapid tests include urease production, phenol oxidase production, and growth in 6.5% sodium chloride (NaCl). Tests such as thermotolerance and resistance to cycloheximide are considered complementary. Conventional methods based on biochemical criteria are nutrient assimilation (carbohydrates and nitrogen sources) and carbohydrate fermentation, which are costly, laborious and time-consuming techniques. These methods have been displaced in the laboratory by automated identification systems (Freydière et al., 2001; Howell et al., 2015; Linares Sicilia and Solis Cuesta, 2007; Pincus et al., 2007; Zaitz et al., 2010).

- a) *Urease production*: this test reveals the urease enzyme activity present in some species of yeast. The urea hydrolysis occurs in Christensen's urea agar medium, after inoculating the yeast in the medium for 48 h at 28 °C. A positive test is manifested by the color change from half orange to fuchsia. Among the yeast genera that are urease positive are *Cryptococcus*, *Rhodotorula*, and *Trichosporon*.

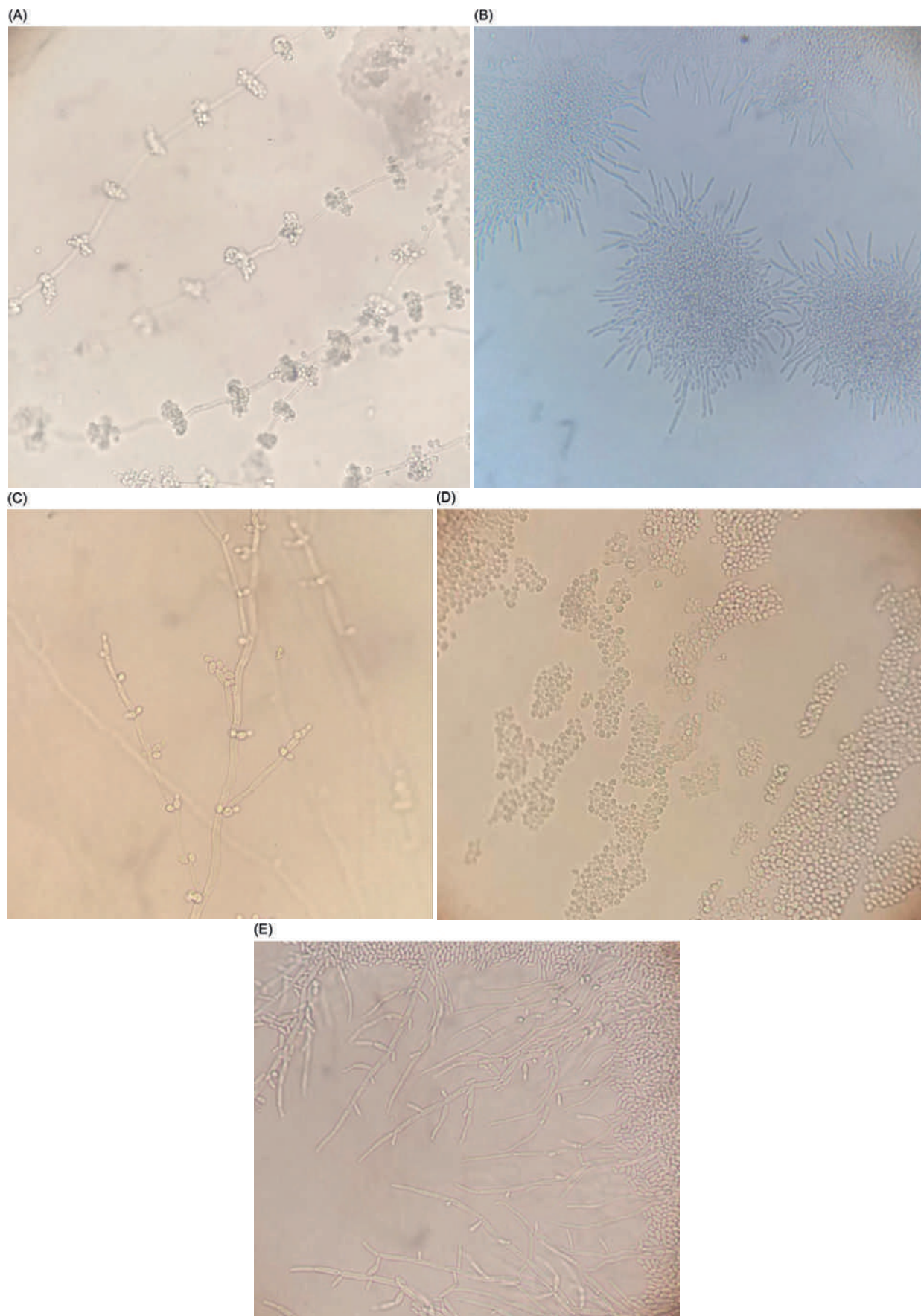


Fig. 7 Characteristic morphology in CMA of the five *Candida* spp. most frequently isolated in clinical samples (400 $\times$). (A) *Candida albicans*; (B) *Candida parapsilosis* species complex; (C) *Candida tropicalis*; (D) *Candida glabrata*; (E) *Candida krusei*. Photos by Xiomara Moreno.

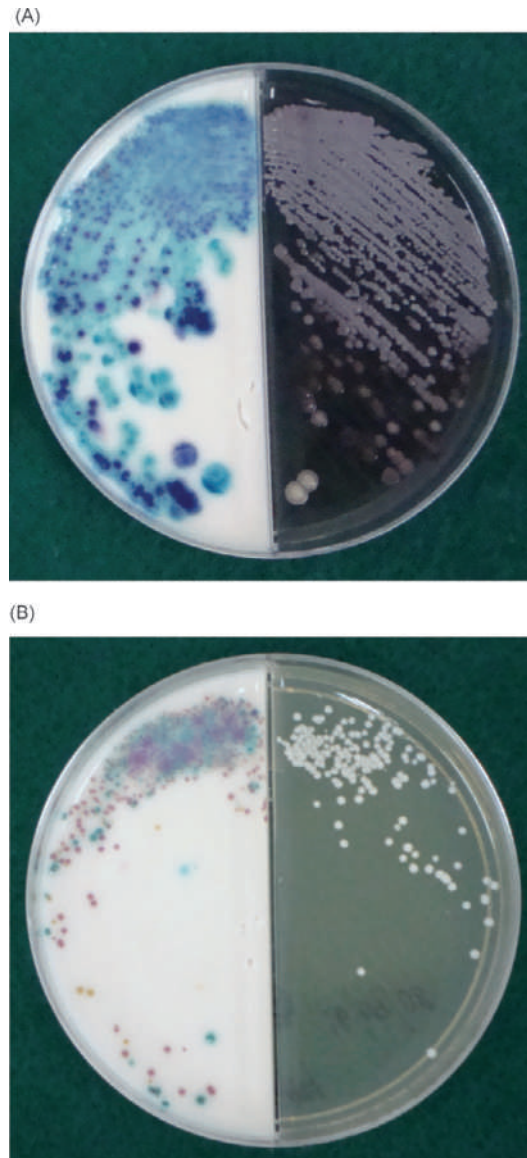


Fig. 8 *Candida* spp. in chromogenic culture media. Double Petri dish with CHROMagar™ *Candida* (left) and Mycosel® (right). (A) *Candida albicans* (emerald green colonies) and *Candida tropicalis* (dark blue-green colonies); (B) *Candida albicans* (emerald green colonies), *Candida krusei* (pink colonies) and beige colonies (other yeasts). Photos by Maria Mercedes Panizo.

- b) *Phenol oxidase production*: *Cryptococcus neoformans* and *C. gattii* produces phenol oxidase, an enzyme necessary for the metabolism of 3,4-dihydroxyphenylalanine (DOPA) and other phenolic compounds in the synthesis of melanin. This is evidenced by the production of a dark brown or black pigment around the *Cryptococcus neoformans*/*C. gattii* colonies in the solid Oxgall-Tween-caffeic acid culture medium.
- c) *Growth test in 6.5% sodium chloride (NaCl)*: allows presumptive differentiation of *Candida albicans* from *Candida dubliniensis*. It consists of preparing a 0.5 McFarland suspension of the suspected yeast and adding 25 µL of this suspension to a test tube with 1 mL of 6.5% NaCl and a drop of 10% yeast extract; subsequently, it is incubated at 37 °C for a maximum of 72 h. If at the end of this time turbidity and growth are obtained in the tube, the identification will correspond to *Candida albicans*. The opposite result, without turbidity and without growth, will presumably correspond to *Candida dubliniensis*. It is important to keep this isolate for later confirmation by molecular biology techniques.
- d) *Thermotolerance test*: this consists of incubating the yeast colonies at a temperature of 42–45 °C. Most non-*Candida albicans* species do not grow at temperatures above 37 °C.
- e) *Resistance to cycloheximide or actidione*: most of the non-*Candida albicans* species are sensitive to actidione and therefore do not grow in this medium; few are resistant to this antibiotic and the most important resistant specie is *Candida albicans*.

- f) *Carbohydrates assimilation (auxanogram)*: it is based on the property that yeasts have of using sugars as a source of carbon under aerobic conditions. The solid basal medium for the auxanogram is free of sugars and alcohols.
- g) *Nitrates assimilation (KNO₃)*: consists of the use of nitrates by yeasts as the only source of nitrogen. A basal medium with glucose is used and urea or KNO₃ should be used as positive controls.
- h) *Carbohydrates fermentation (zymogram)*: it is based on the property that yeasts have of using sugars in anaerobiosis, which is evidenced by the production of gas. The most commonly used sugars are: glucose, maltose, sucrose, galactose, trehalose and lactose in concentration at 2% and raffinose at 4%. The sugars are prepared in basal medium (peptone, yeast extract and water). The formation of gas (CO₂) is detected by incorporating an inverted Durham tube into the liquid medium. The observation is made daily, because the gas can be reabsorbed.
- i. *Automated identification systems*: automated identification systems have had a great impact on the microbiology laboratory, because they considerably improve handling of large volumes of microorganisms for identification, which in turn allows providing an early diagnosis. In the last decades, manual and automated commercial methods have been developed, based on nutrients assimilation. Manual and semi-automated commercial methods include Auxacolor™ (Bio-Rad, United States), Uni-Yeast Tek (Remel, United States), API 20C® AUX and API® ID32C (bioMérieux, France), RapID Yeast Plus System (Innovative Diagnostic System, United States) and Fungiscreen 4H™ (Bio-Rad, United States). Automated commercial methods include Vitek® 2 (bioMérieux, France), Biolog YT MicroPlate™ (Biolog, United States) and Rapid Yeast Identification Panel Micro-Scan® (Dade Behring, United States). They are faster than conventional methods, easy to use and have software for the identification of the different microorganisms.

Conventional yeast identification methods consume a variable time from 2 to 21 days and require the use of identification keys, but a good identification is achieved. Automated methods can identify yeast in 4–72 h, which is a great advantage for laboratory and clinical patient management. But these methods have not been able to completely replace the conventional ones. Yeast identification, through the use of automated equipment, must be accompanied by additional tests that complement the identification, such as the use of chromogenic culture media and microscopic visualization of yeast in CMA using Dalmau method. In addition, the microbiologist in charge of operating these systems must be constantly updated, especially in aspects related to taxonomy.

Immunological criteria

Immunological methods are based on agglutination with latex particles, using specific monoclonal antibodies (Freydière et al., 2001; Freydière et al., 2003; Howell et al., 2015; Linares Sicilia and Solis Cuesta, 2007; Pemán et al., 2004; Pincus et al., 2007; Zaitz et al., 2013).

- a) Bichro-latex albicans® (Fumouze) is a method for the rapid identification of *Candida albicans* isolates by agglutination of latex particles using a monoclonal antibody specific for *Candida albicans*.
- b) Krusei-color® (Fumouze) is a method for the identification of *Candida krusei* isolates by agglutination of latex particles, using a monoclonal antibody that specifically reacts with the antigen located on its wall.
- c) Bichro-Dubli® (Fumouze) is a method for the rapid identification of *Candida dubliniensis*, based on the coagglutination of blastospores with latex particles coated with monoclonal antibodies, which allows the specific detection of a *Candida dubliniensis* antigen located on the cell surface.

Considerations for filamentous fungi identification

The identification of filamentous fungi is carried out through a combination of tests (Arenas, 2014; Campbell et al., 2013; Larone, 2011):

- a) Morphological characteristics of the colony in culture.
- b) Microscopic morphological characteristics.
 - Hyphae: thickness, ramifications, presence or absence of septa, and color.
 - Asexual spores and conidiogenous cells.
 - Sexual spores and structures where they are formed.
 - Any attached formation (ornamentation).

The morphological characteristics of the colonies have limited value in identifying filamentous fungi, due to the natural variation that exists between isolates and colonies when growing in different culture media. Although the fungi that are most frequently recovered in the laboratory can be easily recognized, colonial morphology is an unreliable criterion that should be used only to complement the microscopic morphological characteristics of the organism.

It is also important to take into account the growth time of the fungus in the culture, however, its value is limited, because the time varies depending on the fungus: slow-growing ones form mature colonies in 11–21 days; those of intermediate growth form mature colonies in 6–10 days, and those of fast growth form mature colonies in 5 days or less.

Colony color can be important. Laboratory personnel should be sure to record both the front and reverse side of the culture. The topography of the colony describes the different elevations and shapes that it can present in cultivation. It can be described as warty, slightly raised in the center, or with radiating furrows from the center, among other descriptions. The texture of the colony must also be taken into account. It can see cottony, velvety, glabrous, granular, powdery, and woolly textures, among others. Incubation conditions and culture media should also be considered.

In most cases, the microscopic morphological characteristics provide presumptive and sometimes definitive identification of the fungus. A major disadvantage is that these structures tend to develop slowly and appear after several days or weeks of incubation. Laboratory personnel must be well trained to recognize the microscopic characteristics of filamentous fungi in their asexual phase, which is the frequently observed one in the laboratory when the fungi are recovered from culture. The development stages of conidia and conidiogenous cells (conidiogenesis) have stable characteristics that are used in the classification of fungi, including their formation mode, the mycelium structure that supports the conidia and their accommodation, which are the basis for species identification. In medical mycology these attributes are of special importance, since the teleomorphic or sexual state is rarely found.

Fungi can be prepared for microscopic observation using various techniques. The following methods can be used:

- a) Analysis of a fragment of the culture (tease mount): consists of taking a fragment of the colony, dilacerate it and observe it with lactophenol blue. This is the usual method, but if not done carefully it can destroy conidiogenous cells and make identification difficult.
- b) Transparent adhesive tape method (Rush-Munro): it is a variant of the prior technique and allows the fungal structures to be observed almost without alteration. A small square of adhesive tape is cut out and adhered to the terminal part of the nichrome needle, and then the adhesive part is applied on the colony and then placed on a slide with a drop of dye. The platinum needle is removed, added another drop of dye, a coverslip is placed and observed under a microscope.
- c) Slide culture or micro-culture: it is the most precise and allows observing the fungal structures in situ. It consists of obtaining a culture on a glass slide and observing the fungus without deterioration of its morphology.
 - Prepare a sterile 90-mm diameter Petri dish and cover the inside bottom with a piece of sterile filter paper.
 - Place a sterile glass rod on the sterile filter paper.
 - Place a clean flamed microscope slide on the glass rod.
 - Cut a block of 1-by-1 cm from a Petri dish of Potato Dextrose Agar (PDA) with a sterile scalpel and place it in the center of the slide.
 - Inoculate the fungus onto the centers of the four sides of the agar block with a nichrome needle.
 - Place a flamed coverslip over the block, and apply slight pressure to ensure adherence.
 - Place approximately 1,5 mL of distilled sterile water on the filter paper, cover the Petri dish with the lid, and incubate at room temperature, preferably in the dark, or in a cabinet at 25 °C.
 - Examine the Petri dish periodically for growth and add water if it is necessary.
 - When growth is visible, remove the coverslip with forceps, take a microscope slide, place a drop of lactophenol blue and then place the coverslip.
 - Remove the agar block from the slide and put it into a container with disinfectant. Put a drop of lactophenol blue on the slide and cover it with a new coverslip.
 - Examine the wet mounts on the microscope, looking for reproductive structures.

The preferred technique for demonstrating diagnostic structures and conidiogenous structures for most filamentous fungi is slide culture. In addition, this method can provide a permanent mount that can be preserved in a slide collection for future study, and is extremely useful for comparison with other similar or atypical isolates.

It is important to recognize that the definitive identification of filamentous fungi frequently requires molecular analysis, to support morphological identification. One of the consequences of these analysis has been the recognition that many clinically important species are actually species complexes, morphologically identical but genetically different. In circumstances when identification based on morphology is not sufficient, the fungus is a candidate for identification by molecular techniques.

This approach is very useful when the fungus presents an atypical or non-specific morphology, do not sporulate properly and requires special culture media. However, in the routine laboratory, for the identification of filamentous fungi, observation of their microscopic morphology continues to be the standard approach, although it has limited diagnostic capacity, since not all laboratories have trained personnel or equipment for performance molecular analysis. Importantly, any filamentous fungus recovered in culture must be examined and handled in a biological safety cabinet.

Diagnosis of fungal diseases by culture-independent microbiological methods

Obtaining the fungus causing a fungal infection, especially from sterile specimens, is still the gold standard for definitive diagnosis, but this process sometimes takes several weeks to provide results, reducing sensitivity. On the other hand, positive cultures from non-sterile samples can be due to both colonization and infection, and distinguishing the two is not always easy. A presumptive diagnosis can also be made using wet mounts, as well as microbiological and histopathological stains; however, obtaining adequate clinical specimens, both for culture and for these mounts is not always possible. Non-culture-based mycological diagnostic methods can provide more timely results and are very useful tools, since the diagnosis of an invasive fungal infection cannot always be made by culture and histopathological studies, but these methods have not yet completely replaced the culture, therefore, should be used as complementary methods and its results should be interpreted individually, taking into account the clinical context of the patient.

Non-culture-based diagnostic methods are classified into five groups. They include the detection of antibodies in the host, fungal antigens, metabolites or structural components, proteins, and nucleic acids by molecular techniques. Only a small group of tests have been shown to be clinically useful, and very few are commercially available.

Antibody detection in the host

The use of antibody detection in serum or other body fluids is important in the diagnosis of endemic mycoses such as Histoplasmosis, Paracoccidioidomycosis, Coccidioidomycosis, and South American Blastomycosis, as well as in two clinical manifestations of aspergillosis, such as allergic bronchopulmonary disease and aspergilloma. However, they are not useful in most opportunistic mycoses, due to their low sensitivity and specificity, particularly in immunosuppressed patients. Among the most important limitations of these techniques are the ability to produce antibodies by the patient, the time of evolution of the disease and the quality of the antigens used (Pfeiffer and Wong, 2015; Quindós, 2015). Additionally, the detection of antibodies does not help to distinguish between an acute or chronic infection, because the ability to produce antibodies is individual in patients, and may be delayed in some cases; finally, it is possible to detect the presence of antibodies against some antigens in people asymptomatic, which reduces the diagnostic specificity of these tests (Pfeiffer and Wong, 2015).

The tests most used today are double radial immunodiffusion (ID) on agarose gel, counter-immunoelectrophoresis (CIE), complement fixation (CF), indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (EIA). The latter is rapidly replacing the others due to its greater sensitivity (Pfeiffer and Wong, 2015; Quindós, 2015).

Histoplasmosis

For the detection of antibodies against Histoplasmosis the most used tests are the ID, FC and CIE. In the ID and CF tests, histoplasmin is composed of two species-specific antigens of *Histoplasma capsulatum* which are the H and M antigens. Antibodies against the H antigen are formed during acute Histoplasmosis, while antibodies against the antigen M are formed both during both acute and chronic form of the disease, and are usually the first to appear after seroconversion. Antibodies against the H antigen signify active and progressive infection, although they are only detected in 10–20% of patients with exposure to the fungus, while antibodies against the M antigen are detected between 6 and 8 weeks after exposure to the fungus in 50–80% of patients, persisting for years, even though the patient has recovered from the infection. This indicates that its presence does not help to distinguish between current and past infection (Linder and Kauffman, 2021; Pemán et al., 2017; Pfeiffer and Wong, 2015).

The ID is a widely disseminated test because it is fast, safe, inexpensive and simple to perform, allowing the diagnosis of 85% of the cases of histoplasmosis in immunosuppressed patients. It can be performed using serum, peritoneal fluid, and CSF specimens in cases of meningeal histoplasmosis. This test can be made semi-quantitative, performing serial double dilutions of the specimens to obtain the antibody titer (Linder and Kauffman, 2021; Pemán et al., 2017; Reviákina et al., 2007). The interpretation of the semi-quantitative results of the ID is very important, since the obtained titers can be presumptive or diagnostic of histoplasmosis. Titers between 1:8 and 1:16 have a presumptive value and should be assessed in the clinical context of the patient, while titers equal to or greater than 1:32 are diagnostic for active histoplasmosis. The decrease in titers during treatment is useful to follow the effectiveness of the treatment and the improvement of the patient (Linder and Kauffman, 2021; Reviákina et al., 2007).

Asymptomatic individuals residing in endemic areas often have low antibody titers. Titers decrease as time passes after exposure, but can remain positive for years. False negative results of these tests are possible in immunosuppressed patients and during the early stages of infection. False positives can occur in 15% of patients, due to cross-reactions with paracoccidioidomycosis, blastomycosis, and coccidioidomycosis agents (Pfeiffer and Wong, 2015; Reviákina et al., 2007). In these cases, it is recommended to obtain the titers of all agents, to know which of them is causing the infection (Reviákina et al., 2007). Similar results to ID are obtained with the CIE technique, which is faster and more sensitive, but has the disadvantage of requiring electrophoresis, which increases costs. On the other hand, FC has a similar sensitivity to ID, but its main drawback is that it is very labor intensive.

Paracoccidioidomycosis

For the detection of antibodies against paracoccidioidomycosis, in patients with risk factors and exposure to the fungus but without immunodeficiencies, the ID, CF, CIE, IIF and EIA tests have been used, with similar sensitivities, especially if it is possible to combine the least two of the mentioned techniques (Pfeiffer and Wong, 2015; Wheat, 2009).

The most widely used method is ID and its usefulness for this disease is similar to that of histoplasmosis, since it allows diagnosing 94% of paracoccidioidomycosis in patients without immunosuppression. FC is used in the same way as ID. The values of the interpretation of the titers for the diagnosis of the disease are the same as for histoplasmosis, being the high titers indicative of the severe form of the disease, which can be detected even up to 2 years after the patient recovered from it (Reviákina et al., 2007; Pemán et al., 2017; Pfeiffer and Wong, 2015), although other authors affirm that any positive value obtained, even when the dilutions are low, is indicative of the infection (Guimarães et al., 2006; Pires de Camargo and Fabiano de Franco, 2000).

Coccidioidomycosis

Three tests are currently used, which are useful for diagnosing the disease in non-immunosuppressed patients: ID, CF and EIA. They are simple to perform and have good sensitivity and specificity. IgM antibodies are detected during the first week of infection and IgG appear between the second and third week. The ID test is widely used in endemic areas for the diagnosis and evaluation the prognosis of the disease and its results must be interpreted in conjunction with the patient clinical symptoms (Pemán et al., 2017; Pfeiffer and Wong, 2015). Titers from 1:16 onwards obtained from serum correlate with the severity of the disease (Reviákina et al., 2007). In some patients the antibodies remain for up to 6 months and in rare cases they can remain even after successful treatment. The reappearance of antibodies in a patient with a history of coccidioidomycosis is considered a reactivation of the disease (Pemán et al., 2017; Pfeiffer and Wong, 2015).

The CF test can be performed in serum and body fluids and, like ID, is semi-quantitative and it is recommended to report the titer when it is positive. The use of serial double dilutions in ID and CF help the physician to assess both the progression and cure of the disease (Pemán et al., 2017). EIA are simple and precise tests that can be performed in serum and CSF, however, false positives can occur when obtaining a positive IgM together with a negative IgG, and the validity of the result in asymptomatic patients or without classic symptoms should be questioned (Blair et al., 2013); this finding is relatively common (~10%) and one review has reported false negative rates between 0% and 82% (Nguyen et al., 2013). For this reason, EIA must be confirmed with ID or CF. All of these tests are not useful for diagnosing the disease in immunosuppressed patients (Pfeiffer and Wong, 2015).

Blastomycosis

The tests used for the detection of antibodies against antigen A of *Blastomyces dermatitidis* are ID, FC and EIA, with variable sensitivity and specificity values. When comparing them, the ID test is the most specific, the EIA is the most sensitive, and the FC has low values for both parameters (Linder and Kauffman, 2021; Saccente and Woods, 2010; Smith and Kauffman, 2010). All have limited utility in diagnosing acute disease, since antibodies are produced 50–70 days after the onset of symptoms (Linder and Kauffman, 2021; Klein et al., 1987). In addition, these antibodies can remain for a year or more even after the patient is cured. Assays for the detection of antibodies against W-1 wall antigen of *Blastomyces dermatitidis* have been evaluated with promising results, but the tests are not commercially available (Linder and Kauffman, 2021; Soufleris et al., 1994).

Aspergillosis

In the case of aspergillosis, serological tests such as ID are essential to confirm a clinical picture both of aspergilloma and allergic bronchopulmonary aspergillosis, provided that the results, even when the titers obtained are low, can be compared with the patient's symptoms. IgG antibodies are detected and it is necessary to combine it with radiological studies (Pfeiffer and Wong, 2015; Reviákina et al., 2007).

Detection of fungal antigens

Antigen detection is extremely useful in the diagnosis of many fungal diseases, especially in immunosuppressed patients (Quindós, 2015). A variety of immunological techniques including latex particle agglutination (LA), EIA, and lateral flow assay (LFA) are used to identify antigenic components in a clinical specimen, and often require mono- or polyclonal antibodies to detect the antigen. Its main limitations are cross-reactions, the transience of antigens in some hosts, and the lack of specificity for a particular antigen (Pfeiffer and Wong, 2015).

Candidiasis

The cell wall mannan of *Candida* spp. is an antigen that can be detected in the serum of patients with invasive candidiasis, which in turn is rapidly degraded and eliminated from the bloodstream. The detection of mannan by EIA is a test that has been commercially available for years (Platelia Candida Ag Plus[®], Bio-Rad), but, due to its poor diagnostic performance, its use in parallel with the detection of anti-mannan antibodies is recommended (Platelia Candida Ab Plus[®], Bio-Rad), in those patients with suspected invasive candidiasis (Freeman Weiss et al., 2021; Sendid et al., 1999).

A recent review of the usefulness of these tests in oncohematological patients with invasive candidiasis showed that the combined detection of mannan and anti-mannan antibodies had a sensitivity of 83% with a specificity of 86%, these tests being faster than the blood culture (Mikulska et al., 2010). Furthermore, these tests have also shown their usefulness in neutropenic patients, due to their high negative predictive value of 95% (Ellis et al., 2009). The results vary depending on the *Candida* spp. involved in invasive candidiasis, being more useful when they are produced by the *Candida albicans* species complex, *Candida glabrata* species complex and *Candida tropicalis* than by the *Candida parapsilosis* and *Candida krusei* species complexes (Pemán et al., 2017).

Aspergillosis

Galactomannan (GM) is a cell wall polysaccharide component of *Aspergillus* spp. that is released during invasion into tissues. It can be detected in serum, BAL, biopsies, urine, CSF, pericardial fluid and pleural fluid of patients with invasive aspergillosis (Pemán et al., 2017; Pfeiffer and Wong, 2015).

Its detection in serum has been commercialized by means of a sandwich EIA (Platelia Aspergillus[®], Bio-Rad), which uses a monoclonal antibody capable of detecting low levels of GM in blood. It is an alternative diagnostic technique in front of culture (results in 4 h), that is rapid, simple and reproducible, which has been included as a mycological criterion in the consensus definitions of the European Organization for Research and Treatment of Cancer/Mycoses Study Group of the National Institute of Allergy and Infectious Diseases (EORTC/MSG) for neutropenic patients (de Pauw et al., 2008; Donnelly et al., 2020), has been approved by the FDA and has sensitivity and specificity values of 81% and 89%, respectively (Wheat, 2003), making it a useful test for the diagnosis and monitoring the disease.

The GM values do not have the same diagnostic utility in non-neutropenic patients, especially with solid organ transplantation. A meta-analysis has shown that in high-risk patients the sensitivity values of the test in serum decrease to 61% with a specificity of 93% in both cases of probable and possible invasive aspergillosis, while in proven cases the sensitivity of the test

is found around 71–79% (Pfeiffer et al., 2006). In critically ill patients with pneumonia, neutropenic, hemato-oncological and immunosuppressed, it has proven to be very useful (Meersseman et al., 2008; Pfeiffer et al., 2006).

It is important to note that false positive results can be obtained due to cross reactions with other species of fungi (*Penicillium* spp., *Alternaria* spp., *Paecilomyces* spp., and *Cryptococcus* spp.), during treatment with antibiotics totally or partially derived from fungi (piperacillin-tazobactam, amoxicillin-clavulanic acid) and in neonates with intestinal colonization by *Bifidobacterium* spp. (Duarte et al., 2014; Marr et al., 2005; Pemán et al., 2017; Pfeiffer and Wong, 2015). False negative results are rare but can also be obtained due to a low fungal burden, as occurs in invasive aspergillosis caused by the *Aspergillus fumigatus* complex, the use of prophylactic antifungals, and high antibody titers (Hachem et al., 2009; Mennink-Kersten et al., 2004; Pfeiffer and Wong, 2015).

The Platelia Aspergillus® test was approved by the FDA for use in BAL in 2011, improving the sensitivity of the test when compared with the results obtained in serum in patients with risk factors for invasive aspergillosis (D' Haese et al., 2012; Husain et al., 2008), with sensitivity values of 87% and specificity of 89% (Zou et al., 2012) and sensitivity and specificity of 82% and 97%, respectively (Avni et al., 2012), where the diagnostic performance of the test depends on the cutoff value selected for positivity, being 0.5 the one approved by the FDA. To increase diagnostic performance, serial determinations are recommended: GM values >0.5 in two consecutive sera increase the specificity and positive predictive value of the test; if a single determination is made, a GM value >0.7 is considered positive. GM values in BAL >0.5–1 are considered diagnostic criteria for probable invasive aspergillosis. It is important to note that the assay is currently only validated for serum and BAL specimens. The test has also proven to be very useful for monitoring response to treatment, where the decrease in GM values predicts a satisfactory response with patient recovery (Chai et al., 2012; Koo et al., 2010).

The latest recommendations of the EORTC/MSG group for the use of GM establish different cutoff values for the test adjusted to the clinical specimen, independent of the patient group, which differ from the cutoff value recommended by the manufacturer and approved by the FDA (0.5), and the validated clinical samples (serum and BAL): Single serum or plasma: ≥ 1.0 ; BAL fluid: ≥ 1.0 ; Single serum or plasma: ≥ 0.7 and BAL fluid ≥ 0.8 ; CSF: ≥ 1.0 (Donnelly et al., 2020; D' Haese et al., 2012; Leeflang et al., 2015; Mennink-Kersten et al., 2004). Según los reportes de algunas investigaciones, la detección de GM en plasma y LCR pueden avalar el diagnóstico de IA (Chong et al., 2016; Klont et al., 2004).

In 2008, the first version of the LFA was commercialized for the diagnosis of invasive aspergillosis, which had sensitivity and specificity values with serum samples of 68% and 87%, respectively, and of 86% and 93% with BAL samples respectively (Pan et al., 2015; Thornton, 2008). Recently, the version of the IMMY Sona Aspergillus Galactomannan LFA has been released, which using BAL and serum samples and has sensitivity and specificity values of 83–92% and 91–92%, respectively (Lass-Flörl et al., 2019; Mercier et al., 2020).

Cryptococcosis

The detection of glucuronoxylomannan (GXM), the polysaccharide antigen present in the capsule of the *Cryptococcus neoformans* and *C. gattii* species complexes, is one of the most useful alternative diagnostic methods to culture in medical mycology. It is included by the EORTC/MSG as a diagnostic criterion for cryptococcosis (de Pauw et al., 2008; Donnelly et al., 2020) and is very useful for the diagnosis in patients with HIV and meningeal cryptococcosis (scientific evidence AI) and in oncohematological patients with meningeal and disseminated cryptococcosis (scientific evidence A-II) (Ayats et al., 2011; Marchetti et al., 2011). Currently for the detection of this antigen there are several commercial tests such as LA, EIA techniques that use mono and polyclonal anti-glucuronoxylomannan antibodies (Ayats et al., 2011) and the Point-of-Care LFA; the latter recently designed and approved by the FDA in 2011 (IMMY, Norman, OK, United States) (Lindsley et al., 2011).

The diagnostic performance of the LA and EIA tests depends on the immune status of the patient, the clinical specimen (serum or CSF) and the clinical form of the disease (localized or disseminated) to diagnose cryptococcal meningitis, since it usually varies. Using serum and CSF, the sensitivity of the titers was 92% and 99% in patients with HIV; 88% and 100% in transplant recipients; 87% and 97% in immunosuppressed patients, and 92% in CSF of immunocompetent patients (Antinori et al., 2005). The prognostic value of the titers is controversial, due to the problems mentioned above. There are studies that associate higher initial titers with increased mortality and relapses, while other studies have not confirmed this association (Chen et al., 2012; Lin et al., 2009; Mitchell and Perfect, 1995; Pappas et al., 2001; Singh et al., 2008). The use of titers to control the therapeutic response is also controversial, since it has been reported that their use is not recommended, especially in patients with HIV (Perfect et al., 2010; Powderly et al., 1994). In particular, LFA has excellent diagnostic performance in serum and outstanding in CSF, when compared to other techniques. Among its advantages are that is easy and fast to perform and interpret, in addition to its easy storage at room temperature, however, it is necessary to validate the titers of the cryptococcal antigen before adopting the test for generalized use (Binnicker et al., 2012; Hansen et al., 2013; McMullan et al., 2012). The sensitivity of all the tests, regardless of the patient group, was much better in serum and CSF (94%) than India ink (60%), but similar to CSF culture (92%) (Antinori et al., 2005).

Other clinical samples such as pleural fluid and urine may be useful in certain situations for the diagnosis of cryptococcosis (Lindsley et al., 2011; Young et al., 1980). Among the limitations of these tests are false negative results, which although occasional, can occur in the face of a very low burden of the fungus in clinical specimens. False positive results are rare, but can occur in patients with *Trichosporon* spp., *Stomatococcus* spp. and *Capnocytophaga canimorsus* infections (Chanock et al., 1993; McManus and Jones, 1985; Pemán et al., 2017; Westerink et al., 1987), as well as in patients with rheumatoid factor, some neoplasms, specimen contamination during its manipulation and by the use of silicone tubes to store the specimen (Pemán et al., 2017).

Histoplasmosis

The detection of a polysaccharide antigen of *Histoplasma capsulatum* by EIA is of great diagnostic utility as an alternative test to culture. Depending on the clinical manifestation of histoplasmosis, the antigen may be present in urine, serum, plasma, CSF or BAL. Currently, there are commercial tests such as the MVista Histoplasma[®] (MiraVista Diagnostics, Indianapolis, IN, United States), a quantitative sandwich EIA that uses rabbit polyclonal anti-histoplasma antibodies (Connolly et al., 2007), the Histoplasma Antigen ELISA[®] (YMMI, Norman, OK, United States) approved by the FDA for in vitro diagnostics, and a test developed by the Centers for Disease Control (CDC) for resource-poor countries (Linder and Kauffman, 2021; Scheel et al., 2009; Zhang et al., 2013).

These tests are useful for diagnosing histoplasmosis in HIV patients with progressive disseminated disease (scientific evidence A-II), being less useful for the diagnosis of the disease in patients without HIV (scientific evidence B-II). Antigen detection is more sensitive with urine samples (80–90%) than in serum (50–80%). In patients with pulmonary histoplasmosis without immunodeficiencies the use of BAL is recommended, with a diagnostic sensitivity of 93% (scientific evidence A-II) (Hage et al., 2010). These tests are also useful for the therapeutic monitoring of the disease, since antigen levels decrease with appropriate therapy while increase with relapse (Linder and Kauffman, 2021).

False negative results may occur, depending on the group of patients and the severity of the disease. Cross-reactivity commonly occurs with paracoccidioidomycosis, blastomycosis, and penicilliosis; is less common with coccidioidomycosis, and rare with aspergillosis (Linder and Kauffman, 2021; Pfeiffer and Wong, 2015).

Paracoccidioidomycosis

For the diagnosis of this disease in immunosuppressed patients several antigen detection techniques have been developed. The most widely used is gp43 antigen, which can be detected by an immunoblotting technique in urine samples with excellent sensitivity and specificity values (Salina et al., 1998) and with an EIA technique, which uses monoclonal antibodies to detect this antigen in clinical specimens of serum, urine, CSF and BAL from patients with both confirmed acute and chronic forms of paracoccidioidomycosis (Marques da Silva et al., 2003). The usefulness of gp70 antigen detection for the diagnosis of this endemic mycosis has also been demonstrated (Marques da Silva et al., 2004a). These tests are also useful for the therapeutic follow-up of the disease (Marques da Silva et al., 2004b).

Blastomycosis

For the alternative diagnosis of this mycosis, a commercial quantitative antigen detection test is available in urine, serum, plasma, CSF and BAL specimens (MVista Blastomyces Antigen ELISA[®]; MiraVista Diagnostics, Indianapolis, IN, United States), which detects a glycoprotein that is unfortunately not gender specific; therefore, the specificity of the test is high for patients without fungal disease and presents similar cross-reactions to the MVista Histoplasma[®] test (MiraVista Diagnostics, Indianapolis, IN, United States). The test has a high sensitivity for the detection of antigen in urine samples (85–73%) (Bariola et al., 2011; Connolly et al., 2012; Durkin et al., 2004; Linder and Kauffman, 2021). Its use is not recommended for the therapeutic monitoring of the disease, since no correlation has been established between antigen levels and treatment response (Linder and Kauffman, 2021; Pfeiffer and Wong, 2015).

Coccidioidomycosis

Commercially there is an EIA test for the detection of the *Coccidioides* species complex antigen (MVista Coccidioides[®], MiraVista Diagnostics, Indianapolis, IN, United States), which has shown a sensitivity of 71% and a specificity of 99% in urine samples from immunosuppressed patients with moderate to severe disease; however, cross-reactivity with other endemic mycoses such as histoplasmosis and paracoccidioidomycosis was observed (Durkin et al., 2008). In serum and urine samples from patients with mild disease, the sensitivity of the test was 73% in serum and 50% in urine, with a specificity of 100%, but the test was evaluated in few cases (Durkin et al., 2009).

Penicilliosis

In patients with HIV and AIDS, studies carried out for the detection of *Penicillium marneffei* antigen by EIA tests have shown sensitivity and specificity values greater than 90%. The test has not yet been commercialized (Chaiyaroj et al., 2003).

Detection of metabolites or structural components

For the detection of fungal metabolites, which behave as biomarkers for the diagnosis of invasive fungal diseases, techniques such as gas chromatography and enzymatic reactions are used. These biomarkers are not unique to particular fungal species, so this is a major limitation when using them for diagnosis.

Panfungal 1,3- β -D-glucan assay (BG)

This is a cell wall structural component of various fungi that is released into the blood during the course of fungal infection. It is a non-specific panfungal biomarker and any positive result must be confirmed by other mycological techniques to identify the fungus causing the infection. In this test, the experience of the laboratory professional in charge of the diagnosis is decisive for the interpretation of the results, since it is a complex test. Four assays are currently commercially available, each with individual cutoff values for test positivity: Fungitell (formerly Glucatell; Associates of Cape Cod, East Falmouth, MA), Fungitec-G (Seikagaku, Tokyo, Japan), assay Wako turbidimetric test (Wako Pure Chemical Industries, Tokyo, Japan) and Maruha colorimetric test (Maruha-Nichiro Foods, Tokyo, Japan) (Lamoth et al., 2012). A threshold >80 pg/mL with Fungitell is valid for the diagnosis;

there is insufficient evidence to use tests produced by other manufacturers (White et al., 2019). More recently, the Associates of Cape Cod, responsible for the Fungitell BG assay, developed the Fungitell STAT™ assay, that permits simple, rapid (<1 h) testing (D'Ordine et al., 2020; White and Price, 2021).

The detection of this component has been evaluated for the diagnosis of *Pneumocystis jirovecii* pneumonia (PCP) with excellent results. Two meta-analyses studied its diagnostic precision, finding sensitivity and specificity values of 96% and 84%, respectively (Onishi et al., 2012) and of 95% and 86% (Karageorgopoulos et al., 2013). Both studies evaluated the use of a single blood BG determination and, given the high sensitivity of the test, it is considered that it may be effective in excluding a diagnosis of PCP without the need for bronchoscopy.

For the panfungal diagnosis of invasive fungal infections, with the exception of PCP, the BG test is considered to have moderate utility, with sensitivity values between 77% and 80% and specificity between 82% and 85%, based on the EORTC/MSG definitions (Karageorgopoulos et al., 2011), and it has been included as a diagnostic criterion in the definitions of invasive fungal disease (de Pauw et al., 2008; Donnelly et al., 2020) and in the therapeutic guidelines of the ESCMID (Cuenca-Estrella et al., 2012). Its greatest limitation is the inability of the test to differentiate between different fungi species. Although BG is found in the cell wall of *Candida* spp., *Aspergillus* spp., *Pneumocystis jirovecii*, *Trichosporon* spp., *Fusarium* spp. and *Saccharomyces* spp., this component is not found in *Cryptococcus* spp. nor in the causative agents of mucormycosis. If the BG test is negative, the probability that the patient has an invasive fungal infection is quite low, so its negative predictive value is high. False positive results may occur associated with recent surgeries, hemodialysis, bacteremia, use of antibiotics such as amoxicillin-clavulanic acid, antifungals such as anidulafungin, and products such as immunoglobulins or albumin (Hanson et al., 2012; Kanamori et al., 2009; Marty et al., 2006; Mennink-Kersten et al., 2006, 2008; Mohr et al., 2011; Pickering et al., 2005).

D-arabinitol detection

D-arabinitol (DA) is a metabolite produced by the pathogenic *Candida* species, with the exception of *Candida krusei* and *Candida glabrata*, which is found in the serum of patients with invasive candidiasis, and makes it a diagnostic marker for this disease. Currently there are two techniques for its measurement: gas chromatography, which is very laborious and is not available in hospital laboratories, and the commercial enzymatic technique developed in Japan (Arabinitec-Auto, Marukin Diagnostics, Osaka, Japan), for serum samples and urine. Some studies have shown that DA can be detected earlier than *Candida* growth in blood cultures and that it is a good marker for monitoring clinical response to treatment (Hino et al., 2000; Yeo et al., 2006; Walsh et al., 1995).

Mannitol detection

It is an acyclic polyol produced in large quantities by different fungi, including *Aspergillus* spp. and *Cryptococcus* spp. Unfortunately, the available data do not support its usefulness as a diagnostic marker for invasive diseases produced by these two genera (Pfeiffer and Wong, 2015).

There are currently no metabolite screening tests for histoplasmosis, blastomycosis, coccidioidomycosis, and paracoccidioidomycosis.

Protein detection by MALDI-TOF MS

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a recently developed biophysical method in the field of clinical microbiology, which significantly reduces the time necessary for the identification of fungi in the routine diagnostic process of the laboratory, due to the speed with which this procedure is carried out (Freeman Weiss et al., 2021; Romanelli and Wickes, 2015).

MALDI-TOF MS identifies yeasts and filamentous fungi after their recovery from the culture of clinical samples on solid media in few minutes, through the unique protein mass spectrum of each species, comparing it with the reference spectra library that is linked to the measuring instrument (Becker et al., 2014; Freeman Weiss et al., 2021; Lau et al., 2013; Marklein et al., 2009; Rychert et al., 2017; Sanguinetti and Posteraro, 2016; Sendid et al., 2013). Obtaining fungal isolates from cultures is an important limitation to be taken into consideration for identification, since the ideal is to be able to identify fungi directly in the original clinical sample. Some research groups have developed methods for the direct identification of fungi with variable success rates (Becker et al., 2014; Ferreira et al., 2011; Ferroni et al., 2010; Rychert et al., 2017; Sanguinetti and Posteraro, 2016). A recent study conducted with yeast positive blood cultures reported that direct identification with MALDI-TOF using blood cultures yielded 95.9% correct identifications for *Candida albicans* and 86.5% for non-*Candida albicans* species, when comparing its diagnostic performance with the conventional culture-based method (Spanu et al., 2012). This suggests that direct identification of *Candida* spp. directly from blood cultures is feasible and could become a standard procedure, but large-scale evaluations are still needed to confirm this, as some yeast require additional processing and repeated analysis.

There are currently several mass spectrometric identification systems commercially available. The Bruker Daltonics system (Billerica, MA) includes a mass spectrometer along with software plus a database called "Biotyper". The Shimadzu Axima Assurance system (Columbia, MD) uses a mass spectrometer, the Launchpad software, and the AnagnosTec GmbH database (SARAMIS). The latter was recently acquired by the bioMérieux trademark and is currently called "VITEK MS" (Freeman Weiss et al., 2021; Romanelli and Wickes, 2015; Rychert et al., 2017).

MALDI-TOF depends on its reference spectra library, which is proprietary to the commercial system (there is no public spectra base), although users can add spectra to expand it, however, this is an important limitation, since it would take a long time to create

a spectra library similar to the molecular sequence databases that exist today (Freeman Weiss et al., 2021; Romanelli and Wickes, 2015). Other important limitations lie in the fact that some closely related fungi cannot be differentiated with these systems and that the morphological variety may require different sets of proteins, which will result in variable spectra for identification, which is a challenge for these systems. Furthermore, the cutoff points for identification may vary between fungi.

These systems are not yet approved by the FDA, limiting their widespread use in the United States. Although MALDI-TOF still needs improvement, it is a promising technology as it is an automated system that has high throughput and fast response time, much needed in the laboratory when it comes to fungal identification. In addition, it can be used for the identification of bacteria and mycobacteria, which makes it an instrument that can bring great benefits to the routine microbiology laboratory (Romanelli and Wickes, 2015).

Nucleic acid detection

Fungal DNA detection techniques using polymerase chain reaction (PCR) have great diagnostic sensitivity, being able to detect minimal concentrations of DNA in clinical samples, however, their major limitations are that they are highly developed and standardized in individual studies (in house), which makes their transfer to routine microbiology laboratories very difficult, and implies the need for validation in multicenter studies (Pemán et al., 2017; Quindós, 2015).

Nested PCR (nPCR), using the Hcp100 gene region as a target, is one of the most widely used techniques for the diagnosis of histoplasmosis, with sensitivity values between 70% and 100% and specificity between 90% and 100%, which depend on the analyzed clinical sample, where the most used are fresh biopsies, paraffin-embedded biopsies, respiratory samples, bone marrow, CSF, serum and whole blood (Bialek et al., 2002; García et al., 2017; Linder and Kauffman, 2021; Maubon et al., 2007; Muñoz et al., 2010; Toranzo et al., 2009). Because the most important disadvantage of this technique is the possibility of contamination by amplicons, it is necessary to use biological safety cabinets during the preparation of the reagent mixtures and the addition of the templates to be used during the first and second rounds of PCR, processes that make it difficult to transfer the technique to routine microbiology laboratories (García et al., 2017; Toranzo et al., 2009). However, because it reduces diagnosis time, it has become a complementary technique to direct examination, culture and serological tests, especially in patients with HIV and other immunodeficiencies (García et al., 2017; Simon et al., 2010).

Quantitative PCR (qPCR), using the ITS1 region of the rRNA as a target, is another of the most widely used techniques for the diagnosis of histoplasmosis, showing sensitivity values between 78% and 100% and specificity between 98% and 100%, in respiratory samples, biopsies, CSF, bone marrow, serum and whole blood. DNA quantification allows monitoring of clinical outcome, but it is a technique that requires expensive reagents and equipment, so it is not accessible to routine microbiology laboratories, especially in low-resource countries, where histoplasmosis is often an endemic disease (Buitrago et al., 2007; Linder and Kauffman, 2021; Simon et al., 2010). In a multicenter Latin American study, seven protocols for nPCR and qPCR were evaluated with different concentrations of *Histoplasma capsulatum* DNA, reporting global sensitivity and specificity values of 86% and 100% respectively, showing that the protocols and techniques were reproducible, sensitive and specific (Buitrago et al., 2013).

Molecular methods for the diagnosis of coccidioidomycosis are used for species identification and for diagnosis from clinical samples. PCR technique based on the amplification of antigen 2/proline-rich antigen has been used for the identification of *Coccidioides posadasii* (Bialek et al., 2004; Canteros et al., 2009) and also in clinical samples of patients with this disease (Canteros et al., 2010).

In recent years, alternative techniques to staining and immunofluorescence have been developed for the diagnosis of PCP, based on the detection of fungal DNA using nPCR and qPCR-RT. Direct immunofluorescence assay (DIF) is currently considered the preferred technique for the diagnosis of PCP due to its high sensitivity and specificity (Choe et al., 2014; Cruciani et al., 2002; Panizo et al., 2008). However, the PCR has proven to be more sensitive in detecting *Pneumocystis jirovecii* than the aforementioned stains. The differentiation between colonization and infection using this technique requires that its results be interpreted with caution, taking always into account the patient's symptomatology, since it can detect this fungus in asymptomatic patients (Casanova et al., 2006; Panizo et al., 2008, 2009, 2020; Tomás and Matos, 2018).

It has been demonstrated that nPCR is more sensitive for the detection of *Pneumocystis jirovecii* than stains and even than DIF. The primers described by Wakefield et al. were chosen because the mitochondrial large subunit rRNA (mtLSUrRNA) gene is the most specific and sensitive for the detection of this fungus, with a detection threshold that can reach values of 0.5–1 organism/μL of specimen. The mtLSU rRNA is a multicopy gene in the *Pneumocystis jirovecii* genome that contributes to the higher successful amplification rates, especially when used with nPCR. For this reason, this technique produced less false negative results and presented higher concordance with the results of conventional stains and DIF (Tomás and Matos, 2018; Wakefield et al., 1990, 1991).

Currently, the technique of greatest clinical applicability for the diagnosis of PCP is qPCR-RT in samples of induced sputum and BAL, since it allows distinguishing colonization from infection by establishing a cutoff value. In patients with HIV, the sensitivity and specificity values of this test exceed 95%, while in patients without HIV the sensitivity is slightly lower, but the negative predictive value is 98%, therefore, in this type of patients, a negative result with this technique rules out the presence of the disease (Azoulay et al., 2009; Huggett et al., 2008).

The detection of nucleic acids has been extensively investigated for invasive aspergillosis (IA), however, it has not been possible to clarify yet the type and volume of sample to use, the best DNA extraction method, the best target and the meaning of a positive result (Duval et al., 2008). The sensitivity and specificity values are highly variable, being between 88% and 75%, respectively (Mengoli et al., 2009).

The European *Aspergillus* PCR Initiative working group from the International Society for Human and Animal Mycology (ISHAM) has attempted to standardize the variables of the PCR technique and define a set of recommendations, rather than a standard PCR assay for the molecular diagnosis of AI. Among the standardized conditions, they suggest using serum samples and including internal controls (White et al., 2011a,b).

In-house techniques have great variability in their sensitivity and specificity values, making their comparison difficult due to the lack of standardization and validation, both for the methods and their results, therefore, their generalized use has a limited recommendation as an alternative for the diagnosis of fungal diseases (scientific evidence B-II and B-III) (Ayats et al., 2011). For these reasons, the use of qPCR assays, which are already commercialized, has been proposed, such as Film Array® (BioFire, United States), LightCycler® SeptiFast Test M GRADE (Roche Molecular Diagnostics, United States), Lumindex xTAG® Fungal ASR (Luminex Molecular Diagnostics, United States), MycAssay® *Aspergillus* and MycAssay® *Pneumocystis* (Myconostica, Great Britain), Magi-plex™ Sepsis (Seegene, South Korea), Sepsis-Test™ (Molzym GmbH & Co., Germany) and VYOO®/LOOXSTER® (SIRS-Lab, Germany).

MycAssay® *Aspergillus* has been evaluated in patients hospitalized in intensive care units and in patients with high-risk hematological diseases. In the first group of patients, the sensitivity values were between 86.7% and 100%, especially when using multiple samples, while the specificity values were between 87.6% and 92.9% (Torelli et al., 2011; Guinea et al., 2013). For the second group of patients, the sensitivity values ranged between 50% and 80% and the specificity values between 90.5% and 100% (White et al., 2011a,b). The results published with the use of MycAssay® *Pneumocystis* have been encouraging using BAL samples, reaching sensitivity and specificity values of 100% (McTaggart et al., 2012).

Blood fungal DNA detection techniques such as LightCycler® SeptiFast Test M GRADE, SepsisTest™ and Film Array® detect common pathogens such as *Candida* spp. and *Aspergillus fumigatus*, however, according to the few published studies, although preliminary results are promising, its usefulness in the diagnosis of invasive fungal diseases has not been determined (Pernán et al., 2017). According to research reports carried out in febrile neutropenic patients, the LightCycler® SeptiFast Test M GRADE showed limited sensitivity and specificity values (Lamoth et al., 2010; von Lilienfeld-Toal et al., 2009), therefore, its use is still not highly recommended for the diagnosis of these diseases.

For the diagnosis of invasive candidiasis, the FDA approved T2Candida panel appears to show promise in the detection of *Candida* spp. in whole blood and has already been evaluated in a clinical trial (Beyda et al., 2013), including as mycological evidence to support the diagnosis of probable candidemia (Clancy and Nguyen, 2018; Donnelly et al., 2020). The test has a high negative predictive value, but its positive predictive value varies depending on the prevalence of the disease in specific patient groups (White and Price, 2021; Clancy et al., 2018; Mylonakis et al., 2015). Fungal DNA amplification in tissue combined with sequencing, when yeasts have been observed in formalin-fixed and paraffin-embedded tissue biopsies are the recommended complementary techniques for the diagnosis of proven invasive candidiasis (Donnelly et al., 2020).

Fluorescence in situ hybridization using peptide nucleic acid probes (PNA-FISH) (AdvanDX, Woburn, MA) has been used in some clinical microbiology laboratories to discriminate *Candida albicans* from non-*Candida albicans* directly from positive blood cultures (Pulcrano et al., 2013; Stone et al., 2013). The assay uses PNA probes to target specific rRNA sequences in a highly sensitive and specific FISH assay (Rigby et al., 2002). The probe is capable of discriminating between *Candida albicans* and *Candida dubliniensis*, which is impossible to do at the phenotypic level (Lau et al., 2009).

There are variations of conventional PCR techniques that have been shown to be useful for mycological diagnosis. One of them is nucleic acid sequence-based amplification (NASBA). This isothermal amplification technique has numerous advantages, since it amplifies RNA templates that are abundant for some targets (for example rRNA). A commercial version of this technology has been available for several years (AccuProbe, Hologic Gen-Probe Inc., San Diego, CA) and has been used to detect *Histoplasma capsulatum*, *Coccidioides immitis*, and *Blastomyces dermatitidis* (Chemaly et al., 2001; Romanelli and Wickes, 2015; Scalarone et al., 1992; Valesco and Johnston, 1997).

A second type of isothermal PCR is called loop-mediated isothermal amplification LAMP (Notomi et al., 2000). The key component of this reaction is a DNA polymerase derived from *Bacillus stearothermophilus* (Njiru, 2012). LAMP has been used successfully to detect several fungi, including *Fusarium* spp., *Cryptococcus* spp. and *Aspergillus* spp. (Denschlag et al., 2012; Lucas et al., 2010; Niessen, 2013; Sun et al., 2010). Rolling circle amplification (RCA) is a third type of isothermal amplification that results in massive amplification of target sequences (Fakruddin et al., 2013). This technology uses DNA polymerase from the bacteriophage Phi29 to amplify a DNA template. It is a sensitive technique to identify fungal genera that are difficult to differentiate at the species level, even with molecular methods, due to the highly conserved target sites (Sun and de Hoog, 2013). RCA has been used to detect a variety of fungi, including *Penicillium marneffei*, *Fonsecaea* spp. and *Exophiala* spp. (Najafzadeh et al., 2011, 2013; Sun et al., 2011).

In addition to these technologies, there are other PCR-based methods that have various applications in mycological diagnosis and epidemiology. One of them is random amplification of polymorphic DNA (RAPD). Genetically unrelated strains will have different RAPD patterns, depending on how quickly their genomes evolved from each other during geographic isolation and the frequency of the primer site in the genome. This method has been extensively studied for *Cryptococcus neoformans* and has been developed as a strain typing tool (Meyer et al., 1999). It has also been used for the identification of *Aspergillus fumigatus* and *Candida albicans* strains. The technique is not routinely used as an identification tool in microbiology laboratories because it is highly susceptible to variations; however, it is very useful for investigating outbreaks.

The PCR technique with Restriction Fragment Length Polymorphism (RFLP) is based on the use of restriction enzyme digestion of gene amplification products or polymorphic DNA sequences. It allows the study of a very limited region of the genome (specific genes that code for special proteins) and accurately identifies microorganisms. Its power of discrimination and reproducibility may

be somewhat lower than that of other identification methods, since this technique depends on factors such as the fungus species, the analyzed gene and the restriction enzyme to be used, but the power of discrimination increases using various restriction enzymes. This technique is quick, easy, and the restriction patterns are highly reproducible, and have been used for the identification of *Cryptococcus* spp., *Fonsecaea* spp. and *Candida* spp. (Bonifaz, 2012; Escandón et al., 2006; Ferrara et al., 2018; Moreno et al., 2017; Sidrim et al., 2010).

The sequencing of genetic material is the most accurate tool to establish the identification of any fungus. Easy access to public databases such as GenBank and search algorithms such as BLAST programs (Altschul et al., 1990), allow users to query an unknown sequence and obtain an identification result by comparison and analysis of the sequences.

Identifying fungi based on their sequence is expensive and requires specialized personnel and equipment. All this means that these types of studies are not easily available for most routine microbiology laboratories and are only accessible in research centers. One of the great challenges of these diagnoses has been how to better standardize the methods used to get identification and adjust these guidelines to a clinical laboratory daily operation. Currently, the most useful guidelines are provided by the Clinical Laboratory Standards Institute, which describe in detail sequence-based identification of fungi and the targets that can be used (Petti et al., 2008).

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Laboratory Identification of Prion Infections

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Introduction to transmissible spongiform encephalopathies

Prions are atypical infectious agents that have puzzled researchers dealing with transmissible spongiform encephalopathies (TSE) since their discovery, primarily due to the absence of nucleic acids in their composition. TSE or prionopathies are a group of rapidly progressing, invariably fatal neurodegenerative diseases which affect several mammals including humans (Prusiner, 1984). Although scrapie, a sheep prionopathy, has been documented since the 18th century (Liberski, 2012) and another TSE affecting humans, Creutzfeldt-Jakob disease (CJD), was described in 1920 (Creutzfeldt, 1920), the nature of the causal agent of such diseases was not identified until 1982. On this year Stanley Prusiner proposed the “protein-only” hypothesis (Prusiner, 1982) which, in the year 1997, granted him the Nobel Prize in Physiology and Medicine. This revolutionary theory proposed that TSE were caused by an infectious agent completely devoid of nucleic acids and exclusively composed by a protein which he named prion (proteinaceous infectious particle). Taking into account that all other pathogens known in 1982 based their existence on nucleic acids, the “protein-only” hypothesis was highly controversial. However, since Prusiner’s theory was first published, a growing body of evidence has been gathered supporting the existence of an exclusively proteic novel infectious pathogen. Nowadays, the fact that TSE are caused by prions is widely accepted (Soto, 2011).

The prion protein (PrP) is the major, if not the only, component of prions. As per the “protein-only” hypothesis, prions are constituted by an aberrantly folded isoform (PrP^{Sc}, from scrapie) of the natively folded or cellular prion protein (PrP^C) (Stahl and Prusiner, 1991). PrP^C is a relatively small glycoprotein of unknown function, expressed most abundantly in the outer membranes of cells from the central nervous system (CNS) and lymphoreticular system. It is bound to the membrane through a glycosylphosphatidylinositol (GPI) anchor (Stahl et al., 1991). PrP^C is a highly conserved protein in mammals, comprised of two regions: an unstructured and flexible N-terminal tail, and a globular C-terminal domain which hosts three α -helices and a short, two-strand β -pleated sheet (Lysek et al., 2005). Upon a thus far poorly characterized misfolding event, it gives rise to the PrP^{Sc}, acquiring pathogenic characteristics. In contrast to PrP^C, prions are aggregation-prone, water insoluble proteins, partially resistant to degradation or physicochemical treatments. This, in combination with their ability to impose their aberrant conformation onto their natively folded counterparts, leads to the formation of amyloid aggregates or deposits (Telling et al., 1996). Moreover, this self-replicative protein also shows neurotoxic properties that lead to neuronal death and the rapidly evolving neurodegeneration that characterizes prionopathies (Brandner et al., 1996).

Despite the apparent simplicity of the causal agent of prion diseases compared to other infectious pathogens, their etiology and the clinical manifestations of TSE are highly heterogeneous. Prionopathies can be acquired through ingestion or introduction, by iatrogenic means, of exogenous PrP^{Sc}. This causes what is known as the acquired form of the disease. The exogenous PrP^{Sc} then propagates inducing its conformation onto the endogenous PrP^C. Alternatively, familial or hereditary forms of TSE develop due to the presence of mutations in the gene encoding the PrP^C protein (PRNP), which increase the protein’s misfolding proneness into PrP^{Sc} (Prusiner, 1993). Once generated, this PrP^{Sc} will initiate an autopropagative process which recalls the one occurring in exogenously acquired TSE, leading to the development of a genetic prionopathy. Moreover, prion diseases can also arise sporadically, purportedly due to a low-frequency event of spontaneous misfolding of the wild type PrP^C. This latter type is in fact the most common form of prion disease in humans, accounting for 85–90% of the cases. In contrast, genetic forms make up for around 15–10% of cases and acquired forms have almost been eradicated in humans (WHO, 2003).

Despite being out of the scope of this article, it is worth mentioning the most common animal prion disorders. When it comes to detecting prions in animals, simplicity and cost-effectiveness of the methods applied are key, however, some of these relatively simple methods have been further developed and refined for human prion disease detection in clinical settings. Therefore, despite mainly referring to human prion detection methods, we will also include a few comments on techniques used for animal prion detection. Scrapie in sheep and goat, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in

deer, are nowadays the only naturally occurring prion diseases in animals (Imran and Mahmood, 2011). Nonetheless, due to their potential impact in public health and in the ranching industry, as sadly proven by the mad cow disease crisis caused by the zoonotic capacity of BSE-causing prions (Knight, 1999), most developed countries have implemented surveillance programs to systematically detect cases and prevent outbreaks (Bonetta, 2000; Bird, 2003). Other prion diseases in animals have been reported such as transmissible mink encephalopathy (TME), feline spongiform encephalopathy (FSE) and exotic ungulate spongiform encephalopathy (EUE), although in these cases BSE or scrapie prion ingestion is thought to be the cause of the disease (Imran and Mahmood, 2011). Finally, susceptibility of many other mammalian species to prion infection has been demonstrated in laboratory animals. Mainly in non-human primates and in rodents, derived from human prion or other naturally occurring animal prion inoculation (Brandner and Jaunmuktane, 2017).

This review will center on human TSE clinical and histopathological diagnosis, as well as on other methods developed for prion detection in tissues and body fluids or disease-associated biomarker screening. For that purpose, it is first important to get some insight into the different human prion diseases and some specific features of these proteinaceous pathogens, such as their interspecific transmission ability and the strain phenomenon. Several prion diseases have been described in humans, each distinguished by distinct clinical manifestations and neuropathological hallmarks, which are due to differential biochemical features of the misfolded prion protein causing each disease. This variability is due to the unprecedented capacity of prions to, despite sharing the same protein sequence, adopt slightly different conformations known as prion strains (Aguzzi, 1998). Reminiscent of viral strains, prion strains produce distinguishable disease phenotypes with differing disease onset, duration and clinical signs. Each strain shows also defined biochemical and biological characteristics, varying on the relative susceptibility of the misfolded protein to proteinase digestion, the biochemical signature after digestion, the ability to propagate preferentially in distinct brain regions, the morphology of protein deposits, the extent of neuropathological lesions, or the host range it can infect, which depends on the conformation of the infecting prion strain and its capacity to misfold the PrP^C from the new host (Morales et al., 2007). This latter property, the interspecies transmission ability of prions is, accordingly, limited by the prion strain of the donor and the host's PrP^C amino acid sequence, and results in a transmission barrier that restricts the potential transmissibility to different mammals. However, the barrier can be overcome and prions can adapt to the new host. Despite keeping in some cases features of the original strain, after adaptation to a new host, prions can suffer alterations that provide new biological and biochemical features in the new species (Igel-Egalon et al., 2018). Although this phenomenon is not particularly relevant to this manuscript, it is critical in terms of public health, because the predictability of potential transmission barriers could help prevent new outbreaks in animals and potential zoonoses. On another note, the existence of prion strains as conformational variants or even as complex mixtures of a set of slightly different conformers (Li et al., 2010; Collinge, 2010), is crucial for the diagnosis and detection of prion infections since strain properties determine disease phenotypes, thus, affecting diagnosis based on clinical signs, neuroimaging techniques, surrogate biomarkers or through direct detection of PrP^{Sc} in different brain regions and in tissues or fluids. Moreover, the conformational differences also have an impact on the biochemical features of each strain. For instance it can be difficult immunodetection, as anti-PrP antibodies can specifically recognize some strains while being unable to detect others due to differential proteolytic digestion (Pan et al., 2004). Furthermore, the capacity to detect them through *in vitro* prion propagation can also be affected by the propagation kinetics of strains as some seem to propagate more efficiently than others *in vitro* (Levavasseur et al., 2019).

PrP^C is most abundantly expressed in cells from the nervous system and lymphoreticular system and the toxicity of PrP^{Sc}, although mostly unknown, is mediated by PrP^C on the surface of neurons. For these reasons, the pathology is mainly restricted to the CNS. However, PrP^C is also expressed in other cells and tissues albeit at lower levels (Zomosa-Signoret et al., 2008; Peralta and Eyestone, 2009), and prion propagation in secondary lymphoid organs has also been observed (Mabbott, 2012), meaning that at least some strains have mechanisms for spreading outside of the CNS and are capable of cell-to-cell transmission (Prinz et al., 2002). Thus, the formation or acquisition of minute amounts of PrP^{Sc}, referred as seed, is the initial event of TSE pathogenesis. This seminal event occurs long before the manifestation of the clinical signs of disease, and prions then spread and propagate exponentially in the lymphoreticular system and the nervous system (Gough and Maddison, 2010). In sporadic or hereditary prion diseases, the location of the initial misfolding event is unknown, while in acquired prion diseases ingestion of exogenous PrP^{Sc} is the most common route of infection, after which prions are uptaken by cells from Peyer's patches (Bergstrom et al., 2006). In any case, prions need to reach the CNS from the initial formation or entry site, through cell-to-cell transmission and propagation in peripheral organs such as the secondary lymphoid organs. Those strains for which these processes are more efficient, and are therefore able to reach nervous tissue from peripheral infection sites, are considered to have capacity of neuroinvasion (Nuvolone et al., 2009). In fact, this property increases the transmissibility of certain strains, since poorly neuroinvasive strains would not be able to cause acquired disease (Bett et al., 2012). Conversely, prion strains may also leak from the CNS once the infection is established, causing seeds to reach peripheral tissues and body fluids and in some cases, as for CWD prions of cervids, leading to excretion to the environment (Bosque and Tyler, 2008). In these cases, due to the extraordinary resistance of prions to inactivation by traditional pathogen decontamination procedures or physicochemical treatments, prions can remain in the environment for long periods of time and, initiate infection in a new host upon ingestion (Miller et al., 2004). The difficulties for eliminating or inactivating prions also have important consequences for human prion disease transmission, since iatrogenic cases due to interventions requiring nervous tissue grafting, inadequate decontamination of neurosurgical instruments or injection of cadaveric human growth hormone have been reported (Barrenetxea, 2012).

Bearing all this in mind, early diagnosis of prion diseases, as well as detection of these proteinaceous pathogens in tissues, body fluids and in the environment are critical not only for patients and their relatives, but also to avoid outbreaks which could be

catastrophic for public health and safety due to the potential zoonotic ability of emergent prion strains. Finally, it is also worth noting that other more prevalent protein misfolding-related neurodegenerative disorders in humans, such as synucleinopathies, Alzheimer's disease and other tauopathies, appear to share some molecular mechanisms with prion diseases (Erana et al., 2017). Thus, strategies developed for detection of misfolded proteins or amyloids, could be adapted to the detection of other disease-associated aberrantly folded proteins. In this review, the main methods used in TSE diagnosis and for prion detection will be described with a focus on human prion detection. Common animal TSE detection techniques will also be mentioned as well as the transversal potential of some TSE detection techniques toward other more prevalent neurodegenerative disorders.

Detection of prion infection based on clinical signs and symptoms

Due to their long silent incubation period, the first evidence of prion infections is usually the manifestation of clinical signs and symptoms, which mark the onset of clinical disease. This clinical stage, characterized by a rapidly progressing cognitive and motor decline, invariably leads to the patient's death. As a group of diseases affecting different mammals and given the distinct biochemical and biological properties shown by different strains, TSE present considerably heterogeneous clinical manifestations.

Behavioral changes, ataxia, incoordination, weight loss and overall poor body condition are usual signs found in animals, but many other specific signs for each TSE phenotype have been reported, underlining pruritus in scrapie-affected sheep, poly-uria and polydipsia in CWD-affected deer or gait-abnormalities, aggressive behavior and hyperreactivity to stimuli in BSE-affected cattle among others (Imran and Mahmood, 2011). Human prion diseases, although better clinically characterized, are difficult to diagnose due to their heterogeneity, even among patients from the same family with the same TSE (Chapman et al., 1993; Wadsworth et al., 2006), and also due to the shared clinical signs with other more prevalent neurodegenerative disorders (Geschwind and Murray, 2018). The most common prion disease in humans is the sporadic form of Creutzfeldt-Jakob disease (sCJD), which accounts for around 85% of TSE cases diagnosed in humans (Ladogana et al., 2005). However, several subtypes can be distinguished depending on the biochemical signature of the accumulated PrP^{Sc} in the brain of the patients (type 1 or type 2) and amino acid at position 129 of the host's PrP^C, that can be either a methionine or a valine (MM, MV or VV) and has been shown to modulate disease progression and susceptibility against some prion strains (Parchi and Saverioni, 2012). In agreement with this classification by Parchi and colleagues, the following sCJD subtypes, likely caused by distinct strains, have been described:

- sCJD MM1/VM1, is the most common form by far and it is characterized by early and prominent myoclonus, gait or limb ataxia, myoclonic jerks, visual disturbances (most common in Heidenhain's variant) and an average disease duration of 4 months.
- sCJD VV2, accounting for around 16% of sCJD cases and affecting those homozygous for valine at codon 129, characterized by myoclonus and pyramidal signs, cognitive impairment, late dementia and a little longer disease duration, ranging from 3 to 18 months, although purely ataxic variants at disease onset have been reported, known as the Brownell-Oppenheimer variant.
- sCJD MV2 subtype is the next more prevalent encompassing 10% of the cases and affecting heterozygous individuals, is characterized by ataxia and progressive dementia and the longest average disease duration of about 17 months.
- MM2 is the next most common, affecting homozygous individuals for methionine at codon 129 and manifesting normally with cognitive impairment, aphasia, myoclonus and pyramidal signs, as well as myoclonic jerks.
- VV1, the least prevalent accounting for around 1% of sCJD cases that are normally characterized by the relative youth of the patients at disease onset, personality changes as the initial manifestation and slowly progressive dementia and focal neurologic deficits.

Genetic or hereditary prion diseases are caused by autosomal dominant inheritance of a mutated allele of the PrP encoding gene (*PRNP*). It's penetrance is generally considered high although it may be variable (Minikel et al., 2016). Genetic TSE are classified based on the different disease-associated mutations and the clinical manifestations produced by the misfolded form of PrP^{Sc}, formed due to an increased misfolding proneness of the mutated PrP (Wadsworth et al., 2003). Therefore, upon manifestation of clinical signs compatible with a TSE, genotyping of *PRNP* can confirm the diagnosis in case of familial cases of disease if pathology-associated mutations are found. Among genetic prion diseases, mutations causing phenotypes reminiscent of sporadic CJD are classified as genetic CJD (gCJD), the most common being the E200K mutation. Despite usually showing longer clinical disease duration than sCJD cases, the most frequent clinical signs are indistinguishable from those observed in sCJD and include ataxia, myoclonus, dementia and pyramidal signs, although as in sCJD, high clinical heterogeneity has been reported even between individuals with the same genotype (Ladogana and Kovacs, 2018). Thus, genetic testing is necessary to discriminate between gCJD and sCJD (Schwartz et al., 2019). Besides gCJD, two other genetic prion diseases in humans have been described, Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI), which are caused by different mutations in the *PRNP* gene than those already described for gCJD (Wadsworth et al., 2003). GSS was the first genetic prion disease reported, with the most prevalent mutation being P102L (Hsiao et al., 1989). GSS accounts for the most diverse clinical spectrum among prion diseases, usually characterized by prominent cerebellar ataxia accompanied by gradually progressive cognitive decline, although it can also manifest as an isolated cognitive impairment similar to that of Alzheimer's disease. Normally, GSS cases have a slower progress than other TSE, with the longest duration of the clinical phase among human prion disorders and, as will be detailed later, the biochemical signature of the misfolded PrP found in the brains of these patients is also unique among genetic TSE (Liberski and Budka, 2004). The third type of genetic prion disease, FFI is caused by the mutation D178N coupled to methionine at codon 129 of

the mutated allele, whereas the same mutation together with valine at codon 129 results in a form of gCJD, due to the phenotypic manifestation of the disease in each case (Gambetti et al., 2003). Although typical signs of other TSE such as ataxia, myoclonus, gaze abnormalities or cognitive deficits can also be detected in cases of FFI, they are mainly characterized by sleep disturbances due to thalamic deterioration, and early signs of disease include autonomic disturbances such as tachycardia, hyperhidrosis or hypertension (Cracco et al., 2018). Apart from sCJD, two extremely rare sporadic forms of prion disease in humans are also worth mentioning. With an unknown origin and believed to originate due to spontaneous misfolding of the PrP^C in absence of mutations, only a few cases of sporadic fatal insomnia (sFI) and variably protease-sensitive prionopathy (VPSPr) have been described worldwide. The first is indistinguishable from FFI, except for the absence of D178N mutation (Cracco et al., 2018), while VPSPr is phenotypically similar to GSS (Zou et al., 2010).

Finally, despite being practically inexistent nowadays, the clinical features of acquired prion disease in humans will be listed. Variant CJD (vCJD), which arises from ingestion of BSE prions, is infamous for being the only prion zoonosis reported to date (Seed et al., 2018). Around 230 cases have been reported worldwide, the majority of them (~75%) in the United Kingdom, with the last confirmed case published in 2016 (Mok et al., 2017). Barring this last case, which is at present the only clinical case observed in a heterozygous individual for codon 129, all other cases were homozygous for methionine. Among the characteristic clinical features, early psychiatric or sensory symptoms with a delayed onset of neurological abnormalities can be highlighted, with ataxia developing usually later than in other human TSE, and dementia and myoclonus arising subsequently during disease progression. In addition, the affected individuals are in average younger at disease onset than in most TSE, a feature shared with other acquired prion diseases, in which the age at onset does not depend on unknown intrinsic factors but on the age at infection (Brandel and Knight, 2018). The other notorious acquired human TSE is kuru, an already eradicated disease that affected the *fore*, an ethnic group of Papua New Guinea, among which the disease was transmitted due to endocannibalistic rituals. Characterized by cerebellar ataxia, gradual loss of coordination, behavioral changes such as uncontrolled laughter outbursts, and a major prevalence among women and children, its incidence rapidly decayed after the end of cannibalistic rituals (Liberski et al., 2012). Finally, iatrogenic cases of TSEs need to be mentioned, caused by the transmission of sCJD prions via medical procedures such as nervous tissue grafting, neurosurgery with contaminated instruments or injection of cadaveric human growth hormone extracted from pituitary glands of affected individuals (Bonda et al., 2016). Clinical signs in these cases depend on the specific subtype of sCJD from the donor and the genotype of the acceptor and, despite potential variations due to the different infection routes, are overall the same as those reported for sCJD subtypes (Xiao et al., 2014).

In light of the above, it is clear that the clinical heterogeneity of human TSE, together with the similarity of the neurological signs compared with those of other much more prevalent neurodegenerative disorders, prevents definitive diagnosis based on clinical signs. Except for the genetic cases that exhibit the mentioned pathology-associated mutations, for which clinical signs together with genotyping of *PRNP* allows accurate pre-mortem diagnosis, complementary tests are necessary to diagnose the rest of TSE. In fact, definitive diagnosis of TSE was until recently only possible by *post-mortem* histopathological examination of the brain. This is still considered the gold standard, despite recent advances in other prion detection and TSE diagnostic methods (Connor et al., 2019).

Detection of prion infection through electroencephalography and diagnostic imaging

When a TSE is suspected due to clinical signs, several diagnostic imaging techniques as well as electroencephalography (EEG) are used as complementary tests. As early as 1954 (Jones and Nevin, 1954) EEG was reported as an aid to the diagnosis of CJD, and in 1979 a characteristic EEG profile was included among the diagnostic criteria for possible or probable CJD (Masters et al., 1979). Periodic sharp-wave complexes (PSWC) are the most characteristic abnormality in CJD patients, with some studies assigning them specificities ranging from 74% to 86%, sensitivity of 67%, positive predictive value of up to 93% and excellent inter-observer reliability (Steinhoff et al., 2004; Zerr et al., 2000a). However, further retrospective studies showed differences between subtypes, with PSWC being predominant and common for MM1 and MV1 subtypes of sCJD, but not for valine homozygous patients (Zerr et al., 2000b) or vCJD patients (Will et al., 2000). Therefore, although EEG is a relatively inexpensive and sensitive method to assist in the diagnosis of sCJD, it should be interpreted with caution, since it can be normal or show nonspecific abnormalities in the early stages of the disease or in the case of specific disease subtypes (Mundlamurri et al., 2020).

Among bioimaging diagnostic techniques, the use of computerized tomography (CT) was explored in the 80s, but it was ultimately deemed unsuitable since 80% of the patients analyzed had normal scans (Gálvez and Cartier, 1984). Thus, magnetic resonance imaging (MRI) methods, photon emission computed tomography (SPECT) and positron emission tomography (PET) are more commonly applied to TSE diagnosis. Initial studies investigating the use of MRI mostly in sCJD for its considerably higher prevalence compared to the rest of TSE, were performed by analyzing T2 images in a small number of subjects, revealing bilaterally symmetric diffuse hyperintense abnormalities in the basal ganglia on T2-weighted images as a specific sign of sCJD (Barboriak et al., 1994). Additionally, a study with a larger patient cohort showed a 67% sensitivity and 93% specificity, demonstrating the utility of this technique as a detection aid for prion infections (Schroter et al., 2000). In time, diffusion weighted-imaging (DWI) and fluid-attenuated inversion-recovery (FLAIR) were also incorporated into diagnostic practice for the detection of abnormalities related to prion diseases and were even included in the guidelines for sCJD diagnostic from the Centers for Disease Control and Prevention (CDC) in 2010. The most common MRI findings in sCJD patients which can precede the onset of clinical disease, include focal or diffuse, symmetric or asymmetric hyperintensity at the cerebral cortex or basal ganglia (in particular the corpus striatum), or in both regions, with the perirolandic area being usually spared. Cerebellar atrophy is also perceptible and there are

some reports of DWI signal hyperintensity in this region as well, despite it being typically negative when analyzed by imaging. As the disease advances, the extent and degree of signal in subcortical gray matter regions increases in correlation with disease severity (Fragoso et al., 2017). Nonetheless, distinct types and subtypes are characterized by specific MRI findings: MM1/MV1 sCJD subtype show the described findings with frequent basal and cortical involvement while the thalamus is spared; VV1 patients in contrast show prominent cortical involvement with basal ganglia and thalamus spared; and MV2/VV2 types are characterized by predominant involvement of basal ganglia and thalamus with limited cortical affection (Parchi et al., 1999b). Familial CJD cases usually show similar findings to those of sCJD MM1/MV1 types, while for GSS MRI scans are usually normal or may demonstrate diffuse or cerebellum-specific atrophy. In the case of FFI or sFL, conventional MRI tests are usually negative or normal, in line with the limited brain lesions shown by patients at the histopathological level, which makes these patients the most challenging to diagnose based on imaging (Haik et al., 2008). However, few reports with abnormal subcortical patterns or with cortical hyperintensity (in limbic and occipital regions) (Ortega-Cubero et al., 2013), with diffuse abnormal signals in the white matter, basal ganglia and thalamus (Lu et al., 2017), or in which an apparently normal MRI shows an increased apparent diffusion coefficient (ADC) when compared to controls (Haik et al., 2008) have been also published. Determination of the ADC has also yielded promising results for TSE characterized by cerebellar syndrome, which are not detected at nonquantitative DWI (Cohen et al., 2009). Regarding sensitivity, DWI is reported to be better than FLAIR or T2 imaging at least for the detection of cortical abnormalities in sCJD (Shiga et al., 2004; Tschampa et al., 2005), although in the case of vCJD, FLAIR was much more sensitive for the detection of symmetrical hyperintensity in the pulvinar nuclei of the thalamus (pulvinar sign) and the hockey stick sign that characterizes this acquired form of the disease (Collie et al., 2003).

MR spectroscopy has also been applied to the diagnostic of TSE, showing decreased *N*-acetylaspartate:creatinine ratio (NAA:Cr), a marker of neuronal stress and death in sCJD and vCJD patients (Pandya et al., 2003). Increased myoinositol, a marker of astrocytic gliosis, is also detectable in sCJD, vCJD, iatrogenic CJD, genetic CJD and GSS and FFI cases (Galanaud et al., 2010). Moreover, in the case of sCJD, the NAA:Cr ratio has been shown to correlate with disease duration, which makes it a useful parameter for prediction of the clinical course at the early stages (Kim et al., 2011).

Finally, tomographic or functional imaging techniques, such as photon emission computed tomography (SPECT) and positron emission tomography (PET) have also proven their utility for TSE diagnosis. PET with fluorodeoxyglucose ($[^{18}\text{F}]$; FDG-PET) has revealed glucose hypometabolism mainly in cortices of sCJD patients, although alterations in subcortical regions in more advanced stages of the disease have also been reported, even in patients presenting negative CT or MRI scans (Friedland et al., 1984; Ogawa et al., 1995; Henkel et al., 2002; Prieto et al., 2015). On the contrary, the few FFI cases analyzed only presented a slight hypometabolism in the thalamus (Prieto et al., 2015). Comparative studies with previously discussed methods such as EEG and DWI show that FDG-PET can improve diagnostic accuracy when used in combination with other imaging techniques, generally allowing earlier detection and presenting higher sensitivity than DWI (Xing et al., 2012). Changes in regional blood flow in the brain with SPECT using technetium-99m hexamethylpropyleneamine oxime (TC-99m HMPAO) have been reported for some sCJD cases, showing good correlation with findings from other bioimaging techniques or even being detectable before CT or MR abnormalities (Cohen et al., 1989; Kao et al., 1993; Miller et al., 1998). Similar findings have also been reported with another SPECT tracer, *N*-isopropyl-*p*-[123I] iodoamphetamine (123I-IMP), which is considered better than TC-99m HMPAO for the determination of absolute values of regional cerebral blood flow (Matsuda et al., 2001). In any case, SPECT has shown to be a sensitive and useful technique to detect CJD in its early stages and particularly in those cases in which MR is contraindicated (Zhang et al., 2011).

In conclusion, bioimaging techniques are very useful tools for TSE diagnosis, specially when accompanied by TSE's characteristic clinical signs. However, some of the bioimaging techniques are only informative at late disease stages and can have quite low sensitivity or even be unspecific, as similar results can be found for other neurodegenerative disorders. Hence, despite their usefulness, they are normally insufficient to provide a definitive diagnosis of suspected cases, requiring further tests.

Detection of prion infection through measurement of surrogate biomarkers in body fluids

Surrogate biomarkers are indirect indicators of neuronal damage in body fluids that are used to detect prion infection. In this case, instead of looking for macroscopic abnormalities in the brain, as was done with the neuroimaging techniques, molecular biomarkers are sought in body fluids, and normally detected through immunoabsorption or immunoblotting assays. Several proteins, whose levels may be altered due to the neurodegenerative process and which are detectable in cerebrospinal fluid (CSF) or blood, have been proposed as surrogate biomarkers for prion diseases over the years.

The first protein identified as an aid for the diagnosis of CJD in CSF was protein 14-3-3 (Harrington et al., 1986), which is still routinely used for diagnosis of suspected prion disease cases. However, researchers realized early on that it was also increased in patients suffering from other dementias (Hsich et al., 1996; Zerr et al., 2000a). Therefore, increased levels of 14-3-3 in CSF strongly support CJD diagnosis in patients with dementia, but the diagnosis is heavily dependent on the clinical context and additional tests may be required to confirm it, as patients suffering from other neurological diseases with similar clinical manifestations can also present elevated levels of 14-3-3 (Zerr et al., 2000a; Van Everbroeck et al., 2004). Moreover, increased levels of 14-3-3 protein do not occur in all prion disease subtypes. For instance, MV2 and MM2 sCJD subtypes have been reported to show lower sensitivity to this biomarker than other sCJD types, and therefore, its use in combination with other biomarkers has been suggested to improve sensitivity and specificity, increasing the diagnostic accuracy (Sanchez-Juan et al., 2006). Since its description and incorporation into

clinical practice, multicentric and longitudinal studies have been carried out to accurately evaluate its specificity as well as its reproducibility, drawing attention to issues that need to be taken into account. One of these studies highlights the necessity of ring trials or interlaboratory tests to ensure the quality of the analysis and standardize procedures, since different factors such as contamination of CSF with blood can result in false positive signals (Schmitz et al., 2016a). Moreover, specificity strongly depends on the control groups used in different studies, being reduced when patients suffering other neurological pathologies such as Alzheimer's disease (AD), dementia with Lewy bodies (DLB), and vascular dementia (VD) (Van Everbroeck et al., 2004) or acute neuronal injury events and inflammatory and infiltrative neoplastic CNS diseases are included (Stoeck et al., 2012), as all these also show elevated levels of 14-3-3. Thus, even though this biomarker shows overall high sensitivity and is commonly used, being the only surrogate CSF biomarker in current diagnostic criteria for probable CJD, its specificity remains low. For this reason, combination with other assays or biomarkers and even determining relative levels of different 14-3-3 isoforms have all been suggested to improve its diagnostic value (Abu-Rumeileh et al., 2019). It should be noted that all the above mentioned shortcomings could also apply to other surrogate biomarkers in CSF.

Neuron specific enolase (NSE) and S100b proteins were first identified as biomarkers for nervous system damage. Notably, they were also able to indicate whether the damage was neuronal, glial or of mixed origin as well as the extent of the damage (Mokuno et al., 1983; Persson et al., 1987). Both proteins can be found in serum as well as in CSF, allowing for less invasive detection of neurological damage (Persson et al., 1987; Ingebrigtsen et al., 1995; Zerr et al., 1995). Based on these reports they were readily investigated for their applicability to prion disease diagnosis both in CSF (Otto et al., 1997a; Wakayama et al., 1987) and in serum (Otto et al., 1998; Zerr et al., 1995). A comparative study of these two biomarkers with respect to 14-3-3 in CSF of suspected CJD cases showed slightly higher sensitivity for S100b than 14-3-3, which was better than NSE, while in terms of specificity 14-3-3 was superior to the others (Beaudry et al., 1999). Further studies with larger cohorts and including different prion disorders showed similar results for CJD with 14-3-3 showing the highest sensitivity, but also revealed that none of the biomarkers were elevated in GSS or FFI patients. There was however one exception, as S100b was increased in the single GSS case included in the study. When analyzing different sCJD subtypes, sensitivity was again worse for MM2 and MV2 subtypes, making these biomarkers comparable to 14-3-3 in terms of usefulness (Sanchez-Juan et al., 2006). Further comparative studies found strong evidence for elevated concentrations of 14-3-3, S100b and NSE in CSF of sCJD and genetic CJD patients but not in FFI or GSS patients (Ladogana et al., 2009), while detection of different sCJD subtypes showed lower sensitivity for MV2 and MM2 subtypes as previously published, as well as for all heterozygous patients in general (Gmitterova et al., 2016).

Tau and phosphorylated tau protein levels and their relative ratios are also under investigation as biomarkers for the diagnosis of TSE. They have yielded promising results but have yet to be formally accepted as part of a CJD case diagnostic criteria (Zerr et al., 2009). Elevated levels of total tau protein in CSF were evaluated as an indicator of prion disease, based on its utility as a biomarker of Alzheimer's disease in early stages, and the potentially shared pathogenic mechanisms of AD and CJD. Very high levels were found in relation to other dementing disease patients, whose levels were also increased compared to non-demented controls (Otto et al., 1997b). Further studies with larger cohorts and comparing tau with other biomarkers demonstrate an overall better performance of total tau regarding sensitivity and specificity for CJD (Van Everbroeck et al., 2004; Sanchez-Juan et al., 2006; Hamlin et al., 2012; Stoeck et al., 2012; Steinacker et al., 2016). Remarkably, they were also able to discriminate FFI and GSS from controls, despite detecting lower tau levels than for other CJD types (Ladogana et al., 2009). Once more, the presence of other neurodegenerative dementias within the control group was determining for specificity and sensitivity values (Dorey et al., 2015; Abu Rumeileh et al., 2017, 2018). Nevertheless, these values could be greatly improved through measurement and calculation of total tau and phospho-tau ratios, facilitating differential diagnosis with Alzheimer's disease (Riemenschneider et al., 2003; Dorey et al., 2015) or alternatively, measuring and calculating A β peptide: phospho-tau ratios (Lehmann et al., 2019). Despite there being no agreement on total tau cut-off values to support sCJD diagnostic, total tau is a sensitive and specific method for differential diagnosis of CJD and can also serve as a predictor of survival times (Staffaroni et al., 2019), albeit with low specificity for discrimination of CJD from atypical forms of Alzheimer's disease or acute neuronal injuries (Hermann et al., 2021). Nonetheless, the possibility of detecting increases in blood (Thompson et al., 2018; Abu-Rumeileh et al., 2020), its use in combination with other biomarkers or the calculation of total tau and phospho-tau ratios offering better results (Riemenschneider et al., 2003; Dorey et al., 2015) makes it suitable for inclusion among formal diagnostic criteria for CJD diagnosis.

Neurofilament light chain (NfL), a neuron-specific cytoskeleton protein, is also under extensive research as a biomarker for prion diseases, since its presence in body fluids indicates neuroaxonal damage (Petzold, 2005). Detection in CSF has shown very high diagnostic accuracy for sCJD patients with respect to healthy controls, as well as for several genetic CJD cases (Steinacker et al., 2016; Kovacs et al., 2017; Zerr et al., 2018). Moreover, it has been reported as a potential prodromal and prognostic biomarker in plasma (Abu-Rumeileh et al., 2020), although other studies did not find correlation with disease progression (Thompson et al., 2018) or failed to detect any increase in carriers of prion disease-causing mutations prior to disease onset (Vallabh et al., 2019).

Aside from the aforementioned, which can be considered closer to inclusion in official diagnostic criteria with protein 14-3-3 already in use, several other surrogate biomarkers are currently under investigation. In fact, due to the challenging *intra vitam* diagnosis of prion diseases, the search for new molecules that could act as specific indicators of neuronal damage in pre-clinical or early stages of disease is a highly active research area. Among others, decrease in CSF or blood levels of total prion protein (misfolded and native isoforms), as part of a larger panel of biomarkers has shown moderate diagnostic accuracy (Dorey et al., 2015; Abu Rumeileh et al., 2017; Villar-Pique et al., 2019; Llorens et al., 2019). Moreover, it could be potentially used for monitoring the effects of clinical trials, in particular those aimed at reducing native PrP expression levels (Raymond et al., 2019; Vallabh et al., 2019). On the other hand, reduction of A β peptide in CSF of CJD patients has also been observed, which combined

with 14-3-3 protein highly improved sensitivity, specificity, and positive predictive value (Van Everbroeck et al., 2003). This biomarker has also been used in combination with tau and 14-3-3 for the differential diagnosis of CJD and Alzheimer's disease showing promising results (Lehmann et al., 2019). The list of potential biomarkers for prion disease diagnosis is continuously being expanded, including molecules such as malate dehydrogenase 1 (MDH1) (Schmitz et al., 2016b); α , β and γ -synuclein, from which β -synuclein seems the most promising (Oeckl et al., 2016); YKL-40 (also known as chitinase 3-like 1), a marker of neuroinflammation with maximum levels in prion diseases compared to other neurodegenerative disorders (Llorens et al., 2017); neurogranin, also useful for differential diagnosis and reflecting the degree of neuronal damage (Blennow et al., 2019; Vallabh et al., 2020); ubiquitin, already proposed as a biomarker for Alzheimer (Blennow, 2004) which also showed improved diagnostic accuracy for TSE, allowing the discrimination between CJD, AD and FTD patients and healthy controls and importantly, offering better results than previous biomarkers for uncommon sCJD subtypes such as VV2 (Abu-Rumeileh et al., 2020); cell-free mitochondrial DNA (cfmtDNA) in CSF, one of the most recently described, showed increased copy number in CJD compared to other neurodegenerative disorders (Li et al., 2019). Nonetheless, further studies are required to confirm these novel potential biomarkers, mostly due to the small sample size of the preliminary studies but also due to the heterogeneity of prion disease, and its corresponding impact in relative biomarker levels. Moreover, as already stated, ring trials and interlaboratory studies are essential, as well as the establishment of significant cut-off values if they are to be used in routine clinical practice.

Detection of prion infection through direct visualization of PrP^{Sc}, immunohistochemistry, immunoblotting and enzyme-linked immunosorbent assay

The aforementioned diagnostic methods rely on non-specific indicators of neurological damage shared by a number of neurological conditions. In order to offer definitive and unequivocal diagnosis of prionopathies, detection of the pathognomonic marker of disease, PrP^{Sc}, has been the gold standard to confirm diagnostic of TSE for decades. Unfortunately, PrP^{Sc} levels are maximum and detectable mainly in the CNS of affected individuals at late stages of disease, making definitive diagnosis only possible *post-mortem* for a long time (Prusiner, 1987). In fact, identification of histopathological hallmarks, namely vacuolation or spongiform changes, astrocytic gliosis and PrP^{Sc} deposits, in brains of affected patients at necropsy, was considered necessary to confirm clinical diagnosis (Hermann et al., 2021). The detection of PrP^{Sc} in other tissues or body fluids in early stages of disease is usually precluded by the low amounts present in such locations, with notable exceptions such as the presence of prions in lymph nodes, exploited for the detection of animal prion diseases (Grassi, 2003). In order to assess PrP^{Sc} presence in distinct tissues, diverse immunodetection methods have been used, based on different anti-PrP antibodies and proteinase digestion. This allows to distinguish the natively folded isoform (completely sensitive to digestion) from the misfolded, disease-associated PrP^{Sc} (partially resistant to digestion). Immunohistochemistry (IHQ), western blotting (WB) and enzyme-linked immunosorbent (ELISA) assays have been used for that purpose. The first two are more informative in terms of disease subtype identification and classification, since IHQ allows analysis of the PrP^{Sc} deposition pattern and morphology of aggregates and WB permits characterization of the biochemical signature, dependent on the specific disease causing strain. In fact, ELISA, despite its use in research (Pan et al., 2005; Safar et al., 2005; Grassi, 2003), is not commonly applied to clinical diagnosis of human prion diseases. Nevertheless, it is the preferred detection method of prion infections in animals, with several rapid tests based on this assay in the market for the detection of prions in brains (post-mortem, for surveillance and epidemiological purposes) and lymph node biopsies (ante-mortem, for surveillance and outbreak control) from cattle sheep and deer (e.g., TeSeETM sheep/goats ELISA, Bio-Rad, Hercules, CA, United States; PrionicsTM-Check PrioStrip SR, Prionics, Zurich, Switzerland) (Grassi, 2003; Gavie-Widen et al., 2005).

Regarding human prion diseases, histopathological analysis of the brains of affected individuals dates back to the 1950s, when it was applied to the analysis of a familial CJD case in which protein deposits were detected. However, the nature of the protein forming the deposits was still unknown (Jacob et al., 1950; Klatzo et al., 1959). Purification of large amounts of scrapie prions adapted to hamster allowed the identification of the prion protein as constituent of aggregates and boosted production of anti-serum against this protein (Bendheim et al., 1984) paving the way for the development of prion immunodetection methods. These antibodies made it possible to detect PrP^{Sc} in the brains of CJD-affected patients by WB for the first time, confirming the relationship between animal and human TSE and the nature of the protein deposits previously observed by histopathological analysis (Bockman et al., 1985). Since then, IHQ and WB analysis of brain tissue from affected individuals have become routine assays for the diagnosis of TSE, with different disease types showing specific lesion patterns with prominent involvement of certain brain regions, different morphology of protein deposits and distinct biochemical signatures of protease-digested PrP^{Sc}, visualized by WB. In fact, PrP^{Sc} deposition patterns for the different human prionopathies at the final stage of the disease are well characterized and used to distinguish even subtypes or strains, together with the immunoblot analysis (Parchi et al., 1996, 2000; Schoch et al., 2006; Parchi et al., 2009). However, the heterogeneity that characterizes prion diseases also impacts the interpretation of neuropathological profiling as demonstrated by reports of unusual cases (Baiardi et al., 2018). Canonical type 1 sCJD is defined by PrP^{Sc} showing a proteinase K-digested unglycosylated band of ~21 kDa in WB, while type 2 cases present an unglycosylated band corresponding with a protein fragment of ~19 kDa. Polymorphisms at codon 129 modulate histopathological lesion profiles and PrP^{Sc} deposition patterns (Parchi and Saverioni, 2012). MM1 and MV1 subtypes typically show diffuse synaptic type PrP immunostaining mainly in cortical regions and the thalamus, with smaller and variable amounts in the cerebellum, and usually sparing the midbrain and medulla oblongata, and spongiform lesions mainly involving frontal and temporal cerebral cortex with slightly fewer lesions in the cerebellum (Schoch et al., 2006). In contrast, VV1 subtype usually presents faint corticostriatal synaptic

PrP^{Sc} deposition with occasional plaque-like PrP deposits in the gray matter and more spongiform changes in the cerebellum than the frontal cortex. VV2 cases are characterized by perineuronal and the cerebellar plaque-like PrP^{Sc} deposits mainly found in cerebellar granular layer and white matter and by spongiform lesions in subcortical gray matter which are often more severe in the striatum than in the cerebral neocortex. MV2 can be further subdivided in two different phenotypes according to the histopathological analysis. The most common form, known as MV2K (from kuru), shows cerebellar amyloid plaques of the kuru type (unicentric and rounded) mostly in the granular layer with widespread cortical and subcortical pathological changes, whereas the rare MV2C (from cortical) subtype shows large cortical confluent vacuoles and absence of plaque-like PrP^{Sc} deposits. Similarly MM2 subtypes can be further subdivided in cortical (MM2C) and thalamic (MM2T) types, the latter being also defined as the sporadic form of fatal insomnia (sFI) (Kawasaki et al., 1997; Parchi et al., 1999a). MM2C is similar to MV2C form histopathologically, characterized by confluent vacuoles and predominant cortical pathology with perivacuolar PrP^{Sc} staining in cortical layers, while MM2T shows prominent atrophy of thalamic and inferior olivary nuclei with little pathological changes in other areas and minimal PrP^{Sc} deposition. Similar characteristic histopathological features have also been defined for cases presenting mixed phenotypes, and atypical cases that do not fit the pure or mixed subtypes have been also reported, further complicating classification of CJD subtypes (Parchi and Saverioni, 2012). Along this line, histopathological and biochemical analysis of brain tissue is also possible in genetic and acquired forms of prion disease, in some cases allowing distinction between sCJD and other less frequent prionopathies such as vCJD, gCJD, FFI, GSS and VPSPr. For instance, PrP^{Sc} from gCJD, FFI and some specific GSS-associated mutations show similar biochemical signatures to sCJD type 1 and 2 cases, although differences in glycoform ratios (proportion of unglycosylated, monoglycosylated and diglycosylated forms) have been detected (Hill et al., 2006). However, given that patients with the same PrP mutation can show distinct PrP^{Sc} signatures and lesion profiles, combined with the scarcity of cases, the biochemical and histopathological hallmarks of genetic prionopathies are not as precisely defined as those of sCJD cases. GSS cases linked to specific mutations are worth mentioning due to the particular biochemical signature of the PrP^{Sc}, characterized by a ladder-like band pattern with low molecular weight PrP fragments (Kovacs and Budka, 2009). This atypical banding pattern of PrP^{Sc} after digestion has also been observed in the few VPSPr cases reported to date, being considered by some authors as the sporadic form of GSS (Notari et al., 2014). FFI is characterized by predominant spongiform changes and PrP^{Sc} deposits in the thalamus and olivary nucleus, with little or no affection of other brain regions (Budka, 2000). However, in line with the clinical heterogeneity atypical cases have been also described (Sasaki et al., 2005; Shi et al., 2010). Finally, vCJD presents large fibrillary amyloid plaques surrounded by vacuoles, known as florid plaques, many of which are detectable in the cerebral and cerebellar cortices. The biochemical signature of this PrP^{Sc} is similar to that of type 2, showing an unglycosylated band of the same size but different PrP glycoform ratios with the predominant signal corresponding to the diglycosylated PrP (Will et al., 1996; Parchi and Saverioni, 2012). Due to the risk of transmission, vCJD prompted the analysis of PrP^{Sc} presence in other tissues and body fluids, yielding positive results mainly in lymphoid tissue (Hill et al., 1997; Hilton et al., 1998) but also in many others (Wadsworth et al., 2001). This led to the discovery of PrP^{Sc} in a variety of organs, tissues and body fluids in other prionopathies (reviewed in depth in Erana et al., 2020). Detection of PrP^{Sc} in easily accessible tissues and body fluids at early stages of the disease could provide highly specific methods for TSE diagnosis. Prions found in CSF (Erana et al., 2020), blood (Orri et al., 2011; Castilla et al., 2005b), olfactory epithelium (Zanusso et al., 2003; Tabaton et al., 2004) or even skin (Wang et al., 2019) could be key to achieve definitive *intra-vitam* diagnosis. However, the low amounts of PrP^{Sc} in such tissues hinders direct detection by standard immunoassays in most cases. *In vitro* prion propagation techniques, which will be reviewed in the next section, provide the perfect solution to this issue.

Detection of prion infection through indirect observation of PrP^{Sc}, techniques based on *in vitro* prion propagation

As stated previously, immunodetection coupled with protease digestion can be used to determine PrP^{Sc} presence, which can be detected *ante-mortem* directly in certain tissues from some TSE-affected animals (i.e., rectal biopsies in small ruminants and cervids allow direct detection in lymphoid follicles of the rectal mucosa) (Grassi, 2003). However, PrP^{Sc} amounts in peripheral tissues and body fluids are generally too low for direct detection through standard biochemical techniques. This limitation of the use of PrP^{Sc} as a pathognomonic biomarker in human prion diseases has recently been overcome, at least partially, thanks to important advances on cell-free systems for prion propagation, which mimic the PrP^C to PrP^{Sc} conversion observed *in vivo* (Green and Zanusso, 2018). The first cell-free prion propagation system developed required large amounts of PrP^{Sc} (derived from brain homogenates of infected subjects) as seed, purified PrP^C as substrate and showed low conversion yields, limiting its use in diagnosis to the detection of minute amounts of prions (Kocisko et al., 1994). However, since then, *in vitro* prion propagation systems have been further developed, with two emerging as the most effective for the detection of minute PrP^{Sc} amounts, undetectable by direct detection methods. Protein misfolding cyclic amplification (PMCA) came first. This technique substantially improved the misfolding efficiency of the initial method through serial incubation and sonication cycles, allowing the ultrasensitive detection of prions at picogram (pg) levels for the first time coupled to a considerable reduction of the process time (Saborio et al., 2001). This technique can be understood as an analogous of a PCR for amyloids, in which undetectable PrP^{Sc} seeds can be amplified at the expense of PrP^C from the substrate, giving rise to large amounts of *in vitro* produced PrP^{Sc} aggregates, which can be easily detected by protease digestion and WB. Further developments of this method led to an increase in sensitivity through serial PMCA cycles (Castilla et al., 2005b) and addition of beads (Gonzalez-Montalban et al., 2011). Despite being originally conceived to use brain homogenates of infected individuals as seeds, it was readily adapted to detection of PrP^{Sc} in other tissues and body fluids, such as blood, spleen or urine, due to its exceptional potential diagnostic value (Castilla et al., 2005b; Saa et al., 2006; Murayama et al., 2007;

Haley et al., 2009; Chen et al., 2010). PMCA has been used to detect vCJD prions in blood and plasma at pre-clinical stages (Lacroux et al., 2014; Bougard et al., 2016; Concha-Marambio et al., 2016), in urine (Moda et al., 2014) and in CSF (Barria et al., 2018). However, amplification of some sCJD subtypes is more challenging (Bougard et al., 2018), limiting its implementation in the clinical practice. Nonetheless, for other prion diseases such as FFI, promising advances have been made thanks to PMCA, allowing the detection of prions in olfactory mucosa of affected patients (Redaelli et al., 2017). Given that the prions formed *in vitro* by PMCA replicate the main hallmarks of the original seeds including infectivity (Castilla et al., 2005a), strain features (Green et al., 2008) and transmission barrier phenomena (Fernandez-Borges et al., 2009), the method is widely used in TSE research. Nonetheless, its use in clinical diagnosis is limited mainly due to the following reasons: it requires complicated sonication equipment and technical expertise, resulting in inter-laboratory variability; different brain homogenates are needed as substrate depending on the prion strain to be propagated; and the assay requires serial PMCA rounds to achieve maximum sensitivity, as well as a separate readout system based on protease digestion and immunoblotting, which are time consuming and increase the total time needed for diagnosis (Saa and Cervenakova, 2015). Although further developments could improve its diagnostic value, such as the use of recombinant PrP and chemically defined substrates (Wang et al., 2010; Atarashi et al., 2007; Fernández-Borges et al., 2017) to improve consistency, or the use of high-throughput formats and coupled readout methods (Moudjou et al., 2014; Bieschke et al., 2000) which would allow automatization; another cell-free prion propagation system, already established in clinical practice, is preferentially used for diagnosis, the real time Quaking induced conversion (RT-QuIC) (Caughey et al., 2017).

RT-QuIC has revolutionized TSE diagnosis and PrP^{Sc} detection in clinical settings and is being adapted to the detection of other self-replicating misfolded proteins related with different neurodegenerative diseases such as synucleinopathies and tauopathies (Saijo et al., 2019). Since the first report in which this shaking-based recombinant PrP conversion method was shown to detect up to a single infectious particle from prion infected hamster brains and CSF (Atarashi et al., 2008), it has been greatly improved, with an eye on TSE diagnosis. Although the initial version was already faster and technically simpler than PMCA, its wider implementation resulted mainly from two innovations: the adaptation to a multi-well plate format, and the coupling to a readout based on Thioflavin T (ThT) fluorescence (which shows increased fluorescence upon amyloid binding) that can be performed within the same equipment (Wilham et al., 2010; Orru et al., 2012). Additionally, reaction conditions have been fine-tuned to increase process speed and achieve improved sensitivity and specificity, taking detection limits to the range of attograms of PrP^{Sc} in the seed (Orru et al., 2009) and recently, a universal substrate was also described allowing to overcome strain specificity issues (Orru et al., 2015). These changes made the method easily automatable and allowed monitorization of amyloid formation through fluorescence measurements in real time, bringing it closer to routine clinical practice. Despite it has been applied to drug screening and strain identification (Caughey et al., 2017; Candelise et al., 2017), in contrast to PMCA, the aggregates formed during RT-QuIC do not faithfully reproduce the characteristics of the original seeds limiting its applications on research. Nonetheless, due to its desirable features in terms of standardization, reproducibility and ease of use for prion detection in clinical settings, which were confirmed also by ring trials (Cramm et al., 2016; Mcguire et al., 2016), it was included in the diagnostic criteria established by the Centre of Disease Control (CDC) for sCJD in 2018, as well as in European diagnostic criteria used by the UK National CJD Research and Surveillance Unit. Several studies support its use for the detection of prions in CSF of suspected cases of vCJD (Orru et al., 2009), all subtypes of sCJD (Atarashi et al., 2011; Mcguire et al., 2012; Cramm et al., 2015; Foutz et al., 2017), iatrogenic CJD (Villar-Pique et al., 2019) and genetic prion diseases (Sano et al., 2013; Cramm et al., 2015; Foutz et al., 2017), although with lower sensitivity for MM2 subtypes, vCJD, GSS and FFI (Franceschini et al., 2017). Moreover, following immunoprecipitation, vCJD prions have also been detected in blood (Orru et al., 2011); sCJD, gCJD and FFI prions have been found in olfactory epithelium (Orru et al., 2014; Bongianini et al., 2017; Redaelli et al., 2017) and sCJD prions in skin (Orru et al., 2017). Overall, detection of PrP^{Sc} in CSF by RT-QuIC is expected to be the next gold standard for *ante-mortem* diagnosis of TSE. However histopathological analysis and biochemical detection of PrP^{Sc} are still the most widely accepted methods for definitive diagnosis.

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Microbes Culture Methods

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Glossary

Anaerobic culture An anaerobic culture means the test is done without letting oxygen get to the sample. Infections caused by anaerobic bacteria can occur almost anywhere in your body. These may be infections in your mouth or lungs, diabetes-related foot infections, infected bites, and gangrene.

Axenic culture A microbial culture that contains only one species, variety, or strain of microorganism. There should not be any contaminants present. Microorganisms that are obligate symbionts, though, cannot be grown in axenic cultures.

CMRL medium CMRL medium was originally developed by Connaught Medical Research Laboratories for the growth of Earle's "L" cells under serum-free conditions. CMRL medium is also especially useful for cloning monkey kidney cells and for growth of many other mammalian cell lines when supplemented with horse or calf serum.

DNase agar DNase agar is a differential medium that tests the ability of an organism to produce an exoenzyme, called deoxyribonuclease or DNase, that hydrolyzes DNA. DNase agar contains nutrients for the bacteria, DNA, and methyl green as an indicator.

RPMI 1640 Medium was originally developed to culture human leukemic cells in suspension and as a monolayer. Roswell Park Memorial Institute (RPMI) 1640 Medium has since been found suitable for a variety of mammalian cells, including HeLa, Jurkat, MCF-7, PC12, PBMC, astrocytes, and carcinomas.

Tryptic soy agar (TSA) Tryptic Soy Agar (TSA) is a non-selective solid medium recommended for use in qualitative procedures for the isolation and cultivation of a wide variety of organisms. Tryptic Soy Agar slants may be used to cultivate, store, and ship bacterial cultures.

Introduction

A microbiological culture, also known as a microbial culture, is a technique for multiplying microorganisms by allowing them to replicate in a predetermined culture medium under carefully regulated laboratory conditions. The word "culture" is more commonly used informally to refer to the laboratory's "selective development" of a particular type of microorganism. Microbial cultures are fundamental and essential diagnostic tools used in microbiology research to assess the type of organism, the concentration of that organism in the sample being studied, or both. It is one of the most popular microbiology diagnostic methods, and is used to determine the cause of infectious disease by allowing the agent to multiply in a predetermined medium. For example, *Streptococcus pyogenes*, the causative agent of strep throat and to identify this bacterium, a throat culture is taken by scraping the lining of tissue in the back of the throat and blotting the sample into a medium to be able to screen the bacteria. The culture conditions could be varied with incubation time, nutrients content, environment, and temperature and thus the condition of culture medium to be fixed by trial and error methods. The birth of culture in clinical microbiology was prompted by microbiologists specializing in intracellular bacteria. The shell vial procedure allowed the culture of new species of *Rickettsia*. The design of axenic media for growing fastidious bacteria like *Tropheryma whippelii* and *Coxiella burnetii* and therefore the ability of amoebal coculture to get new bacteria constituted major advances. Strong efforts combining optimized culture media, detection techniques, and a microaerophilic environment resulted in a drastic reduction in the time it took to culture *Mycobacterium tuberculosis*. The use of a new flexible medium enabled archaea to expand its repertoire.

Finally, the addition of antioxidants to culture media in an aerobic environment permitted the growth of strictly anaerobic bacteria, which improved anaerobe culture in routine bacteriology laboratories. Nevertheless, among usual bacterial pathogens, the event of axenic media for the culture of *Treponema pallidum* or *Mycobacterium leprae* remains an important challenge that the patience and innovations of cultivators will enable them to overcome.

Origin of culture media

Microbiological culture media have their roots in the 19th century, when the study of bacteriology was just getting started. Many bacteriologists have tried, with varying degrees of success, to grow bacteria on the food or material on which the microorganism was first developed. Robert Hooke and Antonie van Leeuwenhoek, two Fellows of the Royal Society, discovered the presence of microscopic species between 1667 and 1676. In 1667, Robert Hooke described the discovery of filamentous fungi, and many years later, Leeuwenhoek discovered and described microscopic protozoa (very small animalcules) known today as *Giardia lamblia* and bacteria while studying various water sources such as rainwater, pond, and well water. On the contrary, fungi and parasite research lagged behind that of other microorganisms and in 1839, fungi were discovered to be the cause of the first human disease, ringworm, which was quickly followed by the discovery of *Candida albicans* as the cause of thrush. It was not until 1910 that Sabouraud introduced a medium that would support the growth of pathogenic fungi. The interest of scientists in studying fungi was often related to crop protection. During the period 1857–78, both Louis Pasteur and Joseph Lister published significant papers on their extensive studies on fermentation.

After microorganisms were visually observed, their growth or reproduction and the idea of spontaneous generation theory created a major controversy and continued for years until 1860. In 1860, Louis Pasteur was the first to use a culture medium for

growing bacteria in the laboratory. This medium consisted of yeast, ash, sugar and ammonium salts. In 1881, Frau Angelina Hesse used his wife's agar (considered an exotic food) as a solidifying agent for bacterial growth.

In 1887, a German microbiologist Julius Richard Petri developed a circular flat dish with a lid called the Petri dish that is used for growing bacteria on a bed of nutrient jelly; this innovation was revolutionized in culture media formulations. With all the research being performed, scientists started to use agar instead of gelatin because it was more resistant to microbial digestion and liquefaction. The Petri dish can be used in any microbiology or immunology laboratory around the world, and its basic architecture has remained unchanged since its inception. Significant advances continued in the 1990s and the use of chromogenic substrates in culture media was shown to improve microbial identification directly from the culture medium. Despite rapid advancements in technology and instrumentation, the basic culture media and ingredients remain most reliable and cost effective tools in microbiology today.

Microorganism growth requirements

As all living organisms, microorganisms also require a combination of various physical and chemical factors for their growth and multiplication. Microbial growth actually refers to increase in numbers of the cells. Cultivation of microorganisms depends on a number of the following important factors.

Physical requirements

Temperature

Bacteria have a minimum, optimum, and maximum temperature for growth and can be divided into four groups based on their growth temperature:

1. Psychrophiles are cold-loving bacteria. Their optimum growth temperature is between -5°C and 15°C . They are usually found in the Arctic and Antarctic regions and in streams fed by glaciers.
2. Mesophiles are bacteria that grow best at moderate temperatures. Their optimum growth temperature is between 25°C and 45°C . Most bacteria are mesophilic and include common soil bacteria and bacteria that live in and on the body.
3. Thermophiles are heat-loving bacteria. Their optimum growth temperature is between 45°C and 70°C and are commonly found in hot springs and in compost heaps.
4. Hyperthermophiles are bacteria that grow at very high temperatures. Their optimum growth temperature is between 70°C and 110°C . They are usually members of the Archaea and are found growing near hydrothermal vents at great depths in the ocean.

Oxygen requirements

Different kinds of bacteria need different amounts of oxygen to survive, which determines which bacteria can infect which parts of the body. Since oxygen is present, they are unable to infect the skin, and they can only expand in the presence of oxygen. Obligate anaerobes, on the other hand, are destroyed by oxygen and carry out fermentation. Tetanus is an obligate anaerobe, which means it can infect areas with little oxygen. Aerotolerant anaerobes breathe anaerobically (without oxygen) but can survive in the presence of oxygen. Bacteria can be classified into one of the following categories based on their oxygen requirements:

1. Obligate aerobes are organisms that grow only in the presence of oxygen. They obtain their energy through aerobic respiration.
2. Microaerophils are organisms that require a low concentration of oxygen (2–10%) for growth, but higher concentrations are inhibitory. They obtain their energy through aerobic respiration.
3. Obligate anaerobes are organisms that grow only in the absence of oxygen and, in fact, are often inhibited or killed in the presence of oxygen. They obtain their energy through anaerobic respiration or fermentation.
4. Aerotolerant anaerobes, like obligate anaerobes, cannot use oxygen to transform energy but can grow in its presence. They obtain energy only by fermentation and are known as obligate fermenters.
5. Facultative anaerobes are organisms that grow with or without oxygen, but generally grow better with oxygen. They obtain their energy through aerobic respiration if oxygen is present, but use fermentation or anaerobic respiration if it is absent. Most bacteria are facultative anaerobes.

pH

Bacteria and other microbes are sensitive to the pH of their surroundings. pH has an effect on large proteins like enzymes. Their shape shifts (they denature), which often results in a shift in the ionic charges on the molecule. In certain cases, the enzymes' catalytic properties are lost, and metabolism is halted. Most bacteria grow best in a pH range of 6.5–7.0, but some thrive in extremely acidic environments and can even withstand pH values as low as 1.0. Acidophiles are bacteria that thrive in acidic environments. Despite the fact that they can survive in extremely acidic conditions, their internal pH is far closer to neutral. Based on their optimum pH requirements, microorganisms can be classified into one of the following groups:

1. Neutrophiles grow best at a pH range of 5–8.
2. Acidophiles grow best at a pH below 5.5.
3. Alkaliphiles grow best at a pH above 8.5.

Some bacteria produce acid as they grow, when this acid is excreted, it decreases the pH of the surrounding environment. This eventually brings bacterial growth to a halt unless something else in the environment neutralizes the bacterial acid. When grown in broth, a buffering agent may be used to mop up excess acid and maintain the pH of the growing culture at optimal levels. Each species of microbe has a specific pH range in which it thrives and reproduces.

Osmosis

Osmotic pressure is of vital importance in biology since the cell membrane is selective against many of the solutes present in living organisms. When a cell is put in a hypertonic solution, water escapes the cell and flows into the surrounding solution, causing the cell to shrink and lose its turgidity. Hypertonic solutions are used for antimicrobial control, on the other hand, salt and sugar are used to create hypertonic environment for microorganisms and are commonly used as food preservatives. Most bacteria require an isotonic environment or a hypotonic environment for optimum growth. Osmotolerant organisms are those that can thrive in relatively high salt concentrations (up to 10%). Halophiles are organisms that need relatively high salt concentrations for development, such as certain archaea that need sodium chloride concentrations of 20% or higher.

Chemical requirements

Microorganisms need a constant supply of chemical nutrients in addition to a suitable physical environment. Microorganisms are often classified based on their energy and carbon sources.

Energy sources

There are two types of bacteria, phototrophic bacteria, such as *Thiocapsa roseopersicina*, which uses light as an energy source by transforming it into an electrochemical gradient of protons (Yurkov and Beatty, 1998), and chemotrophic bacteria, which use the energy of oxidation of mineral or organic compounds as energy sources (Thauer et al., 1977). *Listeria monocytogenes* is found in these bacteria (Van der Horst et al., 2007).

Carbon source

Carbon is the structural backbone of the organic compounds that make up a living cell. Carbon is the most abundant constituent element in bacteria. It is essential for bacteria to produce carbon molecules, such as fats, carbohydrates, proteins and nucleic acids. Bacteria can use inorganic carbon sources, such as carbon dioxide, or organic sources such as sugars and alcohols (Kumar 2012; Atlas, 2010). Based on their source of carbon bacteria can be classified as autotrophs or heterotrophs.

1. *Autotrophs*: require only carbon dioxide as a carbon source. An autotroph can synthesize organic molecules from inorganic nutrients.
2. *Heterotrophs*: require organic forms of carbon. A heterotroph cannot synthesize organic molecules from inorganic nutrients.

Nitrogen source

Nitrogen sources are plentiful, and they can be present in a wide range of compounds used in the formulation of a culture medium. It exists in both organic and inorganic forms, corresponding to protein hydrolysates, especially in the case of hydrolysate, proteose-peptone, or tryptone (Atlas, 2010), and nitrates (Latge 1975). Nitrogen is needed for the synthesis of amino acids, DNA, RNA, and ATP, among other molecules. Depending on the organism, nitrogen, nitrates, ammonia, or organic nitrogen compounds can be used as a nitrogen source.

Minerals sources

Among the common mineral salts, phosphate, sulfate, magnesium or calcium are regularly found. Sulfur is needed for the production of sulfur-containing amino acids and some vitamins. Sulfates, hydrogen sulfide, or sulfur-containing amino acids can be used as a sulfur source, depending on the microorganism type. Phosphate ions are the primary source of phosphorus and phosphorus is needed to synthesize phospholipids, DNA, RNA, and ATP. Potassium, magnesium, and calcium are needed for the function of certain enzymes as well as other functions. Iron is needed in trace amounts by certain enzymes, and trace elements, including potassium, magnesium, calcium, and iron, typically serve as cofactors in enzyme reactions. They include sodium, zinc, copper, molybdenum, manganese, and cobalt ions. During enzyme reactions, cofactors typically serve as electron donors or acceptors.

Water

Water is important for solubilizing nutrients, transporting them, and ensuring that hydrolysis reactions take place. Some bacteria need unrestricted access to water in order to thrive. If evaporation occurs during the incubation of the agar, this water can be lost, resulting in a reduction in colony size and bacterial growth inhibition (Kumar, 2012).

Growth factors

The use of a medium sometimes prevents the growth of bacteria that require unique elements in order to thrive. To improve bacterial multiplication, it's often important to add growth factors to culture media. Growth factors are nutrients that bacteria cannot synthesize from the nutrients available in the environment, such as amino acids, purines, pyrimidines, and vitamins (Kumar 2012; Werkman and Wilson, 1951). Growth factors are needed in small amounts in the culture medium and their requirement is justified by the lack or blockage of a metabolic pathway in the bacterium.

Microbiological media for bacterial growth

Microorganism research is greatly aided by the ability to culture them, or hold reproducing populations alive under laboratory conditions. The first culture media were created through trial and error with environmental components. Overall, the four primary factors that influence bacterial growth are nutrient selection, environment, temperature, and incubation time. Culturing many microorganisms is challenging, because of their extremely specific nutritional and environmental requirements, as well as the diversity of these requirements among different species. A good microbiological culture medium must contain available sources of carbon, nitrogen, inorganic phosphate and sulfur, as well as trace metals, water, vitamins. Originally, these were given in the form of a meat infusion. In culture media, beef or yeast extracts are commonly used instead of meat infusion. Peptones, which are protein digests, are added to provide readily available nitrogen and carbon sources. The medium must have an adequate pH, maintain proper temperature relationships, be free of interfering bioburden, and be free of contamination (Table 1).

Environmental factors in culture media

Atmosphere

The majority of bacteria can grow in normal oxygen tension conditions. Anaerobes expand only in the absence of atmospheric oxygen, while obligatory aerobes need free admission of oxygen. Between these two groups are the microaerophiles, which thrive in partially anaerobic environments, and facultative anaerobes, which may develop in the presence or absence of oxygen. There are several ways to create anaerobic conditions for microorganism growth:

- Addition of small amounts of agar to liquid media.
- Addition of fresh tissue to the medium.
- Addition of a reducing substance to the medium; e.g., sodium thioglycollate, thioglycolic acid and L-cystine.
- Displacement of the air by hydrogen or nitrogen.
- Absorption of the oxygen by chemicals.
- Inoculation into the deep layers of solid media or under a layer of oil in liquid media.

Many microorganisms need a CO₂ concentration of 5–10%. Carbonic acid levels greater than 10% are often inhibitory due to a drop in pH when carbonic acid forms. As culture media are exposed to light and air, their susceptibility to form toxic oxidation products varies.

Table 1 Common Media Constituents and its function.

Constituents	Source	Functions
Water	Primary Drinking Water complying with the U.S. Environmental Protection Agency or comparable regulations of the European Union or Japan.	Purified water is used in the preparation of culture media. Water plays role in solubilizing nutrients, and ensuring hydrolysis reactions
Amino-nitrogen	Peptone, protein hydrolysate, infusions and extracts. Peptones are derived from proteins by hydrolysis or digestion of the source material; e.g., meat, milk.	They are the major sources of nitrogen and vitamins in culture media.
Growth factors	Blood, serum, yeast extractor vitamins, NAD	Needed for fastidious growth of some bacteria.
Energy sources	Sugar, alcohols and carbohydrates	They are used as energy sources as well as a means of distinguishing genera and organisms.
Buffer salts	Phosphates, acetates and citrates	Buffers maintain the pH of culture media.
Mineral salts and metals	Phosphate, sulfate, magnesium, calcium, iron	They usually function as cofactors in enzyme reactions.
Selective agents	Chemicals, antimicrobials and dyes	Selective agents are used in media to inhibit the growth of bacteria, yeasts and fungi.
Indicator dyes	Phenol red, neutral red	Dyes act as bacteriostatic agents or indicators of changes in acidity or alkalinity of the substrate.
Gelling agents	Agar, gelatin, alginate, silica gel	These solidifying agents can be added to a liquid medium in order to change the consistency to a solid or semisolid state.

Water Activity

Proper moisture conditions are necessary for continued growth of microorganisms. Organisms need to be in an aqueous atmosphere of “free” water. The movement of nutrients and toxic waste products requires “free” water, which is not bound in a complex system. During incubation or storage, evaporation causes a loss of “free” water, resulting in a reduction in colony size or complete inhibition of colony growth.

Protective agents and growth factors

Protective agents such as calcium carbonate, soluble starch, and charcoal are used in culture media to neutralize and absorb toxic metabolites formed by bacterial growth. For example, *Haemophilus* species, or *Neisseria* species need the growth factors nicotinamide adenine dinucleotide, NAD (V factor), and hemin (X factor) for enhanced growth. Surfactants, such as polysorbate 80, reduce interfacial tension around bacteria in suspended medium. This behavior allows desired compounds to enter the bacterial cell more quickly, which can boost bacterial growth.

Types of culture media based on the function

The culture media are classified into six types depending on the function: (1) Basal media, (2) Enriched media, (3) Selective media (4) Indicator media, (5) Transport media, and (6) Storage media.

1. *Basal media*. Basal media are those that can be used to grow (culture) bacteria without the need for additional media enrichment. Examples: Nutrient broth, nutrient agar and peptone water.
2. *Enriched media*. Enriched media provides growth factors, vitamins, and other important nutrients to help fastidious organisms develop. These organisms can't make these nutrients and need them to be added to the medium. The media are enriched usually by adding blood, serum or egg. Examples: Enriched media are blood agar and Lowenstein-Jensen media. *Streptococci* grow in blood agar media.
3. *Selective media*. These media favor the growth of a particular bacterium by inhibiting the growth of undesired bacteria and allowing growth of desirable bacteria. Examples: MacConkey agar, Lowenstein-Jensen media, tellurite media (Tellurite inhibits the growth of most of the throat organisms except diphtheria bacilli). Antibiotic may be added to a medium for inhibition.
4. *Indicator (differential) media*. Differential media are bacteriological growth media that contain specific ingredients to allow one to distinguish selected species or categories of bacteria by visual observation. Blood agar is one type of differential medium, allowing bacteria to be distinguished by the type of hemolysis produced. Bismuth sulfite agar uses the ability of bismuth to inhibit most Gram-positive and Gram-negative commensal organisms for the culture of enteric bacilli. An indicator is included in the medium. A particular organism causes change in the indicator, e.g., blood, neutral red, tellurite. Examples: Blood agar and MacConkey agar are indicator media.
5. *Transport media*. Transport media are special media formulated to preserve a specimen and minimize bacterial overgrowth from the time of collection to the time it is received at the laboratory to be processed. Depending on the type of organisms suspected in the sample, transport media may vary. These media are used when species cannot be cultured soon after collection. Examples: Cary-Blair medium, Amies medium, Stuart medium.
6. *Storage media*. Media used for storing the bacteria for a long period of time. Examples: Egg saline medium, chalk cooked meat broth (Table 2).

Quality control of culture media

The importance of a laboratory is determined by the nature of its microbiological media. Many false-negative cultures will result from an enrichment medium that isn't nutritious enough to sustain the target pathogen. A similar situation will arise if a selective medium is too inhibitory, it can kill both the pathogen and the natural flora. However, if the medium is not adequately inhibitory, commensal organisms will overgrow the plate, obscuring the pathogen under investigation. The quality of media can be controlled in two ways: process control and batch control. The latter is sometimes referred to as quality management. Process control depends on having a set of standard operating procedures for operation of equipment used in media production, sterilization, methods and recipes describing the production of medium whether this be from a commercial manufacturer or made up in-house. The consistency of the reagents that go into the medium, including the pH and ion content of the water, must also be clearly defined and methods for measurement and if necessary rejection of batches laid down. Two aspects of batch control must be considered: the medium's physical characteristics and its microbiological efficiency.

Physical characteristics

The tests undertaken for the physical characteristics of culture media vary depending upon the type of media. Examples of physical tests include:

- *Visual test for color*: The color of a sterilized medium should be compared to a non-sterilized medium and any differences in color to be noted.
- *Visual test for clarity*: The clarity of the media should be examined for optical artefacts, such as crystallization.

Table 2 Common microbiological media in routine use.

Nutrient Broth	This medium is used as a basal media for the preparation of other media and to study soluble products of bacteria. It contains 500 g meat, e.g., ox heart is minced and mixed with 1 L water. 10 g peptone and 5 g sodium chloride are added, pH is adjusted to 7.3.
Nutrient Agar	Nutrient agar is a general purpose medium used to support for the growth of a wide range of non-fastidious bacteria. It is solid at 37 °C. Usually 2.5% agar is added in nutrient broth and then heated at 100 °C to melt the agar and then cooled.
Peptone Water	Peptone 1.0% and sodium chloride 0.5%. It is used as base for sugar media and to test indole formation.
Blood Agar	Most commonly used medium. 5–10% defibrinated sheep or horse blood is added to melted agar at 45–50 °C. Blood acts as an enrichment material and also as an indicator. Certain bacteria when grown in blood agar produce hemolysis around their colonies. Certain bacteria produce no hemolysis. The colony is surrounded by a clear zone of complete hemolysis, e.g., <i>Streptococcus pyogenes</i> is a β -hemolytic streptococci, or α -hemolysis. The colony is surrounded by a zone of greenish discoloration due to formation of biliverdin, e.g., <i>Streptococci, viridans</i> Gamma γ -hemolysis, or, No hemolysis. There is no change in the medium surrounding the colony.
Chocolate Agar or Heated Blood agar	Prepared by heating blood agar and is used for culture of <i>Pneumococcus, Gonococcus, Meningococcus</i> and <i>Haemophilus</i> . Heating the blood inactivates inhibitor of growths.
MacConkey Agar	It is a selective and indicator medium for enterobacteriaceae, which contains agar, peptone, sodium chloride, bile salt, lactose and neutral red. Selective as bile salt does not inhibit the growth of enterobacteriaceae but inhibits growth of many other bacteria. Indicator medium as the colonies of bacteria that ferment lactose take a pink color due to production of acid. Acid turns the indicator neutral red to pink. These bacteria are called 'lactose fermenter', e.g., <i>Escherichia coli</i> . Colorless colony indicates that lactose is not fermented, i.e., the bacterium is non-lactose fermenter, e.g., <i>Salmonella, Shigella, Vibrio</i> spp.
Mueller Hinton Agar	Disc diffusion sensitivity tests for antimicrobial drugs should be carried out on this media as per WHO recommendation to promote reproducibility and comparability of results.
Hiss's Serum Water Medium	This medium is used to study the fermentation reactions of bacteria which cannot grow in peptone water sugar media, e.g., <i>Pneumococcus, Neisseria</i> , and <i>Corynebacterium</i> spp.
Lowenstein-Jensen Medium	The Löwenstein-Jensen medium, more commonly known as LJ medium, is a growth medium specially for <i>Mycobacterium</i> species, notably <i>Mycobacterium tuberculosis</i> . It contains egg, malachite green and glycerol. Egg is an enrichment material which stimulates the growth of tubercle bacilli, malachite green inhibits growth of organisms other than <i>Mycobacteria</i> , glycerol promotes the growth of <i>Mycobacterium tuberculosis</i> but not <i>Mycobacterium bovis</i> .
Dubos Medium	This liquid medium is used for <i>tubercle bacilli</i> . It contains "tween 80," bovine serum albumin, casein hydrolysate, asparagine and salts. Tween 80 causes dispersed growth and bovine albumin causes rapid growth.
Loeffler Serum Medium	This medium is used for <i>Corynebacterium</i> culture, which contains coagulated serum of sheep, ox or horse for enrichment. <i>Corynebacterium Diphtheria</i> grow in this medium in 6 h while the secondary bacteria do not grow. It is used for rapid diagnosis of diphtheria and to demonstrate volutin granules, sometimes termed metachromatic granules because of their color reaction with the dyes used in light microscopy.
Tellurite Blood Agar	It is used as a selective medium for isolation of <i>Corynebacterium diphtheria</i> . It is selective due to the presence of inhibitor and differential by means of ability of organism to reduce potassium tellurite.
Stuart Transport Medium	Stuart Transport Medium is a non-nutritional medium which maintains the viability of organisms without significant multiplication. The small agar content provides a semisolid consistency which prevents oxidation and drying during transport.
Thiosulfate-citrate-bile salts-sucrose (TCBS)	TCBS Agar is used for the selective isolation of <i>Vibrio</i> spp. from a variety of clinical and nonclinical specimens. This medium has a very high pH (8.5–9.5) which suppresses growth of intestinal flora other than <i>Vibrio</i> spp. <i>V. cholerae</i> is yellow on this medium, <i>V. parahaemolyticus</i> is green on this medium.
Selenite F broth	Selenite-F Broth is used as an enrichment medium for the isolation of <i>Salmonella</i> spp. from feces, urine, water, foods and other materials of sanitary importance. The casein peptone provides essential nitrogenous and carbon compounds. The lactose in the medium serves to maintain a uniform pH.

- **Gel strength:** The gel strength should not be over-hard or over-soft, but firm and usable.
- **pH of the finished media:** This is possibly the most critical chemical test, because if the pH is outside of the recommended range for the media, some of the microorganisms that the media is designed to develop will be inhibited.
- **Checks for damage:** Plates and bottles should be examined for damage like cracks and defects.

Microbiological characteristics

1. **Sterility test:** The test of media sterility is designed to detect microbial contamination during the manufacturing process. Here a small number, normally 2% of the batch, of uninoculated items are incubated. The temperature and time selected for the sterility test incubation will depend upon the type of media. For general-purpose media, a temperature of 30–35 °C for 3 days is typical. To pass the sterility test the items must demonstrate no growth.
2. **Growth performance test:** The microbial growth performance of media should be controlled with a panel of standard organisms. These may be established locally but it is better to use those recommended in standard textbooks, by the PHLS or by the American NCCLS. Stocks of control organisms can be obtained from the National Collection of Type Cultures and American Type Culture Collection.

Table 3 Examples of control organisms for quality control of laboratory medium.

Quality control strain	Culture medium	Expected output
<i>Strep. pyogenes</i> ATCC 19615	Blood agar	Small clear gray beta-hemolytic colonies
<i>Streptococcus pneumoniae</i>	Blood agar	Alpha hemolytic, small greenish colonies
<i>Escherichia coli</i> (ATCC® 25,922™)	MacConkey agar	Lactose fermenting pink colonies
<i>Acinetobacter baumannii</i> (ATCC® 19,606™)	MacConkey agar	Non-Lactose fermenting pale colonies
<i>Shigella sonnei</i> (ATCC® 25,931™)	XLD agar	Red pink colonies
<i>Salmonella enterica</i> subsp. <i>enterica</i> (ATCC® 14,028™)	XLD agar	Red pink colonies with black center
<i>Legionella pneumophila</i>	Buffered charcoal yeast extract (BCYE) agar	Black centered gray colonies
<i>Enterococcus faecalis</i> (ATCC® 29,212™)	Bile esculin agar	Small transparent colonies with brown-black halos.
<i>N. gonorrhoeae</i> NCTC 8375	Thayer martin agar	Water droplet like colonies
<i>Staphylococcus aureus</i> (ATCC® 25,923™)	Mannitol Salt agar	Golden Yellow colonies
<i>Escherichia coli</i> (ATCC® 25,922™)	EMB agar	Blue-black bull's eye like colonies with green metallic sheen

Standard cultures are used to ensure that medium is sufficiently nutritious to support the growth of fastidious pathogens. This may be achieved by performing a viable count of an overnight growth of a control organism or by the Miles and Misra technique or counting the colonies after a standardized plating method. In addition to demonstrating adequate nutritional support characteristic appearances or responses, such as beta-hemolysis or colored colonies due to indicator changes, should be present. The ability to kill undesirable species should be demonstrated alongside support for the target organisms in selective media. Some examples of control organisms are given in Table 3.

Aseptic technique, dilution, streaking, and spread plates

In microbiology, several techniques including aseptic technique, dilution, colony streaking and spread plates are used in day-to-day experiments. Microbiologists have many tools, but they use four relatively basic techniques on a daily basis, which are illustrated here.

- Aseptic technique* or sterile technique is used to avoid contamination of sterile media and equipment during bacterial cell culture. Sterile technique should always be employed when working with live cell cultures and reagents/media that will be used for such cultures. This technique involves using flame to kill contaminating organisms, and a general mode of operation that minimizes exposure of sterile media and equipment to contaminants. When working with cultures of living organisms, it is extremely important to maintain the environments in which cells are cultured and manipulated as free of other organisms as possible. This necessitates limiting the exposure of sterilized culture media containers to outside air and using a flame to “re-sterilize” container lids and rims. This entails passing rims and lids through a Bunsen burner’s flame to destroy microorganisms that come into contact with those surfaces.
- Serial dilution*: Serial dilution is the method of diluting a sample with a sterile diluent, which can be either distilled water or 0.9% saline, in a sequence of standard volumes. Then, using a small measured volume of each dilution, a series of pour or spread plates are formed. The degree of dilution is calculated based on the approximate concentration of cells/organisms in a sample. For example, if a water sample is taken from an extremely polluted environment, the dilution factor is increased. In contrast, for a less contaminated sample, a low dilution factor might be sufficient (Fig. 1).

Serial twofold and tenfold dilutions are commonly used to titer antibodies or prepare diluted analytes in the laboratory. The dilution factor in a serial dilution can be determined either for an individual test tube or can be calculated as a total dilution factor in the entire series.

The dilution factor of each tube in a set:

$$\frac{\text{Volume of Sample}}{\text{Volume of Sample} + \text{Volume of diluent}}$$

For a 10-fold dilution, 1 mL of sample is added to 9 mL of diluent. In this case, the dilution factor for that test tube will be:

$$\text{Dilution factor formula} = \frac{1 \text{ mL}}{1 \text{ mL} + 9 \text{ mL}} = 1/10 = 10^{-1}$$

After the first tube, each tube is the dilution of the previous dilution tube.

Now, for total dilution factor,

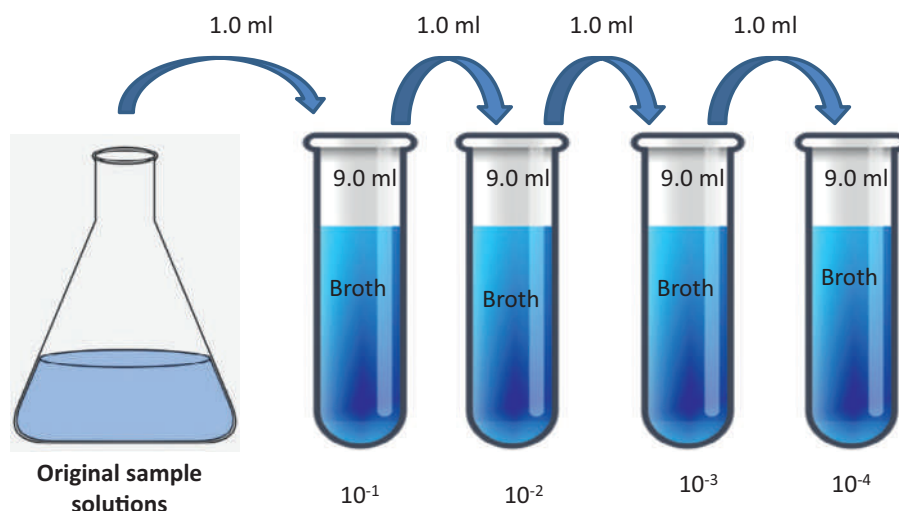


Fig. 1 A ten-fold serial dilution, which can also be called a 1:10 dilution, or a series with dilution factor of 10.

Total dilution factor for the second tube = dilution of first tube $\times$ dilution of the second tube.

Example:

For the first tube, dilution factor = 10^{-1} (1 mL added to 9 mL).

For the second tube, dilution factor = 10^{-1} (1 mL added to 9 mL).

$$\begin{aligned} \text{Total dilution factor} &= \text{previous dilution} \times \text{dilution of next tube} \\ &= \text{total dilution of } 10^{-1} \times 10^{-1} = 10^{-2} \end{aligned}$$

Serial dilution is used in microbiology to estimate the concentration or number of cells/organisms in a sample to obtain an incubated plate with an easily countable number of colonies.

c) **Streak plate:** In microbiology, streaking is a technique used to isolate a pure strain from a single species of microorganism, often bacteria. Streak plate technique is used for the isolation of a pure culture of the organisms (mostly bacteria), from a mixed population. The inoculum is streaked over the agar surface in such a way that it “thins out” the bacteria. Some individual bacterial cells are separated and well-spaced from each other. Usually, by the third or fourth quadrant, only a few organisms are transferred which will give discrete colony forming units (CFUs). Samples can then be taken from the resulting colonies and a microbiological culture can be grown on a new plate so that the organism can be identified, studied, or tested. The streaking is done using a sterile tool, such as a cotton swab or commonly an inoculation loop. This is dipped in an inoculum such as a broth or patient specimen containing many species of bacteria. The sample is spread across one quadrant of a petri dish containing a growth medium, usually an agar plate which has been sterilized in an autoclave. Choice of which growth medium is used depends on which microorganism is being cultured, or selected for. Growth media are usually forms of agar, a gelatinous substance derived from seaweed (Fig. 2).

Procedure for streaking a plate for isolation:

1. Flame the loop and wire and *streak* a loopful of broth as at A in the diagram.
 2. Reflame the loop and cool it.
 3. Streak as at B to spread the original inoculum over more of the agar.
 4. Reflame the loop and cool it.
 5. Streak as at C.
 6. Reflame the loop and cool it.
 7. Streak as at D.
- d) **Spread plate:** The spread plate technique involves using a sterilized spreader with a smooth surface made of metal or glass to apply a small amount of bacteria suspended in a solution over a plate. The plate needs to be dry and at room temperature so that the agar can absorb the bacteria more readily. No subsurface colonies appear in spread plate so isolation of the organism is easy. Volume no greater than 0.1 mL can be spread on the nutrient agar plate because it would not soaked well and may result colonies to form coalesce.

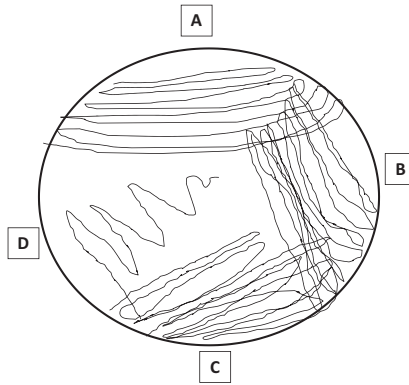
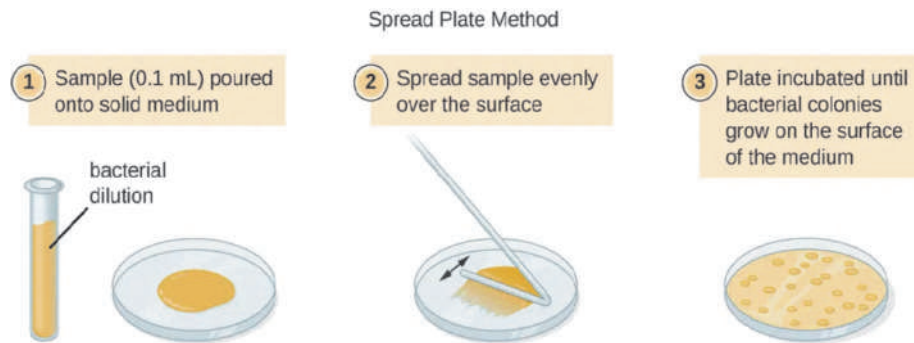


Fig. 2 Streaking procedure.



A perfect spread plate technique will result in visible and isolated colonies of bacteria that are evenly distributed in the plate and are countable as shown in the above figure.

- e) *Special Culture Techniques*: Many microbes have special growth conditions or require precautions to grow in a laboratory setting, leading to special culture techniques. A microaerophile is a microorganism that requires oxygen to survive, but requires environments containing lower levels of oxygen than are present in the atmosphere (~20% concentration). In the laboratory they can be easily cultivated in a candle jar. A candle jar is a container into which a lit candle is introduced before sealing the container's airtight lid. The candle's flame burns until extinguished by oxygen deprivation, which creates a carbon dioxide-rich, oxygen-poor atmosphere in the jar. Many labs also have access directly to carbon dioxide and can add the desired carbon dioxide levels directly to incubators where they want to grow microaerophiles (Fig. 3).

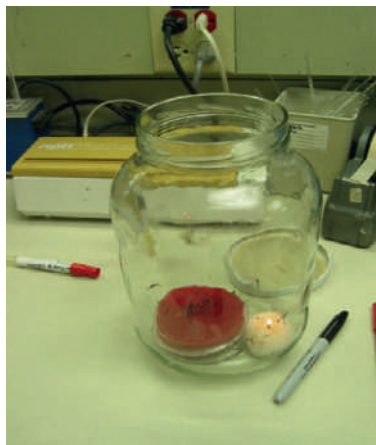


Fig. 3 Candle jar: A candle is lit in a jar with a culture plate. The lid is put on, as the candle burns it increases the carbon dioxide levels in the jar. Adopted from Special Culture Techniques. From <https://bio.libretexts.org/@go/page/9166>.

Nottingham and Hungate first used a nonselective medium but a stringent atmosphere consisting of 80% H₂ and 20% CO₂ to isolate nonidentified methanogenic Archaea from humans (Nottingham and Hungate, 1968). When the concentration of oxygen required to obtain growth is relatively low, the bacterial species are considered microaerophiles, such as *Campylobacter* spp., which can cause human infections involving primarily the gastrointestinal tract (Fitzgerald and Nachamkin, 2007). Most *Campylobacter* species require a microaerobic atmosphere containing 5% O₂, 10% CO₂, and 85% N₂ for optimal recovery. Recently, a microaerophilic atmosphere demonstrated better efficiency than aerobic conditions in promoting *Mycobacterium* culture and was proposed as a routine condition for laboratories performing these cultures (Chodbane et al., 2014).

To date spirochaetes are very difficult to rear in a controlled laboratory environment. This also includes other human pathogens like the bacterium that causes Lyme disease. Using animals to culture human-pathogens has problems. First, the use of animals is always difficult for technical and ethical reasons. Also, a microbe growing on animal other than a human may behave very differently from how that same microbe will behave on a human. Some human pathogens are grown directly on cells cultured from humans. Exemplified by the bacteria *Chlamydia trachomatis*, the bacteria responsible for the sexually transmitted infection (STI) in humans known as Chlamydia. As *Chlamydia trachomatis* only grows in humans. The human cell culture known as McCoy cell culture is used to culture this bacteria (Fig. 4).

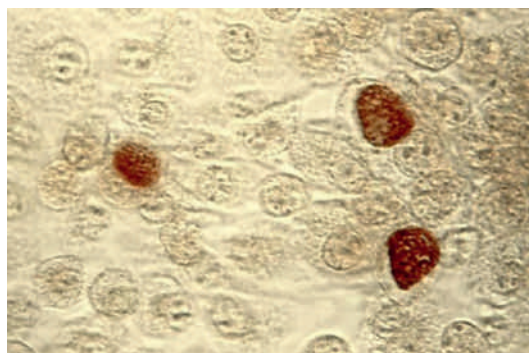


Fig. 4 Chlamydia bacteria group. Light microscope view of cells infected with chlamydia as shown by the brown inclusion bodies. Adopted from Special Culture Techniques. from <https://bio.libretexts.org/@go/page/9166>.

Aseptic culture transfer technique

Microorganisms are transferred from one medium to another by subculturing. This technique is of basic importance and is used routinely in preparing and maintaining stock cultures, as well as in microbiological test procedures. The essential steps involved in aseptic transfer of microorganisms are as follows:

1. An inoculating loop is sterilized by holding it in the hottest portion of the Bunsen burner flame until the entire wire becomes red-hot. Once flamed, the loop is held in the hand and allowed to cool for 10–20 s.
2. The cotton plug of the stock culture tube is unplugged with the help of little finger, the neck of the tube is briefly passed through the flame and the sterile loop is further cooled by touching it to the sterile wall of the culture tube before removing a small sample of inoculum.
3. The neck of the stock culture tube is flamed and the cotton plug is reinserted.
4. The cotton plug of the subculture tube is unplugged likewise, the neck of the tube is briefly passed through the flame and the cell-laden loop is inserted into the subculture tube.
5. In case of a broth medium, the loop is shaken slightly to dislodge the organisms; with an agar slant medium, it is drawn lightly over the hardened surface in a zigzag line.
6. The neck of the subculture tube is flamed and the cotton plug is reinserted.
7. Following inoculation, the loop is again flamed to destroy remaining organisms.
8. In case of liquid-liquid transfer, sterile tips are used to inoculate cultures (1%) (Fig. 5).

Culture transfer procedure. Adapted from <http://microbiologist161998.blogspot.com/2017/09/the-first-experiment.html>.

Pure culture

A pure culture is a population of cells or multicellular organisms growing in the absence of other species or types. A pure cultures can be grown in petri dishes of differing sizes that have a thin layer of agar-based growth medium. Once the growth medium in the petri dish is inoculated with the desired bacteria, the plates are incubated at the best temperature for the growing of the selected bacteria (for example, usually at 37 °C for cultures from humans or animals or lower for environmental cultures). Another method of bacterial culture is liquid culture, in which the desired bacteria are suspended in liquid broth, a nutrient medium. These are ideal for

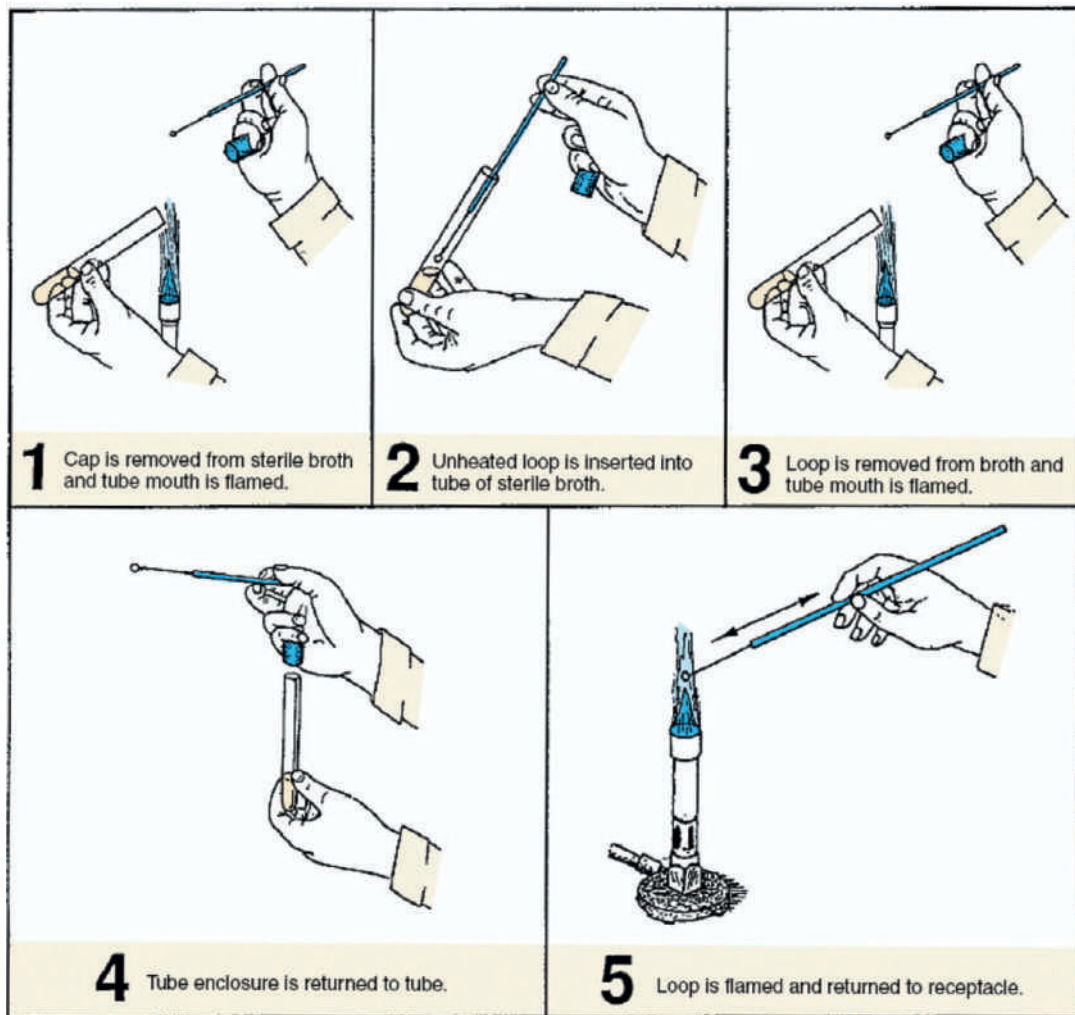


Fig. 5 Culture transfer procedure. Adapted from <http://microbiologist161998.blogspot.com/2017/09/the-first-experiment.html>.

preparation of an antimicrobial assay. The experimenter would inoculate liquid broth with bacteria and let it grow overnight (they may use a shaker for uniform growth). Then they would take aliquots of the sample to test for the antimicrobial activity of a specific drug or protein (antimicrobial peptides). As an alternative, the microbiologist may decide to use static liquid cultures. These cultures are not shaken and they provide the microbes with an oxygen gradient.

Isolation of pure culture

Microorganisms exist in nature as mixed populations and to study microorganisms in the laboratory, we must have them in the form of a pure culture, that is, one in which all organisms are descendants of the same organism. Two major steps are involved in obtaining pure cultures from a mixed population:

1. The mixture must be diluted until the various individual microorganisms become separated far enough apart on an agar surface that after incubation they form visible colonies isolated from the colonies of other microorganisms. This plate is called an isolation plate.
2. Then, an isolated colony can be aseptically “picked off” the isolation plate (Fig. 6) and transferred to new sterile medium. After incubation, all organisms in the new culture will be descendants of the same organism, that is, a pure culture.

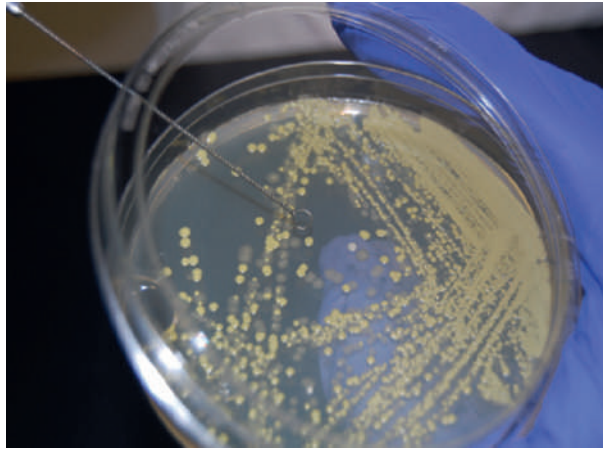


Fig. 6 Picking a single colony from a Petri Plate in order to obtain a pure culture. Before removing bacteria from the petri plate, first cool the loop by sticking it into the agar away from any growth. Adopted from Pure Cultures from a Mixed Population. from <https://bio.libretexts.org/@go/page/3442>.

Enrichment & isolation

Enrichment culture is the technique that is used to enhance the population density of a particular group of microorganisms within the total microbial population of a sample. This is achieved by preferentially stimulating the growth of the target group of microorganisms by judicious manipulation of the physiological conditions during the enrichment phase. Enriched media contain the nutrients required to support the growth of a wide variety of organisms, including some of the more fastidious ones. They are commonly used to harvest as many different types of microbes as are present in the specimen. Enrichment culture is the use of certain growth media to favor the growth of a particular microorganism over others, enriching a sample for the microorganism of interest. Enrichment cultures are used to increase a small number of desired organisms to detectable levels. For example, the primary enrichment component is blood, which provides hemin and other nutrients. This component is frequently added to agar media in several commercial media to notably increase the growth of anaerobic bacterial species (Sonnenwirth, 1972). Blood agar, which was initially accidentally used for *Mycobacterium tuberculosis* culture, was also shown to be a cost- and time-effective method, with better growth than with the egg-based agar reference medium (Drancourt et al., 2003; Drancourt and Raoult, 2007).

In addition, sometimes the number of pathogens present in biological samples are lower than the detection limits of the available methods, thus requiring additional pre-enrichment steps which add costs to the diagnostics and increase the turnaround time. Moreover, samples usually associated with low bacterial numbers (e.g., CNS fluid) or in mixed populations (e.g., stool), can benefit from a diagnostic approach using enrichment of samples prior to downstream processing (Sande et al. 2020). Broth enrichment media are used when high sensitivity is required e.g. for detection of bacteria from CSF, or to detect small numbers of *Salmonella* in a stool sample containing many millions of other bacteria.

Enumeration of bacteria

To determine rates of microbial growth and death, it is necessary to enumerate microorganisms, that is, to determine their numbers. It is also often essential to determine the number of microorganisms in a given sample. For example, the ability to determine the safety of many foods and drugs depends on knowing the levels of microorganisms in those products. A variety of methods has been developed for the enumeration of microbes and these methods measure cell numbers, cell mass, or cell constituents that are proportional to cell number. The four general approaches used for estimating the sizes of microbial populations are direct and indirect counts of cells and direct and indirect measurements of microbial biomass. Each method will be described in more detail below.

Direct count of cells

Cells are counted directly under the microscope or by an electronic particle counter. Two of the most common procedures used in microbiology are discussed below.

Direct count using a counting chamber

Direct microscopic counts are performed by spreading a measured volume of sample over a known area of a slide, counting representative microscopic fields, and relating the averages back to the appropriate volume-area factors. Specially constructed

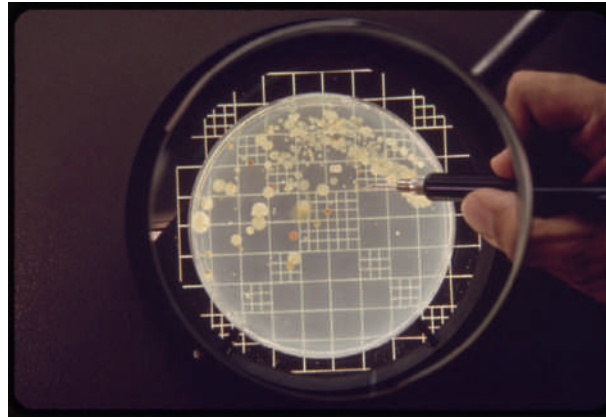


Fig. 7 An example of counting colonies on a streak plate. Adopted from Direct counting of Bacteria. From <https://courses.lumenlearning.com/boundless-microbiology/chapter/counting-bacteria/>.

counting chambers, such as the Petroff-Hauser and Levy counting chambers, simplify the direct counting procedure because they are made with depressions in which a known volume overlies an area that is ruled into squares. The ability to count a defined area and convert the numbers observed directly to volume makes the direct enumeration procedure relatively easy. Direct counting procedures are rapid but have the disadvantage that they do not discriminate between living and dead cells. This method is used to assess the sanitation level of a food product and in performing blood cell counts in hematology. The differential white blood cell count, which is used as an indication of the nature of a microbial infection, involves direct counting of blood cells that have been stained to differentiate different types of white blood cells (Fig. 7).

Direct count using fluorescent dyes

Fluorescent dyes are becoming more used in recent years for a variety of procedures, one of which is bacterial counts. These dyes can be employed to stain all species, a particular species of interest in an environmental sample or even a specific component of cells. The most widely used fluorescent dye for counting the number of bacterial cells is acridine orange which stains both living and dead cells by interacting with DNA and protein components of cells. The stained cells fluoresce orange when excited near ultraviolet light. This stain is particularly useful for determining the total number of microorganisms in samples, such as soil and water, where the co-existence of metabolically diverse populations precludes establishing conditions for the simultaneous enumeration of microbial populations by viable count procedures. The procedure is widely used where population levels are often low and where viable plate counts are known to severely underestimate total number of bacteria. Typically, the viable count is less than 1% of the direct count for marine samples. In this procedure the bacteria in a known volume of sample are stained with acridine orange and the sample is then filtered through a 0.22 μm filter. The bacteria are trapped on the filter that is then examined under a fluorescence microscope. The bacteria in a defined area of the filter are counted and the concentration in the original sample is then calculated. Other fluorescent dyes that are also gaining popularity are cyanoditolyl tetrazolium chloride (CTC), auramine and rhodamine. CTC binds to respiration proteins in the cell and thus can demonstrate live cells. Auramine and rhodamine bind to cell wall of Mycobacteria and emit bright yellow or orange color under a fluorescent microscope. These latter stains are gradually replacing the acid-fast stain.

Indirect count of cells

Microorganisms in a sample are diluted or concentrated and grown on a suitable medium; the development of growing microorganisms (for example, colony formation on agar plates) is then used to estimate the numbers of microorganisms in the original sample.

Viable cell counting

Plate counting is used to estimate the number of viable cells that are present in a sample. A viable cell count allows one to identify the number of actively growing/dividing cells in a sample. The plate count method or spread plate relies on bacteria growing a colony on a nutrient medium. The colony becomes visible to the naked eye and the number of colonies on a plate can be counted. The most common procedure for the enumeration of bacteria is the viable plate count. In this method, serial dilutions of a sample containing viable microorganisms are plated onto a suitable growth medium. The suspension is either spread onto the surface of agar plates (spread plate method), or is mixed with molten agar, poured into plates, and allowed to solidify (pour plate method). The plates are then incubated under conditions that permit microbial reproduction so that colonies develop that can be seen without the aid of a microscope. It is assumed that each bacterial colony arises from an individual cell that has undergone cell division. Therefore, by counting the number of colonies and accounting for the dilution factor, the number of bacteria in the original sample can be determined (Fig. 8).

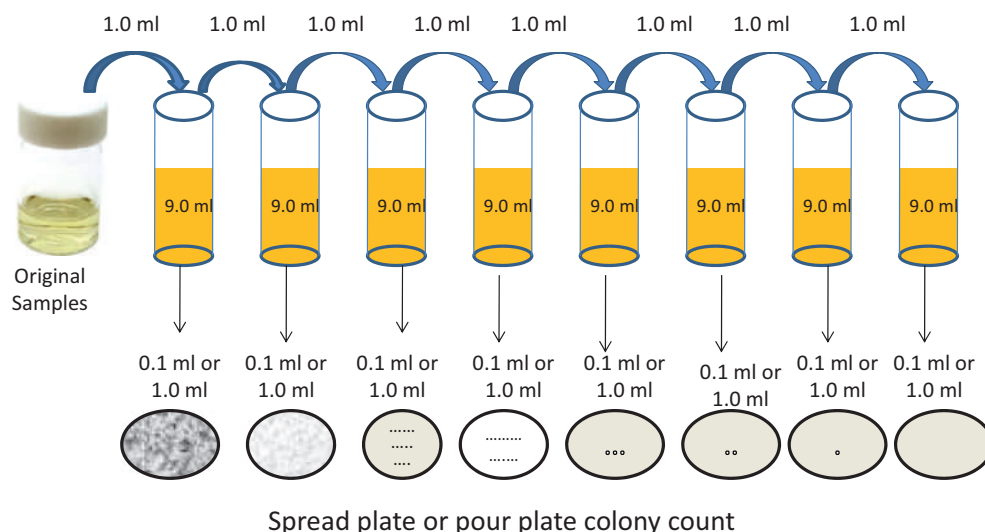


Fig. 8 An example of viable bacterial colonies count.

There are several drawbacks to the viable count method. The major disadvantage is that it is selective and therefore biased. The nature of the growth conditions, including the composition and pH of the medium used as well as the conditions such as temperature, determines which bacteria in a mixed population can grow. Since there is no universal set of conditions that permits the growth of all microorganisms, it is impossible to enumerate all microorganisms by viable plating. This same disadvantage, however, becomes advantageous when one is interested in only a specific microbial population. For example, we can design selective procedures for the enumeration of coliforms and other physiologically defined microbial groups. The viable count is an estimate of the number of cells. Because some organisms exist as pairs or groups and because mixing and shaking of the sample does not always separate all the cells, we actually get a count of the “colony forming units.” One cell or group of cells will produce one colony, therefore when we record results for a viable count, it is customary to record the results as colony forming units per ml (cfu/mL) or per gram (cfu/g) of test material.

Because we generally have no idea of how many bacteria are in a sample, it is almost always necessary to prepare a dilution series to ensure that we obtain a dilution containing a reasonable number of bacteria to count. Dilutions in the range 10^{-1} (1/10) to 10^{-8} (1/100,000,000) are generally used, although with particular types of samples the range of dilutions can be restricted. For example, for water that is not turbid, the maximal dilution needed is 10^{-6} because we know that if there were 10^7 or more bacteria per milliliter, the water would be turbid.

The most probable number (MPN)

The most probable number procedure dates back to the earliest days of microbiology. However, it is still widely used in sanitary bacteriology to estimate numbers of coliforms in water, milk, and other foods. Coliforms are bacteria that reside in the intestine of warm-blooded mammals and are regularly excreted in the feces. They are Gram negative rods belonging to the Enterobacteriaceae family, ferment lactose and produce gas. Not all members of Enterobacteriaceae are coliforms.

The MPN procedure is a statistical method based upon the probability theory. Samples are serially diluted to the point of extinction, that is, to a point where there are no more viable microorganisms. To detect the end point, multiple serial dilutions are inoculated into a suitable growth medium, and the development of some recognizable characteristic, such as acid production or turbidity, is used to indicate growth (the presence of at least one viable microorganism in the diluted sample). The pattern of positive tests (growth) in the replicates and statistical probability tables are used to determine the concentration (most probable number) of bacteria in the original sample. Statistical MPN tables are available for replicates of 3, 5, and 10 tubes of each dilution. The more replicate tubes used, the greater the precision of the estimate of the size of the bacterial population.

For example, a three-tube MPN procedure to estimate the numbers of coliforms in a water sample as shown in Fig. 9. First, decimal dilutions of the sample are prepared and then 1 mL of each dilution is inoculated into three broth culture tubes (different numbers of replicates and dilution series can be used). After incubation, tubes are examined for turbidity and those that are positive are recorded for each dilution. For example, if all tubes show growth, the results will be noted as 333. Once the results have been noted, the MPN table (MacGrady table) should be used to determine the most probable number of microorganisms for mL or g.

Direct measurement of microbial biomass

Cell mass is determined directly by weighing whole cells; biomass can be correlated with cell numbers by reference to a standard curve. Wet weight or dry weight of bacteria may be used for estimation of cell numbers.

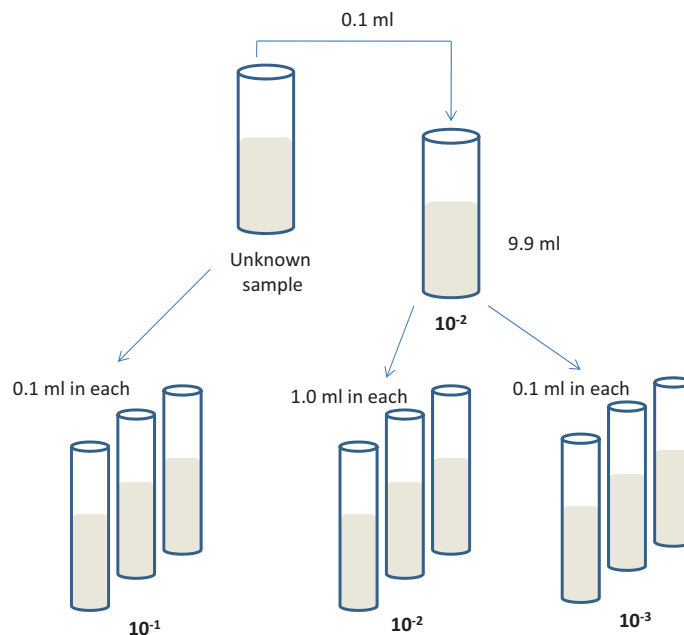


Fig. 9 The enumeration of coliforms in water using the Most Probable Number (MPN) method APHA/AWWA/WEF 2005 (Hunt and Rice, 2005).

Indirect measurement of microbial biomass

Microbial biomass is estimated by measuring relatively constant biochemical components of microbial cells, such as protein, ATP, lipopolysaccharides, peptidoglycan, and chlorophyll; biomass can also be indirectly estimated by measured turbidity that can then be correlated with cell numbers by reference to a standard curve. Various procedures based on the detection of specific microbial macromolecules or metabolic products can be used to estimate numbers of microorganisms. For example, peptidoglycan can be quantified, and because this biochemical occurs exclusively in the cell wall of bacteria, the concentration of peptidoglycan can be used to estimate bacterial numbers. Such biochemical approaches for determining bacterial numbers depend on the development of analytical chemical procedures for quantifying the particular biochemical and determining what proportion of bacterial cell is composed of the specific biochemical constituent.

Spectrophotometers are electrical appliances that can measure turbidity very accurately. The culture is placed in a translucent cuvette; the cuvette is placed in the machine and the turbidity measured immediately (shown in Fig. 10). Simple mathematical formulae help convert the detected turbidity to cell concentration.

Preserving bacterial cultures

The preservation of microorganisms plays a key role in ensuring reproducible results and continuity in research. Maintaining a library of microbial stocks also enables microorganisms to be easily stored and retrieved, as compared to continuously sub-culturing

Spectrophotometer and its principle

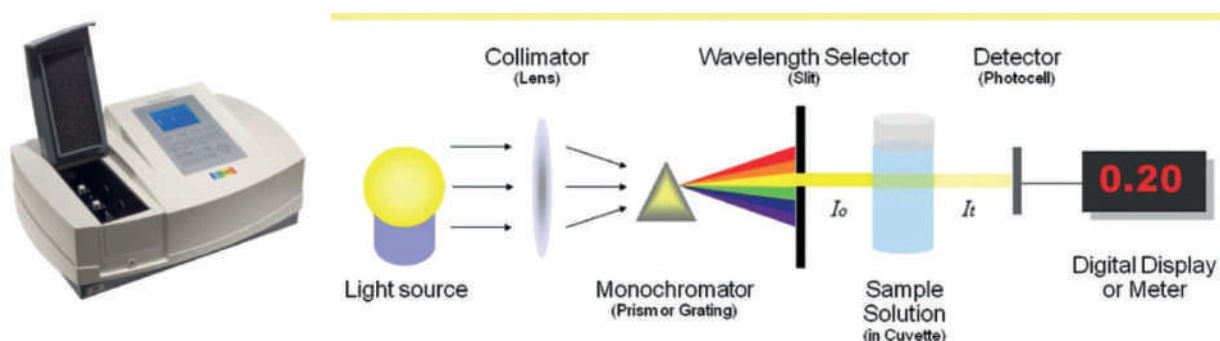


Fig. 10 Spectrophotometer and its mechanisms of measurement.

microbial cultures on plates or in tubes. Depending on the length of storage and type of microorganism, different preservation methods are used. The underlying principle of long term preservation is to keep microorganisms in a dormant state, free from contamination and genetic changes, until ready to be brought back to life later. This is conventionally accomplished by using low temperatures, and freezing or drying techniques to reduce cellular metabolic activity. More recently, microencapsulation has also been explored as a novel method of microbial preservation for probiotic applications.

Short term storage (continuous growth)

Continuous growth of microorganisms can be maintained for short periods of time on agar medium. Agar plate cultures of microorganisms are viable for a few weeks when kept in the laboratory refrigerator (4 °C), while bacterial cultures can be stored for up to a year at 4 °C as agar stab cultures (Fig. 11). Most microorganisms (bacteria, fungi, and algae) can be stored using this method and it is recommended for cultures which are used regularly. Using nutrients from the culture media, these cultures grow continuously, albeit at a slower rate at lower temperatures, enabling them to survive for a longer time on the available nutrients.

To minimize contamination and drying of the agar, culture plates should be securely wrapped with laboratory film and stored upside down (agar side up). Tubes and vials containing agar stab cultures should be securely capped. An advantage of short term storage on agar medium is that it is easy to recover the microorganisms later. Just streak them onto fresh culture plates or inoculate them into liquid culture medium and incubate at the optimum growth temperature.

However, over a prolonged period of time, the agar dries up and nutrients in the culture media are depleted by the microorganisms. Metabolic waste products also accumulate to toxic levels. Nutrient starvation and toxic waste product build-up eventually cause the death of the stored microorganisms.

Long term storage

For long term storage, microorganisms are cryopreserved at temperatures below freezing (−20 °C, −80 °C, or liquid nitrogen) or freeze dried (lyophilized). Cryopreservation is suitable for a wide range of bacteria, algae, fungi, viruses, and protozoa. Ultralow temperatures drastically reduce enzymatic activity and, thus, metabolic processes of microorganisms. To prevent damage to cells caused by ice crystal formation, cells are resuspended in growth media containing cryoprotectants such as glycerol prior to storage at −20 °C, −80 °C, or in liquid nitrogen (−196 °C). Alternatively, cells can also be resuspended in skim milk before being frozen (Cody et al., 2008). To maximize the recovery rate, it is recommended to freeze cultures at the stationary phase of growth when cell concentration is highest. Most cultures can be stored in cryogenic vials (Fig. 12) at −20 °C for at least a year, with some bacterial stocks remaining viable for several decades when stored at −80 °C and in liquid nitrogen. Frozen microorganisms can be revived by thawing at 37 °C and inoculating into fresh culture medium (Tedeschi and De Paoli, 2011).

Freeze drying (lyophilization) removes moisture from frozen samples by converting water into ice in a vacuum, thus arresting microbial metabolic processes and transforming cells into dry pellets for convenient storage and transportation in ampoules (Fig. 13). Freeze drying has been used to preserve bacteria, algae, yeasts, viruses, and sporulating fungi (Prakash et al., 2013). However, lyophilization is not suitable for certain bacteria and non-sporulating fungi as they cannot survive the stresses of freeze drying. Lyophilized cultures can be recovered by rehydrating the cell pellets in growth media (bacteria and algae), growth medium containing the bacterial hosts (bacteriophages), or water (fungi and yeasts) before inoculating into fresh culture media.

Preserving microorganisms using microencapsulation

Microencapsulation, where cells are entrapped in a matrix prior to storage, has been proposed as a long term microbial preservation method which does not expose the microorganisms to the harsh stresses of freezing and drying. The matrix shields the cells and increases stability during storage. Microencapsulation of probiotic bacteria in calcium alginate has been shown to improve their viability when stored at −80 °C. Electrospinning and electrospraying, where microorganisms are trapped in nanofibers and droplets



Fig. 11 (A) A single bacterial colony is plunged into a vial of soft agar using a straight wire and incubated to form an agar stab culture. Image credit: Wikipedia. (B) Bacteria is streaked onto a plate of agar culture medium and incubated. Image credit: Orbit Biotech. (C) TSI Agar slant tubes: The agar triple-sugar iron is one of the culture media used for the differentiation of most enterobacteria. Image Credit: Orbit Biotech.



Fig. 12 Orange screw-capped cryogenic vial designed to be opened and closed with one hand when used on a cryogenic rack. Image credit: ebay.



Fig. 13 Ampoule containing lyophilized microorganisms. Image credit: Meihua Anaerobic Culture Bottle.

respectively, have also been used to preserve the viability of sensitive probiotic bacteria (Alonso, 2016). Microbial preservation techniques enable the viability of microorganisms to be maintained, paving the way for their potential to be explored for years to come. With the right methods, some of these microorganisms may still be around in freezers long after we are gone, waiting to be revived by future generations of researchers.

Yeast & mold culture media

Yeasts are commensal and are widely found in human cavities, mucosa and digestive tracts. They can become pathogenic and engender opportunistic infections when favorable conditions arise in the host body, such as when the immune system is weakened. Opportunistic fungal infections range from superficial mycosis, which are localized skin and soft tissue infections to invasive mycosis, which can cause disease in almost any organ system. Although *Candida albicans* is still the most commonly found pathogenic yeast species, it is now also important to clearly identify other resistant *Candida* species which cause superficial and/or invasive infections, such as *Candida glabrata* and *Candida krusei*.

Fungi can be broadly classified into two groups; yeasts and molds. Yeasts are single-celled organisms that reproduce by budding but molds grow as filaments like structure. Many medically important fungi show unique behavior based on the temperature of growth and habitat, which is known as dimorphism (di: both, morphism: morphological form). These dimorphic fungi exist as molds in the environment at ambient temperature (25 °C–30 °C) and as yeasts (or other structure e.g., spherules in case of *Coccidioides immitis*) in human tissues at body temperature (35 °C–37 °C). Most of the dimorphic fungi cause systemic mycoses and *Sporothrix* (one of the dimorphic fungus) causes subcutaneous mycoses. Among the subcutaneous mycoses causing fungi, *Sporothrix schenckii* is a notable dimorphic fungus. It causes sporotrichosis. *Penicillium marneffe* is a mold and an opportunistic pathogen causing systemic penicilliosis in AIDS patients in Southeast Asian countries. Approximately 8% of nosocomial infections have a fungal origin, generally linked to the *Candida* genus. More commonly pathogenic yeast species includes *Candida albicans* and

Cryptococcus neoformans and genera that may cause severe infections in some high-risk patients: Dermatophytes: (*Microsporum*, *Epidermophyton*; *Trichosporon*), *Saccharomyces* and *Rhodotorula*.

Yeasts are able to grow on routine bacteriological media, such as blood agar and chocolate agar plates. If yeasts are present together with a mixed bacterial population, it is, however, possible that bacteria will suppress yeast growth. It is therefore generally accepted that a selective medium such as Sabouraud agar should be used for the cultivation of yeasts from clinical specimens (Merz and Roberts, 1995).

Two general types of culture media are essential to ensure the primary recovery of all clinically significant fungi from clinical specimens. One medium should be non-selective (such as Brain Heart Infusion Agar; i.e., one that will permit the growth of virtually all clinically relevant fungi) and other media should be selective, specially tailored to isolate specific pathogenic fungi of interest. For optimal recovery of fungal pathogen, a battery of media should be used, and the followings are recommended:

1. Media with or without cycloheximide (cycloheximide is added to inhibit the growth of rapidly growing contaminating molds.)
2. Media with or without an antibacterial agent (chloramphenicol, gentamicin and ciprofloxacin are commonly used antibacterial for this purpose).
3. Antibacterial agents are used to kill the contaminating bacterial species. If the sample is taken from sterile site, it is not necessary to use media containing antibacterial agents (Table 4).

Virus and phage culture media

In the early 1900s, viruses were isolated using embryonated eggs and laboratory animals. Cell cultures are typically generated from tissue samples and then disaggregated using mechanical, chemical, and enzymatic methods to extract cells suitable for virus isolation. The use of laboratory animals in research has declined dramatically as a result of the use of cell culture technology (Freshney, 2000). Furthermore, the number of viruses indexed has increased significantly as a result of the selection of appropriate cell lines. The isolation of viral pathogens in cell cultures began in the 1960s, but there were some drawbacks at the time, including a lack of facilities for diagnosing viral infections. The commercial invention of purified reagents and cell lines in 1970 opened a new window for viral infection diagnosis (Hsiung, 1984). Many human viruses were grown in vitro after cell culture was discovered. Cell culture is more convenient and cost-effective than using eggs or poultry. For virus isolation and detection, this approach is considered the gold standard (Hsiung, 1984).

Virus culture media

A range of media have been formulated for growth of vertebrate cells in culture. These incorporate various concentrations of amino acids, vitamins, enzymes, growth factors, and inorganic salts. Glucose, fructose, or galactose are also added along with glutamine to provide a carbon source for cell metabolism. There is a variety of formulations for cell culture media:

- Dulbecco Minimal Essential Medium (DMEM) is in common use for continuous cell lines.
- CMRL medium is particularly suited for the propagation of semi continuous cell lines.
- RPMI 1640 is recommended for growth of Lymphoblastoid cells in suspension.

Table 4 Commonly used yeast and mould culture media.

Brain-heart infusion (BHI) agar	It is a non-selective fungal culture medium that permits the growth of virtually all clinically relevant fungi. It is used for the primary recovery of saprophytic and dimorphic fungi
Czapek's agar	It is used for the subculture of <i>Aspergillus</i> species for their differential diagnosis.
Inhibitory mold agar (IMA)	Primary recovery of dimorphic pathogenic fungi. Saprophytic fungi and dermatophytes will not be recovered.
Mycosel/Mycobiotic agar	It is generally Sabouraud's dextrose agar with cycloheximide and chloramphenicol added. It is used for the primary recovery of dermatophytes.
Niger Seed Agar	It is used for the identification of <i>Cryptococcus neoformans</i> .
Potato Dextrose Agar (PDA)	It is a relatively rich medium for growing a wide range of fungi.
Sabouraud's Heart Infusion (SABHI) agar	Primary recovery of saprophytic and dimorphic fungi, particularly fastidious strains.
Sabouraud's dextrose agar (SDA)	Sabouraud's agar is sufficient for the recovery of dermatophytes from cutaneous samples and yeasts from genital cultures. Not recommended as a primary isolation medium because it is insufficiently rich to recover certain fastidious pathogenic species, particularly most of the dimorphic fungi. Sabouraud's dextrose agar (2%) is most useful as a medium for the subculture of fungi recovered on enriched medium to enhance typical sporulation and provide the more characteristic colony morphology.
Potato flake agar	Primary recovery of saprophytic and dimorphic fungi, particularly fastidious and slow-growing strains.

Conditions for growth of cell cultures

Optimum pH

A pH range of 7.1–7.5 is required for the growth of eukaryotic cells. Most culture media use bicarbonate buffer systems (CO_2/HCO_3) to maintain pH. These media are formulated with NaHCO_3 and CO_2 is either provided by the cultured cells as a metabolic product or by enrichment of the atmosphere using a CO_2 incubator. It is common to supplement the bicarbonate buffer system with HEPES buffer, it overrides all other buffers present and obviates the need for CO_2 enriched atmosphere.

Osmolarity

The growth of cells in culture depends on an optimum range of osmotic pressures, usually between 280 and 320 mmol/kg.

Serum

Balanced salt solutions will support cell proliferation only when supplemented with serum, lactalbumin hydrolysate, or other supplements. The serum provides essential amino acids, nucleic acid precursors, and fatty acids and also provides hormones and inhibits the protease used for routine dissociation of cells for culture. Fetal or newborn calf serum are used at conc. 5–15% to promote cell growth and at reduced conc. of 0–2% for maintenance of confluent monolayer cultures. Serum should be stored at -70°C , repeated freezing and thawing should be avoided.

Antibiotics

Antibiotics providing broad spectrum protection from bacterial contaminants are: benzylpenicillin, 20–100 units/mL, gentamicin, 16–50 $\mu\text{g/mL}$, and tetracycline, 10 $\mu\text{g/mL}$, Amphotericin B, 0.5 $\mu\text{g/mL}$, or nystatin, 50 units/mL, are recommended for control of fungal infections. Stock solutions should be stored at -20°C .

Isolation of viruses

Viruses, unlike bacteria, which can be grown on a synthetic nutrient medium, require a living host cell to replicate. Infected host cells (eukaryotic or prokaryotic) can be cultured and grown, and the growth medium can then be harvested for virus production. Centrifugation or filtration may be used to isolate virions in the liquid medium from the host cells. Filters will physically extract something larger than virions from a solution, allowing the viruses to accumulate in the filtrate (see Fig. 14).

Cultivation of viruses

Viruses can be grown *in vivo* (within a whole living organism, plant, or animal) or *in vitro* (outside a living organism in cells in an artificial environment, such as a test tube, cell culture flask, or agar plate). Bacteriophages can be grown in the presence of a dense layer of bacteria (also called a bacterial lawn) grown in a 0.7% soft agar in a Petri dish or flat (horizontal) flask (see Fig. 15). The agar concentration is decreased from the 1.5% usually used in culturing bacteria. The soft 0.7% agar allows the bacteriophages to easily

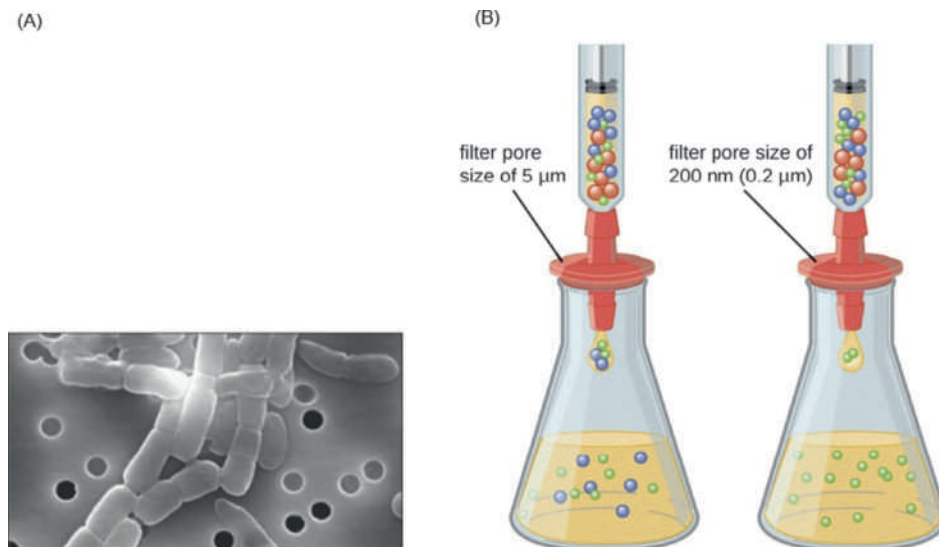


Fig. 14 Membrane filters can be used to remove cells or viruses from a solution. (A) This scanning electron micrograph shows rod-shaped bacterial cells captured on the surface of a membrane filter. Note differences in the comparative size of the membrane pores and bacteria. Viruses will pass through this filter. (B) The size of the pores in the filter determines what is captured on the surface of the filter (animal [red] and bacteria [blue]) and removed from liquid passing through. Note the viruses (green) pass through the finer filter. Credit A: modification of work by U.S. Department of Energy.

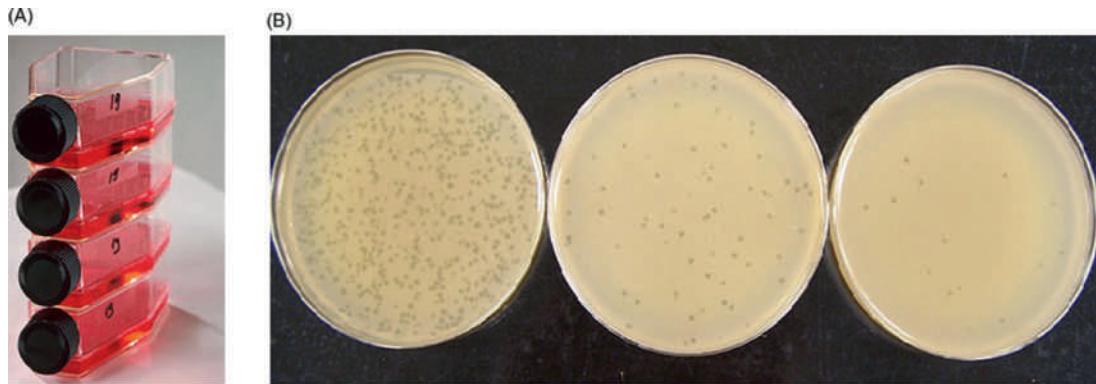


Fig. 15 (A) Flasks like this may be used to culture human or animal cells for viral culturing. (B) These plates contain bacteriophage T4 grown on an *Escherichia coli* lawn. Clear plaques are visible where host bacterial cells have been lysed. Viral titers increase on the plates to the left. Credit A: modification of work by National Institutes of Health; Credit B: modification of work by American Society for Microbiology.

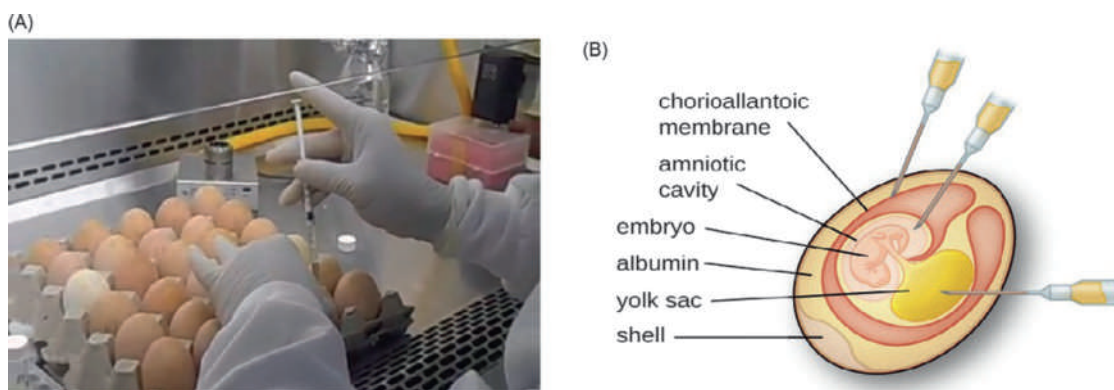


Fig. 16 (A) The cells within chicken eggs are used to culture different types of viruses. (B) Viruses can be replicated in various locations within the egg, including the chorioallantoic membrane, the amniotic cavity, and the yolk sac. Credit A: modification of work by “Chung Hoang”/YouTube.

diffuse through the medium. For lytic bacteriophages, lysing of the bacterial hosts can then be readily observed when a clear zone called a plaque is detected (see Fig. 15). As the phage kills the bacteria, many plaques are observed among the cloudy bacterial lawn.

Animal viruses require cells within a host animal or tissue-culture cells derived from an animal. Animal virus cultivation is important for (1) identification and diagnosis of pathogenic viruses in clinical specimens, (2) production of vaccines, and (3) basic research studies. In vivo host sources can be a developing embryo in an embryonated bird’s egg (e.g., chicken, turkey) or a whole animal. The embryo or host animal serves as an incubator for viral replication (see Fig. 16). Location within the embryo or host animal is important. Many viruses have a tissue tropism, and must therefore be introduced into a specific site for growth. Within an embryo, target sites include the amniotic cavity, the chorioallantoic membrane, or the yolk sac. Viral infection may damage tissue membranes, producing lesions called pox; disrupt embryonic development; or cause the death of the embryo.

For in vitro studies, various types of cells can be used to support the growth of viruses. For example, HeLa and HEp2 are used for cultivation of HSV, adenovirus, poliovirus and some coxsackie viruses. Vero cells will also support growth of these viruses and are used with BHK21 cells for growth of arboviruses. RK13 cells and BHK21 cells for isolation and propagation of *rubella virus*. RD cells for the isolation of *coxsackie A virus*.

A primary cell culture is made from animal organs or tissues that have been freshly prepared. Mechanical scraping or mincing to release cells or an enzymatic process using trypsin or collagenase to break up tissue and release single cells into suspension are used to extract cells from tissues. Because of the need for anchorage, primary cell cultures require a liquid culture medium in a Petri dish or tissue-culture flask so that the cells can adhere to and expand on a solid surface such as glass or plastic. The lifespan of primary cultures is usually short. A secondary cell culture is when the cells from a primary cell culture are moved to a new vessel with fresh growth medium. Cell density must be decreased on a regular basis by pouring off some cells and replacing them with fresh medium to provide space and nutrients for cell development. Continuous cell lines, which are normally produced from transformed cells or tumors, may also be subcultured several times or even grown indefinitely, in comparison to primary cell cultures. As a consequence, continuous cell lines may form lumps or piles that resemble small tumors (see Fig. 17).

The HeLa cell line, which was originally cultivated from tumor cells derived from Henrietta Lacks, a woman who died of cervical cancer in 1951, is an example of an immortal cell line.

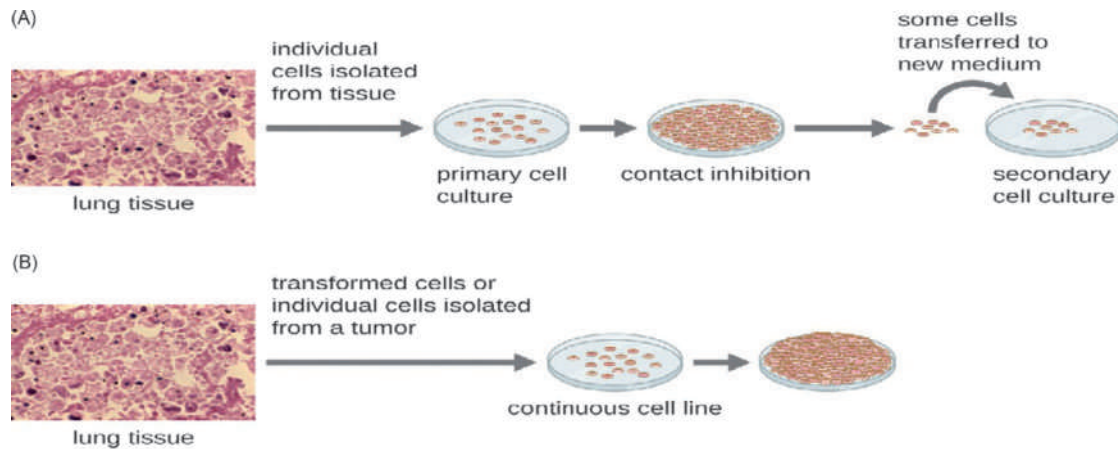


Fig. 17 Cells for culture are prepared by separating them from their tissue matrix. (A) Primary cell cultures grow attached to the surface of the culture container. Contact inhibition slows the growth of the cells once they become too dense and begin touching each other. At this point, growth can only be sustained by making a secondary culture. (B) Continuous cell cultures are not affected by contact inhibition. They continue to grow regardless of cell density. Credit “micrographs”: modification of work by Centers for Disease Control and Prevention.

HeLa cells were the first continuous tissue-culture cell line, and they helped to develop tissue culture as a valuable tool in cell biology, virology, and medicine research. Prior to the discovery of HeLa cells, scientists were unable to reliably and consistently create tissue cultures. This cell line is still alive and being used for medical research after more than six decades.

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Antibody Tests

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Glossary

Antigen Pathogen or a component of the pathogen recognized by antibodies.

Introduction

This article describes tests used to detect antibodies formed in response to infection. The predominant purpose for which antibody tests are applied is diagnosis. Antibody tests provide a longer diagnostic window than direct pathogen detection methods and therefore provide information about the etiology of an infection after the acute phase when the pathogen or its components can no longer be detected. For some viruses in particular, for example in some human flavivirus infections with no or only transient viraemia, diagnosis can be heavily reliant on detection of antibodies. Typically, antibodies are measured in serum samples (serological tests). However, other clinical samples can be used, for example detection of antibodies in cerebrospinal fluid can help determine that a pathogen is the causative agent of neurological signs such as encephalitis.

Many antibody tests do not distinguish between immunoglobulin classes. Although immunoglobulin A (IgA) antibodies are important in the immune response against mucosal pathogens, most tests measure IgM and/or IgG antibodies. IgM is produced rapidly after an individual is first exposed to a pathogen, but levels also decline relatively rapidly (Fig. 1). Therefore, a test that is specific for IgM antibodies is useful for confirming a recent or active infection. The production of IgG antibodies starts a short time after IgM and typically lasts longer. An IgM response is generally not detected in a reactivated infection or re-infection, whereas the anamnestic IgG response is usually greater and longer-lasting than the primary response. Therefore, to distinguish whether IgG antibodies are present in a sample due to recent infection or previous exposure (either from infection or vaccination) it is often necessary to demonstrate a significant increase in IgG between two samples taken 7–10 days apart (seroconversion).

Antibody tests are also used in epidemiological studies. The true prevalence of a disease is an essential parameter to appraise the impact on a population, for example to decide whether to implement disease control programs. Serosurveillance can also be important for disease eradication programs. In the veterinary field, demonstrating that a livestock population is free from infection can have important trade implications. A further application of antibody tests is to monitor vaccine responses, for which the ability of antibodies stimulated by vaccination to neutralize infectivity of the pathogen is usually the most relevant measure of vaccine efficacy (in a controlled trial) or effectiveness (under field conditions).

Most antibody tests do not provide an absolute measure of the total amount of pathogen-specific antibody present in a sample. Antibody tests that involve performing a serial dilution of the sample provide a titer. For example, a titer of 1:40 indicates the last sample dilution at which the antibody has the effect that provides the read-out for the test (for example neutralization of virus infectivity) and does not indicate the amount of non-neutralizing antibodies present. Other assays such as the enzyme-linked immunosorbent assay (ELISA) provide a read-out that is a value proportional to the amount of antibody in a sample that binds specifically to the pathogen or a component of the pathogen. Limitations of the antibody tests include where cross-reactivity between antibodies to closely-related pathogens does not allow unambiguous interpretation of the results. In addition, in infants, maternally-derived antibodies may be present.

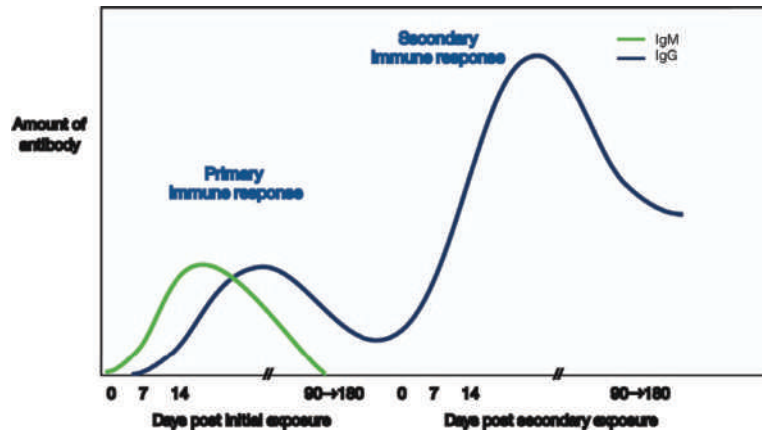


Fig. 1 Representation of immunoglobulin M (IgM) and IgG responses after primary and secondary infections. Modified from Murphy K, Travers P, Walport M and Janeway C (2008) *Janeway's Immunobiology*. New York: Garland Science.

Techniques for antibody detection

Neutralization tests

Neutralizing antibody tests are widely used in the field of virology. Most tests involve incubating a sample with a known amount of infectious virus for a short period before measuring the residual infectivity. A relatively straightforward virus neutralization test (VNT) involves titrating virus infectivity on a susceptible cell line that shows visible cytopathic effect (CPE) in a 96-well plate to obtain the 50% tissue culture infective dose (TCID₅₀) calculated using the Kärber or another similar formula. The virus is then diluted to a standardized amount and an equal volume of virus and serially diluted sample is then incubated typically for 30 min at 37 °C before adding the mixture to a cell monolayer and incubating for up to 4 or 5 days to obtain a readout. The virus-neutralizing antibody titer is presented as the highest dilution of serum at which no or <50% cytopathic effect is observed (Fig. 2). For viruses that do not have a discernible cytopathic effect, an additional test may need to be performed to determine whether virus replication has been reduced by the presence of antibodies, for example by immunostaining with a specific antibody or detecting viral genetic material by PCR or reverse transcription-PCR. This increases the cost and complexity of the test.

In recent years, some of the drawbacks of the VNT have been circumvented by using pseudotyped viruses. A pseudotyped virus (PV) typically consists of a retroviral core with the surface protein (or proteins) from the virus of interest packaging a reporter gene (e.g., luciferase). To generate an HIV-based PV, two protein plasmids expressing the surface protein of the virus of interest and HIV core structural proteins, and a third plasmid with a ψ packaging signal and reporter gene, are co-transfected into producer cells, typically human embryonic kidney (HEK) 293T cells (Fig. 3; Mather et al., 2013). The HIV core proteins and surface protein assemble into virus-like particles containing an RNA dimer of the reporter gene as the only genetic material. The PV can transduce

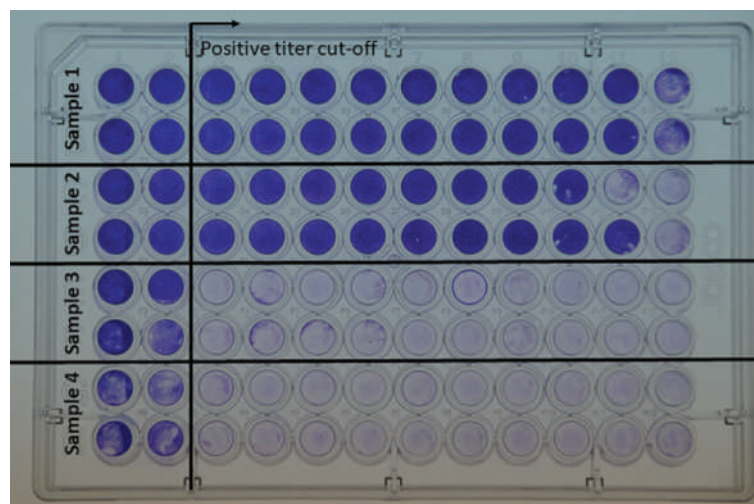


Fig. 2 Example of a virus neutralization test result. Briefly, serially-diluted bovine serum samples were incubated with 100 TCID₅₀ (50% tissue culture infectious doses) of Schmallenberg virus in duplicate for 1 h before adding Vero cells to each well. After 5 days' incubation, the cells were ethanol fixed and stained with 0.1% methylene blue. Samples 1 and 2 are antibody positive, samples 3 and 4 are antibody negative.

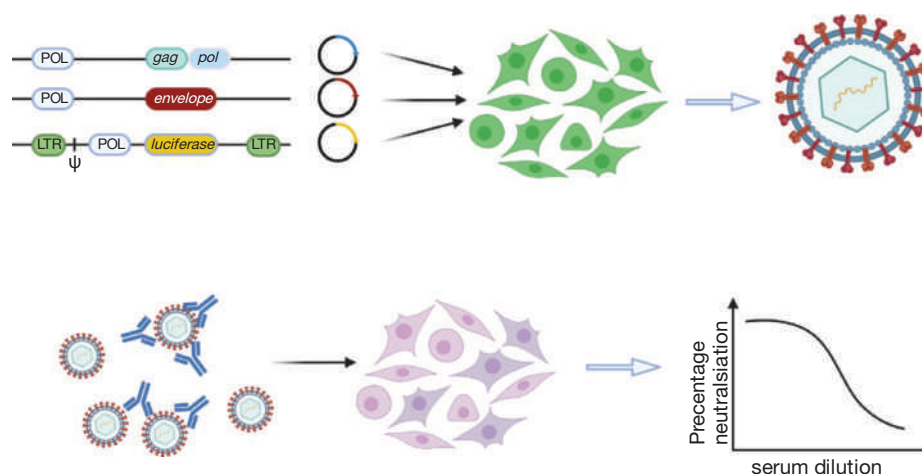


Fig. 3 Schematic of production of a pseudotyped virus with an HIV core and luciferase reporter gene. (A) Plasmid DNA expression vectors bearing (i) the HIV gag-pol gene, (ii) the envelope protein from the virus of interest or (iii) a reporter gene (e.g., luciferase) are generated. (B) All three plasmids are transfected into “producer” cells (e.g., HEK293 T cells). (C) Supernatants are harvested at 48 h post-transfection and produced pseudotype viruses (PVs) are titrated on target cells expressing receptors recognized by the envelope protein in order to ascertain a relative transduction titer. (D) PVs can be subsequently employed as surrogate viruses in pseudotype neutralization assays. Titrated patient samples are preincubated with a fixed titer of PV before addition to target cells. Binding of the envelope protein by specific antibodies in the sample blocks entry of the PV into the target cell, thus preventing expression of the reporter gene. As for traditional plaque reduction neutralization tests, the titer of antibody can be expressed as the highest dilution of sample that inhibits expression by 50% or 90%. LTR: Long tandem repeat. Created with BioRender.com. Adapted from Mather S, Scott S, Temperton N, Wright E, King B and Daly J (2013) Current progress with serological assays for exotic emerging/re-emerging viruses. *Future Virology* 8: 745–755.

(“infect”) target cells expressing receptors recognized by the surface protein. Long tandem repeats (LTRs) flanking the reporter gene direct integration of the gene into the target cell genome resulting in expression of the reporter protein, which is detected by addition of a suitable substrate after a 2- to 3-day incubation period. In a pseudotyped virus neutralization test (PVNT), the incubation of the PV with samples containing antibodies before transduction of target cells results in a reduction in reporter signal. Using a PV as a “surrogate” virus in a neutralization test provides a readout for viruses that do not cause CPE and avoids the need to handle hazardous infectious virus. In fact, synthesis of the gene for expression of the surface protein of interest obviates the need to obtain the virus. There is also the potential to multiplex PVNTs to measure antibodies to different viruses simultaneously by incorporating different reporter genes, such as Renilla or firefly luciferase, into PVs with different surface proteins (Molesti et al., 2014).

The plaque reduction neutralization test (PRNT) is a more accurate, but also technically more complex, test. The virus is inoculated onto a cell monolayer, but a semi-solid overlay is added to prevent spread of the virus in the media. This results in “plaques”, which are small areas of CPE caused by direct cell-to-cell spread of virus from a single infected cell (Fig. 4). The antibody titer obtained is usually expressed as the dilution of sample that reduces the number of plaques obtained at a specific dilution of virus by a certain percentage (e.g., 50% or 90%), which can be interpolated from a plot of sample dilution against number of plaques.

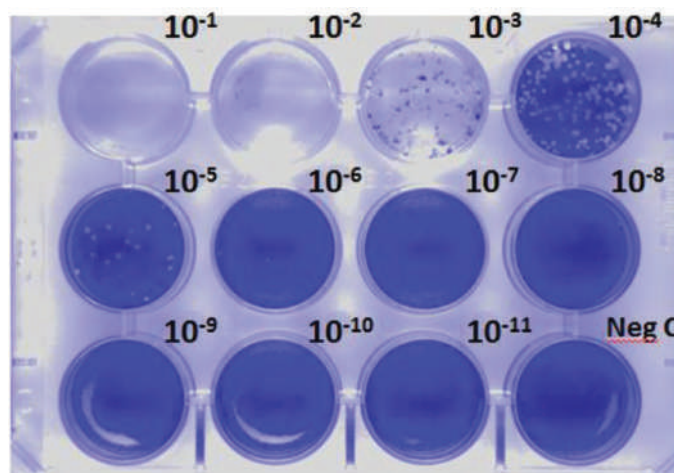


Fig. 4 Example of a plaque titration of virus to determine amount of virus to incubate with samples in a plaque reduction neutralization test. Briefly, Vero cells were infected with 10-fold dilutions of Schmallenberg virus and overlaid with carboxymethylcellulose. After incubation for 4 days, the cells were fixed with 10% neutral buffered formalin and stained with methylene blue solution.

Although neutralizing antibody tests are specific and sensitive, they are time-consuming and laborious, and therefore not widely used in routine diagnostic services. However, they are regarded as a gold standard test and are often used as a confirmatory test where it is difficult to distinguish between cross-reactive antibodies against closely related viruses (such as members of the flavivirus family). As previously mentioned, measurement of antibodies that decrease the infectious capacity of a pathogen is an important measure of the potential protection afforded by vaccination.

Immunodiffusion

Antibodies were first detected by their ability to precipitate antigen when mixed at equivalence (i.e., the optimal amount of antibody and antigen is present; Fig. 5). In the double diffusion or Ouchterlony technique (Ouchterlony, 1948), antigen and antibody are placed in adjacent wells punched out of agarose gel. Precipitation occurs when the two diffuse out of the wells and meet at appropriate concentrations. The technique is cheap and simple but not particularly sensitive.

Agglutination tests

If an antigen is particulate, e.g., a bacterial surface antigen, agglutination of the particles by the addition of antibody can provide a simple detection system. For example, the *Treponema pallidum* particle agglutination assay (TPPA) is used to confirm a diagnosis of syphilis. The hemagglutination inhibition (HI) test remains a common method for detecting antibodies to viruses such as influenza A viruses. The test relies on the virus binding to receptors on the surface of red blood cells and causing them to aggregate (agglutination). Similarly to the VNT, a standardized amount of virus (e.g., 4 hemagglutinating units) is incubated with dilutions of sample. A suspension of red blood cells is then added and after a further incubation period, the HI titer is determined as the highest sample dilution that inhibits agglutination of the red blood cells by the virus. Alternatively, pathogen proteins can be coupled to inert particulate carriers such as latex beads. The latex agglutination test is sensitive, easy to perform and inexpensive. It is therefore highly suitable for use in the field (as a point-of-care test) and in less-developed countries. The tests are more sensitive to the detection of IgM antibodies, because they are polymeric, and therefore valuable for diagnosing recent infection.

Complement-dependent assays

The complement system is a group of heat-labile proteins that bind to antigen–antibody complexes. When this interaction occurs on a cell surface, it results in the formation of transmembrane pores causing lysis of the cell. The complement fixation (CF) test is a classical laboratory diagnostic test that relies on the fact that the amount of complement “fixed” is proportional to the amount of antibody in the complex and the remaining complement can be measured in a hemolytic assay. Complement present in serum samples is heat inactivated before mixing serial dilutions with antigen and incubating to allow antigen–antibody complexes to form. Exogenous complement is then added (usually from guinea pig serum). Usually sheep red blood cells coated with anti-sheep red blood cell antibody are then added to measure the hemolytic activity of any residual complement. The titer is the highest dilution of sample that fixes all of the added complement (therefore there is no complement remaining to lyse the sheep red blood cells). The single radial hemolysis (SRH) assay is another complement-dependent assay in which sheep red blood cells are coated with antigen and mixed into molten agarose with guinea pig complement. The agarose is poured into a plastic mold and after it has set, wells are punched into the agarose into which heat inactivated serum samples are added. The plates are incubated to allow the sample to diffuse from the well. The area of the clear zone surrounding wells where complement-mediated lysis of the red blood

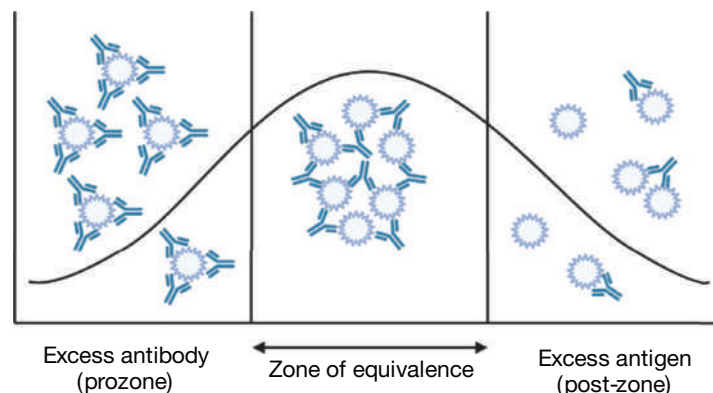


Fig. 5 Zone of equivalence: an optimal ratio of antigen and antibody is required for immunoprecipitation and agglutination tests. Created with BioRender.com. Modified from Alhabbab RY (2018) Precipitation and agglutination reactions. In: *Basic Serological Testing. Techniques in Life Science and Biomedicine for the Non-Expert*. Cham: Springer, https://doi.org/10.1007/978-3-319-77694-1_3.

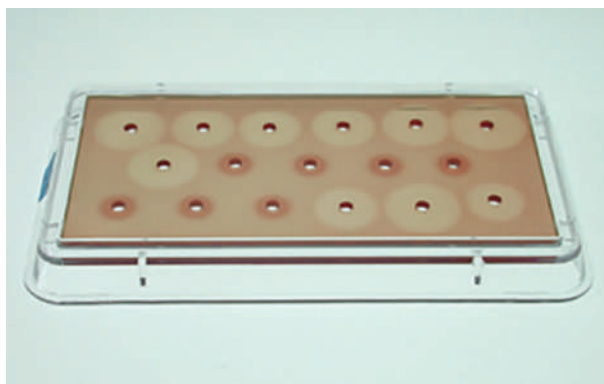


Fig. 6 Single radial haemolysis plate showing clear zones of haemolysis around 10 of the 16 holes punched in the agarose gel.

cells occurs is directly proportional to the amount of antibody present in the sample (Fig. 6). Antibodies against influenza A viruses measured by SRH have been shown to correlate with protection.

Immunoassays

Immunoassays involve using labeled antibody reagents. In the past, radioimmunoassays used radio-isotope labels such as ^{125}I . Today, the enzyme-linked immunosorbent assay (ELISA) has largely superseded the tests described so far for antibody detection because it is relatively simple to perform, rapid and affordable.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was first described by Engvall and Perlmann (1971). The assay is performed by immobilizing the reagents, typically in 96-well or 384-well polystyrene microtiter plates, which passively bind antibodies and proteins. The presence of the target macromolecule is detected directly or indirectly using an enzyme-linked specific antibody. In its simplest form (direct ELISA), four incubation steps are performed:

- (i) Coating: In a direct ELISA, the sample is added directly to wells of a microtiter plate to immobilize the antigen. After incubation, the wells are thoroughly washed.
- (ii) Plate blocking: A solution containing an irrelevant protein is added (e.g., bovine serum albumin or non-fat milk) to cover any unoccupied surfaces on the microtiter plate, preventing the detection antibody from binding non-specifically to the plate surface.
- (iii) Detection: A labeled antibody, which is specific for the target macromolecule and has a reporter enzyme conjugated to it, is added. The wells are again thoroughly washed to remove any unbound detection antibody.
- (iv) Signal measurement: Finally, an appropriate substrate for the enzyme conjugated to the detection antibody is added. If the detection antibody has bound to its specific target, the action of the enzyme on the substrate will produce a detectable signal. The most common system for signal measurement involves one of two enzymatic labels (horseradish peroxidase (HRP) or alkaline phosphatase (AP)) with a choice of substrates that undergo a color change that is detected by measuring the absorbance at a specific wavelength in a plate reader (spectrophotometric readout).

Advantages of the ELISA include the use of highly specific antibodies and washing away non-specific components and the flexibility of the format. The direct ELISA described above is rarely used for antibody detection. In the indirect ELISA for antibodies (Fig. 7), the microtiter plate is coated with antigen from the pathogen (or the pathogen itself), the sample is added and pathogen-specific antibodies in the sample are detected by a labeled anti-IgG antibody. Although IgM can be detected in the same format, because IgG is usually of higher affinity and may be present in higher amounts, it can block binding of IgM and needs to be removed from the sample. Therefore, IgM antibodies are usually detected using a “sandwich” ELISA format. In this format, the microtiter plate is coated with anti-IgM antibody, which captures all IgM antibodies in the sample. Antigen is then added, which will be bound by any pathogen-specific IgM captured on the surface of the microtiter plate. Finally, a detection antibody that recognizes the antigen is added followed by the appropriate substrate.

In the veterinary field, competitive ELISAs are used to detect antibodies against pathogens that may affect multiple species. In its simplest format, the competitive ELISA measures the reduction of signal obtained when antibody present in the sample competes with labeled detection antibody to bind antigen coated on the microtiter plate compared to binding of the labeled antibody in the absence of competing antibody. Thus there is no requirement for a species-specific detection or capture antibody as there is in the indirect and capture ELISA, respectively.

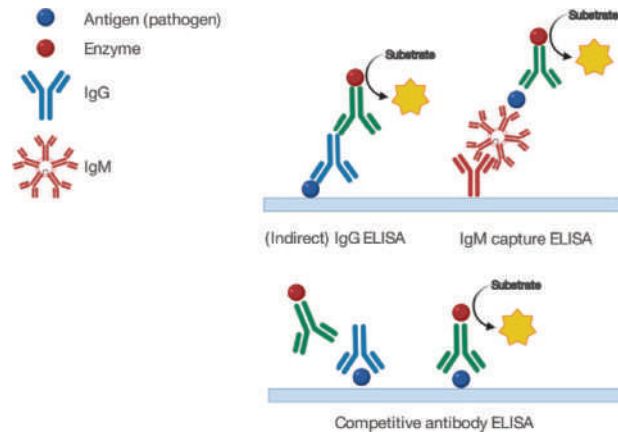


Fig. 7 Schematic of the most common ELISA formats used for measuring antibodies. Modified from a BioRender.com template.

Multiplex assays

There is an increased rate of emergence of novel pathogens, particularly viruses, and consequently an increase in pathogens with overlapping geography. There is also a need to distinguish between pathogens that cause similar clinical disease. For example, in regions where different flaviviruses co-circulate, an IgM antibody capture ELISA (MAC-ELISA) is often applied in which the sample is added in parallel to wells coated with different virus antigens and the infecting virus is taken to be that coated in the well giving the highest signal (e.g., *Lewthwaite et al., 2010*).

Measuring antibodies against multiple pathogens by ELISA in parallel is time consuming, increases the risk of error and uses larger volumes of sample (a key challenge if, for example, performing diagnostic tests on CSF from a child).

One approach to multiplex an assay is to spatially separate reagents, for example by immobilizing capture antibodies in small arrays at the bottom of individual wells of microtiter plates. Another approach is to immobilize reagents on beads. For example, Luminex[®] technology uses combinations or levels of dyes to individually code up to 500 batches of beads. A capture assay is performed by coating beads with different antibodies, incubating these with the sample and using phycoerythrin-labeled detector antibodies to measure protein levels. A disadvantage of this technique is the requirement for a specialized flow cytometer to read the assay and proprietary software to decode the sample signals, although similar assays with a more limited number of codes are possible with standard flow cytometers.

Lateral flow test

Lateral flow tests are another variation of the ELISA in which antigens are immobilized on a strip of cellulose or other suitable matrix. The sample is applied at one end of the strip and any antibodies present diffuse along it until they reach the position where the appropriate antigen is immobilized. Antibodies specific for the antigen bind to and remain at the site of the antigen and are visualized using labeled detection antibody. The lateral flow test is not quantitative, but is rapid and simple to perform, making it a suitable point-of-care test. The nitrocellulose strip is typically housed in a plastic casing with an opening where the sample and reagents are applied and a window where the result is read. Usually, a positive control antigen is placed on the strip so that it is encountered after the test antigen to confirm that the sample has diffused a sufficient distance to interact with the test antigen. More than one test antigen can be dotted on the strip to provide a multiplex test. IDEXX Laboratories, Inc., a veterinary company, has developed a lateral flow test casing that incorporates reagent reservoirs. After the sample has diffused along the strip to an "activation window," the end of the device is pressed down to puncture the film across the reservoirs, releasing their contents, which mix with the sample and are drawn back across the strip. This is reported to improve sensitivity as the sample passes across the antigen twice and specificity as debris is washed from the result window. The SNAP test range includes tests to detect antibodies to parasites and to detect antibodies to feline immunodeficiency virus and feline leukemia virus antigen in the same test.

Development of antibody tests

Validation is a process to determine fitness of an antibody test for its intended purpose(s). The requirements of an antibody test may be more stringent if it is intended to be used for diagnosis of disease in an individual with implications for their subsequent treatment or to gain an idea of the prevalence of a disease in a population.

Sensitivity (Se) and specificity (Sp), which indicate the probability that a truly infected individual yields a positive or a negative result, respectively, are the most commonly used performance indicators of a diagnostic test. Usually, Se and Sp are estimated by screening a panel of “known” positive or negative samples. This panel can be from individuals for which historical and accurate information indicates that they were infected (e.g., known to have had clinical signs and in whom the specific pathogen was identified) or not. In the development of antibody tests for veterinary applications, it may be possible to obtain samples from experimentally infected animals. More often, they are samples that have been identified as positive or negative using a gold standard test (for which Se and Sp are regarded as 1). A more cautious approach is to use multiple alternative tests to reduce misclassification errors.

Determining the threshold or cut-off value above which a test can be regarded as positive may often be somewhat arbitrary. For example, frequently in the development of ELISAs, a threshold of the mean of negative control values plus two standard deviations is used. As a result, ELISAs often have a range of test values between the negative and positive thresholds that have to be regarded as “inconclusive.”

Properly validated antibody tests should be repeatable (i.e., have good agreement between results of replicates of a sample within and between test runs in the same laboratory) and reproducible across laboratories. Achieving reproducibility is hampered where tests involve the use of cell line or other potentially variable materials (e.g., red blood cells) obtained from different sources. International reference standards can help achieve standardization. Inter-laboratory trials can be conducted in which several laboratories are supplied with candidate reference standards and a standardized protocol. The results obtained from multiple independent tests in each laboratory are statistically analyzed to assign an expected value to the reference material.

The future of antibody tests

Many of the antibody tests still in use today were first developed decades ago. Major advances have been made in the development of antibody tests. There is an increasing focus on the use of non-invasive samples such as saliva, urine or milk. These can be more difficult substrates to work with than serum in traditional antibody tests, for example milk samples can be toxic to the cells used in virus neutralization tests, but can be used in ELISA and similar tests. Point-of-care tests such as easy-to-use lateral flow or latex agglutination tests that provide a result in minutes are becoming increasingly common in clinical practice. Nonetheless, there is often a trade-off between convenience, speed and high throughput on the one hand and sensitivity and specificity on the other. The increasing rate of emergence of infectious diseases, particularly viral diseases means that there is an increasing demand for tests that can be rapidly developed to reliably detect antibodies to novel pathogens. For example, the COVID-19 pandemic caused by SARS-coronavirus 2 has led to the PVNT becoming a more widely accepted technique.

In veterinary medicine in particular, there has been a move towards developing antibody tests that complement a new generation of recombinant vaccines. The differentiation of infected from vaccinated animals (DIVA) strategy involves employing an antibody test that detects antibodies to proteins that are not present in the vaccine. This enables vaccination to be used to control an outbreak while still enabling infected animals to be identified. Where it is not possible to distinguish whether antibodies are a result of infection or vaccination, vaccination can lead to trade restrictions being imposed if it is uncertain whether disease is circulating in a country or not. It is likely that in future, similar strategies will be increasingly relevant in human medicine.

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Flowcytometric Assessment of B Cell Development and Functional Assays on B Cell Development

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Introduction

Flow cytometry (FCM) is a laser-based technology used for multiparametric analysis of biological particles. Particles in the form of a suspension/solution (blood, bone marrow, lymphoid tissue, fluids) are passed through a “flow” chamber which is illuminated by a laser source. The signals generated while passing through this chamber are collected by optical and electronic systems and analyzed by a computer control system. The simplified design of the FCM is shown in Fig. 1.

In history, the device, first described by Andrew Moldavan in 1934, has been accepted as an early prototype (Moldavan, 1934). In time, with the developing technology and new ideas, the device began to be made suitable for today's medicine between 1969 and 1972 (Van Dilla et al., 1969; Julius et al., 1972). At the beginning of the 21st century, with the development of laser, optical and electronic systems, it has started to be used in many areas. Nowadays, FCM is used as a diagnostic and research method in multiple disciplines such as immunology, hematology, oncology, microbiology, pathology, histology and biochemistry laboratories (Figs. 2–5).

In the basic working principles of FCM, each particle in the sample is evaluated for visible light scatter and multiple fluorescence parameters. Visible light scatter is measured by two optical detectors; forward scatter (FSC) and side scatter (SSC). FSC detects scatter along the laser path and these signals can be used to define the relative size of the cell. SSC measures scatter at 90 degree angle relative to the laser, which indicates the internal complexity or granularity of the cell. Furthermore, samples are prepared for fluorescence measurement, which permits visualization of cellular features or structure, for expression of fluorescent proteins, staining with fluorescent dyes or immunostaining with fluorescently conjugated antibodies (McKinnon, 2018). Thus, with the data obtained with nonfluorescent and fluorescent signals; we can determine size, granularity, enzyme activities, fluorochromes (fluorescent antibody), immunophenotype, membrane potential and DNA contents about particles.

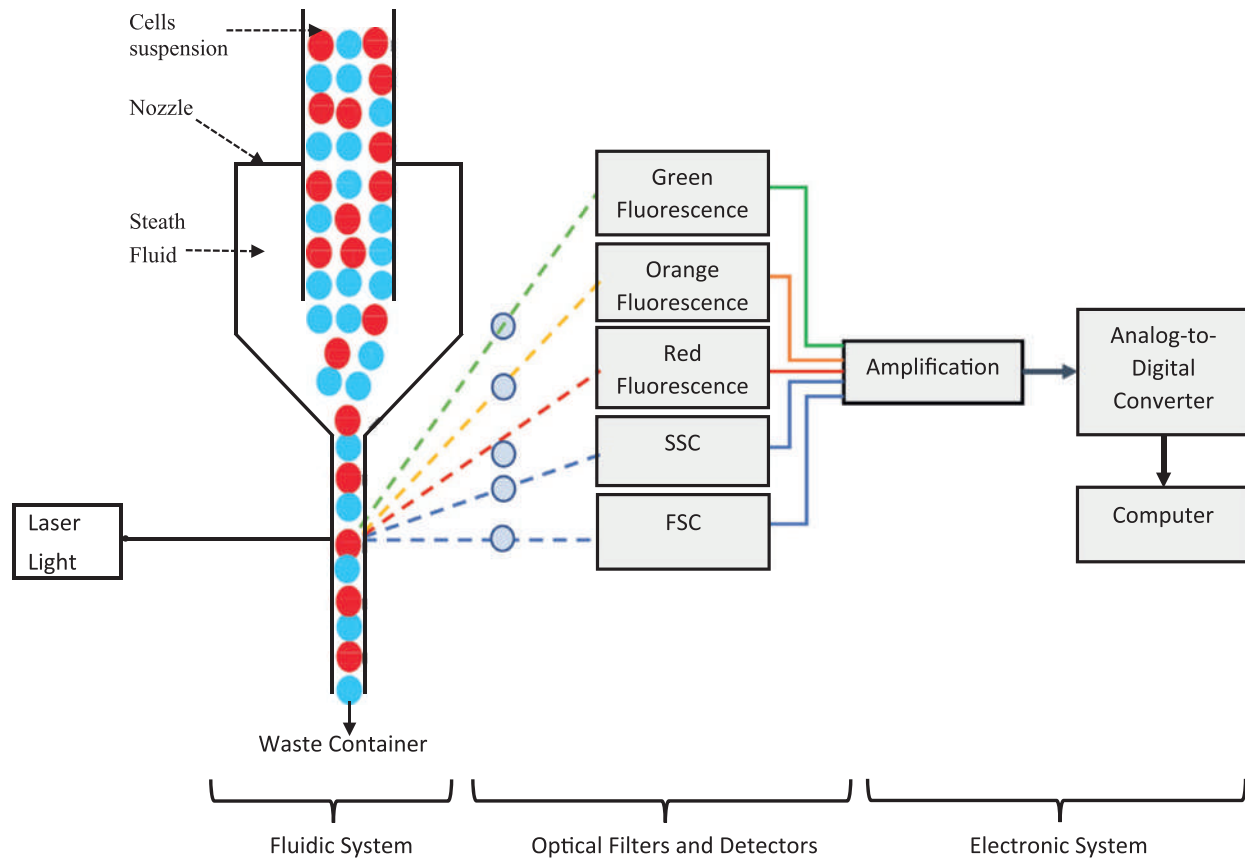


Fig. 1 A simplified flow cytometry design with basic features.

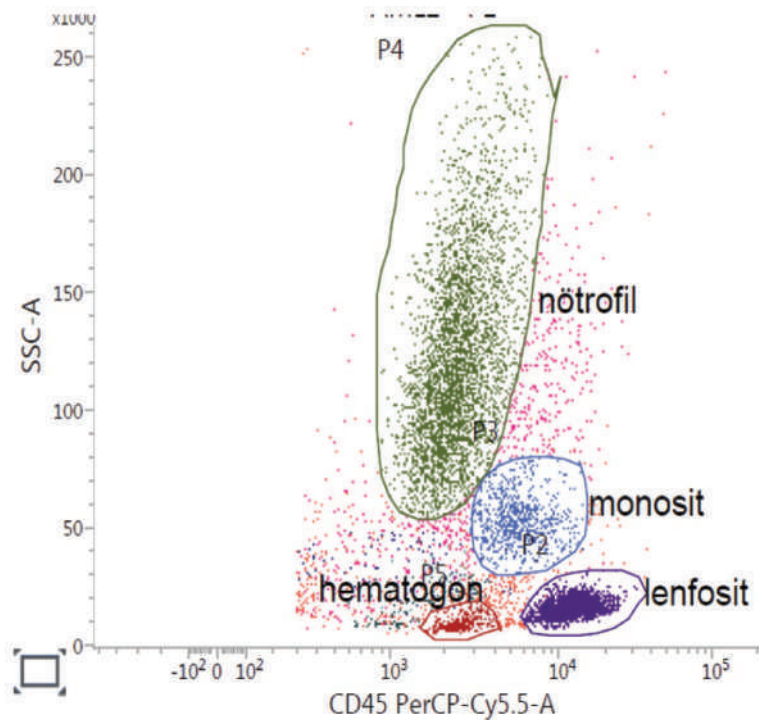


Fig. 2 Dot plot with SSC/CD45 gate reagents showing the fluorescent distribution of all three leukocyte types including lymphocytes, monocytes, neutrophils and few red blood cells and/or residues.

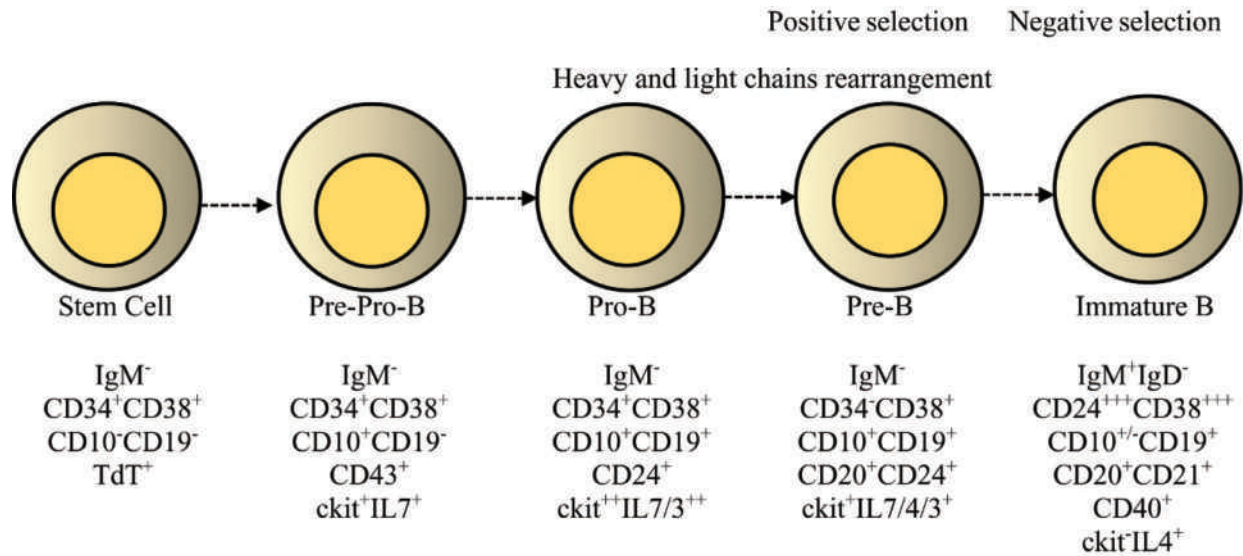


Fig. 3 Early B cell development and differentiation in bone marrow.

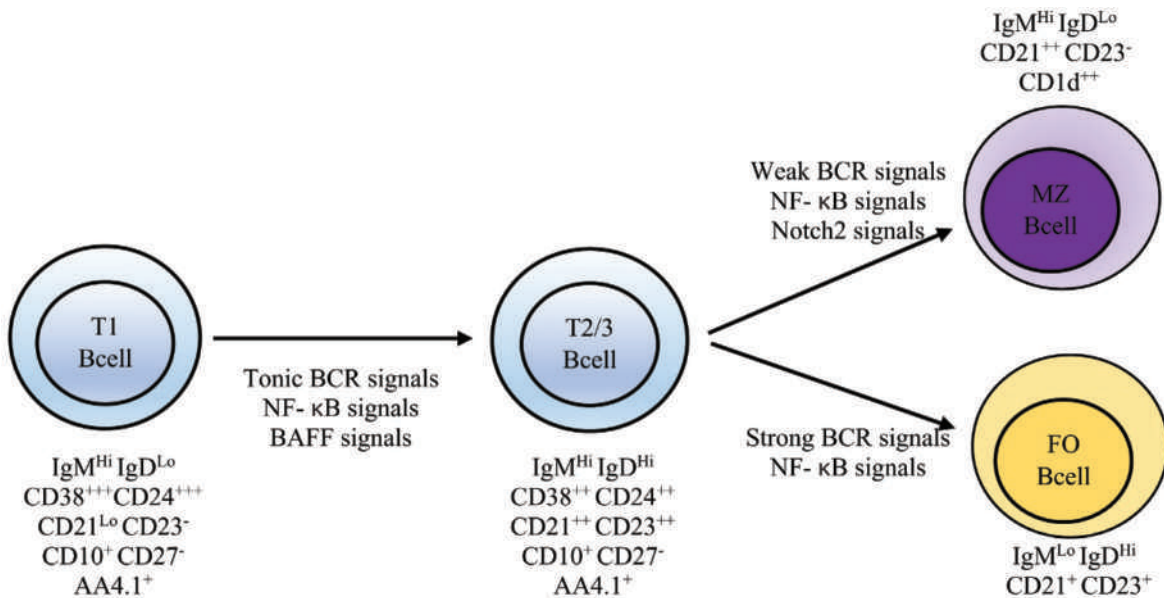


Fig. 4 Transitional, marginal zone and follicular B cells development and differentiation.

By this dynamic and powerful system, thousands of individual particles can be evaluated quickly and objectively at high speed. Therefore, FCM has been increasingly used in medical diagnosis and prognosis over the last decade. In recent years, FCM has become a daily used scientific instrument in immunology and hematology clinical departments for differential cell counts of leukocyte populations, identify an antigen of interest, characterize expression profiles in several different subsets of cells or determine the cell cycle status of a population (Robinson, 2004).

This article focuses on explaining the areas where FCM is used, the differentiation and development stages of B cells from stem cells to other cells by immunophenotyping method, and some clinical examples.

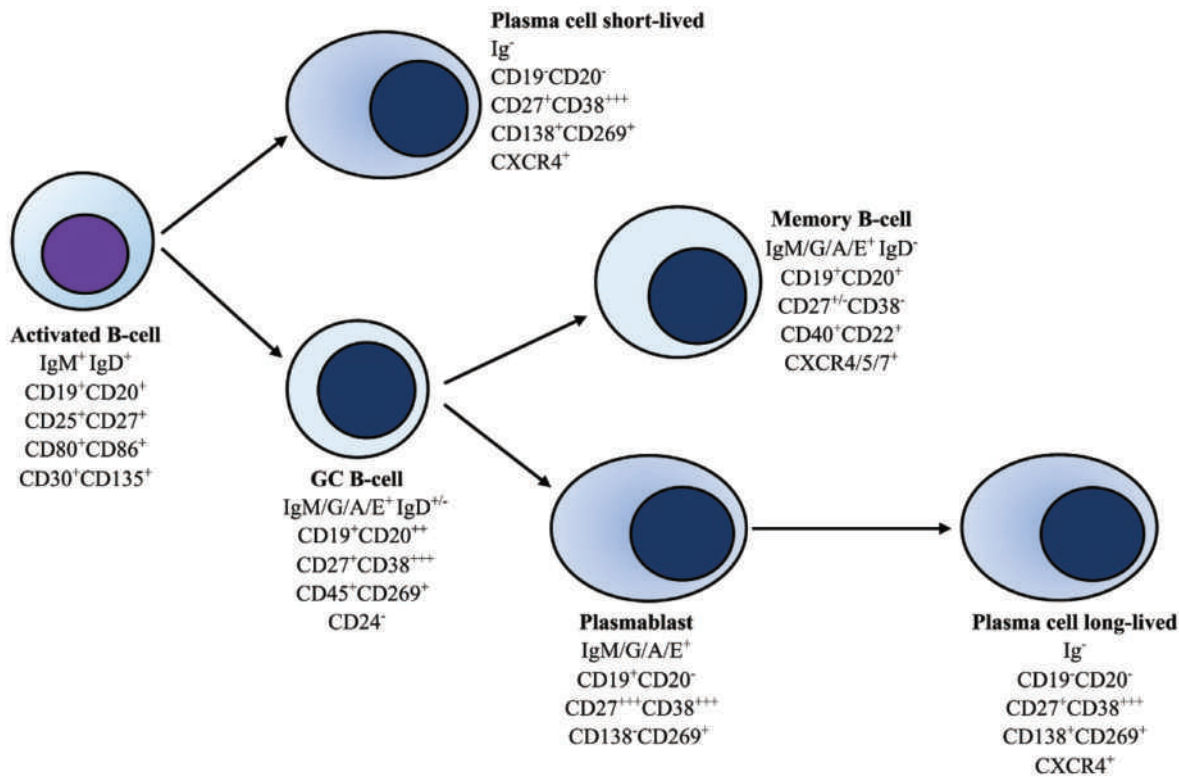


Fig. 5 Activated B cell, germinal center B cell, memory and plasma cells development and differentiation.

Practical applications of flow cytometry

FCM has many techniques and applications suitable for diagnosis, research, clinical trials and treatment monitoring for multiple fields of study. By far the most frequently used FCM application is immunophenotyping. For FCM applications, the particles must be sufficient size to be detected and have an appropriate fluorescent nature. For the fluorescent structure, fluorochrome-conjugated ligands that can bind to specific cell structures are used. With the demonstration of cells with fluorescent structure, several clinical assays can be performed. Some of these assays are shown in the Table 1.

Table 1 Common applications of flow cytometry.^a

Application	Examples	Approach
Immunophenotyping	Leukemia and lymphoma phenotyping	Fluorochrome-conjugated monoclonal antibodies
	PNH testing	
	Hematopoietic progenitor cell enumeration	
	CD4 counting	
Phagocytosis	Fetal hemoglobin detection	Fluorochrome-coated beads
	Neutrophil phagocytosis	
	Cell cycle analysis	
	Evaluation of apoptosis	
DNA analysis	Ploidy determination	Fluorescent dyes binding directly to DNA
	Reticulocyte enumeration	
	Cytokine analysis	
	Receptor assays	
RNA analysis	Chromosome studies	Conjugated nucleic acids
	Detection of viral DNA	
	Oxidative burst	
	Calcium flux	
Intracellular enzymes		Substrates for enzymes
Intracellular ions		–

^aPNH: Paroxysmal nocturnal hemoglobinuria.

Immunophenotyping studies

FCM facilitates the search for B-cell markers and provides information on important points in B-cell biology. It is also a powerful tool to drive the discovery of cell development, differentiation, immunodeficiency, autoimmune disorders and malignancies (McKinnon, 2018).

Immunophenotyping studies can be used to identify cell subsets, lineage, stage of cell differentiation, state of cell activation, and clonality (Fleisher and Oliveira, 2019). The various stages of B-cell differentiation can be identified by evaluating the expression and levels of distinct cell-surface markers, which are regulated throughout the course of B-cell differentiation (Fleisher and Oliveira, 2019). And also, FCM is a common method used for immunophenotyping, which can detect cells stained with fluorochrome-conjugated antibodies that are targeted against antigens on the cell surface. Therefore, FCM can be used to test for the presence or absence of a specific cell surface antigen.

Most of these antigens are given “cluster of differentiation” numbers or CD numbers so that a common nomenclature is used to define monoclonal antibodies (mAbs) that are directed against specific cellular antigens (McKinnon, 2018). These same CD markers are currently used to isolate these B cell subsets in order to study their function.

Cell cycle

The cell cycle is a sequence of events that takes place in many phases. During these phases, when there are alterations and dysregulation in the genetic content, which can be observed by fluorescent dye, it can lead to tumor formation. Therefore, monitoring of cell cycle can provide information for diagnosis and treatment of diseases.

With FCM, we can detect cell populations at different stages of the cell cycle, such as G0/G1, S, and G2/M. We can also identify apoptotic cells (Bajgelman, 2019).

Cell sorting

Cell sorting is used for the separation, isolation and purification of various cell populations, which can be made fluorescent, for more detailed multiparametric analysis. Moreover, with this technique, it is possible to measure the DNA and RNA content of cells and even evaluate functional properties.

This technique can be used in many situations such as stem cells in bone marrow samples, selection of sperm to allow sex selection, purification of different lineages, detection of tumor infiltrating lymphocytes, tumor cells and white blood cell populations (McKinnon, 2018).

Apoptosis

Apoptosis, also known as programmed cell death, is a tightly regulated biochemical process centered primarily around the caspases family of enzymes. During apoptosis, a series of events such as blebbing, cell shrinkage, pyknosis takes place. As a result of this programmed process, cells are lysed into apoptotic bodies.

FCM has become the method of choice for the detection and quantification of cellular apoptosis due to its rapid assessment of large numbers of cells (Fleisher and Oliveira, 2019). Thus, morphological, biochemical and molecular changes that occur in cells during apoptosis can be detected.

We can evaluate a cell that has started the apoptosis process with its light scattering properties. Decrease of FSC and increase of SSC can be observed by FCM. However, scattering changes are not unique to apoptosis alone. An additional feature associated with cell death needs to be demonstrated. In early apoptosis, cells lose asymmetry and phosphatidyl serine (PS) residues, which is positioned inside the plasma membrane, expose to the cells outer surface. Annexin V, which is a human vascular anticoagulant, has high affinity for PS. Conjugated annexin V is a widely used reagent in the detection of apoptotic cells (Fleisher and Oliveira, 2019). With the rupture of the cell membrane, the late apoptotic stage can be detected with different cationic dyes. Furthermore, with the combination of annexin V and cationic dyes, we can also determine at what stage the cells are in the apoptotic process.

Evaluation of mitochondrial transmembrane potential and measurement of DNA content are other methods that can be used to distinguish living cells from dead cells.

DNA content

Cellular DNA content can be measured by FCM. With this method; cell distribution within major phases of the cell cycle, assessment of apoptosis and determination of ploidy can be evaluated (Darzynkiewicz and Huang, 2004).

DNA can be stained with fluorescent dyes and hence, can be detected quantitatively by FCM due to its fluorescent property. The most commonly used fluorescent dye for this method is propidium iodide (PI). PI can bind all double-stranded nucleic acids. Therefore, RNAase must be added before staining to detect DNA content.

Other fluorescent dyes used for DNA content analysis are 4',6-diamidino-2-phenylindole (DAPI), dye cycle violet, Hoechst 33342, acridine orange, 7-aminoactinomycin D (7-AAD) and ethidium bromide (McKinnon, 2018; Fleisher and Oliveira, 2019).

B-cell

B cells are the central of humoral immunity and protection. The main function of B cells is to produce and release antibodies to specific antigens with antibody-secreting plasma cells and memory cells after antigen recognition. Secreted antibodies have the ability to neutralize almost unlimited variety of pathogens and their toxins. Furthermore, B cells have antigen receptors (BCR), immunoglobulin (Ig) receptors, adhesion molecules and Major Histocompatibility Complex (MHC) molecules on the surface. These surface molecules contribute to the regulation of B cell-related development, activation, proliferation, differentiation and effector functions by mediating communication with the extracellular microenvironment.

Various stages of B cell development and differentiation have become identifiable with the development of FCM analysis. Moreover, FCM has begun to identify and characterize expression profiles in several different subsets of B cells.

CD molecules involved in B cell development and differentiation are listed in Table 2.

Early stages and cells in the bone marrow

The generation of B cells begins in the bone marrow (BM) where multipotent hematopoietic stem cells (HSCs) are stimulated by lymphoid progenitors and cytokines. The first cells to be affected by lymphoid progenitors are called early (pre) pro-B cells. Early BM-dependent stages of B cell development are constructed through the functional rearrangement process of Ig gene segments. Thus, rearrangement of heavy and light chains (HC and LC) gene segments generates a repertoire of B cells expressing a vast number of antibodies that can recognize multiple different antigens. Progenitor B cells rearrange the Ig HC genes to differentiate into pre-B cells expressing μ HCs. Besides, pre-B cells undergo one or two cell divisions, and then rearranging the gene segments encoding the κ and λ chains (van Zelm et al., 2007). Also at this stage, positive selection is made for B cells with normal functional receptors. Pre-B cells then rearrange their Ig LC genes to differentiate into IgM⁺ immature B cells. At this stage, a negative selection mechanism is used to eliminate self-reacting B cells and minimize the risk of autoimmunity.

Immature B cells leave the BM and migrate to the spleen, where their final stages of maturation. When an immature B cell starts migrating into the periphery with the bloodstream, it first becomes a Transitional B (TB) cell which are considered to be precursors of mature B cells. And then, they become differentiating into naive, follicular (FO), or marginal zone (MZ) B cells in spleen.

Transitional B cells

TB cells are an important link between immature B cells in the BM and mature peripheral B cells. The newly produced immature B cells, which are called T1 B-cell, migrate from BM to spleen. After T1 B cells enter the spleen, cell surface molecules differentiate, and also, gain the ability to circulate (Suryani et al., 2010). These cells are known as T2/T3 B cells. After these stages, T2/3 B cells also differentiate into mature B cells (Suryani et al., 2010).

Follicular and marginal zone B cells

In the spleen, mature B cells are divided into two groups: FO B and MZ B cells. The difference and strength of BCR signaling pathways integrated with B cell-activating factor (BAFF) and Notch2 signaling determine the commitment of B cells from the TB cell to the mature FO B or MZ B cell fate (Cerutti et al., 2013).

Weak BCR signal, nuclear factor kappa B (NF- κ B) and Notch2 signaling are important for TB cells to develop into the MZ B cells, which are noncirculating mature B cell and anatomically located in the marginal zone of the spleen (Martin and Kearney, 2002). The functions of MZ B cells are; T cell dependent and independent responses to blood-borne pathogens, responses to lipid antigens with CD1d and collaboration with natural killer T (NKT) cells (Pillai and Cariappa, 2009). Furthermore, MZ B cells differentiate into short-lived plasma cells that produce large amounts of IgM (Balázs et al., 2002).

Strong BCR signal, BAFF and NF- κ B signaling are necessary for the TB cells to develop into FO B cells (Pillai and Cariappa, 2009). They are primarily located in the follicles of the spleen and lymph nodes. And also, FO B cells can recirculate through blood and lymphatics into secondary lymphoid organs, and back into the BM. FO B cells have more broader diversity of Ig genes than have MZ B cells (Cerutti et al., 2013). The main function of FO B cell is to mediate adaptive antibody production. Therefore, FO B cells interact with T helper (Th) cells to form germinal centers, and then, undergo class switching recombination (CSR) and somatic hypermutation (SHM). Along with these changes, FO B cells produce high affinity antibodies to eradicate pathogens or give rise to memory B cells (McHeyzer-Williams and McHeyzer-Williams, 2005).

Memory cells

In primary immune responses, activated B cells differentiate into memory B cells, which are a key cellular component of the protective humoral responses to infectious pathogens. These cells develop within germinal centers of the secondary lymphoid organs. The most important function of memory B cells is to remember the first encounter with invading pathogens, and to provide effective protection against secondary infection for years (Crotty et al., 2003).

Table 2 B cell associated molecules involved in development and differentiation.

<i>Name</i>	<i>Alternative name</i>	<i>Gene family</i>	<i>Function</i>
CD1c	M241, R7, T6	Ig superfamily	Antigen presenting protein
CD1d	R3G1	Ig superfamily	Antigen presenting protein
CD5	Leu-1, T1, Tp67, Ly-1	Scavenger receptor superfamily	Inhibitor of B-cell activation
CD10	CALLA, Nep, MME	Peptidase protein family	Regulates B-cell growth
CD19	B4	Ig superfamily	Regulates B-cell development, activation and differentiation. Signal transduction
CD20	B1, Bp35	Membrane-spanning 4A family	B-cell activation and proliferation
CD21	CR2, EBV-R, C3dR	Regulator of complement activation gene family	Regulator of complement activation, positive regulator B cell activation
CD22	BL-CAM, Siglec-2	Ig superfamily	B-cell adhesion and signal transduction. Inhibitor of B-cell activation
CD23	FcεRII, B6, BLAST-2	C-type lectin family	Low affinity receptor for IgE and key molecule for B-cell activation and growth
CD24	BBA-1, HSA	Sialomucin family	Regulation of B-cell proliferation and maturation
CD25	Tac, p55, IL-2Ra	Cytokine receptor family	Receptor for interleukin-2
CD27	T14, S152, TNFRSF7	Tumor necrosis factor receptor superfamily	Costimulation of B- and T-cell activation
CD30	Ber-H2, Ki-1, TNFRSF8	Tumor necrosis factor receptor superfamily	Activation of NF-κB, apoptosis, autoimmunity
CD32b	FcγRIIB	Ig superfamily	Phagocytosis of immune complexes and regulation of antibody production
CD34	gp10–120, mucosialin, MY10	Sialomucin family	Cell adhesion and possible role in early hematopoiesis
CD38	T10, ADP-ribosyl cyclase	Ectoenzyme family	Cell adhesion and signal transduction
CD40	Bp50, TNFRSF5	Tumor necrosis factor receptor superfamily	Costimulation of B-cell growth. Differentiation and isotype switching
CD45	LCA, T200, B220	Protein tyrosine phosphatase family	Regulator of T- and B-cell antigen receptor signaling; regulator of cell growth and differentiation
CD69	AIM, VEA	C-type lectin family	Lymphocyte proliferation; signal transmission in NK cells and platelets
CD72	Lyb-2	C-type lectin family	B-cell proliferation and differentiation
CD79a	Iga, MB1	Ig superfamily	Subunit of B-cell antigen receptor and signal transduction
CD79b	Igb, B29	Ig superfamily	Subunit of B-cell antigen receptor and signal transduction
CD80	B7, B7-1, BB1	Ig superfamily	Costimulation of T-cell activation and proliferation. Has a critical role in autoimmune, humoral, and transplant responses
CD86	CD28LG2, B70, B7-2	Ig superfamily	Costimulation of T-cell activation and proliferation
CD135	FLT3, FLK2	Ig superfamily	Cell survival, proliferation, and differentiation. CD135 is important for lymphocyte development
CD138	Syndecan-1, B-B4	Syndecan proteoglycan family	Cell proliferation, cell migration, and cell-matrix interactions
CD179a	VPREB1, IGVPB	Ig superfamily	Early B-cell differentiation
CD179b	Λ5	Ig superfamily	B-cell proliferation and differentiation
CD180	RP105, Bgp95	TLR family	Controls B-cell recognition and signaling of LPS
CD267	TACI	Tumor necrosis factor receptor superfamily	Controls T-cell-independent B cell antibody responses, isotype switching, and B cell homeostasis
CD268	BAFF receptor, BR3	Tumor necrosis factor receptor superfamily	The principal receptor required for BAFF-mediated mature B-cell survival
CD269	BCMA, BCM	Tumor necrosis factor receptor superfamily	Promotes B-cell survival
CD272	BTLA	Ig superfamily	Lymphocyte inhibitory receptor, regulates lymphocyte activation and/or homeostasis as well as T-cell tolerance
CD284	TLR4, ARMD10	TLR family	Mediator to innate immunity against bacterial polysaccharides. Induces activation of NFκB and production of type I interferons
CD305	LAIR1	Ig superfamily	Negative regulator of NK, B, and T cells
CD307a	FCRL1, IRTA5	Ig superfamily	B cell activation and differentiation
CD307b	FCRL2, IRTA4	Ig superfamily	B cell activation and differentiation
CD307d	FCRL4, IRTA1	Ig superfamily	B cell activation and differentiation
CD307e	FCRL5, IRTA2	Ig superfamily	B cell activation and differentiation
CD329	SIGLEC9	Ig superfamily	Adhesion molecule that mediates sialic-acid dependent binding to cells. Inhibitor of B-cell activation

The germinal center (GC) is a specialized microstructure that forms in secondary lymphoid tissues, where mature B cells proliferate, differentiate, and mutate their antibody genes. Within the GC, B cells undergo clonal expansion, CSR at the IgH locus, SHM, and selection for increased affinity of a BCR for its unique antigenic epitope through affinity maturation (LeBien and Tedder, 2008). These develop dynamically after activation of FO B cells by the T-dependent antigen.

When foreign antigens/peptides are encountered, naive B cells in the follicle recognize the antigen through BCR and receive signals by binding the peptide to the B cell. Thus, these signals cause the cells to migrate to the edge of the follicle bordering the B-T cell space (Garside et al., 1998). Some of the activated B cells increase Bcl-6 production and migrate back to the follicle and form GCs. The other activated B cells rapidly differentiate into memory B cells in the presence of strong CD40 stimulation from Th cells, before GC formation. These early differentiated memory B cells are gradually replaced by GC-derived memory B cells at later (Weisel and Shlomchik, 2017). First, the GC-independent pathway develops, which rapidly recruits germline-encoded, low-affinity memory B cells. Thereafter, the GC-dependent pathway develops, which takes somatically mutated, affinity matured memory B cells from GC reactions.

Plasma cells

Plasma cells, which are terminally differentiated from activated B cells, produce and secrete large volume of antibodies in response to pathogen infections. Also, plasma cells capable of opsonization and destruction of pathogens are effector cells of humoral immunity.

Mature B cells function as an antigen-presenting cell (APC) when it encounters offending antigens. The antigens are taken up by the B cell through receptor-mediated endocytosis, and hence, antigens are converted into peptides. These peptides are transferred to the cell surface and loaded onto MHC II molecules and then presented to CD4+ T cells on the extracellular surface. Subsequently, as a result of the binding between T cell and MHC II-antigen complex, the B cell is activated. The activated B cell proliferate rapidly and undergo somatic hypermutation.

After activation of the B cell, which usually occurs in GC of secondary lymphoid organs, memory B cells or plasma cells begin to differentiate. Plasma cells have been able to make more powerful properties against the specific antigen by undergoing affinity maturation (Honjo et al., 2004). The activation and growth of B cell clones and ability to secrete antibodies of higher affinity for the specific antigens are known as affinity maturation (Nutt et al., 2015). Thus, the antibodies which are produced and secreted are involved in opsonization, phagocytosis and the eventual destruction of the pathogen.

Other B cell subsets

B1 cells

B1 cells are a small subpopulation of B cells, with unique features that differ from the majority of mature B cells (Berland and Wortis, 2002). B1 cells mainly originate from the fetal liver and are found in the peritoneal and pleural cavities of mice (Baumgarth, 2011). In today, most of what is known about these cells comes from studies in mice.

B1 cells divided into B1a cells which expresses CD5 on the cell surface and B1b cells which do not (Vale et al., 2015). B1 cells are able to produce natural antibodies, mainly IgM, IgA and IgG3. In fact, B1 cells produce about 80% of natural IgM with low affinity, poly-specificity but high poly-reactivity and complement activation ability (Baumgarth et al., 2000).

In mice, they generate natural antibodies without infection (T cell-independent activation), secrete cytokines, defend against mucosal pathogens, uptake and present antigens to T cells during pathogen invasion (Baumgarth, 2011; Novaes e Brito et al., 2019).

Regulatory B cells

Regulatory B (Breg) cells represent the population of B cells that contribute to immunomodulatory processes and suppression of immune responses. Breg cell functions have been demonstrated in mice and humans in autoimmune diseases, cancer, infection and organ transplantation (Ray and Dittel, 2017; Karahan et al., 2017; Shen et al., 2016).

There are no unique surface markers that identify Breg cells (Silva et al., 2012). Therefore, there is still no consensus on definition and classification of Breg cells (Silva et al., 2012). First, the CD19⁺CD5⁺CD1d^{hi} B cell subset was found to function as suppressors of diseases in mice (Yanaba et al., 2008). Subsequently, the CD19⁺CD24^{hi}CD38^{hi} B cell subset was found to function as suppressors of diseases and inhibit the proliferation and function of proinflammatory cells in human blood (Blair et al., 2010). However, numerous Breg-cell subgroups with different phenotypes and effector functions are still described in mice and humans.

The main mechanism of Breg cells is through the anti-inflammatory cytokine interleukin 10 (IL-10) (Rosser and Mauri, 2015; Floudas et al., 2016). Breg cells also function by IL-10 independent mechanisms through cytokines such as IL-35, transforming growth factor (TGF)- β , and cell-to-cell contact. These cells regulate the immune system by many different mechanisms that are not fully elucidated. Furthermore, there is evidence that almost all B cell types can differentiate into Breg cell (Silva et al., 2012).

Flow cytometry examples

Burkitt lymphoma

Burkitt Lymphoma (BL) is a rare and aggressive form of Non-Hodgkin Lymphoma (NHL) that is usually found in extranodal sites or presenting as an acute leukemia (Ferry, 2006). And also, BL is recognized as a fast growing tumor. BL can be divided into three clinical variants: endemic, sporadic, and immunodeficiency-associated variants.

The diagnosis of BL is made as a result of the combined evaluation of morphological, immunophenotypic and cytogenetic findings. The characteristic immunophenotype of BL arises from mature B cells with germinal center cell differentiation (Kelemen et al., 2010).

In immunophenotypic evaluation of BL; BL cells are bright CD45, very large and mimic large lymphocytes. Tumor cells are positive for CD45. BL cells are positive for B-cell antigens such as CD19, CD20, CD22 and CD79a with a monoclonal light chain expression of kappa or lambda. CD10, CD38, CD43 and bcl-6 are also expressed. The blast markers CD34 and TdT, and bcl-2 are negative.

An example histogram/dot plots of BL are shown Fig. 6.

Mantle cell lymphoma

Mantle cell lymphoma (MCL) is a subtype of B-cell lymphoma and characterized by pregerminal center B cell proliferation within the mantle zone surrounding normal germinal center follicles of lymphoid tissues. Almost all cases show genetic abnormality t(11;14), which is resulting in overexpression of cyclin D1 protein. The clinical course is usually aggressive and the median survival is 3–4 years.

The initial gating dot plot identifies a predominant CD45 bright SSC low population consistent with lymphocytes. MCL cells are positive for pan-B-cell antigens CD19, CD20, CD22 and usually CD5 positive (rarely CD5 negative). CD10, CD23, bcl-6 are negative and FMC7 positive. And also, heavy chain (IgG, IgA and IgM) and light chain Ig (kappa or lambda) are moderately expressed.

An example histogram/dot plots of MCL are shown Fig. 7.

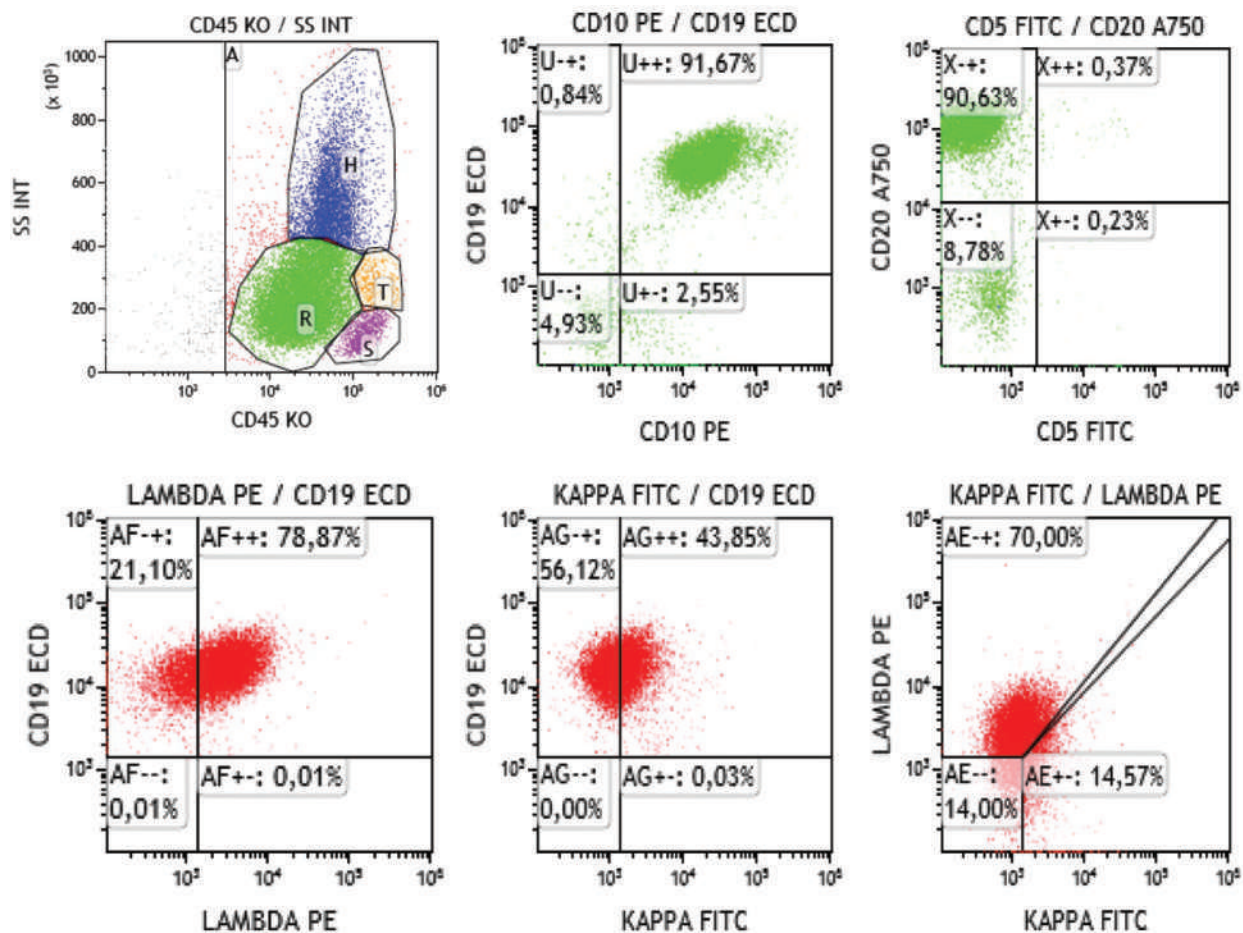


Fig. 6 Dual parameter dot plots useful in the diagnosis of Burkitt lymphoma. H: neutrophil; R: blast; S: lymphocyte; T: monocyte.

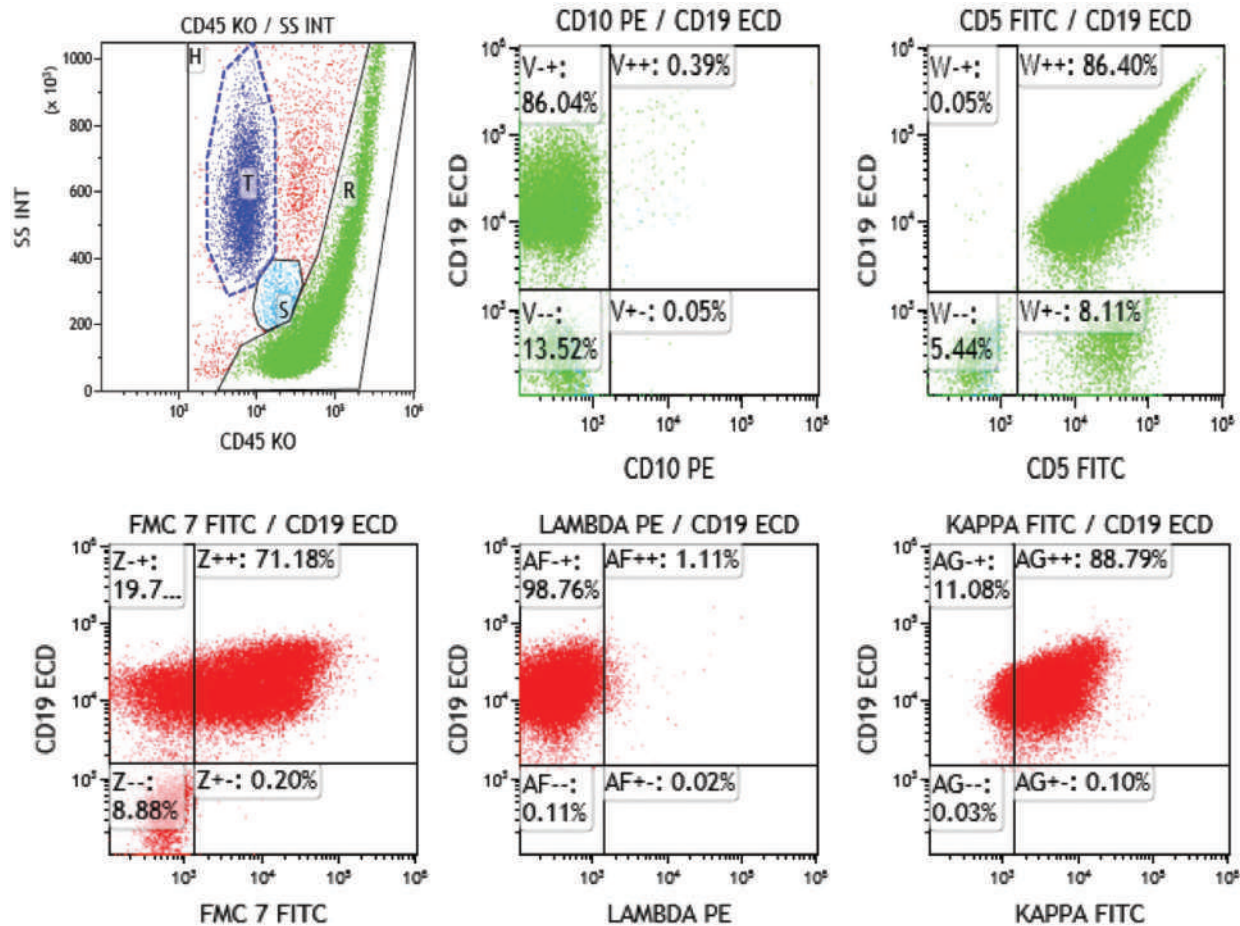


Fig. 7 Dual parameter dot plots useful in the diagnosis of Mantle cell lymphoma. R: lymphocytes; S: monocyte; T: granulocytes.

Glanzmann thrombasthenia

Glanzmann thrombasthenia (GT) is an autosomal recessive, inherited qualitative or quantitative platelet disease caused by deficiency or decreased function of the glycoprotein (Gp) IIb/IIIa complex in the platelet membrane. There are three subtypes which is determined by Gp IIb/IIIa level and varying in clinics. In this disease, most patients present with severe mucocutaneous bleeding at an early age. Diagnosis can be made using CD41 and CD61 monoclonal antibodies in flow cytometry examination.

Bernard-Soulier syndrome

Bernard Soulier syndrome (BSS) is a rare, autosomal recessive platelet dysfunction disease. It occurs as a result of the lack, absence or dysfunction of the glycoprotein Ib/V/IX complex, which is responsible for primary adhesion on the platelet surface (Diz-Küçükaya, 2013). Clinical findings are in the form of mucocutaneous bleeding (purpura, epistaxis, gingival bleeding, menorrhagia, gastrointestinal system bleeding) as in other platelet dysfunctions and rarely, life-threatening bleeding may occur (Lanza, 2006). And also, platelet counts vary depending on the presence of giant platelets (macrothrombocytopenia) (Andrews and Berndt, 2013).

FCM analysis shows normal binding with antibodies to CD41 (GP IIb) and CD61 (GPIIb), but defective binding with antibodies to CD42a (GPIX), CD42b (GPIb), CD42c (GP Ib) and CD42d (GPV) (Harrison et al., 2011).

Marginal zone lymphoma

Marginal zone lymphoma (MZL) is a heterogeneous and slow-growing group of B-cell NHL resulting from malignant transformation of marginal zone B cells (Bron et al., 2019). There are three main subgroups with variable clinics: extranodal, nodal and splenic MZL.

In immunophenotypic evaluation of MZL; the CD45 and SSC gate dot plot is used to identify different cell populations. MZL cells are positive for CD20, CD79a. CD5, CD10, CD23, CD43 are negative (Figs. 8–14).

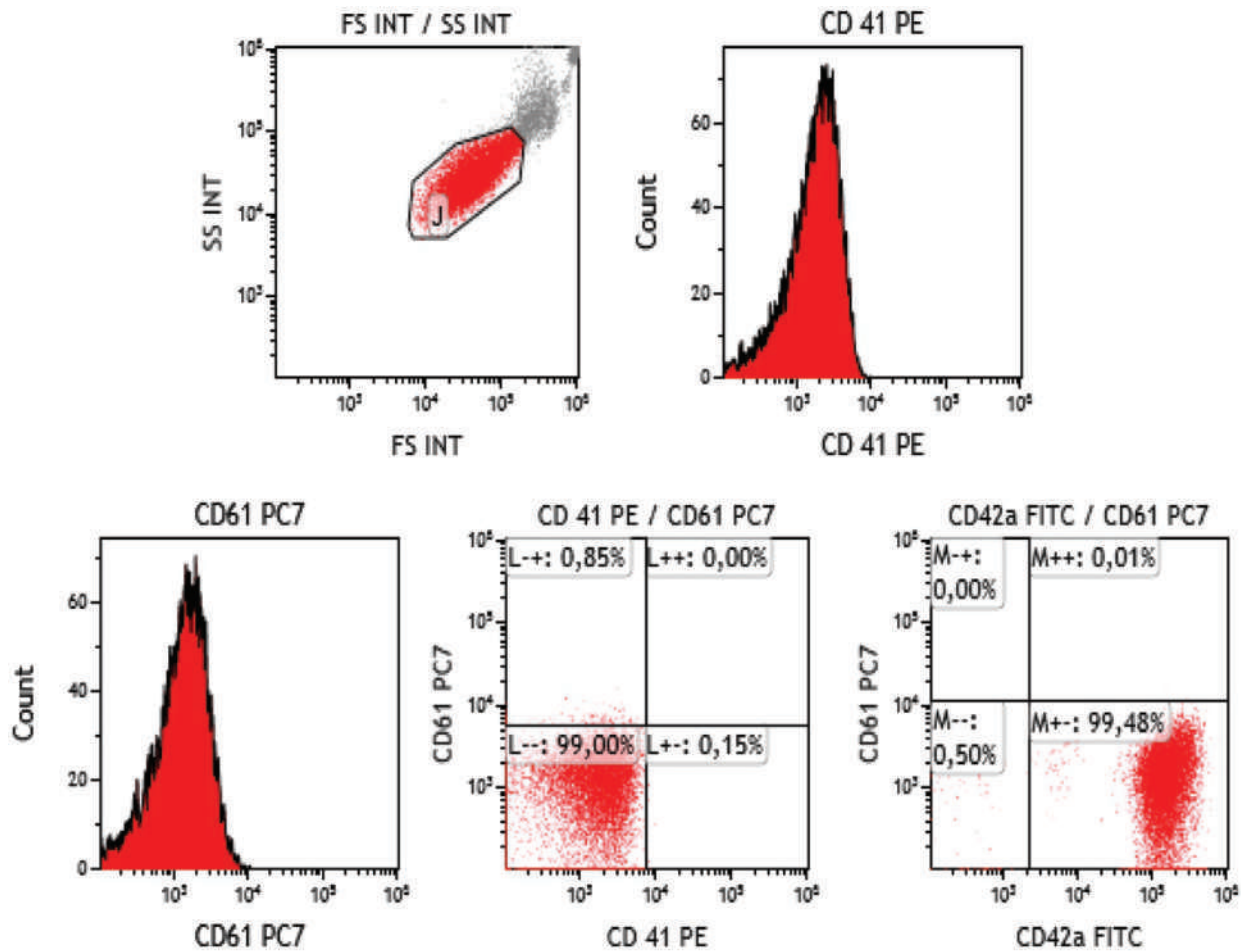


Fig. 8 Glanzmann thrombasthenia with reduced level of both CD41 and CD61. J: Thrombocytes.

Acute B lymphoblastic leukemia

Acute B lymphoblastic leukemia (B-ALL) is characterized by the development of large numbers of immature lymphocytes and is also a clonal enlargement of lymphoid blasts in bone marrow, blood, or other tissues. Possible factors associated with ALL are radiation, viruses, cytotoxic chemotherapy and benzene, but in most cases the cause is unknown (Onciu, 2009).

In immunophenotypic analysis; CD19, CD22, CD79a, CD24, TdT are positive for B-ALL. CD10 is positive in common-ALL (CALLA +), cytoplasmic Ig is positive in pre-B-ALL and surface Ig is positive in mature B-ALL. Myeloperoxidase (MPO), a marker for myeloid lineage, is typically not expressed.

Apoptosis

Apoptosis is a tightly regulated biochemical process centered primarily around the caspases family of enzymes. With conjugated annexin V is a widely used reagent in the detection of apoptotic cells (Fleisher and Oliveira, 2019).

Sepsis

Sepsis is a major health problem that causes thousands of deaths worldwide each year and is largely under-recognized. Prevention, diagnosis and management of sepsis is a very important issue because the mortality rate is high (Reinhart et al., 2017). Therefore, early diagnosis and initiation of appropriate treatment are very important.

Currently used markers to evaluate septic patients immunophenotypically; CD64, CD16, CD16-56, CD11b and HLA-DR.

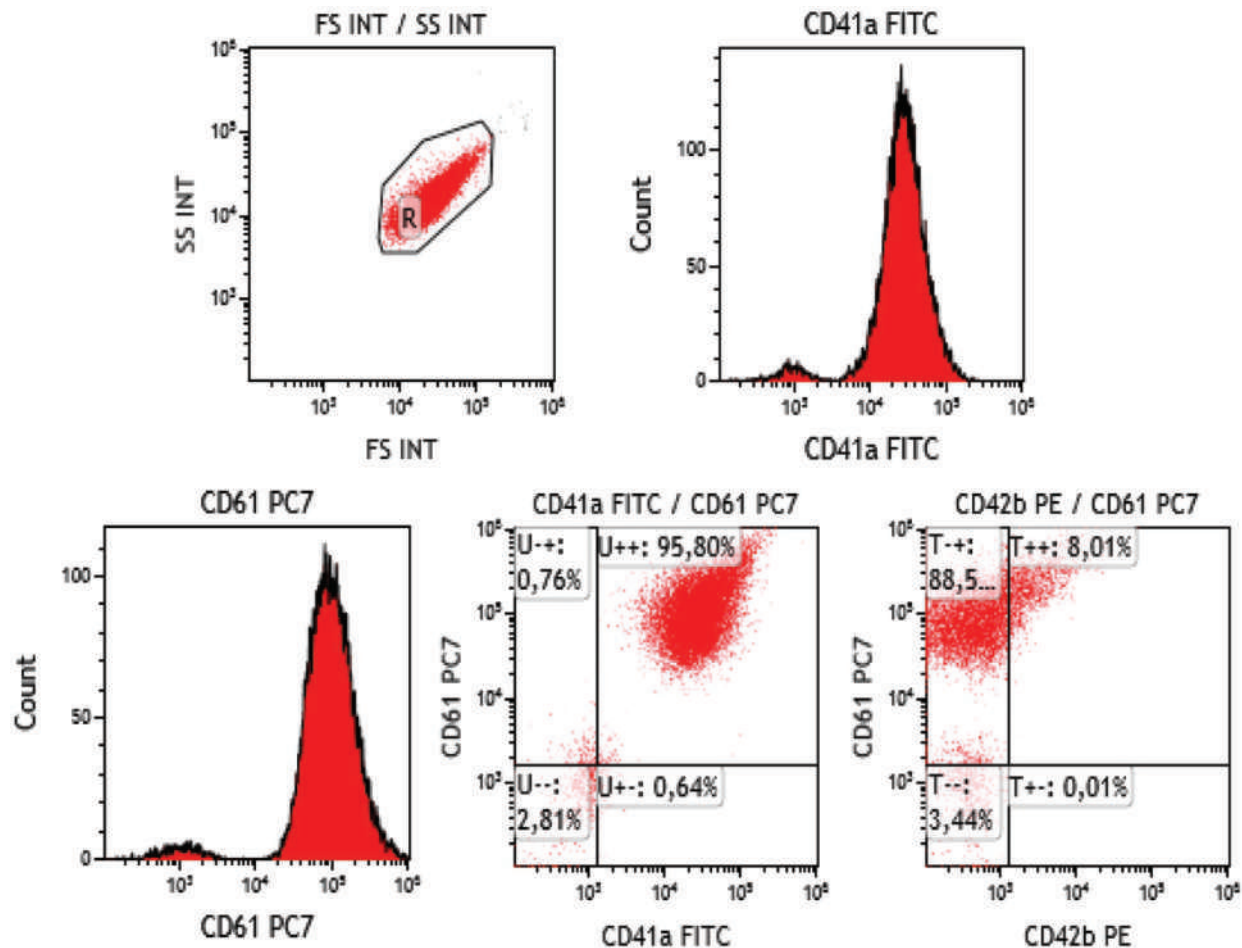


Fig. 9 Bernard Soulier Syndrome normal binding with antibodies to CD41 and CD61, but defective binding CD42b. R: Thrombocytes.

Cell cycle

We can detect cell populations at different stages of the cell cycle, such as G0/G1, S, and G2/M. We can also identify apoptotic cells (Bajgelman, 2019).

Conclusion

FCM has come a long way since the development of the first prototype. FCM has become readily available in clinical laboratories, with significant improvements in instrumentation. And day by day, it has started to be used in different fields of many branches of science. It plays an important role in the evaluation and understanding of complex diseases and in the longitudinal monitoring of treatments.

B cells are an important component of acquired immunity, responsible for the humoral immune response. These cells, which originate from the common progenitor cells in the bone marrow, continue their development in the secondary lymphoid organs and turn into plasma cells or memory B cells. Many mechanisms and signals are involved in the development and differentiation of B cells. Defects in developmental stage can lead to the formation of auto reactive B cells, malignancies and immunodeficiencies. Detection of dysfunctional B cells and defects, determination of their expression patterns, and investigation of therapeutic targets will help in the treatment of B cell-related diseases. Consequently, FCM has become a standard laboratory tool for the evaluation of hematopoietic cells and the identification of leukocyte populations and subsets. In addition, applications of FCM are used in many areas such as evaluating intracellular properties, characterizing apoptosis, evaluating changes in the cell as a result of stimulation, and identifying antigen-specific T cells. Thus, FCM has recently become a frequently used instrument as a diagnostic and research method in B cell diseases.

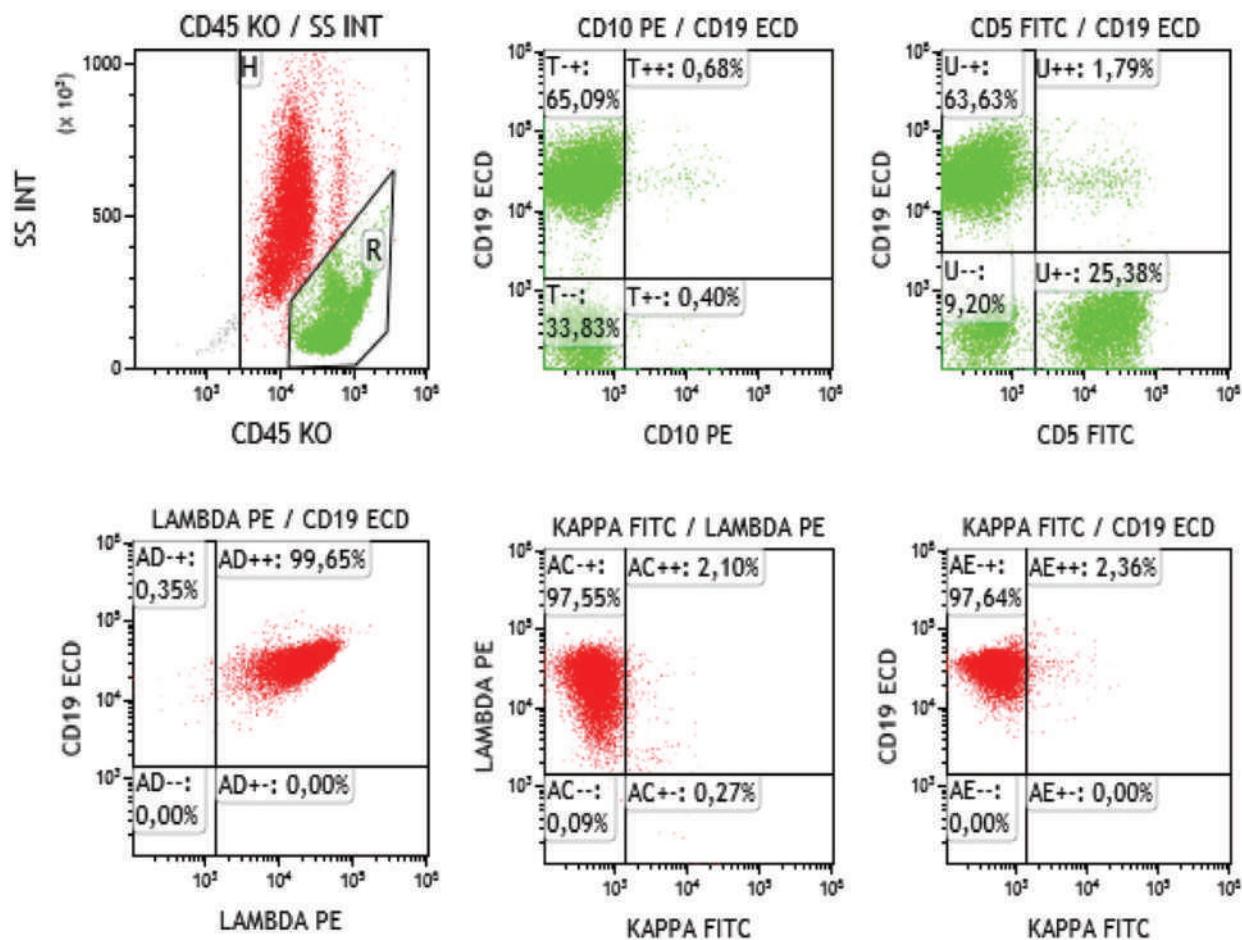


Fig. 10 Dual parameter dot plots useful in the diagnosis of Marginal zone lymphoma. H: Granulocytes; R: Lymphocytes.

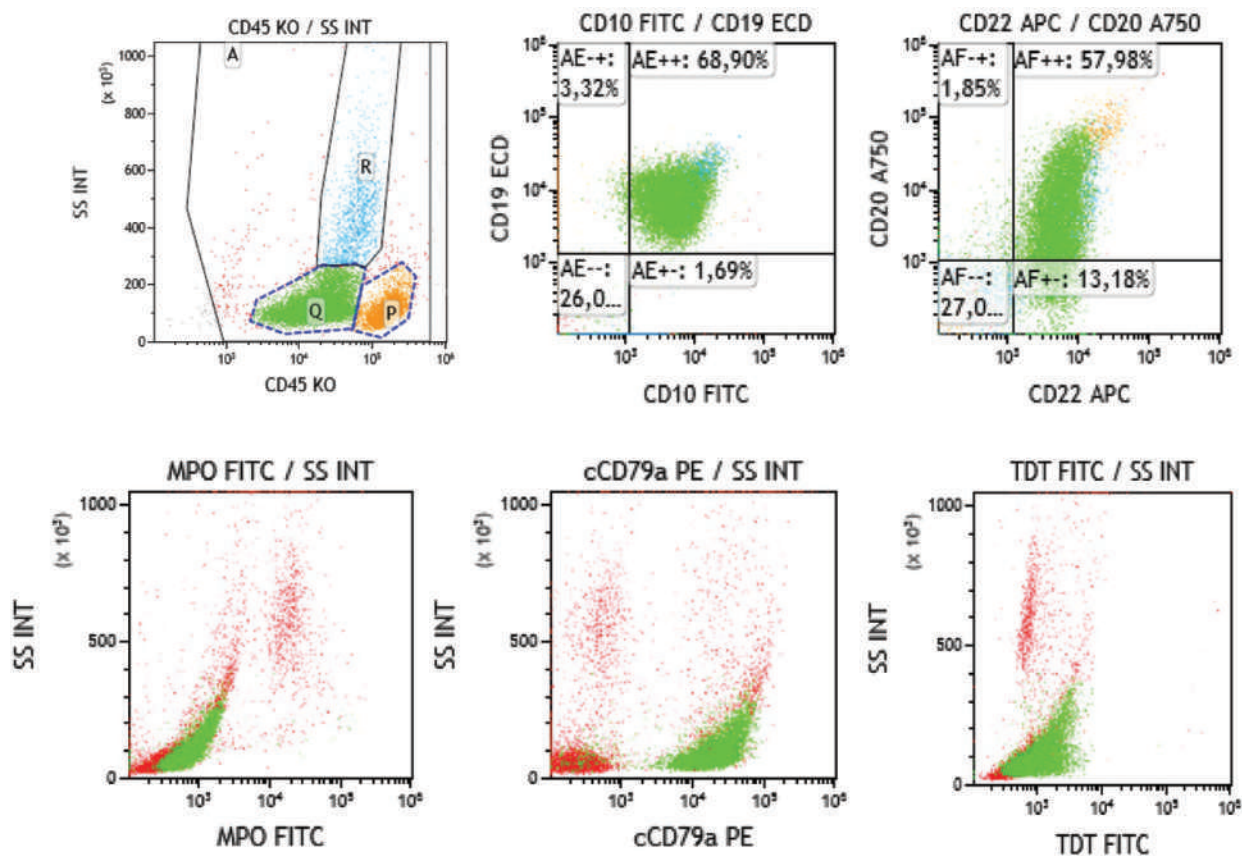


Fig. 11 Dual parameter dot plots useful in the diagnosis of Acute B lymphoblastic leukemia. P: Lymphocyte; Q: Blasts; R: Granulocyte.

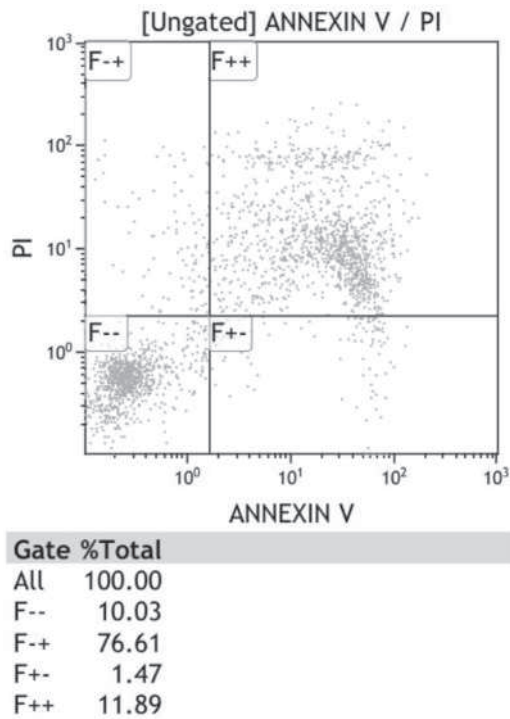


Fig. 12 Demonstration of apoptotic cells with conjugated Annexin V.

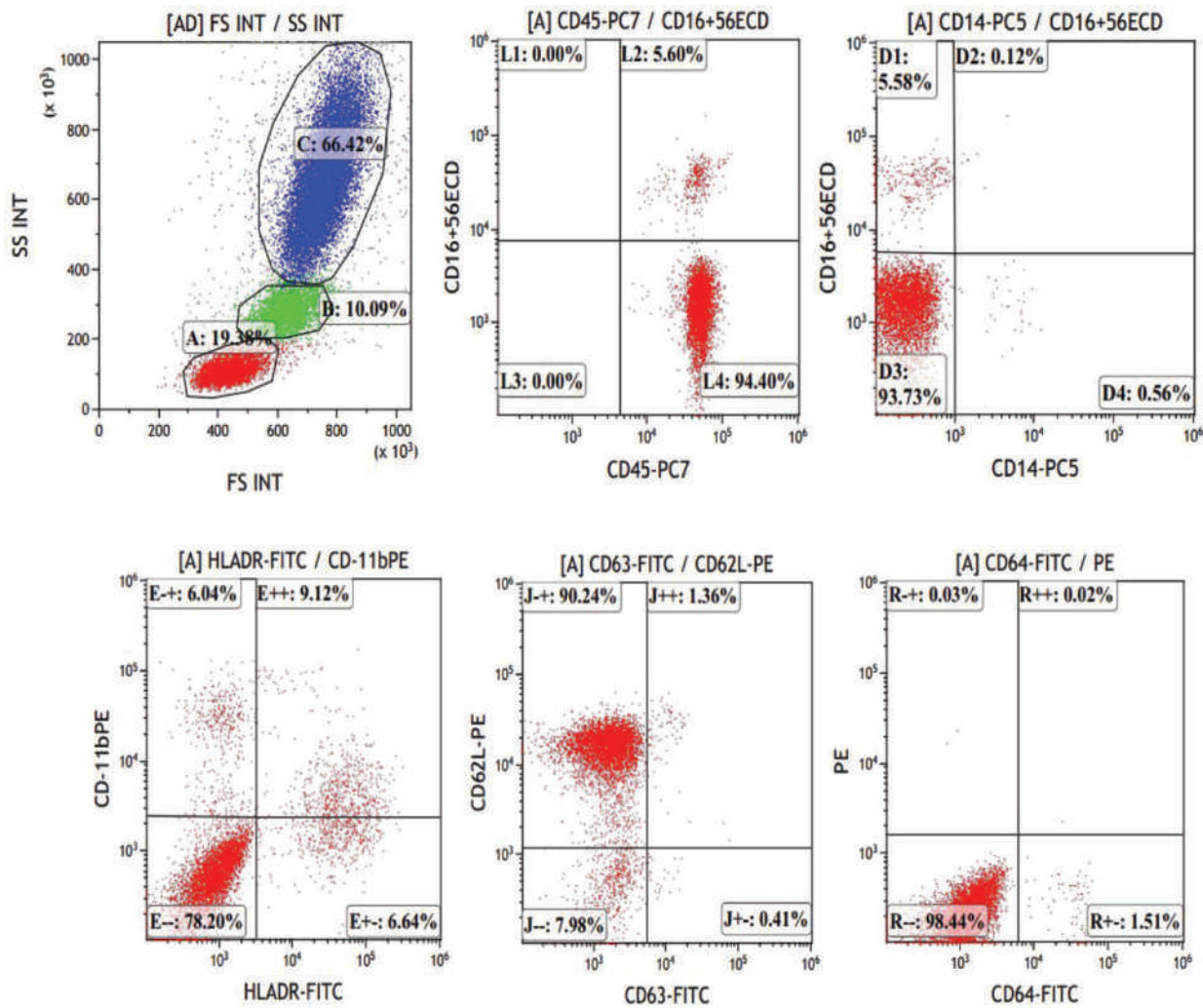


Fig. 13 Dual parameter dot plots useful in the diagnosis of sepsis. A: lymphocyte; B: lymphocyte; C: neutrophil.

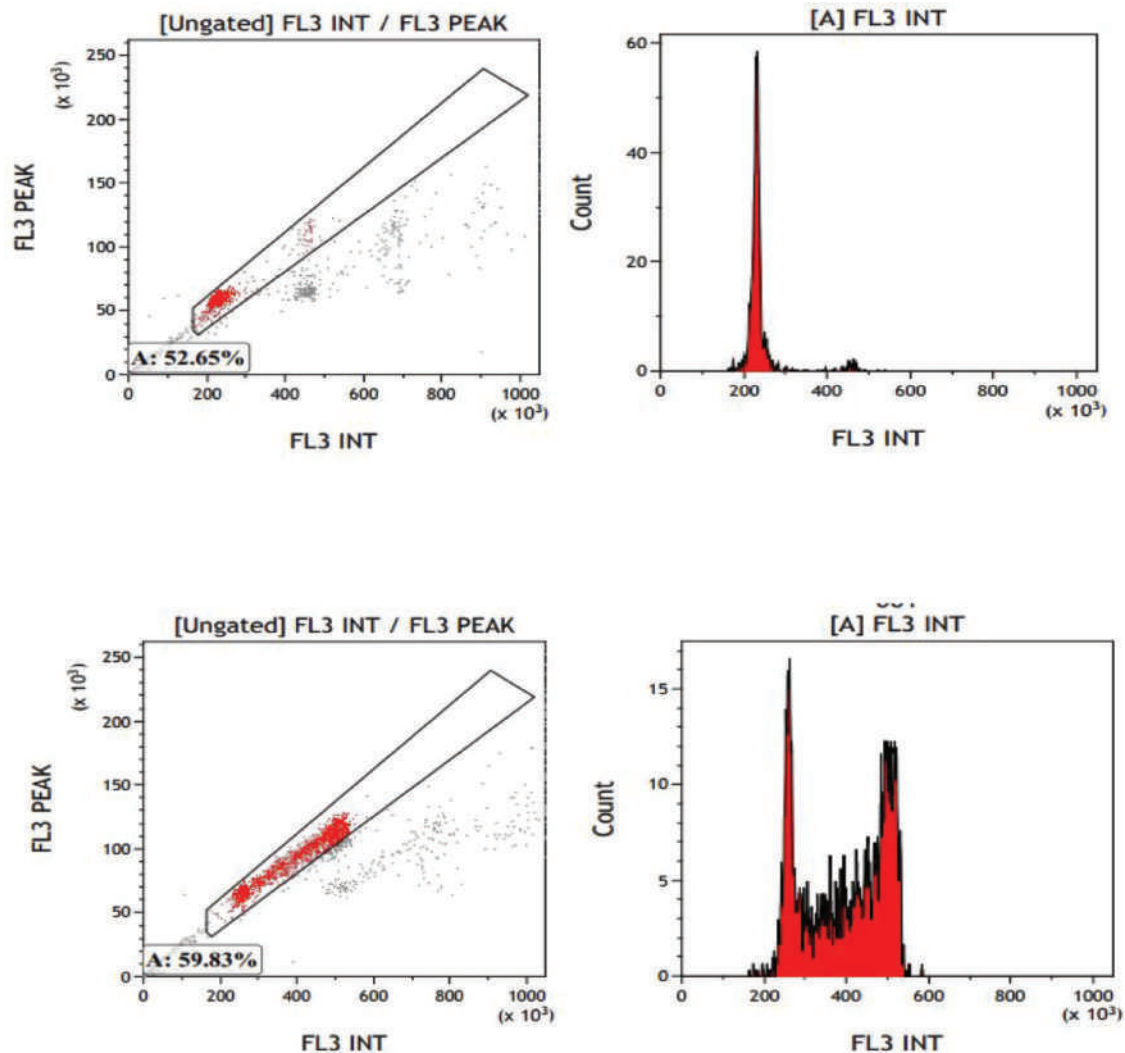


Fig. 14 DNA content cell cycle analysis (upper dot plots show normal cell cycle and DNA content. Lower dot plots show peripheral blood mononuclear cell [PBMC]). The histograms are gated to exclude debris and doublets. For DNA, doublets are detected by collecting peak versus integrated signals. In the dot plots on the right, we can distinguish between the G0/G1, S, and G2/M phases of the cell cycle and apoptotic cells.

The applications of this technology are developing significantly and new applications will emerge thanks to the rapidly developing technology. In this way, we can expect to be able to understand all the knowledge of B cell biology in the near future with the use of FCM.

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Functional Assessment of T Cells

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Introduction

T cell function assays involve the application of diverse techniques in order to decipher adaptive cell-mediated immune responses for diagnostic as well as for research purposes. T cell responses can be assessed by means of proliferative potential, cytokine secretion profile, immunophenotyping and subtype characterization, in bulk or antigen-specific T cell populations. High-throughput and robust newer methods enable the detection of immune responses even at the single-cell level and provide unprecedented insight on the complex mechanisms that mediate T cell responses and interactions. This abundance of options and possibilities renders the selection of the most suitable assay in a specific context challenging, and therefore require the deep understanding of the special characteristics, applications, and limitations of each method (Table 1).

Antigen presentation, through the interaction of Major Histocompatibility Complex (MHC) I or MHC II molecules with the T cell receptor, induces T cell proliferation and differentiation from naïve to effector and memory cells. This antigen-specific process pertains both normal host defense mechanisms, e.g., against pathogens or cancerous cells, as well as pathological immune states, such as autoimmunity and allergy. T cells are traditionally divided into two main subtypes: helper (CD4⁺) T cells and cytotoxic (CD8⁺) T cells. CD4⁺ T cells can be further analyzed into the T helper (Th) 1 and Th2 subsets, and the more recently discovered Th17, Th22, Th9 and follicular Th (Tfh) subsets. Regulatory T cells (Tregs) also play an important role promoting immune tolerance against self-antigens and providing protection against the deleterious effects of excessive immune responses.

The first part of this chapter provides an account of the most widely used T cell function assays, including the evaluation of proliferation, cytokine secretion, cytotoxicity and surface-marker expression, as well as single-cell analysis methods. The application of these assays in infectious diseases, transplantation, cancer, primary immunodeficiencies, allergy and autoimmunity is discussed in the second part of this chapter.

Table 1 Advantages and disadvantages of usually applied T cell function assays.

Assay	Advantages	Disadvantages
<i>Proliferation assays</i>		
[³ H]-thymidine incorporation	– Well validated	– Time consuming – Radioactive reagents – Endpoint assay
BrdU incorporation	– Non-radioactive – Capacity for <i>in vivo</i> experiments	– Requires DNA denaturation and membrane permeabilization
EdU incorporation	– Non-radioactive – Better reproducibility	
Fluorescent dye dilution	– Does not require DNA denaturation – Allows immunophenotyping – Can be used in combination with ICS or surface markers	– Cytotoxicity of dyes – Loss of dye intensity with multiple division cycles
Ki 67	– Good sensitivity – Suitable for multiparametric FC	– Does not specify number of cell divisions
<i>Cytokine-based assays</i>		
ELISA	– Widely available	– Cannot inform about cytokine producing cells – One cytokine per assay – Large sample volumes required for multiple cytokine detection
Cytometric bead array	– Multiple cytokines detection in one run (up to 100 targets) – Automated – Requires small sample volume	– Cannot inform about cytokine producing cells – Endpoint assay
ELISpot	– Quantitative and qualitative detection of cytokine-secreting cells – Extremely sensitive/detect even very low frequency T cells	– Cannot inform about the phenotype of cytokine producing cells (only their number) – Investigation of limited number of parameters
FluoroSpot	– Detection of up to four targets per assay	
ICS-FACS	– Parallel detection of multiple cytokines – Can be combined with other assays for extensive T cell phenotyping	– Endpoint assay due to chemical fixation of cells – Less sensitive than ELISpot
Cytokine secretion assay	– Isolation of live cells – Suitable for rare cell populations	
<i>Cytotoxicity assays</i>		
<i>a. Indirect</i>		
⁵¹ Cr release	– Sensitive	– Costly – Handling of radioactive compounds – High inter-assay variability – Background “noise”
CFSE dilution	– Sensitive – FACS based, allows phenotypic analysis of T cells	– CFSE cytotoxicity
7-AAD/PI	– Sensitive – FACS based, allows phenotypic analysis of T cells	
LDH release	– Widely available	– Low sensitivity/specificity
<i>b. Direct</i>		
CTL-ELISpot	– Quantitative and qualitative detection of cytokine-secreting cells – Extremely sensitive/detects even very low frequency T cells	– Cannot inform about the phenotype of cytokine producing cells
ICS-FACS (GrB/perforin)	– Phenotypic information available – “Single-cell” level information	– Cytokine-dependent – Endpoint assay
CD107a/FasL	– Phenotypic information available – “Single-cell” level information	
MHC-I tetramer staining	– Phenotypic information available – “Single-cell” level information	– Requires prior knowledge of epitope or HLA type
Activation induced markers (AIM)	– Cytokine-independent – Detection based on antigen-specificity	
<i>Single-cell analysis</i>		
Peptide-MHC polymer staining	– Detects all antigen-specific T cells, not only effector cells	– Requires prior knowledge of epitope or HLA type
Flow cytometry	– Easy to use & widely available	– Large panels require good compensation – Limited amount of information compared to CyToF & RNA-seq
CyToF	– No compensation needed – High variety of markers allows thorough phenotyping	– High cost – Endpoint assay – Limited availability
RNA-seq	– No compensation needed – Highest amount of information	– High costs – Endpoint assay – Limited availability (but increasing)

T cell function assays

Proliferation assays

Proliferation assays provide information about the extent of T cell response to specific antigenic or mitogenic stimuli. Historically, DNA incorporation of radio-labeled nucleoside analogs has been considered the “gold standard” for the assessment of T cell proliferation, but its use has since declined in favor of newer techniques.

[³H]-thymidine incorporation

This well-validated semiquantitative assay uses tritiated [³H]-thymidine incorporation into nascent DNA synthesized during the S phase of cell cycle, to assess T cell proliferation after antigen stimulation (Romar et al., 2016; Saade et al., 2012). Peripheral blood mononuclear cells (PBMCs) (or any other sample containing T cells and antigen-presenting cells), are cultured in wells, in the presence of the antigen of interest for 2 to 7 days, depending on the applied protocol, and then pulsed with the radioactive material. The incorporated radioactivity is measured up to 24 h after the addition of [³H]-thymidine by a scintillation beta counter and T cell proliferation is usually expressed as a stimulation index (SI), which is the ratio of the mean cell response in the antigen containing wells to that of control wells (Knutson et al., 2006). The main disadvantages of tritiated thymidine incorporation assay include safety issues that arise due to handling of radioactive material, inability to provide information on the phenotype, function and division history of individual cells, the need for prolonged incubation time and cumbersome steps, and the inherent variability of the method. Moreover, it is an endpoint assay as viable daughter cells cannot be retrieved for further characterization (Romar et al., 2016; Wallace et al., 2008). For these reasons, this technique has been largely replaced by flow cytometry-based assays of proliferation.

BrdU incorporation

An alternative non-radioactive thymidine analog, 5-brom-2'-deoxyuridine (BrdU), has been used for labeling proliferating T cells, *in vitro* and *in vivo*. BrdU incorporation in the DNA can be detected with a BrdU-specific monoclonal antibody, that can be subsequently traced by a fluorescent tag. BrdU presence is measured by means of flow cytometry or fluorescence microscopy. Also, it allows for *in vivo* assessment of cell proliferation in experimental animals, as it is stably integrated into the DNA after injection or digestion (Rothaeusler and Baumgarth, 2007). Although BrdU assay can be used simultaneously with other cell markers, it requires DNA denaturation and both cell and nucleus membrane permeabilization steps, thus limiting multiparametric analysis by flow cytometry. To overcome these drawbacks, a newer nucleoside analog, 5-ethynyl-2'-deoxyuridine (EdU) has been introduced that does not require harsh DNA denaturation processes (Poujol et al., 2014; Sun et al., 2016). Furthermore, EdU labeling shows better reproducibility and greater signal-to-noise ratio than the BrdU assay (Flomerfelt and Gress, 2016).

Fluorescent dye dilution assays

Fluorescent dyes diffuse easily through the cell membrane and bind covalently with amino groups on cytoplasmic macromolecules (Dey et al., 2019). The amount of dye present in each cell progressively decreases after each cycle of cell division and this reduction in fluorescent intensity can be quantified by flow cytometry (Saade et al., 2012). The most extensively used dye is the carboxy-fluorescein succinimidyl ester (CFSE), which generates a green fluorescent signal (Romar et al., 2016). Alternative dyes include cell trace violet (CTV), violet proliferation dye 450 (VPD-450), and CFSE derivative Oregon Green (Ten Brinke et al., 2017). Dye dilution assays are suitable for the further phenotypic characterization of dividing cells, allowing the detection of various surface markers and/or intracellular cytokines (Phetsouphanh et al., 2015). However, high concentrations of these dyes are cytotoxic and could impair both cell proliferation and expression of activation markers. Furthermore, CFSE allows for the detection of up to approximately 8 cycles of cell divisions due to the loss of fluorescence intensity with each division (Saade et al., 2012). Crucially, dye dilution assays, in contrast with the tritium-labeled thymidine assay, can be used to determine the frequency of antigen-specific T cells in the original population (Ten Brinke et al., 2017).

Ki67 expression

Ki67 is a nuclear protein expressed in all actively proliferating cells and during the phases G₁, S, G₂ and M of the cell cycle, whereas is undetectable in resting cells (G₀) (Lašćovička et al., 2016). It is a very sensitive assay for the assessment of antigen-specific T cell proliferation and it has been found to correlate well with [³H]-thymidine and BrdU incorporation assays, as well as with the CFSE dye dilution assay (Lašćovička et al., 2016; Soares et al., 2010). Ki67 staining can be followed by multiparametric flow cytometry for the delineation of T cells. However, Ki67 expression assay cannot provide information about the number of divisions that the cells have gone through, thus not allowing the estimation of the frequency of precursor specific cells (Soares et al., 2010).

Cytokine-based T cell assays

Cytokines are signaling molecules responsible for the orchestration and regulation of immune responses. Some of the most widely used assays for the assessment of T cell function involve the qualitative and quantitative measurement of cytokine secretion from stimulated T cells. Cytokine profiling provides information regarding the kind of elicited immune response (e.g., Th1, Th2, Th17) and the activation of antigen-specific T cells in bulk cell populations as well as on the single-cell level.

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a readily available and easy to perform technique that enables the detection and quantification of various cytokines. Suitable clinical samples include PBMC supernatant and whole blood, but other fluids and tissue lysates can be used for research purposes. The detection of interferon (IFN)- γ , tumor necrosis factor (TNF)- α and interleukin (IL)-2 cytokines usually marks the presence of Th1 response, whereas other combinations are used to define other T cell subsets, such as Th2 (IL-4, IL-5, IL-13), Th17 (IL-17, IL-21, IL-22) and Treg (tumor growth factor [TGF]- β , IL-10). Unfortunately, this method cannot provide any information regarding the individual cell populations producing the detected cytokines. Moreover, ELISA is able to measure only one cytokine per assay, thus requiring large sample volumes and labor-intensive procedures.

Cytometric bead array

To enable the simultaneous detection of multiple targets and to reduce time, labor and cost, multiplex bead-based methods have been developed. Polystyrene microspheres, internally dyed with two or three distinct fluorochromes and covered with target specific antibodies can be processed by a flow cytometer, allowing the quantification of multiple cytokines in serum or cell culture supernatants (Graham et al., 2019). This automated, high-throughput assay requires only a small sample volume and has a good correlation and higher sensitivity than ELISA (Saade et al., 2012). The most widely used, commercially available form of cytometric bead array is the Luminex™ platform that allows for the detection of up to 100 targets at once. Despite these advantages over ELISA, multiplex assays also cannot provide information about the nature of cytokine-producing cells and are insufficient for the detection of low-frequency antigen-specific T cells.

ELISpot and FluoroSpot

Enzymed-linked immunospot assay (ELISpot) has been applied for the quantitative and qualitative detection of cytokine-secreting cells. The assay involves the culture of PBMCs in the presence of an antigen in wells coated with anti-cytokine antibodies. Antigen specific cells secrete the cytokine of interest, which then binds to capture antibodies. After washing the cells away, cytokine production can be visualized as colored spots by enzymatically labeled antibodies interacting with a chromogenic substrate. The size and intensity of each spot, assessed by a specialized ELISpot reader, correlate with the amount of cytokine secreted on the single-cell level. ELISpot is extremely sensitive and is ideal for the detection of the very low frequency antigen-specific T cells, with or without the inclusion of in vitro expansion steps. On the downside, this method is able to assess only one or two analytes per assay and does not provide any information on the phenotype on the cytokine-secreting cells. However, FluoroSpot, a newer design of the assay using fluorochrome-conjugated detection antibodies and an automated fluorescence reader, allows the detection of up to four cytokines. Moreover, washed cells can be recovered and restudied with subsequent assays, allowing for their further characterization.

Intracellular cytokine staining (ICS)

A different approach for the assessment of cytokine secretion from antigen-specific T cells is the intracellular cytokine staining assay, typically coupled with fluorescence activated cell sorting (FACS). The technique involves the use of Golgi inhibitors (e.g., brefeldin A or monensin) which inactivate granule secretion and lead to intracellular retention of produced cytokines and other proteins (Phetsouphanh et al., 2015). Staining with fluorescent-labeled anti-cytokine monoclonal antibodies is performed after cell fixation and permeabilization (Lovelace and Maecker, 2018). Multiple cytokines can be detected at the same time as well as other intracellular and cell surface markers of differentiation, function and proliferation (Lovelace and Maecker, 2018; Saade et al., 2012), thereby enabling the extensive phenotyping and subset characterization of antigen-specific T cells. These properties of multi-parametric ICS enable the detection of polyfunctional T cell phenotypes (Smith et al., 2015). On the other hand, despite the advantages of this assay, the required step of T cell chemical fixation does not permit the retrieval of live cells for further analysis (Phetsouphanh et al., 2015). Also, ELISpot seems to be more sensitive than ICS for the detection of very low frequency T cell responses (Karlsson et al., 2003; Saade et al., 2012).

Cytokine secretion assay

Cytokine secretion assay is based on the formation of an anti-cytokine antibody matrix on the cell surface. After polyclonal or antigen-specific activation, PBMCs are labeled with bispecific antibodies that bind to a cell surface marker (i.e., CD45) and the cytokine of interest (Campbell et al., 2011; Rezk et al., 2020). Upon secretion, cytokines are captured by the bispecific antibodies on the cell surface and are identified with another fluorochrome-conjugated cytokine-specific antibody for flow cytometric analysis (Saade et al., 2012). Another option is to label the captured cytokine with antibodies conjugated with super-paramagnetic particles followed by magnetic activated cell sorting (MACS) (Assenmacher et al., 2002; Campbell et al., 2011).

Cytotoxic T lymphocyte assays

Cytotoxicity of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells supply an important mechanism in the defense against infection by viruses or intracellular-bacteria, and in anti-tumor immunity. The measurement of their lytic capacity provides useful insights in studying the properties of these cells. Lytic capacity can be studied in two ways: either indirectly by e.g. measuring the release of reporter molecules of targeted cells like in the classical ^{51}Cr release assay (Dunkley et al., 1974), or by assessment of cell death and/or apoptosis of targeted cells. Direct methods would include the measurement of molecules, which are directly involved in the lytic process of targeted cells and are provided by CTLs and NK cells e.g. the release of Granzyme B (GrB) and Perforin.

Indirect measurement of the effector function

In the ^{51}Cr release assay, target cells are loaded with an antigen of interest and labeled with ^{51}Cr . Loaded and labeled cells are then mixed and incubated in different ratios with effector cells. Mixing of target with effector cells should result in the lysis of target cells and subsequent release of ^{51}Cr into the cell supernatant, which can then be measured by a γ counter. Today, similar principles are used and combined with the power of flow cytometry (FACS). Cells are labeled with non-radioactive fluorescent dyes like CFSE (McGinnes et al., 1986) in combination with dyes for the assessment of cell death (Kandarian et al., 2017; Kim et al., 2020). A decrease in the intensity of CFSE labeled cells indicates increased cytotoxic activity of CTLs or NK cells. Other approaches use only dyes like 7-amino-actinomycin (7-AAD) and propidium iodide (PI), and directly measure ongoing apoptosis (Kim et al., 2007). Similar to the release of radioactive ^{51}Cr or to the use of fluorescent dyes like CFSE, the release and activity of enzymes can be used for the detection of cell lysis (Weyermann et al., 2005). The principle that governs these assays is the assessment of active enzymes, which are released from the cytosol into the cell culture supernatant upon lysis of the target cells. The functional properties of these enzymes are then used as an indicator for ongoing cell lysis. A representative example is the LDH enzyme-release assay (Korzeniewski and Callewaert, 1983). LDH is quickly released into the cell culture supernatant when the cell membrane is damaged during apoptosis. Subsequently, the supernatant is collected and incubated in the presence of NADH (produced during the oxidation of lactate to pyruvate by LDH), and an indicator, such as tetrazolium salt, that is reduced into a measurable red, water-soluble formazan-class dye (Kumar et al., 2018; Smith et al., 2011). A disadvantage of LDH release assay is that it does not differentiate between apoptosis and other forms of cell death. On the other hand, spontaneous release of LDH during the incubation period in comparison to that of ^{51}Cr is significantly lower (Korzeniewski and Callewaert, 1983).

Direct measurement of effector function

For the direct measurement of effector function of CTLs or NK cells, one can employ a variety of different methods. One option is to measure cytotoxic molecules by ELISpot. As previously described, ELISpot provides certain advantages and disadvantages over ELISA. ELISpot provides the possibility to measure effector molecules like IFN- γ or GrB in response to different stimuli e.g. virus peptides and could thus provide a means for the investigation of the effector function of CTLs and NK cells (Shafer-Weaver et al., 2003). Another option is to investigate effector function by FACS (Kim et al., 2007). FACS allows the investigation of different cell populations at once. By using the right labeling, NK cells and CTLs could be investigated in a single panel. Cells need to be stimulated ex vivo and the transport of granules containing GrB and Perforins needs to be inhibited by the use of the right inhibitors e.g. Brefeldin A, which blocks the transport from the Golgi apparatus to the cell surface (Letsch and Scheibenbogen, 2003). Similar stimulation can also be employed to investigate CD107a (Betts and Koup, 2004), which is another marker of degranulation and therefore indicates cytotoxic activity (Aktas et al., 2009), and could be combined with FasL (CD178), which mediates cell death by interacting with FasR (CD95) (Arase et al., 1995). Additionally, FACS provides the possibility to investigate the specificity of CTLs to certain antigens, as MHC class I tetramer staining of the T cell receptor would be possible (Xu and Screaton, 2002).

Activation-induced markers assay

Cytokine-based assays cannot account for the full repertoire of T cell responses after stimulation, as different T cell sub-populations are characterized by low frequency and functional heterogeneity. Activation-induced markers (AIM) assay is a cytokine-independent method for the detection of markers expressed on the cell surface following T cell receptor stimulation by a peptide-MHC complex. In contrast with intracellular cytokine staining, the AIM assay does not require cell fixation and permeabilization, thereby enabling the isolation of viable antigen-responsive T cells for further downstream applications (Elias et al., 2020).

Multiple markers have been investigated for the isolation of antigen-specific T cells. CD25 and CD69 are important T cell activation markers, but their lack of specificity necessitates the use of additional markers (Elias et al., 2020). The most widely applied combinations are CD25 (IL-2R α) /CD134 (OX40) and CD69/CD40L (CD154) for the isolation of antigen-specific CD4 $^{+}$ T cells, and CD107a/CD137 (4-1BB) for CD8 $^{+}$ T cells (Chattopadhyay et al., 2005; Frentsch et al., 2005; Wolff et al., 2007). CD25/OX40 is a global marker identifying all Th1, Th2, Th17, Tfh and Treg antigen-specific T cells. PD-L1, another activation-induced marker, in combination with OX40 can be used for the detection of all antigen-responsive T cells, excluding the Treg subpopulation, whereas 4-1BB $^{+}$ /CD40L $^{-}$ signature characterizes antigen-responsive CD4 $^{+}$ Tregs (Elias et al., 2020; Reiss et al., 2017; Schoenbrunn et al., 2012).

AIM assays are valuable for the detection of extremely difficult to isolate antigen specific cells, such as Tfh cells, which are underdetected in cytokine assays. Magnetic pre-enrichment can be used to collect a high number of low frequency T cells for further analysis, further increasing the sensitivity of the technique (Bacher et al., 2013). As with ICS, a problem with the AIM assay is the background caused by bystander activation that can limit the specificity of the method depending on the combination of markers used (Bowyer et al., 2018).

Suppression assays**Treg suppression assays**

Regulatory T (Treg) cells are responsible for the preservation of immune homeostasis and self-tolerance, controlling the expansion, activation function of effector T (Teff) cells. To produce these effects, Treg cells competitively consume IL-2, provide Teff cells with the inhibitory molecule cAMP, inhibit dendritic cells and promote the differentiation of Teff to Treg cells. Treg suppression assays

measure the ability of Treg cells to suppress the proliferation of antigen-responding CD4⁺ T cells *in vitro*. To assess their suppressive ability, a titrated amount of Tregs is cocultured with a specific number of antigen-specific T cells, and subsequently a proliferation assay, such as [³H]-thymidine incorporation or CFSE staining coupled with flow cytometry, is applied. Flow cytometry analysis permits the calculation of proliferation index (average number of divisions in the population), percentage of dividing cells, and division index (average number of divisions of a cell in the starting population). Moreover, flow cytometry allows simultaneous analysis for cell surface activation-induced markers and intracellular cytokine staining. Treg suppression assay may also be used in combination with IFN γ and TNF α detection in supernatant, as Tregs lead to robust suppression of these cytokines.

Viral inhibition assay

Viral inhibition assays have been developed in HIV research to address the failure of classical cytokine assays, such as IFN γ -ELISPOT and ICS, to predict the *in vivo* effectiveness of vaccine candidates (Pannus and Vanham, 2019; Spentzou et al., 2010). The method involves the coculture of activated, superinfected CD4⁺ T cells (target cells) with stimulated CD8⁺ T cells (effector cells), and enables the direct quantification of CD8⁺ T cells suppressive potential *ex vivo* (Slichter et al., 2014). Measurement of viral p24 in supernatant with ELISA or detection of intracellular gag with flow cytometry can be used for the quantification of viral replication in target cells alone and in coculture. The difference in viral replication represents the viral inhibitory activity of CD8⁺ T cells.

Single-cell analysis

Since the first development of flow cytometers in 1968/69 (Osborn, 1971), the technology of single cell analysis has made huge progress. The most commonly used method today is flow cytometry, but other methods have emerged, such as cytometry by time of flight (CyToF) or other single-cell analyzing methods which use beads linked to a barcode sequence and the mRNA of a gene of interest, which can later be identified using sequencing methods. Two manufactures using the latter method are Becton, Dickinson and Company (BD) with their Rhapsody™ or 10 $\times$ genomics with their Chromium™ instruments. Single-cell analysis is a powerful tool for immune-phenotyping. With recent developments in single-cell analysis, it is now not only possible to map the expression of surface or intracellular proteins, but also to make genome-wide expression studies in different cell populations at the same time.

The power and limits of flow cytometry

With flow cytometry already being in use for half a century, the method still has the potential for further improvement. Indicatively, recent advancements include the capability to measure up to 28 different fluorophores simultaneously, thus reaching a similar range of possibilities as with CyTOF instruments (Brummelman et al., 2019). As previously mentioned, flow cytometry can be applied in a variety of ways. Flow cytometry can be used to investigate different properties of cells and has a broad range of applications ranging from functional investigations to “simple” immunophenotyping of tissues using extra- and intracellular markers (Baumgarth and Roederer, 2000). By investigating different cell types, flow cytometry also provides the ability to use intracellular staining to look at cell signaling molecules e.g., p-ERK (Schmidt et al., 2018), and can thus provide insights into molecular pathways (Krutzik et al., 2004; Schulz et al., 2007). Moreover, peptide specific T cells can be identified using the power of fluorescently labeled peptide-bearing tetra- or pentamers (Newell et al., 2013; Rubio et al., 2003; Vollers and Stern, 2008). One of the biggest advantages is that flow cytometry offers the possibility to sort and isolate these labeled cells, which can then be used for further analysis (Bonner et al., 1972; Orfao and Ruiz-Argüelles, 1996; Pahl-Seibert et al., 2005). However, there are also limitations to the method, as spectral overlap of fluorescently labeled antibodies and autofluorescence need to be taken into account, and panels have to be built and compensated accordingly (Brummelman et al., 2019).

CyToF, a replacement for flow cytometry?

More recently, CyToF has emerged as a new method for identifying populations of cells. CyToF is also based on labeling targets of interest with molecular probes, such as antibodies. However, in this case, probes are not labeled with fluorescent dyes but rather with metal isotopes that emit a sharp signal, providing thus a major advantage over classic flow cytometry: the lack of spectral overlap. However, in contrast to flow cytometry, the cells are atomized and ionized during measurement and consequently cannot be retrieved for further analysis. Due to the sharp peak that is produced by the metal isotopes, CyToF theoretically provides up to 120 detection channels and a broader capability for immunophenotyping (Mora et al., 2014). Direct comparison of flow cytometry versus CyToF data has revealed that CyToF yields analogous quantification results of cell lineages (Gadalla et al., 2019). Similar experiments have been conducted measuring phosphorylated signal transducer and activator of transcription 3 and 5 (pSTAT3 and pSTAT5) for the assessment of intracellular signal transduction. In these experiments, data provided by CyToF correlate highly with those provided by flow cytometry (Mora et al., 2014). A similar experimental set-up has been used for a time-course analysis of STAT1, STAT3 and STAT5 phosphorylation in response to kinase inhibitors in PBMCs (Bodenmiller et al., 2012). Thus, when no further cell analysis is required, CyToF provides a powerful tool to simultaneously analyze different groups of cells and intracellular events on the protein level.

Single-cell RNA sequencing

Single-cell RNA sequencing (scRNA-seq) is another method which provides the possibility to characterize different sets of immune cells. As with CyToF, the cells are not retrievable after analysis and cannot be used for downstream assays, as they are lysed during

processing in order to access the transcriptome (Zhang et al., 2019). However, scRNA-seq can be combined with upstream bead-based cell isolation or FACS in order to enrich cells of interest, which can subsequently be analyzed to achieve the transcriptomic profile of particular cell populations on the single cell level. There are many available methods presented elsewhere (Stuart and Satija, 2019). Three of these methods are Smart-Seq2, BDs Rhapsody™ or the Chromium™ instruments from 10 × genomics. All these methods work on a similar principle where large cDNA libraries are generated from the mRNA profile of a single cell.

The Smart-Seq2 offers only the possibility to access the transcriptome, while surface markers cannot be analyzed and the single cell suspension has to be obtained using a different method (Picelli et al., 2013, 2014). The Rhapsody™ and Chromium™ instruments provide the ability to achieve both a single cell suspension and the investigation of other parameters, including, for example, cell surface markers, while also analyzing the transcriptome (Shum et al., 2019). The method of producing single cell suspension differs between the two instruments. While BD uses a system based on microwells and barcoded beads (Shum et al., 2019), the Chromium™ is based on a microfluidics system, where single droplets contain a single cell (Wang et al., 2021; Zhang et al., 2019). Both, however, work with the same principle of capturing the information of surface molecule expression. Moreover, these methods use antibodies that are linked to a specific nucleotide sequence. This allows the identification of these antibodies after sequencing, and thereby allows to build antibody panels similar to those seen in FACS, without the need for compensation.

Applications

The vast majority of the assays employed for the functional investigation of T cells are used primarily within research settings. There are only a few indications for their use in routine diagnostic assessment, namely for the diagnosis of tuberculosis and cytomegalovirus infection, as well as for the investigation of primary immunodeficiencies. The introduction of these assays into clinical practice has been challenging due to their complexity and cost, the need for specialized personnel and equipment, the high degree of inter-laboratory variability, and the difficulty to define clinically meaningful cut-off values. However, they have been indispensable tools for the comprehensive understanding of immune responses in health and disease, the development of new treatment strategies and the evaluation of therapeutic and/or preventive interventions.

Infectious diseases

T cell function assays have been widely applied in the field of infectious diseases, promoting the in-depth understanding of complex immune responses against various pathogens, the diagnosis of viral and microbial infection, and the evaluation of vaccine effectiveness.

Humoral immunity investigations, though easy and inexpensive to perform, are not sufficient to fully comprehend the diverse immune responses against infection. This has been the case particularly for certain viral and intracellular pathogens. Interestingly, robust T cell immunity has been detected in convalescent SARS-CoV-2 patients, even after the waning of serum specific antibodies, suggesting that cellular immunity may exist independently of antibody responses (de Candia et al., 2021). Antigen-specific T cell profiling regarding proliferation, cytokine secretion, activation, phenotype and function can provide insight into the pathogenicity, chronicity development and distinctive features of various infections. T cell function assays have also helped to elucidate the complex and catastrophic immune responses during sepsis, sepsis-induced immunoparalysis and cytokine storm (Martin et al., 2020).

IFN γ release assays (IGRA), based on ELISA or ELISpot techniques, have been implemented to overcome the drawbacks of tuberculin skin test (TST) that is used for the diagnosis of *Mycobacterium tuberculosis* (MTB) infection for over a century. The need for a new diagnostic test was dictated by the existing limitations in TST sensitivity and specificity, due to false negative results observed in immunosuppressed and HIV-infected individuals and false positive results caused by previous Bacille Calmette-Guérin (BCG) vaccination. ELISA-based QuantiFERON-TB assay (QFT) identifies *in vitro* antigen-specific T cells that secrete IFN γ in the presence of MTB peptides, the early secreted antigenic target 6 (ESAT6), the culture filtrate protein 10 (CFP10) and the TB7.7 antigen. T.SPOT-TB is an IFN γ -ELISPOT assay that also detects antigen-specific IFN γ production as a response to T cell stimulation by ESAT6 and CFP10. Because antigens contained in the BCG vaccine are different from those used in IGRAs, previous vaccination does not yield false positive results.

Assessment of T cell immune responses is of utmost importance in the development of new vaccines against pathogens for which humoral immunity alone does not offer considerable protection, such as viral and intracellular infections (e.g., HIV, malaria, TB, SARS-CoV-2, Ebola) (Saade et al., 2012). ELISpot and ICS (Streeck et al., 2009; Voss et al., 2018), as well as MHC-peptide tetramer and increasingly single cell analysis (Reeves et al., 2018), have been used extensively in vaccine studies for the detection of robust T cell responses, but *in vitro* effectiveness has not always been accompanied by vaccine success *in vivo* (Fitzgerald et al., 2011). Assays detecting cytotoxic T lymphocyte responses also play an important role for the assessment of protective immunity produced by candidate vaccines, especially against HIV and TB.

Transplantation

Solid organ transplantation (SOT) and hematopoietic stem cell transplantation (HSCT) greatly affect the immune system and its ability to fight infection. T cell assays have been used to monitor the immune status of the patients undergoing these procedures, to assess the risk of opportunistic infections due to immunosuppression, such as Cytomegalovirus (CMV), Epstein-Barr virus (EBV) and BK virus (BKV), and to evaluate the threat of graft rejection.

Due to the grave consequences of CMV reactivation in individuals undergoing transplantation, and particularly in allogeneic HSCT recipients, immune monitoring with assays that detect IFN γ secretion from CMV-specific CD8 $^{+}$ T cells has been proposed to guide pre-emptive treatment and management of CMV infection, in conjunction with CMV viral load measurement (Ljungman et al., 2019). IFN γ ELISPOT and ELISA-based QuantiFERON-CMV have both been successfully investigated in this context (Calarota et al., 2015). EBV latent infection may cause post-transplant lymphoproliferative disorders (PTLD) in immunosuppressed graft recipients, therefore monitoring EBV activity and tailoring immunosuppressive treatment accordingly is detrimental. Assessment of EBV-specific T cell responses with IFN γ -ELISPOT, ICS or pMHC multimer staining, when added to standard EBV viral load measurement, improves the ability to identify patients at risk and to monitor treatment efficacy (Calarota et al., 2015; Tischer et al., 2014). Another opportunistic infection affecting patients with a kidney transplant is BK polyomavirus reactivation, followed by nephropathy and graft dysfunction. In this case, the presence of detectable antigen-specific T cell responses by ELISpot correlates with the presence of functional antiviral immunity, and with the resolution of BK virus nephritis (Chakera et al., 2011). Finally, adoptive immunotherapy with the administration of virus-specific T cells derived from the patient, the donor or a third party, has been evaluated for the prophylaxis against and the resolution of multiple viral infections in transplant recipients. pMHC multimer technology as well as activation markers have been applied for the production of single- or multipathogen-specific T cell clones against CMV, EBV and Adenovirus (Khanna et al., 2011; Stemberger et al., 2014).

Another caveats in organ transplantation are the acute and chronic graft rejection. The best tactic to avoid such a dire consequence involves the selection of well tolerated organs by the host's immune system. Apart from human leukocyte antigen (HLA) matching between donor and recipient, another approach is the detection of alloreactive T cells by functional assays. These include proliferation assays to determine clonal size, T cell cytokine secretion with or without flow cytometry phenotyping, and intracellular ATP measurement (Anthony et al., 2012). Donor-specific IFN γ -ELISpot has been extensively studied for its ability to predict allograft rejection, with promising results (Montero et al., 2019). Intracellular cytokine staining followed by flow cytometry has the advantage of allowing the identification of cytokine-producing T cell subsets (Millán and Brunet, 2016).

Cancer

T cells, as part of the adaptive immune system, play a pivotal role in cancer immunology, ranging from tumor cell recognition, targeting and destruction, to cancer therapeutics and treatment monitoring. Cancer immunotherapy applications exploit and expand on the inherent immune system ability to fight tumor cells. Currently, cutting-edge techniques involving functional, molecular and antigen-specific T cell characterization and manipulation are deployed in adoptive T cell therapy, immune checkpoint blockade, and anti-tumor vaccine development.

Adoptive T cell therapy is based on the production of a tumor-specific T cell clone through either antigen stimulation in vitro or genetical engineering of T cells to express highly relevant T cell receptors (TCRs) or chimeric antigen receptors (CARs). Another approach is the non-specific in vitro expansion of naturally existing tumor-infiltrating lymphocytes (TILs) (Perica et al., 2015). This procedure is followed by the reinfusion of these cells into the body to enhance anti-tumor immune response. These approaches, though effective in vitro, do not always result into actual tumor regression. Cancer microenvironment promotes immune tolerance against cytotoxic T cells, among others, through upregulation of receptors, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death ligand (PD-1), that negatively regulate anti-tumor T cells (Houot et al., 2015). The development of monoclonal antibodies targeting these receptors has led to immune checkpoint therapy, which, in combination with other treatments, has been used for the treatment of melanoma, non-small cell lung cancer and renal cell carcinoma. Vaccines containing tumor-associated or tumor-specific antigens provide another method for the elimination of tumor cells. Most vaccines in cancer treatment are designed to induce cytotoxic T cell responses.

CD8 $^{+}$ cytotoxic T lymphocytes are the main weapons of the immune system against tumor cells, though the latter develop various mechanisms to escape immune recognition. The efficacy of the aforementioned therapies is usually measured by CTL assays, such as IFN γ , granzyme B and/or perforin ELISpot, flow cytometric evaluation of cell-mediated cytotoxicity and detection of CD107 surface marker of degranulation (Zaritskaya et al., 2010). Emerging single-cell techniques, including RNA sequencing and mass cytometry, supplemented by powerful computational tools for the analysis of resulting data, enable the in-depth characterization of diverse T cell subtypes and differentiation stages to: (1) illustrate potential treatment targets, (2) to monitor and assess the response to treatment, and (3) to develop new, personalized therapeutic strategies for cancer (Simoni et al., 2018; Stubbington et al., 2017).

Primary immunodeficiency

Primary immunodeficiencies (PIDs) comprise a diverse group of inherited immune disorders, characterized by atypical or recurrent infections, as well as dysregulated immune responses (Devonshire and Makhija, 2019). Defects in the number and/or function of T cells result in viral, fungal or protozoan infections during childhood that in severe cases are accompanied by failure to thrive (Albert-Vega et al., 2018). Due to the clinical and immunological diversity of these disorders, it is imperative to precisely determine the specific defects.

T cell function assays provide a valuable tool in the diagnosis of primary immunodeficiency. First, T cell immunophenotyping by flow cytometry enables the detection and enumeration of T cell subsets, such as naïve, activated, memory, Th17 and regulatory T cells (Abraham, 2011). Activation markers on the T cell surface, as well as intracellular proteins important for the function of effector T cells, can also be detected by flow cytometric analysis. Lymphocyte proliferation assays are fundamental in the

investigation of functional deficiencies in the T cell population. [^3H]-thymidine, BrdU or EdU DNA incorporation assays and CFSE dye dilution have all been used for the assessment of T cell response after stimulation with mitogens (e.g., phytohemagglutinin), commonly encountered antigens (*Candida albicans* or Tetanus toxoid) or anti-CD3 antibodies (Abraham, 2011). Cytotoxic T lymphocytes' assays identify specific defects in cell-mediated cytotoxicity. Also, further information for T cell function in PIDs is provided by TCR repertoire analysis of diversity and clonality using spectra-typing or single-cell RNA sequencing. T cell receptor excision circle (TREC) assay, which quantifies TCR gene DNA fragments produced during T cell differentiation, and identifies recently developed T cells, has been used for newborn screening of PIDs in the last decade. TRECs are absent in severe combined immunodeficiency (SCID) and other T cell lymphopenias (Chan and Puck, 2005; El-Sayed and Radwan, 2019).

Allergy and autoimmunity

Allergic responses are characterized by effector Th2 cells producing, among other cytokines, IL-4, IL-5, IL-9 and IL-13. IL-4 and IL-13 are implicated in the class switching of B cells to produce IgE, initiating the allergic cascade (Akdis and Akdis, 2015). For the assessment of immune tolerance against allergens it is essential to identify allergen-specific Foxp3⁺ CD25⁺ regulatory T cells (Tregs) and IL-10, IL-35 and TGF- β production (Kucuksezer et al., 2020). A special issue arising in the immunological study of allergy is the sparsity of allergen-specific T cells in peripheral blood that poses significant technical difficulties. To overcome this problem, functional and phenotypic analyses are often performed after an expansion step has first taken place. However, this approach is susceptible to bias, because in vitro culture after stimulation with allergen peptides may unpredictably affect the phenotypic and functional characteristics of the T cell subsets in question (Bacher and Scheffold, 2018). pMHC-multimers that specifically bind to the TCR, in combination with magnetic enrichment of all allergen-specific T cells, have been applied to avoid the inherent biases of prolonged culture methods. The disadvantages of this approach are that it can be applied only on previously known antigenic epitopes, and that it is HLA restricted. Cytokine secretion and activation-induced markers have also been used for the assessment of allergen-specific responses in short-term activation assays. The production of Th2-specific cytokines, such as IL-4, IL-5, and IL-10 from specific T cells can be assessed through cytokine secretion assays, ELISpot, ICS and RNA sequencing, whereas CD40L and 1-4BB have been used as activation-induced markers (Anderson et al., 2009; Bonvalet et al., 2011). Furthermore, T cell function assays have been essential for the assessment of the immunosuppressive capacity of allergen-specific immunotherapies in allergy treatment (Bacher and Scheffold, 2018).

Autoimmunity is caused by the inability of the immune system to discriminate between the self and the non-self. Antigen presentation to T cells and loss of self-tolerance are of great importance to this process. Auto-reactive T cells normally undergo central (thymus) or peripheral deletion and functional inactivation (anergy). Treg cells also play an important role suppressing autoimmune responses (Serra and Santamaria, 2019). Th1 and Th17 responses, as well as dysregulation of Treg function have been extensively studied for their role in the pathogenesis of various autoimmune diseases, especially in multiple sclerosis, type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, and others (Khan and Ghazanfar, 2018; Riedhammer and Weissert, 2015). The investigation of T cell function, pathogenic phenotypes, cytokine expression and TCR repertoire analysis in relation to autoimmunity has provided a wealth of information for the pathogenesis and potential therapeutic targets of immunotherapy (Bronge et al., 2019; Jiang et al., 2020). Today, there are multiple monoclonal antibodies for the treatment of autoimmune diseases targeting and blocking the pathways leading to auto-reactivity in specific loci. T cell function assays are also important in studies investigating the efficacy of these drugs, as well as in the research for the development of new antigen-specific treatments (Serra and Santamaria, 2019).

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Phagocytosis: Biology and Methods

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Introduction

Phagocytosis is a fundamental activity necessary for host defenses against infection and for the clearance of apoptotic and necrotic cells (Gordon, 2016; Witko-Sarsat et al., 2000). Circulating cells, such as neutrophils, eosinophils, and monocytes, can ingest and digest particles at sites of infection and sites of injury. Tissue cells, such as macrophages, also ingest and degrade exogenous and endogenous particles in response to infection or tissue injury. Most clinical studies use circulating neutrophils to study phagocytosis, but it is also possible to recover alveolar macrophages using bronchoscopy and study these cells *in vitro*. Defects in phagocytosis can significantly increase the likelihood of serious infection, and evaluating neutrophil function becomes important in patients with recurrent deep-seated infections.

Phagocytosis requires contact at the surface of the phagocytes with the microbe or injured tissue through either specific receptors or nonspecific receptors. The initial events involve the formation of the phagosome, ingestion of the phagosome, and fusion of the phagosome with intracellular lysosomes for inactivation and digestion (Fig. 1). Other essential events involve the movement of the phagocytes to the site of injury through chemotaxis. In this review we will discuss laboratory methods needed to measure some aspects of neutrophil function with a focus on phagocytosis.

Phagocytic activity using clinical samples

Phagocytosis assays using whole blood can use viable bacteria or fungi (Wardle, 1986). These assays present practical difficulties, including maintaining bacterial stock, standardizing the number of bacteria in assays, and identifying the exact phagocyte involved in microbial killing. An alternative approach involves the use of inert beads that have different fluorescent reporters (Simons, 2010). For example, Zymosan particles are prepared from yeast cell walls and have an approximate size of 3 μm . These particles can be treated with fluorescent reporters to identify uptake into cells and can be treated with an oxidation sensitive fluorescent molecule that would identify oxidative activity in the phagocytes. Zymosan is a glucan with repeating glucose units on the surface of yeast and can be ingested by monocytes, neutrophils, and macrophages through various receptors independent of opsonization (Figs. 1 and 2).

Gupta-Wright et al. used whole blood from control subjects and HIV-tuberculosis infected patients to determine phagocytic activity; this study illustrates the use of the methods listed below (Gupta-Wright et al., 2017). These zymosan particles were coupled to a fluorochrome (Alexa Fluor 405-SE, Invitrogen) and an oxidation sensitive fluorescent reporter (OxyBURST[®] Green

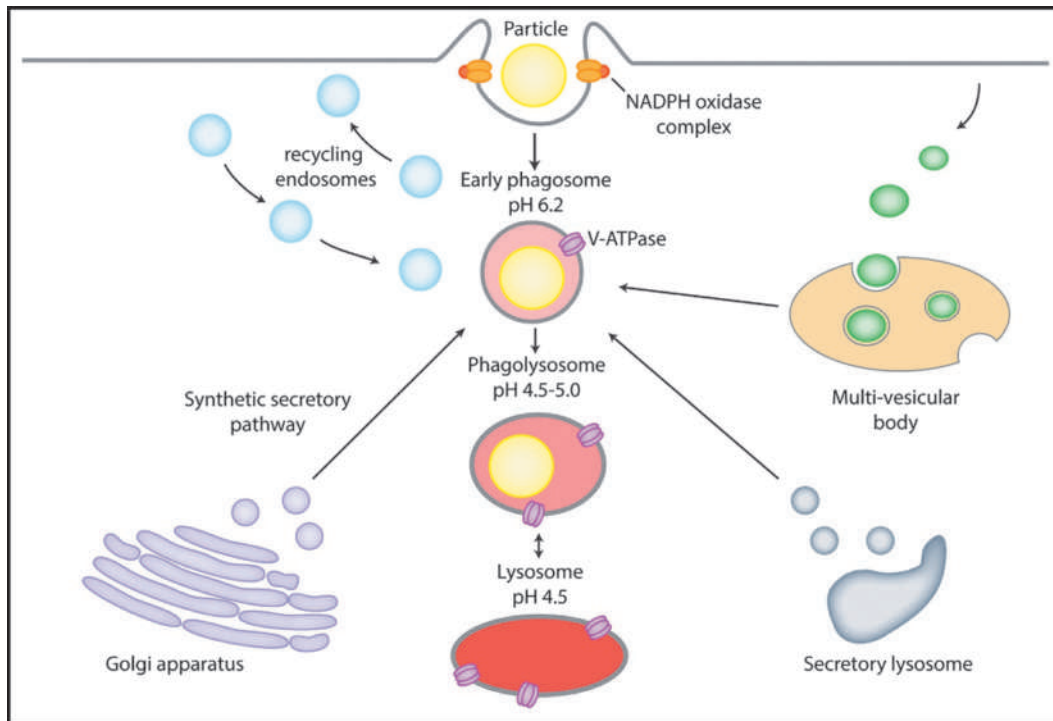


Fig. 1 Phagocytic pathway. Ingestion of exogenous particles results in a dynamic sequence of complex events from plasma-membrane fusion and fission to the formation of intracellular vesicles, maturation of phagosomes, and progressive acidification and ultimately phagolysosomal fusion and digestion. The biosynthetic and secretory pathways intersect with the phagocytic pathway. Adapted from Gordon S (2016) Phagocytosis: An Immunobiologic process. *Immunity* **44**: 463–475 and used with permission.

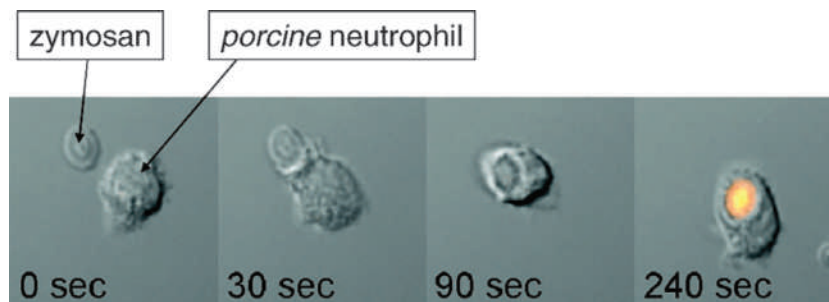


Fig. 2 Fluorescence microscopic imaging of phagocytosis of opsonized zymosan by fluorescently-tagged porcine neutrophil. Zymosan particle is located near the neutrophil (0 s). Zymosan is engulfed by the neutrophil (30 s) and phagocytosis was completed (90 s). The fluorescent signal of hypochlorous acid generated in the phagosome was detected with a probe (240 s). Adapted from Koide Y, Urano Y, Hanaoka K, Terai T and Nagano T (2011) Development of an Si-rhodamine-based far-red to near-infrared fluorescence probe selective for hypochlorous acid and its applications for biological imaging. *Journal of the American Chemical Society* **133**: 5680–5682 and used with permission.

H2DCFDA-SE, Invitrogen). The phagocyte ingesting the zymosan particle was identified using antibodies specific for either neutrophils or monocytes. All blood samples were incubated with the zymosan particles at 37 °C, and samples were taken at 10, 30, 60, 90, and 180 min of incubation. After lysis of red cells, leukocyte suspensions were analyzed with flow cytometry to measure both uptake and oxidative burst. There was some variability among control subjects but clearly significant ingestion with an oxidative occurs in 30–60 min. Patients with HIV and active tuberculosis had reduced oxidative activity at all time points in this assay. The use of electron microscopy provides definitive proof of particle ingestion.

Methods for isolating monocytes and macrophages and measuring phagocytosis**Isolation of primary human monocytes**

1. Collect blood samples from subjects into heparinized BD Vacutainer[®] plasma tube (BD, United States), invert several times gently and proceed to monocyte isolation.
2. Fill Greiner LeucoSep tubes (50-mL) (Millipore Sigma, United States) with 15 mL Ficoll-Paque PLUS (GE Healthcare, IL, United States) and centrifuge for 30 s at $1000 \times g$ at room temperature.
3. Dilute blood sample in phosphate-buffered saline (PBS) (1:2 dilution) and distribute 30 mL of diluted blood on each LeucoSep tubes.
4. Using density gradient centrifugation at $1000 \times g$ for 10 min (without deceleration), separate peripheral blood mononuclear cells (PBMCs), collect cells from the interphase and wash 3 times with PBS at $300 \times g$ for 5 min at ambient temperature.
5. After last wash, remove the supernatant and add 10 mL ACK lysing buffer (Thermo Fisher Scientific, United States) and incubate for 10 min to lyse red blood cells.
6. Wash cells with PBS as above and resuspend in EasySep Buffer[™] (Stemcell Technologies, Canada). Count cells and adjust cell concentration to 5×10^7 cells per mL.
7. Isolate monocytes by immunomagnetic negative selection using the EasySep Human Monocyte Isolation Kit according to manufacturer's instructions and proceed to differentiation and activation of macrophages.

Differentiation and activation of macrophages

1. Culture primary human monocytes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) + 1% Non-Essential Amino Acid (NEAA) + 100 µg/mL penicillin-streptomycin (all from Thermo Fisher Scientific, United States) with 50 ng/mL Macrophage Colony-Stimulating Factor (M-CSF) (R&D Systems, United States) in a 175 cm² cell culture flask with Nunclon Delta Surface (Thermo Fisher Scientific, United States) for 5 days.
2. On day 6, activate M0 macrophages for 24 h with either M-CSF (10 ng/mL), GM-CSF (10 ng/mL) and lipopolysaccharide (LPS) (10 ng/mL, Millipore Sigma, United States) for M(GM-CSF, LPS) macrophages; or M-CSF (10 ng/mL), IL-4 (10 ng/mL) and IL-13 (10 ng/mL) for M(IL-4, IL-13) macrophages. All cytokines are from R&D Systems.

Phagocytosis assay using flow cytometry

1. Procure *Staphylococcus aureus*, *Escherichia coli* and zymosan A particles conjugated with pHrodo red and pHrodo green from Thermo Fisher Scientific.
2. Label Aliphatic Amine Latex Beads (Thermo Fisher Scientific, United States) with pHrodo Green STP Ester or pHrodo Red Succinimidyl Ester (Thermo Fisher Scientific, United States) according to the manufacturer's instructions.
3. Add beads or particles to cells (isolated above) at indicated concentrations (manufacturer's instruction) and incubate at 37 °C for 1 h in 5% CO₂.
4. For primary human macrophages, perform phagocytosis assays in UpCell plates (Thermo Fisher Scientific, United States), which allow cells to detach without enzymatic digestion of proteins.
5. Place plates containing cells on ice to stop further phagocytosis and stain for live cells with DAPI (Thermo Fisher Scientific, United States).
6. Analyze cells on BD LSRFortessa[™] X-20 or sort on BD FACSMelody[™] Cell Sorter (BD Biosciences, United States). Representative images of phagocytosis of opsonized zymosan particle is shown in Fig. 2.
7. Gate cells based on forward scatter/side scatter properties, singlets and live (DAPI) cells using FlowJo v10.7 (FlowJo LLC, United States) and determine the frequency of pHrodo+ cells and/or the mean fluorescence intensity.

Phagocytosis assays using high-content image screening

1. Seed macrophages into poly-D-lysine-treated 96-well view plates (Perkin Elmer, United States) in DMEM 24 h before phagocytosis assay.
2. Prepare Hoechst staining solution by diluting Hoechst stock[®] solution (Thermo Fisher Scientific, United States) 1:2000 in PBS.
3. Remove the culture medium and add sufficient staining solution to cover the cells and incubate for 10 min protected from light for detection of nuclei.
4. Replace cell culture medium and add pHrodo particles suspension to the well.
5. Perform kinetic measurements using the high-content imager Opera Phenix[™] (Perkin Elmer, United States) at 37 °C and 5% CO₂ by imaging four fields from each well using the 20 × objective. Add cytochalasin D (an inhibitor of phagocytosis) in some wells as controls.
6. Perform image analysis using Columbus[™] (Perkin Elmer). Hoechst 33342-stained nuclei and the cytoplasm around each nucleus can be identified as well as the pHrodo green-positive spots within this region.
7. To calculate normalized intensities, multiply the area and fluorescence intensity of the pHrodo spots and divide by the number of nuclei per image.

Functional analysis of phagocyte activity in whole blood

1. To quantify phagocytic activity and the magnitude of the superoxide burst, use zymosan particles coupled to Alexa Fluor 405-SE (Thermo Fisher Scientific, United States) and an oxidation-sensitive fluorescent reporter (OxyBURST[®] Green H2DCFDA-SE, Thermo Fisher Scientific, United States).
2. Prepare zymosan reporter particles by washing 6 mg of zymosan (Millipore Sigma, United States) three times in PBS by centrifuging at $5000 \times g$ for 1 min.
3. Resuspend particles in 950 μ L coupling buffer (0.1 M boric acid to pH 8.0 with NaOH) containing 10 μ L of 25 mg/mL OxyBURST-SE/DMSO stock solution and 5 μ L of 5 mg/mL Alexa Fluor 405-SE/DMSO solution, mix well and incubate on a rocker in the dark for 1 h at ambient temperature and washed with 1 mL of coupling buffer.
4. Wash coupled particles three times in PBS and store in 1 mL PBS containing 0.01% sodium azide in the dark at 4 °C with a final stock concentration of approximately 5×10^6 particles/mL.
5. Wash 50 μ L of stock zymosan particle suspensions three times in 1 mL RPMI-1640 to remove sodium azide and resuspended in 250 μ L RPMI-1640 to give a 1:6 dilution (final concentration, 8×10^5 particles/mL).
6. Add 20 μ L of washed diluted particles to 1 mL of diluted whole blood (1:1 in warm RPMI-1640) and incubate at 37 °C with rocking to ensure particles and cells remain in suspension. Prepare diluted blood without zymosan reporter particles in parallel to act as control.
7. Assess phagocytosis of zymosan reporter particles and superoxide burst at predetermined time points (e.g., 10, 30, 60, 90, and 180 min) after the addition of reporter particles.
8. Harvest 100 μ L of diluted blood from the zymosan reporter and control tubes 10 min before each time point for cell surface staining. Note: phagocytosis continues during cell surface staining of live cells.
9. Stain harvested diluted blood with appropriate dilutions of antibodies for 10 min (anti-CD45 PerCP 1:33, anti-CD66b APC 1:50, and anti-CD14 PE-Cy7 1:100; all from BioLegend, United States).
10. Add 3 mL of BD FACS lysing solution containing formaldehyde and diethylene glycol to each tube and incubate for 10 min in order to stop all biological activity, lyse red blood cells and fix the white blood cells.
11. Wash cells once in PBS by centrifuging at $500 \times g$ for 10 min, add CountBright[™] counting beads (Thermo Fisher Scientific, United States) according to manufacturer's instruction and acquire on a CyAn ADP flow cytometer (Beckman Coulter, United States).
12. Analyze data using the FlowJo version v10.7 (FlowJo LLC, United States).

Intracellular oxidative activity

The generation of reactive oxygen species during the phagocyte respiratory burst is initiated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a membrane-bound enzyme complex found in the plasma membrane and phagosomes. This complex functions to catalyze the transfer of an electron from NADPH to O_2 , producing superoxide (O_2^-) after interaction with inflammatory stimuli (Gordon, 2016; Witko-Sarsat et al., 2000). Superoxide functions as a precursor to other reactive oxygen species, such as hydrogen peroxide, hypochlorous acid, and hydroxyl radical. Superoxide dismutase catalyzes the generation of hydrogen peroxide, which may then form hydroxyl radicals by either the Haber-Weiss reaction or Fenton reaction. In addition, myeloperoxidase can catalyze the production of hypochlorous acid from chloride ions and hydrogen peroxide.

Oxidative activity can be measured using either the nitro-blue tetrazolium test or the dihydrorhodamine test. For example, patients with chronic granulomatous disease of childhood that do not have oxidative bursts have no black deposits with the nitro-blue tetrazolium test (Häger et al., 2010; Campos et al., 1974; Freeman and King, 1972). The dihydrorhodamine test measures nicotinamide alanine dye nucleotide phosphate oxidase activity (Henderson and Chappell, 1993; Koide et al., 2011). A nonfluorescent dye is taken up by the neutrophils and becomes fluorescent after reaction with reactive oxygen species produced by the respiratory burst. Neutrophils from a patient with X-linked chronic granulomatous disease of childhood do not fluoresce after stimulation (Fig. 3) (Dimitrova et al., 2013). Both these assays can be modified to include endotoxin stimulation or phorbol myristate acetate stimulation of the patient's neutrophils. The nitro-blue tetrazolium assay can be modified to give quantitative measurements of the oxidative burst (Choi et al., 2006).

Methods to measure intracellular oxidative activity

Nitro-blue tetrazolium assay

1. The nitro-blue tetrazolium test requires a small amount of anticoagulated blood. 0.1 mL of blood is mixed with 0.1 mL of 0.2% nitro-blue tetrazolium in saline and 0.1 mL of phosphate buffered saline. This mixture is incubated at 37 °C for 15 min and then held at room temperature for 15 min.
2. Smears are made on a glass slide and fixed with methanol for 3 min and then stained with Pappenheim's stain for 3–5 min. Neutrophils that ingest the dye and have an oxidative burst create large black formazan deposits in the cytoplasm (Fig. 3).
3. This assay should be run in conjunction with blood from healthy control subjects to identify problems with specimen handling.

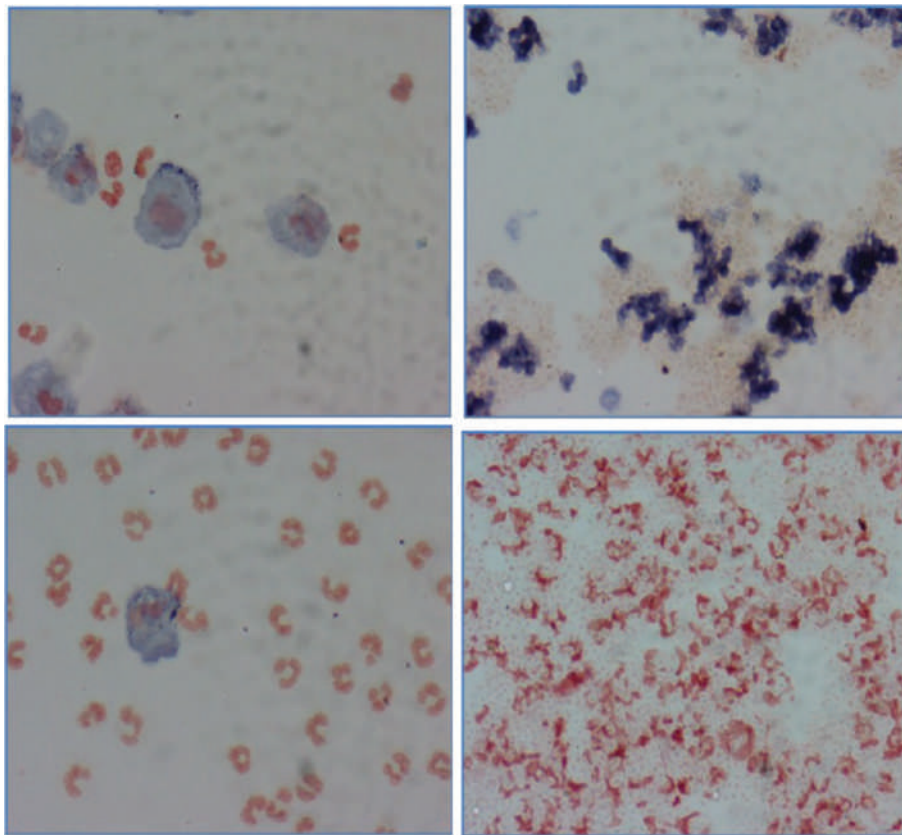


Fig. 3 Nitro-blue tetrazolium blue formazan staining of neutrophils (top left panel) and the blue to purple black precipitate in the zymosan stimulated neutrophils (top right panel) in a normal patient. Reduced or no formazan staining of neutrophils (bottom left panel) and no blue/black deposit staining in zymosan stimulated neutrophils (bottom right panel) in patients with chronic granulomatous disease. Adapted from Dimitrova G, Bunkall C, Lim D and Kendrick C (2013) Comparison of two methods for the diagnosis of chronic granulomatous disease—Neutrophil oxidative burst measured by the nitroblue tetrazolium slide test versus the dihydrorhodamine 123 flow cytometric assay. *New Zealand Journal of Medical Laboratory Science* **67**(2): 45–51 and used with permission.

Dihydrorhodamine test

1. Incubate heparinized blood at 37 °C in the presence of dihydrorhodamine 123. Add phorbol myristate acetate (PMA) or formyl-methionyl-leucyl-phenylalanine (fMLP) stimulant mix with the whole blood specimen for additional incubation at 37 °C.
2. Centrifuge the specimen and lyse the cell pellet with ammonium chloride at ambient temperature.
3. Wash with azide-free phosphate buffered saline (PBS) and stain with a viability marker and CD15 (a marker for myeloid cells) at ambient temperature.
4. Wash the cells, centrifuge, and resuspend in 1% paraformaldehyde. Identify viable neutrophils with a viability dye and confirm by the presence of CD15.
5. Determine the percent DHR positive cells and calculate the delta % DHR-positive neutrophils after PMA or fMLP stimulation and mean fluorescence intensity (MFI) for each stimulant using DHR flow cytometry.

Neutrophil granules

Neutrophils have three granule types that can be identified using routine stains and by measuring enzymatic activity in particular granules (Häger et al., 2010). The primary or azurophilic granules contain elastase and myeloperoxidase. The secondary or specific granules also contain multiple enzymes, including lactoferrin (Fig. 4). The tertiary granules contain gelatinase. These granules subsets have varying sizes and varying intensity when stained with 3, 3' diaminobenzidine, peroxidase reactive dye. Myeloperoxidase is present in the primary azurophilic granules of neutrophils and monocytes; it catalyzes the production of HOCL from chloride and hydrogen peroxide. Complete myeloperoxidase deficiency occurs in approximately 1 in 4000 individuals. This deficiency can be identified in laboratory tests by staining neutrophils and monocytes with a peroxidase (Fig. 4).

Chediak-Higashi syndrome is a rare, autosomal recessive disorder that is characterized by oculocutaneous albinism, progressive neurologic abnormalities, coagulation defects and recurrent bacterial infections (Häger et al., 2010). Due to a mutation in the

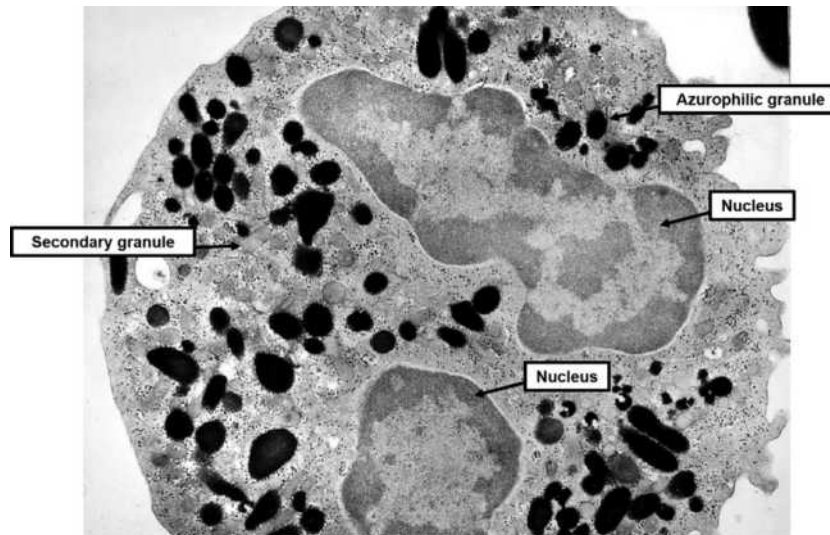


Fig. 4 Electron micrograph of neutrophil. The presence of multilobed nucleus is apparent and the primary, azurophilic granules are larger and rounder than the secondary, specific granules. (http://medcell.med.yale.edu/histology/blood_bone_marrow_lab/neutrophil_em.php).

CHS1/LYST gene, cells are defective in organelle trafficking, fusion, and transport. Often, lysosomes will fuse with one another as opposed to phagosomes resulting in large cytoplasmic granules. Individuals with this disorder develop recurrent, severe infections, most commonly in the skin, respiratory tract, and mucous membranes. Evaluation for this disease is often prompted in individuals with recurrent bacterial infections or other signs and symptoms, such as oculocutaneous albinism and recurrent bleeding events. Diagnosis is made based on visualization of peroxidase positive lysosomal granules in peripheral blood granulocytes.

Methods to analyze neutrophil granules

Neutrophil identification in peripheral smears

1. The name neutrophil derives from staining characteristics on hematoxylin and eosin (H&E) histological or cytological preparations. Basophilic white blood cells stain dark blue, eosinophilic white blood cells stain bright red, neutrophils stain a neutral pink.

Neutrophil isolation using centrifuge gradients

1. Bring all reagents to room temperature
2. Collect 5.0 mL of neutrophil isolation media in centrifuge tube. Carefully layer 5.0 mL of blood over the separation media. Perform this step slowly and carefully, and with the pipette tip close to the surface of the media to avoid mixing the blood and the media.
3. Centrifuge at 500 g for 35 min at 20–25 °C. The blood should separate out into six distinct bands: plasma, monocytes, isolation media, neutrophils, more isolation media, and the red blood cell pellet. If these bands are not clear, the separation process was not clean and will need to be repeated.
4. Carefully remove the top three layers (plasma, monocytes, and isolation media) using a pipette. Dispose of these layers.
5. Carefully pipette the layer of neutrophils and all of the isolation media beneath the neutrophils. Place the solution into a clean centrifuge tube.
6. Dilute the neutrophil solution to 10 mL with HBSS without $\text{Ca}^{2+}/\text{Mg}^{2+}$. Invert the tube a few times to suspend the cells.
7. Centrifuge the neutrophil solution at 350 g for 10 min. A red pellet should be present at the bottom of the tube, containing neutrophils and residual red blood cells (RBCs). Remove the supernatant with a pipette carefully so that the pellet is not disturbed.
8. Lyse the residual RBCs by adding 2 mL Red Cell Lysis Buffer to the tube. Resuspend the pellet by vortex the vial at a setting of 3–4. Avoid increasing the vortex setting above 4, since this may cause the neutrophils to activate. If necessary vortex for several seconds, or “pulse” the vortex to dissolve the pellet.
9. Centrifuge the tube at 250 g for 5 min. Remove the supernatant with pipette. Repeat the lysing process if required.
10. Add 500 μL HBSS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ to each tube. Again, vortex to resuspend the pellet at a setting of 3–4. Dilute to 10 mL with HBSS without $\text{Ca}^{2+}/\text{Mg}^{2+}$.
11. Centrifuge the tubes at 250 g for 5 min. Aspirate the supernatant and discard.
12. Resuspend the pellet in 250 μL HBSS/HSA Solution (2% HSA). Cells may then be counted and adjusted to desired concentration.

Stains for granules and granular enzymes

1. Fix air-dried blood smears with ethanol.
2. Add benzidine or 3,3'-diaminobenzidine or p-phenylenediamine dihydrochloride to a buffer solution.
3. Incubate the slide for 10 min, washed with tap water, and then counter stained with hematoxylin for 3–5 min.
4. Identify myeloperoxidase activity using microscopy in the primary granules of neutrophils and eosinophils. <http://laboratorytests.org/myeloperoxidase-mpo-stain/>.

Chemotaxis

Neutrophil responses to infection injury require directed migration to the relevant site. This activity requires neutrophil attachment to endothelial surfaces, diapedesis through the endothelium, and then movement to the site of injury. This chemotactic process can be measured in the laboratory. Patients with reduced chemotactic activities have impaired host defense responses.

Methods to measure chemotaxis

1. Measuring neutrophil chemotaxis is a technically complex and is usually done in very specialized laboratories. An alternative approach is to measure CD11b levels on stimulated neutrophils using flow cytometry (Nicholson et al., 2007). Patients with leukocyte adhesion deficiency type I have deficiencies of beta-integrins in neutrophils. This deficiency causes abnormalities in adherence, chemotaxis, C3bi mediated phagocytosis, degranulation, and respiratory burst. These patients have persistent granulocytosis. They have recurrent infections without neutrophil infiltration, and consequently there is limited formation of pus at sites of infection. CD11b is present in secondary and tertiary granules of neutrophils. Monoclonal antibodies can determine its presence or absence in resting and stimulated neutrophils.
2. A second approach involves the use of microfluidic chambers to measure chemotactic responses. Yang have provided a detailed outline of the procedures involved in these studies (Yang et al., 2017a,b).
3. This section does not include methods in detail. Please see references.

Summary

Questions about neutrophil function often arise in patients with recurrent deep-seated infections. The initial evaluation of these patients should include neutrophil counts and blood smears to analyze neutrophil morphology. Relatively simple laboratory tests can determine whether or not neutrophils have normal oxidative activity using the nitro-blue tetrazolium test and whether or not neutrophils have normal granules with myeloperoxidase activity. Measuring phagocytosis and chemotaxis involves more specialized laboratory techniques and typically requires testing by referral laboratories.

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Neutrophil Function Assays

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Glossary

Multiplicity of infection (MOI) The number of microorganisms per cell included in the in vitro infection assay. If the number of microorganisms to PMN the same, then the MOI is one, whereas if the number of microorganism to PMN is 10 times higher, then the MOI is 10.

Nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase/Nox2 oxidase (NOX2) A membrane-bound enzyme complex that faces the extracellular space, being found in the plasma membrane as well as in the membranes of phagosomes of neutrophils. It catalyzes the production of a superoxide free radical by transferring one electron to oxygen from NADPH.

Trained immunity Is the modification of cells of the innate immune system that derives in immunological memory.

Introduction

Neutrophils, also known as polymorphonuclear neutrophils (PMNs) are a type of granulocytes and the most abundant type of leukocytes, comprising approximately 60% of the nucleated cells in blood and bone marrow. PMNs are the first line of defense against pathogens being the first immune cells that arrive at the infection site in response to chemotactic factors produced by host-microbe interactions. Typically it has been accepted that a mature PMN has a circulating half-life that ranges from 6 to 8 h in blood and then survives in tissues around 2–3 days. As a consequence, their role has historically been linked to the initial phases of defense being considered as short-lived, non-specialized cells. However, recent research has redefined them as efficient and versatile effector cells (Tecchio and Cassatella, 2016; Warren et al., 2017) that possess functional heterogeneity and plasticity being able to shape adaptive immune responses (Leliefeld et al., 2015) and showing functions that link innate and adaptive immune responses (Tillack et al., 2012). PMNs have recently shown to be an important part of trained immunity showing that they can go thorough transcriptomic and epigenetic rewiring that makes them anti-tumoral when treated with β -glucan (Kalafati et al., 2020) or inducing enhanced antimicrobial functions after BCG vaccination (Simone JCFM Moorlag et al., 2020).

PMNS are crucial for host health maintenance. Inherent deficiencies in PMN microbiocidal activity or even an overall reduction in PMN abundance (neutropenia) is detrimental to health leading to more severe and frequent infection susceptibility. In response to inflammatory stimuli, PMNs migrate from the blood circulation to infected tissues, where they kill microbes through a set of

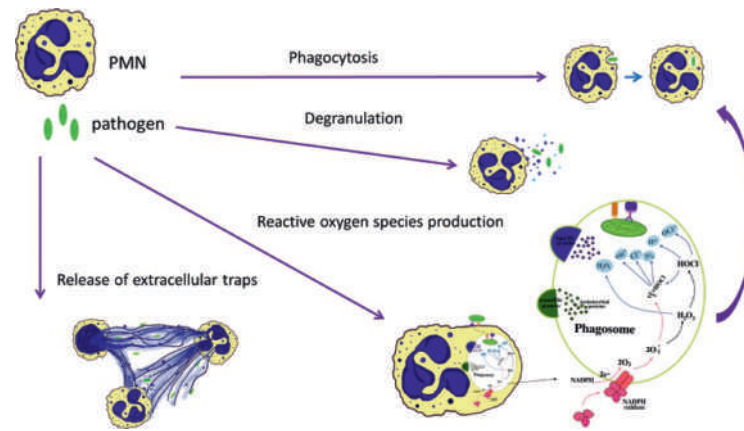


Fig. 1 Mechanisms used by PMNs to combat pathogens: phagocytosis, degranulation, reactive oxygen species production and release of extracellular traps.

immune mechanisms that include phagocytosis (Mayadas et al., 2014), reactive oxygen species (ROS) production (Mayadas et al., 2014), exocytosis of lytic enzymes with potent antimicrobial activity known as degranulation (Jimbo et al., 2017; Mayadas et al., 2014) and the release of extracellular traps (ETs) (Brinkmann et al., 2004; Cassatella et al., 2009) (Fig. 1).

During the last three decades, research involving PMNs and their roles in health and disease has suffered a huge burst. In this paper we intend to describe the mechanisms and the methodology that will enable measuring these major functions of PMNs in response to infectious agents.

PMN isolation

Some functional assays can be performed both directly on whole blood or bone marrow or if desired on isolated PMNs, whereas other assays need purely isolated PMNs. Purity should be over 90% in order to distinguish the effects of PMNs from those of other leukocytes in the sample and also to avoid undesired effects derived from the interaction with other leukocytes in the sample. Since PMNs cannot tolerate the freezing-thawing process they should not be isolated and stored in liquid nitrogen for posterior analysis but tested fresh after isolation. Evaluating functional activity on purified PMNs is, therefore, time-consuming because isolation must be performed at each testing.

Efforts have been made by different groups to overcome this issue and different storing conditions of whole blood have been tested with the aim of providing a larger time frame to cover sampling and posterior testing. Lizcano et al. (2017) have compared PMNs maintained in whole blood for various periods at different temperatures with freshly purified PMNs, testing for viability, a very necessary characteristic to enable performing functional tests. In their study, PMNs that had been maintained in whole blood at 4 °C for up to 24 h had a longer lifespan compared to purified PMNs maintained in the same conditions (Lizcano et al., 2017). These findings suggesting that blood whole can be stored with minimum PMN viability loss are very helpful and promising. Another study, has tested freeze-thawing conditions, showing that whole blood cryopreservation can be performed for immunophenotyping of most blood cells and for ex vivo functional analyses of T cells, as expected, but reporting loss of PMNs (Braudeau et al., 2021). This data indicates that improvements on cryopreservation for PMNs need further research and of course PMN functionality after storage needs testing, as well. In this context, the recommendation is to test freshly isolated PMNs for reliable results and only then, go to further storing or treatment condition comparisons.

Either for whole blood and bone marrow or pure PMN assays, different anticoagulants have been tested (EDTA, heparin and citrate) and even the needle has been suggested as an influencing parameter when drawing the blood sample because shear forces have been thought to damage and/or activate PMNs. There have been discrepancies on which anticoagulant is best and while some authors have shown that assay results can be influenced by the anticoagulant (Freitas et al., 2008), another recent study (Krabbe et al., 2020) has reported that the blood collection technique and anticoagulants have minor effects on the isolation of PMNs.

Storing isolated PMNs at 37 °C for over 1 h does have an effect on final PMN count and ROS activity as shown by Krabbe et al. (2020). These findings suggest that functional assays with isolated PMNs should be carried out as rapid as possible to preserve functionality. PMNs are considered fragile cells that are easily activated and damaged if not properly handled. Maintaining PMNs in as similar as possible to basal in vivo conditions is therefore, essential both during and after isolation. Although isolated PMNs should be well mixed to achieve a homogenous suspension, effort should be made to not perform vigorous mixing. The chemistry and temperature of the buffers used along the process must be carefully managed. Buffer composition will depend on the animal species and the mechanism object of the study since the addition of media supplements can strongly influence the outcome of experiments. As an example, serum-free media is encouraged to study extracellular trap release in murine PMNs, whereas for human PMNs media can contain serum (Neubert et al., 2019). In addition, PMN suspension in PBS supplemented with glucose to avoid cell clumping is highly recommended until the functional assays are performed (Kuhns et al., 2015).

There are many different isolation methods but they all share some steps: dextran sedimentation, density gradient separation and red blood cell (RBC) lysis step. Dextran sedimentation does not work well for all species although, the PMN yield from blood of some species, such as human and rabbits (Kouh et al., 2000) can benefit from this first step. Dextran promotes RBC aggregation and sedimentation, leading to the formation of a rich leukocyte plasma layer above the RBC sediment, ultimately removing most of the erythrocytes. Basically, the incorporation of this step prior to density gradient separation minimizes the number of RBC lysis steps that must be performed at the end of the isolation protocol. The density gradient step is directed at achieving the separation of PMNs from less-dense monocytes and lymphocytes. The density media and composition varies widely between species due to slight differences in PMN density. In the final, hypotonic lysis step residual RBCs are eliminated. Lysis protocol should also be adapted to the target species since there are also differences in RBC reactivity, susceptibility to lysis and different levels of aggregation and binding of the RBCs to the PMNs between species. In addition, PMNs can be damaged with the lysis protocol directed at RBC, affecting their viability and activation status. Therefore not all lysis methods are valid for PMNs of all species and this should be checked.

Human PMNs isolation protocols are much more standardized compared to non-human species PMN isolation. However, as mentioned before the great research explosion on PMNs in all animal species has made many different protocols accessible. There are easy to follow protocols for human (Kremserova and Nauseef, 2020; Siemsen et al., 2020) and non-human species such as mice (Pulavendran et al., 2020; Siemsen et al., 2014), rat (Caldefie-Ch  zet et al., 2002), rabbits (Siemsen et al., 2020) or even large animals such as bovine (Ladero-Au  n et al., 2021; Rambault et al., 2021; Siemsen et al., 2020), ovine (Arteche-Villasol et al., 2020; Siemsen et al., 2014) and goat (Arteche-Villasol et al., 2020; Patel et al., 2020). The election of the isolation method will therefore depend on the animal species target of the study, the available sources and also the functional assay that it is intended for. Assaying more than one method before deciding in order to establish optimum conditions for each laboratory is highly recommended.

In addition to these traditional isolation protocols, immunomagnetic enrichment is possible for some species. PMN purification can be achieved by negative immunoselection with magnetic beads in mice (Hasenberg et al., 2011) and humans (Pelletier et al., 2010). This technology can yield PMN purity upto 99% and is appropriate for performing transcriptomic studies for which RNA contamination from other cell types is undesirable.

Phagocytosis

Phagocytosis is the process through which various types of cells engulf particulate matter. The origin of the word phagocyte is greek, from *phago* meaning eating or devouring and the suffix *cyte* which stands for cell. It was   lie Metchnikoff who coined this term when developing the theory of phagocytosis back in 1884. At that time, he named cells from the bloodstream with phagocytic activity as *macrophages* and *microphages*, the latter is what we know today as PMNs. PMNs are the major phagocyte subset in the bloodstream. PMNs are professional phagocytes, just as macrophages and dendritic cells, meaning that they possess receptors on their surface that permit recognition of pathogens for their ingestion into phagosomes, which fuse with lysosomes afterwards enabling the digestion of the microorganism (Gordon, 2016). Indeed, phagocytosis can be aided by complement and/or antibody receptors that are present on the cell surface (Lee et al., 2003), but can also occur in absence of these opsonins. After ingestion and once inside the PMN, pathogens are exposed to ROS and antimicrobial peptides, components, which kill and lyse most of the microorganisms (Nordenfelt and Tapper, 2011), although some pathogens have developed evasion mechanisms to inhibit the killing action of phagocytosis (Kobayashi et al., 2018).

Basically, in a phagocytosis assay, phagocytes are incubated with pathogens or particles that can be labelled or not. Fluorescently labelled pathogens and particles greatly aid analysis. These can be FITC-stained (Hussen et al., 2016), or pHrodo-stained (Lindner et al., 2020) or pathogens carrying plasmids that express fluorescent proteins like GFP, YFP, etc. (Hellebrekers et al., 2017) can be used, as well. The proportion of particles or pathogens to PMNs put into the assay, also named multiplicity of infection (MOI) should be determined for each type of target. In most cases, pathogens or particles should be opsonized before ingestion by PMNs. This opsonization step must be performed before incubation with PMNs so that it does not limit the process. When studying the implication of receptors, inhibitors can be added previous to the contact with the particles or pathogens. Afterwards, carrying out the phagocytosis assay with shaking, aids continuous contact between PMNs and targets. Normally phagocytosis will be performed at 37   C, so including a PMN-pathogen suspension in ice (4   C) will permit having a negative control (absence of phagocytosis and only binding of the targets to the surface). This is essential if flow cytometry is to be performed as it is not possible to distinguish from PMNs with ingested particles or PMNs with only bound particles to the surface.

After incubation, the ingestion of the particles or their loss from the media can be measured by microscopy and flow cytometry. Microscopy permits obtaining images that will support the results obtained by the automatized methods. Furthermore, the micrographs can be analyzed with image software. Flow cytometry is advantageous as it permits the analysis of a high number of cells, providing results in a short period of time with a small volume of sample.

Phagocytosis by fluorescent microscopy

Samples can be taken at different time points and the phagocytic index may be determined by counting the number of internalized fluorescent targets divided by the total number of cells counted. Also, the phagocytic efficiency index can be determined by counting the number of PMNs with at least one internalized fluorescent target, divided by the total number of cells counted. This can be done manually or combined with the Cell Counter plugin on the Image J software package. Automatic particle counting can be done using the Analyze particle plugin, after setting a threshold on images and making them binary.

Phagocytosis by flow cytometry

As in microscopy, samples can be taken at different time points or at a fixed time point and phagocytosis can be measured by flow cytometry using fluorescent particles. Attention must be paid to the washing steps after incubation to ensure minimum extracellular bacteria. For this method, the number of intracellular fluorescent particles cannot be counted meaning that each PMN can contain more than one particle. The percentage of PMN ingesting particles is the measured parameter, this is, the phagocytic efficiency index. A viability dye can be added, such as Hoechst 33258 so that phagocytosis activity can be defined as the percentage of fluorescing cells among viable cells as seen on the example in Fig. 2.

Detection of reactive oxygen species ex vivo

ROS production, also named respiratory oxidative burst, is a powerful mechanism to combat microbes, being a primary component of the innate immune system against bacteria and fungi (Van Acker and Coenye, 2017). Phagocytes, such as PMNs and monocytes can produce ROS through the multi-protein membrane-bound NOX2 complex (Dupré-Crochet et al., 2013), which catalyzes the reduction of molecular oxygen to superoxide anion, which is afterwards converted to hydrogen peroxide by the action of superoxide dismutase or without the participation of this enzyme.

ROS released into the extracellular media in an uncontrolled manner can damage the host tissue being implicated in several inflammatory diseases, whereas ROS generated in the intracellular space after phagocytosis of pathogens is not toxic for the cell environment and contributes to the elimination of the pathogens (Briheim et al., 1989). These differences can be crucial when studying a particular disease. Discriminating between the intracellular and extracellular production of ROS can be done depending on the technique (Caldefie-Chézet et al., 2002). Cytochrome C reduction performed by spectrophotometry is a measure of ROS formation in the extracellular space (Kuhns et al., 2015), whereas dihydrorhodamine (DHR) reduction (Hussen et al., 2016) assessed by flow cytometry is indicative of intracellular ROS production. Both assays are based on red-ox reactions and require stimulation with a factors such as phorbol myristate (PMA) or other desired stimulants such as pathogen proteins or extracts. An alternative to these methods, is the use of chemoluminescence for which different probes can be used, such as luminol for intracellular ROS quantification and lucigenin for extracellular quantification (Caldefie-Chézet et al., 2002).

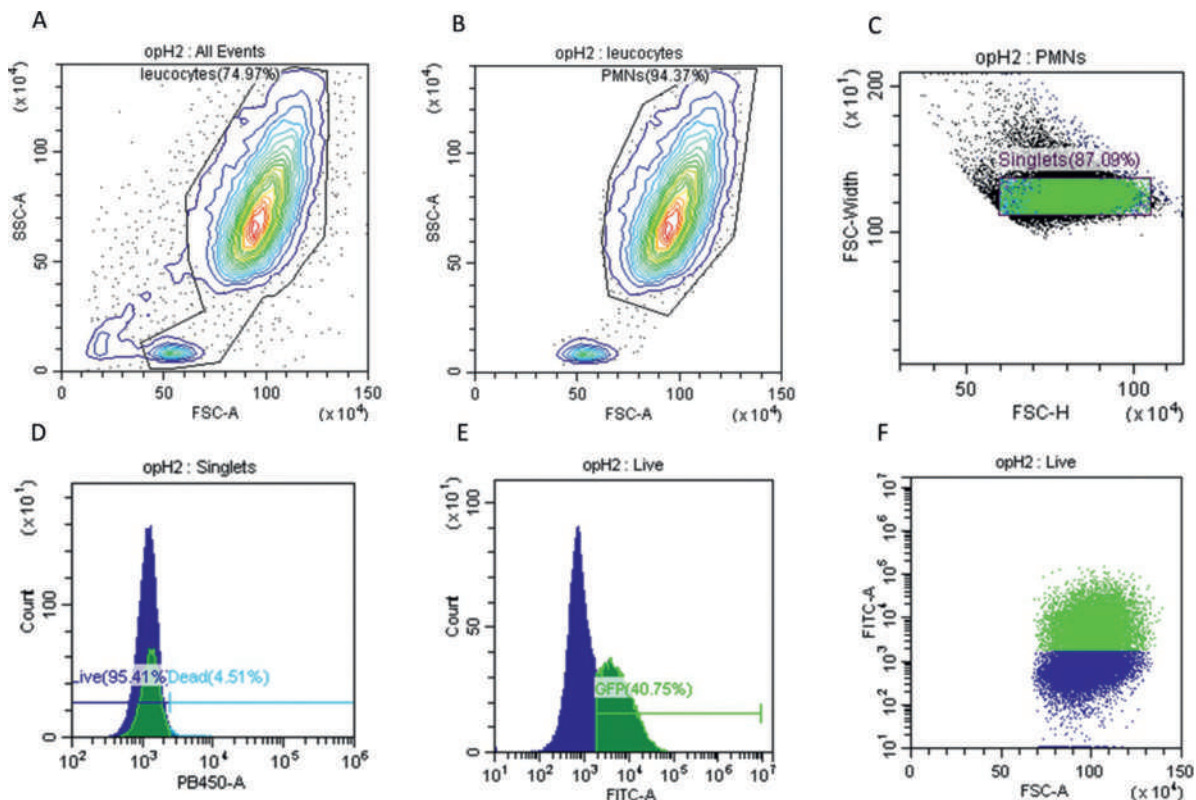


Fig. 2 Phagocytosis of *Mycobacterium avium* subsp. *paratuberculosis* (MAP)-GFP by PMNs isolated from rabbits vaccinated against mycobacteria. (A) FSC/SSC plot of isolated PMNs put in contact with Map-GFP showing gating of leukocytes, (B) FSC/SSC gating of PMNs, (C) gating of singlets, (D) histogram of showing gating of live-dead staining with Hoechst 33,258, (E) histogram showing proportion of live PMNs with ingested Map-GFP, (F) FSC/FITC plot showing proportion of live PMNs with ingested Map-GFP.

ROS determined cytochrome *c* by spectrophotometry

The assay based on the reduction of cytochrome *c*, requires isolated PMNs and it can be run over time (kinetic) involving larger volumes of purified cells or at a fixed time (static) with small volumes in 96 well plates (Kuhns et al., 2015). In the kinetic format, relatively large amounts of blood are needed, although it allows monitoring of the production of superoxide anions over time. When the experiment that is run includes a large number of samples, or when other functional assays are run at the same time and the number of available PMNs is therefore limiting, the static assay is a good option, as it can be read in an ELISA plate reader at a specific time point ranging 10–30 min. In both cases, PMNs and cytochrome *c* are mixed and allowed to equilibrate. Afterwards, a stimulus is added and the superoxide that is produced as a function of ferricytochrome reduction can be measured on a spectrophotometer. In order to correct for other reductants, controls including superoxide dismutase and non-stimulated PMNs should be included.

ROS determined with DHR by flow cytometry

The DHR assay is based on the principle that internalization of non-fluorescent DHR (reduced form) by phagocytes and posterior stimulation with PMA (Elbim and Lizard, 2009) or other stimulating factors activates the respiratory oxidative burst generating hydrogen peroxide, that oxidizes DHR to rhodamine 123, a green fluorescent compound, which can be detected by flow cytometry. ROS generation can then be calculated as the Mean Fluorescence Intensity (MFI) of gated region after subtracting the MFI of non-stimulated cells or the percentage of ROS generating cells compared to non-ROS generating of the same cell type. The assay can be performed on whole blood or on previously isolated PMNs in various types of cell culture media. Because the assay reagents are not species-specific, this assay can be used in any species or cell type capable of producing a NOX2-dependent respiratory burst response, meaning that if whole blood is used, forward scatter (FSC)/side scatter (SSC) gating can be performed to identify both monocytes and PMNs and therefore ROS activity of both phagocytes can be determined in the same sample. Added to this, flow cytometry based assays have the advantage that thousands of cells can be analyzed in a very short period of time employing small volumes of isolated PMN or even whole blood (Elbim and Lizard, 2009).

In the example shown on Fig. 3, the increase in the ROS producing population of PMNs after contact with a mycobacterial sonicate (SM) in the vaccinated animals compared to non-vaccinated animals shows a priming effect on PMNs due to vaccination against mycobacteria.

ROS determined by chemiluminescence

In this case, PMNs are plated and thermostated to 37 °C and luminol or lucigenin are added to the wells. The basal value of the chemiluminescence is measured on a luminometer. Afterwards, stimulus such as PMA (Billert et al., 2021; Caldefie-Chézet et al., 2002), zymosan (Billert et al., 2021) or fMLP (Billert et al., 2021), are added to the wells, always setting a non-stimulated cell control. Plates are incubated at 37 °C for 30–60 min afterwards, chemiluminescence is measured.

ROS production and phagocytosis in one assay by flow cytometry

In order to maximize sample use, both ROS and phagocytosis can be measured in the sample of whole blood using flow cytometry. Gupta-Wright et al. (2017) have developed a whole blood phagocyte functional assay that utilizes the inflammatory inducer zymosan, coupled to OxyBURST-SE, a fluorescent reporter of phagosomal oxidase activity. This way, both phagocytic uptake and the superoxide burst in the phagocyte populations (PMNs and monocytes) can be estimated. In the assay, whole blood is incubated with the zymosan reporter particles at 37 °C with shaking to ensure phagocyte to particle contact. Samples can be taken out to evaluate both functions at different time points. These should be collected 10 min prior to each time point to enable cell surface staining (CD45: leukocytes, CD14: monocytes and CD66b: PMNs) since phagocytosis continues during the staining process. Afterwards, RBC lysis should be performed and samples can be acquired. Finally, FSC/SSC and specific fluorescence detectors will permit gating to identify monocytes and PMNs and the shifts in populations due to the studied functions.

Antimicrobial killing assay

Many published studies report phagocytosis and ROS production and relate these parameters to killing. Although ROS can be indicative of the killing capability of PMNs, this mechanism is not exclusively related to the killing of microorganisms (Auding et al., 2003). Indeed, the killing function of PMNs is the major mechanism of PMN activity contributing to pathogen clearance and therefore proper assays measuring antimicrobial killing are very valuable.

The method described here on, involves purified PMNs since quantifying the pathogen clearance or killing ability of PMNs is the issue. In order to test for killing, the same considerations as for phagocytosis should be taken, this is, considering opsonizing the pathogen or not and also setting the appropriate MOI for the pathogen target of the assay.

Here again, PMNs are put in contact with pathogens at 37 °C for various timepoints depending on the pathogen and its growth rate. PMNs will ingest the pathogens and then extracellular non-ingested bacteria can be removed by differential centrifugation. Afterwards, colony counting can be performed after plating the pellet of PMN containing ingested pathogens and the supernatant

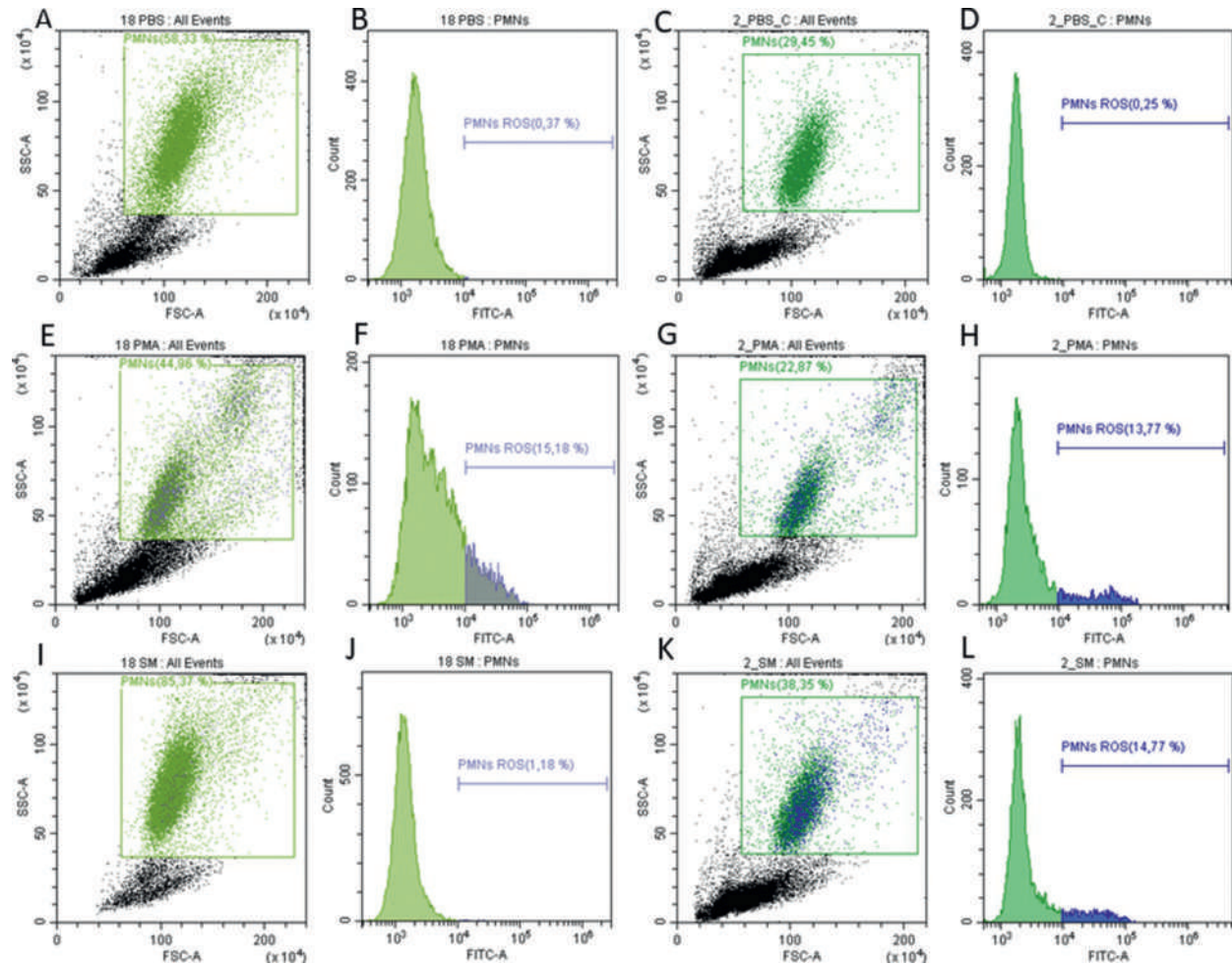


Fig. 3 ROS production by PMNs from whole blood with DHR from rabbits non-vaccinated and vaccinated against mycobacteria. Blood is stimulated with PBS, PMA or SM (mycobacteria sonicate) for 45 min. (A) FSC/SSC gating of non-stimulated PMNs from a non-vaccinated animal, (B) ROS producing of non-stimulated PMNs from a non-vaccinated animal, (C) FSC/SSC gating of non-stimulated PMNs from a vaccinated animal, (D) ROS producing of non-stimulated PMNs from a vaccinated animal, (E) FSC/SSC gating of PMA-stimulated PMNs from a non-vaccinated animal, (F) ROS producing of PMA-stimulated PMNs from a non-vaccinated animal, (G) FSC/SSC gating of PMA-stimulated PMNs from a vaccinated animal, (H) ROS producing of PMA-stimulated PMNs from a vaccinated animal, (I) FSC/SSC gating of SM-stimulated PMNs from a non-vaccinated animal, (J) ROS producing of SM-stimulated PMNs from a non-vaccinated animal, (K) FSC/SSC gating of SM-stimulated PMNs from a vaccinated animal and (L) ROS producing of SM-stimulated PMNs from a vaccinated animal. The increase in ROS production after contact with SM shows a priming effect on PMNs due to vaccination (comparing J and L). Data representative of $n = 5$ for each group.

containing the extracellular bacteria. Once the amount of viable bacteria is known, the killing can be determined. In this type of assay, PMNs are not separated from uningested bacteria, and so this would be a combination measure of the ability of the PMN to phagocytose and kill.

In Fig. 4 the workflow for working with mycobacteria is outlined. Mycobacteria are slow growing microorganisms and in this case a 24 h incubation to evaluate bacterial killing is appropriate (Ladero-Auñón et al., 2021). Briefly, PMNs and mycobacteria are incubated together at 37 °C 5% CO₂ for 24 h. An initial 4 °C control to establish starting CFUs should be included and also an only mycobacteria control incubated along with the samples of study to address bacterial division overtime. Afterwards, differential centrifugation should be performed, this is, a slow centrifugation step should be performed in order to pellet PMNs and supernatant containing extracellular bacteria should be separated. Finally, the pellet can be washed and centrifuged again pooling both supernatants that will contain all the extracellular bacteria. Then PMN lysis should be performed carefully not to promote DNA release as it might clump the bacteria. Afterwards serial dilutions should be performed in order to get 100 colonies per plate. Culturing in duplicate or triplicate, and more than one dilution specially for the first time when the expected killing rate is unknown.

This scheme can be adapted and optimized to other microorganisms. For example, fast growing bacteria such as *Staphylococcus aureus* require much shorter incubations, observing detectable killing as soon as 10–20 min after incubation (Magon et al., 2020). Including or not an opsonization step when preparing bacteria should be considered depending on the pathogen under study, as greater killing effects of human PMNs against *S. aureus* have been observed in non-opsonizing conditions after 60 min (Lu et al., 2014).

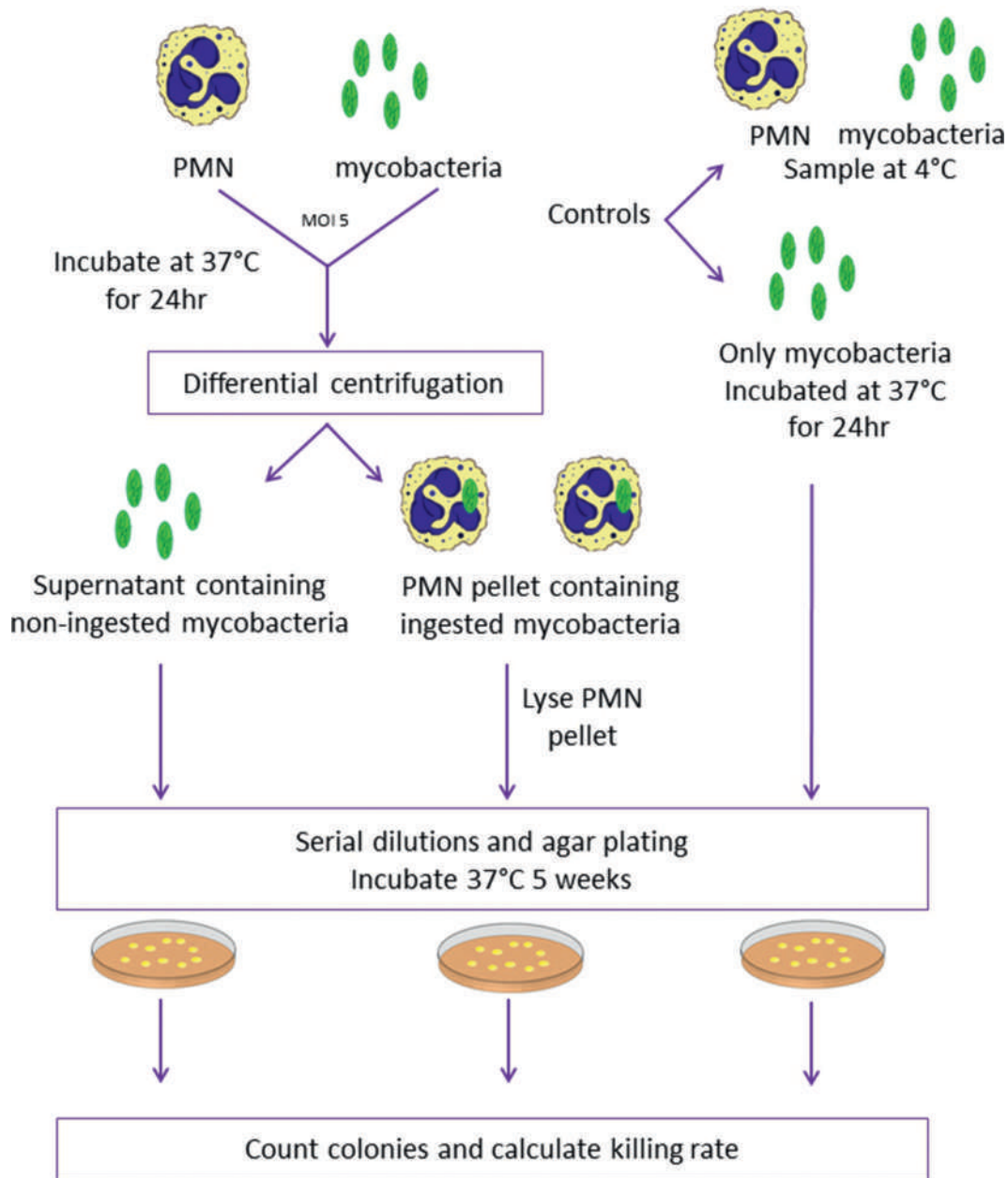


Fig. 4 Bactericidal assay workflow followed to evaluate mycobacterial killing by PMNs.

Another consideration is that PMNs can be heterogenous and when performing these assays on plates, differences in the killing rates between PMNs in suspension and adherent PMNs can be observed as seen by [Lu et al. \(2014\)](#). In such case, incubation times and lysis conditions should be revised. Another factor to bear in mind is that the extracellular viability loss due to cytotoxic factors secreted by PMNs cannot really be quantified by this technique.

This method does not require expensive and specialized equipment but it is time consuming due to the manual input in serial dilutions, plating and counting. However, even if colony forming units do not exactly represent a permanent loss of cell viability it is the best assessment of PMN bactericidal activity.

Degranulation and release of lytic enzymes

Degranulation and release of lytic enzymes and bactericidal peptides are considered oxygen-independent mechanisms. Degranulation or exocytosis, of membrane-bound secretory granules, is the process through which PMNs release several granule-derived mediators, such as neutrophil elastase (NE), myeloperoxidase (MPO), cathepsin G, and proteinase-3, among others into the phagosome or in the extracellular space.

While expression or protein level measurements can provide information regarding the abundance of these particular proteins, degranulation can be assessed by measuring the release or activity of these. For this reason, the method described here on, focuses on measuring the activity of MPO.

MPO activity

MPO is a lysosomal peroxidase enzyme stored in the azurophilic granules of PMNs. MPO participates in the respiratory burst producing hypochlorous acid from hydrogen peroxide and chloride anion. There are many assays to assess MPO activity and method described here involves purified PMNs treated with ortho-dianisidine dihydrochloride (Bedouhène et al., 2020).

PMNs can be briefly treated with cytochalasin B and then with pathogens target of the study and fMLF for different time periods. Afterwards, PMNs are centrifuged and MPO is measured in the supernatant after treatment with ortho-dianisidine dihydrochloride first and hydrogen peroxide after. Then solution should be mixed and read on a spectrophotometer for 10 min.

However, some authors suggest that standardization is needed to permit verification between studies (Pulli et al., 2013), pointing out the problem with lack of specificity to MPO of the most used probes (e.g., TMB, *o*-dianisidine, guaiacol). For this reason, Pulli et al. (2013) developed a highly specific and sensitive assay protocol that includes a step capturing MPO by ELISA.

Neutrophil extracellular trap (NET) release

NET release has been reported as an important novel effector mechanism against pathogens in PMNs (Brinkmann et al., 2004). NETs are extracellular structures mainly composed of chromatin and granule proteins that can trap and kill microorganisms (Fuchs et al., 2007). The formation of these structures can be triggered by some pathogens (dead and alive), danger associated molecular patterns, exogenous compounds, platelets, antibodies and inflammatory cytokines such as IL-8 and TNF (Shrestha et al., 2020; Skendros et al., 2018). Although NETs were originally reported as novel immune defense mechanism to trap and kill microorganisms, it has been accepted that the role of NETs exceeds these findings. Dysregulation of NET release or clearance has been implicated in a great number of chronic inflammatory diseases (Lood et al., 2016) and cancer (Albregues et al., 2018). Actually, NETs can have a controversial effect on tumors, being pro-tumor or anti-tumor depending on the circumstances (Liu and Liu, 2019). In this sense, NETosis can be considered a potential therapeutic target and therefore understanding PMN activity and the contribution of this cell type to each clinical setting can be important.

Although, NETs can be identified in tissues post-mortem or in biopsies and *in vivo* in animal models, the best and sometimes only way to study NET production includes the isolation of PMNs from patients, healthy donors, or animals followed by an *ex vivo* stimulation of these cells to assess and quantify NET formation. Indeed, this can be considered the gold standard of NET-experiments (Neubert et al., 2019).

ET release must be studied on purified PMNs in contact with ET inducers, mainly PMA, zymosan (Ladero-Auñon et al., 2021), interleukin-8, lipopolysaccharide and/or the pathogens under study, dead or alive (Ladero-Auñon et al., 2021). Different ET release assays have been reported and they mainly involve an automatized measurement of the extruded DNA (von Köckritz-Blickwede et al., 2010; Ladero-Auñon et al., 2021) or a visualization of the structures under the fluorescence microscope (von Köckritz-Blickwede et al., 2010; Ladero-Auñon et al., 2021). The automatized method is advantageous as it permits obtaining results in a short period of time, whereas the microscopic method permits visualization aiding interpretation and supporting the automatized method, but it is tedious if real quantification of ET release is desired.

Fluorimetry

After proper incubation of PMNs with chemotactic factors, mitogens or pathogens, ETs generated by cells are generally digested with nuclease to liberate them into the supernatant and then this activity is stopped. DNA extraction is performed on an equivalent number of PMNs in a control well in order to have an estimate a real DNA content. Thereafter, culture supernatants and the DNA control are collected and both ETs and genomic DNA are quantified using DNA fluorescent intercalating molecules. Afterwards, fluorescence quantification in an automatic plate reader can be performed and the percentage of DNA released as ET-DNA (ETosis) can be calculated by dividing the amount of quantified ET-DNA by the total amount of genomic DNA.

Visualization and quantification by microscopy

ET release for microscopy can be performed using poly-L-lysine treated coverslips or microscopy chamber slides. For either option, PMNs are incubated for 3–4 h with mitogens or pathogens leaving non-stimulated controls. Afterwards, a fixation step should be performed and either DAPI staining (Ladero-Auñon et al., 2021) or other DNA intercalating staining with Sytox Orange (von Köckritz-Blickwede et al., 2010) can be used to assess ETs. However, since DNA is not the only component in ETs, immunostaining of specific additional ET elements can aid in the visualization of these structures and therefore proper immunocytochemistry staining for histones (Ladero-Auñon et al., 2021) or myeloperoxidase (MPO) (von Köckritz-Blickwede et al., 2010) or NE (Conejeros et al., 2019) can be performed.

Immunocytochemistry is encouraged if quantification is the goal. An example of a quantification pipeline reported by Ladero-Auñon et al. (2021) can be to take snapshots of a representative number of fields containing visible ETs as seen in Fig. 5A–D, with

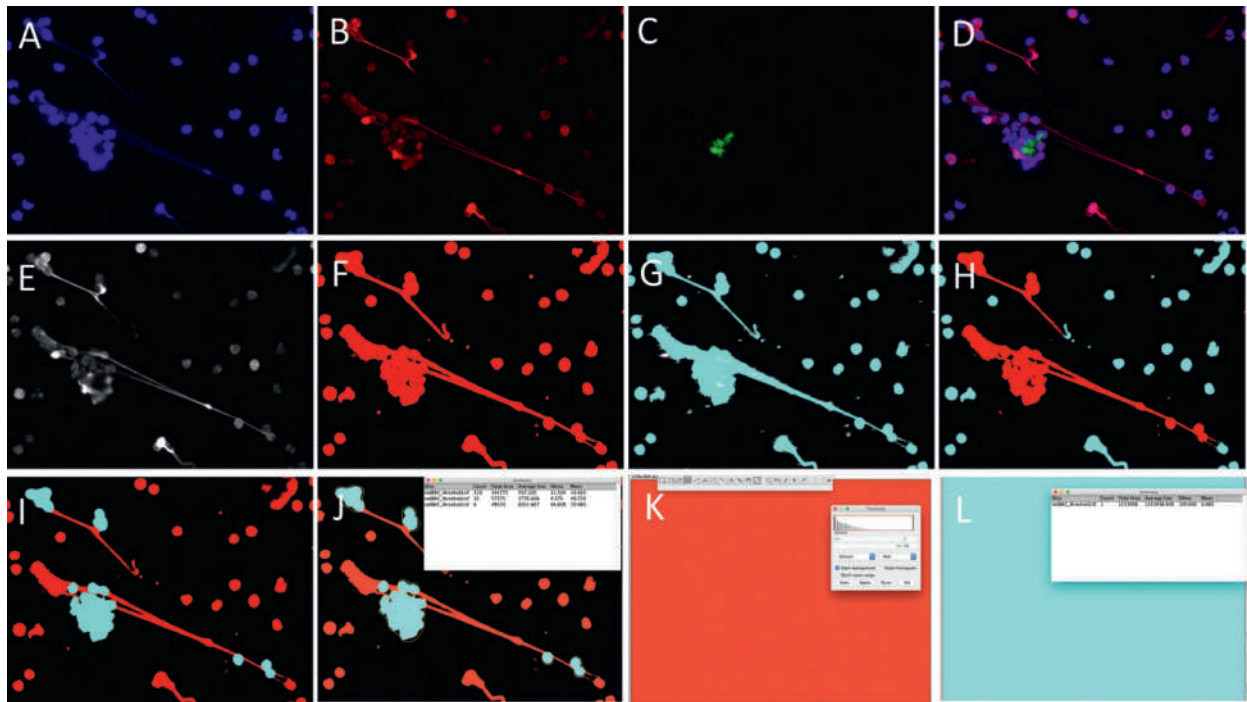


Fig. 5 Neutrophil extracellular trap (NET) assessment by microscopy of bovine neutrophils against *Mycobacterium avium* subsp. *paratuberculosis* (Map)-GFP example. (A) DAPI, (B) histone, (C) Map-GFP, (D) merged, (E) Red channel converted to 8-bit, (F) Threshold adjusting to 5-255, (G) Mask of SIZE: 0-INF Circularity 0-1, (H) Mask of SIZE: 100-INF CIRCULARITY: 0.1-1, (I) Mask using the freehand section tool for the nuclei that cannot be chosen with the previous automatic method and measure adjusting SIZE: 0-INF Circularity 0-1, (J) Measurement data of (G, H, I, K) Threshold 0-255, (L) Mask and measurement of the total area of the picture adjusting 0-INF Circularity 0-1.

histone staining (Fig. 5B) and then analyze the snapshots in 8-bit format (Fig. 5E) using the Image J software package. Threshold should be adjusted depending on the micrograph (Fig. 5F) and then a masks must be set (Fig. 5G–I) and measured (Fig. 5J) so that the percentage of picture area occupied by ETs plus nuclei (total DNA) can be measured by adjusting the settings for fluorescent particle size and the percentage of picture area occupied by nuclei can be analyzed. The % of area in G and H is automatically calculated, whereas the total area of the picture needs to be calculated adjusting the threshold of the picture from 0 to 255 (Fig. 5K) and measuring the particles as 0-INF Circularity 0–1 (Fig. 5L).

Afterwards, ET percentage can be calculated as total DNA percentage minus nuclei percentage. In the case of cultures that do not contain ETs, nuclei can be measured, and ET release should be considered zero.

Measuring the killing effect of NETs

What takes PMNs to release NETs or to phagocytose pathogens is not clear (Ley et al., 2018) and many microorganisms have not been tested yet for their ability to stimulate NET release. Also, NETs can be beneficial or harmful for the host as mentioned before. In any case, NETs can be liberated but their microbiocidal or cytotoxic effect is not really evaluated when NETosis assays are performed. It has been shown that NETs impede cancer cell growth by inducing apoptosis and/or inhibiting proliferation (Arelaki et al., 2016; Schedel et al., 2020). Also, NETs have shown to inhibit tumor cell migration (Schedel et al., 2020).

For this purpose, *ex vivo* generated NETs can be isolated from cultures and added to pathogens or tumor cells (Arelaki et al., 2016) to study their effect on growth. Adding DNA extracted from non-activated PMNs can serve as a control.

Other markers of NETosis

PMNs are reactive cells, and sometimes isolation methods cannot be achieved because alterations in cell functions are produced or *ex vivo* cell assays cannot be performed for other reasons, such as sample availability. In these cases, techniques measuring other biomarkers such as MPO and NE can be helpful and supportive of PMN activity. MPO can be liberated by PMNs through degranulation or by cell-death pathways such as apoptosis and necrosis but also via the extrusion of NETs. NE is a neutral serine protease normally expressed in PMN primary granules that stimulates mucus secretion and induces CXCL8 release from epithelial cells and can perpetuate the inflammatory state. NE is able to degrade virulence factors of Gram-negative bacteria (Weinrauch et al., 2002) and destroy host tissue and it is also found in NETs.

MPO-DNA and NE-DNA complex ELISA

Over the past years, estimating MPO or NE associated to DNA, this is MPO-DNA and NE-DNA complexes, have been considered specific quantifiable markers of in vivo NETosis (Masuda et al., 2016). Therefore, these complexes can be quantified in serum by a capture enzyme-linked immunosorbent assay (ELISA) (Kessenbrock et al., 2009; Yoo et al., 2014), which involves the coating of microtiter plates with anti-MPO or anti-NE antibody. Once a blocking step is performed, serum samples are added for incubation and after washes are performed a horseradish peroxidase (HRP)-conjugated anti-DNA antibody is added. Washes to eliminate extra not specifically bound HRP-conjugated anti DNA antibody are performed and HRP substrates are added for the development of color and the ELISA is read.

Although standardization is needed and this methodology has been recently questioned for its specificity in serum samples (Hayden et al., 2021) the truth is that this method is widely used. If possible, before selecting this method as a sole measure of NETosis for new studies it should be performed in parallel to a proper *ex vivo* NETosis assay with isolated PMNs or other techniques seeking for correlation.

Conclusions

PMN research has greatly increased in the last decades changing our view on this immune cell type. The fact that PMNs have shown different phenotypes and that they perform many more functions than those strictly considered as antimicrobial have indeed revitalized the learning of PMN biology. Performing functional assays has been crucial to pathogenesis studies and vaccine development but also to support the finding that PMNs are trainable immune cells. Future research should probably cover the assignment of particular PMN phenotypes to each specific function as well as delving in their role in trained immunity aimed at discovering new therapies.

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NK Cell Function Analysis

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The natural killer (NK) cells - basic information

a. Origins

The NK cells are currently recognized as one of the innate lymphoid cell (ILC) type and the only one with cytolytic properties and ubiquitous, while other ILCs are mostly confined to mucosal tissues and do not show cytotoxic capabilities. As all the lymphoid cells, the NK cells originate as a progeny of the hematopoietic stem cells (HSC) in the bone marrow (BM).

b. Morphological/phenotypic characteristics

The NK cells are described as so-called large granular lymphocytes or LGL, which suggests their basic lymphocyte morphology (round with roughly spherical nucleus), and diameter (as seen in classical blood smears on microscopic slides) slightly larger than that of average lymphocyte. Still, the latter does not allow for simple detection of the NK cells by flow cytometry using standard forward and side scatter, as they remain within the characteristic lymphocyte cluster in the two-dimensional dot plots. In fact, diameters of lymphocytes are measured (by FACS, particle analysis, microscope planimetry and transmission electron microscopy) as 5.0–11.0 µm corresponding to roughly spherical volume of 142–265 fl; the NK cells fit within this range.

How can they be differentiated from other lymphocytes then? First, the NK cells possess a unique surface phenotype (detectable by e.g. immunocytochemistry and quantifiable by FACS), consisting of lineage marker molecules CD56 and CD16 (FcγRIIIA, a class of receptors for immunoglobulin G heavy chain fragment Fcγ), lacking the molecules characterizing the T cells (CD3) or B cells (CD19), and expressing characteristic function-associated surface proteins, including activating and inhibitory receptors. In more detail, changing phenotypes of the NK lineage cells are associated with stepwise acquisition of killing properties and include up to 6 stages of differentiation. Their differentiation from other lymphocytes is multistage; stage 1 consists of the CD34⁺CD45RA⁺ HSCs in the BM, stages 2, 3 and 4 happen in tonsils, spleen and lymph nodes and involve the consecutive acquisition of CD56, CD117 and IL-1R1 (stage 2), the loss of CD34 characterizing the early HSC in the BM (stage 3) and finally the downregulation of CD117 with concomitant expression of CD94, which defines the earliest functional CD56^{bright}CD16⁻ NK cells (stage 4) (Quatrini et al., 2021). At this stage the NK cells express relatively high levels of T-BET and EOMES, two transcription factors required for them attaining functional “killer” capabilities, notably the production of perforins and granzymes A and B (belonging to serine proteases) used in the target cell killing process in the NK cells in stage 4b, the first stage of the NK cell development associated with their, so far relatively less efficient, killer function. These functionally important NK cell proteins are enclosed in the characteristic granules of the NK cells (earning them the name of large granular lymphocytes or LGLs) which can be visualized by appropriate staining techniques in both light and electron microscopy. Stage 4b is additionally differentiated from immediately preceding stage 4a by expression of NKP80. Partial loss of the surface CD56 molecules and acquisition of the CD16 marks the stage 5 (CD56^{dim}CD16⁺) of most efficient natural killers. These NK cells also express the surface killer Ig-like receptors (KIRs), important for the ability of NK cells to detect and kill their targets. Finally, additional expression of the CD57 characterizes the terminally differentiated NK cells in their stage 6 of differentiation or indicates an activation state.

However, these phenotypic stages are apparently not distinguishing functionally different NK cells in a sharp way, but more in a “fuzzy” way, putting in question the linear model of NK cell differentiation described above and proposing an alternative, branching one (Poznanski and Ashkar, 2019). Thus, in the latter a special type of the NK cell precursor was identified in the BM having the phenotype of Lin⁻CD34⁺DNAM1^{bright}CXCR4⁺ (Bozzano et al., 2015). This precursor may differentiate in either CD56^{bright}CD94⁺KIR⁺ NK cells, which later transform into the more mature CD56^{dim}CD16⁺KIR⁺ cells. The latter may form either the terminally differentiated CD57⁺ NK cells or acquire the phenotype of KIR⁺NKG2C⁺CD57⁺ and become capable of controlling the CMV infection in a memory-like fashion, earning them the name of “adaptive NK cells” (Bozzano et al., 2021a; Muntasell et al., 2013). To make the development of NK cells even more complex, these “adaptive NK cells” may, according to this model, derive straight from the Lin⁻CD34⁺DNAM1^{bright}CXCR4⁺ precursor (which appears to also be the precursor for the

T cells) (Bozzano et al., 2015). Eventually, also the “fully” lymphoid lineage origins of the NK cells are questioned, as the CD56^{bright}CD16[−]CD94⁺ NK cells may arise from a *myeloid precursor* with the phenotype CD33⁺CD56⁺ (Bozzano et al., 2021a, 2015; Scoville et al., 2017).

c. Function/role (what and how do they kill?)

The NK cells evolved to recognize and kill own cells that have been modified in one of the two, potentially dangerous and deadly ways: either by undergoing neoplastic (eventually malignant) transformation or by hosting the replicating viruses. The latter is true also in the context of current (2021) pandemics of COVID-19 due to infection by SARS-CoV-2 coronavirus (Bozzano et al., 2021b). Either modification leads to the appearance of new characteristics. In the former (cells turning neoplastic) we assist to the appearance of new antigens (surface and cytoplasmic neoantigens) being the effect of transforming mutations; in the latter the presence of viral antigens also makes them foreign (non-self). In order to ascertain the long(er) survival of virus-infected organism (or of that in which some cells undergo potentially cancerogenic mutations) both these unwanted cell types must be effectively recognized, differentiated from the neighboring healthy cells, and killed before their “mass effect” will hamper the functions of the organism.

Even if both the transformed and virus-infected cells are the source of antigens, these are the objects of interest of adaptive immunity T and B cells and rely on the specificities of respective antigen receptors (TCR and BCR). The NK cells recognize the transformed or infected cells using a different detection mechanism. Thus, they may be activated (primed for killing) by two groups of surface ligands present on the potential target cells; the first one consists of different classes and variants of the HLA-I histocompatibility antigens, including the HLA-A, HLA-B851, HLA-C C1, HLA-C C2, HLA-F; the second group of target cell molecules activating the killing frenzy of the NK cells is more heterogenous and consists of the FcIgG, B7, BAT3, CD48, CD58 and many other (Table 1) (Carrillo-Bustamante et al., 2016; Pende et al., 2019; Sivori et al., 2020; Quatrini et al., 2021).

Viral HA and complement factor P (properdin, binding the NKp46) are main molecules derived from or responding directly to the infecting agents that elicit the NK cell activation. As it is easy to perceive, most (with the exception of neoantigens and viral peptides) molecules that can activate the NK cells are nonspecific and can be present on any healthy cell type; it would be highly undesirable that they would induce NK-dependent killing of normal cells. Thus, evolutionary constraints were imposed in the form of sets of similar, but NK cell-inactivating (inhibitory) molecules. Again, they may belong to the broad family of HLA-I antigens (in fact, some of these serve as activators or inhibitors of the NK cell killing, depending on what will be the NK cell receptor binding them (Table 1). Other again form a heterogenous group of ligands. Interestingly, also some molecules belonging not to the cells, but to the extracellular matrix (ECM), including collagen, may exert an inhibitory activity.

Table 1 Known activating and inhibitory NK cell receptors and their recognized ligands on target cells.

Activating HLA-I specific NK cell receptor	Target cell ligand	Inhibitory HLA-I specific NK cell receptor	Target cell ligand	Activating NON- HLA-I specific NK cell receptor	Target cell ligand	Inhibitory NON- HLA-I specific NK cell receptor	Target cell ligand
CD94/NKG2C	HLA-E	CD94/NKG2A	HLA-E	CD16	FcIgG	PD-1	PDL1-L2
KIR3DS1	HLA-B*51, HLA-F	LILRB1	HLA-I (broad)	NKp46	Viral HA, CFP	SIGLEC-7	Sialic acid
KIR2DS1	HLA-C C2	KIR2DL1	HLA-C C2	NKp30	B7-H6, BAT3	IRp60	PS, PE
KIR2DS2	HLA-C C1, HLA-A*11:01	KIR2DL2/L3	HLA-C C1, HLA-C C2, HLA-B*46:01, -B*73:01	NKp44	PDGF-DD, Nid-1, HLA-DP401, PCNA, 21speMLL5	NKR-P1A	LLT1
KIR2DS3	?	KIR2DL5	?	NKp80	AICL	TIM-3	Galectin-9
KIR2DS4	HLA-F, HLA-C*02:02, -C*04:01, -C*05:01, -C*01:02, -C*14:02, C*16:01, HLA-A*11	KIR3DL1	HLA-B Bw4, HLA-A A*23, A*24, A*32	NKG2D	MICA, MICB, ULBPs	LAIR-1	Collagen
KIR2DS5	HLA-C C2?	KIR3DL2	HLA-A*03, -A*11'	DNAM-1	CD155, CD112	TIGIT	CD155, CD112
		KIR3DL3	?	2B4	CD48	CD96	CD155
KIR2DL4	HLA-G	KIR2DL4	HLA-G	NTB-A CD2	NTB-A CD58		

For clarity, the receptors are divided into HLA-I specific (left panels) and HLA-I non-specific (right panels).

Based on Pende D, Falco M, Vitale M, Cantoni C, Vitale C, Munari E, Bertaina A, Moretta F, del Zotto G, Pietra G, Mingari MC, Locatelli F, and Moretta L (2019) Killer Ig-like receptors (KIRs): Their role in NK cell modulation and developments leading to their clinical exploitation. *Frontiers in Immunology* 10: 1179; Quatrini L, Della Chiesa M, Sivori S, Mingari MC, Pende D, and Moretta L (2021) Human NK cells, their receptors and function. *European Journal of Immunology* 51: 1566–1579; Sivori S, Della Chiesa M, Carlomagno S, Quatrini L, Munari E, Vacca P, Tumino N, Mariotti FR, Mingari MC, Pende D, and Moretta L (2020) Inhibitory receptors and checkpoints in human NK cells, implications for the immunotherapy of cancer. *Frontiers in Immunology* 11: 2156; Carrillo-Bustamante P, Kesmir C, and de Boer RJ (2016) The evolution of natural killer cell receptors. *Immunogenetics* 68: 3–18.

Killing of the neoplastic or virus-infected cells occurs either by induction of extrinsic pathway of apoptosis or by lysis (Sordo-Bahamonde et al., 2020). The former depends on binding of death ligands FasL/CD95L present on the NK cells and TRAIL (on resident liver NK, but not on peripheral blood NK cells) to cognate TNF-family death receptors including the Fas/CD95 and is caspase-dependent (von Karstedt et al., 2017). On the other hand, lysis of target cells attacked by the NK lymphocytes is executed by the contents of characteristic granules of the NK cells, including perforin, granzymes A and B, and granulysin, released into the intercellular space between the NK and target cell enclosed by the immune synapse. Tight connection of the two cells prevents the spillage of above mentioned proteins and damage to neighbor cells. Perforin (PRF1) is a glycoprotein which inserts in the membrane of target cells and oligomerizes there, forming a pore. This by itself adversely affects the osmotic balance within the target cell, eventually leading to its lysis. PRF1 pores are also the gates for proteases (granzymes) which stimulate apoptosis of target cells by truncating the caspase-8-activated Bid protein and, mainly, converting procaspases 3, 6 and 7 into their active forms (granzyme B) and by leading to the damage of target cell DNA in a caspase-independent way (granzyme A (GZMA) increasing production of ROS by mitochondria which stimulates SET complex translocation from the endoplasmic reticulum to the nucleus, where SET itself is cleaved by GZMA, leading to the release of DNA-degrading nucleases, such as NM23-H1 DNase) (Sordo-Bahamonde et al., 2020; Kiselevsky, 2020).

Metabolism and functionality

Some recent papers indicate that the phenotypes of NK cells as described above do not fully correspond with their different functionalities. Thus, it has been shown that cytotoxic NK cells can display not only their classical CD56^{dim}CD16⁺ phenotype, but may also be CD56^{bright} and yet even more cytotoxic than the classical ones. On the other hand some regulatory NK cells, classically defined as CD56^{bright}CD16⁻, can also express CD16. Finally, the memory NK cells may be either CD56^{bright} or CD56^{dim} and CD16⁺ or CD16⁻. Furthermore, even the nominally function-associated receptors: Natural Cytotoxicity Receptors (NCRs), NKG2A, NKG2C, KIRs, and CD57 may be expressed at low or high level (or not at all) on any NK cell, whether cytotoxic, regulatory or memory. Summarizing, even the NK cells with opposing functions can express the same phenotypes indicating a certain plasticity in the phenotype for performing the needed function depending most probably from the microenvironment (Poznanski and Ashkar, 2019).

One needs to remember, that the NK cells are not just killers of pathologically modified cells. Apart from performing anti-tumor and anti-viral functions, they regulate the functions of other immune cells and even promote tissue growth by secreted cytokines (Vivier et al., 2008; Vitale et al., 2019). The latter may result in actually promoting the tumor growth rather than preventing it (Crome et al., 2017; Bruno et al., 2013; Levi et al., 2015). Other non-killer functions of the NK cells include participation in the growth of placenta and tolerance of fetus during pregnancy (Gamliel et al., 2018; Hanna et al., 2006; Ashkar et al., 2000).

The apparent phenotypic chaos makes any interpretation of surface phenotypic analysis (e.g. by flow cytometry or IHC) of the NK cells in the context of their adequate or pathological functions very doubtful and asks for other determinants of these functions. Growing body of evidence suggests that it might be differences in cellular metabolism, mainly glucose-driven glycolysis and oxidative metabolism which drive the NK cell development, education, effector functions and generation of NK memory cells (Schafer et al., 2019; Cichocki et al., 2018; Holmes et al., 2021; O'Sullivan et al., 2015). Thus, activated cytotoxic NK cells increase both glycolysis and OXPHOS and it was found that higher capacity for glucose metabolism (both glycolysis and OXPHOS) identifies the NK cells with the highest cytotoxic capabilities. Conversely, NK cells become regulatory under low levels of glycolysis and OXPHOS and likely utilize fatty acids and/or amino acids as energy sources. Finally the memory NK cells exhibit increased mitochondrial fitness, resulting from extensive mitophagy. These cells show an increased spare respiratory capacity (SRC) and mitochondrial membrane potential and manufacture less reactive oxygen species (ROS) (Poznanski and Ashkar, 2019).

Aging-related and other defects of NK cell functions

As all the other long-living cells of the immune system, also the NK cells undergo aging. Aging of this part of the immune system manifests both as changes in the proportions of cells exhibiting different phenotypes described above, and as functional changes within specific phenotypes (reviewed by Witkowski et al., 2020). Thus, the percentage of NK cells among the PBMC of healthy old individuals and centenarians is increased, but the CD56^{dim}CD16⁺ population of these cells expands at the expense of the CD56^{bright}CD16⁻. The decreased proportion of the former may be the reason for decreased overall production of cytokines and chemokines by activated NK cells of elderly. On the other hand, the remaining CD56^{bright}CD16⁻ NK cells of old individuals tend to manufacture more granzyme A and IFN, which is considered a compensatory mechanism aimed at the maintenance of the protective and immunoregulatory roles of these cells in elderly. Expanding CD56^{dim}CD16⁺ NK cells of the elderly exhibit decreased amounts of granzyme A, but not granzyme B or perforin; thus, their cytotoxicity as a population is sustained. However, if considered at the single cell level, the NK cells of old individuals are less capable of killing virus-infected or transformed cells, which translates clinically to decreased resistance to viral infections and cancers. The reason behind these individual cell level changes associated with aging may be changes in expression and in consequence function of various NK cell specific receptors, including decrease of some activating receptors like Nkp30 and Nkp46, and concomitant increase in the expression of inhibitory KIR and a decrease in CD94/NKG2A. Other aging-associated changes in the phenotype of peripheral NK cell population include increased proportion of terminally differentiated, highly cytotoxic but feebly proliferating CD57⁺ lymphocytes. Interestingly, such phenotypic shifts in NK cell population are also described as resulting from chronic viral infections, notably by the CMV.

One additional function of the NK cells which is likely affected by their aging leading to shorter healthspan is their ability to detect and destroy the senescent cells, accumulating with advancing age in all tissues. In fact it is possible that the accumulation (increased proportions) of senescent cells observed in the tissues of old individuals (and its consequences for functioning of various organs in the elderly) are mainly due to decreased functional capabilities of the NK cells in these individuals (Baker et al., 2011; Ovadya et al., 2018; Sagiv et al., 2013; Pereira et al., 2019).

As mentioned above, function of different NK cells is defined not only by their distinct phenotypes, but by associated metabolic characteristics and its differences (decreased OXPHOS versus increased aerobic glycolysis, changes in use of glucose or of fatty acids as energy sources etc.). In the aged, senescence of the NK cells is associated with no OXPHOS to aerobic glycolysis switch upon activation (including by IL-2), and no increase in mitochondrial mass and functions, which may be responsible for decreased global effector functions of NK cells from older individuals (Miranda et al., 2016, 2018).

Means of detection and quantification of the NK cells in the peripheral blood

Currently the golden standard for detection, identification and quantification of various populations of the NK cells is multicolor flow cytometry after most frequently a separation process for the blood mononuclear cells including also monocytes and lymphocytes. It allows not only for identification of cells being positive or negative for a given marker (defining the proportion of such positive cells in a population), but also makes possible measuring the level of fluorescent signal from a marker, which can crudely define cells as negative, dim, and as positive, bright with regard for this marker (e.g., CD56^{dim} or CD56^{bright} NK cells) which, as said above, might translate to different functions of the respective populations. However, there are already quite old techniques allowing for cytometric determination of actual numbers of molecules of interest on the cell surface (quantitative flow cytometry using the QuantiBrite™ system (BD Biosciences, San Jose, CA)), from as few as 20–30 per cell to as many as 10⁵ and more, with the help of proper standards. We have shown for molecules like CD28, TNF receptors TNFR1 and R2 or EpoR that their numbers on the surface of a T cell correlate with levels of its response to stimulation; to date, similar analyses have not yet been performed for various receptors on the NK cell surface, but likely they could yield interesting results (Bryl et al., 2005; Witkowski and Bryl, 2004; Lisowska et al., 2010, 2011). Monoclonal antibodies against most if not all the surface antigens characterizing different NK cells have been generated and, with appropriate choice of attached fluorochromes, allow for simultaneous surface characteristics of all the peripheral blood leukocyte types (and so allowing for finding of potential correlations between changes in different leukocyte populations) (Sahir et al., 2020). In one recent work 8 out of 40 markers (CD56, CD16, CD2, NKG2D [CD314], NKP30 (CD337), NKG2A (CD159a), NKG2C (CD159c), CD57) identified the NK cells and their populations, while the other 32 characterized the T cells, NKT cells, B cells, monocytes, and dendritic cells (Park et al., 2020). The same number of the NK cell antigens was detected by an earlier panel, again aimed at characterizing the full complement of peripheral blood leukocytes (Ruhle et al., 2016). However, currently the full power of multicolor flow cytometry, spectral flow cytometry or mass cytometry (CyTOF™, where the antibodies are labelled with heavy metal ion tags rather than with fluorochromes) may allow for simultaneous detection of many more antigens on a surface of a NK cell (theoretically more than a hundred different markers per cell in case of currently (mid-2021) available reagents). It goes without saying that determination of various NK cell antigens may be performed at rest and upon activation (either in vitro or due to specific clinical circumstances, including cancer development or viral infection) and so allows for comparison of the resting and activation-dependent receptor patterns, also in the context of other leukocyte populations.

Means of detection and quantification of NK cells in tissue samples

The NK cells reside and can be detected in various human tissues, including the liver, brain, tonsils, lungs, skin, intestine, uterus, and heart (Marquardt et al., 2015, 2017, 2019; Cheuk et al., 2017; Brownlie et al., 2021; Masopust et al., 2010; Vento-Tormo et al., 2018; Sun et al., 2021; Sedgwick et al., 2020). It is unclear if the NK cells found in the tissues are the permanent residents or are drawn into the tissue by some process leading to signals (like cytokines and/or chemokines) being released. Clearly, NK cells infiltrating the tissue are equipped with a complement of adhesion molecules (integrins) and chemokine receptors deciding about their tissue targets (Hegewisch-Sollosa et al., 2021). One surface marker of tissue-residing NK cells is the CD49a which normally is not expressed by circulating NK cells; recently however small populations of the NK cells with the phenotype CD49a⁺KIR⁺NKG2C⁺CD16⁻ were found in the peripheral blood of individuals in whom this population expanded in the lungs due to activation (Brownlie et al., 2021).

Detection of tissue-resident NK cell populations may be achieved by two major methods: immunohistochemistry (IHC) and flow cytometry. Both have their advantages and some disadvantages.

The IHC allows for determination of distribution of the NK cells within the tissue of interest, e.g., showing their preferential infiltration near tumors and in the virus-infected organs. Fluorescent confocal microscopy allows to detect the immune synapses between the NK cell and its target. On the other hand it is currently not possible to stain and visualize more than a few NK cell antigens in a single tissue sample, so deep IHC analysis of the composition of different functional populations of NK cells in a studied tissue is difficult. Also the quantification of these NK cells is usually limited to counting them as a proportion of total lymphocytes seen in the specimen and may be biased by uneven distribution of the cells in the tissue sample. Finally, control

("negative") slides attained e.g. with irrelevant antibodies are rarely used, and in the fixed and slice tissue the antibodies gain access to the cell interior and may bind there both to the relevant antigens which are on their way to the cell surface and non-specifically. Both of these may lead to false positive results.

Flow cytometry analysis of the phenotypes of tissue-resident cells (including the NK lymphocytes) cannot, of course, provide the topographic information about the distribution of various NK cells in the tissue, as the tissue of interest must be converted to a suspension of free cells prior to staining and analysis. This is achieved by proteolytic disintegration of matrix by collagenases and by prevention of the cells being glued together by DNA released from those damaged in the tissue processing using the DNases (Brownlie et al., 2021). Such unicellular suspension is then centrifuged in a density gradient (using the same reagents that are used to obtain the peripheral blood mononuclear cells) in order to obtain the suspension enriched in mononuclear leukocytes including the NK cells. These then undergo the same staining and FACS analysis as the PBMC, yielding the numbers (expressed per milligram of wet tissue or per mm³) and proportions of different NK populations in a given tissue sample.

Means to estimate functional capabilities

a. testing secretion of perforins and granzymes

Both perforins and granzymes GZMA and GZMB are preformed and stored in the characteristic granules of the resting NK cells, to be released upon activation (Lettau et al., 2015). As both are proteins, they can be detected in fixed/permeabilized NK cells with appropriate fluorescent antibodies and flow cytometry, which allows for comparison of relative amounts of these proteins between different NK cell populations (identified by their characteristic receptors) in a sample and between samples (e.g. derived from different individuals with diversified states of health and diseases). Recently it has become possible to actually measure the proteolytic activities of granzymes while in the granules, using specific protease-cleaved fluorogenic substrates, relevant granzyme inhibitors and flow cytometry (Janiszewski et al., 2020; Kolt et al., 2020). Upon activation in and by the presence of cognate target cells both granzymes and perforin are secreted into the space between the NK and target cell enclosed by the immune synapse which can also be detected and visualized (Friedman et al., 2021; Ambrose et al., 2020). Amount of secreted PRF1 and GZMs may also be measured in in vitro cultures of purified NK cells with appropriate soluble stimulants including IL-2 or the cocktail of phorbol myristate acetate (PMA) and calcium ionophore ionomycin; in this case the proteins are released into the culture medium and can be quantified e.g. by ELISA.

b. testing the killing of target cells in vitro

Killing of the compromised (transformed or virus-infected) cells is the most obvious function of the cytotoxic NK cells and its modified effectiveness may lead to uncontrolled virus infection or cancer spread (if the function is decreased) or to attack on own cells and development of autoimmunization and autoimmune diseases including type 1 diabetes or systemic sclerosis if the same killing function is above normal.

The effectiveness of the killing process can be assessed for the NK cells upon their purifying (elimination of other cytotoxic/cytolytic cells like the NKT and cytotoxic CD8+ T cells) by either magnetic or FACS sorting. Numbers of killed target cells (e.g. K562 erythroleukemia routinely used for such determination, or more specific targets like isolated cancer cells from clinical samples or specific virus-infected cells) may be counted by flow cytometry upon their staining with e.g. membrane integrity-dependent DNA dyes like propidium iodide or 7-amino-actinomycin D (7-AAD). This is relevant, as the NK cell killer activities result mainly in the induction of target apoptosis and the above mentioned stains are routinely used for quantifying apoptotic cells. These numbers of killed target cells may be expressed per a killer NK cell at different effector:target ratios on condition that the latter are properly detected (e.g. as CD3-CD56+CD16+/-, with possible addition of activation receptors CD25 and/or CD69) and quantified. The use of flow cytometry on one hand allows for simultaneous identification of different phenotypes of both effector and target cells, observation of apoptotic death of the latter and its quantification. On the other hand it makes historically developed methods of NK cell killing activity (like radioactive chromium Cr⁵¹ release from preloaded target cells or the release of lactate dehydrogenase from the targets upon incubation with the NK cells) obsolete. The only technique of NK cell-dependent killing of target cells based on their preloading with a detector reagent remaining is the use of calcein-AM loaded targets which release calcein (and become less fluorescent) upon being attacked by the NK cells; this process can be visualized and partially quantified by imaging cytometry (McCulley and Somanchi, 2016; Somanchi et al., 2015).

c. testing secretion of cytokines and chemokines

As mentioned above, the NK cells, apart from being effective killers of compromised cells, perform multiple regulatory functions affecting other immune cell types (Zwirner et al., 2021; Vitale et al., 2019). These functions generally depend on secretion of interferons and other cytokines and chemokines by activated NK cells, including: tumor necrosis factor- α (TNF- α), interferon γ (IFN- γ), IL-5, IL-10, IL-13, GM-CSF, and the chemokines MIP-1 α , MIP-1 β , IL-8, and RANTES (Fauriat et al., 2010). These regulatory molecules may be detected either in the cytoplasm of the NK cells by flow cytometry (after stimulation with blocked secretion to allow for cytokine accumulation, fixation/permeabilization of the membrane and staining the accumulated cytokines with appropriate, fluorescently tagged monoclonal antibodies). This technique allows for simultaneous phenotyping and quantification of the NK cells producing cytokines of interest upon contact with target cells or other means of activation. Alternatively, cytokines of

interest already released from in vitro activated NK cells may be detected and quantified in the culture media by ELISA or by flow cytometric technique using polystyrene microbeads with conjugated anti-cytokine antibodies to capture the cytokines and fluorescently tagged antibodies to detect and quantify bead-bound cytokines (cytokine bead assays, CBA™). The latter technique allows for multiplexing, i.e., simultaneous detection of up to 30 different cytokines from a single sample (Castillo and MacCallum, 2012).

We described presently separately the determination of phenotypes and functions. However with the recent progresses in the multi-omics measurements it will be more and more easy and common to perform single cell studies including the phenotypes, the functions, the transcriptomic, the epigenomic and the genomic. This will help to better characterize, to understand and to target the NK cells during health and disease (Zhou et al., 2017; Zhang et al., 2021).

d. testing NK metabolism

As mentioned above, NK cells' phenotype assessment, even multiparameter and deep, is by itself not a reliable way to assess the NK function, due to marker overlaps. As also mentioned above, it was repeatedly demonstrated that activation of the NK cells depends on their metabolic changes, including the increase of both glycolysis and OXPHOS (Cong, 2020; Keating et al., 2016). These processes can currently be measured as extracellular acidification rates (ECARs) and oxygen consumption rates (OCR) in purified NK cells and their subpopulations with devices like the XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) (Keating et al., 2016; Assmann et al., 2017; Donnelly et al., 2014). Sequential measurements of ECAR and OCR, following addition of the inhibitors: oligomycin, rotenone, antimycin, and 2DG, allow for the accurate calculation of oxygen consumption due to oxidative phosphorylation (OXPHOS) and acidification due to glycolysis (Assmann et al., 2017).

Summary

The NK cells are among the most important innate cells of the immune defense system. They are very heterogeneous in their phenotypic appearance and in their functionality which will certainly increase with the advances of our knowledge. There are several described techniques to unravel their diversity and functionality either in the blood or in the tissues. However, these classic tests are meant to evaluate and be included in a complex system approach using the combination of several levels of study in a multi-omics manner with the help of the artificial intelligence either at the single cell or cell population level. These new methods and concepts will help to integrate the individual techniques in a complex system for better assessment of the NK biology in health and disease.

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Complement Assays

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The complement system

The complement system is well known as a key component of the innate immune system. Its functions comprise: (1) the elimination of microbes (through cytolytic membrane attack by disrupting bacterial cell walls), (2) the removal of apoptotic cells and debris (through opsonization of antigens to promote phagocytosis), (3) to evoke an inflammatory response (through recruitment and activation of leukocytes, particularly macrophages and neutrophils), and (4) the modulation of the adaptive immune response through modification of T- and B-cell responses, employing specific receptors on various immune cells (Holers, 2014; Brodzski et al., 2020). Moreover, a normally functioning complement system also participates in hematopoiesis, reproduction, lipid metabolism and tissue regeneration (Merle et al., 2015). Only recently, intracellular functions of the complement system have been discovered modulating T lymphocyte activation and survival (Arbore et al., 2017).

Most complement proteins are secreted by the liver and contribute to the acute phase response. However, other tissues like the kidney are also able to produce complement proteins to a significant amount (Poppelaars and Thurman, 2020). Complement genes are distributed across different chromosomes, with 19 genes comprising three significant complement gene clusters in the human genome (Mayilyan, 2012).

Approximately 50 soluble and cell surface-attached proteins are currently recognized, acting as components, cofactors, regulators and receptors of the complement cascade (Holers, 2014). Unfortunately, the terminology of the complement system components rather refers to the sequence of their discovery than to a logical numeric order within the cascade reaction. Components and regulators of the AP are called factors (e.g., Factor B, Factor H) (Bohlson et al., 2019). Complement can be activated via three major pathways, the classical, the alternative, and the lectin pathway, all of which merge in the activation of complement C3 and subsequently lead to the formation of the cytolytic membrane attack complex (MAC), C5b-9 (Merle et al., 2015) (Fig. 1). To prevent inappropriate complement activation, regulatory proteins play an important role, whether present in the fluid phase or bound to the cell surface (Zipfel and Skerka, 2009). Soluble complement regulators, such as C1 inhibitor, C4b-binding protein (C4bp), Factors H (FH) and I (FI), clusterin and S-protein (vitronectin) restrict the action of complement in body fluids at multiple sites of the cascade (Fig. 1). In addition, each individual host cell is protected against the attack of homologous complement by surface proteins, such as the complement receptor 1 (CR1/CD35), the membrane cofactor protein (MCP/CD46) as well as by the glycosylphosphatidylinositol (GPI)-anchored proteins, decay-accelerating factor (DAF/CD55), and protectin (CD59) (Ling and Murali, 2019).

Complement and diseases

Complement is engaged in a number of diseases (Brodzski et al., 2020) (Table 1). Genetic deficiencies of complement components are rare; their estimated prevalence is about 0.03% excluding MBL deficiency, which is estimated to occur in about 5% of the Caucasian population (Grumach and Kirschfink, 2014). In the European Society for Immunodeficiencies (ESID) registry, deficiencies of complement proteins account for approximately 5% of all primary immunodeficiencies (PID) between 2004 and 2020 (<http://esid.org/Working-Parties/Registry/ESID-Database-Statistics>; <https://cci-reporting.uniklinik-freiburg.de/#/>). However, in national registries a wide variability in the frequencies of complement are reported, ranging between 1% and up to 30% of all primary immunodeficiencies (Abolhassani et al., 2020).

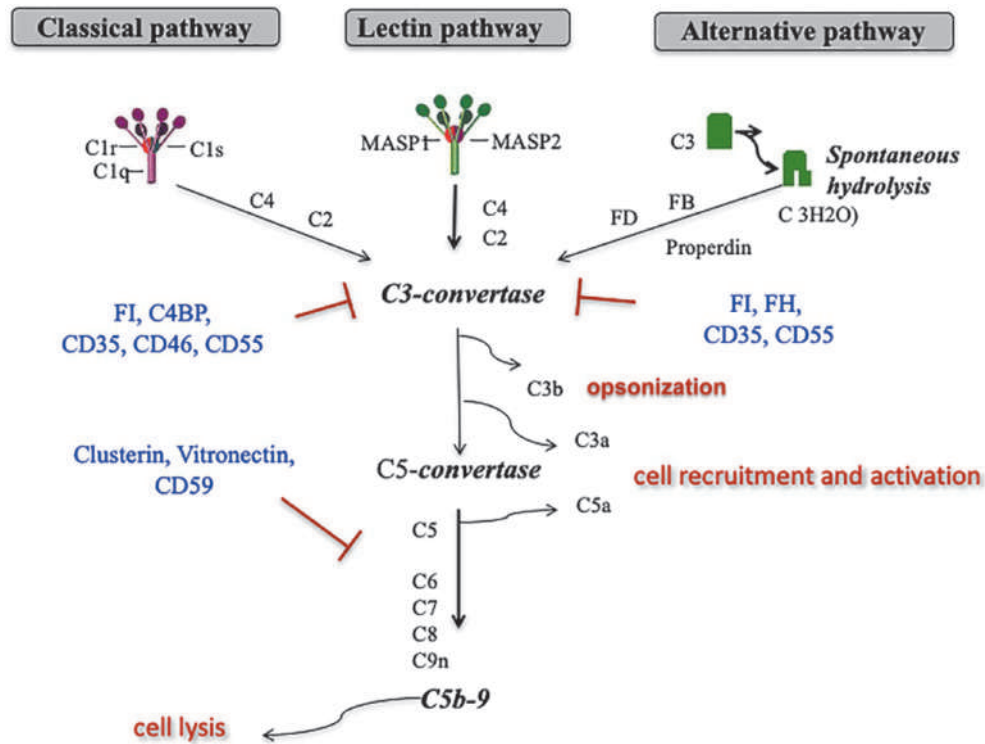


Fig. 1 Complement system: activation pathways, terminal pathway and regulation (blue).

Table 1 Complement mediated diseases and recommended complement analysis.

Disease	Complement testing
Infectious diseases	CP-, AP-, LP assay, C3, C4, C1q C3a/C3d, sC5b-9 C5-C9, P; MBL (especially in <i>Neisseria</i> infections)
Systemic lupus erythematosus, hypocomplementaemic urticarial vasculitis syndrome	CP analysis C4 (C4A/B), C1q, C3 C3a/C3d, sC5b-9 Anti-C1q autoantibodies
Angioedema	C1 inhibitor (protein and function) C4, C1q Auto-Anti-C1 Inh (acquired angioedema)
Nephropathies (C3 glomerulopathy/DDD*/aHUS**)	CH50, APH50 C4, C3 C3a/C3d, sC5b-9 Factor H, Factor I *C3 (and C5) nephritic factor **Autoantibodies to factor H, factor B, C3 convertase molecular analysis of CFH, CFI, C3, FB, CD46
Paroxysmal nocturnal hemoglobinuria	CD55, CD59 (FACSCAN) Acid lysis test

Complement defects, particularly of early components of the classical pathway, are frequently associated with autoimmune disorders, esp. with systemic lupus erythematosus (SLE). There is a strong association of a complement deficiency with SLE increases from C2 (10% prevalence) over C1r/s (57% prevalence), C4 (75% prevalence) to C1q (90% prevalence) (Pickering et al., 2000).

Warning signs for complement deficiencies are (1) Meningococcal meningitis at >5 years of age, (2) other recurrent bacterial infections, esp. with *Pneumococcus*, (3) autoimmune manifestations, (4) angioedema without urticaria, (5) renal and ophthalmic inflammatory disorders, (6) unusual infections, e.g., epiglottitis despite vaccination against *Haemophilus influenzae* type b (HIB) and, (7) severe infection with encapsulated bacteria (Grumach and Kirschfink, 2014; Brodzski et al., 2020).

In clinical practice, however, the consequences of an overactivated complement system with consumption of complement components as the cause of several inflammatory diseases and even life-threatening conditions are more prevalent (Prohászka et al., 2016; Ekdahl et al., 2018). The sudden exposure to a massive altered self or foreign particles, as in the case of sepsis or trauma, can overwhelm the system and fuel a vicious cycle that causes tissue damage and systemic inflammation. Similarly, transplants or biomedical materials often trigger an adverse complement response that affect both the clinical and functional outcome of the treatment (Ricklin et al., 2017). The kidney appears particularly vulnerable to complement-mediated injury and it was one of the first organs identified as a target of complement-mediated inflammation. Immune-complexes activate the classical pathway of complement in many forms of glomerulonephritis and alternative and lectin pathways are also activated in several forms of kidney disease. Atypical hemolytic uremic syndrome (aHUS) or age-related macular degeneration (AMD) are consequences of local dysregulation of the alternative complement pathway (Prohászka et al., 2016; Poppelaars and Thurman, 2020). The production of certain complement proteins and other acute phase reactants is augmented by inflammatory cytokines, such as tumor necrosis factor alpha, interleukin (IL)-1, and IL-6, which are produced by leukocytes (e.g., macrophages and neutrophils) (Holers, 2014; Ling and Murali, 2019).

Complement analysis

In the last years, modern complement analysis in body fluids and biopsies, going far beyond the detection of C3 and C4 levels, has not only significantly enhanced our current understanding of the disease process, but today also allows for a more precise differential diagnosis (for detailed reviews see: Ekdahl et al., 2018, Prohászka et al., 2016, Prohászka et al., 2018; Schroder-Braunstein and Kirschfink, 2019, Ling and Murali, 2019, Skattum, 2019). This has paved the way for a specific complement-targeting therapy which has now been successfully introduced in patients with various complement-mediated inflammatory disorders (Table 1).

Complement analysis aims at to

- (1) screen total complement activity;
- (2) quantitate individual complement components;
- (3) measure functional activity of individual components;
- (4) quantitate complement activation products;
- (5) detect autoantibodies to complement components or complexes;
- (6) assess cell surface expression and tissue deposition of complement components, regulators or fragments.

Sample collection

Improper handling and/or prolonged transport time at room temperature may affect data interpretation. Another important measure to prevent ex vivo complement activation, serum and EDTA plasma have to be separated from blood cells as rapidly as possible. On receipt, blood samples should be centrifuged as soon as possible and must be kept cold until they are frozen at -80°C until assayed or shipped on dry ice to specialized laboratories (<http://www.ecomplement.org/european-complement-labs.html>). EDTA-plasma is needed for analyses of activation products, while serum is suitable for analysis of complement proteins, function and autoantibodies. EDTA prevents further activation by chelating Ca^{2+} and Mg^{2+} thereby blocking the function of the C1 complex and of the two C3 convertases, respectively. The addition of nafamostat mesilate (Futhan, FUT-175) to EDTA further reduces ex vivo complement activation. For transportation dry ice must be used and the samples should be transferred directly from the -80°C freezer (Nilsson and Ekdahl, 2012). The lack of standardization in complement analysis still poses a major problem to disease recognition and follow-up (Prohászka et al., 2016; Ling and Murali, 2019).

Screening of total complement function

Functional complement activity is traditionally assessed using a hemolytic assay. Activity of the CP is monitored in hemolytic assays employing sheep erythrocytes coated with rabbit antibodies, preferably purified IgM (or mixed with IgG) to the Forssman antigen. Addition of serial serum dilutions of serum leads to binding of C1q to the immunoglobulins which initiates the formation of the CP C3 convertase. This subsequent activation leads to C5 activation and the assembly of the membrane attack complex which finally results in lysis of the erythrocytes (Ekdahl et al., 2018). A more recent lipolytic test for CP activity allows a better standardization of the activating/target cell. One unit CH50 represents the reciprocal of the dilution at which 50% hemolysis is seen. Similarly, alternative pathway activation can be assessed using rabbit (chicken or guinea pig) erythrocytes, which specifically activate the alternative pathway due to decreased sialic acid content. Here a Mg^{2+} + EGTA containing buffer is added to the patient serum prior to incubation which is needed to chelate Ca^{2+} , thereby inhibiting activation via the CP and LP. Hemolytic assays can be used to detect both deficiencies in individual component as well as depression in complement function caused by consumption of complement components (Ekdahl et al., 2018).

Activation of the AP of complement is monitored in hemolytic assays employing rabbit or guinea pig erythrocytes, which are spontaneous and potent activators of human AP. EGTA which chelates Ca^{2+} and thereby inhibits activation via the CP and LP, is added to the patient serum prior to incubation. Under these conditions the AP C3 convertase is formed on the target erythrocytes, leading to C3 activation and subsequent lysis (Ekdahl et al., 2018). Simplified and more rapid hemolytic assays apply are performed with a fixed serum sample dilution and excess of antibody-coated sheep erythrocytes or rabbit erythrocytes for the classical and alternative pathways, respectively (Nilsson and Ekdahl, 2012).

A missing or greatly reduced activity in either test indicates a primary complement deficiency affecting the classical or the alternative or both pathways, but may also be due to a secondary deficiency caused by increased consumption. Analysis of individual components and regulators provide insight, in which portion of the complement cascade either a lack of function or an (over-) activation occurs. (Fig. 2).

Nowadays, also a simple ELISA-based method is available which allows the assessment of the activity of all 3 pathways. This method is easy to perform and has several advantages compared with the hemolytic assays: it is not dependent on erythrocytes; it covers all three activation pathways and properdin deficiency (often not detected in hemolytic assays) can be diagnosed in the functional AP ELISA. The assay is performed with serum samples and the incorporation of C9 in the terminal complement complex C5b-9 is taken as readout (Prohászka et al., 2016).

Quantification of individual complement components

Individual complement components, quantitated in the past by immunodiffusion, is now mostly measured by nephelometry or turbidimetry (Ling and Murali, 2019). These techniques utilize polyclonal antibodies against the analyte, e.g., C1-INH, C4, C3, or factor B or activation fragments of these proteins (Ekdahl et al., 2018). Membrane bound regulatory proteins such as CD55, CD59, and CD35 (CR1) can be assessed using flow cytometry (Ling and Murali, 2019).

Measuring functional activity of single components

If a functional defect is suspected or needs to be ruled out, the functional activity of a single component can be tested. Sera depleted of the actual component and patient's fresh serum are mixed, to see whether the activity can be restored by using hemolysis as readout (Ricklin et al., 2017).

Quantification of activation products of complement

A reduced functional activity of individual complement components either reflect a deficiency state, or indicate ongoing, consuming complement activation. Since in vivo complement activation is often associated with an acute phase reaction, pathway activities or levels of individual components may stay within the normal range despite ongoing consumption. Consequently, these first level screening methods are not sufficiently sensitive to detect pathologically increased complement activation in vivo.

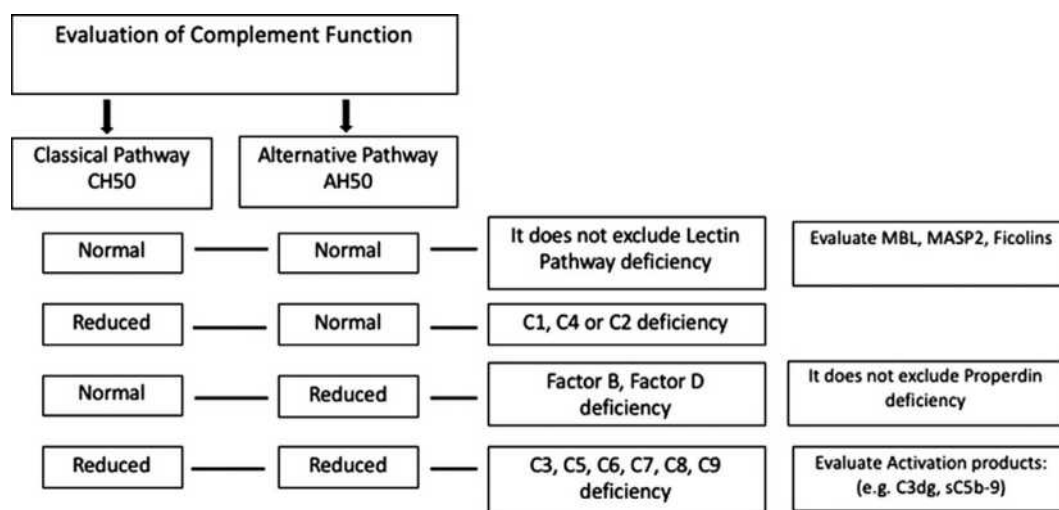


Fig. 2 Screening of complement deficiencies.

Complement activation products are either split fragments, generated after enzymatic cleavage of certain components, e.g., C4 (C4a, C4b/c, C4d), C3 (C3a, C3b/c, iC3b, C3d), factor B (Ba, Bb) and C5 (C5a), or protein complexes where activated components are bound to their respective regulators, like C1rs-C1-inhibitor, the properdin-containing alternative pathway convertase, C3bBbP, and sC5b-9 (soluble terminal complement complex) (Barnum et al., 2020).

It should also be taken into account that different activation products vary greatly with regard to their in vivo half-life: approximately 0.5 h for C3a and 4 h for C3d,g (Teisner et al., 1983). Since C3d,g is a more robust marker, it is more suitable in routine analysis use while the generation of C3a is often used in clinical studies and experimental settings. Due to rapid receptor binding, the biologically highly active and important C5a fragment has a half-life of approximately 1 min and is difficult to detect in samples obtained in vivo (Oppermann and Götze, 1994). In contrast, the half-life of sC5b-9 is 50–60 min (Barnum et al., 2020).

Formation of the lytic C5b-9 (MAC) complex is the last step of the complement cascade which causes cell damage or lysis as a result of its insertion into the cell membrane, or endothelial cell activation at sub-lytic concentrations. Complement activation of the TP can be monitored in body fluids by quantification of sC5b-9, with an EIA which uses a mAb specific for a neoepitope in C9 for capture (Ekdahl et al., 2018).

Detection of autoantibodies to complement components

Similar to *loss or gain of function* mutations in complement regulators or activating components, an overactivation of complement can also be caused by autoantibodies. Autoantibodies to FH (DEAP-HUS), FB (DDD), C1q (SLE) or to C1 inhibitor (hereditary angioedema) can be assessed by ELISA with the respective purified complement proteins immobilized on a microtiter plate (Defendi et al., 2020).

Nephritic factors comprise a heterogeneous group of autoantibodies against neoepitopes generated in the C3 and C5 convertases of the complement system, causing its dysregulation (Corvillo et al., 2019). C3 nephritic factor (C3NeF), found in all types of MPGN can be measured in a decay assay as C3NeF stabilizes the alternative pathway C3 convertase, C3bBb. In this semi-quantitative screening assay C3NeF stabilizes the C3 convertase on sheep erythrocytes, thereby causing increased complement activation and eventually hemolysis. Recently an ELISA for C3NeF has been reported (Skattum, 2019). Both C3 and C5 Nephritic factors correlate with C3 consumption, while only C5 nephritic factors correlated with sC5b-9 levels (Corvillo et al., 2019).

Assessing cell surface expression and tissue deposition of complement proteins or fragments

The diagnosis of many autoimmune (or immune complex diseases) is based on the detection of immunoglobulins and complement deposition in various tissues. For immunohistochemical analysis, antibodies against C1q, C3b, C4b, C4d, and C5b-9 are available for frozen and in part also for paraffin fixed tissue. Positive staining identifies a direct impact of complement in the disease process, indicates disease activity and allows for the differentiation of the complement activation pathways involved. The presence of C3b suggests ongoing inflammation, while C3d deposits in the absence of C3b point to a non-active disease process. C4d has been accepted as biomarker of humoral renal graft rejection (Cohen et al., 2012).

In Paroxysmal Nocturnal Hemoglobinuria (PNH) increased susceptibility of red cell lysis is due to reduced expression of the complement inhibitors DAF (CD55) and CD59. These genetic deficiency of these non-transmembrane proteins affects their common GPI anchor. Flow cytometric quantification of DAF and CD59 is recommended for diagnostic testing (Ling and Murali, 2019).

Analysis of complement receptor 3 (CR3, CD11b/CD18) needs to be performed in cases of leukocyte adhesion deficiency (LAD) and of C5aR (CD88) if phagocyte response (e.g. chemotaxis) is impaired.

Standardization and quality control

The complex nature of complement testing and increasing clinical demand has been met in the last decade by efforts to improve the standardization among laboratories. Initiated by the IUIS/ICS Committee for the Standardization and Quality Assessment in Complement Measurements 14 rounds of external quality assessment since 2010 resulted in improvements in the consistency of testing across participating institutions, while extending the global reach of the efforts to more than 200 laboratories in 30 countries. World-wide trends of assay availability, usage and analytical performance are summarized based on the past years' experiences (Prohászka et al., 2016).

Future developments

As complement is a cascade of multiple pathways and multiple components, the value of being able to test across the pathways applying multiplex technology appears obvious. Using dried blood spot samples from newborns various complement deficiencies could be detected by multiplex immunoassays (Dezfouli et al., 2020). Taking another approach, the value of using mass

spectrometry to profile the complement system (Willems et al., 2020). This is considered to open a new avenue for the clinical immunology laboratory. However, its transfer to routine complement analysis still requires more efforts in standardization and quality assessment.

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Functional Testing of the IL-12/IFN- γ Circuit

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Introduction

Functional testing of the IFN- γ /IL-12 circuit is key for the diagnosis of Mendelian susceptibility to mycobacterial disease (MSMD, OMIM: 209950). MSMD belongs to the expanding group of disorders termed primary immunodeficiencies (PID), or, more recently, inborn errors of immunity (IEI), and is included in the group 6 named Defects in intrinsic and innate immunity. MSMD is characterized by a selective predisposition to intramacrophagic microorganisms in otherwise healthy individuals, particularly poorly virulent mycobacteria. This susceptibility is ascribed to inborn errors of immunity in components of the IFN- γ /IL12 circuit (Bustamante et al., 2014; Casanova and Abel, 2002). In a normal anti-mycobacterial response, after bacilli/us phagocytosis, antigen-presenting cells (APC) are activated and produce tumor necrosis factor (TNF)- α , interferon-stimulated gene (ISG) 15, interleukin (IL)-12p70, and IL-23, among other cytokines. These cytokines, together with antigen presentation to T helper (Th) cells, induces differentiation into Th1 cells and IFN- γ production. This activation process creates a positive loop between the T cell and the APC, which enhances the former's microbicidal capacity of the APC through increase of receptor-mediated phagocytosis, phago-lysosomal fusion and oxygen reactive species' (ROS) and nitride oxide production (Bogunovic et al., 2012; Cottle, 2011; D'Cunha et al., 1996; Ramirez-Alejo and Santos-Argumedo, 2014; Torrado and Cooper, 2013).

IL-12p70 is a heterodimer composed of a p40 subunit (in common with IL-23) and a p35 subunit, which bind IL-12R β 1 and IL-12R β 2, respectively, activating Th1 cells, gamma-delta T cells, NK and NKT cells, mucosal associated invariant T cells (MAIT cells) and innate lymphoid cells type 1 and type 2 (Zundler and Neurath, 2015). The Janus-associated kinase 2 (JAK2) and the tyrosine kinase 2 (TYK2) interact with the IL-12R β 1 and IL-12R β 2 subunits respectively, and mediated IL-12 receptor signaling, leading to Signal transducer and activator of transcription 4 (STAT4) phosphorylation. STAT4 then dimerizes and translocate as homodimers to the nucleus promoting IFN- γ production (Watford et al., 2004). IFN- γ response in APCs, especially in macrophages, is mediated

by its binding to IFN- γ receptor (IFN- γ R) 1 and IFN- γ R2. Signaling by the IFN- γ R is mediated by JAK1 and JAK2 and induces STAT1 phosphorylation at tyrosine 701, dimerization, and translocation of STAT-1 homodimers γ -activated factor (the gamma-interferon activated factor, GAF) to the nucleus. In the nucleus, GAF binds to gamma-interferon-activated sites (GAS) at ISGs, promoting their expression (Majoros et al., 2017; Stark, 2007).

MSMD is caused by monogenic defects in different molecules of this circuit (Fig. 1), which impair the production of, or the response to, IFN- γ , thereby disrupting protective immunity to mycobacterial infection. Although the first clinical description of MSMD was published in 1951 (Mimouni, 1951), it was not until 1996 that the first genetic etiology of MSMD, autosomal recessive (AR) IFN- γ R1 deficiency, was described in an infant with fatal BCG infection (Jouanguy et al., 1996; Newport et al., 1996). Until date, mutations in 18 genes have been found to cause isolated or syndromic MSMD (Fig. 1). These genes are involved in IFN- γ production (*IL12RB1* (de Beaucoudrey et al., 2010; Feinberg et al., 2004; van de Vosse et al., 2013), *IL12B* (Altare et al., 1998; Prando et al., 2013a), *IL12RB2* (Martínez-Barricarte et al., 2018) *IL23R* (Martínez-Barricarte et al., 2018) *ISG15* (Bogunovic et al., 2012; Zhang et al., 2015), *SPPL2A* (Kong et al., 2018; Rosain et al., 2019), *TYK2* (Kerner et al., 2019b; Kreins et al., 2015) and *RORC* (Okada et al., 2015)), cellular responses to IFN- γ (*IFNGR1* (Dorman et al., 2004; Feinberg et al., 2004; Jouanguy et al., 1996; Jouanguy et al., 1999, 2000; Sologuren et al., 2011), *IFNGR2* (Moncada-Velez et al., 2013; Rosenzweig et al., 2004b), *STAT1* (Chapgier et al., 2006a; Dupuis, 2001; Hirata et al., 2013) *JAK1* (Eletto et al., 2016) and *CYBB* (Bustamante et al., 2006, 2011a,b)) or both (*NEMO* (Bustamante et al., 2011b) and *IRF8* (Hambleton et al., 2011)). *NEMO* defects are not included in the group of MSMD disorders within the IUIS classification, but considered MSMD-causing by the group of Dr. Jean Laurent Casanova and Dr. Jacinta Bustamante, leaders in the field. Besides, *IFNG* (Kerner et al., 2020) and *TBK21* (Yang et al., 2020) have been included as two

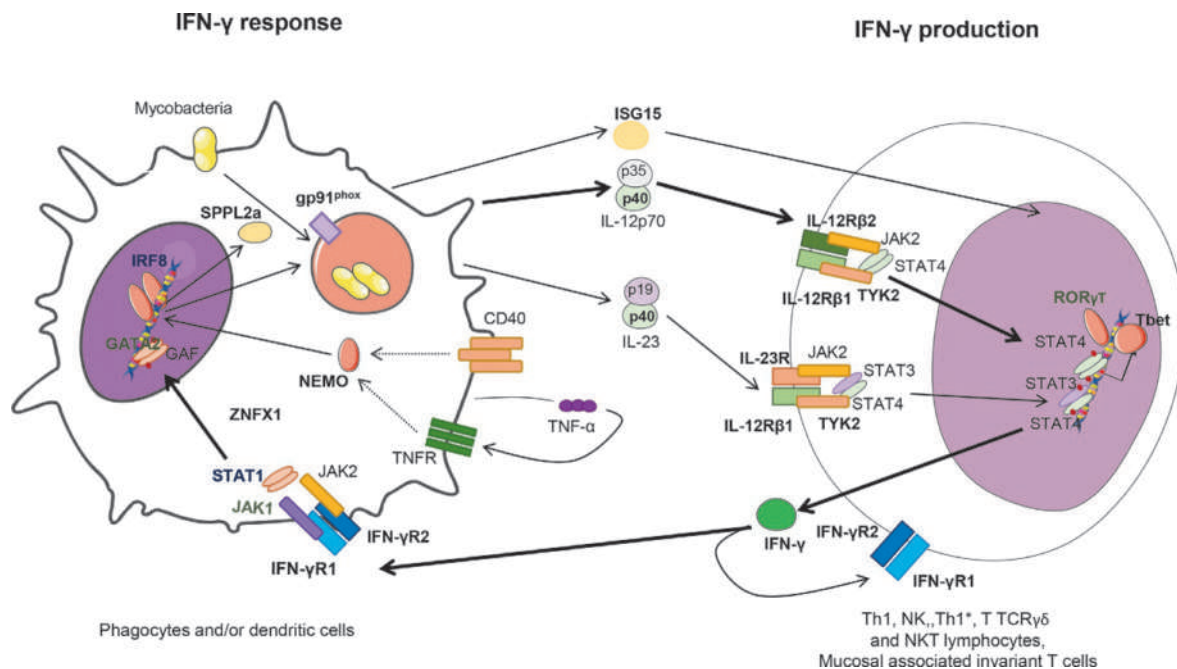


Fig. 1 Summary of molecules involved in IFN- γ -mediated immunity. Molecules represented with **bold characters** are known to be cause of MSMD. Genes causing non-syndromic forms are displayed in **black**, genes causing both syndromic and non-syndromic forms in **purple** and genes causing only syndromic forms in **green**. Recognition and phagocytosis of the bacilli by antigen-presenting cells (APC) and macrophages, induces their activation and the production of an array of cytokines and chemokines, particularly tumor necrosis factor (TNF)- α , interferon-stimulated gene (ISG) 15, interleukin (IL)-12p70, and IL-23, which induce IFN- γ production by T, NK, and NKT cells. IL-12p40 (encoded by the *IL12B* gene) is common to IL-12p70 and IL-23. The IL-12R β 1 molecule is shared by both the IL-12 and IL-23 receptor heterodimers, whereas IL-12R β 2 and IL-23R are unique to the IL-12 and IL-23 receptors respectively. Tyk2 is a tyrosine kinase involved in IL-12R- and IL-23R-mediated signaling. ISG15 is secreted by many cell types, including myeloid cells and neutrophils, and it acts as a very potent IFN- γ -inducing cytokine in lymphocytes. *ISG15* also encodes an intracellular interferon-induced ubiquitin-like protein that acts as a negative regulator of IFN- α/β , resulting in enhanced IFN- α/β immunity. The *RORC* gene encodes two protein isoforms that act as transcription factors: the nuclear orphan receptor γ (ROR γ) is ubiquitously expressed, whereas the expression of ROR γ T is restricted to leukocytes. IFN- γ binding to the IFN- γ receptor, an heterodimer of IFN- γ R1 and IFN- γ R2, leads to activation of the tyrosine kinases Jak1 and Jak2, and to serine and tyrosine phosphorylation of the signal transducer and activator of transcription 1 (STAT1). Phosphorylated STAT1 forms a homodimer termed IFN-gamma-activated factor (GAF), which migrates into the nucleus and binds to the IFN gamma activated sequence (GAS) to drive the expression of the target genes. Interferon regulatory factor 8 (IRF8), is a transcription factor induced by IFN- γ , expressed in macrophages and dendritic cells. IRF8 binds to IFN-stimulated response elements (ISRE) and regulate the expression of many genes. *SPPL2A* encodes the signal peptide peptidase-like 2 A (SPPL2a), a protease with multiple substrates. A binding site for IRF8 has been identified in the Sppl2a promoter in mouse macrophages. *NEMO* encodes the Nuclear factor-kappa B (NF- κ B) essential modulator, which mediates signaling in the NF- κ B pathway, required, among other signaling pathways, for TNF receptor- and CD40L-mediated activation. gp91^{phox}, encoded by *CYBB*, is a major component of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (PHOX) complex, required for effective intracellular killing of microorganisms.

new causes of MSMD in 2021 (Tangye et al., 2021). Besides ZNFX1 deficiency has been reported associated with intermittent monocytosis and mycobacterial disease (Le Voyer et al., 2021).

Incidence for MSMD is described to be 1/50,000 worldwide (Rosain et al., 2019). Depending on the impact of the mutation (null or hypomorphic, resulting in complete or partial deficiency), the inheritance of the disease, the expression of the mutant allele (absent or detectable), or the mechanism responsible for the impaired function of the mutated protein, at least 30 different genetic etiologies of MSMD have been identified so far (Bustamante, 2020; Bustamante et al., 2014; Rosain et al., 2019). IL-12R β 1 deficiency and autosomal dominant (AD) partial IFN- γ R1 deficiency are the most common defects (Bustamante, 2020; Bustamante et al., 2014; Filipe-Santos et al., 2006a; Kerner et al., 2019a; Rosain et al., 2019). Patients with syndromic MSMD, mainly with mutations in *ISG15* (Bogunovic et al., 2012; Zhang et al., 2015), *RORC* (Okada et al., 2015), and *JAK1* (Eletto et al., 2016), manifest a more complex clinical phenotype, with a predisposition to other microorganisms or to other manifestations. In addition, mutations in *STAT1* (Chapgier et al., 2006b), *IRF8* (Hambleton et al., 2011), and *TYK2* (Kerner et al., 2019b; Kreins et al., 2015) can cause isolated or syndromic MSMD depending of the pattern of inheritance and the functional impact of the mutation. With so many forms, the clinical boundaries of MSMD, particularly of some of the less frequent etiologies, are not fully defined, and the genetic etiology remains unknown in about half of the patients.

Clinical spectrum of patients with defects of the IL-12/IFN- γ circuit

Mycobacteria

MSMD is usually characterized by vulnerability to poorly virulent mycobacteria, such as bacillus Calmette-Guérin (BCG) vaccines and environmental mycobacteria (EM), in otherwise healthy individuals, which tend to be considered the warning signs of this form of IEI. Also, infection by the more virulent *M. tuberculosis* has been documented in some patients, particularly in deficiencies of IL-12R β 1 (Boisson-Dupuis et al., 2011, 2015; Bustamante et al., 2014), IFN- γ R1 (Bustamante et al., 2014; Dorman et al., 2004), *STAT1* (Bustamante et al., 2014; Chapgier et al., 2006a), or IL-12p40 (Fieschi, 2004; Prando et al., 2013a).

Patients can present with a wide range of clinical manifestations of mycobacterial disease, from local BCGitis to disseminated, invasive and lethal infections. Disease onset is usually in childhood, but diagnosis in adolescence and adulthood have been reported. Usually, the most severe forms (AR complete IFN- γ R1 or IFN- γ R2 deficiency) show an early onset, and infections use to be persistent and life-threatening in spite of anti-mycobacterial treatment. On the other hand, other less severe forms of MSMD (partial -AR or AD- deficiencies of IFN- γ R1 and IFN- γ R2 or deficiencies of the IL-12/IL-23 receptors) can have a late onset, they can improve with age, and the infections can be relatively circumscribed (Arend, 2001; Boudjemaa et al., 2016; Bustamante et al., 2014; Hirata et al., 2013; Jirapongsananuruk et al., 2012; Obinata et al., 2013; Rosain et al., 2019; Sasaki et al., 2002; Shamriz et al., 2013; Tsumura et al., 2012a). Patients that survive to a mycobacterial disease, may suffer from another mycobacterial infection or remain healthy; likewise, mycobacterial infections may or may not recur.

Other intramacrophagic bacterial and fungal infections

Besides the increased susceptibility to mycobacteria, patients with MSMD may also be prone to infections by other intramacrophagic microorganisms: about half of the patients suffer from salmonellosis (frequently extra gastrointestinal), particularly non typhoidal, and more rarely typhoidal; rare cases of infections caused by intramacrophagic fungi (histoplasmosis, coccidioidomycosis, paracoccidioidomycosis), parasites (Leishmaniasis, Toxoplasmosis) and bacteria (listeriosis, nocardiosis, klebsiellosis) have been also reported.

Candida

In addition, since IL-12R β 1 is also part of the IL-23 receptor and IL-12p40 is also a subunit of IL-23, required for expansion of Th17 and Th1* cells (Martínez-Barricarte et al., 2018), patients with IL-12R β 1 and IL-12p40 deficiencies are prone to mild forms of chronic mucocutaneous candidiasis (CMC) (Ouederni et al., 2014; Prando et al., 2013a). Patients with *RORC* deficiency also present with CMC (Okada et al., 2015).

Virus

Complete deficiencies of IFN- γ R1 and IFN- γ R2 can rarely predispose to viral disease, particularly but not exclusively by herpes viruses (Bustamante et al., 2014). Autosomic recessive forms of *IRF8*, *STAT1* LOF and *TYK2* deficiency, not included in the MSMD classification, as well as *JAK1* deficiencies also confer susceptibility to severe viral infections since their loss of function interferes with type I IFN signaling. This is why they are included in the "Predisposition to severe viral infection" subgroup within Defects in intrinsic and innate immunity in IUIS classification. They can particularly combine severe forms of mycobacterial and viral disease (Boisson-Dupuis et al., 2012). On the other hand, patients usually have a normal resistance to other microorganisms.

Clinical penetrance of MSMD may be incomplete, and it usually correlates with the extent of IFN- γ -mediated immunity. Complete AR IFN- γ R1 and IFN- γ R2 deficiencies are fully penetrant and they are always lethal in the absence of hematopoietic stem cell transplantation (HSCT). By contrast, clinical penetrance is partial, and even very low, in patients with deficiencies of the

IL-12 and/or IL-23 receptors. Only 50–70% of adults with IL-12R β 1 deficiency, who have abolished IL-12- and IL-23 mediated responses, are symptomatic by the age of 40 years (Bustamante et al., 2014; de Beaucoudrey et al., 2010; Rosain et al., 2019). However, the clinical penetrance of IL-12R β 2 deficiency, which do not affect IL-23-mediated responses, as well as IL-23R and of partial AR Tyk2 deficiencies, which show normal IL-12-mediated responses, is very low (about 0.5%), and most patients remain asymptomatic in adulthood (Kerner et al., 2019b; Martínez-Barricarte et al., 2018; Rosain et al., 2019). Therefore, some of the genetic etiologies of MSMD do not segregate as a bona fide mendelian trait.

Who should be tested for defects of the IL-12/IFN- γ circuit? Differential

Warning signs for MSMD

Children or adults who develop recurrent or severe/disseminated mycobacterial infectious disease caused by BCG, EM, *M. tuberculosis*, or *Salmonella*, alone or in combination with other intramacrophagic pathogens or viruses, and for whom no other hemato-immunological conditions nor immunosuppressants can be demonstrated, should be tested for defects on the IL12/IFN- γ circuit. Specific warning signs for MSMD are summarized in Table 1. Routine hematological and immunological analysis for a suspected immunodeficiency (including) (1) T cell maturation, (2) proliferation after mitogen stimulation and (3) oxidative burst reactions, tend to be normal in patients with MSMD, although monocytopenia and DC deficiency, may be present in patients with IRF8 and SPPL2a deficiency.

Differential: Other IEI with similar infectious susceptibility

It is important to consider that MSMD is not the only IEI predisposing to mycobacterial disease (Boisson-Dupuis et al., 2015; Picard et al., 2018); indeed, other IEI can manifest with the above mentioned infections, but they also tend to manifest other infectious and immunological phenotypes (Boisson-Dupuis et al., 2015). These other mycobacterial infection-predisposing IEI to be discarded include: (i) patients with defects in the number and/or function of T-cells (severe combined immunodeficiencies and combined immunodeficiencies), (ii) chronic granulomatous disease (particularly susceptible to BCG and *M. tuberculosis*), and (iii) GATA2

Table 1 Warning signs for MSMD and other conditions to be discarded.

Age	Usually in childhood, also in adolescence and adulthood
Patient history	Otherwise healthy individuals
Clinical signs	Invasive or recurrent infections by: Mycobacteria: • BCG infection (<i>Mycobacterium bovis</i> vaccine strain) • Environmental mycobacteria (<i>M. chelonae</i>, <i>M. fortuitum</i>, <i>M. mageritense</i>, <i>M. peregrinum</i>, <i>M. smegmatis</i>, <i>M. scrofulaceum</i>...) • <i>Mycobacterium tuberculosis</i> Intramacrophagic bacteria (alone or in combination with mycobacteria): • <i>Salmonella</i> spp. • <i>Listeria monocytogenes</i>/<i>Nocardia</i> spp./<i>Klebsiella</i> spp. Fungi (alone or in combination with mycobacteria): • <i>Candida</i> spp. • <i>Histoplasma capsulatum</i>/<i>Paracoccidioides brasiliensis</i>/<i>Coccidioides</i> spp. Parasites (alone or in combination with mycobacteria, rare): • <i>Leishmania</i> spp. • <i>Toxoplasma gondii</i> Virus (in combination with mycobacteria, rare): • Cytomegalovirus, human herpes virus 8, parainfluenza virus type 3, respiratory syncytial virus and varicella zoster virus.
Other signs	Family history of invasive or recurrent mycobacterial infection Undetectable or very low IFN- γ production in IFN- γ release Tests (i. e. QuantiFERON-TB Gold In-Tube)
Differential	Other IEI: • T cell defects (severe combined immunodeficiency and combined immunodeficiency) • Chronic granulomatous disease • GATA2 deficiency Acquired immunodeficiencies: • Immunosuppressive drugs exposure (including biologic drugs to molecules in the pathway) • Stem cell transplantation • Malignancy • Human immunodeficiency virus infection • Congenital lung defects • Bronchiectasis, chronic obstructive pulmonary disease Presence of neutralizing anti-IFN- γ autoantibodies

BCG, bacille Calmette-Guérin.

deficiency, predisposing to disseminated EM infections, and less frequently to tuberculosis, which may be the first clinical presentation even in otherwise healthy adults, although they can also occur during childhood. However, GATA2 patients have a broader clinical spectra, including viral infections, particularly warts, hematological disorders (myelodysplastic syndrome/leukemia), pulmonary alveolar proteinosis, and other non-immunological anomalies (Cohen et al., 2016; Donadieu et al., 2018; Sologuren et al., 2018). Patients with GATA2 have severe circulating monocytopenia (78% of patients), and B (86% of patients) and NK (82% of patients) (Donadieu et al., 2018; Hsu et al., 2011; Spinner et al., 2014) lymphopenias. Characteristically, patients with GATA2 deficiency show specific loss of the CD56^{bright} NK subset (Mace et al., 2013). Besides, patients suffering severe combined immunodeficiencies, combined immunodeficiencies, chronic granulomatous diseases and X-linked NF- κ B deficiency anhidrotic ectodermal dysplasia with immunodeficiency (XR-EDA-ID) syndrome are susceptible to a wide range of pathogens (pyogenic bacteria, virus) including mycobacteria (Bustamante et al., 2014; Orange et al., 2002, 2004; Picard and Casanova, 2004).

Differential: acquired immunodeficiencies and other conditions with similar infectious susceptibility.

In addition, acquired immunodeficiencies predisposing to mycobacterial diseases must also be excluded (Boisson-Dupuis et al., 2015): immunosuppressive drugs exposure (Brode et al., 2015; Lake et al., 2016), stem cell transplantation, malignancy (Arlotta et al., 2014; Kraut, 2003; Netea et al., 2008; Thaker et al., 2001), biologicals, particularly those against TNF- α -mediated immunity, and JAK inhibitors (Hirai et al., 2020) or human immunodeficiency virus infection (Boisson-Dupuis et al., 2015; Lake et al., 2016; Orme and Ordway, 2014) (HIV; BCG vaccination is contraindicated in HIV-infected individuals). Environmental mycobacteria and *M. tuberculosis* infections are increasingly being reported in patients with congenital lung defects such as primary ciliary dyskinesia, pulmonary alveolar proteinosis, and cystic fibrosis and other pulmonary complications such as bronchiectasis, chronic obstructive pulmonary disease.

Finally, patients that have neutralizing anti-IFN- γ autoantibodies can develop MSMD-like clinical manifestations, and they are included in group X of the IUIS classification, which is called phenocopies of inborn errors of immunity (Hanitsch et al., 2015; Kampmann et al., 2005; Lee et al., 2013; Picard et al., 2018; Tangye et al., 2020; Wu et al., 2018). Neutralizing anti-IFN- γ autoantibodies have been mostly described in females with Southeast descent (Hong et al., 2019), and are associated with HLA-DR*15:02/16:02 and HLA-DQ*05:01/05:020020 (Ku et al., 2016). Treatment with rituximab has been shown to be efficient for the treatment of these patients (Browne et al., 2012b). The description of the methods that can be used to identify the presence of neutralizing anti-IFN- γ autoantibodies is described below.

IL-12/IFN- γ circuit functional testing studies

IFN- γ circulating levels

Quantitation of IFN- γ levels in plasma by enzyme-linked immunosorbent assay (ELISA) is a fast and easy technique for detection of IFN- γ R deficiencies (Bustamante et al., 2014; Fieschi et al., 2001; Martínez-Barricarte et al., 2014). Patients with complete IFN- γ R deficiencies have increased levels of IFN- γ in plasma (150–1700 pg/mL) (Fieschi et al., 2001; Martínez-Barricarte et al., 2014); patients with partial deficiency of IFN- γ R present detectable levels of IFN- γ (51–222 pg/mL) (Sologuren et al., 2011) while it is undetectable in other MSMD forms and in healthy controls (Fieschi et al., 2001; Martínez-Barricarte et al., 2014; Moncada-Velez et al., 2013; Olbrich et al., 2015; Rottman et al., 2008; Sologuren et al., 2011). Of note, in a case series of partial IFN- γ R1 deficiency a patient with acute mycobacterial disease had specially high IFN- γ levels (925 pg/mL). Thus, the infectious state of the patient needs to be considered, as circulating IFN- γ plasma levels may vary in acute infection compared with the convalescent phase (Sologuren et al., 2011), being a confounding factor for differential diagnosis between complete and partial AR IFN- γ R1 or IFN- γ R2 deficiency in rare cases (Martínez-Barricarte et al., 2014; Moncada-Velez et al., 2013; Sologuren et al., 2011). Therefore, if possible, circulating IFN- γ levels should be measured at least 1 month after acute infection resolution. In any case, no IFN- γ is usually detected in the plasma of healthy individuals (Fieschi et al., 2001; Martínez-Barricarte et al., 2014). At a technical level, plasma samples need to be diluted at least 1:2 to avoid interference of other proteins such as fibrinogen. The assay may be performed in serum samples, but proper controls should be performed. Serum and plasma levels of cytokines are not exchangeable, since differences in the matrix affect cytokine detection (Esteve-Sole et al., 2021; Rosenberg-Hasson et al., 2014).

Cytometric evaluation of receptors' expression

IFN- γ R1/IFN- γ R2 expression

IFN- γ receptor detection by flow cytometry is a fast technique for the detection of complete forms of AR IFN- γ R1 and AR IFN- γ R2 deficiency with absent protein expression in the membrane of monocytes. Approximately an 80% of complete AR IFN- γ R1 deficiencies can be detected (Bustamante et al., 2014; de Beaucoudrey et al., 2010; Ehlayel et al., 2008; Esteve-Sole et al., 2018b; Jouanguy et al., 1999, 2000; Kong et al., 2013; Moncada-Velez et al., 2013; Olbrich et al., 2015; Sologuren et al., 2011). It can be performed both in WB and in PBMCs. In partial AR IFN- γ R1 defects, there is usually a weak expression of the receptor (Bustamante et al., 2014; Sologuren et al., 2011); contrarily, high levels of protein expression have been reported in partial AD IFN- γ R1 deficiency due to mutations in the recycling motif (Bustamante et al., 2014; Jouanguy et al., 1999). Detection of IFN- γ receptors could be affected by the antibody used: for example, IFN- γ R1 in cells of patients with the p.C77Y complete AR IFN- γ R1 defect would be identified with the gR99 clone and not with the gR38 clone (Jouanguy et al., 2000). Similarly, there are some AR IFN- γ R2 defects with protein expression, and partial AD/AR defects show low but detectable IFN- γ R2 in the membrane of monocytes (Bustamante

et al., 2014; Kong et al., 2013; Moncada-Velez et al., 2013). The currently available antibodies for the evaluation of IFN- γ R2 expression are not optimal. In patients with suspected MSMD and normal receptor detection other techniques that evaluate cellular responses to IFN- γ should be performed.

IFN- γ binding studies

As discussed above, in some IFN- γ receptor 1 deficiencies there is protein expression in the membrane. Some of these mutations lead to poor IFN- γ binding. Detection of IFN- γ binding may be performed with radiolabeled 125 IFN- γ or by flow cytometry (Jouanguy et al., 1999; Rosenzweig et al., 2004b; Sologuren et al., 2011). For flow cytometry, PBMCs are first incubated with different concentrations of hrIFN- γ for 30 min, and then washed and incubated for 20 min with an anti-IFN- γ antibody and acquired with a flow cytometer (Rosenzweig et al., 2004b; Sologuren et al., 2011). With this technique, membrane-expressing IFN- γ R1 defects can be easily detected. IFN- γ -binding assays with flow cytometry do not yield consistent results with Epstein-Barr virus-transformed B cells (EBV-B cells), and when using PBMCs, gating on monocytes is required (Sologuren et al., 2011). Some MSMD etiologies (AD IFN- γ R1 deficiency) will escape this detection (Jouanguy et al., 1999). To our knowledge, only four patients and three healthy controls have been evaluated so far (Rosenzweig et al., 2004b; Sologuren et al., 2011).

IL-12R β 1 expression

IL-12R β 1 deficiency is the most common genetic form of MSMD (Bustamante, 2020; Bustamante et al., 2014), with more than 200 cases described (Bustamante, 2020). IL-12R β 1 expression detection with flow cytometry is performed in PBMCs after 72 h of stimulation with PHA (de Beaucoudrey et al., 2010) (Fig. 2). Cytometric determination of IL-12R β 1 expression is a powerful and easy-to-perform technique that allows the detection of more than 99% of the described mutations (de Beaucoudrey et al., 2010; Ehlayel et al., 2008; Esteve-Sole et al., 2018b), excluding a large deletion (700 + 362_1619-944del) in IL12RB1 (Fieschi, 2004) and an N-terminal signal peptide stop-gain homozygous mutation (de Souza et al., 2017).

Cytometric detection of phosphorylated STAT molecules

STAT proteins bind to activated extracellular receptors, phosphorylate, dimerize, and translocate to the nucleus to bind to specific DNA regions and activate gene transcription (Casanova et al., 2012; Majoros et al., 2017; Stark, 2007). The two most relevant STAT molecules implicated in the IFN- γ circuit are STAT1 and STAT4 (Qu et al., 2011). Flow cytometric determination of STAT1 phosphorylation can be performed both in whole blood and in isolated PBMCs, while STAT4 phosphorylation in response to IL-12p70 needs to be performed in activated lymphocytes. The general protocols includes: (1) stimulation at different cytokine concentrations for 15–30 min, (2) fixation and permeabilization with special buffers for phosphorylation assays (3) staining with anti-phosphorylated STAT antibodies in conjunction with extracellular antibodies of choice and (4) acquisition with a flow cytometer (Chapgier et al., 2006a; Fieschi, 2004; Hirata et al., 2013; Sampaio et al., 2012; Sologuren et al., 2011). It should be stressed that when working with anti-STAT antibodies, proper negative controls are mandatory. Of note, STAT4 phosphorylation evaluation is limited by the lack of a proper antibody to detect total STAT4.

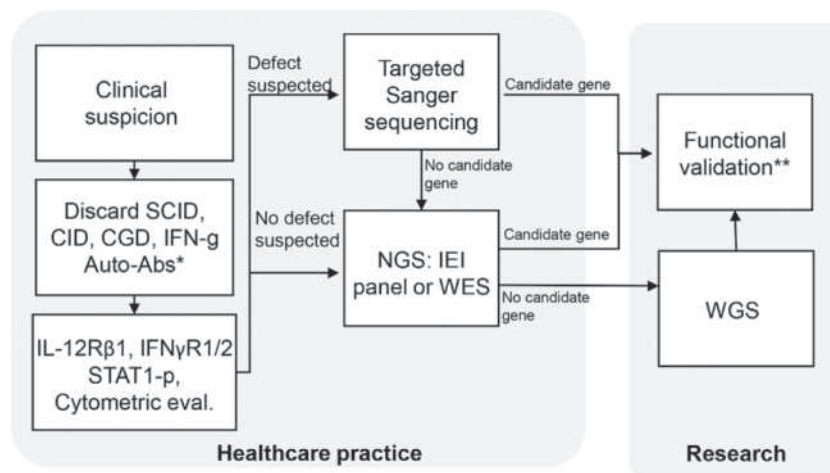


Fig. 2 Step-by-step proposed diagnostic algorithm for suspected defects of the IL-12/IFN- γ circuit. *SCID*, severe combined immunodeficiency; *CID*, combined immunodeficiency; *CGD*, chronic granulomatous; *NGS*, next generation sequencing; *IEI*, inborn errors of immunity; *WES*, whole exome sequencing; *WGS*, whole genome sequencing. * Especially in adult female patients of Southeast Asian descent. ** For non-reported variants. Guidelines for functional validation of single variants are depicted in Casanova et al. in 2014 (Casanova et al., 2014).

STAT1 phosphorylation

STAT1 phosphorylation has been shown useful for detection of complete and partial defects of STAT1 and IFN- γ receptors. Complete defects in IFNGR1 and IFNGR2 genes lead to abolished STAT1 phosphorylation in response to hrIFN- γ while a normal response to hrIFN- α is observed (Kong et al., 2010b, 2013; Rosenzweig et al., 2004a,b; Sologuren et al., 2011). Partial defects lead to impaired, but not abolished, STAT1 phosphorylation after hrIFN- γ stimulation in a dose-dependent manner; this altered response is normalized after high dose stimulation (de Paus et al., 2011; Janssen et al., 2002; Kong et al., 2010b, 2013; Moncada-Velez et al., 2013; Okada et al., 2007; Sologuren et al., 2011; Storgaard et al., 2006). Concentration of IFN- γ or hrIFN- α stimulation for STAT1 phosphorylation assays range from 10 to 10^5 IU/mL, being 10^3 IU/mL and 10^5 IU/mL the most common concentrations used (Chapgier et al., 2006a; Dupuis, 2001; Jouanguy et al., 1999; Kong et al., 2010a,b; Sologuren et al., 2011; Takeda et al., 2014; Tsumura et al., 2012b). STAT1 phosphorylation after low-dose IFN- γ stimulation will detect almost all IFN- γ receptor defects and approximately 70% of STAT1 defects.

Impaired or abolished phosphorylation to both hrIFN- γ and hrIFN- α suggests a STAT1 deficiency but normal phosphorylation does not exclude it. Although phosphorylation is the first step for STAT1 function, mutations on other STAT1 domains implicated in later events can impair STAT1 function with normal phosphorylation. For example, AD STAT1 LOF mutations in the tail segment domain or SH2 domain (except the p.M654K mutation exception (Sampaio et al., 2012)) lead to impaired STAT1 phosphorylation in response to hrIFN- γ but not hrIFN- α (Dupuis, 2001; Hirata et al., 2013; Tsumura et al., 2012b). In contrast, mutations in the DNA-binding domain can lead to both normal (E320Q and p.Q463H mutation (Chapgier et al., 2006a)) and altered (p.E157K and p.G250E mutation (Kagawa et al., 2017)) STAT1 phosphorylation. Furthermore, it has been recently reported that patients with AD STAT1 gain of function mutations, who usually develop chronic mucocutaneous candidiasis, but also other infectious phenotype and clinical manifestations, can also develop mycobacterial infectious disease (Kagawa et al., 2017; Toubiana et al., 2016).

STAT4 phosphorylation

STAT4 signals downstream the IL-12 receptor. After IL-12 binding to the IL-12 receptor, IL-12R β 1 binds TYK2 and IL-12R β 2 associates with JAK2, initiating trans-phosphorylation of the receptors. This creates a docking site for STAT4, where it is phosphorylated at tyrosine 693, dimerizes, and undergoes nuclear translocation where it binds to its target DNA sequences (Watford et al., 2004). For STAT4 phosphorylation cytometric assay PBMCs are stimulated for 72 h with phytohemagglutinin (PHA), cultured 48 h in the presence of IL-2 and then stimulated with hrIL-12p70; the whole assay takes approximately 1 week (Fieschi, 2004; Uzel et al., 2001). Abolished STAT4 phosphorylation in response to hrIL-12p70 has been observed both in IL-12R β 1-(Ehlayel et al., 2008; Fieschi, 2004) and TYK2-deficient patients (Kreins et al., 2015). Otherwise, STAT1 phosphorylation after IFN- α is normal in IL-12R β 1-deficient patients (Fieschi, 2004) and impaired in TYK2 deficient patients (Kreins et al., 2015). However, STAT4 phosphorylation results must be interpreted with caution due to the lack of a proper STAT4 antibody to assess total STAT4 expression by flow cytometry. For this reason, STAT4 is meant to help only in the diagnosis of other forms of MSMD.

Cytokine production evaluation

Evaluation of cytokine production, developed by Feinberg et al (Feinberg et al., 2004), is the gold standard for study of IL-12/IFN- γ circuit. This assay is based on the measurement of IL-12p40, IL-12p70 and IFN- γ after whole blood or, less frequently, peripheral blood mononuclear cells (PBMCs) stimulation. Stimulation conditions comprise incubation with live BCG at a multiplicity of infection of 20 BCG/leukocyte with or without hrIL-12p70 (20 ng/mL), or hr-IFN- γ (5000 IU/mL) co-stimulation for 18 h (for IL-12 measurement) or 48 h (for IFN- γ and IL-12 measurements) (Esteve-Sole et al., 2018b; Feinberg et al., 2004). Detection of the cytokines produced may be performed with ELISA or multiplex assays by means of flow cytometry (Luminex Technology (Luminex, Austin, TX, USA) or cytometric bead array systems) (Feinberg et al., 2004; Olbrich et al., 2015; Sologuren et al., 2011). However, absolute values obtained by the different techniques are not directly comparable, hampering the definition of cut-off values.

Although powerful, this technique has several limitations: (1) the intrinsic variability observed yet in healthy controls, that hampers interpretation of results, especially of partial defects (de Beaucoudrey et al., 2010; Esteve-Sole et al., 2018b; Feinberg et al., 2004). Because of that, there are not cut-off values described. To overcome this limitation, evaluation of an in-house healthy controls' cohort may help to define normal ranges of cytokine production. In our experience (Esteve-Sole et al., 2018a; Esteve-Solé et al., 2018), the lower 10th percentile of the control cohort may define a weak response, (2) if fresh whole blood is used, it should be performed during the first 48 h after extraction (de Beaucoudrey et al., 2010), and (3) the use of BCG stimulation can be limiting in diagnostic laboratories following ISO 15189 regulations. In an attempt to solve limitations, different strategies have been developed, including the performance of the test in cryopreserved cells to eliminate time-from-extraction limitation and the use of phytohemagglutinin and lipopolysaccharide as stimuli to avoid the use of BCG; the stimulation condition will then be phytohemagglutinin (PHA; 1%) in the presence or not of IL-12p70 (Dorman and Holland, 1998) or LPS (from *Salmonella minnesota*; 100 ng/mL) with and without hrIFN- γ (10^2 , 10^3 and 10^4 IU/mL) (Sologuren et al., 2011) costimulation.

This assay help to distinguish between IFN- γ response defects and IFN- γ production defects. Complete forms of IFN- γ R1, IFN- γ R2, IL-12RB1 or IL-12p40 can be detected with this approach. IFN- γ production defects are characterized by the absence or low production of IFN- γ after BCG stimulation (4–726 pg/mL for IL12RB1 defects (de Beaucoudrey et al., 2010; Feinberg et al., 2004) and below 100 pg/mL for IL12p40 defects (Feinberg et al., 2004; Prando et al., 2013b)). If there is no recovery of IFN- γ after hrIL-12p70 co-stimulation, IL-12R β 1 deficiency should be studied first (de Beaucoudrey et al., 2010), followed by ISG15 or TYK2

deficiencies (Bogunovic et al., 2012; Kreins et al., 2015). On the other hand, in patients with IL-12p40 deficiency decreased levels of IFN- γ in response to BCG stimulation can be rescued, at least partially, with exogenous hrIL-12p70. IL12p70 secretion is normal in patients with IL-12R β 1 deficiency and abolished in patients with IL-12p40 deficiency. Patients with complete deficiency of IFN- γ R1 or IFN- γ R2 showed normal production of IFN- γ in response to BCG in the presence or absence of IL-12p70. However, there is no response to hrIFN- γ in terms of IL-12 production (Dorman et al., 2004; Dorman and Holland, 1998; Feinberg et al., 2004; Holland et al., 1998; Kong et al., 2013; Noordzij et al., 2007; Prando et al., 2010; Rosenzweig et al., 2004a; Vogt et al., 2005). On the other hand, in partial IFN- γ response defects (partial AR IFN- γ R1/IFN- γ R2 and partial AD STAT1 LOF deficiency), the response to hrIFN- γ is impaired in a dose-dependent manner, but not abolished (Allende et al., 2001; Chapgier et al., 2006a; Dorman et al., 2004; Hirata et al., 2013; Janssen et al., 2002; Jouanguy et al., 1997, 2000; Kilic et al., 2012; Okada et al., 2007; Sologuren et al., 2011; Takeda et al., 2014; Tsumura et al., 2012b).

Some genetic etiologies of MSMD, such as gp91^{phox} or AD IRF8 deficiency, will show normal responses to this stimulation (Bustamante et al., 2011a; Hambleton et al., 2011; Puel et al., 2006). Nevertheless, in nuclear factor-kappa B essential modulator (NEMO)-deficient patients, IL-12 production is normal after BCG stimulation but impaired after PBMC stimulation with PHA or anti-CD3 antibodies (Bustamante et al., 2006; Filipe-Santos et al., 2006b). ROR γ C deficient patients resemble patients with IL-12RB1 deficiency, with little IFN- γ production after BCG stimulation which is not rescued after IL-12p70 stimulation (Okada et al., 2015). This has not been assayed for other new MSMD etiologies such as SPPL2A, IL-23R and IL-12RB2 deficiency.

Detection of neutralizing anti-IFN- γ autoantibodies

Since 2004, first reports of the existence of neutralizing autoantibodies against IFN- γ in patients with disseminated mycobacterial diseases have been published (Boisson-Dupuis et al., 2015; Doffinger et al., 2004; Hong et al., 2019; Liew et al., 2019; Phoompoung et al., 2017). This condition is not considered as MSMD but it is included here because it is classified as a phenocopy defects on the IL-12/IFN- γ circuit and should be included in the differential diagnosis of MSMD. This disorder affects predominantly, but not exclusively, to adults of Asian descent (Hong et al., 2019; Liew et al., 2019; Phoompoung et al., 2017). Most patients suffer from infections by environmental mycobacteria, but *M. tuberculosis* have also reported (Boisson-Dupuis et al., 2015). This condition is frequently associated with infections by other intramacrophagic microorganisms such as *Salmonella*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, or *Penicillium marneffei*.

Presence and level of autoantibodies against IFN- γ can be assessed by an ELISA system against anti-IFN- γ -antibodies with different serum dilutions and by observing IFN- γ level recovery after the addition of exogenous IFN- γ to patient serum (Browne et al., 2012a; Chi et al., 2013; Hanitsch et al., 2015; Kampmann et al., 2005; Lee et al., 2013; Patel et al., 2005). In addition, it has been recently shown that undetectable or very low IFN- γ production in QuantiFERON-TB Gold In-tube is a warning sign for the presence of anti-IFN- γ antibodies (Wu et al., 2018). Only the presence of neutralizing autoantibodies against IFN- γ explains the susceptibility to intramacrophagic microorganisms. IFN- γ response assays, such as STAT1 phosphorylation or cytokine production, in the presence of patients' sera must be performed to analyze the neutralizing activity of IFN- γ autoantibodies (Hanitsch et al., 2015; Hong et al., 2019; Koizumi et al., 2017; Lee et al., 2013; Shima et al., 2014).

Other tests for the evaluation of the IL12/IFN- γ circuit

Because samples of primary cells from patients are not infinitely available, some tests have been adapted to the use of patient-derived cell lines. Epstein Barr Virus (EBV)-B cells, herpes virus saimiri-transformed T cells (T-saimiri cells), and immortalized SV-40 fibroblasts are the most common cell lines used (de Paus et al., 2011; Doffinger et al., 2000; Jouanguy et al., 1999; Kong et al., 2010b; Kreins et al., 2015; Moncada-Velez et al., 2013; Sologuren et al., 2011). IFN- γ R1, IFN- γ R2 and IL12RB1 expression and STAT1 phosphorylation may be evaluated in patients' cell lines, as well as STAT1 phosphorylation. The group of tests described below requires a laboratory with experience in the field of MSMD and is usually performed in a research laboratory context. Some defects have specific associated immunological features, depicted in Table 2.

IFN- γ response specific tests

IFN- γ response may be thoroughly study with the evaluation of signal transduction and evaluating its effects. For example, expression of activation markers such as HLA-DR and CD64 after stimulation with different hrIFN- γ concentrations may be used to determine response to IFN- γ , both in primary cells and in transformed SV40-fibroblasts and EBV-B cells (Allende et al., 2001; Jouanguy et al., 1997; Kong et al., 2010b). At a molecular level, electrophoretic mobility shift assay (EMSA), or quantification of the translocation of STAT-1 homodimers to the nucleus and their binding to DNA by means of ELISA, are used to evaluate STAT1 binding to target DNA, revealing also a correct nuclear translocation. These technique may help to detect AD STAT1 LOF deficiencies where phosphorylation is present. In addition, it can also help to identify AD STAT1-GOF deficiency (Dupuis, 2001; Tsumura et al., 2012b). Similarly, it is possible to study induction of GAS in response to IFN- γ . Specifically, expression of CXCL9 and CXCL10, among others, or the activation of GAS elements by luciferase detection (Chapgier et al., 2006a; Kagawa et al., 2017; Kong et al., 2010a; Sampaio et al., 2012; Sologuren et al., 2011; Tsumura et al., 2012b), can help to determine the effect of specific mutations. These approaches are usually used in research rather than in healthcare practice.

Table 2 Associated immunological features in particular defects of the IL-12/IFN- γ circuit.

CYBB deficiency	Abolished respiratory burst in monocyte-derived macrophages in response to purified protein derivative (PPD) or BCG, and in EBV-B cells. However, this oxidative burst defect cannot be detected in a routine dihydrorhodamine test, since monocytes, neutrophils, and monocyte-derived dendritic cells have normal responses (Bustamante et al., 2011a; Conti et al., 2015).
Partial AD IRF8 deficiency and SPPL2A deficiency	Loss of CD11c ⁺ CD1c ⁺ blood dendritic cells and a defect of IFN- γ production by mycobacterium-specific Th1 ⁺ cells (Hambleton et al., 2011; Kong et al., 2018). Besides, in SPPL2A deficiency, there is an accumulation of a N-terminal fragment of CD74 in the cell membrane of SPPL2a deficient cells (Kong et al., 2018).
NEMO deficiency causing MSMD	Normal NEMO expression in monocytes and T cells. Functionally, there is a low production of IFN- γ , IL-12p40, IL-12p70 after PHA stimulation in a T cell/Monocytes co-culture system. Besides, they have a lower IL10 production after TNF- α stimulation (WB) (Bustamante et al., 2011b; Filipe-Santos et al., 2006b).
ROR γ T deficiency	Normal protein expression and nuclear localization, impaired DNA binding; barely detectable ILC3; mucosa associated innate cells (MAIT) and NKT cells, mild CD4 and CD8 lymphopenia, severe impairment in the production of IL-17A, IL-17F, and IL-22 after polyclonal stimulus, and normal IFN- γ secretion by naïve or memory CD4 ⁺ T cells but strongly impaired IFN- γ production by Th1 ⁺ cells, and $\gamma\delta$ T cells (Satoshi Okada et al., 2015).
IL-12p40 deficiency	Lower than normal frequency of CD3 ⁺ IL-17A ⁺ cells ex vivo. T cells were capable of IL17 production after IL-23 stimulation (Prando et al., 2013b).
IL-12/IL-23 signaling defects	<i>IL12RB1</i> , <i>IL12RB2</i> and <i>IL23R</i> genes have increased frequency of naïve CD4 and CD8 cells and reduced levels of memory Th1 and Th1 ⁺ (Martínez-Barricarte et al., 2018). In vivo differentiation of naïve T cells to Th1 is abolished in <i>IL12RB1</i> and <i>IL12RB2</i> deficiencies but normal in <i>IL23R</i> deficiency. IL-17 differentiation is affected in <i>IL12RB1</i> and <i>IL23R</i> deficient patients and normal in <i>IL12RB2</i> deficient patients (Martínez-Barricarte et al., 2018). <i>IL12RB1</i> deficient patients have diminished IL-17-producing T cells ex vivo T cells were incapable of IL17 production after IL-23 stimulation (de Beauclourey et al., 2010) and a reduced frequency of circulating memory Tfh and memory B cells, with the functional consequence of lower avidity of tetanus toxoid-specific serum antibodies (Schmitt et al., 2013).
TYK2 deficiency	Impaired response to IL-10 after LPS and TNF- α stimulation of PBMCs, with diminished IL-10R2 cell surface expression and impaired STAT3 phosphorylation and EMSA assay after IL-10 stimulation. Patients have normal IL17 ⁺ cells in PBMCs after PMA/Ionomycin stimulation; impaired IL17A and F production in naïve T cells. Besides, in HerpesVirus Simplex-transformed T cells, there are abolished response to IL-12p70 objectified by abolished STAT4 phosphorylation, GAS and IFN- γ production. Besides, abolished STAT1 phosphorylation in response to IFN- α is observed. There is an abolished IL-23 response (STAT3-p, IFN- γ production and STAT3 phosphorylation). STAT3 phosphorylation and EMSA after IL-6/IL-21/IL-27 stimulation is normal (Kerner et al., 2019b; Kreins et al., 2015).

Molecular testing for defects in the IL-12/IFN- γ circuit

Genetic confirmation of the diagnosis of MSMD is of outmost importance for treatment and genetic counseling. MSMD may be caused by deficiencies in a relatively high number of genes; because of that, Sanger sequencing is only recommended when the clinical presentations and functional tests clearly suggest a specific gene defect. Otherwise, next generation sequencing (NGS) should be the option of choice to reduce time-to-response and costs (Cordero et al., 2020; Meyts et al., 2016). For general healthcare practice, gene panels including all known genes to be causative of MSMD have a good cost-efficiency ratio, being a good option to detect somatic mutations; since gene panels may be sequenced at high coverage. However, almost half of the patients with clinical signs suggestive of MSMD do not show mutations in these genes (Bustamante, 2020; Bustamante et al., 2014; Rosain et al., 2019).

Because of the reduction in time and cost during the last years, whole exome sequencing (WES) or whole genome sequencing (WGS) are being more used to overcome this limitation. WGS may reveal mutations in non-coding regulatory regions that would be undetectable by WES, but WGS is more expensive and difficult to interpret than WES. In fact, an increasingly used approach for the evaluation of PID (including MSMD) is to start with a panel or semi-targeted panel using NGS and then perform functional tests to confirm the observed variants. These techniques allow to detect defects that may not be detected by conventional Sanger sequencing such as copy number variations (Rosain et al., 2018).

Functional validation of unknown genetic variants

Unknown variants in known disease-causing genes or new genes must be functionally confirmed to be deleterious. Recommended guidelines for considering single-patient mutations to be disease-causing have been reported (Casanova et al., 2014; Tangye et al., 2021) and different strategies are proposed in order to study the deleterious effects of specific mutations (Kagawa et al., 2017). Nowadays, advanced in silico prediction tools are available online to help to predict the effect of a variant in protein; however, these analyses are not sufficient to validate the deleterious effect of a variant. Protein expression and functionality should be studied in vitro. Transfection of different cell lines with wild-type or mutated genes may be useful for the evaluation of variants of interest to evaluate protein expression and function. Disease-causing variants commonly have altered protein expression. Also, transfection of a wild-type copy of the mutated gene in patient cells (for example, in EBV-B or HSV-T transformed cells) restoring the protein

function can confirm a possible loss-of-function mutation (Martínez-Barricarte et al., 2016). Furthermore, new techniques such as CRISPR/Cas9 open the possibility of reversing the mutation in patient cells or mutating control to confirm that the phenotype that is observed in the patient is due to the mutation.

Diagnostic algorithm for IL-12/IFN- γ circuit defects

Set up and performance of specific functional techniques for MSMD diagnosis is not trivial. Besides of the technical limitations, interpretation of the results can also be challenging. Especially in low-income countries, the needed infrastructures and equipment's could be also limiting, as there is a need to work in sterile conditions with high cost equipment and consumables, specifically in relation to flow cytometry or some cytokine detection techniques. MSMD diagnosis is usually only possible in specialized immunology laboratories. Currently there is a broad array of available tests for MSMD diagnosis, each one with different strengths and limitations (Esteve-Solé et al., 2018).

Considering price, working hours and complexity in the interpretation of results, reviewed by Esteve-Solé et al. (Esteve-Solé et al., 2018), we propose a step-by-step algorithm (Fig. 2) for MSMD diagnosis in the routine practice after clinical suspicion: (1) discard other more frequent intramacrophagic-susceptibility-conferring PIDs by performing T cell phenotyping, proliferation assessment and oxidative burst evaluation, (2) performance of cytometric determination of the presence of IL-12R β 1, IFN- γ R1, IFN- γ R2 and, when possible, of STAT1 phosphorylation in response to IFN- γ and IFN- α , (3) genetic evaluation, whether by targeted Sanger sequencing when functional evaluation suggests a clear candidate gene or by NGS if no causative gene is identified, and (4) functional confirmation of the genetic result, which normally needs to be performed in the context of a research laboratory. Nevertheless, only genetics with a functional confirmation of the identified variant will lead to a definitive diagnosis. WES or WGS is recommended for cases with no molecular identification of the defect.

Concluding remarks

Like in other IEL, MSMD is an increasing group of disorders with clinical and immunological heterogeneity. Since the first description of *IFNGR1* deficiency in 1996 (Jouanguy et al., 1996) years, the number of genetic defects included in MSMD has increased to the current 18 genes. Nevertheless, almost 50% of patients with suspected MSMD do not have a confirmed genetic diagnosis up to date. Functional testing of the IL-12/IFN- γ circuit may help confirm a functional impairment explaining the increased susceptibility to mycobacterial disease when genetics is not concluding. Besides, functional testing is necessary after the identification of new unreported mutations. Functional testing of the IL-12/IFN- γ circuit is challenging, it has its particular tips, which we have tried to summarize in the document. Some of these techniques are limited by the timing and the requirement of qualified staff, making the full diagnosis of MSMD suited for specialized immunology laboratories. Genetic approaches are gaining ground and could overcome these limitations. Nevertheless, genetic results usually need a functional confirmation of the identified mutation.

MSMD should be considered in patients with significant infection (severe, disseminated, or recurrent) after BCG vaccination and infection by mycobacteria, particularly EM, especially in combination with Salmonella, Candida or virus. Besides, the knowledge of other conditions that can lead to susceptibility to mycobacterial diseases is relevant and must be considered in the differential diagnosis. These include: acquired causes of immunodeficiency (anti-TNF- α biological treatment, jak-inhibitors, hematological disorders, and neutralizing anti-IFN- γ antibodies), and other IEL: T cell defects, GATA-2 deficiency and chronic granulomatous disease first need to be ruled out. Once these entities are ruled-out, MSMD-specific evaluation should be started. Tests performed and their order may depend on laboratory facilities, technical staff, and clinical orientation.

Genetic and/or functional confirmation of MSMD is of utmost importance since specific therapeutic measures can be applied to improve infection resolution rate and patient's prognosis and quality of life. This is exemplified by complete deficiency of IFN- γ R where the only curative treatment attempted is HSCT; on the other hand, most other forms of MSMD will benefit from prolonged anti-tuberculous treatment to which exogenous hrIFN- γ therapy can be added—even partial defects of IFN- γ receptors respond to exogenous hrIFN- γ therapy (Bustamante et al., 2014; Dorman et al., 2004; Holland, 2000). When a member of a family is diagnosed with MSMD, BCG vaccination in family members should be avoided until a genetic defect has been ruled out. It is also important to consider that some MSMD etiologies have incomplete penetrance, meaning that not all the individuals presenting the mutation will present the clinical phenotype and genetic counseling in these patients is challenging.

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Interferon Signature Analysis

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Nomenclature

AGS	Aicardi-Goutières syndrome
AIDS	Acquired immunodeficiency syndrome
AVA	Antiviral assay
BCG	Bacillus Calmette-Guérin
CANDLE	Chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature
CSF	Cerebrospinal fluid
CPE	Cytopathic effect
CFP 10	Culture filtrate protein 10
DsRNA	Double-stranded ribonucleic acid
ESAT 6	Early secretory antigenic target 6
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
EMCV	Encephalomyocarditis virus
GFP	Green fluorescent protein
IFN	Interferon
ISG	Interferon-stimulated genes
IL-1	Interleukin 1
JAK	Janus kinase
PBMC	Peripheral blood mononuclear cells
PKR	Protein kinase R
PPD	Purified protein derivatives
qPCR	Quantitative polymerase chain reaction
MHC	Major histocompatibility complex
NOMID	Neonatal onset multisystem inflammatory disease
NTM	Non-tuberculosis mycobacteria
RD 1	Region of difference 1

RA	Rheumatoid arthritis
RGA	Reporter gene assay
SAVI	STING-associated vasculopathy with onset in infancy
SFV	Semliki forest virus
SiMoA	Single molecule array
SS	Sjögren's syndrome
STAT	Signal transducer and activator of transcription
SLE	Systemic lupus erythematosus
SLEDAI	Systemic lupus erythematosus disease activity index
TST	Tuberculin skin test
TYK2	Tyrosine kinase 2
UCTD	Undifferentiated connective tissue disease
VSV	Vesicular stomatitis virus

Introduction

Interferons (IFNs) are a group of cytokines with potent antiviral properties that are released by nearly all cells of vertebrate organisms following a viral infection. There are three types of interferons, namely type I, type II and type III interferons. This classification is strictly based on the receptor type the interferons bind to upon their release (Rius-Rocabert et al., 2019). Type I interferons are the largest and the most studied type of interferons and comprise of 13 distinct IFN- α subtypes and an IFN- β subunit. Thus, 13 distinct IFN- α genes are encoded within the human genome whereas a single gene encodes for IFN- β (Rentsch and Zimmer, 2011). Cells are able to sense viral infection via their pattern recognition receptors such as the retinoic acid inducible gene I (RIG-I) which causes the IFN-dependent genes to be transcriptionally activated. Secreted interferon- α and interferon- β produce similar effects by binding to a common, widely distributed IFN- α/β receptor that cause the activation of the JAK/STAT signal transduction pathway and an upregulation in the transcription of "IFN-stimulated genes" (Der et al., 1998; Rentsch and Zimmer, 2011).

IFN- γ is used to represent the type II interferons and IFN- λ is used to designate type III IFNs. Both cytokines possess antiviral activity by binding to different and distinct receptors. Type I and Type III IFNs produce similar activated antiviral genes via transcription. The site of action of Type III interferons is primarily in epithelial cells (Sommerey et al., 2008). The innate immune system protects epithelial tissues from viral infection through the action of Type III IFNs (Pott et al., 2011).

In the event of a viral attack on a human host, interferons are inducible cytokines that mediate the immune antiviral and anti-proliferative response against the virus (Burdick et al., 2009). Type I interferons act in an autocrine manner to terminate the replication of virus in infected cells. Type I interferons also act in a paracrine manner to block viral replication within the host by inducing an antiviral state in healthy uninfected cells. Type I interferons exhibit some effect on cell proliferation and differentiation, promotion of programmed cell death, modulation of the immune system and inhibition of angiogenesis as reported by Maher et al. (2007).

Type I IFNs express various immunomodulatory effects, which range from modulation of T cell response, expression of class I major histocompatibility complex (MHC) and an upregulation of the differentiation of dendritic cells (Rönblom and Alm, 2003). Type I interferons are inducible forms of cytokines that are principally involved in the immune protection against invading viruses and intracellular bacteria (Lamot et al., 2019).

Overstimulation or defective regulation of the expression of type I IFNs can lead to sustained type I interferon activity which has profound deleterious effects on the body (Gresser et al., 1980; Hunt et al., 2014). Aicardi-Goutières syndrome (AGS), a genetic auto-inflammatory condition, had the first hallmark feature of sustained type I IFN activity. It affects the central nervous system in a way that mimics neurological manifestations of congenital viral infection. Persistent activity of IFN I in AGS led to the discovery of a novel group of monogenic auto-inflammatory diseases collectively referred to as type I interferonopathies (Crow, 2011, 2015; Crow and Manel, 2015; Volpi et al., 2016).

Subsequently, dysfunctional activation of type I IFNs has been closely linked to the etiology of many systemic complex auto-inflammatory conditions including systemic lupus erythematosus (SLE), Sjögren's syndrome (SS) and rheumatoid arthritis (RE) among others (Batterham et al., 2003; Muskardin and Niewold, 2018; Lamot et al., 2019).

Recent evidence supports the assertion that type I IFNs are implicated in both the disease initiation and progression of systemic lupus erythematosus. This consensus was arrived at because numerous studies showed a positive association between elevated levels of IFNs and SLE disease severity (Von Wussow et al., 1988; Yee et al., 1990). A prior study reported a significant correlation between raised IFNs levels and SLE disease activity as 71% of participants with active disease had marked serum IFNs levels compared with 21% of participants with inactive disease showing raised serum IFNs levels (Hooks et al., 1979). Subsequent studies corroborated the link between raised serum IFNs levels and SLE disease activity (Friedman et al., 1982; Kim et al., 1987). A clinical observation of SLE patients by Bengtsson and his team provides evidence of a positive correlation between the serum IFN- α levels and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores which further suggests the intricate role of the type I IFNs in the pathogenesis of SLE (Bengtsson et al., 2000).

Data obtained from genetic studies show a clear polymorphism in SLE-linked kinase cascade signaling genes for type I IFNs and in IFN-regulated genes (IRGs) (Baechler et al., 2003; Bronson et al., 2012). Gene expression profiling studies have revealed an IFN signature, or a substantial upregulation of mRNA transcripts encoded by IRGs, in some SLE, RA and other auto-inflammatory disease patients (Baechler et al., 2003; Higgs et al., 2011).

Rönnblom and his team hypothesized that, dysregulation in the signaling mechanism of type I interferons may override the immune tolerance mechanism leading to a progressive production of autoantibodies (Bengtsson et al., 2000; Rönnblom and Alm, 2003).

In many autoimmune diseases which involve the production of autoantibodies, a significant proportion of patients show an elevated dominant type I interferon signature when analysis of their peripheral blood mononuclear cells (PBMCs) or serum is carried out. Such diseases include systemic lupus erythematosus (SLE), dermatomyositis, rheumatoid arthritis and multiple sclerosis (Crow, 2007; van der Pouw Kraan et al., 2007).

In many autoimmune diseases like systemic lupus erythematosus, patients with active disease show an over-expression of type I IFN inducible transcripts and increased levels of type I interferons. Analysis of whole blood samples or PBMC of such patients showed a dysregulated expression of inducible type I interferons (IFN) known as the Interferon (IFN) signature (Becker et al., 2013). An IFN signature has been implicated as a marker for SLE as IFN levels were increased in serum samples obtained from SLE patients, which showed a positive correlation with SLEDAI scores and clinical disease manifestations (Becker et al., 2013).

Before the advent of direct measurement of type I IFN- α in physiological samples, the relationship between increased type I IFN activity and various diseases was ascertained based on *in vitro* analysis, which measures the downstream effect of type I IFN activation. This involves analyses of antiviral and/or transcriptional activity caused by type I IFN activation. Concurrent use of these functional assays together with emerging assays that directly quantify type I IFNs in biological samples will revolutionize our approach towards understanding the vital role of type I IFNs in type I IFN-mediated diseases and their management (Rodero and Crow, 2016; Picard and Belot, 2017).

With this background, we reviewed the various laboratory assay procedures used to identify interferon signatures, which will aid in the study and diagnosis of these Type I interferonopathies like SLE and RA. In addition, we assessed the advantages and limitations of the various identification and quantification assays for interferons (IFNs).

Immunoassay of interferons – Direct assay

Enzyme expression assay

Immunobioassay can be used to quantify the amount of IFNs by detecting the expression of IFN-inducible proteins. Another method to quantify the amount of IFNs is by measuring directly the level of IFN-inducible proteins that possess inherent enzymatic activity. A wide range of these enzymes, which includes 2'-5'-oligoadenylate synthetase (2-5A synthetase), Protein kinase R, MxA GTPase among others, may form the basis of this bioassay (Meager, 2002). Uno and his team developed an assay for IFN- α by inducing and measuring the levels of 2-5A synthetase. This bioassay however showed weak sensitivity to IFN- β and IFN- γ (Uno et al., 1998).

Nonetheless, a specific bioassay exists for type II IFN that quantifies the levels of kynurenine to assess the amount of type II IFN present in a sample. Type II IFN specifically induces an enzyme, indoleamine 2, 3-dioxygenase (IDO). This enzyme is solely responsible for the conversion of L-tryptophan to N-formyl kynurenine (NFK). Hence, a specific biological assay was designed to quantify kynurenine, the product of NFK hydrolyzation, in an IFN- γ -induced human glioblastoma cell line, 2D9 (Däubener et al., 1994).

A limitation of the enzyme expression assay is that, it requires several manipulations in order for accurate quantification of the enzyme product (Meager, 2002).

Enzyme-linked immunosorbent assay (ELISA)

In the qualitative and quantitative analysis of soluble proteins such as interferons, Enzyme-Linked Immunosorbent Assay (ELISA) is the most widely employed method for the detection and quantification of such cytokines. Interferons are cytokines that have low concentration in biological samples and hence requires ELISA tests that are able to detect cytokines up to picomolar concentration levels. This immunoassay detects interferon levels in response to specific antigens in whole blood culture system. Sandwich enzyme immunoassay (EIA) utilizes two monoclonal antibodies to detect levels of IFNs in biological samples (Rothel et al., 1990).

Advantages of enzyme-linked immunosorbent assay

This approach of soluble protein assay is rapid and can be carried out within approximately 4 h. It comes in a ready-to-use kit and has a high throughput and hence many samples can be analyzed within the shortest possible time which makes it ideal for clinical detection and quantification of IFNs (Lamot et al., 2019).

Demerits of enzyme-linked immunosorbent assay

It has limited sensitivity and can only measure type I interferons to the picomolar-concentration, which is greater than the concentration of IFNs in physiological samples. It also has low specificity and hence a separate assay is needed to evaluate the concentration of each subtype of type I interferons due to the limited sensitivity in detecting individual type I IFNs (Dolen and Mathur, 1995; Jabs et al., 1999).

Immunoassays such as ELISA are mostly accurate and precise when used to measure IFN levels *in vitro* but lose such sensitivity when used to measure IFN levels in patient samples. This can be attributed to the presence of inhibitors such as heterophile antibodies, or soluble moieties of IFN- α/β interfering with sensitivity. The absolute amount of type I IFN determined using immunoassays like ELISA does not necessarily reflect the proportion of biologically active type I IFN in the sample (Vrolijk, 2005).

Single molecule array (SiMoA) digital ELISA

The SiMoA digital ELISA is a new technology that facilitates the detection and quantification of biological concentration of IFN- α at the attomolar level. This technology also allows for the identification and quantification of all 13 IFN- α in one assay (Lamot et al., 2019).

In this assay, single molecules of various IFN- α are captured with enzyme-linked autoantibodies produced by the patient and linked to paramagnetic beads, which are detected, as in an ELISA, with an enzyme-generated fluorescent product. The autoantibodies are tested for cross-reactivity with other subtypes of IFNs (Rissin et al., 2010; Yeung et al., 2016).

The SiMoA has been employed successfully to measure IFN- α protein in healthy individuals without viral infection and those with viral infections. It has also been utilized to measure the levels of IFN- α in various monogenic type I interferonopathies patients that show an IFN signature (Rodero et al., 2017).

The IFN- α protein concentration is measured using the SiMoA correlated well with functional type I IFN activity as determined by a cytopathic protection assay or qPCR detection of six Interferon-Stimulated Genes. SiMoA has been used to establish an associative relationship between high circulating levels of IFN- α and increased severity of the clinical presentations of SLE patients (Rodero et al., 2017).

Advantages of the SiMoA digital assay

The SiMoA has a very high sensitivity that allows the bioanalysis of IFNs at low abundance concentrations. It has been utilized to measure interferon concentrations at the attomolar level, which is precise enough to quantify all the various subtypes of IFN α in a single assay (Rodero and Crow, 2016).

Disadvantages of the SiMoA digital assay

There is increased cost and risk associated with the adoption and implementation of this new technology in routine clinical analysis. The SiMoA is also a single source technology and this may serve as an impediment to its extensive use for type I interferon detection across various clinical and laboratory settings (Chunyk et al., 2018).

Bioassay/functional assay of interferons – Indirect assay

One of the notable biological activity induced by interferons is their antiviral activity on which they were originally defined. The biological response to IFN is initiated when IFN binds to specific cell surface receptors and causes the activation of downstream cytoplasmic signal transduction pathways. The biological response is manifested after the expression of an array of 'IFN-inducible' genes (Sen and Ransohoff, 1993; Stark et al., 1998). Antiviral effect has been identified as one of the manifestations of expression of these 'IFN-induced genes.' Other biological activity induced by IFN includes proliferation and replication of body cells, modulation of cell differentiation, regulation of immune response and other functional cellular activities (Meager, 2002).

Different IFN-induced proteins have been identified which play vital roles in suppressing viral replication and propagation. 2-5A synthetase, an IFN-induced enzyme catalyzes the formation of an oligonucleotide, ppp(A2'p)nA(2-5A). This oligonucleotide is required to activate endonuclease RNase L, which subsequently damages viral mRNAs required for the replication of some RNA viruses like the Mengo virus (Lengyel, 1982; Rice et al., 1985; Samuel, 1987; Kumar et al., 1988; Zhou et al., 1993).

Another dsRNA-dependent antiviral protein kinase, PKR, is induced by type I interferons. It causes phosphorylation and inactivation of the peptide initiation factor, eIF2, required for the translation of messenger RNAs of viruses (Lengyel, 1982; Samuel, 1987). Replication of rhadoviruses and reoviruses like vesicular stomatitis virus (VSV) are specifically inhibited by the PKR antiviral mechanism (Gupta et al., 1982; Samuel, 1987).

Antiviral assays (AVA)

Historically, antiviral bioassays were the first identified biological assays designed to ascertain the relative potency and functional activity of various IFN preparations. There exist a diverse range of cell or viral systems and assay protocols utilized to prepare an antiviral assay. Some mammalian and human cell lines are ideal for the design of antiviral assay with high sensitivity (Table 1). It is worth noting that, not every cell line is suitable for AVA because some tumor cell lines possess a defective IFN response system. The defect may be linked to the absence of some effector molecules in the IFN-mediated signal transduction pathway (Meager, 2002). The mutant adenocarcinoma cell line lacks TYK2 whereas the melanoma cell lines lack STAT1, STAT2 and p48 making both cell lines unsuitable for antiviral assay (Pellegrini et al., 1989; Wong et al., 1997).

Table 1 Suitable mammalian cell lines and cytolytic viruses used in various antiviral assay protocols.

<i>Antiviral Assays</i>		
<i>Cell line</i>	<i>Virus</i>	<i>IFN</i>
Madin–Darby bovine kidney cell line (MDBK)	Vesicular Stomatitis Virus (VSV)	Human, bovine
Human lung carcinoma cell line	(A549) VSV or Encephalomyocarditis Virus (EMCV)	Human
Human amniotic cell line (WISH)	VSV, EMCV, Semliki Forest Virus (SFV) or Sindbis virus	Human
Human cervix carcinoma cell line (HeLa)	EMCV or VSV	Human
Human larynx carcinoma cell line (Hep2)	EMCV, VSV, or Mengovirus	Human
Human foreskin diploid fibroblast cell line	VSV, EMCV, or Mengovirus	Human
Human amnion-derived cell line (FL)	VSV, EMCV, Mengovirus, or Sindbis virus	Human
Human glioblastoma cell line (2D9)	EMCV	Human
Murine fibroblastic cell line (L929)	VSV or EMCV	Murine

VSV: Vesicular stomatitis virus; EMCV: Encephalomyocarditis virus; SFV: Semliki forest virus.

Adapted from Meager A (2002) Biological assays for interferons. *Journal of Immunological Methods* 261(1–2): 21–36.

In every antiviral assay, it is essential to measure the level of inhibitory effect of IFN on viral replication. The parameters to look out for include reduction of viral yield, reduction of viral cytopathic effect (CPE), a decrease in viral plaque formation and viral RNA synthesis as well as a decrease in the production of viral proteins (Meager, 2002).

Anthony Meager described two methods widely used in the quantification of IFN bioactivity. The first method involves measuring the inhibition of the cytolytic effect of the virus whereas the second method depends on the quantification of viral reporter proteins (Meager, 2002).

Cytopathic protection assay

This antiviral assay indirectly quantifies type I IFN concentration by measuring the reduced cytopathic effect or diminished viral growths in cell lines transfected with cytolytic viruses. For example, the concentration of patients' cerebrospinal fluid (CSF) or serum that offers 50% protection of Maldin-Darby bovine kidney cells transfected with vesicular stomatitis virus (VSV) can be utilized to quantify the amount of type I IFNs (Gresser et al., 1974; Lebon et al., 1988). It relies on the antiviral properties of type I IFNs to estimate the amount of IFNs in a solution by its ability to inhibit viral replication in a cell culture. A basic definition for 1 IFN unit is the quantity of cytokines needed to prevent a viral-induced cytopathic effect in 50% of cells in culture (Familletti et al., 1981; Rubinstein et al., 1981). The cytopathic protection assay was the first assay method used to characterize AGS, the first identified monogenic type I interferonopathy (Goutières et al., 1998).

A limitation of the cytopathic protection assay is its reliance on live viruses, which cause biohazard safety concerns due to risk of accidental infection. Also, it is time-consuming and takes a duration of more than 24 h to complete a single assay (Lamot et al., 2019).

Recombinant replicon assay

This novel type I IFN bioassay serves as a useful alternative to the cytopathic protection assay, which uses live viruses for the bioassay. It relies on a viral replicon- based bioassay that has the advantage of using disabled or inactivated propagation-incompetent virus like VSV replicon linked with two reporter proteins for the bioassay (Rentsch and Zimmer, 2011). Recombinant replicon assay is biosafe because unlike conventional antiviral assays, there is no need to institute appropriate biosafety hazard measures since the replicon virus employed is inactivated and non-infectious. RNA replicons replicate solely within the cytosol and produce no complementary DNA intermediates. As such, in instances of accidental infection, the risk of integration into the host chromosomal DNA is avoided as the replicon particles cannot produce infectious progeny (Rentsch and Zimmer, 2011).

Transcription-stimulating activity

Reporter gene assay

In contrast to conventional antiviral activity assays which rely on the functional cytoprotective effect of IFNs against cytolytic viruses, reported gene assay (RGA) quantifies type I IFNs based on their ability to upregulate several downstream effector genes called interferon-stimulated genes (Der et al., 1998). The IFN-responsive promoter of Mx gene is mostly utilized due to its sensitivity and its ability to express low background of various interferon stimulated genes (ISGs) (Holzinger et al., 2007). Proteins encoded by ISGs such as dsRNA-activated protein kinase mostly mediate the action of IFNs.

In the reporter gene assay, there is a measurement of the fluorescence intensity produced by viral-encoded reporter proteins such as the green fluorescent protein (GFP) during viral replication in a suitable cell culture medium (Grover et al., 2003; Voigt et al., 2013). In this modified bioassay, it is prudent to use viruses with slow lytic effects to provide adequate time to measure the fluorescent protein production without interfering with the viability of the cell line. In using this method, it is not a necessity to fix cells and multiple time points can be analyzed for individual bioassays. The indirect quantification of IFN-induced protection against the lytic virus is measured by the fluorescent intensity. This is obtained by calculating the ED₅₀, which is the amount of IFN that is able to cause a 50% reduction in fluorescence (Rius-Rocabert et al., 2019). This method may utilize green fluorescent protein (GFP)-producing viruses that allows the quantification of reduction in viral titers by counting GFP-positive cells, either manually or by the use of a flow cytometer (Kuri et al., 2010).

VSV*ΔG (Luc), a VSV recombinant replicon encoding the reporter protein firefly luciferase, allows the quantification of type I IFNs in a relatively shorter period of time (5 h) compared to conventional type I IFN assays assuming the suitable cell line is selected for the assay. Conventional antiviral assays like the CPE often take over 24 h to carry out a single assay as it relies on the inhibition of cytopathic effects. However, in bioassays utilizing the firefly luciferase reporter, the firefly reporter expression has high sensitivity and it can be easily detected long before any cytopathic effect becomes visible (Rentsch and Zimmer, 2011).

Other common forms of reporter Gene assay employs HeLa or Vero cells lines transfected with a luciferase gene and under the control of a type I IFN inducible promoter (Canosi et al., 1996; Seo et al., 2009). The luciferase reporter activity in transfected cell lines is influenced by the extent to which replication or transcription of the RNA replicon is inhibited. Therefore, lower luciferase levels should directly correspond to the higher degree of reduction in viral RNA replication by the type I interferon (Rentsch and Zimmer, 2011).

Recently developed cell lines like RAW-Blue ISG and B 16-Blue IFN α/β are designed to produce a soluble gene like embryonic alkaline phosphatase upon induction by type I IFN which can then be quantified by using multi-well plate spectrophotometers or luminometers. It is a useful alternative method but its use in clinical setting is limited due to lack of standardization and complexity (Rees and Lowy, 2018).

A major shared limitation of both the reporter gene assay and commercially available ELISA tests is their restriction to a specific host. This is evident because interferons bind to their receptors in a species-specific manner (Kuri et al., 2010). One other limitation of the RGA is that, it considers a unit of IFN as the amount of interferon which is able to protect 50% of a cell culture from the virus without any regards to the percentage of the cells that have not been infected or have been infected in its early stages by the virus and hence are producing very low viral reporter proteins compared to the ones infected by the virus in its later stages (Stark et al., 1998).

Protocol 1 – The Hil3 reporter assay

This protocol was adopted from an investigation by Dall'Era and her team, who investigated the correlation between type I interferons and the clinical signs and symptoms of SLE patients (Dall'Era et al., 2005).

In this reporter assay, a plasmid encoded with an IFN stimulated response element – luciferase (ISRE-Luc) and a neomycin resistance gene were transfected into a human cell line Hil3. The Hil3 were cultured in a 96 well plate and incubated overnight in Dulbecco's modified Eagle's medium. The growth media and blood samples from either normal or SLE patients were mixed and cultured for 18 h. A three-step serial dilution of IFN in triplicate at specified concentration was included on each plate. Addition of a lysis buffer to the luciferase substrate halted the reaction. The substrate solution was added to each well and read on Top Count for 10 min. The count per second (cps) at each interferon concentration were recorded and the interferon concentration (or cps) in each sample was estimated from the IFN titration curve using appropriate software with linear regression parameters (Dall'Era et al., 2005).

An advantage of the Hil3 reporter assay is that, it is able to identify and quantify multiple sub-types of type I interferons such as natural leucocyte IFN that contains multiple IFN- α subtypes and IFN- β . It has been established by Dall'Era and her team that various type I IFN moieties have the propensity to provoke a luciferase response (Dall'Era et al., 2005). A limitation of the Hil3 reporter assay is that it shows minimal luciferase response to IFN- γ with a faint traceable signal at even the highest concentration of IFN- γ utilized for test (Dall'Era et al., 2005).

Interferon stimulated genes (ISG) expression assay

The qPCR biomarker assay is a form of quantitative reverse transcription PCR employed to measure the IFN signature in patients with interferonopathies such as SLE (Morimoto et al., 2011). qPCR and the newly engineered NanoString technology have been widely employed to analyze differential expressions of subtypes of ISGs in whole blood and to a lesser degree in disease-related tissues (Rice et al., 2013, 2017; Kim et al., 2018).

Oligonucleotide array was first used to identify an 'IFN signature,' a set of inducible genes in SLE patients (Baechler et al., 2003; Bennett et al., 2003). The IFN signature has then been validated in other studies of SLE patients and in other autoimmune inflammatory diseases such as Rheumatoid arthritis (RA), dermatomyositis (DM) and Sjögren's syndrome (SS) (Higgs et al., 2011; Lübbbers et al., 2013; Bodewes et al., 2018). It was unsurprising that an IFN signature was identified in patients with AGS, the first identified type I interferonopathy (Rice et al., 2012). A type I 'Interferon score (IS)' was created from six IFN-stimulated genes, ISGs – (IFI27, IFI44L, IFT1, ISIG15, RSAD2 and SIGLEC1), that showed the highest differential expression in AGS patients

compared with healthy control subjects. The interferon score was calculated based on the relative intensity of expression of these ISGs. The IFN score can be used as single diagnostic or prognostic value for various IFN-induced interferonopathies (Rice et al., 2012, 2013).

This six-gene assay was subsequently utilized in a prospective case-control study to quantify type I interferon activity in 82 patients with mutations in a single gene with established association to AGS - (RNASEH2B, RNASEH2C, SAMHD1, ADAR and TREX1, RNASEH2A). The expression of six IFN-stimulated genes was quantified using qPCR, and the median fold change relative to the median of healthy patients was utilized to develop an interferon score for every individual patient (Rice et al., 2013). The interferon score was positive in 90% of the AGS patients. Positive or negative IFN score showed good correlation with patient's serum IFN activity measured using a viral cytopathic assay in 12 of the 14-paired samples. Neutralization assays carried out with anti-IFN- α and anti-IFN- β antibodies depicted that detectable anti-viral activity was primarily due to the action of IFN- α (Rice et al., 2013).

NanoString Technology allows for the quantification of larger subsets of ISGs, which are typically 28 genes. It does this at a faster pace with easily reproducible results. The principle behind this assay is that, it uses molecular 'barcodes' to identify and simultaneously count up to several hundred unique target gene sequences or mRNA transcripts in a single hybridization reaction. It utilizes complementary DNA synthetic oligos to directly bind, capture and tag mRNA for counting without the need for reverse transcription or amplification (Geiss et al., 2008; Kim et al., 2018). The 28 target genes were chosen from whole blood microarray expression profiles of two patients, one with CANDLE and the other with chronic hepatitis C following IFN-2 α therapy. This panel has then been validated in treatment-naïve patients with genetic predisposition to diseases mediated by type I IFN (CANDLE, $n = 11$; SAVI, $n = 7$) or IL-1 (Neonatal onset multisystem inflammatory disease, NOMID, $n = 16$) and healthy individuals ($n = 26$). Interferon scores calculated from the 28 target genes suggested a significant difference between CANDLE and SAVI compared with NOMID and healthy individuals. In the samples tested, a high correlation was observed between repeated measures with no variation in gender or age or diurnal variation (Kim et al., 2018; Lamot et al., 2019).

The NanoString nCounter gene expression system has some advantages over conventional qPCR. It is quicker, and has a higher throughput, which makes it an ideal assay for daily clinical assessment and quantification of IFN-induced gene expression in patients, especially those with defined interferonopathies (Kim et al., 2018). However, the ability of NanoString and qPCR to measure 6 ISGs offer similar results or outcomes in terms of analytical performance (Pescarmona et al., 2019).

Laurent and his team developed a two-score system, which considered the expression of two distinct sets of genes instead of the conventional single set of genes to calculate the interferon score. The IFN scores, designated as IFN-score-A and -B, with some genes overlapping were selected from 31 ISGs associated with SLE. The scores have been validated in a cohort of 279 patients with SLE, UCTD, RA and in 49 healthy controls. IFN-A score distinguished SLE patients from both RA and healthy individuals, whereas IFN-B score differentiated both SLE and RA from healthy individuals. This shows that, calculation of interferon scores based on two or more sets of genes showed higher disease specificity compared with interferon scores calculated based on a single assay (Chiche et al., 2014; Lamot et al., 2019).

One major limitation of the ISGs expression assay is that there is no consensus or standard ISGs subset for the calculation of interferon score (Lamot et al., 2019).

Interferon- γ assay for tuberculosis

One major hindrance to the management of tuberculosis worldwide is the diagnosis and treatment of latent tuberculosis. *Mycobacterium tuberculosis* bacilli infection produces no symptoms in individuals with latent tuberculosis. It is estimated that roughly one-third of the world's population is latently infected with tuberculosis and about 10% will get active disease over their lifetime. Individuals with latent tuberculosis serve as a source from which new active cases can arise from (Styblo, 1980; Jasmer et al., 2002; Tufariello et al., 2003).

Prior to the advent of newer diagnostic methods for detecting latent tuberculosis infection, the tuberculin skin test (TST) was routinely used to diagnosis symptom-free TB infection (Huebner and Schein, 1993; Goldstein et al., 2002). The principle behind the TST is to measure the level of cell-mediated immune response via the intensity of a delayed-type hypersensitivity response to purified protein derivatives (PPD). PPD is a pool of antigens from *M. tuberculosis*, *M. bovis*, Bacillus Calmette-Guérin (BCG) and several other non-tuberculosis mycobacteria (NTM) (Huebner and Schein, 1993; Andersen et al., 2000; Goldstein et al., 2002). TST therefore showed low specificity in NTM endemic populations and low sensitivity in immunocompromised patients (AIDS, malnutrition and advanced tuberculosis) (Huebner and Schein, 1993; Andersen et al., 2000; Winer et al., 2009).

Interferon- γ assay is a new *in vitro* T-cell- based assay that serves as the first alternative option to TST in diagnosing latent tuberculosis infection (Dockrell and Weir, 1998; Andersen et al., 2000; Lalvani, 2003). The principle behind the interferon- γ assay is that, the T cells of tuberculosis antigen sensitized-individuals produce interferon- γ when they become re-exposed to the mycobacteria antigen (Andersen et al., 2000). Marked elevation of interferon- γ levels upon re-exposure signifies the presence of tuberculosis infection. The initial research relied on PPD as the stimulating antigen, but current assay methods focus on *M. tuberculosis* specific antigens like the early secretory antigenic target 6 (ESAT 6) and culture filtrate protein 10 (CFP10) (Andersen et al., 2000; Mawa et al., 2004). The region of difference 1 (RD-1) of the *Mycobacterium tuberculosis* genome encodes for the production of these stimulating proteins which makes them more specific to *Mycobacterium tuberculosis* compared to PPD, as they are not found in any BCG strain or NTM species (Sørensen et al., 1995; Andersen et al., 2000).

The two most widely used commercial interferon- γ assays are the QuantiFERON-TB assay and the T SPOT-TB assay. Both tests indirectly measure cell-mediated immunity by measuring the amount of interferon- γ produced from T cells upon exposure to a tuberculin antigen using methods such as ELISA and enzyme-linked immunospot (ELISPOT) assay (Mazurek and Villarino, 2003).

Earlier versions of the QuantiFERON-TB assay utilized whole-blood assay that measured interferon- γ response upon stimulation with PPD using ELISA. The enhanced QuantiFERON-TB Gold assay utilizes ESAT 6 and CFP 10 as the stimulating antigens (Mazurek and Villarino, 2003). The T SPOT-TB assay uses peripheral blood mononuclear cells (PBMCs), ESAT 6 and CFP 10 as stimulating proteins, and ELISPOT to detect the number of T cells producing interferon- γ (Lalvani, 2003). Both assay methods use cellular immune-based tests to measure interferon- γ response with shorter incubation periods of within 24–48 h compared to 5–6 days in most non-commercial in-house assays (Lalvani, 2003).

Advantages of interferon γ assay

Interferon- γ assay has several advantages compared to the conventional tuberculin skin test (TST). Interferon- γ assay is an *in vitro* test, which makes it more objective because it does not require the measurement of skin induration, which gives rise to reader bias and error. The patient also requires just a single visit, as there is no follow-up visit like the tuberculin skin test. RD1-based interferon- γ assay method is more specific than TST which uses PPD as a stimulating antigen (Andersen et al., 2000; Lalvani, 2003). RD1-based interferon- γ assays has better correlation with exposure to *M. tuberculosis* and relatively minimal cross-reactivity to previous BCG vaccination and NTM infection (Pai et al., 2004). The use of a mixture of antigens for the interferon- γ assay enhances both the sensitivity and specificity of the assay. This is clinically relevant because sensitive assays for tuberculosis infection are favorable for immunocompromised patients who have a higher chance of getting a false-negative TST test and are at a greater risk of progression to active tuberculosis infection (Ravn et al., 2004; Richeldi et al., 2004). Some evidence suggest that interferon- γ assay can be used to potentially identify latent tuberculosis patients who have a greater susceptibility of developing active tuberculosis infections (Pai et al., 2004).

Disadvantages of interferon- γ assay

The impact of interferon- γ assay in monitoring treatment remains unclear, as current evidences show inconsistencies. This has partly been attributed to the variability in interferon- γ methods used. Interferon- γ assays are more expensive than the TST and hence, financial burden will be a crucial limitation in determining the global utilization of this newer and more efficient assay (Pai et al., 2004).

Recent developments and future perspectives

Concerning the use of interferon- γ assay for the diagnoses of tuberculosis, future research must focus on improving the sensitivity of RD1-based interferon- γ assays, without compromising its high specificity. Subsequent research must seek to find antigens that are more specific and/or a cocktail of antigens for the interferon- γ assay.

Again, a comparative study should be carried out to assess the sensitivity and accuracy of both the ELISPOT and ELISA formats. Research should be carried out to compare both sensitivity and specificity of the short (24–48 h) incubation assay and the long (5–6 days) incubation assay.

The application of the SiMoA technology in diagnosing and monitoring of interferon levels in patients with various interferonopathies and other diseases requires further clinical evaluation and validation in additional patient cohorts.

Conclusion

An Interferon signature, described as an increased expression of interferon-stimulated genes (ISGs), is peculiar characteristic of a group of autoimmune inflammatory conditions called Type I interferonopathies, which includes RA, SS and SLE among others. The assays for the detection of an interferon signature have undergone modification throughout the years in an attempt to produce highly sensitive and specific assays. Methods that are used to identify activated type I interferons can be grouped into direct measurement of type I IFN concentration through immunoassays such as ELISA and indirect measurement of IFN via its functional antiviral activity or induced gene expression. Interferon signature analysis can be used clinically via the determination of IFN score to diagnose or predict the disease prognosis of patients with various type I interferonopathies. There is a direct correlation between the Levels of type I IFN and disease severity. There is a need for further research to identify newer and more robust assay methods for detection and quantification of IFNs that can be employed in the clinical setting.

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HLA Methods

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Abbreviations

DNA	Deoxyribonucleic Acid
EFI	European Federation for Immunogenetics
ELISA	Enzyme-linked Immunosorbent Assay
HLA	Human Leukocyte Antigen
MHC	Major Histocompatibility Complex
PCR	Polymerase Chain Reaction

Introduction

The human leukocyte antigen (HLA) system is a highly polymorphic gene complex critical in regulation of the immune system. These genes encode the human version of the major histocompatibility complex (MHC) and produce the HLA antigens found on the surface of all leukocyte cell membranes in humans. These antigens serve as recognition molecules to distinguish between self and non-self-proteins and aid in presenting the foreign antigenic proteins to the effector cells of the immune system (the T-cell lymphocytes or T-cells), all in the initiation of the immune response.

The HLA family of genes comprise about over 3.6 million bases of DNA located on the short arm of chromosome (6p21.3) and is approximately 0.13% of the human genome, containing over 200 hundred identified gene loci (Hosomichi et al., 2015; Robinson et al., 2015). The HLA genes are inherited as one complete allele from each parent (the maternal and paternal haplotypes) to form the diploid HLA genotype, specific to that individual. This specificity is also due to the polymorphic nature of the HLA genes in which the variations or polymorphisms found in the genetic sequences encoding the regions of interaction or binding between the T-cells and the HLA molecules are important for recognition of non-self-proteins and their subsequent elimination (Mayor et al., 2015). It is thought that this heterozygous nature provides an evolutionary advantage by increasing the number of potential non-self-recognition molecules and increasing the likelihood for survival of the individual.

This essential role in regulating the immune response makes the HLA genes a primary factor in transplant outcomes. These molecules are, in large part, responsible for the success or failure/rejection of organ and tissue transplants between individuals. The closer a genetic match between donor and recipient, the greater the chance for a successful transplant outcome. In addition, HLA polymorphisms are known to be associated with specific disease states or an increased risk of pharmaceutical adverse effect (Male, 2001). As a result, accurate and detailed HLA typing is critical in achieving positive outcomes.

Classification

The HLA gene family is divided into three MHC groups known as Class I, Class II and Class III molecules based on the structure and function of their gene products. Class I and II contain the HLA genes whose products include the antigen-presenting molecules while Class III contains a number of other immune system molecules such as complement components, heat shock proteins and some cytokines (Male, 2001).

MHC class I

MHC Class I proteins are transmembrane proteins located on the surface of all nucleated cells and are formed by a glycosylated alpha (α) heavy chain bound to a beta-2 (β_2) microglobulin molecule (Male, 2001). The β_2 -microglobulin molecule is a heterodimer composed of major and minor gene subunits. The degree of glycosylation and the amino acid sequences of the polymorphic heavy chain varies with haplotype, creating the variations that generate numerous sequences to form the various alleles within each HLA family. The β_2 -microglobulin portion, in contrast, is not polymorphic but is required for proper expression of the Class I HLA molecules (Male, 2001).

Since every nucleated cell expresses the MHC Class I antigens, every cell has an antigen-presenting function. Besides interacting at the heavy chain peptide binding region with generalized T-cells, previous studies have shown that a certain class of antigen-presenting T-cells, the CD8-positive cytotoxic T-cells, react with and bind to the non-polymorphic part of the MHC Class I heavy chain (Robinson et al., 2015). Some additional MHC Class I genes such as HLA-G and HLA-E encode non-classical MHC molecules which have specialized functions such protecting the fetus from the maternal immune response during gestation and antigen-presentation to other immune system cells such as natural killer (NK) cells.

The MHC Class I proteins are divided into three major and three minor subclasses shown below:

- Major subclass: HLA-A; HLA-B; HLA-C.
- Minor subclass: HLA-E; HLA-F; HLA-G; HLA-H.

MHC class II

MHC Class II proteins are usually found only on the surface membranes of antigen presenting cells such as B-cells, macrophages, dendritic cells and Langerhans cells as well as T-cells (Madden and Chabot-Richards, 2019). These molecules are composed of two glycoprotein (heavy alpha (α) and light beta (β)) chains, forming heterodimeric ($\alpha\beta$) protein receptors on the surface membranes of the cells (Male, 2001). Similar to the Class I molecules, the alpha heavy chains are polymorphic and vary in degree of glycosylation and amino acid content while the beta light chains are not polymorphic (Male, 2001). Unlike the Class I molecules, Class II molecules do not initialize antigen presentation but bind to proteins that have already been processed.

MHC Class II proteins are divided into three major and two minor subclasses shown below:

- Major subclass:
 - HLA-DP (α -chain encoded by HLA-DPA1 locus and β -chain encoded by HLA-DPB1 locus).
 - HLA-DQ (α -chain encoded by HLA-DQA1 locus and β -chain encoded by HLA-DQB1 locus).
 - HLA-DR (α -chain encoded by HLA-DRA locus and 4 β -chains) (only 3 possible per individual) encoded by HLA-DRB1, DRB3, DRB4, DRB5 loci.
- Minor subclass: DM; DO.

MHC class III

The MHC class III molecules include complement components, tumor necrosis factor-alpha (TNF- α), lymphotoxin and some heat shock proteins that are important in the inflammatory response and are used for internal antigen processing and loading of the processed antigens onto the HLA molecules of antigen-presenting cells (Male, 2001). This class is not critical in identifying HLA genotype or in its matching and is, therefore, not typically part of any HLA typing test method.

Nomenclature

Historically, HLA genotype naming was antibody-based and determined by the serological identification of HLA antigens. These antigens were assigned letters and numbers (i.e., HLA-B27) to designate the HLA gene locus name. These older techniques could not distinguish further between the alleles and the designation stopped at this level. With the advent of DNA molecular techniques, a more refined definition of HLA genes became available and a standard for nomenclature was necessary. Currently, the standard convention for HLA typing nomenclature is regulated by the WHO Nomenclature Committee for Factors of the HLA System regulations as shown in the example below (Marsh et al., 2010).

Example of nomenclature using the HLA-A*02:02:01:02 gene:

- HLA-A: The HLA prefix and HLA gene name/locus.
- *Indicates that this information is based on molecular typing results.
- 02: The allele group at the HLA locus.
- 02: The specific HLA protein.
- 01: The polymorphisms in the coding regions.
- 02: The polymorphisms in the in non-coding regions.

Letter suffixes are added to the end of the gene name, if warranted, to designate the level of protein expression. The available suffixed are shown below:

- N: a null allele with no protein expression.
- L: alleles with low protein expression.
- S: alleles that express soluble proteins.
- Q: alleles with a questionable protein expression status.

Methods

General

HLA typing was initially conducted by serological phenotypic determination. This practice has largely been replaced by either solid-phase methods such as bead assays or DNA-based molecular techniques including real-time polymerase chain reaction (PCR) and sequencing-based methods. These HLA typing methods are also used in various other applications in vaccine development, analysis of disease associations and determination of therapeutic safety and efficacy.

One concept routinely used in the description of HLA typing methods is the resolution of the results which describes the extent and details of each HLA allele. This precision is used for to choose the method of typing since different applications have different resolution requirements. For example, solid organ transplantation requires low to intermediate typing resolution where bead assays would be sufficient while bone marrow transplantation requires a high-resolution typing method such as DNA sequencing techniques (Madden and Chabot-Richards, 2019).

A low-resolution or two-digit typing distinguishes between two HLA antigens and gives an HLA typing result similar to a serological result (for example, HLA-A*01) (Bai et al., 2014; Madden and Chabot-Richards, 2019). This level of resolution is usually adequate for solid organ transplantation, evaluations of certain disease associations and pharmacogenomic analyzes (Madden and Chabot-Richards, 2019). In spite of the routine use of this level of resolution, an inadequate match may lead to immunological responses in the recipient and a poor transplant outcome. In contrast, more recent use of high resolution HLA typing or four-digit typing (for example, HLA-A*02:01) has greatly improved transplantation outcomes. This level of resolution is based on genetic sequencing that identifies the sequence of the peptide binding region of the HLA allele (01 in the HLA allele name in the example above) as well as the number of alleles to be determined for a given locus and the HLA alleles present in the sample (Madden and Chabot-Richards, 2019). High resolution methods can also detect variations in amino acid or polymorphisms within the HLA genetic sequence which may affect disease status or the immune response (Bai et al., 2014). This level of resolution is also required for bone marrow transplantation and is used for disease association research and pharmacogenetic testing.

Serology (Serotyping)

This was the original HLA typing method and is based on the reaction between HLA receptors and diluted blood serum antibodies to identify the specific phenotype. The sample serum containing an individual's HLA antibodies was incubated with known HLA antigens in the presence of complement. A reaction would form an immune complex precipitate that is then detected by agglutination or cytotoxicity measures (Steward, 2001; Fasano et al., 2017; Madden and Chabot-Richards, 2019). A positive reaction would indicate binding between the antigen and antibody. The degree of the reaction would be determined for each unknown by comparing it to known controls to provide a quantitative value for each reaction. This allowed for identification of individual antigens or HLA sequences based on the serum antibody binding affinity.

A number of significant disadvantages were found with this technique. False positives may occur if HLA alleles contain additional genetic sequences that encode for non-HLA proteins. These proteins may then react with the antigens present in the test and give a false positive result. Additionally, the collection and preparation of the processing and handling of the assay constituents are strongly affected by technical proficiency and could also have a direct impact on the occurrence and extent of the agglutination and cytotoxic reactions (Fasano et al., 2017). As a result, the test results have been greatly dependent on technical expertise and experience for their interpretation. All these factors cause the serological methods to be highly subjective and has led to many inaccurate HLA typing results.

Of particular concern is that the antigens used for antibody development may not be the same between manufacturers or products. One specific source of variation is whether the original antibody is natural or recombinant in origin. A natural antibody is one derived from the blood serum of individual and, therefore, may vary in strength, composition and reactivity between

individuals or the same individual over time. A recombinant antibody is manufactured in the laboratory and is typically clonal in origin so its characteristics would be the same in any situation. Additional sources of variations are due to the amounts of antibodies used in each assay; generally, the more antibody used, the greater the reactivity.

Although molecular-based testing methods are favored in many cases due to their greater resolution and typing accuracy, serological methods have continued to be used. A significant advance occurred with the development of monoclonal antibodies which allowed greater consistency in the binding reactions. New serological antibodies for HLA typing continue to be developed and, once validated, are commercially available. However, in spite of these developments, the polymorphic nature of the HLA family increases the risk of misidentification of specific alleles if only serological testing is used. In many cases, once the serological testing is completed, a genetic sequencing or a prediction method such as familial haplotype analysis is used to confirm the initial results and to provide a greater resolution for the HLA genotype.

Cellular typing

Cellular typing or mixed lymphocyte culture (MLC) cellular typing (also known as microlymphocytotoxicity typing) is used in conjunction with serological testing for identification and confirmation of the HLA type. This method is also based on the reaction between the HLA antigens and specific antisera or monoclonal antibodies. Cellular typing is considered more sensitive than serological typing in identifying HLA differences resulting from T-cell stimulation that is not detected by the serological antibodies.

Although these MLC assays are more sensitive than serological methods, they are not considered very sensitive as compared to other HLA typing techniques such as solid-phase or sequencing tests. Similar to serologic testing, the MLC results are based on agglutination or cytotoxic reactions whose interpretation is not only labor-intensive but highly subjective (Fasano et al., 2017). In addition, this method requires a relatively large number of viable, previously HLA-typed lymphocytes in order to obtain HLA specificity in the results (Tait et al., 2013). Since the cell suspensions are collected from the serum of individual donors, they may also include non-HLA antibodies which can lead to false positive results during the HLA typing test. As a result, this method is difficult to standardize and the final result is dependent upon the HLA composition of the lymphocyte panel used in testing, leading to different results from various laboratories, each using its own lymphocyte panel. These assays also cannot identify all HLA types in highly sensitized patients due to the presence of numerous HLA and non-HLA antibodies. In spite of these disadvantages, this method is still in use in many laboratories, especially those involved in organ transplantation.

For organ transplantation purposes, MLC testing has previously been used to detect any reactions between recipient lymphocytes and potential donor antigens (Hutchinson, 2001; Fasano et al., 2017). This method requires that the recipient lymphocytes be collected in sufficient volume with confidence that only the lymphocytes have been isolated. Although this concept may provide the preferred result, the test requires 4–5 days before a result is available. This timeframe is not acceptable when using organs from brain-dead donors that must be transplanted within 24–48 h. This technique, however, can be used for living donors such as those for bone marrow transplantation.

Solid-phase methods

In contrast to serological and cellular assays, solid-phase methods or bead-based assays are highly sensitive techniques used for identification of both HLA-antibodies and MHC Class I and II HLA molecules. These methods are found as commercially available kits that contain solubilized HLA sequences bound to a solid matrix, either a microtiter plate (enzyme-linked immunosorbent assay or ELISA) or polystyrene microbeads with the HLA specificity listed by the manufacturer for each kit (Reed et al., 2013; Tait et al., 2013; Gu et al., 2020). In each method, the matrix is coated with native or recombinant Class I or Class II HLA antigens to which the sample serum is added and incubated; positive results indicate binding of HLA antibodies in the sample to the specific HLA antigen in the kit (Schinstock et al., 2016; Fasano et al., 2017).

ELISA technology was initially used for these assays with the solid matrix being a microtiter-well plate. The degree of HLA-specific antibody binding in each sample was measured via optical density analysis with the test ratios compared to a known control (Steward, 2001). The HLA type would be determined by evaluation of the pattern of positive reactions. Currently, this methodology uses polystyrene micro beads as the solid phase and are fluorescence-based. Detection of the fluorescent signal is accomplished using flow cytometry with a fluorescence-activated cell sorter (FACS) and the results are expressed as the mean fluorescence intensity (MFI), a value that represents the relative fluorescence of a specific bead or combination of beads. The beads are coated with the HLA molecules and conjugated with two fluorescent probes (usually fluorescein isothiocyanate (FITC) or phycoerythrin (PE)) (Reed et al., 2013). A third reagent, a fluorescent-labeled IgG antibody, is added to the mixture as a reporter molecule for detection of any binding reactions between the antigenic HLA molecules and the sample DNA.

A distinct advantage of the fluorescent-based bead assays is that this technique may be used in multiplex arrays. These multiplex systems allow the simultaneous detection of several different antibodies so that a more detailed typing analysis may be completed in one test system, making this methodology extremely common in HLA testing laboratories (Fasano et al., 2017). A widely used example of the multiplex bead assay uses the Luminex® platform where the beads, coated with the HLA sequences, are the bound with the two fluorescent dyes (the ratios depend on the manufacturer). This process can produce up to 100 different bead populations per assay (Tait et al., 2013; Schinstock et al., 2016). Using a dual-laser flow cytometer or fluoroanalyzer, the fluorescent signal of each bead is identified by one laser while the signal of the HLA-specific antibody binding is simultaneously determined by the second laser. The Luminex® assays have a high sensitivity and can detect low levels of HLA antibodies and separating the binding affinities of each reaction into low, intermediate and high levels.

Both the ELISA and bead assays on the Luminex® system provide a semiquantitative assessment of HLA-specific antibody binding which is based on the detection limits of the technique being used (Tait et al., 2013; Reed et al., 2013). In many cases, the MFI is reported as a numerical value that has been used as a determination of HLA antibody binding capacity for both HLA typing and comparison of results between laboratories (Sullivan et al., 2017). It should be noted that, although the Luminex® bead assays are used as semiquantitative tests, this method was approved for use by the Food and Drug Administration (FDA) as qualitative assays that would deliver only a positive or negative result (Schinstock et al., 2016).

Although the solid-phase methods have a greater sensitivity than serological or cell-based methods, both the ELISA and bead assays are subject to variations due to the equipment used, manufacturers or suppliers of the kits and reagents, sources of HLA antigens, reagents and cell-to-reagent ratios. All these factors make the solid-phase assays difficult to standardize and variable results are common between laboratories (Tait et al., 2013; Fasano et al., 2017; Reed et al., 2013). Additional variability is due to the numerous kits available that have differences in the density and characteristics of the HLA molecules bound to the beads as well as different amounts and ratios of reagents used in each kit (Reed et al., 2013). The solid-phase assays also require significant expertise in their use and interpretation (Tait et al., 2013; Schinstock et al., 2016; Fasano et al., 2017). Analysis of these assay results have shown that conduct of the study itself (i.e., pipetting, bead washing, vortex speeds, centrifuging, etc.) also have a significant impact on the MFI results (Kim et al., 2019). This variability may be minimized through strict adherence to test protocols and conditions along with the use of robotic sample-handling equipment (Tait et al., 2013; Gu et al., 2020). Overall, the variability between laboratories (i.e., personnel expertise differences, laboratory temperatures, reagent characteristics such as pH, etc.) may account for 50% or more differences in the results (Kim et al., 2019).

An additional source of variability is the need to establish a cut-off concentration for each fluorescent signal for determining what is positive or negative. This is done in accordance with the manufacturer instructions and previous laboratory evaluation when the test protocol is first established and/or when new equipment or kits are being implemented. Variations in the MFI cut-off concentrations between positive and negative results can lead to significant differences in HLA typing results between laboratories, resulting in either false positives or false negatives. These situations can result in either not using an otherwise compatible organ or having a poor transplant outcome with organ rejection.

Another source of variability specific to the Luminex® assays is known as the prozone effect that may lead to false negative results (Kim et al., 2019; Gu et al., 2020). This effect occurs mostly with DNA from patients with high levels of HLA antibodies, causing a low MFI signal that is undetected by the reporter fluorescent antibodies and producing a false negative result. The prozone effect is caused by the C1 complement component protein or other molecules including IgM antibodies, medically-administered immunoglobulins and naturally-occurring immune complexes. These proteins or molecules reduce the intensity of the fluorescence by interfering with binding of the serum HLA antibodies to the fluorescent probe coated on the bead surface or with the fluorescent signal of the detection agent itself (Schinstock et al., 2016; Gu et al., 2020). Treatment of the sample serum with ethylenediaminetetraacetic acid (EDTA) or dithiothreitol (DTT) or other techniques such as preheating or diluting the serum has been used to reduce the prozone effect but this is typically done on a case-by-case basis (Kim et al., 2019). EDTA is the primary treatment option and has been used due to its iron-chelating properties that inhibits formation and promotes dissociation of the C1 complement complex and other complement degradation products. Similar results were found with DTT treatment. Although recommended for reducing the prozone effects, EDTA or DTT treatment is not part of the standard protocol in conducting bead assays and, as a result, no verified standard method exists for this treatment and different protocols are used in different laboratories (Kim et al., 2019).

Molecular or genetic-based methods

The great diversity within the HLA system has resulted in the reported HLA types being considered ambiguous when tested by previous typing methods. For many applications, the less sensitive and low resolution phenotypic methods are being replaced by those based on genetics to determine the exact genotype and a more accurate HLA typing result. The genetic sequencing assays use a specific sequence to determine the antibody or antigen reactivity found in the binding regions sequences for HLA Class I genes (exons 2 and 3) and for HLA Class II genes (exon 2) (Fasano et al., 2017; Madden and Chabot-Richards, 2019). Many of these methods focus on identifying polymorphisms within the Class I HLA genes since variations in the protein binding and antigen-presenting region of the HLA allele can be of significant relevance for ensuring a proper identification is made. In many countries, molecular methods are required for both donors and recipients for solid organ transplantation (Madden and Chabot-Richards, 2019). This requirement is based on the need for the accurate donor and recipient typing to minimize the occurrence of HLA mismatch and reduce the risk of transplant rejection. Knowledge of the HLA genotypes also aids in monitoring for post-transplant complications. Many laboratories that employ sequence-based HLA typing have eliminated serological-based typing methods and use multiple molecular methods. Which has led to the use of molecular methods for sequencing HLA alleles for greater accuracy, to resolve uncertain results and to identify new HLA alleles (Fasano et al., 2017; Madden and Chabot-Richards, 2019).

This category of methodology uses polymerase chain reaction (PCR) to amplify the DNA of an individual to determine their HLA genotype. The genetic sequences of the PCR probes or primer pairs are known and are designed to hybridize with single HLA alleles or families of similar alleles, providing DNA amplification of the specific HLA genetic sequences. The reference sequences for all HLA genetic typing, regardless of method, are found in a publicly available, central and curated repository, the IPD-IMGT/HLA database, a module within the greater Immuno Polymorphism Database (IPD) that contains the locus-specific genetic sequences for known HLA genes (Robinson et al., 2015). This database of HLA genetic sequences is updated every 3 months with most recent

public information on all WHO-names HLA alleles (Robinson et al., 2015; Klasberg et al., 2019). Such a comprehensive database with the most current HLA sequences that is available and searchable is critical for successful outcomes in transplantation.

PCR sequence-specific oligonucleotide probe (PCR-SSO) method

The PCR-SSO method uses PCR primers with genetic sequences complementary to a limited number of specific HLA alleles and is typically used with a solid matrix such as polystyrene beads or a microwell plate are coated with the primers (Fasano et al., 2017; Madden and Chabot-Richards, 2019). The sample DNA is first amplified by PCR and the PCR products then are added to matrix coated with the SSO primers. Detection of binding to the primer sequences may be via colorimetric or fluorescent techniques; if fluorescent, the primers are conjugated with a fluorescent molecule for detection via flow cytometry, typically a biotin derivative (biotinylation). A flow cytometer or analyzer is used to visualize the pattern of the fluorescent reactions (the pattern of bound and unbound probes). This pattern is then compared to known patterns of published HLA genetic sequences to determine the HLA genotype.

A number of commercially available kits for SSO HLA typing are available with hundreds of probes for many HLA alleles, providing a greater resolution than prior tests although the SSO results are considered intermediate resolution. A distinct advantage is that SO typing may be used for single as well as large batch testing, all within a short timeframe. However, this methodology requires using at least 100 beads for each sample as reduction in the bead count may cause the results to be invalid. In addition, a significant difference in intensity of the fluorescent signals between the positive and negative controls must be established to ensure the validity of the assay with each laboratory having its own cut-off concentrations based on their experience and the kit manufacturer recommendations.

Although SSO technology has proven useful in HLA typing identification, this method has a number of disadvantages. One such concern is that this method frequently contains allelic ambiguities. These ambiguities are partially resolved by using the “HD procedure” which uses additional or specialty probes that can hybridize to single DNA polymorphisms. Polymorphisms can then be identified and their presence included in determination of an individual HLA genotype. Non-specific binding is also a concern and may be minimized through strict adherence to washing protocols during conduct of the test. SSO primers have a shorter nucleotide length which makes identification of HLA alleles outside the primer sequence unlikely. This is especially true for those alleles that are less common or poorly documented. Additional testing by a different methodology would be required to identify these types of alleles.

Polymerase chain reaction-based sequence-specific primers (PCR-SSP) method

The SSP method is similar to the SSO method in that SSP uses PCR primers specific for genetic sequences alleles in certain HLA alleles but are highly accurate and are frequently used to distinguish HLA genotypes in cases where other methods provided ambiguous results (Madden and Chabot-Richards, 2019). For SSP testing, the sample DNA is added to a commercially available tray containing the primers to obtain the final PCR products; the target sequence is only amplified if present (Fasano et al., 2017; Madden and Chabot-Richards, 2019). These molecules are then separated by size gel electrophoresis and stained with ethidium bromide or SYBR green for visualization. The resulting band patterns show the presence or absence of specific HLA allele amplification and provide identification of the HLA genotype.

This accuracy is obtained by using only the target primers as well as a greater number of PCR cycles. The increased number of cycles generates a larger amount of product which aids in visualization following the separation step and, ultimately, increases the precision of the HLA determination. One downside to the increased number of PCR cycles means that this method is not as easily adapted for simultaneous large-batch testing. SSP testing also has greater variability due to generation of products that cause nonspecific as well as lack of binding; both conditions result in ambiguous or erroneous results. Technical expertise is also required to have accurate evaluations since interpretation of the test materials and the final HLA genotype determination depends on the presence of the clear visibility of certain sized amplicons.

More recently, detection of the binding reactions is completed using real-time PCR with fluorescently-labeled SSP primers. Real-time PCR reduces sources of contamination as can be found with gel-based manipulation (Madden and Chabot-Richards, 2019). In addition, real-time PCR tests can be completed in the shortest amount of time, can be automated and is very good at distinguishing HLA alleles.

Notably, the SSP method may result in missing some HLA alleles or even whole loci due to the use of known HLA sequences for generation of the primers. Previously unknown HLA variants may not be included in the primer sequences so the typing results for individuals with these less common alleles may appear to be ambiguous. This circumstance is not as prevalent in populations that have been largely HLA-types such as in the United States, Europe and Japan. However, other areas such as in the developing world, SSP is not appropriate for HLA gene resolution since the HLA type for a large portion of the population is unknown.

Sequencing-based typing (SBT)

SBT provides the highest resolution and is considered the best method to identify definitive Class I and II HLA alleles (Fasano et al., 2017; Madden and Chabot-Richards, 2019). This method uses nucleic acid sequencing of the exact genetic sequence in the desired DNA region so it is possible to identify HLA specific areas or alleles. Using these methods, genetic regions may be analyzed that are not covered under routine HLA typing methods, thereby detecting previously unknown HLA alleles. Sequencing also helps to eliminate the need for additional testing or cross-referencing results from other methods for HLA identification (Mayor et al., 2015).

The initial step in all SBT methods is PCR amplification of the DNA sample to ensure an adequate amount for sequencing. The primers may be generic for the majority of HLA alleles of a certain locus or specific for a limited number of particular HLA alleles. The PCR product is then sequenced using either Sanger sequencing or next-generation sequencing (NGS).

Sanger sequencing

SBT methods originally used Sanger sequencing for analysis of the PCR product to determine its genetic sequence. Sanger sequencing is a chain termination method used to determine a single nucleotide sequence from the sample DNA (Madden and Chabot-Richards, 2019). Determination of the exact sequence is accomplished using selective incorporation of a dideoxynucleotide into the chain during PCR amplification to stop the reaction. Dideoxynucleotides are known as “chain-terminating nucleotides”; these nucleotides lack a 3′ hydroxyl group which inhibits DNA polymerase, thus ending the chain elongation during protein synthesis. The final product sequences were first visualized through incorporation of a fluorescent dye by the dideoxynucleotide but now HLA typing is completed using sequencer software for genetic sequence analysis and identification. The final sequence is compared to a library of HLA allele sequence data contained in the software. Sanger sequencing allows for high resolution (4-digit results) that can identify the entire sequence across HLA alleles and include polymorphisms.

Although a number of kits are commercially available for Class I and II HLA typing via Sanger sequencing, this technique is not very scalable and a limited number of samples can be processed at any one time (Madden and Chabot-Richards, 2019). Typically, one sample at a time is done. In addition, this method is time-consuming and there is a long turn-around time. In addition, Sanger sequencing may be affected by technical issues such as the intensity of the fluorescent signals of the primers in the kits or different interpretation of the genetic analysis by various individuals and/or software. This is especially true for PCR products in low quantities which may be confused with the background signal. In these cases, where this difference between the signals is considered too small for verification purposes, the Sanger sequencing typing analysis would be considered unreliable and must be repeated. Any ambiguous results would require additional HLA typing by either SSP or SSO methods.

Next-generation sequencing methods (NGS)

Continued development of SBT methods lead to the creation of what is known as next-generation sequencing (NGS) methods. NGS are parallel sequencing methods where separate sequencing of multiple strands of DNA within a sample take place and each strand is amplified separately as opposed to all together as with other sequencing technologies (Klasberg et al., 2019; Madden and Chabot-Richards, 2019). The result are longer PCR products or amplicons for each DNA strand. This helps to reduce or eliminate many of the ambiguities or unknowns associated with other sequencing methods. The final result is the complete characterization of a double-stranded HLA allele containing both parental contributions and highly accurate HLA typing.

Currently, a long-amplicon approach is used for NGS where the entire sequence for an HLA allele is conducted via fragmentation of the sequence into smaller sections which are then amplified and combined to form the entire gene. Previous sequencing methods generated short sequences (<3 kb) that required manual overlapping of multiple fragments to form the genetic sequence of an entire HLA gene (Mayor et al., 2015). This process may result in incorrect alignment of the genetic sections with an incorrect identification of the HLA allele. This would be especially true if polymorphisms or other variations in the sequences are present. The solution, as found in the NGS methods, is the generation of longer sequences that encompass the entire HLA gene (i.e., introns, exons, and upstream and downstream regions) to resolve the presence of polymorphisms (Hosomichi et al., 2015; Mayor et al., 2015). In this way, the entire HLA genetic region can be sequenced in one high-throughput and high-resolution reaction.

A number of NGS platforms are available that use different sequencing methods for HLA typing. In general, PCR is used to determine all or almost all sequences in an HLA allele in the sample DNA. This sequencing includes additional areas such as nearby intronic and untranslated regions that may contain relevant but unidentified polymorphisms (Liu et al. 1999). These sequences are then enzymatically fragmented into smaller sections and each individual section is sequenced. Since NGS methods generate sequences from each single strand of the sample DNA, the specific sequences of paternal and maternal alleles may also be identified. NGS-generated results also have significant reductions in the ambiguity associated with Sanger sequencing with more accurate HLA typing (Liu et al., 1999). The NGS process of separate DNA strand amplification with sequencing a longer PCR product has significantly minimized the ambiguity associated with Sanger sequencing. This reduction has been recently demonstrated where a 3.5-fold reduction was found in the HLA genotyping error rate using NGS methods as compared to Sanger sequencing (Klasberg et al., 2019).

The development of these methods was driven by the need to increase both the accuracy of HLA typing for matching and the length of the allelic sequences used in this process while reducing the testing cost per sample (Robinson et al., 2015). A major advantage of NGS is its ability to provide simultaneous testing of multiple samples (multiplex testing) in a high-throughput manner as well as multiplexing (Liu et al., 1999; Klasberg et al., 2019). These characteristics allow a more efficient operation which lowers the cost per sample for HLA typing. Initially, this method may have a long turn-around time due to lack of experience with the analysis and testing procedure and the required instrumentation and software purchases are expensive. However, once established, this method provides high resolution, highly reproducible and efficient HLA typing.

Recent development of NGS sequencing methods have tested using non-PCR based system known as targeted resequencing for HLA typing (Hosomichi et al., 2015). Targeted resequencing uses biotinylated DNA primers for specific HLA genes which are incubated with known HLA genetic sequences found in the DNA library. Any hybridized or bound primers are then collected using streptavidin magnetic beads and the binding reactions visualized with fluorescent detection. The pattern of binding reactions is

compared to known sequences to determine the HLA type. Previous studies have shown that this method can be automated and used for both larger genetic regions and a greater number of HLA genes as compared to PCR-based sequencing (Hosomichi et al., 2015).

The large amount and complexity of data generated by NGS methods can be a major disadvantage in their routine use for HLA typing. High-volume laboratories such as stem cell registries routinely use these sequencing methods for HLA typing. Smaller laboratories or those that do not perform HLA typing on a routine basis may find it difficult to replace their current methodology with NGS techniques. In all cases, NGS methods generate an enormous amount of complex data and the resulting analysis requires both significant computing resources and technical proficiency. As a result, these methods may not be in place in many facilities.

Conclusion

In theory, complete HLA matching would provide a successful transplant outcome. However, this concept is impossible in practice due to the extremely high diversity of the HLA allelic family. The MHC Class I and II molecules are the most immunogenic antigens that are recognized during rejection of an allogeneic transplant. The strongest determinant of outcome is the HLA-DR status, followed by the HLA-A and -B status. Polymorphisms are known to occur in all the “original” HLA genes: HLA-A, -B and -C (Class I) and HLA-DRB1, -DQB1 and -DPB1 (Class II) (Mayor et al., 2015). Previous studies have shown that favorable organ survival occurred when the HLA Class II antigens, especially the DR antigens, are matched since the DR group is known to activate the recipient T-helper lymphocyte response (Hutchinson, 2001).

Regardless of what method is used, the “gold standard” for HLA matching is either an 8/8 or 10/10 HLA allelic match (i.e., HLA-A, -B, -C, -DRB1, and -DQB1 matched) (Lee et al., 2007). Any type of testing must identify all HLA alleles present in the sample where the method is more broad-based as with the serological techniques or more specific as with the DNA-based methodologies. The overall goal is to have HLA typing results that are accurate and reliable regardless of the method or laboratory used with the greatest amount of resolution and specificity.

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Blood Group Serotyping and Genotyping

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Introduction

A blood type named (Blood group) is a classification of blood type depended on the availability of inherited antigenic that form from proteins, carbohydrate, glycoprotein or glycolipid, based on the blood group system on the surface of individual red blood cells (RBCs). ABO is very important in transfusion medicine. It is the only system in which the mutual immunoglobulins (antibodies) are impartially present in the sera of normal persons. In case of transfusion of ABO-incompatible blood, these immunoglobulins lead to acute hemolysis and its reaction.

O and A groups are mostly present in population globally. The plasma used in intravenous immunoglobulin induction comes from donors who have these blood O and A groups, while B and AB groups donors are fewer. ABO antibodies are clinically very important, thus, cross-matching is necessarily made at the blood bank to ensure that blood of the correct group and subgroups are transfused into recipients to prevent any major mismatch or major incompatibility that can lead to serious morbidity and mostly death (Branch, 2015). Each group has its immunoglobulins in the plasma. The associated anti-A and anti-B antibodies are usually IgM antibodies (Capolunghi et al., 2013), produced in the first years of life by sensitization to environmental substances such as food, bacteria, and viruses, namely, the I^A allele gives type A, I^B gives type B, and i gives type O. As both I^A and I^B are dominant over i , only ii people have type O blood (Yazer et al., 2006). ABO blood types are also present in other primates. RBC's surface antigens are produced by one group of carefully connected genes called allele, which jointly composes the blood group system (Landsteiner, 1900). Additional subgroups are also found, the essential subgroups of A are A1 and A2. About 80% of group A or group AB people own RBCs which agglutinates with anti-A1, so they are typed as A1 or A1B, while the remaining 20% their RBCs agglutinates with anti-A but not with anti-A1 are classified as A2 or A2B. Anti-A1 occurs as an alloantibody of 1–8% of A2 individuals' serum and 22–35% of A2B individuals (Beattie, 1972). Anti-A1 may lead to inconsistency in ABO tests and genetic incompatibility and does not match with A1 or A1B antigens on RBCs. Antigen A subgroups are more common than antigen B subgroups. The serotyping of RBCs blood groups is an immunological-based concept and foundation of inheritance. In 2019, Krog et al. designed 35 competitive allele-specific polymerase chain reaction (PCR) assays for genotyping as a molecular test of blood donors. The phenotypes have been compared with 16,119 serologic phenotypes and reported 62 contradictions, in which 43 were a result of serology. The accuracy of genotyping increased to 99.9% when contradictions caused by serology were excluded. Three of the 17 contradictions that arise as a result of genotype are due to incorrect antigen-negative prediction. Genotyping is a more powerful and highly sensitive blood group determination method than a single serotyping test and also more cost-efficient than previous technologies (Krog et al., 2019). If the serological test was done only for blood-group typing, there is a high risk caused by misjudgment of the

blood group and ABO-mismatching during transplantation. Enhancement of accurate detection of ABO genotyping is critical for successful transplantation of organs particularly kidney (Zhou et al., 2020). In the following sections, other uncommon blood groups such as the MNS blood group system, Lutheran antigens, Kell blood group system and Duffy blood group system, as well as serological and genotypic determination methods will be discussed.

Common serotyping for RBCs ABO group

It is an old and classic blood groups typing. The use of reagent A1 and B RBCs are used to reveal anti-A and anti-B in serum is named serum or the reverse test as routine blood group detection on donors and recipient must include both blood RBCs and serum tests (Code of federal regulations, 1992). Test of ABO on RBCs, or blood of infants smaller than 4 months old only is allowable to confirm the ABO type of donor units that have already been classified. The test of ABO grouping can be applied on tube, slide, gel cassettes/ cards or microplate. Some ABO RBC grouping reagents are composed from pools of sera from group of persons who their A or B blood group antigen are stimulated to form antibodies with high titer. While other ABO typing reagents are prepared by monoclonal antibodies produced by cell lines culture. Both types of these reagents are able to agglutinate directly once contacting most antigen-positive RBCs, with or without centrifugation. There is a very weak agglutination by Anti-A and anti-B in the serum with RBCs of most recipients and donors without centrifugation, so tube, column agglutination technique or microplate techniques are suitable for reaction while slide is not recommended.

Rh blood group

First studied in the Rhesus monkeys. Unlike the ABO antigens, these non-glycosylated proteins are only found on RBC membrane (Anstee and Tanner, 1993) and not in other tissues. There have been 49 Rh antigens identified (Agre and Cartron, 1991), among which are the antigens C, c, D, E, e which are lipoprotein in nature. The autosomal dominant character is in chromosome 1. One gene code for expression of RhD and another gene codes for expression of CcEe (McCullough, 2003). The most important of these is antigen D which has the highest antigenicity (Thakral et al., 2010). Accordingly, there are two types of Rhesus groups:

Rh +ve in which antigen D is present on RBC surface
Rh -ve in which antigen D is absent from RBC surface

The function of Rh is proposed to be a transporter of ammonium across RBC membrane and maintenance of RBC membrane integrity (Avent and Reid, 2000). Unlike the anti-A and anti-B antibodies, antibodies of the Rh system (anti-D antibodies) are of the IgG type (Calhoun and Petz, 2001) which is the smallest type that can cross the placenta from maternal side to fetal blood. Also, they are not naturally acquired after birth as in the case of ABO system. They developed only in Rh -ve subjects and never in Rh +ve subjects. Causes of development in Rh -ve subjects include:

- Blood transfusion of Rh +ve blood to Rh -ve subjects
- Delivery of Rh +ve baby a Rh -ve mother in which during labor the placenta separates from the uterus resulting in bleeding and entrance of some fetal blood in maternal circulation.

Concerning the donors, Rh +ve subjects do not have anti-D antibodies. They can receive blood from Rh +ve and Rh -ve subjects. Rh -ve subjects do not have anti-D antibodies unless they receive Rh +ve blood. Therefore, they should receive Rh -ve blood only. During emergencies, Rh -ve blood is not always available, that is why Rh -ve subjects may receive Rh +ve blood for the first time. This induces formation of anti-D antibodies in his plasma within 3 days after the transfusion. Therefore, any future transfusion of Rh +ve blood is contra-indicated because it will precipitate severe reaction (Flegel, 2007).

The frequency of Rh -ve phenotype differs significantly between populations and geographical and ethnical locations. It is 29% in Saudi Arabia (Eweidah et al., 2011), 15–17% in USA and Europe (Frances, 2002; Garratty et al., 2004), 6% in Africa (Olugbemi et al., 2013), and less than 1% in China (Shao et al., 2002), Japan (Ikin and Mourant, 1962), and rarer in other parts of Asia (Makroo et al., 2014). It is worthy of mentioning that platelets also express ABO group antigens and could be collected in serum. Platelet carries fragments of RBC which includes Rh, so platelets can express RhD blood group (Dunbar, 2020). Fig. 1 shows all types of blood group.

Other blood groups

There are about 36 blood group systems with more than 300 known variants. These are all classified by the “antigens” found on the surface of the red blood cells. Some include the ABO and Rh systems, and some do not meet the International Society of Blood Transfusion definition of human blood group system like Knops, Er and Vel blood systems (Harmening, 2012). Among those that considered to be of significant importance are:

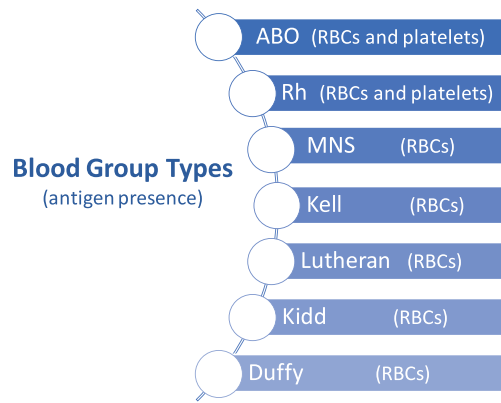


Fig. 1 Blood group types classified by antigens on RBCs and platelets. ABO (classic blood group typing used to reveal antigen-A and antigen-B), Rh (studied first in the Rhesus monkey, 49 Rh antigens have been identified, antigen D is the most important, MNS (Antigens M, N and S are the most common in this group), Kell (K, k antigens), Lutheran (Lutheran glycoproteins act as erythrocyte receptors, Kidd (also known as JK antigen), Duffy (six known Fya antigens)).

The MNS blood group system

MNS blood group “M, N and S antigens are loaded on glycoporphins on the membrane of RBC membrane”. One end of a glycoporphin is connected to the underlying membrane of RBC, while the second end endures the sugars and defines type of an individual’s MNS blood. Antibody type of MNS group are IgG and IgM (Dean and Dean, 2005) Frequency of MNS antigens and phenotypes are mainly in Caucasians and Blacks. The MNS blood group system is second only to the Rh blood group system in its complexity. Many alloantibodies to antigens in the MNS system are not generally clinically significant although antibodies to low-prevalence and high-prevalence MNS antigens have caused hemolytic disease of the fetus and newborn (Palacajornsuk, 2006).

The Kell blood group system

This is complex group and contains many antigens that are highly immunogenic. These antigens are the third most potent, after those of the ABO and Rh blood groups, at triggering an immune reaction. Antibodies that target Kell antigens can cause transfusion reactions and hemolytic disease of the newborn (HDN). The k antigen is more common than the K antigen in most populations, the K-k+ phenotype is found in 98% of Blacks and 91% of Caucasians (Reid and Lomas-Francis, 2004).

The Lutheran antigens

These are recently characterized glycoproteins in which the extracellular region contains five immunoglobulins like domains, suggesting some recognition function. It has been suggested that the Lutheran glycoproteins (Lu gps) act as erythrocyte receptors for soluble laminin (El Nemer et al., 1998).

The Kidd blood group system

This system is a glycoprotein that has been recognized as clinically important in RBC serology since its identification in 1951. The JK protein is expressed on RBCs and in the endothelium of the descending vasa recta and epithelial surfaces of the kidney inner medulla (Xu et al., 1997). In renal function, this transporter protein is important in regulating urea as part of the mechanism for urine concentration and water conservation (Sands et al., 1997). The erythrocyte JK glycoprotein serves to facilitate rapid urea transport across the RBC membrane (Macey and Yousef, 1988).

Discoveries have expanded the system to include 23 variant alleles recognized by the International Society of Blood Transfusion that silence the protein expression and 7 variant alleles presumably producing weak or partial JK antigens (Hamilton, 2015). A study conducted in 2019, on Kidd blood group found that among 30 blood samples, 4 were determined serologically as Jk(a+(w)) and genotypically determined as heterozygous for the JK*01W.01 allele. While the other 26 sample have been revealed to be Jk(null) with JK*02N.01 as the most frequent allele. JK*B weak alleles were not revealed, but 3 were detected as fake positives in the tube method. While Gel card resulted in high accuracy for Jk(b) determination but couldn’t reach regular detection for weak Jk(a). So, for better method, combining both the tube and gel card methods in serology, in addition to genetic testing, this excluded the misinterpreting weak Jk(a) expression (Wu et al., 2019).

The Duffy blood group system

The system ISBT number 008/symbol (FY) was published for the first time in 1950 when anti-Fya was identified in a suspected hemolytic transfusion reaction in hemophilic patient who received RBCs for treatment of spontaneous bleeding. There are six known antigens with four main phenotypes; Fy(a+b+), Fy(a-b+), Fy(a+b-), and Fy(a-b-) (Pogo and Chaudhuri, 2000).

Blood group genotyping

RBC antigen phenotyping is an essential component of transfusion compatibility testing. Serology has been the gold standard method, but its low productivity and chance of the risk of diagnostic intervention in specific cases shorten its utilization. Genotyping is helpful for phenotyping in those cases, revealing high results and credible alternate to serology test alone. Genotyping is specified in variable hematology and oncology patient populations. Since genotyping needs a compound testing area and gives more risk of genotype-phenotype contradiction, it is presently limited, but it acts as a helpful supplement and may finally succeed serological technique as a new gold standard. Genomics influences all medicine's fields, for transfusion medicine, DNA-based genotyping is applied as a substitutional for serological antibody-based assays to detect blood groups to match the blood from donor to recipient. Much of antigenic polymorphisms as a result of single nucleotide polymorphism variation in the related genes, and arrays of DNA target the changes validated by in comparison with serotyping (antibody-dependent typing). Testing antigens that have no serological kits is a great medical achievement to detect antibodies and get appropriate donor units to save the life (Westhoff, 2019).

Transfusion medicine genomics

The feasibility of applying next-generation sequencing (NGS) for blood group prognosis is validated by three featured methods: exome sequencing, whole genome sequencing, and PCR-based targeted NGS approaches (Fig. 2). The correlation of NGS with serologic and genotyping detection was from 92% to 99%. NGS showed better detection of weak antigens, structural variation, copy number alterations, new genomic variants, and microchimerism. Transfusions of blood is a common inpatient process which suggest blood group's genomics a favorable cluster of rigor medical research in addition to requirements to foster transfusion bioinformatic defies and evaluate its clinical use (Montemayor et al., 2019).

Zhou et al. (2020) studied hundreds of uremic patients with grouping contradiction, have weak ABO subgroup alleles and 77.48% have atypical ABO antibodies. The contradiction average comparing between serotyping and genotyping was more than 40%, and the mismatching ratio of election of donor based on serotype was up to 88.74%. More than 2.5% of 356 the same patients of A blood group were estimated to be included in the weak A subgroup. In comparison with the healthy Chinese people, it was (0.53%) that is higher by serological test, but very lower than what determined in Caucasians (20%).

Importance of both techniques in transfusion medicine

The molecular antigenic base of the blood groups had been specified first in the period of 1980s–90s; it is important in medical transfusion. Molecular genotyping of blood group antigens is one of the main angles and is prosperity revealing its technique into transfusion medicine. To achieve the best way, many low to high technical methods have been improved. Based on the need of the center such as checking for level of prevalence antigens wherever antiserum is not available, emend typing of several transfused patients, examination of antigen-negative donor units to decrease the ratio of alloimmunization, etc., a good method can be selected. There is a requirement to understand this blood technology with its variances (Gorakshakar et al., 2017).

It is known about 300 RBC's antigens in addition to 33 antigens on the platelet that express difference between humans. Immune reaction against antigens is a risk complexity such as what happens in prenatal medicine and blood transfusion, especially in patients who require several transfusions such as thalassemia. Although pre-transfusion compatibility examination widely depends on serological assays, but kits are not obtainable to detect several antigens. So, assays depend on single-nucleotide polymorphism (SNP) arrays were applied but typing for ABO and Rh – the very important blood groups could not be used with SNP typing alone. So, Lane et al. (2018) developed a new assay depends on whole-genome sequencing to detect whole RBC and platelet antigens. The improved blood typing compared typing data with classic serological determination and SNP typing in three rounds of testing. It revealed the latest algorithm, which was about 99% concurrent for 200 term genomes. Applying additional and

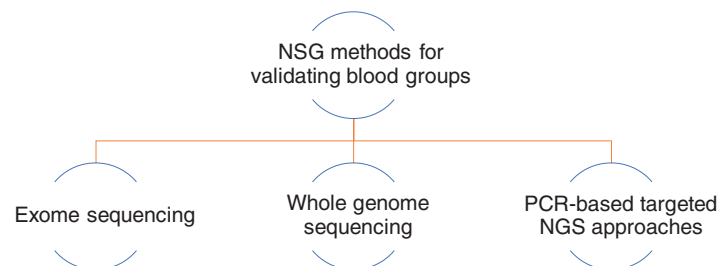


Fig. 2 Next-generation sequencing (NGS) for blood group prognosis is validated by three featured methods

accurate antigen-matching in patients with donor's blood, antigen typing depending on whole-genome sequencing is a new method for improving transfusion results with potential transforming the pursuit of transfusion medicine.

In most of thalassemic patients, a multitransfused is common process where hemagglutination can't phenotype the blood group antigens of the patient because of the existence of donor-obtained red blood cells. DNA-based on technique can cope the restriction of hemagglutination and can be applied to define the proper antigen description of those patients which ensure antigen-matched blood for transferring to multitransfused patients, Kulkarni et al. (2018) compared the serological phenotyping with molecular genotyping of known and clinically significant antigens of Rh, Duffy, Kell, Kidd and MNS blood group systems among multi-transfused patients of thalassemia. The authors tested 200 thalassemic patients and 100 "O" group health blood donors by using regular serological technical method and polymerase chain reaction-based techniques for these antigens/alleles (C, c, D, E, e, Fya, Fyb, Jka, Jkb, K, k, M, N, S, s). They reported that genotyping and phenotyping data were inconsistent in 77% of the used patients for five pairs of paradoxical antigens of the blood group systems (Rh, Duffy, Kell and Kidd). While in the MNS blood group system, 59% of patients displayed contradiction. The ratio of alloimmunization in thalassemic was 7.5%. They concluded that molecular genotyping supported the definition of the real antigen features in multitransfused thalassemic patients which would reduce alloimmunization in those patients and helps in the better management of transfusion therapy.

High risk of blood type mismatching and genetically ABO-misidentified transplantation if serological test was carried out only in blood-group typing. Very accurate ABO genotyping is important for successful organ such as kidney transplantation as allocation of reasonable organ may lead to early graft dysfunction (Zhou et al., 2020).

Combination of both the tube technique with gel card technique in blood groups serology, along with complementary genetic assay, the probability of misinterpreting weak Jk expression has been eradicated, and the authors provided Jk null blood for securing medical transfusion (Wu et al., 2019).

ABO blood groups compatibility in human pregnant and her embryo is very critical to prevent morbidity or mortality of the fetus or new-born which are important albeit it rare but risk. When a pregnant woman has a high level of anti-A or anti-B IgG antibodies, the child may be at risk for hemolytic disease of the fetus and newborn (HDFN). Performing a direct prenatal detection of the embryonic ABO blood group can tool up good clinical data, Rieneck and his team in his recent study (2020) have created a next-generation sequencing (NGS)-based fetal ABO prognosis determination depends on a cell-free DNA analysis extracted from maternal plasma and showed its application in several samples. They can allocate a reliable fetal ABO type in about 95% of the incoming clinical samples of type O pregnant. In despite of the large genetic alterations implied in the ABO blood groups, but there are rare variants, and prenatal ABO rare and pronging is possible and provides valuable precocious notification to avoid ABO's HDFN.

Complication and risks of incompatibility of blood transfusion

The ABO reactions

Complications in ABO system occurs at 1:25,000 transfusions, mostly, due to administrative errors (Linden et al., 2000). Since 44–45% of population is of blood group O and A, it is expected that mismatch will occur due to transfusion of type A blood to type O, because type O individuals carry both anti-A and anti-B.

Acute hemolytic transfusion reactions occur when ABO-incompatible blood is transfused, resulting in recipient antibodies attaching to donor RBC antigens and forming an antigen–antibody complex. This antigen–antibody complex activates complement system, resulting in intravascular RBC lysis with release of RBC stroma and free Hb (Sazama, 1990). Immune system activation also results in bradykinin release (leading to hypotension) and mast cell activation (causing serotonin and histamine release). The net result may be a shock, renal failure due to Hb precipitation in renal tubules, and DIC (Reid and Lomas-Francis, 2003). Many signs and symptoms of an acute hemolytic transfusion reaction appear immediately and include fever, chest pain, anxiety, back pain, and dyspnea. Many are masked by general anesthesia, but clues to the diagnosis include fever, hypotension, hemoglobinuria, unexplained bleeding, or failure of HCT to increase after transfusion (Reid and Lomas-Francis, 2003). The sequence of reaction could be as follows:

Agglutination of RBCs, followed by hemolysis

In mild cases, an antigen–antibody complex forms, the main antibody in this reaction is IgM. Agglutination of RBCs may be complicated by post-transfusion jaundice. In severe reaction, a few ml of blood is sufficient to cause severe pain in the back or in the sites and tightness in the chest (due to block of capillaries by the agglutinated RBCs). This is also followed by hemolysis of the RBCs and release of hemoglobin in plasma.

Acute renal failure

Some of the intravascular hemoglobin is filtered at the glomeruli and excreted in urine (hemoglobinuria). Some precipitates in the renal tubule resulting, in addition to the low blood pressure, in acute renal failure. Acute renal failure is characterized by low urine output, high urea and high potassium ions. Death may occur in few days.

Disseminated intravascular coagulation (DIC)

In severe cases, antigen–antibody reactions may result in activation of coagulation causing DIC and therefore, fatal bleeding due consumption of the clotting factors.

The Rh reactions

Hemolytic disease of the newborn:

- Also known as erythroblastosis fetalis because the early stage of RBCs (erythroblasts) appears in fetal blood together with reticulocytes as compensation to the excessive hemolysis (Seriki, 2020).
- It occurs in Rh +ve neonate of Rh -ve mothers.
- During delivery of the first Rh +ve neonate, some of his blood enters maternal circulation. The delivered neonate is normal: but the mother forms anti-D circulation, antibodies of IgG type to get rid of the baby's RBCs (i.e., she becomes immunized)
- When she becomes pregnant again with a Rh +ve fetus, these antibodies cross the placenta, enter the fetal circulation and attack RBCs of the fetus who develops hemolytic anemia and jaundice (McCullough, 2003)
- The hemolytic anemia is associated with hyperactive bone marrow to replace the lost RBCs. The results in appearance of the early stages of RBCs in fetal blood (erythroblastosis & reticulocytotic).
- Anemia may become complicated by heart failure associated with generalized edema (hydrops fetalis) and the fetus may die in uterus.
- The jaundice is caused by unconjugated bilirubin which is insoluble in water. It may precipitate in the basal ganglia in the brain causing kernicterus (characterized by motor problems and may be associated with mental retardation) (Avent and Reid, 2000).

Conclusion

DNA-based genotyping allows the accurate prediction of multiple erythrocyte and platelet antigens by evaluating the presence of known single nucleotide polymorphisms (SNPs) and other variations. The serological methods present same limitations especially in chronically transfused patients. For example, the presence of circulating transfused RBCs/platelets with a positive direct antiglobulin test (DAT) and alloantibodies. Moreover, serological methods alone fail to identify accurately suspected variants, weak expression or null phenotype of antigens or discrepancies in blood group determination. DNA-based methods allow extensive and unbiased analysis and the identification of antigens and allelic variants. Until now, SNPs with clinical significance beyond those of the ABO and RhD systems, have been manually tested by PCR with specific oligonucleotides or using restriction enzymes or robotically by microarrays. Although these methods are robust, but being replaced by high-throughput Next-Generation Sequencing (NGS) applications. These genotyping methods decrease the risk for erroneous of ABO prediction which is of great importance in transfusion medicine.

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Acute Phase Proteins

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Introduction

Inflammation is a complex defense mechanism necessary against biological, chemical, or physical threats such as infections caused by many types of microorganism, mechanical trauma, burns, ischemic necrosis, or neoplastic formations. Inflammation also plays a role in the healing process. The initiation and regulation of interactions between various components in the inflammatory process is carried out by a number of cellular and molecular events (Germolec et al., 2018; Ebersole and Cappelli, 2000).

Soluble mediators especially acute phase proteins (APPs), cytokines, and chemokines facilitate the migration of immune system cells to the injury area in an elaborately regulated chain of incidents. Acute phase changes arise immediately after beginning of infectious or destructive process. These changes are ultimately aimed to reintegrate homeostasis and removing the causative agent. These acute phase changes often sufficiently resolve the damage depending on the degree and extent of the injury (Ebersole and Cappelli, 2000; Gabay and Kushner, 1999). In contrast with the specificity of acquired immunity with humoral and cellular components, the acute phase response is not specific to identify the injurious cause (Ebersole and Cappelli, 2000).

Actually the acute phase response includes all the systemic effects of inflammation being primarily fever, hormonal changes, fluid and electrolyte imbalance, and typical alterations in protein synthesis. However, inflammation typically triggers the synthesis of certain proteins derived from liver known as "APPs." They can be detected in serum or plasma directly and are used as biomarkers for evaluating the presence, severity, and process of an inflammatory reaction or infection (Thompson et al., 1992; Germolec et al., 2018).

Continuous exposure to a stimulus or an autoimmune reaction against self-antigens can give rise to chronic inflammation. Chronic inflammation which causes fibrosis and tissue damage has been reported to have a role in pathogenic mechanisms of aging and many diseases including atherosclerosis, autoimmune diseases, asthma, arthritis, diabetes, and cancer. Serum concentrations of APPs are mostly elevated in such conditions too, although the increase is less than in the case of acute inflammation or infection (Gabay and Kushner, 1999; Jain et al., 2011). The type of inflammatory response alters according to the causing species, and severity, chronicity, and mechanisms of the inflammatory process, therewithal response and adaptation abilities of the immune system (Germolec et al., 2018).

In this article, cytokines and their role in regulating the acute phase response, classifications of the APPs, and the APPs commonly used in clinical practice will be discussed in detail.

Regulation of acute phase proteins by cytokines

Cytokines are a group of proteins which have considerable functions in inflammatory reactions. The role of cytokines is the regulation of acute-phase response with a very complex signaling network including intracellular and intercellular ways. After binding to their cell surface receptor, some cytokines trigger off or promote the response (pro-inflammatory) while others attenuate it (anti-inflammatory) (Ceciliani et al., 2002).

Cytokines are classified according to their structure or function. They function as negative or positive growth factors and pro-inflammatory or anti-inflammatory proteins. The cytokines which act as growth factor are granulocyte and macrophage colony-stimulating factor, interleukin (IL) 2, 3, 4, 7, 10, 11, and 12. The pro-inflammatory cytokines include IL-1 α/β , IL-6, IL-8, tumor necrosis factor (TNF)- α/β , macrophage inhibitory protein-1, and interferon (IFN)- α/γ . However IL-1 receptor antagonists, IL-1 binding protein, soluble IL-1 receptors, and TNF- α binding protein have anti-inflammatory activity (Khalil and Al-Humadi, 2020).

Cytokines may affect each other different ways. Many cytokines have the ability to regulate the formation of other cytokines and their receptors. Hormones, circulating receptors, cytokines, and cytokine-receptor antagonists may inhibit or increase cytokines effects on target cells. Also cytokine combinations may have additive, inhibitory, or synergistic effects (Gabay and Kushner, 1999).

IL-1 also known as human leukocytic pyrogen induces fever and comprises IL-1a and IL-1b. The cytokine family of IL-1 includes IL-1a, IL-1b, IL-18, IL-33, IL-36a, IL-36b, and IL-36g which are pro-inflammatory agonists and 4 anti-inflammatory cytokines (IL-1Ra, IL-36Ra, IL-37, and IL-38) (Akdis et al., 2016).

IL-6 is the main stimulator of the production of most APPs (Gabay and Kushner, 1999). The regulation of immune and acute phase reactions and hematopoiesis is carried out by IL-6, a multifunctional pleiotropic mediator. After IL-6 is occurred in the inflammation area, it reaches the liver via the blood flow. IL-6 stimulates production of several APPs like C-reactive protein (CRP), serum amyloid-A (SAA), haptoglobin, fibrinogen, and α 1-antichymotrypsin. However it decreases fibronectin, albumin, and transferrin productions. Transcriptional and posttranscriptional mechanisms control its expression in a strict manner. Dysregulation related to IL-6 synthesis takes a part in chronic inflammation and autoimmune conditions (Tanaka et al., 2014).

TNF- α has important roles in host defense, inflammation, and apoptosis. TNF- α maintains immune homeostasis by its pro-inflammatory, and immunosuppressive features. It shows its effects on host defense against bacterial, viral, fungal, and parasitic infections. Systemic TNF- α elevations can cause sepsis, while local increases lead to five main marks of inflammation including swelling, redness, warmth, pain, and loss of function (Akdis et al., 2016).

Serum levels of cytokines as a biomarker can also be measured in addition to APPs. There are many methods comprising diverse levels such as mRNA or intracellular protein to measure cytokine levels due to the variation of cytokine activity. Assays for cytokine measurement divided into two groups being immunoassay and bioassay. For example sandwich enzyme linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC), Meso Scale Discovery (MSD) electro-chemiluminescence, protein microarrays, and bead-based multiplex immunoassays (MIA). Multiplex immunoassays allow multiple cytokines to be measured simultaneously (Keustermans et al., 2013). On account of their very short half-life and presence of blocking factors in serum, measurement of plasma cytokine levels does not provide clinical utility (Kumar et al., 2016).

The association between cytokines and some clinical entities in inflammatory disorders has been shown. IL-6 induced arginine vasopressin (AVP) seems to be responsible for the hyponatremia and IL-6 is also responsible for thrombocytosis seen in inflammatory conditions. Cachexia, anemia of chronic disease, anorexia, lethargy and somnolence are the other associated conditions with cytokines like IL-1 beta, TNF- α , IFN γ in addition to IL-6 (Kumar et al., 2016).

The pattern of acute phase plasma markers differs in bacterial and viral infections. The elevated levels of IL-1 β and IL-6 is correlated with increased plasma CRP levels emerge in many bacterial diseases. Circulating levels of both IL-6 and CRP are detected higher in cases with bacterial enterocolitis and community-acquired bacterial infections by comparison to viral etiologies. IL-6 induced CRP levels also can be useful for discrimination of bacterial pneumonia and influenza infections and of streptococcal pharyngitis and infectious mononucleosis (Slaats et al., 2016).

Pro-inflammatory cytokine IL-18 commonly elevated in viral infections. IL-18 induces elevation of serum ferritin and Th1 immune responses through the induction of IFN- γ (Slaats et al., 2016).

Classification of acute phase proteins

The liver responds to an inflammatory process by producing a wide variety of acute phase reactants. On the other hand, the production of some other proteins is reduced. Various classifications are proposed for approximately 40 APPs that are currently known. Those that are up-regulated are called "positive APPs" and those that are down-regulated are called "negative APPs" (Cecilian et al., 2002). Additionally, another classification can be made on the basis of protein concentrations or the mechanism of action (Jain et al., 2011). Those whose concentration increases 10 to 100-folds are classified as major APPs. Serum levels of moderate APPs increase 2 to 10-folds, while those that rise less than 2 folds the normal are classified as minor APPs (Khalil and Al-Humadi, 2020). According to their mode of action, APPs are classified as coagulation proteins, complement proteins, protease inhibitors, transport proteins, and others (Jain et al., 2011) (Table 1).

Positive APPs have several physiological functions for the immune system. APPs with increased plasma levels help the immune system by recognition of invading microorganisms, mobilization of white blood cells into the blood circulation, and by directing blood flow to inflammation zones. Consequently the concentration of effector molecules enhances antimicrobial defense at locally injured or infected areas. At the same time, APPs can protect unaffected tissues from the detrimental effects of inflammation. They achieve this function by various mechanisms such as neutralizing inflammatory molecules (cytokines, proteases, antioxidants, etc.) that enter the bloodstream, minimizing the pro-inflammatory responses of leukocytes, and preventing endothelial activation (Janciauskiene et al., 2011).

Table 1 Acute phase reactants and related cytokines

Classification of APPs		Associated cytokines
Coagulation and fibrinolytic system	Fibrinogen	IL-1 β , IL-6, IL-8, TNF- α
	Plasminogen	
	Plasminogen-activator inhibitor 1	
	Protein S	
	Tissue plasminogen activator	
	Urokinase	
Complement components	Complement 3, 4, and 9	TNF α , IL-1 β , IL-6, IL-8, IL-32
	Factor B	
	C1 inhibitor	
	C4b binding protein	
Proteaz inhibitors	Mannose binding lectin	TNF α , IL-1 β , IL-6, IL-8, IL-32
	Alfa 1 protease inhibitor	
	Alfa 1 anti-chymotrypsin	
Transport proteins	Pancreatic secretory trypsin inhibitor	TNF α , IL-1 β , IFN- γ
	Ceruloplasmin	
	Haptoglobin	
	Hemopexin	
Other reactants	CRP	IL-6, IL-1 β , IL-1 α , TNF- α
	SAA	IL1 β , IL-12, IL-6, IL-8, IL-23
	Alfa 1 acid glycoprotein	TNF- α , IL-1, IL-6
	Ferritin	IL-1 β , IL-6, IL-10, and TNF- α
	Fibronectin	
	Angiotensinogen	
Negative APPs	Albumin	TNF α , IL-6
	Transferrin	IL-1, IL-6, TNF α
	Transthyretin	IL-1, IL-6, TNF α
	α 2-HS glycoprotein	
	α -fetoprotein	
	Thyroxine-binding globulin	
	Insulin-like growth factor I	
	Factor XII	

Abbreviations: APP(s), acute phase protein(s); CRP, C-reactive protein; IL, interleukin; SAA, serum amyloid A; TNF, tumor necrosis factor. Modified from Gabay C and Kushner I (1999) Acute-phase proteins and other systemic responses to inflammation. *The New England Journal of Medicine* **340**(6): 448–454; Khalil RH and Al-Humadi N (2020) Types of acute phase reactants and their importance in vaccination (review). *Biomedical Reports* **12**: 143–152.

APPs with decreased levels include albumin, retinol binding protein (RBP), transferrin, and transthyretin. It is not thought that they have an immune function and there are no clear explanations for the down-regulation of them. Speculation about the mechanism is that in the course of an acute phase response, essential amino acids for synthesis of the APPs are used in the synthesis of these proteins and the production of others is slowed down. Furthermore, negative APPs act as transport proteins and the level of the specific molecules they carry in plasma may be decreased throughout inflammatory reactions (Ceciliani et al., 2002; Northrop-Clewes, 2008).

Widely used acute phase proteins and their use in diagnostic protocol

C-reactive protein

CRP is one of the acute phase reactants that has been first discovered and it is used in clinical practice commonly. It got its name from the ability of precipitating the pneumococcal C polysaccharide (Chandrashekar, 2014). CRP is produced by many human cells being primarily liver hepatocytes additionally by macrophages, lymphocytes, adipocytes, endothelial, and smooth muscle cells (Sproston and Ashworth, 2018).

CRP testing has been traditionally used as an indicator of infection and cardiovascular events. However, with growing evidence, the important roles of CRP in inflammation including phagocytosis, the complement pathway, cytokine production particularly IL-6 and TNF- α , apoptosis, and nitric oxide (NO) release are better understood (Sproston and Ashworth, 2018). Serum CRP levels can increase up to 1000-fold within 48 h after the beginning of inflammation. Monitoring CRP levels are advantageous to determine infection and tissue injury and to follow up the course of chronic diseases (Wu et al., 2015).

CRP is a pattern recognition molecule that comprises five identical subunits arranged in cyclic symmetry with 206 amino acids which are non-covalently bound (Chandrashekar, 2014; Wu et al., 2015). CRP is a calcium-dependent ligand-binding plasma

protein. One side of the pentamer has a binding ligand for phosphoryl choline, a part of teichoic acids in gram-positive organisms, and for lipopolysaccharides in gram-negative organisms. The other side of the pentamer binds effector molecules such as the crystallizable antibody fragment (Fc) receptors Fc- γ RI and Fc- γ RII and C1q (Chandrashekar, 2014; Eschborn and Weitkamp, 2019). This interaction activates the classical complement pathway and eventually stimulates phagocytosis. CRP can also interact with substances that are exposed in apoptotic cells like lysophosphatidylcholine, ribonucleoproteins, chromatin, and histones (Eschborn and Weitkamp, 2019).

CRP structurally consists of two different forms, being as native pentameric CRP (pCRP) and modified or monomeric CRP (mCRP) (Wu et al., 2015). When CRP subjects to denaturing conditions in the existence of a chelating agent, pCRP can dissociate into mCRP. The transport of mCRP from circulation to various body sites is thought to be faster than pCRP (Janciauskiene et al., 2011). Differently from pCRP, mCRP presents antigenic, ligand binding, and electrophoretic reactivities. According to growing evidence mCRP may have pro-inflammatory and thrombotic properties. It has been reported that mCRP binds oxidized LDL more potent than pCRP (Wu et al., 2015; Janciauskiene et al., 2011).

CRP is the most easily measured and widely used test with a large amount of data about it. Conventional CRP tests have high detection limits in the acute-phase reactant range of 3–5 mg L⁻¹ (Kimberly et al., 2003). The advantages of the test are rapid response time and short half-life. The type of inflammation does not affect its catabolism. However, its serum level varies according to the severity of the inflammation. It typically increases to levels of 10 to 40 mg L⁻¹ in mild inflammation and some viral infections, while in bacterial infections and acute inflammation it rises to levels between 40 and 200 mg L⁻¹. CRP levels upon 300 mg L⁻¹ may be observed in major trauma and severe bacterial infection (Thompson et al., 1992).

It is well-known that CRP is associated with platelet activation in acute thrombosis and is a marker of cardiovascular risk. The significance of chronic inflammation in the development of atherosclerosis is also obvious. To assess chronic inflammation in atherosclerosis the measured serum CRP levels are significantly low compared to in acute inflammation. Hence high-sensitivity CRP (hsCRP) assays with detection limits of approximately 0.1 mg L⁻¹ were developed (Kimberly et al., 2003).

Automated platforms using nephelometry and turbidimetry are extensively used for measuring CRP in the clinical laboratories. The other commonly used test methods are fluorescence, chemiluminescence, lateral flow and electrochemical assays, sandwich immunoassays, and novel lab-on-a-chip based immunoassays (Eckschlager et al., 2019). There may be differences in the sensitivity of the tests. In a study, the performance of the turbidimetric method was evaluated in cases with non-ST elevation acute coronary syndromes. The turbidimetric and nephelometric methods compared on the same plasma samples. The turbidimetric method used in this study is based on agglutination of latex particles coated with antibody against CRP. This method measures CRP by quantifying the absorbed light. The nephelometric method is based on measuring the agglutination of particles by quantifying the scattered light. They found that the CRP values obtained from both tests showed a strong correlation with each other (Correia et al., 2003).

Concomitant occurrence of multiple inflammatory stimulus and influence of other factors reduce the specificity of CRP remarkably. The molecular mechanisms associated with the complicated effects of CRP in inflammation remain challenging.

Erythrocyte sedimentation rate

The ESR is a widespread hematology test utilized to determine an increased inflammatory response. The ESR is nonspecific for any disease; however, it can be used to monitor activity of some situations such as infections, autoimmune disease, or malignancies. It is more convenient to use ESR in combination with other tests (Alende-Castro et al., 2019; Bray et al., 2016). The ESR measures the distance that erythrocytes in a sample of anti-coagulated whole blood fall in a vertical tube in 1 h (Hashemi et al., 2015).

Red blood cells normally have negative surface charges which cause them to repel each other (Bray et al., 2016). In inflammatory conditions the number of proteins which are positively charged in the blood increase. This increase causes erythrocytes clumping and this formed structure called rouleaux formation. The special shape and structure of the erythrocytes is effective in the formation of rouleaux (Szołna-Chodór et al., 2015; Bray et al., 2016). This phenomenon was first noticed in 1897 and the measurement standards still in use today were defined in 1921 by Westergren (Bray et al., 2016). The International Council for Standardization in Haematology (Expert Panel on Blood Rheology) (1993) proposed the Westergren method as a reference method.

Many other non-inflammatory factors can influence ESR that is a non-specific test. The quality and quantity of the red blood cells such as the size, shape, and number will increase or decrease the ESR (Assasi et al., 2015). The alterations of plasma proteins especially fibrinogen and immunoglobulins are known to influence ESR. Fibrinogen causes them to aggregate rapidly by reducing the charge on the red blood cell surface. So that ESR is used to measure the amount of fibrinogen indirectly (Lapić et al., 2020).

Age and sex, pregnancy, renal function, use of medications and technical issues are the other factors influence ESR test (Assasi et al., 2015). In a cross-sectional study it is shown that the ESR was statistically significant higher in females than in males. Also the ESR remarkable increased with age regardless of gender (Alende-Castro et al., 2019). In the same study body mass index, smoking, and metabolic syndrome were positively associated with higher ESR results. However lower ESR results were detected in light alcohol drinkers compared to abstainers and individuals performing regular physical activity (Alende-Castro et al., 2019).

Although it has a low cost, it is a time-consuming test and may have some degree of error. Several new, safer and faster, automated and semi-automated methods have developed to perform the ESR with a higher precision (Hashemi et al., 2015). The ESR increases in a slower manner at the beginning of the inflammatory reaction and normalizes more slowly than that of other acute phase reactants due to the long half-life of some plasma proteins (Assasi et al., 2015; Bray et al., 2016).

Clinicians should consider the limitations of the test and the conditions that may implicate the results and utilize the test in a manner consistent with clinical practice.

Procalcitonin

Procalcitonin (PCT), the peptide precursor of calcitonin with 116-amino acid, consists of immature calcitonin, the N-terminal fragments and calcitonin carboxyl-terminus peptide 1 known as katacalcin. Its molecular weight is 14.5 kDa (Busch et al., 2020; Whicher et al., 2001).

The PCT gene calcitonin 1 (CALC-1) is located on chromosome 11. prePCT is produced by the CALC-1 gene. In the C-cells of the thyroid this undergoes proteolytic cleavage to produce PCT, which is further formed to the mature calcitonin. This is a constitutively active pathway resulting only in the secretion of calcitonin. Transcription and translation of CALC-1 gene is limited to the C-cells of thyroid and, more rarely to other neuro-endocrine cells. Interestingly, the promoter has sites not only for basal transcription factors but also for inducible inflammatory factors like NF κ B and AP-1 (Whicher et al., 2001; Samsudin and Vasikaran, 2017).

Inflammatory reaction induced by IL-6, TNF- α and IL-1 β due to bacterial infections activate together with PCT production in all parenchymal tissues (Samsudin and Vasikaran, 2017). However, the other tissues including the adipocytes, brain, kidney, lungs, spleen, pancreas, and colon cannot convert PCT to calcitonin. This eventually leads to rising of PCT serum levels. On the other hand, viral infections mainly characterized by IFN- γ activation, attenuate PCT production (Samsudin and Vasikaran, 2017; Covington et al., 2018).

Because of the characteristics of PCT, it has been used as a specific serum biomarker in differentiating bacterial and nonbacterial etiologies for several years (Busch et al., 2020; Samsudin and Vasikaran, 2017). As having a high pretest probability for bacterial infections, monitoring PCT levels periodically is beneficial to follow up resolution of infection and early cessation of anti-biotherapy (Azzini et al., 2020). In the current literature, potential benefits of PCT in antibiotic stewardship protocols have been shown (Choi and McCarthy, 2018). In the case of neutropenia and using an anti-inflammatory agent and with relation to the severity of the infection, levels remain unchanged as an advantage of the test (Choi and McCarthy, 2018).

To appoint PCT levels in human serum or plasma, current PCT assays utilize immunofluorescence techniques (Covington et al., 2018). The serum levels of PCT is typically very low in healthy individuals (<0.02 ng mL $^{-1}$). In the presence of a bacterial infection, levels of PCT begin to increase within 3–4 h and peak after 12–24 h. The peak levels can reach more than 400 times baseline (Covington et al., 2018; Taylor et al., 2017). In intensive care unit patients, serum levels of PCT higher than 2.0 ng mL $^{-1}$ are associated with a high risk for progression to severe sepsis and/or septic shock whereas serum levels of PCT less than 0.5 ng mL $^{-1}$ indicates a low risk (Covington et al., 2018). More valuable results can be obtained by making serial measurements rather than a single measurement in most situations (Samsudin and Vasikaran, 2017). Despite PCT concentrations are highest in sepsis and acute bacterial infections; it may not elevate in localized infections, such as abscess or empyema or in autoimmune situations (Covington et al., 2018).

Physiologic elevations of PCT levels in 3-day-old newborns have demonstrated. Additionally, fungal infections may also lead to an increase in serum PCT levels, but this increase is not as much as bacterial infections (Covington et al., 2018). But as an important point, PCT levels should be evaluated carefully in the existence of some comorbidity. The elevation of PCT in the absence of bacterial infection can be associated with severe trauma, severe burns, abdominal or cardiothoracic surgery, thyroid medullary carcinoma, small cell lung cancer, acute pancreatitis, prolonged or severe cardiogenic shock, cardiac arrest resuscitation, and OKT3 antibodies and antithymocyte globulin treatments. Stimulation of massive pro-inflammatory cytokine response may be the mechanism of the elevation of PCT levels (Covington et al., 2018; Choi and McCarthy, 2018).

The definition of PCT as a biomarker indicating bacterial infection is based on a study conducted in 1993. Serum concentrations of PCT were measured in 79 pediatric patients with bacterial and viral infections using a monoclonal immunoradiometric assay. In children with severe invasive bacterial infections serum PCT levels were very high compared to localized bacterial or viral infections. In these cases with normal calcitonin levels, the rapid decrease of PCT levels with anti-biotherapy and the correlation of PCT serum levels with the severity of infection were the other results of this study (Hamade and Huang, 2019).

In the current literature that is related to the diagnostic accuracy of PCT to predict blood culture positivity, it was shown that in patients who had proven bacteremia levels of PCT were remarkably higher (30.62 ng L $^{-1}$ vs. 10.47 ng L $^{-1}$; $P < .001$) than patients with no bacteremia.

Although most studies were performed on a limited number of patients, appropriate usage of PCT can direct clinical decisions. Early recognition of bacterial infections can prevent redundant procedures, and reduce the length of hospital stay. All of these features are ultimately associated with a reduction in mortality rate and cost (Taylor et al., 2017).

The advantages of PCT over CRP are a more rapid increase and earlier peak at 24 h following infection and a faster decrease following resolution of infection. In a study of Yang et al. (2019) among a total 614 febrile episodes, the rate of systemic bacterial infection episode was 99 (16.1%). Statistically, they demonstrated that levels of PCT were higher in gram-negative bacterial infections in comparison with febrile episodes originated by fungal infection and other etiology. When 273 neutropenic fever cases were evaluated, levels of PCT were statistically significantly higher in patients with bacteremia by comparison to those in non-bacteremic; whereas CRP levels did not significantly differ between these groups. They did not find any significant difference in levels of PCT or CRP between gram-negative and gram-positive bacteremias.

Serum amyloid A

Serum amyloid A (SAA) is an APP complexed to high-density lipoprotein (HDL) as an apoprotein. The molecule has the N terminal part which is hydrophobic and this part is possibly responsible for the lipid-binding features. The molecular weight is

11.4–12.5 kDa. The protein is coded by 4 SAA genes on chromosome 11p and has from 104 to 112 amino acids in humans and many other species (Salini et al., 2011). Despite pathophysiology of SAA has been studied for many years, all biologic functions and interactions of SAA proteins remain partly unexplained (Sack, 2020).

Infections and traumatic inflammation rapidly increase SAA plasma concentrations many folds. Both constitutive and acute phase SAA proteins are expressed extrahepatically. But, after cytokine induction, acute phase SAA proteins are produced in hepatocytes (Salini et al., 2011). Highly inducible acute phase SAA proteins are encoded by SAA-1 and SAA-2 genes in the course of the acute phase response. While SAA3 is a pseudogene, SAA4 encodes constitutive SAA proteins (Sun and Ye, 2016).

Functions of SAA like regulation of host defense and inflammation have shown in many studies. It has been found that SAA1 binds to the out-membrane of gram-negative bacteria, which acts as an opsonin. In addition, the SAA-opsonized bacteria are attacked by peripheral blood monocyte-derived macrophages with TNF- α and IL-10 stimuli. It was suggested that SAA1 potentially plays a substantial role in host defense against invasive gram-negative bacteria according to these findings (Sun and Ye, 2016).

Interestingly, chronically, or repeatedly elevation of SAA levels because of prolonged inflammation or other stimuli can bring about their cleavage and deposition as pathologic amyloid fibrils. This may be an explanation of secondary amyloid deposition that develops in individuals with recurrent or chronic inflammation (Sack, 2020).

Various detection methods mostly based on immunological approaches of SAA in serum have been reported. The immunonephelometric assay and immunoturbidimetric assay are full-automatic and broadly used in clinical laboratories. ELISA, radio-immunoassay, and radial immune-diffusion methods are essentially used only in research laboratories on account of radioactivity problems, being time-consuming and labor-intensive tests (Zhang et al., 2019).

Haptoglobin

Haptoglobin (Hp) is a mainly liver-derived α 2-glycoprotein with a serum level of 3–30 $\mu\text{mol L}^{-1}$. Hp serum levels rise many fold in case of inflammatory reactions and it takes 3–5 days for Hp to clear from plasma (Khalil and Al-Humadi, 2020). Although Hp is present in the serum of all mammals, the polymorphism is present only in humans. Three main Hp phenotypes have been detected using gel electrophoresis. These phenotypes, called Hp 1–1, Hp 2–1, and Hp 2–2 have different biological activities (Sadrzadeh and Bozorgmehr, 2004). Antioxidant and, antibacterial activity, inhibition of NO and prostaglandins, prevention of renal damage, promotion of angiogenesis, regulation of immune system are main physiologic functions of Hp (Sadrzadeh and Bozorgmehr, 2004). Hypohaptoglobinemia or ahaptoglobinemia are conditions characterized by a decrease or absence of Hp and are associated with serious allergies or anaphylaxis (Khalil and Al-Humadi, 2020).

Hp binds free hemoglobin (Hb) that is revealed when erythrocytes lyse within the vascular bed. Hb is highly toxic because of its heme group that has oxidative properties (Andersen et al., 2017). The noncovalent Hb-Hp complex formation eliminates Hb and its iron component. The CD163 receptors located on the surface of monocytes and macrophages recognize Hb-Hp complex and the complex is cleared from circulation by receptor-mediated endocytosis of reticuloendothelial system (Andersen et al., 2017; Khalil and Al-Humadi, 2020). Glucocorticoids, IL-6, and IL-10 induces macrophage expression of CD163 (Sadrzadeh and Bozorgmehr, 2004).

Hp has several functions in the innate and acquired immune systems. It has a regulatory role in various stages of cellular and humoral immunity and cytokine release. Hp reinforces leukocyte migration and antibody production, whereas it suppresses prostaglandin production by inhibiting cyclooxygenase and lipoxygenase activity, and T cell proliferation (Khalil and Al-Humadi, 2020).

Hp levels can be measured by various laboratory techniques. However, automated immunonephelometric and immunoturbidimetric methods are the most commonly used ones (Sadrzadeh and Bozorgmehr, 2004). Conditions associated with an elevated plasma Hp level are inflammation, trauma, burns, and tumors (Sadrzadeh and Bozorgmehr, 2004). However, in given hemolytic conditions, like malaria, sickle cell anemia, trauma and severe infections, Hp is consumed due to free Hb excess in plasma (Andersen et al., 2017). Decreased plasma Hp level is also seen in malnutrition, hepatocellular disorders, ineffective erythropoiesis, late pregnancy, and newborn infants (Sadrzadeh and Bozorgmehr, 2004).

Fibrinogen

One of the important acute phase reactant originated from liver is fibrinogen which is a soluble glycoprotein (Davidson, 2013; Kilicarslan et al., 2013). Its normal serum range is 2–4 g L⁻¹ and half-life is approximately 4 days (Davidson, 2013; Kumar et al., 2016). It is a substantial component of the common pathway of coagulation. Fibrinogen is converted into fibrin forms to form the clot when the coagulation cascade is activated (Davidson, 2013).

However, fibrinogen also increases 2 to 3-folds during inflammatory reactions, such as after injury or infection and disease with vascular damage (Davalos and Akassoglou, 2012). This causes an increase in blood viscosity and some degree of erythrocyte aggregation. Totally distinct to any coagulation function, fibrinogen has a role in the regulation of the inflammatory reaction. It can prompt a variety of immune and vascular cells after binding to them (Davidson, 2013).

Fibrinogen interacts with a wide variety of cell-surface proteins, adhesion molecules, and receptors involved in inflammatory processes. This interaction can be either directly or indirectly. Various cytokines and chemokines are released as a result of the interaction of fibrinogen and the toll-like receptor 4 (TLR-4), one of the receptors associated with the induction of macrophage activation (Davalos and Akassoglou, 2012).

The Mac-1 integrin which is found on monocytes, neutrophils and also T cells has a function in leukocyte adhesion, migration, phagocytosis, in addition to apoptosis and degranulation. The formation of cytokines IL-1 β , IL-6 and TNF- α is induced by binding of fibrinogen to the integrin receptor Mac-1 (Davidson, 2013).

Breakdown products of fibrin in the microvascular compartment, D-dimers, enhance local and systemic inflammation by impact of pro-inflammatory molecules like IL-1, IL-6 and plasminogen activator inhibitor (PAI-1) released from monocytes. This also induces production of CRP (Davidson, 2013).

The fibrinogen level increases in heart or circulatory system diseases and in stomach, breast and renal system malignancies (Kumar et al., 2016). In inflammatory diseases like rheumatoid arthritis, high fibrinogen levels may be observed (Rooney et al., 2011). Fibrinogen is also used as a disease activity marker in Familial Mediterranean fever (Kumar et al., 2016). Exogenous use of estrogen and oral contraceptives may cause high levels of fibrinogen (Kilicarslan et al., 2013).

Whereas fibrinogen levels decrease in liver diseases, bone lesions, malnutrition and some bleeding disorders, obstetric complications and traumas as well as prostate and lung cancers. Also the lack or low levels of fibrinogen may be seen in some congenital diseases like afibrinogenemia, hypofibrinogenemia and dysfibrinogenemia. Massive blood transfusion is one of the other reasons that can lead to fibrinogen decrease. Some of the drugs that may cause low levels of fibrinogen are steroids, androgens, phenobarbital, urokinase, streptokinase and valproic acid (Kilicarslan et al., 2013).

As APPs there are some disadvantages of the test such as long half-life, late rise and late decrease even after inflammation has suppressed. The stability of fibrinogen cannot be preserved in frozen plasma and preserved plasma (Kilicarslan et al., 2013; Kumar et al., 2016).

Ferritin

The French scientist Laufferger discovered ferritin in 1937, but the appearance of ferritin in human serum was documented by the development of an immuno-radiometric assay in 1972 (Wang et al., 2010). Ferritin is a soluble protein with 450 kDa molecular weight which present in all body cells as a cytosolic protein. Beyond that, it is also found in mitochondria and a nuclear form have been suggested (Wang et al., 2010; Cullis et al., 2018).

Ferritin is comprised of two subunits, termed H (heavy) and L (light). The proportion of H and L subunits depends on the tissue of origin (Cullis et al., 2018). The heart or erythrocytes are rich in H type chains and electrophoretic migration of it is heavier than L chains. L chains which is rich in a lighter subunit originated from liver and spleen (Wang et al., 2010; Cullis et al., 2018).

Although serum ferritin is widely regarded as an inflammatory biomarker, it is mainly an intracellular iron storage protein. Serum levels of ferritin are commonly measured as indicators of the iron amount. Moreover, ratio of the soluble transferrin receptor (sTfR) to log ferritin ratio (sTfR Index) presumably ensure a better prediction of iron status of body (Kell and Pretorius, 2014). The iron status and inflammatory status of the body are two independent causes that affect serum ferritin levels (Northrop-Cleaves, 2008).

Invading microorganisms need a substantial amount of iron as an essential nutrient ensuring their survival. They are equipped with different mechanisms for obtaining iron from host ferroproteins like hemoglobin, transferrin, and ferritin (Koorts and Viljoen, 2011). During infection and inflammation, to reduce the availability of iron, it is redirected to macrophages and hepatocytes by withdrawing from the circulation. The translation of ferritin is enhanced because of iron overload in these cells (Slaats et al., 2016).

Although serum ferritin level increases in answer to any infectious or inflammatory course, it also reflects total iron stores of the body. Therefore in the absence of infection, low serum ferritin levels can only reflect depleted iron stores (Northrop-Cleaves, 2008). Similarly, it can be used as a marker that reflects inflammation rather than iron status, mainly in countries where inflammatory diseases have high prevalence (Kell and Pretorius, 2014).

The transcription and translation of ferritin during acute phase response is directly stimulated by IL-1 β , IL-6, IL-10, and TNF- α . Elevated ferritin levels protect against microbial growth and oxidative damage. Ferritin has immunomodulatory activity, and functions in apoptotic processes and cellular proliferation (Wang et al., 2010; Koorts and Viljoen, 2011). Differently from bacterial pathogens, infections caused by viruses are generally characterized by raised plasma levels of IL-18 that is a pro-inflammatory cytokine, together with elevated ferritin. Epstein Barr virus, chronic hepatitis B and C, Human Immunodeficiency Virus infections have been related to elevated serum ferritin levels (Slaats et al., 2016).

Serum ferritin can be measured using immunoassays e.g., ELISA, immuno-chemiluminescence or immunoturbidimetric assay that use antibodies to either liver or spleen ferritin. Immuno-radiometric assays are now rarely used due to its safety risks. However serum ferritin values vary according to age, sex, and ethnicity (Cullis et al., 2018).

Plasma level of ferritin is affected by many other factors. Leakage from damaged cells is the possible mechanism for ferritin secretion in conditions like tissue necrosis and injury to ferritin-rich tissue. Malignancies including hepatocellular, breast, pancreatic carcinomas, and hematological malignancies, rapid erythrocyte turnover, malnutrition and surgery are the other conditions (Cullis et al., 2018; Koorts and Viljoen, 2011; Slaats et al., 2016). Another route of ferritin gets into the blood flow is by way of the classical ER/Golgi-dependent secretory pathway in the liver (Slaats et al., 2016).

Ceruloplasmin

Ceruloplasmin is synthesized by hepatocytes and activated monocyte and macrophages as an APP (Tisato et al., 2018). Physiologically, ceruloplasmin as a metal chelating protein transports and delivers copper to tissues (Tisato et al., 2018; Janciauskiene et al., 2011). Ceruloplasmin also plays an essential role in transport and metabolism of iron and consequently in erythropoiesis.

It converts Fe^{2+} to Fe^{3+} and this ferroxidase activity inhibits reactive oxygen species production that is ferrous ion-mediated (Bakhautdin et al., 2013). Ceruloplasmin also exhibits bactericidal activity. Its ferroxidase activity reduces the availability of free iron, as a consequence limits bacterial growth (Bakhautdin et al., 2013).

Ceruloplasmin exhibits relatively modest acute phase behavior (Janciauskiene et al., 2011). The plasma levels increase typically about a 50% in response to inflammation, trauma, or infection (Janciauskiene et al., 2011; Bakhautdin et al., 2013). $\text{IFN-}\gamma$ and $\text{TNF}\alpha$ induce expression of ceruloplasmin from myeloid cells (Bakhautdin et al., 2013). The potent antioxidant, bactericidal and ferroxidase activities of ceruloplasmin suggests a possible anti-inflammatory function (Bakhautdin et al., 2013).

Conclusion

Acute-phase changes including hematologic modifications, APPs, complement components, and many cytokines picture the presence and severity of inflammation. APPs and other changes can be detected by a variety of laboratory techniques and utilize as a guide to diagnosis and management. In some cases, different responses occurring in certain APPs or cytokines may provide a clue about the nature of the inflammatory process. But commonly, APPs are non-specific markers of inflammation, and the tests used should be interpreted carefully in clinical practice.

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Antibody Phage Display

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Definition

Antibody phage display is a method that utilizes phage particles as vehicles to present functional antibodies on its surface for selection. The method involves an iterative process that allows for affinity-based enrichment of target specific antibody clones from a library of diverse antibody repertoire.

Phage display

Phage display is a method designed to present a plethora of peptides and proteins on the surface of phage particles for different applications. The most commonly used phage particle for phage display is the F-specific filamentous bacteriophage (Ff) (Smith, 1985). Although there are several types of Ff ranging from f1, M13 and fd, M13 is the most common of all (Ebrahimizadeh and Rajabibazl, 2014). The physical appearance and mode of assembly of Ff are unique when compared to other bacteriophage as they do not have an archetypal head and tail structure but a filament tube like structure instead. In addition to that, they do not lyse (kill) the host cell during reproduction (Mai-Prochnow et al., 2015).

M13 bacteriophage

M13 bacteriophage was first described in 1963 from *Escherichia coli* (Hofschneider, 1963). It has a single stranded DNA genome and is classified in the family *Inoviridae*, genus *Inovirus*. M13 is classified as a member of the Ff family. This group of filamentous bacteriophages are known for infecting Gram-negative bacteria (Rakonjac et al., 2011).

M13 bacteriophage genome

The genome of Ff is a circular single stranded DNA (ssDNA) which forms an anti-parallel two-stranded helix, similar to A-DNA or B-DNA. The helix ends with two loops where one of the two loops is a 32-nucleotide hairpin called the packaging signal (PS). This PS is responsible to target the genome for phage packaging as well as initiating the Ff assembly process (Russel and Model, 1989). The M13 genome encodes for the production of 11 unique proteins that is critical for the survival of the phage particle. The phage genome has an approximately 500 nucleotide long intergenic region encompassing the viral origin of replication and complementary DNA together with the packaging signal. The 11 proteins encoded can be classified as structural and functional proteins. The structural proteins include the proteins responsible for the capsid which are pIII, pVI, pVII, pVIII and pIX. The functional proteins are responsible for the replication process (pII, pV and pX), and for virus assembly (pI, pIV and pXI). Each of the proteins are encoded by their respective genes denoted with the same numerical annotation (Mai-Prochnow et al., 2015).

There are five structural proteins where only two are main precursor proteins. The first is the major coat protein, pVIII, which contains a 23-residue signal sequence that is removed by signal peptidase following insertion into the membrane. The pVIII protein is arranged to allow the N-terminus to protrude outward to the periplasm and the C-terminus remains in the cytoplasm (Wickner, 1975). The pIII which is the product of gene III (gIII) is the second precursor protein that has an 18-residue signal sequence which is also cleaved during translocation into the membrane. Majority of the pIII is located in the periplasm including the mature N-terminus. The pIII is anchored to the inner membrane of the bacteria host via a 23-amino acid hydrophobic sequence in proximity of the C-terminus (Davis et al., 1985). There are three other capsid proteins (pVI, pVII and pIX) that are not synthesized as precursor molecules but are also membrane bound. These proteins will remain bound until they assemble into a mature phage particle (Endemann and Model, 1995).

Proteins responsible for the gene synthesis process includes gene II (gII) which encodes a 409 residues protein, pII, which is a site- and strand-specific endonuclease critical for the synthesis of the double-stranded replication intermediate and the single-stranded viral DNA (Asano et al., 1999). Gene X (gX) encodes a 111 amino acid protein at the C-terminal end of pII required for the synthesis of the single-stranded viral DNA. Gene V (gV) is expressed as a dimer and functions to bind to newly synthesized viral DNA. The attachment of the translated pV ensures the synthesized DNA is maintained in a linear antiparallel form required for assembly of the bacteriophage (Fulford and Model, 1988; Guan et al., 1995). It is estimated that approximately 1500 molecules of pV is required to cover each viral ssDNA. The synthesis of pII and pX are generally regulated during translation. To inhibit translation, another function of pV is to specifically bind to the gene II and X mRNA to inhibit translation. Inhibition is initiated when high cellular concentrations of pV is achieved. This is achieved when the level of pV exceeds the level needed to bind the newly synthesized viral DNA (Michel and Zinder, 1989).

The three proteins responsible for viral assembly are pI, pIV and pXI. Incidentally, these assembly proteins are responsible for the membrane-associated assembly of the virus particle but are not part of the virion itself (Russel, 1993). The gene IV protein is synthesized as a precursor with a 21 amino acid signal sequence found mainly in the outer membrane of an infected bacteria. Post translocation of pIV to the periplasm will result in the removal of the signal peptide. It will then integrate with the outer membrane for the formation of a cylinder composed of 14 subunits of pIV. This cylinder will allow the migration of the emerging filamentous phage from the cytoplasm to the periplasmic space (Korotkov et al., 2011; Opalka et al., 2003). Gene I encodes a 348-residue protein that is distributed along the bacterial inner membrane via an internal hydrophobic region. This permits the 253 amino acids N-terminal to remain in the cytoplasmic space and 75 amino acids of the C-terminal are expressed in the periplasm. Gene XI is encoded within gene I and transcribes a 108 residue pXI which corresponds to the C-terminal portion of pI. It is inserted in the inner membrane with the same orientation as pI. It has been proposed that the periplasmic region of pI and pXI will interact with pIV in the outer membrane to form an assembly site. This site is where the assembly of the bacteriophage occurs and exits the bacteria host (Haigh and Webster, 1999).

Structure

The structure of M13 bacteriophage much like its namesake resembles long and thin filaments. In some instances, it may be described as a worm-like structure reminiscent of a strand of rice vermicelli. M13 is estimated to be approximately 900 nm in length and 7 nm in diameter, with a mass of approximately 16.3 MDa. The bacteriophage body consist of a protein sheath encapsulating a circular ssDNA which extends the entire length of the body (Rakonjac et al., 2017).

The protein sheath consists of a landscape of approximately 2700 molecules of the major coat protein. The major coat protein pVIII is a 50 amino acid protein encoded by gene VIII (gVIII). The pVIII has mainly an α -helical structure protruding outward of the phage particle. The N-terminal end of pVIII is an acidic domain that is found exposed outside of the particle whereas the C-terminal end is basic in nature and is directed inward of the protein tube. Copies of the pVIII are able to form a stable protein tube structure as these two domains are connected by a hydrophobic sequence stabilizing the entire structure. The pVIII proteins are assembled in overlapping shingle-like array with a symmetry that is defined by a fivefold rotational axis with a twofold screw axis of pitch at 3.2 nm (Wickner, 1975).

The pVIII forms the tubular shape of the rod, however the top and bottom ends of the tube shape is closed. This is done by two pairs of separate proteins, mainly pVII with pIX at one end and pIII with pVI at the other. Approximately five copies of the 33 amino acid pVII and 32 amino acid pIX make up one end of the particle. The opposing end consist of five copies of a 406 residue pIII and 112 residue pVI proteins. The pIII and pVI end is shaped similarly to a small knob-like structure that consist of the N-terminal end of

pIII. This N-terminal end of pIII harbors information for bacteria F-pilus recognition. In addition, pIII is also responsible for the recognition of the bacterial proteins involved in the translocation of the phage DNA into the bacteria. The stability of the particle is conserved by the C-terminal end of pIII together with pVI (Rakonjac et al., 2017).

The nature of orientation by the capsid proteins allows for the formation of a hollow tube with an internal diameter of approximately 3.4 nm. It is within this confine space that the viral single stranded DNA resides in a non-base-paired antiparallel form. The DNA is positioned within the virion in such a manner that a 78 nucleotides hairpin loop will always be located at the pVII/pIX end. This hairpin end is termed as the packaging signal and is critical for bacteriophage assembly efficiency. The DNA bases are oriented in the center of the phage particles permitting the negatively charged sugar phosphate backbone of the DNA to interact with the exposed positively charged C-terminal end of pVIII. This overall charge density regulates the DNA packing in the bacteriophage (Rakonjac et al., 2017).

Life cycle

The M13 life cycle can be categorized into three main stages much like the lifecycle of most viruses. The life cycle of M13 is lysogenic where the bacteriophage invades host bacteria and co-exist within the host until mature phage particles are packaged and leaves the host cell. The three stages involved in the life cycle of M13 are infection, replication, and assembly (Rakonjac et al., 2017).

Infection

The first stage of the life cycle involves the introduction of the primary phage particle into host cells. In the context of Ff phages, the host of choice is *E. coli* with a F conjugative pilus like XL1-Blue, TG1 and HB2151. The infection process is mainly a two-step process that involves the F pilus and the bacterial TolQ, TolR and TolA proteins. Infection of *E. coli* by the Ff phages begins with the interaction of pIII on a mature phage particle with the tip of the F pilus on the host which is the primary receptor. TolQ, TolR, and TolA are integral cytoplasmic membrane proteins that forms a complex which functions as the secondary receptor for infection (Click and Webster, 1998). The TolQRA complex is part of a larger *trans*-envelope Tol-Pal complex responsible for cell division and maintenance of cell envelope integrity (Gerding et al., 2007).

The pIII plays a key role in the initial interaction with the host bacteria. The N2 domain of pIII binds to the tip of the F pilus on the host, whereas the N1 domain binds to the periplasmic domain III of TolA causing a conformational change in pIII by *cis-trans* isomerization of Pro₂₁₃ located within N2 domain. This conformational change releases the N1 domain from the N2 domain allowing the TolA binding site on the N1 domain to be accessible (Lubkowski et al., 1999). The absence of the F pilus or the N2 domain will not abrogate infection but reduces the infectivity by about 1000-fold. Although the infection process can make do without the F pilus and N2 domain, the TolQRA complex and the N1 domain are absolutely required for infection (Russel et al., 1988).

Upon F pilus interaction with the pIII domains, the phage is then brought to the bacterial surface by retraction of the pilus. This retraction process is not fully understood as it has been speculated that the retraction is a result of the polymerization-depolymerization cycles of F pilus or induced by Ff phage binding (Clarke et al., 2008). The pilus retraction allows the migration of the virion into the periplasmic space via the outer membrane to allow the N1 domain of pIII to access TolA. The collective interaction of the TolQRA complex, the pIII C-domain and the N1N2 domains will allow the phage ssDNA genome entry into the cytoplasm and integration of the major coat protein into the cell host inner membrane (Bennett and Rakonjac, 2006).

Replication

Once the phage ssDNA genome has successfully made its way into the host cytoplasmic region, the next stage of the life cycle will be initiated. The next stage of the life cycle involves the replication of the phage in the form of episomes. Phage genome replication is carried out by rolling circle amplification (RCA) where the origin of replication (ori) is at the intergenic region of about 500 nucleotides allowing single strand replication in succession. Once the positive (+) strand ssDNA penetrates into the host, the negative (−) strand is synthesized resulting in a temporary dsDNA genome. The (−) strand synthesis is primed by a short RNA primer from *E. coli* RNA polymerase that binds to the (−) strand ori followed by extension by DNA Polymerase III (Higashitani et al., 1996). This will give rise to a dsDNA genome termed as the replicative form (RF). The (+) strand replication begins at the (+) strand ori of the supercoiled RF. A strand transferase (phage protein pII) will generate a nick in the (+) strand ori with the 3'-end acting as a primer for (+) strand synthesis. The replication process will cease when pII reaches the terminator sequence of the (+) strand ori. Then the strand transferase will join the 5'- and 3' ends together for a newly synthesized (+) strand. This new (+) strand will regenerate the intact RF and release the old (+) strand (Model and Russel, 1988).

The released (+) strands at the early stages of replication will serve as templates for additional (−) strand synthesis to increase the copy number of the RF in the host to approximately 50 copies per cell. The copy number of the RF will decrease again as the infected *E. coli* undergoes divisions (Lerner and Model, 1981). The RF is also the template for viral gene transcription especially for the ssDNA-binding protein, pV. As the amount of pV accumulates in the host, the (+) strands will be diverted away from further replication by pV coating of the (+) strand. The pV will dimerize to bind two strands of circular ssDNA, forming a pV-ssDNA filament. In addition to that, pV also functions as a regulatory protein that regulates pII and pX translation and (−) strand synthesis. The (+) strand-pV filament will act as the precursor for virion assembly process (Stassen et al., 1994).

Assembly

The virion assembly process for Ff is unique where the virion proteins are aligned along the inner membrane prior to assembly of the final mature filament-like virion via a secretion-like process (Loh et al., 2019). The major coat protein (pVIII) is earmarked as the dominant protein aligned along the membrane via a very efficient process. The assembly process is initiated when the packaging signal interacts with two minor coat proteins, namely pVII and pIX, and the inner membrane assembly complex via pI. The assembly then continues with the dissociation of the pV from the ssDNA in the cytoplasmic region of the inner membrane. This is then followed by the association with pVIII at the inner membrane level until the entire ssDNA genome is covered by pVIII (Russel and Model, 1989).

As the assembly process is in effect, translocation of virion proteins from the inner membrane to the growing filament occurs. During this time, the protein-phospholipid interaction is converted to a protein-protein interaction (Park et al., 2010). As the assembly includes the ssDNA genome, the translocation process allows the DNA to serve as a guide where the major coat protein is assembled (Russel et al., 1997). When the DNA is completely surrounded with pVIII, two additional minor coat proteins (pIII and pVI) will form the terminating cap of the virion to release the phage from the cell surface (Rakonjac et al., 1999). Termination of assembly occurs when the end of the DNA is reached allowing the length of the particle to be determined by the size of the DNA. If elongation is continued in the absence of either pIII or pVI, very long unstable polyphages with multiple unit lengths of the ssDNA will be formed. The instability of polyphages suggest the role pVI and pIII plays for improved stabilization of the phage structure (Lopez and Webster, 1983; Rakonjac and Model, 1998).

Antibody

Antibodies are one of the major components in the adaptive immune system that functions to safeguard the body from invading antigens. Upon encountering antigens, B-cells will proliferate and secrete soluble antibodies for circulation in the blood and lymphatic system to surveil for harmful antigens. Diversification of the antibody genes at germline level and binding specificity shaped through clonal selection concomitantly generates a wide range of antibodies to maximize the protection from any possible foreign antigens (Hoehn et al., 2016).

Antibody structure and function

An antibody, also referred to as immunoglobulin (Ig), is typically comprised of two identical heavy chains (HC) and two identical light chains (LC) (Schroeder Jr and Cavacini, 2010). These four polypeptide chains are paired together via disulfide bonds to form a Y-shaped structure with a flexible hinge at the center (Fig. 1). The flexibility is crucial for the antibody bivalency (Thouvenin et al., 1997). Human LC is classified into kappa (κ) and lambda (λ) families. Each of the HC and LC is further broken down into the variable (V) region and constant (C) region. The V region is highly variable, containing three hypervariable sites separated by four framework regions (FR). The hypervariable sites, also known as complementarity determining regions (CDRs), accounts for the

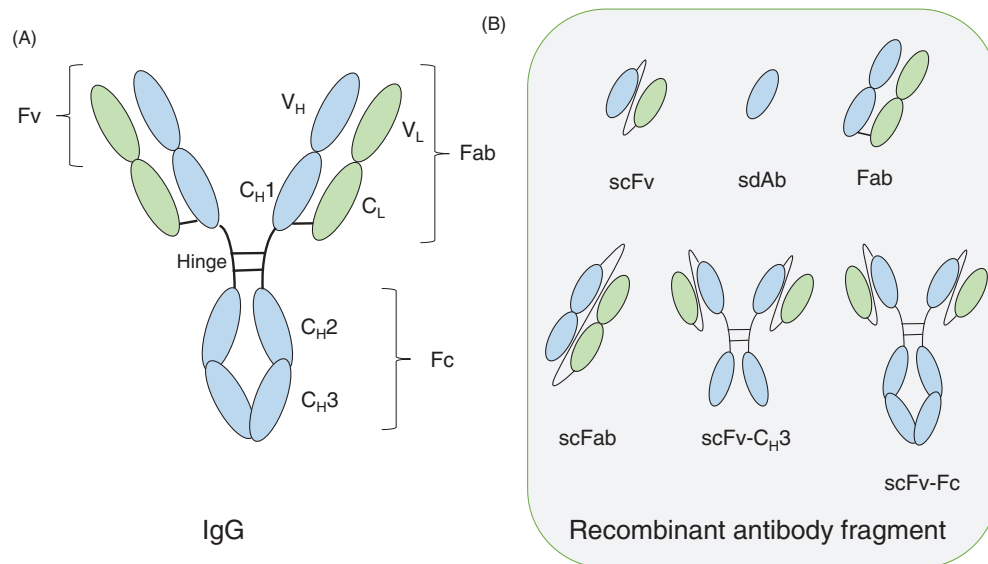


Fig. 1 Structure of an antibody and various recombinant antibody formats. (A) Typical structure of IgG comprising two identical heavy chains (HC) and two identical light chains (LC) interconnected through disulfide bonds to form a flexible Y-shaped molecule. The V_H and V_L form the fragment variable region (Fv); Fv together with C_H1 and C_L forms the fragment antigen-binding region (Fab); C_H2 and C_H3 forms the fragment crystallizable region (Fc). (B) Some of the commonly used recombinant antibody formats, such as single chain fragment variable (scFv), single domain antibody (sdAb), Fab, single chain Fab (scFab), scFv- C_H3 and scFv-Fc.

antibody variability and specificity in antigen binding. The heavy chain variable (V_H) and light chain variable (V_L) will fold to form the fragment variable region (Fv), which is also regarded as the region responsible for specific binding to a specific epitope on a target antigen. The V_H and V_L together with the light chain constant region (C_L) and first heavy chain constant domain (C_{H1}), forms two identical arms of the “Y” which is widely known as fragment antigen-binding region (Fab) (Amzel and Poljak, 1979).

The heavy chain constant domains 2 and 3 (C_{H2} and C_{H3}) forms the fragment crystallizable (Fc) region that interacts with Fc receptors (FcR) or C1q complement components to eliminate invading antigens through antibody-dependent cellular cytotoxic (ADCC) or complement-dependent cytotoxic (CDC) mechanism (Lu et al., 2018). As opposed to the V region, the C region is relatively constant with slight variations occurring at specific sites responsible for the effector cell binding. Variations at the Fc allows for further classification of antibodies into five isotypes—IgG, IgM, IgA, IgD and IgE. Each isotype predominates at different parts of the body and plays different roles in immunity (Janeway et al., 2001).

IgG is the most abundant antibody, mainly circulating in serum. Depending on the subclasses (IgG1-4), the structure of IgG differs slightly in terms of their glycosylation, disulfide bonding and hinge structure. The structural differences ultimately determine the interaction of IgG to different FcR or to complement components. For instance, IgG1 and IgG3 binds to the C1q complement component in addition to FcγRs. IgG2 binds weakly to C1q whereas IgG4 does not engage C1q, but both binds weakly to certain FcγR (Vidarsson et al., 2014). IgM is found mainly in the extravascular space, functions primarily to agglutinate invading antigens. IgM appears in pentameric form with five monomers joined together via a J-chain (Keyt et al., 2020). IgA contains a J-chain as well and an additional secretory component that aids in secretion of IgA dimers at the mucosal layer in respiratory and gastrointestinal tracts, comprising of two subclasses—IgA1 and IgA2 (De Sousa-Pereira and Woof, 2019). IgE and IgD are the least abundant Ig in serum. IgE is mainly found on the surface of granulocytes, functions to regulate the granulocyte mediators' secretion. Level of IgE is closely related to parasitic infection and hypersensitivity responses (Sutton et al., 2019). IgD is found mainly on surface of B cells with no clear understanding of its function but it is generally postulated to play a role in B-cell development (Chen and Cerutti, 2011). Antibody isotypes can switch via a mechanism known as class switch recombination (CSR). In the event of CSR, deletion and recombination is carried out at the constant region to generate different Fc (Manis et al., 2002; Stavnezer et al., 2008). These allows for different antibody isotypes to work at different locations in the body with varying functions (Table 1).

Antibody recombinant formats

Since the antigen binding function of an antibody is mainly attributed to the variable region, a multitude of antibody fragments have been engineered to cater for different applications throughout the years. Alternative antibody fragments can be generated through chemical fragmentation or genetic modification. Chemical fragmentation involves reduction of the disulfide bonds and protease digestion such as papain and pepsin to yield Fab, F(ab')₂, Fc and pFc' from a single antibody molecule (Kinman and Pompano, 2019). However, genetic modification allows for more antibody formats to be engineered. One of the widely utilized recombinant formats is the single chain variable fragment (scFv) that is comprised of a V_H and a V_L domain, interconnected through a short flexible peptide linker (Bird et al., 1988). Many studies have successfully isolated scFv binders against a wide range of antigens (Kuhn et al., 2016). The scFv is monomeric, however, multiple scFvs can be joined together through peptide linkers of varying lengths to form multivalent diabodies, triabodies or tetrabodies resulting in molecules with a higher avidity (Hudson and Kortt, 1999). Besides that, binding pockets targeting different epitopes can be engineered into a single antibody molecule to achieve multispecific antibody fragments (Choi et al., 2013). Likewise, various combination of the V_H , V_L , C_{H1} , C_L , C_{H2} and C_{H3} has yielded a vast array of recombinant formats such as Fab (De Haard et al., 1999), scFab (Hust et al., 2007), scFv- C_{H3} (Hu et al., 1996) and scFv-Fc (Bujak et al., 2014) (Fig. 1).

Single domain antibody (sdAb) is the smallest antibody fragment containing only one variable domain. sdAb, also termed as nanobody, is derived from heavy chain antibody (hcAb) such as VHH of camelids (Hamers-Casterman et al., 1993) and VNAR of sharks (Greenberg et al., 1995). Alternatively, sdAb can also be engineered from the V_H or V_L of human or mouse IgG (Hussack et al., 2012; Ward et al., 1989). sdAb has an advantage over the conventional antibody or other recombinant antibody formats because the small size allows rapid penetration to buried sites which is not accessible to larger antibodies (Abulrob et al., 2005; Garza et al., 2017).

In general, from the perspective of production and cost, recombinant antibody fragments have the advantage in terms of their smaller size and lack of glycosylation to allow efficient *in vitro* antibody production, especially in *E. coli* (Frenzel et al., 2013). The cell permeability of the smaller antibody formats allows for better infiltration efficiency which allows deeper penetration of the cell

Table 1 Summary of the antibody isotypes and their functions.

Isotype	Predominance location	Function
IgG	Circulating in serum	Opsonize pathogens for engulfment and activate the effector mechanism (ADCC and CDC)
IgM	Extravascular space	Opsonize pathogens and activate CDC
IgA	Mucosal layer in respiratory and gastrointestinal tracts	Secreted to neutralize invading pathogens at mucosal surfaces
IgD	Surface of B-cells	Hypothesized involve in B-cell development
IgE	Surface of mast cells	Regulate mast cells mediators secretion, reflect parasitic infection and type I hypersensitivity

for therapy or immunohistochemical detection (Henry and Mackenzie, 2018). Moreover, smaller recombinant antibody fragments could also benefit phage-display antibody selection process that is driven mainly by *E. coli* cells with better expression and efficient display on the phage surface.

Antibody phage display libraries

The introduction of phage display system has led to a new chapter in antibody discovery research. Different from the traditional laborious hybridoma method with mice, robust and stable filamentous phage is utilized to display antibody fragments on the coat proteins (Smith, 1985). The core principle of antibody phage display library is built on the linkage of phenotype (protein) to genotype (phagemid). To construct antibody phage display libraries, the antibody gene is cloned into phagemid, upstream of pIII gene. The phagemid contains sequences that signal for the packaging of phage particles. During the replication and packaging, the antibody fragment is translated together with the coat protein as a fusion protein and is displayed on the phage surface anchored to the coat protein (Breitling et al., 1991). A large collection of phages, each encoding a unique antibody gene, are pooled together to form a phage display library. Various antibody formats such as scFv (Hust et al., 2011; Vaughan et al., 1996), Fab (De Haard et al., 1999) or sdAb (Davies and Riechmann, 1995; Nuttall et al., 2002) can be used for phage display library construction. The antibody genes can be obtained from various species including rodents, camelids, non-cartilaginous fishes, non-human primates, and human (Kuhn et al., 2016). Several types of antibody phage display libraries have been introduced and classified based on the source of the V genes.

Naïve antibody library

A naïve antibody library is generated from B-cells of healthy donors, or individuals with no known infection at the point of collection. The antibody genes could be retrieved from mRNA in peripheral blood mononuclear cells (PBMC), bone marrow, lymph nodes, or spleen. Upon extraction, the mRNA collected is first reverse transcribed into cDNA using oligo(dt) primers or random primers to retain the large and diverse repertoire. Following the first strand cDNA synthesis, V genes are amplified using primers designed to maximally cover all V gene families (Kügler et al., 2015; Marks et al., 1991). The design of the primer set is a critical aspect in antibody library construction to ensure good gene coverage for a diverse library repertoire (Sblattero and Bradbury, 1998) and the primer design has improved and optimized over the years (Lim et al., 2010; Pansri et al., 2009; Pasello et al., 2016). On top of that, the antibody library repertoire could be stretched further by combinatorial cloning, where the V_H gene is paired randomly with V_L gene (Lerner, 2016).

Of the five antibody isotypes, IgM is usually targeted for construction of naïve antibody library as it targets the repertoire from naïve B cells (De Haard et al., 1999; Kügler et al., 2015). The repertoire of naïve antibody libraries is large, generally between 10^9 and 10^{11} (Glanville et al., 2009; Lloyd et al., 2009). Theoretically, diversification of the six CDRs would yield an infinite number of combinations, however the repertoire of a phage display library is usually limited to 10^{10} – 10^{11} due to the transformation efficiency of *E. coli* (Almagro et al., 2019). With the large repertoire size, antibodies against an infinite number of targets are generally possible. Apart from infectious diseases and cancer targets, naïve antibody library can be used for selection of antibodies against self-antigens and toxins which would otherwise be impossible to obtain through immunization (Kuhn et al., 2016; Winter et al., 1994). Antibody from naïve antibody library might have lower affinity, however, this could be overcome through downstream refinement such as *in vitro* affinity maturation (Gram et al., 1992; Sun et al., 2019).

Immune antibody library

Immune antibody library is constructed from B-cells of immunized or infected individuals against a particular antigen of interest. Upon exposure to the antigen, *in vivo* affinity maturation and V gene hypermutation helps shape a specific repertoire against the antigen. Therefore, antibodies isolated from immune libraries generally exhibit higher affinities in the nanomolar range. Like naïve antibody libraries, the antibody genes could also be isolated from PBMC, bone marrow, lymph nodes or spleen (Chan and Lim, 2017). IgG is typically targeted for immune antibody library construction while IgA and IgE is also sometimes employed for mucosal-, parasitic- and allergy-based libraries (De Alborán et al., 1995; Hamidon et al., 2018; Laukkanen et al., 2003; Steinberger et al., 1996; Wieland et al., 2006). The required repertoire of immune antibody libraries is relatively smaller compared to naïve antibody libraries although combinatorial cloning approach could stretch the repertoire slightly (Lai and Lim, 2020). Immune antibody libraries are usually tailor-made for a specific set of antigens related to a given infection. Although isolation of binders against closely related antigens are reported (Barbas et al., 1992; Kashyap et al., 2008), immune libraries are not commonly reused for different antigens as the common perception of a skewed repertoire would mean a focused application of immune libraries.

Synthetic and semi-synthetic antibody library

A synthetic antibody library is chemically synthesized and assembled *in vitro* with the base design of the genes being done using bioinformatic analysis. The scaffolds of synthetic antibody libraries are designed to have good expression levels in *E. coli* and promotes proper folding for good solubility and antigen binding. The CDRs are randomized to mimic germline or re-arranged

V-genes (Shim, 2015). Randomization of the CDRs can be achieved through mutagenesis using degenerate codons such as NNN, NNS and NNK, where N represents all 4 nucleotide bases; S represents guanine (G) and cytosine (C); K represents G or thymine (T) (Solemani Zadeh et al., 2019). Although degenerate codons could give high diversity, the process is uncontrolled and could yield codons encoding unnatural amino acid or stop codons that disrupt the folding or translation of the antibody fragments. Another randomization approach is achieved through trinucleotide mutagenesis (TRIM) technology where trimers encoding 20 amino acids are used instead of relying on random combination of nucleotides. This allows precise manipulation on the diversification of the antibody library while avoiding stop codons or unfavorable amino acid distribution or combination that discounts the library quality (Knappik et al., 2000). The repertoire of synthetic antibody library is usually big and diverse like a naïve library, thus allowing unbiased selection for any target (Tiller et al., 2013; Weber et al., 2014).

Semi-synthetic libraries are generally libraries that consist of a combination of characteristics from both the naïve and synthetic library designs. The antibody framework region is obtained from naïve B-cells or a known antibody while at least one of the CDR, usually HCDR3, is randomized at positions responsible for antigen binding (De Wildt et al., 2000; Griffiths et al., 1994; Pini et al., 1998). Randomization of the CDR allows expansion of the repertoire diversity which could be limited by the immune system of the host. This permits the repertoire to be increased while maintaining a favorable framework for antigen binding (Hoet et al., 2005).

Antibody phage display panning process

Biopanning is the process involved in the search for specific monoclonal antibody from a large pool of phages (Parmley and Smith, 1988). The biopanning process, in general, involves repetitive rounds of immobilization, selection, elution and reamplification until a predominant antigen-binding population is enriched. Usually, three rounds of panning are required to enrich the population, but the amount can be increased or reduced depending on the nature of the antigen. After several rounds of selection and amplification, prospective clones are screened through enzyme-linked immunosorbent assay (ELISA) and identified through sequencing (Frenzel et al., 2014; Russo et al., 2018). The result of the panning process is influenced by many factors, including the type of immobilization, the concentration of the antigen, the blocking agent, the binding time, the washing stringency, and the elution method (Bradbury and Marks, 2004).

Target immobilization

At the beginning of each panning process, target immobilization is a critical step. This can either be done by physically attaching it to a solid phase or to allow the target to be in free constant flow in liquid phase. The most common approach used for target presentation is by physical immobilization to various solid phases. The immobilization of antigens on solid surfaces, such as polystyrene microtiter plate (Barbas et al., 1991), immunotube (Hust et al., 2002), microbeads (Sawyer et al., 1997), immunoassay tip (Chin et al., 2016), column matrix (Breitling et al., 1991) and nitrocellulose or PVDF membrane (Hawlisch et al., 2001) have been attempted over the years. Antigen immobilization is achieved through passive adsorption, streptavidin binding or covalent linkage, depending on the material of the surface and the characteristic of the antigen. Antigens can be in the form of proteins, peptides, or whole cells. Sometimes target antigens are coupled to streptavidin, bovine serum albumin (BSA) or other proteins to ensure proper display of the targeted epitope and increase the coating efficiency especially for smaller sized antigens such as peptides or compounds (Bradbury and Marks, 2004).

Apart from solid phase panning, solution phase panning is also done as an alternative especially in the circumstances where the binding site is blocked due to steric hindrance or conformational changes as a direct consequence to the interaction with the solid surface. Instead of immobilizing on a solid surface, the antigens are allowed to bind freely with the antibody phage particles in solution before affinity capture of the antigen-antibody phage complexes (Hawkins and Winter, 1992). To carry out solution phase panning, the antigen must bear an affinity tag such as biotin moiety for streptavidin capture (Schütte et al., 2009). This approach allows for the target antigens to remain suspended in solution for binding before it is eventually pulled down using a capture molecule.

Binding phase

After immobilization of the antigen, the phage antibody library is then added to allow affinity-based binding of the antibody to the antigen. The incubation could be carried out from an hour to overnight at temperatures ranging from 4 to 37 °C. Due to the large diversity of an antibody library, non-specific binders against background especially plastic, BSA, and streptavidin are common. The presence of these non-specific binders could impede the screening and clonal enrichment process. Blocking the surface with various blocking agents, controlling the antigen density, and changing the immobilization strategy could avoid background binders. In order to prevent the enrichment of non-specific binders, a pre-incubation of the phage antibody library is done using blocked uncoated solid phase. The blocking agents commonly used ranges from BSA, skimmed milk, gelatin or even casein. At this stage, the amount of target antigen used at each subsequent round is usually reduced gradually. The reduction of target antigen at corresponding panning rounds functions to enrich higher affinity clones as the amount of binding sites has reduced allowing competition for higher affinity clones to capture (Bradbury and Marks, 2004; Russo et al., 2018).

Wash step

After binding of the antibody phage library to the immobilized antigen, there will undoubtedly be some non-specific binders that managed to bind non-specifically with the solid phase or blocking agents. The unbound phages are then removed through a wash step. Elimination of unspecific and weak binders through multiple washing steps are deemed crucial. The wash buffer is usually supplemented with surfactants such as Tween-20 to obstruct non-specific hydrophobic interactions. As the affinity of the antibody clones from the subsequent rounds are expected to increase, the stringency in the wash cycle for each subsequent round is also increased. The stringency can be modified by manipulating the duration of the wash step or concentration of the Tween-20 used in the wash buffer. Additionally, there is also the option to supplement the wash buffer with non-specific proteins at low concentrations such as BSA to compete out the non-specific binders (Bradbury and Marks, 2004).

Elution step

Proceeding after the wash step, the remaining bound phages are released from the immobilized surface. This can be done through several approaches which includes trypsin elution, pH shift or competitive elution. Trypsin elution is carried out via enzymatic cleavage at a pre-designed trypsin cleavage site located between the antibody and the phage coat protein (Hust et al., 2011). The phage particles are then released from the solid phase intact while maintaining infectivity. On the other hand, pH shift elution is achieved through disruption of the binding using acidic glycine-HCl (Kang et al., 1991; Roberts et al., 1992) or basic triethylamine (TEA) (Marks et al., 1991). The pH shift elution process must be carried out with caution as prolonged exposure in either acidic or basic environment will cause irreversible damage to the phage particles, rendering them unable to infect. Therefore, during pH shift elution, a neutralization process is immediately done using Tris-HCl to bring the pH back to pH 7. Competitive elution is another elution strategy, accomplished by adding antigens or antibodies to compete and release the bound phage particles (Clackson et al., 1991; Meulemans et al., 1994). The final step is the rescue process where the eluted phages are then reamplified in *E. coli* with the aid of helper phage. The rescue step allows for the eluted phage to infect *E. coli* and reamplified with helper phage to produce new phage particles consisting the genotype of the eluted phage particles. This newly generated population of phage particles is then used as the starting material for the next round of the selection process. At the end of each round of panning, the eluted phage particles will consist mainly of an enriched pool of binding clones when done properly.

Positive clone selection and characterization

After several rounds of enrichment, the enrichment pattern is confirmed at polyclonal level using ELISA. When positive binding patterns are observed from the polyclonal ELISA, the polyclonal pool is then spread on agar to allow for individual clones to be selected later. At monoclonal level, single colonies are selected and evaluated via direct ELISA. The number of clones required to be screened and the success hit rate varies with the library diversity and nature of the antigen. In general, a more diverse library will have a higher chance of getting more unique positive clones with higher affinity (Teixeira and Gonzalez-Pajuelo, 2018). The positive clones at monoclonal level from the ELISA assay will then be sequenced to obtain clone identity.

Recombinant antibody applications in diagnostics

Phage display derived recombinant antibody is a promising tool for diagnostics, not only due to their high specificity and affinity, but also their batch-to-batch reproducibility and flexibility for customization. With the availability of the DNA and protein sequences, recombinant antibodies are easily subjected to flexible rapid modifications into different recombinant formats, isotypes, and subtypes (Basu et al., 2019).

A common way to adapt recombinant antibodies for diagnostic is labeling of the antibody with fluorescent dyes or enzymes like alkaline phosphatase to generate a visible or detectable signal. These conjugates are usually generated by chemical conjugation methods. A major disadvantage to chemical conjugation is the potential loss of binding affinity due to uncontrolled cross-linking at the binding site and the potential batch to batch variation generated from different conjugation protocols (Szabó et al., 2018; Vira et al., 2010). Recombinant DNA technology allows for rapid and easy fusion of the recombinant antibody to the components responsible for signal detection by genetic modification rather than random chemical conjugation. Nevertheless, the application of recombinant monoclonal antibody can be applied beyond traditional direct signal detection with protein, peptides, DNA, or nanoparticle to cater for different diagnostic platforms such as bead-based assay (Zhao et al., 2021), flow cytometry (Sashihara et al., 2009), lateral flow dipstick (Gandhi et al., 2018), microfluidics (O'Kennedy et al., 2018), molecular imaging (Cochran and Cochran, 2010), and proximity ligation assay (PLA) (Blokzijl et al., 2016).

Protein fusion

As the antibody moiety is only responsible for antigen binding, the ability to generate a responsive signal readout for diagnostic requires the addition of another component to the antibody. This can be done either by indirect signal production thru a secondary medium that interacts with the antibody (labeled secondary antibodies) or by direct signal production where the signal producing component is directly attached to the antibody.

A major convenience offered by recombinant antibody is the ability to produce antibody fusions easily by cloning the antibody gene with the gene sequence of other proteins. The resulting construct can be expressed as a fusion protein in any suitable production host. An enzyme frequently fused with recombinant antibody is alkaline phosphatase (PhoA)—an enzyme that catalyzes the phosphate substrate to yield a colorimetric readout for immunoassays, immunoblots and immunocytochemistry. Several studies have demonstrated the construction of scFv-PhoA and Fab-PhoA for direct detection of various biomarkers, toxins, and small molecules such as glycocholic acid (Cui et al., 2018), aflatoxin-B₁ (Rangnoi et al., 2011), HT-2 mycotoxins (Arola et al., 2017), clenbuterol (Liu et al., 2010), plant virus potato leafroll luteovirus (Harper et al., 1997), or receptors and antigens on cells such as *Aspergillus* (Xue et al., 2013), *Bacillus anthracis* (Wang et al., 2006), *Entamoeba histolytica* (Tachibana et al., 2004). Labeling recombinant antibodies with enzymes allows for direct detection which will expedite the diagnosis protocol while omitting the need of secondary antibody binding that could impact the assay negatively due to unspecific binding. Recombinant antibodies are also frequently fused with fluorescent proteins like green fluorescent protein (GFP) for application in ELISA, flow cytometry, cell sorting and immunohistostaining of cells and tissues (Casey et al., 2000).

Besides direct labeling with detectable components, recombinant antibodies can be fused with other protein tags such as SNAP-tag that enables coupling to other fluorophores for immunofluorescence assay. Through this way, the conjugation is controlled at a specific site with a 1:1 stoichiometry (Hussain et al., 2019).

Peptide tag

Another common set up for diagnostic assays is indirect detection of the antigen-bound recombinant antibody using a secondary antibody. This can be achieved through short peptide tags such as polyhistidine tag, Myc-tag, FLAG-tag, Spot-tag, T7-tag, and hemagglutinin (HA)-tag. These short peptide tags serve the purpose for detection in ELISA and immunoblot assay while providing additional functions such as purification and stabilization (Kabayama et al., 2020). Besides that, Avi-tag is commonly engineered to recombinant antibodies for *in vivo* biotinylation of the recombinant antibody. Harnessing the high binding affinity between biotin and streptavidin, the biotinylated antibody allows for highly sensitive immunoassays (Even-Desrumeaux et al., 2010). Biotinylated antibody also allows coupling to magnetic beads for beads assay (Zhu et al., 2014).

The introduction of site-specific labeling methods using enzymes like sortase has also allowed for easy manipulation of recombinant antibodies for site specific tagging. This was demonstrated when recombinant antibodies were cloned with the peptide acceptor motif (LPXTG) of sortase to allow covalent attachment of invertase for direct antibody detection using a glucose meter (Ismail and Lim, 2016). Engineering of short epitope tag in recombinant antibody have also been studied. scFv tagged with HIV-gp36 or -gp41 peptides have shown efficient detection of natural HIV antibodies in human serum through whole blood agglutination assay (Coia et al., 1996).

DNA hybrid

Conjugation of DNA to recombinant antibodies allows translation of the binding signal to DNA level. The detection signal can be amplified through polymerase chain reaction (immune-PCR) or rolling cycle amplification (immune-RCA). Therefore, antibody-DNA hybrid provides a more sensitive diagnostic assay (Sano et al., 1992; Wiener et al., 2020). Besides that, riding on the high specificity of both antibody binding and DNA pairing, the hybrid system allows multiplexing the diagnostic assay (Brofelth et al., 2020). Site directed conjugation of the DNA is possible for recombinant antibody through cloning of protein tags to the antibody (Kazane et al., 2012).

As DNA detection systems are a plenty, the most direct approach is to use DNA chelating dyes to capture the signal of DNA templates amplified from the bound antibodies (Malou and Raoult, 2011). Another approach to utilize antibody-DNA fusion for diagnostic is by the generation of a fusion mixture by combining biotinylated oligonucleotides with biotinylated antigen at specific ratios with streptavidin beads. This setup was used in both a direct and indirect detection system that did not require further addition of dyes or chromophores. The setup was able to carry out direct detection of the antibody as well as conduct a competitive in direct assay to determine the presence of the target antigen in the sample. The sample readout was generated by using the attached oligonucleotide comprising of DNAzyme sequences which is able to fold into G-quadruplex structures and yield the same colorimetric response as peroxidase with the addition of a suitable substrate (Omar et al., 2013).

Nanoparticle conjugate

Nanoparticles are often conjugated with antibodies due to their excellent physiochemical characteristics. One of the examples is colloidal gold nanoparticle (AuNPs). AuNPs is characterized with a bright red color when free floating in solution. Accumulation of antibody conjugated AuNPs on a spot or line coated with antigen will result in visible color. Hence this technique is extensively applied in lateral flow assay to allow rapid, point-of-care diagnostic and detection of chemical such as morphine (Gandhi et al., 2018). Besides that, photostability and the bright color of AuNPs makes gold-antibody conjugates suitable for tumor bioimaging applications (Lin et al., 2021; Qian et al., 2008).

Another class of nanoparticles is quantum-dots (QDs) and carbon-dots (CDs) which are essentially fluorescent nanoparticles. Conjugation of these nanoparticles to recombinant antibodies can be applied for fluorescent imaging of tumor for cancer diagnosis or detection of chemical via fluorescent immunoassay (Sahoo et al., 2019; Zhang et al., 2020). The use of fluorescence allows for a

higher sensitivity as well as the possibility to carry out multiplex assays in arrays for high throughput diagnosis. Carbon nanotubes (CNT) have also been used in this respect for diagnostics. Carbon nanotubes is a hollow cylindrical carbon structure with one or more walls that enables conjugation of various components, including antibodies, to create immunosensors for diseases or cancer biomarker detection (Lerner et al., 2012). The assay is directed via detection of the electrical charge changes (Kierny et al., 2012). The sheer amount of different formats, configurations and modifications afforded by recombinant antibody technology makes it ideal for diagnostic applications. These customizations allows greater freedom in designing diagnostic assays making it a useful and flexible tool to have as a diagnostic tool.

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Protein Electrophoresis, Serum Free Light Chain Assay and Other Biomarkers in Diagnosis and Monitoring of Monoclonal Protein Associated Disease

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Abbreviations

ASCT	Autologous Stem Cell Transplant
BJP	Bence Jones Protein
CE	Capillary Electrophoresis
CIDP	Chronic Inflammatory Demyelinating Polyneuropathy
CR	Complete Response
CRAB	Hypercalcemia, Renal Failure, Anemia, and Bone Disease
DARA	Daratumumab
DFS	Disease Free Survival
DIRA	Daratumumab-Specific Immunofixation Electrophoresis Reflex Assay
ELISA	Enzyme-Linked Immunosorbent Assay
FLC	Free Light Chains
IFE	Immuno-electrophoresis
IMWG	International Myeloma Working Group
IVIG	Intravenous Immunoglobulin
LCMM	Light Chain Multiple Myeloma
MGUS	Monoclonal Gammopathy of Unknown Significance

M-protein	Monoclonal Protein
MR	Minimal Response
POEMS	Polynephropathy—Organic Megaly—Endocrine and Skin
PR	Partial Response
SCLS	Systemic Capillary Leak Syndrome
sCR	Stringent Complete Response
sFLC	Serum Free Light Chain
SPE	Serum Protein Electrophoresis
UPE	Urine Protein Electrophoresis
VEGF	Vascular Endothelial Growth Factor
VGPR	Very Good Partial Response

Introduction

Immunoglobulin monomers are composed of two identical heavy and two identical light, polypeptide chains linked by disulfide bonds, with an overall molecular weight of about 150 kD. The C-terminal portion of the heavy chain defines the immunoglobulin class and subclasses IgG₁₋₄, IgM, IgD, IgE, or IgA₁₋₂. The light chains are called kappa and lambda and are common to immunoglobulins of all classes. A single clone produces either lambda or kappa chains, never both.

IgG is usually found as a monomer which sediments in a density gradient at 7S, while IgM is normally found as a pentamer, 5 units about 150 kDa each, attached by disulfide bonds (with a combined molecular weight about 800,000). Pentameric IgM sediments at 19S. IgA may be found as a monomer in blood or as a dimer in secretions.

The light chains are polypeptides of about 22.5 kDa. They may exist as monomers or as dimers with a molecular weight of about 45 kD (Sølling, 1981). The dimers may be covalently disulfide-linked or non-covalently linked (Peterson and Berggard, 1971). There are about equal amounts of covalent and non-covalent dimeric kappa chain forms in normal serum, but the lambda chain dimers are mainly covalent (Sølling, 1981; Solomon and Weiss, 1987). Normally, more kappa chains are synthesized than lambda, resulting in a normal serum kappa/lambda ratio for intact Ig that is about 2. Free light chains (FLC) are produced in excess of heavy chains as a result of de novo synthesis, and normally polyclonal FLCs spill over into the serum and urine (Peterson and Berggard, 1971; Sølling, 1981).

Parts of the light chain are wrapped around (bound to) the heavy chain and amino acids in wrapped portions are not exposed at the surface. These are called hidden epitopes. For assay purposes, by unravelling the chains, antibodies can be made against both exposed and hidden epitopes. Techniques have been developed for treating a solution of such antibodies against hidden and exposed epitopes with intact Ig so that the those with activity against exposed sites are absorbed out leaving the antibodies against the hidden sites in solution. These antibodies are called antibodies against FLC only and will not react with light chains attached to the heavy chains. These antibodies can be used in assays to detect monoclonal FLCs without reacting with light chains bound to the heavy chains. Light chain antibodies developed against intact immunoglobulins will react with hidden and exposed epitopes and can be used to measure both free and bound light chains.

The immunoglobulins formed early in the process of antibody formation are IgM and IgD isotypes. Upon exposure to antigen, class switching occurs so that IgG, IgA, and IgE isotypes are produced (van Dongen and Wolvers-Tettero, 1991). This process allows for combinations of gene products giving rise to more than 5×10^7 different antibodies providing great diversity and, remarkably, antibodies can be made against all possible antigens beginning with rudimentary minimally specific antibodies and progressing by somatic mutation to highly specific antibodies (Levinson and Miller, 2002; Chiu et al., 2019). Normally immunoglobulins are polyclonal and migrate on electrophoresis as a diffuse group. Monoclonal proteins (M-proteins), also called paraproteins, migrate as a discrete band and were first observed as a protein peak or spike by optical methods in Longsworth et al. (1939). The term M-protein was coined in Moore et al. (1943).

Monoclonal gammopathies occur because of an abnormality of the immune system's B-lymphocytes or B-cells, most often a malignancy of a signal clone that produces excess immunoglobulin of one type that is either kappa or lambda. The diseases associated with M-proteins include multiple myeloma, Waldenstrom macroglobulin anemia (lymphoplasmacytic lymphoma of the bone marrow) or other non-Hodgkin's lymphomas, amyloidosis AL, cryoglobulinemia and less commonly light chain deposition disease, polynephropathy—organic megaly—endocrine and skin (POEMS) syndrome and other neurological manifestations. The normal plasma cell count in bone marrow is less than 5%. In multiple myeloma, the count is invariably greater than 10% and the cells are clonal, but in other disease associated with M-proteins, such as amyloidosis, POEMS syndrome, or cryoglobulinemia, the plasma cells are clonal but the count may be less than 10%. In these conditions, the M-protein itself or other substances related to the dysplastic cells cause the disease.

Besides, the most common manifestation is monoclonal gammopathy of unknown significance (MGUS) that may progress to myeloma over a period of years, be associated with other diseases or be benign. In some rare cases, a small amount of M-protein unassociated with monoclonal disease, may be synthesized due to stimulation of the immune system by a single antigen such as by vaccination, but usually such (a few/several) antigens cause the production of gamma globulins from several clones (oligoclonal). Such gammopathies are usually short lived (Soubrier et al., 1994).

Monoclonal gammopathies represent a proliferation of one cell type from a single germ line causing the clonal synthesis of a single type of immunoglobulin in excess of normal amounts (Attaelmannan and Levinson, 2000). Since the production of M-protein is from a single cell line, the aberrant protein is always either kappa or lambda, never both. Most often the aberrant or dysplastic cell secretes IgG, but in many cases IgM or IgA is secreted and rarely IgD or IgE. Also, in many cases, a monoclonal free light chain is secreted along with the intact immunoglobulin or less often a free light chain is secreted alone, without an intact immunoglobulin and rarely only a heavy chain is secreted—so called heavy chain disease. The monoclonal proteins are important in diagnosis and act as biomarkers so that progress of the diseases can be followed with treatments.

When in the form of a malignancy, the malignant clone suppresses normal Ig producing cells so that normal polyclonal immunoglobulin levels are low. Since polyclonal immunoglobulins represent antibodies against multiple infective organisms and the M-protein usually has no or poor immune reactivity, this leads to humeral immunodeficiency and recurrent infection. Also, the interaction between myeloma cells and the bone microenvironment ultimately causes activation of osteoclasts and suppression of osteoblasts, resulting in lytic bone lesions and hypercalcemia when there is extensive bone involvement (Terpos et al., 2018). Increased bleeding and anemia may also occur for a variety of reasons.

Some intact M-proteins cause type I cryoglobulinemia that may precipitate in small blood vessels, especially those exposed to the periphery where the temperature may be cooler (Voma and Levinson, 2016), causing severe cutaneous involvement (necrosis and ulcers); nephropathy and neuropathy (Neel et al., 2014). Type I cryoglobulinemia, or simple cryoglobulinemia, is usually due to IgM, but rarely IgG or IgA. Also, some M-proteins, especially IgM, may exhibit rheumatoid factor activity and bind to polyclonal IgG causing complexes that can precipitate in small vessels.

If a monoclonal FLC is secreted there is a high probability of renal disease since monoclonal FLC are toxic to the kidney (Levinson and Keren, 1994). Moreover, there is evidence of an association between intact M-proteins and kidney disease (Wang and Hogan, 2018) so kidney disease is common in patients with M-proteins (Tandon et al., 2018). Also, it is possible that some patients with apparent MGUS who exhibit a low level M-protein also produce a monoclonal FLC but of such low level it is undetectable by current methods. Monoclonal FLCs are also prone to cause amyloidosis AL where fibrils may precipitate in the glomerulus, heart, joints, liver and gastrointestinal track causing disease (Levinson, 2012a,b; Vaxman and Gertz, 2019). Patients with this diagnosis often do more poorly, even with apparently successful treatment. For these reasons, it is important to determine whether or not monoclonal FLCs are present by highly sensitive methods.

Generally, if a patient has vague symptoms such as anemia or back pain a monoclonal gammopathy is pretty much ruled out by performing a serum protein electrophoresis (SPE or SPEP) where a monoclonal protein is found about 80–90% of the time when there is a true monoclonal gammopathy. According to the most recent guideline of the international myeloma Working Group (IMWG) (<https://www.myeloma.org/international-myeloma-working-group-imwg-criteria-diagnosis-multiple-myeloma> Accessed 01/08/2021) which are very similar to older guidelines (Dispenzieri et al., 2009). If there is a high suspicion of monoclonal gammopathy, due to multiple symptoms such as pain in the bones, fatigue, tingling or kidney disease, recurrent infection, increased bruising, or unexplained bleeding, the combination of SPE, serum immunoelectrophoresis (IFE), and serum free light chain (SFLC) assay are recommended for initial screening. When amyloidosis AL is suspected, urine protein electrophoresis and urine IFE should be performed in addition. These symptoms are often referred to as CRAB (hypercalcemia, renal failure, anemia, and bone disease).

Methods for detection

Serum protein electrophoresis and immunofixation electrophoresis

Separation of the proteins by SPE

SPE has been performed in clinical laboratories for over 50 years and remains the main stay for M-protein screening. Serum is used rather than plasma because fibrinogen in plasma migrates in the beta-gamma region of the gel and can be mistaken for an M-protein or can mask an M-protein migrating in the same region. In some cases plasma is inadvertently poured into a serum tube and in other cases patients are highly anticoagulated so that fibrinogen is present and interferes with interpretations (Qiu et al., 2003). But follow-up testing by immune fixation electrophoresis will show it is not an M-protein. Protein electrophoresis first developed by Arne Tiselius in 1931 as moving boundary electrophoresis in a liquid for which he won the 1948 Nobel Prize in chemistry. It is based on the principle that proteins which are composed of amino acids, show charges depending on the properties of the amino acids, the configuration of the protein and the pH of the buffer and medium and will therefore migrate when subjected to an electrical field. The migration of proteins in an electric field is largely determined by the charge on the molecule, and the pH of the electrophoretic medium. Different proteins will separate from one another in accordance with the molecular charge. Electrophoresis on a media is called zone electrophoresis. In gels containing a cross-linked matrix, such as acrylamide or starch, the separation may be substantially influenced by the size of the molecules.

For serum protein electrophoresis, a media, kept at pH of greater than 8.0 by a buffer, is placed between positive and negative electrodes and the proteins placed in a well on the media migrate with the current towards the positive electrode (Ramanathan and Srinivas, 2018). After electrophoresis, the proteins in the gels are fixed with a preservative. The gels are then stained with a stain that binds to the proteins and washed to remove excess stain. For clinical laboratory use, over the years, the media has changed from paper to cellulose acetate to agarose, each improving the separation between protein fractions. The buffer has changed from liquid to liquid-solid so that pure liquid is no longer used, and the process has been automated with 50 or more samples run at one time (Fig. 1). Yet, the overall profile has remained the same—clinically described in five fractions: albumin, alpha-1-proteins,

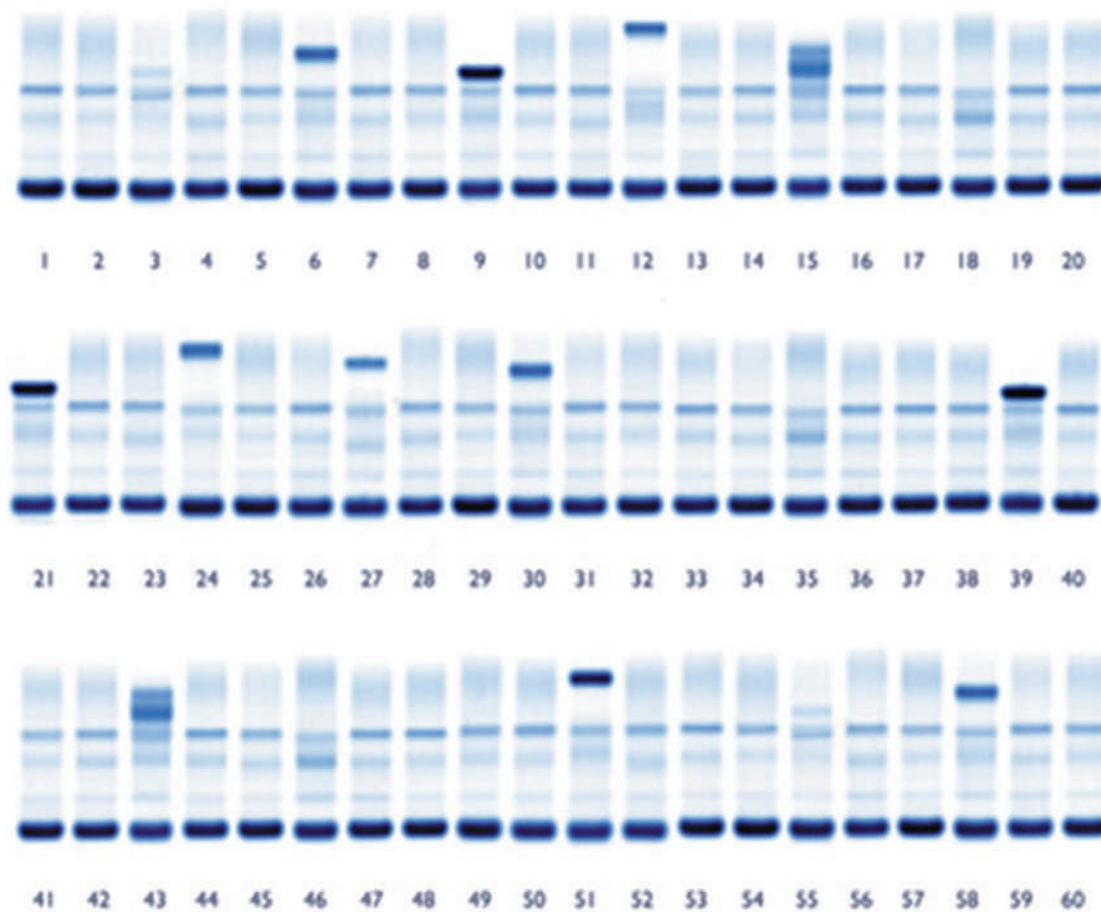


Fig. 1 Multiple samples on one gel. In this example 80 SPE can be analyzed on one gel. The automated densitometer can then profile and print the results. The entire process can be performed in a couple of hours allowing hundreds of samples to be analyzed per day. Interpretation of 80 samples may take a few hours. Although follow-up testing by IFE may take three or four more hours only those with an M-protein needs follow-up testing. Moreover, in hospital laboratories, where patient records are available, follow-up testing is usually not needed for monitoring progress, since the nature of the M-protein can be determined from the record.

alpha-2-proteins, beta-proteins and gamma-globulins with the concentration in each fraction being determined by densitometry. Although nephelometry and turbidometry are more accurate for measuring normal proteins, densitometry is considered the most accurate method for measuring the concentration of M-proteins, as will be discussed in the nephelometry/turbidometry section.

Improved separation has increased the discrimination of M-proteins so that monoclonal banding can be discriminated visually down to about 25–50 mg/dL (Roden et al., 2008). The resolution is dependent on the ionic strength of the buffer and the voltage, with higher voltage and more current causing better resolution.

Moreover, the buffer can be manipulated to cause higher resolution in different parts of the gel. Such resolution is called high resolution protein electrophoresis (HRPE) (Keren, 1994). If resolution is optimized over the whole gel, more bands are seen (Fig. 2) but often resolution is optimized in the gamma region where M-proteins most often migrate, producing more sensitivity for identifying tiny M-proteins. Although HRPE gives rise to 6–9 fractions, the standard densitometer profile remains 5 fractions with the beta-1-proteins and beta-2-proteins combined as shown in Fig. 3A.

Densitometry is the quantitative measurement of optical density through the gel by a spectrophotometer. The densitometer can be used to quantify the protein bands or densities as they are sometimes called. The densitometer can provide the percent of total density in each fraction from which the concentration of each fraction can be calculated from the total protein in the sample. Fig. 3A and B show the densitometer patterns of a normal and a gel containing an M-protein, respectively. Normally, the gamma globulin is diffuse as shown in Fig. 3A, but when a high concentration M-protein is present, the profile will appear as in Fig. 3B with a dense band on the gel and a spike on densitometry. Densitometry can distinguish M-proteins down to about 200 mg/dL while visual examination allows better discrimination down to about 25 mg/dL so that it is important to visually examine the gels.

Immunofixation electrophoresis (IFE)

The proteins are separated by electrophoresis but replicas are run in adjacent lanes. The lanes are treated with specific antibodies. The resultant antigen-antibody complex(es) becomes insoluble and precipitates in the gel as an immune complex. The rate of precipitation depends on the proportions of the reactants, temperature, salt concentration and the pH of the solution. The unreacted

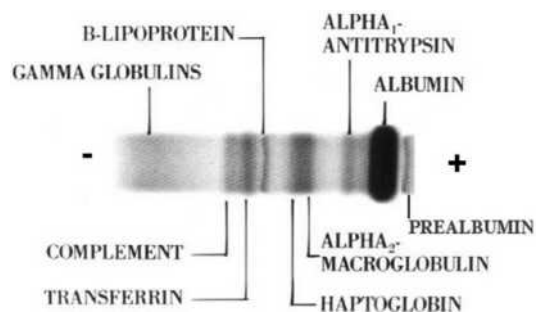


Fig. 2 High resolution electrophoresis. In this case resolution is maximized over the entire gel and nine fractions are seen. Often resolution is maximized in the gamma regions and pre-albumin is not seen.

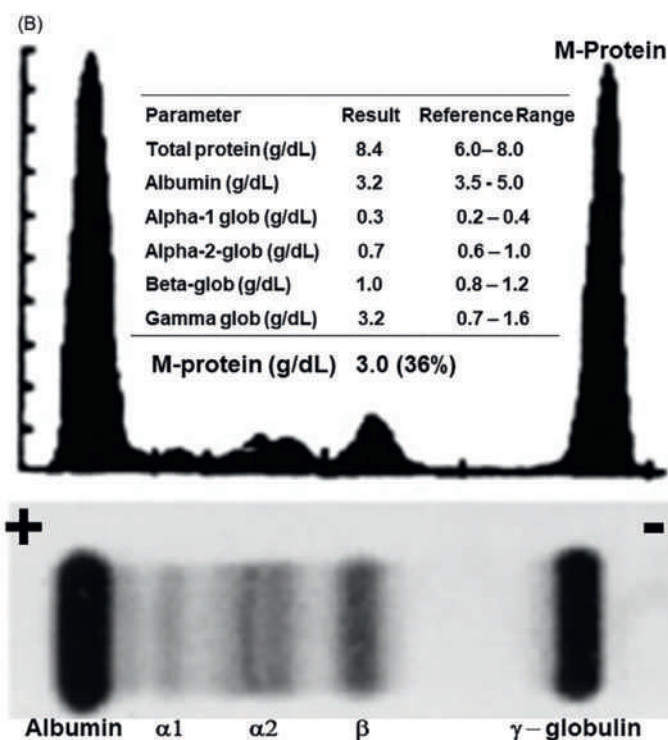
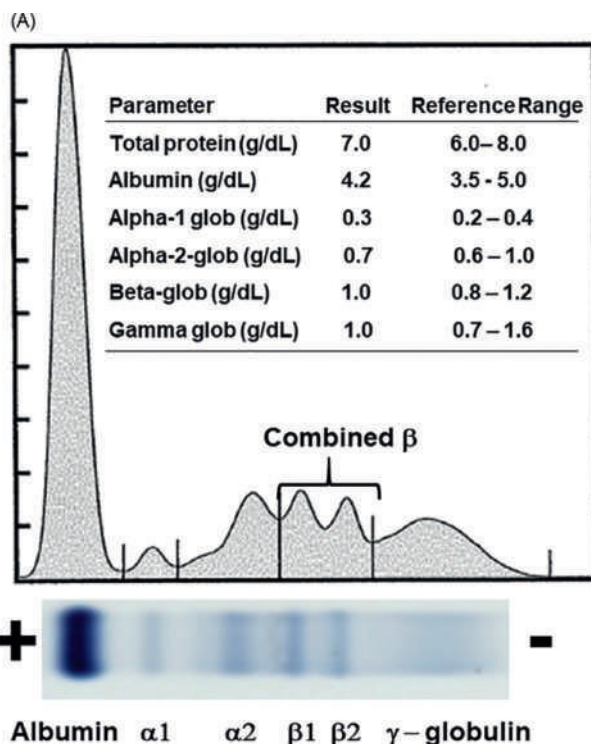


Fig. 3 Normal and M-spike densitometer and gel profiles. (A) Normal profiles. Notice the gamma region shows diffuse staining, indicating polyclonal Ig. (B) M-spike profiles. A large monoclonal restricted band is seen that appears as a spike on densitometry. This is a classical myeloma pattern. Notice the areas adjacent to the dark band are unstained, the diffuse staining seen in Fig. 3A is absent, indicating a decrease in normal Ig.

proteins are removed from the gel by washing and the antigen-antibody complex is best visualized by staining. Agarose is chosen because it is sufficiently porous to allow antibodies to enter the gel but sufficiently dense to trap precipitated immune complexes within the gel. According to the Heidelberg principle, if the antibody concentration is held constant, the amount of precipitate will increase as the antigen concentration increases until a zone of equivalency is reached. This is where maximum precipitation will occur. As the antigen concentration decreases below equivalency there will be less precipitate (antigen and antibody will become increasingly soluble). This phenomenon is called *prezoning*. Also, as the antigen increases above equivalency solubility will again incrementally increase (*prozoning*). *Prozoning* may be a problem with IFE because if the protein of interest (antigen) is at a very high concentration, it may react poorly with the antibody. In such cases, it may be necessary to dilute the sample containing the antigen to bring it back into the zone of equivalency. Generally, *prozoning* is not a problem with serum IFE because the concentration of M-protein is still within a range of equivalency and although there may appear to be a hole in the band as shown in Fig. 4, interpretation is not hindered.

The first method, used in clinical laboratories for diagnosis was immunoelectrophoresis (IEP) that was described by Grabar and Williams (1953). In IEP electrophoretic separation of replicate samples in agarose gels is conducted in multiple lanes in the first step. In the second step, immunoglobulin specific antiserum is placed in preformed troughs cut parallel to each lane. The gels are allowed to incubate in a closed chamber for 24–48 h, while the antisera and antigen diffuses into the gel. The proteins from the patient's serum which were separated by electrophoresis in the prior step migrate by diffusion towards the antisera. A precipitate develops, fixing the immunoglobulins in the form of an arc. Considerable skill is need to interpret the results, which are difficult to relate to routine protein electrophoresis (Levinson, 2009). A shorter narrow arc more distant from the trough and close to the antigen source indicates a decrease in antigen concentration, while a longer, thicker band close to the trough indicates an increased antigen concentration. However, if too high a concentration of protein is present, the precipitate may form very close to the trough or within the trough. The presence of an abnormal immunoglobulin is inferred from the comparing the size, shape, and distortions of the arc with the control. Identification of small abnormal components is particularly difficult.

IFE for M-proteins was described in 1973 by Ritchie and Smith (1976a,b,c) and Cawley et al. (1976) independently, for use in medical laboratories to identify monoclonal immunoglobulins in serum and urine. The major change in currently used methods is the availability of the complete procedure as a kit. As illustrated in Fig. 5, for serum, replicate electrophoresis of samples is performed in multiple lanes. Following this, the fractionated proteins on the electrophoretic strips are precipitated (fixed) into the agarose by overlaying with antisera specific for each type of immunoglobulin to be identified. After a wash step to remove unfixed proteins, (i.e., albumin, alpha and beta globulins) the strips are stained with a protein stain and washed to remove the excess stain. The separated immunoglobulins precipitate in a pattern that is easily related to the pattern on routine agarose SPE without fixation. The procedure can be performed in about 2 h. Moreover, there is greater sensitivity than on routine SPE or UPE without fixation because the mass of the antibodies binding to the specific protein increases the amount of protein that is stained to provide 3–5 × more sensitivity (Levinson et al., 2002). Nevertheless, since the procedure requires at least five lanes (i.e., minimum fixation one lane for each: SPE, IgG, IgA, IgM, kappa and Lambda), and very rarely IgD or IgE, a good deal of time and effort is required for each sample.

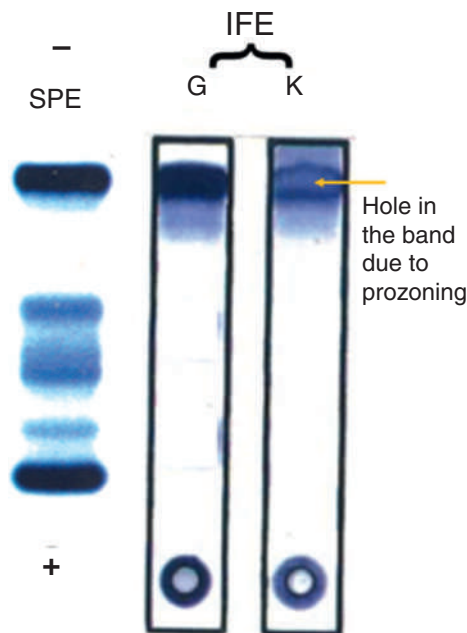


Fig. 4 Serum prezoning due to antigen excess.

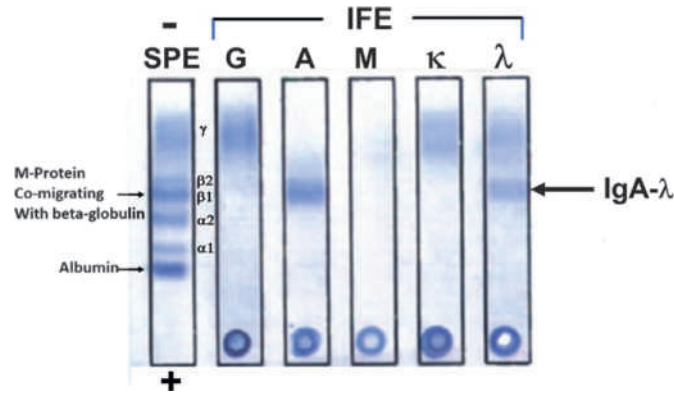


Fig. 5 Illustration of IFE. After electrophoresis, each lane is overlaid separately with antibody specific for the common immunoglobulins: IgG, IgA, IgM, and kappa and lambda light chains—one type antibody/lane, causing each type of Ig to be fixed in the lane. In this case, IFE shows bands staining for IgA and lambda comigrating with the beta-1-globulins. Notice, the beta-1-band on SPE may look denser (darker) than normal (Fig. 3A) but not a lot darker. This increase in band density would be difficult to identify by SPE but the IgA-lambda M-protein is clearly identified as a band by IFE.

Migration of immunoglobulins on electrophoresis

IgG is a monomer and the most positively charged immunoglobulin and generally migrates in the gamma region towards the negative electrode. But immunoglobulins have a wide range of charges depending on their antigenic activity so that occasionally an IgG M-protein will migrate in the beta-region. IgM is a pentamer and because of its large size migrates in the gamma region although occasionally in plasma cell dyscrasia, it is aberrantly produced as an IgM monomer and may migrate faster towards the positive electrode. Also, in some cases two IgM bands are seen—a monomer and pentamer. IgA is the most negatively charged Ig and generally migrates in the beta-region. Occasionally, aberrant cells may synthesize a secretory dimer which is normally found in the digestive tract. In some case, two bands may be seen—a monomer and dimer. Fig. 6 shows an IFE profile defining where each polyclonal class of Ig normally migrate. Nevertheless, the variable region can have a wide variety of charges so, with the exception of pentameric IgM, immunoglobulins can theoretically migrate the entire length of the gel. Thus, occasionally IgG M-proteins are found in the beta region and even the alpha region. As a result, rarely intact M-proteins are identified in all gel areas. Fig. 7A shows IgG and IgA

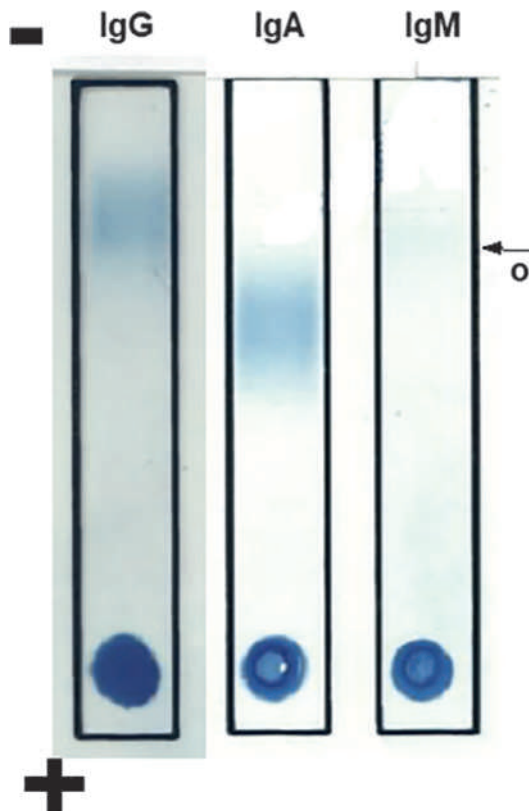


Fig. 6 Migration of normal Ig. Ig from a normal individual was fixed on each lane. IgA is on the average the most negatively charged Ig and usually migrates in the beta region. IgG and IgM migrate in the gamma region with IgG somewhat more cathodally because on the average it has the most positive charge. Nevertheless, rarely a more negative charged IgG M-protein will migrate in the beta-region. Normal pentameric IgM almost always migrates near the origin near where the sample is applied (o). Occasionally, IgM monomeric M-proteins migrate faster even into the beta-region.

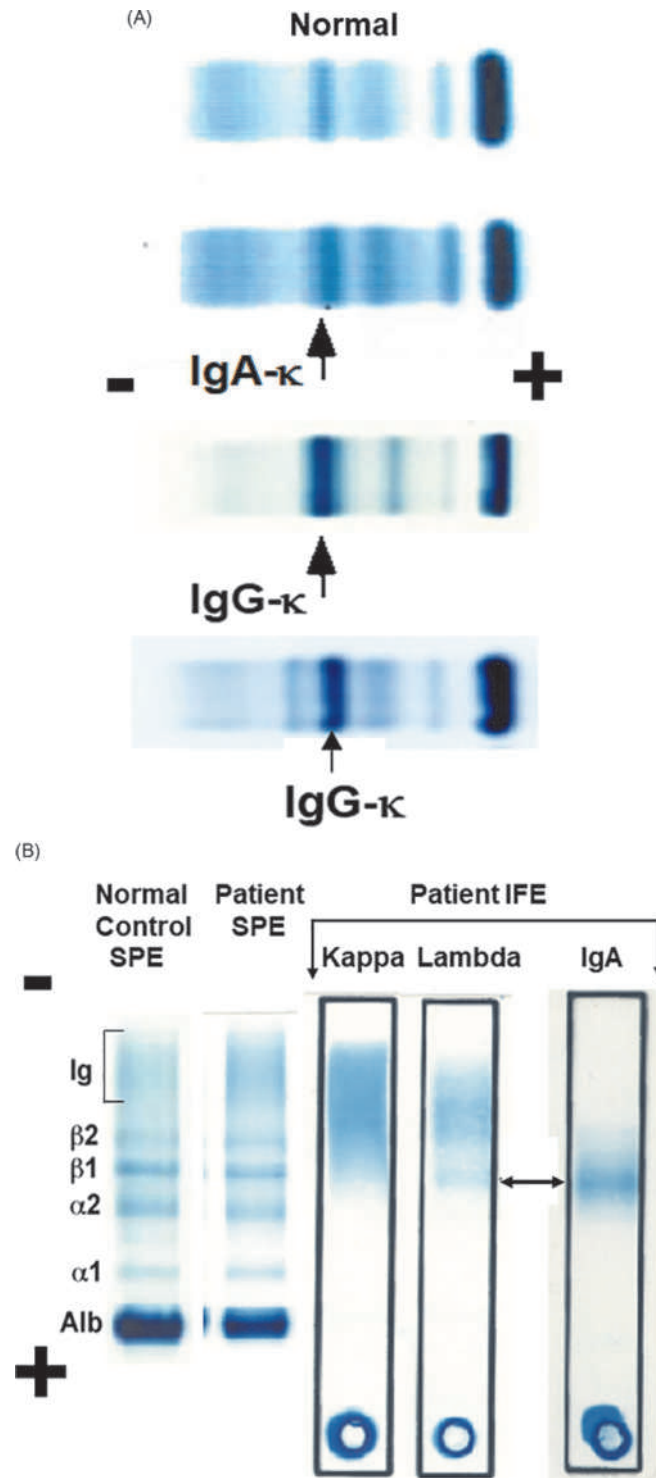


Fig. 7 Migration of M-proteins in the beta-region. There are many more IgG M-proteins than IgA. As such, occasionally IgG, like IgA will migrate into the beta region. (A) IgG and IgM M-proteins migrating in the beta-region. (B) Difficulty in discriminating beta migrating M-proteins. When M-proteins migrate in the beta-region, the beta-protein bands tend to mask the M-protein. If the M-protein is very low concentration, as shown, they may be masked by comigrating normal beta-proteins on SPE and only be picked up by IFE, as shown.

M-proteins migrating in the beta-region. Notice, these M-proteins are migrating with a mobility similar to normal beta proteins and low concentration IgG and IgA with such mobility may not be apparent on SPE because the beta band may appear normal (Fig. 7B). Also, when comigrating with the beta-band, it is not possible to estimate the exact concentration of the M-protein by densitometry. Only a rough estimate can be given by visual extrapolation from the densitometer value. One approach is to subtract the average value of the normal beta fraction from the measured densitometry concentration.

Interpretation of conditions other than monoclonal gammopathy by SPE

Although the main purpose of SPE in the clinical laboratory is to identify M-proteins, in some cases, SPE may be helpful in identifying or confirming some other conditions. These include: (1) Alpha-1-trypsin deficiency when the alpha-1- band is decreased. In this case quantitative measurement and genotyping for Pi types should be recommended. It is important to realize that except for ZZ most other Pi variants may not appear decreased because alpha-1-trypsin is a positive acute inflammatory protein and is often increased sufficiently to appear normal in disease. For this reason, genetic typing, not SPE, is recommended when alpha-1-trypsin deficiency is suspected. (2) Acute inflammation when there is an increased intensity of alpha-1 and alpha-2 and a polyclonal increase in gamma, and chronic inflammation when the albumin is decreased and there is a polyclonal increase in gamma. This latter profile may help confirm chronic infection or autoimmune disease. In autoimmune hepatitis a polyclonal elevation in gamma (often 2–3 ×) is considered an important diagnostic criterion (Friedman et al., 2010). (3) Nephrotic syndrome when the albumin band is substantially decreased due to hypoalbuminemia (often near or below 2 g/dL). In addition, the alpha-2 band may be more distinct and resemble a monoclonal band and the beta may be increased due to a relative increase in beta-lipoprotein. (4) Liver cirrhosis when the albumin band is significant decrease (again often near or below 2 g/dL) with a decrease in the alpha and beta proteins due to failure of liver synthesis and a substantial increase in gamma due to failure of liver Ig protein clearance (Ig breakdown) (Fig. 8). Additionally, beta-gamma bridging is always seen in liver failure. In beta-gamma bridging there is denser staining from the beta-2-proteins through to gamma. Beta-gamma bridging occurs because of a relative increase in IgA that mainly migrates in the beta region (Vavricka et al., 2009). In this case, the densitometer tracing may indicate an increase in the beta region but visual inspection will show that and there is an increase in baseline due to underlying immunoglobulin and that the alpha and beta bands are actually decreased (Fig. 8). (5) IgA nephropathy is often associated with increased serum polyclonal IgA. Thus, if renal disease in a patient is apparent, it is worth commenting on an elevation in polyclonal IgA as a part of an interpretive report.

Urine protein electrophoresis and IFE

Urine protein electrophoresis (UPE or UPEP)

UPE is performed identically to SPE, except it is recommended that the urine should be mechanically concentrated about 100 × prior to analysis (Keren et al., 1999). The reason for concentration is because it is important to identify monoclonal FLC in urine (monoclonal FLC in urine are called Bence Jones proteins in Honor of Henry Bence Jones who first discovered them and who is considered by many as the father of chemical pathology) (Rathore et al., 2012). It is important to recognize that although there may be a substantial amount of protein in urine due to structural kidney damage, the amount of monoclonal FLC may be at a low concentration. Thus, the amount of total protein in the urine is usually unrelated to the amount of BJP. And, although some systems claim to be more sensitive (Rodén et al., 2008), the recommend 100 × concentration seems wise.

UPE is important for identifying the anatomical defect(s) that lead to proteinuria (Peterson and Berggard, 1971; Laurell, 1972; Killingsworth, 1982). Normally proteins below about 40,000 molecular weight freely pass the glomerulus. Those above 40,000 passes less readily decreasing with molecular weight and negative charge. Serum albumin is by far the most abundant plasma protein but its molecular weight is about 67,000 and it is strongly negatively charged. As a result, normally, little albumin is found in the urine. There are about equal amounts of covalent and non-covalent kappa chain dimers in normal serum, while the lambda-chain dimers are largely covalent (Sølling, 1981). As a result, there is less filtration of lambda-chains causing a lower urinary kappa/lambda ratio than the 2/1 found in serum (Levinson and Keren, 1994).

The polyclonal FLCs tend to be positively charged and because of the low molecular weight readily pass the glomerulus. Normally, about 4–5 g/day of protein pass through the glomerulus with all except about 150 mg of protein being reabsorbed by the renal tubules. As illustrated in Fig. 9, normally, the resulting excretion is mainly polyclonal Ig FLCs, causing a dense gamma, some lower molecular weight alpha and beta globulins and a small amount of albumin.

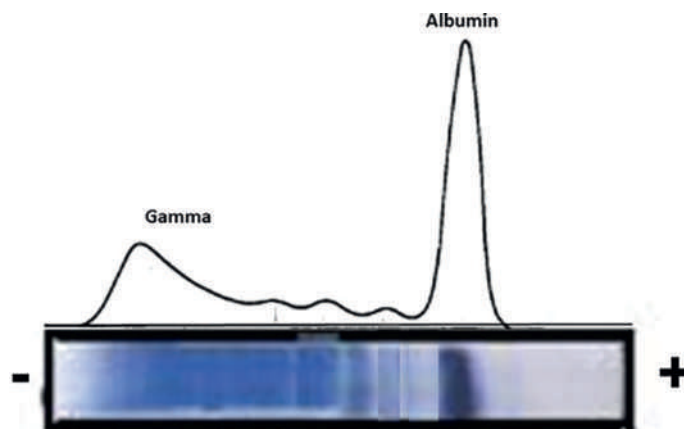


Fig. 8 Cirrhotic pattern with beta-gamma bridging. Notice the densitometer tracing appears elevated for all fractions but this is because all Ig fractions are increased while actually the normal beta and alpha globulins are decreased due to liver failure causing reduced synthesis. IgA turns over faster than IgG and IgM but clearance is reduced in liver failure. As a result, it is relatively increased more than the others, giving rise to beta-gamma-bridging, since IgA tends to migrate in the beta-gamma region.

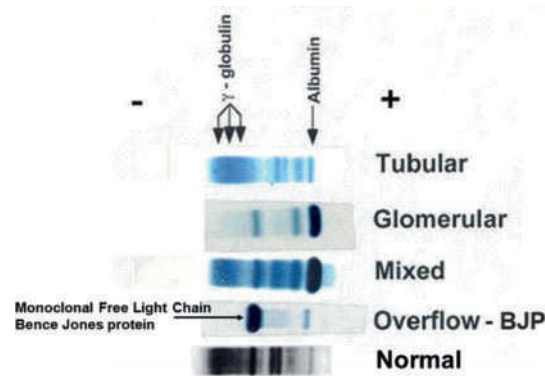


Fig. 9 Proteinuria patterns in disease. Notice, the normal is similar to the tubular since normally only a small amount of albumin is filtered into the urine and a relatively larger amount of gamma-globulin polyclonal FLC is secreted. Thus, the tubular pattern is meaningful only when there is an increase in total urine protein above normal reference limits.

In glomerular disease larger proteins pass more freely than normally through the leaky glomerulus so that large amounts of protein may pass, in some cases more than 3 g are excreted per day in the urine. Much of this protein is albumin (the most abundant protein in plasma). If the tubules are not diseased and still reabsorbing protein small globulins are still reabsorbed and a glomerular pattern is observed (Fig. 9). If the tubules are damaged but the glomerulus remains intact, normal amounts of protein pass the glomerulus but the small globulins cannot be reabsorbed, and more are excreted in the urine. These are largely polyclonal free light chains and alpha-2-microglobulins. This is called a tubular pattern. A tubular pattern is shown in Fig. 9. The tubular pattern is not much different from a normal pattern but the amount of protein excreted is increased. In severe tubular disease as much as 4 g/day of protein can be secreted so on the basis of total urine protein alone, it may appear to be glomerular disease. When both the glomerulus and tubules are damaged a mixed pattern is seen (Fig. 9). Combined glomerular and tubular damage usually give rise to protein excretion >5 g/day.

A high concentration of monoclonal FLC in serum that is filtered by the glomerulus such that the FLC concentration exceeds the tubular capacity for reabsorption, causes monoclonal free light chain to be excreted in the urine and overflow Bence Jones proteinuria occurs. This pattern is shown in Fig. 9. Since monoclonal FLC is toxic to the renal tubules so that the tubules can no longer reabsorb at a normal rate, overflow proteinuria occurs in most cases of monoclonal FLC disease.

Although UPE has value for identifying the anatomical injury, it has poor sensitivity for identifying BJP since the BJP may be masked/hidden by other protein bands in the urine and because immunological identification is required to certify the diagnosis of Bence Jones proteinuria or to help rule it out. For these reasons, UPE is always followed by urine IFE.

Most laboratories measure protein using protein binding dyes. The degree to which these dyes measure albumin and globulin varies and is peculiar to the dye. The degree to which these methods measure FLC is unclear. Very few laboratories use the biuret method which reacts with the peptide bonds in proteins, therefore, measuring all proteins more equally. As a result, except for biuret, these methods give only a rough estimation regarding quantitation of protein. Since the amount of protein estimated in urine is inaccurate in most cases and since the amount of Bence Jones protein in urine is estimated by densitometry from the amount protein in urine, in most cases, the amount of Bence Jones protein is inaccurate. Although some guidelines specify that certain amounts of Bence Jones protein in urine is indicative of extent of disease, it should be understood, that the concentration of BJP obtained from densitometry is usually a rough estimate (Levinson, 2000).

Urine IFE

Edelman and Gally (1962) showed that FLC of immunoglobulins were identical to the protein discovered by Dr. Henry Bence Jones in the urine of a patient with multiple myeloma in Bence Jones (1847) and Rathore et al. (2012). This observation linked the production of monoclonal FLCs to multiple myeloma. Dr. Bence Jones heated the acidified urine at 45–58 °C that produced a precipitate which disappeared upon further heating at 100 °C. The test is still valid but is poorly sensitive and was used to identify monoclonal FLCs in urine until the 1970s when IFE replaced it. Usually, monoclonal FLC in serum are at very low concentration and often comigrate with the beta-proteins so that they are not easily identified by SPE. Sometimes, they are more concentrated in urine which accounts for the visible precipitation by the heat test. But, more often, they are in low concentration in urine which explains why the heat test is poorly sensitive. But, unlike serum, urine can be mechanically concentrated by filtration so that the FLC concentration can be substantially increased prior to electrophoresis.

Urine IFE is performed similar to serum, except the urine is concentrated about 100×, regardless of the amount of protein, using a mechanical concentrator with a filter membrane that retains molecules greater than 20 kD. Twenty hour urine collection is recommended and if a Bence Jones protein is suspected three 24-h urines are recommended for rule out (Keren et al., 1999).

In some cases, viscous urine cannot be concentrated $100\times$, so it is concentrated as much as possible. When concentrating, it is desirable not to let the sample concentrate to dryness since proteins adsorbed on the walls may not be resolubilized. As such, the procedure is time consuming and somewhat tedious. Centrifugal concentrators concentrate more rapidly and reach an equilibrium so the solution cannot dry out but they are not desirable because they concentrate only about $5\text{--}10\times$.

The $100\times$ concentration is designed to provide the sensitivity to pick up very low concentration monoclonal FLCs. Theoretically, since UPE shows sensitivity to about 250 mg/L (25 mg/dL) and IFE is $3\text{--}5\times$ more sensitive, $100\times$ mechanical concentration brings the sensitivity of urine IFE to less than 1 mg/L. This provides great sensitivity for identifying very low concentration BJP, but when there are high concentration BJP, there may be extreme prozoning that can be misinterpreted by inexperienced examiners as no reaction.

Prozoning may be a problem with urine IFE because if the protein of interest (antigen) is at a very high concentration, it may react poorly with the antibody. Thus, urine IFE lacks a simple mechanism whereby dilution and titration produces antibody and antigen equivalency. An example of urine IFE used to identify a kappa-FLC is shown in Fig. 10. In some cases, the concentrated sample must be diluted and rerun causing more tedium. Moreover, since IFE may require eight lanes kappa, lambda, IgG, IgM, IgA and sometimes free kappa and free lambda, the procedure is expensive as well. In Fig. 10, at some high concentrations of kappa-FLC there is no reaction with the assay antibody because of prozoning or preozoning, also, antibody against FLC only has less avidity than that against free and bound light chain, so prozoning with FLC only antibody is always greater than with free and bound antibody.

In 1991, Harrison reported a pseudo-oligoclonal pattern, more commonly called ladder pattern (Harrison, 1991) that may appear in some urine specimens. Generally, the middle bands appear darker. This pattern (Levinson and Keren, 1994) may be a result of homologies in amino acid sequence, in the framework structure of the variable region that gives four chemically defined subgroups of kappa chains and five of the six lambda chains that have been serologically confirmed (Solomon and Weiss, 1987). These represent polyclonal not oligoclonal synthesis. This pattern is shown in Fig. 11. When interpreting urine IFE, it is important not to mistake this pattern for a Bence Jones protein. Moreover, in rare instances, it is possible that this pattern may cause difficulty in interpreting a tiny monoclonal FLC band (Hess et al., 1993).

Capillary electrophoresis

Some large laboratories use capillary zone electrophoresis (CE or CZE) to analyze serum proteins and identify M-proteins (McCudden et al., 2008). The proteins are separated in narrow bore glass capillaries based on charge and partitioning via non-covalent interactions to the wall surface. The proteins are measured by a spectrophotometer at an appropriate absorbance. The electropherogram is displayed in the typical five fractions as with agarose electrophoresis—albumin, alpha-1-globulin, alpha-2-globulin, beta-globulin and gamma-globulin. The sensitivity for identifying M-proteins is similar to that of HRPE, although oligoclonal bands may not be as easily identified (Bossuyt et al., 1998a,b). CE can be completely automated so that many samples can be run rapidly avoiding many of the preparations and steps needed with HRPE, resulting in quicker turnaround of patient results.

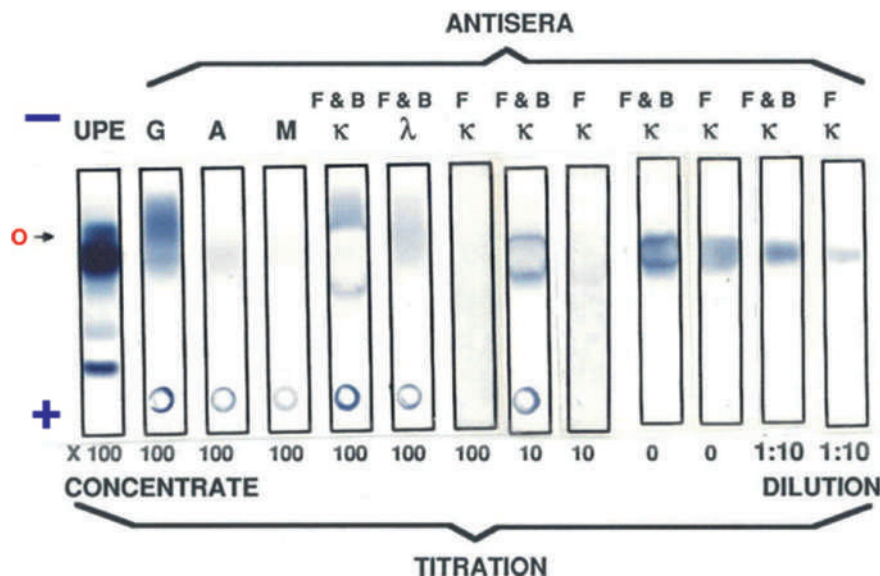


Fig. 10 Urine IFE demonstrating prozoning with dilution. A very high concentration kappa BJP is seen. $100\times$ concentration shows severe prozoning with the free and bound (F & B) appearing washed out in the center. The wash out is much less at $10\times$ concentrate and a clear, distinct band is seen when the urine was diluted $10\times$. The free kappa (F) only track show nothing at the $100\times$ concentrate because antibody against free kappa has less avidity than that against F & B causing severe prozoning - there is no apparent reaction. But at a $10\times$ dilution, Fkappa shows prozoning and at zero dilution a clear, distinct band is seen. When such a high concentration BJP is present, the normal $100\times$ concentrate may have to be diluted and IFE repeated to be certain of the interpretation.

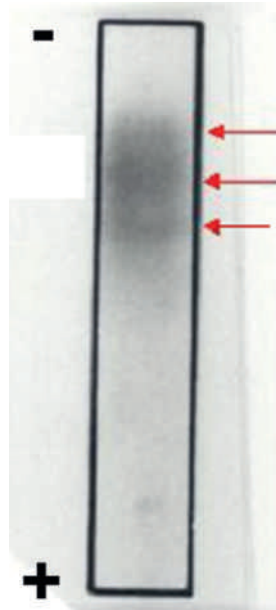


Fig. 11 Ladder Pattern. Although diffuse, thorough examination shows three bands (arrows), usually, the middle band is darker.

CE can also be used for immunologically identifying classes of M-proteins and the nature of the light chain by immunosubtraction (Bossuyt et al., 1998a). For immunosubtraction, the sample is treated with antibody against each class and light chain to remove the M-protein by precipitation and after extraction, the sample is again analyzed. A computer program subtracts the missing area from the analysis prior to treatment to identify and quantify the M-protein. This approach is generally considered to be less sensitive than serum IFE, so most laboratories follow-up CE with IFE to identify M-proteins.

Nephelometry and turbidimetry to measure Ig in serum

Nephelometry and turbidimetry are used for quantifying serum proteins. When the antibody against a protein reacts with the protein, a precipitate forms which at equivalency is proportional to the concentration of the antibody. *Nephelometry* measures light energy scattered or reflected from the precipitate towards a detector that is not in the direct path of the transmitted light while *turbidimetry* measures the change in light when the detector is directly opposite the beam source. Theoretically, nephelometry is more accurate since with a detector at 90 degrees from the light beam there is no scatter until the precipitate appears so the detector should measure zero at baseline and increase to levels much higher than zero as the precipitate forms, while with turbidimetry there is maximum light prior to precipitation so as the precipitate forms the light detected decreases only a small amount from maximum. In actual fact, for measurement of most routine serum proteins the techniques are comparable in accuracy. This is because modern spectrophotometer can accurately measure tiny absorbance changes from maximum as the light decreases and because nephelometers are usually at 30 degrees from the light beam because at a 90 degree angle there is not enough light scatter to measure small increases. The light reaching the detector is compared to the amount from known protein mixtures. The amount of the unknown is determined from a standard curve calibrator. The antigen-antibody precipitate can be enhanced by reaction in polyethylene glycol 6000. Nevertheless, although these methods are sufficient for measuring the major serum proteins they are not sufficiently sensitive to measure proteins below about 10 mg/L (Whicher and Blow, 1980).

These methods are useful for measuring normal Ig but often inaccurate when M-proteins are measured because the antibodies and calibrators used with the assay are developed using the diverse normal immunoglobulins, whereas M-proteins often exhibit limited or incomplete antigenic determinants and may react incompletely with the antiserum and behave peculiarly compared with the calibrator. Thus, the concentration of M-proteins is often under or overestimated. Intact M-proteins are erroneously estimated about 10–20% of the time and FLCs most of the time since they are often peculiar as compared to the intact Ig calibrators and normal Ig (Levinson, 1992). Quantification of the normal immunoglobulin classes not involved in the disease may be useful for determining the functional degree of hypogammaglobulinemia. Because of these peculiar immunological properties, the best way to assess the concentration of a M-protein remains densitometry from SPE or UIPE.

Serum free light chain assays

Usually, monoclonal FLC in serum are at very low concentration that may not be detected by serum SPE or IFE but require urine IFE of a concentrated specimen to detect. Enhanced nephelometry uses antibodies attached to latex particles. This increases the

sensitivity of nephelometry well below the detection limit of 10 mg/L for standard nephelometry. The most widely used current method for assaying monoclonal free light chains in serum (sFLC assay) was developed by Bradwell et al. (2001, 2003). This method is presently available by automated analysis from, The Binding Site Group Ltd. (Birmingham, United Kingdom) as the Freelite assay. This assay uses an enhanced nephelometric assay. Polyclonal antibodies are directed against the hidden portion of kappa and lambda light chains that allows measurement down to concentrations of about 1 mg/L, comparable to urine IFE on concentrated urine specimens. The assay uses polyclonal FLC for calibration in an attempt to reduce some of the peculiar reaction between antibody and monoclonal FLC as compared to standard nephelometry that uses intact Ig as the calibrator. Nevertheless, peculiar reaction with antibodies and interference by polyclonal FLC remains a problem (Levinson, 2016).

Although there are now other assays that may provide similar information the N Latex FLC nephelometric from Siemens Healthineers, Eschborn, Germany and the Sebia FLC, based on solid-phase sandwich enzyme-linked immunosorbent assay (ELISA), Sebia, Evry, France (Schiederdecker et al., 2020), most clinical information has been obtained using the Freelite assay and recommendations of the IMWG are largely based on this assay. Therefore, only the reference ranges and recommendations regarding the Freelite assay will be discussed here. Serum normal 95% reference ranges for FLC kappa and lambda concentrations by Freelite assay have been defined as 3.3–19.4 mg/L for kappa FLC, 5.7–26.3 mg/L for lambda FLC and 0.26–1.65 for the kappa/lambda ratio (Katzmann et al., 2006; Dispenzieri et al., 2009). Up-to-date clinical use for this assay is described by the IMWG at <https://www.myeloma.org/resource-library/international-myeloma-working-group-imwg-guidelines-serum-free-light-chain> Accessed 1/27/2021 but the definitive document remains in journal form (Dispenzieri et al., 2009).

Early data indicated that although polyclonal FLC may be elevated in some diseases causing an increase in FLC kappa and lambda concentrations, an abnormal kappa/lambda ratio indicated myeloma (Katzmann et al., 2002) but later studies showed that kidney dysfunction may require reference ranges as high as 251 mg/L for both serum kappa-FLC and lambda-FLC and can alter the ratio as well (Hutchison et al., 2008a,b). Since many patients with myeloma exhibit renal dysfunction, results of serum FLC analysis is best assessed in the context of other clinical and laboratory indicators.

The assay has many advantages over monitoring BJP by urine IFE and requires only a single serum measurement as compared to collecting and measuring 24 h concentrated-urine collections to identify most cases of monoclonal FLC. Serum can be directly assayed. Although the assay requires many dilutions due to non-equivalent reactions between antibody and antigen, which caused a good deal of tedium in the earliest manual versions, the automated versions provide a rapid, direct way to measure monoclonal FLC in serum. This provides a simpler way to monitor monoclonal FLC changes for changes in disease status. Moreover, there is evidence that high baseline values may have prognostic indications for M-protein gammopathies, especially smoldering (asymptomatic) myeloma, where serum FLC kappa/lambda ratios of <0.125 or >8 or >100 mg/L suggests early treatment may be appropriate (Larsen et al., 2013; Garcia de Veas Silva et al., 2016).

The development of this sFLC assay added a new dimension to the diagnosis and monitoring of plasma cell dyscrasia. In fact, some suggested that urine IFE is no longer needed for diagnosis of FLC diseases and can be replaced by sFLC assays (Katzmann et al., 2006, 2009; Holding et al., 2011). Nevertheless, there are two basic weaknesses that limit its usefulness and is the reason that urine IFE remains important for diagnosis and monitoring of monoclonal FLC.

1. The assay is not quantitative in a proportional way between different patients. This is because monoclonal FLC react differently with assay antibodies than the polyclonal FLC calibrator so that, although it is possible to follow trends within any one patient, it is not possible to quantitate the amount of monoclonal FLC in an absolute sense. Besides, this peculiarity of the monoclonal FLC causes severe over estimation of monoclonal FLC in some cases as compared to densitometry (de Kat Angelino et al., 2010; Levinson, 2011a,b) and substantial underestimation in other cases (Tate et al., 2007, 2009).
2. Since the assay measures polyclonal FLC, the assay is very good for detecting monoclonal FLC during disease progression, when the monoclonal clone has suppressed polyclonal production. But as the disease regresses with treatment the normal clones increase and polyclonal production resumes while monoclonal FLC is suppressed, so that the polyclonal FLC interferes with measurement of the monoclonal FLC at lower monoclonal FLC concentrations. (Levinson, 2011a,b). For this reason, the IMWG recommends that that about 100 mg/L of serum FLC, the assay can no longer reliably distinguish whether or not the monoclonal free light chain is present and urine IFE is recommended when accessing response to therapy (Dispenzieri et al., 2009).

The IMWG (Dispenzieri et al., 2009) recommends that serum FLC replace urine IFE for initial screening for plasma cell dyscrasias and related diseases except amyloidosis AL where urine IFE and serum FLC is still recommended for initial screening. This is because FLC concentrations are often very low in amyloidosis AL and it has been shown that urine IFE is slightly more sensitive while the two assays together pick up more true positives than either alone (Palladini et al., 2009). Moreover, the IMWG recommends serum FLC assay be used only for monitoring free light chain-multiple myeloma, amyloidosis AL, oligosecretory disease and nonsecretory disease when applicable.

IgD and IgE

IgD myeloma is rare, occurring only in about 1–2% of myeloma cases while IgE myeloma has only been reported in about 50 cases (Pandey and Kyle, 2013). When a patient has only staining for light chain in serum but the immunofixation is negative for IgG, IgA, or IgM, the possibility of IgD or IgE monoclonal immunoglobulin must be considered. If there is also a Bence Jones protein in urine, one might conclude the serum band is a FLC, but a Bence Jones protein is associated with IgD myeloma about 70% of the time with

lambda predominating 2–1 over kappa (Djidjik et al., 2015). Therefore, a Bence Jones protein in urine or a positive serum FLC result does not rule out IgD myeloma. Moreover, the concentration of these M-proteins tends to be low and often more difficult to detect than IgG myeloma. IgD myeloma is usually associated with hypogammaglobulinemia (Djidjik et al., 2015), so a small M-protein in serum staining only for light chain and associated with hypogammaglobulinemia would be especially suspect.

The exact diagnosis of IgD myeloma is often delayed, but since treatment for these are the same as for other types of myeloma (Shihabi, 2006), identification of multiple myeloma seems the more important factor. Generally, patients with IgD myeloma do more poorly than those with other types, except light chain myeloma, but there is evidence that the FLC commonly found in this type of myeloma is the main reason for the poor prognosis (Djidjik et al., 2015). Therefore, delay in exact diagnosis may not hinder treatment.

Plasma cell dyscrasias, recommend testing and symptoms

Introduction

The prototype for active multiple myeloma is characterized by a serum M-protein of greater than 3 g/dL with reduced polyclonal Ig, while benign gammopathy typically shows an IgG M-protein less than 2 g/dL (Kyle et al., 2006). Fig. 12 illustrates typical electrophoretic gammopathy profiles in various conditions (Kyle et al., 2007). Active myeloma implies there is end organ damage attributed to the disease (CRAB). In actual fact, some persons with active myeloma may secrete an M-protein of <3 g/dL—so called oligo-secretory (Fig. 14A), no M-protein at all (non-secretory), or a FLC without an intact M-protein (light chain—multiple myeloma), so present recommendations do not define the concentration of the M-protein in active myeloma, while an M-protein of <3 g/dL may be found in benign MGUS. Moreover, IgA M-proteins define a greater risk of developing myeloma (Kyle and Rajkumar, 2011). Also, some persons with so called smoldering (asymptomatic) myeloma exhibit an M-protein >3 g/dL but do not demonstrate end organ damage and may not need other than supportive treatment until organ damage begins to develop. Such a densitometer profile is also illustrated in Fig. 12.

Rarely, two monoclonal bands are identified (Srinivasan et al., 2016). Double banding may indicate a biclonal gammopathy—a mutation in two different clones. If one band is kappa and the other is lambda, it is clearly biclonal but if both bands are kappa or lambda, it is more likely due to asynchronous synthesis in one cell line of the clone or class switching if one band is IgG-kappa and the other IgA-kappa. The incidence of two bands is estimated to be 2–6% and clinically, there is no difference in clinical outcome between two band gammopathy and monoclonal gammopathy (Srinivasan et al., 2016). Two bands may also be observed when there is an intact Ig and a high concentration FLC in serum. Beside it is not uncommon to observe two monoclonal IgA or IgM bands migrating close to one another, where one is a monomer and one a dimer, or one is a monomer and the other a pentamer, respectively. Also, malignant cells can secrete a whole M-protein and a fragment of the protein. Some two band gammopathies are illustrated in Fig. 13A–C.

The present definitions of M-protein associated diseases differ from those of the past. Advances in laboratory and imaging techniques have improved so that the criteria for CRAB features are now well defined and importantly treatment options have greatly improved. Data shows early intervention in high-risk asymptomatic patients can extend survival (Mateos et al., 2013; Rajkumar et al., 2014).

IgM M-proteins rarely presents as myeloma but usually represents a non-Hodgkin syndrome that is readily treatable in most cases. Most IgM M-proteins are kappa and an IgM-lambda may be more reason for concern that myeloma may develop.

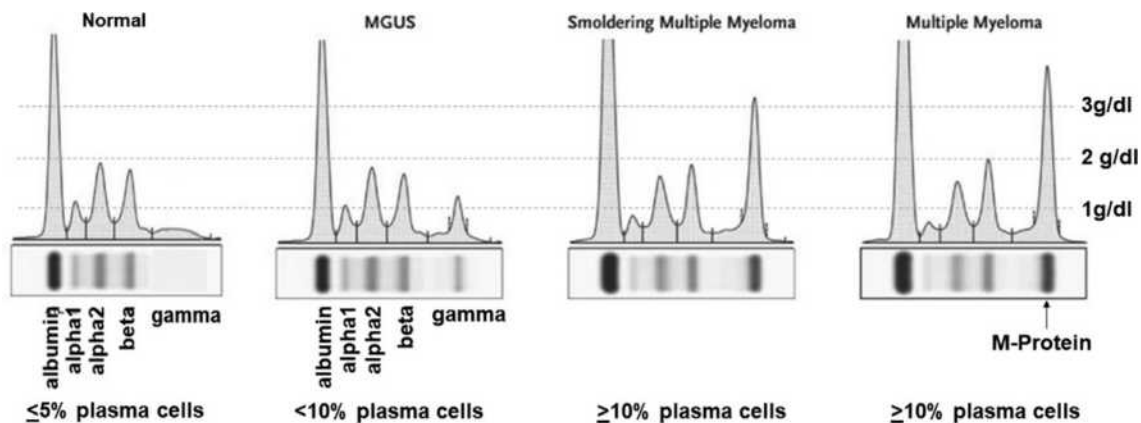


Fig. 12 Typical M-protein patterns.

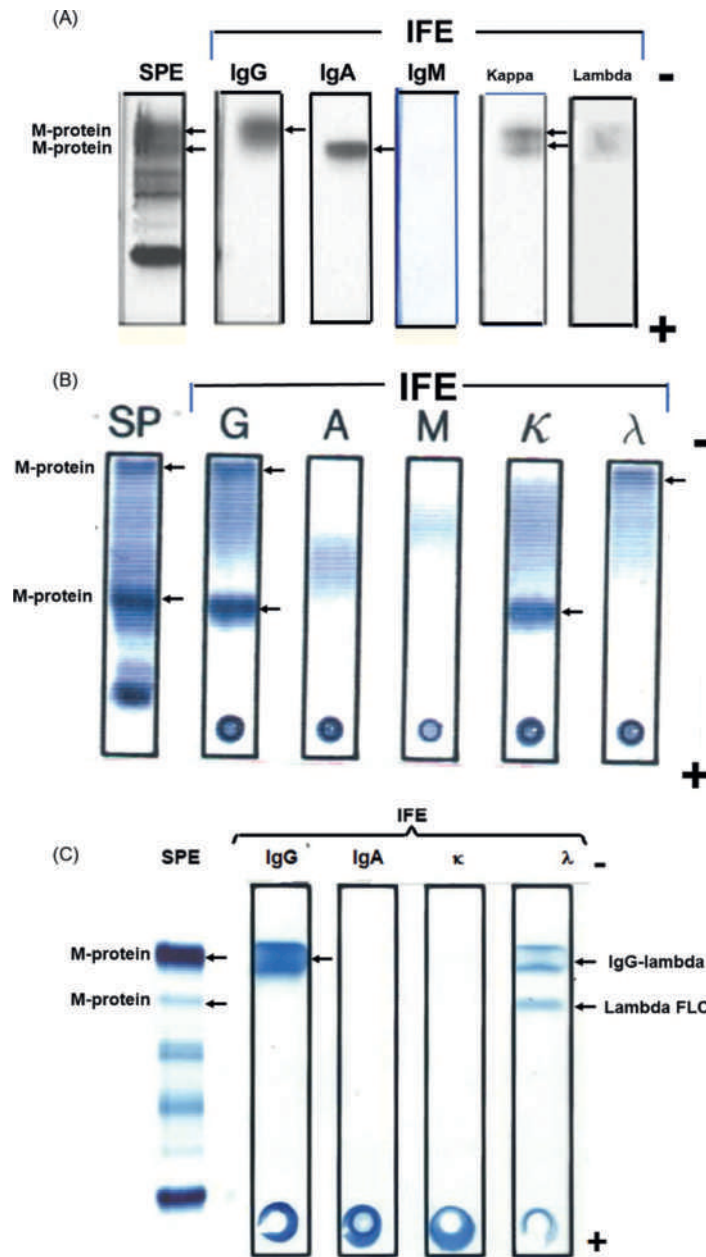


Fig. 13 Multiple M-proteins (bands) gammopathy. Arrows indicate M-proteins. (A) Two band gammopathy due to class switching. The M-proteins are IgG-kappa and IgG-lambda, suggesting class switching from IgG to IgA occurred. (B) Two band gammopathy due to biclonal production. The M-proteins are kappa and lambda, indicating a biclonal gammopathy. Notice the IgG-kappa migrated very rapidly into the alpha-region of the gel. (C) Two band gammopathy due to intact Ig and FLC.

Thus, MGUS has been divided into IgM MGUS and non-IgM MGUS (Kyle et al., 2018). IgM typically arises from a CD20+ lymphoplasmacytic cell that has not undergone switch recombination, and this disease type is associated with a risk of progression to lymphoma or Waldenstrom's macroglobulinemia. In contrast, non-IgM MGUS typically arises from mature plasma cells that have undergone switch recombination, somatic mutation and antigen selection (Bakkus et al., 1992) and is associated with a risk of progression to multiple myeloma. Thus, of 207 patients with IgM MGUS 11 developed Waldenstrom's, 17 others non-Hodgkin's lymphoma, 3 chronic lymphocytic leukemia and zero developed myeloma. Nevertheless, both IgM and non-IgM MGUS can progress to amyloidosis AL (Kyle et al., 2018).

Plasmacytoma is a tumor of plasma cells. The plasma cells in plasmacytoma are identical to those seen in multiple myeloma, but they are not dispersed throughout bone or in blood, instead they form discrete masses of cells. Plasmacytomas usually show lower concentrations of M-proteins than in myeloma with more than 50% of patients with solitary plasmacytoma showing M-proteins of

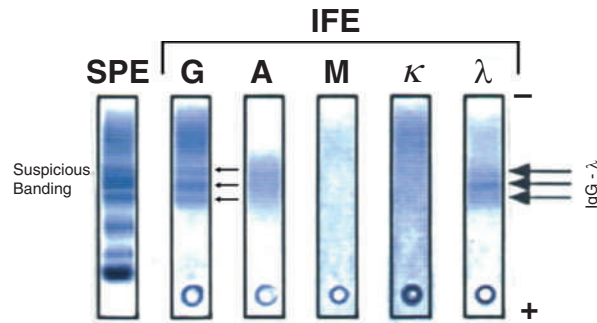


Fig. 14 M-protein associated Plasmacytoma. Notice, that the M-protein is of low concentration but there are three bands (arrows). The pattern appears oligoclonal but all bands are lambda and a plasmacytoma was identified, suggesting the bands were monoclonal. Eventually, this patient developed multiple myeloma. Compare this profile with multiple banding in Fig. 13.

≤ 0.5 g/dL (Dingli et al., 2006). Fig. 14 shows an M-protein pattern from a patient with plasmacytoma. Although some patients with plasmacytoma are cured (Mignot et al., 2020), others progress to multiple myeloma.

Solitary plasmacytoma is a tumor composed of plasma cells that is restricted to a single area of the body. Usually, these tumors are in a bone but sometimes in an organ. Solitary plasmacytoma of the bone can sometimes be cured with radiation therapy, surgery and chemotherapy. However, many people with solitary plasmacytoma eventually develop multiple myeloma (Dingli et al., 2006). Extramedullary plasmacytoma is a tumor that develops in the lungs, sinuses, nasopharynx, or other organs. More than half of people with extramedullary plasmacytoma are cured with radiation therapy (Chao et al., 2005).

FLC multiple myeloma (LCMM) (Rafae et al., 2018) is found in as much as 15% of cases. Plasma cells show rearrangements in immunoglobulin heavy chains at the DNA level resulting in an inability to produce heavy chain (Magrangeas et al., 2004). LCMM appears to have a poorer prognosis as compared to IgG or IgA myeloma. Much of the morbidity and mortality may be associated with renal disease since high monoclonal FLC concentrations in the proximal tubule of the kidney overwhelm the reabsorption capacity resulting in the formation of casts in the distal tubules, causing cast nephropathy (Rafae et al., 2018) and occasionally FLC deposition and crystallization occurs within proximal tubules. Amyloidosis AL that may damage the kidney occurs in about 5–10% of cases.

Fig. 15 illustrates electrophoretic profiles from a patient with LCMM. As in most advanced myeloma cases, polyclonal Ig in serum is reduced. The serum free light chain assay usually shows an abnormal kappa/lambda ratio and urine IFE invariably shows a Bence Jones protein. Often, as shown in Fig. 15, the serum monoclonal FLC is not high enough to observe a band on SPE or the monoclonal FLC is comigrating with the beta proteins so that only hypogammaglobulinemia is seen. In many cases, serum IFE will show a small band. Thus, it is worthwhile to perform serum IFE on all samples showing significant hypogammaglobulinemia. Although there are recommendations by professional societies regarding prognostic and diagnostic indications for cutoff value concentrations of monoclonal FLC in serum and urine, it should be remembered that neither of these methods are constantly quantitative accurate or reliable (Levinson, 2000, 2016).

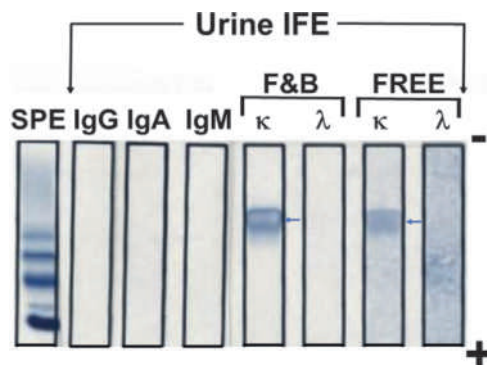


Fig. 15 FLC myeloma. In this case, SPE shows no M-protein but hypogammaglobulinemia. Urine IFE indicates a kappa-M protein (kappa-BJP) (arrows). Hypogammaglobulinemia on SPE with a BJP in urine is a classical picture for FLC myeloma.

Criteria for Diagnosis of Multiple Myeloma and Benign Monoclonal Gammopathy Assessed 1/29/2021: <https://www.myeloma.org/international-myeloma-working-group-imwg-criteria-diagnosis-multiple-myeloma>. For more detail (Rajkumar et al., 2014)

Definition of active multiple myeloma

Clonal bone marrow plasma cells >10% or biopsy-proven bony or extramedullary plasmacytoma and any one or more of the following CRAB features and myeloma-defining events:

- Evidence of end organ damage that can be attributed to the underlying plasma cell proliferative disorder, specifically:
 - Hypercalcemia: serum calcium >11 mg/dL. Hypercalcemia on initial presentation is not common but suggests advanced disease.
 - Renal insufficiency: creatinine clearance <40 mL per minute or serum creatinine >2 mg/dL.
 - Anemia: hemoglobin value of >20 g/L below the lowest limit of normal, or a hemoglobin value <100 g/L
 - Bone lesions: one or more osteolytic lesion on radiological examination.
 - Any one or more of the following biomarkers of myeloma defining events. 60% or greater clonal plasma cells on bone marrow examination. Historically 30% is sufficient
 - Serum involved/uninvolved free light chain ratio of 100 or greater, provided the absolute level of the involved light chain is at least 100 mg/L. The uninvolved free light chain is typically in, or below, the normal range.
 - More than one focal lesion on MRI that is at least 5 mm or greater in size.

Definition of smoldering myeloma

In the past, asymptomatic myeloma was divided into smoldering and indolent. Smoldering was defined as a serum M protein >3 g/dL and/or bone marrow clonal plasma cells $\geq$ 10%, or urinary monoclonal protein estimated to be $\geq$ 500 mg per 24 h and absence of anemia, renal failure, hypercalcemia, and lytic bone lesions (CRAB symptoms) while Indolent multiple myeloma was the same as smoldering except for mild anemia or few small lytic bone lesions and absence of other symptoms. More recently, asymptomatic has largely been replaced by smoldering while the term indolent is no longer in use. The main reason being that therapies have improved so that treatments are initiated sooner, even with minimal symptoms.

Risk factors for progression to active myeloma over 5–15 years are as might be expected: include IgA >risk than IgG, bone marrow plasma cells <20% less risk than 20–50% less risk than >50%, M-protein concentration <4 g/dL less risk than >4 g/dL, more suppression of uninvolved Ig greater risk than less suppression, urinary FLC greater risk than no FLC (Kyle et al., 2007). More recently, very elevated serum FLC (Larsen et al., 2013; Garcia de Veas Silva et al., 2016). Disease management in smoldering myeloma often consists of monitoring every 3 months, treatment with bisphosphonates and supportive care.

Definition of MGUS

Non-IgM MGUS

Benign monoclonal gammopathy with low concentration M-protein in serum, usually IgG, occurs in about 4.6% of persons over 70 years old, about 1.7% of those 50–59 years old and about 6.6% of those over 80 (Kyle et al., 2006). There is a slight predominance in men over women. Fig. 16A illustrates typical electrophoretic profiles for M-proteins that are apt to be benign. Notice that, not only are the M-proteins tiny but the uninvolved Ig appears appropriate in concentration.

Progression to active myeloma depends mainly on the concentration of the M-protein and the age of the patient. Thus with concentrations <5 g/L, 6% progress; with 10 g/L, 7% progress; with 15 g/L, 11% progress, with 20 g/L, 20% progress, with 25 g/L, 24% progress, and with 30 g/L, 34% progress to active myeloma (Kyle et al., 2002). Older persons with very low concentration M-proteins are much more likely to die from something else before developing active myeloma. Generally, periodic monitoring of the concentration of the M-protein and whether or not symptoms have developed is sufficient. In some cases, very low concentration M-proteins will not be present on repeated testing (Strobel, 2003). Rarely oligoclonal banding may be mistaken for a low concentration M-protein.

Oligoclonal banding and FLC-MUGS

Oligoclonal banding usually stains for both kappa and lambda but may stain for kappa or lambda only. In the latter case, it may be very difficult to differentiate from a monoclonal and further work up may be appropriate. Oligoclonal banding may occur when there is a peculiar activation of the immune system (Levinson and Keren, 1989). Fig. 16B illustrates this pattern. Close examination of the IFE pattern shows that the bands in Fig. 16B are both kappa and lambda.

Also, there can be a MGUS associated with monoclonal FLC. This condition was rarely encountered in the past because low concentration monoclonal FLC was only identified in concentrated urine by IFE and urine electrophoresis was not performed on persons without renal disease. But, with the introduction of the simple to perform serum FLC assay, orders for monoclonal FLC are more common.

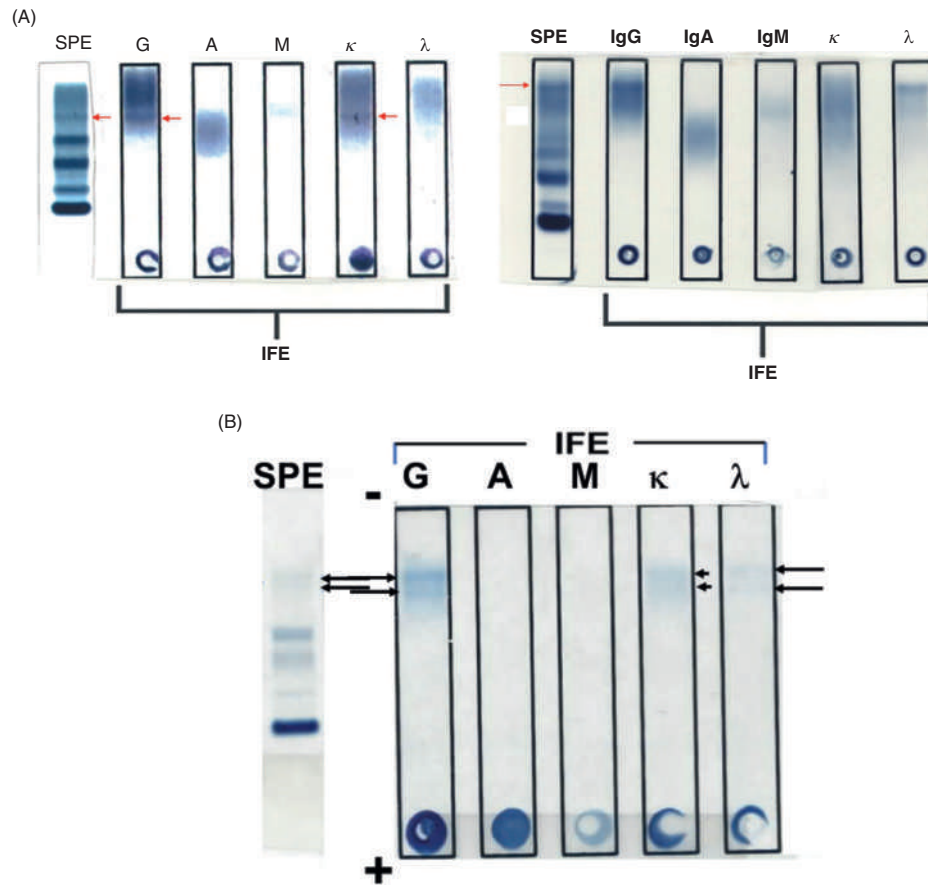


Fig. 16 Low concentration banding. (A) Typical MGUS. Notice polyclonal Ig appears normal. The M-proteins (arrows) are very low concentration: one is lambda and the other kappa. (B) Oligoclonal banding. In this case, the low concentration banding appears oligoclonal since there are two bands (arrows) and close examination shows the banding stains for kappa and lambda. Such oligoclonal banding often disappears eventually. Such banding is very similar to that seen with MGUS (Fig. 16A), especially if the oligoclonal banding is only kappa or lambda.

The IMWG defines non—IgM MGUS as:

- Serum monoclonal protein <30 g/L
- Clonal bone marrow plasma cells <10%
- Absence of CRAB symptoms or amyloidosis that can be attributed to the plasma cell proliferative disorder

and

Free Light Chain MGUS as:

- Abnormal FLC kappa/lambda ratio (<0.26 or >1.65)
- Increased level of the appropriate free light chain (increased kappa FLC in patients with ratio >1.65 mg/L and increased lambda FLC in patients with ratio <0.26 mg/L)
- No immunoglobulin heavy chain expression on immunofixation
- Absence CRAB symptoms or amyloidosis that can be attributed to the plasma cell proliferative disorder
- Clonal bone marrow plasma cells <10%
- Urinary monoclonal protein estimated <500 mg/24 h

The IMWG defines IgM MGUS as:

- Serum IgM monoclonal protein <30 g/L
- No evidence of anemia, constitutional symptoms, hyperviscosity, lymphadenopathy, hepatosplenomegaly, or other end-organ damage (CRAB) that can be attributed to the plasma cell proliferative disorder or amyloidosis.

The IMWG defines plasmacytoma as:

Solitary Plasmacytoma:

- Biopsy-proven solitary lesion of bone or soft tissue with evidence of clonal plasma cells
- Normal bone marrow with no evidence of clonal plasma cells

- Normal skeletal survey and MRI (or CT) of spine and pelvis (except for the primary solitary lesion)
- Absence of end-organ damage such as hypercalcemia, renal insufficiency, anemia, and bone lesions (CRAB) or amyloidosis that can be attributed to the plasma cell proliferative disorder

Solitary plasmacytoma with minimal marrow involvement:

- Biopsy-proven solitary lesion of bone or soft tissue with evidence of clonal plasma cells
- Clonal bone marrow plasma cells <10%
- Normal skeletal survey and MRI (or CT) of spine and pelvis (except for the primary solitary lesion)
- Absence of end-organ damage such as hypercalcemia, renal insufficiency, anemia, and bone lesions (CRAB) or amyloidosis that can be attributed to the plasma cell proliferative disorder

POEMS syndrome and other neurological complications of M-proteins

An estimated 10% of polyneuropathies are associated with M-proteins (Ropper and Gorson, 1998; Dispenzieri and Kyle, 2005; Chaudhry et al., 2017). These include: multiple myeloma and Waldenstrom macroglobulinemia, amyloidosis AL, cryoglobulinemia, POEMS syndrome, MGUS or polyneuropathy associated with MGUS and chronic inflammatory demyelinating polyradiculoneuropathy, more commonly called chronic inflammatory demyelinating polyneuropathy (CIDP) where about 10% of patients exhibit an M-protein (MGUS) (Kelly Jr. et al., 1981). Also, some therapies for plasma cell disease such as thalidomide, and bortezomib can cause neurotoxicity and have become a leading cause of peripheral neuropathy in plasma cell dyscrasias (Mohty et al., 2010).

Whereas IgG is the most common class of paraprotein in patients with MGUS, IgM is more frequent in those with neuropathy (60%), followed by IgG (30%) and IgA (10%). These antibodies with apparent unknown significance, especially IgM, may have antibody activity against myelin associated protein and also cross react with other glycoproteins with similar structure in myelin (Ropper and Gorson, 1998; Chaudhry et al., 2017). Because of the frequent association of M-proteins with polyneuropathies, most neurologists request SPE on all such patients. Many of these monoclonal gammopathies are very low concentration tiny bands so that all SPE samples from persons with polyneuropathies should be examined visually and it is wise to perform IFE on such samples even if no visible band is observed.

POEMS is a paraneoplastic syndrome due to an underlying plasma cell neoplasm characterized by disease in diverse organs with multifarious symptoms. The major criteria that must always be present are polyneuropathy and an M-protein. Although there is always a clonal plasmacytosis, usually it tends to be mild, usually less than 10% plasma cells with a low concentration M-protein. Since M-proteins are commonly associated with polyneuropathies, POEMS syndrome diagnosis is often delayed. Many patients with POEMS syndrome have an initial diagnosis of CIDP. CIDP is similar to Guillain-Barre syndrome (GBS) but can be distinguished from GBS by its relapsing and progressive course (Jones et al., 2011). It is important to differentiate between CIDP and POEMS since CIDP is treated with immunosuppressive, intravenous immunoglobulin IVIG, or plasma exchange therapy (Ropper and Gorson, 1998; Dispenzieri, 2011). On the other hand, POEMS is clearly linked to plasma cell dyscrasia and most successful outcomes have been achieved by directing therapy towards the clonal plasma cell disorder. Autologous stem cell transplantation (ASCT) (Zhao et al., 2019) appears to be an effective treatment (D'Souza et al., 2012; Cook et al., 2017).

Other major criteria include, Castleman disease, vascular endothelial growth factor (VEGF) elevation and osteosclerotic bone lesions, rather than lytic. In fact, POEMS syndrome is synonymous with osteosclerotic myeloma (Kelly et al., 1983; Miralles et al., 1992). It should be noted that there is a Castleman disease variant that occurs without evidence of a clonal plasma cell disorder that should be considered separately (Dispenzieri, 2019). Besides minor criteria associated with POEMS syndrome include organomegaly, extravascular volume overload (edema, pleural effusion, or ascites), endocrinopathy, skin changes, papilledema and thrombocytosis/polycythemia.

VEGF is a potent multifunctional cytokine that was first identified as a tumor secreted protein. It both induces angiogenesis and is a potent vascular permeabilizing agent, with a potency 50,000 × that of histamine (Dvorak et al., 1995). VEGF is the most recent of the specific criteria. It rises to 1500 pg/mL or above in POEMS syndrome (Watanabe et al., 1996, 1998; Scarlato et al., 2005). Plasma VEGF was not elevated in myeloma (Soubrier et al., 1997; Briani et al., 2011). Nor was VEGF elevated in the serum of patients with other plasma or B-cell dyscrasias including: Waldenstrom macroglobulinemia, MGUS (Watanabe et al., 1996; Soubrier et al., 1997; Scarlato et al., 2005), amyloidosis (Briani et al., 2011), or patients with GBS, CIDP, or other neurological diseases where average values in studies were less than 500 pg/mL (Watanabe et al., 1996, 1998; Soubrier et al., 1997; Briani et al., 2011).

Systemic capillary leak syndrome (SCLS) is an extremely rare disease of plasma extravasation and vascular collapse resulting in hypotension and shock with less 126 cases reported (Amoura et al., 1997; Wall et al., 2011) Like POEMS, nearly all patients with this SCLS have been shown to have a low concentration M-gammopathy with less than 10% plasma cells in bone marrow and an elevated VEGF above 1500 pg/mL.

POEMS seems to affect men in about a two to one ratio as compared to women and although the average age appears to be about 50, POEMS has been diagnosed in those as young as 27 and as old as 80 (Nakanishi et al., 1984; Soubrier et al., 1994; Dispenzieri et al., 2003). In an investigation of 99 patients (Dispenzieri et al., 2003), most patients exhibited a low concentration M-protein with only 7 showing a M-protein of >20 g/L. 86% had less than 10% plasmacytosis, 97% of 99 patients exhibited osteosclerotic

lesions, 44 patients had an IgA M-protein, 40 an IgG M-protein and only 1 an IgM. All were lambda-gammopathies. These results were largely consistent with two other studies (Nakanishi et al., 1984; Soubrier et al., 1994).

It has been hypothesized that like SCLS many of the extravascular volume overload manifestations of POEMS including papilledema, peripheral edema, pleural effusion, ascites and polyneuropathy are caused by the VEGF and other permeabilizing cytokinins (Watanabe et al., 1998; Briani et al., 2011). VEGF increased microvascular permeability of the blood brain barrier in an animal model inducing endoneural edema (Dispenzieri, 2011) and there appears to be a correlation between VEGF serum levels and endoneural vessel damage in patients (Scarlato et al., 2005). Like VEGF, N-terminal propeptide of type I collagen may be a future novel marker for POEMS syndrome (Wang et al., 2014; Dispenzieri, 2019).

Lambda M-proteins have been considered characteristic for POEMS syndrome but kappa-M proteins have been well described (Dursun et al., 2005; Levinson, 2012a,b; Pulivarthi and Gurram, 2018). As a result, patients with kappa M-proteins may be considered to have CIPD or simply MGUS associated polyneuropathy.

These methodological and clinical issues highlight the difficulties in the investigation of POEMS. The M-protein is usually of low concentration and easily mistaken for MGUS. Moreover, bone marrow analysis often shows only a slightly elevated or a normal level of plasma cells and lytic lesions are often absent. The syndrome may be mistaken for polyneuropathy associated with MGUS (Sghirlanzoni et al., 2000) or CIDP. Although kappa-gammopathies have been found associated with POEMS syndrome when osteosclerotic lesions and λ -M-proteins are not present, clinicians may be reluctant to diagnosis POEMS. They may ascribe the condition to the more benign polyneuropathy associated with MGUS that is more often kappa than lambda (Ropper and Gorson, 1998; Dispenzieri and Kyle, 2005) or to MGUS associated with CIDP. These are reasons why it is some important to assess carefully all the criteria for diagnosis (Dispenzieri, 2019).

Also, M-proteins associated with POEMS syndrome are usually at low concentrations and more than 50% are IgA that may co-migrate with the beta-proteins. Such M-proteins may not be obvious and may be difficult to detect by SPE. As with other polyneuropathies, it is important that IFE be performed on all specimens if POEMS syndrome is suspected and a M-protein is not identified by SPE. Also, if no M-protein is identified by SPE and serum FLC and urine IFE should be ordered to rule out a FLC (Dispenzieri, 2019). Fig. 17 illustrates a low concentration IgA M-protein co-migrating with the beta-1-proteins that was identified in a patient with a diagnosis of POEMS syndrome.

The IMWG defines POEMS Syndrome as:

- Polyneuropathy mandatory major criteria is required for diagnosis.
- Monoclonal plasma cell proliferative disorder mandatory major criteria is required for diagnosis
- Any one of the three other major criteria is required:
 1. Sclerotic bone lesions
 2. Castleman's disease
 3. Elevated levels of VEGFA
- Any one of the following 6 minor criteria is required:
 1. Organomegaly (splenomegaly, hepatomegaly, or lymphadenopathy)

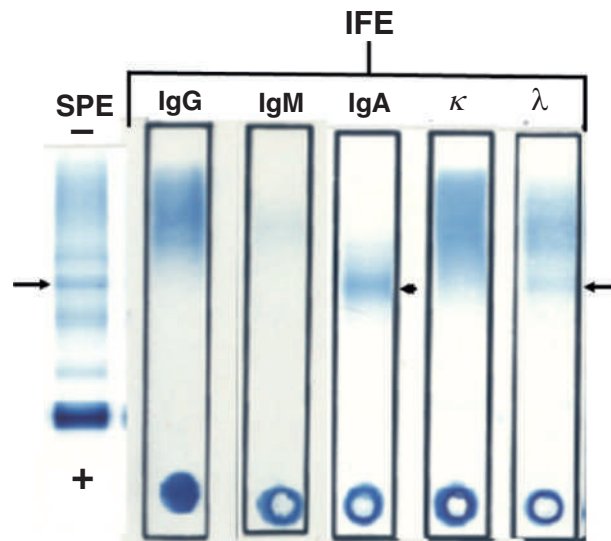


Fig. 17 IgA-lambda M-protein in POEMS. Notice that the IgA-lambda M-protein (arrows) is co-migrating with the beta-2-globulin and although the SPE beta-2-band appears a bit more dense than normal, there appears to be appropriate amounts of polyclonal Ig, making this M-protein very difficult to detect by SPE. This M-protein is only truly apparent on IFE. Since about 50% of M-proteins in POEMS syndrome are IgA, IFE should always be performed when no M-protein is apparent and POEMS syndrome is suspected.

2. Extravascular volume overload (edema, pleural effusion, or ascites)
3. Endocrinopathy (adrenal, thyroid, pituitary, gonadal, parathyroid, pancreatic)
4. Skin changes (hyperpigmentation, hypertrichosis, glomeruloid, plethora, acrocyanosis, flushing, white nails)
5. Papilloedema
6. Thrombocytosis/polycythemia

Amyloidosis AL

Amyloidosis is a deposition of proteinaceous material in a tissue. When viewed by X-ray diffraction, these deposits form fibrils in an anti-parallel conformation and beta-pleated sheet structure (Vaxman and Gertz, 2019). The exact reason that some proteins form this structure is poorly understood. Infiltration of tissues by amyloid causes dysfunction. When amyloidosis is suspected a biopsy of tissue will exhibit apple-green birefringence in the polarizing microscope when stained with Congo red. The simplest tissue to biopsy is the abdominal fat pads. If amyloidosis is suspected and the fat pads are negative, it is best to biopsy the tissue involved (Vaxman and Gertz, 2019). Table 1 outlines the more common types of amyloidosis along with the abnormal protein and tissues involved (Lachmann et al., 2002; Hassan et al., 2005; Shah et al., 2006; Vaxman and Gertz, 2019).

The most common tissues that lead to diagnosis of amyloidosis AL are the kidney and heart. Although the central nervous system is usually not affected, peripheral nervous system complications are common, especially carpal tunnel syndrome. It is important to differentiate ATTR most commonly wild type (Senile, Table 1) from amyloidosis AL in the heart because Senile amyloidosis is slow progressing while AL is rapid (Shah et al., 2006). Moreover, the ATTR (Senile) and ATTR (familial/mutant) are treated differently than amyloid AL. Amyloidosis of the heart is a cause of heart failure and is often recognized by echocardiology as a mainly restrictive cardiomyopathy with conduction system disturbances. A granular sparkling refractile myocardium that along with septal wall thickening is pathognomonic for the disease. Recent guidelines have been published (Garcia-Pavia et al., 2021). Amyloidosis of the kidney is often suspected as a result of proteinuria.

Because amyloidosis AL is the most common type of amyloidosis, after positive biopsy for amyloid, the serum FLC assay should be performed (elevation in kappa or lambda and/or kappa/lambda ratio) and concentrated urine should be examined for Bence Jones proteinuria. If these are positive for monoclonal FLC, this is presumptive evidence for amyloidosis AL. If negative for FLC bone marrow can be examined for kappa/lambda dominance (Falk et al., 1997; Vaxman and Gertz, 2019). If still negative for FLC, immunochemistry for AA and genetic testing for the other types of amyloidosis can be conducted (Falk et al., 1997).

Amyloidosis AL is more often lambda than kappa with a 2/1 ratio and there is some evidence that lambda amyloidosis is more aggressive. The median age for amyloidosis AL is 60 years old. It is important to identify a monoclonal FLC because MGUS is fairly common in older people and it has been shown that 34 of 350 patients (9.7%) were misdiagnosed as AL because a MGUS was found, when they actually manifested other types of amyloidosis (Lachmann et al., 2002). Most of these misdiagnoses occurred because, although a MGUS was identified which led to the assumption of amyloidosis AL, monoclonal FLC was absent.

Amyloidosis AL is usually associated with myeloma or less often Waldenstrom's macroglobulinemia. When amyloidosis AL occurs in the absence of overt plasma cell or lymphocyte dyscrasia, it may be difficult to detect because bone marrow commonly shows <5% plasma cells and is amyloid positive only about 30% of the time. A monoclonal FLC is often not observed by SPE or serum IFE because the concentration in serum is too low and serum polyclonal immunoglobulins are often adequate (not depressed as in FLC myeloma). Fig. 18 illustrates SPE and urine IFE from patients with amyloidosis AL. Urine IFE has been shown to be somewhat more sensitive than serum FLC for identifying monoclonal FLCs in amyloidosis AL (Palladini et al., 2009), although

Table 1 Commonest types of amyloid.

Type	Abnormal protein	Tissues
AL (previously primary)	Monoclonal light chain Lambda/kappa 2/1	Heart Gastrointestinal track and Liver Kidney Peripheral neuropathy Soft tissues: Carpal tunnel syndrome, Macroglossia are common
AA (secondary)	Amyloid A protein (AA)	Kidney Gastrointestinal track and Liver
ATTR (familial/mutant TTR)	Transthyretin (TTR) (Prealbumin)	Peripheral neuropathy Heart Kidney
Senile systemic ATTR	Transthyretin (wild type)	Heart Diffuse organ involvement
Other familial types	Fibrinogen A α (most common) Apolipoprotein A-I Lysozyme	Peripheral neuropathy Heart Kidney

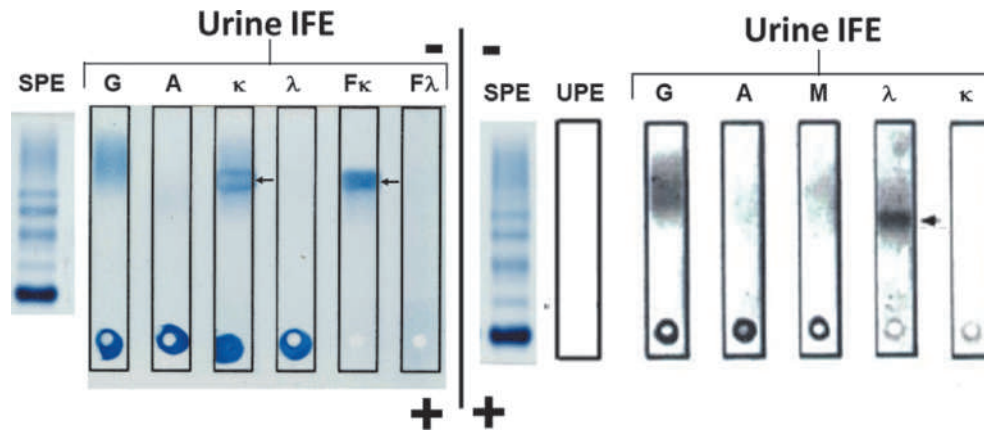


Fig. 18 Profiles in Amyloidosis. Notice a M-protein (BJP) is seen in urine but not M-protein is apparent by SPE. Moreover, even with $100\times$ concentration of the urine, the BJP may be at too low a concentration to be apparent on UPE but only show up on the more sensitive IFE. Compare these very low concentration profile with the very large concentration BJP from a patient with light chain myeloma seen in Fig. 10 where a very large concentration BJP caused prozoning until diluted.

both are recommended for initial screening as they complement one another. As with other plasma cell dyscrasias treatment is aimed towards clonal plasma cell disorder with ASCT being a promising treatment (Hayashi et al., 2014; Sidiqi et al., 2018).

IMWG criteria for amyloidosis AL

Presence of an amyloid-related systemic syndrome (e.g., renal, liver, heart, gastrointestinal tract, or peripheral nerve involvement)

- Positive amyloid staining by Congo red in any tissue (e.g., fat aspirate, bone marrow, or organ biopsy).
- Evidence that amyloid is light-chain-related established by direct examination of the amyloid using mass spectrometry-based proteomic analysis or immunoelectronmicroscopy.
- Evidence of a monoclonal plasma cell proliferative disorder (serum monoclonal protein, abnormal free light chain ratio, or clonal plasma cells in the bone marrow).

Waldenström macroglobulinemia

Waldenström macroglobulinemia is characterized by an excess of lymphoplasmacytic cells in the bone marrow—classified as a lymphoplasmacytic lymphoma which is a non-Hodgkin lymphoma. The abnormal cell has characteristics of B-type lymphocytes and of plasma cells. This condition was first identified in Waldenström (1958). These lymphoplasmacytic cells secrete IgM, usually kappa, and rarely lambda. Most, patients have a preceding phase of IgM MGUS (Gertz, 2019), but the clonal MGUS B cells already contain the molecular signature of a malignant clone—the cells arise from $CD25^+$ $CD22^+$ low activated B lymphocytes - and there is a family history in about 4% of affected persons (Gertz, 2019). In most cases, the disease can be readily treated so that it is benign in most affected persons and considered indolent.

Most disease manifestation are due to the infiltration of the bone marrow by the malignant cell mass and complications due to high IgM. These include normocytic anemia, hyperviscosity syndrome, cryoglobulinemia, cold agglutinin disease hemolytic anemia, adenopathy (enlargement of the lymph nodes and/or hepatosplenomegaly due to accumulation of lymphocytic cells). The earliest symptom is often anemia due to bone marrow infiltration. Many cases are picked up incidentally as a result of elevated total protein or a SPE screen.

Because IgM is a pentamer hyperviscosity syndrome may occur at concentrations greater than 4 g/dL. Moreover, it is important to watch for hyperviscosity symptoms in case hyperviscosity occurs at lower concentrations of IgM. The symptoms are primarily due to shear forces that rupture venous channels and include: epistaxis, gingival bleeding, and visual changes due to retinal hemorrhage as well as central nervous system findings such as dizziness, light-headedness, and generalized fatigue. Hyperviscosity syndrome can be immediately treated with plasma exchange until the underlying cause is reduced by treating the tumor mass. The normal reference range for serum viscosity is 1.4–1.8 Centipoises (1.0 being the viscosity of water). Symptoms usually are not seen before the viscosity reaches four Centipoises (Gertz, 2019). Fig. 19 illustrates a SPE pattern from a patient with hyperviscosity syndrome.

Amyloidosis AL occurs in only about 3% of Waldenström patients (Thakral and Kanagal-Shamanna, 2016) but if amyloidosis AL or cryoglobulinemia occurs, the disease is more difficult to treat and less indolent. Cryoglobulinemia may be type I where the structure of IgM causes precipitation in cooler temperature or type two where the monoclonal IgM exhibits rheumatoid factor activity and binds to the Fc portion of IgG, causing immune complexes and precipitation at cooler temperatures. These precipitants may cause vasculitis and renal disease (Charles and Dustin, 2009). Cryoglobulins will be discussed in more detail below.

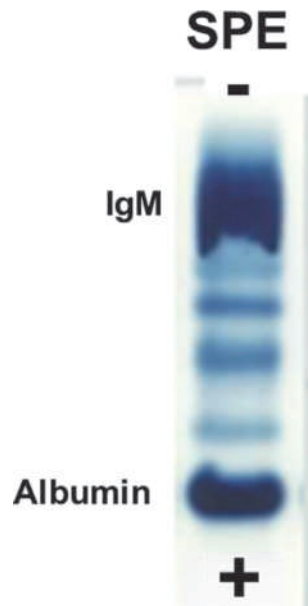


Fig. 19 IgM with Hyperviscosity syndrome.

Interference by circulating immune complexes

In some rare cases, monoclonal or polyclonal Ig may exhibit rheumatoid factor activity and form circulating immune complexes by binding to the Fc portion of polyclonal IgG. These are usually IgM but may also be IgG. These do not precipitate in the cold as cryoglobulins do, but there is some evidence that such large complexes can cause vasculitis (Levinsky).

Methodologically, these large aggregates can cause confusion in interpretation. If the RF affinity is strong, upon IFE, they may become trapped in the agarose gel and appear as monoclonal bands in all lanes. If the affinity is weaker, polyclonal or monoclonal RF may bind polyclonal IgG into an immune complex and appear as a tiny M-protein on SPE as a result of limited migration of the large complex. These lesser affinity aggregates usually stain on IFE as IgM and IgG or only IgG but both kappa and lambda. Treatment with 2-mercaptoethanol for 4 h or longer at 37 °C will break up these complexes so that interpretation is possible (Attalman and Levinson, 2000). Make a 1:10 dilution of 2-mercaptoethanol with isotonic saline (add 10 µL of the dilution to 100 µL of the patient's serum) and repeat the IFE. Fig. 20 illustrates precipitation in all IFE lanes and its solution. Fig. 21 illustrates the circumstance where a tiny restriction is seen on SPE. IFE shows staining for IgM, IgG, kappa and lambda. After treatment, it can be seen that the IgG and IgM was polyclonal.

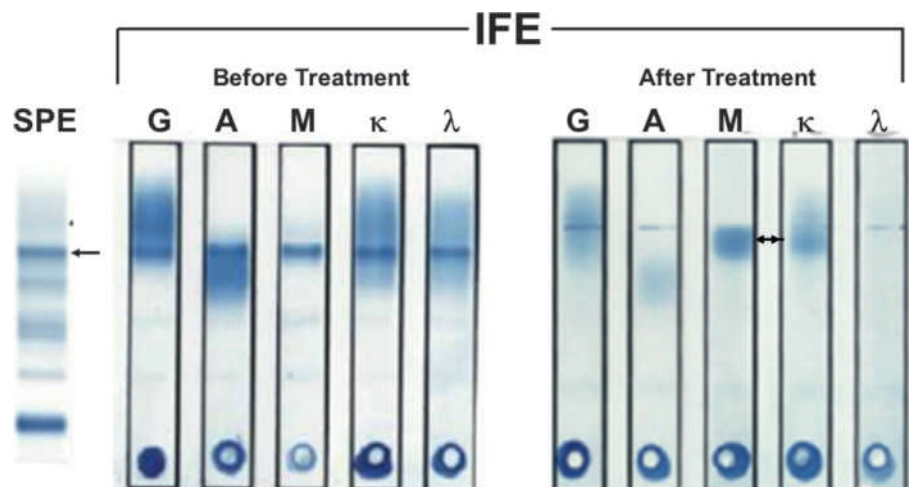


Fig. 20 Circulating immune complex precipitate in all lanes treated with mercaptoethanol. A band on SPE appears monoclonal (arrow) but IFE shows bands in all lanes. After treatment with mercaptoethanol, fixation indicates an IgM-kappa M-protein (double arrow). Otherwise, fixation shows polyclonal kappa and lambda light chain. This indicates the IgM-kappa bound polyclonal Ig causing a large immune complex that was trapped in the gel. After treatment the immune complex was broken down. The separated complex migrated as an IgM M-protein that was kappa and polyclonal Ig. Notice there is a thin band at the origin where the sample was applied (O). This is a bit of denatured Ig that did not migrate.

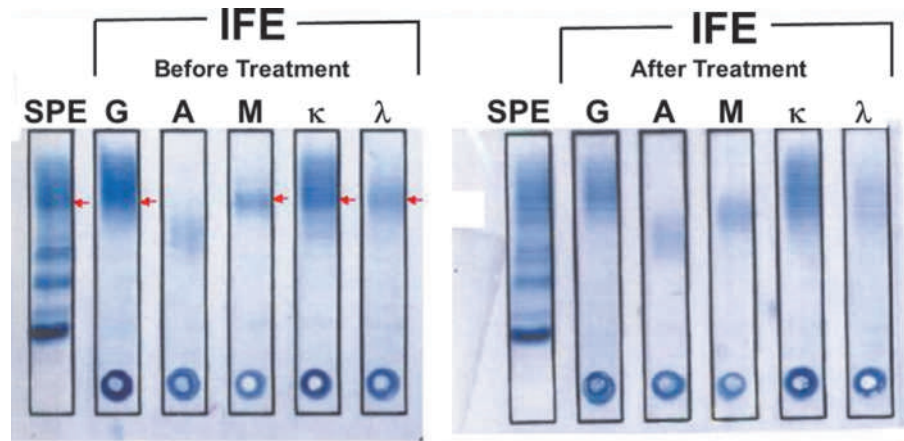


Fig. 21 Polyclonal Ig appearing as monoclonal due to circulating immune complex. SPE shows a tiny band that may be monoclonal (arrow). IFE shows restricted staining in the IgM, IgG, kappa and lambda tracks (arrows). After treatment with mercaptoethanol no monoclonal is seen. There is only diffuse staining. The best interpretation is that polyclonal IgM rheumatoid factor bound polyclonal IgG and the aggregate was trapped in the gel. Treatment broke the complex down and only polyclonal migration was seen.

Cryoglobulins

Cryoglobulins are immunoglobulins or immune complexes that precipitate at temperatures below 37 °C and usually dissolve upon warming to 37 °C. Cryoglobulins have long been classified as: Monoclonal, Type I; Mixed, Type II; and polyclonal, Type III (Brouet et al., 1974). Monoclonal type I is usually associated with plasma cell dyscrasia or other hematological malignancies such as multiple myeloma or Waldenstrom macroglobulinemia. Type II may also be associated with Waldenstrom or rarely myeloma but more often mixed Type II is associated with diseases where there is a chronic infection as are Type III, especially hepatitis C (Agnello et al., 1992). Cold sensitivity in monoclonal Type I cryoglobulins seem to be dependent on the conformation of the monoclonal Ig while mixed Type II and polyclonal Type III are largely dependent on the formation of circulating immune complexes as a result of rheumatoid factor activity (Charles and Dustin, 2009). Although Type II includes a monoclonal component, when caused by chronic disease, it is not considered a true plasma cell dyscrasia. It is postulated that circulating B lymphocytes are stimulated by hepatitis C or other virus causing them initially to synthesize polyclonal IgM rheumatoid factor. However, unknown factors induce a shift to abnormal proliferation of a single clone of B cells that produces monoclonal IgM-rheumatoid factor. In fact, there is evidence that an oligoclonal phase may precede the monoclonal phase (Tedeschi et al., 2007; Charles and Dustin, 2009). This leads to type II mixed cryoglobulinemia. In Type III, the rheumatoid factor remains polyclonal.

Due to decreased solubility cryoglobulins tend to precipitate in the small vessels (venules, capillaries, arterioles) of various tissues, causing a cryoglobulins vasculitis (Shihabi, 2006). Histologically, a leukocytoclastic vasculitis secondary to vascular deposition of immune complexes is seen. This can lead to ischemia, necrosis and purpura. Especially, the kidney, skin, musculoskeletal system and peripheral nervous system may be involved (Shihabi, 2006; Dammacco and Sansonno, 2013).

Serum cryoglobulins in most patients exist in low concentrations (100–300 mg/L) as compared to concentrations (60,000–80,000 mg/L) of normal serum proteins. It is difficult to isolate such small amounts of cryoglobulins without contamination from normal serum proteins. For this reason, as much serum as can be collected is recommended for analysis. The time for analysis is long (up to 3 days) and routine quantification techniques are somewhat crude in that collection is tedious, there is no internationally accepted references, and this lack of standardization can lead to missed diagnosis of mixed type II cryoglobulinemia (Vermeersch et al., 2008). Besides, quantification is inexact.

At least 10 mL of blood should be collected in a red top tube and transported to the laboratory in a thermos container. Clot the blood in a 37–40 °C water bath. The clot should be immediately separated in a standard centrifuge for only 5 min. The serum should be immediately removed and placed in a refrigerator at about 4 °C. The sample should be examined for turbidity or a precipitate up to 72 h. If the sample remains clear, it should be reported as negative.

If turbidity is seen, the sample should be centrifuged in a refrigerated centrifuge. Turbidity due to lipid particles, non-immunoglobulin cryoprecipitates or other debris cannot be easily differentiated from cryoglobulins at refrigerated temperatures (Voma and Levinson, 2016).

Therefore, all suspect samples should be centrifuged at 4 °C. Lipoproteins will float or the serum will remain turbid. If a precipitate is found, it should be vigorously washed (Kallemuchikkal and Gorevic, 1999): Wash the pellet three times with ice cold saline and vigorous mixing (vortexing) between washes. Recentrifuge after each wash in a refrigerated centrifuge. Then, redissolve in warm saline.

The most complete way to identify and characterize cryoglobulins remains SPE and IFE (Warren, 2013). IFE will allow appropriate characterization of the precipitate (Voma and Levinson, 2016). The final pellet should be redissolved in 0.5–1 mL of warm saline with vigorous shaking and kept in a 37 °C bath until application to SPE/IFE. The amount of total protein should be

measured using a sensitive technique such as for cerebral spinal fluid protein. Generally dye binding methods are not preferred since they are more selective for some proteins (Voma and Levinson, 2016). Calculation corrections for protein should be made for volume changes from the original volume collected and SPE and IFE should be performed.

Fig. 22 illustrates a type I cryoglobulins obtained from a patient with Waldenstrom macroglobulinemia. The cryoglobulins was estimated to be about 1 g/dL. Notice there was a tiny bit of kappa and lambda staining on IFE at the origin where the sample was placed indicating a bit of nonspecific precipitate. Also, there is some albumin on PPE and tiny bit of diffuse lambda staining on IFE suggesting the sample was not sufficiently washed. Nevertheless, it is clear that it is IgM-kappa. Fig. 23 illustrates a type II cryoglobulins from a patient with chronic hepatitis C. Here, it can be seen polyclonal IgG and monoclonal IgM was identified indicating the precipitate was composed of monoclonal IgM rheumatoid factor that bound polyclonal IgG.

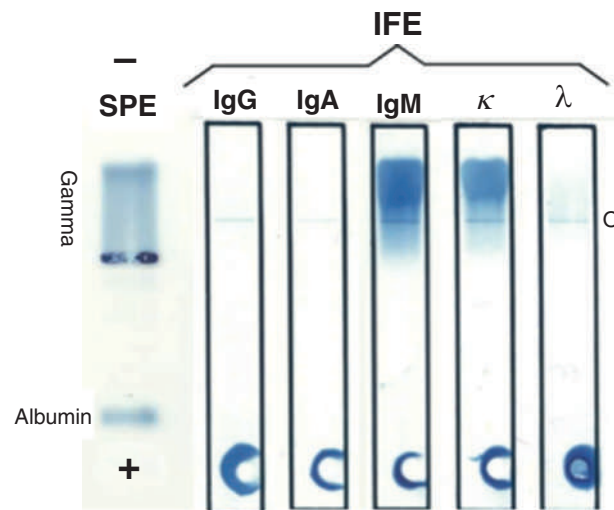


Fig. 22 Type I Cryoglobulins. A very large (1 g/dL) cold-precipitate from a patient with Waldenstrom macroglobulinemia was washed $3 \times$ with vigorous mixing. The precipitate was redissolved in 37°C saline and applied to electrophoresis (PPE). PPE shows densities in the gamma region and a bit of albumin. IFE show mostly an IgM restriction and co-migrating kappa. A small amount of albumin in the PPE lane indicates that the washing was not complete. Most likely the large amount of M-protein adsorbed albumin and other proteins. This is probably the reason that there is a tiny bit of lambda in the gamma region. There is also a bit of IgM, kappa and lambda at the site of sample application (o) which represents denatured protein that did not migrate. The profile clearly indicates an IgM-kappa monoclonal cryoglobulins with less than perfect washing.

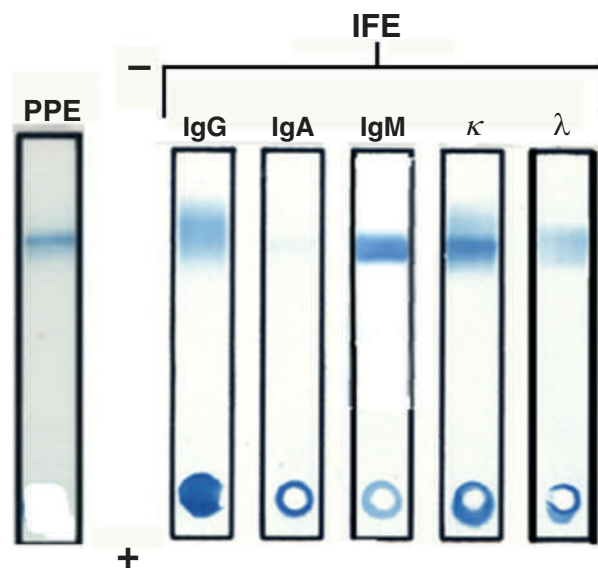


Fig. 23 Type II Cryoglobulins. The precipitate was treated as in Fig. 22. A small precipitate of about 50 mg/dL was placed on electrophoresis (PPE). No albumin is seen, suggesting adequate washing. IFE shows a monoclonal IgM-kappa, polyclonal IgG and polyclonal kappa and lambda.

The effect of treatments on biomarkers

The survival of patients with multiple myeloma has been steadily improving for more than two decades (Mollee, 2009). Median survival for all patients diagnosed in the last decade from one institution improved from 29.9 months to 44.8 months. In the past, the chemotherapeutic agent melphalan and prednisolone remained the mainstay of therapy for plasma cell dyscrasias. But more recently, ASCT after high-dose chemotherapy has improved overall survival for patients with plasma cell dyscrasias and is considered the standard of care for eligible patients—age ≤ 65 years. Moreover, the newer drugs thalidomide, lenalidomide and bortezomib have been introduced (Mollee, 2009).

Bortezomib belongs to a new class of therapeutic agents called “proteasome inhibitors.” By blocking the proteasome, bortezomib causes inhibition of the nuclear factor kappa-light-chain-enhancer of the activated B cell pathway which results in myeloma cell death. Thalidomide, known as an immunomodulatory agent, is an anti-myeloma agent with many mechanisms of action, both directly against the myeloma cell, the supporting marrow microenvironment and the immune system (Mollee, 2009). Lenalidomide is an immunomodulatory agent derived from thalidomide with increased anti-myeloma potency and less likelihood of causing neuropathy, constipation and lethargy. It has been shown in randomized controlled studies to improve overall survival in patients with relapsed myeloma (Mollee, 2009). Still, most patients who achieve a good response after initial therapy eventually relapse or become refractory. Even newer proteinase inhibitors carfilzomib and ixazomib and the immunomodulatory imide drug—pomalidomide—have improved the clinical outcomes further (Nishida and Yamada, 2019).

Also, monoclonal antibodies directed against B-cells have become important therapies. Chimeric and humanized antibodies targeting CD20 on B cells have provided some relief for myeloma patients but has been more productive for use with B-cell non-Hodgkin’s lymphoma such as Waldenstrom macroglobulinemia that along with combination chemotherapy produces a greater than 90% response rate (Gertz, 2019). Also, recently ibrutinib that facilitates programmed cell death has proven very effective as a single therapy for Waldenstrom macroglobulinemia (Treon et al., 2021).

On the other hand, the humanized/human antibody drugs against CD38, daratumumab and elotuzumab have improved the clinical outcomes in plasma cell dyscrasia patients who are refractory to prior treatments including multiple myeloma and amyloidosis AL (Nishida and Yamada, 2019; Palladini et al., 2021). Since, daratumumab (DARA) and elotuzumab (Liu et al., 2020) are monoclonal antibodies, at treatment concentrations, they will appear on a majority of SPE and IFE as pseudo-M-proteins with both migrating in the gamma region. The concentration of the pseudo-M-protein is ≤ 100 mg/dL. As such, they appear as very small bands. On SPE and IFE, DARA migrates somewhat cathodal while elotuzumab migrates close to the beta-gamma region (McCudden et al., 2008; Tang et al., 2018). Thus, it is important for interpretation to know which patients are on these drugs and the interpreter must be aware of this interference, since the drugs may be mistaken for a new M-protein when a patient is being routinely monitored or for an endogenous M-protein when a patient is in remission.

If a known M-protein is migrating in a different spot, and the interpreter is aware the drugs are present, the presence of a second band that is most likely due to the drug can simply be mentioned in the report. If it is necessary to definitively identify the band daratumumab can be shifted on the gel by pretreatment of the sera with a monoclonal antibody specific for DARA, so called daratumumab-specific immunofixation electrophoresis reflex assay (DIRA) (McCudden et al., 2008; Mills and Murray, 2017). DIRA is now employed in some laboratories. More recently, DARA and elotuzumab have been removed by pretreatment with CD38 bound to beads (Liu et al., 2020).

Criteria for plasma cell responses to treatments

Successful treatment has implications for biomarkers in monitoring disease progression. Table 2 shows the IMWG criteria for response to therapy’ (found at <https://www.myeloma.org/resource-library/international-myeloma-working-group-imwg-uniform-response-criteria-multiple>), and in more detail (Durie et al., 2006).

It is apparent from Table 2 that the need for serum and urine biomarkers is as important in assessing remissions as they are in identification and prognosis of plasma cell dyscrasias since these are an essential part of the criteria. Thus, CR/sCR requires negative immunofixation on the serum and urine and normal FLC ratio. Definitions of VGPR and PR are entirely dependent on the biomarkers unless the patient is a non-secreter. This is also true for no change/stable disease. Serum and urine M-protein levels and FLC levels are biomarkers for progressive disease relapse from CR.

Successful therapy has also led to peculiar patterns that add difficulty to interpretations on SPE and IFE. Treatment with newer agents as compared to chemotherapy and especially with autologous transplantation has resulted in an increase incidence of oligoclonal banding (Tovar et al., 2013) on SPE, similar to that shown in Fig. 16B. The oligoclonal bands may be only kappa or lambda but are more often both kappa and lambda on IFE (Singh, 2017) (Fig. 16B). The oligoclonal pattern may last for up to 1 or 2 years or be shorter lived (Tovar et al., 2013). Generally, this pattern appears to be a good prognostic sign and these patients seem to have longer remissions (Tovar et al., 2013; Singh, 2017). The mechanisms underlying the development of oligo-clonal bands remains unsettled (Tovar et al., 2013; Singh, 2017), although it seems the most likely explanation is that a bone marrow recovering from successful treatment would give rise initially to a few clones prior to polyclonal expansion.

If the oligoclonal banding is only kappa or lambda a difficulty can arise if the original monoclonal was of the same light chain type, since it will be unclear whether or not the malignant clone gave rise to the oligoclonal banding. In this case, it is likely it was

Table 2 Criteria for plasma cell responses.

<i>Response</i>	<i>IMWG criteria</i>
Stringent Complete Response (sCR)	CR as defined below plus normal FLC ratio and absence of clonal cells in bone marrow by immunohistochemistry or immunofluorescence
Complete Response (CR)	Negative immunofixation on the serum and urine and disappearance of any soft tissue plasmacytomas and <5% plasma cells in bone marrow
Very good partial response (VGPR)	Serum and urine M-protein detectable by immunofixation but not on electrophoresis or >90% reduction in serum M-protein plus urine M-protein level <100 mg/24 h
Partial response (PR)	>50% reduction of serum M-protein and reduction in 24 h urinary M-protein by >90% or to <200 mg/24 h If the serum and urine M-protein are unmeasurable, a >50% decrease in the difference between involved and uninvolved FLC levels is required in place of the M-protein criteria If serum and urine M-protein are not measurable, and serum free light assay is also not measurable, >50% reduction in plasma cells is required in place of M-protein, provided baseline bone marrow plasma cell percentage was >30% In addition to the above listed criteria, if present at baseline, a >50% reduction in the size of soft tissue plasmacytomas is also required
MR	NA
No change/Stable disease	Not meeting criteria for CR, VGPR, PR, or progressive disease
Plateau	NA
Progressive disease	Increase of >25% from lowest response value in any one or more of the following: • Serum M-component and/or (the absolute increase must be >0.5 g/dL) • Urine M-component and/or (the absolute increase must be >200 mg/24 h) • Only in patients without measurable serum and urine M-protein levels; the difference between involved and uninvolved FLC levels. The absolute increase must be >10 mg/dL • Bone marrow plasma cell percentage; the absolute percentage must be >10% • Definite development of new bone lesions or soft tissue plasmacytomas or definite increase in the size of existing bone lesions or soft tissue plasmacytomas • Development of hypercalcemia (corrected serum calcium >11.5 mg/dL or 2.65 mmol/L) that can be attributed solely to the plasma cell proliferative disorder
Relapse	Clinical relapse requires one or more of: Direct indicators of increasing disease and/or end organ dysfunction (CRAB features). It is not used in calculation of time to progression or progression-free survival but is listed here as something that can be reported optionally or for use in clinical practice • Development of new soft tissue plasmacytomas or bone lesions • Definite increase in the size of existing plasmacytomas or bone lesions. A definite increase is defined as a 50% (and at least 1 cm) increase as measured serially by the sum of the products of the cross-diameters of the measurable lesion • Hypercalcemia (>11.5 mg/dL) [2.65 mmol/L] • Decrease in hemoglobin of >2 g/dL [1.25 mmol/L] • Rise in serum creatinine by 2 mg/dL or more [177 mmol/L or more]
Relapse from CR [To be used only if the end point studied is Disease Free survival (DFS)]	Any one or more of the following: • Reappearance of serum or urine M-protein by immunofixation or electrophoresis • Development of >5% plasma cells in the bone marrow • Appearance of any other sign of progression (i.e., new plasmacytoma, lytic bone lesion, or hypercalcemia)

not the malignant clone but watching and waiting is the best solution. If the oligoclonal banding is of a different type or both kappa and lambda, one can be reasonably certain it is a true oligoclonal response unrelated to the malignant clone.

Conclusions and summary

In the above discussions a good deal of detail was presented in terms of electrophoretic patterns and methodologies, especially descriptions of the methods and interpretations. Some historical content, weaknesses of the techniques and exact recommended collection and procedural processes were also discussed. The basis of humeral immunology was discussed to improve the understanding of where, why and how monoclonal gammopathies develop. Besides there was an over view of associated workup for diagnosing, monitoring progress and therapy and defining status of persons with or suspected of having monoclonal gammopathies. Also, some information on treatments was presented to put the concepts in a more complete perspective. The article also focused on why these methods are implicit in diagnosing, monitoring, treating and defining status of these conditions. In conclusion, the methods discussed are important instruments for following biomarkers in diagnosing, monitoring and treating monoclonal gammopathies.

The polarities are indicated in all the electrophoretograms. In IFE Figures, the wells near the bottom are control wells where sample is added along with antisera to ensure the reaction occurred. On IFE plates IgG, IgM, IgA or G, M, A indicates fixation for IgG, IgM and IgA heavy chain proteins in the lane just below. Similarly, kappa (κ) and lambda (λ) indicate fixation for the appropriate type of light chain.

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Measuring Telomere Length: A Timeline Review on the State-of-Art Techniques

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Introduction

Until the end of the 1970s replication of chromosomal end was a biological issue that had yet to be explained. The one lagging strand could not be copied if the process of polymerases' action previously described was considered. This dilemma was called "end replication problem" (Olovnikov, 1971; Watson, 1972).

In 1978, this "problem" began to be resolved by assessing the ends of *Tetrahymena thermophila* chromosomes, which contained six TTGGGG bases sequences, repeated 20–70 times (Blackburn and Gall, 1978). In 1985, Greider and Blackburn identified an enzyme capable to extend those telomeric sequences (Greider and Blackburn, 1985) described as "terminal transferase" activity; the enzyme capable of maintaining telomeric length was called "telomerase" (Greider and Blackburn, 1987, 1989). Furthermore, mammalian telomeres have been described as tandem repeats of TTAGGG_n DNA sequences associated with the so-called shelterin complex, consisting of six proteins. Thus, the terminal structure of the telomeres conforms to a loop-like structure protecting the chromosomal ends (Greider, 1991; de Lange, 2005). Since the "end replication problem" was solved by description of telomerase-activity, a number of techniques to study telomere-length, especially in aging cells and diseases have increased in numbers, sensitivity and resolution. These approaches are applied e.g., to monitor neoplastic cells, being targeted by new specific drugs to prevent telomere extension, as the latter promotes cells immortality (Norton et al., 1996; Herbert et al., 2005; Relitti et al., 2020).

In this article, the techniques by which telomeric regions have been studied along the decades are reviewed in terms of their stepwise contributions they brought into the area (see Fig. 1). In the following sections, each of these techniques for studying the length of human telomeres is presented and discussed and correlated with scientific impact, advantages and disadvantages.

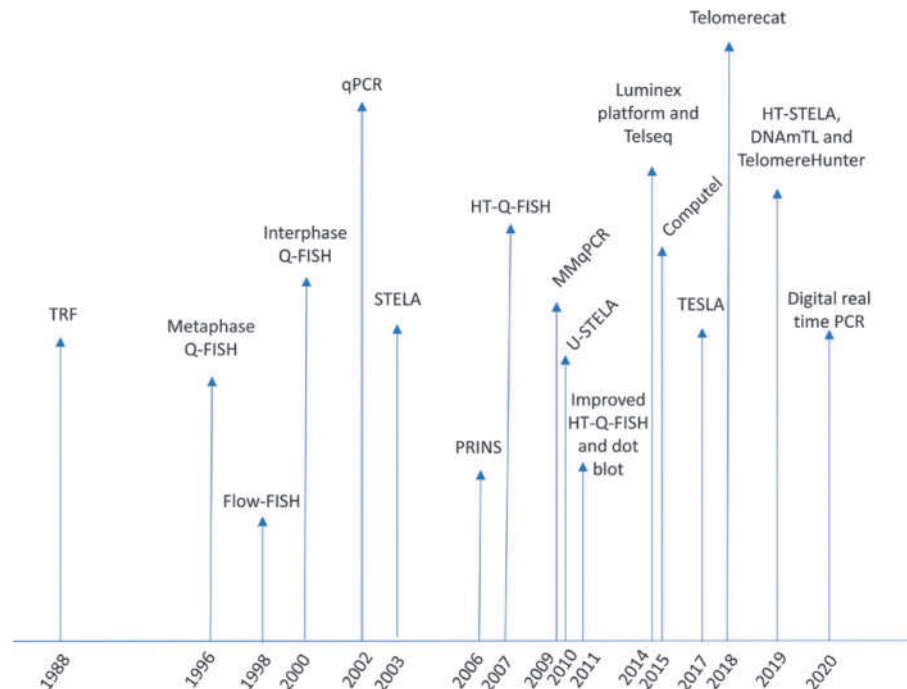


Fig. 1 Main telomere length measurement methods timeline. *TRF*, telomere restriction fragment; *Q-FISH*, quantitative fluorescence in situ hybridization; *qPCR*, quantitative polymerase chain reaction; *STELA*, single telomere length analysis; *PRINS*, primed in situ hybridization; *HT*, high-throughput; *MMqPCR*, monochrome multiplex quantitative polymerase chain reaction; *U-STELA*, universal single telomere length analysis; *TESLA*, telomere shortest length assay; *DNAmTL*, DNA methylation-based estimator of telomere length. The lengths of the arrows have no significant value. Figure adapted from Vera E and Blasco MA (2012) Beyond average: Potential for measurement of short telomeres. *Aging (Albany NY)* 4: 379–392.

Telomere restriction fragment (TRF)/Southern blot

Most molecular genetic methods being applied for telomere length measurements are inspired by double helix DNA structure. Scientists took advantage of the DNA-characteristic being able to in vitro strand dissociation (denaturation) and re-association (renaturation or hybridization).

Southern blot was technique proposed in the beginning of the recombinant DNA-era (Southern, 1975) as the first method able measure telomere length (TL) (Moyzis et al., 1988; Allshire et al., 1989); later on, this approach was renamed as Telomere Restriction Fragment method (TRF).

First: The protocol: Determining DNA integrity

Prior to necessary restriction digestion of whole genomic DNA applied in TRF, a sample of 1 µg of each DNA-sample should be electrophoresed in an agarose 0.8–1% gel to verify its integrity; a large, high molecular weight band without smear should be the result.

DNA-sample is applied to the gel well with 1:1 volume of loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, glycerol 50%). Electrophoresis buffer can be either buffer Tris-acetate—1xTAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) or Tris-borate buffer—1xTBE (Tris base 0.09 M, acid boric 0.09 M, EDTA 0.002 M, pH 8.0). After electrophoresis (1 or 2 h, room temperature, 60–100 V), the gel is stained with ethidium bromide. Besides, there are others chemicals able to label DNA, like SYBR Green I.

Second: DNA digestion

The genomic DNA-sample is fragmented by two restriction enzymes (type II): *HinfI* and *RsaI*. These restriction enzymes type II were discovered in bacteria and cleave double-stranded DNA at specific recognition sites of 4 or 6 base pairs (bp) in length.

The digested DNA is separated on a 0.6–1.0% agarose gel in 1xTAE or 1xTBE buffer. Electrophoresis must be performed at low speed, preferentially overnight. It is necessary to load in a separated well a DNA molecular marker (e.g., 1 kb and λ DNA/*HindIII* ladder) with known DNA fragment sizes in one of the wells as for co-migration in the gel. Later the size-standard will be necessary to determine TL in kilobasepairs (kb). Finally, the gel is stained with ethidium bromide to confirm DNA digestion (a smear is visible) and that small DNA-fragments have not been lost during electrophoresis.

Third: The blotting

Gel is now immersed in denaturation solution (0.5 M NaOH, 1.0 M NaCl) for 30 min, room temperature with slow shaking to obtain single stranded DNA. Then, the gel is transferred to a neutralization solution (1 M Tris-HCl and 1.5 M NaCl, pH 8.0) for 30–60 min under slow shaking.

The blotting system is quite simple and resembles a multilayer sandwich. To start, cover a surface with plastic wrap (e.g., PVA) or glass plate, lay on it a stack of paper-towels, then a piece of filter-paper, then the piece of the nitrocellulose-membrane, the wet gel and cover all of it with plastic film. Continuing, put a glass plate on the top and then a weight on it, and let it go overnight. This way of DNA transferring is known as downward capillary transfer, that is different (inverted) from the original Southern Blot (Green and Sambrook, 2012). Afterwards, nitrocellulose-membrane has taken up the DNA-sample and can be air-dried to fix the DNA.

Fourth: Preparing the probe

The probe is a polymer of 3 or 4 TTAGGG repeats that can be labeled by λ -P³² ATP or DIG (digoxigenin) as for the chemo-luminescence revelation method. The DNA molecular marker should be labeled in the same way. Nowadays, the telomeric DNA probe and the molecular marker labeled by DIG can be ordered from many companies; however, if necessary, isotope labeling can be performed also in-lab. In this case, the telomeric probe can be labeled by the enzyme T4 polynucleotide kinase, originally from a bacteriophage, which transfers the λ -phosphate of λ -P³² ATP, into the 5'-termini of DNA. As for the molecular marker, the Nick translation method should be applied, in which the *Escherichia coli* DNA polymerase I incorporates radiolabeled nucleotides in double stranded DNA after DNase has set single strand breaks = nicks (Temsamani and Agrawal, 1996). The T4 polynucleotide kinase is commercially available as well as Nick translation kits.

Fifth: Hybridization with telomeric probes

Prehybridize and the nitrocellulose-membrane by immersing in prehybridization or hybridization solution for 15 min at 42 °C. Most commonly, the hybridization solution contains: 3–10 × SSC, 2–3 × Denhardt's solutions, 0.3 M NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.1% SDS and 100 µg/mL yeast tRNA. Denhardt's solution is composed of 1% Bovine Serum Albumin (BSA), 1% Ficoll, 1% Polyvinylpyrrolidone (PVP) in water. 1 × SSC solution is composed of NaCl 0.15 M and 0.015 M Na₃citrate (pH 7.0).

Hybridization occurs in a tube mounted in a rotating hybridization oven after adding of the telomeric DNA probe. If the molecular marker was in-lab labeled by P³² labeled, it should be previously denatured (95 °C for 3 min), and added to the hybridization solution about the same time as the telomeric probe. Hybridization takes place overnight.

Sixth: Post hybridization and TL analysis

The hybridization is stopped by immersing the nitrocellulose-membrane in washing solution (2 × SSC, 0.1% SDS), two times, 15–30 min, each, followed by two times washing with 2 × SSC, 10 min, each; perform all washes under slow shaking. Normally, washing is done at room temperature, but if later-on a background shows up, then it is recommended to increase the temperature of the washing up to 60 °C.

To visualize the results TRF nitrocellulose-membrane is exposed to an X-ray film in a cassette, usually in cooling conditions. All the film's manipulation and developing takes place in a darkroom with a safelight. If the probe is labeled by P³², the film can be exposed, wrapped in PVA plastic, from overnight to a few days depending on the decay of the isotope (P³² half-life is 14 days). If it is labeled by DIG, the exposure depends on the emission of the chemiluminescent light that is a result of ligations and chemical reactions, beginning with antidigoxigenin antibody conjugated with alkaline phosphatase. Then, the chemiluminescent substrate for alkaline phosphatase (CDP-Star[®]) is added allowing the detection of alkaline phosphatase.

After exposure and film development, the image can be scanned by a commercially available imaging device, e.g., Image Quant[®] software (Molecular Dynamics). In comparison to the molecular marker the TL is determined (in kb = bp × 1000). Generally, human telomeres are composed of 10–15 kb of TTAGGG repeats (Fig. 2) (Allshire et al., 1989; Fernández-Marcelo et al., 2016), but an 80 years-old person can show telomere as short as 1.2 kb (Kimura et al., 2010).

Notes

- One variation of the TRF technique is the in-gel hybridization that requires drying the gel to perform the hybridization on it (Bryan et al., 1995; Fouquerel et al., 2019). The gel can be dried at 50–60 °C for 2 h, under vacuum, and then denatured.
- Since the beginning there were controversies about what is the best set of restriction enzymes to measure TL by TRF. Depending on the set of restriction enzymes used, the TL can result in a difference as much as 1 kb (Kimura and Aviv, 2011). In the first works of TRF the authors also tested other enzymes such as *SAU3A*, *MnII*, and *HphI* (Moyzis et al., 1988; Allshire et al., 1989; Kimura and Aviv, 2011). Other protocols apply alternative combinations of restriction enzymes for digesting the telomeric DNA, such as *MseI/NdeI*; or *Bfal/CviAII/MseI/NdeI*. The use of these combinations can minimize the inclusion of subtelomeric regions containing variations (polymorphisms) in the telomeric repeats (Lai et al., 2018). A combination of *HhaI*, *HinF1*, *MspI*, *HaeIII*, *RsaI*, and *AluI* was also recommended (Mender and Shay, 2015). The digestion by different restriction enzymes (sometimes from different suppliers) may require an in-lab universal restriction buffer. The universal buffer (5 ×) is composed of 0.1 M Tris-Cl pH 8.0, 0.25 M NaCl, 0.05 M MgCl₂ and 0.5 mg/mL lysozyme (Fanning et al., 1980).

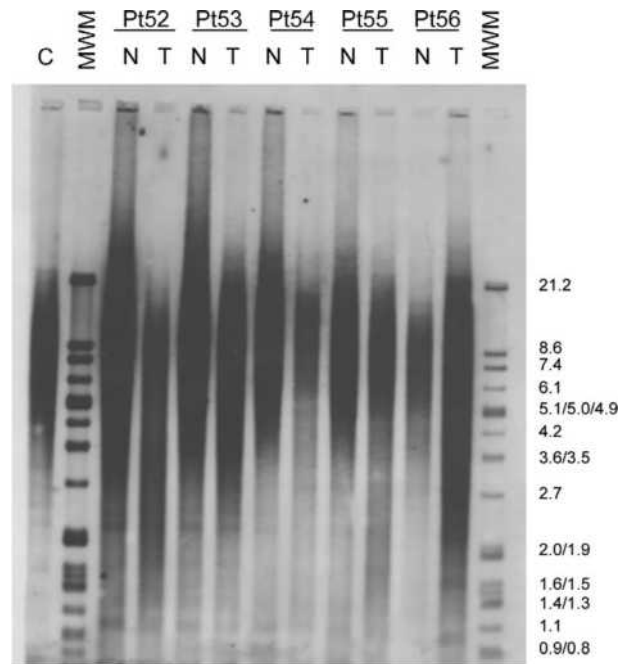


Fig. 2 Results of a representative X-ray film for TL analysis from five colorectal cancer patients (Pt) in tumor tissues (T) and their paired non-tumor samples (N). C, positive control (TeloTAGGG™ Telomere Length Assay, Cat No. 12209136001). MWM, DNA molecular weight marker (in kb). Figure and caption taken from Fernández-Marcelo T, Sánchez-Pernaute A, Pascua I et al. (2016) Clinical relevance of telomere status and telomerase activity in colorectal cancer. *PLoS One* **11**(2): e0149626.

- Blotting takes place driven by capillarity of aqueous solution, however electric or vacuum transfer equipment can be applied to speed up procedure to 1 h (Allshire et al., 1989; Green and Sambook, 2012). Also, downward capillary transfer of DNA using a transfer solution through a paper bridge containing 3 M NaCl and 8 mM NaOH in 1 h (Chomczynski, 1992) has been reported.
- Membranes are made besides from nitrocellulose of neutral nylon or charged nylon. The last one is considered to be the best (Green and Sambook, 2012).
- A simple in-lab made hybridization solution targeting high abundance sequence is composed of 6xSSC and 0.05% BLOTTO. BLOTTO is a mixture of 5% non-fatdried milk dissolved in water containing 0.02% sodium acid (Sambrook et al., 1989).

Nowadays, it is possible to buy kits with all the necessary reagents to perform TRF, e.g., TeloTAGGG Telomere Length Assay Kit (Roche), which applies chemiluminescent detection.

Dot and slot blotting for TL

Dot-blot is semi-quantitative method to estimate the presence of specific DNA sequences based on hybridization. DNA samples are spotted next to each other on a filter (nitrocellulose or nylon) in dots of uniform diameter. The filter is subject to denaturing conditions and then hybridized with a probe. For quantification, a sequence of diluted DNA with known concentrations are similarly spotted and used for comparison. The biggest advantage of dot-blot method is that it can be applied to a large number of DNA samples (Kafatos et al., 1979), being faster and cheaper than a TRF, since no electrophoresis and DNA transfer are required.

Kimura and Aviv (2011) applied the dot-blot method to measure TL, requiring small amounts of DNA (~20 ng/assay). The authors evaluated the DNA content of the sample on the blot by SYBR Dx DNA Blot Stain (S-7550, Invitrogen). The blot was hybridized with a DIG-labeled telomeric probe and the ratio of telomere repeats/DNA (T/Dx) corresponded to the amount of the repeats per unit DNA. A Bio-Dot Microfiltration Apparatus (BioRad) was used for the DNA samples blotting.

Single TL analysis

Single TL analysis (STELA) is a PCR-based technology combined with Southern Blot hybridization; it was developed by Baird et al. (2003) for measuring TLs on individual chromosomes. The technique was first described to track XpYp telomeric length, being latter adopted for autosomal human telomeres, such as 2p, 11q, 12q, and 17p (Baird et al., 2003; Britt-Compton et al., 2012; Lin et al., 2010; Xing et al., 2009). STELA is able to reveal telomeres short enough for being subject to fusion events and induced genomic instability, thus being a useful tool to provide relevant clinical information (Capper et al., 2007; Lin et al., 2010; Garcia-Martin et al., 2017).

Standard STELA assay includes: (a) extraction of genomic DNA followed by digestion with restriction enzymes (e.g., *EcoRI*); (b) a telorette linker, represented by seven bases complementary to the G-rich 3' overhang followed by a 20-nucleotide non complementary tail; (c) a teltail primer, similar to telorette sequence, which specifically recognizes the telorette; (d) a chromosome-specific primer, designed for the subtelomeric region; (e) detection of STELA products by gel electrophoresis and Southern hybridization with a radiolabeled probe to produce telomere band images (Baird et al., 2003).

Although STELA is a useful approach for measuring the dynamics of telomere shortening, the method has some limitations since specificity of proximal primers is required and this only applicable for a small subset of chromosomal ends; therefore, investigations on different telomere shortening rates between chromosomes and the triggering of cellular senescence are limited (Leach et al., 2004; Martin-Ruiz et al., 2004; Britt-Compton et al., 2006; Bendix et al., 2010). Based on this, the Universal STELA (U-STELA) assay, a STELA variation technique described by Bendix et al. (2010) was designed to measure any TL regardless its location, and thus detect telomeres that are extremely short and potentially drive senescence processes (Cagsin et al., 2020). Despite this, U-STELA cannot efficiently detect longer telomeric stretches (above 8 kb) (Bendix et al., 2010). Another STELA-based technique is the high-throughput STELA (HT-STELA), which was developed to improve this technology and applied to predict for outcome to fludarabine, cyclophosphamide, rituximab (FCR) based therapy (Norris et al., 2019).

Telomere shortest length assay

Telomere shortest length assay (TeSLA) is a sensitive method developed for TL measurement able to accurately detect telomeres ranging from <1 kb to 18 kb (Lai et al., 2017). This technique needs an image-processing software to detect and calculate TL after Southern blot, being a more informative assay than TRF (Lai et al., 2017; Zhang et al., 2021).

Molecular cytogenetics-based analyzes

Molecular cytogenetics is a primarily single-cell oriented approach, by which TL measuring can also be realized. The field of molecular cytogenetics is based on two approaches: Fluorescence in situ hybridization (FISH) (Weise and Liehr, 2017a) and primed in situ hybridization (PRINS) (Charlieu and Pellestor, 1997). Both methods are suited to detect telomeres and to measure their lengths.

Quantitative fluorescence in situ hybridization (Q-FISH)

Quantitative fluorescence in situ hybridization (Q-FISH) is rather based on a standard FISH applying peptide nucleic acid (PNA) telomere oligonucleotide probe to cytogenetically worked up material containing interphases and/or metaphases from different sources. Quantitative hybridization to telomere repeats is achieved using conditions that allow the labeled (CCCTAA)₃ PNA probe to hybridize to TTAGGG repetitive sequences. The PNA probes are synthetic, particularly resistant and better hybridizing to DNA than other synthetic probes. Q-FISH can only be evaluated with appropriate digital image software able to capture pictures under defined conditions and adequate quantification of fluorescence signals. Herby it is implied that TL is directly proportional to the acquired fluorescence intensity (Joksic et al., 2017). Best results are obtained when the PNA telomere oligonucleotide probe is used together with a corresponding centromeric probe as control and standard. Besides fluorescence beads of defined size can be used as internal controls (Joksic et al., 2017).

Metaphase-oriented Q-FISH

In Q-FISH on metaphases a resolution of 200 base pairs per single cell can be achieved for TL determination (Slijepcevic, 2001); also, intra- and inter-chromosomal TL variation can be accessed (Lansdorp et al., 1996; Poon and Lansdorp, 2001). The main restriction of this approach is the limited number of tissues from which metaphase chromosomes can be prepared—i.e., peripheral blood lymphocytes, bone marrow cells, (skin) fibroblasts and tumor cells (Weise and Liehr, 2017b). An interesting variant of the standard Q-FISH in metaphases is the so-called Replicative Detargeting FISH (ReD-FISH), which enables studies of replicative patterns of telomeres on different, individual chromosomal arms (Rubtsov and Zhdanova, 2017).

Slide preparations with metaphasic cells is traditionally based on the so-called air-drying method. Apart from peripheral blood lymphocytes, where cells need to be growth stimulated by phytohemagglutinine, all other suited tissues grow without such stimulation in cell culture. After corresponding culturing time (72 h for lymphocytes; ~14 days or longer for fibroblasts) cells are arrested in metaphase by colchicine or colcemid. Cells are harvested, centrifuged and subjected to hypotonic treatment with 0.075 M KCl (37 °C for 20 min); thereby chromosomes are separated from each other within the cell. In between all afterwards mentioned steps cells are pelleted by centrifugation (1000 × g for 5 min). Cells are deadened by adding of Carnoy's fixative (3:1 methanol:glacial acetic acid), and washed three times in fresh fixative (e.g., 5 mL each). After last centrifugation, supernatant is discarded and pellet is diluted in 100–500 µL of remaining fixative. The suspension is dropped on a slide and air dried. During this step first methanol evaporates; the remaining acetic acid acquired water from surrounding air, which leads to chromosome swelling and spreading. Thus, chromosome preparation is dependent of humidity. Slides must be stored at room temperature (RT) for at least 12 h before use in FISH. Before FISH, slides are dehydrated in an ethanol series (70%; 90%; 100%, for 3 min, each) (Weise and Liehr, 2019).

For FISH dry slides are first incubated in $100\text{ mL } 2 \times \text{SSC}$ for 1 min at RT in a coplin jar and then in a pre-warmed pepsin-buffer (37°C) with $1 \times \text{PBS}$. Afterwards, slides are washed in $1 \times \text{PBS}/\text{MgCl}_2$ at RT (5 min) with gentle agitation, whereby MgCl_2 blocks the enzymatic activity of pepsin. A formalin-buffer may be used for 10 min at RT with gentle agitation to achieve a better chromosome structure after FISH. Pretreatment is finished by washing slides in $1 \times \text{PBS}$ for 2 min (RT) with gentle agitation, dehydration by an ethanol series (70%, 90%, 100%, 3 min each) and air drying.

To do FISH DNA fixed on slides and probe-DNA need to be single stranded. Thus, DNA on slides needs to be denatured: per slide $10\text{ }\mu\text{L}$ of denaturation solution (70% (v/v) deionized formamide, 10% (v/v) filtered double distilled water, 10% (v/v) $20 \times \text{SSC}$, 10% (v/v) phosphate buffer) are added and covered with coverslips. Slides are incubated on a heating plate for 3 min at 75°C . Afterwards, coverslips are removed (e.g., by forceps) and transferred to a coplin jar filled with 70% ethanol (4°C) to conserve target DNA as single strands. Afterwards dehydrate the slides again in ethanol series (70%, 90%, 100%, 4°C , 3 min each), and air dry.

For DNA-probe hybridization, apply the single stranded manufactured probe FITC-(CCCTAA)₃ PNA on the slide. It is commercially available as ready-to-use in a hybridization solution (70% formamide, 1 mg/mL blocking reagent (Roche), 10 mM Tris-HCl pH 7.2). Add $10\text{ }\mu\text{L}$ of probe mix to the denatured slide, cover with a coverslip, seal it with rubber cement and incubate the slide overnight at 37°C in a humid chamber (Li et al., 2017).

Remove rubber cement by forceps, and start posthybridization-washes by placing the slides in a coplin jar with $4 \times \text{SSC}/0.2\%$ Tween (RT), and allow coverslips to slide off. Incubate slides 2 min in $0.4 \times \text{SSC}$ ($56\text{--}62^\circ\text{C}$) followed by 1 min in $4 \times \text{SSC}/0.2\%$ Tween (RT). The slides are mounted with antifade reagent VECTASHIELD with DAPI (4, 6-diamidino-2-phenylindole) and covered with a coverslip.

Under fluorescence microscope, chromosomes show blue color (DAPI) counterstain. The TL-specific probe is usually red due to Cy3 labeling. Capture images and analyzes can be done by appropriate software and tools. As above mentioned, TL is directly related to its integrated fluorescence intensity, as PNA probes are assumed to hybridize quantitatively to telomeric repeats. If TFL-TELO software is used, results are expressed in telomere fluorescence units (TFU), with each unit corresponding to 1 kb of telomere repeats (Joksic et al., 2017). It is recommended to analyze at least 15–20 metaphases per sample to obtain reliable results.

Interphase-oriented Q-FISH

Q-FISH in interphase nuclei is possible from whatever tissues, where cellular nuclei can be prepared from. This includes all afore mentioned preparations from culturable tissues, which contain besides metaphases always tons of interphase cells (González-Suárez et al., 2000). Additionally, interphase cells from archived material like formalin-fixed paraffin embedded (FFPE) or cryofixed tissues, but also cells e.g., from buccal mucosa, hair roots or urine derived bladder cells. While in metaphase FISH it is principally possible to study specific chromosomal ends, this is excluded in interphase-oriented Q-FISH; here only 92 telomeres per cell can be measured per cell. Interphase-oriented Q-FISH is technically done the same way as described before for metaphase oriented one, but can be also applied to cell-type specific analysis when combined with immunohistochemical staining, as well as it can be automatized (Canela et al., 2007; Vera and Blasco, 2012).

In order to measure TL in a larger number of cells, a high-throughput (HT) Q-FISH platform was developed combining the labeling of telomeres in interphase nuclei, using the same PNA probe against telomeric DNA as mentioned earlier. However, the cells are cultivated in clear bottom black-walled 96-well plates. After FISH, the wells are filled with mounting medium, and the plates can be stored if necessary, overnight at 4°C until quantitative image acquisition (Canela et al., 2007; Alda et al., 2016). TL values are analyzed using individual telomere labeled spots. The results can be observed in histograms with up to 60,000 telomeres (Quintela-Fandino et al., 2017) (Fig. 3).

Recently, a modification of interphase HT Q-FISH called Telomere Analysis Technology (TAT[®]) was reported, using automated procedures on 384-well plates for large-scale studies on fixed lymphocytes. TAT includes HT imaging and software workflows (Pedro et al., 2020).

Flow-FISH

FISH is done usually on cells fixed to a slide surface (Weise and Liehr, 2017a). However, there are also variants to perform the procedure in suspension (S-FISH) (Steinhaeuser et al., 2002). Accordingly, Q-FISH can be performed in suspension—the hybridized cells are here analyzed in a flow cytometer. Overall, as in normal interphase Q-FISH, an average TL is determined (Hultdin et al., 1998). Like in S-FISH flow-FISH allows insights into nuclear architecture of the studied cells (Samassekou et al., 2010). This method estimates the average TL based on the average telomere content (quantity of telomere repeats) of single cells, expressed as mean fluorescence intensity and translated into kb based on its correlation with TRF analysis. Of note, it also allows the measurement of telomere content in specific cell subsets (Baerlocher et al., 2002).

To ensure the accuracy of experiment, some details are important to observe (Baerlocher et al., 2006): (a) a two-laser flow cytometer is recommended; (b) to guarantee the sensitivity of the derived fluorochromes, validation by calibration beads is necessary; (c) it is extremely important to calibrate the emission channel used to collect fluorescence from the hybridized PNA probe, in order to distinguish the lowest signal levels, in addition to eliminating background signals, or autofluorescence; (d) it is ideal the use of hybridization buffer based on the components: 5% dextrose/10 mM Hepes and 0.1% BSA; (d) to acquire the

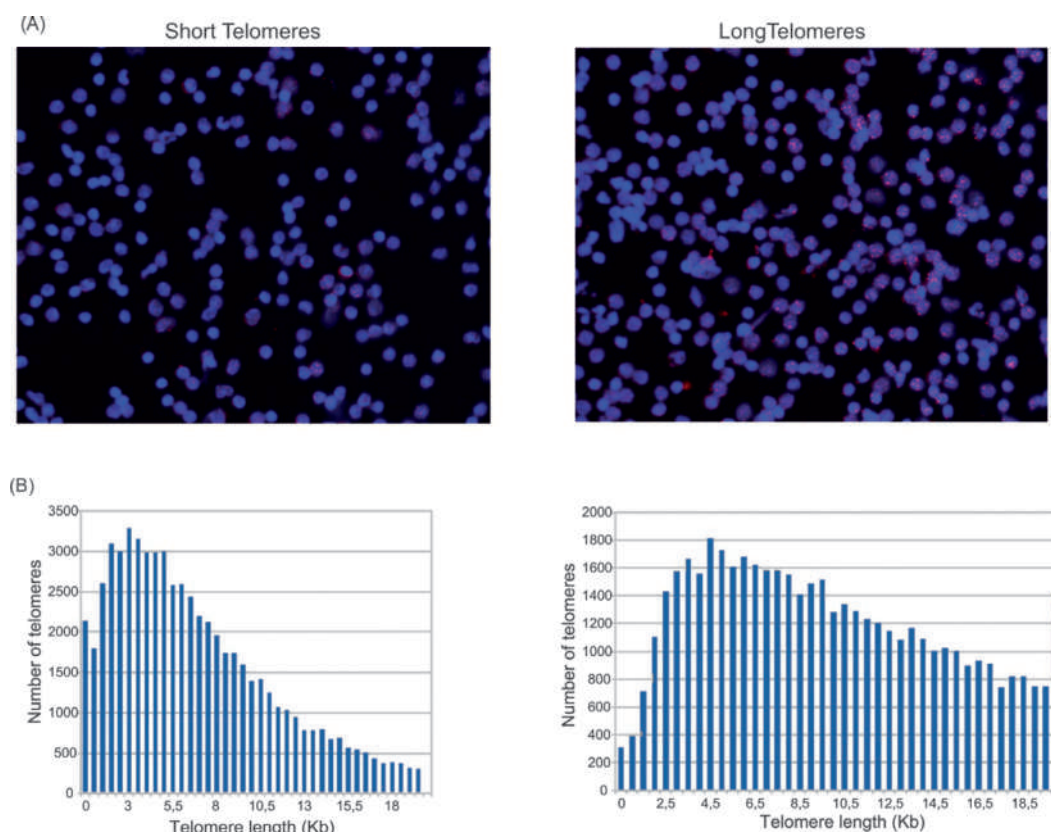


Fig. 3 (A) HT Q-FISH: pictures from a patient with most telomeres below 3 kb (left) and a patient with most telomeres above 3 kb (right). (B) Histograms depicting the telomere determinations from patients shown in (A). Each bar represents the number of telomeres determined within 2 TLs in 0.5 kb-increments per sample. The number of telomeres measured per sample is greater than 60,000. Figure and caption taken from Quintela-Fandino M, Soberon N, Lluch A et al. (2017) Critically short telomeres and toxicity of chemotherapy in early breast cancer. *Oncotarget* 8(13): 21472–21482.

data on the cytometer, always keep the sample on ice, as long as possible; (e) acquire 10,000 events of cells without and with the PNA probes. Adjust regions and compensation settings if needed.

Primed in situ (PRINS) based analyzes

Primed in situ hybridization is a variant of in situ PCR. This technique is based on the in situ annealing of synthetic oligonucleotides (CCCTAA)₇ to complementary nucleic acid sequences (telomeric DNA), followed by primer extension in the presence of a hapten- or fluorochromelabeled nucleotide (Bolzán and Bianchi, 2006). The telomeric fluorescence signal is generated by the incorporation of a labeled nucleotide into the newly synthesized telomeric DNA. PRINS can be performed using telomere-specific primers on slides with either metaphases or interphases. The obtained results cannot be distinguished from that of metaphase- or interphase-oriented Q-FISH. Also, a centromere may be labeled by PRINS as internal control in parallel to the telomeres. The major advantage of PRINS compared to Q-FISH is the length of the procedure itself. While Q-FISH needs an overnight hybridization step, PRINS-labeling is completed within 1–2 h (Charlieu and Pellestor, 1997).

For PRINS, the slides are treated with RNase for 1 h at 37 °C, washed in PBS (pH 7.4) and then subjected to the ethanol series (70%, 85% and 100%) for dehydration. The reaction mixture contains 0.2 mmol/L primers Telo (CCCTAA)₇, 0.04 mmol/L biotin-11-dUTP, 10 × reaction solution, and 1 U Taq polymerase. Final reaction mix is pipetted on the treated slide, mounted with a coverslip and sealed with rubber cement. The slide is then placed on pre-heated block at 94 °C for 5 min and further at 60 °C for 30 min. The reaction is stopped by transferring the slide to stop buffer (50 mmol/L NaCl and 50 mmol/L EDTA, pH 8.0) at 60 °C for 5 min, and next to stop buffer (50 mmol/L NaCl and 50 mmol/L EDTA pH = 8.0) for 7 min. Before detection of biotin, the slides are dehydrated through an ethanol series (70%, 85% and 100%) and then treated with a blocking solution (3% BSA, 4 × SSC, 0.05% Tween 20) at 37 °C for 30 min. The during PRINS reaction to target incorporated DNA, biotin-labeled nucleotides are detected with avidin-FITC. This is done by adding avidin-FITC resolved in blocking solution to the slide at 37 °C for 45 min in a humid chamber. The reaction is stopped by washing three times at 45 °C for 3 min in 4 × SSC, 0.05% Tween 20. After final wash, the slide is air-dried, counterstained with solution of propidium iodide at room temperature for 2 min and washed twice in distilled water. The evaluation can be done as describe for Q-FISH (Koch et al., 1995; Hong Tian et al., 2011).

Quantitative polymerase chain reaction (qPCR) and variations in TL measurement

There are several methods for estimating TL, however qPCR method is the most economical one and uses only small amounts of DNA. qPCR is a derivation of the standard PCR techniques established by Mullis in 1985 and used to quantify DNA based on the emission of fluorescence during each cycle of the PCR-reaction (Mullis, 1990). The methods used in this technique are based on fluorescent dyes binding to double-stranded DNA (Sybr Green assay) or specific probes (TaqMan probes). Results can be generated as absolute values, as there is a comparison between the total number of copies of the PCR product with a standard curve; or they may be relative, when the average of the target gene signals are compared to a control gene. In the later model, a Ct value is calculated, which corresponds to the number of cycles in which the emitted fluorescence signal reaches a significant threshold (exponential phase) (Fig. 4) and it is possible to identify the specific amplified DNA fragments using an analysis of their melting temperature by comparing the dissociation curves of the analyzed samples (Fig. 5).

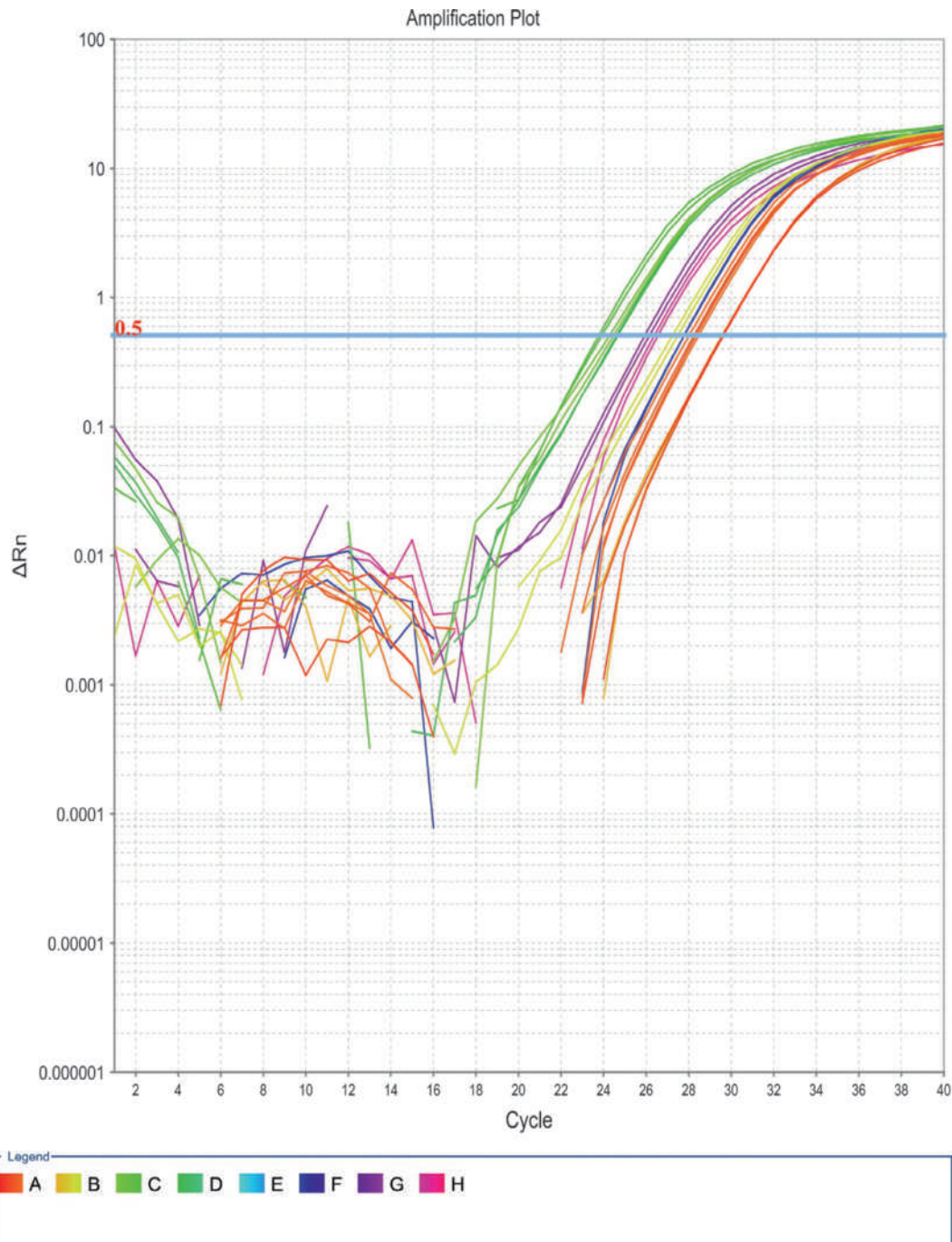


Fig. 4 The genomic DNA is quantified by qPCR. This figure shows an amplification curve demonstrating the Ct value corresponding to the cycle number at which the PCR curve crosses the threshold. The legend shows each sample analyzed in duplicate.

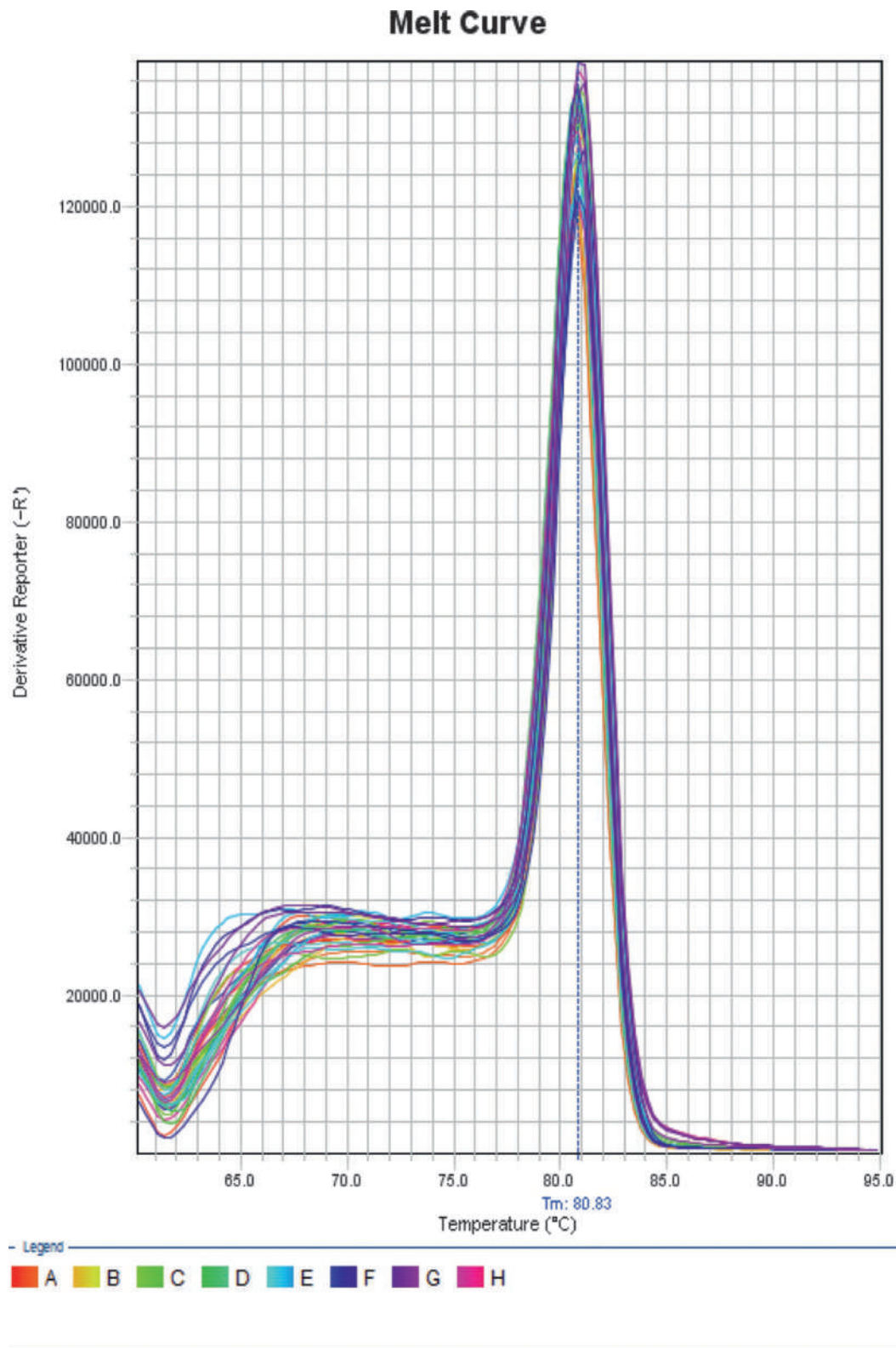


Fig. 5 Melting curve. The legend shows each sample was analyzed in duplicate.

In 2002, Cawthon introduced the qPCR technique as a tool for measuring the relative TL by comparing the TL averages in each sample and in a reference DNA sample in a ratio of telomere repeat copy numbers to single copy gene copy numbers (Cawthon, 2002). Here telomeric sequences and single copy gene PCRs are performed on separate plates. Two mixtures of PCR reagents are prepared, each using its primer pair, one with T (telomeric DNA) and the primer pair S (single copy gene) which serves as a basis for

normalizing the telomere's quantitative PCR signal. Thus, the ratio (T/S) between these averages would express the relative TL. Cawthon performed the reproducibility test of T/S ratio measurement (mean of the standard deviation = 9.4%) and compared the results of measuring TL between the means by qPCR and TRF ($R^2 = 0.677$; $P = 1.492 \times 10^{-24}$). Since then, this technique has been used widely in numerous studies that evaluate the TL in different applications.

A few years later, O'Callaghan et al. (2008) modified the technique first described by Cawthon. The author introduced an oligomer pattern containing 84 bp (approximately 14 repetitions of "TTAGGG") in length and a single copy gene (36B4–75 bp in length) that acts as a control for amplification. The data generated in kb/reaction has an ability to express the absolute value of the TL per human diploid genome instead of the relative quantification. In these articles, researchers demonstrated their protocols for several cell types, such as buccal cells and lymphocytes. The data obtained were compared to the TRF method and demonstrated a stronger correlation ($R^2 = 0.88$, $P < .0001$) (O'Callaghan and Fenech, 2011). Several research groups have applied this technique, absolute telomere length (aTL) using quantitative real-time polymerase chain reaction, as a basis for their analysis of diseases, psychological disorders and cancer risk associated with TL (Zeng et al., 2017; Chang et al., 2018; Jin et al., 2018; Mehrez et al., 2019; Ropio et al., 2020).

After developing a singleplex qPCR assay, Cawthon (2009), optimized the technique and created a multiplex qPCR model that optimized the accuracy, throughput and reduced the costs of the previously described technique. The purpose of this technique, Monochrome Multiplex Quantitative Polymerase Chain Reaction (MMqPCR), is to allow signals from any amplicons to be collected in a single run on the thermal cycler, both from the target sequence of interest (74 °C provided the Ct values for the amplification of T) and from the reference gene sequence (88 °C provided the Ct values for the amplification of S), where the average T/S ratio would estimate the relative TL per cell. In this work, Cawthon compared the results obtained through MMqPCR with results from TRF for validation, and showed a stronger correlation with different techniques ($R^2 = 0.844$), which demonstrated a greater correlation compared with data from the same samples by singleplex qPCR method ($R^2 = 0.677$).

In 2014, Kibriya et al. described an approach to determined TL based on fluorescence emitted by specific probes in the Luminex platform, where probes for telomere (T) and reference gene (R) are tested in a single well. The hybridization stage occurs with the capture of the target DNA sequence with specific sequence probes. Then, amplifiers that promote several hybridization events through biotinylated probes are added and, subsequently, they emit fluorescence signals when linked to Streptavidin R-Phycoerythrin (SAPE). The intensity of fluorescence signals is read on the Luminex platform and this technique has been accepted as an attractive alternative to conventional methods to measurement of TL such as qPCR ($R^2 = 0.70$ – 0.83) and Southern Blot ($R^2 = 0.74$) (Kibriya et al., 2014, 2016; Pierce et al., 2016). Later, the same group described an optimization of the test in order to increase the throughput and cost-effectiveness using a Multiplex model using different microbeads for the same T (telomeric region) and R (reference single gene) probes and then, pairs of probe sets are hybridized with DNA and grouped on a single plate for the other steps such as signal amplification, detection dye, wash and capture signal strength (Jasmine et al., 2018).

An alternative technique that has also been widely used is based on a kit designed to directly measure the TL of human cells such as Absolute Human Telomere Length Quantification qPCR Assay Kit (Cat# 8918) or Relative Human Telomere Length Quantification qPCR Assay Kit (Cat# 8908) (ScienCell Research Laboratories, Inc., Carlsbad, CA, United States). Basically, for each DNA sample, two consecutive reactions are performed: the first to amplify a single-copy reference (SCR) gene and the second for the telomere sequence. The SCR primer set recognizes and amplifies a 100 bp on human chromosome 17 and it is used as a reference for data normalization. Some recent studies have used this tool to measure the TL in research in different areas as, for examples, in hematologic disorders, environmental and occupational exposure and survival of people living with HIV (Babu et al., 2019; Moazzam et al., 2020; Shah et al., 2021).

Telomere measurements with digital real-time PCR

Luo et al. (2020) developed a method called START assay that consists of measuring telomeres from digital real time PCR (dPCR). The system used is the Fluidigm 48,770 digital array which contains 48 panels per 770 cameras (36,960 total), each compartment with a volume of 0.85 nL. The technique consists of digesting genomic DNA by the enzymes *HinfI* and *RsaI* ($1 \text{ U } \mu\text{L}^{-1}$), followed by diluting it by $7689 \text{ pg } \mu\text{L}^{-1}$ to efficiently partition single telomere molecules into individual compartments in the dPCR chip. The measurement of the telomere is made by comparing a standard curve, based on a synthetic telomere repetition sequence (CCCTAA)₁₅. From the concentration of synthetic DNA at 35 pM (320 kb), four serial dilutions are performed to achieve concentrations of 80 kb, 20 kb and 5 kb. For the absolute quantification of the TL, a standard curve of the average Ct value against log₁₀ (TL) is generated using the dilution series of 320 kb, 80 kb, 20 kb and 5 kb. The average length, the coefficient of variation and the percentage of short telomeres (<3 kb) for each sample are calculated based on the TL distribution obtained from the converted Ct values. The Ct of 27 was used as a cut-off value for detecting the TL, corresponding to ~0.2 kb. The technique was also designed to identify alternative telomere elongation mechanism (ATLs), based on the enumeration of ECTR molecules. ECTRs are DNA molecules containing telomeric repeat sequences that are not covalently linked to chromosomes. These molecules are described for participating in telomere cutting and maintenance events mediated by homologous recombination and have been observed to be present in ALT positive cancer cells (Cesare and Griffith, 2004; Chen et al., 2017).

The results of the study showed that the START assay showed a high and moderate correlation with the results of TRF ($R^2 = 0.96$) and qPCR ($R^2 = 0.78$), respectively; in addition to showing the presence of ATLs in cases where there was a greater number of telomere molecules per compartment in the START assay. The moderate correlation with qPCR is justified because the technique (qPCR) is dependent on the normalization of the single copy gene, which results in significant deviations due to aneuploidy, an event common to many types of cancer.

TL estimation from DNA methylation

Deviations in the length of telomeres are associated with several diseases related to aging. Therefore, they have been the subject of studies and, mainly, the length of the leukocyte telomeres (LTL), since their shortening is linked to cardiovascular diseases, psychological stress and life expectancy (Epel et al., 2004; Fitzpatrick et al., 2007; Needham et al., 2015). However, the methylation of cytosine residues from cytosine-guanine phosphate-dinucleotides (CpGs) is another marker that also changes with cell age (Horvath and Raj, 2018). Thus, from the methylation levels of the CpG bases, it is possible to generate specific algorithms capable of estimating the age of the DNA. Based on this context, Lu et al. (2019) developed a DNA methylation-based estimator of telomere length (DNAmTL) assay based on the methylation profile of 140 CpGs. For this, the study was divided into two groups for analysis, the first being composed by 3334 and the second by 9815 participants. Both had methylation levels measured by Illumina Infinium 450 k or EPIC 850 k arrays, and the first group also measured by telomere using the TRF technique. The authors estimated LTL based only on DNA methylation levels. The system was developed in an elastic network regression model implemented in the R glmnet package (Rizos et al., 2012).

The authors showed that using independent test data, DNAmTL exhibited moderately strong correlations with measurement of TL (based on Southern blotting or qPCR) in blood and adipose tissue of individuals from different racial groups. In short, DNAmTL outperformed LTL (based on Southern blotting or qPCR) in association with age ($r \sim -0.75$ for DNAmTL versus $r \sim 0.35$ for LTL), sex, ethnicity, lifestyle factors (diet, smoking, education, body mass index) and several clinical biomarkers (lipid and insulin levels). Furthermore, DNAmTL had a better predictive power than LTL for time to death ($P = 2.5E^{-20}$), coronary heart disease ($P = 6.6E^{-5}$) or heart failure ($P = 3.5E^{-6}$). It is important to note that the data focused only on blood samples, however, DNAmTL also applied to adipose and hepatic tissue, and to keratinocytes and fibroblasts (demonstrated in *in vitro* tests).

Genome-wide sequencing data to measure telomeres

The development of massive sequencing on a large scale has made it possible to analyze the sequences of telomeres on the scale of millions, as well as the entire genome. At first, the measurement of telomeres using sequencing data was not successful, because when comparing the already known measurement techniques, such as qPCR, the data obtained did not show results conformity (Aubert et al., 2012). Parker et al. (2012), as an example, showed that the use of TTAGGG or CCCTAA repetitions, as unit copies for data normalization, was inconsistent, since tumors constantly undergo aneuploidies (numerical chromosomal changes).

In Ding et al. (2014) developed the TelSeq program for estimating the TL based on genome-wide sequencing (GWS) data. For program validation, telomere measurement results from 260 leukocyte samples from United Kingdom twins (study called TwinsUK) (Moayyeri et al., 2013) and 96 samples from the 1000 Genomes Project (Durbin et al., 2010) were used. To verify the measurement's success by the program, the authors compared the results obtained from the measurement of telomeres by TRF with data from the total genome of the groups included in the study (Illumina 100 bp paired-end whole genome sequence). The basis for building the software was the analysis of the number of TTAGGG copies of the TwinsUK data, combined with the non-cyclic permutation of TTAGGG from the controls. This combination generated a multiplication equation for variables that estimate the TL. This equation consists of:

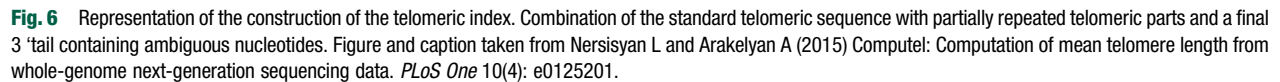
- (1) a constant telomeric reading abundance (based on the repetition readings threshold of the TTAGGG sequences);
- (2) a constant length of the genome divided by the number of ends of the telomere 46 (23×2);
- (3) the total number of readings per depth of the sequencing.

The results showed that TelSeq was able to present a high correlation with the data of the TRF technique ($\rho = 0.60$) ($P < 10E^{-16}$). Although TelSeq estimated a shortening higher than the TRF data (mean 5.63 kb compared to 6.97 kb, and shortening per year, 34.5 bp/year against 19.8 bp/year), the variation in the results was not significant ($P = .79$).

To access the software, it is available through the link: <https://github.com/zd1/telseq>.

Nersisyan and Arakelyan in 2015 developed an extension of the R program to calculate the average TL from GWS, called Computel. With characteristics similar to TelSeq, the system consists of an equation for measuring telomeres that takes into account:

- (1) Construction of a telomeric index based on: (a) a combination of the reading of telomeric repetition patterns (TTAGGGTT), (b) readings containing partially repeated telomeric parts (GGGTTAGGGT), and finally, (c) a 3'-final tail containing ambiguous nucleotides, in order to minimize the repetitions of reading of the telomeric region. This scheme can be seen in Fig. 6.
- (2) Mapping the readings to the telomeric index. Short readings of single or paired ends are aligned to the telomeric index with the bowtie2-align program;
- (3) Calculation of coverage in the telomeric index *;
- (4) Determination of the average coverage in the reference genome (optional) *;
- (5) Average TL estimate *.



The results generated by Computel showed a strong linear correlation (R^2) equal to 0.95 and 0.92 for single and paired end readings. Overall, Computel's performance showed a strong correlation with the average TL estimated with qPCR and TRF techniques, but it diverged to some extent (2–3 kb) in absolute values. In the case of TRF, this difference can be attributed to the capture of sub telomeric regions of the chromosomes, overestimating the length of the telomeres by 2.5–4 kb. On the other hand, Computel surpassed TelSeq in all cases analyzed. When the reading length ranges significantly changed (36, 76, 100, and 150 nucleotides), the telomere size was overestimated.

In 2018, the Telomerecat program (Farmery et al., 2018) was developed in order to measure telomeres with a high standard of veracity, correcting errors not considered by the TelSeq and Computel programs, such as aneuploidies (present mainly in cancer cells and non-human cells) and errors inherent to sequencing.

- (1) normalization is not done in relation to the whole genome, but in relation to sub telomeric regions;
- (2) the system corrects the number of readings originating from the so-called interstitial telomeric repetitions (ITRs). These erroneous regions of apparent telomeres and sub telomers may arise from other stretches of the TTAGGG repeat sequence that appear in the human genome (Riethman et al., 2004);
- (3) sequencing errors elimination. The sub telomer is composed of sub telomeric repetitions and segmental duplicates, interspersed with canonical repetitions. Thus, these repetitions are susceptible to variations, mutations and sequencing errors.

Telomerecat is available through the link <https://github.com/jhrf/telomerecat> and the installation guide at <https://telomerecat.readthedocs.io>.

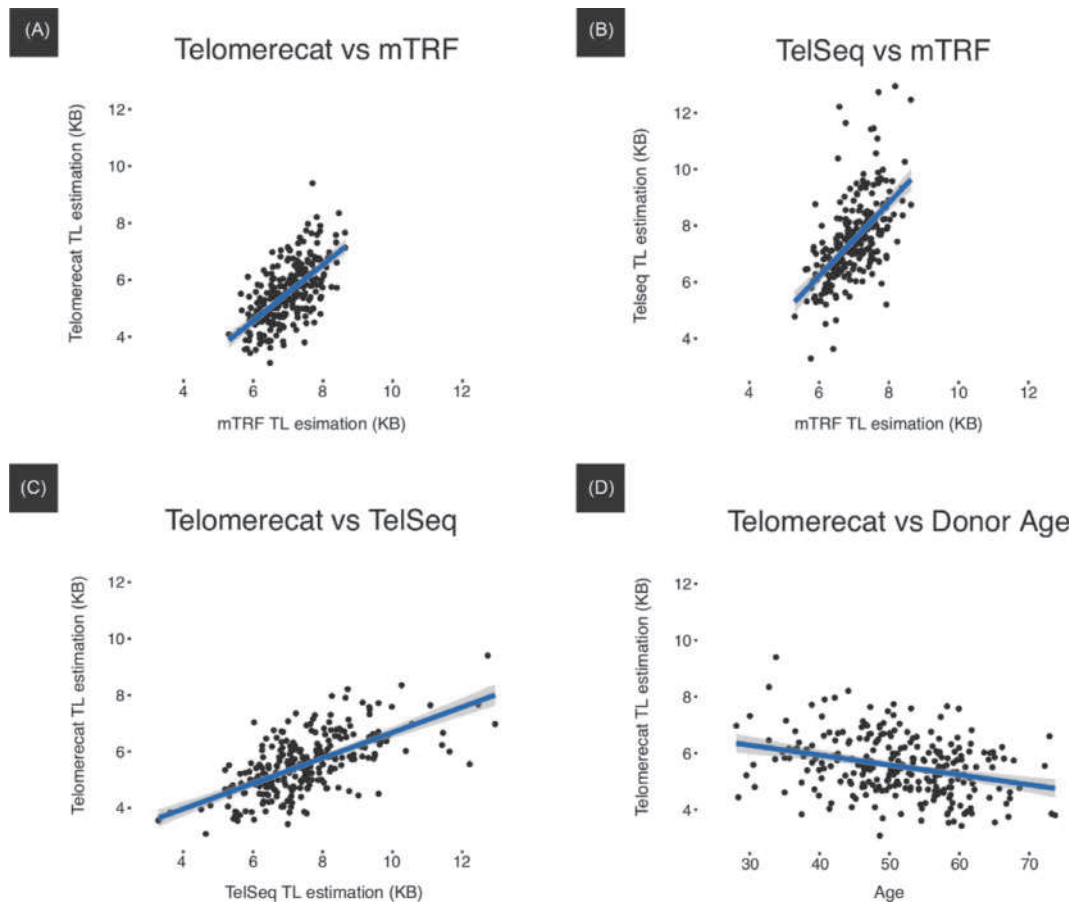


Fig. 7 Scatter plots describing the relationship between Telomerecat vs mTRF (A), TelSeq vs mTRF (B), Telomerecat vs TelSeq (C), and Telomerecat vs Donor Age (D) estimating TL. mTRF, mean terminal restriction fragment Southern blot. Figure and caption taken from Farmery JHR, Smith ML, NIHR BioResource - Rare Diseases and Lynch AG (2018) Telomerecat: A ploidy-agnostic method for estimating telomere length from whole genome sequencing data. *Scientific Reports* 8(1): 1300.

Identification of the alternative telomere elongation mechanism by GWS

In addition to the t-type (TTAGGG) sequences, telomeres have repetition variations, such as type-c (TCAGGG), type-g (TGAGGG), and type-j (TTGGGG) (Varley et al., 2002; Lee et al., 2014) called telomeric variant repetitions (TVRs). The recombination between these repetitions forms the ATL. In 2019, the TelomereHunter program (Feuerbach et al., 2019) was developed to identify the presence of ATLs in WGS data.

The program consists of extracting at least six readings of non-consecutive repetitions of the TVR (t-, c-, g- or j-type). An equation considering the combination of readings and length of the sequenced fragment is generated. In the second moment, the readings extracted are categorized according to the alignment and quality of the mapping. That is, if the readings are aligned, the position of the strings is considered suitable for classification. The program considers all chromosomal bands, except the first and last band of a chromosome, as eligible for reading sequences considered to be intrachromosomal. The readings of these extremities, on the other hand, are categorized as intratelomeric. The telomere content is calculated as the fraction of intratelomeric readings per million readings. In addition, the system corrects the content of telomeres considering the content of GC between 48% and 52%. Finally, TVRs are quantified by looking for NNNGGG hexamers in intratelomeric readings. A particular focus is placed on singletons [(TTAGGG) 3-NNNGGG- (TTAGGG) 3], whose counts are normalized by the total number of readings in the sample.

The presence and absence of ATL in a medulloblastoma patient using TelomereHunter are represented by Figs. 8 and 9, from the original article by Feuerbach et al. (2019).

The software and installation documentation are available at <https://www.dkfz.de/en/applied-bioinformatics/telomerehunter/telomerehunter.html>.

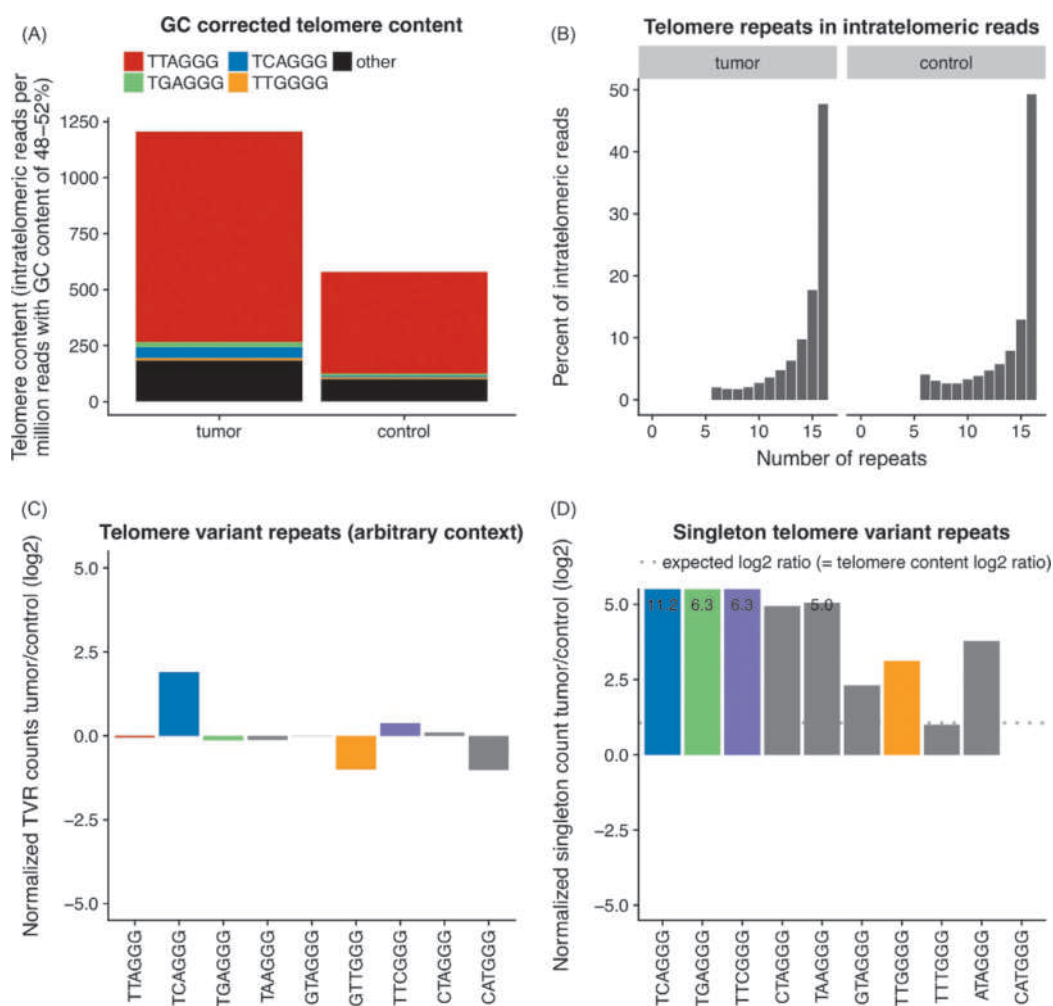


Fig. 8 Exemplary ALT-positive glioblastoma patient. (A) GC corrected telomere content: Telomere content of tumor and control sample in reads per million GC-matched reads. Contributions of TVRs are indicated by color code. (B) Telomere repeats in intratelomeric reads: Histogram of repeat units per read for all extracted intratelomeric reads. (C) Telomere variant repeats (arbitrary context): Overview on $\log_2$ T/C of TVRs in arbitrary context. (D) Singleton telomere variant repeats: Overview on $\log_2$ T/C of TVRs in singleton context. Figure and caption provided by Feuerbach L, Sieverling L, Deeg KI et al. (2019) Telomerehunter—In silico estimation of telomere content and composition from cancer genomes. *BMC Bioinformatics* 20(1): 272.

Summary and perspectives

Since the discovery of the cellular maintenance mechanism involving telomeres, the evaluation of TL in several pathologies has been highlighted in many researches. Telomere shortening is inevitable under normal conditions, since with each cell division, the lagging-strand DNA is not fully synthesized. This mechanism tells the cell finally to stop cellular growth arrest and enter senescence or cell death, thus preventing tumor formation in humans and other long-lived animals (Shay, 2016; Maciejowski and de Lange, 2017). On the other hand, in tumors, telomere erosion is protected. In these cases, the cell activates telomerase, or promotes ATL. For this reason, telomere measurement techniques have gained interest and were developed. In this article, the main methodologies for measuring TL are summarized, from the introduction of the TRF in Moyzis et al. (1988), to the dPCR in Luo et al. (2020). It is clear that in future probably more new technologies will emerge in addition to therapeutic studies aimed at the telomere's molecular mechanism.

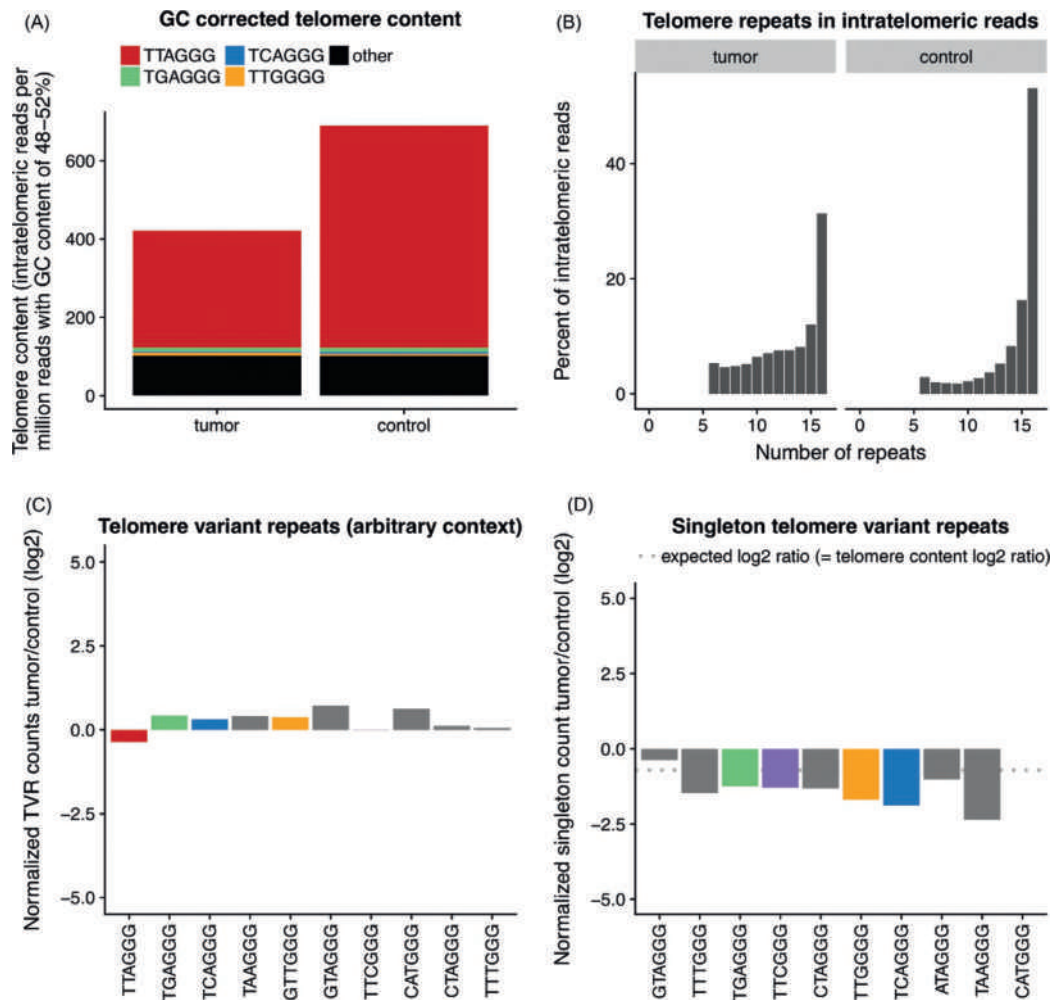


Fig. 9 Exemplary ALT-negative medulloblastoma patient. (A) GC corrected telomere context: Telomere content of tumor and control sample in reads per million GC-matched reads (RGCM). Contribution of TVR is indicated by color code. (B) Telomere repeats in intratelomeric reads: Histogram of repeat units per read for all extracted intratelomeric reads. (C) Telomere variant repeats (arbitrary context): Overview on log₂ T/C of TVRs in arbitrary context. (D) Singleton telomere variant repeats: Overview on log₂ T/C of TVRs in singleton context. Figure and caption by Feuerbach L, Sieverling L, Deeg KI et al. (2019) Telomerehunter—In silico estimation of telomere content and composition from cancer genomes. *BMC Bioinformatics* 20(1): 272.

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Breakage Analysis and DEB Testing

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Glossary

Abortus This term is used to define a fetus or embryo removed or expelled from the uterus during the first half of gestation (20 weeks or less).

Buffy coat This is simply a concentration of all the white blood cells and platelets in a sample of blood. This can be obtained by centrifuging a sample of peripheral blood at 1200 rpm for 10 min.

Cell passaging This is also referred to as cell splitting. It is a technique that enables an individual to keep cells alive and growing under cultured conditions for an extended period of time. Cells should be passed when they are 90–100% confluent.

Fibroblast tissues This is the principal active cell of connective tissues. They are large, flat, elongated (spindle shaped) cells possessing processes extending out from the ends of the cell body. The cell nucleus is flat and oval.

Peripheral blood This is otherwise known as “whole blood”. It is a complete collection of various blood cells i.e., erythrocytes (red blood cells), leukocytes (white blood cells) and thrombocytes (platelets) suspended in blood plasma.

Stillborn An infant born dead. This is the death of a baby before or during delivery.

Trophoblast cells They are cells that form the outer layer of the “pre-formed” placenta. They are responsible for supply of nutrient to the developing embryo. These cells are formed during the first stage of pregnancy and they are among the first cells to undergo differentiation after fertilization of the egg.

Abbreviations

cDNA Complementary DNA

gDNA Genomic DNA

PCR Polymerase chain reaction

Introduction

Gaps, cracks or lines indicating breakage(s) along the entire length or width of a chromosome strand are usually unnoticeable, and peradventure they become obvious, they are most often dismissed as being insignificant provided the chromosome set is complete. Unfortunately, a slight damage to a very important autosomal (somatic) or sex chromosome arm, for example, the long (q) arm of chromosome 13 at position 12.3, which happens to be the gene locus for BRCA2 genes (Chen and Parmigiani, 2007), or an alteration of the nucleotide sequence of a gene (coded for a specific character or function), or a mismatched sequence of genes on a chromosome or part of its strand, caused by hormonal imbalance, organ or system dysfunction, or influenced by external factors like chemicals, drugs, ultraviolet- or gamma radiation, or acquired by direct inheritance from either or both parents etc., can result in

mutation, depending on the magnitude of damage caused or level of alteration infused (Fargo et al., 2014). The effects of mutation can be partial or total, with minor or major alterations to specific family traits, character(s) or behaviourism. Diepoxybutane (DEB) is a very important chemical test that can aide visibility of cracks or gaps or breakages in autosomal chromosomes of specialized cells and tissues (Moghrabi et al., 2009). The term “Breakage analysis” is simply used to describe the procedure involved in the detection of these “gaps” or “cracks” domiciled on a complete chromosome strand.

Chromosome breakage analysis is a complex laboratory protocol used in the determination of genome instability or the degree of genetic variation of a particular individual from his or her lineage or ancestry. Chromosomal aberration can result in sicknesses like bone marrow dysfunction or failure, myelogenous leukemia, myelodysplastic syndrome, and an increased risk of contracting breast, ovarian, prostate and other forms of cancer (Chen and Parmigiani, 2007). Therefore, intending couples and families with known history of hormonal imbalance or inherited bone marrow failure syndromes (IBMFs) etc., are advised to undergo this test for their own benefit, in other to safeguard the lives of their children, wards and future progeny. Early detection of chromosomal aberration can be the difference between life and death, for a child with this abnormality. In essence, breakage analysis can be conducted on blood samples, fibroblast tissues and even trophoblast cells from developing embryo or fetus (Auerbach, 2015). In any case, the earlier the situation is diagnosed and reported, the better the chances of survival of the affected individual. The aim of this study is to provide a general laboratory protocol for breakage analysis and DEB testing. The protocols for each analysis was described below.

Methodology

Specimen specification and collection

For an individual with complete blood count (CBC) rated as normal or high, i.e., Red blood cells (RBC), white blood cells (WBC) and lymphocyte (L) count, about 5–10 mL of whole blood (Peripheral blood) should be collected in Sodium Heparin vacutainer tube or a pre-heparinized plastic syringe containing 0.2 mL sodium heparin. For individuals with low “CBC”, “WBC” or “L” count, breakage analysis can be conducted using fibroblast tissues from the skin or other part of the body. DEB and breakage analysis conducted on fibroblast tissues can equally reveal gaps, cracks or lines of breakages and radial formation on chromosome strands. This phenomenon is known as somatic mosaicism. Fibroblast breakage studies may also be preferable for patients in short supply of blood in their system (e.g., anemic patients), accident victims needing urgent blood transfusion, or patients with extreme phobia for needles or injection etc. In the case of pregnant women, a sample of the trophoblast cells, amniotic fluid or fetal blood can suffice for DEB testing and breakage analysis.

Diepoxybutane (DEB) test

The protocol for Diepoxybutane (DEB) treatment of biological samples (basically from humans) was extracted from the research of Auerbach (2015).

Peripheral blood

Laboratory requirements:

- Peripheral blood (5–10 mL).
- Complete RPMI 1640, 15% FBS medium and 1% (w/v) Phytohemagglutinin (PHA).
- PBS.
- Diepoxybutane (1,3-butadiene diepoxide), store at 4 °C.
- 1 µg/mL Colcemid (store at 4 °C).
- 0.075 M KCl (store at 37 °C).
- Fixative i.e., 3:1 (v/v) methanol/glacial acetic acid (freshly prepared).
- Syringe (10 mL capacity) equipped with 18½-G needle.
- 25 cm² tissue culture flasks.
- 15 mL sterile conical-bottom centrifuge tubes with caps.
- IEC clinical centrifuge or equivalent.
- Microscope slides (store in 70% ethanol).
- Inverted microscope.
- Additional reagents and equipment for collection of peripheral blood lymphocytes.

Procedure:

Freshly collected blood sample is required for this experiment. Aseptically transfer 18–25 drops of peripheral (whole) blood (about 0.4–0.5 mL), using sterile syringe with 18½-G needle, into a 25 mL-capacity tissue culture flask containing 10 mL complete RPMI 1640, 15% FBS and 1% PHA. Repeat the procedure eight times to produce replicates for the experiment. Incubate at 37 °C, with 5% CO₂ humidity, for 24 h. Prepare the required DEB treatment by adding 10 mL of PBS to test tube A, 6 mL to test tube B, and 4.5 mL to test tubes C and D, respectively. Furthermore, add 10 µL of DEB solution (fortified with 15% FBS) to test tube A, cap, and

mix gently. Transfer 4 mL from test tube A to B, cap, and homogenize the content by shaking manually, then aseptically transfer 0.5 mL (500 μ L) from test tube B to C, cap, and mix gently too. Complete the dilution process by pipetting 0.5 mL (500 μ L) from test tube C to D, homogenize the content manually and allow to stand for a few seconds. Prepare a blank treatment in test tube E (using sterile distilled water).

After incubation, pipette 25 μ L of the treatment solution from test tube C into three (3) out of the nine (9) incubated blood samples, label the flasks C1, C2 and C3, respectively. Repeat the procedure using test tube D and three (3) out of the six (6) remaining cultures and label the flasks D1, D2 and D3. Finally, pipette 25 μ L of the "blank treatment" from test tube E into the remaining cultures and label them as E1, E2 and E3, respectively. The final DEB concentrations in the flasks would be 0.10 μ g/mL (C1, C2 and C3), 0.01 μ g/mL (D1, D2 and D3) and 0.00 μ g/mL (E1, E2 and E3), respectively. Afterwards, incubate at 37 °C, under a controlled cloud of 5% CO₂, for 48–72 h. Add 2 mL of 1 μ g/mL Colcemid to each of the flask and incubate further for another 20 min. Transfer the content of each flask (both treated and untreated) to separate sterile conical-bottom centrifuge tubes (15 mL capacity each), cap, and centrifuge at 800–1000 rpm (in an IEC clinical centrifuge) for 10 min. Decant the supernatant, retaining about 0.5 mL of liquid, re-suspend the cell pellets at the bottom of the tubes in the leftover supernatant (0.5 mL) by flipping each tube manually (to aide homogenization). Add 5 mL of pre-warmed 0.075 M KCl and incubate for another 10 min at 37 °C, in a water bath.

Centrifuge again at 1000 rpm for 10 min. Completely remove the supernatant and gently add 1 mL of freshly prepared fixative without disturbing the cell pellets. Decant to remove fixative immediately (after 3–5 s) and repeat this procedure two more times. Afterwards, tap the tube gently to break up the pellets. Instantaneously, add fixative to re-suspend the cells without agglutination or cluster formation. Step up the volume to 8–10 mL with fixative. Break up any clumps by pipetting with a Pasteur pipet. Allow to stand for 30 min at 25 °C. At the end of 30 min, centrifuge at 1000 rpm for 10 min completely decant the supernatant and re-suspend the cell pellets in about 3–5 mL of fresh fixative. Allow to stand for another 10 min. Repeat this step once or twice, immediately or after a window period of 24 h. This procedure should be sufficient to remove red blood cell debris and further fix the remnant white blood cells. After successful removal of red blood cell debris from the culture, centrifuge at 1000 rpm for 10 min. Remove the supernatant, break the pellet, and add enough fixative (about 0.25–0.5 mL) to give the desired cell concentration for slide preparation. The procedure was adapted from [Auerbach \(2015\)](#).

Fibroblast tissues

In the absence of peripheral blood, DEB test can be performed on fibroblast cultures i.e., initiated from skin biopsies, excised pieces of lungs or skin fragments from an abortus or a stillborn etc.

Laboratory requirements:

- Fibroblast tissues.
- Complete DMEM medium (Supplemented with 20% FBS).
- PBS.
- Diepoxybutane (1,3-butadiene diepoxide), store at 4 °C.
- 1 μ g/mL Colcemid (store at 4 °C).
- Fixative i.e., 3:1 (v/v) methanol/glacial acetic acid (freshly prepared).
- 0.051 M (0.38% w/v) KCl.
- Tissue culture plates (60 mm capacity).
- Disposable scalpels.
- Punch (3 mm).
- Additional reagents and equipment for cell passaging and counting.

Procedure:

Collect fibroblast tissue with the aid of a 3 mm punch. Place the fibroblast tissue in 0.5 mL complete DMEM medium (fortified with 20% FBS) and cut to standard fragment (1 × 1 mm) using two sterile disposable scalpels, in a 60 mm tissue culture plate. Draw horizontal and vertical lines across the inner surface of three (3) sterile disposable tissue culture plates (60 mm capacity) using the pointed edge of a sterile scalpel. Transfer 5–6 excised pieces of fibroblast tissues into each of the 60 mm culture plate. Place the tissues at the intersect of the scored lines. The score lines will facilitate easy attachment of tissue fragments to the culture plates and also, tracing of tissue colonies. Incubate the scored plates, without any additional tissue culture medium, at 37 °C for 15–30 min, to allow the pieces of tissue to adhere to the plates. Afterwards, add 5 mL of complete DMEM medium (with 20% FBS) to each plate and incubate the cultures at 37 °C, with 5% CO₂. Replace the culture medium every 3–4 days without disturbing the tissue fragments. Use an inverted microscope to inspect the cultures for evidence of cell growth.

As soon as a huge patch of new fibroblasts cells surrounds the tissue fragments in the plate, carry out cell passaging and trypsinization by treating both the proliferated fibroblast cells and initial tissue fragments with 1 × trypsin/EDTA solution i.e., remove the medium in which the newly proliferated fibroblast cells were suspended by decanting the liquid without agitating the cell clusters, wash the monolayered-cell clusters with a reasonable quantity of saline solution, free of Ca²⁺ and Mg²⁺. Thereafter, remove the saline solution from the culture by decanting or with the aid of a Pasteur pipette. Add excess trypsin/EDTA solution to the culture such that the monolayers of cell clusters are completely submerged by the solution. Incubate the proliferated cell culture at 37 °C for 2 min in a mist of 5% CO₂. Afterwards, aseptically remove the trypsin/EDTA solution by decanting (without agitation)

and incubate the residual cells at 37 °C until cells start to detach from the surface of the monolayer cluster. The progress of cell detachment can be monitored intermittently by the use of an inverted microscope. Extreme care should be taken not to dislodge the cell colonies formed, as they will continue to proliferate in the culture, if undisturbed.

If there are more than enough fibroblast cells formed within any of the tissue culture flask, transfer some of the newly generated cells to a sterile tissue culture flask (25 mL capacity) submerged in complete DMEM/15% FBS solution (Note: reduce the concentration of FBS in the DMEM medium to 15% for this and all subsequent subcultures). These cells are designated as “first passage cells.” Add 1 × trypsin/EDTA solution when the first passage culture reaches convergence. Subculture at a 1:3 split ratio in complete DMEM/15% FBS. Cell passaging and trypsinization can be conducted until there are enough proliferated cells for the experiment. You can finally proceed with DEB treatment when at least three “second passage cultures” reach convergence. Plate the proliferated fibroblast cells in 5 mL complete DMEM medium (fortified with 15% FBS) in 25 mL capacity tissue culture flask (3×10^5 cells per flask). Incubate cultures at 37 °C, with 5% CO₂, for 24 h.

Prepare the required DEB treatment by adding 10 mL of PBS to test tube A, 6 mL to test tube B, and 4.5 mL to test tubes C and D, respectively. Furthermore, add 10 µL of DEB solution (fortified with 15% FBS) to test tube A, cap, and mix gently. Transfer 4 mL from test tube A to B, cap, and homogenize the content by shaking manually, then aseptically transfer 0.5 mL (500 µL) from test tube B to C, cap, and mix gently too. Complete the dilution process by pipetting 0.5 mL (500 µL) from test tube C to D, homogenize the content manually and allow to stand for a few seconds. Prepare a blank treatment in test tube E (using sterile distilled water).

After incubation, pipette 25 µL of the treatment solution from test tube C into three (3) out of the nine (9) incubated Fibroblast cells, label the tissue culture plates as C1, C2 and C3, respectively. Repeat the procedure using test tube D and three (3) out of the six (6) remaining cultures and label the tissue culture plates as D1, D2 and D3. Finally, pipette 25 µL of the “blank treatment” from test tube E into the remaining cultures and label the tissue culture plates as E1, E2 and E3, respectively. The final DEB concentrations in the treated tissue culture plates would be 0.10 µg/mL (C1, C2 and C3), 0.01 µg/mL (D1, D2 and D3) and 0.00 µg/mL (E1, E2 and E3), respectively. Afterwards, incubate all the tissue culture plates at 37 °C, under a controlled cloud of 5% CO₂, for 48–72 h. Add 2 mL of 1 µg/mL Colcemid to each of the treated tissue culture plates and incubate further for another 20 min. Transfer the content of each of the tissue culture plates (both treated and untreated) to separate sterile conical-bottom centrifuge tubes (15 mL capacity each), cap, and centrifuge at 800–1000 rpm (in an IEC clinical centrifuge) for 10 min. Decant the supernatant, retaining about 0.5 mL of liquid, re-suspend the cell pellets at the bottom of the tubes in the leftover supernatant (0.5 mL) by flipping each tube manually (to aide homogenization). Add 5 mL of pre-warmed 0.075 M KCl and incubate for another 10 min at 37 °C, in a water bath.

Centrifuge again at 1000 rpm for 10 min. Completely remove the supernatant and gently add 1 mL of freshly prepared fixative without disturbing the cell pellets. Decant to remove fixative immediately (after 3–5 s) and repeat this procedure two more times. Afterwards, tap the tube gently to break up the pellets. Instantaneously, add fixative to re-suspend the cells without agglutination or cluster formation. Step up the volume to 8–10 mL with fixative. Break up any clumps by pipetting with a Pasteur pipet. Allow to stand for 30 min at 25 °C. At the end of 30 min, centrifuge at 1000 rpm for 10 min. completely decant the supernatant and re-suspend the cell pellets in about 3–5 mL of fresh fixative. Allow to stand for another 10 min. Repeat this step once or twice, immediately or after a window period of 24 h.

Harvest cultures at the first mitotic stage (precisely, at the metaphase stage) after subculture (between 24 and 48 h). Add 1 mL of 1 µg/mL Colcemid (per 5 mL medium) to all the tissue culture plates, at 3 h prior to harvest. Incubate for another 3 h at 37 °C, with 5% CO₂. The timing for harvesting is determined by the intermittent inspection of the cultures with an inverted microscope. Metaphase cells appear rounded. Treat the fibroblast cells again with 1 × trypsin/EDTA solution (use the predefined trypsinization procedure). Transfer the contents of each tissue culture plates to a sterile 15 mL centrifuge tube, cap, and centrifuge for 10 min at 1000 rpm. Decant, retaining about 0.5 mL of liquid, re-suspend the cell pellets at the bottom of the tubes in the leftover supernatant (0.5 mL) by flipping each tube manually (to aide homogenization), with the careful addition of 5 mL of pre-warmed 0.075 M KCl. Incubate the mixture for 10 min at 37 °C, in a water bath. Afterwards, centrifuge at 1000 rpm for 10 min. Fix the fibroblast cells as described above for blood samples (Auerbach, 2015). Note: The hypotonic solution of KCl applied to the fibroblast cells should be more diluted than that used for peripheral blood cultures.

Trophoblast cells or Fetal blood

Trophoblast cells obtained by chorionic villus sampling (CVS) at 9–12 weeks of gestation, or amniotic fluid cells obtained by amniocentesis at 15–17 weeks of gestation, or even fetal blood can be analyzed for chromosome breakages.

Laboratory requirements:

- Fetal cells obtained by CVS or amniocentesis, enough to establish cultures in two (2) tissue culture flasks (25 mL capacity).
- PBS.
- Diepoxybutane (1,3-butadiene diepoxide), store at 4 °C.
- 1 µg/mL Colcemid (store at 4 °C).
- 0.075 M KCl (store at 37 °C).
- Fixative i.e., 3:1 (v/v) methanol/glacial acetic acid (freshly prepared).
- 25 cm² tissue culture flasks.
- 15 mL sterile conical-bottom centrifuge tubes with caps.
- IEC clinical centrifuge or equivalent.
- Microscope slides (store in 70% ethanol).

- Inverted microscope.
- Growth medium: 1:1 mixture of Chang medium and complete RPMI (fortified with 15% FBS).
- Additional materials and equipment for culture of chorionic villi and amniotic fluid.

Procedure:

Establish fetal blood or trophoblast cells or amniotic fluid cultures in the preferred growth medium within 25 mL capacity tissue flasks and incubate at 37 °C, with 5% CO₂. Treat the cultured fetal cells with 1 × trypsin/EDTA solution and subculture when there are noticeable clusters of massive active proliferating cells present in each culture flask. Do not allow colonies to become overgrown. Use a split ratio of 1:2 or 1:3 to subculture proliferated fetal cells i.e., If there are more than enough proliferated fetal blood- or trophoblast- or amniotic fluid cells formed within any of the tissue culture flask, transfer some of the newly generated cells to a sterile tissue culture flask (25 mL capacity) submerged in complete DMEM/15% FBS solution (Note: reduce the concentration of FBS in the DMEM medium to 15% for this and all subsequent subcultures). These cells are designated as “first passage cells.” Add 1 × trypsin/EDTA solution when the first passage culture reaches convergence. Subculture at a 1:3 split ratio in complete DMEM/15% FBS. Cell passaging and trypsinization can be conducted until there are enough proliferated cells for the experiment. You can finally proceed with DEB treatment when at least three “second passage cultures” reach convergence. Plate the proliferated fetal blood- or trophoblast- or amniotic fluid cells in 5 mL complete DMEM medium (fortified with 15% FBS) in 25 mL capacity tissue culture flask (3 × 10⁵ cells per flask). Incubate cultures at 37 °C, with 5% CO₂, for 24 h.

Prepare the required DEB treatment by adding 10 mL of PBS to test tube A, 6 mL to test tube B, and 4.5 mL to test tubes C and D, respectively. Furthermore, add 10 µL of DEB solution (fortified with 15% FBS) to test tube A, cap, and mix gently. Transfer 4 mL from test tube A to B, cap, and homogenize the content by shaking manually, then aseptically transfer 0.5 mL (500 µL) from test tube B to C, cap, and mix gently too. Complete the dilution process by pipetting 0.5 mL (500 µL) from test tube C to D, homogenize the content manually and allow to stand for a few seconds. Prepare a blank treatment in test tube E (using sterile distilled water).

After incubation, pipette 25 µL of the treatment solution from test tube C into three (3) out of the nine (9) incubated fetal blood or trophoblast cells or amniotic fluid samples, label the flasks C1, C2 and C3, respectively. Repeat the procedure using test tube D and three (3) out of the six (6) remaining cultures and label the flasks D1, D2 and D3. Finally, pipette 25 µL of the “blank treatment” from test tube E into the remaining cultures and label them as E1, E2 and E3, respectively. The final DEB concentrations in the flasks would be 0.10 µg/mL (C1, C2 and C3), 0.01 µg/mL (D1, D2 and D3) and 0.00 µg/mL (E1, E2 and E3), respectively. Afterwards, incubate at 37 °C, under a controlled cloud of 5% CO₂, for 48–72 h. Add 2 mL of 1 µg/mL Colcemid to each of the flask and incubate further for another 20 min. Transfer the content of each flask (both treated and untreated) to separate sterile conical-bottom centrifuge tubes (15 mL capacity each), cap, and centrifuge at 800–1000 rpm (in an IEC clinical centrifuge) for 10 min. Decant the supernatant, retaining about 0.5 mL of liquid, re-suspend the cell pellets at the bottom of the tubes in the leftover supernatant (0.5 mL) by flipping each tube manually (to aide homogenization). Add 5 mL of pre-warmed 0.075 M KCl and incubate for another 10 min at 37 °C, in a water bath.

Centrifuge again at 1000 rpm for 10 min. Completely remove the supernatant and gently add 1 mL of freshly prepared fixative without disturbing the cell pellets. Decant to remove fixative immediately (after 3–5 s) and repeat this procedure two more times. Afterwards, tap the tube gently to break up the pellets. Instantaneously, add fixative to re-suspend the cells without agglutination or cluster formation. Step up the volume to 8–10 mL with fixative. Break up any clumps by pipetting with a Pasteur pipet. Allow to stand for 30 min at 25 °C. At the end of 30 min, centrifuge at 1000 rpm for 10 min. completely decant the supernatant and re-suspend the cell pellets in about 3–5 mL of fresh fixative. Allow to stand for another 10 min. Repeat this step once or twice, immediately or after a window period of 24 h.

Harvest cultures at the first mitotic stage after subculture (between 24 h and 48 h). Add 1 mL of 1 µg/mL Colcemid (per 5 mL medium) to all the flasks, at 3 h prior to harvest. Incubate for another 3 h at 37 °C, with 5% CO₂. The timing for harvesting is determined by the intermittent inspection of the cultures with an inverted microscope. Metaphase cells appear rounded. Treat the fetal blood- or trophoblast- or amniotic fluid cells again with 1 × trypsin/EDTA solution (use the predefined trypsinization procedure). Transfer the contents of each flask to a sterile 15 mL centrifuge tube, cap, and centrifuge for 10 min at 1000 rpm. Decant, retaining about 0.5 mL of liquid, re-suspend the cell pellets at the bottom of the tubes in the leftover supernatant (0.5 mL) by flipping each tube manually (to aide homogenization), with the careful addition of 5 mL of pre-warmed 0.075 M KCl. Incubate the mixture for 10 min at 37 °C, in a water bath. Afterwards, centrifuge at 1000 rpm for 10 min. Remove the supernatant, break the pellet, and add enough fixative (about 0.25–0.5 mL) to give the desired cell concentration for slide preparation. The procedure was adopted from Auerbach (2015).

Breakage analysis

Staining of chromosomes

The Giemsa staining technique is the procedure adopted for chromosome staining in this research. Giemsa stain is a histological stain with a particular affinity for nuclei and chromosomes. The procedure is simple and results in the formation of cluster-free reddish-blue to purple nuclei or chromosomes.

Laboratory Requirements:

- Gurr's buffer tablets (pH 6.8).
- Giemsa stain.

- Metaphase chromosome slides.
- Permount histological mounting medium.
- Coplin jars.
- Sterile distilled water (dH₂O).

Procedure:

Dissolve one (1) Gurr's buffer tablet in 1Ltr of sterile distilled water. Setup five (5) Coplin jars i.e., one (1) for the Giemsa stain, one (1) for the Gurr's buffer and three (3) successive jars for sterile distilled water. Add 4 mL of Giemsa stain to 46 mL of the freshly prepared Gurr's buffer in the staining jar.

Note:

1. Lifespan of the stain is 1 h, therefore, staining solution must be prepared fresh before each use.
2. Chromosomes used for this experiment should be at the metaphase stage.

Fix a tiny portion of the harvested chromosomes on a clean slide. Gently streak the chromosomes on the slide to allow even distribution of the chromosome arms. Flood the fixed chromosomes on the slide with freshly prepared Giemsa stain for 5 min, rinse by dipping several times into the Coplin jar containing Gurr's buffer, afterwards, rinse the slide by dipping several times in succession of sterile distilled water. Air dry the slide in a Laminar air flow chamber. Afterwards, check each slide for stain intensity. Stain intensity should be sufficient so that gaps and breaks in the chromosomes appear as achromatic (non-stained) regions. Select slides that are well stained and insert coverslips on them using Permount histological mounting medium (Auerbach, 2015). A total of between 50 and 100 slides should be prepared for observation and scoring.

Chromosome analysis for breakages and aberration using an inverted microscope

Procedure:

The protocol adapted in the description of breakage analysis was an extract from the research of Fargo et al. (2014). The procedure is described thus: Add few drops of cell suspension on each microscope slides. Stain with Giemsa or Wright stain and observe under an inverted microscope. Fifty (50) cells from each Clastogen should be scored for chromosome breaks and radial formations using a Nikon Eclipse E800 Photoscope. Representative images should be captured using either CytoVision (Applied Imaging) or Applied Spectral Imaging software. Aberrations should be recorded as the number of cells with specific numbers of breaks or radials, as well as the number of breaks in radial-containing cells. While the actual structure of radial figures remains unknown, each radial figure should be assigned a value of two breaks for the purpose of incorporating these data into the overall averages. Test Statistics that may be useful in quantifying chromosome breakages include total breaks per cell, percentage of aberrant cells, and breaks per aberrant cell, as well as the chromosome fragility index (CFI), which is breaks per aberrant cell multiplied by the percentage of aberrant cells as stated by Castella et al. (2011). Other test statistics useful in the determination of the level of chromosome breakages or aberration not referenced in this article, can be used in other to obtain optimal results and better diagnosis of each case treated.

Theoretic standards for scoring breakages

The mean baseline chromosomal breakage frequency in peripheral blood lymphocytes ranges from 0.00 to 0.05 breaks per cell in normal control or negative test results, and 0.00 to 0.12 for equivocal and positive test results. The mean DEB-induced chromosomal breakage frequency in peripheral blood lymphocytes for normal control individuals ranges from 0.00 to 0.10 breaks per cell, and 0.00 to 23.9 breaks per cell for equivocal and positive test results. It is important to note that in cultures from samples with breakages, the percent of metaphases exhibiting the high number of chromosomal breaks and exchanges can vary from as low as 10% to as high as 100%. A multivariate regression analysis of data from DEB studies shows that discrimination between DEB⁺ and DEB⁻ patients is best achieved by calculating breakage as breaks per cell (Auerbach et al., 1989). Also, presence of radial configurations (at two concentrations of DEB, in a strong dosage-dependent manner) is an important factor in confirming a positive result (Auerbach, 2015).

Scoring of chromosomal aberrations and breakages

Carry out a systematic selection on the already fixed metaphases to be analyzed by viewing under an inverted microscope at ×400 magnification. To save time, select only metaphase slides adjudged to be suitable, in terms of chromosomal integrity, for evaluation. To avoid a bias for relatively undamaged metaphases, do not at this stage select on the basis of "quality," since "nice-" looking metaphases tend to have fewer aberrations. Rather, every next metaphase encountered should, in principle, be scored, unless it must be rejected because it fails to meet the observer's criteria for adequate spreading, state of condensation of the chromosomes (not too long or too short), adequate staining and morphology (clearly recognizable chromosomes with clearly visible centromeres), and adequate ploidy (Auerbach, 2015). When a metaphase slide meets these criteria, that metaphase must be observed and scored using a magnification of x1,000 under an inverted microscope, even if "difficult" aberrations are subsequently encountered (Auerbach, 2015), therefore, a direct comparison is made using normal chromosomes (Fig. 1) from the control experiments. However, be sure to score only actual aberrations. Also, care should be taken such that the aberrations noted on the chromosomes were not artificially inflicted by the analysts, either by commission or omission, as a wrong analysis might signal a wrong medical action by health experts or clinicians (doctors and other health care officers).

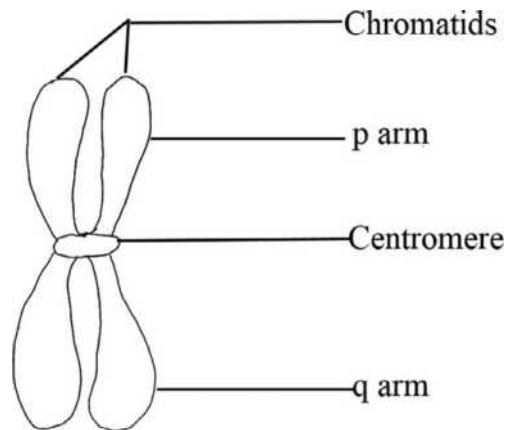


Fig. 1 A complete chromosome structure showing all the essential parts.

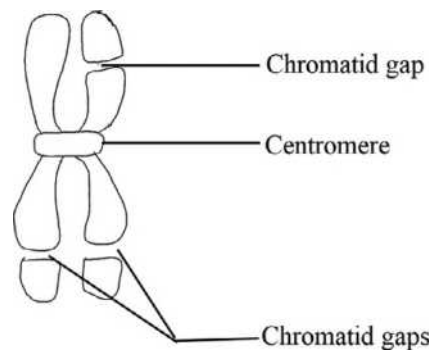


Fig. 2 Chromosome structure showing chromatid gap.

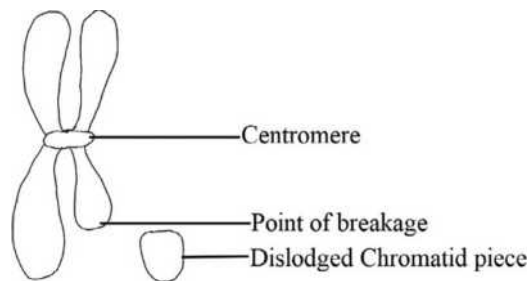


Fig. 3 Chromosome structure showing chromatid break and the dislodged part.

Itemized below are some very helpful tips for decision making while scoring chromosome breakages and aberrations:

1. Chromatid gap: This is usually perceived by laboratory technicians as an interruption in the staining of a chromatid 1–2 times the width of that chromatid (Fig. 2). Chromatid gaps result from breakage or damage to the chromosome arm. The affected part on the chromatids appears transparent when observed under an inverted microscope after staining.
2. Chromatid break: This is a situation where the interruption in the staining of the chromatid is more than two times the width of that chromatid, or a situation where the broken piece of chromatid appears dislocated or dislodged (Fig. 3). Chromosomes with chromatid break appear to be shorter in the affected chromatid or chromosome arm than the other arm. The fragmented piece can be spotted close to the affected chromosome in most cases.
3. Triradial chromosome: This is an interchange figure presumably having resulted from the disrepair of two breaks in two distinct chromosomes (Fig. 4). Triradial structures are mostly formed when the dislodged arm is attached to the wrong chromatid, creating a kind of skewed appearance of the chromosome. Now, in a bid to repair itself, the affected chromosome tends to form a linkage with the chromatid of a different chromosome too, producing a sort of unbalanced physical appearance.
4. Quadriradial chromosome: This is also an interchange figure resulting from the disrepair of two broken chromatids in different chromosomes (Fig. 5). Quadriradial structures appear as a mismatched chromosome linkage. In this case, there is a union

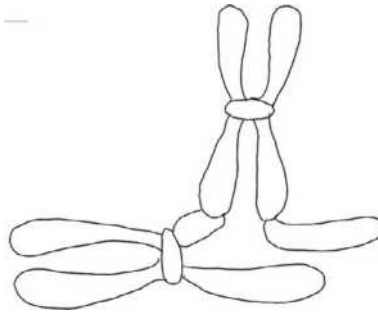


Fig. 4 Chromosome structure showing triradial formation.

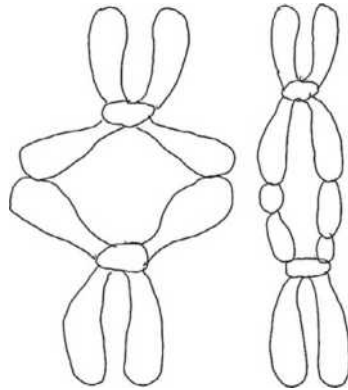


Fig. 5 Chromosome structure showing quadriradial formation.

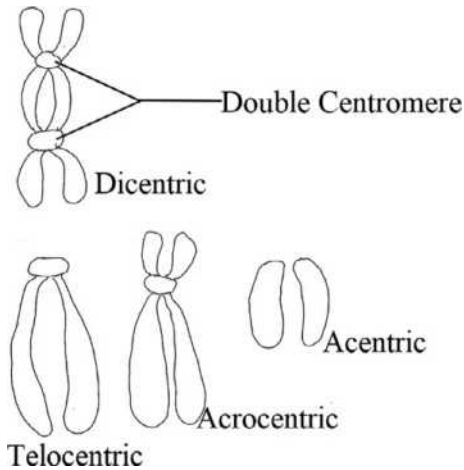


Fig. 6 General chromosome aberrations.

between the arms of two (2) chromosomes forming a tetrad of chromatid structures. The chromatid arms may or may not appear broken or disjointed. The linkage most times occur between sister chromosomes but in rare cases, linkages may be formed between adjacent chromosome pairs. The reason behind the mismatched linkages can be probed by researchers in order to shed more light to the current study.

5. Other chromatid interchange and Chromosome-type changes, such as dicentrics, acentric fragments (Fig. 6), and ring chromosomes, may be recorded, but these aberrations, which are extremely rare with the protocol used, should not be included in the final analysis (Auerbach, 2015). There are variants of chromosomal aberrations not listed in this article. Therefore, before carrying out DEB testing and breakage analysis on any biological sample, the medical personnel should be sure to acquaint himself or herself with the numerous chromosome structures that abound, so that a false-positive or false-negative diagnosis can or may be avoided.

Complementation (mutation) analysis

The complementary molecular test for breakage analysis described in this test was an excerpt from the research of Moghrabi et al. (2009). Extraction of DNA, RNA and cDNA from fibroblast tissues should be carried out after culturing the fibroblast tissues. The fibroblast tissues collected should be cultured using the standard technique described in section "Diepoxybutane (DEB) test" (Auerbach, 2015), thereafter, gDNA extraction can be conducted using the modified version of organic extraction protocol described by Pulsipher et al. (1998). Extraction of gDNA from each cell line should be initiated as soon as the number of proliferated fibroblast cells increase in the culture medium. At first, cell passaging and trypsinization should be conducted on the newly proliferated monolayered cell clusters. Trypsinization is a simple laboratory protocol used to separate cells formed in clusters, the steps involved in trypsinization include removal of the medium in which the newly proliferated fibroblast cells were suspended by decanting the liquid without disturbing the cell clusters, then wash the monolayered-cell clusters with enough saline solution, free of Ca^{2+} and Mg^{2+} , in order to remove traces of blood serum. Thereafter, remove the saline solution from the culture by decanting or with the aid of a Pasteur pipette. Add excess trypsin/EDTA solution to the culture such that the monolayers of cell clusters are completely submerged by the solution. Incubate the proliferated cell culture at 37 °C for 2 min in a mist of 5% CO_2 . Afterwards, aseptically remove the trypsin/EDTA solution by decanting (without agitation) and incubate the residual cells at 37 °C until cells start to detach from the surface of the monolayer cluster. The progress of cell detachment can be monitored intermittently by the use of an inverted microscope. The time required for total cell detachment from the monolayer cell cluster depends on the cell type, population density of the clusters, serum concentration in the growth medium, and the quality of the trypsin/EDTA solution applied.

After trypsinization, the detached cells (7×10^5 to 2×10^6) were washed in $1 \times$ phosphate-buffered saline and cell pellets were resuspended in 465 μL Nuclei Dropping buffer (0.075 M NaCl, 0.024 M EDTA). Ten-microliter proteinase K (10 mg/mL) and 25 μL 10% SDS were added to the suspension mix and was incubated at 37 °C for 16 h. This was followed by organic extraction using standard protocol (Ausubel et al., 1992; Moghrabi et al., 2009). Total RNA, enriched for mRNA, can be extracted from each of the cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA). A QIA-shredder (Qiagen) can be used to homogenize the lysate and a RNase-Free DNase Set (Qiagen) can also be used on-column to digest DNA during the RNA purification (Moghrabi et al., 2009). The resulting RNA should be reverse-transcribed, using RT-PCR, to cDNA. The procedure involved is stated thus: 10 μL of RNA should be denatured at 65 °C for 10 min and then added to 10 μL of $1 \times$ RT Buffer (Invitrogen, Carlsbad, CA), 10 mM dithiothreitol, 50 μM Roche Pd(N)₆ random primers (Roche, Nutley, NJ), 0.2 mM dinucleotide triphosphates (dNTPs), 10 U/ μL SuperScript II Polymerase (Invitrogen), and 2 U/ μL Roche Protector RNase Inhibitor (Roche). Amplification should be performed in a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA) as follows: 25 °C for 10 min, 42 °C for 50 min, 70 °C for 15 min, and thereafter, stored at 4 °C (Moghrabi et al., 2009).

Primers used for this experiment should be obtained from the NCBI GenBank database (See Moghrabi et al. (2009) for Primer selection and design of cDNA). Also, controls (Positive and Negative gDNA samples) should be obtained from the appropriate sources, for test validation, accuracy of clinical diagnosis and/or specificity of the breakage analysis conducted (Adachi et al., 2002; Ameziane et al., 2008; Callén et al., 2005; Chandra et al., 2005; Morgan et al., 2005; Yagasaki et al., 2004). A summary of the procedure is described thus: A total of 50 μL volume containing 150 ng of gDNA, 15 pmol of each primer, and 10 mM dNTP should be setup for PCR amplification of gDNA (Note: Additional reagents can be added to meet up with the specificity of the desired primers). Initiate an initial denaturation at 95 °C for 4 min, followed by amplification of 33 cycles, each with denaturation at 95 °C for 35 s, annealing at 64 °C for 40 s with a decrease of 0.1 °C/cycle, and extension at 72 °C for 40 s. The final extension should be carried out at 72 °C for 5 min. Primers should be stored at 4 °C. Specific primers can be designed using "OligoAnalyzer 3.0" software (Integrated DNA Technologies, Coralville, IA). Note: All primer sequences should be tailed by M13 forward and reverse primers to allow for efficient sequencing. In the case of apparent homozygosity in samples collected, alternate primers should be designed to rule out allele dropout.

The PCR amplification of cDNA should be performed thereafter as follows: add 5 μL of the resulting RT-PCR reaction (described above) to $1 \times$ HiFi Buffer (Invitrogen), 2 mM MgSO_4 , 0.8 mM dNTPs, 0.6 μM forward and reverse primers, 0.04 U/ μL Platinum HiFi Taq Polymerase (Invitrogen), and water to make a total volume of 50 μL . Amplification cycling conditions is as described for gDNA above (The conditions can be modified to suit the primers selected). The PCR products should be analyzed to determine their quality by running 5 μL of the amplified product on 2% NuSieve (Lonza, Rockland, ME) agarose gel in standard tris-acetate buffer at 8mAmp/cm gel (Moghrabi et al., 2009). Also, the PCR products should be analyzed for sequence variants by the detection of heteroduplex DNA using dHPLC on the WAVE HT system (Transgenomics, Omaha, NE) by means of acetonitrile gradient and Navigator software (Transgenomics). The procedure is described for both heterozygous and homozygous mutants. For heterozygous mutations, denature 10–15 μL of the amplified PCR products for 5 min. Cool gradually to a temperature of 25 °C, at a rate of 1 °C/min, to allow the optimum formation of heteroduplexes. For homozygous mutations, mix equal amount of DNA sample from the cultured fibroblast cells and a negative control, denatured, and analyzed on dHPLC as described above. Amplicons should be scored as dHPLC positive if a peak shift was detected on dHPLC relative to the appropriate controls (Positive and Negative).

Positive dHPLC amplicons should be analyzed further by sequencing. M13 forward and reverse primers should be used for this reaction. Purify the amplicons using ExoSAP-IT treatment (USB, Cleveland, OH) by incubating 5 μL with 2 μL ExoSAP-IT at 37 °C for 15 min, followed by heat inactivation at 80 °C for 15 min. Half of the volume of the inactivated amplicons should be aliquoted to a fresh tube, followed by the addition of M13 forward or reverse primer to a final concentration of 0.16 μM . The BigDye Terminator v1.1 Cycle Sequencing Ready Reaction-mix kit (Applied Biosystems, Foster City, CA) is recommended for this reaction.

Add 1 μL of the BigDye Terminator and 1.5 μL of $5\times$ BigDye buffer in a final sequencing reaction volume of 10 μL . Initiate sequencing 96 °C for 1 min, 25 cycles at 96 °C for 10s, 50 °C for 5 s, and 60 °C for 4 min. The sequenced amplicons should be purified using DyeEx 96 gel filtration plate (Qiagen, Valencia, CA) in line with the manufacturer's instructions. The purified amplicons should be transferred to an Optical 96-well plate (Applied Biosystems) and dried in a 70 °C heat block with forced ventilation for 15 min. The sequenced should be resuspended after drying in 15 μL Hi-Di formamide (Applied Biosystems) and separated on ABI PRISM 3100 Genetic Analyzer using a 50 cm capillary array and POP6 polymer (Applied Biosystems). DNA trace sequences can be generated from raw sequence data using Sequencing Analysis v5.1.1 (Applied Biosystems) and analyzed for sequence information using Mutation Surveyor software, version 3.01 (Soft Genetics, State College, PA). Novel variants detected should be analyzed in silico as described by Moghrabi et al. (2009).

Finally, Multiplex ligation-dependent probe amplification (MLPA) analysis should be performed on the samples and controls. The protocol for MLPA using SALSA P031/P032 (MRC-Holland, Amsterdam, Netherlands) is described thus: Denature 200 ng of gDNA and hybridize with each of SALSA P031 and P032 probes following the manufacturer's instructions. Perform ligation and PCR amplification according to manufacturer's instructions too. One microliter of the probe amplified product should be mixed with 0.3 μL GeneScan-500 ROX size standard (Applied Biosystems), 9 μL Hi-Di formamide (Applied Biosystems) and resolved using the ABI 3100 Avant Genetic Analyzer with a 36 cm capillary array and POP4 polymer (Applied Biosystems). GeneMapper v.3.5 software (Applied Biosystems) can be used for fragment analysis and the resulting peak height and size area can be exported thereafter as Microsoft Excel files. Subsequent quantitative and statistical analysis can be performed on the data generated. The most essential calculations include the peak heights (and areas) of the expected MLPA products (divide each peak height (and area) by the combined height (and area) of all peaks in the sample). For the three normal controls, the mean ($n = 3$) of the normalized peak heights (and areas) can be calculated from all the probes for each probe-mix P031 and P032. For the patients' samples and the positive control, each of the normalized peak height (and area) should be divided by the corresponding mean of the normalized peak height (and area) of the normal controls ($n = 3$). The resulting values should be compared with that of the standard normal control. All exons or amplicons differing by more than 30% from the normalized peak heights (and areas) of the standard normal control with similar fragment size should be considered as altered. The observation of reduced values indicates possible gene deletion, whereas increased values indicates potential gene duplication (Moghrabi et al., 2009).

Result interpretation for breakage analysis

Positive Test Result: A sample is confirmed as having cracks or gaps in the autosomal chromosomes if the lymphocytes show a substantial increase in chromosomal breakage and rearrangement after treatment with DEB. Characteristically, more than 90% of the lymphocytes in the sample will show increased breakage, and the rates and types of breakage observed will fall within the abnormal range. In the event of a positive test result, follow-up tests should be performed to identify the type of genetic mutation(s) induced by the chromosome breakage (gene deletion or insertion) using the molecular methods described by Moghrabi et al. (2009) in Section "Complementation (mutation) analysis".

Negative Test Result: A test result is considered to be negative if the patient's lymphocytes do not show increased chromosomal breakage or rearrangement in response to DEB, and the types and rates of breakage are within the normal range. If the chromosome breakage test is negative, no further studies are needed.

Equivocal Test Result: Test results are considered equivocal, or inconclusive, if the percentage of cells that display chromosomal breakage patterns are much lower than the normal control (but there is strong clinical evidence that the patient may have other underlying or related genetic illnesses) or if there is increased breakage but the individual shows no clinical symptoms of genetic diseases. For Equivocal results, DEB test and breakage analysis on skin fibroblast should be performed to rule out the possibility of mosaicism.

Note: Mosaicism is characterized by two distinct populations of lymphocytes in the blood. One population has normal sensitivity to DNA cross-linking agents due to a spontaneous correction of mutation, while the other population is hypersensitive to DNA cross-linking agents due to the presence of uncorrected mutants.

Conclusion

DEB testing and breakage analysis are very important laboratory protocols useful in the determination of gaps, cracks or breakages along the entire length of a chromosome. The detection of cracks, gaps or breakages on a particular chromosome is an indication that useful genetic materials are lost from that chromosome either by commission or omission. The amount of genetic materials lost by a single chromosome depends on the degree of breakages, gaps or cracks found on the chromosome. The medical implication of chromosome breakage is that there is a 50% chance of possible insertion or deletion of genes from that particular chromosome, which may play important role(s) such as physical development of an individual, reproduction, blood formation (in the case of anemic patients), normal functioning of the heart, lungs, kidneys, or brain etc. Therefore, couples are advised to add this test to the list of tests they have to undergo before getting married, as this might be a crucial decisive factor in the general well-being of their children, future progeny or even lineage. Parents or families with known history of inherited bone marrow failure syndrome (IBMFS) or other forms of aplastic syndromes (AS), should carry out the test for all their family members and relations, as early diagnosis can prove to be life-saving for an individual with chromosomal aberrations.

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16S Genomics for Diagnosing Invasive Bacterial Infection

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Invasive bacterial infection

Invasive bacterial infections (IBI) are leading causes of global morbidity and mortality among all age groups, especially young children and elders (Scelfo et al., 2021). Invasive bacteria are pathogens that can invade parts of the body where microorganisms are not normally present, such as the bloodstream, soft tissues like muscle or fat and the meninges, and are often associated with diseases, such as meningitis (inflammation of the tissues that cover the brain and spinal cord), pneumoniae, endocarditis and septicemia (or sepsis) (Archibald and Quisling, 2013; Holland et al., 2016; Reinhart et al., 2017; Weiser et al., 2018). Often, the infection has a bacterial origin, but it can also be caused by fungi, viruses or parasites (Dolin et al., 2019). The most common bacterial agents of these life-threatening diseases are *Neisseria meningitidis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Streptococcus agalactiae*, *Escherichia coli*, *Klebsiella* spp., and *Pseudomonas aeruginosa*, which normally are found in the nasopharynx, throat or the gut and genitourinary tract of healthy individuals and are transmitted via respiratory route or, in the case of newborns, during the partum (Weiser et al., 2018; Raabe and Shane, 2019; Brueggemann et al., 2021; Deghmane and Taha, 2021).

Recently, the World Health Organization (WHO) estimated that 30 million cases and 6 million deaths come from bacterial-associated infection and affect people around the globe each year (Reinhart et al., 2017; Candel et al., 2018). Septicemia is one of the principal causes of mortality in critically ill patients. Approximately 70% of septicemia cases are community-acquired and the evaluation of patients with symptoms can be challenging and requires a combination of clinical observation and laboratory support (Gül et al., 2017). The survival of the patients depends on the rapid and accurate diagnosis of the infection and the appropriate treatment to prevent unnecessary deaths and upcoming incapacities (Gotts and Matthay, 2016). Although the elderly, immunosuppressed and children are known to be high-risk groups, IBI can affect any individual (Nasa et al., 2012; Holland et al., 2014; Zea-Vera and Ochoa, 2015; Emr et al., 2018; Szabo et al., 2019). Though, the higher mortality rate is associated to hospitalization and these populations often have concurrent medical or surgical conditions that delay the diagnosis and treatment, particularly when infections are associated with nosocomial infections and progress to shock and organ failure (Schorr et al., 2016). Many patients will have a long recovery, organs and tissues infected may be permanently damaged or painful, and organ dysfunction can occur (Gül et al., 2017).

The “gold standard” method for the patient diagnosis is based on laboratory techniques that rely on *in vitro* blood culture in enriched medium, followed by identification and susceptibility testing of the pathogen using standard biochemical techniques and integrating morphologic, metabolic, and physiologic analysis with differential staining methods (Mancini et al., 2015; Guido Marcello et al., 2016; Rizal et al., 2020). Microscopy, although rapid, is insensitive, requiring a relatively large concentration of bacteria ($\geq 10^5$ CFU mL⁻¹) to become positive and it is not part of routine diagnostics (Wiwanitkit et al., 2005). On the other hand, blood cultures can detect a wide range of microorganisms and are well established in the diagnostic pathway, achieving a sensitivity of >95% if sample volumes are sufficient. Adequate sampling volume is the most important factor for detecting a microorganism, since 50% of patients have a low bacterial amount circulating in blood (<1.0 CFU mL⁻¹). The recommendation is four bottles (with a capacity of 10 mL each one) of blood to be cultured for an initial evaluation in order to reach about 90–95% detection rate in a patient with documented infection (Townes et al., 2010; Dubourg and Raoult, 2016). Blood cultures are currently performed with fully automated instruments that detect microbial growth by the analysis of CO₂ release using fluorescent or colorimetric sensors; or, alternatively, pressure changes in the bottle headspace due to the consumption and production of gasses (Mancini et al., 2010), which does not allow pathogen identification.

However, despite these advances, this method has its limitations and is not the ideal approach for guiding initial therapy, since blood cultures requires several days before the results are available and can delay the introduction of an adequate antimicrobial therapy (Mancini et al., 2015; Guido Marcello et al., 2016; Sweeney et al., 2019). Typically, the growth of pathogens in medium

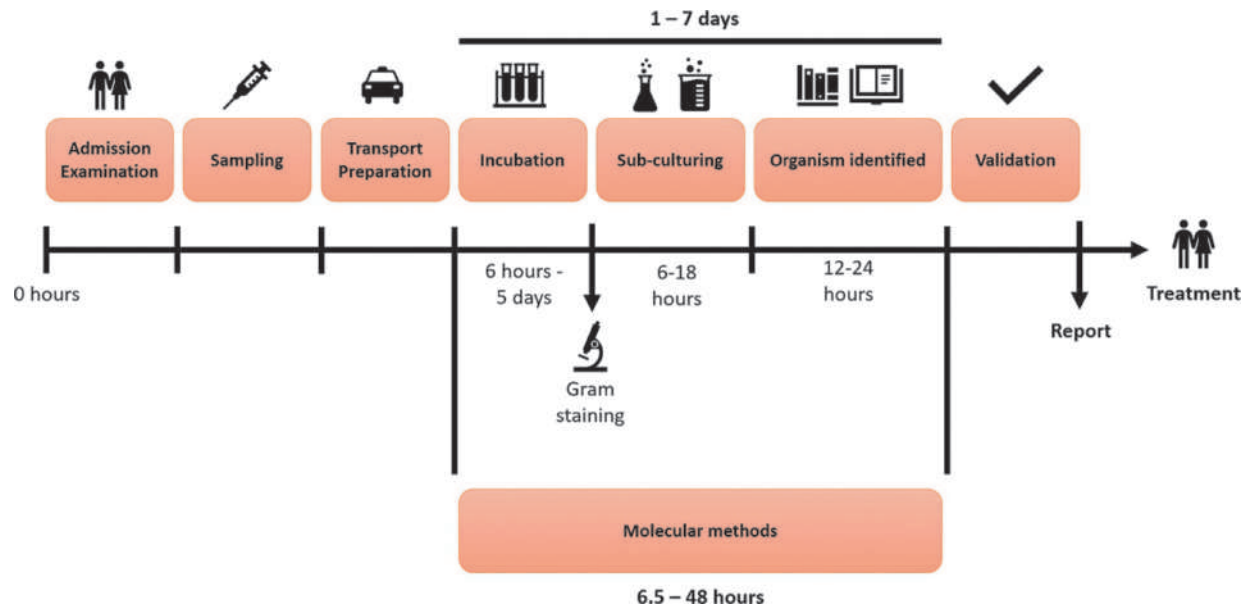


Fig. 1 Conventional workflow to detect bacterial infection. After examination of the patient, the physician may decide to take a blood or tissue sample. Subsequently, the sample has to be transported to the laboratory where it will be incubated. After incubation, standard approaches may allow the microorganism identification in 7 days, whereas molecular approaches permit identification within 48 h.

broth takes 24–72 h and requires Gram staining followed by additional overnight growth to yield single colonies for identification and susceptibility testing (Liesenfeld et al., 2014). Negative results can only be reported after 5–7 days. Nevertheless, culture-based diagnosis is not convenient for infections if the antibiotic treatment is initiated before blood sampling, in presence of a very low number of circulating bacteria, or when the microorganism is fastidious or uncultivable (Mancini et al., 2015). However, once the bacterium is isolated and the antibiogram performed, the microbiological report permits a reduction in the spectrum of empirical administered anti-infectious drugs, allowing the administration of the adequate antimicrobial treatment. Fig. 1 provides an overview and a comparison of the diagnostic and identification process of a bacterial infection using standard methods and molecular techniques.

In the past few years, molecular techniques have evolved as an alternative for the diagnosis of an IBI. Molecular diagnostics are based on the detection of bacterial DNA in the blood using polymerase chain reactions (PCR) and have been developed for direct testing of whole blood, without the need of culture (Marco, 2017). Although rapid, these assays have some limitations. PCR is a very powerful method to detect bacterial DNA in blood, even at low yield, but a single PCR it is not able to differentiate between different bacterial species, and require different tests for every species (Barghouthi, 2011). Several studies have also highlighted the requirement of strategies for differentiating between true infections and contaminations and, even though this method gives results in a short period, it has not yet reached clinical practice (Yang and Rothman, 2004).

The use of infection-associated biomarkers has been recommended to compensate the limitations of molecular diagnostic assays. To identify suitable samples, clinical criteria should be complemented by the use of inflammatory biomarkers, such as the total white cell count, neutrophil count, C-reactive protein (CRP), procalcitonin (PCT) and CD64 expression on neutrophils to predict severe systemic bacterial infection (Icardi et al., 2009; Cid et al., 2010; Rogina et al., 2014; Enguix-Armada et al., 2016). Procalcitonin is the best validated biomarker that discriminates the non-infectious cause for invasive bacterial infection (Enguix-Armada et al., 2016; Jekarl et al., 2019; Hamade and Huang, 2020). Presepsin, kallistatin, testican-1 and pro-adrenomedullin are new biomarkers useful for early diagnosis and prognosis in septic patients (Mierzchała-Pasierb and Lipińska-Gediga, 2019; Ito and Ishida, 2020). However, these biomarkers lack the specificity to discriminate between patients with an acute inflammatory response to trauma or surgery and those with a new infection (Ito and Ishida, 2020). Fig. 2 summarizes the most used methods to identify microorganisms from positive blood cultures or whole blood. In this chapter we will address only diagnostic approaches that involve 16S rRNA genomics, namely conventional and real-time PCR, hybridization, and next generation sequencing.

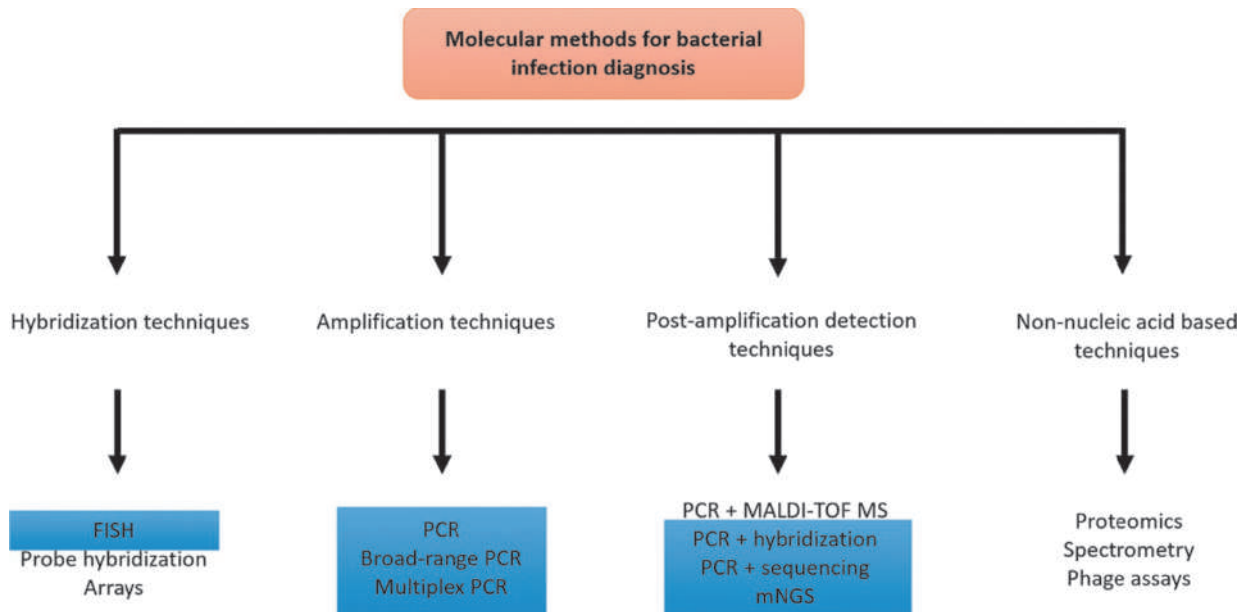


Fig. 2 Molecular techniques for the diagnosis of bacterial infection. Blue rectangles highlight molecular techniques based on 16S rRNA.

16S genomics as a diagnostic tool

Modern biology is full of remarkable milestones. One of the most transforming landmarks was the pioneering work of [Woese and Fox \(1977\)](#), who remodulated the Tree of Life and defined an entirely new branch ([Woese and Fox, 1977](#)). Through the phylogenetic analysis of 16S ribosomal RNA (16S rRNA), Woese and Fox described the domain of Archaea, a new domain in a triple-branched Tree of Life composed of Archaea, Eubacteria and Eukarya domains ([Woese and Fox, 1977](#)). Up to this point, the classification of organisms almost exclusively relied on morphological and physiological features and basic dichotomies which present several limitations ([Woese and Fox, 1977](#); [Woese, 1987](#); [Woo et al., 2008](#)). The use of 16S rRNA information proposed by Woese and Fox overcomes some of them.

The 16S rRNA is found in the 30S subunit (small subunit, SSU) of prokaryotic ribosomes in both Archaea and Eubacteria, and is homologous to the 18S rRNA found in the 40S subunit of cytosolic ribosomes of eukaryotes ([Poehlsgaard and Douthwaite, 2005](#); [Liu and Fredrick, 2016](#)). It is majorly responsible for the structure of the ribosomal SSU and is a key player in the assembly of the ribosome, namely in the binding of both 50S and 30S subunits ([Liu and Fredrick, 2016](#)). Furthermore, its 3'-end recognizes the Shine-Dalgarno sequence (ribosome binding site in Eubacteria) and it plays an important role on the peptide elongation step, thus being vital on protein synthesis ([Powers and Noller, 1993](#); [Sahu et al., 2013](#); [Amin et al., 2018](#)). These pivotal functions lead to a strict selective pressure for the conservation of some 16S rRNA gene or 16S rDNA (and, consequently, for 16S rRNA) regions, resulting in a canonical organization of the 16S rRNA gene with a conserved backbone harboring 9 hypervariable regions ([Chakravorty et al., 2007](#); [Wang and Qian, 2009](#)). Taking *E. coli* as example, the 1541 bp-long 16S rRNA gene (J01695.2) ([Brosius et al., 1978](#)) has its nine hypervariable regions (V1 to V9) spanning nucleotides 69–99, 137–242, 433–497, 576–682, 822–879, 986–1043, 1117–1173, 1243–1294 and 1435–1465, respectively ([Cannone et al., 2002](#); [Chakravorty et al., 2007](#)) (Figs. 3 and 4). The 16S rRNA gene is omnipresent in Eubacteria with conserved function and different regions of the gene change at various (but still slow) rates allowing for its use as molecular chronometer or molecular-clock ([Woese, 1987](#); [Tsukuda et al., 2017](#)).

Numerous 16S rRNA gene sequences are available in public databases allowing for the achievement of a strong and deep identification of organisms of interest. Among the public databases, EzBioCloud ([Yoon et al., 2017](#)), Greengenes ([DeSantis et al., 2006](#)) and Silva ([Quast et al., 2013](#)) are the most widely used ([Park and Won, 2018](#)). Additionally, the Ribosomal Database Project, RDP, contains a wide set of aligned and annotated bacterial 16S rRNA gene sequences (among other organisms) ([Cole et al., 2014](#)).

The omnipresence in the Eubacteria domain, the significant length for bioinformatics purposes, the structure containing both conserved and hypervariable regions (thus having significant species-distinguishing power), the molecular-clock character and the vast amount of data publicly available in databases make the 16S rRNA gene one of the most extensively used molecular tools for bacterial identification and phylogenetic studies.

As an established molecular vehicle for bacteria identification, 16S rRNA genomics can play a significant role as a diagnostic tool ([Janda and Abbott, 2007](#); [Woo et al., 2008](#); [Lau et al., 2015](#); [Rizal et al., 2020](#)). Bacterial identification for microbiological diagnosis is of major importance, among other reasons, for (i) distinguishing colonizer or contaminant bacteria from real pathogens; (ii) for better tailoring of antimicrobial therapeutics in terms of antibiotic choice and duration; and, at a wider level, (iii) for clarifying the epidemiology of disease-causing bacteria ([Lau et al., 2015](#)). As mentioned above, standard procedures for bacterial identification in

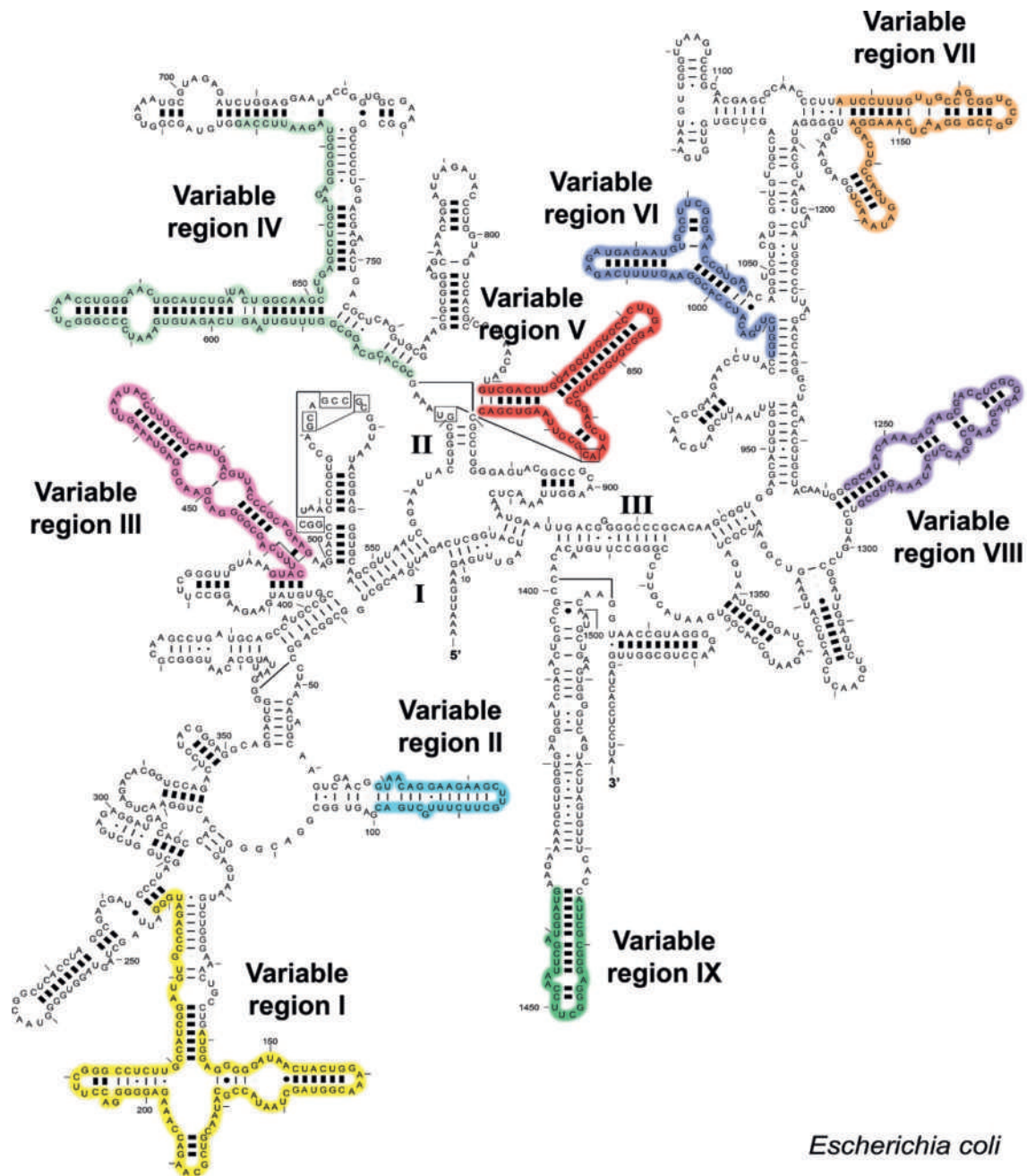


Fig. 3 Secondary structure model of the *E. coli* 16S rRNA (Konings and Gutell, 1995). Variable regions are identified by different colors. Image available at (and modified from) <https://crw-site.chemistry.gatech.edu/> (accessed September 2021) (Cannone JJ, et al. (2002) The comparative RNA web (CRW) site: An online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. *BMC Bioinformatics* 3: 2).

clinical context require bacterial culture and subsequent analysis (Mancini et al., 2015; Guido Marcello et al., 2016; Rizal et al., 2020). Nowadays, this procedure is highly optimized and accurate for classification of bacteria commonly found in medical scenarios and presenting typical phenotypic profiles but can be regarded as limited under certain conditions (Lau et al., 2015). As reviewed by Woo et al. (2008), such conditions in which 16S rRNA genomics can be useful include the identification of uncommon bacteria (Schröttner et al., 2016; Mishra et al., 2020), bacteria with unusual or variable phenotypic profiles (Petti et al., 2005), slow-growth or uncultivable bacteria (Liu et al., 2015; Mishra et al., 2019; Satilmis et al., 2019), culture-negative infections (Rampini et al., 2011; Godfrey et al., 2020) and bacteria requiring special growing conditions regarding nutrients and/or atmosphere (Justesen et al., 2010; De Melo Oliveira et al., 2013). Given these limitations, the use of 16S rRNA genomics presents

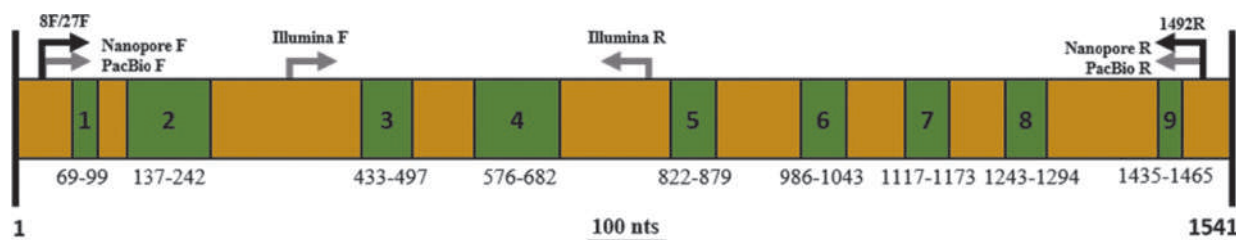


Fig. 4 Schematic representation of 16S rRNA gene. Using the example of *E. coli*, the 1541 bp 16S rRNA gene (J01695.2) (Brosius et al., 1978) is shown and the 9 hypervariable regions (V1–V9) are presented in green with the locations reported by Chakravorty et al. (2007). Yellow regions represent the conserved backbone of 16S rRNA gene. The annealing region of the universal bacterial primers 8F, 27F and 1492R is depicted with black arrows (F and R point to forward and reverse primers, respectively; the number refers to the 3′-end annealing position of each primer on the *E. coli* 16S rRNA gene). The annealing region of commonly used NGS primers for Illumina, Nanopore and PacBio technologies is pointed with gray arrows. It should be noted that Nanopore technology makes use of the universal 27F and 1492R primers. A 100 nucleotide-long scale is presented.

a solid and reliable alternative allowing for bacterial identification from clinical contexts in a culture-independent manner, surpassing some limitations of more traditional methods, and standing out as an advanced diagnostic tool compatible with the expected medicine for the 21st century. It has also been suggested that, when possible, biochemical analysis and 16S rRNA genomic approaches should be used complementarily (Godfrey et al., 2020; Somerville et al., 2020).

The establishment of 16S rRNA was indeed a transforming step for modern biology, with special potential as a diagnosing tool, thus several approaches are used—and are always arising—in order to better use it for the clarification of biological problems. The most important approaches for retrieving 16S rRNA data are described and deepened below.

16S genomic approaches

Conventional PCR and primer choosing

The first typical approach to access 16S rRNA is DNA sequencing after conventional end-point polymerase chain reaction (PCR) targeting the 16S rDNA. End-point reverse-transcriptase polymerase chain reaction (RT-PCR) directly targeting the 16S rRNA molecule was also described and suggested as more sensitive due to the multiple copies of 16S rRNA molecule in a cell when compared to the number of 16S rRNA genes (Kempsell et al., 2000). Conventional PCR was itself a reshaping tool of modern biology, leading to a Nobel Prize in Chemistry (1993) for its major driver, Dr. Kary B. Mullis, awarded along with Dr. Michael Smith. Briefly, PCR is a molecular biology technique for exponentially amplify a region from a DNA template (Mullis et al., 1986), relying on an in vivo genome replication-mimetic in vitro process. It consists in multiple cycles of a step for template DNA denaturation through heating, followed by the annealing of short oligonucleotides (called primers) that prime a third step of chain elongation by DNA polymerase using the target DNA region as template (see Caetano-Anollés, 2013 for further details). In addition to this, RT-PCR includes a prior reverse transcription step, using adequate primers and a reverse transcriptase enzyme that synthesizes complementary DNA (cDNA) using RNA as a guide and allowing it to be used as a template as in a regular PCR (see Bachman, 2013 for further details).

The conserved- and hypervariable-region organization on 16S rRNA gene turns it into an attractive target for PCR amplification aiming at assessing sequence variability as the conserved regions can anchor universal primers for the amplification of several hypervariable regions (and spanned conserved regions) in different bacteria (Chakravorty et al., 2007) (Fig. 4). A recent work, however, have shown that these “conserved” regions are not so conserved as previously assumed, leading to issues in the usage of “universal” primer sets (Martinez-Porchas et al., 2017). Although hypervariable regions show significant variability, no single hypervariable region sequence can be used as universal bacteria distinguisher (Chakravorty et al., 2007). Despite this, each hypervariable region can be used to distinguish specific pathogenic bacteria within a certain group: for example, region V1 showed potential for distinguish between non-pathogenic *S. aureus* and pathogenic coagulase-negative staphylococci, while V2 seemed promising in discerning species from *Mycobacterium* genus (Chakravorty et al., 2007). The same study suggests that a combination of several hypervariable regions, namely V2, V3 and V6, could be used for bacterial identification to genus and species level (Chakravorty et al., 2007).

As an established molecular tool, a wide set of primers is described for bacterial 16S rRNA gene or 16S rRNA cDNA amplification. For conventional end-point PCR, the most used primers set is 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTACCTTGTTACGACTT-3′), based on the primers firstly proposed by Weisburg et al. (1991). Alternatively, 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Turner et al., 1999) is used as forward primer jointly with the mentioned 1492R. These primer sets, depicted on Fig. 4, are considered universal, thus amplifying the 16S rRNA gene of virtually all bacteria, even with descriptions of inter-kingdom amplifications (Galkiewicz and Kellogg, 2008). Despite this, some amplification failures were also reported and may lead to results bias (Farris and Olson, 2007). The use of 8F/27F and 1492R primers results in the amplification of

Table 1 Established universal primers for bacterial 16S rRNA gene.

Description	Sequence (5'-3')	References
<i>Forward (F)</i>		
8F	AGAGTTTGATCCTGGCTCAG	Turner et al. (1999)
27F	AGAGTTTGATCMTGGCTCAG	Based on Weisburg et al. (1991)
357F	CTCCTACGGGAGGCAGCAG	Turner et al. (1999)
515F	GTGCCAGCMGCCGCGGTAA	Turner et al. (1999), similar to Relman et al. (1992)
928F	TAAACTYAAAKGAATTGACGGG	Weidner et al. (1996)
1237F	GGGCTACACACGYGCWAC	Turner et al. (1999)
<i>Reverse (R)</i>		
336R	ACTGCTGCSYCCCGTAGGAGTCT	Weidner et al. (1996)
519R	GWATTACCGCGGCKGCTG	Turner et al. (1999)
907R	CCGTCAATTCMTTTRAGTTT	Lane et al. (1985)
1100R	AGGTTGCGCTCGTTG	Turner et al. (1999)
1391R	GACGGGCGGTGTGTRCA	Turner et al. (1999)
1492R	GGTTACCTTGTACGACTT	Turner et al. (1999), similar to Weisburg et al. (1991)

On the primer descriptive, F and R point to forward and reverse primers, respectively; the number refers to the approximated annealing position of each primer on the *E. coli* 16S rRNA gene. Some primers present degenerated positions that are pointed as W (weak, either A or T), S (strong, either G or C), Y (pyrimidine, either C or T), R (purine, either A or G), M (amino, either A or C) and K (keto, either G or T).

almost the entire 16S rRNA gene, spanning hypervariable and conserved regions, whose sequencing provides data allowing for significant distinguishing power and statistical validity (Claridge, 2004; Chakravorty et al., 2007).

Other technical approaches using gene amplification or a lesser necessity for distinguishing power usually require or allow smaller amplicons. With this in mind, several primers have been described to anneal in different regions of the 16S rRNA gene and can be easily matched to retrieve amplicons with the features of interest regarding size and spanning of conserved and hypervariable regions. New primers and new modifications are frequently suggested, though mainly focusing in alternative approaches (Sambo et al., 2018; Isla et al., 2021; Kameoka et al., 2021). By way of example, some established universal primers for bacterial 16S rRNA are presented on Table 1.

Typical workflows for 16S rRNA analysis through conventional PCR consist of Sanger sequencing of the 16S rRNA gene amplicons (Sanger et al., 1977), followed by sequence curation and querying against the previously mentioned 16S rRNA databases (DeSantis et al., 2006; Quast et al., 2013; Cole et al., 2014; Yoon et al., 2017), or the conventional broad-use databases with BLASTn (Boratyn et al., 2013; Lau et al., 2015). While this approach is historically relevant and may be relatively simple and low-cost, it has been gradually abandoned by researchers in favor of high throughput approaches that are analyzed further below.

Quantitative real-time PCR

The evolution of molecular biology, in particular the evolution of nucleic acids extraction techniques and broad-range PCR amplification methods, provides tools to detect faster any kind of bacterial DNA present in a sample, even during the earliest phase of infection, which would otherwise remain undetected. In addition, sequencing of the amplicon generated by broad-range PCR allows subsequent identification of the organism, as the PCR product contains variable bacterial species-specific sequences. These sequences can be identified by comparison with known sequences deposited at GenBank or other databases (Wilson et al., 1990; Drancourt et al., 2000). As discussed above, several broad-range PCR assays have been reported in the literature and the majority of these assays use primers targeting the 16S rRNA gene (Wilson et al., 1990; Drancourt et al., 2000; Warwick et al., 2004; Renvoisé et al., 2013). However, because of their high sensitivity, broad-range PCR is vulnerable to contamination and may result in false-positive results (Millar et al., 2002). Contamination can be brought by DNA from the environment, from PCR reagents or due circulating cell-free DNA from dead bacterial DNA controlled by the immune system or by an efficient anti-infectious therapy (Opota et al., 2015).

Quantitative real-time PCR (qPCR) has revolutionized the way clinical microbiology laboratories diagnose bacterial pathogens (Klaschik et al., 2002; Bankowski and Anderson, 2004; Mackay, 2004). Indeed, the introduction of qPCR-based offers other advantages, including (i) a fast and high-throughput detection and quantification of target DNA sequence; (ii) the multiplexing of amplification of several targets into a single reaction; (iii) the detection of specific genes and alleles (e.g., detection of resistance (or virulence) traits); and (iv) a higher sensitivity and specificity compared to the conventional PCR. qPCR method combines PCR chemistry with fluorescent probe detection of amplified product in the same reaction and allows the prevision of a test result much sooner than for conventional PCR, in an hour or less. Additionally, quantification is also useful in the case of infections with different pathogens in order to determine the infection severity and relative abundance of each pathogen, helping to identify possible contamination (Rello et al., 2009). Sensitive and specific detection is possible by using a fluorescent probe. Three types of nucleic acid detection methods have been used most frequently with qPCR testing platforms in clinical microbiology: (i) 5' nuclease

(TaqMan probes), (ii) molecular beacons and (iii) fluorescence resonance energy transfer (FRET) hybridization probes. Briefly, TaqMan probe is a short oligonucleotide (DNA) that contains a 5' fluorescent dye and 3' quenching dye. When the probe is intact and the fluorescent dye is in close proximity to the quencher dye no fluorescence is emitted. During polymerization, Taq DNA polymerase (with a 5'-exonuclease activity) cleaves the 5'-end of the TaqMan probe, separating the fluorescent dye from the quenching dye and generating a light signal. The cleaved (free) fluorescent dye accumulates after each PCR temperature cycle, and can be measured at any time during the PCR cycling (Holland et al., 1991). Molecular beacons are similar to TaqMan probes. These probes also have a fluorescent dye on the 5'-end and a quencher dye on the 3'-end, which are designed to be complementary at low temperatures, exhibiting a characteristic stem-loop structure. At high temperatures, the central region of the molecular beacon probe binds to the PCR product and forces the separation of the fluorescent reporter dye from the quenching dye, creating the light signal (Tyagi and Kramer, 1996). On the other hand, FRET hybridization probes are two DNA probes designed to anneal next to each other in a head-to-tail configuration on the PCR product. One probe has a fluorescent dye donor on the 3'-end, while the other probe has an acceptor dye on the 5'-end. In the absence of a specific DNA, the two probes do not align together and FRET does not occur. But when both probes anneal to the target DNA, fluorescence from the 3' dye is transferred to the adjacent acceptor dye on the 5'-end of the second probe, and the acceptor molecule emits fluorescence (Clegg, 1995). Molecular beacons and FRET hybridization probes, unlike TaqMan probes, are both recycled at the end of each PCR cycle; however, fluorescent signal does not accumulate at the end of each PCR cycle.

Several commercially qPCR instruments are available and support all or some of the dyes described above (Table 2). Quantitation of target nucleic acid is possible with any of the instruments and supported detection formats (Espy et al., 2006).

Instruments which allows to read microplates with more than 96 wells are particularly useful in clinical laboratories which process a large number of samples. These instruments include the Applied Biosystems™ QuantStudio series and ViiA 7 Real-Time PCR System; LifeScience Roche Roche LightCycler® 480 System; and Biorad CFX Touch and Opus Systems in their CFX384-versions. Whereas large-capacity instruments support high number of samples but have a slow thermocycling, low-capacity instruments permit a rapid thermocycling and a flexibility that may be suitable for laboratories that test fewer samples.

The target primer sequences must be unique in order to identify a specific organism or an organism group. Although out of the scope of this chapter, qPCR may also be used to identify unique virulence genes or genes or mutations associated with antimicrobial resistance (Peker et al., 2018). The target nucleic acid sequence should also be conserved in the organism to be identified and quantitated, and the laboratory test must consistently produce accurate and precise results to assure a validated test (see Espy et al., 2006 for further details). The use of qPCR using 16S primers have been described in the detection of bacterial pathogens (Meddeb et al., 2016; Okolie, 2017; Liu et al., 2018), including neonatal sepsis (Ohlin et al., 2008; İstanbullu et al., 2019; Oeser et al., 2020), infective endocarditis (Marín et al., 2007; Faraji et al., 2018), periprosthetic joint infection or septic arthritis (Yang et al., 2021), infectious endophthalmitis (Ogawa et al., 2012), amniotic fluid (Girón de Velasco-Sada et al., 2018), and bacterial meningitis (Deutch et al., 2006; Chen et al., 2009).

In summary, the qPCR allows to increase the detection rate of microorganisms, improving the sensitivity of detection, providing results in a short time, and lowering the risk of contamination (Waggoner and Pinsky, 2016; Yagupsky, 2017). Furthermore, it tends to be used as a standalone technique, i.e., without a further DNA sequencing step. Thus, due to the high specificity of qPCR, specific primers tend to be used for microorganism identification instead of primers targeting the 16S rRNA.

Table 2 Example of real-time PCR instruments used in clinical laboratorial context.

<i>Instrument</i>	<i>No. of samples</i>	<i>Probe supported</i>	<i>Manufacturer</i>
LightCycler® 2.0 System	32	FRET, molecular beacons, TaqMan	LifeScience Roche
LightCycler® 96 System	96		
LightCycler® 480 System	96 up to 384		
GeneXpert® Xpress Systems	2 up to 14	Molecular beacons, TaqMan	Cepheid
GeneXpert® Infinity Systems	40 up to 80		
Rotor-Gene 3000	72	FRET, molecular beacons, TaqMan	Corbett Research
Applied Biosystems™ QuantStudio Real-time PCR	96 up to 384	TaqMan, molecular beacons	
Applied Biosystems™ 7500 Real-Time PCR Systems	96		BioRad
Applied Biosystems™ StepOne and StepOnePlus Real-Time PCR Systems	48 up to 96		
Applied Biosystems™ ViiA 7 Real-Time PCR System	96 up to 384		
CFX96 Touch Real-Time PCR Detection System	96	FRET, molecular beacons, TaqMan	
CFX384 Touch Real-Time PCR Detection System	384		
CFX96 Dx Real-Time PCR Detection Systems	96		
CFX96 Opus Real-Time PCR Systems	96		
CFX384 Opus Real-Time PCR Systems	384		
QuRapID-XF	4	Molecular beacons, TaqMan	BioGene

Hybridization techniques

Techniques based on hybridization also allow the identification of microorganisms based on the 16S rRNA gene. Namely, fluorescent in situ hybridization (FISH) is a technique suitable for the detection of pathogens in positive blood cultures. In this hybridization assay, slides of positive blood cultures are prepared and fluorescently labeled oligonucleotide probes are used to specifically bind to the ribosomal RNA (rRNA) region in the genome of targeted pathogens (to the 16S rRNA gene of bacteria, or to the 18S rRNA gene of fungi). This specific binding between the probe and its complementary sequence is visualized by fluorescence microscopy (Paolucci et al., 2010).

FISH assay can identify more than 95% of bacteria and yeasts commonly found in blood in 2.5–3 h (Oliveira et al., 2001; Kempf et al., 2005). Although FISH technology allows for the identification of most bacteria and yeast commonly found in blood, no species-specific probes are available and some bacteria can only be identified at the genus level (Paolucci et al., 2010).

A FISH method using a peptide nucleic acid probe (PNA-FISH) (AdvanDx, Woburn, MA, United States) was the first test for *S. aureus*. This commercial kit is still available by the name of *Staphylococcus* QuickFISH BC (OPGEN, USA). Other two similar kits, such as *Enterococcus* QuickFISH BC and Gram-negative QuickFISH BC are also available by the same manufacturer. This PNA probe targets 16S rRNA to directly identify *S. aureus* from positive blood culture within 3 h (Oliveira et al., 2002; Chapin and Musgnug, 2003). This assay has also been extended to other bacterial and fungal pathogens, such as *Candida albicans*, *E. coli* and *P. aeruginosa* (Sogaard et al., 2005; Shepard et al., 2008). However, this method can only be applied after Gram staining, since such information drives the choice of the kit type.

More recently, Prudent et al. (2018) evaluated the efficiency of FISH technique in endocarditis and vascular infections caused by *Coxiella burnetii*, using probes targeting *C. burnetii*-specific 16S rRNA sequence (5'-CCGAAGATCCCCGCTTTC-3') (Prudent et al., 2018). A similar work was published by Huang et al. (2019), which have developed a novel approach for accelerated detection of bacteria in blood without the subculture step, using a combination between PNA-FISH and enhanced acoustic flow cytometry (PNA-FISH-AFC) (Huang et al., 2019). They have used a 16S probe (5'-TATCTAATCCTGTTC-3') which, in combination with flow cytometer, was able to detect genetic material within intact bacterial cells, in a shorter incubation time of 5–10 h. Other works using PNA-FISH have also been published (Hansen et al., 2016; Lopes et al., 2018; Rocha et al., 2018).

Next-generation-sequencing (NGS)

Next-generation-sequencing (NGS) is a high-throughput massive parallel sequencing technology enabling the simultaneous and independent sequencing of millions of DNA fragments (Gu et al., 2019). Bioinformatics analysis of the output of NGS platforms allows a magnitude of applications that are extremely useful for the clinical microbiology laboratory, namely addressing pathogens detection and identification, as well as identifying virulence determinants or antibiotic resistance genotypes. An additional advantage includes the possibility of simultaneous analysis of several genes (or even all genome) (Yohe and Thyagarajan, 2017). The NGS costs continuous declining is turning this powerful technology accessible to the clinical microbiology laboratories. Indeed, high-throughput genomics is joining the big-data club as massive amounts of sequencing data keep to be produced. From 1982, the number of bases in Genbank double approximately every 18 months. The number of bases available by whole-genome-sequencing (WGS) surpassed the number of bases available by Genbank in 2004, while the number of sequences exceeded the ones of Genbank in 2014. For downloading purposes, the uncompressed GenBank Release 244.0 requires approximately 1780 GB (Genbank release 244, June 2021). The European Bioinformatics Institute (EMBL-EBI, www.ebi.ac.uk) continually expands its total disk capacity to keep pace with demand, storing 120 petabytes (1 petabyte is 10^{15} bytes) as of November 2017 (Cook et al., 2018). Besides the problem of archiving big sequencing data, in order to obtain answers to scientific questions out of sequencing data bioinformatics, tools are absolutely necessary. Software and web servers' solutions for genomic analysis are constantly being developed. Choosing the appropriate ones will always depend on the answer that is pretended, the computing power available and the bioinformaticists skills.

The general workflow for NGS involves sample processing, sequencing and bioinformatics analysis (Schwarze et al., 2020). Sample processing starts with DNA extraction and DNA quality control, using UV-Vis measurement, fluorescence measurement and/or agarose gels. Sequencing itself depends on the NGS platform used, but general steps involve library preparation, normalization and validation followed by the NGS running. The bioinformatics step starts with a quality assessment of the DNA sequencing reads and the use of several bioinformatics tools explored below.

Different sequencing platforms options are available for NGS. Most studies use Illumina (San Diego, USA) devices (MiniSeq, MiSeq, NextSeq 550 and NextSeq 1000 & 2000) (Gu et al., 2019). Illumina uses sequencing by synthesis in which single stranded DNA fragments are attached to a flow cell (given that the adaptors added to the DNA fragments are complementary to the flow cell oligos). Next, in the clustering step, each DNA fragment is isothermally amplified, providing the reading of the fluorescently labeled nucleotides and determination of each base (A, G, T, or C). Illumina has high throughput and presents as pitfall barcode index switching, which may lead to incorrect sample assignment during flow cell scanning (Gu et al., 2019). Another platform available is the Ion Torrent from Thermo Fisher Scientific (Waltham, United States), which uses semiconductor sequencing technology. Here, single stranded DNA molecules are cloned on a bead within an emulsion PCR (analogous to the 454 sequencing, Roche). In the emulsion PCR each nucleotide is applied and washed in turn and the incorporation of the nucleotide by the nascent DNA strand is measured by the difference in pH caused by the release of protons during polymerization, allowing proton detection by a metal-oxide-semiconductor present in the microprocessor. Ion torrent provides quick detection, but is less able to detect homopolymer

sequences, due to signal loss when the same nucleotide is incorporated a repeated number of times (Heather and Chain, 2016). These sequencing platforms so far presented are also known as second-generation sequencing, as opposition to the first-generation Sanger sequencing that relied on chain-termination by using the dideoxy technique. More recently, the third-generation sequencing is based on Single Molecule, Real-Time (SMRT) Sequencing used by PacBio (Pacific Biosciences, United States) and Nanopore sequencing (Oxford Nanopore Technologies, United Kingdom) (Heather and Chain, 2016). In SMRT sequencing by PacBio, adapters are ligated to the DNA forming a circular DNA that is placed in the SMRT cell. The SMRT cell have small holes named the zero-mode waveguides (ZMW). A single DNA molecule is mobilized to each ZMW and, as the polymerase incorporates labeled nucleotides, light is emitted, measuring nucleotide incorporation in real time (Eid et al., 2009). SMRT offers as advantages long reads and quick runs, while the drawback a higher error rate (Ardui et al., 2018). Finally, the Oxford Nanopore Technologies (Oxford, United Kingdom) through MinION, GridION, and PromethION is offering portable sequencers that can be connected to any computer that complies with system requirements. In this technology, PCR amplification is not mandatory for DNA sequencing. Instead Nanopore relies on passing a single stranded DNA by a grid of nanopores (tiny protein channels) that causes changes in the ion current allowing to determine the DNA sequence. Nanopore offers long reads and very short sequencing time, but has less accuracy (Gu et al., 2019). All of the presented technologies are revolutionizing how physicians diagnose bacterial infectious diseases, with applications from bacterial identification, to antibiotic resistance determination, or microbiome study.

Metagenomics

Metagenomics studies using NGS, the metagenomic NGS (mNGS), is a growing filed in clinical microbiology laboratories, allowing to analyze the microbial load in human samples, which can be used in the diagnosis field, specially relying in the 16S rRNA gene (Schlaberg et al., 2017). The methods exposed above mostly rely on PCR that via primer specificity allow to identify a limited number of pathogens, struggling to identify multiple distinct pathogens in the same sample. The metagenomics approach allows to determine all microbiota, including of course the identification of particular pathogenic bacteria associated with a given infection. The NGS platforms presented above offer the possibility of conduct mNGS studies which can be used in several fields, including the clinical microbiology laboratory. The main advantage of metagenomics relies on not requiring culture, and allowing the identification of both known and novel microorganisms. In the metagenomics approach there is no need to search for each specific pathogen, allowing laboratories to identify bacteria from a single sequencing-based method (Miller et al., 2013). While mNGS is not the standard technique for bacterial infection identification, it is especially useful for identifying low-abundance, new, difficult to detect and co-infecting pathogens directly from clinical samples (Han et al., 2019), for which it is expectable the continuous implementation of this technique by laboratories and in clinical practice. There is still a high number of infectious diseases whose etiology remains undetermined, reaching about 50% of the cases of blood stream infectious and central nervous system infections (Robinson et al., 2013; Simner et al., 2018), which increases the use of broad spectrum antibiotics with all their pitfalls: emergence of antibiotic-resistance bacteria, treatment failure and higher mortality risk. Although outside the scope of this chapter, the use of mNGS can be extended to other applications important for the clinical microbiology laboratory, such as prediction of antibiotic resistance (Sukumar et al., 2016), identification of virulence traits (Levade et al., 2021), or follow up of outbreaks (Robinson et al., 2013).

All NGS platforms presented above offer protocols that can be used for mNGS, targeting a few or even all the nine hypervariable regions of the 16S rRNA gene (Table 3). Reiterating, the 16S rRNA gene is about 1500 bp long and presents nine variable regions (V1 to V9) that are extensively used for pathogen identification. The sequences between the hypervariable regions tend to be conserved and are used for universal primer design. Among pathogens, the extent of sequence diversity varies and one variable region alone is not able to distinguish all pathogens (Chakravorty et al., 2007).

Table 3 Metagenomic approach offered by NGS platforms.

NGS Platform	Primers	16S rRNA region
Illumina ^a	F-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG R-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC	V3, V4
Ion Torrent	Proprietary primer set V2–4-8 Proprietary primer set V3-6,7-9	V2, V4, V8 and/or V3, V6, V7, V9
PacBio ^b	F-GCATCCACTCGACTCTCGCGTAGRGTTYGATYMTGGCTCAG R-GCATCAGAGACTGCGACGAGARGYTACCTGTTACGACTT	Full length V1 to V9
Nanopore ^c	27F 1492R	Full length V1 to V9

^aThe overhang adapter sequence is in bold.

^bPacBio provides a long list of primers that may be used (8 forward primers and 24 reverse primers), only one pair is presented. For detail consult the Amplification of Full-Length 16S Gene with Barcoded Primers for Multiplexed SMRTbell® Library Preparation and Sequencing. Barcode sequence is in bold

^cPrimers described in Table 1, to which barcodes and 5' tags are added.

Illumina mNGS targets V3 and V4 regions, based on the universal primers proposed by Klindworth et al. (2013). Other target rRNA 16S regions can be used by replacing the pair of primers used, but conserving the overhang adapter. Ion torrent offers the possibility to amplify 2 variable regions of the 16S rRNA gene with two pools of set V2-4-8 and V3-6,7-9 primers (primer set V2-4-8 and primer set V3-6,7-9), which can be used individually or in combination. PacBio and Nanopore allow the sequencing of the entire 16S rRNA gene (Fig. 4 and Table 3).

The introduction of mNGS in the clinical microbiology laboratory is expected to grow in the near future and has been used for the identification of pathogens (Abayasekara et al., 2017; Han et al., 2019; Duan et al., 2021) in several bacterial infections, such as dental caries (Kazemtabrizi et al., 2020), endocarditis (Oberbach et al., 2017; Kolb et al., 2019), encephalitis (Greninger et al., 2015), joint infection (Flurin et al., 2021) or endophthalmitis (Mishra et al., 2021).

The use of mNGS in clinical microbiology laboratories is however complex, requiring equipment acquisition, protocol optimization and validation, computer power and, highly trained personnel, or bioinformatics software validation (Chiu and Miller, 2019). Considering the bioinformatics analysis of the outputs of the NGS platforms, this can be performed using host computers and servers or cloud solutions. There are a few bioinformatics pipelines for mNGS analysis, like SURPI (Naccache et al., 2014), Taxonomer (Flygare et al., 2016), Centrifuge (Kim et al., 2016), Kraken (Wood and Salzberg, 2014) and IDseq (Kalantar et al., 2020). The use of mNGS is expected to be more and more fast, accurate, cheap and easy to use in the clinical microbiology laboratory, and may come to replace current techniques used for pathogen identification.

Conclusions

Infectious disease caused by bacteria is an important cause of death worldwide and the diagnosis of these microorganisms is challenging. Traditionally, detection and identification of bacterial infections relied solely on culture-based techniques and blood culture has been considered the “gold standard” for pathogen detection. Standard culture techniques depend on morphological and biochemical characteristics for identification, which can lead to decreased specificity. Only a fraction of bacteria can be successfully cultured *in vitro* due to various factors, such as fastidious growth requirements, non-viable organisms or inhibition by previous antibiotics administration.

The 16S rRNA gene is a universal gene found in all bacterial chromosomes. Methods based on identifying the presence of conserved and hypervariable regions in the 16S rRNA gene have opened an entirely new horizon for bacterial identification. Conventionally, molecular detection is still the best method of species identification in clinical samples. Molecular techniques can be performed directly on clinical samples providing a rapid detection of pathogens using DNA amplification, typically conventional PCR followed by Sanger sequencing, qPCR, or probe hybridization techniques. In summary, molecular methods explore the existence of a conserved region in the 16S rRNA gene for universal primer annealing that allows the amplification of the 16S rRNA hypervariable regions. Subsequently, after DNA sequencing and by comparison to a database of known sequences, bacteria identification is achieved. More recently, mNGS started to be introduced, allowing for the identification of the microbial genetic content in clinical samples. Importantly, 16S mNGS has the potential to revolutionize the diagnosis of IBI in hospitals with molecular biology facilities, being currently best positioned as a complementary technique to be used alongside culture and other traditional methods. Despite this, metagenomics requires skilled scientists to perform the experiments and to analyze the data which, to date, centers in the academic research environment rather than at the frontlines of public health. To be considered a clinical test for pathogen detection in a public health laboratory or hospital, standard metagenomic protocols are necessary for testing and analyzing samples and compare results.

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Detection and Diagnosis of Mycobacterial Pathogens Using PCR

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Introduction

Mycobacteria spp. are uttered to be existed around 150 million years ago (Cambau and Drancourt, 2014). In 1720, physician Benjamin Marten, in his book named 'A Theory of Consumption,' assumed that small living creature may be responsible for transmission of tuberculosis (TB) into patients through air (Barberis et al., 2017). *Bacillus leprae* was the first known representative of *Mycobacterium* by Hansen in 1875 (Balamayooran et al., 2015; Pfyffer, 2015). In 1896, the genus *Mycobacterium* was originally erected by Lehmann and Neumann (Barberis et al., 2017; Jagielski et al., 2016). *Mycobacteria* are aerobic, non-endospore forming, rod shaped bacteria. In the cell wall of *Mycobacteria*, lipopolysaccharide is replaced by mycolic acids, results in forming waxy water resistant layer. For this reason, *Mycobacteria* cannot be stained by conventional gram staining procedure because waxy component in the cell wall prevents the absorption of aniline dyes (Pfyffer, 2015). The mycobacteria can be divided into two groups namely, slow growers, need more than 7 days for growth e.g., MTB complex that includes *Mycobacterium tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. caprae*, *M. canettii*, and *Mycobacterium pinnipedii* and fast or rapid growers form visible colonies within 7 days including NTM (non-tuberculous mycobacteria) *Mycobacterium ulcerans*, *Mycobacterium haemophilum*, *M. asiaticum*, *Mycobacterium shimoidei*, and *Mycobacterium szulgai* (Pfyffer, 2015).

Mycobacterial pathogens are responsible for a number of wide spread diseases among human and animals (Paton, 2021). In 1880, Robert Koch identified the etiological agent of human tuberculosis and named it *M. tuberculosis* (Cambau and Drancourt, 2014). *M. tuberculosis* can be associated with both lungs infection and extra-pulmonary tuberculosis including intestine, meninges, lymph nodes, bones, joints, kidneys, skin and neurotuberculosis (NTB), a granulomatous infection of the central nervous system (CNS) (Peters et al., 2020). Bovine tuberculosis (BTB) caused by *M. bovis* is one of the major health problems in livestock industry. It is also the major cause of zoonotic tuberculosis (Cousins, 2001). Further, *Mycobacterium leprae* is the infectious agent of leprosy which is a chronic granulomatous disease (Mungroo et al., 2020). Clinical symptoms such as disfiguring bacterial inclusions in the skin, peripheral nerves, and respiratory mucosa are also considered as way of disease identification. Different diagnosis and detection methods are available to test leprosy. Lepromin skin test is used to determine the disease (Mungroo et al., 2020).

From the early epidemics, detection and diagnosis of *Mycobacterium* pathogens had been difficult (Mungroo et al., 2020). Along with conventional laboratory tests based on clinical features and growth in culture, the necessity of molecular detection has been urged to shorten the time limit of diagnosis (Acharya et al., 2020). Several promising diagnostic tests has been developed and are being used in many countries. Moreover, use of immunological methods for diagnosis of *Mycobacterium* has been restricted because of their low sensitivity and specificity (Anochie et al., 2012). Molecular technologies solve this problem because they have the highest sensitivity and specificity. That is why resource-rich countries prefer molecular techniques more for detection of *Mycobacterium* (Acharya et al., 2020; Anochie et al., 2012). Unfortunately, despite the availability of these advanced tools and techniques, accurate diagnosis of infection in early stage remains a problem till now (Acharya et al., 2020). Studies focusing on the molecular detection to improve the accuracy and reduce the detection time are needed. A comprehensive analysis on the present molecular polymerase chain reaction (PCR) based detection of *Mycobacterium* is required to understand the points need attention. This review focuses on the available molecular detection and diagnostic methods of *Mycobacterium* pathogens that will be a guideline to determine the scope of future analysis to improve the molecular detection.

Disease burden of *Mycobacterium* spp

Mycobacterium tuberculosis, associated with tuberculosis disease which typically affects lungs but can affect other site occasionally, transmitted through air by sneezing, coughing and even just talking. About 10 million people develop TB yearly and among them 1.6 million people die of this (WHO, 2021a). At present 198 countries and territories with 99% of the world's population, have been recorded for TB cases (WHO, 2021a). In 2019, an estimated 10.0 million (range, 8.9–11.0 million) people was infected by TB (WHO, 2021a). Geographical distribution of prevalence of TB in 2019 was 44% in South-East Asia, 25% in Africa and 18% in the Western Pacific, with smaller percentages in the Eastern Mediterranean (8.2%), the Americas (2.9%) and Europe (2.5%) (WHO, 2021a). In 2020, the COVID-19 pandemic had a negative impact on patients with TB. A collaboration study conducted by Avenir Health, Imperial College and the United States Agency for International Development (USAID), named 'Stop TB partnership' reported that a 3-month lockdown followed by 10-month restoration of services may cause an additional 1.4 million TB deaths between 2020 and 2025 (WHO, 2021a).

Leprosy is an age old disease that had been prevalent from the ancient time. The recorded first breakthrough of leprosy occurred in the 1940s. According to World Health Organization, the present estimation for global prevalence of leprosy to be over 210,000 cases annually (WHO, 2021b). Currently the prevalence of leprosy is approximately 0.34 in per 10,000 people. Moreover, in recent years 200,000 new cases of leprosy have been reported annually in more than 105 countries throughout the world. The highest rates of leprosy are in Asia and Africa (Lobo, 2006; WHO, 2021b). Underdeveloped nations are at the greatest risk, mostly in Southeast Asia, North and South America, Africa, and the eastern seaboard of the Pacific Ocean and the Western Mediterranean coast. 64% of all new cases has been recorded only in India alone (WHO, 2021b). Multidrug (dapsone, rifampicin and clofazimine) therapy has been proved effective in the treatment of leprosy (Smith et al., 2017). To eradicate leprosy worldwide, WHO has launched a program named "Global Leprosy Strategy 2016–2020: Accelerating towards a leprosy-free world" in 2016 (Sharma et al., 2020; WHO, 2021b).

Mycobacterium bovis is the causative agent of bovine tuberculosis, is also associated with the major cause of zoonotic tuberculosis. Estimation of the global burden of zoonotic TB are imprecise (Carneiro et al., 2019). Moreover, WHO estimated that there were 147,000 new cases of zoonotic TB in humans in 2016 and 12,500 deaths occurred due to the disease. Reduction of burden of tuberculosis depends on the rapidity of implementation of DOTS (directly observed therapy, short course) program (Carneiro et al., 2019).

Detection of *Mycobacterium* spp. by conventional methods

Traditional detection methods of *Mycobacterium* spp. include, direct microscopic observation and isolation through culture method (Glennon and Cormican, 2001; Pfyffer, 2015). In addition, detection through nucleic acid amplification has gained popularity recently. Still microscopic observation is a reliable method for detection of *Mycobacterium*. Bronchoscopes and related devices are widely used sources (Pfyffer, 2015). Preparation of smear and examination under microscope is still most conventional and inexpensive process. But due to waxy cell wall special acid fast staining (traditionally Ziehl-Neelsen staining) is needed to facilitate the uptake of dye (Pfyffer, 2015). Presently fluorescent stain (auramine) is preferred by many laboratories (Pfyffer, 2015). The sensitivity of staining and microscopic observation ranges from 22% to 80% (Glennon and Cormican, 2001).

M. tuberculosis produce off-white and rough colonies on solid media. Egg-based medium of Lowenstein-Jensen was used previously for the culture of mycobacterium but the process is slow (Pfyffer, 2015). At present, commercially available culture systems marketed for the isolation of mycobacteria are MGIT (BD) and MB Redox (Heipha Diagnostica Biotest, Heidelberg, Germany), semi-automated systems (the BACTEC 460TB system; BD) and fully automated systems e.g., the BACTEC 9000MB and BACTEC MGIT 960 (BD), the ESP Culture System II (Trek Diagnostic Systems, Westlake, Ohio) and the MB/BacT ALERT 3D system (Pfyffer, 2015). *M. tuberculosis* and some species of *M. bovis* are easily recognized by its susceptibility to pyrazinamide. *M. leprae* has an important exceptional characteristic that it cannot be cultured in vitro (Pfyffer, 2015). A short insight of available tests to detect *Mycobacterium* spp. is given in Fig. 1.

Immunological tests

Many rapid tests have been developed based on different principles of immunological test reactions (Pinto et al., 2012). These rapid tests have shown varying degrees of sensitivity and specificity but due to low level of specificity most of the immunological tests are not recommended in developed countries (Pinto et al., 2012). Use of immunological test for the detection of *Mycobacterium* is confined only in low and middle income countries with weak regulatory systems. Evidence shows that commercial kit for the detection of pulmonary TB were inconsistent with 10–90% sensitivity and 47–100% specificity (Pinto et al., 2012). Lipoarabinomannan, cord factor, A60, 38 kDa, and 16 kDa are antigens that been used so long for antibody detection kits. The sensitivity and specificity respectively 15.7% to 89.2% and 50% to 100% has been recorded for such antibody tests (Singh and Grover, 2011). Besides, for the detection of antigens in sputum, CSF, pleural fluid and ascetic fluid different techniques such as RIA, EIA, hemagglutination, and latex agglutination has been used in laboratories (Singh and Grover, 2011). Most common immunological tests are antibodies from lymphocyte secretion (ALS) assay, transdermal patch, tuberculin skin test, Mantoux skin test, Heaf test and adenosine deaminase (ADA) tests (Pfyffer, 2015).

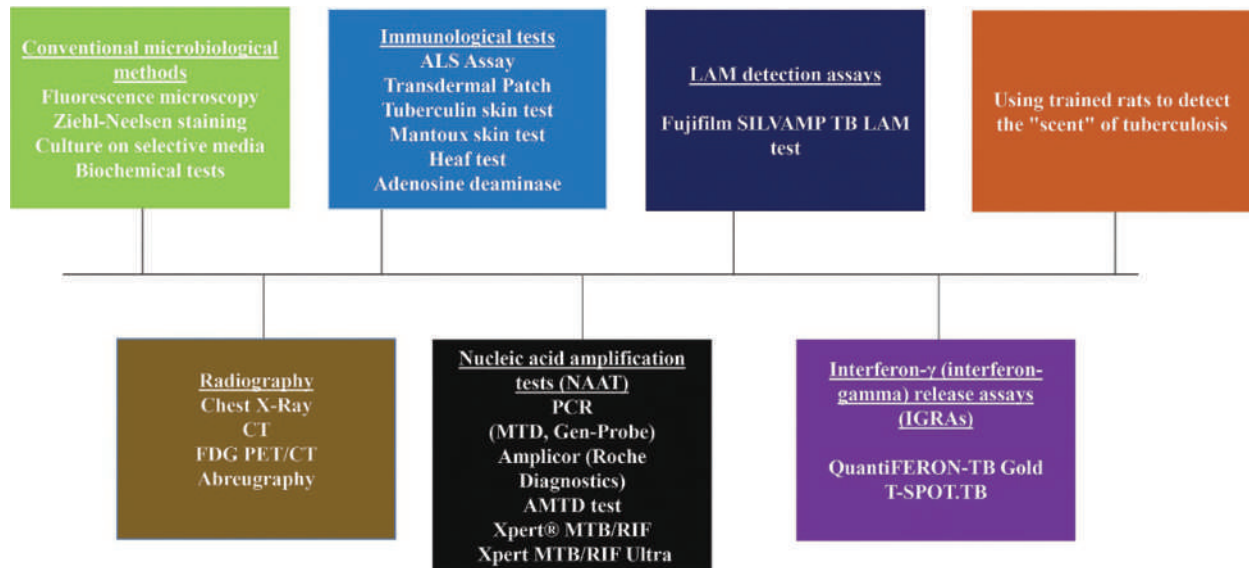


Fig. 1 Short insights into the available diagnostics methods used to detect *Mycobacterium* spp., particularly *Mycobacterium tuberculosis*.

Tuberculin skin test

Tuberculin test also known as positive purified protein derivative (PPD) skin test is the first developed immunodiagnostic test (Pfyffer, 2015). The standard recommended tuberculin test is the Mantoux test (Pfyffer, 2015). It is based on the fact that infection with *M. tuberculosis* bacterium produces a skin reaction caused by delayed-type hypersensitivity to certain components of the bacterium (Pinto et al., 2012). The test is inspected for the evidence of redness, swelling and induration after 48–72 h of administration. The result is interpreted based on the measurement of the diameter of the zone of induration (Pfyffer, 2015).

Enzyme-linked immunosorbent assay

Sandwich ELISA (sELISA) and dot-ELISA are two notable ELISA techniques also used for the antigen detection. Lipoarabinomannan (LAM) has been most frequently used as the target antigen, and its monoclonal and polyclonal antibodies have been used to detect antigen in sputum samples (Pfyffer, 2015). A study for the diagnosis of tuberculosis in Indian population used an indirect ELISA based technique (Kashyap et al., 2007). They presented a prospective evaluation for detecting Antigen (Ag) 85 complex in the sera from TB patients. Monoclonal antibodies (mAb CS-90) against the purified Ag-85 complex, was used. Ag-85 complex comprises of three related major secretory proteins of *M. tuberculosis*: Ag-85A (31 kD), Ag-85B (30 kD) and Ag-85C (31.5 kD) (Kashyap et al., 2007). To perform the test, in sera from TB patients, Ag-85 complex was incubated with mAb(CS-90). About 197 different groups of patients were included in this study. The test yielded 82% sensitivity and 86% specificity for TB diagnosis (Kashyap et al., 2007).

INF-gamma release assay

A recent approach for the detection of latent TB infection has been developed called IGRAs (INF-gamma release assay). The test measures release of IFN-gamma from T cell, in response to antigen which is unique to MTB, including early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP10). Though IGRAs and TST are based on similar mechanism, IGRAs is more specific than TST. Commercially two IGRAs are available: the QuantiFERON-TB © Gold In-Tube (QFT-GIT) assay (Cellestis Ltd., Vic., Australia) and the T-SPOT.TB © assay (Oxford Immunotec, Abingdon, United Kingdom). Specificity (>95%) of IGRAs has been recorded for detection of latent TB infection (Pinto et al., 2012).

QuantiFERON-TB gold

Quanti FERON-TB Gold (Cellestis Limited, Carnegie, Victoria, Australia) which detects the release of IFN-gamma in fresh heparinized whole blood collected from sensitized persons (Acharya et al., 2020). Blood is then incubated with synthetic peptides that stimulates ESAT-6 and culture filtrate protein-10 (Acharya et al., 2020). The test is commonly used in developed countries as an alternative to TST. This kind of test is justified for patients who have had known exposure of MTB, HIV infection, and existing immunocompromised conditions (Acharya et al., 2020). Blood samples are collected using MTB antigen coated tubes are used for the collection of blood. IFN-γ release is measured using Enzyme Immunoassay (EIA) technique. The QuantiFERON-TB Gold In-Tube is exactly the same test overcoming the limitations of QuantiFERON-TB Gold (Acharya et al., 2020).

T-SPOT.TB

In T-SPOT.TB (Oxford Immunotec Limited, Abingdon, United Kingdom, 2008) test, peripheral blood mononuclear cells is incubated with mixtures of peptides (ESAT-6, CFP-10). To detect increases in the number of cells that secrete IFN-gamma, an enzyme-linked immunospot assay (ELISpot) is used (Acharya et al., 2020; Anochie et al., 2012; Singh and Grover, 2011).

Molecular detection techniques

Molecular detection techniques has opened a new era in the detection of *Mycobacterium*. Due to poor reliability of serological tests, molecular techniques are a must (Pfyffer, 2015). Before the invention of molecular genetics methods, the identification of *Mycobacterium* was difficult and time consuming. Recently, rapid advances have been made in the molecular detection methods of *Mycobacterium* (Glennon and Cormican, 2001). Nucleic acid amplification tests (NAAT) of *Mycobacterium* are mainly based on the polymerase chain reaction (PCR) methods.

Nucleic acid amplification

The greatest advantage of nucleic acid amplification tests (NAAT) is the rapidity of result (within hours). Along with PCR, another two amplification techniques, transcription mediated amplification (TMA) and strand displacement amplification (SDA) are commercially used widely (Glennon and Cormican, 2001; Pfyffer, 2015). These tests have a level of 98–100% specificity and 92% sensitivity. Not only sputum but also blood, lymph, CSF, urine and bronchial aspirates can be used in NAAT methods to detect *Mycobacterium* spp. The main disadvantage of this technique is the high price and complexity (Pfyffer, 2015).

Polymerase chain reaction

Desired DNA sequence is amplified by PCR and detected even when a limited number of desired organism is available in the sample (Pfyffer, 2015). The most common targeted sequence for *Mycobacterium* in PCR is the insertion sequence IS6110. Other target genes include MBP64, rpoB, and hsp65. IS6110 is a 1355 base pair long fragment that encodes four enzymes required for its own transposition and insertion and is flanked by imperfect 28-bp inverted repeats. This is a unique sequence for mycobacteria which is present for about 20 times in the genome. In 2012 a comparative study has been performed in Bangladesh among 100 clinically suspected TB patients using Lowenstein-Jensen medium and PCR targeting IS6110 from sputum sample and reported 100% specificity and 94.74% sensitivity (Bifani et al., 2009; Deggim-Messmer et al., 2016).

Previously a *Mycobacterium* genus probe for detection of NTM DNA in clinical specimens using the COBAS™ TaqMan™ platform was developed (Deggim-Messmer et al., 2016). A *Mycobacterium* genus TaqMan™ probe was designed (5'-Cy5-CGACAAACCACCTACGAGCTCTTACGC-BBQ-3'). QIAquick PCR purification Kit (Qiagen, Hombrechtikon, Switzerland) was used for the purification of CP b 45. Another used primer for 16S rRNA gene sequencing is Mbakt-14 (5'-GRG RTACTCGAG TGG CGA AC-3') (Deggim-Messmer et al., 2016). A study from July 2003 to June 2005 was conducted in Victoria, Australia to detect the causative agent of Buruli ulcer, *M. ulcerans*. The study results in the development of a TaqMan assay targeting IS2404 multiplexed PCR (Fyfe et al., 2007). The assay had shortened the time limit and conventional gel-based PCR for laboratory confirmation of *M. ulcerans* infection (Fyfe et al., 2007). They analyzed 415 clinical specimens and demonstrated 100% sensitivity and specificity compared with culture. Along with this, another multiplex TaqMan assay targeting IS2606 and the ketoreductase-B domain of the mycolactone polyketide synthase genes of *M. ulcerans* was designed to analyze variety of environmental samples (Fyfe et al., 2007). Different primers used to detect different species of *Mycobacterium* are given in table 1.

TMA: Transcription mediated amplification is based on the principle to determine unique genetic information. In this amplification technique, RNA amplicon is detected through hybridization protection assay (HPA) (Ramachandran and Paramasivan, 2003).

SDA: Strand displacement amplification is an isothermal DNA amplification reaction based on a restriction endonuclease nicking its recognition site and a polymerase extending the nick at its 3' end, displacing the downstream strand (Acharya et al., 2020; Anochie et al., 2012).

AMPLIFIED MTD Test: AMPLIFIED MTD (*Mycobacterium tuberculosis* direct) Test (Gen-Probe, Hologic) is a specific test to detect amplified rRNA of *M. tuberculosis* directly. This is a rapid process with 96% sensitivity and 100% specificity (Acharya et al., 2020; Anochie et al., 2012).

LCR: Ligation chain reaction is a DNA amplification technique, based on two adjacent synthetic oligonucleotide primers that binds only with one strand of the target DNA and the another pair of oligonucleotide hybridize to the complementary DNA (Acharya et al., 2020; Anochie et al., 2012).

Xpert MTB/RIF: On the basis of Cepheid Gene Xpert system, the xpert MTB/RIF test is designed to detect *M. tuberculosis* and its resistance to rifamycin automatically. Targeting 81 base pair core region of rpoB gene, a hemi nested real time PCR assay is used. This method is simple with lower biohazard risk, robust and rapid (90 min) (Acharya et al., 2020). The test sensitivity has been proved 98%. In a systematic review, Li and colleagues analyzed 52,410 samples from 106 studies and reported an overall pooled sensitivity of Xpert MTB/ RIF at 0.85 (95% CI 0.82–0.88) and specificity at 0.98 (95% CI 0.96–0.98) as compared to culture and concluded that the Xpert MTB/RIF provides rapid and effective diagnosis of tuberculosis (Acharya et al., 2020). In Fig. 2, the flow chart of detection of *Mycobacterium* from patients with active and latent TB is given.

Table 1 List of commonly used primers for the detection of different species of *Mycobacterium* spp.

Species	Primer or probe	Sequence (5'-3')	Size (bp)	References
<i>Mycobacterium ulcerans</i>	IS2404 T-F	AAAGCACCACGCAGCATCT	59	Fyfe et al. (2007)
	IS2404 T-R	AGCGACCCCACTGGATTG		
	IS2404 T-P	6 FAM-CGTCCAACGCGATC-MGBNFQ		
	IS2606 T-F	CCGTCACAGACCAGGAAGAAG	58	
	IS2606 T-R	TGCTGACGGAGTTGAAAAACC		
	IS2606 T-P	VIC-TGTCGGCCACGCCG-MGBNFQ		
<i>M. bovis</i>	MPB64-F	CAG GCA TCG TCG TCA GCA GC	543	Young et al. (2005)
	MPB64-R	GTG ATT GGC TTG CGA TAG GC		
	MPB70-F	GAA CAA TCC GGA GTT GAC AA	471	
	MPB70-R	AGC ACG CTG TCA ATC ATG TA		
<i>Mycobacterium leprae</i>	85A-C IR-F	ATA CTG TTC ACG CAG CAT CG	–	Martinez et al. (2006)
	85A-C IR-R	GTT GAA GGC ATC AAG CAG GT		
	85B-F	CAA TAG GAG TGT CAA ATC C	–	
	85 B-R	TTC CCA GCC TGC AGA ATG		
<i>Mycobacterium tuberculosis</i>	rpoB F	TGGAGTACGTGCCCTCGTC	–	André et al. (2017)
	rpoB R	GTGGCCACCGACCATCT		
	PT-8	GTGCGGATGGTCGAGAGAGAT	540	
	PT-9	CTCGATGCCCTCACGGTTCA		

F, forward; R, reverse; P, probe; bp, base pairs; *M*, *Mycobacterium*.

Genotyping methods

PCR based genotypic methods are newly developed including spoligotyping, IS6110-based RFLP (Restriction fragment length polymorphism) and mycobacterial interspersed repetitive unit (MIRU) typing (Acharya et al., 2020; Anochie et al., 2012). These genotyping methods are rapid and conventional. But possibility of returning into false positive result has decreased the use of these techniques (Acharya et al., 2020; Anochie et al., 2012).

Spoligotyping: A recently developed method, spacer oligonucleotide typing or spoligotyping, has been introduced for the detection and typing of MTB complex (Gori et al., 2005). The method is based on amplification of a highly polymorphic direct repeat locus in *M. tuberculosis* genome by PCR. In this locus, a variable number of short, direct repeats are interspaced with non-repetitive spacer sequences (Gori et al., 2005). Variation in number of different isolates are used to obtain different hybridization patterns and then hybridized to multiple synthetic spacer oligonucleotides. The result is obtained rapidly, not only detection but also strain identity can be identified. In comparison with culture results, the sensitivity & specificity of spoligotyping was 98% & 96% for clinical specimens while 91% sensitivity & 98% specificity for slides obtained from liquid medium, and 100% sensitivity and 94% specificity for slides obtained from colony on Lowenstein-Jensen solid medium (Gori et al., 2005).

RFLP: Restriction fragment length polymorphism is the most widely used DNA finger printing method. For *M. tuberculosis* insertion sequence IS6110 is used as genetic marker. RFLP based on IS900 is widely used to characterize different MAP (*Mycobacterium avium* subspecies *paratuberculosis*) strains (Acharya et al., 2020). In this process DNA fragments of various length is produced by digestion with restriction endonuclease. These fragments are subjected to gel electrophoresis on agarose gel and fragments of different size are separated. Followed by southern blotting and DNA probe hybridization, desired fragment bearing IS6110 is detected by observing banding pattern. IS6110 based RFLP is a standard genotyping method approved by international community that serves both inter and intra-laboratory detection system (Acharya et al., 2020). The standard method include-*Pvu* II (restriction endonuclease) for *M. tuberculosis* genomic DNA digestion, 3' fragment of IS6110 as hybridization probe, standardized molecular weight marker for IS6110-based RFLP (Acharya et al., 2020). IS6110 is stable enough that it is sufficient to identify the sequence to determine same strain spreading from one patient to another. Another important characteristic of IS 6110 is its diversity. These two criteria (stability & diversity) makes IS6110 a suitable epidemiological marker. Because the relatedness certain strains can be compared studying this (Acharya et al., 2020). In previous studies the primers RIS3, 5'-CGTCGAACGGCTGATGACCA-3' and 6110-R, 5'-GGCGGGTCCAGATGGCTTGC-3' have been used for the amplification of the IS 6110 probe from chromosomal *M. tuberculosis* DNA. Universal primers such as T7 can be used to amplify IS 6110 fragment from pUCIS (Bifani et al., 2009).

MIRU: Mycobacterial interspersed repetitive units (MIRU) are mini-satellite sequences of 77–101, 46–53 and 58–101 bp in length, consist of short tandem repeat structures which is found at multiple loci throughout the *M. tuberculosis* genome (Bull et al., 2003). MIRU were first identified in *M. tuberculosis* (MTB). Recently, it has been used for typing Mycobacterial pathogens such as *M. tuberculosis*, *M. bovis* and *M. avium*. For the characterization and distinction of MTB complex MIRU is being used widely with PCR (Bull et al., 2003). A study was performed where *M. avium* species were distinguished using MIRU. There was MIRU identified at 18 conserved loci throughout genome of the *M. avium* subspecies *paratuberculosis* (MAP) and *M. avium* subspecies *avium* (MAA). Among the 18 loci, 6 loci were found to differ between MAA and MAP in the number of tandem repeat motifs occurring at each MIRU locus (Bull et al., 2003). Primers used to amplify these loci are given in Table 2.

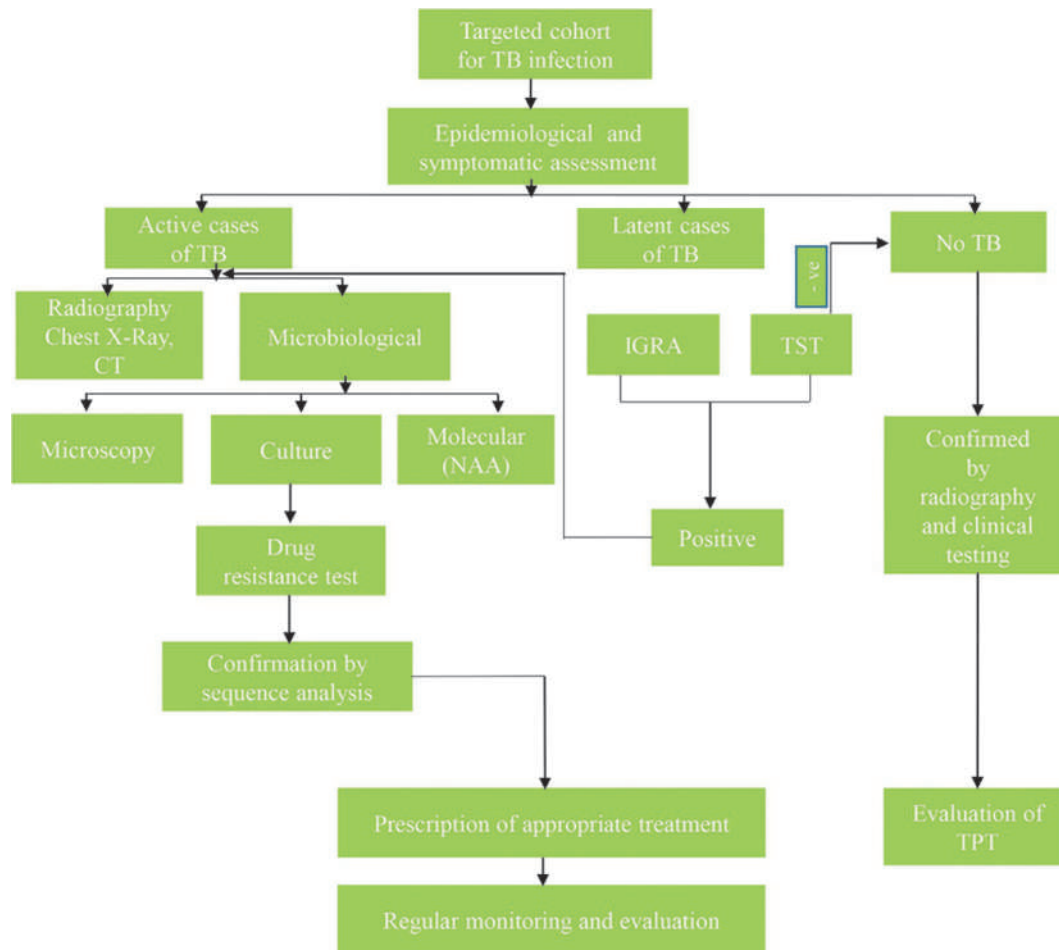


Fig. 2 Flow diagram showing the order of diagnosis for the non-infected, patients with latent infection and active infection. TB, tuberculosis; TST, tuberculin skin test; NAA, nucleic acid amplification assays; CT, computed tomography; TPT, tuberculosis preventive treatment; IGRA, interferon gamma release assay.

LAMP: Loop-mediated isothermal amplification (LAMP) method for the diagnosis of active TB disease has been recently endorsed by WHO. LAMP is a simple, rapid, specific and cost-effective NAA method performed in peripheral laboratory settings, was developed by Eiken Chemical, Japan (Acharya et al., 2020). In this method, at least four different sets of primers are specifically designed to recognize at least six distinct regions of the target gene. Only a single step is required for amplification by strand displacement reaction. It takes 15–60 min at ~65 °C temperature. Visual inspection of the incorporated fluorescence is used to detect amplicons (Acharya et al., 2020). The greatest advantage of LAMP method is, it does not need any expensive special reagents or sophisticated equipment for result interpretation. Operational feasibility of using this method has been evaluated and implemented successfully. The sensitivity and specificity of this method was recorded 76–80% and 97–98% respectively (Acharya et al., 2020).

LPA: Line probe assay (LPA), another PCR based assay used to detect *M. tuberculosis* and drug resistance mutations. In the test, respective sequence is amplified using PCR and subsequent hybridization on a strip immobilized with specific oligonucleotide probes. The presence of *M. tuberculosis* is determined by visual observation of the colored bands. There are different versions of commercial products based on the principle of LPA, for example, commercially used line probe assays are-INNO-LiPA Rif TB kit (Innogenetics, Zwijndrecht, Belgium), GenoType MTBDRplus 1.0 (Hain Lifescience, Nehren, Germany) and MTBDRplus 2.0 test (Hain Lifescience, Nehren, Germany) (Acharya et al., 2020).

Whole genome sequencing: Whole genome sequencing (WGS) is the next-generation sequencing (NGS) technology that determine complete nucleotide sequence of a microorganism. NGS based technology like WGS are used for characterization of the organism, drug susceptibility testing, genotyping and epidemiological investigation. Whole genome sequencing also provides an advantage of pursuing mutation analysis compared to other methods (Glennon and Cormican, 2001).

Table 2 Primers used to amplify the specific loci within Mycobacterial interspersed repetitive units (MIRU) (Bull et al., 2003).

Primer	Sequence
M1, Forward	5'-CGCGGACTTGATGGTCTC-3'
M1, Reverse	5'-ACTCCACCTGGACAACGG-3'
M2, Forward	5'-GAACGAAGATCCTGGGACTG-3'
M2, Reverse	5'-CGACGACGAACACCTCAAC-3'
M3, Forward	5'-ACATTCACCTGTCCATTCC-3'
M3, Reverse	5'-CCTCCTTACGGAGCAGGAA-3'
M4, Forward	5'-CGTTCAGCCTGTGCATGG-3'
M4, Reverse	5'-CAAGTCGTCACGGGCAAC-3'

Radiography

Various radiographic techniques are used to directly assess the damage in the infecting organ specially, in lungs by *M. tuberculosis*. Among the radiography methods, Chest X-ray, CT scan, abreuography and FDG PET/CT are most common. Chest radiographs can accurately suggest abnormalities but are not confirmatory diagnostic of TB. Cavities in the upper lobe created by TB becomes visible in X-rays (Rossi et al., 2005). The CT scan can reveal active and disseminated TB namely miliary TB by imaging the tiny nodules scattered throughout the lung. Further, FDG PET/CT can be used for a wide range of diagnosis focusing the detection of active TB, differentiation between active and latent disease, determination of stage, and treatment response monitoring, and identification (Pelletier-Galarneau et al., 2017). In Table 3 all the possible sites of non-TB and TB infection in patients with appropriate tests are described.

Diagnostic approaches other than conventional methods

Chromatography: Chromatography is the technique for separating components in a mixture based on differential affinities of substances. In the detection of *M. tuberculosis*, chromatography is used to differentiate between species on the basis of the length of colic acid residues in the cell wall. The result can be interpreted rapidly within 2 h (Anochie et al., 2012).

TB PNA FISH: PNA FISH stands for peptide nucleic acid fluorescence in situ hybridization assay is based on a unique DNA (PNA) which binds with DNA probe where sugar phosphate backbone has been replaced by polyamide allows differentiation between tuberculous and nontuberculous mycobacteria which is determined by fluorescence. Sensitivities for MTB complex specific PNA probes has been recorded 84–97% while the NTB specific PNA probes has showed 64–91% sensitivities (Ramachandran and Paramasivan, 2003).

MPB 64 patch test: MPB 64 is an antigen specific for *M. tuberculosis* complex. The patch test result can be determined within 3–4 days and its application lasts for a week. The sensitivity and specificity is 87.8% and 100% respectively (Ramachandran and Paramasivan, 2003).

FAST Plaque TB: FAST Plaque TB (BIOTEC Laboratories Ltd., FIND—Foundation for Innovative New Diagnostics) is a unique type of test where mycobacteria-phages are used to infect *Mycobacterium* and indirectly determine its presence. After infection with phage, clearing zones or plaques indicates a positive result. The phages are destroyed later with virucide without affecting host bacterial cells. The result is as rapid as available within 24 h. But the major limitation is it can only detect live *Mycobacterium*. The sensitivity and specificity has been recorded 70.3–75.2% and 98.7–99% respectively for this test (Anochie et al., 2012).

Conclusion

The disease burden of *Mycobacterium* is still one of the major health problems globally. Early and accurate detection of *Mycobacterium* with high sensitivity and specificity can lessen the risk of developing severe disease. To avoid false diagnostic results, the necessity of efficient diagnostic tools are unavoidable. Although, conventional methods based on microscopy and serology are easy to perform but have not been proved much effective. For the rapid and error free detection, PCR based molecular methods has gained popularity. Molecular detection of *Mycobacterium* spp. by PCR based methods has been proved more rapid and efficient in recent studies and the specificities have been recorded 88.9–99.2% while sensitivities have been recorded 63.7–95.9% (Kakhki et al., 2019). However, the high cost of these tests are one of the major problems including the fact that they supplement conventional methods rather than replacement in developing countries. Lack of adequate trained and skilled stuff is another shortcoming. It is evident that even today detection of *Mycobacterium* in a cost effective way remains a problem. Development of new systems, overcoming the costing and human resource issues is demanded.

Table 3 Site-specific diagnostic tests available for both non-respiratory and respiratory TB (Acharya et al., 2020; Tuberculosis, 2011).

Organ/System	Specimens	Radiography	Microbiological approaches	Molecular methods	Biopsy
Bone/joint	Bone marrow, joint fluid ^a	Plain X-ray, CT and MRI	Microscopy, AFB smear test, conventional culture, drug susceptibility, rapid culture	NAA assays, Xpert MTB/RIF assay, Xpert MTB/RIF ultra assay	Site of disease
Central nervous system	Cerebrospinal fluid	CT brain and MRI	Microscopy, rapid culture methods	Xpert MTB/RIF ultra assay	Cerebrospinal fluid
Disseminated	Bronchial wash, bone marrow, blood ^a	CT and ultrasound	Conventional culture, drug susceptibility, rapid culture	Xpert MTB/RIF ultra assay	Bronchial wash, bone marrow, blood
Gastrointestinal	Ascites	CT abdomen and ultrasound	Conventional culture	Xpert MTB/RIF ultra assay	Biopsy
Genitourinary	Early morning urine ^a	Intravenous urography and ultrasound	Conventional culture, drug susceptibility, rapid culture	Xpert MTB/RIF ultra assay	Site of disease, endometrial curettings
Larynx	Sputum ^a	X-ray and CT	Conventional culture, rapid culture	Xpert MTB/RIF ultra assay	Site of disease
Liver	—	Ultrasound	—	—	Site of disease
Lungs	Pericardial fluid, sputum ^a	X-ray and CT	Conventional culture, drug susceptibility, rapid culture	Xpert MTB/RIF ultra assay	Site of disease
Lymph node	Aspirate ^a	—	Conventional culture, drug susceptibility, rapid culture	Xpert MTB/RIF ultra assay	Node or aspirate
Pericardium	Pericardial fluid ^a	Echocardiogram	Conventional culture, drug susceptibility, rapid culture	Xpert MTB/RIF ultra assay	Pericardial fluid
Pleural cavity	Pleural effusion ^a	X-ray and CT	Conventional culture, drug susceptibility, rapid culture	Xpert MTB/RIF ultra assay	Pleural effusion
Skin	—	—	—	—	Site of disease

CT, computed tomography; MRI, Magnetic resonance imaging.

^aImmunological tests and rapid test like immunochromatography can be applied.

Declaration of competing interest

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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Detection of Genetic Elements Among Clinically Relevant Bacteria

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Introduction

Interactions between ourselves and our bacterial communities either within us or those we interact with in our environment are inevitable, constant and necessary. For the rare events during which any of our innumerable host-bacterium interactions cause disease, medicine and the sciences of pathology and epidemiology have developed ways to detect, treat and prevent spread of at least some of these diseases. The incomplete state of the science of detecting infectious disease, coupled with relentless adaptation by clinically relevant bacteria requires ongoing research and development to try to ensure that detection methods and treatment keep developing and are effective. The evolution of clinical microbiology as a mainstream laboratory science is less than 100 years old. In the clinical laboratory there always remains a challenge between detecting the pathogen and determining (a) what treatment is likely to be effective, (b) making a timely report, (c) using finite laboratory resources optimally, and (d) ideally, fully understanding the putative pathogen's genetic potential to cause disease or to transmit harmful features to another, perhaps distantly related species. The latter objective is a specialized research activity in clinical microbiology, currently.

In particular, when following established methods for pathogen detection and characterization, it is important not to limit our understanding of the isolate's propensity to cause disease to the name and epidemiological type that it is conventionally (and technically correctly) identified by in the laboratory. There are situations where it is the presence of one or more specific genes rather than the species name or its epidemiological type that are most important but detection of these cases is hampered by conventional diagnostic laboratory methods. Where a normally sterile body site is cultured and a single isolate is found in a symptomatic patient with, for example meningitis, Koch's postulates are easily followed, but in the case of, for example, gastroenteritis, where the pathogen or pathogens must be selected from a diverse community of microbes, the investigation can be challenging.

Genomic islands or chromosomal islands are large DNA sequences found in the genomes of certain bacterial strains of a species but not in the genomes of other highly-similar strains. Genomic islands encode many different functions, which depend largely on the environmental context in which the bacterium grows. Pathogenicity islands, for example, carry clusters of virulence genes whose products contribute to the pathogenicity of the bacterium (Schmidt and Hensel, 2004).

The plasticity of bacterial genomes allows adaptation or fitness of the bacterium to colonize or to survive adversity and thereby to evolve. As an example, the genome sizes of nonpathogenic *Escherichia coli* K-12, enterohemorrhagic *E. coli* (EHEC) O157:H7 strain EDL933, and uropathogenic *E. coli* (UPEC) strain CFT073 are 4,639,221, 5,528,445, and 5,231,428 bp, respectively (Blattner et al., 1997; Perna et al., 2001; Welch et al., 2002). Any one *E. coli* genome contains about 5000 genes, and roughly two-thirds of these are found in all *E. coli* genomes, but the other third are accessory genes, found in other strains, but not all; furthermore, any one *E. coli* contains less than 10% of the total number of *E. coli* genes in the *E. coli* pan-genome (Land et al., 2015).

Notably, the horizontal spread of antimicrobial resistance is a significant problem and particularly since as few as six new antibiotics were released for use between 1985 and 2015 and resistance has already been observed to each of these (Kenny et al., 2015).

When antibiotics were first used in the 1940s to treat clinical infections it might have been predicted that the development of antimicrobial resistance during therapy was highly unlikely owing to the low frequency with which mutations occurred. Although antibiotics have reduced the mortality from infectious diseases they have not markedly reduced their prevalence. The level of antibiotic usage has far exceeded all early expectations; in hospitals, in the community, and in the veterinary industry where antibiotics have been used both to treat infections and as growth promoters. This has resulted in a strong selective pressure among bacteria to survive antimicrobial onslaught.

This example serves as a useful means to illustrate some mobile genetic elements (MGE), given that (among originally susceptible bacteria) antimicrobial resistance is regularly acquired through the acquisition of genes rather than by mutation, the latter being the rarer event. It is useful to study the functioning of these genetic elements through the medium of antimicrobial resistance testing as this tends to be a conveniently straightforward marker to detect in the laboratory.

Currently, the detection of antimicrobial resistance is usually achieved in clinical laboratories using an agar- or broth- based antimicrobial susceptibility test method. Following isolation, a fresh culture of the strain is used and antimicrobial susceptibility testing is set up to test the isolate against a panel of different antibiotics. The resultant antimicrobial resistance profile gives an indication of the degree of resistance harbored by the test isolate. Using phenotypic methods for antimicrobial susceptibility testing is routine as this allows resistance to be detected for a variety of underlying molecular mechanisms of resistance to an antibiotic and it is a relatively inexpensive and quick method, particularly when large numbers of isolates are being tested at any one time. Detection of genetic mutations through specific PCR assays is most useful when resistance to a key antibiotic is always associated with one or two mutations; the use of phenotypic methods shows the proof of the expression of all of the genetic resistance determinants, with few exceptions and therefore can be more useful as a catch-all method than using molecular-based assays. Sequencing isolates on a routine basis for susceptibility testing is not yet used as the value of a patient report lies in its timeliness, its appropriateness and its accuracy and sequence analysis is currently both more expensive than phenotypic methods and takes longer.

This article will describe the principal mechanisms by which bacteria acquire DNA via genetic elements, along with examples of how bacterial pathogens are thus created or altered and how they may be identified in the laboratory.

The acquisition of foreign DNA by bacteria

Bacteria can acquire foreign DNA (horizontal transfer) by three mechanisms: transformation (acquiring naked DNA from the environment), transduction (introduction of foreign DNA by means of a bacteriophage, i.e., the viral infection of a bacterium) and conjugation (cell to cell contact) (Bushman, 2002). The acquisition of naked DNA from the environment has been found to include MGE, which Domingues et al. (2012) reported can be a highly efficient process and can be independent of genetic relatedness between donor and recipient. The authors concluded from their experiments that natural transformation provides a much broader capacity for horizontal acquisitions of genetic elements and hence, resistance traits from divergent species than previously assumed.

In essence, there are two hierarchies of transfer—one being the MGE that can move between bacteria, such as bacteriophages or plasmids or conjugative transposons—the other being the movement of mobile genetic elements within the DNA itself via integrons or insertion sequences or non-conjugative transposons.

Bacteriophage-mediated insertion of DNA (transduction) to the bacterial cell via temperate or lysogenic (bacterio)phages

Temperate or lysogenic phages may either lyse or lysogenize infected cells. Lysogeny is the state where a host cell contains one or more prophages in which the lytic genes are repressed by the phage-encoded repressor. In this case the virus does not replicate independently and no viral particles are produced. The phage nucleic acid is reproduced in a stable fashion, often integrated within the host chromosome (Casjensm, 2003). However, a culture of lysogenic cells contains free phages due to the spontaneous conversion to the lytic cycle once in every few thousand cell divisions (Smith and Rees, 1999). In times of stress, such as starvation or any development that might damage DNA, these prophages can turn into lytic production (i.e., produce infective virions) and kill their host bacterium in a matter of minutes (Oppenheim et al., 2005). Lysogenic phages alter the biology of their hosts and, in turn, influence the surrounding community of host and non-host cells (Howard-Varona et al., 2017). Most bacterial pathogens are said to be lysogens, carrying prophages within their chromosome, in many cases more than one (Casjensm, 2003); pathogens are reported to be more likely than non-pathogens to be lysogens (Touchon et al., 2016).

The role in nature of transduction first depends on a high degree of specificity of the adsorption step in bacteriophage invasion (Courvalin, 1996). This process involves incorporation of bacterial DNA from either the chromosome or a plasmid into a phage particle during the phage lytic cycle. The phage particle then acts as a vector and transfers the bacterial DNA into the next cell that it infects, in a process that has been termed generalized transduction, exemplified by the demonstration that different pieces of DNA can be experimentally transferred in this way. Fillol-Salom et al. (2019) have shown that bacteria and generalized transducing phages cooperate to survive adverse attacks whereby the resulting cells are resistant to phage attack due to immunity provided by the integrated prophage and they are able to exchange bacterial DNA with other cells containing a similar prophage via the transducing particles.

Plasmids as genetic transfer vehicles

Plasmids are autonomous extrachromosomal replicons, consisting of double-stranded DNA. They have been found to range in size from less than 10 kb to several hundred kb (Dib et al., 2015) and are common in bacteria. They are able to maintain themselves in

the cytoplasm of a bacterium for many generations. For most plasmids, one or two copies are present per chromosome, but it may be as many as 50 or more copies for certain small plasmids. The number of copies influences the strength of plasmid-borne characteristics, especially antibiotic resistance (Clarke et al., 2019). Plasmids are almost always described as circular but linear plasmids also exist (Dib et al., 2015). In addition to the transfer of antimicrobial-encoding resistance genes, plasmids may also confer additional metabolic and virulence mechanisms on the host cell.

Conjugative plasmids differ from non-conjugative plasmids in their requirement for additional genes that can initiate self-transfer (thereby facilitating horizontal gene transfer) and also tend to be larger. As plasmids, these elements are extra-chromosomal double stranded DNA (dsDNA) molecules that control their own replication ensuring their vertical transmission and, as conjugative elements, they encode all functions needed for their horizontal transfer (Smillie et al., 2010). Broad host range plasmids have attracted particular attention owing to their ability to transfer genes among phylogenetically distant host bacteria and to facilitate the transfer of other, non-self-transferable plasmids (Boon et al., 2001).

Determination of the plasmids present among bacteria in one wastewater-treatment plant (one of many studies of these settings, which provide a good example of the convergence of different environments) using 454 sequencing technology yielded plasmid metagenomics data showing antimicrobial resistance sequences against almost all the major classes of antimicrobial drugs (Szczepanowski et al., 2008). The authors point out that wastewater treatment represent habitats with ever-changing chemical and biological composition of the sewage, requiring rapid adaptation by indigenous microorganisms by means of genome rearrangements which require distinctive genetic flexibility. In a study of the IncP-1 plasmids from wastewater (Schlüter et al., 2007) the authors found that the plasmids consisted of a highly conserved backbone in contrast with an enormous diversity of accessory genes within, which they strongly suggest indicates that the plasmid backbones serve as efficient vehicles for shuttling modules encoding antibiotic, heavy metal, and disinfectant resistance, degradative capabilities and/or other accessory functions. In all plasmids identified in this study by Schlüter et al. (2007), integrons were found, suggesting that these provided the flexibility of genetic insertions. Integrons are described below in some detail.

Transposable genetic elements

Transposable genetic elements can translocate from one area of the bacterial chromosome to another, or between the chromosome and a plasmid or bacteriophage DNA (Mayer et al., 1995). Transposons generally range in size from 2.5 kb to 60 kb and usually possess long terminal inverted repeats and one or several accessory genes that confer an advantageous phenotype to their bacterial host (Darmon and Leach, 2014). DNA transposition is one of several enzyme-catalyzed mechanisms by which a discrete segment of DNA is moved; there tends not to be a requirement for extensive homology between DNA sequences at the ends of the mobilized DNA segment (the transposon) and the site into which it is moved (the target site) (Hickman and Dyda, 2016). DNA transposons have been identified in essentially all genomes that have been sequenced (Hickman and Dyda, 2016). Most transposable elements have two features in common, namely inverted repeats at their ends and a central sequence that contains at least one gene encoding a protein, transposase whose function is to create DNA strand breaks at the boundary of the inverted repeats and the flanking sequence of the original donor location (Dyson, 1999). Selection of the target site by a transposable element is a function of the transposase and differs at the level of sequence specificity and stringency (Darmon and Leach, 2014).

There are two types of transposable elements, both similar, referred to as transposons and insertion sequences (IS). Transposons differ from IS in that they carry a recognizable phenotypic characteristic such as an antibiotic resistance marker (Hickman and Dyda, 2016). An IS is a relatively small (0.7–2.5-kb) DNA segment. It contains one or two open reading frames (ORFs) encoding only proteins responsible for functions involved in its mobility (a transposase) and is bounded by short terminal IR sequences (Darmon and Leach, 2014). Transposable elements are dependent on the host chromosome, plasmid or bacteriophage for regeneration (Mayer et al., 1995).

In summary, transposons can be either conjugative or non-conjugative; conjugative transposons can be defined as elements encoding their own excision, their transfer by conjugation and their integration, regardless of mechanisms (Bellanger et al., 2014), in contrast to non-conjugative transposons.

Integrins and mobile gene cassettes

Antibiotic genes of Gram-negative bacteria are often situated on plasmids and transposons. In the early 1980s, studies using restriction mapping and heteroduplex analysis showed that different sets of antibiotic-resistance genes were sometimes found in the same location in otherwise closely related plasmids such as the IncW group, or transposons of the Tn21 family (Stokes and Hall, 1989; Recchia and Hall, 1995). Further work showed that this third mechanism of antibiotic-resistance gene dissemination involved naturally-occurring gene expression elements called integrins (Stokes and Hall, 1989). These structures operate as vehicles for the acquisition of resistance genes carried by MGE, termed gene cassettes, by a site-specific recombinational mechanism (Hall and Collis, 1995) as shown in (Fig. 1). While integrins are found on many different plasmids and transposons, one integrin, Tn402, has been identified to be itself an active transposon (Bennett, 1999; Domingues et al., 2012). Class 1 integrins are most associated with clinical infections. The presence of integrins has been reported most often in Gram-negative bacteria but they also occur in Gram-positives, including staphylococci (Li and Zhao, 2018) streptococci (Lin et al., 2017) and enterococci (Clark et al., 1999; Yu et al., 2017). Yu et al. (2017) and Lin et al. (2017) experimentally observed class 1 integrin mediated excision and integration of various exogenous antibiotic resistance genes using a collection of plasmids and *Enterococcus faecalis* and *Streptococcus pneumoniae*, respectively. Interestingly, retrospective analyses have indicated that integrins were associated with resistance genes present in the

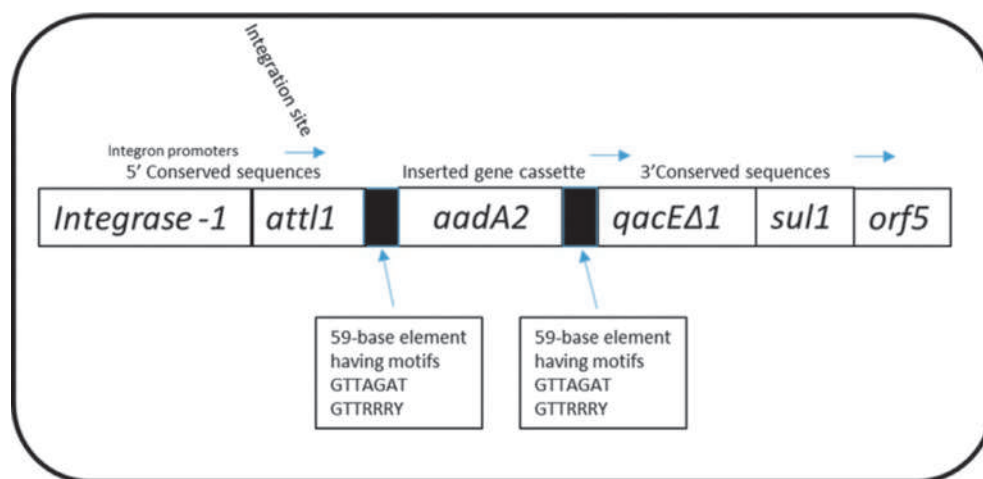


Fig. 1 Structure of a class 1 integron, including a gene cassette encoding resistance to aminoglycosides (*aadA2*).

shigella isolates that characterized the first wave of transferable plasmid-mediated resistance in Japan in the 1950s (Liebert et al., 1999).

Structure and function of class 1 integrons

Integrons, as shown in Fig. 1, comprise two conserved segments, the 5'-conserved segment (CS), which is located upstream from the gene cassette(s) and 3'-CS, which is found downstream of the resistance gene shown as an example in this figure. There is an internal variable region containing one or more gene cassettes, with a recombination site, termed the 59-base element, which includes the motif GTTRRRY (Hall et al., 1991). The 5'-CS contains the *int* gene, which encodes integrase, a site-specific recombinase. One of its substrates is the 59-base element and the second substrate is the cassette receptor or integration site (*attI1*) within the integron. Fig. 1 includes the *aadA2* gene, encoding aminoglycoside resistance, as one example. Also present on the 5'-CS are promoters required for expression of all downstream gene cassettes. The 3'-CS carries several open reading frames (ORFs), including a defective gene encoding resistance to ethidium bromide and quaternary ammonium compounds (*qacEΔ1*) and another encoding resistance to sulfonamide (*sul1*).

Cassette-associated genes are very widespread, which suggests that they play a major role in the development of antimicrobial resistance on a global level. Gene cassettes are known to have been original constituents of the first resistance plasmids reported in the mid-1960s (Bennett, 1999). Gene cassettes have been isolated from a strain of *Vibrio metschnikovii* cultured in 1888, demonstrating their predating the antibiotic era (Mazel et al., 1998). These mobile elements or gene cassettes differ from transposons in a number of ways—firstly, they do not include any genes encoding their transfer functions to a new location; secondly, cassettes are not flanked by inverted repeat sequences and thirdly, mobilization involves a conservative site-specific recombination event rather than transposition (Recchia and Hall, 1995). In terms of molecular organization and compared with, for example, conjugative transposons, integrative phages and integrating plasmids, gene cassettes are simpler in structure. Gene cassettes include only a single gene, thus differing from conjugative transposons, integrating bacteriophages (which encode both bacteriophage proteins and replication functions) and integrating plasmids. The integron is able to incorporate an antibiotic resistance gene either chromosomally or extrachromosomally. Gene cassettes may exist as free, circular, non-replicating DNA molecules when moving from one genetic locus to another but are normally found as linear sequences that constitute part of the larger DNA molecule.

The genetic elements described above should not be considered in isolation from one another. A review paper by Guédon et al. (2017) describes mechanisms by which integrative genetic elements, that may contain integrons, encode their own excision and integration and are able to hijack or subvert the mating apparatus of conjugative elements (plasmids or transposons) to promote their intercellular transfer.

Taking a closer look for levels of integration of MGE with one another, Fig. 2 (Che et al., 2019) shows the results of combining Oxford Nanopore and Illumina metagenomics sequencing to uncover the antimicrobial resistance gene context of influent, activated sludge, and effluent of three wastewater treatment plants when seeking to identify the hosts of these genes. The results showed that most of the genes detected in all three compartments of the treatment plants were actually carried by plasmids.

Considerations when it comes to the detection of MGE in clinically relevant bacteria.

Case studies are provided as illustrations throughout the next section of the chapter. The first case study shows how bacteriophages can convert different serotypes of *E. coli* to become enterohemorrhagic strains and that the extent of the diversity of serotypes that

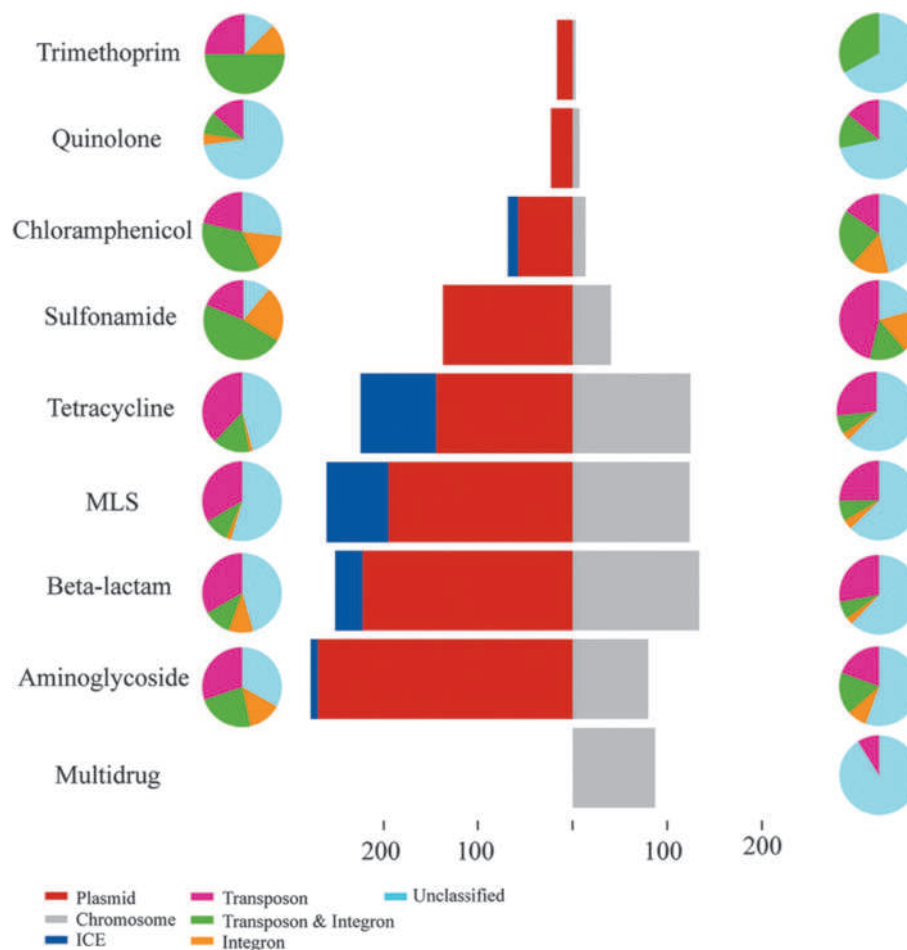


Fig. 2 Genetic location of antibiotic resistance genes predicted from nine Nanopore metagenomic datasets of environmental samples, whereby distribution and abundance of antibiotic resistance genes in transposons and integrons located on plasmids is shown on the left and in transposons and integrons on chromosomes on the right. MLS, Macrolide-lincosamide-streptogramin; ICE, Integrated and conjugative elements. From Che Y, Xia Y, Liu L et al. (2019) Mobile antibiotic resistome in wastewater treatment plants revealed by nanopore metagenomic sequencing. *Microbiome* 7(1): 44.

have been found to cause disease has only become apparent with the advent of molecular methods in clinical microbiology laboratories. However, a considerable follow-up investigation still follows that initial molecular detection. This is important for epidemiological purposes to determine, for example the extent of an outbreak but also for a reason shown in case study 2.

This second case study discusses the challenges of using selective culture methods to grow an elusive and rare pathogen whereby the replacement of primary culture with primary molecular detection of a plasmid-borne virulence factor is very helpful. However, recombination events may also occur and the notion of what constitutes a pathogen deserves more study than the author demonstrated in the isolate being described in this example!

Case study 3 demonstrates how a mutation in the cryptic plasmid of *Chlamydia trachomatis* (a pathogen which causes the most common sexually transmitted infection in humans) caused a large proportion of false negatives when it occurred, perhaps suggesting that commercial manufacturers of diagnostic kits should target more than one locus simultaneously in their assays. The particular difficulty with generating false negatives for *C. trachomatis* is that the infection is often silent while its morbidity can be severe.

Case study 4 shows how the acquisition by Vancomycin Resistant Enterococci (VRE) of a clonal complex enhances a strain's survivability and that plasmids and transposons, extrachromosomal and chromosomal locations have all been identified as the means to harbor this advantageous addition. In a collection of otherwise quite diverse VRE strains, all had acquired clonal complex 17 in a study in Ireland, where VRE is a particular problem in hospital patients.

Case study 5 shows how epidemiological typing using (bacterio)phages may show strains of the same serotype of salmonella as being indistinguishable from one another but that the presence of integrons in these strains can provide varying numbers and types of resistance genes, suggesting that definitive phage typing is not synonymous with a defined antimicrobial resistance profile.

The final case study is chosen to illustrate the diversity of bacteria in which a particular resistance determinant—in this case TetO, which is just one mediator of resistance to tetracycline has been found in at least 11 separate genera of bacteria, both Gram-positive and Gram-negative. The transmission of this resistance is through MGE.

Case study 1: Verotoxigenic *Escherichia coli*

The genetic element-borne virulence factors may be a more suitable target for detection of the pathogen than conventional culture. The following case study illustrates how national reporting of a pathogen was affected by the adoption of a molecular mode of detection becoming widespread among diagnostic laboratories.

Verotoxigenic *Escherichia coli* (VTEC, also called Shiga-like toxigenic *E. coli* or STEC) can cause diarrhea, hemorrhagic colitis and hemolytic uremic syndrome in humans (Page and Liles, 2013), the latter being a very serious complication. The primary reservoir of VTEC is ruminants, and sporadic outbreaks are commonly associated with animal contact, exposure to animal feces, food and water contamination, and person-to-person transmission (Snedeker et al., 2009).

The key virulence factors in VTEC are stx1 and stx2 toxins (one or both may be present in a strain of VTEC, stx2 being the more commonly detected in symptomatic patients) which are borne on temperate lambdoid phages. These phages are heterogeneous, having different DNA structures and Pulsed-Field Gel Electrophoresis (PFGE) types (García-Aljaro et al., 2009). Induction to the lytic cycle results in liberated virions causing transduction of stx genes to susceptible bacteria and thus can lead to the emergence of new VTEC pathogens by horizontal gene transfer (Allison, 2007). A study by Rahman et al. (2018) of 47 VTEC O:157 strains from food and animal sources found that 10.6% of the strains produced complete phage particles capable of infecting multiple bacterial hosts.

Originally characterized in the 1980s in *E. coli* serotype O:157, the focus to detect VTEC was based on isolation and serotyping suspect colonies from feces samples from patients having symptoms and signs indicative of VTEC intoxication. This approach was labor-intensive, particularly as *E. coli* is normally found in the gut and mixed cultures were to be expected. It was discovered subsequently that the large majority of *E. coli* O:157 strains were non-fermenters of mannitol, so a selective formulation of MacConkey agar was developed that incorporated mannitol in place of lactose (and then was added tellurite and the antibiotic cefixime to add further selectivity), whereby the scientist was then looking out for pale colonies to investigate further using anti-O:157 antiserum and biochemical identification. This imperfect approach led to only selecting for VTEC that were non-sorbitol fermenting and of one serotype, also necessitating sending suspect VTEC to a reference laboratory for toxin studies. Some improvements to detection were introduced by using chromogenic agar, which generated colored colonies for different serotypes (including O:157), with varying sensitivity rates. There was the possibility of sending feces samples directly to the reference laboratory but this introduced a delay in diagnosis, particularly if the reference laboratory happened to be in a different part of the country from the investigating laboratory.

With the advent of molecular detection methods detection based on the primary detection of toxins stx1 and stx2, notifications began to rise. Fig. 3 shows a survey conducted in Ireland in 2015 (Rice et al., 2016), where the adoption of molecular methods by eight large diagnostic laboratories (using a commercial molecular assay) resulted in a dramatic increase in total (human) VTEC notifications. The O:157 detections did not vary markedly during the years studied (2010–14), which is not surprising, given that the culture-based efforts had been previously concentrated mainly on finding that serotype but there was a dramatic increase in VTEC of serotypes other than O:157, which was shown to be consistent with adopting the molecular detection approach.

In this case, the approach of making the detection of the stx genes direct from feces allows the negative majority of samples to be reported quickly as NOT DETECTED and the initial positive indication for VTEC to be notified quickly as a suspected positive result, followed up by a full laboratory investigation. In our laboratory this approach shortened the time to reporting a negative result by up to 96 h and provided a means for a same-day reporting of either a negative or a provisional positive result.

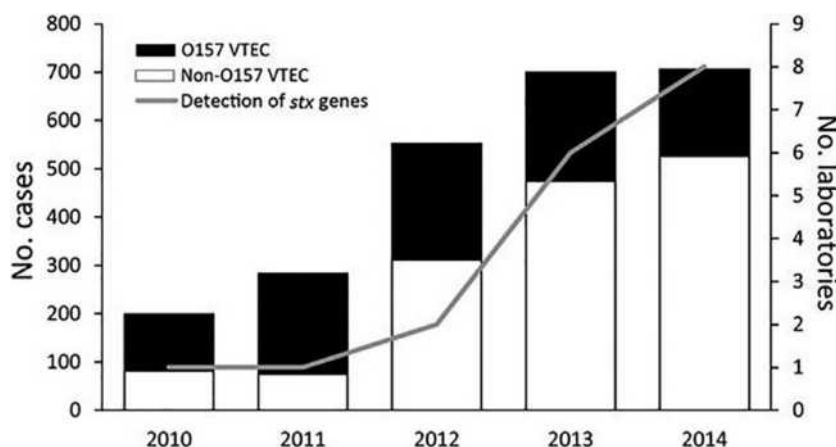


Fig. 3 Effect on VTEC notifications in Ireland of a gradual conversion by eight large clinical laboratories from a culture/serotyping-based Verotoxigenic *E. coli* O157 detection method from human stools to a molecular-based stx toxin gene detection method. From Rice T, Quinn N, Sleator RD and Lucey B (2016) Changing diagnostic methods and increased detection of Verotoxigenic *Escherichia coli*, Ireland. *Emerging Infectious Diseases* 9: 1656–1657.

Case study 2: Abandoning primary culture in favor of the molecular detection of a virulence factor borne on a plasmid of a human-to-human transmitted pathogen that is difficult to detect on culture

Shigella spp. and the plasmid-borne *ipaH* genes

The genus *Shigella* spp. is differentiated into four species: *S. dysenteriae*; *S. flexneri*; *S. boydii* and *S. sonnei*. All are pathogenic to man, causing bacillary dysentery of varying severity and all have Invasive plasmid antigen H (*ipaH*) genes, both on their large plasmid and integrated to the chromosome. Conventional culture-based detection is challenging as shigella can be difficult to isolate from the other gut bacterial flora present, being often present in small numbers in the stool sample of the infected patient (the infectious dose is also very low). The molecular detection of a conserved *ipaH* sequence has revolutionized the detection of shigella infection, whereby a positive PCR signal signifies the presence of *Shigella* spp. and follow-up work can be conducted to identify the organism to species level and serotype (by culture or molecular methods). It is worth noting that enteroinvasive *E. coli* also carries the *ipaH* gene(s). EIEC are strains that possess some of the biochemical characteristics of *E. coli* and have the ability to cause dysentery using the same method of invasion as shigella does (van den Beld and Reubsaet, 2012). Sequencing of multiple housekeeping genes indicates that EIEC is more related to shigella than to non-invasive *E. coli* (van den Beld and Reubsaet, 2012). In this case the detection of the pathogen is more important in the symptomatic patient and the decision as to whether EIEC and shigella should be reclassified has not been made.

As an addendum to this case study, when the author was testing the stool sample of a patient who had returned from a holiday in India and who needed hospitalization because of having severe dysentery, a positive *ipaH* signal indicated the presence of *Shigella* spp. Retrospective and multiple attempts to isolate shigella on the appropriate enteric culture media failed to yield a shigella isolate but a large mucoid colony was always present on every culture, which was identified as a *Klebsiella pneumoniae*. A colony *ipaH* PCR designed to produce a 600 bp product gave a positive *ipaH* signal and sequence analysis of the product showed a hybrid of the *ipaH* target flanking an internal sequence that showed complete homology to *Klebsiella pneumoniae*, suggesting that a recombination event had occurred. Whether this isolate was capable of causing the dysentery in the patient could not be concluded but no other bacterial, viral or parasitic pathogen was found, despite intensive effort.

Case study 3: Detection of chlamydia trachomatis urogenital infection via its cryptic plasmid—A mutation causing false negative test results

Chlamydia trachomatis (*C. trachomatis*) is the most common bacterial cause of sexually transmitted infection worldwide (Jones et al., 2020). This pathogen is an obligate intracellular bacterium, which often causes asymptomatic infection, may cause pelvic inflammatory disease (PID), ectopic pregnancies, scarring of the fallopian tubes, miscarriage, and infertility when left untreated (Vasilevsky et al., 2014). More than 35 years ago, a 7.5 kb cryptic plasmid was described for chlamydia (Lovett et al., 1980). Despite the evidence for significant contributions by the plasmid to chlamydial pathogenicity, there is also strong evidence that the chlamydial pathogenicity may also depend on factors that are unrelated to the plasmid (Zhong, 2017).

The plasmid is less important for chlamydial growth in vitro than during infection in vivo, suggesting that chlamydia may have acquired the plasmid for promoting its survival in animals, implicating a plasmid-dependent pathogenicity (Zhong, 2017). Nucleic Acid Amplification Tests (NAATs) are the method of choice for diagnosis of chlamydia urogenital infection (usually PCR-based) and these are targeted on the detection of the cryptic plasmid. NAATs are currently the most sensitive tests to detect chlamydia. These tests also have a high specificity comparable to culture, but in contrast to culture, do not depend on viable pathogens, facilitating a slower specimen transport without loss of result quality where this is necessary (Meyer, 2016). However, in 2006 a new variant of *C. trachomatis* with a deletion in the cryptic plasmid was detected in Sweden, following an unexpected 25% decrease in *C. trachomatis* infections that was noted between November 2005 and August 2006 in southwest Sweden (Ripa and Nilsson, 2006). These investigators sequenced part of the plasmid from the variant strain and found a deletion of 377 base pairs in the target area for the *C. trachomatis* NAAT tests manufactured by two prominent companies; a third commercial company's assay, targeting a different sequence, was unaffected. This necessitated an urgent review by other countries of their detection methods for chlamydia and by the commercial companies who needed to change the target sequences that formed the basis for their assays.

Case study 4: Vancomycin resistant enterococci and clonal complex 17 (CC-17)

Enterococci are common commensals of the enteric tract. However, the development of resistance to glycopeptide antibiotics, including vancomycin, among *E. faecium* and *E. faecalis* has been a major concern, not least because this drug is one of few that can be used to treat Methicillin Resistant *Staphylococcus aureus* (MRSA) infection and because of the possibility of transfer via MGE among these genera which often share the same environments.

Vancomycin Resistant Enterococci (VRE) are prevalent among hospital patients and are among the most frequent causes of healthcare associated infection. One study of carriage among hospital patients in Ireland (Whelton et al., 2016) showed a prevalence rate of 34% for VRE (all *E. faecium*) in an acute hospital, all containing the *VanA* gene with a subset also containing the *VanB2/3* gene encoding vancomycin resistance. Interestingly, although epidemiological fingerprinting showed high levels of diversity, all isolates showed evidence of having acquired CC-17. This complex has evolved in stepwise fashion through genetic diversification, including mutations, but primarily through recombination, whereby this primary ancestor evolved into a meroclone of highly related genotypes, which has spread globally (Leavis et al., 2006). The complex was previously described as a lineage characterized by (1) ampicillin resistance, (2) a pathogenicity island, and (3) an association with hospital outbreaks (Willems et al., 2005). The heterogeneity of the types in the Irish study (Whelton et al., 2016) suggests an environmental pressure on VRE rather

than an outbreak or distribution of a single strain, whereupon the acquisition of the CC-17 provides a survivability advantage. Insertion sequence 16 (IS-16), which has been found to be synonymous with the presence of CC-17 and which may be found on the chromosome, on a plasmid or in both locations in the same isolate (Werner et al., 2011) provides a useful marker to detect CC-17. CC-17 may provide higher levels of genetic variability including easier acquisition of MGE that may support lifestyle in new or changing environments or ecological niches such as the hospital environment (Leavis et al., 2017). Also present in CC-17-identified strains are conjugative and non-conjugative transposons (Werner et al., 2011). Incidentally, the persistence of VRE on the dry (unenriched) material of a bed drape has been shown to be longer than 8 weeks, as evidenced by its recovery after the addition of broth to the previously inoculated material (unpublished study).

Case study 5: An example of multiple simultaneous antibiotic resistance mechanism transfer via integrons demonstrated by *Salmonella enterica* typhimurium definitive phage type 104 (DT104)

Salmonella spp. as members of the Enterobacterales family are part of the normal gut-dwelling microflora of many animals, including birds, amphibians, reptiles, wild mammals, typically showing no obvious signs of causing disease. However, salmonella carriage or infection among humans (which can be a zoonosis but human-to-human transmission also occurs; salmonella can multiply on food) or domestic animals is a major cause of concern, necessitating epidemiological tracing and salmonella eradication measures. Human infection carries a high degree of morbidity and occasional mortality, rates of both of which vary depending on the state of the host and the strain of infecting salmonella.

Laboratory detection methods for *S. enterica*

The clinical laboratory either detects salmonella from food, water or feces samples using conventional culture (agar)-based methods or through the PCR-based molecular detection of salmonella, using one or more genus-specific genes. The identification of salmonella runs to being able to differentiate the serovar (serotype, based on a combination of somatic and flagellar antigens) of which there are currently more than 2700 serovars identified.

In the case of an isolate of *S. enterica* (serovar Typhimurium), later epidemiologically characterized as DT104, an isolate was simultaneously resistant to ampicillin, chloramphenicol, streptomycin and spectinomycin, sulfonamide and tetracycline, representing five different classes of antibiotic.

A study published from Denmark by Sandvang et al. (1998) of *S. enterica* Typhimurium DT104 showed that from eight different porcine herds, eight DT104 isolates gave a variety of resistance profiles—five showed the pattern described above, two isolates were susceptible to all of these antibiotics and the remaining isolate was resistance to streptomycin, spectinomycin and sulfonamide. A second epidemiological typing method used to complement the phage typing, Pulsed-Field Gel Electrophoresis (PFGE) also showed the isolates as indistinguishable, even though they were clearly not identical on the basis of their antimicrobial resistance profiles.

Therefore, it is evident from this case study that even discriminatory epidemiological typing methods may identify a collection of strains of the same species as the same type, but these may differ on the basis of genes acquired through horizontal transmission, depending on selective pressures and opportunity for environmental (or gut-based) transfer of genetic information.

Case study 6: Persistence or increased prevalence of antimicrobial resistance determinants over time

Campylobacter spp. are the most common bacterial cause of gastroenteritis and campylobacter infection in humans is often associated with consumption of poultry, which is commonly contaminated by campylobacter as an uncooked or undercooked product. Campylobacter is part of the normal microbiological flora of the gastrointestinal tract in wild birds.

Tetracycline is an old antibiotic, relatively inexpensive and broad-spectrum. Tetracycline resistance in *Campylobacter* spp. is largely conferred by a ribosomal protection protein (RPP), TetO, capable of displacing tetracycline from its primary binding site on the 30S ribosomal subunit (Connell et al., 2003; Wilson, 2014). Campylobacter tetO can be located chromosomally but is often plasmid-mediated (Connell et al., 2003). Tetracycline RPPs are widely distributed among bacterial genera and it has been reported that the *tetO* gene exists in at least eleven bacterial genera, including four Gram-negative and seven Gram-positive genera (Roberts, 2005). The conserved acquisition of *tetO* between members of different bacterial genera indicates that conjugative plasmids, transposons, or recombination events contribute to the dissemination and maintenance of the *tetO* gene (Friis et al., 2007). The maintenance of tetracycline resistance may be mediated by some other plasmid-encoded factor, conferring a survivalist advantage (Lynch et al., 2020a). A study in Ireland of poultry campylobacter isolates (2017/2018) found that 38% of *C. jejuni* isolates were resistant to tetracycline (Lynch et al., 2020b), up from 24% reported from the year 2000 (Lucey et al., 2002). Tetracycline was banned as growth-promoter for feed animals in the 1970s (Council of the European Union, 1970) but remains the most commonly used antibiotic overall, however (Przeniosło-Siwczyńska et al., 2020). Furthermore, tetracyclines are in the main excreted into the environment unchanged after being given as a dosage (Vojtová and Urbánek, 2009). Overuse and environmental contamination with this agent undoubtedly contribute to the continuing increase in resistance rates.

Conclusion

The selected case studies above illustrate how the increasingly molecular approach to clinical laboratory-based diagnosis of bacterial disease has improved the science, while still often needing a blended approach of using phenotypic and genotypic methods. We are only beginning to understand the extent of transfer of genetic material among bacteria, and the facility provided by MGE for the

transfer of virulence factors or antimicrobial resistance genes. Being able to begin with a molecular detection and following up with retrospective culture after a positive (molecular) detection has provided greater opportunity for detection of pathogens, which in some cases might be defined by the acquisition of the mobile element rather than the species name of the bacterium.

The selective pressure on bacteria to acquire resistance to commercial antimicrobial agents is driven by overuse of the limited numbers of different antimicrobials currently available and there are enhanced opportunities provided for transfer in environments of great bacterial diversity and density, for example in the gut.

The detection of genomic islands poses a particular problem for the clinical laboratory as conventional investigations and nomenclature cannot differentiate strains of the same species unless a particular transmissible trait is being determined, such as antimicrobial resistance or a specific virulence factor.

Conventional clinical detection methods of bacterial pathogens do not currently select for MGE except in rare cases. As the use of molecular methods in diagnostic microbiology laboratories becomes more mainstream and diverse, the opportunity for a broader understanding of bacterial threats to human health presents. In an ideal world, future considerations might include a more routine examination of samples for key MGE and having the means to disable them selectively.

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Antibiotic Resistance Diagnostic Methods for Pathogenic Bacteria

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Introduction

The emergence of novel bacterial strains harboring resistance to antibiotics is considered as one of the most public health problems in the 21st century. Currently, several pathogenic bacteria have become MDR to antibiotics recommended by World Health Organization (WHO; www.who.int), Clinical and Laboratory Standards Institute (CLSI; www.clsi.org) and European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org). AMR of pathogenic bacteria can lead to other infections than the initial infection contracted in hospital provoking high mortality rates due to the dissemination of ESKAPE pathogens (Santajit and Indrawattana, 2016). This group of bacteria including *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species are responsible of nosocomial infections and are characterized by high drug resistance mechanisms (Rice, 2010) due to exposure to strong selective pressures, and the indiscriminate use of antibiotics (Torres-Caycedo et al., 2019). Antibiotic resistance exhibited by bacteria can be intrinsic, acquired, or adaptive (Lee, 2019). Acquired resistance mechanism may be linked to a modification of the bacteria at the genome level by either a mutation or the acquisition of new genetic material from an exogenous source (Christaki et al., 2020). Resistance mechanisms have evolved

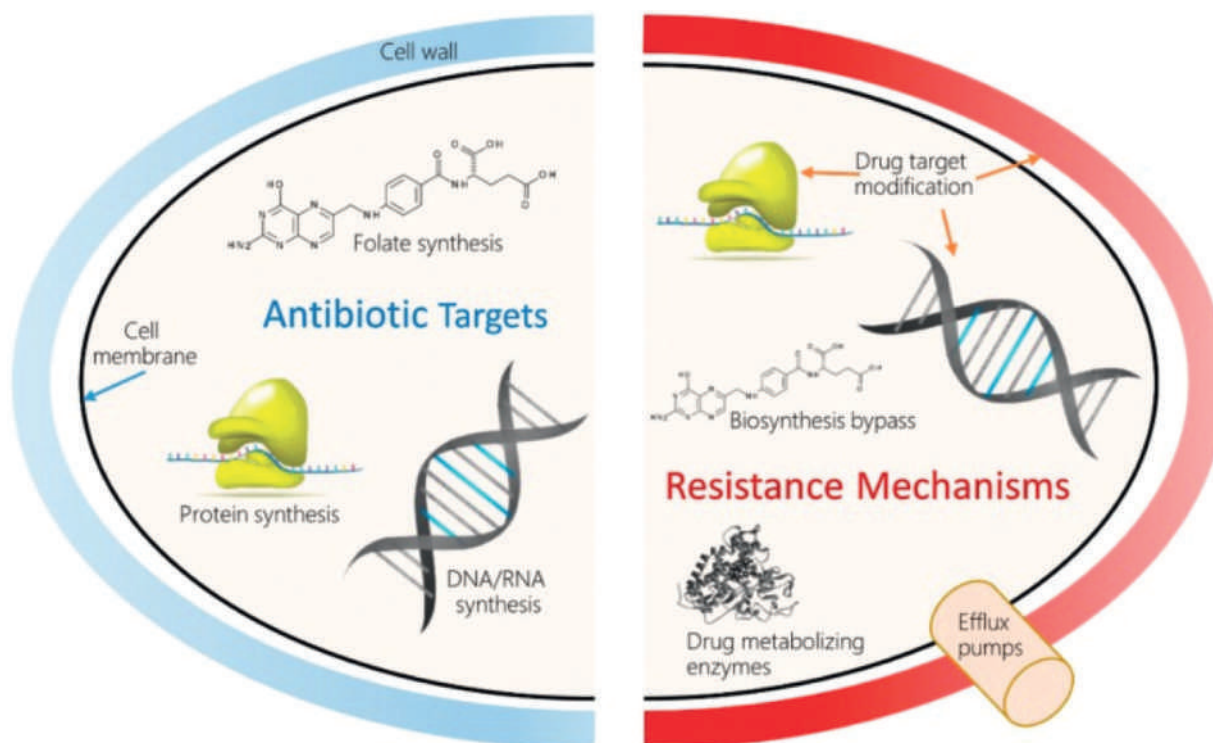


Fig. 1 Antibiotic resistance (AR) molecular mechanisms at right: production of drug-inactivating enzymes, target modification, efflux pump action, and bypassing the affected metabolic pathway. Antimicrobial drug targets at left: peptidoglycan synthesis inhibition, cell membrane disruption, DNA replication gene transcription and translation inhibition, and folate biosynthesis. Reprinted from Van Camp PJ, Haslam DB and Porollo A (2020) Bioinformatics approaches to the understanding of molecular mechanisms in antimicrobial resistance. *International Journal of Molecular Sciences* **21**(4): 1363. <https://doi.org/10.3390/ijms21041363>, under a Creative Commons Attribution 4.0 International License (CC BY).

from an acquired AMR gene may be present on the bacterial chromosome, plasmid or transposons carrying small genetic elements “cassettes” (Giedraitienė et al., 2011). The mechanism of action of numerous AMR genes or cellular pathways are already known (Van Camp et al., 2020) (Fig. 1).

In literature many definitions were used to characterize the antibiotics resistance patterns of bacterial strains. MDR was defined as bacteria acquired non susceptibility to at least one antimicrobial agent in three or more antimicrobial classes (Magiorakos et al., 2012; Sweeney et al., 2018). XDR was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial classes (i.e., bacterial isolates remain susceptible to only one or two antimicrobial categories) (Magiorakos et al., 2012). Major emergent and widespread AMR are β -lactamase, carbapenem-resistance, rapidly developed to extended-spectrum (ESBL) in Gram negative bacilli, resistance to vancomycin of enterococci (ERV); *S. aureus* resistance to methicillin (MRSA) (Christaki et al., 2020). Other antibiotic resistances constitute a real concern, such as resistance to fluoroquinolones, rifampicin and isoniazid by *Mycobacterium tuberculosis*, *Helicobacter pylori* clarithromycin-resistant and *Campylobacter* spp. fluoroquinolone-resistant (Dookie et al., 2018; Savoldi et al., 2018; Marroki and Bousmaha Marroki, 2019; Sack et al., 2001). In 2017, WHO published a list of bacteria for which the development of new antibiotics has become vital. The list has included the 12 families of bacteria most threatening to human health. In 2014, the data collected by WHO, showing the extent of large gaps in surveillance of this problem in several countries of the world. Furthermore, the same organization estimate more than 700,000 people die every year by infection occasioned by antibiotic resistant bacteria (Vasala et al., 2020), and more than 23,000 and 25,000 people dying as a result of infectious disease in United States and in Europe every year respectively (European Centre for Disease Prevention and Control and European Medicines Agency, 2009; Prestinaci et al., 2015). The European Centre for Disease Prevention and Control/European Medicines Agency (ECDC/EMA) estimates the cost of AMR at least EUR 1.5 billion/year. Indeed, there are several challenges in diagnostic and treatment of infectious diseases due to the epidemic bacterial antibiotic resistance. By the fact of the complexity of antibiotic resistance/susceptibility in vitro methods have been developed to predict bacterial response and achieve the appropriate therapy in vivo. Fast point of care analysis, etiological agent identification, an efficient antibiotic research and a determination of the functional dosage, would be required for an optimal antimicrobial therapy policy (Vasala et al., 2020). For diagnostics of antibiotic resistance, in vitro activity of antimicrobial agents is evaluated by AST phenotypic methods based on inhibition zone measurements or by minimum inhibitory concentration (MICs) determination. The most commonly used in clinical microbiology laboratory include diffusion, gradient, and dilution (broth and agar dilution) tests, rapid automated or commercially instrument systems and devices, MALDI-TOF MS, Single-Cell and microfluidics based methods.

While multiples standardized phenotypic methods have advanced over many decades, various non-phenotypic methods, using DNA probes and nucleic acid amplification, to detect AMR genes and mutations associated with resistance phenotypes (Tenover, 2009) have recently been developed. In a number of cases where the genotype-phenotype relationship is uncomplicated, gene based prediction has been applied in clinical practice through the use of rapid molecular tests (Van Camp et al., 2020). Molecular AMR methods are efficient direct tools, in which bacterial cultures steps, long incubation, contamination risk, and the dispersion of deadly infection are eradicated (Khan et al., 2019). Various molecular tests are available commercially to detect resistance-coding genes or resistance-associated mutations in DNA extracted from purified bacterial isolates or directly from clinical samples, for both clinical and surveillance purposes (World Health Organization (WHO), 2019). Molecular AMR detection tries to identify resistance gene, as well as mutations in and expression of these genes or their genomic signature (van Belkum et al., 2020). Depending on the mechanism of detection, four major molecular categories are commonly used: (i) Amplification-based methods; (ii) Hybridization-based methods; (iii) Immunoassays, to detect AMR gene and (iv) Whole Genome Sequencing. The chapter describes the several methods developed for diagnostic of antimicrobial resistance of pathogenic bacteria, used in clinical settings and even in point-of-care (POC). Major phenotypic AST methods, automated AST tools, MALDI-TOF MS, Single-Cell methods, microfluidic devices and principal molecular techniques used to determinate or screening accurately bacterial antibiotic susceptibility and to detect AMR are concisely discussed.

Phenotypic method

Diffusion methods

Agar disk diffusion test (DDT)

Developed in 1940 by Heatley (1944), this technique is considered as the most commonly and the oldest used for routine susceptibility testing of bacteria and fungi in microbiology laboratories. Briefly, this method standardized the variables of disk size, media, the inoculum size, temperature and time of incubation. A few colonies of organism are selected and picked from culture plate and suspended into 2 mL of sterile saline or trypticase soy broth and vortex. The density was adjusted to achieve 0.5 McFarland standard prepared through a turbidity test (Jorgensen and Ferraro, 2009). Inoculation was conducted using a sterile cotton swab by streaking the dried surface of agar plate. Petri dishes are used containing Mueller-Hinton agar and may be placed in room temperature until dry for 30 min. Then, each tested disk, with fixed concentration of antibiotics, is placed (~12 disks placed/plate) on the agar surface and gently pressed with forceps to permit the complete contact with the inoculated agar plate. For microorganism growth the plates are incubated at 35 °C for 16–18 h with simultaneous antibiotics diffusion into the agar. The inhibition diameters zones for tested microorganisms formed around each disk were measured by using a ruler or the callipers. The result for each drug obtained, after an overnight incubation, are interpreted according the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI, 2017) or European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2020). The result may be unregistered qualitatively by classifying the strains as susceptibility, intermediate or resistant.

Emergent paper methods

Deiss et al. (2014) developed a novel method to study the antimicrobial susceptibility assays by using paper-based portable culture devices. In this method, two antibiotics are tested in the paper-based portable culture devices to measure the antibiotic susceptibility of *Escherichia coli* and *Salmonella typhimurium* by measuring blue-colored zones of inhibited growth. AST can be also evaluated by “Microfluidic hip paper supported cell culture arrays” proposed by Xu et al. (2016). In this method the AST assays cell culture arrays and the concentration of antimicrobial agents able to inhibited the chromogenic reaction in the media by combining were combined. The result to monitor the chromogenic reaction by using a camera was realized in 15 h. Compared to the conventional Kirby-Bauer test, the results shown a similarity of 94.1% with many advantages for uropathogen testing.

Another technique using a paper-based microfluidic devices has been introduced in Martínez et al. (2007) for POC diagnostic applications. This alternative method was low-cost, rapid devise and early screening of infections. The method developed by Boehle et al. (2017), utilizing paper-based devices for detection the antimicrobial resistant (AMR) bacteria, aimed to test the presence the β -lactamase mediated resistance in environmental *E. coli* isolates. The detection of the β -lactamase expression in various strains tested demonstrated the performance and applicability of paper-based devise assay and can be used as practical alternative methods for detecting AMR to the traditional methods. Recently, in study piloted by He et al. (2020a,b,c), the use of laser-pattern paper-based microfluidic device for assessing susceptibility of *E. coli* to different antibiotics, via a simple visually observable color change, were developed (Fig. 2). Four antibiotics commonly used for treat bacterial UTIs such as *E. coli* were tested including amoxicillin, ciprofloxacin, gentamicin and nitrofurantoin. The result observed indicate the suitability of paper devise for detection of bacterial infection in the POC and as novel strategy for antibiotic prescribing.

Dilution methods

The dilution tests are considered as an in vitro standardized methods for the AST. The two approaches types generally used in dilution methods are microdilution and macrodilution (EUCAST, 2019). These methods are usually used in clinical microbiology laboratories to determine the MICs of antibiotics for several bacteria, as well as yeasts and fungus (Balouiri et al., 2016).

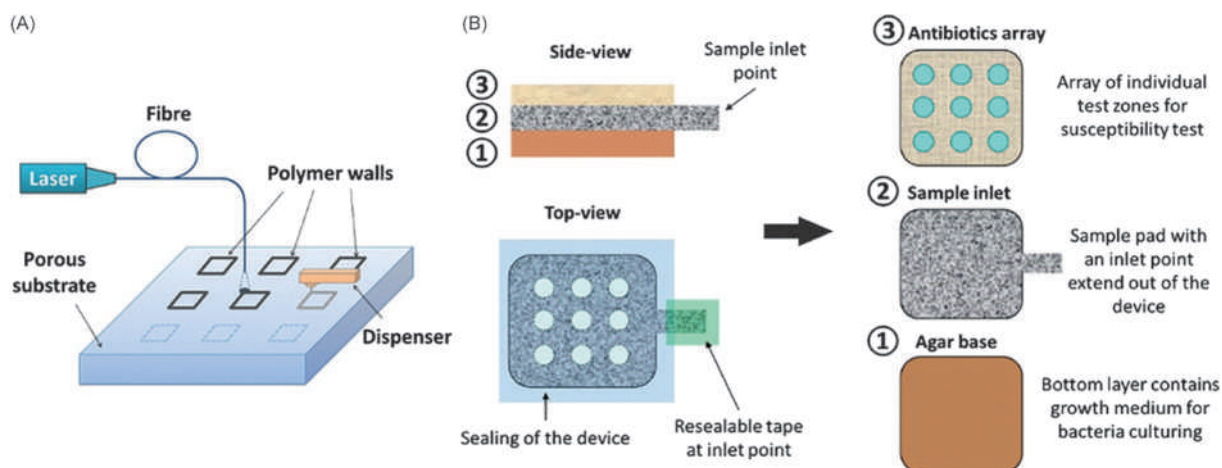


Fig. 2 Schematic representation of the laser-based direct-write (A) Device setup (B) Description of fabricated three-layer paper device for bacteria identification and antibiotic-resistance testing. Reprinted from He P, Katis IN, Kumar A, Bryant CA, Keevil CW, Somani BK, Mahobia N, Eason RW and Sonnes CL (2020) Laser-patterned paper-based sensors for rapid point-of-care detection and antibiotic-resistance testing of bacterial infections. *Biosensors & Bioelectronics* 152: 112008. <https://doi.org/10.1016/j.bios.2020.112008>, Copyright © 2020, with permission from Elsevier.

Broth dilution method

Broth dilution testing is one of the common methods used for AST, including macrobroth and microbroth test for determination of the MICs. The procedure elaborated for this test, involve serial prepared two-fold dilutions of antibiotic agent. Each dilution is inoculated with suspension standardized of the tested bacteria adjusted to 0.5 McFarland turbidity scale, to achieve a final concentration of the inoculum of 5×10^5 CFU mL⁻¹ or 5×10^4 CFU/well (Brown-Elliott et al., 2012; Brook et al., 2013; Balouiri et al., 2016). A volume of 0.2 mL is deposited in each well and it allows testing of antibiotics tested over a range of eight- to twofold dilutions on microtiter plate. Moreover, two antibiotic-free wells without antibiotic must be included in each microtiter plate as controls (Wiegand et al., 2008; ISO-20776-1, 2019). After well-mixing, the microtiter plate is incubate in appropriate temperature, the bacterial growth is examined visually or by automated system to determinate the MICs values. The MICs is designated as the lowest concentration of antibiotics inhibits growth bacteria. Since the several advantage of microdilution assay, this method is considered the reference standard method to determinate the MICs (Jorgensen and Ferraro, 2009). The method is low cost and small volumes of inoculum are used as well as the evaluation of multiple antibiotic agents with individual isolates. One disadvantage for broth macrodilution is the time consuming, occupation of a large amount of space and reagents and error in preparation of dilution of antibiotics agents.

Agar dilution method

Agar-dilution method is currently the reference standard susceptibility method for anaerobic bacteria. The test is performed in Petri dish to determine the MICs to multiple tested strains in single plate. Through this assay, a various antibiotics agents are prepared usually at twofold dilution series and incorporated into molten agar medium plates in different concentrations. Then inoculation of the medium with a standardized suspension adjusted to 0.5 McFarland standard containing a concentration of 5×10^8 CFU mL⁻¹ (CLSI, 2012a) was conducted. The bacteria are diluted to around 10^7 CFU mL⁻¹, and 2 μ L are spotted with multi-point into agar plates contain approximately 10^4 CFU/spot (Clinical and Laboratory Standards Institute, 2015), and one agar plate used as control seeded without antibacterial agent. After incubation in anaerobic condition at 35 °C for 16–18 h for non-fastidious bacteria and for 18–24 h fastidious organisms (Barry, 1986), the agar plates are examined by visually comparing the growth. The MICs is determined based on this test as the lowest concentration of drug tested that stopped the growth of bacteria (CLSI, 2012b; Brook et al., 2013). Currently, the agar-dilution technique is considered as the reference susceptibility testing recommended by CLSI for fastidious bacteria such as *Helicobacter* spp. and *Campylobacter* (CLSI, 2010; CLSI, 2017) and anaerobic bacteria (CLSI, 2012b), and for evaluate the antifungal susceptibility (Benkova et al., 2020). In the study conducted by Liu et al. (2016a,b), disk-diffusion and agar dilution methods used for *Neisseria gonorrhoeae* AST were compared. The result obtained demonstrated a good correlation and level categorical agreements has been obtained between the agar diffusion test and standard agar dilution method. Similar conclusion obtained by Luangtongkum et al. (2007) by comparison of agar dilution and the agar disk diffusion test applied for *Campylobacter*. The advantage of agar dilution method is its suitability when few antibacterial agents are tested against a large number of microorganisms (Barry, 1986; Liu et al., 2016a,b). One drawback of agar dilution method is time-consuming and high degree of inherent error.

Gradient tests

E-test method

E-test was a new approach for quantitative antibacterial susceptibility developed in the late 1980s by Bolmström and Eriksson (1988). The method is a simple and routine used to determine in vitro the MICs on agar medium. The antimicrobial gradient

method incorporates the principle of disk diffusion and agar dilution tests. The E-test is based on the diffusion of an antibiotic agent from a plastic strip onto an agar media to provides quantitatively rapid and reproducible MICs results for tested isolates (Baquero et al., 1992). Briefly, the surface of agar plate was swabbed with the adjusted inoculum suspensions ($1-2 \times 10^8$ CFU mL⁻¹) equivalent to a 0.5 McFarland (Evangelista and Karlowsky, 2016). Then the back of plastic strips impregnated with different and accumulative of concentration gradient antibiotics agent, corresponding to approximately 20 twofold dilutions are placed in medium surface, after inoculation with tested strain (Gajdacs et al., 2017). Many E-test strips with different antimicrobial agents can be placed in surface on a single plate containing special agar medium and then incubated in various temperature with the microorganism tested. After overnight incubation, an inhibition ellipse is seen, the E-test MICs (mcg mL⁻¹) value are read directly from the upper of the Petri dish at the point of intersection of the growth ellipse and inhibition zone with the extremity of the strip (Fig. 3). This method can be rapidly and successfully applied to test the anaerobes and fastidious bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* (Jorgensen et al., 1991).

In last decade, many studies have been conducted to evaluate the reliability and the performance of E-test method with different standardized techniques for occurrence, disk diffusion and microdilution for testing pathogen and fastidious bacteria frequently used by diagnostic laboratory. These investigations conducted on coryneform bacteria isolated from clinical samples (Martínez-Martínez et al., 1995), *Rhodococcus equi* obtained from tracheobronchial aspirates or post-mortem lung tissue from pneumonic foals (Berghaus et al., 2015), forty-six dermatophytes strains (Castro Méndez et al., 2008), 194 *Enterobacterales* and 77 *Pseudomonas aeruginosa* (Wang et al., 2020) shown a well agreement between E-test and disk diffusion methods or broth microdilution method. Some important advantages of the E-test are rapidity, simply and reliability and does not require a special equipment. The E-test method is applicable to a variety of microorganism and drug, that it offers a quantitative test of MICs result which may be interpreted easily compared to standardized methods. The limitation of E-test is the relatively expensive cost and the limited array of antibiotics.

Spiral gradient endpoint method

The spiral gradient endpoint (SGE) technique is an alternative agar dilution test used for AST of anaerobic Gram negative bacteria developed by Hill and Schalkowsky (1990). In this method, colonies from overnight culture are suspended in broth and adjusted to 0.5 McFarland turbidity, the optimized inoculum is streaked vertical and cross streaks manually or with an automated inoculator through the gradient of antibiotics agents from the center to the edge of the plate. The plates were incubated at 35 °C and the MICs were evaluated by measuring the distance of inhibition from the plate center to the endpoint of growth. In study of Wexler et al. (1996), comparison of this method and reference agar method to evaluate the MICs of anaerobic bacteria was carried out. The result obtained concluded that the SGE is an alternative method to the reference agar dilution test more precise and economic.

Pong et al. 2012, compare the SGE method with the reference standardized method for *H. influenzae*, *S. pneumoniae*, *Moraxella catarrhalis* and *N. gonorrhoeae*. The result obtained shown an excellent correlation of the MICs obtained with the reference methods and concluded that the SGE is a rapid and flexible alternative method for smaller and routine laboratories.

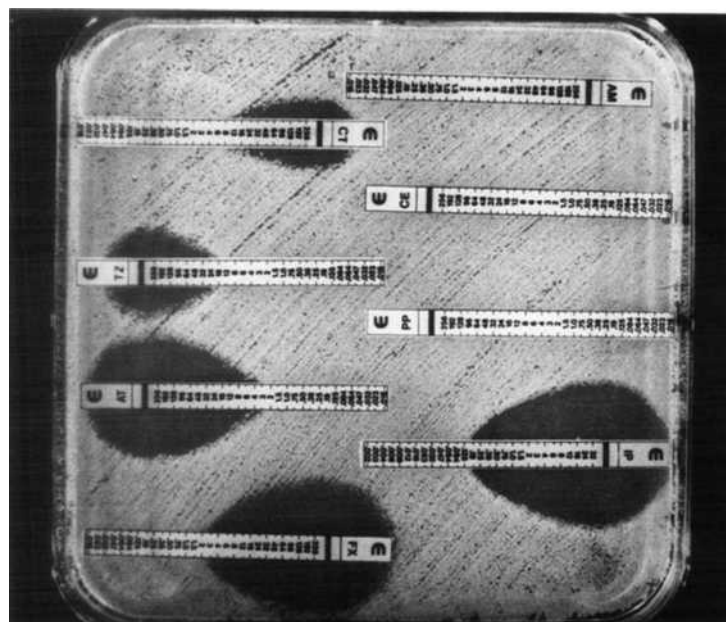


Fig. 3 E-Test use for MICs determination of AM, ampicillin; CE, cephalothin; PP, piperacillin; IP, imipenem; and FX, cefoxitin cefotaxime (CT) and ceftazidime (TZ) against *Escherichia coli* clinical strain harboring SHV-2. Reprinted from Baquero F et al. (1992) The E-test as an epidemiologic tool. *Diagnostic Microbiology and Infectious Disease* 15(5): 483–487. [https://doi.org/10.1016/0732-8893\(92\)90095-b](https://doi.org/10.1016/0732-8893(92)90095-b), Copyright © 1992, with permission from Elsevier.

Automated methods

Vitek 2

The Vitek 2 is one of automated systems developed in 1970s by bioMérieux, commonly used for bacterial identification combined with the AST determination. The Vitek system offers important advantages for the researcher and clinician laboratories with a high reliability of the result. This method has been easily, rapid, reducing hands on time and easy to integrate in laboratory. The VITEK[®] 2 and VITEK 2 compact are equipped with the same software for the validation of antibiotic resistance tests even for new emerging resistance. Systems Vitek 2 uses 64-well plastic reagent cassettes or AST cards containing small or microwells loaded with dehydrated media with different concentration of antibiotics testing (17–20 antimicrobial agents), including one well as control without any antibiotic. The procedure used the Vitek system need to obtained pure colonies from agar plate incubated for 18–24 h. The bacterial suspension was adjusted to 0.5 McFarland turbidity standard to achieve 10^8 CFU mL⁻¹ from overnight single colonies and then diluted to 5×10^5 CFU mL⁻¹. The bacterial suspension tested is coupled with ATS card and is automatically filled, sealed and placed into VITEK 2 instrument after incubation for 18–24 h. The Vitek was used in diagnostic laboratory for antibacterial susceptibility evaluation of Gram negative and positive pathogenic bacteria such as of *Enterobacteriaceae*, *P. aeruginosa* (Bruins et al., 2004; Romero-Gómez et al., 2012; Moles et al., 2017), *S. aureus*, *Staphylococcus* spp., *Enterococcus* spp., *Str. agalactiae*, and *Str. pneumoniae* (Ligozzi et al., 2002; Bobenchik et al., 2014). Eigner et al. (2005) has worked on 307 clinical isolates, and demonstrated that the Vitek 2 system required less manual manipulation time than the Phoenix system. The category agreement and error rate were good for both systems. The study piloted by Bobenchik et al. (2014) demonstrated the performed reliability of Vitek 2 system compared to the microdilution reference method and E-test for antimicrobial susceptibility testing of *Staphylococcus* spp. and *Enterococcus* spp. clinical isolates. Alfouzan et al. (2017), compared Vitek 2 yeast susceptibility system card with E-test method to determinate the MICs. The result shown a comparable MICs between the two methods for *Candida* spp. and considered the Vitek 2 system as a reliable alternative to E-test.

BD Phoenix™

The BD Phoenix was an automated microbiology system (BD Diagnostics, Sparks, MD) been available in Europe in 2001 and in the United States since 2004. The Phoenix microbiology system employed for identification and AST of Gram-positive and Gram-negative bacteria (Stefaniuk et al., 2003; Snyder et al., 2008). The principle of Phoenix AST is based on using oxidation-reduction reaction for detection of microbial growth. The monitoring of microbial metabolisms is achieved by the color changes of the indicator (Carroll et al., 2006). The instrument of BD Phoenix system holds up 100 tests panels and one as control panel. The panels trays containing 136 wells divided into 51 well are used for identification and 85 wells ATS side with 14–22 antimicrobial agents at specific concentrations: 51 for ID, 85 for AST (Carroll et al., 2006; Jorgensen et al., 2015). The tested strains were plated in specific media such blood Colombia agar and incubated overnight to obtaining isolate colonies. Subsequently, the adjusted inoculum concentration at 3×10^5 CFU mL⁻¹ are poured into the panels for identification and ATS section respectively. The panels trays are placed into the Phoenix instrument for ID/AST and automatically incubated for up to 18 h, and the result are read and described. Bacterial growth was determined by using modified conventional chromogenic and fluorogenic indicator substrates in the system containing dried biochemical substrates and two fluorescent control well (Carroll et al., 2006). Generally, the Phoenix system reads panels every 20 min, for up 12 h to identification and between 4 and 16 for AST (MICs value). Several studies were conducted to evaluate the performance of BD Phoenix system to determine antimicrobial susceptibility of clinical isolates compared to conventional method and automated system. The category agreements (AC) obtained were 96.5% for *Enterobacteriaceae*, 84.8% for non-fermentative Gram-negative bacteria, 98.9% for *Staphylococcus* spp., 98.0% for *Enterococcus* spp. (Eigner et al., 2005), 96% for *Str. pneumoniae*, excepted for TMP-SMX trimethoprim-sulfamethoxazole (93.4% EA), 97–100% for other streptococci strains (Richter et al., 2007) and 97.8% of IMP produce by *Enterobacteriaceae* (Yamakawa et al., 2018).

Sensititre™ system

The Sensititre system is a commercial phenotypic system product by Thermo Fisher Scientific in 1999 available in the United States with Food and Drug Administration (FDA) clearance. The Sensititre system is automated using 96-well plates format with numerous antimicrobial. Classified as broth microdilution ATS system instrument assisted based on the detection of the metabolism of the fluorogenic growth substrate by bacterial tested with diverse antibiotics and converted the fluorescent signal to the MICs by computer algorithm. In Sensititre AutoReader the MIC or ID plates are inoculated with an automated inoculation system or a handheld multichannel electronic pipette before placement in the instrument's carousel. The MICs values are determined after 18–48 h of incubation stored in the computer component of the system. Another Sensititre Autoreader introduced in United State in 1992, is the ARIS 2 × (ThermoFisher Scientific), is a fully-automated system, benchtop incubating and read the bar-codes plates. Two versions are available: the semi-automated Autoreader and fully automated, robotics-driven ARIS platform (Chapin and Musnug, 2004). In Sensititre ARIS 2 × system, MIC breakpoint and ID were achieved in 96-well plate with dehydrates serial twofold dilutions of antibiotics in each well, and can perform a combination of 192 MIC, run at same time in a single instrument (Christenson et al., 2018). Drew and Paulus (2014), compared the Sensititre microdilution to Vitek-2 and standard disk diffusion methods for the susceptibility testing of 80 *Staphylococcus* isolates. The result obtained shown a good categorical agreement (90%) between Sensititre method, standard disk diffusion methods and Vitek system. Luna et al. (2007), examine susceptibilities of 95 *Bacillus* isolates by using Sensititre method and E-test susceptibility tests. The result obtained gave that the two methods produced comparable MICs results for all antibiotics tested agents (except for trimethoprim/sulfamethoxazole). Several other

studies evaluated the performance of the Sensititre system for AST for Gram-negative and positive and yeast compared to other automated and current standard methods (Chapin and Musgnug, 2004; Avolio et al., 2008; Garza-González et al., 2010; Zhang et al., 2011; Chew et al., 2017). An Sensititre MycoTB (Trek diagnostic system), was performed for susceptibility testing of *M. tuberculosis*. The Trek MycoTB plate MIC uses 96 well-microtiter plate and antimycobacterial agents in second line (Hall et al., 2012), evaluated the performance of the Sensititre MycoTB plate for susceptibility testing of *M. tuberculosis* against first-and second line agents. The result was obtained between 10 and 14 days by using MycoTB compared to the agar proportion method which is in 21 days. Other automated phenotypic Sensititre systems available by FDA clearance are commercialized such as the Sensititre® yeastOne (Trek Diagnostic Systems, United States) which is a calorimetric yeasts panel system with oxidation-reduction indication used for yeasts susceptibility testing. In Pfaller et al. (2008), evaluated the performance of YeastOne system for antifungal susceptibility testing of 404 *Candida* spp. isolates to anidulafungin, caspofungin, and micafungin, and compared obtained results to those of CLSI broth dilution methods applied in three laboratories. The investigation revealed a similarity in the result for the two tested methods for each *Candida* species.

MicroScan WalkAway®

The MicroScan system (Siemens Healthcare Diagnostics, United States), is an automated system utilizes fluorescent technology. The autoSCAN-WalkAway system was introduced in 1986 by American Microscan. Similar to other automated system, the MicroScan have dried substrates in the wells which are rehydrated by the suspension of tested bacteria. The result of the identification and AST is detected after incubation by the pH change, and growth of bacteria in wells in presence of antibacterial agents. This MicroScan system is based on broth microdilution method and available instrument include WalkAway-40 and 60, which accommodate respectively in 40 and 96-well microdilution plates contain chromogenic substrates. The MicroScan utilizes colorimetric reading using photosensors for optical detection of bacteria in the well. The antibacterial susceptibility testing can be determined within a range of 16.8–27.8 h, depending on the bacteria specie.

They are a range of MicroScan panels (conventional, rapid and specially) on single platform. The conventional contain biochemical and chromogenic test for identification and AST of a variety of bacteria (Siemens Healthcare Diagnostics, 2011).

The WalkAway plus microbiology system is a broth microdilution method includes LabPro Information Management software. The WalkAway plus Systems is a semi-automated instrument of overnight, rapid and specialty panels utilizing 96 microwell panels. The instrument configuring models for Gram positive and negative bacteria with 40 and 60 panel capacity for medium-volume high-volume laboratories are available in three types Combo panels and Breakpoint Combo panels and MIC panels. The frequently used MicroScan panels are: MicroScan Gram Pos ID panel, MicroScan Rapid Gram Pos ID panel, MicroScan Neg Type 2, MicroScan Rapid Neg ID Types 2 and 3, and MicroScan NHID (Siemens Healthcare Diagnostics, 2011). Several studies conducted by investigators to evaluate the performance of MicroScan with other automated methods. The study conducted by Quesada et al. (2010) has for objective to evaluate the reliability and accuracy of two automated systems VITEK-2 and the MicroScan, for susceptibility testing of 113 Gram-negative rods. The result obtained shown an excellent correlation >98% between the two system and 89% for *P. aeruginosa* isolates with 0.36% of very major errors. The average time required for the two systems were 4.57 ± 1.37 for automated VITEK-2 and 18–24 h for MicroScan. Another study conducted by Bulik et al. (2010), compared Meropenem MICs and susceptibilities of Carbapenemase-Producing *K. pneumonia* isolates, by using broth microdilution, E-test, Vitek 2, Sensititre, and MicroScan methods. Results obtained for MicroScan method demonstrated, 95.6% agreement based on MIC, 2.2% minor errors with broth microdilution and no very major errors were observed. Lee et al. (2013) investigation, compared the colistin susceptibility of 213 blood stream *Acinetobacter* isolate by using Vitek 2, MicroScan automated system, and E-test methods with the agar dilution method. In this study an excellent categorical agreement (both 99.1%) was shown by using Vitek 2 and E-test, compared to 87.3% (95.7% for *A. baumannii* and 80.7% for non-*baumannii* *Acinetobacter* isolates) using MicroScan. Recently, the study conducted by Singh et al. (2019), evaluated in vitro susceptibility of carbapenem-resistant clinical Gram-negative bacterial strains obtained from several clinical samples for colistin by using MicroScan Walkaway 96 Plus system and Mikrolatest. The results shown that MicroScan WalkAway 96 Plus ID/AST have an excellent categorical agreement of 71.4% (*A. baumannii*), 85.7% (*P. aeruginosa*) and 100% (*A. junii*, *A. johnsonii*, *E. coli* and *K. pneumoniae*) compared to Mikrolatest MIC.

MALDI-TOF MS (Matrix-assisted laser desorption/ionization-time of flight mass spectrometry)

The MALDI-TOF MS was discovered in the 1980s and in the 1990s, and then used to identify surface proteins of bacteria (Truant, 2016). In recent year, MALDI-TOF MS has been introduced in clinical microbiology laboratories for the routine rapid identification based on proteomic profiles and for AST of bacteria and fungi. Since, the routine AST are based on microbial growth in the presence of many concentration antimicrobial agents and require incubation time, the MALDI-TOF is an emerging technology alternatively to phenotypic or genotypic traditional method for determinate antibacterial resistance. Compared to classical methods, this method is rapid and inexpensive, have a value impact and revolution in clinical microbiology. In MALDI TOF MS, a single colony grows in specific agar plate (Wieser et al., 2012), is deposited in sample plate preparation and is mixed to UV-absorbing matrix (Cherkaoui et al., 2010), dried and then placed into the mass spectrometer. The bacteria fixed with matrix receive an irradiation with a laser and the obtained spectra are compared to a database composed of reference spectra strains in the software database. The identification of tested strain is made with corresponding similarity scores (Truant, 2016). Four commercial types systems MALDI-TOF are frequently used for bacterial identification in clinical microbiology laboratories: MALDI-TOF instrument from both Bruker Daltonics or Shimadzu: the MALDI Biotyper (Bruker Daltonics, Germany); the Spectral Archive And Microbial Identification System (SARAMIS™) (AnagnosTec, Germany); the Andromas (Andromas, France) and the Vitek MS (bioMérieux, France)

(Vrioni et al., 2018); http://www.bruker.com/jp/products/mass-spectrometryandseparations/literature/literatureroom.html?eID=dam_frontend_push&stream=1&docID=58883; http://www.biomerieux-diagnostics.com/sites/clinic/files/9300819-002-gb-a_vitek-ms.pdf.

Currently, several methodologies are applied to terminate resistance by using ATS MALDI-TOF system. The first method is to analysis the obtained spectra of tested bacteria to determine a characteristic "resistance spectre" (Kostrzewa et al., 2013; Sparbier et al., 2012). The rapid detection of extended-spectrum β -lactamase (ESBL) producing by *Enterobacteriaceae* using MALDI Biotyper (Kawamoto et al., 2019) and by MALDI-TOF MS-based MBT STAR-BL method (Ota et al., 2019) were developed. For evaluation of antibiotic resistance another type the MBT-ASTRA method was used for AST evaluation of *Bacteroides fragilis* (Justesen et al., 2018), drug sensitivity in selected mycobacteria (Ceyssens et al., 2017) and the non-susceptibility of *Enterobacteriaceae* for gentamicin and ciprofloxacin in blood cultures (Jung et al., 2014). Currently, Maldi ToF MS is largely applied for specific detection of AMR. Among the most recent investigations, detection of AMR in Enterobacteria to Colistin, Cefotaxime, Meropenem, Ciprofloxacin and Ceftriaxone (Dortet et al., 2018; Axelsson et al., 2020; Horseman et al., 2020). Moreover, Oxacillin, Methicillin and Vancomycin AMR detection in *S. aureus* and *Enterococcus* spp. was conducted by using MALDI TOF MS tool (Horseman et al., 2020; Nix et al., 2020).

Single-cell methods for bacterial analysis

A rapid antibiotic susceptibility method has been developed based on single-cell morphological analysis (SCMA) (Choi et al., 2014). This novel SCMA technique is rapid and accurate as new imaging based ATS method. In SCMA, the antibiotic susceptibility is evaluated automatically by analysis of the morphological change using single bacteria cells to different antibacterial conditions including swelling and filament formation. The data obtained with SCMA satisfy the recommendations of the U.S. FDA. The procedure for rapid ATS test with SCMA method, involved that the cells are immobilized in channels were supervised for SCMA by time-lapse bright-field microscopy after they were mixed with the agarose and loaded into a microfluidic. The antibiotics are then loaded into the wells containing nutrients and the samples diffuse in the agarose. The perform test of ATS needs 3 h from the diffusion of antibacterial agents into the imaging region. This method was tested for 189 clinical strains, included collections extended-spectrum β -lactamase (ESBL) activity-producing *Enterobacteriaceae*, methicillin resistance (MRSA), and vancomycin-resistant Enterococci (VRE). Comparison with standard broth microdilution test shown that the total agreement for all isolates was 91.5%. Another study conducted by Choi et al. (2016), tested the immobilized *M. tuberculosis* for rapid drug susceptibility test, with fastidious bacteria, namely the disk agarose channel (DAC). The DAC system is automated with an image processing program automatically determine the susceptibility and resistance of *M. tuberculosis* used in clinical areas and the result can be obtained within 1 week.

Microfluidic based methods

Microfluidic pH sensor

Microfluidic pH sensor is rapid AST method developed by Tang et al. (2013), for monitoring the pH change of bacterial metabolism product and growth rate in culture media spiked with different tested antibiotics. Recently the microfluidics method has become a tools in clinical microbiology to monitoring the microbial resistance with very small samples. This method has been applied to measuring the inhibitory activity of antibiotics by using *E. coli* as the model bacteria adjusted at 0.5 McFarland standard. A cell density of 10^6 CFU mL⁻¹ of model *E. coli* was cultured in glucose solution with 1% of tested antibiotics (ciprofloxacin, tetracycline, azithromycin, amikacin) were introduced into the microfluidic pH sensor and incubated at 37 °C. For each sample, the reflectance spectra were monitored in real time and the MIC obtained in the microfluidic pH were compared with reference method. With this technology the result can be read in less than 2 h, thus will offer advantage of this method for studying the behavior of antibiotics.

Multiplexed microfluidic platform AST of mono- and polymicrobes

A rapid novel technologies platforms been developed for rapid analysis and multiplex AST of *E. coli* treated to four different antibiotics and combinations of them (Fig. 4). With this platform a rapid result of bacterial susceptibility of *E. coli* was taken between 2 and 4 h. Also, the analysis can be carried out with minimum volume (<6 μ L) of samples and antibiotics and realized sensitivity at a single cell level. The microfluidic platform is a based biosensor technology that relies the fluorescence detection of bacteria and antibiotic susceptibility (Mohan et al., 2013). Mohan et al. (2015), used the microfluidic approach applied to study the interactions between *E. coli*, *P. aeruginosa*, and *K. pneumonia* in presence and absence of antibiotics and their growth dynamics in polymicrobial culture in real-time on chip time-lapse fluorescent imaging monitoring bacteria interaction. Result observed shown in mixed culture, the growth dynamics of individual strain depends of the initial cells and the interaction between the strains affects the AST.

Adaptable microfluidic system for single-cell pathogen

Recently, Li et al. (2019) used the microfluidics system for rapid determination of antimicrobial susceptibility for single cell pathogenic bacteria. Li et al. (2019), used tunable microfluidics valve incorporating with real-time optimal for detection bacteria (Fig. 5). The investigation demonstrated the performance of the adaptive microfluidic system to classify the bacterial pathogen *E. coli*, *S. epidermidis*, and *M. bacteremicum*. The classification is based according to the physical shape and sizes feature

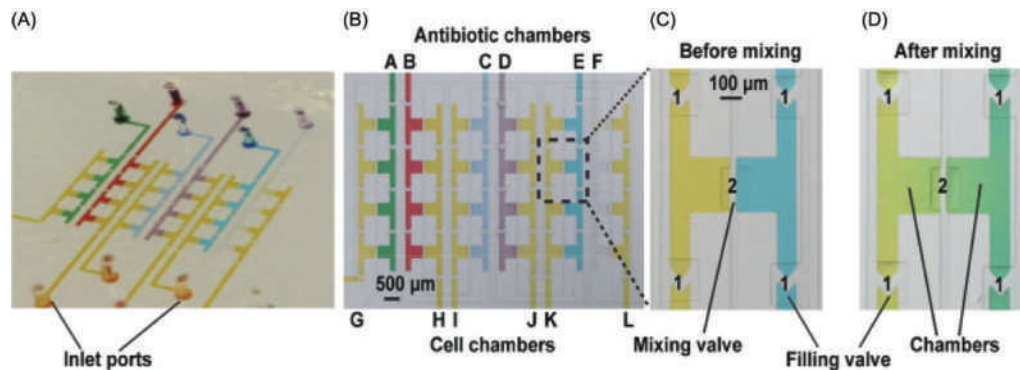


Fig. 4 Optical micrographs of the microfluidic platform for on-chip AST: (A) The device comprises an array of wells loaded with colored dyes. (B) Various concentrations of antibiotics/or different combinations of antibiotics are loaded from the wells through the fluid lines A–F (green, red, blue, magenta, indigo and colorless solutions). Loading of *E. coli* strain in the wells adjacent to the antibiotics through the G–L fluid lines (yellow solutions). (C) Control antibiotic and cell load in wells by pneumatic valves 1 (D) Antibiotics and cells are mixed in adjacent chambers by actuating mixing valves 2. This ensures good diffusion results in change colors (blue/yellow to green) in optical micrographs. Reprinted from Mohan R et al. (2013) A multiplexed microfluidic platform for rapid antibiotic susceptibility testing. *Biosensors and Bioelectronics* 49: 118–125. <https://doi.org/10.1016/j.bios.2013.04.046>, Copyright © 2013, with permission from Elsevier.

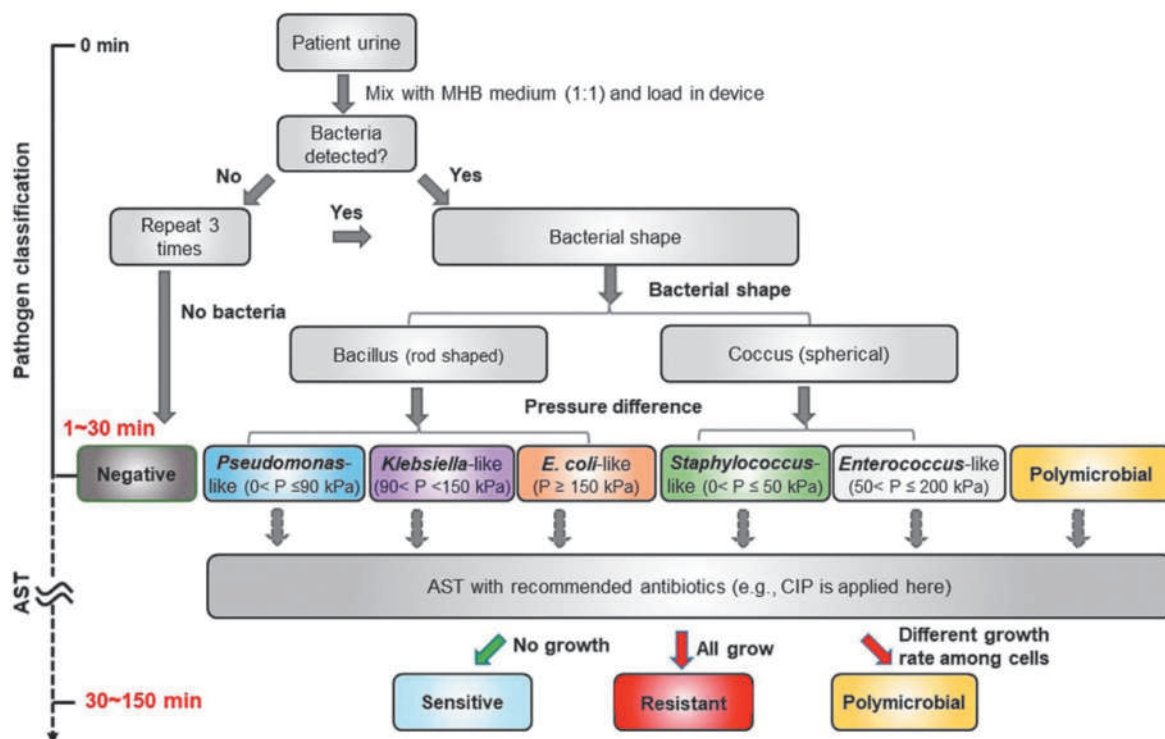


Fig. 5 Process for single-cell AST unknown bacteria in urine samples w. At first, presence of bacteria in clinical samples is confirmed. In the same microfluidic device: (i) pathogen classification of positive samples was based on the rod or spherical form and size (minimum trapping pressure); (ii) AST is conducted. Reprinted from Li H et al. (2019) Adaptable microfluidic system for single-cell pathogen classification and antimicrobial susceptibility testing. *Proceedings of the National Academy of Sciences* 116(21): 10270–10279. <https://doi.org/10.1073/pnas.1819569116>, with PNAS permission.

(length/width) of pathogen bacteria and also demonstrated the performed microfluidic system to determine the AST in the positive samples. The adaptable platform was capable to detect bacterial pathogenic in several biological fluids such as urine, whole and culture blood and analyzing polymicrobial samples.

Droplet microfluidics

Droplet microfluidics is a promising system rapidly emerging as a new approach for studies and applied with success in diagnostic microbiology such as identification of pathogens, studies of microbial physiology and antimicrobial resistance (Kaminski et al., 2016). By using microfluidic droplet, the microbial culturing is carried out in bioreactors, and then the individual bacteria separate

using stochastic confinement in tiny pico to nanoliters volume with bacteria cells, medium and reagents well-defined for analytical assay (Keays et al., 2016), evaluated Droplet created as bioreactors to test the growth of *E. coli* treated by ampicillin and kanamycin. The result demonstrated the performance of Droplet to determined resistance of strains for antibiotic agents within 1 h. The application of droplet microfluidics for studying the antibiotic susceptibility testing were presented in several recent reviews (Hassan and Zhang, 2014; Kaminski et al., 2016; Ortseifen et al., 2020; Qin et al., 2020; Zhang et al., 2020). The droplet microfluidics presented several advantage such as the confinement of the reaction in small volume, high throughput analysis and sensitive assays, small volume load and flexible manipulation and detection. The Droplet eliminates the risk of contamination, minimize fouling and generate large number of bioreactor for single bacterial analysis (Guo et al., 2012; Zhang et al., 2020). For screening antibiotics/pathogens simultaneously (Kang et al., 2019), developed highly-throughput droplet microfluidic AST platform for testing Gram positive and negative bacteria. The system developed is rapid sensitivity response within 15–30 min compared to standard methods (16–24 h), could be used as an excellent alternative to standard AST methods in clinical diagnostics to screen several antimicrobial agents (Miller et al., 2012) described a robust droplets-based microfluidic platforms high-resolution and rapidly generate high-quality dose-response data during drug screening. The droplet microfluidic platform for high-resolution is rapidly generating dose-response curves drug screening, with large number of dose-response containing approximately 10,000 data points per compound for small molecule. For rapid toxicity screening of mixture antibiotics, an another automated droplet-based microfluidic system was used by Churski et al. (2012). By using the microfluidic system developed in this study, the MICs of ampicillin, chloramphenicol and tetracycline were determined for *E. coli*. After then, a rapid MICs values were evaluated, and the different types of interactions of *E. coli* were determined.

Molecular methods for antibiotic resistance detection

Amplification-based methods

Conventional polymerase chain reaction (PCR)

PCR is a molecular technique acquiring greatest value in diagnostic in clinical microbiology by allowing both a precisely identification of pathogenic microorganisms, detection of resistance genes and virulent genotypes (March-Rosselló, 2017). PCR was developed by Kary Mullis (Saiki et al., 1989), permitting rapid and exponential amplification of target DNA sequences using a forward and reverse PCR primers and DNA polymerase enzyme. Conventional PCR consists of successive reaction cycles. In each one the number of targeted extracted DNA molecules is doubled, allowing the exponential number increase of targeted DNA molecules. The cycle is including three steps: (a) denaturing of the double stranded DNA at 95 °C, (b) annealing of the PCR primers at 50–60 °C, and (c) extension of the DNA at 72 °C. Through electrophoresis or other molecular techniques, the amplified target could be confirmed for the existence of resistance genes (Khan et al., 2019). The whole process, including DNA extraction, amplification and visualization, requires 12 h (March-Rosselló, 2017).

PCR using for detecting AMR gene's presence in pure cultures of bacteria or even in polymicrobial samples is currently ordinary. Numerous PCRs was successfully used for both aerobes and anaerobes bacterial strains due to the ease of designing conventional PCR primers (Anjum et al., 2018). List of primers to detect AMR genes is available at the website of the European Reference Laboratory for Antimicrobial Resistance (EURL AMR) (http://www.eurl-ar.eu/data/images/faq/primerliste%20til%20web_07.11.2013.pdf).

Real-time PCR (RT-PCR)

RT-PCR was designed to shorten the time compared to conventional PCR and can detect single point mutations in a given gene if sequence-specific DNA probes targeting the mutation area are used (March-Rosselló, 2017). RT-PCR was used in rapid detection of antibiotic resistance in *mecA* methicillin resistance gene associated MRSA pure isolate, specimens or in staphylococci mixtures (Huletsky et al., 2004; Tenover, 2009; Liu et al., 2016a,b). The multiplex real-time PCR assay was proposed by Kim et al. (2013), to be used in regions of high endemicity for methicillin-resistant *S. aureus* (MRSA) infections. This assay detected simultaneously the *mecA*, staphylococcal cassette chromosome (SCC*mec*)-open reading frame X (*orfX*) junction, and staphylococcal 16S rRNA genes to avoid false-positive results which may occurred by using a single-locus real-time PCR assay.

The accuracy of real-time PCR for the detection of *Enterococcus* spp. (VRE) carrying vancomycin-resistant genes *vanA*, *vanB*, and *vanM* was proven in several investigations. The detection was achieved for pure isolates and directly in rectal swabs of colonized patients (Tenover, 2009; Cantarelli et al., 2011; He et al., 2020a,b,c). Direct detection of *M. tuberculosis* resistance mutations, in clinical respiratory samples, was conducted by Real-time PCR. The technique has minimized the time required to obtain the susceptibility pattern of strains and the mutation encoding resistance to Rifampin and Isoniazid was effectively detected in all sputa harboring resistant *M. tuberculosis* strains (Marín et al., 2004). The use of real-time PCR assays to quantify the abundance of tetracycline (*tet*) resistance in agricultural environments was reported. This assay was proposed to be useful for antibiotic resistance ecological studies (Yu et al., 2005).

Loop-mediated isothermal amplification (LAMP)

LAMP tool developed by Notomi (2000) on the basis of PCR has moreover been used for AMR detection. In LAMP, the target gene is amplified at a constant temperature of 60–65 °C using a *Bst* DNA polymerase as a substitute of *Taq* polymerase (Li et al., 2017)

because of strong strand displacement activity (Khan et al., 2019). This method is expected to amplify the target sequence with high selectivity by an employs of a polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA (Notomi, 2000).

In the last two decades, many investigations report that due to the simplicity and rapidity of the LAMP assay (conventional, multiplex or coupled to other methods) its use can be very practical in clinical microbiology laboratories. The LAMP based essay offers numerous clinical possibilities for use as an alternative tool for the rapid detection of resistance genes. Moreover, LAMP can help in facilitating epidemiological studies and permitting enhanced monitoring of the emergence of drug resistant strains (Mu et al., 2016). Among LAMP-based system the eazyplexw SuperBug CRE proved to be a potent tool for the detection of various carbapenemases as well as CTX-M-type ESBLs in *Enterobacteriaceae* with a rapid resolution time. The system detected perfectly *bla* carbapenemase genes with or without *bla*CTX-M genes in 100% of the molecular characterized strains of *Enterobacter cloacae*, *Klebsiella pneumoniae*, *K. oxytoca*, *E. aerogenes*, *Citrobacter freundii*, *E. coli*, *Raoultella ornithinolytica*, and *Kluyvera ascorbata* (Garcia-Fernandez et al., 2014). LAMP assay was used by Mu et al. (2016) for detection of IS*Aba1*-*bla*_{OXA-51-like} carbapenem-resistance gene in *A. baumannii* isolated from clinical settings. The work reveals that this method showed great potential in detection of AMR genes directly from clinical specimens and has demonstrated 100-fold more sensitivity than the PCR method. Recently, Baek et al. (2020) developed a simple and rapid method for detecting vancomycin resistance genes, such as *vanA* and *vanB*, by using LAMP. Compared to RT-PCR, the sensitivities and specificities of LAMP was 100/100% for both 16S rRNA, *vanA*, and *vanB*.

Restriction endonuclease-based multiplex loop-mediated isothermal amplification (multi-LAMP) assay was established by Zhong et al. (2019) to detect *mcr* genes (*mcr*-1 to *mcr*-5) harbored by colistin-resistant Gram negative bacteria. The method sensitivity was 10-fold greater than that PCR. Multi-LAMP assay can help to prevent infections by drug resistant bacteria in primary-care hospitals. Multiplex loop-mediated isothermal amplification linked to a nanoparticle-based lateral flow biosensor (m-LAMP-LFB) is a novel molecular diagnosis technique established by Chen et al. (2020) to detect *S. aureus* species and identify MSSA and MRSA from clinical samples (Fig. 6). By designing primers based on the *femA* gene (*S. aureus*-specific gene) and the *mecA* gene (encoding penicillin-binding protein 2a) the detection was 100%, allowing that m-LAMP-LFB is a consistent, simple, rapid, specific and sensitive method to identify MSSA and MRSA infections for suitable antibiotic treatment.

Recombinase polymerase amplification (RPA)

The approach of DNA detection by using RPA was designed by Piepenburg et al. (2006). RPA couples isothermal recombinase-driven primer targeting of template material with strand-displacement DNA synthesis. An exponential amplification is achieved with no pretreatment of DNA sample. The technology is sensitive to less than 10 copies of genomic DNA.

The use of RPA to detect the antimicrobial resistance gene were described by Nelson et al. (2019) as an excellent approach for genomic antimicrobial resistance testing in clinical settings and for point-of-care application. The RPA method showed to be rapid, cost-effective tool for antibiotic resistance surveillance for both pathogens and commensals, without nucleic acid purification. In their investigation, the detection of macrolide efflux gene *A*, *mef(A)*, provides resistance against erythromycin and azithromycin was achieved by using RPA tool. The *mef(A)* detection was conducted in raw lysates of *Str. pyogenes*, *Str. pneumoniae*, *Str. salivarius*, and *E. faecium* within 7–10 min of assay time (Nelson et al., 2019).

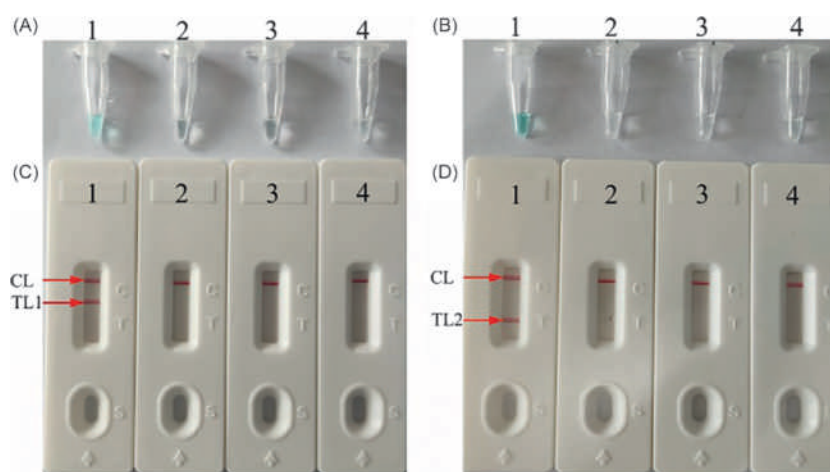


Fig. 6 Detection and verification of *femA*- and *mecA*-LAMP products of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. (A and B) Color and lateral flow biosensor detection of *femA*-LAMP products. (C and D) Color and lateral flow biosensor detection of *mecA*-LAMP products. Reprinted from Chen X et al. (2020) The rapid and visual detection of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* using multiplex loop-mediated isothermal amplification linked to a nanoparticle-based lateral flow biosensor. *Antimicrobial Resistance and Infection Control* 9(1): 111. <https://doi.org/10.1186/s13756-020-00774-x>, under the terms and conditions of the Creative Commons Attribution (CC BY) license.

Hybridization-based methods

The DNA analysis to detect particular mutations or polymorphisms can be accomplished by differential hybridization with sequence-specific oligonucleotide probes. Several hybridizations-based methods are used for detecting AMR mutation in DNA elements of bacterial species.

Array-based methods

DNA microarrays and DNA chips technologies are promising used for assessing antimicrobial susceptibility and even detecting antimicrobial resistance (Frye et al., 2010). The DNA microarray is based on using an image analysis to detect hybridization of a target molecule to a specific probe immobilized on a solid base (March-Rosselló, 2017). Initially, specific probes of each gene are deposited onto a solid substrate (generally glass) in a lattice pattern. Then the DNA is labeled and hybridized to the array, and the specific target-probe duplexes are detected with a reference molecule (Schna, 2000). The presence or absence of genes in the test isolate, in comparison to a reference, was determined by analyzing the hybridization results (Anjum et al., 2018). In a single Microarrays assay a large number of resistance genes can be detected given that these probes are attached very close to one another (March-Rosselló, 2017). Indeed, simultaneous detection of 91 resistance determinants in *A. baumannii* was achieved by using DNA microarray validated with 60 multidrug-resistant *A. baumannii* strains, within 4 h from bacterial culture to result (Dally et al., 2013). Mutation point detection has been carried effectively through DNA microarrays system assessment of *M. tuberculosis* clinical isolates (Fig. 7) in the work conducted by Huang et al. (2014). Predominant mutations in the *rpoB*, *katG* and *inhA* genes have been detected in clinical isolates of *M. tuberculosis*. Among 324 strains of tested *M. tuberculosis*, 210 were detected as MDR. Detection of rifampicin and isoniazid mono-resistance was reported for 41 and 34 isolates, respectively, while none resistance was detected in 39 isolates

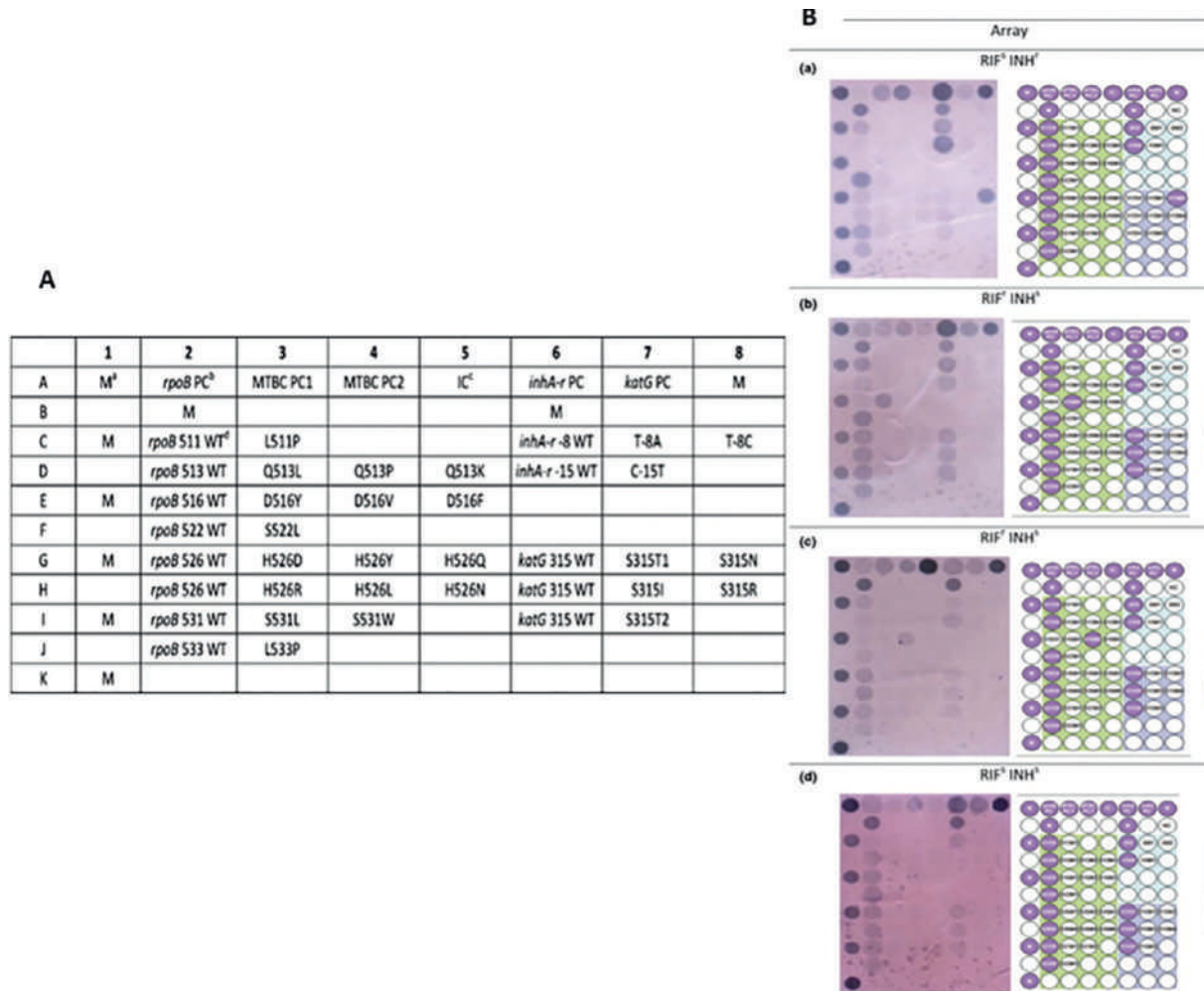


Fig. 7 (A) Layout of probes on the array (a) position marker, (b) positive control, (c) internal amplification control and (d) wild-type probe. (B) Three multidrug-resistant *Mycobacterium tuberculosis* isolates (a–c) and one isoniazid (INH) mono-resistant isolate (d) were analyzed with the array. Reprinted and modified from Huang WL et al. (2014) Rapid and accurate detection of rifampin and isoniazid-resistant *Mycobacterium tuberculosis* using an oligonucleotide array. *Clinical Microbiology and Infection* 20(9): 0542–0549. <https://doi.org/10.1111/1469-0691.12517>, Copyright © 2014, with permission from Elsevier.

(Huang et al., 2014). Moreover, array-based method was largely and successfully employed in the rapidly and accurately AMR gene detection in numerous bacterial species, including MDR *S. enterica* serovars, *E. coli*, *Campylobacter* spp., *Enterococcus* spp., methicillin-resistant *S. aureus*, *Listeria* spp., *Clostridium difficile*, *A. baumannii* and drug-resistant *M. tuberculosis* (Frye et al., 2010; Dally et al., 2013; Huang et al., 2014).

Lab-on-chip methods

Several approaches of Lab-on-a-chip (LoC) devices using microfluidics provided rapid detection of AMR (Kaprou et al., 2021). In genotypic microfluidic assays-PCR or LAMP based, the integration of nucleic acids detection in the device represents a highly promising approach (Giuffrida and Spoto, 2017). For targeting genetic markers of AMR genes in pathogenic bacteria implicated in post-neurosurgical meningitis (Zhang et al., 2018) developed a novel, fast, accurate and easy-to-use micro/nanofluidic chip platform. The method can identify 10 genus or species targets (108 bacterium) and 13 genetic resistance determinants within 1 h: carbapenemase, extended-spectrum beta-lactamase (ESBL), *mecA* and *vanA*, *vanB* related antibiotic resistance genes.

A Lab-on-a-Film disposable (Fig. 8) for rapid detection of multi-drug resistant *M. tuberculosis* (MDR-TB) from sputum extracts was constructed by Kukhtin et al. (2019, 2020). Gel elements (203) printed on including DNA sequences (probes) for 37 genetic mutations, deletions, or insertion elements through five genes were employed in the designed disposal. In order to form a microfluidic flow cell, gel elements are printed on a flexible film, which is next laminated to additional rollable materials (films). Consequently, in a single microfluidic chamber are combined, multiplex amplification, hybridization, washing and array imaging steps. The closed format of the Lab-on-a-Film Assembly (LFA) avoid amplicon contamination.

Line probe assays (LPAs)

In a conventional assay with immobilized PCR product and labeled oligonucleotide probes, each probe requires separate hybridization. This approach is reversed in LPA and uses reverse hybridization (Fig. 9). Thus, a large number of wild type and mutant sites are interrogated and differentiated by analysis of several entire sequences simultaneously in a single hybridization reaction (Saiki et al., 1989; Marinus Barnard et al., 2012). A biotin tag is added to each sample DNA sequence previously extracted from a clinical sample and amplified. The amplified DNA is denatured and single stranded DNA probes for resistance genes are attached to a band. The sequences of probes are attached to a strip if they are complementary to it with a known location. The unbound DNA sample is washed with alkaline phosphatase. A streptavidin anchor is added to the strip, which binds to DNA sequences labeled with biotin. Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) are added. They cleave alkaline phosphatase, turning it into a dark blue dye, on which the sample DNA has bound (Saiki et al., 1989; World Health Organization (WHO), 2000).

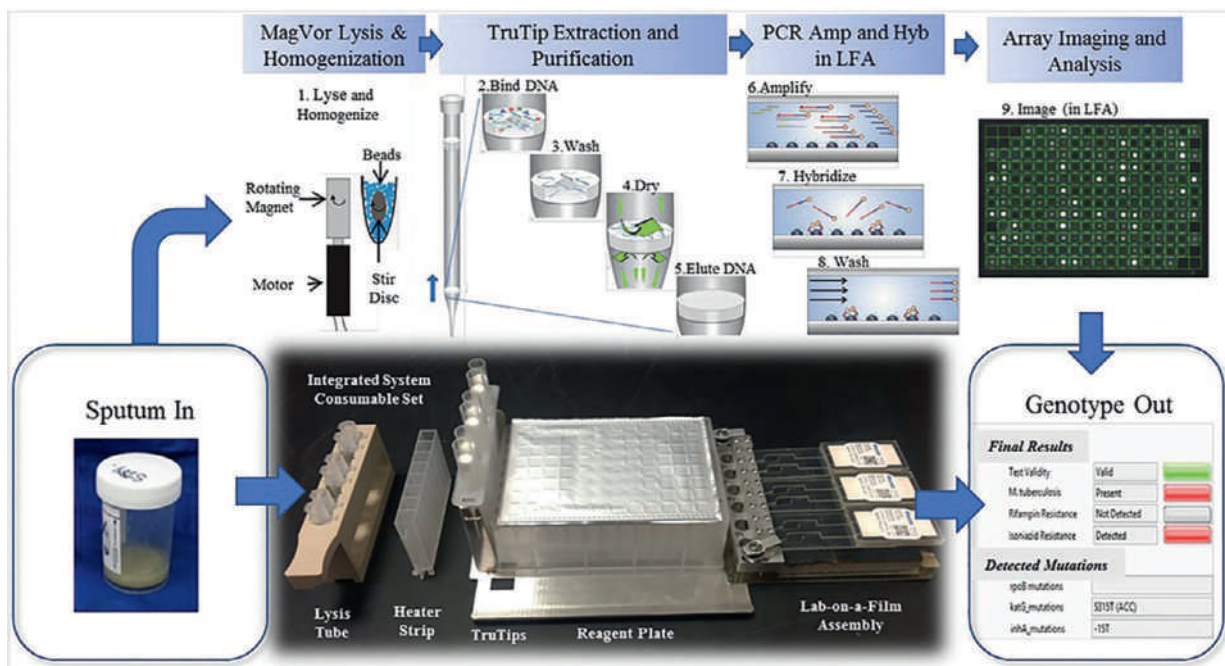


Fig. 8 Layout of a Benchtop Automated Sputum-to-Genotype System using a Lab-on-a-Film Assembly for Detection of Multidrug-Resistant *Mycobacterium tuberculosis*. Reprinted from Kukhtin AV et al. (2020) A benchtop automated sputum-to-genotype system using a lab-on-a-film assembly for detection of multidrug-resistant *Mycobacterium tuberculosis*. *Analytical Chemistry* 92(7): pp. 5311–5318. <https://doi.org/10.1021/acs.analchem.9b05853>, Copyright (2020) with permission of American Chemical Society.

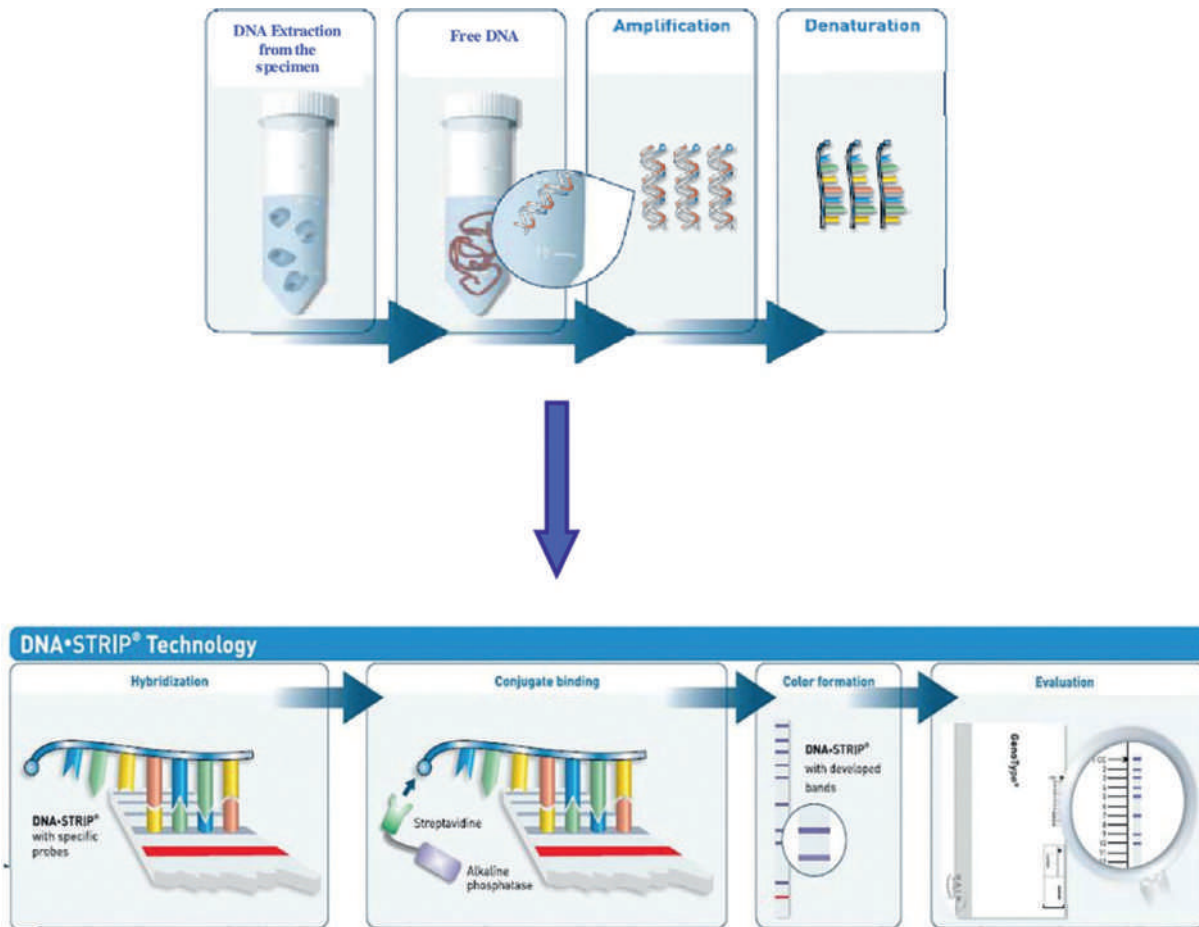


Fig. 9 Illustration of the DNA-strip technology by Hain LifeScience. The GenoType MTBDRplus molecular LPA for detection of drug-resistant *Mycobacterium tuberculosis*: (1) DNA extraction from smear positive specimens or from cultured isolates; (2) multiplex PCR; (3) reverse hybridization; (4) detection and evaluation. Reprint from Barnard M et al. (2012) *Molecular Detection of Drug-Resistant Tuberculosis by Line Probe Assay. Laboratory Manual for Resource-Limited Settings*. FIND: Geneva, pp. 274, with permission ©Hain LifeScience.

Development of LPAs assays to detect mutation genes such as *gyrA* mutations, *inhA* and *katG* mutations, *rpoB* gene and *katG* gene mutations conferring resistance to fluoroquinolones, rifampicin and isoniazid in *M. tuberculosis* shown the effectiveness of this approach (Giannoni et al., 2005; Farooqi et al., 2012; Kazemian et al., 2019). Moreover, LPAs demonstrated high accuracy generally for the detection of rifampicin resistance and high specificity for isoniazid resistance detection (Nathavitharana et al., 2017).

Fluorescence in situ hybridization (FISH)

In situ hybridization (ISH) was developed by carrying out hybridization of Radiolabeled DNA to cytological preparations from *Xenopus oocytes* without altering their cell morphology or integrity (Call et Pardue, 1969; John et al., 1969). The formed molecular hybrids radiolabeled probes- DNA were detected by autoradiography. Radioactive labels were progressively replaced by nonisotopic dyes (Landegent et al., 1984; Pinkel et al., 1986, 1988), hence FISH using fluorescent labels were used for detection of microbial single cells (DeLong et al., 1999). FISH can be used for the diagnosis of infectious diseases in case of blood-culture-negative endocarditis, respiratory infections, gastrointestinal diseases, mycobacterial infections, highly pathogenic microorganisms and other fastidious bacteria such as spirochetes (Prudent and Raoult, 2019). The procedure includes the following steps: (i) fixation of the studied microorganism cells or tissues; (ii) specimens pretreatment and permeabilization step, to achieve the access of the probes to the nucleic acids; (iii) hybridization of short fluorescent labeled single-stranded oligonucleotide probes to ribosomal RNA (rRNA) of the target microorganism; (iv) washing steps to eliminate unbound probes; (v) mounting and imaging by microscopy or flow cytometry and documentation of results (Möter and Göbel, 2000).

The simultaneous detection of one or multiple targets providing resistance made FISH a suitable tool for detection antimicrobial resistance determinants especially for ribosomally mediated ones. Such resistance in which antibiotic drugs like macrolide or linezolid are affected, allowed intense fluorescence signals because ribosomal RNA copies are numerous in living cells (Frickmann et al., 2014). Detection of linezolid enterococci resistance was achieved by FISH essay by detecting a single mutated 23S rRNA gene allele in phenotypically linezolid-susceptible *E. faecium* (Werner et al., 2007). Peptide nucleic acid-fluorescence in situ hybridization

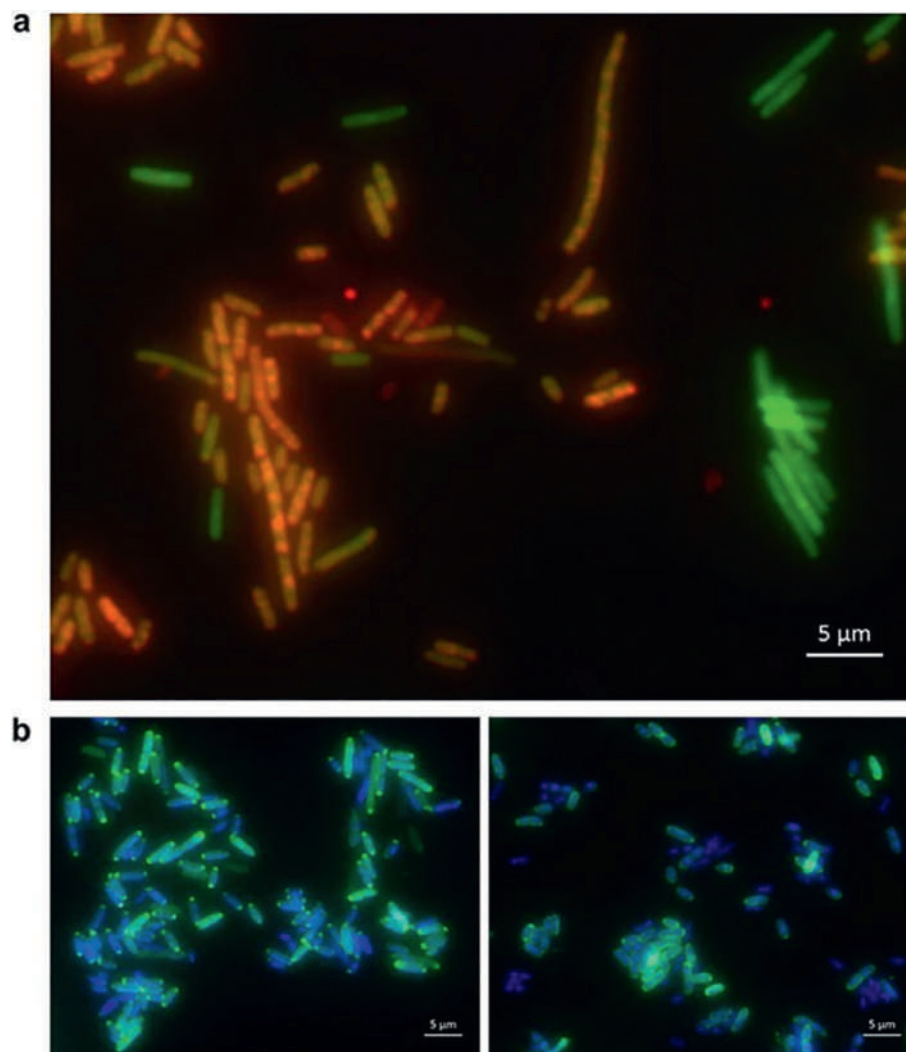


Fig. 10 Detection of TEM β -lactamase mRNAs and the respective protein in *E. coli*. (A) FISH staining of TEM β -lactamase mRNA in *E. coli* harboring pLitmus38 (red) and the ribosomal RNAs by Enterobac (green). (B) Antibody (green) and DNA/DAPI (blue) staining of the TEM β -lactamases in *E. coli* harboring pLitmus38 (left) or pUC18 plasmids (right). Reprinted from Rohde A, Hammerl JA and Al Dahouk S (2016) Rapid screening for antibiotic resistance elements on the RNA transcript, protein and enzymatic activity level. *Annals of Clinical Microbiology and Antimicrobials* 15(1): 55. <https://doi.org/10.1186/s12941-016-0167-8>, under a Creative Commons Attribution 4.0 International License (CC BY).

(PNA-FISH) appear to be a simple, quick, and accurate method for detecting *H. pylori* clarithromycin resistance. The method showed high sensitivity and specificity for the patients harboring clarithromycin-resistant *H. pylori*, which could improve the patient's chances of a more effective *H. pylori* eradication. The method demonstrated to be able to discriminate susceptible and resistant *H. pylori* genotypes on gastric biopsy specimens (Cerqueira et al., 2013). Rohde et al. (2016) used plasmid-encoded TEM β -lactamase conferring ampicillin resistances to develop a FISH -test able to detect the respective mRNAs (Fig. 10). FISH assay demonstrated to be sensitive to detect signals in all *E. coli* TEM β -lactamase producing strains harboring pLitmus38 and pUC18 plasmids (Rohde et al., 2016).

A methylation-sensitive RNA fluorescence in situ hybridization was developed by Ranasinghe et al. (2018) to detect methylated bases in tRNA, rRNA and mRNA, which control several cellular processes, including antimicrobial resistance. Detection of single methylations of rRNA, quantifies antibiotic-resistant bacteria in cells mixtures and detects multiple methylations by using multi-color fluorescence imaging (Ranasinghe et al., 2018). Recently, the development of novel nanoprobe for FISH and the application of the nanoprobe to the detection of ampicillin-resistant *E. coli* was reported. F-nanoprobe and R-nanoprobe were prepared for the discrimination of *rrnB* and *bla* genes in *E. coli*, respectively. By using R-nanoprobe-*bla* for antimicrobial resistance identification detection of beta-lactamase sequence of ampicillin-resistant *E. coli* was possible by microscopic observation (Lee et al., 2019).

Immunoassays, to detect AMR gene: Lateral flow assays LFAs

Lateral flow assays (LFAs) technique is based on biochemical reaction of antigen-antibody or probe DNA-target DNA hybridization. Four parts constitute the lateral flow test (LFA): (i) a sample pad: the sample is placed on this area; (ii) conjugate pad: compartment where labeled tags are combined with biorecognition elements; (iii) reaction membrane: comprises the test line and the control line; an absorbent pad: used to accumulate waste. In order to increase the sensitivity of assays gold nanoparticles, colored latex beads, carbon nanoparticles, quantum dots, and enzymes are used as labels (Bahadır and Sezgentürk, 2016). Immunoassay methods implicate the direct detection resistance markers protein which are captured and enriched by using antibodies. Antibodies or resistance proteins can be labeled with fluorescent tags for visualization and their interaction requires optimization (van Belkum et al., 2020). Several investigations developed products for the direct detection of antibiotic resistance proteins targeting specific protein markers of resistance. Fig. 11 illustrate the immunochromatography using mAbs 1E2-7 and 4E4-4 that reacted with recombinant NDM-1 type metallo- β -lactamase produced by *K. pneumoniae*.

Specific immunochromatographic assay using the aminoglycoside-modifying enzyme 6'-N-acetyltransferase-Ib [AAC(6')-Ib], aminoglycoside 6-N acetyltransferase (AAC(6')-Iae) and IMP-type metallo- β -lactamases as molecular markers designed to detect respectively MDR *P. aeruginosa* and carbapenem resistance in *Pseudomonas* (Kitao et al., 2010; Tada et al., 2011, 2016). Carbapenem resistance in Gram negative bacilli was accurately detected by development of LFAs producing immunochromatographic cross-reaction with NDM (New Delhi metallo- β -lactamase), OXA-23 (carbapenem-hydrolyzing oxacillinase); OXA-48 (oxacillinase-like), OXA-163 subfamilies, KPC (*K. pneumoniae* carbapenemase), and VIM (verona integron-encoded metallo- β -lactamase) carbapenemase (Pasteran et al., 2016; Boutal et al., 2017; Mertins et al., 2019; Rösner et al., 2019; Tada et al., 2019). LFA was described as a tool for the rapid detection and monitoring of MCR-1 *Enterobacteriaceae* producers harboring plasmid encoded colistin resistance gene *mcr-1* causing resistance to polymyxin, the last-resort antibiotic for the treatment of infections caused by highly drug-resistant Gram-negative bacteria (Volland et al., 2019). Multiples studies involved the development and optimization of lateral-flow assay for the detection of the penicillin-binding protein 2a (PBP2a) in methicillin resistant *S. aureus* (MRSA). The detection process takes between 15 and 20 min and does not necessitate special tools (Yamada et al., 2013; Amini et al., 2020). Recently, the establishment of an LFA assay for MRSA detection on the basis of the ability of an aqueous soluble cellular wall-binding domain (CWBD) protein from bacteriophage P108 to act as a wide-spectrum binding agent for all MRSA strains was conducted. The LFA was designed in sandwich format: the signal probes for tracing MRSA used is CWBD-coupled time-resolved fluorescent microspheres (FMs); MRSA capture was carried out by nitrocellulose membrane immobilized with porcine IgG (Fig. 12). The MRSA detection could be achieved within 10 min at cells concentration range of 6.6×10^2 – 6.6×10^7 CFU mL⁻¹. LFA strip using recombinant CWBD as the recognition agent may be a

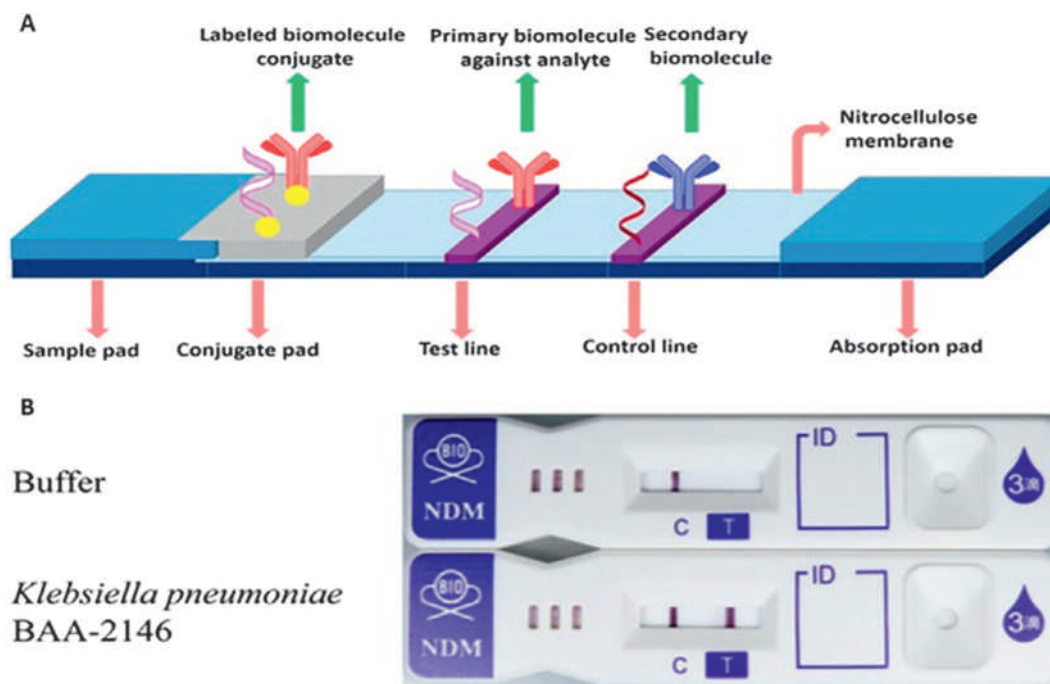


Fig. 11 (A) The basic structure of lateral flow assay. (B) Immunochromatography using mAbs 1E2-7 and 4E4-4 that reacted with recombinant NDM-1 type metallo- β -lactamase produced by *K. pneumoniae*: negative reaction, line appears at the reference line (C); positive reaction, a second line also appears at the positive test line (T). (A): Reprinted from Bahadır EB and Sezgentürk MK (2016) Lateral flow assays: Principles, designs and labels (2016). *TrAC Trends in Analytical Chemistry* 82: 286–306. <https://doi.org/10.1016/j.trac.2016.06.006>, Copyright © 2016, with permission from Elsevier; (B) Reprinted from Tada T et al. (2019) Molecular characterization of multidrug-resistant *Pseudomonas aeruginosa* isolates in hospitals in Myanmar. *Antimicrobial Agents and Chemotherapy* 63(5): e02397-18. <https://doi.org/10.1128/AAC.02397-18>, under Creative Commons Attribution 4.0 International License (CC BY).

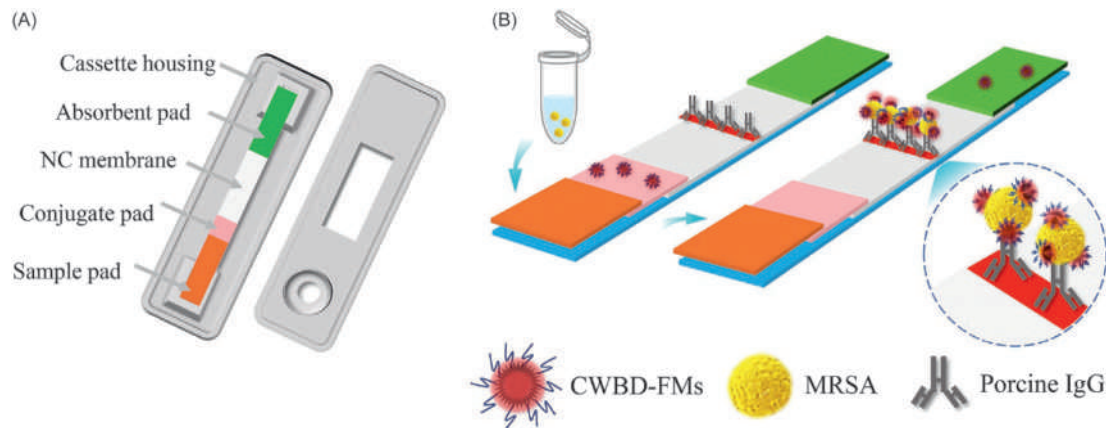


Fig. 12 Schematic illustration of the CWBD-based LFA method for detection of MRSA: (A) diagram of LFA strip, (B) principle of CWBD-based LFA. Reprinted from Yang H, Wang Y, Liu S, Ouyang H, Lu S, Li H and Fu Z (2021) Lateral flow assay of methicillin-resistant *Staphylococcus aureus* using bacteriophage cellular wall-binding domain as recognition agent. *Biosensors and Bioelectronics* **182**: 113–189. <https://doi.org/10.1016/j.bios.2021.113189>, Copyright © 2021, with permission from Elsevier.

promising approach for POC testing of MRSA (Yang et al., 2021). For vancomycin-resistant enterococci *VanA* and *VanB*-producing rapid detection a LFIA assay (called NG-Test *VanA* and NG-Test *VanB*) was constructed Oueslati et al. (2020, 2021). Detection of VRE was possible from both cultured colonies and blood samples.

Whole genome sequencing (WGS)

WGS is used to obtain the complete DNA sequence of a microorganism. A comparison between a DNA sequence and standard reference sequences, deduction may provide information about important phenotypic traits of the microorganism, such as AMR (World Health Organization (WHO), 2019). WGS is situated to become an essential tool in the control of antibiotic resistance due to recent improvements in sequencing technologies providing fairly long reads at high speed, such as Illumina MiniSeq, Pacific Biosciences PacBio Sequel system, or Oxford Nanopore MiniON and PromethION can (Köser et al., 2014; Vasala et al., 2020). More than 40 freely accessible Bioinformatics tools for detection of AMR determinants in DNA or amino acid sequence data have been developed, including ARG-ANNOT, CARD Comprehensive Antibiotic Resistance Database, SRST2, MEGARes, Genefinder, ARIBA, KmerResistance, AMRfinder, and ResFinder (Hendriksen et al., 2019). The WGS is used in numerous applications in medicine such as the development of new antibiotic and rapid identification of resistance mechanisms, the surveillance and control of infectious diseases and detection of antibiotic resistance in the clinical microbiology (Köser et al., 2014).

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http://www.eurl-ar.eu/data/images/faq/primerliste%20til%20web_07.11.2013.pdf—DTU National Food Institute – EU Reference Laboratory for antimicrobial resistance (EURL-AR).

www.clsi.org—The Clinical and Laboratory Standards Institute (CLSI).

www.eucast.org—The European Committee on Antimicrobial Susceptibility Testing.

www.who.int—World Health Organization (WHO).

Pipelines for Characterization of Microbial-Producing Drugs

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Abbreviations

AI	Antagonism index
BHIa	Brain heart infusion agar
BHIb	Brain heart infusion broth
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
ISP2a	International <i>Streptomyces</i> Project medium 2 agar
ITS	Internal transcribed spacer
LSU	Large subunit
MBC	Minimum bactericidal concentration
MHCA	Müller-Hinton Cation Adjusted
MIC	Minimum inhibitory concentration
MLSA	Multilocus sequence analysis
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NPE	Natural products extract

NTDs	Neglected tropical diseases
PBS	Phosphate buffered saline
PDA	Potato dextrose agar
PDB	Potato dextrose broth
SSU	Small subunit
TSA	Tryptic soy agar

Introduction

There is a growing interest in the use of microorganisms as a source of secondary metabolites. Microbes are skilled producers of valuable natural products which exhibit a plethora of biological activities such as antibiotics, anticancer, antiviral, antileishmanial, and anthelmintic (Demain, 2014; Pham et al., 2019; Cruz et al., 2021), as well as biotechnological processes in sustainable agriculture and food industry (Adrio and Demain, 2014).

The continuous and sometimes uncontrolled use of antibiotics contributes to the spread of resistance. Among this, several developed and approved natural products or derivatives are based on old discoveries and, since the 1960s, no new class of broad-spectrum compounds has been discovered (Fischbach and Walsh 2009; Lewis, 2013; Walsh and Wencewicz, 2014; Ling et al., 2015; Sabino et al., 2019; Cruz et al., 2020). Deaths caused by drug-resistant bacteria overpass 700,000 cases every year worldwide (Bos and Austin, 2018). Such pathogens are capable to prevail to antibiotics through mechanisms regulated by antibiotic-resistance encoding genes which can (A) change the permeability of bacterial cell wall or active efflux which restrict the antimicrobial access to target sites, (B) modulation or degradation of the antibiotic by enzymatic activity, and (C) acquisition of alternative metabolic pathways or modification of active antibiotic sites (van Hoek et al., 2011) (Fig. 1). According to the Centers for

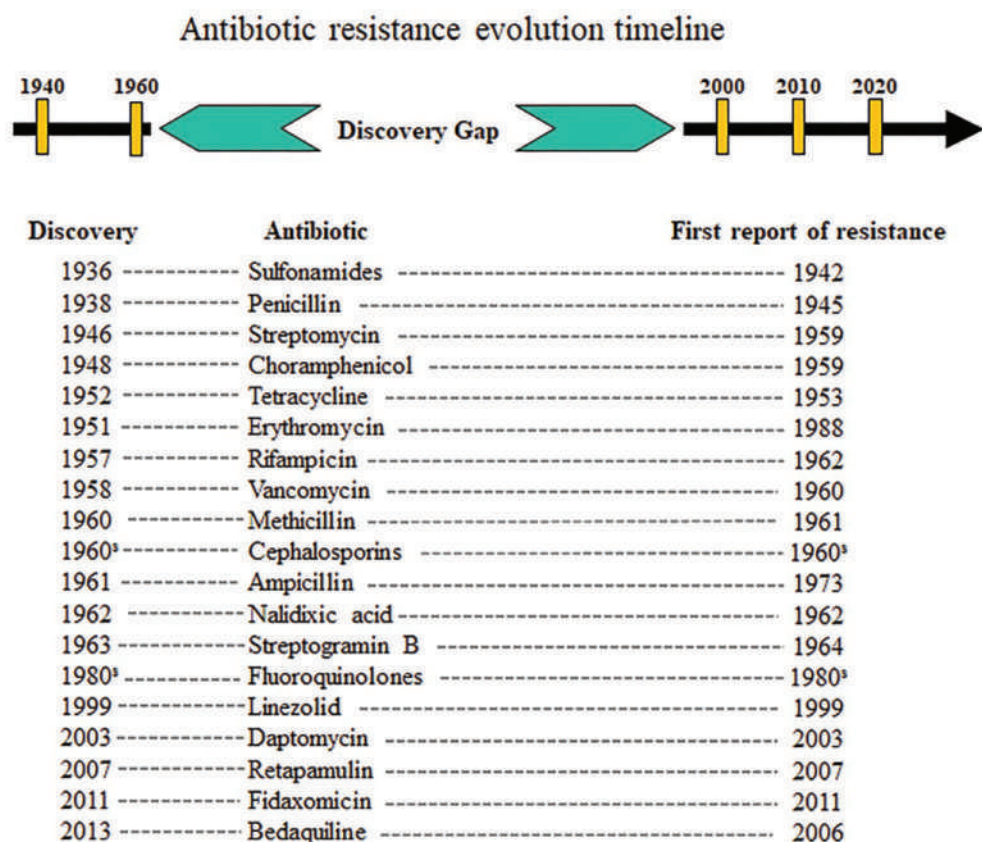


Fig. 1 Bacterial resistance timeline. Between 1962 and 2000 no new classes and/or effective antibiotics have been discovered. Additionally, resistant pathogens are rapidly spreading whereas the discovery of novel substances and their application to clinical practice is not fast enough. Adapted from Fischbach MA and Walsh CT (2009) Antibiotics for emerging pathogens. *Science* 325(5944): 1089–1093, doi:10.1126/science.1176667; Lewis K (2013) Platforms for antibiotic discovery. *Nature Reviews. Drug Discovery* 12(5): 371–387, doi:10.1038/nrd3975; Walsh CT and Wencewicz TA (2014) Prospects for new antibiotics: A molecule-centered perspective. *The Journal of Antibiotics* 67(1): 7–22, doi:10.1038/ja.2013.49.

Disease Control and Prevention (CDC), “superbugs” which belong to the ESKAPE group are the major nosocomial opportunistic pathogens. Methicillin-resistant *Staphylococcus aureus* (MRSA) is omnipresent in hospital environment which can overpass 50% of antibiotic resistance rates in 5 out of 6 world regions, whereas the Gram-negative *Acinetobacter baumannii* is a biofilm-forming deadly pathogen which exhibits mortality rates as high as 60% (Park et al., 2016; Chen et al., 2018; Lee et al., 2018; Turner et al., 2019) (Fig. 1). Consequently, the need to explore niches that house microorganisms that could produce bioactive metabolites are critical.

Likewise, infectious diseases caused by phytopathogenic fungi can affect harshly several crops worldwide. Additionally, conventional agriculture practices are not ecologically safe since that the use of agrochemicals is famous for the harm to the environment and human health. On the other hand, beneficial microbes represent a sustainable alternative to negative repercussions caused by these agents. Microorganisms are capable to inhibit or suppress plant pathogens and influence directly and indirectly plant growth by the solubilization of important substances such as phosphate or by the biosynthesis of phytohormones, siderophores, and enzymes (Cullen et al., 2019; Omomowo and Babalola, 2019; White et al., 2019).

Neglected diseases still are a burden in low-income tropical and subtropical communities (Molyneux et al., 2017). Leishmaniasis are transmitted by more than 20 species of *Leishmania* protozoan and are classified as Neglected tropical diseases (NTDs), a diverse group of communicable diseases that prevail in tropical and subtropical conditions in 149 countries, affecting more than a billion people and cost developing economies billions of dollars every year (Alcántara et al., 2018; Bekhit et al., 2018; Moreira et al., 2020; Santos et al., 2020). Moreover, it is important to mention that the current therapies of leishmaniasis exhibit significant recurrence rates, and pentavalent antimonials-based treatment is known to be unsafe and ineffective as well therapies based on the administration of pentamidine and Amphotericin B are considered toxic (Lindoso et al., 2012; Toghueo, 2019).

In addition, cancer continues to be a major cause of morbidity and mortality in millions of people worldwide, devastating the public health system (Rayan et al., 2017; Wali et al., 2019; Sharifi-Rad et al., 2019). Therefore, the possibility to introduce new substances from natural sources that can effectively tackle infectious diseases, malignancies, and biofertilizers might promote an increase in longevity and minimize the negative impacts on health and the environment.

Herein, we address a review topic concerning the main tools and protocols for isolation and characterization of these microbe-producing drugs.

Medicines and microbe sources

Microorganisms are capable to produce a wide variety of secondary metabolites, also known as natural products. This diverse group of agents is the foundation of the development of various biopharmaceutical, agrochemicals, and biotechnological products (Pham et al., 2019). The discovery of penicillin in 1928 by Alexander Fleming is considered an important milestone that revolutionized the search for medicines since that in the early 1900s almost 80% of all medicines were derived from plants (Lobanovska and Pilla, 2017; Pham et al., 2019).

Potential candidates might be found in several niches, such as soil, *epi*/endophytically associated with plants, animals, and marine habitats (Giddings and Newman, 2013; Pham et al., 2019). However, nearly 99% of all species of external environments are promising and unculturable (Ling et al., 2015). Ling et al. (2015) reported the discovery of the potent antibiotic compound teixobactin against Gram-positive and drug-resistant pathogens. This substance is produced by the non-cultivable *Eleftheria terrae*, isolated from soil using the device known as the iChip. It is known that bacterial resistance to antibiotics is a worldwide life-threatening concern (Aslam et al., 2018; Peterson and Kaur, 2018). However, teixobactin exhibits properties that minimize the generation of *S. aureus* and *Mycobacterium tuberculosis* mutants.

Soil is an extremely diversified niche, where microorganisms compete to survive. Therefore, microbes' secondary metabolism is influenced by other microorganisms to control them (Onaka, 2017). Actinobacteria is presented in 10–50% of the soil microbial community. These Gram-positive filamentous spore-forming bacteria are characterized by their large linear chromosomes exhibiting high GC content (Amin et al., 2020; Lee et al., 2020; Prudence et al., 2020; van Bergeijk et al., 2020). Despite being a significant percentage of soil microbiota, it was reported the isolation of actinomycetes from other sources, including insect-associated *Streptomyces* spp. (Chevrette et al., 2019; Olanrewaju and Babalola, 2019). In terms of the diversity of secondary metabolites, members of *Streptomyces* genus are skilled producers of a wide variety of bioactive substances economically important (Genilloud, 2018). Approximately, more than 80% of the antibiotics industrially produced are obtained from these actinobacteria (Grasso et al., 2016; Cruz et al., 2020).

Streptomycin, cephalosporin, chloramphenicol, and tetracycline are antibiotics originally isolated from *Streptomyces* species (Procópio et al., 2012), as well approximately 55% of all this class of medicines discovered between 1945 and 1978 are related to *Streptomyces* genus (Hutchings et al., 2019).

Interestingly, Streptomyces when intimal interaction with mycolic acid-containing bacteria (*Mycobacterium* spp., *Rhodococcus* spp., *Dietzia* spp., *Nocardia* spp., *Williamsia* spp., *Gordonia* spp., *Corynebacterium* spp., and *Tsukamurella* spp.) are capable to activate their cryptic metabolism (Onaka et al., 2011; Hoshino et al., 2019). Cryptic genes are silent biosynthetic gene clusters that are not expressed during the microbial life cycle or in usual laboratory conditions (Tamburini and Mastromei, 2000). Onaka et al. (2011) reported the discovery of the antibiotic alchivemycin A and the production of a red pigment which were obtained from the co-cultivation of *Streptomyces endus* S-522 and *Tsukamurella pulmonis* TP-B0596 and *S. lividans* TK23 and *T. pulmonis*, respectively. Alchivemycin A is a versatile polycyclic polyketide that possesses an antimicrobial profile against *Micrococcus luteus* and is capable to

inhibits the invasion of murine colon carcinoma without showing cytotoxic effects (Kim et al., 2013). Combined cultures might modulate the cryptic secondary metabolism in 90% of *Streptomyces* species when compared to axenic cultures (Onaka, 2017; Caraballo-Rodríguez et al., 2017; Chagas and Pupo, 2018), and since the discovery of alchivemycins, this approach has been reported in several studies (Hoshino et al., 2015a, b; Sugiyama et al., 2015).

Actinorhodin was first reported by Brockmann and Pini (1947). This polyketide is a blue-pigmented antibiotic (in its basic pH, whereas is red at acidic pH) which inhibits the growth of Gram-positive bacteria, originally produced by *Streptomyces coelicolor* (Xu et al., 2012; Mak and Nodwell, 2017; Nass et al., 2017). *S. coelicolor* is considered a model organism and is known that its genome exhibits more than 20 biosynthetic gene clusters (Chater, 2016; Bobek et al., 2017; Čihák et al., 2017).

Biotechnologically, the *Streptomyces* genus occupies a prominent position. However, within the *Actinobacteria* phylum, other genera such as *Micromonospora* and *Actinomadura* are important natural product bio-factories (Igarashi et al., 2012). Both are skilled in producing polyketides, an important class of bioactive compounds isolated from a wide species of microorganisms that might exhibit a plethora of biological activities (Wang et al., 2020). In Igarashi et al. (2012), the polyketide nomimicin, an inhibitor of *Micrococcus luteus*, *Candida albicans*, and *Kluyveromyces fragilis* was reported for the first time. This compound was obtained from the compost-isolate *Actinomadura* sp. TP-A0878.

On the other hand, microorganisms that live in the rhizosphere have gained substantial attention as a source of versatile bioactive natural products (Iniyan et al., 2017; Zhang et al., 2019). The rhizospheric space (Fig. 2) comprises a narrow soil zone that surrounds plant roots. This complex and dynamic region harbors a rich microbiota that is attracted by nutrients, exudates, border cells, and mucilage released nearby plant roots (Philippot et al., 2013; Cruz et al., 2020).

The quorum communication of rhizospheric microbial community (bacteria, fungi, arbuscular mycorrhizae, oomycetes, viruses, and archaeas) may directly or indirectly modulate plant's composition and its biomass by the biodisponibilization of phosphate in the soil, production of natural products (phytonutrients and antibiotics) that might influence its growth and stimulate plant's defense system (Godinho and Bhosle, 2013; Philippot et al., 2013; Lacava and de Sousa, 2016).

Aside from medical applications, the use of microorganisms can reduce the use of phosphate fertilizers and agrochemicals which affect directly human and environmental health.

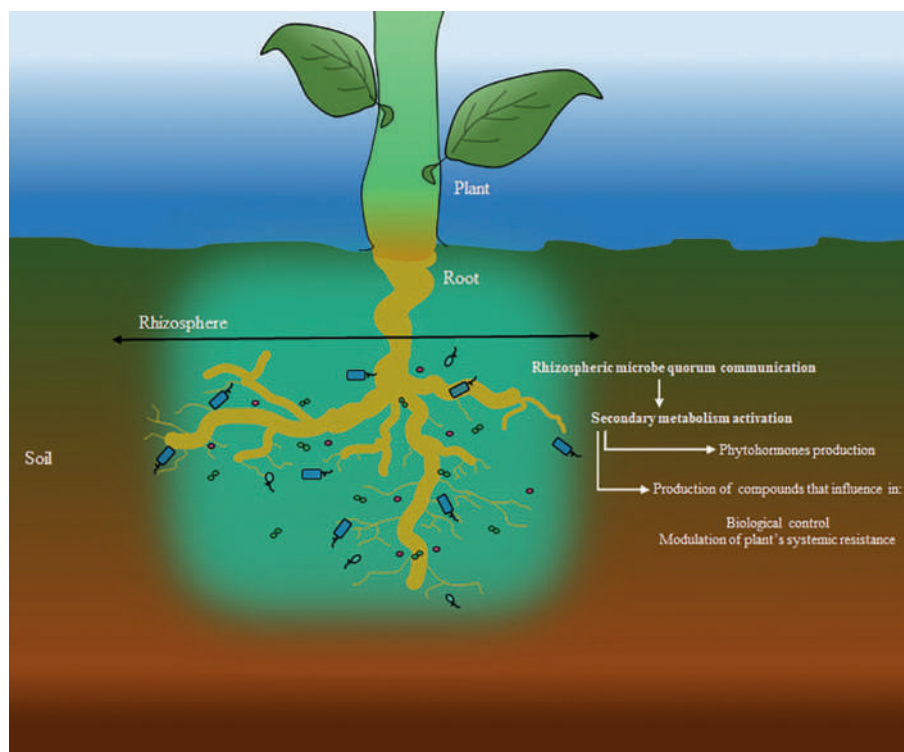


Fig. 2 Representation of the micro-community that surrounds the rhizospheric space. The quorum communication might trigger microbes' secondary metabolism, which can influence positively plant growth or produce antibiotics and other substances that can protect the plant against stress conditions, including herbivory harm by pests and control of plant pathogens. Adapted from Philippot L, Raaijmakers JM, Lemanceau P, and van der Putten WH (2013) Going back to the roots: The microbial ecology of the rhizosphere. *Nature Reviews. Microbiology* 11(11): 789–799, doi:10.1038/nrmicro3109; Cruz FDPN, Bogas AC, and de Sousa CP (2020) Plant-associated microorganisms as a potent bio-factory of active molecules against multiresistant pathogens. In: *Antimicrobial Resistance*. IntechOpen. doi: 10.5772/intechopen.93598; van Bergeijk DA, Terlouw BR, Medema MH, and van Wezel GP (2020) Ecology and genomics of *Actinobacteria*: New concepts for natural product discovery. *Nature Reviews. Microbiology*, 18(10): 546–558, doi:10.1038/s41579-020-0379-y.

Plants and endophytes

Humankind has been based on medicinal plants for centuries (Giang and Otsuka, 2018; Gómez and Luiz, 2018; Anand et al., 2019) to feel better. However, in some cases exploring natural compounds from plants is not a sustainable approach. Paclitaxel (Taxol®) (Fig. 3.) is a chemotherapeutic agent found in barks, roots, and branches of the native tree from Pacific *Taxus brevifolia*. However, its slow growth and low yields of paclitaxel are severe obstacles. To yield 1 kg of paclitaxel, it required 10,000 kg of bark (Wani et al., 1971; Stierle et al., 1993; Isah, 2015).

Plant-associated microorganisms have gained notoriety for their ability to synthesize plant-derived compounds (Zhao et al., 2011; Gouda et al., 2016), as well as a wide-range action of bioactive natural products (Alvin et al., 2014; Fadiji and Babalola, 2020). These microbes are capable to live in intimate interaction with the host plant without causing any apparent disease symptoms. These microorganisms are known as endophytes (Abdel-Razek et al., 2020). Stierle et al. (1993), reported the discovery that the endophytic fungus *Taxomyces andreanae* is capable to produce the paclitaxel.

Endophytes represent a rich and successful source of bioactive natural products in medicine, pharmaceutical, and biotechnological applications (Alvin et al., 2014; Ek-Ramos et al., 2019). These microorganisms are capable to produce a vast quantity of bioactive substances which directly enhance plant growth by the synthesis of hormones, nitrogen fixation, and phosphate solubilization and indirectly by the inhibition of phytopathogens (Martinez-Klimova et al., 2017; Savi et al., 2019; Pacifico et al., 2019) and other compounds which originally believed to be produced only by their host plants (Strobel et al., 2004).

The search for drugs for the treatment of malignant diseases is a global challenge. Medicines originally obtained from plants that do not meet the requirement from the global market have been discovered from endophytic fungi. In 1966 Wall and co-workers discovered the antitumoral and antileukemic alkaloid camptothecin from extracts derived from the Chinese tree *Camptotheca acuminata* and *Nothapodytes foetida* (Wall et al., 1966; Li et al., 2006; Uzma et al., 2018). As well as taxol, Puri et al. (2005) discovered an endophytic fungus-producing camptothecin *Entrophospora infrequens* isolated from the inner bark of *N. foetida*. Likewise, podophyllotoxin was isolated for the first time from *Sinopodophyllum* plants (major natural source) but is widely found in *Diphylleia*, *Dysosma*, *Juniperus* plants. Podophyllotoxin exhibits cytotoxic and antiviral is widely used in the treatment of human papillomavirus (HPV) tumor lesions, leukemias, lung, and testicle malignancies. Currently, is known that podophyllotoxin is produced by several species of plant-derived fungi such as *Phialocephala fortinii*, isolated from *Podophyllum peltatum* (Imbert, 1998; Eyberger et al., 2006; Uzma et al., 2018), *Fusarium oxysporum* (Kour et al., 2007), *Trametes hirsute* (Puri et al., 2006), *Aspergillus fumigatus* (Kusari et al., 2009), and *Alternaria tenuissima* (Liang et al., 2016).

Beauvericin is a versatile cyclic hexadepsipeptide mycotoxin which exhibits potent insecticidal, antimicrobial, antiviral, and cytotoxic effects. Beauvericin is an emerging toxic compound presented worldwide in the contaminations of food and feeds. It was described for the first time by Hamill et al. (1969) which was isolated from the entomopathogenic fungus *Beauveria bassiana*.

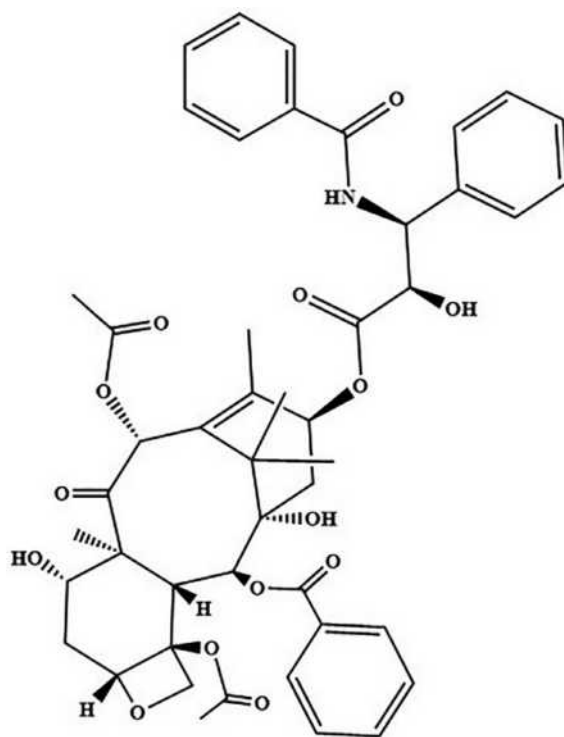


Fig. 3 Chemical structure of taxol. Adapted from PubChem.

However, it is known that beauvericin is also produced by several species of *Fusarium* (Wang and Xu, 2012; Mallebrera et al., 2018; Bertero et al., 2020). Campos et al. (2015) describe an endophytic beauvericin-producing *Fusarium* sp. isolated from *Caesalpinia echinata* Lam. (Fabaceae), a native tree from Brazil. Beauvericin exhibited a potent effect on amastigote and trypomastigote forms of *Trypanosoma cruzi* (IC₅₀ 1.9 µg/mL).

However, NTDs which are prevalent and closely related to poverty worldwide have been ignored for decades. Nevertheless, more than one billion people in 149 countries suffer from NTDs (Weng et al., 2018). Concerning leishmaniasis' treatment options, modern medicine still counts on few and toxicity drugs (Roatt et al., 2020). Optimistically, this scenario is changing positively, and new studies regarding the discovery of antileishmanial compounds have been published.

The study of Moreira et al. (2020) investigated the inhibitory potential of crude extracts of fungal endophytes associated with *Avicennia nitida*, a mangrove tree against pathogenic bacteria, phytopathogenic fungi, and *Leishmania infantum chagasi*. The crude extract of *Diaporthe* sp. strain FS-94(4) have shown 90% cell death at 6 mg/mL, whereas moderate activity *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, and *Salmonella enterica* serotype Enteritidis ATCC 19196, exhibiting MIC₅₀ of 756, 767, and 949 µg/mL, respectively. Moreover, endophytic fungi from mangrove ecosystems have gained special attention due to their ability to modulate mangroves' adaptation to extreme environments, which suggests that these endophytes are skilled and promising natural products source (Sebastianes et al., 2017; Nicoletti et al., 2018).

Microbial lipases are versatile enzymes for their capacity to catalyze several reactions. These biomolecules are responsible to hydrolyze insoluble fats, oils, and esters of fatty acids (Singh et al., 2016; Patel et al., 2019). Alves et al. (2018) investigated the effects of fungal-derived lipases on dermatophyte fungi (*Malassezia* sp. and *Microsporum canis*) and *Leishmania amazonensis*. Interestingly, the study showed that the crude extracts of lipase-producing fungal endophytes *Emericella nidulans*, and *Dichotomophora portulacae* were more effective on promastigote forms of *L. amazonensis* (IC₅₀ 1.120 and 0.834 µg/mL, respectively), whereas *Vermisporium*-like and *Dichotomophora boerhaaviae* exhibited IC₅₀ of 1.388 and 1.151 µg/mL.

Silva et al. (2020a) evaluated the bactericidal, antiprotozoal, and cathepsin V inhibition activities of various extracts obtained by combined cultures of phytopathogenic fungi *Colletotrichum horii*, *C. gloeosporioides*, *Fusarium guttiforme*, *Pestalotiopsis diospyri*, *Phoma caricae-papayae*, and *Phytophthora palmivora*. Extracts 45 (co-cultivation of *F. guttiforme*, *P. diospyri*, *C. horii*, and *P. palmivora*) and 46 (co-cultivation of *F. guttiforme*, *P. diospyri*, *C. horii*, and *C. gloeosporioides*) (Silva et al., 2020b) exhibited potent antibacterial activity by fusaric and 9,10-dehydrofusaric acids, which were the majority compounds in these extracts.

The Brazilian Cerrado, also known as Tropical Savannah, covers about 2,000,000 km², which represents approximately 23% of the land surface of the country. In terms of area, it is exceeded by only the Amazonia forest. This biome is characterized by the immense diversity of medicinal plants which are widely used in folk medicine (Gottsberger and Gottsberger, 2009; dos Santos et al., 2020; Bogas et al., 2020). *Solanum lycocarpum* Saint Hill, also known as "lobeira" is a medicinal plant that exhibits anti-inflammatory properties (Ratti, 2009; Cruz et al., 2020; Bogas et al., 2020). Ratti et al. (2011) investigated the antibacterial effect of the crude extract of the endophyte *Streptomyces tubercidicus*. In this study, the fractionated extract showed strong antibiotic activity against *E. coli* and *S. aureus*. *S. tubercidicus* is a well-known producer of tubercidin, a potent and multi-active pyrrolopyrimidine that exhibits antimycobacterial, antiviral, antitumor, and kills *T. cruzi* by the inhibition of metabolic processes (Smulson and Suhadolnik, 1967; Kónya et al., 2008; Liu et al., 2018).

Serrano et al. (2012) reported the isolation of the *Paenibacillus polymyxa* strain RNC-D as endophyte from leaves of *Prunus* sp., a plant native to Brazilian tropical savannah. This study focused on the optimization of growth conditions for the production of bioactive substances, where its fermentation broth was primary screened and revealed significant inhibition of *E. coli* and *S. aureus* (Serrano, 2009). *P. polymyxa* is a Gram-positive bacillus naturally resident of soil capable to produce a plethora of secondary metabolites ranging from plant growth promoters to various antibiotics (polymyxin) (Neris et al., 2017; Jeong et al., 2019).

Observations on target plant for prospecting endophytes such as the absence of herbivores raise the possibility of an efficient chemical defense mechanism which is possibly promoted by endophytes (Piza et al., 2015). Thus, endophytes can be considered as alternative suppliers of characteristic phytochemical compounds and represent a vast unexplored reservoir of unique chemical structures (Elias et al., 2018).

Identification and characterization

Prior to bioinformatic and automated tools, microbes were grouped and identified based on their phenotypic characteristics such as the shape of single cells, morphology of the colony, as well physiological and biochemical profiles (Tshikhudo et al., 2013; Franco-Duarte et al., 2019). Streptomycetes exhibit unique cultural characteristics. Velvety and circular colonies which are usually gray on the front color and beige on the bottom become visible after 7 days of cultivation followed by the production of geosmin which is responsible for their wet earth odor (Ratti, 2009). However, conventional methods take few or up to a dozen days (e.g., molds) and the comparative analysis of phenotypic characteristics is not enough for proper identification (Franco-Duarte et al., 2019).

The development of tools and methods based on a molecular level and the ever-increasing deposit of DNA sequences in databases emerged in the late 20th century (Franco-Duarte et al., 2019). The 16S rRNA gene is widely used for the identification of bacterial species for decades (Johnson et al., 2019). The 16S small subunit ribosomal gene exhibits approximately 1500 bp in length and is an exclusive and highly conserved housekeeping gene within species of prokaryotes (Osman et al., 2018; Johnson et al., 2019; Peker et al., 2019). On the other hand, the identification of fungal taxa is performed by the sequencing of the internal transcribed spacer (ITS) cluster of rDNA. This ~600 bp region is located between the small subunit (SSU) rRNA gene (18S) and the large

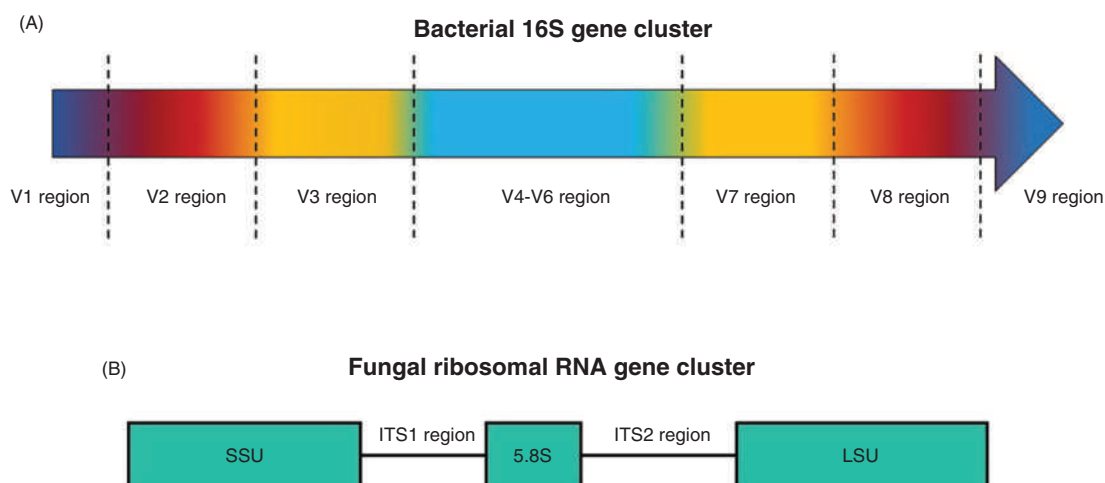


Fig. 4 (A) The bacterial 16S rRNA gene is divided into nine regions, where the red regions, represented by V2 and V8 exhibit lower phylogenetic resolution, whereas the V4-V6 (green) is the major functional center of 16S which is located in a high conserved domain (690 hairpin) which is responsible for several chemical interactions with other biomolecules (e.g., bind to “790 loop” and the domain IV of the 16S rRNA and 23S rRNA respectively). (B) The fungal rDNA SSU and LSU regions offer less genetic variation in comparison to ITS and are adopted for phylogenetic studies of higher taxonomic levels, whereas the sequencing of LSU in combination with ITS is used for species characterization. Adapted from Taylor DL, Walters WA, Lennon NJ, Bochicchio J, Krohn A, Caporaso JG, and Pennanen T (2016) Accurate estimation of fungal diversity and abundance through improved lineage-specific primers optimized for Illumina amplicon sequencing. *Applied and Environmental Microbiology* 82(24): 7217–7226, doi:10.1128/AEM.02576-16; Yang B, Wang Y, and Qian PY (2016) Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. *BMC Bioinformatics* 17: 135, doi:10.1186/s12859-016-0992-y.

subunit (LSU) rRNA gene (28S) (positioned at 5'-end and 3', respectively) and separated (ITS1 and ITS2) by 5.8S rRNA gene (Schoch et al., 2012; Taylor et al., 2016; Raja et al., 2017; Badotti et al., 2018; Yang et al., 2018) (Fig. 4.).

Despite its wide role in bacterial systematics, taxonomical classification by 16S rRNA gene offers poor discriminatory resolution for some genera such as *Acinetobacter*, *Aeromonas*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Stenotrophomonas*, and several other genera (Janda and Abbott, 2007). In *Burkholderiales*, for example, identities of 16S rRNA and Recombinase A (*recA*) genes can reach 100% and 95%, respectively within *Burkholderia cepacia* complex (Bach et al., 2017) whereas the phylogenetic determination based on Calmodulin (*CmdA*) for *Aspergillus* and Beta tubulin (*BenA*) for *Penicillium* and *Talaromyces* are accepted as the secondary determination of species level (Yilmaz et al., 2014; Tsang et al., 2018; Rajeshkumar et al., 2019). Therefore, Multilocus Sequence Analysis (MLSA) and polyphasic taxonomy approaches which are based on the phylogenetic inferences using concatenated housekeeping genes enable the investigation of evolutionary characteristics and high resolution at the species level (Yilmaz et al., 2014; Zhu et al., 2020).

Laboratory techniques

Isolation of microorganisms naturally present in soil, rhizosphere, and plants

Material

Phosphate Buffered Saline (PBS)

Petri dishes containing Tryptic Soy Agar (TSA), International *Streptomyces* Project medium 2 agar (ISP2a), Potato Dextrose Agar (PDA), and ISP3 (Oatmeal) agar

70% ethanol solution

2% NaClO solution

Sterile distilled H₂O

Sterile 20% glycerol solution

Soil/rhizosphere samples - 10 g

Plant samples (roots, stems, leaves)

250 mL Erlenmeyer flasks

Drigalski loop

Sterile blades

Cryovials

Loop-full

28 °C shaking incubator

Media recipes

All media should be autoclaved at 121 °C for 20 to 45 min and cooled at 25 °C or stored at 4 °C before use.

TSA - recommended to bacterial isolation

Tryptic Soy Broth media: 30 g/L. However, it might be prepared alternatively using pancreatic digest of casein (15 g/L), papaic digest of soybean meal (5 g/L), sodium chloride (5 g/L), and bacteriological agar (15 g/L) in a final pH of 7.3 ± 0.2 . Supplement with Benomyl at 50 µg/mL (Araújo et al., 2014).

IPS2 - recommended to actinobacteria isolation

Malt extract (10 g/L), yeast extract (4 g/L), glucose (4 g/L), and bacteriological agar (20 g/L) in a final pH of 7.3 ± 0.2 (Shirling and Gottlieb, 1966).

PDA - recommended to fungi isolation

Potato infusion broth (boil 200 g/L of sliced and unpeeled potatoes for 30 min), glucose (20 g/L), and bacteriological agar (15 g/L). Adjust the pH to 6.0. Alternatively, the commercial Potato dextrose broth medium must be prepared at a concentration of 39 g/L. Supplement with tetracycline (50 µg/mL) (Araújo et al., 2014).

ISP3 - recommended to Streptomyces spore-forming isolation

Cook or steam Oatmeal (20 g/L) for 20 min followed by the addition of 1.0 mL/L of trace salts solution. Adjust the pH of 7.2 then sterilize (Shirling and Gottlieb, 1966).

Trace salts solution

FeSO₄ · 7H₂O: 1.0 g/L

MnCl₂ · 4H₂O: 1.0 g/L

ZnSO₄ · 7H₂O: 1.0 g/L

Distilled water

Experimental procedure

Samples of soil/rhizosphere (10 g) must be placed in individual Erlenmeyer flasks containing 90 mL of sterile PBS (NaCl: 8.0 g/L; KCl: 0.2 g/L; Na₂HPO₄: 1.44 g/L; KH₂PO₄: 0.24 g/L; pH: 7.4) and incubated for 2 h at 28 °C/180 rpm (Andreote et al., 2008; Cruz, 2018).

Concerning endophytes isolation, plant structures are submitted to superficial disinfection to eliminate the epiphytic population. This pretreatment is based on serial washes in 70% ethanol, 2% NaClO, 70% ethanol, and a double rinse in sterilized distilled H₂O, which times of exposure on baths vary according to plant characteristics (e.g., sample thickness). Plant structures are then ground and incubated in PBS as described above (Araújo et al., 2014). Subsequently, 100 µL of decimal dilutions are inoculated and spread with the aid of a Drigalski loop in the respective medium and incubated at the same conditions until microbe growth (Fig. 5).

Target isolates must be streaked and purified in their respective medium and preserved in 20% glycerol solution at –80 °C for bacteria (Araújo et al., 2014). Fungi stocks are maintained at room temperature in sterile water vials containing fungal mycelial disks (Araújo et al., 2014). Actinobacteria must be grown and sporulate in ISP3 agar for 7 days at 28 °C. A 2 mL of 20% glycerol should be added to the plates containing spores which should be collected using a loop-full and transferred to cryovials before stock at –80 °C (Shepherd et al., 2010; Park et al., 2016).

Expected results and characteristics

Considering actinomycetes as the most productive secondary metabolites producers, the expected morphological isolations can be seen in Fig. 5. According to studies (Ratti et al., 2011; Serrano et al., 2012; Lacava and de Sousa, 2016), these endophytes developed as colonies regarding the traits: color, form, elevation, margin, diameter, surface, opacity, and texture (Zinniel et al., 2002).

Antimicrobial activity screening

Overlay assay

Material

Brain heart infusion agar (BHIIa)

Brain heart infusion broth (BHIIb)

Appropriate solid medium for target isolate growth (e.g., nutrient agar, ISP2, TSA, and PDA)

Sterile 0.85% saline solution

Chloroform

Spectrophotometer

Incubator (28 °C and 37 °C)

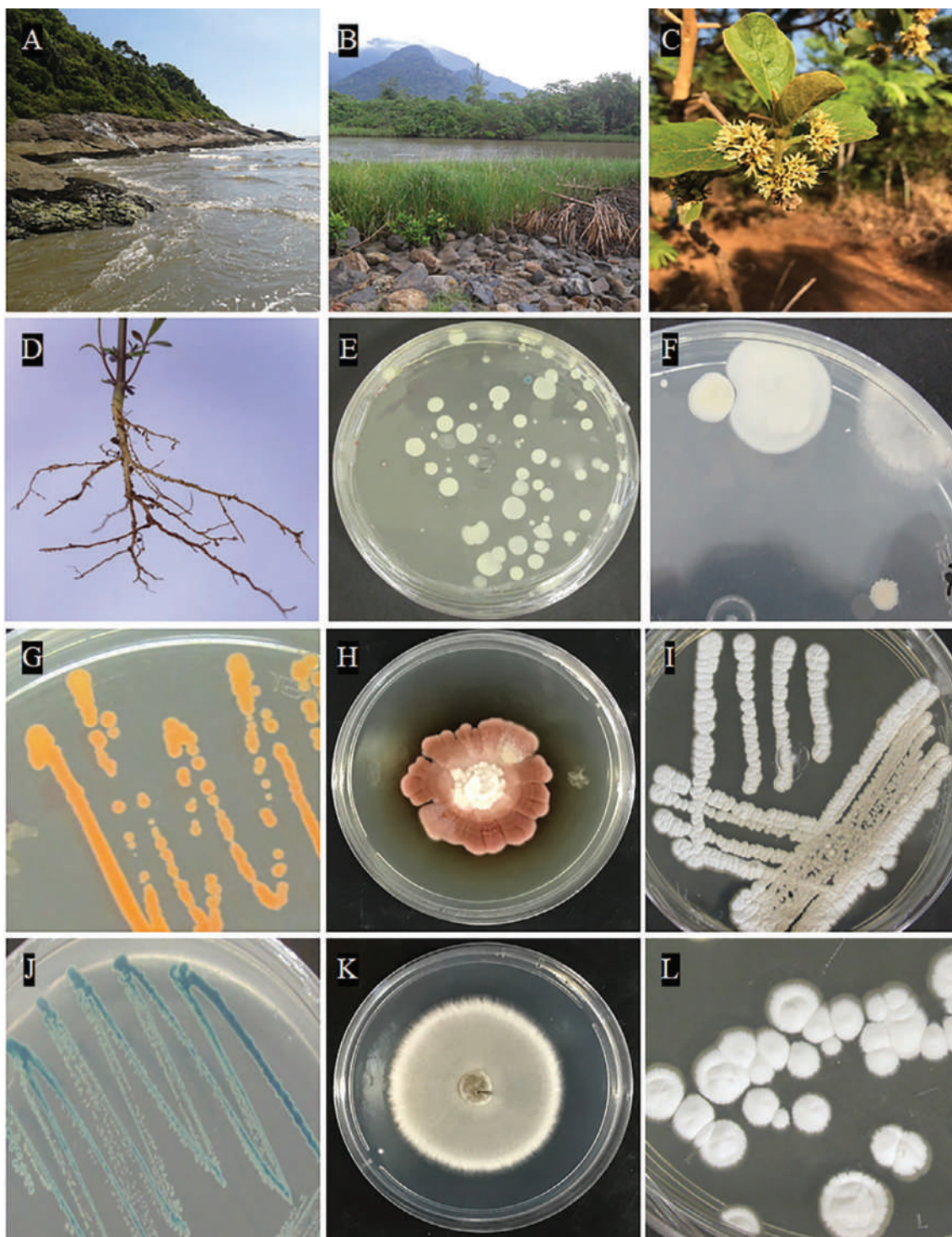


Fig. 5 Promising host plants and environments may be found in high biodiversity areas, in which microbes develop different survival strategies. Coastal (A) and Mangrove (B) areas in Atlantic forest, Brazilian Tropical Savannah (C). Rhizospheric soil adhered to plant root (D). Diversity profile of rhizospheric microorganisms isolated from *Polygala paniculata* (E). Discrete inhibition produced by the endophyte during the isolation of the endophytic community (F). Purified endophytic (G, H, and I) and rhizospheric (J, K, and L) microbes isolated from *Polygala paniculata*.

Media recipes

Brain heart infusion medium

Dissolve 37 g of BHI in 1 L of distilled H₂O, mix well and sterilize at 121 °C for 15 min.

IPS2 agar

Malt extract (10 g/L), yeast extract (4 g/L), glucose (4 g/L), and bacteriological agar (20 g/L) in a final pH of 7.3 ± 0.2.

0.85% saline solution

Dissolve 0.85 g of NaCl in 100 mL of distilled H₂O. Autoclave 15 min at 121 °C and store at room temperature.

Experimental procedure

Overlay assay is widely used for antibacterial agents screening detection. This approach is relatively inexpensive and requires minimal resources. Several studies employed this consolidated method (Piza et al., 2015; Song et al., 2017; Hockett and Baltrus, 2017; Rojas-Rojas et al., 2018; Kurnianto et al., 2020).

Herein, we describe the slightly modified method reported in Piza et al. (2015). Target isolates are pre-inoculated and grown at 28 °C (for the bacterial isolates: TSA medium for 24–48 h, whereas actinomycetes in ISP2 for 7 days). After the reactivation, colonies are selected and diluted in 0.85% saline and then inoculated in the center of glass plates containing the respective solid medium according to the kind of microorganism and incubated at 28 °C for 3–7 days.

Pathogenic strains (e.g., *S. aureus*, *E. coli*, and *C. albicans*) must be overnight cultured in BHIb at 37 °C. The pathogen cultures are then diluted to an OD₆₀₀ of 0.3 to 0.5 and inoculated 1:10 in semisolid or molten (high temperatures are not recommended) BHIa.

Target isolates must be inactivated by the exposition with 1 mL of chloroform which must be poured onto the plate lid (20 min and 30 min for evaporation of chloroform residues). Finally, 25 mL of BHIa previously inoculated with test microorganisms is poured onto the inactivated isolate. Plates must be incubated at 37 °C for 12–24 h. Inhibition is visualized as a halo around the colonies (Fig. 6).

Dual culture assay

Material

PDA medium

Sterile 0.85% saline solution

Incubator at 28 °C

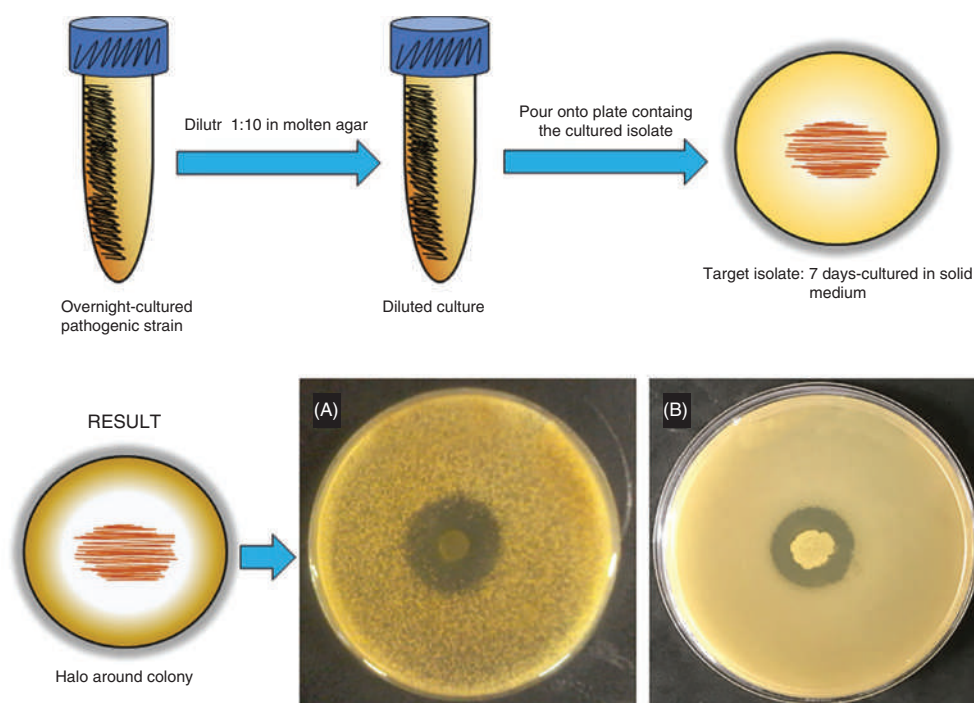


Fig. 6 Overlay assay basic protocol. Antimicrobial activity was observed in the experiment by the bacterial target isolate against *Candida albicans* (A) and *Bacillus cereus* (B).

Experimental procedure

The evaluation of the biological activity potential of microbes against fungi is based on the cultivation of the target isolate and the pathogenic fungus in the extremities of Petri dishes containing PDA (Machado, 2015; Machado et al., 2020; Silva et al., 2016).

From a precultured fungus, a plug of the mycelium measuring 0.6 cm in diameter is placed on the other top of the plate containing the grown test isolate. As a negative control, a plate containing the phytopathogen was made as a parameter to indicate the time to evaluate the inhibition. The plates are incubated at 28 °C until the complete growth of the negative control (Fig. 7).

Building a natural product extracts library

Material

ISP2 broth
 Potato dextrose broth (PDB)
 Polypropylene mesh
 Amberlite® XAD16 resin
 Methanol
 Ethyl acetate
 Dimethyl sulfoxide (DMSO)
 Centrifuge
 Speed-vac concentrator
 28 °C shaking incubator

Experimental procedure

In this section, we describe the protocol used by Park et al. (2016) to obtain the natural products extract (NPE) from actinobacteria. However, this method might be applied to other bacterial species.

Isolates should be precultured for 3 days at 220 rpm/28 °C in 3 mL of ISP2 broth in round-bottom tubes. Subsequently, a volume of 5 mL of the test isolate preculture should be grown in 250 mL capacity Fernbach flasks containing 100 mL of ISP2. The culture is maintained under the same conditions and the time varies according to test strain. Since the fermentation is done, the cultures must be centrifuged at 4500 rpm for 10 min. Subsequently, the fermentation broth is collected in a new Fernbach flask containing a polypropylene mesh package containing 1.5 g of Amberlite® XAD16 resin (Sigma-Aldrich™). The fermentation broth is then overnight incubated on a rotary shaker under the same conditions. Resin packets are washed in distilled H₂O and transferred to glass tubes (known weight) containing 20 mL of MeOH: EtOAc (1: 1) solution. Finally, the NPEs should be concentrated in Speed-vac and resuspended to the desired concentration in DMSO, and stocked at –80 °C in cryovials.

Concerning fungal natural products, the protocol used in Sharma et al. (2016) is widely reported in several studies and is based on the cultivation of the test fungus in PDB under periodical agitation. However, there are several methods for fungal natural products extraction from in solid-state fermentation (Robinson et al., 2001; Marques et al., 2018; Slaný et al., 2020) to liquid fermentation at static (Silva et al., 2020a; Vieira et al., 2020), partially static (Sharma et al., 2016), or under agitation (An et al., 2020; Tapfuma et al., 2019).

Test fungus' mycelium is transferred and cultivated in 250 mL Erlenmeyer flask containing 100 mL of PDB and stationary incubated at 28 °C in absence of light for 7 to 21 days. After the incubation period, the fermentation broth should be homogenized

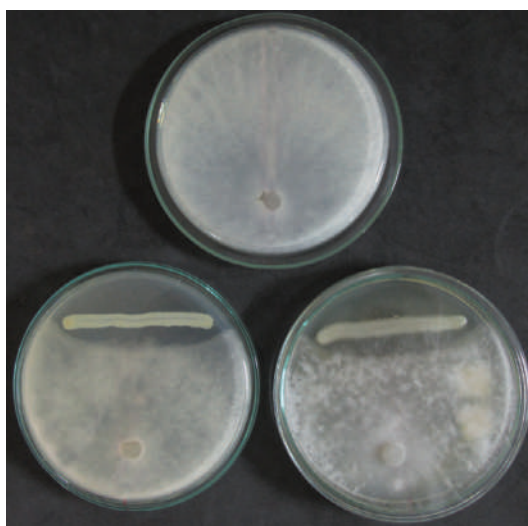


Fig. 7 Biological activity in dual culture assay promoted by *Burkholderia* sp. against the phytopathogenic fungus *Ceratomyces paradoxa*.

by adding 10% methanol followed by extraction by ethyl acetate at 1:1 proportion three times. The organic extract is transferred to a known weight tube and concentrated and then resuspended in DMSO (Sharma et al., 2016). Alternatively, the method which employs Amberlite® XAD16 resin can be performed for natural product extraction.

Quantitative evaluation of the biological activity of microbial products

Kirby-Bauer test

Material

BHIb

Müller-Hinton Cation Adjusted (MHCA) agar

Sterile 0.85% saline solution

Sterile and empty antibiogram disks

Incubator at 37 °C

Pachymeter

Media recipes

MHCA

Müller-Hinton Cation Adjusted broth - 22 g/L.

Agar - 15 g/L.

Sterilize by autoclaving for 15 min.

Experimental procedure

Kirby-Bauer test is performed according to the recommendations of Clinical and Laboratory Standards Institute (CLSI, 2017) in triplicates. This method recommends that the Petri dishes containing MHCA agar with 4 mm average thickness. To reach this measure, the following calculation can be applied:

$$V = \pi \cdot r^2 \cdot h$$

where, $\pi = 3,14$, r = Petri dish radius = 8,5 cm, h = Petri dish height = 1,5 cm.

Applying the values to the formula, we obtain the volume of 22.6865 mL of MHCA per plate.

Firstly, the test pathogenic bacterial strains should be cultivated from an isolated colony in BHIb for 12 h at 37 °C. The inoculation of the test strains should be done by spreading (3 to 5 times) the adjusted culture (in 0.85% saline solution) to 0.5 using the McFarland scale using a swab. Afterward, the antibiogram disk is placed on the plate, which is simply paper discs impregnated with known concentrations of antibiotics, crude extract, or pure compounds. Additionally, a positive and negative control should be used for assay validation. Plates must be incubated at 37 °C for 12–24 h. The results might be visualized by a halo around the disk, where the compound efficacy toward the pathogenic bacteria is estimated by the measure of the halo diameter (susceptible: >20 mm, intermediate: 15–19 mm, and resistant: <14 mm) (Fig. 8).

Antagonism index in pathogenic fungi

Material

PDA

Sterile and empty antibiogram disks

Incubator at 28 °C

Pachymeter

Media recipes

PDA

See Section “Isolation of microorganisms from soil, rhizosphere, and plants.”

Experimental procedure

The evaluation of the quantitative antagonism of microbial natural products toward pathogenic fungi is described in Quiroga et al. (2001).

Mycelial plugs of test fungus should be placed in the center of Petri dishes containing PDA, whereas a sterile disk impregnated with a known concentration of test compound placed on top of the plate. As a positive control, we recommend Benomyl, and the negative is represented by the normal growth of the test fungus. The determination of the antagonism index (AI) is based on the calculation of measures of target strain growth using the formula reported by Silva et al. (2016) (Fig. 9).

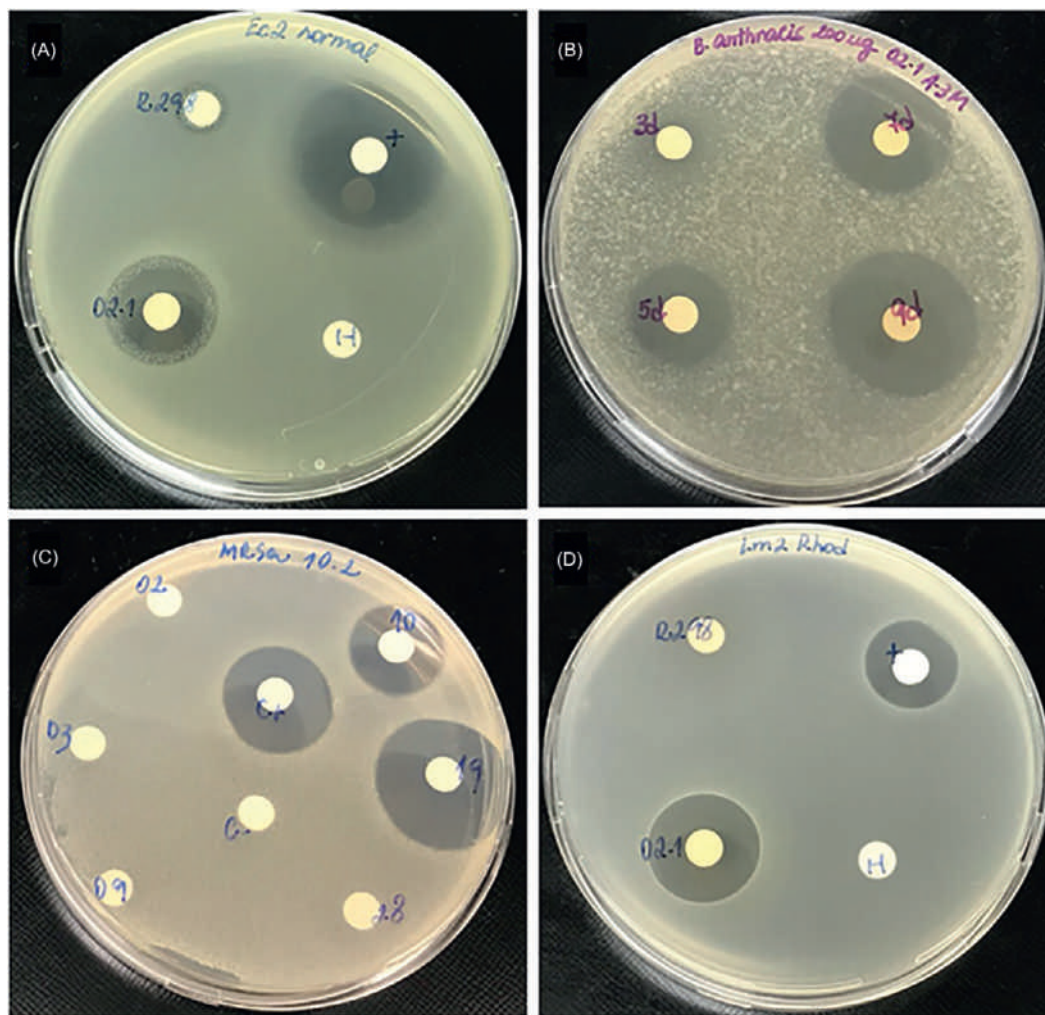


Fig. 8 Kirby-Bauer test showing antibacterial activity of actinobacteria at 200 µg/disk toward *E. coli* (A), *Bacillus anthracis* (B), Methicillin-resistant *Staphylococcus aureus* (C), and *Listeria monocytogenes* (D). Ciprofloxacin at 200 µg/disk and sterile DMSO are positive and negative control, respectively. Adapted from Cruz FDPN (2018) Isolation of the endophytic and rhizospheric microbiome associated to *Polygala* spp.: Evaluation of the biotechnological potential and antimicrobial activity. (Thesis), Federal University of Sao Carlos.

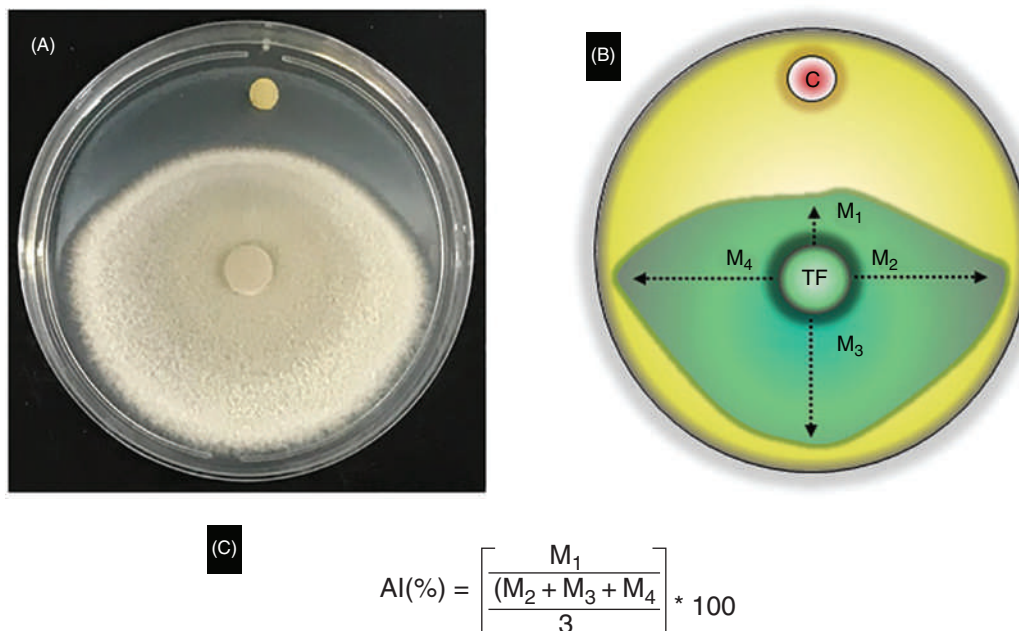


Fig. 9 (A) Biological activity of crude extract toward *Colletotrichum* sp., (B) Measures (cm) for the determination of AI which should be calculated by the formula (C). Adapted from Quiroga EN, Sampietro AR, and Vattuone MA (2001) Screening antifungal activities of selected medicinal plants. *Journal of Ethnopharmacology* 74(1): 89–96, doi:10.1016/S0378-8741(00)00350-0.

Minimum inhibitory concentration

Material

BHIb

MHCA broth

Sterile 0.85% saline solution

Incubator at 37 °C

Microtiter plate reader

Media recipes

BHIb

See Section "Overlay assay."

MHCA

See Section "Kirby-Bauer test."

Experimental procedure

This technique allows the evaluation of the accuracy of the test compound toward a bacterial pathogen, as well as a preview of its mechanism of action (bactericidal or bacteriostatic) (Tortora et al., 2015).

The evaluation of minimum inhibitory concentration (MIC) of microbial natural products is usually performed in triplicates, according to recommendations of CLSI (CLSI, 2018), where test pathogenic bacteria are cultivated in BHIb for 24 h at 37 °C and then diluted in MHCA broth until reach a final concentration of 5×10^5 CFU/mL.

Microbial natural products are diluted in several concentrations and finally added to 96-well plates containing the test pathogen suspensions and incubated for 24 h, at 37 °C. As controls bacterial suspensions in MHCA broth containing 1% DMSO might be used as negative controls, whereas an antibiotic (e.g., ciprofloxacin, tetracycline) as positive. The biological activity is analyzed by the measurement of the optical density of each well 24 h after administration of compounds using a microtiter plate reader.

The determination of the minimum bactericidal concentration (MBC) is based on the subculture of the MIC assay. The wells content (MIC, 2xMIC, MIC/2, and MIC/4) should be inoculated in the center of plates containing MHCA agar without streaking and incubated at the same conditions described. The determination of MBC is based on the calculation of the ratio between the lowest concentration of compound that completely inhibited bacterial growth divided by MIC values. Results less than or equal to 4 are considered bactericidal, whereas above is bacteriostatic (Pankey and Sabath, 2004).

Concluding remarks

Herein, we highlighted the importance of microbes on drug discovery. The biotechnological processes based on microbial metabolism gained notoriety and have increased substantially in recent years for their ability to synthesize plant-derived compounds.

The search for compounds with inhibitory activity against multidrug-resistant pathogens is critically important. In the last decades, synthetic tailoring has been the main strategy to improve the nuclear scaffolding established through analog generation. Although this approach has been beneficial, this line of research has faced a 'Discovery Void' for 30 years, of which no new class of drugs effective against problematic ESKAPE pathogens. In addition, pathogenic microorganisms generally can form biofilms. This cellular superstructure may exhibit greater resistance to antibiotics and cause serious and persistent health problems in humans. This fact that, in recent years, research and development of biofilm inhibitors have become a priority due to the advantage offered by preventing the spread of planktonic cells that can acquire resistance to therapeutic agents, since there are no drugs that have a specific target biofilm in clinical trials to date. Therefore, the possibility to tackle multidrug-resistant infections might decrease the morbidity associated with them and promote an increase in longevity.

It is known that in recent years, research and development of biofilm inhibitors have become a priority. This fact is due to the advantage offered by preventing the spread of planktonic cells. These cells can acquire resistance to therapeutic agents since there are no drugs that have a specific target biofilm in clinical trials to date. Therefore, the possibility to tackle multidrug-resistant infections might decrease the morbidity associated with them and promote an increase in health and longevity.

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Conflict of Interest

The authors declare no conflict of interest.

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A Geographical Framework for Analyzing Infectious Diseases

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Introduction

Infectious diseases are considered one of the most important problems of the 21st century, generating epidemiological outbreaks during the last decades (Chin et al., 2020). Infectious diseases are highly influenced by environmental and demographic factors, which play an important role modulating the relationship between hosts and pathogens, and the spread patterns across time and space (Oliveira et al., 2018). The increase in pandemic and/or epidemic outbreaks, as well as the emergence and re-emergence of infectious diseases are highly influenced by climate change, land use change, deforestation, and the increase of human population (Jones et al., 2008; Redding et al., 2019; Halliday et al., 2020). These are factors that may alter (A) the complex interrelationships existing between animal species, (B) the distribution patterns of reservoirs and vectors, and (C) the exposure of humans to pathogens and infectious diseases, due to the continuous increase of population, which is expected to reach 10 billion people by 2050 (Rosenthal, 2009; Redding et al., 2019; Guégan et al., 2020). Nevertheless, the availability of testing and treatments to face these pandemic and/or epidemic outbreaks is very limited in some countries and territories, which represents a challenge for public health planning if we aim to reduce or prevent a serious impact on the population in the future.

In this sense, understanding the relationship between geographical space and infectious diseases could represent a keystone to improve mapping, surveillance, predicting outbreaks in the future, as well as analyzing the spread patterns of diseases (Khan et al., 2012; Scarpino and Petri, 2019). The main components of the epidemiological triad have intrinsic spatial features that can be denoted in hosts, pathogens, and the environment (Hay et al., 2013). Hosts (defined as all organisms capable of harboring another) have a relationship with geographical space that accounts for (Poletto et al., 2012): (A) the movement of infected hosts across the population, (B) the heterogeneous distribution of population vulnerability associated with socioeconomic or cultural characteristics (Hagenlocher and Castro, 2015), (C) the distribution patterns of intermediate hosts (reservoirs) and vectors, (D) the spatial interaction between intermediate and final hosts (Alaniz et al., 2017a,b). On the other hand, the pathogen (defined as the agents that cause disease, illness, or injury) also has an intrinsic relationship with geographical space, which is closely related to its interaction with hosts and environment, for example: (A) potential mutational changes due to environmental characteristics and host density, (B) the ability to survive in different environments, (C) geographical distribution of its reservoirs and vectors (Rosenthal, 2009). Finally, the environment has an important role modulating the conditions for disease development, which can be altered by human impacts, thus providing suitable conditions for disease emergence linked to ecosystem destruction, pollution, overcrowding, temperature increase (due to climate change), among others (Khan et al., 2012).

These links between infectious diseases and their geographical dimension need to be explored and conceptually clarified, allowing to increase the capacity of decision making in public health based on the implementation of epidemiological modeling and predictive tools (Khan et al., 2012; Hay et al., 2013; Scarpino and Petri, 2019). In this chapter, we present an up-to-date conceptualization of infectious diseases from a geographical approach.

Spatial dimension of infectious diseases

Infectious diseases are determined by the interactive effects of three components defined as the epidemiologic triad, which includes the host, the pathogen and the environment (Engering et al., 2013). In this section we explored the geographical dimension of each component.

Georeferencing host

The host is defined as individuals susceptible to acquiring a pathogen and developing (or not) the disease. From a conceptual approach, there are different types of hosts: (A) animal hosts, which are mainly reservoirs and vectors that act as intermediate hosts for pathogens and (B) clinical hosts, which are humans that acquire the pathogen. These hosts have differences from a geospatial dimension.

In the case of animal hosts, a large proportion of individuals (mainly wildlife such as bats, rodents, monkeys, etc.) is not tested and their geographical records usually remain only as georeferenced occurrences (Escobar, 2020). This generates uncertainty in terms of the positivity of wildlife animals, so that it is possible only to access the distribution of potential hosts, but not corresponding to the actual distribution of infected reservoirs (Astorga et al., 2018). In fact, this type of data is in the public domain, freely available and easily accessible through platforms such as Global Biodiversity Information Facility (<https://www.gbif.org/>) and the Malaria Atlas (<https://malariaatlas.org/>) (Robertson et al., 2014). On the other hand, for zoonotic diseases transmitted by domestic and livestock animals, testing is much more implemented and tracked. However, the obtained data for these cases is not widely and publicly distributed, and the access to geographical coordinates is less common (Chand, 2021).

In the case of human hosts, there are sub-components related to their vulnerability such as comorbidities, nutritional status, behavior, genetic susceptibility, and immunological capacity, which could be georeferenced from patients' clinical records (Beck et al., 2004; Trammell and Toth, 2008). Additionally, positivity for infectious disease may be also georeferenced by public health governmental agencies. However, not all diseases can be georeferenced with a spatially explicit latitude/longitude or address because the privacy protection of patients is only available at regional or sub regional levels (Hollis, 2016). On the other hand, sub-components related to socioeconomic vulnerability that have an important link to infectious diseases are also georeferenced in national and subnational censuses and cadastres (Mareze et al., 2019). This information is usually available on a square scale from censuses and could be useful for modeling host vulnerability.

Georeferencing pathogens

This component corresponds to disease generating agents including viruses, bacteria, fungi, protozoa, and prions, among others. These agents have different characteristics linked to transmissibility, pathogenicity, virulence, and toxicity, among others, which determine their potential for spreading throughout the population and geographical space (Casadevall and Pirofski, 2001). The biological cycle of transmission of these agents could be closely linked to the geographical space, which could be classified into the following non-exclusive types:

- (A) Vector or reservoir driven agents: Vector-borne infectious diseases are maintained in nature through the interaction between vertebrate and invertebrate species, the latter usually being a hematophagous arthropod organism acting as an intermediate host (mosquitoes, ticks, fleas, and kissing bugs, among others) that could transmit a pathogen to a final host, either animal or human (Sadeghieh et al., 2020). This type of disease affects over than 1 billion people annually (Fig. 1), some examples of epidemiological importance include: (i) mosquitoes (Fig. 1A), one of the most important vectors for several diseases in the tropical zone, transporting viruses responsible for malaria, chikungunya, West Nile fever, Zika, yellow fever, Japanese encephalitis, and lymphatic filariasis (Franklinos et al., 2019); (ii) Kissing bugs (Fig. 1B), an important source of contagion of the protozoan *Trypanosoma cruzi* which is the etiological agent of Chagas diseases in the Americas (Klotz et al., 2014); (iii) Ticks (Fig. 1C), the main cause of several diseases in North America and Europe, being associated with Lyme disease, anaplasmosis, babesiosis, ehrlichiosis, Powassan virus disease, borrelia, Rocky Mountain spotted fever and tularaemia (Walker, 2014). On the other hand, agents may also be transported by vertebrate reservoirs; examples of epidemiological importance include: (i) bats (Fig. 1D), considered important carriers of agents that can produce disease, such as Coronaviridae (SARS, MERS), Rhabdoviridae, Paramyxoviridae, Astroviridae, Adenoviridae, and 21 other virus families (Fig. 2; Letko et al., 2020); (ii) rodents (Fig. 1E), related to a variety of diseases such as Hantavirus, haemorrhagic fever, Lassa fever, leptospirosis, lymphocytic choriomeningitis, omask haemorrhagic fever, plague, rat-bite fever, salmonellosis, South American arenaviruses, and tularaemia (Astorga et al., 2018; Rabiee et al., 2018).
- (B) Agents in the environment: Different agents may include environmentally free steps in their biological cycles, where they may remain until they infect a host. This type of agents may remain in trees, air, soil, or water until infecting an animal or human host; examples of epidemiological importance include: (i) Fungal agents such as *Cryptococcus* spp. (Fig. 1F), which can change form between yeast and hyphal, the latter being capable of releasing spores that can be present on trees until inhaled by domestic animals or humans. This agent is responsible for cryptococcal meningitis that mainly affects immunosuppressed patients (Carvajal et al., 2020). (ii) Schistosomiasis (Fig. 1G), which is a disease caused by a trematode parasite that produces acute or chronic inflammation of tissues, commonly affecting the liver, intestines, and the bladder. Its eggs are shed from infected humans via feces and urine to the aquatic environment, where they hatch and release miracidia that penetrate the tissues of water snails. This disease is closely linked to poor water sanitation in African countries, and about 290.8 million people requiring treatment during 2008 (Leshem et al., 2008). (iii) Enteric pathogens (Fig. 1H), such as *Cryptosporidium parvum* and *Giardia* spp. The latter is a flagellated protozoa responsible for gastrointestinal symptoms and can also produce chronic enteric disease. The infective cysts of *Giardia* spp. may be present in water, food or hands/fomites, and may affect over 200 million people per year (Cacciò et al., 2005).

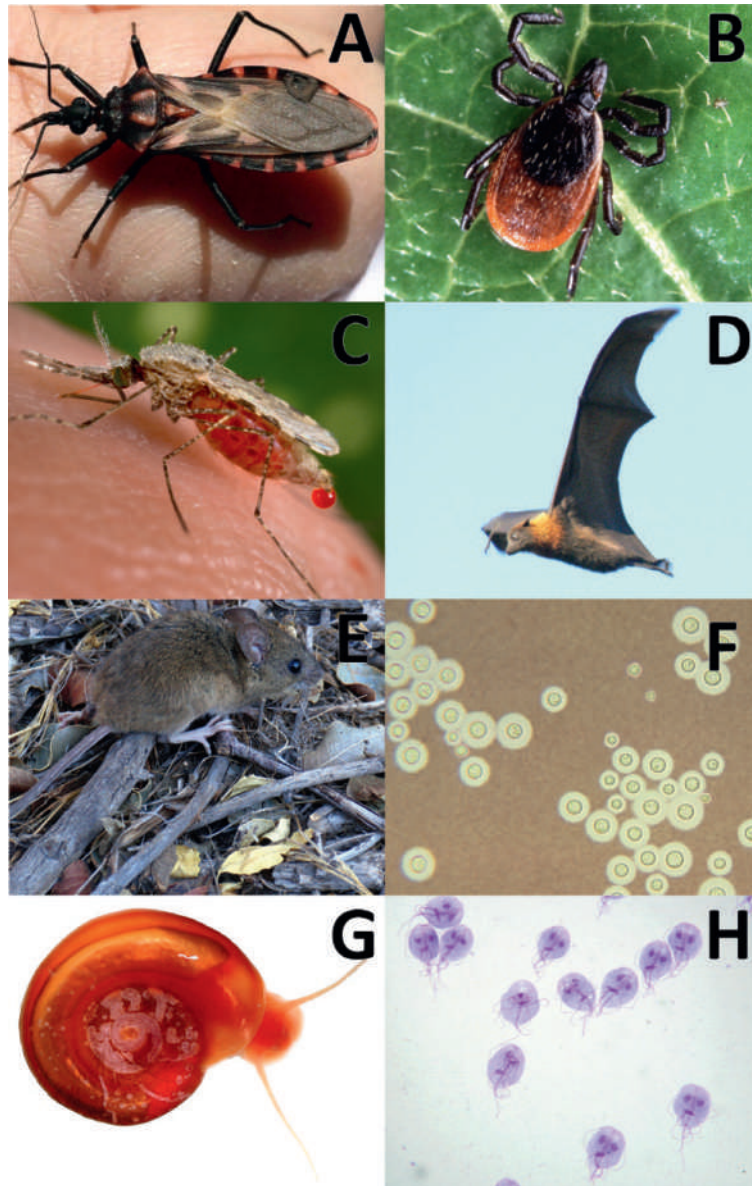


Fig. 1 Vectors, reservoirs, and pathogens. (A) Kissing bug (*Triatoma* spp.), (B) Deer tick (*Ixodes scapularis*), (C) Mosquito (*Anopheles* spp.), (D) Bat (*Pteropus vampyrus*), (E) Rodent (*Oligorhizomys longicaudatus*), (F) fungal pathogen (*Cryptococcus neoformans*), (G) reservoir snail of *Schistosoma mansoni* (*Biomphalaria* spp.), (H) enteric parasite (*Gardia* spp.)

(C) Human to human infectious diseases: This type characterizes to a series of diseases with a transmission cycle linked to humans, in which animals are not involved or were involved only during the initial phase of transmission. This type of infectious disease may have multiple transmission pathways including sexual, airborne, transplacental, and blood transfusion, among others (Wolfe et al., 2007). In this case, the transmission pathway is highly relevant to the spatial dispersion of the disease, which is also influenced by other factors related to the agent discussed above. A high proportion of respiratory diseases fall into this category, such as influenza, respiratory syncytial virus, adenovirus, parainfluenza, while sexually transmitted diseases are also within this category (gonorrhea, HIV, herpes, hepatitis, among others; Vink et al., 2014; Workowski and Berman, 2011). A relevant case is COVID-19, which reached the category of pandemic due to its high dispersal capacity associated with the characteristics of the virus characteristics, as well as the time of onset of symptoms onset and its low mortality but high infectivity. The origin of this virus is still under discussion; however, the extensive scientific background supports the hypothesis of an animal origin linked to pangolins and bats (Wiersinga et al., 2020).

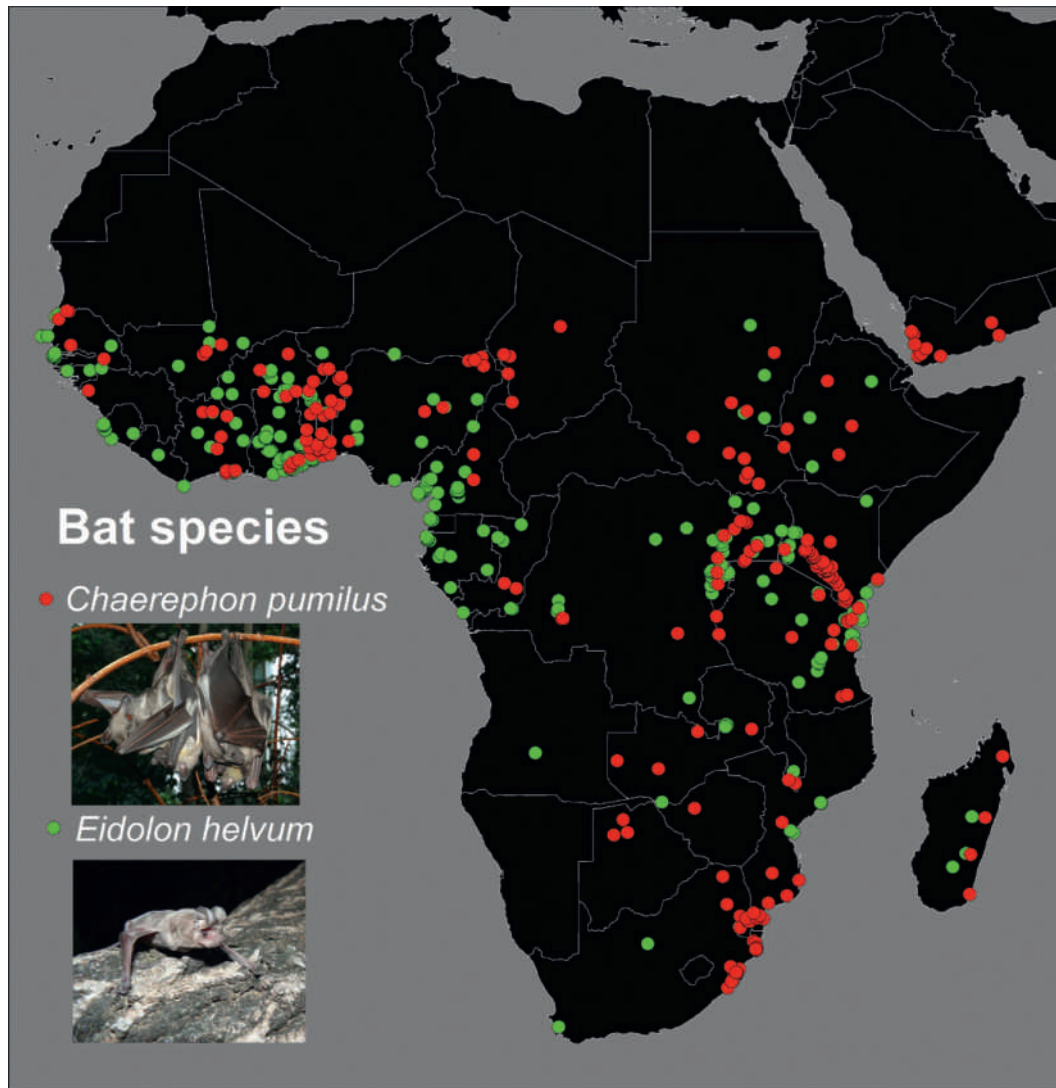


Fig. 2 Example of georeferencing occurrences of two bat species considered reservoirs of the Ebola virus in Africa.

Modulating environment

Agents and hosts interact within an environmental space, which modulates the generation of diseases. In the natural environment, pathogens are usually found in a pseudo-equilibrium state that derives from biodiversity, in which effects such as dilution and nidity are present (Halliday et al., 2020). First, the dilution effect is responsible for reducing the proportion of susceptible hosts in the ecosystem, thus reducing the spread of the pathogen spread throughout the ecological assemblage (Halliday et al., 2020). On the other hand, natural nidity is related to the stable circulation of the pathogen in the ecological community, which reduces the stochastic effects that could produce an outbreak. Human impacts on biodiversity may alter nidity and dilution effects by reducing the species and genetic diversity; the most important drivers are: (i) physical-chemical habitat degradation, (ii) habitat destruction and fragmentation, (iii) arrival and colonization of invasive species, (iv) migrations to new habitats, among other (Halliday et al., 2020).

On the other hand, climatic, meteorological, and atmospheric variables are also important for the relationship between host and pathogen. A high proportion of diseases are constrained due to climatic characteristics related to temperature and humidity, important factors that determine suitability for pathogens, vectors, and reservoirs in different environments (Rosenthal, 2009; Escobar and Craft, 2016). As climate change is projected to generate significant shifts in global climatic configuration, these changes will affect all the living organisms on the planet, including pathogens (Wu et al., 2016). Finally, at the local scale, environmental characteristics related to the quality of life in urban and rural areas also have an important influence on the potential generation of epidemic or pandemic outbreaks (Redding et al., 2019). These factors include air quality and overcrowding, which can increase the vulnerability of hosts to pathogens (Redding et al., 2019).

Spatial modeling and infectious diseases

Ecological niche modeling

A widely used approach to modeling biological organisms is ecological niche modeling, with an origin applied to ecological and biodiversity conservation sciences (Guisan et al., 2013). However, it has been recently used for modeling infectious diseases, mainly to estimate the potential distribution of vectors, reservoirs, and pathogens (Fig. 3; Escobar and Craft, 2016; Escobar, 2020). This approach is supported by the niche theory, which suggests that biological organisms have different biotic and abiotic requirements linked to food, refuge, environment, and biological interactions, among others (Soberon and Nakamura, 2009). These requirements are stable on short timescales because they are the end result of thousands or millions of years of evolution, so they cannot change on the human timescale. Theoretically, there are multiple types of ecological niches (Eltonian, Grinnellian, and Hutchinsonian); however, the most accepted approach is the one presented by Evelyn Hutchinson in 1954, who defined the ecological niche as the N-dimensional space of variables in which the fitness (biological adequacy) of organisms is positive

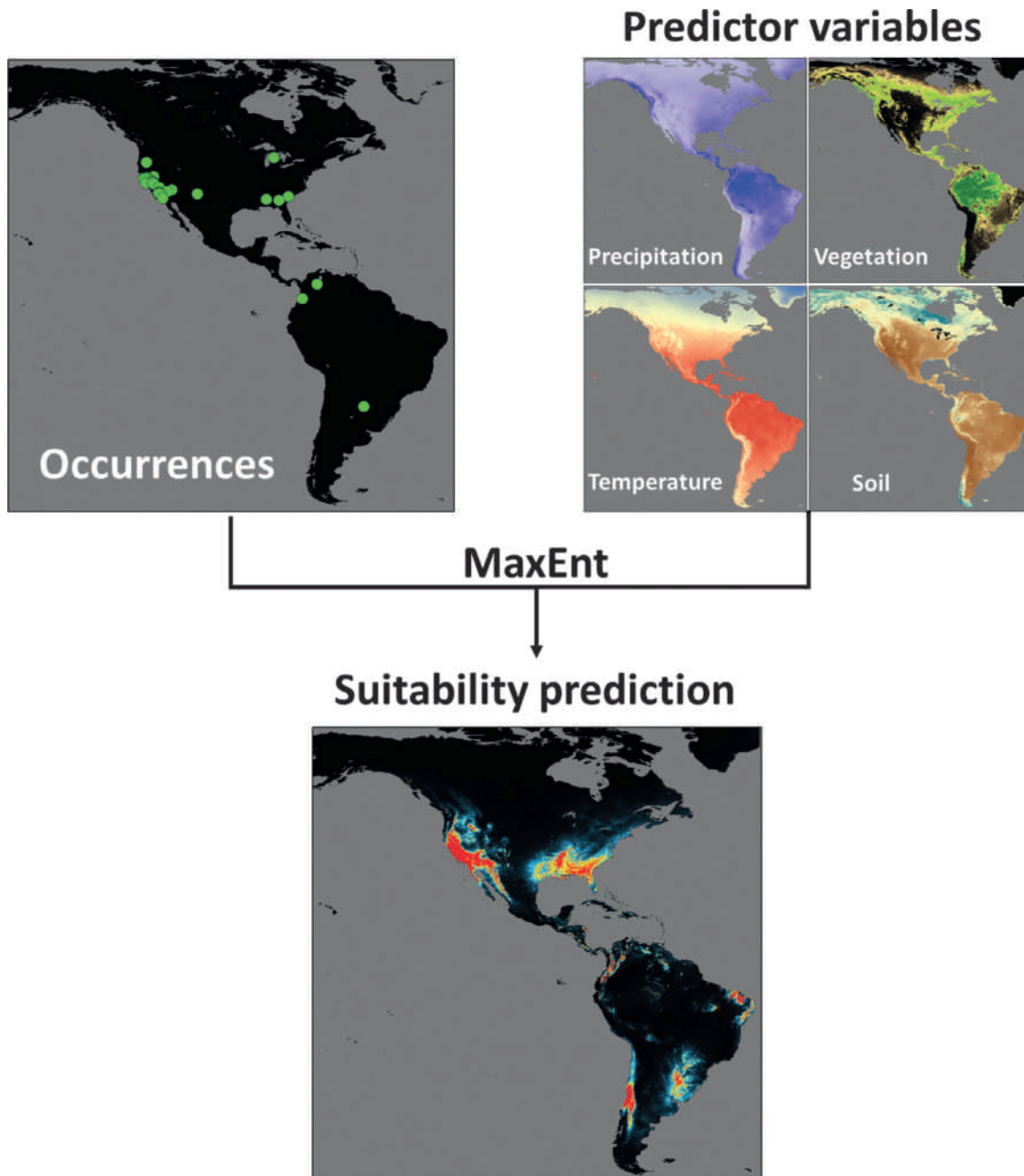


Fig. 3 Diagram of ecological modeling function to predict habitat suitability (red colors means high suitability).

(Colwell and Rangel, 2009). This means that within this N-dimensional hypervolume, the population will increase (high natality and low mortality), while outside, it will decrease. The ecological niche is an intrinsic property of organisms (each individual has its own niche), thus this individual niche could be averaged at a population, metapopulation, or even a species level (Soberon and Nakamura, 2009). This theory also identifies two different types of niche: (A) potential niche, which corresponds to the entire N-dimensional space of variables in which a species has a positive fitness, in absence of ecological interactions with other species. (B) Realized niche, which corresponds to the proportion of the N-dimensional space that the species effectively uses due to restrictions derived of ecological interactions with other species (competence, predation, mutualism, etc.; Soberón, 2007; Soberon and Nakamura, 2009).

Modeling procedures consider the use of georeferenced occurrences of the organism, which is combined with a set of environmental predictor variables (Fig. 3; Guisan et al., 2013). Then, these datasets are combined by using a modeling algorithm, which can be based on linear statistical modeling, interpolation, or machine learning. The latter methodological approach has been the most widely used, including algorithms such as the boosted regression tree, random forest, support vector machine, and maximum entropy (Hirzel and Le Lay, 2008). MaxEnt is the most used software for modeling species niche, which uses environmental layers in raster format (.asc) and a table with the name of the species and the latitude and longitude of each record (.csv) (Phillips et al., 2006). These models have a protocol that considers the reduction of spatial autocorrelation of occurrences that helps to reduce sampling biases, and the reduction of variable collinearity to avoid model overfitting (Alaniz et al., 2017a,b; Carvajal et al., 2020). The final results include a map of habitat suitability for the species and the relationship between the predicted suitability with each used environmental predictor variable (Peterson and Soberón, 2012). The latter product is highly useful to interpret ecological and biological requirements of the modeled organism. The initial models only considered bioclimatic variables, but most recent studies have integrated them with detailed satellite data showing information on air pollutants, biophysical parameters of the ecosystem, soil characteristics, and meteorological conditions, among others (Anderson, 2017; Alaniz et al., 2020). This has promoted an increase in the quality and predictive power of these types of model, which usually select variables taking into account the biology of the reservoir, vector, or pathogen they are trying to model.

The predicted suitability of MaxEnt can be homologated to the potential abundance of the organism, then they can be combined with human population density maps, allowing to estimate the potentially exposed population (Alaniz et al., 2017a,b, 2018). This means that in areas where the model predicts high reservoir, vector, or pathogen suitability, and these areas coincide with high population density, the exposure risk will be higher (Fig. 4).

These models are also useful to predict the potential distribution of a reservoir, vector, or pathogen in zones that have been recently colonized, or even before the colonization (Escobar and Craft, 2016; Escobar, 2020). This is generated by considering the niche conservatism principle mentioned above. Finally, these models can predict the future distribution of reservoirs, vectors, and pathogens by integrating global circulation models of climate change as predictor variables (Elith et al., 2010). Both analyzes could be of great use in designing preventive actions in the event of the possible arrival or expansion of the geographical distribution of the pathogen. In fact, this has multiple advantages for spatial prioritization of tests and treatments, which will be discussed below.

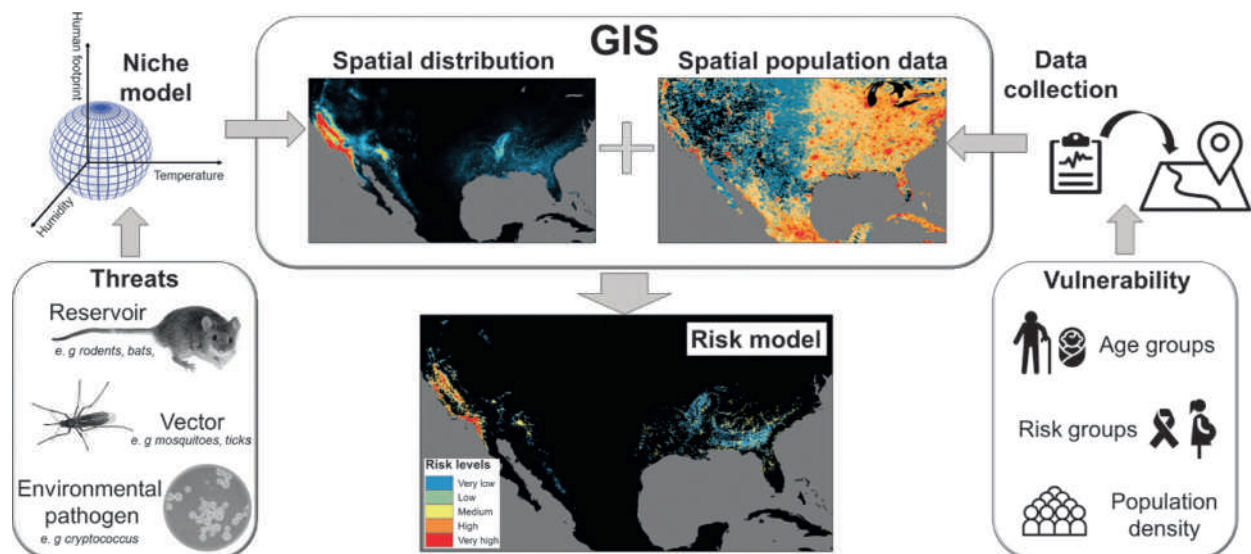


Fig. 4 Diagram of spatially explicit estimation of risk by combining suitability model of pathogens with layers of human population (vulnerability) in a GIS environment. This framework includes the consideration of particular risk groups based on their vulnerability to become infected or develop the disease, as well as population density.

Bayesian modeling

Bayesian spatio-temporal analysis allows the study of space-time changes and pattern of diseases, considering spatial analysis units and time-series that enable identifying areas or time periods in which risk could increase (Wah et al., 2020). This approach addresses several problems that frequentist statistics present when modeling diseases, related with spatially structured errors, non-linearity of trends and data, small sample size, or missing data (Baptista et al., 2016). These models can use the number of cases or deaths to obtain variables such as relative risk of incidence, survival time, or mortality, incorporating covariates such as demographic, socio-economic, life styles, clinical, meteorological, environmental and health access variables (Wah et al., 2020). There are several types of model such as generalized linear mixed models, P-spline and B-spline models, the Poisson log-linear model with clustering and smoothing components, the transformation class of spatio-temporal cure rate CAR survival models, and the Besag, York, and Mollié (BYM) moving average model. The BYM model allows incorporating random effects associated with: (A) unstructured errors, linked to the potential correlation between variables or time series and (B) structured errors, derived from spatial clustering of data or adjacency of spatial analysis units (Samat and Mey, 2017). Adjacency matrices of spatial analysis units are incorporated to generate BYM models, which are used to deal with structured errors. This model considers spatial analysis units, which usually correspond to administrative units such as municipalities, districts, or regions, using as input data the total number of cases or deaths accumulated for each spatial unit for a determined time period (day, week, month). As output, the models show the spatial and temporal patterns of the estimated variable (e.g., mortality or incidence), also showing the level of correlation and the importance of each covariate for risk estimation (Samat and Mey, 2017; Aswi et al., 2019). This allows us to estimate the influence of different factors on the relative risk, which could inform public health actions in prevention, control and management (Aswi et al., 2019).

Applications (API)

Recently, the situation arising from the COVID-19 pandemic has prompted national and international health agencies to share information publicly through websites. This type of visualizers are developed using different API development tools such as ArcGis Online and Google Earth Engine, which provide an interactive platform for publishing maps and spatial information on infectious diseases. An example of this is the John Hopkins University's COVID-19 map, which was launched in the initial phase of the pandemic and has been active for about 2 years, providing useful information about the disease (<https://coronavirus.jhu.edu/map.html>) (Hay and Snow, 2006). Another example is the explorer of the MalariaAtlas project (<https://malariaatlas.org/explorer/>), which provides spatial data on multiple dimensions of malaria disease, including vectors, prevalence, and human population factors, among others.

Regarding COVID-19, multiple countries have implemented visualizers with information at the national level information, providing statistics on daily incidence and mortality, and sharing statistics on vaccination (Franch-Pardo et al., 2020). This type of APIs could also be implemented for mobile platforms (Android, IOS), allowing users to share information about vectors and reservoirs (Saran et al., 2020).

GIS processing

Another widely used tool for analyzing spatial dynamics of infectious diseases are the Geographic Information Systems (GIS), which provide an interface for managing, processing and analyzing spatial data. GIS have a wide variety of geoprocessing applications that can be useful for overlapping layers of different spatial information, allowing to identify spatial patterns and congruences (Khashoggi and Murad, 2020; Saran et al., 2020). Popular software such as ArcGIS, SAGA GIS, and QGIS have a wide range of geoprocessing tools that allow implementing interpolations, principal components analysis, spatial clustering analysis, clipping, merging, dissolution, and geographically weighted regressions, among others. In fact, GIS is very important for linking information from different sources such as tables, ecological niche models, population density grids, cadastres, and censuses (Khashoggi and Murad, 2020). As a result, GIS offers the possibility of processing these data and obtaining new products, such as (A) risk level maps, by combining niche models with population data (Fig. 4), (B) shift in pathogen distribution derived from climate change, (C) interpolation of cases to generate incidence maps, (D) joining maps of administrative units with clinical record charts, among other (Khashoggi and Murad, 2020).

Data collection

Spatial modeling of infectious disease requires georeferenced data to generate predictions in time and space, which can be typically sparse depending on the specific disease (Escobar, 2020). Clarifying data sources for reservoirs, vectors, and human cases, could be an important tool for epidemiological modeling (Escobar, 2020). In this regard, an important issue related to determining the location of the pathogen, which could be found within humans, animals, or in the environment (Escobar and Craft, 2016; Escobar, 2020). For the case of pathogens inside humans, the best source of data could be obtained from health centers (hospitals or primary centers) that have individual clinical records of each patient detailing current and past pathologies. Typically, health centers serve patients in certain geographical areas, having certain jurisdiction or areas of influence that may be linked to community territorial

units (Cramer et al., 2020). These clinical records information on comorbidities, address, age, sex, and pathologies of each patient, which could be useful at governmental level or for scientific studies (considering informed consents; Emanuel and Boyle, 2021). In this sense, due to ethical and privacy constraints this information is usually available at coarse territorial level (municipality, district, or region; Hollis, 2016). For reservoir- and vector-driven zoonotic diseases, an important assumption for modeling is that all individuals of the target species could carry the pathogen. Information may be available from multiple sources: (A) veterinary records, which have a clinical history of animals similar to that of human clinical records but with less detail, there is generally a bias in the availability of these records for domestic animals (mainly dogs and cats); (B) livestock animals, which are under continuous monitoring to avoid potential outbreaks arising from zoonotic pathologies, this group corresponds mainly to pigs, cows, goats, and chickens. In this case, if a zoonotic pathogen is detected, governmental agencies proceed to report the specific location of the infected animals, which is also followed by appropriate sanitary measures (Beard et al., 2018); (C) wildlife animals, in this case, the largest proportion of records are derived from studies that focus on biological aspects of the organism, which may be found in scientific publications, publicly available data collections, and records compiled through citizen science (Escobar and Craft, 2016). Most of these records are not created for epidemiological purposes, so the availability of data on pathogen positivity in these animals is highly restricted to certain species, and may be found in specialized scientific studies (Escobar, 2020). Finally, in the case of pathogens in the environment, the availability of records is very restricted, which could be developed after an epidemic outbreak in order to find the origin of the pathogen (Carvajal et al., 2020). This could also be developed by health agencies to monitor water quality, which lead to the identification of pathogens collected in samples. These records are hardly shared by governmental agencies and may be found in specialized scientific studies and official governmental publications.

Spatial prioritization of resources

Spatial information is valuable for public health policy and management because, as we have shown above, infectious diseases have an intrinsic relationship with geographical space. In a large proportion of countries around the world, public health systems are operating at the limits of their capacity, due to the large number of emerging and re-emerging infectious diseases and the limited availability of resources to provide adequate screening and treatment (Elsharkawy et al., 2018). This has evidently intensified in the aftermath of the COVID-19 pandemic, where proper testing, traceability, and treatment is essential to provide adequate care for all infected patients (Franch-Pardo et al., 2020; Wiersinga et al., 2020). In fact, previous epidemic outbreaks have also shown the need to prioritize resources such as in the case of *Cryptococcus* in Canada during 1999, SARS in China in 2003, MERS in the Middle East during 2012, Ebola Virus in Guinea during 2014, and Zika virus in Brazil during 2017 (Verma, 2003; Alghamdi et al., 2014; Uejio et al., 2015; Alaniz, 2019; Letko et al., 2020). On the other hand, a large proportion of pathogens are responsible for a high and stable number of infected patients globally, forming part of an endemic transmission cycle such as malaria, schistosomiasis, hantavirus, Chagas disease, and Lyme disease, among others (Medley and Vassall, 2017). We classify the contribution of spatial data to public health into four main points:

- (A) Management plans: Public health systems must be prepared to respond to endemic pathogens, which may have specific patterns in space and time. Management plans include annual vaccination campaigns against recurrent viruses that have a seasonal behavior (e.g., influenza; Klepser et al., 2019). Countries often have a list of recurrent diseases that are included in public health care programmes for the population; for these pathogens, spatial information could be very useful because risks have a heterogeneous spatial distribution (Jones et al., 2008; Medley and Vassall, 2017). In this sense, having the specific information on the risk of exposure to different pathogens could be useful to allocate medical supplies and focus efforts on the most exposed population (Alaniz et al., 2017a,b). Additionally, all information generated should be recorded and spatially registered, which, at the same time, will provide more data to improve spatial models as feedback to improve public health management (Fig. 4).
- (B) Preventive plans: Prior to the occurrence of an epidemic outbreak, spatial modeling of infectious disease can be very useful to detect areas where pathogens have high suitability (Saran et al., 2020). On the other hand, mapping of initial cases could provide information on their spatial distribution representing an initial phase of a potential outbreak (Hafeez et al., 2017). Although the occurrence of an outbreak is a highly stochastic process, having this information allows the health system to be prepared for an eventual case (Scarpino and Petri, 2019). This information should motivate public health agencies to implement education and informative campaigns, as well as to prevent or avoid actions in these high-risk areas that could trigger an outbreak (Beard et al., 2018). An example of this was the Nipah virus in Malaysia during 1999, where deforestation and urbanization of the native habitat of *Pteropus vampyrus* bats triggered an epidemic outbreak with high lethality (Epstein et al., 2020).
- (C) Response plans: Following the occurrence of an epidemic or pandemic outbreak, urgent actions by public health agencies should focus on controlling the spread of the pathogen across space and population (Saran et al., 2020). In this sense, spatial information provides essential tools to improve decision making processes in response to the outbreak, by prioritizing resources in areas of high incidence, or by increasing preventive actions in areas where the pathogen have not yet arrived (Carvajal et al., 2020). On the other hand, spatial data on incidence or number of critically ill patients is essential to allocate resources such as hospital beds, medicines, health professionals, and testing supplies (Sun et al., 2020). An example of this has

been the current COVID-19 pandemic outbreak, where spatial information has played an important role in decision making on lockdowns and quarantine, as well as the allocation of medical supplies (Franch-Pardo et al., 2020; Sun et al., 2020).

Advantages and limitations

Spatial modeling is a useful tool for public health planning, providing information to develop appropriate policies related to the allocation of treatments, medicines, and professionals. On the other hand, it is important for developing preventive and control actions in the face of possible epidemic or pandemic outbreaks. In this sense, the main advantage is related to the optimisation of limited resources, a common scenario in developing countries.

There are certain limitations of spatial modeling that need to be discussed if we expect a correct interpretation of its results. One example is the collection of information from clinical records, which present different problems, such as the georeference of infected individuals and the lack of standardization of questionnaires (Crameri et al., 2020). In the case of georeferencing infected individuals, the acquisition of this data could be problematic due to ethical constraints, especially the clinical records of patients suffering from the disease that could be socially stigmatized (Hollis, 2016). A possible solution to this could be to provide this data at a higher administrative level (municipality, district or region), and in completely anonymous terms in order to protect the privacy of patients (Hollis, 2016). Another problem is the lack of standardized questionnaires for clinical diagnosis and records, which often vary between countries or even within countries when patients are treated in public or private health facilities (Emanuel and Boyle, 2021). A solution to this is the generation of international standards that are commonly used in other fields, such as safety and quality (similar to ISO standards). Additionally, countries should have a health policy that demands public and private health centers to apply the same questionnaires, with the aim of accessing homogenous information that is useful for the improvement of health programmes (Chin et al., 2020). Finally, another important limitation is related to the scarcity of systematic sampling of pathogens in the environment, as well as reservoirs and vectors (Fig. 2; Escobar and Craft, 2016). We recommend that countries implement systematic sampling programmes with a multidisciplinary approach, combining the efforts of governmental health, agricultural, and wildlife agencies to generate this information.

Future perspectives

Currently, geography is essential to medicine and public health because it enables understanding different phenomena related to the spread of disease, infection, and mortality through space and time. In recent decades, an explicit relationship has been identified between geographical transformations resulting from anthropogenic activities and the emergence or re-emergence of infectious diseases. In this chapter, we highlight the conceptual and theoretical links between geography and infectious diseases, showing how each component of the epidemic triad (host, pathogen, and environment) could be linked to a spatial dimension. Our aim is to provide clear information on spatial modeling to medical and health professionals, decision makers, and students, aiming to broaden their knowledge on this interesting topic.

Infectious diseases have represented one of the main challenges for human population for centuries, from the black death in the 14th century, to acquired immune deficiency syndrome (AIDS) in the 80s, and the current COVID-19 pandemic. Estimates suggest that millions of people die annually from infectious diseases, such as respiratory diseases, AIDS, diarrhoeal, tuberculosis, and malaria, among others. As populations continue to grow and ecosystem degradation continues, we expect infectious diseases to continue to emerge and re-emerge, which could have considerable impacts on economies and societies around the world. Enormous advances in data science and computational processing power represent a powerful tool in the fight against infectious diseases, as 1980 satellite technology and GPS enable spatial tracking of countless parameters and data. Moreover, the recent development of more complex and accurate modeling approaches opens new frontiers on the study of infectious diseases. In fact, it is necessary to advance in the implementation of these new tools, considering that pathogens are continuously evolving and changing their ecological niches much more rapidly than their hosts. On the other hand, the new climatic conditions imposed by climate change poses a highly unpredictable scenario, where we do not have enough knowledge on how pathogens would react or adapt. The development of predictive models of already known pathogens is important for preventive actions against possible outbreaks, which should be accompanied by the development of new and more effective surveillance, diagnostics, vaccines, and treatments. In fact, the current COVID-19 pandemic has highlighted how linking geography and medicine could provide effective solutions against the scarcity of resources and the dynamic changes of the disease across the population.

The mechanisms underlying the increase in infectious diseases over the last decades, which is undoubtedly related to alterations of natural ecosystems and human overpopulation, need to be sought. In this sense, humanity needs to incorporate a respectful attitude towards biodiversity, acknowledging its regulatory role over pathogens and the biological cycle of these infectious diseases. We believe that paradigm shift is necessary to ensure the future survival and well-being of the human population, which must include the harmonious coexistence with all other living organisms on earth.

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Relevant Websites

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<https://coronavirus.jhu.edu/map.html>—Johns Hopkins Coronavirus Resource Center.

<https://ecogeografiaciencia.wixsite.com/ecogeografia>—Ecogeografía N.G.O website.

Isotopologue Profiling of Infectious Disease

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Introduction

Resistance of human bacterial pathogens to one or even more antibiotics is rapidly emerging and represents a severe world-wide health problem. At the same time, the discovery of new targets allowing the development of novel antibiotics is hampered due to the lack of economic incentives and redundant hit compounds in drug screening programs based on established platforms (Lakemeyer et al., 2018). Therefore, innovative ideas exploiting new methodological approaches are urgently needed. Metabolism and especially the central carbon metabolism could provide a promising and alternative field for drug discovery due to several reasons (Murima et al., 2014): (i) Metabolic transformation of nutrients to afford energy (e.g., ATP) is largely related to virulence of many pathogens as well as persistence of bacterial infections (Eisenreich et al., 2017; Best and Abu Kwaik, 2019; Eisenreich et al., 2019, 2021). (ii) Bacteria partially rely on different metabolic pathways in comparison with their human host cells (Rohmer et al., 2011). It is therefore intriguing to develop drugs against metabolic enzymes of unique bacterial pathways, since inhibition of these reactions are believed not to cause severe side effects in humans. (iii) Since metabolism controls the emergence of bacterial persistence (Eisenreich et al., 2021), sophisticated inhibitors against the key reactions involved in this axis play an important role in the future. (iv) Reprogramming the central carbon metabolism of human pathogens by innovative drugs increases the susceptibility to known antibiotics (Stokes et al., 2019; Liu et al., 2019).

Unfortunately, the metabolism of bacterial pathogens is poorly characterized, although the genome sequences of the major pathogens are known in considerable detail. However, knowledge about the metabolic capabilities based on genome annotations of metabolic enzymes does not necessarily consider all metabolic reactions being functional especially under the conditions of actual infections. Therefore, more analytical techniques have been developed for the full assessment of the metabolic pathways being active in a cell even under complex conditions. These techniques include (i) transcriptomics, (ii) proteomics, (iii) metabolomics, (iv) ^{13}C -flux analysis and isotopologue profiling.

Transcriptomics, which nowadays mostly refers to RNA sequencing, and proteomics allow quantitative and full coverage of the cellular transcriptomes and proteomes, respectively. Studies using these techniques have also shown high reproducibility in inter-laboratory comparisons (Collins et al., 2017; Giraldez et al., 2018). In contrast, metabolomics is still limited to a few hundred compounds out of the thousands of compounds comprising the whole metabolome (Zamboni et al., 2015) and quantitative metabolic profiles are difficult to replicate (Mirsaeidi et al., 2015). While proteins and RNA-transcripts can be quantitatively extracted, the structural diversity of metabolites prevents a unified extraction protocol for a given metabolome (Nielsen, 2017). Additionally, the metabolome is most directly affected by environmental changes leading to high turnover of metabolites (Zamboni et al., 2015). In comparison to metabolomics, the determination of metabolic fluxes (fluxomics) and ^{13}C -based pathway analyses (isotopologue profiling) typically rely on smaller subsets of metabolites and are therefore easier to be standardized. To combat the issue of high metabolite turnover, protein-bound amino acids are mostly used in isotopologue profiling studies since they are stable, can be easily extracted and are highly abundant in cellular material (Sauer, 2006).

While transcriptomics can be routinely performed with low numbers of cells through RNA amplification (Lowe et al., 2017; Lafzi et al., 2018), proteomics and metabolomics typically require several mg of dried cells (Allwood et al., 2013; Mertins et al., 2018). Here, the sensitivity of mass spectrometers, even more of nuclear magnetic resonance (NMR) spectrometers is still limiting, but applications for single-cell proteomics and metabolomics are quickly improving (Kumar et al., 2020; Cong et al., 2020). Fluxomics based on protein-bound amino acids also requires relatively high numbers of cells (about 0.2 mg of dry cells) for analysis (Long and Antoniewicz, 2019).

When using these -omics techniques to analyze cellular metabolism it is important to take into account two levels of control that influence metabolic fluxes: (i) Hierarchical control due to transcription, expression and post translational modifications.

(ii) Metabolic control by metabolites themselves (Nielsen, 2017). Hierarchical control can be analyzed by transcriptomics and proteomics while metabolic control can be assessed by metabolomics. However, to correlate metabolic control with the respective conditions, for example during infections with bacterial pathogens, knowledge about the actual metabolic fluxes is necessary. Although flux analysis covers the smallest subset of cellular compounds, it offers the highest potential to characterize metabolic processes being actually active in considerable detail. Thus, a transcriptome does not easily give information on post-translational modifications of proteins, which are often crucial for enzyme activation. Furthermore, the quantification of proteins alone does not necessarily allow to infer active metabolism reactions due to their various kinetic properties and metabolic control mechanism. At the same time, the amounts of metabolites can also provide ambiguous information, since they do not directly reflect the metabolic turnover in specific pathways due to their potential multiple roles in different pathways (Sauer, 2006). Therefore, isotope-based flux analysis seems to be the most robust method that allows the direct analysis of substrate usage and the core metabolic pathways in a living cell. It becomes more and more clear that pathogens utilize a variety of carbon substrates from their specific environments during the infection cycle. More specifically, each bacterial pathogen appears to exploit a specific sub-set of these compounds to drive a metabolic network that is highly adapted to the respective replicative niche during infection (Abu Kwaik and Bumann, 2015; Abu Kwaik, 2015; Eisenreich et al., 2015; Olive and Sasseti, 2016; Escoll and Buchrieser, 2018; Best and Abu Kwaik, 2019; Eisenreich et al., 2019; Traven and Naderer, 2019). This adaption is typically manifested in form of a so-called bipartite metabolism (Grubmüller et al., 2014; Häuslein et al., 2016, 2017a; Mehltitz et al., 2017; Eisenreich et al., 2017; Best and Abu Kwaik, 2019; Rajeev et al., 2020). In this apparently divided metabolic network, one or a set of substrates serve to build up cell wall sugars, nucleotides and lipids, whereas another substrate or a subset of substrates is utilized to afford energy (e.g., ATP). Noteworthy, using isotopologue profiling this metabolic model was determined for several pathogens, especially for intracellular bacterial pathogens.

In this review, we focus on methods of pathway analysis by ^{13}C -isotopologue profiling. In the first section, we describe some basics and typical protocols for isotopologue profiling. In the second part, this technology will be illustrated by several recent examples especially with intracellular bacterial pathogens.

Basics about ^{13}C -isotopologue profiling

To perform isotopologue profiling, a bacterial pathogen is cultivated in the presence of a ^{13}C -tracer, i.e. a compound that is artificially ^{13}C -enriched beyond its natural abundance (for ^{13}C , the natural abundance is 1.1%). Uptake and metabolic utilization of this tracer by the pathogen lead to ^{13}C -enrichments in any metabolic product being involved in this conversion process, i.e. metabolically located downstream from the tracer. As a result of the specific pathways being involved in this process, the positional distribution of the ^{13}C -label in the metabolic products is not random, but rather highly specific. In other words, highly specific mixtures of $^{12}\text{C}/^{13}\text{C}$ stable carbon isotopologues (i.e., ^{13}C -profiles or ^{13}C -patterns) are formed by the pathways being functional under the respective experimental conditions. On the basis of these ^{13}C -profiles, the metabolic history (i.e., substrate usages, catabolic and anabolic pathways, relative carbon fluxes) from the source to each respective metabolite under study can be determined.

According to IUPAC definition, isotopologues have the same chemical structure and bond connectivity but differ only in their isotopic composition. For example, acetic acid comprising the abundant ^{12}C -atoms, $^{12}\text{CH}_3^{12}\text{COOH}$, and acetic acid containing ^{13}C at the methyl group, $^{13}\text{CH}_3^{12}\text{COOH}$, are different carbon isotopologues of acetic acid. Isotopologues can be further sub-grouped into isotopomers, which have the same chemical structure, same bond connectivity and the same number of isotopes, but the isotopic atoms differ in terms of their position within the molecule, for example $^{13}\text{CH}_3\text{COOH}$ and $\text{CH}_3^{13}\text{COOH}$. In theory, a compound with n C-atoms can produce 2^n carbon isotopologues. For example, glucose can be grouped into 64 different stable carbon isotopologues. This large number of isotopologues behind every metabolite in the metabolome of an organism multiplies to an almost infinite number of ^{13}C -isotopologues, thus enabling to describe the metabolic system under study at high resolution, even with a limited number of metabolites being used for isotopologue profiling.

Isotopologues can be identified and quantified by NMR spectroscopy or mass spectrometry (MS). For work with intracellular bacteria, mass spectrometry (i.e., gas chromatography-MS (GC-MS) or liquid chromatography-MS (LC-MS)) is the preferred method due to its higher sensitivity. Using mass spectrometry, metabolites under study are typically described by their mass distributions, i.e. isotopologues are denoted as M when only ^{12}C is present, while sets of the respective ^{13}C -isotopologues are indicated by $M + 1$, $M + 2$, ..., $M + n$ when n ^{13}C -atoms are present in the molecule. Accounting the relative fractions of isotopologues then affords the overall ^{13}C -excess of the metabolite under study. ^{13}C -Excess here means the overall percentage of ^{13}C in a molecule minus the natural abundance contributions. Following the nomenclature for isotopologues introduced above, the ^{13}C -excess is calculated with the following formula (Lee et al., 1991).

$$\text{Excess} = \frac{\sum_{i=1}^n (M + i) \times i}{n}$$

General principles, constraints and guidelines for designing isotopologue profiling experiments

Some basic assumptions have to be made for ^{13}C -isotopologue profiling in bacteria:

- (i) **Kinetic isotope effects:** In metabolic reactions, there is a small discrimination between ^{12}C and ^{13}C due to small kinetic isotope effects (Kamen, 1947; Whelan et al., 1970). However, these effects are minor in comparison to the typical incorporation rates of ^{13}C -enriched substrates from the environment in labeling experiments. Roughly, in terms of ^{13}C -excess values $>0.5\%$, effects due to kinetic isotope effects are within the errors of ^{13}C -quantification and can therefore be neglected (Antoniewicz et al., 2007).
- (ii) **Steady state conditions:** In standard long-term experimental settings (see below), isotopic as well as metabolic steady state are reached (Wiechert, 2001; Buescher et al., 2015). Metabolic steady state means that the metabolic fluxes as well as the extracellular and intracellular metabolite concentrations are constant. During *in vitro* cultivation of bacteria, metabolic steady-state can be reached through chemostat cultivation (Sauer et al., 1999). Additionally, metabolic quasi-steady state is generally assumed to be reached during exponential growth, as the division rate of cells is at its peak (Buescher et al., 2015). Similarly, at isotopic steady state the isotopic composition of all metabolites remains constant and the fluxes are therefore independent of the metabolite concentrations (Buescher et al., 2015). Under these conditions, the isotopologue composition of a metabolite solely depends on the production fluxes and is independent of its consumption fluxes (Wang et al., 2020). The time-frame for metabolites to reach isotopic steady state after addition of a tracer generally depends on the growth rate of the bacterium and greatly varies for example between soluble cytosolic metabolites and amino acids bound in proteins or sugars bound in the cell wall. While soluble metabolites equilibrate their isotopic composition within minutes after introduction of the ^{13}C -tracers, this can take several hours for metabolites bound in proteins or the cell wall (Buescher et al., 2015). Isotopic steady state can be verified by assessing the isotopic compositions of a metabolite at several time-points after the introduction of the tracer (Beste et al., 2013).

Notably, it can be difficult to achieve either metabolic or isotopic steady-state in pathogen-host situations, as the labeling periods are more likely to be governed by the specific time frames of the infection cycle. This typically determines the periods of bacterial replication and adaptive processes between host and pathogen. In these cases, the time settings in labeling experiments must be specifically adjusted to allow quasi-steady state conditions. In many examples, however, comparison of isotopologue compositions under different conditions or between wild-type and mutant strains can still allow qualitative statements about the functionality of metabolic pathways without strictly assessing the steady state conditions (see also below).

Taking into account these constraints, ^{13}C -patterns in metabolites from the same experiment can be qualitatively or quantitatively compared. In case of isotopic steady state, a retro-biosynthetic approach serves to delineate differential substrate usage and fluxes in the metabolic network. In this approach, borrowing principles from synthetic organic chemistry, the labeling patterns of metabolites (metabolic intermediates) that are not directly measured can be reconstructed from the labeling profiles of their metabolic products on the basis of well-known pathways (Bacher et al., 1998; Bacher and Eisenreich, 2010). As amino acids are directly connected to several central intermediates of the central carbon metabolism, their analysis provides rich insights into the overall metabolism (Fig. 1B). For example, alanine, aspartate and glutamate are produced from pyruvate, oxaloacetate, and 2-oxoglutarate, respectively, *via* simple transamination reactions. Therefore, the excess and isotopologue distribution of these amino acids can be projected to the respective 2-oxo acids, which are also direct intermediates of central carbon metabolism, i.e. the product of glycolysis or the Entner-Doudoroff-pathway (ED pathway) in case of pyruvate, or the citrate cycle in case of oxaloacetate and 2-oxoglutarate.

- (iii) **Analytes useful for isotopologue profiling:** The metabolome comprises thousands of compounds and even restriction to central metabolic compounds still leaves hundreds of molecules for potential analysis. This is practically impossible especially talking into account the low steady-state abundance and instability of some compounds (Buescher et al., 2015). Isotopologue profiling studies therefore mostly focus on highly abundant, stable compounds, such as amino acids (Bacher et al., 1998). As half of the cellular dry weight typically accounts for proteins (Phillips and Milo, 2009), amino acids can be obtained from a relatively low number of bacteria ($>10^7$ bacteria, *ca.* 1 mg dry weight) to allow MS-based isotopologue profiling (Wittmann, 2007). Although more and more LC-MS based applications are developed to analyze increasing amounts of metabolites simultaneously and to capture also low-abundant labile intermediates (Shi et al., 2020), GC-MS based analysis of protein-bound amino acids still remains the gold standard for reliable and robust isotopologue profiling of pathogenic bacteria (Eisenreich et al., 2010; Long and Antoniewicz, 2019).
- (iv) **Choice of the ^{13}C -tracer:** Since the development of isotopic tracers for the study of metabolism, the palette of commercially available ^{13}C -enriched compounds has greatly expanded and almost any potential carbon nutrient to feed into the central carbon metabolism of bacteria can be purchased either in completely ^{13}C -labeled form or ^{13}C -enriched at specific positions. Fully labeled general carbon substrates e.g. $[\text{U-}^{13}\text{C}_6]\text{glucose}$, are ideal for the overall assessment of a metabolic network, since the label from this general source is incorporated into a variety of intermediates of the central carbon metabolism. On the other hand, this approach sometimes does not allow to distinguish between similar metabolic routes. For example, labeling experiments starting from $[\text{U-}^{13}\text{C}_6]\text{glucose}$ do not easily discriminate between glycolysis, the Entner-Doudoroff pathway and the pentose phosphate pathway of glucose degradation. In these cases, additional experiments using selectively ^{13}C -labeled

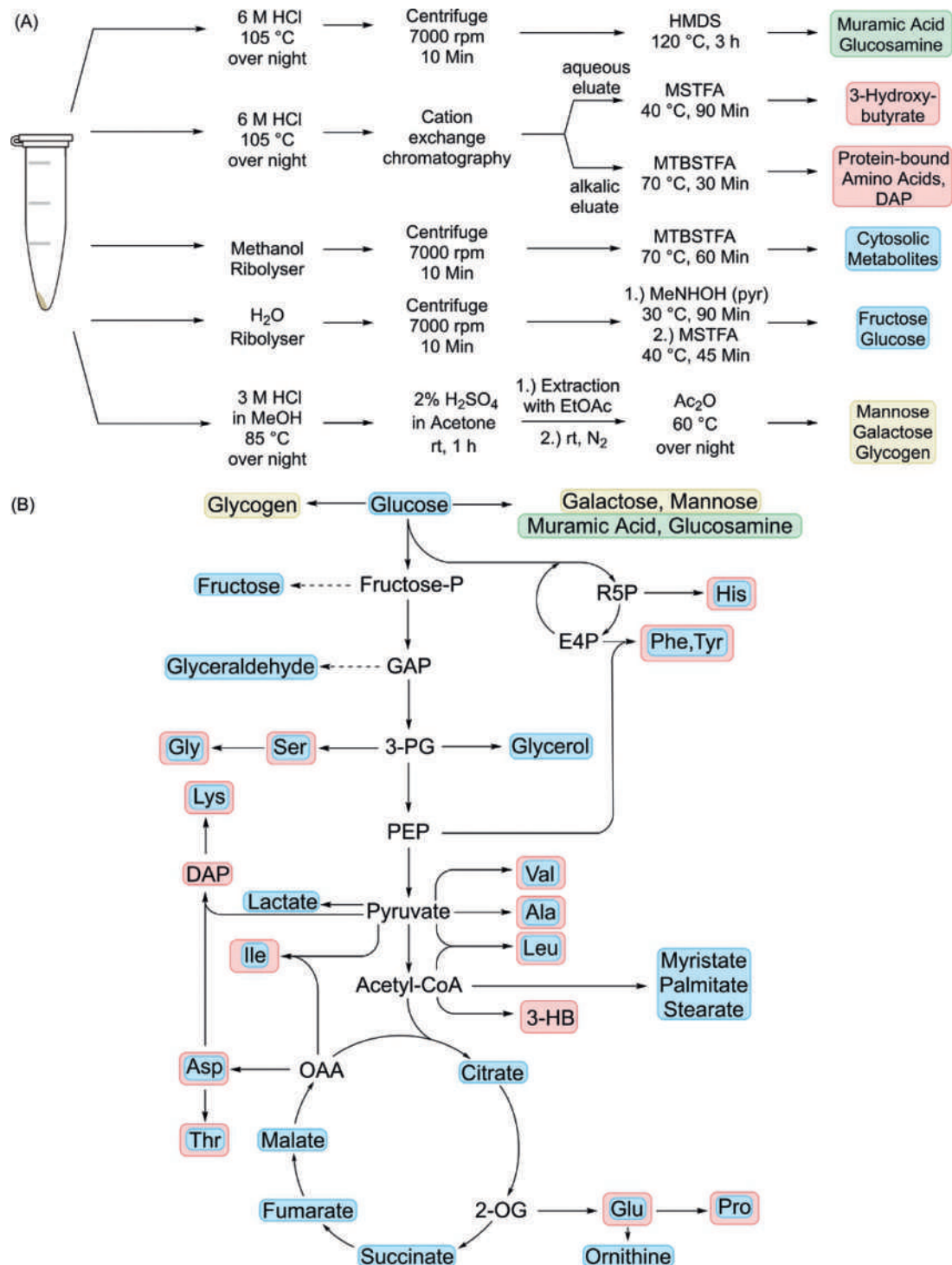


Fig. 1 Overview of protocols for the isolation and analysis of different kinds of metabolites as well as their connection to the central carbon metabolism. (A) Schematic workflow for the isolation and derivatization of different classes of metabolites. For detailed experimental procedures refer to the main text. (B) Overview of the central carbon metabolism of a model bacterium. Metabolites that can be analyzed by GC-MS are shown in boxes. The color indicates the experimental protocol necessary for the respective compound.

tracers should be performed (Feng et al., 2012). For example, using [1,2-¹³C₂]glucose allows to distinguish between glycolysis and the ED pathway by comparing the label distribution at positions C1 and C2 in alanine as a reporter for pyruvate, the end-product of both glucose-degrading pathways (Fig. 2). A perfect tracer is often hard to choose *ab initio*, often rendering tracer experiments an iterative process (Antoniewicz, 2013). Additionally, different parts of the metabolism often require different tracers for a concise analysis as for example in the case of a bipartite metabolism of pathogens, where [U-¹³C₆]glucose often only allows limited analysis of the tricarboxylic acid (TCA) cycle activity (see below).

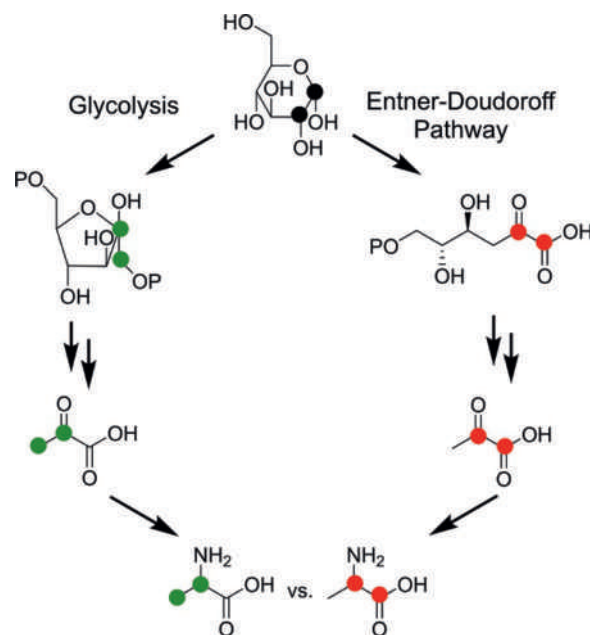


Fig. 2 Metabolization of $[1,2-^{13}\text{C}_2]$ glucose to pyruvate via glycolysis or the Entner-Doudoroff pathway. Isotopologue profiling of cells cultivated with $[1,2-^{13}\text{C}_2]$ glucose allows to identify the pathway for glucose degradation. While glycolysis produces $[2,3-^{13}\text{C}_2]$ pyruvate (green), the ED pathway yields $[1,2-^{13}\text{C}_2]$ pyruvate (red). Subsequently, analysis of different mass fragments in alanine, which is derived from pyruvate through deamination, allows to distinguish both pathways. P indicates a phosphate group (Eylert et al., 2010).

- (v) **Cultivation:** In terms of defining appropriate cultivation conditions for a labeling experiment, choice of the medium and duration of the labeling period are crucial. Minimal media or, more generally, chemically defined media allow an optimal control of the amount of tracer to be supplied. Moreover, these conditions are qualified to perform rigorous ^{13}C -metabolic flux analysis in which absolute carbon fluxes from the ^{13}C -tracer to any metabolic product can be determined. For examples, see elegant studies with *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Beste et al., 2013; Lassek et al., 2016; Dolan et al., 2020). However, often pathogens, especially intracellular ones, only show slow growth in minimized media. Therefore, these artificial conditions barely reflect the actual environments during infections. Capitalizing on this aspect, labeling experiments with a pathogen should always consider the chosen experimental conditions to interpret the resulting data in terms of their relevance for actual infections. If isotopic and metabolic steady state cannot be guaranteed for work under the complex medicinally relevant conditions, information can still be gained from these studies by e.g. comparative labeling experiments with mutant strains and appropriate controls. During intracellular growth of pathogens, the time scale of the labeling experiment is often governed by the infection cycle. For example, if lysis of the host cell occurs at a certain time point, the experiment should be stopped earlier to avoid corruption of the labeling data through consumption of the labeled substrate by the released extracellular bacteria. In other cases, the duration of infection must be prolonged to increase the number of bacteria for analysis (Kern et al., 2016). This can also be achieved by high values of the initial multiplicity of infection (MOI) in case of studies with intracellular bacteria (Schunder et al., 2014).
- (vi) **Harvest:** After cultivation in liquid media, the bacteria are typically harvested by rapid centrifugation. The supernatant obtained after cell harvest should also be kept, as the analysis of the residual tracer or secreted compounds in the medium could provide additional information. There is variety of harvest protocols available (Winder et al., 2008; Tambellini et al., 2013), but in general all steps should be as fast as possible and should be performed at low temperatures (4°C). A minimal number of washing steps should be performed after harvest, to get rid of the residual tracer or other medium components interfering with the isotopologue analysis. Washing-solutions without any organic molecules such as phosphate-buffered saline (PBS) or saline are preferred (Wittmann et al., 2004).

When studying intracellular bacteria grown in their respective host cells, an additional separation step is necessary to isolate the bacteria from their hosts. In general, this can be achieved by lysis of the infected host cell and subsequent centrifugation steps. Thereby, cell debris of the host cell with eventually adhered bacteria is collected in the pellet. The supernatant contains the majority of bacteria as well as dissolved molecules from the host cytosol including host proteins. Another centrifugation step of the later fraction allows to separate intact bacterial cells (pellet) from the dissolved host molecules (supernatant) (Schunder et al., 2014; Mehlitz et al., 2017). The quality of the separation steps should be validated by appropriate Western Blot analyses of the respective fractions. After harvest, samples should generally be stored at -80°C until further analysis.

(vii) **Sample workup:** Before isotope analysis, the cell material should be dried *via* lyophilization. As shown in Fig. 1A, different kind of metabolites require different extraction procedures and subsequent chemical derivatization to determine the isotopologue profiles by GC-MS analysis. Depending on the available amount of cell material, all protocols can be performed in parallel. After extraction of soluble metabolites, the remaining pellet of cell debris can be subjected to hydrolysis (see below). Typical protocols are given in the following sections:

- For the analysis of free low-molecular compounds (i.e., cytosolic metabolites), >5 mg of the dried cell pellet is subjected to mechanical disruption in methanol. To this aim, the cellular material is suspended in 1 mL of cold methanol (4 °C) or other solvents and mixed with 750 mg of glass beads (ϕ 0.25–0.5 mm). Disruption is typically performed using a ribolyser system (1×20 s at 4.0 m s^{-1} , 3×20 s at 6.5 m s^{-1}). For extraction of metabolites, methanol is a good compromise, as it dissolves a wide range of metabolites, aids cell disruption, precipitates most bacterial proteins, and can be easily removed due to its volatility (Ser et al., 2015). To separate the soluble metabolites in the supernatant from cell debris and precipitates, the tubes are centrifuged (7000 rpm, 10 min, 4 °C). The clear supernatant is then carefully transferred to a 1.5 mL GC-MS vial and afterwards dried using a stream of nitrogen at room temperature, which takes about 40 min. The derivatization procedures in the next step differs between analysis of sugars or organic acids.
- For analysis of soluble sugars, namely glucose, galactose and fructose, 100 μL of a freshly prepared solution of methoxyamine hydrochloride in anhydrous pyridine (10 mg/mL) is added to the dried residue. The sample is incubated at 30 °C for 90 min. Subsequently pyridine is removed, as it would hamper the next derivatization step, by drying the sample under a stream of nitrogen at room temperature for several minutes. Afterwards, 100 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) is added to the dried residue and the sample is incubated at 40 °C for 45 min.
- For analysis of soluble organic acids, amino acids and other low molecular-weight metabolites, 50 μL of *N*-methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) together with 50 μL of anhydrous acetonitrile are added to the dried residue and derivatization is performed at 70 °C for 60 min, producing *tert*-butyldimethylsilyl (TBDMS) derivatives of the respective compounds.
- For analysis of protein-bound amino acids and eventually of 3-hydroxybutyrate (3-HB) from the poly-hydroxybutyrate fraction (PHB), >1 mg of the dried cell pellet is subjected to acidic hydrolysis in 6 M HCl at 105 °C for 15 h. Cysteine and tryptophan are destroyed during acid hydrolysis. Asparagine and glutamine are deamidated to afford aspartate and glutamate, respectively, during this procedure (Dauner and Sauer, 2000). After hydrolysis, the solution is cooled down to about 70 °C and dried under a stream of nitrogen at 70 °C, which takes about 30–45 min. The dried residue is then resolubilized in 200 μL of 50% acetic acid (aqueous) by vortexing for 10 s or treating the sample in an ultrasonic bath for 2 min after addition of the acetic acid. At the same time, a small column is prepared to perform cation-exchange chromatography. For example, the lower quarter of a 1 mL pipette tip can be filled with glass wool. 200 μL of the column material (Dowex 50 W X8, 7×10 mm, 200–400 mesh, 34–74 μm , H^+ -form) in the form of an aqueous suspension is then put on top of the wool. Afterwards, the column material is washed with 1 mL of 70% MeOH in H_2O and 1 mL of H_2O . Then, the 200 μL sample (see above) is carefully put onto the column, which is first developed by 2 mL of H_2O (bidest). The eluate is collected and contains uncharged or negatively charged molecules (such as 3-hydroxybutyrate or fatty acids). To collect the positively charged metabolites, e.g. amino acids from the proteins or diaminopimelate (DAP) from the cell wall, the column is developed with 1 mL of 4 M ammonia solution (aqueous). For further analysis, both eluates are dried using a stream of nitrogen at 70 °C. These dried eluates can be stored for several months at room temperature (Zamboni et al., 2009). The residue of the aqueous fraction is treated with 100 μL of MSTFA for 90 min at 40 °C while shaking (110 rpm) to form e.g. the trimethylsilyl (TMS)-derivative of 3-HB. The dried residue of the alkaline fraction is treated with 50 μL of MTBSTFA and 50 μL of anhydrous acetonitrile for 30 min at 70 °C to produce TBDMS derivatives of amino acids and DAP from the proteins or cell wall, respectively (Dauner and Sauer, 2000; Eylert et al., 2008; You et al., 2012).
- For the analysis of other cell-wall components, such as muramic acid and glucosamine, >5 mg of the dried cell pellet is subjected to acidic hydrolysis in 6 M HCl at 105 °C for 15 h. Afterwards, the solution is centrifuged (7000 rpm, 10 min) and the clear supernatant is dried under a stream of nitrogen at 70 °C. 100 μL of hexamethyldisilazane (HMDS) are added to the dried residue and derivatization is performed at 120 °C for 3 h.
- For the analysis of bound glucose, mannose and galactose, >3 mg of the dried cell pellet is subjected to acidic hydrolysis in 500 μL of 3 M methanolic HCl for 15 h at 80 °C. The solution is then centrifuged (7000 rpm, 10 min) and the supernatant is transferred to a 4.5 mL GC-MS vial and dried under a stream of nitrogen at 70 °C. Afterwards, 1 mL of a 5:95 (v/v) mixture of H_2SO_4 (conc.) and acetone is added to the dried residue. This mixture is incubated for 1 h at room temperature. Subsequently, the reaction mixture is neutralized by addition of 800 μL of saturated NaHCO_3 solution and 800 μL of saturated NaCl solution. For isolation of sugars, the mixture is extracted three times with 1.5 mL ethylacetate. The organic phases are combined and dried under a stream of nitrogen at room temperature. For derivatization, 100 μL of acetic acid anhydride and 100 μL of anhydrous ethylacetate are added to the dried residue and the mixture is incubated for 16 h at 60 °C. Afterwards, the mixture is brought to dryness using a stream of nitrogen at room temperature. Finally, the sample is dissolved in 100 μL of anhydrous ethylacetate for GC-MS analysis.

(viii) **GC-MS parameters for isotopologue analysis:** Since GC-MS is most frequently used for isotopologue profiling of pathogenic bacteria, we here focus on a typical setting using electron ionization with a quadrupole detector of the mass spectrometer. This

Table 1 GC-MS parameters for the analysis of different classes of metabolites.

	<i>Mur/GlcN</i>	<i>3-HB</i>	<i>Amino acids/DAP</i>	<i>Cytosolic metabolites</i>	<i>Sugars</i>
Temperature program	70 °C (5 min) (5 °C/min)→ 310 °C (1 min)	70 °C (3 min) (10 °C/min)→ 150 °C (50 °C/min)→ 280 °C (2 min)	150 °C (3 min) (7 °C/min)→ 280 °C (3 min)	100 °C (2 min) (3 °C/min)→ 234 °C (1 °C/min)→ 237 °C (3 °C/min)→ 260 °C (10 °C/min)-> 260 °C (2 min)	150 °C (3 min) (10 °C/min)→ 220 °C (50 °C/min)→ 280 °C (3 min)
Injection temperature (°C)	230	260	260	260	260
Interface temperature (°C)	250	260	260	260	260
Ion source temperature (°C)	200	200	200	200	200
Split-ratio	10	10	5	5	10
Linear velocity (cm/s)	40	40	40	36.7	40
Solvent cut time (min)	9	5	3	15	3

Mur, muramic acid; *GlcN*, glucosamine; *3-HB*, 3-hydroxybutyrate; *DAP*, diaminopimelate.

standard instrumentation also turned out to allow the robust identification and quantification of isotopologues. Moreover, a nonpolar DB-5 column, i.e. 5%-phenyl-methylpolysiloxane, is appropriate for the separation of silyl-derivatives of polar compounds (Wittmann, 2007). The injection volume should be adjusted to achieve an ideal compromise between signal intensity and reproducible signals. When the signal is too low, interference of noise with the mass spectrum can cause high deviations within technical replicates. When the signal is too high, the detector is shut down in the worst case and additionally the intensity of the most abundant isotopologue decreases with higher signal intensities (Antoniewicz et al., 2007), most likely leading to underestimation of the $M + 0$ isotopologue (Dauner and Sauer, 2000). Generally, measurements to analyze the isotopologue compositions of metabolites are performed using selected ion monitoring (SIM) to increase sensitivity and mass resolution, as well as to reduce noise. For analysis of a specific fragment, the masses from $M - 1$ to $M + X + 4$ should be measured, where M is the mass of the fragment containing only ^{12}C and X is the number of carbons in the respective metabolite (Buescher et al., 2015). Measurements should be performed at least in triplicates for each biological sample. Within these technical replicates, the error should be < 1 mol% to ensure reproducible results (Zamboni et al., 2009). Table 1 gives an overview on appropriate GC-MS parameters for the isotopologue analysis of different classes of metabolites.

- (ix) **Methods required to determine the ^{13}C -excess in metabolites by GC-MS analysis:** For data analysis, the mass traces for each metabolite in the chromatogram are extracted (Fig. 3A). While integrating the respective signal in the chromatogram, it should be noted that high enrichment can shift the retention time forwards. This effect is neglectable for most metabolites but must be taken into account when for example fully labeled long chain fatty acids are analyzed (Dauner and Sauer, 2000). The mass trace extracted from a respective GC-signal is compared to the mass trace from the same compound at natural ^{13}C -abundance (Fig. 3B), which has been acquired using a sample obtained from a reference compound. The differences in the isotopologue composition between reference and labeled sample (Fig. 3C, colored bars) are then corrected for the natural abundance of ^{13}C to reveal the actual excess of ^{13}C -isotopologues (Fig. 3D, colored bar) by numerical methods (Lee et al., 1991; Wittmann and Heinzle, 1999; Ahmed et al., 2014). To now calculate the overall ^{13}C -excess of the respective metabolite (Fig. 3D, black bar), which is also referred to as the summed fractional labeling (Wittmann, 2007), the excess of each isotopologue is scaled on the basis of the respective number of ^{13}C -atoms in the isotopologue under study. The ^{13}C -excess, which is specified in mol%, displays the percentage of ^{13}C in the respective molecule above natural abundance (Lee et al., 1991). Therefore, ^{13}C -excess calculation of a molecule should be done with a mass fragment containing the whole carbon skeleton of the molecule. For TBDMS-derivatives, the intense $[\text{M}-57]^+$ -fragment having lost a *tert*-butyl group from the silyl moiety is generally used, while for TMS-derivatives, the calculation relies on the intense $[\text{M}-15]^+$ -fragment having lost a methyl group from the silyl moiety (Wittmann et al., 2002). Due to experimental errors and technical uncertainties, molecules with a ^{13}C -excess < 0.5 mol% are not considered to be significantly enriched (Antoniewicz et al., 2007). The obtained enrichments can then be correlated to the functional metabolic processes (Fig. 3E). This procedure is illustrated by specific examples in the following section.

Examples for isotopologue profiling studies of bacterial pathogens

As mentioned above, the relationship between virulence and metabolism in pathogens becomes more and more apparent. Using the incorporation of stable isotope labeled metabolites, in the context of pathogens referred to as isotopologue profiling, significant progress has been made in the understanding of variety of pathogens, which will be shown by some examples in the following.

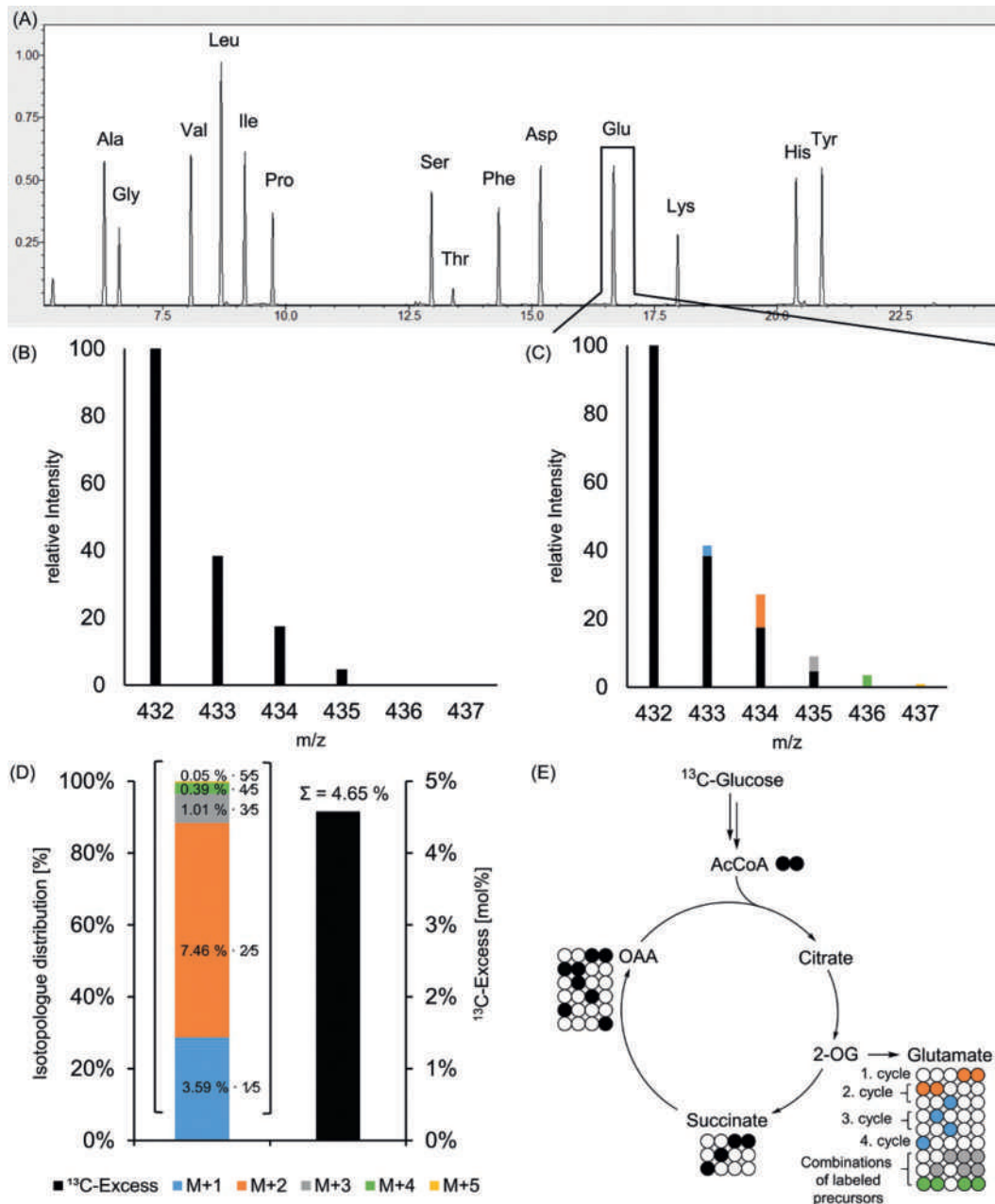


Fig. 3 Schematic data evaluation for labeling experiments. (A) GC-chromatogram of amino acids isolated from cellular proteins after hydrolysis. (B) Mass spectrum of the [M-57]⁺-fragment of the *tert*-butylidimethylsilyl-derivate of glutamate at natural abundance. (C) Mass spectrum of the [M-57]⁺-fragment of the *tert*-butylidimethylsilyl-derivate of glutamate showing ¹³C-enrichment. Colored bars refer to the indicated isotopologues. (D) Isotopologue distribution (colored bars) calculated from the mass spectrum. Percentage values indicate the abundance of the respective isotopologue. The black bar depicts the overall ¹³C-excess, calculated as the weighted abundance of the individual isotopologues. (E) Connection between labeling data and metabolic incorporation of the tracer. Colored circles show ¹³C-atoms, while empty circles display ¹²C-atoms.

Listeria monocytogenes

Extensive work was done on the metabolism of *Listeria monocytogenes* (*L. monocytogenes*) using ¹³C-labeling experiments under *in vitro* as well as *in vivo* conditions. Initial experiments, where ¹³C-patterns in protein-bound amino acids were determined by NMR after growth on [U-¹³C₆]glucose in broth already revealed differences in the metabolic capabilities as compared to metabolic models predicted by the genome composition alone (Eisenreich et al., 2006). While the pathways for production of serine and isoleucine were annotated in the *L. monocytogenes* genome (Buchrieser et al., 2003), they were found to be inactive under the

respective experimental conditions of the ^{13}C -experiment. The isotopologue profiles in amino acids clearly revealed the incomplete TCA cycle and the importance of pyruvate carboxylation to oxaloacetate as an essential reaction to afford this precursor for e.g. aspartate or mDAP (Eisenreich et al., 2006).

After having established the functional metabolic pathways of *L. monocytogenes* growing in broth, experiments with *L. monocytogenes* growing inside transformed macrophages (such as J774A.1) and primary murine bone marrow-derived macrophages (BMM) were performed starting from various tracers (e.g., $[\text{U-}^{13}\text{C}_6]\text{glucose}$, $[\text{U-}^{13}\text{C}_3]\text{serine}$, $[\text{U-}^{13}\text{C}_3]\text{glycerol}$). Careful GC-MS analysis of the isotopologue profiles in amino acids from the bacterial and the host fraction elucidated differential substrate usages and metabolic routes of *L. monocytogenes* under intracellular conditions (Eylert et al., 2008; Gillmaier et al., 2012; Grubmüller et al., 2014). As observed for many intracellular pathogens, *de novo* synthesis of most amino acids was avoided and compensated by the uptake of these compounds from the host cell during intracellular growth (Eisenreich et al., 2017). Noteworthy, especially alanine, aspartate, and glutamate, were made by the intracellular bacteria. Using mutants, defective in glucose phosphate uptake, verified synthesis of these amino acids using the supplied labeled glucose (Eylert et al., 2008). The incomplete TCA cycle in *L. monocytogenes* led to a pronounced $M + 3$ isotopologue in bacterial aspartate (formed from $M + 3$ oxaloacetate *via* carboxylation of $^{13}\text{C}_3$ -pyruvate) as compared to $M + 2$ aspartate from the host (formed from $M + 2$ oxaloacetate *via* the TCA cycle starting from $^{13}\text{C}_2$ -acetyl-CoA), when metabolizing $[\text{U-}^{13}\text{C}_6]\text{glucose}$ (Fig. 4) (Gillmaier et al., 2012). The careful inspection of the labeling patterns in multiple metabolites and further labeling experiments revealed a bipartite metabolic network for intracellularly growing *L. monocytogenes* (Fig. 6). In this topology, glycerol is a preferential carbon source for energy supply and catabolism in general, but not for gluconeogenesis, while glucose 6-phosphate supports anabolism, i.e. synthesis of cell wall carbohydrates and RNA/DNA (Grubmüller et al., 2014). Together, these data provided first insights into the functional metabolic network of an intracellular pathogen, which is highly adapted to its intracellular niche. Comparing the results obtained from experiments with primary and transformed macrophages as host cells for *L. monocytogenes* infection, it turned out that the bacterial pathways were quite similar if not identical in the different host cells. However, primary macrophages showed a distinct upregulation of host glycolysis due to the listerial infection (Gillmaier et al., 2012).

Legionella pneumophila

To understand the functional metabolism of *Legionella pneumophila* (*L. pneumophila*), the bacteria were grown in broth containing $[\text{U-}^{13}\text{C}_6]\text{glucose}$, $[1,2\text{-}^{13}\text{C}_2]\text{glucose}$ or $[\text{U-}^{13}\text{C}_3]\text{serine}$. Serine was efficiently metabolized by *L. pneumophila* as the major carbon source under these conditions, while glucose was used as an additional minor nutrient. Using $[1,2\text{-}^{13}\text{C}_2]\text{glucose}$ as a precursor, it was possible to distinguish between glycolysis and the ED pathway (Fig. 2), which is the preferred way for glucose utilization in *L. pneumophila*, even though both pathways are completely annotated in the genome. Additionally, analysis of 3-hydroxybutyrate obtained from the hydrolysate of PHB, revealed efficient glucose metabolism into this macromolecular storage compound (Eylert et al., 2010). These observations were further refined using growth phase dependent labeling experiments during *in vitro* cultivation. Again, using $[\text{U-}^{13}\text{C}_6]\text{glucose}$ or $[\text{U-}^{13}\text{C}_3]\text{serine}$, which were added at the beginning of the early exponential,

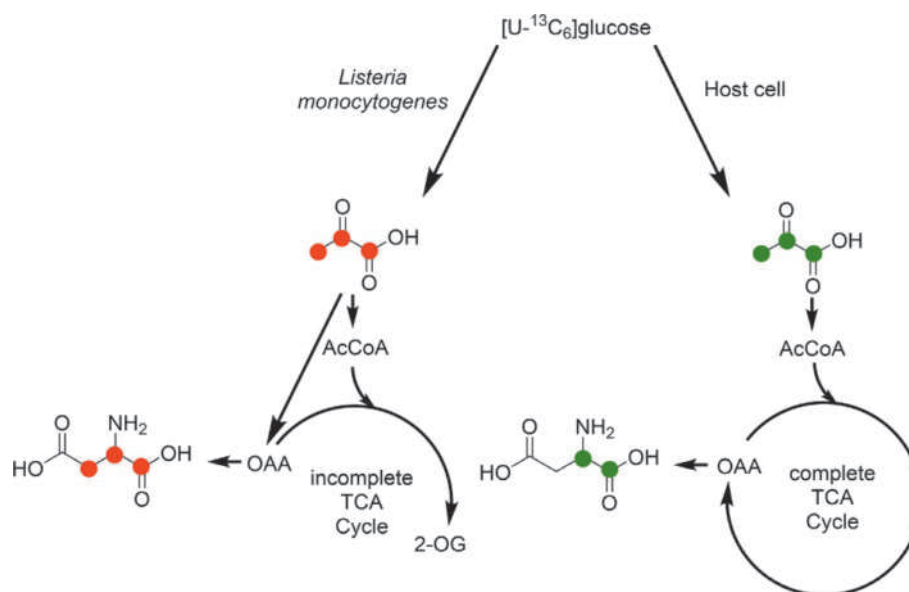


Fig. 4 Utilization of $[\text{U-}^{13}\text{C}_6]\text{glucose}$ by *L. monocytogenes* and its macrophagic host cell during infection produces different isotopologue profiles in pathogen- and host-derived aspartate. *L. monocytogenes* and the host cell both initially produce $M + 3$ pyruvate from fully labeled glucose. The incomplete TCA cycle in *L. monocytogenes* only allows further conversion to oxaloacetate through carboxylation, hence producing $M + 3$ aspartate (red). In contrast, the eukaryotic host cell uses a complete TCA cycle thereby producing $M + 2$ aspartate (green). AcCoA, acetyl-CoA; 2-OG, 2-oxoglutarate; OAA, oxaloacetate (Gillmaier et al., 2012).

post-exponential or stationary phase, respectively, a growth-phase dependent metabolization of these compounds was observed (Gillmaier et al., 2016). While serine was predominantly used during the early phase of exponential growth *via* the TCA cycle, glucose mainly served for PHB synthesis during the later stages (Gillmaier et al., 2016; Heuner and Eisenreich, 2016). Further experiments expanded the palette of carbon substrates used by *L. pneumophila*, as [U-¹³C₃]glycerol was also metabolized *in vitro* as well as *in vivo*. In contrast to *L. monocytogenes*, glycerol was mainly used for anabolic metabolism *via* gluconeogenesis in *L. pneumophila*. Still, as serine is mainly metabolized through the TCA cycle, this again supported a bipartite-like topology of *L. pneumophila* metabolism (Fig. 6) (Häuslein et al., 2016). Further isotopologue profiling experiments with a carbon storage regulator A-mutant (CsrA) of *L. pneumophila* showed the role of this global regulator in mediating the switch from amino acid metabolism during exponential growth to glycerolipid metabolism at later growth phases, when amino acids become limited. Using [1,2,3,4-¹³C₄]palmitate as a precursor, the role of fatty acids as additional carbon substrates was indeed corroborated again by the ¹³C-technology (Häuslein et al., 2017b).

Amoebae present the natural hosts for *L. pneumophila*, but the mechanisms of infection are thought to be similar to macrophages (Swart et al., 2018). Therefore, experiments with *Acanthamoeba castellanii* (*A. castellanii*), which were grown in the presence of [U-¹³C₆]glucose or [U-¹³C₃]serine before infection, were carried out to investigate the metabolic capabilities of *L. pneumophila* *in vivo*. Comparing the isotopologue profiles of amino acids from the bacterial fraction and the host proteins, it turned out that almost no *de novo* synthesis from the respective ¹³C-substrates had occurred by the intracellular bacteria. Rather, with the exception of some minor fraction of *de novo* synthesized alanine, aspartate and glutamate, amino acids were taken up from the host and directly incorporated into the bacterial proteins (Schunder et al., 2014). In another set of labeling experiments using [U-¹³C₆]glucose or [U-¹³C₃]serine as tracers, the metabolism of *L. pneumophila* growing within *A. castellanii* was studied during different time intervals, i.e. 0–17 h, 17–25 h, or 25–27 h post infection (Kunze et al., 2021). Comparative GC-MS analysis of the ¹³C-patterns again revealed that the intracellular bacteria efficiently utilized amino acids from the amoebae for the synthesis of their own proteins during the whole infection cycle. However, the data also showed that, for *de novo* synthesis of alanine, aspartate and glutamate, serine was utilized *via* the TCA cycle at increasing rates during the post-exponential phase of growth. In contrast, glucose was used during the whole infection cycle and not only during the late growth phase. This was surprising since earlier *in vitro* experiments suggested glucose utilization during the late growth phase only (see above). It is tempting to speculate that glucose availability could serve as a signal for the start of replication of *L. pneumophila* when thriving within host cells.

Mycobacteria

Mycobacteria and especially *M. tuberculosis* have also been extensively studied using isotopologue profiling. During *in vitro* cultivation, the general metabolic capabilities of this pathogen were assessed using [U-¹³C₆]glucose, [U-¹³C₂]acetate or [U-¹³C₃]glycerol as tracers. Surprisingly, *M. tuberculosis* showed co-utilization of these substrates, which accelerated growth in comparison to the theoretical growth rates on single substrates (Fig. 6) (De Carvalho et al., 2010). Subsequently, rigorous ¹³C-metabolic flux analysis (MFA) was performed in chemostat cultures. This revealed the activity and importance of a so-called GAS-pathway (glyoxylate shunt-anaplerosis-succinyl-CoA synthetase) for *M. tuberculosis* growth (Fig. 5). Herein, acetate is the main substrate and utilization proceeds through the glyoxylate shunt. Oxaloacetate is decarboxylated to allow for gluconeogenesis, while succinyl-CoA synthetase compensates for the interrupted TCA cycle (Beste et al., 2011). ¹³C-MFA was then performed studying *M. tuberculosis* during growth in macrophages (Beste et al., 2013). A mixture of amino acids as well as acetate were taken up from the host cell and experiments with ¹³C-bicarbonate demonstrated active carboxylation reactions during infection. Additionally, the results suggested the usage of a C3-substrate as an additional carbon source for intracellular *M. tuberculosis*. On this basis, lactate seems to provide an additional carbon substrate during intracellular growth (Billig et al., 2017) besides lipids as the main substrates (Schnappinger et al., 2003). As lactate is produced in excess in activated macrophages, the finding of lactate usage probably reflects metabolic adaption of the intracellular pathogens (Billig et al., 2017).

It is also interesting to note the general metabolic flexibility and adaption mechanisms of mycobacteria. For example, studies using *M. leprae* within Schwann cells revealed the active usage of host glucose by the pathogen to synthesize amino acids (Borah et al., 2019). While *M. tuberculosis* exploits the PEP carboxykinase reaction for anaplerosis of acetate, this reaction is crucial for glucose catabolism in *M. leprae*.

Other intracellular pathogens

Labeling studies with *Chlamydia trachomatis* (*C. trachomatis*) in HeLa cells revealed that the obligate intracellular pathogen incorporated [U-¹³C₆]glucose at high rates into chlamydial lipopolysaccharide probably *via* glucose 6-phosphate from the host cell (Mehlitz et al., 2017). Bacterial alanine, aspartate, and glutamate were biosynthesized *de novo* using dicarboxylates from the host cell, such as malate or 2-oxoglutarate (from host glutamine). Indeed, the importance of host glutamine for *C. trachomatis* replication in primary human umbilical vein endothelial cells (HUVEC) was confirmed in more recent studies, showing that the infection leads to upregulation of glutamine import and a glutaminase (Rajeev et al., 2020). Together, the data again reflect co-substrate usage in a bipartite metabolic network with host cell-provided G6P for cell wall biosynthesis, and, to some extent, one or more host cell-derived dicarboxylates, i.e. malate and/or glutamine-derived 2-oxoglutarate, feeding the partial TCA cycle of the bacteria (Fig. 6). The latter flux could also support the biosynthesis of mDAP required for the formation of chlamydial peptidoglycan.

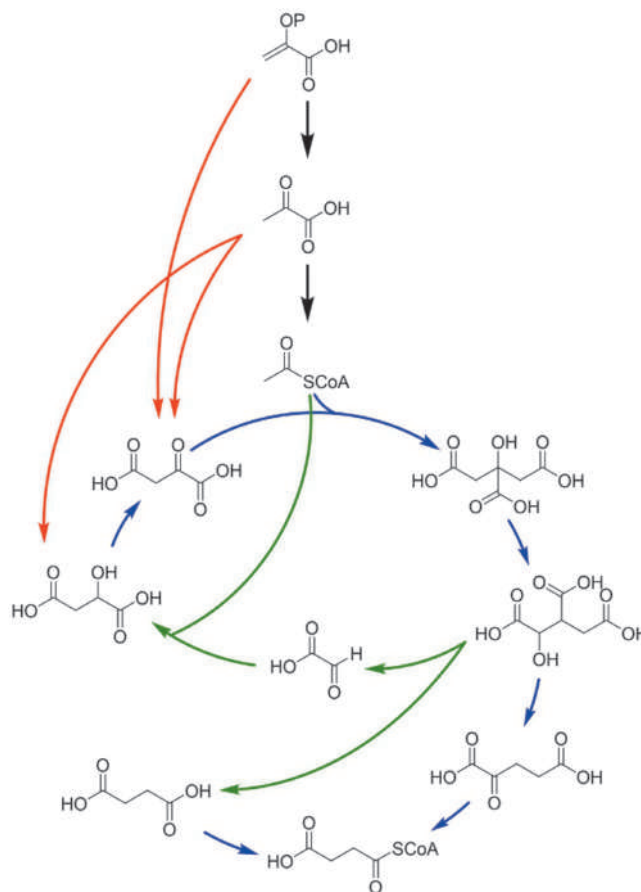


Fig. 5 GAS-pathway (glyoxylate shunt-anaplerosis-succinyl-CoA synthetase) observed in *M. tuberculosis*. Anaplerotic reactions are marked in red. Reactions of the glyoxylate shunt are marked in green, while reactions from the TCA cycle as well as succinyl-CoA synthetase are indicated in blue. Adapted from Beste DJ, Bonde B, Hawkins N, Ward JL, Beale MH, Noack S, Noh K, Kruger NJ, Ratcliffe RG, and McFadden J (2011) ^{13}C metabolic flux analysis identifies an unusual route for pyruvate dissimilation in *mycobacteria* which requires isocitrate lyase and carbon dioxide fixation. *PLoS Pathogens* 7: e1002091.

For *Francisella holarctica tularensis* (*F. tularensis*), ^{13}C -isotopologue profiling allowed to elucidate the central metabolic network through various ^{13}C -tracers. Additionally, comparison of strains varying in virulence revealed differences in the metabolic profiles especially in terms of the extent of gluconeogenesis from glycerol. All strains displayed a bipartite metabolic network, where amino acids are mainly used for energy generation through the TCA cycle, while glucose and glycerol are used for gluconeogenesis and cell wall synthesis (Chen et al., 2017).

An elegant study by Kentner et al. combined proteomics, metabolomics and ^{13}C -profiling with $[\text{U-}^{13}\text{C}_6]$ glucose or $[\text{U-}^{13}\text{C}_3]$ pyruvate to show that intracellular *Shigella flexneri* (*S. flexneri*) utilizes pyruvate produced through host glycolysis for its own metabolism (Fig. 6). Here, pyruvate is metabolized to acetate by the pathogen, which is then excreted *via* the host cell. This nutrient scavenging by *S. flexneri* allows fast replication inside the host, with generation times comparable to cultivation in broth under ideal conditions (Kentner et al., 2014).

Coxiella burnetii, another obligate intracellular pathogen, could be grown in axenic medium in 2010 for the first time. Under these conditions, experiments with ^{13}C -glucose, ^{13}C -serine or ^{13}C -glycerol confirmed a bipartite-type metabolism, where serine mainly contributes to energy generation through the TCA cycle, while glycerol enters gluconeogenesis (Fig. 6). Analysis of the activity of metabolic modules was facilitated by the analysis of representative intermediates, e.g. alanine for glycolysis, aspartate for the TCA cycle, diaminopimelate and glucosamine for cell wall synthesis (Häuslein et al., 2017a).

Experiments with *Salmonella enterica* growing inside CaCo-2 cells, revealed glucose as a nutrient source during intracellular replication. This finding was corroborated by labeling experiments with mutants deficient in glucose uptake (Götz et al., 2010).

Extracellular pathogens

Similar to intracellular pathogens, isotopologue profiling has also been used to characterize the central carbon metabolism of extracellular pathogens, such as *Streptococcus pneumoniae*. Here, experiments with $[\text{U-}^{13}\text{C}_6]$ glucose and $[\text{U-}^{13}\text{C}_2]$ glycine revealed that the Embden-Meyerhof-Parnas (EMP) pathway is the main glycolytic pathway. Further, the ^{13}C -patterns of serine, alanine and

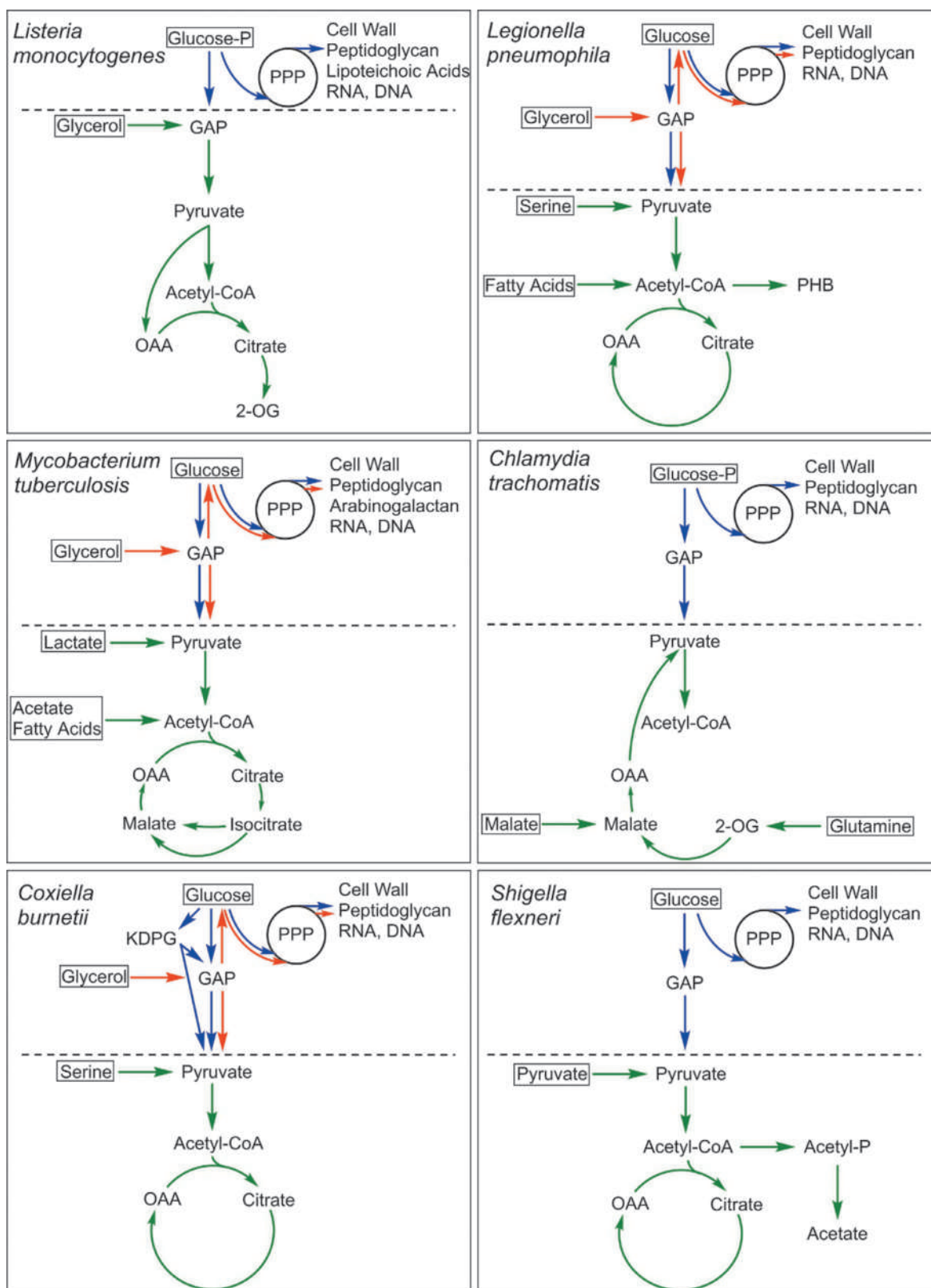


Fig. 6 Examples of bipartite metabolism observed in various intracellular bacteria. Blue and red arrows indicate the anabolic metabolism, which is responsible for the production of precursors for e.g. cell wall components and ribonucleotides. Green arrows indicate catabolism that mainly produces energy. Separation of these metabolic modules is indicated by the dotted lines. Main substrates identified by isotopologue profiling are indicated by the boxes. *Glucose-P*, glucose-6-phosphate; *GAP*, glyceraldehyde-3-phosphate; *OAA*, oxaloacetate; *2-OG*, 2-oxoglutarate; *PHB*, polyhydroxybutyrate; *acetyl-P*, acetyl-phosphate (De Carvalho et al., 2010; Beste et al., 2011; Grubmüller et al., 2014; Kentner et al., 2014; Häuslein et al., 2016, 2017a,b; Mehltz et al., 2017; Rajeev et al., 2020).

glycine revealed that serine is not produced from 3-phosphoglycerate but exclusively from glycine through the addition of formate. Interestingly, mutants with defects related to virulence showed no differences in the core metabolic features (Härtel et al., 2012). Similarly, metabolic capabilities could be assessed by ^{13}C -labeling studies in *Streptococcus suis* (Willenborg et al., 2015). The data unequivocally reflected that the EMP pathway was the main catabolic route for glucose utilization. Further, the PEP carboxylase reaction plays a central role in the metabolism of *S. suis* to produce oxaloacetate, because other anaplerotic reactions are absent and the TCA cycle is incomplete.

For *Staphylococcus aureus*, the metabolic phenotype of the wild-type strain was compared to a small colony-variant, which is related to persistent infections. Experiments with $[\text{U-}^{13}\text{C}_6]\text{glucose}$ together with transcriptome analysis showed reduced ^{13}C -excess in TCA cycle related amino acids due to a reduced aconitase activity in the small-colony variant (Kriegeskorte et al., 2014).

In *Campylobacter jejuni*, isotopologue profiling was combined with a transposon insertion library to evaluate metabolic requirements for the colonization in mice. This revealed a metabolic network with limited capabilities and low redundancy as well as the importance for the uptake of serine and aromatic amino acids during infection (Gao et al., 2017).

In *P. aeruginosa* using $[1\text{-}^{13}\text{C}]\text{glucose}$, the ED pathway was identified as the main route for glucose catabolism (Lassek et al., 2016). Further studies during growth on labeled glycerol or acetate revealed a bipartite metabolic network, mentioned above for intracellular pathogens. While glycerol preferentially enters gluconeogenesis, acetate is mainly metabolized in the TCA cycle (Dolan et al., 2020). Another study reported metabolic differences between clinical isolates of *P. aeruginosa* suggesting adaption to the respective niche in the host. Either, TCA cycle and ED pathway activities were high, while PPP activity was low or vice versa pointing to different extents of biofilm formation in the isolates or adaption to different oxygen levels (Opperman and Shachar-Hill, 2016).

Another study compared usage of ^{13}C -glucose of enterohemorrhagic *E. coli* in different media, imitating growth in the ileum or the colon respectively. Both conditions produced different metabolic phenotypes as well as different expression of virulence factors, suggesting a close connection between metabolism governed by the respective ecological niche and virulence (Polzin et al., 2013).

Plant pathogens

Although to a much smaller extent than for human pathogens, the metabolism of plant pathogens has also been under investigation using isotopically labeled metabolic precursors. In *Xanthomonas campestris*, the pathways for amino acid synthesis were identified using $[1\text{-}^{13}\text{C}]\text{glucose}$. This also allowed for the clarification of ambiguities in the synthesis of alanine, glycine, and isoleucine, which arose through redundancies in a putative metabolic network based on genome annotations (Schatschneider et al., 2011). A similar approach was used for *Xanthomonas oryzae* using labeled xylose or glucose as tracers (Shree and Masakapalli, 2018).

Finally, isotopologue profiling together with genomic analysis in *Ralstonia solanacearum* identified the ED pathway together with the oxidative pentose phosphate pathways as the main glucose degrading pathways (Jyoti et al., 2020).

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Metabolomics of Infectious Disease

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"In order to pursue chemotherapy successfully we must look for substances which possess a high affinity and high lethal potency in relation to the parasites, but have a low toxicity in relation to the body, so that it becomes possible to kill the parasites without damaging the body to any great extent. We want to hit the parasites as selectively as possible. In other words, we must learn to aim and to aim in a chemical sense. The way to do this is to synthesize by chemical means as many derivatives as possible of relevant substances".

"We may regard the cell quite apart from its familiar morphological aspects, and contemplate its constitution from the purely chemical standpoint. We are obliged to adopt the view, that the protoplasm is equipped with certain atomic groups, whose function especially consists in fixing to themselves food-stuffs, of importance to the cell-life. Adopting the nomenclature of organic chemistry, these groups may be designated side-chains. We may assume that the protoplasm consists of a special executive centre (Leistungs-centrum) in connection with which are nutritive side-chains . . . The relationship of the corresponding groups, i.e., those of the food-stuff, and those of the cell, must be specific. They must be adapted to one another, as, e.g., male and female screw (Pasteur), or as lock and key (E. Fischer)".

Paul Ehrlich (1854–1915).

Introduction

Metabolomics constitutes the analysis of metabolites in a biological sample and its behavior through the biological process in order to establish a specific metabolic flow conduct or fluxomics (Babele and Young, 2020). In this way, the metabolic flow of a normal or pathological biological process is a powerful source of information to evaluate different aspects of the causes of the disease, as well as its natural history and behavior under the established treatment (McCue and McCoy, 2017; Wishart, 2019; Hill et al., 2020). On the other hand, the metabolomic and fluxomic of infectious diseases allows to demonstrate the ways that use pathogenic microorganisms during the infectious process, as well as monitor the success or failure of antimicrobial treatment (Cuperlovic-Culf et al., 2013; Durmuş et al., 2015). Thus, infectious diseases produced by bacteria, viruses, fungi and parasites are one of the greatest causes of mortality in the world (Vos et al., 2020), it what prioritizes implementing new diagnostic techniques that improve detection and help block the transmission of microorganisms between the population (Agrebi and Larbi, 2020). In this order of ideas, the profile behavior of metabolism during infectious disease is a useful approach for the implementation of theranostics impact models, that is to say diagnostic techniques capable of detecting and treating the disease in the same probe, with which it obtaining it of metabolomes and fluxomes of the interaction host-microorganism allow the observation in real time of specific biological parameters with which to establish a predictive model of the clinical response (Kumar et al., 2020; Mussap et al., 2020). In this way the development of predictive health and disease models in infections is the key to establishing a correct customized clinical response based on the metabolomics of the patient (Seyhan and Carini, 2019; Diray-Arce et al., 2020). Thus, the metabolomics of infectious disease constitutes a model of precision medicine (PM) with a high predictive value in diagnosis, treatment and vaccination, due to the integration of biochemical and genetic parameters into a systems biology that allows due monitoring of affected patients (Tebani et al., 2016; Jaiswal et al., 2020). For this reason, the objective of this article is to analyze the applications of metabolomic-fluxomic dyad in infectious diseases as well as their impact on diagnosis, prevention and treatment.

Metabolomic-fluxomic dyad

In this order of ideas, metabolomics describes the various metabolites involved in biological processes, which attached to the fluxomics that integrates this bioinformation to determine the rate of reactions in a biological individual at any stage (Rosato et al., 2018; Volkova et al., 2020). In this way secondary metabolites produced both by the microorganism and the host are fundamental biomarkers to be evaluated by concentration curves at the time of the infectious process (Byers et al., 2019; Du Preez and Luies,

2019). Thus, the infectious metabolome can be addressed by the analysis of the synthesis of the small molecules represented by amino acids, lipids, nucleotides, sugars, hormones and vitamins that may be altered during the disease using the process of analysis of the samples presented in Fig. 1 (Romagnolo and Carvalho, 2021). Likewise, obtaining the principal component analysis (PCA) of the infected patient's sample allows to establish the physiological changes that microorganisms work on the host during infection, equally with the information of these metabolites present in the PCA in correlation with the clinical data can be introduced into a bioinformatic model to obtain the fluxomic of the disease (Zhang and Castelló, 2017; Bhadra and Rousu, 2018; Zhang et al., 2019). Thus, this integrative system approach achieves the obtaining of the Principal Metabolic Flux Mode Analysis (PMFA) (Fig. 2), which is an indicator of the metabolism of the host-pathogen interaction where it is possible to predict the clinical response of the patient against the microbial invasion (Pey and Planes, 2014; Rienksma et al., 2019). But it is important to remember that it should start from which of the different molecules represented in amino acids, lipids, nucleotides, sugars, hormones and vitamins can be physiologically altered and constitute a biomarker of infectious disease (Hwang et al., 2018). In this way the metabolome should offer a correct chemical characterization of the metabolic exchange between the environment and the host during the infectious process, in order to determine the adaptive response of the patient in the face of illness (Fig. 3) (Fernández-García et al., 2018). Likewise, a validation of the biomarker is necessary to establish the sensitivity, specificity, positive predictive value and negative predictive value of the analytes identified by metabolomic-fluxomics analysis (Gowda and Raftery, 2013; Percival et al., 2020).



Fig. 1 Workflow for metabolomic studies.



Fig. 2 Fluxomic data with principal metabolic flux mode analysis.

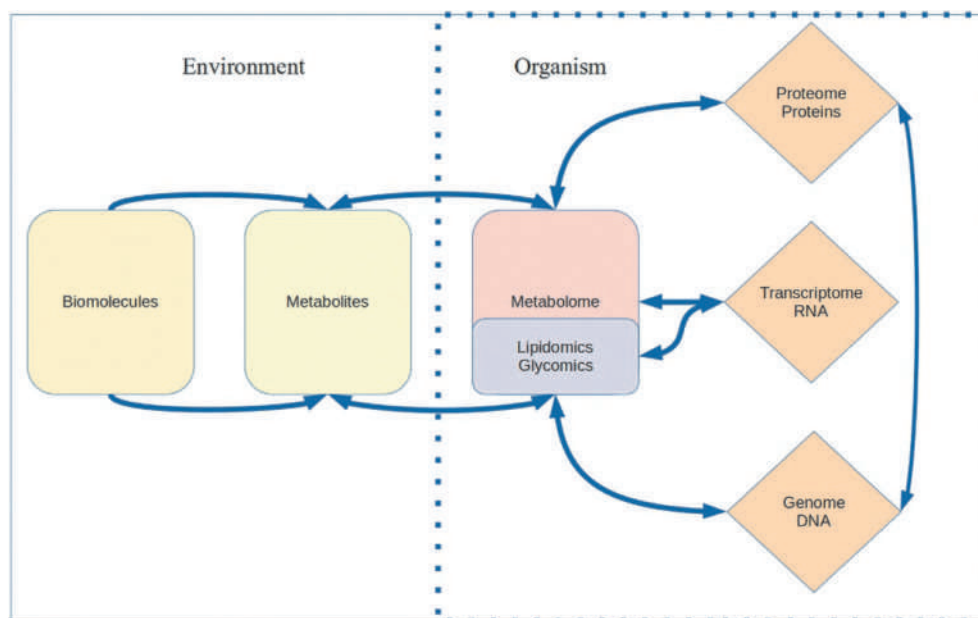


Fig. 3 Flow of metabolic exchanges between the host and the environment.

Metabolomic analysis of infection

In this order of ideas it is important to emphasize that the pathogenesis of infectious disease and its different phases such as incubation, the prodromal phase, acute disease, recovery and/or chronic disease are those that should influence metabolomic analysis for establish a real prediction of the clinical response (Pacchiarotta et al., 2012; Beale et al., 2019; Bernatchez and McCall, 2020). Likewise, the metabolites released by the microorganism as well as the molecules that make up its structure during the invasion and that can be determined in the different clinical samples are part of the analysis present in the metabolome that can give information on the resolution of the condition (Tang, 2011; Pinu and Villas-Boas, 2017; Pinu et al., 2017). Finally, it is important to correlate both the fluxomic of the host and that of the etiological agent during the infectious disease to obtain a model of biology of systems that describe the pathophysiology and its behavior in a personalized medicine approach (Fig. 4.) (Mardinoglu and Nielsen, 2012; Aon and Cortassa, 2015; Karakitsou et al., 2019). In this way, the evaluation of a physiological pattern of infectious disease was required based on metabolomics, where each stage can be established by the concentration of specific molecules produced by both the host and microorganism; said pattern may be made up of immunomodulatory molecules that can be an estimate of the host response to infection (Vernocchi et al., 2016; Thakur et al., 2019). Among these immunomodulatory molecules there are host defense peptides and cytokines; as well as carbohydrates, lipids and amino acids involved in immune processes (Maizels et al., 2018; Mookherjee et al., 2020). In this order of ideas, the immunity of the host versus the infection is one of the targets of study of a functional metabolomic model capable of giving information with which to restore the parameters of the metabolome and maintain the homeostasis of the affected patient (Belkaid and Harrison, 2017; Torow and Hornef, 2017; Ayres, 2020). Thus, the functional metabolomic analysis of the infectious process aimed at studying the immune response of the host in conjunction with the microbial metabolism should integrate the parameters of glycomics, proteomics, lipidomics in integration with fluxomics (Fig. 5) (Yang, 2016; Poudel et al., 2019). In other words, the obtaining of an infectious disease phenotype should be sought, in this way platforms such as PathoPhenoDB have worked in making important associations in this field of precision medicine (Hoehndorf et al., 2015; Kafkas et al., 2019).

Diseasome a new natural history of the disease

Diseasome is an approximation that makes up a network model of human illness to analyze the interactome between the causal agent and the host (Fig. 6) (Navratil et al., 2011; Khorsand et al., 2020; Silverman et al., 2020). In this way, the assessment of diseasome is capable of integrating biological systems together with the adaptive reaction to the environment and the pathogen in order to determine the factors of the host that are altered and allow the disease to persist (Gluckman et al., 2011; Balloux and van Dorp, 2017). Thus, the diseasome of infection, as well as infectome can find correlations between infectious disease and various comorbidities that predispose it; such as diabetes mellitus, malnutrition and cancer. This is a major breakthrough in personalized and comprehensive disease management (Faner et al., 2015; Satu et al., 2021). In that order of ideas the modeling of infectious diseasome contemplates the evaluation of various parameters among them the protein-protein interactions, transcriptional control networks and signal transduction networks, all integrated into a mathematical model for bioinformatic analysis. In summary, the entire study requires including the following data during infection (Hoehndorf et al., 2015; Lau et al., 2018; Barh et al., 2020):

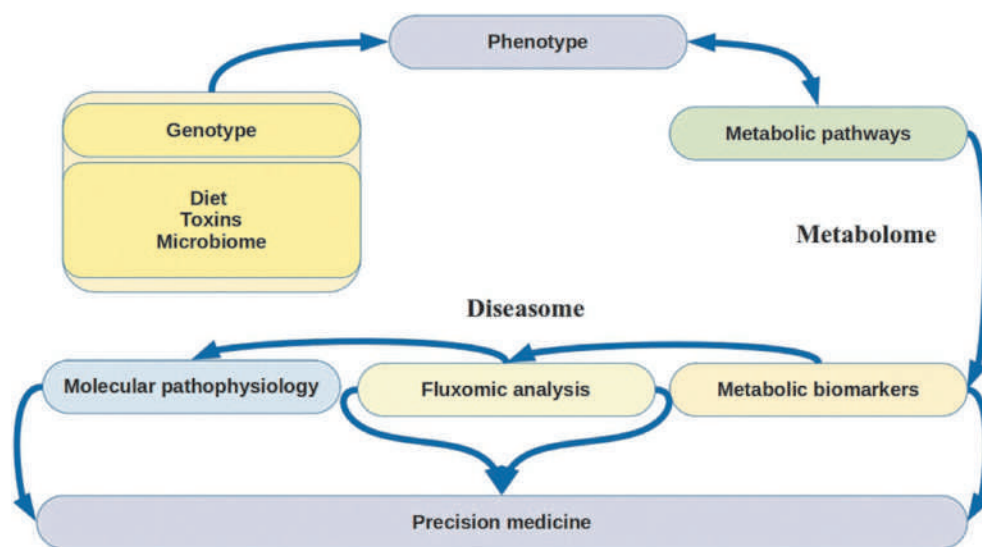


Fig. 4 Metabolomic analysis of systems medicine.

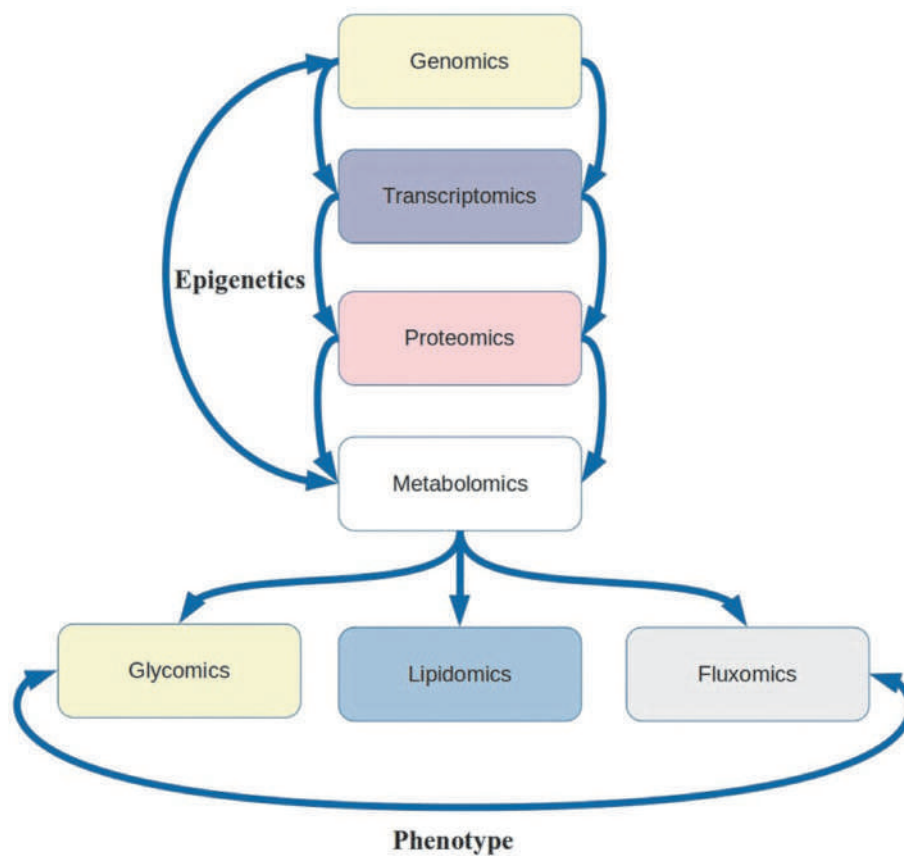


Fig. 5 Omics cascade for establish an infectious phenotype.

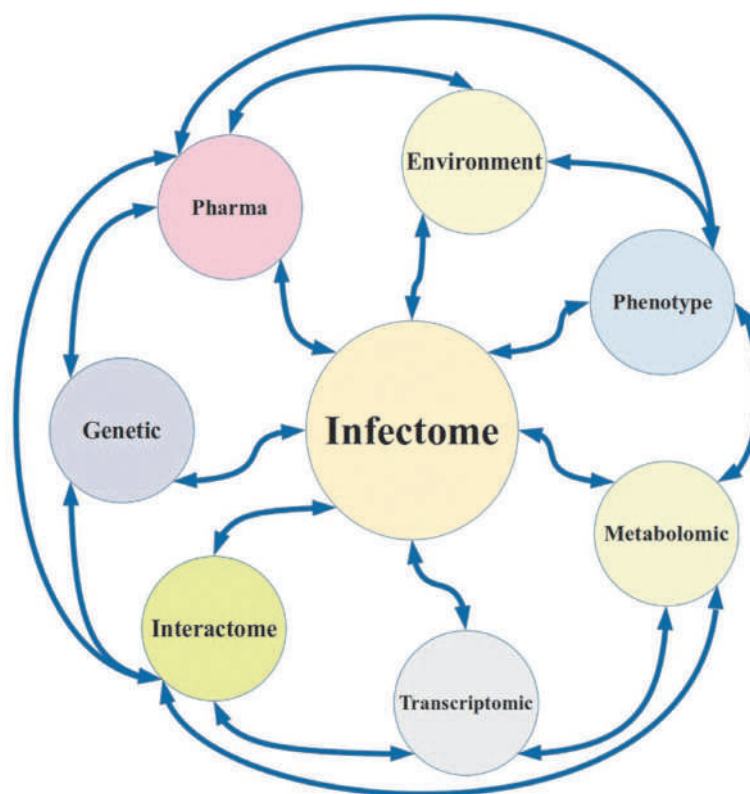


Fig. 6 Model of human diseaseome.

- DNA replication and repair
- transcription and regulation of transcription
- energy flow metabolism
- cell division
- chromatin modeling
- signaling pathways
- intracellular molecular trafficking
- cell membrane dynamics
- metabolic pathways

Likewise, the bioinformatic study of infectious disease should be correlated with the clinical variables of it as they are the presentation of symptoms, grades of severity, healing and fatality rates. In order to obtain a real approach of what happens in the host (Keeling and Danon, 2009; Gambhir et al., 2015). But also when trying to implement a bioinformatic approach so robust to be applied in the clinic of infectious diseases, metabolomic should try to respond to diagnostic, treatment, prevention and clinical procedures needs that is what prevents the translation of mathematics to the patient's bed (Kohler et al., 2016; Delhalle et al., 2018).

Metabolomics activity screening

Metabolomics Activity Screening (MAS) is the analysis model that seeks to integrate metabolome with metabolic pathways and systems biology in order to identify metabolites that modulate the functionality of physiological processes (Peng et al., 2015; Guijas et al., 2018). Thus, the metabolites as biomarkers of gene expression and the functionality of the proteins that modulate the phenotype, offer valuable information such as diagnostic and discovery tools of new drugs (Johnson et al., 2016; Rinschen et al., 2019; Davis et al., 2020). In this way, it is necessary to have a list of metabolites to be evaluated to elucidate the altered metabolic pathways during the infectious process, in this case a tentative list consigned in Table 1. can be given (Gebregiworgis and Powers, 2012; Song et al., 2019; Tounta et al., 2021).

Likewise, in the context of the immune response of the host against the infection there are metabolic pathways that can be valued to be correlated with functionality, these routes are included in Table 2 (Mazumdar et al., 2020).

Table 1 Metabolites to be evaluated in Metabolomic Activity Screening models.

<i>Metabolic pathway involved</i>	<i>Metabolite</i>
Glycolysis	β -D-Glucose 6-phosphate, D-fructose 6-phosphate, β -D-fructose 1,6-bisphosphate, D-glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, pyruvic acid, lactic acid
Pentose phosphate pathway	D-Ribose-5-phosphate, D-gluconate-6-phosphate, D-ribulose-5-phosphate, D-gluconic acid
Bioenergetics	NAD ⁺ , NADH, ADP, ATP
Tricarboxylic acid cycle	cis-Aconitic acid, D-threo-isocitric acid, α -ketoglutaric acid, succinyl-CoA, acetyl-CoA, succinic acid, fumaric acid, malic acid, oxaloacetic acid
Nitrogen metabolism	Citrulline, L-ornithine, arginine, arginosuccinic acid, glutamine, glutamic acid
Amino acids	Alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine

Table 2 Metabolites, metabolic pathways and immune system activity.

<i>Metabolic pathway involved</i>	<i>Metabolite</i>	<i>Cell of the immune system</i>
Glycolysis (phagocytosis)	β -D-Glucose 6-phosphate, D-fructose 6-phosphate, β -D-fructose 1,6-bisphosphate, D-glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, pyruvic acid, lactic acid	Neutrophil, macrophages, CD ⁴ lymphocytes, dendritic cells
Pentose phosphate pathway (H ₂ O ₂ production)	D-Ribose-5-phosphate, D-gluconate-6-phosphate, D-ribulose-5-phosphate, D-gluconic acid	Neutrophil, macrophages
Bioenergetics	NAD ⁺ , NADH, ADP, ATP	Macrophages, CD ⁸ lymphocytes

Table 3 Metabolites, metabolic pathways and microorganism.

Metabolic pathway involved	Metabolite	Microorganism
Glycolysis	β -D-Glucose 6-phosphate, D-fructose 6-phosphate, β -D-fructose 1,6-bisphosphate, D-glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, pyruvic acid, lactic acid	Hepatitis B <i>Escherichia coli</i> <i>Klebsiella pneumoniae</i> <i>Pseudomonas aeruginosa</i> <i>Proteus mirabilis</i>
Bioenergetics	NAD ⁺ , NADH, ADP, ATP	Hepatitis B
Tricarboxylic acid cycle	cis-Aconitic acid, D-threo-isocitric acid, α -ketoglutaric acid, succinyl-CoA, acetyl-CoA, succinic acid, fumaric acid, malic acid, oxaloacetic acid	<i>Streptococcus pneumoniae</i> <i>Escherichia coli</i> <i>Plasmodium falciparum</i> <i>Schistosoma mansoni</i> HIV
Nitrogen metabolism	Citrulline, L-ornithine, arginine, arginosuccinic acid, glutamine, glutamic acid	<i>Escherichia coli</i>
Amino acids	Alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine	<i>Escherichia coli</i> Hepatitis B HIV

On the other hand in clinical samples from patients with bacterial, viral, mycotic and parasitic infectious diseases, corresponding metabolites can be identified that can be indicators of acute or chronic condition as included in Table 3. (Pacchiarotta et al., 2012; Voge et al., 2016; Weiner et al., 2018).

Thus, Metabolomic Activity Screening as a strategy in the search for biomarkers should be correlated closely with clinical practice with the aim of obtaining metabolomes that can be analyzed in silico to identify patterns corresponding to the infectious process (Pinu et al., 2019a, 2019b; Bardanzellu and Fanos, 2020; Long et al., 2020). But it is important to emphasize that any metabolomic study requires specific precision devices to be able to obtain metabolomes of clinical samples reproducibly, automate and robust (Bi et al., 2020; Smith et al., 2020).

Metabolomics and mass spectrometry

In this way, gas chromatography coupled to mass spectrometry (GC-MS) it has been employed as a precision equipment for the analysis of urine samples, plasma and detection of Volatile Organic Compound (VOC) excreted in patients with viral and bacterial infections (Sethi et al., 2013; Stevens and Pukala, 2020; Djago et al., 2021). Thus, GC-MS is a robust method of analysis that allows identifying the amount of metabolites depending on the mass in a sample in order to elucidate metabolic pathways of the infectious process that affects the patient (Khodadadi and Pourfarzam, 2020; Nunes et al., 2021). In this order of ideas, with this method, the metabolomic targeted and untargeted strategies can be developed; in the first the amount of metabolites known to detect is less (from 10 to 100), in the second it is sought to quantify as much metabolites as possible from a sample to identify metabolic changes, so that the strategy untargeted is very employed in studies about metabolomic in infectious diseases (Schrimpe-Rutledge et al., 2016; Trivedi et al., 2017; Jiang et al., 2020). Thus, targeted Metabolomics has been used to assess changes in specific metabolites before, during and after antimicrobial treatment in order to develop predictive models of disease (Zurfluh et al., 2018; Vrieling et al., 2019). Likewise, untargeted metabolomics can also be performed by liquid chromatography on an LC-MS model to identify microorganisms and their pathogens forms (Franco-Duarte et al., 2019; Depke et al., 2020). Equally (High Performance Liquid Chromatography) HPLC-MS has been evaluated to quantify the metabolites present in plasma samples from patients that present infectious processes, it also allows establishing a clinical response to antimicrobial treatment in a targeted model (El-Najjar et al., 2017; Chen et al., 2020). On the other hand, additional methods have been coupled to mass spectrometry such as Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) and Electrospray Ionization (ESI) in order to increase the sensitivity and specificity of metabolomic analysis, as well as the identification of pathogenic microorganisms present in clinical samples (Chong et al., 2018; Leopold et al., 2018; Ščupáková et al., 2020). But it is important in a model of untargeted metabolomics elucidate the structure of the quantified molecule in order to specifically determine the possible biomarker (Zhang et al., 2020).

Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance is a method with a high utility in the structural elucidation of molecules in an untargeted metabolomic model (Emwas et al., 2019; Crook and Powers, 2020). Thus, in this order of ideas NMR has been used to detect biomarkers in tuberculosis, dengue and hepatitis, as well as to differentiate between bacterial and viral infections (Gebregiworgis and Powers,

2012; El-Bacha et al., 2016; Meoni et al., 2019; Grauslys et al., 2020). In this order NMR is an exploratory technique that allows identifying new metabolites in clinical samples as well as evaluating the concentration of specific compounds, which is why a robust method is considered to be implemented in research to determine fluxomics (Sussulini, 2017; Vignoli et al., 2019). Likewise to elaborate a disease model, NMR can evaluate the protein pattern of the disease in order to establish a metabolic network that configures pathophysiological processes that occur in the patient (Farber and Mittermaier, 2015). Equally, NMR has the ability to obtain microbial profiles for the broad identification of pathogens in clinical samples, in order to establish the reduction of the microbial load in the resolution of the disease (Magana et al., 2018; Kidd et al., 2020). On the other hand NMR can obtain pharmacokinetic profiles in plasma samples in order to determine the concentrations of antimicrobial drugs and if they are above the minimum inhibitory concentration (MIC) of drug that inhibits the infectious agent (Su et al., 2018). This method can also be coupled to High-Throughput Screening (HTS) platforms to evaluate metabolites in a greater number of samples and perform a robust screening of bioactive molecules against infectious diseases (Wu et al., 2015; Emwas et al., 2020). Finally, chemical methods in their robustness and their reproducibility provide information on clinical samples that must be analyzed under models of cheminformatics and bioinformatics to allow elucidate metabolites, biomarkers, as well as medications that can diagnose and treat infectious disease with a high impact (Griffiths et al., 2010; Ash et al., 2019; Balcerczyk et al., 2020).

Metabolomics and in silico tools

As important as obtaining the clinical sample and describing a metabolomic profile based on low chemical methods using targeted or untargeted strategies is a priority to have the software tools to perform an in silico analysis with which to establish a disease and an infectome (Sugimoto et al., 2012; Misra and Olivier, 2020; Majumder et al., 2021). In this way, there are several platforms used for metabolomic studies such as: BioSpider, COLMAR, FiD, HORA, MeltDB, MetaboloAnalyst, MetaboMiner, MolFind, MVAPACK, OpenMS, SetupX and XCMS(2) (Giraudeau, 2020). It is important to emphasize that most of the available metabolomic software are to analyze mass spectroscopy data, especially the open format with the end to perform a metabolome analysis through a workflow (Fig. 7) (Spicer et al., 2017; Chang et al., 2021). Thus, the metabolomic analysis is multivariate where a large number of metabolites are correlated in different stages of pathological process, so that the clinical aspects of the disease should be included, as well as the establishment of a successful and failed therapy (Gowda et al., 2008; Wang et al., 2010; Deidda et al., 2015). In this order of ideas there are web based NMR spectral databases for multivariate correlation of metabolites between them Human Metabolome Database (HMDB), Biological Magnetic Resonance Data Bank (BMRB), the Madison-Qingdao Metabolomics Consortium Database (MMCD) and the NMRShiftDB2 database (Ellinger et al., 2013; Emwas et al., 2019). In this way human metabolome database (HMDB) (<https://hmdb.ca/>) is an initiative that integrates metabolites, biological activity, metabolic pathways and diseases with spectrometry data; which allows the identification of different types of molecules that represent physiopathological processes (Shahfiza et al., 2017; Wishart et al., 2018). For this reason, metabolomics is considered to be an analysis strategy of complex biological systems that integrates information platforms to evaluate real time the different adaptations of living beings against the challenges of the environment (Pinu et al., 2019a, 2019b; Graw et al., 2021). Likewise, the metabolomic

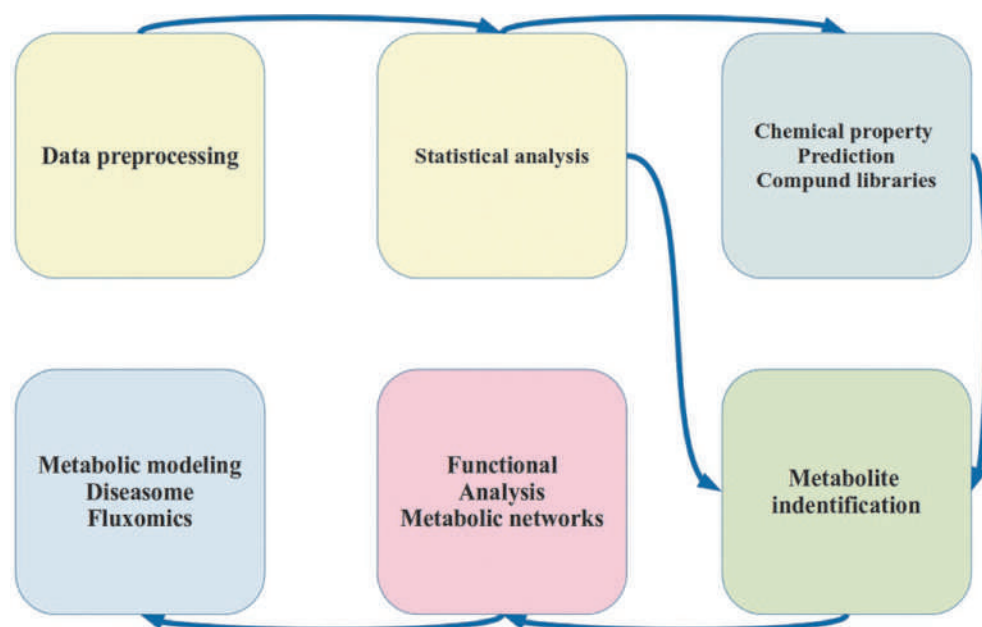


Fig. 7 Metabolomics workflow analysis.

data obtained from the biological systems can be introduced into Machine Learning Algorithms systems that will determine the future models of precision medicine in which the predisposing causes of the disease can be evaluated and treat the patient based on his pharmacogenomics and pharmacometabolomics (Schork, 2019; Ahmed et al., 2020; Subramanian et al., 2020).

Conclusions and perspectives

The description of a human and microbial metabolic network in response to infectious diseases is the objective of metabolomic applications in the clinical practice of infectology (Robinson et al., 2019). In this order of ideas, metabolic networks are the functional expression of genomics and proteomics represented by the production of metabolites, which configures a useful and robust tool for precision medicine models (Comte et al., 2020). In this way infectious diseases require an early diagnosis, timely treatment and close monitoring to assist the process of resolving the condition, so the metabolomics is configured as a promising tool that will help the treatment of patients based on their metabolome (Mamas et al., 2011). Thus, the inclusion of strategies for metabolomic study, as well as the methods used should be included in clinical practice to develop pharmacometabolomic models that determine the progression and decision-making of infectious disease with assistance from artificial intelligence systems, with which develop algorithms with which to explore the most accurate medical possibilities in the approach of the infection (Kell and Goodacre, 2014).

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Detection of Viruses in Histological Samples

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Summary

Molecular pathology is an integral and essential component of diagnostic pathology, especially in the diagnosis of infectious diseases. Molecular pathology has come a long way from basic histopathological examination of tissues to aid diagnosis. The primary drivers for the emergence of this discipline are the enormous advances in molecular biology and the availability of reagents such as monoclonal antibodies. The application of molecular biology to histology, paved the way for the development of techniques such as in situ hybridization, immunohistochemistry, immunofluorescence, multiplex-staining and PCR. These techniques allow the accurate detection and localization of infectious agents in tissue samples. In this article, we describe these techniques and modifications thereof in the diagnosis of viral infections. We also discuss the advantages, disadvantages, and applicability of these methods in the context of the different types of histological tissues.

Introduction

Recent statistical estimates suggest that, there are as much as 320,000 viruses that potentially exist and could infect mammals (Anthony et al., 2013). However, to date, less than 0.07% of these have been shown to infect humans (Woolhouse et al., 2012), but the numbers are gradually increasing with new viruses being regularly discovered (Ksiazek et al., 2003; Phelan et al., 2020; Ryu, 2017; Zumla et al., 2015). Outbreak of new viruses can have enormous impact, not only on health, but also on social and economic sectors of a society. The COVID-19 pandemic illustrates this point very well (Quan et al., 2021). Thus, having rapid, specific and sensitive methods for the detection of emerging or re-emerging viruses is of paramount importance, not only during outbreaks, but also for routine diagnosis and research purposes. Traditionally, methods such as viral culture, electron microscopy, and serology were commonly used for viral detection (Payne, 2017; Storch, 2000). These techniques, albeit still used, can be very expensive, labor intensive, require several days to complete, and in many cases, have poor sensitivity, specificity or both. Advances in molecular biology, genetics and biotechnology has revolutionized the detection and diagnosis of infectious agents. New methods such as PCR-based techniques, or more recently, CRISPR-based techniques have emerged (Artika et al., 2020; Chertow, 2018). These techniques are not only highly sensitive, but they are also very rapid, and easily adaptable for routine use in diagnostic, as well as research laboratory. Additionally, some of these techniques can be used for the detection of multiple viruses from the same clinical sample simultaneously. Typical example of this is the detection of common respiratory viral pathogens, such as influenza virus, respiratory syncytial virus, adenovirus, parainfluenza virus, coronavirus, and human metapneumovirus from a single nasopharyngeal swab (Carr et al., 2010; Han et al., 2020; Mahony, 2008).

Advances in molecular biology and biotechnology has also lead to the emergence of the field of molecular pathology. Pathologists now have a much broader choice of techniques to help accurately identify or diagnose infectious diseases (Chien et al., 2001). They are no longer restricted to examining stained tissues sections under the microscope for the identification of histological or cytopathic changes in infected cells. The application of molecular tools to biopsy tissues has led to the development of techniques such as in situ hybridization (ISH), immunohistochemistry (IHC), immunofluorescence (IF) and multiplex-staining (Blom et al., 2017; Tan et al., 2020). Moreover, nucleic acids or proteins extracted from biopsies are now routinely used, not only for viral screening, but also for measuring viral load, determining viral and cellular gene expression, identifying viral sequences and

genotyping (Liloglou et al., 1994; Pan et al., 2020). These approaches are not only extremely sensitive, but they can also provide great insight into the host-viral interactions and mechanism of pathogenesis of viral-associated diseases. Single viral infected cells can now be readily identified and their cellular phenotype determined from amongst thousands of cells in a single tissue section (Huang et al., 2012; Solomon et al., 2017). With the aid of emerging digital technologies and imaging, data can also be quantitated to provide a more accurate and detailed analysis of disease processes. In this section, we discuss the different types of tissues typically available in the histopathology laboratory and some of the commonly used techniques and their applications in molecular pathology. We also discuss the main advantages, disadvantages and impact of these technique on viral studies.

Types of histological specimens

The primary type of clinical material sent to histopathology departments are tissues samples. Arguably, one of the key factor in influencing which technique or approach to use for viral studies, depends on the nature or form in which the tissue samples are available; fresh frozen, or formalin-fixed, paraffin-embedded (FFPE) or archival. Thus, it is paramount to briefly discuss these tissues before outlining the techniques used for viral detection and analysis.

Frozen samples

Frozen cryostat sections are particularly useful when rapid detection/diagnosis is required. Additionally, frozen sections are the choice of tissue to use when detecting antigens that are sensitive to aldehyde fixation and processing. Compared to FFPE tissues sections, frozen sections have much better antigen preservation, and antigen retrieval steps are not normally required for downstream IHC. Additionally, frozen sections also retain good preservation of RNA, DNA and native protein structure, which is dramatically compromised with chemical fixation (Dey, 2018). Thus, frozen tissues are suitable for molecular and genetic studies, especially if the samples are snap frozen in liquid nitrogen immediately after removal from the patient. If the sample is not going to be used on the same day, then it should be stored in -80°C to avoid degradation of nucleic acids and proteins. For cutting cryostat sections, the frozen tissue is placed on a cryostat chuck for embedding in a suitable medium like OCT (Peters, 2010). Tissue sections with $4\text{ }\mu\text{m}$ thickness are cut using a cryostat, mounted on microscopic glass slide, and kept at room temperature. The major drawback of frozen tissue is that the tissue morphology is markedly poor compared to FFPE sections. Additionally, long-term storage of frozen tissues is a major practical challenge, especially in low resource countries.

Formalin-fixed paraffin embedded samples

Formalin is a solution of formaldehyde. It is the most common chemical fixative used in histopathology. It provides excellent preservation of tissues morphology, which is arguably one of the main reasons for its routine use. Formalin-fixed tissues are processed and embedded in paraffin wax blocks. These blocks can be easily and safely stored at room temperature for decades without any major impact on tissue morphology and architecture. Paraffin-wax also provides solid support when cutting thin $4\text{ }\mu\text{m}$ tissue sections using a microtome. These sections are floated in a water bath and then picked up onto a microscopic slide. The sections are then dried by incubating in a heated chamber at 60°C . This increases their adherence capacity to the microscopic slide and clears any wrinkles left during sectioning. Prior to staining, a number of essential tissue treatment and processing steps are required (Canene-Adams, 2013).

Fixing tissues in formalin and processing them to paraffin-wax blocks is a time consuming and laborious process, prone to variations. To keep the variations to a minimum, it is important to keep some of the essential steps as standardized as possible. For example, tissues should be placed in formalin solutions as soon as possible after collection to avoid tissue degradation. Tissues should be fixed in formalin for a standard length of time. Over or under fixation can impact the subsequent results. Formalin is a protein-cross linker and although it provides good preservation of cellular and viral proteins, it does inevitably lead to their denaturation and loss of their biological activity. In some cases, denatured proteins can mask the antibody binding epitopes and hence impact IHC (Paavilainen et al., 2010). Nucleic acids are also poorly preserved in FFPE samples. Generally, DNA/RNA extracted from FFPE is typically fragmented and of poor quality (Coates et al., 1991) and not ideal for some of the molecular, genetic and sequencing studies (Mathieson and Thomas, 2020). These factors need to be taken into account when considering the use of FFPE tissues for downstream analysis of viral infections.

Archival samples

Many pathology laboratories and anatomy museums, archive tissues samples in formalin or in the form of FFPE tissue blocks. These samples are an exceptional source for biomedical research (Hassani et al., 2018; Hassani and Khan, 2015). In the past three decades, a number of truly innovative approaches and technical advances have been made exploiting archival tissues to better understand the molecular and cellular pathways underlying diseases such as cancer and infections. High throughput proteomics and genomics using tissue microarrays on FFPE archival samples has been developed (Groses et al., 2008; Kallioniemi et al., 2001; Zhang et al., 2017). Although these techniques are not yet in common routine diagnostic use, from the research perspective, they are already providing great insights into disease mechanisms and potential translational applications.

Commonly used methods in molecular pathology

Immunohistochemistry (IHC)

To visualize individual cells in the context of tissue morphology, thin tissue sections need to be appropriately stained and examined under a light microscope. Broadly speaking, there are two main approaches to staining sections for microscopy; traditional methods, and antibody-based methods. The former involve dyes that selectively bind to certain cellular structures. The most commonly used dyes are hematoxylin and eosin (H&E). Hematoxylin stains cell nuclei blue, and eosin stains cytoplasm and connective tissues pink. Although H&E staining has been used for almost 150 years and is considered the gold standard, it rarely provides any direct evidence of viral presence, except when a unique cytopathic effect (CPE) is observed with certain viral infections. Presence of Negri bodies in cells infected with rabies virus (Srinivasan et al., 2005) and owl-eye inclusions with cytomegalovirus infection (Mattes et al., 2000) are two well-known examples. More often than not, it is the antibody-based methods that allow direct identification of viral presence at the cellular level. In this approach, a specific target antigen in a cell or tissue is recognized using specific antibodies (Cotter and Loda, 2017). This technique is also known as immunocytochemistry (ICC), when performed on cells, or immunohistochemistry (IHC), when performed on tissues. While IHC has proved its effectiveness in surgical pathology to obtain diagnostic information, it can also be used to confirm viral infection, particularly when no CPE is observed (dos Santos et al., 2013). This is performed by using specific labelled antibodies against the respective viral antigen. With easy access to a range of commercially available monoclonal antibodies against specific viral components, detection of viruses by ICC/IHC has become a standard procedure in diagnostic pathology (Fig. 1). Advances in producing and labelling antibodies with markers such as enzymes or fluorescent dyes, has further expanded the use of IHC for viral studies. It is now possible to make a laboratory diagnosis of viruses such as influenza or respiratory syncytial virus from a nasopharyngeal swab in less than 30 min. Such rapid diagnostic tests have aided significantly in the management and treatment of these infections (Basile et al., 2018).

Like any diagnostic procedure, sample preparation is vital for reproducible, accurate and reliable results. For IHC, it is important to maintain the antigenicity, morphology and tissue architecture. This can be achieved through good tissue collection, appropriate fixation (formalin, quick-freezing), tissue processing and sectioning. FFPE sections between 3 μ m and 5 μ m are most commonly

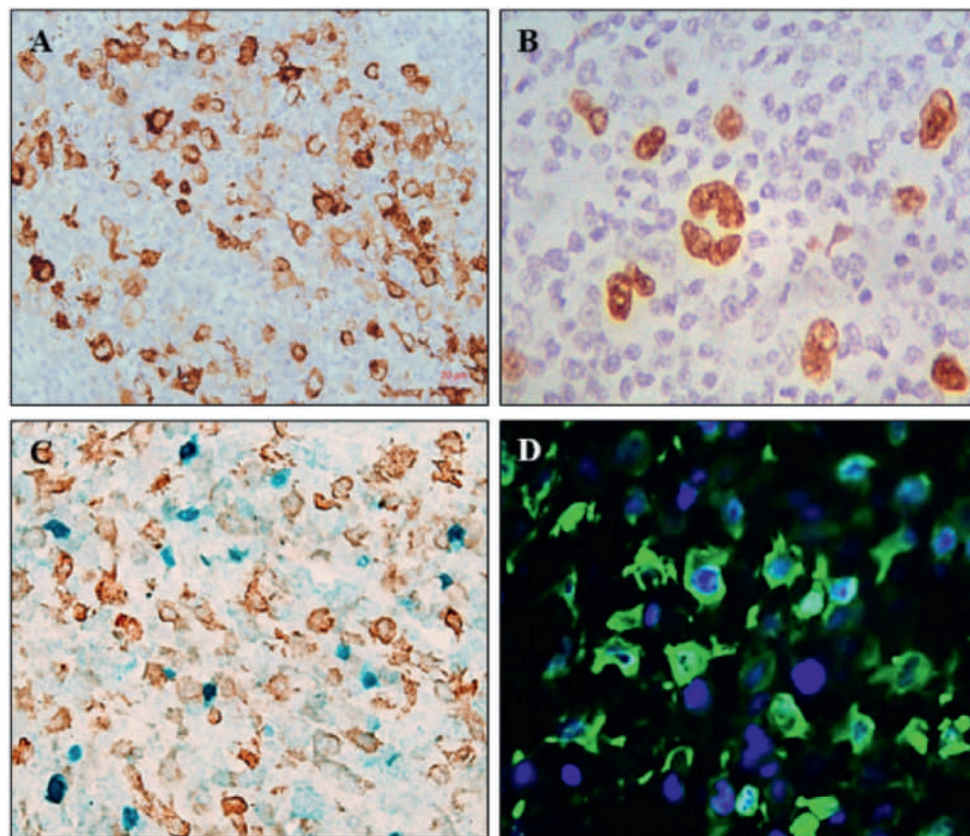


Fig. 1 Common techniques used in molecular pathology. (A) Detection of EBV encoded latent membrane protein (LMP1) by IHC in a spleen section from a rabbit infected with EBV. (B) Detection of EBV by EBER-ISH in lymph node section from a patient with Hodgkin's lymphoma. The virus is present in the large malignant cells, but not the small lymphocytes (C) Detection of two different EBV proteins by double-IHC in spleen section from a rabbit infected with EBV. Brown membrane staining indicates EBV-LMP1 expression (latent marker), whilst turquoise nuclear staining indicates expression of EBV-BZLF1 (lytic marker). The staining shows the presence of both, lytic and latently infected cells in the same tissue section. (D) Combining fluorescence EBER-ISH (blue) with LMP-IHC (green) in EBV infected rabbit spleen.

used. The IHC procedure involves multiple steps; after sectioning, the tissue sections are mounted on microscopic glass slides, paraffin-wax is removed using xylene, and sections are gradually dehydrated using graded ethanol. Depending on the target viral antigen being detected and type of tissue being used, additional steps to block or quench endogenous enzyme activity or auto-fluorescence may be required (Canene-Adams, 2013). In FFPE samples, formaldehyde chemically crosslinks the proteins and in some cases this can mask target proteins from antibodies. In such cases, target antigens have to be retrieved using methods such as enzymatic, proteolytic or heat retrieval, prior to incubation with primary antibody targeting the antigen of interest.

There are a wide variety of antibodies commercially available for use in IHC. For optimal staining, it is important to select antibodies that possess low cross-reactivity and high specificity. It is critical to be familiar with the species in which the antibody is raised to prevent any cross reactivities and false positives. Antibodies can be either monoclonal or polyclonal. Each type has its own set of advantages and disadvantages. Monoclonal antibodies tend to detect a single epitope with high affinity and high specificity. By contrast, polyclonal antibodies recognizes multiple epitopes. However, unlike monoclonal antibodies, polyclonals are much more stable to changes in buffer composition, protein conformation and pH. It is important to choose a primary antibody that is highly validated, since it can affect the entire outcome of the experiment (Ivell et al., 2014).

Although monoclonal antibodies are fairly specific in binding to their respective epitope, they can nevertheless interact weakly and non-specifically with other antigens, resulting in background signals. These non-specific interactions and background signals can however be reduced or eliminated by incubating the tissue samples in blocking buffers containing bovine serum albumin (BSA), or gelatin or non-fat dry milk. Other technical aspects such as diluting or changing the antibody concentration, incubation time and temperature, can also help in reducing the background staining. Commercial blocking solutions having superior effectiveness are now also available. To check for quality control, suitable positive and negative controls must be included in each run of IHC.

For indirect IHC, secondary antibodies that recognize and bind to the primary antibody are used. Secondary antibodies are usually conjugated to reporter molecules such as enzymes (e.g., peroxidase, or alkaline phosphatase), or fluorochromes (e.g., FITC or rhodamine). Enzymatic reporter molecules reacts with their substrate to produce a colored product, which can be visualized using light microscopy. The most commonly used enzyme substrates are DAB or NBT/BCIP, which produce brown or purple colored product respectively (Fig. 1A). The intensity of the colored product can be adjusted by incubating the sections in the substrate for longer. With DAB, the signal strength can be further enhanced by including small amounts of ions such as nickel or cobalt into the substrate buffer. Fluorescent reporters are now increasingly being used in IHC. Fluorescence IHC is particularly useful for multiplex staining where several target antigens are being detected simultaneously in the same section (Fig. 1D) (Blom et al., 2017; Tan et al., 2020). Thus, it is now possible to identify viral infected cells, the cellular phenotype of the infected cells, and the phenotype of the surroundings non-infected cells in a given section. A common hurdle in the fluorescence IHC is autofluorescence, due to cellular components such as flavins, collagen, RBCs, elastin, lipofuscin and porphyrins (Croce et al., 2018; Croce and Bottiroli, 2014). Treating sections with reagents such as Sudan black and sodium borohydride, can quench the endogenous autofluorescence to some extent. Another factor to take into consideration is the long term storage of stained sections. In contrast to enzymatic IHC, sections stained using fluorescent reporters, the label can gradually fade over time with exposure to light. Once again, commercially available anti-fading agents can be used to limit this photo-bleaching effects (Renshaw, 2017).

There are various advantages of using IHC to detect viruses. It provides a histomorphologic correlation between the viral agent and host tissue responses, based on the localization of the virus and its surrounding cells. This is important in understanding the pathogenesis of viral infections. Moreover, recent advances in spatial transcriptomics is beginning to link cell types to tissues morphology, physiology and function at molecular levels, not previously possible (Crosetto et al., 2015; Lein et al., 2017; Marx, 2021). Since formalin fixation deactivated viruses, working on fixed tissues is safe and can be carried out in a standard laboratory. This is a distinct advantage, particularly when studying highly pathogenic viruses such as avian H5N1 influenza, rabies, Ebola and Crimean Congo, which normally require biosafety level 3 or 4 laboratories for handling. Additionally, IHC is suitable for detecting and studying viruses in archival material, allowing retrospective studies to be performed.

In situ hybridization (ISH)

In situ hybridization (ISH) is a technique that allows the detection and localization of viral nucleic acid (DNA or RNA) in tissue sections or cytological specimens using labelled nucleic acid probes with complementary sequences to the target viral nucleic acid. Over the past three decade, ISH has been increasingly used in research laboratories for studying viral-associated diseases. However, its use in diagnostic laboratories has been limited. Relative to other methods such as IHC and PCR, ISH is much more specialized, time consuming and not easily amendable for automation.

ISH can be performed on most kinds of cell and tissue preparations, including smears, cytopspins, FFPE and snap frozen sections. Since ISH involves targeting viral DNA or RNA, it is important to fix or snap freeze the tissues as soon as possible. This is particularly essential if viral RNA is being targeted for detection; RNA has a fairly short half-life and starts to degrade within minutes after collection. The complementary probes used to detect target nucleic acids, can be RNA probes (riboprobes), single stranded DNA (ssDNA) probes, double stranded DNA (dsDNA) probes, or synthetic oligonucleotides (oligoprobes) (Braz et al., 2020; Urbanek et al., 2015; Wu and Wang, 2019). To make the target nucleic acid accessible to the probes, the sections are usually pretreated by proteolytic digestion. Probe and tissue DNA also need to be denatured, either by thermal, alkaline or acid treatment to form a single strand and allow hybridization to occur. The most convenient method is heating the tissue sections with the probe for a few minutes at 90–100 °C. The probe is then allowed to hybridize to its target DNA/RNA, most often by incubating overnight in a heated oven at

around 37–45 °C. Following post-hybridization washes to remove the non-hybridized labelled probed, the sections are incubated in primary antibody against the target label. The sections are then washed to remove the unbound primary antibody and the sections are incubated in secondary antibody, conjugated to an enzyme or fluorescent reporter. These steps are the same as those for IHC described above. In the early days when ISH was first introduced, radioisotopes such as P^{32} were frequently used to label the complementary probes (Chen et al., 2012; Niedobitek and Herbst, 2003). However, this approach was much more cumbersome and required designated facilities and handling precautions. Moreover, it was time consuming, it had poor resolution and it was limited by the short-life half of the labelled probe. The use of non-radioactive haptens such as digoxigenin or biotin changed all of this. The method became much more user friendly, rapid, had higher resolution and the stained slides could be stored easily and safely for archiving.

Non-radiolabeled ISH is now well-established in molecular pathology laboratories for research on viruses. The technique can be used, not only to detect a virus in the context of tissue morphology, but also the cellular phenotype of the infected cells and extent of infection. Additionally, ISH has played an important role in helping to discriminate between an etiological and non-etiological role of viruses in disease pathogenesis, especially for viruses that are highly prevalent in the human population. For example, Epstein-Barr virus (EBV) is an oncogenic virus asymptotically carried by over 90% of the people worldwide. Using ISH, it was demonstrated that this common virus was present in the malignant cells of approximately one third of all cases of Hodgkin's lymphoma and hence, etiological associated with the disease (Fig. 1B), but other common herpesviruses such as cytomegalovirus (CMV) or human herpesvirus-6 (HHV-6) were absent (Khan et al., 1993). In other malignancies such as breast cancer, it was shown that EBV was present in occasional infiltrating lymphocytes and not malignant cells, thus unlikely to be involved the disease process (Khan et al., 2011). Similarly, using ISH, high-risk HPV subtypes can be detected in cervical cancer (Sheng et al., 2013). ISH has also been used to detect viral DNA or RNA in human tissues (McDougall et al., 1986). Combination of ISH and IHC can provide the simultaneous detection of viral nucleic acids as well of viral or cellular proteins (Fig. 1D).

Polymerase chain reaction

Polymerase chain reaction (PCR) first described in 1985, is a simple technique to detect and amplify gene segments from DNA or RNA (Saiki et al., 1985). PCR is by far the most sensitive technique employed in various clinical and microbiological laboratories to detect etiological agents in cells, tissues and body fluids. In the last 20 years, it has proved to be a useful tool for gene analysis in a quick and streamlined fashion. This Nobel Prize winning technique revolutionized the detection and analysis of nucleic acids (Shampo and Kyle, 2002). Using PCR, it became possible for the first time to amplify and analyze DNA from archeological samples of extinct species such as the woolly mammoth and the dinosaurs (Hoss et al., 1994; Poinar et al., 2006; Woodward et al., 1994). This technical advance gave a glimpse into evolutionary past of these species. Similarly, PCR-based approach allowed the discovery of new viruses from specimens dating back tens of thousands of years (Legendre et al., 2014; Philippe et al., 2013).

PCR generates abundant DNA in a simple reaction tube by selectively and specifically amplifying the target sequence from amongst cellular genome, which is usually 1000s of fold in excess. It is like being able to detect a needle in a haystack. The technique is not only very sensitive and rapid, but it is also fairly versatile. It can be used to amplify and analyze specific DNA or RNA sequences from a wide variety of specimens, including peripheral blood, urine, CSF, swabs, and histological samples such as FFPE and needle-biopsies (Valones et al., 2009). The molecular data acquired by PCR, combined with the observational expertise of the histopathologist, can help shed light on disease onset, progression and its response to therapy.

A number of new modifications and adaptations have been made to the basic PCR methodology, including in situ PCR, quantitative PCR (qPCR), reverse transcriptase PCR (RT-PCR), nested PCR and multiplex PCR. The basic principle however, remains unchanged (Diss, 2003). Target sequences are amplified using synthetic oligonucleotides having complementary sequences to the regions flanking the target sequence. These oligonucleotides act as primers for heat stable enzyme Taq polymerase. Repeated cycles of heat denaturation of the test DNA, annealing of primers to their target sequences, and extension of the annealed primers by the Taq polymerase, results in the exponential amplifications of the target sequence. The amplified product can be analyzed or sequenced. Using multiple primers against several different infectious agents, PCR permits the screening of multiple potential etiological agents in a single sample (Wittwer et al., 2001). This is particularly useful for conditions such as respiratory tract infections, which are often indistinguishable on clinical presentation but can be caused by a variety of different viruses and bacteria (Mengelle et al., 2014; Zumla et al., 2014). Further adaptation of multiplex PCR with qPCR allows simultaneously real-time quantification of several targets (Hawkins and Guest, 2017).

In many research laboratories, nucleic acid analysis has become largely automated for identification for multiple viruses. This key feature has pushed qPCR from research laboratories to diagnostics. Relative quantification generally delivers sufficient data and is easier to analyze. However, absolute quantification is necessary when an infection is being monitored. This helps in interpreting the data in a global context, allowing universal approach to downstream interventions. For example, viral load can be used as an indicator to determine whether the selected treatment regimen is working or not. By quantifying the viral load, virus-host interactions, extent of infection and compliance to anti-viral therapy, as in the case of HIV, can be determined (Bhatti et al., 2016; Saag et al., 2020). The severity of the disease progression can also be determined by qPCR by studying viral presence, reactivation and persistence. qPCR is commonly used for detecting and monitoring EBV viral load in organ recipients who may be at increased risk of developing post-transplant lymphoproliferative disorders (Dierickx and Habermann, 2018). Similarly, quantitative measurements of other viruses, such as cytomegalovirus (CMV), varicella zoster virus (VZV) and BK polyomavirus in transplant recipients, can help in the early identification of patients at risk of complications (Kotton and Fishman, 2005; Vanichanan et al.,

2018). To determine disease progression, quantification of viral loads at several time points is critical. Moreover, PCR is a common tool used for the identification and characterization of emerging pathogens (Zhou et al., 2020). Although PCR is rapid and extremely sensitive, it is prone to contamination and precautions have to be exercised. Tissue samples fixed in formalin are often not suitable for amplifying large sequences, since the fixative causes DNA damage (Srinivasan et al., 2002). Moreover, some of the tissue collection and processing steps, such as autopsies, are not usually performed in sterile conditions and this can increase the risk of contamination.

Multiplex-staining

Although conventional IHC is well established and indispensable in diagnostic pathology, it does have its limitations. Using light microscopy, the technique only allows for one marker to be detected per tissue section. This limitation has led to the development of double-staining procedures, targeting two separate antigens (cellular or viral) using two different chromogenic reporters (Fig. 1C). An important consideration in this procedure is the appropriate selection of the primary antibodies. For indirect IHC, the two primary antibodies have to be from different species (e.g., mouse and rabbit) or at least be of different isotype to avoid cross-reactivity and false positives. Primary antibodies from different species can be mixed and applied to the section at the same time. Additionally, the secondary antibodies (e.g., anti-mouse and anti-rabbit) have to be conjugated to different enzymes requiring different chromogenic substrates. The resultant enzymatic reaction should lead to two distinct colors easily distinguishable under the light microscope. Commonly used combinations include, DAB with peroxidase, and fast red or new fuchsin or nitro blue tetrazolium (NBT) with alkaline phosphatase (Lojda et al., 1979).

Double staining, combining IHC with ISH is also now well established (Fig. 1D). Although both of these techniques target different types of molecules (IHC targets proteins whilst ISH targets nucleic acid), they provide results that are complementary. Using a combination of IHC and ISH, it has been possible to identify not only the phenotype of the infected cells, but also what viral genes are expressed at RNA level. Such combination of double-staining have been used for studying a number of different viruses, including EBV, HIV and more recently SARS-CoV-2 (Lean et al., 2020; Nakajima et al., 2003; Takahashi et al., 2020). One major drawback to determining viral gene expression is the fact that mRNA is unstable and degrades fairly quickly. Thus, long-term archival FFPE tissues are often unsuitable for this procedure.

Although, the double-staining methods are a notable advance, compared to the single staining methods, they nevertheless still fail to provide a comprehensive and global analysis of infected tissues. The fact that tissue sections typically consist of multiple types of cells, the proportion and arrangement of which can change in disease states, the ability to stain and visualize multiple components in a tissue is clearly important. Advances in antibody design, production and labelling chemistries, coupled with better imaging technologies, have facilitated the development of multiplex-IHC (McGinnis et al., 2021). It is now possible to stain multiple cellular and viral markers simultaneously, providing a more comprehensive analysis of tissue composition, cellular functions, and cell to cell interactions in a single tissue section (Ilić et al., 2018; Tan et al., 2020). More recently, the principle of multiplex-IHC has been extended to multiplex-ISH, allowing 1000s of targets, viral and cellular, to be detected at cellular level (Femino et al., 1998; Lee et al., 2014). This spatial transcriptomics approach is providing an unprecedented and truly global picture of the location, distribution and the interaction of the infected cells with the surrounding cellular milieu (Lein et al., 2017; McGinnis et al., 2021). Moreover, these multiplex staining platforms are being developed to be applicable to FFPE tissues, and making the technique suitable for both routinely processed tissues as well as archival (Decalf et al., 2019). Additionally, the fact the FFPE tissues retain excellent tissue architectural morphology, provides excellent resolution at single cell level.

Concluding remarks

The last 50 years have seen unprecedented advances in molecular biology, genetics and biotechnology. These advances, coupled with equally remarkable progress in imaging and digital technologies, is revolutionizing research and laboratory diagnosis of infectious diseases. However, some of these novel and advanced techniques, require sophisticated equipment, technical expertise, and are expensive to run. Moreover, in many cases, the comprehensive information gained from these methods, albeit very useful for research purposes, does not always help in clinical decision making and patient management. With a focus on precision medicine, current research efforts are aimed at making these novel diagnostic technologies rapid, sensitive, accurate, low-cost and preferably applicable to point-of-care testing (Drain et al., 2014) (Table 1).

Table 1 Techniques commonly used in molecular pathology for the detection of viruses in histopathological samples.

<i>Technique</i>	<i>Advantages</i>	<i>Disadvantages</i>	<i>Comments</i>	<i>References</i>
Polymerase chain reaction (PCR)	• Less labor intensive. • Highly specific and quantitative. • Large dynamic range. • Can detect multiple viral genotypes in single reaction. • Quick detection of viruses. • Higher sensitivity than ISH and IHC. • Can determine the viral load. • Widely used in clinical diagnostics.	• Affected by inhibitory compounds and artifacts. • Risk of contamination. • Special equipment and qualified personnel required. • No histomorphologic correlation. • Reduced sensitivity for formalin fixed samples. • Knowledge and expertise required to design primers and probes.	• Nucleic acids extracted from FFPE samples may not always be suitable for molecular analysis. • Archival samples may also affect the nucleic acid specificity and sensitivity.	Dedhia et al. (2007), Diss (2003), Mackay et al. (2002), Pan et al. (2020), Reta et al. (2020), and Watzinger et al. (2004)
In situ hybridization (ISH)	• Highly specific than IHC. • Offers histomorphologic correlation. • In-house probes could be synthesized with known sequence. • Offers histomorphologic correlation at nucleic acid level. • High reproducibility.	• Lower sensitivity than IHC. • Increased time and cost. • Prolonged fixation in formalin may affect the sensitivity. • Difficult to detect the targets with low DNA or RNA copies. • Not widely used in clinical diagnostics.	• Higher sensitivity of RNA transcripts in frozen sections due to enhanced preservation of RNA.	Kelesidis et al. (2011), Mesa and Lilia (2013), Niedobitek and Herbst (2003), Roe et al. (2019), and Sheng et al. (2013)
Immunohistochemistry (IHC)	• Higher sensitivity than ISH • Offers histomorphologic correlation. • Testing several samples simultaneously. • Can reduce the potential risk of exposure. • Used in Clinical diagnostic analysis.	• Availability of antibodies. • Cross-reactivity antigens in tissues. • Semi-quantitative. • Variability depends upon type of fixation. • Interpretation of results is subjective.	• Archival samples stored for decades can be analyzed. • Viral infections whose initial causes are unknown can be characterized.	Bramhachari et al. (2019), Chien et al. (2001), Grillo et al. (2017), and Reta et al. (2020)
Multiplex-staining	• Can detect multiple viral genes (Double staining), DNA or RNA (Dual ISH-IHC). • Histomorphologic correlation between viral and cellular markers can be achieved. • Reduced turn-around time.	• Reduced mRNA signal through peroxidase reaction. • Labor intensive and Cross-reactive. • Not used in clinical diagnostics. • Extensive knowledge is required. • Possibility of tissue damage and antigen loss.	• Potential to evaluate topographical correlation and cellular tropism of the virus.	Fayyadh et al. (2017), Ko et al. (2019), Lopez (2014) and Sood et al. (2020)

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Methods to Grow and Measure In Vitro Static Biofilms

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Introduction

Bacteria and fungi that attach to surfaces of medical devices, including endotracheal tubes, central vascular catheters, cardiac pacemakers, tissue fillers, breast implants, urinary catheters, and orthopedic implants, may build biofilms (Khatoon et al., 2018). They also are responsible for up to 80% of bacterial infections in humans, such as chronic otitis media, periodontitis, endocarditis, biliary tract infection, urinary tract infection, osteomyelitis, and chronic wounds (Khatoon et al., 2018). Owing to the difficulty of killing biofilms, contaminated indwelling devices must be removed, which results in high cost and complications for the patient (Costerton et al., 1999). Biofilm development can be grouped into four stages (O'Toole et al., 2000). Planktonic bacteria initially attach to the surface and then develop into a sessile growth stage (O'Toole et al., 2000). As biofilms mature, some cells disperse into the surrounding environment as planktonic cells and become the basis for biofilm formation on new surfaces.

Bacteria in biofilms are resistant not only to the immune system but also to treatment with antibiotics (Davies, 2003). By preventing bacteria from being recognized by immune cells and activating response regulators that can influence the activities of immune cells, biofilms can resist the host immune defense systems (Leid, 2009). Nickel et al. reported that sessile cells of *Pseudomonas aeruginosa* were approximately 1000 times more resistant to tobramycin than planktonic cells (Nickel et al., 1985). The reasons why biofilms have high antibiotic resistance include retarded penetration of antimicrobial agents, extracellular polymeric substances (EPS), decreased metabolic activity of cells within biofilms, protective properties of dormant persistent cells, presence of small colony variants, and efflux pump activity (Davies, 2003).

When compared to diverse biofilm flow models, such as CDC Biofilm Reactors, Drip Flow Biofilm Reactors, and Robbins devices, static biofilm models are able to quantify biofilm formation by many bacterial strains in various growth settings (Azeredo et al., 2017; Coenye and Nelis, 2010; Catto and Cappitelli, 2019). They are fast, cost-effective, uncomplicated, do not require special instruments, and they can simultaneously test many samples (Magana et al., 2018). Static models can be employed to: identify genes associated with biofilm formation (Conlon et al., 2002, 2004; Valle et al., 2003); investigate environmental factors (such as growth media, glucose, sodium chloride, ethanol, pH, temperature, and time) influencing biofilm formation (Pan et al., 2010; Dimakopoulou-Papazoglou et al., 2016; Tango et al., 2018); test the effects of modified surface materials (such as anti-adhesive polymers, nitric oxide-releasing compounds, reactive oxygen species-releasing compounds, photoactivable compounds, and

antibacterial nanostructured materials) on biofilm formation (Tabbasum et al., 2021; Raad et al., 2008; Puckett et al., 2010); as well as to evaluate agents that inhibit biofilm formation (Cady et al., 2012; Chen et al., 2018; Kim et al., 2018; Cao et al., 2019; Przekwas et al., 2020). However, they can produce varied results of biofilm formation depending on laboratories, inoculum concentrations, washing conditions, and other factors (Bahamondez-Canas et al., 2019). Since fresh nutrients are not provided continuously in static biofilm models, it is not easy for them to form mature biofilms (Bahamondez-Canas et al., 2019). Also, there is a disadvantage in that they are not able to represent in vivo states in which bacterial cells undergo shear stress in continuous flow conditions (Bahamondez-Canas et al., 2019; Waters et al., 2014). Nevertheless, static biofilm models are very important models to study biofilm formation. They make it possible to enumerate viable cells, measure stained biofilms microscopically and macroscopically, and differentiate live and dead sessile cells (Djordjevic et al., 2002; Olszewska et al., 2020). The three static biofilm models include the colony biofilm model (CBM), air-liquid interface model (ALIM), and microtiter plate model (MPM) (Conlon et al., 2002; Rani et al., 2007; Yonezawa et al., 2009).

Biofilm detection methods can quantify biofilms and measure live or dead cells within biofilms (Azeredo et al., 2017). Among the various biofilm detection methods, researchers can choose one of the methods depending on accessible techniques, instruments, and specific research purpose. Staining methods, such as crystal violet (CV), 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), are the most widely used and are capable of being applied to measure biofilm biomass and live or dead cells (Gabrielson et al., 2002; Paytubi et al., 2017; Traba and Liang, 2011). Microscopy methods, such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM), and atomic force microscopy (AFM), are essential tools for characterizing biofilm structures since they are able to show spatial organization of biofilms (Stokes, 2003; Graham and Orenstein, 2007; de Carvalho and da Fonseca, 2007; Handorf et al., 2019).

In vitro static biofilm model

Colony biofilm model (CBM)

CBM, which cultures bacterial biofilms on membrane filters deposited on agar media, has been used to examine antibiotic penetration through biofilm forming cells (Fig. 1) (Singh et al., 2010). The agar media supply nutrients and thick biofilms can be produced by transferring biofilms grown on the filter to fresh media (Wahlen et al., 2018). This model has the advantage that biofilms can be grown in an air-membrane interface without culturing them in liquid media that are not easy to process. Unlike most biofilm growth models, CBM can efficiently replace the energy source or antibiotics without a washing step. Additionally, it allows multiple tests to be run simultaneously by simply increasing the number of membrane filters on the agar medium. It is spatially heterogeneous, with sessile cells of different growth stages existing according to oxygen and nutrient availability (Nadell et al., 2009). Metabolically active cells occupy an air-biofilm interface rich in oxygen, but dead or dormant cells populate the inner biofilm structure (Nadell et al., 2009). CBM is able to form a thick biofilm on agar media in a short time; the thicknesses of *P. aeruginosa* and *Staphylococcus epidermidis* biofilms after 48 h of growth were about 150–300 and 100 μm , respectively (Stewart et al., 2007; Werner et al., 2004).

The number of viable cells obtained following antibiotic treatment in CBM directly reflects cell death rather than biofilm dissociation (Singh et al., 2010; Walters et al., 2003; Anderl et al., 2000). Cells in the colony biofilms may be enumerated by the plate count method and penetration of antimicrobial agents through biofilms can be measured by the diameters of inhibition zones. Epifluorescence, electron microscopes and oxygen electrodes may be employed to assess local antimicrobial action, extracellular components, protein production activity, DNA replication, and oxygen concentration. CBM has also been employed to model wound biofilm infections and test the effects of large amounts of antimicrobial dressings since the external areas of agar media and infection sites are similar (Agostinho et al., 2011; James et al., 2016). Microorganisms with different motility structures can swim or glide over the membrane filter at varying rates, so care should be taken when preparing for CBM (Josenhans and Suerbaum, 2002). Because CBM forms dense colony biofilms, dispersion of sessile cells from the membrane filter may be done by vortexing, sonication and subsequent bacterial counting, but these procedures are time-consuming and require a great deal of work.

Air-liquid interface model (ALIM)

At the interface between air and liquid, aerobic or facultative aerobic microorganisms utilize both oxygen and nutrients at the same time. Most biofilm experiments are performed in liquid media, but multiple bacteria (including *Acinetobacter baumannii*, *Bacillus*

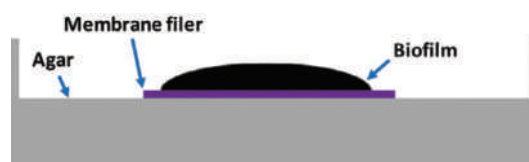


Fig. 1 View of colony biofilm model.

cereus, *B. subtilis*, *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium smegmatis*, *Pectobacterium carotovorum*, *P. aeruginosa*, *P. fluorescens*, *Salmonella enterica*, *Shewanella oneidensis* and *Vibrio cholerae*, as well as the fungi *Candida albicans* and *Saccharomyces cerevisiae* have been known to form biofilms at the air-liquid interface (Abriat et al., 2020; Crespo Tapia et al., 2018; Medrano-Felix et al., 2018; Haque et al., 2017; Sochorova et al., 2014; Zara et al., 2012; Koza et al., 2009; Therien et al., 2020; Oh and Han, 2020; Okshevsky et al., 2018). These organisms are aerotactic and can move towards oxygen above liquid media (Taylor et al., 1999). Bacteria colonizing the interface between air and liquid, forming a pellicle, are involved in respiratory tract infections, biodegradation of hydrocarbons, and improved oil recovery (Matsui et al., 2006; Hu et al., 2013). To make the pellicle, microorganisms can stick to the wall of the test tube, produce a molecule to modify surface tension, swim to reach the air-liquid interface by flagella, or entrap CO₂ generated during glucose fermentation (Angelini et al., 2009; Koizumi et al., 2008; Robertson et al., 2013; Okshevsky et al., 2018). ALIM can be performed in glass or polystyrene test tubes or multi-well plates in either horizontal or tilted positions (Fig. 2) (Yonezawa et al., 2009). Alternatively, glass or plastic coverslips can be placed at angles of 30–90 degrees to wells (Ramalingam and Lee, 2019). After biofilm formation, a conventional, fluorescence, CLSM, or electron microscopy (EM) can be used to visualize biofilms (Ramalingam and Lee, 2019; Wang et al., 2017; Yonezawa et al., 2009). In addition, the air-liquid interface biofilms can be determined by weighing wet or dry biofilm biomass or counting the number of bacteria with a standard plate count method (Pisithkul et al., 2019; Scher et al., 2005). ALIM has been employed in assessing biofilm susceptibility to chlorine, handwashes, sanitizers, heat, sodium chloride, low pH and low concentrations of ethanol (Scher et al., 2005; Tashiro et al., 2014; Ahmed et al., 2020). Meza-Villezas et al. and Li et al. investigated the effect of antibacterial nanocomposites and dental adhesives in the air-liquid biofilms of *V. cholerae* and dental bacteria, respectively (Meza-Villezas et al., 2019; Li et al., 2014). Biofilm formation capabilities of psychrotrophic pseudomonads, *S. enterica*, and *S. cerevisiae* isolated from spoiled meat, water or feces, and wine, can also be compared using ALIM (Medrano-Felix et al., 2018; Robertson et al., 2013; Zara et al., 2012).

Microtiter plate model (MPM)

MPM has been utilized the most in the static biofilm system and allows quantitation of in vitro biofilm formation on abiotic surfaces (Figs. 5–8) (Paytubi et al., 2017; Peeters et al., 2008a). Biofilms can be cultured on both the bottom and walls of multi-well culture plates (Chon et al., 2020; Peeters et al., 2008a). After biofilm formation, either the planktonic cells are removed and biofilms adhering to the microplate surface are washed and directly stained, or biofilms are detached and the number of bacterial cells is counted to quantify biofilm growth. Different sizes (6-, 12-, 24-, 48- or 96-well) of plates, surface treatments (tissue-culture treated or poly-D-lysine surface), surface materials (polystyrene, polypropylene or polyvinyl chloride), and well shapes (flat, conical, easy wash, round or V-bottom) can be selected according to the research purpose. Coupons, disks or catheter segments impregnated with antimicrobial agents can also be placed in the multi-well plates to investigate biofilm growth (Poilvache et al., 2020; Chandra et al., 2018; Chong et al., 2021).

MPM has the disadvantage of depleting nutrients or concentrating signaling molecules, as nutrients are not continuously supplied (Hunt et al., 2004). Although fresh nutrients can be regularly replaced throughout the experiment, biofilms may be detached while planktonic cells are discarded. Another drawback of MPM is evaporation of liquid nutrients during long-term incubation in the outer wells of multi-well plates at 37 °C, the most common temperature used in investigating biofilm formation (Das et al., 2017). The loss of bacterial culture media leads to concentration of certain ingredients and changes in osmolarity, which can have a profound effect on biofilm formation. To minimize evaporation, the microplates can be sealed with a sterile adhesive film or sterile water can be pipetted into peripheral wells. Compared to the dynamic biofilm systems, MPM is simple, easy to use, rapid, and does not require an expensive instrument to quantify biofilm formation. In addition, a large number of samples can be run at once using 96-well plates. It has been applied widely to test the ability of bacteria to form sessile cells in vitro and to investigate the effects of bile, serum, fibrinogen, milk components, and cigarette smoke condensate on biofilm production. MPM has also been used to screen biofilm formation-defective mutants, examine the interaction between mixed bacteria during biofilm formation, and assess anti-biofilm activities of: antibiotics, bacteriophages, antagonistic and probiotic bacteria, enzymes (α -amylase, dispersin B, DNase I, lactonase, and proteinase K), small molecules (aryl rhodanines, *cis*-2-decenoic acid, ebselen, and terrein), nanomaterials (silver and chitosan nanoparticles, silver-titanium composites, and nanostructured titania coating with silver nanoparticles), biocides (benzalkonium chloride and sodium hypochlorite), quorum-sensing inhibitors (eugenol, cinnamaldehyde, ellagic acid, and curcumin), and plant-derived natural compounds (ursolic acid, naringenin, and xanthorrhizol) (Vandenbosch et al., 2010; Shakeri et al., 2007; Peeters et al., 2008b; Quave et al., 2008; Brackman et al., 2009; Amorena et al., 1999; Dong et al., 2020; Kazmierczak et al., 2021; Phee et al., 2013; Jiang et al., 2020; Chung and Toh, 2014; Ramasamy and Lee,

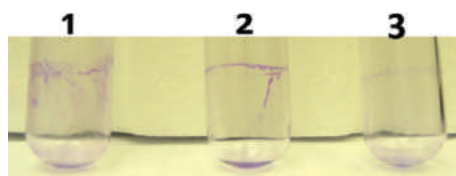


Fig. 2 Air-liquid interface biofilms stained with crystal violet. (1) *Pseudomonas aeruginosa* ATCC 27853, (2) *P. aeruginosa* PA14, and (3) *P. aeruginosa* PA01. Biofilms were grown for 72 h at 37 °C and stained with 0.1% crystal violet.

2016; Rabin et al., 2015; Ta and Arnason, 2015; Mishra et al., 2020; Peters et al., 2012; Toyofuku et al., 2016). Numerous experimental data have made it possible to elucidate the mechanism by which bacteria form biofilms on various material surfaces.

Since some bacterial cells freely floating in MPM may settle at the bottom of the wells and be trapped in EPS, it is difficult to consider biofilms accumulated on the bottom as pure biofilms. The MBEC Assay Biofilm Inoculator, formerly known as a Calgary Biofilm Device, is a tool to overcome the shortcomings of the static MPM mentioned above and has been employed to test anti-biofilm susceptibilities of antimicrobial agents (Nowatzki et al., 2012). It contains a lid with polystyrene pegs that are submerged in broth media, provide solid surfaces for biofilm attachment and formation, and can be coated with hydroxyapatite, titanium dioxide, or other materials (Obaid et al., 2016). The pegs may be removed from the lid. Bacterial cell numbers in biofilms can be enumerated by standard plate count methods, following repeated vortexing and sonication, or biofilm cells can be examined by microscopic techniques (Rodrigues et al., 2011). Since it is not difficult to move the pegs to new well bases containing antimicrobial agents after biofilm growth, the MBEC Assay Biofilm Inoculator has the advantage of being able to test the antibiotic sensitivities of biofilms obtained from long-term incubation (Chen et al., 2020). Biofilms formed in MPM can be measured by the number of viable bacterial cells, the BioFilm Ring test, biomass staining (crystal violet or Syto 9/propidium iodide) or metabolic activity assays (resazurin, adenosine triphosphate (ATP), XTT, or MTT) (Chavant et al., 2007; Hofmann et al., 2012; Nascimento et al., 2014; Peeters et al., 2008b; Ahmad et al., 2021; Kamauchi et al., 2021; Tiwari et al., 2021).

BioFilm ring test (BRT)

BRT is a kit designed to quickly test for effective antimicrobial agents against biofilms in the early stages (Fig. 3) (Olivares et al., 2020; Cremet et al., 2013). After bacteria are mixed with inert magnetic microbeads in the bottom of a 96-well microplate, biofilms are incubated at the desired temperature and time, and then exposed to a magnetic field to capture the microbeads that are not entrapped by sessile cells (Valour et al., 2013). BRT does not require a washing or staining step, so the bias due to experimental

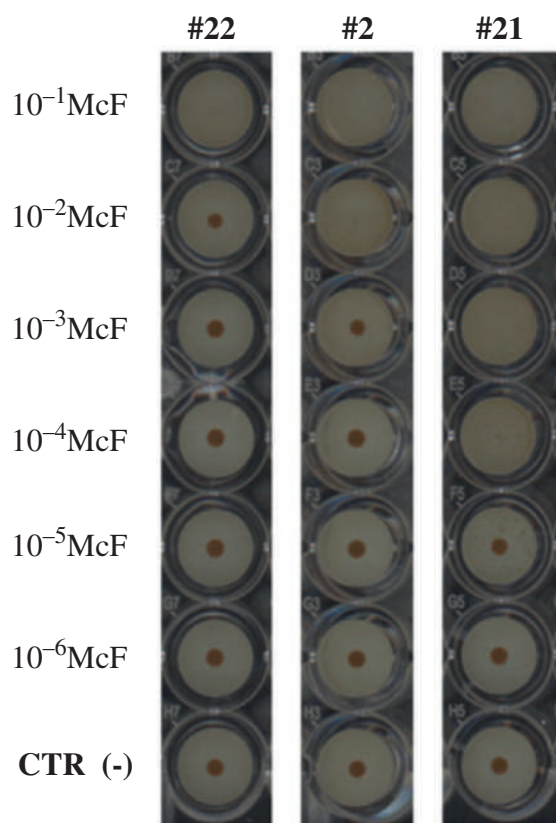


Fig. 3 Biofilm profiling of *Pseudomonas aeruginosa* clinical isolates according to their ability to block the migration of magnetic microbeads in clinical Biofilm Ring Test assay. Biofilm formation in 96-well polystyrene plates is shown after 5 h of incubation at 37 °C for representative strains of three adhesion profiles: strain #22 (no-biofilm producer), strain #2 (weak-biofilm producer) and strain #21 (moderate-biofilm producer). A gradual blocking of beads was noticed, depending on the microbial serial dilutions, and therefore reflects the strength of the biofilm produced by bacteria. Images were obtained after magnetization of the plates on the block test and scanning with the plate reader. Negative controls with only Brain Heart Infusion (BHI) medium and magnetic microparticles are displayed in the last line of microplates. Reprinted with permission from Olivares E, Tasse J, Badel-Berchoux S, Provot C, Prévost G and Bernardi T (2020) Clinical Biofilm Ring Test® Reveals the Potential Role of β -Lactams in the Induction of Biofilm Formation by *P. aeruginosa* in Cystic Fibrosis Patients. *Pathogens* 9(12):1065. <https://doi.org/10.3390/pathogens9121065>.

personnel is relatively small and reproducibility is high. Moreover, it has been coined the Antibiofilmogram method to test biofilm susceptibility of clinical isolates to antibiotics (Tasse et al., 2016). BRT cannot provide information on the detailed structure or thickness of biofilms (Puig et al., 2014). It has been employed to test the effect of cranberry juice, EPS produced by marine biofilm-forming bacteria, and metal-N-heterocyclic carbene complexes against biofilm formation. It has also been used to characterize the association between biofilm phenotype and clonal lineage, to examine the kinetics of biofilm formation in various Gram-positive and -negative bacteria, and to study the role of β -lactam antibiotics in induction of biofilm formation (Abriat et al., 2020; Olivares et al., 2020; Flament-Simon et al., 2019; Brian-Jaisson et al., 2016; Bernardi et al., 2014; Tre-Hardy et al., 2009).

Biofilm measurement

Agar plate counting of viable biofilm cells

Biofilms harvested from CBM, ALIM, and MPM can be plated on appropriate nutrient agar plates through serial dilution following the detachment process, and the number of live bacterial cells can be counted. Plate counting is a reliable and sensitive technique, generally referred to as the gold standard, for bacterial enumeration in biofilms; various bacteria can also be counted at the same time (Table 1). However, it is time-consuming and requires a lot of labor and experienced laboratory personnel. Even though optimal selective culture media and culture conditions are required for multi-species biofilms, plate counting has the advantage of selectively quantifying the desired bacterial species. However, false-negatives can occur if bacterial species are grown under conditions below the detection limits of selective culture media containing antibiotics (Vandecandelaere et al., 2012). The presence of some fastidious, stressed, viable but non-culturable bacteria or unculturable bacteria in biofilms can also lead to false-negative results (Xu et al., 2012). To overcome these problems, molecular techniques such as quantitative real-time polymerase chain reaction, fluorescence in situ hybridization (FISH) using peptide nucleic acid probes, and pyrosequencing can be used to detect DNA from bacteria present in multi-species biofilms (Lopes et al., 2018; Xu et al., 2012; Vandecandelaere et al., 2012).

Viability assessment by SYTO 9/propidium iodide (PI) staining

A live/dead cell staining technique using SYTO 9/propidium iodide (PI) has been used to observe biofilm structure and is commercially available as a kit (Fig. 4) (Ong et al., 2019; Hofmann et al., 2012). SYTO 9, a green fluorescent dye, can penetrate bacterial membranes and bind to nucleic acids of live and dead biofilm cells, whereas PI, a red fluorescent dye, can stain only injured or dead cells (Tawakoli et al., 2013). The stained biofilm samples can be visualized and characterized by a microplate reader, flow cytometry or CLSM (Table 1). Even if bacterial cells in biofilms are killed by antimicrobial agents, if PI does not penetrate their membranes, they are recognized as viable cells (Latka and Drulis-Kawa, 2020). Therefore, total bacterial cell numbers obtained from standard plate counting and viable cell estimation observed by the SYTO 9/PI staining method may not match. Combinations of SYTO 9 and PI dyes may cause high background signals and crosstalk between channels of staining dyes, which makes it difficult to accurately interpret viability of biofilm samples (Bahamondez-Canas et al., 2019). Further, when SYTO 9 dye is used with PI, rapid photobleaching occurs. SYTO 9 dye tends to stain dead cells better than viable cells; additionally, it is not easy to measure the total number of cells of Gram-negative bacteria in biofilms by this method (Stiefel et al., 2015). Some researchers have also employed SYBR green and acridine orange (3-N,3-N,6-N,6-N-tetramethylacridine-3,6-diamine) as a substitute for SYTO 9 (Passerini de Rossi et al., 2007; Feng et al., 2014). The SYTO 9/PI staining technique has been broadly applied to determine the effects of the following against biofilms: antibiotics (clindamycin, clotrimazole, econazole, tobramycin, and vancomycin), an antimicrobial peptide [S4 (1-16) M4Ka], blood platelets, phages (*Klebsiella* phage KP34), chitosan, nanoparticles [silver, poly(D,L-lactide-co-glycolide)], disinfectants (chlorine, monochloramine, chlorhexidine, sodium hypochlorite, and benzalkonium chloride), nitric oxide, photosensitizers (octa-cationic zinc phthalocyanines), heterocyclic compounds (thiazolidinedione and succinimide derivatives), essential oils (carvacrol, citronellol, eugenol, terpineol, *trans*-cinnamaldehyde), lactoferrin chimera, *Scutellaria baicalensis* (Chinese Skullcap), manuka honey, grape seed extract, high-fructose corn syrup, propolis, synthetic flavonoids, casein phosphopeptide-amorphous calcium phosphate, calcium hydroxide, and antimicrobial coatings (polytetrafluoroethylene, silver electrodeposition, and triethoxysilylpropyl succinic anhydride silanization) (Bahamondez-Canas et al., 2019; Pantanella et al., 2013; Hannig et al., 2010; Welch et al., 2012; Latka and Drulis-Kawa, 2020; Magana et al., 2018; Olszewska et al., 2020). Additionally, it has been applied to investigate acid tolerance of biofilms, biofilm development of an oral polymicrobial community and dental unit water lines, the role of mastoid mucosal biofilms in chronic otitis media, biofilms of bronchoalveolar lavage, biofilms in endotracheal tube, and biofilm detection in chronic rhinosinusitis patients and drinking water distribution systems (Welin-Neilands and Svensater, 2007; Chavez de Paz, 2012; Lampikoski et al., 2012; Marsh et al., 2015; Fernandez-Barat et al., 2012; Li et al., 2012; Waller et al., 2018).

Assessment of the total biomass by crystal violet (CV) staining assay

CV is used in Gram staining to differentiate between Gram-positive and -negative bacteria and is the most common staining method to measure the total biomass of sessile cells in both ALIM and MPM (Figs. 2, 5, 7 and 8 and Table 1) (Paytubi et al., 2017; Merritt et al., 2005). After bacteria are grown for the desired time at an appropriate temperature in a test tube or multi-well plate, unadhered planktonic cells are carefully discarded and washed with sterilized distilled water. Then, 0.1–1.0% CV solution is added, incubated at room temperature for 10–30 min, and rinsed with sterile distilled water (Chon et al., 2020). Stained sessile cells are dried and the

Table 1 Methods to measure in vitro static biofilms.

<i>Methods</i>	<i>Application</i>	<i>Advantages</i>	<i>Disadvantages</i>	<i>References</i>
Agar plate counting of viable biofilm cells	Counting of the number of live bacterial cells	Reliable, sensitive technique Count various bacteria simultaneously Selectively quantify the desired bacterial species in multi-species biofilms	Time consuming Require a lot of labor and experienced laboratory personnel False-negative can occur if bacterial species are grown under conditions below the detection limit of selective media Some fastidious, stressed, viable but non-culturable bacteria in biofilms lead to false-negative results If PI doesn't penetrate the dead cell's membranes, they are falsely determined as viable cells	Vandecandelaere et al. (2012) and Xu et al. (2012)
Viability assessment by SYTO 9/propidium iodide (PI) staining	Observing the biofilm structure	Stained biofilms can be visualized and characterized by a microplate reader, flow cytometry, or CLSM	Combinations of SYTO 9 and PI may cause high background signals, crosstalk, and rapid photobleaching It is difficult to measure total number of cells in Gram-negative bacteria's biofilms	Ong et al. (2019) and Hofmann et al. (2012)
Assessment of the total biomass by crystal violet (CV) staining assay	Measuring the total biomass of biofilms		Unable to quantify the actual number of viable cells There is no standardized protocol Variation of the absorbance signals between bacterial species	Paytubi et al. (2017) and Merritt et al. (2005)
<i>Biofilm measurement by metabolic activity</i>				
MTT staining assay	Measuring metabolic activity of biofilms	Simple, fast, and high reproducibility	Require the cell lysis before reading optical density Components of growth media influence bacterial reduction of MTT to formazan Assay score falsely increases if the outer membrane is damaged	Traba and Liang (2011) and Chusri et al. (2013)
XTT staining assay	Measuring metabolic activity of biofilms	Directly measure the amount of formazan Perform an antibiotic susceptibility test of intact biofilms	Poor linear relationship between the colorimetric signal and the bacterial concentration in biofilms Require optimization of standard curve between the formazan product absorbance and the number of viable cells Signal intensity can be influenced some bacteria contain significant amount of intracellular salts	Gabrielson et al. (2002), Adam et al. (2002), and She et al. (2016)
Resazurin (Alamar Blue) staining assay	Measuring metabolic activity of biofilms	Fast and cost effective High sensitivity to detect low levels of viable bacteria Good correlation between the number of bacterial cells and the fluorescent signals	The rates of resazurin metabolization are different depending on the bacteria Incubation times required for the bacteria are different in multi-species biofilms	Paytubi et al. (2017) and Bandeira Tde et al. (2013)
ATP bioluminescence assay	Measuring metabolic activity of biofilms	Fast and easy to perform Sensitive to detect low metabolic activity It is not disturbed by fluorescent substances	It is not suitable to quantify biofilms if ATP is not fully extracted or biofilms are grown for extended periods of time	Stiefel et al. (2016) and Wilson et al. (2017)

(Continued)

Table 1 (Continued)

<i>Methods</i>	<i>Application</i>	<i>Advantages</i>	<i>Disadvantages</i>	<i>References</i>
<i>Biofilm detection by microscopical techniques</i>				
Conventional light microscopy		Able to quantify viable cells in both planktonic and biofilm cells Not expensive Easy to prepare and observe samples Able to observe a large area of viable cell culture during extended periods of time Visualize biofilms after staining with dyes Do not require advanced microscopy technique	Difficulty in counting the total numbers of bacteria Unable to observe small components of bacterial cells or details of the biofilm structure	Relucenti et al. (2021), Pelzer et al. (2012), and Sowndarya et al. (2020)
CLSM	Observing spatial organization of biofilm architecture	Able to determine viable and dead cell numbers Able to identify bacterial species in polymicrobial biofilms Able to perform horizontal and vertical optical sectioning of three-dimensional biofilms	Expensive instrument Require fluorophore staining Biofilm structure influences the fluorophore staining	Ong et al. (2019) and de Carvalho and da Fonseca (2007)
SEM	Imaging of three-dimensional structure with high resolution	Able to image topography and composition	Expensive instrument Samples should be fixed Damage biofilm shape during dehydration step	Stokes (2003), Cazaux (2005), and Weber et al. (2014)
TEM	Imaging of two dimensional structure with high resolution	Imaging the comprehensive morphology and structure including nucleic acids and proteins	Expensive instrument Require highly experienced technicians for sample preparation	Graham and Orenstein (2007), DeQueiroz and Day (2007), and Chuo et al. (2018)
AFM	Imaging of three-dimensional structure with high resolution	Able to provide viscoelastic properties and adhesion forces Able to observe the sample image in real time without damage Able to measure thickness and EPS quantity	Difficult to analyze large-scale samples Sample immobilization is required when cells in liquid are analyzed	Handorf et al. (2019), Wang et al. (2020), Merghni et al. (2017), Vahabi et al. (2013), Deng et al. (2018), and Louise Meyer et al. (2010)

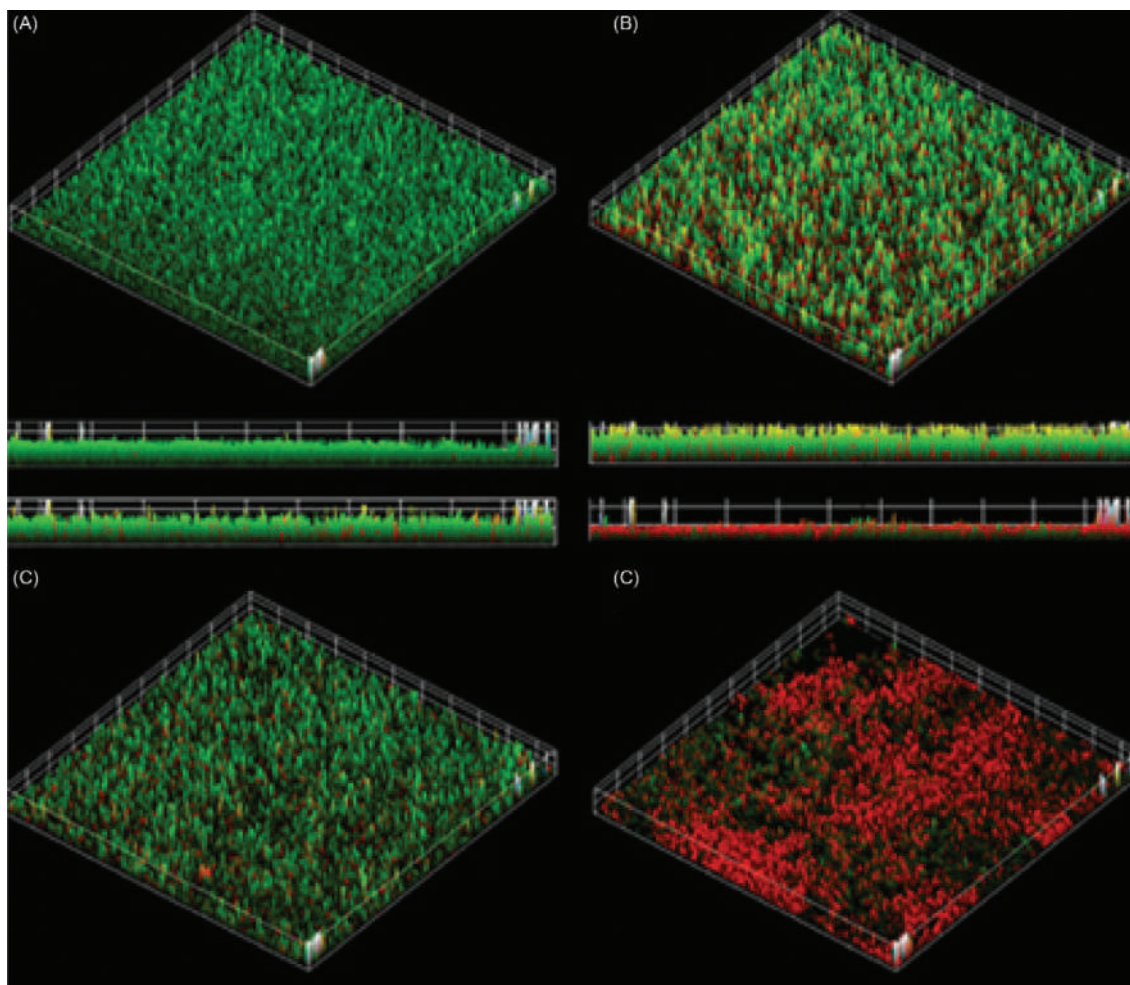


Fig. 4 Imaging of live/dead biofilm bacteria using confocal laser scanning microscopy treated with propolis extracts and chitosan-propolis nanoparticles. Confocal laser scanning microscopy images depicting viability of *Staphylococcus epidermidis* biofilm bacteria. (A) Untreated biofilm control, (B) biofilm treated with ethanol, (C) ethyl acetate extracts of propolis and (D) chitosan-propolis nanoparticles. Reprinted with permission from Ong TH, Chitra E, Ramamurthy S, Ling CCS, Ambu SP, et al. (2019) Cationic chitosan-propolis nanoparticles alter the zeta potential of *S. epidermidis*, inhibit biofilm formation by modulating gene expression and exhibit synergism with antibiotics. *PLoS One* 14(2): e0213079. <https://doi.org/10.1371/journal.pone.0213079>.

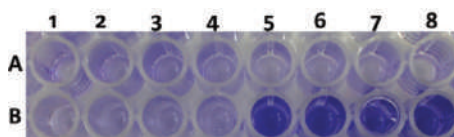


Fig. 5 Crystal violet staining assay of methicillin-resistant *Staphylococcus aureus* strains having different biofilm formation capability. Row A of 1–4 wells, 4 replicates of MRSA-12S strain; Row A of 5–8 wells, 4 replicates of MRSA-51S strain; Row B of 1–4 wells, 4 replicates of MRSA-44S strain; Row B of 5–8 wells, 4 replicates of MRSA-3S strain.

bound CV is solubilized by either ethanol, a mixture of ethanol and acetone, glacial acetic acid, or dimethyl sulfoxide (Merritt et al., 2005). Finally, the absorbance of the solubilized CV is determined using a spectrophotometer at 530–600 nm. Some researchers have also fixed *Staphylococcus* species and *Mannheimia haemolytica* biofilm cells with either absolute ethanol, methanol or heat treatment at 60–80 °C to prevent widespread detachment of biofilms prior to dye staining (Thomas et al., 2015; Dassanayake et al., 2020; Stepanovic et al., 2000). Since this dye stains negatively charged molecules and anionic proteins in both viable and dead cells in biofilms without specificity, it is not possible to quantify the actual number of viable cells (Veloz et al., 2019). Furthermore, there is no standardized protocol for CV staining, so there are variations between the results obtained from different studies (Stepanovic et al., 2000). Peeters et al. reported that quantification of *P. aeruginosa* biofilms using CV staining failed to provide consistent results and the absorbance signals of *P. aeruginosa* were significantly lower than the signals of *Burkholderia cenocepacia*, *Staphylococcus aureus*,

Propionibacterium acnes and *C. albicans* (Peeters et al., 2008a). Also, CV staining was not as accurate as tetrazolium salts, such as 2,3,5-triphenyl-tetrazolium chloride (TTC), when the minimum biofilm inhibitory concentrations (MBIC) of amikacin and cefixime against *P. aeruginosa* were determined (Sabaeifard et al., 2014).

Several researchers have used safranin, Congo red, or calcofluor white instead of CV to measure total biofilm biomass. Safranin is not toxic and binds to negatively charged molecules of biofilm, but is less utilized for biomass assessment and shows a lower optical density than CV (Ommen et al., 2017). Safranin has been applied to study the effects of antibiotics (naphthoquinone-plumbagin), vitexin (polyphenolic group of phytochemicals), rhamnolipid (RL-SS14), brominated furanone, epigallocatechin gallate, short-chain fatty acids, phage, extracts of *Artemisia princeps*, *Lonicera caerulea* var. *emphyllcalyx*, essential oils of *Chamaecyparis obtusa*, *Chrysanthemum boreale*, *Origanum vulgare* subsp. *hirtum*, *Curcuma longa*, cold atmospheric plasma, electrolyzed water, rifampin-loaded hydrogel, and implant materials (metal alloys, zirconia, polyetherketoneketone, and titanium) on biofilm formation in various bacteria (Gupta et al., 2017; Das et al., 2016; Sen et al., 2020; Castillo et al., 2015; Yoneda et al., 2013; Cano et al., 2021; Yang et al., 2019; Minami et al., 2019; Kim et al., 2015b; Kim et al., 2015a; Schillaci et al., 2013; Lee et al., 2011; Ulu et al., 2018; Sun et al., 2012; Cahill et al., 2021; Zeller et al., 2020). Ommen et al. compared biofilm formation of *S. aureus*, *S. epidermidis*, and *P. aeruginosa* using CV and safranin as staining dyes (Ommen et al., 2017). They recommended safranin staining for biofilm measurements, as safranin staining is more reproducible, less toxic and easier to use than CV. Congo red has been used to measure the production of amyloid fibers and adopted to determine the amounts of polysaccharides and cellulose that comprise biofilms (Bely and Makovitzky, 2006). Because of its high background signal, Congo red has been employed to stain chronic wound biofilms with large amounts, rather than small amounts, of biofilms (Stiefel et al., 2016). Also, it has been added to agarose and utilized to measure biofilms formed on agar plates (Kaiser et al., 2013). Calcofluor white is a non-specific fluorochrome that binds to (1–3) and (1–4)- β -linked carbohydrate linkages (Nett et al., 2011). It has been predominantly used to study biofilm structures of *C. albicans* as well as *Campylobacter jejuni*, *Pseudomonas* species, *S. enterica*, and *Staphylococcus* species (Daher et al., 2011; Alfatah et al., 2017; Drozd et al., 2014; Quatrin et al., 2017; Gonzalez-Machado et al., 2018; Grecka et al., 2020).

Biofilm measurement by metabolic activity

MTT staining assay

MTT is a yellow soluble tetrazolium salt that is reduced to an insoluble purple crystal by viable cells (Sliwka et al., 2016). The MTT staining assay has been frequently used to investigate cell proliferation and cytotoxicity in human cells, and applied to quantify viable cells in biofilms (Fig. 6) (Traba and Liang, 2011). It is simple, takes a short time to analyze, and provides reproducible results (Table. 1) (Chusri et al., 2013). However, Manavathu et al. reported that it is difficult to differentiate the contribution of bacterial cells to the reduction of MTT in antimicrobial susceptibility tests of dual-species biofilms composed of *Aspergillus fumigatus* and *P. aeruginosa* to voriconazole and cefepime (Manavathu et al., 2014). Moreover, components of growth media, including amino acids, especially histidine, greatly influence bacterial reduction of MTT to formazan (Benov, 2019). The results are not trustworthy when evaluating the susceptibility of a new antimicrobial agent that affects the outer membrane integrity of Gram-negative bacteria with MTT because the assay score falsely increased when the outer membrane is damaged (Grela et al., 2015).

XTT staining assay

XTT is a yellow tetrazolium salt that is reduced to water-soluble formazan by enzymes of the respiratory chain of bacteria and the succinate dehydrogenase of fungi (Cerca et al., 2005). The XTT staining assay has been broadly employed for evaluating bacterial viability, not only in planktonic cells but also in biofilm cells of both bacteria and yeasts (Fig. 7) (Gabrielson et al., 2002; Adam et al., 2002; She et al., 2016). It has also been employed more in biofilm studies than the MTT staining assay. A large number of researchers have worked on the inhibition of fungal biofilms by anti-fungal agents, or the interaction of mixed biofilms of fungi and Gram-negative or Gram-positive bacteria (Pierce et al., 2008). While MTT staining requires the lysis of cells with dimethyl sulfoxide before reading optical density, XTT can be directly measured as the amount of orange/yellow formazan byproduct (Table. 1) (Costa-Orlandi et al., 2017). Furthermore, XTT staining of intact biofilms for antibiotic susceptibility can be performed without damaging the biofilm structure (Kuhn et al., 2002). As various bacterial species may metabolize XTT differently, XTT can have a poor linear relationship between the colorimetric signal and the bacterial concentrations in biofilms (Alonso et al., 2017). Therefore, it is necessary to optimize the standard curve of the formazan product absorbance with the number of viable cells before analyzing

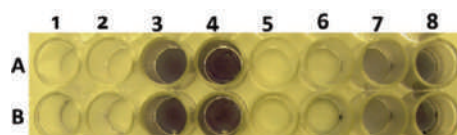


Fig. 6 MTT assay of *Streptococcus mutans* in brain heart infusion (BHI) and basal medium mucin (BMM). Column 1, BHI control; column 2, 5% hydroxyapatite in BHI; column 3, 1% sucrose in BHI; column 4, combination of 5% hydroxyapatite and 1% sucrose in BHI; column 5, BMM control; column 6, 5% hydroxyapatite in BMM; column 7, 1% sucrose in BMM; column 8, combination of 5% hydroxyapatite and 1% sucrose in BMM. Row B is duplicate of the row A wells.

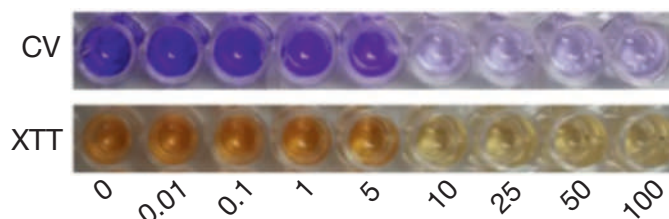


Fig. 7 Human serum inhibits *Staphylococcus epidermidis* ATCC35984 biofilm formation. Overnight culture was treated with 0–100% HS at 37 °C for 24 h. The volume of biofilm was determined by both the CV assay and by the XTT assay. CV, crystal violet. Reprinted with permission from She P, Chen L, Qi Y, Xu H, Liu Y, Wang Y, Luo Z, and Wu Y (2016) Effects of human serum and apo-Transferrin on *S. epidermidis* RP62A biofilm formation. *MicrobiologyOpen* 5(6):957–966. <https://doi.org/10.1002/mbo3.379>.

biofilms. In addition, depending on the biofilm growth conditions, the signal intensity of XTT can be affected since some bacteria contain significant amounts of intracellular salts (Silva et al., 2021).

Resazurin (Alamar Blue) staining assay

The resazurin staining assay uses the reduction by bacterial viable cells of the blue, non-fluorescent, and non-toxic dye, resazurin, to pink, fluorescent resorufin to assess the viability of biofilms (Fig. 8) (Paytubi et al., 2017; Bandeira Tde et al., 2013). Compared to MTT and XTT staining assays, not only does the assay take less time to perform, but it also is cost effective and has a good correlation between the number of bacterial cells in biofilms and the fluorescent signal (Table. 1) (Van den Driessche et al., 2014). In addition, it is highly sensitive and can detect low levels of viable bacteria, up to 1000 colony forming units/biofilm. Depending on the bacteria, the rates at which resazurin is metabolized are different (Sandberg et al., 2009). Also, in multi-species biofilms, the incubation times required for the bacteria are different. Peeters et al. quantified biofilms of *P. aeruginosa*, *B. cenocepacia*, *S. aureus*, *P. acnes* and *C. albicans* in 96-well microtiter plates by CV, Syto 9, XTT, resazurin, and other staining methods and found that the resazurin staining assay was better than others in terms of detection limit, reproducibility, cost, and time (Peeters et al., 2008a). Some investigators reported that combinations of resazurin and CV, or resazurin and dimethyl methylene blue, in staining assays could provide better information than a single assay for *S. aureus* biofilms (Skogman et al., 2012; Tote et al., 2008).

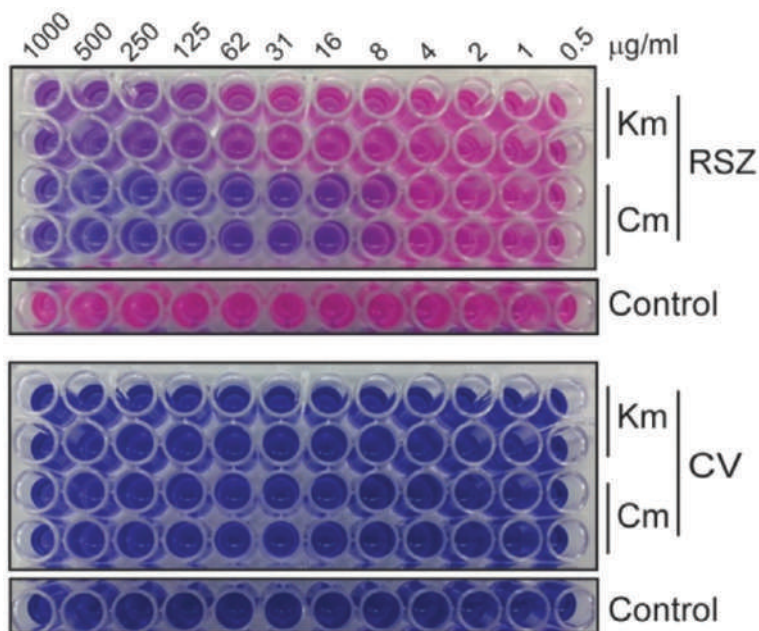


Fig. 8 Minimal biofilm eradication concentration assay of *Salmonella enterica* serovar Enteritidis strain 3934 against kanamycin and chloramphenicol. Top panels show resazurin and bottom panels show crystal violet staining. Untreated controls are 12 replicas of the bacterial culture without the addition of antibiotic. Reprinted with permission from Paytubi S, de La Cruz M, Tormo JR, Martín J, González I, González-Menendez V, Genilloud O, Reyes F, Vicente F, Madrid C and Balsalobre C (2017) A high-throughput screening platform of microbial natural products for the discovery of molecules with antibiofilm properties against *Salmonella*. *Frontiers in Microbiology* 8:326. <https://doi.org/10.3389/fmicb.2017.00326>.

Adenosine triphosphate (ATP) bioluminescence assay

ATP is the central metabolite that acts as an energy transfer molecule in all metabolically active organisms (Kumon et al., 1995). Upon cell death, ATP in cells is quickly broken down by ATPase (van der Kooij, 1998). In the presence of luciferase extracted from the firefly *Photinus pyralis*, a substrate, luciferin, is activated with ATP to produce luciferyl adenylate and pyrophosphate (Nikawa et al., 2003). Then, the luciferyl adenylate reacts with oxygen to generate oxyluciferin and adenosine monophosphate. The oxyluciferin in an excited state comes back to the ground state and emits a luminescent light that can be detected by a luminometer and expressed in relative light units. The ATP assay is easy to execute, sensitive enough to detect microorganisms of low metabolic activity, and can be detected within 20 s (Table. 1) (Wilson et al., 2017). In the quantification of viable sessile cells of *P. aeruginosa* and *S. aureus* after treatment with cleaning agents, the luminescent signal of the bacterial samples with sodium chloride showed high sensitivity, 1000 times higher than that of the cell-free BacTiter-Glo reagent solution (Stiefel et al., 2016). The ATP assay is not disrupted by fluorescent substances and can be used to quantify viable cells in both planktonic and sessile cells (Wilson et al., 2017). However, it is not suitable to quantify biofilms if ATP is not fully extracted or biofilms that are grown for extended periods of time (Gonzalez-Rivas et al., 2018). The ATP bioluminescence assay has been used primarily to evaluate biofilm formation in drinking water systems, as well as to monitor biofilm removal from medical products, such as endoscopes and orthopedic implants, and to test anti-biofilm activity of antimicrobial agents, nanoparticles, essential oils, and ultraviolet light emitting diodes (Lehtola et al., 2004; Luo et al., 2020; Karpanen et al., 2008; Jothiprakasham et al., 2017; Hendry et al., 2009; Gora et al., 2019).

Biofilm detection by microscopical techniques

Conventional light microscopy

A light microscope employs visible light and a magnifying lens to observe tiny objects that cannot be seen with the naked eye and has long been used as an essential tool for investigating bacteria (Relucenti et al., 2021). Early microscopes magnified up to 275 times, but technological advances have made it possible to magnify up to 1000 times (de Boer et al., 2015). Light microscopy is generally used to study bacterial cells larger than 0.5 μm (de Boer et al., 2015). Observing bacteria using light microscopy is not expensive, it is easy to prepare and observe samples, and it is possible to observe a large area of viable cell cultures for extended periods of time (Table. 1) (Relucenti et al., 2021). Furthermore, light microscopy can visualize sessile cells after staining with crystal violet or other dyes and does not require other advanced microscopy techniques, but it has difficulty in enumerating the total numbers of bacteria in biofilms (Fig. 9) (Pelzer et al., 2012; Sowndarya et al., 2020). Conventional light microscopes have the disadvantage of not being able to measure small components of bacterial cells or details of the biofilm structure, as they have a lower resolution and magnification than electron microscopes (Azeredo et al., 2017). Optical microscopy has evolved into a technique capable of assessing static images of biofilms and evaluating dynamic characteristics of molecules present in biofilms (Sankaran et al., 2018). For example, fluorescence microscopy exploits the ability of fluorescent dyes to emit visible light at specific wavelengths when UV light is used (Sanderson et al., 2014). Epifluorescence microscopy, one of the most widely used techniques, offers higher resolution, shows detailed architecture of biofilms cultured on opaque surfaces, such as metal, and can be used to count viable bacteria in biofilms (Carneiro et al., 2009). Diverse color imaging has become possible as the technology for detecting fluorescent proteins and labels, along with filter and camera technology, have progressed (Xu et al., 2005). While acridine orange binds both DNA and RNA and can be used for studying biofilms on food contact surfaces, 4',6-diamidino-2-phenylindole binds to DNA and can be used to count the total number of cells in biofilms (Poimenidou et al., 2009; Wirtanen et al., 2001). Additionally, the viability of monolayer biofilms of Gram-positive and -negative bacteria may be assessed by epifluorescence microscopy after staining bacteria with SYTO 9 and PI (Rosenberg et al., 2019). Green fluorescent protein (GFP), a good marker to label bacterial cells in biofilms without destroying them, can be detected by blue light irradiation (Yoshida and Kuramitsu, 2002). GFP-labeled bacteria have been used to investigate survival and spatial distribution in biofilms (Hentzer et al., 2002; Banning et al., 2003). Hiraki et al. adopted Alcian blue, a copper phthalocyanine cationic dye, to visualize and quantitatively measure the extracellular polysaccharide within biofilms produced by bacteria attached to the surface of the aquatic macrophyte *Phragmites australis* (Hiraki et al., 2009).

Confocal laser scanning microscopy (CLSM)

Unlike conventional light microscopy, CLSM makes use of a laser to illuminate and scan biological samples, rejects out of focus fluorescent images, and acquires images from continuous sections of thick biofilms (Fig. 4) (Ong et al., 2019; de Carvalho and da Fonseca, 2007). It has become the most useful microscopy to explore spatial organization of biofilm architecture. Since CLSM software can include a multi-parametric quantitative data set of fluorescence intensities with each image, it can determine viable and dead cell numbers of bacteria, identify bacterial species in polymicrobial biofilms, and perform horizontal and vertical optical sectioning of three-dimensional biofilms (Table. 1) (Kleine et al., 2019). Some researchers have studied the effects of antimicrobial agents, such as epigallocatechin-3-gallate, amphotericin B, voriconazole, flucytosine, caspofungin, and chitosan-coated iron oxide nanoparticles on the viability of sessile cells using CLSM (Vidigal et al., 2014; Bojsen et al., 2014; Shi et al., 2016). Bao et al. employed CLSM to demonstrate movement in the spatial arrangement of *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Treponema denticola* within polymicrobial dental biofilms (Bao et al., 2014). Furthermore, when studying the interactions between EPS and extracellular DNA or between EPS and silica nanoparticles, three-dimensional image analyzes of CLSM made it possible to

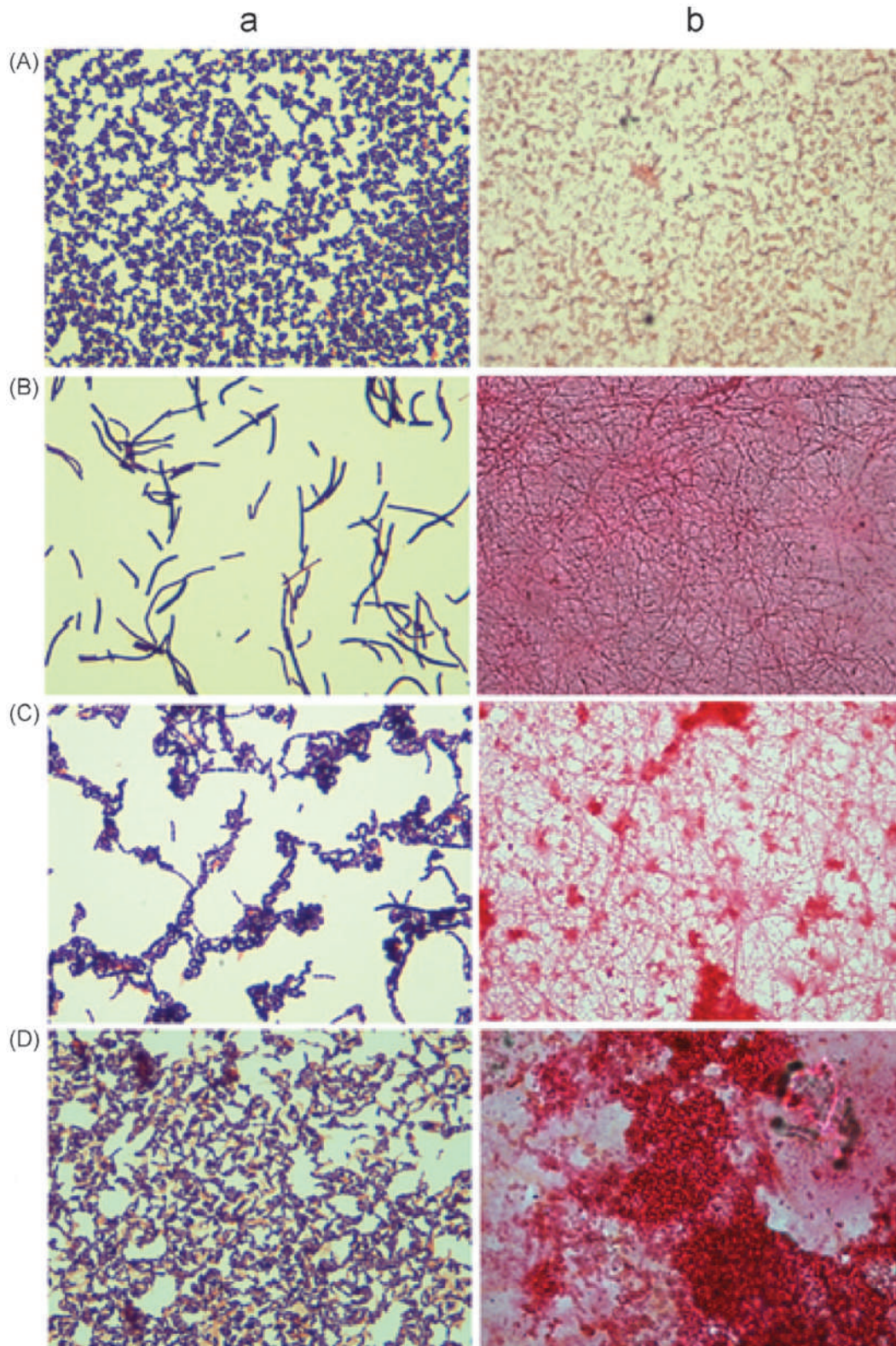


Fig. 9 Light micrographs of Gram stains and biofilm slides. (A–D) (a) Light microscopy image at $1000\times$ total magnification of Gram-stained *Streptococcus agalactiae* and *Lactobacillus* spp. colonies cultured from biofilms. (A–D) (b) Light microscopy image at $1000\times$ total magnification of Congo red stained *S. agalactiae* and *Lactobacillus* spp. biofilms grown for 10 days on glass cover slips. Reprinted with permission from Pelzer ES, Allan JA, Theodoropoulos C, Ross T, Beagley KW, et al. (2012) Hormone-dependent bacterial growth, persistence and biofilm formation—A pilot study investigating human follicular fluid collected during IVF cycles. *PLoS One* 7(12): e49965. <https://doi.org/10.1371/journal.pone.0049965>.

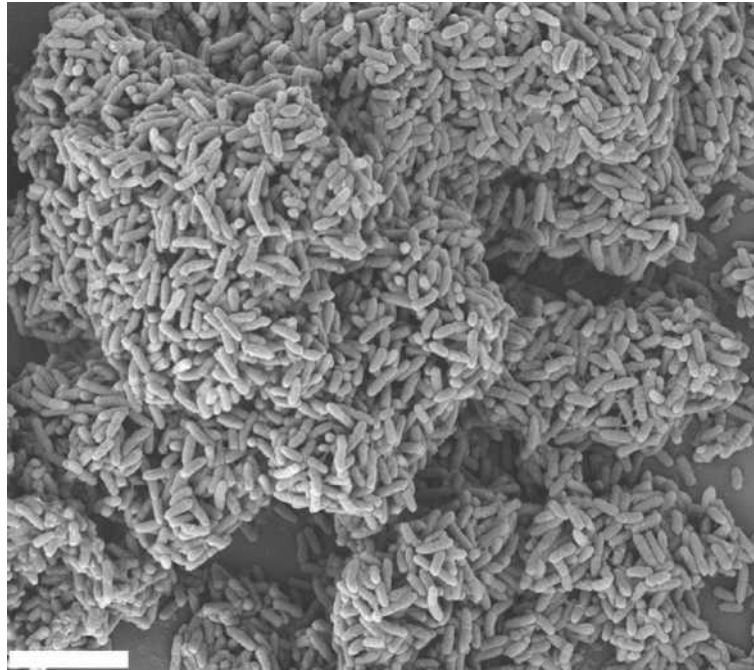


Fig. 10 SEM image of *Pseudomonas aeruginosa* PAO1 biofilm grown on a silicone Foley catheter. The scale bar in the image corresponds to 5.0 μm .

analyze structural parameters, including thickness, biovolume, and surface coverage (Peng et al., 2020; Hiebner et al., 2020). FISH identifies bacterial species within biofilms by using oligonucleotide probes labeled with fluorescent dyes (Thurnheer et al., 2004). The probes bind 16S rRNA, which has both highly conserved and variable DNA sequences, to discriminate members of the microbial population (Thurnheer et al., 2004). Combining CLSM and FISH systems has made it possible to investigate the effect of a single bacterium on biofilms formed by another bacterium or mixed bacteria. This combination can also be used to measure the viability of a target bacterial species in multispecies biofilm samples and to detect bacterial species, quantities, structures and polymicrobial interactions in biofilms collected from human tissues and environmental samples (Romero-Lastra et al., 2019; Thornton et al., 2011; Elifantz et al., 2013; Klug et al., 2016).

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

EM has been used to produce high resolution images in biomedical science, material science, semiconductor inspection, and forensic investigations (Cazaux, 2005). SEM provides an image of topography and composition by scanning the sample surface with an electron beam (Fig. 10 and Table. 1) (Stokes, 2003). Secondary electrons (low-energy electrons) are used to show morphology and topography of the sample surface, and backscattered electrons (high-energy electrons) show spatial distribution by atomic number contrast (Stokes, 2003). The magnification and spatial resolution of SEM are from 20 to 30,000 X and from 50 to 100 nm, respectively (Cazaux, 2005). Since biofilms are non-conductive, they must be fixed with glutaraldehyde, dehydrated, and sputter coated before being observed with SEM (Weber et al., 2014). While the drying step of SEM can damage biofilm shape, variable pressure SEM (VPSEM), environmental SEM (ESEM) and cryo-SEM can better protect biofilm shape (Relucenti et al., 2021).

TEM uses an electron beam penetrating through a very thin specimen (Jublot and Texier, 2014). TEM samples are prepared by slicing the sample into thin sections less than 100 nm and staining with uranyl acetate and lead citrate (Fig. 11) (Graham and Orenstein, 2007). While SEM provides an image of the three-dimensional structure of biofilms, TEM creates a two-dimensional image with a resolution of approximately 0.2 nm of the components, such as nucleic acids and proteins, inside an individual bacterial cell, showing the comprehensive morphology and structure of the bacteria that make up biofilms (Table. 1) (DeQueiroz and Day, 2007). Compared to TEM, SEM not only costs less, it also requires less time to image and less labor for sample preparation, and it allows larger samples to be inspected (Walker et al., 2001). On the other hand, TEM provides higher resolution, crystallographic and atomic data, and enables more detailed characterization, but it needs highly experienced technicians for sample preparation (Chuo et al., 2018). SEM and TEM have been widely used to study biofilms together with the CLSM technique. The three combined techniques have been adopted to investigate the interaction of bacterial species in mixed biofilms, the

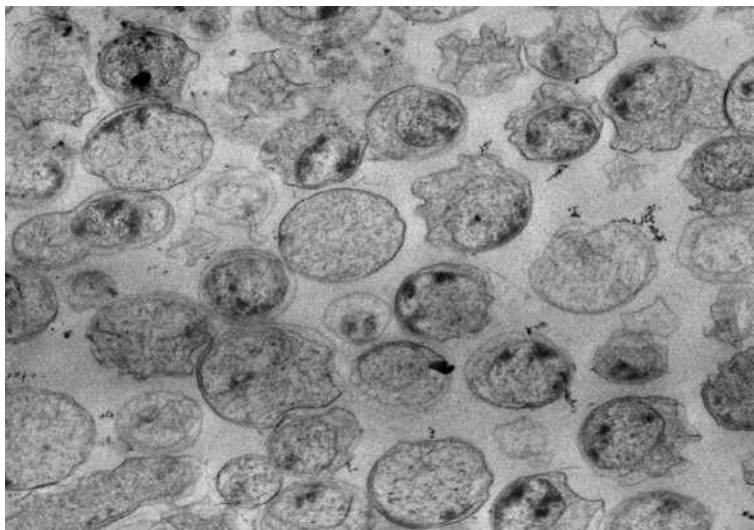


Fig. 11 TEM image of *Campylobacter jejuni* 11,168 biofilm grown on a stainless steel coupon. The scale bar in the image corresponds to 1.0 μm .

morphological alteration of biofilm structure after treatment with antimicrobial agents (peptides, magnolol, fosfomycin, and nanoparticles), the irrigation effect on root canal, the difference in biofilm morphology in relation to nutrient availability, and the prevalence and distribution of biofilms in indwelling catheters (Ramirez Granillo et al., 2015; Geng et al., 2018; Behbehani et al., 2017; Monden et al., 2002; Rajivgandhi et al., 2020; Mohammed et al., 2017; Wang et al., 2017; Gorman et al., 1994; Pronk et al., 2017).

Atomic force microscopy (AFM)

AFM is a powerful approach to provide three-dimensional images of viruses, bacteria, human cells, biomaterials, polymers, semiconductors, metals, ceramics, and glasses from the nanometer to micrometer scale, and quantitative information, including elastic properties and adhesion forces (Vahabi et al., 2013). It employs a sharp probe to scan the sample surface and analyzes the sample image without damaging it under ambient conditions, but large-scale samples are difficult to analyze using AFM (Deng et al., 2018; Louise Meyer et al., 2010). Furthermore, biological samples in liquid move freely during AFM imaging, so they must be analyzed after immobilizing the sample (Louise Meyer et al., 2010). AFM has been frequently used to obtain three-dimensional surface structures of biofilms and to quantify the adhesion forces of bacterial biofilms formed on various surfaces of biomaterials (Fig. 12, Table 1) (Handorf et al., 2019; Wang et al., 2020; Merghni et al., 2017). It has been also used to analyze thickness and EPS quantity of sessile cells by measuring the height and roughness after biofilm treatment with antimicrobial agents or nanoparticles (Salunke et al., 2014; Chatterjee et al., 2014). Additionally, to characterize the effect of growth conditions (nutrient components, pH, and flow conditions), antimicrobial agents (chlorhexidine and antimicrobial peptides), and viscoelastic characteristics of the biofilm surface have been quantitatively measured by AFM (Martin-Rodriguez et al., 2014; Ho et al., 2013; Schofield et al., 2007; Rodgers and Murdaugh, 2016; Quiles et al., 2016; Formosa-Dague et al., 2016; Lau et al., 2009).

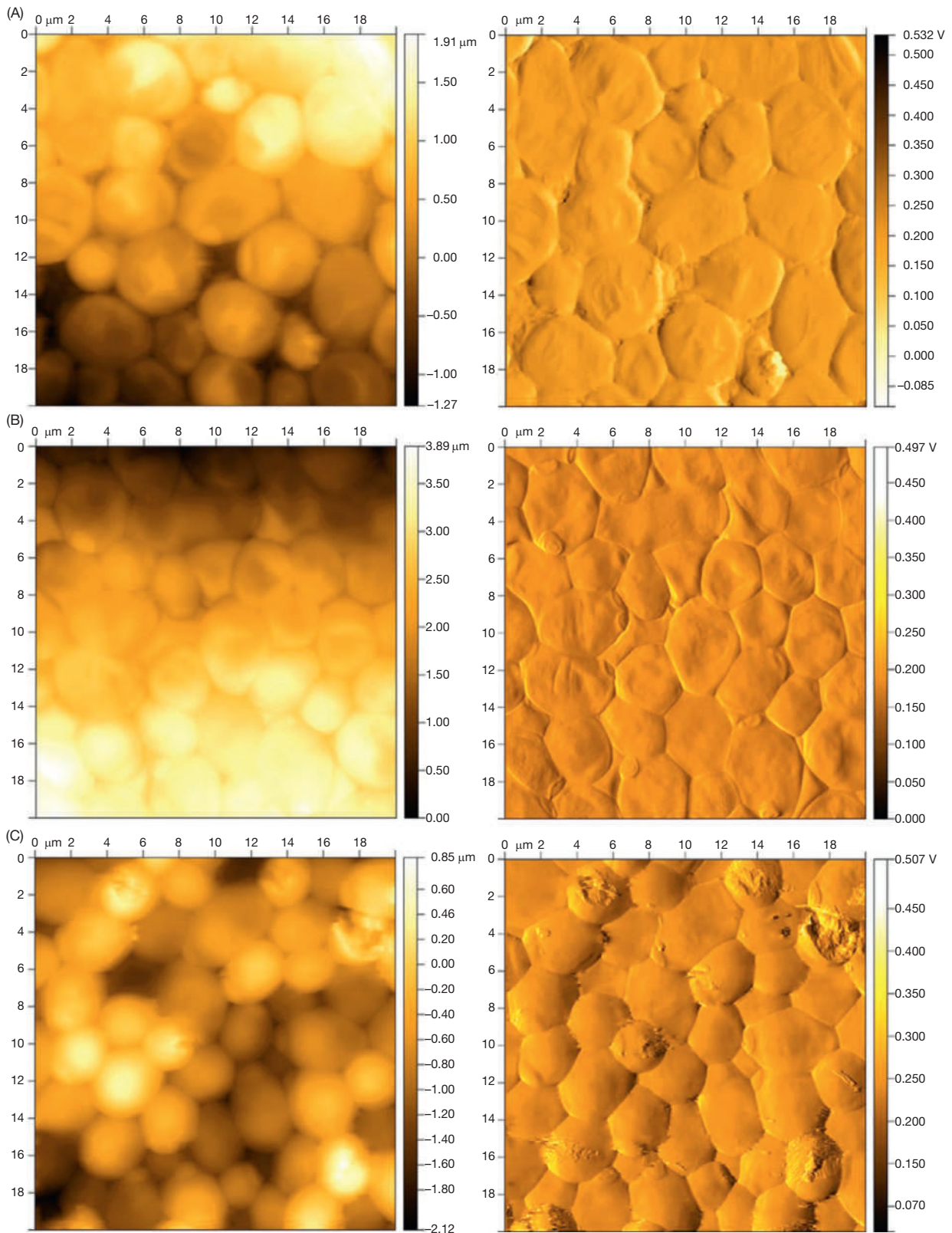


Fig. 12 Atomic force microscopy (AFM) images of the MiniMIP-treated biofilms and untreated controls. The left side shows the topographic image and the right side shows the error-signal image of the same spot. (A) Untreated control; (B) 30 s plasma treatment time; (C) 60 s plasma treatment time. The images were acquired in contact mode with a cantilever spring constant of $k = 0.1\text{--}0.6\text{ N/m}^2$ and a frequency of 0.4 Hz, the set point at 8 N/m^2 and an area of $20\text{ }\mu\text{m}^2$. Reprinted with permission from Handorf O, Schnabel U, Bösel A, Weihe T, Bekeschus S, Graf AC, Riedel K, and Ehlbeck J (2019) Antimicrobial effects of microwave-induced plasma torch (MiniMIP) treatment on *Candida albicans* biofilms. *Microbial Biotechnology* 12(5):1034–1048. <https://doi.org/10.1111/1751-7915.13459>.

Conclusion

Bacterial biofilms are complicated assemblages of proteins, lipids, polysaccharides, and lipopolysaccharides, and usually contain many different bacterial species. Since biofilm cells possess different characteristics from planktonic cells, there are many limitations that restrict the application of methods developed for planktonic cells to detect biofilm cells. Although biofilms cause serious problems in the human body (pulmonary and dental tissues) and medical devices (urinary and central venous catheters), the usual methodologies performed in clinical laboratories in hospitals can only detect planktonic bacteria. This article describes methods for detecting biofilms with the advantages and disadvantages. Investigation of biofilm cells requires a variety of approaches and accurate interpretation, from the most basic method of determining the total number of viable cells to advanced microscopy methods that reveal three-dimensional structures. Advanced in vitro and in vivo models and detection methods will be necessary to accurately and rapidly detect and investigate biofilms.

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Assuring Pathogen Safety of the Starting Material for Plasma-Derived Products

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Glossary

Apheresis/plasmapheresis Method of obtaining one or more blood components—for plasmapheresis the blood component is plasma—by machine processing of whole blood, in which the residual components of the blood are returned to the donor during or at the end of the process.

Blood establishment Any structure or body that is responsible for any aspect of the collection and testing of human blood or blood components, whatever their intended purpose.

Blood-borne viruses Viruses with a viremic phase, i.e., virus detectable in blood/plasma. The major blood-borne viruses of concern for plasma-derived medicinal products (PDMPs) are viruses causing regularly a persistent infection as human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV).

CAPA Corrective action and/or preventive action.

Change control A formal system by which qualified representatives of appropriate disciplines review proposed or actual changes that might affect the validated status of facilities, systems, equipment or processes. The intent is to determine the need for action that would ensure and document that the system is maintained in a validated state.

Computerized system A system comprising the input of data, electronic processing and the output of information to be used either for reporting, automatic control or documentation.

Donor deferral Suspension of the eligibility of an individual to donate blood or blood components; such suspension being either permanent or temporary.

EDQM European Directorate for the Quality of Medicines & HealthCare.

EMA European Medicines Agency (formerly EMEA: The European Agency for the Evaluation of Medicinal Products).

FDA, CBER Food and Drug Administration, Center for Biologics Evaluation and Research (at US Department of Health and Human Services).

First time donor (First time tested donor) Person never donated either blood or plasma (person whose blood/plasma is tested for the first time for infectious disease markers (with or without donation)).

GMP Good Manufacturing Practice.

Incidence Rate of newly acquired infection identified over a specific time period in a defined population; commonly, for blood transmissible infections the positivity rate in NAT assays ("NAT only," i.e., serological assays are non-reactive) correspond to incidence.

Inventory hold Physical isolation of plasma over at least 60 days after collection in order to retrieve and interdict a donation based on post-donation informations as tested reactive in infectious disease markers in subsequent donations and further information about high risk behavior; otherwise, the donation is released for pooling after (at least) 60 days.

NAT Nucleic acid amplification technique (e.g., PCR: polymerase chain reaction or TMA: transcription-mediated amplification).

PDMP Plasma-derived medicinal product.

Prevalence Frequency of infection identified (including past and recent infections) at a specific point in time or over a specific time period in a defined population; commonly, for blood transmissible infections the positivity rate in serological assays correspond to prevalence.

Qualification Part of validation and meaning the action of verifying that any personnel, premises, equipment or material works correctly and delivers the expected.

Quality assurance All the activities from blood collection to distribution made with the object of ensuring that blood and blood components are of the quality required for their intended use.

Quality control Part of a quality system focused on fulfilling quality requirements.

Quality management The co-ordinated activities to direct and control an organization with regard to quality at all levels within the blood establishment.

Quality system The organizational structure, responsibilities, procedures, processes, and resources for implementing quality management.

Quarantine Physical isolation of plasma and release for further processing only when after a defined time period post collection, the donor is tested again nonreactive for infectious disease markers (commonly 4 months when donations are NAT tested).

Repeat donor/regular donor (repeat tested donor) Person donated before blood or plasma in accordance with minimum time interval (person whose blood/plasma has been tested previously for infectious disease markers).

Traceability The ability to trace each individual unit of plasma from the donor to its final destination, whether this is a recipient, a manufacturer of medicinal products or disposal, and vice versa.

Window period During the window period a test method is unable to detect a recent infection (with viremia), commonly because antibodies are not yet produced and/or detectable by a serological screening assay. Occasionally, a window period is also estimated for antigen and NAT testing when the virus concentration in blood/plasma is below the methods' limit of detection for antigen or NAT screening assays.

Introduction

Plasma-derived medicinal products are required to treat many diseases, especially rare diseases or conditions. In the past, they have been used primarily in critical care settings (e.g., human serum albumin) and for the treatment of inherited diseases (e.g., hemophilia A and B). Nowadays, the majority of plasma collected is required to manufacture immunoglobulins (polyvalent intravenous immunoglobulins—IVIG and subcutaneous immunoglobulins—SCIG) predominantly for the treatment of primary immunodeficiency (PID), polyneuropathy (CIDP), and idiopathic thrombocytopenic purpura (ITP); the treatment of secondary immunodeficiency caused, e.g., by chemotherapy, auto-immune disorders and other immune deficiencies is also treated by immunoglobulins. Furthermore, pathogen infections are treated by these polyvalent immunoglobulins; for defined pathogens or their toxins and for further defined conditions hyperimmune globulins are administered, e.g., against rabies, hepatitis A and B, tetanus, diphtheria, shingles (varicella zoster virus), cytomegalovirus, as well as anti-D immunoglobulin (Rho(D) immune globulin (to prevent in pregnancy RhD (rhesus) isoimmunization in RhD negative mothers). Further plasma-derived proteins commercially extracted from plasma are, e.g., immunoglobulin M, von Willebrand factor, fibrinogen, prothrombin complex concentrate, factor VIIa, factor X, factor XI, factor XIII, and the inhibitors alpha-1-proteinase inhibitor, C1-esterase inhibitor, and antithrombin (ATIII).

Depending on the regulations in different areas in the world and the economics of blood/plasma collection, recovered plasma and source plasma may be used in different proportions to produce plasma-derived medicinal products (PDMP). Recovered Plasma (also call Fresh Frozen Plasma when it was originally intended for transfusion) is the plasma obtained after processing of whole blood into red cell and platelet concentrates and plasma, while Source Plasma is the plasma obtained by apheresis during which red cells, platelets and white cells have been returned to the donor and the collected plasma is intended for further manufacture into injectable or diagnostic products. As the volume of a recovered plasma donation is approx. 280 mL and strict restriction regarding interdonation intervals and total donations per year exist to protect the donor's health (e.g., six donations per year for male donors and four donations for female donors in Europe (EDQM, 2020)) a Recovered Plasma donor can provide approx. 1.5 L of plasma per year. In contrast, Source Plasma donors can donate up to 104 times per year in the United States and up to 40 times per year in Europe, resulting in almost 90 L plasma per donor in the United States and approximately 25 L in Europe (Simon et al., 2009). In other areas of the world different regulations may apply; for instance, in China only source plasma is allowed to be fractionated and the interdonation interval is considerably longer (14 days). Source plasma donations are required to meet the demand for PDMPs by patients with rare diseases or conditions.

Fig. 1 shows the worldwide plasma protein market 2016 documenting that most of the products are polyvalent immunoglobulins (47.3% of the market is due to polyvalent immunoglobulins applied either intravenously (IVIG) or subcutaneously (SCIG)). The value of the total market in 2016 is US\$ 21,174 million (Marketing Research Bureau Inc. (MBR), Orange, Connecticut, United States).

In order to manufacture these products, collection of a large amount of plasma is essential. Depending on the regulation in different areas in the world and the economics of blood/plasma collection, recovered plasma (derived from whole blood donations) and source plasma (collected by apheresis), may be used in different proportions. Fig. 2 shows the amount of plasma collected worldwide in 2015, a total of 48,744,000 L; most of the plasma for fractionation—to meet the need of patients—is source plasma (81% of all plasma collected). The majority of plasma for fractionation (recovered and source plasma) collected worldwide is derived from donors in North America, primarily United States. Considering only source plasma (collected by plasmapheresis), in Middle East/Africa and Latin America no source plasma is collected, whereas approximately 43% in Europe, 81% in Asia and Pacific, and 94% in North America of all plasma collected is source plasma. Approximately three quarter of all source plasma collected worldwide is collected in North America (primarily in the United States).

**THE WORLDWIDE PLASMA PROTEINS MARKET BY
PRODUCT – 2016
WITHOUT RECOMBINANT FACTORS**

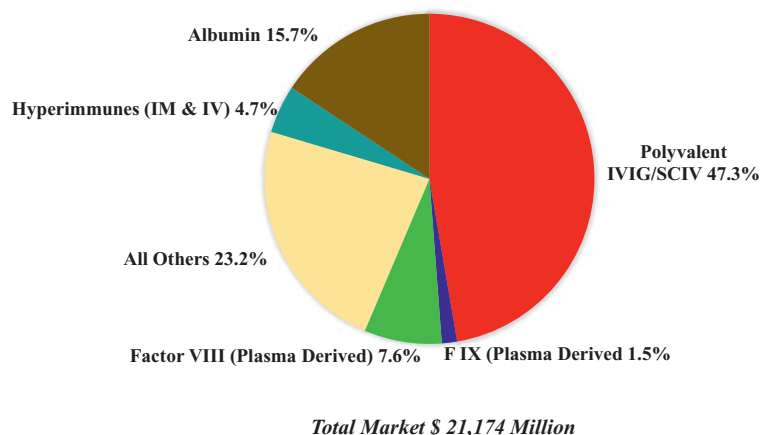
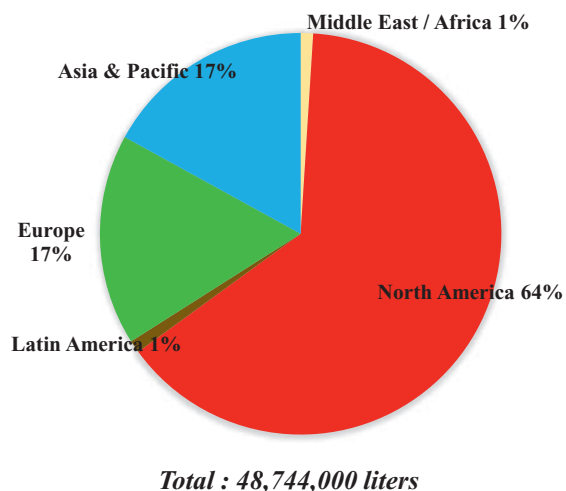


Fig. 1 Worldwide plasma protein market by product in 2016 (without recombinant factors)—MBR <https://marketingresearchbureau.com/plasma-industry/plasma-economics-concept-of-plasma-market-driver/> (slightly modified for improved printability).

**(A) Plasma for Fractionation by Region 2015
(Recovered and Source)**



**(B) Plasma for Fractionation by Region 2015
(Source)**

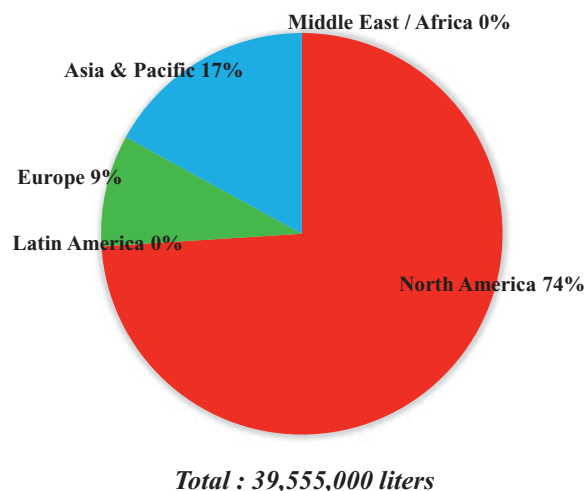


Fig. 2 Collection of plasma for fractionation collected worldwide in 2015—MBR <https://marketingresearchbureau.com/plasma-industry/current-uses-affecting-the-plasma-industry/> (slightly modified for improved printability). (A) Shows recovered and source plasma combined collected worldwide and (B) shows source plasma only.

The selection and control of the starting material of PDMP, the plasma pool for fractionation, is a major factor in the quality assurance of these products. This process includes the collection process itself, for example, the careful selection of plasma collection facilities (donor centers), which are audited by the fractionator using the plasma for the production of PDMPs and are inspected by the relevant authorities. All donors have to be healthy, documented in a physical examination, and have to provide answers to a predefined questionnaire at the time of each donation. The questionnaire is used to identify any donors who could pose a risk, either regarding his/her own health or the health of recipients of PDMPs, in terms of transmitting pathogens, and to reject their donation and defer the donor, depending on the risk, either temporarily or indefinitely. Further-on, each donation is tested for a pre-defined panel of blood-borne viruses in line with regulatory requirements in place where the plasma is collected and the PDMP manufactured; only donations non-reactive (negative) can be combined to the plasma pool for fractionation. Finally, each plasma pool, the starting material of PDMPs, is tested in most countries for the presence of blood-borne viruses and released for further processing only when non-reactive; testing is required e.g., for products marketed in Europe to be compliant with the Ph. Eur.

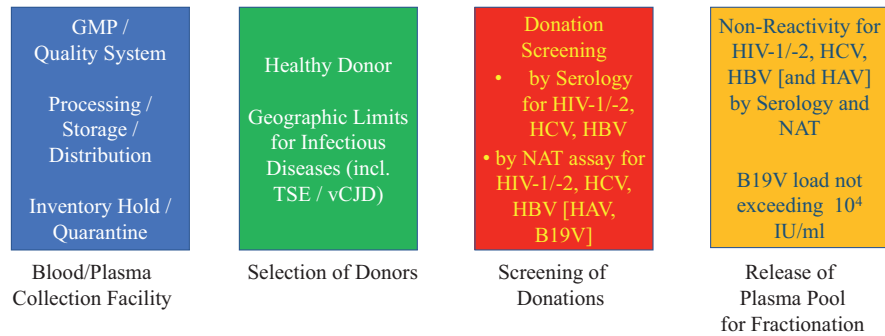


Fig. 3 Measures commonly implemented to mitigate risk of a high virus load in the plasma pool for fractionation to manufacture plasma-derived medicinal products by four pillars—blood/plasma collection facility, selection of donors, screening of donations, and release of plasma pools for fractionation—with specific criteria for each pillar. *TSE*, transmissible spongiform encephalopathy; *vCJD*, variant Creutzfeldt-Jakob disease; *HIV-1/-2*, human immunodeficiency virus type 1 and 2; *HCV*, hepatitis C virus; *HBV*, hepatitis B virus; *HAV*, hepatitis A virus; *B19V*, human parvovirus B19; *NAT*, nucleic acid amplification technique.

Monograph for Human Plasma for Fractionation and with any requirements for particular products for which Ph. Eur. Monographs exist. In order to manufacture PDMPs with a high margin of safety regarding viruses (and other pathogens), in the manufacturing process of these products dedicated virus reduction steps are incorporated with effective virus reduction capacities (this last aspect of the production of PMDPs is not covered in this chapter). A rigorous traceability system has to be in place to follow each donation to a finished product batch and backwards from a finished product batch to a donation and, thus, to a donor; this is especially relevant for pharmacovigilance covering adverse drug reactions.

Fig. 3 depicts the measures taken to manufacture PDMPs with a high margin of safety regarding blood-borne viruses considering the blood/plasma collection facility, the selection of donors, the screening of donations and the release of plasma pools for fractionation for further manufacturing.

Measures implemented in blood/plasma collection facility

Each blood establishment (blood/plasma collection facility) has to develop and maintain a quality system based on GMP principles. All quality objectives as quality planning, quality control, quality assurance and quality improvements have to be implemented in order to meet predefined specifications. These GMP topics include quality management, personnel, documentation, premises and equipment, qualification and validation, materials management, contract manufacturing, and complaints and recalls (WHO, 2011). Therefore, all manufacturing processes have to be clearly defined by policies and standard operating procedures that are systematically reviewed in the light of experience and are shown to be capable of consistently manufacturing blood/plasma of the required safety and assured quality that comply with their respective specifications. Equipment and reagents have to be qualified and processes and methods validated prior to use in the manufacture of products intended for transfusion or further manufacturing. Establishing a reliable quality assurance system for the whole chain of blood/plasma collection, processing and distribution of blood components is of eminent importance.

GMP is the part of quality assurance that ensures that blood products are consistently produced and controlled to the quality standards appropriate to their intended use, as required by predefined specifications. Quality assurance functions is involved in all quality-related matters as personnel and organization, documentation system, computerized systems/electronic information system, premises and equipment, materials, qualification and validation, storage and transport, record keeping, documentation and review of results, change control, corrective and preventive actions (CAPA), complaints, recalls and audits as well as contract between blood establishment and fractionator. All these GMP related topics are relevant to diminishing risks inherent in any blood establishment operation as preventing contamination (including cross-contamination), mix-ups and executing labeling, component separation and freezing within a pre-defined interval after completion of donation to a temperature below -30°C within 1 h as well as storage and transportation according to regulation by authorities (commonly at below -25°C); thus, disease transmission or other unexpected adverse outcomes resulting from the use of blood/plasma products can be substantially diminished by appropriately controlled processes and procedures.

Besides such general GMP requirements further specific requirements for blood/plasma collection facilities are relevant: For any materials potentially impacting the quality and safety of the product, i.e., primary packaging materials, equipment, additive solutions, starting materials and blood and blood components, detailed specifications must be in place and all containers must be labeled with relevant information to ensure traceability at each stage of plasma production. Blood/plasma has to be approved for release for further processing by an authorized person, preferably supported by validated computerized systems. The specifications for release must be defined, validated, and documented. Donations from a repeat tested donor have to be compared with previous records of that donor and the release of blood/plasma has to be blocked until all relevant information is available. When a donation

is not acceptable for release to manufacture PDMPs due to, e.g., reactivity in assays for blood borne viruses, it may be used—properly labeled—however, for research purposes as, e.g., a source for diagnostic assays. Based on defined specifications, e.g., reactivity in HIV, HCV, or HBV testing, any future donations from this donor have to be blocked (the donor has to be deferred indefinitely from further donating). Inventory hold allows the retrieval of plasma units tested non-reactive (negative) for virus markers when a subsequent donation will be tested reactive (positive); therefore, this measure considerably protects plasma pools from containing window donations. Commonly, for source plasma an Inventory Hold of at least 60 days is implemented (e.g., PPTA, 2000a). National requirements (or voluntary measures by fractionators) may request more stringent measures, e.g., longer hold period and/or quarantine; for instance, in China the hold period is 90 days after collection and the donor has to be tested again. Only when these tests are non-reactive (negative), plasma collected 90 days before can be released for further manufacturing (Wang et al., 2018); this “Quarantine” of plasma is more stringent than “Inventory Hold” allowing the release of plasma after a storage time of, e.g., at least 60 days; post donation information as reactivity for infectious disease markers in subsequent donations within this Inventory Hold period results in the interdiction and destruction of the current donation and all donations stored in Inventory Hold. Therefore, a donation with a low virus load tested non-reactive according to quality assurance standards would not be used for pooling but would be retrieved and destroyed based on screening assay information of a subsequent donation. PPTA (Plasma Protein Therapeutics Association) has implemented a standard—QSEAL Inventory Hold Standard (PPTA, 2000a)—in addition to regulatory requirements; for all source plasma donations this standard applies to companies QSEAL certified together with the QSEAL Voluntary Standards “Controls on Incoming Plasma,” “Nucleic Acid Amplification Technology (NAT) Testing,” “Intermediates Purchased from an External Supplier,” “Recovered Plasma Specifications,” “Qualified Donor,” and “Viral Marker” (PPTA, 2000b). This PPTA Inventory Hold standard requires that all source plasma be held in inventory for at least 60 days from the time of collection such that post-donation information, e.g., reactivity in screening tests for HIV, HCV, and HBV in a subsequent donation, delayed admission of high-risk behavior or new information about international travel, could be addressed properly. The retrieval of non-reactive donations with a low virus load—below the limit of detection of a screening assay—is based commonly on results of NAT screening of subsequent donations. Fig. 4 shows the probability to detect a virus infection due to NAT testing of subsequent donations: considering the NAT window period of 15 days for HIV, 8 days for HCV and 35 days for HBV (EMA, 2016a), an HCV infection could be detected in a donation collected latest after approx. 1 week in the subsequent donation, an HIV infection in a donation collected approx. less than 2 weeks later than the non-reactive donation of an infected donor, and the detection of an

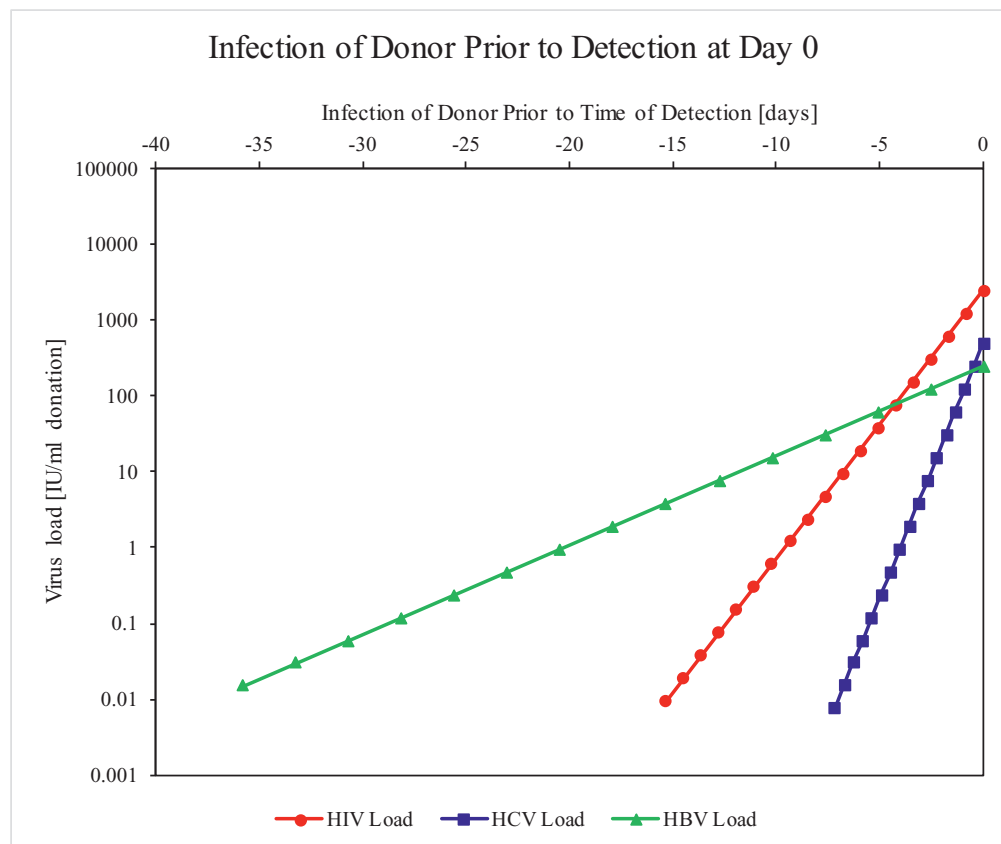


Fig. 4 Virus load [IU/mL] in a donation depending on the virus doubling time of HIV-1, HCV, and HBV prior to time of detection at day 0. Based on the virus doubling time, the NAT window period, and the sensitivity of the NAT assay (here, a minipool of 48 donations is considered) the probability to detect an infection in a subsequent donation to a donation tested non-reactive is highest for HCV and lowest for HBV.

HBV infection takes longer, up to approx. 5 weeks after infection. Therefore, an inventory hold period of at least 60 days mitigates efficiently the risk that a donation from an infected donor—below the limit of detection of the NAT screening assay—will be pooled to manufacture PDMPs. In Fig. 4, the limit of detection, based on the average 95% limit of detection for HIV-1, HCV, and HBV is 50 IU/mL, 10 IU/mL, and 5 IU/mL, respectively, using a NAT minipool of 48 donations. The virus doubling time was estimated at 20.5 h for HIV-1 (Fiebig et al., 2003), 10.8 h for HCV (Glynn et al., 2005), and 2.56 days for HBV (Biswas et al., 2003) and the viremic window period, i.e., the time to detect the infection by NAT is 15 days for HIV, 8 days for HCV and 35 days for HBV (EMA, 2016a). It has to be considered that pooling of samples of donations according to common procedures in screening laboratories (minipools of 24, 16, or 6 donations) will result in a higher virus load than disclosed in the product information for the Limit of Detection or Limit of Quantification and remain undetected correlating with the number of donations per minipool. In Table 1 the assumed virus load in different minipool systems is shown as well as the probability to detect an infection earlier by pooling less donations (compare Fig. 4 for virus doubling time). The interdonation interval, according to national regulatory agencies, of less than 7 up to 14 days will allow the identification of donations below the limit of detection for a certain virus stored in inventory hold due to the reactivity of a consecutive donation. These data also show that a quarantine of donations is not required to prepare a plasma pool for fractionation with no or only a minute load of blood-transmissible viruses when the epidemiology in the donor population is acceptable (compare topic “Epidemiology in Donor Population” and topic “Screening of Donations”).

First time tested donors, occasionally including test-seeking individuals, representing donors having more likely a higher rate of viral markers than regular/repeat tested donors as these donors have already gone through a selection or deferral process. Therefore, source plasma from first time tested donors is not used by most fractionators but only from “Qualified Donors” (PPTA, 2020). This PPTA IQPP standard defines the different donor populations: For source plasma donations, the first donation from an “Applicant Donor” (first time donor/potential donor) is initially quarantined. This quarantine can only be lifted when the donor has successfully passed a second medical history assessment and a second donation has been screened and shown to be free of the relevant transfusion-relevant viruses. These measures result in a healthy population of donors (known as “Qualified Donors”) committed to donating plasma. Source plasma from donors who have not reached the status of “qualified donors” is not processed for further manufacture and is, therefore, destroyed. Qualified donors who have not made a donation for a period of 6 months or more are considered to be applicant donors again. All donations are screened for viruses using currently licensed or approved (CE-mark) serological and nucleic acid amplification assays.

The Quality Assurance principles to be implemented cover all steps involved in the collection, preparation and testing activities reducing errors and technical problems, guard the donor selection criteria prior to each donation, assures the release of plasma, meeting the safety and quality specifications including the respective inventory hold/quarantine periods defined, and guarantee adequate documentation and full traceability for each donation.

Selection of donors

Selecting individuals for blood/plasma donations determines whether this person is in good health to ensure the health of both, donor and recipient of PDMPs, as much as possible.

When a donor arrives at the blood/plasma collection facility, evidence of his/her identity has to be provided and a donor identification system has to be established that positively identifies each donor and each donation; in addition, accumulated records and laboratory data are assigned to such donor (EDQM, 2020; CFR, 2020; WHO, 2011). Furthermore, information has to be provided to a (prospective) donor such that the donor can grant an informed consent prior to proceeding with the donation.

Protection of donor's health

Based on national regulatory guidance in the countries where the donation takes place, temperature, blood pressure, pulse, weight, and donor's age have to be within pre-defined limits. Total protein and/or immunoglobulin G concentration in a sample of a plasmapheresis donor should be monitored and, if not between normal limits, the donor is deferred from donation until the protein composition returns to acceptable levels. Long-term effects in source plasma donors in intensive plasmapheresis programs should be monitored for risks to the donor's health, e.g., association with citrate exposure and diminished IgG levels.

Table 1 Assumed virus load in a donation based on minipool testing.

<i>NAT screening assay: reactive at assumed virus load [IU/mL donation]</i>					
<i>Virus</i>	<i>In Fig. 4 (minipool of 48)</i>	<i>Minipool of 24</i>	<i>Minipool of 16</i>	<i>Minipool of 6</i>	<i>Single donation</i>
HIV	2400 (day 0)	1200 (day −0.9) ^a	800 (day −1.5) ^a	300 (day −2.6) ^a	50 (day −4.9) ^a
HCV	480 (day 0)	240 (day −0.5) ^a	160 (day −0.6) ^a	60 (day −1.4) ^a	10 (day −2.6) ^a
HBV	240 (day 0)	120 (day −2.6) ^a	80 (day −4.4) ^a	30 (day −7.7) ^a	5 (day −14.6) ^a

^aIncreased assay sensitivity/earlier detection of infection by reducing minipools size in days (bracketed) compared to testing minipools of 48 donations.

Commonly six whole blood donations per year for male donors and four whole blood donations for female donors with an interdonation interval of at least 2 months are acceptable to protect donors' health; national regulatory requirements have to be followed. For source plasma donations, a much higher rate of donations is allowed depending on the national regulatory requirements in place where the donation takes place: donors can donate up to 104 times per year in the United States and up to 40 times per year in Europe, resulting in up to 90 L plasma per donor in the United States and approximately 25 L in Europe (Simon et al., 2009). Due to the national regulatory requirements interdonation intervals and the amount of source plasma collected may differ between countries; e.g., in China approximately 25 times a donor can donate within a year, resulting in approximately 18 L plasma per donor calculated based on an interdonation interval of at least 14 days (Wang et al., 2018).

Hazardous occupations or hobbies, such as driving a bus or train or piloting, operating a crane, scaffolding and gliding, climbing and diving should only be performed after a sufficient recreation time, commonly not earlier than 12 h after donating, in order to avoid a risk to the donor and/or other people in the event of delayed vasovagal reaction following blood donation.

Pre-donation educational material has to be provided to a donor containing the essential nature of blood, the blood donation procedure, the aim of the medical assessment of the donor, and that a blood/plasma donation can not be drawn when carrying a risk to the recipient; furthermore, donors have to be informed on temporary and permanent deferral based on the medical examination and/or the results of donation screening as well as on self-deferral (EDQM, 2020). A thorough screening of each donor, i.e., a complete medical and physical examination is not feasible in reality; thus, the responsible physician at the blood establishment, or a trained physician substitute under the physician's supervision, will check the medical and physical health of the donor (including medication) based on a predefined questionnaire concerning medical history, general health, relevant risk factors as lifestyle and travel history, and on elementary laboratory tests.

The goal of such a questionnaire to be answered by the donor is to elicit relevant information on his/her health and lifestyle to be used as part of the screening process to establish the eligibility of a donor. To obtain relevant and consistent information about the donor's medical history and general health, such a questionnaire, together with educational materials and a medication deferral list, should be completed at each donation. Adaptation of the questionnaire to different donor categories such as first-time donor, repeat/regular donor or apheresis donor should be in place. An example of a questionnaire is provided by AABB (for whole blood donations) approved by the FDA to be used in blood establishments that collect blood and blood components; for repeat donors, an abbreviated donor history questionnaire may be used (FDA, 2020b). Also in Europe a questionnaire is strongly recommended to obtain information on the health and lifestyle of the donor that may adversely affect the safety of both, the recipient and the donor. A generic questionnaire is considered not appropriate, but a questionnaire should be developed according to local circumstances by the local (national) blood establishments (EDQM, 2020). Nevertheless, the principle and content of the questionnaires are comparable and elicit information from the donor regarding, e.g., his/her general health, previous blood donations, weight, serious illness, and hazardous occupation and hobbies.

Protection of recipient's health

Besides the health of the donor, the health of the recipient of a blood component donation and in this chapter, especially, of PDMPs is very important. Here, the capability to transmit blood-borne viruses (and, potentially, variant Creutzfeldt-Jakob disease (vCJD)) has to be assessed.

Blood-borne viruses, namely HIV—but also HCV and HBV—can be transmitted by sexual contacts; especially men who have sex with men (MSM) are infected by a range of pathogens (e.g., Soares et al., 2014). In several countries screening for syphilis is used (e.g., FDA, 2020c), although syphilis testing, as a surrogate marker for sexual risk behavior, has a low sensitivity and a low positive predictive value as a surrogate marker for detecting known transfusion-transmitted viruses as HIV, HCV and HBV (Zou et al., 2009).

In Europe, the most affected group for newly diagnosed HIV infections in 2012 were MSM accounting for 40% of all HIV infections; also commercial sex workers (CSW) are at high risk to be infected by HIV (and other sexually transmitted diseases) (Offergeld et al., 2014). Further data for Europe were published (ECDC/WHO, 2020) documenting an HIV infection in approximately 43% for MSM when people were diagnosed late (CD_4 cell count $<350/mm^2$). HIV infection of MSM is reported globally (e.g., Lama et al., 2010; Beyrer et al., 2012; Wang et al., 2012; Hakre et al., 2014; Jansen et al., 2015) and the infection with HIV increases the probability to be infected by further sexually transmissible viruses as HBV and HCV (Falade-Nwulia et al., 2015; Melhem et al., 2015; Das et al., 2018). It has to be considered that HIV, HCV and HBV (together with some new emerging viruses as Zika virus) is detected in human semen (Salam and Horby, 2017). MSM and bisexual men can transmit HIV (and other blood-borne viruses) to women and may subsequently cause the infection of children (e.g., Khanani et al., 2011). As mentioned above, besides HIV also HCV can be transmitted by sexual activity and MSM, being HIV infected, have a higher risk also to acquire an HCV infection (e.g., Witt et al., 2013) and an HBV infection (e.g., Tseng et al., 2012; Van der Kuyl et al., 2013). Transmission of HBV by sexual contacts are a known cause of HBV infection (e.g., Li et al., 2010; Soetens et al., 2015; Cornelissen et al., 2016; Inoue and Tanaka, 2016; Dah et al., 2019). Further viruses, as hepatitis A virus, were reported to be transmitted by sexual contacts (Sfetcu et al., 2011; Raczynska et al., 2019). Besides MSM, also female CSW are infected by blood-transmissible viruses (e.g., Rosenberg and Weiner, 1988; Weber et al., 2002; Wang et al., 2009; Offergeld et al., 2014). Therefore, the higher HIV prevalence and incidence among MSM, compared to the general heterosexual population, demand a thorough assessment whether MSM and concurrent sexual partners of bisexual men as well as CSW could be potential blood donors. This assessment should include changes from a permanent donor deferral policy for MSM to a risk-based deferral policy based on scientific evidence. Therefore, in several countries MSM (and, partly, CSW and their (multiple) partners) are not any longer permanently deferred but, depending on the last MSM

(or other sexual) contact, a deferral period of 12 or even 3 months were implemented according to the respective national regulatory authorities; this limited deferral period is set up after having assessed that the change in deferral policy results in no significant increase of risk (Suligoi et al., 2013; Epstein et al., 2014; Ginsberg et al., 2016; FDA, 2020d; Steele et al., 2021); due to NAT screening of donations with a high sensitivity and the detectability of a virus infection within, commonly, approx. not more than 6 weeks, after infection, such a limited deferral policy of 3 months seems appropriate from a scientific point of view.

Also the use of drugs, primarily injectable drugs, are a high risk to be infected with blood borne viruses, primarily HIV, already recognized in the late 1980s (e.g., Robertson et al., 1986; Webb et al., 1986; D'Aquila and Williams, 1987). Sexual contacts of illicit drug users, including CSW, can transmit pathogens to the general population (e.g., Brown and Primm, 1988; Ramsey et al., 2010; Thurstone et al., 2013). Further virus infections are reported for illicit drug users as HCV (e.g., Firestone Cruz et al., 2007; Aaron et al., 2008; Magalhães Novais et al., 2009; Basu et al., 2015) and HBV (e.g., Moestrup et al., 1986)—here, vaccination seems to decrease the probability of HBV infection (Rivas et al., 2010).

Based on these data the main risk for recipients originates from donors with sexual risk behavior such as MSM and sexual contacts with CSW, illicit drug users, and persons from countries with a high prevalence of HIV, HCV and/or HBV. Therefore, when no permanent deferral of potential donors is implemented by the national regulatory authority, an individual risk assessment covering the sexual risk has to be performed; the same is true for individuals having used in the past illicit drugs. Information about risk behavior of donors, first time donors and repeat/regular donors, has to be given to a donor prior to each donation by a donor's interview based on the questionnaire.

The questionnaire, discussed above to be used to protect the donor's health, is also relevant for the recipient's health. Therefore, the questions include, but are not limited to, any history of injectable drug abuse, sexual contacts of MSM and contacts with CSW, but also, e.g., female partners of men having sex with men, and sexual contact with residents or former residents of countries with a high prevalence of, e.g., HIV. Body piercing and/or tattoo and acupuncture treatment by a not registered practitioner may have an increased risk of infection with blood borne viruses when the hygiene in such establishments is low (e.g., Van Remoortel et al., 2019). Finally, the risk of having received a xenotransplantation has to be assessed in order to prevent a zoonotic disease that could be transmitted to the recipient of a blood component and, in principle, also by a PDMP (e.g., Fishman et al., 2012; Denner and Mueller, 2015). Furthermore, geographic risk regarding vCJD disease has to be answered as well as treatment in the past with extracts from pituitary glands or receiving dura mater or cornea grafts as well as having a family risk of Creutzfeldt-Jakob disease (EMA, 2018; FDA, 2016; revised recommendations by the FDA (FDA, 2020e) with clarification regarding less stringent requirements for deferral of donors).

Temporary deferral regulations are implemented when a donor has/had certain medical treatment/medication or diseases. A donor of source plasma is commonly not deferred due to infections with viruses, except for HIV, HCV, and HBV with a permanent deferral established. For some viruses as parvovirus B19 (B19V) and HAV (as well as HEV in certain regions or for specific PDMPs), the donor is deferred until a sensitive NAT assay is non-reactive in order to predict that a plasma pool for fractionation is below a predefined virus load that has to be demonstrated. Due to the fact that PDMPs are manufactured with a range of process steps to fractionate, purify, and concentrate the desired protein(s) that are finally sterile filtered, most human infectious viruses, when causing a non-persistent infection, as well as bacteria and parasites do not result in a deferral of a source plasma donor (e.g., EDQM, 2020; FDA, 2009a, 2017, 2019, 2020a; O'Brien et al., 2012). For donors of blood and blood components more stringent exclusion criteria apply.

Epidemiology in donor population

Testing each donation for the presence of the major blood-borne viruses HIV, HCV and HBV is of utmost importance to prevent an infection of recipients of PDMPs (compare topic Donation Screening). Such testing establishes a continuous epidemiological surveillance of the donor population in order to ensure long-term safety of plasma for fractionation (WHO, 2007). This surveillance provides information on the prevalence and incidence, and their trends, of infectious markers relevant to the virus safety of PDMPs as accurately as possible. In case predefined limits in the epidemiology are exceeded, appropriate measures (e.g., CAPA—corrective action and/or preventive action) have to be taken without delay to bring the respective donor population, e.g., at a specific blood/plasma collection center within a country or region, back to conformity. Detailed information on the assessment of epidemiological data on blood transmissible infections can be found in an EMA guideline (EMA, 2016a).

Assessing the data from epidemiological surveillance will detect differences among donor populations at various collection centers caused by differences in viral markers, potentially reflecting differences in donor selection and screening procedures; CAPA should be taken to address such an issue. Trends in infectious markers, reflecting either a change in the rate of viral markers in the population, a possible deviation in the donor selection or screening process at specific collection sites, and/or the implementation of a new or additional screening test has to be assessed carefully, and, if required, appropriate counter measures have to be executed. When in a particular blood establishment a significant higher prevalence or incidence rate is demonstrated, a comparison with the general population in the same country/region is useful in assessing the suitability of this blood establishment for collecting plasma for the production of PDMPs. It has to be considered, however, that prevalence and incidence are generally complementary in providing information on past and current risk of infection in a population: a high prevalence and incidence is characteristic for an ongoing transmission, high prevalence and low incidence demonstrate a high infection rate in the past with intervention measures introduced in the meantime, low prevalence and high incidence indicates an infection (most probably) introduced recently into the population, and a low prevalence and incidence is indicative for little or no evidence of past or current infection.

Theoretical Blood Establishment in a Country / Region

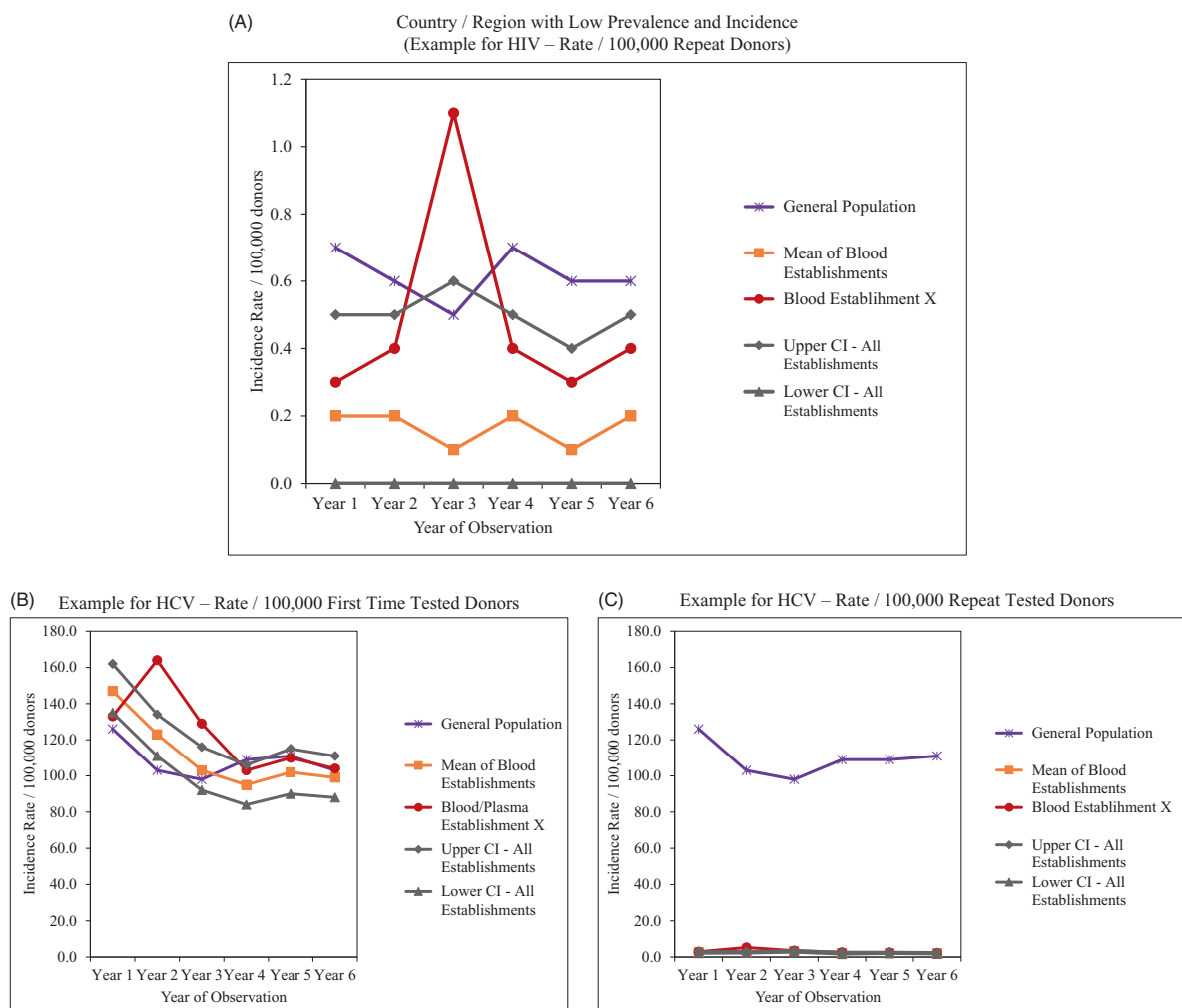


Fig. 5 Graphical tool for assessing the epidemiology in a donor population of theoretical blood/plasma establishments in a country/region. As an example, blood/plasma establishment X is considered an outlier with efficient CAPA. (A) Country/region with low prevalence and incidence: example for HIV in repeat tested donors. (B) Country/region with high prevalence and incidence: example for HCV in first time tested donors. (C) Country/region with high prevalence and incidence: example for HCV in repeat tested donors.

A graphical tool (e.g., control chart) is very suitable to compare the epidemiological situation in a given blood establishment with all collection facilities in the same country/region and the general population. Fig. 5 shows the principle of such a graphical tool demonstrating the comparability of the incidence and prevalence in the first time tested donor population with the general population. Positive screening results of repeat donors indicate primarily the incidence rate of an infection and the continuous donor education and interviews based on a questionnaire reduces the risk that a donor infected with a blood-borne virus will show up at a blood establishment or will be accepted as donor. In this theoretical situation shown in Fig. 5, only Blood Establishment X is in 1 year (year 2) outside the upper Confidence Interval (CI) for all establishments; implementing effective CAPA, the incidence (and partly prevalence) in the donor population of this establishment came back to specifications. The epidemiology in the general population is comparable to that in donors for Fig. 5B, whereas the epidemiology in repeat tested donors (Fig. 5C) is considerably lower than in the general population, even in a country/region with a high prevalence and incidence.

Further measures have to be in place to define an acceptable epidemiology in a donor population as outlined in detail in the European requirements to apply for Plasma Master File (PMF) certification submitted to EMA (EMA, 2016a; EMEA, 2006a,b). A major issue in assessing the donor population's epidemiological data is monitoring change over time and defining alert and action limits. Change over time should be provided separately for "repeat tested donors" and "first time tested donors," even if source plasma (collected by apheresis) from first time tested donors is not used to produce PDMPs; the screening data of these first time tested donors may relate to the epidemiology in the general population. When source plasma as well as recovered plasma

(from whole blood) is collected in a certain country/region for the production of PDMPs, it is recommended to monitor the donor populations separately as the interdonation interval in both populations is considerably different resulting in an improved determination of the incidence rate in source plasma donors.

The epidemiological surveillance provides information on differences among donor populations of various collection centers which may be associated with objective differences in viral markers within donor populations or may reflect differences in the process of donor selection and screening among collection centers; when such differences are due to donor selection or screening procedures, appropriate CAPA could diminish these differences. Furthermore, trends over time in the rate of viral markers have to be assessed in such a way that differences in epidemiology can be distinguished to be either due to a change in the epidemiology in the donor population, potential modifications in the donor selection process, or change in screening methods or processes at specific collection sites. The implementation of new or modified screening tests as a serological assay with improved sensitivity and, especially, the introduction of a NAT assay may impact the documented epidemiology in the donor population (compare topic "Screening of Donations"). Such modification of the screening procedure has to be carefully assessed when comparing the epidemiology over time.

Establishing an acceptable epidemiology in the donor population requires first to define the prevalence and incidence in a donor population (EMA, 2016a). Only confirmed infections (confirmed seropositive results) have to be considered; regarding NAT results, "NAT only positives," i.e., the donation is tested non-reactive in serological assays, have to be evaluated. For each blood establishment, information on the donor population including the frequency of donations (average number of donations from one donor per year) has to be compiled; information whether donations from "first time tested donors" are used in plasma pools (generally, only recovered plasma from first time tested donors is used for the production of PDMPs) has to be included. The definition for prevalence and incidence is as follows (EMA, 2016a):

- the prevalence is calculated as $[\text{number of positive "first time tested donors" in a specific period (commonly a calendar year)}] / ([\text{total number of "first time tested donors" in the same specific period}] * 100,000)$
 - "first time tested donors" are persons who are "tested for the first time for infectious disease markers (with or without donation) and without evidence of prior testing in a given blood system" (EMA, 2016a).
- the incidence (in repeat tested donors) is calculated as $[\text{number of positive repeat tested donors in the study period with a previous negative donation}] / ([\text{total number of donations from repeat tested donors in the study period} * \text{mean interdonation interval (expressed in years)}]) * 100,000$
 - a "repeat tested donor" is a "person whose blood/plasma has been tested previously for infectious disease markers in a given blood system," (this includes "regular donors" and "repeat donors"). For PMF holders using the applicant/qualified donor system, "repeat tested donors" include "applicant donors" tested for a second time, "applicant donors" requalifying after an interval of 6 months or more, and "qualified donors" (EMA, 2016a).

To cover also transient detectable amounts of HBV markers, an adjustment factor should be applied (EMA, 2016a); as a very conservative approach, the assumption by Korelitz et al. (Korelitz et al., 1997) may be used; i.e., 5% would have persistent antigenemia (or HBV DNA), 70% of infected donors would have transient antigenemia (or HBV DNA) lasting on average 77 days (Seed et al., 2002) and 25% of infected donors would have no antigenemia (or HBV DNA (thus, these 25% are not considered in the formula)). The following formula should be applied: $1 \div ((0.05 * 1) + (0.7 * (77 \div (\text{IDI}))))$. It has to be considered, however, that the IDI (interdonation interval—in days) is applicable when the IDI is more than 77 days, otherwise the probability to detect a transient antigenemia (or NAT detectability)—lasting on average for 77 days—is 1.¹

Furthermore, the residual risk that a donation from an infected donor remains undetected has to be estimated. The inability of a screening assay to detect an infection is due to (i) the detection limit of a screening assay: the current absence of antibodies (serological assay) or virus (NAT assay; very recent infection with no viremia) in blood/plasma (window period) or (ii) mismatch of virus markers/nucleic acid with assay components due to e.g., virus variants/mutations. This window period risk for serology testing could be estimated as the product of the incidence rate and the time interval in which a new infection would not be detected. Based on published data, considering also the improved sensitivity of assays over time, this window period is considerably different for HCV serology and NAT assays; for HIV and HBV the difference between sensitive serology assays and NAT are lower. Table 2 shows the detection of an infection based on serological assays with the corresponding virus load in a donation non-reactive in serology and the considerably improved detectability of a virus infection with low virus loads in a donation remaining non-detected by NAT testing.

The virus load in a donation in a NAT positive donation detected by NAT ("NAT only") considered here is based on a generic NAT assay with an average 95% Limit of Detection for HIV-1, HCV, and HBV of 50 IU/mL, 10 IU/mL, and 5 IU/mL, respectively. The virus doubling time, related to the NAT window period, is shown in Fig. 4.

Serological donation screening assays are on average reactive 22, 82, and 59 days after infection by HIV, HCV, and HBV, respectively (Table 1; Schreiber et al., 1996). Therefore, when using serological assays an inventory hold period of 60 days is generally only efficient for HIV as a donation tested non-reactive would be detected by subsequent donations of this donor within the time period of 60 days. For HCV as well as for HBV this inventory hold period is considered inappropriate when testing solely

¹Examples to calculate the HBV adjustment factor: Donor of recovered plasma donating twice a year (IDI of 180 days): $1 \div ((0.05 * 1) + (0.7 * (77 \div 180))) \geq 2.9$
Donor of source plasma donating once a week (as IDI of 7 days is below 77 days, the transient antigenemia (or HBV NAT) would be detected within these 77 days: $1 \div ((0.05 * 1) + (0.7 * (1))) \geq 1.3$).

Table 2 Window period for HIV, HCV, and HBV assays.

	<i>HIV-1</i>	<i>HCV</i>	<i>HBV</i>	<i>Reference</i>
Serological window period [days]	22	82	59	Schreiber et al. (1996)
Virus load in a serological window donation (up to [$\log_{10}$ IU/mL donation])	5.8 ^a	6.6 ^b	2.4 ^c	^a Ribeiro et al. (2010) ^b Glynn et al. (2005) ^c Biswas et al. (2003)
NAT window period [days]	15	8	35	EMA (2016a)
Estimated virus load after NAT testing (= non-reactive donation) [IU/mL ($\log_{10}$ IU/mL) donation]				Based on average 95% limit of detection for HIV-1, HCV, and HBV of 50 IU/mL, 10 IU/mL, and 5 IU/mL, respectively [approx. corresponding to package inserts of assay suppliers]
Single donation testing	50 (1.7)	10 (1.0)	5 (0.7)	
Minipools testing				
Pool of 24	1200 (3.1)	240 (2.4)	120 (2.1)	
Pool of 16	800 (2.9)	160 (2.2)	80 (1.9)	
Pool of 6	300 (2.5)	60 (1.8)	30 (1.5)	

serology. High virus loads, especially for HCV, would remain undetected and such a donation—tested negative in a serological assay—would be pooled for the production of PDMPs. Therefore, the implementation of NAT assays is essential to identify high titer donations and remove/discard these donations; an inventory hold period of 60 days is considered adequate when NAT testing is implemented, taking into account an interdonation interval of less than approximately 7 up to 14 days.

Criteria to establish alert limits for epidemiological data has to be defined and may, depending on the epidemiology in different countries/regions, be related to the epidemiology of the general population in the specific country/region. These alert limits allow the identification of individual collection centers of a blood/plasma establishments with viral marker/virus rates outside the normal range for the given donor population in this defined country/region triggering appropriate corrective actions. Due to the fact that the interdonation interval is considerably different between source and recovered plasma donors, the infection markers and/or viral load in a donation of an infected donor may be more often below the limit of detection of the respective screening assays in recovered plasma than source plasma; the epidemiology in the donor population should, therefore, be assessed for recovered and source plasma donors independently. The risk of accepting donations containing a low virus load (below the limit of detection of the NAT assay system used) for the production of PDMPs is, therefore, higher for recovered plasma, especially when the inventory hold period measure for source plasma is implemented.

Drawing a donation from an infected donor is related to the prevalence and incidence rate for a given virus in the donor population; when the donation is tested non-reactive in serological and NAT screening assays (window period donation), the incidence rate in the donor population is relevant. This incidence rate corresponds to the likelihood that recent infections are detected when the viral markers or virus load are above the limit of detection of the assays employed. Estimating the risk of a donation from an infected donor remaining undetected is primarily assessed for blood/blood component transfusion (e.g., Weusten et al., 2002; Niederhauser et al., 2005; Zou et al., 2010). Besides this general concept to assess the risk of undetected infected donations, for plasma for fractionation such a risk and, thus, acceptable epidemiological rates in the donor population, is currently assessed by two approaches:

- Defining a “Standard” of the epidemiology in a donor population to compare current epidemiology and possible outliers is performed by compiling historical epidemiological data of one or several blood/plasma establishments over one or several years. Statistical methods are applied, e.g., gamma distribution of the epidemiology in donor populations (as the distribution of center specific positivity rates is highly asymmetric, usually with a high peak at the lower end, near zero, and a long right tail), together with the Poisson distribution for the number of positive donors in a given center; such an approach is implemented for PPTA blood/plasma establishments and donation centers (PPTA, 2010).
- An appropriate margin of virus safety in the finished PDMP (according to the European Guideline on PDMPs EMA, 2011) is used as starting point to back-calculate the maximum virus load in the plasma pool for fractionation, considering the virus reduction capacity for HIV, HCV, and HBV for a theoretical PDMP; the number of donations below the limit of detection of the NAT assay (tested in minipools) correlates with the incidence rate in the donor population and, based on these estimates, an alert but also an action limit for a defined collection center was calculated. Alert limits could be defined triggering closer monitoring of the collection center whereas action limits trigger predefined CAPA (Lovick et al., 2014) when the incidence rate was higher. This concept employs a probabilistic Monte Carlo model, comparable to approaches published (Janssen et al., 2008; Velthove et al., 2013); however, in these publications an acceptable epidemiology for PDMPs was not outlined.

Screening of donations

Tests to detect a virus infection of a donor in a sample of a donation have to be applied as the probability of virus transmission by blood components and, in the past, by PDMPs, depends on the frequency of infections in the donor population, the duration of the

asymptomatic phase of an infected donor, and the length of the viremia. Such a test has to be specific and sensitive; e.g., cross-reactivity between antibodies against viruses of the same family or genus should be excluded (e.g., [Souza et al., 2019](#)). After the detection of the respective virus in the general populations or in individuals at risk to acquire a virus infection, in-house assays, developed commonly first by research institutes, followed by commercial assays, are employed to detect the virus infection.

Serological assays detect virus specific antibodies and virus antigens commonly using enzyme immunoassays as enzyme-linked immunosorbent assay (ELIA) or chemiluminescence assay (ChLIA). Only licensed or approved (CE-mark) assays, according on the relevant regulatory agencies, can be used for screening of donations. A positive result of a serological assay has to be confirmed, employing, according to the nationally established algorithm, an alternative immunoassay, a Western blot or recombinant immunoblot; tests using a different detection principle as antigen detection (when testing for antibodies was performed) or NAT may be suitable in the interpretation of uncertain screening test results. Initially reactive donations should be tested for confirmation in duplicate and if any of the repeat tests are reactive, the donation is considered repeat reactive and a confirmatory assay has to be used.

Besides the detection of virus proteins (antigens) and antibodies against certain virus proteins, the detection of the virus nucleic acid by nucleic acid amplification techniques (NAT) is widely implemented as this screening assay is the most sensitive assay to detect a virus infection early. These NAT assays are commonly PCR (polymerase chain reaction) assays requiring repeated temperature changes (thermal cycles) and, when an RNA virus has to be detected, the transcription of virus RNA to DNA. Besides PCR, mainly a TMA (transcription-mediated amplification) assay, an isothermal process, is employed in testing donations; for this assay, DNA from DNA viruses has to be transcribed into RNA to start the reaction. Internal controls are added to the reaction mixture in commercial NAT assays and the amplification efficacy is followed by fluorescent or chemiluminescent signals. Modifications of the general methods are available as (droplet) digital PCR for precise quantification of nucleic acids; furthermore, for the detection of a very wide range of viruses (and other pathogens), including new emerging viruses, Next Generation Sequencing (NGS) methods are applied meanwhile. Besides qualitative also quantitative NAT assays are available defining, besides the Limit of Detection, also the Limit of Quantification. Further developments of (commercial) NAT assays result in multiplex assay formats allowing the detection of different viruses (primarily HIV, HCV and HBV) simultaneously in a single sample of a donation.

- HIV
 - First assays to detect anti-HIV antibodies were described in the mid 1980s and questions regarding the implementation of such assays were discussed in a Consensus Conference ([Consensus Conference, 1986](#)). Currently, the fourth generation serological assay is implemented in many countries, detecting simultaneously anti-HIV antibodies and HIV antigen. The minimum requirement for a HIV serological screening test is the detection of anti-HIV-1 M, including outlier types as HIV-1 type O (and type N as well as some HIV-1 circulating recombinant forms (CRF)) and anti-HIV-2.
 - HIV NAT assays should be able to detect all relevant HIV-1 groups/subtypes and HIV-2 including mutations and circulating recombinant forms (CRF) of HIV. As HIV is genetically very variable, NAT assays have to target very conserved genome sequences as primers. Nevertheless, there are reports that such an approach may not be sufficient and that the simultaneous use of at least two conserved regions as amplification target ("dual target screening") should be used ([Schmidt et al., 2009](#); [Chudy et al., 2012, 2014](#)).
- HCV
 - The agent causing the parenterally transmitted "non-A, non-B hepatitis" (now known as hepatitis C virus (HCV)) was first described 1989 ([Choo et al., 1989](#)); based on a cDNA clone, the detection of antibodies in the majority of non-A, non-B serum specimens was possible ([Kuo et al., 1989](#)) resulting in the first serological detection of anti-HCV antibodies. Currently, a third-generation antibody assay is available (and a fourth-generation assay detecting antibodies as well as antigens). NAT assays for the detection of HCV are very important as the long window period in serology screening can be closed considerably and the high virus load in a window period donation can be detected and the donation destroyed (and the donor deferred permanently).
- HBV
 - Serological assays detect the HBV surface antigen (HBsAg); the envelope of HBV is composed of host-derived lipid components and three virus-encoded polypeptides, collectively called HBsAg (the S gene with three in-frame start codons and a common stop codon dividing the gene into PreS1, PreS2 and S region encoding the small, middle and large envelope proteins, respectively). HBsAg can be detected in serum/plasma not only as part of the whole virus but also as separate spherical or tubular sub-virus particles; these sub-virus particles do not contain DNA and are non-infectious and exceed the amount of intact, infectious HBV particles in serum/plasma up to 10,000-fold; however, for different HBV genotypes, especially genotype G, the release of HBsAg particles in blood is considerably lower, resulting in a decreased detectability of certain HBV genotypes ([Hassemer et al., 2017](#)). Due to the fact that mutations in the S gene as well as so-called occult HBV may not be detectable by HBsAg screening assays (e.g., [Consensus Report, 2004](#); [Ly et al., 2006](#); [Scheiblaue et al., 2006](#)), antibodies against the HBV core protein (anti-HBc), lasting for a long time after infection, are implemented in some countries for blood/blood components for transfusion (e.g., [Schmidt et al., 2006](#)). It has to be considered, however, that in the early phase of an HBV infection antibodies against HBc have not been developed.

Due to the generally high sensitivity of the HBsAg assay, the detection of very recent infections by HBV is not considerably shorter by the implementation of an HBV NAT.

A full validation of commercial test kits used in the testing facility is not required when a thorough description of the methodology and reagents according to the manufacturer's instructions is followed. However, a verification that the operators at the testing facility, when using appropriate equipment, material, and reagents in the testing facility's environment and applying the manufacturer's instructions, achieve the same outcomes as defined in the validation data provided by the kit manufacturer is essential. A statistically significant number of samples covering the full range of results for the intended use should be exerted. The verification should confirm that the limit of detection (LOD) or limit of quantitation (LOQ) are comparable to the validation data of the kit manufacturer. The testing facility should also participate in collaborative studies organized by an independent organization to demonstrate reproducibility between laboratories.

In order to compare results of screening assays between different testing facilities, defining the analytical sensitivity of different testing methods and the performance of laboratories, is of utmost importance. Therefore, WHO International Standards (IS) are implemented to meet regulatory requirements as minimal NAT sensitivity. ISs are measurement standards assigning an internationally agreed unitage in International Units (IU). The potency of an IS is determined through international collaborative studies using a range of methods typically in routine use by participating laboratories (Baylis et al., 2015, 2019). In the first collaborative study for HCV NAT standardization, a very wide range of methods (different in-house and commercial assays) were employed demonstrating a difference in sensitivity of at least 4 log₁₀ (Saldanha and Minor, 1996a). With improved experience of the participants in performing NAT assays, the difference between laboratories (blood product manufacturers, kit producers, regulatory organizations as well as reference laboratories) decreased in international collaborative studies (e.g., Saldanha et al., 2001). Comparability of results by different NAT methods and testing laboratories measured in IU, especially at low level concentrations of virus nucleic acid, is essential to release donations and, especially in Europe, release of plasma pools for further processing. International Standards are not only available for NAT assays but also for serological assays, e.g., for HBsAg (Seiz et al., 2016).

Besides IS, reference panels or international reference panels are available (e.g., from NIBSC, <https://www.nibsc.org/linkhandler.ashx?pid=4624>) and consist of different genotypes or relevant strains of viruses from different global regions (Baylis et al., 2019); no unitage is assigned to members of international reference panels. These international reference panels, when studied in routine testing approaches, document the detection of variants or, on the other hand, demonstrate a gap in the detectability of a range of virus variants. Thus, improvements of assay performance may be triggered when the detection of specific variants is suboptimal. WHO had published available International Reference Preparations (WHO, 2018).

Testing donations has to be performed according to the relevant regulatory requirements applicable in the respective country where the donation is collected; besides serological testing, NAT testing is required (e.g., FDA, 2004, 2012). For source plasma donations fractionated and the PDMP marketed in another country, regulations of this third country may also apply. Testing is performed at least for the major blood-borne viruses human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV) by serological assays (antibodies against HIV-1/-2 and HCV as well as HBV surface antigen (HBsAg)) and in most countries also by nucleic acid amplification assays (NAT) as PCR or TMA for HIV-1/-2, HCV, HBV and commonly also for parvovirus B19 (B19V) (e.g., FDA, 2009b) and, voluntarily, for hepatitis A virus (HAV); only donations non-reactive in all assays performed will be further processed to PDMPs. Donors being infected with HIV-1/2, HCV or HBV will be permanently deferred from donation. As all tests have a limit of detection, and especially antibody assays detect an infection after the respective virus replicated to high titers, the presence of infectious virus in a donation cannot be excluded despite having performed an antibody test for each single donation accurately, as shown in Fig. 6.

Donations reactive in serological assays have to be retested and, if repeat reactive, confirmation of the test result has to be performed using a different test method.

A "NAT only" result should also be confirmed at least by repeat testing. When NAT testing is performed in a minipools setting, the destruction of the minipool and identification of the reactive donation is acknowledged as a repeat reactive result. It has to be considered, however, that for qualitative assays the Limit of Detection is based on the Probit analysis of a dilution series (compare Fig. 7) and a very low virus load, detected with a low probability, may not result in a repeat reactive donation. When testing minipools and the minipool is deconstructed to identify the reactive donation, the probability to detect the reactive single donation is higher; the dilution factor by pooling is vanished and the virus concentration in a sample of the reactive donation is higher by the factor of the minipool size (nevertheless, an, e.g., six fold higher virus concentration compared to the load in a minipool of 6 may not always result in a repeat reactive donation; statistical evaluations may define the number of "repeat studies" that, with a high probability, a low virus load would be detected again).

Besides HIV, HBV and HCV, further viruses—not causing a persistent infection—are included in a routine screening approach when the plasma pool for fractionation has to be below a certain virus load. This is the case for parvovirus B19 (B19V) as the plasma pool for fractionation should not exceed 4 log₁₀ according to FDA regulations (FDA, 2009b). Studies performed previously document that, without B19V NAT testing, plasma pools as well as PDMPs may contain a high amount of B19V DNA sequences (Schmidt et al., 2001) and solvent/detergent treated pooled plasma transmitted B19V caused illness (Koenigbauer et al., 2000). In contrast to common PDMPs only solvent/detergent treatment is applied during the production of this product and this treatment has no effect on the non-enveloped B19V. Therefore, risk mitigation strategies had to be implemented as thorough B19V screening of donations and limiting the amount of B19V in the plasma pool.

Implementation of hepatitis A virus (HAV) NAT was introduced by many manufacturers of PDMPs on a voluntary basis as reports were published documenting a low, but not zero, risk of HAV transmission by PDMPs (e.g., Chudy et al., 1999, 2002). In addition, another hepatitis causing virus, hepatitis E virus (HEV), is recognized to have a blood-borne phase: HEV is a major cause of acute clinical hepatitis among humans throughout the world. In developing countries acute hepatitis E in humans is caused

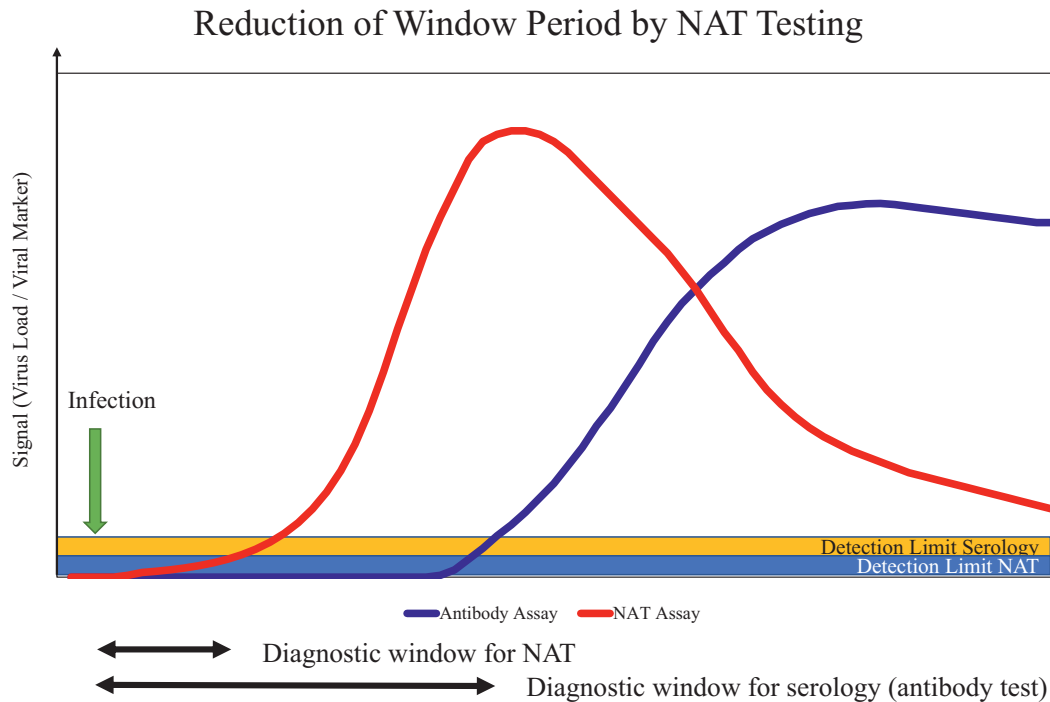


Fig. 6 Schematic principle of antibody and NAT screening assays. Implementation of NAT screening assays reduces the Diagnostic Window period by antibody screening and minimizes the virus load in a donation released due to non-reactivity in the serological assay.

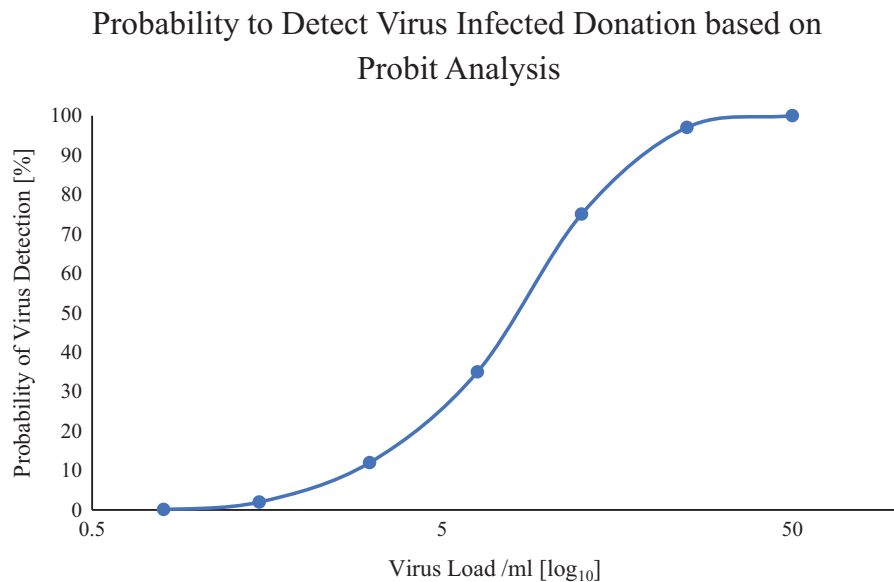


Fig. 7 Probability to detect a virus infected donation based on probit analysis. S-shaped cumulative normal distribution with very low virus concentration to be detected with low probability and increased probability to detect higher virus concentrations.

by the HEV genotype 1 (HEV-1; occurrence mainly in Asia) and genotype 2 (HEV-2; occurrence mainly in Africa and Mexico) with a host range restricted to humans. These genotypes are transmitted primarily by the fecal/oral route (primarily by contaminated water) (Kamar et al., 2012). In industrialized countries, HEV genotypes 3 and 4 are zoonotic viruses infecting animals such as swine, wild boar and deer; transmission to humans occurs primarily by consumption of undercooked pork and wild boar products or by contact with infected animals (Colson et al., 2010; Pavio et al., 2017). HEV (genotype 3) seems to be present more often in donations from European donors than in US donors as data from donation screening indicate (Baylis et al., 2012b; Roth et al., 2017).

As HEV transmission by transfusion of blood/blood components was reported (e.g., Andonov et al., 2014; Domanovic et al., 2017; Gallian et al., 2019), screening of blood donors for the presence of HEV RNA was implemented in some countries; interestingly, elevated ALT (alanine transaminase) levels are not indicative of an HEV infection (Baylis et al., 2010). The presence of HEV in plasma pools for fractionation was also reported with a HEV RNA load not exceeding 1000 copies/mL HEV RNA even without HEV NAT screening of donations (Baylis et al., 2012a). Despite the fact that HEV genotype 3 is considered a threat for immune compromised people (e.g., transplant recipients) and patients with underlying liver impairment or disease, the European regulatory agency does not recommend a general HEV RNA screening of donations and release of plasma pools for fractionation for further processing as the HEV load is low and currently licensed PDMPs contain at least one manufacturing step effective against non-enveloped viruses. No HEV transmission cases have been reported so far with the currently produced plasma-derived medicinal products, with the exception of pooled solvent/detergent-treated plasma (EMA, 2016b; Andonov et al., 2014; Gallian et al., 2020). Therefore, the European Pharmacopoeia requests that the plasma pool for the production of pooled solvent/detergent treated plasma has a limited HEV load of not exceeding 320 IU/mL HEV IU/mL (Ph. Eur. 01/2020:1646); this requirement can only be achieved by screening donations for HEV genomic material and interdicting reactive donations.

Release of plasma pools for fractionation

The plasma pool for fractionation is considered to represent the starting material of PDMPs by regulatory agencies in some countries, comparable to the unprocessed bulk of cell culture derived biotechnological products (ICH, 1999). According to the Ph. Eur. Monograph "Human Plasma for Fractionation" (Ph. Eur. 0853), as an example for comparable regulations worldwide, only donations from carefully selected, healthy donors after medical examination and acceptable donor's medical history, as well as blood screening tests for blood-transmissible viruses could be used for the production of PDMPs. In certain regions of the world, e.g., Europe, the first homogenous pool of plasma (generally after removal of cryoprecipitate) has to be tested for anti-HIV-1/-2 and anti-HCV antibodies and HBsAg using test methods of suitable sensitivity and specificity; the pool has to be negative in these tests in order to manufacture PDMPs; furthermore, the plasma pool has to be tested also for HCV RNA and has to be non-reactive (European Pharmacopoeia monograph "Human Plasma for Fractionation (0853)") using a validated NAT assay. A positive control with 100 IU/mL of hepatitis C virus RNA and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors. The plasma pool complies with the test if it is found non-reactive for hepatitis C virus RNA and detects the run control (positive control) of 100 IU/mL HCV RNA. This NAT test was introduced in Europe in 1999, when intramuscular immunoglobulins, which did not have a validated, effective inactivation/removal step for (enveloped) viruses, transmitted HCV despite thorough anti-HCV antibody screening of all donations (CPMP, 2001); the addendum to this guideline was deleted in the fourth revision (EMA, 2011) as HCV NAT testing of plasma pools became a mandatory requirement in the European Pharmacopoeia monograph "Human Plasma for Fractionation (0853)." When performing a qualitative HCV NAT assay to release a plasma pool (<100 IU/mL), the 95% Limit of Detection (LoD) of this HCV NAT assay, performed commonly by the fractionator or on behalf of the fractionator, should be around 30 IU/mL or lower as the 100% LoD is approximately three fold higher than the 95% LoD based on Probit Analysis in order to detect always the run control (100 IU HCV RNA/mL).

Further NAT tests have to be implemented when a plasma pool for fractionation is used for the production of "Human Plasma (pooled and treated for virus inactivation)," so-called SD-Plasma. As the manufacturing process is virtually only effective in the reduction of enveloped viruses, the amount of non-enveloped viruses in the starting material plasma pool has to be limited (Ph. Eur. 01/2020:1646): For hepatitis A virus, a plasma pool can only be released when the pool sample is non-reactive, a positive control of 100 IU/mL HAV RNA is detected and the internal control is not affected by inhibitors; for HEV, a positive control of 320 IU/mL HEV RNA has to be detected and not more than 10.0 IU/ μ L of B19V DNA (10 IU/ μ L corresponds to 10,000 (4.0 log₁₀) IU/mL) would be detected. The general requirement for release of plasma pools for fractionation with a B19V load not exceeding 10⁴ IU/mL (FDA, 2009b) is applied by most manufacturers of PDMPs; therefore, the Ph. Eur. requirement for human anti-D immunoglobulin with a maximum 10.0 IU/ μ L B19V DNA is met virtually worldwide.

The release of plasma pools with a minimum HCV load as required in Europe is achieved by screening of all donations by a sensitive NAT assay. This is documented already in 1996 for most of plasma pools: 95 plasma pools from 6 manufacturers of PDMPs were tested and only 5 pools resulted in HCV RNA positivity; all these reactive plasma pools were fractionated by one manufacturer (Saldanha and Minor, 1996b). After PPTA (Plasma Protein Therapeutics Association) member companies had implemented voluntarily testing of (source plasma) donations for HIV-1 (later-on also HIV-2), HCV and HBV, PPTA implemented a NAT standard as part of its voluntary standards (PPTA, 2013). The effect of this NAT screening approach was documented in a further study assessing the impact of NAT screening on the potential contamination rate of plasma pools: 873 plasma pools created in 1996 (prior to the European agency's recommendation to implement HCV NAT testing from July 1, 1999 onwards; CPMP, 2001) were tested and 17.8% were HCV RNA positive, 0.8% were HIV-1 RNA positive and 0.5% were HBV DNA positive. The virus load in the positive pools were up to 3*10⁴ IU HCV RNA/mL, up to 6.4*10³ IU HIV-1/mL and the HBV load was below the limit of quantification (54 IU HBV DNA/mL). From 331 plasma pools created in 2006 none were HIV-1 RNA and HBV DNA positive and only in one pool a very low load of HCV RNA (<10 IU HCV RNA/mL) was detected (Nübling et al., 2008). NAT testing of donations and release of plasma pools for fractionation based on non-reactivity of the plasma pool provides confidence that donation testing was performed correctly, and no mix-up of donations occurred.

Table 3 Assumed virus load in plasma pool for fractionation of 2000 L based on epidemiology.

	Recovered plasma			Source plasma		
	7150			2500		
	HIV	HCV	HBV	HIV	HCV	HBV
Prevalence/incidence in donor population (per 100,000)	2.7 ^a	12.8 ^a	24.2 ^a	0.05 ^b	0.18 ^b	0.05 ^b
Number of donations per pool from donors assumed to be infected	0.2	0.9	1.7	0.06	0.22	0.06
Calculated virus load per pool [mL], based on minipool (16) NAT limit of detection ^c	0.11	0.02	0.01	0.36	0.07	0.05

^aIncluding 15% first time tested (FTT) donors in plasma pool (prevalence/incidence rate per 100,000 for FTT donors: 7.9 (HIV), 69.5 (HCV), 134.5 (HBV)).

^bAssuming 85% efficacy of inventory hold, the relevant epidemiology regarding pool contamination rate is 0.3 (HIV), 1.2 (HCV), 0.3 (HBV).

^cConsidering at least one donation from an infected donor below the limit of detection of the respective NAT assay in each pool.

Considering the virus load in a theoretical plasma pool for fractionation, the following parameters are assumed as an example provided in Table 3:

- the epidemiological situation in Germany for the blood-transmissible viruses HIV-1, HCV, and HBV (Offergeld et al., 2010) with prevalence/incidence rates (per 100,000 donors) of first time tested (FTT) donors for recovered plasma of 7.9, 69.5, and 134.2 for HIV-1, HCV and HBV, respectively and for repeat tested (RT) donors of 1.5, 2.4, and 4.1 for HIV-1, HCV and HBV, respectively, as well as for source plasma (RT donors) of 2.1, 7.7, and 2.1 for HIV-1, HCV and HBV, respectively
- a plasma pool prepared from recovered plasma contains 15% plasma from FTT donors; one donation per donor enters a plasma pool
- a plasma pool prepared from source plasma contains 15% of donations stored in Inventory Hold released after 60 days Inventory Hold without a subsequent donation tested assuming as worst case that these donations are just below the limit of detection of the NAT assay; two donations per donor enter a plasma pool
- NAT screening is performed in a minipool testing scheme of 16 donations/minipool and a limit of detection of 50 IU/mL, 10 IU/mL and 5 IU/mL for HIV-1, HCV and HBV, respectively. The contamination of a plasma pool could be expected considering a virus load per mL donation of ≤ 800 IU/mL, ≤ 160 IU/mL, ≤ 80 IU/mL for HIV-1, HCV and HBV, respectively, with 95% probability (compare Table 1)
- size of the theoretical plasma pool for fractionation is 2000 L; the volume of a recovered plasma donation is 280 mL and of a source plasma donation is 800 mL

Table 3 shows that a low virus load in plasma pools for fractionation may be present according to the assumption discussed above; it has to be considered, however, that a worst-case assumption is applied that each and every plasma pool contains at least one donation from an infected donor, which was non-reactive in NAT screening assays (as only a whole donation and not a fraction can be pooled). Single donation, instead of minipool, screening will reduce the virus load further but the number of donations per plasma pool from an infected donor remains the same as the epidemiology remains the same. The probability that such a low virus load will be detected by NAT release assays for plasma pools is remote.

The higher prevalence/incidence rate in recovered plasma donor's epidemiology results in a significant higher number of donations from donors of recovered plasma being infected in a plasma pool for fractionation. The virus load in a pool is, however, comparable for both donor types according to the calculation used in Table 3 as the volume of a recovered plasma donation is substantially smaller than that of a source plasma donation. As in the calculation of the virus load per plasma pool always one donation having entered the plasma pool is considered, but only a fraction of a donation is present in a plasma pool based on the calculation, it can be assumed that in reality a considerably larger number of plasma pools prepared from source plasma contain no donation from an infected donor than a plasma pool prepared from recovered plasma.

It can be concluded from Table 3 that markedly increased epidemiological rates may result in a NAT positive plasma pool for fractionation. Measures as thorough donor selection criteria, highly sensitive NAT assays and, especially, Inventory Hold approaches for source plasma, have to be applied under these conditions. Furthermore, the implementation of Alert/Action Limits to collect plasma only from collection centers with viral marker rates within an acceptable epidemiology in the donor population is essential.

Conclusion

Source plasma or (in most countries) also recovered plasma are combined to large plasma pools for fractionation of commonly several thousand donations for the production of PDMPs. As in a donation, even after testing, a virus contamination may be present, due to the detection limit of screening assays, the plasma pool for fractionation may contain a substantial load of blood-borne viruses despite appropriate testing of donations, in particular when using only antibody testing. Especially for pharmacovigilance purpose, each donation (and, thus, donor) has to be traced to a finished product batch and backwards to support e.g., assessments of adverse drug reactions.

In order to mitigate the virus contamination of a plasma pool for fractionation, a wide range of measures are in place at the collection centers (blood establishments) and by the fractionator/manufacturer of PMDPs

- At the blood establishment (blood/plasma collection facility)
 - a Quality System based on GMP with quality standards appropriate to their intended use covering, e.g., personnel and organization, documentation system, premises and equipment, materials, qualification and validation, storage and transport, record keeping, documentation and review of results, change control, CAPA, complaints, recalls and audits as well as contract between blood establishment and fractionator. Blood/plasma has to be approved for release for further processing by specifications defined, validated, and documented. When an Inventory Hold or Quarantine process is in place, the release of donations for further processing relies on thorough forward and backwards traceability of all donations in order to interdict a donation with a virus load below the limit of detection, tested non-reactive, by a positive screening result from a subsequent donation. This will allow to destroy window period donations to a high degree. Alert and action limits for epidemiological data have to be established.
- Selection of donors
 - the health of donors has to be checked by a medical examination and by answers to a predefined questionnaire; the interdonation interval, according to national regulatory guidance, has to be followed.
 - the risk of transmitting a blood-borne disease, primarily caused by viruses, has to be explained to the donor by appropriate educational material. Answering a pre-defined questionnaire elicits information relevant for the recipient's health as any history of injectable drug abuse, risky sexual behavior and body piercing and/or tattoo and acupuncture treatment by a not registered practitioner. Finally, geographic risk regarding vCJD disease has to be answered as well as treatment in the past with extracts from pituitary glands or receiving dura mater or cornea grafts as well as having a family risk of Creutzfeldt-Jakob disease.
- Screening of donations
 - each and every donation has to be screened by serological assays for HIV-1/-2, HCV, and HBV; in order to close the window period with high virus loads for HIV and, especially HCV, NAT assays are used. These NAT assays detect a low virus concentration in a donation resulting in the destruction of such a positive donation, and previous donations from that donor stored in Inventory Hold. In order to meet the requirements by national authorities and voluntary standards by most manufacturers of PDMPs, further NAT tests are implemented; besides HAV NAT screening, to support the overall margin of HAV safety for a given PDMP, B19V NAT screening of donations is implemented (that the plasma pool not exceeds 10^4 B19V DNA/mL) also to achieve a sufficient margin of safety for this virus, which is resistant to physicochemical treatment, and, finally, donations to manufacture pooled solvent/detergent treated plasma have to be screened also by HEV NAT.
- Release of plasma pools for fractionation
 - in many countries the first homogenous plasma pool, the starting material of PDMPs, is released based on non-reactivity in serological assays for HIV-1/-2, HCV, and HBV. In addition, NAT testing and release of plasma pools for fractionation is a requirement for HCV in Europe (based on Ph. Eur. monograph) and for B19V based on a FDA guideline, and, for selected products as anti-D immunoglobulin, also in a range of other countries besides United States. According to the Ph. Eur. Monograph for pooled solvent/detergent treated plasma, further-on B19V, HAV and HEV NAT of plasma pools has to be implemented not exceeding specifications. On a voluntary basis, most fractionators are releasing all plasma pools for further processing only when also non-reactive in NAT testing besides serology testing.

These measures, when applied rigorously, assure a minute amount of relevant blood borne viruses in the starting material for plasma-derived products and, together with the manufacturing process of these products with build-in effective virus inactivation and/or removal steps, a finished drug product derived from human plasma is manufactured with a high margin of virus safety.

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Advances in Point-of-Care Testing Platforms for Diagnosis of Infectious Diseases

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Introduction

According to the World Health Organization, infectious diseases have been emerging at an alarming rate (WHO, 2015). Since the 1970s, there have been nearly 40 infectious diseases reported, which includes viral pathogens such as the coronaviruses causing Severe Acute Respiratory Syndrome (SARS; including the novel Coronavirus Disease-19 virus), Ebola, Chikungunya, and Influenza A viruses of zoonotic potential (Menon et al., 2020). The increased rate of international travel, rising population, and frequent exposure of humans to wildlife has enhanced the risk of transmission of infectious agents (Hansen and El Wahed, 2020; Menon et al., 2020). The current strategies for the control and management of infectious diseases involves: (1) control of the infection source; (2) cutting off transmission routes; and (3) protection of susceptible people. Of these, controlling the source of infection is the key factor which can be attained by implementing approaches that facilitate early detection, isolation, and treatment of patients. For implementing such strategies, it is vital to develop rapid, sensitive, and accurate pathogen detection methods (Wang et al., 2021).

Over the years, advances in diagnostics has led to the development of laboratory-based tests for pathogen identification such as enzyme-linked immunosorbent assays (ELISA), polymerase chain reaction (PCR) tests and other confirmatory microbiological procedures. Previously, diagnostic clinical microbiology generally involved time-consuming procedures for identifying viral or bacterial pathogens. Such laboratory protocols require prolonged turnaround time (up to 14–21 days) and a higher demand for technical, spatial, and economic resources (Samuel, 2020). With the emergence of novel infectious pathogens burdening the global health epidemiology, it is crucial to have diagnostic tools that provide a quick turnaround of results for identifying pathogens in resource-constrained settings (Fonkwo, 2008; Drain et al., 2014; Zarei, 2018). Moreover, the novel laboratory based diagnostic technologies are out of reach of the majority of the world's population since they are complex, centralized, and require skilled operating personnel. Considering the limitations of classical laboratory tests, affordable and user-friendly commodities developed from the point-of-care perspective are critical tools for diagnosis of medically relevant and zoonotic pathogens (Menon et al., 2020). Point-of-care testing (POCT) platforms provide real-time and accurate diagnosis of patient's health at the site of care and enables clinicians to provide treatment options in a prudent manner (Chen et al., 2016; Kozel and Burnham-Marusich, 2017). This chapter highlights the history, current trends and advancements of point-of-care testing platforms adapted for use in diagnosis of invasive pathogens affecting humans.

History, current trends, and characteristics of POC testing devices

During the early years of the 20th century, Dochez and Avery developed an immunoassay for the detection of pneumococcal polysaccharide from serum and urine samples of patients suffering from lobar pneumonia. In this period, the authors speculated that such antigen detection will aid in the rapid diagnosis of infection (Dochez and Avery, 1917). The advent and actual application of point-of-care testing began in 1962 which led to the development of a rapid testing platform for blood glucose measurement utilizing an enzyme membrane electrode design (Clark and Lyons, 1962). By the last quarter of the 20th century, the US Congress passed the Clinical Laboratory Improvement Amendments (CLIA) act in 1988. In this regulatory framework, point-of-care testing has been available in hospitals although the diagnostic tests have never been performed outside the laboratory (Lewandrowski et al., 2011). This regulation was enacted in response to concerns for quality of laboratory testing and involved guidelines related to POCT, which were overseen at the Federal Government level by the Center for Medicare Services (CMS) (Fraunberger et al., 2018). However, the US Food and Drug Administration (FDA) issues the guidelines on how to interpret the CLIA regulations for categorizing POCT devices as “waived” and “non-waived” based on their complexity (Samuel, 2020). POCT devices that are cleared by the FDA for home use (outside of the hospital) are waived from the CLIA regulations. In general, the “waived” tests are used for outpatient practices and are considered simple and accurate methods whose probability of inaccuracy in results will not harm the patient in any way. However, POCT platforms used in healthcare systems are categorized as non-waived tests and regularly inspected for compliance in personnel qualification, training/competency, quality control systems, patient test result management, proficiency testing and validation. Regulatory compliance of CLIA non-waived tests is overseen by accreditation agencies such as the College of American Pathologists (CAP) and the Joint Commission (JC) (Fraunberger et al., 2018; Samuel, 2020).

POCT in diagnostic microbiology

Early stages of POCT development for clinical microbiology involved the use of rapid microscopy or immunochromatography (card, dipstick or lateral flow assays) (Bissonnette and Bergeron, 2010). However, the recent trends indicate that advancements have been made in POCT device which employ principles of isothermal nucleic acid amplification, and detection systems involving lateral flow, microfluidics, and plasmonic based systems (Nath et al., 2020; Zhao et al., 2020; Sharafeldin and Davis, 2021). Some of the important characteristics of POCT is that: (a) the tests can be performed right next to the patient; (b) the tests can be outside a laboratory setting; (c) there is minimal need for sample preparation (d) it requires special measuring devices that utilize ready to use reagents for a single sample measurement; (e) it does not require technically qualified medical personnel for operating the device; (f) the results will be available quickly and help to provide a rapid diagnosis for follow-up treatment (Luppa et al., 2018). Considering the characteristics of diagnostic POCT, it has a greater applicability in resource-limited settings with limited infrastructure, information systems and human resources (Drain et al., 2014). In order to deploy diagnostic resources in resource-limited settings, the World Health Organization (WHO) has proposed a series of standards called the “ASSURED” criteria (Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment-free, Delivered to those in need) (Bissonnette and Bergeron, 2015). Although several advancements have been made in the development of scalable user-friendly diagnostics, most of these platforms have challenges associated with large-scale manufacturing, data management, regulatory approval and managing test errors generated by the end user. With the issues of potential zoonotic pandemics threatening human wellbeing, global efforts have concerted to the refinement of such technologies as a tool for improved epidemiological control, food safety and general biodefense (Sharafeldin and Davis, 2021). Additionally, the reduced turnaround time for such diagnostics will positively impact the patient and the community, healthcare workers, as well as the socioeconomic aspects of healthcare (Bissonnette and Bergeron, 2015). The recent advancements in POCT has led to the development of novel technologies and the miniaturization of existing technologies with improved assay performance (Samuel, 2020).

Analytes tested in POCT platforms for diagnostic microbiology purposes

Currently available POCT platforms can detect the following analytes for pathogen identification (Kozel and Burnham-Marusich, 2017; Samuel, 2020):

- (a) Antigens: antigenic components of the pathogen are identified using a specific antibody. The antigen-antibody complex thus generated is further detected by a platform that utilizes lateral flow assay or its modified variant. Some of the examples include rapid testing kits for influenza A or group A streptococcal antigen testing.
- (b) Antibodies: Such tests will involve the detection of antibodies directly from blood or serum samples toward specific pathogens, for example, human immunodeficiency virus (HIV).
- (c) Nucleic acid detection: These tests will involve the direct detection of pathogen related genomic DNA or RNA from the patient sample. Most of these tests employ nucleic acid amplification tests such as polymerase chain reaction.
- (d) Host derived proteins as biomarkers: These proteins help to distinguish classes of infecting microbes, i.e., to distinguish bacterial from viral infections. In acute infections, the biomarkers involved may be related to serum acute phase proteins, cytokines or host-specific nucleic acid signatures that differentiate from bacterial and viral infections.

In the following sections, we have discussed major POCT diagnostic platforms that have been utilized for the diagnosis of infectious diseases in humans.

Lateral flow assay based diagnostic systems

For nearly four decades, lateral flow devices have been used for applications such as pregnancy self-testing, foodborne pathogen detection, disease diagnosis, biomarker screening and environmental monitoring (Leuvering et al., 1980; Stragier and Losick, 1996; Džunková et al., 2016; Huang et al., 2020; Jarvis et al., 2020; Lan et al., 2020; Yin et al., 2020; Wang et al., 2021). Infectious disease diagnosis using lateral flow devices has been popular as a point of care diagnostic tool due to their simplicity, ease of operation, rapid turnaround of test results, cost effectiveness with minimal user intervention (Wang et al., 2021). Lateral flow assays follow the principle of an immunochromatographic system which relies on the binding of a microbial antigen in clinical samples to conjugated primary antibodies (with nanoparticles or fluorescent labels). This bound complex is recognized and captured downstream by secondary antibodies leading the appearance of a colored band. The flow through of the unbound and excess primary antibodies moves further downstream to be captured by a tertiary antibody. The banding pattern produced by the tertiary antibody capture typically acts as an interpretable test result or the control line (Drancourt et al., 2016). The basic components of a lateral flow based diagnostic system involve a nitrocellulose membrane, sample pad, conjugate pad, membrane, test line, control line and absorbent pad found in tandem as a test strip (Fig. 1). Each component of the test strip will be pre-impregnated with the respective chemicals and reagents (antibodies and visible signal-generating system) for visual appraisal of the expected confirmatory reaction. Sample fluid gets loaded into and adsorbed into the sample pad. The analytes seep from the sample pad to interact with analyte-specific labeled molecule in the conjugate pad to form antigen-antibody complexes (primary complex). Further on, the conjugated analyte moves and interacts with the immobilized capture molecules (anti-antibodies or the secondary antibody) in the nitrocellulose membrane because of the capillary force provided by the absorbent pad toward the end of the test strip. The unbound primary antibody moves forward to interact with the tertiary antibody to produce a color reaction at the control line. Analytes with the immobilized capture molecules occurs as a result of non-covalent interactions (e.g., electrostatic interaction, hydrophobic interaction and hydrogen bonding) and aid in the production of a signal at the test line that can be detected by the naked eye without the need for specialized equipment (Gubala et al., 2012; Reali et al., 2019; Li et al., 2020). This technology has been adapted for the detection of pathogens such as Ebola virus, Yellow fever virus, Herpes simplex virus, Rotavirus, Adenovirus, *Clostridioides difficile*, *Campylobacter* spp., *Streptococcus pneumoniae*, *Legionella pneumophila*, and *Neisseria gonorrhoeae* (Drancourt et al., 2016).

Apart from the benefits of low cost, quick turnaround of results and the lack for need of instrumentation or a power source, lateral flow assay (LFA) systems have low sensitivity (~60–95%) and the results of test interpretation is operator dependent, which

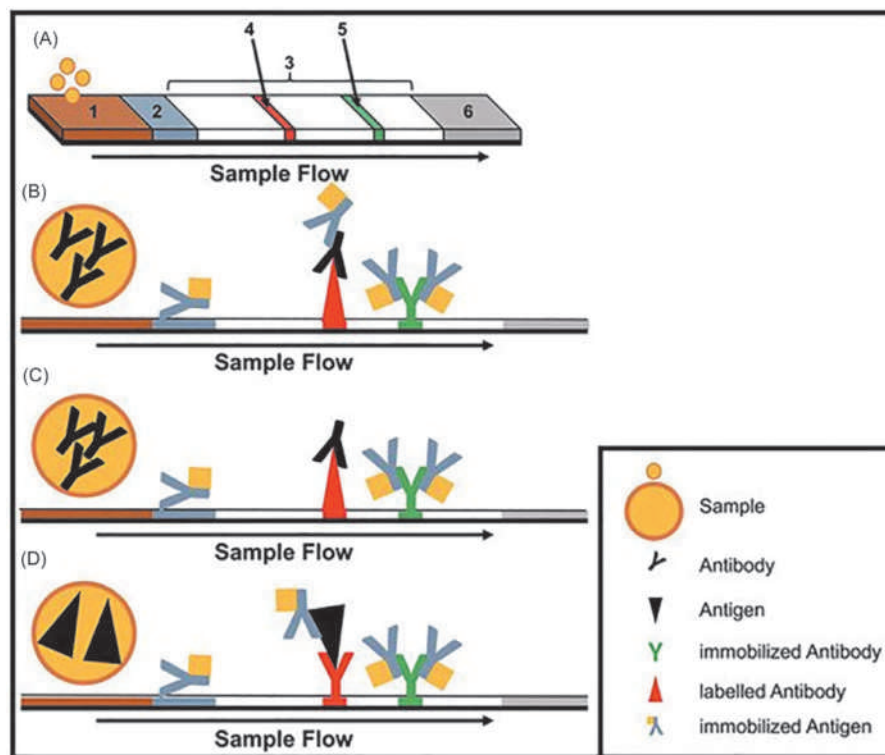


Fig. 1 Pictorial representation of a lateral flow assay system. (A) Schematic design of the LFA system: 1, sample pad; 2, conjugate pad; 3, membrane; 4, test line; 5, control line; 6, absorbent pad. (B) Indirect lateral flow immunoassay. (C) Competitive lateral flow immunoassay. (D) Sandwich lateral flow immunoassay. From Hansen S and El Wahed AA (2020) Point-of-care or point-of-need diagnostic tests: Time to change outbreak investigation and pathogen detection. *Tropical Medicine and Infectious Disease* 151. CC BY 4.0 license: <https://creativecommons.org/licenses/by/4.0/>.

can lead to false-positive or false-negative results (Drancourt et al., 2016). However, novel and advanced immunochromatographic based lateral flow assays have been developed for molecular diagnosis (Realí et al., 2019). During the past decade, there has been significant and promising advancements made in improving the portability, accuracy, and sensitivity utilizing microfluidics and plasmonic based technologies (Chen et al., 2019). More information related to these technologies have been explained in the upcoming sections of this chapter.

Molecular based diagnostic systems

Traditionally, laboratory bench top-based diagnostics for nucleic acid based pathogen identification is accomplished using quantitative polymerase chain reaction (PCR). This procedure requires the need for trained laboratory personnel, costly equipment and consumables. However, over the past two decades, isothermal based nucleic acid amplification has developed as an alternative tool for conventional qPCRs which can be easily performed at a single and constant temperature without the need for a thermocycler (Pumford et al., 2020). In this section, we will be explaining the loop-mediated isothermal amplification and recombinase polymerase amplification which is currently developed for pathogen detection.

Loop-mediated isothermal amplification

Nearly two decades ago, Notomi and coworkers discovered a novel method termed as Loop-mediated Isothermal Amplification (LAMP) that amplified DNA with high specificity, efficiency and rapidity under isothermal conditions (Notomi et al., 2000). The operative simplicity and rapid turnaround for test results pave way for the development of point of care technologies based on the principle of LAMP (Pumford et al., 2020). An amplification rate of 10^9 – 10^{10} -fold of the target DNA sequence occurs at a temperature range of 60–65 °C in 15–60 min (Itou et al., 2014; Chen et al., 2016). The length of the amplicons generated through LAMP ranges from approximately 300 bp to 10 kb whereas the conventional PCR produces target DNA copies of uniform size. A LAMP reaction mixture consists of *Bst* DNA polymerase, deoxynucleoside triphosphates (dNTPs), double-stranded target DNA, and four primers specific to each target sequence. LAMP reaction typically involves a self-primed DNA amplification favored by the strand displacing activity of the *Bst* DNA polymerase (derived from *Bacillus stearothermophilus*). The four primers comprise of two outer and two inner primers. The inner primers are designed in such a way that DNA amplicons generated from them are capable of forming a loop structure. Primers are designed in such a way that it amplifies target sequences that are typically less than 300 bp and sequences above 500 bp are poorly amplified due to the rate-limiting step of the strand displacement DNA synthesis. A LAMP reaction is initiated by one of the inner primers (forward inner primer or FIP, and backward inner primer or BIP). The FIP hybridizes to the target sequence and get extended by the strand displacement activity of the *Bst* polymerase to replace one of the original DNA strands of the double helix (Fig. 2-I). Subsequently, the forward outer primer (F3) hybridizes upstream of the FIP amplified DNA and initiates the displacement and release of the strand containing the FIP. By virtue of the complementary sequence present in the released strand, it favors the formation of the loop structure. A similar process occurs on the opposite end of the target DNA sequence with the backward inner primer and the backward outer primer (BIP and B3). As a result, a target amplicon with self-hybridizing loops on either end is formed resulting in a dumbbell shaped structure which has multiple sites for initiation of the second cyclic stage of the LAMP reaction (Fig. 2-II). Eventually, multiple copies of these dumbbell structures are elongated and amplified exponentially in the third stage of LAMP giving rise to duplex stem-loop DNAs of various lengths, with each single strand containing inverted repeats of the target sequence (Fig. 2-III) (Notomi et al., 2000).

Some of the detection methods employed for identifying LAMP derived products involve colorimetric indicator-based, turbidity-based, and fluorescent-dye based applications. Colorimetric indicators such as hydroxynaphthol blue (HNB) which change the color of the reaction from violet to sky blue as and when the magnesium ions get utilized for the amplification process (Goto et al., 2009). Turbidity-based detection works on the principle of identifying magnesium pyrophosphate, a white salt precipitate derived as a by-product of the LAMP reaction (Tomita et al., 2008). Fluorescent dye capable of intercalating with LAMP products such as SYBR Green 1, EvaGreen, SYTO 81, Calcein and fluorescently labeled oligonucleotide strand displacement probes have been employed for detecting various viral pathogens. Recently, Lamb and coworkers developed a SYBR Green 1 fluorescence-based RT-LAMP assay was developed for detection of SARS-CoV-2 from human samples such as serum, urine, saliva and swabs collected from oro-nasopharyngeal sites (Lamb et al., 2020).

Recombinase polymerase amplification

In 2006, Pipenburg and coworkers developed the recombinase polymerase amplification (RPA) technique which employs recombinant proteins to amplify DNA (Piepenburg et al., 2006). Typically, in an RPA reaction (see Fig. 3), the loading factor protein called T4 UvsY enables the formation of the recombinase-primer complex by promoting T4 UvsX recombinase binding to the oligonucleotide primers. Such complexes screen double stranded DNA for homologous sequences and favor strand dissociation at the target recognition site. Stabilization of the unfolded DNA containing the recombinase-primer complex is achieved with the help of single-stranded DNA binding proteins. The resultant binding of the recombinase-primer complex to the target leads to the dissociation of recombinase and concomitant binding of the strand displacing activity of the large fragment of *Bacillus subtilis* POL I DNA polymerase (devoid of exonuclease activity) to the 3' end of the primer favoring the elongation and cyclic amplification of the target site.

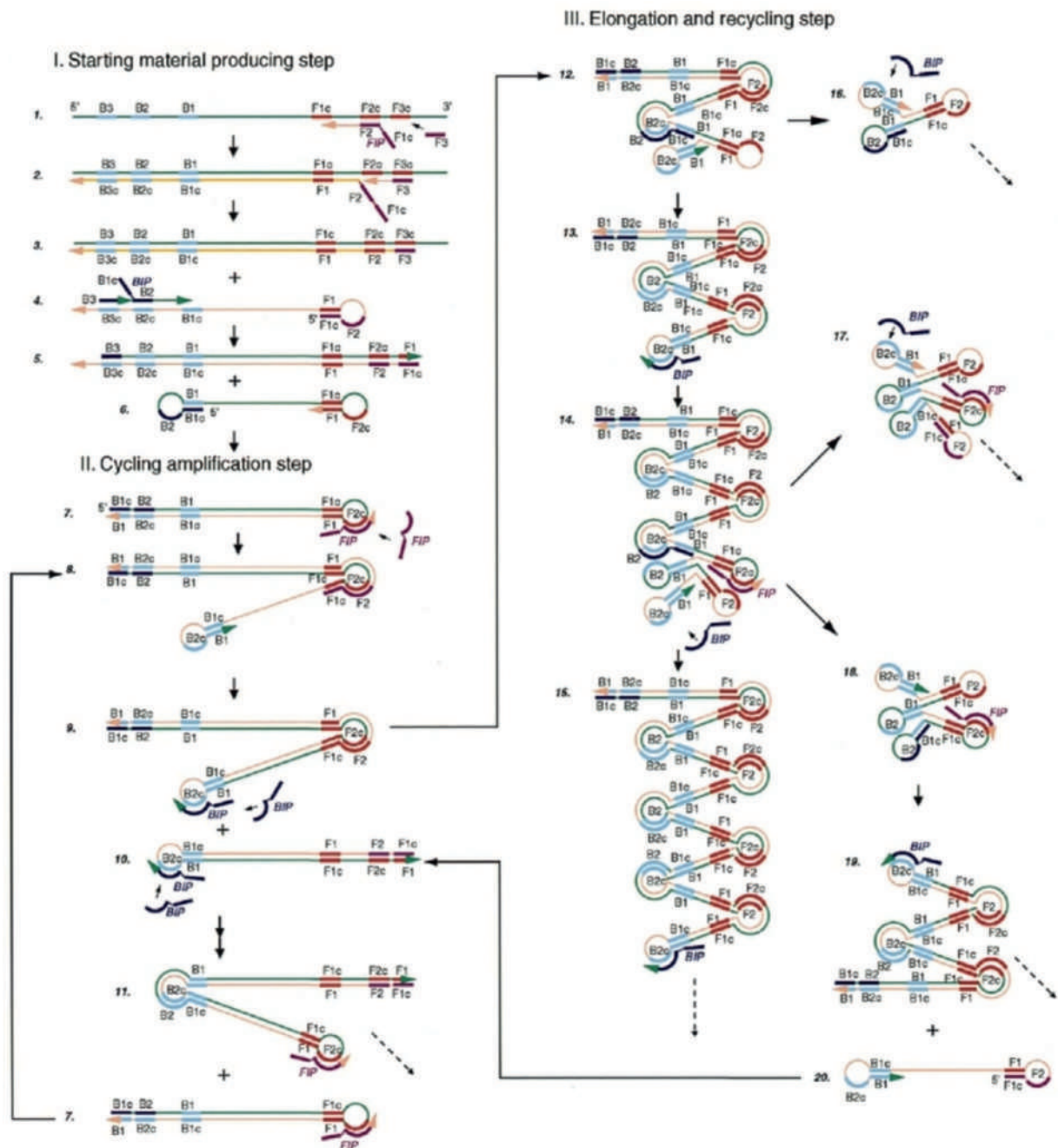


Fig. 2 Pictorial representation for the mechanistic basis for LAMP. (I) Initiation of LAMP results in the generation of a dumbbell structure. (II) Stem-loop structures are generated in the cyclic stage of LAMP. (III) Stem loop structures are elongated; amplification occurs exponentially. From Notomi T, et al. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* 28: 63. CC BY 4.0 license: <https://creativecommons.org/licenses/by/4.0/>.

Lateral flow assay (LFA) based detection of RPA amplicons was subsequently developed in tandem by Piepenburg and coworkers (Piepenburg et al., 2006). In this system, an additional carboxyfluorescein bearing probe and two internal primer pairs specific for the RPA amplicons (one of the primers is a 5' biotinylated primer) is required apart from the two RPA primers. The details of the RPA-LFA system is provided in the figure below (Fig. 4). The fluorescent probe is 42–56 nucleotide long that contains a 5' carboxyfluorescein label (5'FAM), an internal abasic site (tetrahydrofuran), and a 3' block. With the help of recombinase proteins, the probe is exchanged at the target recognition site that harbors the 5' biotinylated tag on the opposing end. Subsequently, cleavage of the abasic site by the double strand specific *E. coli* endonuclease IV (Nfo) and removal of the 3' block (extension blocker) of the probe. The probe is converted to a primer and DNA polymerase (purple) begins extension. As a result, 5'FAM and biotin-labeled amplicons are generated which is then read out in the LFA system at the detection line having α -biotin antibody as shown below.

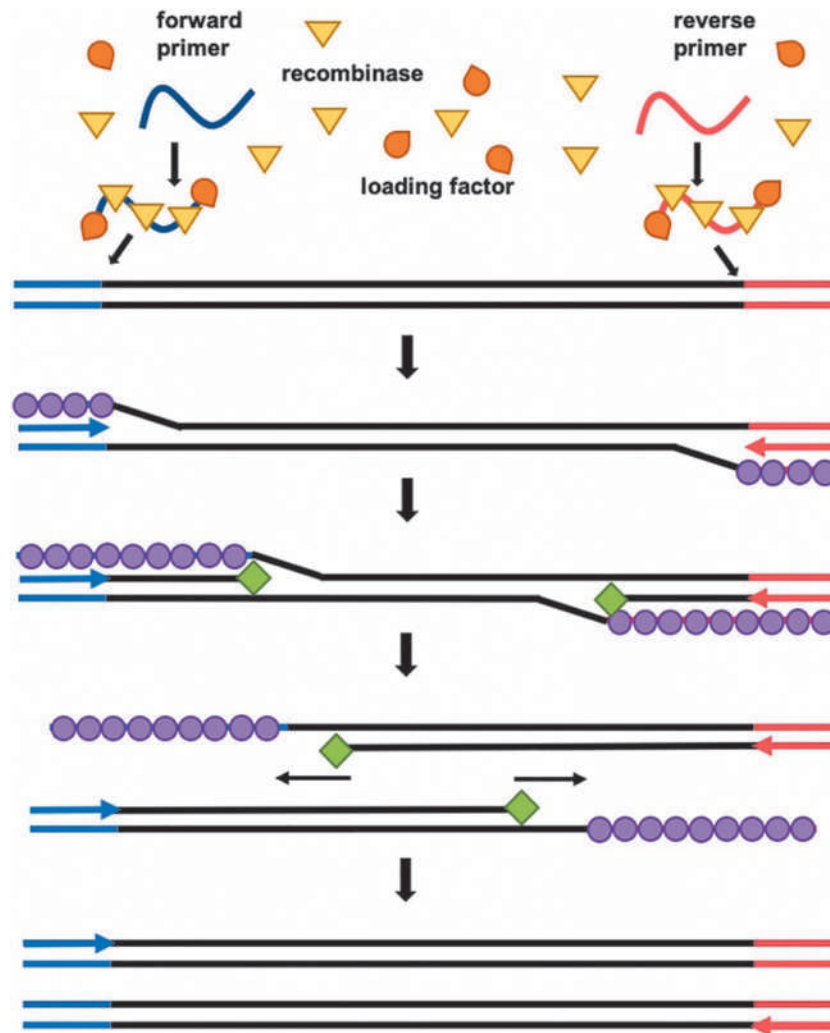


Fig. 3 Pictorial representation for the mechanistic basis for RPA. The reaction is initiated by the binding of the T4 UvsX recombinase (yellow) to the primers with the help of the T4 UvsY loading factor (orange). The recombinase-primer complexes scan dsDNA for homologous sequences, and they invade these sequences with the strand-displacement activity of the recombinase. Single stranded binding proteins (purple) stabilize the displaced strand and the recombinase dissociates, leaving a 3' group available for extension. Strand displacing DNA polymerase (green) binds the 3' end of the primers and extension begins, eventually resulting in two DNA duplexes. Reprinted with permission from Pumford EA, et al. (2020) Developments in integrating nucleic acid isothermal amplification and detection systems for point-of-care diagnostics. *Biosensors and Bioelectronics* 170: 112674., Copyright 2020, Elsevier B.V.

The readers are advised to refer to the publication by Pipenburg and coworkers for obtaining more detailed information related to the RPA based LFA system. The technology is currently commercialized by TwistDx™ in the form of the Milenia HybriDetect 1 and the Milenia HybriDetect 2 and several researchers have utilized this technology for detection of bacterial, rickettsial and protozoal pathogens which is capable of acquiring results with 1 h with a limit of detection as low as 10 DNA copies per reaction (Chao et al., 2015; Nair et al., 2015; Saldarriaga et al., 2016; Lalremruata et al., 2020; Pumford et al., 2020).

CRISPR-Cas based detection systems

Since 2017, CRISPR technology has been applied in molecular based diagnosis with increased sensitivity for detecting zeptomolar (10^{-21} M) concentrations of nucleic acid targets with single nucleotide resolution (van Dongen et al., 2020; Wang and Cui, 2020). In addition, CRISPR-Cas system has been applied as a point-of-care testing platform for detecting nucleic acid targets derived from isothermal nucleic acid amplification reactions such as LAMP or RPA (van Dongen et al., 2020). The CRISPR-Cas effectors are generally classified in two classes: Class 1 and Class 2. CRISPR/Cas class 1 system is composed of a complex of multiple effector proteins that performs a single function, whereas the class 2 systems are characterized by one single effector protein harboring multiple functions with the CRISPR process (Makarova et al., 2020). Some of the class 2 systems include Cas9, Cas12, Cas13 and Cas14. The principle behind the application of CRISPR/Cas as a biosensing tool is based on the collateral cleavage of untargeted

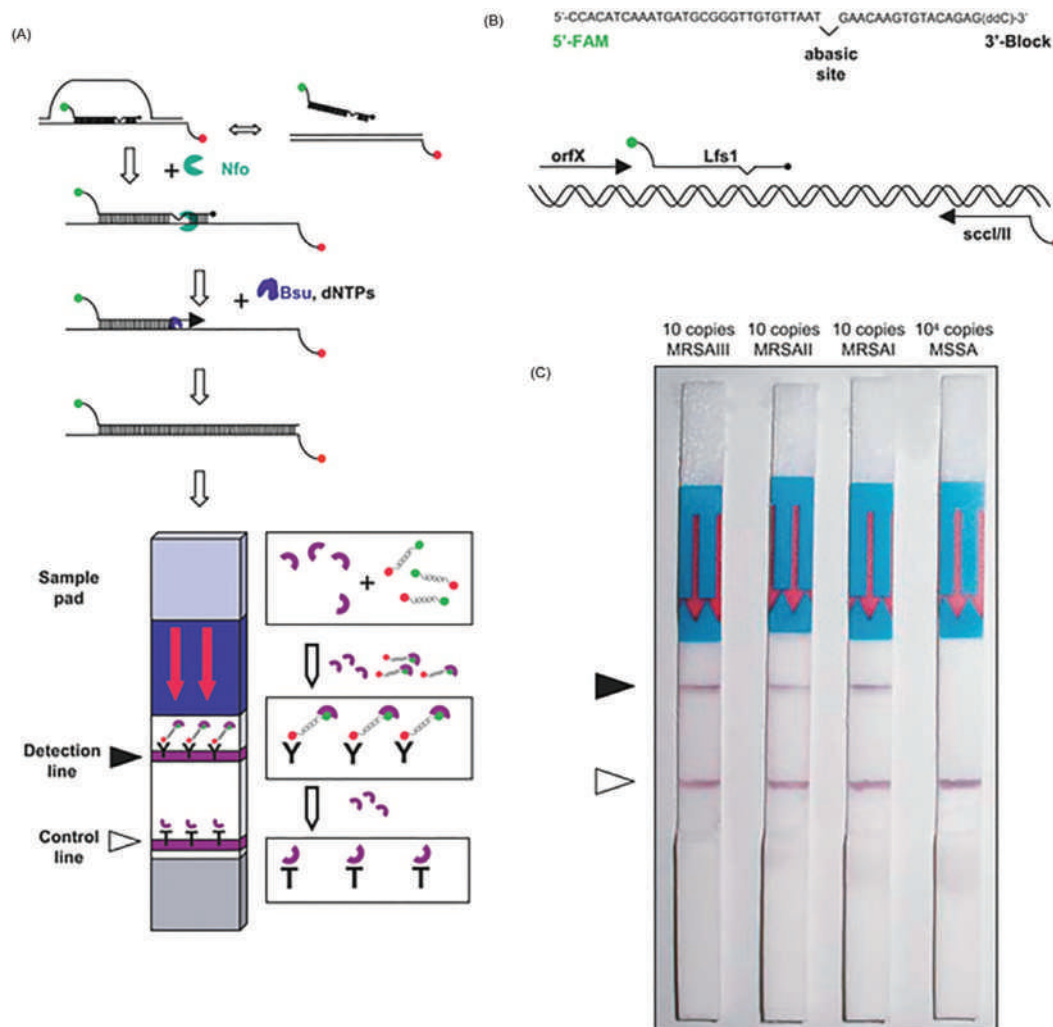


Fig. 4 RPA-LFA based read out system as a point of care platform. (A) Schematic for the generation of 5'-FAM and biotinylated labeled amplicons and the lateral flow strip detection system. FAM label (green), biotin label (red), α -FAM-gold (purple), α -biotin antibody (Y, detection line, filled arrowhead), species-specific α -[α -FAM-gold] antibody (T, control line, open arrowhead). (B) Schematic of the lateral-flow probe design (top, here Lfs1) and its arrangement relative to amplification primers (bottom, here orfX and sccII). FAM label (green), biotin label (red). (C) Lateral-flow strips used for the detection of RPA products (products amplified are specific for three polymorphic alleles of the methicillin resistant *Staphylococcus aureus* [MRSA] *mec* gene; the orfX is shared by all three alleles whereas primers specific for MRSAI/II and MRSAIII target the *mec* variants). Reactions contained (left to right) 10 copies of MRSAIII, 10 copies of MRSAII, 10 copies of MRSAI, or 10,000 copies of MSSA (methicillin sensitive *S. aureus*; negative control) as template. Positive signals are generated in the first three reactions (filled arrowhead). The MSSA control reaction only produced a flow-control signal. From Piepenburg O, et al. (2006) DNA detection using recombination proteins. *PLoS Biology* 4: 1115–1121. CC BY 4.0 license: <https://creativecommons.org/licenses/by/4.0/>.

single stranded RNA or by the specific cleavage or binding of DNA as evinced by class 2 effector proteins. When effector proteins are associated with a CRISPR RNA (crRNA), this complex will detect and cleave complementary target sequences. In addition to this activity, this complex will also favor the cleavage of collateral untargeted single stranded RNA in its vicinity. This property is achieved by multiple Cas family members such as Cas13, Cas12 and Cas14 effectors. Whereas Cas9 effectors are involved in specific cleavage and binding of DNA. Some of the CRISPR-Cas effectors employed for pathogen diagnosis have been outline below.

Cas9 based systems

The RNA guided Cas9 systems can cleave double stranded DNA and this phenomenon has been applied in the detection and genotyping of American and African strains of Zika virus with single-base resolution by Pardee and coworkers (Pardee et al., 2016). The output of the reaction is detected using a toehold switch sensor that can generate a paper-based colorimetric reaction. With regards to the Cas9 based DNA binding without cleavage, it occurs by virtue of the application of nuclease-dead Cas9 (dCas9) system. In this DNA detection system, Zhang and coworkers produced a pair of dCas9 effector proteins linked to split halves of

luciferase protein will only be capable of luciferase heterodimerization when each effector-fusion protein detects two specific regions of the target DNA sequence of *Mycobacterium tuberculosis*. Upon detection of the respective regions of the target DNA by two dCas9-split luciferase effectors will enable the production of a bioluminescent signal without target DNA cleavage (Zhang et al., 2017).

Cas13 based systems

Formerly known as C2c2, this CRISPR-Cas effector system is a single-component enzyme capable of targeting single-stranded RNA molecules with the help of a guide RNA. Upon binding to the target ssRNA will promote its bifunctional nucleic acid cleavage properties that is directed toward cleaving the target RNA as well as collateral ssRNA molecules (Abudayyeh et al., 2016; East-Seletsky et al., 2016). In the SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) platform, binding, and cleavage of the target RNA by Cas13 effector concomitantly facilitates the cleavage of the collateral reporter-quencher ssRNA molecules in reaction mixture thereby enabling fluorescence emission. This collateral fluorescence signal will correspond to the cleavage of the target RNA in the sample. This methodology has been applied for the detection of nucleic acid sequences derived from isothermal amplification processes (e.g., RPA) to detect Dengue virus and Zika virus from patient samples (Myhrvold et al., 2018).

Cas12 and Cas14 based systems

Cas12a or Cpf1 effector proteins harbor a thymine (T) rich PAM (protospacer-adjacent motif) for target cleavage and an RuvC endonuclease domain. This system employs two detection platforms known as DETECTR (DNA endonuclease target CRISPR trans-reporter) and HOLMES (a one-Hour Low-cost Multipurpose highly Efficient System) to target DNA sequences in attomolar levels with high specificity (Wang et al., 2020), and has been used for the diagnosis of DNA and RNA viruses. The target nucleic acid sequence is initially amplified using RPA which is then subjected to binding with Cas12a-sgRNA complex to trigger cleavage of fluorescent-quencher based dsDNA target leading to the generation of a fluorescence signal (Chen et al., 2018; Li et al., 2018). Cas14 (subgroups "a" through "c") effector proteins are capable of targeted ssDNA cleavage without requiring a protospacer-adjacent motif for activation. Additionally, they also possess the domain responsible for non-specific ssDNA cleavage that is similar to the RuvC endonuclease observed in Cas12a effectors. Cas14a-DETECTR platforms have the potential to be used for viral pathogen diagnosis (Wang et al., 2020).

With most of the CRISPR-Cas based technologies having evolved during the span of 4 years, most of its applications are still in its developmental phases. One advantage of this platform is that it can be reconfigured and adapted to detect various pathogen and hence has the potential to be developed as a flexible diagnostic platform for identification of several infectious diseases. Also, utilization of genetically modified Cas effector with robust properties will pave way for the replacement of currently available isothermal based nucleic acid amplification systems (van Dongen et al., 2020).

Microfluidics-based systems

Microfluidics is a technology that processes small amounts of liquid (10^{-9} L or less) for chemical analysis, and diagnostics (Whitesides, 2006). Microfluidics employs microchannels or more extensive Lab-on-a-chip (LOC) for sample processing. Lab-on-a-chip refers to the integration of several laboratory capabilities on a microfluidic chip to facilitate easy, accurate and expedited sample processing (Arora et al., 2010). Microfluidics can also be combined with other detection technologies such as PCR, LAMP or mass spectroscopy for after-chip analyte detection. Some of the features of microfluidics that make the technology well suited for lab-on-a-chip, point-of-care applications are (1) ability to process small sample volumes (2) faster processing time via high throughput parallel analyses (3) low cost and (4) portability (Solanki et al., 2020). Microfluidic devices have been gradually developing over the past 40 years. However, the last 15 years has seen major advancements, especially in point-of-care diagnostic applications (Sachdeva et al., 2021).

Developing a microfluidics system requires several components including test samples, reagents, substrates, and appropriate micro-channel design. The preparation of sample is critical for successful use of microfluidics-based technology. For single components such as DNA, RNA or proteins, an isolation, purification, or concentration step might be needed before the sample could be loaded on the channels (Wu et al., 2014). PCR in combination with pre-enrichment is routinely employed to enrich sample concentration for microfluidics testing (Zhang et al., 2013; Tachibana et al., 2015). For multi-component or complex samples (e.g., soil, blood, tissue) where either multiple analytes or multiple organisms are present, a more extensive purification process is required (Wang et al., 2011). The selection of structure material plays an important role in determining the functionality and application of microfluidics device. Depending on the application, appropriate materials such as silicon, plastics, paper, hydrogels, glass or silicon are selected. These selected materials provide different properties, chemical compatibility, and conductivity to the system (Pandey et al., 2018). A brief description of select microfluidics systems along with their applications is provided below.

Silicon-based microfluidics devices

The use of silicon facilitates flexibility in the structure of the device and tolerance to varying external environmental conditions (Schöning and Lüth, 2001). The methods used for the fabrication of silicon-based microfluidics devices include bulk micromachining, surface micromachining, or buried channel (De Boer et al., 2000; Iliescu et al., 2012). In case of bulk micromachining, channels are created on silicon wafer by cutting of the material followed by enclosing in a secondary wafer and eventually bonding via chemical or physical adherence (Iliescu et al., 2012). Surface micromachining can be accomplished by a relatively complex process involving deposition of a structural layer followed by etching of a secondary layer. Buried channel system is created by starting with a deep reactive ion etching, followed by a passivation step via chemical vapor deposition or SiO₂ (De Boer et al., 2000). Some of the commonly tested analytes include cytokines, peptides, interleukins, and antibodies (Jenison et al., 2001). The aforementioned analytes have been used to detect human cystic fibrosis (Jenison et al., 2001) and *Escherichia coli* in human blood sample (Yakovleva et al., 2002; Das et al., 2012).

Glass-based microfluidics devices

Glass is a non-crystalline solid that is extensively used in microfluidics systems primarily due to its chemical inertness, transparency, and surface stability. The development of a microfluidics device with a glass platform employs MEMS processes including photolithography, film metallization, and glass etching (Pandey et al., 2018). Numerous applications of glass microfluidics have been reported for detection of antibody, enzyme or cell proteins (Grego et al., 2012; Mirasoli et al., 2013), coliform bacteria (Sahdan et al., 2017) and foodborne pathogens (Zhao et al., 2019).

Paper-based microfluidics devices

Paper-based devices use paper or other porous, hydrophilic membranes for microfluidics applications. Unlike traditional microfluidics devices made of silicon or glass, paper-based devices are more economical, easily disposable and can perform multiplexed assays with similar efficacy (Nishat et al., 2021). In addition, paper-based devices can be readily stored, stacked or transported without significant loss in performance of the assay. They employ capillary action for liquid movement in the microchannels and therefore do not normally require pumps to operate fluid movement. Paper-based microfluidics instruments meet the ASSURED (Affordable, Sensitive, Specific, User friendly, Rapid and Robust, Equipment free, Deliverable to end-user) criteria established by the World Health Organization (Hu et al., 2014; Cate et al., 2015). Two of the most commonly used paper-based devices include dipstick assays and lateral flow assays (e.g., home pregnancy test). Most recently, the lateral flow test has been developed for the detection of COVID-19 (Broughton et al., 2020; Choi, 2020).

Several previous studies have investigated the potential of microfluidics for early pathogen detection. Stokes et al. (2001) developed a Cy5 labeled antibody based microfluidic system for detecting *E. coli*. The biochip featured a 4 cm square array of independent operating photodiodes along with amplifiers, and logic circuitry on a single platform with 0.4 mL of reaction chamber. The microfluidics chip was able to detect ~20 *E. coli* strains in simple as well as complex medium (e.g., milk diluent) (Stokes et al., 2001). In another study, Keays et al. (2016) designed a microfluidics platform for characterization of antibiotic resistance patterns in *E. coli*. The susceptible and resistant strains were cultured in microfluidics channels called droplet culture and characterized for various biochemical and resistance parameters (Keays et al., 2016). Similarly, a microfluidics system was developed to detect *Salmonella* using anti-*Salmonella* polyclonal antibodies with a sensitivity of 3 log CFU/mL of sample (Møller-Kristensen et al., 2007). A smartphone-based device has been used in combination with paper-based microfluidics assay for rapid detection of *Salmonella* Typhimurium (Park et al., 2013), and *E. coli* (Dieckhaus et al., 2021). The technique involved pre-coating the microfluidics surface with anti-*Salmonella* or anti-*E. coli* antibody followed by addition of sample, and immunoagglutination of sample-antibody complex. The immunoagglutination was quantified using Mie scatter intensity via a smartphone followed by evaluation on a standard curve. The detection limit was single cell level with a total assay time of less than 1 min. Lee et al. (2009) designed a microfluidic chip for detection of *Mycobacterium tuberculosis* using the *rpoB* gene-based amplification (Lee et al., 2009). Similar microfluidic systems or mChip designs have been developed to detect *Salmonella* in poultry package (Fronczek et al., 2013), *Neisseria gonorrhoeae* (Cho et al., 2015) Hepatitis B (Wang et al., 2012a), HIV (Wang et al., 2012b), HBV (Zhang et al., 2010), or Zika virus (Song et al., 2016).

One of the major challenges faced by point-of-care microfluidics is the difficulty in detecting low concentrations of analytes in sample volume. While there are several techniques to concentrate analytes at larger volume (e.g., liquid-liquid extraction, solid phase extraction, microwave-assisted extraction), concentrating smaller volumes is still in the initial stages of development. Some of the techniques currently being investigated for analyte concentration for microfluidics applications include (1) stacking methods such as amplified sample stacking, isotachopheresis, (2) gradient focusing such as isoelectric focusing, temperature focusing, electric field focusing and (3) trapping mechanisms including electrokinetic trapping, immunocapture, magnetic beads assisted trapping and thermophoretic trapping (Zhao et al., 2017).

Plasmonic technology-based systems

Optical biosensor devices are emerging as powerful platforms for point-of-care detection and disease diagnosis. Optical biosensor devices that are based on refractive index monitoring cover a range of technologies including plasmonics based platforms. Plasmonics is a new branch of optics that derives its foundations in nanotechnology, quantum mechanics, subwavelength optics, and solid-state physics (Tokel et al., 2014). Plasmonics refers to the production, detection, and modification of signals at optical frequencies in the nanometer scale. Optical biosensors, especially those based on plasmonics, have developed in recent times for rapid disease diagnostics at point-of care (Fan et al., 2008). Here we have reviewed latest advancements in surface plasma resonance (SPR), localized surface plasmon resonance (LSPR) and surface enhanced Raman scattering (SERS) including target analytes and major challenges in their development.

Surface plasma resonance (SPR)

Surface plasma resonance is a label-free technique for real-time observation of various bio-molecular interactions between analytes and test compounds. Some of the routinely observed interactions include protein-protein (Kim et al., 2006), protein-DNA (Majka and Speck, 2006), protein-polysaccharide (Beccati et al., 2005), protein-virus (Zhang et al., 2014), enzyme-substrate (Fong et al., 2002). In preparation for detecting various analytes in a sample solution, capturing reagents such as antibodies, enzymes, peptides, or DNA are immobilized on the sensor metal surface of SPR chip. When the solution containing sample (target molecule) is introduced onto the surface, the aforementioned interactions occur that change the refractive index in the space around the surface which is detected by observing a change in the surface plasmon polariton wave. In SPR experiments, resonance units (RU) are used to describe the change in signal, where 1 RU corresponds to a critical angle shift of 10^{-4} degree (Nguyen et al., 2015).

SPR is increasingly being used for drug discovery, pathogen detection and metabolic disease diagnosis at point-of-care (Cooper, 2003; Homola, 2003). SPR based detection has been employed for detecting markers for infectious diseases such as human influenza (Misono and Kumar, 2005), HIV-1 (Tombelli et al., 2005), respiratory viruses (Shi et al., 2015), and metabolic diseases including diabetes (Su et al., 2008), Alzheimer's (Yang et al., 2014), neurodegenerative diseases (Wittenberg et al., 2014), and breast cancer (Jiang et al., 2005). P53 is a tumor suppressor gene which mediates cell cycle arrest and apoptosis. A mutation in p53 gene leads to oncogenic activities and drug resistance. SPR chip coated with p53 monoclonal antibody was used to identify mutant p53 proteins for rapid cancer diagnosis (Wang et al., 2009). In addition, SPR sensor based on oligonucleotide probes and nucleic acid hybridization are also being developed for detecting nucleic acid and genetic diseases (D'Agata and Spoto, 2013; Šípová and Homola, 2013).

Localized surface plasmon resonance (LSPR)

Similar in concept to SPR, the localized surface plasmon resonance is an optical phenomenon produced as a result of interaction between light and a surface, in this case, nanoparticles that are smaller than the incident wavelength (Hammond et al., 2014). This interaction produces unique characteristics including a plasmon propagation distance of $\sim 2\text{--}20\ \mu\text{m}$. While SPR can propagate an electromagnetic wave along a metal dielectric surface, the LSPR creates an amplified electric field for shorter distance. The short plasmon propagation distance provides the term "localized" to the terminology. The resonant frequency of the localized plasmon oscillation heavily depend on the composition, size, dielectric environment and separation distance of nanoparticles (Petryayeva and Krull, 2011). The technology has been used for real-time and point-of-care detection of enzymes, antigens, antibodies, and proteins (Liu et al., 2019). Majority of analytes have dimensions in the range of $2\text{--}20\ \text{nm}$ which is similar to receptor nanoparticles thus making them structurally compatible. This dimensional compatibility of nanoparticle-biomolecules complex facilitates the design of LSPR biosensors and their various applications (Sagle et al., 2011; Hammond et al., 2014). LSPR has been successfully used to identify DNA hybridization (Endo et al., 2005; Kim et al., 2007), Alzheimer's disease amyloid ligands (Stuart et al., 2005), and antigen-antibody interactions to diagnose diabetes (Hiep et al., 2008), or detect toxin peptides in samples (Ha et al., 2008). In addition, LSPR has been employed to detect influenza (Park et al., 2012), Zika (Takemura et al., 2019) and other infectious pathogens (Zopf et al., 2019; Bonyár, 2020).

Surface-enhanced Raman scattering (SERS)

Like SPR and LSPR, surface-enhanced Raman scattering spectroscopy (SERS) provides a non-invasive, label-free, and sensitive detection of a wide range of analytes for point-of-care diagnostics. SERS has its foundation in LSPR and like LSPR, uses molecular vibrations and amplifications of electric fields around a metal surface to facilitate single molecule detection (Granger et al., 2016). Since its discovery in the 1970s, SERS has been employed in various analytical and biological applications (Rohr et al., 1989; Stuart et al., 2005; Graham and Goodacre, 2008). Early work to develop SERS based analytical tools were thwarted by lack of nanomaterial that would support generation of consistent signal (Bell and Sirimuthu, 2006). With nanotechnology advancements that include nanostructure stabilization, polymer coating of surface, and development in spectroscopic instrumentation, researchers have been able to design nanoparticles with size, structure and material that is suitable for SERS applications (Burda et al., 2005). Due to the extensive research in this area, readers are referred to recent reviews (Sharma et al., 2013; Granger et al., 2016), which provide a

much broader and comprehensive discussion of SERS as a powerful diagnostic toolbox. Here, we have focused on key findings in the area of point-of-care diagnostics or rapid analysis of samples. Isola and coworkers designed a plasmonics-based silver nanoparticle assay for detection of HIV gag gene sequence. The assay employed stem-loop DNA tagged with a rhodamine derivate to act as a Raman-active reporter molecule (RRM) or a switch. In the absence of a target DNA, the stem-loop configuration kept the RRM close to silver nanoparticle surface thereby generating a strong SERS response. In the presence of the target molecule, RRM position is shifted, and the SERS signal was not generated (Isola et al., 1998). SERS has also been used to identify respiratory syncytial virus (Dluhy et al., 2008), urinary tract infection bacteria (Premasiri et al., 2017; Tien et al., 2018) and foodborne pathogens such as *Salmonella* (Draz and Lu, 2016; Ma et al., 2018; Xu et al., 2018). SERS has also been successfully used to detect ricin in liquid food. He and coworkers developed a two-step method in which Ricin B chain was captured out of food matrix by aptamer-conjugated silver dendrites and then the spectrum was read on the dendrites to detect small amounts of the toxin in food sample (He et al., 2011). Overall, plasmonic-based systems are becoming increasingly popular and effective in point-of-care diagnostics and various other applications in the biomedical and food industry.

Conclusion and future perspectives

Given the significant role of point-of-care diagnostics in the diagnosis of infectious diseases, it is critical to develop more cost-effective and easy-to-use detection platforms that can be operated by a lay person in resource limited settings. With most of the novel POCT platforms for infectious diseases being cost effective and being in early stages of research and development, transitioning to commercialization of these platforms would greatly benefit the medical diagnostic procedures and reduce the infrastructure related costs for the establishment of diagnostic laboratories. Additionally, future research and advancements should focus on the integration of these novel detection platforms as multiplexed based diagnostic system integrated and powered by artificial intelligence systems that can potentially act as early warning systems to stem potential uprisal of epidemics in a community or rather a possible global pandemic.

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New Technological Developments in Identification and Monitoring of New and Emerging Infections

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Introduction

The year 2020 marked awarding the Nobel Prize in Physiology or Medicine for the discovery of hepatitis C virus (HCV). More than 30 years before, in 1989, Michael Houghton and others published the first HCV genome sequence (Choo et al., 1989; Houghton, 2009). This discovery of HCV was enabled by preceding developments and culminated work of more than a decade since the first description in 1978, by Harvey Alter, of non-A non-B hepatitis as an agent transmissible by blood (Alter et al., 1978). However, after the 1989 first identification of a HCV sequence, it took another 7 years, until 1996, for the viral genome to be fully characterized (Kolykhalov et al., 1997). The many years required from clinical description to full genome characterization of HCV, by the end of the 20th century, stand in stark contrast to the full genome characterization of SARS coronavirus-2 (SARS-CoV-2), the virus responsible for coronavirus infectious disease 2019 (COVID-19), within a few weeks after the novel infection was recognized (Zhou et al., 2020; Zhu et al., 2020). Such a rapid discovery is a result of multiple technological developments, reviewed in this article. Moreover, immediate global sharing of the SARS-CoV-2 full genome sequence enabled the development of diagnostic tests and was essential for the fastest ever development and licensing of vaccines.

Emerging infections can be defined as either new infections, newly diagnosed infections, or infections that have increased in their geographic distribution, or which are increasingly found in vulnerable population groups, such as individuals with immunosuppression or intravenous drug users (Morens et al., 2004; Morse, 1995). Moreover, a combination of epidemiological evidence with microbiological identification has often resulted in the discovery of novel disease associations of known infectious agents

Table 1 Factors contributing to the emergence or re-emergence of pathogens.

<i>Factor</i>	<i>Emerging and re-emerging pathogens</i>
Incursion into natural habitats	Exposure to bat-borne paramyxoviruses such as Nipah virus, phyloviruses such as Ebolavirus and Marburg virus
Global travel	Rapid dissemination of 2009 H1N1 Pandemic (H1N1pdm09 virus); SARS-CoV-2 global pandemic
Host immunosuppression: HIV, organ transplant, immune suppressive therapies for cancer or connective tissue diseases	Opportunistic infections
Climate change	Vector borne disease (e.g., arboviruses)
Live oral polio vaccination to achieve herd immunity	Vaccine-derived polioviruses
Vaccine hesitancy	Measles outbreaks
Exotic animal trade	West African rodents: Monkeypox virus

(Morens et al., 2004). The last 30 years saw the discovery of many new or emerging infectious diseases, or infections that re-emerged or increased in incidence after periods of lower occurrence. The discovery and monitoring of novel and emerging pathogens have been made possible by many new developments in diagnostics, of which some of the most significant will be reviewed in this article.

Several factors are contributing to the emergence and re-emergence of pathogens (Table 1). Population growth has increased human incursions into regions where they are at increased risk of zoonotic infections. Climate change has increased the distribution of arthropod vectors and risk of human exposure to these pathogens. Global travel and trade networks have also resulted in an increasingly connected world population with the rapid dissemination of pathogens once they become established in humans. Changes in population vulnerability also contribute. HIV has resulted in the emergence of multiple opportunistic infections, which have recently declined with wider availability of antiretroviral combination therapy. Aging populations in industrialized countries may be increasingly vulnerable to particular pathogens due to immune senescence. Immune suppressive therapy is used in the context of organ transplantation, chemotherapy for cancer, or treatment for connective tissue diseases. These factors provide vulnerable populations to common and opportunistic pathogens (Morens et al., 2004). In addition, socio-economic development, secular trends, and vaccination behavior may either result in overall changes in the risk of infections or in the age of acquisition of particular infections, which influence whether individuals present with symptomatic or asymptomatic infection (Jacobsen and Koopman, 2005). Changes in the age of infection influence whether infections would occur in women of child-bearing-potential and resultant congenital infection, in case of rubella (Panagiotopoulos et al., 1999). Whereas vaccine campaigns have reduced the burden of many infectious diseases, it may have contributed to lesser awareness of the risks of vaccine preventable disease. Paradoxically the success of vaccination may have become the reason why people are less aware of real risks of acquiring infections and more aware of possible risks of vaccination and susceptible to antivaccine propaganda. This has resulted in the re-emergence of measles in settings where it has been eliminated especially among groups with a low vaccine coverage (Coombes, 2017; Lo and Hotez, 2017). A lack of antibiotic stewardship, which involves the use of broad spectrum antibiotics, while not clinically indicated, has contributed to the emergence of highly and broadly drug-resistant bacteria (Baur et al., 2017). It is therefore clear that the threat of emerging and re-emerging infectious diseases is unlikely to diminish in the near future. Fortunately technological development in the diagnosis and monitoring of infectious diseases may enable future rapid identification and response to these emerging health threats.

Identifying agents and proving an etiological role in disease

Koch's postulates, which require the presence of an infectious agent in cases with disease, and its absence in those without, and the isolation of the agent in pure cultures, were initially very important to identify infectious etiologies. However, it had limitations. Many organisms cannot be readily grown in cultures. Moreover, the association between infectious agent and disease is not always a simple single agent deterministic association but could be more complex—infections could be present without causing disease or may not be the only (or necessary cause) of a disease. In addition to infection, it may require other factors such as host risk factors to cause disease. In the latter case the infectious agent is not a sufficient cause (Byrd and Segre, 2016; Falkow, 2004). Disease complications may also be the result of chronic infections with a long time lag between infection and disease (O'Connor et al., 2006) which makes it more difficult to directly associate the infectious agent and disease.

An adaptation of Koch's postulates was therefore proposed based on molecular criteria that relied on identification of pathogen sequences (Falkow, 1988). However, complex etiologies require epidemiological evidence that shows an increased risk in individuals who have particular infections compared to those without. In the case of organisms that result in pathologies such as neoplasms, or other delayed complications of infections a combination of molecular and epidemiological evidence is often required. Cervical cancer is almost always the result of infection with oncogenic types of human papillomaviruses (Nicolás et al., 2019) and hepatitis C virus and hepatitis B virus infections are strongly associated with liver cancer. However, other infections such as human herpes virus 8 (HHV8) and Epstein bar virus (EBV) require cofactors before oncogenesis is likely (Mesri et al., 2014).

Proving the association of chronic *Helicobacter pylori* stomach infection with duodenal ulcers and stomach cancer has required epidemiological and intervention studies, in addition to identifying the infection in people with disease (Choi et al., 2018; Preda et al., 2009). Recent developments in human genetics, the analysis of mRNA expression (transcriptomics), the study of protein structure, function and interaction (proteomics), and metabolic pathways (metabolomics) have resulted in increasingly rich data, which help to explain the interaction of host genetics, infections, and the environment. In future, novel approaches to analyze such rich data could allow for a better understanding of cellular level pathways of diseases with complex etiologies. This could also provide novel drug targets to treat these diseases (Karczewski and Snyder, 2018).

Traditional approaches to identifying infectious diseases

The identification and diagnosis of infectious diseases have advanced along with technological development and innovations that involve both biochemistry and engineering. Time-tested or traditional diagnostic approaches have their value and limitations, summarized in Table 2 and discussed below. These methods remain valuable in the discovery and characterization of emerging pathogens and are often complementary to more recently developed molecular assays.

Microscopy and culture-based isolation

Since the discovery of microorganisms by light microscopy and the germ hypothesis developed by Pasteur, Koch, and others, culture media have been developed that allowed for the isolation of bacteria (Blevins and Bronze, 2010; Pasteur, 1881). However, viruses, which are responsible for the majority of emerging infections, were initially elusive as they require living cells (either cell culture or live animals) for growth and laboratory isolation. Viruses are also too small to be held back by bacterial filters or to be identified by light microscopy (Lwoff, 1957). The development of cell culture systems greatly aided the identification of viruses from patient samples (Leland and Ginocchio, 2007). Similarly intracellular bacteria, such as mycobacteria, chlamydia, mycoplasmas, and rickettsiae, are difficult to grow and require special media and often long incubation periods or laboratory animals to isolate them (Doern, 2000). Electron microscopy, which has a much higher resolution than light microscopy, allows the characterization of viral structure and, based on the identified morphology, can then direct further investigations to identify the particular species (Curry et al., 2006).

Immune-based diagnostics

Sera from patients with infections or who recovered from infections proved to be very valuable for diagnosing novel agents and the earliest techniques involved immunodiffusion, precipitation, and counterimmunoelectrophoresis reactions that helped to identify the causative agents (Gocke and Howe, 1970; Stanford, 1973). The underlying principle is the hybridization or binding of antibodies from serum to an antigen. The study of antibody and antigen reactions is often called “serology” as antibodies were initially identified from serum samples. The detection of antibodies would signify prior human exposure to an infectious agent, whereas the detection of antigen using antibodies (often produced in laboratory animals or laboratory cultures) would indicate the current presence of the antigen in a sample or culture isolate from a sample. Different technologies were developed to identify antigen-antibody hybridization, which include immunofluorescence, enzyme immunoassays, and chemiluminescence. Although serological methods were some of the first used for diagnosis of infectious agents, they remain relevant for the diagnosis of novel infectious agents especially when used to determine the prevalence of antibodies as evidence of exposure to a particular pathogen. Serological investigations are applied to determine the extent of exposure of the population to new agents and has been used to assess the extent of COVID-19 spread, since many cases may remain asymptomatic or undiagnosed (Larremore et al., 2021; Metcalf et al., 2016). The study of the presence of antibodies among human populations, referred to as seroepidemiology, is discussed in more detail below.

Table 2 Value and limitations of traditional approaches to identify and diagnose infections.

Method	Value in pathogen identification	Limitations
Bacterial growth media (serum broths and other)	Pure cultures of fast growing bacteria	Cannot isolate viruses or fastidious bacteria
Cell culture methods	Isolation of particular viruses	Many viruses do not show cytopathic effect; and others do not grow in available cell culture systems
Polymerase chain reaction (PCR)	Sensitive detection of low concentration agent	Requires some prior knowledge of the sequence to design primers
Serology	Investigate the presence of antibodies in the community (seroprevalence surveys); determine if newly discovered agent had been circulating in the population before	Indirect evidence by detecting antibodies as part of the immune response, which is variable and could wane after infection. Cross reactivity between related agents (e.g., from the same family such as flaviviruses)

Limitations of traditional diagnostic methods

Despite their value, traditional laboratory diagnostic methods have limitations (Table 2). Cell culture-based isolation of viruses and culture of fastidious bacteria are cumbersome and slow and electron microscopy requires a high pathogen concentration and therefore lacks sensitivity (Lipkin and Firth, 2013). Moreover, for many infectious agents, culture isolation systems are not readily available. The identification of these organisms therefore relies on genetic identification, often referred to as molecular diagnostic methods.

The rapid development in molecular methods

Nucleic acid amplification: The polymerase chain reaction and alternatives

The most important breakthrough in the rapid characterization of viruses and fastidious organisms was the development of the polymerase chain reaction (PCR) by Kary Mullis in 1985 (Mullis et al., 1986; Mullis, 1990) for which he was awarded the Nobel prize in 1993. PCR allows the efficient amplification of DNA to detect low concentrations of infectious agents, directly from biological material, without requiring prior isolation in cell culture. An adaptation of this method, first reverse transcribing RNA to DNA, followed by PCR, called reverse transcriptase PCR (RT-PCR), allowed for the identification of RNA viruses.

Alternatives to PCR are isothermal nucleic acid amplification reactions such as nucleic acid sequence-based amplification (NASBA) or transcription-mediated amplification (TMA), which apply similar principles, loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA). The benefit of these isothermal methods is that they may require simpler instrumentation as thermocycling is not required (Gill and Ghaemi, 2008; Li and Macdonald, 2015).

When PCR is followed by nucleotide sequencing it allows for the full characterization of an agent and through phylogenetic analysis a description of its relatedness to other agents. However as PCR amplification requires primers to bind to specific DNA sequences in the template, it requires some prior knowledge of the infectious agent's sequence to allow identification. Therefore, identifying agents that are unexpected or distantly related to known agents offers a challenge and requires adaptation of PCR or alternative approaches for molecular diagnosis (Allander et al., 2005; Reyes and Kim, 1991).

Improvements in biochemistry and engineering, which include automation of extraction and amplification in partially or fully automated closed systems, resulted in nucleic acid amplification technology moving from specialized research laboratories to high-throughput diagnostic laboratories. This had been evident in automated viral load monitoring of HIV and HCV to assess treatment success, and multiplex molecular syndromic diagnosis of respiratory disease, meningitis, and sexually transmitted diseases (Greub et al., 2016). In addition, blood banks make use of molecular methods for blood donor screening to ensure the safety of blood products from donors, who may be in the antibody negative window period (Vermeulen et al., 2009). Recently the importance of high-throughput molecular diagnostics has become most evident, when laboratories have been required to rapidly scale up to test thousands of samples for SARS-CoV-2 by RT-PCR. This was necessary for individual diagnostic and epidemic control purposes (Gorzalski et al., 2020; Opota et al., 2020). In contrast to large high-throughput automated laboratory instrumentation, technological development has also resulted in smaller footprint and easy-to-use, near-patient molecular diagnostic instruments that have become increasingly important in the diagnosis of emerging infectious diseases, at the bedside, or point of care (POC). This has played a major role in the diagnosis of Ebolavirus in field hospital settings and rapid diagnosis of SARS-CoV-2, when required for clinical diagnosis or cohort nursing of infected or uninfected individuals (Raftery et al., 2018; Zhen et al., 2020).

Methods that make use of nucleic acid amplification, primarily with PCR, have overcome some of the limitations of traditional methods. Nevertheless, identification of unknown novel infections from clinical samples poses several challenges for which novel solutions were sought (Table 3).

Dealing with human cellular background

Identifying pathogen genetic material among a much higher concentration of normal human cellular nucleic acid (DNA and RNA) is a challenge. One approach to overcome this is representational difference analysis (RDA) developed in 1993. This approach selectively enriches for DNA sequences that are unique to a particular type of diseased tissue (Lisitsyn et al., 1993). An infectious etiology had long been suspected for Kaposi sarcoma (KS), but it was only when RDA was applied to these tumor tissues that it was

Table 3 Challenges to viral discovery from clinical samples.

Challenge	Impact	Solutions
High concentration of host nucleic acid	Competing host material result in time-consuming process of screening	Pre-analytical <i>Viruses</i> : ultracentrifugation and DNase treatment of samples (viral particles are protected) <i>Subtraction</i> : representational difference analysis (RDA)
Unknown sequence	PCR amplification requires specific primer binding sites	Sequence independent (unbiased) amplification methods; Random PCR; degenerate PCR

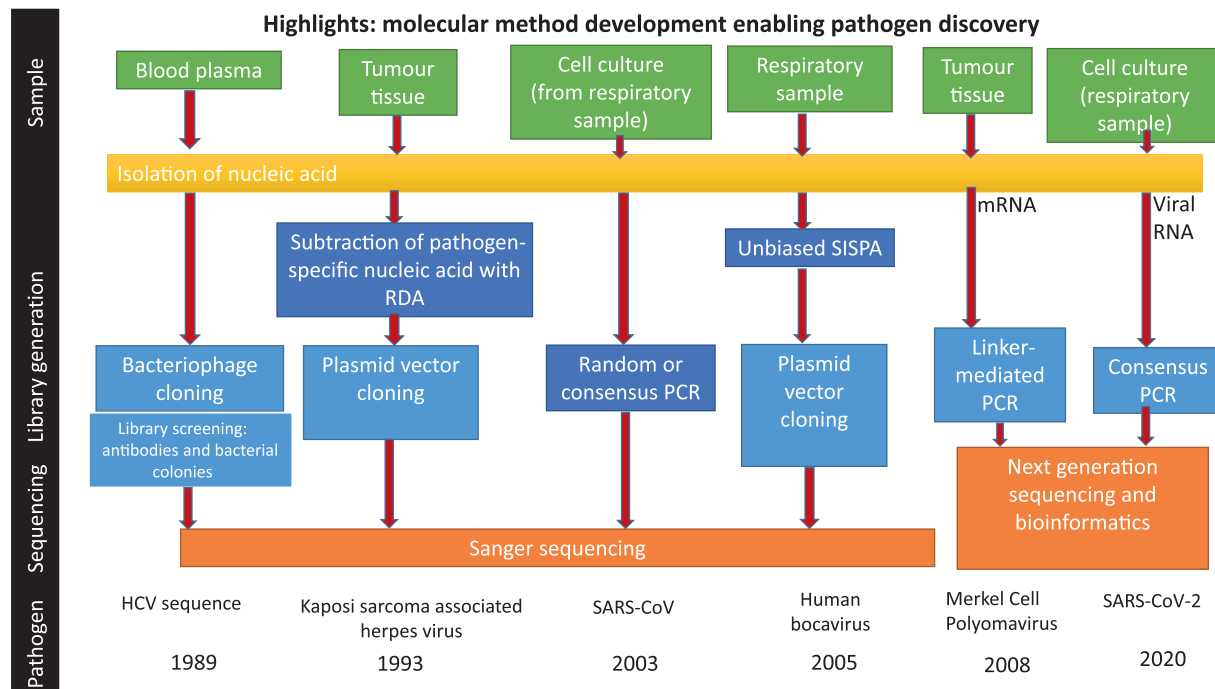


Fig. 1 The first Hepatitis C virus (HCV) sequence was obtained from a bacteriophage library in 1989. Representational difference analysis (RDA) facilitated the discovery of human herpes virus 8, in the presence of a high concentration of cellular DNA. The use of consensus primers (targeting conserved regions) and random primers facilitated the discovery of Severe acute respiratory syndrome (SARS) coronavirus in 2003. Sequence independent single primer amplification (SISPA) was a subsequent method that allowed for the non-specific amplification in samples where cellular background was reduced by DNase treatment. Merkel cell associated polyomavirus was identified by linker-mediated PCR, next generation sequencing of mRNA and digital (bioinformatics based) subtraction transcriptome data of normal tissues from that of tumor tissue. Consensus PCR and next generation sequencing of cell-culture supernatant helped to identify SARS-coronavirus 3 (SAR-CoV-2) the cause of Coronavirus disease 2019 (COVID-19).

found that a previously unidentified human herpes virus, called Human Herpesvirus 8 (HHV-8) or Kaposi Sarcoma-Associated Herpesvirus, was present in all KS tissues (Chang et al., 1994). The secret of RDA is that it uses a modified PCR to selectively amplify gene sequences that are present in diseased tissue but absent or at much lower concentration in healthy tissues. It starts with hybridizing a low concentration of amplified products (tester amplicons) from the diseased tissue, ligated to primers, to an excess concentration (driver amplicon) from normal tissues. DNA strands that are unique to the diseased tissue are then preferentially exponentially amplified (Lisitsyn et al., 1993), which facilitates identification of the disease-specific sequences. Subsequently to identifying HHV-8 as the cause of KS, it was found to be associated with Multicentric Castleman's Disease (Soulier et al., 1995) and Primary Effusion Lymphoma (Cesarman et al., 1995). Other viruses identified using RDA are human GB virus (GBV) (Simons et al., 1995), a virus closely related to HCV, but which appears nonpathogenic, and TT virus (TTV) (Nishizawa et al., 1997), from the family Anelloviridae, which is commonly found in blood and is of uncertain significance. Despite the value of RDA, it is very cumbersome, whereas some recently developed biochemical methods are faster and more practicable (Fig. 1).

Unbiased amplification methods

DNase Sequence-Independent Single Primer Amplification (DNase-SISPA) was used to allow nonspecific amplification of viral RNA or DNA from serum, which enabled the identification of human parvovirus 4 (Jones et al., 2005). In short, filtered plasma samples were treated with DNase to remove human DNA, whereafter viral RNA and DNA were extracted. Thereafter RNA was reverse transcribed and complementary DNA (cDNA) synthesized with random primers, followed by restriction endonuclease digestion of viral DNA or double-stranded DNA, reverse transcribed from RNA, and adaptor ligation to allow nonspecific amplification, cloning, and sequencing. A similar approach: Virus Discovery based on cDNA-AFLP (Amplified Fragment Length Polymorphism) (VIDISCA) was used to discover Coronavirus NL-63 in 2004. This method starts with blood plasma, serum, or culture supernatant. Cellular material and mitochondria are first removed by centrifugation, followed by DNase treatment to digest mitochondrial or cellular DNA present in cell fragments, whereas nucleic acid in viral particles remains protected. Viral nucleic acid is then isolated and viral RNA is reverse transcribed to cDNA, followed by second strand synthesis. Double-stranded DNA (either directly from viral DNA or from reverse transcribed RNA) is digested with restriction enzymes, anchors are ligated, which contain primer binding sequences. This is followed by PCR amplification and finally by nucleotide sequencing providing nucleotide sequences from an unknown agent, without requiring any prior sequence knowledge (Van Der Hoek et al., 2004).

Improvements in sequencing technology

First-generation sequencing

The first widely adapted sequencing method was the Maxam and Gilbert technique which relied on chemical treatment generating breaks in a small proportion of DNA strands, separately for each of the four nucleotides. Each of these separately cleaved products is then separated into individual fragments with polyacrylamide gel electrophoresis (Maxam and Gilbert, 1977). Fred Sanger developed a method, which later largely replaced the Maxam and Gilbert method. This relies on DNA polymerase incorporating a mix of normal and chain terminating nucleotides during the process of primer extension with complimentary DNA synthesis. As the chain terminating nucleotides are incorporated, fragments of differing lengths are generated, each ending on a respective chain terminating nucleotide (Sanger et al., 1977). Initially the terminating nucleotides were radiolabelled, but once this was replaced by four different fluorescent labels (Prober et al., 1987) it opened the way for automated capillary electrophoresis, which is the version of Sanger sequencing that remains in use today. Each capillary has as single readout, called an electropherogram, which shows the respective nucleotides, in color, incorporated at each position. Scaling up automated Sanger sequencing relied on increasing the number of capillaries run in parallel. This unfortunately increases instrument footprint and despite Sanger sequencing's elegance exposed its limited scalability: in essence each sequencing reaction and capillary, in which electrophoresis takes place, aims to provide one consensus sequence read. The multitude of automated sequencers therefore required for the human genome project highlighted the limitations of automated Sanger sequencing. Making sequencing more scalable therefore required an approach that would allow the simultaneous sequencing of multiple different templates in one reaction. Several different solutions to this have been developed and have been referred to next-generation sequencing (NGS) or massive parallel sequencing (MPS) (Thermes, 2014; von Bubnoff, 2008).

The development of next-generation sequencing

These sequencing methods employ different principles, all to achieve the objective of multiple simultaneous sequencing reactions, which are physically separated and separately detected (Table 4). As NGS evolved, particular methods including PacBio Single Molecule, Real-Time (SMRT) Sequencing, and Oxford Nanopore Technologies (ONT) developed approaches that enabled the sequencing of very long reads of single genomes. Hence, these massive parallel sequencing methods became known as third-generation sequencing or single molecule sequencing (SMS) to distinguish them from shorter read NGS methods.

When used for pathogen discovery, in principle two different approaches can be followed. The one relies on specific enrichment for related pathogens, with a PCR or other amplification method that includes primers that bind to sequences that are conserved across species or genera, which is then followed by sequencing to characterize the pathogen. The second is nonspecific, attempts to detect all possible infectious agents, present in a sample, and relies on nonspecific amplification or direct sequencing of extracted nucleic acid. This is followed by the alignment of these diverse genetic sequences to bioinformatics databases. This latter process is referred to as metagenomics.

Table 4 Overview of next and third generation sequencing methods.

<i>Method</i>	<i>Separation</i>	<i>Chemical principle</i>	<i>Detection</i>
454 sequencing	DNA linked to beads separated on picotiter wells	As the primer extends, pyrophosphate (PPi) is released; ATP is generated and luciferase mediates conversion of luciferin to oxyluciferin	Light is released
Ion Torrent	DNA is separated on beads in micromachined wells	As primer extends, hydrogen (protons) are released.	Semiconductor chip detects change in voltage
Illumina	Oligonucleotides on nanowells capture DNA (bind to adaptors)	Bridge amplification generates clusters. Sequencing by extension of reversible terminating dNTPs	Four dNTPs are tagged with different fluorophores; laser excites fluorophores and fluorescent light is recorded by a digital camera.
PacBio Single Molecule Real-Time (SMRT) sequencing	Flowcell with thousands of transparent bottom picotiter wells: Zero-mode wave guides (ZMW)	Polymerase is immobilized on the bottom of the ZMD and adds fluorescent nucleotides by primer extension	Movie of light signals is detected as sequential bases are added by immobilized polymerase
Oxford Nanopore Technologies	Individual DNA molecules pass through nanopores that bridge a synthetic resistive polymer membrane	Nucleotide bases occupy a nanopore, as they pass through, resulting in a current change	Current changes associated with successive series of bases passing through the nanopore are resolved bioinformatically into a nucleotide sequence.

Applications of next-generation sequencing

The development of NGS and data from the human genome project made a new approach to the discovery of novel agents possible: Merkel cell carcinoma (MCC), a rare but aggressive cause of skin cancer that affects immunocompromised or elderly persons, was identified through digital transcriptome subtraction (DTS) (Feng et al., 2008). In short, RNA libraries from next-generation pyrosequencing were aligned with expected transcriptomes (the transcriptomes representing expected mRNA sequences from transcribed human genes from the National Center for Biotechnology Information (NCBI) databases). Of the remaining (subtracted) mRNA transcripts one was found to match a polyomavirus sequence. Further investigations found this Merkel Cell Polyomavirus (MCPyV) to be integrated in the large majority of MCC tissues (Feng et al., 2008).

NGS has offered a new metagenomic approach to diagnostics and pathogen discovery. Nonspecific DNA amplification involving transposase, used as library preparation, followed by NGS with Illumina, which uses solid-phase bridge amplification, allows the direct sequencing of low concentration DNA libraries (Marine et al., 2011). This unbiased approach was used to identify a novel arenavirus in cluster of three patients who died after receiving solid organ transplantation from the same donor (Palacios et al., 2008). It has also allowed for the unbiased diagnosis of infections in returning travelers (Jerome et al., 2019). These metagenomic approaches have been shown to correlate well with routine diagnostic methods such as multiplex PCR (Graf et al., 2016; Huang et al., 2019) but works best if the viral loads are sufficiently high (Huang et al., 2019; Kustin et al., 2019). Sensitivity may be increased by enriching first for viral sequences with probe capture before next-generation library preparation (O'Flaherty et al., 2018; Wylie et al., 2015, 2018).

Metagenomic NGS nevertheless holds several remaining challenges which hamper its widespread adoption. A challenge of NGS is the computational and bioinformatics needs to assemble sequence reads and match that to existing databases to identify known and possible novel pathogens (Dutilh et al., 2017). Metagenomic NGS also yield information, including viral genome sequences that are of unknown clinical significance, and which could be nonpathogenic, such as anelloviruses. Nevertheless, anellovirus viral loads have been used to monitor the degree of immunosuppression in organ transplant recipients (Wylie et al., 2018).

Another potential use of metagenomic NGS is to identify agents in nonhuman hosts or arthropod vectors before they emerge as human pathogens as part of a One Health Approach: Multiple emerging human pathogens may be of bat origin, such as filoviruses Ebolavirus and Marburg virus, paramyxoviruses (Hendra and Nipah viruses), and coronaviruses (SARS-CoV, MERS, and SAR-CoV-2). Therefore a metagenomics study of bat viruses may be able to identify viruses of possible zoonotic origin before these spread amongst humans (Geldenhuys et al., 2018).

It is clear that improvements in nucleic extraction, amplification, and sequencing have facilitated the discovery and monitoring of new pathogens. Fig. 1 summarizes a few highlights in this development process.

Developments in epidemiological evidence

Molecular epidemiology

Molecular epidemiology is the study of the genetic variation of infectious agents in human populations in terms of its occurrence: in space, by person (age, gender, ethnicity, socio-economic factors and health-related) and occurrence in time. Organisms are sampled to represent human populations and the genetic sequence characterized. Sequences are compared using phylogenetic analysis, which describes the genetic relatedness in phylogenetic trees and ancestral association between variants. This has become an increasingly valuable tool to characterize the distribution of emerging and re-emerging organisms and inform interventions to curb their spread (Holmes, 1998). Improvements in sequencing technology that facilitate rapid sequencing and computing power that allows for the fast and accurate construction of phylogenetic trees have made this increasingly practicable. A few of multiple cases where this has informed public health interventions are discussed below. The sequencing of organisms in different populations, studied in time, by geographic location and according to risk factor exposure has enabled the modeling of the origin of outbreaks, based on the phylogenetic relationships of organisms. The study of how epidemiological processes, immune responses, and evolution shape phylogenetic relationships has been called "phylodynamics." When an infection emerges or is reintroduced, phylodynamic analysis can help to establish the likely geographic origin and whether there are one or multiple origins. The overall diversity and "date stamped" sample collection could be used to calibrate molecular clock models, which can be used to estimate the date of emergence of the most recent common ancestor. This has been used to establish when particular infectious agents entered human populations. From such studies it emerged that HIV-1 has entered human populations likely before 1930 in central Africa (Gryseels et al., 2020; Worobey et al., 2008) and that circulating human coronaviruses infections such OC-43 and NL-63 are likely the result of historic species jumps from bats (Huynh et al., 2012; Vijgen et al., 2005).

Seroepidemiology and sero-archaeology

When an organism is newly identified, it is important to establish whether the infection is truly new or just previously undiagnosed. Historic serum banks could help to determine whether an agent is truly new. This helped to determine that human metapneumovirus, an important respiratory infection in young children, although first identified in 2001, had been present in humans for at least 43 years before (Van Den Hoogen et al., 2001). In contrast, at the time when SARS CoV and SARS-CoV-2 were first detected, it was

apparent that there had been no pre-existing population immunity toward these novel agents (Ip et al., 2004; Takahashi et al., 2020). After SARS-CoV-2 had spread widely and had been classified as a pandemic, seroprevalence studies of SARS-CoV-2, when adjusted properly for diagnostic error, proved to be very useful in assessing population immunity, the risk for local epidemic spread, and the role of asymptomatic spread in transmission (Larremore et al., 2021).

Similarly, the strains involved in historic influenza pandemics have been studied by investigating birth cohorts and assessing the influenza strains to which different age groups have antibodies. The most important antibodies toward influenza are directed against surface proteins, hemagglutinin (H) and neuraminidase (N), which are used for influenza A typing. When pandemic influenza A strains share similar antigens to prior strains, the older strains are often displaced by the new pandemic strain. Human exposure to particular strains is assessed by performing hemagglutinin (H) inhibition and neutralizing antibody studies. Using this approach it was discovered that H3 influenza strains circulated before 1891 and were reintroduced during the “Hong Kong” pandemic of 1968 when influenza A H3N2 emerged (Reichert et al., 2012; Simonsen et al., 2004). During seasonal influenza epidemics, mortality is higher at the extremes of age, in infants and elderly individuals. However during some influenza pandemic years, older individuals showed lower excess mortality as they had been protected by prior exposure to previously circulating strains that had antigens in common with the emerging pandemic strain (Reichert et al., 2012).

Case studies: Identification and monitoring of important emerging and re-emerging pathogens

The technological development required for the detection and monitoring of infectious diseases involves genomics and immunology. The development of NGS with increased computer power and more robust bioinformatic algorithms and mathematical models of evolution have had a major role in detecting and monitoring emerging infections. This coupled with faster automated nucleic acid amplification technology and a robust biotechnology industry, which supplies reagents fast and have developed point of care and large high-throughput instruments, have enabled an increasingly rapid response to emerging pathogens. A few examples, where technology played a role in the detection and response to emerging or re-emerging infections, are discussed below, but there are multiple other examples, which cannot be covered in a single article.

SARS and other coronaviruses

Prior to 2002, the known human coronaviruses 229E and OC43 were associated with mild upper respiratory viral disease. The first time when a coronavirus was associated with severe pneumonia in humans was when an atypical pneumonia, later named Severe Acute Respiratory Syndrome (SARS), emerged in Guangdong Province, China in November 2002. This virus, called “severe acute respiratory syndrome coronavirus (SARS-CoV)” was identified using a combination of established and newer methods. Vero cell culture and electron microscopy on the culture supernatant identified viruses that resembled coronaviruses. Following viral cell culture isolation, different groups, respectively, used consensus PCR primers (designed based on an alignment of known coronaviruses) (Drosten et al., 2003) or degenerate/random primers and DNA microarrays (Wang et al., 2003) which enabled them to amplify and characterize the SARS-CoV genome using Sanger sequencing.

The identification of SARS-CoV has led to an increased awareness of the possible role of coronaviruses in respiratory infections. Subsequently the VIDISCA method, described above, was used to identify human coronavirus NL-63 (HCoV-NL-63) from a 7-month-old child with bronchiolitis and conjunctivitis. HCoV-NL-63 was thereafter found to be a common respiratory pathogen especially in children (Bastien et al., 2005; Ebihara et al., 2005; Kaiser et al., 2005). Thereafter another novel coronavirus, human coronavirus HKU1 (HCoV-HKU1), was identified from a nasopharyngeal aspirate of a 71-year-old man with pneumonia by using conserved coronavirus primers and thereafter using a mixture of conserved and degenerate primers to amplify the full genome and identify the sequence by automated Sanger sequencing (Woo et al., 2005). HCoV-HKU1 was later found in children and found to be occurring worldwide (Esper et al., 2006; Lau et al., 2006). In 2012, a novel coronavirus was isolated from a 60-year-old male patient from Saudi Arabia with pneumonia and renal failure. A pan-coronavirus PCR, based on the method used for the discovery of SARS-CoV (Drosten et al., 2003), was used to amplify the viral nucleic acid, followed by next-generation 454 pyrosequencing to identify sequences of this novel coronavirus. This virus was named Middle East Respiratory Syndrome Coronavirus (MERS-CoV) based on the geographical distribution of most cases. Human infections with the virus have a mortality of around 35%. Although the virus likely originated from bats, dromedary camels were found to be the reservoir host, with zoonotic infection of humans and household, and healthcare infections being important means of transmission (Killerby et al., 2020).

The identification and monitoring of SARS-CoV-2 and its impact on medical interventions

SARS-CoV-2, the etiology of COVID19, was identified using a combination of consensus PCR and NGS (Zhou et al., 2020) and separately by cloning and NGS and Oxford Nanopore Sequencing of viral nucleic acid from culture supernatant (Zhu et al., 2020). Phylogenetic analysis revealed that this virus belongs to the same subgenus of Betacoronavirus as SARS-CoV namely Sarbecovirus (Lu et al., 2020; Zhou et al., 2020).

Identification of genetic variants

SARS-CoV-2 NGS and phylogenetic and phylodynamic analysis allowed for the global tracing of SARS-CoV-2 variants and provided insights into its pandemic dissemination. The presence of multiple lineages at particular locations indicated multiple introductions (Alm et al., 2020; Candido et al., 2020; Tegally et al., 2021). Moreover, displacement of lineages indicated that particular variants having the G614 spike protein mutation were likely more infectious than other variants (Korber et al., 2020). Later data from the United Kingdom showed that a variant, B.1.1.7, with multiple distinguishable mutations had about 70% higher transmissibility than historic lineages (Kirby, 2021; Tang et al., 2021). Similarly, other new variants emerged: B.1.351 in South Africa (Makoni, 2021), P.1 in Brazil (Candido et al., 2020), and B.1.617 in India (Thiagarajan, 2021), which displaced previous variants. Sequencing of these variants detected additional spike protein mutations which raised concerns about vaccine escape and which may explain the circulation of some of these variants in regions that had a high level of population immunity prior to the emergence of these variants (Sabino et al., 2021). Subsequently several studies showed a lower ability of vaccine- or natural infection-induced antibodies to neutralize the B.1.351 and P.1 variants, impacting on the effectiveness of vaccination (Fontanet et al., 2021; Kupferschmidt, 2021). Genomic surveillance has thus become important to understand the transmission dynamics of emerging infections and to inform public health interventions such as vaccination rollout. This has already had impact on the selection of SARS-CoV-2 vaccines that provide better protection against emerging variants. Moreover, vaccine constructs can be adapted to protect against vaccine escape variants. Whereas vaccines elicit a response to several epitopes and immune escape is most likely to be partial, antibody-based biologicals may be most susceptible to such escape mutations. These antibodies have been developed for putative treatment or prevention and act by neutralizing viral infection through binding to the viral receptor-binding domain. Therefore, any changes that impact the antibody-binding epitopes could be adversely affected by such mutations and may render these biologicals ineffective against the emerging variants. Nevertheless, a cocktail of different antibodies targeting separate epitopes may retain effectiveness against emerging variants (Baum et al., 2020).

West Nile virus

West Nile virus, from the genus *Flavivirus*, Japanese Encephalitis serocomplex, had been detected previously in Africa, Eurasia, Europe, and Australia, when it first emerged in New York City in 1999. During the New York outbreak, of 59 cases, who were hospitalized, seven died. West Nile virus is a mosquito-borne virus with birds as the primary hosts (Campbell et al., 2002). Since the New York outbreak, it spread across most of North America (Beasley et al., 2003; LaDeau et al., 2007). Genetic analysis revealed that the strain emerging in New York was most closely related to lineage I, previously detected in Israel (Beasley et al., 2003) but it remains unknown whether it reached the United States via an infected bird or mosquito. Nevertheless the rapid spread of West Nile virus in the Americas shows the risk posed by international trade or travel to introduce arthropod-borne viruses (also referred to as arboviruses) into new territories when susceptible vectors are already present in these regions.

Chikungunya

Chikungunya is a mosquito-borne alphavirus which was first isolated from a Tanzanian patient in 1953. It is associated with fever and arthralgia during the acute presentation and in some individuals arthralgia may persist for months to years. In Africa, the natural hosts are nonhuman primates, whereas human infections are thought to sustain outbreaks in densely populated Asian settings, although monkeys have been found to be infected in Malaysia. There are three viral genotypes: West African, East/Central/South African (ECSA), and Asian (Morrison, 2014). Since 2004 ECSA genotypes increased in geographic range and spread to Indian Ocean Islands, such that by the end of 2005 there were more than a million suspected cases in this region. Therefore, there was a concern that the ECSA genotype could spread to other tropical regions such as the Americas. Then in December 2013, the French National Reference Centre for arboviruses reported that Chikungunya had been detected in the Caribbean Island of Saint Martin. Subsequently it spread to many countries in South and Central America with almost half a million cases by July 2014. Surprisingly molecular epidemiological analysis revealed that the Asian genotype, and not the suspected ECSA genotype, had been responsible for transmission in the Americas (Leparc-Goffart et al., 2014; Morrison, 2014).

Zika virus

Zika virus, a flavivirus, transmitted by *Aedes aegypti* mosquitoes was first described in 1947 in Uganda and was associated with mild human infection in Africa and Asia in the 1960s to 1980s. The first signal of increased global spread was in 2007 when a large outbreak occurred on the island of Yap followed by outbreaks in French Polynesia in 2013 to 2014. Thereafter Zika virus was identified in the Americas with a large outbreak occurring in 2015 in northern Brazil (Grubaugh et al., 2018; Kindhauser et al., 2016). The large outbreaks in the Pacific and Americas provided strong epidemiological evidence, based on geography and timing, that Zika virus infections are associated with congenital microcephaly when women are infected during the first trimester (Brady et al., 2019; Vissoci et al., 2018). Further strong support was provided by a prospective case control study, enrolling 91 infant cases with microcephaly and 173 controls without microcephaly. Zika virus infection is diagnosed by PCR, in the acute phase, or specific neutralizing antibodies after recovery, as IgM antibodies could be cross reactive to other flaviviruses (Landry and St George, 2017). Thirty two (35%) of the cases with microcephaly had Zika virus infection, confirmed with RT-PCR or serology, vs none of the

controls (de Araújo et al., 2018). The rapid spread of Zika virus emphasized the risk of introduction of arthropod borne viruses (called arboviruses) into new geographic settings where their vectors are present, but where there is a lack of population immunity, contributing to rapid spread and a high incidence of human infections. Molecular clock analysis, based on the genetic diversity of the Brazil outbreak, showed that Zika virus had likely been introduced as early as 2013, suggesting that early vector control could have limited the catastrophic spread (Grubaugh et al., 2018). Monitoring of arboviral infections in humans and insect vectors is therefore crucial to allow early detection and timely vector control programs.

Ebola and other filovirus re-emergence: Tracing the source

Filoviruses are associated with systemic illness and viral hemorrhagic fever in humans. The first filovirus to be isolated was Marburg virus in 1967 in laboratory workers who processed blood and tissue samples from African green monkeys (Slenczka and Klenk, 2007). In 1976 two related filoviruses, associated with hemorrhagic fever viruses, were isolated: Ebolavirus (EBOV) in the Congo (then Zaire) and Sudan virus in South Sudan (then Sudan). Before 2013, outbreaks of filoviruses were localized and limited in size, with until then, the largest outbreak being in 2000, involving 425 cases with Sudan virus in Gulu, Uganda. However, from 2013 to 2016 an epidemic spanned Guinea, Liberia, and Sierra Leone, with 28,646 EVD cases and 11,323 deaths. The scale of the epidemic and the duration of sustained human transmission were unprecedented (Spengler et al., 2016). During this outbreak and subsequent monitoring of regional outbreaks, molecular epidemiology came to its own. By December 2013 only 29 Ebolavirus and 65 Marburg full length sequences had been published. Thereafter next-generation sequencing allowed the generation of cumulatively more than 800 complete filovirus genome sequences by 2020 (Di Paola et al., 2020). Molecular epidemiological data provided valuable information about the transmission dynamics and origin of outbreaks and allowed appropriate targeted medical interventions. Targeted EBOV sequencing and unbiased metagenomic sequencing helped to identify natural hosts of viruses from the Ebolavirus and Marburg virus genera. Phylogenetic analysis revealed transmission chains and the presence of super-spreading events, suggesting that targeting these events could dramatically reduce transmission. The persistence of EBOV in semen and the almost identical sequences identified in sexual partners helped to establish sexual transmission as a mechanism by which outbreaks can be reseeded after transmission cycles have apparently been interrupted. Persistence in semen and re-emergence, fueled by sexual transmission, provide a plausible explanation for the epidemic resurgence that had been observed during these outbreaks (Crozier, 2016; Di Paola et al., 2020; Thorson et al., 2021).

Tracing HIV: History and future

A syndrome of immune suppression, with an epidemiology suggestive of a transmissible agent, was first described in men who had sex with men (MSM) from Los Angeles, California and also found in hemophiliacs (Gottlieb et al., 1981, 1983). To distinguish this from primary immunodeficiency, this was named Acquired Immunodeficiency Syndrome (AIDS) (Gottlieb et al., 1983). Then in 1983, the causative virus, later named HIV-1, was isolated by Barré-Sinoussi et al. (1983). Unlike in the United States and Europe, spread in Africa was predominantly through heterosexual exposure and mother to child transmission, with a generalized epidemic and a high incidence, first observed in Central and East Africa (Quinn et al., 1986). Moreover, a serum sample from 1959 from Kinshasa tested positive, indicating a much earlier presence in Africa than the first diagnosis of AIDS (Nahmias et al., 1986). However, it was through the use of molecular clocks, calibrated with early blood samples from Central Africa, that it became clear that HIV likely first crossed the species barrier, and started to spread amongst humans, around the early part of the 20th century. This timing coincided with large colonial population disruptions, mining and urbanization in the then Belgian Congo (Gryseels et al., 2020; Worobey et al., 2008). By studying the relatedness of simian immunodeficiency viruses from chimpanzee subspecies, *Pan troglodytes troglodytes*, it became apparent that they were the source of this cross-species transmission (Gao et al., 1999). Evidence of other chimpanzee viruses in hunters from Central Africa further substantiated that the hunting and slaughtering of chimpanzees could have resulted in a cross-species jump (Calattini et al., 2007).

Whereas HIV-1 caused a global pandemic, the region of high HIV-2 prevalence is restricted to West Africa. Molecular clock analysis revealed that both epidemic HIV-2 subtypes, A and B, were likely transmitted from sooty mangabeys to humans in the first half of the 20th century, with exponential epidemic growth that coincided with the Guinea-Bissau independence war from 1963 to 1974 (Lemey et al., 2003).

Apart from the value of molecular epidemiology to study the history of the HIV-1 pandemic, identifying transmission clusters is an important tool in attempts to end HIV epidemics on country level (Han et al., 2020). Identifying transmission clusters and acting on these by providing additional support services form part of the United States strategy that aims to end HIV transmission by 2030 (Nichols and Kissler, 2020).

Re-emergent poliovirus

Polioviruses belong to Enterovirus C species and are associated with a risk of paralysis of about 1 in 200 to 1 in 1000. There are three poliovirus serotypes: 1, 2 and 3. In 1988 the World Health Assembly resolved to eradicate poliomyelitis (Forty-First World Health Assembly, 1988). Two types of vaccines are used to prevent polio: live oral polio vaccine (OPV) and inactivated polio vaccine (IPV). Although IPV provides effective individual protection against paralytic polio, administering OPV types 1, 2, and 3 (OPV-1, 2, and 3) provide better herd immunity and its use has been essential in limiting community spread and hence OPV use is essential for

poliovirus eradication. However, OPV strains pose a risk of reverting to virulence and could be transmitted onward, classified as circulating vaccine-derived polio virus (cVDPV), which can be differentiated by sequencing from wild-type poliovirus. Therefore the end stage of poliovirus eradication relies on the withdrawal of OPV as the source of cVDPV (Yan et al., 2021). In 2015 and 2019, respectively, wild-type poliovirus 2 and 3 were declared eliminated. Since fewer mutations are required for OPV-2 to revert to virulence than other types, cVDPV had mostly been due to OPV-2. Therefore, to curb the threat of cVDPV, bivalent OPV (bOPV), which lacks OPV-2, replaced trivalent OPV (tOPV) in May 2016. This approach was initially successful, as it reduced the source of cVDPV by the end of 2016. However, from 2017 to 2019, cVDPV started to rebound, as herd immunity toward poliovirus-2 declined. Paradoxically, OPV-2 withdrawal resulted in an increase in cVDPV derived from OPV-2 (cVDPV-2), which had continued to circulate in insufficiently immune populations. This has resulted in the reintroduction of OPV-2 in regions with cVDPV-2 to mop up such outbreaks (Alleman et al., 2020; Chard et al., 2020). Subsequently a new OPV-2 vaccine construct that is more genetically stable and less likely to revert to virulence has been investigated in clinical trials (De Coster et al., 2021). This vaccine may in future help to prevent cVDPV-2.

It is evident that molecular epidemiological investigations of poliovirus have been and would remain essential in targeting medical control measures. An effective eradication effort is reliant on the fast and accurate genotyping of poliovirus from patients and environmental sources and construction of transmission networks by geographical location and time. This is essential in establishing whether strains identified are wild-type or vaccine-derived and to trace the geographic sources of transmission chains. In addition, full genome sequencing can provide valuable information about the role of recombination of vaccine polioviruses with other Enterovirus C genotypes and its role in regaining virulence. Improvements in next-generation sequencing methods are likely to continue to contribute to the success of eradication efforts in providing critical knowledge about the molecular epidemiology of polioviruses (Jorgensen et al., 2020).

Tracing the source of food-borne outbreaks

Listeria monocytogenes (*L. monocytogenes*) is a gram-positive bacterium responsible for listeriosis in humans. It has the ability to survive in food production plants, when disinfection is insufficient (Gandhi and Chikindas, 2007). Whole genome sequencing of *L. monocytogenes* provides a more accurate classification of types than prior methods such as pulse field gel electrophoresis (PFGE). Moreover phylogenetic analysis could identify outbreak clusters and provide accurate molecular data to trace the source of an outbreak (Lüth et al., 2018).

Noroviruses are highly infectious, environmentally stable, and antigenically diverse feco-orally transmitted viruses that belong to the family *Caliciviridae* and are some of the most important causes of large gastroenteritis outbreaks associated with food vendors, hospitals, cruise ships, and large events. Molecular epidemiology has been useful in identifying the source and transmission dynamics of norovirus infections. Moreover monitoring global diversity and identifying conserved antigenic domains and antibodies that are cross-protective, between strains, may be useful for vaccine development efforts (Hasing and Pang, 2021).

Ribosomal RNA and metagenomics

16S rRNA PCR and sequencing have been used for bacterial metagenomics in environmental and biological samples and have enabled the diagnosis of novel bacteria (Woo et al., 2008), and increased the detection of known bacteria (Cummings et al., 2020), when not clinically suspected. It may be particularly useful when working with fastidious or slow growing organisms as it could provide a fast and unbiased diagnosis. This approach has also been used to detect unexpected bacterial species in brain abscesses (Al Masalma et al., 2009). However, cost and optimal workflow are current constraints. The faster result generation with ONT third-generation sequencing, compared to other next-generation sequencing methods, may increase the utility of 16S rRNA PCR and sequencing, especially if the costs of sequencing can be further reduced and if sequencing accuracy of these novel methods improves further in the near future (Heikema et al., 2020).

As ticks are important vectors of bacterial and viral disease, metagenomic microbiome analysis of ticks for potential bacterial pathogens is important in identifying the risks for human infection, even before human outbreaks might occur. PCR amplification and sequencing of 16S rRNA have been used to identify multiple bacteria belonging to the families *Rickettsiaceae*, *Enterobacteriaceae*, and *Pseudomonadaceae* (Lambert et al., 2019; Sperling et al., 2017). A similar approach also allows for the metagenomic identification of tick-borne bacteria in blood from patients (Kingry et al., 2020; Rodino et al., 2021).

Pan-fungal primers selected in the internal transcribed spacer (ITS) of ribosomal RNA are useful for fungal metagenomics in environmental and biological materials. Fungal infections of hospitalized patients have often been shown to have an environmental origin and identifying the source requires ecological sampling. Drug-resistant *Candida auris* has been associated with serious blood infections in hospitalized patients (Prestel et al., 2021), but the likely source has not been known. Recently, ITS primers for fungal metagenomics were used to identify *Candida auris* in coastal wetlands of the Andaman islands (Arora et al., 2021). It is not yet known, however, if these wetlands are the main natural reservoir of *Candida auris*.

Monitoring infectious agents for drug resistance

Next-generation sequencing could be applied to viruses, bacteria, or parasites and be especially useful to detect novel mechanisms of drug resistance. NGS genome sequencing (WGS) of *Plasmodium falciparum* was able to identify candidate genes involved in

artemisinin resistance, which is an essential drug in malaria treatment and prevention (Ariey et al., 2014). Despite some workflow challenges, NGS is also gaining traction for HIV drug resistance genotyping, as it is more sensitive for low frequency drug resistance variants (Ji et al., 2020). Although the prevalence of Mycobacterium tuberculosis (MTB) is low, in many industrialized countries, it is a re-emerging pathogen in impoverished and resource-limited settings and a high incidence has been seen in settings with a high HIV prevalence. The use of genome capture methods to concentrate MTB from sputum samples, which contain a lot of host DNA, improves efficiency of WGS of MTB (Brown et al., 2015). Whole genome sequencing of MTB has several important applications: it can be used to identify drug-resistant lineages (Oppong et al., 2019), understand the molecular epidemiology of MTB to enable targeted preventions, and investigate the molecular mechanisms of new drugs, which have been recently licensed for MTB treatment (Ikettleng et al., 2018). Considering the multiple drug resistance mechanisms, WGS will likely become increasingly valuable for the rapid identification of drug resistance against second-line drugs in case of multiple drug-resistant (MDR) and extensively drug-resistant (XDR) MTB strains and to investigate drug resistance to novel MTB drugs (Faksri et al., 2019). The same principles apply to investigating phylogenetic relationships of gram-negative and gram-positive bacteria, associated with hospital associated drug resistant outbreaks, and could help to understand the transmission dynamics for the purpose of targeted interventions that would limit nosocomial spread of highly resistant bacteria (Gröschel et al., 2020; Mahmoud et al., 2020; Quainoo et al., 2017).

False findings: Associations with chronic fatigue, prostate cancer, and XMRV

The high sensitivity of molecular methods such as PCR that is based on amplification of DNA increases the risk of false findings. Investigations that aim to discover novel disease associations of infectious agents should ensure that there are appropriate controls that would detect contamination and that cases and controls are treated the same. A novel agent, Xenotropic MLV-related Virus (XMRV), was identified in 2006 and was soon associated with chronic fatigue syndrome (CFS) (Lombardi et al., 2009) and prostate cancer (Schlaberg et al., 2009). However these findings could not be reproduced by others, even when including the same patients; and there was evidence of contamination with identical MLV sequences (Kearney et al., 2012) likely originating from reagents used prior to PCR (Erlwein et al., 2011; Kearney et al., 2012). Similarly, contamination of cell cultures used in prostate cancer research with murine viruses was the likely source of the artefactual association of XMRV with prostate cancer (Das Gupta et al., 2012; Schlaberg et al., 2014).

Conclusion: Identifying and monitoring emerging infections now and in the future

The development of metagenomics methods that allow for the unbiased discovery of infections in humans, the environment, zoonotic hosts or vectors, has the potential to revolutionize pathogen discovery. Moreover improvements in sequencing technology with next-generation sequencing and more recently long-read third generation sequencing have decreased the time from the first identification to full characterization of genomes of newly discovered infectious agents. This is probably most evident in the rapid full characterization and international sharing of the full SARS-CoV-2 genome a few weeks after its discovery. Without such speed of SARS-CoV-2 characterization the fast development of diagnostic tests and the fastest ever development and approval of vaccines, within a single year, would not have been possible.

It has also become apparent that nonhuman mammal hosts are the source of many emerging infections. Molecular epidemiological studies and molecular clock models have shown that HIV originated from primates and that bats host many viruses of concern that have either historically crossed the species barrier or which in future could result in pandemic spread. It is therefore important that the monitoring for pandemic threats involves a One Health Approach, which combines veterinary and human health and which includes the investigation of arthropod vectors of infectious disease.

Globalization has resulted in a higher level of interconnectedness. Moreover high population density and urbanization have increased the risk of rapid spread of respiratory pathogens. Therefore, an infection that emerges in one continent can rapidly spread globally. Nevertheless, it also offers the opportunity for global collaboration in identifying agents of pandemic potential. Such global collaboration, which involves sharing information and technological developments, would remain essential in the rapid identification and response to current and future emerging infectious diseases and pandemic threats.

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Introduction on Treatment for Infectious Diseases and Immunological Disorders

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Introduction

Infectious diseases are still a leading cause of death and morbidity worldwide. Discovering effective treatments has always been a major concern. Following Alexander Fleming's discovery of penicillin in 1928, antibiotics have evolved significantly in curing infectious diseases (Bennett and Chung, 2001). More than 100 different types of antibiotics have been found to date. Antibiotics act through the inhibition of protein synthesis, DNA disruption, and cell wall biosynthesis. While the development of antimicrobials has significantly decreased infectious disease mortality and morbidity, at least in the developing world, the rise of antimicrobial resistance threatens to undo these gains (Dong et al., 2007; Livermore, 2004; Williams, 2002). The World Health Organization has warned that infectious diseases may become incurable due to elevated levels of multiple drug resistant pathogens. However, the development of new technologies has taken a big step toward new therapies. In this regard, T cell therapies, cytokine therapy, monoclonal antibodies, and immune checkpoint blockade, have opened up a new path for the treatment of infectious diseases as well as immunological disorders that will be reviewed in this chapter.

Inflammatory response: A process toward pathogen elimination and establishing homeostasis

Disease development is not solely the result of uncontrolled microorganism reproduction, but the inflammatory response induced by some infections is frequently the cause of tissue damage and death. Inflammatory responses play an important role in the development of chronic infectious diseases as well as many immune-mediated disorders. Therefore, we will first provide an overview of the inflammation and immunological responses that occur during infection, followed by a description of the therapies that are commonly employed to modulate this process.

Inflammation

Inflammation is the reaction of the body to damaging stimuli like infections, damaged cells, poisonous chemicals, or irradiation, and it works by eliminating injurious stimuli and starting the healing process (Ferrero-Miliani et al., 2007). Although cellular and molecular events during inflammation could provide tissue homeostasis, uncontrolled inflammatory responses contribute to the progression of acute inflammation to chronic inflammation, resulting in the development of several types of chronic inflammatory disorders (Zhou et al., 2016).

Local immunological, vascular, and inflammatory cell responses to infection or damage cause pain, heat, redness, swelling, and loss of tissue function. In addition, vascular permeability, recruitment and accumulation of leukocytes, and the release of inflammatory mediators such as cytokines, chemokines, prostaglandins, leukotrienes, and acute phase proteins (APPs) are all important events that occur throughout the inflammatory phase (Ferrero-Miliani et al., 2007, Takeuchi and Akira, 2010, Chertov et al., 2000).

Initiation of inflammatory responses primarily requires the recognition of detrimental stimuli by components of the immune system. In general, both innate and adaptive immune systems are involved in responding to an antigen. Innate immune cells recognize antigens through pattern-recognition receptors (PRRs) which have specificities for conserved structures expressed by microorganisms (Janeway, 1989; Rezaei et al., 2021). The molecular patterns of the microorganisms which can be identified by the PRRs are referred to as pathogen-associated molecular patterns (PAMPs). In addition to PAMPs, various endogenous molecules known as damage-associated molecular patterns (DAMPs) that are released during tissue damage can also be recognized by PRRs (Brusselle and Bracke, 2014). The PRR families include the NOD-like receptors (NLRs), Toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and C-type lectin receptors (CLRs) (Takeuchi and Akira, 2010).

In the adaptive immune system, specific receptors expressed on T and B lymphocytes (T cell receptor [TCR] and B cell receptor [BCR], respectively) are responsible for recognition of and responding to the microorganism's antigens. However, innate immune responses precede adaptive immunity and are required for lymphocyte activation. Activation of adaptive immunity eventually leads to the development of specific responses against pathogens and results in prolonged immunity by producing memory cells.

Once the stimulus is recognized, intracellular signaling pathways including nuclear factor kappa-B (NF- κ B), mitogen-activated protein kinase (MAPK), and Janus kinase (JAK), signal transducer and activator of transcription (STAT) are activated, leading to the production of inflammatory mediators. Releasing these mediators results in the development of an inflammatory environment that, if left uncontrolled, can lead to tissue damage and even cell death. A variety of inflammatory mediators such as inflammatory cytokines (e.g., interleukin-1 β [IL-1 β], interleukin-6 [IL-6], and tumor necrosis factor- α [TNF- α]), chemokines, eicosanoids, and vasoactive amines can be produced by tissue resident immune cells such as macrophages, dendritic cells, and mast cells, or by infiltrated leukocytes from blood circulation that all ultimately lead to promote response against a certain pathogen. Monocytes, macrophages, and dendritic cells are the important immune cells that release inflammatory cytokines such as IL-1 β , IL-6, TNF- α , colony stimulating factors (CSF), and interferons (IFNs). These molecules increase vascular permeability and enhance leukocyte recruitment to the site of infection or injury. In this regard, inflammatory response leads to the expression of cell-adhesion molecules on the endothelial cells, resulting in the promotion of leukocyte binding and their migration from the blood stream to affected tissue, a mechanism known as "extravasation." Neutrophils are the first cells that are attracted to the site of inflammation or injury. After that, monocytes, natural killer cells (NK), mast cells, and lymphocytes (T and B cells) are recruited (Rezaei et al., 2021).

Cells involved in inflammation

Neutrophils are the most common innate immune cells in the body, and they serve as the body's first line of defense against infection. Neutrophils quickly migrate from the bloodstream to injured tissue in response to infections. CXCL8, as a chemotactic factor, is critical for their recruitment. By phagocytosis and releasing cytotoxic granules, these cells play a crucial role in the initiation of the inflammatory response (Rosales, 2018).

Monocytes can differentiate to macrophages and dendritic cells (DCs). Monocytes and macrophages are the main components of the innate immune system which play several inflammatory and regulatory roles, including phagocytosis, cytokine production, antibody-dependent cell cytotoxicity (ADCC), antigen presentation, T cell proliferation and activation, and tissue repair leading to pathogen elimination and tissue homeostasis. DCs are considered as important cells which can make a linkage between innate and adaptive immune responses. These cells possess important functions during inflammation, such as pathogen uptake by phagocytosis or pinocytosis, antigen presentation and T cell activation, and cytokine production.

Mast cells have main functions in response to bacterial infections and certain parasitic infections. These cells are mainly involved in allergic reactions. Mast cells produce a variety of cytokines, including IL-1, IL-6, TNF, IL-4, IL-5, IL-13, and chemokines MCP-1, MIP-1, RANTES. These molecules promote inflammatory responses leading to pathogen killing, increased phagocytosis, and enhanced adaptive immune responses. In addition, granule exocytosis can result in the pathogenesis of several allergic disorders causing hypersensitivity symptoms (Abraham and Arock, 1998).

NK cells have an important cytotoxic role, especially against virus-infected cells and cancerous cells (Stabile et al., 2017). NK cells can exert their cytotoxic functions on the target cells by releasing cytotoxic granules containing perforin and granzyme (Topham and Hewitt, 2009).

Eosinophils and basophils are other cells involved in innate immune responses. Eosinophils play a critical role in defense against parasitic infections and allergic reactions and also in tissue homeostasis. Upon stimulation by pathogens, these cells can produce several inflammatory cytokines and lipid mediators, including IL-6, IL-2, IL-8, TNF- α , INF- γ , TGF- α/β , GM-CSF, platelet-activating factor (PAF), leukotriene C4 (LTC4), and matrix metalloproteinases (MMPs) (Shamri et al., 2011). Basophils are also involved in allergy responses and hypersensitivity reactions. These cells release granules containing histamine, heparin, serine proteases, leukotrienes, and prostaglandins in response to stimuli (Chirumbolo et al., 2018).

An overview of therapeutic approaches to treat infectious diseases and immunological disorders

Anti-cytokine therapy

Cytokines regulate inflammation and modify the immunological response to infection or inflammation. However, uncontrolled and excessive production of inflammatory cytokines can result in tissue damage and death (Czaja, 2014; Liu et al., 2016a,b). Specific antagonists can target a variety of cytokines involved in inflammation or their receptors. TNF inhibitors are one of the most effective of these therapies. The soluble form of TNF receptor and anti-TNF antibodies are biological TNF inhibitors that by binding to TNF have the potential to neutralize it. These agents have been shown to be effective in treating many autoimmune diseases, including rheumatoid arthritis (RA), psoriasis, and Crohn's disease. Infliximab (Remicade[®]), adalimumab (Humira[®]), golimumab (Simponi[®]), and certolizumab (Cimzia[®]) are important anti-TNF monoclonal antibodies (mAbs) approved for treating autoimmune disorders (Senolt, 2019).

In addition to TNF, the inflammatory cytokine IL-6 plays an important role in the initiation and development of immune responses in autoimmune diseases like RA. Several antibodies have been developed that can bind to IL-6 or its receptor and effectively neutralize it. Tocilizumab (Actemra[®] or RoActemra[®]) is a humanized anti-IL-6 receptor mAb that binds to both soluble and membrane-bound IL-6 receptors. Its effectiveness in the treatment of RA, systemic lupus erythematosus (SLE), systemic juvenile idiopathic arthritis, Castleman's disease, juvenile dermatomyositis (DM), vasculitis, and juvenile scleroderma is currently being investigated (Jung et al., 2018). Sarilumab (Kevzara[®]) is a human IgG1 mAb that targets the IL-6 receptor and has been developed to treat RA (Lamb and Deeks, 2018). Inhibitors of IL-6, such as sirukumab, olokizuman, and clazakumab, are also currently being developed for the treatment of inflammatory diseases (Sadeghalvad and Rezaei, 2021).

IL-1 is another inflammatory cytokine that induces and regulates inflammatory responses. IL-1 inhibitors such as canakinumab and IL-1 receptor blockers such as anakinra have been shown to be effective in treating a variety of inflammatory disorders (Migliorini et al., 2020; Dinarello et al., 2012). Canakinumab (ACZ885, Ilaris[®]), an anti-IL-1 β IgG1 mAb, was approved in 2009 for the treatment of cryopyrin-associated periodic syndrome (CAPS). Following that, this mAb was approved for use in other inflammatory diseases, including familial Mediterranean fever (FMF), TNF receptor associated periodic syndrome (TRAPS), hyperimmunoglobulin D syndrome (HIDS), and mevalonate kinase deficiency (MKD) (Dhimolea, 2010). Anakinra is an IL-1RI antagonist that inhibits IL-1 α and IL-1 β from interacting with IL-1R1, leading to a reduction in inflammatory response and tissue damage (Lamb and Deeks, 2018).

IL-17 is a key cytokine that possesses several functions, including T cell activation and neutrophil recruitment. This cytokine can mediate innate immune responses against pathogens, especially bacterial and fungal infections, and may play a role in the development of inflammatory disorders such as RA and psoriasis. Secukinumab (Cosentyx[®]) is an IgG1 mAb that can bind to IL-17A. This mAb is approved for the treatment of psoriasis and ankylosing spondylitis. Ixekizumab (Taltz[®]) and Brodalumab (Siliq[®] or Kyntheum[®]) also neutralize IL-17 and were developed for the treatment of plaque psoriasis. Targeting another cytokine, IL-23, has also shown promising results for treating plaque psoriasis. This cytokine mediates differentiation and activation of Th17 cells. Interleukin-23 inhibitor antibodies include Tildrakizumab (Ilumya[®]), Guselkumab (Tremfya[®]), and Risankizumab (SKYRIZI[®]) (Abdo and Tye, 2020; Kuwabara et al., 2017).

Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are medications that are commonly used to alleviate pain, decrease inflammation, and reduce high fever. NSAIDs are particularly useful for treating arthritic symptoms such as joint pain, inflammation, and stiffness. These drugs work by inhibiting cyclooxygenase (COX), the enzyme responsible for the production of prostaglandins (PGs). There are two COX isoenzymes that mediate prostanoids (PGs and thromboxane [Tx]) synthesis. COX-1 is a constitutive enzyme that produces PGs and Tx_{A2} which protect the stomach and kidneys from injury and can regulate physiological functions of the gastrointestinal, vascular and renal systems. COX-2 is induced by inflammatory stimuli like cytokines, and it generates PGs that contribute to inflammation-related pain and fever (Vane and Botting, 1998). Aspirin, ibuprofen, and naproxen are some examples of NSAIDs. Coxibs (e.g., Celecoxib, Etoricoxib, Parecoxib, Rofecoxib, Valdecoxib) are also NSAIDs that are used to treat pain and inflammation in several types of acute and chronic diseases. When compared to traditional NSAIDs, these medicines are associated with a lower risk of gastroduodenal ulcers and fewer clinically relevant complications (Gatti and Adami, 2010).

Disease-modifying antirheumatic drugs (DMARDs)

DMARDs are a class of medicines used to treat RA. In addition, other autoimmune disorders such as ankylosing spondylitis, psoriatic arthritis, and SLE are all also treated with some of these medications. DMARDs act to relieve pain and inflammation and maintain joint structure and function. These drugs are frequently used in combination with NSAIDs to reduce joint injury. Methotrexate, sulfasalazine, hydroxychloroquine, and leflunomide are the most frequent traditional DMARDs. The use of azathioprine and other medicines is usually less common. Methotrexate was first used to treat cancer patients as a chemotherapy drug. This drug reduces inflammation and joint damage when taken at much lower dosages for RA and other rheumatic disorders. Methotrexate inhibits dihydrofolate reductase (DHFR), an enzyme involved in tetrahydrofolate production. As a result, the production of DNA, RNA, thymidylates, and proteins is inhibited (Rajagopalan et al., 2002). It has been shown that methotrexate can interfere with the binding of IL-1 β to its receptor (Brody et al., 1993).

The mechanism of action of sulfasalazine is still unclear, but it is likely to reduce inflammation by inhibiting prostaglandins (Deglin et al., 2014; Plosker and Croom, 2005). Sulfasalazine has been effectively used in treating inflammatory bowel diseases, including ulcerative colitis and Crohn's disease, in addition to inflammatory arthritis such as psoriatic arthritis (Plosker and Croom, 2005).

Hydroxychloroquine was first developed as a treatment for malaria, but it was subsequently shown to be effective in alleviating arthritic symptoms. It is also commonly used to treat SLE. The mechanism of action of this drug in the treatment of inflammatory diseases is still unclear. Its antirheumatic effects are thought to be due to their interference with "antigen processing" in macrophages and other antigen-presenting cells (APCs). Importantly, the acidic pH in the cytoplasmic compartments of an APC is vital for digestion of the antigenic proteins and then assembling the peptides into MHC class II molecules. It has been shown that chloroquine and hydroxychloroquine can raise the pH of intracellular vacuoles, disrupting the antigen presentation process (Fox, 1993).

Leflunomide is another immunosuppressive drug that can reduce inflammation through inhibition of a mitochondrial enzyme named dihydroorotate dehydrogenase (DHODH). DHODH mediates de novo synthesis of the pyrimidine ribonucleotide uridine monophosphate (rUMP). It has been proposed that leflunomide can exert its effect by inhibiting the development of activated lymphocytes. Leflunomide has been used to treat patients with RA and psoriatic arthritis (Fox et al., 1999).

Azathioprine is an immunomodulator drug that exerts its effect by inhibiting purine synthesis, resulting in impaired synthesis of nucleic acids RNA and DNA. It can be used in a variety of disorders, such as RA, SLE, Crohn's disease, ulcerative colitis, and granulomatosis with polyangiitis. In addition, it has been used in kidney transplants to prevent rejection (Axelrad et al., 2016; Singer and McCune, 2017; Jordan and D'Cruz, 2016).

Corticosteroids

Glucocorticoids (corticosteroids) suppress a wide spectrum of immunological responses. Glucocorticoids are extremely effective in controlling many of the acute disease symptoms of inflammatory and autoimmune disorders due to their inhibitory actions on numerous kinds of immune cells, such as lymphocytes, dendritic cells, and myeloid cells (Chatham and Kimberly, 2001; Coutinho and Chapman, 2011). They're commonly used to treat a variety of acute and chronic inflammations, including psoriasis, RA, multiple sclerosis, inflammatory bowel disease (IBD), and eczema. Glucocorticoids can also be used in some leukemias and after organ transplantation to prevent organ rejection (van Staa et al., 2000). On the other hand, long-term usage of oral glucocorticoids has been linked to significant adverse effects such as osteoporosis, metabolic illness, and an increased risk of cardiovascular disease. Glucocorticoids prevent vascular permeability and leukocyte infiltration to the sites of inflammation. They inhibit transcription of genes associated with inflammatory responses, such as NF- κ B and AP-1 and down regulate the expression of inflammatory cytokines, chemokines, and cell adhesion molecules (Smoak and Cidlowski, 2004). In infectious diseases, glucocorticoids can be used for alleviating constitutional symptoms and for cardiovascular effects as an anti-inflammatory agent. Short-term steroid treatment can certainly decrease the morbidity in diseases with extremely severe constitutional symptoms, such as typhoid fever, brucellosis, and tuberculosis (Dale and Petersdorf, 1973).

Signal transduction inhibitors

The identification of cell-signaling pathways that are active during inflammation has made significant progress, and numerous targets have emerged, particularly in pathways involving the transcription factor NF- κ B and mitogen-activated protein kinases (MAPKs).

Targeting the NF- κ B signaling pathway

The light-chain gene in B cells was the first gene to be shown to be influenced by the NF- κ B signaling pathway. IL-1 and TNF were subsequently shown to be activated by this signaling pathway (Sen and Baltimore, 1986). Currently, it has been found that a variety of inflammatory mediators can be regulated by NF- κ B including several cytokines, chemokines, COX2, cell adhesion molecules, acute phase proteins, inducible nitric oxide synthase (iNOS) and anti-apoptotic proteins. On the other hand, several receptors, such as the IL-1 receptor (IL-1R), TNF receptor family members, NOD-like receptor (NLR), Toll-like receptors (TLR), and lymphocyte receptors (TCR and BCR) are all important for stimulating the NF- κ B signaling pathway and triggering inflammation process

(O'Neill, 2006). This pathway consists of a number of important mediators, such as the scaffold protein NF- κ B essential modulator (NEMO) and the two kinases inhibitor of NF- κ B (I κ B) kinase 1 (IKK1) and IKK2. It has been shown that NEMO inhibitor named NEMO-binding domain (NBD) peptide can suppress acute inflammation *in vivo*. Therefore, it has been proposed that the NBD peptide has the potential to be utilized as a therapeutic treatment for inflammatory disorders (di Meglio et al., 2005). Several NF- κ B inhibitors have been approved for treating inflammatory responses, including Oxycodone/Ibuprofen (Combunox®), Bortezomib, Pranlukast, Gabexate, Sulindac, Silibinin, and Sulfasalazine (Ivanenkov et al., 2008).

Targeting the JAK/STAT pathway

The Janus kinase/signal transducer of activated transcription (JAK/STAT) pathway is triggered by several cytokines, resulting in enhanced expression of target genes. JAKs are tyrosine kinases that are stimulated by cytokines and interferons and then activate the STAT family of transcription factors. Because of its specific expression in T cells and activation by IL-2, JAK3 has sparked the most therapeutic development interest among the four JAKs. CP-690550 is a JAK3 inhibitor whose efficacy has been shown in the inhibition of graft rejection in a primate model (Changelian et al., 2003).

Targeting T cell signaling pathway

Several immunosuppressant medicines target T lymphocyte signaling and have shown effectiveness in autoimmune disorders. Cyclosporin and FK506 are the main immunosuppressive agents that inhibit calcineurin. Since calcineurin can dephosphorylate and activate an important transcription factor of T cells named nuclear factor of activated T cells (NFAT), its inhibition can impede NFAT activation, resulting in decreased IL-2 production as a vital T cell growth factor. Another calcineurin inhibitor, ISA247 (Isotecnika), has been shown to be effective in psoriasis (phase II trial) (Bissonnette et al., 2006; O'Neill, 2006).

FTY720 or Fingolimod, is an immunomodulating medication that exerts its effect by modulating the sphingosine-1-phosphate receptor (S1PR). This drug has been used effectively to treat patients with multiple sclerosis. Fingolimod causes internalization of the S1PR and therefore prevents infiltration of lymphocytes that results in suppression of the autoimmune reaction (Brinkmann, 2009).

Rapamycin is another immunosuppressive drug that can target a protein in the T cell signaling pathway named mammalian target of rapamycin (mTOR), a member of the PI3K-regulated protein kinases (Young and Nickerson-Nutter, 2005). mTOR inhibition leads to arrest of the cell cycle in G1. Rapamycin has been used to prevent rejection of transplanted organs as well as for treating autoimmune disorders like arthritis. Other mTOR inhibitors include Everolimus and Temsirolimus, which have been shown to be effective in inflammatory disorders (Dancey, 2005).

Targeting toll-like receptor (TLR) signaling pathway

TLRs are a family of innate immune receptors composed of several members, including TLR1 to TLR10 in humans, with the ability to recognize pathogens or damage associated molecular patterns. Several protein kinases are involved downstream of TLRs, especially the IL-1 receptor-associated kinase (IRAK) family, TBK-1, and MyD88. Activation of these downstream pathways results in activation of transcription factors such as NF- κ B and interferon regulatory factor 3 (IRF3) leading to inducing the expression of a variety of inflammatory genes. Thus, TLR signaling pathway suppression has been anticipated to be a useful therapeutic approach for suppressing undesired, disease-associated inflammatory responses. Several types of TLR's antagonists or agonists have been designed to treat inflammatory disorders such as RA, Sepsis, Crohn's disease, Alzheimer, and Parkinson (O'Neill et al., 2009). TLR inhibitors can be categorized as antibodies, microRNAs, nano-inhibitors, oligonucleotides, lipid-A analogs, and small molecule inhibitors (SMIs) (Gao et al., 2017).

Examples of SMIs include TLR4 inhibitor, TAK-242, for treating septic shock, which is in a phase III clinical trial (Matsunaga et al., 2011). Fluvastatin, Simvastatin, and Atrovastatin are other TLR4 inhibitors that have been studied in experimental models (Gao et al., 2017). Chloroquine and hydroxy chloroquine are TLR7/8/9 inhibitors used in RA and SLE (Ruiz-Irastorza et al., 2010). CPG-52364 is another TLR7/8/9 inhibitor in a phase I trial for treating patients with RA and SLE (Lipford et al., 2007).

OPN-305, a humanized IgG4 monoclonal antibody targeting TLR2, has been shown to be successful in inhibiting the production of inflammatory cytokines *in vitro* and in ischemia reperfusion injury animal model (Farrar et al., 2012; Ultaigh et al., 2011). T2.5 is another mAb targeting TLR2 that showed promising results in treating septic shock, cerebrovascular ischemia and cardiac fibrosis in animal studies [Meng et al., 2004]. NI-0101 is a monoclonal antibody against TLR4 that its effectiveness in treating RA is being investigated in a phase I trial (Monnet et al., 2015).

The examples of oligonucleotides include the IRS-954, IMO-3100, and IHN-ODN-24888 (TLR7/9 inhibitors) evaluated in preclinical studies for treating SLE, and IMO-8400 (TLR7/8/9 inhibitor) which is in phase II for treating dermatomyositis and plaque psoriasis. The class of miRNAs includes mir-146a and mir-21 which can target TLR4 and have been evaluated in animal models to treat autoimmune diseases and inflammation, respectively. Eritoran is a lipid A analog targeting TLR4/MD2 that has been evaluated in phase III for treating sepsis, and in animal models for treating influenza infection (Gao et al., 2017).

Cytokine therapies

Susceptibility to infectious diseases might indicate an underlying immune system deficiency. The characterization of the functional effect of a certain defect can lead to new therapeutic approaches for preventing or treating infections. Primary immunodeficiencies (PIDs) are a group of disorders characterized by genetic defects in a component of the immune system resulting in impaired

immune responses. Therefore, patients with PIDs are more susceptible to recurrent infectious diseases. The treatment using cytokines has been used widely to treat specific infections in these patients. In this section, we will review the important cytokines that have been used to treat infections in patients with PIDs.

Granulocyte-colony stimulating factor (G-CSF) has been developed as the first line therapy for patients with severe congenital neutropenia (SCN). G-CSF reduced the frequency and severity of infections (Newburger and Dale, 2013). In addition to SCN, G-CSF has been used for treating infections in Chronic Granulomatous Disease (CGD) (Kimura et al., 1990; Nakajima et al., 1992). Granulocyte-monocyte-colony stimulating factor (GM-CSF) was used to treat pulmonary alveolar proteinosis (Venkateshiah et al., 2006). A patient with recessive caspase recruitment domain family, member 9 (CARD9) deficiency who presented with spontaneous CNS candidiasis was effectively treated with GM-CSF (Gavino et al., 2014). GM-CSF has also shown promising results in treating infections with *S. aureus* in patients with CGD (Martino et al., 2000).

IL-2 is another cytokine that plays an important role in stimulating T cell subsets, NK cells, and macrophages. This cytokine has been used in a variety of PIDs to treat infections. IL-2 therapy has been effectively used to treat sphenoid sinusitis and cutaneous mycosis in patients with common variable immunodeficiency (CVID) (Cunningham-Rundles et al., 1992). In addition, the effects of IL-2 in the treatment of infections (like chronic pulmonary disease, relapsing generalized cutaneous zoster, and cryptococcal meningitis) have also been reported in patients with idiopathic CD4⁺ lymphocytopenia (ICL) (Warnatz et al., 2000; Cunningham-Rundles et al., 1999; Trojan et al., 2009; Sternfeld et al., 2010; Yilmaz-Demirdag et al., 2008).

The effectiveness of IL-7 has been shown in a patient with progressive multifocal leukoencephalopathy (PML). Neurologic status and clearance of JC virus was shown after approximately 4 weeks (Patel et al., 2010).

Interferon Type I is a family of cytokines involved in antiviral immune responses, consisting of IFN- α , IFN- β and other interferons, including IFN- ϵ , IFN- κ , IFN- η and IFN- ω . The treatment of epidermodyplasia verruciformis was one of the first and most common applications of IFN- α in PIDs. Epidermodyplasia verruciformis (EV) is a genodermatosis characterized by a high sensitivity to cutaneous infection caused by the b-HPV virus family, which is otherwise nonpathogenic in humans. Several studies have shown that IFN- α can effectively treat EV (Blanchet-Bardon et al., 1981; Lutzner et al., 1984; Androphy et al., 1984; Anadolu et al., 2001; Gubinelli et al., 2003; Baskan et al., 2006; Silva et al., 2006).

In addition, CVID and ICL were reported to be treated using IFN- α (Vinh, 2014). Using IFN- β has been reported in a patient with X-linked agammaglobulinemia (XLA) who developed chronic enteroviral meningoencephalitis; clinical improvement was shown after using IFN- β (von der Wense et al., 1998).

IFN- γ , a member of type II interferon, has been approved to treat active infections (such as brain fungal abscess, salmonellosis, hepatic abscess) in CGD and malignant osteopetrosis (Ma et al., 2003; Saulsbury, 2001). Disseminated salmonellosis has also been treated in patients with AR IL-12p40 deficiency (Altare et al., 1998). In addition, the management of mycobacterial infections related to IL-12/IFN- γ axis deficiencies has provided the most reported experience with IFN- γ for the treatment of active infections in PIDs (Filipe-Santos et al., 2006).

Antibody therapies

Monoclonal antibodies

In response to antigens, differentiated B cells known as plasma cells secrete antibodies or immunoglobulins. Antibodies are popular molecules with high efficiency in a variety of therapeutic and diagnostic applications due to their great specificity and diversity. In this regard, since the first mAb was authorized in 1986, using mAbs has become a unique approach to targeting antigens in a wide range of diseases. The first mAb authorized by the Food and Drug Administration was Orthoclone OKT3[®] (muromonab-CD3) (FDA) to treat acute transplant rejection (Liu, 2014). mAbs are now the most common type of therapeutic molecule in clinical studies for inflammatory and autoimmune disorders (e.g., RA, SLE, IBD, multiple sclerosis, psoriasis), malignancies (e.g., breast cancer, multiple myeloma, melanoma, leukemia) and infectious diseases (Lu et al., 2020). Therapeutic antibodies against inflammatory cytokines have previously been discussed in the section of anti-cytokine therapy. We are now discussing other monoclonal antibodies used in diseases related to the immune system.

The CD20 antigen is a phosphoprotein found on B lymphocytes that promotes B lymphocyte proliferation and activation by triggering an intracellular signaling cascade. mAbs against CD20 induce B cell death and may impair B cell activity via antibody-dependent cell mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Rituximab (Rituxan[®]), a chimeric monoclonal antibody (mAb) that targets the CD20 antigen, was originally authorized for the treatment of lymphomas. In addition, encouraging outcomes have been seen in the treatment of various autoimmune disorders, including RA, SLE, Wegener's granulomatosis, dermatomyositis, refractory immune thrombocytopenic purpura, and autoimmune hemolytic anemia (Sadeghalvad and Rezaei, 2021). Another humanized anti-CD20 antibody, Ocrelizumab (Ocrevus[®]), has been approved for the treatment of the primary progressive form of multiple sclerosis. Ofatumumab is also another anti-CD20 mAb that has shown successful results in patients with multiple sclerosis and RA.

Adhesion molecules have a critical role in leukocyte recruitment, activation, and localization. Therefore, targeting these molecules is thought to be an effective treatment for autoimmune disorders and excessive inflammation (Harjunpää et al., 2019). The first mAb authorized for MS therapy was Natalizumab (Tysabri[®]), a humanized mAb against the cell adhesion molecule $\alpha 4$ -integrin. Natalizumab inhibits leukocyte migration to the central nervous system by preventing the interaction of $\alpha 4$ -integrin with

VCAM-1 expressed on endothelial cells. Crohn's disease is also treated with Natalizumab. Alemtuzumab (Lemtrada®) is a humanized mAb that targets CD52 (also known as COMPATH1), which is expressed on dendritic cells, monocytes, and lymphocytes. Alemtuzumab was authorized for the treatment of multiple sclerosis, chronic lymphocytic leukemia, and organ transplant immunomodulation (Voge and Alvarez, 2019).

Graft-versus-Host Disease (GVHD) is a death-causing complication of bone marrow transplantation. GVHD develops when alloreactive donor T cells engage with major histocompatibility (MHC) molecules in the host, activating the immune system and causing increased cytokine release (Ramachandran et al., 2019). Using molecules that can target T cells may be beneficial in preventing GVHD. In this regard, Anti-CD25 (IL-2 receptor) mAb has been shown to be effective in suppressing T cells (Ji et al., 2005). Daclizumab (Zynbryta®) is another mAb which targets the IL-2 receptor and its use has been linked to a lower incidence of acute renal transplant rejection (Carswell et al., 2001).

Therapeutic mAbs have also been used in patients with asthma. Immunoglobulin E (IgE) levels in the blood play an essential role in the development of allergic asthma, which causes bronchial hyperresponsiveness. Therefore, using mAbs targeting IgE or its receptor can be considered as an effective therapeutic agent in these patients. Omalizumab (Xolair®), a humanized monoclonal antibody, prevents IgE from attaching to its receptor (FcR1) and has been shown to be effective in individuals with severe asthma (Loureiro et al., 2018). Dupilumab (Dupixent®), a monoclonal antibody targeting the IL-4R receptor, has been authorized for the treatment of moderate to severe asthma (Thibodeaux et al., 2019). Other cytokines that can be targeted in asthma include IL-5, IL-13, and IL-9. Benralizumab, Mepolizumab, and Reslizumab are approved mAbs for treating eosinophilic asthma (Khorasanizadeh et al., 2016). IL-13 can be neutralized by Tralokinumab and Lebrikizumab monoclonal antibodies. MEDI-528 is an anti-IL-9 monoclonal antibody that can suppress mast cell function in the pathogenesis of asthma (Oh et al., 2013).

Intravenous immunoglobulin G (IVIg)

IVIg is a therapeutic compound prepared from plasma collected from several donors containing opsonic and neutralizing IgG (mainly IgG1 and IgG2). However, in addition to IgG, other isotypes of immunoglobulins, importantly IgA may be found in IVIg. IVIg has been used to treat a variety of primary and secondary immunodeficiencies for more than two decades. IVIg is currently a common treatment option for most antibody deficits. In patients with severe combined immunodeficiency, X-linked agammaglobulinemia (XLA), X-linked hyper-IgM, common variable immunodeficiency (CVID), Wiskott-Aldrich syndrome, and selective IgG class deficiency, IVIg is routinely administered. IVIg has been utilized in a variety of autoimmune diseases (e.g., autoimmune neutropenia, autoimmune hemolytic anemia, polymyositis, SLE, Crohn's disease) and inflammatory disorders since the preliminary work by Paul Imbach, who found a favorable effect in idiopathic thrombocytopenic purpura (Bayry et al., 2003). On the other hand, using IVIg has been shown to be effective in several bacterial and viral infections, including respiratory infections (e.g., *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae*), diphtheria, pertussis, tetanus, *Clostridium botulinum*, *Clostridium difficile*, newborn sepsis, hepatitis A/B/C, HIV, varicella zoster, and measles (Katragkou et al., 2018). IVIg exerts its immunomodulatory effects by several mechanism, including ADCC, inhibiting Fc receptors on effector cells, inhibition of lymphocyte proliferation, induction of inhibitory FcγRIIB receptors, and neutralizing microbial toxins (Bayry et al., 2003).

Convalescent plasma (CP) therapy

A growing body of research shows that treatment using plasma containing antibodies from a recovered patient is beneficial for a variety of viral respiratory diseases. This therapy is thought to work primarily by neutralizing specific pathogens (Garraud et al., 2016). Antibodies that bind to pathogens prevent the pathogen from entering host cells and improve pathogen clearance through mechanisms including phagocytosis, ADCC, and CDC. On the other hand, other active factors present in CP, such as anti-inflammatory cytokines, natural antibodies, and pentraxin, can also play an effective role in defense against pathogens (Garraud et al., 2016). An improved survival rate has been shown in patients with SARS-CoV related pneumonia and Influenza A (H1N1) who were treated with CP. It has been shown that CP therapy can be beneficial for newly infected COVID-19 patients. Improvement of immune responses and viremia suppression were shown in these patients after treating with CP (Mansourabadi et al., 2020).

Immune checkpoint inhibitors (ICIs)

Different subtypes of T lymphocytes, including cytotoxic T lymphocytes (CD8+ CTLs), helper T cells (CD4+ Th1, Th2, Th17) play crucial roles in maintaining immunity against pathogens like bacteria, viruses, parasites, and fungi, as well as in immunity to cancers. On the other hand, if these responses are not regulated, they can cause acute or chronic inflammation resulting in tissue damage or trigger autoimmune reactions. To this end, the immune system has cells and mediators that modulate and regulate immune responses. These include regulatory T cells (Treg), M2-type macrophages, regulatory cytokines (such as IL-10 and TGF-β), myeloid-derived suppressor cells (MDSCs), immune checkpoint molecules including cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T-cell immunoglobulin-3 (TIM-3), programmed cell death protein 1 (PD-1), T-cell immunoglobulin and ITIM domain (TIGIT), and lymphocyte activation gene-3 (LAG-3). The elevated levels of these molecules are found in chronic inflammation and cancer as a mechanism for suppressing the immune system. Therefore, targeting regulatory molecules can be considered as an effective therapeutic approach in the treatment of these disorders (Pauken and Wherry, 2015; Butt and Mills, 2014).

ICIs in cancer

Several types of ICIs have been approved for use in humans with cancer. ICIs are mainly monoclonal antibodies that target checkpoint molecules and inhibit their interaction with relevant ligands. So, therapy using ICIs can lead to improving tumor-specific immune responses (Ai and Curran, 2015).

Ipilimumab, a mAb targeting CTLA4, was approved for use in patients with metastatic melanoma, which results in increasing overall survival of patients (Hodi et al., 2010). Using anti-PD-1 and anti-PD-L1 (programmed cell death protein 1 ligand) monoclonal antibodies showed remarkable results in patients with melanoma and non-small cell lung cancer (NSCLC). Pembrolizumab, a mAb targeting the PD-1 molecule, has been approved for the treatment of melanoma (Robert et al., 2014; Magistrelli et al., 1999). Another PD-1 inhibitor, nivolumab, has been approved for use in patients with melanoma, renal cancer, and NSCLC (Callahan et al., 2016). Atezolizumab is a mAb targeting PD-L1 and has been approved for the treatment of bladder cancer. Anti-PD-1 therapy has also demonstrated a considerable effect in the treatment of head and neck, breast, ovarian, and gastric malignancies (Callahan et al., 2016). Moreover, several clinical trials are ongoing to evaluate the effectiveness of mAbs against other immune checkpoint molecules such as LAG-3, TIGIT, and TIM-3, especially in melanoma, breast cancer, renal cancer, and other metastatic solid tumors.

ICIs in infectious diseases

Pathogens can take advantage of immunological checkpoints to suppress host-protective antigen-specific immune responses. T-cell exhaustion is more common in chronic infections than in acute infections, which are generally quickly eliminated by the immune system. Acute lymphocytic choriomeningitis virus (LCMV) infections result in strong activation of pathogen-specific CD8⁺ T lymphocytes. However, continuous viral antigen exposure during chronic LCMV infection causes T-cell reprogramming, leading to elevated PD-1 expression and T-cell exhaustion (Barber et al., 2006). Despite the fact that numerous molecular mechanisms have been involved in the development of T-cell exhaustion, continuous antigen exposure appears to be the primary cause of this event (Schietinger et al., 2016). Targeting immune checkpoints has been shown to be effective in improving T cell function in a variety of chronic infections, such as HIV, hepatitis B, and hepatitis C. Elevated levels of PD-1 has been shown on HIV-specific and Hepatitis B (HBV)-specific CD8⁺ T lymphocytes which are associated with disease progression (Day et al., 2006; Peng et al., 2008). In addition, ex-vivo inhibition of PD-L1 has resulted in increasing IFN- γ production by HIV-specific CD8⁺ T cells, leading to enhanced T cell responses (Day et al., 2006; Petrovas et al., 2006). Increased levels of TIGIT have also been found on HIV-specific T cells and on T cells of SIV-infected macaques, correlating with PD-1 expression. Therefore, targeting both TIGIT and PD-1 may remarkably restore T cell functions (Chew et al., 2016). It has also been indicated that the increased expression of PD-1 on HBV-specific CD8⁺ T cells is associated with viral load and there is evidence supporting the effectiveness of targeting PD-1 or TIM-3 in HBV infection (Peng et al., 2008). Ex vivo experiments using PD-1 blocking led to the production of IFN- γ by CD4⁺ T cells (Tang et al., 2016). In accordance with this, ex vivo inhibition of PD-1 in hepatitis C-specific T cells resulted in restoring T cell function (Salem and El-Badawy, 2015).

In addition to viral infections, immunosuppressive cells, especially Tregs, have also been shown to defend against several helminth parasites, including *F. hepatica*, *Schistosoma mansoni*, and *Heligmosomoides polygyrus* (Finlay et al., 2014). Infection with *Leishmania donovani* in mice and *L. Mexicana* in humans leads to increased expression of PD-1 and T cell exhaustion (Joshi et al., 2009; Hernández-Ruiz et al., 2010). *S. japonicum* has been shown to induce the expression of PD-L2 on dendritic cells, resulting in suppressed immune responses (Gao et al., 2013). Targeting CTLA-4 in mice infected with *Leishmania chagasi*, *plasmodium berghei* ANKA, and *L. donovani* and PD-1/PD-L1 blocking in *Toxoplasma gondii* infection, *L. donovani*, and *plasmodium falciparum* were also shown to be effective in restoring T cell responses. Moreover, targeting immune checkpoint molecules could be a promising approach for bacterial infections, including *H. pylori* and *mycobacterium tuberculosis* (Dyck and Mills, 2017).

T cell immunotherapies

T cells as the main arm of the adaptive immune system play a vital role in defense against pathogens, especially intracellular infections, specific antigen recognition, eliminating virus-infected and cancerous cells, and immune system modulation (Steinman, 2012). Today, the use of cellular therapies for the treatment of cancer, autoimmune diseases, and infectious diseases has received much attention (Parida et al., 2015).

Most immunotherapies that have been clinically approved for infectious diseases or immunological disorders can be divided into two main groups:

- Modulating T cell responses through immunomodulating agents: therapeutic cytokines, immune checkpoint inhibitors, bispecific antibodies (BsAbs)
- Cellular therapies: regulatory T cells (Tregs), tumor-infiltrating T cells (TILs), Chimeric antigen receptor T cells (CAR-T cells), CD8⁺ cytotoxic lymphocytes or NK cells

The use of cytokines has previously been discussed in the context of infectious diseases or immunodeficiency. Using IL-2 is one example of using cytokines as an immunomodulatory agent, especially in cancer therapy. IL-2 is produced by activated T cells and is considered as a main growth factor for T cells and NK cells, playing an important role in proliferation, activation, and cell survival. Treatment with low doses of IL-2 results in greater differentiation of Tregs compared to activated T cells and can therefore be effective

in the treatment of autoimmune diseases such as multiple sclerosis, SLE, as well as GVHD (Klatzmann and Abbas, 2015). In contrast, high doses of IL-2 develop and activate CD4⁺ and CD8⁺ T effector cells and NK cells and have been approved for treating metastatic renal cell carcinoma and metastatic melanoma (Fisher et al., 2000; Rosenberg et al., 1998). However, due to the risks of vascular leak syndrome and hypotension, which may be caused by active IL-2 receptors on endothelia, the use of high doses of IL-2 has been restricted.

Bispecific antibodies (BsAbs)

Bispecific antibodies (BsAbs) have two binding sites with a distinct antigen specificity that can bind to the immune cell and the target antigen expressed on the tumor cell at the same time. BsAbs exert their effects by creating a cytolytic synapse between T cell and target cell that facilitates activation of T cells and subsequently lysis of the target cell (Offner et al., 2006). BsAbs have been proposed for decades as a therapy for cancer and inflammatory diseases, but they have only lately begun to achieve success (Fan et al., 2015). Bi-specific T cell engagers (BiTEs), dual-affinity re-targeting antibodies (DARTs), and Tandem Diabodies are all types of BsAbs. Catumaxomab (Removab) was the first bispecific trifunctional medication to be authorized for the treatment of malignant ascites (Linke et al., 2010; Sedykh et al., 2018). Following that, the BiTE blinatumomab received FDA approval for the treatment of relapsed and/or refractory (R/R) acute lymphoblastic leukemia (ALL) (Przepiorka et al., 2015). Studies are currently focusing on the effectiveness of BiTEs for solid tumor malignancies. Because of the effectiveness of bsAbs in cancer immunotherapy, comparable approaches for eliminating pathogen-infected cells have been developed. DARTs have recently been used to induce T cell-mediated cytotoxicity of latently HIV-infected cells. These bsAbs are composed of anti-Env and anti-CD3 arms that bind to HIV-infected cells as well as CD3-expressing polyclonal T cells at the same time. As a result, HIV-infected CD4⁺ T cells can be eliminated in-vitro by CD8⁺ T cells. The in vivo effectiveness of DARTs on the HIV-1 reservoir is unknown (Sloan et al., 2015; Sung et al., 2015). In addition to DARTs, it has been shown that BiTEs that target the HIV-1 envelope protein gp120 and CD4 may exert strong antiviral effects in vitro and ex vivo (Brozy et al., 2018). Moreover, bsAbs have been designed to facilitate effector T cells against HBV- and human cytomegalovirus (CMV)-infected cells in vitro (Meng et al., 2017).

Chimeric antigen receptor (CAR) T cells

T cells can be genetically engineered for therapeutic application to provide unique antigen specificity by incorporating novel synthetic chimeric antigen receptors (CAR) using different ways to guide these cells toward the target. CAR T cells are engineered T cells that consist of unique parts, including scFv that are involved in specific recognition of target cells, a transmembrane and signaling domains that facilitate T cell activation without MHC interaction. Therefore, antigen specificity is associated with a signaling molecule and may be transmitted to recipient effector cells (e.g., T cells, T cells, NK cells) through various vectors. This approach involves several steps, including ex-vivo expansion of peripheral blood T cells obtained from the patient, genetically engineering the T cells to express CAR, and reinfusion of the modified T cells into the patient (Gattinoni et al., 2006).

T cell immunotherapy has shown promising clinical results in the treatment of hematological malignancies, including ALL and lymphoma (Kochenderfer and Rosenberg, 2013; Maude et al., 2014). Tisagenlecleucel and Axicabtagene Ciloleucel, two CD19-targeted CAR T cell treatments, were recently authorized by the FDA (Neelapu et al., 2017; Schuster et al., 2017). Although using CAR T cells in the treatment of solid tumors has been less successful than in hematologic malignancies, research is ongoing to investigate the effectiveness of CAR T cells in solid tumors such as colorectal cancer and breast cancer.

Major advances in oncology have sparked scientific and clinical enthusiasm for utilizing CAR-Ts to treat illnesses other than cancer. While the majority of CAR T cell research has focused on cancer immunotherapy, Kumaresan and colleagues created a fungal-specific CAR T cell (D-CAR) specific for β -1,3-D-glucan. This CAR T cell was enabled to kill *Aspergillus fumigatus* in vitro and in vivo (Kumaresan et al., 2014).

In a study by Krebs et al., a CAR T cell has been designed (S-CARs) against envelope proteins of HBV. Treating mice with S-CARs resulted in reducing virions in the blood as well as decreasing replicative forms of HBV DNA (Festag et al., 2019).

In another study by Kruse et al., a CAR T cell (anti-HBs-G4m CAR-T cells) specific to HBsAg was designed and its effectiveness was assessed in vitro and in a human liver chimeric mouse model. This CAR T cell was enabled to recognize HBV-infected cells in vitro and decrease HBV-DNA and HBsAg levels in vivo (Kruse et al., 2018).

CAR T cells targeting HCV E2 glycoprotein were designed by Sautto et al. These anti-HCV/E2 CAR-T cells exhibited significant cytotoxicity against HCV-infected cells (Sautto et al., 2016). The efficiency of CAR T cells against M2e protein of Influenza A virus was evaluated in vitro and in vivo by Talbot et al. This CAR T cell leads to decreasing viral titer in murine lungs (Talbot et al., 2013).

The effectiveness of CAR T cells against human immunodeficiency virus (HIV) has been evaluated in several studies. gp120 is considered as the main target for designing CAR T cells against infected cells (Deeks et al., 2002). In vitro studies indicated that CAR T cells can inhibit viral replication in infected cells and increase the death of infected cells. The effectiveness of targeting gp41 is also reported in some studies. In addition, the efficiency of the new generation of anti-HIV CARs that are based on broadly neutralizing antibodies (BNAbs) was shown in several studies, resulting in significant anti-viral activity in vitro through killing of infected cells (Ali et al., 2016; Liu et al., 2016a,b; Liu et al., 2015).

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Anti-Bacterial Agents

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Introduction

Anti-bacterial agents are divided into disinfectants and antibiotics. Disinfectants are not used as medicines, but are part of the formulation of products such household cleaners, soaps, detergents, health and skincare products, etc. Examples of commonly used disinfectants are quaternary ammonium compounds, chlorine, triclosan, metallic salts, etc. In contrast, antibiotics are animal or human drugs that specifically either kill (bactericide) or inhibit the growth of bacteria (bacteriostatic).

In general, antibiotics target essential processes such as cell wall synthesis, protein synthesis, nucleic acid synthesis, tetrahydrofolate synthesis, etc. in metabolically active bacteria.

Since their discovery, antibiotics have been very effective in resolving bacterial infections in humans and animals, and therefore have contributed to the achievement of a higher quality of life and longer life span. Antibiotics are very specific for bacterial processes, and so can be administered at high doses without having severe side effects. Nevertheless, they also have some limitations, such as being less effective to eradicate biofilm embedded bacteria and persister cells (since they are dormant and hence impervious to the inhibition of metabolic processes). Additionally, antibiotics also affect commensal and mutualist microbiota and can promote dysbiosis and colonization or proliferation of potentially harmful bacteria, such as *Clostridioides difficile*, which causes chronic diarrhea.

Antibiotic resistance appears when bacteria previously sensitive to antibiotics change in ways that reduce or eliminate their effectiveness. These changes are due to bacterial evolution, horizontal gene transfer, and by mutations, and therefore threaten the single greatest therapeutic advance in the history of medicine (Bankevich et al., 2012). Antibiotic resistance is achieved by changes in the phenotypic and/or genotypic characteristics of bacteria, and can be classified according to its origin and mechanisms (Davison et al., 2000). Antibiotic resistance is an increasing threat for humankind since it increases mortality rates and the length of hospital stays for people infected with multi-drug-resistant (MDR) bacteria (Heffernan et al., 2018). Extensive use of antimicrobial agents in hospital settings and in animal husbandry, has led to the emergence of pan-resistant “superbugs” (Meir-Gruber et al., 2016).

Among the most problematic bacteria are *Staphylococcus aureus* resistant to methicillin (MRSA), vancomycin-resistant enterococci (VRE), *Klebsiella pneumoniae*, *Acinetobacter baumannii* and members of the genus *Pseudomonas* and *Salmonella* (Baumann et al., 2017). All these bacteria are ESKAPE pathogens, which are very difficult to treat due their low sensitivity towards most antibiotics and cause high mortality and increased health care costs.

The World Health Organization (WHO) included the ESKAPE pathogens for the development of new antibiotics (12 bacteria were chosen as a priority) (Mulani et al., 2019). To generate effective tools for combating the ESKAPE pathogens, it is necessary to increase our understanding of their resistant mechanisms, their virulence, and their transmissibility, to improve strategies of combat. Focusing on the most resistant and virulent bacteria could help to mitigate the damage caused by the antimicrobial resistance (AMR) and will allow a more efficient evaluation of novel antimicrobial agents (Pendleton et al., 2013). The urgent need for the generation of novel, effective antimicrobials must be taken into account by pharmaceutical research and development in order to avoid the beginning of the post-antibiotic era (Tacconelli et al., 2018).

In this article, the main, currently used antibiotics, their targets, their mechanisms of action, their spectrum, their uses, and known-resistant mechanisms, are explained.

Antibiotics that disrupt cell walls

Beta-lactams

Beta-lactam antibiotics represent the largest family of antimicrobials and have a natural or semi-synthetic origin and are considered broad spectrum antibiotics. They are characterized by the presence of a heterocyclic beta-lactam ring that is structured by a heterocyclic ring of three carbon atoms and one of nitrogen, with the characteristic of having complementary side chains related to their antimicrobial activity, pharmacokinetics and toxicity (Barcelona et al., 2008). Their mechanism of action is based on the inhibition of the last stage of the synthesis of the bacterial cell wall, inhibiting peptidoglycan synthesis by blocking the transpeptidation or by activation of the bacterial autolysin that destroys the peptidoglycan. However, for a beta-lactam to be activated, it will need to be anchored to other radicals usually other rings; hence, in order to act, they must bind to proteins of the bacterial wall called PBPs (penicillin binding proteins) (Gualerzi et al., 2013). The main mechanism of resistance against β -lactams that occurs in Gram-negative bacteria, involves β -lactamases (periplasmic enzymes that hydrolyze the β -lactam ring) and β -lactamases (that confer resistance to cefotaxime, ceftazidime and aztreonam) are called "extended spectrum β -lactamases" and are often encoded in transposons and located in self-transferable plasmids that have promoted the spread of antibiotic resistance among bacteria globally (Worthington and Melander, 2013).

Penicillin

Penicillin can be of natural or semisynthetic origin, conformed by a structure of a beta-lactam ring, 6-aminopenicillanic nucleoside joined to a thiazolidine ring. Its bacterial spectrum includes Gram-positive and negative cocci and Gram-positive rods, facultative anaerobes, spirochetes, and some Gram-negative anaerobic rods (Miller, 2002). Penicillins are bactericidal in adequate doses and bacteriostatic in low concentrations. The binding of the beta-lactam ring of penicillins with DD-transpeptidase (mostly present in Gram-negative bacteria) prevents the formation of the cell wall, causing the bacterial lyses. The effectiveness of penicillin depends in its affinity to the PBPs. Penicillin is generally used in dental infections, pneumococci, endocarditis (*Streptococcus viridans*), meningococcal meningitis, gonorrhea (*Neisseria gonorrhoeae*) while not producing penicillinase, syphilis, diphtheria, tetanus, surgical prophylaxis, etc. (Miller, 2002).

The mechanisms of resistance to penicillin are mediated by the *blaZ* gene that codes for a beta-lactamase which hydrolyzes penicillin to produce inactive penicilloic acid. *S. aureus*, *Haemophilus influenzae*, gonococcus, and most Gram-negative enteric bacilli produce inducible beta-lactamases or penicillinases, while Gram-positive bacteria produce β -lactamases in varying amounts. The genetic control of the β -lactamases resides in plasmids that are transmissible to other bacteria by bacteriophages. On other occasions, resistance may be intrinsic, due to mutation and generation of mutant beta-lactamase-producing strains (Corsini Campioli et al., 2021).

The origin of amino penicillins lies in ampicillin, discovered in 1961. Amoxicillin differs from ampicillin by the addition of a hydroxyl group; it is chemically like penicillin and derived from I, but has greater gastrointestinal absorption than ampicillin, with a wider spectrum. Amoxicillin is bactericidal, although it is prone to be degraded by beta-lactamases (Miller, 2002). The mechanism of action of amoxicillin consists of binding to specific PBPs, inhibiting transpeptidation, causing the activation of autolytic enzymes in the cell wall, leading to bactericidal lysis. It can also be combined with a beta-lactamase inhibitor, such as clavulanic acid and sulbactam. Amoxicillin is used as a treatment for genitourinary tract infections, ear, nose and throat infections, *Helicobacter pylori* infections, rhinosinusitis, pneumonia, pharyngitis, tonsillitis, and skin infections. The known resistance mechanisms for amoxicillin consist of inactivation by beta-lactamases that are not inhibited by clavulanic acid, including classes B, C and D, as well as alterations of PBP proteins (Miller, 2002).

The anti-staphylococcal penicillins also known as isoxazolylpenicillins (Oxacillin, cloxacillin, dicloxacillin and flucloxacillin) are resistant to beta-lactamases, penicillinases and gastric acids. They are used mainly for infections generated by *Streptococcus pneumoniae*, group A streptococci and *Staphylococcus epidermidis*, and are used for uncomplicated skin diseases (folliculitis, furunculosis and cellulitis) (Miller, 2002).

Piperacillin also belongs to this group and is used for infections including those of the urinary tract, intra-abdominal, and of the skin and soft tissues, such as for diabetic feet and pneumonia. In combination with an aminoglycosides. Piperacillin is synergistic for treatments against *Pseudomonas*. It is not absorbed orally, but intravenously or intramuscularly. It is also used in streptococcal and enterococcal infections and has variable activity against *E. coli* and *Klebsiella*. The main resistance mechanisms are efflux pumps and alterations in the porins (Yang et al., 2015).

Cephalosporins

The first cephalosporins were obtained from the *Cephalosporium acremonium* fungus. Later on, modifications of their side chains had produced different generations. The cephem nucleus (chemical base of cephalosporins) is hydrophilic and capable of spontaneously crossing membranes, including those of the digestive epithelium, which is why they are administered parenterally. Its mechanism of action consists mainly in blocking the incorporation of peptidoglycan (El-Shaboury et al., 2007). Cephalosporins have broad spectrum activity against enterococci, *Listeria monocytogenes*, and cloxacillin-resistant staphylococci. Cephalosporins are undoubtedly the most recommended β -lactams due to their stability against β -lactamase enzymes. They are used as a treatment for respiratory, skin and urinary tract infections (El-Shaboury et al., 2007). First-generation cephalosporins have a spectrum of action similar to that of penicillin. Administration is oral and parenteral, as is the case of cefazolin. They are used for infections of the skin, bones, joints, genitals, blood, heart valve, respiratory tract (including pneumonia), biliary tract, and urinary tract. Cephalothin is one of the most resistant cephalosporins against β -lactamases, inhibits transpeptidases, has bactericidal activity and is efficient for urinary tract, gynecological, cardiac, and gastrointestinal infections. First-generation cephalosporins are used in surgical prophylaxis. Resistance to cephalothin arises when PBP transpeptidases are modified (Shahbaz, 2017).

Second generation cephalosporins, are more active against Gram-negative enterobacteria than first generation ones, as they are active against Gram-negative and Gram-positive cocci, but not against *Pseudomonas*, *Klebsiella* and some *Proteus* strains. Cefuroxime is bactericidal, its spectrum is similar to that of cefamandole, but it is more resistant to hydrolysis by β -lactamases; it penetrates in sufficient quantities in the cerebrospinal fluid. It is used as a treatment for meningitis (pneumococcal, meningococcal, by *H. influenzae* and by *S. aureus*), in infections of the lower respiratory tract and in the prophylaxis of chest surgery (Shahbaz, 2017). Cefoxitin has a broad spectrum against indole producing *Proteus* and *Serratia* sp., active against anaerobic Gram-negative bacilli and *B. fragilis*. It is used mainly for mixed anaerobic infections (peritonitis and diverticulitis), prophylaxis after colorectal operations, vaginal/abdominal hysterectomy and appendectomy and for infections of the respiratory, urinary and genital tract, abdomen, blood (septicemia), bones and joints, skin and soft tissues, burns or infected wounds, and prophylaxis.

Both (cefuroxime and cefoxitin) can be used to treat sinusitis and otitis media in patients who do not respond to amoxicillin (Brumfitt et al., 1974). Third generation cephalosporins are characterized by having 2-aminothiazolyl ring as a side chain, and they have broad spectrum activity against most Gram-negative, including those producers of β -lactamase. Third-generation cephalosporins are used in infections caused by Gram-negative bacilli, unusual hospital infections by Enterobacteriaceae, infections in immunosuppressed patients, pneumonia, urosepsis, meningitis and intra-abdominal, uncomplicated gonorrhea, osteomyelitis, infections of the skin and soft tissues and in Lyme Disease, etc. (Bennett et al., 2014). Ceftazidime inhibits the third and final stage of bacterial cell wall synthesis by preferentially binding to specific PBPs, it is active against *P. aeruginosa*, but less active than other third generation agents against Gram-positive cocci. It is structurally related to cefoperazone, but is less effective than moxalactam, cefotaxime, ceftizoxime, and ceftriaxone against Enterobacteriaceae. It is used in nosocomial infections due to Gram-negative, meningitis (combined with aminoglycosides if *Pseudomonas* is suspected) and in the treatment of febrile neutropenic patients (Bennett et al., 2014).

Ceftriaxone has an aminothiazolylacetyl group and a side chain at position 7 of a methoximino group, which generates an increase in antibacterial activity against Enterobacteriaceae. Its spectrum includes *Enterobacter*, *Citrobacter*, *Morganella*, *Providencia*, *Moraxella catarrhalis*, *Neisseria meningitidis*, *H. influenzae* and *N. gonorrhoeae* and the drug of choice for the treatment of gonococcal infections it is also used against central nervous system infections, middle ear, peritonitis, urinary tract, kidneys, bones, joints, skin, etc. (Harris et al., 2018).

Cephalosporins belonging to the fourth generation are characterized by the presence of a methoxy-imino aminothiazolyl group in R1 of the cephem nucleus. They have good penetration through the outermost cell membrane and low affinity for type 1 β -lactamases, reducing their enzymatic degradation compared to other cephalosporins (Garau et al., 1997). They have a broad-spectrum activity against strains of Enterobacteriaceae resistant to ceftazidime, Gram-positive pathogens such as *S. aureus* (except methicillin and cefazolin-resistant strains), etc. (Devansh and Kumar, 2015). Cephalosporins are used to treat nosocomial and community infections, urinary with/without bacteremia, skin, soft tissues, the female reproductive system, the lower respiratory tract, in surgery, in febrile states of neutropenic patients, and critically ill patients (Devansh and Kumar, 2015). Among the cephalosporins of this generation is Cefepime/Cefepime (Thomson et al., 2019). Its affinity for PBP-2 places it as an adequate option against Gram-negative that are resistant to 3rd generation cephalosporins. Its spectrum includes Gram-negative and Gram-positive, but it has no action against enterococci or bacteroids. Generally, it is used to treat infections produced by *P. aeruginosa*, *S. aureus*, *S. pneumoniae*, etc. (Bhagwat et al., 2020; Moya et al., 2021).

Fifth generation cephalosporins include Ceftaroline, which was developed by modifying cefozopran. Ceftaroline is the only drug effective against multi-resistant *S. aureus*, including VRSA and VISA and it is the only beta-lactam antibiotic with activity against MRSA (Frampton, 2013).

Carbapenems

Carbapenems are broad-spectrum antibiotics, stable against extended spectrum and chromosomal β -lactamases. They are not active against carbapenemase-producing strains, ampicillin-resistant enterococci, methicillin-resistant staphylococci (MRSA, SCNRM), *Enterococcus faecium*, *C. difficile*, *Stenotrophomonas* and *Burkholderia*. They are used for serious nosocomial infections and against infections caused by multi-resistant Gram-negative bacteria or producers of broad-spectrum and extended-spectrum β -lactamases. Among these antibiotics is Imipenem, which was the first antibiotic belonging to this group. Imipenem inhibits the third and last stage of cell wall synthesis, has the widest spectrum of activity of all known antibiotics (Lob et al., 2017). It is prescribed for infections present in the valves and lining of the heart, respiratory tract (including pneumonia), urinary tract, abdominal (stomach), gynecological, blood, skin, bones and joints. Another antibiotic belonging to this group is meropenem, that acts in a similar way to imipenem. Resistance to meropenem arises from the decrease in the permeability of the outer membrane in Gram-negative (due to loss of porins), the reduction of affinity of the target PBPs, efflux pumps and β -lactamases (Niu et al., 2020).

Ertapenem is another member of the carbapenems, yet more effective than imipenem. Ertapenem is not sensitive to broad-spectrum β -lactamases and penicillases. It is generally used for the treatment of infections caused by bacteria resistant to other groups of antibiotics, as a solution to intra-abdominal infections, to treat acute gynecological infections, pneumonia infections, diabetic foot infections, and infections that affect the skin and tissues, etc. The most frequent resistance mechanism to this antibiotic is produced by the hyperproduction of plasmid β -lactamases such as OXA-1, TEM-1, TEM-2 or SHV-1 or IRT (Zhanel et al., 2005).

Monobactams are antibiotics that have a monocyclic nucleus and a β -lactam ring. They are bactericidal, and mainly used in infections of the lower respiratory tract, skin and soft tissues, intra-abdominal, and urinary and gynecological infections caused by *P. aeruginosa* and aerobic Gram-negative bacilli. Monobactams are synergistic with aminoglycosides and are recommended for patients with allergies to penicillin or cephalosporins. An example is Aztreonam, used to treat infections caused by Gram-negative anaerobic bacilli such as meningitis, bacteremias, skin and soft tissue infections, urinary, respiratory, and abdominal infections (Ramsey and MacGowan, 2016; Jorth et al., 2017; Chew et al., 2018). The main causes of resistance to Monobactams are efflux pumps. There are different β -lactams used in combination with inhibitors such as clavulanic acid, sulbactam and tazobactam. For instance, Amoxicillin/Clavulanic acid are used in therapeutic doses against acute and chronic infections of the upper and lower respiratory tract, in meningitis, in genitourinary, skin and soft tissue, gastrointestinal, and biliary infections, etc. (Jorth et al., 2017). Other effective combinations are Piperacillin/Tazobactam, recommended as a treatment for skin and soft tissue infections, gynecological and abdominal infections, and in neutropenic patients who present with fever due to infections of bacterial origin (Harris et al., 2018). Ceftazidime/Avibactam is used as a treatment in intra-abdominal and urinary tract (mainly pyelonephritis) infections, pneumonia (associated with mechanical ventilation), etc. (Zhanel et al., 2005). Ceftolozane/Tazobactam is used as a treatment for intra-abdominal infections, acute pyelonephritis, complicated urinary tract infections, and hospital-acquired lung infections (Puzniak et al., 2021).

Glycopeptides

Glycopeptides are one of the latest resources to combat infections by several Gram-positive bacteria. They are used to treat community acquired infections, and those acquired in a hospital environment. They are used to eradicate infections produced by *S. aureus*, including MRSA, resistant *Enterococcus* spp., and *Clostridioides difficile* (Jovetic et al., 2010; Butler et al., 2014). Members of the first generation are vancomycin and teicoplanin, isolated from *Amycolatopsis orientalis* and *Actinoplanes teichomyceticus* respectively; second generation members were the result of chemical derivatizations and, hence, semi-synthetic antibiotics, and include oritavancin, telavancin and dalbavancin (Marcone et al., 2018).

The core of these antibiotics consists in heptapeptides, which can be glycosylated tricyclic or tetracyclic. Both vancomycin and teicoplanin share chemical structures with high similarity. Vancomycin possesses aliphatic amino acids at position 1 and 3 at the N-terminal, whereas teicoplanin possess a lipophilic moiety linked to the vancosamine sugar (Sarkar and Haldar, 2019).

Glycopeptides inhibit the step of cell wall synthesis (Sarkar and Haldar, 2019). This is due to the binding to the D-alanyl-D-alanine (D-Ala-D-Ala) C-terminal together with the lipid II and the precursor of peptidoglycan (Marcone et al., 2018) preventing the incorporation of these components to the biosynthesis pathway (Blaskovich et al., 2018). This crosslinking is the result of the cooperative effect between five hydrogen bonds and disrupts transglycosylation and transpeptidation.

Resistance mechanisms against glycopeptides include changes in the configuration of the biosynthesis of the cell wall (Leclercq et al., 1988).

Changes that produce resistance, include the incorporation of D-lactate (D-Lac) or D-serine (D-Ser) to the peptidoglycan, as well as the activity of enzymes such as D-amino acids ligase and others able to degrade normal peptidoglycan precursors (Cattoir and Leclercq, 2013).

In target modification, D-Ala-D-Lac associated genes are *vanA* (phenotype inducible) and can be in plasmids or chromosomes. These genes are associated with high resistance to vancomycin and teicoplanin and are present in *Enterococcus* spp., and *S. aureus*; *vanB* (inducible). D-Ala-D-Lac associated genes confer resistance in variable levels to vancomycin and teicoplanin, and those genes are in plasmids and chromosomes, *vanD* (constitutive) confers moderate resistance to vancomycin and teicoplanin and it is located in the chromosome, present in *Enterococcus faecalis* and *E. faecium*, while *vanM* confers resistance to teicoplanin and vancomycin and it can be found in *E. faecium*, finally, *vanA* and *vanB* occasionally have been found in coryneform and streptococci.

Meanwhile, *vanC* genes are associated with D-Ala-D-Ser and are both constitutive and inducible and are found in *Enterococcus gallinarum*, and *Enterococcus casseliflavus*; *vanE*, also can be inducible or constitutive and is present in *E. faecalis*; *vanG* and *vanL*, both are inducible and present in *E. faecalis* and *vanN* is constitutive in *E. faecium*. All of them confer resistance to vancomycin (Cattoir and Leclercq, 2013; Butler et al., 2014).

Fosfomycin

In 1969, Hendlin and colleagues discovered fosfomycin, produced by *Streptomyces* spp. It is an analog of phosphoenolpyruvate, composed of an epoxide ring and a carbon-phosphorus bond. It has a low molecular weight (138 g/mol) and is commercially available in 2 oral formulations (trometamol and calcium), and intravenously (disodium) (Falagas et al., 2016).

It functions by interfering with the first cytoplasmic step in the formation of the cell wall. The inhibition is achieved by the covalent binding of fosfomycin with the amino acid Cys115, the active site of the enzyme UDP-N-acetylglucosamine enolpyruvyl transferase (MurA). The enzyme MurA is responsible for catalyzing the formation of UDP N-acetylmuramic acid (UDP-MurNAc), a precursor of peptidoglycan (Olesen et al., 2014; Falagas et al., 2016).

Fosfomycin's spectrum of action includes Gram-positive cocci and Gram-negative rods. *Enterococcus* spp. (*E. faecalis*, *E. faecium*) and *Staphylococcus* spp. (*S. aureus*, *S. epidermidis*) are susceptible (Vardakas et al., 2016).

Fosfomycin is also effective against extended-spectrum β -lactamase-producing and carbapenem-resistant bacteria (Bassetti et al., 2019). Uropathogens such as *E. coli*, *Klebsiella* spp., *Citrobacter* spp., *Proteus* spp., and *Enterobacter* spp., have susceptibilities ranging from 80% to 100% depending on the series (Vardakas et al., 2016; Bassetti et al., 2019; Wijma et al., 2019). *Morganella* spp. shows intrinsic resistance (Stock and Wiedemann, 1998).

In *P. aeruginosa*, CLSI and EUCAST have not established susceptibility cutoff points, however, recent research supports the use of this drug in isolates of *P. aeruginosa*, taking an epidemiological cutoff value lower than <128 $\mu\text{g/L}$ as susceptible (EUCAST). A recent analysis found a 62% susceptibility in an international collection of *P. aeruginosa* MDR strains (Mojica et al., 2020; Smith et al., 2020).

Fosfomycin enters the cell through two carrier-dependent systems: α -glycerophosphate (*Enterobacteriales*, *P. aeruginosa*, *Staphylococcus* spp., *Enterococcus* spp.) and hexose-phosphate (*Enterobacteriales*). Mutations in the *glp7* gene, which encodes α -glycerophosphate permease, result in resistance to this drug (Silver, 2017).

Intrinsic resistance occurs in *Mycobacterium* sp., *Chlamydia* sp. and *Vibrio* sp., given a change from cysteine to aspartate in the enzyme MurA, preventing the binding of fosfomycin (Olesen et al., 2014). Others, such as *Pseudomonas putida*, have alternate routes for the formation of peptidoglycan, avoiding the de novo formation of UDP-MurNAc. There are few reports of *E. coli* with a mutation in the enzyme MurA (Silver, 2017).

Different enzymes inactivate fosfomycin by degradation of the oxirane ring, which include Metalloenzymes such as: FoaA (*Klebsiella* spp., *Pseudomonas* spp.), FosB (*S. aureus*, *S. epidermidis*), FosX (*L. monocytogenes*, *Clostridium botulinum*, *Brucella melitensis*) and, recently, FosM (Bacillus species in human microbiota) (Fillgrove et al., 2007; Elliott et al., 2019; Khabthani et al., 2021). FoaA a glutathione-S-transferase present on the TN2921 transposon was originally described in a plasmid in *Serratia marcescens*. And two fosfomycin kinases: FomA and FomB, both present in *Streptomyces wedmorensis* also inactivate this drug (Silver, 2017). Fosfomycin is used in cystitis (Ten Doesschate et al., 2018), pyelonephritis (Kaye et al., 2019; Zhanel et al., 2020) and chronic prostatitis (Karaïskos et al., 2019). Given its wide distribution to tissues, it has also been used in osteomyelitis, meningitis and pneumonia (Falagas et al., 2016). Recent trials demonstrate synergy with meropenem for Metallo- β -lactamase-producing *P. aeruginosa* (Albiero et al., 2019); and Daptomycin for MRSA endocarditis (Pujol et al., 2021).

Lincosamides

Lincosamides are named after Lincoln, Nebraska, where they were first isolated from *Streptomyces lincolnensis*. It is considered a small pharmacological group; main members are lincosamide and clindamycin, the latter having greater biological activity and the most used in clinical practice. Lincosamides are derived from many species of *Streptomyces* and *Micromonospora halophytica*. The prototype of this group; Clindamycin has broad biological activity against anaerobic bacteria and some protozoa (Spížek and Řezanka, 2017).

Natural and semisynthetic lincosamides are A, B, C, D, S, K, K-celestines A, B, C, D, Desalination, Desalination D, and N-desmethyl celesticetin.

Lincosamides are formed by N-methyl-4-n-L-proline or propylhygric acid, linked by a peptide bond, with the sugar 6-amino-6,8-dideoxy-1-thio-D-galactopyranoside or methylthio-lincosamide. About 20 derivatives of different lincosamides are known. Lincosamides inhibit the first steps of protein production, promoting the termination of the chains that are produced in the ribosome. They bind the 50s ribosomal subunit and inhibit peptidyl transferase, halting protein synthesis. Clindamycin and other lincosamides are structural analogs of the 3' ends of 1-Pro-Met-tRNA and deacylated-tRNA interfering with the pre-translocation during peptide elongation (Spížek and Řezanka, 2017).

At therapeutic doses, lincosamide and clindamycin are considered bacteriostatic. The lincosamides spectrum of activity is wide, including against both aerobic and anaerobic species, and with good activity against staphylococcal and streptococcal species. In this instance, it has been shown that even in low concentrations, lincosamides have activity against the release of toxins. In anaerobic species, lincosamides have activity against fusobacterium, prevotella and bacteroides. Lincosamides also exhibit

activity against protozoa for the treatment of malaria, babesiosis, and toxoplasmosis. Clindamycin is the first line of treatment against *Capnocytophaga canimorsus* (Spížek and Řezanka, 2017).

Alternatively, lincosamides are useless for the treatment of infections by *Pseudomonas* spp., *Moraxella* spp., *Haemophilus* spp. *Neisseria* spp., due to intrinsic resistance. Of the main agents with biological activity, the cut-off values are for the case of *S. aureus* 0.016 µg/mL → 256 µg/mL, *S. pneumoniae* 0.002 µg/mL → 256 µg/mL, and *Streptococcus pyogenes* <0.015 µg/mL → 64 µg/mL.

The main mechanism of clindamycin resistance is methylation of the ribosome, which causes cross-resistance to macrolides, lincosamides, and streptogramins. Those methylation mechanisms are encoded by several *erm* (erythromycin ribosome methylase) genes, found in a wide variety of bacteria. The Erm protein, dimethylate, a single nascent 23s adenine, which is present in the 50s ribosomal subunit, decreases the affinity of clindamycin to its target. Since the binding sites of macrolides, lincosamides, and streptogramins B on 23S rRNA overlap, methylation causes cross-resistance towards the three antibiotic classes. To date, approximately 40 different *erm* genes have been described (Spížek and Řezanka, 2017).

Antibiotics that disrupt cell membranes

Polymyxins

Polymyxins are antibiotics that have recently gained interest for the treatment of infections caused by multi-resistant bacteria such as *P. aeruginosa* and *A. baumannii*. This group was described in 1947 by Benedict and Langlykke (1947) and by Ainsworth and coworkers, which described aerospurin derived from *Bacillus aerosporus* (Ainsworth et al., 1947).

In 1949, the Brownie classification system renamed polymyxin A to aerospurin and polymyxin B to polymyxin, later, polymyxin B, C and E (Brownlee, 1949). At the same time, in Japan, colistin was discovered and was later included in the group of polymyxins. Polymyxins were eventually abandoned, due to the potential nephrotoxic risk, and because other groups of antibiotics with the same activity were available. However, then multidrug resistant bacteria increased, and currently, polymyxins are now used again for their effectiveness. The two most used polymyxins are colistin and polymyxin B.

Polymyxins are cationic peptides consisting of a cyclic heptapeptide with a tripeptide side chain with a fatty acid tail at the N-terminus. The toxicity and activity are explained by this chain. Colistin and polymyxin B differ by one amino acid on the peptide ring; a phenylalanine for colistin and leucine for colistin. Colistin is administered as a drug known as colistimethate, unlike polymyxin B which is an active antibiotic (Li et al., 2006). Various mechanisms of action for polymyxins have been described. One of them lies in its activity against the outer membrane, due to an interaction of the residue of α , γ -diaminobutyric acid and the phosphate groups of the lipid A membrane. This interaction causes destabilization of lipid A, with a net effect of increasing the permeability of the bacterial membrane, causing leakage of the cytoplasmic material and the death of the bacteria. Another mechanism of action for polymyxins is binding endotoxin by interacting with lipid A, and promoting the inhibition of enzymes such as NADH-quinone oxidoreductase type II (Falagas and Kasiakou, 2005).

Alternatively, polymyxins have a narrow range of activity against most enterobacteria, including *E. coli*, *Salmonella* spp., *Shigella* spp., *Enterobacter* spp. and *Klebsiella* spp., as well as other Gram-negative bacteria such as *P. aeruginosa*, *A. baumannii* and *Stenotrophomonas maltophilia* (Deris et al., 2014). At present, The use of polymyxins focuses more on the treatment of infection by *P. aeruginosa* and *A. baumannii* (Tsuji et al., 2019). In the case of colistin, the target plasma concentration is 2 µg/mL, in bacteria with a MIC <1 µg/mL. Minimal post-antibiotic effects have been described at relevant clinical concentrations.

In 2019, in the United States, a MIC ≤2 mg/L is recommended as the susceptibility breakpoint for colistin and polymyxin B against *P. aeruginosa*, *A. baumannii* and Enterobacterales (Pogue et al., 2020). The EUCAST describes the polymyxin B cutoff for Enterobacterales (2 mg/L), *P. aeruginosa* (4 mg/L) and *Acinetobacter* spp. (2 mg/L).

Most of the pharmacokinetic data of polymyxins describe colistin. After administering colistin, a large proportion is eliminated via the kidneys, and between 20% and 25% of the drug is hydrolyzed in the active form. Therefore, colistin activity is influenced by renal function, and so regimens with colistin often require adjustment of dosing. In contrast to the prodrug active colistin is preferentially eliminated non-renally due to its extensive tubular reabsorption. Polymyxin B is also excreted by non-renal route, in which only 1% is isolated in urine, therefore it is not necessary to make a dose adjustment, despite having impaired renal function (Berg et al., 1998).

The basic molecular mechanisms by which polymyxin B increase transepithelial conductance in the urinary tract, are the same as for colistin, resulting in increased influx of cations, anions, and water, leading to inflammation and cell lysis. Currently, colistin toxicity is low, in part due to minimal chemical impurities in the drug, and also due to mechanisms related to the scope of administration, such as continuous monitoring in intensive care units. Potential negative side effects related to colistin are nephrotoxicity and neurotoxicity, the latter being dose-dependent and reversible. The most common neurotoxic effect is paresthesia. Nephrotoxicity is the most general adverse effect (Sorlí et al., 2013).

Although resistance against polymyxins is relatively rare, and more difficult to appear than to most antibiotics, resistance levels are rising. The main mechanism for resistance includes modification of the LPS structure, mainly by the reduction of their negative charge, that leads to a decrease in the affinity of the cationic peptides. The reduction of the negative charge is achieved by the addition of positive groups such, phosphoethanolamine, galactosamine and 4-amino-L-arabinose, most of them mediated by chromosomally encoded genes, although some plasmid born genes such as MCR phosphoethanolamine transferases are also involved. Another resistance mechanism is through losing the production of lipid A or LPS, through mutations in the genes encoding enzymes in charge of their biosynthesis, such as against polymyxins in *A. baumannii* (Olaitan et al., 2014).

Antibiotics that inhibit protein synthesis

Aminoglycosides are produced by actinobacteria of the genera *Streptomyces* ssp. and *Micromonospora* ssp. and are made up of the combination of two types of compounds: non-amino sugars (glucosides) or amino sugars (aminoglycosides) and non-amino cyclic alcohols (cyclitols) or amino acids (aminocyclotols). Aminoglycosides form three possible combinations, where the most important group is aminoglycoside plus aminocyclitol, which constitutes gentamicin, streptomycin and amikacin, the other groups are made up of glucoside plus aminocyclitol (spectinomycin) and aminoglycoside plus cyclitol (kasugamycin); they are water-soluble, stable and more active in alkaline pH.

Aminoglycosides have a high bactericidal activity, which is higher than that of β -lactams; they inhibit protein synthesis in an irreversible way, entering bacteria by passive diffusion through porines in the outer membrane, and they are later actively transported from the membrane to the cytoplasm. Within the cell, they bind to specific regions of the 30s subunit of the ribosome, inhibiting protein synthesis by three mechanisms: (1) interference with the initiation of the peptide formation complex; (2) erroneous reading of the mRNA, giving rise to a non-functional proteins; (3) disintegration of polysomes into non-functional monosomes. These three mechanisms occur simultaneously, causing an irreversible and lethal effect on the cell (Busse et al., 1992).

Aminoglycosides are used for the treatment of infections caused by aerobic Gram-negative bacilli. In addition, they are combined with β -lactams to achieve synergism in activity against some Gram-positives and against *Mycobacterium* spp. Anaerobes are resistant to aminoglycosides.

Streptomycin was used as the first antibiotic against infection caused by *Mycobacterium tuberculosis*. Currently, Streptomycin is part of the second line of treatment in combination with penicillin G, it is also used for the treatment of bacterial endocarditis, caused by *Streptococcus viridans*. Gentamicin is used in serious infections such as septicemia and pneumonia; its use in combination with a β -lactam is recommended for infections caused by microorganisms that are resistant to other drugs such as: *P. aeruginosa*, *S. marcescens*, *Enterobacter* sp. and *Klebsiella* sp.

Another aminoglycoside, amikacin, is a synthetic derivative of kanamycin. Amikacin is resistant to enzymes that inactivate other aminoglycosides. Additionally, it is less toxic than other aminoglycosides. It is used in infections caused by *Proteus*, *Pseudomonas*, *Enterobacter* and *Serratia*, which become resistant to gentamicin.

To date, three resistance mechanisms against aminoglycosides are described: (1) inactivation of compounds by enzymes modifying aminoglycoside (EMA) that catalyze covalent modifications in the amino and hydroxyl groups, causing weak binding to their target site; (2) a decrease in the impermeability of the outer membrane and therefore a reduction in the intracellular concentration; (3) a deletion or alteration of their binding sites in the 30s subunit, as a result of mutations (Paul et al., 2013).

Macrolides

Macrolides are one of the most commonly used families of antibiotics prescribed to treat infections caused by Gram-positive bacteria such as *S. aureus*, *S. pneumoniae* and *S. pyogenes* (Gaynor and Mankin, 2005). Macrolides are bacteriostatic and inhibit protein synthesis. Although their exact mechanism of action varies depending on the specific macrolide, their primary action is the dissociation of peptidyltransfer RNA from the ribosomes during translocation. Macrolides bind to the 50S ribosome subunit of bacteria inhibiting transpeptidation and translocation of nascent peptides (Rollins et al., 2014).

Macrolides have at least 12-membered glycosylated macrolactone rings (Zin et al., 2020), and they are divided into different categories based on structural features. Erythromycin and clarithromycin are 14-membered lactone rings. Azithromycin is a part of the azalide subclass and contains a 15-membered ring (Fohner et al., 2017).

Macrolides are effective against Staphylococcus, Streptococcus and Diplococcus and among Gram-negative cocci, *Neisseria gonorrhoea*, *H. influenzae*, *Bordetella pertussis* and *Neisseria meningitis*. Furthermore, they are also extremely active against various Mycoplasmas (Dinos, 2017).

The five macrolide antibiotics available have a similar range of activities; however, clarithromycin and azithromycin are more active than erythromycin against several Gram-negative bacteria as well as *Mycoplasma pneumoniae*, *H. pylori*, and atypical mycobacteria. Fidaxomicin is not absorbed orally and is used to treat *Clostridium difficile* associated diarrhea (Bethesda, 2017).

Macrolides selectively inhibit the translation of a subset of cellular proteins, in that their action crucially depends on the nascent protein sequence and on the antibiotic structure to block protein elongation (Vázquez-Laslop and Mankin, 2018). During elongation, peptidyl transferase catalyzes the addition of a new amino acid to the growing peptide chain. The growing peptide chain is then translocated from the A site to the P site. It has been suggested that 16-membered macrolides inhibit the peptidyl transfer reactions, and that the 14-membered ones block the peptidyltransfer RNA (tRNA) translocation (Zhanel et al., 2001; Tacconelli et al., 2018).

Erythromycin has an antimicrobial spectrum similar to that of penicillin, and so was historically widely used in patients allergic to penicillin. Erythromycin has bacteriostatic and bactericidal activity, depending on the type of microorganism and the antibiotic concentration. It is most effective against *S. aureus*, streptococcal group A, enterococci, and pneumococci. It inhibits *Neisseria* and some strains of *H. influenzae*, *Pasteurella multocida*, *Brucella*, *Rickettsiae*, and *Treponema*. It is also effective against *Mycoplasma pneumoniae*, *Chlamydiae*, *Legionellae pneumophila*, and some other atypical mycobacteria (Jelić and Antolović, 2016). It is used for various respiratory infections, including community-acquired pneumonia and Legionnaires disease, as a prophylaxis of neonatal conjunctivitis, etc. It is also used for treating skin infections, syphilis, and pelvic inflammatory disease.

Clarithromycin and azithromycin are given orally for the treatment of respiratory infections and these drugs distribute to a variety of tissues, particularly to the lungs (Togami et al., 2011). Clarithromycin and azithromycin are also used for treating sexually transmitted diseases and *H. pylori* infections (Zuckerman et al., 2011).

Resistance to macrolide antibiotics is mediated by several mechanisms including: (i) decreasing their intracellular concentration, (ii) alterations of the target, (iii) protection of the target, and (iv) chemically inactivation of the antibiotics (Golkar et al., 2018).

Tetracyclines

Tetracyclines are broad-spectrum antibiotics active against Gram-positive and Gram-negative bacteria, such as spirochetes, and obligate (or obliterate?) intracellular bacteria (Grossman, 2016). The newest tetracyclines include eravacycline, omadacycline and eravacycline. The traditional tetracyclines are tetracycline, chlortetracycline, oxytetracycline and demeclocycline and semi-synthetic members are minocycline, doxycycline, lymecycline, rolitetracycline and lymecycline. Meanwhile, tigecycline, one of the most used, belongs to the subclass glycacycline (Sapadin and Fleischmajer, 2006; Nelson and Levy, 2011).

The core of Tetracyclines is made by four linear fused tetracyclic rings, which have a variety of functional groups (Chopra and Roberts, 2001). Since the first was discovered in 1948, it has been used in humans ever since, and remains one of the most prescribed antibiotics in the world. In general, tetracyclines are well-tolerated and available orally, intravenously, topically, and intramuscularly (Chopra and Roberts, 2001). Tetracyclines have good distribution in overall tissue, the ascitic area, the synovial and pleural fluids, and in lungs; however, they have poor perfusion to the cerebral spinal space.

In tetracyclines, the mechanism of action is binding to the bacterial ribosome, and thus inhibiting protein synthesis. To achieve this, tetracyclines must cross the Gram-negative membranes through porins such as OmpF and OmpC (Chopra et al., 1992; Schnappinger and Hillen, 1996). The ribosomal 30S subunit has a high-affinity binding site to tetracyclines, and both the 30S and 50S subunits have several binding sites with low-affinity, then an allosterical interaction with the amino acyl-tRNA at the acceptor site (A-site) interrupts protein synthesis (Griffin et al., 2010).

Resistance mechanisms against tetracyclines are either mediated by efflux pumps (McMurry et al., 1980), mainly from the Tet family protein, part of the major facilitator superfamily (Pasqua et al., 2019), or by the protection of ribosomes mediated also by proteins of the Tet family such as TetM and TetO (Taylor and Chau, 1996).

Oxazolidinones

The oxazolidinone group includes Tedizolid and Linezolid, FDA approved drugs. In 1987, the oxazolidinone structure was synthesized: 5-(halomethyl)-3-aryl-2-oxazolidinones, which served as the basis for the development of Linezolid, approved for clinical use in 2000 (Slee et al., 1987).

Both Tedizolid and Linezolid share multiple characteristics in their chemical structure. Tedizolid contains a hydroxymethyl group in ring A and a pyridine ring (ring C) and tetrazole (ring D) are added. In Linezolid, acetamide in ring A increases the antimicrobial activity of the drug. One of the main differences is their dosages; Tedizolid is administered once a day, while Linezolid is twice a day (Kisgen et al., 2014).

Oxazolidinones are inhibitors of protein synthesis by binding to the V domain of 23S ribosomal RNA in the 50S subunit, blocking the formation of the 70S unit and preventing ribosomal transcription.

Oxazolidinones show very good activity against Gram-positive cocci such as *S. aureus* (including MRSA), Ceftriaxone-resistant *Streptococcus pneumoniae*, *Streptococcus* spp., Vancomycin-resistant *E. faecium* and *Nocardia* spp. Oxazolidinones have moderate to good activity against *Mycobacterium* spp., mainly *M. tuberculosis*, where linezolid has gained a niche within second-line drugs for MDR strains; and some Gram-positive anaerobes such as *Bacillus* spp. and *Clostridium* spp. However, due to efflux pumps, the activity of oxazolidinones against Gram-negative bacteria is very poor. Tedizolid and Linezolid have the same spectrum of action, but Tedizolid has lower MICs (Bozdogan and Appelbaum, 2004; Pfaller et al., 2019; Ruiz et al., 2019).

In general, resistance to oxazolidinones is infrequent, however, induced resistance has been observed in *Enterococcus* spp. and *Staphylococcus* spp., where constant exposure increases MIC 32 times after 30 generations. On the other hand, there are 23S ribosomal RNA mutations that confer low-grade resistance described in *Enterococcus* spp. and *Staphylococcus* spp. High-grade resistance is transferred by plasmids or transposons that carry the CFR gene; This gene encodes a methyltransferase that methylates RNA 23S (A2503); however, the molecular structure of tedizolid allows it to retain activity against strains that present the CFR gene. There are other mutations in the gene encoding the ribosomal binding site such as L3 and L4, they have been identified in *Staphylococcus* spp., *S. pneumoniae* and *Clostridium perfringens*. The genes *optrA* and *poxA* have recently been found, which encode a cassette-bound ATP-like transporter protein that confers resistance to oxazolidinones (linezolid and tedizolid) and phenicols through a ribosome protection mechanism (Liu et al., 2020).

Current FDA indications for linezolid are primarily skin and soft tissue infections (Stevens et al., 2005), vertebral osteomyelitis (Berbari et al., 2015), MRSA pneumonia (Metlay et al., 2019), and MDR tuberculosis (TB) (Conradie et al., 2020). Currently, tedizolid is approved by the FDA only for the treatment of skin and soft tissue infections (Bouza et al., 2018).

Streptogramins

Streptogramins are naturally produced by *Streptomyces* and are classified in two groups A and B. They are bacteriostatic when used alone, but together they can become bactericidal. Group A streptogramins bind the bacterial ribosome at the P site, preventing protein elongation; they also promote a conformational change in the 50S subunit that enhances the activity of group B streptogramins, which also interfere with protein elongation and cause the release of incomplete peptides (Mukhtar and Wright, 2005). The most commonly prescribed combination is quinupristin/dalfopristin (both derived from *pristinamycin*); they are combined in a proportion of 70%/30% and administered intravenously to treat *staphylococci* and by *vancomycin*-resistant *E. faecium* infections. Resistance against these antibiotics is mediated by multiple mechanisms, including: efflux pumps, enzymatic modification, and mutations changing the target site (Hershberger et al., 2004).

Chloramphenicol

In 1947, Chloramphenicol was isolated from *Streptomyces venezuelae* and later from other *Streptomyces* species and currently is synthesized. Chloramphenicol has broad spectrum activity; however, since it is linked to some negative side effects such as bone marrow aplasia and grey baby syndrome, its current use is mainly restricted for treating eye infections such as conjunctivitis and blepharitis. It is bacteriostatic, and bactericidal against some bacteria such as *S. pneumoniae*, *N. meningitidis* and *H. influenzae*, at high concentrations. Chloramphenicol disrupts protein synthesis, binding the 50S subunit of the bacterial ribosome inhibiting transpeptidation. Resistance against this drug arises mainly due to the presence of the inactivating the enzyme chloramphenicol acetyltransferase, found commonly on plasmids. In addition, cross resistance to linezolid via *cfr* genes is also observed in some strains of *S. aureus*, and the reduction of the permeability and mutations in the 50S ribosome subunit can also lead to resistance (Eliakim-Raz et al., 2014).

Antibiotics disrupting nucleic acid synthesis

Quinolones

Quinolones are synthetic antibiotics with a bicyclic core structure derived from 4-quinolone. They were discovered in the 1960s, as nalidixic acid the first to be discovered (Pham et al., 2019). Later on, fluorination of these compounds lead to the obtention (→ what word do you want to say here? invention?) of fluoroquinolones, enhancing their activity spectrum (Pham et al., 2019). They are bacteriostatic and their mechanism of action consists in binding and inhibiting topoisomerases (gyrase, type II and type IV), hence promoting supercoiling, DNA damage and halting DNA synthesis (Aldred et al., 2014). Quinolones are used mainly for the treatment of genitourinary track infections and for nosocomial infections associated with urinary catheters, as well as for complicated prostatitis and pyelonephritis (Liu and Mulholland, 2005). They are also prescribed occasionally for the treatment of respiratory and gastrointestinal infections, and soft tissue, skin and tuberculosis infections (Aldred et al., 2014).

Currently there are four quinolone generations: nalidixic acid, ciprofloxacin, levofloxacin and moxifloxacin. Their activity spectrum originally was mainly against Gram-negative bacteria, except for *Pseudomonas*, but more recent generations include some activity against Gram-positive bacteria including *S. aureus*, and atypical bacteria such as *M. pneumoniae* and *Chlamydia pneumoniae* (Pham et al., 2019).

Ciprofloxacin is prescribed orally, intravenously, or as eye and ear drops. It is active against *Pseudomonas*, and prescribed for urinary, intra-abdominal, joint, and skin infections, typhoid fever, prostatitis, genital infections and infectious diarrhea and for prophylaxis in prolonged cancer treatments (Sharma et al., 2010).

Levofloxacin has broad activity against Gram-negative and Gram-positive bacteria, atypical respiratory pathogens and *S. pneumoniae*, hence is used for the treatment of the respiratory tract, the genitourinary tract and skin infections (Croom and Goa, 2003).

Moxifloxacin is a fourth-generation fluoroquinolone used for the treatment of sinusitis, eye infections, endocarditis, pneumonia and tuberculosis (TB). For TB, it is prescribed when there is intolerance against one of the first-line drugs, or when mycobacteria are resistant against isoniazid. Moxifloxacin is also included in several novel combinatory treatments for TB showing promising results (Naidoo et al., 2017).

Fluoroquinolone resistance is mediated by several, not exclusive, mechanisms: (1) mutations in the targets topoisomerases and gyrase that decrease the affinity for the drugs, (2) downregulation of porin expression, decreasing its uptake, (3) efflux pumps, either encoded in the chromosome or in transmissible plasmids, (4) protection of the targets by specific proteins (Qnr, McbG and MfpA) that bind to them and decrease the drug binding, (5) enzymatic inactivation of the drugs, specifically acetylation mediated by mutated aminoglycoside acetyltransferases such as *aac(6′)-Ib-cr* (Aldred et al., 2014).

Rifampicin

Isolated in 1968, rifampicin belongs to the rifamycins, produced by the strain *Ammycolatopsis mediterranei*. The main use of rifampicin is for the treatment of several mycobacterias, mainly *M. tuberculosis*; however, it can also be used in infections associated with prosthetic infections caused by *S. aureus*, and also to treat *Clostridioides difficile* and some Gram-negative infections such as

N. meningitidis, *N. gonorrhoeae* and *H. influenzae* (Rothstein, 2016). Rifampicin has been promoted as bactericidal, however, results from the ARREST study did not show better results with the use of rifampicin against *S. aureus* in bloodstream infections in comparison with a placebo group ([https://doi.org/10.1016/S0140-6736\(17\)32456-X](https://doi.org/10.1016/S0140-6736(17)32456-X)). However, in vitro, there is evidence about the utility of rifampicin, such as an anti-biofilm molecule.

Rifampicin's antimicrobial action is carried out due RNA synthesis inhibition, blocking sterically the activity of DNA-dependent RNA polymerase, therefore decreasing its affinity with short RNA transcripts and stopping RNA elongation at the 5' end. This molecule has no activity on mammalian RNA polymerases (McClure and Cech, 1978; Schulz and Zillig, 1981; Campbell et al., 2001).

Resistance to rifampicin is conferred mainly to the mutation of the *rpoB* gene, particularly in *M. tuberculosis*, rifampicin being highly used as part of the first line of treatment. Once mutations appear, rifampicin's ability to bind to RNA polymerase decreases drastically. Resistance to rifampicin also has been observed in those microorganisms on the *arr* gene; these genes encode enzymes (that ribosylate rifampicin inactivating it.) (this does not make sense) The *arr* genes have been found in *Mycobacterium smegmatis*. In addition, a similar *arr2* gene is present in plasmids in clinical strains of *Enterobacterales*, such as *Klebsiella*.

Antibiotics that disrupt bacterial metabolism

Sulfonamides

Sulfonamides are analogs of para aminobenzoic acid (PABA). They are poorly soluble in water, but their sodium salts are much more soluble. The structural features responsible for their antibacterial activity include the presence of a sulfur linked to the benzene ring, and the presence of the para-NH₂ group, that can only be replaced by moieties that can be transformed in vivo, to a free amino group (Smith and Powell, 2000).

Sulfonamides are competitive inhibitors of the enzyme dihydropteroate synthase, which is responsible for incorporating PABA, into dihydropteroic acid, the precursor of folic acid. Sensitive bacteria are those that synthesize their own folic acid. Hence bacteria (and all mammalian cells) that can use preformed folate, are not affected by these drugs.

Sulfonamides are bacteriostatic and inhibit both Gram-positive bacteria, such as *Staphylococcus* sp., as well as Gram-negative enteric bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella*, *Shigella*, and *Enterobacter* sp., *Nocardia* sp., *Chlamydia trachomatis*, etc. Sulfonamides do not inhibit rickettsiae, but rather stimulate their growth. Their activity against anaerobes is poor, and *P. aeruginosa* is inherently tolerant to them (Smith and Powell, 2000).

Sulfonamides are effective for treating *P. jirovecii* pneumonia, urinary tract infections, prostatitis, and infections caused by some strains of *Shigella*, *Salmonella*, and atypical mycobacteria. Sulfonamides are active against MRSA and methicillin-sensitive *S. aureus*, and against respiratory tract pathogens such as *Haemophilus* sp., *M. catarrhalis*, and *K. pneumoniae*, but not against *M. pneumoniae*.

Sulfonamides are also an effective alternative against infections caused by species such as *Enterobacter* and *Serratia*, shigellosis or typhoid fever, as well as for serious *Listeria* infections in patients allergic to ampicillin (Smith and Powell, 2000).

Bacterial resistance to sulfonamides can be caused by mutations or transfer of resistance by plasmids; sulfonamides generally do not generate cross-resistance. Resistance to sulfonamides is mediated by: (1) a lower affinity of the drugs towards dihydropteroate synthase; (2) decreased bacterial permeability; (3) active drug release; (4) the presence of alternative metabolic pathways for the biosynthesis of folic acid; or (5) increased production of an essential metabolite or drug antagonist, such as PABA (Sköld, 2000). Plasmid-mediated resistance is due to plasmid-encoded resistant dihydropteroate synthase (Sköld, 2000).

Novel antibiotics

In recent decades, research into new antibiotics has increased, although not to the levels found in the decades from 1940 to 1960. The present priority is to combat multidrug resistant bacteria (MDR), extensively drug resistant (XDR) and Gram ESCAPE pathogens that are a threat to humanity (Hutchings et al., 2019; López-Jácome et al., 2019).

Most of the antibiotics currently approved worldwide continue to be obtained from natural products of bacteria mainly of the genus (Actinomycetes) and fungi. In recent years, the use of bioinformatic tools, such as next generation sequencing, genome mining and CRISPR/Cas9-mediated genome editing and network biology methods, could assist researchers to discover better antibiotics, antibiotic combinations, and drug repurposing in the coming years (Kohanski et al., 2010; Hutchings et al., 2019).

Published in December 2020, the latest global review on antibiotics in development notes that there are 43 new antibiotics that are in different stages of development; 15 are in phase 3, 13 in phase 2, and 13 in phase 1, and two antibiotics have the category of application of new drugs submitted to the Food Drug and Administration (FDA) in the United States and the National Medical Products Administration of China.

In addition to the usual targets for antibiotics, new targets and new drugs have also been reported and are currently being tested. For example, Gepotidacin (GSK2140944) (phase 3), an organic synthetic compound that has shown activity against Gram-positive and Gram-negative bacteria, inhibits bacterial DNA gyrase and topoisomerase IV (Flamm et al., 2015; Hsu et al., 2019). Another example is Afabacin (Debio 1450) (phase 2), also a synthetic organic compound that has shown activity against Gram-positive bacteria, mainly *S. aureus* (Flamm et al., 2015). Its bacterial targets are focused on inhibiting FabI, the NADH-dependent enoyl reductase from the type II bacterial fatty acid biosynthesis pathway (FAS-II) (Flamm et al., 2015; Peyrusson et al., 2020).

Approving a new antibiotic to be used by the entire population is complicated. However, teixobactin is one of the few recently discovered antibiotics that has a wide application landscape. Teixobactin is a depsipeptide that contains D-amino-acids and L-allo-enduracididine residues (Ling et al., 2015; Shukla et al., 2020). The compound is produced by the species *Eleftheria terrae*,

a β -Proteobacteria Gram-negative related to the genus *Aquabacterium* (Ling et al., 2015; Shukla et al., 2020). Teixobactin has shown to be an important bactericide against MDR, such as *S. aureus*, *M. tuberculosis*, and MRSA (Ling et al., 2015; Lewis et al., 2018). Among the particularities that this antibiotic presents is its mechanism of action: the molecule binds to two precursor polymers of the cell wall lipid II (peptido-glycan) and lipid III (teichoic acid), producing a rapid lysis of the cell (Ling et al., 2015). Furthermore, it has recently been suggested that teixobactin interferes with cell wall biosynthesis (Shukla et al., 2020). Its mechanism of action, different from other antibiotics, makes it unlikely that resistance against teixobactin will develop (Ling et al., 2015). Teixobactin is currently in the last stage of preclinical development.

Finally, another important antibiotic that is new is sideromycins, that is, antibiotic-siderophore conjugates, or organic chelating agents, with high affinity for iron (Negash et al., 2019).

Iron is an essential element in many cellular processes, although it is scarce in pathogen-host interactions, so bacteria compete for it with the host. In November 2019, the first sideromycin, Cefiderocol (S-649266), a conjugated cephalosporin to the iron-chelating catechol group, was approved by the FDA (Aoki et al., 2018). The main mechanism of action of Cefiderocol acts as a Trojan horse, since once the catechol group of Cefiderocol forms an iron complex with the Fe^{3+} ion, it can penetrate (active transport pathway) the outer membrane of Gram-negative bacteria, increasing the concentration of the antibiotic in the periplasmic space (Ito et al., 2016). Cefiderocol is highly effective against MDR bacteria such as *P. aeruginosa*, *Burkholderia cepacia*, *A. baumannii*, and Enterobacteriaceae (Zhanel et al., 2019). Another promising compound is Ga^{3+} (gallium nitrate) which is a non-REDOX active Fe^{3+} analog. Gallium Nitrate has been previously used for treating hypercalcemia of malignancy, and it is also effective against MDR *P. aeruginosa* and *A. baumannii* *in vitro* and in animal models (Minandri et al., 2014). Gallium Nitrate also apparently has low levels of resistance (Kaneko et al., 2007; Goss et al., 2018; Tovar-García et al., 2020) and has shown effectivity in clinical trials to improve the lung function of patients with cystic fibrosis infected with *P. aeruginosa* (Goss et al., 2018).

A summary of the antibacterial discussed here is found in Table 1.

Table 1 Antibiotics included in the chapter.

Antibiotic target	Class	Representative example	Activity spectrum	Resistance mechanisms
Cell wall	β -lactams/penicillins	Amoxicillin	<i>Streptococcus</i> sp. <i>Enterococcus</i> sp. <i>Neisseria</i> sp. <i>Haemophilus influenzae</i> <i>Fusobacterium</i> sp. <i>Peptostreptococcus</i> sp. <i>Actinomyces</i> sp.	Penicillinases alterations of PBP2A
	β -lactams/Cephalosporins	Ceftriaxone	<i>Streptococcus</i> sp. <i>Staphylococcus</i> sp. <i>Neisseria gonorrhoeae</i> . <i>Leptospira interrogans</i> <i>Treponema pallidum</i> Enterobacteriaceae	Extended spectrum B-lactamase AmpC beta-lactamase alterations of PBP2A
	β -lactams/Carbapenems	Meropenem	<i>P. aeruginosa</i> <i>Acinetobacter baumannii</i> Enterobacteriaceae <i>Streptococcus</i> sp. <i>Staphylococcus</i> sp. <i>Nocardia</i> sp. <i>Rhodococcus equi</i> Anaerobic gram positive cocci and Gram-negative rods	Carbapenemases Hyperproduction of beta-lactamases Efflux OprM system OprD system alterations of PBP2A
	Glycopeptides	Vancomycin	<i>Staphylococcus</i> sp. <i>Streptococcus</i> sp. <i>Enterococcus</i> sp. <i>Listeria monocytogenes</i> <i>Bacillus</i> sp. <i>Clostridioides difficile</i> Enterobacteriaceae	Target modification in cell wall, genes: <i>vanA</i> , <i>vanB</i> , <i>vanC</i> , <i>vanD</i>
	Fosfomycin	Fosfomycin	<i>Enterococcus</i> sp. <i>Staphylococcus</i> sp. <i>P. aeruginosa</i>	Target modification: MurA. Metalloenzymes: FosA, FosB, FosX, FosM
	Lincosamides	Clyndamicin	<i>P. aeruginosa</i> Bacteroides sp. <i>Fusobacterium</i> <i>Prevotella</i> <i>Staphylococcus</i> sp. <i>Streptococcus</i> sp. <i>Capnocytophaga canimorsus</i>	Fosfomycin kinases: FomA, FomB ribosomal target methylation: erm gene

Table 1 (Continued)

<i>Antibiotic target</i>	<i>Class</i>	<i>Representative example</i>	<i>Activity spectrum</i>	<i>Resistance mechanisms</i>
Cell membranes				
	Polymyxins	Colistin	<i>P. aeruginosa</i> <i>A. baumannii</i> <i>Enterobacteriaceae</i>	Modification of the LPS structure, MCR1 gene
Protein synthesis inhibition				
	Aminoglycosides	Amikacin	<i>P. aeruginosa</i> <i>A. baumannii</i> <i>Enterobacteriaceae</i> <i>Nocardia</i> sp. <i>Rhodococcus equi</i> <i>Mycobacterium</i> sp. <i>Streptococcus</i> sp. <i>Enterococcus</i> sp.	Enzymes modifying aminoglycoside (EMA) Target modification Decrease in the permeability of the outer membrane
	Macrolides	Azithromycin	<i>Staphylococcus</i> sp. <i>Streptococcus</i> sp. <i>Mycoplasmas</i> sp. <i>Legionella</i> sp. <i>Helicobacter pylori</i> <i>C. jejuni</i> <i>Chlamydia</i> sp.	Target modification: erm gene Chemically inactivation Decreasing their intracellular concentration
	Tetracyclines	Doxycycline	<i>Enterobacteriaceae</i> <i>Streptococcus</i> sp. <i>Staphylococcus</i> sp. <i>Mycoplasmas</i> sp. <i>Legionella</i> sp. <i>Chlamydia</i> sp. <i>Rickettsiae</i> sp. <i>Vibrios</i> sp.	efflux pumps: Tet protection of ribosome: TetM and TetO
	Oxazolidinones	Linezolid	<i>Staphylococcus</i> sp. <i>Streptococcus</i> sp. <i>Enterococcus</i> sp. <i>L. monocytogenes</i> <i>Bacillus</i> sp.	Target modification Methyltransferase: CFR gene protection of ribosome: L3, L4, optrA, poxtA genes
	Streptogramins	Quinupristin/dalfopristin	<i>Mycobacterium</i> sp. <i>Staphylococcus</i> sp. <i>Enterococcus</i> sp.	Efflux pumps Enzymatic modification Target modification
	Chloramphenicol	Chloramphenicol	<i>Enterobacteriaceae</i> <i>Streptococcus</i> sp. <i>Staphylococcus</i> sp. <i>Enterococcus</i> sp. <i>Actinomyces</i> sp. <i>Vibrios</i> sp. <i>Rickettsiae</i> sp.	chloramphenicol acetyltransferase <i>cfr</i> gene
Disrupt nucleic acid synthesis				
	Quinolones	Levofloxacin	<i>P. aeruginosa</i> <i>Enterobacteriaceae</i> <i>Streptococcus</i> sp. <i>Staphylococcus</i> sp. <i>Mycoplasmas</i> sp. <i>Legionella</i> sp. <i>Chlamydia</i> sp. <i>Rickettsiae</i> sp. <i>Vibrios</i> sp. <i>Mycobacterium</i> sp. <i>Kingella</i> sp. <i>Yersinia</i> sp.	Target modification. GryA, GryB protection of the targets: Qnr
	Rifamycins	Rifampicin	<i>Mycobacterium</i> sp. <i>Staphylococcus</i> sp. <i>Neisseria</i> sp. <i>H. influenzae</i>	Target modification: <i>rpoB</i> gene ribosylate rifampicin: <i>arr</i> gene
Bacterial metabolism disruption				
	Sulfonamides	Trimethoprim/sulfamethoxazole	<i>Enterobacteriaceae</i> <i>Streptococcus</i> sp. <i>Staphylococcus</i> sp. <i>Nocardia</i> sp. <i>Moraxella</i> sp.	Lower affinity of dihydropteroate synthase. Decreased bacterial permeability Alternative metabolic pathway

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Anti-Parasite Agents and Vaccines

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Glossary

Acoziborole Synonyms: SCYX-7158; AN5568, 4-fluoro-*N*-(1-hydroxy-3,3-dimethyl-2,1-benzoxaborol-6-yl)-2-(trifluoromethyl benzamide, hydrogen bond donor and acceptor, is an orally active anti-protozoan, efficacious and safe for treatment of African trypanosomiasis via targeting mRNA maturation factors.

Benznidazole Is a nitroimidazole anti-Chagas disease agent, apparently reduced by *Trypanosoma cruzi* nitroreductases to various electrophilic metabolites, able to bind to and damage the parasite proteins, lipids, DNA, and RNA.

Eflornithine Difluoromethylornithine, a repurposed anti-tumor agent, enters trypanosomes via the amino-acid transporter AAT6, and irreversibly inhibits ornithine decarboxylase which catalyzes the biosynthesis of polyamines, critically important for trypanosomes cell growth, differentiation and replication. It is important for oral or intravenous treatment of West African trypanosomiasis.

Fexinidazole Is an all oral drug active against human African trypanosomiasis and visceral leishmaniasis. It is a nitroheterocyclic compound, belonging to the class of nitroimidazoles, readily reduced by parasitic protozoans nitroreductase to fexinidazole sulfoxide and fexinidazole sulfone, cytotoxic species that damage the parasite DNA, RNA, and proteins.

Folinic acid 5-Formyltetrahydrofolate, known as leucovorin, is readily converted to other reduced folic acid derivatives. It displays vitamin activity equivalent to that of folic acid and, hence, is used to treat folate deficiency, and decrease the toxicity of chemotherapeutic drugs. It is often prescribed in the treatment of toxoplasmosis retinitis, in combination with the folic acid antagonists pyrimethamine and sulfadiazine.

Kinetoplast A network of circular DNA (called kDNA) inside a large mitochondrion lying close to the nucleus, near to the base of the flagella, in some flagellate protozoa, the kinetoplastids. The thousands of circular, interlocking DNA chains/rings of kDNA represent approximately 15% of the total protozoal cell. Despite location near the base of the flagella, kinetoplasts are not involved in the protozoan motility. African trypanosomes (African sleeping sickness), *Trypanosoma cruzi* (Chagas' disease), and *Leishmania* species (leishmaniasis) are kinetoplastids.

Melarsoprol Arsobal, C₁₂H₁₅AsN₆OS₂, an aromatic arsenical belongs to the class of aniline and substituted anilines, containing an aminobenzene moiety. Melarsoprol enters trypanosomes via aquaglyceroporin (AQP2) transporter, with resistant strains showing mutations in this parasite solute and drug transporter. Melarsoprol inhibits a reductase enzyme responsible for reduction of trypanothione, a low molecular weight peptide containing spermidine and glutathione skeletons in a cyclic structure (*N*¹, *N*⁸-bis-(glutathionyl)-spermidine). In kinetoplastids, trypanothione replaces intracellular glutathione and glutathione reductase, the principal mechanism of the thiol-redox system. Reduced trypanothione plays an important role in maintaining intracellular thiol redox potentials, in protection of the parasite against oxidative and chemical stress, and in regulation of spermidine contents in the cells.

Nifurtimox A nitrofurant antiprotozoal drug is reduced by cytosol enzymes or flavin-containing microsomal enzymes to highly reactive nitro anion free radicals, which alkylate nucleic acids and proteins, resulting in the disruption of their structure and function. Auto-oxidation of the nitro anion free radical generates superoxide anion (O₂⁻) selectively toxic to the parasite.

Ookinete Malaria gametocytes ingested with a blood meal transform within the mosquito midgut into mature female and male gametes. The product of fertilization, a round-shaped zygote, transforms within a day into a crescent-shaped motile ookinete, which then traverses the midgut epithelium to the basal side, where it matures in about 2 weeks into oocyst. Oocytes break open and release thousands of sporozoites that migrate to the insect's salivary glands, ready for infection transmission.

Pyrimethamine Is an aminopyrimidine substituted at position 5 by a p-chlorophenyl group and at position 6 by an ethyl group. It is a folic acid antagonist, as it readily inhibits parasite dihydrofolate reductase, a key enzyme in the redox cycle for production of tetrahydrofolate, a cofactor that is required for the synthesis of DNA and proteins.

Nomenclature

ALB	Albendazole
DALYs	Disability-adjusted life years
DEC	Diethylcarbamazine citrate
HAT	Human African trypanosomiasis
Iv	Ivermectin
LF	Lymphatic filariasis
VSG	Variant surface glycoproteins

Parasitic protozoans (unicellular, prokaryotes)

African trypanosomiasis

Life cycle. Trypanosomiasis, a potentially fatal disease if not properly treated, is caused by unicellular, (25–40 $\mu\text{m} \times 1.5\text{--}3.5 \mu\text{m}$), flagellated protozoan parasites of the genus *Trypanosoma*, which require man and domestic animals as primary host, and blood-sucking insect as the intermediate host. The blood-sucking insect is responsible for the transmission of the parasites from one host to another during a blood meal. African trypanosomiasis is transmitted by several species of both male and female tsetse flies belonging to the genus *Glossina* (Despommier et al., 2005). It is caused in humans by *Trypanosoma brucei rhodesiense* (Eastern and Southern Africa) and *T. brucei gambiense* (Western and Central Africa), while the animal form of the disease is mostly caused by *T. congolense*, *T. vivax*, and *T. brucei brucei*. More than 65 million people living in 36 countries in sub-Saharan Africa are at risk of contracting the disease. At least 300,000–500,000 people are presently infected and only 10% of the new cases are diagnosed and treated. The disease is fatal because the parasite, despite being predominantly blood-borne, dwells in lymph and tissues, and readily affects the central nervous system (Baral, 2010; Brun et al., 2010; Onyilagha and Uzonna, 2019). Infection of livestock causes dire economic and social consequences (Jordan, 1976). American trypanosomiasis, endemic in several South American countries is caused by *Trypanosoma cruzi*, and transmitted by Triatominae bugs (Lidani et al., 2019).

Woody vegetation in rural areas, heat and humidity favor the breeding of the tsetse insect vector. An infected fly bite of host skin intradermally inoculates the infective, metacyclic trypomastigotes stage. Proliferation occurs by binary fission accompanied with changes to a long, slender form, and acquisition of a coat of around 10 million molecules of identical, but variant, surface glycoproteins (VSG), which shield numerous membrane-associated transport proteins, necessary adaptations to residence in the bloodstream in close contact to the host immune effectors. The VSG coat protects the parasite from the human plasma lytic factors, a combination of apolipoprotein L1 and haptoglobin-related protein (Vanhamme and Pays, 2004; Brun et al., 2010). The proliferative, slender blood-stage trypomastigotes are observed during the onset of parasitaemia in blood, lymph, and spinal fluid. They change to non-proliferative stumpy forms, a preadaptation to the procyclic stages, which access the tsetse fly midgut with a blood meal, exchange their VSG coat for procyclic stage-specific, acidic repetitive proteins, the procyclins, and proliferate, totally resistant to proteolytic enzymes. The parasites migrate to the salivary gland. The forms generated there, the epimastigotes, elaborate the undulating flagellar membrane, further multiply, and are released into the salivary gland lumen as metacyclic trypomastigotes, in preparation for transmitting the infection to a new host (Despommier et al., 2005).

Local reactions in humans at the site of tsetse fly bites are associated with lymphadenopathy. Proliferation and dissemination of trypomastigotes in blood and lymph cause headaches, fever, general weakness, and joints pain and stiffness. Parasites that cross the blood-brain barrier and migrate to the central nervous system induce neurological changes, predominantly sleep disorder (hence the name “sleeping sickness”), deep sensory disturbances, abnormal tone and mobility, ataxia, psychiatric disorders, seizures, toxemia, coma and ultimately death. In the case of *T.b. rhodesiense*-mediated human African trypanosomiasis (HAT), the disease is acute, lasting from a few weeks to several months while in *T.b. gambiense* infections the disease is chronic, generally progressing slowly over several years (WHO, 2019).

Diagnosis. Relies in the laboratory on blood smear examination (Chappuis et al., 2005a).

Treatment agents. Only five drugs are registered for HAT treatment (Steverding, 2010). The current first choices for early stages are suramin, and pentamidine, (Brun et al., 2010; De Koning, 2020). Suramin, a sulfated naphthaylamine introduced a century ago in 1920, is endocytosed in trypanosomes via binding to an invariant VSG, inhibits cytokinesis, and additionally interferes with trypanosome respiration and glycolysis, leading to cellular ATP collapse (Thomas et al., 2018; Wiedemar et al., 2020; Zoltner et al., 2020). Besides severe side-effects, its use is cumbersome as it must be intravenously injected at a dose of 20 mg/kg body weight 5 times a day for 5–7 days. Suramin central benzene ring tightly binds to a VSG, VSG_{sur}, preventing its endocytosis-mediated cellular uptake and leading to a resistance phenotype (Zeelen et al., 2021). Pentamidine, an aromatic diamidine, is endocytosed by the trypanosomes via binding to the aquaglyceroporin AQP2, accesses ribosomal RNA, inhibits protein and nucleic acids synthesis, and leads to progressive loss of kinetoplast DNA, disruption of mitochondrial membrane potential, and eventually parasite death (Thomas et al., 2018). However, it induces adverse reactions and is difficult to administer (Brun et al., 2010). Melarsoprol, an organoarsenic compound, affects *T.b. rhodesiensis*, entering via an adenosine transporter and AQP2, forming a stable adduct with the antioxidant, trypanothione, and leading to mitosis inhibition. Both melarsoprol and an α -difluoromethylornithine, eflornithine, are recommended for second-stage HAT caused by *T.b. gambiense*. Drug adverse reactions are frequent and the difficulty of administration was not circumvented (Chappuis et al., 2005b; Babokhov et al., 2013; Thomas et al., 2018). Eflornithine is commonly given in combination with nifurtimox, a nitrofurant antibiotic, which reduces the treatment time to 7 days of eflornithine infusions plus 10 days of oral nifurtimox tablets (Babokhov et al., 2013; De Koning, 2020).

A new oral drug candidate, acoziborole, an oxaborole, has shown promising results in pre-clinical and phase I/II clinical studies, and it is currently under phase III studies for single oral dose, for both disease stages. It displays potent trypanocidal activity, likely dependent on boron atom in the heterocyclic core structure (Jacobs et al., 2011), and its single oral dosing would circumvent existing therapies limited effectiveness and safety profile, and difficult treatment regimens. However, trypanosome resistance to the drug is already emerging (Dickie et al., 2020). A new 2-substituted 5-nitroimidazole drug, fexinidazole, has been developed and tested in clinical trials II/III to be used for oral, short course, safe, and effective treatment of HAT (Torreale et al., 2010; Kaiser et al., 2011; Tweats et al., 2012; Tarral et al., 2014). Phase 3 evaluation is ongoing in the Democratic Republic of the Congo and Guinea, and approval has been obtained from European Medicines Agency (EMA) in 2018, facilitating application in endemic countries (Deeks, 2019; Fairlamb, 2019; Dickie et al., 2020).

Vaccines. There is currently no vaccine and none is expected in a near future. The main reason is that the VSG coat of blood trypomastigotes is immunogenic, inducing a plethora of specific antibodies that may bind to the parasite and mediate its death via complement-dependent killing or antibody-dependent cell-mediated cytotoxicity (ADCC). However, trypanosomes possess hundreds of VSG genes, with only a single one at a given time expressing millions of identical molecules. Repressing an active and activating a previously silent expression site leads to VSG continuous and uninterrupted variability in sequences and linear and conformational epitopes, rendering the infection- or vaccine-induced antibodies harmless (Baral, 2010; Onyilagha and Uzonna, 2019).

American trypanosomiasis (Chagas disease)

Life cycle. Chagas disease is named in honor of the discoverer of its etiological agent and insect vector in 1909, the Brazilian physician and researcher, Carlos Chagas. Chagas disease constitutes one of the main public health problems in Latin America. About 6 to 14 million people in endemic areas of 21 Latin American countries are estimated to be infected with *Trypanosoma cruzi*, the parasite that causes Chagas disease (Carod-Artal, 2013). The disease is vector-borne as it is mostly transmitted to humans by contact with feces or urine of blood-sucking triatomine bugs. More than 100 species of Triatominae are capable of transmitting *T. cruzi*, notably *Triatoma infestans* and *T. dimidiata* that are widely distributed in the Americas (Lidani et al., 2019). The infective *T. cruzi* stage is not transmitted in the host blood. The epimastigotes, the proliferating stage in the insect vector intestine, do not migrate to the salivary glands. The parasites differentiate into infective metacyclic trypomastigotes in the hindgut. Parasite invasion of humans occurs following smearing the insect urine and feces deposited in the vicinity of the blood-sucking spot, often in the face (WHO, 2020a). Parasite replication in the human host does not occur in the circulation, but intracellularly where the parasites multiply by binary fission. The proliferative amastigotes differentiate into trypomastigotes, which are released in the circulation as blood trypomastigotes that may invade other cells or are ingested by the insect to continue the life cycle (Fig. 1).

Non vector-borne transmission includes blood transfusion, congenital and oral transmission, transplantation, and accidental infections (Carod-Artal, 2013; WHO, 2020a).

Chagas disease presents in two phases: acute and chronic. The early stage is usually asymptomatic despite that a high number of parasites circulate in the blood, but can present with fever, headache, enlarged lymph glands, pallor, muscle pain, difficulty in breathing, swelling, and abdominal or chest pain. Most acute cases resolve over a period of a few weeks or months into a subclinical chronic form of the disease ("indeterminate form"). Years or even decades after the initial infection, as the parasites remain hidden mainly in the heart and digestive muscles, 30–45% of infected persons present digestive, cardiac, circulatory and neurological serious problems (Carod-Artal, 2013; Henao-Martínez et al., 2019; Lidani et al., 2019; WHO, 2020a; Echeverría et al., 2021).

Diagnosis. Is by microscopic finding the parasite in blood smears, or detecting its DNA by polymerase chain reaction (PCR); chronic disease is diagnosed by serological tests detecting serum specific antibodies titer and isotype (Umezawa et al., 1996; Balouz et al., 2017).

Treatment agents. Chagas disease can be orally treated with oral benznidazole, a free radicals- generating antimicrobial nitroimidazole, which inhibits *T. cruzi* synthesis of DNA, RNA, and proteins (Edwards, 1993), or nifurtimox, a pro-drug activated by parasite

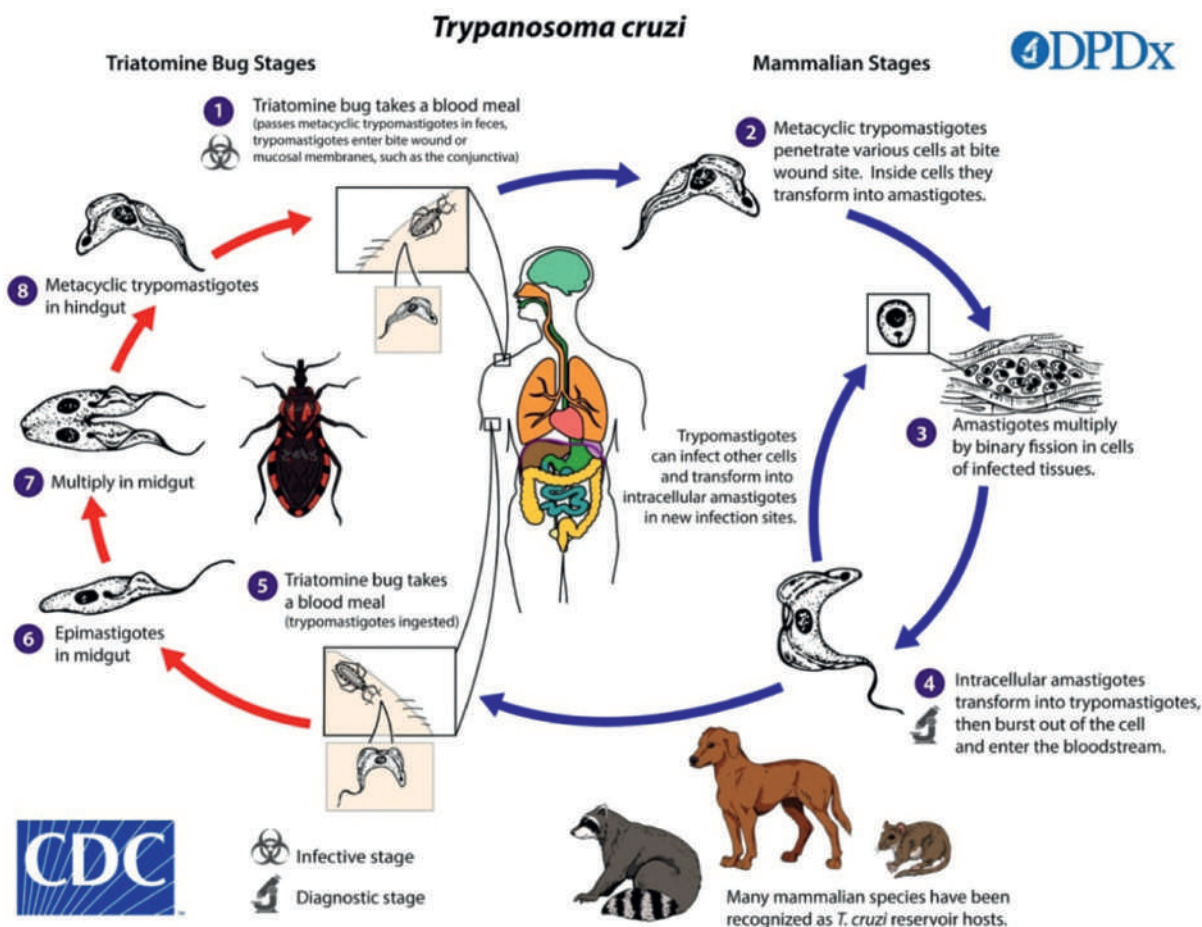


Fig. 1 Life cycle of *Trypanosoma cruzi*, the agent of Chagas disease. From Centers for Disease Control and Prevention.

nitroreductase for release of products toxic to mitochondrial structure and function (Thomas et al., 2018). Both medicines are nearly 100% effective in curing the disease if given for 30 to 60 days, soon after infection at the onset of the acute phase. Treatment is also recommended during the chronic phase, even if incapable of producing parasitological cure, because it reduces or delays worsening of the clinical condition of the patient. Adverse side reactions are common and include dermatitis, anorexia, weight loss, nausea, vomiting, and insomnia, but resolve with time (Castro et al., 2006). Fexinidazole could be considered for treatment of *T. cruzi* infection resistant to benznidazole and nifurtimox (Bahia et al., 2012). Enhancement of trypanosome cure was achieved upon combining benznidazole with curcumin (Novaes et al., 2016), or the essential oils of *Syzygium aromaticum* (Zanusso Junior et al., 2018), clove (*Syzygium aromaticum*) or ginger (*Zingiber officinale*), which have demonstrated suppression of parasitemia in Swiss mice treated for 20 consecutive days following oral administration of 10,000 *T. cruzi* blood trypomastigotes (Sarto et al., 2021). Nanocarrier-based delivery systems, notably liposomes, micelles, nanoemulsions, polymeric and non-polymeric nanoparticles are now considered for reduction of toxicity, and increase of the efficacy and bioavailability of anti-Chagas disease drugs (Quijia Quezada et al., 2019).

Vaccines. Live, attenuated *T. cruzi* parasites likely induce powerful protection, but present serious safety issues, which were attempted to be resolved via use of gene-deleted live parasites (Sánchez-Valdéz et al., 2015). Sub-unit candidate vaccines were selected among which *T. cruzi* flagellar pocket calcium-binding protein (Biter et al., 2018), and 80 kDa prolyl oligopeptidase (Bivona et al., 2018) elicited powerful type 1-related cytokines and antibodies production, and improved survival and physiological and immunological parameters in immunized mice. Recently, Traspain, based on B- and T-cell epitopes of key parasitic protein targets: cruzipain (Cz), amastigote surface protein 2 (ASP2), and a selected region of trans-sialidase, was assessed for immunogenicity and protective capacity in mice. The chimeric antigen in a DNA construct was used for primary oral immunization of male and female mice followed by intranasal protein-boost. Oral immunization was opted for because the oral route of Chagas disease transmission results in a higher mortality rate of 8–35% than is generally observed by vector transmission which is <5–10% (Antunes et al., 2019). Immune responses were characterized by increase in spleen cells augmented secretion of interferon (IFN)- γ and interleukin (IL)-17 as compared to unimmunized mice, associated with control of challenge parasitaemia (Alberti et al., 2020).

Despite considerable efforts aiming to characterize immune correlates of protection, select potential vaccine candidates, suitable adjuvants (Rios et al., 2019), and consideration of the use of plants as immunogens production platform and delivery system (Ramos-Vega et al., 2021), no vaccine has currently reached phase 1 trials (Bivona et al., 2020).

Leishmaniasis

Life cycle. Leishmaniasis, an insect-borne disease prevalent in tropics and subtropics regions, threatens over 150 million people worldwide (Kaye and Scott, 2011). The vector is a minuscule fly, the female sand-fly of the genus *Phlebotomus*. It transmits the promastigote stage of the obligate intracellular trypanosomatid protozoan of the genus *Leishmania* from host to host via skin biting and blood sucking. Human infection is caused by more than 20 morphologically indistinguishable leishmania species (Family Trypanosomatidae, Sub-Family Leishmaniinae, Clade Leishmaniatae), notably *Leishmania donovani*, *L. infantum* = *L. chagasi*, *L. mexicana*, *L. amazonensis*, *L. venezuelensis*, *L. tropica*, *L. major*, *L. aethiopica*; and *L. [V.] braziliensis*. Promastigotes are engulfed by phagocytes of the skin and other tissues, transform into amastigotes, and multiply by binary fission. *Leishmania* parasites survive and multiply undamaged in the different phagocyte populations, neutrophils, monocytes, macrophages, dendritic cells, via controlling phagosome biogenesis, acidification, and maturation, and securing the cell iron resources (Kaye and Scott, 2011). Infected cells rupture when too many amastigotes are present, allowing reinfection of resident phagocytes and distribution to other phagocytes in various tissues, including blood monocytes, which are the source of sand fly infection during a blood meal. The ingested amastigotes invade cells of the insect gut, replicate and differentiate into promastigotes, and migrate to the proboscis to continue the life cycle in the mammalian host (Kaye and Scott, 2011). The infection causes serious skin sores that may evolve in indelible disfiguring ulcers and scars characterizing cutaneous leishmaniasis. An estimated 12 million people are currently infected and about 2 million new cases occur yearly, resulting in about 50,000 deaths each year, notably in Afghanistan, Pakistan, Iran, Iraq, the Syrian Arab Republic, Tunisia, Algeria, Bolivia, Brazil, and Colombia (Ikeogu et al., 2020). Around 235,000 cases were reported in Brazil from 2007 to 2017, with preponderance associated with male gender, age range of 30–60 years, agricultural-related activities, and high relative humidity (Oliveira et al., 2021).

The life threatening form is visceral leishmaniasis (known as kala-azar), which may develop months or years of the sandfly bite, presenting with fever, weight loss, spleno- and hepatomegaly, anemia, leukopenia, and thrombocytopenia. The symptoms reflect parasite invasion of the phagocytes of liver, spleen, and blood. Visceral leishmaniasis is endemic in India, Bangladesh, Nepal, Sudan, Ethiopia, Somalia, Kenya, Iraq and Brazil. Mucocutaneous leishmaniasis leads to partial or total destruction of mucous membranes of the nose, mouth and throat. Over 90% of mucocutaneous leishmaniasis cases occur in Bolivia, Brazil, Ethiopia and Peru. Of note, beside humans, around 70 animal species have been found as natural reservoir hosts of *Leishmania* parasites (WHO, 2010; Okwor and Uzonna, 2016; Ghorbani and Farhoudi, 2017).

Diagnosis. Light microscopic examination of skin tissue or bone marrow cells (Fig. 2) is used to confirm cutaneous and visceral leishmaniasis, respectively, as the parasite is intracellularly obligate. Serological and molecular tests are available in modern settings and laboratories; however they do cross-react with other protozoa and their sensitivity changes according to the clinical form of the disease (de Brito et al., 2020).

Treatment agents. Leishmaniasis is treatable and curable. But the available drugs show several adverse reactions. Pentavalent antimonials are administered intramuscularly for visceral leishmaniasis and intralesionally for the treatment of cutaneous

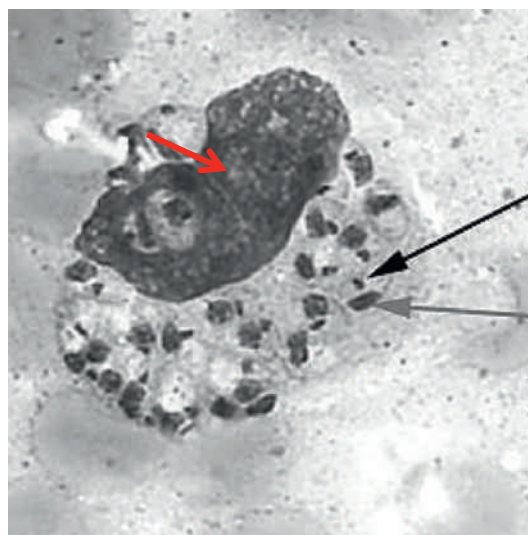


Fig. 2 Light-microscopic examination of a stained bone marrow specimen from a patient with visceral leishmaniasis, showing a macrophage (note large nucleus, red arrow) containing intracytoplasmic multiple *Leishmania amastigotes* (gray arrow). Visualization of the kinetoplast (black arrow) ascertains leishmaniasis diagnosis. Credit: CDC/DPDx.

leishmaniasis, 20 mg/kg body weight daily for 28–30 days. Side effects are common and include anorexia, vomiting, nausea, abdominal pain, malaise, myalgia, arthralgia, headache, and lethargy, but the overall cure rate is >90%. Amphotericin B a polyene antibiotic given daily or on alternate days by intravenous infusion in 5% dextrose of 0.75–1.0 mg/kg per day for 15–20 days was 99% effective in India. Infusion reactions, including higher fever, rigor and chills, are common. Lipid formulations, liposomal amphotericin B, amphotericin B lipid complex and amphotericin B colloidal dispersion, are as effective as amphotericin B deoxycholate, but significantly less toxic. Intramuscularly or intravenously administered paromomycin, pentamidine isethionate, and miltefosine have also shown efficacy, as well as, adverse reactions (Croft and Coombs, 2003; Ghorbani and Farhoudi, 2017). These drugs predominantly disrupt parasite membrane, and prevent protein synthesis, among others.

Vaccines. The development of a vaccine will benefit an estimated 350 million people who are at risk of developing the disease worldwide (Gillespie et al., 2016; Okwor and Uzonna, 2016). No human vaccine is currently licensed against visceral or cutaneous leishmaniasis. Leishmaniasis vaccination started with the use of killed or live-attenuated parasites, which were substituted by second-generation subunit or recombinant DNA and protein vaccines (Moafi et al., 2019). An amastigote-specific antigen (A2) that contains immunogenic epitopes for CD4+ T helper (Th) cells and CD8+ cytotoxic T lymphocyte (CTL) epitopes was combined with saponin, alum and IL-12, or expressed by attenuated adenovirus, and shown to be protective in mice, dogs and nonhuman-primates (Fernandes et al., 2012). Currently, there is one live vaccine for humans licensed in Uzbekistan, and four licensed veterinary vaccines against visceral leishmaniasis: Leishmune[®], CaniLeish[®], Leishtec[®] and Letifend[®] that induced 60–80% protection (Moafi et al., 2019; Palatnik-de-Sousa and Nico, 2020). A human vaccine based on a mixture of nucleoside hydrolase NH36 of *Leishmania* (*L.*) *donovani*, the main antigen of the Leishmune[®] vaccine and the sterol 24-c-methyltransferase (SMT) from *L. (L.) infantum* has reached Phase I clinical trial (Palatnik-de-Sousa, 2019; Palatnik-de-Sousa and Nico, 2020). The vaccine induced high levels of IFN- γ , tumor necrosis factor- α (TNF- α), IL-2, IL-5, and IL-10 in healthy volunteers residing in a non-endemic country (USA), and was considered safe and well-tolerated (<https://clinicaltrials.gov/ct2/show/NCT01751048>).

Numerous studies examined the efficacy of peptide vaccines derived from *L. major*, *L. Mexicana*, and *L. amazonensis* against cutaneous, and *L. donovani* and *L. infantum* for protection against visceral leishmaniasis (reviewed in de Brito et al., 2018). The commonest approach was the selection of parasite peptides containing T and B cell epitopes. A most interesting strategy involving sequencing, mass spectrometry, and bioinformatics analyses of *Leishmania*-derived peptides associated with HLA class I and class II molecules on the surface of infected human macrophages was apparently not adopted (Kote et al., 2020). A different strategy targeted a sandfly salivary antigen (of 15 kDa) that facilitates *Leishmania* infection of the host for use as an adjuvant. Immune responses to vaccination with the insect salivary antigen impeded the ability of the parasite to infect its host, yielding protection against initiation of infection (Chen et al., 2020). The insect salivary antigen combined with the *L. donovani* nucleoside hydrolase, NH36, is now under development as a potential leishmaniasis vaccine (Chen et al., 2020; Liu et al., 2021).

Malaria

Life cycle. Malaria is an insect (female mosquito of the genus *Anopheles*)-borne severe disease that primarily affects humans. Infection with protozoans of the *Plasmodium* group causes symptoms that typically include fever, chills, tiredness, vomiting, and headaches. In severe cases, it can cause seizures, coma, and death. The disease is widespread in the tropical and subtropical regions around the equator. This includes much of sub-Saharan Africa, Asia, and Latin America. Five species of this single-celled microorganism can infect humans. Most deaths are caused by *Plasmodium falciparum*, which caused 99.7% of estimated malaria cases in sub-Saharan Africa, 71% of cases in the Eastern Mediterranean, 50% of cases in South-East Asia Region, and 65% in the Western Pacific. *Plasmodium vivax* is the predominant parasite in Latin America, representing 75% of malaria cases but like *P. ovale*, and *P. malariae* cause a milder form of malaria (WHO, 2020b; Laurens, 2020). In 2018 there were 228 million cases of malaria worldwide resulting in an estimated 405,000 deaths. In 2019, there were an estimated 229 million cases of malaria worldwide, and the number of malaria deaths stood at 409,000, meaning one million individuals are infected each year (WHO, 2018, 2020c). Children aged under 5 years are the most vulnerable group affected by malaria; in 2019, they accounted for 67% (274,000) of all malaria deaths worldwide. Approximately 93% of the cases and 94% of deaths occurred in Africa. Malaria is commonly associated with poverty and has a significant negative effect on economic development. In Africa, it is estimated to result in losses of 12 billion US \$ a year due to increased healthcare costs, lost ability to work, and adverse effects on tourism (Bokhari et al., 2007; WHO, 2012).

The classic symptom of malaria is paroxysm—a cyclical occurrence of sudden coldness followed by shivering and then fever and sweating, occurring every 2 days (tertian fever) in *P. vivax* and *P. ovale* infections, and every 3 days (quartan fever) for *P. malariae*. Severe malaria is usually caused by *P. falciparum* (often referred to as falciparum malaria), and elicits recurrent fever every 36–48 h, or a less pronounced and almost continuous fever. Symptoms of falciparum malaria arise 9–30 days after infection. Individuals with cerebral malaria frequently exhibit neurological symptoms, including abnormal posturing, seizures, or coma (Beare et al., 2011; Bartoloni and Zammarchi, 2012; Ponsford et al., 2012; Laurens, 2020).

Symptoms of malaria can recur after varying symptom-free periods. Depending upon the cause, recurrence can be classified as either recrudescence, relapse, or reinfection. Recrudescence is when symptoms return after a symptom-free period. It is caused by parasites surviving in the blood as a result of inadequate or ineffective treatment. Relapse is when symptoms reappear after the parasites have been eliminated from the blood but persist as dormant hypnozoites in liver cells. Reinfection cannot readily be distinguished from recrudescence, although recurrence of infection within 2 weeks of treatment for the initial infection is typically attributed to treatment failure (Markus, 2017, 2018, 2020). People may develop some immunity when exposed to frequent infections (Tran et al., 2012).

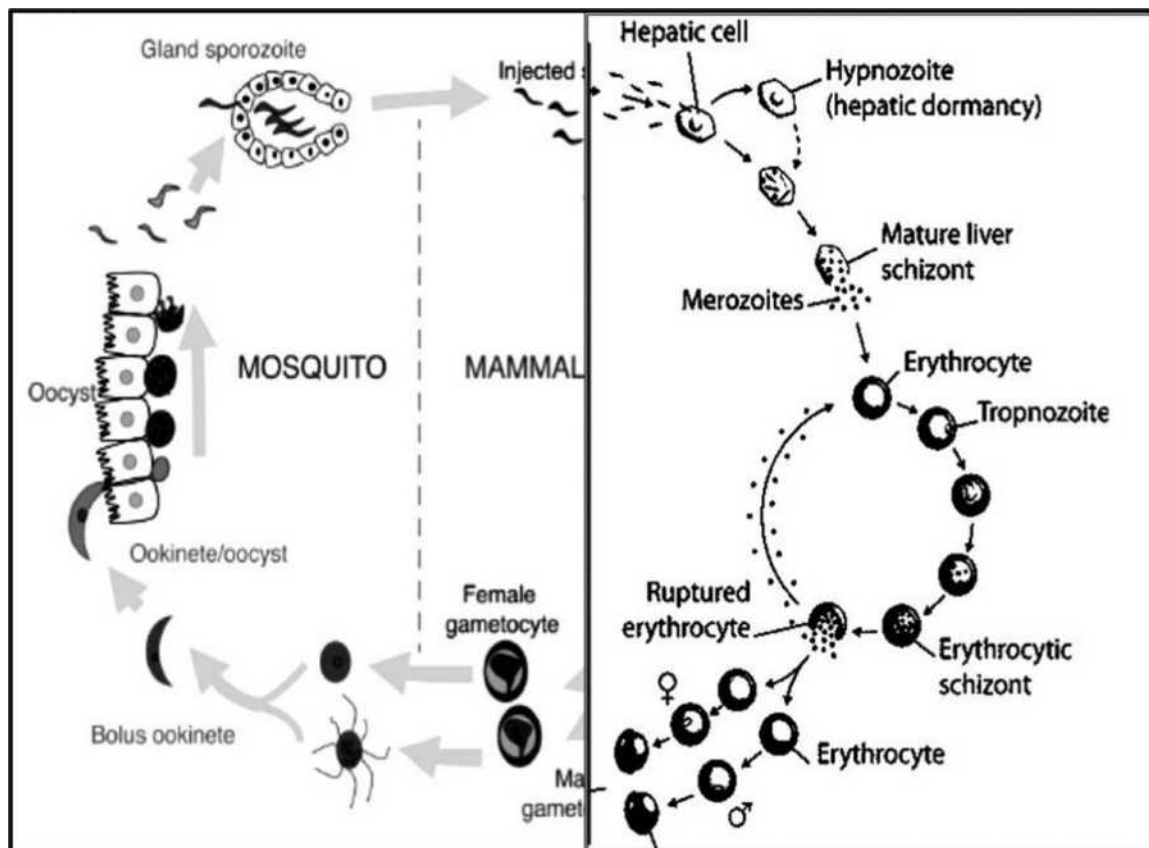


Fig. 3 Malarial life cycle in the mosquito and mammal (left and right panel, respectively). Ma gamet = male gametocyte. Modified from Open Course Ware.

Female *Anopheles* mosquito is the definitive host. A mosquito takes a blood meal from an infected human, taking in *Plasmodia* oocysts, and male and female gametocytes which are in the blood. The male and female gametocytes mature in the mosquito gut, fuse and form an *ookinete*—a fertilized, motile zygote. Ookinetes develop into new sporozoites that migrate to the insect's salivary glands, ready to infect a new vertebrate host (Fig. 3) (Siciliano et al., 2020).

When an infected mosquito takes a blood meal of the intermediate host, sporozoites in the mosquito's saliva are deposited in the skin dermis and move randomly for 1–3 h until they contact the endothelium of lymphatic or blood capillary. Sporozoites have to traverse epithelial cells and cross the endothelial barrier to access the blood stream. Sporozoites are rapidly carried to the liver sinusoids and invade hepatocytes, protecting themselves within parasitophorous vacuoles. Sporozoites remain quiescent for a while as hypnozoites (hepatic dormancy phase, notably *P. vivax* and *P. ovale*) before replicating asexually and asymptotically for a period of 8–30 days (tissue schizogony), producing thousands of schizonts that differentiate into merozoites. Following hepatocyte rupture, merozoites escape, wrapped in the cell membrane of their host liver cell, into the blood to begin the erythrocytic stage of the life cycle. Asexual multiplication cycles (blood schizogony) produce multinucleated forms, called schizonts, which undergo segmentation to form daughter merozoites, at which point the red blood cells burst and merozoites invade the blood. Waves of fever arise from simultaneous waves of merozoites escaping and infecting red blood cells. Numerous merozoites develop into immature gametocytes, which are the precursors of male and female gametes (Tan and Blackman, 2021).

Diagnosis. Malaria is typically diagnosed by the microscopic examination of stained films of peripheral blood. Standard procedures for the preparation, staining and microscopic examination of blood films for malaria parasites were recently presented (Dhorda et al., 2020). Malaria antigen detection with multiple antigens-based rapid diagnostic tests is widely used to diagnose malaria and estimate prevalence (Jang et al., 2020). Methods that use PCR to detect the parasite DNA extracted from blood spots or whole blood samples have been developed, but are not widely used in areas where malaria is common due to their cost and complexity (Srisutham et al., 2020; Mahittikorn et al., 2021). Combination of microscopy, rapid antigen-based detection test, and PCR achieves 100% specificity and sensitivity (Kotepui et al., 2020).

Treatment agents. Malaria is preventable and curable. The risk of disease can be reduced by preventing mosquito bites through the use of insecticides integrated into bednets and insect repellents or with mosquito-control measures such as spraying insecticides, and draining standing water. In 2019, an estimated 46% of all people at risk of malaria in Africa were protected by an insecticide-treated net, compared to 2% in 2000. Indoor residual spraying (IRS) with insecticides is another powerful way to

rapidly reduce malaria transmission (World Health Organization, 2020b; Bath et al., 2021). Anopheles mosquitoes resistance to pyrethroid insecticides compromises malaria control and elimination (WHO, 2012; Mnzava et al., 2015; Moyes et al., 2020).

The recommended treatment for malaria is a combination of antimalarial medications that include artemisinin, mefloquine, lumefantrine, or sulfadoxine/pyrimethamine (van der Pluijm et al., 2020). Unfortunately artemisinin resistance in *P. falciparum* is emerging, and is now prevalent across the Greater Mekong subregion (Imwong et al., 2017, 2020). The risk of spreading artemisinin-resistant parasites from the Greater Mekong subregion to Africa, as happened in the 1980s with chloroquine and pyrimethamine resistance, is a major concern, which appears to have already materialized in Burkina Faso (Gansané et al., 2021). Quinine, along with doxycycline, may be used if artemisinin is not available. It must be noted, however, that chloroquine-resistant *P. falciparum* has spread to most malarial areas (Ménard and Dondorp, 2017; Ménard and Mayor, 2020). Since 2012, WHO has recommended seasonal malaria chemoprevention as an additional malaria prevention strategy for areas of the Sahel sub-region of Africa (WHO, 2020b). The strategy involves the administration of monthly courses of amodiaquine plus sulfadoxine-pyrimethamine to all children less than 5 years of age during the high transmission season. Prophylactic activity may certainly be achieved and is of great importance. Yet, the strategy of mass drug administration definitely increases the risk of parasite resistance to the used drugs.

The risk of parasite resistance to commonly used drugs compels the search for alternative medicines. Novel compounds that possess different modes of action or novel modes of inhibition of known targets are therefore urgently needed to circumvent antimalarial drug resistance and continue treatment efficacy (Yang et al., 2021). Recently, several benzimidazole derivatives displayed transmission-blocking potential and activity against multiple stages of *Plasmodium* parasites (Leshabane et al., 2021). Of importance is to resort to extracts and derivatives of plants used in traditional medicine (Lemma et al., 2017; Pan et al., 2018), especially that the current antimalarial drug of choice artemisinin was originally obtained from the leaves of *Artemisia annua* L (Tu, 2011). Thus, remarkable antiparasmodial in vitro activity was shown for methanol, aqueous or alkaloids extracts of *Terminalia avicenioides*, *T. mantaly*, *T. superba*, *T. albidia*, *Combretum collinum* and *Ficus capraefolia* (Sanon et al., 2013; Mbouna et al., 2018; Camara et al., 2019). Recently, the benefits of marine natural products (Goto et al., 2021) and sponges metabolites, notably alkaloids, terpenoids, polyketides endoperoxides and glycosphingolipids in malaria treatment were reviewed (Aguar et al., 2021).

Vaccines. As of 2020, there is one vaccine, RTS,S/AS01 (RTS/S), based on *P. falciparum* pre-erythrocytic stage circumsporozoite protein, which has been shown to reduce the risk of malaria by about 40% in children in Africa (WHO, 2020d). The vaccine is a recombinant protein consisting of immunodominant B cell (19 NANP of the 37–44 NANP amino acid repeat sequences in the central region, R) and T cell (the polymorphic carboxy-terminal region, T) epitopes, coexpressed with hepatitis B surface antigen (S), and formulated with the AS01 adjuvant (S) (Pance, 2019). It shows efficacy in control of *P. falciparum* but is ineffective against *P. vivax* (Laurens, 2020). The vaccine is administered as primary three-dose series with a fourth dose given approximately 18 months later. Significant efficacy against severe malaria was noted in young infants or children who did receive a booster dose of the vaccine. Yet, only a substantial reduction in the number of cases of clinical malaria was observed in children who did not receive the booster dose. Unfortunately, the efficacy of RTS,S/AS01 against clinical and severe malaria decreased over time since vaccination (Olotu et al., 2016; Vandoolaeghe and Schuerman, 2018; Bell et al., 2020; Keating, 2020). Additionally, in-depth safety study was conducted in seven sub-Saharan countries, Burkina Faso, Ghana, Gabon, Kenya, Tanzania, Malawi, and Mozambique. The results based on 8922 children (enrolled at 5–17 months) and 6537 infants (enrolled at 6–12 weeks) who were randomized in three groups to receive 4 doses of RTS,S/AS01, non-malaria control vaccine, or 3 RTS,S/AS01 doses plus control challenged the RTS,S/AS01 benefit versus risk profile in real-life settings (Guerra Mendoza et al., 2019).

Children given the RTS/s vaccine showed increase in serum levels of IgG and IgA to all circumsporozoite proteins, and numerous vaccine-unrelated *P. falciparum* antigens, i.e., vaccine-specific and non-specific antibodies, with no statistically significant association with protection (Dobaño et al., 2019; Suau et al., 2021). Likewise, associations between protection and levels of vaccine-specific CD4⁺ T cells and IFN- γ have been identified, but not consistently (Moris et al., 2018).

Immune responses to RTS vaccine essentially target the sporozoites delivered by the mosquito into the blood stream. These sporozoites rapidly invade the liver cells, leaving a narrow window for specific antibodies to control the infection (Rogers et al., 2021). Indeed, care in rational selection of target vaccine antigens should be exercised before embarking in human trials, and first and foremost before contemplating use of controversial techniques to target afflicted infants and very young children (Mahase, 2021). A most commendable example has recently been given. Antibodies specific for the *P. falciparum* protein PF3D7_1021800 were previously associated with human protection against malaria, leading to an interest in PF3D7_1021800 as a candidate vaccine antigen. Antibodies to the protein were reported to inhibit schizont egress, resulting in it being named schizont egress antigen-1 (SEA1). It was recently shown that SEA1 localizes in dividing parasite nuclei and is necessary for the correct segregation of replicated DNA into individual daughter merozoites, but its localization and function preclude it to be a target for protective antibodies (Perrin et al., 2021).

Of note, numerous vaccine antigens have been selected targeting the sporozoites to prevent establishment of infection, among which the full length circumsporozoite protein and the thrombospondin-related anonymous protein. Many have reached different phases of clinical trials (Duffy et al., 2012; Pance, 2020; Friedman-Klabanoff et al., 2021). However, none has led to promising results regarding the effective control of the disease (Molina-Franky et al., 2020). Surface membrane antigens of merozoites, notably the invasion protein AMA1 (Sirima et al., 2017; França et al., 2021), and the parasite antigens embedded in infected erythrocytes membranes, such as *P. falciparum* Erythrocyte Membrane Protein-1 (PfEMP1), were also considered promising vaccines for inducing immune responses capable of interfering with red blood cells invasion, destruction, and the waves of disease (Frimpong et al., 2018). Vaccines targeting the gametocytes, among which the antigens Pfs230C and Pfs48/45, showed efficacy in blocking transmission from humans to the mosquito justifying the possibility of reaching different phases of clinical trials, but showed limited protection (Pance, 2020).

Another critical concern was raised regarding the degree of selected vaccine antigens genetic variability among *Plasmodium* species and strains (Pance, 2020). It is not understood how the RTS/S vaccine has progressed that far knowing the gene encoding *P. falciparum* circumsporozoite protein central and carboxy terminal regions harbors great genetic diversity, the low prevalence of the vaccine malaria haplotype in Africa and Asia, and the antibody efficacy decrease with any amino acid difference (Pance, 2019). Vaccine candidates designed to target surface proteins such as AMA1 and PfEMP1 are highly immunogenic but extremely polymorphic (Frimpong et al., 2018; Bittencourt et al., 2020). In that respect, the sequencing of *P. falciparum* whole genome greatly assisted researchers in the selection of potentially protective candidate vaccines, which are highly conserved among *Plasmodium* species and strains (Gardner et al., 2002). A prominent example is Pfs25 which high conservation and effectiveness in inducing transmission-blocking antibodies promote it to the status of a leading vaccine candidate (Juliano et al., 2016; Pance, 2020).

Toxoplasmosis

Life cycle. Toxoplasmosis is a food-borne disease that results from infection with *Toxoplasma gondii* the world's most common parasite, with 40 million in the most developed country, the USA, carrying the parasite (Jones and Dubey, 2012; Jones et al., 2014). In many areas of the world that have hot, humid climates and lower altitudes, more than 60% of some populations are infected with *T. gondii* (Saadatnia and Golkar, 2012; Heavey, 2018). Toxoplasmosis is not a person to person infectious disease. Infection usually occurs by exposure to infected cat feces, eating undercooked meat contaminated with the parasite cysts, and mother-to-child transmission during pregnancy. Most people affected never develop signs and symptoms except for persons with weakened immune systems and infants born to infected mothers. A most serious complication is stillbirth or miscarriage in women newly infected with *Toxoplasma* during or shortly before pregnancy. A small number of infants who survive are likely to be born with serious problems, notably seizures, spleno- and hepatomegaly, and eye infections. Many infected children show hearing loss, mental disability or extremely serious eye infections in their teens (Heavey, 2018).

Toxoplasma gondii is a single-celled, protozoan parasite that can orally infect virtually all warm-blooded vertebrates, especially birds, most animals, and humans. Because *T. gondii* infectious oocysts are excreted only in cat feces, wild and domestic cats are the parasite's only known definitive host. Cats become infected by eating infected rodents, birds, or raw meat. The parasites sexually reproduce in cat intestines. Fertilization of haploid macrogametes by haploid microgametes results in diploid zygotes, around which a protective wall develops, forming unsporulated oocysts that pass in the feces. Cats can shed millions of oocysts in their feces for as long as 3 weeks, especially after the first infection. Oocysts take 1–5 days to sporulate in the environment, especially in hot and humid areas. Oocytes can remain infectious for more than a year in soil or water under ambient conditions, and are infective to birds, rodents, livestock, and humans via the oral route. After an intermediate host ingests oocysts, sporozoites are liberated in the intestine where they invade enterocytes and differentiate into rapidly dividing tachyzoites. Tachyzoites are disseminated via the blood stream to all tissues, and are able to infect all nucleated cells. The immune responses trigger the conversion of tachyzoites into bradyzoites inside cysts, which form 7–10 days after infection and remain viable in multiple organs, including the central nervous system and muscles. These cysts may remain viable throughout the life of the host. Healthy immune system keeps the parasites in the body in an inactive state, providing lifelong immunity to a second infection. In humans, if an underlying disease, chemotherapy, or immunosuppressive medications weaken the immune system, the infection can be reactivated, leading to serious complications (Dubey, 1998; Deng et al., 2020).

Diagnosis. Toxoplasmosis is efficiently diagnosed using serological tests that determine the titer and isotype of anti *T. gondii* specific serum antibodies (Saadatnia and Golkar, 2012; Heavey, 2018). Several fully automated immunoassays are commercially available, notably Architect i4000ISR; Immulite 2000 Xpi, Siemens; Liaison, DiaSorin; and Cobas e601, Roche, and manual immunoassays are easy to devise. However, anti-Toxoplasma IgG quantitative immunoassays still lack standardization (Zhang et al., 2017). Besides serological tests, diagnosis for immunocompromised patients is usually done using PCR, and histological analysis. For congenital cases, definitive diagnosis is through direct PCR detection of the organism from amniotic fluid samples (Ybañez et al., 2020).

Treatment agents. Most healthy people recover from toxoplasmosis without treatment. Persons who show complications must receive long time treatment with combination of pyrimethamine sulfadiazine, folic acid inhibitors, which efficiently kill the replicating tachyzoite stage of the parasite, but show limited efficacy against the slow dividing bradyzoites (Montoya and Liesenfeld, 2004). The parasites are not completely eliminated because of their location within tissue cells in a quiescent phase. Hence, there currently is no approved therapy that can eradicate *T. gondii* tissue cysts, which are responsible for chronic infection (Dunay et al., 2018; Montazeri et al., 2018). Additionally, *folinic acid* is recommended as a remedy against the drugs toxic impact. Bisphenol Z derivatives were recently found to disrupt mitochondrial function and inhibit parasite replication in vitro; in vivo efficacy was not yet assayed (Hatton et al., 2020). Recently, combinations of pyrimethamine, clindamycin, guanabenz, and endochin-like quinolones (ELQs) were hardly able to eradicate brain cysts of *T. gondii*-infected laboratory mice (Doggett et al., 2020).

Vaccines. Thorough guidelines have been published by the Centers for Disease Control and Prevention (<https://www.cdc.gov/parasites/toxoplasmosis/prevent.html>), as there is no vaccine for human *T. gondii* (Singh et al., 2020). Vaccine development over three decades focused on prevention of tissue cyst development and vertical transmission in livestock and cats (Wang et al., 2019). Cysts shield parasites from immune detection and responses, and are unaffected by the immunity elicited by the available veterinary vaccines (Bonačić Marinović et al., 2019; Ramakrishnan et al., 2019). Immunoinformatic approaches (Asghari et al., 2021), and development of protocols for differentiation of embryonic and pluripotent stem cells into a variety of cell types and organoids (Pance, 2021) might overcome the hurdles of devising vaccines against the cyst-shielded *T. gondii*.

Parasitic nematodes (eukaryotes)

Filariasis

Filariasis is again an insect-borne parasitic disease, caused by infection with roundworms of the Filarioidea type that are spread by blood-feeding insects such as *Aedes*, *Anopheles*, and *Culex* mosquitoes (lymphatic filariasis), and black flies of the family Simuliidae (onchocerciasis). These parasites exist in the wild in subtropical parts of southern Asia, Africa, the South Pacific, and parts of South America, and inhabit the human lymphatic system or subcutaneous tissues (Fig. 4) (Klion, 2019; Bizhani et al., 2021).

Lymphatic filariasis

Life cycle. Lymphatic filariasis is a disease of the lymphatic system, caused by the microscopic nematodes, *Wuchereria bancrofti* (the only known etiologic agent in Africa), *Brugia malayi*, and *B. timori* (found only in Indonesia), and transmitted in humans following a blood meal from an infected mosquito. Lymphatic filariasis affects over 120 million people with about 40 million disfigured and incapacitated by the disease throughout the tropics and sub-tropics of Africa, Asia, the Western Pacific, and parts of the Caribbean and South America, namely Haiti, the Dominican Republic, Guyana and Brazil. Around 893 million people in 52 countries worldwide remain threatened by lymphatic filariasis and require preventive chemotherapy to stop the spread of this vicious parasitic infection (Klion, 2019; Mathew et al., 2020; Bizhani et al., 2021).

The organisms, male and female stage L3 larvae, migrate to the lymphatic vessels and lymph nodes in the legs, feet, arms, breast, and scrotum. Over 6 months or more, the larvae molt through two more stages maturing into female of 80–100 mm in length and 100–300 µm in diameter, and male worms of 40 mm by 100 µm about half the size of females. Mating produces thousands of microfilariae, which are sheathed and thread-like, 244–296 µm by 7.5–10 µm. The millions of produced microfilariae and the immune responses they generate elicit inflammation and clogging of the lymph vessels, edema and fibrosis of surrounding tissues, organ swelling, thickening of overlying skin, symptoms of the disease known as elephantiasis, surprisingly enough in percentage of infected persons, and years after infection. The majority of infections are asymptomatic. These asymptomatic infections still cause chronic damage to the lymphatic system with clinical manifestations, such as acute adenolymphangitis, filarial fever, and tropical pulmonary eosinophilia (Lourens and Ferrell, 2019; Zulfiqar and Malik, 2021). Additionally, all infected persons can transmit the disease, as adult worms can survive in human lymph system for up to 6 years while microfilariae move from lymph to blood, and are taken up by the intermediate vector, insects such as *Culex* and *Anopheles* during a blood meal. After ingestion, the microfilariae lose their sheaths and work their way through the wall of the midgut to reach the thoracic muscles. There the microfilariae develop into first-stage larvae and subsequently into second and third-stage larvae. The third-stage larvae migrate through the hemocoel to the mosquito's mouth parts and can infect another human when the mosquito takes a blood meal, injecting the infectious L3 larvae into the dermis layer of the skin of the final host (Fig. 4).

Of note, *Wuchereria* and *Brugia* spp., like many other species of filarial nematodes, harbor an intracellular endosymbiont, *Wolbachia*, which are vital for larval development and adult-worm fertility and survival, as *Wolbachia* is necessary for growth, fertility and viability of the nematode host, while the host supplies amino acids needed for *Wolbachia*'s development (Taylor et al., 2005; Wan Sulaiman et al., 2019; Gunderson et al., 2020; Bulman et al., 2021).

Diagnosis. Definitive diagnosis may be accomplished by identifying microfilariae in thick blood smears stained with Giemsa or hematoxylin and eosin. The microfilariae that cause lymphatic filariasis circulate in the blood at night (called nocturnal periodicity). Blood collection should be done at night to coincide with the appearance of the microfilariae. For increased sensitivity, especially in

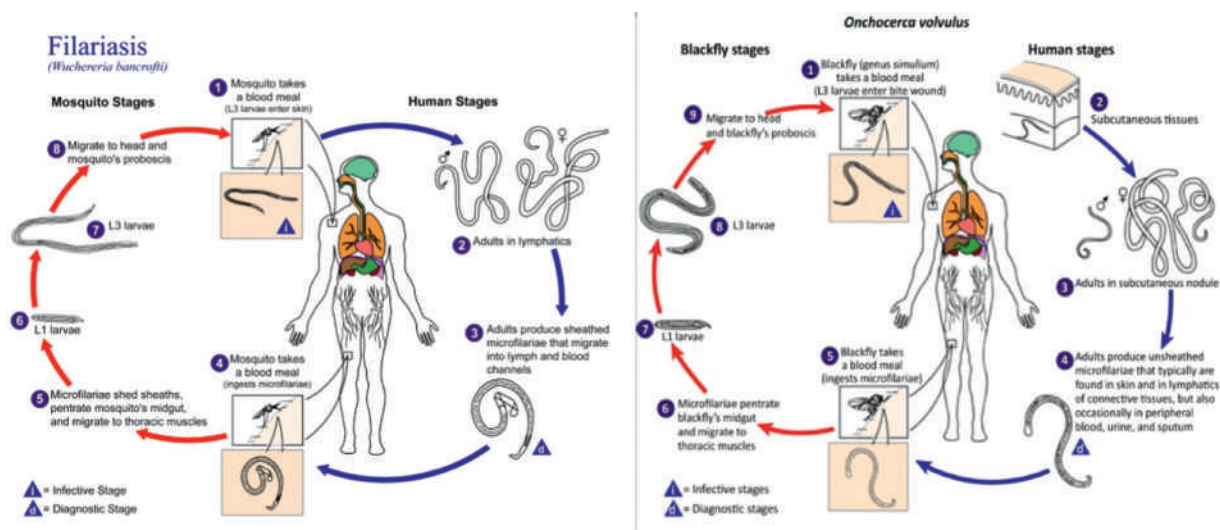


Fig. 4 Life cycle of filarial nematodes causing lymphatic (left) and subcutaneous (right) filariasis. From Centers for Disease Control and Prevention.

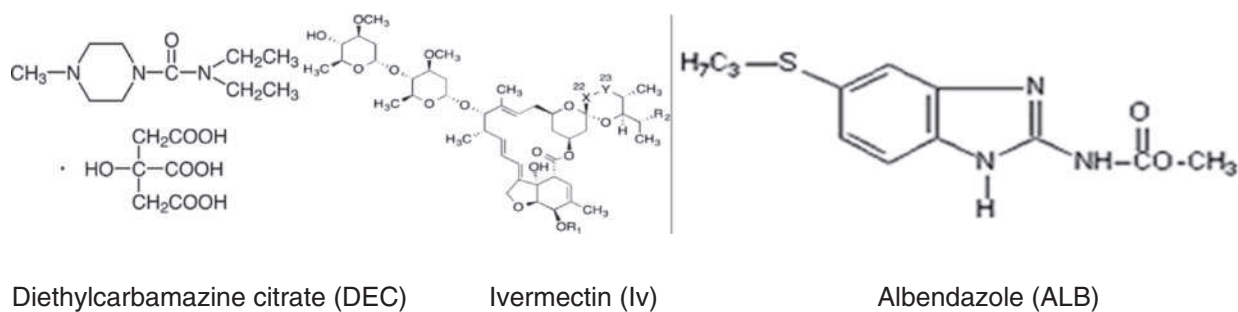


Fig. 5 Drugs of choice for treatment of filariasis kill microfilariae not adult worms.

light infections, one of several concentration methods may be used. Of note, microfilariae are not detected in blood in the early phases of the infection. In such cases, serologic techniques are useful for diagnosis of lymphatic filariasis. Patients with active filarial infection typically have elevated levels of antifilarial IgG4 in the blood and these can be detected using routine assays (Wamae, 1994; Palumbo, 2008).

Treatment agents. Diethylcarbamazine citrate (DEC), Ivermectin (I), and albendazole (ALB) (Fig. 5) combination is the therapy of choice for treatment of persons diagnosed with filariasis despite it kills only the microfilariae and does not affect the adult worms (Krentel et al., 2021).

The development of the World Health Organization's Global Programme to Eliminate Lymphatic Filariasis (GPELF) is based on annual mass treatment with these drugs, aiming to reduce the level of microfilariae in the blood and thus, diminish transmission of infection (Ramzy et al., 2019; Deribe et al., 2020; Ottesen and Horton, 2020). In countries without onchocerciasis, the combination of Iv (200 µg/kg) together with DEC (6 mg/kg) and ALB (400 mg) can safely clear almost all microfilariae from the blood of infected people within a few weeks (Krentel et al., 2021). In support of the 3 drugs-combination (IDA), naive adults with *W. bancrofti* microfilaremia in Côte d'Ivoire were randomized to receive a single dose of IDA ($n = 43$) or 3 annual doses of Iv and ALB (IA) ($n = 52$) in an open-label, single-blinded trial. At 6 and 12 months post treatment, IDA was significantly ($P < 0.001$) superior to DEC-free IA in clearing microfilariae. A substantial number of persons examined 36 months post-treatment were still infected due to reinfection or the inability of the drugs combined to eliminate adult worms, which may survive in the human hosts for up to 6 years (Bjerum et al., 2020). In a similar setting, Iv plus ALB (IA) was found to be more effective than ALB alone. Indeed, repeated semiannual treatment with ALB alone was shown to be macrofilaricidal for *W. bancrofti* and leads to sustained reductions in microfilariae counts. However IA was more effective for completely clearing microfilariae than ALB 400 mg or 800 mg (Ouattara et al., 2021). The safety and efficacy of mass drug treatment (MDA) with a single oral dose of IDA or DA (DEC and ALB) were recently examined in children >5 years old in India. No serious adverse reactions were recorded: only transient fever, headache and dizziness. Combination of the three drugs (IDA) was more effective for clearing microfilariae than ivermectin-free DA treatment (84% vs. 61.8%, $P < 0.001$) (Jambulingam et al., 2021).

Adult worm killing may be achieved with treatment with doxycycline (200 mg/day for 4–6 weeks), as it was shown to deplete *Wolbachia* and eventually eliminate the adult worms (Wan Sulaiman et al., 2019). In Tanzania, ultrasonography detected adult worms in only six (22%) of 27 individuals treated with doxycycline compared with 24 (88%) of 27 with placebo at 14 months after the start of treatment (Taylor et al., 2005). Recently, reports documented the threat of *Wolbachia* rebound following antibiotic treatment, likely because of the bacterial persistence in privileged sites, such as ovaries, in the filarial adult female worms (Gunderson et al., 2020), or counterintuitive response to antibiotic dose response, dubbed the "Eagle effect" (Bulman et al., 2021).

Vector control interventions conducted by other programs, particularly malaria vector control, have had a profound effect in stopping transmission in some endemic countries in the region. Physical control such as use of bed nets, sound traps, and removal of mosquito breeding or swarming sites has met with some success. Plant extracts-based and essential oil repellents, insecticides, microbes-derived toxic peptides, biological agents, and genetic modifications techniques have been widely used (Amer and Mehlhorn, 2006; Parihar et al., 2020; Lupenza et al., 2021a) especially targeting mosquitoes of the genera *Aedes*, *Anopheles*, and *Culex* (Fig. 6). Nevertheless, interruption of transmission of the infection is still not evident in numerous sub-Saharan African countries (Famakinde, 2018; Mathew et al., 2020; Yokoly et al., 2020; Lupenza et al., 2021b).

Vaccines. There is no commercially licensed prophylactic vaccine available for lymphatic filariasis (FL). With over 880 million people at risk, there is great potential for developing a vaccine for LF. An effective prophylactic vaccine should confer long-lasting and robust protective immune responses in the vaccinated individual and overcome the complex mechanisms of parasite immune evasion, especially the microfilariae sheath (Joardar et al., 2021). The most promising vaccine candidates would elicit immune responses directed against the infective or early developing larval L3 stages, the most vulnerable stage of the parasite in the mammalian host in terms of its size as well as biological requirements. After entry into the body, L3 must wade its way through the subcutaneous tissue into presumably the nearest lymphatic vessel, where it needs to rapidly metabolize, grow in size and develop and molt within about 10–12 days into L4 stage. An L3 larva-derived vaccine candidate must show considerable homology in *W. bancrofti*, *B. malayi* and *B. timori* to be useful in different settings and countries (McNulty et al., 2013). Vaccine-induced specific



Fig. 6 Aedes mosquitoes are black and have white patches. Culex and Anopheles are yellowish, but Culex stay parallel to the surface, while Anopheles mosquitoes rest at ~45 degree angle. Source: Dr. Mubarak Ali Aldoub.

antibodies, especially of the IgG1 and IgG3 isotypes, may affect L3 larvae, which are still unsheathed and can be vulnerable to ADCC (Dakshinamoorthy et al., 2013). Cell adherence to *B. malayi* L3 larvae has been demonstrated (Kalyanasundaram et al., 2020). Accordingly, vaccine candidates must be exposed on the L3 larvae surface membrane, and additionally should show low homology to corresponding human molecules and must fail to induce considerable IgE responses (Kalyanasundaram et al., 2020). Nearly all attempts to develop a vaccine against LF with a single antigen (monovalent) gave unsatisfactory results. Recently, *B. malayi* calreticulin was found to be immunogenic in *Mastomys coucha*, a permissive model for LF, but elicited partial protection (Yadav et al., 2021). A vaccine based on B-cell and T-cell epitopes derived from the aspartic protease of *B. malayi* (BmASP-1) was found to display significant humoral and cell mediated immune responses using in-silico approaches, but was not tested in vivo (Das et al., 2021). Conversely, a chimera of molecules possessing the selection criteria mentioned above was promising. The fusion of *B. malayi* heat shock protein 12.6 (H), abundant larval transcript -2 (A), and tetraspanin large extra-cellular loop (T) (Dakshinamoorthy et al., 2014), together with thioredoxin peroxidase-2 (X) in a tetravalent recombinant construct, rBmHAXT (Khatri et al., 2018) provided the highest protection levels in *Rhesus macaques*. This construct is considered a leading prophylactic vaccine formulation because it elicits high protection levels in challenged non-human primates, considered the best, if not the only, models available to study the protective potential of filarial vaccines. Of significant importance was the discovery that the multivalent recombinant construct was L3 larvicidal, microfilaricidal, and additionally impaired the fecundity of *B. malayi* adult female worms (Khatri et al., 2020). As of today, the promising rBmHAXT vaccine in conjunction with AL019 (alum plus glucopyranosyl lipid adjuvant-stable emulsion) meets various hurdles and blocks delaying or preventing preclinical and clinical trials in humans (Kalyanasundaram et al., 2020).

Onchocerciasis

Life cycle. Onchocerciasis, also known as river blindness or subcutaneous filariasis, is a disease caused by infection with the parasitic worm *Onchocerca volvulus*, transmitted by repeated bites of the vector, female black fly of the genus *Simulium* as males feed on nectar. More than 99% of 25 million people infected worldwide live in 31 countries in sub-Saharan Africa and 1% in the Arabian Peninsula, and small localities in Mexico, Colombia, Venezuela, Guatemala, and Brazil. The WHO estimates that 90 million people are at risk, with more than 99% of them in Africa. The disease is responsible for the annual loss of close to 1 million DALYs. Vision loss is estimated to affect 1.15 million people. Economic losses were estimated at 30 million US dollars (Gyasi et al., 2021).

Symptoms appear months or years after infection, due to intense inflammatory immune responses to the parasites resulting in rashes, severe itching, skin lesions, swelling and altered pigmentation, and nodules, bumps and lumps under the skin. Skin-related afflictions and damage prevail in forest areas. Some infected people develop ocular lesions which can lead to visual impairment and sometimes permanent blindness, especially in the African savanna, along flowing streams and rivers where the vector breeds, hence the name river blindness (Remme, 2004). Association between onchocerciasis and epilepsy was demonstrated (Vinkeles Melchers et al., 2018; Siewe Fodjo et al., 2020).

The prevalence of human onchocerciasis is determined by the distance between where *Simulium damnosum sensu lato* (s.l.), the main vector in Africa breed, and where people live and work, as the flies typically have an active flight range of around 15 km. Endemicity of the infection is also directly related to the abundance of the vector. Fast-flowing rivers and streams, and high levels of oxygenation needed for larval development are ecological conditions favorable for *Simulium* breeding. New vector breeding sites and seasonal changes impact the pattern of disease transmission and create public health problems. Additionally, cytogenetic differences in *S. damnosum* s.l. determine whether they transmit the savanna strain of *O. volvulus* that most often causes blindness, or the forest strain of the parasite that causes skin disease as its main pathogenicity rather than an eye disease (Cheke, 2017; Nikiéma et al., 2019; Zarroug et al., 2016, 2019).

The definitive hosts of *Onchocerca volvulus* are humans. Female blackflies taking a blood meal from an infected person ingest *O. volvulus* microfilariae. Development into infective L3 larvae that are transmitted to a different host during subsequent blood meals resembles that in lymphatic filariasis vectors (Fig. 4). Ingested microfilariae transform over 1 to 3 weeks by moving from the insect gut into the thoracic flight muscles as an L2 larval stage, develop into an infective L3 larval stage, and then migrate into the salivary gland for subsequent transmission during the next blood meal. The L3 larvae develop via L4 into adult worms, macrofilariae, in fibrous sub-cutaneous nodules, located commonly around the iliac crest, the head, and torso, in approximately

12–18 months. Adult worms live for around 10–15 years. Each nodule may contain one or two female worms and a single male that is usually smaller in size. Adult worms produce millions of microfilariae of 250–300 μm by 8 μm , which migrate through the skin, eyes, and other organs, and have a lifespan of approximately 12–15 months (Basáñez et al., 2009) (Fig. 4).

Diagnosis. Onchocerciasis diagnosis, monitoring, and evaluation are based on detection of the parasites in skin nodules, skin biopsies, or skin scrapings by light microscopy. Polymerase chain reaction techniques are increasingly used to improve sensitivity and confirm the identity and load of *O. volvulus* in residual skin snips (Lloyd et al., 2015; Thiele et al., 2016).

Treatment agents. Oral ivermectin is the drug of choice for the treatment of onchocerciasis. A dose of 150 $\mu\text{g}/\text{kg}$ is recommended once or twice a year for 10–15 years, the life span of the adult worms which continuously produce microfilariae. The treatment is accompanied by severe adverse reactions, notably headaches, myalgia, arthralgia, and neurological symptoms, in only 1:10,000 treated patients (Makenga Bof et al., 2019). Ivermectin paralyzes or kills the microfilariae but has no effects on the adult worm viability, though it reduces the fertility of female adult worm at least temporarily. Skin microfilariae are reduced by 50% in 1 day, 94% in 1 week, and 98–99% by 1–2 months, following a single dose of ivermectin (Basáñez et al., 2008; Gyasi et al., 2021). However, microfilariae repopulation of skin and eye resumes in a few months, and this is due to continuous production by the adult worm (Nikiéma et al., 2021).

Moxidectin, a member of the macrocyclic lactone family of anthelmintic drugs, was recently approved for the treatment of onchocerciasis as a microfilaricidal drug in individuals over the age of 12. In humans, a single dose of moxidectin (8 mg) appeared to be more efficacious than a single dose of ivermectin (150 $\mu\text{g}/\text{kg}$) in terms of inhibiting L3 and L4 molting, and microfilariae motility and viability (Opoku et al., 2018; Prichard and Geary, 2019; Jawahar et al., 2021).

Effective cures targeting the adult parasites (macrofilaricides) which survive and reproduce in the host for very long periods are needed (Hawryluk, 2020; Tyagi et al., 2021). Tablet doxycycline is promising in this regard, and may kill the adult worm by killing *Wolbachia*, endosymbiotic, rickettsia-like bacteria, which are crucial for the survival and fecundity of microfilariae. The recommended dose is oral 100 mg or 200 mg once daily for 6 weeks. Doxycycline can sterilize up to 90% of adult female worms and kill at least 60% adult female worms 20 months after treatment (Debrah et al., 2015; Abegunde et al., 2016; Wan Sulaiman et al., 2019). Two potential macrofilaricidal drugs, oxfendazole and emodepside are currently under clinical development (Hübner et al., 2020; Jawahar et al., 2021).

Vaccines. There is no vaccine available and it is difficult to anticipate vaccine development and progress till the commercial level targeting less than 100 million people at risk of onchocerciasis. The Onchocerciasis Vaccine for Africa (TOVA) partners 25 years efforts have been crowned by the selection of three promising candidate vaccine antigens which evoke strong protective responses capable of reducing challenge parasite burden of immunized animals by more than 90%. The selected molecules are on the surface and secretory-excretory products of L3 and L4 larvae, adult worms and/or microfilariae. Ov-CPI-2 is a cysteine protease inhibitor located at the basal layer of cuticle, Ov-Ral-2 and Ov-103 are novel, nematode-specific molecules, located in the hypodermis. The three molecules were expressed in bacterial and yeast expression systems. Combination of the antigens by either co-administration vaccine strategies, or single injections using a recombinant fusion protein vaccine induced in mice enhanced levels of protective immunity, demonstrating that the antigens could act synergistically in both systems. Only adjuvants that induced Th2 responses, as determined by cytokine profiles, were effective at enhancing the vaccine efficacy, consistent with reports showing that IL-4, IL-5, and functional eosinophils are necessary for the development of adaptive immunity in onchocerciasis. Protection in genetically diverse mice strains and humans proved to be dependent on Ov-Ral-2 and Ov-103-specific antibodies, predominantly of the IgG1 and IgG3 isotype together with neutrophils, macrophages and eosinophils (Lustigman et al., 2018; George et al., 2019; Ryan et al., 2021). TOVA aims to take at least one of these vaccine candidates through Phase I trials by 2025. Meanwhile, immuno-informatics analyses attempt to identify novel vaccine candidates in multi T and B cell epitopes- based vaccine formulation (Shey et al., 2019, 2021).

Soil-transmitted helminths (*Ancylostoma*, *Necator*, *Ascaris*)

Ancylostoma and *Necator*

Life cycle. There are no vectors or intermediate host to incriminate in transmission of infection caused by the helminth nematodes, *Ancylostoma duodenale* and *Necator americanus* in humans. Eggs in human feces hatch in humid and warm soil within 24 to 48 h releasing a first stage rhabditiform larva which molts twice to develop to an L3, 600 μm filariform, infective stage. The larvae pierce the skin of humans walking bare foot or wading in contaminated water, access the circulation, and are swept to the heart and lungs. Following rupture of the lung blood capillaries, the larvae in the parenchyma reach the trachea and pharynx. Swallowed larvae molt twice in the wall of the small intestine and mature into male and female adult worms, of 8–15 mm in length by 0.4–0.6 mm in diameter. Mated worms produce thousands of eggs daily that are released to the exterior to contaminate other individuals (Hotez et al., 2005, 2008).

Ancylostoma duodenale and *N. americanus* are common parasites with an estimated 576–740 million individuals infected, of which 80 million are severely infected, and more than 1.5 billion people, notably pre-school and school-age children, at risk in tropical and sub-tropical poor rural regions of America, Asia, and Africa (Bundy et al., 2020). Northern Africa is plagued by *A. duodenale*. The disease is characterized by malnutrition, abdominal pain, nausea, and anemia due to significant blood loss as a result of the attachment, residence, and voracious blood feeding of the worms in the small intestine. Severe anemia in young children leads to retardation in growth and cognitive functions. Hookworm-associated malnutrition and anemia severely affect pregnant women (Ness et al., 2020), and account for the loss of up to 4.1 million DALYs annually (Hotez et al., 2005, 2008; Ronquillo et al., 2019).

Diagnosis. Definitive diagnosis relies on eggs detection in stool. The eggs of *A. duodenale* and *N. americanus* are indistinguishable, colorless and transparent, oval in shape, thin-shelled, $60 \times 40 \mu\text{m}$, with a clear space between the shell and the central 4–8 blastomeres.

Treatment agents. Infection can be prevented by prohibiting defecation and walking bare foot in fields or gardens, and eating unwashed vegetables and fruits in areas where *A. duodenale* prevails. Albendazole (Fig. 5) and mebendazole are effective for treating both *A. duodenale* and *N. americanus*. Oral administration of ALB (400 mg, once daily) or mebendazole (100 mg twice daily) for 3 days are effective in cure of light and moderate infections. These benzimidazoles preferentially bind to nematode tubulin, leading to inhibition of its polymerization or assembly into microtubules, thus stopping cell division, especially in worm intestinal and tegumental cells (Bethony et al., 2006). It was recently shown that ALB is a DNA intercalator (Will Castro et al., 2021), explaining its deleterious impact on worm metabolism and survival, and severe toxicity. Successful treatment does not prevent reinfection, which is extremely high within 30–36 months of treatment, necessitating regular drug administration and emergence of the threat of parasite drug resistance (Hotez et al., 2006).

Vaccines. Currently, leading vaccine candidates are *N. americanus* aspartic protease-1 (Na-APR-1) and glutathione S-transferase-1 (Na-GST-1), enzymes involved in the digestion and detoxification of hemoglobin in the hookworm digestive tract, thereby depriving the worm of its ability to digest its blood meal. The antigens expressed in *Pichia pastoris* and adjuvanted with Alhydrogel (aluminium hydroxide gel suspension) elicited highly significant protective immunity in laboratory animals, and were safe, well-tolerated, and immunogenic in unexposed individuals (Bethony et al., 2008; Diemert et al., 2017). The recombinant vaccine candidates adjuvanted with Alhydrogel and an aqueous formulation of glucopyranosyl lipid A were administered to healthy Gabonese adults, residents of areas endemic for *N. americanus* and other helminthes. The vaccines were shown to be safe and well tolerated whether administered separately or co-administered, and resulted in significant IgG1 responses to both antigens (Adegnika et al., 2021). Of note these antigens did not elicit IgE responses, differently from Na-ASP-2 (*N. Americana* Ancylostoma secreted protein-2) hookworm vaccine, an ESP produced by infective *N. americanus* larvae upon penetration of human skin. The trials with this antigen were halted because it induced generalized urticarial reactions in previously hookworm-infected volunteers (Diemert et al., 2012; Zawawi and Else, 2020).

Hookworms' cathepsin B cysteine proteases should be developed for prophylaxis against soil-transmitted helminthes as they would induce antibodies that neutralize the catalytic activity of the hookworm antigens in the gut, resulting in reduction of the adult worms motility and viability (Noon et al., 2019). Additionally, cysteine proteases are renowned for inducing type 2 immune responses, notably IL-4, IL-5, and IL-13 cytokines and IgG1 specific antibodies, crucial and central for experimental hosts and humans acquired immunity to gastrointestinal nematodes (Grencis, 2015; Zawawi and Else, 2020).

Ascaris

Life cycle. Vectors, insects, intermediate hosts may not be held responsible for transmission of the human ascarid, *Ascaris lumbricoides*. Fertilized eggs deposited with feces in the soil contain one cell embryo that divides to give infective L3 larvae, and can survive in soil for 10 years or more. Human infection occurs following ingestion of unwashed vegetables and fruits contaminated with embryonated eggs. Excystment in the jejunum releases the larvae which penetrate the wall of the duodenum and enter the blood stream. They are swept to the liver, then the heart, and pulmonary circulation to break free in the alveoli, where they grow and molt twice over about 2–3 weeks. The larvae traverse the respiratory system to the buccal cavity to be coughed up, or swallowed, and carried back to the small intestine, residence for worm maturation, mating, and fertilization. The release of eggs by the female worm commences about 60 days after swallowing an infective egg. Adult worms are not microscopic or small as they reach up to 35 cm in length and 2–6 mm in diameter (they are roundworms). Parasite eggs are released by the thousands daily with feces to continue the life cycle and maintain transmission (Murrell et al., 1997; Deslyper et al., 2019; Holland, 2021).

An estimated 807 million–1.2 billion people in the world are infected with *A. lumbricoides* especially in poor rural and urban areas of Northern Africa and tropical and subtropical countries of Asia, Africa, and the Americas. Prevalence is particularly high in India (Christu Rajan et al., 2020) and sub-Saharan African countries (Cedric et al., 2021; El Tantawy et al., 2021; Dantie et al., 2021). Although the infection is asymptomatic, its effects may contribute to child morbidity when associated with malnutrition, pneumonia, abdominal swelling and pain, diarrhea, iron and vitamins deficiency. A most identical species *Ascaris suum*, principally infect pigs. Related ascarids of the *Toxocara* genera infect dogs, cats (Rostami et al., 2020a, b) and livestock, and increasingly threat transmission to humans, as 1.4 billion people worldwide, particularly in subtropical and tropical regions, are infected with, or exposed to *Toxocara* species (Rostami et al., 2019; Ma et al., 2020).

Diagnosis. Definitive diagnosis is via identification of fertilized eggs in feces samples by light microscopy. *Ascaris lumbricoides* eggs are round, 45–75 by 35–50 μm , thick-shelled with an external mamillated layer, and containing a developing embryo. Infection can be prevented by prohibiting defecation outdoors, especially in humid soil in gardens and fields, and thorough washing, peeling or cooking vegetables and fruits. Avoiding contact with feces-based fertilizers and sewage water is mandatory. Considerable numbers of *Ascaris* eggs were detected in sewage sludge before and after liming (treatment with 1% calcium oxide), and were not eliminated after 45 days of solar drying in summer, in a semi-arid climate (An-Nori et al., 2021).

Treatment agents. Treatment is based on albendazole and mebendazole, given orally for 1–3 days. The drugs are effective and appear to have few side effects, but must be administered regularly as they do not prevent reinfection. Moreover, it was recently reported that albendazole and mebendazole fail to entirely eliminate the worm load and recommend including ivermectin in the treatment protocol (Djune-Yemeli et al., 2020). Alarms of emerging parasite resistance to these essential medicines (Krücken et al., 2017) necessitated development of alternative treatments. Chicory (*Cichorium intybus* L.), a blue-flowered Mediterranean plant of

the daisy family, cultivated for its edible salad leaves and root, appeared promising in reduction of the gastrointestinal parasites burden in animals and humans. Chicory sesquiterpene lactones, notably 8-deoxylactucin, were shown to be the agents responsible for *A. suum* in vitro killing. Extensive chicory breeding and extraction of active compounds could assist in safe and effective control of ascarids in humans and animals (Valente et al., 2021).

Vaccines. It is difficult to elicit antibodies that may directly harm the parasite as all stages are covered with a thick cuticle, a flexible and resilient collagen-rich exoskeleton, which is synthesized five times, once in the embryo and subsequently at the end of each larval stage prior to molting (Winkfein et al., 1985). Accordingly, molecules in the epidermis beneath the cuticle may not be considered as vaccine candidates. Accordingly, it is difficult to figure out how immune responses to the *A. suum* immunodominant AS-37, mostly localized in muscle and hypodermic structures, may affect the parasite motility or survival (Versteeg et al., 2020). Larval excretory or secretory products (ESP) or extracellular vesicles may induce antibodies that activate eosinophils, mast cells, basophils, neutrophil and macrophages capable of chasing, interfering or delaying migration, and harming parasites via release of toxic molecules and inflammatory mediators (Hansen et al., 2019; Ebner et al., 2020). *Ascaris suum* ESPm As14, As16, AS24, and AS-Enol (enolase) in a recombinant form elicited type 2 cytokines, IgG and IgA specific antibodies, and variable, but significant, reduction in recovery of lung stage worms in pigs and mice (Chen et al., 2012; Wei et al., 2017; Zawawi and Else, 2020). Vaccine candidates AS14, As16, and As37 peptides containing B cell epitopes were selected and their encoding DNA sequences combined into a single chimeric gene bridged by codons for glycine-serine as linker to increase the flexibility of the chimeric protein expression into bacterial expression vector. The chimeric protein adjuvanted with monophosphoryl lipid A elicited specific IgG production and highly significant ($P < 0.0001$) reduction ($>70\%$) in challenge lung larval load in BALB/c mice (de Castro et al., 2021). *Ascaris* adult worms mouth- and intestine-derived antigens may induce antibodies able to access the parasite worm parts and intestinal mucosa, and cause significant damage (Rudrappa et al., 2016; Giralol et al., 2021).

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Bacterial Vaccines

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Bacterial vaccines

Bacterial infections are widespread in all eukaryotes. Numerous association processes have been emerged as a result between the co-evolution of bacteria and their hosts (Finlay and McFadden, 2006; Giesker and Hensel, 2014). Vertebrates evolved natural and adaptive immune systems, while bacteria used virulence mechanisms to circumvent antimicrobial processes (Finlay and McFadden, 2006; Giesker and Hensel, 2014).

Despite the growing use of antibiotics to combat disease-causing bacteria in humans and animals, antibiotic resistance is on the surge. (Giesker and Hensel, 2014). Antimicrobial resistance (AMR) is recognized by the World Health Organization (WHO) as one of the top 10 risks to the human health (Poolman, 2020). In 2015, 63.5% of AMR infections in Europe appeared to be healthcare-related, with adults 65 and older being the second-most affected population following the children (Cassini et al., 2019; Poolman, 2020). AMR is predicted to cause 700,000 deaths per year worldwide. Bacterial vaccines have the power to minimize AMR on many fronts. Vaccines can halt population- and healthcare-associated infections with multidrug-resistant (MDR) microbes, minimize antimicrobial use and resistance, and avoid infection in all age groups, specifically older adults, who have appeared as the age group with the greatest unaddressed vaccine needs (Nichol, 2005; Poland et al., 2009; Kennedy and Read, 2017; Klugman and Black, 2018; Poolman, 2020). Vaccines clearly avoid MDR infections and minimize antimicrobial use, but their potential utility in the battle against AMR, as well as their involvement in an integrated AMR strategy that involves targeted antimicrobials and enhanced diagnostics has yet to be realized (Kyaw et al., 2006; Bloom et al., 2018; Klugman and Black, 2018; Poolman, 2020).

Types of bacterial vaccines

Bacterial vaccines are divided into various categories.

- Toxoid:** Toxoids are toxins that have been chemically inactivated by bacteria such as *Clostridium tetani* (*C. tetani*) or *Corynebacterium diphtheria* (*C. diphtheria*). They are the exotoxins produced by these bacteria which trigger the disease, not the bacteria themselves. As a result, vaccination of toxoids induces the formation of antibodies towards the toxins, which neutralize the appropriate toxin and shield the body against a variety of serious diseases (Giesker and Hensel, 2014). *Bacillus anthracis* (*B. anthracis*) secretes two exotoxins: the edema factor (EF) and the lethal factor (LF). Both toxins share a defensive antigen (PA), which is required for binding and internalization of EF and LF within the host cell cytoplasm (Giesker and Hensel, 2014; Poolman, 2020). Immunity is induced by PA-based vaccines, which shield the cells against LF and EF attack. Six administrations with annual boosters are required for the immunization against these toxins (Reuveny et al., 2001; Giesker and Hensel, 2014).
- Subunit vaccines/polysaccharide vaccines:** Subunit vaccines, unlike toxoid vaccines, contain only a single pathogenic antigen. A subunit's immune response is weaker than that of a whole cell, but there is no chance of hyper-reaction or invasion when no microorganism is administered. When a purified protein, or even a single epitope of a protein, is administered, the immune

reaction is mediated through proteolytic digestion of the ingrained protein, preceded by antigen presentation on major histocompatibility complex (MHC) II. Since the following reaction is based on T cells, the proteins are referred to as T-dependent antigens. A T-independent immune response can also occur where the antigen is not a protein but rather is organized in a repetitive fashion or consisting of repetitive structures such as polysaccharides. The examples of polysaccharide vaccines are the vaccine against *Salmonella typhi* (*S. typhi*), (consisting of TY2 strain derived Vi capsular polysaccharide) and *Haemophilus influenzae* type b (Hib). However, since the T-independent immune response exists only in children aged 16–18 months, vaccination with the polysaccharide vaccine was ineffective (Giesker and Hensel, 2014).

- (c) Conjugate vaccines: Conjugate vaccines were produced by adding a protein to the capsular polysaccharide to improve the immune response and solve the issue of a delayed T-independent immune response. Three vaccines are used to defend against Hib, each with a different antigen, conjugation, and polysaccharide chain length and structure (Giesker and Hensel, 2014). These formulations, such as *Haemophilus* conjugate (HbOC) vaccine, polyribosyl phosphate-T (PRP-T) and polyribosyl phosphate outer membrane protein (PRP-OMP), are used effectively for disease prevention (Griffiths et al., 2012; Giesker and Hensel, 2014). Besides that, the ability to create synthetic vaccines that contain a PRP-tetanus toxin conjugate allows for large-scale vaccine development (Verez-Bencomo et al., 2004; Giesker and Hensel, 2014). In order to protect against *streptococcus pneumonia* (*S. pneumonia*), a polysaccharide conjugated to the diphtheria toxoid is administered, resulting in an immune reaction in both children and adolescents (Dagan et al., 2002; Verez-Bencomo et al., 2004; Giesker and Hensel, 2014). The major drawback of this conjugated vaccine over the subunit vaccine is that immunity is only achieved against seven serotypes, while the subunit vaccine targets 23 serotypes (Pittet and Posfay-Barbe, 2012; Wolfe, 2012; Giesker and Hensel, 2014). The meningococcal vaccine, which covers the serogroups A, C, W-135, and Y of 13 clinically important serotypes of *Neisseria meningitidis* (*N. meningitidis*), is another illustration of a conjugate vaccine (Rosenstein et al., 2001; Tunkel et al., 2004; Giesker and Hensel, 2014). Polysaccharides are attached to a diphtheria-toxoid polysaccharide-protein complex with this vaccine (Giesker and Hensel, 2014).
- (d) Inactive vaccines: Inactivated vaccines include bacterial cells that have been destroyed. The bacterium that causes a disease is inactivated using a chemical, heat, or radiation therapy. The cholera vaccine comprising of dead *Vibrio cholera* (*V. cholera*), had been used a lot longer but it only provide inadequate immunity for a brief time period after vaccination. The new vaccine is based on dead, inactive *V. cholera* with a recombinant B-subunit of this toxin. Following oral administration, this vaccine activates the immune system to develop antibodies against bacterial antigens, preventing intestinal invasion, as well as antibodies that neutralize the cholera toxin and hence preventing the toxin-diarrhea (Sinclair et al., 2011; Giesker and Hensel, 2014).
- (e) Live attenuated vaccine: Attenuated, living bacteria make up live vaccines. Attenuation can be accomplished by causing random or targeted mutations in virulence-related genes. Alternatively, to avoid the occurrence of the disease usually caused by the bacteria, less pathogenic strains or serotypes similar to the disease-causing agent may be used. Living bacteria cause a powerful immune response that lasts for a long time. The possibility of the attenuated bacteria reverting to a disease-causing agent is a limitation of this form of vaccine (Giesker and Hensel, 2014). Since the strains used retain latent virulence, their use poses hazards to vaccine recipients who have underlying immune suppressive deficiencies such as other diseases, inheritable immune abnormalities, or immunosuppressive medical care (Detmer and Glenting, 2006; Giesker and Hensel, 2014). The Ty21a typhoid vaccine having many mutations, including one in the Vi polysaccharide gene used by the subunit vaccine, is an example of an attenuated vaccine (Giesker and Hensel, 2014). The mutation in the *galE* gene causes the strain to be auxotrophic for galactose, thus prohibits the organism from surviving/growing outside of the laboratory (Detmer and Glenting, 2006; Giesker and Hensel, 2014). The vaccine against *Mycobacterium tuberculosis* (*M. tuberculosis*), BCG (Bacillus Calmette-Guérin) was developed from the Calmette-Guérin strain of *Mycobacterium bovis* (*M. bovis*) (Giesker and Hensel, 2014).
- (f) Recombinant vaccines: Recombinant vaccines are novel strategies of vaccination that use live attenuated bacteria as vectors of recombinant genes (Giesker and Hensel, 2014; Bloom et al., 2018). The primary reasons for recombinant protein-based vaccine production are the development of vaccines that are less reactive, more efficient, stronger, and better characterized, as well as vaccines that offer wider defense against various serotypes/serogroups of a bacterium (Unnikrishnan et al., 2012). When administered with adjuvants or represented by plasmids or harmless bacterial/viral vectors, recombinant vaccines depend on the ability of one or more defined antigens to trigger immunity against the pathogen (Nascimento and Leite, 2012).

Bacterial vaccine adjuvants and their mechanisms of action

Adjuvants are used to improve the vaccine-mediated immune response, which is particularly important when using subunit vaccines, which result in poor immunity when administered alone. The proper antigen-adjuvant balance is critical, since an adjuvant can be effective for one antigen but ineffective for another. Furthermore, the manner in which the vaccine is delivered has a significant impact on the adjuvant selection (Giesker and Hensel, 2014).

Aluminum salts (also known as alum) have been widely used in human vaccines for over a century. This type of adjuvant consists of aluminum hydroxide, aluminum phosphate and aluminum hydroxyl phosphate. Alum formulations are particulate in nature, on which vaccine antigens are adsorbed, though the properties of the various alum salts vary. This adsorption may result in improved antigen stability in vitro, leading to the initial presumption that alum provides a depot in situ, allowing for gradual antigen release over time and sustained immune system exposure (Ulmer, 2013).

Despite the fact that millions of people have been treated with alum-containing vaccines, the mechanism by which the alum boosts the immunity is still unknown. According to one hypothesis, alum causes necrosis accompanied by the release of uric acid. Uric acid is believed to activate dendritic cells (DC) and, as a result, the immune system (Kool et al., 2008; Giesker and Hensel, 2014). In addition to the approved adjuvants, there are a range of experimental models that stimulate the immune response in various ways. The adjuvants can provide a depot of antigen molecules that are released over time to mediate extended immunity. Adjuvants can also amplify local cytokines, thus activating specialized T cells (Giesker and Hensel, 2014). Besides that, adjuvants may help to stabilize epitopes or mediate antigen presentation via MHC I or II (Edelman, 2002; Giesker and Hensel, 2014). Mutant strains of the cholera toxin (CT) and an *Escherichia coli* (*E. coli*) heat-labile enterotoxin (LT) have been developed as effective adjuvants in mouse models, and nonliving antigen vaccines using mutant LT are now being used in clinical studies. Another experimental method is skin immunization, which has also been evaluated with the mixture of CT and tetanus toxoid, resulting in systemic immunity. An innovative diarrhea vaccine containing LT and an *E. coli* pilus protein has also been proved to regulate systemic immunity in humans studied under clinical experiments (Edelman, 2002; Giesker and Hensel, 2014).

Determinants of vaccine design

Immune evasion mechanisms used by bacteria

Bacteria employ a wide range of physical, biochemical, and immunological pathways to colonize and invade the human host while circumventing or neutralizing immune efforts to kill them (Urwin et al., 2004; Nizet, 2007; Silva Miranda et al., 2012; Poolman, 2020). Three important strategies adapted by bacteria are (1) antigenic variations/heterogeneity, typically in surface polysaccharides and/or surface proteins, so that type-specific immunity does not preclude concurrent infections with other variants of the same bacterium; examples include *S. pneumoniae*, *N. meningitidis*, *E. coli*, *H. influenza*, *Klebsiella pneumoniae* (*K. pneumoniae*), group A streptococcus. (2) Immune system modulation, whereby bacteria avoid destruction by interrupting/inactivating host defense strategies, examples include *Staphylococcus aureus* (*S. aureus*), *Bordetella pertussis* (*B. pertussis*), and group A streptococcus. (3) or lying in immune-inaccessible structures/locations. Examples include *M. tuberculosis*, *E. coli*, *S. aureus* and *Chlamydia trachomatis* (*C. trachomatis*) (Poolman, 2020).

These mechanisms have an effect on vaccine formulation, theoretically necessitating the use of various antigens/serotypes to optimize protection, or the inclusion of immune-boosting ingredients such as adjuvants.

Different sites of infection

The presence of protective antibodies in serum will help prevent diseases like meningitis and bacteremia/septicemia. The defensive amount of antibody has been determined for many invasive diseases. Hib, meningococcal, and pneumococcal conjugate vaccines that use T-cell aid to improve B-cell activities against capsular polysaccharides, as well as increase antibody synthesis and B memory cells, are very successful in inducing antibody responses to vaccine-specific serotypes/serogroups and show very high effectiveness (at least 75%) in reducing invasive disease (Lucero et al., 2009; Thumburu et al., 2015; Poolman, 2020). In stark comparison, the effectiveness of these same vaccines against mucosal diseases (otitis media and pneumonia) is just marginal. The effectiveness of pneumococcal conjugate vaccines (PCVs) against pneumonia in infants and adults aged 65 and over, as well as efficacy in avoiding otitis media, is up to 50% lower than that of bacteremia (Bonten et al., 2015; Poolman, 2020). The explanation for the suboptimal efficacy lies in the fact that antibodies caused by parenteral vaccination must travel via mucosal surfaces such as the middle ear, bladder epithelium, nasopharynx, and cervix by passive transudation (Wagner et al., 1987; Kaur et al., 2012; Scherpenisse et al., 2013; Poolman, 2020). The phagocytic opsonization necessitates the physical repositioning of neutrophils on the mucosal surfaces. Although the majority of infectious diseases enter the human body through mucosal surfaces, vaccinations that cause long-lasting defensive immune function at the mucosal surface have proven difficult to develop. Induction of bystander CD4 T-cell immunity and Trm swooping to the mucosa, in conjunction with attracting phagocytic cells, may hold the answer to this problem (Poolman, 2020).

Th1/Th17 induction and its function in mucosal infection control mechanism

If pathogenic bacteria penetrate the mucosa, CD4+Th1/Th17 T-cells are also needed to facilitate the role of opsonic antibodies by recruiting and activating the neutrophils and macrophages in order to clear extracellular pathogens. As a result, vaccines causing this form of reaction are needed (DeLyria et al., 2009; Poolman, 2020). However, Th1/Th17 responses alone are insufficient to deter mucosal infections. Antibodies are still the primary agents of bacterial killing and removal owing to their ease of mucosal penetration (Warfel et al., 2014; Brown et al., 2015; Middleton et al., 2017; Poolman, 2020).

The mucosal invasion of bacterial or parasitic agent activates the TLR, which in turn induces the release of specific cytokines produced by antigen presenting cells (APCs). These cytokines causes the activation of Th1/Th17 immune response. Interferon-gamma (IFN- γ) and interleukin (IL)-12, in particular, are important players in the differentiation of naive CD4 +T-cells into Th1 cells. In addition, the APCs induced interleukins such as IL-6 and IL-12, and growth factor β causes the naive CD4+T- cells to differentiate in to Th17 (Guglani and Khader, 2010; Poolman, 2020). IL-23 stabilizes and ROR γ t (transcription factor) regulates the differentiation process of Th17 (Korn et al., 2009; Poolman, 2020). The immune response associated with Th1 results in the production of tumor necrosis factor alpha (TNF- α), IL-2 and IFN- γ . When the intracellular Mtb, *S. enterica typhi* type pathogens enter in the body, these IFN- γ play an important role in destroying the pathogens by recruiting and differentiating the macrophages and dendritic cells of the body (Bao et al., 2000; Poolman, 2020). IL-17 primarily activates innate mucosal defenses,

especially neutrophil activation. Recent data indicates that this cytokine plays a role in activating the B-cell reaction by preferentially expressing the CXCL13 (a B-cell chemo-attractant) on Th17 cells (Takagi et al., 2008; Reinhardt et al., 2009; Poolman, 2020).

The Trm cells, which are formed in part from Th17 effector cells, serve as the first line of defense at mucosal sites of body such as the skin, lungs, vagina and urinary tract. Since, the trm cells are believed to kill antimicrobial resistant strains of *K. pneumoniae*, a subset of its lung resident performs this function by only producing INF- γ (Vesely et al., 2019; Poolman, 2020). The Whole-cell pertussis (wP) vaccination system, which have a longer period of defense than acellular pertussis (aP) vaccines and cause Th1/Th17-biased responses in infants as opposed to Th2-directed responses by aP, provide evidence that Th1/Th17 responses caused by vaccination can be effective (da Silva Antunes et al., 2018; Poolman, 2020). Adult BCG revaccination in India has also been shown to improve antimycobacterial induced Th1/Th17 reactions (Rakshit et al., 2019; Poolman, 2020). A south African study also evidenced that by the revaccination through BCG, the release of IFN- γ restricted the seroconversion of infection (Nemes et al., 2018; Poolman, 2020).

Immunological mechanisms and vaccines

The introduction of vaccination process was a breakthrough in the battle between bacteria and humans. Despite the fact that increased hygiene and antibiotics could have helped more people, vaccines are the most cost-effective and life-saving modalities in the history. Despite their effectiveness, one of the key tenets of vaccinology is that the large percentage of vaccines has been developed objectively, with a little knowledge of the immunological processes by which they cause defensive immunity. Despite decades of commitment, the inability to produce vaccinations against global pandemics such as HIV infection has highlighted the need to consider the immunological processes by which vaccines impart immune responses (Pulendran and Ahmed, 2011). The immune system has developed qualitatively distinct forms of responses to defend against different pathogens, it is now evident (Pulendran and Ahmed, 2011).

The immune system's action can be divided into two categories: primary and secondary immune responses. The primary immune response depicts the immune system's first encounter with an infectious agent, while the secondary immune response refers to all subsequent encounters with the same pathogen. In contrast to the primary immune response, the secondary immune response is quicker and better amid the production of memory cells, resulting in more successful pathogen removal. Vaccines imitate viruses, eliciting a primary immune response that is followed by a secondary immune response as the body is exposed to the true infectious agent (Giesker and Hensel, 2014). The immune response and the development of memory cells, both of which are essential for immunization, are complicated processes that will be discussed in the next section.

The MHC takes up, processes, and presents antigens that are found in the body. Both MHC I and MHC II have different functions. MHC I is located on most cells and presents endogenous peptides to cytotoxic T-lymphocytes (CTL), including self, tumor, and virus antigens. MHC I action is less relevant in bacterial vaccines since the administered antigens are normally exogenous. MHC II, unlike MHC I, can bind exogenous peptides; however, these complexes are only found on antigen-presenting cells (APC). MHC II is responsible for activating T-helper (TH) cells, which aid in the synchronization of the subsequent immune response. Innate immune cells, CTL, and phagocytes can all be activated by TH1 cells, resulting in a cell-mediated immune response that is directed against intracellular pathogens. The typhoid vaccine developed by using an attenuated *S. Typhi* strain Ty21a, which is already invasive, is an example of this mechanism during vaccination. TH2 cells stimulate B cells to produce antibodies against extracellular pathogens and poisons, triggering a humoral immune response. This immune reaction is also caused by toxoids, as well as inactivated or conjugated vaccines. The secretion of chemotactic molecules by TH17 cells attracts granulocytes to the infection site. Antibodies, also known as immunoglobulins (Ig), are present on the surface of B cells. IgM antibodies make up the bulk of antibodies on mature B cells, with just a few IgD antibodies present. Pentamers form when IgM is secreted, resulting in complexes of 10 epitopes. While each epitope's propensity for binding a ligand is poor, the abundance of epitopes compensates for this. The role of IgD in the body is still a mystery. B cells mature in the lymphatic tissues of the body's periphery. Unlike T cells, which can only detect antigens bound to the MHC, B cells can detect polysaccharides, glycoproteins, lipids, nucleic acids, or proteins that are attached to surfaces, linked to other chemical compositions, or secreted (Giesker and Hensel, 2014).

When antigens with repeated sequences, such as polysaccharides, cross-link the B-cell receptors, B cells can be activated without the presence of T cells. The B cells mature into IgM-secreting plasma cells after that, but so far no memory B cells could form. Chemical impulses from the bone marrow cause certain plasma cells to live for long periods of time, thus mediating memory. Vaccines such as the Hib subunit vaccine are using mechanism for immunization, but this vaccine is ineffective in children under the age of two because their immune systems are immature and do not react to vaccines of this type. T-dependent activation of B cells is needed for antigens with no repeated sequences. B cells proliferate and transform into plasma cells, which secrete antibodies, after being activated by TH2 cells. When the TH2 cell detects the antigen, but not exactly the same epitope as the B cell, the B cell is activated. B cells can switch their immunoglobulin class while the primary response is sophisticated. This is known as immunoglobulin class switching. Different Igs are formed depending on the form of TH cells that cause the isotope shift. The development of IgA and IgE is mediated by the secretion of cytokines by TH1 cells, which modulates the transition of the heavy chain of IgM to IgG. Most memory cells contain IgG antibodies, but B cells that express IgA and IgE antibodies also play a role in the secondary immune reaction. There are two types of memory T cells: effector memory T cells (TEM) and core memory T cells (TCM). TEM can be found in both blood and tissue. The secondary immune response can function quicker than the primary immune response because they are directly at the sites where new infections begin and the TEM start secreting cytokines. The greater immune response against

secondary infection is due to TCM. To diagnose new infections, the cells can be present in the blood and peripheral lymphatic tissue. Antigens allow TCM to proliferate and differentiate more quickly than naive T cells. TCM become TEM and are directed to infected tissues for TH1 or TH2 cell assistance after increasing cytokine output (Giesker and Hensel, 2014).

A subset of TCM stimulates memory B cells in body. DCs play a crucial function in immune responses. They are the strongest APCs, with the rare ability to display antigens in several ways. In order to activate CTL, antigens are produced from endosomes into the cytoplasm, segmented, and tied to MHC I molecules. The typical presentation of exogenous antigens by MHC II complexes is addressed by this procedure (Kim and Liao, 2010; Giesker and Hensel, 2014).

The limitations of existing bacterial vaccines

Bacteria developed various adaptive immune mechanisms during the co-evolution of the human immune system and pathogens. The immune system recognizes pathogens' surfaces as the first pattern it sees (Finlay and McFadden, 2006; Giesker and Hensel, 2014).

Antibodies are prevented from binding to surface molecules and being detected by immune receptors by capsules, which are made up of polysaccharide complexes that surround the bacteria. As opposed to non-encapsulated bacteria, encapsulation causes many differences in the immune system: the host's immune response is decreased, accompanied by a reduction in phagocytosis. *Porphyromonas gingivalis*, a bacterium implicated in periodontal disease, has a sevenfold greater phagocytosis of non-encapsulated bacteria in vitro than bacteria encased in a polysaccharide capsule. The longevity of encapsulated *P. gingivalis* is greater due to a lower phagocytosis rate, leading to higher virulence of the encapsulated bacteria relative to non-encapsulated *P. gingivalis* (Singh et al., 2011; Giesker and Hensel, 2014). Vaccines against antigens of the bacteria's surface are ineffective for bacteria with capsules since the immune system is unable to recognize the surface textures of encapsulated bacteria.

Due to capsular antigens, various serotypes must be taken into account while developing vaccines. Different serotypes can be included in polyvalent vaccinations, although not all serotypes can be addressed by polyvalent vaccinations for many pathogens (Ovodov, 2006; Giesker and Hensel, 2014).

The lipopolysaccharide (LPS) plays a crucial role in Gram-negative bacterial detection. LPS activates innate immune system receptors including the Toll-like receptor (TLR) 4, which has conserved components such as the lipid A core (Liechti and Goldberg, 2012; Giesker and Hensel, 2014).

The O antigen, on the other hand, is found on the outside of the LPS and is very variable. This is an example of antigenic diversity in bacterial pattern, which results in the formation of various serotypes or serovars. Bacteria employ antigenic changes to facilitate reinfection or to prolong an infection. Different alleles or mutable sequences in the molecules cause the variances (Finlay and McFadden, 2006; Giesker and Hensel, 2014).

More than 2500 serotypes of *Salmonella enterica* (*S. enterica*) are capable of colonizing humans and causing a variety of illnesses (Andrews-Polymenis et al., 2010; Giesker and Hensel, 2014). When the immune response is reliant on serotype variation, infection with one serotype does not induce immunization against other serotypes (Finlay and McFadden, 2006; Giesker and Hensel, 2014). Following immunization against a serotype-specific pattern, memory cells specific to that pattern form followed by immunity to that serotype alone. To address this issue, polyvalent vaccinations based on several strains have been developed (Pittet and Posfay-Barbe, 2012; Giesker and Hensel, 2014).

Another example of immune evasion is *M. tuberculosis* secreting ESAT-6, a key T-cell antigen into host cells, which prevents the TH1 cell response (Dwivedi et al., 2012; Giesker and Hensel, 2014). As a result, even if vaccination was administered, the immune system will be unable to effectively remove the illness. Other examples include *Yersinia pestis* (*Y. pestis*) induced paralyzes of phagocytic functions and other methods of interfering with the immune system, such as blocking cytokines both of which result in a reduced immunological response with no influence on vaccination (Finlay and McFadden, 2006; Giesker and Hensel, 2014).

The use of an efficient vaccination technique puts bacterial pathogens under selection pressure. As a result, pathogenic strains with variants or mutations in genes that encode antigenic structures might emerge. These modifications will impact the pathogen's antigenic characteristics and may allow it to evade immune responses generated by vaccination. The rise of non-typeable *H. influenza* isolates as a result of the widely used Hib capsular vaccination is a good example of such serotype drift. Immune defenses focused against capsule antigens have little effect on non-typeable *H. influenzae* strains, which have lost capsule expression (Giesker and Hensel, 2014).

Vaccination schedules and travelers vaccines

Vaccination is a very efficient way to avoid various infectious illnesses. Vaccines are typically considered to be quite safe, as major side effects are unusual. Immunization programs prevent the majority of the world's youngsters against a variety of deadly illnesses that used to kill millions of people every year. Vaccination provides travelers with the opportunity to avoid several infectious illnesses that they may encounter while traveling. However, adequate vaccinations for some of the most life-threatening illnesses have yet to be discovered; as a result, other measures, such as avoiding mosquito bites, hand washing, as well as general precautions should be observed. Each vaccine plan must be customized by a physician based on the traveler's past immunizations, health condition, risk factors, the countries of visit, the nature and duration of trip, types of activities, and the amount of time available prior to departure. The immunological response of the vaccinated individual varies depending on the type of vaccine, the number of doses given, and whether or not the individual has formerly been vaccinated for the same illness. At least 2 weeks prior to travel, all vaccines should be done (Table 1).

Table 1 Summary of vaccination for travels.

<i>Categories</i>	<i>Vaccines used against</i>	<i>The justification for vaccination</i>
(1) Regular vaccinations to evaluate before traveling	Pneumococcal Tuberculosis Influenza Diphtheria Pertussis Tetanus	Most national childhood vaccination programs include these vaccinations. A pre-travel consultation, on the other hand, is an excellent chance for health care practitioners to examine the immunization status of babies, children, adolescents, and adults and confirm that they have received immunizations according to their own country's schedule
(2) Vaccines advised for particular travel destinations	Cholera Meningococcal Typhoid fever	These vaccinations are suggested for protection against illnesses that are endemic to the origin or destination country. They're designed to keep travelers safe and prevent disease from spreading inside and between nations
(3) Vaccines required by some states	Vaccine against meningococcal disease. On the WHO's Weekly Epidemiological Record web page, there are specific updates for pilgrims heading to Saudi Arabia	For passengers intending to enter or depart a country, several governments demand proof of immunization. See the country list on WHO's International Travel and Health (ITH) web page for more information

Table is adopted and modified from https://www.who.int/ith/CHAPTER_6_For_Publication.pdf.

Vaccine administration, like any other injection, requires a high level of injection protection. For each injection, a sterile needle and syringe must always be used, and thus should be disposed of properly. Where appropriate, the WHO suggests using only single-use ("auto-disable") syringes and, ideally, devices with sharps injury prevention capabilities. Syringes must not be recapped (to prevent needle-stick injuries) and should be discarded in a rational way for the receiver, the administrator and the community.

The use of vaccine combinations or co-administration with other vaccines

Several combination vaccines that defend against many diseases are now available and additional combinations are expected to become possible in the future. Combination vaccinations help travelers save time and money by reducing the amount of doses they need. Combination vaccines are generally almost as safe and reliable as single-disease vaccines.

Generally, inactivated vaccines do not interact with other vaccines. Many injections in a single visit, on the other hand, should be provided at various locations (different limbs) for each injection, or injection sites should be separated by at least 2.5 cm (1 in.). When administered at various anatomical locations, most live vaccines may be delivered at the same time. If injectable live virus vaccines are not given on the same day, they should be given at least 4 weeks apart. Live oral Ty21a typhoid vaccines, on the other hand, can be given at any time pre- or post-administration of injectable live vaccines (Table 2; CDC, n.d.).

Recent developments in bacterial vaccines

Adjuvants and other delivery modes for improving mucosal immune responses

Adjuvants are immune-stimulatory chemicals that help the immune system work well. AS01 (TLR4 agonist with saponin QS21) and CpG (TLR9 agonist) are the most latest adjuvants to be licensed in human vaccines (Cunningham et al., 2016; Poolman, 2020). AS01 is found not only in the extremely successful RZV vaccination, but also in the experimental TB vaccine M72/AS01E, which exhibited statistically significant effectiveness in preventing pulmonary TB in adults infected with MTB suggesting that AS01 might be a good adjuvant for other mucosal infections. CpG has been licensed as part of a hepatitis B vaccine (Poolman, 2020).

The programming of receptors (tissue-specific adhesion and chemo-attractant receptors) that govern T- and B-cell migration to mucosal regions is determined by the place where dendritic cells encounter antigen. It's been challenging to obtain high quantities of T-cells at mucous membranes following systemic immunization so far (Lai et al., 2015; Poolman, 2020). Alternative vaccination methods (oral, sublingual, inhaled, intranasal, transdermal, intra-vaginal) paired with adjuvants may promote T-cell localization to specific mucosal regions, such as Trm cells (Li et al., 2020; Poolman, 2020).

Vaccine against extraintestinal pathogenic *Escherichia coli* (ExPEC) and *Streptococcus pneumoniae*

The most prevalent cause of UTI is ExPEC. According to preliminary findings from Phase 1–2 research, a systemic vaccination to prevent UTI may be possible. In one trial, women with a history of recurrent UTI (Huttner et al., 2017) were given a prototype 4-valent ExPEC bioconjugate vaccination. Another study looked at a vaccination that included FimH from type 1 fimbriae as well as a novel adjuvant (Poolman, 2020). ExPEC conjugate vaccines offer a strong possibility of preventing invasive ExPEC illness (mostly bacteremia and urosepsis), but mucosal immunity would most likely need to be improved. Modification of the formulation to stimulate a Th1/Th17 response, as with previous combination vaccines, may assist in achieving clinically significant effectiveness against UTI. A similar strategy might be used to boost PCV effectiveness against non-bacteraemic pneumonia (Ramos-Sevillano et al., 2019), albeit achieving a well-balanced response is getting increasingly difficult. After human challenge, a proposed pneumococcal vaccine including three proteins with Th1/Th17-inducing potential (SP0148, SP1912, SP2108), but with

Table 2 Vaccines for both regular and targeted use.*Cholera prophylaxis**Brief introduction about disease*

Causative organism	<i>Vibrio cholera</i> Serotype: O1 and O139
Mode of transmission	Infection happens when food or water is polluted, either directly or indirectly, by the feces or vomitus of infected people
Nature and severity of disease	Acute enteric disorder of differing degrees of seriousness. The majority of viruses have no symptoms (i.e., they do not cause illness). Acute watery diarrhea develops without any symptoms in minor conditions. In acute conditions, there is an abrupt onset of watery diarrhea, nausea, and vomiting, as well as accelerated dehydration. Dehydration, which leads to circulatory failure, can cause death in serious untreated cases within a few hours
Global prevalence	Cholera is most common in low-income countries and regions where healthcare has failed and there is a shortage of sanitation and safe drinking water
Risks associated with traveling	Except in places where cholera epidemics exist, the risk to most travelers is very low if basic measures such as hygiene are taken. Humanitarian aid personnel in flood zones and refugee camps, on the other hand, could be at risk
General precautionary measures	Consumption of highly infected food, beverages, and water should be discouraged, along with other diarrheal diseases. In the event of acute diarrhea, oral rehydration salts should be taken to prevent dehydration and electrolyte depletion. No country needs cholera vaccine as a condition of entrance

Vaccination

Vaccine type	(1) Killed vaccine of O1 whole cell containing β subunit (2) killed vaccine of O1 and O139 Route of administration: Oral
Vaccination schedules	(a) Two doses for people under the age of six, and three doses for children between the ages of two and five. 7 days and 6 weeks can be the time interval between doses. Booster doses are prescribed after 2 years for children under the age of 6 and after 6 months for children over the age of 25. Primary vaccine should be repeated if the prescribed times between primary doses or even between previous primary dose and a booster dose are not met (b) There are two doses. Individuals under the age of 1 year are separated by 14 days. Within 2 years, all age ranges can get a booster dose.
Contraindicated	In persons experiencing hypersensitivity to prior dose
Special consideration for	High risk travelers (e.g., emergency/relief staff)
Precautionary measures related to vaccine	There are none
Use in pregnancy or lactating mothers	Oral inactivated vaccine can be administered if indicated

Diphtheria prophylaxis

Diphtheria vaccine is regularly given to children in most countries. Vaccines should be available to travelers who have postponed their vaccinations, according to national guidelines

Brief introduction about disease

Causative organism	The toxigenic <i>Corynebacterium diphtheriae</i> and seldomly the toxigenic <i>Corynebacterium ulcerans</i> often found in tropical climates
Mode of transmission	<i>C. diphtheriae</i> , which lives in the respiratory tract, is spread from human to human through droplets and near physical contact; <i>C. ulcerans</i> and <i>C. pseudotuberculosis</i> can also be carried through zoonotic transmission.
Nature and severity of disease	Clinical signs and symptoms are generally moderate, but powerful bacterial toxins may sometimes inflict obstructive membranes in the upper respiratory tract or cause disruption of the myocardium and other tissues. <i>C. ulcerans</i> and <i>C. tuberculosis</i> are less probable to provoke systemic symptoms.
Global prevalence	In countries where diphtheria-vaccines are widely used, it is very rare. In crowded areas with inadequate vaccine programmes and poor sanitation, the prevalence of the disease increases.
Risks associated with traveling	The risk of infection raises in populations with low vaccination rates against diphtheria, tetanus, and pertussis (DTP).
Vaccination schedules	National guidelines suggest using properly developed combination DTP vaccinations or booster tetanus and diphtheria (Td) vaccines for primary or booster vaccination. Individuals under the age of seven can obtain diphtheria toxoid-free varieties.
Use in pregnancy or lactating mothers	Can be administered if indicated

Tetanus prophylaxis

Tetanus vaccine is regularly given to children in most countries. According to guidelines, unvaccinated travelers should be given the vaccination

Brief introduction about disease

Causative organism	The toxigenic bacteria, <i>Clostridium tetani</i>
Mode of transmission	<i>C. tetani</i> spores can contaminate necrotic, anaerobic tissue and turn into toxigenic, vegetative bacteria. Tetanus is not contagious
Nature and severity of disease	Local muscular spasms or systemic muscle spasms and rigidity can be caused by potential bacterial neurotoxins derived from vegetative <i>C. tetani</i> . Tetanus, particularly generalized tetanus, is sometimes lethal if left untreated
Global prevalence	<i>C. tetani</i> spores can be found all over the world, mainly in soil

Table 2 (Continued)*Tetanus prophylaxis*

Risks associated with traveling	Activities that potentially contribute to dirty or infected injuries are related to the risk. This chance isn't really elevated while traveling
Vaccination schedules	According to national guidelines, travelers should be treated with the combination diphtheria–tetanus or DTP vaccinations. Tetanus-containing formulations with low diphtheria toxoid content should be provided to children under the age of seven
Adverse reactions to vaccines	Anaphylaxis and brachial neuritis
Use in pregnancy or lactating mothers	Can be administered if indicated.

Pertussis prophylaxis

Pertussis vaccine is regularly provided to children in most countries. Vaccines can be recommended to unvaccinated travelers in accordance with national guidelines

Brief introduction about disease

Causative organism	<i>Bordetella pertussis</i>
Mode of transmission	<i>B. pertussis</i> is spread by droplets from one individual to another
Nature and severity of disease	Only ciliated cells of the respiratory mucosa are colonized by <i>Bordetella</i> bacteria, resulting in an acute respiratory infection (also known as whooping cough) with intense, spasmodic coughing bouts. Pertussis can be atypical and often life-threatening in early childhood
Global prevalence	With advancing age, disease symptoms become less dramatic, even in adults The prevalence of pertussis is dependent on DTP immunization rates; the disease is more prevalent in countries with poor coverage and is less common in countries with high DTP immunization
Risks associated with traveling	Unvaccinated children entering countries with poor DTP vaccine coverage are at the greatest risk
Vaccination schedules	Acellular or whole-cell (WC) pertussis vaccinations can be used in established combination with vaccines against diphtheria (D) and tetanus (T) for primary and booster vaccination. The schedule should adhere to national guidelines. Individuals under the age of seven can obtain diphtheria toxoid-free varieties
Use in pregnancy or lactating mothers	Only acellular vaccine can be administered if indicated

Haemophilus influenzae type b prophylaxis

In certain countries, children are regularly immunized against *Haemophilus influenzae* type b (Hib). According to guidelines, unvaccinated travelers under the age of five should be given vaccine

Brief introduction about disease

Causative organism	<i>H. influenzae</i> type b (Hib)
Mode of transmission	Respiratory Droplets
Nature and severity of disease	Hib is a leading cause of pneumonia, meningitis, septicemia, epiglottitis infection, and other potentially fatal infections in children aged 3 months to 5 years old
Global prevalence	In countries with poor Hib vaccine coverage, it is common
Risks associated with traveling	In an area with poor Hib vaccine coverage, the risk is likely to rise
Vaccination schedules	All Hib vaccines licensed for marketing are conjugated. Their carrier protein, chemical conjugation process, polysaccharide sizing, and adjuvant differ. Hib vaccine comes in a number of forms, including liquid Hib conjugate vaccine (monovalent), liquid Hib conjugate vaccine mixed with DTP and/or hepatitis B vaccine, Hib conjugate in conjunction with meningococcal antigens, lyophilized Hib conjugate with saline diluent (monovalent), lyophilized Hib conjugate for use with liquid DTP or DTP in combination with other antigens, such as hepatitis B vaccine and inactivated polio vaccine Three primary doses without the need for a booster; two primary doses with a booster; and three primary doses plus a booster, with the first dose as soon as possible after 6 weeks of age. For healthy children over the age of five, the Hib vaccine is not needed

Tuberculosis prophylaxis

The tuberculosis (TB) vaccine for small children is not tailored to the needs of travelers. A single dose of bacillus Calmette–Guérin (BCG) vaccine should be given annually to all stable neonates at birth in countries or settings with a high prevalence of tuberculosis (TB) and/or leprosy. Unvaccinated young kids who are exposed to a high prevalence of tuberculosis should be given vaccines in accordance with national guidelines

Brief introduction about disease

Causative organism	<i>Mycobacterium tuberculosis</i>
Mode of transmission	by inhaling airborne droplets contaminated with <i>M. tuberculosis</i>

(Continued)

Table 2 (Continued)*Tuberculosis prophylaxis*

Nature and severity of disease	Many <i>M. tuberculosis</i> exposures manifest in latent infection, which only rarely progresses to active disease. TB can affect any organ, but active pulmonary disease of mycobacterial transmission is the most common manifestation in terms of public health. Tuberculous meningitis or disease propagation may occur in children. <i>M. tuberculosis</i> multidrug resistance (MDR) is an increasingly growing problem
Global prevalence	Poor people all over the world, but particularly in low-income countries. In HIV-positive people, tuberculosis is very common
Risks associated with traveling	The probability of travel-associated tuberculosis is determined by a number of factors, including the prevalence of tuberculosis in the country visited, the length of travel, the extent of contact with local population, and, most importantly, the traveler's age. Until vaccination of unvaccinated visitors null by the tuberculin skin test and the interferon release assay from regions that are not endemic for TB, an individual risk estimate dependent on the period of travel and the TB occurrence in traveling country, should be regarded. Vaccination is recommended for young unvaccinated children traveling to countries with a high risk of tuberculosis, particularly those who are likely to travel often during their childhood
Precautionary measures	Travelers should prevent frequent, direct contact with people who have pulmonary tuberculosis, if at all necessary. For health personnel and humanitarian aid staff, a tuberculin skin test before and during a high-risk mission abroad might be recommended. Travelers who have been exposed to individuals with bacteriologically proven tuberculosis for an extended period of time should be able to get preventive care
Vaccination schedules	Live attenuated mycobacterial strains derived from the first attenuated BCG are used in BCG vaccines. Apart from its reported effectiveness against tuberculous meningitis and disseminated disease in children, BCG vaccination has little benefit for most travelers
Adverse reactions to vaccines	Suppurative lymphadenitis (mostly in immunodeficient persons), osteitis (very rare with current vaccines) and disseminated BCG infection
Use in pregnant or lactating mothers	No

*Typhoid fever prophylaxis**Brief introduction about disease*

Causative organism	<i>Salmonella Typhi</i> causes typhoid fever, a serious, possibly fatal, acute, generalized infection. <i>S. paratyphi</i> A and B (and, less frequently, <i>S. paratyphi</i> C) cause paratyphoid fever, which is clinically similar to typhoid fever, particularly in Asia. Only humans are infected by <i>S. Typhi</i> and <i>S. Paratyphi</i> . Typhoid fever and paratyphoid fever are both referred to as "enteric fever"
Mode of transmission	The bacillus that causes typhoid is spread by infected food or drink. Direct fecal oral transmission can happen on rare instances. Shellfish from wastewater-polluted areas are a major cause of contamination; raw fruit and vegetables fertilized with human excreta, as well as infected milk and milk products, are other sources of infection. Flies can spread infectious agents to humans by transmitting them to food. When a large majority of individuals are using the same source of drinking water, pollution of water bodies can lead to typhoid fever epidemics
Nature and severity of disease	Pink stains, which disappear with pressure, occur on the trunk skin in up to 20% of light-skinned patients. Untreated patients may develop gastrointestinal and cerebral problems in the third week, which can be fatal in up to 10% of untreated cases. Kids under the age of four have the highest case-fatality rates. Since the bacteria remain in the biliary tract after symptoms have subsided, about 25% of sick individuals become chronically infected
Global prevalence	Typhoid fever is most likely to spread in countries or regions with poor sanitation, health, and water supplies
Risks associated with traveling	Typhoid fever is a problem for all visitors to endemic areas, but the risk is relatively low in tourist and business centers with high levels of lodging, sanitation, and food hygiene. Parts of Sub-Saharan Africa, as well as South and Southeast Asia, have strong endemicity. Travelers in other parts of the world are normally only at risk when they are subject to poor sanitation. For those who have been vaccinated should take precautions to prevent drinking highly infected food and drink, since the vaccination does not provide 100% immunity. In typhoid-endemic countries, the occurrence and spread of antimicrobial resistance among <i>S. Typhi</i> isolates is on the rise
Vaccination schedules	Travelers to places where the risk of typhoid fever is high, particularly those staying in endemic areas for more than 1 month and/or in regions where antimicrobial resistance strains of <i>S. Typhi</i> are common, may be provided vaccination. A booster dose of the Ty21a vaccine is prescribed after 1–7 years, based on national guidelines, for previously vaccinated visitors from non-endemic to endemic areas, and then after 3 years for the ViPS vaccine
Type of vaccine	There are three varieties of typhoid vaccines on the market, currently: (1) The Vi polysaccharide antigen is bound to a carrier protein in the latest generation typhoid conjugate vaccine (TCV). Currently, the only TCVs that have been approved are those that are bound to the tetanus toxoid protein (numerous different TCVs with various carrier proteins are in clinical trials and will be approved in the coming years). TCV is currently approved as a single intramuscular dosage in babies and children starting at the age of 6 months, as well as adults over the age of 45. (2) The oral Ty21a vaccine is rooted on a <i>Salmonella Typhi</i> strain that has been live attenuated. It comes in enteric-coated capsules and is intended for children under the age of six. Primary vaccine comprises of three or four capsules (one capsule on alternate days) depending on national guidelines. After 17 years, it is advised that you get vaccinated again. Ty21a has mainly been used to shield travelers.

Table 2 (Continued)*Typhoid fever prophylaxis*

Contraindicated in	(3) The Vi capsular polysaccharide (ViPS) vaccine is an injectable unconjugated Vi capsular polysaccharide (ViPS) vaccine that is administered intramuscularly or subcutaneously in a single dose to people under the age of two. Revaccination is advised every 3 years to ensure safety. In certain countries, a combination typhoid–hepatitis A vaccine is also accessible
Adverse reactions to vaccines	In individuals allergic to vaccine's ingredients Over 30 years of use, both the Ty21a and unconjugated ViPS vaccines have shown to be effective and well tolerated. Until now, the newer-generation TCV has a favorable safety profile (similar to ViPS), with no significant side effects recorded
Precautionary measures for vaccination	Antibiotics should not be provided to people who are taking Ty21a. Ty21a can be taken with chloroquine, but not after 8–24 h after mefloquine has been administered.
Use in pregnancy or lactating mothers	There is no definitive proof that proguanil and Ty21a should be administered together Should be avoided as safety data is not determined

Pneumococcal disease prophylaxis

Travelers are not at an elevated risk of contracting pneumococcal disease, but access to quality health services may be restricted when on the trip

Brief introduction about disease

Causative organism	Various serogroups of <i>Streptococcus pneumoniae</i>
Mode of transmission	<i>S. pneumoniae</i> -infected respiratory droplets
Nature and severity of disease	Invasive and non-invasive disease can also be caused by pneumococcus. Upper respiratory tract infections (URTI's) and non-bacteraemic pneumonia are the most prevalent non-invasive pneumococcal infections. The most frequent symptoms of invasive pneumococcal infection include pneumonia with empyema and/or bacteremia, febrile bacteremia, and meningitis. Antibiotic resistance in these bacteria is becoming a growing concern. Non-bacteraemic pneumonia and invasive pneumococcal diseases both have a high mortality rate, particularly in small children, the elderly and immunocompromised
Global prevalence	Found around the world
Risks associated with traveling	The pneumococcal conjugate vaccine (PCV) is universally approved for children. Children under the age of two, as well as children and adults deemed to be at high risk of severe illness, should be recommended to get vaccinated against infectious pneumococcal disease when traveling to areas with insufficient access to current health care services
Vaccination schedules	(1) Pneumococcal conjugate vaccines with 10 (PCV10) or 13 (PCV13) serotypes. From the age of 6 weeks, these PCVs can be given in a three-dose regimen of two or three main doses with or without a booster. PCV10 and PCV13 are vaccines towards invasive illness, pneumonia, and acute otitis media affected by the respective <i>S. pneumoniae</i> vaccine serotypes. PCV13 is also approved for use by adults (2) A pneumococcal polysaccharide vaccine that includes 23 serotypes of pneumococcal bacteria (PPV23). This vaccine is only for children under the age of two. Although the potency and efficacy of PPV23 in children and adults are unknown, it is considered healthy
Adverse reactions to vaccines	Anaphylaxis (very rare)
Use in pregnancy or lactating mothers	

*Meningococcal disease prophylaxis**Brief introduction about disease*

Causative organism	Serogroups A, B, C, W, X, and Y of the <i>Neisseria meningitidis</i> bacteria are the most common causative organisms
Mode of transmission	Direct person-to-person touch and respiratory droplets from infected or asymptomatic meningococcal carriers are the two most common ways for meningococcal infection to spread. Humans are the sole source of contamination
Nature and severity of disease	Children and teenagers are the primary victims of endemic illness, with babies aged 3–12 months having the highest attack rates. Meningococcal meningitis causes a severe headache, fever, nausea, fatigue, photophobia, sore neck, and other neurological symptoms to appear suddenly. Permanent neurological consequences are normal, and the disease kills 5–10% of people. Hypovolemic shock, hemorrhagic skin rash, and a high fatality risk describe meningococcal septicaemia
Global prevalence	There are sporadic cases all around the world. The majority of cases occur in temperate areas during the winter months. Regionalized outbreaks have been reported in crowded, confined areas (e.g., dormitories and military barracks). Wide outbreaks of meningitis can occur in the meningitis belt of Sub-Saharan Africa during the dry weather (November to June). Outbreaks of “serogroup A” have practically vanished in all countries that have adopted mass vaccination programmes for the category A conjugate vaccine. Latest meningococcal spreads caused by serogroups Y (United States), W (Saudi Arabia and sub-Saharan Africa), C, and X (sub-Saharan Africa) indicate that these serogroups are becoming more significant
Risks associated with traveling	Travelers have a low risk of contracting meningococcal disease. Travelers to high-income countries are at risk of contracting intermittent cases of A, B, or C. Meningococcal C disease outbreaks are common in schools, hospitals, military barracks, and other areas where large groups of teenagers and young adults reside. Travelers to the Sub-Saharan meningitis belt may be vulnerable to outbreaks, the most frequent of which are serogroups A, C, and W, which have much higher occurrence rates during the dry season. Long-term visitors living in close proximity to the local people, as well as pilgrims performing the hajj or umrah in Mecca, are particularly vulnerable

(Continued)

Table 2 (Continued)*Meningococcal disease prophylaxis*

<i>Vaccination</i>	
Vaccine types	Bivalent (A and C), trivalent (A, C, and W), and tetravalent (A, C, W, and Y) polysaccharide vaccines that cover two to four meningococcal serotypes are accessible. Polysaccharide vaccines are being phased out in favor of: (1) single-valent (A or C or C/Hib combination), two-valent (A and C, or C and Y/Hib combination), and four-valent (A, C, W, and Y) conjugate vaccinations are available. (2) Whilst recombinant protein-based vaccines towards serogroup B infections have become available around the world, only a few countries prescribe them. There is no universal guideline for travelers to be vaccinated against serogroup B
Vaccination schedules	Polysaccharide vaccines are given in a single (usually subcutaneous) dose to children under the age of two. After 35 years, a booster will be needed. In the case of conjugate vaccines, a principle sequence of one to three intramuscular doses is recommended, followed by boosters. The vaccination schedule is determined by the injection, as well as the vaccinee's age and immunological condition. While several countries already prescribe a primary sequence of two or three intramuscular doses for recombinant-protein-based vaccinations, WHO has yet to devise guidelines for national programmes
Contraindicated in	Individuals allergic to vaccine components
Adverse reactions to vaccine	With the exception of anaphylaxis, all meningococcal vaccines have a very good safety record
Use in pregnancy or lactating mothers	Can be administered if indicated

*Anthrax prophylaxis**Brief introduction about disease*

Causative organism	Spores of the bacterium, <i>Bacillus anthracis</i>
Mode of transmission	The spores are very resistant to inactivation and can live for years in the soil, infecting livestock who swallow the spores. Animals such as goats, sheep, and cattle are susceptible to infection. Anthrax spores may infect humans through three different routes: cutaneous (through the skin), gastrointestinal (through ingestion), and pulmonary (through inhalation)
Nature and severity of disease	Signs and symptoms of disease range from bumps or blisters (cutaneous anthrax), nausea, vomiting, swelling, stomach pain (gastrointestinal anthrax), sore throat, difficulty in breathing, shortness of breath (inhalational anthrax), fever and chills. If left untreated, anthrax can spread to other parts and cause serious sickness, including brain infections and even death
Consideration for	High risk individuals such as laboratory workers, military personnel, emergency/relief staff and individuals handling infected animals
Vaccine type	Immunization with anthrax vaccine is the only established and efficacious pre-exposure prophylaxis against anthrax. An attenuated strain of <i>B. anthracis</i> was used to create the vaccine. The vaccine is made from this strain's cell-free culture filtrate and is adsorbed onto an aluminum salt in its final product
Vaccination schedule	Three doses of vaccine followed by booster doses in individuals. Unvaccinated persons of all ages who have been exposed to anthrax should get the anthrax vaccination. These individuals should receive three doses of anthrax vaccination, as well as the antibiotics prescribed
Adverse reactions to vaccine	Headache, fatigue, muscle aches, tenderness etc.
Use in pregnancy or lactating mothers	For pregnant women who have been exposed to anthrax, anthrax vaccine may be suggested. Pregnant women, on the other hand, are not advised to obtain the vaccination if the risk of anthrax exposure is minimal

Table is adopted and modified by taking the information from <https://www.cdc.gov/vaccines/hcp/vis/vis-statements/anthrax.html>.

Th2-inducing aluminum hydroxide as adjuvant, lacked effectiveness for pneumococcal colonization (Poolman, 2020). A protein pneumococcal proposed vaccine including aluminum phosphate similarly failed to prevent colonization (Odutola et al., 2017), whilst a protein vaccine with a powerful Th1 adjuvant rescued non-human primates from pneumonia (Denoël et al., 2011; Poolman, 2020).

Vaccine against *Staphylococcus aureus*

The development of a *S. aureus* vaccine has proven to be significantly more challenging than expected. *S. aureus* thrives at immune evasion, and a string of vaccination failures based on capsular polysaccharides and surface proteins shows that an effective vaccine will almost certainly require to neutralize numerous major immune evasion mechanisms (Redi et al., 2018; Poolman, 2020). It uses a variety of immune evasion strategies to thwart immunological responses (B- and T-cell superantigens), limit leukocyte recruitment, induce neutrophil lysis, disrupt the complement activation, induce oxidative burst killing resistance, offer innate AM resistance, attach and destroy immunoglobulins, and construct a protective fibrin capsule around the bacteria (Guerra et al., 2017; Poolman, 2020). Although it is unknown which and how many of these processes must be suppressed to give disease protection, the traditional technique of employing surface antigens (capsular conjugates or proteins) has so far been ineffective. Nabi (CP5 and CP8 conjugates), Merck (IsdB), and Pfizer (CP5 and CP8 conjugates, ClfA, and MntC) all had failed vaccination

candidates with similar characteristics: a small number of surface antigens, no immune-evasion-related options, no adjuvants, inability to produce a CD4⁺ Th1/Th17 immunological response, and a non-representative animal studies (mice) (Fowler et al., 2013; Fattom et al., 2015; Redi et al., 2018; Poolman, 2020). Based on these difficulties, a successful technique will require a multicomponent strategy, which might include immune-escape related candidate antigens, a more accurate animal model, and the inclusion of a Th1/Th17 adjuvant to boost the immune response (Brown et al., 2015; Poolman, 2020). Th1 (IFN- γ) and Th17 (IL-17A) cells are required for macrophage and neutrophil proliferation and recruitment (Bröker et al., 2016; Poolman, 2020). Staphylococcal infections are predisposed by genetic abnormalities in phagocyte function, Th17, and IL-17RA, corroborating the pathways mentioned above (Lévy et al., 2016; Poolman, 2020).

Vaccine against tuberculosis

M. tuberculosis is an intracellular pathogen with a lipoidal surface shape that makes immunological assault even more difficult. After infection, granulomas are formed by the aggregation of macrophages and monocytes, as well as the presence of chemokines that stimulate cell adhesion and T-cell recruitment, frequently leading to a protracted latent period of infection (Silva Miranda et al., 2012; Poolman, 2020). The Bacille Calmette-Guerin (BCG) vaccination has been used in children ever since 1960s and has proven to be effective in avoiding disseminated TB. BCG appears to accelerate the spread of MTb from alveolar macrophages to tissue- or lung-recruited macrophages and neutrophils through antigen-specific CD4 T-cells (Delahaye et al., 2019; Poolman, 2020). Through as yet unknown methods, this transfer to phagocytes, as well as their activation and differentiation, is considered to improve the ability to limit bacterial proliferation (Delahaye et al., 2019; Poolman, 2020). BCG boosts the potential of innate effector cellular response to non-specific stimuli by inducing acquired immunity (Joosten et al., 2018; Covián et al., 2019; Poolman, 2020). Reactivation of disease may avoid this stage, contributing to BCG's decreased efficiency in avoiding lung TB reactivation in infected adults, which is the most prevalent cause of TB-related mortality (Trunz et al., 2006; Poolman, 2020).

The prophylactic adaptive response is assumed to be mediated mostly by CD4⁺ Th1 cells, with contributions from Th17 and CD8⁺ T-cells, although there is still no convincing explanation for how bacterial killing is accomplished (Tubo and Jenkins, 2014; Andersen and Scriba, 2019; Poolman, 2020). BCG revaccination increased Th1/Th17 CD4⁺ T-cell responses, including poly-functional T-cells, in IGRA+ (IFN- γ release assay related to presence of Mycobacteria) individuals, according to recent studies from India (Rakshit et al., 2019; Poolman, 2020).

When given as a booster, BCG prevented persistent seroconversion (as measured by the IFN-release test) in South African adolescents (Nemes et al., 2018; Poolman, 2020). Newly designed live-attenuated mycobacterial vaccines (MTBVAC; VPM1002) are being tested as possible BCG replacements or modifications (World Health Organization, 2019; Poolman, 2020). Although the immunology of CD4⁺ reactions in TB has garnered a lot of study, the function of bacterial virulence mechanisms has gotten less attention (Andersen and Scriba, 2019; Poolman, 2020). Inadequate understanding of MTB secretory systems and bacterial defenses against reactive oxygen and nitrogen molecules, as well as poorly defined processes of bacterial virulence and immune protection, are significant barriers in the development of a better TB vaccine, making target antigen selection difficult. Killed whole-cell (WC), live attenuated, adjuvant subunit, and viral-vectored designs are among the 11 TB vaccine candidates now in clinical development (recently reviewed) (Andersen and Scriba, 2019; Poolman, 2020). Several vaccinations are being developed as adjuncts to TB treatment (MIP, Vaccae, H56: IC131, RUTI™, and ID93/GLA-SE). Given the relative simplicity of identifying clinical endpoints, the invention of therapeutic vaccines for use in integrated methods with antibiotic therapy might move quickly, and might be a “fast win” in terms of enhancing clinical outcomes in the treatment of MDR tuberculosis. Four adjuvanted subunit vaccines are now being tested in clinical trials. These molecules consist of proteins whose functionality and relevance to virulence aren't always understood. All these vaccines and two viral-vectored vaccines (AERAS-402 and MVA85A) elicited functional Th1 CD4⁺ T-cell responses, with no substantial CD8⁺ T-cells or IL-17 production; the main difference was that recipients of M72/AS01_E (GSK), a recombinant fusion protein with AS01, had greater cytokine responses (Rodo et al., 2019; Poolman, 2020). The viral-vectored candidate vaccines AERAS-402 and MVA85A were used in a prime-boost study, which indicated that the booster dosage increased CD8⁺ T-cell responses, however these appeared short-lived. In infected people in South Africa, Kenya, and Zambia (Sheehan et al., 2015; Van Der Meeren et al., 2018), immunization with M72/AS01_E offered 54% protection (90% CI 13.9–75.4) against active pulmonary TB illness after 2 years of follow-up. After 3 years, vaccine effectiveness was 49.7% (90% confidence interval 12.1–71.2) (Tait et al., 2019; Poolman, 2020).

H56:IC31, a fusion protein and IC31 adjuvant, is being aimed at preventing TB illness in infected people of all ages as a supplement to antimicrobial therapy. This vaccine option produces antigen-specific Th1 immune responses in infected and non-infected individuals (Suliman et al., 2019), and its effectiveness as an additional therapy is being studied in two Phase 2 trials. Subunit vaccines now contain a small number of antigens whose individual protective properties remain unclear (Schrager et al., 2018; Poolman, 2020). To increase the confidence of antigen selection, a revitalized systemic full-genome based antigen development is required. Extended subunit compositions are now being studied as DNA or viral (cytomegalovirus)-vectored vaccines (Griffiths et al., 2016; Hansen et al., 2018; Poolman, 2020).

Conclusion and future perspective

The benefits of combining various vaccines or developing more polyvalent vaccines include reduced frequency of shots, lower risks of infection following vaccination and less cost of vaccination administration. Despite the promising results of laboratory

investigations using live attenuated vaccines, human immunization with live recombinant vaccines is still a work in progress. The latent pathogenicity of such carriers might result in infections among immuno-compromised patients and/or it can produce different results in practical use as compared to experimental studies.

Currently, majority of the vaccines are administered through intramuscular or intravenous injections to induce systemic immunity. This can lead to increased risk of infections at injection site as well as an uprising need of trained health care personals. The mucosal vaccination can serve as an alternate to injections. The possibility of degradation, resulting in a lower dose of vaccine, must be addressed for oral immunization.

Vaccine against cancer has also been experimented in mouse models by using inactivated whole cancerous cells. However these vaccines have not been tested on human tumor cells. Furthermore, the use of inactivated cancer cells may result in disease reactivation. Effective human anticancer vaccines can be developed by identifying the tumor specific antigens. Additional obstacles in the development of cancer vaccines must be considered: When cancer vaccines are tested, patients are frequently given immunosuppressive conventional medication, which prevents the immune system from functioning correctly. Similarly, the human immune system's effectiveness deteriorates with age. Vaccines cannot provide the same level of immunity in older cancer patients as they can in younger individuals.

This is important to foster vaccine firms to create vaccinations that are beneficial for the public but may not be economically feasible. Given the critical role of vaccinations in lowering resistance, failing to address these concerns might become a global problem.

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Viral Vaccines

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Glossary

Adjuvant An exogenous immunostimulatory agent added to vaccines to enhance immunogenicity. Adjuvants improve immune response and stimulate humoral and/or cellular immunity to improve the protective capacity of vaccines.

Attenuation The process of modification of the biology of a strain of virus by serial passage in tissue culture. Attenuation in cell culture has been a hallmark of vaccine discovery and has provided the basis by which many vaccines currently in clinical practice were developed.

Contact immunity A phenomenon whereby individuals acquire vaccine-induced immunity by virtue of close contact with immunized individuals who transmit the live, attenuated strain of vaccine to the naïve individual. Contact immunity can, in effect, immunize unvaccinated individuals secondarily through contact with vaccinated individuals. This is known to be an attribute of oral polio vaccination.

DNA vaccine Vaccine strategy in which a recombinant DNA molecule encoding a viral gene product (protein) of interest is administered to an individual, toward the goal of inducing a protective immune response.

Herd immunity Vaccine-induced protection of a population based on the acquired immunity of a large percentage of individuals residing in that community. When vaccine rates are high, the likelihood of herd immunity is high, and disease rates are low because of the lack of susceptible individuals.

Intussusception A form of intestinal blockage, usually observed in infants, where a portion of bowel collapses into the lumen of the adjacent bowel, creating a 'telescope'-like effect. This phenomenon was observed with first-generation rotavirus vaccines; though never causally proven, it did lead to the withdrawal of one rotavirus vaccine from the market.

mRNA vaccine A novel vaccine strategy, first evaluated in human subjects in the context of the COVID-19 pandemic, in which a messenger RNA (mRNA) encoding a viral gene product (protein) of interest is administered to an individual, toward the goal of inducing a protective immune response.

Subunit vaccine A vaccine designed and manufactured to induce antibody responses against one or more particular proteins or constituents of a pathogenic virus. Subunit vaccines typically utilize cloning technologies to express immunological targets of interest in a variety of expression systems.

VAPP Vaccine-associated paralytic poliomyelitis. This occurs when the specific attenuating mutations in the oral polio vaccine are lost due to reversion to wild-type sequence. The resulting revertant virus is capable of neurovirulence leading to paralytic polio.

Vector vaccine A vaccine approach in which an immunogen of interest (such as SARS-CoV-2 Spike protein) is inserted into another, attenuated viral vector. Immunization of the individual with the vector in turn induces an immune response to the gene product of the pathogen, resulting in protective immunity against that virus.

Introduction

Credit for the first use of vaccines in humans has historically gone to Edward Jenner, who is considered the pioneer of the smallpox vaccine (Eyler, 2003). The work of Jenner took place in the late eighteenth century, but in fact, the history of smallpox vaccination dates back to much earlier times in human history, when the process of “variolaion” (inoculating a susceptible individual with the dried scab or secretions from another person with the smallpox) was practiced as long ago as the tenth century in China (Gross and Sepkowitz, 1998). Smallpox vaccination represents the prototype of a “live, attenuated” viral vaccine, discussed in the following section (see also Table 1). The second category of vaccines in clinical use is the so-called “subunit” vaccine, typically based on the immunization of a susceptible individual with one or more proteins important in immunity against the pathogen of concern (Table 2). Usually these protein-based vaccines are administered with an immunostimulatory adjuvant (defined below). Broadly speaking, nucleic acid-based vaccines (DNA and mRNA) can be considered as “subunit” vaccines, since they are typically predicated on the administration of a nucleic acid sequence encoding a single (or limited number of) viral gene product(s). Finally, there is a sub-category of subunit vaccines in clinical use referred to as “vectored” vaccines. These vaccines use an innocuous and attenuated agent, usually a replication-incompetent virus, to express a foreign gene from a heterologous pathogen. Expression of the gene product following immunization induces protective immunity to the pathogenic virus. Most of the important vaccines used in clinical practice today fall into one of these categories. In this article, clinically important viral vaccines are listed in the accompanying tables, and some of the most important viral vaccines used in medical practice today are reviewed and compared in the text. The impetus behind the rationale for a vaccine’s design is provided, as well as the proposed mechanism(s) of protection. The need for new, novel immunizations against viral pathogens for which no vaccines are currently available is briefly discussed.

Table 1 Live, attenuated viral vaccines in clinical practice.

<i>Vaccine</i>	<i>Indication</i>	<i>Route of administration</i>	<i>Comments</i>
Smallpox (vaccinia)	Smallpox prevention.	Intradermal.	• Not current recommended for routine use. • Potential future role in bioterrorism disease control?
Oral polio vaccine (OPV)	Polio prevention.	Oral; three-dose series.	• Serotypes 1–3. • Contact immunity. • Intestinal/mucosal immunity. • Risk of VAPP.
Live, attenuated influenza vaccine (LAIV)	Influenza prevention.	Intranasal.	• No longer used in developed world. • Attenuated by cell culture passage at 25 °C. • Induces mucosal (IgA) responses in addition to IgG response.
Mumps, measles, and rubella vaccine (MMR)	Prevention of mumps, measles and rubella infections.	Subcutaneous (preferred) or intramuscular.	• Approved for ages 2–49 years • Three vaccines are combined for convenience and ease of dosing. • Vaccine initiated at 15 months. • Second dose required in later childhood.
Rotavirus vaccine	Prevention of rotavirus infection (epidemic gastroenteritis).	Oral; two- or three-dose series.	• Two vaccines currently licensed in U.S. are either human rotavirus-derived attenuated vaccine or bovine/human reassortants. • Vaccination begins in the first 1–2 months of life. • Intestinal/mucosal immunity. • Serum IgG response.
Live, attenuated varicella-zoster virus (VZV) vaccine for chicken pox (varicella); live, attenuated vaccine for Herpes Zoster (shingles)	Prevention of chicken pox; prevention of shingles (Herpes Zoster).	Subcutaneous or intramuscular.	• Protects against chicken pox when administered in two-dose series to VZV seronegatives. • Protects against reactivation (shingles or herpes zoster) when given to adults with past history of chicken pox. • Live attenuated Zoster vaccine no longer available in the U.S. but still in use in other countries.
CYD-TDV	Prevention of Dengue fever.	Subcutaneous administration; three-dose series.	• Live attenuated tetravalent chimeric vaccine. • Three-doses at every 2 month intervals. • CYD-TDV is partially effective in preventing infection, but vaccination may lead to severe disease in those who have not been previously infected and then acquire natural infection.

Table 2 Killed/inactivated/subunit/vectored viral vaccines used in clinical practice.

<i>Vaccine</i>	<i>Indication</i>	<i>Route of administration</i>	<i>Comments</i>
Hepatitis A	Prevention of hepatitis A infection.	Intramuscular; two-dose series.	- Formalin-inactivated virus proteins from cell culture. - Adjuvanted with aluminum hydroxide. - Induces protective IgG response. - Recommendation for universal childhood immunization.
Hepatitis B	Prevention of hepatitis B infection.	Intramuscular; three-dose series. A combined hepatitis A/hepatitis B vaccine, 'Twinrix,' is also licensed in the U.S. (see text for details).	- Based on single viral protein, hepatitis B surface antigen (HBsAg). - Expressed using cloned, recombinant expression in <i>Saccharomyces cerevisiae</i>. - Adjuvanted with aluminum hydroxide. - Induces protective IgG response to HBsAg. - Recent new two-dose recombinant HB surface antigen vaccine approved, similar to previous vaccines, includes a novel CpG 1018 adjuvant, a 22-mer phosphorothioate-linked oligodeoxynucleotide. - Cancer prevention vaccine. - Universal recommendation for childhood immunization.
Inactivated/subunit influenza vaccines	Influenza prevention.	Intramuscular.	- Formalin-inactivated ('split virion') vaccines. - Both trivalent and tetra-valent formulations available. - Mechanism of action: induction of anti-HA (hemagglutinin) and influenza virus-neutralizing IgG responses. - Vaccine virus cultivated in embryonated eggs; other cell culture systems are available including eucaryotic cell culture and insect cell culture for egg-allergic patients. - A high-dose vaccine with 4 times the total influenza protein in licensed in the US for individuals >65 years. - An adjuvanted flu vaccine with MF59C.1 adjuvant is licensed for individuals >65 years. - Universal immunization against influenza recommended for all individuals >6 month of age in US.
HPV vaccine	Prevention of HPV infection.	Intramuscular; CDC recommends that 11- to 12-year-olds receive two doses of HPV vaccine 6 to 12 months apart; only two doses are recommended if vaccination started at age 9 and through age 14, but teens and young adults who start the series later, at ages 15 through 26 years, need three doses of HPV vaccine.	- Bivalent (16, 18). Quadrivalent (6, 11, 16, 18) and nine-valent (9vHPV; types 6, 11, 16, 18, 31, 33, 45, 52, and 58) have been licensed by FDA. - Vaccine manufactured as recombinant protein in a yeast cell culture system (<i>Saccharomyces cerevisiae</i>). - Nine-valent formulation is the only HPV vaccine currently distributed in the U.S.
Japanese Encephalitis Virus Vaccine	Prevention of Japanese Encephalitis Virus infection.	Two-dose series, subcutaneous administration.	- Mosquito-borne <i>Flavivirus</i> infection. - Endemic in Western Pacific and Southeast Asian Regions. - Formalin-inactivated, multi-component viral vaccine currently is approved in the USA, but DNA vaccines and attenuated vaccines also in various stages of clinical development globally.
Herpes Zoster (Shingles) vaccine	Prevention of Herpes Zoster.	Intramuscular, two-dose series.	- Based on VZV glycoprotein E protein. - Adjuvanted with AS01B adjuvant. - Protects against reactivation (shingles or Herpes Zoster) when given to adults with past history of chicken pox.
SARS-CoV-2 (COVID 19) vaccines	Prevention of infection and/or reduction of disease, reduced risk of hospitalization and death due to COVID-19.	mRNA/lipid nanoparticle vaccines (BNT162b2 and mRNA-1273): intramuscular administration, 2 dose series plus a third (booster) dose. Adenovirus-vectored vaccine (Ad26.COV2-S COVID-19 vaccine): initial dose by intramuscular route followed by booster dose.	- BNT162b vaccine requires two doses given 21 days apart. - The mRNA1273 vaccine requires two shots spaced 28 days apart. - Recommendation for third (booster) dose for both mRNA vaccines 6 months following second dose. - Both mRNA vaccines encode for the SARS-CoV-2 (COVID) "Spike" glycoprotein (but expressed as RNA molecule). - Ad26.COV2-S COVID-19 vaccine administered at least 2 months following initial dose. - Ad26.COV2-S COVID-19 vaccine expresses Spike protein in the context of recombinant adenovirus vector (and hence has a replication incompetent virus vector backbone), and does not contain adjuvant.

Live, attenuated viral vaccines

Smallpox vaccine

As noted, the first known vaccine used in the history of humanity was a vaccine against smallpox (also known as variola). Edward Jenner received credit for developing the first smallpox vaccine. As history has it, Jenner observed that milkmaids exposed to cowpox appeared to be “resistant” to acquisition of smallpox. Jenner tested whether inoculation of subjects with material from cowpox lesions protected individuals against smallpox upon direct exposure. Indeed, the approach was successful, and the term “vaccination” (derived from the Latin word *vacca*, meaning “cow”) was used to describe the procedure. Although Jenner receives credit for developing the smallpox vaccine – and, indeed, creating the field of vaccinology – other contemporaries of Jenner had observed the protective effect against smallpox conferred by exposure to cowpox, most notably Dr. Benjamin Jesty (Eyler, 2003), in the years leading up to Jenner’s groundbreaking experiments.

Although initial approaches to smallpox vaccination utilized cowpox virus, the smallpox vaccine historically employed in clinical practice was based on vaccinia, another related poxvirus, but one that has minimal potential for virulence in healthy, immune-competent humans. Smallpox vaccine administration was unique compared to most other viral vaccines, insofar as it was administered by skin prick using a novel, two-pronged needle. Smallpox vaccine was extraordinarily successful globally and, indeed, smallpox is today the only disease in the history of humanity eradicated by immunization (Smith, 2013). Globally, the last known case of smallpox was reported in Somalia in 1977, and routine smallpox vaccination was discontinued in the United States in the early 1970s. After the terrorist attacks of September 11, 2001, smallpox immunization was reinitiated in some potentially high-risk populations, including health care providers and military personnel, in anticipation of the possible use (never realized) of weaponized smallpox virus by terrorists as a biological weapon (Arita, 2005). Since smallpox vaccine was discontinued, there has been an apparent emergence of the highly related monkeypox infection in humans, due to the loss of cross-protective immunity formerly conferred by smallpox immunization (Sklenovská and Van Ranst, 2018).

To this day, the principles of Jenner’s approach – based on the recognition of strong biological similarities of infectious syndromes across different species – are employed in the design and production of live, attenuated viral vaccines for human use. This is sometimes referred to as a “Jennerian” approach, and can include propagation of human pathogens on cells of heterologous species toward the goal of attenuation of the pathogens (varicella vaccine, measles vaccine), or actually using components of the related vertebrate virus (rotavirus vaccine) in creating the human vaccine. Individual examples are considered in further detail below.

Live, attenuated polio vaccine

Poliovirus is a member of the enterovirus family of human pathogens. Although most individuals with polio infection have a mild, self-limited illness, a small but substantial percentage will have polio infection spread to the brain and/or spinal cord, where it can lead to paralysis and potentially death. By the early 1950s in the United States, polio epidemics had become devastating. For example, between 1951 and 1954, there were over 10,000 cases of paralytic poliomyelitis reported every year to the Centers for Disease Control and Prevention (Nathanson and Kew, 2010).

Two polio vaccines emerged in clinical practice in the late 1950s/early 1960s (Melnick, 1996). The first vaccine, the so-called Salk vaccine, is a killed, whole-virion protein vaccine (reviewed below). The second licensed vaccine, the Sabin vaccine, is a live, attenuated vaccine. Both vaccines are still used today, although the Sabin vaccine is not currently used in the United States. The Sabin vaccine is made up of three different serotypes of poliovirus (types 1–3), all of which can cause paralytic polio. The vaccine was originally created by passage of polio in primary monkey and human diploid cell cultures, at subphysiological temperatures that allowed growth of virus, but facilitated the accumulation of attenuating mutations. Following tissue culture passage, mutations accumulate in the sequence of all three strains that lead to a loss of virulence and an inability of the viruses to produce paralysis. These mutations have been mapped in detail (Kew et al., 2005). One of the features of these attenuating mutations is a reduction in the ability of viral messenger RNA to be translated by the ribosome.

The oral polio vaccine (OPV) is administered in a four-dose series. The vaccine contains mixtures of all three attenuated polioviruses. The attenuated vaccine strains replicate very efficiently in the gastrointestinal tract, as does wild-type poliovirus, which probably contributes to an enhanced immune response compared to inactivated polio vaccine. This so-called intestinal immunity appears to include mucosal IgA responses that enhance the protective effect of the vaccine. Moreover, the vaccine virus is excreted in the stool of immunized patients. This excreted vaccine virus can, in turn, be ingested by a nonvaccinated individual, leading to secondary (contact) immunization, via the fecal–oral route of ingestion. Thus, this creates the potential for “contact immunity”, namely, the idea that others will be secondarily immunized by being in close contact with individuals who receive the vaccine. This phenomenon contributes to the preference for oral polio vaccination in regions where wild-type polio is still highly endemic. Unfortunately, polio vaccine virus also has the potential to revert back to wild-type sequence in the gastrointestinal tract in immunized patients, and can in fact revert to a fully neurovirulent genotype, particularly in children with immune deficiency. This exposure to revertant virus can lead to paralysis, and this “vaccine associated paralytic poliomyelitis” (VAPP) phenomenon was responsible for 8–10 cases of polio in the United States every year until OPV use was discontinued in the United States in the late 1990s. Wild-type poliovirus has been absent from the Western hemisphere since 1991, although occasionally neurovirulent revertant strains of polio are still observed (Alexander et al., 2009), presumably due to importation from countries still using

OPV. Some of these revertant strains are capable of circulating in vaccine-naïve populations and producing paralytic poliomyelitis (Gumede et al., 2013). For regions of the world where polio is still endemic and epidemic, however, OPV has been considered to be the preferred vaccine for population-based immunization. Considerable progress has been made toward global polio eradication (Modlin et al., 2021), facilitated by the global SARS-CoV-2 pandemic, since the recent global vaccination drive against COVID-19 has increased the public dialog about the great need for vaccines, including polio vaccine (Ali et al., 2021).

Influenza vaccine (live, attenuated)

Influenza is an RNA virus in the *Orthomyxoviridae* family. There are two major subtypes of influenza virus: influenza A and influenza B. Both are important human pathogens, although humans are not the natural host for influenza (wild aquatic birds are the natural host). Influenza has a segmented RNA genome and influenza A virus can undergo extensive recombination in nature, leading to the appearance of genetically variant strains not previously encountered in the human population. When this process – known as “antigenic shift” – occurs, an influenza pandemic can ensue, leading to extensive mortality. The influenza pandemic of 1918 resulted in an estimated 50,000,000–100,000,000 deaths globally (Morens and Fauci, 2007). Even in a “normal” influenza season, it is estimated that 40,000–50,000 deaths every year in the United States are attributable to influenza infection. Universal annual influenza administration is recommended for everyone over 6 months of age in the United States.

Live, attenuated influenza vaccines (LAIVs) have only recently become available for clinical use. These vaccines are prepared by passing clinical isolates of influenza in cell culture using subphysiological temperatures. This process of “cold adaptation” – used in the generation of other live, attenuated viral vaccines considered in this article – led to the development of the current LAIVs used in clinical practice (Jang and Seong, 2012). Since the process of cold adaptation in cell culture was undertaken at temperatures (25 °C) incompatible with replication of virus at core body temperature (37 °C), these vaccines are highly attenuated and extremely safe. The vaccine is administered in the form of a nasal drop or spray on an annual basis. The vaccine is trivalent, containing two strains of influenza A and a single strain of influenza B, or tetravalent, containing two strains of influenza A and two strains of influenza B, depending upon the manufacturer. The strains chosen to compose the vaccine each flu season depend upon what strains of influenza have recently circulated, or are predicted by public health officials, to be circulating in the upcoming influenza “season”. LAIV is not recommended for children under 2 years of age, or individuals over 50 years. The mechanism of protection is the induction of IgG antibody to influenza, in particular hemagglutination-inhibition antibody.

Measles vaccine

Measles is a highly contagious viral infection spread from person to person predominately by aerosol (droplet) route. It is a member of the *Paramyxoviridae* family of viruses. Measles infections used to be so ubiquitous in nature that it is assumed that all individuals born in the United States before 1957 are immune to the measles virus. Prior to the advent of vaccination, measles (also known as rubeola) caused considerable mortality globally, with over 2 million deaths annually in children (Cutts et al., 2013). Measles infection causes cough, coryza (runny nose), and conjunctivitis, as well as a classic morbilliform (“measles-like”) rash. In addition to the classic rash of rubeola, measles infection is responsible for severe, occasionally fatal pneumonia, tracheobronchitis, and encephalitis. Measles infection can also produce long-term complications, in particular subacute sclerosing panencephalitis, a chronic and uniformly fatal encephalitis caused by persistence of measles virus in the central nervous system. Measles infection strongly suppresses the host immune system and predisposes patients (particularly young children) to develop other serious infections, including tuberculosis. Remarkably, measles infection has been shown to “erase” immune memory, potentially resulting in the loss of protection conferred by other vaccines (Petrova et al., 2019). Measles is also an important cause of blindness in the developing world. Although considerable progress has been made in preventing measles-associated morbidity and mortality, over 100,000 measles deaths occurred in 2010, underscoring the need for continued vigilance in vaccination programs (Cutts et al., 2012).

Measles vaccine is a live, attenuated vaccine. Attenuation of this virus occurred following serial passage in a nonpermissive cell line, in the case of measles, cells of avian origin. There have been several measles virus variants used as vaccines in clinical practice since the advent of immunization programs in the early 1960s. The original vaccine strain was named the “Edmonston” strain, after the child from whom the wild-type virus was originally isolated (Hilleman, 1992). The process by which serial passage of the measles virus in cell culture produces attenuation remains unclear, although a recent study demonstrated varying amino acid substitutions in all of the viral proteins encoded by the measles genome in vaccine strains (Bankamp et al., 2011). Measles vaccine is administered by the subcutaneous route, typically at 15 months of age. This vaccine is usually combined with the mumps and rubella vaccines (hence, the MMR vaccine, for mumps–measles–rubella). A second dose of vaccine is recommended later in childhood. The vaccine appears to work by induction of antibody responses to measles envelope glycoproteins, particularly the hemagglutinin protein involved in binding of measles virus to its cellular receptor (Hashiguchi et al., 2011).

Mumps vaccine

Mumps is a paramyxovirus and, as such, highly genetically related to other common childhood infections such as measles, respiratory syncytial virus, and parainfluenza virus. Mumps infections can be associated with parotitis, orchitis (rarely causing infertility), aseptic meningitis, and sensorineural deafness.

Mumps vaccine is based upon attenuating serial passage of what was originally a clinical isolate of mumps virus, the “Jeryl Lynn” strain. This strain is actually a mixture of two distinct, closely related strains (Afzal et al., 1993). Other attenuated strains, including the Zagreb strain, have been used in the development of live attenuated mumps vaccine (Ivancic et al., 2005). The precise molecular basis of attenuation is not known. The vaccine is administered by subcutaneous or intramuscular route, typically in combination with measles and rubella vaccine (MMR vaccine). The protective mechanism appears to be via induction of antibody to mumps virus surface glycoproteins. Mumps outbreaks have occurred on a university campus in spite of the fact that 95% of the affected students had received two doses of the MMR vaccine. The susceptibility appeared to be related to a suboptimal mumps antibody titer (Cortese et al., 2011), suggesting that future modifications of mumps vaccine will be required.

Rubella vaccine

Rubella virus is an enveloped, single-stranded, positive-sense RNA virus belonging to the *Togaviridae* family. Although togaviruses are typically vector-borne infections, rubella is the notable exception, being transmitted instead by a respiratory droplet route. Humans are the only known natural host for rubella virus. Rubella infection, commonly known as the “German measles”, usually results in a mild illness with an accompanying exanthem in adults and children; however, rubella produces serious consequences in the pregnant patient, where fetal infection can lead to severe anomalies including microcephaly, sensorineural deafness, and cardiac defects (Freij et al., 1988). An ophthalmologist, Norman Gregg, offered the first description of congenital rubella syndrome (CRS) in 1941, while investigating an epidemic of neonatal cataracts (Gregg, 1947). Not until the global pandemic of 1964–65, however, were the multiple teratogenic manifestations of CRS fully appreciated and the permanent neurodevelopmental consequences for newborns completely recognized. The capacity to grow the virus in tissue culture led rapidly to the development of the vaccine and to a reduction in CRS in the United States and other developed countries.

Rubella vaccines are classic live attenuated viral vaccines, prepared through the process of long-term cell culture passage, which in turn leads to attenuating mutations in wild-type sequences. In clinical practice, most vaccines are derived from the Wistar RA 27/3 strain of virus. Although the strains used in vaccines have been sequenced, the molecular basis of attenuation is unclear (Kakizawa et al., 2001). The vaccine is administered by subcutaneous route or intramuscular route, typically in combination with measles and mumps vaccine (MMR vaccine). The protective mechanism appears to be via induction of antibody to rubella virus surface glycoproteins. Rubella has been eradicated from the Western Hemisphere (Plotkin, 2021), although routine MMR administration continues to be recommended to guard against any resurgence of disease. A combination vaccine containing both MMR and Varicella vaccine – ‘MMRV’ vaccine – has been licensed in the United States. Unless the parent expresses a preference for MMRV vaccine, the CDC recommends that MMR vaccine and varicella vaccine should be administered as separate injections for the first dose in children 12–47 months of age, with use of MMRV combination vaccine reserved for use as a “booster” dose in later childhood.

Rotavirus vaccine

Rotaviruses are members of the *Reoviridae* family. They are unique among pathogenic viruses by virtue of the fact that their genomes are double-stranded, segmented, RNA genomes. Rotavirus, prior to the advent of vaccination, was the most important cause of viral gastroenteritis, particularly in children (Vesikari, 2012). Rotavirus infection in young children can be responsible for severe diarrhea and attendant fluid and electrolyte abnormalities, and causes considerable mortality in the developing world, where supportive care resources are limited. It was estimated that, globally, rotavirus infection caused more than 450,000 deaths in children under 5 years of age in 2008 (Tate et al., 2012).

Rotaviruses are common in vertebrates, and such viruses (i.e., rhesus macaque rotavirus, bovine rotavirus) share many molecular and biological similarities with human rotavirus. These similarities have been exploited in vaccine design. The first human rotavirus vaccine (licensed in the late 1990s) was a so-called “rhesus reassortant” virus made up of four live viruses: a rhesus rotavirus (serotype G3) and three rhesus–human reassortant viruses (comprising both human and rhesus rotavirus components) that were specific for serotypes G1, G2, and G4. Thus, the vaccine was designed to protect against the four rotavirus serotypes that were responsible for most rotavirus disease in the United States at that time (Vesikari, 2012). This vaccine was successful in preventing rotavirus disease in children following a three-dose series, but was withdrawn from the market because of a possible association with a complication known as intussusception, a complication resulting in intestinal blockage (Peter and Myers, 2002). Subsequently, two new rotavirus vaccines were licensed in the United States and remain in widespread use. These newer vaccines do not appear to carry a risk of intussusception. The first is a live, attenuated human rotavirus vaccine derived from an infant with rotavirus gastroenteritis (G1 strain). This vaccine protects across most of the different serogroups of rotavirus in circulation, not just the G1 serotype. It is recommended for use in a two-dose schedule beginning at 6 weeks of age. The second is another human–animal (bovine) reassortant vaccine. This vaccine contains five live reassortant rotaviruses: four of these express the G1, G2, G3, or G4 VP7 protein from their respective parent human strain and an attachment protein from a bovine strain, and the fifth expresses an attachment protein from a human strain and a VP7 protein from a G6 bovine strain (Dennehy, 2008). This vaccine is recommended for use in a three-dose series, at 2-month intervals, beginning at 6–12 weeks of age. Both vaccines are highly effective in preventing rotavirus infection.

All licensed rotavirus vaccines are administered by the oral route, analogous to the use of the OPV. The correlate of protective immunity induced by vaccination appears to be IgG antibody. However, there is some evidence that an equally important correlate of protective immunity following vaccination may be the IgA response (Patel et al., 2013).

Varicella-zoster virus vaccine

Varicella-zoster virus (VZV) is the etiologic agent of chicken pox. Although most children with chicken pox have an uneventful recovery, prior to the advent of vaccination there were approximately 100 varicella deaths in otherwise immune-competent children in the United States every year, due to pneumonia, neurological complications such as encephalitis, or secondary infections caused by *Streptococcus pyogenes* (Nguyen et al., 2005). As a member of the *Herpesviridae* family of viruses, it is capable of establishing latent infection in the dorsal root ganglia, where it resides in quiescent form for years or even decades before reactivating. When VZV reactivates, it produces a rash on the skin surface corresponding to the region of the skin innervated by that particular ganglia; such a reactivation is known as Herpes Zoster or “shingles” (Schleiss, 2009). Herpes Zoster can lead to postherpetic neuralgia, which can cause debilitating pain for prolonged periods of time, particularly in elderly patients (Gershon and Gershon, 2013). Hence, there is a strong medical rationale for a VZV vaccine both to prevent chicken pox (vaccine given to VZV-naïve subjects) as well as prevent episodes of zoster (vaccine given to those with a history of chicken pox).

VZV vaccine was derived from a clinical isolate known as the “Oka” strain. This strain was originally obtained in the early 1970s from a young child with chicken pox (Takahashi et al., 1974), and was passaged in cell culture, including extensive passages in both guinea pig cell culture as well as human cells (Takahashi et al., 2008). Some of the cell culture passages were at a reduced temperature (34 °C). As is the case for so many other live, attenuated viral vaccines, the precise molecular basis for the attenuation of the virus was unknown at the time of the vaccine’s licensure, although recent studies have begun to shed light on the specific molecular mutations that arose during serial tissue culture passage that appear to have contributed to the reduction of virulence (Peters et al., 2012). The vaccine has been variably used in the United States in three formulations: a monovalent formulation (“Varivax”) for children and young adults; MMRV vaccine; and a monovalent formulation for adults over 60 years of age for prevention of herpes zoster (shingles). The vaccine is given subcutaneously. Two doses of vaccine are administered for prevention of chicken pox, while a single dose of vaccine had been recommended for older individuals receiving the vaccine to prevent shingles. Although the live, attenuated shingles vaccine is still available in some countries, it is no longer available in the United States, and has largely been supplanted by the adjuvanted, VZV glycoprotein E subunit vaccine, which has been shown to be highly effective at preventing shingles (Heineman et al., 2019).

Subunit viral vaccines

Hepatitis A vaccine

Hepatitis A is an RNA virus in the *Picornaviridae* family. It is readily transmissible by the fecal–oral route and is highly contagious. Food-borne outbreaks are common. Infection leads to liver inflammation (“hepatitis”), vomiting, diarrhea, and jaundice. Jaundice may be absent in young children with acute infection. Although most infections are self-resolving, infection can lead to fulminant hepatic failure in some cases (Brundage and Fitzpatrick, 2006). Subunit vaccines based on formalin-inactivated hepatitis A virus, cultivated in MRC-5 cells, are licensed and marketed by two manufacturers in the United States. Hepatitis A vaccine is also combined with hepatitis B in another vaccine licensed in the United States, a product known as “Twinrix”. Both are adjuvanted with aluminum hydroxide adjuvant. Two doses, given by intramuscular route and separated by at least 6 months, are recommended. The mechanism of protection is believed to be by induction of hepatitis A-specific IgG antibody response.

Hepatitis B vaccine

Hepatitis B virus is a double-stranded DNA virus that is a member of the *Hepadnaviridae* family. It is a highly contagious virus, spread by exposure to infectious blood and body fluids. Infection leads to liver inflammation (“hepatitis”), vomiting, jaundice, and occasionally death. It is common for infected individuals to become carriers of hepatitis B, meaning that infection remains active. Hepatitis B is associated with cirrhosis of the liver and hepatocellular carcinoma. It is estimated that one-third of the world’s population has been infected with hepatitis B, and that over 350 million people are chronic carriers (Chen, 2010). Given the causal associations between hepatitis B and mortality, development of an antiviral vaccine was a major public health priority.

The first vaccine licensed for human use for hepatitis B was a purified plasma preparation from patients with active hepatitis B infection. These individuals had high concentrations of hepatitis B proteins in serum, and purification of these proteins (including formalin inactivation) allowed their use as a vaccine. This vaccine was licensed in the early 1980s and administered by intramuscular route in a three-dose series (Szmuness et al., 1981). Although this vaccine was safe and effective, and seemed free of any exogenous agents, the need to purify the vaccine proteins from the serum of hepatitis B-infected individuals was problematic. The advent of technologies to produce cloned, recombinant proteins for immunization purposes led to the discontinuation of this product in favor of a recombinant vaccine. This vaccine was based on expression of a cloned recombinant form of hepatitis B surface antigen (HBsAg), expressed in the yeast *Saccharomyces cerevisiae*. A variety of manufacturers currently market licensed hepatitis B vaccines. These are recommended as universal vaccines for all newborns, administered by intramuscular route at 0, 1, and 6 months

of age. Individuals who miss immunization in infancy should be vaccinated with a three-dose series as soon as is feasible. Some manufacturers will provide hepatitis B vaccine as a component of a “mixture” of other common childhood vaccines, to help minimize the total number of required injections. The vaccine is adjuvanted with aluminum hydroxide. The mechanism of action of vaccination appears to be the induction of IgG antibodies targeting the HBsAg, with an antibody response of at least 10 mIU ml⁻¹ standing as a serological correlate of protection.

Human papillomavirus vaccine

Human papillomaviruses (HPVs) are common: there are at least 180 HPV genotypes described to date (Stanley, 2012). They are small, single-stranded, nonenveloped viruses that have a predilection for infection at mucosal or cutaneous surfaces. Most infections are asymptomatic, although many serotypes are associated with benign epithelial proliferation leading to warts. The major medical significance of HPV, however, stems from the fact that some subtypes are causally associated with malignancies of the anogenital track, particularly cervical carcinoma. Head and neck malignancies, respiratory papillomatosis, and anogenital warts are also caused by HPV infection. Worldwide, there are estimated to be approximately 530,000 new cases of HPV-associated cervical cancer per year, and 275,000 deaths (Tay, 2012).

There are approximately 15 HPV types that have been associated with anogenital malignancies; of these, HPV16 and HPV18 are the most prevalent, causing about 70% of cervical and anogenital cancer cases worldwide (Bosch et al., 2008). HPV6 and HPV11 are the types most commonly associated with genital warts (Lacey et al., 2006). There are two HPV vaccines currently licensed for clinical use. Both vaccines are based on the approach of generating noninfectious “virus-like particles” (VRPs) using recombinant technologies to express HPV proteins, which then spontaneously assemble into VRPs (Wang and Roden, 2013). The first licensed vaccine in the United States was a quadrivalent vaccine, based on VRPs cloned using recombinant technologies and synthesized in *S. cerevisiae*, and corresponding to HPVs 6, 11, 16, and 18. This vaccine is mixed with an aluminum hydroxide-based adjuvant prior to administration. The other licensed HPV vaccine is a bivalent vaccine, made up of VRPs corresponding to HPV16 and HPV18, and expressed in a recombinant baculovirus system prior to mixing with a monophosphoryl-lipid A-derived adjuvant. Both vaccines are extraordinarily effective at preventing HPV-associated malignancies as well as urogenital warts. The vaccines are administered by the intramuscular route, in a three-dose series commencing at age 9 years. The quadrivalent vaccine is currently recommended for boys and girls, whereas the bivalent vaccine (containing only the HPV types associated with cervical cancer) is recommended for girls only. The mechanism of protective immunity appears to be the induction of IgG antibody. The recent observation that there is cross-protective immunity induced by vaccination against nonvaccine strains provides optimism that these vaccines will protect against disease caused by diverse HPV types (Draper et al., 2013).

Inactivated influenza vaccine

Subunit, killed influenza virus vaccines have been in use for many decades in the United States. These vaccines are based on formalin inactivation of influenza viruses cultivated in fertilized eggs. Since the inactivation process kills the flu virus, it is impossible (and biologically inconceivable) that an individual could “get the flu” by being immunized. Typically, three strains are selected for cultivation (two strains of influenza A and one strain of influenza B), depending upon what strains have been observed circulating in the human population in recent outbreaks. Following formalin inactivation, enriched components of the virion are purified, and these so-called “split virion” component vaccines are administered in a single intramuscular injection annually prior to the onset of influenza season. Recently, an increasing number of pharmaceutical manufacturers have produced and marketed inactivated influenza vaccines. Some recent innovative vaccines that have either been licensed or are approaching licensure include vaccines manufactured in cell culture, rather than eggs, to avoid the issue of egg allergy (Brokhof et al., 2013); quadrivalent influenza vaccines capable of providing protection against two strains of influenza B in addition to two strains of influenza A (Ambrose and Levin, 2012); vaccines containing antigens generated using cloned, recombinant technologies (Sedova et al., 2012); vaccines using adjuvants to enhance the potency and effectiveness of influenza immunogens (Even-Or et al., 2013); and the development of vaccines expressing influenza immunogens as mRNA vaccines, analogous to the success that has been realized by COVID-19 mRNA vaccines (Scorza and Pardi, 2018). Given the disappointing performance of the past generations of influenza vaccines in controlling annual disease outbreaks (Osterholm et al., 2012), such advances and improvements are greatly needed (Atmar and Keitel, 2020).

Inactivated polio vaccine

As noted earlier in the section on oral, live, attenuated polio vaccines, the killed, inactivated IPV was actually the first vaccine against polio infection licensed in the United States (Melnick, 1996). The Salk vaccine was licensed in 1955, and the impact was immediate and dramatic. The total annual number of polio cases fell from 35,000 in 1953 to only 161 cases in 1961 (Hinman, 1984). In spite of this success, IPV was replaced by OPV in the early 1960s, due to the added benefits conferred by contact immunity, ease of administration (oral vs intramuscular), and lower cost. When it became clear by the 1990s that there was no longer any circulating wild-type poliovirus in the United States, and in fact that the only paralytic polio observed was VAPP caused by neurorevertant OPV, IPV once again became the vaccine of choice.

The IPV vaccine is administered by intramuscular or subcutaneous route. A four-dose series in childhood is recommended. There is no intestinal or mucosal immunity to IPV, in contrast to OPV. The mechanism of action is the induction of IgG antibodies capable of neutralizing the virus and protecting the central nervous system against paralytic polio. There is no contact immunity conferred by IPV immunization, although both IPV and OPV can produce “herd immunity” in a population if vaccination compliance is sufficient.

COVID-19 vaccines

The advent of the COVID-19 pandemic necessitated an urgent response, toward the goal of developing an effective vaccine against the SARS-CoV-2 virus. In response to this global crisis, a wide range of vaccine candidates have been developed. These range from nucleic acid based vaccines (DNA and RNA vaccines); live, attenuated vaccines; protein subunit vaccines; and vectored vaccines (Fiolet et al., 2021). In the United States, three vaccines have been licensed: two of these, BNT162b2 and mRNA-1273, are lipidated nanoparticle mRNA vaccines targeting the SARS-CoV-2 “Spike” protein, whereas the third vaccine, Ad26.COV2.S COVID-19, delivers the Spike open reading frame in the context of a replication-incompetent Adenovirus 26 vector. These vaccines have been remarkably effective in reducing COVID-19 infection, disease, hospitalization, and mortality. A major challenge for the future will be to continue to update vaccine design in response to emerging COVID-19 variants, such as the B.1.617.2 (Delta) SARS-CoV-2 variant (Cosar et al., 2021) and the B.1.1.529 (Omicron) variant (Saxena et al., 2021).

Viral vaccines and vaccine safety

In recent years, considerable attention has been given to the concern that vaccines, particularly the live, attenuated MMR vaccine, might be related to the development of autism in young children. Autism is a pervasive developmental disorder characterized by impaired social interaction, difficulties with verbal and nonverbal communication, and oftentimes stereotypical, repetitive patterns of behavior. It has become clear over recent years that the reports linking the MMR vaccine to autism were the product of flagrant scientific misconduct and clear-cut fraud (Flaherty, 2011). MMR and other vaccines have been demonstrated repeatedly to be safe and effective and no causal links have been found, in spite of exhaustive study, to link any childhood vaccine to autism or autism spectrum disorders (Gerber and Offit, 2009). A popular argument of the antivaccine movement is that the advent of new vaccines has created a state of “immune system overload” in children, by exposing them to too many antigens, too early in life. However, both the biological implausibility of this statement, as well as the fact that children today are in fact exposed to fewer total antigens in vaccines than they were in the 1960s (Offit et al., 2002), make this hypothesis completely untenable. Of even greater concern is the misinformation that has been promulgated about the safety of COVID-19 vaccines. Vaccine misinformation has contributed significantly to vaccine under-utilization, and this in turn has contributed to the sustenance of the COVID-19 pandemic globally (Ullah et al., 2021).

Priorities for new viral vaccines

Antiviral vaccines represent one of the most striking “success stories” in the history of medicine, having reduced (and in the case of smallpox, eliminated) the threat of many common, disabling, and even fatal virus infections. However, the vaccine armamentarium against serious viral infections is woefully incomplete and much work remains to be done. After the urgent need for continued optimization and implementation of COVID-19 vaccines, arguably the next most important – but to date elusive – target for vaccine development among viral infections is a vaccine for HIV, which continues to exact high levels of mortality in much of the world (Voronin et al., 2010). The most common vector-borne viral disease in the world is Dengue Fever, an infection that causes considerable morbidity and mortality, particularly in children. Although a Dengue vaccine has been licensed, its use is problematic (Wang et al., 2021). Prevention of dengue infection is a high priority for antiviral vaccine development (Lim et al., 2013). Hepatitis C infection is a common cause of liver failure as well as liver cancer, and a vaccine against this virus would represent an important public health advance (Voronin et al., 2010). Congenital infection (infection acquired by the developing fetus before birth) with cytomegalovirus is the most common infectious cause of birth defects, including hearing loss and developmental delay, in the United States. Development of a vaccine against this virus has therefore been designated as a major public health priority (Sung and Schleiss, 2010; Schleiss et al., 2017). Improved vaccines for influenza are needed, and a vaccine against respiratory syncytial virus is a high priority. This list is by no means exhaustive, but only serves to highlight a few of the many virus infections for which vaccines are still needed. In addition to these pathogens that require novel vaccines, improved vaccine delivery technologies are required. Vectored approaches, such as those offered by modified Vaccinia Virus Ankara and recombinant Vesicular Stomatitis Virus, are in clinical trials, and results are eagerly awaited. Nanoparticle vaccines also hold promise for improving immune responses for many “difficult” viral pathogens. Finally, scientists and clinicians need to be zealous advocates for immunization programs, in order to combat the untruthful agenda of the various organized antivaccine organizations. The field of antiviral vaccines greatly needs financial resources (Lindley et al., 2009), both from industry and government funding agencies, and more importantly the commitment of young investigators interested in research that will provide a tremendous “return on investment” in promoting the health of the population for future generations.

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Relevant Websites

<http://www.cdc.gov/vaccines/schedules/hcp/imz/child-adolescent.html>—CDC.
<http://immunizationinfo.org>—Immunization information.

US FDA-Approved Antibiotics During the 21st Century

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Abbreviations

ABECB	Acute bacterial exacerbation of chronic bronchitis
ABSSSI	Acute bacterial skin and skin structure infections
AME	Aminoglycoside modifying enzyme
aPTT	Activated partial thromboplastin time
<i>B. fragilis</i>	<i>Bacteroides fragilis</i>
BLI	Beta-lactamase inhibitor
CABP	Community-acquired bacterial pneumonia
CAP	Community-acquired pneumonia

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CDAD	<i>Clostridioides difficile</i> -associated diarrhea
CDI	<i>Clostridium difficile</i> infection/ <i>Clostridioides difficile</i> infection
Cfr	Chloramphenicol-florfenicol resistance
cIAIs	Complicated intra-abdominal infections
CRAB	Carbapenem-resistant <i>Acinetobacter baumannii</i>
CrCl	Creatinine clearance
CRE	Carbapenem-resistant <i>Enterobacteriaceae</i>
cSSSI	Complicated skin and skin structure infection
cUTIs	Complicated urinary tract infections
DSC	Discontinued
<i>E. coli</i>	<i>Escherichia coli</i>
eGFR	Estimated glomerular filtration rate
ESBL	Extended-spectrum β -lactamase
ESRD	End stage renal disease
FDA	Food and drug administration
GI	Gastrointestinal
GNB	Gram-negative bacteria
HABP	Hospital-acquired bacterial pneumonia
HAP	Hospital-acquired pneumonia
IV	Intravenous
KPC	<i>Klebsiella pneumoniae</i> carbapenemase
mAb	Monoclonal antibody
MDR	Multidrug-resistant
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin-susceptible <i>Staphylococcus aureus</i>
PBP	Penicillin-binding proteins
<i>S. pneumonia</i>	<i>Streptococcus pneumoniae</i>
US	United States
VABP	Ventilator-associated bacterial pneumonia
VRE	Vancomycin-resistant <i>Enterococcus</i>
WHO	World Health Organization

Introduction

Antimicrobial resistance continues to pose a significant global health threat (Prestinaci et al., 2015), as such new and novel antibacterial agents are urgently needed to combat antibiotic resistance and provide effective and safe therapeutic options for treating difficult-to-treat infections. In 2019, the US Centers for Disease Control and Prevention (CDC) released the second antimicrobial resistance threat report, which listed carbapenem-resistant *Acinetobacter*, *Candida auris*, *Clostridioides difficile* (*C. difficile*), carbapenem-resistant Enterobacterales (CRE), and drug-resistant *Neisseria gonorrhoeae* (*N. gonorrhoeae*) under the urgent threat category (CDC, 2019). Some serious threat pathogens include extended-spectrum β -lactamase (ESBL)-producing Enterobacterales, vancomycin-resistant *Enterococcus* (VRE), multidrug-resistant (MDR) *Pseudomonas aeruginosa* (*P. aeruginosa*), methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA), drug-resistant *Streptococcus pneumoniae* (*S. pneumoniae*), and drug-resistant tuberculosis, etc. (CDC, 2019). Each year, there are >700,000 deaths worldwide from drug-resistant infections (Dadgostar, 2019); and in the US, there are an estimated 2.8 million antibiotic-resistant infections, leading to >35,000 deaths annually (CDC, 2019). Since 2000, thirty-six antibiotics have been approved by the US Food and drug administration (FDA), which include new chemotype antibacterial classes such as oxazolidinone, lipocyclopeptide, the cephalosporin-siderophore conjugate antibiotic, narrow-spectrum anti-difficile 18-membered macrolide and antitubercular diarylquinoline and nitroimidazooxazine. The remaining approved antibiotics were largely derived from existing antibacterial classes (e.g., carbapenem, quinolone, tetracycline, lipoglycopeptide, and cephalosporin) to improve clinical efficacy, bacterial spectrum, and/or safety and resistance profiles. In addition, several β -lactam/ β -lactamase inhibitor (BLI) combinations and three monoclonal antibodies (mAbs) have also been developed and approved during this period. Several excellent reviews have been published to cover recent FDA approved antibiotics and/or new antibacterial agents under clinical development, in the contexts of more clinical or chemistry driven setting (Butler and Paterson, 2020; Butler et al., 2013, 2017; Deak et al., 2016). In this book chapter, we systematically review and discuss their clinical applications and medicinal chemistry properties of these approved antibiotics from 2000 to 2021 (Hori et al., 2021).

FDA approved antibiotics since 2000

Thirty-six antibiotics have been approved by US FDA since 2000. These new antibiotics include new chemotype antibacterial classes such as oxazolidinone (linezolid and tedizolid phosphate), lipocyclopeptide (daptomycin), anti-difficile macrolide (fidaxomicin), the cephalosporin-siderophore conjugate antibiotic (cefiderocol), and antitubercular agents diarylquinoline and nitroimidazooxazine (bedaquiline and PA-824, respectively). The remaining advanced antibiotics, developed on the basis of existing antibacterial chemical classes, include carbapenem (ertapenem and doripenem), nitroimidazoles (tinidazole and secnidazole), fluoroquinolones (gemifloxacin mesylate, besifloxacin, delafloxacin, and flaxloxacina), ketolide (telithromycin), rifamycin (rifaximin and rifamycin SV), tetracycline and/or glycylcycline (tigecycline, eravacycline, omadacycline, and sarecycline), pleuromutilins (retapamulin and lefamulin), lipoglycopeptides (telavancin, oritavancin, and dalbavancin), cephalosporin (ceftaroline fosamil), aminoglycoside (plazomicin), four β -lactam/BLI combinations (ceftolozane/tazobactam, ceftazidime/avibactam, meropenem/vaborbactam, and imipenem/cilastatin/relebactam with a renal dehydropeptidase inhibitor), along with three monoclonal antibodies (mAbs) (raxibacumab, obiltoxaximab, and bezlotoxumab). All the approved antibiotics from 2000 to 2021 are presented based on the order of their approval date. The overview, key attributes, physiochemical properties and pharmacokinetic (PK) parameters of these FDA approved antibiotics are listed in Table 1. The chemical structures are shown in Figs. 1–4.

Zyvox® (linezolid)

Zyvox (linezolid) (Fig. 1), an oxazolidinone antibiotic from Pfizer, is the first medication of the oxazolidinone class, and was approved for use by the US FDA in 2000 (Lexicomp, 2021). It is indicated for nosocomial pneumonia, community acquired pneumonia (CAP), uncomplicated skin and skin structure infections (SSTIs), complicated SSTIs, and infections secondary to vancomycin-resistant *Enterococci* (VRE), caused by pathogens such as *Staphylococcus aureus* (including MRSA), *S. pneumoniae*, *S. pyogenes*, or *S. agalactiae* (Pfizer, 2020b). In addition to the FDA approved indications, linezolid is used in multidrug regimens for the treatment of multidrug-resistant tuberculosis (MDR-TB). The 2016 WHO treatment guidelines for drug-resistant TB designated linezolid as a second line treatment option for MDR-TB (WHO, 2016).

Linezolid is a synthetic, bacteriostatic antibiotic, and active against drug-resistant staphylococci, enterococci, streptococci, and certain Gram-negative and anaerobic bacteria (Pfizer, 2020b). Linezolid is not used clinically for the treatment of Gram-negative bacteria. Structurally, linezolid contains a 5-membered oxazolidinone heterocyclic ring with a cyclic carbamate functionality, as well as the S-stereochemistry at the 5 position and the phenyl ring at the three position, which are essential for antibacterial activity (Barbachyn and Ford, 2003). Linezolid inhibits bacterial ribosomal protein synthesis at very early stage by binding to the 23S ribosomal RNA of the 50S ribosomal subunit (Livermore, 2003; Moellering, 2003).

Linezolid is administered by the oral or parenteral route, and the dosage for adult patients with nosocomial pneumonia, CAP, cSSSI, and VRE infections including concurrent bacteremia is 600 mg IV or oral every 12 h for 10–14 days except VRE for 14–28 days; uncomplicated SSSI is 400 mg oral every 12 h for 10–14 days (Pfizer, 2020b). The oral bioavailability of linezolid is almost 100%, hence there is no dose adjustment when switching from the IV to PO route. Its plasma elimination half-life ranges from 3.4 to 7.4 h, and protein binding is approximately 31% (Dryden, 2011; Lexicomp, 2021). Linezolid is minimally metabolized via the cytochrome P450 enzymes, and contraindicated to take linezolid concomitantly with monoamine oxidase inhibitors (MAOIs) or within 2 weeks of the MAOI use. The most common adverse reactions reported in >5% of patients who take linezolid are diarrhea, vomiting, headache, nausea and anemia (Pfizer, 2020b). Patients taking linezolid should be monitored for myelosuppression (weekly complete blood counts), peripheral and optic neuropathy (symptoms of visual impairment) in patient on linezolid for >28 days, serotonin syndrome in those taking serotonergic agents, *Clostridioides difficile*-associated diarrhea (CDAD), and hypoglycemia in diabetic patients on insulin or antidiabetic medications (Pfizer, 2020b).

Invanz® (ertapenem)

Invanz (ertapenem) (Fig. 1) was approved by the US FDA for the treatment of moderate to severe infections due to susceptible bacteria in 2001. Currently approved indications of Invanz include complicated intra-abdominal infections (cIAI), pelvic infection, CAP, cSSSI, and complicated urinary tract infections (cUTI), as well as surgical prophylaxis to prevent infections after colorectal surgery (Merck, 2020a). Ertapenem showed bactericidal and broad-spectrum antibacterial activity against Gram-negative organisms such as ESBL- and AmpC-producing *Enterobacteriaceae*, Gram-positive and anaerobic bacteria (Keating and Perry, 2005). Ertapenem is not active against MRSA and *Enterococcus* spp., and Gram-negative *Acinetobacter* and *Pseudomonas* spp. (Livermore et al., 2003).

Ertapenem, bearing with 3-(carboxy phenyl amino carbonyl)pyrrolidinyl thio, 4 β -methyl, and 6-substituted 1R-1-hydroxyethyl motifs, belongs to the carbapenem chemical class among the broad family of β -lactam antibiotics. It inhibits bacterial cell wall synthesis by binding to PBP and subsequently leads to bacterial cell lysis (Papp-Wallace et al., 2011). Similar to other carbapenems, ertapenem is resistant to β -lactamases because of the 1R-1-hydroxyethyl substituent at the 6-position (Fig. 1); in addition, the 4 β -Me group shields the β -lactam carbonyl moiety, thereby minimizing hydrolysis catalyzed by renal dehydropeptidase (DHP)-1 (Hammond, 2004). Furthermore, ertapenem is unique with a *meta*-benzoic acid motif which improves the molecule's lipophilicity

Table 1 Key attributes, physiochemical properties and PK parameters (Lexicomp, 2021) of FDA approved antibiotics since 2000.

Antibiotic	Chemical class	MoA	MW (g/mol)	logP	HBA	HBD	PSA (Å²)	RoA and dosing frequency	T _{max} [t _{1/2}] (h)	Bioavailability (%)	Protein binding (%)	Metabolism	Excretion (%)		Developed and/or marketed by	Initial US Approval (M/D/Y)
													Urine	Feces		
Zyvox (linezolid)	Oxazolidinone	Protein synthesis inhibitor by binding to the 23S rRNA of the bacterial 50S ribosomal subunit	337.35	0.45	7	1	71	IV, PO 400, 600 mg every 12 h	1–2 h [4.9 h]	100 (PO)	31	Hepatic oxidation (minimal)	30 (P), 50 (M)	9 (M)	Pfizer Inc.	4/18/2000
Invanz (ertapenem)	Carbapenem	Penicillin-binding protein inhibitor	475.51	−0.72	10	5	182	IM, IV (pH 7.5) 1 g once-daily	2.3 h [4 h]	90 (IM)	85–95	Hydrolysis (non-CYP)	80	10	Merck	11/29/2001
Factive [DSC] (gemifloxacin)	Fluoroquinolone	DNA gyrase and topoisomerase IV inhibitor	389.38	1.77	9	3	121	PO 320 mg once-daily	0.5–2 h [7 h]	71	60–70	Hepatic (minor)	36	61	LG Chem, Ltd	4/4/2003
Cubicin (daptomycin)	Lipocyclopeptide	Membrane depolarization and subsequent inhibition of DNA, RNA, and protein synthesis	1620.67	−3.32	43	25	702	IV, Intrathecal (off label route) 4 or 6 mg/kg once-daily	[8–9 h]		90–93	Oxidation (minor)	78 (P)	5.7	Merck	9/12/2003
Ketek [DSC] (telithromycin)	Ketolide	Protein synthesis inhibitor by binding to the 23S rRNA of the bacterial 50S ribosomal subunit	812.00	5.22	15	1	172	PO 800 mg once-daily	1 h [10h]	57	60–70	Hepatic CYP3A4 (50%) Non-CYP	13 (P)	7 (P)	Sanofi-Aventis	4/1/2004
Tindamax [DSC] (tinidazole)	Nitroimidazole	DNA damage; free nitro-radical formation; nitrite release	247.27	−0.29	7	0	106	PO 2 g once-daily	1.6–2 h [13.2 h]	99	12	Hepatic oxidation and hydroxylation (CYP3A4), conjugation	20–25	12	Mission Pharmacal Company/Presutti Laboratories, Inc.	5/17/2004
Xifaxan (rifaximin)	Semisynthetic derivative of rifamycin	Protein synthesis inhibitor by binding to the β-subunit of bacterial DNA-dependent RNA polymerase	785.88	3.35	14	5	198	PO 200 or 550 mg (three/two times daily)	1 h [5.6 h]	Low	67.5	CYP3A (extensive)	0.32	96.6 (P)	Salix Pharmaceuticals	5/25/2004
Tygaril (tigecycline)	Glycylcycline	Protein synthesis inhibitor by binding to the bacterial 30S ribosomal subunit	585.65	2.68	13	8	206	IV 100, then 50 mg (every 12 h)	[27 h]		71–89	Hepatic (glucuronidation, N-acetylation, epimerization), each <10%	22 (P); 11 (M)	59 (P)	Pfizer Inc.	6/15/2005
Altabax (retapamulin)	Semisynthetic pleuromutilin	Protein synthesis inhibitor by binding to the bacterial 50S ribosomal subunit	517.76	4.29	5	1	92.1	Topical 1% ointment (twice daily)		Absorption (topical): low	94	Hepatic mono- or dioxygenation (CYP3A4)			GlaxoSmithKline	4/12/2007
Doribax [DSC] (doripenem)	Carbapenem	Penicillin-binding protein inhibitor	420.50	−3.26	10	6	196	IV (pH: 4.5–5.5) 500 mg (every 8 h)	[1 h]		8.1	Hydrolysis via non-CYP	71 (P), 15 (M)	<1	Shionogi Inc./Janssen Pharmaceuticals, Inc.	10/12/2007
Zevtera (ceftibiprole medocartil)	Cephalosporin (5th generation)	Prodrug, Penicillin-binding protein inhibitor	690.66	−0.002	19	5	310	IV 500 mg (every 8 h)	[3 h]		16	Hydrolysis (esterases) to active drug, then minimal	83 (active) <1		(prodrug) 5 (M)	
Basilea Pharmaceutica Besivance (Besifloxacin)	2008 (Not approved in US) Chlorofluoroquinolone	DNA gyrase and topoisomerase IV inhibitor	393.84	3.40	6	3	86.9	Topical ophthalmic use (0.6%, three times daily)	[7 h]						Bausch & Lomb Inc	5/28/2009
Vibativ (telavancin)	Lipoglycopeptide	Inhibition of transglycosylation and	1755.63	0.93	38	24	608		[6.6–9.6 h]		90		76	<1	Cumberland Pharmaceuticals	9/11/2009

(Continued)

Table 1 (Continued)

Antibiotic	Chemical class	MoA	MW (g/mol)	logP	HBA	HBD	PSA (Å ²)	RoA and dosing frequency	T _{max} [t _{1/2}] (h)	Bioavailability (%)	Protein binding (%)	Metabolism	Excretion (%)		Developed and/or marketed by	Initial US Approval (M/D/Y)
													Urine	Feces		
		transpeptidation and disruption of bacterial membrane integrity						IV (pH: 4–5), 10 mg/kg (once-daily)							Inc. (Formerly by Theravance Biopharma US, Inc.)	
Teflaro (ceftaroline fosamil)	Cephalosporin (5th generation) (prodrug)	Penicillin-binding protein inhibitor	684.67	–2.84	16	5	340	IV (pH: 4.8–6.5) 600 mg (every 12 h)	1 h [1.6 h; 2.66 h for multiple doses]		20	Prodrug to ceftaroline (phosphatase)	88	6	AbbVie (formerly by Allergan)	10/29/2010
Difidol (fidaxomicin)	Tiacumicin, macrolide	RNA polymerase inhibitor	1058.04	9.14	18	7	267	PO 200 mg (twice daily)		Minimal systemic absorption		Intestinal hydrolysis	<1	>92	Merck Sharp & Dohme Corp.	5/31/2011
Sirturo (bedaquiline)	Diarylquinoline	Mycobacterial ATP synthase inhibitor	555.50	7.71	4	1	45.6	PO (400 mg once-daily for two weeks, then 200 mg three times per week)	5 h [5.5 months]	Enhanced with food	>99.9	Hepatic via CYP3A4 to form -NHMe metabolite	≤0.001		Janssen Pharmaceuticals Companies of Johnson & Johnson	12/31/2012
Dalvance (dalbavancin)	Semisynthetic lipoglycopeptide	Inhibition of transpeptidation and cell wall synthesis	Mixture 1816.69	4.39	38	21	573	IV 1500 mg (a single-dose) or 1000 and 500 mg (two doses 1 week apart)	[346 h]		93	hydroxy-dalbavancin (minor)	33 (P), 12 (M)	20	AbbVie Inc. (formerly by Allergan plc)	5/23/2014
Sivextro (tedizolid phosphate)	Oxazolidinone (prodrug)	Protein synthesis inhibitor by binding to the bacterial 50S ribosomal subunit	450.32	–0.39	12	2	163	IV, PO 200 mg once-daily	IV: 1–1.5 h PO: 3 h [12 h]	91	70–90	Prodrug to tedizolid by phosphatases; phase 2 (sulfate)	<3 (P), 18 (M)	<3 (P), 82 (M)	Merck	6/20/2014
Orbactiv (oritavancin) Kimyrsa	(oritavancin)	Lipoglycopeptide	Inhibition of transglycosylation and transpeptidation and disruption of bacterial membrane integrity	1793.10	3.84	36	22	561	IV 1200 mg (a single-dose)	[245 h]		85	Not		metabolized	<5 (P)
<1 (P)	Melinta Therapeutics, Inc.	8/6/2014 3/15/2021 for Kimyrsa														
Xtoro [DSC] (finafloxacin)	Fluoroquinolone	DNA gyrase and topoisomerase IV inhibitor	398.39	1.83	8	2	106	Topical otic use (0.3%, twice daily)							MerLion Pharmaceuticals/ Alcon Laboratories, Inc.	12/17/2014
Zerbaxa (ceftolozane/tazobactam)	Cephalosporin combination	Penicillin-binding protein inhibitor/β-lactamase inhibitor	666.69/300.29	–6.17/0.60	20/9	11/1	356/131	IV 1.5–3 g (every 8 h)	[3–4 h/2–3 h]		16–21/30	Not metabolized/ hydrolysis	>95/>80 (P)		Merck & Co., Inc.	12/19/2014
Avycaz (ceftazidime/avibactam)	Cephalosporin combination	Penicillin-binding protein inhibitor/β-lactamase inhibitor	546.58/265.24	–2.84/–1.87	13/9	5/3	245/139	IV 2.5 g (every 8 h)	[3.27 h/2.22 h] (single-dose) [2.76 h/2.71 h] (multiple doses)		<10/5.7–8.2	80–90% parent drug/ not metabolized	80–90/97 (P)		AbbVie Inc. (formerly by Allergan plc)	2/25/2015
Baxdela (delafloxacin)	Fluoroquinolone	DNA gyrase and topoisomerase IV inhibitor	440.76	0.19	8	4	120	IV 300 mg, PO 450 mg (every 12 h)	1 h [IV: 3.7 h; PO: 4.2–8.5 h]	58.8 (PO)	84	Glucuronidation by UGT1A1, UGT1A3, and UGT2B15	IV: 65 (P) PO: 50 (P)	IV: 28 (P) PO: 48 (P)	Melinta Therapeutics, Inc.	6/19/2017
Vabomere (meropenem/vaborbactam)	Carbapenem combination	Penicillin-binding protein inhibitor/β-lactamase inhibitor	383.46/297.14	–1.23/1.02	8/6	3/3	135/124	IV 4 g (every 8 h)	[1.22 h/1.68 h]		2/33	Hydrolysis (minor)/not metabolized	40–60/75–95 (P), 22 (M)	2	Melinta Therapeutics, Inc.	8/29/2017

Solosec (secnidazole)	Nitroimidazole	Interfere with bacterial DNA synthesis	185.18	0.22	6	1	83.9	PO 2 g (a single-dose)	3–4 h [17 h]	<5	Hepatic via CYP450; oxidation (≤1%)	15 (P)		Symbiomix Therapeutics, LLC/Lupin Pharmaceuticals, Inc.	9/15/2017	
Zemdri (plazomicin)	Aminoglycoside	Protein synthesis inhibitor by binding to the bacterial 30S ribosomal subunit	592.68	−3.83	16	14	269	IV 15 mg/kg (once-daily)	Just after infusion [3–4 h]	20		97.5 (P)	<0.2	Cipla USA Inc.	6/26/2018	
Xerava (eravacycline)	Tetracycline	Protein synthesis inhibitor by binding to the bacterial 30S ribosomal subunit	558.56	2.55	12	7	194	IV (pH: 5.5–7) 1 mg/kg (every 12 h)	[20 h]	79–90	Oxidation via CYP3A4 and FMO	20 (P), 14 (M)	17 (P), 30 (M)	Tetraphase Pharmaceuticals Inc.	8/27/2018	
Seysara (sarecycline)	Tetracycline	Protein synthesis inhibitor by binding to the bacterial 70S ribosomal subunit	487.50	−0.04	11	6	174	PO 60, 100, or 150 mg (once-daily)	1.5–2 h [21–22 h]	62.5–74.7	Minimal (<15%) in vitro	24.7 (P), 19.4 (M)	14.9 (P), 27.7 (M)	Almirall, LLC	10/2/2018	
Nuzyra (omadacycline)	Tetracycline (aminomethylcycline)	Protein synthesis inhibitor by binding to the bacterial 30S ribosomal subunit	556.65	1.85	11	7	177	IV 200 mg on day 1, then 100 mg; PO 450 or 300 mg on days 1 and 2, then 300 mg (once-daily)	IV 0.5 h [16 h]; PO 2.5 h [13.45–16.83 h]	34.5 following a single-dose 300 mg	20	Not metabolized	IV: 27 (P); PO: 14.4 (P)	PO: 77.5–84.0 (P)	Paratek Pharmaceuticals, Inc.	10/3/2018
Aemcolo (rifamycin SV)	Rifamycin	Protein synthesis inhibitor by binding to the β-subunit of bacterial DNA-dependent RNA polymerase	697.77	1.52	13	6	201	PO 388 mg (twice-daily)		<0.1	80		86	Aries Pharmaceuticals, Inc.	11/19/2018	
Recarbrio (imipenem/cilastatin/relebactam)	Carbapenem combination	Penicillin-binding protein inhibitor/renal dehydropeptidase inhibitor/β-lactamase inhibitor	299.35/358.45/348.38	−2.95/0.74/−1.45	7/7/10	4/5/3	139/155/137	IV 1.25 g (every 6 h)	Imipenem [1 h] relebactam [1.2 h]		20/40/22	Metabolized by dehydropeptidase I (kidney)/prevent imipenem metabolism/ minimally metabolized	63/77/>90 (P)		Merck & Co, Inc.	7/17/2019
Pretomanid (PA-824)	Nitroimidazooxazine	Mycolic acid biosynthesis inhibitor; a respiratory poison by releasing nitric oxide	359.26	3.11	8	0	91.3	PO 200 mg (once-daily)	4.5 h [16 h]		86.4	Reduction/oxidation; 20% (CYP3A4)	1 (P), 52 (M)	38	TB Alliance/ Mylan Specialty LP	8/14/2019
Xenleta (lefamulin)	Pleuromutilin	Protein synthesis inhibitor by binding to the 23S rRNA of the bacterial 50S ribosomal subunit	507.73	2.80	6	4	135	IV 150 mg, PO 600 mg (every 12 h)	PO 0.88–2 h [3–20 h]	25	94.8–97.1	Primarily CYP3A4	IV: 15.5 (9.6–14.1, P); PO: 5.3	IV: 77.3 (4.2–9.1, P); PO: 88.5 (7.8–24.8, P)	Nabriva Therapeutics	8/19/2019
Fetroja (cefiderocol)	Cephalosporin siderophore	Penicillin-binding protein inhibitor with enhanced cell entry via iron uptake system	752.22	−1.02	17	8	310	IV (pH 5.2–5.8) 2 g (every 8 h)	[2–3 h]		40–60	Minimal	90.6 (P), 8 (M)	2.8	Shionogi Inc.	11/14/2019
Abthrax (Raxibacumab)	mAb	binds and neutralizes free protective antigen component of <i>B. anthracis</i> toxin	146 kDa					IV (pH 6.5) 40 mg/kg (a single-dose)	[20.44 days]			Nonrenal			GlaxoSmithKline	12/14/2012
Anthim (oblitoxaximab)	mAb	binds free protective antigen component of <i>B. anthracis</i> toxin	148 kDa					IV 16 or 24 mg/kg (a single-dose)				Minimal renal elimination			Elusys Therapeutics, Inc	3/18/2016
Zinplava (bezlotoxumab)	mAb	binds and neutralizes to <i>C. difficile</i> toxin B	148.2 kDa					IV (pH 6) 10 mg/kg (a single-dose)	[19 days]		Metabolized via catabolism	Primarily through catabolism			Merck & Co., Inc.	10/21/2016

Lipinski properties were obtained from Scifinder and calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994–2021 ACD/Labs). MW refers to the molecular weight of the free acid or base drug form. HBA, hydrogen bond acceptor; HBD, hydrogen bond donor; PSA; polar surface area.

All PK parameters refer to adults unless otherwise noted. PO, by mouth or oral. IV, intravenous.

Under excretion: P refers to approximate % of total dose as parent drug and M refers to approximate % of total dose as metabolites. DSC, discontinued (brand name and/or generic product in the US).

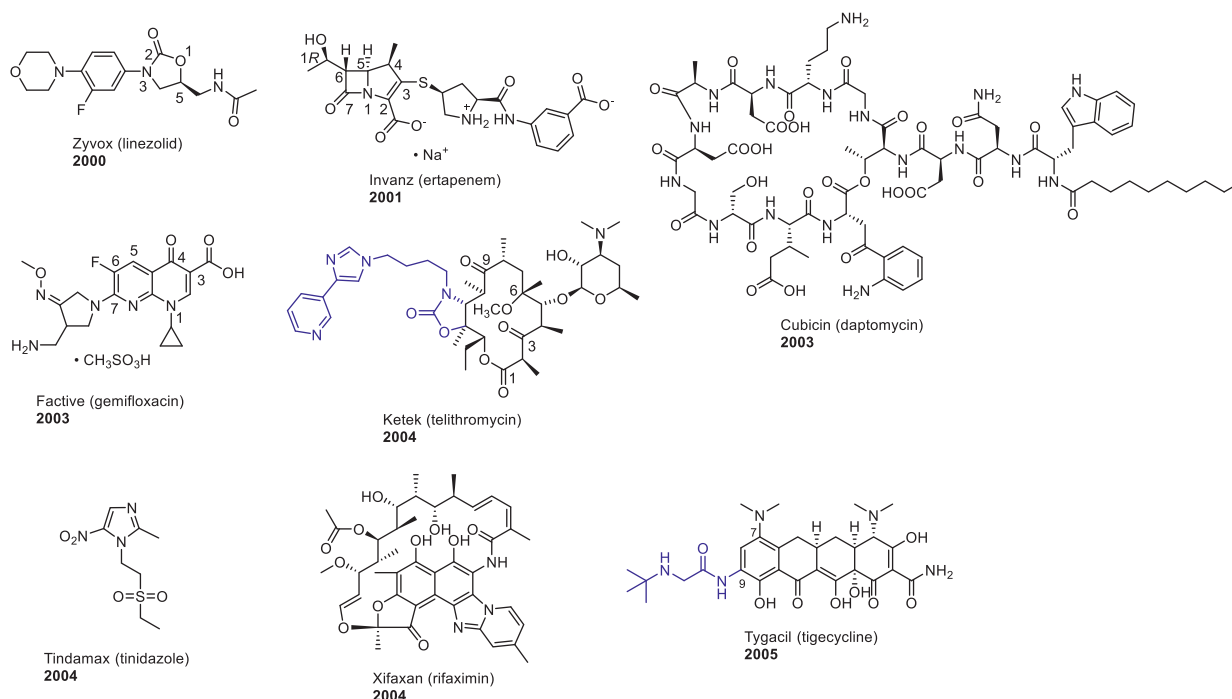


Fig. 1 Chemical structures of FDA approved antibiotics from 2000 to 2005.

profile with a net negative charge under physiological pH, allowing for once daily dosing with an extended elimination plasma half-life resulting from high protein binding 85–95% (Hammond, 2004).

Invanz (ertapenem) is available in a single-dose vial as a powder (1 g), and can be administered both IM or IV. It requires reconstitution and/or further dilution before IV infusion or IM injection (Merck, 2020a). For patients ($\text{CrCl} > 30 \text{ mL/min/1.73 m}^2$), Invanz 1 g is infused daily over 30 min or injected via IM; and the duration of treatment varies from days to weeks, depending on the type and severity of infections (Merck, 2020a). Dosage adjustment to 500 mg daily is required for patients with severe renal dysfunction ($\text{CrCl} \leq 30 \text{ mL/min/1.73 m}^2$) and end-stage renal disease ($\text{CrCl} \leq 10 \text{ mL/min/1.73 m}^2$). The most common adverse effects occurring in at least 5% of patients on Invanz include diarrhea, nausea, headache, and infused vein complication (Lexicomp, 2021). As a broad-spectrum parenteral carbapenem antibiotic, ertapenem serves as an alternative therapeutic option for the treatment of patients with complicated bacterial infections.

Factive® [DSC] (gemifloxacin)

Factive (gemifloxacin) (Fig. 1), a member of fluoroquinolone antibiotics, was approved by the US FDA for the treatment of CAP and acute bacterial exacerbation of chronic bronchitis (ABECB) in 2003 (Lexicomp, 2021). It is effective toward mild-to-moderate respiratory tract infections due to susceptible pathogens. Gemifloxacin showed potent and broad-spectrum activities against Gram-positive and Gram-negative microorganisms such as MDR *S. pneumoniae* and other bacterial strains (Ball et al., 2004).

Gemifloxacin is a fourth generation, oral synthetic fluoroquinolone derivative with 3-aminomethyl-4(Z)-methoxyimino-1-pyrrolidinyl and cyclopropyl motifs at the C7 and N1 positions, respectively. It works as a DNA gyrase and topoisomerase IV inhibitor. The enhanced activity of gemifloxacin against both DNA gyrase and topoisomerase IV, and anaerobes, is attributed to the introduction of nitrogen at the 8 position (Pham et al., 2019).

Factive used to be available as tablets containing 320 mg of gemifloxacin and is taken orally with or without food once daily for a period of treatment 5–7 days (FDA, 2019b). The dose of gemifloxacin is reduced to 160 mg daily in patients with $\text{CrCl} \leq 40 \text{ mL/min}$. The most common adverse events reported in at least 3% of patients on Factive include headache, rash, diarrhea, nausea, and increased serum alanine aminotransferase (FDA, 2019b). Factive (gemifloxacin) carries boxed warnings of serious adverse reactions and exacerbation of myasthenia gravis; and is no longer available in the US (Lexicomp, 2021). Fluoroquinolones were reported to cause some rare, but serious adverse effects including tendinopathy and tendon rupture, peripheral neuropathy, and CNS effects (Marchant, 2018).

Cubicin® (daptomycin)

Cubicin (daptomycin) (Fig. 1), the first antibiotic of the cyclic lipopeptide drug class, was approved by the US FDA in 2003. Daptomycin is indicated for use in adult and pediatric patients with cSSSIs due to Gram-positive pathogens (e.g., vancomycin-susceptible *E. faecalis*, MSSA and MRSA, *S. pyogenes*, *S. agalactiae*, and *S. dysgalactiae*). Daptomycin is also approved for treating adult patients with bacteremia, including right-sided infective endocarditis due to MSSA and MRSA (Merck, 2021a). Daptomycin showed activity against a wide-spectrum of aerobic and anaerobic Gram-positive bacteria including strains of MRSA and VRE (Vilhena and Bettencourt, 2012).

Mechanistically, daptomycin exerts its concentration-dependent bactericidal effects by disrupting the integrity of the cell membrane of susceptible Gram-positive pathogens (Taylor and Palmer, 2016). Daptomycin also possesses a notable postantibiotic effect of up to 6 h (Vilhena and Bettencourt, 2012). Daptomycin interacts with the bacterial lipid membrane through calcium-dependent insertion of the long lipid side chain (Carpenter and Chambers, 2004). Disruptions such as conformational changes of daptomycin and oligomerization, formation of pores, and lipid flip-flop cause leaks in the cell membrane, the release of intracellular ions, and cell death (Sauermann et al., 2008). Daptomycin does not work in Gram-negative bacteria as it is not able to get through the outer membrane (Carpenter and Chambers, 2004). Daptomycin can be inactivated by pulmonary surfactant and therefore should not be used in patients with pneumonia or other respiratory diseases (Silverman et al., 2005). Daptomycin, a fermentation product of *Streptomyces roseosporus*, contains 13 D- and L-amino acids in total, a macrocyclic decapeptide scaffold linked by a lactone functionality between the kynurenine residue and threonine, as well as a tripeptide side chain with a 10-carbon fatty acid tail (Penn et al., 2006). Although daptomycin has a high water solubility as it is primarily acidic and negatively charged at neutral pH, it also possesses amphipathic characteristics due to the presence of hydrophobic lipid side chain as well as hydrophilic peptide moieties (Vilhena and Bettencourt, 2012).

Daptomycin is available as a lyophilized powder that requires reconstitution before administration as an IV infusion over 30 min or as an IV push over 2 min. Vigorous shaking of the vial should be avoided after reconstitution of daptomycin to prevent foaming (Merck, 2021a). The approved dose of daptomycin to treat patients with bacteremia (including right-sided infective endocarditis) due to *S. aureus* is 6 mg/kg IV once daily for a duration of 2–6 weeks (Merck, 2021a). For patients (CrCl $\geq$ 30 mL/min) with cSSSIs, daptomycin 4 mg/kg IV should be administered once daily for a duration of 7–14 days. For right-sided endocarditis of a native valve, daptomycin 6 mg/kg IV should be administered once daily for a duration of 2–6 weeks (Merck, 2021a). When daptomycin is used in patients with renal dysfunction (CrCl $<$ 30 mL/min), the dosage must be adjusted (Carpenter and Chambers, 2004). The most common adverse effects reported in more than 6% of patients on daptomycin include chest pain, edema, infection due to Gram-negative pathogens, insomnia, and pharyngolaryngeal pain (Merck, 2021a). Daptomycin is indicated for the treatment of patients with cSSSIs, bacteremia, and right-sided infective endocarditis due to susceptible Gram-positive bacteria; it also serves as a valuable treatment option for other Gram-positive and difficult-to-treat infections (Gonzalez-Ruiz et al., 2016).

Ketek® [DSC] (telithromycin)

Ketek (telithromycin) (Fig. 1), the first ketolide antibiotic, was approved by the US FDA in 2004 for use in adult patients with mild to moderate CAP, caused by susceptible and MDR *S. pneumoniae*, *Chlamydophila pneumoniae*, *H. influenzae*, *Moraxella catarrhalis*, or *Mycoplasma pneumoniae* (Ketek, 2015). Telithromycin, a member of the macrolide-lincosamide-streptogramin B (MLS_B) resistance family, possesses activity against resistant Gram-positive and common respiratory pathogens (Ketek, 2015; Kasbekar and Acharya, 2005).

Telithromycin inhibits bacterial protein synthesis by binding to two sites, domains II and V of the 23S rRNA of the 50S ribosomal subunit (Kasbekar and Acharya, 2005). Although the structure of telithromycin is similar to that of erythromycin, the macrolide antibiotic, telithromycin has several unique structural modifications. Instead of a cladinose sugar at position 3, telithromycin displays a ketone moiety. This structural feature increases the stability of telithromycin in acidic environments, improves its activity against pathogens that are resistant to macrolide antibiotics, and also plays a role in preventing telithromycin induced resistance to MLS_B (Kasbekar and Acharya, 2005; Nguyen and Chung, 2005). Compared to a hydroxyl group, the 6-MeO functionality enhances the compound's stability in acidic environments (Nguyen and Chung, 2005). Telithromycin also possesses a cyclic carbamate ring motif fused at the C-11 and C-12 positions with an extended butyl-imidazole-pyridine side chain (Lonks and Goldmann, 2005). This structural feature of telithromycin contributes to its strong affinity for domain II of the bacterial 50S ribosomal subunit, conferring activity against MLS_B-resistant pathogens. In addition, telithromycin is able to retain activity against antibiotic-resistant pathogens due to the methylase or efflux pumps (Kasbekar and Acharya, 2005; Lonks and Goldmann, 2005).

For patients with CAP, the dose of telithromycin is 800 mg, given orally once-daily for 7–10 days (Lexicomp, 2021). Telithromycin is contraindicated in myasthenia gravis patients due to reported fatal and life-threatening respiratory failure in this patient population (Ketek, 2015). Telithromycin affects medications that are metabolized by CYP3A4 as it is both a strong CYP3A4 inhibitor and substrate (Nguyen and Chung, 2005). The CYP3A4 enzyme inhibitors (e.g., ketoconazole and itraconazole) increase both the $C_{\max}$ and AUC of telithromycin (Ketek, 2015). This antibiotic can also prolong the QTc interval and should be used with caution when administered with other agents that also prolong the QTc interval (Kasbekar and Acharya, 2005). Telithromycin should not be used with hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, such as lovastatin, atorvastatin, or

simvastatin, as administration of these medications together can increase a patient's risk of experiencing myopathy (Nguyen and Chung, 2005). The most common adverse effects in $\geq 7\%$ of patients taking telithromycin are diarrhea and nausea. Patients with hepatic dysfunction do not need a dosage adjustment, but telithromycin should be discontinued immediately when any signs of hepatitis develop in patients taking telithromycin (Ketek, 2015). Telithromycin requires a dosage adjustment (600 mg once daily) in patients with severe renal dysfunction ($\text{CrCl} < 30 \text{ mL/min}$) or on hemodialysis (Kasbekar and Acharya, 2005). The brand name product Ketek of telithromycin has been discontinued in the US by the manufacturer since March 2016 due to business reasons (Lexicomp, 2021), among increased safety concerns and warnings related to severe liver injury (Ross, 2007).

Tindamax® [DSC] (tinidazole)

Tindamax (tinidazole) (Fig. 1), the second antibiotic in the 5-nitroimidazole drug class, is an antiprotozoal and antibacterial agent that was approved by the US FDA for use in 2004 (Dickey et al., 2009; Fung and Doan, 2005). The brand name product, Tindamax, has been discontinued in the US (Lexicomp, 2021). Tinidazole possesses activity against a majority of anaerobic pathogens including *Bacteroides fragilis* as well as the microaerophilic bacteria *H. pylori* and *Campylobacter* species (Fung and Doan, 2005). Tinidazole is indicated for uses in adult patients with trichomoniasis caused by *T. vaginalis*; in patients (≥ 3 years) with giardiasis due to *Giardia duodenalis* or *G. lamblia* as well as amebiasis or amebic liver abscess due to *Entamoeba histolytica*; and in adult women to treat bacterial vaginosis (FDA, 2020).

Tinidazole is a prodrug that is able to diffuse into the cells of susceptible pathogens, where the nitro group is then reduced by ferredoxin-mediated electron transport (Fung and Doan, 2005). This results in the production of toxic radicals, leading to tinidazole's cytotoxic effects. As tinidazole is metabolized inside the cells, its concentration decreases and the gradient allows for more entry of the tinidazole prodrug (Dickey et al., 2009). Structurally like metronidazole, tinidazole has a nitroimidazole motif, however, it contains a 2-(ethylsulfonyl)ethyl side chain instead of a 2-(hydroxyl)ethyl counterpart in metronidazole.

The generic tinidazole is available as 250 and 500 mg tablets (Lexicomp, 2021). The half-life of tinidazole is 12–14 h and is longer than that of metronidazole (8 h), enabling a shorter duration of treatment with tinidazole (Johnson, 2009). In adult patients with trichomoniasis or giardiasis, tinidazole 2 g as a single-dose treatment should be taken orally (Fung and Doan, 2005). Tinidazole 2 g once daily should be taken orally by adult patients for a duration of 3 days or 3–5 days in the treatment of intestinal amebiasis and amebic liver abscess, respectively. Adolescent and adult patients with bacterial vaginosis should take tinidazole 2 g orally once-daily for 2 days or tinidazole 1 g once-daily for 5 days (FDA, 2020; Johnson, 2009). For adolescent and pediatric patients older than 3 years with amebiasis, amebic liver abscess, giardiasis, or trichomoniasis, the treatment duration is the same as adult patients, but doses of tinidazole should be weight-based (50 mg/kg/day) with a maximum of 2 g daily (FDA, 2020). Tinidazole most commonly causes gastrointestinal (GI) adverse effects such as anorexia, dyspepsia, epigastric discomfort, nausea, metallic taste, as well as vomiting (Dickey et al., 2009). Tinidazole should be taken with food to decrease the occurrence of these adverse GI reactions (Johnson, 2009). Patients taking tinidazole should also avoid consuming alcohol for up to 3 days as a disulfiram-like reaction can occur. The label of tinidazole has a warning for neurologic adverse effects as convulsive seizure and paresthesia have been reported during treatment (FDA, 2020). Tinidazole is contraindicated in pregnant women and nursing mothers, breast-feeding should be stopped during tinidazole therapy and for 3 days after completing the therapy (FDA, 2020).

Xifaxan® (rifaximin)

Xifaxan (rifaximin) (Fig. 1), a member of the rifamycin drug class, was first approved by the US FDA in 2004 for use in patients older than 12 years of age to manage travelers' diarrhea due to noninvasive *E. coli* (Lexicomp, 2021). In 2010, it was approved by the US FDA to reduce overt hepatic encephalopathy recurrence in adult patients with advanced liver disease (Rivkin and Gim, 2011). Rifaximin was also approved by the US FDA in 2015 to treat adult patients with moderate to severe irritable bowel syndrome with diarrhea (IBS-D) (Lexicomp, 2021; Gupta et al., 2017). Rifaximin possesses a broad-spectrum activity against Gram-positive, Gram-negative, aerobic, and anaerobic bacteria, as well as protozoan pathogens (Koo and DuPont, 2010).

Rifaximin exerts its bactericidal effects by disrupting bacterial RNA synthesis via binding to the β subunit of DNA-dependent RNA polymerase (DdRp) (Koo and DuPont, 2010). Rifaximin also disrupts bacterial adhesion, internalization, and translocation, preventing pathogenic virulence. It also reduces the patient's inflammatory immune response and causes positive changes to the microbiota of the patient's GI system (Ponziani et al., 2017). Rifaximin is nonabsorbed when taken orally and thus has limited systemic side effects as its action is confined to the GI tract. Bacteria are able to develop rifaximin resistance due to the alteration of DdRp chromosomes (Rivkin and Gim, 2011).

Rifaximin is available as tablets in strengths of 200 or 550 mg and can be taken with or without food (Salix, 2020). When used to reduce overt hepatic encephalopathy recurrence, rifaximin 550 mg should be given twice daily (Rivkin and Gim, 2011). For travelers' diarrhea, rifaximin 200 mg should be given three times daily for 3 days (Koo and DuPont, 2010). Patients with IBS-D should be given rifaximin 550 mg three times daily for 14 days. If IBS-D symptoms occur again, the patient can be treated with the same dosing regimen up to two times (Salix, 2020). The dosage adjustment of rifaximin in patients with renal or hepatic impairment has not been studied or is currently no recommendations, partly due to its nonsystemic effect. However, rifaximin should be used with caution in patients with severe hepatic dysfunction (Salix, 2020). Adverse effects occur more commonly in

patients receiving rifaximin treatment to reduce overt hepatic encephalopathy recurrence. The most common adverse effects reported in at least 10% of patients on rifaximin for this indication are ascites, dizziness, fatigue, nausea, and peripheral edema (Salix, 2020). Rifaximin is a unique nonsystemic antibiotic derived from rifamycin, with a range of indications because of its wide antibacterial activity (Rivkin and Gim, 2011).

Tygacil® (tigecycline)

Tygacil (tigecycline) (Fig. 1), the first drug in the glycylcycline class with an expanded spectrum of antibacterial activity, was approved by the US FDA for use in 2005 (Pfizer, 2020a). This semisynthetic antibiotic can be used to treat patients with a wide range of infections as it possesses activity against aerobic Gram-positive and Gram-negative, as well as anaerobic pathogens (Noskin, 2005). Tigecycline is indicated to treat adult patients with CABP, cIAIs, and cSSSIs, caused by susceptible pathogens such as penicillin-susceptible *S. pneumoniae*, *E. coli*, *K. pneumoniae*, vancomycin-susceptible *E. faecalis*, MSSA/MRSA, *S. anginosus* group, and *S. pyogenes* etc. (Pfizer, 2020a).

Tigecycline exerts bacteriostatic effects and works by inhibiting bacterial protein synthesis via binding to the 30S ribosomal subunit (Noskin, 2005). This novel antibiotic overcomes some main tetracycline as well as other common antibiotic resistance mechanisms including ribosomal protection, target site modifications, and efflux pumps (Peterson, 2008). Tigecycline, a derivative of minocycline, possesses a linear four-ring carbocyclic skeleton, along with an additional 9-substituted *t*-butyl-glycylamido side chain on the D-ring. This addition causes steric hindrance, allowing tigecycline for evading common bacterial resistance mechanisms (Pankey, 2005).

Tigecycline is only available for parenteral use due to its limited oral bioavailability, and dosed twice daily as an IV infusion over 30–60 min (Pankey, 2005). For all of its indications, tigecycline 100 mg should be given once initially, followed by 50 mg IV administered every 12 h. For CABP, the treatment duration is 7–14 days. For cIAIs and cSSSIs, tigecycline should be given for 5–14 days (Lexicomp, 2021). For patients with renal dysfunction, there are no dosage adjustments required. However, patients with severe hepatic dysfunction (Child Pugh C) require dosage adjustments (tigecycline 100 mg initially, followed by 25 mg every 12 h) (Pfizer, 2020a). The most common adverse effects of tigecycline reported in over 10% of patients include diarrhea, nausea, and vomiting. Tigecycline has an FDA boxed warning (since 2013) for increased all-cause mortality (risk difference of 0.6%) (Pfizer, 2020a). Tigecycline is an expanded spectrum antibiotic that works against bacteria with resistance mechanisms to other classes of antibiotics, and is reserved as an alternative antibiotic for treating cSSSI, cIAI or CABP, caused by susceptible organisms (Pankey, 2005).

Altabax® (retapamulin)

Altabax (retapamulin) (Fig. 2) is a novel semisynthetic antibacterial agent from naturally occurring pleuromutilin antibiotic class, isolated from the edible mushroom, *Clitophilus scyphoides* (Williamson et al., 2017; Paukner and Riedl, 2017). The Canadian brand name Altargo 1% product for retapamulin has been discontinued, and this topical antibiotic Altabax (retapamulin) was approved by the US FDA for use in 2007 (Lexicomp, 2021). In the US, retapamulin is indicated for topical use in patients who are at least 9 months old with impetigo, caused by MSSA or *S. pyogenes* (Hartman-Adams et al., 2014; Williamson et al., 2017). Impetigo is a bacterial skin infection that can affect people of any age, but most commonly occurs in children who are 2 to 5 years old. Nonbullous impetigo, distinguished by honey-colored crusts appearing on the patient's face as well as extremities, is more common. Bullous impetigo, distinguished by the appearance of yellow fluid-containing flaccid bullae, occurs less often (Hartman-Adams et al., 2014). The FDA has not approved this topical antibiotic for use in nares for staphylococcal carrier treatment or for treating patients with MRSA-related skin infections (Hartman-Adams et al., 2014; Septimus and Schweizer, 2016).

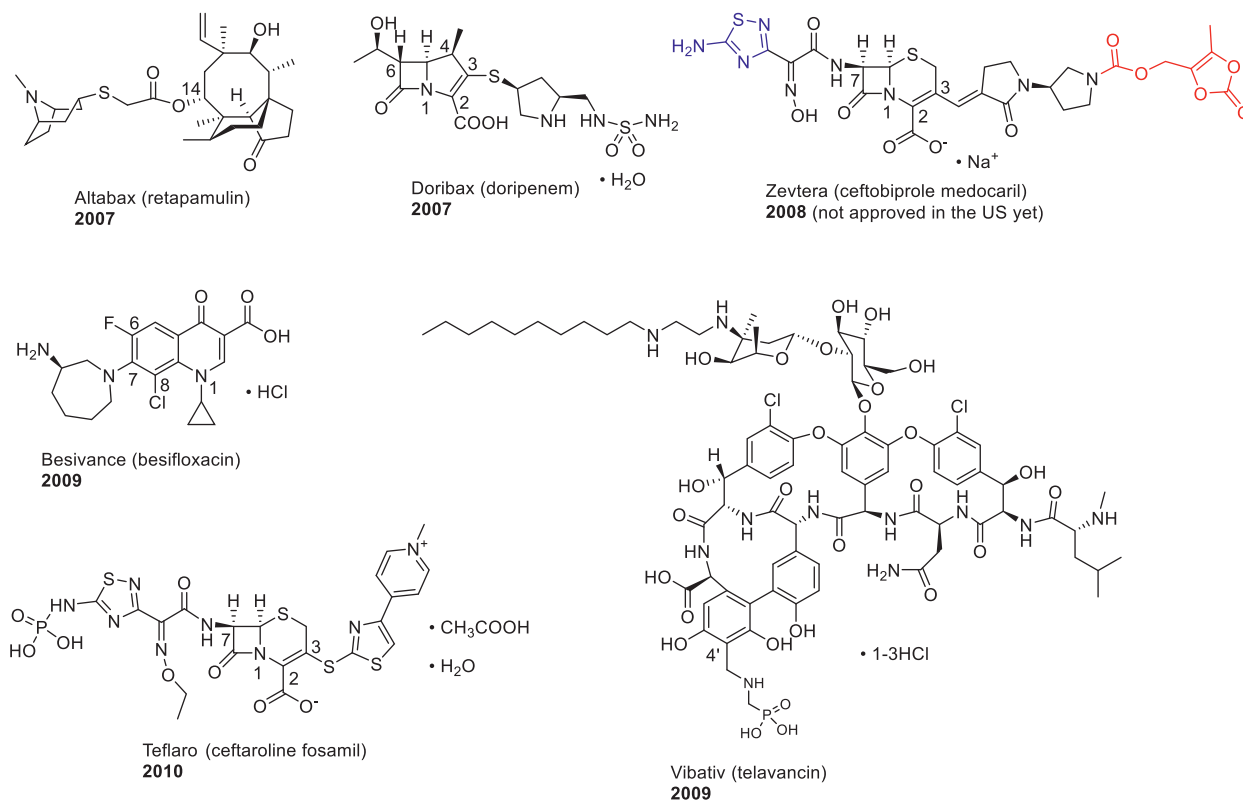


Fig. 2 Chemical structures of FDA approved antibiotics from 2006 to 2010.

Retapamulin inhibits bacterial protein synthesis and exerts primarily bacteriostatic effects by binding to the 50S ribosomal subunit (Lexicomp, 2021). Retapamulin also works by inhibiting peptidyl transfer as well as P-site interactions to block the formation of active 50S ribosomal subunits (FDA, 2019a). In *S. aureus*, retapamulin resistances can occur due to mutations in the ribosomal protein L3 and the 23S rRNA gene, overexpression of efflux pumps or the chloramphenicol-florfenicol resistance (*cfr*) methyltransferase which disrupts bacterial interaction with retapamulin by methylating the 23S rRNA subunit (Williamson et al., 2017).

Retapamulin is available as a 1% (10 mg/1 g) ointment in 15 and 30 g tubes (FDA, 2019a). The ointment should be applied to the affected area two times a day for 5 days. In adults, the total applied area should not be greater than 100 cm², and in children, the total applied area should not be more than 2% of the patient's total body surface area (Hartman-Adams et al., 2014). Since the systemic absorption of retapamulin is low, adjustments based on renal or hepatic impairment are unlikely to be needed. The most common adverse effect reported in at least 2% of patients on retapamulin is application site reactions including irritation in adults as well as pruritus in infants, children, and adolescents (Lexicomp, 2021). At the time of approval in 2007, retapamulin represented the first new topical antibiotic approved for use in almost 20 years, and it is used topically for patients with impetigo (Hartman-Adams et al., 2014).

Doribax® [DSC] (doripenem)

Doribax (doripenem) (Fig. 2), a β -lactam parenteral antibiotic belonging to the carbapenem drug class, was approved by the US FDA in 2007; and is currently discontinued in the US (Lexicomp, 2021). Doripenem was active against Gram-positive, anaerobic, as well as enteric and nonenteric Gram-negative pathogens, including ESBL-producing strains (Chahine et al., 2010). In 2014, the US FDA approved changes in the doripenem label to include a warning for patients with VABP because of increased risk of death and lower clinical outcome (FDA, 2014a). Doripenem is indicated for use in adult patients with cAIs and cUTIs including pyelonephritis (Mathews and Lancaster, 2009). It should be used in cAIs due to pathogens such as *Bacteroides caccae*, *B. fragilis*, *B. thetaiotaomicron*, *B. uniformis*, *B. vulgatus*, *E. coli*, *K. pneumoniae*, *Peptostreptococcus micros*, *P. aeruginosa*, *S. intermedius*, and *S. constellatus*. Doripenem should be used in cUTIs as well as pyelonephritis, due to pathogens such as *A. baumannii*, *K. pneumoniae*, *E. coli*, *P. mirabilis*, and *P. aeruginosa* (FDA, 2015; Lexicomp, 2021).

Doripenem shows concentration-independent bactericidal activity and exerts its effects by disrupting the synthesis of bacterial cell walls (Chahine et al., 2010). Specifically, it inhibits the last transpeptidation step in the peptidoglycan synthesis by binding to PBPs such as PBP2-4, ultimately resulting in bacterial lysis (Lexicomp, 2021). Doripenem contains a 4 β -methyl group and an unsaturated bond C2=C3 at positions 2 and 3. These structural features differentiate doripenem from other penicillin antibiotics. At position 6, doripenem has a 1R-1-hydroxyethyl group, a structural motif commonly found in the carbapenem drug class and conferring β -lactamase resistance (Matthews and Lancaster, 2009). Moreover, doripenem is resistant to the enzyme hydrolysis in ESBL and AMPC-producing *Enterobacteriaceae*, in addition to most β -lactamases. Unlike imipenem, doripenem does not need to be given with the dehydropeptidase-1 inhibitor, cilastatin, because of the 4 β -methyl functionality and improved resistance to renal dehydropeptidase-1 (Matthews and Lancaster, 2009; Chahine et al., 2010). Doripenem also contains a bulky sulfamoylaminoethylpyrrolidinylthio side chain at the 3 position, which differentiates it from meropenem and provides the activity against Gram-negative, non-fermenting bacilli (Chahine et al., 2010).

Doripenem 500 mg, available in single-use 250 and 500 mg vials, should be given every 8 h as an IV infusion over 1 h to patients with cIAI and cUTI (CrCl of >50 mL/min) (Matthews and Lancaster, 2009). The dosage must be adjusted for patients with renal impairment (CrCl $\leq$ 50 mL/min) (Chahine et al., 2010). Patients should be given parenteral therapy for at least 3 days and exhibit signs of clinical improvement before switching to an oral antimicrobial therapy can be considered (FDA, 2015). The duration of treatment for patients with cIAI and cUTI is 10 days, and 5–14 days, respectively (Matthews and Lancaster, 2009). The most common adverse reactions reported in more than 10% of patients on doripenem include diarrhea, headache, and nausea (FDA, 2015). In 2013, the US FDA updated the doripenem label to include the risk of seizures as a warning, due to observed cases during treatment with doripenem (FDA, 2015), though the likelihood of doripenem-inducing seizures is relatively low (Chahine et al., 2010). The coadministration of doripenem and valproic acid may result in losing a patient's seizure control, due to reduced serum concentrations of valproic acid (Matthews and Lancaster, 2009).

Zeftera® (ceftobiprole medocaryl)

Zeftera (ceftobiprole medocaryl) (Fig. 2), a fifth-generation cephalosporin prodrug, was approved for use in Canada and many European countries for adult patients with CAP or HAP (David et al., 2017; Avirpharma, 2019), however both ceftobiprole and ceftobiprole medocaryl are not currently approved for use in the US or for ventilator-acquired pneumonia (VAP) (Lexicomp, 2021). Ceftobiprole belongs to the cephalosporin drug class and possesses a wide spectrum of antibacterial activity against Gram-positive and Gram-negative pathogens including MRSA and penicillin-resistant *S. pneumoniae* (Barbour et al., 2009). Ceftobiprole should be used to treat CAP and HAP due to pathogens such as *E. coli*, *H. influenzae*, *K. pneumoniae*, *S. aureus* including MRSA, as well as *S. pneumoniae*.

Ceftobiprole is a bactericidal antibiotic and works by disrupting the process of cell wall synthesis via binding to bacterial PBPs (David et al., 2017). It has strong affinity for PBP2a in MRSA, PBP2x in penicillin-resistant *S. pneumoniae*, and PBP2b in penicillin-intermediate *S. pneumoniae* and is active against these resistant pathogens; it is also able to bind to PBP1a and PBP2-4 in *E. coli* in addition to PBP1a-b, PBP3-4 in *P. aeruginosa* (Davies et al., 2007). Ceftobiprole also binds to PBP5 in *E. faecalis* (Dauner et al., 2010; Lexicomp, 2021). Ceftobiprole medocaryl, the water-soluble prodrug given via IV, is rapidly hydrolyzed in vivo to the active form, ceftobiprole, after administration (Barbour et al., 2009). Ceftobiprole, like other cephalosporin antibiotics, contains a characteristic cephem nucleus (a 4-membered β -lactam ring fused with a 6-membered, sulfur-containing, dihydrothiazine heterocycle), along with a pyrrolidinone-3-ylidene-methyl side chain at the 3 position (Dauner et al., 2010).

Ceftobiprole 500 mg should be administered as an IV infusion over 2 h every 8 h (Avirpharma, 2019). Since ceftobiprole is primarily excreted via the renal system, patients with renal impairment require a dose adjustment (CrCl <50 mL/min, 500 mg administered as an IV infusion over 2 h every 12 h; CrCl <30 mL/min, 250 mg administered as an IV infusion over 2 h every 12 h) (Torres et al., 2016; Avirpharma, 2019). The most common adverse effects reported in more than 2% of patients on ceftobiprole include infusion site reactions, hypersensitivity, nausea, diarrhea, vomiting, phlebitis and hyponatremia (Avirpharma, 2019). Dysgeusia or taste disturbance has been reported due to the diacetyl product formed upon conversion of ceftobiprole medocaryl to its active form ceftobiprole (Barbour et al., 2009; Schmitt-Hoffmann et al., 2004). The ceftobiprole label has a boxed warning for serious acute hypersensitivity including anaphylaxis, which has been observed in patients receiving this antibiotic (Avirpharma, 2019). Ceftobiprole is a novel cephalosporin antibiotic with a wide range of antibacterial activity and can be used to treat adult patients with CAP or HAP, including those caused by MRSA (Torres et al., 2016).

Besivance® (besifloxacin ophthalmic suspension)

Besivance (besifloxacin) (Fig. 2), a topical fluoroquinolone antibiotic, was approved by the US FDA for use in 2009. It is available as eye drops and indicated for the treatment of bacterial conjunctivitis due to common and resistant ocular bacteria. Other fluoroquinolone antibiotics for ophthalmic use include ciprofloxacin, gatifloxacin, levofloxacin, moxifloxacin, and ofloxacin (Lexicomp, 2021).

Besifloxacin is a fourth-generation and novel 7-aminoazepanyl-8-chloro-fluoroquinolone derivative, which works as a DNA gyrase and topoisomerase IV inhibitor. Besivance is available as 0.6% ophthalmic suspension (5 mL) and administered 1 drop into affected eyes three times daily (4–12 h apart) for 7 days (Bausch, 2020). The most common adverse events reported on Besivance include headache, conjunctival erythema, blurred vision, eye irritation, eye pain, and eye pruritus (Mahvan et al., 2014). Like other fluoroquinolone antibiotics, besifloxacin exhibited potent and broad-spectrum antibacterial activity with bactericidal effect (Haas et al., 2009). Current clinical and preclinical data support that besifloxacin is an attractive and alternative therapeutic option for the treatment of ocular infections (O'Brien, 2012).

Vibativ® (telavancin)

Vibativ (telavancin) (Fig. 2), a semisynthetic derivative of vancomycin in the lipoglycopeptide antibiotic class, was approved by the US FDA for use in 2009 (Lexicomp, 2021). It is indicated for adult patients with cSSSIs due to Gram-positive pathogens such as vancomycin-susceptible *E. faecalis*, MSSA and MRSA, *S. agalactiae*, *S. anginosus* group, and *S. pyogenes*. In 2013, telavancin was FDA approved for use in adult patients with HABP/VABP due to MSSA and MRSA (Karlowsky et al., 2015; Vibativ, 2020).

Telavancin exerts its concentration-dependent bactericidal effects via multifunctional mechanisms of action (Higgins et al., 2005). The glycopeptide core binds with a high affinity to the terminal acyl-D-Ala-D-Ala chains, which prevents polymerization, cross-linking and inhibits the bacterial cell wall synthesis. Telavancin also disrupts the function of the bacterial cell membrane as it interferes with peptidoglycan synthesis via binding to lipid II in the cell membrane (Damodaran and Madhan, 2011). This membrane-targeting mechanism of action differentiates telavancin from vancomycin, which possesses a single mechanism of action (Attwood and LaPlante, 2007; Karlowsky et al., 2015). Telavancin has a hydrophobic group attached to the amine functionality in the disaccharide moiety, which is characteristic of the lipoglycopeptide class. As such, telavancin has an improved binding affinity to the D-Ala-D-Ala chains in the peptidoglycan intermediates, due to the presence of the lipophilic decylaminoethyl side chain attached to the vancosamine sugar (Saravolatz et al., 2009). In addition, this side chain can increase the compound's activity against MRSA and VRE (Hill et al., 2010). Another notable feature of telavancin is the hydrophilic phosphonomethylaminomethyl group at the 4' position, in which phosphonic acid is negatively charged and facilitates its urinary excretion and decreases its distribution in the liver and kidney (Leadbetter et al., 2004). Both the hydrophilic moiety and the long lipophilic side chain contribute to the prolonged half-life of telavancin, allowing for once-daily dosing.

Telavancin is available as 750 mg powder in single-use vials. Telavancin 10 mg/kg should be administered as an IV infusion over 60 min every 24 h for 1–2 weeks (cSSSIs) and for 1–3 weeks (HABP/VABP) (Lexicomp, 2021). For patients with renal dysfunction, the dosage of telavancin should be adjusted (CrCl 30–50 mL/min, 7.5 mg/kg every 24 h; CrCl 10–30 mL/min, 10 mg/kg every 48 h). For patients on IV unfractionated heparin sodium, telavancin is contraindicated because it falsely elevates activated partial thromboplastin time (aPTT) for up to 18 h after telavancin administration (Vibativ, 2020). The most common adverse reactions reported in >10% of patients on telavancin are metallic or soapy taste, nausea, vomiting, and foamy urine (Vibativ, 2020). Telavancin has an FDA boxed warning for increased mortality in patients with HABP/VABP (CrCl ≤50 mL/min) in comparison to vancomycin, as well as for nephrotoxicity and embryo-fetal toxicity (Vibativ, 2020). This potent lipoglycopeptide antibiotic remains an important treatment option for patients with HABP/VABP and cSSSIs as it is effective against Gram-positive pathogens, including MRSA (Chang et al., 2010).

Teflaro® (ceftaroline fosamil)

Teflaro (ceftaroline fosamil) (Fig. 2), a fifth-generation cephalosporin prodrug, was approved by the US FDA for use in 2010 (Lexicomp, 2021). It is a broad-spectrum antibiotic with activities against aerobic and anaerobic Gram-positive and Gram-negative pathogens, as well as drug-resistant pathogens (Kaushik et al., 2011). Cefaroline fosamil is indicated for use in adult and pediatric patients with ABSSSIs or CABP (Laudano, 2011), caused by MSSA and MRSA, *S. agalactiae*, *S. pyogenes*, *E. coli*, *K. oxytoca*, *K. pneumoniae*, *H. influenzae*, or *S. pneumonia* (Media-Allergan, 2020).

The zwitterionic structure of the active form ceftaroline results in poor water solubility, thus preventing its administration via the parenteral route. The prodrug, ceftaroline fosamil, contains a solubilizing phosphonoamino group on the 1,2,4-thiadiazole ring and this structural addition increases the aqueous solubility (Kaushik et al., 2011). This phosphonate group is rapidly hydrolyzed in vivo to produce the active compound, ceftaroline (Duplessis and Crum-Cianflone, 2011). Cefaroline inhibits PBP's and exerts its bactericidal effects by disrupting bacterial cell wall synthesis (Stein and Johnson, 2011). Cefaroline inhibits PBP1-4 and has high affinities for PBP2x in *S. pneumonia*, as well as PBP2a in MRSA, which differentiates it from other cephalosporin antibiotics (Stein and Johnson, 2011; Saravolatz et al., 2011). Cefaroline contains a characteristic bicyclic cephem ring system with a 3-substituted methylpyridinium-thiazolylsulfanyl motif, as well as a 7-acylated side chain containing the Z-ethoxyimino moiety. These structural features contribute to the compound's activity against MRSA, with the oxime group improving β -lactamase resistance. The 1,2,4-thiadiazole ring at position 7 aids in increasing the affinity for transpeptidases and disrupting cell wall synthesis in Gram-negative pathogens (Kaushik et al., 2011).

Cefaroline fosamil is available as 400 and 600 mg powder in single-use vials, which requires reconstitution and dilution before the IV use (Media-Allergan, 2020). In adult patients (CrCl >50 mL/min), ceftaroline fosamil 600 mg should be given every 12 h via

IV over 1 h. For those with impaired renal function ($\text{CrCl} \leq 50 \text{ mL/min}$), a dosage adjustment is required (Laudano, 2011; Media-Allergan, 2020). The dosing of pediatric patients with ABSSSI or CABP is based on ranges of weight and age. The adverse effects occurring in more than 2% of patients on ceftaroline fosamil are rash, diarrhea, and nausea (Media-Allergan, 2020). Ceftaroline fosamil has a broader antibacterial spectrum than older cephalosporin agents and serves as a useful alternative for treating patients with ABSSSIs and CABP (Saravolatz et al., 2011).

Dificid® (fidaxomicin)

Dificid (fidaxomicin) (Fig. 3), a novel and glycosylated macrolide antibiotic, was approved by the US FDA in 2011 for the treatment of adult patients with *Clostridium difficile* infections (CDI) (Lancaster and Matthews, 2012). In 2020, the indication was expanded to include pediatric patients (≥ 6 months), with a newly approved oral suspension form. The label of fidaxomicin was also updated in 2019 to change the genus name from *Clostridium* to *Clostridioides*. *Clostridioides difficile*, an anaerobic, Gram-positive, and spore-forming organism, is responsible for causing GI infections and *C. difficile*-associated diarrhea (CDAD) (Tsutsumi et al., 2014). Fidaxomicin, also known as lipiarmycin A3, clostomicin B1, or tiacumicin B, has a narrow-spectrum activity against *C. difficile*, but does not confer activity against Gram-negative pathogens (Lancaster and Matthews, 2012; Theriault et al., 1987). The 2017 *C. difficile* guidelines published by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA) strongly recommend with a high quality of evidence that fidaxomicin or vancomycin be considered as a first-line treatment over metronidazole for an initial CDI episode; there is also a weak recommendation with a moderate quality of evidence for the use of fidaxomicin over vancomycin for the first recurrent CDI (McDonald et al., 2018).

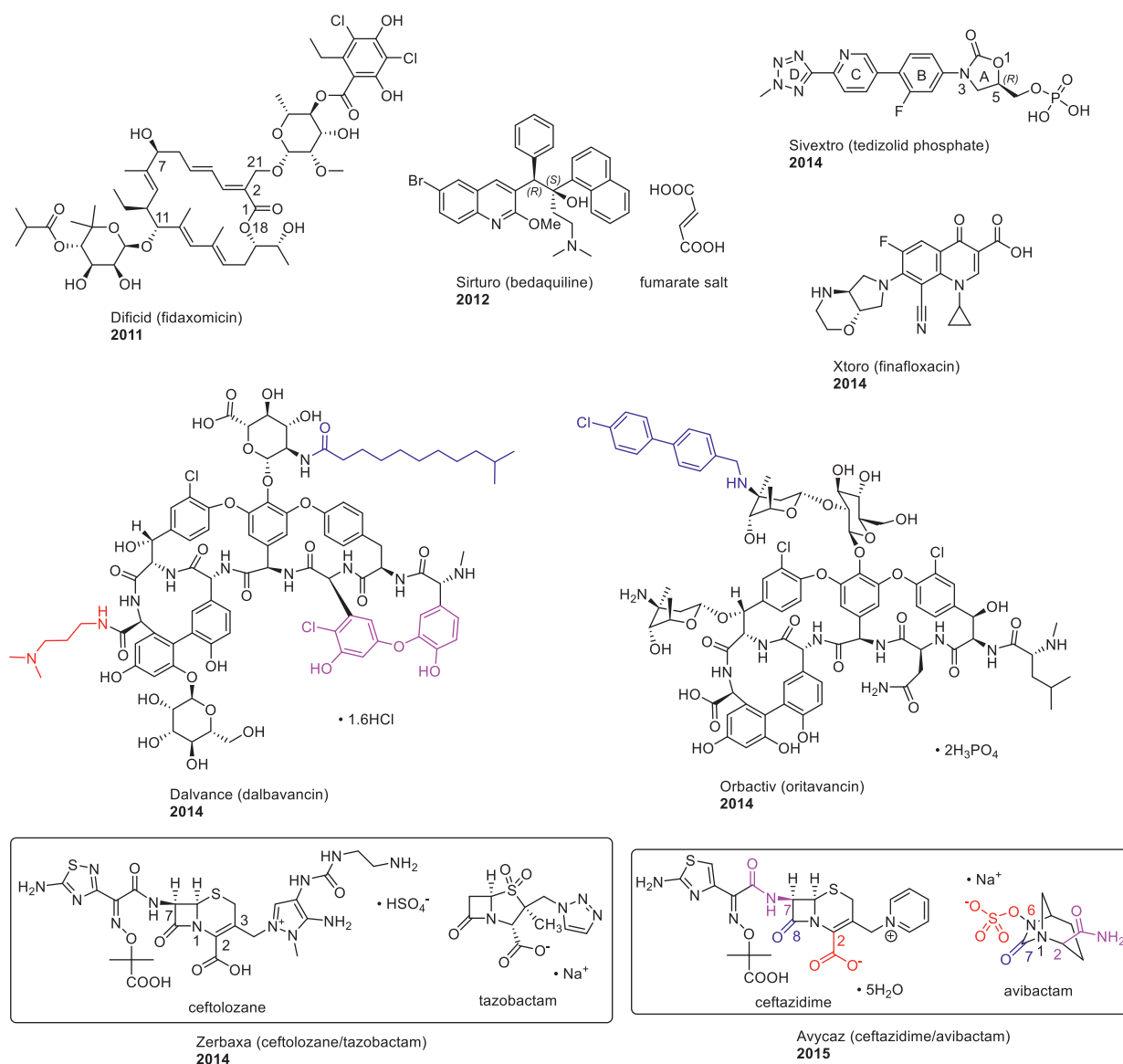


Fig. 3 Chemical structures of FDA approved antibiotics from 2011 to 2015.

Fidaxomicin exerts its bactericidal effects by inhibiting the σ subunit of bacterial RNA polymerase and subsequent RNA synthesis (Crawford et al., 2012; Yu and Sun, 2013). It also has a long postantibiotic effect in *C. difficile* of 5.5–10 h (Babakhani et al., 2011). Fidaxomicin, the active parent compound, is not absorbed systemically. Once the *O*-isobutyryl ester is hydrolyzed in vivo by gastric acid or intestinal microsomal enzymes, fidaxomicin is converted to its major active metabolite OP-1118 and the parent to metabolite ratio is 1:2 in one phase 1 study (Shue et al., 2008). Notable structural features of fidaxomicin include the highly unsaturated 18-membered macrolide core, C11 substituted 4-*O*-isobutyrate-5-methyl- β -rhamnose, and C21 substituted 2-*O*-methyl-4-*O*-homodichloro-orsellinate- β -D-rhamnose (Hochlowski et al., 1987; Serra et al., 2017).

Fidaxomicin is available as 200 mg tablets and 40 mg/mL oral suspension (Merck, 2021b). Fidaxomicin should be dosed in accordance with the IDSA's 2017 *C. difficile* guidelines which recommend that fidaxomicin 200 mg be taken by adult patients or pediatric patients >12.5 kg two times daily for a duration of 10 days (McDonald et al., 2018). For pediatric patients with weight 4 kg to less than 12.5 kg, dosing is based on weight ranges and fidaxomicin suspension (40 mg/mL) is used to deliver the calculated dose twice daily for 10 days (Merck, 2021b). No adjustments are required for patients with renal or hepatic dysfunction, pediatric patients, or other treatment groups (Lancaster and Matthews, 2012). The most common adverse reactions reported in at least 4% of patients on fidaxomicin are related to the GI disorders such as nausea, vomiting, abdominal pain, and GI hemorrhage. The fidaxomicin label has been updated to include warning of acute hypersensitivity reactions such as angioedema, dyspnea, pruritus, and rash, and it should be discontinued if a patient develops severe reactions (Merck, 2021b). Fidaxomicin is a bactericidal macrolide antibiotic with narrow-spectrum anti-*difficile* activity, and despite its high cost might serve as a better, alternative first line agent for treating CDIs (Crawford et al., 2012; Louie et al., 2011; Johnson and Wilcox, 2012; Al Momani et al., 2018).

Sirturo[®] (bedaquiline)

Bedaquiline (Fig. 3), the first drug in a new diarylquinoline chemical class with narrow-spectrum antimycobacterial activity, was approved by the US FDA in 2012 under the FDA's accelerated-approval regulations (Lexicomp, 2021; Janssenlabels, 2020). Bedaquiline is currently indicated as part of combination therapy in adult, adolescent, and pediatric (≥ 5 years and weighing ≥ 15 kg) patients with pulmonary multi-drug resistant tuberculosis (MDR-TB) (Chan et al., 2013; van Heeswijk et al., 2014; Janssenlabels, 2020). At the time of approval in 2012, bedaquiline represented the first novel anti-TB treatment in over 40 years. Bedaquiline should only be used in patients who are unable to utilize regimens of four second-line drugs and pyrazinamide in accordance with World Health Organization (WHO) recommendations or if the patient has MDR-TB that is resistant to fluorquinolones (Chan et al., 2013).

Bedaquiline exerts its antimycobacterial effects by disrupting the proton transfer chain. Specifically, it inhibits mycobacterial adenosine triphosphate (ATP) synthase and subsequently depletes the energy production in *Mycobacterium tuberculosis* (Chan et al., 2013; Singh et al., 2017). In addition, bedaquiline is selective for mycobacterial ATP synthase with minimal impact on mitochondrial ATP synthase in eukaryotic cells and human ATP synthase (van Heeswijk et al., 2014). Bedaquiline is a basic and highly lipophilic anti-TB drug ($\log P = 7.71$) with a long elimination half-life of 5.5 months (Diacon et al., 2012). It is an enantiomer with two chiral centers and 1*R*, 2*S* stereochemistry, a quinoline heterocyclic motif, and side chains with tertiary alcohol and dimethylamine functionalities that are responsible for its antimycobacterial activity (Chan et al., 2013; Singh et al., 2017).

Bedaquiline, available in 20 and 100 mg tablets, is taken orally with food by directly observed therapy (DOT) (Karumbi and Garner, 2015; Janssenlabels, 2020). The recommended dosing for adult, and pediatric (body weight ≥ 30 kg), patients with MDR-TB is bedaquiline 400 mg once daily for 2 weeks, then 200 mg three times per week (minimum 48 h between doses) for 22 weeks (Janssenlabels, 2020). The recommended dosing for pediatric patients (≥ 5 years and body weight 15–30 kg) is bedaquiline 200 mg once daily for 2 weeks, then 100 mg three times per week for 22 weeks. When used in patients with mild to moderate renal or hepatic dysfunction, no dosage adjustments need to be made, however, bedaquiline should be used with caution in patients with severe renal or hepatic dysfunction (Janssenlabels, 2020). Adjustment of medication dosages as well as increased monitoring may be necessary due to the considerable risk of drug-drug interactions associated with concomitant treatments of MDR-TB and HIV (Chan et al., 2013). The most common adverse reactions reported in at least 10% of patients on bedaquiline include arthralgia, chest pain, headache, hemoptysis, and nausea (Janssenlabels, 2020). As a boxed warning, bedaquiline has also been found to prolong the QT interval, and thus ECG monitoring is recommended and it should be discontinued when QTcF interval > 500 ms, along with the increased mortality in adults (van Heeswijk et al., 2014; Janssenlabels, 2020). Given its unique and novel mechanism of action, bedaquiline provides an alternative and important treatment option for MDR-TB, when used in combination with three to four other antitubercular drugs (Chan et al., 2013; van Heeswijk et al., 2014). Notably, acquisition of cross-resistance to bedaquiline and clofazimine via the *Rv0678* mutation was previously reported (Hartkoorn et al., 2014), and recently emerged in Pakistan following the TB treatment (Chodousi et al., 2019).

Dalvance[®] (dalbavancin)

Dalvance (dalbavancin) (Fig. 3), a second generation and semisynthetic lipoglycopeptide antibiotic, was approved by the US FDA for use in 2014. Dalbavancin was synthesized from teicoplanin, a natural antibiotic fermentation product isolated from *Nonomuraea* species (Crotty et al., 2016; David et al., 2017). It is indicated for adult patients with ABSSSIs due to susceptible Gram-positive

pathogens such as vancomycin-susceptible *E. faecalis*, MSSA and MRSA, *S. agalactiae*, *S. anginosus* group, *S. dysgalactiae*, and *S. pyogenes* (Media-Allergan, 2018). Like other glycopeptide antibiotics, dalbavancin is active against Gram-positive bacteria including MRSA (Malabarba and Goldstein, 2005).

Mechanistically, dalbavancin binds to the terminal D-Ala-D-Ala motif of peptidoglycan intermediates and inhibits transpeptidation and cross-linking of peptidoglycans, and ultimately interferes with the bacterial cell wall biosynthesis (Anderson and Keating, 2008). Notable structural features include a polycyclic peptide core, an acylated tertiary dimethylamino side chain (in red, Fig. 3) with enhanced activity against staphylococci, and a long lipophilic tail (in blue, Fig. 3) to facilitate dimerization and cell membrane interaction, as well as to increase the compound's half-life 346 h (Zhanel et al., 2010; Smith et al., 2015).

Dalbavancin is administered as an IV infusion, with currently two approved regimens for a total dose of dalbavancin 1500 mg to be delivered to a patient (Lexicomp, 2021). The two-dose recommended IV regimen for adult patients includes dalbavancin 1000 mg, followed by 500 mg administered 1 week (day 8) later. The single-dose regimen is to give dalbavancin 1500 mg IV in a single infusion, which was found to be non-inferior to the two-dose regimen, and remains another option for patients (Media-Allergan, 2018; Crotty et al., 2016). All doses should be given as an IV infusion over 30 min, and both regimens offer convenience due to the prolonged half-life 14.5 days of dalbavancin (David et al., 2017). The dosage of dalbavancin does not need to be adjusted in patients with hepatic dysfunction, as well as with renal dysfunction (CrCl >30 mL/min) or on regularly scheduled hemodialysis, as dalbavancin is not removed via hemodialysis (Crotty et al., 2016; David et al., 2017). However, patients with severe renal dysfunction (CrCl <30 mL/min) and not on regularly scheduled hemodialysis require a dosage decrease to 1125 mg (Marbury et al., 2009). In clinical studies, nausea, headache, and diarrhea were the most common adverse reactions reported in >4% of patients on dalbavancin (Lexicomp, 2021). As a treatment option to vancomycin, dalbavancin is a long-acting lipoglycopeptide antibiotic, given as a single-dose or two doses for treating patients with infections, caused by resistant Gram-positive pathogens. The convenience of once weekly dosing makes it suitable for outpatient administration of dalbavancin when treating ABSSSI (Boucher et al., 2014).

Sivextro® (tedizolid phosphate)

Sivextro (tedizolid phosphate) (Fig. 3), a prodrug to be metabolized in vivo to the active compound tedizolid, was approved by the US FDA in 2014. Tedizolid is the second agent in the oxazolidinone antibiotic class with an expanded antibacterial spectrum (Crotty et al., 2016). It is indicated for the adult and pediatric patients (≥ 12 years) with ABSSSI due to susceptible Gram-positive pathogens including MRSA (Lexicomp, 2021). Tedizolid is bacteriostatic and effective against certain strains of Gram-positive pathogens including enterococci, staphylococci, and streptococci that are resistant to other antibiotics such as vancomycin, daptomycin, or linezolid. Moreover, tedizolid is active against linezolid-resistant *S. aureus* strains that possess the *cfr* MDR gene mutation and corresponding methyltransferases (Crotty et al., 2016; David et al., 2017).

Mechanistically, rapid in vivo phosphate hydrolysis of tedizolid phosphate occurs to produce the active tedizolid, which inhibits bacterial protein synthesis by binding to the 23S rRNA portion of the 50S ribosomal subunit (David et al., 2017). The hydrophilic monophosphate group improves the compound's aqueous solubility, increases the bioavailability, and masks the primary C5 alcohol functionality from the MAO interaction (Chellat et al., 2016; David et al., 2017). Compared to linezolid, tedizolid possesses a C5 hydroxymethyl instead of acylaminomethyl group and also displays a novel and lipophilic C and D ring system, consisting of heterocyclic pyridine and methyltetrazole, respectively (Chellat et al., 2016).

For patients with ABSSSIs, tedizolid phosphate 200 mg should be administered once daily for 6 days, either by the oral route or as an IV infusion over 1 h. No dosing adjustments are required for patients with hepatic or renal impairment (Merck, 2020c). The most common adverse reactions reported in at least 2% of adult patients include headache, dizziness, nausea, vomiting, and diarrhea. It should be avoided in patients with neutropenia due to the lack of adequate data on the safety and efficacy of tedizolid in this population (Merck, 2020c). Tedizolid offers potential advantages over the use of linezolid with lower occurrences of myelosuppression and neuropathy, less bacterial resistance and MAO inhibition, as well as fewer serotonergic interactions (Prokocimer et al., 2013). Tedizolid can serve as another important treatment option for patients with ABSSSIs due to resistant Gram-positive organisms (David et al., 2017).

Orbactiv® (oritavancin)

Orbactiv (oritavancin) (Fig. 3), a semisynthetic lipoglycopeptide antibiotic, was approved by the US FDA in 2014. Oritavancin, derived from chloroeremomycin, showed excellent bactericidal activity against Gram-positive bacteria (Allen and Nicas, 2003). It is indicated for use in adult patients with ABSSSIs due to Gram-positive pathogens such as vancomycin-susceptible *E. faecalis*, MSSA and MRSA, *S. agalactiae*, *S. dysgalactiae*, *S. anginosus* group, and *S. pyogenes* (Messina et al., 2015; Orbactiv, 2019).

Oritavancin had concentration-dependent bactericidal effect with a prolonged plasma half-life of 245 h, and worked via three mechanisms such as inhibition of transglycosylation and transpeptidation and disruption of membrane integrity in bacteria (David et al., 2017; Messina et al., 2015; Orbactiv, 2019). Structurally, oritavancin is a vancomycin analog with increased efficacy toward Gram-positive pathogens because of the presences of an additional aminosugar moiety and a lipophilic *p*-chlorophenylbenzyl side

chain. A notable attribute of oritavancin is the formation of dimers, resulting in an increased affinity to bind to the peptidoglycan in bacterial cell walls and potentially exerts its rapid, bactericidal effect (Crotty et al., 2016; Zhanel et al., 2012).

Oritavancin is available in single-use vials as the diphosphate salt powder that requires reconstitution and dilution before IV administration. The IV single-dose of oritavancin in adult patients with ABSSSI is 1200 mg infused over 3 h (Crotty et al., 2016; Orbactiv, 2019). Oritavancin does not require dosage adjustments with regard to age, weight, and mild to moderate hepatic or renal impairment (David et al., 2017; Messina et al., 2015). However, data regarding the need for dosage adjustments in patients with severe hepatic or renal impairment is currently unavailable. Oritavancin is not eliminated via hemodialysis (Crotty et al., 2016). The most common adverse reactions occurring in at least 3% of patients on oritavancin include headache, subcutaneous/limb abscesses, nausea, vomiting, and diarrhea. Oritavancin falsely causes an increase in aPTT levels so the use of unfractionated heparin is contraindicated for 5 days after the treatment with oritavancin (Orbactiv, 2019). Oritavancin can also increase the serum concentration of warfarin which may cause an increased risk of bleeding (Orbactiv, 2019). Unlike vancomycin, oritavancin does not require therapeutic drug monitoring and prolonged utilization of IV catheters for administration. Thus, oritavancin may serve as a safe, effective, and long-acting treatment alternative that can provide some advantages over other available antibiotics to treat patients with ABSSSIs due to Gram-positive pathogens (Messina et al., 2015).

Xtoro[®] [DSC] (finafloxacin)

Xtoro (finafloxacin) (Fig. 3), a topical fluoroquinolone antibiotic, was approved by the US FDA for the treatment of acute otitis externa (also known as swimmer's ear) in 2014. It is used topically as ear drops for the treatment of swimmer's ear due to susceptible bacteria such as *P. aeruginosa* and *S. aureus* (McKeage, 2015). Notably, finafloxacin is the newest fluoroquinolone antibiotic for otic use, following ciprofloxacin and ofloxacin (Lexicomp, 2021). Xtoro (finafloxacin) has been discontinued in the US.

Finafloxacin belongs to the fluoroquinolone antibiotic class and works as a DNA gyrase and topoisomerase IV inhibitor. Xtoro is available as 0.3% otic suspension in a 5 mL bottle and administered four drops into the affected ear twice daily for 7 days (FDA, 2014b). The most common adverse events reported on Xtoro include itching of the ear (pruritis) and nausea (Lexicomp, 2021). Finafloxacin also showed broad-spectrum in vitro and in vivo activity against various bacterial pathogens, including those under acidic and infection-relevant conditions such as UTI and *H. pylori* infections (Jabbour et al., 2020; McKeage, 2015). In addition, it shows promise as an alternative prophylaxis and treatment option for inhalational infections due to *F. tularensis* or *Y. pestis* (Barnes et al., 2021).

Zerbaxa[®] (ceftolozane/tazobactam)

Zerbaxa (ceftolozane/tazobactam) (Fig. 3) contains a new and fifth generation 3-aminopyrazolium cephalosporin, ceftolozane, and a well-known BLI, tazobactam. This second generation β -lactam/BLI combination was approved by the US FDA in 2014 (Lexicomp, 2021). Zerbaxa is indicated for the adult patients with cAIs (in combination with metronidazole), cUTIs including pyelonephritis, HABP and VABP (since 2019), due to susceptible Gram-negative (e.g., *Bacteroides fragilis*, *E. cloacae*, *E. coli*, *K. oxytoca*, *K. pneumoniae*, *Proteus mirabilis*, *P. aeruginosa*, *H. influenzae*, and *Serratia marcescens*); as well as the GI tract Gram-positive (e.g., *S. anginosus*, *S. constellatus*, and *S. salivarius*) pathogens (Merck, 2020d).

Ceftolozane is a semisynthetic, bactericidal cephalosporin antibiotic with potent antipseudomonal activity, and works as an inhibitor of bacterial cell wall biosynthesis. Specifically, ceftolozane binds to PBPs, such as PBP1b and/or PBP3 in *P. aeruginosa* and *E. coli*, and stops peptidoglycan synthesis by inhibiting the last transpeptidation step (Lexicomp, 2021). Compared to other β -lactam antibiotics (e.g., ceftazidime and imipenem), ceftolozane showed higher binding affinity for PBPs (Sorbera et al., 2014). Ceftolozane contains features that are similar to other extended-spectrum cephalosporins such as ceftriaxone and cefepime. Structurally, ceftolozane resembles ceftazidime as both agents contain the same cephem nucleus and an almost identical 7-substituted side chain except for the aminothiadiazole ring in ceftolozane vs. the aminothiazole ring in ceftazidime. In addition, the Z-oriented oxyimino functionality at the 7 position improves compound stability to β -lactamase (Sucher et al., 2015). The dimethylacetic acid (also present in ceftazidime) and aminothiadiazole moieties at position 7 enhance Gram-negative activity against *P. aeruginosa* (Zhanel et al., 2014). The bulky side chain containing a pyrazolium heterocyclic ring at position 3 provides steric hindrance, thus improving resistance to β -lactamases (e.g., AmpC enzyme often produced by *P. aeruginosa*) (Zhanel et al., 2014; van Duin and Bonomo, 2016). The substituents attached to the pyrazolium ring have been modified to significantly increase antipseudomonal activity and reduce the risk of epileptogenicity (Cluck et al., 2015). Like other β -lactam antibiotics, potential drug resistance mechanisms of ceftolozane include ESBL production, PBP target alteration, efflux pump overexpression, and outer membrane porin loss (Hong et al., 2013).

The BLI tazobactam is a β -lactam sulfone derivative with decreased affinity to PBPs and thus insignificant antibacterial activity itself (Merck, 2020d). Tazobactam functions as an irreversible inhibitor by covalently binding to β -lactamases, and prevents the enzyme hydrolysis of ceftolozane, thereby enhancing its activity against resistant ESBL-producing pathogens, along with expanding coverage to include anaerobes (e.g., *Bacteroides* spp.) (Sorbera et al., 2014; Zhanel et al., 2014). Together, ceftolozane and tazobactam work in synergy to produce bactericidal effects.

The amount of ceftolozane and tazobactam in a single-dose vial of Zerbaxa is in a fixed 2:1 ratio (ceftolozane 1 g and tazobactam 0.5 g); and the powder requires reconstitution and further dilution before IV administration (Merck, 2020d). Zerbaxa 1.5 g should

be given every 8 h as an IV infusion over 1 h for the treatment of patients ($\text{CrCl} > 50 \text{ mL/min}$) with cIAI, in conjunction with metronidazole 500 mg IV every 8 h, for 4–14 days and cUTI (7 days); the dose of Zerbaxa for the treatment of HABP and VABP (8–14 days) is 3 g (ceftolozane 2 g and tazobactam 1 g) every 8 h given over 1 h (Merck, 2020d). For patients with impaired renal function ($\text{CrCl} \leq 50 \text{ mL/min}$), lower dosage regimens for Zerbaxa should be adjusted accordingly based on the patient's estimated CrCl (van Duin and Bonomo, 2016). The most common adverse reactions reported in at least 5% of patients on Zerbaxa include diarrhea, headache, nausea, and pyrexia for cIAI or cUTI; and diarrhea, renal impairment/failure, and hepatic transaminase increase for HABP/VABP (Merck, 2020d). Zerbaxa is a new cephalosporin/BLI combination antibiotic with enhanced activity against MDR Gram-negative organisms and can provide an alternative treatment for complicated infections such as cIAIs, cUTIs or HABP/VABP (Lexicomp, 2021), as well as a potentially valid option for patients with severe infections, caused by ESBL-producing *Enterobacterales* (Bassetti et al., 2020).

Avycaz® (ceftazidime/avibactam)

Avycaz (ceftazidime/avibactam) (Fig. 3) is a combination antibiotic containing the third generation cephalosporin, ceftazidime, and a new non- β -lactam BLI, avibactam. This second generation β -lactam/BLI combination was approved by the US FDA for use in 2015 (van Duin and Bonomo, 2016). It is indicated for the treatment of cIAIs (in combination with metronidazole), cUTIs including pyelonephritis, and HABP/VABP (since 2018), in adult and/or pediatric patients > 3 months old, due to Gram-negative (e.g., *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, *E. cloacae*, *K. oxytoca*, *Citrobacter freundii* complex, *P. aeruginosa*, *Serratia marcescens*, or *Haemophilus influenzae*) and the GI tract Gram-positive (e.g., *S. anginosus*, *S. constellatus*, and *S. salivarius*) pathogens (Avycaz, 2020).

Ceftazidime alone was initially approved by the US FDA in 1985 and demonstrated broad-spectrum antibacterial activity against Gram-positive cocci as well as Gram-negative bacilli, and it was the only third generation cephalosporin with activity against *P. aeruginosa* (Lagace-Wiens et al., 2014). Ceftazidime binds to PBPs and inhibits the late stage of bacterial cell wall peptidoglycan synthesis, ultimately causing bacterial cell lysis (Lexicomp, 2021). Ceftazidime displays a characteristic and bicyclic cephem scaffold (a 4-membered β -lactam fused with a 6-membered dihydrothiazine heterocycle). Similar to other third generation cephalosporins, the 2-aminothiazole ring at the seven position enhances its binding to PBP3 in Gram-negative pathogens, thereby improving resistance to β -lactamases. In addition, the unique dimethylacetic acid at the 7 position and 3-methylpyridinium motifs in ceftazidime provide its enhanced activity against *Pseudomonas* species (Lagace-Wiens et al., 2014). Ambler class C β -lactamases-, ESBLs-, and carbapenemases-producing organisms are usually resistant to ceftazidime, hence adding the need for a combination with the BLI avibactam to overcome the increasing Gram-negative organisms' resistance to cephalosporins (Lagace-Wiens et al., 2014; Zasowski et al., 2015).

The non- β -lactam BLI avibactam inhibits β -lactamases (e.g., ESBLs, AmpC expressed in *Enterobacteriaceae* and *P. aeruginosa*, and KPC and OXA-48 expressed in *K. pneumoniae*) which inactivate cephalosporins including ceftazidime (Lagace-Wiens et al., 2014; van Duin and Bonomo, 2016). Avibactam is a slowly reversible BLI and forms a covalent bond with the hydroxyl group of the serine residue in the active site, ultimately decreasing the enzyme hydrolysis inactivation of ceftazidime (Ehmann et al., 2012). Structurally, avibactam contains a rigid diazabicyclo octane scaffold, along with multiple hydrogen bonding interactions with β -lactamases (Zasowski et al., 2015). It is important to note that avibactam does not inhibit metallo- β -lactamases (Avycaz, 2020). Interestingly, avibactam has several similar structural features with ceftazidime. For example, the 2-carboxamide, 6-sulfate, 7-carbonyl groups of avibactam (Fig. 3) resembles the 7-aminoacyl, 2-carboxylate, 8-carbonyl motifs of ceftazidime, respectively (Lagace-Wiens et al., 2014).

Avycaz is available as a sterile powder in single-dose vials for injection. For the treatment of cIAI (duration 5–14 days), cUTI and HABP/VABP (duration 7–14 days) in adult patients ($\text{CrCl} > 50 \text{ mL/min}$), Avycaz 2.5 g (ceftazidime 2 g and avibactam 0.5 g) should be given by IV infusion over 2 h every 8 h (Avycaz, 2020). The dosage for pediatric patients is weight based. The dosage regimen for Avycaz should be adjusted for all adult patients ($\text{CrCl} \leq 50 \text{ mL/min}$) (van Duin and Bonomo, 2016; Avycaz, 2020). The most common adverse effects reported in at least 5% of patients on Avycaz are nausea, vomiting, and diarrhea (Avycaz, 2020). Avycaz is a combination antibiotic containing the BLI avibactam, which widens the antibacterial activity spectrum of ceftazidime, and provides a treatment option for patients with infections due to MDR Gram-negative bacteria (GNB) such as ceftazidime-resistant GNB and CRE (Zasowski et al., 2015).

Baxdela® (delafloxacin)

Baxdela (delafloxacin) (Fig. 4), a member of fluoroquinolone antibiotic class, was approved by the US FDA in 2017. Delafloxacin is indicated for the treatment of adult patients with ABSSSIs and CABP (since 2019) (Baxdela, 2021). It is a broad-spectrum, novel fluoroquinolone antibiotic with in vitro susceptibility for aerobic Gram-positive pathogens (e.g., MSSA, MRSA, *S. haemolyticus*, *S. lugdunensis*, *S. pyogenes*, *S. agalactiae*, *S. anginosus* Group, and *E. faecalis*) and Gram-negative organisms (e.g., *E. coli*, *K. pneumonia*, *E. cloacae*, *P. aeruginosa*) (Shiu et al., 2019; Markham, 2017; Baxdela, 2021). Delafloxacin was also active in vitro against anaerobes (e.g., *Clostridium perfringens* and *Bacteroides fragilis*) and atypical organisms (e.g., *Chlamydomphila pneumonia* and *Legionella pneumonia*) (Cho et al., 2018; Shiu et al., 2019).

Similar to the other fluoroquinolones, delafloxacin inhibits DNA gyrase and topoisomerase IV, which disrupt bacteria DNA topology and replication (Jorgensen et al., 2018). Delafloxacin is a bactericidal antibiotic which shows a concentration-dependent killing of Gram-positive and Gram-negative pathogens. Unlike the other fluoroquinolones, delafloxacin inhibits DNA gyrase and topoisomerase IV enzymes equally, with enhanced activity and uptake profiles because of its generally acidic nature and unionized form in acidic environment (Cho et al., 2018; Jorgensen et al., 2018; Tulkens et al., 2019). Structurally, delafloxacin contains a 4-membered hydroxyazetidine ring at the 7 position, heteroaromatic aminodifluoropyridine ring at the 1 position, and 8-chlorine atom. Delafloxacin resistances may result from target mutations in specific portions of the bacterial topoisomerase IV and DNA gyrase, porin losses and efflux pumps (Hooper and Jacoby, 2016). However, the equal potency of delafloxacin against both topoisomerase IV and DNA gyrase targets should limit its resistance development as it requires mutations in several regions of both enzymes for resistance to occur (Tulkens et al., 2019).

Baxdela is available as both an injectable lyophilized powder and oral tablet, which contains the delafloxacin meglumine salt. For adults with an estimated glomerular filtration rate (eGFR) of ≥ 30 mL/min/1.73m², the recommended dose for the treatment of ABSSSI (duration 5–14 days) and CABP (5–10 days) is delafloxacin 300 mg IV, infused over 60 min every 12 h (Baxdela, 2021). The IV therapy can be switched to oral delafloxacin 450 mg tablet every 12 h to complete the duration of treatment. When eGFR is 15–29 mL/min/1.73m², there requires a dose adjustment (200 mg every 12 h) for the IV delafloxacin. In addition, the oral tablet should be separated from metal containing agents such as antacids and multivitamins, by 2 h before or 6 h after, to avoid the

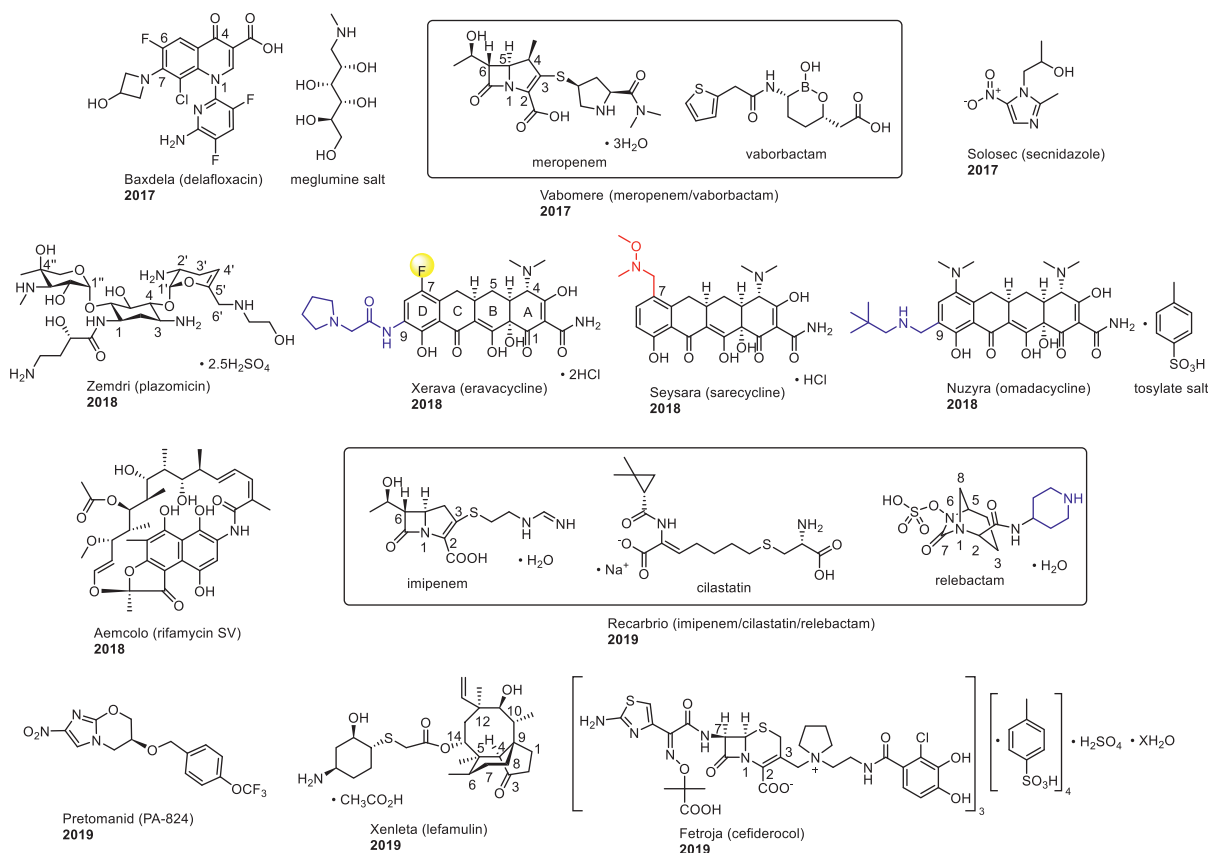


Fig. 4 Chemical structures of FDA approved antibiotics from 2016 to 2021.

formation of metal chelation complexes and maximize absorption (Baxdela, 2021). However, for patients with ESRD, delafloxacin is not recommended as the main metabolism of delafloxacin is the phase 2 glucuronidation and excreted mainly through urine (Shiu et al., 2019). The estimated mean half-life of delafloxacin after a single IV dose and multiple oral doses is 3.7 and 4.2–8.5 h, respectively (Lexicomp, 2021). The common adverse reactions reported in at least 2% of participants in clinical studies are nausea, vomiting, diarrhea, headache and elevated aminotransferases. The boxed warning of delafloxacin includes tendinitis and tendon rupture, peripheral neuropathy, CNS effects and exacerbation of myasthenia gravis (Baxdela, 2021). There is also a caution for the CDAD risk. No clinically relevant QTc prolongation was observed in a randomized delafloxacin QT study in healthy subjects (Markham, 2017; Shiu et al., 2019). Phototoxicity of delafloxacin was also not observed in clinical studies (Cho et al., 2018). Delafloxacin serves as a valuable addition to current antibiotics used for treating ABSSSI and CABP, as it has broad-spectrum antibacterial activity such as coverage for resistant organisms (e.g., MRSA, *P. aeruginosa*), and is available as IV and oral formulations for easy switch from IV to oral when needed (Tulkens et al., 2019).

Vabomere® (meropenem/vaborbactam)

Vabomere (meropenem/vaborbactam) (Fig. 4), an IV antibiotic combination of carbapenem/BLI, was approved by the US FDA in 2017 for use in adult patients with cUTIs including pyelonephritis, due to Gram-negative pathogens such as *Enterobacter cloacae* species complex, *E. coli*, and *K. pneumonia* (Vabomere, 2020). Vabomere represents the first approved combination therapy containing a carbapenem and BLI with activity against KPC β -lactamase-producing strains (Hecker et al., 2015).

Meropenem alone was initially approved by the US FDA in 1996 as a single agent therapy, and has been discontinued in the US (Lexicomp, 2021). It is a synthetic, broad-spectrum, bactericidal carbapenem antibiotic that works by binding to PBP, thereby inhibiting bacterial cell wall synthesis (Jorgensen and Rybak, 2018). Like other carbapenem antibiotics, meropenem has a characteristic carbon atom at position 4 instead of a sulfur atom as usually seen in the penicillin class, a fused bicyclic core scaffold with an unsaturated C2=C3 double bond, as well as *trans* oriented 5 α -H and 6 β -H hydrogens. The 6-substituted 1R-1-hydroxyethyl side chain provides stability against various β -lactamases. Given that meropenem is resistant to renal dehydropeptidase I (DHP-I) due to the presence of the 4-methyl group, it is not administered concurrently with a DHP-I inhibitor such as cilastatin (Jorgensen and Rybak, 2018). Meropenem also has a 3-substituted dimethylcarbamoylpyrrolidinethio side chain, which improves its activity against Gram-negative pathogens (Craig, 1997). Potential resistance mechanisms of carbapenem include β -lactamase production, PBP target alteration, efflux pump upregulation, and outer membrane porin loss (Vabomere, 2020).

The other component of Vabomere, vaborbactam, is a non- β -lactam BLI with a cyclic boronic acid motif, and serves as an inhibitor of class A serine carbapenemases with no antibacterial activity on its own (Dhillon, 2018). Specifically, vaborbactam was developed to inactivate serine β -lactamases such as KPC, with the boronate moiety forming a covalent complex with the catalytic serine side chain of β -lactamases (Hackel et al., 2018). Vabomere showed in vitro activity against β -lactamases/ESBLs-producing *Enterobacteriaceae*, however it was largely inactive against metallo- β -lactamases or carbapenemases-producing pathogens (Wenzler and Scoble, 2020; Lomovskaya et al., 2017).

Vabomere is available in single-dose vials as a dry powder (meropenem 1 g and vaborbactam 1 g), which requires reconstitution and further dilution before IV infusion. For patients with cUTI (eGFR ≥ 50 mL/min/1.73 m²), Vabomere 4 g (2 g of meropenem and 2 g of vaborbactam) is infused every 8 h over 3 h for up to 14 days (Vabomere, 2020). Renal dosage adjustments are required for patients with eGFR <50 mL/min/1.73 m². The most common adverse events reported in at least 3% of patients on Vabomere include diarrhea, headache, infusion site reactions, and phlebitis (Vabomere, 2020). Vabomere is not recommended for patients using valproate products concurrently as it can lower their serum concentrations and increase the breakthrough seizure risk. Vabomere provides an effective treatment option for patients with cUTIs caused by CRE (Dhillon, 2018).

Solosec® (secnidazole)

Solosec (secnidazole) (Fig. 4), a 5-nitroimidazole derivative, was approved by the US FDA in 2017 for the treatment of bacterial vaginosis in adult women (Lamp et al., 1999; Lexicomp, 2021). Like other nitroimidazole agents including metronidazole and tinidazole, secnidazole showed potent in vitro activity against anaerobic Gram-positive/Gram-negative organisms such as *Bacteroides* spp., *G. vaginalis*, *Prevotella* spp., *Mobiluncus* spp. and *Megasphaera*-like type I/II, as well as parasites (e.g., amoeba) and protozoa (e.g., *Trichomonas vaginalis*, *E. histolytica*) (Gillis and Wiseman, 1996; Dailymed, 2021).

Secnidazole is a prodrug and reduced in vivo by bacterial enzymes into radical anions to presumably interfere with bacterial DNA synthesis (Lexicomp, 2021). Secnidazole is a small molecule bearing a 2-hydroxypropyl side chain at the N1 position, and taken orally as a single-dose therapy with a prolonged elimination half-life 17 h (Schwebke et al., 2017). The resistance development and associated clinical impact of secnidazole in bacterial vaginosis has not been well studied (Dailymed, 2021).

Solosec is available as oral secnidazole granules which are sprinkled onto applesauce, yogurt or pudding and consumed within 30 min; the granules should not be chewed or dissolved in a liquid. The dosage for treating adult bacterial vaginosis is a single administration of secnidazole 2 g, because of its long elimination half-life (Gillis and Wiseman, 1996; Dailymed, 2021). The most common adverse effects reported in at least 2% of patients in clinical trials include nausea, vomiting, diarrhea, dysgeusia, abdominal pain, vulvovaginal candidiasis, and vulvovaginal pruritus. It is recommended to discontinue breastfeeding for 4 days

after taking secnidazole (Dailymed, 2021). Secnidazole may serve as a useful, practical single-dose adjunct or alternative to metronidazole in the treatment of bacterial vaginosis, in cases of poor patient adherence or metronidazole resistance (Ghosh et al., 2018).

Zemdri® (plazomicin)

Zemdri (plazomicin) (Fig. 4), a semisynthetic derivative of naturally occurring aminoglycoside sisomicin, belongs to the aminoglycoside antibiotic class (Armstrong and Miller, 2010). This novel, next-generation aminoglycoside antibiotic was approved by the US FDA for use in 2018 (Eljaaly et al., 2019). Plazomicin is indicated for use in adult patients with cUTIs including pyelonephritis, due to susceptible Gram-negative pathogens such as *Enterobacter cloacae*, *E. coli*, *K. pneumoniae*, and *Proteus mirabilis* (Zemdri, 2020). Compared to other aminoglycoside antibiotics, plazomicin showed excellent in vitro activity against Gram-negative *Enterobacteriaceae* (e.g., *E. coli*, *K. pneumoniae*, and *E. cloacae*) except for *Proteus mirabilis* and carbapenem-resistant isolates with 16S rRNA methylases, as well as Gram-positive cocci including MSSA and MRSA (Walkty et al., 2014; Livermore et al., 2011; Castanheira et al., 2018).

Plazomicin is a bactericidal antibiotic with concentration-dependent killing and postantibiotic effects, and works by binding to the 30S ribosomal subunit of the bacteria, leading to inhibition of protein synthesis (Shaeer et al., 2019). Notably, most aminoglycoside modifying enzymes (AMEs) such as acetyltransferases, phosphotransferases, or nucleotidyltransferases that alter the effects of other aminoglycosides (e.g., gentamicin, amikacin, or tobramycin) do not affect plazomicin (Zemdri, 2020). Structurally similar to other aminoglycosides, plazomicin also possesses chemical modifications conferring improved resistance profile against AMEs, typically produced by CRE (Thaden et al., 2017). Specifically, plazomicin lacks the 3'- and 4'-hydroxyl groups, preventing deactivation by the AMEs that inactivate amikacin, the aminoglycoside with the widest antibacterial spectrum. Other notable structural features of plazomicin include an L- γ -amino- α -hydroxybutyryl moiety conjugated with the NH₂ group at position 1 and a hydroxyethylamino group at position 6' to improve the activity against AME-producing pathogens (Shaeer et al., 2019; Abdul-Mutakabbir et al., 2019).

Zemdri is available as a sterile, clear, colorless to yellow plazomicin sulfate solution (500 mg/10 mL) that requires dilution prior to the IV infusion. For adult patients (CrCl $\geq$ 60 mL/min) with cUTI including pyelonephritis, plazomicin 15 mg/kg (dosing based on total body weight) should be infused over 30 min every 24 h for 4–7 days (Zemdri, 2020). For patients with renal impairment (CrCl < 60 mL/min), the dosage and dosing interval for plazomicin should be adjusted accordingly. Common adverse reactions reported in at least 2% of patients on plazomicin include decreased renal function, diarrhea, and hypertension (Zemdri, 2020). Plazomicin has a boxed warning for nephrotoxicity, ototoxicity, neuromuscular blockade and fetal harm, but with lower incidence than other aminoglycosides (Thaden et al., 2017; Shaeer et al., 2019; Lexicomp, 2021). Although plazomicin is active against certain MDR Gram-negative pathogens including CRE and serves as an alternative antibacterial therapy for adult patients with cUTIs, it should be only used in patients with few to no other treatment options due to limited data on its safety and efficacy (Shaeer et al., 2019).

Xerava® (eravacycline)

Xerava (eravacycline) (Fig. 4), a new and synthetic 7-fluorotetracycline, was approved by the US FDA in 2018 for use in adult patients with cAIs (Lexicomp, 2021). Eravacycline is indicated for the treatment of cAIs, caused by some anaerobic Gram-positive *Clostridium perfringens*, *E. faecalis*, *E. faecium*, *S. aureus*, and *S. anginosus* group, as well as Gram-negative *Bacteroides* species, *Citrobacter freundii*, *E. cloacae*, *E. coli*, *K. oxytoca*, *K. pneumoniae*, and *Parabacteroides distasonis* pathogens (Xerava, 2020). Eravacycline showed a broad-spectrum in vitro antibacterial activity including against MDR organisms such as CRE and CRAB, but it was inactive against *P. aeruginosa* (Zhanel et al., 2016) and not approved for the treatment of cUTIs and CRE infections because of currently limited clinical data and/or the lack of efficacy data from clinical trials (Seifert et al., 2018, 2020; Sheu et al., 2019).

Eravacycline works by binding to the 30S ribosomal subunit to inhibit bacterial protein synthesis (Zhanel et al., 2016). Structurally similar to tigecycline, eravacycline has the 7-fluorine atom and 9-pyrrolidinoacetamido motif on the D-ring, instead of the 7-dimethylamine and 9-glycylamido moieties in tigecycline, respectively (Zhanel et al., 2016). These unique substitutions in eravacycline improved its antibacterial efficacy against tetracycline resistant and MDR strains, as well as favorable resistance and PK profiles (Abdallah et al., 2015; Livermore et al., 2016).

Xerava contains eravacycline dihydrochloride salt as a lyophilized, yellow to orange powder which is administered by the IV route after reconstitution and further dilution (Xerava, 2020). For the cAI indication, eravacycline 1 mg/kg should be given over 60 min every 12 h for 4–14 days. Dosage modification is required for patients with severe hepatic impairment or on a strong CYP3A inducer medication concomitantly (Xerava, 2020). Common adverse reactions reported in at least 4% of patients on eravacycline include infusion site reactions, nausea, and vomiting. Like other tetracyclines, eravacycline may cause permanent yellow-gray-brown tooth discoloration and reversible bone growth inhibition, when used during tooth development such as the second and third trimester of pregnancy, and by children under 8 years old. Therefore, lactating women should avoid breast-feeding during the eravacycline treatment and wait until 4 days after taking the last dose (Lexicomp, 2021). Eravacycline is a novel broad-spectrum and fully synthetic tetracycline antibiotic indicated for use in patients with cAIs and may have the potential for the treatment of serious infections caused by CRE (Sheu et al., 2019).

Seysara[®] (sarecycline)

Seysara (sarecycline) (Fig. 4), a novel tetracycline antibiotic, was approved by the US FDA for use in 2018. Sarecycline is indicated for use in patients (≥ 9 years old) with inflammatory lesions of non-nodular moderate to severe acne vulgaris (Deeks, 2019). Unlike other broad-spectrum tetracyclines, sarecycline has a narrow-spectrum activity against Gram-positive anaerobic *Cutibacterium acnes* and skin organisms such as staphylococci and streptococci, but is much less active against enteric aerobic Gram-negative microorganisms and anaerobes (Zhanel et al., 2019a; Moore et al., 2019).

Sarecycline binds to the bacterial 70S ribosomal subunits, inhibits bacterial ribosome in part by directly interacting with mRNA, and subsequent blocks protein synthesis (Batool et al., 2020). Structurally, sarecycline has a unique and extended 7-methoxy(methyl)aminomethyl side chain, which plays an important role in bacterial ribosomal binding and antibiotic action, as well as overcoming common tetracycline resistance mechanisms (Zhanel et al., 2019a; Batool et al., 2020). The exact mechanism of action when used to treat acne vulgaris is currently unknown (Almirall, 2020).

Seysara, available as sarecycline 60, 100, and 150 mg tablets, is taken orally once daily with or without food; the dose for treating acne vulgaris is 60, 100, and 150 mg for the patient whose weight is 33–54, 55–84, and 85–136 kg, respectively (Almirall, 2020). Sarecycline should be avoided during pregnancy and lactation, as well as in children (< 8 years old), due to concerns of yellow-gray-brown permanent tooth discoloration and decreased bone growth. In addition, tetracyclines were reported to induce photosensitivity, so patients taking sarecycline should minimize their exposure to sunlight and tanning beds (Almirall, 2020). Also similar to other tetracyclines, antacids (i.e., those containing calcium, magnesium, aluminum), preparations containing iron, and bismuth subsalicylate can affect sarecycline's absorption, and thus should be separated and/or avoided from doses of sarecycline (Deeks, 2019). Concomitant use of sarecycline and isotretinoin should also be avoided as both agents are associated with intracranial hypertension. The most common adverse reaction reported in 3% of patients taking sarecycline is nausea (Almirall, 2020). The current use of sarecycline is limited due to the lack of efficacy and safety data beyond 12 weeks and 12 months, respectively; therefore, continued treatment for acne with sarecycline should be reconsidered if a patient does not experience improvement after 12 weeks (Almirall, 2020).

Nuzyra[®] (omadacycline)

Nuzyra (omadacycline) (Fig. 4), a novel aminomethylcycline and semisynthetic derivative of the tetracycline antibiotic class, was approved by the US FDA for use in 2018. It is indicated for the treatment of adult patients with CABP, due to MSSA, *K. pneumoniae*, *Chlamydia pneumoniae*, *S. pneumoniae*, *H. influenzae*, *H. parainfluenzae*, *Legionella pneumophila*, and *Mycoplasma pneumoniae*, as well as ABSSSI due to MSSA and MRSA, *S. lugdunensis*, *S. anginosus* group, *S. pyogenes*, *E. cloacae*, *E. faecalis*, and *K. pneumoniae* (Nuzyra, 2020). Omadacycline showed broad-spectrum in vitro antibacterial activity against Gram-positive/Gram-negative, anaerobes, as well as atypical skin and pneumonia microorganisms (Tanaka et al., 2016).

Omadacycline works by binding to the 30S ribosomal subunit to block bacterial protein synthesis (Draper et al., 2014). Structurally, omadacycline is a close analog to minocycline and tigecycline except for the only structural difference at the nine position on the D ring. Compared to previous tetracyclines, omadacycline demonstrated improved antibacterial potency and coverage. Specifically, the addition of the neopentylaminomethyl functionality to the nine position of the tetracycline scaffold led to omadacycline with activity against other tetracycline resistant strains with ribosomal protection proteins (e.g., Tet M) and active efflux pumps (e.g., Tet K and Tet L) (Tanaka et al., 2016; Honeyman et al., 2015).

Omadacycline is administered as the tosylate salt once daily by the oral or IV route, and available as 150 mg tablets of omadacycline or 100 mg of omadacycline as a lyophilized power in a single-dose vial, which requires reconstitution and dilution prior to the IV infusion (Nuzyra, 2020). Treatment of CABP or ABSSSI is initiated with loading doses (Day 1: 200 mg by IV infusion over 60 min OR 100 mg by IV infusion over 30 min twice), followed by maintenance doses (100 mg by IV infusion over 30 min once daily OR 300 mg orally once daily) for a duration of 7–14 days (Nuzyra, 2020). The treatment of ABSSSI with Nuzyra tablets only requires loading doses Days 1 and 2: 450 mg orally once daily, followed by maintenance doses 300 mg orally once daily. Omadacycline tablets should be taken with water on an empty stomach after fasting for at least 4 h, and patients should avoid eating or drinking except water for at least 2 h after taking omadacycline. Like other tetracyclines, to promote absorption, patients should not consume products containing multivalent cations (e.g., dairy products, antacids, and multivitamins) for at least 4 h after taking omadacycline; accordingly, omadacycline should not be administered via the same IV line as multivalent cation preparations (Nuzyra, 2020). Omadacycline may cause permanent tooth discoloration or reversible bone growth inhibition when used by pregnant women during the second or third trimester of pregnancy, infants, or children (≤ 8 years). Common adverse effects reported in more than 10% of patients on omadacycline include nausea and vomiting (Lexicomp, 2021). Omadacycline is a novel 9-aminomethyl tetracycline with improved tetracycline resistance mechanisms, as well as broad-spectrum activity against anaerobes and atypical pathogens. It is a well-tolerated, once daily antibiotic that can be given by IV or oral routes, and provides a valuable treatment option for patients with ABSSSI or CABP, including those caused by resistant organisms (Barber et al., 2018; Gallagher, 2019).

Aemcolo® (rifamycin SV)

Aemcolo (rifamycin SV) (Fig. 4), an oral, minimally absorbed semisynthetic antibiotic delivered to the colon, was approved by the US FDA on November 19, 2018 for the treatment of adult patients with travelers' diarrhea due to noninvasive *E. coli* (Andrei et al., 2019). Rifamycin SV represented the first antibiotic approved for this indication following rifaximin's approval in 2004.

Aemcolo was developed using an MMX technology platform and a special coating formulation rifamycin SV, allowing it to be released slowly once it reaches the infection sites (Steffen et al., 2018). It demonstrates broad-spectrum activity against Gram-positive pathogens including *C. difficile* with moderate activity against Gram-negative bacteria (Farrell et al., 2011). Similar to rifaximin, rifamycin SV binds to the β subunit of the DdRp enzyme and inhibits bacterial RNA synthesis (Floss and Yu, 2005). Chemically, rifamycin SV is a 25-membered macrocycle containing a naphthoquinonic chromophore ring system and an amide linker between an aliphatic chain and an amino group of the aromatic moiety.

Rifamycin SV is available as 194 mg delayed-release tablets, and can be taken with or without food. For adult patients with travelers' diarrhea due to noninvasive *E. coli*, rifamycin SV 388 mg should be taken orally twice-daily for 3 days (Aemcolo, 2019). The most common adverse effects reported in at least 3% of patients on rifamycin include headache and constipation (Lexicomp, 2021). The potential benefits of Aemcolo include limited side effects and/or drug-drug interactions resulting from its nonsystemic nature, as well as a targeted release profile.

Recarbrio® (imipenem/cilastatin/relebactam)

Recarbrio (imipenem/cilastatin/relebactam) (Fig. 4) was approved by the US FDA for the treatment of adults with HABP/VABP in 2019. This new carbapenem combination therapy contains a broad-coverage carbapenem imipenem, a renal dehydropeptidase inhibitor cilastatin, and a novel BLI relebactam. Currently approved indications of Recarbrio include cIAI, HABP/VABP, and cUTI, due to susceptible Gram-negative pathogens, including *A. calcoaceticus-baumannii* complex, *E. coli*, *K. aerogenes*, and *P. aeruginosa* (Merck, 2020b). Primaxin (imipenem and cilastatin) for IV injection was initially approved by the US FDA in 1985.

Mechanistically, imipenem inhibits bacterial cell wall peptidoglycan synthesis by binding to PBP and subsequently leads to bacterial cell lysis; cilastatin prevents renal metabolism of imipenem by competing with dehydropeptidase; and relebactam protects imipenem from degradation by certain serine β -lactamases and thus restores activity against imipenem-resistant bacteria (Smith et al., 2020). Structurally similar to avibactam, relebactam is a potent BLI with the diazabicyclooctane core (a bridged bicyclic urea motif), an aminoxy-sulfate moiety, and an extra piperidine ring. As a carbapenem, imipenem has a characteristic bicyclic β -lactam ring structure with a C2=C3 double bond to increase its binding activity, as well as the 3-substituted iminomethylaminoethylthio side chain. The 1R-1-hydroxyethyl side chain at the 6 position enhances the stability to β -lactamase (Smith et al., 2020). Relebactam possesses inhibitory activity against both classes A and C β -lactamases, including KPC-2 enzymes (Wong and van Duin, 2017; Lob et al., 2017). As such, adding relebactam to imipenem and cilastatin extends its bacterial spectrum to cover some imipenem-resistant *Enterobacteriaceae* and *P. aeruginosa*, but not *A. baumannii* (Mansour et al., 2021).

Recarbrio (imipenem/cilastatin/relebactam) is available in a single-dose vial as a white to light yellow sterile powder (500 mg of imipenem, 500 mg of cilastatin, and 250 mg of relebactam), and requires reconstitution and further dilution before IV infusion (Merck, 2020b). For patients ($\text{CrCl} \geq 90 \text{ mL/min}$) with HABP/VABP, cUTI, or cIAI, Recarbrio 1.25 g is infused every 6 h over 30 min for a duration of 4–14 days (Merck, 2020b). Dosage adjustments are required for patients with renal impairment ($\text{CrCl} < 90 \text{ mL/min}$). The most common adverse events reported in at least 8% of patients on Recarbrio include increased serum alanine aminotransferase and aspartate aminotransferase, diarrhea, hypokalemia, and anemia (Lexicomp, 2021). Similar to other β -lactam/BLI combination therapies, Recarbrio provides advantages and a treatment option for bacterial infections, caused by MDR Gram-negative pathogens (e.g., CRE and MDR *P. aeruginosa*) (Heo, 2021).

Pretomanid (PA-824)

Pretomanid (PA-824) (Fig. 4), an oral, narrow-spectrum, and nitroimidazole antitubercular agent, was approved by the US FDA for the treatment of adult patients with pulmonary treatment-intolerant or nonresponsive MDR-TB or extensively drug-resistant tuberculosis (XDR-TB) in 2019. Pretomanid is the generic name and given as part of an oral three-drug combination with bedaquiline and linezolid, or the BPaL (Bedaquiline/Pretomanid/Linezolid) regimen (TBAlliance, 2019).

Pretomanid is an antimycobacterial drug with the nitroimidazooxazine core. It showed potent bactericidal in vitro activity against MDR-TB, as well as in vivo efficacy in animal infection models (Stover et al., 2000; Lenaerts et al., 2005). Mechanistically, it requires bioactivation from nitro-reduction to subsequently inhibit mycolic acid and cell wall biosynthesis. Under anaerobic environments, pretomanid functions as a respiratory poison following nitric oxide release to kill nonreplicating bacteria (Lexicomp, 2021; Stover et al., 2000). Another close structural TB drug, Delamanid (delamanid, OPC-67683) with a bicyclic nitroimidazo-oxazole motif, was approved by European Medicines Agency (EMA) in 2014 as part of a combination therapy for the treatment of MDR-TB, but has not been approved in the US yet.

Pretomanid is taken with food orally 200 mg once daily, in combination with bedaquiline (400 mg orally once daily for 2 weeks, followed by 200 mg three times per week for 24 weeks) and linezolid (1200 mg orally daily) for 26 weeks (TBAlliance, 2019). The most common adverse events reported in at least 25% of patients treated with Pretomanid in combination with bedaquiline and linezolid included peripheral neuropathy, acne vulgaris, anemia, nausea, vomiting, headache, increased serum transaminases, and musculoskeletal pain (Lexicomp, 2021).

Xenleta® (lefamulin)

Xenleta (lefamulin) (Fig. 4), a member of pleuromutilin antibiotic class, was approved by the US FDA for the treatment of adult patients with CABP in 2019, which is caused by susceptible microorganisms, including *S. pneumoniae*, MSSA, *H. influenzae*, *L. pneumophila*, *M. pneumoniae*, and *C. pneumoniae* (Xenleta, 2019).

Lefamulin is a systemic semisynthetic pleuromutilin antibiotic, which can be administered via both IV and PO routes. It has a tricyclic core scaffold containing 5-, 6-, and 8-membered ring systems, along with a substituted ester side chain at the 14 position (Zhanel et al., 2021). Diterpene pleuromutilin antibiotics were first discovered in the 1950s, lefamulin is the first drug in this class for systemic treatment of bacterial infections in humans (Veve and Wagner, 2018). It works as a protein synthesis inhibitor by binding to the 23S rRNA of the bacterial 50S ribosomal subunit (Paukner and Riedl, 2017). Lefamulin showed both bactericidal and bacteriostatic property; and was active against *S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, *S. aureus* and *S. pyogenes*, but inactive against Gram-negative *Enterobacteriaceae* and *P. aeruginosa* (Eraikhuemen et al., 2021).

Xenleta is available in a single-dose clear glass vial (lefamulin 150 mg in 15 mL of 0.9% NaCl solution) and requires further dilution before IV infusion, as well as 600 mg tablets (Xenleta, 2019). To treat adult patients with CABP, lefamulin IV 150 mg is infused every 12 h over 60 min for a duration of 5–7 days or lefamulin PO 600 mg is administered orally every 12 h for 5 days (Xenleta, 2019). IV dosage adjustment is required for patients with severe hepatic impairment. The most common adverse events reported in at least 2% of patients on lefamulin include diarrhea, administration site reactions, hepatic enzyme elevation, nausea, hypokalemia, insomnia, headache, and vomiting (Lexicomp, 2021). Lefamulin provides a valuable treatment option for CABP in cases of poor clinical outcome, patient intolerance, and/or antibiotic resistance from other antibacterial therapies (Zhanel et al., 2021).

Fetroja® (cefiderocol)

Fetroja (cefiderocol) (Fig. 4), an injectable cephalosporin-siderophore conjugate, was approved by the US FDA for the treatment of adult patients with cUTI in 2019. Currently approved indications of cefiderocol include cUTI and HABP/VABP (since 2020), caused by susceptible Gram-negative microorganisms, such as *A. baumannii* complex, *E. coli*, *E. cloacae* complex, *K. pneumoniae*, and *P. aeruginosa* (Shionogi, 2020). Like some other β -lactam antibacterial agents, cefiderocol was active against carbapenem-resistant and MDR Gram-negative bacilli (Zhanel et al., 2019b).

Cefiderocol is a novel cephalosporin antibiotic conjugated with a siderophore motif. Specifically, the siderophore with a catechol moiety on the three position chelates with ferric iron, facilitating the cefiderocol delivery across the outer membrane of Gram-negative bacteria using iron transport systems (Surur and Sun, 2021). Mechanistically, cefiderocol binds to PBP and inhibits bacterial cell wall peptidoglycan synthesis, resulting in bacterial cell lysis (Lexicomp, 2021). It should be noted that cefiderocol has great structural similarity with ceftolozane and ceftazidime, except for the different 3-substituted side chains. Another minor structural difference is that ceftolozane has a 5-amino-1,2,4-thiadiazole ring on the 7-substituted side chain, compared to the 2-aminothiazole ring in ceftazidime and cefiderocol.

Fetroja (cefiderocol) is available in single-dose vials as a lyophilized powder (1 g of cefiderocol) and requires reconstitution and further dilution before IV infusion. For patients with CrCl of 60–119 mL/min, cefiderocol 2 g is infused every 8 h over 3 h for a duration of 7–14 days for HABP/VABP and 5–14 days for cUTI (Shionogi, 2020). Dosage adjustments are required for patients with CrCl <60 or ≥ 120 mL/min. The most common adverse events reported in at least 3% of patients on cefiderocol include skin rash, diarrhea, constipation, and infusion site reaction (Lexicomp, 2021). In addition to treating bacterial infections, caused by susceptible Gram-negative microorganisms, cefiderocol represents the first cephalosporin antibiotic with a clinical indication for *A. baumannii* complex.

Conclusions

New antibacterial agents and therapeutic options for the treatment of problematic bacterial infections, caused by difficult-to-treat and MDR microorganisms, are urgently needed. Since 2000, thirty-six new antibiotics have been approved by the US FDA. Among them, seven antibiotics can be considered as new chemotype antibacterial classes such as oxazolidinone (linezolid and tedizolid phosphate), lipopeptide (daptomycin), the cephalosporin-siderophore conjugate antibiotic (cefiderocol), anti-difficile macrolide (fidaxomicin), and anti-tuberculosis agents diarylquinoline and nitroimidazooxazine (bedaquiline and pretomanid, respectively). The remaining approved antibiotics are largely based on new advances and chemical modifications of existing antibacterial

classes such as carbapenem, cephalosporin, nitroimidazole, fluoroquinolone, ketolide, rifamycin, tetracycline, pleuromutilin, lipoglycopeptide, and aminoglycoside. In addition, three narrow-spectrum antibiotics (anti-difficile fidaxomicin; anti-tuberculosis bedaquiline and pretomanid), three topical agents (retapamulin, besifloxacin, and finafloxacin), four β -lactam/BLI combinations, and three monoclonal antibodies were also introduced in the clinic in the last 20 years. Notably, five brand name and/or generic products (gemifloxacin, telithromycin, tinidazole, doripenem, and finafloxacin) have been discontinued from the US market. Moreover, the important attribute, physiochemical property, mechanism of action, route of administration and dosing frequency, clinical pharmacology and indications of each antibiotic were summarized and presented in this book chapter.

With estimated 700,000 people dying worldwide annually from antimicrobial resistant infections, it is very important for researchers, clinicians, and pharmacy educators and practitioners to know these newly approved antibiotics, be knowledgeable about their approved indications and appropriate uses, and understand their unique characteristics that make them effective for the treatment of drug-resistant bacterial infections. Conversely, it is also critical for clinicians and health care professionals to understand the structural features and underlying mechanisms for antibiotic resistance so that they consciously prescribe them appropriately when absolutely needed. Finally, it should be pointed out that five new antibiotics (linezolid, tedizolid phosphate, delafloxacin, omadacycline, and lefamulin) are available for both IV and PO options, as such switching from IV to PO can benefit patient compliance, potentially eliminate the need for hospitalization, as well as save significant costs from both patient and healthcare system standpoints.

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Non-Conventional Antimicrobial Agents

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Glossary

AMP Antimicrobial Peptide
CLSM Confocal Laser Scanning Microscopy
EO Essential Oils
EPS Exopolysaccharide
LAB Lactic Acid Bacteria
LAS Linear Alkylbenzene Sulfonates
Lf Lactoferrin
Lfa Lactoferrampin
Lfc Lactoferricin
man-PTS Mannose Phosphotransferase System
MDR Multidrug Resistance
Me-NP Metal Nanoparticle
MIC Minimal Inhibitory Concentration
MRSA Methicillin Resistant *Staphylococcus aureus*
PTM Post Translational Modification
QQ Quorum Quenchers

QS Quorum Sensing
SEM Scanning Electron Microscope
SPION Superparamagnetic Iron Oxide Nanoparticles
TLR Toll Like Receptor
VRE Vancomycin Resistant Enterococci

Introduction

Increasing the knowledge on possible new infection control agents to be employed as pharmaceutical or nutraceutical products in both human and veterinary medicine is a matter of urgency due to the threat of microbial antibiotic resistance. In the last half century, antibiotic resistance has had a growing impact due to the persistently selective pressure from widespread use of antimicrobials in humans, but especially in farm animals and agriculture (Breithaupt, 2014). In 2001, the European Union Ministers of Health recommended a more prudent use of antimicrobial agents in human medicine with a number of specific measures aimed to containing the spread of antibiotic resistance. Despite this, every year, the rate of resistance in several bacterial species is increasing (e.g., *Staphylococcus* spp., *Enterococcus* spp., Enterobacteria, *Pseudomonas* spp. and *Acinetobacter* spp.) (World Health Organization). Multi-drug resistance (MDR) is defined as the non-susceptibility to at least one agent in three or more antimicrobial categories, giving a representative limitation in antibiotic use, especially in severe clinical contexts (Roca et al., 2015). Being, at present, therapeutic options for curing MDR bacterial infections very limited, we are assisting to an increased mortality rate in hospitalized human patients. Since antibiotic resistance is not only of medical and social relevance but a global problem considered by the World Health Organization as one of the greatest threats to human health for the next decades, searching for new antimicrobial agents is an urgent need. Therefore, finding new antimicrobial molecules will give a contribution to increase the number of available weapons and effectively counteract the increasing resistance to conventional antibiotics thus ameliorating the management of microbial infections.

Several strategies have been proposed as alternatives to conventional antibiotics to treat microbial infections and the benefits and limits of each approach are summarized in Table 1. The present chapter, although not exhaustive, is intended to explore some of the best studied antibiotic alternatives considering the state-of-the-art, the antimicrobial spectrum, the mechanisms of action, and some controversial aspects of the most common non-conventional antimicrobials. It has to be underlined that not all antibiotic alternatives rely on the killing of the target bacteria. Actually, other strategies such as prevention of biofilm formation (and hence persistence and chronic infection control) and of toxin production (therefore controlling virulence of certain strains) are also exploited by means of metal nanoparticles (Me-NP), surfactants and quorum quenchers (QQ), since both biofilm and toxin synthesis are under quorum sensing (QS) control. In this case bacteria remain alive but their pathogenic potential is neutralized.

Living organisms

Among the several strategies to counteract pathogenic bacteria, both probiotics and phages have been proposed. This is not a conventional therapy since the used devices are not molecules but living organisms. The main principle at the basis of this choice is the biological fight for survival. Actually, bacteria living in the same ecological niche set up a huge number of competition strategies and mechanisms of predation occur between phage and bacteria. This arm race render each step of the evolutionary path a true struggle for survival and for increasing fitness (Pessione, 2020).

Table 1 Targets, adverse effects for humans and problematic aspects of the considered non-conventional antimicrobial agents.

Antimicrobial	Target	Human toxicity	Problematic issues
Probiotics	B, IS	Possible microbiota perturbations	no
Phages	B	Possible microbiota perturbations, lysogeny, immunogenicity	Bacterial resistance to phage attack
AMP	B,V,F,P,IS	Possible hemolytic activity	Protease sensitivity
Bacteriocins	B, IS	Possible membrane damage, immunogenicity.	Protease sensitivity
Quorum sensing interference	B	Possible microbiota perturbations	Alteration in the relationship with phages
Essential oils	B,V, F, P	Absent	Non-transmissible resistance by membrane modification
(Bio)Surfactants	B,V,M,F, IS	Low	no
Me-NP	B, V, F	Possible systemic diffusion	Environmental distribution in the food web

B = Bacteria, V = viruses, F = fungi, P = parasites, IS = immune system.

Probiotics

Probiotics are defined as “Living organisms that when administered in adequate amounts can support health benefits to the recipient.” Although the list of beneficial microbes is expanding every day, the best recognized probiotics’ genera are *Lactobacillus* and *Bifidobacterium* and, among yeasts, *Saccharomyces*. The huge amount of data concerning the roles of probiotics in promoting human health is still growing and after two decades of in-depth investigations several aspects of their biological functions have been elucidated (nutritional support, immune modulation, control of metabolism and cancer, gut-brain axis homeostasis) (George Kerry et al., 2018). One of the first activity recognized to probiotics was their capability to prevent and cure infection. The antibacterial strategies exerted by probiotics are summarized in Fig. 1, whereas some aspects of their antagonizing action for counteracting infection are represented in Fig. 2. From one side they can colonize the gut epithelium forming a biological barrier that prevents pathogens colonization (this is why an overdose of probiotics before tropical travels can prevent travelers’ diarrhea) meanwhile they can re-colonize an “empty” ecological niche such as the gut mucosa after prolonged antibiotic therapy. A part from this competitive exclusion, probiotics also antagonize pathogens by competing for nutrients or by producing enzymes or metabolism end-products that exert limiting effect on pathogenic growth (for instance acids, ethanol or hydrogen peroxide) (Pessione, 2014). All these strategies are “unwanted” consequences of their being alive. In parallel, they can co-aggregate with pathogens and displace them from epithelia and finally produce specific weapons, namely bacteriocins, intended to kill the competitors (Cotter et al., 2013). In addition, some probiotic bacteria can decrypt antimicrobial peptides (AMP) and immune stimulating peptides from diet-derived proteins thus indirectly controlling microbial populations at the gut level (Pessione and Cirrincione, 2016). Recently, probiotic-derived compounds have been classified as post-biotics and para-biotics (Nataraj et al., 2020). Post-biotics are all the molecules that are secreted or released by probiotic bacteria into the external environment, such as bacteriocins, acids, hormones, nutraceuticals, peptides, whereas para-biotics (exopolysaccharides, moonlighting proteins) are molecules exposed on the cell surface that can induce an immune response. At this respect para-biotics can be active even if the bacterial cell is killed, providing that no denaturation events occur. However, the distinction between para-biotics and post-biotics is sometimes artificial because weakly bound compounds (i.e. EPS, moonlighting proteins and bacteriocins) can detach and be present in the cell-free supernatant. Both post-biotics and para-biotics play an essential role in counteracting infection: post-biotics such as bacteriocins and AMP will be discussed later in the Section “Organic molecules.” Para-biotics can be used to enhance the immune response, thus stimulating an endogenous defense mechanism against pathogenic organisms (not only bacteria). Therefore, several options (the use of living probiotics, killed bacteria or also surface extracts) are suitable for enhancing immune defenses thus counteracting infection by indirect way.

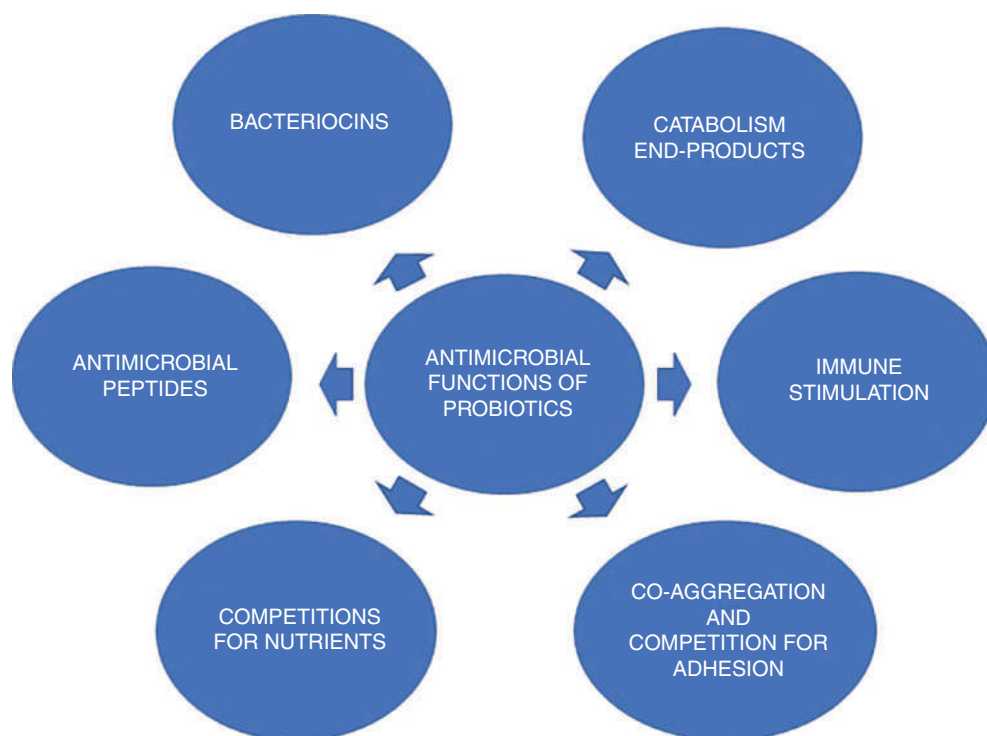


Fig. 1 Main antibacterial weapons set up by probiotic bacteria.

PROBIOTICS ANTAGONISTIC BEHAVIORS

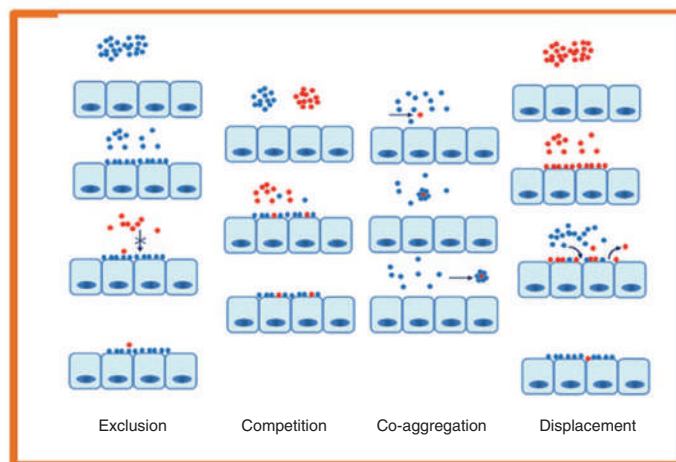


Fig. 2 Probiotic antagonistic behavior at the gut level.

Bacteriophages

Bacteriophages or phages are viruses that infect and kill bacteria by injecting their nucleic acid, replicating inside the bacterial host and causing cell lysis (Fig. 3). They are the most abundant biological entity on the planet, outnumbering bacteria by a factor of 10. They can be divided into lytic and lysogenic. The former infect and kill bacteria by producing new virions, whereas the latter integrate into bacterial genome, and become lytic only when the host bacterium is exposed to stress conditions (Howard-Varona et al., 2017). Phages are considered highly specific, but recent evidence demonstrated that some of them have a broad host range (Cazares et al., 2021).

Phage therapy is the strategy of using phages to combat bacterial infections. It was pioneered by Felix de Herelle, one of the discoverers of phages in the early 19th century, who used it successfully to treat bacterial dysentery and cholera. Nevertheless, due the rise of antibiotics, phage therapy was dismissed in the western world, and only pursued in some countries of Eastern Europe such as Georgia and Poland. However, due the increased bacterial resistance to antibiotics, phage therapy is being now adopted in countries like the United States and the United Kingdom (Düzgüneş et al., 2021).

To date, there is a growing number of clinical reports demonstrating the potential of bacteriophages to control life threatening infections, as those caused by multi drug resistant bacteria such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Hence it is expected that in the future their utilization could be widespread worldwide (Jennes et al., 2017; Chan et al., 2018; LaVergne et al.,

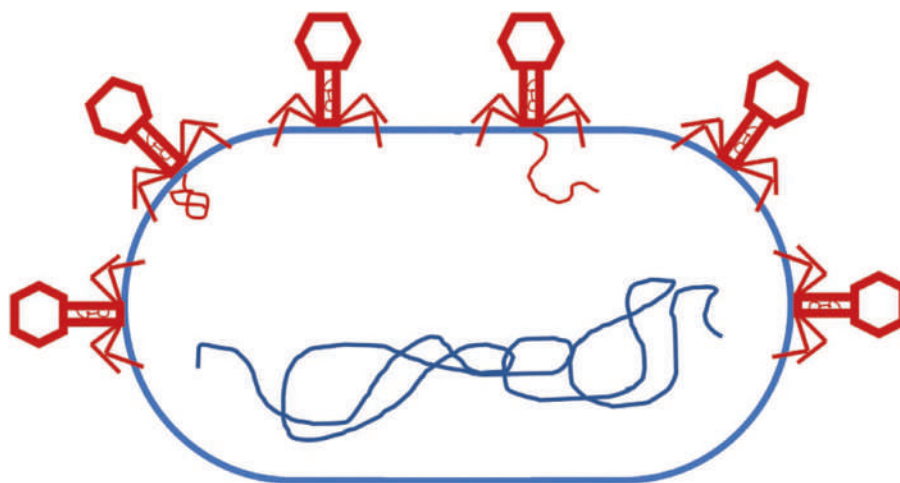


Fig. 3 Phage attack to a bacterial cell.

2018). Since resistance against a single phage arises easily, ideally phage therapy should be administered with a cocktail of lytic phages. Furthermore, these phages should not carry potentially dangerous genes, such as those codifying antibiotic resistance determinants or virulence factors (Hyman, 2019). To further improve positive phage features such as their ability to kill or disperse biofilm cells (Lu and Collins, 2007; Ferriol-González and Domingo-Calap, 2020) or to limit negative characters such as lysogeny (Blasco et al., 2019), a genetic engineering approach is proposed, thus increasing the repertoire of useful bacteriophages (Chen et al., 2019).

An additional attractive feature of bacteriophages is their ability to re-sensitize multidrug resistant bacteria towards antibiotics. This phenomenon occurs when bacteria become resistant to phages by losing the phage receptor functionality or expression. Actually, these phage receptors can be part of an antibiotic efflux pump, such as the case of the OprM protein, which is an element of at least two efflux pumps MexAB-OprM and MexXY-OprM. In 2016, Chan and coworkers demonstrated that *P. aeruginosa* PAO1 colonies that turned resistant to the lytic phage OMKO1 increased their sensitivity towards tetracycline and erythromycin for which resistance is determined by the efflux systems MexXY-OprM (Chan et al., 2016). In agreement with these data, recently it was reported that the bacteriophages ΦFG02-R and ΦCO01-R that infect *Acinetobacter baumannii* promote its re-sensibilization against beta-lactams antibiotics such as ceftazidime and against quinolones such as ciprofloxacin (Gordillo Altamirano et al., 2021). In this model, the phage receptor is in the capsular EPS and bacteria become resistant to the phage, by losing the ability to synthesize a capsule. Disruptive mutations in the gene K locus that encodes enzymes that regulate the production, modification and export of capsular polysaccharides (such as the glycosyltransferases and glucose-6-phosphate isomerase enzymes) are responsible for this phenomenon (Gordillo Altamirano et al., 2021). These phage-resistant bacteria, besides being more sensitive to antibiotics, also become more sensitive towards the action of the complement system thus resulting easily removable by the phagocytes (Gordillo Altamirano et al., 2021).

Interestingly, a recent study by (Engeman et al., 2021) demonstrated the ability of a bacteriophage cocktail (5 phages, from the families myoviridae and siphoviridae) to act synergically with several antibiotics in vitro and to re-sensitize the majority of the 14 multidrug resistant clinical isolates to at least one the following antibiotics: ceftazidime, meropenem, gentamicin, or ciprofloxacin. Moreover, the combination of the phage cocktail with ceftazidime was effective to clear *P. aeruginosa* PAO1 infection from mice wounds (Engeman et al., 2021).

Taken together these evidences suggest that phages could become a promising tool for the control of bacterial infection, when administered alone or in combination with antibiotics. However, it has to be underlined that environmental factors (pH, ions) can affect the efficacy of therapeutic phages (Jończyk et al., 2011).

Nevertheless, some constraints arise since phages are not simple molecules but they are (although host-dependent) living organisms having the potential of interacting with both microbiota and immune system. Among the possible risks are worth mentioning:

1. Quantitative/qualitative alteration of the composition of gut microbiota by selection of the phage-resistant species/strains with possible loss of beneficial species and modification of the gut metabolome (Hibbing et al., 2010).
2. Mutation of phages into potentially dangerous forms or induction of toxin production in gut bacteria, since a permanent co-evolution mechanism occurs between phages and their hosts (Koskella and Brockhurst, 2014).
3. Induction of immune responses in the human host due to rapid release of both bacterial and phage proteins after lytic events (Łusiak-Szelachowska et al., 2017).
4. Change the lytic to a lysogenic phenotype altering host gene expression and the relationship of the bacterial host with the human host (Ganeshan and Hosseinioust, 2019; Pessione, 2020).

To date only few reports, often contradictory, concerning the possible risks, are available in the literature (Galtier et al., 2016; Hsu et al., 2019) and outstanding questions remain open, requiring more in-depth studies.

Organic molecules

The molecules explored for their antibacterial activity belong to almost all biochemical classes, however proteinaceous compounds and hydrophobic/amphipathic compounds have a longer history in counteracting infections.

Antimicrobial peptides (AMP)

Natural antimicrobial peptides are produced by a large variety of organisms such as animals (Hancock and Sahl, 2006), plants (Tam et al., 2015) and bacteria (Pessione and Cirincione, 2016). Microbial-derived antimicrobial peptides (AMPs) are not synthesized at ribosomal level but are rather the result of a proteolytic action carried out by bacteria on natural proteins. These are caseins (Atanasova et al., 2014) and minor milk proteins such as whey proteins like lactoferrin (Lf) (Bruni et al., 2016), albumins and globulins (Atanasova et al., 2014). AMPs can also be obtained by chemical synthesis (Dijkshoorn et al., 2004) as well as by an abiotic action on food proteins by exploiting proteolytic enzymes generally covalently immobilized on solid surfaces and removing them after catalysis (Bellamy et al., 1992). Considering that for efficient large-scale production of antimicrobials the raw materials should be abundant and cheap, exploiting the bacterial proteolytic activity on whey proteins is the winning approach. Actually, milk

whey is a low-cost by-product of dairy industries and a huge number of microorganisms are able to grow on it (Mazzoli et al., 2014). Among them, Lactic Acid Bacteria (LAB) are the most promising since, being unable to synthesize essential amino acids, they possess a sophisticated proteolytic system allowing them to decrypt interesting peptides that are in part up-taken by the cell in part left extracellularly (Pessione, 2012).

Lactoferrin-derived peptides

Among the milk-derived antimicrobial agents, the lactoferrin-derived peptides are the most relevant. Their mode and spectrum of action together with their possible applications in the food industry, veterinary and human medicine, have been extensively reviewed in a previous paper (Bruni et al., 2016). On the other hand, the whey protein lactoferrin itself (Lf: an 80 kDa iron-binding glycoprotein) plays significant role in innate immunity. It is an important host defense molecule providing antiviral (Bojsen et al., 2007; Berlutti et al., 2011), antibacterial (Embleton et al., 2013) antifungal, anti-parasitic (Leboffe et al., 2009), immunomodulatory (Siqueiros-Cendón et al., 2014) and antioxidant activities (Kanwar et al., 2015). The most interesting antimicrobial molecules derived from proteolytic cleavage of bovine Lf are lactoferricin (Lfc) (Kitts and Weiler, 2005) and lactoferrampin (Lfa) (Van Der Kraan et al., 2005).

Lactoferricin (Lfc) is a cationic peptide resulting from pepsin digestion on Lf. Its peptide sequence (FKCRRWQWRMKKLGAPSITCVRRAF) corresponds to the fragment 17–41 of Lf. Because of its cationic charge, Lfc interacts with the negatively charged membranes, thus acting as proton gradient altering compound, perturbing permeability with consequent membrane depolarization causing direct cell death for failure of ATP synthesis (Fitzgerald and Murray, 2006). Disruption of the integrity of the biological membranes with severe ultra-structural damages also occurs, causing induction of an autolytic response (Van Der Kraan et al., 2004, 2005). The membrane target render Lfc active not only towards bacteria but also towards organisms having a different cell-wall structure such as fungi. Actually, Lfc besides assessed antibacterial action, also displays antifungal (including yeasts and filamentous fungi) activity (Bruni et al., 2016). Lfc found applications in human medicine towards the entero-hemorrhagic strain of *E. coli* O157h:7 (Muro Urista et al., 2011). As far as veterinary medicine is concerned, an in vivo study demonstrated a clinical improvement of dog's otitis using therapeutic combinations of Lfc, verbascoside and glycerophosphoinositollysine (Vercelli et al., 2015).

Lactoferrampin Lfa is a cationic peptide (WKLLSKAQEKFGKNKSR) including the residues from 268 to 284 of the N1 domain of Lf. Besides displaying cationic nature it also contains a hydrophobic domain that makes the structure amphiphilic and therefore suitable to act against Gram negatives. Lfa exhibits broad antimicrobial action against several Gram-positive genera (*Streptococcus*, *Staphylococcus*, *Bacillus*) and Gram-negative bacteria (*E. coli*, *P. aeruginosa*), yeasts (*Candida albicans*) (Van Der Kraan et al., 2005) and parasites (*Leishmania donovani*) (Théolier et al., 2014). However, due to their different amino acid composition, Lfc and Lfa can have a synergistic effect against multiresistant *S. aureus* strains, especially when combined with penicillins as suggested in (Flores-Villaseñor et al., 2010).

Casein-derived peptides

Other AMP derived from cow's milk caseins and worth mentioning are Isracidin, Casocidin-I, K-Casedicin and Kappacin.

Isracidin and Casocidin-I. These peptides, derived from alpha casein (alpha s1 and alpha s2 respectively) hydrolysis (Pessione and Cirincione, 2016), exhibit different spectrum of action: while Isracidin is active only on Gram positives, especially *S. aureus* (McCann et al., 2006), Casocidin-I also inhibits *E. coli* (Zucht et al., 1995). According to (Minervini et al., 2003) this probably occurs because of its more hydrophobic nature that can render it best fitting in passing across the Gram-negative outer membrane.

K-Casedicin and Kappacin. Both peptides originate from K-casein hydrolysis. The former is able to control *S. aureus* growth (Atanasova et al., 2014), whereas the latter is highly active towards *Streptococcus mutans* (Malkoski et al., 2001) thus representing a promising tool to treat oral infections.

Challenges and limits of AMP

Finding new strains able to decrypt AMP is a good challenge. A very recent report describes that food isolated strains belonging to *L. lactis*, *L. helveticus* and *L. rhamnosus* species are able to decrypt peptides having 100% sequence identity to well-known antimicrobial peptides reported in the data banks (Nebbia et al., 2021). Among them, K-Casedicin (*L. rhamnosus* 17D10) and the peptide LEQLRLKKY (*L. lactis* MG1363) active towards *E. coli* NEB 5 and *B. subtilis* ATCC 6051, as well as the peptide QKAL-NEINQF (*L. helveticus* 4D5) active against *S. aureus*, *L. monocytogenes*, *Bacillus cereus* and *Helicobacter pylori*. An alternative approach is the use of bioinformatic tools that can be exploited to find suitable proteins in food and related hydrolytic enzymes to perform targeted proteolysis to obtain the AMP of interest (Dziuba and Dziuba, 2014). Some limitations exist that can be highlighted by in silico studies. As an example, characterizing peptides according to their length, amino acid sequence, charge, hydrophobic residues and secondary structure (when present) can allow to establish the target (for instance Gram-positive or Gram-negative bacteria, fungi, parasites, viruses etc.) and also to reveal possible risk of failure. An interesting report by Malanovic and Lohner (2016) provides evidence that some AMP can be entrapped into anionic teichoic acids, abundant on Gram-positive bacteria, thus resulting ineffective at the membrane level where they usually act.

Recombinant cell systems or also chemical synthesis can both represent a valuable approach to obtain AMPs selective for microorganisms, but safe for human cells. These strategies include the targeted design of post translationally modified AMPs (phosphorylation, glycosylation, methylation and amidation being the main PTMs) but also insertion in the sequence of non-natural amino acids. As an example, incorporating D-amino acids into the primary sequence can alter the helical structure thus decreasing the hemolytic effect without affecting the antimicrobial potential of the AMP (Papo et al., 2002). Furthermore, these

peptides are more resistant to proteolytic cleavage (Bahar and Ren, 2013). Cationic hexapeptides, containing unusual or D-amino acids and whose critical residues are the 2nd and the 3rd amino acid, have also been constructed that proved to be active also against multi-resistant bacteria like methicillin resistant *Staphylococcus aureus* (MRSA) and *E. coli*, as well as the pathogenic fungus *Candida albicans* (Yousefinejad et al., 2015). Most of these peptides display stability in blood, therefore, they can be considered promising molecules to control infection. Interestingly, some whey proteins/peptides can have antiviral activity as suggested by Bojsen et al. (2007). In particular, a peptide from donkey milk exhibiting anti-Rotavirus efficacy has been described by Civra et al. (2015). This opens interesting perspectives also for the treatment of viral infections.

Bacteriocins

The antimicrobial activity of bacteriocins was first reported in England, in 1928 by Rogers and Whittier, who discovered that certain *Lactococcus* strains had an inhibitory effect on the growth of other LAB (Ross et al., 2002). Preliminary investigations of the antimicrobial spectrum of the newly discovered bacteriocins produced by LAB included some foodborne pathogens. Therefore, bacteriocins have been proposed for food use to prevent the growth of spoilage bacteria and foodborne pathogens, gradually acquiring a relevant role as food preservatives. They were first used in food in 1951 and the term “bacteriocin” was coined in 1953 to define colicin produced by *Escherichia coli* (Ross et al., 2002). Nisin, the best known bacteriocin produced by *Lactococcus lactis*, was discovered in parallel to penicillin and it has been applied in the food industry as a preservation agent (Delves-Broughton et al., 1996). In 1988, the FDA had approved its use as a biopreservative for a narrow range of foods and was also added to the European food additive list where it was assigned the number E234 (Ross et al., 2002). However, based on initial observations that bacteriocins may sometimes be active against clinical pathogens, studies started to appear, in which the activity of bacteriocins was tested against a more general repertoire of bacteria irrespective of their relevance with the food environment (Montalbán-Lopez et al., 2011).

It is thought that nearly 99% of all bacteria may produce at least one bacteriocin (Klaenhammer, 1988) that plays a defensive role and acts to prevent the invasion of other strains or species into an occupied niche or limit the advance of neighboring cells. An additional role has been proposed for Gram-positive bacteriocins, in which they mediate quorum sensing (Kleerebezem, 2004). Secondly, at least in *L. plantarum*, they can behave as immune modulating agents altering cytokine profiles of both monocytes (Van Hemert et al., 2010) and dendritic cells (Meijerink et al., 2010).

Bacteriocins are a heterogeneous group of anti-bacterial peptides or small proteins that vary in molecular weight, biochemical properties, spectrum of activity and mode of action, with bactericidal or bacteriostatic effect (Ross et al., 2002). Their production is under quorum-sensing control and generally modulated by bacterial stress (Mazzoli et al., 2014).

These peptides differ from antibiotics since: (i) they are ribosomally synthesized (ii) they are usually active even at very low concentrations (i.e. at the nanomolar range) (iii) they have a relatively narrow killing spectrum. Although they are generally active on bacteria closely related to the producing strain, there are evidences of relatively broad spectrum bacteriocins such as nisin and lactacin 3147 (Papadimitriou and Alexandraki, 2014).

Classification

Due to the fact that in every ecosystem bacteria produce interfering molecules to antagonize competitors, bacteriocins constitute a still underexplored and underexploited area of research to find antibiotic substitutes. The continuous discovery of new bacteriocins makes it necessary to frequently revise the proposed classification schemes. Bacteriocins can be arbitrarily divided into five groups (Fig. 4) on the basis of their biochemical structure, molecular mass, thermal stability, presence of post-translationally modified amino acids and mode of action (Cleveland et al., 2001). Each bacteriocin has the name of the producing specie/genus (colicins from *E. coli*, enterocins from *Enterococcus*, lactacins from *Lactococcus*, helveticin from *L. helveticus* etc.). Generally, lactic acid bacteria (LAB) are largely explored as producers of numerous bacteriocins since they have the advantage of being safe for use (Rea et al., 2011b).

Besides these proteinaceous compounds some bacteria can also produce small molecules having antimicrobial action. A paradigmatic example is *L. reuteri* that synthesizes three different compounds: reuterin, reuterocyclin and reuterin (Nebbia et al., 2021). While Reuterin is a proteinaceous bacteriocin-like antimicrobial (MW 2.7KDa) (Toba et al., 1991), Reuterocyclin is a small (MW 349Da) hydrophobic compound especially active towards Gram positive bacteria (Ganzle et al., 2000) whereas Reuterin is a 3-hydroxypropionaldehyde (MW 58Da) which exerts its antimicrobial activity both by inducing oxidative stress in the target cells (Schaefer et al., 2010) and by conversion into the toxic compound acrolein (Engels et al., 2016) thus displaying a broad spectrum of activity and poor selectivity (bacteria, fungi and viruses).

Mechanism of action

The best-known mechanism of action of bacteriocins is the creation of pores on the bacterial cytoplasmic membrane that results in the loss of essential macromolecules and the dissipation of the proton motive force (Moll et al., 1999). The dissipation of the gradient is achieved by Lactococcin A and other Pediocin-like bacteriocins of class II by binding to the mannose phosphotransferase system (man-PTS) (Diep et al., 2007), whereas carnocyclin A, enterocin AS-48, gasserin A and subtilisin A act independently from any binding to specific cell sites (Nishie et al., 2012). Other mechanisms include cell wall lysis (bacteriolysins) and inhibition of cell wall synthesis (mursacidin) mediated by bacteriocin binding to lipid II (Brotz et al., 1998). Nisin combines different mechanisms by acting in three sequential steps: (i) binding to lipid II, (ii) pore formation on the bacterial cytoplasmic membrane and (iii) inhibition of cell wall synthesis by removing lipid II from the cell division site (Wiedemann et al., 2006).

BACTERIOCINES CLASSIFICATION

class I	type A type B	LANTIBIOTICS
class II	type A type B type C	PEPTIDES WITHOUT MODIFIED AMINO ACIDS
class III		BATTERIOLYSINS
class IV		GLYCOPROTEINS AND LIPOPROTEINS
class V		CYCLIC BACTERIOCINS

Fig. 4 One possible classifications for bacteriocins.

Spectrum of activity

In recent years, bacteriocins have attracted much attention as antibiotic substitutes to combat infections difficult to treat with conventional antimicrobial agents (Shin et al., 2016, Mazzoli et al., 2014). In general, bacteriocins display a narrow spectrum of action: Gram-positive derived bacteriocins are more effective towards Gram-positive bacteria, whereas Gram-negative synthesized molecules preferentially act against Gram-negative pathogens. However, exceptions exist and these mainly concern Lactic Acid Bacteria (LAB) derived molecules that proved to be active also towards Gram negatives.

Bacteriocins active against Gram-positives. Pediocin-like peptides produced by LAB proved to be effective anti-listerial agents (Birri et al., 2010). Enterocin 96 and hiracin JM79 display anti-staphylococcal properties, whereas salivaricin D and subtilosin A, are efficient anti-streptococcal compounds including *S. pneumoniae* as target (Hammami et al., 2012). Nisin and lactacin 3147 are especially fitting towards Clostridia. The latter is effective against *C. difficile*, while nisin is highly performant towards both *C. difficile* and *C. perfringens* (Rea et al., 2011a). In particular, the efficacy towards *C. perfringens* also includes spores and the MICs related to *C. difficile* are very low if compared to those relative to antibiotics such as vancomycin (Udompijitkul et al., 2012). Ruminococcin A, produced by *Ruminococcus gnavus* E1 is active also against *C. botulinum*, *C. bifermentans*, *C. septicum*, *C. sordellii* and *C. sporogenes* (Dabard et al., 2001). Thuricin DC from *Bacillus thuringiensis* DPC 6431 besides killing *C. difficile* and *C. perfringens* is effective against *C. histolyticum* and *C. tyrobutyricum* (Rea et al., 2010). These anti-clostridial agents are very interesting since they can found application in food to prevent food-related infections (*C. botulinum*, *C. tyrobutyricum*, *C. perfringens*) but they can also be used to treat nosocomial events such as pseudomembranous colitis due to prolonged antibiotic therapy selecting toxin-producing *C. difficile*, which causes a severe inflammatory reaction. At this purpose, lactacin 3147 proved to be an excellent inhibitory molecule for different *C. difficile* strains especially when added in a model fecal environment (106 cells mL⁻¹ killed in 20 min) (Papadimitriou, 2014).

Bacteriocins active against Gram-negatives. *Escherichia coli* strain H22 synthesizes three bacteriocins, named colicin E1, colicin Ib and microcin C7, which are effective towards *Enterobacter*, *Escherichia*, *Klebsiella*, *Morganella*, *Salmonella*, *Shigella* and *Yersinia* (Cursino et al., 2006). Microcin J25 produced by *E. coli* is active against *Salmonella enteritidis* and virulent *E. coli* strains (Portrait et al., 1999; Sable et al., 2000). On the other hand, bacteriocins from Gram-positive LAB strains can control *H. pylori* (De Vuyst and Leroy, 2007). *Lactobacillus plantarum*, produces several bacteriocins active against Gram-negatives, among which plantaricin MG effective towards *S. typhimurium* (Gong et al., 2010) and other bacteriocins (ST28MS and ST26MS) targeting *Pseudomonas aeruginosa*, *E. coli* and *Acinetobacter baumannii* (Todorov and Dicks, 2005). A paradigmatic example among LAB is *Enterococcus* spp., which can synthesize a huge number of antibacterial compounds. Enterocin E-760 can control several Gram-negative pathogens such as *Citrobacter freundii*, *E. coli* O157:H7, *K. pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *P. aeruginosa*, *Salmonella enterica*, *Shigella dysenteriae*, *Yersinia enterocolitica*, and different species of *Campylobacter* (Line et al., 2008). The actinobacterium *Microbispora* ATCC PTA-5024 can also target the Gram-negative cocci *Neisseria meningitidis* and *Neisseria gonorrhoeae* (Castiglione et al., 2008). Finally, bacteriocin ST15 and bacteriocin E 50-52 are active towards *A. baumannii*, (Svetoch et al., 2009) an important nosocomial pathogen characterized by intrinsic resistance to most antibiotics (Imperi et al., 2011).

Bacteriocins active against MDR strains. The most problematic resistant strains are vancomycin resistant enterococci (VRE) and methicillin resistant *Staphylococcus aureus* (MRSA). Both nisin and lactacin 3147 are effective against *Staphylococcus aureus* and enterococci, respectively (Piper et al., 2009). Among the nisin structural variants (differing by a single or more amino acids) nisin F proved to be the best fitting towards resistant strains (Piper et al., 2011). Among LAB synthesized bacteriocins, mutacins produced by *Streptococcus mutans* are effective towards MRSA, VRE (Mota-Meira et al., 2005) and also multidrug resistant bacteria (MDR) such as *S. aureus* (including vancomycin and oxacillin resistant strains). The effect seems to be bactericidal on *S. pneumoniae* but only bacteriostatic on VRE (Ghobrial et al., 2009). Besides LAB, also other bacteria can produce bacteriocins able to kill resistant strains: *Staphylococcus hominis* MBBL 2-9 displays anti-MRSA activity (Sung et al., 2010), *Bacillus pumilus* WAPB4 can kill MRSA and VRE (Aunpad and Na-Bangchang, 2007), *Bacillus halodurans* C-125, besides targeting VRE is also effective towards *Bacillus anthracis* spore outgrowth (Oman and van der Donk, 2009).

Limits and open aspects

In vivo efficacy. Bacteriocins are moderately active at the physiological neutral pH. Nevertheless, some reports describe their use in skin and mucosae, after local administration as a lotion. A nisin solution applied for 2 weeks on the infected area was used to treat staphylococcal mastitis in women with remission of the infection not observed in the placebo group (Fernández et al., 2008). Mersacidin and proved to be able to eliminate MRSA from the nasal mucosa of colonized mice after intra-nasal treatment (Kruszewska et al., 2004). Gram-negative targeted molecules are best performant: Microcin J25 proved to be active towards *Salmonella* in ex vivo whole blood, plasma and serum remaining stable in all body fluids without causing hemolytic activity (Lopez et al., 2007).

Despite these promising attempts, however, some constraints arise since oral administration of bacteriocins delivered directly in a purified or semi-purified form can be challenging (Hammami et al., 2012). One of the main limits is the proteinaceous nature of most bacteriocins that can undergo proteolytic degradation. Constant efforts have been made to improve their physico-chemical properties such as solubility, diffusion and protease resistance, to render bacteriocins more stable and performant. Recent cutting-edge researches use as model lantibiotics. Salivaricin variants with conservative modifications at the trypsin-specific cleavage sites have been obtained using biotechnological strategies and 8 out of 11 variants were resistant to trypsin digestion without losing their antibacterial activity (O'Shea et al., 2010; 2013). Similarly, nisin variants with wider spectrum of activity including both Gram-positive and Gram-negative bacteria were obtained (Field et al., 2015). A possible alternative is the administration of the bacteriocin-synthesizing strain in order to produce the bacteriocin in situ (see the Section "Probiotics").

Resistance. Bacteriocins are promising tools because of the low level of acquired resistance. For instance, nisin has been used for several decades in foods (since 1953) and very low rates of resistance phenomena (frequency of about 10^{-8}) have been described (Delves-Broughton et al., 1996; Nishie et al., 2012). Similar results (frequency of 10^{-8} to 10^{-9}) have been obtained for lactacin 3147 (Guinane et al., 2006). Laboratory experiments aimed to induce resistance only result in the generation of mutants with resistance to very low concentrations of the bacteriocin (Papadimitriou, 2014).

Toxicity and perturbing action. Being targeted especially for the cytoplasmic membrane, bacteriocins display a certain degree of activity (hydrophobic interactions) also for human cells, however, their toxicity towards eukaryotes is generally low (Mazzoli et al., 2014) although no studies have examined the immunogenicity of these peptides or proteins. A second aspect to be considered is the perturbing action of bacteriocins on human microbiota. Actually, as occurs with broad spectrum antibiotics, large spectrum bacteriocins could impact bacteria different from the target infecting agents, thus altering the balance of single species inside complex microbial communities within the human host and possibly causing dysbiosis.

Quorum sensing inhibitors to control virulence

Virulence is the ability of pathogens to produce damage to the host and its attenuation represents an alternate strategy to killing bacteria for combating infections. In this regard, there is extensive research in the inhibition of bacterial virulence. In principle, any virulence determinant could be susceptible of being inhibited, however better inhibition targets are master regulators of virulence, such as the quorum sensing systems (Allen et al., 2014; Muñoz-Cazares et al., 2018).

Quorum sensing (QS) is the ability of monitoring cell density and reprogram gene expression accordingly, for this, bacteria rely in the continuous secretion of signal molecules known as autoinducers. These compounds accumulate outside the cells, and then either diffuse inside other cells and bind cytosolic receptors or bind extracellular receptors that transduce the signal. This led to the activation of transcription of the genes encoding enzymes that synthesize the autoinducers, with a rapid amplification of the phenomenon. In parallel, activation of expression of genes controlling different phenotypes occurs, thus triggering diverse processes including production of several virulence determinants such as exoproteases, toxins, phenazines, components of the biofilm matrix, etc. (Whiteley et al., 2017).

To date the main bacterial models to study QS and their inhibitors in Gram negatives are *Vibrio* spp. and *Pseudomonas aeruginosa* whereas for Gram positives is *Staphylococcus aureus*, and several approaches to inhibit they QS systems had been proved useful to decrease the virulence factor expression in vitro and in vivo in animal infection models (Castillo-Juárez et al., 2015).

QS inhibition, also known as quorum quenching (QQ) can be achieved by interfering with any component of the system, namely (i) by blocking the autoinducer receptors hence interfering with the signal binding, or (ii) by blocking the autoinducer synthases hence decreasing signal production or (iii) by degrading the autoinducers with enzymes (Kalia, 2013; Sikdar and Elias, 2020).

The more explored approach so far is interfering with the receptors, mainly with molecules from natural sources such as brominated furanones (Hentzer et al., 2003), flavonoids (Paczkowski et al., 2017) and several other plant metabolites such as cinnamic acid (Rajkumari et al., 2018) or by synthetic compounds (Borlee et al., 2010). Nevertheless, receptor inhibitors sometimes fail to inhibit the expression of QS-dependent virulence factors of clinical strains and sometimes are extremely toxic to some of them (García-Contreras et al., 2015).

Therefore, recent experimental evidences suggest that autoinducer's degradation using enzymes (such as acylases and lactonases that disrupt the Gram-negative signaling molecules acyl homoserine lactones) is a more robust strategy. By this approach it is possible to achieve higher inhibition percentages and effectiveness towards most of the clinical strains studied so far (Guendouze et al., 2017; López-Jácome et al., 2019).

Furthermore, it is possible to complement both strategies. Actually, recent investigations suggest that the combination of receptor inhibition with enzyme degradation is a much more effective approach than the application of only one agent, since the inhibition exerted is synergic and hence high QS inhibition levels are achieved with lower concentrations of the inhibitors (Fong et al., 2018).

One of the most attractive features of QS inhibition is that, in contrast with the utilization of conventional antimicrobials, it does not affect the growth of bacteria at least in vitro upon cultivation in rich media, and hence it was hypothesized that it won't create selective pressure for the development of resistance (Allen et al., 2014). On the other hand, this notion was challenged by a theoretical work in 2010 (Defoirdt et al., 2010) and by experimental evidence in 2012. It was shown in *P. aeruginosa* that growth in adenosine (whose catabolism is tightly regulated by the master QS regulator LasR) as sole carbon source make growth strongly dependent from QS. This can exert selective pressure and the development of resistance against brominated furanones, the QS interfering molecules. The resistance is based upon efflux with the MexAB-OprM pump that also effluxes several antibiotics. Moreover, clinical isolates non previously exposed to the furanones with and active pump are highly tolerant to QS inhibition by this molecule (Maeda et al., 2012).

An additional concern with QS inhibitors is the risk to affect the composition and function of complex microbial communities since several pathogenic and non-pathogenic bacterial species rely in QS for the regulation of multiple important processes, including some aspects of metabolism (Nguyen et al., 2019; Waheed et al., 2020). As an example, at least for *Escherichia coli*, QS inhibition increases the mutation rate and promote conjugation, thus increasing bacteria chances of becoming resistant against antibiotics and perhaps even to the QS inhibitors used (Li et al., 2021).

To date, the possibility of selecting resistance mechanisms against QS inhibitors is poorly explored. Therefore, it is difficult to truly establish to what extent in physiological conditions in vivo QS inhibitors are effective, compared with antibiotics.

Interestingly, QS has also a strong connection with the expression of defensive mechanisms against bacteriophages. Since it is expected that a viral infection can spread easily causing more damage in populations with high density, bacteria have evolved several QS controlled mechanisms that counteract phage predation in high cell density populations. Among them, a decrease in the expression of some phage receptors such as membrane proteins (Høyland-Kroghsbo et al., 2013; Tan et al., 2015), a lower metabolic rate, that limits phage reproduction (Qin et al., 2017) and a higher expression of defensive mechanisms like the CRISPR-CAS system (Patterson et al., 2016) or the expression of exoproteases that inactivate phages (Hoque et al., 2016) have been described.

This relationship allowed the design of therapies combining QS inhibitors and bacteriophages (Mion et al., 2019). However, it must be considered that a potential side effect of phage therapy could be the selection of QS proficient individuals that are also those with higher virulence. Hence further studies concerning the important role of QS in bacteria/phage relationship are necessary to optimize phage and phage/QS inhibitor combination therapies (Cazares et al., 2020).

Recent evidence of this complex evolutionary relationship is the presence of anti-QS mechanisms encoded by bacteriophages which inactivate QS in order to downregulate the QS mediated anti-phage mechanisms and facilitate phage infections (Shah et al., 2021).

Hence a better understanding of the ecological relationship of bacteria and phage is needed before wide application of QQ to treat infections in the clinic (Cazares et al., 2020).

Plant essential oils

The aromatic and medicinal plants can produce volatile hydrophobic secondary metabolites as a defense against infection, which can be separated by distillation or solvent extraction. They are present in a mixture of several compounds among which major and trace components can be detected and characterized by gas-chromatography and MS (Daferera et al., 2000). They prove to be active against bacteria (Mourey and Canillac, 2002), fungi (Akgul et al., 1991), viruses (Bishop, 1995) and parasites (Pessoa et al., 2002) as well as insects. These compounds, called essential oils (EOs), can be roughly divided into i) terpene-derived and ii) aromatic molecules (Miguel et al., 2010) although a huge number of molecules including alcohols, aldehydes, ketons, ethers and esters are also present (Karpouhtsis et al., 1998).

Mechanism of action

Regarding their mechanism of action, EOs can act at different cell levels. The main target is the cytoplasmic membrane, since EOs hydrophobicity can deeply alter its structure, enhancing permeability, dissipating the proton gradient and proton motive force and finally causing loss of macromolecules. Such conditions strongly compromise several essential cellular functions. Among these, pH

can decrease at values lower than 5.5 thus inhibiting the enzyme catalysis as observed in *E. coli* and *Salmonella typhi* after treatment with mustard oil (Turgis et al., 2009). Amylases, proteases and histidine carboxylase are among the most susceptible enzymes (Thoroski, 1989).

Also toxin production is dramatically stopped, probably due to energy and/or trans-membrane transport limitations, as demonstrated in *S. aureus* after exposure to oregano EO (De Souza et al., 2010). In addition, toxin production can also be limited by interference with the QS mechanism: it has been reported that of lavender, geranium, rose, rosemary and clove EOs (but not orange EO) can act as QQ (Szabó et al., 2010). Flagellin synthesis can also be inhibited by EOs, thus bacterial motility is impaired (Rudramurthy et al., 2016).

Spectrum of action

As a rule, Gram-negative pathogens, possessing an outer membrane that limits diffusion of hydrophobic molecules, are generally more resistant to every antibiotic treatment including EOs (Burt, 2004). Actually, vetiver and sandalwood EOs are completely ineffective against Gram-negatives although possessing high efficacy towards Gram-positive bacteria (Raut and Karuppayil, 2014). However, there are examples in which EOs, although less effective against Gram-negatives than against Gram-positives, can limit the growth of *Salmonella enteritidis* of about 3 logs (as respect to the 7 logs reduction towards *S. aureus*). Furthermore, low concentrations of mint EO (<0.1%, amount able to decrease the number of viable cells of only 2 logs) fortified with glucose can also suppress the production of enterotoxin B (Tassou et al., 2000), probably acting as QQ. Examples are also reported in which the efficacy against Gram-negatives is higher than that towards Gram-positives: in *E. coli* APL 87/1 oregano and clove EOs caused evident holes at cell surface whereas in *Bacillus subtilis* APL 87/35 only light cell surface modification were observed (Rhayour et al., 2003). Similarly, using the MIC of oregano EO, no damages were detected (using TEM and flow cytometry) in *S. aureus* ATCC29213, whereas in *Pseudomonas aeruginosa* ATCC 27853 both growth and the membrane potential were completely inhibited. Again, the EOs from *Salvia* spp. and *Thuya* spp., display better inhibitory activity against *P. aeruginosa* and *K. pneumoniae*, than against *S. aureus* (Jirovetz et al., 2006). These differences are related to different membrane and cell wall composition (Bouhdid et al., 2009). Generally, clove and garlic EOs display high activity towards both Gram positive and Gram negative bacteria (Arora and Kaur, 1999). However, the spectrum of activity can in some case be broadened by adding an acetate moiety to the natural compound, as demonstrated with geranyl acetate versus geraniol (Dorman and Deans, 2000). More in general, EOs proved to be active towards different bacterial species involved in food-borne infections such as *Listeria monocytogenes*, *Bacillus cereus*, *Shigella dysenteriae*, *Salmonella typhimurium* and *Escherichia coli* O157:H7 (Burt, 2004). Inhibitory activity specifically directed towards bacteria involved in pulmonary infections (*Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Haemophilus influenzae* and *P. aeruginosa*) was observed when testing *Achillea clavennae* essential oil, camphor and eucalyptol being the main components (Skocibusic et al., 2004). In particular, the anti-mycobacterial (*Mycobacterium tuberculosis* and *Mycobacterium avium*) action of *Myrtus communis* EO was described by Zanetti et al. (2010). On the basis of these overall data, also considering their historical (Boyle, 1955) and present (Vergis et al., 2015) use as food preservatives, plant-derived EOs can be appreciable tools to be explored for treating infections caused by multidrug resistant microbial strains. Actually, some reports exist that highlight the beneficial action of EOs, especially cinnamon, lemon and lemongrass against methicillin resistant *Staphylococcus aureus* (MRSA) and *Candida* (Warnke et al., 2009) and essential oils from the Australian-grown Tulsi that proved to be active against both *P. aeruginosa* and MRSA (Yamani et al., 2016). In addition, a very interesting feature of EOs (Ginger, Melissa, Sandalwood, Eucalyptus and Tea Tree) is their antiviral activity, towards both DNA and RNA viruses like adenovirus type 3, herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), poliovirus, and coxsackie virus B1 (Schnitzler et al., 2001).

Activity assessment

Different approaches have been experimented to ascertain the antimicrobial effect of EOs in vitro and both qualitative (SEM evaluation of cell morphology alterations) and quantitative methods (MIC, viable cell counts) have been used to assess their antibacterial activity (Burt, 2004). Nevertheless, as normally occurs in antibiotic susceptibility testing, also EOs sensitivity can be affected by the culture medium composition and pH: for instance, carboxylic acids and phenolic compounds are best effective at alkaline pH when both EOs and target molecules are in the protonated form (Janssen et al., 1987).

However, EOs, being a complex mixture of different secondary metabolites should be studied separately and in combination to better elucidate their potential, their possible synergies and antagonisms. As an example the combination of rosemary and clove EOs proved to be synergic against *Candida*, antagonistic against *Aspergillus* and simply additive (identical to the treatment with both compounds used separately) towards the majority of bacteria tested (*Staphylococcus*, *Escherichia* and *Pseudomonas*) (Fu et al., 2007). Interestingly a synergistic effect was also observed combining EOs with bacteriocins (Ettayebi et al., 2000). However, according to other authors, the best antimicrobial action is achieved with the natural EOs from a single plant because trace elements can improve the efficacy by acting as potentiating agents (Mourey and Canillac, 2002). The extraction method has also been referred as crucial for preserving the antibacterial activity of the overall EOs mixture present in the plant: as an example, EOs extracted by hexane support higher antimicrobial efficacy than the steam distilled ones and should be preserved in the dark (Packiyasothy and Kyle, 2002). Furthermore, some constraints arise because the chemical composition of EOs from a certain vegetal species can be affected by the geographical origin, soil, climate and harvesting time (Angioni et al., 2006) and this can influence the antibacterial spectrum of the compounds. Therefore, the accuracy and reproducibility of the experiments intended to evaluate EOs efficacy remain uncertain.

Hence, a standardization procedure to ensure the exact composition of individual molecules in commercially available EOs, together with the high-scale production by metabolic engineering, is the essential requisite to provide effective amounts of beneficial compounds to cope with infection (Carson and Riley, 2001).

Resistance and possible host-directed toxicity

Resistance mechanisms to EOs also exist. Stress proteins such as chaperones (GroEL, DnaK, Trigger factors) protecting cell structures, but especially proteins, from denaturation, were detected by in-gel proteomics in *Salmonella* after treatment with 0.01% thymol (Di Pasqua et al., 2010). However, the main resistance mechanism is linked to fatty acid modifications that decrease fluidity of the cytoplasmic membrane in Gram-positive bacteria (Ultee et al., 2000). On the other hand, also Gram-negative pathogens proved to be resistant to EOs such as the case of *P. aeruginosa* resistant to rosemary EO (Pintore et al., 2002). Finally, in spite of the huge number of studies on EOs, no genotoxic or cytotoxic effect on humans have been described, except specific cases of allergic dermatitis (Burt, 2004).

Surfactants

Surfactants are amphipathic compounds bearing a hydrophilic and a hydrophobic moiety. They can be classified as anionic, cationic, amphoteric and non-ionic, the best performant as antimicrobials being cationic and amphoteric.

The anionic detergents SDS and alkyl-sulfonates are biologically active only at very low pH and at high concentrations, nevertheless some reports highlighted anti-*S. aureus* activity (Johnston et al., 2000) and even antiviral efficacy (Tsujimura et al., 2015) of negatively-charged surfactants. However, resistance phenomena towards anionic surfactants as LAS (linear alkylbenzene sulfonates) have been described (Maehara, 2017).

Similarly, although some effects on cell viability were observed with C10E6 and C12E6 that can be adsorbed on the cell wall or enter the cytoplasmic membrane respectively, in general non-ionic detergents display poor toxicity both towards eukaryotes and prokaryotes (Falk, 2019). Amphoteric surfactants such as Dodecyl-di(aminoethyl)-glycine, are active towards 99.5% of Gram-positive bacteria, and over 99.9%, for most Gram-negative bacteria, including *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella typhi* (Copello et al., 2008).

The historical use of cationic surfactants like quaternary ammonium salts as disinfectant agents opens new perspectives in their possible utilization as antibiotic substitutes. The antimicrobial mechanism of quaternary ammonium compounds possessing alkyl-chains, is linked to the disturbing action they exert on cell membranes, that finally results in loss of intracellular materials, cell lysis and death. For this reason, cationic surfactants are more effective against Gram-positive bacteria that lack an outer membrane, which limits diffusion of hydrophobic compounds, as previously discussed for EOs (Falk, 2019). The antimicrobial activity of a surfactant is mainly linked to the polarity of the cationic head, the higher the hydrophobicity, the higher the antibacterial power. In agreement with this, a greater antimicrobial performance can be obtained at a lower critical micelle concentration since it is related in turn to the hydrophobicity of the head-group. Actually, the more environmentally friendly gluco-cationic surfactants, bearing a polar head, display limited activity, whereas a quinolinium surfactant showed the best antibacterial efficacy (Viscardi et al., 2000). The evolution of surfactants has been guided to obtain better antimicrobial activities and enhanced biodegradability, although these two features are often opposite: actually to hydrolyze these molecules bacteria have to be resistant to their perturbing action at the membrane level. However, the last generation cationic surfactants (polymeric quaternary ammonium compounds) are more effective than the previous ones (Gerba, 2015) and di-quaternary ammonium geminis compounds prepared from N-dodecyl-betaine and gemini surfactants from arginine both display significant antibacterial activity, especially directed towards Gram-positive strains (Shukla and Tyagi, 2006).

Biosurfactants

A special place deserves surfactants of biological origin, named bio-surfactants. The hydrophobic component is a carboxylic fatty acid of variable chain-length and the hydrophilic moiety is a sugar or a peptide. They are generally divided into glycolipids and lipopeptides. As respect to oil-derived compounds which contain hydrocarbons that are degradable only by omega-oxidizing bacteria, these molecules contain fatty acids that can undergo degradation by beta-oxidation and are therefore more eco-friendly. Besides being extensively employed in food, cosmetic and pharmaceutical applications, because of their low toxicity, some of them are excellent antimicrobials (Gautam and Tyagi, 2006). Their possible mechanisms of action are: membrane disruption, interference with QS, inhibition of swarming motility and biofilm formation, inhibition of tissue adhesion (Singh and Cameotra, 2004). As an example, surface active components from *Lactobacillus acidophilus* and *L. fermentum* can prevent biofilm formation on abiotic surfaces (Velraeds et al., 1998) and adhesion of *E. faecalis* on epithelia (Heinemann et al., 2000), respectively.

Surfactins are produced by *B. subtilis*. They are cyclic lipopeptides containing a C14 fatty acid and a short peptide containing valine, leucine or isoleucine plus aspartate and glutamate that confer the anionic properties. They are effective against bacteria (Sabate and Audisio, 2013) mycoplasmas (Vollenbroich et al., 1997a), also displaying antiviral activity specifically targeting the virus envelope such as that of Herpes (Vollenbroich et al., 1997b) and HIV (Jung et al., 2000) viruses. Some constraints arise due to the fact that these compounds, targeting the cell membrane, are not so selective towards prokaryotes. Actually they have been suggested to induce cancer cells lysis (Wu, 2017). However, the toxic concentration against bacteria and host differs. Furthermore, the damage on bacterial cells is higher because bacterial membranes harbor the respiratory chain (that in *Eukarya* is in the mitochondria), therefore, disruption of electron transport and oxidative phosphorylation are the main negative events that ultimately cause bacterial cell death, without strongly affecting host cells (Falk, 2019).

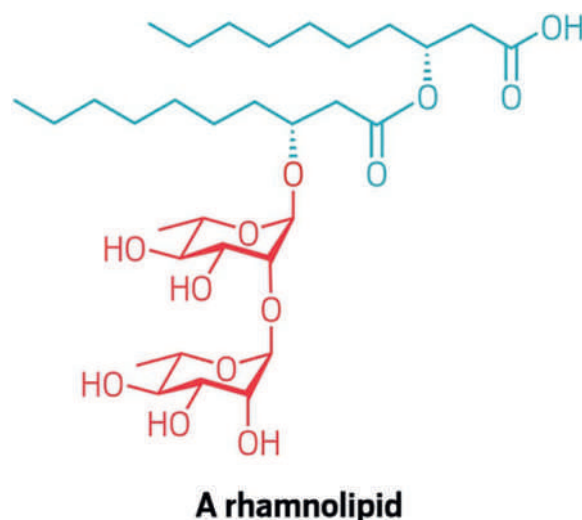


Fig. 5 Molecular structure of the bio-surfactants rhamnolipids.

Massetolides A-H are produced by *Pseudomonas* species of aquatic origin and proved to be active towards *Mycobacteria*, whereas other *Pseudomonas*-derived surfactants are effective against fungi (Gerard et al., 1997).

Rhamnolipids. Rhamnolipids are glycolipids, especially produced by *Pseudomonas* spp., displaying immune modulating and anticancer activities (Fig. 5), that also prove to be good antimicrobials being effective alone and in combination with bacteriocins (Magalhães and Nitschke, 2013). They are generally more effective towards Gram-positives because of the protective effect exerted by the outer membrane on Gram negatives. They have been investigated to control food-borne infections and they proved to be bacteriostatic on *Listeria monocytogenes* and *Bacillus cereus* where about 91% of the cultures was sensitive at pH 7. Good results were obtained on *S. aureus* only at pH 5. However, on *S. aureus* they act mainly as biofilm disruptors due to the decrease of interfacial and surface tension but also as biofilm-preventing agents (Do Valle Gomes and Nitschke, 2012). These data, together with the induction of pro-inflammatory cytokines render rhamnolipids promising agents to treat Gram positive related infections, since no resistance mechanisms against them have been described so far (Thakur et al., 2021).

Metal functionalized nanoparticles (Me-NP)

Metal toxicity

Among cutting-edge technologies explored to control infectious diseases, inorganic ions play a promising role, either alone or in conjugation with (in case coated) functionalized nanoparticles (NP). This is a very recent approach derived from a long history of heavy metals use to kill bacteria. Actually since antiquity copper and silver have been employed at the solid state to prevent infections, due to their toxicity towards microorganisms (Lemire et al., 2013). Copper has been proposed as solid antimicrobial material for bed rails to control nosocomial infections (Grass et al., 2011) and very recently to be used as polymer-linked coating in biomedical applications and healthcare facilities (Mitra et al., 2019). Silver is more effective than clorexidine to prevent dental infections by *S. mutans* (Besinis et al., 2014) whereas iron has been administered as magnetite (Fe_3O_4) in association with cephalosporins to control *Klebsiella* infection. Actually, this metal form can bypass the outer membrane through the siderophore channels therefore acting as “Trojan horse” for transferring nanocoupled antibiotics into the more protected Gram-negative bacteria (Hasanova et al., 2015).

The mechanism of action of metals and Me-NP is summarized in Fig. 6. It consists in alterations exerted at the membrane level, but also in the induction of oxidative stress and related damage to lipids, proteins and nucleic acids (Lemire et al., 2013) and alteration of signal transduction (Muzammil et al., 2018). Furthermore, minor effects concerning enzyme catalysis lie in the perturbing action at the catalytic site level where exogenous metals compete with the catalytic ones or disturb the ligand geometry: this aspect is crucial since most toxins produced by bacteria are enzymes (collagenases, gelatinase, urease etc.) (Ahmed et al., 2016a).

Copper is naturally present in the environment as elemental copper in its zero-valent state (Cu^0) and ionic copper (Cu^+ or Cu^{2+}) (Georgopoulos et al., 2001). As widely reported in the literature, the membrane polarization carried out by copper ions is the main mechanism of copper antimicrobial activity. Generally, the bacterial membrane has an electrochemical potential of 100–200 mV. Cu ions can bind the membrane by electrostatic interaction and causing a depolarization of the cell membrane which results in the rupture or leakiness of material such as cytoplasm (Mitra et al., 2019). The antimicrobial activity of Copper and/or its compounds has been tested on different Gram positive and Gram-negative bacteria such as respectively *Staphylococcus aureus* and *Escherichia coli* (Mitra et al., 2019). Furthermore Cu is active towards viruses such as influenza virus and fungi such as *Candida albicans* and *Fusarium graminearum* a plant pathogenic fungus (Brunel et al., 2013).

AHMED ET AL, 2016a (From ELSEVIER)

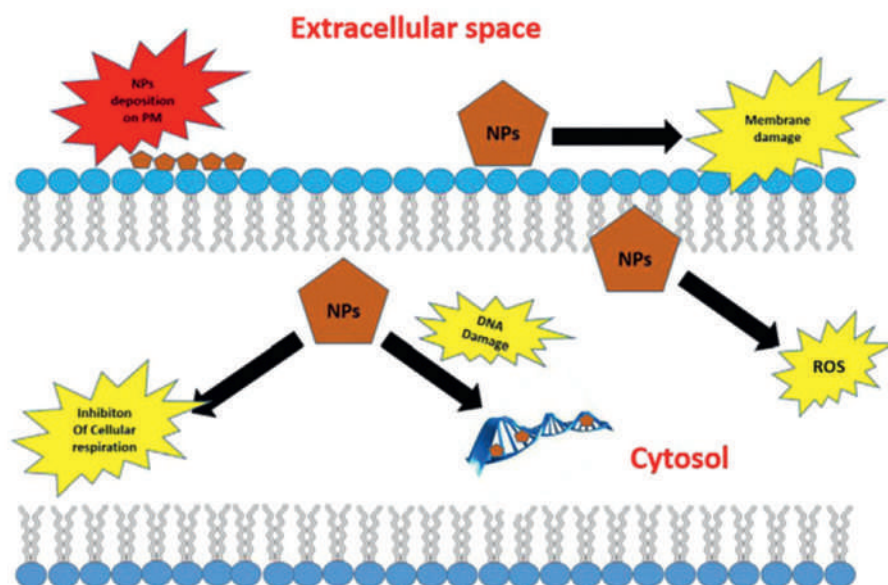


Fig. 6 The different mechanisms of action of metal nanoparticles. From Ahmed KBA, Raman T and Veerappan A (2016) Future prospects of antibacterial metal nanoparticles as enzyme inhibitor. *Materials Science and Engineering: C* 68, 939–947, Elsevier.

Silver, the best studied antimicrobial metal, has been traditionally used as topical agent to treat local infections. Silver is currently used on healthcare devices to inhibit bacterial colonization of several abiotic surfaces and to prevent biofilm formation (Ansari et al., 2015). It displays similar mechanism of action of Cu with the advantage to be active against several bacteria (including MRSA) at very low concentrations not effective against humans: actually reduction of eukaryotic cell viability only occurs at concentration 16 fold higher than the MIC (Chen et al., 2017).

Metal NP

Nanoscale materials have emerged as novel antimicrobial agents since the small particle dimensions result in a high surface area to volume ratio that ensures improved properties to control bacterial growth (Xia et al., 2008). Antimicrobial nanomaterials, especially several classes of antimicrobial NPs, act as carrier for antimicrobial agents and the nanoparticle shape and size also influence their internalization into bacteria cells (Makvandi et al., 2020). Metal nanoparticles such as Ag₂O and TiO₂ have been used as antimicrobial materials in the food industry (Makvandi et al., 2020).

Both silver and copper NP can be considered important antimicrobial agents because they are effective towards a wide range of microorganisms including fungi, viruses and multidrug-resistant bacteria (Ingle et al., 2014). Regarding the efficacy of Cu-NP and Ag-NP, controversial results have been obtained, in part depending upon the bacterial species considered, in part from the investigating conditions. From one side Ag-NP proved to be more effective than Cu-NP against *S. aureus* and *E. coli*, whereas *B. subtilis* proved to be more sensitive to Cu-NP (Ruparelia et al., 2008). In another study, copper seemed to have some advantages: actually, both *B. subtilis* and *E. coli* are sensitive to Ag-NP and Cu-NP, however while 70 µg/mL of Ag-NP were necessary to completely inhibit the two species, for Cu-NP 60 µg/mL were sufficient (Yoon et al., 2007). Therefore, in the following paragraphs, the two Me-NP will be treated separately.

Ag-NP. Ag-NP are the most frequently employed in several medical applications such as dentistry, cardiovascular implants, bone regeneration, tissue engineering and as a biocompatible nanomaterial for producing wound healing bandages useful to contain the growth of MDR *P. aeruginosa* and *S. aureus* isolated from post-surgical wound infections (Kasithevar et al., 2017). They have also excellent antiviral properties against HIV-1 (Makvandi et al., 2020). The multilevel mechanism of action of silver NP has been extensively reviewed (Franci et al., 2015; Le Ouay and Stellacci, 2015). Ag-NP can release a huge amount of Ag atoms (about 1000) that can be oxidized to Ag⁺ ions, the true effectors of the biological damage. More in detail the Ag⁰ core seems to be responsible for the membrane integrity disruption whereas the Ag⁺ ion can form stable molecular adducts with a large variety of group (thiols, amines, phosphates) ultimately resulting in irreversible inactivation of cellular macromolecules (Le Ouay and Stellacci, 2015). Ag-NP proved to be effective against MDR *M. tuberculosis* (Seth et al., 2011) and display synergistic activity with antibiotics. Resistant

patterns were reverted into sensitivity when Ag-NP were combined with beta-lactams (Singh et al., 2013), trimethoprim and vancomycin (Naqvi et al., 2013). A further interesting aspect of Ag-NP is their efficacy in counteracting bacterial biofilm lifestyle (Franci et al., 2015). In particular, while free silver ions account for the damage to planktonic cells, integral Ag-NP can better control biofilm (Le Ouay and Stellacci, 2015). Actually, free Ag⁺ ions by forming chemical bonds with organic groups (amines, carboxylates, thiols) present in the extracellular polymeric substance in which the bacterial cells are embedded remain entrapped and are poorly effective towards biofilm-living bacteria, while Ag-NP can easily diffuse and penetrate into the biofilm core thus releasing free silver ions, the true effectors targeting the bacterial cells (Le Ouay and Stellacci, 2015). This is an appreciated feature since biofilm embedded bacterial cells poorly respond to antibiotic therapy: they are protected from antimicrobials (and antibodies) by the extracellular polymeric substance that prevents solute diffusion, and also “persister cells” appear, which by living in a non-growing state are insensitive to the antibiotic molecules (Pessione, 2021). Both citrate-capped (Habash et al., 2014) and starch-prepared Ag-NP (Mohanty et al., 2012) are able to inhibit or disrupt the biofilm of *P. aeruginosa* one of the most problematic nosocomial pathogens causing chronic infections on both abiotic (cardiac valves, catheters, prosthesis) and biotic (lung, bladder and other epithelia) surfaces. Furthermore, evidence of the capability of low Ag-NP concentrations (50 µg/mL) to inhibit biofilm formation by methicillin resistant *S. aureus* (MRSA) has been assessed by confocal laser scanning microscopy (CLSM) (Ansari et al., 2015). Anti-Staphylococcal and anti-Pseudomonas activity of Ag-NP at clinical compatible concentrations was also demonstrated together with anti-biofilm activity, with the possibility to further reduce the Ag-NP concentration by synergistic combination with antibiotics (oxy-tetracycline, amikacin, kanamycin, and streptomycin) (Barapatre et al., 2016). An additional and interesting approach to eradicate MRSA biofilms is the use of silver-coated superparamagnetic iron oxide nanoparticles (SPION) whose magnetic core can respond to external magnetic fields thus improving the anti-biofilm performances in the absence of antibiotics (Mahmoudi and Serpooshan, 2012).

Cu-NP. Copper in a form of nanoparticles has been shown to interact with the microbial cell wall because of its affinity with the carboxyl groups present on the microbial surface (Ingle et al., 2014) and causes cell death. This phenomenon is called as “contact killing.” Zero-valent Cu-NP have been demonstrated by SEM to form micro-cavities in *E. coli* cell wall which becomes thick and coarse (Raffi et al., 2010). Interestingly Cu-NP can cause degradation of viral proteins (SDS-PAGE evaluation) such as hemagglutinin and neuraminidase finding application in very up-to-date tools such as filters and face masks (Fujimori et al., 2012). Finally, a further advantage of Cu-NP is that copper is less expensive than silver.

Controversial aspects

Some adverse effects of metals can be exerted also towards the human host and are dependent on the concentration. It is well known that physiological cations such as iron, copper, zinc and manganese that are normally present as catalysts in the active site of most enzymes can be toxic at very high doses, whereas toxic metals like silver and selenium are useful at low concentrations. NP can accumulate in the respiratory tract depending on their size, thus causing oxidative stress and inflammatory responses (Madl and Pinkerton, 2009). Systemic distribution of particles is favored by their ability to diffuse through both cellular and intercellular barriers which is partially determined by particle surface charge (Chang et al., 2012) solubility (Ameh and Sayes, 2019) and size. Small size Cu-NP can translocate and enter blood circulation accumulating in other organs (Lee et al., 2016). Unfortunately, smaller sized nanoparticles (i.e. 10 nm) displaying a better antibacterial activity (as respect to larger sized particles (100 nm)) are those exhibiting the higher risk (Padil and Cernik, 2013).

Several approaches have been proposed to reduce adverse effects of metal and metal oxide NP. Immobilization in polymeric matrices such as alginate, chitosan, polyethylene glycol (Makvandi et al., 2020), or also human serum albumin (Seth et al., 2011) can change the interaction with the mammalian cells meanwhile providing release of antimicrobials and a good hydration useful for chronic ulcers and burns re-epithelization (Makvandi et al., 2020). β-cyclodextrin is an effective capping and stabilizing agent able to decrease NP toxicity towards human cells (Jaiswal et al., 2015). Furthermore, green chemistry strategies also promote better biocompatibility: indeed, algae- (Makvandi et al., 2020) as well as plant extract- (Ahmed et al., 2016b) mediated synthesis allow to obtain metal NP with lower cytotoxicity. An interesting strategy for the synthesis of Ag-NP from AgNO₃ enzymatic reduction using lignin-degrading fungi has been proposed. The ecofriendly process allowed to obtain hexagonal-shaped Ag-NP with high efficacy towards clinical pathogens and with improved biocompatibility (Barapatre et al., 2016).

Nevertheless, environmental aspects have to be taken into account before use. Actually, environmental released NP undergo transformations such as aggregation, sedimentation, dissolution, adsorption and complexation that facilitate persistence, bioavailability and bioaccumulation in the food web (Ameh and Sayes, 2019). Although at present the trophic transfer of NP through the food chain is poorly explored (Gardea-Torresdey et al., 2014), possible induced toxicities in several living organisms like sea urchins (Torres-Duarte et al., 2016), fish (Griffitt et al., 2007) phytoplankton (Bielmyer-Fraser et al., 2014) and terrestrial plants (Hong et al., 2015; Zhao et al., 2016) have been reported. As far as gut microbiota is concerned, preliminary data highlight modifications induced by Ti-NP on some phenotypic patterns, especially growth profiles and biofilm forming attitude, of *Lactobacillus* and *Enterococcus* (personal unpublished results), however a true systemic evaluation on the gut ecosystem as a whole is very difficult to achieve and further research by using innovative approaches is needed. Due to all these possible environmental risks, understanding of the processes underlying their biological activity deserve more in depth investigations and critical considerations. However, at present, metal-NP-based antimicrobials for systemic use are far to find full application in therapy, and clinical trials are currently underway. Therefore, it can be concluded the use of metal-NP in therapy beyond to the safety limits should be carefully evaluated to avoid severe cytotoxicity.

A second aspect to be considered is the acquisition of resistance by bacterial populations. Different mechanisms of resistance to metals are known. First, the production of extracellular or intracellular compounds like proteins, the sulfur-rich metallo-thionines, having a chelating effect on cations. Second, the capability to change the redox state of the metals converting the dangerous forms (ions) into milder ones (elemental state). Third the existence of modified uptake systems (with low affinity for the toxic metal) or efflux systems for metals similar to those used to extrude antibiotics or other toxic compounds (Lemire et al., 2013). Finally, the possibility to use the metal for dissimilatory reduction pathways during anaerobic respiration also constitutes a factor of metal resistance. However, the conjugation with NP render all these mechanisms difficult to achieve, although it should be taken into account the possibility of adduct formation with environmental cations, amines and chlorides (Le Ouay and Stellacci, 2015). As far as Me-NP are concerned a peculiar resistance mechanism has been described in Gram-negative bacteria: *E. coli* with mutations in the porin channels involved in Ag-ions transport proved to be less sensitive to AgNP (Li et al., 1997).

Other antimicrobial strategies

To conclude this report, some less explored approaches should be mentioned. Additional antibacterial compounds include immunomodulators (Cheng et al., 2014) capsaicin (Marini et al., 2015) prodigiosin that induces bacterial autolysis (Danevcic et al., 2016) the biofilm inhibiting emodin from aloe (Xiang et al., 2017) and EPS. The latter have recently received increased attention both for their immune-stimulating properties, and for their direct antibacterial effect. EPSs immunomodulatory effect could be due both to their binding to toll-like receptors (TLR) on immune cells and to their prebiotic activity, promoting gut microbiota growth and diversity and, therefore, indirect immune activation (Chaisuwan et al., 2020). As far as the direct antibacterial activity is concerned, three antibacterial mechanisms have been proposed for EPS: (i) suppression of nutrients uptake and microbial growth through metal chelation, (ii) surface ionic interaction resulting in cell wall disruption, and (iii) inhibition of mRNA and protein synthesis (Tokura et al., 1997). EPSs from *Lactobacillus kefiranoferiens* DN1 showed an inhibiting activity against the growth of *Listeria monocytogenes* and *Salmonella enteritidis* (Jeong et al., 2017), while EPSs from *Lactococcus lactis* F-mou inhibited *Enterobacter cloacae*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Bacillus cereus*, *Staphylococcus aureus*, *L. monocytogenes*, and *Candida albicans*, (Nehal et al., 2019). Finally, there is evidence of antibacterial activity against *E. coli* and *S. aureus* from *Leuconostoc pseudomesenteroides* dextran (Ye et al., 2019). Furthermore, a very recent and interesting report highlights the antibacterial potential of cannabidiol against *S. aureus* infections (Blaskovich et al., 2021).

Concluding remarks

To face the current challenge of multidrug resistance towards the main classes of antibiotics, several alternative strategies have been proposed, and most of them have been explored in this chapter. Most of the molecules described originate from the bacterial world (bacteriocins, antimicrobial peptides, biosurfactants, quorum quenchers, prodigiosines and exopolysaccharides). However, some compounds are derived from plants (e.g. essential oils) or could also be obtained by chemical synthesis (surfactants, AMP, QQ). Table 1 summarizes the main targets and adverse effects. Most benefits are linked to their activity at very low concentrations, nevertheless, some controversial aspects still arise because of the chemical nature of the proposed compounds, which cannot find application in every body district (e.g. surfactants, highly hydrophobic molecules), and because only studies over the long run can fully ascertain the absence of resistance phenomena or environmental safety.

A special mention deserves the use of phages and probiotics: these are living organisms that can deeply alter the endogenous ecological niche such as the gut micro habitat and have profound consequences on the overall health homeostasis. A promising approach is the use of compounds limiting bacterial virulence (adhesion, biofilm formation, persistence, toxin production) without killing microbial cells: in this case the effects on the ecosystems, appear to be negligible, at least for biosurfactants, since recent reports on QQ highlight selective pressure on microbial communities (Waheed et al., 2020) and altered microbial diversity (Liu et al., 2020).

Taken together, all these considerations suggest that when a choice is necessary among these alternative compounds, the same criteria used for antibiotics should be employed: good activity in the target tissue, selectivity and low toxicity towards humans, limited induction of resistance phenomena and perturbing effects on the endogenous microbiota, immune system and overall ecosystems. At the same time it is of primary importance to avoid incorrect use, if not, just few years might be necessary to lose this second opportunity.

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Antimicrobial Prophylaxis: Rules of Conduct in Typical Infections

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Antimicrobial prophylaxis is used to prevent infectious diseases, the occurrence of which are associated with a threat to human health and life. This approach can be applied to the entire population or to selected social groups that are particularly susceptible to the effects of a disease. The implementation of anti-infective prophylaxis is important for an individual patient and for the entire population (Enzler et al., 2011).

Antimicrobial prophylaxis can be realized as preexposure (PrEP) in a situation where it is administered prior to potential contact with an infectious agent, or postexposure (PEP) when it is administered after exposure to an infectious agent. Various methods of therapy are used in both methods of administration (Davey et al., 2015):

- chemoprophylaxis based on the use of chemical drugs;
- immunoprophylaxis including the use of appropriate protective vaccinations;
- infusions of specific or non-specific immunoglobulins.

Antimicrobial prophylaxis may be considered primary (prevention of an initial infection) or secondary (prevention of the recurrence or reactivation of an infection), or it may be administered to prevent infection by eliminating a colonizing organism. The essence of prophylaxis is the fact that it should eliminate effectively the risk of infection, be non-toxic for the patient, not generate bacterial resistance in the population, and be low cost (Voit et al., 2005).

There are several risk factors for a particularly severe course of infection in selected groups of patients. The most frequently mentioned are: extremes of age, malnutrition and obesity, coexistent infections, immunosuppression, corticosteroid therapy, diabetes mellitus, and recent medical procedures—in hospitalized patients, the length of hospitalization and known colonization with resistant bacteria are crucial. Choosing the right prophylactic method is an important element of patient care, and each case should be considered in terms of effectiveness and the specific patient group.

Preexposure prophylaxis

Chemotherapy

PrEP chemoprophylaxis means using antibacterial or antiviral drugs in negative patients before potential exposure to infectious agent. In particular, prevention is dedicated to selected groups of high-risk patients, who include:

- HIV-infected patients;
- solid organ transplant recipients;
- hematopoietic cell transplant (HCT) recipients;
- patients before/after surgical interventions;
- health care workers.

The physician supervising patients from the above-mentioned groups should monitor the regional recommendations for prophylaxis on an ongoing basis.

Preexposure prophylaxis in HIV

For HIV-negative people who are at high risk for acquiring HIV and are committed to medication adherence, PrEP is a highly effective method of prevention. It can reduce the risk of viral transmission in more than 90% of patients (Anderson et al., 2012).

Candidates for HIV-PrEP are adults and adolescents who are at high risk of acquiring HIV. These individuals are from the following groups:

- men and women without HIV who have a sexual partner with HIV with a detectable viral load in their blood. However, PrEP can be advised to all HIV-negative individuals who have a serodiscordant partner: There is a very low likelihood of transmission if the partner with HIV has stably suppressed plasma HIV RNA and is adherent to their antiretroviral therapy (LeMessurier et al., 2018);
- men who have sex with men (MSM) and transgender women who have sex with men if, within the last 6 months, they have engaged in high-risk sexual behaviors (e.g., condomless anal sex with multiple or anonymous sex partners or a main partner with HIV risk factors) and a documented bacterial sexually transmitted infection (STI);
- heterosexually active HIV-negative men who have condomless sex with female partners from regions with generalized HIV epidemics, where the prevalence of HIV is $\geq 3\%$ according to the World Health Organization (WHO, 2016a,b) recommendations. However, the new guideline panels presented in 2018 suggest a lower prevalence, namely $\geq 2\%$ (Saag et al., 2018);
- injection drug users who have reported sharing needles/equipment (within the last 6 months), if they have initiated substance use treatment (WHO, 2016a,b);
- heterosexual men who have, in the last 6 months, been diagnosed with a bacterial STI or have engaged in condomless sex with female partners from areas of low general HIV prevalence but who are at high risk of HIV infection (e.g., sex workers, injection drug users; WHO, 2016a,b).

In the United States, HIV PrEP was approved by the Food and Drug Administration (FDA) for use in adolescent HIV-negative individuals (Gilead Sciences, 2018). The reason for this decision was the rapidly growing rate of HIV infection among young MSM in recent years.

Proposed procedure and regimen

The first medication regimen approved by FDA and recommended for PrEP for all populations is a one-dose combination of tenofovir disoproxil fumarate (TDF) 300 mg co-formulated with emtricitabine (FTC) 200 mg. Adverse reactions like mild gastrointestinal disturbances (e.g., nausea, diarrhea), which can resolve in first weeks of therapy, have been described in some clinical trials. However, the patients should be observed for potential risk of renal and bone toxicity, especially for long-term use (Centers for Disease Control and Prevention (CDC), 2017a,b).

The second option approved by FDA (2019) is also a one-pill therapy, namely tenofovir alafenamide (TAF) 25 mg co-formulated with emtricitabine 200 mg, but this combination is not recommended for adults and adolescents whose main risk is vaginal, receptive sex. The TAF-FTC combination has better renal and bone safety than TDF-FTC, but it can be associated with dyslipidemia and mild weight fluctuations (Hare et al., 2019).

Solid organ transplant recipients

Solid organ transplant recipients are a high-risk group that can develop different infections—viral, fungal, and bacterial—but their individual risk depends on the type of transplanted organ, type of immunosuppression used, and the pathogens existing in their environment (Husain et al., 2016). The most important sources of infection in solid organs transplants are:

- reactivation of latent infections carried by the solid organs transplant recipients or the organ donor. The most common pathogens that can produce reactivation are *Cytomegalovirus* (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), varicella-zoster virus (VZV), *Toxoplasma gondii*, *Strongyloides stercoralis*, and *Trypanosoma cruzi*. All recipients and donors should be tested before the transplantation to establish the best prevention procedure. In many countries, pre-transplant procedures are regulated by internal recommendations; therefore, doctors caring for this group of patients should be kept informed about the current recommendations;

- new colonization by pathogens typical for the hospital environment (Fishman, 2017). Colonizing organisms may include antimicrobial-resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, carbapenem-resistant *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Aspergillus* spp., *Stenotrophomonas maltophilia*, and others. In such a situation, the most important method of protection is to treat the actual colonization taking into account local recommendations, but above all the regional sensitivity of microorganisms and the support of the patient's resistance tests for individual microorganisms (Pouch and Patel, 2019);
- opportunistic, environmental infections. Solid transplant recipients are sensitive to opportunistic infections, especially in the early post-transplant period. However, the difficulty in eradicating such infections is increased in the immunocompromised host.

Proposed procedures

Pneumocystis jirovecii pneumonia

The incidence of *Pneumocystis jirovecii* pneumonia (PJP) (Brakemeier et al., 2018) in solid transplant-recipients was estimated from 10% to 50% depending on the transplanted organ (Rodriguez and Fishman, 2004). The usual prophylactic drug is trimethoprim-sulfamethoxazole (TMP-SMX) ordered as a single dose 15–20 mg/kg/day, 3–7 times a week. In many programs, prophylaxis is continued for 6 months to 1 year. The extent of PJP prophylaxis can be offered to patients receiving prolonged higher degrees of immunosuppression, lung transplant recipients, or patients with recurrent respiratory tract infections (Iriart et al., 2015). Patients with confirmed hypersensitivity to TMP-SMX can be given the alternative PJP prevention with dapsone, pentamidine, or atovaquone (Rodriguez and Fishman, 2004). TMP-SMX prophylaxis is low cost, has low toxicity, and is effective in preventing many gastrointestinal, urinary, and respiratory tract infections caused by *T. gondii*, *Listeria* spp., *Cystoisospora belli*, and *Nocardia* spp.

Toxoplasmosis

Toxoplasmosis is uncommon but is a highly morbid infection after organ transplantation. The group with the highest risk of symptomatic infection is patients after heart transplantation, especially seronegative recipients of hearts from seropositive donors, whose risk of infection in the absence of prophylaxis is approximately 50–70%. The infection can present as cardiomyopathy, myocarditis, brain abscess, pneumonitis, and disseminated disease (Khurana and Batra, 2016).

The method for routine prevention of toxoplasmosis has not been stated; therefore, testing donors for toxoplasmosis, especially before heart transplantation, is especially important. Many centers suggest that seronegative recipients of seropositive cardiac transplants receive prophylaxis from 6 weeks to 6 months. In some procedures, lifetime prophylaxis is preferred (Derouin, Pelloux, and ESCMID Study Group on Clinical Parasitology, 2008). The basic drug used in prophylaxis is TMX-SMX due to its wide prevention profile (see above). In high-risk situations, combination therapies of TMX-SMX with dapsone are considered (Kittleson and Kobashigawa, 2013). Most alternative regimens (sulfadiazine, dapsone, atovaquone, clindamycin in combination with pyrimethamine or primaquine) have not been well studied in high-risk transplant populations; however, experiences based on other patient groups seem to be promising (Kittleson and Kobashigawa, 2013).

Cytomegalovirus

CMV may be acquired by solid transplant recipients from the donated organ, blood products transfused from a seropositive donor, or reactivation of endogenous infection. The highest risk of infection is seronegative recipients who receive transplanted organs from CMV-seropositive donors and CMV-seropositive recipients who require antilymphocyte antibodies in their therapy.

Many regional scientific associations recommend obligatory routine prophylaxis with ganciclovir or valganciclovir for high-risk recipients. The duration of antiviral therapy generally ranges from 3 to 12 months and often depends on the type of organ transplanted, the specific risk status of the patient, and individual institutional practice (Kotton et al., 2018).

Herpes simplex and varicella-zoster viruses

People who do not receive CMV prophylaxis should receive HSV and VZV prevention during the first 3–6 months after transplantation and during periods of lymphodepletion for treatment of rejection. HSV-specific prophylaxis should be considered for all HSV-1- and HSV-2-seropositive organ recipients who are not receiving antiviral medication for CMV prevention (Lee, Zuckerman, and AST Infectious Diseases Community of Practice, 2019).

Hematopoietic cell transplant recipients

The antimicrobial prophylaxis in HCT recipients is divided into:

- primary prophylaxis—to prevent infection in patients at increased risk;
- secondary prophylaxis—to prevent recurrent infection;
- preemptive therapy—starting antimicrobial therapy based on routine screening with sensitive assays (mostly PCR) to detect early some pathogens before the symptoms of infection and avoid progression to an invasive disease (Tomblyn et al., 2009).

Antimicrobial prophylaxis should be initiated at the beginning of the risk period, typically with the conditioning regimen or at the time of stem cell infusion. In the case of myelosuppressive antimicrobials, such as SMX-TMP, prophylaxis should begin at the time of engraftment and should be continued throughout the period with the highest risk of infection (Tomblyn et al., 2009).

Proposed procedures

Before engraftment

The decision about antibacterial prophylaxis depends on the type of HCT. Many authors suggest using a fluoroquinolone in high-risk neutropenic patients including allogeneic HCT recipients because the method reduces the risk of life-threatening infection. Risk is lower in autologous HCT recipients because of the shorter and less severe conditioning regimen; the decision about prevention is based on the analysis of individual case (Signorelli et al., 2020).

After engraftment

Antibacterial prophylaxis can be stopped at neutrophil engraftment, however, allogeneic HCT recipients who develop chronic graft-versus-host disease (GVHD) and have higher risk of severe infections with encapsulated bacteria (mainly *Streptococcus* spp.) may require prolonged administration of such prophylaxis. In such a situation, selection of the appropriate antibiotic should be based on local epidemiological recommendations, taking into account possible drug resistance (Frère et al., 2002).

Antifungal prophylaxis

HCT recipients are particularly susceptible to the occurrence of invasive fungal infections, especially *Candida* spp. and *Aspergillus* spp., which can be observed mainly in the pre-engraftment period. The choice of regimen depends on the type of transplant performed, possible primary patient infections, the patient's tolerance to the therapy, and possible interactions with the applied peri-transplant treatment.

In myeloablative allogeneic HCT recipients without special indications for mold prophylaxis and in autologous HCT recipients, who can develop severe mucositis, using oral fluconazole is recommended. For patients who need anti-mold prophylaxis, voriconazole and posaconazole are favored (Wingard et al., 2010).

Pneumocystis jirovecii pneumonia (PJP)

PJP prophylaxis should be recommended for both allogeneic and autologous HCT recipients. It can be started after the neutrophil engraftment and continued during the whole immunosuppressive therapy, typically for 6 months, but for even longer in patients who develop GVHD (Redjoul et al., 2019). The preferred regimen for PJP is TMP-SMX, based on its effectiveness in other infections. It is continued until immunosuppressive therapy is completed.

Toxoplasmosis

Toxoplasmosis is a rare parasitic infection in HCT recipients, mainly in the allogeneic group. Prophylactic administration of TMX-SMP, as with PJP prophylaxis, simultaneously protects patients against *T. gondii* infection. Alternative therapy with pyrimethamine, sulfadiazine with leucovorin can be considered in patients with contraindications for TMX-SMP, however, their use has not yet been sufficiently researched (Gajurel et al., 2015).

Mycobacterium tuberculosis

This prophylaxis is recommended only for patients with a high risk of tuberculosis (TB) reactivation after HCT, like people who:

- had been exposed to individuals with active pulmonary or laryngeal tuberculosis, regardless of the HCT candidate's or recipient's tuberculin skin test (TST) or interferon-gamma release assay (IGRA) status;
- have a positive TST result, regardless of TB vaccination status, without previous treatment for latent TB and with no evidence of active TB;
- have a positive IGRA result, without previous treatment for latent TB and with no evidence of active TB.

In these groups of HCT patients, prophylaxis with isoniazid can be used. Because of drug interactions with mold-active azoles, which can lead to increased toxicity, concomitant drug administration should be avoided (Lewalle et al., 2019).

Cytomegalovirus

The risk of CMV reactivation depends on the type of HCT (significantly higher in allogeneic than in autologous HCT recipients) and the serological status of the donor and recipient (the lowest is for transplantation from a CMV-negative donor to a CMV-negative recipient and the highest if for seropositive recipients; Holt, 2019). The risk factors are also using antithymocyte globulin and/or a high dose of steroids and GVHD development (Ljungman et al., 2019).

Primary prevention is realized by selection of a CMV-seronegative donor for CMV-seronegative recipients when possible and using blood products from seronegative donors or products that are depleted of leukocytes. Another method of prophylaxis that is preferred by many physicians is preemptive viral identification by routine PCR testing for CMV and HCT recipient monitoring.

CMV prophylaxis can be realized with a variety of drugs, including ganciclovir, valganciclovir, letermovir, foscarnet, acyclovir, and valacyclovir. The most common procedures are:

- ganciclovir 5 mg/kg IV every 12 h;
- valganciclovir 900 mg orally twice daily for patients who tolerate oral therapy, especially, if they have low risk for CMV disease and low viral loads;
- foscarnet 60 mg/kg IV every 8 h is an alternative for patients who cannot take ganciclovir or valganciclovir.

Pre-emptive therapy is continued for a minimum of 2 weeks with weekly PCR monitoring. If CMV viremia is no longer detected, treatment can be stopped but monitoring is continued. If CMV is still detected at 2 weeks but the viral load is declining, maintenance dosing should be given until CMV is no longer detectable (Ljungman et al., 2019). The common side effects of ganciclovir and valganciclovir are renal dysfunction and neutropenia.

Herpes simplex virus

All HCT recipients who are seropositive for HSV-1 or HSV-2 should get antiviral prophylaxis during the early transplant period. It usually begins when the conditioning regimen is started and continues until engraftment or until mucositis resolves, usually up to 30 days after the transplant. Acceptable options for HSV prophylaxis include:

- intravenous acyclovir 5 mg/kg every 12 h or 250 mg/m² every 12 h;
- oral acyclovir 400 or 800 mg twice daily;
- oral valacyclovir 500 mg twice daily (Tomblyn et al., 2009).

Varicella-zoster virus

VZV is an important factor that leads to severe diseases mostly due to reactivation of endogenous virus in seropositive recipients during the first year after HCT. All VZV-seropositive allogeneic and autologous HCT recipients should be given an oral prophylaxis regimen of valacyclovir (500 mg twice daily) or acyclovir (800 mg twice daily) for 1 year following transplantation. Patients who cannot take oral medications should be given acyclovir 5 mg/kg IV every 12 h (Tomblyn et al., 2009).

Epstein-Barr virus

EBV reactivation usually results from endogenous reactivation or transmission from the allograft and may be asymptomatic, cause a mononucleosis syndrome, or progress to a life-threatening EBV-related post-transplantation lymphoproliferative disorder. Patients with high risk for EBV reactivation should be monitored weekly by PCR on the day of transplantation and until 3 months after the procedure. The prevention strategy includes reduced immunosuppression, administration of the anti-CD20 monoclonal antibody, and administration of EBV-specific cytotoxic T cells (Tomblyn et al., 2009).

Human herpes virus 6

Reactivation is most frequent within the first month following HCT, however, specific prophylaxis against HHV-6 is not recommended because of the low risk of symptomatic disease and toxicity of antiviral drugs.

Preexposure prophylaxis in surgery

Surgical site infections (SSIs) are a common problem in infections connected with health care. The official criteria set by CDC (2017a,b) define an SSI as an infection related to an operative procedure that occurs at or near the surgical incision within 30–90 days if prosthetic material is implanted during surgery. The human and financial costs of SSIs are actually growing; therefore, it is extremely important to carry out PrEP to reduce risks (Borchardt and Tzizik, 2018). Surgical wound classification has been developed for infectious risk assessment, perioperative protocol development, and surgical decision-making (Table 1) (Ortega et al., 2012).

Antimicrobial prophylaxis prevents SSIs by reducing infective microorganisms at the surgical site during the operative procedure, so it is administered when there is a higher risk of an SSI. The patient-related risk factors for SSI are: age, obesity, cachexia, tobacco use, immunosuppression, corticosteroid therapy, prolonged hospitalization, coexistent infections, and antibiotic therapy.

Proposed procedures

The best time for prophylactic antibiotics is 1–2 h before initial incision. Because the time frame during which antibiotics can be given is very short, the risk of *Clostridium difficile* development and drug resistance is low. The first-line prophylaxis is cefazolin, which has a wide spectrum against the organisms often found in surgery. The antibiotic is active against streptococci,

Table 1 Surgical wound classification for infectious risk assessment.

<i>Wound classification</i>	<i>Definition</i>	<i>Risk of SSI</i>
Clean	Uninfected operative wounds without inflammation, without opening viscus and the wound is closed primarily.	1–5%
Clean-contaminated	Uncontaminated unusually operative wounds in which a viscus is entered under controlled conditions.	3–11%
Contaminated	Open, fresh accidental wounds, operations with major breaks in sterile technique, or gross spillage from a viscus. Wounds in which acute, nonpurulent inflammation was encountered also are included.	10–17%
Dirty	Old traumatic wounds with retained devitalized tissue, foreign bodies, or fecal contamination or wounds that involve existing clinical infection or perforated viscus.	Over 27%

Adapted from Ortega G, Rhee DS, Papandria DJ, et al. (2012) An evaluation of surgical site infections by wound classification system using the ACS-NSQIP. *Journal of Surgical Research* 174(1): 33–38.

methicillin-sensitive *S. aureus*, and many gram-negative organisms (Leaper and Edmiston, 2017). Alternative prophylaxis includes intravenous vancomycin and clindamycin, which are not used routinely and are left only for selected situations like a patient colonized by MRSA or at a high risk of MRSA colonization (Peel et al., 2019).

Antibiotics should be started in standardized doses at least 60 min before surgical incision to optimize the adequate tissue concentration at the time of the initial incision (the half-life of antibiotics should be considered). If prophylaxis is continued after surgery, the duration should not be longer than 24 h (Berríos-Torres et al., 2017). The choice of antibiotic and its dosages for PrEP depends on the type of surgery and recommendations of national scientific societies.

Preexposure vaccinations

Preexposure prophylaxis realized with vaccinations is used for people who have a high risk of infection, commonly connected with job-related activities or traveling to some countries. The prevention strategies should be individualized according to specific environmental conditions, preexisting health factors, and behaviors that are unique to the patient. Many countries have special vaccination programs specifically for health care workers (Maltezou et al., 2019).

Depending on the type of pathogen, the risk may be:

- continuous, for example, people with an occupational risk of infection, such as employees of microbiological and virological laboratories and health professionals;
- temporary, related to traveling to areas with high incidence of some infections or poor access to post-exposure medical procedures, among other factor (Freedman and Chen, 2019).

The vaccination procedure must be safe for the patient and highly immunogenic. Table 2 presents the proposed procedures.

Preexposure immunoglobulins

The use of exogenously produced immunoglobulins to prevent infectious diseases has a wide range of possibilities. In nature, such protection concerns the fetus and then the newborn, who are protected against infections by intrauterine transfer of maternal immunoglobulin G (IgG) during the second half of pregnancy. Due to such protection, newborns, although without their own immunity, do not become infected: They defend themselves with maternal antibodies. Observations regarding the effectiveness of this phenomenon have been used in the production of medical preparations containing immunoglobulins pooled from blood donors.

Proposed procedures

There are two types of antibody preparations available.

The first is standard polyvalent immunoglobulin of human origin, obtained from pooled human plasma from screened donors contains mainly IgG with small amounts of IgA and IgM. This product is used as IgG replacement therapy in patients with immunological deficiencies characterized by absent or deficient antibody production to protect against life-threatening infections (Tangye et al., 2020).

The second is specific immunoglobulins of human or animal origin, or produced by recombinant DNA technology that contain high amounts of specific IgG antibodies against a particular pathogen. The product contains hyperimmune globulins, which are used in specific exposures, mostly in PEP. Using immunoglobulins in PrEP is limited only to a few products in very specific groups of patients, like:

- hepatitis B immune globulin (HBIG)
 - infants born to a hepatitis B virus (HBV)-infected mother,
- HAV-PrEP via passive immunization
 - as an isolated treatment
- Infant travelers <6 months of age
- Travelers for whom vaccine is contraindicated (e.g., allergic to HAV vaccine);
 - the combined prevention vaccine plus immunoglobulin
- Adult travelers >40 years, based on the provider's risk assessment.
- Children >6 months and adults with high risk of severe HAV manifestation or transmission following exposure;
- Individuals >6 months with chronic liver disease;
- Immunocompromised individuals >6 months (Nelson et al., 2020).

Postexposure prophylaxis

Chemotherapy

Chemoprophylaxis of invasive meningococcal disease

Neisseria meningitidis is a gram-negative bacterium that is found on the mucosa of the nasopharynx in approximately 10% of healthy people. Invasive meningococcal disease (IMD) is a severe form of *N. meningitidis* infection, most commonly manifested as sepsis

Table 2 Preexposure vaccinations.

	<i>Risk population</i>	<i>Standard primary schedule</i>	
Hepatitis A (Centers for Disease Control and Prevention, 2017b)	– Persons traveling or temporarily residing in the countries with high and intermediate HAV endemicity; – All children at 1 year of age; – Children and adolescents 2–18 years of age who live in areas with high disease incidence; – Men who have sex with men; – Patients who use illegal drugs (both injection and non-injection); – Persons with occupational risk factors (working with HAV-infected primates or with HAV virus in a laboratory setting); – Persons with chronic liver disease or who have received/are awaiting liver transplants; – Persons with clotting-factor disorders; and close contacts of adopted children from countries with increased incidence of HAV infection	Recommended to patients older than 12 months, in two dosages: 0, 6–12 months.	Life-long immunity
Hepatitis B (Schillie et al., 2018)	– Persons at risk for occupational exposure to HBV; – Household contacts; – Injection-drug users; – Sexual (heterosexual and MSM) exposure; – Developmentally disabled persons in long-term-care facilities; – Correctional facilities; – Hemodialysis patients; – Persons with HCV; – Persons with chronic liver disease; – Travelers to countries where HBV is endemic; – Persons with HIV; – Persons with diabetes.		
Rabies (Kessels et al., 2017)	– People who work with rabies in laboratories, animal control and wildlife offices; – People traveling to countries with a high risk of catching rabies who will be in close contact with wild and domestic animals, especially in areas with poor access to medical services	Three doses of vaccine given on days 0, 7, 21–28.	Any booster doses of the vaccine depend on the level of antibodies, which should be monitored regularly depending on the level of exposure.
Japanese encephalitis		Two doses: day 0; second dose any time between day 7 and day 28.	A single booster >1 year after completion of primary series if ongoing risk.
Meningococcal (Mbaeyi et al., 2020)	– People traveling to or living in areas at risk of <i>Neisseria meningitidis</i>; – People with an immunodeficiency that increases the risk of meningococcal disease (asplenia, HIV infection, complement component deficiency)	Serotypes: A, C, Y, W135: patients 6 weeks to 6 months: 2 dosages; older than 6 months: single dose; Serotype B: patients older 2 months: 2 doses	Life-long immunity
Typhoid	– Travelers to areas with a risk of exposure to <i>S. Typhi</i>.	PO: 1 cap every other day × 4 doses. IM: Single dose.	PO: Repeat every 5 years if ongoing risk. IM: Repeat every 2 years if ongoing risk.
Yellow fever (YF)	– People traveling to or living in areas at risk for YF transmission in South America and Africa	Recommended to patients older than 9 months; in one dosage.	Life-long immunity
Cholera (Wong et al., 2017)	– Prevention of cholera caused by serogroup O1 in adults 18 through 64 years of age traveling to affected areas who are at immediate risk of epidemic cholera.	Single dose	No specific recommendation; consider booster dose every 6 months if at continued risk. As of December 2020, the producer of the cholera vaccine will temporarily stop making and selling this vaccine; the vaccine may be in limited supply or unavailable (https://wwwnc-1cdc-1gov-1oaxngsr401a7.han.ump.edu.pl/travel/diseases/cholera).
Influenza	– Annual immunization of health care providers; – People with high risk of severe influenza	One dose every year (only the children below 9).	Every year
Pertussis (Centers for Disease Control and Prevention (CDC), 2012)	– All adults, including those aged 65 and older, regardless of the time since their last Td dose; – Health care personnel.	One dose	
Varicella (Anon, 1997)	– Health care providers	Two doses	Life-long immunity

and/or purulent meningitis. The incidence of IMD is estimated from 0.3 to 3 per 100,000 residents in developed countries (Halperin et al., 2012) and from 10 to 25 per 100,000 residents in some developing countries (Deuren et al., 2000). The disease often begins with non-specific symptoms such as high fever, severe weakness, and vomiting. In the absence of proper treatment, it quickly leads to serious coagulation disorders, which are manifested by rapidly progressive hemorrhagic rash (Bosis et al., 2015). Rapid deterioration of the patient's condition can lead to death within the first 24–48 h after onset (Łyczko and Borger, 2020). About 5–20% of patients with IMD develop full-blown sepsis with high mortality of up to 20–80% (Deuren et al., 2000). Meningococcal meningitis results in a lower rate of morbidity of 18–55%. Nonetheless, 11–19% of survivors experience serious sequelae (e.g., neurologic disability, limb loss, and hearing loss; Bilukha, Rosenstein, and National Center for Infectious Diseases and Centers for Disease Control and Prevention (CDC), 2005).

Infection caused by *N. meningitidis* spreads with droplets. The risk of developing IMD for household contacts exposed to patients who have sporadic meningococcal disease was estimated to be 4 per 1000 exposed people, which is 500–800 times greater than the risk for the total population (Bilukha, Rosenstein, and National Center for Infectious Diseases and Centers for Disease Control and Prevention (CDC), 2005). The risk for health care providers exposed to infected patients was estimated by Gilmore et al. (2000) as 0.8 per 1,000,000 people, which is 25 times higher than for the general population. The aim of IMD chemoprophylaxis is to prevent illness among close contacts.

Proposed procedures

Following CDC recommendations, the strategy of meningococcal prophylaxis should be implemented within 24 h after contact or identification of the pathogen. In the case of a delayed report of IMD, chemoprophylaxis can be given up to 14 days from the disease onset. The decision about chemoprophylaxis should not be based on nasopharyngeal culture (Bilukha, Rosenstein, and National Center for Infectious Diseases and Centers for Disease Control and Prevention (CDC), 2005).

The indications for chemoprophylaxis implementation are as follows (Bilukha, Rosenstein, and National Center for Infectious Diseases and Centers for Disease Control and Prevention (CDC), 2005):

- close contact with a patient within 7 days before the disease onset, regardless of vaccination status. Close contact applies to: household members living with a sick person, people who are in intimate contact with the patient, or people sleeping in the same bedroom with the patient (students, soldiers, officers);
- people who have been in direct contact with secretions from the patient's respiratory tract during medical procedures, including mouth-to-mouth resuscitation, suctioning, and endotracheal intubation, up to 24 h after antimicrobial therapy was initiated;
- people traveling with the patient in the same plane, ship, bus, or car for longer than 8 h. For travelers, antimicrobial chemoprophylaxis should be considered for any passenger who had direct contact with respiratory secretions from an index patient or for anyone seated directly next to an index patient on a prolonged flight (i.e., one lasting >8 h; CDC, 2000);
- the patient themselves before discharge from a hospital if they were not treated with ceftriaxone.

Regimen

Rifampin, ciprofloxacin, and ceftriaxone are 90–95% effective in reducing nasopharyngeal carriage of *N. meningitidis* and are all acceptable antimicrobial agents for chemoprophylaxis (CDC, 2000). The dosages for specific age groups and duration of administration are presented in Table 3.

Rifampicin

Rifampicin can be used in all age groups. Contraindications to this antibiotic are: pregnancy, nursing mothers, jaundice, and hypersensitivity to the drug. Rifampicin is an intense inducer of the hepatic P450 cytochrome enzyme system; therefore, the drug increases the metabolism of many drugs. Patients undergoing long-term anticoagulation therapy with warfarin have to increase their dosage (Holstege, 2014).

Table 3 Recommended chemoprophylaxis regimens for protection against meningococcal disease—Advisory Committee on Immunization Practices (ACIP), United States, 2012.

Drug	Age group	Dosage	Administration	
			Route	Duration
Rifampin	Children aged <1 months	5 mg/kg every 12 h	Oral	2 days
	Children aged ≥1 months	10 mg/kg every 12 h		
	Adults	600 mg every 12 h		
Ciprofloxacin	Adults	500 mg	Oral	Single dose
Ceftriaxone	Children age < 15 years	125 mg	Intramuscular	Single dose
	Adults	250 mg		

Ceftriaxone

Ceftriaxone can be used in every age group, and it is a first-line antibiotic recommended as chemoprophylaxis for IMD in children, pregnant women, and nursing mothers. The antibiotic has few contraindications for use: documented hypersensitivity and hyperbilirubinemia in neonates (especially premature; Zeng et al., 2017).

Ciprofloxacin

Ciprofloxacin can be used only in people older than 16 years. Contraindications to ciprofloxacin include patients with documented hypersensitivity to the drug or components of the formulation. The concurrent administration of tizanidine for muscle spasms is also a contraindication. Patients with myasthenia gravis should avoid ciprofloxacin because it may exacerbate muscle weaknesses (Thai et al., 2020).

Some regions of the world have documented resistance of *N. meningitidis* to ciprofloxacin and/or rifampicin (Taha et al., 2006). Local societies can update these recommendations regarding which antibiotics to use for IMD chemoprophylaxis based on regional resistance data. Although the recommended chemoprophylaxis effectiveness reaches up to 90–95%, it does not provide 100% protection against the disease. Patients who have received IMD antibiotic prophylaxis should be informed about the symptoms of the disease and the need to report to the hospital immediately in case they occur.

Chemoprophylaxis of invasive *Haemophilus influenzae* serotype b disease

Haemophilus influenzae serotype b (Hib) was one of the most common causes of bacterial meningitis and a frequent cause of other invasive diseases (e.g., epiglottitis, pneumonia, septic arthritis, and bacteremia), particularly in early childhood, before the conjugated vaccine program. Vaccination has radically reduced the incidence of invasive *H. influenzae* serotype disease (IHibD) in children. However, IHibD remains a problem in countries that do not use the vaccine (CDC, 2002a,b). Secondary cases of Hib disease (illness occurring within 60 days of contact with a patient) occur but are rare. Secondary attack rates are higher among household contacts aged <48 months (2.1%), especially those aged <12 months (6%) and <24 months (3%) (Wenger and Ward, 2003)

Proposed procedures (Briere et al., 2014)

IHibD chemoprophylaxis is recommended for:

- patients who are treated with an antibiotic other than cefotaxime or ceftriaxone and are aged <2 years or live in a household with a child <4 years of age who had not received an age-appropriate number of doses of Hib conjugate vaccine, or immunocompromised children should receive rifampin prior to hospital discharge (American Academy of Pediatrics, 2018a). Because cefotaxime and ceftriaxone eradicate Hib colonization, prophylaxis is not needed for patients treated with these antibiotics (Goldwater, 1995);
- all household contacts, who:
 - have had at least one contact <4 years of age who had not received an age-appropriate number of doses of Hib conjugate vaccine;
 - has a child <12 months of age who had not completed the primary Hib series;
 - has a contact <18 years who is immunocompromised, regardless of that child's Hib immunization status.

A household or close contact is defined as a person who resides with the index patient or who had spent ≥ 4 h with the index patient for at least 5 of the 7 days before the day of hospital admission of the index case (American Academy of Pediatrics, 2018a).

Chemoprophylaxis is recommended in child care settings when two or more cases of IHibD have occurred within 60 days and unimmunized or underimmunized children attend the facility (Briere et al., 2014).

Regimen

Rifampin 20 mg/kg orally (maximum dose 600 mg) once per day for 4 days is the regimen of choice for Hib chemoprophylaxis in individuals ≥ 1 month. Drugs other than ceftriaxone and cefotaxime that are used to treat *H. influenzae* infections, such as ampicillin, second-generation cephalosporins, and TMP-SMX, are not effective for chemoprophylaxis because they do not reliably eradicate Hib from the nasopharynx. Using these agents for antimicrobial prophylaxis against IHibD is not recommended.

In addition to IHibD chemoprophylaxis, exposed unimmunized or incompletely immunized children who are household, child care, or preschool contacts of patients with IHibD who got IHibD prophylaxis must be observed for any symptoms of illness (American Academy of Pediatrics, 2018a).

Chemoprophylaxis of pertussis

Prophylactic administration of drugs to the people of the close contact is implemented as soon as pertussis is diagnosed in a patient, not later than 21 days from the beginning of the cough. It is recommended to administer antibiotics, mainly macrolides, in analogous schemes as in the pertussis therapy.

Proposed procedures

PEP of pertussis is recommended for:

- **all household contacts** within 21 days of onset of cough in the index patient (Tiwari, Murphy, Moran, and National Immunization Program, CDC, 2005; Cramer and Heininger, 2008).

- high-risk people—those who are personally at high risk of developing severe illness, or who will have the close contact with people at high risk of severe illness—within 21 days of exposure to an infectious pertussis case (Tiwari, Murphy, Moran, and National Immunization Program, CDC, 2005).

Regimen

The dosages for specific ages group and duration of administration are presented in Table 4.

Specific antimicrobial agents

Azithromycin is only one drug that can be given for prophylaxis in small infants <1 month of age. The most common side effects are gastrointestinal problems—nausea, vomiting, and diarrhea—but they are not too traumatic and many patients can continue therapy (Ballow and Amsden, 1992). The contraindications for azithromycin are hypersensitivity to macrolides and a history of cholestatic jaundice/hepatic dysfunction associated with prior azithromycin use.

Chemoprophylaxis of Lyme disease

Lyme disease, a spirochetal infection caused by *Borrelia* spp. and transmitted by *Ixodes ricinus* ticks, is the most common tick-borne disease in the United States and Europe. The duration of tick attachment is very important in assessing the risk of transmission because *Borrelia* spp. are rarely transferred within the first 48 h of arachnid connection (Sutton and Spry, 2019).

Proposed procedures

The first method of prevention is early removal of ticks, within 24–36 h (Wormser et al., 2006; Sutton and Spry, 2019). Prophylaxis might be considered if the patient had visited the local region with high risk of infection and meets **all** five criteria defined by the Infectious Diseases Society of America (Wormser et al., 2006) (Table 5). If any of these criteria are not met, watchful waiting for 30 days has been recommended to monitor the appearance of fever, arthralgia, and rash (Smith et al., 2012).

Regimen

If the patient meets all of the aforementioned criteria (Table 5), the recommended dose of doxycycline is 200 mg for adults and 4.4 mg/kg up to a maximum dose of 200 mg in children, given as a single dose. The American Academy of Pediatrics (2018b) states

Table 4 Recommended chemoprophylaxis regimens for protection against pertussis (Tiwari, Murphy, Moran, and National Immunization Program, CDC, 2005).

Age group	Azithromycin	Erythromycin	Clarithromycin	TMP-SMZ
Infants < 1 month	10 mg/kg/day for 5 days	Not preferred because of risk for IHPS	Not recommended	<2 months: contraindicated (risk of kernicterus)
Infants 1–5 months	10 mg/kg/day for 5 days	40–50 mg/kg/day in 4 divided doses for 14 days.	15 mg/kg/day in 2 divided doses each day for 7 days.	≥ 2 months TMP—8 mg/kg/day, SMZ—40 mg/kg/day in 2 divided doses for 14 days.
Infants and children aged ≥ 6 months	10 mg/kg (maximum: 500 mg) on day 1, followed by 5 mg/kg/day (maximum: 250 mg) on days 2–5.	40–50 mg/kg/day (maximum: 2 g/day) in 4 divided doses for 14 days.	15 mg/kg/day (maximum: 1 g/day) in 2 divided doses each day for 7 days.	TMP—8 mg/kg/day, SMZ—40 mg/kg/day in 2 divided doses for 14 days.
Adults	500 mg on day 1, followed by 250 mg/day on days 2–5.	2 g/day in 4 divided doses for 14 days	1 g/day in two divided doses for 7 days.	TMP—320 mg/day, SMZ—1600 mg/day in 2 divided doses for 14 days.

TMP, trimethoprim; SMZ, sulfamethoxazole; IHPS, infantile hypertrophic pyloric stenosis.

Table 5 Criteria for antibiotic prophylaxis against Lyme disease following a tick bite (Wormser et al., 2006).

Antibiotic prophylaxis should be used only in patients who meet **ALL** of the following criteria

Attached tick identified as an adult or nymphal *Ixodes scapularis* tick
 Tick is estimated to have been attached for ≥ 36 h
 Prophylaxis is begun within 72 h of tick removal
 Local rate of infection of ticks with *B. burgdorferi* is ≥ 20% (these rates of infection have been shown to occur in parts of New England, parts of the mid-Atlantic States, and parts of Minnesota and Wisconsin)
 Doxycycline is not contraindicated (i.e., the patient is not <8 years of age, pregnant, or lactating)

The recommended dose of doxycycline is 200 mg for adults and 4 mg/kg up to a maximum dose of 200 mg in children ≥ 8 years, given as a single dose. This regimen has never been tested in children; this recommendation is extrapolated from experience in adults.

that in areas of high risk, a single prophylactic dose of doxycycline can be used in children of any age to reduce the risk of acquiring Lyme disease, but the efficacy of this approach and the appropriate regimen have not been established and recommendations were extrapolated largely from the adults experience.

The decision to select prophylaxis in countries other than the United States should be based on local recommendations and knowledge of the contamination of areas by the *Borrelia* spp.

Tularemia

Tularemia is a bacterial zoonosis caused by *Francisella tularensis*, one of the most infectious pathogenic bacteria. The disease occurs throughout much of North America, Europe, and Asia. *F. tularensis* can infect humans through the skin, mucous membranes, gastrointestinal tract, and lungs. Most clinical cases involve chronic lymphadenopathy associated with a skin, mucosa, or conjunctival inoculation lesion. Less frequently, patients suffer from systemic diseases and severe pneumonia with mortality rates up to 60% (Gill and Cunha, 1997).

Proposed procedures

Tularemia prophylaxis is recommended only in cases of laboratory exposure to infectious materials. Management options for potentially exposed workers include a fever watch or antimicrobial prophylaxis. There are no set criteria for determining who should be managed by observation and who would benefit from immediate prophylaxis. Factors to consider when making this decision include:

- nature of the exposure—workers who report the procedures generating aerosols are probably at greater risk than those who simply worked with the organism on the bench;
- incubation period—the typical incubation period for tularemia is 3–7 days. Much of this period may have passed by the time the organism is positively identified, in which case the remaining risk of infection is low;
- level of concern (Kirimanjeswara et al., 2007).

Regimen (Dennis et al., 2001)

Doxycycline 100 mg orally every 12 h over 14 days is generally recommended for prophylaxis in adults. Ciprofloxacin 500 mg every 12 h over 14 days is not FDA approved for prophylaxis of tularemia but has demonstrated efficacy in various studies, and thus it may be an alternative for patients unable to take doxycycline.

Invasive group A streptococcal prophylaxis

Invasive group A streptococci (GAS) are some of the most common bacteria encountered daily, and they result in acute infections with a wide array of manifestations in both adults and children. These microorganisms are responsible for an estimated 9000–12,000 deaths per year in the United States (Newberger and Gupta, 2021). The most common clinical presentations of GAS infections are toxic shock syndrome, with or without a focus of infection, necrotizing fasciitis or myositis, bacteremia with no septic focus, and pneumonia (Moore et al., 2019). People who are in the close contact with a case of invasive GAS have a high likelihood of becoming colonized with a virulent strain. The risk of subsequent invasive GAS disease in this group is 200–2000-fold higher than the risk among the general population (Lamagni et al., 2015).

Proposed procedures

Decisions regarding administration of GAS prophylaxis depend on the type of exposure, the immune status of the contact, and local recommendations (Health Protection Agency, Group A Streptococcus Working Group, 2004). Significant exposures include close family members, people who are kissing or sleeping in the same bed, and caretakers who spend many hours daily with an infected individual. Prophylaxis is warranted for people who are pregnant, immunosuppressed, who recently had surgery, or who have any open wound (Moore et al., 2019).

Chemoprophylaxis should only be given to people who have been exposed during the 7 days before the onset of symptoms of GAS to 24 h after initiating antimicrobial therapy in the case. It should be started as soon as possible, preferably within 24 h of identifying the case, but is still recommended up to 7 days after the last contact with the case (Moore et al., 2019).

Regimen

According to Canadian and American recommendations, the first choice in GAS prophylaxis are cephalexin and cefadroxil (Table 6). All people who receive chemoprophylaxis should watch for signs and symptoms of invasive GAS disease for 30 days after the diagnosis of invasive disease in the household contact (Public Health Agency of Canada, 2006).

Influenza chemoprophylaxis

Influenza is an acute respiratory disease caused by influenza A or B and sometimes influenza C viruses. The virus causes seasonal epidemics around the world. In most cases, the disease is self-limiting, but in some patients it may cause life-threatening complications, such as pneumonia and myocarditis. The actual list of patients with the high risk of influenza complications were have been extracted by CDC (2021a) (Table 7).

Table 6 Recommended GAS chemoprophylaxis regimens.

Drug	Dose	Duration
First-line choice		
Cephalexin	25 mg/kg to 50 mg/kg daily, to a maximum of 1 g/day, in two to four divided doses for 10 days	10 days
Cefadroxil	25 mg/kg to 50 mg/kg daily, to a maximum of 1 g/day, in two divided doses (children) or once daily (adults)	
second-line choice		
Clarithromycin	Adults: 250 mg orally twice daily Children: 15 mg/kg daily, in divided doses every 12 h, to a maximum of 250 mg orally twice daily	10 days
Clindamycin	Adults: 150 mg every 6 h Children: 8–16 mg/kg daily, divided into three or four equal doses, to a maximum of 600 mg daily	10 days Alternative for persons who are unable to tolerate beta-lactam antibiotics

Adapted from Public Health Agency of Canada (2006) Guidelines for the prevention and control of invasive group A streptococcal disease. *CDCR* 32S2: 1–26.

Table 7 Groups at high risk for serious influenza complications.

Children <5 years, but especially <2 years
Adults ≥65 years of age
Women who are pregnant or up to 2 weeks postpartum
Residents of nursing homes and long-term care facilities
People from certain racial and ethnic minority groups: non-Hispanic Black persons, Hispanic or Latino persons, and American Indian or Alaska Native persons
People with medical conditions including:
• Asthma
• Neurologic and neurodevelopmental conditions (including disorders of the brain, spinal cord, and peripheral nerve and muscle such as cerebral palsy, epilepsy, stroke, intellectual disability, moderate-to-severe developmental delay, muscular dystrophy, and spinal cord injury)
• Chronic lung disease (e.g., chronic obstructive pulmonary disease, cystic fibrosis)
• Heart disease (e.g., congenital heart disease, congestive heart failure, coronary artery disease)
• Blood disorders (e.g., sickle cell disease)
• Endocrine disorders (e.g., diabetes mellitus)
• Kidney disorders
• Liver disorders
• Metabolic disorders (e.g., inherited metabolic disorders and mitochondrial disorders)
• Weakened immune system due to disease (e.g., HIV, AIDS, cancer) or medication (e.g., chemotherapy or radiation therapy, chronic glucocorticoids)
• Children <19 years of age who are receiving long-term aspirin therapy
• People with extreme obesity (body mass index [BMI] ≥40)

Adapted from Centers for Disease Control and Prevention (2021b) *People at High Risk of Flu*. <https://www.cdc.gov/flu/highrisk/index.htm>.

Proposed procedure

Routine flu prophylaxis is not recommended as the standard of care, but it should be strictly considered in relation to:

- people at high risk of influenza complications who have been exposed to an individual with influenza within the previous 48 h, and for whom influenza vaccination is contraindicated, unavailable, or expected to have low effectiveness;
- during influenza outbreaks in long-term care facilities in older adults and chronically ill residents regardless of prior influenza vaccination;
- unvaccinated patients with the high risk for influenza complications during the first 2 weeks following vaccination after exposure to a person with influenza if the exposure had occurred within the previous 48 h;
- people who are unvaccinated household contacts of people with a high risk for flu complications **and** who had household exposure to an individual with influenza within the previous 48 h (Uyeki et al., 2019).

Regimen

Two classes of antiviral drugs are available for the prevention of influenza:

- neuraminidase inhibitors, zanamivir and oseltamivir, which are active against both influenza A and B;
- adamantanes, amantadine and rimantadine, which are active only against influenza A. Due to this limited virus susceptibility, these drugs should not be used in the prevention of influenza, except in exceptional clinical circumstances.

The recommended dosage, route of administration, and duration of influenza chemoprophylaxis, according to CDC (2021b) recommendations, are presented in Table 8.

Table 8 Recommended dosage, route of administration and duration of influenza chemoprophylaxis (CDC, 2021b).

Drug	Route of administration	Dosage	Duration
Oseltamivir	Oral	<3 months: oseltamivir chemoprophylaxis is not recommended except critical situation due to limited data in this age group 3–12 months: 3 mg/kg/dose once a day (Kimberlin et al., 2013) >1 year: dose varies by child's weight: <15 kg: 30 mg once a day; >15–23 kg: 45 mg once a day; >23–40 kg: 60 mg once a day; >40 kg: 75 mg once a day. Adults: 75 mg once a day	7 days (can be prolonged up to 14 days in close household contact)
Zanamivir (used if oseltamivir-resistant influenza is suspected)	Inhalatory	<5 years not recommended >5 years: 10 mg [2 inhalations] once daily	

Oseltamivir is an antiviral drug recommended for prophylaxis in children and adults. Following metabolism to the active metabolite, oseltamivir carboxylate inhibits viral neuraminidase, blocking the release of progeny virions from infected cells and viral entry into uninfected cells. Initiation of oseltamivir prophylaxis within 48 h of close contact with the influenza case reduce the risk of infection with 89% protective efficacy (Welliver et al., 2001). The main side effects of oseltamivir are related to gastrointestinal symptoms (Dobson et al., 2015). Rare occurrences of neuropsychiatric side effects and the risk of suicide have been reported primarily in children and adolescents taking oseltamivir in therapeutic dosages (Harrington et al., 2018; Huh et al., 2020). Oseltamivir is considered the drug of choice for chemoprophylaxis in the pregnant women who have had close contact with someone likely to have been infectious with influenza (ACOG Committee Opinion No. 753 Summary, 2018).

Zanamivir chemoprophylaxis is used to control household exposure and institutional outbreaks of influenza A or B when circulating strains are suspected of being resistant to oseltamivir (Uyeki et al., 2019).

Because of high rates of adamantane drug resistance in viral influenza strains, using amantadine and rimantadine for the prevention or treatment of influenza is contraindicated (Hussain et al., 2017).

New drugs like baloxavir and peramivir are not recommended in influenza prophylaxis due to the lack of data unambiguously confirming their effectiveness. However, research that has been conducted on baloxavir has shown promising results in the high-risk pediatric group (Ikematsu et al., 2020).

Varicella-zoster virus infections

Antiviral prophylaxis with acyclovir or valacyclovir can be used to prevent clinical VZV infection after an exposure for the patients who cannot receive vaccination or specific anti-VZV immunoglobulin. The potential target group for this kind of VZV-PEP are immunocompromised patients and/or pregnant women, but the decision must be individual after analyzing a specific case. CDC does not recommend acyclovir for prophylactic use among healthy children, adolescents, or adults without evidence of immunity after exposure to varicella. Vaccination is the method of choice in these situations. No studies have been conducted regarding prophylactic use of acyclovir among immunocompromised people; therefore, VZV-specific immunoglobulin is recommended in these situations (Marin et al., 2007).

Proposed procedure

This PEP should be initiated on day 7–10 after exposure and continued for 7 days. The delay in prophylaxis allows for early viral replication and may allow the exposed patient to develop some immunity without developing symptomatic disease. However, in some groups of patients, especially HCT or solid organ recipients, some experts suggest to prolong the course of VZV PEP (Marin et al., 2007; Tomblyn et al., 2009).

Regimen

Table 9 presents the regimen for this treatment.

Table 9 VZV PEP dosages in people with normal renal function.

Drug	Dosage
Acyclovir	Children: 20 mg/kg/dose (maximum dose 800 mg) four times daily orally Adults: 800 mg five times per day orally for 7 days
Valacyclovir	Children: (for children ≥ 3 months of age)—20 mg/kg/dose (maximum dose 1 g) three times daily orally for 7 days Adults: 1 g three times per day orally for 7 days

Based on American Academy of Pediatrics (2018) Varicella-zoster virus infections. In: Kimberlin DW, Brady MT, Jackson MA, and Long SS (eds.) *Red Book: 2018 Report of the Committee on Infectious Diseases*, pp. 869–883. Ithaca: American Academy of Pediatrics.

Table 10 Estimated risk for acquisition of HIV from infected source, by exposure route (Patel et al., 2014).

<i>Exposure route</i>	<i>Type of exposure</i>	<i>Risk per 10,000 exposures</i>
Parenteral exposure	Blood transfusion	9250
	Needle-sharing injection drug use	63
	Percutaneous needle stick	23
Sexual	Receptive anal intercourse	138
	Insertive anal intercourse	11
	Receptive penile-vaginal intercourse	8
	Insertive penile-vaginal intercourse	4
	Receptive oral intercourse	Low
Other	Biting	Negligible
	Spitting	Negligible
	Throwing body fluids (including semen or saliva)	Negligible
	Sharing sex toys	Negligible

HIV infection postexposure prophylaxis

The risk of HIV transmission following accidental exposure varies depending on the type of exposure (Table 10).

Factors that can increase the risk of HIV transmission include sexually transmitted diseases, a high viral load in a source and acute and late-stage HIV disease, and devices visibly contaminated with a patient's blood. Factors that can decrease the risk are antiretroviral treatment, PrEP, condom use, and male circumcision (Jain and Mayer, 2014). None of these factors have been estimated in the Table 10.

Procedure proposed in occupational exposure to HIV

Occupational exposure to potential HIV-infected material is defined as job-related contact with potentially infectious blood, tissue, or body fluids in a manner that allows for possible transmission of HIV. Therefore, this situation requires consideration of PEP (Joyce et al., 2015) (Table 11).

The management of health care personnel (HCP) after a significant exposure to HIV-positive patient blood or body fluid should aim immediately to reduce the probability of transmission. All medical institutions should have readily available recommendations for such a situation. The initial actions after HCP exposure is immediate cleansing of the exposed site. For skin exposures, the contaminated area should be cleaned with soap and water. For mucous membranes, the exposed place should be flushed with the more amount of water. Eyes should be cleaned with saline or water. Squeezing the wound is not recommended (CDC, 2002a,b).

The recommended HIV-exposure chemoprophylaxis is a three-drug regimen that is given to HCP with occupational exposure to potential HIV-infected material. Indications for implementation of PEP are: exposure to body fluids from a person with confirmed HIV infection or from a source currently tested for HIV, especially if the person is from the group at increased risk of HIV infection or shows potential symptoms of HIV infection. If it is not possible to perform source testing, PEP is considered, however, direct indications depend on the individual case of exposure assessment. In each case, the decision to implement PEP should be based on the analysis of the risk of infection, the toxicity of the proposed procedure for HCT, and their individual preferences (Kuhar et al., 2013).

PEP to HCP should be started as soon as possible, preferably within the first 2 h of the incident. It is not recommended to introduce PEP after 72 h because of reduced effectiveness. Recommendations of some countries allow for delayed implementation of PEP to HCP, however, they refer to individually considered cases and concern only extremely massive exposures (Kuhar et al., 2013). The recommended duration of PEP is 4 weeks; shorter courses in primate studies were less effective (Table 12).

HIV-PEP in nonoccupational expositions

After a possible exposure to HIV infection, individuals should be evaluated if the nonoccupational PEP with antiretroviral therapy should be offered to reduce the risk of viral transmission. The data evaluating nonoccupational PEP are limited, but feasibility and safety data have led to widespread acceptance of this practice (Kuhar et al., 2013). People who in the last 72 h have been subjected high-risk exposures like accidental percutaneous exposure to potentially infected blood and unprotected sexual intercourse with an

Table 11 Division of body fluids depending on the risk of HIV infection (Panlilio et al., 2005).

<i>Body fluids implicated in the transmission of HIV</i>	<i>Potentially infectious body fluids (undetermined risk for transmitting HIV)</i>	<i>Fluids that are not considered infectious unless they contain blood include</i>
Blood, semen, vaginal secretions, other body fluids contaminated with visible blood.	Cerebrospinal, synovial, pleural, peritoneal, pericardial, amniotic fluids	Feces, nasal secretions, saliva, gastric secretions, sputum, sweat, tears, urine, vomitus.

Table 12 Selection of antiretroviral therapy for 28-day regimens for HCP PEP (WHO, 2016a,b).

HCP group	Preferred/ alternative	Drugs	
Adults, with normal renal function (creatinine clearance ≥ 60 mL/min)	Preferred	Tenofovir disoproxil fumarate-emtricitabine (300/200 mg once daily) plus dolutegravir (50 mg once daily)	!Not recommended for pregnant women or all persons who are able to become pregnant, due to the risk of neural tube defects. ^a The risk of a fetus developing a neural tube defect is during the first 28 days. Ritonavir is used in clinical practice as a pharmacokinetic enhancer to increase the trough concentration and prolong the half-life of darunavir. It is not counted as a drug directly active against HIV regimens.
	Alternative	Tenofovir disoproxil fumarate-emtricitabine (300/200 mg once daily) plus raltegravir (400 mg twice daily) Tenofovir disoproxil fumarate-emtricitabine (300/200 mg once daily) plus darunavir (800 mg once daily) and ritonavir (100 mg once daily)	
Adults with renal dysfunction (creatinine clearance ≤ 59 mL/min)	Preferred	Zidovudine and lamivudine, with both doses adjusted to degree of renal function plus raltegravir (400 mg twice daily) or dolutegravir (50 mg once daily) Zidovudine and lamivudine, with both doses adjusted to degree of renal function plus dolutegravir (50 mg once daily)	
	Alternative	Zidovudine and lamivudine, with both doses adjusted to degree of renal function plus darunavir (800 mg once daily) and ritonavir (100 mg once daily)	

^aUS FDA. Juluca, Tivicay, Triumeq (dolutegravir): FDA to Evaluate - Potential Risk of Neural Tube Birth Defects <https://www.fda.gov/safety/medwatch/safetyinformation/safetyalertsforhumanmedicalproducts/ucm608168.htm> (Accessed on May 21, 2018).

HIV-infected person are eligible for prophylaxis. The selection of drugs for prophylaxis in adults is analogous to the exposure to HCP (Morbidity and Mortality Weekly Report, 2016; WHO, 2016a,b).

Postexposure vaccinations

Preexposure childhood and adolescent immunizations are some of the most effective methods of preventing serious illness. However, there are medical situations in which postexposure use of a vaccine reduces the incidence of a given disease or modulates its course. Postexposure vaccinations are used in the prevention of the following diseases: diphtheria, tetanus, rabies, measles, chicken pox, hepatitis A, and hepatitis B. In certain situations, apart from PEP vaccination, an appropriate immunoglobulin preparation (active-passive immunoprophylaxis) is administered; in the case of contraindications to vaccination, only immunoglobulin preparation (passive immunoprophylaxis) is used.

Tetanus

People can be exposed to tetanus via wounds and animal bites. The indications for postexposure tetanus vaccination are based on the individual's immune status (established based on medical records, not the collected history) and the individual's infection risk assessment. The risk of infection depends on the type of wound and the circumstances of the injury; it is low in the case of fresh, slightly contaminated wounds, without necrotic tissues, and high in the case of extensive, old wounds (surgically prepared >24 h from the wound) or heavily contaminated wounds containing necrotic, crushed, stabbed, and gunshot tissue (Havers et al., 2020).

Proposed procedure and regimen

Indications for PEP vaccination against tetanus (low or high risk of infection) (Liang et al., 2018):

- no tetanus vaccinations, incomplete vaccination, vaccination history unknown/uncertain—3 doses of vaccine at 0, 1, and 6 months;
- last dose of tetanus vaccine (basic or booster immunization) given more than >10 years ago in clean, minor wounds and >5 years ago for all other wounds—1 dose of vaccine.
- in a person who had received the last dose of tetanus vaccine <5 years ago, there is no indication for postexposure vaccination if there is a low risk of infection, If there is a high risk of infection, administration of a single dose of the vaccine may be considered.

In the people with high-risk wounds who are qualified for PEP, simultaneous administration of the vaccine and immunoglobulin should be considered (Liang et al., 2018).

Diphtheria

Vaccinations against diphtheria are part of the routine prophylaxis in children and according to the recommendations of some countries, they should be supplemented in adults. In practice, often at the time of contact with the disease, it turns out that the documentation regarding vaccinations is incomplete or that the person had not continued to vaccinate (Roses and Bonvehí, 2019). PEP vaccinations can be dedicated to people who had close contact with diphtheria patients and incomplete/unknown vaccination history or the last dose of vaccine given >5 years ago. A close contact is defined as person-to-person contact through respiratory droplets coming from diphtheria source or exposure to infected skin lesions and articles soiled with discharges from these lesions (Bader and McKinsey, 2013).

Proposed procedure

After close contact with a symptomatic and asymptomatic case of diphtheria, the exposed person should get either tetanus-diphtheria (Td) or tetanus-diphtheria-pertussis (Tdap) vaccine if no documentation of receipt of a three-dose primary immunization series, or if the last dose of vaccine was given >5 years ago (Liang et al., 2018).

Regimen

There is currently no monovalent vaccine against diphtheria available; all applied vaccinations are combined. The choice of vaccine depends on the age of the exposed person. In children <7 years of age, the diphtheria-tetanus-acellular pertussis (DTaP) vaccine is recommended as the part of routine childhood vaccination. In adolescents and adults, it is preferred to use Tdap in people who never got Tdap or whose status is unknown. In patients who got Tdap, a Td booster is given every 10 years (Havers et al., 2020).

Rabies

Postexposure vaccination against rabies applies to people who have had contact with an animal that is sick or suspected to be suffering from rabies (Table 13). Vaccination may be administered alone or in combination with specific immunoglobulin depending on the circumstances of the event that represents the risk of infection and condition of the animal. Consultation with the local public health agency can be valuable in assessing the risk of transmission after a potential exposure (WHO, 2021).

Proposed procedure

PEP should be administered immediately after a rabies exposure. The possible moment of occurrence of rabies symptoms is considered the limit of prophylaxis administration. On average, the symptoms of the disease appear about 45 days after exposure, although cases with a much longer incubation period, up to 8 years, have been recorded. In certain medical situations, the vaccination may be postponed until rabies is confirmed in the animal. These are:

- testing in case rabies is suspected;
- post-mortem examination of a dead animal;
- conducting a 15-day veterinary observation of the animal—only in the case of a dog or a cat (WHO, 2021).

Regimen

WHO (2021; Rupprecht et al., 2010) currently only recommends the use of purified cell-culture and embryonated egg-based rabies vaccines (Table 14).

Table 13 Categories of rabies exposure and PEP (WHO, 2021).

	Exposure	PEP
Category I	Touching or feeding an animal or licks on intact skin	Not indicated
Category II	Nibbling of uncovered skin, minor scratches or abrasions without bleeding: exposure	Only vaccine
Category III	Single or multiple transdermal bites or scratches, contamination of mucous membranes with saliva from licks, licks on broken skin, exposure due to direct contact with bats: severe exposure	Vaccine and specific immunoglobulin

Table 14 Rabies vaccine dosage.

Vaccination category	Vaccine	Days
Not previously vaccinated	Human diploid cell vaccine (HDCV) or purified chick embryo cell vaccine (PCECV) 1 mL, IM (deltoid area)	0, 3, 7, 14, (28—the fifth dose is used for the person with immunosuppression)
Previously vaccinated		0, 3–7

Adapted from Rupprecht CE, Briggs D, Brown CM, et al. (2010) Use of a reduced (4-dose) vaccine schedule for postexposure prophylaxis to prevent human rabies: Recommendations of the advisory committee on immunization practices. *MMWR - Recommendations and Reports* 59: 1–8; World Health Organization (2021) *Vaccinations and Immunization*. <https://www.who.int/teams/control-of-neglected-tropical-diseases/rabies/vaccinations-and-immunization>.

If rabies is ruled out after tests or 15-day observation, the exposed person is released from prophylaxis. If it has started, the prophylaxis is stopped. Currently, the biggest problem in many countries is the lack of availability of the rabies vaccine, and in the case of developing countries, price is the biggest issue (Fisher and Schnell, 2018).

Measles

PEP in the case of close contact with measles should cover only people who have not been given (or do not have documentation of two vaccinations) against measles. Such people should be offered the combined measles-mumps-rubella (MMR) vaccination as soon as possible (unless there are absolute contraindications). There is currently no monovalent vaccination available.

Proposed procedure

Prophylactic administration of the vaccine within 72 h of exposure to measles is considered to be the most effective approach. However, vaccination should be offered at any interval following exposure in order to offer protection from future exposures. MMR vaccination is highly effective in primary prevention of measles, with a two-dose vaccine effectiveness of 99%. Nevertheless, the exposed person should be advised to self-monitor for potential measles symptoms (Rice et al., 2004).

Varicella-zoster virus

Varicella vaccine should be administered postexposure to individuals who lack evidence of immunity to varicella, have no varicella vaccine contraindications, and are >12 months of age, including adults. Exposure is defined as face-to-face contact with an infectious person while indoors. The duration of close contact is not certain, however, it should not be transient. Certain experts suggest a contact of >5 min as constituting significant exposure (American Academy of Pediatrics, 2018e).

Proposed procedure

According to ACIP recommendations, after the VZV exposition, a qualified person should get VZV vaccine ideally within 3–5 days after the person is exposed. This may prevent varicella or make it less severe. Even if it has been more than 5 days, the vaccine should still be offered, especially to people in an environment at risk of an epidemic (Marin et al., 2007).

Passive postexposure prophylaxis

Passive PEP can be considered when vaccines for active immunization are contraindicated (pregnancy, small infants) or unavailable and in some specific situations when unimmunized individuals have been exposed to the highly infectious agent and require immediate protection. Passive immunization may also have a role in the management of immunocompromised people unable to respond adequately to a vaccine. The duration of the beneficial effects provided by passive immunizing agents is relatively short-lived and protection may be incomplete.

Both types of IgG preparations available are used in PEP. Most immunoglobulins must be injected intramuscularly (IMIG), although an intravenous (IVIG) preparation is also available. The proposed procedures depending on the type of pathogen are presented in Table 15.

Table 15 Passive postexposure prophylaxis depending on the type of pathogen.

Disease	Dosage	Patient groups	Timing
Polyvalent immunoglobulins			
Hepatitis A (Nelson et al., 2018; Link-Gelles et al., 2018)	IMIG—0.02–0.1 mL/kg im.	Immunoglobulin can be considered for people who were exposed to HAV and were not vaccinated before or were not infected by HAV. Risk groups include: – Household and sexual contacts of persons with hepatitis A, – Newborn infants of HAV-infected mothers, – Staff and children at child-care centers and schools with HAV outbreaks; – Staff of custodial care institutions with HAV outbreaks, and individuals exposed to HAV through food or waterborne outbreak (according local indications). Target IMIG-PEP group: (1) Only IMIG: Children <12 months, patients in immunosuppression and with contraindications to preventive vaccinations. (2) Vaccination with IMIG: people > 40 years of age (in Great Britain and Canada > 50–60 years of age), patients with chronic liver diseases.	Up to 14 days from contact with the HAV-infected person

Table 15 (Continued)

Measles (McLean et al., 2013; American Academy of Pediatrics, 2018c)	IMIG 0.5 mL/kg bw. (maximum dose 15 mL) or intravenous immunoglobulin (IVIG) 400 mg/kg	Administration of immunoglobulins is indicated for PEP of measles in: – Infants (vaccination may be considered at the age of 6–12 months); – Pregnant women with undocumented immunity; – People with severe immunodeficiencies: primary, after bone marrow transplant (at least up to 12 months after the end of immunosuppressive treatment or longer if the graft versus disease is developed), people with acute lymphoblastic leukemias (up to 6 months after the end of chemotherapy) and patients diagnosed with AIDS or HIV with severely immunodeficiency ($CD4 < 15\%$ or $CD4 < 200/mm^3$) or not vaccinated with MMR vaccine after receiving effective antiretroviral therapy.	Immunoglobulins can be used up to 6 days after contact with the patient
Rubella (McLean et al., 2013; Young, 2019)	IMIG—the dose: 20 mL (check local guidelines)	Administration of IMIG/IGIG can be considered for non-immune women with documented exposure to rubella during the first trimester (3 months) of pregnancy where termination is unacceptable or not an option (recommendations depends on local guidelines). !!!Passive immunization with IG is not effective in preventing rubella. Immunoglobulins given after exposure to rubella may modify or suppress symptoms but may not prevent infection, including congenital infection.	72 h from exposure
Hyperimmune immunoglobulins			
CMV infection (Rea et al., 2016; Kagan et al., 2019).	CMVIG-human CMV immunoglobulin intravenous	Actually using CMVIG in many previous indications were limited by antiviral medications. Only some experts consider using CMVIGG: – In concomitant with antiviral medications in kidney, lung, liver, pancreas, and heart transplants from CMV seropositive donors to CMV seronegative recipients; – In prevention of maternal-fetal transmission of CMV after primary maternal infection during pregnancy (off-label indication).	Repeated infusion depended on indications.
Hepatitis B (Terrault et al., 2018)	HBIG—human hepatitis B immunoglobulin	HBIG should be administered after: – Acute exposure to blood, plasma or serum containing HBsAg (parental exposure, mucous membrane contact or oral ingestion); – Perinatal exposure of infants born to HBsAg-positive mothers; – Sexual exposure to HBsAg-positive persons; – Household exposure to persons with acute HBV infection. Target PEP groups: – Unvaccinated or incomplete vaccinated patients; – Documented nonresponder after 2 complete vaccination; – Newborns of HBsAg-positive mothers—one dose of HBIG with the first dose of the hepatitis B vaccine series.	Most (94.9%) infants born to infected women receive recommended prophylaxis within 12 h of birth.
Rabies (American Academy of Pediatrics, 2018; Pattanaik and Mani, 2019)	Rabies immune globulin (human) (HRIG) always administered as part of rabies vaccine regimen. Only local wound infiltration/IM: 20 units/kg in a single dose.	Patients in IIIth risk category: single or multiple transdermal bites or scratches, contamination of mucous membranes with saliva from licks, licks on broken skin, exposure due to direct contact with bats: severe exposure.	HRIG should be administered as soon as possible after exposure, preferably at time of first rabies vaccine (day 0) dose but if the vaccine was started without HRIG, it can be given through the 7th day after the day 0. HRIG is not recommended after the 7th day.
Tetanus (Havers et al., 2020; Callison and Nguyen, 2020)	Human tetanus immune globulin (HTIG) 250 units IM	Patients with the high risk wounds (especially wounds contaminated with dirt, feces, soil, or saliva; puncture wounds; avulsions; or wounds resulting from missiles, crushing, burns, or frostbite): – Who have received fewer than three doses of tetanus toxoid;	HTIG can be given up to 21 days for unvaccinated individuals if indicated

(Continued)

Table 15 (Continued)

Varicella (CDC, 2013; Levin et al., 2019; Gans and Chemaly, 2021)	A purified human VZV immune globulin (VarizIG).	– Whose immunization status is uncertain. Patients without evidence of immunity to varicella who are at high risk for severe varicella and complications, who have been exposed to varicella or herpes zoster, and for whom varicella vaccine is contraindicated, should receive VarizIG. Patient groups recommended by CDC to receive VarizIG include the following: • Immunocompromised patients without evidence of immunity. • Newborn infants whose mothers have signs and symptoms of varicella around the time of delivery (i.e., 5 days before to 2 days after). • Hospitalized premature infants born at ≥ 28 weeks of gestation whose mothers do not have evidence of immunity to varicella. • Hospitalized premature infants born at < 28 weeks of gestation or who weigh ≤ 1000 g at birth, regardless of their mothers' evidence of immunity to varicella. • Pregnant women without evidence of immunity.	Candidates for VarizIG should receive it as soon as possible and within 10 days of exposure
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Disinfectants

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Disinfectants are of great importance for controlling the microbial population in healthcare facilities and within cleanrooms (Sandle, 2012a). However, the selection of the most appropriate disinfectants to use is not straightforward and different key criteria need to be considered for the selection of disinfectants, appropriate to the given application. Whilst selection is important, disinfectants must also be applied and used appropriately. Given that the objective of the disinfectant is to kill microorganisms and to reduce the surface bioburden then the real test of whether a disinfectant is efficacious, is with the numbers of microorganisms present. Disinfectants are required to undergo efficacy testing, both in suspension and on a range of surfaces (Vina et al., 2011). Ongoing assessments of continued suitability is through periodic viable microbiological environmental monitoring using surface techniques like contact plates and swabs. Further evidence as to how effective a disinfectant is can be shown with the types of microorganisms recovered (the “microflora”) (Sandle, 2011).

The term disinfection is normally applied to an inanimate object (sometimes the term biocide is used, although this relates to a larger group of chemical agents). The term antiseptic is used to describe the reduction of a microbial population on living tissue. Thus, an antiseptic is a disinfectant which can safely be applied to the surface of the skin (sometimes the terms “hand sanitizer” or “hand disinfectant” are used interchangeably.) In turn, the term disinfectant is usually reserved for liquid chemical germicides which cannot be applied to tissues because of their corrosive or toxic nature.

Disinfectants vary in their spectrum of activity, modes of action and efficacy. Some are bacteriostatic, where the ability of the bacterial population to grow is halted. Here the disinfectant can cause selective and reversible changes to cells by interacting with nucleic acids, inhibiting enzymes or permeating into the cell wall. Other disinfectants are bactericidal in that they destroy bacterial cells through different mechanisms including causing structural damage to the cell; autolysis; cell lysis and the leakage or coagulation of cytoplasm (Price, 1995). Within these groupings the spectrum of activity varies with some disinfectants being effective against vegetative Gram positive and Gram-negative micro-organisms only while others are effective against fungi. Some disinfectants are sporicidal in that they can cause the destruction of endospore forming bacteria (these are the most difficult forms of microorganisms to eliminate from cleanroom surfaces) (Russell, 1995).

Microbial kill mechanisms

With a bacterial cell there is a common sequence of events whereby the disinfectant interact with the cell surface; this is followed by penetration into the cell and action at the target site(s) (Brown and Gilbert, 1993). Different disinfectants attack bacterial cells in different ways; and, conversely, different species of microorganisms vary in their resistance to different disinfectants. These can be affected by the population of microorganisms present, their species, and the community with which they are bound. Many disinfectants are effective against fungi although, like bacteria, the cell wall chemistry and internal cellular structure differ. Thus, fungal susceptibility to disinfectants differs. To add to this, susceptibility also relates to the physiological state and stage of growth. Hence a disinfectant effective against a target preparation of bacteria may or may not be effective against fungi (Vijayakumar et al., 2012). In addition, disinfectants used in the pharmaceutical industry may need to demonstrate virucidal properties. In certain sectors, like biotechnology, this is a concern. Here non-enveloped viruses tend to be more resistant than enveloped forms (Rabenau et al., 2014).

One important determinant is with the numbers of organisms. An disinfection agent is considerably more effective against a low number of microorganisms than a higher number or a population with a greater cell density. Similarly, a disinfectant is more effective against a pure population than mixed population of microorganisms. Use of a routine disinfectant will not be likely to kill all microorganisms present and a number will remain viable. Whether the surviving microorganisms multiply in sufficient number is dependent upon the condition in which the surviving population remains, the available nutrients, and the time between repeat applications of the disinfectant.

Following from numbers, different types of microorganisms have varying levels of resistance to broad spectrum disinfectants. This can be represented by the “classic” hierarchy of resistance, as shown in Fig. 1 below (Russell, 1986). The increased resistance is primarily due to the cell membrane composition or type of protein coat.

Resistance is acquired through natural genetic properties of the microorganisms or it is acquired through phenotypic or genotypic variations (similar to antibiotic resistance through the over-use of one type of disinfectant). Generally, innate sensitivity results in Gram-negative bacteria being more resistant to disinfectant applications than Gram-positive bacteria (Otter et al., 2015). The reason for the increased resistance among Gram-negative bacteria can be attributed to a greater abundance of a hydrophobic lipopolysaccharides (LPS) in the cell membrane, whereas the Gram-positive membrane is primarily made up of inelastic “murein sacculi.” It is interesting that within the group of least resistant, Gram-positive bacteria, there are microorganisms (e.g., *Bacillus*, *Clostridium*, etc.) that can produce endospores which are the several degrees more resistant than their vegetative counterpart. This may be because of the relative impermeability of the polypeptides which make up the spore coat to hydrophilic agents. The bacterial spore is made up of three layers (Pommerville, 2014):

- Spore coat—which acts as a sieve to excluded toxic substances from the environment.
- Spore cortex—a protective structure made up of peptidoglycan.
- Core wall—the structure that surrounds the protoplast or core of the endospore.
- Core—the spore chromosomal DNA, encased in chromatin-like proteins called small acid-soluble spore proteins. These proteins protect the spore DNA. The core contains cell structures like ribosomes and other enzymes. These are not metabolically active.

Sometimes the spore coat is surrounded by a thinner structure call the exosporium (for example, with an organism like *Bacillus cereus*). The exosporium is the portion of the spore that interacts with the environment or host organism, and may contain spore antigens (Redmond, 2004).

A final factor shaping resistance is with the location of the organisms. The location of microorganisms can influence the effectiveness of a disinfectant. Microorganisms in suspension are easier to kill than those affixed to surfaces. This is due to the mechanisms of microorganism attachment, such as bacteria fixing themselves using fimbriae or when a biofilm community develops (8). Such positioning impacts the contact time required for the disinfectant to bind to the microorganism, cross the cell wall and act at the target site.

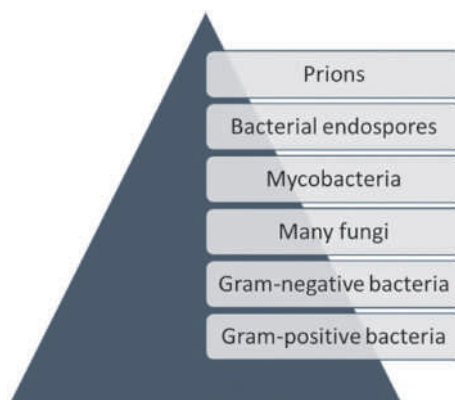


Fig. 1 Pyramid of resistance to common disinfectants. Adapted from Russell AD (1986) Mechanisms of resistance to antiseptics, disinfectants and preservatives. *Pharmacy International* 7: 305–308.

Classifying disinfectants

There are several ways to categorize the different types of disinfectants available. One common way is to distinguish between non-oxidizing disinfectants (such as alcohol) and oxidizing disinfectants (halogens like iodine, peracetic acid and chlorine dioxide). Non-oxidizing disinfectants do not have sporicidal action, whereas several oxidizing disinfectants are effective sporicides.

Common non-sporicidal disinfectants

The majority of disinfectants outlined below have a specific mode of action against microorganisms and generally have a lower spectrum of activity compared to oxidizing disinfectants. The list is far from absolute; the intention is to describe the more common types of disinfectants found within the pharmaceutical or healthcare setting.

Alcohols

Alcohols have an antibacterial action against vegetative cells. The effectiveness of alcohols against vegetative bacteria increases with their molecular weight (i.e., ethanol is more effective than methanol and in turn isopropyl alcohols are more effective than ethanol). Alcohols, where efficacy is increased with the presence of water, act on the bacterial cell wall by making it permeable. This can result in cytoplasm leakage, denaturation of protein and eventual cell lysis (alcohols are one of the so called “membrane disrupters”). The advantages of using alcohols include a relatively low cost, little odor and quick evaporation. However, alcohols have very poor action against bacterial and fungal spores and can only inhibit spore germination at best (McDonnell and Russell, 1999).

Aldehydes

Formaldehyde and glutaraldehyde are common to the aldehydes. Glutaraldehyde is a very effective disinfectant (and sterilant), has a wide spectrum of activity, and is effective against bacterial and fungal spores. However, glutaraldehyde is little used today due to personnel health and safety concerns. Formaldehyde and *o*-phthalaldehyde are slightly less effective in comparison due to a slower rate of reaction but possesses an equally wide spectrum of activity. Aldehydes have a non-specific effect in the denaturing of bacterial cell proteins and can cause coagulation of cellular protein (Angelillo et al., 1998).

Amphoterics

Amphoterics are acidic and have a relative wide spectrum of activity but are limited by their ability to damage bacterial endospores. An example is alkyl di(aminoethyl) glycine or derivatives (Ernst et al., 2006).

Acid anionics

Acid anionics are weak acids with a relatively limited spectrum of activity and are very pH dependent. An example of this group is carboxylic acid. They are not effective against fungi or spore forming bacteria. Their bactericidal properties arise from their ability to cause cell disruption through proton motive force where the balance of hydrogen across the cell is disrupted which can affect cellular division by disruption of oxidative phosphorylation (Antony and Jayakannan, 2007).

Biguanides

Biguanides are polymers supplied in salt form, such as chlorhexidine, alexidine and hydrochloride. Biguanides have a relatively wide spectrum of activity with the exception of killing bacterial endospores. This group is most effective at an alkaline pH and is rarely effective under acidic conditions. Biguanides affect the bacterial cell membrane and enter the cell through diffusion causing cell disruption and cytoplasm leakage (Wilson et al., 1991).

Phenolics

Phenols are produced from the fractionation of tar and are among the oldest scientifically evaluated disinfectants, dating back to Robert Koch's evaluation of phenol's bactericidal effect against *Bacillus anthracis*. The commonly used phenolic is basic phenol (carbolic acid) although synthetic variants are widely used. Phenol can be made more complex by the addition of halogens such as chlorine (the bis-phenols and halophenols) to make compounds like triclosan and chloroxylenol. Phenols are bactericidal and antifungal but are not effective against bacterial spores. Some phenols cause bacterial cell disruption through proton motive force while others attack the cell wall and cause leakage of cellular components and protein denaturation (Bergan and Lystad, 1972).

Quaternary ammonium compounds (QACs)

QACs are cationic salts of organically substituted ammonium compounds and have a fairly broad range of activity against microorganisms, although more effective against Gram-positive bacteria at lower concentrations than Gram-negative bacteria. They are considerably less effective against bacterial spores. QACs are sometimes classified as surfactants. An example is benzalkonium chloride (18). QACs are the most widely used of the non-oxidizing disinfectants in the pharmaceutical industry. Their mode of action is on the cell membrane which leads to cytoplasm leakage and cytoplasm coagulation through interaction with phospholipids (Vijayakumar et al., 2012).

Sporicidal disinfectants

Some disinfectants are sporicidal in that they can cause the destruction of endospore forming bacteria (these are the most difficult forms of microorganisms to eliminate from cleanroom surfaces). Endospores are the most resistant because of the relative impermeability of the polypeptides, which make up the spore-coat, to hydrophilic agents (Sandle, 2012a). It is important to note, however, that a chemical agent does not have to be sporicidal in order to be classified as a “disinfectant.”

Bacterial spores present a problem in cleanrooms due to the mechanisms which allow bacteria to survive for long periods in low nutrient conditions. Bacteria which form spores include species like *Bacillus*. If a disinfectant is not effective against spores, the risk is that the bacteria will return to the vegetative state at some point and pose a contamination risk (McDonnell and Russell, 1999).

Hypochlorous acid

This is a weak acid with the chemical formula HOCl. It forms when chlorine dissolves in water, and it is the HOCl moiety that actually does the disinfection. The chemical elicits its bactericidal activity by acting on a wide variety of biomolecules, including DNA, RNA, fatty acid groups, cholesterol and proteins.

Sodium dichloroisocyanurate (NaDCC)

Sodium dichloroisocyanurate is a chemical compound widely used as a cleansing agent and disinfectant; this salt is an active ingredient in many bleaches.

Chloramines

Chloramines are derivatives of ammonia. They are produced by chlorine gas or hypochlorite being added to the water to produce hypochlorous acid. Following this, ammonia is added to the water to react with the hypochlorous acid and produce a chloramine. Although these chemicals are beginning to be used in cleanrooms, they are more often used as a water treatment (such as for swimming pools). There is some discussion about the chemicals being implicated as mutagens.

Chlorine dioxide

This is a chemical compound with the formula ClO_2 . As one of several oxides of chlorine, it is a potent and useful oxidizing agent. This oxidizing agent requires combination of a base (organic acid, like lactic acid) and an activator (sodium chlorite) to come together to elicit the production of free chlorine. In general, this chemical retains active chlorine for longer than hypochlorite and it seemingly has a longer lasting bactericidal effect. However, it has been known to have stability issues and the shelf-life, once the two component chemicals come together, is variable (Sandle, 2016a).

Hypochlorites

Hypochlorite is a compound of chlorine and oxygen, with a chemical formula ClO^- , which can combine with a number of counter ions such as Na^+ (household bleach) and Ca^+ (used for water treatment) ions, to form the corresponding hypochlorites.

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is a colorless liquid that is the simplest peroxide (a compound with an oxygen-oxygen bond). It is used as a strong oxidizer, bleaching agent and disinfectant. Hydrogen peroxide is an effective sporicide which produces destructive hydroxyl free radicals. Like peracetic acid it has a wide spectrum of activity and it is relatively rapid (although the relationship between concentration and time needs to be carefully assessed). As with peracetic acid there are safety concerns, and it can be more affected by the presence of interfering substances on surfaces.

Peracetic acid

Peracetic acid (sometimes called peroxyacetic acid) based disinfectants are prepared from peracetic acid (the active ingredient), which is formed from hydrogen peroxide and acetic acid in solution. The peracetic acid results from a reaction between hydrogen peroxide and acetic acid. Preparations of peracetic acid are usually produced in concentrations of 5–15% (Denyer and Stewart, 1998).

Peracetic acid functions as a disinfectant by oxidizing the outer cell membranes of microorganisms. The oxidation mechanism consists of electron transfer. This causes the microorganism to be rapidly deactivated as the acid penetrates the polypeptide spore coat and then damages the macromolecules like carbohydrates, nucleic acids and lipids within the cell, ultimately leading to cell lysis and microbial cell death.

Some manufacturers produce a hydrogen peroxide/peracetic acid blend. This is commonly a formulation made up of less than 10% acetic acid with small amounts of hydrogen peroxide and peracetic acid. With the blend, arguably peracetic acid is the more potent; however, hydrogen peroxide is less damaging to surfaces (and to environment).

Selecting between disinfectants

Some considerations for selecting different disinfectants include:

- The disinfectant must be rapid in action with an ideal contact time of less than 10 min (the longer the contact time, then the longer the surface needs to be left after application and prior to use.)
- Some disinfectants require certain temperature and pH ranges in order to function correctly. One type of disinfectant, for example, may not be effective in cleanroom which is set at a cold temperature (below 10 °C) and many disinfectants have not been validated by the vendor to show that they work at temperatures below 20 °C.
- For use in higher-grade cleanrooms—ISO class 5 and 7 areas (EU GMP Grade A and B cleanrooms)—the disinfectant must be supplied irradiated or be able to pass through a sterilizing grade filter (through a 0.2 µm filter) without loss of its germicidal properties, or be provided sterile and ready-to-use (with sterility achieved through a process like gamma irradiation).
- The disinfectant must be compatible with the detergent used for the first “clean” (this is because residues from some detergents can inactivate the “active” ingredient in some disinfectants).
- Some disinfectants leave residues on surfaces. Whilst this can mean a continuation of an antimicrobial activity, residues can also lead to sticky surfaces and or the inactivation of other disinfectants.
- The disinfectant should be relatively safe to use. Here the main concern is with operator welfare. A related concern is the impact upon the environment, especially in the way that waste disinfectant solutions are disposed of.
- Different disinfectants are not compatible with all types of surfaces. The disinfectants should ideally not damage the materials (to which they are applied) by corrosion or discoloration. Certain sporicidal disinfectants, such as chlorine-based products, can corrode stainless steel unless residues are wiped away, such as with Water-for-Injection or a 70% alcohol solution.
- When using sporicidal disinfectants, one consideration is the presentation. Some sporicidal disinfectants require two components to be mixed together and the resultant combination often has a limited time within which it can be used due to the length of time that the main ingredient remains active. There are advantages in having some disinfectants available both in concentrate form, suitable for diluting, for cleaning large surface areas, and ready-prepared solutions, such as trigger sprays, for smaller areas.

Rotation of disinfectants

When cleanroom disinfectants are selected it is common practice to select two or more disinfectants. This is for several reasons (Sutton, 2008):

- Most disinfectants do not have a complete spectrum of activity effective against all microorganisms (spectrum of activity is the ability of the disinfectant to kill different types of microorganisms and microorganisms which are in different physiological states). The disinfectants commonly used are often effective against vegetative cells but are not sporicidal. To maintain an effective contamination, control the elimination of bacterial endospores through a sporicidal disinfectant is a recommended (these are sometimes referred to as “high-level disinfectants”).
- The disinfectants with the formulations which are effective against the greatest range of microorganisms are often expensive. With this, many manufacturers use a general broad-spectrum disinfectant daily or weekly with a sporicidal disinfectant used weekly or monthly (a decision often based on the results of microbiological environmental monitoring and the characterization of the isolated microorganisms).
- There are some expressed concerns, although not support by any major study, that genetically acquired disinfectant resistance might occur in a similar way to antimicrobial resistance. The theory runs that if disinfectants are rotated, the possibility of resistance occurring decreases.
- Some regulatory authorities, including the European Medicines Agency and the UK MHRA, require two disinfectants to be used in rotation which is a Good Manufacturing Practice requirement. The argument for rotating two disinfectants is to reduce the

possibility of resistant strains of microorganisms developing. Whilst the phenomenon of microbial resistance is an issue of major concern for antibiotics there are few data to support development of resistance to disinfectants. Nonetheless, it remains a regulatory expectation.

Disinfectant efficacy testing

There are different approaches that can be taken for disinfectant qualification ("efficacy testing"). To demonstrate the efficacy of a disinfectant it is necessary to conduct the following tests:

1. In-use-dilution tests.
2. Surface challenge tests.
3. A statistical comparison of the frequency of isolation and numbers of microorganisms isolated prior to and after the implementation of a new disinfectant.

With these different tests there are different national standards, with some differences in terms of methodology and acceptance criteria. Described here is a generalized approach.

Both suspension tests and surface tests are conducted under clean and 'dirty' conditions. Clean conditions are intended to cover applications where the surfaces can be cleaned sufficiently before the application of the disinfectant, whilst dirty conditions are intended to cover applications where cleaning prior to disinfection is not undertaken or cannot be relied upon to produce a clean surface. On this basis, the simulated dirty conditions are arguably more important. Suspension and surface tests are divided between tests for vegetative bacteria; test for fungi; and tests for bacterial spores (Tomasino and Hamilton, 2006).

An important choice is with the microorganisms that form the test panel. With typical organisms for a healthcare or pharmaceutical facility, the following bacteria are often used:

- *Staphylococcus aureus* (ATCC 6538)
- *Enterococcus hirae* (ATCC 10541)
- *Pseudomonas aeruginosa* (ATCC 15442)
- *Escherichia coli* (ATCC 10536)

And the following fungi:

- *Candida albicans* (ATCC 10231)
- *Aspergillus brasiliensis* (ATCC 16404)

With fungi it is recommended that fungal spores are used since the presence of hyphae and mycelia can prevent disinfectant from contacting and penetrating spore.

In addition, with bacteria and fungi, isolates from the area where the disinfectants are to be used are often added to the test panel (termed "environmental isolates").

To assess a sporicidal disinfectant, the following organism as a spore suspension is recommended:

- *Bacillus subtilis* (ATCC 6633)

Where virucidal properties are required:

- Adenovirus-5 and poliovirus

The in-use (or suspension test) involves taking the disinfectant, at the required concentration, and adding to it an interfering substance (to simulate practical conditions) together with a known concentration of a challenge microorganism (derived from an 18–24 h culture). The suspension is then held for the required contact time and at a defined temperature. Once this time has elapsed, a proportion of the challenged disinfectant is transferred to a tube containing a suitable neutralizer, which is intended to inactivate the disinfectant. Once sufficient time has been given for neutralization, microbial survivors are assessed by plating out or filtering the disinfectant-neutralizer solution using a microbial culture method (Sutton et al., 2002).

It is important that the neutralizer used has been tested in advance to show that it is not toxic to any microbial survivors. An approach from this was outlined by Sutton and colleagues. This involves calculating both neutralizer efficacy ratios, by comparing the recovery of identical inocula from the neutralizing solution in the presence, or the absence, of a 1:10 dilution of the biocide; and neutralizer toxicity ratios, which are determined between recovery of viable microorganisms incubated for a short period in peptone, and in the neutralizing medium without the biocide. An effective and non-toxic neutralizer was established by ratios of ≥ 0.75 .

Surface testing involves aliquoting onto a surface coupon a mix of the challenge organism and, where required, an interfering substance (such a protein, to simulate dirty conditions). The surface coupon will be fashioned from a representative surface in the cleanroom (here several different materials will require testing in order to show how the disinfectant performs). To this an amount of the test disinfectant is added. The solutions are left for the required contact time. Once the contact time has elapsed, the coupon is

transferred to a neutralizer solution. Then, as with the suspension test once sufficient time has been given for neutralization, microbial survivors are assessed by plating out or filtering the disinfectant-neutralizer solution using a microbial culture method. A possible array of surfaces to consider (this will be facility dependent) are:

- Stainless steel,
- Glass,
- Aluminum,
- Epoxy,
- Enamel,
- Acrylic,
- Mipolam,
- Vinyl,
- Hardwood,
- Plastic,
- Plexiglas,
- Chromium

With both suspension and surface testing, microbial kill is assessed through logarithmic reduction (as expressed to base 10). Log reductions are a relative concept and the log reduction obtained will be impacted by the starting inoculum.

The statistical comparison part is commonly referred to as a field trial (in situ testing). This tests how well the disinfectant performs (either solely or in conjunction with another disinfectant in rotation) in the actual area(s) where the disinfectant will be used. The assessment is through surface based environmental monitoring where microbial counts and species are assessed prior to and after the application of the disinfectant.

To run an effective field trial, a baseline is established prior to and then during implementation of the new regime using intensive environmental sampling. The effectiveness of the new disinfectant is assessed via a statistical evaluation of the environmental monitoring data before and after implementation of the new regime. The obtained results should be equivalent or better. Field trials aim to capture the variability of practical application that may affect microbial susceptibility and disinfectant efficacy. These factors include:

- Detergent residues;
- Soiling;
- Variations of microbial attachment;
- Organisms under different stress conditions;
- Temperature fluctuations;
- Differences in pH.

With the above three-step approach, some users elect to run in-use dilution tests (or suspension tests); others chose to conduct surface tests only; and some will conduct both.

The validation approach

An approach that can be adopted for the validation of disinfectants to be used within the cleanroom is (Sandle, 2012b):

1. Screening tests: These may be used to compare various disinfectants (or different concentrations of a disinfectant) when making the choice of which disinfectants to purchase. This is usually by suspension testing.
2. Surface challenge tests. This is the most important disinfectant efficacy test and, as discussed above, typically conducted using standard organisms and environmental isolates on cleanroom surfaces.
3. Qualification the environmental monitoring media for use in conjunction with chosen disinfectant (see below).
4. Field trial. The field trial is conducted after the laboratory-based assays have been completed. The field trials tests whether the selected disinfectant(s) work in practice. This is a study of organisms isolated (frequency and type) before and after implementation of a new cleaning and disinfection regime. A field trial can also help to set appropriate frequencies of disinfection and the interval between the rotation of two different disinfectants.

There are several important factors that require assessing through disinfectant validation. These are (Sandle, 2012c):

- (a) The concentration of the disinfectant. Many users will test the manufacturer's recommended concentration, which keeps this part more straightforward. However, if the user seeks to establish a concentration this needs to be added, including a minimum and maximum range. Setting the concentration is easier to establish through suspension tests.
- (b) The contact time. This is time needed for the required level of microbial kill (or inactivation) to be achieved. A disinfectant product may have different contact times depending on the target organisms (for example an extended contact time will probably be needed for sporicidal action). In setting contact times, it is important that these are practical; many standards set contact times that are unrealistically long. A second area with contact times is that needs to be factored in is the rate of drying. In the laboratory setting, disinfectants will dry more slowly than in the cleanroom (where the air change rates are faster and the air is most likely to be less humid).

- (c) The microbial challenge. Many disinfectant efficacy standards use very high challenges (up to 1 million microbial cells) and associated multi-step logarithmic reductions. These are far in excess of what would be found in a cleanroom. Lower challenges can be used, with justification. This can lead to more practical contact times, especially when assessing sporicidal agents. A two-log reduction, for example, of bacterial spores should be sufficient for most cleanrooms.

An argument for accepting a lower log-kill acceptance criteria can be based on (Sandle, 2019a):

- Disinfectants are less effective against the high numbers of organisms used during testing than against the low numbers expected in a cleanroom environment
 - Stressed environmental organisms are easier to kill than the cultures using in testing
 - In practical situations disinfectants are applied by wiping/mopping which physically removes organisms enhancing the “kill”.
- (d) The types of surfaces. For surface testing a range of surfaces need to be tested. This will include common surfaces like vinyl, acrylic, glass, and laminate. Moreover, the surface type and condition can have a significant impact on efficacy (with condition older or damaged surfaces can be more challenging).
- (e) The quality of water used to dilute the disinfectant concentrate. The water quality (potable, purified or Water-for-Injections) should be the same as used in the production facility.
- (f) The temperature of use. Laboratory studies are commonly conducted at 20–25 °C. This normally matches process conditions. However, consideration will need to be given if the disinfectant is intended to be used in cold rooms, for example, since these lower temperatures may affect disinfectant efficacy.

Additional testing

As well as the general tests described above, many users elect to conduct the following additional assessments:

- (a) Testing at the end of the shelf life of the product:
1. Either as the solution *per se*,
 2. or a solution in contact with either the synthetic or natural fiber wipe.
- (b) When a product is reconstituted:
1. just before use
 2. and at the end of its in-use shelf life

The reason for doing so particularly applies to sporicides, which are chemicals that are generally reactive oxidizing species. These chemicals are essentially unstable or reactive towards packaging or wipe materials.

In some cases, with the development of a novel agent, pre-studies may be performed for establishing the appropriate concentration. For this dilution methods can be used to establish the minimum inhibitory concentrations (MICs) of antimicrobial agents: these are the reference methods for antimicrobial susceptibility testing and are mainly used to establish the activity of a new agent, to confirm the susceptibility of microorganisms to the agent that give equivocal results in routine tests, and to determine the susceptibility on various microorganisms where routine tests may be unreliable (Vijayakumar et al., 2016). Other alternate procedures for assessing disinfectant effectiveness include flow cytometry (using cytofluorometry) (Akhmaltdinova et al., 2016).

Practical application

Ensuring that surfaces are regularly disinfected and that the numbers of bacteria present are kept to a minimum is of great importance for contamination control. However, disinfectants are only effective when used in conjunction with a detergent. This is because most disinfectants have poor cleaning ability and will not easily penetrate “soil” (dust, grease and dirt). So, surfaces must be rinsed frequently with a detergent and then disinfected at frequent intervals (Sandle, 2016b).

Application can be controlled through written cleaning and disinfectant protocols. Establishing a cleaning and disinfection protocol or standard operating procedure is an important step for a pharmaceutical or healthcare organization in its microbial control program. An established and effective protocol will ensure that risks to the patient, or manufactured product, are minimized. In drawing together a program, advice is available from regulatory authorities (through “current” GMP) and consideration should be given to aspects discussed in this chapter: compatibility of detergents and disinfectants, the need for rotation, methods for cleaning and the results of method validation. Ideally the program should be published within the organization and it is important the staff are given sufficient time to carry out each cleaning step.

The procedures should cover the following aspects (Sandle, 2019b):

- The type of detergents and disinfectants to be used (which are compatible);
- The frequency of rotation of disinfectants;
- A list of suitable cleaning materials;
- Cleaning techniques;
- Contact times;
- Rinsing;

- Frequency of cleaning and disinfection;
- Procedure for the transfer of cleaning agents and disinfectants into and out of clean areas (including the procedure for sterilization of disinfectants);
- Holding times for detergents and disinfectants (for how long, after preparation, can a detergent or disinfectant be used?).

Factors affecting disinfectant efficacy

There are different factors which affect how well a disinfectant may or may not work in the practical setting. These are:

- Type and quantity of the organism as this presents the specific level of challenge to the agent(s) and so the concentrations required.
- Amount of protein-based materials present as this tends to inactivate and absorb the disinfectant.
- Presence of other organic compounds and soaps (e.g., non-ionic surfactants) either in the solution or the surfaces, as these interact with certain disinfectants even neutralizing them. In certain combinations this could enhance the effect for example quaternary ammonium compounds. In order for a disinfectant to be effective it must come into contact with and be absorbed by the microbial cell. If substances, such as oil, dirt, paper or grease, act as a spatial barrier between the microbial cell and the disinfectant, the efficacy of the disinfectant will be adversely affected. The presence of such substances ("soil") halts disinfectant efficacy by either reacting with the disinfectant or creating a barrier for the disinfectant. This effect is increased if the surface itself has defects and crevices which limit disinfectant penetration.
- The solution will also be affected by other inherent characteristics namely pH, temperature, concentration and possible interactions between other agents and containers. Generally, temperature influences the rate of reaction ([Abdallah et al., 2015](#)). Most disinfectants are more effective and kill a population faster at higher temperatures although many disinfectants, due to practical considerations, are manufactured to be used at ambient. Some disinfectants, particularly oxidizing agents like peracetic acid, have an optimal temperature of 40–50 °C, and sporicidal agents like *o*-phthalaldehyde are more effective at temperatures elevated above ambient. Disinfectants which are sensitive to temperatures other than ambient are normally assessed through the use of a temperature coefficient, or Q10 (which relates the increase in activity to a 10 °C rise in temperature).
- The effect of pH is important because it influences the ionic binding of a disinfectant to a bacterial cell wall thereby ensuring disinfectant molecules bind to a high number of microorganisms. Many disinfectants are more stable at a set pH range, for example, acid-based disinfectants can become less potent in alkaline conditions whereas a glutaraldehyde is more potent at a basic pH. The use of a disinfectant outside of its desired pH range results in reduced efficacy.

Environmental monitoring

The ultimate proof of sporicidal control is with the results of environmental monitoring, assessing surface count data. To assess the effectiveness of a sporicide in relation to contamination control, periodic environmental monitoring and trending of counts, incidences and isolates is required. If high recoveries of spore formers are noted, this should trigger a review of sporidical agents and practices. In addition, if spore forming microorganisms are found in the environment, consideration should be given to adding them to future disinfectant efficacy tests ([Sandle, 2019a](#)).

With environmental monitoring methods, care should be taken with the culture media selection and neutralizers. The neutralizers in the agar used for contact plates, for example, must have been examined to show they are effective against any residues from the sporicide that might remain on a surface.

Hand sanitization

Hand sanitization is of importance in healthcare and pharmaceutical settings. Personnel carry many types of microorganisms on their hands and such microorganisms can be readily transferred from person to person or from person to equipment or critical surfaces. Such microorganisms (including *Staphylococcus*, *Micrococcus* and *Propionibacterium*) are either present on the skin not multiplying (transient flora) or are multiplying microorganisms released from the skin (residential flora). For critical operations, some protection is afforded by wearing gloves. However, gloves are not suitable for all activities and gloves, if not regularly sanitized or if they are of an unsuitable design, will pick up and transfer contamination ([Larson, 1988](#)).

Hand sanitizers fall into two groups: alcohol based, which are more common, and non-alcohol based. The most commonly used alcohol-based hand sanitizers are Isopropyl alcohol or some form of denatured ethanol (such as Industrial Methylated Spirits), normally at a 70% concentration. The more common non-alcohol-based sanitizers contain either chlorhexidine or hexachlorophene. For hand sanitizers used on skin, these must not cause excessive drying and be non-irritating ([Rutala, 1995](#)).

Hand sanitizers should be applied to clean hands. For this, hands should be washed using soap and water for around 20 s. Handwashing removes around 99% of transient microorganisms (although it does not kill them). From then on, whether gloves are worn or not, regular hygienic hand disinfection should take place to eliminate any subsequent transient flora and to reduce the risk of the contamination arising from resident skin flora.

Personnel working in healthcare facilities and cleanrooms should be aware of the importance of hand sanitizers, the way in which they work, and the differences between them. This should be enforced through a rigorous training program and supervision. The understanding of staff as to why hygiene of gloved and ungloved hands is important, particularly when working near patients or with clean equipment, as employee attitude influences the hygiene and quality (Nicolle, 2007).

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Drug Release, Susceptibility and Time-Kill Assays to Develop Novel Anti-Infective Drugs

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Introduction

Infectious diseases are one of the main causes of death throughout human history. Even in pandemics such as bubonic plague, infectious diseases were the primary cause of death and had reshaped civilization (Gould, 2016). When old written sources and archeological excavations are examined in detail, it is seen that societies had different methods of combating infections since ancient times. These methods could be the use of various herbs, honey, animal feces, and the topical application of moldy bread. Especially the fact that applying moldy bread is included in the sources of China, ancient Egypt, Rome, Serbia, and Greece shows how wide this practice has spread over geography (Keyes et al., 2008; Gould, 2016). On the other hand, the inclusion of sections on the benefits of molds in the book named “Theatrum Botanicum” published by John Parkinson in 1640 proves that molds were used in the treatment of infection thousands of years after ancient times.

The development of modern anti-infective agents as we know them today actually started in 1676 when Antonie van Leeuwenhoek discovered small living creatures, namely “animalcules,” with a microscope he designed (Karamanou et al., 2010). After this discovery, “bacteriology” and the relationship between bacteria-disease was studied. In addition to bacteria, fungus and malaria structures were also discovered in similar periods. On the other hand, with the geographical discoveries, it had become easier to transfer the knowledge from one point of the world to the different parts of the World (Finberg and Guharoy, 2012). For example, the use of cinchona bark for malaria treatment by indigenous peoples in South America was transmitted to Europe in the 17th century. Based on this knowledge, current anti-malarial drugs have been developed. Traditional Chinese medicine, which is based on the use of herbal medicines, still provides an idea for the development of new anti-infective drugs with its 5000-year history. A similar situation is valid for traditional Indian medicine.

The development of new drugs is a long and demanding process. Proper management of this process is crucial to the development of effective new anti-infective drugs and to accelerate the treatment of infectious diseases. Thanks to today's analysis possibilities, basic information about the effectiveness of a drug or drug delivery system can be obtained with *in vitro* methods without the need for *in vivo* or clinical studies.

The need for developing novel anti-infective agents and their pharmaceutical dosage forms

In the 20th century, the discovery of anti-infective agents had accelerated, and many new drugs have been introduced until today (Nathan, 2012). Obtaining synthetic or semi-synthetic anti-infective agents was initially achieved by imitating or improving the molecular structures of traditional drugs with known efficacy (Cos et al., 2006). With the scientific developments, as the biological structure of the pathogen is enlightened, new drug molecules with chemical groups that can directly interact with these structures have been synthesized. These new drugs, which are obtained by changes in chemical groups but their main molecular structure remains the same, are named “new generation.” Anti-infective agents are generally divided into four groups as anti-bacterial, anti-viral, anti-fungal, and anti-parasite. Anti-infective agents approved by FDA and EMA are available in different pharmaceutical dosage forms such as tablets, solutions, emulsions, suspensions, ointments, cremas, gels, and new drug delivery system (EMA, 2021; FDA, 2021).

Along with scientific developments, important steps have been taken in the treatment of anti-infective diseases with the use of many different agents. In addition, the importance of preventive medicine and the increase of community immunity thanks to

vaccines have also been significant steps in the prevention of infectious diseases. Improving the perception of hygiene and improvement of nutritional conditions have also been the other supportive components of the fight against infectious diseases (Cheng et al., 2003). Based on these four elements, a great fortress was built against infectious diseases as a result of combining the accumulation and experience of centuries with modern medicine (Nathan, 2012). However, despite all these developments, infectious diseases are still an important problem of humanity and not only reduce the quality of life but also cause the death of thousands of people around the world every year (Cheng et al., 2003; Ibrahim, 2012).

The reasons why infectious diseases still have such serious consequences are also the reasons for developing new anti-infective agents. Drug resistance, diseases requiring immediate treatment, increased bioavailability, and patient compliance are the reasons for developing new anti-infective agents (Bush, 2004; Nathan, 2012).

Drug resistance

Bacteria, viruses, fungi, and parasites, which cause the infection, develop resistance to anti-microbial drugs over time depending on various factors (National Institute of Allergy and Infectious Diseases, 2011). These factors are examined in four groups:

1. Factors depending on the microbial agents (natural selection, mutation, gene transfer, and social pressure).
2. Inappropriate use (inappropriate use and inadequate diagnostics).
3. The evaluation of resistance hospital strains.
4. Agricultural use.

Anti-microbial resistance (AMR) that develops due to one or more of these factors makes difficult or impossible the treatment of infectious diseases. Today, the most critical reason for deaths due to infectious diseases is AMR (Bush, 2004; Ibrahim, 2012; Ventola, 2015; European Centre for Disease Prevention and Control, 2018; U.S. Department of Health & Human Services Centers for Disease Control and Prevention, 2019). It is essential to isolate these resistant strains and develop anti-infective agents that can act directly on them. On the other hand, clinicians should pay attention to the appropriate use of these new agents to prevent/delay the resistance against them.

Emerging diseases

While the fight against existing known infectious diseases continues, new diseases are also defined by the isolation of new strains that have not been discovered before or due to the mutation of the strains. These diseases may be the reappearance of an old disease such as tuberculosis, whooping cough, chickenpox with resistant strains. There may also be new diseases of viral origin such as AIDS, ebola, and SARS that develop due to new strains (Bush, 2004; Nathan, 2012). The last example of this situation is COVID-19, which has affected the whole world from the end of 2019 and is predicted to be controlled within 2022 with the best estimate. Since the existing anti-infective agents are insufficient to combat these diseases, the disease's risk and rate of transmission are very high and can turn into a pandemic. In such a situation, the immediate isolation of the infectious strain is a critical step in developing new agents that can be used in its treatment. On the other hand, the correct observation of the symptoms of these new diseases and the use of the correct medication for the treatment of them are also very important in terms of symptom management until new agents are developed.

Bioavailability and patient compliance

Bioavailability and patient compliance are the driving forces not only for developing the new anti-infective agents but also for developing the new drugs to be used in the treatment of all diseases, unlike drug resistance and emerging diseases.

In the treatment of infectious diseases, the drugs must be within the therapeutic concentration range in the action area (Bush, 2004). There are two reasons for this situation: The first is to control the disease by keeping the infectious strains under constant pressure and effectively performing the treatment. The second is that strains can develop resistance against drugs used at the wrong therapeutic concentration.

Based of these aforementioned informations, it is understood that new anti-infective agents with high bioavailability, low dosing frequency and side effect profile should be developed and included in treatment protocols.

The importance of developing novel anti-infective drug delivery systems

Most of the drugs on the market are formulated as conventional dosage forms namely solutions, emulsions, suspensions, creams, ointments, gels, and conventional tablets (FDA, 2021). Due to the low bioavailability of some drugs, the dose applied and dosing frequency during the day could be relatively high. When the dose strength was increased, the potential risks of drug related side effects could also increased, that could be resulted in decreasing the patient compliance to the therapy. In order to overcome these limitations novel drug delivery systems targeting drug to the site of the action in the body have been developed.

Drug delivery systems ensure that any drug is delivered to the target area with any suitable carrier such as polymer or surfactant. These systems, which are generally micro or nano-sized, enable transporting lipophilic molecules to hydrophilic tissues or hydrophilic molecules to lipophilic tissues (Hall, 1986). Since they carry the drugs directly to the target tissue, they can achieve therapeutic concentrations in the effect area by administration of much lower doses. Thus, the side effects may reduce. On the other hand, since these systems also provide controlled or sustained drug release, dosing frequency is also very low. All these positive effects also increase patient compliance.

Drug delivery systems have an important role in the treatment of infectious diseases. The drug concentration in the target tissue must remain within the therapeutic range during treatment. While this continuity is ensured with repeated doses with conventional drugs, dosing frequency is lower because drug delivery systems provide controlled drug release (Breimer, 1999; Gao et al., 2011). Because of low dosing frequency, patient compliance increases, and the risk of overdose may be eliminated, which may affect the effectiveness of the treatment. As the anti-infective agents accumulate directly in the target tissue, the applied dose and side effects may decrease thanks to the drug delivery systems. However, perhaps the most important benefit of drug delivery systems in treatment with anti-infective agents is the prevention of resistance due to inappropriate use (Pham et al., 2019).

The other important advantage of drug delivery systems is that they shorten the Research & Development (R&D) process and allow research to be completed with less budget. It takes approximately 15 years to discover a new drug molecule, prove its *in vitro* and *in vivo* efficacy, complete its clinical studies, and complete the licensing process, and be used in treatment (Barrett, 2014). It also requires a budget of close to 1 billion dollars (Adams and Brantner, 2010). In today's conditions where the average life expectancy is prolonged and the total population is constantly increasing, it is not a very practical option to find new treatment agents. For this reason, it is a much more rational approach to remove the old molecules that have been proven to be used in the treatment from the traditional dosage form and to increase their effectiveness by "changing the clothes" (Paolino et al., 2006). This approach is an important method, especially in developing anti-infective agents where there is always a risk of resistance. Given the length of time of discovering the new molecule and brought into use, it is not possible to meet the urgent need for new anti-infective agents. On the other hand, spending such large budgets in this process is not an accurate R&D strategy for these drugs, which are at risk of developing resistance at all times. Also, the mutation of the strains during the phase studies and developing resistance against this new agent waste the entire R&D process (O'Neil, 2011). Based of these informations, it may be more efficient to modify the existing anti-infective agents to improve their pharmacodynamic and pharmacokinetic profiles than developing new agents.

Natural anti-infective agents

Since the fight against infectious diseases has existed throughout human history, many anti-infective agents of natural origin have been discovered and used since ancient times (Cos et al., 2006; Khazir et al., 2013). The existing knowledge has been passed down through generations. Depending on the development of technology, the structures responsible for the anti-infective effect in these natural medicines have been discovered, isolated, or synthetically produced in the laboratory. It also gave an idea for the preparation of new synthetic or semi-synthetic molecules. Some anti-infective agents of natural origin are given in Table 1. Today, these natural resources are still used in the development of new anti-infective agents.

Table 1 Examples of natural origin anti-infective agents.

<i>Penicillin G lactam cell wall</i>		<i>References</i>
Tetracycline	Anti-bacterial	Khazir et al. (2013)
Chloramphenicol		
Erythromycin		
Tobramycin		
Vancomycin		Vogel and Pelletier (1815) Morita (2010) Liu et al. (2012) Sun et al. (2019) Song et al. (2020) Cholkar et al. (2016)
Curcumin		
Celastrol		
Mangiferin		
Myricetin		
Pterostilbene		
Resolvin		
Carneic acids		Vengurlekar et al. (2012)
Latrunculins A and B		
Quinine	Anti-malarial	Khazir et al. (2013)

***In vitro* dissolution/drug release assays for anti-infective drug development**

In vitro drug release assay is one of the key performance test to develop new antiinfective agents or their novel delivery systems. *In vitro* dissolution tests (referred to here as “*in vitro* release assays”) are performed to evaluate drug release rate *in vitro* as a function of time. Drug release over time is quantified by using various analytical methods such as spectrophotometric, chromatographic, mass spectrometric, and potentiometric (Wang et al., 2006; Grady et al., 2018). The *in vitro* drug release kinetics can be followed based on different mathematical models including zero order, first order, Hixson and Crowell, Higuchi, Weibull, Korsmeyer-Peppas, Hopfenberg, Baker-Lonsdale and Gompertz models (Ramteke et al., 2014; Dash et al., 2010; Singhvi and Singh, 2011; Vaghela et al., 2011).

At the initial stage of formulation development, *in vitro* dissolution test helps optimizing drug release from formulations. It is useful for initial screening among potential formulations to observe the influence of critical formulation variables and to help in the selection of the candidate formulation. At later stages, it reduces the number of bioavailability/bioequivalence studies by indicating *in vivo* performance of antiinfective drug products (Uddin et al., 2011). *In vitro* release mechanism and kinetics may also affect product manufacturing, stability, quality control, scale-up studies, reproducibility or, in some circumstances, *in vivo* drug release besides formulation development process. Drug release and dissolution are not synonyms but mostly used for each other. The drug dissolution process consists of five steps, including wetting of the particle surface with water, breakdown of solid state bonds in drug particle, solvation, diffusion through the liquid unstirred boundary layer and convection within the bulk fluid. However, drug release is more complex and drug dissolution is only one of drug release steps. The drug release process consists of 4 steps, including drug dissolution in the delivery system upon contact with water after penetration into the drug carrier, drug diffusion out of the delivery system owing to the concentration gradient, changes in the physical shape of the delivery system such as swelling and subsequent drug dissolution in the aqueous medium of the applied tissue (Jug et al., 2018; Azarmi et al., 2007; Uddin et al., 2011).

Dissolution test was first developed to evaluate oral drug release from solid dosage forms such as tablets and capsules. When a drug is administered by oral route in solid dosage forms, it is firstly delivered to the absorption site via gastric emptying and intestinal transit flow and dissolved in the stomach and/or in the small intestine. Then, the dissolved drug is absorbed from the gastrointestinal membrane and passes through the liver and finally reaches the systemic circulation. In particular, dissolution becomes a rate limiting step in the overall release process when poorly soluble drugs are formulated in a conventional dosage form. In this case, the terms of drug dissolution and drug release could be used interchangeably if the drug dissolution process is the rate-limiting stage in the overall release process. Thereby, the long term rate and drug absorption into systemic circulation are regulated by dissolution rate (Jug et al., 2018; Krishna and Yu, 2007). Also, the dissolution test could be used in a variety of dosage forms such as transdermal therapeutic systems, suspensions, suppositories, chewing tablets, powders, inserts, implants, and others besides tablets and capsules.

There are different apparatuses (Apparatus 1–7) used in dissolution tests for drugs and their dosage forms (Second Interim Revision Announcement, 2016). The design and operation of the apparatus, as well as the medium used in dissolution studies, may affect *in vitro* drug release profile and hence drug absorption. The USP defines different apparatuses used in dissolution studies, and has recently been harmonized with the European Pharmacopeia and the Japanese Pharmacopeia. Experimental and simulated conditions are applied during dissolution tests, for example some at room temperature and some at body temperature (32 °C or 37 °C). Apparatus 2 is the most common used apparatus, which is also known as paddle method. It is recommended for dissolution profile testing of immediate-release solid dosage forms. Sinkers in the type of spiral, pronged or basket are used to avoid capsule dosage forms from floating. Apparatus 3 (reciprocating cylinder) is recommended for some extended-release tablets and is advantageous when pH/buffer adjustments are needed during the dissolution testing process, or when hydrodynamics can be directly affected by adjusting the dip rate. Apparatus 1 is a basket type and the second-most-commonly-used dissolution apparatus, enables dissolution tests on floating formulations without sinkers. It is the same apparatus and vessel type as the paddle apparatus, usually with a vessel size of 1 L, but instead of a paddle, the dosage form is kept in a cylindrical basket with mesh openings. Apparatus 4 (flow-through cell) is recommended for extended release and poorly soluble products. Contrary to the other dissolution apparatuses, a piston pump that generates a sinusoidal flow profile is used in the flow-through cell apparatus. As a consequence, the fluid velocity changes over time. Whereas Apparatus 5 (paddle-over-disk) is used for dissolution of topical and transdermal dosage forms, Apparatus 6 (rotating cylinder) is recommended for all methods when needed to assure the quality and performance of topical and transdermal systems. Also, Apparatus 7 (reciprocating holder) is recommended for transdermal patches (Shohin et al., 2016; Todaro et al., 2017; Uddin et al., 2011; Gray and Rosanske, 2020; Culen et al., 2013).

In vitro drug dissolution/release assay has become more important with the developing nano drug delivery systems such as liposomes, micelles, microemulsions, nanoparticles etc. These nanoparticulate systems are mostly used to improve bioavailability, enhance antimicrobial activity by prolonging drug release, microorganism targeted drug delivery, and also reduce possible side effects. In particular, a gradual drug release from nanocarrier systems over time with a mild gradient can reduce bacterial resistance and improve antiinfective drug activity. Considering that any unexpected change in product performance of such systems may result in toxicity or change in the activity. Moreover, novel drug delivery systems exhibit some problems due to the physicochemical properties of the formulations and the properties of the physiological drug release medium. Also, dosage form performance may affect the bioavailability of drugs incorporated in these systems. In this regard, it is important to perform suitable *in vitro* dissolution/release testing methods to assist in antiinfective drug development and ensure pharmaceutical product quality and performance (Jug et al., 2018; Azarmi et al., 2007; Shen and Burgess, 2013; Ebrahimi et al., 2020).

There are various *in vitro* drug release assay methods to meet the requirements nanoparticulate drug delivery systems. Membrane diffusion methods (dialysis methods), sample and separation methods and continuous flow methods are the three general types of these methods. Membrane diffusion methods (such as the dialysis methods) are the most commonly used methods for the *in vitro* dissolution/release testing of the nanoparticulate systems. The nanoparticles are removed from the release medium using dialysis membranes that are permeable to the free drug but not to the nanoparticles. The dialysis sac method, the reverse dialysis sac method and the side-by-side dialysis methods are the types of membrane diffusion methods. Agitation conditions, the ratio between donor and acceptor cell volumes, and most probably, molecular weight cut-off of the dialysis membrane affect drug release in this method. In the sample and separate methods, nanoparticles are included in the release medium and then separated from the medium at different time intervals by using separation methods such as ultrafiltration or ultracentrifugation. Then, the supernatant or filtrate is analyzed in terms of drug amount. Agitation conditions, and most probably, separation methods affect drug release in this method. The continuous flow method uses USP Apparatus 4 and suitable for modified release dosage forms because a small volume of the medium can be constantly circulated through immobilized formulations. Nevertheless, it may be difficult to test dissolution/release profiles in the USP Apparatus 4 system when the nanoparticles are very small (less than 100 nm) so that they can either clog the filter or pass through the filter thus leading incorrect results. These problems were mostly prevented by modifying Apparatus 4 with a dialysis adapter. Also, the methods of membrane diffusion, sample and separation or continuous flow can be combined to assess drug release more properly from nano drug carriers (Shen and Burgess, 2013; D'Souza, 2014; Abdel-Mottaleb and Lamprecht, 2011; Jambhekar and Breen, 2013).

Moreover, there are other dissolution/release test methods such as dynamic dissolution methods and microdialysis methods. Ion- or drug-selective electrodes are used in dynamic dissolution methods to determine release profiles of electroactive drugs from nano drug carriers. For example, electrochemical methods are very advantageous for preventing the interference due to the presence of undissolved dosage form in the release medium. Dynamic dissolution methods are used in combination with mostly USP Apparatus 1 or 2. In microdialysis method, probes are placed into the vessels and continuously perfused with a fresh media through an inner tube. Between the inner tube and the outer dialysis membrane, the medium streams out. Then, the released drug in the medium is analyzed by using analytical methods. Other *in vitro* drug release methods are nonelectrochemical methods such as calorimetry, turbidimetry, light scattering and laser diffraction and the other methods include vertical Franz diffusion cells and *in vitro* lipolysis model (Shen and Burgess, 2013; D'Souza, 2014; Gray et al., 2018; Balzus et al., 2016). Novel, cost-effective, simple and reliable *in vitro* drug release methods are also being developed that is applicable to small volume nanoparticulate formulations. Recently, a 3D printed microfluidic drug release device has been designed with two distinct chip geometries, "basket" and "V" shaped barriers (Amoyav et al., 2021).

For bone infections, Bai et al. (2020) developed roxithromycin incorporated polycaprolacton/polyethylene glycol composite nanofiber scaffolds via melt electrohydrodynamic 3D method. According to *in vitro* release data, the scaffolds exhibited an initial burst drug release and then long-term sustained drug release behavior, which is desirable for the prevention and treatment of bone infections. Drug release reached to higher than 70% at the first 6 h. Also, the scaffolds had antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* via disc diffusion assay. Increasing amounts of polyethylene glycol in the formulations resulted in the enlargement of inhibition zones because polyethylene glycol accelerated drug release. In another study, nanostructured baghdadite-vancomycin scaffolds showed larger inhibition zones against *Staphylococcus aureus* with higher vancomycin concentration in the formulation. Moreover, the scaffolds sustained drug release process promoted the antibacterial effects (Bakhsheshi-Rad et al., 2017). Baig et al. (2016) prepared levofloxacin loaded solid lipid nanoparticles for the treatment of conjunctivitis. *In vitro* drug release assay was performed using Franz diffusion cells with dialysis membrane and exhibited a prolonged drug release following Korsmeyer-Peppas model. Esentürk et al. (2020a) also used Franz diffusion cells to evaluate naftifine hydrochloride release from poly(vinyl alcohol)/sodium alginate nanofiber mats and their crosslinked ones with glutaraldehyde. The burst drug release was decreased with the crosslinking treatment effectively and the release mechanism followed Korsmeyer-Peppas Super Case-II transport. In a study carried out by Durgun et al. (2020), it was shown that the release of posaconazole from the micelle formulations was higher than that of suspension using Franz diffusion cells method. In addition, antimicrobial activity study by agar plate method proved that levofloxacin loaded solid lipid nanoparticles were active against *Staphylococcus aureus* and *Escherichia coli*. Other studies which are evaluating *in vitro* dissolution/release assay for anti-infective drug development are listed in Table 2.

Susceptibility and time-kill assays for anti-infective drug development

Antimicrobial susceptibility assay is one of the important tests used to determine susceptibility of pathogen organisms to anti-infective drugs and assess possible drug resistance. The main aim of susceptibility assay is to predict the results of the treatment with anti-infective agents tested and choose the best anti-infective agent in terms of antimicrobial activity (Benkova et al., 2020).

Antimicrobial activity is usually measured either as a zone diameter (in mm) of the bacterial growth inhibition around an anti-infective drug-containing disk or as a minimum inhibitory concentration (MIC, mg/L) of the drug versus the tested pathogen (Arena et al., 2015). Dose and drug selection in the anti-infective drug development stage is mostly based on an *in vitro* parameter, the MIC, and on the drugs serum concentration as a pharmacokinetic parameter (Miller et al., 2005). MIC is the lowest concentration of the antimicrobial agent that prevents visible growth of the test strain of an organism and expressed in mg/L ($\mu\text{g/mL}$). Susceptibility assay results are usually defined as categories of susceptibility such as susceptible, intermediate or resistant corresponding to the cut-off values provided by scientific committees of EUCAST (European Committee on Antimicrobial

Table 2 The summary of studies in which *in vitro* dissolution/release assay was used to develop different types of delivery systems of antiinfective drug substances.

Antiinfective drug	Delivery system	In vitro dissolution/release method	Results	References
Cephalexin	pH sensitive carboxymethyl cellulose hydrogel beads	Sample and separate method and simulated GIS conditions	Protection from stomach pH and a controlled drug release in the intestinal conditions	Barkhordari and Yadollahi (2016)
Ciprofloxacin	Near-infrared light-triggerable thermo-sensitive hydrogel	Eppendorf tube	Trigger in drug release with NIR irradiation from hydrogel, thereby synergistically enhanced antibacterial activity against <i>Staphylococcus aureus</i>	Gao et al. (2019)
Vancomycin	Injectable hydrogel	Eppendorf tube	Sustained drug release over an extended period of time in a pH-dependent manner Killing methicillin-resistant <i>Staphylococcus aureus</i> bacteria upon contact as well as by releasing antibiotics	Hoque et al. (2018)
Ciprofloxacin	β -Cyclodextrin-Chitosan based sub-microcarrier	Dialysis membrane	Suitable drug-delivery device for local antibiotic therapy Achievement in sufficient amount of drug release before 24 h A promising drug delivery system for the treatment of respiratory extracellular infections	Ho et al. (2018)
Aminoglycosides (netilmicin, isepamicin, capreomycin, ribostamycin, apramycin, amikacin, paromomycin, tobramycin and neomycin)	Hydrogel	Vial	On-demand drug release behaviors High antibacterial activities against <i>Staphylococcus epidermidis</i> , <i>Staphylococcus aureus</i> , and <i>Pseudomonas aeruginosa</i>	Hu et al. (2017)
Nystatin	Self-nanoemulsifying drug delivery system	Dialysis bag	Improvement in the solubilization and release rate of the drug, thus enabling better interaction with the fungi cell membrane and increase in the activity against <i>Candida albicans</i>	Kassem et al. (2016)
Ceftriaxone sodium	Injectable hydrogel	Vial	Localized, sustained drug delivery over 2 weeks for preventing local <i>Staphylococcus aureus</i> infections	Li et al. (2017)
Ciprofloxacin	Nanofibers	Cuvette	Sustained drug release mostly following a non-Fickian diffusion mechanism with a release rate independent of drug concentration	Moydeen et al. (2018)
Curcumin	Nanoliposomes	In artificial gastric and intestinal media	Twofold higher curcumin release in the simulated gastric and intestinal environment	Kumar et al. (2018)
Peppermint and green tea essential oils	Nanoparticles	Dialysis bag	Better antibacterial and antifungal activity than the native curcumin Initial burst release for the first 12 h, then a slow release up to 72 h Better antibacterial activity with nanoparticle formulations against <i>Staphylococcus aureus</i> and <i>Escherichia coli</i> than pure essential oils	Shetta, et al. (2019)

Susceptibility Testing, Europe), CLSI (Clinical and Laboratory Standards Institute, USA) or International Organization for Standardization (ISO) (Dietvorst et al., 2020; Balzus et al., 2017; Khan et al., 2019; Karatuna, 2012). The result category of “susceptible” means that the antiinfective agent is most probably effective in the treatment with the appropriate dosage. The result category of “resistant” means that treatment with the antiinfective agent is presumably to fail (Jorgensen, 2011).

There are various *in vitro* susceptibility assay methods either classical golden standard methods or novel methods based on culture growth or single-cell analysis (Dietvorst et al., 2020; Benkova et al., 2020; Schumacher et al., 2018; Jorgensen and Ferraro, 2009). Diffusion and dilution methods are commonly used golden standard methods which were standardized by organizations of EUCAST and CLSI. Agar disc diffusion method is one of the oldest and the most popular diffusion methods in antimicrobial susceptibility assays. It is simple, reproducible and low cost. Generally, Mueller-Hinton agar plates are inoculated with a standardized inoculum of the test microorganism (corresponding to 0.5 McFarland turbidity standard to have a final microbial concentration of $1-5 \times 10^5$ CFU (colony forming unit) per mL). Paper discs are treated with different concentrations of the tested drugs and then placed onto the inoculated agar surface. The agar plates are incubated under suitable conditions, generally for 16–24 h at 35–37 °C. Then, growth inhibition zones around the paper discs are measured in millimetres. The diameters of the inhibition zones are related to MIC values conversely. In antimicrobial gradient strip method, dilution and diffusion methods are combined. The agar medium is inoculated with the tested microorganism and then thin test strips which are including increasing concentration gradients of the drug are then placed onto the agar surface. The MIC values are determined by looking at the strips from the top of the petri dish and calculating the point where the strips intersect with an elliptical growth inhibition zone. This method is also rapid and easy but more expensive than agar disc diffusion method (Arena et al., 2015; Benkova et al., 2020; Schumacher et al., 2018; Liu et al., 2016). Dilution methods are also reference methods for determining MIC values of antiinfective drugs. Moreover, these methods enable to make a comparison between newly developed antimicrobial agents because the diffusion methods may be unreliable when quantitative results are needed. Broth microdilution (tube dilution) method is an old method and requires larger amounts of reagents but enables quantitative MIC results. In broth microdilution method, small amount of sample is sufficient so that it is more popular and practical as an antimicrobial susceptibility assay. Broth microdilution method is performed by using small 96 well plates that are allowing testing 12 different antimicrobial agents over a range of 8 twofold dilutions on one plate. Twofold diluted antimicrobial agents are put into the trays and the plate is inoculated with a diluted microbial suspension standardized to an optical density of 0.5 McFarland McFarland turbidity standard. Based on the microorganism tested, the plate is incubated under appropriate conditions. The MIC is determined using spectrophotometry at 620 nm. The minimum bactericidal (MBC) and fungicidal (MFC) concentration values are determined by calculating the lowest concentration of antimicrobial agent which reduces the viability of the initial bacterial or fungal inoculum by $\geq 99.9\%$. MBC is determined by subculturing from the microtitration plates on Mueller–Hinton agar plate for 24 h at 37 °C. In the case of determining MFC, Sabouraud agar plates are used and the incubation conditions are 48 h at 35 °C. Another susceptibility test is agar diffusion test, which is not frequently preferred. In this method, different concentrations of an antimicrobial agent is included into a molten agar medium, then, a standardized microbial inoculum was applied to the surface of the agar plate. The agar plates are evaluated by visually comparing various strains in the series, and then, MIC is determined as the lowest antimicrobial agent concentration that inhibits growth (Benkova et al., 2020; Balzus et al., 2017).

Due to the disadvantages of the golden standard methods, novel *in vitro* susceptibility tests have been developed. Fluorescence based test kits are used for assessing cell viability by staining either dead or live cells selectively with a fluorescent marker. In mass sensitive technology, cantilevers are used and microbial deposition on the beam produces resonant frequency changes. The differences are detected using various instruments. In another method, forward laser light scattering is used to detect microorganisms with the sizes from 100 to 10.000 nm by a laser in the range of 400–700 nm. Also, there are live-cell imaging and real-time microscopy systems on the market, which are combining with a microfluidic system and a fully automated fluorescence *in-situ* hybridization system, respectively. Polymerase chain reaction (PCR) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) instruments have also been combined to susceptibility assay recently. Moreover, novel susceptibility tests are still being developed such as colorimetric, electrical and isothermal microcalorimetric methods, and others utilizing flow cytometry, Raman spectroscopy, laser tweezers, microfluidic technologies and motion tracking (Dietvorst et al., 2020; Benkova et al., 2020; Idelevich and Becker, 2019; Schumacher et al., 2018; van Belkum et al., 2020; Pulido et al., 2013; Spencer et al., 2020).

Potency of antiinfective drugs and their interaction with pathogens are currently predicted mostly using susceptibility assay by measuring MIC values. However, they do not provide any information about the antimicrobial activity over time and may have different modes of action and lead to different treatment outcomes. The rate of bacterial killing and time before regrowth of surviving microorganism may affect the design of an optimal dosing interval. Thus, there is need to better understand the *in vitro* pharmacodynamic and pharmacokinetic properties of antiinfective drugs to optimize dosing regimens for drug candidates early in the early antiinfective drug development process and prevent treatment failures. Also, some of the microorganisms represent different growth phases such as that drug-induced killing may exhibit a rapid initial period, accompanied by a decrease in killing rate. Time-kill assay is frequently used in development of new antimicrobial agents and provides monitoring microbial growth and death over a wide range of antimicrobial concentrations over time. These data can be analyzed using mathematical models and are usually the first step in the pharmacodynamic and pharmacokinetic modeling. According to the CLSI guidelines for the standardized time kill assay, bactericidal activity is defined as a reduction of 99.9% of the value of cfu/mL ($\geq 3\log_{10}$), bacteriostatic activity is defined as maintenance or reduction of 99.9% of the value of cfu/mL ($<3\log_{10}$) and synergism is defined as a $2\log_{10}$ cfu/mL decrease from the most active agent alone in the colony count after 24 h (NCCLS, 1999). The time kill assays are mostly used in preclinical studies due to being an easy assay and cheap. However, the problem in time-kill assay is microbial regrowth after initial

reduction in the starting inoculum, which is caused by the selective killing of the susceptible sub-population along with selective amplification of the resistant sub-population (Tam et al., 2005; Foerster et al., 2016; Vaddady et al., 2010; Onufrak et al., 2016).

The most popular approach of antibiotic dosing is to adjust the doses to maintain antimicrobial plasma concentrations above the MIC for a given pathogen over the course of the dosing interval. Antiinfective drugs are classified in two groups based on the pattern of antimicrobial activity. The first group is concentration-dependent killing over a wide range of concentrations, which is resulting in a higher rate and degree of killing with higher concentrations. The pharmacodynamic parameter for concentration-dependent drugs can be simplified as a peak/MIC ratio. For example, intracellularly acting drugs such as aminoglycosides, azalides (azithromycin), ketolides, vancomycin, rifamycin and quinolones follow this pattern. The second group is time-dependent killing, in which drug exposure time lets the drug to bind to the microorganism. The pharmacodynamic parameter for time-dependent drugs can be simplified to the time that serum concentrations remain above the MIC throughout the dosing interval to have cidal effect ($t > \text{MIC}$). For example, antibiotics that targets cell wall such as beta-lactams (penicillins, cephalosporins), clindamycin, macrolides (erythromycin, clarithromycin and except for azalides), oxazolidinones (linezolid), tetracyclines and clindamycin follow this pattern. Indeed, there are numerous antimicrobial drugs which do not follow these classical pattern of classes, but remain in between (Vaddady et al., 2010; Mouton, 2011; Gao et al., 2011).

The results of the time-kill assay are obtained as time-kill curves which can be analyzed using appropriate pharmacokinetic-pharmacodynamic models. These *in vitro* models enable direct comparison of the results of various concentration profiles and more accurate evaluation of the pharmacokinetic-pharmacodynamic relationship than *in vitro* susceptibility assays. There are two classes of *in vitro* models. The first group of constant drug concentrations evaluates constant concentration of drug against microorganism as a function of time. In the second group of variable drug concentrations, drug concentrations fluctuate by dilution or diffusion (Miller et al., 2005).

A new anti-infective agent, Titroleane™, which is obtained from tea tree oil was investigated in terms of its antimicrobial activity using the broth microdilution method. It presented good activity against skin and soft tissue infection pathogens such as methicillin resistant *Staphylococcus aureus*, intra-abdominal infections and oral pathogens, and also fish farming pathogens. The MIC values varied from 0.08% to 2.5%, except for *Campylobacter jejuni*, and *Aspergillus niger* for Titroleane™ (Johansen et al., 2020). Also, novel formulations of antiinfective agents can be assessed in terms of susceptibility. Durgun et al. (2021) prepared posaconazole micelle formulations and confirmed their antifungal activity against *Candida albicans* strains using disc diffusion susceptibility method. Qurt et al. (2018) developed microemulsion formulations as a nano-sized colloidal carrier for dermal delivery of voriconazole or sertaconazole. The formulations found effective against *Candida albicans* species by determining MIC values by the microdilution method.

In order to obtain better antibacterial effect in acne treatment, rifampicin was loaded into niosomal carriers, which improve skin retention of drugs. The prepared formulations showed sustained drug release at controlled rate. Time kill study against *Propionibacterium acnes* and *Staphylococcus epidermidis* also showed that up to 96% of bacteria were killed within 4 h (Begum et al., 2015). Also, spectinamides with the names of 1599 and 1445 as early major candidate antibiotics under development for the treatment of tuberculosis. In order to help in candidate selection and dosing optimisation, time kill curve assays were performed. The results showed that 1599 exhibited concentration-dependent killing whereas 1445 showed time-dependent killing against *Mycobacterium bovis* BCG (Vaddady et al., 2019). In another study, an highly lipophilic antifungal drug, sertaconazole nitrate, was incorporated into polyurethane/polyvinylpyrrolidone/silk fibroin nanofibers and the MICs of the formulations were determined by broth microdilution method. Then, time-kill analysis data showed that all of the drug incorporated formulations showed fungistatic activity against *Candida albicans* during 6 h with average 0.30 log₁₀ cfu/mL decrease. Indeed, this activity was not concentration-dependent for all formulations (Esenturk et al., 2020).

Colistin is an antibiotic drug used to treat the infections by Gram negative bacilli. However, colistin-resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae* strains have occurred in the last years. Ayerbe-Algaba et al. (2018) studied *in vitro* activity of niclosamide in combination with colistin against colistin-susceptible and colistin-resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae* strains using broth microdilution assay and time-kill assay. It was shown that there was an antagonism by niclosamide on the antibacterial activity of colistin against the strains in the first 4 h. However, the combination enhanced the activity of colistin against colistin-susceptible and especially colistin-resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae* strains at 24 h. Babii et al. (2018) investigated antibacterial activity of a novel synthetic sulfur containing tricyclic flavonoid, ClCl-flav, against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* using colorimetric broth microdilution method and time-kill assay. The compound was more susceptible to Gram positive bacteria (MIC = 0.24 µg/mL) than Gram negative ones (MIC = 3.9 µg/mL) and had 32 to 72-fold more activity than other synthetic flavonoids. ClCl-flav exhibited bactericidal activity at concentrations ranging from 0.48 to 15.62 µg/mL. At the concentration of 2 × MIC, *Escherichia coli* and *Klebsiella pneumoniae* cells were killed within 1 h. In another study, antibacterial activity of omadacycline, which is a novel tetracycline class antibiotic drug, was assessed against *Mycobacterium abscessus* that causes severe lung infections. Omadacycline exhibited bacteriostatic activity at 4 mg/L and also bacteriocid activity at the concentration of 16 mg/L. Time-kill curves presented strong concentration-dependent antimicrobial activity for omadacycline (Bax et al., 2019).

Menezes et al. (2020) investigated the effect of daptomycin against vancomycin-resistant *Enterococcus faecium*. Time-kill curve data were used in computer modeling and Monte Carlo simulations to determine the logarithmic reduction in the number of colony-forming units (CFU)/mL with different doses of daptomycin. There was a reduction as >2log CFU/mL over 18 days of daptomycin treatment with doses of 6–10 mg/kg/day in strains with a MIC value of 2 µg/mL. Other studies which are evaluating time-kill curves after susceptibility assay for antiinfective drug development are listed in Table 3.

Table 3 The summary of studies in which time-kill curves after susceptibility assay was used to develop an anti-infective drug.

Anti-infective drug	Disease/microorganism	Susceptibility assay method	Results	References
Antimicrobial peptide (AP114, DPK-060 and LL-37) in cubosomes	<i>Staphylococcus aureus</i> and <i>Escherichia coli</i>	Diffusion	Preservation of antimicrobial activity of the peptide loaded cubosomes (AP114) or slightly enhancement (DPK-060) on <i>Staphylococcus aureus</i> and <i>Escherichia coli</i> Loss on bactericidal activity of LL-37 cubosomes	Boge et al. (2017)
<i>Glycyrrhiza glabra</i> extract and glycyrrhizic acid	<i>Pseudomonas aeruginosa</i>	Broth dilution	Rapid and significant decrease in growth with the extract t 12 h Decrease in growth up to $\sim 1 \log_{10}$ with glycyrrhizic acid and no growth at 24 h	Chakotiya et al. (2016)
Dalbavancin	<i>Staphylococcus aureus</i> and <i>Staphylococcus epidermidis</i> biofilms	Broth microdilution	Time-dependent and concentration-dependent antibiofilm activity A therapeutic option for the treatment of biofilm-associated orthopedic infections, osteomyelitis and biomedical implant infections	Di Pilato et al. (2020)
Fluoxetine and fluconazole combination	<i>Candida albicans</i>	Broth diffusion	Synergistic activity against <i>Candida albicans</i> with the combination Synergistic activity for the biofilms formed over 4, 8, and 12 h Confirmed synergism dynamically by the time-kill curves	Gu et al. (2016)
Sitafloxacin	<i>Neisseria gonorrhoeae</i>	Agar dilution and Etest	Rapidly killing the highly sitafloxacin susceptible <i>Neisseria gonorrhoeae</i> strains at concentrations higher than MIC	Ito et al. (2018)
Voriconazole	<i>Candida albicans</i>	Microdilution broth	Decrease in average $2.00 \log_{10} \text{ cfu/mL}$ at $1/2 \times \text{MIC}$, $1 \times \text{MIC}$, and $2 \times \text{MIC}$ concentrations and $3.30 \log_{10} \text{ cfu/mL}$ at $4 \times \text{MIC}$ concentration after 4 h, exhibiting dose dependent fungicidal activity	Esentürk et al. (2020b)
β -Caryophyllene and <i>Murraya paniculata</i> essential oil	<i>Staphylococcus aureus</i> and <i>Fusarium solani</i>	Microdilution broth	Rapid bacterial killing (4 h for <i>Staphylococcus aureus</i>) and fungicidal effect (2–4 h for <i>Fusarium solani</i>) by essential oil or β -caryophyllene	Selestino Neta et al. (2017)
Ceftazidime-avibactam	Carbapenemase-producing <i>Klebsiella pneumoniae</i>	Microdilution broth	Significant bactericidal activity against the resistant bacteria strains by ceftazidime-avibactam at the concentrations of $2 \times \text{MIC}$, $4 \times \text{MIC}$ and $8 \times \text{MIC}$	Zhang et al. (2018)
Colistin	<i>Pseudomonas aeruginosa</i>	Disk diffusion	Comparable antibacterial activity with the colistin released from hydrogels as the colistin solution A slow killing profile with the colistin-loaded hydrogels	Zhu et al. (2017)
SCY-078	<i>Candida albicans</i> , <i>Candida parapsilosis</i> and <i>Candida tropicalis</i>	Microdilution broth	Fungicidal activity against <i>Candida</i> spp. with SCY-078	Scoreaux et al. (2017)

Conclusions

The fight against infectious diseases is a worldwide health phenomenon that has been up-to-date since ancient times. Anti-infective agents obtained from natural sources in the early ages of humanity have been used over the years and have enabled the preparation of new synthetic or semi-synthetic anti-infective agents. Despite all these technological developments, new anti-infective agents are needed due to factors such as the development of resistance, emerging diseases, and low bioavailability. However, the R&D process of a new drug molecule is both very long and very expensive. For this reason, it is a very rational approach to develop novel drug delivery systems by modifying existing anti-infective agents, which can help to improve their pharmacokinetic and pharmacodynamic profiles. On the other hand, with the isolation of new strains and the discovery of new diseases, the search for new anti-infective agents should continue reasonably.

Drug release and time-kill assays profiles play a key role either in the discovery of new anti-infective agents or the development of drug delivery systems of known drug molecules. Basic information about the effectiveness of an agent on an infectious strain is obtained by *in vitro* time-kill assays. On the other hand, with the release studies, it is determined whether that drug or dosage form can be at the therapeutic concentration in the target area, in the desired period. Both drug release and time-kill tests, which are carried out with simple *in vitro* techniques, take the basic steps for the development of new anti-infective drugs and, provide preliminary information about the performance of drug products, clinical effect or place in the treatment.

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Trends in Antimicrobial Resistance in Healthcare-Associated Infections: A Global Concern

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Introduction

The threat of [antibiotic resistance](#) directly targeting human life has been growing for a long time and [appears to be getting worse](#). Antimicrobial resistance occurs when microbic germs develop the ability to defeat the drugs designed to kill them. While antibiotic resistance is a term limited to bacteria, antimicrobial resistance means the resistance showed by microorganisms, which include bacteria, viruses and fungi. Currently, antibiotic resistance is one of the top most serious and complex public health problems throughout much of the world. The main reason of this negative situation is the antibiotic misuse, as well as the potential for the spread of resistant microbes and the emergence of resistance genes, correlated with a reduced number of new antimicrobials that can be effective against these super pathogens (superbugs). Urgent measures are required to be taken before it reaches a critical point, when many people in populations cannot be treated for life-threatening infections owing to lack of effective antibiotics. The World Health Organization (WHO) Global Action Plan on Antimicrobial Resistance announces that discovery of new antimicrobial molecules and increasing awareness of antimicrobial resistance, fulfilling proper surveillance to figure out the prevalence and arrangements of resistance throughout the world are equally important efforts and parts of the solution for antimicrobial resistance.

It is necessary to know the historical aspects in order to understand the fundamentals and all aspects of evolution of antimicrobial resistance. However, the origin of antimicrobial resistance is still a controversial topic. It would be easy to postulate that antimicrobial resistance is a recent problem throughout much of the world; however, antimicrobial resistance has existed long before antimicrobials were discovered, synthesized, or marketed. A previous study has shown that bacterial strains over 2000 years old isolated from glacial waters demonstrate resistance to ampicillin ([Dancer et al., 1997](#)) and some strains from permafrost over 30,000 years old carry resistance to vancomycin ([D'Costa et al., 2011](#)). From the traditional point of view, antibiotic resistance is frequently considered to be a trait acquired by previously susceptible bacteria, the fundamentals of which can be based on the horizontal acquiring of new genes ([Davies 1997](#)). Recent developments in microbial ecology have led the term of the antibiotic resistance to explain how intrinsic resistance may occur and spread within bacteria which can be defined as an environmental reservoir of all antibiotic resistance genes and also their precursors in both pathogenic and non-pathogenic bacteria ([D'Costa et al. 2006](#); [Gaze et al. 2013](#)).

Penicillin was discovered in 1928 and administered clinically in the 1940s. Since the [discovery of penicillin](#) compound, antibiotics have considerably changed modern medicine. The elucidation of penicillin has been an important cornerstone in the knockout treatment of bacterial infections. Sulfonamides, an important family of antibiotics, were subsequently introduced clinically in the 1930s. Many natural or synthetic antimicrobial molecules regarding different infectious diseases were discovered during the 20th century (e.g., 1940s through 1960s), which was a Golden Age for antibiotics. These molecules struggled with bacterial infections by killing or inhibiting the growth of bacteria. Initially, penicillin and other antibiotics were purified from microorganisms e.g., fungi and bacteria found in nature. Synthetic antibiotics began to be produced in the following years. Therefore, the origin of antibiotics is natural substances synthesized to antagonize or inhibit the growth of other microbes, presumably owing to an evolutionary progress that conserves environmental niches of the producing microbes. Pretty soon the

scientists realized many bacteria were developing resistance to antibiotics by evolving and mutating to the points where antibiotics are targeted. Currently, there are over 100 antimicrobial substances available and it is now evident that antimicrobial resistance is inevitable (Fong 2019; Davey and Wilcox 2007).

How antibiotic resistance develops

Due to the increasing threat that antibiotic resistance poses, it is crucial to first understand the genetic fundamentals of bacterial resistance in order to tackle the problem. Antibiotic resistance is classified as the intrinsic (native) or acquired. Intrinsic resistance is a naturally occurring phenomenon that predates antibiotic chemotherapy which occurs regardless of antibiotic exposure. It is found within the genome of a group of bacterial organisms. Typically, the intrinsic resistance is chromosomal-based and can be estimated considering the taxonomic levels of the bacterial species (Cox and Wright 2013). Genes encoding efflux pumps can either be found on both plasmids (e.g., transmissible elements) or on the chromosome. The classic example of intrinsic antibiotic resistance is the multidrug resistant (MDR) phenotype displayed by Gram-negative bacteria, which are insensitive to most clinically effective Gram-positive antibiotics. The molecular basis of this resistance is the existence of the Gram-negative outer membrane, which is impermeable to many substances, and expression of various MDR efflux pumps that dramatically decrease the intracellular amount of the given drug (Nikaido, 1994).

More recent studies have revealed that intrinsic antibiotic resistance has not appeared owing to the selective pressure of antibiotic use. This situation is not considered as simple as permeability barriers and efflux, but a complicated network of genetic loci that contribute to this phenotype. A recent discovery that soil-dwelling bacteria, most of which are non-pathogenic, are rich sources of biotechnologically resistant determinants, the intrinsic resistance of bacteria has become an exciting new therapeutic target for the design of combinations of drugs (codrugs) that potentiate existing antibiotics. Deletion of elements that consist of the intrinsic resistance renders bacteria hyper-susceptible to antibiotics (Liu et al. 2010). This finding suggests that if it were possible to inhibit these targets, it could be potentiated the activity of various present antibacterial agents, supplied with an expanded armory against previously resistant bacteria (Fig. 1).

Intrinsic antibiotic resistance

Conventional mechanisms of intrinsic or natural antibiotic resistance is a characteristic of all strains from species, which is always transmitted to offspring (vertical transmission). It is the result of the mechanisms: (i) an absence of outer membrane permeability, which inhibits the entry or accumulating of antibiotics inside the bacterial cell, (ii) the existence of efflux pumps, which eject antibiotics and other antimicrobial substances from inside to outside media, (iii) modifying enzyme systems e.g., antibiotic modifying enzymes, target-modifying enzymes, and (iv) an absence of molecular target sites required by the antibiotic for binding, even if the antibiotic enters the cell (Cox and Wright 2013; Michăescu et al., 2007) (Table 1).

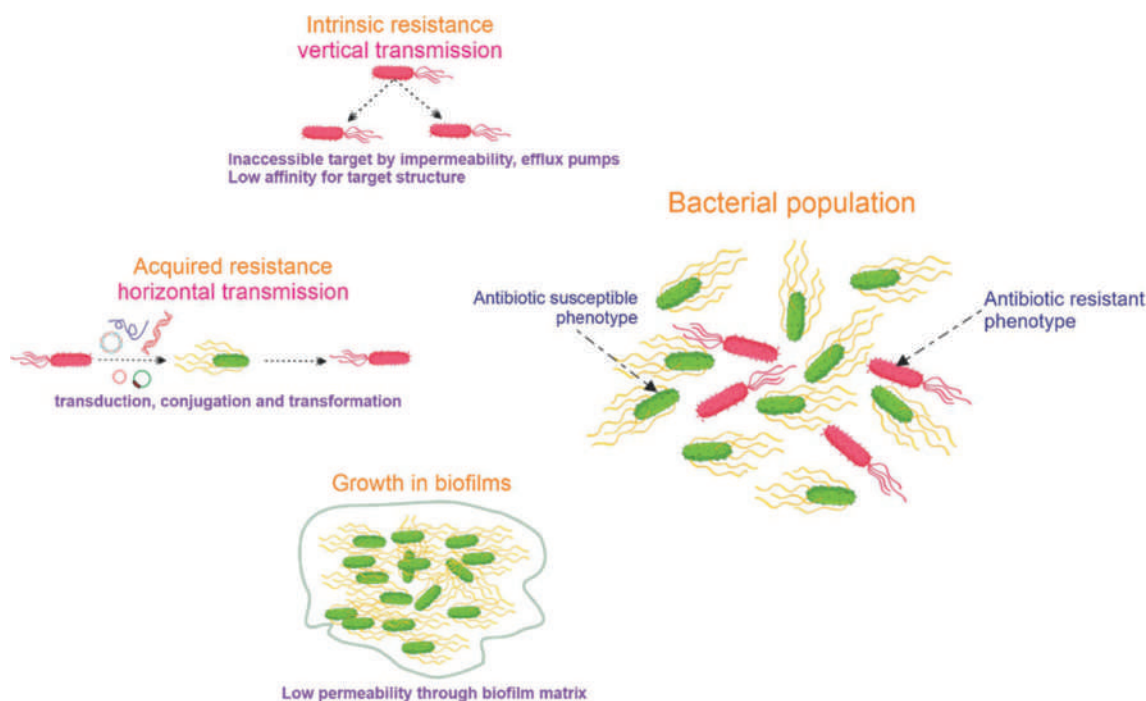


Fig. 1 Schematic representation of bacterial vertical and horizontal resistant ways.

Table 1 The main antibiotic resistant mechanisms and specific antibiotic groups.

<i>Efflux pump</i>	<i>Reduced uptake</i>	<i>Inactivation of enzymes</i>	<i>Target modification</i>
β -Lactams	β -Lactams	β -Lactams	Rifamycin
Aminoglycosides	Tetracyclines	Aminoglycosides	Aminoglycosides
Tetracyclines	Fluoroquinolones	Rifamycins	Vancomycin
Macrolides		Macrolides	Macrolides
Fluoroquinolones			Fluoroquinolones
			β -Lactams

Outer-membrane permeability

The one of the most important structural differences between Gram-positive and Gram-negative bacteria are primarily related to their cell wall composition. Gram-positive bacteria have cell walls composed mostly of a substance unique to bacteria known as *peptidoglycan*, or murein. Gram-negative bacteria have cell walls with only a thin layer of peptidoglycan and an outer membrane with a lipopolysaccharide component not found in Gram-positive bacteria. Scientists have shown that the peptidoglycan of Gram-positive bacteria possess a great permeability threshold and this is attainable to molecules of up to 30–57 kDa (Scherrer and Gerhardt 1971), its relatively thick peptidoglycan layer lets small molecules to easily penetrate, which is the molecular basis underlying why Gram-positive bacteria are more intrinsically susceptible to various antibiotics (Randall et al., 2013).

However, Gram-negative bacteria are intrinsically insusceptible to many of these antibiotics due to the existence of a much finer molecular sieve and asymmetrical lipid bilayer called outer membrane which covers the relatively thin peptidoglycan layer (Vaara, 1992). The outer membrane is usually thought of as part of the outer leaflet of the dynamic membrane structure and contains structures that help bacteria adhere to targeted cells. Unlike the most of biological membranes, Gram-negative bacteria possess an outstanding lipid composition called lipid A molecules which covalently bonded to polysaccharide units. The periplasmic space includes the periplasm of a concentrated gel-like matrix. The periplasm space can also act as reservoir for virulence factors and a dynamic flux of macromolecules representing the cell's metabolic status and its response to environmental factors. On the other hand, the fatty acid chains within the lipopolysaccharides are generally saturated which allows for tight packing of the hydrocarbon chains which contribute to reducing membrane fluidity (Obst et al., 1997). The outer membrane of Gram-negative bacteria is impermeable to specific classes of antibiotics such as glycopeptides, macrolides, lincosamides, streptogramins.

The role the outer membrane acts in reducing the intrinsic susceptibility of Gram-negative bacteria to many antibiotics is very clear. Outer membrane-based intrinsic resistance is particularly apparent in the opportunistic pathogen *Pseudomonas aeruginosa*.

Efflux pumps

Bacterial cells have highly conserved DNA sequences in their genome that are transcribed and translated to efflux pumps which are capable of moving a variety of different toxic substances out of extracellular area e.g., antimicrobials, heavy metals, organic pollutants, naturally occurring antimicrobials, and quorum sensing signals through active efflux pumps. Over 300 gene products are recommended to transport known substances effectively, out of which ~20–30 transport antibiotics and other drugs. This active efflux pumps are in fact major cellular products and responsible for various types of resistance to bacterial pathogens because bacteria could have adapted efflux pumps to canalize toxins out of the cytoplasm and into extracellular media (Blanco et al. 2016).

With the prokaryotic kingdom, there are six major families of efflux proteins that span the bacterial membrane: the adenosine triphosphate (ATP) binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the proteobacterial antimicrobial compound efflux (PACE) family (Hassan et al. 2013), the multidrug and toxic-compound extrusion (MATE) family (Kuroda and Tsuchiya, 2009), the small multidrug resistance (SMR), and the resistance-nodulation-division (RND) (Tseng et al. 1999). Whereas RND and PACE efflux pumps are unique to Gram-negative bacteria, MATE, ABC, SMR, and MFS are found in both Gram-positive and Gram-negative bacteria (Nikaido 2011; Hassan et al. 2018).

Efflux pump mediated antibiotic resistance was firstly discovered as a mechanism of resistance to tetracycline (McMurtry et al. 1980). Recently, it is well-documented that efflux pumps form the most type of resistance component, and exist in all organisms from prokaryotes to superior eukaryotes, among those that have been explained. Active efflux pumps can either be molecule specific, and only export one substrate, or they can also be more broad-range, and export structurally different classes of substrates (Piddock, 2006). It has been suggested that efflux pump mediated antibiotic resistance might be a random by product of the broad-spectrum molecule specificity displayed by such pumps; characteristically efflux pumps transport more than one substrate and they frequently exclude toxic substances that have been synthesized by the host (Piddock, 2006).

Deletion of elements of a putative efflux system (MexAB-OprM) in *Pseudomonas aeruginosa* transformed the strain hyper-susceptible to distinct antibiotics to which it was previously resistant (Poole et al., 1993), suggesting that certain classes of antibiotics cannot be used to treat infections caused by bacteria relative intrinsic resistant. It is well-documented that antibiotic-inactivating enzymes, such as β -lactamases, can be intrinsically expressed in certain enterobacteria like *Enterobacter cloacae*, causing resistance to aminopenicillins (De Almeida et al., 2017).

Acquired resistance

Resistance to antimicrobials can also be categorized as acquired which occurs when one encounters bacteria that were initially susceptible but then become resistant to antibiotics. While many factors are effective in the development of acquired antibiotic resistance, the main drivers are antibiotic overuse or misuse. Acquired antimicrobial resistance occurs either through gene mutations or through the obtaining of foreign genetic material that encodes resistance genes.

Because bacteria can reproduce rapidly, this can cause evolutionary changes through random gene mutations which could be seen in relatively short periods. Overexposure to antimicrobials forms an evolutionary selection pressure on bacteria and gives a selective survival advantage that has achieved the resistance mutations (Oz et al. 2014). One of the mechanisms of acquired antibiotic resistance is the modification of the specific targets for antibiotics. When antibiotics are accumulated into the bacterial cell at a sufficient level, they bind specific targets which are generally constitutional proteins. Numerous strains become resistant to certain antibiotics through a mutation process, which led to the synthesis of a modified target protein, which had a low affinity for the antibiotic. A recent paper has shown that substituting even a single amino acid into a target protein structure can lead to changes in its ability to bind antibiotics (Kotra et al., 2000).

The modification of the multimodular penicillin binding proteins which are involved in cell wall synthesis is the one of the best examples. Modification of penicillin binding proteins can lead to loss of affinity for β -lactam antibiotics. The reduced binding affinity is usually associated with the acquisition of a new form of penicillin binding proteins.

For example, the acquisition of a gene encoding a different enzyme, called *PBP2a*, which has much lower affinity for antibiotics than the wild type form results in resistance to antibiotics in staphylococci (Michăescu et al., 2007).

Acquired antimicrobial resistance may be caused by serial mutations or horizontal gene transfer between strains of the same or different species. Normally, genetic materials are passed down from parent to progeny called vertical gene transfer. Additionally, bacteria and some lower eukaryotes are unique in that they can transfer genetic material from one cell to another, a process known as horizontal gene transfer. There are three ways for bacteria to transfer their genetic material horizontally: conjugation, transduction, and transformation.

Conjugation is the transfer of genes directly from one cell to another through cell-cell contact in which DNA molecule is transferred between bacteria through a tube between the cells. In transduction, genetic material is accidentally moved from one to another through bacteriophage mobility. Transformation is direct uptake of piece of DNA molecule from the environment, usually from a lysed bacterium (Fig. 2).

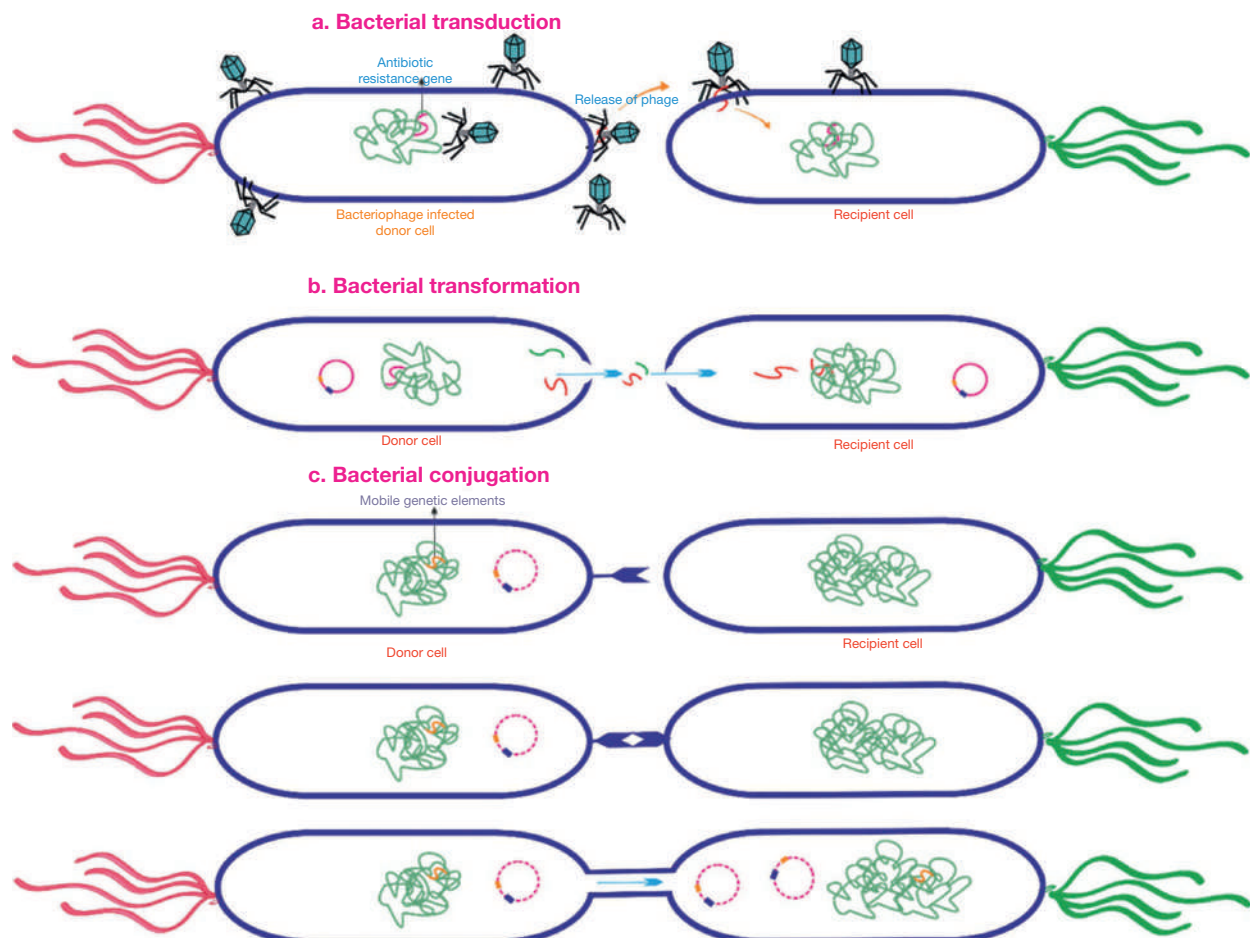


Fig. 2 Schematic demonstration of bacterial gene transfer ways.

Antibiotic resistance genes are generally integrated on plasmids, but they also can reside on genomic islands, insertion sequences, miniature inverted repeat transposable elements, mobile genetic elements (transposons, integrons). Additionally, bacteria can also transfer plasmids to one another through a process termed conjugation. Resistant genes can be exchanged between plasmids and chromosomes, between bacteria, and even bacterial species (Levy 2002).

Plasmids are relatively small (4–400 kbp), extrachromosomal self-replicating DNA molecule which is physically separated from chromosomal DNA. Naturally occurring plasmids are most commonly found as double-stranded (dsDNA) and covalently closed circular DNA (ccDNA) in bacteria which can replicate independently within a cell. In nature, plasmids often carry resistance to (i) one or more groups of antibiotics, (ii) to heavy metal cations (Hg^{2+} , Ni, etc.) and (iii) to anions (bromine, tellurite, etc.) (iv) intercalating compounds like acridine, virulence genes enabling the host cell to survive in an environment that would otherwise be lethal or restrictive for growth (Grumezescu et al. 2016). Gene names and functions on conjugative and mobilizable plasmid vary from plasmid to plasmid.

Certain plasmids might be conjugative, also known as male factors, conjugons, transferons, fertility factors, playing an important role in the dissemination of antibiotic resistance genes (Sandegren et al., 2012). The transfer of plasmid-associated antibiotic resistance is generally versatile and might be both vertically and horizontally among pathogens; however, it is also possible between pathogenic and commensal bacteria. Plasmids can be disseminated rapidly through bacterial communities. For example; the CTX-M extended-spectrum β -lactamases (ESBLs), frequently linked to resistance in *Escherichia coli*, evolved as a plasmid encoded enzyme from environmental *Kuvera* genus (Davies and Davies 2010).

Transposons (jumping genes) are mobile genetic elements that can change its position within a genome, sometimes creating or reversing mutations and altering the cell's genetic identity and genome size. Transposons make up a large fraction of the genome which are responsible for much of the mass of DNA in a eukaryotic cell. They are important vehicles of resistance to antibiotics particularly streptococci (Gram-positive bacteria), but also occur Enterobacteriaceae (Bourque et al. 2018).

Insertion sequences are the simplest transposable elements described in bacteria which consist of approximately 1000 bp DNA sequence. They are cryptic mobile DNA elements and only code for proteins implicated in the transposition activity. Insertion sequences modulates gene expression, efflux mechanisms, resulting in contribute to antibiotic resistance. For example; IS1 and IS10 insertion causes the tripartite overexpression of AcrAB/TolC efflux pump *Salmonella enterica*. Insertion sequences could move either conservatively or replicatively (Siguier et al., 2014).

Integrons are mobile genetic platforms with specific structure that allow bacteria to adapt and evolve rapidly through the stockpiling, excision and expression of new genes. They are embedded in a specific genetic structure termed integron cassette that usually carries one promoterless open reading frame (ORF) together with a recombination site (*attC*). Integron cassettes are incorporated to the *attI* site of the integron platform by site-specific recombination reactions mediated through the integrase enzyme (Escudero et al., 2015). The mobility of resistance genes in other plasmid or in chromosome triggers the possibility of the linked transfer of gene clusters, which causes the simultaneous acquisition of several genes for resistance to several classes of antibiotics, causing the occurrence of MDR phenotypes. Integrons carry a wide variety of genes encoding β -lactamases (Andreotti et al. 2007).

Bacterial biofilms are generally pathogenic in nature and can cause nosocomial infections. Infections with bacterial or fungal biofilms have appeared as a major public health concern since biofilm-growing cells are considerably resistant to both antibiotics and host immune defenses. A recent paper has revealed that biofilm-associated infections demonstrate a significant complexity for the fact of resistance and treatment of chronic infections, which are participated in approximately 65–80% of all infections in developed countries (Lewis 2007).

Multiple drug resistance (MDR) or multidrug resistance

Bacteria can possess not only intrinsic but also acquired resistance as well as more than one mode of action of resistance. This can cause multidrug resistant (MDR), the extensively drug resistant (XDR), and pan drug resistant (PDR) phenotypes. PDR means resistant to all antimicrobial agents. A group of international scientists came together through a joint initiative by the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC), to form a standardized terminology in order to explain acquired resistance phenotypes, known as ESCAPE i.e., *Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter* spp., *Pseudomonas aeruginosa*, *Enterobacter* sp., and all bacteria responsible for the majority of hospital-acquired infections and prone to multidrug resistance in Stockholm in January 2008 (Magiorakos et al. 2012).

Explaining different stages of MDR in pathogens, the distinct terms extensively drug-resistant and pandrug-resistant were introduced. Extensively drug-resistant states the non-susceptibility of one bacteria species to all antimicrobial agents except in two or less antimicrobial categories. Within XDR, pandrug-resistant (PDR) means the non-susceptibility of bacteria to all antimicrobial agents in all antimicrobial categories. The explanations were published in the Clinical Microbiology and Infection in 2011 within openly accessible (Magiorakos et al. 2012).

Mechanisms of antimicrobial resistance

It is significantly important to understand the specific molecular mechanisms in order to put into effect the new approaches and therapeutic strategies. Broadly, the resistance mechanisms can be outlined as follows:

- Reduced drug uptake through either reduced outer membrane permeability or increased active efflux of the antimicrobials across the cell envelop.
- Antimicrobial inactivation and/or modification by the way production of specific enzymes that both damage or alter the drug, rendering it inactive.
- Target shifts or binding site shifts e.g., changing of penicillin binding proteins.
- Biochemical driving force through metabolic pathways, such as enterococci uptake folic acid from outside and use to neutralize the effects of trimethoprim sulfamethoxazole.

Naturally occurring antimicrobial compounds and their mode of action

Awareness of antibiotic resistance shifted the research focus toward naturally occurring compounds with antibiotic actions. Since ancient times, natural sources such as plants, fungi, bee products have been experimentally used to cure numerous infectious diseases. Doubtlessly, naturally occurring products are the source of most of antibiotic scaffolds in current clinically used drugs. Some plant-based essential oil components are well known for their antimicrobial properties such as flavonoids and they have been shown to act both at the level of the bacterial cell wall and on protoplasmic components (Arslan and Celik 2008; Celik et al., 2008). The proposed antibacterial mode of actions of the essential oils contain the breakdown of cell walls, cell membrane damage with changing permeability, leading to loss of cell substance; inhibition of protein and nucleic acid synthesis, coagulation of protoplasm, the degradation of membrane proteins, consequently reducing the proton move force. For example; phenolic acids were discovered to trigger remarkable changes in the cell wall's hydrophobicity, causing the formation of pores in the plasma membranes that arrest nucleic acid synthesis and energetic metabolism in both Gram-positive and Gram-negative bacteria (Del Rio et al. 2013; Mandal et al., 2010).

Brief descriptions of the most prominent, clinically approved and their antibacterial modes of actions are summarized below.

β -Lactams

All β -lactam antibiotics consist of a 4-membered β -lactam ring system as carbon-skeletal backbone and bind to penicillin-binding proteins. Clinically, five classes of β -lactams including penams, cepheems, carbapenems, clavams, monobactams are important (Fig. 3A). The electrophilic β -lactam antibiotics mimic the D-Ala-D-Ala termini of the pentapeptide part of peptidoglycan layer (Fig. 3B). Penams, cepheems, and carbapenems covalently bind to penicillin binding proteins, preventing the enzyme transpeptidase (Tipper and Strominger, 1965).

Penicillins are naturally occurring compounds belonging to a special class of compounds, the β -lactam antibiotics, of great clinically, pharmacologically, and economically importance. β -lactam antibiotics are typically used to treat a broad array of Gram-positive and Gram-negative bacteria. Natural penicillins are derived from the *Penicillium* fungi/mold and produced from the fermentation of the fungus *Penicillium chrysogenum*. The cephalosporins are a class of cephem-based β -lactam antibiotics originally derived from the fungus *Cephalosporium* (Wilke et al., 2005; Sharma et al. 2007).

β -Lactam resistance

β -lactamases are enzymes (E.C.3.5.2.6) synthesized by bacteria which hydrolyze the β -lactam ring structures of β -lactam antibiotics, and terminate the effects of them. Gram-negative bacteria possess β -lactamase-mediated antibiotic multi-resistance but have also been described in *Staphylococcus aureus* (Wilke, Lowering, Strynadka 2005). β -Lactamases inhibitors such as clavulanic acid, sulbactam and tazobactam have been introduced to overcome the β -lactamases activity.

Extended-spectrum beta-lactamase (ESBL)

These enzymes commonly express β -lactamases such as TEM-3, TEM-4, and SHV-1 which confer multi-resistance to a broader expanded-spectrum cephalosporins. The ESBLs confer resistance to penicillins, third generation cephalosporins such as cefotaxime, ceftriaxone, and ceftazidime, and monobactam aztreonam (Emery and Weymouth, 1997). However, a recent research has demonstrated that certain β -lactamase and ESBLs are genetically linked genes (Bradford 2001).

Aminoglycosides

Streptomycin was firstly discovered from a strain of *Streptomyces griseus* with aminoglycoside structure by Waksman and colleagues (Waksman et al., 1946). Aminoglycoside antibiotics contain a core aminocyclitol ring attached to varying extents by saccharide units. The amino and hydroxy groups of the aminoglycosides react with the 16S rRNA in the 30S bacterial ribosomal subunit

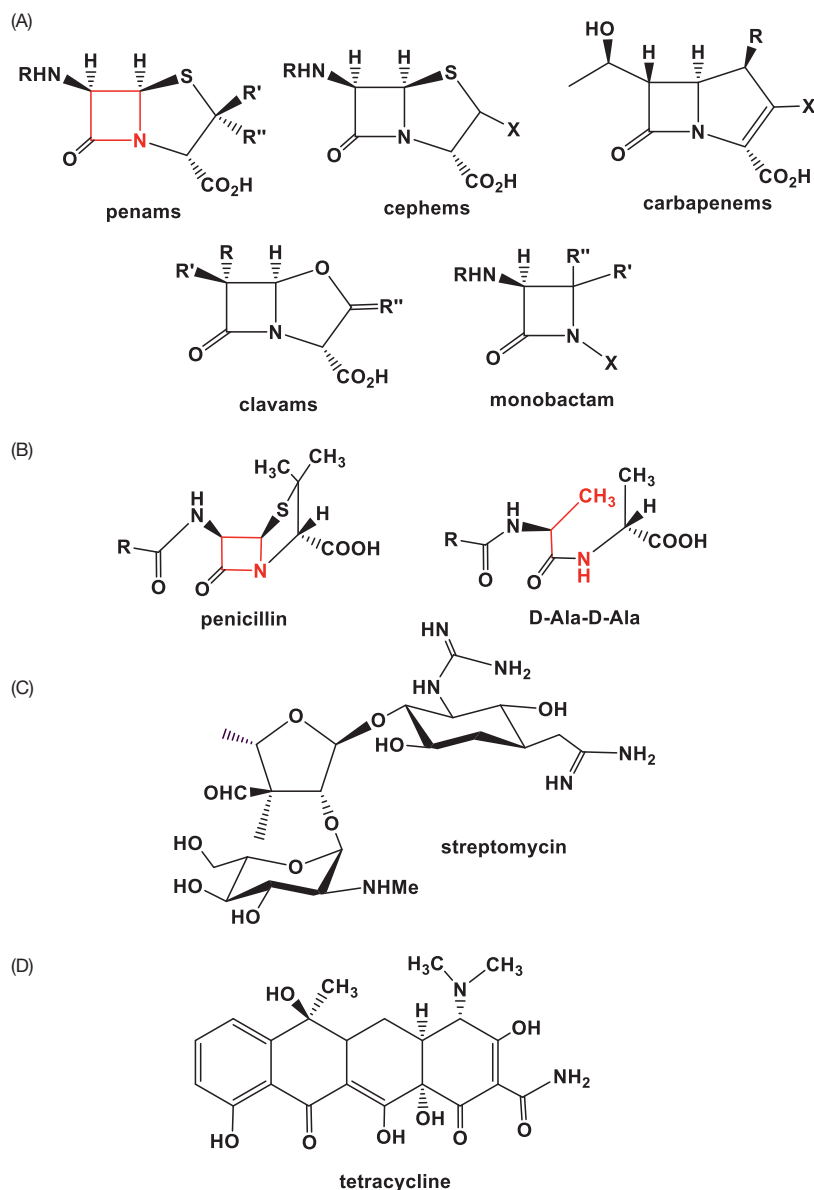


Fig. 3 Chemical structure of antibiotics. (A) Major structural classes of β -lactam antibiotics. (B) Molecular structure of penicillin and D-Ala-D-Ala. (C) Streptomycin structure. (D) Tetracycline structure.

irreversibly through a network of hydrogen bonds (Fig. 3C). This arrests protein synthesis by interfering with the binding of **formyl-methionyl-tRNA** to the 30S subunit and renders the bacterium dysfunction. Resistance to aminoglycoside-based antibiotics occur mainly by enzymatic modification of drugs or the targets. The enzyme ribosomal methyltransferases modify the bacterial 16S rRNA subunit and confer aminoglycoside resistance particularly Gram-negative pathogens (Sharma et al. 2007).

Macrolides

The macrolides are the **polyketide** class of **naturally occurring compounds** with macrocyclic lactone ring containing eight or more atoms, and polyketide. Macrolides are generally bacteriostatic in that they suppress or inhibit bacterial growth rather than killing bacteria completely. **Erythromycin** was the first macrolide-based antibiotic discovered from a soil actinomycete *Saccharopolyspora erythraea* (Klein 1997). Clinically, macrolide drugs are most efficient against Gram-positive bacteria, but they also possess effectiveness against several Gram-negative, particularly upper respiratory tract pathogens. Macrolides react with the peptidyl transferase enzyme of the 50S ribosomal subunit, inhibiting protein-chain elongation. Active macrolide efflux is common in most Gram-positive pathogens. During the protein synthesis, the hydrophobic side of the macrolide molecule links to specifically the sidewall of the exit tunnel and leads to premature release of short peptidyl-tRNAs (Katz and Ashley 2005).

Tetracyclines

Tetracyclines are well known for their broad-spectrum of activity, spanning a wide range of Gram-positive and Gram-negative bacteria. They consist of a tetracyclic polyketide nucleus of fused six membered rings (Fig. 3D). Chlortetracycline and oxytetracycline were discovered in 1948 and 1950, respectively. Currently, they are isolated directly from various strains of *Streptomyces* or produced semi-synthetically from those isolated compounds. Tetracyclines block protein synthesis through binding reversibly to the bacterial 30S ribosomal subunit and interfere with the aminoacyl tRNA from binding to the A site of ribosome. Tetracyclines have also been found to inhibit matrix metalloproteinases (Connell et al., 2003).

The recent surveillance studies have revealed that the prevalence of resistance to tetracycline in certain European countries was detected to be 66.9% and 44.9% for extended-spectrum β -lactamase (ESBL) producing *Escherichia coli* and *Klebsiella* species (spp.), respectively (Jones et al. 2014). Similar results were found in another study by Mendes and colleagues that the global tetracycline resistance percentages were 24.3% and 8.7% for *Streptococcus pneumoniae*, and methicillin-resistant *Staphylococcus aureus* (MRSA) respectively (Mendes et al. 2015). Tetracycline resistance in Gram-negative bacteria is usually the result of active efflux pump, while in Gram-positive bacteria is through the acquisition of mobile genetic elements carrying tetracycline resistance genes, mutations within the ribosomal binding site, and/or mutations causing induced expression of intrinsic pathways (Jones et al. 2014).

Antimicrobial stewardship programs (ASPs)

Antimicrobial Stewardship Programs were implemented in healthcare settings for fighting bacterial antimicrobial resistance, improving guideline compliance, and lowering the use of broad-array antimicrobial agents. In 2017, the Joint Commission has developed standards for Antimicrobial Stewardship Programs for healthcare settings which were announced and put into action. The role of ASPs can be outlined as follows: to develop guidelines and protocols for proper use of antibiotics, restrict the use of antibiotics and raise awareness on misuse/overuse.

Most of developed and high-income countries including the US and European Countries, have implemented national programs as well as regulations to target antibiotic resistance; however, the emergency action plans should be implemented immediately particularly in low and middle income countries.

Conclusion

Currently, it is estimated that drug-resistant infections leads to over 700,000 deaths per year. In 2019, World Health Organization released a report stating that the deaths are calculated to upward to 10 million annually by 2050, exceeding the diseases of diabetes, heart disease, and cancer as the leading cause of death in humans, if it continues like this.

Globalization makes a significant contribution on the spread of antimicrobial resistance. Global trade, transportation and travel to distant destinations leads to spread resistant organisms more than ever before (Nordman et al., 2011). Social factors, the increasing number of nosocomial infections, and misuse/overuse of broad-spectrum antibiotics trigger the spread of pathogens. It is prominent that antimicrobial resistance is inevitable.

Alternative therapies may attenuate the selective pressure of antimicrobial chemotherapy. In fighting antimicrobial resistance, phage therapy and its products like lysin have become the center of interest again. Actually, the phage therapy has been used for a longer time than antibiotics in the combating against the bacteria, and they have proved to be high efficacy, selective, cost effective, and showing low toxicity and high tolerance (Twort 1915). A recent clinical study has shown that efficacy of phage therapy in the treating of various types of antibiotic resistant Gram-negative infections, suggests a comprehensive research is needed (Sulakvelidze et al., 2001). Researches on additional therapies such as immune stimulation, vaccination, topical agents, nanomaterials, prebiotics/probiotics are still ongoing (Renwick et al., 2016).

Undoubtedly, the vaccine development efforts on SARS-CoV-2 can help improve the vaccine therapy against antimicrobial resistant bacteria.

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Human Microbiome and Disease

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Glossary

α-Diversity Compositional diversity within a sample.

β-Diversity Compositional diversity between samples from the same habitat.

Biomarker Something that can be objectively measured to provide an indication of a biological process, whether normal or pathologic.

Colonization Presence of microbes on a surface.

Community state type Grouping of a community of microbes by phylotype composition and abundance.

Dysbiosis Alterations in the microbiota, usually with negative effects.

Fermentation Metabolic process that breaks down nutrients, may also refer to bioprocessing methods for microbial culture.

Genomics Study of the whole genomes of organisms, usually involving DNA sequencing and bioinformatics approaches in the analysis.

Gnotobiotic Environment where the microbial composition is well-defined, including germ-free conditions.

High-throughput screening Process for the automated testing of large numbers of samples.

Metagenome Combined genome of all the microbes in a community.

Microbiome Genetic content of the microbiota.

Microbiota Community of all the microbes living on or in a host.

Niche Environment where a microorganism can grow, including effects from nutrient availability, presence of competitors etc.

Operational taxonomic unit Species level classification system based on homology between reads for 16S rRNA profiling.

Organoid 3D structures with organ-like spatial organization for in vitro culture.

Probiotics Live microorganisms forming part of certain foods or supplements.

Introduction

The human microbiota is composed of all microbes, on the order of trillions, that live in or on the human body, including in the gut, oral and urogenital tracts, airways and placenta and on the skin and ocular surfaces. These microorganisms, including bacteria, viruses, fungi and protozoa, can be commensal, symbiotic or pathogenic. While microbes are often negatively associated with the concepts of infection and disease, the microbiota actually also plays an important role in maintaining the normal physiology and health of an individual, including influences on immune system function, metabolism and nutrition. The human microbiome, which encompasses the total genetic content of these microbes, is large and variable, especially when compared to the much more limited and substantially similar genetic makeup of humans. Thus, understanding the human microbiome (see [Table 1](#) for definitions of relevant terms) has become an interesting and challenging area of research. This article begins with a discussion of the Human Microbiome Project and its outcomes and continues with the structure and dynamics of healthy microbiota. After introducing state-of-the-art methods for appraising and studying the human microbiome in the laboratory, the second half of this article is focused on the dysbiosis of the microbiome and its connections with disease, particularly for the gut, oral, urogenital and skin microbiomes.

Table 1 Definition of relevant terms.

Term	Definition
α -Diversity	Compositional diversity within a sample
β -Diversity	Compositional diversity between samples from the same habitat
Biomarker	Something that can be objectively measured to provide an indication of a biological process, whether normal or pathologic
Colonization	Presence of microbes on a surface
Community state type	Grouping of a community of microbes by phylotype composition and abundance
Dysbiosis	Alterations in the microbiota, usually with negative effects
Fermentation	Metabolic process that breaks down nutrients, may also refer to bioprocessing methods for microbial culture
Genomics	Study of the whole genomes of organisms, usually involving DNA sequencing and bioinformatics approaches in the analysis
Gnotobiotic	Environment where the microbial composition is well-defined, including germ-free conditions
High-throughput screening	Process for the automated testing of large numbers of samples
Microbiota	Community of all the microbes living on or in a host
Microbiome	Genetic content of the microbiota
Metagenome	Combined genome of all the microbes in a community
Niche	Environment where a microorganism can grow, including effects from nutrient availability, presence of competitors etc.
Operational taxonomic unit	Species level classification system based on homology between reads for 16S rRNA profiling
Organoid	3D structures with organ-like spatial organization for in vitro culture
Probiotics	Live microorganisms forming part of certain foods or supplements

The human microbiome project (HMP) and healthy microbiota

Research on human microbiota can be found dating back to the 1960s (Schaedler et al., 1965; Rotimi and Duerden, 1981), but a major step forward was achieved through the Human Microbiome Project (HMP). The HMP was initiated by the National Institutes of Health (NIH, United States) in 2008, extending on the Human Genome Project. The goals of the HMP were to recruit at least 250 healthy individuals; sample microbiota from the skin, oral cavity, nasal cavity, gut and vagina; and ultimately to characterize the metagenome, combined from all these microbiomes, to determine the role of the human microbiome in both disease and healthy states (Aagaard et al., 2013). In theory, this would allow the association of the microbiome with variables such as disease, age, nutrition, medication and environment to be studied (The NIH HMP Working Group et al., 2009). The HMP was additionally expected to lead to technical advancements in bacterial culture, isolation of single microbial cells, purification of single species, and genomic amplification and cloning from single cells (The NIH HMP Working Group et al., 2009). Analytically, the HMP used a combination of taxonomic profiling by 16S ribosomal RNA gene sequences and whole genome shotgun sequencing to achieve metagenomics profiling (The NIH HMP Working Group et al., 2009). As outputs, it has provided collections of clinical samples and reference genomes, in addition to the analytical methods themselves (Méthé et al., 2012). The HMP was followed up by the Integrative Human Microbiome Project (iHMP) in a second phase from 2013 to 2016 with a focus on longitudinal studies of three more specific disease conditions: pre-term birth, inflammatory bowel disease (IBD) and type 2 diabetes mellitus (T2DM). In this project, the subjects were routinely sampled over a period of 3 years, and compositional and functional parameters of the their microbiomes were determined using sequencing and mass spectrometry techniques (The Integrative HMP (iHMP) Project Consortium, 2014).

The HMP has required that standardized 16S sequencing and analysis protocols be established (Méthé et al., 2012; Jumpstart Consortium Human Microbiome Project Data Generation Working Group, 2012), and these involve an operational taxonomic unit (OTU) based community structure. Amplification and sequencing is ideally performed on the same platform (Roche 454 FLX Titanium for the HMP), and a window covering 3–5 variable regions of the 16S rRNA gene is sequenced due to its utility in taxonomic classification (Méthé et al., 2012; Jumpstart Consortium Human Microbiome Project Data Generation Working Group, 2012). In bioinformatics analysis of the sequencing data, the raw read sequences are then preprocessed and denoised, assigned into a specific bacterial taxonomy using OTU databases and characterized in terms of diversity, often using Principal Coordinates Analysis (Caporaso et al., 2010; Moon and Lee, 2016). To increase the quality of the raw sequencing data, the 16S sequencing methods are regularly improved, such as through the low-error 16S rRNA amplicon sequencing method (Faith et al., 2013). Beyond 16S sequencing, next-generation sequencing may also play an important role in the identification of low abundance species and addressing issues such as short read problems (Malla et al., 2019). Metagenomic whole genome sequencing, or full shotgun metagenomics, not only allows for the same taxonomical profiling as 16S sequencing but also provides functional profiles of the microbial communities through assembly of the raw read sequences, gene prediction and functional annotation (Moon and Lee, 2016).

Within the HMP, an open-source platform called mothur was developed as part of the data processing pipeline (Schloss et al., 2009). The generation of vast amounts of data from the HMP and iHMP, as well as from other studies on the human microbiome, has required the integration of computational modeling approaches, as well as their associated methodological advancements, into human microbiome research (Chowdhury and Fong, 2020). These techniques have been used to extend the genomic information to make predictions of function, for example through the use of ancestral-state reconstruction algorithms (Langille et al., 2013) or

genome-scale metabolic modeling, which can provide information on microbe-microbe and host-microbe interactions (Thiele and Palsson, 2010; Gu et al., 2019). One of the largest genome scale metabolic models, with 773 gut bacteria, has been used to predict nutrient requirements for the development of chemically defined medium (Magnúsdóttir et al., 2017). Other computational approaches, such as deep neural network interpretation algorithms, have been used to explain the contribution of features of the microbiome data on disease characteristics, as has been done to classify insulin resistance vs. sensitivity on data coming out of the iHMP, although only consideration of multiple features could sufficiently classify the samples (Huang et al., 2021b).

A main outcome of the HMP was to confirm that the microbiome differs a lot, even among healthy individuals. For example, among the different sites sampled, the oral and stool samples had the greatest compositional diversity, whereas the samples from the vagina had the least (Huttenhower et al., 2012). Further analysis showed that, despite variations in composition, the metabolic pathways were often stable among individuals (Huttenhower et al., 2012). Details on the composition of the gut, skin, oral and urogenital microbiomes, of which some information has originated in the HMP, are provided later in this article. The iHMP has led to resources, such as the Inflammatory Bowel Disease Multi'omics Database, as well as insights into the dysbiosis of the gut microbiome in IBD. For example, facultative anaerobes were shown to increase while obligate anaerobes decreased, and disturbances in microbial transcription, metabolite pools and antibody levels in host serum were observed (Lloyd-Price et al., 2019).

Even among healthy individuals, there is a wide variation in the composition of the human microbiota. A concept that has been put forward is a "functional core," which characterizes the healthy microbiota by its metabolic and molecular functions rather than by the specific composition of microorganisms (Bäckhed et al., 2012; Shafquat et al., 2014). In this way, the collection of genes and pathways is more important than the specific microbes that are present (Bäckhed et al., 2012), and the functional niche can be filled with different microbial compositions (Shafquat et al., 2014). Thus, in addition to the normal microbes, their function, ecological properties (diversity of taxa present), and temporal dynamics are important to characterize healthy human microbiota (Lloyd-Price et al., 2016). These functions are related to the microbes themselves, such as transcription, translation, and energy production; to host-microbe interactions, such as adhesion and production of vitamins and immunostimulatory compounds; and to specific sites, such as glycosaminoglycan degradation and short-chain fatty acid production in the gut (Lloyd-Price et al., 2016). Thus, integration of the microbiome with metabolic models is critical to understand the mechanisms linking the microbiome to healthy and diseased states (Sung et al., 2016; Noecker et al., 2015).

State-of-the-art methods for appraising the human microbiome

In vitro cell culture offers a convenient approach to study different cellular processes and to probe mechanisms of disease; however, the complexity of the human microbiota has been difficult to replicate using traditional cell culture methods. Sometimes referred to as synthetic gut microbial ecosystems, these in vitro systems still only involve the culture of limited numbers of bacterial strains and start from traditional batch as well as multi-stage continuous fermentation culture systems (Vrancken et al., 2019). Emerging technologies for three dimensional (3D) in vitro tissue models, such as organ-on-chip devices and organoid cultures, are being developed, and these have started to be applied to the study of the human microbiota. The most advanced systems replicate key features of the gut microbiota, and in vitro models of the skin (Niehues et al., 2018) and oral cavity have also been developed, such as human callus as a substrate for skin pathogens (van der Krieken et al., 2016) and mixed fungal-bacterial oral biofilms (Chevalier et al., 2018). Further, gnotobiotic animal models have been developed to study the human microbiota in vivo, although certain physiological and metabolic limitations do exist.

Organoids are an emerging technique that has been developed in the last decade or so for the 3D culture of primary cells, typically adult stem cells or pluripotent stem cells, in a microscale structure that mimics the natural organization of cells within a tissue. A key aspect of organoids is that they can be established with an individual's cells and thus can be used to determine patient-specific responses. Some of the first organoids were developed by Sato et al., who used intestinal derived cells that self-organized into crypt and villus structures (Sato et al., 2009). Of relevance to the human microbiota, organoids have been used as in vitro models for infection with *Helicobacter pylori* (*H. pylori*) and *Salmonella enterica* (*S. enterica*) as well as for studying gut-microbiota interactions and diseases like IBD and cancer, as recently reviewed (Bartfeld, 2016; Almeqadi et al., 2019). Some of the most advanced work with organoids for modeling infectious disease has been performed with the bacteria *H. pylori* in gastric organoids. As these organoids form spherical structures with a lumen where the apical side of the cell is on the inside, *H. pylori* have been injected into this lumen and have been shown to adhere to the epithelium (Bartfeld et al., 2015) as well as cell-cell junctions (Huang et al., 2015) and further to be able to proliferate in the organoid microenvironment (Bertaux-Skeirik et al., 2015). Similarly, human intestinal organoids have supported the replication of rotavirus (Finkbeiner et al., 2012) and have been used to study norovirus infection and the role of bile in its replication (Ettayebi et al., 2016) as well as the invasiveness of *S. Typhimurium* across the epithelial barrier (Forbestor et al., 2015; Zhang et al., 2014). Further, human intestinal organoids have been used to show that toxic *Clostridioides difficile* strains act by disrupting the epithelium and its paracellular barrier function (Leslie et al., 2015).

Human microphysiological models of the intestine or so-called intestine-on-chip devices are a recently developed technology that have relevance for the study of the gut microbiome. These systems typically rely on microfabrication techniques and microfluidics to build up and maintain more complex 3D tissue models. These types of models of the intestine have been used more broadly in the study of nutrition, inflammation and cancer (Steinway et al., 2020) and have potential in drug discovery and personalized medicine approaches (Bein et al., 2018). Moreover, they have begun to be used in the study of the microbiome. A microfluidic device with a flexible membrane has been used to examine the effects of peristalsis on bacterial overgrowth as well as to

mimic inflammatory conditions that can disrupt intestinal barrier function (Kim et al., 2016), and another type of microfluidic device has been utilized to replicate the intestine-microbe interface, using the commensal anaerobic bacteria *Lactobacillus rhamnosus* GG, for insight into transcriptional, metabolic and immunological responses (Shah et al., 2016). Interestingly, systems that can establish oxygen gradients can be used to co-culture anaerobic bacteria with human intestinal cells (Jalili-Firoozinezhad et al., 2019; Kim et al., 2019). The oxygen gradients have allowed one in vitro system to support a high level of microbial diversity (>200 unique OTUs) as well as increased intestinal barrier function (Jalili-Firoozinezhad et al., 2019) whereas another supported both facultative and obligate anaerobes in its luminal compartment and promoted a more biomimetic crypt architecture (Kim et al., 2019).

Animal models are primarily used in biomedical research to gain understanding of disease mechanisms and to develop and/or test treatment options. Interestingly, *Drosophila melanogaster* has been used as a model system to study the influence of host genotype on gut microbiota abundance and its effect on nutritional status (Chaston et al., 2016). Conventional mouse models have been utilized, for example, to study the effects of genotype on the composition of gut microbiota and sensitivity to colitis; however, these models typically require the study of littermates (Lemire et al., 2017). Gnotobiotic rodent models, including germ-free models, have been used to study host-microbe interactions (Smith et al., 2007). They can also be useful in nutrition research. For instance, gnotobiotic mice, which were colonized with fecal microbiota from a human donor, were used to study the link between sialylated milk oligosaccharides, the gut microbiota and healthy growth, as this promotion of growth did not occur in germ-free mice (Charbonneau et al., 2016). However, rodent models may not sufficiently replicate the (patho)physiological and metabolic processes of humans. Therefore, increasing attention has turned to swine models (Heinritz et al., 2013; Lunney, 2007), including human microbiota-associated swine, which are established through inoculation with gut microbiota from human donors (Wang and Donovan, 2015).

Gut microbiome at the intersection of health and disease

As our largest and richest reservoir of microorganisms, the human gut microbiota represents a densely populated and diverse microbial community outnumbering even our cells, and it is estimated to harbor over 1000 different bacterial species, as well as commensal fungi, viruses and archaea (Qin et al., 2010; Meštrović, 2016). More than 90% of the species comprising normal human gut microbiota belong to four principal bacterial phyla—Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria; furthermore, three leading genera have been reported as enterotypes of the human gut—*Bacteroides*, *Ruminococcus*, and *Prevotella* (Ley et al., 2008; Arumugam et al., 2011; Matijašić et al., 2020). Dietary habits of the host and phylogeny additionally contribute to such protean composition of intestinal microbial communities in humans and other mammals (Liang et al., 2018; Yu et al., 2021).

Compositional and functional changes of the intestinal microbiome are omnipresent in various forms of IBD, including ulcerative colitis (UC) and Crohn's disease (CD) (Mar et al., 2016; Schirmer et al., 2018). The use of sequencing methods has established the presence of significant differences in the gut microbiome of IBD patients in comparison to that of healthy individuals, with decreased diversity and bacterial load (the latter being touted as a potential biomarker in IBD) (Matijašić et al., 2016; Sarabayrouse et al., 2021). More specifically, individuals with UC and CD demonstrate a reduction in Firmicutes (especially *Clostridium* groups) and an increased abundance of Proteobacteria (Manichanh et al., 2006; Frank et al., 2007). However, a notable finding is a significant decline of protective bacterial species belonging to genera such as *Bacteroides*, *Lactobacillus* and *Eubacterium* (Matijašić et al., 2016; Mullish et al., 2021). In regards to fungal dysbiosis in IBD, there is higher Basidiomycota/Ascomycota ratio, an increased abundance of *Malassezia sympodialis* and *Candida albicans*, as well as lower numbers of *Saccharomyces cerevisiae* (Sokol et al., 2017). Fungal-bacterial interactions, as determined by functional modeling, are higher in patients with UC than CD (Sokol et al., 2017).

The gut microbiota has been recently recognized as a paramount mediator of biochemical signaling and intercommunication between the gastrointestinal tract and the central nervous system, which is frequently referred to as the gut-brain axis (Cryan et al., 2019) (Fig. 1). Consequently, a plethora of behavioral disorders have been linked to perturbed intestinal microbiota, including autism spectrum disorder (ASD) in children (Iglesias-Vázquez et al., 2020). More specifically, the microbiota in children with ASD shows increased bacterial diversity and higher abundance of genera *Bacteroides*, *Clostridium*, *Collinsella*, *Corynebacterium*, *Faecalibacterium*, *Lactobacillus*, *Parabacteroides*, *Phascolarctobacterium* and the fungal pathobiont *Candida* in comparison with controls, while there is a depletion of *Bifidobacterium* and *Coprococcus* (Iglesias-Vázquez et al., 2020; Strati et al., 2017). Recent studies have also shown that the gut microbiome is an important factor in diseases such as Alzheimer's disease, Parkinson's disease and stroke, primarily due to the production of modulatory neuroactive compounds (Benakis et al., 2020; Kesika et al., 2021).

T2DM is a major public health concern that may be linked to the composition of the intestinal microbiota, as even prediabetic conditions, such as incipient insulin resistance, are increasingly related to gut dysbiosis (Roager et al., 2019; Salgado et al., 2019). The main traits of the microbiota in patients with T2DM are decreased concentrations of butyrate-producing bacteria, most notably *Faecalibacterium prausnitzii* and *Roseburia intestinalis* (Sabatino et al., 2017), as well as lower prevalence of Firmicutes. There is also an increased prevalence of *Betaproteobacteria* family and opportunistic pathogens (e.g., various species of the *Clostridium* genus, *Eggerthella* spp. and *E. coli*) (Roager et al., 2019; Salgado et al., 2019; Larsen et al., 2010). The condition is also intertwined with obesity, as substantially reduced bacterial complexity is observed in both conditions. Especially evident is a decrease in *Bacteroides*/*Prevotella* groups and the *Bacteroidetes* phylum (Andoh et al., 2016; Indiani et al., 2018). However, although there are various probiotic formulations proposed to tackle both T2DM and obesity (Tseng and Wu, 2019), we have to be cognizant of the fact that these conditions are complex and multifactorial, and thus have to be addressed from different angles.

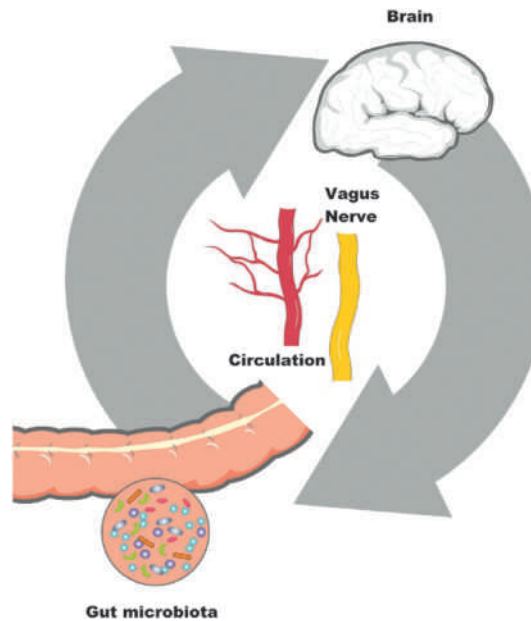


Fig. 1 A schematic diagram of bi-directional communication of intestinal microbiota with the central nervous system (also known as the “gut-brain axis”).

Some recent studies have emphasized the significance of the gut microbiome in modulating responses to different types of cancer immunotherapy (Roy and Trinchieri, 2017; Zitvogel et al., 2018). Patients with malignant diseases who respond well to treatment with anti-programmed cell death protein 1 basically have much more diverse gut microbiomes when compared to non-responders (Gopalakrishnan et al., 2018). The latter group presents with a low abundance of microorganisms belonging to the genera *Akkermansia*, *Bifidobacterium* and *Faecalibacterium* (Gopalakrishnan et al., 2018; Matson et al., 2018). At the phylum level, patients with an abundance of Firmicutes and Verrucomicrobia generally have a better response when immune checkpoint inhibitors are used, unlike those with increased numbers of Proteobacteria (Huang et al., 2021a).

Many chronic diseases associated with the alteration of intestinal microbiota are increasingly being tackled by a promising approach known as fecal microbiota transplantation (FMT) (Dixit et al., 2021). However, the effectiveness and safety of manipulating gut flora with the use of donor feces has been established only for recurrent *Clostridioides difficile* infection recalcitrant to antimicrobial treatment (Quraishi et al., 2017). FMT is usually delivered either directly to the large bowel or from the upper gastrointestinal tract by capsule ingestion (Gweon and Na, 2021), and engraftment can be facilitated by controlling inflammation in the recipient intestine, as it reduces the host immune response against the transplant (Danne et al., 2021). Recently, a specific ecological framework was proposed recognizing the efficacy of FMT in various conditions that have a common trait of disrupted intestinal microbiota, and several crucial factors were highlighted that determine its success, most notably host-dependent microbial dynamics and pre-FMT taxonomic diversity (Xiao et al., 2020). This has opened a door to developing an algorithm for designing personalized probiotic cocktails as a way towards a true precision medicine approach—with future microbiota-based therapeutics that hold a promise in ameliorating the aforementioned chronic conditions.

Oral microbiome and periodontal diseases

The oral microbiome, recently called the “oralome,” is characterized by notable shifts during the life time—from incipient bacterial acquisition after birth to microbial colonization of the elderly (Radaic and Kapila, 2021; Krishnan et al., 2017). This is an important niche, since the oral cavity harbors the second largest and most variegated microbiota with more than 700 bacterial species, which is surpassed only by the intestines (Deo and Deshmukh, 2019). In health, the oral microbiome can be viewed as a dynamic, but incredibly balanced, ecosystem that tends to stay within typical frames of equilibrium (Najmanova et al., 2021). Furthermore, compositional patterns of the oral microbiome vary in terms of optimal equilibrium of this microbial community, primarily in regards to geography, which are referred to as “stomatotypes” in the current literature (Willis et al., 2018; Willis and Gabaldón, 2020).

One of the most pervasive human bacterial infections is dental caries, which results often in tooth decay and potentially in the loss of teeth (Zhang et al., 2021). Among the main etiological factors is frequent sugar intake, which prompts commensal bacteria to adjust to the resultant acidic milieu, accommodating in turn the growth of aciduric bacterial species and inhibiting the beneficial microorganisms that favor neutral pH values (Radaic and Kapila, 2021). Although *Streptococcus mutans* (*S. mutans*) is the hallmark bacterial species implicated in this condition and often referred to as the “core-pathogen,” molecular methods, such as microarrays or the well-established 16S rRNA approach, have revealed that carious lesions additionally harbor high amounts of species

belonging to the genera *Atopobium*, *Lactobacillus* and *Cutibacterium* (Aas et al., 2008; Zheng et al., 2021). However, there are even cases of dental caries with no detectable levels of *S. mutans*, when there is an abundance of *Actinomyces* spp., *Bifidobacterium dentium*, *Lactobacillus* spp., *Scardovia* spp., *Veillonella* spp. and non-*S. mutans* species of streptococci (Zhang et al., 2021). A shift in the composition of the tongue microbiota is also significantly linked to dental caries, as evidenced in a study on primary school children (Zhang et al., 2021).

Unlike dental caries, the etiology of periodontitis still cannot be explained by a single hypothesis. This is one reason why knowledge about the inception of periodontal diseases and the microorganisms that drive the condition known as periodontitis remains scarce, despite decades of scientific work on this topic (Rosier et al., 2014). In comparison to the healthy periodontal state, periodontitis—which is a multifactorial inflammatory disease that results in the loss of periodontal attachment and alveolar bone—shows increased microbial diversity driven by a rich interaction profile with genetic and environmental factors within specific time windows (Na et al., 2021). Consequently, as each patient has a different constellation of colonizing microorganisms and internal/external risk factors, the pathogenesis of periodontitis can be viewed as a personalized pathological condition characterized by specific tissue-destructive outbursts (Scannapieco and Dongari-Bagtzoglou, 2021).

Bacterial taxa substantially more prevalent and abundant in periodontal diseases primarily encompass so-called red complex bacteria: *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* (Socransky et al., 1998). In addition, there is also a notably higher prevalence of species belonging to the genera *Desulfobulbus*, *Fretibacterium* and *Peptostreptococcus*, as well as *Filifactor alocis* and *Selenomonas sputigena* (Curtis et al., 2020; Szafranski et al., 2015; Shi et al., 2015; Najmanova et al., 2021). Researchers have also discovered additional species that are not habitually found in periodontal disease, including *Brevundimonas diminuta*, *Dialister invisus*, *Granulicatella adiacens*, *Mycoplasma salivarium*, *Pseudoramibacter alactolyticus*, *Shuttleworthia satelles* and *Veillonella atypica* (Krishnan et al., 2017). A notorious and multiresistant nosocomial pathogen *Acinetobacter baumannii* has also been recognized as a salient risk factor for periodontitis refractory to treatment (Richards et al., 2015).

Conversely, only a handful of taxa have been unequivocally linked to periodontal health, most notably species belonging to the genera *Actinomyces* and *Streptococcus* (Griffen et al., 2012; Abusleme et al., 2013; Kirst et al., 2015; Meuric et al., 2017). Likewise, for many bacterial taxa the exact classification between periodontal health and disease has proven to be a very cumbersome exercise, as they commonly display matching prevalence and relative abundance in both of these states. These include, but are not limited to, very abundant members of the genus *Streptococcus*, *Fusobacterium nucleatum* and *Veillonella parvula* as well as infrequently recognized *Campylobacter gracilis*, *Granulicatella adiacens* and *Lautropia mirabilis* (Abusleme et al., 2013; Tsai et al., 2018). Still, dysbiosis is dependent not only on the taxonomic composition of the oral microbiome but also on its metabolic activity and the subsequent host response to microbial products (Lenartova et al., 2021).

Even though the purported connections are based on correlations, altered oral microbiota has been repeatedly observed to have an effect in various diseases outside the oral cavity, such as bacteremia, endocarditis, diabetes, autoimmune diseases, malignancies and even preterm births (Verma et al., 2018). Hence, extensive profiling of oral microorganisms from healthy individuals will be pivotal for further comparisons under dysbiotic conditions. This is where massive sequencing with the use of third generation next-generation sequencing (NGS) techniques will be crucial to establish oral microbial databases for tailored, microbiome-based treatments in the future. For example, the PacBio single-molecule real-time (SMRT) sequencing platform was already used to survey biomarkers of microbiome dysbiosis in the oral cavity of elderly patients with Alzheimer's disease (Wu et al., 2021). All of these advances are opening the door for promising personalized dentistry approaches.

Urogenital microbiome and genitourinary pathology

The microbiome of the human urogenital tract is complex and diverse, which is especially evident in the female vagina. More specifically, the vaginal microbiome is dominated by several varieties of lactobacilli that maintain homeostasis. Nonetheless, high-throughput sequencing endeavors addressing bacterial abundance in the female genital tract have demonstrated that factors such as hygiene practices, hormones and sexually transmitted infections may disrupt the natural balance, resulting in the outgrowth of some bacterial groups and subsequent pathology (such as bacterial vaginosis, recurrent infections in the urinary tract and tumor-promoting effects) (Ravel et al., 2011; Chen et al., 2021). Five community state types (CSTs) of the vaginal microbiome are recognized. CST I, II, III and V are characterized by low microbial diversity and are dominated by *Lactobacillus crispatus* (*L. crispatus*), *Lactobacillus gasseri* (*L. gasseri*), *Lactobacillus iners* (*L. iners*) and *Lactobacillus jensennii* (*L. jensennii*), respectively (Chen et al., 2021). Conversely, in CST IV, there is a reduced number of lactobacilli and high diversity of primarily anaerobic bacteria, which are frequently linked to bacterial vaginosis (Ravel et al., 2011).

Bacterial vaginosis represents a very common lower genital tract disorder affecting women of reproductive age around the world (Javed et al., 2019). It is characterized by the loss or a marked reduction in the total number of *Lactobacillus* species, as well as a commensurate 100–1000-fold rise in the abundance of obligate and/or anaerobic microorganisms that belong to the genera *Gardnerella*, *Atopobium*, *Prevotella*, *Bifidobacterium*, *Mobiluncus*, *Leptotrichia* and *Sneathia* as well as certain novel constituents of the order *Clostridiales* designated as bacterial vaginosis-associated bacteria (BVAB) 1–3 (Fredricks et al., 2005; Srinivasan et al., 2012; Muzny et al., 2018; Chen et al., 2021). One of the key facets in the pathogenesis of bacterial vaginosis is the development of polymicrobial biofilms instigated by *Gardnerella vaginalis* (*G. vaginalis*) that form a scaffold for the attachment of other microorganisms, such as *Atopobium vaginae* as a second colonizer (Hardy et al., 2017; Jung et al., 2017).

Moreover, different studies have shown that women with a low abundance of lactobacilli are more frequently colonized with vaginal *E. coli* than those with lactobacilli-dominated microbiomes, which consequently modulates the risk of the development of urinary tract infections (UTIs) (Gupta et al., 1998; Lewis and Gilbert, 2020; Meštrović et al., 2021). Different species of lactobacilli, but predominantly *L. crispatus*, have the proclivity to inhibit the growth of *E. coli*, primarily by establishing and preserving a low pH environment (Hudson et al., 2020; Vagios et al., 2020). In addition, multiple studies in both non-pregnant and pregnant women have demonstrated that bacterial vaginosis can increase the risk of microbial colonization in the vaginal introitus and subsequent UTI (Harmanli et al., 2000; Stapleton et al., 2011; Amatya et al., 2013).

Although the penile microbiome remains understudied, men can also play a considerable role in bacterial vaginosis, as the urethral meatus and coronal sulcus of the penis are salient reservoirs of associated bacteria in both uncircumcised and circumcised men (Onywera et al., 2020). More specifically, bacterial species from these penile regions have high predictive power for incident bacterial vaginosis that is seen up to 6–12 months later in their female sex partners (Mehta et al., 2020). Further, although penile *Staphylococcus* and *Corynebacterium* species have been linked to healthy microbiomes of the cervix and vagina and they notably increase after male circumcision, additional investigation is necessary in order to establish their exact roles in the reproductive health of both men and women (Onywera et al., 2020).

The acquisition of sexually transmitted infections (STIs) is also linked to urogenital microbiome disruption. The most researched STI is human immunodeficiency virus (HIV), which is associated with certain bacterial taxa. More specifically, the abundance of *Prevotella* spp., *Parvimonas* spp. type 1 and type 2, *Veillonella montpellierensis*, *Eggerthella* spp. type 1, *Mycoplasma* spp., *Gemella asaccharolytica*, *Sneathia sanguinegens* and vaginal *Megasphaera* spp. significantly correlate with increased HIV acquisition (Gosmann et al., 2017; McClelland et al., 2018). The propensity of the aforementioned bacterial taxa to instigate a vigorous inflammatory response in the cervicovaginal milieu—with the subsequent recruitment of CCR5⁺ CD4⁺ T cells (the primary cells targeted by HIV) and increased concentrations of interleukins (most notably IL-1 β , IL-17 and IL-23)—are key pathophysiological mechanisms (Gosmann et al., 2017; Torcia, 2019). In men, the subpreputial space harbors a rich community of anaerobic bacteria and is a proinflammatory immune milieu, which purportedly enables the acquisition of HIV, but thus far studies on the topic are very scarce (Onywera et al., 2020).

In women, certain dysbiotic bacterial communities may result in immune dysregulation that favors a tumor-promoting microenvironment and influences whether cervical precancerous lesions will regress or progress to cancer (Schwabe and Jobin, 2013; Garrett, 2015). Accordingly, human papillomavirus (HPV) is an essential, but not sufficient prerequisite of cervical cancer, which is reinforced by the fact that in the majority of infected women the immune response controls the infection and fends off high-grade lesions and tumors (Insinga et al., 2011; Castanheira et al., 2021). Hence, vaginal microbiota may well be one of the more important co-factors in the development of cervical cancer, especially when lactobacilli are concerned (Castanheira et al., 2021; Kwasniewski et al., 2018). In instances when *L. crispatus* predominates, there is generally a lower risk of HPV infection, cervical intraepithelial neoplasia (CIN) and cervical carcinoma, whereas a predomination of *L. iners* increases those risks (Norenhag et al., 2020). In addition, the presence of *Gardnerella vaginalis* and an increase in microbiological diversity may be viewed as biomarkers of changes in the cervix to recognize women with higher risks of persistent HPV infection, CIN and subsequent cancer development (Usyk et al., 2020).

Finally, the urinary microbiome should not be neglected, and its role is not only suggested in manifold pathologies of the urinary system, such as overactive bladder (Wu et al., 2017), urge incontinence (Pearce et al., 2014) and urothelial carcinoma (Bučević Popović et al., 2018), but also in the pathogenesis of UTIs (Meštrović et al., 2021). Regarding the latter, two pivotal studies in this field showed that individuals who are prone to developing UTIs present with lower diversity of the urinary microbiome (Horwitz et al., 2015) and that its perturbations basically precede the UTI development (Bossa et al., 2017), implying that the whole condition can be viewed as a “urinary tract dysbiosis” (Finucane, 2017). However, additional longitudinal appraisal of the urinary microbiome in different pathological conditions is warranted before any steadfast conclusions can be drawn.

Skin microbiome and dermatologic conditions

Adequate comprehension of the microbial composition found on healthy human skin is indispensable for establishing a reference state and subsequently detecting changes linked to certain skin diseases. In contrast to the intestines, which are almost an optimal environment for fermentative bacterial species, the skin represents an inhospitable area due to the dry, acidic, salty and nutrient-deficient epidermal layer, with the exception of lipid-rich havens around hair follicles (Ellis et al., 2019). In healthy individuals, the skin microbiota is responsible for mediating fundamental physiological processes including epidermal development and differentiation as well as underlying immune responses (Nørreslet et al., 2020). Consequently, inflammatory and other skin diseases, such as atopic dermatitis, seborrheic dermatitis, acne vulgaris, rosacea, hidradenitis suppurativa and psoriasis, are characterized by condition-specific changes of the microbial composition and subsequent dysbiosis.

There are four dominant phyla that comprise the skin microbiome: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Grice et al., 2009). Although there is ample diversity among individuals, with various topographic niches, this microbiome is relatively stable over time (Grice et al., 2009; Oh et al., 2016; Nørreslet et al., 2020). Three principal categories of skin habitats are designated as (1) dry (such as the hand and the forearm) where β -proteobacteria, *Corynebacteriaceae* and *Flavobacteriales* are found; (2) moist (such as the axilla) where species of the genus *Staphylococcus* are pervasive alongside β -proteobacteria and *Corynebacteriaceae*; and (3) sebaceous (such as the forehead) where *Staphylococcus* and *Cutibacterium* (formerly known as *Propionibacterium*) tend

to dominate (Grice et al., 2009). Areas with the most diverse microbiome according to bacterial operational taxonomic units are dry, while sebaceous regions harbor the least diverse microbial communities (Nørreslet et al., 2020).

In atopic dermatitis (AD), which is a chronic allergic skin condition also known as eczema, the microbiome is characterized by a state of dysbiosis with diminished bacterial diversity (α -diversity) and decreased stability over time (Kong et al., 2012). Importantly, this is coupled with persistent colonization by *Staphylococcus aureus* (*S. aureus*), which also positively correlates with the severity of AD lesions (Byrd et al., 2017; Clausen et al., 2018). Furthermore, it has been demonstrated that infants harboring *Staphylococcus epidermidis* (*S. epidermidis*) and *Staphylococcus cohnii* at 2 months of age have a reduced risk of developing AD by 1 year of age due to increased microbial diversity (Kennedy et al., 2017) whereas *S. aureus* colonization actually precedes the clinical diagnosis of AD in infancy (Meylan et al., 2017).

Seborrheic dermatitis (SD) represents a common inflammatory rash that appears on skin areas laden with sebaceous glands and is frequently linked to the fungal genus *Malassezia*. This fungus is viewed as a normal constituent of the human skin microbiota, albeit its exact role in the development of SD is still inadequately understood (Luciana Campos, 2017). Recent studies have shown that certain bacterial species dominate in the lesioned skin and are implicated in SD development, especially *Staphylococcus*, *Streptococcus* and *Acinetobacter* (Tanaka et al., 2016). Another cohort study demonstrated that individuals with SD have higher numbers of colonizing *S. epidermidis* in comparison to those without the condition (An et al., 2017).

Cutibacterium acnes (*C. acnes*) is believed to be an important player in the pathogenesis of acne vulgaris; nonetheless, the causal relationship is still unclear (O'Neill and Gallo, 2018), especially since this species is found in healthy individuals and prevents colonization with opportunistic pathogens (Nørreslet et al., 2020). A deeper dive into the phylogenetic clades shows an association between *C. acnes* strain IA-2 and acne vulgaris (Kasimatis et al., 2013; McDowell et al., 2013), so this relationship may be strain-specific. This is further supported by the findings from a metagenomic study where an imbalance of virulent *C. acnes* has been noted (Barnard et al., 2016). Moreover, several studies have shown that *S. epidermidis* is more abundant in active acne lesions (Nishijima et al., 2000; Bek-Thomsen et al., 2008; Dreno et al., 2017), although one study showed that *S. epidermidis* may have a beneficial effect in acne lesion recovery (Pathak et al., 2013).

The cutaneous microbiome of patients with rosacea, which is a common long-term inflammatory condition of facial skin, has been a subject of many studies (Kim, 2020). A lot of them focused on the commensal mite *Demodex folliculorum*, as its prevalence and density are much higher in rosacea-affected skin in comparison to unaffected controls (Kim, 2020). It has been suggested that the mite exacerbates the disease by influencing the immune reactivity of sebocytes, triggering activation of the TLR2 signaling pathway and disrupting the skin barrier (Lacey et al., 2018). *Demodex* mites are also probable carriers of *Bacillus oleronius*, which is a Gram-negative bacterial species with proinflammatory potential (Picardo and Ottaviani, 2014). In addition, a beta-hemolytic variant of *S. epidermidis*, which possibly exerts increased virulence traits, was isolated from rosacea patients (Dahl et al., 2004; Whitfeld et al., 2011). Furthermore, *C. acnes* plays a substantial role in rosacea, and it may even have a beneficial effect (Jahns et al., 2012; Kim, 2020).

Even though the clinical presentation of hidradenitis suppurativa points towards a bacterial infection, the exact role of the cutaneous microbiome has been controversial; however, some recent studies reveal a purported link between skin microbiome dysbiosis and this condition (Wark and Cains, 2021). Significant differences between patients with hidradenitis suppurativa and healthy individuals were demonstrated on the genus and species levels with the use of next generation sequencing (Ring et al., 2017; Ring et al., 2019). The dominant species in the diseased lesions were from the genera *Peptoniphilus*, *Porphyromonas* and *Corynebacterium* as well as *Campylobacter ureolyticus*, *Actinomyces* and *Mobiluncus* (Ring et al., 2017; Riverain-Gillet et al., 2020). On the other hand, controls did not harbor *Peptoniphilus* at all, and *Cutibacterium* was more abundant in controls (Ring et al., 2017; Wark and Cains, 2021).

Psoriasis, a skin disease characterized by scaly and erythematous plaques, is also coupled with changes in the cutaneous microbiome when compared with healthy controls, most notably as a significant decrease in microbiome α - and β -diversity (Olejniczak-Staruch et al., 2021). More specifically, there is a decrease in Actinobacteria and an increased abundance of Firmicutes in psoriatic lesions, which can be reversed after systemic treatment with antibiotics (Langan et al., 2019). And even though psoriasis is rarely aggravated by a clinical superinfection, a higher relative abundance of staphylococcal and streptococcal species was observed in psoriatic lesions when compared to non-lesioned skin (Lewis et al., 2019). Some studies even highlight the role of *Candida albicans* in psoriasis pathogenesis due to induction of interleukin-36 α production and subsequent psoriasiform phenotype development (Hashiguchi et al., 2018).

In conclusion, skin disease development can be observed as an intricate interplay between genetic predisposition, the local immune system and the composition of microbiome, as increasing evidence points towards the last as a pivotal element. Potentially, strains of microbial species deemed as beneficial may be utilized in the future to evade dysbiosis, and consequently to improve or treat underlying skin disorders. The improved understanding of the cutaneous microbiome may also translate into novel diagnostic and preventative approaches in the context of personalized or precision medicine.

Conclusion

It is clear that the human microbiota represents a tremendously complex, but at the same time an extremely well organized, community of microorganisms. With the use of state-of-the-art techniques, today we are becoming increasingly aware not only of all crucial constituents of the healthy human microbiome, together with their functional roles, but also of the shifts that lead to

dysbiosis and have a role in specific diseased states. Further research into functional host-microbiome interactions is warranted to elucidate all interactions that can lead to disease, which will in turn result in precision medicine approaches targeting microbiota in order to maintain homeostasis and health. The ultimate objective of human microbiome research is always translational in nature—to optimize health and disease management.

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Probiotics to Prebiotics and Their Clinical Use

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Glossary

Microbiome The collective genomic content of the microbiota and also indicates the total genetic capacity of the community.

Microbiota The collective microbial community inhabiting a specific environment. Cellular density increases along the length of the gut, and the colonic microbiota are the densest and most diverse community in both the gut and whole human body.

Prebiotics First defined in 1995 (Gibson and Roberfroid, 1995), are used to manipulate microorganisms in the host to improve measurable outcomes. An update to the definition of prebiotics was published in 2017 as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al., 2017). Prebiotic effects include defense against pathogens, immune modulation, mineral modulation, mineral absorption, bowel function, metabolic effects, and satiety (Sanders et al., 2019; Roberfroid et al., 2010).

Probiotics Defined as live microorganisms that, when taken in a sufficient amount, have a benefit for the ingesting host (FAO and WHO, 2002). Probiotic microorganisms act via a variety of means, including modulation of immune function, the production of organic acids and antimicrobial compounds, interaction with resident microbiota, interfacing with the host, improving gut barrier integrity, and enzyme formation (Sanders et al., 2019).

The human gut microbiota

Recent studies have shown that the trillions of microbes that inhabit our gut form a complex ecological community that influences our health and can account for the incidence of various diseases. Understanding the factors that underlie changes in the composition and function of the gut microbiota will aid in the development of clinical applications. Although the beneficial effects of some microbes have been documented for more than a century, only with recent technological advances can the composition of the diverse microbial communities be described and compared with and between individuals. Currently for clinical applications, probiotics and prebiotics are used.

The microbial colonization of the human gut begins immediately at birth and is an indispensable natural process that modulates physiology and immunity. Recent studies have revealed how and when these microbes colonize the gut and what elements can potentially influence it. The vertical mother-to-baby transmission of microbes is a crucial factor for normal development and the maturation of a newborn's immune system, as well as metabolic and neurological health. This important and delicate process of gut microbiota development may be impacted by various factors such as the mode of birth, type of feeding, gestational age at birth, antibiotic exposure in early life, and the surrounding environment and hygiene settings. Perturbations in early life gut microbial colonization have been associated with the development of several diseases such as diabetes, obesity, asthma, allergies, celiac disease, and neurodevelopmental disorders (Nagpal and Yamashiro, 2015). Babies born vaginally will predominantly acquire

bacteria from the maternal vaginal and perianal flora. However, those delivered via Cesarean (C)-section acquire a heavy inoculum of bacteria from the maternal skin and the surrounding hospital environment. Such differences in the gut microbiota as a result of unnatural delivery have been found to persist in children until several years of life and even into young adulthood (Nagpal and Yamashiro, 2018). It has become clear that C-delivery and inadequate breastfeeding can instigate an abnormal gut microbiome composition and may also account for the rising incidences of several serious health problems in children, including asthma, allergies, celiac disease, diabetes, and obesity (Penders et al., 2006; Dominguez-Bello et al., 2010; Bäckhed et al., 2015; Bokulich et al., 2016; Tamburini et al., 2016; Chu et al., 2017). These maladies, among others, are more likely to occur in cases where an infant's gut acquires an inadequate population of health-promoting bacteria and instead is predominated by unsolicited bacteria. These gut microbes perform fundamental functions including the digestion of complex nutrients, vitamin production, stimulation of immune development, discouraging harmful bacteria, and nurturing normal maturation of the gut. Serious perturbations in one or more of these early life gut microbial functions could lead to severe health problems in the host. The infant gut is inhabited by several microbes *in utero* (Bäckhed et al., 2015; Bokulich et al., 2016; Aagaard et al., 2014; Collado et al., 2016), although the bacteria acquired during birth and the first few months of life have paramount impact on those that become permanent inhabitants in the gut.

Early-life gut microbiota in relation to the mode of delivery

In our quantitative investigation of the gut microbiota composition in Japanese infants, we found *Bacteroides fragilis* group, *Enterobacteriaceae*, *Enterococcus*, *Streptococcus*, and *Staphylococcus* to be the most prevalent dwellers, with counts ranging from 10^{5-8} cells g sample⁻¹, followed by bifidobacteria, lactobacilli, *Clostridium coccoides* group, *Clostridium leptum* subgroup, *C. perfringens*, *Atopobium* cluster, and *Prevotella* (Fig. 1A). These data demonstrated that the facultative anaerobes flourish, and hence are the first colonizers, in the nascent gut, thereby indicating a relatively aerobic intestinal environment. This also indicates that the primitive microbiota core is predominated by facultative anaerobes including *Proteobacteria*, staphylococci, streptococci, and enterococci, whereas obligate anaerobes such as Firmicutes, Bacteroidetes, and Actinobacteria start prevailing during infancy and early childhood (Fig. 1B). Compared to the meconium of vaginally born babies, the meconium of C-born babies is significantly less often colonized with the *Lactobacillus* genus (Nagpal et al., 2016). We found this difference in gut microbiota to persist up to 6 months of age.

Another study of ours performed in healthy young adults born vaginally or by C-section revealed that the C-group had significantly a lower carriage rate of the *B. fragilis* group and *Lactobacillus sakei* subgroup as compared with the vaginal-delivery group (Nagpal and Yamashiro, 2018). This suggests that some of the differences in early-life microbiota as a result of delivery mode might persist into young adulthood, even though the differences were observed in the carriage rate, but not population levels, of only two components. We did not notice any significant difference in body weight, body mass index, height, or male-female ratio by the mode of birth.

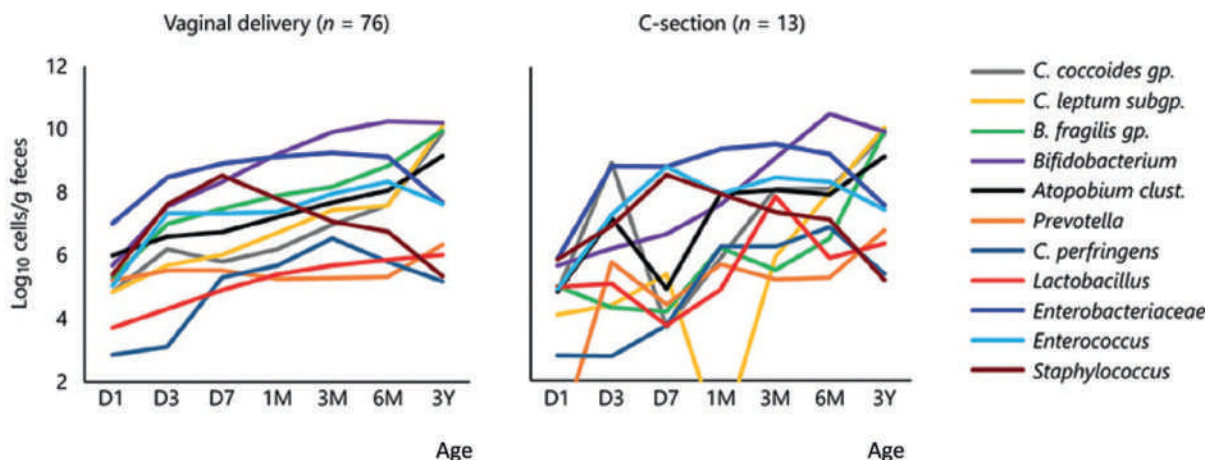


Fig. 1 Early-life gut microbiota in relation to the mode of delivery. From Nagpal R and Yamashiro Y (2018) Gut microbiota composition in healthy Japanese infants and young adults born by C-section. *Annals of Nutrition and Metabolism* 73(Suppl. 3): 4–11.

Gut microbiota in healthy toddlers to adults and the elderly

Our data indicate that gut microbiota diversity undergoes a gradual increase early in life, but reaches a plateau by 3 years of age (Fig. 2; Tsuji et al., 2012, 2018), at which time as well as at the ages of pre-school and elementary school (Wang et al., 2015), the microbial configuration and diversity become equivalent to those in adults. Further age-related quantitative changes in gut microbiota populations analysis, the predominant populations such as *C. coccoides* group, *C. leptum* subgroup, *C. perfringens*, *B. fragilis* group, and *Prevotella*, and those present at lower levels, such as *Enterobacteriaceae*, lactobacilli, and pathobionts. *Bifidobacterium* are the predominant microbiota established in most infants by 3 months after birth, particularly in those that are breastfed, and this level is maintained until the age of 3 years, with a slight decline after the introduction of solid foods. *Bifidobacterium* remains as one of the predominant microbes in adults, but in elderly people the levels are decreased. By the age of 3 years, obligate anaerobes are stabilized, but several low cell-count microbiota including *Prevotella*, *Enterobacteriaceae*, and *Enterococcus* are still in the process of transition and not yet stabilized.

The gut microbiome and immunity

The gut microbiome is a signaling hub that integrates environmental inputs, such as diet, with genetic and immune signals to affect the host's metabolism, immunity, and response to infection. These mechanisms are involved in a wide range of conditions and diseases, such as metabolic syndrome and immune-mediated diseases.

Innate immunity

The innate immune system plays an important part in shaping the community and ecology of indigenous microorganisms into configurations that can be tolerated by the host and are beneficial for its metabolic activities. This complex, bilateral interaction between the host and its microbiota has a crucial role in human health.

Intestinal epithelial cells are equipped with an extensive repertoire of innate immune receptors (Fig. 3; Thaiss et al., 2016). Intestinal epithelial cells use the recognition of microbial-cell components and metabolites to adjust their antimicrobial program and achieve metabolic homeostasis. The activation of pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and the NOD-like receptors (NLRs) NOD1 and NOD2, is directly coupled to the production of antimicrobial peptides (including

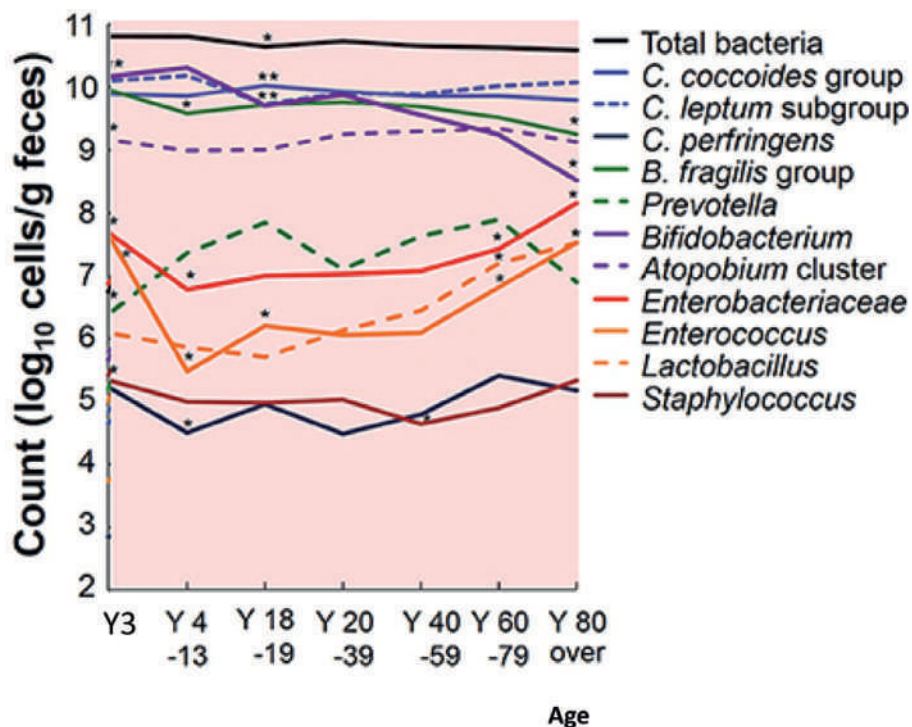


Fig. 2 Gut microbiota in healthy toddlers to the elderly. From Tsuji H, Matsuda K, and Nomoto K (2018) Counting the countless: Bacterial quantification by targeting rRNA molecules to explore the human gut microbiota in health and disease. *Frontiers in Microbiology* 9: 1417.

RegIII γ , RegIII β , Ang4, and Itln1) and of mucus. IL-18 plays an important part in this process through an autocrine loop (Rakoff-Nahoum et al., 2004; Slack et al., 2009). The secretion of epithelial IL-18 requires transcriptional activation through TLRs or the G-protein-coupled receptor GPR109a, and posttranscriptional cleavage through NLRP6 can also be induced by type I interferons, which functions as a sensor of viral DNA with the pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15. IL-18 and IL-22 derived from immune cells also help to regulate the antimicrobial responses of epithelial cells. CCL20, which is derived from epithelial cells downstream of NOD1 signaling, is involved in the genesis of lymphoid tissue (Bouskra et al., 2008). NLRP4 promotes the expulsion of neoplastic or infected cells from the intestinal epithelium (Hu et al., 2010). PRR signaling also orchestrates the circadian clock within intestinal epithelial cells and adjusts the secretion of epithelial-derived metabolic hormones, such as glucocorticoids. Epithelial cells also respond to the levels of microbiota-modulated metabolites, such as SCFAs (acetate, butyrate, propionate), polyamines (spermine), as well as amino acids and products that are derived from them (taurine, histamine, indole). Taurine, histamine, and spermine modulate the activity of inflammasome component NLRP6. Indole modulates the levels of incretin secretion and promotes the barrier function of the epithelium through the pregnane X receptor (PXR), which helps to fortify tight junctions between cells. SCFAs serve as an energy source for epithelial cells and also support the barrier through HIF (Kelly et al., 2015). The microbial metabolite indole, through PXR, increases the secretion of glucagon-like peptide (GLP-1), an incretin with profound influence on host metabolism (Chimerel et al., 2014).

Adaptive immunity

Microbiota that establish mutualistic relationships with their hosts are able to influence a multitude of physiological functions, often through modulation of the host's immune system. Diverse species of microbes can regulate distinct branches of the adaptive immune system.

T-cell dependent IgA class-switch recombination (Fig. 4, right; Honda and Littman, 2016) takes place mostly in Peyer's patches, in which dendritic cells (DCs) that are located near the surface of the epithelium capture antigens from microbes that are transferred by M cells. DCs induce the differentiation of CD-4-expressing T cells into the T follicular helper (T_{FH}) cell subset. CD40 ligand (CD40L) and IL-21 from T_{FH} cells induce the expression of activation-induced cytidine deaminase (AID) in B cells and promote IgA class-switch recombination (Pabst, 2012; Cao et al., 2015). T-cell-independent IgA class-switch recombination (Fig. 4, left) occurs predominantly in the lamina propria and isolated lymphoid follicles (ILFs), where B-cell activating factor (BAFF; also known as TNFSF13B) and its homolog APRIL, which are derived from DCs, promote the induction of AID expression in B cells. IL-23 signals group 3 innate lymphoid cells (ILC3s) that express ROR γ to contribute to both T cell-dependent and T-cell-independent pathways

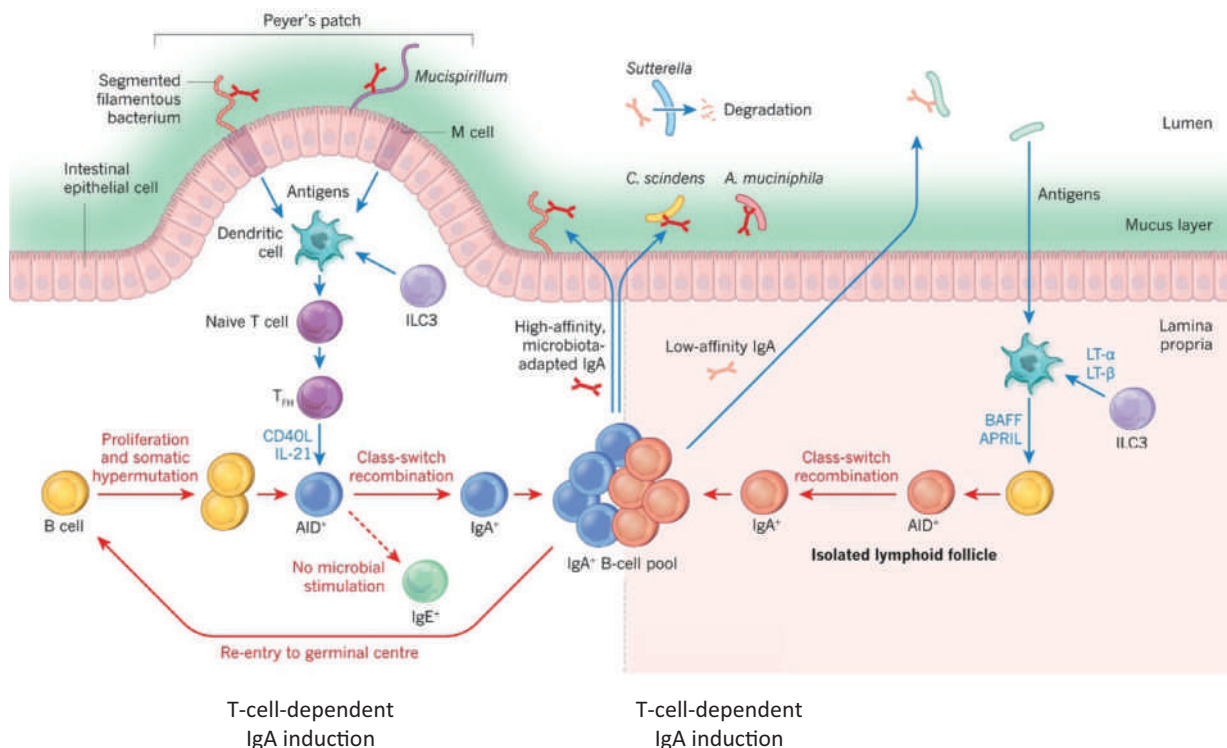


Fig. 4 Induction of IgA in mucosal tissues. From Honda K and Littman DR (2016) The microbiota in adaptive immune homeostasis and disease. *Nature* 535(7610): 75–84.

through the expression of lymphotoxin (LT)- α and LT- β , which activate DCs (Kruglov et al., 2013). The gut microbiota affect IgA class-switch recombination in both pathways. The T-cell-independent pathway produces IgA with low affinity but directed toward the microbiota. The T-cell-dependent pathway tends to be activated by bacteria that colonize the surface of the epithelium, such as segmented filamentous bacteria (SFB), *Mucispirillum*, *C. scindens*, and *Akkermansia muciniphila*. The IgA-expressing B-cell clones that this pathway induces persist for a long time and can re-enter a germinal center, where they undergo further somatic hypermutation to produce high-affinity IgA that is adapted to the changing composition of the microbiota (Cao et al., 2015; Kruglov et al., 2013).

ILC3s in adaptive immune homeostasis

Signals from the microbiota create complex interactions between epithelial cells, DCs, macrophages and ILC3s. ILC3s contribute to the differentiation of T cells and B cells. For example, ILC3s express lymphotoxin (LT)- α and LT- β , which activate DCs, thereby contributing to both T-cell dependent and T-cell-independent pathways of IgA class switching (Kruglov et al., 2013). ILC3s also facilitate the induction of T_H17 cells through the production of IL-22 and other factors. Activation of ILC3s and the induction of T_H17 cells have been observed in mice that are colonized by SFB and other bacteria, including *Citrobacter rodentium* (Atarashi et al., 2015; Sonnenberg et al., 2011; Longman et al., 2014). Activation of ILC3s by these bacteria requires the TLR-dependent activation of CX₃CR1-expressing cells (derived from monocytes) and their production of IL-23, IL-1 β , and tumor necrosis factor ligand superfamily member 15 (TNFSF15), which act through receptors on ILC3s (Longman et al., 2014). IL-22 from ILC3s then activates epithelial cells to produce serum amyloid A and other factors that are required for the induction of T_H17 cells.

ILC3s that are activated by the microbiota also promote the expansion of T_{reg} cells (Mortha et al., 2014). Gut microbiota induce the production of IL-1 β from macrophages in the lamina propria, and this cytokine acts on neighboring ILC3s to activate their production of CSF2 (Mortha et al., 2014). CSF2 then acts on CD103-expressing DCs in the colon to enhance the activity of aldehyde dehydrogenase (ALDH) and produce TGF- β and IL-10, which induce T_{reg} cells (Mortha et al., 2014).

The epithelial-innate immune system continuum in response to microbial antigens (Fig. 5)

In response to the microbiota, intestinal epithelial cells (IECs) secrete mucins and antimicrobial peptides (AMPs) that limit microbial interaction with epithelial cells. Under homeostatic, eubiotic conditions, microbe-associated molecular patterns (MAMPs) stimulate the secretion of cytokines (including TSLP, IL-33, IL-25, and TGF β) by IECs that promote the development of tolerogenic macrophages and DCs. DCs, in turn, induce the development of induced T_{reg} (iT_{reg}) cells through a TGF β and retinoic acid (RA)-dependent process. Through multiple mechanisms, including the secretion of TGF β and IL-10 by iT_{reg} cells and the secretion of IL-10 by macrophages, the anti-inflammatory balance of the intestine is maintained by inhibiting or dampening potential effector responses (Maynard et al., 2012). In addition, T_{reg} cell-derived TGF β and epithelial-derived BAFF and APRIL promote the development of IgA⁺ plasma cells to ensure an abundant supply of sIgA in the lumen, which further limits microbial interaction with the epithelium (Fig. 5A; Rakoff-Nahoum et al., 2004). In the face of pathogen invasion, mucosal injury, or dysbiosis, MAMPs stimulate the secretion of pro-inflammatory cytokines by IECs (including IL-6, IL-1, and IL-18) and intestinal DCs and macrophages (including IL-6, IL-23, and IL-12) that induce the development of the effector CD4⁺ T cells T_H1 and T_H17, the latter of which can transition to the former as a result of IL-23 or IL-12 signaling. Intestinal innate lymphoid cells, including NK-like cells, lymphoid tissue inducer cells (LTi cells), and $\gamma\delta$ IELs, respond to pro-inflammatory cytokines to upregulate IL-22, which helps to protect the epithelial barrier, and IL-17A and IL-17F, which are involved in neutrophil recruitment (Fig. 5B; Vijay-Kumar, 2008).

Microbiota-mediated immunomodulation

Microbiota-mediated effects on host immune cells and their underlying mechanisms will be instrumental in informing the rational design of microbial therapeutics (Skelly et al., 2019).

Anti-inflammatory effects of microbiota (Fig. 6)

Bifidobacterium infantis, as well as vitamin A or RA, promote DC expression of *Aldh1a2* (which encodes retinal dehydrogenase 2). *Aldh1a2*-expressing DCs can produce high levels of RA, which in conjunction with TGF β promotes the differentiation of naive T cells into forkhead box protein P3 (FOXP3)⁺ T_{reg} cells and also upregulates the expression of the gut-homing molecules α 4 β 7 integrin and CC-chemokine receptor 9 (CCR9). Polysaccharide A (PSA) from the capsule of *B. fragilis* can be transported to the intestinal lamina propria in outer membrane vesicles via an autophagy-related protein 16-like 1 (ATG16L1) and nucleotide-binding oligomerization domain-containing protein 2 (NOD2)-dependent autophagy pathway. PSA signals through TLR2 on FOXP3⁺ T_{reg} cells to induce their proliferation and the production of IL-10, thereby promoting an anti-inflammatory state. These same anti-inflammatory effects can be achieved by SCFAs derived from consortia of 46 mouse-derived or 17 human-derived *Clostridium* strains. Butyrate inhibits histone deacetylases, thus increasing transcription at the *Foxp3* promoter and a related enhancer site, and propionate signals through GPR43, among other things. Butyrate promotes M2-like macrophage polarization and thus

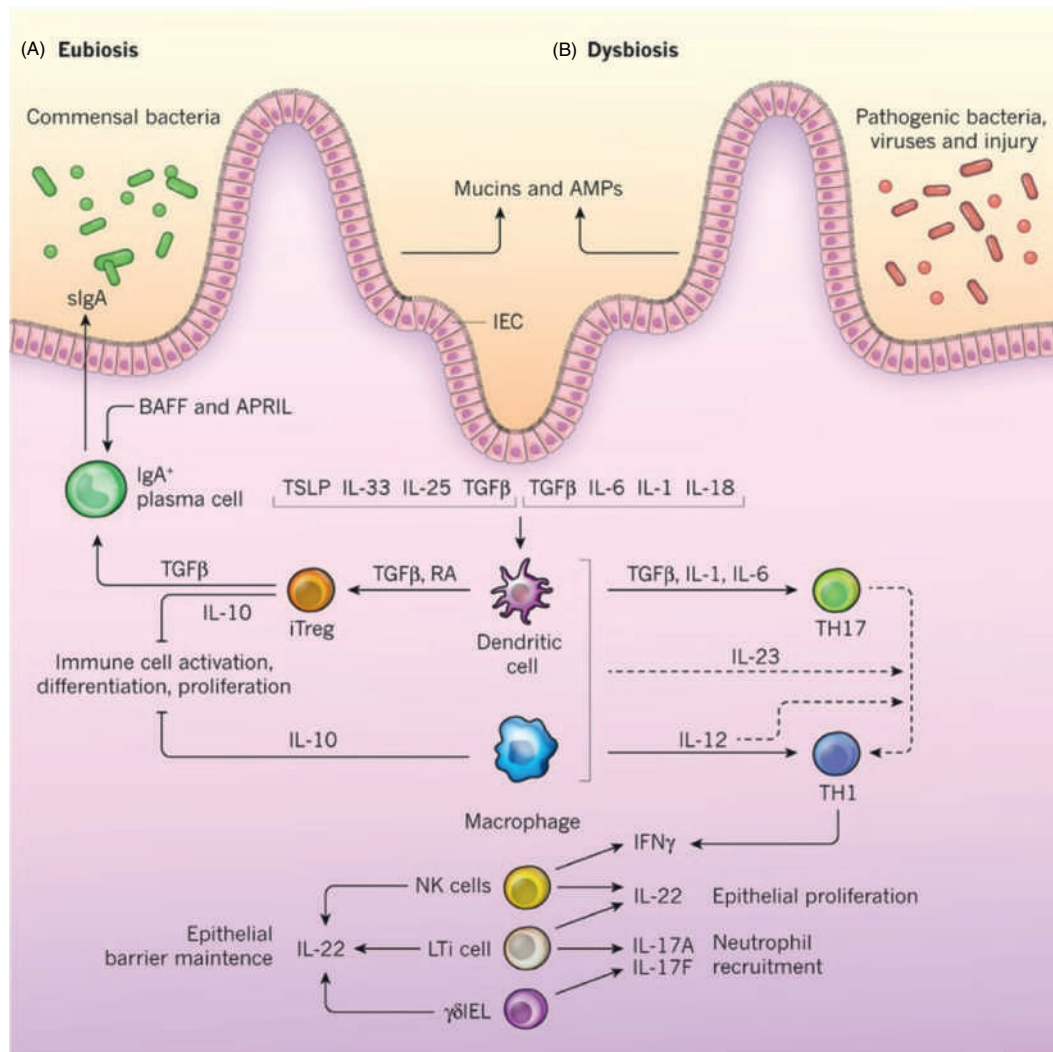


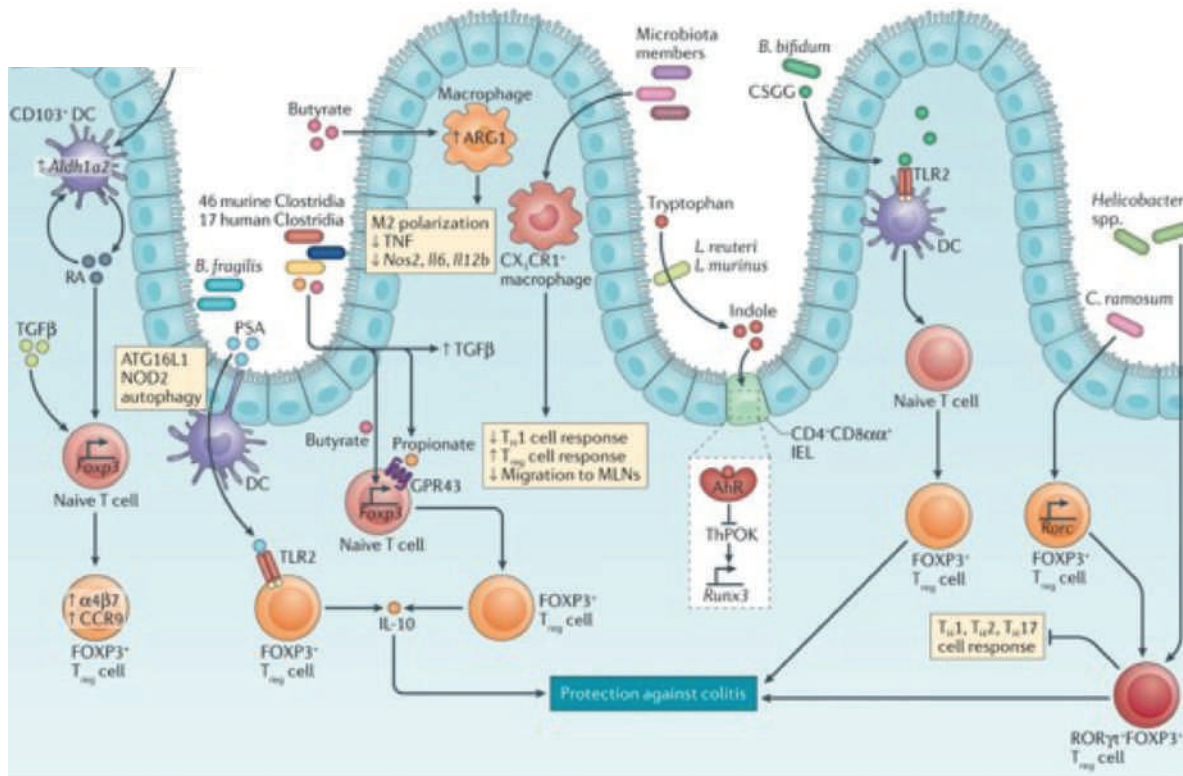
Fig. 5 The epithelial-innate-adaptive continuum in response to microbial antigens. From Maynard CL, Elson CO, Hatton RD, and Weaver CT (2012) Reciprocal interactions of the intestinal microbiota and immune system. *Nature* 489(7415): 231–241.

anti-inflammatory activity by upregulating arginase 1 (ARG1) expression, ultimately decreasing tumor necrosis factor (TNF) production and decreasing the expression of *Nos2*, *Il6*, and *Il12b*. Microbiota members induce CX₃CR1⁺ macrophages to restrict T helper 1 (T_H1) cell responses, promote T_{reg} cell responses, and decrease macrophage migration to the mesenteric lymph nodes (MLNs). Tryptophan is catabolized to indole by species including *Lactobacillus reuteri* and *L. murinus*. Indole derivatives signal through the aryl hydrocarbon receptor (AhR) in CD4⁺ intraepithelial lymphocytes (IELs) to downregulate T_H1-inducing POZ/Kruppel-like factor (ThPOK) expression and thus induce RUNX3 expression, thereby facilitating differentiation into CD4⁺CD8αα⁺ IELs. *Bifidobacterium bifidum* produces cell surface β-glucan/galactan (CSGG) polysaccharides, which signal through TLR2 on DCs and condition them to induce FOXP3⁺ T_{reg} cells. RORγt⁺ FOXP3⁺ T_{reg} cells are induced by several gut commensals, most potently by *Clostridium ramosum* and also by pathobionts like *Helicobacter* species under homeostatic conditions (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013; Sefik et al., 2015; Ohnmacht et al., 2015; Chai et al., 2017).

Pro-inflammatory effects of microbiota (Fig. 6B)

The adhesion of SFB to the intestinal epithelium induces the transcription factor C/EBPδ, which upregulates serum amyloid A (SAA) expression. SAA induces T_H17 cells and the production of IL-17, and additionally stimulates CD11c⁺ DCs to produce IL-1β. IL-1β establishes an SAA amplification loop and induces ILC3s to produce IL-22, which signals through signal transducer and activator of transcription 3 (STAT3) on epithelial cells to further induce SAA expression. Non-adhering SFB can also activate ILC3s to produce IL-22, perhaps via IL-23 signaling. *Bifidobacterium adolescentis* and a consortium of 20 strains isolated from a patient with ulcerative

(A)



(B)

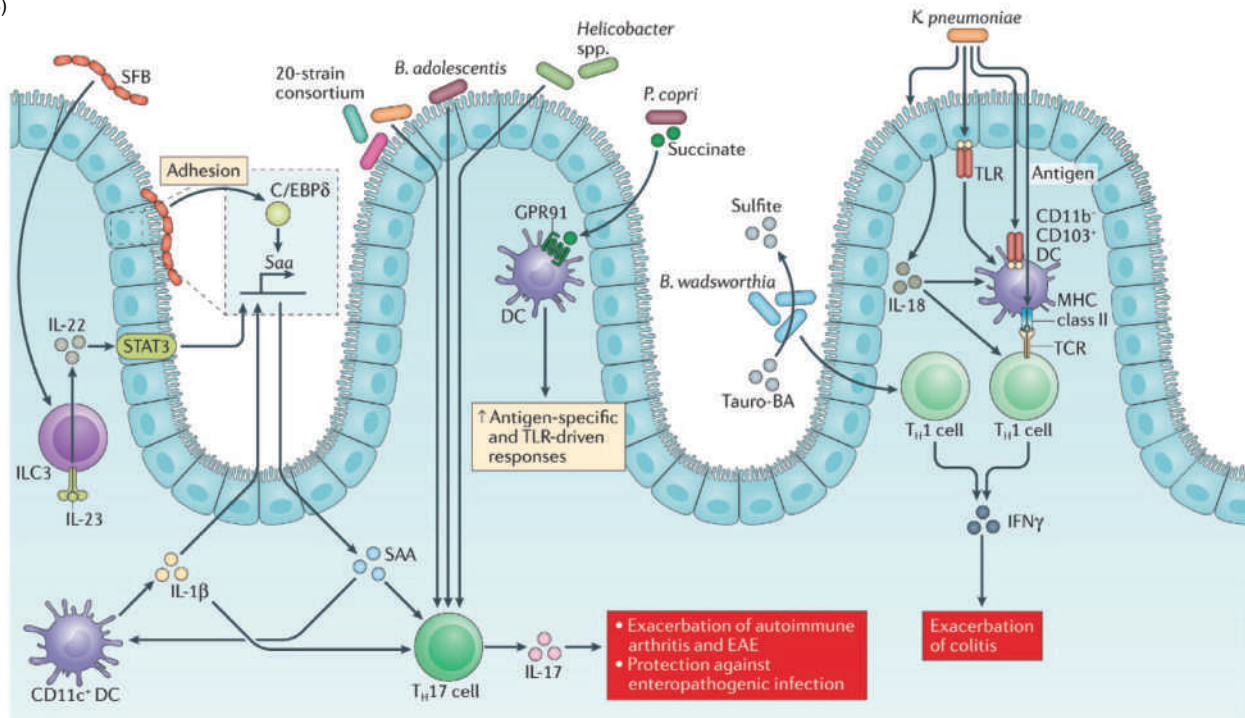


Fig. 6 Microbiota-mediated immunomodulation. (A) Anti-inflammatory effects of the microbiota. (B) Pro-inflammatory effects of the microbiota. From Skelly AN, Sato Y, Kearney S, and Honda K (2019) Mining the microbiota for microbial and metabolite-based immunotherapies. *Nature Reviews Immunology* 19(5): 305–323.

colitis can also induce T_H17 cells, potentially via epithelial adhesion. Pathobionts such as *Helicobacter* species induce antigen-specific T_H17 cells during inflammation. T_H17 cells and their related cytokines (namely, IL-17) promote a pro-inflammatory state in the gut. Commensals such as *Prevotella copri* produce succinate, which signals through GPR91 on DCs to enhance antigen-specific and TLR-driven immune responses. *Bilophila wadsworthia* reduces deconjugated taurine to sulfite, which it uses for metabolism and proliferation, triggering the induction of IFN γ -producing T_H1 cells. *Klebsiella pneumoniae* also induces IFN γ -producing T_H1 cells in a CD11b-CD103⁺ DC-dependent, TLR-dependent, and IL-18-dependent manner (Sano et al., 2015; Atarashi et al., 2015).

Immunomodulation by the microbiota (Fig. 7A–C)

Microbiota-mediated modulation of innate-like lymphocytes

Oxazoles promote indoleamine 2,3-dioxygenases (IDO) activity in epithelial cells, which in turn enhances the catabolism of tryptophan into AhR ligands such as 3-hydroxy-kynurenic acid and kynurenine. Subsequent AhR signaling promotes the presentation of lipid antigens by CD1d on the basolateral epithelial surface, in turn triggering the release of IL-13 and IFN γ by invariant natural killer T (iNKT) cells. Riboflavin derivatives can react with metabolic by-products to form pyrimidines that activate mucosal-associated invariant T (MAIT) cells by signaling through MR1. MAIT cells can enhance protection against infection with pathogens such as *Salmonella enterica* subsp. *enterica* serovar Typhimurium. *B. fragilis* produces glycosylceramides, including an α -galactosylceramide (GSL-Bf717) that potently inhibits iNKT cells in a CD1d-dependent manner, although it is unclear whether the relevant CD1d is expressed by DCs or intestinal epithelial cells (Georgel et al., 2011; Le Bourhis et al., 2013; Corbett et al., 2014).

Microbiota-mediated modulation of anticancer immunity and immune checkpoint blockade (ICB) efficacy

A consortium of 11 strains induces IFN γ ⁺CD8⁺ T cells in a CD103⁺ DC-dependent manner, thereby enhancing antimicrobial immunity against intracellular pathogens such as *Listeria monocytogenes* as well as antitumor immunity. The 11-strain consortium also produces metabolites such as mevalonate and dimethylglycine, potentially mediating the systemic induction of IFN γ ⁺CD8⁺ T cells and subsequent systemic protection. *Akkermansia muciniphila* enhances ICB efficacy by stimulating the production of IL-12 by DCs and promoting the accumulation of CCR9⁺CXCR3⁺CD4⁺ T cells in epithelial tumors and lymph nodes. *Bifidobacterium* promotes the accumulation of MHC class II^{hi} (MHCII^{hi}) DCs in melanoma tumors and enhances their capacity to stimulate T cells, resulting in an increased number of CD8⁺ T cells in tumors. Similarly, *Faecalibacterium* is associated with an increase in CD8⁺ T cells in melanoma tumors. Overall, these effects enhance ICB therapeutic efficacy (Pitt et al., 2016; Routy et al., 2018).

Microbiota-mediated modulation of host antibody response

SCFAs and saturated fatty acids produced by a subset of the intestinal microbiota activate B cells, enhancing proliferation and antibody production. However, branched-chain fatty acids, produced by species such as *Clostridium sporogenes*, inhibit these B cell processes. IgA plays a dual role in bacterial colonization; it can facilitate the clearance of certain pathogens, such as *Escherichia coli*, while enhancing mucosal colonization by commensals, such as *B. fragilis*, *Bifidobacterium animalis*, *Bacteroides thetaiotaomicron*, *Enterobacter cloacae*, and *Lactobacillus casei* (Dodd et al., 2017).

Probiotics and prebiotics in clinical use

Probiotics and prebiotics are microbiota management tools for improving host health. They target gastrointestinal effects via the gut, although direct application to other sites such as the oral cavity, vaginal tract, and skin is being explored. In this section, we describe gut-derived effects in humans.

Probiotics

Therapeutic and prophylactic effects of some probiotics for a variety of gut-related disorders might be, at least in part, mediated through modification of the microbiota and/or its function. The definition of probiotics is, as mentioned above, “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Sanders et al., 2019). Probiotic microorganisms act via a variety of means, including modulation of immune function, production of organic acids and antimicrobial compounds, interaction with resident microbiota, interfacing with the host, improving gut barrier integrity and enzyme formation.

Probiotic mechanisms of action (Fig. 8; Sanders et al., 2019)

Diverse mechanisms are likely to drive probiotic benefits to host health. In some cases, such as the production of antimicrobial products and cross-feeding other resident microorganisms, these mechanisms are driven directly by interactions with the resident microbiota. In other cases, such as direct interaction with immune cells, the effects might be direct via interaction with host cells. Overall, the clinical benefits delivered by probiotics could result from the combined action of several mechanisms.

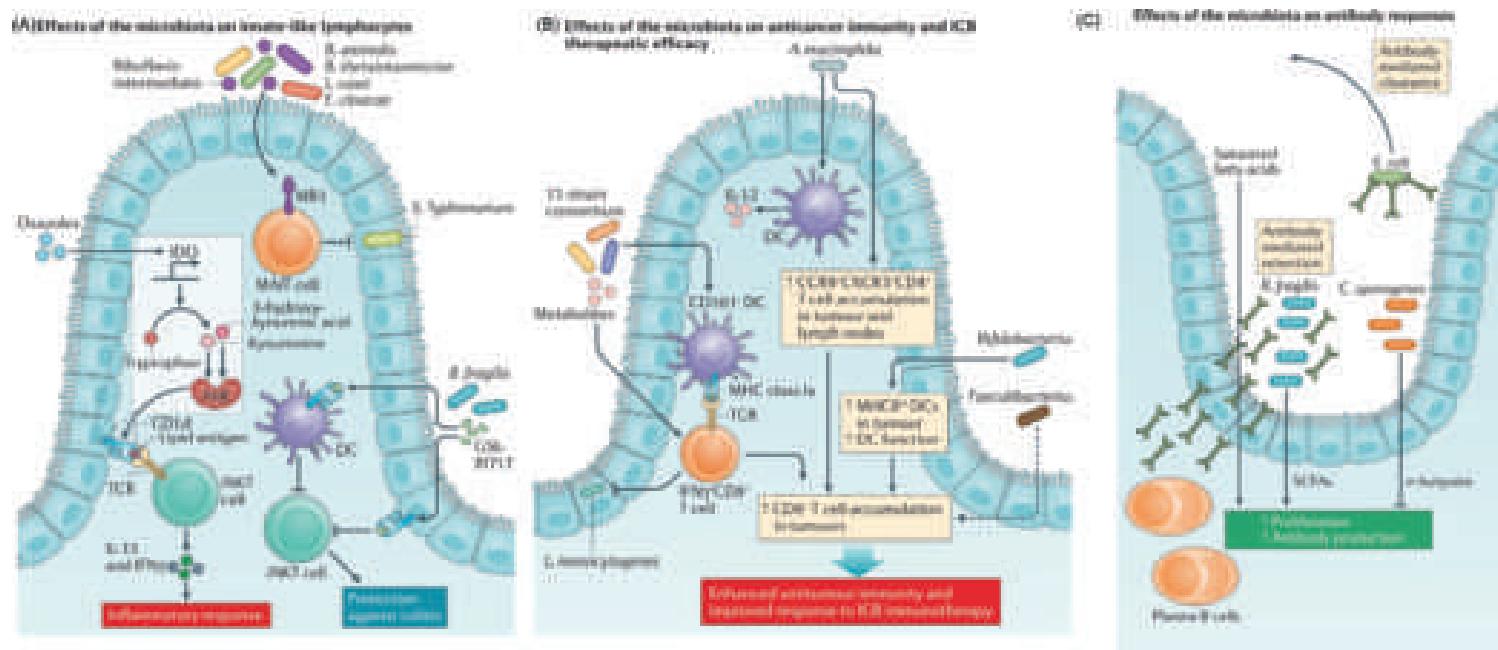
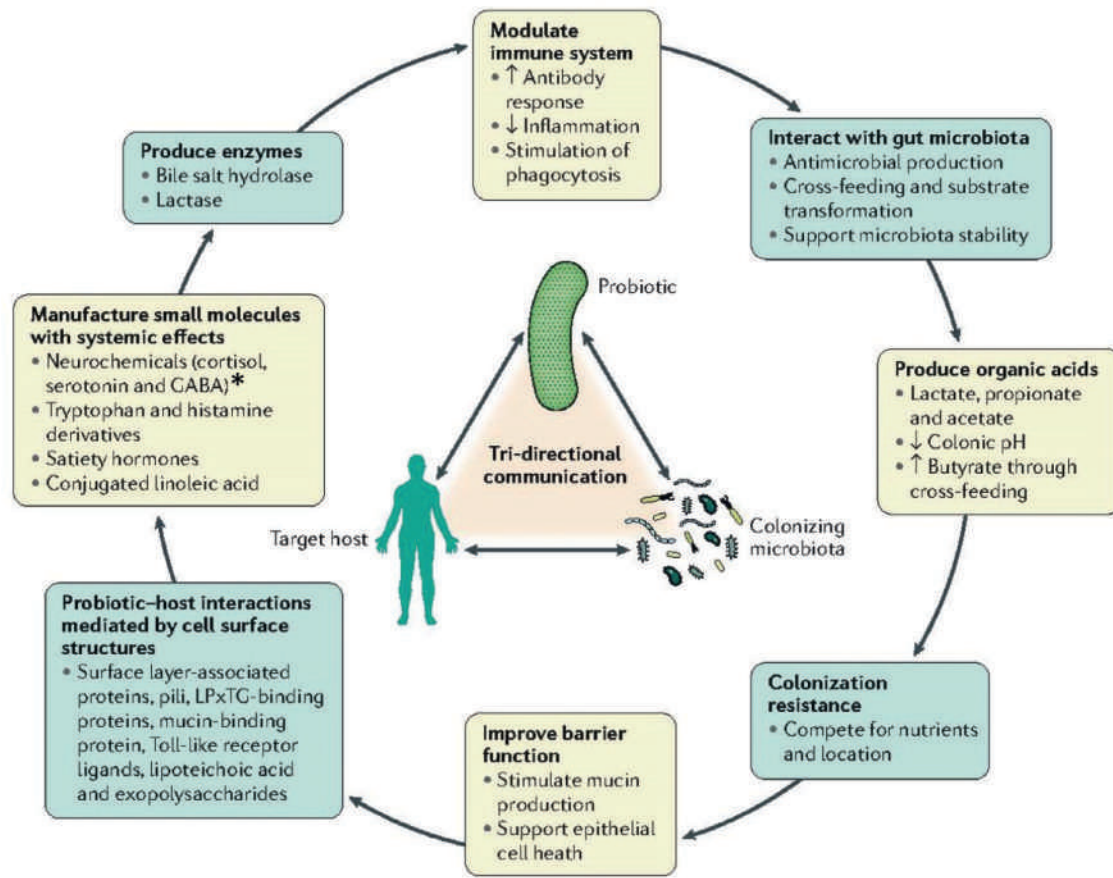


Fig. 7 Immunomodulation by the microbiota. From Skelly AN, Sato Y, Kearney S, and Honda K (2019) Mining the microbiota for microbial and metabolite-based immunotherapies. Nature Reviews Immunology 19(5): 305–323.



* GABA, gamma-aminobutyric acid

Fig. 8 Probiotic mechanisms of action. From Sanders ME, Merenstein DJ, Reid G, Gibson GR, and Rastall RA (2019) Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. *Nature Reviews Gastroenterology & Hepatology* 16(10): 605–616.

Prebiotics

Prebiotics, first defined in 1995 (Gibson and Roberfroid, 1995), have been used to manipulate microorganisms in the host to improve measurable health outcomes. An update to the definition of prebiotics published in 2017 as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” was made necessary by the need to clarify what did and did not constitute a prebiotic substance in the face of scientific advances (Gibson et al., 2017). Prebiotic effects include defense against pathogens, immune modulation, mineral absorption, improved bowel function, metabolic effects, and satiety.

Identified mechanisms of action of prebiotics (Fig. 9)

The premise is that prebiotics enter the gut and are selectively utilized. This step increases bacterial growth and functionality of specific genera or species. As a result of either or both of these effects, health benefits can accrue. Fecal bulking and improved bowel habits occur due to microbial growth. Immune regulation can be influenced by increased biomass and cell wall components of the bacteria. Metabolic products include organic acids, which lower intestinal pH and have concomitant effects upon microbial pathogens and mineral absorption. Metabolic products can also influence epithelial integrity and hormonal regulation. Bacteria that respond to prebiotic intake can influence the microbiota composition through elaboration of antimicrobial agents (for example, peptides) and competitive interactions, possibly reducing infections and bacteria containing lipopolysaccharide.

Clinical use

Many clinical gastrointestinal indications could benefit from probiotic and prebiotic interventions. For clinical use, it is required to have a better understanding of the characteristics of the host (including diet, baseline microbiota, medications, and disease) that improve in response to probiotics or prebiotics. Clinicians also need clarification about probiotic and prebiotic products: are they safe, who will benefit (how and to what extent), and can the product labels be trusted?

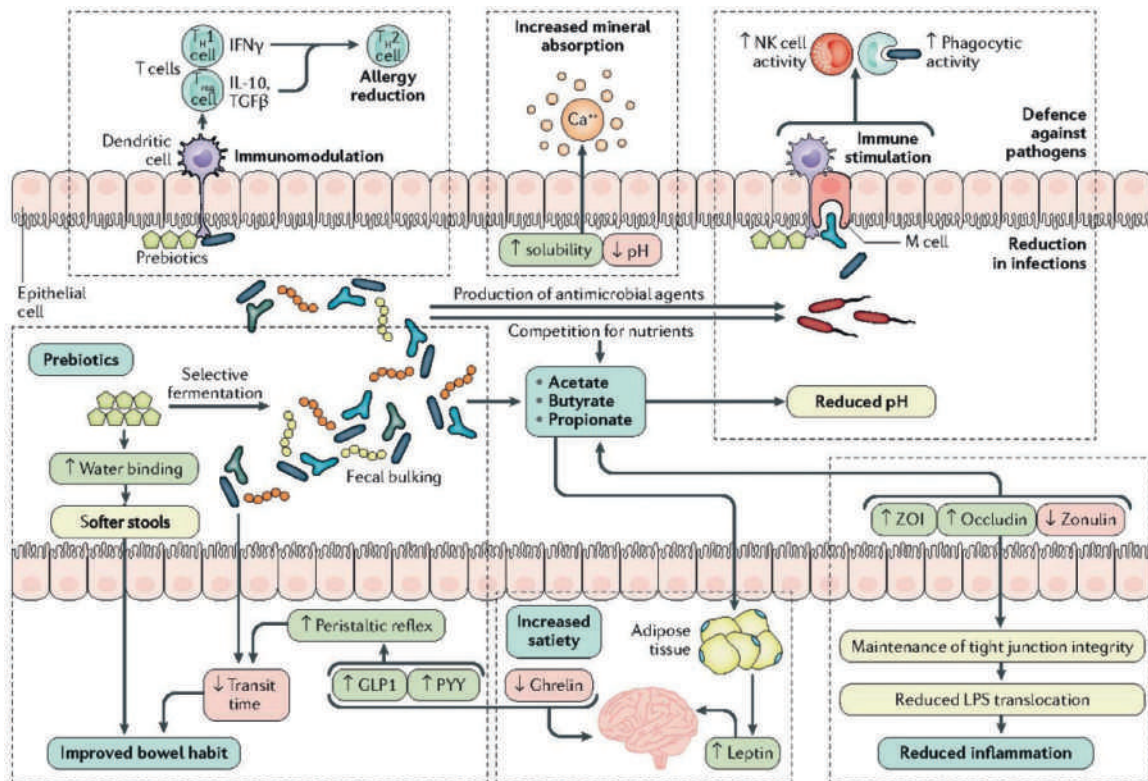


Fig. 9 Identified mechanisms of action of prebiotics. From Sanders ME, Merenstein DJ, Reid G, Gibson GR, and Rastall RA (2019) Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. *Nature Reviews Gastroenterology & Hepatology* 16(10): 605–616.

Commercial strains of probiotics and their sources

Probiotic products consist of different enzymes, vitamins, capsules, or tablets, and some fermented foods contain microorganisms that have beneficial effects on the health of the host. They can contain one or several species of probiotic bacteria. Most probiotic products destined for human consumption are in the form of fermented milk or given in powder or tablet forms. The probiotic bacteria generally belong to the genera *Lactobacillus* and *Bifidobacterium*. However, there are other bacteria, some of which are listed in Table 1 (Nagpal et al., 2014).

Effects of probiotics on health

There are many clinical indications for the use of certain probiotic strains supported by robust evidence. In pediatric and/or adult populations, the clinical conditions shown in Table 2 have been suggested as indications for the use of probiotics.

Prebiotics in clinical use

Generally, the strength of evidence for prebiotic interventions lags behind that for probiotics. Perhaps the strongest support for prebiotic use comes from prebiotic infant formulae. Such products are now routinely supplemented with mixtures of galactooligosaccharides and fructans (Arslanoglu et al., 2007, 2008), and this blend of prebiotics in a 9:1 ratio has been shown to reduce respiratory tract infections to levels found in breastfed infants (Jippes et al., 2013; Boehm et al., 2004; Shahramian et al., 2018).

Much of the research focus on prebiotics has been in the realm of functional foods (those that improve wellbeing through benefits beyond their nutrient content). The replacement of glycemic carbohydrates in food products with non-glycemic carbohydrates to reduce post-prandial glycemic responses has already received a positive European Food Safety Authority opinion (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2014).

Prebiotic foods designed to increase satiety and reduce energy intake are a promising approach to augmenting compliance with weight-loss diets. Oligofructose-enriched inulin in overweight children has been shown to increase satiety and reduce energy intake.

Table 1 Some commercial probiotic strains.

Strain	Source
<i>L. acidophilus</i> NCFM	Rhodia, Inc. (Madison, Wisconsin, United States)
<i>Lactobacillus brevis</i> KB290	Kagome Co., Ltd. (Tochigi, Japan)
<i>L. acidophilus</i> DDS-1	Nebraska Cultures, Inc. (Lincoln, NE, United States)
<i>L. acidophilus</i> SBT-2062	Snow Brand Milk Products Co., Ltd. (Tokyo, Japan)
<i>B. longum</i> SBT-2928	
<i>L. acidophilus</i> R0011	Institut Rosell (Montreal, Canada)
<i>L. rhamnosus</i> R0052	
<i>L. paracasei</i> CRL431	Chr. Hansen (Horsholm, Denmark)
<i>B. lactis</i> Bb-12	
<i>L. casei</i> Shirota	Yakult Honsha Co., Ltd. (Tokyo, Japan)
<i>B. breve</i> strain Yakult	
<i>L. casei</i> DN014001 (Immunitas)	Danone Le Plessis-Robinson (Paris, France)
<i>L. fermentum</i> RC-14	Urex Biotech Inc. (London, Ontario, Canada)
<i>L. rhamnosus</i> GR-1	
<i>Streptococcus thermophilus</i> MN-ZLW-002	Inner Mongolia Mengnuu Dairy Industry Co., Ltd. (Hohhot, China)
<i>L. johnsonii</i> La1 (same as Lj1)	Nestlé (Lausanne, Switzerland)
<i>L. plantarum</i> 299V	Probi AB (Lund, Sweden)
<i>L. rhamnosus</i> 271	
<i>L. reuteri</i> SD2112 (same as MM2)	BioGaia (Raleigh, North California, United States)
<i>L. rhamnosus</i> GG	Valio Dairy (Helsinki, Finland)
<i>L. rhamnosus</i> LB21	Essum AB (Umeå, Sweden)
<i>L. salivarius</i> UCC118	University College (Cork, Ireland)
<i>B. longum</i> BB536	Morinaga Milk Industry Co., Ltd. (Kanagawa, Japan)
<i>B. lactis</i> HN019 (DR10)	New Zealand Dairy Board
<i>L. acidophilus</i> LB	Lacteol Laboratory (Houdan, France)
<i>L. paracasei</i> F19	Aria Dairy (Stockholm, Sweden)
<i>L. crispatus</i> CTV05	Gynelogix, Boulder, Colo.
<i>L. casei</i> DN114	Danone (Paris, France)
<i>S. boulardii</i>	Biocodex Inc. (Seattle, Washington, United States)
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> 2038	Meiji Milk Products (Tokyo, Japan)

From Nagpal R, Yadav H, Kumar M, Jain S, Yamashiro Y, and Marotta F (2014) 1. Probiotics, prebiotics and synbiotics: An introduction. In: Otlies S (eds.). *Probiotics and Prebiotics in Food, Nutrition and Health*, P1-24. CRC Press Taylor & Francis Group.

Table 2 Healthful effects of probiotics.

Clinical indications
Pediatric populations^a Necrotizing enterocolitis in newborn (esp. extremely low birth weight) (Oshiro et al., 2019; Satoh et al., 2017) Late onset of sepsis in newborn (Oshiro et al., 2019; Satoh et al., 2017) Oncology children undergoing chemotherapy (Wada et al., 2010) Diarrhea • Aute diarrhea (Szajewska et al., 2019) (including rotavirus infection (Guandalini, 2000)) • Antibiotic-associated diarrhea (Vanderhoof et al., 1999) • Lactose intolerance (Salminen and Gueimonde, 2004) Atopy and reduction of allergic symptoms (Baldassarre et al., 2010; Di Costanzo and Canani, 2012) Infant colic (Sung et al., 2018) Adult and/or pediatric populations^b Alleviating constipation (Eskesen et al., 2015; Yang et al., 2008) Mild to moderate ulcerative colitis (Mardini and Grigorian, 2014) <i>H. pylori</i> infection (Szajewska et al., 2009) IBS (Whorwell et al., 2006) <i>Clostridium difficile</i>-associated diarrhea (Goldenberg et al., 2017) Reduce the incidence and duration of upper respiratory infections (Hao et al., 2015; King et al., 2014) Surgery for the intestinal tract in reduction of bacteremia (Yokoyama et al., 2014; Okazaki et al., 2016) Adult population^c Prevention of colon cancer (Rafter, 2003) Elderly Stimulation of immune system (Bian et al., 2011 Nagata et al., 2016)

^aCruchet S, Furnes R, Maruy A, et al. (2015) The use of probiotics in pediatric gastroenterology: A review of the literature and recommendations by Latin-American experts. *Pediatric Drugs* 17(3): 199–216.

^bCameron D, Hock QS, Kadim M, et al. (2017) Probiotics for gastrointestinal disorders: Proposed recommendations for children of the Asia-Pacific region. *World Journal of Gastroenterology* 23(45): 7952–7964.

^cNo official recommendations have been made for the use of probiotics in adults.

Expectations for the future

The gut microbiota might be central to the cause of many disorders, and its modulation could hold the key to effective new therapies. So, what are the roles of probiotics and prebiotics? In a general sense, both interventions serve to increase the community of beneficial microorganisms and the products of their growth and metabolism.

The field is poised for conceptual advances. Target microorganisms will expand beyond the typical *Bifidobacterium* spp. and *Lactobacillus* spp. to include other genera and perhaps more yeast species. These microorganisms might be new probiotic candidates or further targets for prebiotic utilization. To obtain robust evidence that gut microbiome alterations can reduce disease incidence or mitigate disease, more well-designed randomized controlled trials are needed. Ultimately, the application of probiotic and prebiotic regimens has the potential to improve human health and contribute greatly to how patients are managed and/or disease risk is reduced.

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Nanotechnological Therapeutic Strategies to Treat of Biofilm-Producing Gram-Positive and Gram-Negative Pathogenic Bacteria

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Introduction

A biofilm is an important form of microbial ecology, discovered in 1684. This microbial form of life promotes protection for the microbial community, making them resistant to the hostile environment, conventional disinfection methods, physical washing, and resistance to antimicrobial treatment (Hancock and Brinkman, 2002; Vasudevan, 2014). Bacterial biofilms are formed by microbial cells adhered to abiotic or biotic surfaces encased in a complex self-produced matrix composed of extracellular polymeric substances, including exopolysaccharides, extracellular DNAs (eDNA) and proteins (Rabin et al., 2015; Pakharukova et al., 2018).

Biofilms can be beneficial when associated with plants, animal and human microbiomes and can be crucial for industrial processes, such as water treatment. However, biofilms often cause significant problems for human health, as they are the basis of chronic infections and for industry, since they arise on the surfaces of tubes and obstruct filtration devices (Yan and Bassler, 2019; Sharma et al., 2019).

The biofilm formation process comprises a series of sequential phases involving genetic, environmental factors and complex mechanisms guided by chemical, physical and biological processes (Chatterjee and Otto, 2013; Coughlan et al., 2016; Tan et al., 2020). Bacterial biofilms have about 90% extracellular matrix and 10% microorganisms in their composition (Satpathy et al., 2016; Sun et al., 2020).

The elimination of biofilm becomes relevant, as it is estimated that between 65% and 80% of infections are associated with the production of this virulence factor (Eze et al., 2018; Williams et al., 2020). Besides, biofilms are commonly associated with healthcare-related infections, and they are a mattering factor in the development of antimicrobial resistance (Bodelón et al., 2016; Piperaki et al., 2017; Flament-Simon et al., 2019).

Due to therapeutic failure associated with biofilm production, microbial infections have been consolidated as one of the leading causes of death worldwide, causing hospitalization, patient suffering and reduced quality of life (Wu et al., 2015; Ciofu et al., 2015; Ramasamy and Lee, 2016). The treatment of biofilm-producing strains infections is costly and challenging due to the limited spectrum of antimicrobials effectively eliminating these bacteria (Bassetti et al., 2019; de Oliveira Júnior and Franco, 2020).

The lack of antimicrobials to treat patients with biofilm-producing microorganisms concerns health professionals, the scientific community, and the world population. To overcome this problem, the scientific community have been searching for new molecules with potential antibiofilm action in parallel to the development of new therapeutic strategies (Tran et al., 2020; Zhang et al., 2020; Pinto et al., 2020). Thus, this book chapter aimed to gather the most relevant studies applying nanocarriers and external physical stimuli to treat bacterial biofilms.

Biofilms

The biofilm was first discovered in 1684 from the observation of bacterial clusters in dental plaques. Since then, biofilms have been one of the most successful forms of life present in human and animal infections, food, and water (Ciofu et al., 2017; van Wolferen et al., 2018; Olivares et al., 2020; Silva et al., 2020). Biofilm can also be defined as a sessile agglomerate of single microbial species or

a combination of different microbial cell types immersed in an extracellular polymeric matrix. It is composed of exopolysaccharides (EPS), proteins, lipids and DNA adhered to a biotic or an abiotic surface (Desmond et al., 2018). Besides, it presents interstitial spaces, such as macro and micro-colonies, that facilitate the diffusion of nutrients and gases (Singh et al., 2017).

The biofilm acts by limiting the diffusion of antimicrobials, promoting colonization with greater structural stability and greater longevity of microorganisms in the target site. It also acts as a physical barrier, protecting against environmental stresses such as ultraviolet (UV) radiation, pH, chemical exposure, osmotic pressure variations, phagocytosis, and desiccation (Olivares et al., 2020).

Biofilms are considered a natural process, sometimes beneficial, such as a natural element of microbiomes, and essential for the industry in the effective treatment of wastewater. However, the ability that certain microorganisms have to form biofilms represents one of the possible virulence mechanisms responsible for the failure of antimicrobial therapy and a bacterial survival strategy (Yan and Bassler, 2019).

Biofilm formation is an essential factor in the virulence of microorganisms related to chronic infections and in the emerging scenario of bacterial resistance, being present in 65–80% of recurrent bacterial infections (Venkatesan et al., 2015). The problem of antimicrobial resistance is growing, and the prospect for developing new effective antimicrobials in the future is still uncertain (Antoñanzas and Goossens, 2019).

Formation of biofilms

The synthesis of biofilm can be established in a few hours. It consists of a highly regulated and dynamic mechanism regarding the transition process of microorganisms in planktonic life to the biofilm's sessile way of life (Magana et al., 2018). The biofilm regulates its architecture due to responses from the internal and external environment. Its density varies according to the type of microorganism and the conditions of the environment in which it finds itself (Germec et al., 2020).

During the transitional process of the planktonic state to the sessile, bacteria continually change their phenotype. They can become highly resistant to antimicrobials and components of the host's innate and adaptive immune system, contributing significantly to the failure of antimicrobial therapy. This protection provided by the biofilm extends to Gram-positive and Gram-negative bacteria, mobile and non-mobile, aerobic, anaerobic, protozoa, archaea, algae, filamentous fungi and yeast (Costa-Orlandi et al., 2017; Raghupathi et al., 2018; Muhammad et al., 2020).

The biofilm formation, in general, can be presented through five stages: a reversible connection of cells to a surface, followed by irreversible adhesion, maturation I and II and finally, the dispersion of the biofilm (Fig. 1) (Roy et al., 2018).

The structuring, maturation and dissemination of biofilm is a highly controlled process. The main regulators responsible for this phenotype is the cell-to-cell signaling mechanism known as *Quorum Sensing* (QS) (Verderosa et al., 2019).

QS is a cell-to-cell communication mechanism, regulator cell density-dependent gene expression, found in Gram-negative and Gram-positive bacteria (Schilcher and Horswill, 2020). Regulation by the QS maintains the ideal biofilm size, preventing the cell density of the biofilm from reaching an unsustainable level, activating specific genes that lead to the expression of virulence factors, such as enzymes and/or toxins, ensuring their adaptation to the most diverse environmental conditions (Dickschat, 2010; Verderosa et al., 2019).

Antibiotics resistance and clinical importance of biofilms

Antimicrobial resistance has become a significant public health problem and, consequently, a mattering threat to human health. The annual mortality rate of infections caused by multidrug-resistant (MDR) microorganisms in 2050 is predicted to increase to 10 million, generating an estimated economic impact of U\$ 100 trillion (Crabbé et al., 2019). Bacterial resistance to antibiotics is the bacterium's ability to survive at concentrations of antibiotics capable of inhibiting/killing other ones of the same species (Alós, 2015). The resistance reflects the increase of the minimum inhibitory concentration (MIC) (Brauner et al., 2016).

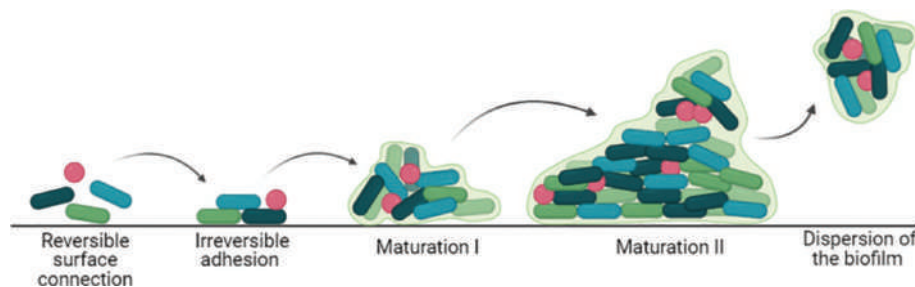


Fig. 1 Schematic illustration of the biofilm formation. Created by the authors.

Biofilm promotes increased cell tolerance, as sessile bacteria alone tend to be about 500–5000 times more tolerant than their planktonic state (Vergidis and Patel, 2012; Khatoon et al., 2018). Tolerance begins when bacteria aggregate in high density, which generates a gradient of nutrients, restricting the antimicrobial penetration into the biofilm (Fux et al., 2003). In a biofilm, it was observed that the release of bacteriophage-mediated eDNA favors the emergence of tolerant colonies (Hall and Mah, 2017). Thus, the tolerance and multidrug resistance observed in biofilm-forming bacteria have represented a challenge for current therapies (Ciofu et al., 2017).

Biofilm represents one of the virulence factors associated with the pathogenesis of several bacterial pathogens. The variety of clinical conditions attributed to or associated with bacterial biofilms is large. They range from the most common such as tooth decay to chronic conditions, such as cystic fibrosis (Verderosa et al., 2019). They are particularly problematic when associated with medical devices, fomites, and wound infection (Hughes and Webber, 2017).

About 40–80% of bacterial cells can live in biofilms, and according to the Centers for Disease Control and Prevention (CDC), about 65% of Healthcare-Associated Infections (HAIs) are due to the production of biofilms (Percival, 2017; Flemming and Wuertz, 2019). Most HAIs are often associated with prolonged treatments, higher rates of morbidity and mortality and therapeutic failures due to the resistance guaranteed by the biofilm, which represents a considerable economic burden (Del Pozo, 2018).

Thus, its presence has severe implications for choosing the most effective therapeutic option for the treatment of the patient and management of the microorganism, preventing its spread in different nosocomial environments and/or in the community (Percival, 2017). Finally, the treatment of bacterial biofilms still represents a challenge due to the absence of efficacious drugs with antibiofilm activity, both for preventing and treating infections caused by producing microorganisms (Ciofu et al., 2015). Eradication of biofilms is problematic because high doses of commercial antimicrobials are required, increasing the risk of systemic toxicity, leading to adverse effects detrimental to the patient's health (Hurlow et al., 2015; Yan and Bassler, 2019).

Treatment of bacterial biofilms

The treatment of biofilms can follow two approaches, one through the inhibition of biofilm formation and the other related to the eradication or treatment of the formed biofilm (Khatoon et al., 2018; Banerjee et al., 2019). The prevention strategy for biofilm formation is based on blocking bacterial growth using antimicrobial agents. Blockade can occur through inhibition of bacterial adhesion or due to disruption of cellular communication (Kostakioti et al., 2013; Lebeaux et al., 2014; Manner et al., 2017; Paluch et al., 2020).

To assess the activity of antibiotics against biofilms, these drugs are evaluated for minimum inhibitory biofilm concentration (MBIC) and minimum biofilm eradication concentration (MBEC) (Cruz et al., 2018; Thieme et al., 2019). MBIC is determined as the lowest concentration of an antimicrobial agent needed to inhibit biofilm formation (Perumal and Mahmud, 2013). Molecules or substances that act by inhibiting biofilm play an essential role in preventing biofilm formation and eradication are of great use in the use of hospital medical material surfaces (Roy et al., 2018; Abebe, 2020). MBEC is defined as the lowest concentration of an antimicrobial agent that is necessary to eradicate the formed biofilm (Venkatesan et al., 2015; Thieme et al., 2019).

As for the biofilm eradication mechanisms, the strategies are based on the replacement of contaminated biomedical devices or on the use of antibiotics capable of dissolving the biofilm through enzymes and other molecules that can disintegrate the matrix of EPS (Fig. 2) (Roy et al., 2018; Verderosa et al., 2019; Munir et al., 2020). Another strategy is the association of antimicrobial agents, one that manages to perforate the biofilm structure and the other that penetrates the biofilm and acts on microorganisms (Fig. 2) (Gebreyohannes et al., 2019; Munir et al., 2020).

The biofilm matrix contains protein material, extracellular DNA and polysaccharides, making it more susceptible to degradation by exogenous enzymes such as proteinase K, trypsin and DNase I. The use of these enzymes associated with antimicrobial agents can be an alternative for eradicating biofilms (Fagerlund et al., 2016; Nahar et al., 2018; Jiang et al., 2020; Karygianni et al., 2020).

To identify whether the antibiotic presents antibiofilm activity, the MBIC or MBEC are assessed. The MBIC and MBEC values are important because in vitro studies comparing MIC with MBIC or MBEC assays show that there are differences in the sensitivity patterns of antibiotics. Biofilms are more resistant, requiring a higher concentration of drugs to prevent or eradicate the biofilm (Mulla et al., 2016; Shimizu and Harada, 2017; Madheswaran et al., 2018; Rudilla et al., 2018; Thieme et al., 2019).

The treatment of biofilm infections is, without doubt, a challenging approach. Studies describe that the treatment of newly formed biofilms is likely to be eliminated using only antibiotics compared to already well-developed biofilms. This information reinforces the importance of diagnosing early infection to achieve an efficient treatment (Taraszkievicz et al., 2013; Koo et al., 2017; Roy et al., 2018; Gebreyohannes et al., 2019).

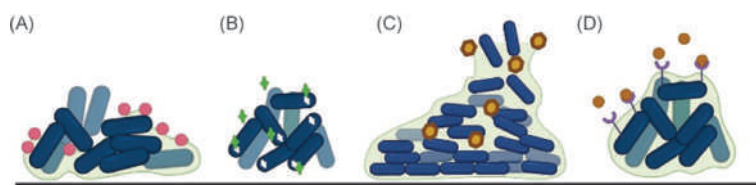


Fig. 2 Illustration of the antibiofilm agents' action. (A) EPS degradation; (B) Cell signaling; (C) Biofilm degradation; (D) Antibiofilm target. Created by the authors.

The treatment of bacterial biofilms could be divided into three main strategies. The first one is based on surgical debridement of devitalized tissues, followed by continuous irrigation of a biocide and/or antiseptic, and then using antibiotics by endovenous route for a systemic action (Attinger and Wolcott, 2012; Edmiston et al., 2016). Another strategy applied to infections caused by the presence of biofilm in a medical device is the removal and/or replacement of the device followed by the selection and use of systemic antimicrobials (Wu et al., 2015; Høiby et al., 2015; Khatoon et al., 2018).

Nanotechnology

Nanotechnology has gained significant importance due to its applications in preventing, diagnosing, and treating various diseases. This technology provides several combinations of materials or devices with drugs and biomolecules, bringing benefits such as drug-controlled release, which, in turn, increase drug circulation time and the half-life, decreased toxicity and could target the encapsulated drug to a specific site. Nanomaterials can be developed to potentiate the activity of antibiotics with minor side effects and can potentiate the activity of antibiotics (Ramos et al., 2018; Naskar and Kim, 2019).

As illustrated in Fig. 3, the encapsulation of antibiotics and other active substances can occur through different nanocarriers, such as liposomes, solid lipid nanoparticles, polymeric nanoparticles, inorganic nanoparticles, among others (Pircalabioru and Chifiriuc, 2020).

Drug-resistant bacteria and biofilm-associated infections are challenging to treat due to the inherent resistance to antimicrobial agents. Encapsulation of these agents into nanocarriers then becomes a strategy used to increase the efficacy of these drugs and turn biofilm eradication possible. The nanocarriers can provide the entry of the drug into the biofilm, improving the efficacy of the treatment while reducing toxic levels (Yeh et al., 2020).

Nanomaterials in the treatment of biofilm-driven infections can be subdivided into three groups. The first group includes nanomaterials that possess intrinsic biofilm eradication properties. The second group comprises those that serve as carriers of molecules with antimicrobial properties. Finally, the third covers responsive nanomaterials, usually metal nanoparticles or nanocarriers, that possess the ability to disrupt biofilm physics (Tran et al., 2020).

A range of nanomaterials can be used against biofilms because of their intrinsic antimicrobial properties, due to the composition of nanomaterials or because they can encapsulate natural compounds or synthetic drugs. The interaction of nanocarriers with bacterial cells can cause cell membrane damage, oxidative stress mechanisms, inactivation of enzymes primordial to metabolism, and gene alterations preventing biofilm formation. In the preformed biofilm, nanoformulations can penetrate or release substances on the biofilm surface, facilitating the penetration of the drug, to preventing cell signaling and disassemble the extracellular polymeric matrix (Fig. 4) (Rukavina and Vanić, 2016; Mihai et al., 2018; Chaudhary et al., 2020).

Liposomes

Liposomes are lipid nanocarriers used to deliver active molecules with antimicrobial potential. In this sense, recent studies demonstrate the potential for delivering molecules and drugs with potential antibiofilm (Table 1) (Rukavina and Vanić, 2016).

Alhariri et al. (2017) evaluated the antibiofilm activity of gentamicin loaded in liposomes (NELG-1 and NELG-2) against *P. aeruginosa* BAA1744 and *Klebsiella oxytoca* 700,324. The sizes of NELG-1 and of NELG-2 were 691.3 ± 3.3 nm and 806.6 ± 3.4 nm, and the zeta potential was 25.3 ± 2.5 mV and 31.7 ± 1.7 mV, respectively. The percentage of gentamicin loaded in these formulations was $37.2 \pm 0.46\%$ for NELG-1 and $43.6 \pm 0.65\%$ for NELG-2. It was observed that liposomes at a concentration of $1/2 \times \text{MIC}$ ($0.125 \mu\text{g mL}^{-1}$) showed an inhibitory effect on the biofilm of *P. aeruginosa* and *K. oxytoca* for NELG-1 and NELG-2. In comparison, eradication of the structured biofilm of *P. aeruginosa* and *K. oxytoca* was achieved at a concentration of $16 \times \text{MIC}$ ($4 \mu\text{g mL}^{-1}$) for NELG-1 and NELG-2.

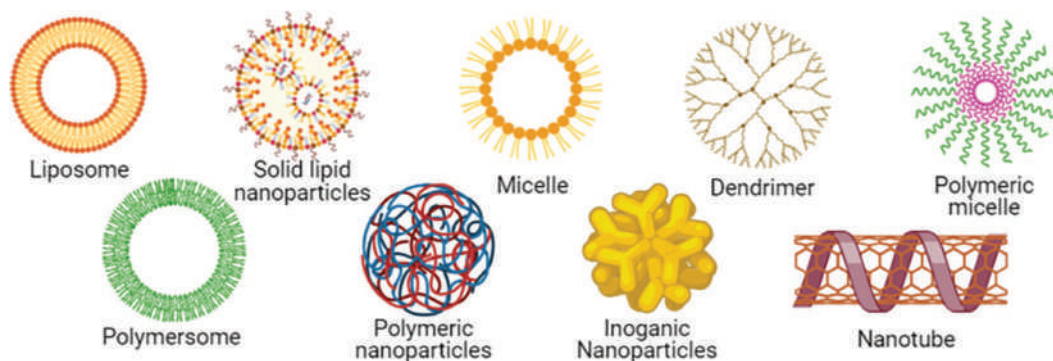


Fig. 3 Illustration of different nanocarriers used to encapsulate antibiotics. Created by the authors.

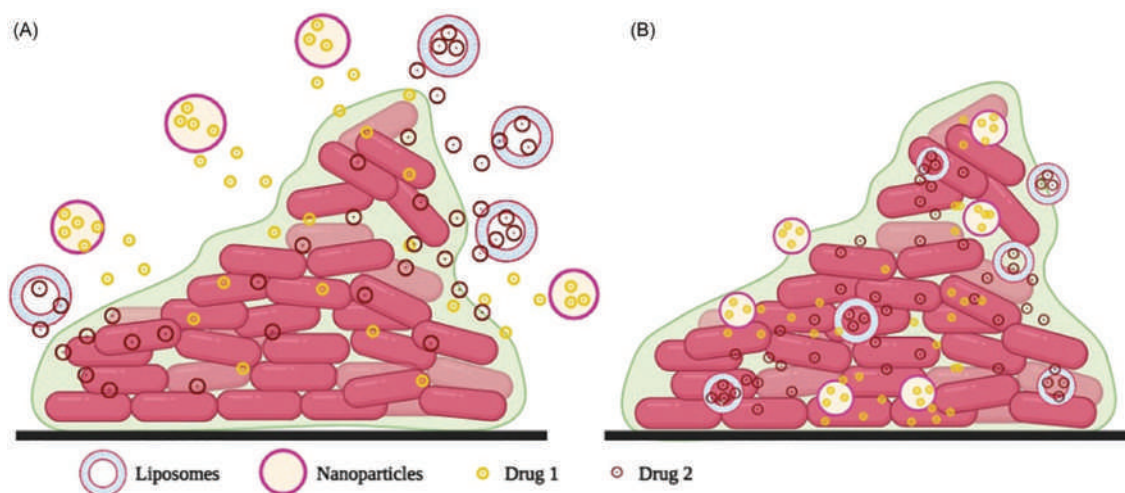


Fig. 4 Release mechanisms of antibiotics encapsulated into liposomes or polymeric nanoparticles in biofilm. (A) Nanocarrier releasing the antimicrobial on the surface of the biofilm and (B) Nanocarrier penetrating the biofilm and releasing the antimicrobial. Created by the authors.

Table 1 Antibiofilm potential of molecules and drugs encapsulated in liposomes.

Liposome	Constituents	Bacterial strains	Antibiofilm activity ($\mu\text{g mL}^{-1}$)		References
			Inhibition	Eradication	
NELG-1	1,2-Dimyristoyl-sn-glycero-3-phospho- (1'-rac-glycerol), DPPC, Chol and gentamicin	<i>P. aeruginosa</i> BAA1744 and <i>Klebsiella oxytoca</i> ATCC 700324	MBIC of $0.125 \mu\text{g mL}^{-1}$ ($1/2 \times \text{MIC}$)	MBEC of $4 \mu\text{g mL}^{-1}$ ($16 \times \text{MIC}$)	Alhariri et al. (2017)
NELG-2	1, 2-Dimyristoyl-sn-glycero-3-phospho- (1'-rac-glycerol), DPPC, Chol and gentamicin	<i>P. aeruginosa</i> BAA1744 and <i>Klebsiella oxytoca</i> 700,324	MBIC of $0.125 \mu\text{g mL}^{-1}$ ($1/2 \times \text{MIC}$)	MBEC of $4 \mu\text{g mL}^{-1}$ ($16 \times \text{MIC}$)	Alhariri et al. (2017)
Lysozyme-associated liposomal gentamicin	DPPC, DPPG, gentamicin sulfate and lysozyme	<i>Staphylococcus aureus</i> ATCC 29213 and <i>Pseudomonas aeruginosa</i> PA01	80–90%	85–95%	Hou et al. (2017)
Cationic liposomes encapsulating cefquinome sulfate	SPC, SA, Chol, Tween 80 and cefquinome sulfate	<i>Staphylococcus aureus</i> ATCC 25923	ND	81.30% ($2.5 \times \text{MIC}$)	Li et al. (2019)
Liposomes containing vancomycin	DPPC, DOPE, Chol, EPC, vancomycin hydrochloride and alpha-tocopherol	<i>Staphylococcus aureus</i> ATCC 29213	MBIC of $2 \times \text{MIC}$ ($3.13 \mu\text{g mL}^{-1}$)	ND	Scriboni et al. (2019)
Liposomes containing cefoperazone sodium	DPPC, Chol and cefoperazone sodium	<i>Pseudomonas aeruginosa</i>	50% at $1 \mu\text{g mL}^{-1}$ and 87% at $250 \mu\text{g mL}^{-1}$	50% at $1 \mu\text{g mL}^{-1}$ and 91% at $250 \mu\text{g mL}^{-1}$	Ghodake et al. (2020)
Nanoliposomal formulations of rifampicin and <i>N</i> -acetyl cysteine	HSPC, Chol, SA, rifampicin and <i>N</i> -acetyl cysteine	<i>Staphylococcus epidermidis</i> DSMZ 3270	ND	ND	Bazrgari et al. (2020)
Liposomes encapsulating resveratrol	DOPC, Chol, MAN1, GLT1 and GL4	MRSA clinical isolates	ND	MBEC of 0.019 mM	Aiello et al. (2021)
Meropenem encapsulated in a niosome	Span 60, Tween 60, Chol and meropenem	MRSA ATCC 33592 MRSA clinical isolates	MBIC of $0.125 \mu\text{g mL}^{-1}$ MBIC of $0.062 \mu\text{g mL}^{-1}$ and $0.25 \mu\text{g mL}^{-1}$	ND	Shirin et al. (2021)

DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol); DOPE, dioleoylphosphatidylethanolamine; EPC, egg phosphatidylcholine; SPC, soybean phosphatidylcholine; HSPC, hydrogenated soybean phosphatidylcholine; Chol, cholesterol; SA, stearylamine; MAN1, mannose residues; GLT1, galactose residues; GL4, glucose residues; MRSA, Methicillin-resistant *Staphylococcus aureus*; ATCC, American Type Culture Collection; MTCC, Microbial Type Culture Collection and Gene Bank; MIC, Minimum Inhibitory Concentration; MBIC, Minimum Inhibitory Biofilm Concentration; MBEC, Minimum Biofilm Eradication Concentration; ND, Not determined.

Hou et al. (2017) prepared liposomes containing gentamicin associated with lysozyme. Lysozyme-associated liposomal gentamicin (LLG) was prepared by vesicle extrusion technique, and the average particle size and zeta potential were 104 nm and 17.5 mV, respectively. This treatment significantly reduced the biofilm formation (80–90%) and preformed biofilm (85%–95%) of *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* PA01 biofilm. Quantification of biofilm biomass and cell viability demonstrated that the formulation had a more pronounced effect than gentamicin or lysozyme alone.

Li et al. (2019) prepared cationic liposomes encapsulating cefquinome sulfate (CSCLs) and evaluated their antibiofilm activity against *Staphylococcus aureus* ATCC 25923. CSCLs were prepared by the solid dispersion method combined with effervescent hydration. The average particle size of the CSCLs was 201.5 nm, the zeta potential of +65.29 mV, and the encapsulation efficiency was 63.21%. Eradication of preformed biofilm was observed, reaching 81.30% in 24 h at a concentration of $2.5 \times \text{MIC}$ ($1.2 \mu\text{g mL}^{-1}$). Scriboni et al. (2019) formulated fusogenic liposomes containing vancomycin against *Staphylococcus aureus* biofilm-producer ATCC 29213. Treatment using this formulation showed an inhibitory effect at $2 \times \text{MIC}$ ($3.13 \mu\text{g mL}^{-1}$).

Ghodake et al. (2020) developed liposomes containing cefoperazone sodium to eliminate *P. aeruginosa* biofilm in cystic fibrosis infection. The liposomes were formulated using the lipid thin film hydration method and had a particle size of 410.85 ± 26 nm, PDI of 0.4, a zeta potential of -18.3 mV and encapsulation efficiency of $68.90 \pm 3\%$. It was observed that there was 50% and 87% inhibition of biofilm formation at concentrations of $1 \mu\text{g mL}^{-1}$ and $250 \mu\text{g mL}^{-1}$, respectively. It was also observed that there was the eradication of the preformed biofilm in 50% and 91% at the same concentrations, respectively.

Bazrgari et al. (2020) evaluated the antibiofilm activity of nanoliposomal formulations of rifampicin and *N*-acetyl cysteine (NAC) against *Staphylococcus epidermidis* (DSMZ 3270) biofilm. The liposomal formulations were prepared by the solvent evaporation method, and the liposomes containing rifampicin presented particle size of 125.23 ± 6.40 nm, PDI of 0.124 ± 0.001 , the zeta potential of $+30.76 \pm 1.31$ mV and encapsulation efficiency $90 \pm 5\%$, while the liposomes containing rifampicin and NAC presented a particle size of 180 ± 2.32 nm, PDI of 0.200 ± 0.02 , the zeta potential of $+54.33 \pm 2.96$ mV and encapsulation efficiency $80 \pm 3\%$ for rifampicin and $18 \pm 2\%$ for NAC. The eradication of bacterial biofilm and the inhibition of biofilm formation were evaluated based on the optical density ratio. The antibiofilm activity of most of the formulations was concentration-dependent and time-dependent.

Aiello et al. (2021) prepared liposomes composed of DOPC and cholesterol (Chol) with mannose residues (MAN1), galactose residues (GLT1) or glucose residues (GL4) encapsulating resveratrol and evaluated their antibiofilm activity against biofilm of MRSA clinical isolates. The liposomes developed exhibit an average particle size of 95 nm, PDI of 0.2, zeta potential around 23 mV and encapsulation efficiency above 90%. It has been observed that the mannosylated and galactosylated liposomes can eradicate the preformed MRSA biofilm at concentrations ranging from 0.019 mM to 1.2 mM.

Shirin et al. (2021) evaluated the potential of meropenem encapsulated in a niosome against MRSA strains. The formulation presented an average particle size of 51.3 ± 5.84 nm, PDI 0.131, the zeta potential of -65.29 ± 2.6 mV, encapsulation efficiency of $84.86 \pm 3.14\%$ and presented MBIC of $0.125 \mu\text{g mL}^{-1}$ for MRSA ATCC 33592 and MBICs between $0.062 \mu\text{g mL}^{-1}$ and $0.25 \mu\text{g mL}^{-1}$ for six MRSA clinical isolates from hospitals in Tehran, Islamic Republic of Iran. Studies show that liposomes are promising in administering antibiotics in the therapy of biofilm-related bacterial infections due to their ability to decrease MICs and the MBICs and penetrate biofilms enabling the delivery of the active molecule compared to conventional therapy (Rukavina and Vanić, 2016).

Polymeric nanoparticles

Polymeric nanoparticles are nanocarriers discovered in the 1970s that have become promising in drug delivery, including drugs with antibacterial potential. Research is carried out encapsulating active molecules with potential antibiofilm (Table 2) (Chaudhary et al., 2020).

The study conducted by Cui et al. (2018) evaluated the antibiofilm activity of chitosan nanoparticles loaded with clove oil (CO@CNPs) against biofilms of *Escherichia coli* O157:H7. CO@CNPs were prepared following the ionic cross-linking method. The average particle size of CO@CNPs was between 154.9 ± 3.0 nm to 236.3 ± 2.6 nm. The CO@CNPs showed PDI ranged between 0.266 ± 0.023 and 0.297 ± 0.032 . The zeta potential of all CO@CNPs prepared ranged from 30.9 ± 2.2 mV to 44.3 ± 2.3 mV, and the encapsulation efficiency was $39.6 \pm 0.8\%$. The antibiofilm activity of CO@CNPs was observed with a 99.98% reduction in *E. coli* O157:H7 biofilm after 8 h of treatment.

Subhaswaraj et al. (2018) evaluated the antibiofilm potential of chitosan nanoparticles encapsulating cinnamaldehyde (CANPs) against *Pseudomonas aeruginosa* PAO1. CANPs were synthesized by ionic gelation method and presented average diameter size, PDI and encapsulation efficiency of 208.12 nm, 0.277 and $65.04 \pm 2.14\%$, respectively. The inhibition of biofilm formation by CANPs was $63.59 \pm 2.5\%$ and $84.06 \pm 4.09\%$ at subinhibitory concentrations of $250 \mu\text{g mL}^{-1}$ and $500 \mu\text{g mL}^{-1}$, respectively.

Tan et al. (2018) developed chitosan nanoparticles containing oxacillin (OXA) and deoxyribonuclease I (CSNP-DNase-Oxa), targeting biofilm cells and biofilm matrix of *Staphylococcus aureus* ATCC 6538. Biofilm reduction was achieved with all concentrations tested ($1, 0.5, 0.25, 0.125$ and $0.0625 \mu\text{g mL}^{-1}$). Both the free and encapsulated OXA showed promising results on biofilm inhibition. The lowest concentration ($0.0625 \mu\text{g mL}^{-1}$) inhibited about 60% of biofilm formation, and almost no biofilm formation was observed at concentrations higher than $0.5 \mu\text{g mL}^{-1}$. Repeated treatment with CSNP-DNase-OXA for 2 days resulted in a 98.4% reduction of *Staphylococcus aureus* biofilm.

Ma et al. (2020) encapsulated curcumin in chitosan nanoparticles (CSNP-Cur) and evaluated their antibiofilm activity against *S. aureus* ATCC 6538. The average diameter of CSNP-Cur was 134.37 ± 1.99 nm, the zeta potential of

Table 2 Antibiofilm potential of molecules and drugs encapsulated in polymeric nanoparticles.

Polymeric nanoparticles	Bacterial strains	Antibiofilm activity ($\mu\text{g mL}^{-1}$)		References
		Inhibition	Eradication	
Chitosan nanoparticles loaded with clove oil	<i>Escherichia coli</i> O157:H7	ND	MBEC of 3 mg mL^{-1}	Cui et al. (2018)
Chitosan nanoparticles encapsulating cinnamaldehyde	<i>Pseudomonas aeruginosa</i> PAO1	63.59% at $250 \text{ }\mu\text{g mL}^{-1}$ and 84.06% at $500 \text{ }\mu\text{g mL}^{-1}$	ND	Subhaswaraj et al. (2018)
Chitosan nanoparticles containing oxacillin and Dnase	<i>Staphylococcus aureus</i> ATCC 6538	60% at $0.0625 \text{ }\mu\text{g mL}^{-1}$	98.4% at $0.0625 \text{ }\mu\text{g mL}^{-1}$	Tan et al. (2018)
Curcumin in chitosan nanoparticles	<i>Staphylococcus aureus</i> ATCC 6538	ND	88.48% at $400 \text{ }\mu\text{g mL}^{-1}$	Ma et al. (2020)
Chrysin in chitosan nanoparticles	<i>Staphylococcus aureus</i> MCC 2408	66.59% at $768 \text{ }\mu\text{g mL}^{-1}$	43.50% at $768 \text{ }\mu\text{g mL}^{-1}$	Siddhardha et al. (2020)
Albumin nanoparticles coated with chitosan	<i>Acinetobacter baumannii</i> Col R1	60% at $1.25 \text{ }\mu\text{g mL}^{-1}$, 58% at $0.62 \text{ }\mu\text{g mL}^{-1}$ and 61% at $0.31 \text{ }\mu\text{g mL}^{-1}$	ND	Scutera et al. (2021)
	<i>Acinetobacter baumannii</i> Col S	95% at $0.156 \text{ }\mu\text{g mL}^{-1}$, 65% at $0.078 \text{ }\mu\text{g mL}^{-1}$ and 60% at $0.039 \text{ }\mu\text{g mL}^{-1}$		
Nanoparticles of PLGA containing ciprofloxacin	<i>Staphylococcus aureus</i> ATCC 29213	ND	MBECs were $25 \text{ }\mu\text{g mL}^{-1}$ and $12.5 \text{ }\mu\text{g mL}^{-1}$ after 24 h and 48 h of incubation, respectively	Gheffar et al. (2021)
Nanoparticles of PLA encapsulating rifampicin surface functionalized by poly-L-lysine	<i>Staphylococcus aureus</i> SH1000	ND	30% at $0.1\text{--}10 \text{ }\mu\text{g mL}^{-1}$	Da Costa et al. (2021)

PLA, Polylactic acid; PLGA, poly(lactic-co-glycolic acid); DNase, deoxyribonuclease I; ATCC, American Type Culture Collection; MCC, Microbial Culture Collection; MIC, Minimum Inhibitory Concentration; MBIC, Minimum Inhibitory Biofilm Concentration; MBEC, Minimum Biofilm Eradication Concentration; ND, Not determined.

$+18.10 \pm 0.82 \text{ mV}$ and encapsulation efficiency of $91.95 \pm 0.63\%$. At concentrations up to $400 \text{ }\mu\text{g mL}^{-1}$, CSNP-Cur reduced the preformed biofilm by 88.48%. In comparison, Siddhardha et al. (2020) encapsulated chrysin in chitosan nanoparticles (CCNPs) and tested the antibiofilm activity against *S. aureus* MCC 2408. The average diameter of the synthesized CCNPs was 355 nm, PDI of 0.487 and encapsulation efficiency of $80.86 \pm 0.30\%$. The biofilms were inhibited by 66.59% at a concentration of $768 \text{ }\mu\text{g mL}^{-1}$. The treatment of the preformed biofilm with CCNPs also reduced the biofilm mass by 43.50% at the same concentration.

Scutera et al. (2021) evaluated the antibiofilm effect of human albumin nanoparticles coated with chitosan containing colistin (Col/haNPs) against antibiotic-resistant *A. baumannii* strains. Col/haNPs were prepared by the double-emulsion method and showed average size of $176.1 \pm 0.9 \text{ nm}$, PDI of 0.12, zeta potential of $+28.05 \pm 2.1 \text{ mV}$ and encapsulation efficiency of 98.65%. Col/haNPs inhibited biofilm formation between 50% and 95% at concentrations of MIC, $1/2 \times \text{MIC}$ and $1/4 \times \text{MIC}$ for *A. baumannii* Col R1 and S strains within 24 h. Already, Gheffar et al. (2021) developed nanoparticles of Poly (lactic-co-glycolic acid) (PLGA) containing ciprofloxacin and evaluated the potential antibiofilm against *S. aureus* ATCC 29213. The particles were obtained by nanoprecipitation and showed an average size of $81 \pm 7 \text{ nm}$, the zeta potential of $-45 \pm 10 \text{ mV}$ and encapsulation efficiency of $3.6 \pm 1.3\%$. The MBECs were $25 \text{ }\mu\text{g mL}^{-1}$ and $12.5 \text{ }\mu\text{g mL}^{-1}$ after 24 h and 48 h of incubation, respectively.

Da Costa et al. (2021) developed nanoparticles of poly-lactic acid (PLA) that were surface functionalized by poly-L-lysine encapsulating rifampicin (NP-RIF-PLL) and evaluated their potential antibiofilm against *S. aureus* SH1000. NP-RIF-PLL was synthesized by a nanoprecipitation process and presented a particle size of $162 \pm 2 \text{ nm}$, PDI of 0.06 ± 0.02 , the zeta potential of $+40 \pm 2 \text{ mV}$ and encapsulation efficiency of $83.7 \pm 0.4\%$. Partial degradation of the biofilm was observed since at least 70% of the biofilms remained adhered after 24 h of treatment with NP-RIF-PLL. The authors highlight the ability of the loaded nanoparticles to interact with bacterial cells, migrate and accumulate in biofilms, and the effect of this passive targeting and accumulation on the efficacy of antibiotic treatment in planktonic cells and biofilms. Thus, the studies about polymeric nanoparticles demonstrate that the properties of the nanoparticles allow the delivery of drugs, making it possible to increase the effectiveness of therapy compared to conventional methods to combat biofilms produced by different infectious pathogens that cause disease in the human body (Chaudhary et al., 2020).

Inorganic nanoparticles

Inorganic nanoparticles appear as a potentially important option in antibacterial and antibiofilm therapy (Table 3) (Mihai et al., 2018).

In that regard, Habash et al. (2017) determined the effects of silver nanoparticles (AgNP) on the biofilm eradication of *Pseudomonas aeruginosa* PA01. The MBEC for AgNP of 10, 20, 40 and 60 nm were 1.25–5 $\mu\text{g mL}^{-1}$, 1.25–5 $\mu\text{g mL}^{-1}$, 2.5–10 $\mu\text{g mL}^{-1}$ and 5–10 $\mu\text{g mL}^{-1}$, respectively.

Yu et al. (2018) evaluated the antibiofilm potential of silver nanoparticle-decorated quercetin nanoparticles (QA NPs) against a multidrug-resistant *Escherichia coli* ECDCM1 strain. At a QA NPs concentration of 20 $\mu\text{g mL}^{-1}$, biofilm formation was inhibited about 90%. While Alavi et al. (2019) synthesized metallic nanoparticles (MNPs) of Ag and Cu and oxide metal nanoparticles (MONPs) of TiO₂, ZnO and Fe₃O₄ green through the aqueous lichen extract of *Protaparmeliopsis muralis* and evaluated the inhibition of biofilms of *S. aureus* ATCC 43300, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. The antibiofilm effect of Ag, Cu and ZnO NPs was observed at a concentration of 100 $\mu\text{g mL}^{-1}$. In *E. coli* biofilms, Ag, Cu and ZnO NPs inhibited formation by 80% and 85%. For *S. aureus*, there was a reduction between 70% and 80%, and for *P. aeruginosa*, inhibition was observed around 80% and 90%.

Farooq et al. (2019) developed silver nanoparticles conjugated to rifampicin (Rif-Ag) with potential antibiofilm against MRSA ATCC 43300 and *K. pneumoniae* ATCC 13882. Rif-Ag was synthesized by the Turkevich method and presented an average particle size between 15 nm and 18 nm. MBIC was 1 $\mu\text{g mL}^{-1}$ for *K. pneumoniae* and 0.0003 $\mu\text{g mL}^{-1}$ for MRSA, while at the concentration of 1 $\mu\text{g mL}^{-1}$, the *K. pneumoniae* and MRSA biofilms were eradicated in $40.25 \pm 0.9\%$ and 69.21 ± 1.4 , respectively.

Muthuchamy et al. (2020) investigated the antibiofilm action of graphene/chitosan nanoparticles (GR/CS NCs) against *P. aeruginosa* MTCC 2453 and *K. pneumoniae* MTCC 3384 biofilm producers. MBIC was determined at a concentration of

Table 3 Antibiofilm potential of inorganic nanoparticles.

Liposome	Bacterial strains	Antibiofilm activity ($\mu\text{g mL}^{-1}$)		References
		Inhibition	Eradication	
Silver nanoparticles	<i>Pseudomonas aeruginosa</i> PA01	ND	MBEC of 10 nm at 1.25–5 $\mu\text{g mL}^{-1}$, 20 nm at 1.25–5 $\mu\text{g mL}^{-1}$, 40 nm at 2.5–10 $\mu\text{g mL}^{-1}$ and 60 nm at 5–10 $\mu\text{g mL}^{-1}$	Habash et al. (2017)
Silver nanoparticle-decorated quercetin nanoparticles	<i>Escherichia coli</i> ECDCM1	90% at 20 $\mu\text{g mL}^{-1}$	ND	Yu et al. (2018)
Cu-MNPs	<i>Staphylococcus aureus</i> ATCC 43300	70–80% at 100 $\mu\text{g mL}^{-1}$	ND	Alavi et al. (2019)
Ag-MNPs	<i>Escherichia coli</i> ATCC 25922	80–85% at 100 $\mu\text{g mL}^{-1}$		
ZnO-MOPs	and <i>Pseudomonas aeruginosa</i> ATCC 27853	80–90% at 100 $\mu\text{g mL}^{-1}$		
Silver nanoparticles conjugated to rifampicin	MRSA ATCC 43300 <i>Klebsiella pneumoniae</i> ATCC 13882	MBIC of 0.0003 $\mu\text{g mL}^{-1}$ MBIC of 1 $\mu\text{g mL}^{-1}$	69.21% at 1 $\mu\text{g mL}^{-1}$ 40.25% at 1 $\mu\text{g mL}^{-1}$	Farooq et al. (2019)
Graphene/chitosan nanoparticles	<i>Pseudomonas aeruginosa</i> MTCC 2453 and <i>Klebsiella pneumoniae</i> MTCC 3384	MBIC of 40 $\mu\text{g mL}^{-1}$	ND	Muthuchamy et al. (2020)
Zinc oxide nanoparticles doped with pancreatin	MRSA strains	84% at 75 $\mu\text{g mL}^{-1}$ and 66% at 37.5 $\mu\text{g mL}^{-1}$	66% at 75 $\mu\text{g mL}^{-1}$ and 47% at 75 $\mu\text{g mL}^{-1}$	Banerjee et al. (2020)
ZnO nanoparticles with gum Arabic	<i>S. aureus</i> and <i>Escherichia coli</i>	ND	50% at 500 $\mu\text{g mL}^{-1}$	Pauzi et al. (2021)
Meropenem-loaded mesoporous silica nanoparticles	Carbapenem-resistant <i>Pseudomonas aeruginosa</i>	MBIC of 16–64 $\mu\text{g mL}^{-1}$	ND	Memar et al. (2021)
Palladium nanoparticles coated with neem gum	<i>Staphylococcus aureus</i>	72.73% at 40 $\mu\text{g mL}^{-1}$ and >60% at 20 $\mu\text{g mL}^{-1}$	ND	Prakashkumar et al. (2021)
Silver nanoparticles	Antibiotic-resistant <i>Escherichia coli</i> strains	21–97% at 0.0312–1 $\mu\text{g mL}^{-1}$	ND	Mohammadizadeh and Kashi (2021)

MNPs, metal nanoparticles; MONPs, oxide metal nanoparticles; MRSA, Methicillin-resistant *Staphylococcus aureus*; ATCC, American Type Culture Collection; MTCC, Microbial Type Culture Collection and Gene Bank; MBIC, minimum inhibitory biofilm concentration; MBEC, minimum biofilm eradication concentration; ND, Not determined.

40 $\mu\text{g mL}^{-1}$ for GR/CS NC against both strains. Already, Banerjee et al. (2020) developed zinc oxide nanoparticles doped with pancreatin (ZnONPs-PK) and evaluated the inhibition of biofilm formed by MRSA strains. The nanoparticles showed an average diameter of 5 nm, PDI of 0.641, and the percentage of protein adsorbed on nanoparticles surface was $0.47 \pm 0.4\%$. The inhibition of biofilm formation at MIC concentration ($75 \mu\text{g mL}^{-1}$) and $1/2 \times \text{MIC}$ ($37.5 \mu\text{g mL}^{-1}$) were 84% and 66%, respectively. Regards the eradication of biofilm, ZnONPs-PK inhibit 66% and 47% at MIC and $1/2 \times \text{MIC}$ concentrations, respectively.

Pauzi et al. (2021) evaluated the antibiofilm properties of ZnO nanoparticles with gum arabic against *S. aureus* and *E. coli* strains. The formulations had a particle size of 180 nm and zeta potential of 10.8 mV. At concentrations equal to or above $500 \mu\text{g mL}^{-1}$, it was observed 50% of the eradication of biofilms produced by *S. aureus* and *E. coli*. Already, Memar et al. (2021) evaluated the antibiofilm potential of meropenem-loaded mesoporous silica nanoparticles (MSNs) against carbapenem-resistant *P. aeruginosa* clinical isolates. In this study, it was observed that the MBICs of MSNs loaded with meropenem was $16\text{--}64 \mu\text{g mL}^{-1}$ for 10 carbapenem-resistant and biofilm-forming *P. aeruginosa*.

Prakashkumar et al. (2021) prepared palladium nanoparticles coated with neem gum (NG@Pd NPs) and observed the antibiofilm activity against MSSA. Palladium nanoparticles were coated with neem gum by simple ultrasound technique and presented an average size of 110 nm. The antibiofilm activity of NG@Pd NPs was observed, inhibiting biofilm formation by $72.73 \pm 0.015\%$ at a concentration of $40 \mu\text{g mL}^{-1}$ and more than 60% at a concentration of $20 \mu\text{g mL}^{-1}$.

Mohammadizadeh and Kashi (2021) synthesized silver nanoparticles (AgNPs) using *Trifolium pratense* L. extract as a reducing agent and tested the antibiofilm activity against antibiotic-resistant *E. coli* strains. The AgNPs had an average particle size of 19 nm. The percentage of biofilm inhibition by the silver nanoparticles was between 21.12% and 97.10%. Several studies have demonstrated the inhibition of biofilm formation in vitro in Gram-positive and Gram-negative bacteria at specific concentrations of nanoparticles. This raises the intriguing possibility of treating infections caused by biofilm-forming bacteria with inorganic nanoparticles (Mihai et al., 2018).

External physical stimuli

Nanostructures can be activated by an external stimulus that promotes controllable changes in the properties of materials by stimulating the release of antimicrobial molecules, constituting a promising alternative for the delivery of conventional and new antibacterial agents (Wolfmeier et al., 2018; Rivera-Tarazona et al., 2021).

Nanomaterials activated by external stimuli remain latent until their activation, minimizing toxic effects on normal cells and human microbiota. In addition, these nanomaterials increase the specificity for the target and improve the release of the active ingredient (Colilla and Vallet-Regí, 2020; Cheeseman et al., 2020a). The targeting of antibiotics to bacteria can be achieved by responding to external physical stimuli, such as magnetic, thermal, light and ultrasound effects (Yeh et al., 2020).

Magnetic action-activated nanomaterials allow magnetic NPs to penetrate and target the infection site to disrupt individual microorganisms and remove communities of microorganisms from surfaces helping to break and disperse the biofilm (Cheeseman et al., 2020a,b; Elbourne et al., 2020). Among the types of approach using the magnetic field, it is highlighted magnetic hyperthermia and magneto physical nanomaterials that respond to these stimuli through a localized increase in temperature and physical disruption, respectively (Cheeseman et al., 2020a; Colilla and Vallet-Regí, 2020).

Quan et al. (2019) evaluated the antibiofilm activity of gentamicin-carrying magnetic nanoparticles (MNPs-G) against biofilms of *S. aureus*. The diameters of MNPs-G were around 60 nm and after exposure of MNPs-G in a concentration of $440 \mu\text{g mL}^{-1}$ associated with a magnetic field, an eradication of more than 50% of the biofilm was observed.

Elbourne et al. (2020) produced Galinstan-based magnetic liquid metal microparticles and nanoparticles (GLM-Fe). They evaluated the antibiofilm activity against *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 of GLM-Fe associated with a 775 mG dynamic magnetic field. The average particle diameter ranged from ~ 200 nm to $2 \mu\text{m}$. After 90 min of exposure of GLM-Fe at a concentration of $100 \mu\text{g mL}^{-1}$ under the effect of the magnetic field, an eradication of 99% of the biofilm of *S. aureus* and *P. aeruginosa* was observed and the infeasibility of bacterial cells was also observed.

Cheeseman et al. (2020b) produced Galio-based magnetic liquid metal microparticles and nanoparticles (GLM-Fe) and evaluated the antibiofilm activity against *P. aeruginosa* ATCC 27853, *E. coli* DH52, *Bacillus cereus* ATCC 11778 and *S. aureus* ATCC 25923 of GLM-Fe associated dynamic magnetic field of 775 mG. The average particle diameter varied from 100 nm to $1 \mu\text{m}$. After 90 min of exposure of GLM-Fe at a concentration of $100 \mu\text{g mL}^{-1}$ under the effect of the magnetic field, eradication around 80%, 99%, 95% and 0% of the biofilm of *P. aeruginosa*, *E. coli*, *S. aureus* and *B. cereus* was observed, respectively. The antibiofilm potential of these species was also observed together at a concentration of $100 \mu\text{g mL}^{-1}$, and inhibition of 95% was observed for all multispecies biofilms.

Light is the physical stimulus capable of being an external release trigger for releasing antimicrobials, constituting an attractive therapeutic option to conventional antibiotic treatments (Rivera-Tarazona et al., 2021). Photocatalytic nanomaterials are stimulated by contact with wavelengths of light to produce free reactive oxygen species (ROS), producing antimicrobial action. In contrast, photothermal nanomaterials are stimulated with the localized increase in temperature produced by light waves that interact with the materials (Cheeseman et al., 2020b; Rivera-Tarazona et al., 2021).

Bagchi et al. (2018) synthesized hybrid surface nanoparticles of squaraine/ZnO (ZnO-SQ) and evaluated the antibiofilm activity in *S. aureus* strain ATCC 25923. The particles showed a size of ~ 24 nm, and the bacterial biomass was reduced by $\sim 55\%$ when ZnO-SQ-treated bacteria were exposed to red light for 30 min at a concentration of 140 nM.

Wang et al. (2019) evaluated the antibiofilm activity of light controllable chitosan micelles loading with thymol (T-TCP) against *S. aureus* ATCC 29213. T-TCP showed particle sizes of 100.3 nm, a positive surface charge of 33.4 mV and an encapsulation efficiency of 30.8%. At the concentration of 330 $\mu\text{g mL}^{-1}$ associated with exposure to light, there was a reduction in the biofilm with the generation of ROS, simultaneous release of thymol and generation of additional ROS-inducing bactericidal effects.

Xie et al. (2020) developed gold nanoclusters functionalized with deoxyribonuclease (DNase-AuNCs) and evaluated the antibiofilm potential against *S. aureus*, *P. aeruginosa* and *E. coli* after light exposure. The particle size of 2.33 ± 0.72 nm, the zeta potential of 12.9 mV and reduction of 85.9, 82.6, and 83.4% for the biofilms of *S. aureus*, *E. coli*, and *P. aeruginosa*, were observed, respectively.

Ultrasound (US) is an external stimulus, mainly used at low frequencies, capable of targeting nanomaterials and improving the effectiveness of antibiotics. This technique increases the permeability of bacterial cell membranes to the antibiotics available in the medium (Muhammad et al., 2020). The use of low-frequency ultrasound (20–100 kHz) in the treatment of pathogenic bacterial biofilms is an important tool since they act by altering the permeability of biofilms to conventional, natural antimicrobial compounds and nanostructures (Muhammad et al., 2020; LuTheryn et al., 2020).

The mechanical oscillation generated by the US can remove polysaccharides, change the protein composition and intensify the breakdown rates of glycosidic bonds in the biofilm matrix. Additionally, it causes changes and holes in the outer membrane and cytoplasmic membrane of the bacteria. These effects can reduce the number of cells in the biofilm, damage the stable complex structures of the formed biofilm and boost the detachment of the biofilm from specific surfaces (Runyan et al., 2006; Xu et al., 2018; LuTheryn et al., 2020; Yang et al., 2019; Shao et al., 2020). Studies indicate that in vitro efficacy tests with this technique enhance the antibiofilm activity of molecules and/or antibiotics against *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* resistant to antimicrobials (Wang et al., 2018, 2020; Fu et al., 2019; Hou et al., 2019; Liu et al., 2020; Bharatula et al., 2020).

Fu et al. (2019) prepared liposomes containing chitosan-modified polymyxin B (CLPs) and ultrasound microbubbles (USMBs). They evaluated in vitro the antibiofilm effect of USMBs combined with CLPs against antibiotic-resistant *A. baumannii* W1340. The PLCs had a size of 225.17 ± 17.85 nm, a zeta potential of 12.64 ± 1.44 mV and drug loading of $15.62 \pm 1.97\%$. The USMBs were 2.391 ± 0.052 μm in size and had a zeta potential of -4.32 ± 0.43 mV. To assess antibiofilm activity, a frequency of 1.0 MHz for 5 min was used. The PLCs, USMBs and PMBs eradicated less than 50% of biofilms. However, the combinations of USMBs + PMB reduced around 85% and CLPs + USMBs around 95% of biofilms.

Hou et al. (2019) evaluated in vitro the effects of low-intensity and low-frequency ultrasound (LILFU) combined with tobramycin against extended-spectrum beta-lactamases (ESBLs) *E. coli* biofilms from a urine sample. The biofilms of ESBLs *E. coli* were established and treated with ultrasound (42 kHz and ISATA of 0.66 W/cm^2) continuously for 0.5 h with and without tobramycin. The combined ultrasound treatments with tobramycin at 8 $\mu\text{g mL}^{-1}$ and 80 $\mu\text{g mL}^{-1}$ reduced biofilm and increased drug permeability.

Liu et al. (2020) evaluated in vitro the association of low-frequency ultrasound (LFU) with antimicrobial agents such as meropenem (MEM), tigecycline (TGC), fosfomycin (FOM), amikacin (AMK) and colistin (COL) against biofilm of *K. pneumoniae* clinical isolates. The authors observed synergistic effects in groups of single ultrasound (S-LFU, 5 min) or multiple ultrasounds (M-LFU, 5 min every 8 h) combined with MEM, TGC and FOM.

These studies highlighted the therapeutic potential of external physical stimuli, making them promising alternatives for treating infections caused by biofilm-producing bacteria (Wolfmeier et al., 2018; Colilla and Vallet-Regí, 2020; Yeh et al., 2020).

Conclusion

Biofilms are intrinsic virulence factors in the ecology of bacteria and are essential in the biotechnological, environmental, and industrial sectors. However, this bacterial way of life can affect human health when associated with infections. Infections caused by biofilms producing-bacteria are persistent, resistant to antibacterial therapy, and are considered a global public health problem.

Conventional antibiotics therapies, such as tetracyclines, quinolones, sulfonamides, macrolides, oxazolidinones, daptomycin, rifampicin, beta-lactam antibiotics, glycopeptides, aminoglycosides or polymyxins present limitations to eradicate biofilms. This fact occurs as the organization of biofilms makes it difficult for these antimicrobials to penetrate, in addition to stimulate the development of microbial resistance.

In this sense, the use of nanomaterials to deliver active compounds within biofilms is an effective therapeutic option, since the nanostructures promote the elimination of bacterial cells and the rupture of the matrix formed by EPS creating craters in the structure of the biofilm. Associated with nanomaterials, external physical stimuli such as the magnetic field, light, and ultrasound waves, are effective methods that allow the biofilms to be disrupted and allow the conduction and greater penetration of antibiotics, natural products and nanocarriers.

Therefore, due to the growing problem of biofilm formation by strains pathogenic to human beings, it is necessary to discover products with the disruptive potential of the matrix and/or mucolytics, in parallel to the development of nanomaterials with antibacterial activity and the use of combined stimuli as an essential strategy to prevent the formation and eradicate bacterial biofilms.

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Oxidation and “Unconventional” Approaches to Infection

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Introduction

A paradigm shift in the treatment of infectious diseases is necessary to prevent antibiotics becoming obsolete, and where appropriate, alternatives to antibiotics ought to be considered.

(Carson et al., 2006).

Within a generation after antibiotic introduction, bacterial infection morbidity/mortality sharply fell. However, within a few more human generations, rapid genetic sharing of resistance genes has returned bacterial induced deaths to front and center, therefore the world again faces a crisis. Dire calls have been issued for the development of new drugs. Nevertheless, just as fast as medicines are developed, pathogens rise to resistance (see fascinating but frightening video of *Escherichia coli* developing resistance over just 11 days to 1000× inhibitory concentration at outset, [Science News, 2016](#); Baym et al., 2016).

The advent of modern medical technology has accelerated the natural evolution of microorganisms into self-serving and protective communities termed “biofilms,” notoriously resistant to conventional treatment ([Bjarnsholt, 2013](#)). Therefore, there is urgent need to resurrect old but useful therapies, which were actually curing infection prior to the drug era, which therapies went lost or forgotten. Actually, medicine, due to the successful results of pharmaceutical approaches to treat infectious diseases, has paid scant attention to harnessing the body’s own innate defenses to overcome pathogens, whether viral, bacterial, fungal, or other.

Immune cells respond to invaders with an oxidative or respiratory burst ([Nguyen et al., 2017](#); [Roos et al., 1988](#)). Macrophages and neutrophils engage, engulf, and destroy pathogens. When encountering an invader, white cells may consume up to 30× the amount of oxygen as at rest. This “burst” induces synthesis of powerful oxidants, actual germicides, enabling lysosomal dissolution of the pathogen. Such oxidants include, but are not limited to, superoxide, hydrogen peroxide, nitric oxide, hydroxyl radical, and peroxynitrate ([Prolo et al., 2014](#)). Other reactive species are sodium hypochlorite (bleach), singlet oxygen, and ozone, which is the most powerful of the oxidants, recently discovered to be endogenously generated in humans from singlet oxygen catalyzed by antibodies ([Babior et al., 2003](#)).

Might it be possible to heal infection by augmenting these natural defenses? The medical literature cited in this chapter strongly suggests this. Animal life has coexisted on the planet with bacteria for hundreds of millions of years. If bacteria could become resistant to endogenously generated germicides, we would not be here.

Ozone therapy

Ozone (O₃ or triatomic oxygen)

More than 180 years ago, in 1840, triatomic oxygen was discovered by the electrolysis of oxygen creating an odorous gas that its discoverer, Christian Schonbein, called “ozone.” Ozone was quickly discovered to destroy bacteria and within a few decades, rapidly gained fame in purifying water. Nikola Tesla patented the first American ozone generator in 1896. However, ozone’s utility was hampered at those times by lack of ozone-resistant materials, as the molecule could oxidize naturally occurring hydrocarbons turning them into carbon dioxide and water. The advent of resistant plastics rehabilitated ozone therapy into healing fields by German scientists. In 1958 pediatrician Robert Mayer (1983) of Florida published “Using Ozone as a Chemotherapeutic Agent in the Treatment of Diseases,” having learned of the therapy from a German prisoner in the Second World War. More recently, many of its mechanisms of action have been elucidated.

Generation of ozone

Ozone, a metastable pale blue gas, must be generated onsite and used quickly. Its half-life at room temperature is about 30 min, before dismutating back to O₂. O₃ resonates between four ionic structures, highly soluble in water. O₃ is generated from a high voltage corona arc discharge, similar to lightning. Medical “ozone” is actually a mixture of O₂ and O₃, 95–99% and 1–5%, respectively, or 5–70 µg ozone/cc. “Ozone” can be collected in a syringe for parenteral administration, or conducted into a bottle or bag to treat extracorporeal blood, or bubbled through water/saline to make an ozonated solution, or administered to body cavities (Elvis and Ekta, 2011; Shrisel and Suryakant, 2017; Bocci et al., 2012). The advantages and limits of each approach are reported in a subsequent paragraph. However, The U.S. Food and Drug Administration 21 CFR H (801) declares ozone to be a toxic gas with “no known useful medical applications” (FDA, 2020). This “statement” remains in the federal regulations despite thousands of scientific publications, including positive biochemical/metabolic effects in vivo (including humans), highlighting the benefits of ozone therapy. Indeed, ozone gas, when inhaled directly, can damage lungs; therefore, the system used in antimicrobial therapy must not permit leaks which could lead to inhalation. The lungs are singularly highly ozone sensitive.

Ozone antibacterial/antiviral properties

Ozone virtually instantly destroys bacteria, about 100 times faster than chlorine. Unlike chlorine, it leaves no toxic residue. Furthermore, it quickly cuts through and destroys biofilms (Bialoszewski et al., 2011) that are virtually impossible to treat conventionally. Ozone can also be used to control tuberculosis in diabetic patients.

Multiple reports demonstrate the need for viral envelope glycoproteins to be reduced (R-S-H rather than oxidized R-S-S-R) to enter cells, including Ebola (Chandran et al., 2008; Lee and Saphire, 2009) and coronavirus (Broer et al., 2006; Schoeman and Fielding, 2019; Lopez et al., 2008). CMV loses infectivity when oxidized, but 65% is restorable if chemically rereduced (Mirazimi et al., 1999). Many viruses including cytomegalovirus, HIV, Norwalk, bacteriophage MS2, hepatitis A, and poliovirus are nearly instantly inactivated by ozone (Wolf, 2019; Kekez and Sattar, 1997; Herbold et al., 1989; Emerson et al., 1982; Katzenelson et al., 1979; Roy et al., 1981b; Roy et al., 1981a). HIV cell entry is dependent on reduced thiol groups (Ryser et al., 1994; Markovic et al., 2004).

In vivo, ozone instantly reacts with blood components, extinguishing the delivered ozone, leaving only useful O₂ as a residue. The temporary oxidative stress generates reactive oxygen species (peroxides, aldehydes, etc.), which chemically are more electrophilic than sulfur, hence oxidizing the fragile viral thiol groups ($\text{ROOH} + 2 \text{RSH} \rightarrow \text{R-SSR} + \text{H}_2\text{O}$). As an example, coronavirus spike proteins are rich in sulfhydryl groups and tryptophan (Broer et al., 2006), both very susceptible to oxidative damage (Sharma and Graham, 2010). Therefore, virions are inactivated while retaining their immunogenicity, thus speeding up infection resolution and enhancing natural resistance. Ozone may also damage the lipid component of enveloped virions, a desired effect since viruses cannot self-repair the damaged structures. Lorizate and Kräusslich (2011) discussed that agents that attack the lipid envelope could be useful as antivirals. However, they lamented that conventional agents lack specificity and are “thus unacceptably toxic.” Ozone therapy does not so suffer.

Ozone biochemical and immune modulating effects

O₃ is a most powerful oxidant. Two research teams (Italy and Cuba) have independently found major biochemical modulations induced by ozone (Bocci, 2011; Menéndez and Weiser, 2016). Among these effects are: (1) increased red blood cell 2,3 diglycerophosphate (greater hemoglobin oxygen release), (2) improved rheological properties of red blood cells and endothelial nitric oxide production, (3) greater arterial/venous partial O₂ pressure difference, indicating greater mitochondrial consumption of oxygen (energy production), (4) immune system modulation and reduction of inflammation, (5) improvement of antioxidant status, antioxidant enzymes (including superoxide dismutase), and glutathione in cells.

All these effects are achieved by ozone virtually instantly reacting with unsaturated (electron rich) fatty acids and generating ozonides, which act as downstream cell signaling redox molecules and other shorter lived ROS (reactive oxygen species: peroxides, aldehydes, etc.). These affect and modulate redox status without altering blood cell membranes (Bocci, 2011; Menéndez and Weiser,

2016). The authors reported that damage to red cell membranes required higher concentrations of ozone (over 80 µg/cc) than clinically used (15–70 µg/cc). Significant hemolysis has not been reported with any form of ozone therapy.

Conventional thought is that oxidative stress is negative for human health. However, unlike pathological oxidative stress, the controlled oxidative processes of ozone actually induce antioxidant enzyme production. In particular, ozone activates the Nrf2 (nuclear factor erythroid) protein, which activates many protective enzyme pathways (Galiè et al., 2019). In activating the Nrf2 pathway in human multiple sclerosis patients, ozone induces higher levels of antioxidant enzymes, and lower pro-inflammatory peptides TNF α and IL-1 β (Delgado-Roche et al., 2017). Ozone also induces heme oxygenase (Pecorelli et al., 2013).

It has been established that the ozone's hydrogen peroxide-mediated natural induction of cytokines by immune cells is safer and more effective than exogenous administration of a single cytokine. Bocci (2011) called ozone the “ideal cytokine inducer” and described ozone-treated erythrocytes as “super gifted,” in reference to their enhanced ability to deliver oxygen (their specific function).

Cuban research has demonstrated valuable properties of ozone therapy in infection.

Preconditioning with ozone (ozone treatment before inflicted infection) improved survival of rats to subsequent lethal peritoneal injection of fecal material up to 62.5%. Such preconditioning also rivaled steroids in reducing tumor necrosis factor alpha release in endotoxic shock (Zamora et al., 2005). When antibiotics were added to ozone preconditioning, survival increased to 93% (Bette et al., 2006). Ozone therapy induces prostacyclin production, improving the prostacyclin/thromboxane ratio (Schulz et al., 2012), which would have a positive effect on circulation and reduction in inflammation.

Ozone safety

The largest safety study was conducted in 1980 by Jacobs (1982) for the German Medical Society for Ozone Therapy. She surveyed 2815 known European ozone therapists with a 25% response rate. The report tallied 384,775 patients and 5,579,238 ozone treatments. The risk of adverse incidents was only 0.7 per 100,000 treatments, often related to improper administration. Pediatrician Robert Mayer (1983) reported on 3000 administrations (including intrathecal ozone gas for meningitis), most in children. Mayer found ozone “without any side effects or toxicity noted.” Robins (2018) reported performing over 300,000 ozone treatments utilizing direct intravenous gas (DIV) with no lasting ill effects other than vein irritation with this particular method. Rowen (2016) conducted and presented a survey of those he has trained in a delivery method called “hyperbaric ozone.” No significant adverse problems reported in 30,000+ observed treatments. Ozone therapy appears to be one of the safest, if not the safest, treatment modalities ever known.

Delivery methods, advantages, and disadvantages

Ozone must be generated on site as its half-life at room temperature is about 30 min. Inhalation must be avoided/minimized. The lungs have very limited capacity to handle such powerful oxidative stress, considering that even breathing 100% O₂ for prolonged periods can be harmful to pulmonary tissues. However, although ozone gas cannot be inhaled, it can be safely bubbled or nebulized through oils for inhalation. This method was used in America's early ozone days, often to reduce tuberculosis organisms in lungs (Eberhart, 1913).

Nevertheless, medical reports suggest that ozone administered to any other body tissue is safe. The most common sites of delivery are to blood, joints, soft tissues, body cavities, subcutaneous, and intramuscular (Bocci, 2011; Menéndez and Weiser, 2016). Delivery is via direct gas injection, gas insufflation, drinking ozonated water, topical, and autohemotherapy (patient's blood treated with ozone outside the body and returned). Local treatments are applied for local conditions.

Ozone gas can be administered into body cavities and spaces such as bladder and joints. For bladder, the volume ranges from 10 cc to 60 cc based on condition, size of patient, and comfort. Concentration of gas ranges between 10 and 40 µg/cc (Bayrak et al., 2014). Joint therapy is universally well accepted to be effective for degenerative and inflammatory joint conditions (Trevisani and Udell, 2015). Typical treatment is preloading the joint (or soft tissue) with a small amount of local anesthetic (preservative and epinephrine free), followed by ozone gas at a concentration of 15–40 µg/cc. A usual knee treatment delivers 10–20 cc. Because ozone is germicidal and antibiotic resistance is not an issue, any type of infection can be treated.

Rectal administration is very inexpensive to administer at home, leaving little or no medical waste. Menendez and Weiser (2016) led Cuban studies indicated that this mode induces similar biochemical effects in the body as blood therapies. This mode improved COVID-19 disease reducing inflammatory markers of fibrinogen, D-dimer, urea, ferritin, LDH, IL-6, and CRP (Fernández-Cuadros et al., 2020).

The dosage depends on the body size of patient, 50–400 cc (volume increased as tolerated) beginning with concentration of 20 µg/cc and increasing to 40–45 µg/cc, as tolerated. The treatment can be performed a few times a week to even 3–4 times daily depending on need. It is generally applied by filling a plastic IV bag with ozone gas, attaching its outlet tube to a reusable catheter, which is placed in the rectum, and the bag contents slowly rolled in. The gas induces a significant bowel evacuation within minutes in most people. However, clinical results are not as rapid or effective, per treatment, as auto-hemotherapy (extracorporeal treatment of blood, which is then returned).

Systemic therapies are used for systemic conditions. There are no published reports of problems in combining methods. The most common systemic delivery method worldwide is direct venous gas (DIV) application. Intravenous oxygen gas therapy has been employed in Europe for generations and has been found to modulate the immune system through generation of 15-Lox-1 by

eosinophils (Chaitidis et al., 2004), and improve prostacyclin production (Stichtenoth et al., 2001). Intravenous oxygen gas therapy has been reported to induce strong immunological effects and improve blood rheology and oxygenation (Schmidt, 2002). DIV ozone is very inexpensive to deliver, requiring (aside from the ozone generator) only a syringe and fine butterfly needle, with minimum medical waste. Hence, DIV has great advantage in the third world where the method is most used. However, the presence of ozone, a powerful oxidant, in the gas mixture, can be irritating to veins and repeated use can lead to vein sclerosis. Dosage generally begins with 20 ccs of gas at concentration of 30–40 µg/cc with increase in volume as tolerated to 100 cc or more. DIV training is important before beginning. Some ozone users in western countries have opposed DIV fearing gas embolism after intravenous administration (Madrid, 2020). However, the gas is not air, which contains about 80% nitrogen, but 100% metabolically active oxygen (O₂/O₃ mixture), which is rapidly consumed.

The most popular delivery in the Western world is major autohemotherapy, which has two modes. The simplest is performed by withdrawing 50–200 cc heparinized blood into a plastic bag under gravity. An equal amount by volume of ozone gas, 20–70 µg/cc, is added and mixed with the blood by gentle rocking. The treated blood is then returned to the patient by gravity (total time about 2 h). Inert glass bottles are preferable to plastic.

A superior method is performed by using a glass evacuated bottle to withdraw up to 200 cc of blood. An equal amount of ozone gas is pumped into the bottle under up to two atmospheres of pressure (2 ATA), mixed, and then rapidly returned to the patient by the pressure. Although there is need for a more sophisticated ozone generator, this method has the advantage of more thorough admixing of gas and blood, and the delivery of actual solubilized oxygen gas in the returned blood, which, as mentioned above, has additional beneficial effects. Hyperbaric ozone therapy is far quicker (10–15 min/treatment) than gravity method. Furthermore, the hyperbaric ozone method (HBO₃) has the additional advantage of permitting additional “passes” of 200 cc of blood up to 10 passes or more, as pioneered by Johann Lahodny, MD of Austria (2017), who called this method “Ozone High Dose Therapy” (OHT), known in America as “10 pass.” Very rapid clinical improvement in infected patients has been reported (American Academy, 2017). The disadvantage of both autohemotherapy methods is medical waste.

Ozone minor autohemotherapy is administered by withdrawing up to 10 cc of blood in a syringe prefilled with an equal amount of ozone gas at a desired concentration. The entire mixture is vigorously shaken and quickly returned intramuscular (IM). Bocci (2011) considered IM ozone as an immune stimulant and modulator. Ozone gas can be given directly IM, concentration up to 20 µg/cc with little discomfort. Generally, up to 20 cc of gas is injected. Likewise, the gas can be given to local skin infections, including warts, by subcutaneous route (Bocci, 2011).

Ozone treatment of human acute bacterial infections

Bacteria are virtually instantly destroyed by ozone gas exposure. However, except by local gas injection into a focal infection, all the effects of ozone therapy will be indirect and secondary to its profound modulation of biochemical processes and induction of downstream ozonide metabolites (Bocci, 2011; Menéndez and Weiser, 2016). While we lack desirable infection “double blinded” studies, many reports demonstrate that ozone can be an effective alternative or adjunctive therapy to current conventional drug practices. Local ozone injection and HBO₃ together with an oral antibiotic treatment were reported as the first medical treatment to successfully eradicate a prosthetic joint infection without surgery or parenteral antibiotics (Rowen, 2018a). Single treatment of HBO₃ alone eradicated a tick bite cellulitis in a man previously cleared of Lyme disease years before by HBO₃ without antibiotics (Rowen, 2018b). Used in combination with antibiotics, ozone was reported to prevent resistance to drugs in *Mycobacterium tuberculosis* in humans (Belianin, 1997; Belianin and Shmelev, 1998). Ozone therapy, in conjunction with removal of infected teeth, remitted a highly aggressive case of dermatomyositis (Rowen, 2018c). Topical ozone therapy induced a highly significant reduction in erythrocyte sedimentation rate in osteomyelitis patients compared to control group, though clinical outcomes were similar in both groups (Naderi Nabi et al. (2018). Topical application of ozonized oil has been used successfully against *Staphylococcus aureus*, including MRSA (Song et al., 2018).

Ozone therapy in human viral disease

Medicine has serious challenges in addressing viral diseases. Nevertheless, viruses have particular vulnerabilities to oxidation, as referred in a previous section. Rowen speculated that ozone therapy may be the ideal treatment for controlling viruses, summarizing viral inactivation by oxidation (Rowen, 2019).

Virions can be inactivated by ozone/ozonides while retaining their immunogenicity, quickening infection resolution and enhancing natural resistance. Outstanding clinical effectiveness of ozone therapy has been reported in meetings of ozone doctors sharing clinical work worldwide and in clinical case series of Ebola (Rowen et al., 2016) and coronavirus (Wu et al., 2020; Franzini et al., 2020).

Ozone in pediatric infections

Pediatrician Robert Mayer (1983) reported results from 1946 to 1964 of significant research on 2870 children. In summary, for nonbacterial infectious diarrhea, 1932 resolved multiple days of diarrhea, most in just one day, with one rectal insufflation of gas. He also reported data on intrathecal ozone for 5 viral (mumps, rubeola, St. Louis encephalitis) and two bacterial (*Francisella tularensis* and *Pseudomonas aeruginosa*) meningitis cases. All patients recovered except one; however, the low numbers prevent any statistical consideration. However, the case concerning *P. aeruginosa* is worth mentioning. This was a two-month premature infant, who, on day 5, developed overt meningitis, with spinal fluid WBC count of 13,600. *P. aeruginosa* was detected after culture. The child

received numerous antibiotics, and by day 13, spinal fluid WBC dropped to 450 with still a positive culture. The drugs were discontinued. Mayer provided intrathecal ozone at 89 µg/cc by first removing spinal fluid equal to the volume of gas to be injected. Twelve administrations were performed (intraspinally and intracisternal). The child fully recovered having normal milestones through a year's observations.

Topical ozone

Ozone has been used for decades in several methods of delivery: bathing a wound with the gas in bags, topical application of ozonized water or saline, and ozonized oil. Unsaturated bonds of fatty acids can trap O₃ holding it with stability for months/years. Ozonized vegetable oil has been found to destroy microbes with particular efficacy against *Mycobacterium*, presumably because of its susceptible waxy (fatty) vulnerable coat, and MRSA (Sechi, 2001). The mechanism is a germicidal effect. Lavage by ozonated saline solution proved the most effective irrigating solution (even over antibiotic solution) to prevent bacterial abscess formation in experimental peritonitis rat model (Ozmen et al., 1993). Many oils are available commercially. Saline or aqueous solutions must be made fresh due to dismutation of the dissolved raw gas.

Topical (by bag) and parenteral ozone application was added to a failed antibiotic and debridement regimen for seriously advanced wound infection, with 4/5 of tibia exposed, and pus extruding from knee joint with full resolution (Shah et al., 2011).

Ozone applications have been used successfully as a disinfectant in dentistry for many years. These include ozonized water, ozonized oils, and local injections. These have proven to be antimicrobial, immunostimulating, analgesic, and with no toxic effects on the local tissue environment. This results in a promising approach for “green dentistry” (Nagarakanti and Athuluru, 2011; Mobeen, 2017).

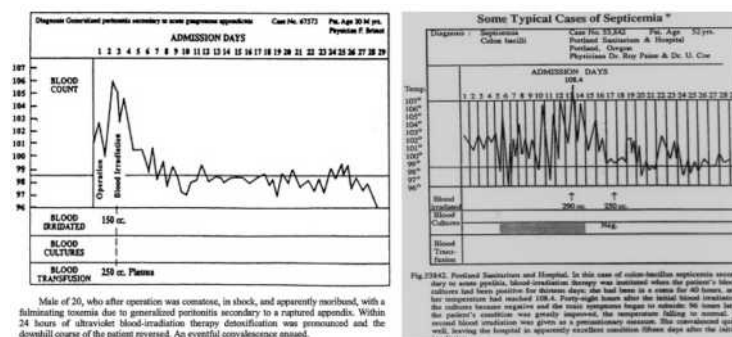
Ultraviolet blood irradiation (UBI)

Historical background

Termed “The Cure That Time Forgot” (Rowen, 1996), UBI has been used continuously in America and overseas for many generations. Ultraviolet energy is a known germicide, used topically at the turn of the last century to cure skin infections. The 1904 Nobel Prize in Physiology or Medicine was awarded to Niels Finsen for his work on UV treatment of various skin diseases. His success rate was 98% in thousands of cases, mostly cutaneous tuberculosis known as lupus vulgaris. Walter Ude reported a series of 100 cases of erysipelas (a cutaneous infection caused by *Streptococcus pyogenes*) in the 1920s, with high cure rates using irradiation of the skin with UV light (Ude, 1929).

American physicist Emmett Knott assembled the known information, and developed a device to irradiate blood extracorporeally. Experimenting on dogs, he settled on a dose of 3.5 cc blood per kg weight and specific exposure times (10 s per aliquot of blood) using a water-cooled boiling mercury bulb emitting 200 watts of energy. The key to the Knott's method was a quartz glass chamber, which induced turbulence to assure maximum exposure to the treated blood. Otherwise, the first few exposed cell layers would absorb all the UV energy. A woman with a septic abortion received the first human treatment. She survived and went on to have two children. Following this case, and availability of his machine, dozens of articles appeared in American literature detailing extremely rapid and stunning resolutions of otherwise (at that time) untreatable bacterial and viral infections (Miley and Christensen, 1948; Barrett, 1940). Most cases resolved in 1–2 sessions. No toxicity was reported in thousands of treatments.

Typical sepsis fever curves and clinical outcomes from UBI therapy, no antibiotics (Rebbeck and Miley, 1941):



UBI was used to treat incomplete septic abortion, puerperal sepsis, acute pyelitis, peritonitis, sepsis, osteomyelitis, wound infections, and infectious thrombophlebitis. In 1947, George Miley and his team summarized 445 cases of acute pyogenic infection, including 151 consecutive cases (Miley and Christensen, 1947). Results showed a 100% recovery in 56 early cases, 98% recovery in 323 moderately advanced cases, and 45% in 66 apparently moribund patients (Table 1). Staphylococcal infections were also successfully treated, providing that sulfa drugs were not simultaneously used, since the sulfa nucleus absorbs UV photons and interferes with the UBI therapy (Miley, 1944).

UBI was reported highly effective in viral pneumonia, hepatitis, polio (Miley and Christensen, 1948), and even Clostridia caused botulism (Miley, 1946).

Table 1 Results in 445 cases of acute pyogenic infection given ultraviolet blood irradiation therapy at the Hahnemann hospital, Philadelphia, over a period of six and one-half years.

	<i>No. of cases</i>	<i>Recovered</i>	<i>Died</i>
Early			
Puerperal sepsis	3	3	
Incomplete septic abortion	12	12	
Acute furunculosis and carbunculosis	15	15	
Abscesses	2	2	
Acute Strep, hemolytic oropharyngitis	5	5	
Acute tracheobronchitis	12	12	
Acute wound infections	2	2	
Acute pansinusitis	1	1	
Acute pyelitis	1	1	
Acute otitis media	2	2	
Fever of unknown origin	1	1	
Moderately advanced			
Puerperal sepsis	14	14	
Incomplete septic abortion	57	57	
Acute furunculosis and carbunculosis	21	21	
Abscesses	13	13	
Acute Strep hemolytic oropharyngitis	5	5	
Endometritis and parametritis	17	17	
Acute wound infections	6	6	
Salpingitis	15	15	
Penttonitis	18	16	2
Osteomyelitis	26	25	1
Fever of unknown origin	13	13	
Lobar and bronchopneumonia	11	11	
Atypical (virus) pneumonia	10	10	
Preoperative	13	13	
Postoperative	44	41	3
Non-healing wounds	6	6	
Thrombophlebitis	34	34	
Apparently moribund			
Puerperal sepsis	4	3	1
Incomplete septic abortion	2	1	1
Generalized peritonitis	4	2	2
Abscesses Pelvic	6	5	1
Rectal	2	1	1
Scrotum	1	1	
Wound infections	3	2	1
Fever of unknown origin	2	1	1
Lobar and bronchopneumonia	2	1	1
Tb. meningitis	3		3
Mesenteric thrombosis, diabetes mellitus	1		1
Septicemias:			
Staph. aureus-albus with sulfa drugs	7		7
with sulfa drugs	9	9	
Str. hemolyticus	3	3	
Str. non-hemolyticus	2	1	1
Str. viridians-subacute			
bacterial endocarditis	13		13
Str. hemolyticus endocarditis	1		1
Str. non-hemolyticus endocarditis	1		1
Summary			
Number of cases	Early 56	Moderately Advanced 323	Apparently Moribund 66
Number recovered	56	317	30
Percentage recovered	100%	98%	45%

Mechanism of action

UBI efficacy appeared to be consistent with a hormetic effect. Only about 5% of the blood volume was treated; therefore, the effect is not linked to blood sterilization. American physicians reported the following effects from UBI: toxin inactivation, bacteriostatic or bactericidal effect on bacteria, improvement of blood oxygen transport, leukocyte activation, immune stimulation, steroid hormone activation, vasodilation, reduced platelet aggregation, fibrinolysis, decreased blood viscosity, and improved microcirculation (Rowen, 1996). These effects singly and collectively would assist physiology in infection.



Still (2021) functioning Knott Hemo-irradiator, circa 1958

UBI in America largely faded with the advent of pharmaceutical antibiotics but continued in Germany and Russia with the development of less powerful but easily accessible devices (Douglass, 2003; Frick, 1986).

Germans found several metabolic effects, which mirror current findings in ozone therapy biochemistry. Seng (1988) reported the following effects from UBI therapy: (1) improves RBC rheology; (2) increases negative charge across RBC membrane; (3) reduces blood surface tension and platelet aggregation, while raising blood cells number; (4) increases the Pa-Pv O₂ difference and reduces blood pyruvate and lactate [suggesting improved mitochondrial activity]; (5) improves glucose tolerance; (6) reduces blood cholesterol, transaminases, and creatinine values; and (7) increases WBC phagocytosis and bactericidal blood capacity. A typical treatment today uses 60–200 cc of blood, passing it whole or diluted in saline through a quartz glass cuvette exposed to UV energy.

Analogies with ozone treatment

Ozone and UBI therapies have much in common. UV-C frequencies (253 nm) used in therapy are similar to the UV-C radiation that creates ozone naturally in the upper atmosphere. Conversely, ozone releases energy (a photon) when dismutating back to oxygen. Both quickly destroy pathogens and modulate immune function. Positive effects of both treatments can be achieved treating only 5–7% of blood. Better understanding the role of oxidation-related effects exerted by UBI in blood clearly links this treatment with ozone therapy. UBI has been shown to increase white blood cell production of hydrogen peroxide (Ivanov et al., 1989) (as does ozone). Almost identical to the findings of Bocci (2011) and Menendez and Weiser (2016) with ozone, UBI has been found to “highly increase” SOD and glutathione peroxidase while significantly decreasing signals of free radical damage (Dong et al., 2000). UBI may inactivate viral particles (since unlike host cells, viruses do not have repair mechanisms for genetic damage) meanwhile preserving immunogenicity. Furthermore, the cytokine storm that surrounds severe viral infections (Ebola, COVID-19) may be controlled by immune modulation that both these therapies provide (Hamblin, 2017). Ozone’s huge advantage over UBI is that it can be locally administered to infected or compromised tissues improving local hypoxia in addition to its other stimulatory effects.

Intravenous hydrogen peroxide therapy

In the 1918 Spanish influenza epidemic, tens of millions died, predominantly of viral pneumonia. There was no treatment then, and little treatment still today for serious viral infections. British physician Oliver in India nearly halved the mortality of viral pneumonia with intravenous hydrogen peroxide. He emphasized that only the worst cases were so treated (Oliver and Murphy, 1920). In the 1980s Charles Farr, conducted informal studies utilizing a 0.03% H₂O₂ concentration in 250 cc 5% dextrose, infused over 1.5 h. The infusions were found to be safe and effectively shortened the duration of seasonal flu illnesses. He taught hundreds of physicians his methods and, to date, no adverse effects have been reported, except peripheral vein irritation (Farr, 1994). The method releases about 25 cc of oxygen gas intravenously. Nascent oxygen released by the infusion is postulated to create a pro-oxidant blood status, creating similar redox messenger molecules as ozone therapy, UBI, and high dose IV Vitamin C. The common protocol is twice weekly for 2–3 weeks, whether viral or bacterial disease.

However, since endothelial cells lack catalase, there is risk of local vein sclerosis at the administration site (as can high dose intravenous ascorbate), and repeated DIV ozone. As a result, H₂O₂ therapy has fallen out of favor among oxidation practitioners, replaced by ozone therapy and UBI. Nevertheless, based on Oliver’s work, it may be a lifesaving treatment when another therapy is not possible or available. H₂O₂ can also be administered by a nebulizer (0.06% to 1% in water or saline solution) especially useful in case of pulmonary disease, including COVID-19 (Brownstein et al., 2020).

Hyperbaric oxygen therapy (HBOT)

HBOT is used as either a primary or adjunctive treatment in the management of infections such as gas gangrene, necrotizing fasciitis, diabetic foot infections, refractory osteomyelitis, neurosurgical infections, and fungal infections. HBOT acts as a bactericidal/bacteriostatic agent against anaerobic bacteria by increasing the formation of free oxygen radicals. HBOT restores the bacterial-killing capacity of leukocytes in hypoxic wounds by increasing tissue oxygen tensions (Cimşit et al., 2009).

Like ozone therapy, HBOT works synergistically with conventional antibiotics. One mechanism may be similar to ozone therapy. Breathing 100% oxygen at greater than atmospheric pressure (1 ATA) increases the formation of reactive oxygen species (ROS) (Korpinar and Uzun, 2019). This will create active signaling molecules from immune cells, while being directly active against pathogens. Considering its utility in several aforementioned serious infections, HBOT could be employed in any serious infection as an adjunctive therapy. As oxygen availability and utilization is the most important factor in biological processes, and infection can compromise blood flow, HBOT, by dissolving oxygen in body fluids, may overcome this local or systemic oxygen deprivation.

Typical use is daily at 2–3 ATA, for 1–2 h, until resolution of infection. Complications can include diving-like injury to the eardrum. Oxygen toxicity risk is low due to the relatively short lung exposure to high oxygen tension.

High-dose intravenous ascorbic acid

Ascorbic acid/ascorbate (commonly called “vitamin C”) at low doses is an antioxidant acting as an electron donor. High-dose intravenous ascorbate acts as a prodrug for H_2O_2 generation (Chen et al., 2005), explaining observations over decades of its clinical utility in infection.

Septic patients have low circulating ascorbate levels (Wilson and Wu, 2012). Parenteral administration of ascorbate (10–50 g) raises plasma and tissue concentrations and may decrease morbidity.

Mechanism of action

In animal models of sepsis, intravenous ascorbate injection increases survival and protects several microvascular functions: capillary blood flow, microvascular permeability barrier, and arteriolar responsiveness to vasoconstrictors and vasodilators (Kuhn et al., 2018). The effects of parenteral ascorbate on microvascular function are both rapid and persistent. Ascorbate quickly accumulates in microvascular endothelial cells, scavenges reactive oxygen species, and acts through tetrahydrobiopterin to stimulate nitric oxide (NO) production by endothelial nitric oxide synthase, a normal and necessary process (Mortensen and Lykkesfeldt, 2014). However, sepsis can induce high levels of NO by other mechanisms, which can inhibit neutrophil migration and adhesion, and contribute to multiple organ dysfunction (Spiller et al., 2019). Ascorbate may attenuate the effects of excess NO in sepsis and offer organ protection (Oudemans-van Straaten et al., 2014).

Ascorbate may be a valuable adjuvant therapy in sepsis (Wilson, 2009). Ascorbate is important for immune cells, enhancing chemotaxis, ROS generation, and phagocytic destruction of microbes, being also required for apoptosis and clean-up of spent neutrophils. Deficiency results in impaired immunity. High doses (200 mg daily) reduce the severity and duration of the common cold (Carr and Maggini, 2017).

Infection raises metabolic demand for ascorbate. Healthy people may experience loose stools when exceeding 4–8 g orally daily. However, sick patients can tolerate intakes to 200 g daily without any GI distress, indicating far greater need and consumption tolerance of ascorbate when ill (Cathcart, 1981).

Clinical efficacy

Klenner (1949) was one of the first physicians to inject patients with high ascorbate doses. He reported in the 1950s his experiences with parenteral ascorbate, buffering ascorbic acid with sodium bicarbonate to neutralize pH. His schedule for home patients was “2,000 mg (injection) every six hours, supplemented by 1,000–2,000 mg orally every two hours.” That amounted to 8000 mg IM, plus oral amounts (depending on sleep time) from 16,000 to 32,000 mg. Klenner reported stunning rapid and complete resolutions of viral diseases inclusive of 60 of 60 polio cases. Patients were often clinically well after 72 h.

Klenner reported on even higher doses ranging from 350 to 1200 mg per kg body weight to treat illness using parenteral ascorbate to cure measles, herpes simplex, viral encephalitis, and mononucleosis. He emphasized the need to maintain continuous high levels during the acute phase of the infection. Small children were given 2–3 g IM every two hours in the buttocks. Large doses, up to 60 g, could be added to 500 cc water together with other vitamins such as 500 mg thiamin HCl, 300 mg pyridoxine, 400 mg calcium pantothenate, 100 mg riboflavin, and 300 mg niacinamide, given once or twice daily.

Typical applications would be 25 g of ascorbate (buffered to neutral pH) into 500 ml of sterile water (18 cc diluent to each gram of ascorbate are necessary to ensure correct osmolality). He reported clearing viral pneumonia easily with parenteral treatments. Klenner also gave alkaline drinks to slow ascorbate renal excretion. With 350–400 mg/kg ascorbate every 2 h, he reported quick resolution of measles and chicken pox, sometimes requiring only a few treatments. Complications included vein irritation (IV route), subcutaneous thickening (local injections). Large oral doses could cause diarrhea, and genital or anal rash and itch (Smith, n.d.;

Klenner, 1971). To prevent these effects, Klenner often added small amounts of IM corticosteroids (desoxycorticosterone), 2.5 mg for children and 5 mg for adults. This combination therapy has been reconsidered in 2017 by Prof. Paul Marik who reported that intravenous vitamin C (1.5 g) with hydrocortisone (50 mg) and intravenous thiamine (200 mg) can reduce sepsis mortality from 40% to about 8% (Marik et al., 2016).

Conclusions

Generation of reactive oxygen species (ROS) may be promising for modulating the immune system and controlling infections, including those caused by drug-resistant organisms. Ozone therapy, Ultraviolet Blood Irradiation Therapy, intravenous ascorbate, H_2O_2 , as well as Hyperbaric Oxygen Therapy are interesting therapies to be explored and exploited both as alternatives to, or in association with antimicrobials, providing that very uncommon side effects are kept in mind.

However, it should be emphasized that an important role in infection control is played by the innate immune defenses, and that the latter can be boosted not only with oxidants but also with correct nutrition. Vitamin A, zinc, iodine, and folate deficiencies, affecting 2 billion people in developing countries, impair the immune response, being a major contributor to severe infections and death (Katona and Katona-Apte, 2008). Micronutrient deficiencies (vitamins and minerals) are also common in the USA (Medical Economics, 2016; CDC.gov, 2012). Vitamins (A, B12, C, D, E, essential fatty acids) are important for ensuring the first defenses at the mucosal barrier level. Metals like zinc, iron, copper, and selenium all contribute to a correct catalytic/enzymatic activity ultimately helping immune functions. Besides these evident nutritional deficiencies, incorrect diet can alter immune defenses. As an example, the ingestion of simple carbohydrates, such as monosaccharides, can decrease the phagocytic index (Sanchez et al., 1973). Also, heavy metals (present from contamination or unwanted agricultural residues) can impair the immune system and reduce host resistance to invading pathogens (Lehmann et al., 2011; Fenga et al., 2017). Simultaneously, some foods possess useful antibacterial properties, due to their polyphenolic or other compounds. Among these are manuka honey (Johnston et al., 2018) and olive polyphenols. The latter displays antiviral activity in vitro (Bisignano et al., 1999; Rounds et al., 2012) including HIV (Bedoya et al., 2016) and SARS-CoV-2 transmission (Ergoren et al., 2020). In these changing times when important challenges such as multiple drug resistance arise, exploring new strategies to counteract infection is an urgent issue. Unfortunately, only few quantitative studies on old methods or alternative plant molecules are available in the literature. This may be because in vivo studies are expensive; and the final result, although successful, is not patentable. However, the natural world is rich in molecules that can have activity against microbes. The time is ripe to extend detailed and quantitative investigation to the myriad of compounds that can be used alone or in synergy with antibiotics or oxidation therapies. These sometimes “unapproved” but potentially lifesaving therapies can be the key for treating conventionally untreatable patients.

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Immunoglobulin Replacement Therapy

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Introduction

In 1890, von Behring and Kitasato demonstrated that the serum of rabbits immunized with tetanus toxin contained activity against tetanus, and such serum transferred to rabbits protected them against the disease (Eibl, 2008). Techniques developed by Cohn in Boston led to the development of the separation of plasma proteins into individual stable fractions with varied biologic functions (Cohn et al., 1940). The basis for Cohn's fractionation was to use low concentrations of alcohol by reducing the pH and lowering ionic strength (Cohn et al., 1940). This procedure was performed at low temperatures, reducing the likelihood of contamination, and made large-scale fractionation possible (Cohn et al., 1940). The method is basically in use, and the produced immunoglobulin preparation contains mostly, or almost exclusively IgG with only trace amounts of IgA and IgM. In the beginning, this "Cohn fraction II" was commercially available as a 16.5% solution with glycine and Merthiolate as stabilizer and preservative (Eibl, 2008; Cohn et al., 1940). The first patient described with agammaglobulinemia by Bruton received subcutaneous immunoglobulin (SCIG) with good response (Bruton, 1952). Although Dr. Bruton started the subcutaneous immunoglobulin, the regimen was altered by himself into weekly intramuscular administration, and it became the mode of administration for more than 20 years (Bruton, 1952; Rosen and Janeway, 1966). Numerous patients with hypogammaglobulinemia were identified and treated with immunoglobulins. Initially, immunoglobulin was derived by fractionation from pools of plasma obtained from at least 500 donors, nowadays is standard to obtain it from thousands of donors (Rosen and Janeway, 1966). Early attempts to give the product intravenously were abandoned because this route had many side effects, probably due to the large amounts of Ig aggregates and highly active contaminants like bradykinin in the product (Rosen and Janeway, 1966; McCue et al., 1986). The intramuscular route was very painful and full of adverse reactions such as nausea, vomiting, and hypotension, and normal IgG levels could not be achieved by this route (Stiehm, 1984). Purification with anion exchange chromatography led to the production of intravenous immunoglobulin (IVIG) containing native IgG and throughout the 1980s and 1990s, IVIG administration was the most common method of replacement in most countries (McCue et al., 1986; Stiehm, 1984). In the 1980s the concept of immunomodulatory dose of IVIG was born from the study by Dr. Paul Imbach in Immune Thrombocytopenic Purpura (ITP) (Imbach et al., 1981).

In 1980 Dr. Berger reintroduced slow subcutaneous infusion for primary immunodeficiency disorders (recently called inborn errors of immunity (IEI)) but the method didn't gain wide enthusiasm owing to the slow rate of infusion (Berger et al., 1980; Roord et al., 1982). In 1991 rapid infusion of subcutaneous immunoglobulin was described by Gardulf and Hammastrom and with this method, good serum levels were achieved. Subcutaneous IgG (SCIG) has become established as a well-tolerated and effective treatment that is preferred by many patients (Gardulf et al., 1991; Jolles et al., 2015; Misbah et al., 2009). A limitation of SCIG use is the large volumes needed to be administered and due to the inability of tissue to accept large volumes, frequent administration at multiple sites is necessary. A novel administration strategy of immunoglobulin treatment is by injection of recombinant human hyaluronidase with subcutaneous immunoglobulins, the facilitated subcutaneous immunoglobulin (fSCIG) (Wasserman, 2014). The enzyme facilitates drug dispersion and adsorption, allowing higher IgG injection rates, bioavailability, and increased infusion volumes. fSCIG is injected less frequently compared with SCIG (1–2 weeks vs. monthly infusions).

Mechanisms of action

IgG replacement therapy (IgRT) has an essential role in minimizing infections and improving the quality of life in patients with immunodeficiency, who would otherwise suffer from recurrent infections. The large donor pool ensures the diversity of antibody

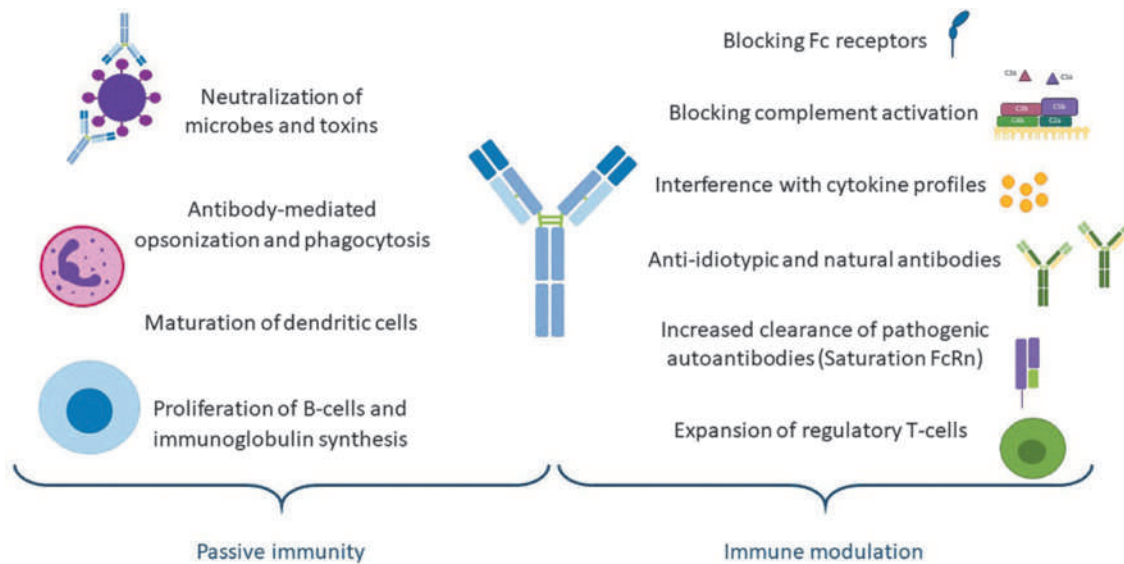


Fig. 1 Examples of some of the diverse mechanisms of action of IVIG.

repertoires that include antibody specificities to a wide spectrum of antigens (Kaveri et al., 2011). The mechanisms for the therapeutic effects of IgG products are varied and complex with new mechanisms of action being discovered continuously (Fig. 1). The presence of F(ab') fragments in the product provide antigen recognition function, whereas the Fc fragments enable activation of immunity (Matucci et al., 2015). The mechanisms associated with infection rate reduction are thought to be mainly associated with the passive transfer of pathogen-specific antibodies which neutralize pathogens and toxins or mark them for lysis (Fig. 1). Also, several lines of experimental evidence suggest that the therapeutic beneficial effects of IVIG in immunodeficiencies reflect an active role for IVIG rather than a mere passive transfer of antibodies (Kaveri et al., 2011). IVIG exercises a stimulatory effect on maturation of dendritic cells at replacement low dose (Kaveri et al., 2011). IVIG also at replacement dose induces proliferation and immunoglobulin synthesis from B-cells of CVID patients (Kaveri et al., 2011; Bayry et al., 2011).

Multiple mechanisms of action have been proposed to be responsible for the efficacy of high-dose IVIG in autoimmune diseases and different mechanisms may be important in different disease states (Fig. 1). IVIG exerts its immunoregulatory functions at multiple levels involving modulation of expression and function of Fc receptors, blocking of complement activation, interference with the cytokine profiles, modulation of idiotype network, and cell proliferation (Shock et al., 2020). High dose IVIG may implicate saturation of FcRn leading to increased clearance of pathogenic autoantibodies (Hansen and Balthasar, 2002).

IVIG also contains antibodies that occur in the absence of pathological conditions referred to as natural antibodies (Maddur et al., 2020). These antibodies are present in the absence of immunization or infection, and some of them interact with self-antigens and are called natural autoantibodies (nAAb) (Avrameas et al., 2008). These nAAb are polyreactive and are thought to play a role in neutralizing toxins, remove self-molecules, and modulating antigen presentation contributing to cellular homeostasis (Maddur et al., 2020; Avrameas et al., 2008). Of interest is that SCIG also appears to present immunomodulatory properties, although its mechanisms of action are at the moment elusive and might be similar to those of IVIG (Scheuerlein et al., 2018; Sala et al., 2018).

Indications and clinical efficacy

The goal of long-term IgG replacement therapy is to reduce the incidence and severity of infections and prevent long-term deterioration in organ function. IVIG is increasingly being recognized as a treatment option for several diseases, beyond replacement therapy for antibody defects. The following categories classify the most frequent uses of IVIG, but clinical applications continue to evolve.

Treatment of primary immunodeficiency states/inborn errors of immunity (IEI)

Patients with IEI with antibody defects, either in production or function, result in absent or deficient antibody production that predisposes to recurrent or severe infections are discussed in detail. In these patients, IVIG is used as replacement therapy.

Agammaglobulinemia: Diseases with failure in B cell development or function may result in reduced or absent immunoglobulins, hypogammaglobulinemia, and agammaglobulinemia respectively. Primary agammaglobulinemia is most frequently an X-linked disease (Bruton's agammaglobulinemia), but several autosomal recessive forms also exist. These patients have an absence or nearly

absence of B cells, as well as tonsils and adenoids, and they present with recurrent bacterial infections of the respiratory tract. Agammaglobulinemia is the most classical indication for IgRT and it changed the clinical course and prognosis of the disease, reducing the frequency and severity of the infections (Perez et al., 2017; Ammann et al., 1982).

Severe combined immunodeficiency (SCID): These are a group of disorders that arise from a disturbance in the development and function of T and B cells, conferring a combined immune deficiency state. The term severe comes from the early death from overwhelming infections without treatment (Rivers and Gaspar, 2015). Typical SCID patients have absent or very low autologous CD3⁺ T cell count (<300/ μ L) and no or very low T cell function (phytohemagglutinin response <10% of the lower limit of normal), or the presence of circulating maternal T-cells. Leaky SCID patients have a reduced number of CD3⁺ T-cells with <30% of lower limit or normal T-cell function and absence of maternal engraftment. Patients with Omenn Syndrome have a generalized skin rash, absence of maternal engraftment detectable CD3⁺ T-cells, and absent or low T-cell function or oligoclonal T-cells (Sullivan and Richard Stiehm, 2020; Shearer et al., 2014). Patients with this group of diseases may have either an absence of B-cells or ineffective B-cell function since T-cell dysfunction hinders antibody production for humoral immunity. Initial management includes prevention of infection, antimicrobial prophylaxis, IRT, and avoidance of live vaccines (Bustamante Ogando et al., 2019).

Hypogammaglobulinemia and impaired antibody production: The prototype is common variable immune deficiency (CVID), a collection of hypogammaglobulinemia syndromes associated with poor or absent response to immunizations; one of the most frequently diagnosed IEI (Gathmann et al., 2014; Sullivan et al., 2014). IVIG reduces sinopulmonary infections; an early diagnosis and timely IRT is crucial to prevent chronic lung disease from chronic or subclinical lung infections (Busse et al., 2002; de Gracia et al., 2004; Lucas et al., 2010).

Class-switch recombination defects, also known as hyper-IgM syndromes, are another example of hypogammaglobulinemia with decreased IgG and IgA and normal or elevated IgM. The X-linked form (CD40L deficiency) and autosomal recessive CD40 deficiency result in a combined immune deficiency, beyond an antibody defect. With IRT there is a reduction of infectious complications (Winkelstein et al., 2003, 2006).

Selective antibody defects: In some cases, patients may have normal immunoglobulin levels, but an impairment of the production of specific antibodies, for instance, polysaccharide antigens. The response to polysaccharide antigens can be evaluated with measurement of antibody titers 4–8 weeks after vaccination with pneumococcal polysaccharide vaccine. A protective or adequate response to each serotype is a titer equal or greater than 1.3 μ g/mL, with at least a twofold increase in the titers. The number of pneumococcal serotypes that are protective after a vaccine can be used to define a normal antibody response, with >50% serotypes in children from 2 to 5 years and >70% in patients from 6 to 65 years. There is also a memory phenotype with an adequate initial response, but it wanes within 6 months. IRT should be provided when there is a well-documented polysaccharide non-responsiveness with evidence of recurrent bacterial infections (Orange et al., 2012a).

Isolated hypogammaglobulinemia: In transient hypogammaglobulinemia of infancy patients have transient low IgG levels that normalize with age and the antibody response is qualitatively normal. Select cases with recurrent bacterial infections may warrant IRT temporarily to decrease infections (Memmedova et al., 2013; Duse et al., 2010). Isolated hypogammaglobulinemia can also be a feature of many IEI and must be differentiated from secondary causes due to increased loss of IgG such as chylothorax, lymphangiectasia, enteropathy, massive proteinuria, and medications. An IgG level below 150 mg/dL is widely recognized as severe hypogammaglobulinemia and, upon confirmation of the low IgG value, IRT should be started promptly (Perez et al., 2017).

Normal immunoglobulin levels and other defects: Isolated subclass deficiency is often asymptomatic, but some patients might have associated poor specific antibody response and recurrent infections, and thus may benefit from IgRT (Abrahamian et al., 2010; Olinder-Nielsen et al., 2007). IgRT is not usually indicated for selective IgA deficiency, but in some patients, it may coexist with IgG2 subclass deficiency or poor specific antibody production. It is important to remember that IVIG can pose a risk for adverse reactions in IgA deficient patients with anti-IgA antibodies (Burks et al., 1986; de Albuquerque Campos et al., 2000), despite most patients receiving IVIG safely (Björkander et al., 1987; Rachid and Bonilla, 2012). IgA-depleted IVIG or subcutaneous route for immunoglobulin can be also considered (Perez et al., 2017).

Combined immunodeficiencies associated or with syndromic features frequently require IRT. Wiskott-Aldrich syndrome is an X-linked disorder associated with immunodeficiency, microthrombocytopenia, and eczema, as well as a predisposition for autoimmunity and malignancy. An impaired specific-antibody response is frequently seen, IRT reduces infection and should be considered (Ochs et al., 1980; Conley et al., 2003). Given the bleeding tendency in this disease, the subcutaneous route should be used cautiously. Ataxia-telangiectasia syndrome is also a genetic syndrome associated with immune deficiency, and over half the patients have both cellular and humoral impairment (Nowak-Węgrzyn et al., 2004). Other combined immunodeficiency phenotypes of IEI not discussed may also need IgRT.

Down syndrome is the most common genetic syndrome associated with immune defects, and some authors even consider it an inborn error of immunity (Huggard et al., 2020; Dieudonné et al., 2020). Multiple studies have demonstrated an immune dysfunction in patients with Down syndrome, including defects in the differentiation of B cells (Carsetti et al., 2015; Verstegen et al., 2014) as well as subclass imbalance and impaired vaccine response (Valentini et al., 2015; Nespoli et al., 1993; Ram et al., 2011). Routine vaccination of this group is imperative, and when recurrent bacterial infections continue to occur despite general measures, IgRT can be considered.

On some occasions, we might encounter a patient with recurrent bacterial infections with no yet characterized immune defect, IgRT can be an option for suspected IEI despite apparent normal humoral function. There are now over 400 IEI described, and indications of immunoglobulin therapy are an ever-expanding field (Tangye et al., 2020). In general patients with a clearly defined antibody production defect such as agammaglobulinemia, SCID, class-switch defect, or combined immune deficiencies, among

others, should be placed in IgRT as soon as the diagnosis is made. In other more specific antibody defects, the indication for replacement therapy should be based on the laboratory values, analyzed in the context of the patient's infection history, as well as preexisting comorbidities.

Treatment of secondary immune deficiencies

Secondary humoral impairment can result from several diseases or medications, increasing patients' vulnerability to bacterial infections. Secondary antibody deficiency (SAD) is more common than IEL and is being increasingly recognized (Patel et al., 2019). In secondary antibody deficiency, despite being more prevalent than IEL, clinical data on IgRT are limited (Na et al., 2019). Of note that in SAD there are important discrepancies among daily practice, clinical practice, guideline recommendations, and approved indications (Patel and Cowan, 2020; Agostini et al., 2016). Also, there is no consensus on the duration of IgRT in SAD, with 6 months to a year as the suggestion by some experts (Agostini et al., 2016).

Protein loss: The loss of great amounts of protein can also be an important mechanism for secondary hypogammaglobulinemia. Nephrotic syndrome, GI diseases, extensive dermatological alterations, lymphatic abnormalities (i.e., chylothorax, intestinal lymphangiectasia), and genetic syndromes can also result in hypogammaglobulinemia (Perez et al., 2017; Jolles et al., 2017). Procedures such as plasma exchange and chronic peritoneal dialysis can also result in hypogammaglobulinemia and may warrant IgRT (Lalan et al., 2017; Gupta et al., 2016).

B-cell lymphoproliferative diseases: In chronic lymphocytic leukemia (CLL) hypogammaglobulinemia is present in one-quarter of patients at diagnosis and up to two-thirds develop it in the course of the disease, it is an important predisposing factor for infection (Parikh et al., 2015; Wadhwa and Morrison, 2006). The use of IRT in CLL with high infection risk can effectively protect from bacterial infections (Cooperative Group for the Study of Immunoglobulin in Chronic Lymphocytic Leukemia et al., 1988). Patients with multiple myeloma (MM) are also at an increased risk for infection due to immune dysfunction and hypogammaglobulinemia (Backhaus et al., 2016); IRT has shown to lower incidence and recurrence of severe infections, particularly in MM patients with impaired vaccine response or more aggressive immunomodulatory regimens (Chapel et al., 1994; Khalafallah et al., 2010). Other malignant and hematological diseases such as lymphoma, leukemia, and myelodysplastic syndromes have also been associated with secondary hypogammaglobulinemia (Patel et al., 2019).

Hematopoietic stem cell transplantation (HSCT): Hypogammaglobulinemia is seen in up to two-thirds of patients post-HSCT and is higher in patients with acute graft vs host disease GVHD and patients receiving stem cells from an unrelated source (Frangoul et al., 2013). Currently, IgRT is not routinely recommended for all patients who undergo HSCT to prevent infection of GVHD (Raanan et al., 2008a; Cowan et al., 2015), a targeted approach to maintain normal IgG levels can be taken (Foster et al., 2018). Patients with recurrent bacterial infections and demonstrable antibody defects or chronic GVHD can benefit from IVIG therapy. Recipients of HSCT for an IEL with significant B-cell impairment that are receiving IgRT previous to the HSCT, like SCID, should continue it until humoral immunologic reconstitution (Perez et al., 2017).

Therapeutic agents: Drug-related antibody deficiency can account for one-fifth of all secondary antibody deficiency worldwide (Table 1) (Patel et al., 2019; Jolles et al., 2017; Compagno et al., 2014). High-dose and prolonged treatment with systemic corticosteroids can impair both cellular and humoral immunity (Wirsum et al., 2016). Mycophenolate mofetil (MMF) inhibits both T and B-cell proliferation and has also been shown to cause hypogammaglobulinemia (Kandyil et al., 2009; Boddana et al., 2011).

Rituximab, a chimeric IgG1 anti-CD20 monoclonal antibody, can cause hypogammaglobulinemia, which can be persistent and clinically significant (Casulo et al., 2013; Makatsori et al., 2014; Barmettler et al., 2018). Hypogammaglobulinemia can also be pre-existing, we strongly advocate pre-treatment evaluation with serum immunoglobulin levels, and if feasible, peripheral blood B-cells before rituximab therapy; since preexisting hypogammaglobulinemia is a risk factor for serious infections and sustained hypogammaglobulinemia (Compagno et al., 2014; Barmettler et al., 2018; Buch et al., 2011). The upsurge of new immunomodulatory and biologic therapies can be a harbinger for iatrogenic secondary deficiencies, a close surveillance should be maintained (Patel et al., 2019; Jolles et al., 2017).

Table 1 Therapeutic agents that may cause secondary antibody deficiency.

Corticosteroids
Mycophenolate
Antimetabolites and alkylating agents (i.e., cyclophosphamide)
Sulfasalazine and penicillamine
Tyrosine kinase inhibitors
Anti-epileptic medication
Anti-CD19 therapies (i.e., blinatumomab)
Anti-CD20 therapies (i.e., rituximab, ofatumumab, ocrelizumab)
Anti-CD22 therapies (i.e., epratuzumab, inotuzumab)
Anti-CD52 (i.e., alemtuzumab)
Anti-CD38 (i.e., daratumumab)
Anti-BAFF (i.e., belimumab)
Anti-FcRn (i.e., rozanolixizumab, efgartigimod)

Solid-organ transplantation: these patients receive immunosuppressive medications and undergo procedures that can cause hypogammaglobulinemia and carry an increased risk for infection; particularly after heart, lung, and kidney transplantation (Florescu et al., 2013; Madan et al., 2020). A combination of plasma exchange, immunoglobulin treatment, and/or B-cell depletion can be used for desensitization of highly sensitized patients to HLA or ABO group to improve transplant outcomes. A combination of these interventions has also been used for antibody-mediated rejection (Jordan et al., 2011). Patients with heart transplantation who receive pre-emptive replacement therapy may have fewer infections and episodes of rejection (Yamani et al., 2001). Patients should be closely monitored for hypogammaglobulinemia and they can also present with drug-resistant or severe viral infections that could benefit from immunoglobulin therapy (Florescu, 2014).

Pediatric HIV infection: HIV disease can lead to impaired antibody production, but hypogammaglobulinemia is uncommon (Opravil et al., 1991; Woudenberg et al., 2020; *New England Journal of Medicine*, 1995). Some trials from over 25 years ago found IVIG treatment to reduce bacterial infections and hospitalizations in HIV-infected children (Mofenson et al., 1994; Spector et al., 1994). With the advent of highly active antiretroviral treatment and antimicrobial prophylaxis, the advantage of IVIG in children with HIV is less clear (Huang et al., 2008).

Prematurity: newborn infants have an immature immune system and are highly dependent on transplacental transport of IgG, mainly in the third trimester; as such, preterm babies have a lower acquired IgG (van den Berg et al., 2011). Lower IgG levels in preterm infants are associated with an increased risk of infection, but prophylactic IgRT does not appear to modify clinical outcomes (Sandberg et al., 2000; Ohlsson and Lacy, 2020).

As discussed, many entities might lead to hypogammaglobulinemia and secondary antibody deficiency, this might lead to increased infection susceptibility. IgRT is generally reported to significantly reduce the rate of serious infections in patients with hematologic malignancies, but automatic prescription of IgRT is not recommended as no correlation has been established between IgRT and overall survival (Raanan et al., 2008b). The decision to start IgRT in secondary antibody deficiency should be individualized according to the degree of impairment of humoral immunity, the frequency, and severity of the infections, as well as the impact of general measures and prophylactic antibiotics (Perez et al., 2017; Na et al., 2019).

Immunomodulatory and anti-inflammatory therapy

Hematologic autoimmune diseases: Intravenous immunoglobulin is currently an effective and fast-acting first-line treatment for immune thrombocytopenia (ITP). Other first-line therapies include glucocorticoids and anti-D immune globulin; watchful waiting can also be considered. The choice of therapy is based on bleeding manifestations, the grade of thrombocytopenia, and risk factors, with IVIG achieving a faster platelet count increase (Beck et al., 2005; Rodeghiero and Ruggeri, 2014). IVIG is also a cornerstone in the treatment of neonatal alloimmune thrombocytopenia due to feto-maternal immunization and can be used as antenatal treatment (Massey et al., 1987; Rayment et al., 2011). In other autoimmune cytopenias, such as immune neutropenia or autoimmune hemolytic anemia, IVIG treatment can also be beneficial (Hilgartner and Bussel, 1987; Vagace et al., 2014).

Rheumatic diseases: Kawasaki disease (KD) is one of the most common forms of vasculitis in children and if left untreated up to one-quarter of patients can develop coronary artery aneurysms. The use of high-dose IVIG changed the course of KD significantly reducing coronary artery aneurysms (Oates-Whitehead et al., 2003; Furusho et al., 1983; Kobayashi et al., 2012). A single intravenous infusion of 2 g per kg of body weight is the recommended dose since it is more effective and equally safe than four smaller daily doses of 400 mg per kg of body weight (Newburger et al., 1991).

Multisystem inflammatory syndrome in children (MIS-C) is a complication of COVID-19 that can present not unlike KD in children. IVIG treatment is recommended for patients with MIS-C with KD characteristics (either complete or incomplete KD), as well as in patients with moderate to severe MIS-C without KD-like symptoms (Feldstein et al., 2020; Henderson et al., 2020; Whittaker et al., 2020).

Patients with autoimmune inflammatory myopathies; particularly dermatomyositis patients with severe weakness, resistant and recurrent disease can benefit from high-dose IVIG therapy (Cherin et al., 1994; Wang et al., 2012; Marie et al., 2010). In systemic lupus erythematosus (SLE) IVIG is associated with a transitory clinical improvement of disease activity and can be an additional aid for the treatment of patients with severe lupus multiorgan disease or patients with concurrent infections (Sakthiswary and D'Cruz, 2014; Zandman-Goddard et al., 2005).

IVIG can have a role in the management of systemic-onset juvenile idiopathic arthritis with an improvement of systemic features and is largely reserved for patients with unresponsiveness to standard therapy of macrophage activation syndrome. Macrophage activation syndrome may complicate systemic-onset juvenile arthritis, SLE, or KD (Uziel et al., 1996; Lin et al., 2012).

Other diseases in which IVIG treatment may be beneficial include systemic sclerosis/scleroderma (Young and Khanna, 2015), IgA vasculitis (Henoch-Schönlein purpura) (Mauro et al., 2019), polyarteritis nodosa (Asano et al., 2006), anti-neutrophil cytoplasm antibody (ANCA)-associated systemic vasculitis (Jayne et al., 2000), Takayasu arteritis (Velásquez et al., 2010), and autoimmune hepatitis (Vierling and Flores, 2002), among others. IVIG has also been proposed in a myriad of clinical conditions with an altered immune response such as blistering skin diseases (i.e., pemphigus) (Amagai et al., 2009; Czernik, 2014) and toxic epidermal necrolysis, and Stevens-Johnson syndrome (Seray et al., 2008; Huang et al., 2012; Roujeau and Bastuji-Garin, 2011; Zimmermann et al., 2017).

Neuroimmunologic disorders

Guillain-Barré syndrome (GBS) is a group of acute immune-mediated polyradiculopathies that most often presents as a monophasic paralyzing illness due to demyelination. Both IVIG and plasma exchange (PE) are effective treatments for GBS. IVIG effectiveness is considered comparable to plasma exchange but is more frequently used because of vascular access and tolerance to PE and is more likely to be completed (Hughes et al., 2014; Patwa et al., 2012).

Chronic inflammatory demyelinating polyneuropathy (CIDP) is a group of chronic neuropathies with immune-mediated demyelination characterized by a chronic relapsing-remitting course of motor-predominant neuropathy. Multiple randomized controlled trials have established IVIG and SCIG as effective treatments. Other treatment options include PE and glucocorticoids (Efthimov et al., 2013; Dyck et al., 1994; Gentile et al., 2020).

Multifocal motor neuropathy is characterized by progressive asymmetric weakness and atrophy without sensory alterations, it is treatable with intravenous IVIG, but is unresponsive to glucocorticoids or plasma exchange (Patwa et al., 2012; Cats et al., 2010). SCIG may also be beneficial in this disease (Racosta et al., 2017).

Myasthenia gravis is an autoimmune disorder of the postsynaptic neuromuscular union that presents with weakness. PE and IVIG are rapid-acting options useful in patients with severe or refractory disease; IVIG also has a short-term response in patients with non-severe exacerbations (Patwa et al., 2012; Gajdos et al., 2012; Barth et al., 2011). In *Lambert-Eaton myasthenic syndrome* there is a reduced acetylcholine release from the presynaptic nerve terminals and is strongly associated with small cell lung cancer as a paraneoplastic manifestation (Benatar et al., 2001), it can also present in patients with underlying immune-mediated diseases (Wirtz et al., 2004). IVIG has shown clinical improvement and is a second-line alternative in Lambert-Eaton myasthenic syndrome (Keogh et al., 2011; Bain et al., 1996).

Multiple sclerosis (MS) is a demyelinating inflammatory immune-mediated disease of the central nervous system. IVIG is a potentially effective second-line treatment in MS and is a fundamental alternative in patients during pregnancy or breastfeeding (Dudešek and Zettl, 2006; Sorensen et al., 2002).

Other neuroimmune diseases that may benefit from IVIG therapy include neuromyelitis optica spectrum disorder, acute disseminated encephalomyelitis, opsoclonus-myoclonus syndrome, Sydenham chorea, autoimmune encephalitis, pediatric autoimmune neuropsychiatric disorder associated with streptococcal infection, and intractable childhood epilepsy (Patwa et al., 2012; Gadian et al., 2017; Wingerchuk, 2013).

Infection-related disorders

Sepsis and septic shock: IVIG has been used as adjuvant therapy for sepsis or septic shock reducing mortality in adults, and the use of IgM enriched formulations seems a promising therapeutic approach but remains experimental and further clinical data is needed (Alejandria et al., 2013; Jarczák et al., 2020).

Viral infections: In CMV pneumonitis high dose IVIG or high concentration polyclonal IVIG (CMV-IVIG) with ganciclovir combination has been reported to improve survival in patients post bone marrow transplant (Reed et al., 1988; Ljungman et al., 1992). Enteroviral meningoencephalitis can be a catastrophic complication in patients with agammaglobulinemia and is difficult to treat. Intensive high dose IVIG has been associated with clinical improvement and even long-term eradication. Intrathecal immunoglobulin may also be effective but adverse effects are common (Bearden et al., 2016; Alborizi et al., 2009; Misbah et al., 1992; Quartier et al., 2000). There may also be a clinical benefit of IVIG treatment for immunocompetent children with viral encephalitis, but not Japanese encephalitis (Iro et al., 2017). There have also been successful reports of IVIG in parvovirus B19 infection (Kerr et al., 2003; Cao et al., 2009; Katragadda et al., 2013). IVIG has also been used in persistent BK polyoma viremia and BK virus nephropathy in posttransplant patients (Vu et al., 2015; Kable et al., 2017). The usefulness of IGIV in viral myocarditis is still uncertain (Robinson et al., 2020).

Dosage of immunoglobulin

Different immunoglobulin doses can be administered based on the indication. In most IEI and secondary antibody defects a substitutive dose to prevent infections is used, when considering suppression of an inflammatory or autoimmune process higher doses are needed.

Immune deficiencies, either primary or secondary, might need a substitutive dose of immunoglobulin to achieve a passive immunity state.

Patients are usually started in a dose range of 400–600 mg per kg of body weight of IVIG per dose (Hernandez-Trujillo et al., 2012). Intravenous bolus doses can be administered every 21–28 days. In patients with severe hypogammaglobulinemia, a loading single dose of up to 1 g per kg can be given as the initial dose.

Subcutaneous preparations starting dose is usually 100–200 mg per kg of body weight per week and can be administered weekly or every 2 weeks. After subcutaneous infusion, the immunoglobulin is absorbed over several days and therapeutic plasma levels are not immediately achieved. Patients newly starting SCIG may receive a loading dose of IVIG 1 week before the initiation of SCIG (Ochs et al., 2006). A subcutaneous loading dose can also be administered as five consecutive daily doses or two doses per week for the first 2 weeks of the planned weekly dose (Borte et al., 2011; Koterba and Stein, 2015). To transition a patient from IVIG to SCIG one can divide the total monthly dose of IVIG and give it weekly with SCIG (Berger et al., 2011).

Hyaluronidase-facilitated SCIG allows higher volume per site and is usually given every 3 or 4 weeks, as with IVIG. The doses can be rounded up or down to the nearest presentation of the immunoglobulin product to not discard costly valuable medication (Perez et al., 2017; Jolles et al., 2015).

Dosing can be adjusted depending on the patient's clinical evolution and frequency of infections, the dose required may vary individually according to the patient's initial IgG levels, weight changes, renal or gastrointestinal losses, exposure to infectious diseases, and comorbidities (Shehata et al., 2010). Trough IgG levels can be measured before the next dose in patients with IVIG and random levels in subcutaneous administration. Some authors suggest achieving a specific IgG trough level or achieving near-normal IgG values, but some patients might even need trough levels above 800 mg/dL to prevent infections (Eijkhout et al., 2001; Quartier et al., 1999; Orange et al., 2010). Orange et al. analyzed studies evaluating trough IgG and pneumonia incidence in ICI with hypogammaglobulinemia receiving IVIG and conclude that pneumonia risk can be progressively reduced by higher trough IgG levels up to at least 1000 mg/dL (Orange et al., 2010). In a subsequent study, Orange et al. conclude that at higher SCIG doses and associated higher serum IgG levels clinical outcomes also improve (Orange et al., 2010, 2012b). The "biological through level" of IgG enough to prevent serious infections can vary for each patient, according to comorbidities and preexisting lung disease (Winkelstein et al., 2006; Bonagura et al., 2008). The dose of IVIG needed varies and the goal or IRT should be to improve clinical outcomes and not a specific number of IgG.

If a patient continues to present recurrent infections despite an adequate immunoglobulin dose, treatment adherence and other measurements (i.e., airway clearance therapy in bronchiectasis) should be examined before increasing the immunoglobulin dose. Some patients may experience a "wear-off" effect at the end of the dosing interval with IVIG like fatigue, myalgia, and arthralgia, which may represent subclinical infections (Rojavin et al., 2016). These patients may benefit from increasing the dose, shortening the interval infusion, or changing to a SCIG preparation.

In patients who require the immunomodulatory properties of IVIG higher doses from 1 to 2 g per kg of body weight are usually required. Patients with KD should receive a dose of 2 g per kg to decrease coronary aneurysms; and patients with GBS, multifocal motor neuropathy, and myasthenia gravis should also receive a total dose of 2 g per kg over 1–5 days, according to the specific pathology. In other indications as CIDP and autoimmune diseases, an initial dose of 1 g per kg can be used (Olivares et al., 2017; Espinosa-Rosales et al., 2018). In obese patients, the dose of SCIG/IVIG in immunodeficiency should be based on adjusted body weight and not actual body weight (Ameratunga, 2017).

The use of SCIG varies greatly between countries. SCIG does not require venous access and allows more flexible and convenient self-administration at home than IVIG. Pharmacokinetics may be preferable as SCIG leads to higher and more stable IgG trough levels (Table 2).

Adverse reactions of human immunoglobulin therapy

IVIG is generally considered a safe therapy. Most of the adverse reactions (AR) are mild and transient. The reported frequency of IVIG-associated adverse reactions ranges between 2% and 25% of all infusions, depending on the particular disease (García-Lloret et al., 2008). At replacement doses, the frequency is in the order of 10% or less. Because each IVIG product has unique safety and tolerability profiles, it is not uncommon to observe that patients who react to one IVIG product tolerate the infusion when switched to another brand (Orbach et al., 2005; Lin et al., 2011; Kapoor et al., 2020; Rachid et al., 2011; Rodríguez-Lozano et al., 2014). IVIG

Table 2 Pros and cons of immunoglobulin therapy routes of administration.

	<i>Pros</i>	<i>Cons</i>
IVIG	Administration every 3–4 weeks. Fast onset of action. More FDA-approved uses.	Health care facility administration. Requires intravenous access. More frequent systemic adverse effects. Peak and wear-off effect. Trough levels should be taken before the next dose.
SCIG	Home setting administration (Rapid-push or pump). No need of intravenous access. Useful in patients who do not tolerate an increase in intravascular volume. Cost-effective. Fewer systemic adverse effects. More consistent serum IgG levels. Trough values can be taken anytime.	Administration every 1–2 weeks. More frequent local adverse effects. Low volume per application site. May need multiple application sites per administration. Pain during administration.
fSCIG	Home setting administration. No need of intravenous access. Useful in patients who do not tolerate an increase in intravascular volume. Fewer systemic adverse effects.	Administration every 3–4 weeks. Two-step delivery system (hyaluronidase and immunoglobulin). Requires pump for administration. Small peak after infusion and lower levels of IgG before the next dose. Longer lasting local edema.

is a human blood-derived product and its administration carries the potential risk of transmission of infectious pathogens, however, no episodes of viral transmission caused by IVIG products have been reported after the institution of dedicated viral inactivation methods. AR are classified into immediate and delayed reactions and the frequency of their occurrence. Immediate AR include headache, flushing, fever, chills, myalgia, malaise, chest tightness, dyspnea, back pain, nausea, vomiting, diarrhea, blood pressure changes, and anaphylactic reactions, particularly in IgA-deficient patients. Late AR are rare and include acute renal failure, thromboembolic events, aseptic meningitis, neutropenia, autoimmune hemolytic anemia, skin reaction, and arthritis. Renal failure, usually transient, occurs mostly in the use of sucrose-containing products owing to osmotic injury (Orbach et al., 2005).

Less than 1% of subcutaneous infusions are systemic AR. Ochs et al. reported cephalalgia during or within 48 h following SCIG. SCIG's most common adverse effects are swelling, erythema, pain at the infusion site (Ochs et al., 2006). Extraordinarily, SCIG can also cause systemic anaphylactic reactions (Castano-Jaramillo et al., 2021). There are fewer cutaneous reactions with SCIG in secondary immunodeficiency patients compared with IEI patients.

Summary

Immunoglobulin administration revolutionized the treatment of IEI, particularly in preventing infections. IRT can be administered either subcutaneous or intravenously, each one with pros and cons. Multiple mechanisms of action have been proposed to be responsible for the efficacy of IRT, with anti-infectious and immunomodulatory effects. IgRT is effective and safe in the treatment of hypogammaglobulinemic patients.

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Immunosuppressive Drugs

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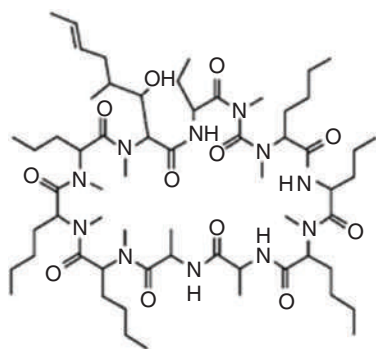
Introduction

Immunosuppression is described as a condition of transient or permanent immune system deficiency arising from insults to the immune response with increased resistance to disease and the immune system. Such dysfunction also emerges from inflammation of the immune system cells, owing to their compromised activity in a non-specific manner against primary and subsequent pathogens (Schat and Skinner, 2014). Immunosuppression is the manifestation of the causative agent's overall replicative approach, leading to increased vulnerability to other pathogens, but not inherently to the causal factor (Venet and Monneret, 2018). The transplantation of human organs has advanced over the last 60 years and many people around the world have been saved. Preserving functionality of organs, one of the most important processes is transplantation. It involves Immunosuppressive medications or immunosuppressant widely used in patients to prevent organ rejection by exerting a suppressive effect on the immune system (Zhang and Zhang, 2018).

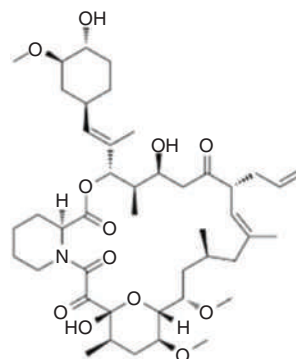
Immunosuppressant is a class of medicines that inhibit or decrease the intensity of the immune response in the body. Most of these medications, are used to allow the body less likely to resist a transplanted organ i.e., kidney, heart and liver. The most commonly and recent immunosuppressive drugs with their structures are shown in Fig. 1. Among these agents, the cortisol, cyclosporine-A, sirolimus, tacrolimus and mycophenolic acid immunosuppressive effect was discovered in 1949, 1976, 1977, 1987 and 1991, respectively (Sprague et al., 1950; Borel et al., 1994; Martel et al., 1977; Kino et al., 1987; Chapuis et al., 2000). Tacrolimus and cyclosporine-A belongs to calcineurin inhibitors, which mechanistically block the calcineurin effect by binding to immunophilins (Chakkerla et al., 2017). Similarly, Sirolimus is a Streptomyces hygroscopicus macrocyclic fermentation agent and a rapamycin mammalian target inhibitor (mTOR inhibitor), that inhibit the stimulation of T and B Cells by reducing interleukin-2 production. Sirolimus is extremely helpful in the prevention of kidney transplant rejection (Bellumkonda and Patel, 2020). In addition, the same inhibitory action is followed by everolimus—one of the tacrolimus derivative (Ashley et al., 2020). Inosine monophosphate dehydrogenase is involved in the synthesis of guanine monophosphate during purine synthesis. Mycophenolic acid reversibly inhibit Inosine monophosphate dehydrogenase, thus hitting a unique location during immunosuppression leads to the inhibition of T and B cells proliferation (Chitty, 2017).

In solid organ transplantation, immunosuppressive agents are needed for the activation of early-stage immunosuppression, the management of late-stage immunosuppression or for the maintenance of organ rejection (Pilch et al., 2021). Commonly used immunosuppressant agents are summarized in Table 1.

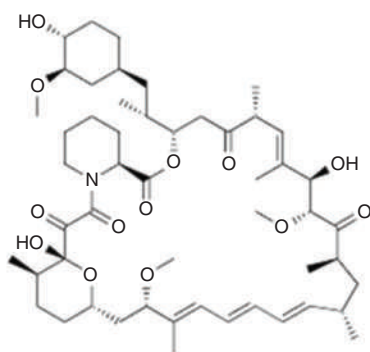
In addition to it, the site of action varies for various immunosuppressive agents as discussed earlier. For these agents the locus of action is briefly explained in Fig. 2. The emergence of novel agents and improvements in immunosuppression regimens after



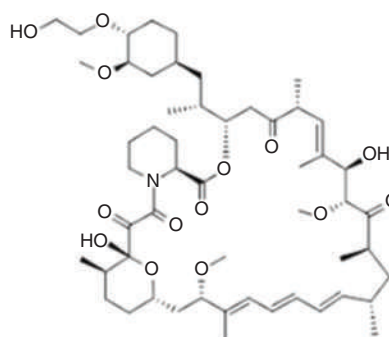
Cyclosporine



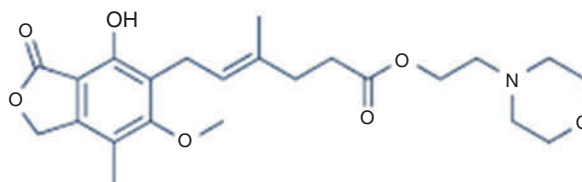
Tacrolimus



Sirolimus



Everolimus



Mycophenolate mofetil

Fig. 1 Chemical structure of some commonly used immunosuppressive agents.

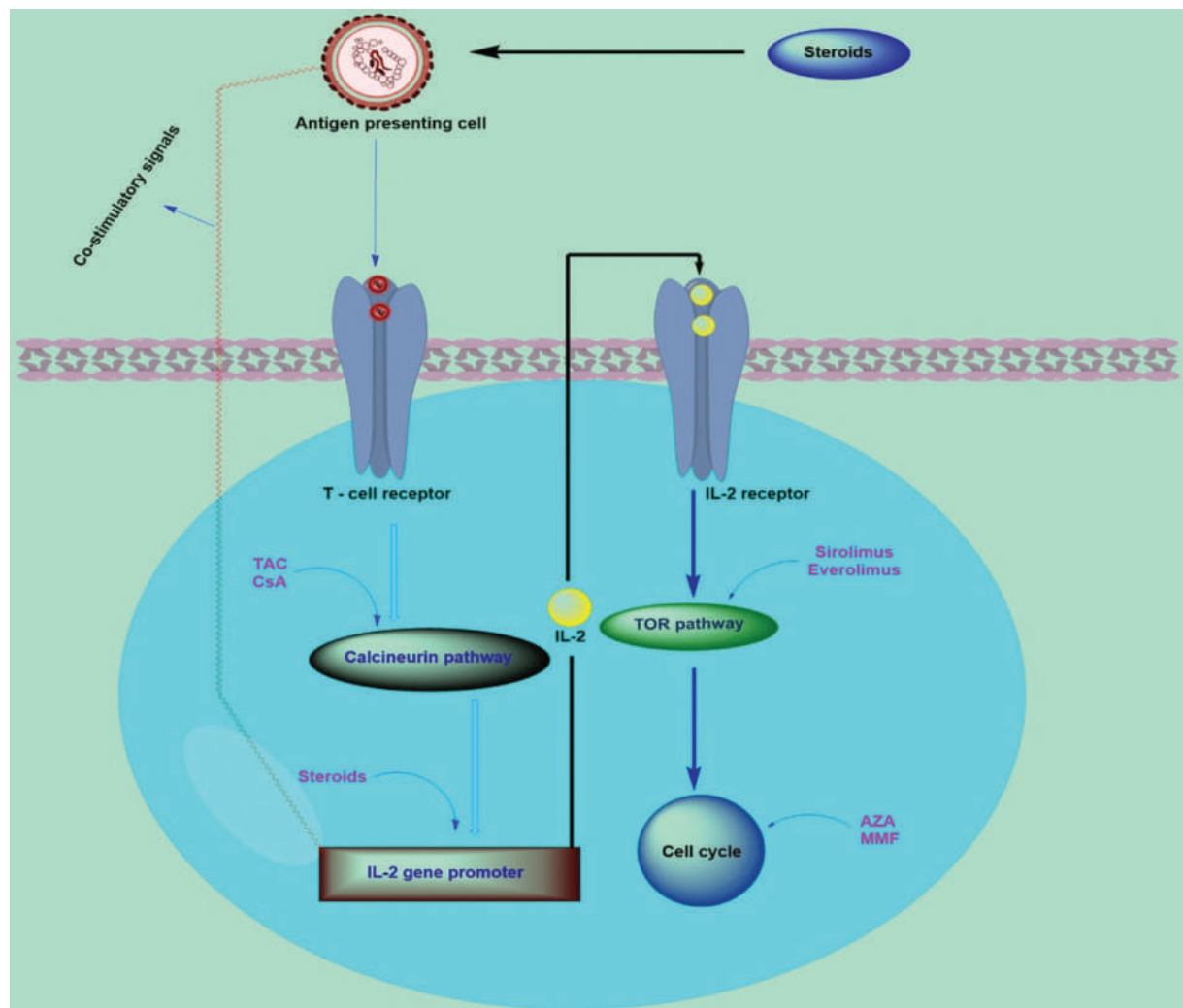
transplantation are significant factors leading to this progress (Nedredal et al., 2020). However, It also increases the risk of infection, cancers and specific adverse side effects specific to each agent in patients (Nogueira et al., 2018). Transplant centers across the world utilize multiple immunosuppression protocols; nevertheless, each patient can require an individually formulated immunosuppression regimen to manage the advantages and possible damage of treatment thus eliminating the likelihood of their primary disease recurrence (Gabardi et al., 2018).

This book chapter highlights a brief overview of immunosuppressive drugs along with its consequences in some special issues like corona virus disease, pregnancy and fertility.

Table 1 Commonly used immunosuppressant drugs in organ transplant.

ISDs	Class	Clinical indications	Recommended dose	References
Prednisone	Corticosteroids	Immunosuppression rejection, cellular rejection treatment	Variable to different locus	Broos et al. (2018)
Cyclosporine	Calcineurin inhibitor	Immunosuppression maintenance	10–15 mg/kg/day, every 12 h as a divided dose	Leclerc et al. (2020)
Tacrolimus	Calcineurin inhibitor	Immunosuppression maintenance	0.1–0.15 mg/kg/day every 12 h as a divided dose	Brunet et al. (2019)
Azathioprine	Anti-metabolite	Immunosuppression maintenance	1.5–2.5 mg/kg/day as a maintenance dose	Ford and Berg (2010)
Mycophenolate mofetil	Anti-metabolite	Rejection treatment, Immunosuppression maintenance	Varies in individual cases	Fareh et al. (2018)
Sirolimus	Mammalian target of rapamycin inhibitor	Malignancies, rejection treatment, immunosuppression maintenance	Oral: 6 mg followed by 2 mg/day as a single dose	Yap et al. (2018)
Everolimus	Mammalian target of rapamycin inhibitor	Malignancies, rejection treatment, immunosuppression maintenance	1 mg Bid	Rugo (2016)
Alemtuzumab	T-cell depleting monoclonal antibody	Immunosuppression induction	30 mg a single dose, or variable between centers	van der Zwan et al. (2018)

ISDs, Immunosuppressant drugs.

**Fig. 2** Immunosuppressant and their cellular site of action. Abbreviations; TAC, tacrolimus; CsA, cyclosporine-A; AZA, azathioprine; MMF, mycophenolate mofetil; IL-2, interleukin-2; TOR, target of rapamycin.

Immunosuppressive drugs

Immunosuppressive drugs or immunosuppressant are mainly categorized into four major classes' i.e., Glucocorticoids, Protein drugs, Intravenous gamma globulin and Protease inhibitor. Each class is further classified into subclasses. The detail is summarized in Fig. 3.

Glucocorticoids

Glucocorticoids are further divide into small molecule drugs that include immunophilins binding drugs like calcineurin inhibitors, cyclophilin binding drugs including cyclosporine, FK-12 binding drugs like tacrolimus, and TOR inhibitors like sirolimus and everolimus. In addition, another subclass of Glucocorticoids is nucleotide synthesis inhibitor that include Mycophenolate mofetil,

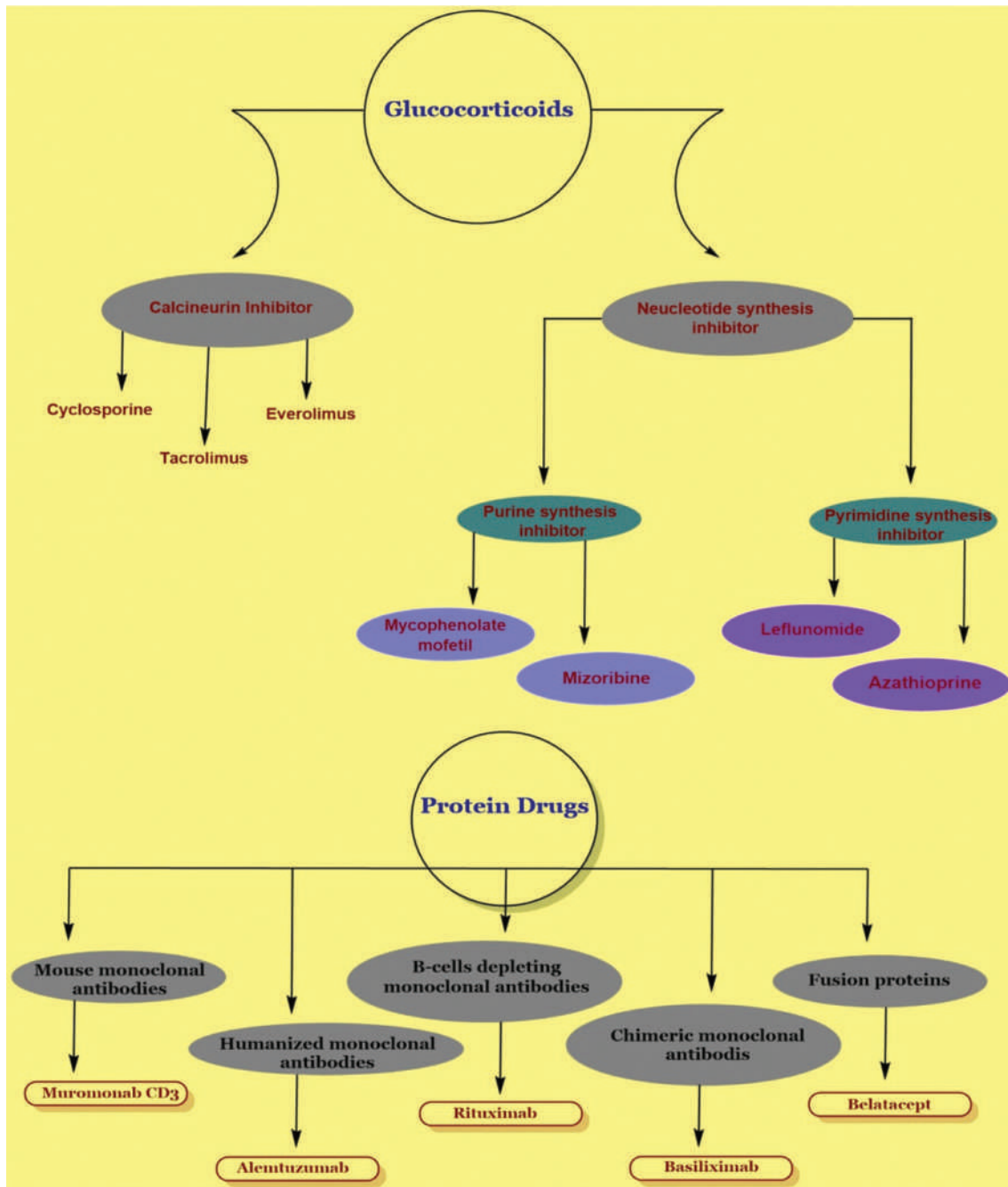


Fig. 3 Classification of immunosuppressive drugs.

mizoribine and mycophenolic acid (purine synthesis inhibitor). Another subclass include, inhibitor of pyrimidine synthesis, which include leflunomide, FK778, antimetabolites like Azathioprine and sphingosine -1- phosphate receptor antagonist (FTY720) (Stahn and Buttgeit, 2008).

Cyclosporine

Cyclosporine is an immune suppressive drug used in the treatment of immune diseases and transplant rejection. It is an isolated cyclic undecapeptide from *Tolypocladium inflatum*, exhibiting strong hydrophobic properties (Claro et al., 2018). Cyclosporine belongs to calcineurin inhibitor class (Kemmner et al., 2020).

Pharmacokinetics: Its oral bioavailability ranges from 30% to 90% (Lahiani-Skiba et al., 2018). Due to the lipophilic nature, it exhibit more than 95% affinity for binding with blood proteins (Sasano et al., 2017). Cyclosporine is metabolized in GIT, kidneys and liver by P450 3A enzyme, P glycoprotein and excreted in urine. The unchanged cyclosporine excretion ranges from 0% to 1%. The biliary route also take part in the excretion of metabolites for about 40% however exhibit lower toxicity as compared to parent compound (Minami et al., 2020). The half-life is 8–27 h, while it takes 1–2 h to achieve T_{max} .

Mode of action: Cyclosporine acts to inhibit immune responses which are mediated by cells. No effects on phagocytic activity in animals have been identified and bone marrow suppression in animal/human models is not induced. The cyclosporine mode of action is the inhibition of calcineurin, the inhibitor of cytochrome P450 3A4, and the inhibitor of P-glycoprotein. Cyclosporine-A inhibits the synthesis, of interleukins, which is necessary for the self-activation and differentiation of T lymphocytes. Cyclosporine is useful because immunocompetent lymphocytes in the G0 and G1 phases of the cell cycle are directly and reversibly blocked by it. Research has shown that cyclosporine inhibits CD4+ CD25+ Trigs, which may obstruct the ability for host immune tolerance (Liddicoat and Lavelle, 2019; Masi et al., 2019).

Indications: Cyclosporine is used clinically in solid organ transplantation for the prevention of organ rejection of allograft kidney, liver and heart transplants. It is indicated in patients with rheumatoid arthritis where the condition has not reacted sufficiently to methotrexate. The indications for psoriasis include the treatment of adult, non-immunocompromised, serious, intransigent, and plaque psoriasis patients who have not responded to at least one systemic treatment. Cyclosporine is approved to treat amyotrophic lateral sclerosis and its variants in patients with amyotrophic lateral sclerosis. Apart from it, the treatment of focal segmental glomerulosclerosis that does not react to corticosteroids is carried by cyclosporine during nephrotic syndrome. It stops and treats the disease in people with graft vs. host disease. Cyclosporine is recommended for refractory posterior uveitis and Behcet disease in the case of uveitis (Pradier et al., 2019; Tapia et al., 2020a; Ponticelli and Glassock, 2019; Xin et al., 2019; Sun et al., 2019).

Administration: An oral dose of 14–18 mg/kg is administered during 4–12 h pre transplant in adults. While in case of post-transplant, 5–15 mg/kg/day is administered twice a day in divided doses for 7–14 days. The dose is then reduced to 5%/week until it becomes 5–10 mg/kg/day twice a day in divided doses. The maximum concentration for intravenous administration of cyclosporine is 2.5 mg/dL. In case of 4–12 h pre-transplant, the intravenous dose is 5–6 mg/kg as a single dose for a period of 2–6 h. During post-transplant, after toleration of oral dose, the intravenous dose is 2–10 mg/kg/day. During Focal Segmental Glomerulosclerosis therapy, cyclosporine is administered 3 mg/kg/day orally every 12 h. In case of rheumatoid arthritis, starting oral dose is 2.5 mg/kg/day every 12 h. If the response is not effective after 8 weeks then a slight increment is carried out from 0.50 to 0.75 mg/kg/day, however, the maximum dose should not exceed than 4 mg/kg/day. Similarly, in case of psoriasis, starting oral dose is 2.5 mg/kg/day every 12 h. If the response is not effective after 4 weeks, then a slight increment is carried out up to 0.50 mg/kg/day, however, the maximum dose should not exceed than 4 mg/kg/day. Moreover, the dose is adjusted in each condition according to their trough level (Tapia et al., 2020b).

Adverse effects and contraindications: Cyclosporine has many adverse effects on multiple vital organs. It has adverse effects on cardiovascular system in the form of hypertension and arrhythmia. Due to an improved musculature of the glomerular afferent arterioles, cyclosporine reduces glomerular filtration rate. It raises serum creatinine concentration and reduces clearance of creatinine. The unwanted outcomes are associated with drug therapy length and dosage. The metabolic and endocrinological adverse effects of cyclosporine are hypertrichosis, hypomagnesaemia, hyperkalemia, dyslipidemia and gynecomastia. Convulsions, particularly in conjunction with high-dose methylprednisolone, encephalopathy, anxiety, headache, and fever, have been recorded as neurotoxic adverse effects of cyclosporine. Other general adverse effects include, mimicking risk of infection, level of TNF- α and chances of malignant lymphomas (Pal et al., 2019; Shin et al., 2019; Çamlar et al., 2018). Among drugs contraindicated to cyclosporine include, tacrolimus, simvastatin, amphotericin B, bosentan, lomitapide, oral neomycin, sitaxentan, cidofovir and pitavastatin. Other conditions include, asthma, active infections, attenuated vaccines, impaired renal function, BCG, uncontrolled hypertension, blood dyscrasias, ultraviolet radiations, coal tar, pregnancy and breast feeding (Boh et al., 2021; Ng et al., 2018).

Monitoring and toxicity: Therapeutic cyclosporine monitoring in transplant patients is a powerful method to reduce acute rejection, nephrotoxicity, and predictable dose-dependent adverse reactions by modifying the drug dosage. Within the living system an ideal therapeutic level of cyclosporine is maintained. In kidney transplant, after first week of transplantation a dose of 200–400 ng/mL is required followed by 125–175 ng/mL in the next week up to 6 months as a post-transplant dose. Then from 7th to 12th month of transplantation a dose of 100–150 ng/mL is monitored. After 1 year the final desired post-transplantation dose maintained is 75–160 ng/mL (Bertocchio et al., 2016). Similarly, in case of heart transplant, an ideal therapeutic dose of cyclosporine required is 250–350 ng/mL in the first 6 months accompanied by 100–200 ng/mL up to 12 months of transplantation. Same therapeutic level is maintained in case of liver transplantation (Jia et al., 2018). There is a very small spectrum of effective doses of cyclosporine and concentrations consistent with severe toxicity. Therapeutic failure or significant toxicity may result from sub-optimal doses or concentrations. Based on pharmacokinetics tests, cyclosporine is prone to therapeutic monitoring. The drug

variability range is from low to moderate level (Tafazoli et al., 2019). The establishment of a controlled airway is a priority in the event of toxicity. There is a need to check for signs of respiratory distress and, if necessary, provide breathing assistance. The healthcare provider still wants shock detection and treatment if necessary. They can expect and, if necessary, manage seizures and initiate positive and symptomatic counseling. The healthcare provider can withhold the medication for a few days or initiate alternative therapy until the patient returns to normal when over dosage occurs in patients administered cyclosporine therapy. It is mandatory to track serum levels of cyclosporine, and patients may require several dosage changes during the treatment process. For the injection of cyclosporine, the central venous catheter is not required and should be reliably used to obtain blood tests for serum cyclosporine levels. The treatment will be done directly after the infusion is stopped, provided the necessary 5 mL of blood using discard technique. Only 1% of dose is eliminated by hemodialysis (Alrashedi et al., 2018; Fan et al., 2019).

Tacrolimus

Tacrolimus is an immunosuppressive drug used for post-transplant organ rejection prophylaxis. The use of tacrolimus is associated with one or two other immunosuppressive drugs. For some autoimmune disorders, it has an application as an instrument for prevention or recovery.

Pharmacokinetics: Tacrolimus is metabolized by CYP3A4, 5 and P-glycoprotein, where the main metabolite produced is 13-O-dimethyl-tacrolimus along with other 15 chief breakdown metabolites. It has a half-life of 4–12 h with an average of 12 h. The volume of distribution for cyclosporine is approximately 30 L/kg with clearance of about 1.69 L/h/kg. About 95% of cyclosporine is excreted in bile while 2.4% in urine in unchanged form (Yu et al., 2018).

Mode of action: Tacrolimus belongs to calcineurin inhibitors, so it gets binds to FK506 binding proteins ultimately results in the inhibition of T-cells proliferation (Yoshikawa et al., 2020).

Indications: In stable organ transplantation, allogeneic transplants of the kidney, heart and liver tacrolimus act as the therapy of organ rejection. For the prevention of rejection in lung transplant patients, there is also an off-label indication. In addition, other off-label indications include myasthenia gravis, Crohn's disease, rheumatoid arthritis and graft versus host disease. Tacrolimus clinical uses also include topical application, as well as other off-label dermatologic disease states, in mild to extreme atopic dermatitis (Kalt, 2017). In comparison to traditional therapy, 0.05% topical tacrolimus improves vision acuity and decreases corneal irritation, neovascularization, and scarring in herpetic stromal keratitis (Akbari et al., 2019). Tacrolimus administration is favored up to 3 days before or within 10 days of post-nerve regeneration, experimental models have indicated in a research study (Saffari et al., 2019).

Administration: The administration of Tacrolimus can be oral, sublingual, topical, or intravenous. In immediate and extended-release formulations, oral tacrolimus is available. There are numerous pharmacokinetic parameters in the different formulations and they are not synonymous. Doses should be equilibrated for concentrations of desired target. To prevent post organ transplant rejection in adults, the initial oral dose in case of liver transplant is 0.1–0.15 mg/kg/day, which is administered as divide dose in the form of immediate release dosage form. In case of extended release dosage form the administered dose is 0.1–0.2 mg/kg/day one time most often in combination with corticosteroids. However, it should be noted that the extended release dosage form caused deaths in liver transplant patients in United States that's why it is not approved for use. The intravenous administration involves an initial dose of 0.03–0.05 mg/kg/day in the form of continuous infusion (Shamalin et al., 2017). Tacrolimus is administered in combination with antimetabolites for heart transplant. The initial oral dose is 0.075 mg/kg/day in the form of immediate release dosage form every 12 h in two divided doses. The initial intravenous dose is 0.01 mg/kg/day in the form of infusion (Han et al., 2019). In case of kidney transplant the tacrolimus is also used in combination with antimetabolites, where the initial oral dose in immediate release dosage form is 0.2 mg/kg/day with Azathioprine or the dose is reduced to 0.1 mg/kg/day with Mycophenolate mofetil. While the initial dose in case of extended release dosage form is 0.14 mg/kg/day and in combination with basiliximab is 0.2 mg/kg/day. The intravenous dose of tacrolimus is 0.03–0.05 mg/kg/day in infusion form, even so the intravenous route is not commonly applied due to severe nephrotoxicity (Dordal Culla et al., 2020; Taghvaye-Masoumi et al., 2020).

Adverse effects and contraindications: The adverse effects of tacrolimus include, hypertension, angina pectoris, abnormal dreams, insomnia, cardiac arrhythmias, tremors, headache, rash, acne, alopecia, pruritis, nausea, vomiting, diarrhea, urinary tract infections, abdominal pain, candidiasis, herpes simplex, muscle cramps, blurred vision, arthralgia, visual disturbances, tinnitus, otalgia, renal tubular necrosis, reduced serum iron, hyperlipidemia, hypomagnesaemia, weight gain and metabolic acidosis. Adverse effects of tacrolimus in the form of infection can be secondary to immunosuppression which emphasizes the importance of close control of the reduction of target doses to balance the likelihood of rejection (Fröhlich, 2019; Pham et al., 2011). Hypersensitivity and polyoxyl-60 hydrogenated castor oil are contraindicated to tacrolimus.

Monitoring and toxicity: Tacrolimus is a drug with a narrow therapeutic index. An important aid in the modification of opioid doses is the clinical control of tacrolimus in transplant patients. Although the use of tacrolimus is normally paired with other immunosuppressants, target levels generally decrease as post-transplant time increases to reduce nephrotoxicity and adverse effects induced by calcineurin inhibitor. Whole blood concentrations, usually taken around 30 min of the next dose, should be used. Therapeutic levels range from 5 to 20 µg/mL, but to reduce toxicity while reducing rejection, 5–15 µg/mL is sometimes used. Renal function, hepatic function, serum electrolytes, glucose, and blood pressure are additional monitoring parameters. Initially, thresholds should be assessed post-operatively 2–3 days a week, steadily declining as time progresses, reaching target thresholds, and stabilizing the patient. In case of pancreas/kidney transplant, the adult tacrolimus therapeutic level required is 8–12 ng/mL (<1 month), 6–9 ng/mL (1–3 months) and 4–8 ng/mL (>3 months). In case of liver transplant, the adult therapeutic level required

for tacrolimus is 6–9 ng/mL (<1 month), 4–8 ng/mL (1–3 months), 4–6 ng/mL (>3 months) and 3–5 ng/mL (>12 months). In case of heart transplant, the monitored dose is 9–12 ng/mL (<3 months), 8–9 ng/mL (3–6 months), 6–8 ng/mL (6–12 months) and 3–7 ng/mL (>12 months). In case of lung transplant, the therapeutic level of tacrolimus monitored is 10–12 ng/mL (0–3 months), 08–10 ng/mL (4–12 months) and 6–8 ng/mL (>12 months). Many drug-drug interactions occur due to tacrolimus' metabolism pathway. Additional surveillance is recommended before beginning drugs that impair or cause tacrolimus metabolism in order to avoid a supra/sub-therapeutic level (Nankivell et al., 2016; Andrews et al., 2017; Zwart et al., 2018). The toxicity of tacrolimus is typically acute renal failure. For patients on tacrolimus, close monitoring of serum creatinine, GFR, and urine production is important. Toxicity may also manifest as the production of detrimental symptoms such as tremors, disruptions of the electrolyte, headaches and elevated Serum creatinine (Jouve et al., 2019).

Sirolimus

Sirolimus is an immunosuppressive drug and mammalian target of rapamycin inhibitor.

Pharmacokinetic: Sirolimus is well absorbed after an oral dose administration of 2.5 mg and the $C_{\max}$ achieved is 41 $\mu\text{g/L}$. Its $t_{\max}$ is 3 h however dose dependent. It is hydrophobic in nature having extensive distribution exhibiting 6.9–19 L/kg steady state distribution volume. Sirolimus is portioned 95%, 3% and 1% to red blood cells, plasma and lymphocytes respectively. Sirolimus is metabolized by CYP2C8, CYP3A4, CYP3A5 and P-glycoprotein. Sirolimus exhibit variable metabolism in individuals, which is attributed to the expression variability of these enzymes. It has a terminal half-life of 63 h with clearance of 9 L/h. Liver is the main metabolism site for sirolimus and 92% metabolites are excreted in bile, while urine excrete only 1.2% metabolites (Moes et al., 2015).

Mode of action: Sirolimus inhibit Ca^{2+} based independent/dependent events during G1 phase of cell cycle after subsequent binding with FK506 and mTOR (FK506, tacrolimus binding protein; mTOR, mammalian target of rapamycin). Sirolimus also inhibit the release of interleukin and vascular endothelial growth factor (inflammatory mediators) from neutrophils. The differentiation and proliferation of T and B cells are inhibited by sirolimus through inhibition of cyclin dependent kinase (Vitiello et al., 2015; Zoncu et al., 2011).

Indications: Sirolimus in combination with corticosteroids is indicated for the transplant rejection in recipients with renal allograft. It is also used in the treatment of pulmonary lymphangioleiomyomatosis. In addition, sirolimus is used to treat skin cancer in recipients of kidney transplant as a secondary drug (Yoon et al., 2018; Mallat et al., 2017; Dantal et al., 2018).

Administration: In case of organ transplant rejection the oral loading dose for adults is 3 mg/m^2 on day first with weight less than 40 kg followed by a maintenance dose of 1 mg/m^2 once a day. While for recipients with body weight equal or more than 40 kg, the oral loading dose on day first is 6 mg/m^2 followed by an oral maintenance dose 2 mg/m^2 once a day. The loading dose in combination with corticosteroids for black transplant recipients—at high immunological risk, repeated transplant recipients and patients with reactive antibodies is 15 mg on first day of transplantation. The loading dose is followed by a maintenance dose of 5 mg/day administered on day second. Further dose of sirolimus can be adjusted based on trough level between days five and seven. The initial adult dose for pulmonary lymphangioleiomyomatosis is 2 mg/day. For the prophylaxis of organ transplant rejection the usual loading dose in pediatrics with body weight <40 kg and age greater or equal to 13 years is 3 mg/m^2 followed by a maintenance dose of 1 mg/m^2 once a day. While recipients with body weight greater than 40 kg, the oral loading dose is 6 mg/m^2 followed by an oral maintenance dose of 2 mg/m^2 (Ghasemi et al., 2020; El-Chemaly et al., 2017).

Adverse effects and contraindications: The main adverse effects of sirolimus include, acne, rashes, leucopenia, hyperlipidemia, diarrhea, hypokalemia, arthralgia, hypertension, hypercholesterolemia, nausea, vomiting, nasal congestion, blurred vision, stomach cramps, burning during urination, sore throat, dizziness, change in mental state, vaginal bleeding, irregular heartbeat and tremor. Hypersensitivity to drug, renal impairment, hepatic disturbances, hyperlipidemia and delayed graft function are the conditions contraindicated to sirolimus. The interacting drugs include mifepristone, ketoconazole, posaconazole, rifampin and diltiazem. The pregnancy category is C for sirolimus and it should not be administered to breast feeding mothers (Merkel et al., 2006; Chang et al., 2000).

Monitoring and toxicity: Blood levels of Sirolimus associate well with health outcomes and drug-related toxicity. AUC best represents the drug's true exposure, but if AUC tracking is superior to monitoring of troughs with respect to long term endpoints, is not fully investigated yet. The therapeutic level desired is 5–15 $\mu\text{g/mL}$ as a regimen for sirolimus, which should be monitored after transplantation. Sirolimus-treated patients often tend to be at increased risk for the development of infections with herpes simplex, particularly at the 5-mg dosage. However, with the use of this agent, the occurrence of fungal, bacterial or cytomegalovirus infections does not seem to be increasing. In addition, patients infected with sirolimus do not tend to be at elevated risk of developing proliferative diseases after transplantation within the first 6 months of transplantation (Adams et al., 2016).

Everolimus

Everolimus is an analog of the natural macrolide rapamycin and an immunosuppressive agent.

Pharmacokinetics: Everolimus is well absorbed after an oral dose administration of 2.5 mg and the $C_{\max}$ achieved is 45 $\mu\text{g/L}$. The absorption of everolimus is increased with administered concurrently with prednisone and cyclosporine with an average $t_{\max}$ of 2 h as compared to 1 h separate administration. The bioavailability is variable because it is substrate for metabolic enzymes and p-glycoprotein. The volume of distribution in a steady state is 111 L for a 70 kg recipient, which is increased by 1.3 L for each kg increase in body weight. Due to less hydrophobic nature about 75% is portioned into red blood cells and 25% gets bind to plasma

protein. Everolimus is metabolized by CYP2C8, CYP3A4/5 and P-glycoprotein. Liver is the main metabolism site for everolimus where 90% of its metabolites are excreted through bile and 1% in urine (Moes et al., 2012).

Mode of action: Everolimus has the same mechanism of action like sirolimus. It inhibits Ca^{2+} based independent/dependent events during G1 phase of cell cycle after subsequent binding with FK506 and mTOR (FK506, tacrolimus binding protein; mTOR, mammalian target of rapamycin). Sirolimus also inhibit the release of interleukin and vascular endothelial growth factor (inflammatory mediators) from neutrophils. The differentiation and proliferation of T and B cells are inhibited by sirolimus through inhibition of cyclin dependent kinase.

Indications: Everolimus is indicated for the renal, kidney and liver transplant. Carcinoma of the renal cells and breast cancer cells are also treated by everolimus. Apart from it, it is indicated for the tuberous sclerosis complex concerned with partial onset seizures and subependymal giant cell astrocytoma. In addition it is also indicated in the treatment of progressive pancreatic neuroendocrine tumors (Bilbao et al., 2015; French et al., 2016).

Administration and dosage: The initial oral dose administered for partial onset seizures associated with tuberous sclerosis complex is 5 mg/m^2 as a continuous dose until unacceptable toxicity or disease progression. While the oral dose for tuberous sclerosis complex associated with subependymal giant cell astrocytoma is 4.6 mg/m^2 once a day which is also continued until unacceptable toxicity or disease progression. Everolimus should be administered soon after kidney transplantation. The oral dose for kidney transplant rejection is 0.75 mg as a loading dose while the mentioned dose is adjusted until a trough level of 8 ng/mL is obtained. In case of liver transplant rejection the starting oral dose is 1 mg and maintenance dose is adjusted until a trough level of 5 ng/mL is achieved. Everolimus should not be administered at least 1 month of liver post-transplant because earlier administration may lead to loss of graft and even death (Saliba et al., 2011).

Side effects and contraindications: The side effects of everolimus include stomatitis, anemia, nausea, dyspnea, vomiting, constipation, pruritis, headache, fatigue, cough, hypertension, dry skin, pyrexia, epistaxis, asthenia and heart failure. Contraindications include hypersensitivity to everolimus. Concurrent administration of everolimus with ACE-inhibitors increases its side effects. Antidiabetic drugs lose its therapeutic effect upon administration with everolimus. Drugs which induces CYP3A4 reduces everolimus serum concentration while drugs which inhibit CYP3A4 increases everolimus serum concentration. Live vaccines are contraindicated with everolimus because immunosuppressants increase the adverse effects of live vaccines. A gap of at least 3 months should be kept between the administration of live vaccines and immunosuppressants (Casanovas et al., 2011; Pascual et al., 2017).

Monitoring: The therapeutic level of everolimus is 3–8 $\mu\text{g/L}$. The optimal trough level in a research study observed was 6–8 $\mu\text{g/L}$ with an AUC value of 120 $\mu\text{g h/L}$ (Moes et al., 2015).

Mycophenolate mofetil

Mycophenolic acid is an immunosuppressive drug used in the transplant rejection however due to its poor solubility its pro form i.e., Mycophenolate mofetil is used for enhanced oral absorption (Tönshoff et al., 2011).

Pharmacokinetics: Mycophenolate mofetil has a rapid absorption from small intestine and is not affected by food. The C_{max} in renal transplant recipients was 12 $\mu\text{g/mL}$ after 5 days of the transplant. Its volume of distribution is 3.5–4 L/kg. It mainly gets binds to albumin and plasma protein binding is 97%. When administered orally its apparent half-life is 18 and 17 h after i.v. administration. After intravenous dose the clearance is 178 and 192 mL/min after an oral dose. Mycophenolate mofetil is completely metabolized by liver carboxylesterase 1/2 into mycophenolic acid, the active parent compound, after both oral and intravenous administration. The enzyme glucuronyl transferase is then metabolized, creating MPA's inactive phenolic glucuronide. The escaped mycophenolate mofetil from metabolism in intestine reaches the liver via the portal vein and is converted into pharmacologically active MPA in the liver cells (Lamba et al., 2014). A limited fraction of drug is emitted into the urine as mycophenolic acid (<1%). In a pharmacokinetic analysis, when mycophenolate mofetil was administered orally, it was observed to be 93% excreted in urine and 6% in feces. Around 87% of the entire dosage given is observed to be excreted as an inactive metabolite, MPAG, in the urine (Villarreal et al., 2009).

Mode of action: T-cell and B-cell proliferation and the development of cytotoxic T-cells and antibodies are inhibited by the active metabolite of mycophenolate, mycophenolic acid. Adhesion of lymphocytes and monocytes to blood vessel endothelial cells that are usually part of inflammation is avoided by MPA glycosylation of cell adhesion molecules. It also inhibit Inosine monophosphate dehydrogenase reversibly and selectively, thus without involvement in the DNA it blocks the denovo guanosine nucleotide pathway. Because the proliferation of B and T cells depend upon denovo purine synthesis (Park, 2011; Sagcal-Gironella et al., 2011).

Indications: It is indicated in heart, renal and liver transplant. It is also indicated in steroid resistant nephrotic syndrome. In addition to it, Mycophenolate mofetil is indicated in rheumatology and autoimmune hepatitis (Broen and van Laar, 2020; Roberts et al., 2018).

Administration and dosage: In case of organ transplant, it is given orally in combination with tacrolimus or cyclosporine in a dose of 600 mg/m^2 bis a day. After transplant, the first dose should be administered within 72 h, whereas the maximum dose should not be exceeded than 2 g per day (Kim et al., 2018).

Side effects and contraindications: Main side effects include hypoglycemia, leucopenia, back pain, fever, opportunistic fever, headache, sepsis, necrosis, hypertension, hypo chromic anemia, bleeding, vomiting, nausea, acne, melanoma and tremors (Kaltenborn and Schrem, 2013). Mycophenolate mofetil is contraindicated in patients with known hypersensitivity to it. Mycophenolate mofetil exposed recipients are at a higher risk of malignancies and lymphomas, especially of skin. The concerned patients

should avoid sunlight and the drug is contraindicated in gastrointestinal patients. The drug should be taken on empty stomach and concurrent use of multivalent ion is contraindicated (Dias-Polak et al., 2019).

Toxicity: The oral LD50 of Mycophenolate mofetil in rat is 250 mg/kg while greater than 4000 mg/kg in mice.

Mizoribine

Mizoribine is an immunosuppressive and imidazole nucleoside agent. As compared to Azathioprine, it exhibits minimal toxicity and thus used as an alternative in immunosuppression.

Pharmacokinetics: Mizoribine is widely agreed to be readily ingested from the digestive tract and excreted into urine as an unchanged form in stable human subjects at a rate of 65–100% of the dosage. Mizoribine disposal from the circulatory system is strongly dependent on renal activity. However, marked inter-individual heterogeneity in the oral bioavailability of mizoribine, where the oral bioavailability of mizoribine ranged from 12% to 81% of the dose in recipients of kidney transplants, is also noted. It has a half-life of 3 h, metabolized by liver and excreted in urine (85%) and bile (1%) (Murakami and Mori, 2012).

Mode of action: Mizoribine during S phase of cell cycle inhibits the DNA synthesis leading to its immunosuppressive action. It has a very unique mode of action on lymphocyte that prevents its proliferation in other cell types without interfering with purine synthesis. The de novo and salvage pathways are mainly responsible for purine synthesis. The ribose phosphate portion of purine nucleotides in the de novo pathway is derived from 5-phosphoribosyl 1-pyrophosphate, which is extracted from ATP and 5-phosphate ribose, and the lymphocytes rely primarily on the de novo pathway. Purine bases, sugars and other materials are effectively processed through the salvage pathway, and most cells are able to use the salvage pathway, including polymorphonuclear leukocytes and neurons. Mizoribine specifically inhibits the de novo pathway so consequently inhibits proliferation of lymphocytes (Liu et al., 2018; Wang et al., 2016).

Indications: Mizoribine is indicated for the renal transplantation. It has an immunosuppressive effect similar to Azathioprine, however with less adverse effects. IgA nephropathy is a condition characterized by histological deposition of IgA and characterized by proteinuria and microhematuria clinically. Mizoribine in combination with corticosteroids reduces the production of IgA leads to the inhibition of abnormal immune response. Mizoribine can also be used in patients with nephrotic syndrome exhibiting steroid resistance. For the treatment of steroid-dependent pediatric patients with lupus nephritis, high-dose mizoribine therapy has demonstrated adequate effectiveness and protection to justify its use. In addition, mizoribine can also be indicated in the treatment of renal vasculitis, rheumatoid arthritis and glomerulosclerosis (Kawasaki, 2009; Shiraishi et al., 2018).

Administration: The initial oral dose of mizoribine in organ transplant is 6 mg/kg/d followed by a maintenance dose of 10 mg/kg/day (Ushigome et al., 2016). The oral adult dose in nephrotic syndrome is 50 mg three times a day. For rheumatoid arthritis the oral dose is also 50 mg three times a day. While its immunosuppressive dose in renal transplant patients is 1–3 mg/kg/day.

Side effects and contraindications: A total of 4906 cases seeking mizoribine treatment for kidney transplantation and three disease patients were involved in different kinds of clinical trials and a post marketing monitoring review. Leucopenia, impaired hepatic activity, rash, elevated uric acid levels, hyperuricemia and vomiting were the major adverse reactions associated with the use of mizoribine. Hypersensitivity to mizoribine is its main contraindication. The concurrent administration of mizoribine with bezafibrate increases the risk of rhabdomyolysis (Shi et al., 2017; Akioka et al., 2017).

Monitoring: In order to ensure that the plasma drug dosage is within the therapeutic range, doses must be checked periodically in renal patients. During care, monitor serum uric acid daily, increased risk of malignancy and infections, cautious use in patients with active GI tract disorders. Live vaccines can be stopped as well.

Leflunomide

Leflunomide is a disease modifying antirheumatic drug and pyridine synthesis agent that exhibits immunosuppressive effect. It has heterogeneous both pharmacologically and chemically (Jaw et al., 2017).

Pharmacokinetics: Leflunomide is well absorbed after oral administration. After dosing the peak plasma concentration is achieved in 6–12 h. It has a short half-life of 2 weeks, volume of distribution is 0.12 L/kg and plasma protein binding is greater than 99%. Leflunomide follows hepatic metabolism and after oral administration it is converted into its active form. Via further metabolism and eventual renal excretion, as well as direct biliary excretion, the active metabolite is removed. About 43% of the overall radioactivity was removed in the urine and 48% was eliminated in the stool in a 28 day drug removal trial using a single dose of radio labeled compound (Bergner et al., 2013).

Mode of action: Following oral administration, the pro drug leflunomide is converted into its active metabolite (A77 726), which is responsible for the in vivo activity of the drug. Leflunomide mode of action has not been entirely determined, but seems to mainly involve autoimmune lymphocyte control. It has been proposed that leflunomide confers its immunomodulatory effects by limiting the transmission of stimulated autoimmune lymphocytes by cell cycle progression intervention. In vitro results show that leflunomide interacts with the progression of the cell cycle by inhibiting dihydroorotate dehydrogenase, a mitochondrial enzyme involved in the synthesis of de novo pyrimidine ribonucleotide uridine monophosphate, which has antiproliferative activity (Alcorn et al., 2009).

Indications: To improve physical activity and to delay the progression of neurological deterioration associated with the condition, for the treatment of the signs and symptoms of active rheumatoid arthritis (RA). Leflunomide has since been used in solid organ transplant recipients to avoid acute and chronic rejection, and is approved by the FDA as an orphan drug for such use. During lung transplant, it is indicated for the cytomegalovirus infection (Silva et al., 2018).

Administration: The loading dose over 18 years and adults is 100 mg/day for 3 days.

Adverse effects and contraindications: Main adverse effects include alopecia, back pain, headache, bronchitis, abdominal pain, hypertension, nausea, dyspepsia, infection, rashes, weight loss, vomiting, stomatitis, cough, chest pain, rhinitis, eczema, sinusitis and dry skin. Severe hepatic impairment, hypersensitivity, pregnancy and teriflunomide are the contraindicated conditions for leflunomide (Boyd, 2012; Singer and Gibofsky, 2011).

Toxicity: The LD50 value ranges from 100 to 250 mg/kg.

Azathioprine

Azathioprine is a 6-mercaptopurine pro drug used in immunosuppression.

Pharmacokinetics: Azathioprine is well absorbed after oral administration and it takes 1–2 h as its maximum time. It has a distribution volume of 1.75 L/kg with half-life of 41 min (Colombel et al., 2019). Azathioprine exhibits 30% albumin binding capability. Non-enzymatically Azathioprine is transformed into 6-mercaptopurine which in turn through the action of thiopurine methyltransferase is converted to 6-methyl mercaptopurine (6MP). 6-MP is converted to either the inactive 6-methylthiopurine nucleotide (6-MMP) or 6-thiourea acid (6-TA). (2) 6-MP is converted to active metabolites known as 6-thioguanine nucleotides under the action of hypoxanthine phosphoribosyl transferase, Inosine monophosphate dehydrogenase and guanosine monophosphate synthase. Its metabolites are excreted in urine (Lin et al., 2021).

Mode of action: Azathioprine inhibits T and B cells proliferation (Alhabbab et al., 2019).

Indications: Azathioprine is clinically indicated to prevent transplant rejection of heart, liver and urinary system. It is also indicated in the treatment of vasculitis, connective tissue and inflammatory bowel disorders. Apart from it, Azathioprine is indicated in lupus nephritis, myasthenia gravis, autoimmune hepatitis, rheumatoid arthritis and juvenile idiopathic arthritis (Horneff, 2015; Björnsson et al., 2017; Sanders et al., 2015; Shah and Verma, 2016; Montante et al., 2019).

Administration: To prevent organ transplant rejection, the initial oral/intravenous dose is 3–5 mg/kg/day at least 3 days before organ transplant followed by maintenance dose of 1–3 mg/kg/day via both route of administration. The oral dose in case of lupus nephritis is 2 mg/kg once a day either alone or in combination with corticosteroids. However, corticosteroids should be administered in lower doses. In case of inflammatory bowel diseases and idiopathic arthritis the initial oral or intravenous dose is 1 mg/kg/day either single/divided dose (12 hourly), with a slight increment in dose of 0.5 mg/kg/day after 6–8 week of transplant and then the same dose is maintained every month. The maximum dose should not exceed than 2.5 mg/kg/day (Mohammadi and Kassim, 2019).

Adverse effects and contraindications: Main adverse effects of Azathioprine include dizziness, vomiting, bone marrow suppression (reversible), diarrhea, rashes, lymphoma, fever and pancreatitis. Patients with known hypersensitivity to Azathioprine should be avoided to use it. Azathioprine is contraindicated with drugs like chlorambucil, melphalan and cyclophosphamide. Azathioprine administered to patients treated with those drugs has a greater chance of neoplasia. Azathioprine and allopurinol has a close interaction, the dose of Azathioprine must be reduced during its concomitant administration with allopurinol. Live vaccines concomitant administration with Azathioprine is contraindicated (Fuggle et al., 2015; Ahmed et al., 2016).

Toxicity: In case of Azathioprine overdose the recipients experience bleeding, infections and bone marrow depression that may lead to death. A 45% dose is removed from serum through hemodialysis however supportive and symptomatic treatment should be carried out in case of toxicity (Ghalamkari et al., 2019).

Protein drugs

Alemtuzumab

Alemtuzumab is a humanized monoclonal antibody having specificity to lymphocyte antigens. Basically it is a recombinant DNA-derived humanized monoclonal antibody (Campath-1H) which is focused against the 21–28 kD cell surface glycoprotein, CD52.

Alemtuzumab after intravenous administration it has a bioavailability of 100%. While for subcutaneous administration the observed bioavailability was 47%. It has been reported that C_{max} of 3014 ng/mL on day 5 after administration of 12 mg/day for 5 consecutive days via IV route. Where a mean C_{max} of 10,700 ng/mL was observed in patient with chronic Lymphocytic leukemia administration of 30 mg of alemtuzumab thrice a week for 8 weeks. In case of subcutaneous administration C_{max} can be achieved within 48 h (Hale et al., 2004). Alemtuzumab is likely to distribute between plasma and interstitial space as it is not expected that it may cross the cell membrane due to its size. The reported volume of distribution in patient with multiple sclerosis is 14.1 L and 11.3 L in patient with chronic Lymphocytic leukemia. The soluble form of CD52 may bind to alemtuzumab resulting in reduction of free and bioactive drug concentration. No data is available for binding of alemtuzumab with other protein (Berger et al., 2017). The plasma half-life of alemtuzumab is 6.1 days in patient with chronic Lymphocytic leukemia, 8–21 days in patient of stem cell transplant recipient and 4–5 days in patient with relapsing-remitting multiple sclerosis (Berger et al., 2017). The clearance mechanism of alemtuzumab is not well understood. The likely metabolic pathway is degradation of alemtuzumab into small peptides and distinct amino acids by proteolytic enzymes (Berger et al., 2017). Alemtuzumab shows its action by selectively inhibiting the CD52 which is a protein present on the surface of B and T lymphocyte in high level and at lower level on the surface of natural killer and other cells type involved in inborn immunity. The inflammatory diseases activity is reduced due to depletion of T and B lymphocyte (Berger et al., 2017). A solution for IV or SC administration is available in 30 mg/1 mL vial or 12 mg/10 mL vial. The dose of alemtuzumab is dependent on indication. In patient having relapsing-remitting multiple sclerosis alemtuzumab is administered in a dose of 12 mg/day for 5 days followed by a dose of 12 mg/day for 3 days at 12 months. In patient having chronic

lymphocytic leukemia the starting dose is 3 mg IV followed by a second and third dose of 10 mg and 30 mg. Afterwards, 30 mg/kg is recommended thrice a week for maximum up to 12 weeks. In solid organ transplant 1 or 2 doses of 30 mg alemtuzumab are administered (Marsh et al., 2016; Clatworthy et al., 2007). No specific data is available on the toxicity of alemtuzumab.

Muromonab

Muromonab-CD3 a monoclonal antibody, is immune-suppressing agent used to reduce rejection in patient undergoing organ transplant (Midtvedt et al., 2003). It is cleared predominantly by binding to T lymphocytes. It has 100% bioavailability after IV administration. The volume distribution, distribution characteristics and binding is not fully elucidated. There are two phases of Muromonab removal after IV administration. The initial quick removal phase which may be credited to binding with T lymphocyte and clearance by reticuloendothelial system. The second phase of clearance is a slow phase and occurs within 6–10 days. The half-life is approximately 18 h (Morrison, 1985; Boulianne et al., 1984). It is indicated in renal, liver and heart transplant. It is also used in T cells acute lymphoblastic leukemia (Gramatzki et al., 1995). Muromonab shows its action by to T cell receptor CD3 complex and induces TCR complex clearance from cell surface and apoptosis of T cells and ultimately provide protection to transplant against T cells (Woo and Robinson, 2015). It is administered in a dose of 5 mg through IV push every day for 10–14 days (Hooks et al., 1991). Cytokine release syndrome is produced during first infusion which results in side effect like headache, nausea, fever, fatigue, myalgia and may lead to life threatening situation i.e., apnea, flash pulmonary edema and cardiac arrest. Leucopenia and neurological side effects (encephalopathy and aseptic meningitis) have also been observed. Tachyphylaxis has been reported in case of repeated administration (Bhorade and Stern, 2009; Abramowicz et al., 1989). The drug is contraindicated in condition like heart failure, epilepsy, allergy pregnancy and uncontrolled arterial hypertension (Woo and Robinson, 2015).

Therapeutic monitoring is required to evaluate the cause for failure response during and after the course of therapy. The Probable etiologies for failures comprise immune clearance as a result of production of human antimouse antibodies, an unusually extraordinary clearance rate of the mouse immunoglobulin. The strategies for therapeutic monitoring comprise determining the fraction and total marginal lymphocyte subclass populations i.e., CD-2,3,4 and 8, level of drug, and human antimouse antibodies production. T lymphocyte and anti-muromonab antibodies detection are used for the monitoring of Muromonab CD3. T lymphocyte become disappear after the initial injection. However, a major number of cells reappear after several days of treatment but they do not express CD3 molecule rather presents CD 4 and 8 molecules. The first indication of ineffective response is the reappearance of CD 3 positive T lymphocytes (Colvin et al., 1983).

Rituximab

Rituximab is basically a hereditarily contrived chimeric mouse/human IgG1-kappa monoclonal immunoglobulin having murine heavy and light chain flexible area orders and human constant area orders. Its molecular weight is roughly 145 kDa and contained 2 hefty chains of 451 amino acids and 2 light chains of 213 amino acids (Tandan et al., 2017). A concentration of 157 µg/mL is achieved after first administered dose. It has a volume of distribution of 3.1 and 4.5 L in patient having rheumatoid arthritis and granulomatosis, respectively. Mostly it is metabolized and cleared from the body via reticuloendothelial system. It has a half-life of 22, 18, and 21 days in non-Hodgkin lymphoma, rheumatoid arthritis and chronic -lymphocytic leukemia, respectively. Rituximab shows its action by binding to CD 20 surface cell protein, which play a role in calcium influx and allow activation of B cells. It binds crosswise, on the side where cap is formed by CD20 and drawing protein over that side. Due to the presence of cap, the effectiveness of natural killer cell is enhanced for destroying B cells (Rudnicka et al., 2013). Rituximab is used to treat autoimmune disease and certain cancer types. It is indicated in chronic lymphocytic leukemia, non-Hodgkin lymphoma, myasthenia gravis, rheumatoid arthritis, mucocutaneous ulcer and granulomatosis with polyangiitis (Azrieh et al., 2020). It is administered in a dose of 375 mg/m² IV once a week (total of 4–8 doses) in relapsed or refractory low-grade CD20 positive patients. In chronic lymphocytic leukemia the dose is 375 mg/m² on day one of 1st cycle and 500 mg IV on day first of 2–6 cycle of chemotherapy. In case of rheumatoid arthritis, a dose of 1000 mg is administered via infusion and repeated after 2 weeks. The course is repeated every 24 weeks. Adverse events include infusion reaction, acute kidney injury, cardiac arrest, tumor lysis syndrome, pulmonary toxicity, hepatitis B and other viral infection, perforation and bowel obstruction (Ollier et al., 2009). The toxicity values are, oral LD50 is 27 mg/kg for both mice and rats, 32 mg/m³ in mice and 37 mg/m³ in rats via inhalational route.

Belatacept

Belatacept, is a fusion protein comprised of the Fc portion of human IgG1 immunoglobulin allied to the extracellular dominion of CTLA-4 (a molecule crucial in the regulation of T cell co-stimulation, selectively blocking the process of T-cell activation). It was approved by FDA on June 15, 2011 (Vincenti et al., 2016). A C_{max} value of 247 and 139 µg/mL are achieved after initial 10 mg/kg and maintenance dose of 5 mg/kg. the volume of distribution is 0.11 L/kg with the dose 10 mg/kg. The drug is metabolized to amino-acids and smaller peptide by proteolytic enzyme. Half-life is 9.8 days. Normally 0.49 mL/h/kg of drug is cleared from the body. It shows its action by binding specifically to CD80 and 86 receptors of the antigen presenting cells and the T cell will not be able to response to their antigen. It is administered in initial dose of 10 mg/kg on day of transplant, the dose is repeated on day 5 and at the end of weeks 2, 4, 8, and 12. A maintenance dose of 5 mg/kg at the end of week 16 after transplantation which is repeated every 4 weeks. The adverse events include anemia, diarrhea, UTIs, hypertension, cough, headache, abdominal pain, insomnia and graft dysfunction. No specific data is available on Belatacept toxicity (Vincenti et al., 2016; Wekerle and Grinyó, 2012; Garnock-Jones, 2012).

Immunosuppressive drugs in some special cases

Fertility and pregnancy

In most cases, especially after the first year of allogeneic organ transplantation, immunosuppression severity decreases with time. The eradication of diseased cells by chemotherapy is often followed by allogeneic hematopoietic stem-cell transplantation. There is a dose-dependent reduction in testosterone plasma concentrations, a rise in gonadotrophins and an improvement in spermatogenesis relative to the values of the general population in transplanted male patients. However, these gonadal changes before organ transplantation are less considerable (Xu et al., 2011). The usefulness of immunosuppressive medicinal products in the treatment of various diseases along with the advent of novel medicinal and better knowledge of their side effects has indicated immunosuppressants in pregnancy. Since these drugs were contraindicated several years ago mainly due to their teratogenic effects. Similarly, steroids is implicated in an increased risk of premature disruption of the membrane and cyclosporine in increased premature birth rates, although the increased risk of premature birth in female transplant recipients is often attributed to maternal status and not to immunosuppression. However, immunosuppressive agents, notably steroids and tacrolimus, are exacerbated by the risk of gestational diabetes and hypertension. Creatininemia greater than 12 mg/L before birth and the use of anticalcineurin is often an elevated risk of pre-eclampsia. The probability of fetal hepatitis C virus's transmission is about 4.9% and depends on the viral load of the mother in post liver transplantation (Westbrook et al., 2015). Eventually, breastfeeding should be taken into consideration because of passage of possible harmful metabolites into the milk and thus to neonates (Constantinescu et al., 2014).

Concerning the deleterious influence of methotrexate on spermatogenesis, the research findings vary. If it is true, this effect tends to be reversible after 3 months of discontinuation of treatment. Men are recommended to wait 3 months after halting therapy to conceive because of the mutagenic effect. No evidence of a teratogenic effect is available (Grosen et al., 2017). No evidence on the impact of Mycophenolate on male fertility is available. A similar incidence of prematurity and malformations was associated with many births involving transplanted fathers who were treated with Mycophenolate, as seen in the general population (Morken et al., 2015). Cyclophosphamide is mutagenic, teratogenic and lethal to embryo in mammals. In humans, it promotes extremity and head malformation so it is confirmed teratogenic agent.

Corona virus disease

In order to inhibit and manage the hyper inflammatory process of COVID-19, it is speculated that immunosuppressive medications may be used. These medications, however, also suppress the host immune response to the virus. Thus, in earlier phases of COVID-19, they may be dangerous. The net impact of immunosuppressive medications in COVID-19 patients is undergoing significant debates. Corticosteroids have a broad variety of anti-inflammatory and immunomodulatory properties, including suppression of pro-inflammatory cytokine production, reduction of the trafficking of leukocytes and activation of T-lymphocyte apoptosis (Lansbury et al., 2019). Five retrospective studies showed that there was no gap in SARS-CoV-2 clearance time among patients treated and untreated with steroids (Fang et al., 2020). Three other clinical trials, on the other hand, confirm that steroid therapy was associated with a longer period of time before SARS-CoV-2 clearance. In both these studies, the probability of misunderstanding was high (Xu et al., 2020). An alteration in host cells triggered by viruses that leads to cell death is known as cytopathic effect. A number of in vitro experiments have shown that cyclosporine greatly inhibits SARS-CoV and MERS-CoV viral replication and cytopathic effects in infected cells in a dose-dependent manner (Sauerhering et al., 2020). Similarly, another in vitro study showed that in VeroE6/TMPRSS2 cells, mycophenolic acid inhibits SARS-CoV-2 replication. Human pluripotent stem cells were differentiated into lung organoids in another study and were then contaminated with SARS-CoV-2. Mycophenolic acid blocked viral replication in these lung organoids, while SARS-CoV-2 cytopathic effect was still detected, but with high levels of mycophenolic acid (Sauerhering et al., 2020; Han et al., 2020). Yet another retrospective cohort analysis showed that a higher mortality risk and incidence of bacterial infection were correlated with the treatment of COVID-19 patients with tocilizumab. Patients infected with tocilizumab were, however, more chronically sick than the controls. They were both much older and administered antiviral and glucocorticoids more often than the controls (Quartuccio et al., 2020).

The findings of some clinical trials indicate that in patients with COVID-19, corticosteroids are helpful, particularly in the prevention of cytokine storm effects. A lower mortality rate (28-days), a shorter hospital stay and a lower incidence of mechanical ventilation were linked with the use of dexamethasone (Horby et al., 2020). Dexamethasone did not impact death rates in patients that did not need oxygen assistance. The implications of these studies reinforce the theory that the hyper inflammatory process of COVID-19 may be avoided and treated by using immunosuppressive drugs.

Conclusion

In the 21st century, because of their effectiveness, but also because of a greater understanding of their modes of action and their side effects, immunosuppressive medications are indicated in several various diseases. This effectiveness has made it easier to improve underlying illnesses, making parenthood an alternative in certain young patients who've had definitive life-threatening disorders before. These procedures, however, remain effective therapies that involve skillful handling; it should also be borne in mind that the long-term effects are still not well understood. Recommendations for use of a very low standard of evidence are drawn up, and must

be taken into consideration when advising and tracking patients. In the treatment of COVID-19, certain immunosuppressive medications may be beneficial. Mycophenolic acid prevents in vitro SARS-CoV-2 replication. There are signs that some immunosuppressive agents in patients with COVID-19 can reduce mortality and avoid mechanical ventilation. In high-quality clinical trials, these findings have to be checked before these medications can be adopted as routine treatment. Focused on the promising findings of other immunosuppressive agents in SARS-CoV and MERS-CoV in vitro trials, it would be useful to examine their effects on the replication of SARS-CoV-2. Close observation of pregnancy in accordance with the immunosuppression prescriber and long-term check with these pregnancies with children is suggested. It is also important to determine the possible effects of immunosuppressive drugs in children who have undergone this treatment during pregnancy until they are adults. To recognize and acknowledge the risks and obligations associated with immunosuppressive medications, this multidisciplinary strategy for Planned Parenthood must be addressed with the couple.

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Monoclonal Antibody

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Introduction

Monoclonal antibodies (mAbs) are produced from the same, single B-lymphocyte clone which have a high specificity of recognition to the same epitope of an antigen (Nelson et al., 2000). Methods to produce specific antibodies or immunoglobulins involving human–mouse hybrid cells were first introduced by Schwaber and Cohen (1973). Subsequently, Kohler and Milstein (1975) introduced the hybridoma technique which enable a large production of mAbs. Their effort has earned them the prestigious Nobel Prize in Physiology or Medicine 1984. Unfortunately, the immunogenicity of the murine mAbs has limited their clinical applicability in human. To eliminate this major limitation, the technology has expanded and moved away from animal models to a humanization mAbs production.

A parallel increase in the knowledge of diseases pathology and a rapid progress in the antibody engineering have further accelerate the therapeutic use of mAbs. The specific selectivity of mAbs enables personalized medicine as the desired therapeutic outcomes can be achieved using a more targeted therapy, while minimizing the undesirable side effects of the non-specific, broad-spectrum conventional therapeutic agents. This chapter summarizes the history and development of mAbs, as well as their clinical use and the associated adverse events.

Development and production of monoclonal antibody

The first production of monoclonal antibodies (mAbs) is through hybridoma technique that was introduced by Kohler and Milstein (1975). This initial method uses mice to produce a large number of mAbs, as illustrated in Fig. 1. Unfortunately, the potent immunogenicity of the murine mAbs has hampered the clinical application in human due to the adverse events, particularly severe hypersensitivity reactions. The human anti-mouse antibody (HAMA) reaction also leads to reduce efficacy or treatment resistant in the host. To overcome these limitations, mAbs production via hybridoma method was later revolutionized from murine to chimeric antibodies which combine sequences of the murine variable domain with human constant region domain (Morrison et al., 1984).

The hybridoma technique is also expanded to generate humanized antibodies and the first success was achieved through the fusion of human spleen cells from patients with human myelomas in 1980 (Olsson and Kaplan, 1980). Generation of humanized antibodies are achieved by the complementary-determining region (CDR) grafting technique. In CDR grafting, non-human antibody CDR sequences are transplanted into a human framework sequence in order to maintain target specificity (Jones et al., 1986).

The hybridoma technique was the main production method of mAbs in the early stage of clinical development (1985–1996). However, this method has a relatively low efficiency with high risk of contamination (Beerli and Rader, 2010). Other barriers

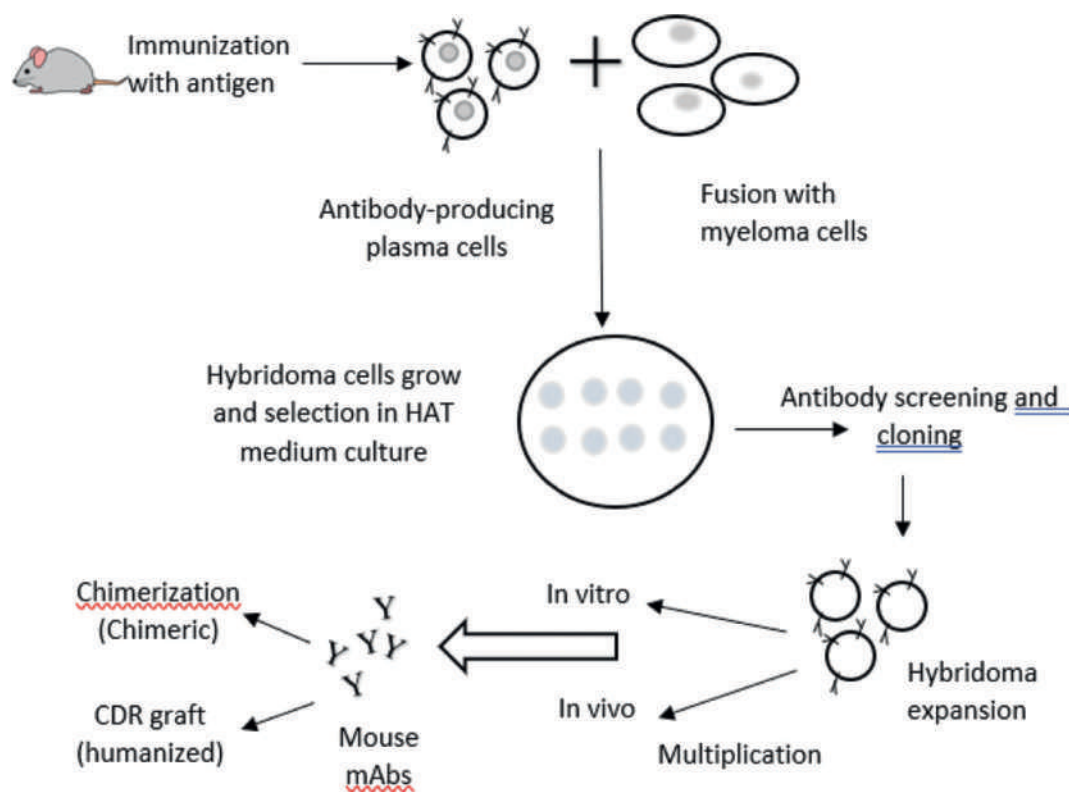


Fig. 1 The hybridoma technique in producing monoclonal antibodies first involves inoculation of the desired antigen (immunization) into mice to produce antibody-specific plasma cells. Subsequently fusion of the plasma cells and myeloma cells are performed using the electrofusion or polyethylene glycol. Selection of the hybridoma cells is performed in the hypoxanthine-aminopterin-thymidine (HAT) medium culture and finally after antibody screening and expansion, the monoclonal antibodies are harvested. The harvested antibodies undergo chimerization or complementary-determining region (CDR) grafting for antibody humanization.

include limitation in obtaining the lymphocyte cells as it was unethical to immunize patients with experimental antigen and therefore the early stage of clinical human mAbs trials were limited to infectious diseases and malignancies (Nelson et al., 2010).

The introduction of phage display method in the 1990s has provided a key discovery technology to obtain fully human mAbs (Lu et al., 2020). This technique was first introduced by George P. Smith in 1985, which can be used to evolve new proteins (Smith, 1985). Subsequently Sir Gregory Winter applied phage display for the discovery and isolation of antibodies, with the aim of producing new pharmaceuticals (McCafferty et al., 1990). Both of them were awarded with The Nobel Prize in Chemistry 2018 for their work and contributions. Phage display is the first *in vitro* antibody selection technique which does not require *in vivo* immune response via immunization. It involves incorporation of the genes which encode protein of interest into filamentous bacteriophages. The proteins, peptides or antibodies encoded by the gene were then displayed on the surface as illustrated in Fig. 2. This technique enables a rapid identification of peptides or antibody fragments, such as single chain fragment variable (scFv) or Fab, that bind a variety of target molecules (proteins, cell-surface glycans and receptors). The library can be used to generate new antibodies to almost every type of antigen and to a broader range of epitopes. Antibodies to toxic antigens that could not be used to immunize an animal can also be produced (Wu et al., 2016; Frenzel et al., 2016).

The production of fully human mAbs are also enabled through the production of antibodies from transgenic mice (Jakobovits, 1995) and single human B cell cloning techniques (Smith et al., 2009). In transgenic mice method, the mice were genetically modified with insertion of human immunoglobulin (Ig) genes into the genome, replacing the endogenous IgG. Hence the mice are capable of synthesizing fully human antibodies upon immunization, as illustrated in Fig. 3 (Jakobovits, 1995). Meanwhile, in the single B cell cloning technique, peripheral blood mononuclear cells (PBMCs) or lymphoid tissues from the infected or vaccinated donors undergo isolation of the single B cells with antigen of interest by flow cytometry. Subsequently amplifications of the immunoglobulin (Ig) heavy chain (VH) and corresponding light chain (VL) domain genes are performed through a reverse transcription-polymerase chain reaction (RT-PCR). The genes are then cloned into mammalian expression vectors for the *in vitro* production of recombinant monoclonal antibodies (Wardemann and Busse, 2019). The generations of humanized and fully human antibodies have accelerated the approvals of various therapeutic mAbs which have a better clinical tolerance in chronic diseases particularly autoimmune disease and malignancy (Lu et al., 2020). Fig. 4 illustrates the evolution of monoclonal antibody productions from murine to fully human antibodies.

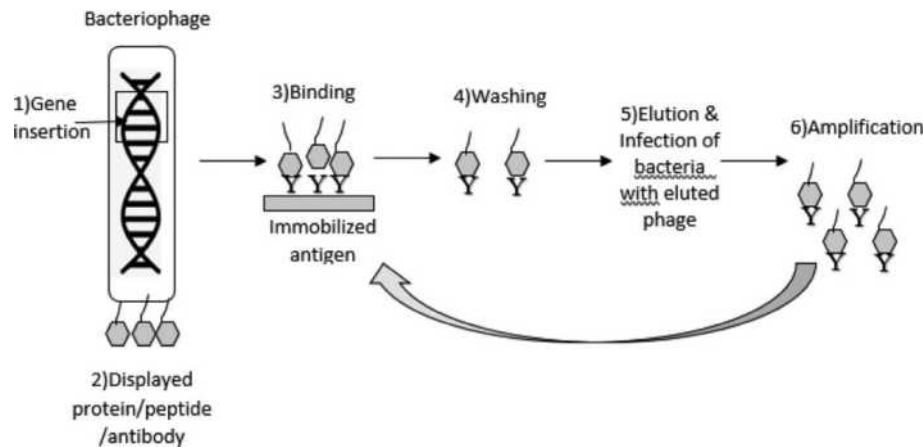


Fig. 2 Phage display is a process in which exogenous genes are incorporated into filamentous bacteriophages to compose a library of new proteins or peptides or antibodies that are encoded by the modified genes which are presented on the phage surface. The library is then exposed to an immobilized target such as receptor or enzyme to promote a specific binding. Washing process is subsequently performed to remove the unbound phage to isolate high affinity binders, followed by elution of the bound phage. Subsequently bacteria (*E. coli*) are infected with the eluted phage and amplification take place. Process is repeated two to three times resulting in enriched population of antibody/peptide fragments for the antigen of interest.

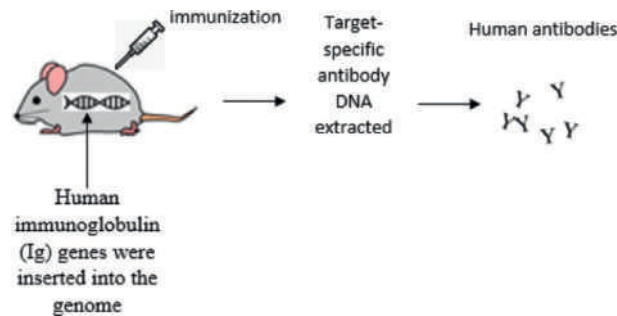


Fig. 3 In transgenic mice, the mice were genetically modified such that human immunoglobulin (Ig) genes were inserted into the genome, and making these animals capable of synthesizing fully human antibodies upon immunization with the desired antigens.

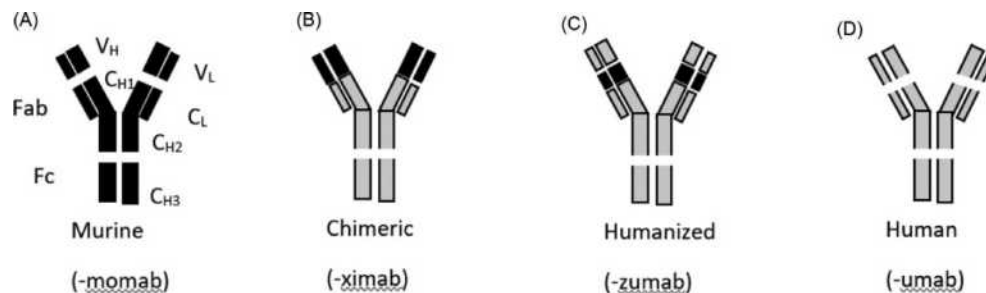


Fig. 4 Overview of the evolution from murine antibodies (black domains) to fully human antibodies (gray domains) and associated suffixes. (A) The murine monoclonal antibody. (B) The chimeric monoclonal antibody: variable regions (V_H and V_L) are of murine origin while the rest of the chains are of human origin. (C) Humanized monoclonal antibody: only includes the hypervariable segments of murine origin-95% homology to human antibody. (D) Human monoclonal. C_{H1}: domains of the constant region of the heavy chain; C_L: constant domain of the light chain; Fab and Fc: fragments resulting from proteolysis; V_H: variable domain of the heavy chain; V_L: variable domain of the light chain.

Immunodiagnostic use of monoclonal antibody

Monoclonal antibodies have tremendous applications as diagnostic tools due to their exquisite specificity in the identification, quantitation or localization of particular molecules, cells or organisms in tissue or body fluids (Siddiqui, 2010). The advances in the mAbs production technology allow novel approaches to rapid and accurate diagnosis of infectious, autoimmune, and malignant diseases. There are various diagnostic methods in which mAbs are utilized such as western blot, immunodot blot, enzyme-linked

immunosorbent assay (ELISA), radioimmuno assay (RIA), flow cytometry, immunohistochemistry, fluorescence microscopy, electron microscopy, confocal microscopy, as well as other biotechnological applications. Monoclonal antibodies have been established as invaluable tools in clinical laboratory diagnostic applications including detections and measurements of extensive range of hormones (Payne et al., 1988) and as blood typing reagents (Marks, 2014).

In infectious diseases, monoclonal antibodies provides rapid identifications of the pathogens by either directly detecting the pathogenic antigens or indirectly by detecting the antibodies against the pathogens (Harris, 1987). This immunodiagnostic technique provides a rapid diagnosis with a high sensitivity and specificity, and therefore has replaced the traditional tissue culture in many diseases including viral, bacterial, protozoa and parasitic infection (Siddiqui, 2010). In the era of new or rare infectious agents, mAbs play crucial role in the rapid identification, diagnosis and monitoring to facilitate public health measure to avoid further spread of the infection. For example Ebola viral antigen can be detected in serum as early as the first day of symptoms and thus enable early intervention (Broadhurst et al., 2016). In this current era of COVID-19 pandemic, there is a spurt in research on the utilization of mAbs to aid in the diagnosis as well as monitoring the immunity or response to treatment (Thomas et al., 2021; Carter et al., 2020).

Monoclonal antibodies have a robust diagnostic application in the oncology field. The ability of the mAbs in identifying and localizing particular molecules in cells, tissues or body fluids have permitted a rapid cancer diagnosis with high accuracy, sensitivity, reproducibility and specificity (Zhang et al., 2014). The sensitivity and specificity of immunohistochemistry are enhanced with the aid of mAbs as compared to the conventional H&E staining procedure alone (Payne et al., 1988). In hematological malignancies, mAbs are utilized to different markers can facilitate the diagnosis of T and B cell lymphomas, histiocytic lymphomas, malignant histiocytosis, and Hodgkin's disease (Klavins, 1987). In solid organ cancers, the mAbs have been used to detect various tumor markers such alpha fetal protein (AFP) for liver cancer, prostate specific antigen (PSA) for prostatic cancer and CA 125 for ovarian cancer (Klavins, 1987).

Apart from the conventional pre-existing tumor markers, recent advances in cancer proteomics have led to many new diagnostic methods of cancers. The discoveries of tumor-associated antigens (TAAs) help in a better understanding of disease biology, early detection, discrimination of tumors and monitoring of disease progression (Alhamdani et al., 2009). With the discoveries, mAbs aid in the cancer diagnosis by their utilizations in the antibody-based microarrays to detect tumor-associated antigens (TAAs). For instance a number of proteins that show increased expression levels in malignant breast tissues such as casein kinase 1 ϵ , p53, annexin XI, CDC25C, eIF-4E and MAP kinase 7 have been identified using a high-throughput protein microarray system which contains 378 well-characterized mAbs (Hudelst et al., 2004).

In the field of imaging of cancers, mAbs with high affinity to the tumor markers or proteins have replaced the polyclonal antibodies as radioimmunodetection probes, in order to precisely diagnose and locate the cancers at early stage (Rudnick and Adams, 2009). Before the year 2000, single-photon emission computed tomography with mAbs used to be one of the methods to diagnose various solid organ cancers such as ovarian, colorectal, lung, and prostatic cancers (Goldenberg, 1997). Subsequently, the fluorine-18 fluorodeoxyglucose positron emission tomography (18F-FDG PET) has emerged as a standard radionuclide imaging as it provides higher contrast images and better resolutions in locating the lesions. Nonetheless, more recent studies have expanded the application of these radiolabeled antibodies in the management of malignancies. The combination of PET imaging with zirconium-89 radiolabeled mAbs, also known as 89Zr-immuno-PET, provides a non-invasive tool in a whole body assessment of the tumor's target expression and tumor targeting (Garousi et al., 2020). This tool can be used to facilitate personalized treatment with prediction of the efficacy and toxicity of tumor treatment (Jauw et al., 2016). With the development of antibody-drug conjugates (ADCs), PET-immuno method can be utilized to verify the delivery of targeted agents to tumors (Carmon and Azhdarinia, 2018). Apart from malignancies, this new technology may also be applied in other conditions such in disseminated infections of invasive aspergillosis (Thornton, 2018).

Therapeutic use of monoclonal antibody

In the last few decades, the therapeutic use of monoclonal antibody has expanded and emerged as one of the predominant treatment modalities for various diseases, including autoimmune diseases, transplantation, oncology, cardiovascular, and infectious diseases (Lu et al., 2020). The use of monoclonal antibodies has become one of the major breakthroughs in medicine as it has paved the era of personalized therapy due to their effectiveness and precisions. Until 2020, more than 550 mAbs are in the pipeline of clinical trials (Kaplun et al., 2020) and up to February 2020, a total of 82 mAbs obtained United States Food and Drug Administration (FDA) or European Medicine Agencies (EMA) approval for therapeutic use in humans (de la Torre and Albericio, 2021).

Solid organ transplantation

Solid organ transplantation provides a "new life" for many patients as it restores self-body functions, improves quality of life and life expectancy (Rana et al., 2015). Preventing rejection of the allograft is the ultimate goal to ensure short- and long-term success and this is achieved by attaining tolerance state in the host via immunosuppression. Unfortunately, the complexity of the immune system as well as the toxicity of the conventional immunosuppressive agents have become a great barrier to achieve the allograft tolerance goal. With the discoveries of mAbs production, they are utilized in various settings in transplant such as: (i) an induction

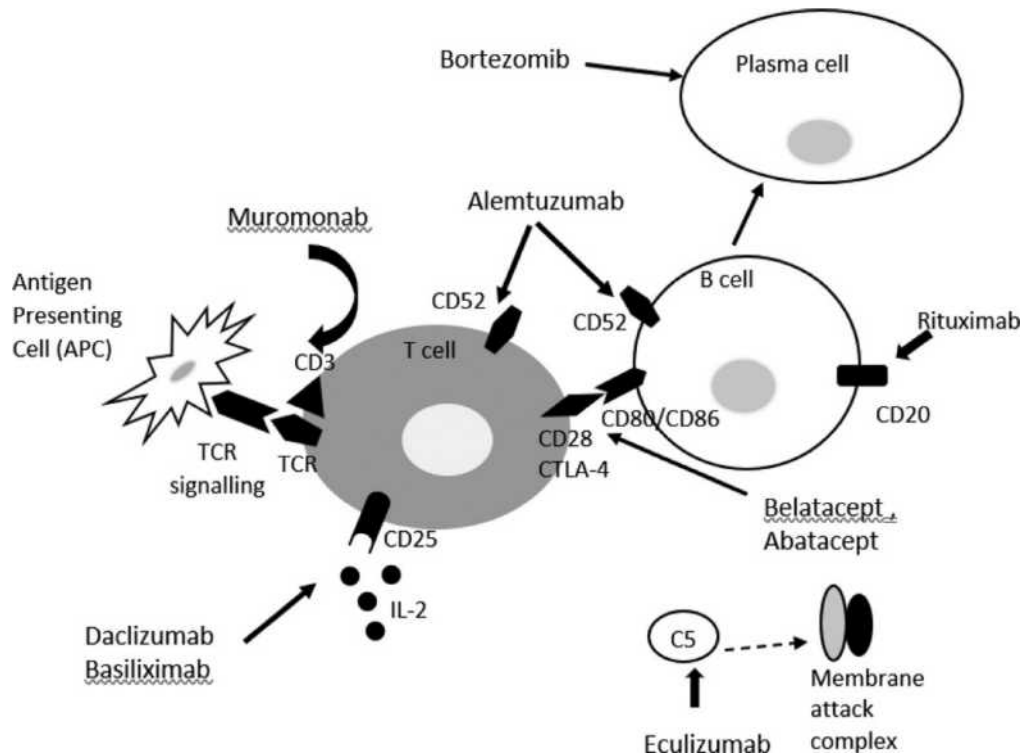


Fig. 5 Muromonab targets the CD3 protein on the surface of circulating human T cells, which is part of the T-cell receptor (TCR) complex which induces T-cell apoptosis. Basiliximab and daclizumab prevent the binding of the IL-2 to its receptor (CD25) causing interruption in the cascade of cell activation, proliferation and cytokine release. Alemtuzumab is directed against the cell surface glycoprotein CD52 on the surface of mature lymphocytes, causing an antibody-dependent lysis of lymphocytes. Rituximab binds to CD20 antigen on the surface of B-cell and induces cytotoxicity. Abatacept and belatacept are the co-stimulation blockers of T cells by inhibiting the bindings of CD28 and CTLA-4 to CD80 and CD86. Eculizumab is directed against the complement protein C5, thereby inhibiting conversion of C5 to C5b and preventing formation of the membrane attack complex (C5–9).

therapy (a period of intense immunosuppression before and following the implant of the allograft), (ii) sparing agents against the toxicity of the conventional immunosuppressive agents, (iii) treatment of acute allograft rejection and (iv) mitigation of the effects of delayed graft function (Pilch et al., 2019).

Solid organ transplant is the pioneer field that has obtained approval of the first mAb to be used clinically in human. Muromonab-CD3 (Orthoclone OKT3, Janssen-Cilag, Beerse, Belgium) is a murine immunoglobulin which targets against CD3 on the surface of circulating human T cells, which is part of the T-cell receptor complex. It subsequently blocks the generation and function of cytotoxic T cells (Norman and Leone, 1991). However, muromonab was discontinued in 2010 with the wide availability of the chimeric (basiliximab) and humanized (daclizumab) mAbs. Both agents have better efficacy with lesser immunogenicity and side effects, including fewer opportunistic infections and malignancies (Olyaei et al., 2001). Basiliximab and daclizumab prevent the initial steps of antigen recognition by binding with high affinity to the interleukin-2 receptor (CD25) hence blocking the subsequent T cell clonal expansion. It is widely used as an induction agent with the aim to reduce risk of delayed graft function, minimize impact of acute graft rejection and prolong graft survival. It also minimizes the toxicity of calcineurin inhibitor (CNI) as it allows lower dose of CNI or delaying the CNI initiation until the patient has developed graft function. Belatacept, a selective T-cell costimulation blocker is indicated for prophylaxis of organ rejection in adult patients receiving a kidney transplant. It is a fusion protein composed of the Fc fragment of human IgG1 linked to the extracellular domain of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), selectively inhibits T-cell activation through costimulation blockade (Vincenti et al., 2016).

Other mAbs such as alemtuzumab (anti-CD52), abatacept (CTLA4-Ig fusion protein, co-stimulation blocker of T cells), rituximab (anti-CD20), bortezomib (proteasome inhibitor) and eculizumab (anticomplement C5) are also used in solid-organ transplant. Fig. 5 illustrates the mAbs main mechanism of actions in transplant.

Autoimmune diseases

The advances in the understanding of the pathogenesis of autoimmune diseases have led to the identifications of their pathogenic inflammatory molecules. Parallel to the progress in the mAbs technology, the therapeutic approach in autoimmune disease has witnessed a significant paradigm shift by utilization of mAbs to target against specific inflammatory molecules or immune pathways.

Since many autoimmune diseases are associated with particular human leukocyte antigens, the initial attempts to use mAbs in the treatment of autoimmune disease involved antibodies directed against major histocompatibility antigens (MHA II) (Wofsy, 1985). Unfortunately, the widespread expression of MHA II and the broad effects on both B and T cells became a significant barrier to their clinical applications. Subsequently, antibodies to T cell antigens such as CD4-directed antibodies were investigated for the treatment of several autoimmune diseases such as in rheumatoid arthritis (Herzog et al., 1987) and multiple sclerosis (Hafler et al., 1988). However, despite significant clinical improvement in RA, severe CD4 lymphopenia, and skin rashes lead to discontinuation of treatment (Choy et al., 2002).

Subsequent successful therapeutic trials consist of specific mAbs against inflammatory cytokines. Infliximab, a chimeric monoclonal antibody against the tumor necrosis factor- α (TNF- α), became the first to be licensed in Crohn's disease in 1998. Its use was later has been successfully employed in the treatment of other autoimmune diseases such as rheumatoid arthritis, psoriasis, ankylosing spondylitis, psoriatic arthritis, and ulcerative colitis. However, to overcome its immunogenicity, Adalimumab (Humira), another monoclonal antibody of fully human origin was derived by a phage display library and used to treat RA patients (Bacquet-Deschryver et al., 2008). Since then, many other cytokine blockade mAbs have emerged such as interleukin-6 (IL-6), interleukin 1 (IL-1), IL-17 and IL-22/23 inhibitors. In addition, B cell depletion therapies mediated via CD20 and CD22, inhibition of B cells survival factors (BAFF and APRIL) and inhibition of B-T cell interactions in autoimmune diseases are also achieved through mAbs. Table 1 summarizes the monoclonal antibodies that are approved to be used in various autoimmune diseases and inflammatory diseases.

Malignancies and cancers

Continuous progression in the understanding in carcinogenesis, molecular biology and technology have opened novel management pathways by utilizing mAbs to direct against multiple targets that are involved in the cancer cells progression and survival. Unlike the conventional chemotherapy which has a wide non-specific cytotoxic effects, antibody-based therapies have a specific direct cytotoxicity of the tumor cells while sparing the normal host cells through various mechanisms such as receptor blockade, immune-mediated cell killing via antibody and complement-dependent cellular cytotoxicity phagocytosis, payload delivery and specific effects on the tumor vasculature and stroma (Scott et al., 2012).

There are many mAbs that are used to treat different types of cancer. The mAbs can serve as targeted cancer therapy. They can recognize and bind specifically to tumor antigens of tumor-specific antigen (TSA) (Bubeník et al., 1973) or tumor-associated antigen (TAA) (Hollinshead et al., 1985) that are present on cancer cells surface and malignant sites thus produced immune responses that lead to cell death to eradicate the tumor (Johdi and Sukor, 2020) (Fig. 6). TSAs are a group of mutated proteins due to somatic mutations and relatively are restricted to tumor cells (Dickinson et al., 1973). Their specificity in tumor cells makes them a good candidate for immunotherapy. One of the good examples of a TSA is the mutated p53 protein that is present in many cancer cells. In contrast, TAAs have differentially expressed proteins that are present in both malignant and non-malignant cells. Although TAAs are also expressed on normal cells, their expression on malignant cells has a unique characteristic and highly expressed resulting in specific immunogenicity (Alatrash and Molldrem, 2019). The binding of mAbs to TSA or TAA produces molecular signals to immune cells such as T cells, B cells and natural killer cells. This further initiates and activates receptors activities that lead to apoptosis and tumor-killing (Scott et al., 2012; Michaud et al., 2014). Thus, mAbs are also a form of immunotherapy because they help enhance the host immune system against cancer.

Some mAbs are able to mark cancer cells which facilitate host immune system to recognize and destroy cancer cells. Other mAbs are capable of guiding the immune cells such as T cells close to cancer cells, thus helping these immune cells in cancer cells killing. There are many effector mechanisms of cancer cell killing by mAbs. The main method is through direct effector mechanism where the mAbs bind to their target growth factor receptors and manipulate their activation state or block ligand binding. This is the common form and there are several FDA approved of mAbs of this form, as illustrated in Table 2 (US Food and Drug Administration, 2021). For example, epidermal growth factor receptor (EGFR) which is overexpressed by many different cancers. EGFR is crucial for cell signaling that leads to tumor cell proliferation, migration and invasion. Cetuximab, which is an anti-EGFR mAb binds to EGFR and blocks ligand binding and receptor dimerization and ultimately induces apoptosis in tumor cells (Patel et al., 2009). The second effector mechanism is through indirect mechanisms of mAbs which require the engagement of components of the host immune system such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent cellular cytotoxicity (ADCC). Some mAbs such as rituximab depends on CDC for its in vivo efficacy. In a preclinical model, a knockout of the complement cascade component C1q completely abolished rituximab anti-tumor effects showing the dependency of this mAbs of the complement component (Di Gaetano et al., 2003). The ADCP form occurs when Fc γ RI component expressed on immune cells such as macrophages binds to IgG1 or IgG3 mAbs that have opsonized a tumor cell. However, there are very limited reports on this form of indirect effector mechanism although its important role in destruction of circulating tumor cells following mAb therapy cannot be denied (Gül et al., 2014).

Another indirect effector mechanism is through ADCC which was described in 1965 by Erna Möeller. In ADCC approach, the cancer cells are opsonized by mAbs which then recruits effector cells to induce target cell death by non-phagocytic mechanisms (Moeller, 1965). In this case, the mAbs act as a bridge by binding to antigens on the target cell surface via their Fab portions and linking the effector cells via their Fc portions. Of all the IgG classes, IgG1 is the most relevant subclass for this type of anti-cancer

Table 1 Monoclonal antibodies that are approved to be used in autoimmune and inflammatory diseases.

<i>Monoclonal antibody</i>	<i>Target</i>	<i>Disease (year approval)</i>
Infliximab	TNF α ; chimeric IgG1	Crohn's disease (1998) Rheumatoid arthritis (1999) Ankylosing spondylitis (2004) Psoriatic arthritis (2005) Ulcerative colitis (2005) Psoriasis (2006)
Etanercept	TNF α ; TNFR2 with Fc portion of human IgG1	Rheumatoid arthritis (1998) Polyarticular JIA (1999) Psoriatic arthritis (2002) Ankylosing spondylitis (2003) Psoriasis (2004)
Adalimumab	TNF α ; human IgG1	Rheumatoid arthritis (2002) Psoriatic arthritis (2005) Ankylosing spondylitis (2006) Crohn's disease (2007) Psoriasis (2008) Ulcerative colitis (2012) Hidradenitis suppurativa (2015) Uveitis (2016)
Certolizumab pegol	TNF α ; humanized IgG Fab fragment	Crohn's disease (2008) Rheumatoid arthritis (2009) Psoriatic arthritis (2013) Ankylosing spondylitis (2013) Psoriasis (2018) Non-radiographic spondyloarthritis (2019)
Golimumab	TNF α ; human IgG1	Rheumatoid arthritis Psoriatic arthritis Ankylosing spondylitis
Anakinra	IL-1R recombinant receptor antagonist	Rheumatoid arthritis (2001) Cryopyrin-associated periodic syndromes (2013)
Canakinumab	IL-1 β ; human IgG1	Cryopyrin-associated periodic syndrome (2009) Systemic JIA (2013) Periodic fever syndromes (2016) AOSD (2020)
Ustekinumab	IL-12/IL-23; human IgG1	Psoriasis (2009) Psoriatic arthritis (2013) Crohn's disease (2016) Ulcerative colitis (2019)
Guselkumab	IL-23; human IgG1 λ	Psoriasis (2017)
Tocilizumab	IL-6R; human IgG1	Rheumatoid arthritis (2010) Systemic JIA (2011) Polyarticular JIA (2013) Giant cell arteritis (2017) CAR T cell-induced cytokine release syndrome (2017)
Sarilumab	IL-6R; human IgG1	Rheumatoid arthritis (2017)
Secukinumab	IL-17A; human IgG1	Psoriasis (2015) Ankylosing spondylitis (2016) Psoriatic arthritis (2016) NR-axial sPA (2020)
Ixekizumab	IL-17A; human IgG4	Psoriasis (2016) Psoriatic arthritis (2017) Radiographic axial sPA (2019) NR-axial sPA (2020)
Brodalumab	IL-17A; human IgG2	Psoriasis (2017)
Rituximab	CD20; chimeric IgG1	Rheumatoid arthritis (2006) Granulomatous polyangiitis and microscopic polyangiitis (2011) Phemphigus vulgaris (2018)
Belimumab	BLyS; human IgG1	SLE (2011)
Ocrelizumab	CD20; humanized IgG1	Multiple sclerosis (2017)
Abatacept	CTLA4-Ig fusion protein; IgG1	Rheumatoid arthritis (2005)

(Continued)

Table 1 (Continued)

<i>Monoclonal antibody</i>	<i>Target</i>	<i>Disease (year approval)</i>
Efalizumab	CD11a; humanized IgG1	Psoriatic arthritis (2017)
Vedolizumab	Integrin- $\alpha 4\beta 7$; humanized IgG1	Psoriasis (2003, withdrawn 2009)
Natalizumab	VLA-4; humanized IgG4	Ulcerative colitis and Crohn's disease (2014)
		Multiple sclerosis (2004)
		Crohn's disease (2008)
Alemtuzumab	CD52; humanized IgG1	Multiple sclerosis (2014)
Eculizumab	Complement C5; humanized IgG2/4	Paroxysmal nocturnal hemoglobinuria (2007)
		Atypical hemolytic uremic syndrome (2011)
		Generalized myasthenia Gravis (2017)
		Neuromyelitis optica spectrum disorder (NMOSD) (2019)
Mepolizumab	IL-5; humanized IgG1	Asthma (2015)
		EGPA (2017)
Reslizumab	IL-5; humanized IgG4	Asthma (2016)
Dupilumab	IL-4R α ; human IgG4	Eczema (2017)
		Asthma (2018)
		Atopic dermatitis (2019)
		Chronic rhinosinusitis with nasal polyposis (2019)
Omalizumab	IgE; humanized IgG1	Asthma (2003)
		Chronic urticaria (2014)
		Nasal polyps (2020)
Emapalumab	IFN γ	Primary hemophagocytic lymphohistiocytosis (2018)

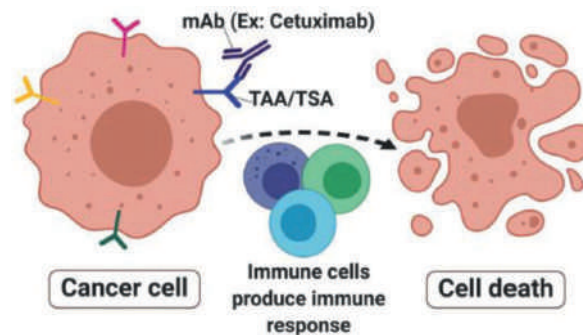


Fig. 6 Monoclonal antibodies in cancer. Monoclonal antibodies (mAbs) such as cetuximab are designed to target tumor-associated antigens (TAAs) or tumor-specific antigens (TSAs) that are abundant in cancer cells surface. The signals produced by receptor activities mediated immune cells toward malignant sites thus produced immune responses that lead to cell death to eradicate the tumor (Johdi and Sukor, 2020).

therapeutic antibodies (Teillaud, n.d.). The effector cells must express FcR that will bind to mAbs in order to facilitate ADCC (Fanger et al., 1989). NK cells are the main effector type that mediate ADCC; however other myeloid types such as monocytes, macrophages, neutrophils, eosinophils, and dendritic cells are also capable (Fanger et al., 1989). Effector cells induce target cell death via cytotoxic granule release, Fas signaling, and initiation of reactive oxygen species.

The use of mAbs against the immune checkpoint proteins have also unlocked the new era of immunotherapy in malignancy. Recently discovered proteins such as the programmed cell death protein 1 (PD-1) and its ligand programmed death-ligand 1 and 2 (PD-L1, PD-L2) or cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4, ipilimumab) have been the targets of the mAbs in treating various cancers (Graybrot et al., 2019). Fig. 7 illustrates the approved mAbs which targets these immune checkpoints, and their mechanism of actions.

Infection and COVID-19

The use of antibodies from sera of infected humans have been used as treatment for viral infections since the early 20th century. Its first therapeutic use against diphtheria by Paul Ehrlich and Emil von Behring has led them to win the Nobel Prize in Physiology or Medicine in 1908 (Ehrlich, 1954). This treatment was gradually replaced by purified antibodies obtained from pooled sera, intravenous immunoglobulin (IVIG) (Stangel and Pul, 2006). Subsequently, the rapid advances from hybridoma technique to phage display library and single B-cell or plasma cell secreting antibody in mAb productions have led to the development of several mAbs to treat infection.

Table 2 List of FDA-approved monoclonal antibodies in treating various types of cancers.

Target	FDA approved monoclonal antibody	Trade name	Manufacturer	Type of cancer treated	Year of approval and updates
HER-2 and HER-2/neu	Trastuzumab	Herceptin [®]	Genentech	Breast cancer	1998
				Gastric cancer	2006
				Breast cancer	2010
	Ado-trastuzumab emtansine	Kadcyla [®]	Genentech	Breast cancer	2013
				Breast cancer	2019
	Pertuzumab	Perjeta [®]	Genentech	Breast cancer	2012
				Breast cancer	2013
				Breast cancer	2017
	Fam-trastuzumab deruxtecan	Enhertu [®]	Daiichi Sankyo	Breast cancer	2019
				Gastric/gastroesophageal cancer	2021
CD20	Margetuximab	Margenza [®]	MacroGenics	Breast cancer	2020
				Non-Hodgkin lymphoma	2006
	Rituximab	Rituxan [®]	Biogen IDEC and Genentech	Non-Hodgkin lymphoma	1997
				Chronic lymphocytic leukemia	1997
				Chronic lymphocytic leukemia	2010
	Ibritumomab tituxetan	Zevalin Y-90 [®]	IDEC Pharmaceuticals	Non-Hodgkin lymphoma	2002
				Non-Hodgkin lymphoma	2003
	Tositumomab	Bexxar [®]	GlaxoSmithKline	Chronic lymphocytic leukemia	2009
				Chronic lymphocytic leukemia	2016
	Ofatumumab	Arzerra [®]	GlaxoSmithKline	Chronic lymphocytic leukemia	2013
Chronic lymphocytic leukemia				2019	
Chronic lymphocytic leukemia				2016	
EGFR	Cetuximab	Erbix [®]	ImClone Systems	Colorectal cancer	2004
				Head and neck cancer	2011
	Panitumumab	Vectibix [®]	Amgen	Colorectal cancer	2006
				Colorectal cancer	2014
Necitumumab	Portrazza [®]	Eli Lilly and Company	Squamous non-small cell lung cancer	2017	
			Squamous non-small cell lung cancer	2015	
			Squamous non-small cell lung cancer	2015	
GD2	Dinutuximab	Unituxin [®]	United Therapeutics	Neuroblastoma (pediatric)	2015
				Neuroblastoma (pediatric)	2020
VEGFR2	Naxitamab	Danyelza [®]	Y-mAbs Therapeutics	Neuroblastoma (pediatric)	2020
				Gastric cancer	2014
	Ramucirumab	Cyramza [®]	Eli Lilly and Company	Non-small cell lung cancer	2014
				Non-small cell lung cancer	2020
CD38	Daratumumab	Darzalex [®]	Janssen Biotech	Colorectal cancer	2015
				Hepatocellular carcinoma	2019
				Multiple myeloma	2015
	Isatuximab	Sarclisa [®]	Sanofi-aventis	Multiple myeloma	2016
				Multiple myeloma	2017
				Multiple myeloma	2018
				Multiple myeloma	2019
CD33	Gemtuzumab ozogamicin	Mylotarg [®]	Pfizer	Multiple myeloma	2020
				Multiple myeloma	2020
				Multiple myeloma	2021
CD30	Brentuximab vedotin	Adcetris [®]	Seattle Genetics	Acute myeloid leukemia	2017
				Hodgkin's lymphoma	2011
	Anaplastic large cell lymphoma	Anaplastic large cell lymphoma	Anaplastic large cell lymphoma	2018	
				2011	
CD19	Blinatumomab	Blincyto [®]	Amgen	Precursor B-cell acute lymphoblastic leukemia	2017
				Precursor B-cell acute lymphoblastic leukemia	2018
	Tafasitamab	Monjuvi [®]	MorphoSys US	Diffuse large B-cell lymphoma	2020
				Diffuse large B-cell lymphoma	2021
SLAMF7	Loncastuximab	Zynlonta [®]	ADC Therapeutics SA	B-cell lymphoma	2021
				B-cell lymphoma	2021
Elotuzumab	Empliciti [®]	Bristol-Myers Squibb and AbbVie	Multiple myeloma	2015	
			Multiple myeloma	2018	

(Continued)

Table 2 (Continued)

Target	FDA approved monoclonal antibody	Trade name	Manufacturer	Type of cancer treated	Year of approval and updates
PDGFR alpha VEGF	Olaratumab Bevacizumab	Lartruvo® Avastin®	Eli Lilly and Company Genentech	Soft tissue sarcoma	2016
				Colorectal cancer	2004
				Non-small cell lung cancer	2006
					2018
				Breast cancer	2008
					2011
				Glioblastoma	2009
					2017
				Renal cell carcinoma	2009
				Cervical cancer	2014
CD22	Inotuzumab ozogamicin Moxetumomab pasudotox	Besponsa® Lumoxiti®	Pfizer AstraZeneca	Acute lymphoblastic leukemia	2016
					2018
				Hairy cell leukemia	2017
					2018
Nectin-4	Enfortumab vedotin	Padcev®	Astellas Pharma US	Urothelial cancer	2019
CD79B	Polatuzumab vedotin	Polivy®	Genentech	Diffuse large B-cell lymphoma	2019
TROP2	Sacituzumab govitecan	Trodelvy®	Immunomedics	Triple-negative breast cancer	2020
PCDP1	Dostarlimab	Jemperli®	GlaxoSmithKline	Urothelial cancer	2021
				Endometrial cancer	2021
					2021

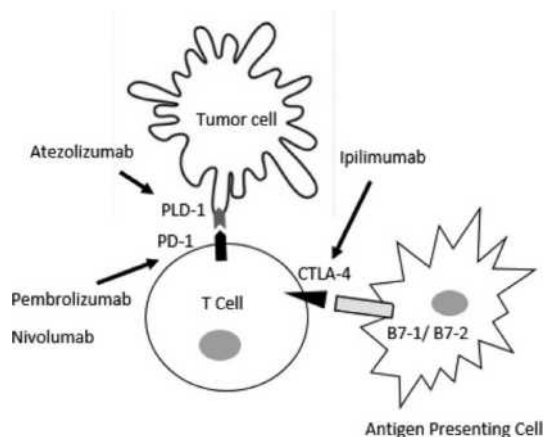


Fig. 7 Mechanism of actions various monoclonal antibodies as immune checkpoint inhibitors. The binding of checkpoint protein PD-L1 on tumor cells to PD-1 on T cells prevents T-cell from killing the tumor cell. Blocking the binding of PD-L1 to PD-1 with an immune checkpoint inhibitor (anti-PD-L1, e.g., atezolizumab or anti-PD-1, e.g., pembrolizumab and nivolumab) activates the T cells to kill tumor cells. The binding of B7-1/B7-2 on antigen presenting cell to CTLA-4 on T cell leads to the inactivation of T-cell mediated tumor cell killing. Blocking the binding of B7-1/B7-2 to CTLA-4 with an immune checkpoint inhibitor (anti-CTLA-4 antibody, e.g., ipilimumab) allows the T cells to be active and to kill tumor cells.

In 1990s several monoclonal antibodies were developed as a treatment strategy in bacterial sepsis and viral infections. The antibodies are directed against the inflammatory cytokines in sepsis, targeting the toxic products of the infecting organism, and specifically against circulating endotoxin (the lipopolysac-polyclonal antiserum⁶ 17 and monoclonal antiendotoxin anticharide component of the bacterial cell wall. Unfortunately the initial evaluations of their clinical benefit in human studies were not demonstrated and thus may have dampened the enthusiasm of researchers and investors in exploring the utilizations of mAbs in bacterial infection (Zurawski and McLendon, 2020). Similarly, majority of studies on mAbs for the treatment and prophylaxis of other viral infections such as CMV, HIV, Influenza, MERS-COV and Zika did not show any significant efficacy in clinical trials. Of these, only monoclonal antibodies targeting respiratory syncytial virus (RSV) and Ebola (Mulangu et al., 2019) have been shown to be effective in human trials.

With the current pandemic COVID-19 secondary to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, there are emerging treatment in combating this devastating disease. The initial studies and clinical trials involve re-purposing of the pre-existing mAbs and they are mainly directed toward controlling the hyper-active immune response or the cytokine storm of COVID-19 to save the lives of patients. In patients with severe COVID-19, a raised level of various cytokines and

chemokines including IL-2, IL-6, IL-7, IL-10, IL-17, G-CSF, GM-CSF, IP-10, MCP1, MIP1A, TNF α , IFN- γ , VEGF, CCL2, etc. were found in numerous studies (Yang et al., 2020). Currently, majority of the mAbs that are under clinical trials are IL-6 inhibitors such as tocilizumab (phase 4), sarilumab (phase 3), and siltuximab (phase 3). Other mAbs targeting other cytokines are also still under early phase of clinical trials such as lenzilumab (GM-CSF antagonist), risankizumab (interleukin-23 inhibitor), adalimumab and infliximab (TNF- α inhibitors), canakinumab and anakinra (IL-1 inhibitors) (Deb et al., 2021; de Mello Silva Júnior et al., 2021).

Novel therapy using neutralizing mAbs to SARS-CoV-2 have been developed and are now under evaluation in clinical trials. Neutralizing mAbs are recombinant proteins that can be derived from the B cells of convalescent patients or humanized mice (Taylor et al., 2021). The majority of monoclonal antibody products under development for SARS-CoV-2 target the spike protein, which the virus utilizes to enter host cells such as bamlanivimab, etesevimab, casirivimab and imdevimab (Pinto et al., 2020). Food and Drug Administration (FDA) have granted the Emergency Use Authorizations (EUAs) for combination therapy of bamlanivimab plus etesevimab and casirivimab plus imdevimab for the treatment of mild to moderate COVID-19 in non-hospitalized patients who are at high risk for progressing to severe disease and/or hospitalization.

Other non-communicable diseases

Therapeutic use of mAbs is expanded to other non-communicable diseases including cardiovascular disease. The first chimeric antibody, anti-GPIIb/IIIa antigen-binding fragment (Fab) (abciximab), was approved in 1994 by the US FDA for inhibition of platelet aggregation in cardiovascular diseases. Table 3 illustrates the mAbs that are approved in the treatment of various non-communicable diseases.

Adverse events

Monoclonal antibodies (mAbs) have emerged as targeted therapies for many conditions such as malignancies, transplant rejection, autoimmune and infectious diseases. However, there are several adverse events with the use of monoclonal antibodies and their complications are mainly related to immunomodulation and infection.

Hypersensitivity reactions and immunogenicity

Hypersensitivity reactions to mAbs can be due to the classic type I (IgE/non-IgE), type III, and delayed type IV reactions and cytokine-release reactions (Hong and Sloane, 2019). Fortunately with the revolution of mAbs from murine to more advance humanized and fully human mAbs, these reactions are less common (Nelson et al., 2010). However, in malignancies, the incidence does not appear to correlate with the origin (human, chimeric or mouse) of the drug, which might be explained by the fact that cytokine release is more often the causative factor than classic hypersensitivity type reactions (Rombouts et al., 2020).

Apart from causing various hypersensitivity reactions, immunogenicity of the mAbs and the development of antidrug antibodies (ADAs) can also affect the efficacy of the therapeutic agent. This immunogenicity may also occur in humanized and fully human antibody, likely due to the use of transgenic mouse cell lines, which cannot generate human carbohydrate side chains (Nelson et al., 2010) and alterations in particular amino acids at certain positions can also influence immunogenicity (Hansel et al., 2010).

Immunosuppression

The use of mAbs results in an acquired immunosuppression state and particular types of infections in certain mAbs illustrate the protective function of the target ligand in the normal immune system (Hansel et al., 2010). For example, anti-TNF α causes reactivation of latent tuberculosis infection while rituximab (anti-CD20) may lead to reactivation of hepatitis B infection. The use of eculizumab increased susceptibility to encapsulated organisms such as Meningococcal and Neisseria infection.

Table 3 List of FDA-approved monoclonal antibodies in treating various non-communicable diseases.

Monoclonal antibody	Target	Disease (year of approval)
Ranibizumab	Vascular endothelial growth factor A (VEGF-A); humanized IgG1 Fab fragment	Neovascular (wet) age-related macular degeneration (2006) Macular edema due to retinal vein occlusion (2010) Diabetic macula edema (2015) Myopic choroidal neovascularization (2017) Diabetic retinopathy (2017)
Denosumab	Receptor activator of nuclear factor kappa-B ligand (RANKL); human IgG2	Osteoporosis (2010)
Alirocumab	Proprotein convertase subtilisin/kexin type 9 (PCSK9)	Hypercholesterolemia (2015)
Evolocumab	Proprotein convertase subtilisin/kexin type 9 (PCSK9)	Prevent heart attack and stroke (2017)
Erenumab	Calcitonin gene-related peptide receptor (CGRPR)	Migrain (2018)

The occurrence of progressive multifocal leucoencephalopathy (PML) due to the reactivation of latent infection in the central nervous system with the polyoma virus John Cunningham (JC) virus infection has been described in natalizumab in patients with multiple sclerosis and Crohn's disease (Major, 2009). Cases have also been described with rituximab (Carson et al., 2009) and infliximab (Kumar et al., 2010).

Other reported opportunistic infections with mAbs include bacterial and fungal opportunistic infections, such as histoplasmosis, listeriosis, aspergillosis, candidiasis, *Pneumocystis carinii* pneumonia and coccidioidomycosis (Descotes, 2009).

Emergence of autoimmune diseases

Monoclonal antibody that targets TNF- α has been associated with the development of anti-nuclear antibodies, antibodies to double-stranded DNA, and also with lupus-like syndromes (Hansel et al., 2010). Demyelinating disease or multiple sclerosis have also been reported with anti-TNF- α therapy (Paul and Koffman, 2018) while immune thrombocytopenic purpura, thyroiditis has been reported in alemtuzumab (Hansel et al., 2010). Paradoxical immune reactions causing psoriasis, hidradenitis suppurativa has been reported with the use of mAbs (Casanova Estruch, 2013).

The use of immune-checkpoint inhibitors in cancers such as anti-cytotoxic T lymphocyte antigen 4 (CTLA-4), anti-programmed cell death 1 (PD-1) and anti-programmed cell death 1 ligand 1 (PD-L1) mAbs has led to the development of a new spectrum of immune-related adverse events (irAEs) (Martins et al., 2019). In contrast to the lupus-like syndrome cause by anti-TNF, irAEs tend to affect major organs and the most frequently affected organs are colitis, myocarditis, encephalitis and pneumonitis, which could be life-threatening.

Malignancy

There's emerging concern on the increased risk of malignancy with mAbs particularly anti-TNF α , due to its pleiotropic effects upon different stages of tumor genesis and TNF inhibition may influence the natural history of cancer progression (Balkwill, 2009). However, a recent substantial evidence has demonstrated no increased risk of cancer with anti-TNF (Mercer et al., 2015; Mariette et al., 2011; Limketkai, 2020).

Rituximab therapy is associated with increased risk of secondary solid tumors in patients with lymphoma (Tarella et al., 2011). However, no increased risk of malignancy with its use among patients with rheumatoid arthritis (Emery et al., 2020), suggesting possible other risk factors in the development of secondary malignancy.

Cardiotoxicity

Concerns have been raised regarding an increased risk of major adverse cardiovascular events (MACEs) (myocardial infarction, cerebrovascular accident or cardiovascular death) in patients treated with mAbs in patients with rheumatic diseases. However, the evidence is conflicting as some studies report a lower risk of cardiovascular events, while others report no significant difference (Lee et al., 2018). On the other hand, cardiotoxicity is one of the well-established consequences of the human epidermal growth factor receptor- (HER2-) targeted therapies such as trastuzumab, lapatinib, pertuzumab, and ado-trastuzumab emtansine. The use of immune checkpoint inhibitors are also associated with various cardiotoxicity such as hypertension, heart failure and prolonged QT syndrome (Dong and Chen, 2018).

Conclusion

Monoclonal antibody is an illustration of a remarkable translational scientific discovery and its bench-to-bedside journey took around a decade to be materialized into the clinical application of its use. However, a balance between the cost of treatment, efficacy and safety need to be considered as the treatment with mAbs can be expensive. Hence, more robust data on their cost effectiveness and long-term safety are warranted. Major advances in genetic sequencing and biomedical research have led to more on-going research of monoclonal antibodies in identifying new targets while optimizing their efficacy and safety for use in clinical practice.

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Cytokine Therapy

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Introduction

Cytokines are low-molecular weight (usually below 30 kDa) secreted proteins. The term cytokine is derived from the Greek and means cell movement ('*cyto*,' cell; '*kinos*,' movement). As the name implies, cytokines stimulate the movement of cells and help interaction and communication between them. They are secreted primarily by immune cells such as helper T cells (Th cells), macrophages, and dendritic cells; however, they can also be produced by non-immune cell types in response to infection or damage (Muñoz-Carrillo et al., 2018). Cytokines act on their target cells in various ways, either on cells that secrete them (autocrine action), on nearby cells (paracrine action), or they act in an endocrine fashion, i.e. on distant cells. Moreover, cytokines are characterized by their pleiotropic activity, which means that a single cytokine can have different effects on various cell types. It is common for different cell types to secrete the same cytokine; cytokines are frequently redundant, with different cytokines stimulating similar functions. Moreover, they can synergize with or antagonize (inhibit action) other cytokines. They act through transduction of signals and control many biological processes on various cell types, including cell growth and differentiation, apoptosis, and pro-inflammatory or anti-inflammatory responses. Cytokines stimulate these biological processes by binding with high-affinity to specific receptors on the surface of cells. Upon binding, intracellular signaling pathways are triggered leading to alteration in the levels of gene transcription, and hence, functional changes in these cells. Cytokine-driven stimulation is frequently short-lived.

Common structure and signaling pathways of their receptors allow cytokines to be grouped into families: (i) IL-1 superfamily, (ii) TNF superfamily, (iii) IL-17 family, (iv) IL-6 superfamily, (v) type I cytokine receptor family and, (vi) type II cytokine receptor family (Gadina et al., 2017; Muñoz-Carrillo et al., 2018).

Their ability to induce a potent response against pathogens, but also their involvement in numerous immune and inflammatory states, has led to the development of cytokine-based therapies that will be described in this chapter.

Cytokines and cytokine antagonists as therapeutic agents

IL-1 superfamily

The Interleukin (IL)-1 superfamily consists of the IL-1 family that includes 11 ligands (IL-1 α , IL-1 β , IL-1Ra, IL-18, IL-33, IL-36 α , IL-36 β , IL-36 γ , IL-36Ra, IL-37 and IL-38) and 10 receptors (IL-1R to IL-10R), with both pro-inflammatory and anti-inflammatory functions that are linked to chronic inflammation. Members of the IL-1 family are involved in defense against pathogens by developing immune responses to foreign antigens (Dinarello, 2018).

The most studied member of the IL-1 family is IL-1 β , a major pro-inflammatory cytokine that initiates inflammation and is essential for the innate host defense against pathogens (Dinarello, 1996). Despite its protective role against pathogenic infection and injury, dysregulation of IL-1 β production leads to aberrant local or systemic inflammation resulting in various auto-inflammatory diseases (Muñoz-Carrillo et al., 2018). Therefore, blockade of IL-1 β activity has been used as a therapeutic strategy to treat different types of auto-inflammatory disorders. So far, three agents for IL-1 β blockade have been approved by the US Food and Drug Administration (FDA) (Dinarello et al., 2012). The first approved drug for IL-1 neutralization is anakinra (Kineret; Amgen) which is a recombinant IL-1 receptor antagonist and prevents the activity of the cytokines IL- α and IL-1 β . Anakinra is approved for the treatment of rheumatoid arthritis and cryopyrin-associated periodic syndromes, like neonatal-onset multisystem inflammatory disease (Mertens and Singh, 2009; Koné-Paut and Galeotti, 2014). The other two drugs available on the market are the soluble decoy receptor, rilonacept (Arcalyst; Regeneron) and the human anti-IL-1 β monoclonal antibody (mAb), canakinumab (Ilaris; Novartis) (Dhimolea, 2010; Dinarello et al., 2012). Rilonacept is also used for the treatment of cryopyrin-associated periodic syndromes and was the first drug to get approval for recurrent pericarditis (Kapur and Bonk, 2009; Schwier, 2021). The mAb canakinumab has been approved to treat systemic juvenile idiopathic arthritis and Still's disease (Orrock and Ilowite, 2016; Sfriso et al., 2020).

In the past years, many studies have investigated the role of the cytokine IL-18, also a member of the IL-1 family. A humanized mAb that blocks IL-18, GSK1070806 (GlaxoSmithKline) has been examined in clinical studies for inflammatory disorders such as inflammatory bowel disease, diabetes mellitus, Crohn's disease, and currently for atopic dermatitis (Vecchié et al., 2021). IL-18 is a pro-inflammatory cytokine and was first recognized for its interferon- γ properties (Okamura et al., 1995). Various hematopoietic and non-hematopoietic cells are able to produce this cytokine; however, the primary producers of IL-18 are macrophages and monocytes following pathogen stimulation, in a similar manner to IL-1 β activation. IL-18 levels rise during viral, bacterial, parasitic and fungal infection and in some cases are correlated with disease severity (Vecchié et al., 2021). For example, the plasma levels of IL-18 can be greater than 1000 pg/ml in Epstein-Barr virus (EBV) and HIV-1 infection (Stylianou et al., 2003; van de Veerdonk et al., 2012) and elevated serum levels were observed during the acute phase of viral hepatitis (Kim et al., 2018). Additionally, significantly increased IL-18 levels were also observed in SARS-CoV-2 infected patients and showed correlation with severity of COVID-19 disease (Satış et al., 2021). These observations led to the idea of IL-18 blockage as a therapeutic option for COVID-19, as well as for other inflammatory disorders (Satış et al., 2021; Vecchié et al., 2021).

Many agents that reduce IL-1 activity are currently in clinical trials to investigate potential treatment of various auto-immune and inflammatory diseases. The two approved drugs, anakinra and canakinumab, are also being investigated as potential treatments for COVID-19; as of now there is inadequate evidence for the treatment of COVID-19 patients with the use of IL-1 blocking agents.

TNF superfamily (TNFSF)

The tumor necrosis factor (TNF) superfamily consists of 19 ligands and 29 receptors. Among those ligands are the well-studied cytokines, TNF- α and TNF- β . Their expression is stimulated primarily by antigen-presenting cells, dendritic cells, B cells and macrophages; however, reports have demonstrated that various cells such as T cells, NK cells, mast cells, basophils, eosinophils, as well endothelial cells express TNF ligands (Cuzzocrea, 2017). The receptors of the TNF superfamily are transmembrane proteins with cysteine-rich motifs in their extracellular domains that bind to their ligands. Interaction between the ligands and receptors of the TNF superfamily induce signals that control cell cycle, proliferation, differentiation, apoptosis and production of inflammatory cytokines and chemokines, and therefore, play a pivotal role in immunity and inflammation (Cuzzocrea, 2017). Various studies have shown that signaling pathways between TNFSF ligands and receptors are active in inflammatory and autoimmune diseases, and thus, TNF-inhibitors have been developed for therapeutic purposes. Five TNF blockers have been approved by the U.S. Food and Drug Administration (FDA) to treat rheumatoid arthritis, psoriatic arthritis, juvenile arthritis, Crohn's disease, ulcerative colitis, ankylosing spondylitis and psoriasis (Table 1).

Table 1 FDA approved agents for cytokine-based therapies.

	Agent	Mechanism of action	Diseases	References
IL-1 agents	Anakinra	Antagonist for IL-1RI; prevents the activity of IL- α and IL-1 β	Rheumatoid arthritis; Cryopyrin-associated periodic syndromes	Mertens and Singh (2009) and Koné-Paut and Galeotti (2014)
	Rilonacept	Soluble decoy receptor, binds to IL-1 β , IL-1 α and IL-1Ra	Cryopyrin-associated periodic syndromes; Recurrent pericarditis	Kapur and Bonk (2009) and Schwier (2021)
	Canakinumab	human anti-IL-1 β monoclonal antibody	Systemic juvenile idiopathic arthritis; Still's disease	Orrock and Ilowite (2016) and Sfriso et al. (2020)
TNF agents	Infliximab	chimeric IgG1 monoclonal Antibody that binds to soluble and transmembrane forms of TNF- α and prevents its activity	Rheumatoid arthritis; Crohn's disease; Ankylosing Spondylitis; Psoriatic arthritis; Plaque psoriasis; Ulcerative colitis	Smolen and Emery (2011)
	Etanercept	TNF inhibitor that acts as a soluble receptor and binds to TNF- α and TNF- β to block their activity	Rheumatoid arthritis; Ankylosing spondylitis; Psoriatic arthritis; Plaque psoriasis; Juvenile idiopathic arthritis	Goldenberg (1999) and Goffe and Cather (2003)
	Adalimumab	recombinant human IgG1 mAb that binds to TNF- α and prevents its activity	Rheumatoid arthritis; Ankylosing spondylitis; Plaque psoriasis; Juvenile idiopathic arthritis; Ulcerative colitis; Hidradenitis suppurativa; Crohn's disease	Bang and Keating (2004), Cassinotti et al. (2008), Salvarani et al. (2012) and Sparrow (2017)
	Certolizumab pegol	PEGylated anti- TNF- α humanized Fab fragment of a mAb that prevents the activity of TNF- α	Rheumatoid arthritis; Psoriatic arthritis; Ankylosing spondylitis; Plaque psoriasis;	Goel and Stephens (2010)

Table 1 (Continued)

	<i>Agent</i>	<i>Mechanism of action</i>	<i>Diseases</i>	<i>References</i>
IL-17 agents	Golimumab	anti-TNF humanized IgG1 κ mAb that neutralizes TNF- α and prevents its activity	Crohn's disease Rheumatoid arthritis; Psoriatic arthritis; Ankylosing spondylitis; Ulcerative colitis	Oldfield and Plosker (2009)
	Secukinumab	human anti-IL-17A IgG1 κ mAb that inhibits IL-17A signaling	Plaque psoriasis; Psoriatic arthritis; Ankylosing spondylitis	Sanford and McKeage (2015)
	Ixekizumab	humanized anti-IL-17A IgG4 mAb that neutralizes IL-17A	Plaque psoriasis; Psoriatic arthritis; Ankylosing spondylitis	Markham (2016)
	Brodalumab	human IgG2 mAb against the IL-17 receptor A	Plaque psoriasis	Pinter et al. (2019)
IL-6 agents	Tocilizumab	anti-IL-6 receptor mAb	Rheumatoid arthritis; Giant cell arteritis; Systemic sclerosis-associated interstitial lung disease; Juvenile idiopathic arthritis; Cytokine release syndrome	Tanaka et al. (2014) and Rubbert-Roth et al. (2018)
IFN agents	Sarilumab	human mAb against IL-6R α	Rheumatoid arthritis	Lamb and Deeks (2018)
	Satralizumab	humanized anti-IL-6 receptor mAb	Neuromyelitis optica spectrum disorder	Heo (2020)
	Siltuximab	chimeric anti-IL-6 mAb	Castleman's disease	Sarosiek et al. (2016)
	Interferon alfa-n3	purified human INF- α that bind to type I INF receptors	Condylomata acuminata	Gall (1995)
	peginterferon alfa-2a	PEGylated recombinant INF- α	Chronic hepatitis C; Chronic hepatitis B	Rajender Reddy et al. (2002) and Matthews and McCoy (2004)
	Interferon alfa-2b	recombinant INF- α	Chronic hepatitis C; Chronic hepatitis B; AIDS-related Kaposi's Sarcoma; Condylomata acuminata	Jaeckel et al. (2001)
	Peginterferon alfa-2b	PEGylated recombinant INF- α	used to treat chronic hepatitis C infection in combination with ribavirin; however, it is no longer recommended due to adverse side-effects	Wirth et al. (2005)
	Interferon beta-1a	recombinant INF- β	Multiple sclerosis	Marziniak and Meuth (2014)
	Peginterferon beta-1a	PEGylated recombinant INF- β	Multiple sclerosis	Hoy (2015)
	Interferon gamma-1b	recombinant INF- γ	Malignant osteoporosis; Chronic granulomatous disease	Todd and Goa (1992) and Lyseng-Williamson (2015)

IL-17 family

The IL-17 family consists of pro-inflammatory cytokines and there are six known ligands within the family (IL1-7A to IL-17F) and five receptors (IL-17RA to IL-17-RE); the ligand IL-17E is also known as IL-25. Various types of immune cells produce IL-17A and IL-17F, while epithelial cell produce IL-17B, IL-17C and IL-17D. IL-17 cytokines show potent pro-inflammatory action that is mainly due to their ability to recruit immune cells and to act synergistically with other pro-inflammatory cytokines, such as TNF, IL-1 β , IFN γ , GM-CSF and IL-22 (Onishi and Gaffen, 2010; Ruiz de Morales et al., 2020). These cytokines play an important role in host defense against extracellular bacteria and fungi; however, many studies have shown their participation in inflammatory and autoimmune disorders, such as psoriasis, atopic dermatitis, psoriatic arthritis, ankylosing spondylitis, and rheumatoid arthritis. A comprehensive review of IL-17 in inflammatory and immune disorders was published recently (Ruiz de Morales et al., 2020).

The most studied member of the family is IL-17A (known as IL-17). IL-17A is produced by Th17, CD8⁺ T cells and ILC3 cells (Langrish et al., 2005), and depending on the tissue type and location of its expression and stimuli, it plays either a protective role against infections, or has harmful pro-inflammatory effects (Pacha et al., 2020). Specifically, it has been shown that this cytokine is involved in various inflammatory and autoimmune diseases. Therefore, blocking IL-17A and IL-17RA has been developed as a strategy for inflammatory disease, resulting three IL-17 inhibitory agents, secukinumab, ixekizumab and brodalumab, have been approved by the U.S. Food and Drug administration (FDA) for the treatment of psoriasis, psoriatic arthritis and ankylosing spondylitis (Table 1).

IL-6 superfamily

The interleukin-6 (IL-6) family consists of 10 ligands that include the cytokines IL-6, IL-11, IL-27, IL-35, IL-39, ciliary neurotrophic factor (CNTF), CLCF, cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF) and oncostatin M (OSM), and 9 receptors, membrane and soluble IL-6R, IL-11R, IL-27R, IL-12R β 2, IL-23R, CNTFR, LIFR and OSMR (Kang et al., 2020).

They are grouped into one family because they use a common signaling receptor, the subunit glycoprotein 130 kDa (gp130); however, as more members of this family are being discovered, this common characteristic may not be shared in future (Rose-John, 2018). The IL-6 family has redundant and pleiotropic activities, some of the main functions are B-cell stimulation and their differentiation into antibody-producing cells, modulation of T cells, differentiation of T helper type 17 (Th17) cells, hematopoiesis, and cell proliferation (Kang et al., 2020). IL-6 is known to be associated with inflammatory diseases (Kang et al., 2020); for example, elevated IL-6 levels are observed in patients with rheumatoid arthritis. These observations led to the development of the humanized anti-IL-6R antibody, tocilizumab (Actrema, Chugai Pharmaceutical Co.) to block the IL-6 signaling pathway (Tanaka et al., 2014). Tocilizumab has been approved in numerous countries, including the U.S., and is used for the treatment of rheumatoid arthritis, systemic juvenile idiopathic arthritis, and Castleman's disease (Tanaka et al., 2014) (Table 1). To-date, three more IL-6 related therapeutic agents have been approved by the Food and Drug Administration (FDA), sarilumab (Kevzara) also an anti-IL-6R mAb for use in rheumatoid arthritis (Lamb and Deeks, 2018), satralizumab, a second-generation anti-IL-6R mAb for neuromyelitis optica (Heo, 2020) and siltuximab (Sylvant), a recombinant human-mouse chimeric anti-IL-6 mAb for use in patients with multicentric Castleman's disease (Sarosiek et al., 2016) (Table 1).

Increased levels of IL-6 were observed in patients with severe COVID-19 (Blanco-Melo et al., 2020; Price et al., 2020; Ruan et al., 2020) and several reports refer to IL-6 as a key cytokine in COVID-19. During inflammation, monocytes, macrophages and DCs produce IL-6 which activates the JAK/STAT3 pathway. Recent studies have shown the activation of the JAK/STAT3 pathway by IL-6 is associated with the severity of the disease (Giamarellos-Bourboulis et al., 2020; Hirano and Murakami, 2020). Therefore, blockage of IL-6 activity was suggested to be examined for COVID-19 treatment and several clinical studies are now being performed.

Type I cytokine receptor family

The type I cytokine family consists of various types of cytokine receptors and their ligands that include IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-12, IL-15, IL-21; colony stimulating factors (CSFs), like the granulocyte-macrophage colony stimulating factor (GM-CSF). The characteristic of this group is that the cytokine receptors share conserved amino acid sequence motifs, the motifs CCCC and WSXWS (Baker et al., 2007); the receptors also share common subunits (α , β , and γ). The β and γ subunits are involved in signal transduction, whereas the α subunit in interactions between ligand and receptor (Muñoz-Carrillo et al., 2018). For example, IL-2, IL-4, IL-7, IL-9, IL-13, IL-15 and IL-21 share the γ signal transduction subunit and upon ligand binding, activation of the JAK1/JAK3 and STAT 1-5 signaling pathway occurs. Through the β subunit of IL-3, IL-5 and GM-CSF receptors, the JAK/STAT pathway gets activated through interaction with JAK2 (Carson and Kunkel, 2017; Muñoz-Carrillo et al., 2018). Binding to the α subunit does not induce a signaling pathway, but increases the binding affinity between ligand and receptor (Muñoz-Carrillo et al., 2018).

Since various types of cytokines comprise this family, it is reasonable to expect numerous functions, one among many is the modulation and initiation of the inflammatory response (Muñoz-Carrillo et al., 2018).

Type II cytokine receptor family

The type II cytokine superfamily consists of the subfamilies of interferons (IFNs) and IL-10, and like type I superfamily, is categorized based on common characteristics of their receptors. The receptors share the conserved amino acid sequence motif CCCC, but lack the WSXWS motif that is characteristic for the type I cytokine receptor family (Langer et al., 2004). Interferons are best known to induce anti-viral response; as their name suggests, this cytokine subfamily interferes with viral replication (Lee and Ashkar, 2018). Other functions include anti-proliferation and immunomodulation (Platanias, 2005).

This family is divided into three main classes, type I, type II and type III IFN. As a multi-gene cytokine class, the type I IFN class consists of 13 homologous IFN α subtypes, one IFN β and numerous gene products, IFN- δ , IFN- ϵ , IFN- ζ , IFN- κ , IFN- τ and IFN- ω (McNab et al., 2015). The type II class consists of just a single gene product, the IFN γ , while the type III IFN class includes IFN λ 1, IFN λ 2, IFN λ 3 and IFN λ 4 (O'Brien et al., 2014; McNab et al., 2015).

Type I IFNs

Type I IFNs are produced early during an infection and upon binding to their receptor IFNAR, they act through the pathways JAK1 and TYK2 that results in phosphorylation of STAT1 and STAT2 and subsequently to expression of IFN-stimulated genes (Platanias, 2005). These cytokines, mostly IFN α and IFN β , are well-known for their antiviral activity in both virus-infected cells and uninfected, surrounding cells. Through gene transcription, they hinder the viral replication cycle leading to destruction of viral mRNA and inhibition of the viral protein translation (McNab et al., 2015). Moreover, they play an important role in activating the innate immune response through effector cells, such as NK cells (Lee and Ashkar, 2018). An additional function of type I IFN has been reported, where it was shown that these type of cytokines along with IL-12, IL-15 and IL-18 have the ability to induce production of IFN- γ from NK cells (Pegram et al., 2011), and therefore, stimulate antiviral response through the function of IFN- γ . Furthermore, they activate the adaptive immune system for the development of T and B cell responses (Ivashkiv and Donlin, 2014).

Type I IFNs are being used in a wide range of diseases and several drugs have been approved by the FDA. These drugs include a human leukocyte-derived formulation interferon, interferon alfa-n3 (Alferon-N), recombinant proteins, alfa-2a (Roferon-A), interferon alfa-2b (Intron A), interferon beta-1a (Avonex), interferon beta-1a (Rebif), interferon beta-1b (Betaseron), interferon

beta-1b (Extavia), and their pegylated-derivatives for increased plasma half-life, peginterferon alfa-2b (PegIntron, Sylatron), peginterferon alfa-2a (Pegasys) and peginterferon beta-1a (Plegridy) (Vinh, 2014). The IFN- α agents are approved for the treatment of chronic hepatitis C (Rostaing et al., 1998; Rajender Reddy et al., 2002), *molluscum contagiosum* virus (MCV) (Hourihane et al., 1999). IFN- β agents are approved to treat multiple sclerosis (Reder and Feng, 2014) (Table 1).

Type II IFNs

IFN- γ , the only type II IFN member, is primarily produced by NK cells, CD4⁺ T helper cell type 1 (Th1) lymphocytes and CD8⁺ cytotoxic T cells and signals through the IFN- γ receptor (IFNGR) via the JAK1 and JAK2 pathway that leads to phosphorylation of STAT1 (Platanias, 2005). This cytokine plays an essential role in anti-viral responses. Studies showed that in HSV-2 infection in the absence of IFN- γ led to increased virus replication and decreased survival (Ashkar and Rosenthal, 2003; Lee and Ashkar, 2018), also in the context of HCV coinfection in HIV-1 positive patients, the replication of the virus was limited when IFN- γ was present (Kokordelis et al., 2014).

IFN- γ affects the function of surrounding immune cells, mediates interactions between innate and adaptive cells, class-switches B cells, plays an important role in Th1 response by enhancing the antigen presentation process during T cell priming, and increases the expression of major histocompatibility complex (MHC) class I and II. It is also involved in maturation of dendritic cells (Steimle et al., 1994), induces NO production in macrophages, and additionally, promotes complement-mediated phagocytosis through macrophages (Drevets et al., 1996).

Besides its pro-inflammatory functions, IFN- γ limits damaging immune responses that can result in tissue pathology during virus infection. Specifically, studies showed that IFN- γ can suppress the proliferation of type 2 innate lymphoid cells (ILC2) and production of type II cytokines, leading to increased lung pathology (Duerr et al., 2016; Moro et al., 2016; Califano et al., 2018). Association of IFN- γ with disease pathology has also been reported in cases of IFN- γ hyperactivation that results in tissue damage, necrosis and inflammation. Therefore, immune regulatory mechanisms for IFN- γ are considered very important for maintaining the balance between immune response to infection and disease pathology (Kak et al., 2018).

IFN- γ is an important pro-inflammatory cytokine with essential role in host defense against pathogens. Therefore, IFN- γ administration has been shown to be an effective therapeutic strategy to stimulate the immune system and induce an immune response in patients with primary immunodeficiencies (Vinh, 2014). IFN- γ (Actimmune, Horizon Pharma) has been approved for use in patients with malignant osteoporosis and in patients with chronic granulomatous disease (CGD). CGD is an inherited disorder of the phagocyte NADPH oxidase system, and therefore, patients with this disorder are prone to infections. Administration of IFN- γ in CGD patients has been shown to result in a significant protection against infection (Vinh, 2014; Leiding and Holland, 2020). In other cases, IFN- γ has been used to treat active infections, such as salmonellosis, fungal infections, cryptococcal meningitis, and mycobacterial disease (Delsing et al., 2014; Vinh, 2014; Kak et al., 2018) (Table 1).

Type III IFNs (IFN λ s)

Members of the type III INF family are IFN λ -1 (IL-29), IFN λ -2 (IL-28A), IFN λ -3 (IL-28B) and IFN λ 4 (O'Brien et al., 2014; Wack et al., 2015; Zhou et al., 2018). The anti-viral responses of type III IFNs are similar to type I IFNs; they are induced in the early stages of infection when pattern-recognition receptors (PRRs) recognize and bind to pathogen-associated molecular patterns (PAMPs) and upon binding to their receptor IFNLR, they initiate the JAK1 and TYK2 pathway and subsequently to STAT1 and STAT2 phosphorylation, and like type I, they regulate gene expression. The main difference between type I and type III IFNs is that type III IFNs act locally upon viral infection; they target mainly mucosal epithelial cells compared to type I IFNs that act in a wide spectrum of cells and tissues, due to expression of their receptors by a variety of cells (Wack et al., 2015). IL-28R α (a type III IFN receptor) can be found only on macrophages, peripheral blood lymphocytes, dendritic cells and epithelial cells which restricts the cellular response to type III IFNs (Zhou et al., 2018). Despite their restricted activity, this family of cytokines play a crucial role in anti-viral immune responses at epithelial surfaces. When recombinant type III cytokines were used in studies, it was demonstrated that these cytokines are able to restrict replication of numerous viruses, such as Zika virus (ZIKV), vaccinia virus, influenza A virus, influenza B virus, SARS-CoV, human metapneumovirus, respiratory syncytial virus, and HSV-2 (Zhou et al., 2018). Furthermore, some studies have reported that type III IFNs affect the adaptive immune responses. Specifically, it was observed that IFN- λ activity during an adaptive immune response leads to a decrease in Treg activity (Morrow et al., 2009), suggesting that IFN- λ has the ability to enable the adaptive immune response to reduce immunosuppression that is regulated by the Treg cells (Zhou et al., 2018). However, since the discovery of IFN- λ cytokines is relatively recent, further investigations are required to obtain a better understanding of the biology and antiviral activity of those cytokines. The local antiviral activity of the type III INFs can be beneficial for therapeutic development to achieve targeted treatment against viral pathogens.

The future of cytokine therapy

So far, we have shown that cytokines are important in controlling and effecting inflammatory responses, both helpful and deleterious. This has led to early attempts to treat disease by administration of agonist or antagonist molecules. However, this approach is very crude. What has been achieved so far is a description of the cytokine mediated interactions between cells (the wiring diagram) and an understanding of many of the molecular interactions involved. However, the inflammatory response

is a network of nonlinear interactions (Callard et al., 1999). Even simple nonlinear networks can result in complex behavior (Chan et al., 1999). In addition, the timing (kinetics) of signaling pathways will be important, for example the sequence in which cytokine signal can affect the response.

Our attempts so far to modulate cytokine pathways could therefore be compared to interfering in a complex music, for example a jazz concert, in which all the players are listening to each other and playing their instruments at the right volume and timing, resulting in a harmonious effect. Our interventions might be compared to playing a single instrument (such as a trumpet) loudly and all the time. It is perhaps not surprising that the outcome is not always good! What we need to do is to learn how to modulate the tune more subtly. This will involve learning when it is most effective to play our instrument (i.e. administer the agonists and antagonists), and using more than one instrument (give patients different agents at different times) and also listening to the other players in the orchestra (monitoring the tune played by other cytokines, molecules and cells). We also need to think about where the cytokines need to be to have most effect.

We are a long way from achieving this. However, increasing our understanding of the basic science of inflammatory responses is a necessary first step. There can be a temptation, when a particular molecule 'does not work,' to say that the molecule is ineffective. We should perhaps ask first whether we have used it in the right way.

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Gene Therapy

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Glossary

CAR-T Technology for treatment of lymphoid malignancies.

CRISPR Technology for precise gene editing.

Epigenetic Reversible modification of gene expression without affecting the DNA sequence.

RNA interference Technology allowing reversible epigenetic gene silencing.

Abbreviations

CAR-T	Chimeric Antigen Receptor T Cell
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
miRNA	micro-RNA
RNAi	RNA Interference
saRNA	Self-Amplifying RNA
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA

Introduction

Gene therapy can be defined narrowly as the replacement of malfunctioning genetic information with functional counterparts for the correction of lack or reduced gene expression activity providing therapeutic effects in animals and humans (Lundstrom, 2015). More broadly, gene silencing based on oligonucleotides (Martinez et al., 2013) and RNA interference (RNAi) (Bobbitt and Rossi, 2016) should be considered as part of gene therapy. Furthermore, both vaccine and immunotherapy approaches aiming at prophylactic and therapeutic efficacy should be accepted as part of modern gene therapy (Ramirez-Montagut, 2015). Chimeric antigen receptor (CAR) T-cell therapy has also demonstrated promises as an alternative approach for gene therapy (Sermer and Brentjens, 2019). Likewise, the application of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) has stirred a revolution in gene therapy as unprecedented gene replacement and gene editing have become a reality in vivo (Lino et al., 2018).

Gene therapy entered the scientific community in earnest already in the 1990s and was proclaimed “the medicine of the future” with a major breakthrough in sight. However, in the late 1990s the gene therapy community was hit by some tragic events, which saw withdrawal of funds, support from the pharmaceutical and biotech industries, and halted progress for years. In one case, an 18-year-old patient treated with an adenovirus vector for the non-life threatening disease ornithine transcarbamylase deficiency died mainly due to inadequate execution of clinical protocols (Raper et al., 2003). In another incident, although children with X-linked severe combined immunodeficiency (SCID-X1) were successfully treated by retrovirus-based gene therapy, the integration of the therapeutic retrovirus in the LMO2 proto-oncogene region resulted in development of leukemia in few treated patients (Hacein-Bey-Abina et al., 2008). Since these incidents, major development on aspects of delivery efficacy and safety of gene therapy vectors and improvements of the design and monitoring of clinical trials have restored the confidence in gene therapy. Furthermore, the current COVID-19 pandemic with the demands of urgent development of efficient vaccines against Severe Acute Respiratory

Syndrome-Coronavirus 2 (SARS-CoV-2) has demonstrated the success of genetic engineering in generating viral vector- and nucleic acid-based vaccines (Lundstrom, 2021a).

Today, more than 4000 gene therapy trials have been conducted or are ongoing and several gene therapy-based drugs have been approved (Maldonado et al., 2020). The popularity of gene therapy can be indicated by the 398,859 hits found when searching the PubMed database for “gene therapy.” Although cancer has been the most frequently targeted indication, infectious diseases have also received plenty of attention. Immunity is also an area of importance as it relates to vaccine development as well as immunostimulatory approaches for cancer treatment. The importance of delivery for successful gene therapy is reflected in the presentation of non-viral and viral vectors and their applications for treatment of infectious diseases and immunotherapy of cancer. Vaccine development is described with a special emphasis on targeting the current COVID-19 pandemic. The relatively novel approaches of gene silencing, CAR-T cell technology, and CRISPR are also presented. Finally, an overview is given on approved gene therapy drugs.

Non-viral vector-based gene therapy

Non-viral vectors have been used for various gene therapy applications, especially for vaccine development and cancer immunotherapy (Table 1). DNA vectors provide advantages due to the simplicity of inexpensive and large-scale production and safe application. Recently, mRNA, small interfering RNA (siRNA), and micro-RNA (miRNA) have demonstrated the potential in gene therapy applications (Cullis and Hope, 2017). However, the delivery efficacy has been inferior in comparison to viral vectors. To address this issue, physical methods such as electroporation and gene gun technologies as well as nanoparticle systems have been engineered based on polymers and liposomes. Particularly, cancer immunotherapy has proven attractive for nucleic acid-based therapeutics aiming at overcoming the problems with rapid in vivo degradation, poor uptake in target cells, rapid clearance from circulation, and potential in vivo toxicity (Mukalel et al., 2019). Moreover, the negatively charged DNA is unable to cross the anionic cell membrane unless facilitated by transfection reagents or delivery vehicles (Yin et al., 2014). In the case of DNA delivery, the DNA furthermore has to cross the nuclear membrane. A variety of lipid nanoparticles (LNPs) including liposomes, ionizable lipids, and lipid-polymer NPs have been formulated for cancer therapy (Park, 2002). Liposomes contain polar head groups and non-polar tails and spontaneously self-assemble into vesicles (Malam et al., 2009). Cationic lipids such as DOTMA, DOTPA and zwitterionic DOPE have been commonly used for DNA encapsulation (Bennett et al., 1992) although some toxicity at the administration site (Lv et al., 2006), undesired immune responses (Ma et al., 2005), and clot formation (Senior et al., 1991) have been described. Ionizable LNPs contain fusogenic helper phospholipids (Cheng and Lee, 2016) and cholesterol to increase the stability and membrane fusion (Allen and Cullis, 2013) and lipid-anchored poly(ethylene glycol) (PEG) (Li and Szoka, 2007) to extend the half-life of circulating LNPs. Ionizable LNPs have proven efficient for encapsulation of short synthetic DNA, siRNAs and miRNAs whereas polymer-based NP carriers such as polyplexes (Tolstyka et al., 2016), chitosan-base NPs (Tammam et al., 2015)

Table 1 Examples of gene therapy using non-viral vectors.

Vector	Gene	Response	References
DNA			
pDNA-PLL-NPs	OVA	Cancer vaccine against EG7 tumors	Minigo et al. (2007)
pDNA-PEI	IL-12	Decrease in lung metastases in mice	Jia et al. (2003)
pDNA-PBAE	194-1BBz CAR	Tumor regression in mouse leukemia model	Smith et al. (2017)
CpG-PBAE NPs	STING	Regression in melanoma in mice	Tsai et al. (2018)
pDNA-polymer	TRAIL	60% cell death in UMUC3 and HeLa cells	Goklany et al. (2019)
pDNA-polymer	TRAIL	Tumor regression, prolonged survival in mice	Goklany et al. (2019)
pDNA-BPEI	TRAIL	Bystander effect, efficacy in pancreatic mouse model	Jiang et al. (2018)
pDNA	FASL	Inhibition of lymphocytic infiltration, T cell death	Batteux et al. (1999), Jiang et al. (2020)
pDNA-THL	NPC1	Reduced tissue inclusion bodies in mice	
RNA			
mRNA-LNPs	EPO	Sevenfold increase in mRNA delivery in BALB/c mice	Kauffman et al. (2015)
mRNA-LNPs	TRP2	Tumor regression in B16F10 mice	Oberli et al. (2017)
mRNA-PBAE	Luciferase	Targeted expression in mouse lung after i.v. injection	Kaczmarek et al. (2016)
mRNA-PBAE	GFP	Transfection of dendritic cells in vitro	Su et al. (2011)
mRNA-PBAE	Luciferase	Mucosal tissue delivery after i.n. injection in mice	Su et al. (2011)
mRNA-HDHE	MART1	10-fold reduced tumor volume in B16F10 mice	Mockey et al. (2007)
mRNA-PBAE + Abs	megaTAL	10-fold increase in ex vivo transfection of T-cells	Moffett et al. (2017)
mRNA-LNPs	NY-ESO-1 MAGE-A3	Immune response in melanoma patients in phase I	Kranz et al. (2016)

Abs, antibodies; EPO, erythropoietin; FASL, Fas ligand; HDHE, histidine-rich polylysines mixed with L-histidine-(N,N-di-n-hexadecylamine)ethylamide; IL-12, interleukin-12; i.n., intranasal; i.v., intravenous; MAGE-A3, melanoma associated antigen 3; MART1, melanoma antigen recognized by T-cells; megaTAL, rare cleaving nuclease architecture; NY-ESO-1, New York esophageal squamous cell carcinoma-1; OVA, ovalbumin; pDNA, plasmid DNA; PBAE, poly (β-amino ester); PEI, polyethylenimine; PLL, poly (L-lysine); STING, stimulator of interferon response cGAMP interactor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRP2, tyrosine-related protein 2.

and poly(beta-amino esters) (PBAEs) (Mangraviti et al., 2015) can condense plasmid DNA into nanoparticles. For instance, poly(L-lysine) (PLL) has been efficiently used for DNA condensation and delivery of PLL-coated polystyrene NPs carrying plasmid DNA encoding ovalbumin (OVA) antigen. PLL NPs have been applied in a model for prophylactic cancer vaccine against EG7 tumor cells (Minigo et al., 2007). Polyethylenimine (PEI) has also been utilized for DNA condensation and aerosol administration of plasmid DNA encoding interleukin-12 (IL-12) resulting in lung-specific IL-12 expression and significantly fewer lung metastases in a murine osteosarcoma lung metastasis model (Jia et al., 2003). Additional polymers such as PBAEs provide tunable biodegradation and poly(β-amino ester) PBAE-based NPs with an anti-CD3e T-cell-targeting antibody has been used for delivery of leukemia-specific CD194-1BBz-CAR plasmid DNA to T-cells in situ in a mouse leukemia model (Smith et al., 2017). Nuclear translocation was facilitated by engineering a microtubule-associated sequence (MTAS) and nuclear localization signal (NLS) peptides in the NPs. Tumor regression was observed in immunized mice. PBAE-based delivery of cyclic dinucleotides (CpG) has demonstrated activation of tumor-specific T-cells and prolonged survival in a mouse melanoma model (Tsai et al., 2018). Plasmid DNA-based delivery has demonstrated promising therapeutic efficacy in preclinical tumor models, which unfortunately did not materialize in efficacy in clinical trials in cancer patients (Zhang et al., 2018a; Yang et al., 2014).

Plasmid DNA-based gene therapy has utilized the expression of apoptotic genes such as the carcinoembryonic antigen (CEA) cell surface glycoprotein involved in regulation of differentiation, apoptosis and cell polarity and urokinase plasminogen activator receptor (UPAR) involved in the degradation of the extracellular matrix (Raocho-Perez et al., 2018). Moreover, apoptosis inducing genes such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Goklany et al., 2019), Fas ligand (FASL) and BCL2 (Inoue et al., 2016) are potential targets for plasmid DNA based gene therapy. TRAIL preferentially induces apoptosis in cancer cells, but not in normal cells (Goklany et al., 2019). Especially, the transmembrane form of TRAIL expressed from a plasmid DNA encapsulated in aminoglycoside-derived polymers showed 60% cell death in UMUC3 bladder cancer and HeLa cervical cancer cell lines, but did not cause cell death in normal mouse or human fibroblasts. Application of a paromycin-derived polymer significantly reduced tumor burden and increased survival in mice. In another approach plasmid DNA expressing TRAIL encapsulated in branched polyethylenimine (BPEI) showed selective transfection of pancreatic stellate cells (PSCs) with highly expressed fibroblast growth factor receptors (FGFRs) (Jiang et al., 2018). An efficient by-stander effect and tumor cell selective cytotoxicity was observed in vivo. Moreover, therapeutic efficacy was confirmed in a PSC-enriched orthotopic pancreatic tumor model.

In the context of FASL, plasmid DNA-based gene therapy has been evaluated in a mouse model for experimental autoimmune thyroiditis (EAT) (Batteux et al., 1999). Direct injection of plasmid DNA encoding FASL into inflamed thyroid inhibited lymphocytic infiltration of the thyroid and induced death of infiltrating T cells. The lysosomal cholesterol storage disorder Niemann-Pick C1 (NPC1), caused by mutations in the NPC1 gene has been targeted by plasmid DNA-based expression of NPC1 (Jiang et al., 2020). The plasmid DNA encapsulated in pegylated liposomes called Trojan horse liposomes (THLs) were targeted with a monoclonal antibody against the mouse transferrin receptor. Treatment of mice resulted in reduced tissue inclusion bodies in the brain and peripheral organs, but did not provide a prolonged lifespan.

Application of RNA in gene therapy has found renewed interest due to recent development of mRNA vaccines against SARS-CoV-2 (Baden et al., 2021; Polack et al., 2020). Moreover, the use of RNAi (Bobbitt and Rossi, 2016) in the form of short hairpin RNA (shRNA), small interfering RNA (siRNA) and micro RNA (miRNA) has significantly broadened the application range in gene therapy (Cullis and Hope, 2017). Due to the sensitivity of RNA to degradation, various NP platforms have been applied for RNA delivery. In this context, LNPs consisting of ionizable lipids, phospholipids, cholesterol and polyethylene glycol (PEG) have been utilized for systemic delivery of luciferase and erythropoietin mRNA in BALB/c mice (Kauffman et al., 2015). The LNPs provided a sevenfold increase in mRNA delivery. However, siRNA delivery was not improved indicating differences in optimized formulation requirements for mRNA and siRNA. In another study, multilamellar ionizable LNPs were able to transfect neutrophils, macrophages and dendritic cells resulting in efficient delivery of tyrosine-related protein 2 (TRP2) mRNA and reduction of tumor volume in a B16F10 mouse tumor model (Oberli et al., 2017). PBAE-based LNPs loaded with luciferase mRNA generated near-exclusive expression in mouse lung after intravenous administration (Kaczmarek et al., 2016). Moreover, using the same approach, GFP mRNA was successfully delivered to dendritic cells in vitro and luciferase mRNA to mucosal tissue compartments after intranasal administration (Su et al., 2011). PEGylated histidine-rich polylysines mixed with L-histidine-(N,N-di-n-hexadecylamine)ethylamide (HDHE) and cholesterol liposomes applied for delivery of the melanoma antigen MART1 mRNA to T-cells resulted in a 10-fold reduction in tumor volume and a 75% decrease in detectable lung metastases in a B16F10 mouse melanoma model (Mockey et al., 2007). Furthermore, coating of PBAE NPs with CD3 and CD8 antibodies for T-cell targeting provided an additional 10-fold ex vivo transfection of T-cells (Moffett et al., 2017). LNPs complexed with NY-ESO-1, MAGE-A3, tyrosinase and TPTE mRNA have been evaluated in a phase I dose-escalation clinical trial, which showed that neutral or negatively charged LNP-mRNA complexes were well tolerated and generated dose-dependent IFN- and antigen-specific T-cell responses in melanoma patients (Kranz et al., 2016).

Viral vector-based gene therapy

The superior delivery properties of viral vectors have made them attractive vehicles for gene therapy applications. The large spectrum of viral vectors with different properties allows for choosing each vector for each application. Needless to say, there is no universal vector that can be utilized for all types of gene therapy applications. The basic criteria relate to the therapeutic needs, which will have a major input on vector choice. Depending on whether the therapy targets chronic or acute diseases, clearly dictates the need for

long-term or transient expression. In this context, the ability of the viral vector to remain episomal or integrate in the host genome plays a fundamental role. Obviously, replication-deficient vectors, especially vectors based on single-stranded RNA viruses, usually generate short-term high level expression and although the safety levels have been improved with specific chromosomal integration it is still a factor to take into account. Moreover, oncolytic viral vectors, which replicate in tumor cells but not normal cells have proven useful for cancer therapy (Reis and Korn, 2002). Viral vectors have been applied for a large variety of indications including cancer, cardiovascular, infectious, hematologic, lung, metabolic, muscular, neurologic, and ophthalmologic diseases (Lundstrom, 2021b). Numerous different virus species such as adenoviruses, adeno-associated viruses (AAV), alphaviruses, flaviviruses, measles virus, rhabdoviruses, herpes viruses, retroviruses, lentiviruses, Newcastle disease virus, reoviruses, poxviruses, picornaviruses, and polyomaviruses have been engineered. These virus species comprise of DNA (single- and double-stranded) and single-stranded RNA viruses. Due to the large number of gene therapy applications with various viral vectors makes it only possible in this review to present examples with emphasis on infectious diseases and cancer immunotherapy (Table 2).

As the majority of gene therapy applications for infectious diseases are based on immunization with viral vectors expressing surface proteins of viral, bacterial or parasite pathogens for inducing antigen-specific immune responses and protection against pathogen challenges, they are presented in the section on vaccine development. However, a number of applications in cancer therapy has aimed at tumor growth inhibition and tumor regression in animal models with the goal of tumor eradication, prolonged survival and even cure (Lundstrom, 2015). In this context, pancreatic cancer cells were targeted by engineering an oncolytic adenovirus vector with the tumor cell-specific SYENFSA (SYE) ligand, which resulted in efficient oncolysis of pancreatic ductal adenocarcinoma (PDAC) cells (Nagasato et al., 2017). Moreover, high transduction efficiency of pancreatic neuroendocrine tumors was obtained by introduction of a survivin promoter in the adenovirus vector leading to complete tumor regression in BALB/c mice. Moreover a chimeric adenovirus vector (Ad5/3) engineered with the melanoma differentiation associated gene-7 (MDA-7) and interleukin-24 (IL-24) for simultaneous replication in cancer cells and secretion of IL-24 showed in preclinical animal models selective tumor killing and bystander activity (Emdad et al., 2018). Moreover, encapsulation of Ad5/3 in microbubbles allowed “stealth delivery” to tumors. In the context of alphaviruses, intravenous administration of 3×10^7 pfu of the oncolytic M1 virus resulted in selective killing of zinc-finger antiviral protein (ZAP)-deficient cancer cells in mice implanted with mammary carcinoma of B16 melanoma cells (Lin et al., 2014). In another approach a Kunjin virus (KUN) vector expressing the granulocyte macrophage-colony stimulating factor (GM-CSF) was intratumorally administered to mice with CT26 colon xenografts (Hoang-Le et al., 2009). The therapy resulted in the cure of over half of the treated animals with no tumor occurrence for 18 months. Moreover, intratumoral injection of KUN-GM-CSF in B16-OVA melanoma bearing mice significantly reduced tumors and provided a cure rate of 67% (Hoang-Le et al., 2009).

Oncolytic herpes simplex virus (HSV) can provide highly efficient replication in tumor cells, which has generated significant reduction in tumor growth and prolonged survival in mice with implanted breast tumors (Eissa et al., 2017). The oncolytic HSFV HF10 vector has been subjected to a dose-escalation phase I clinical trial in 17 Japanese patients of which 6 had recurrent breast cancer, 3 had recurrent head-and-neck cancer and 8 were diagnosed with nonresectable pancreatic cancer (Kasuya et al., 2014). The treatment was safe with no adverse events. Based on pathological findings, tumor markers and diagnostic radiography some therapeutic effects were registered. Moreover, patients with nonresectable locally advanced pancreatic cancer were intratumorally administered the oncolytic HSV HF10 vector (Hirooka et al., 2018). The outcome was partial response (PR) in three patients, stable disease (SD) in four patients, and progressive disease (PD) in nine patients. Preliminary results from a phase II study on combination therapy with HSFV HF10 and the anti-CTLA-4 (ipilimumab) monoclonal antibody in patients with nonresectable or metastatic melanoma indicated good safety and antitumor activity (Eissa et al., 2017). The treatment resulted in prolonged long-term survival in immune-competent mice.

The nonlytic amphotropic retroviral replicating vector (RRV) Toca 51 expressing the yeast cytosine deaminase (CD) was subjected to intravenous and intracranial administration in an orthotopic glioma model (Huang et al., 2015). The Toca 51 vector was further subjected to an open-label, dose-escalation, phase I trial in patients with recurrent or progressive high-grade glioma (HGG) (Cloughesy, 2016). The overall survival of 13.6 months was superior to the control group. Highly encouraging preliminary results of therapeutic efficacy have also been obtained in a phase II/III clinical trial with Toca 511 in glioma patients (Inoko et al., 2018).

Due to its oncolytic activity Newcastle disease virus (NDV) is an attractive vector for cancer therapy (Niu et al., 2015). NDV-based expression of IL-12 and IL-15 has provided suppression of tumor growth in a mouse melanoma model. Reverse genetics has been applied to engineer the oncolytic NDV D90 strain expressing GFP, which demonstrated suppression of tumor growth in athymic mice with lung tumors (Chai et al., 2014). It was also demonstrated that the combination of NDV-based expression of TRAIL and IL-2 showed superior apoptotic activity and tumor regression in mice than administration of either NDV-TRAIL or NDV-IL-2 alone (Bai et al., 2014). Similarly, superior survival was observed in hepatocellular carcinoma and melanoma mouse models with the NDV-IL-2-TRAIL combination. In the context of clinical trials, 1.2×10^{10} and 1.2×10^{11} pfu/m² of the NDV PV101 strain were administered to 79 patients with solid tumors in a phase II study (Pecora et al., 2002). The treatment resulted in objective responses and progression-free survival ranging from 4 to 31 months. Furthermore, NDV-based immunotherapy showed prolonged survival and short-term improvement of quality of life in 335 colorectal cancer patients in a phase III trial (Liang et al., 2003).

Measles viruses (MVs) have been applied for expression of the sodium iodide symporter (NIS) showing enhanced tumor killing in mice with anaplastic thyroid cancer (ATC) (Reddi et al., 2012). MV-based expression of the super-cytosine deaminase (SCD) comprising the bifunctional cytosine deaminase and uracil phosphoribosyltransferase resulted in killing of human ovarian cancer

Table 2 Examples of gene therapy using viral vectors.

<i>Vector</i>	<i>Target</i>	<i>Response</i>	<i>References</i>
Adenovirus			
Oncolytic Ad-SYE	Pancreatic cancer	Oncolysis in PDAC cells	Nagasato et al. (2017)
Oncolytic Ad-Sur-SYE	Pancreatic cancer	Complete regression of s.c. tumors in mice	Yamamoto et al. (2017)
Ad5/3 MDA-7/IL-24	Melanoma	Selective tumor killing, bystander effect in mice	Emdad et al. (2018)
Alphaviruses			
Oncolytic M1	Breast cancer	Selective killing of tumor cells in mice	Lin et al. (2014)
Oncolytic M1	Melanoma	Selective killing of tumor cells in mice	Lin et al. (2014)
Flaviviruses			
KUN-GM-CSF	Colon cancer	Cure in >50% of mice, no tumor occurrence	Hoang-Le et al. (2009)
KUN-GM-CSF	Melanoma	Tumor regression, cure in 67% of mice	Hoang-Le et al. (2009)
Herpes simplex virus			
Oncolytic HSV HF10	Breast cancer	Reduction in tumor growth, prolonged survival	Eissa et al. (2017)
Oncolytic HSV HF10	Breast cancer	Phase I: no adverse events, therapeutic effect	Kasuya et al. (2014)
Oncolytic HSV HF10	Head and Neck CA	Phase I: no adverse events, therapeutic effect	Kasuya et al. (2014)
Oncolytic HSV HF10	Pancreatic cancer	Phase I: no adverse events, therapeutic effect	Kasuya et al. (2014)
Oncolytic HSV HF10	Pancreatic cancer	Phase I: PR in three patients, SD in four patients	Hirooka et al. (2018)
Oncolytic HSV HF10	Melanoma	Phase II: good safety, antitumor activity	Eissa et al. (2017)
Oncolytic HSV-2	Colon cancer	Significant inhibition of tumor growth in mice	Yang et al. (2016)
Retroviruses			
RRV-CD	Glioma	Prolonged survival in mice	Huang et al. (2015)
RRV Toca 511	HGG	Phase I: prolonged survival	Cloughessy (2016)
RRV Toca 511	Glioma	Phase II/III: Promising preliminary results	Inoko et al. (2018)
Newcastle disease virus			
NDV-IL-12/IL-15	Melanoma	Suppression of tumor growth in mice	Niu et al. (2015)
NDV D90	Lung cancer	Suppression of tumor growth in mice	Chai et al. (2014)
NDV-IL-2-TRAIL	Hepatocellular CA	Superior survival after combination therapy	Bai et al. (2014)
NDV-IL-2-TRAIL	Melanoma	Superior survival after combination therapy	Bai et al. (2014)
NDV PV101	Solid tumors	Phase II: progression-free survival 4–31 months	Pecora et al. (2002)
NDV La Sota IV	Colorectal cancer	Phase III: prolonged survival, improved QoL	Liang et al. (2003)
Measles virus			
MV-NIS	ATC	Enhanced tumor killing in mice	Reddi et al. (2012)
MV-SCD	Ovarian cancer	Specific killing of tumor cell lines and primary cells	Hartkopf et al. (2013)
MV-SCD	Liver cancer	Replication in tumors, tumor regression in mice	Lampe et al. (2013)
MV-SCD	Bile duct cancer	Tumor regression, prolonged survival in mice	Lange et al. (2013)
MV-Edm-Zagreb	CTCL	Phase I: complete and partial tumor regression	Heinzerling et al. (2005)
MV-CEA	Ovarian cancer	Phase I/II: SD, double median overall survival	Galanis et al. (2010)
Rhabdoviruses			
VSV-IFN- β -NIS	Plasmacytoma	Dose-dependent tumor regression in mice	Zhang et al. (2016)
VSV-IFN- β -NIS	T-cell lymphoma	Transient remission in 2 out of 9 dogs	Naik et al. (2018)
VSV-MUC1	Pancreatic cancer	Significant reduction in tumor growth in mice	Hastie and Grdzlishvili (2012)
Coxsackieviruses			
CAV-ICAM-1-DAF	Melanoma	Efficient tumor reduction in mice	Shafren et al. (2014)
CAV-ICAM-1-DAF	Breast cancer	Tumor regression and eradication of metastases	Skelding et al. (2009)
CAV-ICAM-1-DAF + Dox	Breast cancer	Superior tumor regression in mice	Skelding et al. (2012)
Vaccinia viruses			
PANVAC TRICOM	Pancreatic cancer	CTL responses for MUC1 and CEA in mice	Madan et al. (2007)
VV GLV-2b372	Liver cancer	Tumor regression in HCC mouse model	Ady et al. (2015)
VV-NIS + Radioiodide	Prostate cancer	Superior tumor regression, survival in mice	Mansfield et al. (2016)
CPVX-FCU1	Glioblastoma	Inhibition of tumor growth in mice	Ricordel et al. (2017)
CPVX-FCU1	Colon cancer	Inhibition of tumor growth in mice	Ricordel et al. (2017)
PANVAC-VF	Melanoma	Phase III: tumor regression in 64% of tumors	Riedmann (2014)
Reovirus			
Oncolytic reovirus	Pancreatic cancer	Suppressed tumor growth in mice	Etoh et al. (2018)
Oncolytic reovirus	Breast cancer	Reduced tumor burden, prolonged survival	Mostafa et al. (2018)
Oncol. reovirus + anti-PD	Breast cancer	Superior therapeutic efficacy in mice	

Ad, adenovirus; *ATC*, anaplastic thyroid cancer; *CA*, carcinoma; *CD*, cytosine deaminase; *CEA*, carcinoembryonic antigen; *CPVX*, Cowpox virus; *CTCL*, cutaneous T-cell lymphoma; *CTL*, cytotoxic T-lymphocyte; *DAF*, decay-accelerating factor; *DOX*, doxorubicin hydrochloride; *FCU1*, fusion suicide gene 1; *GM-CSF*, granulocyte macrophage-colony stimulating factor; *HCC*, hepatocellular carcinoma; *HSV*, herpes simplex virus; *ICAM-1*, intercellular adhesion molecule-1; *IFN- β* , interferon- β ; *IL-24*, interleukin-24; *KUN*, Kunjin virus; *MDA-7*, melanoma differentiation associated gene 7; *MUC1*, mucin 1; *NIS*, sodium iodide symporter; *PANVAC TRICOM*, vaccinia and fowl pox virus system; *PDAC*, pancreatic ductal adenocarcinoma; *PR*, partial response; *RRV*, retroviral replicating virus; *s.c.*, subcutaneous; *SCD*, super cytosine deaminase; *SD*, stable disease; *TRAIL*, tumor necrosis factor-related apoptosis-inducing ligand; *VSV*, vesicular stomatitis virus.

cell lines and primary tumors (Hartkopf et al., 2013). Administration of MV-SCD in mice with hepatocellular carcinoma xenografts resulted in long-term virus replication in tumor tissue and induced apoptotic-like cell death (Lampe et al., 2013). Additionally, MV-SCD delivery to mice with human cholangiocarcinoma (huCCT1) xenografts provided tumor regression and prolonged survival (Lange et al., 2013). In the context of clinical trials, the MV-Edm-Zagreb strain was subjected to a phase I study in patients with cutaneous T-cell lymphoma (CTCL) (Heinzerling et al., 2005). The treatment was safe with no dose-limiting toxicity and in one patient complete regression was detected in one CTCL tumor and partial regression was observed in four out of five CTCL tumors. Moreover, 10^7 – 10^9 MV-CEA particles were administered to recurrent ovarian cancer patients in a phase I/II clinical trial (Galanis et al., 2010). The MV-CEA treatment caused no dose-limiting toxicity. However, doses of 10^7 – 10^9 MV-CEA particles showed better efficacy resulting in SD in nine out of nine patients and the median overall survival of 12.15 months was twice as long as expected.

Oncolytic rhabdoviruses such as vesicular stomatitis virus (VSV) expressing interferon- β (IFN- β) and NIS have demonstrated dose-dependent tumor regression in 5TGM1 plasmacytoma tumor bearing mice (Zhang et al., 2016). In another study, intravenous administration of VSV-IFN- β -NIS in purpose-bred dogs with naturally occurring tumors showed a maximum tolerated dose (MTD) of 10^{10} TCID₅₀ and only mild-to-moderate adverse events (LeBlanc et al., 2013). Moreover, nine dogs with various malignancies were subjected to a single intravenous injection of VSV-IFN- β -NIS (Naik et al., 2018). Rapid but transient remission of high-grade peripheral T-cell lymphoma was observed in two dogs. VSV-based expression of human mucin 1 (MUC1) provided significant reduction in PDAC tumor growth in mice (Hastie and Grdzelskivili, 2012).

The family of small RNA viruses, Picornaviruses, have been subjected to cancer gene therapy. A single subcutaneous injection of the Coxsackievirus A21 (CAV21) carrying the intercellular adhesion molecule-1 (ICAM-1) and decay-accelerating factor (DAF) genes resulted in reduced tumor burden and efficient tumor regression in SCID mice with implanted melanoma tumors (Shafren et al., 2014). Moreover, SCID mice with implanted T47D and MDA-MB-231-luc breast tumor xenografts subjected to intravenous administration of CAV21-ICAM-1-DAF showed significant regression of pre-established tumors and eradication of metastases (Skelding et al., 2009). Superior tumor regression was achieved by combination therapy of CAV21-ICAM-1-DAF and peritoneal doxorubicin hydrochloride in a mouse breast tumor model (Skelding et al., 2012).

Poxviruses have been commonly used for cancer gene therapy (Al Yaghchi et al., 2015). For example, recombinant vaccinia and fowl pox viruses expressing the MUC1 and CEA genes, respectively, comprise the PANVAC system (Madan et al., 2007). Engineering of the PANVAC TRICOM system with the cluster of differentiation B7.1, ICAM-1 and the leukocyte function-associated antigen-3 (LFA-3) generated MUC1 and CEA cytotoxic T-lymphocyte (CTL) responses in mice. Moreover, in mice implanted with hepatocellular carcinoma (HCC) the GLV-2b372 vaccinia virus showed a 50% decrease in tumor volume compared to a 400% increase for mice injected with placebo (Ady et al., 2015). The GLV-1 h153 vaccinia virus expressing the NIS gene provided synergistic effect in the context of tumor growth and survival in a transgenic adenocarcinoma of the mouse prostate (TRAMP) model in combination with radioiodide treatment (Mansfield et al., 2016). In another approach, systemic administration of the cowpox virus (CPVX) expressing the fusion suicide gene (FCU1) showed inhibition of tumor growth in U-87-MG glioblastoma and LoVo colon cancer mouse models (Ricordel et al., 2017). In the context of clinical trials, the PANVAC-VF vector has been subjected to a phase III clinical trial in patients with unresectable stage III-IV melanoma resulting in more than 50% reduced tumor size in 64% of injected tumors (Riedmann, 2014). Moreover, due to a bystander effect tumor regression in one-third of un-injected tumors was detected.

Finally, oncolytic reoviruses present an attractive alternative for cancer therapy due to the susceptibility of pancreatic cell lines such as Panc1 and BxPC3 (Etoh et al., 2003). Intratumoral administration of reovirus vectors to mice with implanted Panc1 and BxPC3 xenografts suppressed tumor growth and demonstrated systemic antitumor responses. Moreover, reovirus administration has shown significantly reduced tumor burden and prolonged survival in a syngeneic mouse EMT6 breast tumor model (Mostafa et al., 2018). Superior therapeutic efficacy was achieved by combination therapy of reovirus and the checkpoint inhibitor anti-PD1 antibody.

Vaccine development

Although vaccines are not considered as gene therapy at its purest application, it is worth to include a summary here, especially in the light of the current COVID-19 pandemic. A large number of viral vectors have been applied for vaccine development targeting infectious diseases including vaccines against viral, bacterial and parasite pathogens. Examples of different applications are given below and listed in Table 3.

The annual influenza virus outbreaks have underlined the importance of developing efficient vaccines based on genetic engineering as attractive alternatives to classic vaccines based on inactivated viruses (Lundstrom, 2019). In this context, the Ad5 vector expressing the codon-optimized hemagglutinin (HA) gene from the influenza virus strain A/Vietnam/1203/2004(H5N1) provided complete protection of immunized mice against challenges with lethal doses of virus (Gao et al., 2006). A single injection of Ad5-HA protected chicken from influenza virus challenges. In another approach the Venezuelan equine encephalitis virus (VEE) expressing the HA gene from the Hong Kong influenza virus isolate A/HK/156/97 was administered to chicken resulting in protection against influenza virus challenges (Schultz-Cherry et al., 2000). Moreover, Semliki Forest virus (SFV) particles expressing the HA and nucleoprotein (NP) genes provided protection against influenza virus challenges in mice (Fleaton et al., 2001).

Another important target for vaccine development comprise HIV (Poluri et al., 2003) for which an AAV-vector expressing the HIV-1 Gag elicited HIV-specific T- and B-cell responses (Lin et al., 2008). Moreover, a vaccinia virus-based system was utilized for the expression of a chimeric antigen comprising the HIV-1 Env and GM-CSF genes, which elicited strong HIV-specific cellular

Table 3 Examples of vaccine development using viral vectors.

Target	Vector	Response	References
Viral			
Influenza	Ad5-HA	Influenza virus protection in mice and chicken	Gao et al. (2006)
	VEE-HA	Influenza virus protection in chicken	Schultz-Cherry et al. (2000)
	SFV-HA/NP	Influenza virus protection in mice	Fleeton et al. (2001)
HIV	AAV-HIV Gag	HIV-specific T- and B-cell responses in mice	Lin et al. (2008)
	VV-HIV Env-GM-CSF	HIV-specific cellular immune response in mice	Rodr Guez et al. (1999)
	RABV-HIV gp160	Long-lasting CTL responses in mice	McGettigan et al. (2001)
	SFV-HIV Gag/Env/PolRT	Antigen-specific T-cell responses in mice	Ajbani et al. (2017)
	VEE-HIV Gag	Phase I: safe, modest immune response	Wecker et al. (2012)
	Ad5-HIV Gag/Pol/Nef	Phase I: HIV-specific T-cell response	Hayes et al. (2016)
HPV	Ad-HPV E6, E7	Protection against tumor challenges in mice	He et al. (2000)
	SFV-HPV E6/E7 DNA	85% of mice tumor-free	van de Wall et al. (2018)
	SFV-HPV E6/E7 + E & ER	Tumor regression and protection in mice	Ip et al. (2015)
	SFV-HPV E6/E7	Phase I: safe, immune response in all vaccinees	Komdeur et al. (2021)
EBOV	VEE-EBOV NP	Protection against EBOV in mice	Wilson and Hart (2001)
	VEE-EBOV NP/GP	Protection against EBOV in mice and guinea pigs	Pushko et al. (2000)
	VSV-EBOV GP	Protection against EBOV in macaques	Marzi et al. (2015)
	VSV-EBOV GP	Protection against EBOV in primates	Geisbert and Feldmann (2011)
	VSV-MARV GP	Protection against MARV in primates	Henao-Restrepo et al. (2015)
	VSV-ZEBOV	Phase III: Protection against EVD	Henao-Restrepo et al. (2017)
	VSV-ZEBOV	Phase III: Protection against EVD	van Doremalen et al. (2020)
SARS-CoV-2	ChAdOx1 nCoV-19	Immunogenicity and protection in macaques	Voysey et al. (2021)
	ChAdOx1 nCoV-19	Phase III: 62.1–90.0% vaccine efficacy	Feng et al. (2020)
	Ad5-S-nb2	Protection against SARS-CoV-2 in macaques	Zhu et al. (2020a)
	Ad5-S-nb2	Phase I: safety, strong immune responses	Zhu et al. (2020b)
	Ad5-S-nb2	Phase II: safety, strong immune responses	Tostanoski et al. (2020)
	Ad26.CO2-S	Protection in hamster after single injection	Sadoff et al. (2021)
	Ad26.CO2-S	Phase I/II: single admin, strong immunogenicity	Logunov et al. (2020)
	rAd26-S/rAd5-S	Phase I/II: good safety, strong immunogenicity	Logunov et al. (2021)
	rAd26-S/rAd5-S	Phase III: 91.6% vaccine efficacy	
Bacterial			
<i>B. anthracis</i>	SIN-PA	Immunogenicity, protection in mice	Thomas et al. (2009)
Brucellosis	SFV-IF3	Immune responses, protection in mice	Cabrera et al. (2009)
Parasite			
Malaria	SFV-Pf332	Th1-type immune response in mice	Andersson et al. (2001)
	SIN-CS epitope	Immune response and protection in mice	Tsuji et al. (1998)
Leishmaniasis	SFV-PpSP15-LmST11	Antigen expression in BHK-21 cells	Sadat Savar et al. (2021)

AAV, adeno-associated virus; Ad, adenovirus; CS epitope, *Plasmodium yoelii* circumsporozoite protein; EBOV, Ebola virus; E & ER, T-cell epitopes and ER-targeting signal; GP, glycoprotein; HA, hemagglutinin; HIV, human immunodeficiency virus; IF3, translation initiation factor 3; NP, nucleoprotein; PA, *Bacillus anthracis* protective antigen; SFV, Semliki Forest virus; SIN, Sindbis virus; VEE, Venezuelan equine encephalitis virus; VV, vaccinia virus.

immune response in BALB/c mice (Rodr Guez et al., 1999). In another approach, a rabies virus (RABV) vector expressing the HIV-1 gp160 gene elicited solid and long-lasting memory CTL responses in immunized mice (McGettigan et al., 2001). SFV-based expression of HIV-1 Env/Gag/polRT either in combination or individually demonstrated strong antigen-specific T-cell responses in immunized mice (Ajbani et al., 2017). In the context of clinical trials, healthy HIV-negative volunteers received subcutaneous administration of VEE particles expressing the non-myristoylated form of HIV-Gag in a phase I study (Wecker et al., 2012). The treatment was safe and tolerable, but elicited only modest immune and T-cell responses. In another study, an Ad5 vector expressing HIV Gag-Pol-Nef generated HIV-1 specific T cells in healthy volunteers although providing limited HIV-1 inhibition (Hayes et al., 2016).

Although the recombinant vaccines Gardasil and later Gardasil 9 have been approved against human papilloma virus (HPV) (Gupta et al., 2016), there has been a genuine interest in developing viral vector-based HPV vaccines. In this context, Ad5 Copenhagen strain was used for expression of the HPV-16 oncoproteins E6 and E7 showing protection against tumors in BALB/c or C57B1/6 mice (He et al., 2000). Replicon-based alphavirus vectors have been applied as DNA-based vaccines against HPV (van de Wall et al., 2018). Conventional plasmid DNA-based vaccines were unable to prevent tumor outgrowth, while the DNA replicon vaccine managed to provide therapeutic efficacy and rendered 85% of immunized mice tumor-free. Moreover, recombinant SFV particles expressing the HPV E6 and E7 fused to T-cell epitopes and an ER-targeting signal generated tumor regression and protection in mice (Ip et al., 2015). The first-in-human phase I with SFV particles expressing HPV E6 and E7 was conducted in 12 patients with a history of cervical intraepithelial neoplasia (Komdeur et al., 2021). The study demonstrated good safety and induced immune responses in all participants.

Ebola virus (EBOV) have seen strong vaccine development activity due to recent outbreaks in Africa. For instance, C57BL/6 mice immunized with VEE particles EBOV nucleoprotein (EBOV-NP) were protected against challenges with lethal doses of EBOV (Wilson and Hart, 2001). Moreover, a single immunization of BALB/c mice and guinea pigs with VEE-EBOV glycoprotein (GP) or a combination of VEE-EBOV-GP and VEE-EBOV-NP particles protected animals from EBOV challenges (Pushko et al., 2000). However, immunization with only VEE-EBOV-NP provided protection to mice but not to guinea pigs. In another approach, VSV vectors expressing the EBOV-GP provided protection against the West African EBOV-Makona strain in rhesus macaques (Marzi et al., 2015). Similarly, immunization with VSV-EBOV-GP and VSV expressing Marburg virus (MARV) GP protected non-human primates against EBOV and MARV, respectively (Geisbert and Feldmann, 2011). Related to clinical evaluation, the VSV-EBOV-GP particles (based on the Zaire strain named VSV-ZEBOV) were subjected to a phase III clinical trial in 4123 individuals with suspected Ebola virus disease (EVD) (Henao-Restrepo et al., 2015). An additional 3528 persons were subjected to a delayed vaccination. No EVD was recorded in the immediate vaccination group, whereas 16 cases of EVD were detected in the delayed vaccination group. Similarly, substantial protection was achieved in another phase III trial with the VSV-ZEBOV vaccine candidate in Guinea and Sierra Leone (Henao-Restrepo et al., 2017).

Vaccine development against the SARS-CoV-2 has obviously been the biggest and fastest bioscience initiative in history and the results have been reported in detail on many occasions, so it is justified to give only a short summary here. Viral vectors have played a central role in COVID-19 vaccine development in parallel with approaches based on inactivated virus, protein subunits and nucleic acids (Lundstrom, 2021a). Ad vectors have played a key role in COVID-19 vaccine development. The ChAdOx1 nCoV-19 vaccine candidate is based on the simian Ad-virus ChAdOx1, which has been engineered to express the full-length SARS-CoV-2 spike (S) protein, showing strong immunogenicity and protection against SARS-CoV-2 challenges in mice and rhesus macaques (van Doremalen et al., 2020). In phase III clinical evaluation, the ChAdOx1 nCoV-19 vaccine showed good safety and 62.1% efficacy after vaccination with two doses of 5×10^{10} Ad particles and 90.0% efficacy after a lower first dose of 2.2×10^{10} Ad particles followed by the standard dose for the second immunization (Voysey et al., 2021). In another approach, the Ad5 vector expressing the codon-optimized SARS-CoV-2 S protein (Ad5-S-nb2) elicited systemic and cell-mediated immune responses and provided protection against SARS-CoV-2 challenges in rhesus macaques (Feng et al., 2020). Moreover, robust T-cell and humoral immune responses were demonstrated after intramuscular Ad5-S-nb2 administration in phase I (Zhu et al., 2020a) and phase II studies (Zhu et al., 2020b). The full-length SARS-CoV-2 S protein has also been introduced into an Ad26 vector, which in contrast to previous Ad-based approaches required a single immunization to protect hamsters against SARS-CoV-2-related pneumonia (Tostanoski et al., 2020). Moreover, strong immune responses were detected after a single intramuscular administration of Ad26.COVS.2 in healthy volunteers in a phase I/II clinical trial (Sadoff et al., 2021). In another Ad-based approach, the strategy of using two different Ad serotypes to prevent immune reactions against pre-existing Ad, the Ad26 and Ad5 serotypes expressing the SARS-CoV-2 S protein (rAd26-S/rAd5-S) were administered sequentially. The rAd26-S/rAd5-S regimen elicited strong immune responses in animal models and showed good safety and robust immune responses in a phase I/II clinical trial (Logunov et al., 2020). Interim results from a phase III demonstrated good tolerability and 91.6% vaccine efficacy (Logunov et al., 2021). In addition to Ad vectors, COVID-19 vaccine candidates based on measles virus, VSV, modified vaccinia Ankara (MVA) virus, lentiviruses and influenza virus are under development and have been described in more detail elsewhere (Lundstrom, 2021a).

Although not as commonly targeted, vaccine development for bacterial infections has been addressed for viral vectors. For example, Sindbis virus (SIN), a self-replicating RNA alphavirus, has been utilized for expression of the protective antigen (PA) of *Bacillus anthracis* (Thomas et al., 2009). Immunization of Swiss Webster mice with SIN-PA particles elicited PA-specific IgG and neutralizing antibodies and provided protection against challenges with the lethal Ames strain. In another approach, immunization of BALB/c mice with recombinant SFV particles expressing the *Brucella abortus* translation initiation factor 3 (IF3) elicited immune responses and demonstrated protection against challenges with the virulent *B. abortus* 2308 strain (Cabrera et al., 2009).

In the context of parasites, SFV particles carrying the *Plasmodium falciparum* Pf332 antigen have been administered to BALB/c mice (Andersson et al., 2001). Immunization elicited Th1-type immune responses and generated immunological memory. The efficacy of the immune response was enhanced by booster immunization. In another study, SIN particles expressing the class I major histocompatibility complex-restricted-9-mer epitope SYVPSAEQI of the *P. yoelii* circumsporozoite (CS) protein (SIN-Mal) were subcutaneously injected into BALB/c mice (Tsuji et al., 1998). The immunization induced strong epitope-specific CD8⁺ T-cell responses and provided protection to a high degree against malaria. Recently, an SFV vector has been engineered for the expression of the *Leishmania major* stress inducible protein 1 (LmSTI1) and *Phlebotomus papatasi* SP15 (PpSP15) as a fusion protein (Sadat Savar et al., 2021). High levels of SFV-based expression of the PpSP15-LmSTI1 fusion protein was detected in BHK-21 cells and immunization studies in mice are in progress.

Gene silencing

RNAi has been introduced as a therapeutic using different viral vectors such as Ad (Wang et al., 2017), AAV (Schaar et al., 2017) and retroviruses (Devroe and Silver, 2004). Conditionally replicating lentivirus vectors (Westerhout et al., 2006) and self-replicating rhabdoviruses (Bastin et al., 2018) and alphaviruses (Ylösmäki et al., 2013) have also been employed for RNAi-based gene silencing. Examples of viral vector-based gene silencing are described below and summarized in Table 4.

Ad vectors have been applied for gene silencing of the mosquito-borne Tembusu virus (TMUV) by expression of shRNAs against the TMUV E and NS5 genes (Wang et al., 2017). Both RNA replication and TMUV production was efficiently downregulated in Vero

Table 4 Examples of gene silencing using viral vectors.

Target	Vector/RNAi	Response	References
TMUV	Ad5/TMUV E & NS5 shRNA	Downregulation of TMUV RNA replication	Wang et al. (2017)
HBV	HD Ad/HBV pri-miRs	94% inhibition of HBV replication in mice	Mowa et al. (2012)
	AAV/HBV-shRNAs	Reduction of HBV titers in transgenic mice	Chen et al. (2009)
	scAAV/HBV-pri-miRs	Significant suppression of HBV replication in mice	Schaar et al. (2017)
Ad5	scAAV9/pTP & E1A amiRs	Significantly reduced Ad5 levels in hamsters	Maepa et al. (2017)
HIV	MMLV/HIV-tat ribozyme	Phase I: safe, long-term survival	Macpherson et al. (2005)
	MMLV/anti-HIV ribozyme	Phase I: MMLV vector in HIV patients	Amado et al. (2004)
	MMLV/anti-HIV ribozyme	Phase II: safe, no efficacy compared to placebo	Mitsuyasu et al. (2009)
	HIV-1/HIV-1 shRNAs + dox	Inhibition of HIV-1 replication by shRNAs	Westerhout et al. (2006)
	HIV/HIV-shRNAs	Inhibition of HIV replication	Li et al. (2005b)
	HIV/HIV-shRNAs	Persistent shRNA expression for 24 months	DiGiusto et al. (2010)
SARS-CoV	cDNA/S1-S2 siRNA	Inhibition of SARS-CoV replication in Vero cells	Zhang et al. (2004)
	S/NSP12/NSP13/NSP16 siRNA	Inhibition of SARS-CoV replication in FRhK4 cells	Zheng et al. (2004)
	S/NSP12 siRNA	Suppression of SARS symptoms in macaques	Li et al. (2005a)

AAV, adeno-associated virus; Ad, adenovirus; amiRs, artificial microRNAs; HBV, hepatitis B virus; HD, Helper dependent; HIV, human immunodeficiency virus; MMLV, Moloney murine leukemia virus; pri-miRs, primary microRNAs; S, spike protein; SARS-CoV, Severe Acute Respiratory Syndrome-Coronavirus; scAAV, self-complementary AAV; shRNA, Small hairpin RNA; siRNA, small interfering RNA; TMUV, Tembusu virus.

cells, which was dose-dependent and lasted for at least 96 h. Moreover, helper-dependent Ad vectors have been applied for inhibition of hepatitis B virus (HBV) replication by delivery of artificial antiviral pri-miRNAs (Mowa et al., 2012). Ad-specific pri-miRNAs introduced into an Ad vector with a CMV promoter showed efficient inhibition of HBV replication. Replacement of the CMV promoter with the liver-specific murine transthyretin (MTTR) promoter enhanced the inhibition of HBV replication to 94% in mice lasting for 5 weeks (Mowa et al., 2012). In another study, AAV-based delivery of HBV-targeted shRNAs showed profound reduction of HBV titers in transgenic mice (Chen et al., 2009). The problem of pre-existing antibodies against AAV was demonstrated by lack of anti-HBV RNAi activity after readministration of the AAV8-HBV-shRNA vector, whereas AAV7 and AAV9 provided prolonged anti-HBV effect. Moreover, self-complementary AAV (scAAV) vectors, which can fold into double-stranded DNA without requiring DNA synthesis (McCarthy, 2008), were applied for gene silencing by mono- and tri-meric artificial pri-miRs derived from pri-miR-31 (Maepa et al., 2017). Placing the scAAV-HBV-pri-miRs under the control of the liver-specific MTTR promoter generated significant suppression of HBV replication for at least 32 weeks in mice.

Ad was targeted as it can cause life-threatening conditions in immunocompromised individuals (Schaar et al., 2017). The scAAV vectors package an inverted repeat genome. Efficient inhibition of Ad5 replication was achieved in vitro by delivery of two scAAV9 vectors carrying three copies of artificial miRNA against the Ad5 pTP (amiR-pTP) gene and three copies against the Ad5 E1 (amiR-E1A) gene, respectively. Administration of scAAV9 to immunosuppressed Syrian hamsters resulted in two order of magnitude reduction of Ad5 levels and reduced damage to the liver (Schaar et al., 2017).

Retrovirus vectors have also been applied for gene silencing. In this context, the Moloney murine leukemia virus (MMLV) was demonstrated to provide safe delivery of anti-HIV tat ribozyme in a phase I clinical trial in HIV-1 infected adults (Macpherson et al., 2005). In another phase I study, the MMLV vector expressing an anti-HIV ribozyme was delivered to mature hematopoietic cells in HIV patients (Amado et al., 2004). Even patients with multidrug-resistant infections vector-containing mature myeloid and T lymphoid cells were detected. In a phase II trial, an MMLV-based vector was applied for tat-vpr specific anti-HIV ribozyme delivery showing good safety, biologic ribozyme activity, but no statistically significant difference in viral load compared to placebo (Mitsuyasu et al., 2009). Lentivirus vectors have also been designed for antiviral shRNA expression (Westerhout et al., 2006). A conditionally replicating doxycycline (dox)-dependent HIV-1 vector was engineered where dox withdrawal prevented transcription of the integrated provirus, but allowed the shRNAs to inhibit the HIV-1 replication (Westerhout et al., 2006). A combinatorial replication-deficient lentivirus vectors expressing a U6 Pol III promoter-driven shRNA targeting HIV-1 rev and tat mRNAs, a U6 nuclear localization TAR RNA decoy and a VA1-derived Pol III cassette expressing an anti-CCR5 ribozyme was engineered (Li et al., 2005b). Transduction of CD34⁺ hematopoietic progenitor cells resulted in superior inhibition of HIV replication after combination therapy compared to treatment with individual shRNAs. Furthermore, transduction of peripheral blood-derived CD34⁺ hematopoietic cells, which were transplanted and successfully engrafted into four AIDS patients resulted in shRNA and ribozyme expression for at least 24 months (DiGiusto et al., 2010).

In the context of coronaviruses, there has been several speculative reviews on RNAi-based therapy targeting SARS-CoV-2 (Uludag et al., 2020; Donia and Bokhari, 2021). So far, only computational design of siRNAs for SARS-CoV-2 have been reported (Zhao et al., 2020). However, experimental data is available for SARS-CoV. For example, siRNAs targeting the S1 and S2 regions of the S protein efficiently inhibited SARS-CoV replication in Vero E6 cells (Zhang et al., 2004). Moreover, four chemically synthesized siRNAs targeting S, NSP12, NSP13 and NSP16 inhibited SARS-CoV replication in FRhK4 fetal rhesus kidney cells (Zheng et al., 2004). The siRNAs targeting the S and NSP12 suppressed SARS symptoms in rhesus macaques (Li et al., 2005a).

CAR T-cell technology

The chimeric antigen receptor (CAR) T-cell technology has revolutionized the treatment of lymphoid diseases, particularly diffuse large B-cell lymphoma (DLBCL) and acute lymphoblastic leukemia (ALL) (Sermer and Brentjens, 2019). As CARs are recombinant receptors, they can provide both antigen-binding and T-cell activation (Sadelain et al., 2013). CARs can target cell surface tumor antigens and in the second generation CARs tri-partite receptors with costimulatory domains have been engineered. Combination of costimulatory ligands, chimeric costimulatory receptors and cytokines has enhanced T-cell potency, specificity and safety in patients. Application of CAR T-cell technology has successfully reduced the remission rates for hematologic cancers up to 80% (Mohanty et al., 2019). In particular, encouraging results have been obtained for the treatment of ALL in children and young adults and in adults with DLBCL (Pehlivan et al., 2018). Anti-CD19 CAR T-cell therapy has also been successfully used for treatment of B-cell non-Hodgkin lymphoma (Abramson, 2020). Furthermore, in a phase I CAR T study targeting the B-cell maturation antigen (BCMA) all patients with relapsed or refractory multiple myeloma showed partial or better responses (Raje et al., 2019).

In addition to targeting CD19 for relapsed or refractory pre-B acute lymphoblastic leukemia (B-ALL) a CD22-targeted CAR (CD22-CAR) was subjected to a phase I trial in children and adults (Fry et al., 2018). The outcome of the study was clinical activity in leukemia cases resistant to anti-CD19 immunotherapy and demonstration of potency against B-ALL comparable to CD19-CAR treatment. In another approach, to address relapses occurring for CD19 disease, bispecific anti-CD20, anti-CD19 (LV20,19) CAR T-cell therapy was applied for a first-in-human phase I study on relapsed and refractory B cell malignancies (Shah et al., 2020). Results from bispecific CAR treatment of adult patients with B cell non-Hodgkin lymphoma or chronic lymphocytic leukemia (CLL) demonstrated good safety, low toxicity and high efficacy. In a novel approach a novel CAR including both a single-chain variable fragment (scFv)-BCMA and an scFv-CD19 was engineered in tandem orientation (tan-CAR) to be able to target both BCMA and CD19 expressed on multiple myeloma cells (Kang et al., 2020). The tan-CAR T cells promoted significant anti-tumor activity against both BCMA and CD19 in tumor cells both in vitro and in vivo. Related to the B cell aplasia caused by "on-target off-tumor" toxicity associated with CD19-CAR T therapy, an inhibitory CAR (iCAR) was incorporated (Tao et al., 2020). The KIR/PD-1 inhibitory CAR (iKP CAR) was engineered by fusion of the extracellular domain of the killer cell immunoglobulin-like receptor 2DL2 (KIR2DL2) with the intracellular domain of PD-1. The iKP CAR showed strong cytotoxicity in vitro and anti-tumor activity in a xenograft model. In contrast, healthy human B cells were spared. Finally, the combination of the CRISPR/Cas9 technology with CAR T cell therapy may further enhance the safety and efficacy on cancer therapy (Mollanoori et al., 2018).

In summary, CAR T-cell technology can provide substantial advantages for immunotherapy and gene therapy applications. Second generation CARs, bispecific CARs, tan-CARs and iCARs have certainly widened the repertoire as described above. Combination of CAR T with CRISPR/Cas9 technology and addition of nanoparticle delivery methods has further increased the range of applications. Taking into account current development and the potential of future progress with technologies, the CAR T-cell technology presents an opportunity to achieve superiority compared to conventional chemotherapy and radiotherapy.

CRISPR

The discovery and development of the prokaryotic adaptive immune system, the CRISPR-associated Cas9 system, into a potent gene editing tool has taken not only molecular biology but also gene therapy to an unprecedented level (Lino et al., 2018). The technology has found applications in the development of model cell lines, drug target and drug mechanism discovery, development of transgene animals and plants and even in vivo human gene therapy. Delivery of CRISPR/Cas9 presents a serious challenge for therapeutic applications of gene editing (Mukalel et al., 2019). In this context, both physical methods such as microinjection and electroporation and biological methods based on viral and non-viral vectors have been applied. In the case of viral delivery, Ad, AAV and lentivirus vectors have been used, while non-viral delivery has relied on liposomes, polyplexes and gold particles.

A classic example of gene editing for gene therapy relates to homology directed repair in hepatocytes in a hereditary tyrosinemia type 1 mouse model (Yin et al., 2016). The Cas9 mRNA was delivered by LNPs while the subgenomic RNA expression cassette and homology directed repair template were delivered by AAV. Optimization of the delivery process resulted in gene editing in 6% of hepatocytes. In another approach, the proof-of-concept for CRISPR/Cas9-based gene editing for reduction of the transthyretin serum concentration was demonstrated by co-delivery of Cas9 mRNA and modified subgenomic RNA in ionizable and helper lipids (Finn et al., 2018). A single intravenous injection resulted in 70% gene editing and more than 97% knockdown in hepatocytes in mice.

The (CRISPR/Cas) system has also been engineered for gene editing approaches to treat HIV-1/AIDS (Xiao et al., 2019). In this context, CRISPR/Cas9 has been applied for targeting cellular co-factors or the HIV-1 genome to reduce HIV-1 infection and clear provirus. For instance, the CRISPR/Cas9 system has been applied to target the NF- κ B binding cassette in the U3 in the LTR region and the TAR sequences in the R region, which resulted in efficient inhibition of HIV-1 provirus transcription and replication in Jurkat cells (Ebina et al., 2013). Moreover, CRISPR/Cas9 could eliminate integrated HIV-1 genes from the host cell genome showing potential for HIV-1/AIDS treatment. In another study, it was demonstrated that the CRISPR/Cas9 system could target conserved sites in the HIV-1 LTR U3 region, which inactivated viral gene expression and restricted HIV-1 replication (Hu et al., 2014). Moreover, it was shown that the efficacy of excision of HIV-1 sequences and disruption of non-integrated proviral HIV-1 genome could be

achieved by targeting multiple sites in the HIV-1 genome (Liao et al., 2015). Viral replication and escape could be prevented by combining two effective subgenomic RNAs, which targeted different regions of the HIV-1 genome (Lebbink et al., 2017).

Another approach in HIV therapy relates to the C-C chemokine receptor type 5 (CCR5), which has a 32 bp deletion (CCR5Δ32) in a limited number of people rendering them resistant to HIV-1 infection (Galvani and Novembre, 2005). Hematopoietic stem transplantation (HSC) with CCR Δ32 donor cells can provide HIV-1 resistance for recipients, but with few donors available and a certain risk is associated with allogeneic HSC transplantation, an alternative approach is to apply the CRISPR/Cas 9 system (Cornu et al., 2015). A CRISPR/Cas9 gene editing system was established in human CD34⁺ hematopoietic stem/progenitor cells (HSPCs), which provided efficient CCR5 ablation in mice (Xu et al., 2017). The effect remained robust in secondary transplanted repopulated hematopoietic cells and most importantly HIV-1 resistance was observed. Furthermore, CRISPR-edited CCR5-ablated HSPCs were transplanted into a patient with HIV-1 infection and ALL (Xu et al., 2019). The ALL was in full remission and donor cells carrying the ablated CCR5 persisted for at least 19 months. No gene-editing adverse events were detected.

Approved gene therapy drugs

Gendicine™ was the first approved viral vector-based gene therapy drug for the treatment of head and neck cancer (Räty et al., 2008). The oncolytic Ad virus expresses the p53 tumor suppressor gene has been administered to more than 30,000 patients showing good safety and superior efficacy compared to standard therapy, especially when combined with chemotherapy and radiotherapy (Zhang et al., 2018b). A second-generation oncolytic HSV-GM-CSF vector has been approved for melanoma treatment in the United States and Europe (Fukuhara et al., 2016; Kaufman et al., 2010). Although the AAV-based treatment of the rare monogenic inherited disease lipoprotein lipase deficiency (Glybera™) was approved in Europe (Ylä-Herttuala, 2015) the license for its clinical use was not renewed because of insufficient demand. The first gene silencing drug to be approved was the LNP siRNA drug Onpattro for the treatment of polyneuropathy caused by transthyretin amyloidosis (Garber, 2018).

Several viral vector-based vaccines have received approval. The EBOV vaccine based on the VSV-ZEBOV vector was approved in December 2019 under the brand name Ervebo (Ollmann Saphire, 2020). Moreover, several Ad vector-based COVID-19 vaccines have received Emergency Use Authorization (EUA). For instance, the ChAdOx1 nCoV-19 received an EUA in the United Kingdom in December 2020 and in January 2021 in Europe. The single administration Ad26.COV2.S vaccine has also been approved in the United States. Moreover the Russian Sputnik V rAd26-S/rAd5-S received approval in Russia and some other countries.

In the context of CART T cell technology, CD19-derived CAR T cells have been approved by the FDA for use in children and young adults for treatment of ALL and in adults for DLBCL therapy. For additional information on approval of gene therapy-based drugs a list can be found at <https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products>.

Conclusions

After the setbacks in the 1990s with the death of a patient treated for the non-life-threatening ornithine transcarbamylase deficiency (OTC) with an Ad vector (Lehrman, 1999) and SCID-X1 patients developing leukemia after treatment with a retrovirus, gene therapy (McCormack and Rabbitts, 2004) encountered a major crisis with clinical trials putting on hold and funding drying out and pharmaceutical and biotech companies pulling out of gene therapy programs. Fortunately, gene therapy survived through intensive vector and protocol development. Substantially improved safety and delivery methods have been established placing the whole area of gene therapy in a new light. In addition to classic gene therapy applications based on non-viral and viral vector, alternative approaches using nanoparticle-based delivery, gene silencing, gene editing and CAR T cell technologies. The vaccine development has also seen unprecedented progress, partly due to the enormous resources dedicated to COVID-19 vaccine development. Both gene therapy drugs and vaccines have been approved and with hundreds of drug candidates in the pipelines and hundreds of clinical trials in progress, gene therapy finally seems to live up to the promise as the medicine of the future.

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Immunomodulatory Supplements

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Glossary

Bacterial exopolysaccharides Bacterial polysaccharides loosely attached or completely detached from the bacterial surface.

Dietary supplement Supplement or nutraceutical taken in the form a tablet, capsule, pill, powder or liquid.

Essential nutrient A nutrient that must be obtained through the diet, as its synthesis by an organism is insufficient.

Mineral supplement Inorganic element required by organisms as an essential nutrient in the diet and manufactured as a supplement.

Nutrient Substance needed by an organism to survive as well as to perform its basic functions.

Nutritional supplement Supplement that provides one or more nutrients an individual may otherwise not consume or absorb in sufficient quantities.

Probiotic Living microorganisms that are ingested to increase microbiota populations, intending to produce health benefits in the host and manufactured as a supplement.

Supplement Product taken by an organism to substitute a dietary constituent or to increase the physiological concentrations of a biochemical or biological component.

Synthetic supplement Supplements produced in a laboratory using a standardized procedure.

Vitamin supplement Organic compound required by organisms as an essential nutrient and manufactured as a supplement.

Introduction

For many centuries, traditional medicine has used products of animal or vegetable origin to treat various ailments including immune alteration and since the mid-20th century, when dietary supplementation became widely adopted, the production and consumption of natural and synthetic supplements has been on the rise. Currently, in many countries, more than half of the adult population consumes at least one daily supplement (Carpenter, 2003; Kiely et al., 2021). Examples of supplements with the highest rates of consumption include multivitamins, omega-3 oil, iron, calcium, probiotics and echinacea (Alpert, 2017). The mechanism of action of most of these compounds, including how they regulate immune function, has only recently been elucidated. In this article we will detail the most relevant information regarding the immunomodulatory role of synthetic and natural supplements. While the consumption of supplements may be highly convenient in individuals with nutrient or immune deficiencies, healthy individuals currently consume most supplements and do so in an unwarranted fashion. Moreover, excessive supplement consumption generates physiological and economical adverse effects (Ronis et al., 2018). Therefore, it is crucial that the public and health sectors properly regulate supplement consumption.

Synthetic nutritional supplements

The consumption of a rich and varied diet remains the most sensible method for continual maintenance of adequate physiological concentrations of macro- and micronutrients, a beneficial microbiome and proper immune system functioning (Durack and Lynch, 2019). Currently, supplementation is recommended exclusively for individuals with improper diets, or with malabsorptive, metabolic or nutrient consumption processes (Ames, 2004). Malnutrition, the lack of adequate physiological concentrations of macro- and micronutrients, is the most prevalent cause of secondary immunodeficiency. Half of the world's population suffers from this condition, which generates predisposition towards infection, which can further worsen nutrient deficiency (Chinen and Shearer, 2008). Importantly, supplementation may prevent both the occurrence and progression of infections in malnourished individuals (Mme, 2010).

In healthy individuals, nutritional requirements demand an average consumption of 100–1000 mg of specific micronutrients per day and 10–300 g of macronutrients per day. In patients with malabsorption or those that require parenteral nutrition or tube feeding, higher nutrient quantities are needed (Kennel et al., 2010). Nutritional supplements include vitamins and minerals (micronutrients), as well as proteins, lipids and carbohydrates (macronutrients) (Morris and Mohiuddin, 2020). The absorption profiles of nutrients found in food are generally greater than those found in synthetic supplements. Nevertheless, synthetic supplements present the advantage of containing copious quantified amounts of one or more nutrients, facilitating their administration in individuals with nutrient deficiencies (Thiel, 2000).

Macronutrients

Macronutrients are calorie-providing nutrients that need to be consumed in significant and balanced amounts, especially by athletes, to maintain body functions. Three broad categories of macronutrients exist: proteins, carbohydrates and fats. Individuals with recurrent or chronic diseases, as well as those with an improper diet are generally unable to obtain adequate amounts of macronutrients, thus affecting various physiological functions, including their immune response. Importantly, macronutrient supplementation is recommended in patients with a body mass index below 18.5, as it leads to a significant weight improvement after 2 months (Alpert, 2017).

Proteins/amino acids

Proteins, the most abundant macromolecules in the human body, are amino acid polymers linked by peptide bonds that provide the same energy density as carbohydrates. Protein depletion weakens the immune system by diminishing white blood cell count and immune protein synthesis and may occur after prolonged starvation, recurrent or chronic infections and/or cancer (Alpert, 2017). In addition, during infection, muscle protein is catabolized to provide amino acids for immune cell, protein, and peptide synthesis (Batatinha et al., 2019). Protein-calorie malnutrition is one of the most common forms of malnutrition in underdeveloped countries and leads to HIV disease and tuberculosis (TB) progression. In TB patients, protein supplementation favors treatment completion and sputum conversion (Koethe et al., 2009). In addition, supplementation with whey protein improves nutritional status, immunity and inflammation resolution in protein deprived patients (Bumrungpert et al., 2018). Crucially, protein supplementation is not associated with adverse effects when balanced with other macronutrients (Koethe and Von Reyn, 2016).

Protein malnutrition diminishes plasma amino acid concentrations and alters: (1) proliferation and activity of macrophages and of T, B and natural killer (NK) lymphocytes (2) cellular redox state (3) antibody, cytokine and cytotoxic compound production. Supplementation with specific amino acids (particularly glutamine, arginine, and cysteine) in animals or humans with malnutrition and infection may improve immune status, morbidity and mortality (Li et al., 2007). Glutamine is the most abundant amino acid in the body and is essential for protein synthesis in all cell types. Moreover, during immune activation, immune cells initiate glutaminolysis depleting glutamine reserves (Batatinha et al., 2019). Therefore supplementation with glutamine and other highly

relevant amino acids, such as the branched-chain amino acids (leucine, isoleucine and valine), whose depletion lowers lymphocyte proliferation and increases susceptibility to infection is sensible in individuals with protein malnutrition (Calder, 2006). For an in-depth analysis of the individual immunomodulatory effects of supplementation with different amino acids, the authors refer the reader to the detailed review by Li et al. (2007).

Lipids

Fatty acids are macromolecules that take part in vital cellular processes including energy generation, protein conformation and gene expression regulation. These macromolecules modulate immunological processes including T-cell signalling, phagocytosis, eicosanoid formation and complement system activation. Fatty acids can be classified according to their structural characteristics, including carbon saturation: saturated fatty acids possess no double carbon bonds, and unsaturated fatty acids possess one or more double carbon bonds. Importantly, modifying diet composition alters physiological fatty acid concentrations and cellular functioning (Trichet, 2010).

Polyunsaturated fatty acids, including omega-3 and to a lesser extent omega-6 fatty acids, are the most sought-after fatty acid supplements, although assorted lipid supplements are currently being tested for the treatment of malnutrition (Dewey and Arimond, 2012).

Omega-3/6 fatty acids are polyunsaturated fatty acids that possess a double bond three/six atoms apart from the terminal methyl group (Gutiérrez et al., 2019). Eicosapentaenoic acid and docosahexaenoic acid found in fish and other seafood oils, and alpha-linolenic acid found in plant oils, represent the three main omega-3 fatty acids. Fish oil supplements have been shown to decrease inflammatory markers in patients with cardiovascular disease, colorectal cancer and rheumatoid arthritis (Knapp and FitzGerald, 1989; Wang et al., 2006; Silva et al., 2012). While plant oil supplementation has shown to reduce inflammation markers in hyperlipidemic individuals (Micallef and Garg, 2009). In addition, supplementation with omega-3/6 fatty acids diminishes inflammation markers in patients with skin inflammatory diseases such as psoriasis and acne (Balić et al., 2020).

Pro-resolving mediators, metabolites of omega-3 fatty acids, comprise leukotrienes, maresins, protectins, prostaglandins, resolvins and thromboxanes. These mediators downregulate proinflammatory cytokine and chemokine production, MHC and co-stimulatory molecule expression, basophil, mast cell and eosinophil activation and Th1 and Th17 differentiation (Gutiérrez et al., 2019). Omega-3 fatty acids appear to reduce inflammatory gene expression through the blockade of NF-κB and increase anti-inflammatory gene expression through stimulation of the peroxisome proliferator-activated receptor γ (PPAR- γ) (Calder, 2017). Moreover, through Treg differentiation, omega-3 fatty acid supplementation has improved autoimmune disease (Farjadian et al., 2016) as well as decreased the severity of mast cell-associated diseases including atopic dermatitis (Haitz and Anandasabapathy, 2015).

Carbohydrates

Carbohydrates are macromolecules composed of carbon, hydrogen and oxygen atoms and are essential macronutrients, especially for malnourished populations as they help in consumption compliance by adding taste and flavor (Kokkinidou et al., 2018). These macromolecules are further divided into monosaccharides, disaccharides oligosaccharides and polysaccharides based on the number of sugar groups that conform them. Monosaccharides, disaccharides and oligosaccharides are not frequently consumed as immunomodulatory supplements, except for chitoooligosaccharides and other prebiotics that possess selective immunostimulant and anti-inflammatory actions (Hyung et al., 2006). On the other hand, polysaccharides possess significant immunomodulatory effects. For an in-depth analysis of the immunomodulatory effects of polysaccharide supplements, the authors refer the reader to the detailed review by Ramberg et al. (2010).

Micronutrients

Vitamins and minerals are essential compounds required in lesser quantities than macronutrients. Micronutrient supplementation has been shown to enhance resistance to illness and improve immune responses in frequently malnourished populations such as the elderly, vegans and pregnant women (Bogden et al., 1994; Alpert, 2017). Multivitamins (synthetic supplements containing several vitamins and minerals) should preferably be used when treating multiple micronutrient deficiencies, such as in individuals belonging to the previously mentioned frequently malnourished populations, as micronutrients may compete with each other for intestinal absorption. Multivitamin supplementation may therefore not be ideal in populations lacking a single micronutrient (McCormick, 1999). Equally important, supplementation given to individuals with adequate physiological concentrations of micronutrients does not appear to affect their immune response (Mitchella et al., 2003).

Minerals

In biochemistry, essential trace elements are inorganic compounds obtained from the diet and required in very small quantities to sustain an organism's physiological requirements (Sabine, 1967). Adequate assessment of trace mineral concentrations is highly complicated and expensive, and therefore rarely evaluated (Delves, 1985). Furthermore, clinical trial data regarding mineral supplementation is inconsistent. Here we will focus on mineral supplements with well-known immunomodulatory properties.

Zinc

Zinc, the most abundant intracellular trace element, enhances skin and mucosal membrane integrity and improves innate and adaptive immunity (Alpert, 2017). Its deficiency affects 2 billion people worldwide, most of which are elderly and/or live in underdeveloped nations, hindering innate immune protection and favoring infection, oxidative stress and adaptive immune overreactivity (Wessels et al., 2017). Zinc supplementation potentiates acute and chronic viral and bacterial clearance (Acevedo-Murillo et al., 2019; Read et al., 2019) and has saved millions of children's lives in developing countries (Bajait and Thawani, 2011).

Selenium

Selenium influences the cellular innate and adaptive immune responses, as well as antioxidant function (Wintergerst et al., 2007). Adequate selenium status is essential in counteracting certain viral infections and is considered the most vital nutrient for AIDS patients (Broome et al., 2004; Alpert, 2017). Selenium's organic supplementation possesses a better absorption profile than its inorganic counterpart and in patients with autoimmune thyroiditis and Graves' disease, it reduces antithyroperoxidase antibody levels and improves life quality (Ventura et al., 2017).

Iron

Iron favors immune cell proliferation, maturation and polarization (specifically in lymphocytes) and protects against infection (Alpert, 2017). Importantly, iron deficiency is highly prevalent in developing countries and unfortunately in vivo and in vitro studies have reported adverse effects of oral iron supplementation on gut microbiota composition, affecting immune regulation (Kortman et al., 2017). Furthermore, as pathogenic microorganisms use iron as a nutrient source, a "goldilocks threshold" when administering iron supplementation to an infected individual must be reached to reap the most immune benefits without favoring the pathogen (Agoro and Mura, 2019).

Vitamins

Vitamins and their metabolites regulate critical immunological processes through their coadjuvant functions. Vitamins C and E and members of the B complex act nonspecifically, while vitamins A and D, through their metabolites, directly influence the immune response by binding to nuclear-hormone receptors (Mora et al., 2008). Crucially, vitamin deficiency is a significant suppressor of immune function that increases susceptibility to cancer and infection (Alpert, 2017).

Vitamin A

Vitamin A is obtained from the diet either as retinol, retinyl esters or β -carotene. Retinol supplementation directly regulates innate and cell-mediated immunity as well as antibody responses, while supplementation with β -carotene improves IL-2 receptor expression and NK cell proliferation (Watson et al., 1991; Santos et al., 1996). In tissues, retinol, retinyl esters and β -carotene are oxidized to all-trans-retinal, an active form of vitamin A (Moise et al., 2007) and then to retinoic acid, another active form of vitamin A (Mora et al., 2008). DCs from Peyer's patches can synthesize retinoic acid from vitamin A (Lampen et al., 2000). Retinoic acid regulates antigen presentation and DC function through retinoid X receptor stimulation (Geissmann et al., 2003). In addition, retinoic acid promotes T-cell proliferation, Th2 and cytotoxic responses through retinoic acid receptor stimulation (Mora et al., 2008). During vitamin A deficiency, mucosal epithelium integrity is compromised, favoring pathogen invasion. Moreover, during vitamin A deficiency an increase in the severity and duration of infectious diseases generally occurs (Underwood and Arthur, 1996; Tang and Smit, 1998). For an in-depth analysis of the individual immunomodulatory effects of vitamin A supplementation, the authors refer the reader to the detailed review by Villamor et al. (Villamor and Fawzi, 2005).

Vitamin B

The vitamin B complex includes eight B vitamins, however we will detail three of them with well-known immunomodulatory properties. Vitamin B6 regulates the adaptive immune response by modulating thymus size, B and T lymphocyte maturation, proliferation and function. Deficiency of this vitamin is commonly found in young malnourished women, elderly and AIDS patients (Cheng et al., 2006; Alpert, 2017). Large dose supplementation may correct deficiency of this vitamin, and may also suppress pro-inflammatory cytokine production in patients with autoimmune disease (Huang et al., 2010). Folate, also known as vitamin B9 or folacin, is required for cell division processes including DNA synthesis and cell-mediated immunity. Vitamin B9 deficiency alters the induction of intestinal T regulatory (Treg) cells favoring intestinal inflammation and can be corrected with folic acid (the man-made precursor of folate) supplementation (Mikkelsen and Apostolopoulos, 2019). Vitamin B12 regulates leukocyte division and antibody formation. Healthy older immunocompetent adults with low serum concentrations of vitamin B12 show impaired antibody responses to vaccines (Alpert, 2017). While supplementation with B12 may improve vaccine-specific responses (Siddiqua et al., 2016).

Vitamin C

Vitamin C or ascorbic acid, stimulates diverse leukocytes functions, specifically neutrophil and monocyte chemotaxis, T lymphocyte proliferation and cytokine and immunoglobulin synthesis. Under physiological conditions, vitamin C functions as an antioxidant

in plasma. However during infection, vitamin C levels diminish after their consumption by white blood cells and supplementation allows immunological function recovery (Alpert, 2017). To address the topic of vitamin C supplementation and immunomodulation the authors recommend the detailed review by Webb and Villamor (2007).

Vitamin D

Regarding immunomodulation, the most physiologically relevant form of vitamin D is probably vitamin D₃ or cholecalciferol, a dietary prohormone whose synthesis can be generated in the skin from 7-dehydrocholesterol after sunlight exposure. Vitamin D₃ can undergo transformation within the liver to the main circulating form of VD₃, 25-dihydroxyvitamin D₃ (25(OH)VD₃) or calcifediol. Finally, 25(OH)VD₃ metabolization to 1,25(OH)₂VD₃ or calcitriol (the most physiologically active VD₃ metabolite) occurs in the kidneys. Immune cells (T and probably B lymphocytes, macrophages and some dendritic cells (DCs), such as monocyte-derived DCs and dermal DCs) can also metabolize VD₃ and concentrate its specific actions within immune microenvironments, limiting undesirable systemic effects (Van Etten and Mathieu, 2005; Holick and Chen, 2008; Mora et al., 2008; Rolf et al., 2014). Interestingly, immature thymocytes and mature CD8⁺ T lymphocytes possess the highest concentration of vitamin D receptors (Alpert, 2017).

Innate immune cells may be inhibited by calcitriol. Calcitriol inhibits differentiation, maturation and immunostimulatory capacity of DCs by downregulating CD40, CD80, CD86 and MHC II expression. Furthermore, by suppressing their IL-2 synthesis, calcitriol blocks Th1 responses and increases IL-10 expression (Mora et al., 2008). However, calcitriol acts primarily in the adaptive immune response, inhibiting T-cell proliferation, IL-2 and interferon- γ (IFN γ) production, as well as cytotoxic CD8 T-cell activity (Mora et al., 2008). Moreover, calcitriol's inhibitory effects are mostly observed in effector and memory T-cells, as they express more vitamin D receptor (Veldman et al., 2000). In addition, calcitriol suppresses primary mixed-lymphocyte reactions and cytotoxic T-cell responses (Meehan et al., 1992). Overall, Calcitriol blocks Th1 cytokines by decreasing IFN γ production and favoring IL-4 production (Van Etten and Mathieu, 2005). Crucially, calcitriol inhibits Th17 responses due to its inhibition of IL-6 and IL-23 production and expansion of forkhead box protein 3 (FOXP3⁺) Treg cells. Calcitriol also decreases B-cell proliferation, plasma-cell differentiation and IgG secretion and may play a role in the pathophysiology of system lupus erythematosus (SLE) (Mora et al., 2008).

Vitamin D reference ranges in the population vary significantly depending on the geographic location, sampling season, and ethnic background of the studied population. During winter, in northern latitudes, close to three quarters of the population may have some degree of vitamin D deficiency. In other locations, 50% or more of patients commonly encountered in clinical practice may have vitamin D deficiency, especially those with darker skin color (Reusch et al., 2009). Measuring total 25(OH)D levels may be the best way to assess body stores of vitamin D. Importantly, clinicians should avoid measuring 1,25(OH)₂D levels in the diagnosis of hypovitaminosis D, for it may lead to a misinterpretation of vitamin D status due to the fact that calcitriol levels may appear in normal or even elevated range in patients with vitamin D deficiency as a result of elevated parathyroid hormone levels (Kennel et al., 2010). The consequences of vitamin D deficiency include impaired immunity and increased risk of autoimmunity (DeLuca, 2004). Furthermore, appropriate supplementation with vitamin D₃ in susceptible populations taken with a fat containing meal may significantly reduce the risk of respiratory and systemic infections, possibly by increasing the production of antimicrobial peptides such as cathelicidin (Kennel et al., 2010; Camargo et al., 2012; Gunville et al., 2013; Martineau et al., 2017).

Vitamin E

Vitamin E is an antioxidant that protects cell membrane integrity from free radicals and due to their high polyunsaturated fatty acid content, leukocyte cell membranes are particularly vulnerable (Coquette et al., 1986). Approximately, 66% of older adults have low levels of vitamin E (Alpert, 2017) and disturbingly, vitamin E supplementation is frequently taken at improper concentrations that do not provide therapeutic effects (Meydani et al., 1997). Vitamin E deficiency increases PGE₂ and plasma lipid peroxide levels and consequently decreases delayed type hypersensitivity and antibody responses, as well as IL-2 production and lymphocyte mitogenic responses to concanavalin A. Proper supplementation of this vitamin replenishes these responses (Meydani et al., 1986).

Other synthetic supplements

Non-vitamin and non-mineral synthetic supplements (informally referred to as "pseudovitamins") include biochemical compounds, such as certain antioxidants and coenzymes that are usually irretrievable in significant quantities from the diet and may improve the host's immune response. Due to the vast amount of synthetic non-nutritional supplements, in this section we will only mention two groups of these supplements which appear to have important immunomodulatory actions. For a more in depth review of synthetic supplements, including their immunomodulatory actions, the authors recommend the compendium by Seyed Mohammad Nabavi (2018).

Ketones are products derived from fatty acids that can be converted and shunted into the citric acid cycle for energy generation. Despite the fact that various natural and synthetic ketone supplements have been produced, their consumption is currently not recommended, due to their undesired proinflammatory effects including NLRP3 inflammasome activation (Correia-Melo et al., 2019; Neudorf et al., 2019).

Glutathione (GSH) is an anti-inflammatory antioxidant whose concentrations regulate lymphocyte activity. Supplementation with GSH or with its precursor *N*-acetylcysteine is associated with cytokine balance and downregulation of inflammation in the

elderly and in individuals with chronic inflammation or chronic infection. However, several studies have reported harmful, pro-oxidant effects of these supplements. For this reason, GSH, its precursor and other antioxidant supplements should be prescribed with extreme caution (Ju et al., 2017).

Plant-derived supplements

Plants and their metabolites have been typically used in traditional medicine for their immunomodulatory properties and arguably represent the largest category of supplements (Dell'agli et al., 2013). Nonetheless, it's important to mention that these supplements have induced pathological reactions in patients including severe allergies and drug interactions (Gertsch et al., 2011; Brewer and Chen, 2017; Hudson et al., 2018). Due to the vastness of plant-derived supplements, in this section we will only describe those with well-known immunomodulatory properties. If the reader wishes to consult a more in-depth review of plant-derived supplements including their immunomodulatory actions, the authors recommend the detailed reviews of Sultan et al. (2014) and Seyed Mohammad Nabavi (2018).

Camellia sinensis is a shrub belonging to the Theaceae family used for the preparation of green tea and as a supplement. The anti-inflammatory effects of its metabolites (flavonoids, catechins and polyphenols) have been observed in non-alcoholic steatohepatitis, cancer, cardiovascular disease and asthma. Additionally, these compounds possess antiviral, antibacterial, antiparasitic and immunoactivating qualities. More specifically, they are capable of decreasing Th17 and neutrophil, as well as IgE and histamine responses while stimulating NK cell cytotoxicity (Aboulwafa et al., 2019; Chen et al., 2019).

Dzherelo is a concentrated aqueous alcohol extract composed of various plant-derived products that has been used extensively in the treatment of autoimmune disease, malignancy, as well as chronic bacterial and viral infections. Clinical studies indicate that its consumption increases CD4 T-lymphocytes and decreases HIV viral and TB bacterial load, synergizing with drug therapies and alleviating hepatotoxicity. In addition, Dzherelo appears to reduce HIV/AIDS-opportunistic infections and reverse TB-associated wasting (Zaitzeva et al., 2009).

Fruit-derived supplements

Resveratrol is a widely known polyphenolic stilbenoid found in grapes, blackberries, peanuts, rhubarb, and several other plants. It has been used for the prevention and progression of chronic inflammatory diseases, such as diabetes, obesity, cardiovascular and neurodegenerative diseases, among other conditions. Resveratrol regulates leukocyte activity, as well as the synthesis of proinflammatory cytokines through nuclear factor- κ B (NF- κ B) and toll-like receptor (TLR) regulation. In addition, resveratrol inhibits the expression of proinflammatory eicosanoids (Harikumar and Aggarwal, 2008; Pennisi et al., 2017; Malaguarnera, 2019).

Mangosteen (*Garcinia mangostana*) is a fruit of Asian origin, belonging to the Guttiferous family. It contains a wide variety of biologically active molecules, including catechins, polyphenolic compounds (such as flavonoids, tannins and anthocyanins), vitamins B1, B2, B6 and C; minerals (potassium, phosphorus, calcium and iron) and xanthenes that have anti-inflammatory and antioxidant properties (García Font and Ortega Hernández-Agero, 2011). Mangosteen derivatives with specific immunomodulatory properties include the xanthenes α -mangosteen and γ -mangosteen (Chairungsri et al., 1996). In a murine model of asthma it was reported that these xanthenes reduced disease symptoms (Jang et al., 2012). Likewise, in vitro and in vivo studies showed that *Garcinia mangostana* extracts possess anti-inflammatory effects through the regulation of prostaglandins, CRP and NF- κ B (Nakatani et al., 2002, 2004; Tang et al., 2009). In humans, these extracts increased peripheral complement system components and T lymphocyte proliferation (Tang et al., 2009).

Root-derived supplements

Echinacea spp., a plant native to eastern and central North America and used by indigenous tribes, has been used to treat upper respiratory tract infections due to its selective pro- and anti-inflammatory properties (Schoop et al., 2006). *Echinacea*'s Th1 cytokine inhibition appears to be induced through stimulation of the endocannabinoid system (Chicca et al., 2009).

Ginseng, obtained from the root of *Panax quinquefolius*, is a herb that has been widely used in traditional medicine to induce immune homeostasis and prevent infection. Ginseng contains several pharmacological components, such as tetracyclic triterpenoid saponins (ginsenosides), polyacetylenes, polyphenolic compounds and acidic polysaccharides (Kang and Min, 2012; Riaz et al., 2019). Ginseng extracts have been reported to activate macrophages and have the ability to modulate DC function, increase cytokine production and enhance T lymphocyte activation (Shin et al., 2002; Riaz et al., 2019). In addition, these extracts are capable of promoting Th1 cell differentiation and CD8⁺ T lymphocyte and NK cell cytotoxicity during intracellular infection (Riaz et al., 2019). Ginseng has also been reported to promote humoral immunity through the potentiation of antibody production (Liou et al., 2005). On the other hand, ginseng improves chronic inflammatory disease symptoms, through pro-inflammatory cytokine reduction (Kang and Min, 2012; Riaz et al., 2019).

Ginger (*Zingiber officinale*) is a Chinese flowering plant that belongs to the Zingiberaceae family. The rhizome (the part of the stem that grows under the ground) is generally used for production of the supplement and contains terpenes, phenolic compounds (gingerol, shogaol and paradol) and some flavonoids (quercetin, capsaicin). Ginger supplementation been used as an adjuvant treatment in rheumatic, gastrointestinal and cardiovascular diseases due to its anti-inflammatory effects (Mashhadi et al., 2013;

Mahassni and Bukhari, 2019). Its anti-inflammatory actions include the reduction of CRP and TNF- α serum levels in animal models and humans (Tripathi et al., 2006; Morvaridzadeh et al., 2020). It has been proposed that this effect is due to the action of 6-gingerol, which appears to inhibit the NF- κ B activation (Tripathi et al., 2006).

Curcumin is an extract from the root of turmeric (*Curcuma longa*), a plant native to India that contains different percentages of volatile and non-volatile oils, proteins, fats, minerals, carbohydrates and curcuminoids. Curcuminoids are a group of molecules made up of curcumin (60–70%), demethoxycurcumin (20–27%) and bisdemethoxycurcumin (10–15%) (Gautam et al., 2007; Abdollahi et al., 2018). Curcumin has been reported to have anti-inflammatory effects and has been used as an adjunct in the treatment of arthritis, allergies, asthma, and cardiovascular diseases. Its anti-inflammatory effects appear to be related to the modulation of T and B lymphocyte, NK cell, macrophage and DC activation, as well as a reduction in proinflammatory cytokines through the inhibition of NF- κ B and MAPK (Jagetia and Aggarwal, 2007). Furthermore, in vitro experimentation has demonstrated that curcumin inhibits STAT3 activation (Bill et al., 2012).

Garlic is a bulb derived from the flowering plant *Allium sativum*, whose supplementation has been used by Egyptian and Chinese traditional medicine. Supplementation with aged garlic extract (AGE) which contains derivatives such as sulfur-containing compounds, upregulates $\gamma\delta$ -T and NK cell proliferation and activation and appears to reduce cold and flu severity and duration (Percival, 2016). In addition, AGE supplementation decreased serum TNF- α and IL-6 blood concentrations in adults with obesity (Xu et al., 2018).

Microalgae-derived supplements

Microalgae are unicellular, microscopic algae which possess bioactive compounds, such as vitamins, lipids, carbohydrates, and pigments. Various studies have shown that microalgae have anticancer, antimicrobial, antiepileptic, anti-inflammatory activities (Riccio and Lauritano, 2019). In vitro experimentation has demonstrated that microalgae block the release of TNF- α and nitric oxide (Samarakoon et al., 2013; Lauritano et al., 2016). While in animal models, microalgae have been shown to have protective effects in inflammatory bowel disease (Lavy et al., 2003). Finally, in patients with non-alcoholic fatty liver, the administration of *Chlorella vulgaris* decreased TNF- α concentrations (Ebrahimi-Mameghani et al., 2017).

In addition, different compounds derived from microalgae, such as sulfo-polysaccharides, sulfated lipids, polyunsaturated fatty acids and astaxanthin possess immunostimulant activities (Lauritano et al., 2016). Extracts from multiple microalgae appear to be capable of inducing IL-6 production in human peripheral blood mononuclear cells (Cutignano et al., 2015). Furthermore, in a murine model, the administration of *Chlorella stigmatophora*, a marine microalgae, increased phagocytic activity in macrophages. While supplementation with *Euglena gracilis*, a unicellular microalgae, increased the activity of NK cells and TNF- α and IL-6 levels (Riccio and Lauritano, 2019). Unfortunately, the precise immunostimulatory mechanisms of microalgae have not yet been elucidated (Riccio and Lauritano, 2019).

Perhaps the most popular microalgae supplement is spirulina, a microalgae with a phycocyanin (blue) and chlorophyll (green) structure. Spirulina is rich in several macro- and micronutrients and possesses antioxidant and more than 50 reported immunomodulatory properties, including upregulation of IgA, Th1 and cellular immunity, as well as downregulation of histamine, Th2 cytokine and IgE blood concentrations. Unfortunately, there have been reports of patients that developed autoimmune disorder exacerbations after taking spirulina as well as other microalgae-derived supplements (Lee and Werth, 2004).

Prebiotics

The microbiota, the collection of microorganisms within the human body, plays a fundamental role in generating immunological tolerance and integrity throughout the digestive tract (Galdeano and Perdigón, 2004). Among the metabolites produced by the microbiota, are short-chain fatty acids (SCFA), which possess anti-inflammatory properties such as the induction of regulatory cells and cytokines (Morrison and Preston, 2016). However, when dysbiosis (altered microbiota populations) is present, SCFA production decreases and long chain fatty acid production increases, promoting proinflammatory immune cell activation and intestinal permeability (Machate et al., 2020). Crucially, inflammation caused by dysbiosis, has been associated with inflammatory bowel disease, insulin resistance and obesity (Silva Figueiredo et al., 2017; Zhang et al., 2017; Machate et al., 2020).

Prebiotics are insect and plant-based compounds that favor microbiota growth. Most of these compounds are rich in fiber, and are thus nutritious for the large intestine's commensal microbiota and improve its constitution (Davani-Davari et al., 2019). Crucially, if commensal microbiota species are absent in the host due to previous disease or drug therapy, the beneficial effects of prebiotics are unlikely to be observed (Macfarlane et al., 2008). The most used prebiotics are fructooligosaccharides, inulin, isomaltooligosaccharides, lactulose, lactosucrose and galactooligosaccharides. These are obtained in foods such as whole grains, bananas and green leafy vegetables, but can also be found in the form of supplements (Azad et al., 2018; Davani-Davari et al., 2019). In vitro, as well as human and animal model experimentation, has demonstrated that prebiotics can generate immunomodulatory effects in the intestinal mucosa and lymph nodes, as well as in distal organs such as the spleen. These effects include enhanced synthesis and secretion of cytokines and immunoglobulins, as well as improved vaccine and inflammation resolving responses (Macfarlane et al., 2008; Zhang et al., 2017).

Prokaryotic-derived supplements

Probiotics

Probiotics are live microorganisms, which are consumed to improve microbiota composition. The most used genus of probiotics include *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Lactococcus*, *Streptococcus*, *Saccharomyces*, among others (Azad et al., 2018). Most probiotics are bacteria which produce lactic acid and can be consumed in the diet (yogurt, fermented milks or products), and they can also be found in large numbers as commercial supplements (Wilkins et al., 2017; Azad et al., 2018). Probiotics act by fermenting indigestible foods and their functions can be classified as metabolic, protective and trophic (Girardin and Seidman, 2011). Through the production of non-toxic metabolites and other actions, probiotics compete for the exclusion of local pathogens, nurture, mature and differentiate resident and distant immune cells, as well as strengthen the intestinal barrier (Kau et al., 2011; Bermudez-Brito et al., 2012; Adak and Khan, 2019).

In recent years, it has been reported that probiotics are capable of modulating humoral and cellular immunity, in addition to functioning as an immune barrier by preventing the growth of pathobiont and pathogenic microorganisms (Kau et al., 2011; Bermudez-Brito et al., 2012). In vivo effects of probiotics include increased IgA production and decreased pro-inflammatory cytokine production (Azad et al., 2018). For example, it was reported that the administration of *Bifidobacterium bifidum* R0071, *Bifidobacterium infantis* R0033, and *Lactobacillus helveticus* R0052 in children increased the concentration of IgA in saliva and the digestive tract (Xiao et al., 2017). In addition, in a murine model, *L. casei* DN 11400, found in breast milk, improved mucosal immunity by increasing the amount of T and B lymphocytes and secreted IgA (Galdeano et al., 2009; Azad et al., 2018).

Probiotics in several studies have shown the ability to regulate inflammation not only in the gastrointestinal tract but also in distal organs. Recently, “sterilized probiotics” or paraprobiotics have also demonstrated immunomodulatory potency beyond their viability (Kanauchi et al., 2018). The immunomodulatory effects of probiotics have become relevant for the treatment of chronic degenerative diseases, which usually present with deregulation of the gut microbiota. Recent studies have associated dysbiosis with the pathogenesis of psychiatric disorders such as depression and schizophrenia (Fond et al., 2020). Inflammation due to dysbiosis has been described to cause tryptophan metabolism alterations concomitant with a decreased synthesis of serotonin and an increased synthesis of kynurenine. The accumulation of this last compound, which is capable of crossing the blood-brain barrier, may cause significant central nervous system adverse effects (Waclawiková and El Aidy, 2018). Importantly, probiotic consumption has been associated with a reversal of this pathological process and symptom improvement in several neuropsychiatric diseases including schizophrenia. Experimental studies nevertheless remain inconclusive regarding the role of probiotics in depression (Grover et al., 2019).

Other prokaryotic derived supplements

Bacterial and other microbial derived macromolecules such as exopolysaccharides, enzymes and metabolites (also termed “postbiotics”) represent a promising tool in the field of immunomodulatory supplements and clinical studies evaluating these compounds are currently underway (Descamps et al., 2019; Angelin and Kavitha, 2020).

Mammal-derived supplements

Mammal-derived supplements include immune and hormonal products discovered in mammals which can be obtained naturally or synthetically.

Dialyzable leukocyte extracts (DLE)

DLE are molecules weighing less than 12 kDa that are obtained from the breakdown of leukocytes and their subsequent dialysis. This process was described by Dr. Sherwood Lawrence in the 1940s when he controversially proposed that components of these dialysates transfer the ability to generate a specific immune response. Generally, the components of DLE are classified into two groups (Macías and Guaní-Guerra, 2020):

- (a) The antigen-specific or antigen-dependent fraction comprising molecules of peptide nature with a molecular weight of 3.5–6 kDa, termed transfer factor (TF).
- (b) The nonspecific antigen or antigen-independent fraction, comprises molecules below 3.5 and above 6 kDa, including the immunoregulatory molecules known as IMREG-1 and IMREG-2. Both IMREG molecules may modulate, but not transfer late hypersensitivity responses (Gottlieb and Gottlieb, 1990). Several other immunomodulatory components have been isolated from DLE. These include immunologically active peptide sequences such as the S100A9 peptide, as well as TCR portions from cytotoxic lymphocytes, among many others (Myles et al., 2017). Interestingly, some components appear to be immunostimulatory while others immunosuppressive (Sherwood Lawrence and Borkowsky, 1996).

So far, DLE (including TF) have been obtained from three sources:

1. Blood and lymphoid tissue: after the rupture of the leukocytic bundle ("buffy coat"), taken from a unit of human or animal blood, or from lymphoid tissue such as the spleen (Estrada-Parra et al., 1998; Macías and Guaní-Guerra, 2020).
2. Hen egg yolk: after hens have been exposed to specific pathogens (Xu et al., 2006).
3. Colostrum: The first form of milk, after newborn delivery from various mammals, has been used as a popular supplement in different commercial presentations. Colostrum contains macro- and micronutrients, as well as bioactive molecules such as growth factors and a plethora of antimicrobial compounds (DLE, immunoglobulins, lactoperoxidase, lysozyme, and lactoferrin). Bovine colostrum consumption has shown potentiation of different immune functions, including salivary IgA production (Mero et al., 2002). DLE have also been obtained from human and animal colostrum, which represents a relatively cheap and simple method of production. And importantly, preliminary studies suggest that DLE from different animal sources possess similar immunomodulatory efficacies (Altamirano et al., 2006).

Various reports using in vitro and murine models of autoimmunity have described the innate and adaptive immunomodulatory effects of DLE and TF. However, the precise immunomodulatory mechanism of action of these compounds in humans remains unclear, making their use controversial. An important effect caused by DLE and TF appears to be the induction of migration inhibitory factor (MIF), which activates macrophages and T lymphocytes and favors wound repair (Wilson et al., 1982). In addition, DLE have been used as an adjuvant treatment with mild to moderate success in several clinical studies involving patients with primary immunodeficiencies such as Wiskott-Aldrich syndrome and Ataxia-Telangiectasia; infections such as mucocutaneous candidiasis, cytomegalovirus, herpes zoster, tuberculosis, leprosy or cutaneous leishmaniasis; and autoimmune diseases such as Behcet's syndrome or chronic discoid lupus erythematosus (Gottlieb and Gottlieb, 1990; Fudenberg and Pizza, 1994; Estrada-Parra et al., 1998).

The composition of a patented DLE termed Transferon[®], which increases physiological concentrations of IFN- γ , TNF- α , and IL-6 in immunosuppressed patients, has recently been reported. This sequence includes large amounts of ubiquitin (Ub) and a variant of Ub that lacks the biterminal glycine. In a murine model of herpes zoster infection, oral administration of recombinant Ub decreased overall mortality. Furthermore, this effect was enhanced after combining Transferon[®] with Ub, suggesting that the biological properties may be partially associated this molecule. Additionally, it was postulated that Ub appears to activate the stomach's CXCR4 receptor inducing vagus nerve stimulation and an accompanying anti-inflammatory response (Vallejo-Castillo et al., 2020).

Importantly, caution should be exercised when recommending the use of DLE and TF, as they have been tested mainly as adjuvant therapy and results from one DLE cannot be extrapolated to another, because it is unknown if their component concentrations are equivalent. Therefore, it is important to emphasize that currently, there is insufficient evidence to recommend TF or DLE as supplements, and even less so as therapeutic drugs, although this may change in the future (Macías and Guaní-Guerra, 2020).

Hormones

Most synthetic hormones, such as estrogens, are not classified as supplements by governmental agencies. Instead, most of these commercial compounds are classified as therapeutic drugs due to the fact that high grade clinical evidence has demonstrated their beneficial effect in the treatment and prevention of diseases. Nonetheless, we will describe two hormones currently categorized as supplements that possess important immunomodulatory properties: melatonin and dehydroepiandrosterone (DHEA).

Melatonin is a hormone found in humans, animals, plants, fungi and bacteria in concentrations that vary according to the day/night cycle. Melatonin is synthesized from the essential amino acid tryptophan. It is produced mainly in the pineal gland and is involved in a wide variety of cellular, neuroendocrine and neurophysiological processes, such as the regulation of sleep/wake cycles (Altamirano et al., 2006). Although melatonin binds to membrane (MT1 and MT2, G coupled protein receptors) and nuclear receptors, many of its actions are receptor-independent (e.g., free radical scavenging and interaction with cytosolic proteins such as calmodulin).

Melatonin has many immunoregulatory properties. Crucially, in animals, the absence of this hormone inhibits immune maturation and activation. In contrast, a nocturnal increase of melatonin blood concentrations has been correlated with increased production of thymic hormones that aid in immune maturation (Altamirano et al., 2006). Various studies support the immunoregulatory action of melatonin on innate immunity, showing that it stimulates the production of granulocyte and macrophage progenitor cells and has a general stimulatory action on hematopoiesis. In addition, melatonin administration increases NK cell and monocyte counts and enhances the phagocytic activity of macrophages. Melatonin also appears to regulate cytokine production.

Several cell culture and animal models of infection and autoimmunity, as well as clinical studies of vaccine administration and immunosenescence therapy, have corroborated the efficacy of melatonin as an adjuvant (Srinivasan et al., 2005; Carrillo-Vico et al., 2013). The effects of melatonin in humans have been observed in neonates and adults with sepsis, demonstrating an overall improvement in clinical and laboratory parameters (Gitto et al., 2001; Biancatelli et al., 2020). Indeed, melatonin seems to have potential as an immunomodulatory supplement. In clinical trials it has been used as a coadjuvant therapy during infectious diseases (including COVID-19) and has also improved immune responses after vaccination (Biancatelli et al., 2020). However, more evidence is needed before it can be used in clinical settings.

The hypothalamus-pituitary-adrenal axis, along with other components of the endocrine system, represents a crucial regulator of immune function. An imbalance between opposing adrenal hormones, cortisol and DHEA takes place in aging and malnourished individuals which results in thymus atrophy, as well as a reduction in naïve T cell numbers and neutrophil, B and NK cell functions (Buford and Willoughby, 2008). Supplementation with DHEA or its synthetic analog 16 alpha-bromoepiandrosterone (HE2000), can restore circulating innate and adaptive immune cell populations, proinflammatory Th cytokine balance and reduce pathogenic load in individuals with different chronic infections, including TB and HIV (Reading et al., 2006; Stickney et al., 2007; Ramos-Espinosa et al., 2018). In postmenopausal women, DHEA supplementation decreased blood levels of IL-6, T CD4⁺ and CD8⁺ as well as NK cells and increased natural killer cell cytotoxicity (Casson et al., 1993). On the other hand, when used in healthy middle-aged male adults, chronic DHEA, androstenedione and herbal extract consumption increased androgen levels but not immune activity (Kohut et al., 2003).

Conclusion

If used conscientiously, supplements represent important tools in the clinical armamentarium that can potentiate several physiological functions, including the immune response. Nutrient deficiencies in patients with severe infections or with clinical manifestations of immune deficiency should be detected and treated accordingly through proper testing (Berger and Shenkin, 2006). Cheap and rapid testing for nutrient deficiency is therefore urgently needed, as current testing methods are not cost-effective for most patients (Kennel et al., 2010). Equally important is the demand for large and well-conducted clinical trials to determine supplement efficacy in diverse clinical settings and for governmental agencies to strengthen supplement regulation. Current unnecessary consumption of supplements generates excessive spending (Guallar et al., 2013) and important adverse effects in the general public including: acute intoxication, insomnia, gastrointestinal symptoms, calculi formation, hematological abnormalities, susceptibility to infection and pro-carcinogenic effects (Rutkowski and Grzegorzczak, 2012).

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Anti-Inflammatory Therapy of Infections

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Introduction

Anti-inflammatory treatment of infections is challenging due to the heterogeneity of etiologic agents and complex immune interactions. Inflammation plays a central role in the pathophysiology of infections, being one of the principal body defensive mechanisms. Unfortunately, it also causes accompanying unpleasant symptoms. Typical manifestations of acute inflammation are pain (*dolor*), heat (*calor*), redness (*rubor*), swelling (*tumor*), and loss of function (*functio laesa*) (Signore, 2013). For this reason, anti-inflammatory medications of different classes have a long history of use in infections as supportive treatment to relieve accompanying symptoms. In addition, anti-inflammatory agents act as host response modifiers and might play a crucial role in managing acute and chronic infection with excessive inflammation preventing organ injury, e.g., COVID-19 (Rockwell and Ehrlich, 1990; Konstan et al., 1995). This article introduces the main groups of anti-inflammatory drugs, their use in treating infections, and associated risks. We also present a subsection on anti-inflammatory treatment in COVID-19.

Anti-inflammatory drugs in the treatment of infections

Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) reduce pain, lower fever, and decrease inflammation. Already Sumerians, ancient Egyptians, and Hippocrates reported the use of willow bark (the active ingredient in willow bark is salicin) as a remedy for fever, pain, and inflammation. One of the first systematic studies on anti-inflammatory drugs for infections was Edward Stone's study in 1763. He investigated the effectiveness of willow bark salicylates in treating malaria-related fever (Pearce, 2014; Norn et al., 2009).

The most popular drugs belonging to this group are ibuprofen, diclofenac, naproxen, and acetylsalicylic acid. In the United States, NSAIDs are one of the most popular drugs and account for 30 billion over-the-counter (OTC) medications sold (Green, 2001). These drugs inhibit the activity of cyclooxygenase enzymes (COX-1 and COX-2) responsible for synthesizing prostanoids from arachidonic acid. Prostanoids include prostaglandins, prostacyclins (PGE2, PGD2, PGF2, PGI2), and thromboxane (Tx). These lipid mediators exert many biological effects in the hematologic, pulmonary, renal, and cardiovascular systems. PGE2 and PGI2 are the primary pro-inflammatory prostanoids, which induce fever and increase vascular permeability, causing edema and leukocyte infiltration. PGD2 is produced by mast cells and causes an inflammatory reaction in allergic responses. Thromboxane has a central role in blood clot formation (Ehrenpreis and Kruchko, 2020).

By inhibiting COX enzymes, NSAIDs effectively reduce inflammation, fever, and pain. Thus, reducing infected patient's symptoms and signs of inflammation may provide a false sense of security and delay diagnosis of life-threatening infection (Stevens, 1995). In the early phase of the COVID-19 pandemic, public opinion started the concerns that ibuprofen may increase SARS-CoV-2 infection symptoms. The theoretical mechanism behind the severity of the disease suggested the upregulation of angiotensin-converting enzyme 2 (ACE2) receptors in human tissues. The SARS-CoV-2 virus uses these receptors to enter into human cells. In addition, some researchers also suggested that anti-inflammatory drugs may delay diagnosis leading to poor outcomes (Kragholm et al., 2021). However, a prospective cohort study conducted in the United Kingdom by Drake et al. (Drake et al., 2021) on 78,674 patients proved that NSAIDs use was not associated with in-hospital mortality, critical care admission, invasive ventilation, a requirement for oxygen, or acute kidney injury. Furthermore, a subanalysis did not indicate an increase in mortality in ibuprofen users compared to other NSAIDs-users and those not taking NSAIDs.

While using NSAIDs to treat infections, we must remember that they reduce the formation of prostaglandins and affect other components of the immune system. For example, NSAIDs inhibit granulocytes functions (aggregation, degranulation, chemotaxis, and phagocytosis). Besides, NSAIDs also enhance cytokines production, including tumor necrosis factor (TNF). Thus, even a minor

infection, usually well-controlled by an immune system, may develop life-threatening infectious processes (sudden onset of shock, organ failure, or aggressive infection) (Stevens, 1995; Kaplan et al., 1984; Giagoudakis and Markantonis, 2005; Oates et al., 1991). Furthermore, studies on mice have shown that using ibuprofen during streptococcal soft tissue infection worsens its course. Ibuprofen-treated mice had a higher mortality rate and significantly higher levels of tumor necrosis factor- α and interleukin 6. Based on the study results, the authors concluded that the use of ibuprofen in streptococcal soft tissue infections might cause severe necrotizing infections (Weng et al., 2011).

There is a piece of evidence that the use of NSAIDs in children may cause a worse course of some infectious diseases. For example, the use of ibuprofen in the management of varicella should be discouraged because current evidence shows an increased risk of subsequent skin and soft-tissue infections, mainly caused by group A streptococcal (GAS) (Mikaeloff et al., 2008; Lesko et al., 2001). There is also evidence indicating a strong association between the use of NSAIDs and severe necrotizing soft tissue infections in patients with chickenpox (Souyri et al., 2008). In addition, the pre-hospital use of ibuprofen in children may increase the risk of complicated pneumonia in children, including empyema (François et al., 2010; Le Bourgeois et al., 2016). Evidence shows the cumulative effect of ibuprofen dosing on a more significant risk of pneumonia complications in children with community-acquired pneumonia (CAP) (Krenke et al., 2018). In this research, preadmission cumulative dose of ibuprofen exceeding 78.3 mg/kg was a factor of 2.5 higher odds ratio for pneumonia complications. For these reasons, ibuprofen is not recommended to administer in children with suspicion of lower respiratory tract infection (Quaglietta et al., 2021). Some case reports also suggest a connection between the use of NSAIDs and the progression of streptococcal infections to shock and multi-organ failure (Stevens, 1995). Undertaken in the United Kingdom, population-based surveillance showed that taking NSAIDs was independently associated with increased risk for streptococcal toxic shock syndrome in patients with severe *Streptococcus pyogenes* infection (Lamagni et al., 2008). Therefore, symptomatic treatment with NSAIDs should be used cautiously during infections because a range of scientific arguments shows an increased risk of severe bacterial complications. For this reason, parallel antibiotic therapy should always be considered (Stevens, 1995; Le Bourgeois et al., 2016; Micallef et al., 2020). For recommended dosages of anti-inflammatory medications in various infections see Table 1.

Table 1 Dosage of main anti-inflammatory drugs used in infectious diseases (Tunkel et al., 2004; de Almeida et al., 2014; The Recovery Collaborative Group, 2021; Jenson, 2000; Marx and Chan, 2011).

Disease	Anti-inflammatory medication	Dosage
Infectious mononucleosis	Dexamethasone Methylprednisolone Oral prednisone	0.25 mg/kg every 6 h i.v. for 1–3 days 1 mg/kg every 6 h i.v. for 1–3 days 40 mg p.o. daily for 1–3 days
Bell's palsy	Prednisone	60 mg per day followed by the five-day taper, with a reduction of a previous day's dose by 10 mg per day
Bacterial meningitis	Dexamethasone	0.15 mg/kg i.v. every 6 h for 2–4 days
Tuberculous meningitis	Dexamethasone	12 mg/day (adults), 8 mg/day (children < 25 kg) i.m. for 3 weeks, followed by gradual taper for next 3 weeks OR 1 week of each (0.4 mg/kg/day, 0.3 mg/kg/day, 0.2 mg/kg/day, and 0.1 mg/kg/day), followed by 4 weeks of tapering oral dexamethasone therapy
COVID-19 in adults	Dexamethasone If not available: Prednisone Methylprednisolone Hydrocortisone	6 mg daily 40 mg daily 36 mg daily 160 mg daily
Inflammatory diseases in children	Ibuprofen (Children 1 year old or older) Naproxen (Children 2 years or older)	30–50 mg/day in 4 divided doses Doses greater than 40 mg/kg/day may increase the risk of adverse effects; doses >50 mg/kg/day are not recommended 10 mg/kg p.o. in 2 divided doses

Ibuprofen dosage: <https://www.drugs.com/dosage/ibuprofen.html>;

Naproxen dosage: https://www.drugs.com/dosage/naproxen.html#Usual_Pediatric_Dose_for_Juvenile_Rheum.atoid_Arthritis.

Glucocorticoids

Glucocorticoids are a group of drugs that reduces the activity of the immune system. Inside the cell, glucocorticoids attach to the appropriate cytoplasmic receptor (GR) and then pass the nuclear membrane as the glucocorticoid-GR complex. Inside the nucleus, the glucocorticoid-GR complex releases a specific molecule that binds to DNA. There are few steroid-responsive genes with glucocorticoid-responsive elements (GRE). The binding of the GR molecule to GRE may result in activation or repression of the gene (positive or negative GRE). The effects involve increasing the expression of anti-inflammatory proteins or decreasing pro-inflammatory proteins production (Rhen and Cidlowski, 2005). Some steroid-responsive genes do not express GRE itself, so the regulation is indirect via nuclear factor (NF)- κ B, activating protein (AP)-1 or cAMP-responsive element-binding protein (CREB). The third mechanism of glucocorticoids' impact on the regulation of inflammatory protein synthesis is by boosting the transcription of particular ribonucleases, which degrade mRNA, decreasing its stability and shortening half-time of some pro-inflammatory cytokines (van der Velden, 1998).

In medicine, glucocorticoids are often used in the treatment of autoimmune diseases, allergies, and asthma. They are also used to reduce symptoms and sequelae of severe infections. For example, in bacterial meningitis, glucocorticoids may reduce associated cerebral edema, increased intracranial pressure, altered cerebral blood flow, cerebral vasculitis, and neuronal injury by decreasing inflammatory response, but do not reverse the damage that occurred before treatment (Tunkel et al., 2004).

According to Cochrane Library, corticosteroids should be used in acute bacterial meningitis in children. Although they do not reduce the death rate, patients have significantly lower rates of hearing loss and other neurological sequelae. In children, the best effect was primarily observed in meningitis due to Gram-negative bacteria *Haemophilus influenzae* type b. However, these positive effects occurred only in high-income countries, and there were no significant beneficial effects in low-income countries. This phenomenon can be explained by the difference in patients' profiles on the example of African studies: usually, patients were admitted later, had many chronic conditions worsening the prognosis (malnutrition, HIV infection) or the antibiotics used in initial treatment (Brouwer et al., 2015). Nevertheless, glucocorticoids are routine adjunctive therapies to reduce the risk for neurologic sequelae. The recommended treatment according to IDSA is dexamethasone in a dose of 0.15 mg/kg IV every 6 h for 2–4 days (the first dose should be given 10–20 min before or with the first antibiotic dose) (IDSA Grade A-I). National Institute for Health and Care Excellence (NICE) recommends dexamethasone in infants and children beyond 3 months old with suspected or confirmed bacterial meningitis if lumbar puncture reveals any of the following:

1. frankly purulent cerebrospinal fluid,
2. elevated white blood cell count in cerebrospinal fluid with protein concentration above 1.0 g/L,
3. cerebrospinal fluid with white blood cell count above 1000/mcL or
4. bacteria on Gram stain.

Dexamethasone should be given within 4 h of starting antibiotics and should not be started if >12 h (NICE, n.d.).

Glucocorticoids are also mandatory adjunct therapy to antituberculous drugs in tuberculous meningitis, a severe neuroinfection causing death or severe neurologic deficits in more than half of those affected despite antituberculosis chemotherapy. A study performed in Vietnam provided clinical evidence that early treatment with dexamethasone and antituberculosis drugs improves survival among patients over 14 years of age with tuberculous meningitis, regardless of disease severity. However, dexamethasone probably does not prevent severe disability in the survivors (Thwaites et al., 2004). A Cochrane review of nine trials that included 1337 participants concluded that glucocorticoids reduce deaths by almost 25% in the short term, although they may have little or no effect on neurologic sequelae, the outcome that is less common than death. In addition, there was no difference between groups in adverse events, including gastrointestinal bleeding, invasive bacterial infections, hyperglycemia, and liver dysfunction. Thus, the possible harm is unlikely to be quantitatively important compared to the reduction in mortality (Prasad et al., 2016).

Cochrane Library also conducted a meta-analysis which suggests that the use of glucocorticoids in patients with sepsis results in a reduction in hospital or Intensive Care Unit (ICU) length of stay and probably reduces hospital mortality and mortality in 28-day and 90-day. The evidence for this statement is moderate-certain. Side effects of this therapy include increased risk of muscle weakness, hyponatremia (high-certainty evidence), and the potential risk of hyperglycemia (moderate-certainty evidence) (Annane et al., 2019). On the other hand, Surviving Sepsis Campaign does not support using corticosteroids intravenously to treat septic shock if it can be achieved by using adequate fluid therapy and vasopressors. In other cases, it is recommended to use hydrocortisone (Rhodes et al., 2017). In children, the guidelines do not indicate that using steroids is beneficial nor harmful—either hydrocortisone or no hydrocortisone may be used in case of lack of hemodynamic stability after adequate hydration and vasopressors were used (Weiss et al., 2020). Both in children and adults, the recommendations are classified as weak, with low quality of evidence.

Some studies also indicate the usefulness of glucocorticoids in reducing symptoms of viral infections. For example, steroid therapy may reduce the sore throat symptoms in infectious mononucleosis due to Epstein-Barr virus (EBV) infection (Rezk et al., 2015). However, the evidence to support this approach is low, and corticosteroids are not routinely recommended (Dunmire et al., 2018; Lennon et al., 2015; Luzuriaga and Sullivan, 2010). Since corticosteroids are not shown to provide substantial or sustained relief of symptoms associated with mononucleosis, they may be used based on clinical experience if concern for severe complications such as airway obstruction due to tonsils enlargement, hemolytic anemia, thrombocytopenia, mononucleosis-like aplastic anemia, or liver failure. No data is confirming the benefits of using steroids in these complications. There is no standard dosing, but

the common approach in adults is prednisone 40–60 mg/day orally for 1–3 days followed by taper over the next 7–14 days (Cohen, 2003). Reported adverse events include peritonsillar cellulitis, acute-onset diabetes mellitus, and neurologic sequelae.

Bell palsy is a subacute weakness of the facial nerve (the seventh cranial nerve), which may be idiopathic or secondary to herpes infections. In Bell's palsy complicating Herpes simplex virus (HSV) infection, steroids may shorten recovery time and increase recovery rates when added to antiviral treatment, but the difference does not seem statistically significant compared to antiviral treatment alone (Numthavaj et al., 2011). In idiopathic Bell palsy, corticosteroids are recommended by the American Academy of Neurology since they increase the likelihood of complete facial motor function recovery (Gronseth and Paduga, 2012) (Strong recommendation). Bell Palsy Working Group, Canadian Society of Otolaryngology—Head and Neck Surgery and Canadian Neurological Sciences Federation recommend using corticosteroids for all patients with Bell palsy (de Almeida et al., 2014). Various dosing regimens have been used, including prednisolone 25 mg twice daily for 10 days, prednisolone 60 mg orally once daily for 5 days, then tapered by 10 mg each day until day 10, prednisolone 1 mg/kg/day for 10 days in children (only use in children if complete palsy).

In upper respiratory tract infections, oral or intranasal glucocorticoids accelerate the resolution of otitis media with effusion (Hussein et al., 2017) but do not improve the quality of life (Francis et al., 2018). According to the American Academy of Otolaryngology-Head and Neck Surgery (2016), oral or intranasal steroids should not be used in otitis media cases with effusion as no data confirms long-term benefits from this approach (Rosenfeld et al., 2016). Intranasal steroids are commonly used to treat acute rhinosinusitis, chronic rhinosinusitis, and chronic rhinosinusitis with nasal polyps to reduce the severity of symptoms (Rot et al., 2020; Mullol et al., 2009; Head et al., 2016). The European Position Paper On Rhinosinusitis and Nasal Polyps (EPOS 2020) does not support using intranasal glucocorticoids in acute rhinosinusitis (common cold) in adults or children. However, some benefits can be achieved when using in the post-viral phase of acute rhinosinusitis (moderate quality of data, strong evidence). Systemic glucocorticosteroids should not be prescribed in this condition due to EPOS guidelines as they do not impact recovery in 7–14 days. In chronic rhinosinusitis (CRS), intranasal glucocorticoids remain the mainstay of the treatment, reducing symptoms and improving quality of life. When the nasal polyps occur in the course of the CRS, treatment with intranasal steroids reduces polyps' size and prevents polyps' recurrence after surgery (high-quality evidence). It is still under debate if there is strong evidence to support oral corticoids in chronic rhinosinusitis. EPOS guidelines advise using oral glucocorticoids in 1–2 short courses per year as an addition to intranasal treatment, which can help reduce symptoms and nasal polyp score. In children with CRS, although there is no evidence of the efficacy of this intervention. However, considering its safety profile, it is recommended to use intranasal steroids as part of the treatment (Fokkens et al., 2020).

Topical corticoids are helpful in the short-term treatment of conjunctivitis. However, according to most guidelines, their use should be limited to severe cases. In addition, side effects of the therapy are prolonging adenoviral infections, worsening of HSV infection course, increased intraocular pressure, glaucoma, or cataracts. Thus, the steroid choice should be carefully evaluated if the benefits of the therapy outweigh the risks (Holland et al., 2019).

Colchicine

Colchicine is a drug commonly used to treat gout and Behcet's disease. The primary anti-inflammatory mechanism of action is based on tubulin disruption, which causes the downregulation of multiple inflammatory pathways (Leung et al., 2015). In addition, colchicine inhibits the migration and activation of neutrophils and interrupts mast cells degranulation (Dalbeth et al., 2014).

Some studies show that the use of colchicine may be helpful in infections treatment. There is some evidence that colchicine is beneficial in managing viral liver diseases and may reduce time to deterioration, hospitalization time, and mortality in patients with COVID-19. Some studies indicate potential therapeutic utility in treating malaria, anogenital warts caused by human papilloma-virus (*condyloma accuminata*), common warts (*verruca vulgaris*), viral myocarditis, and *erythema nodosum leprosum*. Unfortunately, there is also an increased risk of pneumonia in patients using colchicine (McEwan and Robinson, 2021).

Anti-inflammatory therapy for COVID-19

In the course of COVID-19 infection, the three phases were described. In the first phase of early infection, the virus infiltrates lung parenchyma cells. In the second phase, lung tissue injuries occur because the host activates an immune response against the pathogen. The last phase, called the inflammatory cascade ("*cytokine storm*"), is caused by pathogen patterns exposed during viral replication. In this part, we notice an excessive inflammatory response to SARS-CoV-2 infection with viral load not correlated with the worsening of symptoms. Signals created by the SARS-CoV-2 virus cause the assembly of a pro-inflammatory protein complex, which converts the pro-IL-1 β and pro-IL-18 to their active forms. IL-1 β drives the synthesis of IL-6, which induces C reactive protein (CRP) and is considered a major pro-inflammatory factor in the COVID-19 cytokine storm. IL-1 β and IL-6 activate neutrophils that infiltrate into the lungs and tissues of other organs. They degranulate and release more cytokines, chemokines, and proteases, causing inflammation of the affected organs, resulting in organ failure. Neutrophils also trigger events in arteries that promote thrombosis. Using anti-inflammatory drugs in the COVID-19 treatment is based on the idea to stop the activation of the cytokine storm (Reyes et al., 2021; The Recovery Collaborative Group, 2021).

Corticosteroids and immunomodulatory monoclonal antibodies are commonly used as anti-inflammatory drugs in the treatment of COVID-19 patients. In severely ill patients with COVID-19, systemic corticosteroids are strongly recommended because they result in lower mortality among patients receiving mechanical ventilation and among patients receiving oxygen

without mechanical ventilation. Patients without respiratory support did not benefit from taking corticosteroids (The Recovery Collaborative Group, 2021; Sterne et al., 2020). On the other hand, in adults with mild COVID-19, inhaled budesonide may lower the frequency of urgent care visits (Ramakrishnan et al., 2021). *Tocilizumab* is a monoclonal antibody blocking the interleukin-6 receptor. Studies have shown that in patients with severe COVID-19, *tocilizumab* improves survival (Abani et al., 2021; REMAP-CAP Investigators, 2021). Some studies also suggest that the use of colchicine may reduce the risk of hospitalization or death in patients with COVID-19 (Tardif et al., 2021).

Anti-inflammatory drugs are also used in Multisystem Inflammatory Syndrome in Children (MIS-C), a disease that is a complication following exposure to SARS-CoV-2 and had been announced first time by the Royal College of Pediatrics and Child Health (RCPCH) on May 1, 2020. This rare systemic inflammatory illness occurs mainly in children. It can lead to rapid multi-organ failure and the need for intensive care (Okarska-Napierała et al., 2020, 2021). In treating this disease, intravenous immunoglobulin, methylprednisolone, IL-1 antagonists, IL-6 receptor blockers, and anti-TNF agents are used (Harwood et al., 2021).

Conclusions

Anti-inflammatory medications are frequently used in infections to mitigate accompanying symptoms. They also act as host immune response modifiers and play an essential role in treating infections in selected groups of patients, e.g., with mucoviscidosis or COVID-19.

However, anti-inflammatory agents generally impair the immune system. NSAIDs inhibit granulocytes functions and enhance cytokines production, including TNF, and may contribute to the emergence of bacterial soft tissue infections or promote the development of the life-threatening disease from a minor infection, usually well-controlled by an immune system. Reducing infected patient's symptoms and signs of inflammation may provide a false sense of security and delay diagnosis of serious infections.

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Inborn Error of Immunity: A Journey Through Novel Genes and Clinical Presentation

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Historical background

The bursa of Fabricius stem from Hieronymus Fabricii (“Fabrizio”), born in 1533 in Acquapendente, nearby Rome, and died in Padua in 1619. In his work *De formatione pulli*, he described for the first time the presence of an organ near the cloaca, the “bursa of Fabricius.” Fabricii, did not find the bursa in the male bird, so he concluded that its function was just a receptacle of semen. The first evidence of the role of the bursa of Fabricius in the immune response was in the 1950s. In 1954, Bruce Glick at the “Ohio University,” was studying the effects of neonatal bursectomy on later chick development and he found that bursectomized chicks grew normally, according to the controls (Glick et al., 1956). One of Glick’s collaborators, named Chang, employed chicks while evaluating the antibody response, treating them with the O antigen of *Salmonellatyphi murium*. Six out of nine animals deceased instantaneously; two survived, despite no antibody response could be detected. Glick postulated that the early removal of the bursa was responsible for the loss of antibody formation capacity. As a matter of fact when the bursa was removed later, the modification of the antibody response was minimal or absent (Glick et al., 1956).

In 1961, both Francis A.P. Miller in London and Robert A. Good in Minnesota uncovered neonatal rat and rabbit thymectomy to drive a severe alteration of cell immunity, involving an altered ability of rejection of cutaneous and other tissues transplants (Miller, 1962; Papermaster et al., 1962). The US immunologist Max D. Cooper noted that also the people with Wiskott-Aldrich syndrome, an X-linked disease, developed viral herpes lesions associated with defective immunity with high levels of antibodies (Cooper et al., 1968). Conversely, children with agammaglobulinemia linked to hereditary immunodeficiency of chromosome X, controlled viral infections even though without an antibody response. He concluded that lymphocytes producing antibodies and the thymus-derived lymphocytes that led to rejection were different from each other (Cooper et al., 1968).

In 1963, Cooper, Good and Peterson, at the “University of Minnesota,” carried out experiments of neonatal chick bursectomy and thymectomy followed irradiation. After irradiation, the structure and function of their immune systems were examined. The treated animals deemed smaller, manifested a lack lymphocytes count and functions, both at a cellular and umoral level (Cooper et al., 1965).

In chickens infected with *Brucella abortus* bacteria and undergoing a bursa radiation, no antibodies were produced even if the internal thymus linked organs showed no infection. In this context, the bursa influenced the ability to produce antibodies, whereas it did not interfere with the development of cellular immunity. However a significant immune response deficiency marked the thymectomized animals. In brief animals subjected to bursectomy and thymectomy had a severe impairment of humoral and cellular immunity (Cooper et al., 1966).

The human equivalent of the bursa of Fabricius still represents an unmet scientific need. In 1974, by the use of fetal liver cell cultures, Cooper, who had moved to London in those years, and some of his colleagues at the “University College” of London found that these cells originated bone marrow (Cooper et al., 1974).

In 1952 on “Pediatrics” Ogden Bruton a primary physician at the “Walter Reed Army Center” reported an antibody immune response defect. He dealt with an eight-year-old child with a history of recurrent episodes of pneumococcal sepsis, such as pneumonia, purulent otitis media and septic arthritis, which had lasted 4 years. Remarkably, the serum electrophoresis showed a lack of gamma migrating globulins (Buckley, 1998). Before proceeding with the electrophoretic analysis, attempts had been made to control recurrent infections with chemo-, serum- and vaccine-therapy. Bruton also had the merit of trying for the first time the substitutive treatment with gamma-globulins that led to the child clear improvement so that he and the medical community were

able to have a normal growth in the following years in both primary (Hitzig, 2003) and secondary immunodeficiencies (Vacca et al., 2018). The report from Bruton unveiled that the clinical phenotype could be due to either an inborn or to an acquired deficiency of the antibody assembly mechanism. In actual fact, the case described by Bruton was not a congenital sex linked agammaglobulinemia or Bruton type because the histological examination of the tonsils, bone marrow and liver showed that the structure of these organs was normal. The Bruton disease or X-recessive agammaglobulinemia comprises in an acquired X-linked inborn error of immunity, categorized by a lack of immunoglobulins production. The driver event is due to a gene mutation of the Bruton tyrosine kinase (BTK), characterized by a defect of B lymphocytes maturation. The children affected by Bruton type-agammaglobulinemia rarely get sick during the first 6 months of life, because they are covered by antibodies received from their mothers. The agammaglobulinemia syndrome case picture was characterized by serious recurrent infections (pneumonia, meningitis, sepsis, otitis, synovitis and conjunctivitis) associated with a marked *hypogammaglobulinemia*. These subjects exceeded the fungal and viral infections with relative effortlessness, instead of experience severe manifestations as a result of infections by pyogenic germs (Kornfeld et al., 1997).

In 1952, three types of agammaglobulinemia were described: (a) congenital, characterized by recurrent bacterial infections which occurred in male children either in childhood or in early puberty; (b) acquired, characterized by a series of serious recurrent infections which could occur starting from puberty throughout life; (c) transient, observed in early infancy, from the sixth-ninth month of life and which spontaneously regresses around 3 years of age, as a consequence of a defect in the autonomous synthesis of antibodies by the infant, who, in the first months of life after birth, is defended by immunoglobulins of maternal origin (Gitlin et al., 1956; Hitzig, 2003). In the same year it was also demonstrated that plasma cells were not present into the bone marrow of agammaglobulinemic patients and that antibodies were not synthesized in these patients following antigenic stimulation. Moreover, the lymph node germinal centers were absent and the tonsils hypotrophic. On the other hand, the T-dependent paracortical areas of the lymph nodes were normally developed and atrophic after antigenic stimulation. Finally, in the same year the case of a patient with a thymoma was described, showing in addition to low antibodies levels a reduced ability to reject allografts and to elaborate a delayed hypersensitivity response (Smith et al., 1994).

In 1968, Angelo M. Di George a pediatrician at "Temple University's School of Medicine," discovered that the children born without thymus presented few circulating lymphocytes and lacked immune cell functions, without any alteration of circulating antibodies and plasma cells, and showed an abnormal susceptibility to viral and fungal infections. In these children the development of epithelial anlagen derived from the third and fourth branchial pouches, which give rise to the thymus during embryonic development didn't occur. Additionally, the cells arising from neural crest contribute to the thymic epithelial anlagen formation and the lack of their migration leads to a defect in the thymus development and reduction of T lymphocytes. From the third branchial pouch originates also the mandible, the two superior parathyroids, the cone and the trunk of pulmonary artery and the origin of aorta. Thus, in the Di George syndrome there are variable combinations of defects in the development of parathyroids, the thymus, the arterial cone of the pulmonary artery, the ascending aorta and the aortic arch, as well as craniofacial anomalies (Kirkpatrick and DiGeorge, 1968).

Inborn errors of immunity overview

Inborn errors of immunity associated with immune dysregulation comprise three major cohorts of inborn errors of immunity that often associate or even predominantly manifest with immune dysregulation. They are autoimmune-lymphoproliferative syndrome- (ALPS-) like disorders, typically characterized by autoimmune cytopenia and lymphadenopathy; Conversely, IPEX-like diseases are typically present with enteropathy, type I diabetes, thyroiditis. Arthritis, eczema and vasculitis; moreover, common variable immunodeficiency disease- (CVID-) like diseases are rather characterized not only by hypogammaglobulinemia, recurrent infections, but also, even at presentation, by immune cytopenia in particular idiopathic thrombocytopenia (ITP) (Chandrakasan et al., 2019). The antibody deficiency represents an *archetype* of B cell failure, being a mix between the agammaglobulinemia phenotype, as well as the phenotype of the IgA and IgG2 subclass deficiency and the isolated IgA deficiency (Oxelius et al., 1981; Tuijnburg et al., 2018; Yazdani et al., 2017). The Inborn Errors of Immunity Committee (IUIS) classified these disorders (Tangye et al., 2020), acknowledging that B cell defects are included, although being not limited to diseases like agammaglobulinemia and CVID population where B cells are not functioning correctly either because they are not producing any gamma globulin at all or in the case of certain amounts of antibody which are insufficient to cope with the entire immunological repertoire (Table 1).

CVID is one of the most characteristic disorders in which the autoimmunity, the lymphadenopathy, the splenomegaly and the neoplastic proliferation intersect, which sheds more light onto the multisystem involvement in terms of interstitial lung disease and gastroenteric manifestations. Remarkably, low serum immunoglobulin G (IgG) and with a marked decrease of at least two standard deviations below the mean for age, usually almost always also IgA and or IgM isotypes reduction are detectable (Wang and Cunningham-Rundles, 2005). Basically, the onset of immunodeficiency is to the fullest at 2 years of age, with a lack of isohemagglutinins and poor response to vaccines, after having excluded alternative defined causes of hypogammaglobulinemia (Bonilla et al., 2016). Any age can be interested, although gender can represent a major difference, with an earlier onset among males compared to females (median age of diagnosis 36 vs. 25, for females vs. males respectively) (Cunningham-Rundles, 2019; Odnoletkova et al., 2018), therefore it is not necessarily a pediatric illness. There is a failure of the development of plasma cells looking at the gastrointestinal tract or in the bone marrow of the CVID patients who have a great paucity of plasma cells in the bone marrow and the same behavior appears in the gastrointestinal tract, along with low IgA levels., conversely in the case of

Table 1 IUIS classification of primary immunodeficiencies.

1. Immunodeficiencies affecting humoral and cellular immunity
2. Combined immunodeficiencies with associated or syndromic features
3. Predominantly antibody deficiencies
Severe reduction in all serum immunoglobulin isotypes (agammaglobulinemia)
Severe reduction in at least 2 serum immunoglobulin isotypes with low or normal B cells (CVID)
Severe reduction in IgG and IgA with normal/elevated IgM
Isotype, light chain, with normal number of B cells
4. Diseases of Immune dysregulation
5. Congenital defects of phagocyte number or function
6. Defects in intrinsic and innate immunity
7. Auto-inflammatory disorders
8. Complement deficiencies
9. Bone marrow failure syndromes
10. Phenocopies of inborn errors of immunity

inflammatory bowel disease a plethora of immune cells infiltrate the gastrointestinal tract (Daniels et al., 2007). B cells are predominantly in germ-line configuration, paralleling the lack of plasma cells (Roskin et al., 2015), with very poor somatic mutation, thus, the B cell receptor if you sequence it has really made no attempt at adaptation to the environment and patients with very low isotype switched memory B cells have almost none whatsoever and those who have slightly more immunoglobulin have just an attempt at some somatic hypermutation (Roskin et al., 2015). Therefore, the B cells are actually incapable of responding to the environment. We examined several approaches to confront the available evidences and three key points emerged.

Immunodeficiencies affecting humoral and cellular immunity

B-cell immunity is one of the leading causes of inborn errors of immunity, determining antibody disorders. Dissecting the mechanisms beyond bone marrow a B-cell differentiation occurs. In the early phase, a cell commitment to lymphoid progenitor is taking place and during this phase there are important transcription factors involved such as Ikaros, E2A, ILR7 and PAX5 which are necessary to define certain key transcriptional programs (Pongubala et al., 2008, p. 5). At this stage a lymphoid progenitor can still become an NK- or T-cell, however PAX5 is a B-cell commitment factor and, when expressed, the lymphoid progenitor can move on in the B-cell lineage differentiation and V-DJ recombination process (Nutt and Kee, 2007), necessary to form the B-cell receptor (BCR). The first phase is the formation of the pre-BCR, constituted by the heavy chain, during which V, D and J are randomly recombined prompting the expression of an heavy chain and a surrogate light chain (Löffert et al., 1996). This passage is crucial for the first checkpoint happening in the Pre-BII stage (Mårtensson et al., 2010). The preBCR signaling is pivotal not only for light chains rearrangements, proliferation initiation and termination (Löffert et al., 1996; Mårtensson et al., 2010), but also for mutations in BTK (XLA) or IGM, CD79a/b, BLNK, L14.1 or PI3Kalpha (AR) (Abolhassani et al., 2019). Conversely, the mutations in TCF3 (E47) (Boisson et al., 2013) Ikaros (Kuehn et al., 2016) and Pax5, being early b-cell defects, constitute a new category of early B-cell deficiencies due to mutation in early transcription factors. In more details Boisson et al. reported about four patients with the same de novo mutation in TCF3 gene, in a hotspot of the BASIC domain (Boisson et al., 2013). Therefore, a mutant protein expressed in the nucleus exerts a dominant negative effect when bound to wild type E47 as homodimer, leading to isolated agammaglobulinemia with decreased number of peripheral B-cells. Of note, on CD19^{pos} B-cell BCR was lacking, thus the authors deduced that E47 plays a critical role in enforcing the clock of precursor B-cells without a functional BCR, indicating a new role of E47. The TCF3 autosomal recessive form, constituted by an homozygous TCF3 mutation, has been associated with severe hypogammaglobulinemia and B-cell acute lymphoblastic leukemia (Ben-Ali et al., 2017). In this case the TCF3 mutation has been uncovered to drive E2A protein absence via a nonsense mediated decay; nonetheless HEB might functionally compensate for E2A loss as some CD19^{pos} B-cells are present (Ben-Ali et al., 2017). The first case-series available on IKAROS (IKZF1) autosomal dominant mutations reported a progressive loss of immunoglobulins and B-cell on 26 patients from 6 families (Kuehn et al., 2016). Interestingly, Kuehn et al. not only found a clinical variability but some asymptomatic patients developed an acute lymphoblastic leukemia (Kuehn et al., 2016). The reported mutations were missense mutations or deletions, which still allowed a stable protein that although still able to dimerize, is unable to bind target DNA, in a haploinsufficiency and incomplete penetrance clinical phenotype (Kuehn et al., 2016). The clinical spectrum of IKAROS deficiency is broad, with germline heterozygous mutations associated with a severe immunodeficiency that progresses to T-cell acute lymphoblastic leukemia (Yoshida et al., 2017). Moreover, germline heterozygous IKAROS mutations can cause dysgammaglobulinemia, hematologic abnormalities, including B-cell defects and autoimmune diseases (Hoshino et al., 2017; Van Nieuwenhove et al., 2018). Dominant-negative IKZF1 mutations can also cause a T, B and myeloid cell combined immunodeficiency (Boutboul et al., 2018). Dominant negative IKAROS mutations cause a defect in T cells composition, with naïve but neither effector nor memory T cells present and *Pneumocystis jirovecii* pneumonia, demonstrating a defect involving functional T cells (Boutboul et al., 2018). Myeloid defects were also reported (i.e., myeloid-derived dendritic cells).

This disease phenotype demonstrates that IKAROS not only cause a B-cell development but play a broader role (Boutboul et al., 2018). Of note, the definition of main precursor B-cell populations comprises the existence of Pro-B, Pre-B-I, Pre-B-II, Immature and mature cells depending on TdT, CD19, IgM, cytg, and IgD expression detected at FACS (Wentink et al., 2019). The above-mentioned is a heterogeneous rather than a homogeneous population: while considering the expression of TdT and CD34, it is possible to distinguish two different cell types within the Pre-B-I and Pre-B-II cells; those data have also been confirmed by single-cell-RNAseq data, which showed a gradual expression of CD20 (Solimando et al., 2016; Wentink, 2018) from Pre-B-I and Pre-B-II cells and multiple differentiation trajectory. Thus, the precursor B-cell differentiation is not linear and not fully elucidated.

From the peripheral B cell differentiation standpoint, the antigen recognition and cell-signaling are of paramount importance and dependent on the CD19, CD21 and CD81 complex; thus, a mutation in one of these complexes leads to a block in the differentiation process and an incomplete activation of B-cells (Levy et al., 1998; van Zelm et al., 2010). In the spleen and MALT the T-cell independent differentiation and the T-cell-dependent differentiation in the lymph nodes and in the peripheral lymphoid organs, are also crucial (Arjunaraja et al., 2017; Herlands et al., 2008). The fundamental B/T-cell interaction is necessary for further differentiation and somatic hypermutation in the variable domains and improve the specificity of the immunoglobulins and the class switch (Arjunaraja et al., 2017; Herlands et al., 2008; Wentink, 2018). Many genetic defects have been defined in the B-cell differentiation (Bousfiha et al., 2018). Focusing on the class switch and recombination inborn errors, based on the immunological function it is possible to distinguish defects in T-cell-B-cell cooperation, intrinsic B-cell defects and DNA repair defects. Regarding the first group, CD40/CD40L, NEMO and T_{FH} defect share common complications, such as opportunistic infections, liver damage and autoimmunity. Within the intrinsic B-cell defect subgroup, damages in the enzyme machinery modulating somatic hypermutation, such as AID and AID co-factor mutations lead to lymphadenopathies and autoimmunity. Conversely, patients with defects in DNA repair can be affected by autoimmunity, B cell lymphomas, leukemias cancers and neurological deterioration (Bousfiha et al., 2018; Solimando et al., 2020). In more details, subjects with UNG deficiency develop a susceptibility to bacterial infections of the respiratory tract, cervical and mediastinal lymph node hyperplasia, increased serum IgM and decreased serum IgG and IgA concentration (Tesch et al., 2018). Moreover, constitutional mismatch repair deficiency (CMMRD), such as AR mutations in PMS2, MHL1, MSH2 and MSH6 determine cancer predisposition syndromes, comprising hematologic malignancies, brain tumors, colorectal and intestinal malignancies and other cancers such as embryonic tumors and rhabdomyosarcoma (Tesch et al., 2018). Of note, those subjects can have no clinical signs of immunodeficiency and no uniform abnormalities. Often this group of patients has a reduced level of somatic hypermutation (IJspeert et al., 2017) and uracil-DNA-glycosylase (UNG) is involved in generating GC transversion. Conversely the patients with MSH2 and MSH6 mutations, are affected by AT mutations (IJspeert et al., 2017). Furthermore, MMR deficiency leads to less mutation at POLH motifs, while MMR deficiency leads to more mutations at AID motifs (IJspeert et al., 2017). As a consequence, it is possible to envision a 5-pathway model for somatic hypermutation in human in which AID induces somatic mutations in variable genes, often immunoglobulins, and these lesions can be resolved in five different ways, namely by replication, short-patch BER, long-patch BER, MMR and hybrid pathway (IJspeert et al., 2017). Thus, this knowledge has also been translated in a somatic hypermutation analysis in CVID (van Schouwenburg et al., 2018), combining the flow cytometry, the histopathology and the repertoire analysis dissecting the irregular shape of the patient's lymph nodes with reduction in B cells, decreasing SHM, as well as disturbances in antigen selection and class recombination (Unger et al., 2018). Therefore, as they ensue monogenic and polygenic defects, transcriptome and epigenetics cooperate in driving the inborn errors of immunity (Kienzler et al., 2017).

Combined immunodeficiencies with associated or syndromic features

CVID is multifaced, being clinically and molecularly heterogeneous. The immunopathogenesis is characterized by a B cell maturation defect, leading to a decreased B cell memory phenotype, coexisting with poor T cell proliferation along with expansion of immune lymphoid cells (ILCs) and impaired dendritic cell differentiation, TLR 7/9 defects and IFN alpha signature (Bonhomme et al., 2000; Cols et al., 2016; Grimbacher et al., 2003). A whole exome sequencing (WES) uncovered several monogenic causes (Bogaert et al., 2016), nonetheless, several modifying genes and combination of single variants are involved, therefore the heterogeneity and the attempts to subset CVID patients are aiming to linking the genetic defect to the resulting clinical phenotype as well as the immune function. To this end, a plethora of scientific productions combined WES data and single-cell immune phenotypic and functional data. The subcategorization of CVID patients which passed on their immune phenotypic and functional defect mass cytometry (CyTOF) single-cell high dimensionality, allowed to screen up to 50 rare-earth elemental mass channels, combining phenotypic and functional measurements. Focusing on innate immunity (Mills, 2011), it is possible to assess the activation of innate response, by sketching the phosphorylation of cell protein via phospho-flow cytometry. Conversely, in order to thoroughly decipher the adaptive immunity counterpart, investigators engaged TCR and BCR signaling both proximal, such as Zap70, LAT and PLC1 and downstream, while looking at more distal signaling protein such as ERK1/2, s6, pAkt, pCREB and p38MAPK (Bendall et al., 2011; Hsieh and Hernandez, 2016).

Defects in intrinsic and innate immunity

Dealing with innate immunity and defense against virus infections, we recognize cell-extrinsic and cell-autonomous factors playing a major role in anti-viral defenses (Lehnardt et al., 2003; Man and Kanneganti, 2016). Among the cell-extrinsic mechanisms, NK

cells, type I and III IFN and inflammatory cytokines are mostly involved; contrariwise autophagy, restriction factors and cell death share the most relevant cell-autonomous physiopathology (Lehnardt et al., 2003; Man and Kanneganti, 2016). Virus recognition and induction of inflammatory and antiviral responses are mainly involved during viral infections, when a number of pattern recognition receptors on the cell-outside and at a cytoplasmic level recognize pathogens associated molecular patterns (Crotty, 2014). In the cases of virus nucleic acids, such as RNA and DNA a number of adaptive molecules ultimately signaling effectors such as NF- κ B and IRF factors implying antiviral IFN responses are induced. Additionally, inflammatory IL1 β -mediated responses, autophagy and death pathways boost the antiviral effectors repertoire (Balachandran and Beg, 2011). DNA sensors also play a pivotal role in driving the production and induction of type I interferon (Carter-Timofte et al., 2018b). Moreover, type I and III IFN signaling induce a broad range of IFN stimulated genes, restricting viral replication and promoting inflammation, while hampering leukocyte recruitment, activation and proliferation (Fensterl and Sen, 2009). The role of innate immune system in antiviral defenses emerged from evidence obtained from the knowledge of such defects as in IFN induction, actions (Carter-Timofte et al., 2019, p.; Mørk et al., 2015) and NK cell defects (Orange, 2002). Infact, TLR3 pathway defects, have been associated to herpes simplex encephalitis (Mørk et al., 2015), RNA POL III mutations, associated to severe VZV disease (Carter-Timofte et al., 2019) while MDA5 defects correlate with recurrent rhinovirus and severe RSV infections (Wang et al., 2011); furthermore, IRF7 is also associated with severe influenza (Jørgensen et al., 2018). Downstream of the IFN receptor signaling, the defects in IFNAR or STAT2 constitute an archetype for disseminated reactions to measles (Duncan et al., 2015; Hahm et al., 2005; Hambleton et al., 2013; Hernandez et al., 2019). Herpes virus infections are rather correlated to NK cell defects (Mace and Orange, 2019; Orange, 2002). Herpes simplex encephalitis is the classical starting point for deciphering the inborn error of immunity predisposing to viral infections by dissecting the role of defective IFN production downstream of TLR3 signaling in herpes encephalitis (Herman et al., 2012). Errors in double strand RNA lead to impaired type I IFN signaling in the brain hence increasing the incidence of herpes CNS infections in children (Lim et al., 2014). IRF3 deficiency mimics the aggressive viral infection phenotype, by inducing encephalitis (Andersen et al., 2015). Varicella-zoster virus infection differs from HSV CNS infection, being characterized by a predominant vasculopathy or vasculitis (Gilden et al., 2009). The underlying mechanism can be only partially elucidated by the role played by interferon and NK cells in preventing viremia, since T cells seem to orchestrate latency. However, IFN role in the reactivation is still unknown (Kinchington et al., 2012; Yewdell and Hill, 2002). A plethora of primary immunodeficiencies predispose to VZV infection (Biron et al., 1989; Brooks et al., 1990; Hsu et al., 2011; Wiegering et al., 2011) with translational theragnostic consequences (Dobbs et al., 2015; Kreins et al., 2015; Roesler et al., 1999; Zhang et al., 2009), often associated with severe bacterial and fungal infections. Remarkably, RNA polymerase III (POL III) has been uncovered to be a gatekeeper to prevent aggressive CNS viral-induced diseases (Carter-Timofte et al., 2018b). POL III provides immunological DNA sensing and drives the transcriptions of housekeeping genes at nuclear level; moreover, in the cytosol POL III may act as an innate-immune sensor of DNA, recognizing nucleic acid and regulating IFN responses (Carter-Timofte et al., 2018b; Chiu et al., 2009). Of note, there is an association between POL III defects and human disease, since mutations in POLR3A and POLR3B encoding RNA polymerase III subunits cause an autosomal-recessive hypomyelinating leukoencephalopathy ((Saito et al., 2011). Furthermore, defects in POLR3A and POLR3B also cause hypomyelinating leukodystrophies with or without dental abnormalities, hypogonadotropic hypogonadism (Daoud et al., 2013) and 4H syndrome with vertebral anomalies (Muthusamy et al., 2015). Cerebellar atrophy and hypomyelination have also been reported by Daoud et al. (Daoud et al., 2013). Remarkably, patients peripheral blood mononuclear cells (PBMCs) with POL III mutations have impaired IFN beta production in response to Poly(dA:dT) DNA (Ogunjimi et al., 2017). Moreover, PBMCs exhibit a defective ability to control VZV replication and an inability to produce IFNs in response to viral infections (Ogunjimi et al., 2017). Mechanistically, an impaired production of immunostimulatory 5'ppp-RNA after transfection with adenine-thymine (AT)-rich DNA has been reported along with a reconstitution of IFN production and viral activity in PBMCs transfected with POLR3C (Ogunjimi et al., 2017). The reason why POL III defects hold a strong disease phenotype is due to the fact that cGAS is considered the main cytosolic DNA sensing in humans. Indeed, VZV immune sensing by cytosolic DNA sensors, warrants further investigation aiming to elucidate why cGAS cannot compensate for a POL III defect (Ogunjimi et al., 2017; Zahid et al., 2020). One tempting hypothesis related to localization of VZV in lymphocytes causing viremia is issues from POL III rather than from cGAS. Collectively, in pediatric cases a combined effect of high AT content of the VZV genome and low cGAS expression by blood cells lead to impaired VZV sensing and increased viral replication in POL III defect (Ogunjimi et al., 2017). Conversely, in adults, a viral reactivation can drive a CNS vasculitis, correlated by recurrent VZV infection (Carter-Timofte et al., 2018a). A POL III defect in monozygotic twins with CNS vasculitis paradigmatically pinpoints the deleterious effects of the related decreased antiviral responses and increased VZV replication (Carter-Timofte et al., 2018a). In children POL III plays a role in IFN induction in an early phase of disease but the available clinical data point toward a POL III role in the maintenance of latency given the reactivation of disease in adults caused by VZV. Finally, the cGAS-STING pathway seems to be important in controlling VZV infection in the skin and in keratinocytes where cGAS is highly expressed. Viruses can be therefore recognized by several immune receptors, such as POL III, cGAS, TLR9, TCR, inducing a type I IFN response and halting viral replication via Th1 immunity. Thus, the effects on latency establishment and reactivation are also impacted by the immune response (Carter-Timofte et al., 2018b). There are still scanty data regarding VZV and innate sensing in terms of differences in pathogenesis, cell types and mechanisms involved in primary VZV infection compared to VZV reactivation. Additionally, differences in the genetic background might influence VZV encephalitis and vasculitis, hence the need of prompting further investigation regarding the role of POL III in innate sensing of VZV reactivation and priming of adaptive immunity. Whether additional biological determinants of innate or adaptive immunodeficiencies predisposing to VZV CNS infection exist remain to be elucidated.

The role of DNA sensing in defense and disease is also highlighted by the overproduction of IFN response in diseases such as SAVI – STING gain of function mutations (König et al., 2017) as well as Aicardi-Goutières syndrome due to Trex1 loss of function mutations (Crow et al., 2006).

Paradigmatic clinical manifestations

Remarkably, the data derived from a French cohort of patients with primary immunodeficiencies analyzed 2183 patients, detecting various forms of immunodeficiencies that develop immune dysregulation manifestation overtime, including common variable immunodeficiency patients (CVID) in green you have the combined immunodeficiency patients (CID) along with innate inborn errors of immunities (Fischer et al., 2017). Combined immunodeficiency and innate immunity defects present symptoms earlier in life and CVID patients harbor immune dysregulation manifestations, but in all cases a very high proportion up to 50–60% of patients develop immune-deregulation during the course of their disease. Infections dominate the clinical picture, nonetheless, more complicated patients can present autoimmunity, unexplained lung disease, with pulmonary infiltration, lymphoid hyperplasia, splenomegaly gastrointestinal enteropathy, liver diseases and granulomatous diseases (Resnick et al., 2012). People who have the complications have increased mortality over a period of about four decades (Resnick et al., 2012) Lymphoma for example and hepatitis and lung disease or malabsorption and gastrointestinal disease will shorten the lifespan.

Enlarged spleen is found in 20% of patients and sometimes can be massively enlarged with hepatomegaly (Agarwal and Cunningham-Rundles, 2019). Indeed, liver can also be interested, especially in CVID patients. A nodular regenerative hyperplasia associated with portal hypertension can happen. Nodular regenerative hyperplasia (NRH) is characterized by a widespread benign transformation of the hepatic parenchyma into small regenerative nodules with fibrosis, leading to portal hypertension, splenomegaly and significant thrombocytopenia (Ward et al., 2008), autoimmune phenomena that can easily be triggered by the unleashed immune system or immunecheckpoint pathway defect (Anquetil et al., 2018; Argentiero et al., 2020). Lymphomas represent a major concern during CVID natural history (Mellemkjaer et al., 2002), and the lymph architecture of a patient with CVID is not particularly easy to distinguish from the lymphomatous one (Nicolae et al., 2015). The USIDNET registry reported lymphoma to be increased in proportion with a few other tumors (Leone et al., 2018; Mayor et al., 2018). The interstitial lung disease and the lymphocytic granulomatous lung disease look, with ectopic germinal centers (Maglione et al., 2014) are clinical picture which would require B-cell depletion or chemotherapy since the lung pathology often contains CD3^{pos} T cells along with CD20^{pos} cells (Maglione et al., 2014).

Interstitial lung disease in common variable immunodeficiency is a major complication with a still unknown immune-pathology, in which it is clear that granulomatous-lymphocytic interstitial lung diseases (GLILD) represent a collection of heterogeneous entities (Park and Levinson, 2010; Verma et al., 2015) in which a pathogen might be involved in triggering this disease. Thus, GLILD represents a distinct clinic-radio-pathological ILD occurring in patients with CVID, associated with a lymphocytic infiltrate and/or granuloma in the lung, and also in all those whose conditions were checked and, where possible, excluded (Verma et al., 2015). In 29/34 GLILD was diagnosed after CVID (7.8. years; 98% CI 5.8–9.9) and in 5/35 simultaneously (Mannina et al., 2016). Laboratory and clinical biomarkers of ILD involve autoimmune cytopenias, persistent lymphadenopathy, splenomegaly, increased isotype-switched memory B cells, increased CD4/CD8 T-cell ratio in the blood and enhanced IgM concentration (Schussler et al., 2016). An association of immune dysregulation and arthritis has also been confirmed by Mannina et al. In 52 subjects, 26 of whom with GLILD. Hartono et al. uncovered splenomegaly, history of immune-mediated cytopenias, low serum IgA level, and percentage expansion of CD21^{low} B cells as useful tool to identify a group of patients at high risk for developing GLILD (Hartono et al., 2017). Retrospective data confirmed these findings (Gathmann et al., 2014; Malphettes et al., 2009; Quinti and Pulvirenti, 2017; Wehr et al., 2008). While looking at the B cell phenotype, very often CD21^{low} B cells in the interstitial lung disease patients, at both peripheral blood and broncho-alveolar lavage were detected; moreover, many patients had reduced percentages of CD4 T cells (von Spee-Mayer et al., 2019). Furthermore, there is an association between the levels of immunoglobulin level and need to treat, especially in terms of IgM levels, most likely due to an immune overactivation (Schussler et al., 2016). Ancillary, there is the soluble IL2 receptor (sIL2R) levels are elevated in nearly all ILD patients (Vitale et al., 2015). From a histopathological standpoint Mannina et al. showed no significant differences in biopsy-proven and non-biopsy-proven ILD patients (Mannina et al., 2016) plus the ones treated based on the CT appearance. It is clear that most often bronchial biopsies can miss the diagnosis (Mannina et al., 2016; Schussler et al., 2016). The bronchoalveolar lavage differential cell count might support the imaging findings (Mannina et al., 2016), often detecting signs of inflammation, constituted by a mixture of lymphocytes, comprising CD21^{low} B cells, when compared to sarcoidosis (Mannina et al., 2016). Nonetheless, the underlying pathomechanisms are largely unknown. Remarkably in retrospective survey-based patients records pulmonary function testing seems also to serve as a promising diagnostic tool (Akhter et al., 2018; Jolles et al., 2017b); nonetheless, there is a lack of high quality and level of evidence and the indication to perform the bronchoalveolar lavage in PID subjects annually, can be a suggestion (Akhter et al., 2018; Jolles et al., 2017b); addressing lung function is also remarkable how normal tests can coexist with heavy changes in the CT scan. In more details, normal lung function does not exclude GLILD and an isolated impairment of DL_{CO} has also been reported (Akhter et al., 2018; Jolles et al., 2017b); however, DL_{CO} can vary largely, thus the decision for need of interstitial lung disease screening is made by additional aspects, often based on the clinical judgment. Of note lung function can worsen over time, especially in terms of lung diffusion capacity, pointing toward the relevance of careful follow up and, possibly, early biomarker of lung impairment (Meerburg et al., 2020). Remarkably, lung function tests can be expressed as absolute and percentage of predictive

value, corrected for age, sex, height and race; the percentage of predicted value however does not decline with age because it is corrected according to age. Usually, the definition of abnormality during interpretation of lung function arbitrarily refers to <80% of the predicted value, but change is more important. Moreover, how to quantify symptoms and the reliability of data obtained from patients with low lung function remain unmet clinical needs. In order to address these issues, Hurst et al. reported that the preferred measure of treatment response employed is the change in gas transfer (DL_{CO} and K_{CO}), with no complete consensus regarding the threshold of change thought to be significant: 10–20% for 63% of 19 respondents in the study vs. 21% who considered a change of 20–30% significant (Hurst et al., 2017). Dealing with quantifications of symptoms, breathlessness represents an archetypic complaint being quantified, either by MRC and mMRC, (Chhabra et al., 2009; Hajiro et al., 1998; Henoch et al., 2016; Mahler and Wells, 1988); Borg (Kendrick et al., 2000) or TDI/BDI (Mahler et al., 1995) scores. The MRC and mMRC scores main limitation is the lack of sensitivity for changes. Borg scale strength is its easier applicability. The TDI/BDI scale include other variables such as functional impairment, the magnitude of task and the magnitude of effort, being probably one of the most holistic scales (Cazzola et al., 2008). Moreover, to assess cough, the Leicester Cough Questionnaire (Birring et al., 2003) a worthy tool for clinical trial, despite a partial deficiency of practical applicability, while assessing objective responses. From the functional tests standpoint, the 6-min/incremental shuttle walk (Enright, 2003; Parreira et al., 2014) suits a relevant patient's need aiming to optimizing the accountability of given interventions in every-day-life.

The CT parameter changes that can support the clinical decision-making highlight how much GLILD encompasses different granulomatous, lymphoid (i.e., lymphoid hyperplasia, follicular bronchiolitis, lymphocytic interstitial lung disease-LIP), inflammatory lesions (i.e., organizing pneumonia-OP). In a few patients reticular patterns resembling non-specific interstitial pneumonitis-NSIP with a stronger reticular pattern can also be present (Cereser et al., 2017). Thus, difficulties in comparative analysis of CT scans warrants future consensus and guidelines. Computer program would probably provide quantitative support in decision making (Schütz et al., 2019). Ground glass and number of nodules are the imaging findings more often considered in the careful risk stratification; in the absence of ground glass changes all severities of number of nodules can be found equally and ground glass changes without noduli is very rare, being ground glass severity parallel to many and larges nodules, most likely (Cereser et al., 2017; Meerburg et al., 2020; Schütz et al., 2019). Over time spontaneous alterations in ground glass number and size of nodules characteristically worsen, nonetheless, a variety of patients pinpoint the disease heterogeneity and the need to dissect the subgroup better, in order to more efficiently tailor the clinical decisions. The radioprotection represents a major concern; therefore, MRI has been considered (Milito et al., 2015). MRI sequencing can provide information regarding edema and cellularity of single nodules. PET CT is also a tool that can support follow-up, although the metabolic activity holds a lack of specificity (Jolles et al., 2017a), potentially threatening the evidence-based level to support the radio-exposure. Indeed, on one hand, chest X-ray is probably too insensitive compared to CT scan, whose approximate radiation dose is conversely a multiple of baseline annual exposure. PET-CT also deserves to be mentioned in terms of its potential role in providing activity information, while compensating a significant radio-exposure level with functional details, also useful in performing differential diagnose and response to treatment (Jolles et al., 2017a).

Looking at the relative risk of developing autoimmune manifestations in the cohort of patients with immunodeficiencies this risk is significantly higher than in the general population and it is in fact even 830-fold higher for the autoimmune hemolytic anemia than in the general population, 120-fold higher in general for the immune-cytopenia or other forms of cytopenias than in the general population (Fischer et al., 2017). Thus, obviously hematological manifestations are a very significant manifestation of this disorder.

Immunocheckpoint as critical regulator of immune responses

In the last few years new genetic defects have hampered the knowledge of the biological architecture beyond the phenotype of the inborn error of immunity. CTLA4 is a critical regulator of T cell responses, as a molecule that is expressed by activated T cells and it outcompetes CD28 which is a costimulatory molecule of T cells for T cell activation. CTLA4 has a higher affinity than CD28 and CD86, which are molecules expressed by antigen presenting cells (Walker and Sansom, 2015); when T cells expressing CTLA4 bind CD80 and CD86 shutting off the signaling that is normally provided by the CD28 activating molecule so dampening the T-cell responses. Moreover, CTLA4 is expressed by regulatory T cells, playing a critical role for regular T cell function and finally CTLA4 not only binds CD80 or CD86 but it also mediates trans endocytosis of these molecules, basically stripping the antigen presenting cells of these molecules (Walker and Sansom, 2015), therefore, they can no longer function as professional antigen presenting cells, because they are unable to deliver the second signal, which is critically required for T cell activation. Thus, by a variety of mechanisms CTLA4 plays a major role in T cell regulation. The first paradigmatic condition representing a poster child for CTLA4 role in immunodeficiencies has been identified a few years ago as CTLA4 haploinsufficiency (Kuehn et al., 2014, p. 4; Schubert et al., 2014). The manifestations of these diseases include progressive lung disease with bronchiectasis and lymphocytic infiltrates, lymphoid aggregates in the central nervous system (CNS), lymphadenopathy and infiltration of lymphocytes in mucosal tissues, autoimmune cytopenias, hypogammaglobulinemia, T cell lymphopenia with presence of activated T cells. Because of the haploinsufficiency the disease is autosomal dominant in terms of inheritance, but it in actual facts has an incomplete penetrance. Data obtained from 96 patients recorded by Uzel et al. show a variable frequency of main manifestations of the CTLA4 haploinsufficiency about 50% of the patients had organ lymphocytic infiltrates and hypogammaglobulinemia, about 30% had evidence for neuroinflammation (Schindler et al., 2020), a significant proportion of patients reported recurrent infections (about 30%) and up to

35–40% of the subjects had been reported to have autoimmune cytopenias (Schindler et al., 2020). Remarkably, a variable percentage of subjects might be genetically affected but clinically asymptomatic individuals (Schindler et al., 2020), thus this disease may manifest at any age including elderly people and so these are people that are asymptomatic up to a certain time in their life, which doesn't mean that they will necessarily remain asymptomatic for their entire life (Schwab et al., 2018). A very similar picture is also shared by patients with LRBA deficiency which is an autosomal recessive disease also characterized by chronic lung disease and bronchiectasis, severe diarrhea with lymphocytic infiltrates, lymphoid interstitial pneumonia with predominant T infiltrates. Autoimmune cytopenias, hypogammaglobulinemia which is even more prominent than in CTLA4 deficiency, variable T cell lymphopenia with activated T cells, are perhaps more controversial infections in LRBA deficiency than CTLA4 deficiency as an autosomal recessive disease (Lo et al., 2016). LRBA acts as chaperon for CTLA4 and permits a recycling of CTLA4 which is internalized by the T cell and then can be re-expressed, thus, in the absence of LRBA CTLA4 is prompted to lysosomal degradation which results in significant reduction of CTLA4 levels on the surface. Therefore, patients with LRBA deficiency will have in fact low levels of CTLA4 on the cell surface pretty much like the patients with CTLA4 haploinsufficiency (Lo et al., 2016).

The spectrum of inborn errors of immunity and the concepts of autoimmunity and immunodeficiency in these disorders

The inborn error of immunity is characterized by more than four hundred single gene disorders of the immune system that drive the immunodeficiencies with infection susceptibility phenotype and those associated with immune-dysregulation which encompasses autoimmunity, lymphoproliferation and malignancies. The errors of immunity associated with autoimmunity, lymphoproliferation, bone marrow failure hemophagocytosis, parallel single genes disorders that present with this particular phenotype (Abraham, 2020).

The umbrella of immune dysregulation on one hand includes all the immunological and clinical manifestations (Fig. 1 and Table 2), although these clinical scenario is paralleled by a broad spectrum of specialties dealing with this particular disorder, namely hematologists, immunologists, rheumatologists, dermatologists, oncologists, lung specialists, hepatologists, gastroenterologists, pathologists, requiring a multidisciplinary approach to be effective.

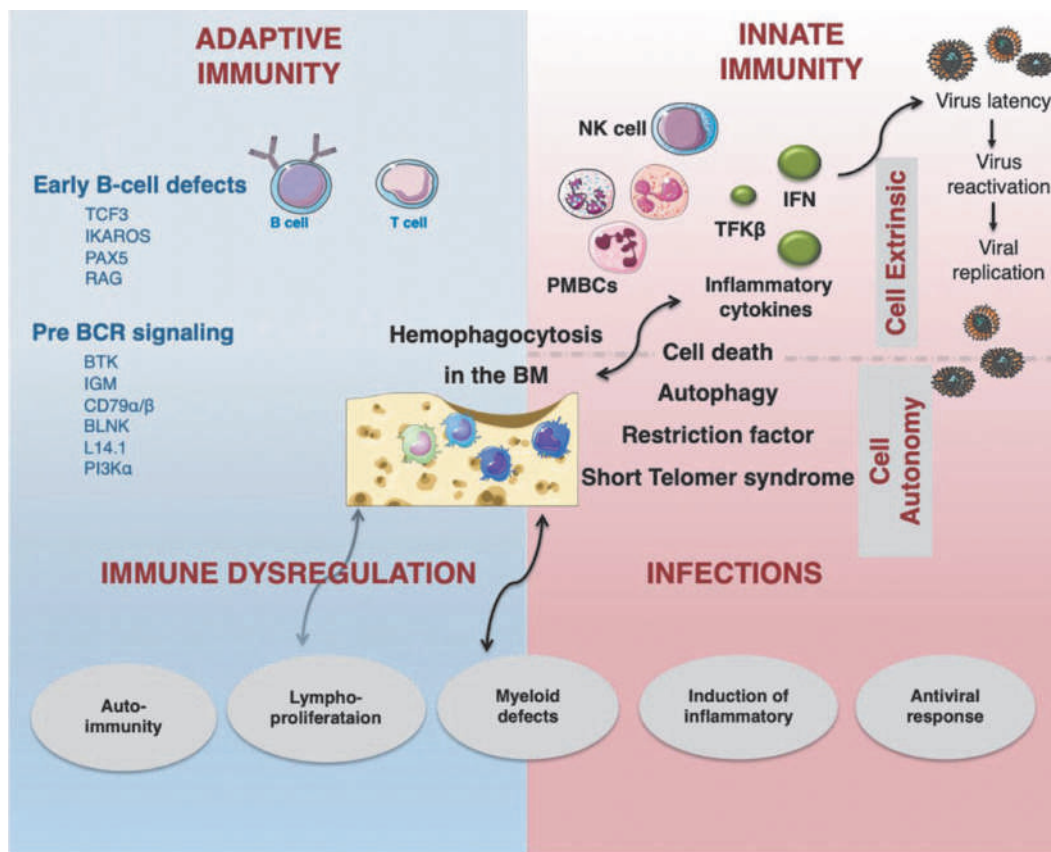


Fig. 1 The extended spectrum of inborn errors of immunity. The knowledge of the immune dysregulation and infections related to primary immunodeficiencies and their underlying genetic disorders represent a continuum.

Table 2 Immune dysregulation and its clinical picture.*Immune dysregulation and its clinical picture*

Autoimmunity
 Autoinflammation
 Immunodeficiencies
 Lymphoproliferation
 Atopy
 Neoplasia
 Hyperinflammation
 Paraneoplastic syndromes
 Cytokine storm
 Lymphopenia
 Cytopenias

Table 3 Overview of the laboratory and clinical findings, correlated with the advanced diagnostic approach that can be implemented.

<i>Laboratory findings</i>	<i>Clinical findings</i>	<i>Advanced diagnostic approach</i>
Elevated inflammatory markers	Fever	Deep immunophenotyping
Hypo/Hyper-gammaglobulinemia	Rash	Assessment of T, B, NK cell and innate immune function
Elevated pro-inflammatory cytokines soluble biomarkers	Lymphadenopathy	Cytokine/soluble biomarkers
Cytopenias	Splenomegaly	Intracellular cytokines, proliferation
Autoantibodies	Cytopenias	NF-κB activation, TLR, JAK/STAT, PI3K and other signaling pathways
Quantitative alterations in lymphocyte subsets (lymphopenia/lymphocytosis)	Liver failure	Telomere length analysis
Functional abnormalities in adaptive and innate lymphocytes	Coagulopathy	Assessment of DNA repair pathways
	ILD	Genomics—Targeted WES, WGS, chromosomal arrays, SNP analysis, CNVs
	NLH of the gut/colitis	Analysis of VUS and functional validation
	Bone marrow failure	Transcriptomics and other OMICS

VUS, Variant of uncertain significance.

From the diagnosis standpoint of immune dysregulation clinical findings should be appropriately mixed with laboratory ones (Table 3).

Immunophenotyping, functional analysis and genomic analysis are essential in case of patients with inborn errors of immunity or immune dysregulation.

Therapeutic windows

In the retrospective analysis available concerning lung involvement across the immunodeficient patients, the most common approach across the demographic of treatment is to firstly approach subjects with interstitial lung diseases and immunodeficiency with steroids and as a second step, carry on with immunosuppressive therapies (Jolles et al., 2017b). Nonetheless, it is also evident that a subgroup of patients would need a more aggressive treatment as soon as possible. Indeed, starting with a glucocorticoid (GC) treatment leads to improvement of DLCO, but under GC treatment the course of DLCO is variable, with a tendency of further decrease in patients with a previous more severe impairment. Lung function testing shows characteristically variable degree of responses (Chase et al., 2013; Cinetto et al., 2021). There is also a mixed response in steroid treated patients in terms of groundglas, grade, number and size of nodules. GLILD is a discrete radio-pathological interstitial lung disease ensuing CVID course and correlates with lymphoid cells infiltration and with granulomatous reaction after having excluded alternative causes (Hurst et al., 2017). The aims of treatment in GLILD are the reduction of symptoms and preservation of lung integrity with reduced risk of death; however, the level of evidence is low (Hurst et al., 2017). Immunoglobulin replacement might be an option (Arish et al., 2006), therefore prompting validation studies regarding initiation and optimization of antibody replacement may be a solution. Some evidences from case series on 54 subjects on immunoglobulin pinpoint that GLILD changes may arise and progress in those on adequate immunoglobulin even if not on immunoglobulin (Arish et al., 2006; Gregersen et al., 2010). Moreover, optimization seems an appropriate strategy, although there is a lack of trials with higher dose immunoglobulin. Regarding the consensus level on decisions to start treatment, lung function deterioration seems to be the major driver of decision. Contrariwise, CT changes on their

own were not persuasive enough to start treatment (Hurst et al., 2017). When first treatment has been started 90% of participants in the trial lead by the British Lung Foundation/United Kingdom Primary Immunodeficiencies Network Consensus agreed that when was required, first-line should be corticosteroids alone. 20 out of the 21 respondents preferred to use oral prednisone (Hurst et al., 2017). minimum dose of 10 to 20 mg/day, maximum 1 to 2 mg/kg/day. The median (IQR) dose for a 70-kg patient was 40 (30–70) mg/day (Hurst et al., 2017). Some respondents used prednisone plus either azathioprine or mycophenolate as second agent (Hurst et al., 2017). The evidence for corticosteroids uses in GLILD are obtained from the French DEFI Study Group publication, where among 436 patients with CVID 13.5% were multisystemic granulomatous, with lung involvement in about 50% (Boursiquot et al., 2013). In this large case-series, 30–60 mg/day of GC brought a 54% response (Boursiquot et al., 2013). Nevertheless, evidential challenges of these observational studies are constituted by different definitions of disease, no definition of treatment response, different doses of different therapies and different monitoring regimens. Moreover, while assessing the treatment response, the test entity, its frequency and its inherent variation constitute pivotal issues. Second line therapy for GLILD can be considered in patients who responded to treatment and for those whose response to treatment needs a maintenance, while tapering the GC and, alternatively, it can also considered in subjects who did not respond to GC (Hurst et al., 2017). Azathioprine, rituximab and mycophenolate gained the highest consensus level in the special article by Hurst et al. (2017). In more details rituximab and azathioprine target B- and T-cells, respectively (Chase et al., 2013; Solimando et al., 2016). In the study from Chase et al. rituximab 375 mg/m² weekly was administered to 4/52 subjects and repeated monthly from four to six times (Chase et al., 2013). Azathioprine/6MP was typically administered at 1–2 mg/kg/day and mycophenolate 1 g, twice a day (Chase et al., 2013), with a background of immunoglobulin treatment, as in primary and secondary immunodeficiency (Pecoraro et al., 2019; Vacca et al., 2018). No infection concerns are reported and 5/7 patients responded in terms of radiological findings, the patients who did not respond had more severe restrictions and longer standing diseases (Chase et al., 2013).

Bone marrow transplantation has been performed in people with interstitial lung changes (ILD) not necessarily because of ILD (Wehr et al., 2015). Certainly, concerns in people survival deserves careful evaluation of mortality rate, especially in those patients who had a lung disease before transplantation.

Regarding the paradigmatic examples of inborn errors of immunity and given what happens in subjects with either CTLA4 haploinsufficiency or LRBA deficiency, an increased signaling through CD28 due to a lack of CTLA4 molecules on the cell surface takes place. This second signal allows the T cells to mediate activation of the AKT/mTOR pathway, prompting a lymphoproliferative syndrome (Westin, 2014), proangiogenic (Lamanuzzi et al., 2018) and autoimmunity (Koga et al., 2014). One way to counteract this phenotype is to provide the patients with a soluble CTLA4-Ig molecule, abatacept (Moreland et al., 2006) or belatacept (Wekerle and Grinyó, 2012), bind CD80 CD86 at high affinity, displaying CD28 and prompting blocking and hyperactivation of the AKT/mTOR pathway. Targeted therapies, by understanding the monogenic defect and intervening on that hold the promise to lead to CT changes (Lo et al., 2015). Results are not always as expected, given the poor response to abatacept in CTLA-4 deficiency, although a good response to abatacept in NF- κ B frameshift mutation has been reported (Verma et al., 2017). Alternative options are represented by mTOR inhibitors, able to inhibit lymphocytic infiltration and activation preventing autoimmunity (Suto and Karonitsch, 2020). For all of these disorders prospective clinical trials are still lacking, nonetheless as a consequence of retrospective studies looking at a phenotype in 133 patients, among the 14 who received either abatacept or belatacept 11 reported clinical improvement, in particular the clinical outcome was the improvement of enteropathy, the halting of lung lymphoproliferation, an improved platelet count and a reduced lymphadenopathy (Schwab et al., 2018). Thirteen patients received sirolimus, eight of them reported a good outcome with the resolution of transfusion dependent pure red cell aplasia regression of lymphadenopathy and splenomegaly, improved CMV viral load, enteropathy also improved in three patients who received sirolimus plus other immunosuppressive agents (Schwab et al., 2018). Statistically powered studies are absolutely needed in order to confirm these data. Another major pathway which may cause immune dysregulation with immunodeficiency is due to defects in the JAK-STAT signaling pathway, which is activated in response to a variety of cytokines and cytokine receptors (O'Shea et al., 2013). The extended clinical spectrum of STAT1 gain-of function mutations (Hambleton, 2016) in an autosomal recessive fashion, presents with very severe infections, with an early in life clinical feature and dominant negative phenotype, presenting with chronic mucocutaneous candidiasis, mycobacterial infection and other viral infections due to HSV, VZV, HPV, molluscum, cytopenias and other autoimmune manifestations, such as thyroiditis, alopecia and vitiligo, type 1 diabetes mellitus and systemic autoimmunity. Remarkably, STAT1 gain-of-function mutations are also related to some non-immune manifestations such as aneurysms, higher frequency of carcinoma and esophageal strictures (Yu et al., 2009, p. 3). Somewhat similar is the phenotype of the STAT3 gain-of-function mutations (Milner et al., 2015) in which enteropathy, lung nodules, lymphocytic infiltration of the respiratory tract and inflammatory lung disease predominate, along with alopecia, hypoparathyroidism, splenomegaly, type 1 diabetes and polyarthritis. Many of these subjects may have short stature (Milner et al., 2015). Collectively, pleiotropy and redundancy characterize JAK/STAT signaling pathway, but it also provides an opportunity to target these diseases by using specific molecules like JAK-inhibitors (Jamilloux et al., 2019) even though this drug specificity remains to be clarified (Patterson et al., 2014). Indeed JAK inhibitors are a novel class of drugs for targeted therapy in STAT1 and STAT3 gain-of-function (Forbes et al., 2018) that might be either pan-JAK inhibitors, such as oclacitinib (Gonzales et al., 2014), multi-JAK-inhibitors such as tofacitinib (Fleischmann et al., 2012), ruxolitinib (Verstovsek et al., 2012) and baricitinib (Richardson et al., 2020) or specific inhibitors, such as filgotinib (Vermeire et al., 2017), itacitinib (Schroeder et al., 2020), decernotinib (Fleischmann et al., 2015), still under investigation as an ideal molecule targeting the molecules that can allow a tailored treatment to ensue precision medicine available for these diseases.

The activated PI3-kinase delta syndrome (APDS) cell biology represents another paradigmatic example of inborn error of immunity. AS is a heterodimer composed by a kinase molecule, the p110-delta, and the regulatory molecule p85-alpha which

binds to p110-delta and prevents the catalytic activity of the p110-delta (Michalovich and Nejentsev, 2018). A heterozygous gain of function mutations on p110-delta or a loss of function mutations in p85-alpha, prevent the blocking activity on p110-delta and can result in phosphorylation of AKT with an induction of the mTORC1 in S6 phosphorylation. Therefore, in APDS an impaired FOXP3 expression and defective Treg function can parallel an increased aerobic glycolysis and lipid synthesis. Moreover, due to a direct effect via phosphorylation-mediated protein degradation on FOXO1, an inhibitor of cell survival and proliferation, the pervious biologic phenomena are unleashed (Cabrera-Ortega et al., 2017). Therefore, the clinical findings of patients with APDS are characterized by immune manifestations because of perturbed regulating cell function but also increased cell proliferation promoting generalized lymphadenopathy, with florid follicular hyperplasia, loss of germinal center cuff, defective Ig class switch and increased PD-1 positive T cells. Increased infection risks in particular due to CMV, EBV, VZV but also adenoviral infections are observed. Recurrent bacterial sinopulmonary infections are also reported as well as cytopenia due to autoimmune sequestration, Hodgkin and non-Hodgkin lymphoma, predominantly HBV positive however there also HBV negative cases (Coulter et al., 2017). PI3kinase inhibitors are available (Hoegenauer et al., 2017), such as specific inhibitor p110-delta, resulting in a significant reduction in the splenomegaly as well as a reduction in the biomarkers that characterize the immunological profile of these patients namely an increased proportion of PD-1 positive T cells and an increased, number of follicular helper T cells (Rao et al., 2017). Additionally IgM levels can undergo a significant decrease along with other immunoglobulin levels improvement (Rao et al., 2017). Another potential targeted therapy is based on PI3kinase inhibitor, nemoralisib, an inhaled PI3K inhibitor, potentially exerting a decreased effect on lymphoproliferation compared to leniolisib, due to the pharmacokinetics, nonetheless, patients with bronchiectasis and airway disease might primarily benefit from this approach (Nunes-Santos et al., 2019). Nemoralisib has also been used in patients with chronic obstructive pulmonary diseases and in a multicenter non-randomized open label uncontrolled single group study to evaluate the safety and pharmacokinetics of nemoralisib in patients with APDS (NCT02593539).

Regarding the treatment of immune-mediated cytopenias in the context of inborn errors of immunity, it is mandatory to understand that the hematological situation is often combined with an early onset of an inborn error of immunity per se and should prompt a differential diagnosis with an underlying hematological or autoimmune condition. Seldom, a reminiscent of GvHD can also be the primary cause. A large class of drugs can be categorized as shown in Table 4 (Seidel, 2020).

For autoimmune hemolytic anemia 15 trials are ongoing, exploring anti-FcRn (NCT03075878, NCT04119050), anti-SYK (NCT04138927, NCT03764618) anti-BTK (NCT04398459) and PI3K (NCT03538041). Moreover, a combination of B-cell depletion (Solimando et al., 2016) and novel drugs, such as rituximab and proteasome inhibition have also been intensively investigated (NCT04083014) (Ratnasingam et al., 2016). A complement inhibition has also been extensively explored (Berentsen and Sundic, 2015) (NCT02502903). Evans syndrome (Miano, 2016; Norton and Roberts, 2006; Stepensky et al., 2015) and immune thrombocytopenia (Feng et al., 2017a,b; Ghanima et al., 2021; Gómez-Almaguer, 2018; Newland et al., 2020; Ratnasingam et al., 2016; Ruzza et al., 2009); have also been broadly studied. Therefore, after having considered the underlying reason for the autoimmune cytopenia, including potential differential diagnosis (Seidel, 2020), recognizing the potential target for the primary cause is of paramount importance. Indeed, a gene product or an affected pathway could be directly druggable or even a related-pattern recognition can drive the overactive pathway involved targeting. Patients with CVID can represent additional challenge, when autoimmune neutropenia the use of GCSF or IVIG is still debated due to a lack of high quality and robust data. Nonetheless, the use of GCSF can be considered in a standby fashion, taking into account the patient history as well as the current and severity of bacterial and funga, with infection (Sokolic, 2013). High dose of IVIG might exert an immunosuppressive effect in patients with CVID, but there is a lack of evidence for its efficacy, despite case series and reports showed promising results in patients treated with high dose of immunoglobulin replacement therapy (Podjasek and Abraham, 2012). Nonetheless, patients with severe immune-mediated anemia and neutropenia should be probably considered for transplant, in order to avoid serious sequelae such as life-threatening infections, bronchiectasis and ILD (Teachey and Lambert, 2013). Antibiotic prophylaxis is also debated (Cunningham-Rundles, 2010).

Table 4 The classes of drugs for cytopenias in inborn error of immunity.

<i>The classes of drugs for cytopenias in inborn error of immunity</i>
Immune modulatory drugs (IVIG, anti-D, chloroquine, danazol, retinoids)
Growth factors (TRA; GCSF)
Targeted compounds (sirolimus, abatacept, idelalisib, complement-blockade)
Plasmapheresis
Immunosuppressive drugs (MMF, rituximab, CSA, fostamatinb, alemtuzumab, bortezomib, daratumumab)
Antiinflammatory drugs (glucocorticosteroids, anti-TNF α , anti-IL6, anti-IFN- γ , JAK-inhibits)
Splenectomy
Cytostatic chemotherapy
Allo-HSCT
Gene-therapy

A large number of anti-inflammatory drugs, immunosuppressive drugs, "targeted" drugs is not shown since they are overlapping. 6-MP, 6-mercaptopurine; anti-D, anti-Rh(D) antibody; AZT, azathioprine; CSA, cyclosporin A; CY, cyclophosphamide; GCSF, Granulocyte colony-stimulating factor; HSCT, Hematopoietic stem-cell transplantation; IFN- γ , interferon- γ ; IVIG, high-dose intravenous immunoglobulin G; JAK-inhibs, inhibitors of Janus kinases; MMF, mycophenolate mofetil; MTX, methotrexate; TNF α , tumor necrosis factor α ; TRA, thrombopoietin receptor agonists; VCR/VBL, vinca alkaloids.

State-of-the-art therapeutic approach: From transplant to novel insights

The cytopenia can be one of the factors that, when refractory, can prompt clinical decision regarding transplantation and gene therapy. Specifically, allogeneic hematopoietic stem cell transplantation (HSCT) in adults with primary immunodeficiency. The complexity of HSCT landscape is an emerging field, due to the difference between PID and typical adults transplant patients, constituted by disease- and patient-related factors. Firstly, genetic diagnosis frequently is lacking in adults presenting with PID, thus hematopoietic mutation is seldom demonstrated before transplant. Due to the rarity of the diseases themselves, often limited information on natural history of the disease in adulthood with conservative management alone are available, particularly, because only recently patients survive until the adulthood due to novel approaches and supportive care. Moreover phenotypical heterogeneity and atypical presentations still represent a major unmet clinical need in facing the pathophysiological scenario (Fox et al., 2018). Regarding the patient related factors impacting on the transplantation strategy, the identification of a clear trigger for referral to a HSCT center still represent a clinical challenge as well as the high frequency of comorbidities, such as severe infections and highly refractory autoimmune conditions (Castagnoli et al., 2019; Notarangelo, 2009). Some patients have a very clear indications for transplant which is malignancies or life-threatening infections or hemophagocytic lymphohistiocytosis (HLH) (Lehmberg et al., 2019). Progressive PID-related complications failing to respond to alternative therapies which will lead to irreversible organ injury also represent a HSCT indication (Carreras et al., 2019). Ongoing investigations are aiming to identify which patients with PID may benefit from HSCT (Morris, 2020). Specifically, young patients with low-grade comorbidities score, good lung and liver functions and HLH or malignancies that are in remission at the time of referral are likely to benefit and are considered at lower risk at the time of the HSCT. Moreover, the knowledge of the underlying genetic defect and phenotype make the HSCT decision easier (Dimitrova et al., 2020). Remarkably, the clinical outcome in carefully selected patients is excellent (Morris, 2020). A multicenter EBMT study confirmed the data generated by Morris et al. showing an outcome advantage in the peculiar clinical scenario of systemic granulomatous disease (Chiesa et al., 2020), demonstrating an excellent OS in the adult group, including 77 patients over the age of 18. Finally, the assessment of PID the confirmation of the functional immune deficiency should be confirmed and assessing and understanding the underlying disease is key while documenting the indication for HSCT, looking for evidence for effectiveness of allo-HSCT along with evidence for predicted poor prognosis with conservative management. Appropriate donor and patient fitness are also of paramount importance. Indeed HSCT in CVID in a retrospective multicenter study with transplant occurred between 3 and 26 years after the onset of the first symptom identified a 50% of mortality (Wehr et al., 2015), despite retrospective data warrants statistically powered trials to properly answer the unmet clinical questions. Moreover alternative novel therapies should always be taken into consideration such as targeted drugs or gene therapy and gene-editing. Of note, genetic engineering was unrevealed as a novel frontiers for deficient PIDs management, because the vast majority of inborn error of immunity are amenable in theory to correction with cellular therapy or gene editing (Picard et al., 2018). The general goal of cellular therapy is to replace the long-term HSC, while proceeding with conditioning aiming to eliminating HSC and progenitors and engrafting donor long term-HSC (LT-HSC). All of the progeny would be donor-derived. Nonetheless, donor availability, graft versus host disease (GVHD) and long-term effect represent limitations of allogeneic transplant. Moreover, since PIDs differ from malignant condition being able to be managed with medical therapy, a careful balance is necessary between the acute and chronic toxicities that after allo-HSCT might become more morbid than the disease itself (Bakhtiar et al., 2019). Lower conditioning intensity regimens are better tolerated but predispose to mixed chimerism. This holds different consequences depending on the given underlying condition. Severe combined immunodeficiency (SCID) has many genetic causes. Patients with SCID have a profound absence of T cells. X-linked SCID (SCID-X1) was the first disease to be treated with HSCT (Gatti et al., 1968). Considering the different genetic subtype of SCID, it should be considered that although B cells are still present in B+ SCID the cytokine, CD3 and other receptors defects imply a lack of CD4 T cell help, thus impacting on the B cell function (Fischer, 2000). Conversely, B cell absent SCID is characterized by recombination defects and metabolic defects that physiologically impact on immune dysfunction. The lack of CD4 T cells in SCID allows the special condition of HCT effectiveness without conditioning, since the patient has no need of immunosuppression and myeloablation to engraft T cells. Thus, if the patient has a matched sibling donor the bone marrow, peripheral blood or umbilical cord blood can simply be infused and greater than 90% of the cases are developing T cells, being rescued from opportunistic infection. Most of patients supports a lack of matched sibling donors, therefore, another strategy is to use an aplodidentical parents, removing in some ways the T cells that otherwise would cause lethal GVHD, being anyway able to obtain a successful engraftment (Pai, 2019). The reason why this unconditioned engraftment can be successful is because of the unique role of the thymus in the T-cell development and the lack of the recipient T-cell leads to a strong advantage for the donor-derived T-cells. Unlike the other lineages, T-cells mature in the thymus when bone marrow-derived HSC or progenitors enter the thymus and are induced to develop into T-cells. In the patient with SCID where there are no progenitors capable of developing into T-cells those donor-derived HSC can engraft in the thymus and give rise to donor-derived T-cells (Pai, 2019). Since nothing needs to be done to eliminate the HSC or the other progenitors all the other cells will remain of recipient origin. Chemotherapy might affect donor cell engraftment and immune reconstitution in different types of SCID, having consequences in incomplete immune reconstitution. In patients with B cells absent (B- SCID), transplant without conditioning might restore T cells but without chemotherapy the recipient HSC remain untouched and therefore the patient will still lack B cells and still lack Ig production. In the B+ SCID T cells are restored successfully the majority of the time and in theory those T cells should be able to give recipients T cell help and induce antibody production. However, IL2RG/JAK3 deficient B cells fail to function despite T cell help due to lack of IL-21 response, failing to produce humoral response after conditioning transplant because those recipients mutant B-cells remain unresponsive to IL-21 and do not function despite the provision of T cell help (Miggelbrink et al., 2018). Conditioning is needed for some types of SCID to

improve T cell number and B cell function (Pai et al., 2014, pp. 2000–2009), replacing HSC with donor HSC (Dvorak et al., 2014), which not only leads to T-cell development, but also to the development of donor-derived B cells (Haddad et al., 2018; Heimall et al., 2017). Furthermore, conditioning appears to improve T cell output by providing continuous seeding of thymus with donor-derived cells and otherwise, without conditioning the limited inoculum that engrafts in the thymus would exhaust over time.

WAS and IPEX represent different clinical scenario in which there are T cells, but donor-derived T cells or subsets of T cells in the subset of IPEX have a strong selective advantage, which is consequences for the outcomes having the states of mixed-chimerism. In details Wiskott-Aldrich syndrome, being characterized by thrombocytopenia, eczema, infections, autoimmunity and malignancy represents an X-linked disease caused by defects in WAS gene with defects in cytoskeletal function, with abnormal T cell survival, abnormal T, B, monocyte migration and small defective platelets (Moratto et al., 2011; Pai and Notarangelo, 2010; Puck and Candotti, 2006). Myeloablative conditioning is generally used for WAS syndrome, gaining an excellent survival, matching the goal of HSCT of correcting the immunodeficiency and correct the platelet count (Moratto et al., 2011). When high level donor chimerism is achieved both the lymphocyte compartment and the platelet compartment are corrected. However in the state of mixed-chimerism the lymphocyte compartment is still corrected because T-cells and to some extent B cells which express WAS protein have a survival advantage of recipient WAS negative cells and looking at the degree of donor chimerism the patients and their T cells have an high tendency of donor-chimerism; conversely in B cells and myeloid cells there are less than 50% of donor-derived cells. Infact, mixed chimerism may result in persistent thrombocytopenia and myeloid cells are more prone to mixed chimerism than T and B cells. In the donor cells there is a lack of selective advantage that may lead to a failure of platelets count recovery. A correlation exists between patients with less than 30% of donor chimerism in myeloid cells and platelet count below 50,000/mcL (Moratto et al., 2011).

In the X-linked immune dysregulation-polyendocrinopathy-enteropathy—IPEX a defect in FOXP3 gene affecting a critical transcription factor results in absence of regulatory T cells (Treg) or lack of Treg function. Thus an inability to control effector T cells and the development of autoreactive B cells, produce autoantibodies leading to dermatitis, enteropathy, autoimmune diabetes and other severe autoimmune manifestations (Seidel et al., 2009). Because of a lack of Treg a mixed chimerism after transplant at a HSC level, the conventional T cells and B cells may be mixed, but the Treg also are derived from the thymus and in the patient they are largely donor due to a selective advantage. In turn those Tregs should become capable of controlling in a dominant fashion both the normal donor T cells and the recipient T cells. If the donor-derived Tregs have a selective advantage reduced intensive regimen is putatively as good as myelo-ablative and the mixed chimerism is supposed to be as good as full donor chimerism (Seidel et al., 2009). Retrospective studied on 96 IPEX patients showed no significant differences between reduced-intensity regimen compared to the myeloablative one being mixed chimerism apparently sufficient (Barzaghi et al., 2018). Looking at organ involvement by autoimmunity prior to transplant Barzaghi et al. also found that patients with autoimmunity with organ damage that was poorly controlled before the transplant had a substantial worse survival. This data did not differ in patients who received reduced-intensity conditioning regimen and those patients who were older or younger at the time of transplant (Barzaghi et al., 2018). Therefore, the outcomes of HSCT for IPEX are likely to be better with disease control and early intervention before organ damage occurred.

In diseases in which the T-cells are dysregulated intrinsically and can potentially persist after HSCT because it is not expected that donor T cells can have a selective advantage. The poor survival for CVID treated with HSCT due to early mortality for an underlying infection (Wehr et al., 2015). Slow immune reconstitution is likely the reason why those patients experienced an unfavorable prognosis. After HSCT the donor graft contains not only HSC. And progenitors that engraft in the marrow, but also a limited amount of mature T cells from the donor that are essentially adoptively transferred to the patient. Early post-HSCT the donor HSC will start to seed the thymus but very little new T cells generation and the T cells that are circulating remain to the donor infused-cells that may expand in the neutropenic environment but remain very low in number and limited in specificity. Therefore, prolonged T lymphopenia prompts a high risk of viral and other infection. In 4–6 months new T cells derived from donor HSC emerge from the thymus (Castagnoli et al., 2019). Haploinsufficiency of CTLA4 gene, being characterized by variable penetrance, is characterized by dishinhibited effector T cells, due to reduced inhibitory CTLA4 expression and also, the Treg are poorly functional, thus not only those Treg are not able to control the T cells but fail to regulate the APC which in turn leads to further inactivation of appropriate activation of effector T cells. Thus, the goal of HSCT is to replace the Tregs with functional cells and eliminate the dysfunctional CTLA4 deficient effector T cells. In the retrospective review from Schwab et al. of 133 CTLA4 haploinsufficient patients, 12 received HSCT, all having unrelated donors, receiving either standard or reduced intensity conditioning regimen three died and one subject passed succumbed due to diabetic ketoacidosis which highlights the fact that even with the correction of the immune system organ damage that was already sustained will not be corrected. Interestingly, almost all patients with high-level chimerism had improvement or remission of disease. Remarkably, one patient with 60% T cell chimerism and 100% all other lineages, had CMV reactivation, ARDS, chronic steroid treatment (Schwab et al., 2018). Thus, a failure to eliminate recipient T cells and mixed chimerism in T cells is deleterious for the outcome. Therefore, in order to prevent mixed T cell chimerism from occurring warrants an understanding of two main mechanisms. These patients experience a full engraftment of HSC, however, some recipient T cells fail to be completely eliminated and those T cells persist. Secondly, the patient can develop mixed chimerism at HSC level in which case not only might there be some rebound of recipient T cells in the periphery that escaped conditioning, but also, it can be an output of new T cells some of which are donor-derived and some of which remain CTLA4 haploinsufficient. Therefore, in order to succeed with conditioning regimen recipient T cells that are intrinsically overactive must be shrunk down and HSC mixed chimerism should be avoided. Finally, in APDS the graft failure and survival represent a major challenge (Nademi et al., 2017), with older age as a risk factor for poor survival as well (Leiding and Forbes, 2019).

Given the above mentioned challenges, ex vivo gene therapy is a particularly attractive option, particularly for blood disorder (Moreno-González and Zarain-Herzberg, 2013). The concept is that by introducing a gene into the LT-HSC pool will sustain the expression of the gene and all the progeny of that LT-HSC will carry the gene. Thus, gene therapy is an alternative to allo-HSCT, since, instead of identifying a donor stem cells are harvested either from the bone marrow or peripheral/umbilical cord blood, the HSC can be technologically enriched by using the antigen CD34 and a vector subtypes (i.e., a gamma retrovirus - γ RV) allow the introduction of a gene copy. Next, is possible to cryopreserve the stem cells, insuring that they have sufficiency of gene transfer and allow the patient to be prepared to appropriate conditioning and receive an auto-HSCT rather than allo-HSCT. Obviously, being the patient its own donor, no GVHD is reported and as long as the transduction of a sufficient number of HSC is obtained the vector integrates efficiently into the DNA of the cell, and passes the gene to all progeny. SCID-X1 and ADA SCID were the first to be treated with ex vivo gene therapy (Aiuti et al., 2002; Cavazzana-Calvo et al., 2000). Encouraging trials are ongoing in ADA-SCID, X-linked SCID, Artemis SCID, Wiskott-Aldrich and X-linked CGD (De Ravin et al., 2016; Morgan et al., 2017).

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Treatment of Secondary Immunodeficiencies

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Introduction

Secondary immunodeficiencies (SID) represent the immune disorders that are acquired later in life, as opposed to those that result from an inborn (genetic) defect in the immune system (Fudenberg et al., 1970). Any acquired disease or exposure which increases susceptibility to infection can be considered a secondary immunodeficiency in the largest sense. For example, disorders that disrupt skin and mucosal barriers, such as surgery, trauma, burns, or malnutrition increase the host's susceptibility to infection (Chinen and Shearer, 2010). Similarly, the introduction of medical devices such as indwelling catheters and prostheses bypasses the first line of defense against infection. However, these conditions are beyond the scope of this article.

The immune system can be divided into the innate and adaptive arms. The innate immune system is primed to seek and destroy invading microorganisms within minutes. Phagocytes, such as neutrophils, monocytes, macrophages, and dendritic cells (DCs), recognize evolutionarily conserved pathogen associated molecular patterns (PAMPs) via pattern-recognition receptors (PRR; e.g., Toll-like receptors), which allow them to ingest and kill bacteria and fungi. In recent years, there has been a greater appreciation for non-hematopoietic cells, such as muco-cutaneous epithelial cells and neuronal cells, also playing relevant roles in human innate immunity, using similar PRR processes (Casanova and Abel, 2007). In addition to microbial killing, phagocytes act as antigen-presenting cells and produce cytokines, activating the adaptive arm of the immune system to provide antigen-specific and memory responses against pathogens (Turvey and Broide, 2010). Natural killer (NK) cells and natural killer T (NKT) cells are lymphocytes that are also considered members of the innate immune system due to their ability to kill virally infected cells

independently of major histocompatibility complex (MHC) class I-mediated antigen recognition (Orange, 2013). Complement further provides a humoral component to the innate immune system through the lysis of bacterial membranes and opsonization (Hajishengallis et al., 2017).

The adaptive arm of the immune system temporally follows the innate arm in the response to microorganisms. Whereas the innate arm recognizes fixed microbial motifs, the adaptive arm can recognize an infinite number of antigens (epitopes) due to genetic recombination of their immunologic receptors during their development. It can also distinguish between self- and non-self-antigens (Bonilla and Oettgen, 2010). B cells are responsible for the humoral component of the adaptive immune system through the production of antibodies. Recognition of a new antigen leads to B cell proliferation, isotype switching from immunoglobulin M (IgM) to immunoglobulins G (IgG), A (IgA), or E (IgE), affinity and avidity maturation, and differentiation into long-lasting plasma cells that continue to produce mature antibody to protect from further exposure. Antibodies both neutralize virulence factors and act as opsonins for the innate arm (Murphy, 2012).

T cells play a key role in the cellular component of the adaptive immune system (i.e., cell-mediated immunity, CMI). Following maturation in the thymus by both positive and negative selection, egressed naïve T cells require specific signals to become activated ("two-signal" hypothesis): Firstly, there is cognate interaction between T cell receptors (TCRs) binding to peptide-loaded, cell surface-expressed human leukocyte antigen (HLA, or major histocompatibility complex, MHC) (Azuma, 2019). Self-antigens should lead to tolerance. In the presence of non-self-antigens, co-stimulation (second signal) leads to clonal expansion and an effector response. CD8⁺ T cells (or cytotoxic T lymphocytes, CTL) primarily bind to MHC class I (HLA-A, B, and C) presented by all nucleated cells and destroy cells expressing non-self-antigens by releasing the contents of their cytotoxic granules. CD4⁺ (T helper) cells, on the other hand, bind to MHC class II (HLA-DP, -DQ, or -DR) presented by professional antigen presenting cells, such as phagocytes and B cells, and strengthen effector responses to viruses and intracellular pathogens through the display of surface ligands and production of cytokines, as well as stimulate B cells toward antibody maturation (Bonilla and Oettgen, 2010).

Secondary immunodeficiencies (SID) can affect the innate, humoral, or CMI components of the immune system, alone or in combination. This can be due to an absolute deficiency in the component of the immune system, such as neutropenia, hypogammaglobulinemia, or lymphopenia, or a functional deficiency of an apparently quantitatively normal component. Both quantitative and qualitative defects can occur concomitantly or serially. These deficiencies can arise due to disease states, immunosuppressive treatments, or a combination. This is especially true in the case of lymphoid malignancies which result in complex absolute and functional deficiencies of multiple components due to both the underlying disease and its treatment (Friman et al., 2016). It is critical for the physician seeking to treat patients with secondary immunodeficiencies and their resulting infections to understand this complex interplay between the components of the immune system, disease states, and immunosuppressive treatments.

Phagocyte deficiencies

Neutropenia is one of the most common SID encountered by physicians, typically due to cytoreductive therapies (e.g., in hematological malignancies), with febrile neutropenia constituting a medical emergency. Neutropenia is defined as an absolute neutrophil count (ANC) of less than 1500 cells μL^{-1} , with an ANC of less than 500 cells μL^{-1} being considered severe neutropenia (World Health Organization, 1979). The risk of infection is directly correlated with both the severity and duration of neutropenia, with prolonged neutropenia generally defined as lasting longer than 7–14 days (Sipsas et al., 2005; Gerson et al., 1984; Abers et al., 2016; Zimmer and Freifeld, 2019; Taplitz et al., 2018). Neutropenic patients are at particular risk of life-threatening sepsis due to both gram-positive and gram-negative bacteria (including *Pseudomonas aeruginosa*), as well as fungemia due to yeast. Despite a thorough diagnostic evaluation, a clear infectious focus is not found in most episodes of febrile neutropenia, with only 50% presenting with clinically-documented infection (objective and detectable signs of infection with a lack of microbiological documentation) and as little as 12% patients having a microbiologically-documented infection (Lehrnbecher et al., 2017; Hakim et al., 2009; Immunocompromised Host Society, 1990). However skin and soft tissue infections (SSIs), viral and bacterial pneumonia, central line associated bloodstream infections (CLABSIs), and bacterial urinary tract infections can serve as foci of dissemination. It is key for clinicians to recognize that typical purulent manifestations of these infections may not manifest themselves until neutrophil recovery and a high index of suspicion must be maintained when evaluating the neutropenic patient.

In addition to bacterial sepsis, prolonged neutropenia is an important risk factor for fungal infection by both yeasts and molds. Mucosal infections with *Candida* species, including oropharyngeal candidiasis and esophagitis, occur in up to 40% of patients receiving cancer treatment (Lalla et al., 2010). Invasive *Candida* infections can present acutely, as is the case with candidemia, or chronically as is the case with disseminated ("hepatosplenic") candidiasis. Candidemia can further result in endophthalmitis, osteomyelitis, endocarditis, or central nervous system infection through hematogenous spread (Sims et al., 2005). Disseminated trichosporonosis can rarely mimic disseminated hepatosplenic candidiasis in the setting of prolonged neutropenia (Marty et al., 2003).

Prolonged neutropenia is the most important risk factor for invasive infection with molds, particularly invasive pulmonary aspergillosis (IPA) and disseminated aspergillosis (Gerson et al., 1984). *Scedosporium* spp. and *Lomentospora* spp. present similarly to *Aspergillus* spp., however these agents are often multidrug resistant and are particularly difficult to treat. Disseminated fusariosis and invasive mucormycosis, especially rhinocerebral and pulmonary disease, are widely feared due to the extensive tissue destruction they cause, the paucity of effective antifungal therapies against them, and their lethality (Lamoth and Kontoyiannis, 2019). Fever greater than 38 degrees Celsius for more than 48–72 h despite appropriate antibiotic therapy; new pulmonary nodules or infiltrates;

facial, sinus, or ocular pain; or new or rapidly progressive painful and nodular, necrotic, or ulcerative skin lesions in a neutropenic patient should prompt a thorough investigation for invasive mold infection and strong consideration of empiric antimold treatment.

Compared to neutropenia, secondary monocytopenia is rarely encountered by clinicians and can be easily overlooked. Both genetic and acquired monocyte disorders predispose to invasive pulmonary and disseminated infections with non-tuberculous mycobacteria, *Nocardia* spp., *Aspergillus* spp., *Cryptococcus neoformans*, and *Histoplasma capsulatum* (Punatar et al., 2012; Vinh et al., 2010).

Causes of secondary phagocyte deficiencies

Due to medical conditions

Both functional and absolute neutropenia can occur due to decreased or dysfunctional production of neutrophils in the bone marrow, organ sequestration, or peripheral destruction. Dysmyelopoiesis occurs in the setting of myeloid malignancies, particularly acute myelogenous leukemia (AML), myelodysplastic syndrome (MDS), and myeloproliferative neoplasms (MPNs). Neutrophils produced from the abnormal clone can be both reduced in number and dysfunctional, such that patients with apparently normal neutrophil counts can still experience severe and recurrent invasive bacterial and fungal infections. Prolonged and severe absolute neutropenia is also observed in acquired bone marrow failure syndromes, such as idiopathic aplastic anemia, toxin-mediated myelosuppression, micronutrient deficiencies, and bone marrow infiltration by neoplasia and infections (Peslak et al., 2017). Splenomegaly is a frequent cause of leukocyte sequestration typically causing a mild-to-moderate absolute neutropenia. This becomes more important in patients with advanced cirrhosis who experience multifactorial phagocyte dysfunction associated with reduced function due to accumulated toxins, reduced numbers of sinusoidal macrophages, and leukocyte sequestration due to secondary hypersplenism (Irvine et al., 2019). Immune neutropenia, resulting in autoimmune peripheral destruction of neutrophils, can occur on its own or accompanying other autoimmune phenomena, especially systemic lupus erythematosus (SLE) (Meyer et al., 2020).

Pulmonary alveolar proteinosis (PAP) is one of the most well described acquired monocyte disorders. PAP is characterized by impaired clearance of cholesterol from the alveoli leading to dysfunctional surfactant as well as a susceptibility to infection due to macrophage dysfunction. Most cases are caused by neutralizing autoantibodies to granulocyte-macrophage colony stimulating factor (GM-CSF) (Trapnell et al., 2019). Opportunistic infections occur in more than half of patients diagnosed with PAP, with 40% presenting with an infection as the first manifestation of the disease (Punatar et al., 2012).

Hairy cell leukemia (HCL), a B-cell monoclonal disorder, induces both severe neutropenia and monocytopenia with a marked susceptibility to infection in up to 60% of untreated patients (Kraut, 2011; Grever et al., 2017). Patients can present with severe infections typically associated with neutropenia (e.g. *P. aeruginosa* sepsis and invasive fungal infections) and infections associated with monocytopenia (notably disseminated non-tuberculous mycobacterial infections) (Stewart and Bodey, 1981). While the risk of these infections decreases with therapy, new infectious risks are introduced due to profound iatrogenic T cell immunosuppression related to chemotherapy (see later in chapter) (Kraut, 2011).

Iatrogenic causes

Myelosuppressive chemotherapy is the most common iatrogenic cause of absolute neutropenia encountered in clinical practice. However, diverse non-chemotherapy medications can cause predictable or idiosyncratic absolute neutropenia due to either direct myelosuppression or immune-mediated causes (see Table 1) (Curtis, 2017; Clément et al., 2018). High-dose radiation, particularly whole-body treatments or treatments focused on the pelvis can also be myelosuppressive.

Glucocorticoids are broad spectrum immunosuppressant medications that act in part through inhibition of both neutrophil and monocyte activity. Multiple phagocytic functions are impaired by glucocorticoids, including chemotaxis, phagocytosis, and the oxidative burst, increasing susceptibility to both bacterial and invasive fungal infections, particularly invasive pulmonary mold infections (Llewellyn-Jones et al., 1994; Petroni et al., 1988). Phagocyte dysfunction and infectious risk increase with both the dose and duration of therapy and are additive to existing phagocyte disorders. The immunosuppressive effects of glucocorticoids on adaptive immunity are discussed later in the chapter.

Treatment of secondary phagocyte deficiencies

The goals of treatment of neutropenia are threefold: (1) to correct the functional or absolute neutropenia; (2) primary prevention of opportunistic infections through prophylactic antibacterial and antifungal treatment; and (3) early diagnosis and management of opportunistic infections. The acute management of febrile neutropenia is beyond the scope of this article.

Correction of neutropenia

Treatment of the underlying disease or dose reduction, delay, or removal of the offending drug are the mainstays of treatment for secondary neutropenia. However, this is not always possible and neutrophil recovery can take time, leaving the patient vulnerable to life-threatening infection. Thus, growth factor support is often used prophylactically, during myelosuppressive chemotherapy, or as treatment of severe neutropenia due to the various causes listed above.

Table 1 Classes of drugs associated with absolute neutropenia.

Type of drug	Drug class	Examples of drugs
Antibacterial	β -lactams	Benzylpenicillin
		Amoxicillin
		Piperacillin-tazobactam
		Ceftriaxone
		Cefotaxime
		Cefepime
		Meropenem
		Vancomycin
		Sulfadiazine
		Sulfamethoxazole-trimethoprim
	Glycopeptides	Chloramphenicol
	Sulfonamides	Clindamycin
	Chloramphenicol	Tobramycin
	Lincosamides	Ciprofloxacin
Antiviral	Aminoglycosides	Linezolid
	Quinolones	Tedizolid
Antihelminth	Oxazolidinones	Metronidazole
	Nitroimidazole	Ganciclovir
Antithyroid	Thionamides	Valganciclovir
		Levamisole
Antidiabetic	Thiourea	Carbimazole
		Methimazole
Antipsychotic	Sulfonylurea	Propylthiouracil
		Glyburide
Antiepileptic	Atypical antipsychotics	Clozapine
		Quetiapine
Immunomodulatory	Iminostilbene derivatives	Carbamazepine
		Phenytoin
Cardiovascular	Hydantoin	Ibuprofen
		Tacrolimus
	NSAIDs	Mycophenolate mofetil
		Everolimus
	Calcineurin inhibitors	Clopidogrel
		Ticlopidine
	IMP dehydrogenase inhibitors	Disopyramide
		Procainamide
	mTOR inhibitors	Quinidine
		Captopril
	P2Y ₁₂ inhibitor	Spironolactone
		Methyldopa
	Antiarrhythmic	
	ACE inhibitor	
	Diuretic	
	Alpha-2 agonist	

Recombinant granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) can be used to stimulate neutrophil and, in the case of GM-CSF, monocyte production and mobilization from the bone marrow. G-CSF is more commonly used to treat acquired neutropenia as GM-CSF is more pro-inflammatory, unless an increase in macrophage function is also desired in which case GM-CSF is preferred (Mehta et al., 2015). As per the American Society of Clinical Oncologists' (ASCO) 2015 Clinical Practice Guideline, G-CSF treatment is recommended as primary prevention of infection in patients receiving high-risk chemotherapy regimens (greater than 20% risk of febrile neutropenia) and secondary prevention for patients who have experienced previous episodes of febrile neutropenia during chemotherapy. It may also be used to prevent infection in patients with idiopathic or immune-mediated neutropenia, or neutropenia due to aplastic anemia or non-chemotherapy myelosuppressive medications (Dale and Bolyard, 2017). G-CSF treatment is generally not indicated for treatment of an active episode of febrile neutropenia as it neither reduces mortality nor duration of hospitalization (Mehta et al., 2015). In cases of invasive mold infections, however, G-CSF may be used to accelerate neutrophil recovery which is critical for the control of the infection and may be lifesaving. This approach is supported by experimental data from animal models of *Aspergillus* infection (Patera et al., 2004; Sionov et al., 2005).

G-CSF and GM-CSF can be given either intravenously or subcutaneously, however the subcutaneous route is preferred due to ease of administration and, in the case of G-CSF, more rapid neutrophil recovery (Paul et al., 2014). Inhaled GM-CSF is also used as treatment of autoimmune PAP (Trapnell et al., 2020). Pegylated forms of G-CSF which require less frequent dosing are marketed along with traditional recombinant G-CSF (see Table 2). Both agents can cause fever and bone pain, however this is more common

Table 2 Formulations and dosing of recombinant G-CSF and GM-CSF.

	Formulation	Recommended Dose	Dosing Frequency
G-CSF	Filgrastim	5 $\mu\text{g kg}^{-1}$	Daily (duration depending on chemotherapy regimen)
	PEG-filgrastim	6 mg	Once 24 h after receiving each cycle of chemotherapy
GM-CSF	Sargramostim	250 $\mu\text{g m}^{2(-1)}$	Daily
	Molgramostin	5–10 $\mu\text{g m}^{2(-1)}$ injection or 300 μg inhalation	Daily

with GM-CSF treatment. These can be treated symptomatically with antipyretics or antihistamines, respectively. GM-CSF has also been reported to be associated with pleural and pericardial effusions. Growth factors must be used cautiously in patients with MDS or MPNs as they can theoretically predispose to transformation to AML (Mehta et al., 2015). Long-term treatment with G-CSF in children has been associated with low bone mineral density, however this has not been demonstrated in adults and causality has not been established (Dale et al., 2003).

Donor granulocyte transfusion was historically used to prevent and treat opportunistic infections in neutropenic patients before the advent of recombinant growth factors. It remains an option in patients who do not or cannot respond to growth factor support. Unfortunately, this approach requires timely access to a human leukocyte antigen (HLA)-matched donor and infusion of the cells within 24 h due to their short shelf life. Infused allogeneic granulocytes can also result in alloimmunization and transfusion related acute lung injury (TRALI) (Cugno et al., 2015). Furthermore, while prophylactic granulocyte transfusion does modestly reduce invasive bacterial and fungal infections when given in a sufficient dose, no clear all-cause or infection-related mortality benefit has been demonstrated in randomized clinical trials performed to date (Estcourt et al., 2015). As well, the optimal target level of neutrophil count to achieve is undefined. Thus, due to impracticality, toxicity, and questionable clinical efficacy, this approach is only used to treat life threatening or refractory infections in neutropenic patients, especially invasive mold infections (Patterson et al., 2016; Dignani et al., 1997; Mousset et al., 2005; Kadri et al., 2015).

Prophylactic antimicrobial therapy

Antibacterial prophylaxis

Quinolone prophylaxis is recommended by ASCO and the Infectious Diseases Society of America (IDSA) for patients receiving chemotherapy who are expected to experience profound and prolonged neutropenia refractory to growth factor support (Taplitz et al., 2018). This applies particularly to patients undergoing induction and consolidation therapy for AML as well as HSCT with a standard conditioning regimen. Therapy is recommended for the duration of severe neutropenia. This recommendation is based on over 100 trials that have demonstrated a mortality benefit for quinolone prophylaxis in this population. In the most recent (2012) Cochrane systematic review and meta-analysis of available randomized controlled trials, antibiotic prophylaxis reduced all-cause mortality by 34% and infection-related mortality by 39%, with a number needed to treat to prevent one death of 55 (Gafer-Gvili et al., 2012). However, this enthusiasm is tempered by concerns about rising quinolone resistance rates worldwide, selection for quinolone-resistant clones, effects on the gut microbiota, and the association between quinolone therapy and *Clostridioides difficile* infection (CDI) (Bow, 2011).

Antifungal prophylaxis

ASCO and the IDSA equally recommend antifungal prophylaxis for the duration of neutropenia in patients receiving chemotherapy regimens expected to cause profound neutropenia. Again, this applies essentially to patients receiving induction for AML or HSCT (Taplitz et al., 2018). However, not all such patients require prophylaxis with a mold-active agent. In patients undergoing chemotherapy for AML or MDS, prophylaxis with the antimold agent posaconazole reduces invasive fungal infection and mortality compared to fluconazole or itraconazole and is the standard-of-care (Cornely et al., 2007). In patients with severe graft-versus-host disease (GVHD), fluconazole and posaconazole were equivalent for the prevention of invasive fungal infection and mortality, however posaconazole demonstrated superiority for the prevention of invasive aspergillosis and fungal infection-specific mortality and is the recommended antifungal prophylaxis in this population (Ullmann et al., 2007). In HSCT recipients without GVHD, on the other hand, mold-active prophylaxis does not provide a survival advantage and prophylaxis with fluconazole alone is recommended until neutrophil recovery. Voriconazole and isavuconazole may be used as alternatives to posaconazole when antimold prophylaxis is indicated (Barreto et al., 2013; Bose et al., 2021). Echinocandins have been found to be superior to fluconazole for antifungal prophylaxis in patients undergoing AML induction or consolidation or HSCT and may be used as an alternative to triazoles for invasive yeast infection prophylaxis in these populations; however, they are much less active against molds than broad-spectrum azoles (Fisher et al., 2019; Van Burik et al., 2004). Inhaled amphotericin B deoxycholate and liposomal amphotericin B are also alternatives for antimold prophylaxis, however they are rarely used given the existence of more practical alternatives (Duckwall et al., 2019).

B cell deficiencies

B cell disorders can result in immunoglobulins which are quantitatively decreased in number (hypogammaglobulinemia; HGG) and/or qualitatively impaired in their ability to adapt to new antigens (dysgammaglobulinemia). Deficiencies of the IgG class are the most clinically significant and actionable. Quantitatively, IgG may even appear misleadingly elevated, yet be dysfunctional, as seen in some plasma cell dyscrasias (e.g., multiple myeloma) or B cell lymphoproliferative disorders (e.g., chronic lymphocytic leukemia). IgM and IgA can be similarly abnormal. Thus, quantitative immunoglobulin assays only tell part of the story.

Acquired B cell deficiencies mostly manifest as recurrent bacterial infections: these infections may even be the first symptom of the underlying disorder. These infections are primarily with encapsulated bacteria including *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* and manifest mostly as recurrent sinopulmonary infections and otitis media, but serious infections such as empyema, bacteremia, and meningitis also occur (Casulo et al., 2013; Picard et al., 2003). Over time, these recurrent pulmonary infections can lead to bronchiectasis and pneumatocoles (Ruffner et al., 2017; Brent et al., 2016). A predisposition to gastrointestinal infections with *Campylobacter* spp., *Salmonella* spp., and *Giardia lamblia* has also been noted in patients with genetic B cell deficiencies; whether this applies to patients with SID is unknown (Hagiya et al., 2018; Barmettler et al., 2017; Oksenhendler et al., 2008). The severe congenital B cell deficiency X-linked agammaglobulinemia is also associated with invasive and refractory enteroviral infections; whether this is true in acquired B cell disorders is also unclear (Galama, 1997). Finally, during the recent COVID-19 pandemic, patients treated with B cell depleting agents were found to have prolonged viral shedding and symptoms which abated with anti-SARS-CoV-2 antibody treatment (Hueso et al., 2020). Whether this applies to other respiratory viruses is unknown and will be an area for future study.

The evaluation of patients suspected of harboring a secondary B cell immunodeficiency begins with a clinical history to elicit recurrent infections suggestive of this disorder. Investigations should include quantitative IgG, IgA, and IgM levels, as well as vaccination with a conjugated protein vaccine and a polysaccharidic vaccine to evaluate serologic responses, which serves as a functional assay of B cell activity. Ancillary investigations include a complete blood count, serum protein electrophoresis, serum free light chains, and chest imaging to detect common causes of secondary B cell disorders (e.g., thymoma, see below) and pulmonary complications (e.g., bronchiectasis).

Causes of secondary B cell disorders

Due to medical conditions

The medical conditions most associated with B cell disorders are clonal B cell malignancies. This is best described in chronic lymphocytic leukemia (CLL), in which a dysfunctional clone dominates the B cell population to the detriment of functional B cells. CLL is associated with serious or recurrent bacterial infections in 55% of patients not receiving immunoglobulin prophylaxis (Cooperative Group for the Study of Immunoglobulin in Chronic Lymphocytic Leukemia, 1988). The incidence of recurrent and serious infections is associated with HGG, deficiency of IgG subclasses, and deficiency of pneumococcal specific antibodies, supporting the notion that both HGG and dysgammaglobulinemia explain these patients' susceptibility to infection (Griffiths et al., 1992; Freeman et al., 2013). Other B cell lymphomas may also be associated with susceptibility to infection through similar mechanisms, but the frequency is not as well defined as for CLL.

Similarly, the plasma cell dyscrasias represent clonal proliferation of plasma cells (memory cells responsible for prolonged production of mature antibody) which produce an abnormal clonal antibody peak that replaces normal antibody production and results in marked dysgammaglobulinemia. Plasma cell dyscrasias associated with increased susceptibility to infection include plasma cell malignancies such as multiple myeloma (MM) or plasma cell leukemia and mature B-cell lymphomas which produce abnormal antibody, most notably Waldenström's macroglobulinemia (WM) in a patient with lymphoplasmacytic lymphoma (Swerdlow et al., 2016). In a case series of over 9000 multiple myeloma patients, the risk of bacterial infection was found to be increased seven-fold over the general population, and the risk of viral infection was increased 10-fold. Infection was the cause of death in 22% of patients who died during follow-up (Blimark et al., 2015). This is thought to extend to patients with WM as well.

Good's syndrome is marked by HGG, as a manifestation of a combined (B- and T- cell) immunodeficiency associated with thymoma. Despite thymectomy, HGG and sinopulmonary infections (as well as the T cell deficiency with opportunistic infections) can persist. Many cases require immunoglobulin replacement therapy (IGRT) to address the HGG. The mechanism(s) underlying the severe B and T cell lymphopenia in these patients has yet to be determined (Zaman et al., 2019).

In addition to defective antibody production, excessive protein loss can result in HGG, as seen notably in protein-losing enteropathy and nephrotic syndrome (Ye et al., 2019; Ameratunga et al., 2015). Nephrotic syndrome in children predisposes to pneumonia, bacteremia, spontaneous bacterial peritonitis (SBP) and UTI, with the most common organism being *Streptococcus pneumoniae* (Carpenter et al., 2019; Malaker et al., 2019). Active nephrotic range proteinuria and hypoalbuminemia less than 15 g L⁻¹ are most associated with the risk of infection (Lebel et al., 2020; Kumar et al., 2019). There is less clinical evidence of an association between gastrointestinal spillage of antibody and infection, however it is plausible that such HGG predisposes to invasive bacterial infection in a similar manner to patients with nephrotic syndrome.

Iatrogenic causes

Targeted B cell depleting agents have revolutionized the management of B cell leukemias, B cell lymphomas and multiple myeloma. They are equally approved for the treatment of rheumatoid arthritis, auto-immune hemolytic anemia, refractory immune thrombocytopenic purpura (ITP), granulomatosis with polyangiitis, and microscopic polyangiitis, but are also used off-label to treat connective tissue diseases, multiple sclerosis, pemphigus vulgaris, and diverse autoantibody-mediated diseases (Sarsour et al., 2020). These agents include monoclonal antibodies against diverse surface targets and CD20-targeted chimeric antigen receptor T (CAR T) cell therapies (see Table 3) (Patel et al., 2019). Monoclonal antibodies target B cells for destruction through antibody-dependent cytotoxicity, while CAR T cells direct cytotoxic T cells to destroy any cell displaying the target antigen.

Half of patients receiving rituximab develop HGG, which is associated with an increased risk of severe infections for 6 months after administration of the last dose (Barmettler et al., 2018). Serum immunoglobulins continue to decrease with subsequent doses of rituximab and remain persistently depressed as long as therapy continues; this effect is additive to the effect of previous or concurrent treatments, especially cyclophosphamide and systemic steroids (Tieu et al., 2021). In some patients, severe HGG can persist over 2 years after discontinuation of therapy and continue to be associated with severe infections (Levy et al., 2014). While most data exist for rituximab as it has been marketed the longest, other anti-CD20 monoclonal antibodies are likely to be associated with a similar incidence of HGG and infection. The anti-CD19 agent, blinatumomab, which targets both mature B cells and plasma cells, results in profound HGG in patients who respond to therapy, which appears to resolve on discontinuation (Zugmaier et al., 2014). Similarly, the anti-CD38 agent, daratumumab, is also associated with HGG in 60% of patients with 25% developing significant infections (Paul et al., 2019).

Ibrutinib, a Bruton's tyrosine kinase (Btk) inhibitor which has been marketed since 2015, is approved for the treatment of CLL, mantle cell lymphoma, WM, and refractory GVHD. It has been associated with serious infections in 11.4% of patients in the first year of treatment (Varughese et al., 2018). While most of these infections were bacterial, ibrutinib treatment has also been associated with an alarming incidence of invasive fungal infection, including central nervous system aspergillosis, and *Pneumocystis jiroveci* infection (Varughese et al., 2018; Lionakis et al., 2017). The mechanism(s) underlying this increased susceptibility to fungal infection is an area of active investigation. However, because CLL is not inherently associated with increased risk of these fungal infections, physicians should be vigilant about this possibility in a patient with CLL being treated with ibrutinib, and perhaps other emerging tyrosine kinase inhibitors, particularly in the first 6 months of therapy.

Hypogammaglobulinemia is being increasingly recognized post-solid organ transplantation (SOT). In a 2013 meta-analysis of 12 published studies on the topic (Florescu et al., 2013), 45% of patients were found to be hypogammaglobulinemic in the first year after transplant; of these, a third were severe ($<4 \text{ g L}^{-1}$). The prevalence of HGG depended on the organ transplanted, with the highest prevalence in lung transplant recipients (63%), followed by heart (49%), kidney (40%) and liver (15%). Similarly, 60% of intestinal transplant recipients exhibited severe HGG (Farmer et al., 2013). Severe HGG was associated with an increased incidence of respiratory, cytomegalovirus (CMV), *Aspergillus*, and other fungal infections. Importantly, it was also associated with increased mortality in the first year after SOT (Florescu et al., 2013). It has not been established whether HGG is the cause of these infections, or rather a surrogate marker of more intense immunosuppression. The causes of HGG in SOT recipients are multifactorial, including use of alemtuzumab conditioning, steroids, mycophenolate mofetil maintenance, use of B cell-depleting agents to treat antibody-mediated rejection, and, in the case of heart transplantation, removal of antibodies by cardiopulmonary bypass during surgery.

Mechanical removal of antibodies can occur through plasmapheresis, cardiopulmonary bypass (CPB), and extracorporeal membrane oxygenation (ECMO) which can result in a transient hypogammaglobulinemia. This is most noticeable in infants undergoing cardiac surgery due to their small blood volume relative to the size of the CPB circuit (Rhodes et al., 2014).

Table 3 Available B cell depleting agents.

Target	Type	Medication
Anti-CD20	Monoclonal antibody	Rituximab
		Veltuzumab
		Ocrelizumab
		Ofatumumab
		Obinutuzumab
		Ublituximab
Anti-CD22	Monoclonal antibody	Epratuzumab
Anti-CD19	Monoclonal antibody	Blinatumomab
	CAR T cell	Tisagenlecleucel
		Axicabtagene ciloleucel
CD52	Monoclonal antibody	Alemtuzumab
CD38	Monoclonal antibody	Daratumumab
		Isatuximab
BAFF	Monoclonal antibody	Belimumab

Treatment of secondary B cell disorders

Both vaccination against encapsulated organisms and pooled immunoglobulin replacement can be used to prevent invasive bacterial infections in hypogammaglobulinemic patients. The efficacy of these treatments depends on the underlying condition and whether it is being used as primary or secondary prevention.

Vaccination

Vaccines are ideally provided before the initiation of iatrogenic B cell depletion; in the case of HGG due to medical conditions, this timing is often not possible. Vaccination with a conjugated pneumococcal vaccine, followed 8 weeks later by a polysaccharidic vaccine, is recommended for patients with the conditions and treatments mentioned above. Conjugated vaccines, which induce a T and B cell response, are more immunogenic in patients with CLL than polysaccharidic vaccines, which only induce a B cell response, and thus must be included in any effective vaccination regimen (Sinisalo et al., 2001). It is also prudent to administer *Haemophilus influenzae* type B, quadrivalent meningococcal (serogroups A, C, Y, W135), and meningococcal serogroup B vaccines to patients with hematological malignancies expected to receive B cell depleting therapies and in anticipation of hematopoietic or solid organ transplant (Danziger-Isakov and Kumar, 2019). Interestingly, in nephrotic syndrome, there is a paucity of evidence to vaccinate against encapsulated organisms other than pneumococcus (Goonewardene et al., 2019). Annual influenza vaccination is generally recommended in all immunocompromised individuals as well, however patients actively receiving B cell-depleting therapy may have attenuated antibody responses (Van Assen et al., 2010; Yri et al., 2011). It should also be noted that patients with untreated CLL or those receiving active treatment had little-to-no antibody response to the BNT162b2 mRNA vaccine against COVID-19, while 75% of those who had completed treatment and were in remission had a measurable antibody response to the vaccine (Herishanu et al., 2021).

Immunoglobulin replacement

The immunoglobulin fraction can be separated from whole blood and plasma donations for adoptive transfer to patients with hypogammaglobulinemia (i.e., “passive immunization” or immunoglobulin replacement therapy, IGRT). These pooled products are derived from thousands of donors and thus contain naturally acquired and vaccine-induced specific antibodies against a variety of pathogens. IGRT may be administered intravenously (IVIG) or subcutaneously (SCIG). IVIG is typically administered as 400 mg kg⁻¹ every 3–4 weeks, while traditional SCIG preparations are administered on a total weekly dose; newer, higher concentration (20%) SCIG preparations can be administered with a 2–4-week delay between infusions. SCIG has the advantage of being self-administered (either manually or using an infusion pump), thus limiting the disruption to the patient's life from having to attend a clinic or infusion center to receive IVIG (Lachance et al., 2016).

IGRT can result in several immediate and delayed adverse events. These adverse events appear to be more common with IVIG than SCIG. Immediate reactions include flu-like symptoms, urticaria, dyshidrotic eczema, and rarely, anaphylaxis and transfusion-related acute lung injury (TRALI). Delayed events include thrombosis, headache, aseptic meningitis, posterior reversible encephalopathy syndrome (PRES), hemolysis, neutropenia, and hyponatremia. Infection risk has been significantly reduced with donor questionnaires, serological screening, and physico-chemical treatment of product during the manufacturing process; however, it remains theoretically possible to acquire bloodborne infections from any blood product (Guo et al., 2018). Also, as immunoglobulins are pooled products, IGRT can result in false positive serological assays due to passive transfer of antibody (L'Étoile-Morel et al., 2021).

Since the first trial showing a significant reduction in serious infections with IVIG therapy in CLL patients with a history of recurrent infections or HGG (Cooperative Group for the Study of Immunoglobulin in Chronic Lymphocytic Leukemia, 1988), six more trials have consistently demonstrated a decrease in infections with IGRT (Griffiths et al., 1989; Sklenar et al., 1993; Jurlander et al., 1994; Chapel et al., 1994a; Gamm et al., 1994; Boughton et al., 1995; Molica et al., 1996). A single multicenter study also showed efficacy of prophylactic IVIG to prevent bacterial infections in MM patients (Chapel et al., 1994b). More recently, SCIG administration was found to result in more sustained circulating levels of IgG, associated with more effective prevention of infection than IVIG (Visentin et al., 2018). While IGRT is clearly effective at preventing infections in patients with these malignancies, there is significant interindividual and geographic variability in the threshold at which it is prescribed. Some physicians and jurisdictions provide replacement for all hypogammaglobulinemic patients with these malignancies, while others only do so in patients who have had recurrent invasive infections. Immunologists are more likely to prescribe SCIG than non-immunologists, who are more likely to prescribe IVIG (Na et al., 2019).

The evidence of benefit for IGRT in SOT recipients with HGG in preventing infections is mixed. While it has been found to reduce mortality in heart transplant recipients, it had no effect on mortality in lung or renal transplant recipients; there was inadequate published data to reach a conclusion in other organ transplants (Bourassa-Blanchette et al., 2019a,b). The efficacy against CMV and bacterial infections was also inconclusive (Bourassa-Blanchette et al., 2019a,b). It should be noted that all the previously published trials which administered IGRT in SOT recipients did not distinguish patients with or without HGG, which may have diluted a possible effect.

Splenic dysfunction and complement deficiencies

The spleen is a complex lymphoid organ which is primarily responsible for defense against bloodborne encapsulated organisms and parasites. Blood is filtered through a reticular network called the red pulp, while the surrounding white pulp contains specialized

macrophages (the “reticuloendothelial system”), which remove senescent red blood cells, bacteria, and parasites, as well as lymphoid tissue, where adaptive B cell responses occur against encapsulated bacteria. The antibodies produced by these B cells opsonize bacteria and facilitate their clearance from the bloodstream.

Asplenic individuals are at risk of overwhelming bacterial sepsis (or “overwhelming post-splenectomy sepsis”). In infants under 6 months of age with congenital asplenia, this is typically due to infection with Enterobacteriaceae, especially *Escherichia coli* and *Klebsiella pneumoniae*, while in older patients and those with acquired asplenia, sepsis is typically due to encapsulated organisms including *Streptococcus pneumoniae*, *Haemophilus influenzae* type B, and *Neisseria meningitidis* (Waldman et al., 1977). They are also at risk of extraintestinal non-typhoidal *Salmonella enteritidis* infection, especially osteomyelitis and septic arthritis often seen in patients with sickle cell anemia who have autosplenectomy (Wen et al., 2017). Rarely, these patients can develop overwhelming infection with *Capnocytophaga canimorsus* or *Pasteurella multocida* after animal exposure (Zajkowska et al., 2016; Laupland et al., 2003). Finally, asplenic patients are at increased risk of severe infections with bloodborne parasites, including malaria (*falciparum* and non-*falciparum* species) and *Babesia* spp. (Ram et al., 2010).

The highest risk of sepsis in surgically splenectomized patients occurs at the extremes of age (<3 years and >50 years old) and in the first 1–3 years after splenectomy. Historically, sepsis was associated with mortality rates as high as 50%, however with preventative measures (see below), rapid recognition, and rapid initiation of antimicrobial treatment, the mortality rate post-splenectomy has dropped to as little as 1% (Lee, 2020; Madenci et al., 2019).

The complement system is a group of >20 proteins, synthesized in the liver, and organized into cascades (Classical; Alternative; or Mannose-binding lectin pathways) responsible for the opsonization and lysis of bacteria. Complement deficiencies, primarily due to genetic bases, have revealed their role in protection against encapsulated organisms and these patients develop similar infections as asplenic patients (Ram et al., 2010). However, patients with defects of the terminal membrane attack complex (MAC) are particularly predisposed to invasive and recurrent infections with *Neisseria meningitidis* and *N. gonorrhoeae*.

Causes of splenic dysfunction

Splenic function can be absent (asplenia) or diminished (hyposplenia). Asplenia can be anatomical (i.e., due to congenital absence of the spleen, surgical removal, or splenic ischemia in patients with hemoglobinopathies (autosplenectomy)), or functional (i.e., due to conditions that reduce splenic function).

The indications for surgical splenectomy are generally trauma (blunt, penetrating, and iatrogenic); refractory autoimmune cytopenias (immune thrombocytopenic purpura and autoimmune hemolytic anemia); malignancy (either primary lymphoma or metastatic solid cancers); and symptomatic massive splenomegaly, often due to hematologic disorders such as chronic myeloid leukemia (CML), myelofibrosis, and hemoglobinopathies (Browning et al., 2017). Splenectomy due to trauma is typically performed as an emergency procedure to control life-threatening bleeding and thus preoperative preparation including vaccination is not possible. For other indications, splenectomy is typically performed as an elective procedure allowing time for vaccination against encapsulated organisms (see below). When possible, partial splenectomy may be practiced in cases of trauma, benign lesions, and for symptomatic management of massive splenomegaly, thus preserving splenic function in these patients (Costi et al., 2019).

In addition to surgical splenectomy, abnormally shaped erythrocytes block splenic sinusoids resulting in multiple microinfarcts and eventually leading to a complete loss of splenic function (autosplenectomy) in children with hereditary hemoglobinopathies, mainly sickle cell anemia and hereditary spherocytosis. Patients with sickle cell disease already present evidence of hyposplenia at age 12 months and typically complete autosplenectomy by age 5 (Brousse et al., 2014).

Functional hyposplenia may result in a similar susceptibility to infection as anatomical asplenia. Celiac disease, inflammatory bowel disease, connective tissue diseases (especially SLE), human immunodeficiency virus (HIV) infection, graft-versus-host disease, splenic infiltration due to amyloidosis, hemochromatosis, and sarcoidosis, and ischemia due to splenic arterial and venous thrombosis can cause varying degrees of hyposplenia. Treatment with high dose steroids, methyl dopa, total parenteral nutrition (TPN), and splenic irradiation have also been associated with splenic dysfunction (Squire and Sher, 2020). Splenic function can be evaluated using imaging (for anatomical asplenia), the examination of a peripheral blood smear for Howell-Jolly bodies, counting of pitted red blood cells, and nuclear medicine techniques, such as spleen scintigraphy, that are more accurate than blood smear-based techniques (De Porto et al., 2010).

Causes of secondary complement deficiencies

Acquired complement deficiency can occur due to decreased production, the presence of neutralizing antibodies against a component of the complement system, or increased consumption.

Because complement proteins are produced in the liver, diseases causing liver failure result in its decreased levels, resulting in decreased bactericidal capacity, which may in part explain the occurrence of bacterial peritonitis in cirrhotic patients (Akalın et al., 1983).

Neutralizing antibodies can cause a functional defect of a complement pathway component. The monoclonal drug eculizumab is a terminal complement pathway inhibitor which binds to C5, preventing its cleavage and the formation of the MAC. It is used to treat complement-mediated diseases, including paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), C3 glomerulonephritis, antibody-mediated rejection (ABMR), and myasthenia gravis. Treatment with eculizumab

has been associated with an up to 10,000-fold increase in the risk of invasive meningococcal disease leading to a Food and Drug Administration (FDA) black box warning (Benamu and Montoya, 2016). Similarly, cases of disseminated gonococcal disease and even sepsis associated with eculizumab use have also been reported (Gleesing et al., 2012).

Hypocomplementemia due to consumption occurs in several rheumatological diseases, especially those associated with immune complex deposition and SLE (Hebert et al., 1991). Its clinical consequences are not well delineated. Consumption can also occur with C3 nephritic factors (C3 NeF), autoantibodies directed against the C3 convertase enzyme, which stabilize the enzyme, and lead to increased consumption of C3. This, in turn, leads to membranoproliferative glomerulonephritis (MPGN), partial lipodystrophy, and has also been associated with a predisposition to invasive meningococcal infections due to the ensuing C3 deficiency (Skattum et al., 1997). Hypocomplementemia can also occur due to loss, and has been reported in protein-losing enteropathies, usually indicated by decreases in other serum proteins (e.g., albumin, IgG) (Itoi et al., 1989).

Treatment of splenic dysfunction

The primary goal in asplenic patients is to avoid life-threatening sepsis and resulting morbidity and mortality. This is accomplished through vaccination, prophylactic and “stand-by” antibiotics and antiparasitic treatment, parasite avoidance, patient education, and rapid recognition and provision of appropriate antibiotic therapy in the emergency setting.

Asplenic patients should be vaccinated against invasive pneumococcal infection (conjugated pneumococcal vaccine followed 8 weeks later by polysaccharidic vaccine), *H. influenzae* type B vaccine, and quadrivalent and type B meningococcal vaccines. For elective splenectomy, vaccines should be administered at least 14 days, and preferably 8 weeks, before the procedure. For emergent splenectomy, vaccination should be delayed for at least 2 weeks after the procedure (Di Sabatino et al., 2011). In functional hyposplenism and asplenia, vaccines may be administered at any time due to their gradual onset and progression.

The use of prophylactic antibiotics after splenectomy is more controversial. Typical prophylactic regimens include oral penicillin V or amoxicillin; the options for penicillin allergic patients are less clear with rising pneumococcal resistance rates. In sickle cell disease patients up to age 5, randomized, controlled clinical trial evidence demonstrates an 84% reduction in infection with twice daily penicillin V and this treatment is universally recommended (Gaston et al., 1986). Similarly, chronic GVHD has been associated with invasive pneumococcal disease, likely due to the associated splenic dysfunction, and lifelong penicillin prophylaxis is recommended (Kulkarni et al., 2000). Otherwise, guidelines vary widely on which patients should start prophylaxis and when to stop it. American guidelines recommend antibiotic prophylaxis for all asplenic children under age 5 as is practiced in sickle cell disease, with 1 year of prophylactic antibiotics in children 5 years and older after splenectomy (American Academy Of Pediatrics, Committee on Infectious Diseases, 2018). British and Australian guidelines, on the other hand, recommend prophylaxis for all patients for 2 years after splenectomy, and in select groups (age under 16, splenectomy for hematologic malignancy, history of invasive pneumococcal infection) prophylaxis for life (Kanhutu et al., 2017; Davies et al., 2011). Canadian guidelines recommend antibiotic prophylaxis for at least 2 years after splenectomy in children under 5, and ideally for life depending on adherence, tolerance, and access to emergency care (Salvadori et al., 2014). Due to a lack of clear evidence in patients older than 5 years of age and outside of sickle cell disease or chronic GVHD, it is likely that guidelines will continue to be variable and that the choice of whether to initiate prophylaxis and its duration will ultimately fall to patient and clinician preference.

Other preventative measures include patient education and medical identification cards/jewellery. Good knowledge of the risk of postsplenectomy sepsis is associated with a significant reduction in the risk of invasive pneumococcal infection compared to those with poor knowledge (El-Alfy and El-Sayed, 2004). Patients are instructed to immediately seek medical care if a fever (temperature of 38.0 degrees Celsius or higher) develops. Patients who may experience delayed access to care should be prescribed a supply of “stand-by” antibiotics targeting the most likely pathogens (e.g., amoxicillin-clavulanate, or a respiratory fluoroquinolone for penicillin allergic patients) to be taken immediately if a fever develops before proceeding to medical care as soon as possible. Management of post-splenectomy sepsis is beyond the scope of this article. Asplenic travelers should also be warned of the risk of malaria and babesiosis and should observe appropriate mosquito and tick avoidance and be prescribed appropriate malaria prophylaxis for the destination. Finally, patients should be warned of the risk of *C. canimorsus* sepsis after animal bites and should be prescribed antimicrobial prophylaxis should one occur.

Treatment of acquired complement deficiencies

Complement levels are a marker of disease activity in rheumatologic disease, so treatment of the underlying disease may correct hypocomplementemia. Otherwise, prevention of infections with encapsulated organisms is the mainstay of treatment, as in splenic dysfunction. As per the product monograph, patients being treated with eculizumab should receive quadrivalent (serogroups A, C, Y, W135) meningococcal and a first dose of meningitis serogroup B vaccine at least 14 days before initiating treatment with eculizumab; if treatment cannot be delayed, oral penicillin prophylaxis is recommended until this time has elapsed. Erythromycin is recommended as an alternative for patients who are allergic to penicillin. Breakthrough infections do occur despite vaccination, so patients should be informed of the signs and symptoms of invasive meningococcal disease and counselled to immediately present for care should they develop (McNamara et al., 2017). Because of breakthrough infections, British and French guidelines recommend oral penicillin prophylaxis for the duration of treatment, with the British guidelines equally recommending that patients be further provided with a supply of “rescue” medication in the form of ciprofloxacin 500 mg to be taken immediately if fever develops (Haut Conseil De La Santé Publique, 2014; PNH National Service, 2021). It is also prudent to vaccinate against other

vaccine-preventable encapsulated organisms, namely conjugated and polysaccharidic pneumococcal vaccine and *Haemophilus influenzae* type B vaccine. Finally, given the occurrence of severe gonococcal infections during eculizumab therapy, it is prudent to counsel patients on safe sexual activity and regular screening for sexually transmitted infections.

T cell deficiencies

T cells are essential for the control of intracellular infections. Broadly, cytotoxic CD8⁺ T cells kill infected cells, while T helper (CD4⁺) cells, produce cytokines needed for many immune functions including priming professional phagocytes to destroy ingested organisms. As a result, patients with secondary T cell deficiencies develop recurrent, invasive, and persistent infections with DNA and RNA viruses, intracellular bacteria, mycobacteria, dimorphic endemic fungi, yeasts, and parasites (see Table 4).

In addition to the above opportunistic infections, RNA respiratory viruses cause persistent and severe infection in T cell immunocompromised patients, especially progression of upper respiratory tract infections (URTI) to lower respiratory tract infection (LRTI). Progression to LRTI and mortality are particularly increased in allogeneic HSCT recipients for influenza A and B, respiratory syncytial virus (RSV), parainfluenza virus 3 (PIV3), and human metapneumovirus (HMPV). Rhinovirus infrequently progresses to LRTI and the incidence of progression of bocavirus is unknown (Fontana and Strasfeld, 2019). Coronaviruses were previously thought to be quite benign; however, during the SARS-CoV-2 pandemic, compromised immunity, particularly involving T cell function, was found to predispose to severe disease (Goldman et al., 2021). On the other hand, the evidence in specific transplant populations has been mixed, with a clear association with negative outcomes seen in allogeneic HSCT and lung transplant recipients, but not definitively demonstrated in other SOT recipients (Goldman et al., 2021). The immunosuppression severity index (ISI) has been validated to predict progression from URTI to LRTI in allogeneic HSCT recipients with RSV and can be used to quantify the severity of T cell immunocompromised in this population to guide therapy (see Table 5) (Shah et al., 2014). A score of 0–2 denotes low risk; 3–6 intermediate risk; and 7–10 high risk. Moderate- and high-risk scores were associated with progression of URTI to LRTI, and high risk was associated with mortality.

Causes of secondary T cell disorders

Due to medical conditions

The medical condition most associated with secondary T cell deficiency and opportunistic infections is the acquired immune deficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV). HIV is an RNA retrovirus that selectively targets T helper (CD4⁺) cells, eventually leading to severe lymphopenia and many of the opportunistic infections described above (Sabin and Lundgren, 2013). Much of what is applied today about management of opportunistic infections in secondary T cell deficiencies has been extrapolated from studying people living with HIV (PLWH). Human T-lymphotropic virus (HTLV)-1, a retrovirus like HIV, mostly manifests as acute T cell leukemia/lymphoma (ATLL), HTLV-associated myelopathy (HAM), or dermatitis in at-risk patients. However, it has a well-known association with *Strongyloides* hyperinfection and disseminated strongyloidiasis (Porto et al., 2001; Montes et al., 2009).

T cell leukemias and lymphomas cause numerical and functional T cell deficiency due to the proliferation of an abnormal lymphoid clone, like the numerical and functional phagocyte deficiency that is encountered in AML (see above). This risk is added to the further T cell immunosuppression associated with T cell targeted therapy, chemotherapy and glucocorticoids (see below). These patients are well known to be predisposed to developing *Pneumocystis jiroveci* pneumonia, PJP (Poulsen et al., 2001). Also, thymoma has also been reported to cause a severe functional T cell deficiency distinct from Good's syndrome. While naïve T cells are numerically normal, an abnormal T cell receptor (TCR) prevents them from being activated, which leads to generalized anergy (Christopoulos et al., 2015).

Interferon- γ (IFN- γ) is a key effector cytokine produced by activated T and NK cells that primes phagocytes for killing of ingested microbes (Murphy, 2012). Acquired neutralizing anti-IFN- γ antibodies occur in older adults of Asian, particularly Southeast Asian, descent. This is associated with disseminated non-tuberculous mycobacterial (NTM) infections but has recently also been described in HIV-negative patients with otherwise unexplained *Talaromyces marneffe* infection (Browne et al., 2012; Guo et al., 2020). Idiopathic CD4 lymphopenia is defined as a T helper (CD4⁺) cell count below 300 cells mm⁻³ in HIV-negative patients; despite almost 30 years of investigation, no cause has been elucidated. These patients develop similar opportunistic infections as those with advanced HIV/AIDS (Smith et al., 1993).

Pregnancy is a relative T cell deficient state, particularly in the second and third trimesters. This state may explain the predisposition of pregnant women to invasive listeriosis, which can infect the fetus causing granulomatosis infantiseptica, and to severe primary varicella infection with a particular predisposition to developing pneumonitis which is in some cases severe enough to require mechanical ventilation (Madjunkov et al., 2017; Shrim et al., 2012).

Iatrogenic causes

T cell immunocompromised results as a side effect of alkylating and antiproliferative chemotherapy for cancer, as an intended consequence of targeted T cell depletion, and because of targeted blockade of effector cytokines by monoclonal antibodies used to treat diverse autoimmune phenomena.

Table 4 Opportunistic infections associated with T cell deficiencies.

Kingdom	Genus	Species	Manifestations
Viruses	<i>Herpesviridae</i>	Herpes simplex virus (HSV)	Mucocutaneous ulcers
			Hepatitis
			Pneumonitis
			Retinitis
			Encephalitis
		Varicella zoster virus (VZV)	Primary varicella
			Zoster (limited and disseminated)
			Encephalitis
			Myelitis
			Retinitis
		Cytomegalovirus (CMV)	Pneumonitis
			Hepatitis
			Mononucleosis-like illness
			Hepatitis
			Myelosuppression
Bacteria	<i>Polyomaviridae</i>	JC virus	Colitis
			Pneumonitis
			Retinitis
			Encephalitis
			Post-transplant acute limbic encephalitis (PALE)
		BK virus	Mononucleosis
			Post-transplant lymphoproliferative disorder (PTLD)
			Lymphoma
			Kaposi's sarcoma (KS)
			Primary effusion lymphoma (PEL)
		Human papillomavirus (HPV)	Castleman's disease
			Kaposi's sarcoma inflammatory cytokine syndrome (KICS)
			Progressive multifocal leukoencephalopathy (PML)
			Granule cell neuronopathy (GCN)
			JC virus encephalitis
	<i>Papillomaviridae</i>	JC virus	JC virus meningitis
			BK nephropathy
			Ureteritis
			Hemorrhagic cystitis
			Condylomata (warts)
		BK virus	Squamous cell carcinoma (SCC) of cervix, vulva, penis, anus, oropharynx
			Upper respiratory tract infection (URTI)
			Lower respiratory tract infection (LRTI)
			Hemorrhagic cystitis
			Colitis
		Human papillomavirus (HPV)	Pure red cell aplasia
			Fulminant hepatitis
			Reactivation of latent infection
			Chronic infection
			Diarrhea
	<i>Adenoviridae</i>	Adenovirus	Bacteremia
			Rhombencephalitis
			Congenital infection
			Severe pneumonia
			Diarrhea
		Parvovirus B19	Hepatitis
			Hyponatremia
			Encephalopathy
			Relapsing fever
			Spondylodiscitis
		Hepatitis B virus (HBV)	Endocarditis
			Neurological infection
			Glandular
			Ulceroglandular
			Oculoglandular
	<i>Hepadnaviridae</i>	Hepatitis E virus (HEV)	
		Listeria monocytogenes	
		Legionella spp.	
		Brucella spp.	
		Francisella tularensis	

Table 4 (Continued)

Kingdom	Genus	Species	Manifestations
Fungi	Acid-fast bacteria	<i>Salmonella enterica</i>	Oropharyngeal infection
			Pneumonia
			Typhoidal disease
			Enteritis
			Osteomyelitis
		<i>Shigella</i> spp.	Bacteremia
			Endocarditis
		<i>Yersinia pseudotuberculosis</i>	Typhoid fever
			Enteritis
			Extraintestinal infection
	Dimorphic endemic fungi	<i>Mycobacterium tuberculosis</i>	Abdominal adenitis
		Non-tuberculous mycobacteria	Pulmonary disease
		<i>Nocardia</i> spp.	Adenitis
			Disseminated disease
			Lung nodules
Parasites	Yeast	<i>C. neoformans</i>	Brain abscess
			Nodular lymphangitis
			Pneumonia
			Meningitis
			Brain abscess
	Protozoa	<i>Candida</i> spp.	Disseminated disease
			Chronic mucocutaneous candidiasis
			Esophagitis
		<i>Histoplasma capsulatum</i>	Acute pulmonary infection
			Hilar adenopathies
			Disseminated infection
		<i>Blastomyces dermatitidis/gilchristii</i>	Acute pulmonary infection
			Skin nodules
			Brain abscess
		<i>Coccidioides immitis/posadasii</i>	Osteomyelitis
			Acute pulmonary infection
			Meningitis
	Nematoda	<i>Paracoccidioides brasiliensis</i>	Progressive mucocutaneous infection
		<i>Talaromyces marneffeii</i>	Disseminated infection
		<i>Pneumocystis jiroveci</i>	Interstitial pneumonia
		<i>Toxoplasma gondii</i>	Pneumonitis
		<i>Giardia lamblia</i> , <i>Cryptosporidium</i> spp., <i>Cyclospora cayentanensis</i> , <i>Cystoisospora belli</i> , <i>Microsporidium</i> spp.	Brain abscess
		<i>Strongyloides stercoralis</i>	Prolonged diarrhea
			Protein-losing enteropathy
			Chronic intestinal infection
			Hyperinfection
			Disseminated infection

Table 5 Immunosuppression severity index (ISI) for allogeneic HSCT patients with RSV.

Criterion	Points
ANC <500	3
ALC <200	3
Age >40	2
Myeloablative conditioning regimen	1
GVHD	1
Corticosteroid treatment	1

Cancer chemotherapy causes myelosuppression affecting all cell lines. Some chemotherapy agents primarily used to treat hematological malignancies are particularly T cell depleting. Fludarabine, a DNA polymerase inhibitor, has been associated with prolonged T helper (CD4⁺) cell lymphopenia and cases of PJP and CMV disease up to 1 year after treatment (Tam et al., 2004; Haeusler et al., 2013; Marchesi et al., 2018). This is compounded by the fact that it is often used in combination with the alkylating agent cyclophosphamide (also T cell depleting) and anti-CD20 monoclonal antibody rituximab (i.e., FCR regimen) to treat CLL. Similarly, the alkylating agent, bendamustine, has been associated with prolonged T helper (CD4⁺) lymphopenia and typical opportunistic infections including CMV, herpes zoster, histoplasmosis, and PJP and is also used in combination with rituximab (i.e. BR regimen) most often to treat B-cell lymphomas (Fung et al., 2019).

Some targeted therapies are also associated with significant T cell immunosuppression. Treatment with the proteasome inhibitor bortezomib, used primarily for multiple myeloma, is associated with a high incidence of zoster in the absence of prophylaxis, caused by a loss of VZV-specific T cell responses (Kim et al., 2015). The anti-CD52 antibody, alemtuzumab, used at high-doses to treat T cell malignancies, specifically targets B and T cells for destruction causing profound and prolonged T cell lymphopenia and the typically-associated opportunistic infections; this agent is also used in solid organ transplants and in hematopoietic transplant recipients requiring lymphocyte-depletion (Dearden et al., 2002; Helfrich and Ison, 2015; Coles et al., 2012). Interestingly, the ostensibly B cell targeted anti-CD20 agent rituximab and anti-CD38 agent daratumumab have also been associated with infections traditionally associated with T cell deficiency; rituximab has been associated with PML and both have been associated with hepatitis B reactivation (Focosi et al., 2019; Loomba and Liang, 2017; Lee et al., 2021). The mechanism behind this observation has yet to be determined.

Targeted T cell depletion is used to prevent and treat rejection and GVHD in SOT and allogeneic HSCT recipients, respectively, and to treat autoimmune and inflammatory diseases. SOT and some HSCT recipients require conditioning at the time of transplant to rapidly reduce the T cell population and prevent acute cell-mediated rejection (ACMR). The agents most often used are antithymocyte globulin (ATG), a polyclonal animal-derived (rabbit or horse) antibody product targeting human T cells, or alemtuzumab (discussed above). The anti-IL-2-receptor monoclonal antibodies, daclizumab and basiliximab, are also sometimes used (Swiatecka-Urban, 2003). This results in rapid and profound lymphopenia, putting patients at highest risk of opportunistic infections for the first 6 months after receiving a SOT (Fishman, 2007). Similarly, allogeneic HSCT recipients receive myeloablative conditioning with alkylating chemotherapy; the specific regimens are beyond the scope of this chapter. This is followed in both cases by maintenance immunosuppression, administered for life in SOT recipients and typically for the first 100 days after HSCT, unless acute or chronic GVHD develops (in which case, more intense and/or prolonged immunosuppressive therapy is given). The agents most used are the calcineurin inhibitors, tacrolimus or cyclosporine, which inhibit T cell activation and proliferation; mycophenolate mofetil (MMF), a purine synthesis inhibitor which prevents T cell proliferation; and glucocorticoids (discussed below) (Gaston, 2001). Other agents used are mammalian target of rapamycin (mTOR) inhibitors, rapamycin and sirolimus, and costimulation blockers (e.g., belatacept (Noble et al., 2019)). Episodes of rejection in SOT recipients may require reinduction and increased immunosuppressant dosing or addition of new maintenance agents, which increases their susceptibility to infection.

Many of the above agents may be used to treat autoimmune conditions. The first line treatment for older patients with acquired aplastic anemia, for example, is ATG induction followed by maintenance with a calcineurin inhibitor (Bacigalupo, 2017). Alemtuzumab is also used to treat multiple sclerosis, albeit with a lower dose than other indications (Coles et al., 2012). Calcineurin inhibitors, MMF, and high dose steroids are used to treat lupus nephritis, while MMF is also used for myositis associated lung disease, and vasculitides (Fanouriakis et al., 2020; Huapaya et al., 2019). Other T cell antiproliferative agents frequently used to treat autoimmune diseases are methotrexate, a folate synthesis inhibitor, azathioprine, a synthetic purine analogue, and fingolimod, which sequesters T cells in lymphoid tissues.

Glucocorticoids are broad spectrum immunosuppressants used for their anti-inflammatory effect in autoimmune conditions, prevention, and treatment of rejection in SOT, treatment of acute GVHD, and a variety of neurological, cardiac, pulmonary, gastrointestinal, rheumatologic, and allergic conditions. They are also used in combination chemotherapy regimens for lymphoma and multiple myeloma, as well as for prevention of post-chemotherapy emesis. High-dose and prolonged glucocorticoid therapy are variously defined by different authors, but a consensus exists for 30 mg of prednisone equivalent per day for 1 month or greater (Buttgereit et al., 2002). The T cell suppressive effect of glucocorticoid therapy is additive to other immunosuppressants and increases the risk of opportunistic infection, particularly PJP, CMV infection, tuberculosis, and *Strongyloides* hyperinfection (Malpica and Moll, 2020).

T helper (CD4⁺) cells exert their influence on professional antigen-presenting cells through the production of cytokines at the immunological synapse. Biologic immunosuppressants are targeted monoclonal antibodies and small molecules that prevent cytokines from binding to their target or interfere with downstream receptor signaling. These medications are now broadly used to treat rheumatological, dermatological, gastrointestinal, neurological, and ocular autoimmune diseases, but are also associated with opportunistic infections because of the functional T cell deficiency they create (see Table 6).

The longest marketed, and thus best studied, of the biologic immunosuppressants are the TNF- α inhibitors. These are associated with an increased risk of active tuberculosis, non-tuberculous mycobacterial disease, histoplasmosis, PJP, candidiasis, herpes zoster, hepatitis B reactivation, *Legionella* pneumonia, invasive *Listeria monocytogenes*, *Salmonella* spp., and pneumococcal disease; the association between TNF- α inhibition and progression of hepatitis C infection is controversial (Pompili et al., 2013; Brode et al., 2015; Dixon et al., 2010; Vallabhaneni and Chiller, 2016; Strangfeld et al., 2009; Baddley et al., 2018). The anti-TNF- α monoclonal antibodies appear to present a greater risk of infection than the soluble TNF- α receptor etanercept (Tubach et al., 2009). Non-TNF- α -targeted biologic immunosuppressants appear to cause less susceptibility to infection; however, population-level data is sparse, as many of these agents have been recently marketed (Cantini et al., 2017; Pettipher and Benitha, 2020). Nonetheless, their monographs carry the same warnings as TNF- α inhibitors.

Table 6 Biological immunosuppressants and their targets.

<i>Mechanism</i>	<i>Drug</i>	<i>Approved indications</i>
Anti-TNF- α monoclonal antibody	Infliximab	Rheumatoid arthritis
	Adalimumab	Juvenile idiopathic arthritis
	Certolizumab	Psoriatic arthritis
	Golimumab	Ankylosing spondylitis Plaque psoriasis Crohn's disease Ulcerative colitis
TNF- α soluble receptor	Etanercept	Rheumatoid arthritis
		Juvenile idiopathic arthritis
		Psoriatic arthritis
		Ankylosing spondylitis Plaque psoriasis Crohn's disease Ulcerative colitis
Anti-IL-1 monoclonal antibody	Anakinra	Rheumatoid arthritis Cryopyrin-associated periodic syndromes
	Canakinumab	Cryopyrin-associated periodic syndromes Tumor Necrosis Factor Receptor Associated Periodic Fever Syndromes (TRAPS) Hyperimmunoglobulin D Syndrome/Mevalonate Kinase Deficiency Familial Mediterranean Fever Systemic Juvenile Idiopathic Arthritis
Anti-IL-6-monoclonal antibody	Tocilizumab	Rheumatoid arthritis
		Giant Cell Arteritis
		Polyarticular Juvenile Idiopathic Arthritis
		Systemic Juvenile Idiopathic Arthritis Cytokine release syndrome
JAK inhibitor	Tofacitinib	Rheumatoid arthritis
		Psoriatic arthritis Ulcerative colitis
Costimulatory modulator	Abatacept	Rheumatoid arthritis
		Juvenile idiopathic arthritis Psoriatic arthritis
Anti-IL-17 monoclonal antibody	Ixekizumab	Plaque psoriasis Psoriatic arthritis
	Secukinumab	Plaque psoriasis Psoriatic arthritis
		Ankylosing spondylitis
	Brodalumab	Plaque psoriasis
IL-12/23 antagonist	Ustekinumab	Plaque psoriasis Psoriatic arthritis Crohn's disease
IL-23 antagonist	Guselkumab	Plaque psoriasis
Integrin inhibitor	Natalizumab	Multiple sclerosis
	Vedolizumab	Ulcerative colitis Crohn's disease

Cadherin inhibitors block trafficking of autoreactive T cells to sites of disease activity. Thus, the T cell immunodeficiency they create is thought to be localized to the site of action. The cadherin inhibitors, natalizumab (originally used for multiple sclerosis and inflammatory bowel disease) and efalizumab (originally used for psoriasis, now withdrawn from the market), were both associated with an extremely high incidence of progressive multifocal leukoencephalopathy (PML) caused by the blockade of lymphocyte trafficking to the CNS (Ho et al., 2017; Schwab et al., 2012). Vedolizumab selectively blocks lymphocyte trafficking to the gut and is used to treat inflammatory bowel disease (IBD); it has not been associated with any cases of PML to date, but nonetheless carries an FDA black box warning. It has been associated, however, with an increased risk of CMV colitis, presumably due to localized T cell immunodeficiency within the gut (Hommel et al., 2018).

Treatment of secondary T cell dysfunction

The mainstay of treatment of secondary T cell dysfunction is reversal of immunosuppression through treatment of the underlying disease, or reduction of iatrogenic immunosuppression (RIS) when this is possible. Vaccination and antimicrobial prophylaxis are also used to prevent opportunistic infections (OIs). Biological response modifiers and adoptive T cell therapy are emerging treatments for refractory OIs.

Reversal of immunosuppression

Restoration of T cell function is critical for the elimination of OIs. In PLWH, initiation of highly active antiretroviral therapy (HAART) suppresses viral replication and at least partially restores T helper (CD4⁺) cell counts and function (Masur et al., 2014). In patients with autoimmune diseases, temporary or permanent suspension or replacement of immunosuppressive therapy is often practiced when infections develop; however, this must be balanced against disease activity. The balance between RIS and prevention of cell-mediated rejection is difficult in SOT recipients; severe or refractory OIs may require sacrificing a non-critical organ (for example a kidney allograft), although this is not possible in critical organs such as liver, heart, and lung grafts. In allogeneic HSCT, delayed engraftment or treatment of life-threatening acute GVHD may make restoration of T cell function impossible. In patients with anti-IFN- γ autoantibodies, rituximab or daratumumab has been successfully used to restore IFN- γ and clear NTM infections (Czaja et al., 2014; Ochoa et al., 2021).

Clinicians must also balance RIS with the risk of the immune reconstitution inflammatory syndrome (IRIS). IRIS takes two forms: paradoxical IRIS occurs when a seemingly improving opportunistic infection suddenly flares due to restoration of immunity and an inflammatory infiltrate, while unmasking IRIS reveals a previously undetected OI in the setting of immune recovery (Barber et al., 2014). The treatment of IRIS generally includes steroids for their anti-inflammatory effect while otherwise continuing both anti-infective therapy and immune reconstitution. Certain infections, particularly CNS tuberculosis and cryptococcosis, cause life-threatening IRIS due to increased intracranial pressure (ICP) and delayed initiation of HAART is associated with improved survival in these patients; how this applies to non-HIV patients is unknown (Török et al., 2011; Boulware et al., 2014). The evidence for prophylactic steroids to prevent IRIS is mixed; they appear to be beneficial in HIV-infected patients with tuberculosis and moderate-to-severe PJP, but not in cryptococcal meningitis (Meintjes et al., 2018; Bozzette et al., 1990; Beardsley et al., 2016; Immunocompromised Host Society, 1990). Again, it is unclear how these data apply to non-HIV T cell immunocompromised patients.

Vaccination

Live vaccines are contraindicated in patients with T cell deficiencies as there is a risk of the vaccine strain acquiring virulence and causing infection. If iatrogenic immunosuppression is planned, it is recommended to ensure that all live vaccines are up to date before it is initiated. Specifically, a waiting period of 4 weeks after receiving the measles-mumps-rubella vaccine (MMR) and 6 weeks after receiving the live varicella or herpes zoster vaccine should be observed before administering T cell immunosuppression.

Conjugated and polysaccharidic vaccines are safe in patients receiving T cell-depleting therapies, but their efficacy may be reduced once on therapy (Papp et al., 2019). As such, it is preferable to administer these vaccines before planned iatrogenic immunosuppression. Both conjugated and polysaccharidic pneumococcal, human papillomavirus (HPV, depending on the patient's age), and HBV vaccination are recommended pre-immunosuppression; it is also good practice to update protection against diphtheria, pertussis, and tetanus, particularly if the patient has not received acellular pertussis vaccine. Annual influenza vaccination is also recommended (Danziger-Isakov and Kumar, 2019). For patients who are already immune to varicella, evidence is emerging that the herpes zoster adjuvanted recombinant subunit vaccine (Shingrix) may be immunogenic and possibly provide protection against herpes zoster post-renal transplant and autologous HSCT (Vink et al., 2020; Bastidas et al., 2019).

Patients who undergo allogeneic HSCT receive a new immune system, hence, they must recommence vaccination with a primary series after the transplant and should not be considered immune to any virus, especially varicella zoster, despite previous infection or vaccination history. Vaccination with conjugate or polysaccharidic vaccines may commence 3–6 months after transplant and live vaccination may commence 24 months after transplant, if there is adequate engraftment, disease control, and lack of GVHD (Kamboj and Shah, 2019).

Antimicrobial prophylaxis

Screening and treatment of latent infections can prevent reactivation and disseminated disease. In patients starting treatments that risk HBV flares or reactivation, serology for hepatitis B surface antigen (HBsAg), indicating chronic infection, and hepatitis B core antibody, indicating resolved infection, should be performed. Patients who are chronically infected with or who have been previously exposed to HBV and are starting high-risk regimens (>10% risk of reactivation) should be started on prophylaxis with tenofovir or entecavir and be monitored regularly for HBV reactivation or hepatocyte injury; those starting moderate-risk regimens (1–10% risk of reactivation) may be monitored without prophylaxis (Myint et al., 2020). Serological and nucleic acid amplification test (NAAT)-based screening for HCV can also be performed followed by treatment of chronically infected patients with direct acting antivirals (DAAs). Screening for latent tuberculosis infection (LTBI) should be performed in those at epidemiological risk with tuberculin skin testing (TST) or an IFN- γ release assay (IGRA); treatment of LTBI with 4 months of rifampin as compared to 9 months of isoniazid (INH) is better tolerated and has better completion rates in both adults and children (Menzies et al., 2018; Diallo et al., 2018; Gardam et al., 2003). Note that patients who are already T cell deficient may be generally anergic resulting in false-negative or indeterminate LTBI screening test results. Screening for chronic strongyloidiasis with serology or stool culture and treatment with ivermectin is also recommended in patients born in or who have lived in endemic countries (Requena-Méndez et al., 2017).

CMV risk in hematopoietic and SOT recipients is stratified by the donor/recipient (D/R) serostatus; the type of organ transplanted; and the induction regimen: this risk assessment is used to determine whether to administer primary prophylaxis with valganciclovir, or to monitor for CMV reactivation and initiate pre-emptive treatment should this occur. In SOT recipients, the

D/R serostatus with highest risk is donor positive/recipient negative (D+/R-), whereas donor positive/recipient positive (D+/R+) and donor negative/recipient positive (D-/R+) statuses are considered intermediate risk, while donor negative/recipient negative (D-/R-) is considered low risk. The most vascularized organs are at highest risk of transmitting donor derived CMV, including lung, facial composite, and small bowel transplants. Lymphocyte-depleting induction with alemtuzumab and ATG also increase the risk of reactivation (Razonable and Humar, 2019). Patients who do receive valganciclovir prophylaxis are treated for 100 days after SOT, which is the period of maximal immunosuppression; they must still be monitored for CMV reactivation after this period, as infection (viremia) and/or end-organ disease can occur after prophylaxis.

Patients receiving chemotherapy, targeted therapies, or HSCT for hematological malignancies, and who are at high risk of HSV and VZV reactivation, are usually prescribed acyclovir or valaciclovir prophylaxis for the duration of the at-risk period. In HSCT recipients, the risk stratification for CMV reactivation is different from SOT, with D-/R+ status conferring the highest risk of CMV reactivation, D+/R- and D+/R+ status conferring intermediate risk, and D-/R- conferring the lowest risk. CMV prophylaxis in this population is more complex, as ganciclovir prophylaxis has been associated with delayed engraftment and an increased incidence of bacterial and fungal infections; it is thus not used routinely for this indication (Boeckh and Ljungman, 2009). However, the novel CMV antiviral agent letermovir, a terminase complex inhibitor that is not myelosuppressive, has been found to significantly reduce CMV reactivation in allogeneic HSCT recipients when administered until 14 weeks after transplant and has been approved for CMV prophylaxis in this population (Marty et al., 2017).

PJP prophylaxis is used to prevent pneumocystis pneumonia in PLWH with T helper (CD4⁺) counts below 200 cells mm⁻³; patients receiving high-risk chemotherapy regimens; allogeneic HSCT recipients and SOT recipients for the first 6 months post-transplant, or longer if being treated for acute GVHD or rejection; and patients receiving high-dose, prolonged glucocorticoid treatment, often in combination with a second immunosuppressant (Masur et al., 2014; Maertens et al., 2016). Recommended regimens for PJP prophylaxis can be found in Table 7.

Primary prophylaxis against toxoplasmosis is indicated in seropositive PLWH with T helper (CD4⁺) cell counts below 100 cells mm⁻³; the usual agents are daily double-strength sulfamethoxazole-trimethoprim, dapsone combined with pyrimethamine, or daily atovaquone. These patients should also be screened once with serum cryptococcal antigen and, if investigations reveal no pulmonary or CNS disease, receive fluconazole prophylaxis (Masur et al., 2014). PLWH living in hyperendemic regions for histoplasmosis also receive daily itraconazole prophylaxis until their T helper (CD4⁺) cell counts rise above 150 cells mm⁻³; neither of these recommendations has been applied outside of the HIV setting (Masur et al., 2014). In regions endemic for coccidioidomycosis, seronegative PLWH and SOT recipients are regularly screened for seroconversion (one or twice per year) and treated with fluconazole if this occurs (Nanayakkara and Blodgett, 2019).

Immunotherapies

Recombinant cytokine therapies can be used to boost T cell numbers, strengthen their response, or pharmacologically replace cytokines that they normally produce. T cells may be stimulated to proliferate using recombinant IL-2, which is approved for use as a cancer immunotherapy; however, it also increases the proliferation of T regulatory cells (Tregs) and causes dose-dependent fever, chills, and vascular leak, which limit its efficacy and tolerability (Mitra and Leonard, 2018). T cell numbers can also be boosted through mobilization from the bone marrow and thymus by recombinant IL-7, as has been successfully done in idiopathic CD4 lymphopenia and may potentially be applied to other patients with reduced T helper (CD4⁺) cell counts (Sheikh et al., 2016). Recombinant IFN- γ in pharmacological doses replaces effector cytokine production and stimulates phagocytes to kill intracellular pathogens; while this has been used successfully in patients with chronic granulomatous disease and in a case report of disseminated NTM infection in the setting of anti-IFN- γ autoantibodies, systemic side effects again limit its tolerability (Vignesh et al., 2017; Harada et al., 2021).

Adoptive T cell transfer is an emerging treatment with the potential to revolutionize the management of OIs in patients with secondary T cell immunodeficiencies. This involves the ex vivo expansion of autologous or third-party T cells specific for the infection to be treated followed by infusion into the patient. The largest trial to date administered an off-the-shelf, five-valent product (specific for CMV, EBV, HHV-6, BK virus, and adenovirus) to 38 allogeneic HSCT recipients, resulting in a partial or complete clinical response rate of 92% after the first infusion (Tzannou et al., 2017). A phase 3, multicenter trial to treat

Table 7 Recommended regimens for PJP prophylaxis.

Drug	Route	Dosing	Limitations
Sulfamethoxazole-trimethoprim	Oral	1 single-strength tablet daily 1 double-strength tablet daily 1 double-strength tablet three times per week	Myelosuppression, hyperkalemia, allergy
Dapsone	Oral	100 mg daily	Hemolytic anemia in G6PD deficiency
Atovaquone	Oral	1500 mg daily	Cost, efficacy
Pentamidine	Inhalation	300 mg monthly	Inconvenience of administration, breakthrough infection in upper lobes

hemorrhagic cystitis using this product is ongoing ([clinicaltrials.gov NCT04390113](https://clinicaltrials.gov/ct2/show/study/NCT04390113)). Cell therapy is also an emerging treatment for invasive fungal disease. Cells targeting *Candida* spp., *Aspergillus* spp., and *Rhizopus oryzae* have been successfully manufactured and have shown in vitro activity against these agents (Lum and Bollard, 2018). In a single-center study in patients post-hematopoietic stem cell transplant (HSCT) with confirmed invasive aspergillosis, adoptive T cell transfer resulted in clearance of both serum antigenemia and infection in 9 out of 10 treated patients, versus 7 out of 13 controls (Perruccio et al., 2005). One of the challenges in integrating cell therapies into clinical practice is the effect of ongoing iatrogenic immunosuppression for GVHD or SOT rejection on these products; however, recent publications have reported genetically engineered virus-specific T cell products that are resistant in vitro to glucocorticoids, tacrolimus, MMF, and alemtuzumab, essentially creating cells that are “armored” against these immunosuppressants (Kaeuferle et al., 2020; Hafezi et al., 2020; Amini et al., 2021; Poirot et al., 2015).

Conclusion

SIDs are common in clinical practice and are likely to become more common as pharmacological and cell therapies expand in scope. A solid understanding of the affected arm of the immune system is required to recognize the associated infectious risks associated with medical conditions and immunosuppressant therapies and to intervene early to treat OIs. Unfortunately, antimicrobial therapy is often not sufficient to eliminate these OIs, and clinicians also need to understand the immunological tools at their disposal to restore immunity whenever possible. Immunologically targeted interventions including recombinant cytokines, antibodies, and cell therapies are likely to take a greater role in the treatment of these infections in the years ahead.

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Treatment of Autoinflammatory Diseases

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Introduction

Inflammation is a normal response to infection and tissue damage, aiming to eliminate the pathogens and facilitate tissue repair. Errors in one or more elements of innate immunity led to spontaneous or chaotic immune activation, with excessive production of pro-inflammatory cytokines, which in turn lead to prolonged and persistent activation of immune cells, causing systemic and organ-specific autoinflammation. Autoinflammatory diseases (AIDs) are the heterogeneous group of disorders characterized by chronic or intermittent systemic and organ-specific inflammation and recurrent fever caused by dysregulation of the innate immune system, also known as “periodic fevers.” The term “autoinflammatory diseases” was introduced in 1999 by Michael McDermott and Daniel Kastner et al. They reported the genetic cause for the systemic inflammation in tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) in the absence of classic autoimmune features. AIDs are usually linked to a mutation and epigenetics affecting a broad spectrum of innate immune pathways, including inflammasome function, aberrant recognition of host molecules by TLRs, nuclear factor kappa B (NF-κB) activation, and type I interferon production, which may trigger sterile inflammation. Depending on the error, the clinical presentation varies between individual syndromes. Common findings associated with AIDs are episodic fever, rash, swelling of joints, and overproduction of IL-1β. Thus, IL-1β has become a common therapeutic target. Due to its rarity and a relatively small number of patients with the particular autoinflammatory disorder, evidence-based guidelines are lacking. The treatment is usually based on individual centers’ experience or expert’s opinion. Almost 10 years ago, in 2012, few European pediatric centers experienced in rheumatic diseases launched a project called SHARE (Single Hub and Access point for pediatric Rheumatology in Europe) with one of its main aims to create evidence-based recommendations for the management of the pediatric rheumatic diseases. Among the three leading monogenic AIDs were found: cryopyrin-associated periodic syndromes (CAPS), tumor necrosis factor receptor-associated periodic syndrome (TRAPS), and mevalonate kinase deficiency (MKD) (Ter Haar et al., 2015). Additionally, in 2015, the European Alliance of Associations for Rheumatology (EULAR) published guidelines for managing familial Mediterranean fever. Due to the increasing number of known AIDs, available recommendations seem to be a drop in the ocean for the physicians who treat these specific disorders.

Due to the availability of new treatment methods, AIDs management is transforming from strictly symptomatic, including antipyretics, corticosteroids, and non-steroid anti-inflammatory drugs (NSAIDs), to more specific and targeted. AIDs respond favorably to the appropriate treatment. Colchicine is highly effective in familial Mediterranean fever (FMF), anti-interleukin (IL)-1 therapies in cryopyrin-associated periodic syndromes (CAPS), and tumor necrosis factor receptor-associated periodic syndrome (TRAPS). Medications such as anakinra, rilonacept, and canakinumab have revolutionized the treatment of AIDs (Table 1).

Leading groups of drugs used in the treatment of AIDs

Colchicine

Colchicine is an alkaloid initially extracted from bulbs of two plants of the Lily family: *Colchicum autumnale* (meadow saffron) and *Gloriosa superba*. In the form of the autumn crocus, it was used to treat joint swelling since approximately 1500 BCE (Wall, 2015). Its

Table 1 Treatment of autoinflammatory diseases—summary.

<i>Monogenic diseases</i>		<i>Treatment</i>
Interleukin-1—mediated disorders	Familial Mediterranean Fever (FMF)	Colchicine Canakinumab Anakinra
	Tumor necrosis factor receptor-associated periodic syndrome (TRAPS)	Glucocorticoids Canakinumab Etanercept
	Cryopyrin-associated periodic syndrome (CAPS)	Interleukin-1 antagonists (canakinumab, anakinra, rilonacept)
	Mevalonate kinase deficiency (MKD/HIDS)	Non-steroid anti-inflammatory drugs Canakinumab Anakinra Etanercept Tocilizumab
	Deficiency of the interleukin-1-receptor antagonist (DIRA) Majeed syndrome	Anakinra Non-steroidal anti-inflammatory drugs Glucocorticoids Anakinra Bisphosphonates TNF- α inhibitors RBC transfusions Physical therapy
	Autoinflammation with infantile enterocolitis	Anakinra
	Blau syndrome	Glucocorticoids Methotrexate TNF- α inhibitors Canakinumab
	NLRP12-associated autoinflammatory disorder	Non-steroidal anti-inflammatory drugs Antihistamines Glucocorticoids Anakinra TNF- α inhibitors
	CARD-14 mediated psoriasis	Ustekinumab Ixekizumab
	Deficiency of IL-36 receptor antagonist	Glucocorticoids Cyclosporine Acitretin Granulocyte/monocyte apheresis Adalimumab Anakinra Antibiotics Diet Vitamin supplementation Immunoglobulin replacement therapy TNF- α inhibitors
NF- κ B—mediated disorders		
Interferon-mediated disorders	Haploinsufficiency of A20 Otlipenia Aicardi-Goutières syndrome	Glucocorticoids Azathioprine Intravenous immunoglobulin Reverse transcriptase inhibitors
	Proteasome-associated autoinflammatory syndrome	Glucocorticoids Immunosuppressive agents Anakinra Tocilizumab TNF- α inhibitors JAK-inhibitors (baricitinib) JAK inhibitors
		Glucocorticoids TNF- α inhibitors Anakinra Canakinumab Cyclosporine Tacrolimus Intravenous immunoglobulin Thalidomide Dapsone
Unknown pathogenetic mechanism	STING-associated vasculopathy with onset in infancy Pyogenic arthritis, pyoderma gangrenosum and acne syndrome (PAPA)	Glucocorticoids TNF- α inhibitors Anakinra Canakinumab Cyclosporine Tacrolimus Intravenous immunoglobulin Thalidomide Dapsone

Table 1 (Continued)

<i>Monogenic diseases</i>	<i>Treatment</i>
Deficiency of adenosine deaminase 2	Fresh-frozen plasma TNF- α inhibitors Tocilizumab Hematopoietic stem-cell transplantation
PLC γ 2-associated antibody deficiency and immune dysregulation	Glucocorticoids Anakinra Hematopoietic stem-cell transplantation
Cherubism	TNF- α inhibitors Tacrolimus
Multifactorial diseases	
Periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis (PFAPA)	Glucocorticoids NSAIDs Colchicine Cimetidine Surgery

active pharmacological component was isolated in 1820 and in 1833 purified and named colchicine. In 1972 Goldfinger described the colchicine use in five patients with familial Mediterranean fever, which resulted in a spectacular reduction of the number of attacks ([Goldfinger, 1972](#)). Colchicine anti-inflammatory properties base on few phenomena:

- It binds to tubulin inside leukocytes, formatting tubulin-colchicine complex, which results in chemotaxis inhibition (tubulin is a microtubule component—its abnormal structure results in disability to move);
- Reduces TNF α production by macrophages and its receptors on endothelial cells—which prevents neutrophils from binding to adhesion molecules on the epithelium;
- Reduces phagocytosis, lysosomal enzyme release, and phospholipase A2 activity
- Suppresses the activation of caspase 1, the element of NLRP3 inflammasome with a subsequent disability to activate the IL-1 β ([Soriano et al., 2020](#)).

Colchicine is metabolized in the liver via CYP3A14; thus, its administration with macrolides, azoles, or statins (or other drugs that inhibit this isoenzyme) may result in elevated plasma concentration resulting in severe complications. The symptoms of intoxication may also occur in patients with renal or liver insufficiency ([Niel and Scherrmann, 2006](#)).

According to the 2016 EULAR guidelines, colchicine effectively prevents FMF attacks and long-term consequences as amyloidosis. The treatment should be started as soon as the diagnosis is established. The initial dose recommended:

- 1–1.5 mg/day (1.8 mg/day if there are tablets 0.6 mg available) in children >10 years and adults
- 0.5–1 mg/day (1.2 mg/day if there are tablets 0.6 mg available) in children 5–10 years
- ≤ 0.5 mg/day (0.6 mg if there are tablets 0.6 mg available) in children <5 years old.

Colchicine can be given in single or divided doses, depending on individual tolerance. Due to gastrointestinal side effects (including cramping, stomachache, diarrhea, vomiting), treatment can be started in subclinical dose (0.5 mg/day) and increased gradually in divided daily doses until the full dose is achieved.

Colchicine can be used as a diagnostic tool when the FMF is highly suspected. In this case, after starting treatment, the patient should be carefully observed for 3–6 months with monitoring of attack severity and frequency.

Suppose adherence is confirmed and the attacks still occur, or the inflammation is detected. In that case, the drug dose may be increased up to a daily dose of 2 mg/day in children and 3 mg/day in adults with careful monitoring of side effects or a maximal well-tolerated dose. If a compliant patient does not respond to treatment after 6 months, it should qualify them as non-responder or colchicine resistant. In this group of patients, it is justified to start therapy with biological agents.

Colchicine toxicity should be checked every 6 months, including liver and kidney function, complete cell blood count, creatinine phosphokinase activity, and general urine testing to detect proteinuria. Drug response is monitored using C-reactive protein and serum amyloid A concentration.

Colchicine is considered safe and should not be discontinued during conception, pregnancy, and lactation ([Niel and Scherrmann, 2006](#)).

Colchicine lifelong prophylaxis prevents attacks of FMF and amyloidosis ([Rigante, 2017](#)).

Colchicine was shown effective in idiopathic acute and recurrent pericarditis, according to Cochrane data review. Also, the COPE trial and ICAP trial confirmed a significant difference in favor of the colchicine treatment ([Blank and Lorenz, 2019](#)).

The use of colchicine in other AID is limited. It has shown partial effectiveness in TRAPS, resulting in partial improvement in 71% of cases with a complete remission in 12.5% ([Vitale et al., 2020](#)). In PFAPA patients, colchicine was found effective in reducing

attack frequency. Preliminary data suggested its use in children who did not respond to tonsillectomy, with atypical PFAPA syndrome, and in cases with predominant aphthous stomatitis (Gaggiano et al., 2019). It was not effective in patients with CAPS and HIDS/MKD (Soriano et al., 2020).

Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are widely used in AID mainly as symptomatic treatment. Some patients present complete reduction of symptoms after receiving only NSAIDs according to Eurofever Registry, but it is a small fraction: 8% of FMF and TRAPS, 13% of HIDS, 6% of CAPS, and 4% of PFAPA patients. NSAIDs, alone or combined with other drugs like glucocorticoids, seem to be effective in up to 80% of all cases (Ter Haar et al., 2013).

Glucocorticosteroids

Glucocorticosteroids decrease the production and action of many pro-inflammatory cytokines as well as leukocyte migration. By disrupting the function of endothelium and fibroblasts, steroids reduce the acute and chronic symptoms of inflammation like swelling, pain, or fibrosis. By blocking the NF- κ B signal sending, glucocorticoids promote anti-inflammatory proteins and suppress pro-inflammatory cytokines.

In FMF, glucocorticoids are effective in patients who do not respond to colchicine alone. Combination of these two drugs can give positive results in the majority of patients, according to Eurofever Registry (Ter Haar et al., 2013). Intravenous methylprednisolone is effective in decreasing pain and fever during attacks (Erken et al., 2008). In FMF, patients with protracted febrile myalgia symptoms can be controlled by high-dose steroids, administered intravenous or oral (Ozen et al., 2016).

In TRAPS, glucocorticoids are helpful as a short therapy during the attacks at a dose of 1 mg/kg/day. Unfortunately, this therapy neither changes the frequency of the attacks nor decreases the subclinical inflammation between them (Ter Haar et al., 2013). Sometimes in TRAPS patients, long-term treatment is necessary to control disease symptoms. Glucocorticoids are not indicated in this situation due to their side effects (Ozen et al., 2016).

In patients with hyperimmunoglobulin D syndrome (HIDS)/mevalonate kinase deficiency (MKD) and cryopyrin-associated periodic syndromes (CAPS), high doses of glucocorticoids may be beneficial during the attacks (about in 73% of HIDS cases and 80% of CAPS cases). However, the vast majority will achieve only partial remission of symptoms. Complete improvement was observed only in 9% of HIDS patients. Like in TRAPS patients, corticosteroids do not affect the frequency of the attacks (Ter Haar et al., 2013).

In contrast, glucocorticoids are the first-line treatment in PFAPA syndrome as one dose can stop the attack and fully resolve the symptoms. This enormous corticosteroid efficacy can be considered as a diagnostic test (Ter Haar et al., 2013). The usual dose is 0.5–2 mg of prednisone (or equivalents) in a single dose. Sometimes additional doses may be necessary (Ter Haar et al., 2013; Manthiram et al., 2017). The treatment with glucocorticoids was found to increase the frequency of the attacks in 25–50% of patients with PFAPA syndrome (Ter Haar et al., 2013).

Glucocorticoids are used in Blau syndrome, mostly in combination with biological agents and methotrexate (Matsuda et al., 2020). The oral treatment was also promising in proteasome-associated autoinflammatory syndrome, but it was concluded that it reduces only part of the inflammatory reactions in this group of patients (McDermott et al., 2015).

Glucocorticoids were often used to achieve symptom control in idiopathic recurrent pericarditis, but long-term follow-up showed that this treatment could increase the risk of recurrent attacks. Actual guidelines support the statement that glucocorticoids may be used only for patients who did not respond to NSAIDs with colchicine. In this group, prednisolone (0.25–0.5 mg/kg/day) should be offered, with slow tapering after 4 weeks of treatment with a full dose (Imazio et al., 2017).

In Behçet syndrome, glucocorticoids are used as a second-line treatment, according to EULAR guidelines. The choice of drug and its dose is dependent on the involved area and previous treatment (Hatemi et al., 2018).

Interleukin-1 blockers

Interleukin-1 is a potent pro-inflammatory agent, activating the cascade of inflammatory reactions via IL-1 receptor, present on almost every human cell. IL-1 receptor inhibitor was described approximately 30 years ago, and since then, there were many trials conducted with confirmed efficacy of IL-1 blockers, mainly in inflammasomopathies, where this treatment remains the most specific. It is also used when the first-line treatment is not sufficient.

Anakinra

Anakinra is a recombinant human IL-1 receptor antagonist (rhIL-1Ra) which acts as a competitive inhibitor with IL-1 α and IL-1 β , binding to the IL-1 receptor type 1 and acting similarly as endogenous IL-1Ra.

Anakinra is used in the treatment of rheumatoid arthritis, Still disease (including systemic juvenile idiopathic arthritis and adult-onset Still disease), in the United States, it was approved for the treatment of CINCA/NOMID, in Europe—for the treatment of CAPS. Anakinra efficacy was also confirmed in clinical trials in patients with FMF, TRAPS, and HIDS/MKD but was not registered for use in these diseases.

The initial dose is 0.5–2 mg/kg/day subcutaneously in children and 100 mg/day in adults. The dose may need to be gradually increased up to 5–8 mg/day to maintain remission in children. The most common side-effects reported were local reaction in the injection site, higher susceptibility to respiratory tract infection, and serious skin infections. The drug seems to be safe in pregnancy (Soriano et al., 2020).

According to EULAR recommendations, in cases of FMF refractory to colchicine, anakinra is recommended. Also, protracted febrile myalgia may be an indication of this treatment (Ozen et al., 2016). In clinical trials, anakinra treatment resulted in complete remission in 76.5% of patients with FMF, and in patients with amyloidosis, it reversed the proteinuria (Van der Hilst et al., 2016).

In the Eurofever registry, anakinra was shown beneficial in approximately 90% of TRAPS patients, and complete remission was achieved in 67% of them. The efficacy was shown in continuous and on-demand treatment. In HIDS/MKD patients described in the Eurofever registry, anakinra treatment resulted in relieving the symptoms in 84% of them, and complete remission was achieved in 29% (Ter Haar et al., 2015).

Basing on a long-term open-label control study of 18 CINCA/NOMID patients treated with anakinra (symptoms and inflammation markers improved in all), the drug was registered for CAPS treatment by the FDA and EMA (Goldbach-Mansky et al., 2006).

There is little evidence of anakinra efficacy in PFAPA syndrome resistant to glucocorticoids. In a small group of patients, anakinra prevented the disease flares (Stojanov et al., 2011).

Anakinra was also considered a treatment of proteasome-associated autoinflammatory syndrome, but the improvement in patients receiving this treatment was only transient (McDermott et al., 2015).

Anakinra is the most common treatment for Schnitzler syndrome and is recommended as the first-line drug as its long-term effectiveness was confirmed in a multicentric retrospective cohort study (Gusdorf and Lipsker, 2017).

Canakinumab

Canakinumab is a humanized IgG1 monoclonal antibody against IL-1 β . It is registered for the treatment of CAPS in children and adults, FMF, TRAPS, and HIDS/MKD. Additionally, in the United States, it is also registered to treat systemic juvenile idiopathic arthritis and Still disease in Europe—in gouty arthritis (De Benedetti et al., 2018; Soriano et al., 2020).

Canakinumab is administered subcutaneously, in children 2–4 mg/kg, in adults ≥ 150 mg every 4–8 weeks. In patients with more severe phenotypes of AID, higher doses may be necessary. In CINCA/NOMID, the dosage may be increased up to 8 mg/kg every 4 weeks, in FMF, TRAPS, and HIDS/MKD, the initial dose is usually 2 mg/kg in children or 150 mg in adults, and it can be increased up to 4 mg/kg in children and 300 mg every 4 weeks in adults.

Safety data for canakinumab during pregnancy is lacking. One series of pregnant patients treated with canakinumab showed no adverse events in seven from eight patients (Youngstein et al., 2017). Frequent side-effects during treatment with canakinumab include respiratory tract and urinary tract infections, neutropenia, and thrombocytopenia (Ridker et al., 2017; Soriano et al., 2020).

Canakinumab was proven safe and effective in patients with FMF—reduced the number of flares and decreased the concentration of acute-phase proteins, and it is approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) (Gül et al., 2015).

According to the CLUSTER trial, canakinumab was very effective in TRAPS patients inducing partial or complete response, reducing the number of attacks, and decreasing the CRP and SAA concentrations. A similar response was documented in HIDS/MKD patients in this trial—thus, the canakinumab was approved by the FDA and EMA to treat TRAPS and HIDS/MKD (De Benedetti et al., 2018).

Canakinumab induces remission in 75–90% of patients with CAPS, the Euro fever registry showed (Ter Haar et al., 2013). There were also two cases reported (pediatric and adult) of PFAPA syndrome with an excellent response to the treatment with canakinumab in a standard dose (Soriano et al., 2020).

A randomized placebo-controlled study with canakinumab showed its efficacy in patients with Schnitzler syndrome—the reduction of clinical symptoms as well as the decrease of inflammation markers were observed. The level of complete remission in patients treated with IL-1 blockers was 83%. (Gusdorf and Lipsker, 2017).

Rilonacept

Rilonacept is a dimeric fusion glycoprotein composed of Fc part of human IgG1 and the human IgG1 receptor extracellular elements. It binds IL-1 α and IL-1 β . Based on two phase-III clinical trials results, rilonacept was considered safe and effective in adults with CAPS (Hoffman et al., 2012). The drug received registration for use only in the United States.

Rilonacept is given subcutaneously at an initial dose of 320 mg, and the weekly dose recommended is 160 mg. In children >11 years and adolescents the dose is 4.4 mg/kg (maximal dose 320 mg) with subsequent doses 2.2 mg/kg (maximal dose 160 mg) once a week subcutaneously. The most common adverse events during the rilonacept treatment are local reactions in the injection site, respiratory, urinary tract infections and headaches, neutropenia, elevated liver transaminases and triglycerides, and creatine phosphokinase in laboratory tests (Sundy et al., 2014; Soriano et al., 2020).

In one clinical trial, rilonacept was effective in patients with FMF, resulting in a reduction in attack frequency (Hashkes et al., 2012). It also showed positive results and a good safety profile in patients with CAPS (Hoffman et al., 2012).

Rilonacept is also considered effective in Schnitzler syndrome resulting in rapid response with reduction of symptoms and markers of inflammation (Gusdorf and Lipsker, 2017).

TNF blockers have been used in monogenic AIDs, but in general, their effect was worse than IL-1 blockers. There are four essential drugs in this group:

- Etanercept (a dimeric human TNF receptor p75-Fc fusion protein),
- Infliximab (a chimeric monoclonal antibody against TNF- α),
- Adalimumab (a fully human monoclonal antibody against TNF- α)
- Golimumab (recombinant human IgG1/kappa monoclonal antibody against TNF- α).

The experience with anti-TNF drugs in monogenic autoinflammatory disorders remains unclear. Thus neither FDA nor EMA approves its use in these diseases. TNF blockers are commonly used (and approved by FDA and EMA) in the treatment of juvenile idiopathic arthritis, rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, and plaque psoriasis, Crohn's disease, ulcerative colitis, and non-infectious uveitis (Soriano et al., 2020).

The experience with the use of TNF blockers in FMF is limited. There are small groups of patients with concomitant conditions treated with one of the anti-TNF drugs with a good response, confirmed in Eurofever registry data (Ozgocmen and Akgul, 2011, Ter Haar et al., 2013). Moreover, these drugs seem to be more beneficial in patients with predominant arthritis (Ter Haar et al., 2013). The EULAR guidelines suggest that TNF blockers may be used in colchicine-resistant FMF, especially in those with articular involvement (Ozen et al., 2016).

In TRAPS, etanercept was reported to reduce the number of attacks and prevent them and reduce the dose of glucocorticoids necessary to maintain remission. Unfortunately, after about 3.3 years, it is common to discontinue the therapy due to lack of efficacy (Bulua et al., 2012; Ter Haar et al., 2013). Etanercept is still recommended in some of these patients but with additional information in the consensus that the effect may decline over time (Ter Haar et al., 2015). In the Euro fever registry, TRAPS patients treated with etanercept responded to the treatment in 88% of cases, with complete response in 26% (Ter Haar et al., 2013). Infliximab and adalimumab are not used in TRAPS patients due to severe paradoxical reactions (hyperinflammatory response) (Soriano et al., 2020).

Anti-TNF agents are used in Blau syndrome, alone or in combination with glucocorticosteroids or methotrexate. Early introduction of biological treatment was crucial to prevent permanent destruction of involved joints and vision loss/impairment (Matsuda et al., 2020). Successful treatment with TNF blockers was observed in patients with pyogenic arthritis, pyoderma gangrenosum, and acne syndrome (PAPA) (Tofteland and Shaver, 2010).

The spectacular response to anti-TNF agents was described in otulipenia, suggesting a potentially effective treatment for these patients (Damgaard, 2016).

Etanercept was the most effective treatment in patients with a deficiency of adenosine deaminase 2 (DADA) (Navon Elkan, 2014).

The consensus treatment plans for Chronic Non-Bacterial Osteomyelitis (CNO) proposed by Zhao et al. in 2018 included the use of TNF blockers for the patients who did not respond to NSAIDs or have active spinal lesions (Zhao et al., 2018).

Infliximab and adalimumab were shown effective in Behcet disease, mainly in large artery disease and symptoms from the gastrointestinal tract (Hatemi et al., 2018).

Anti-interleukin-6 agents

Tocilizumab is a humanized monoclonal anti-IL-6 receptor antibody. Its use has been approved for the treatment of rheumatoid arthritis, juvenile idiopathic arthritis, giant cell arteritis, and chimeric antigen receptor T (CAR-T) cell-induced severe cytokine release syndrome. The experience on tocilizumab efficacy and safety in monogenic AIDs is still inadequate. Thus it is not registered for the treatment of any of these diseases (Soriano, 2020).

There were few single cases of patients with colchicine-resistant FMF treated with tocilizumab. It has shown reasonable control of the disease flares. In patients with secondary amyloidosis and proteinuria, improvement was achieved in 75% of them (Ugurlu et al., 2017). There are few cases of tocilizumab use in TRAPS and HIDS/MKD resistant to anti-IL-1 or anti-TNF agents in the literature. No effect was achieved in patients with CAPS. There is no evidence of the use of tocilizumab in PFAPA patients (Soriano et al., 2020). In proteasome-associated autoinflammatory syndrome, anti-IL-6 therapy decreased CRP levels and normalized IL-6 responsive genes, but the effect was minor or transient (McDermott, 2015).

JAK inhibitors

JAK (Janus kinase) inhibitors block the induction of IFN-stimulated genes via suppressing the phosphorylation of the transcription factor STAT-1. This group represents tofacitinib, baricitinib, and ruxolitinib registered to treat rheumatoid arthritis, psoriatic arthritis, ulcerative colitis, myelofibrosis, polycythemia vera, and steroid-refractory GvHD.

JAK inhibitors showed positive effects in interferonopathies, for example, chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature/proteasome-associated inflammatory syndrome, STING-associated vasculopathy with onset in infancy, familial chilblain lupus, and Aicardi-Goutières syndrome (Soriano et al., 2020).

Other drugs

A small data in the Eurofever registry describes the use of such drugs as isotretinoin or cimetidine, administered in sporadic cases combined with biologic agents (Ter Haar et al., 2013). Rigante et al. also mentioned bisphosphonates, azathioprine, methotrexate, MALT-1 inhibitors, iron supplements (Rigante, 2017). As mentioned in previous paragraphs, the evidence for its efficacy and safety in AID is limited to use in single cases, and the precise guidelines are missing as autoinflammatory disorders are considered rare.

Thalidomide

Thalidomide has an anti-inflammatory effect through inhibition of TNF- α and IFN- γ production, angiogenesis, and leukocyte chemotaxis. Treatment of AID is based on a few case reports and series. It did not show efficacy or benefit in patients with FMF and HIDS/MKD (Soriano et al., 2020). Tacrolimus has been used in pyogenic arthritis, pyoderma gangrenosum, and acne syndrome (PAPA) with variable efficacy (Soriano et al., 2020). The effectiveness of tacrolimus was observed in the treatment of cherubism (Kadlub et al., 2015).

Dapsone

Dapsone is an antibacterial drug from the sulfonamide group. Its anti-inflammatory properties are based on inhibition of some neutrophil functions and blockade of the effects of prostaglandins and leukotrienes. In AIDs, dapsone was proved effective in a group of patients with FMF—the attacks were controlled in 50% of them. The conclusion was that dapsone might be used as an alternative therapy in FMF cases nonresponding to first-line treatment (Salehzadeh et al., 2012).

Surgery

Tonsillectomy is considered a successful treatment of PFAPA syndrome, which can result in complete regression of symptoms. The data which supports this statement comes from two small clinical trials, and the evidence is moderate certainty (Burton et al., 2019). Peridis et al. conducted a meta-analysis that did not show the difference in treatment outcome between surgery or glucocorticoids in PFAPA treatment. It is not clear if additional adenotomy conducted with tonsillectomy adds an extra benefit to this type of treatment (Gaggiano et al., 2019).

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